STRUCTURE-ACTIVITY STUDIES OF SEMIOCHEMICALS FROM *Caladenia plicata* FOR SEXUAL DECEPTION

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**Abstract**— Sexually deceptive orchids attract specific pollinators by mimicking insect sex pheromones. Normally this mimicry is very specific and identical compounds have been identified from orchids and matching females of the pollinators. In this study, we conduct a detailed structure-activity investigation on isomers of the semiochemicals involved in the sexual attraction of the male pollinator of the spider orchid *Caladenia plicata*. This orchid employs an unusual blend of two biosynthetically unrelated compounds, (S)-β-citronellol and 2-hydroxy-6-methyldacetophenone, to lure its *Zeleboria* sp. thynnine wasp pollinator. We show that the blend is barely attractive when (S)-β-citronellol is substituted with its enantiomer, (R)-β-citronellol. Furthermore, none of the nine possible alternative hydroxy-methyldacetophenone regioisomers of the natural semiochemical are active when substituted for the natural 2-hydroxy-6-methyldacetophenone. Our results were surprising given the structural similarity between the active compound and some of the analogues tested, and results from previous studies in other sexually deceptive orchid/wasp systems where substitution with analogues was possible. Interestingly, high-level *ab initio* and density functional theory calculations of the hydroxy-methyldacetophenones revealed that the active natural isomer, 2-hydroxy-6-methyldacetophenone, has the strongest intramolecular hydrogen bond of all regioisomers, which at least in part may explain the specific activity.

**Key Words**— Caladenia, sexual deception, pollination, structure-activity, isomers, hydrogen bonding.

Acknowledgements: BB, GRF, and RP acknowledge the Australian Research Council for funding (DE160101313, FT110100304, LP130100162 and DP150102762). The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. Alyssa Weinstein is thanked for field and laboratory assistance. RP was hosted as a visiting fellow by UWA while completing this study.
INTRODUCTION

Sexual deception is a highly specialised pollination strategy where insect pollinators are sexually lured to flowers by pheromone mimicry. This strategy is most commonly found among the Orchidacea (Ayasse et al. 2011; Borg-Karlson 1985; Franke et al. 2009; Gaskett 2011; Johnson and Schiestl 2016; Phillips et al. 2014; Schiestl et al. 200) where it has evolved independently in many unrelated genera (Ayasse et al. 2011; Gaskett 2011; Phillips et al. 2014, Bohman et al. 2016). Despite comprehensive early studies of the pollination biology of some sexually deceptive orchids in Europe and Australia (Coleman 1929; Kullenberg 1950), chemical ecology investigations leading to identification of the semiochemical(s) involved commenced much later (Bohman et al. 2012a; Bohman et al. 2012b; Bohman et al. 2014; Bohman et al. 2017a; Borg-Karlson 1987; Borg-Karlson 1990; Cuervo et al. 2017; Franke et al. 2009; Gervasi et al. 2017; Schiestl et al. 1999; Schiestl et al. 2000; Schiestl et al. 2003; Xu et al. 2017).

Despite the successful elucidation of the chemistry of sexual deception in a growing number of cases (see the recent review of Bohman et al. (2016) for a summary), these represent just a fraction of the several hundred orchids using this pollination strategy. Nonetheless, it is already evident that an extraordinary diversity of semiochemicals is involved, and that more often than not, the chemical cues consist of unusual compounds, or even new classes of compounds on their initial discovery (see references above). Furthermore, so far, the orchid semiochemicals involved precisely match constituents of the sex pheromone of the pollinators (Bohman et al. 2016). Such examples include Ophrys sphegodes (Schiestl et al. 1999; Schiestl et al. 2000), Ophrys speculum (Ayasse et al. 2003), Chiloglottis trapeziformis (Peakall et al. 2010; Schiestl et al. 2003), Drakaea glyptodon (Bohman et al. 2014), Caladenia crebra (Bohman et al. 2017a) and Caladenia plicata (Xu et al. 2017).

