1 2-(Tetrahydrofuran-2-yl)acetic Acid and Ester Derivatives as Long-range

2 Pollinator Attractants in the Sexually Deceptive Orchid *Cryptostylis ovata*

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ABSTRACT: Sexually deceptive orchids achieve pollination by luring male insects to flowers through chemical and sometimes visual mimicry of females. An extreme example of this deception occurs in *Cryptostylis*, one of only two genera where sexual deception is known to induce pollinator ejaculation. In the present study, bioassay-guided fractionations of *Cryptostylis* solvent extracts in combination with field bioassays, were implemented to isolate and identify floral volatiles attractive to the pollinator *Lissopimpla excelsa* (Costa) (Ichneumonidae). (*S*)-2-(Tetrahydrofuran-2-yl)acetic acid [(*S*)-**1**] and the ester derivatives methyl (*S*)-2-(tetrahydrofuran-2-yl)acetate [(*S*)-**2**] and ethyl (*S*)-2-(tetrahydrofuran-2-yl)acetate [(*S*)-**3**], all previously unknown semiochemicals, were confirmed to attract *L. excelsa* males in field bioassays. Chiral-phase GC and HPLC showed that the natural product **1** comprised a single enantiomer, its (*S*)-configuration being confirmed by synthesis of the two enantiomers from known enantiomers of tetrahydrofuran-2-carboxylic acid.

Pollination via sexual deception is achieved when male insects display copulatory or pre-copulatory behavior with flowers mimicking female insects.[1] This pollination strategy is most widely employed in the Orchidaceae, where several hundred plant species are known to be involved.[2] In orchids, the sexual attraction of male pollinators is usually achieved by species-specific blends of semiochemicals.[2] Thus, each orchid species is typically pollinated by only one pollinator species, although rare cases of multiple pollinators or pollinator sharing between orchids are known.[3,4] Members of the Hymenoptera are the most widely exploited pollinators, with well-known cases involving male bees, wasps, sawflies, and winged ants.[5-7] Pollination by sexually attracted male fungus gnats (Diptera) has also been recorded,[8,9] and may be widespread in some orchid genera.

Cryptostylis is unique among sexually deceptive orchids as the only genus where pollination by male ichneumonid wasps has been recorded.[5,10] Furthermore, in an unusual case of pollinator sharing, *Lissopimpla excelsa* is exploited by all five Australian species of *Cryptostylis*.[11-15] Additionally, *Cryptostylis* is one of only two genera in which sexually deceived pollinators have been observed to ejaculate during attempted copulation at the flowers.[8, 16,17]

To date, most studies on the semiochemicals involved in the pollination of Australian orchids have focused on thynnine wasp pollinators.[4, 18-24] Despite the unusual pollination biology of Australian *Cryptostylis*,[10,25-31] only one study has investigated the chemical signals mediating pollinator attraction.[32] This study by Schiestl et al. focused on detecting electrophysiologically active compounds from *C. subulata* and *C. erecta.* While the two species emitted different floral odor bouquets, they were found to share an unidentified compound that was electrophysiologically active to *L. excelsa* males.[32] In other chemical studies, unrelated to pollinator attraction, multiple alkaloids known as cryptostylines have been extracted from the leaves of several Asiatic species of *Cryptostylis*.[33-35]

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There have been few investigations into the sexual pheromones used by members of the Ichneumonidae, despite being one of the most diverse families in the Hymenoptera.[36-38] There are only two species of ichneumonids where the identification of sexual pheromone constituents have been confirmed by bioassays. In the first case, Robacker and Hendry[38] applied chemical methods to characterize the functional groups of extract constituents of female ichnuemonids. They found the sex pheromone of *Itoplectis conquisitor* to be composed of several unsaturated aldehydes or ketones, and showed that both neral and geranial elicited male sexual activity in field bioassays. In the second case, Eller et al. identified the sex pheromone of *Syndipnus rubiginosus* by using large-scale extraction of females, column chromatography, and microderivatization, to identify a single compound, ethyl (*Z*)-9-hexadecenoate as an attractant for conspecific males.[39] Interestingly, in another ichneumonid, *Pimpla disparis,* instead of using a sex pheromone, the males locate mates by co-opting non sex-specific eclosion pheromones (pheromones accompanying emergence), relying on the 50% likelihood that an emerging wasp will be female.[40]

Herein, more than 90 years after the landmark discovery of sexual deception in *Cryptostylis,*[12] we investigated the semiochemicals used by *Cryptostylis ovata* R.Br. to attract *Lissopimpla excelsa*. Two parallel methodologies, semi-preparative gas chromatography and liquid chromatography, both in combination with field bioassays, were employed to identify floral compounds mediating long-range attraction of pollinators. NMR spectroscopy and GC-MS were used to confirm the structure of the isolated compound and two additional bioactive derivatives. Synthesis of authentic standards, and comparison of their retention times and spectra, was used to determine the absolute configuration of the main attractant.

