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A specific blend of drakolide and hydroxymethylpyrazines – an unusual pollinator sexual attractant used by the endangered orchid *Drakaea micrantha*

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Abstract:

Bioactive natural products underpin the intriguing pollination strategy used by sexually deceptive orchids. These compounds, which mimic the sex pheromones of the female insect, are emitted in particular blends to lure male insect pollinators of specific species. By combining methods from field biology, analytical chemistry, electrophysiology, crystallography, and organic synthesis, we report that an undescribed β-hydroxylactone, in combination with two specific hydroxymethylpyrazines, act as pollinator attractants in the rare hammer orchid *Drakaea micrantha*. This discovery represents an unusual case of chemically unrelated compounds being used together as a sexual attractant. Furthermore, this is the first example of the identification of pollinator attractants in an endangered orchid, enabling the use of chemistry in orchid conservation. Our synthetic blend is now available to be used in pollinator surveys to locate suitable sites for plant conservation translocations.

Pollination by sexual deception is a highly specialized pollination strategy used by hundreds of plant species, where the flower typically attracts male insects with chemical compounds that mimic the female sex pheromone of the pollinator species.[1] Most examples of this pollination strategy are from orchids, where sexual deception is known from over 20 genera.[1a] So far, there has only been a few studies where the compounds involved in orchid pollination have been elucidated and their biological function confirmed. The active compounds include *n*-alkanes and alkenes, along with hydroxy- and keto acids in *Ophrys,*[2] cyclohexane-1,3-diones in *Chiloglottis*,[3] alkyl- and hydroxymethylpyrazines in *Drakaea*,[1e] (methylthio)phenols in *Caladenia*[1h] and tetrahydrofuranyl acids in *Cryptostylis*.[4] Most systems involve multiple compounds, usually derived from similar biosynthetic origins. Only one example of a mixed pheromone/pollinator attractant system originating from distinct biosynthetic pathways has been reported: the orchid *Caladenia plicata* attracts its *Zeleboria* sp. C thynnine wasp pollinator with a specific mixture of the terpene citronellol and a likely polyketide-derived acetophenone.[1h]

Drakaea (hammer orchids) is an Australian genus with flowers characterized by highly reduced tepals and a hinged insectiform labellum, which are pollinated by sexual deception of thynnine wasps. Previous studies have shown that hydroxymethylpyrazines in *D. livida* are detected by the wasp pollinators.[1b, 1c] In *D. glyptodon*, another set of alkyl- and hydroxymethylpyrazines were confirmed as pollinator attractants in field bioassays.[1e] Five of the ten known species of *Drakaea* are endangered, with one additional species poorly known and possibly extinct.[5] The rarity of hammer orchids, in combination with their reliance on specific species of thynnine wasp pollinators, potentially raises great conservation challenges.

Here, we investigate the pollination chemistry of the rare *Drakaea micrantha*, the smallest species (flower size ca. 4 mm x 20 mm), and the only member of the genus pollinated by a *Zeleboria* thynnine wasp. We demonstrate that pollination of *D. micrantha* requires a specific blend of 2-hydroxymethyl-3,5,6-trimethylpyrazine (**1**), 2-hydroxymethyl-3,5-dimethyl-6-ethylpyrazine (**2**) and the unique β-hydroxylactone 4-hydroxy-3-methyl-6*S*-(pentan-2*S*-yl)-5,6-dihydro-2*H*-pyran-2-one (**3c**, here termed drakolide). Key tools facilitating the identification of the compounds included gas chromatography-electroantennography (GC-EAD), where insect antennae were employed as the detector, and semi-preparative gas chromatography. GC-EAD repeatedly detected two compounds (Figure 1), identified as hydroxymethylpyrazines **1** and **2** by synthesis,[6] GC-MS[7] and co-injection on two GC columns. The most abundant EAD-active compound **1,** has also previously been reported as EAD-active to the *Catocheillus* wasp that pollinates some populations of *D. livida*.[1b] The homologue **2** (in lower abundance than **1** in *D. micrantha*) is identified here for the first time as a natural product, having already been synthesized as part of a mass spectrometry study.[7] Compounds **1** and **2**, although EAD-active, did not elicit strong attraction of the *Zeleboria* pollinator in preliminary field tests

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(SI, Figure S1). Therefore, to find the missing component(s) of the semiochemical blend not detected by GC-EAD, bioassay-guided semi-preparative GC of floral extracts was conducted.