In all documented cases so far, precise matches between insect sex pheromones and floral pollinator attractants have been observed (Bohman et al. 2016, Bohman et al. 2017, Xu et al. 2017), raising the question as to whether this tight specificity is a general characteristic of sexual deception. Scope for substitution of natural semiochemicals with synthetic structural analogues in sexually deceptive pollination systems has previously been tested in two cases of Australian hammer orchids (Bohman and Peakall 2014; Bohman et al. 2015). In the first case involving a Catocheilus wasp pollinator of Drakaea livida, it was shown that 3-
(3-methylbutyl)-2,5-dimethylpyrazine, a close analogue to the floral pollinator attractant 2-(3-methylbutyl)-3,5,6-trimethylpyrazine, was equally active in attracting the pollinator. In the second case, 2-hydroxymethyl-3,6-diethyl-5-methylpyrazine, one of two components of the sex pheromone of *Zaspilothynnus trilobatus* and the attractant from *Drakaea glyptodon*, was substituted with a set of structural analogues. One analogue, 2-hydroxymethyl-3,5-dimethyl-6-ethylpyrazine, exhibited comparable activity to the natural product. These two studies illustrate that in these particular wasp-semiochemical interactions some changes in the positions of methyl and ethyl substituents around an aromatic ring can be tolerated with little or no change in the biological activity. Such active homologues could function as parapheromones and may have advantages for the producer, in this case the orchid, over the natural product in terms of ease of synthesis or improved physicochemical properties. From an evolutionary perspective, less structural specificity may allow evolutionary flexibility and increased sustainability.

Despite *Caladenia* being a very large orchid genus comprising >350 species, with many of these species sexually deceptive (Bohman et al. 2017b), the pollination chemistry was until recently, unknown. In *C. crebra* and *C. attingens*, (methylthio)phenols are now confirmed to be the chemical cues for pollinator attraction (Bohman et al. 2017a, Bohman et al. 2017b). *Caladenia plicata*, employs an unusual combination of two biosynthetically unrelated compounds, (S)-β-citronellol and 2-hydroxy-6-methylacetophenone, to sexually lure the males of a single thynnine wasp species (*Zeleboria* sp.) as pollinator. A 1:4 blend elicits the strongest sexual response with attempted copulation rates equivalent to that observed at flowers, when compared with 1:1, 20:1 and 1:20 blends (Xu et al. 2017).

In this present study, we investigate in detail the semiochemical requirements of this interaction between *Caladenia plicata* and its *Zeleboria* sp. pollinator. First, the requirement of enantiomeric specificity of β-citronellol was evaluated by comparing pollinator attraction between the (R)- and (S)-enantiomers in blends with the natural 2-hydroxy-6-methylacetophenone. Second, by synthetically preparing all ten possible regioisomers of hydroxy-methylacetophenone, the effects of the position of all ring substituents were evaluated by blending with (S)-β-citronellol. Unexpectedly, none of the alternative acetophenones were biologically active. Similarly, (R)-β-citronellol was significantly less attractive compared to the naturally occurring enantiomer. To better understand the strict specificity requirements for the acetophenone compounds, we estimated the structural and energetic characteristics of each regioisomer generated by high-level *ab initio* and density
functional theory calculations to provide clues towards understanding the basis of this extreme structural specificity of the antennal receptor.

METHODS AND MATERIALS

Bioassays
To assess the level of pollinator sexual attraction when (S)-β-citronellol was replaced by (R)-β-citronellol, 1a was replaced by 1b at the optimal 1:4 ratio, in field bioassays that followed Xu et al. (2017). In short, spiked dummies made up of black plastic dressmakers pins (4 mm diameter pinhead) attached to bamboo skewers (25 cm in height) were prepared for sequential experiments. Each experiment consisted of a series of two-phase trials. In phase one, the test blend of 1b and 2a was presented for 3 min. Phase 2 commenced with the addition of the control blend of 1a and 2a on a second dummy ca. 1 m from the test blend dummy, perpendicular to the wind direction. Four replicate experiments each with new dummies and chemical preparations, and consisting of four or more trials, were conducted over two successive days, with each day including one morning and one afternoon experiment.

To evaluate the wasp response to the various regioisomers of the hydroxy-methyl acetophenone, 2a was replaced with one of 2b-2j or 3 (Fig 1) in the blend with 1a in a series of field bioassays. We initially began tests of the regioisomers in 2014 at blend ratios of 1:1, before we had experimentally established that the optimal blend ratio was 1:4 (1a:2a). For these experiments, up to four different test blends and a control blend were made up simultaneously each morning or afternoon. Subsequently, sequential two-phase trials (as above) were conducted, with a single test blend evaluated for 3 min, followed by the second phase choice test involving the addition of the control blend (~1 m away) for a further 3 min. Each of the test blends were randomly allocated to successive trials, ensuring each test blend was evaluated twice over the course of either a morning or afternoon session.

None of the regioisomers (other than the natural product) were found to be active at the 1:1 ratio. However, following our discovery that the optimal blend ratio was 1:4, in 2015 and 2016 we re-tested the regioisomers at this ratio to ensure the lack of response was not due to a suboptimal blend. By this point in time, we had also firmly established that the wasps respond rapidly (within 1 min) to the optimal blend. Therefore to expedite the number of
experiments that could be conducted, the second phase choice test was limited to just one minute.