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RESULTS AND DISCUSSION

By conducting an experiment where orchid flowers were hidden from the pollinators' view with a screen, but where volatiles were still able to disperse, it was confirmed that long-range pollinator attraction to *Cryptostylis ovata* flowers is mediated by chemical cues. No wasps approached the screen in the absence of the orchid (as a negative control), and there was no significant difference between the total number of wasps responding to the screened flower (84 responding wasps, 15 trials, 5.6 ±1.2 responses per trial) and the total number of wasps responding to the flower alone (108 responding wasps, 15 trials, 7.2 ±1.5 responses per trial, Mann Whitney U-test, $W = 135.5$, $P = 0.35$). These results are in agreement with the experiment reported in 1930 for the related *C. erecta*, where muslin cloth was used to obscure visual signals.[15]

In preliminary experiments (Supporting Information, Table S1), flowers were extracted using solvents of different polarity, ranging from water to hexanes. These experiments showed that extracts made with polar or semi-polar solvents were significantly more attractive than non-polar hexane extracts. Based on these findings, bioassay guided fractionation was conducted using two separate methods in parallel: solid phase extraction (SPE, C_{18}, from floral extract in water) and semi-preparative GC (from floral extract in MeOH) (Figure 1). Both methods independently led to the isolation of a single pollinator-attracting fraction, which when compared by GC, was shown to contain the same main compound. Since the amount of material obtained in the GC purified fraction was too low for further spectroscopic analysis, semi-preparative HPLC was used to purify the active compound from the bioactive SPE fraction.

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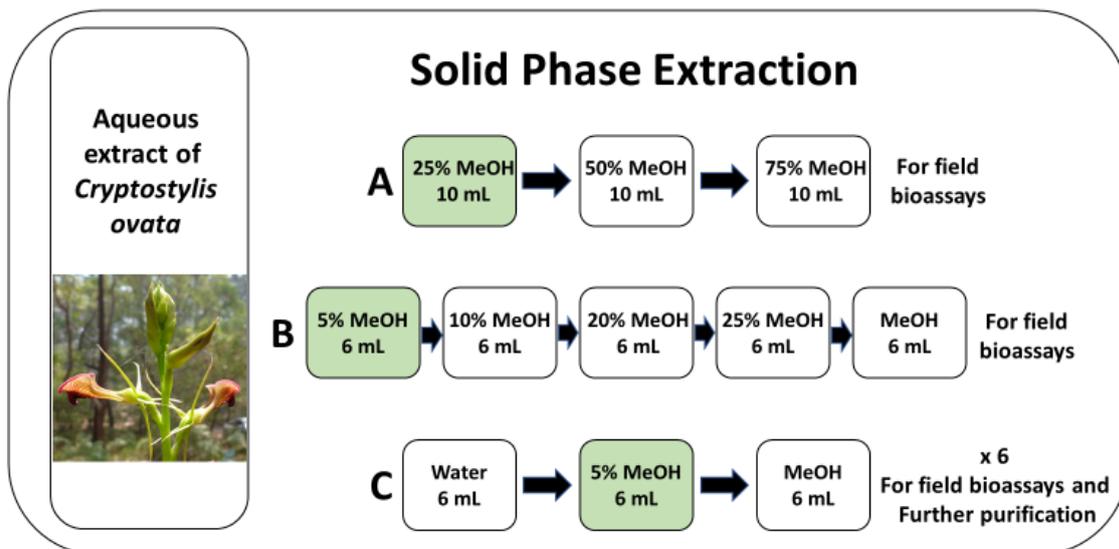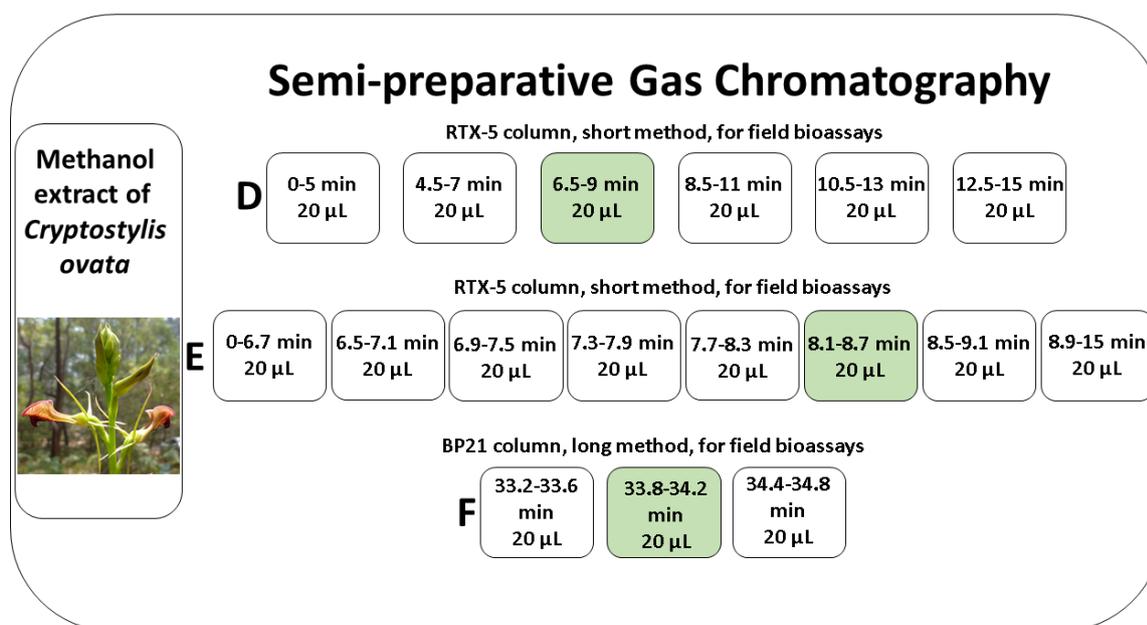
Figure 1. Bioassay guided fractionation of solvent extracts of *Cryptostylis ovata.* Bioactive