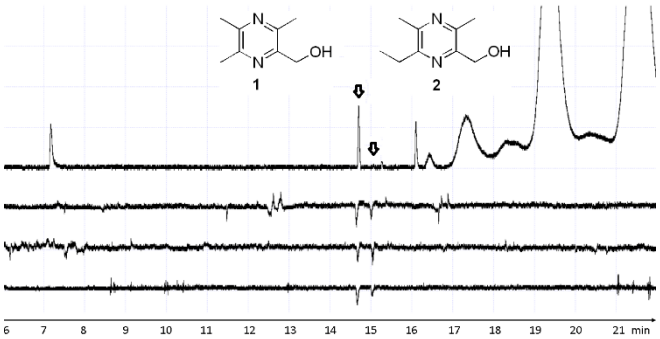
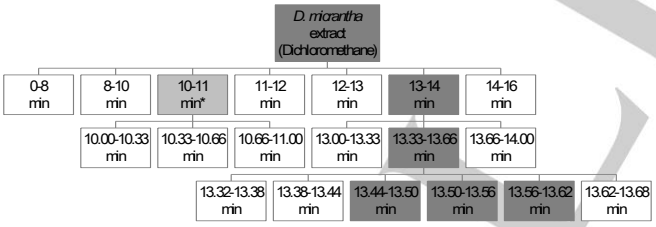


Figure 1. GC-FID chromatogram (top trace) of the floral extract (dichloromethane) of *Drakaea micrantha*. GC-EAD (three replicates, lower traces) of the antennal responses of the *Zeleboria* pollinator to the floral extract of *D. micrantha*. Hydroxymethylpyrazines **1** and **2** are indicated with arrows.

As *D. micrantha* is an endangered species, we used a protocol to minimize the number of flowers collected. We first confirmed that an extract of a single *D. micrantha* flower in dichloromethane added to a dressmaker pin head, a method successfully used for *D. glyptodon*,[1e] attracted and elicited pseudocopulation by the pollinator in field experiments. Next, we prepared an extract from 20 flowers (combined from several populations, collected over two seasons to comply with permit requirements), which was subjected to semi-preparative GC using a bioassay-guided fractionation approach. This extract was separated in increasingly smaller fractions, which were tested together with synthetic compound **1** (the main EAD-active compound, Scheme 1). Following the activity in field trials, three active fractions, containing the same one main peak, remained.



Scheme 1. Semi-preparative GC separation of a 20–flower dichloromethane extract of the flowers of *D. micrantha*. Fractions in grey boxes elicited pollinator responses in field experiments. (* = weakly active fraction. No activity when the fraction was further separated.)

Aside from the rarity of the plant, the identification of bioactive compounds was also complicated by the difficulty in locating females of the pollinator, an undescribed species referred to as *Zeleboria* sp. A, currently unknown from museum collections.[5] Despite extensive searches for pairs *in copula*, we were unable to locate any females. Thus, we had no means of comparing floral attractants and female sex pheromones (which are often identical, or very similar to the active compounds in sexually deceptive flowers) to help pinpoint candidate compounds. Identification of the main compound **3** in the bioactive fraction relied on electron impact mass spectrometry (EIMS, mass spectrum, Figure 2b), as NMR was not possible due to the limited floral material available. High resolution GC-MS (GC-HRMS) revealed a compound with molecular formula $C_{11}H_{18}O_3$ (*m/z*: 198.1260, calcd. 198.1256). The base peak, with formula $C_6H_7O_3$ (*m/z* = 127.0393, calcd. 127.0395) indicated 3 double bond equivalents (DBE) and a loss of a saturated hydrocarbon fragment of C_5H_{11}, supporting the presence of at least one ring in the molecule along with a high degree of oxygenation.