All field bioassays were conducted at a study site in natural heathland near the small township of Peaceful Bay (S35.025°, E116.935°) where male Zeleboria sp. wasps were known to be locally abundant (Xu et al. 2017) in September-October 2014-2016. The experiments were restricted to warm (>18°C) and sunny conditions with limited wind between the hours of approximately 10 am and 2 pm, when male thynnine wasps commonly are known to be actively patrolling for mates (Peakall 1990). These stringent weather conditions considerably restrict the number of days available for conducting bioassays during the short peak flying period of the wasp (~3-4 weeks), particularly in this southern coastal location with highly variable spring weather. The number of wasps that approached, landed and attempted to copulate were recorded for each phase and treatment. Individual trials were aborted as unsuccessful if there was no wasp response to the control blend in phase 2.

Given the typical rapid decline in pollinator responses over the 3 min experiments, and results from mark-recapture studies demonstrating that other thynnine wasps do not revisit baits on the same day, the risk of pseudo-replication, within or between trials, is considered unlikely (Peakall 1990; Bohman et al. 2014; Whitehead and Peakall 2014). Consequently, as in previous studies, the total number of approaches, lands and attempted copulations were pooled across trials for each treatment, with G-tests performed in GenAlEx 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) to compare the proportion of total responses among control and treatment in phase 2 of the sequential trials.

**General chemical procedures.** The two enantiomers of β-citronellol (1a and 1b) and 2-hydroxyacetophenone (3) were purchased from Sigma Aldrich (Australia). 3-Hydroxy-4-methylacetophenone (2g) was purchased from Astatech Inc (USA). All other isomers of hydroxy-methylacetophenones were prepared according to literature procedures (2a, 2b-2d, 2j (Wang et al. 2012), 2e-2f, 2h-2i (Lafleur et al. 2009)). NMR spectra were consistent with previously reported (2a, 2c-2d (Seidel et al. 1990), 2b, 2j (Fischer and Henderson 1981), 2e, 2h (Lafleur et al. 2009), 2f (Baldwin and Lusch 1982), 2i (Fischer et al. 1978)).

**Ab initio Calculations.** Without an experimental structure of the olfactory receptors of thynnine wasps, which can serve as a starting point for theoretical simulations, we were unable to model specific ligand-receptor interactions. Rather, to gain a better chemical understanding of the observed strict structural specificity, we have optimised the geometries
of all the methyl-hydroxyacetophenone isomers (Fig. 1), looking for structural differences not obvious to the “naked eye”, which may explain the otherwise incomprehensive observations. We show the there are some structural differences between the isomers that may play a role in their biological activity. In addition, we also carried out high-level ab initio calculations in order to energetically characterise the methyl-hydroxyacetophenone isomers. The high-level ab initio and density functional theory calculations were performed using the Gaussian 09 program suite (Frisch et al. 2009). Geometries and vibrational frequencies were obtained at the B3LYP/6-31G(2df,p) level of theory. Zero-point vibrational energy, enthalpic, and entropic corrections have been obtained from such calculations. The equilibrium structures were verified to have all real harmonic frequencies. Gas-phase Gibbs free energies at 298 K were obtained using the G4(MP2) thermochemical protocol (Curtiss et al. 2007).

RESULTS

Bioassays: Neither (S)-β-citronellol (1a) nor 2-hydroxy-6-methylacetophenone (2a) could be substituted with their enantiomer (1b) or regioisomers (2b-2j) and 3 respectively and retain pollinator attraction comparable to the control blend (Fig. 2 and Table 1).

A total of 151 wasp responses were recorded across the 4 replicate experiments evaluating the wasp response to (R)-β-citronellol (1b) as a substitute for (S)-β-citronellol (1a) in the 1:4 optimal blend with 2-hydroxy-6-methylacetophenone (2a). Of these responses, only 19 responses (13% of total) were recorded to 1b in phase 1, while in the second phase with the test and control choice, only 4 of the 132 visits (2.1%) were recorded to the test blend (Fig. 1). While barely attractive, it is evident that the presence of the (R)-enantiomer in the phase 2 choice test is not inhibitory at the scale tested (~1 m from the (S)-enantiomer blend). Further, it should be noted that commercial samples of β-citronellol (99% purity) were used. Thus, traces of the opposite enantiomer, difficult to detect by enantioselective gas chromatography due to poor separation, may have attributed to the weak attraction observed.