fractions are indicated as shaded boxes. Top: SPE-fractionation; protocols A, B, C, eluent

composition (MeOH/water) and volume displayed in boxes. Bottom: Semi-preparative GC;

protocols D, E, F, retention time and solvent extract volume (MeOH) displayed in boxes.

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The GC retention time and mass spectra of the purified compound from semi-preparative HPLC were confirmed to match those of the active compound that was isolated through semi-preparative GC. The active compound was analyzed by HRMS and NMR, including 2D experiments (COSY, HSQC, and HMBC, see Supporting Information, Figure S1-S6). From the HRMS data, the molecular formula was indicated as $C_6H_{10}O_3$, which was supported by the ^{13}C NMR spectrum showing the presence of six unique carbon environments (Supporting Information, Figure S3). A carbonyl signal was observed at δ_C 175, which showed HMBC correlations to a methylene group at δ_H 2.52 and a methine at δ_H 4.25, which were connected due to the observation of a ^1H-^1H COSY correlation (Supporting Information, Figure S6). Further consideration of the remaining alkyl signals, including a second methylene group at δ_C 68.8, suggested a tetrahydrofuran system was present with substitution at C-2. The MS fragments, m/z = 60, and m/z = 112 (M-H_2O, Figure 2), in conjunction with the GC peak shape, and the presence of the compound in aqueous extracts were consistent with a substituted acetic acid assignment. Hence the active compound was tentatively identified as 2-(tetrahydrofuran-2-yl)acetic acid (**1**). Co-injection with the commercially available racemate of **1** confirmed this identification. When presented in the field, the racemic synthetic compound **1** attracted a total of 98 male *Lissopimpla excelsa* wasps to within 5 cm of the pin, across two field experiments of four 2-min trials each.
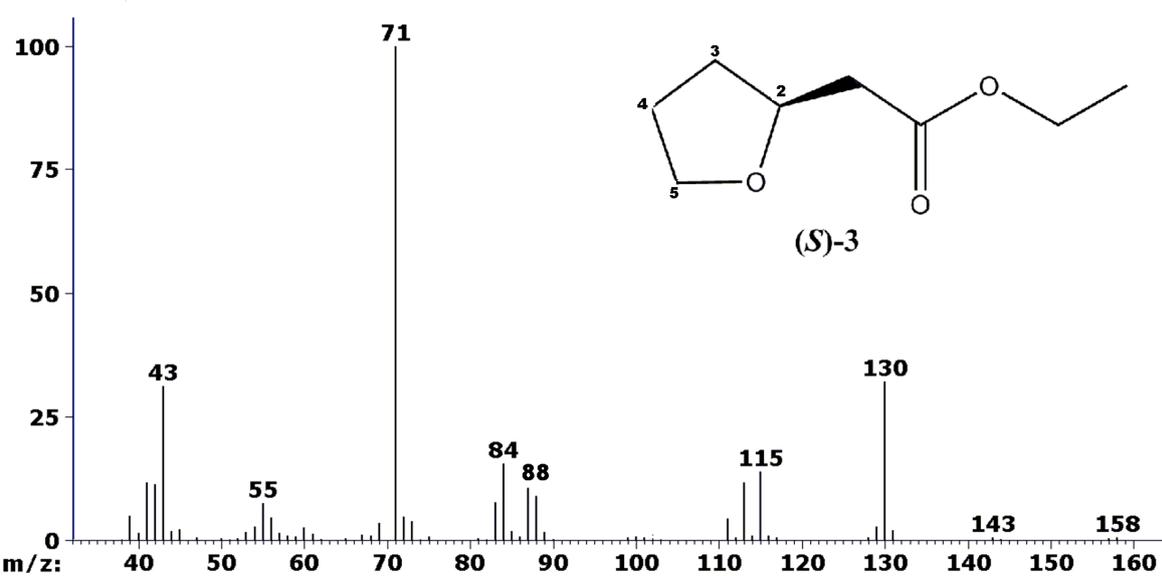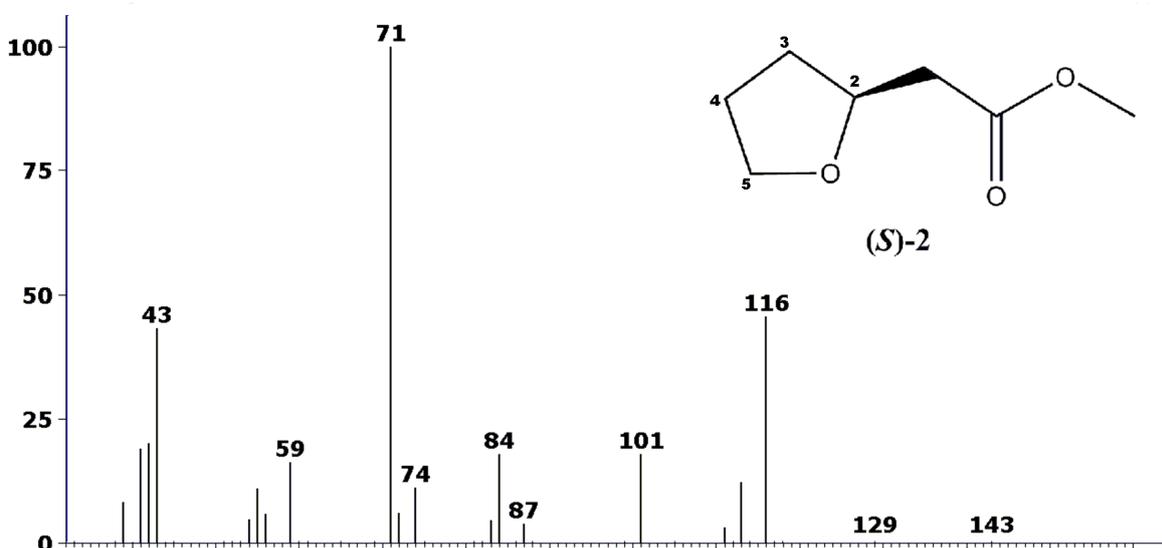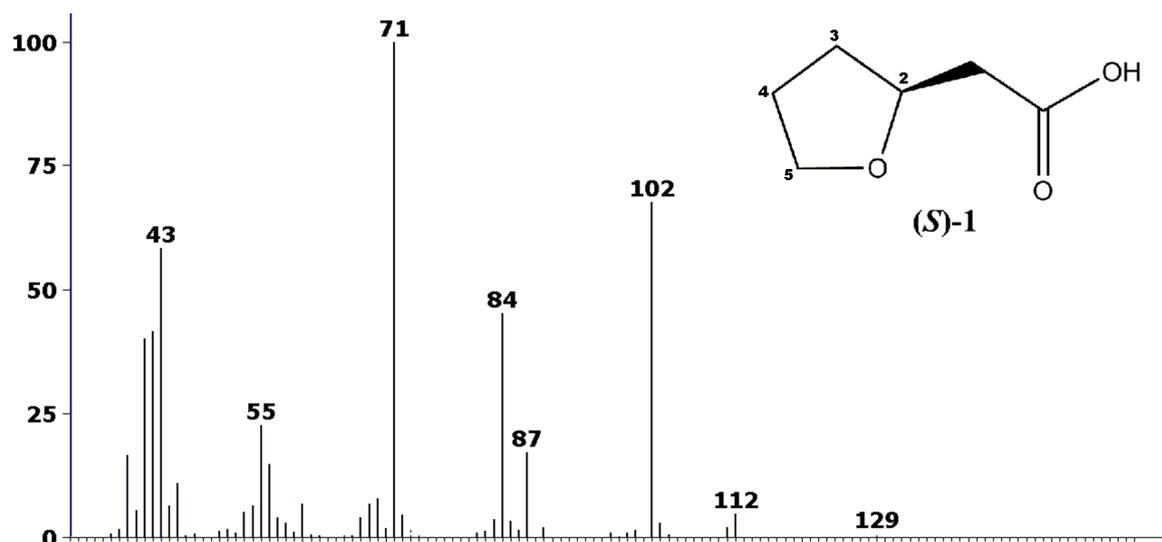
Figure 2. Mass spectra of selected peaks from GC-MS analysis of a methanol extract of *C. ovata*, with the corresponding identified bioactive compounds **1**, **2**, and **3**.