Since our mass spectrometry data (Figure 2b) did not align with any compounds related to the semiochemicals previously identified from *Drakaea*,[1a] we searched for clues from bioactive compounds in other pollination systems involving sexual deception of thynnine wasps. Most similar to the unsaturated oxygen containing semiochemical in *D. micrantha*, are the cyclohexane-1,3-dione (chiloglottone) sex pheromones of *Neozeleboria* thynnine wasps,[3] which pollinate *Chiloglottis* orchids, a genus closely related to *Drakaea*. However, comparisons of mass fragmentations of various chiloglottones[3] revealed no obvious similarities to **3**. A search of the literature revealed some synthesised β-hydroxylactones with mass spectra similar to our unknown **3**. In particular, 4-hydroxy-6-pentyl-5,6-dihydro-2*H*-pyran-2-one[9] showed several common mass losses observed at *m/z* ions that were 14 mass units (i.e. -CH_2) lower than in **3.** Ion fragments of *m*/*z* 113, 129 and 184, in similar ratios, matched those of *m*/*z* 127, 143 and 198 of our unknown. The base peaks of *m*/*z* 113 and 127, corresponding to a loss of C_5H_{11} from the molecular ion, were also in good agreement, collectively guiding us to explore this class of compounds further.

First, we investigated a methyl group in the 3-position of 4-hydroxy-6-pentyl-5,6-dihydro-2*H*-pyran-2-one, to yield 4-hydroxy-3-methyl-6-pentyl-5,6-dihydro-2*H*-pyran-2-one. After synthesis and comparison of GC retention data and mass spectra, it was evident that this compound showed strong mass spectral similarities to the natural product, although the data were not identical. Next, all 3-methyl-6-(branched pentyl)-isomers were subsequently synthesised via condensation reactions of β-oxoacid esters with aldehydes following a modified procedure from Lokot *et al*. (Figure 2a).[10] By evaluating GC-MS data, the natural product was confirmed as a stereoisomer of 4-hydroxy-3-methyl-6-(pentan-2-yl)-5,6-dihydro-2*H*-pyran-2-one, by co-injection on two GC columns and mass spectral comparisons.

A mixture of the four stereoisomers **3a**-**3d** was prepared from racemic 2-methylpentanal and ethyl-2-methyl-3-oxobutanoate (Figure 2a). Field bioassays confirmed that this product, in a specific blend with the hydroxymethylpyrazines **1** and **2**, elicited pseudo-copulation by the male thyninne wasps. With the use of chiral-phase GC fitted with a cyclodextrin γ column, we separated the four stereoisomers **3a** - **3d**. To determine the absolute configuration of the natural isomer, an asymmetric synthesis was conducted, employing enantio-enriched aldehydes in the condensation reaction, thereby fixing the stereocenter in the pentan-2-yl side chain.

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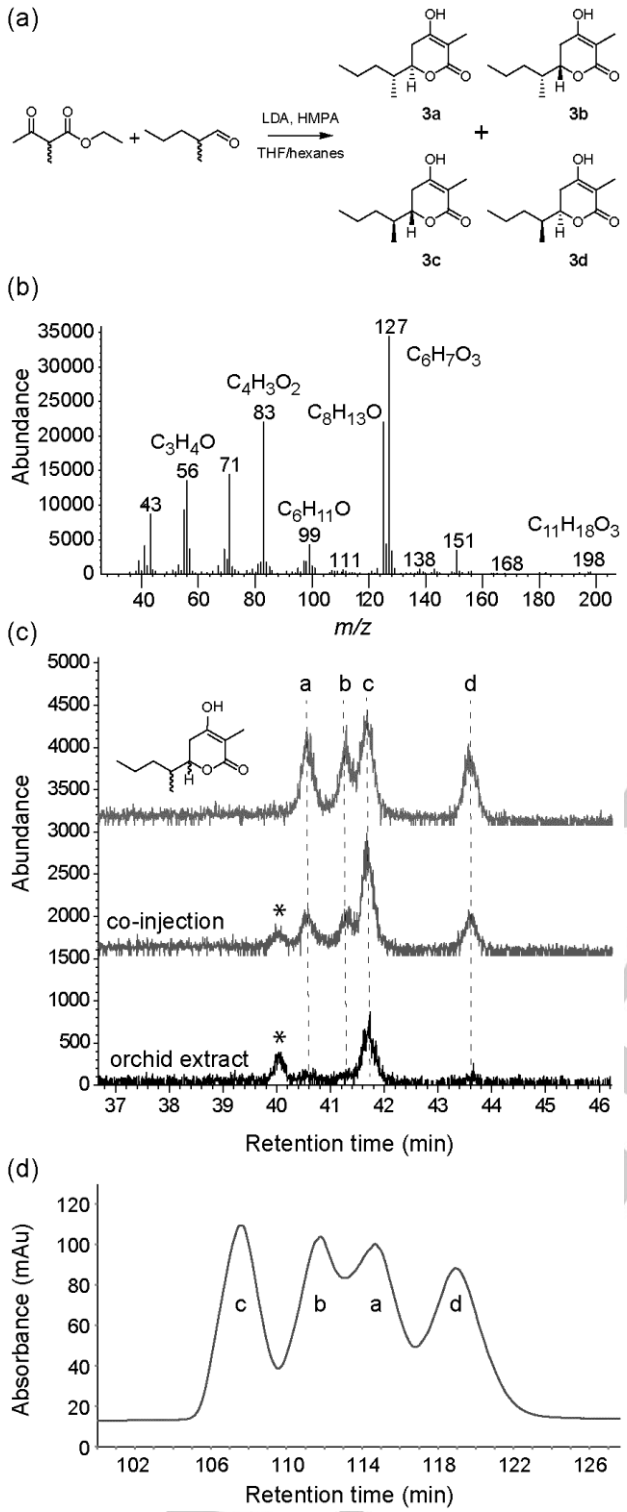