No wasp responses were recorded over the 3 min of phase 1 to any test blend combination of 1a and any single regioisomers 2b-2j or 3 in either the 1:1 or 1:4 blend (Table 1). There was also no response to these test blends observed in phase 2. By contrast, as expected, wasps responded rapidly to the control in phase 2. For example, for the 1:1 blend, at total of 23 responses were recorded across the replicate trials for each of the 9 tests in the first min (mean±se responses per replicate control for each test=2.56±0.50), with a total of 89
responses over all replicate 3 min trials (mean±se responses per replicate control for each test=9.89±0.92). For the 1:4 blend, a total of 49 responses were recorded at the control (mean±se responses per replicate control for each test=5.44±0.55) in the first min (Table 1).

**Ab initio calculations.** All acetophenone derivatives 2a-2j and 3 in (Fig. 1) appear structurally similar to each other, in particular 2a-2d and 3, with the hydroxy positioned adjacent to the carbonyl, yet only compound 2a is biologically active. High-level calculations using the G4(MP2) thermochemical protocol were performed to probe the structural and energetic differences between compounds 2a–2j. Fig. 3 shows the optimised lowest-energy conformations of the ten regioisomers.

In the active pheromone (2a), the acetyl and hydroxyl groups are in adjacent positions on the phenyl ring such that there is a hydrogen bond between the hydrogen of the hydroxyl and the carbonyl of the acetyl group. The length of this bond is 1.557 Å, indicating it is a relatively strong hydrogen bond. It is possible to estimate the strength of a similar intramolecular hydrogen bond by considering the two conformations of 3 shown in Fig. 4. In one conformation the acetyl and hydroxyl groups are pointing towards each other forming a hydrogen bond (Fig. 4a), whilst in the other conformation they point away from each other (Fig. 4b). On the Gibbs-free energy surface at 298K (∆G298), the conformation with the hydrogen bond is lower in energy than the one without the hydrogen bond by 31.1 kJ mol⁻¹. We note that the length of the hydrogen bond in 3 is longer than that in compound 2a by 0.098 Å, indicating that the hydrogen bond in compound 2a is slightly stronger than in 3. We also note that the length of the hydrogen bond in compound 2a is slightly shorter than that in the remaining compounds with intramolecular hydrogen bonding capacity, 2b, 2c, and 2d (see Fig. 4). This might arise from steric repulsion of the acetyl methyl group with the adjacent aromatic methyl group in 2a, in effect pushing the carbonyl oxygen closer to the hydroxyl hydrogen.

Given that the intramolecular hydrogen bond in the active pheromone 2a is estimated to be relatively strong, this structural feature might be important for the interaction of the receptor with compound 2a. In compounds 2e–2j, the acetyl and hydroxyl groups are not positioned on adjacent carbons, so lack this hydrogen bonding arrangement and are biologically inactive. However, compounds 2b–d do contain this hydrogen bonding arrangement, but are also biologically inactive. On the other hand, 2a is unique in that it is the only compound with effectively adjacent methyl groups (Fig. 2), which might be important for binding in the receptor site.
DISCUSSION

Only the two natural product semiochemicals isolated from *Caladenia plicata* and its *Zeleboria* sp. thynnine wasp pollinator, acted as strong sexual attractants. It is perhaps not surprising that the natural enantiomer of β-citronellol is required for bioactivity, as the conformational space for these two enantiomers would be rather different. Perhaps more surprisingly is that every structural analogue to 2-hydroxy-6-methylacetophenone tested failed to attract any male wasps. Thus, even very minor modifications in the ring substitution pattern of this compound, such as the movement or removal of a methyl group, appears to have completely turned off any function as a semiochemical in this system. Such strong specificity would seem to indicate a very well defined active site of the receptor(s) in the wasp antennae (for example see Brookes et al. 2009), although this remains to be determined in the future.

To obtain more details about the specificity of interaction with the unknown receptor, and whether the specificity was purely due to steric factors or also electronic effects, we conducted some theoretical studies. By calculating the energetic differences between the series of analogues and determining the optimised lowest-energy confirmations, we could observe a distinct difference in the strengths of the internal hydrogen bonds among the compounds analysed. It is well known that non-covalent C–H•••π interactions can play important roles in catalytic processes in the gas-phase (Wagner and Schreiner 2015; Neel et al. 2017). We therefore infer that the presence of the methyl group in the position adjacent to the acetyl makes the hydrogen bond stronger through steric repulsion, which might play an important role for the biological activity of compound 2a. Furthermore, the proximity of the two methyl groups, resulting from the hydrogen bonding arrangement, might also be important for the observed bioactivity, via increased localized lipophilicity. This rationale is supported by the fact that 2-hydroxyacetophenone (3) and analogues 2b-d are biologically inactive.