Analysis of floral extracts by chiral-phase GC-MS (Supporting Information, Figure S12) showed only the (*S*)- enantiomer of **1** to be present in *C. ovata* flowers. Field tests of (*R*)-**1** and (*S*)-**1** (separated using enantioselective HPLC) revealed that the naturally occurring (*S*)-**1** was significantly more attractive than (*R*)-**1** (Mann Whitney U-test, W = 100, *p* = 0.0001). Over 10 trials across three days and at two different sites, only three *L. excelsa* males approached (*R*)-**1**, while 53 approaches were observed to the naturally occurring (*S*)-**1** .

It should be noted that while the presentation of (*S*)-**1** across multiple trials of 2 min duration regularly led to the rapid attraction of male *L excelsa* to within 5 cm of the compound, only one individual landed on the pin, and no copulatory behavior (as regularly observed on flowers) was observed. Dose-response experiments (Supporting Information, Table S1) showed that (*S*)-**1** elicited close approaches in amounts from 20 ng to 100 µg, which is in the same range as measured in the floral extracts and applied on the pins in the bioassays.

To further explore the possibility that additional compounds were required to elicit pseudocopulation (a step essential for pollination at the flower), solvent extracts of *C. ovata* were screened for related compounds, as experience from other sexually deceptive orchids and pollinators suggests that it is common for the floral attractants and sex pheromones to contain a series of related active compounds.[2] Indeed, the methyl and ethyl esters **2** and **3** of (*S*)-**1** were found in small amounts when floral extracts (MeOH and CH_2Cl_2) were analyzed in detail. Chiral-phase GC showed that as with **1,** only the (*S*) enantiomers of **2** and **3** were present in the flower. The new semiochemicals (*S*)-**2** and (*S*)-**3** were prepared by Fischer esterification of (*S*)-**1**. The (*S*)-enantiomers of **1**, **2**, and **3** were compared in seven field trials conducted across three days at two sites. Across the trials, at 2 µg, (*S*)-**1** attracted 29, (*S*)-**2** 15, and (*S*)-**3** 25 wasps. There was no significant difference between the mean rank of wasp responses to each compound (Kruskal-Wallis Rank Sum test, *H* = 1.15, df = 2, *p* = 0.56).

While low wasp availability meant that only two combinations could be tested, it is worth noting that in additional trials neither lands nor attempted copulations were observed when combinations of **1**, **2**, and **3** were tested in 50:0:0, 50:50:50, and 50:5:5 (µg). In 10 trials over two days, a total of 63, 64, and 65 wasps were attracted per treatment, with no significant difference between treatments observed (Kruskal-Wallis Rank Sum test, $H = 0.64$, df $= 2$, $p = 0.73$).

Despite the use of various extraction and chromatography methods, none of the isolated fractions from these protocols led to sexual attraction as strong as the whole crude extracts (Supporting Information, Table S1), possibly indicating that some active compounds are lost in the separation process. Furthermore, none of the crude extracts, despite the use of different solvents (water, MeOH, CH_2Cl_2 and hexanes) and doses, were comparable to the flower in attracting *L excelsa*.[10] Additionally, when aliquots of all four extracts were combined on the same pin, no significant enhancement of attraction was achieved (Supporting Information, Table S1). These findings are in contrast to our earlier studies of Australian hammer and spider orchids,[2,23] where using similar methodology we have successfully isolated semiochemicals that induce strong sexual behavior, including frequent attempted copulation at rates similar to that observed with the flowers. For *C. ovata*, while it is clear that we have successfully isolated long-range pollinator attractants, further work is required to elucidate the missing piece of the puzzle - what triggers pseudocopulation in *L. excelsa*.

To rule out the possibility that (*S*)-**2** and (*S*)-**3** were simply artefacts of using MeOH or EtOH as solvents, it was confirmed that extracts prepared using only CH_2Cl_2, without any exposure to alcohols, still contained similar levels of **2** and **3**. Some discrepancies were noted in the spectroscopic data reported for **1**, **2**, and **3** prepared by organic and chemoenzymatic synthesis compared with our data.[41,42] For example, Laxmi and Iyengar reported the ^{1}H NMR spectra for compound **1** and **2**, where in **1** the H-2 proton was reported as δ_H 4.10[41] compared

184 with δ_H 4.23 in this study (both in CDCl$_3$), while the corresponding signal in **2** was in

185 agreement (δ_H 4.24 vs 4.25).[41] Bellur et al. later reported ^{1}H and ^{13}C NMR spectra for **1 - 3**,

186 although neither the NMR nor the MS data are in agreement with this study.[42] For example,

187 in **1**, C-2 was reported at δ_C 75.0 (vs δ_C 76.9) and the protons on the α-carbon to the carbonyl

188 were reported at δ_H 2.58-2.60 (vs δ_H 2.49). For compound **3**, the carbonyl carbon was

189 reported at δ_C 166.7 (vs δ_C 171.3). All EI-MS spectra were fundamentally different to ours,

190 suggesting different compounds. In this study, both enantiomers of **1** were prepared from

191 enantiopure tetrahydrofuran-2-carboxylic acid, where the absolute configuration has been

192 assigned.[43] NMR data of the isolated natural products, purchased *rac*-**1,** and the synthetically

193 prepared products are identical and in full agreement with the most recent studies.[44,45]

194 Despite their structural simplicity, there are only a few examples of oxygenated

195 tetrahydrofuran derivatives as floral volatiles or pheromone components. One example is

196 pityol, which was originally identified from bark beetles[46] and later found to be present in

197 various other beetle species (for example Birgersson, et al.[47] and Pierce, et al.[48]). Additional

198 examples of tetrahydrofuran derivatives are linalool oxides and lilac alcohols/aldehydes,

199 which are known to attract moth[49] and fungus gnat pollinators.[50] Thus far there are no

200 examples of tetrahydrofuran compounds as orchid semiochemicals, hence our discovery adds

201 another compound class to a growing list of semiochemicals used by orchids to achieve

202 pollination by sexual deception.[2]

203 The attraction of male *Lissopimpa excelsa* to the tetrahydrofuran derivatives **1-3** marks

204 the first identification of semiochemicals in the genus *Cryptostylis* and the first identification

205 of floral semiochemicals that attract an ichneumonid wasp. To date, pollinator attractant

206 compounds have only been experimentally confirmed from four other genera of sexually-

207 deceptive orchid; alkenes, cyclohexanediones, and acyclic hydroxy-acids in *Ophrys,*[51-54]

208 chiloglottones in *Chiloglottis,*[4] pyrazines in *Drakaea,*[22,55] and methylthiophenols,

209   acetophenones, and monoterpenes in *Caladenia*.[18,24,56] The discovery of **1-3** as pollinator

210   attractants in *Cryptostylis* highlights the diversity of chemical systems employed by sexually

211   deceptive orchids.