Figure 2 (a) Synthesis of 4-hydroxy-3-methyl-6-(pentan-2-yl)-5,6-dihydro-2*H*-pyran-2-one stereoisomers **3a** - **3d**.[10] (b) Electron impact mass spectrum of the bio-active natural product **3** with fragments for major ions presented. (c) Chiral-phase GC-MS analysis; synthetically prepared **3a** - **3d**, co-injection of **3a** -**3d** with *D. micrantha* solvent extract, and *D. micrantha* solvent extract alone (***** = unrelated compound). (d) Chiral-phase HPLC chromatogram of **3a** - **3d**.

Co-injection of the floral extract with the epimer-enriched products, confirmed that the sidechain configuration was *S* (Figure 2c).

All stereoisomers from the racemic synthetic mixture were also separated by chiral-phase HPLC, using a semi-preparative cellulose dimethylphenyl carbamate (DMP) column (Figure 2d). The four separated stereoisomers were analysed by chiral-phase GC and compared with the natural product. The fraction matching the natural product was the first eluting compound by chiral-phase HPLC. This and the epimer (eluting last), also with *S*-side chain configuration, were collected and recrystallised for X-ray crystallography studies (Figure 3 and SI, Table S1).

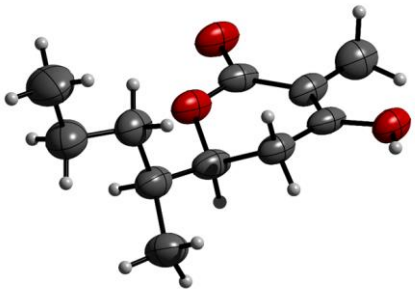

Figure 3: Structure of a single molecule from the crystal structure for drakolide **3c**. Colour scheme: O, red; C, grey; H, white. Ellipsoids for C and O are drawn at 50% probability. H-atoms are drawn as solid spheres.

By combining the results from the analysis of the asymmetrically synthesised products and the relative configuration obtained by X-ray crystallography, the natural product was confirmed to be 4-hydroxy-3-methyl-6*S*-(pentan-2*S*-yl)-5,6-dihydro-2*H*-pyran-2-one (drakolide, **3c**). Under the crystallization conditions used, the enol-tautomer of **3c** was obtained (Figure 3). NMR studies were in agreement when using deutero-methanol as solvent. However, when deutero-chloroform was used, the keto-tautomer was exclusively obtained (SI, Figure S3-S6).

Under suitable conditions, male thynnine wasps respond rapidly to orchid flowers and synthetic attractants in field bioassays. Typically, the responses decline within minutes of initial presentation, but a renewed response is obtained after moving to other positions in the landscape.[11] To confirm semiochemical activity, we employed a sequential two-phase bioassay design in which phase 1 involves a single test blend, whereas phase 2 provides a dual-choice test against a control orchid flower or known active semiochemical blend.[1a,1e,1h]

We commenced with tests involving blends of one of the two hydroxymethylpyrazines (**1** or **2**) with drakolide (as a mixture of stereoisomers **3a**-**3d**) at a one-to-one ratio (Figure 4a). Blends of **1** or **2** with drakolide lead to frequent lands and attempted copulation (L+C) by the male *Zeleboria* wasps in phase 1.