The requirement of highly specific semiochemicals, or blends of semiochemicals, is well documented in the literature, even if the number of examples is limited. Such examples include the butterfly *Pieris napi*, which employs citral as the male sex pheromone. Here it was shown that females only showed acceptance behaviour in the presence of citral but not to the individual components, geranial and neral, demonstrating the importance of the specific
blend (Larsdotter-Mellström et al. 2016). The pine sawfly *Neodiprion sertifer* (Geoffroy) uses the acetate or propionate of (2S,3S,7S)-3,7-dimethyl-2-pentadecanol (diprionol) as pheromone components. Anderbrant et al. (2010) tested the attraction of males to the acetates of three analogues of diprionol, each missing one methyl group. None of the analogues alone, or in combination with diprionol acetate, were attractive in Sweden, while in Japan, the acetate of (2S,3S)-3-methyl-2-pentadecanol attracted males when tested in amounts 10–20 times higher than the acetate pheromone component, showing that even geographic variation in sex pheromone specificity is possible (Anderbrant et al. 2010). Perhaps the most striking example of sex pheromone specificity is in the honey bee. Blum et al. tested 19 structural analogues to the sex pheromone 9-oxo-*trans*-2-decenoic acid, without detecting activity for any compound but the natural product (Blum et al. 1971).

All these findings can be compared with the results from previous structure-activity studies of sexually deceptive *Drakaea* orchid semiochemicals. In those studies, structural analogues of the biologically active hydroxymethylpyrazine in *D. glyptodon* and *D. livida*, in contrast, were biologically active (Bohman and Peakall 2014; Bohman et al. 2015). It is noteworthy that when similar calculations were performed on the pyrazines evaluated as attractants to *Zaspilothynnus trilobatus*, the pollinator of *Drakaea glyptodon*, the energetic differences were negligible, perhaps providing evidence as for why the natural semiochemicals could be substituted with other synthesised analogues and still retain similar activity. The results from the *Drakaea* studies correlated well with the results from studies in insects such as the citrus mealybug *Planococcus citri* (Dunkelblum et al. 1987), the astigmatid mites *Aleuroglyphus ovatus* and *Acarus immobilis*, (Shibata et al. 1998), and the lichen moth (Muraki et al. 2014), where structural pheromone analogues were active.

In conclusion, we report on the highly structurally specific requirements for the *Zeleboria* sp. wasp sex pheromones and *C. plicata* pollinator attractants. Our results reveal for the first time in a sexually deceptive orchid-pollination system that extreme pollinator specificity is finely controlled by chemical structure.
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Fig. 1 Pollinator attractants (1a and 2a) and structural analogues (2b-2j and 3) to the female sex pheromone of the thynnine wasp *Zeleboria* sp.
Fig. 2. Outcomes of sequential bioassays comparing the pollinator wasp response to \((R)\)-\(\beta\)-citronellol (1b) as a substitute for \((S)\)-\(\beta\)-citronellol (1a) at the optimal 1:4 ratio. Phase 1 consisted of the presentation of compounds 1b and 2a for 3 min. This was followed by phase 2, where both the initial blend and the control blend of 1a and 2a was presented for 3 min. Pollinator responses are shown as mean proportions of the total (± se) across replicate experiments, further partitioned into approach A, land L and attempted copulation C (left panel). The total count across experiments is shown separately for phase 2 (right panel).
Fig 3 B3LYP/6-31G(2df,p) optimized structures of the lowest-energy conformations of compounds 2a–2j (atomic colour scheme: H, white; C, grey; O, red). The single dashed lines represent hydrogen-bond interactions (H-bond distances are given in Å).
Fig. 4 B3LYP/6-31G(2df,p) optimised structures of two conformations of 2-hydroxyacetophenone (3) (atomic colour scheme: H, white; C, grey; O, red). The single dashed line represents a hydrogen-bond interaction and its distance is given in Å.
Table 1. Outcomes of sequential bioassays comparing the pollinator wasp response to (S)-β-citronellol (1a) with each of the other nine regioisomers (2b-2j or 3) individually, indicated by R in the table header) against the control blend of 1a and 2a. Results are shown for tests at 1:1 and 1:4 blend ratios. Phase 1 consisted of the presentation of test blend for 3 min. This was followed by phase 2, where both the initial test blend and the control blend was presented for up to 3 min. The total wasp responses over two trials, after 1 min and 3 min respectively (where applicable), are shown for each phase.