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213   **EXPERIMENTAL SECTION**

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215   **General Experimental Procedures.** Optical rotations were acquired on a Kruss

216   Optronic P-8000 polarimeter. Electronic circular dichroism spectra were recorded on a Jasco

217   J-810 spectropolarimeter (using the collected fractions from the chiral-phase HPLC

218   separation, i.e. in 4% isopropanol/hexanes at ca. 0.5 mg/mL). NMR spectra were acquired on

219   a Bruker Avance 500 MHz or 600 MHz (with a 1.7 mm TXI microprobe) spectrometer with

220   either $CDCl_3$ or methanol-*d*4 as solvent. Chemical shifts were calibrated to resonances

221   attributed to residual solvent signals. HR-MS (EI, 70 eV) were recorded on a Waters GCT

222   Premier TOF-MS equipped with a BPX5 column [(5% phenyl polysilphenylene-siloxane), 30

223   m × 0.25 mm × 0.25 μm film thickness, SGE Australia], using helium as a carrier gas. EI-MS

224   (70 eV) were recorded on an Agilent 5973 mass detector connected to an Agilent 6890 GC

225   also equipped with a BPX5 column (30 m × 0.25 mm × 0.25 μm), or an HP 5972 mass

226   detector connected to an HP5890 GC equipped with a Restek Rt-GammaDex sa column (30

227   m × 0.25 mm × 0.25 μm) using helium as a carrier gas. The scan range was *m/z* 33-300. High

228   performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC

229   system, equipped with a photodiode array detector (PDA) and fraction collector. Solvents for

230   extractions and purifications were of HPLC grade unless otherwise stated.

231   **Plant Materials and Insects.** *Cryptostylis ovata* flowers were sourced from

232   populations in South-West Western Australia near Margaret River (33°58'02.21"S,

233   115°00'58.37"E), Boyanup (33°28'30.9"S 115°45'26.2"E), and Capel (33°35'29.69"S,

234   115°32'31.77"E) in November 2015 - January 2019. Flowers were kept on ice in cooler boxes

235   (ca 4 °C) during transportation to the laboratory where they were extracted either in MeOH or

236   CH_2Cl_2 for semi-preparative gas chromatography, or frozen within 24 h of collection for

237   subsequent liquid chromatography separations. Additional small-scale extracts of three

238   flowers were conducted individually in four solvents (water, MeOH, CH_2Cl_2, and hexanes)

239   for preliminary studies comparing pollinator attraction between solvent extracts. Preparations

240   were presented to *L. excelsa* wasps at two sites in suburban Perth; Mosman Park

241   (32°01'02.3"S 115°45'18.0"E) and Kings Park and Botanic Garden (31°57'44.5"S

242   115°50'18.5"E), where wasps are known to occur in suitable numbers for experiments.[10,57]

243   Bioassays were conducted between 6 am and 10 am to coincide with the period of highest

244   wasp activity.[57] Voucher specimens of *C. ovata* are held at the Western Australian Herbarium

245   (voucher number PERTH 06731481).

246   **Extraction and Isolation.** All bioassay-guided fractionation methods were based on

247   the results from preliminary experiments (Supporting Information, Table S1), showing that *C.*

248   *ovata* extracts in polar and semi-polar solvents were more attractive to *L excelsa* males than

249   non-polar extracts. Two independent methods were implemented in order to maximize the

250   likelihood of discovering multiple semiochemicals. To target polar compounds in the aqueous

251   floral extracts, reverse phase solid phase extraction (SPE) in combination with HPLC was

252   employed. For semi-polar compounds detected in the MeOH extract, semi-preparative gas

253   chromatography was used. Three fractionations (below A, B, & C) of *Cryptostylis ovata*

254   crude water extracts were conducted with a C_{18} solid phase extraction column (Waters Sep-

255   Pak Classic C18, WAT051910 [360 mg, 55-105 μm, SPE]) according to the following

256   procedure: For each SPE column, 15 frozen flowers were defrosted in a 5-mL conical

257   extraction vial, after which they were crushed with a glass rod. The resulting floral extract (ca

258   1 mL) was separated from the floral debris with a pipette and transferred to a new vial. Each

259 column was preconditioned with MeOH (5 mL) followed by water (10 mL). The aqueous

260 floral extract was loaded onto the column, and fractions eluted with a set of solvents of

261 decreasing polarity (Figure 1).

262   For fractionations A and B, all fractions were field tested, while in C sub-samples were

263 field tested and the remains of the active fraction were retained for further purification and

264 instrumental analysis. Each eluted fraction was concentrated to ca 0.5 mL by a gentle stream

265 of nitrogen at room temperature and stored at 4 °C for subsequent analysis or bioassays. For

266 semi-preparative HPLC and subsequent NMR analysis, fractionation C was scaled up to

267 obtain a pooled sample from six columns in parallel.