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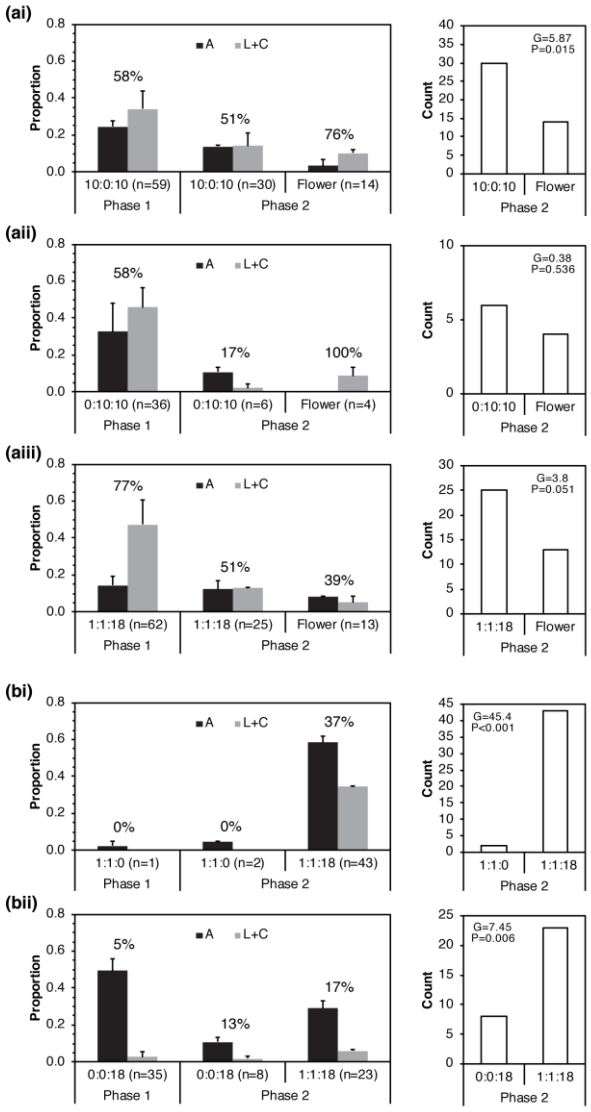

Figure 4. Outcomes of sequential two-phase bioassays showing the response of male *Zeleboria* thynnine wasps to three different blends of synthetic compounds **1**:**2**:**3** in phase 1, with *Drakaea micrantha* flowers as the control in the dual-choice phase 2. (ai) Compounds **1** and **3** (10:0:10); (aii) Compounds **2** and **3** (0:10.10) (aiii): Compounds **1**, **2**, and **3** (1:1:18). Also shown are wasp responses to **1** and **2** only (bi), or **3** only in phase 1 (bii), with the active blend (1:1:18) as the control in the dual-choice phase 2. Compound **3** was always tested as a mixture of stereoisomers **3a**-**3d**. In the left panels, wasp responses are partitioned into approaches (A, black bars) versus lands only, or lands followed by attempted copulations (L+C grey bars with % given above the columns), and shown as the proportion of the total of phase 1 and 2 responses, averaged across replicate experiments. The right panels show the total counts across replicate experiments for phase 2, and the G-test outcomes. All ratios are given in the order of compounds (**1**:**2**:**3**).

In phase 2, the (**1**+**3**) and (**2**+**3**) blends were equally or more attractive than the *D. micrantha* flower. Given that the ratios of **1**, **2** and **3** varied among floral extracts, we also tested blends with proportionally less pyrazines, while holding the total amount constant (Figure 4aiii, SI Figures 1-2). In phase 1, the 1:1:18 blend (**1**+**2**+**3**) elicited the strongest response of 77% L+C (Figure 4aiii), compared with 58% each for the other two blends (Figure 4ai - aii, see also SI Figure 2 for the results at test blends 5:5:10 and 2:0:18 with L+C of 50% and 62%, respectively).

Next, it was tested whether all three components were necessary for full copulatory behaviour (Figure 4b). These bioassays confirmed that **1** and **2** in combination were barely attractive, and never enticed any wasps to land (Figure 4bi). In phase 2, the control blend (1:1:18) was significantly more attractive than the pyrazines only (Figure 4bi). Conversely, drakolide alone led to more than 40% of the total responses in phase 1, but most of these responses were approaches only, with L+C comprising just 5% of responses (Figure 4bii). In phase 2, the control blend (1:1:18) was significantly more attractive than the drakolide alone (Figure 4 bii). In total, more than 400 wasp visits were recorded to synthetic blends (Figure 4, SI Figure 1, SI Figure 2, SI Video), with the overall results showing that neither **1** nor **2** were strongly active on their own or in combination, and that a strong sexual response required the combination of **1** and/or **2** with **3**.