268   **Semi-preparative Gas Chromatography.** All semi-preparative gas chromatography

269 experiments were performed on an HP 5890 GC, equipped with a three-way glass splitter

270 separating the gas flow post column into the FID and the collector. An Rtx-5 column, 30 m ×

271 0.53 mm id × 5 µm film (Restek, USA) or BP21 column, 30 m × 0.32 mm id × 0.25 µm film

272 (SGE, USA) was used. Samples of 3 µL were injected in splitless mode (1 min) and helium

273 was used as carrier gas. A manual fraction collector was used, with samples collected in glass

274 capillaries (100 x 1.55 mm id, Hirschmann Laborgeräte, Eberstadt, Germany) positioned in

275 an aluminum holder submerged in a dry ice/acetone bath. All fractions were eluted with

276 CH_2Cl_2 or MeOH (as appropriate) and stored at -20 °C until field-tested or further analyzed.

277   In the initial fractionation of the crude MeOH extract (for bioassay methods, see

278 below), a short GC method (1 min 50 °C, then programmed to 280 °C at a rate of 15 °C/min,

279 and held for 3 min) was used with the Rtx-5 column (see above). A sample of 48 flowers was

280 extracted in MeOH (5 mL) for 24 h and the extract was concentrated to 0.5 mL under a gentle

281 stream of nitrogen. Aliquots of this concentrated extract were injected (3 µL) and six

282 fractions, each with 30 s overlap (i.e. two injections per complete set of fractions were

283 performed, allowing overlapping fractions to be collected per pair of runs), were collected to

284 ensure that no bioactive compounds would be lost (Figure 1). The fractions were

285 subsequently eluted with MeOH (20 µL). In total, eight injections (24 µL) were conducted

286 for each set of fractions for field bioassays (i.e. in total 16 injections). The activity within the

287 first fractionation series was confined to the fraction eluting at 6.5 – 9 min. Therefore, this

288 fraction was sub-fractionated to create a further eight 0.6-min fractions. Field tests revealed

289 that the fraction at 8.1-8.7 min retained activity. This fraction contained two distinct peaks,

290 which could not be separated on this column, even with a longer method. However, the two

291 peaks could be separated using the more polar BP21 column (5 min 40 °C, then programmed

292 to 200 °C at a rate of 5 °C/min, then to 230 °C at a rate of 15 °C/min and held for 1 min).

293 Field bioassays confirmed the active compound to be present in the fraction at 33.8-34.2 min,

294 which contained the main peak from the non-polar column. The minor peak from the non-

295 polar column was not active in field bioassays and was discarded.

296 **Semi-preparative HPLC Purification.** The 5% MeOH SPE fraction (**C** Figure 1, 36

297 mL combined) was concentrated to ca. 2 mL under reduced pressure and purified further by

298 semi-preparative HPLC. Separation was achieved using a 250 x 10 mm i.d., 5 µm, Apollo C_{18}

299 reversed phase column (Grace-Davison Discovery Sciences, Melbourne, VIC, Australia) with

300 a 33 mm x 7 mm guard column of the same material. The column was eluted at 4 mL/min

301 with 5% (v/v) MeOH/water increasing to 40% (v/v) MeOH/water over 30 min, and then to

302 100% MeOH at 35 min and held for 5 min. Injection volumes of 500 µL were used (x 4) and

303 UV absorbance was monitored at wavelengths of 220, 254, and 280 nm. Fractions were

304 collected every minute for 40 min and these were monitored by GC-MS for the main active

305 compound isolated by semi-preparative GC. The active compound eluted in the fractions

306 collected between 16-18 mins retention time, which were combined and evaporated to

307 dryness under reduced pressure. This purified sample was sufficiently pure for NMR studies

308 (Supporting Information, Figure S1-S6).

Enantiomer Separation and Determination of Absolute Configuration. As the preparation of **1** from tetrahydrofuran-2-carboxylic acid by Arndt-Eistert homologation (see below) unavoidably resulted in some epimerization, chiral-phase HPLC was used to obtain (*R*)-**1** and (*S*)-**1** in >99% e.e. for field bioassays. Separation of the two enantiomers of **1** was achieved using semi-preparative HPLC with an Astec® Cellulose DMP chiral-phase HPLC column (250 mm × 10 mm × 5 µm, Supelco, Bellefonte, PA, USA). An isocratic solvent mixture of 4% isopropanol/hexanes at a flow rate of 2 mL/min with 200 µL injection volumes of 10 mg/mL **1** (in 1:1 isopropanol/hexanes), provided enantiopure samples of (*R*)-**1** (R_t = 25.5 min) and (*S*)-**1** (R_t = 29.2 min).

The absolute configuration of the natural products was confirmed by preparing (*R*)-**1** and (*S*)-**1** from tetrahydrofuran-2-carboxylic acid[58-60] of known configuration,[43] purchased from Enamine Ltd, Ukraine. The specific rotation of the (*R*) and (*S*)-enantiomers of tetrahydrofuran-2-carboxylic acid, respectively were confirmed beforehand: $[\alpha]^{22}_D$ + 16.0 and – 15.6 ($CHCl_3$) respectively. As the optical rotation of (*R*)-**1** and (*S*)-**1** was weak, and we only had access to limited amounts of these compounds in pure form, electronic circular dichroism (ECD) spectra were recorded rather than optical rotation (Supporting Information, Figure S11).