We report here the first case where pollinator attractants of a threatened species of orchid have been identified. Locating sites with high pollinator availability can be critical for the success of plant conservation translocations, where new populations are established to reduce the risk of extinction.[12] While sexually deceived thynnine wasps can be readily surveyed using orchid flowers as baits,[12,13] our synthetic blend can now be used to survey for pollinators of the rare *D. micrantha* without requiring any picked flowers. Synthetic attractants will also allow study of the pollinators outside of the orchid flowering season, and with controlled quantities of attractant. Given that several genera of sexually deceptive orchids are characterised by a high incidence of threatened species,[14] this approach may prove broadly applicable to a large number of species.

The combination of hydroxymethylpyrazines and drakolide is only the second known example of a mixed sex pheromone/pollinator attractant system where the compounds likely arise from different biosynthetic precursors. While hydroxymethylpyrazines **1** and **2** are structurally similar, drakolide is to our knowledge not closely related to any known natural product. The most similar are the 3-acyl-substituted 4-hydroxy-5,6-dihydro-2*H*-pyran-2-ones (podoblastins) from the May Apple *Podophyllum peltatum*[15] and *Serratia plymuthica* bacterial cultures,[16] and the 3,6-dialkyl-4-hydroxy-2-pyrones from various *Pseudomonas* spp.,[17] both of which are bioactive antibiotics.

Our discovery highlights the variety of bioactive compounds involved in pollination among Australian sexually deceptive orchids. Within two *Zeleboria* pollinated orchids alone, citronellol and 2-hydroxy-6-methylacetophenone in *C. plicata*,[1h] and now two hydroxymethylpyrazines and 4-hydroxy-3-methyl-6-(pentan-2-yl)-3,6-dihydro-2*H*-pyran-2-one in *D. micrantha,* have been identified. These discoveries raise some fascinating but challenging theoretical questions: First, is this cross-kingdom use of unusual semiochemicals underpinned by convergence at the biosynthetic and molecular levels? Second, what are the mechanisms that underpin the evolution of pheromone systems

comprising combinations of biosynthetically unrelated compounds that are shared among kingdoms? While some common secondary metabolites are known to be produced by both plants and insects, examples of the cross-kingdom convergent evolution of semiochemical use are few.[18] The exceptions include bark beetles and symbiotic fungi, where both produce bark beetle aggregation signals,[19] and plant cyanogenic glucoside anti-feedants sequestered by caterpillars for their own anti-predator defence[20] or even synthesised *de novo* by the burnet moth for the same purpose.[21] Nonetheless, the shared use of the highly unusual compounds uncovered in the *Zeleboria* wasp/orchid systems appears to be unprecedented.[1h]

In summary, for the first time we have identified pollinator attractants of an endangered orchid. With the use of semi-preparative GC, GC-EAD, chiral-phase GC, chiral-phase HPLC, and single crystal X-ray crystallography, we identified the novel drakolide and two hydroxymethylpyrazines, of which one is a new natural product, from sub-microgram quantities in floral extracts. In field bioassays, a blend of these compounds attracted sexually excited pollinators at a similar level as the orchid flowers, and is now available for pollinator surveys at candidate conservation translocation sites for *D. micrantha*.

Acknowledgements

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Keywords: Sexual deception • drakolide • semiochemicals • natural products • pollination

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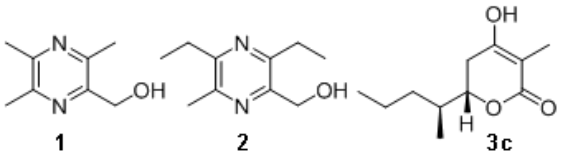

Duping Drakolide: The novel natural product Drakolide **3c**, together with the two hydroxymethylpyrazines **1** and **2,** act as pollinator attractants in the endangered sexually deceptive hammer orchid *Drakaea micrantha*.

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A specific blend of drakolide and hydroxymethylpyrazines – an unusual pollinator sexual attractant used by the endangered orchid *Drakaea micrantha*