Chemicals. Racemic **1** was purchased from Princeton Bio (New Jersey, USA) and the enantiomers were separated by chiral-phase HPLC (see above). The methyl- and ethyl esters (*S*)-**2** and (*S*)-**3**, were prepared from (*S*)-**1** on a small scale (ca. 3 mg) by Fischer esterification with MeOH and EtOH respectively.[61] The chemical purity was confirmed to >95 % by GC-MS.

(S)-2-(Tetrahydrofuran-2-yl)acetic acid ((S)-1). ^{1}H NMR (600 MHz) δ 4.23 (m, 1H), 3.85 (m, 1H), 3.73 (m, 1H), 2.49 (m, 2H), 2.10 (m, 1H), 1.93 (m, 2H), 1.58 (m, 1H); [13]C

333   NMR (150 MHz) δ 175.1, 76.9, 68.8, 41.3, 32.2, 26.4; HREIMS found 130.0627 ($C_6H_{10}O_3$

334   calcd. 130.0630).

335   *Methyl (*S*)-2-(tetrahydrofuran-2-yl)acetate ((*S*)-2).* ^{1}H NMR (500 MHz) δ 4.24 (m,

336   1H), 3.87 (m, 1H), 3.75 (m, 1H), 3.69 (s, 3H), 2.59 (m, 1H), 2.48 (m, 1H), 2.08 (m, 1H), 1.90

337   (m, 2H), 1.55 (m, 1H); ^{13}C NMR (125 MHz) δ 171.8, 75.3, 68.0, 51.7, 40.5, 31.3, 25.6;

338   HREIMS found 144.0788 ($C_6H_{10}O_3$ calcd. 144.0786).

339   *Ethyl (*S*)-2-(tetrahydrofuran-2-yl)acetate ((*S*)-3).* ^{1}H NMR (500 MHz) δ 4.24 (m, 1H),

340   4.15 (q, *J*= 7.1 Hz, 2H), 3.87 (m, 1H), 3.74 (m, 1H), 2.58 (m, 1H), 2.45 (m, 1H), 2.08 (m,

341   1H), 1.90 (m, 2H), 1.55 (m, 1H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz) δ 171.3, 75.3,

342   68.0, 60.5, 40.7, 31.2, 25.6, 14.2; HREIMS found 158.0949 ($C_6H_{10}O_3$ calcd. 158.0943).

343

344   **Field Bioassays.** To determine whether long distance pollinator attraction in *C. ovata* is

345   chemically mediated, experiments were conducted with picked flowers hidden from the view

346   of the pollinator. The flowers were concealed by a non-porous black screen, which had a

347   small opening at the top to allow floral volatiles to disperse. The total number of wasp

348   approaches to within 5 cm for each of three treatments (screen alone, flower alone, and

349   flower concealed inside screen) was recorded. Treatments were presented individually in

350   random order for trials of 3 min duration until a total of 15 trials had been completed per

351   treatment. Owing to the data being non-normally distributed (Shapiro-Wilk Normality test, *p*

352   < 0.001), the non-parametric Mann-Whitney U-test was conducted to test for differences in

353   responses between treatments in R v3.4.0 (R Core Team, 2017).[62]

354   The field bioassays using fractions or synthetic compounds broadly followed the

355   experimental 'wasp baiting' bioassay methods of Bohman et al.,[63] with the exception that the

356   standard 4 mm diameter black colored pin head was replaced by a larger 6 × 10 mm red

357   colored map pin to increase similarity with the color and dimension of the *C. ovata* flower

358 and the female wasp. Each baiting trial was conducted at least 10 m from the previous baiting

359 location to renew the pollinator response.[1] For GC-fractions, the solvent (10 µL) was allowed

360 to evaporate on the map pin before fractions were tested in trials of 2 min duration.

361 Experiments tested multiple fractions from SPE or GC, and synthetic (*R*)-**1**, (*S*)-**1**, (*S*)-**2**, and

362 (*S*)-**3**, with each experiment consisting of a series of trials in which a single fraction or a

363 synthetic compound was presented for 2 min, with the test fractions or synthetic compounds

364 presented in random order within each experiment, with the exception of the enantiomeric

365 comparison experiment. In this experiment, where the two enantiomers of **1** were tested, the

366 aim was to test whether the (*R*)-enantiomer was comparable with the naturally occurring (*S*)-

367 enantiomer. Therefore, (*R*)-**1** was presented for 2 min, before being replaced with (*S*)-**1** as the

368 positive control.

369 In a preliminary experiment (Supporting Information, Table S1), flowers (n=3 for each

370 solvent) were extracted in four separate solvents: water, MeOH, CH_2Cl_2, and hexanes. Each

371 set of flowers was extracted in 2 mL of solvent for 24 hours, before the extracts were

372 concentrated to ca 100 µL under a gentle stream of nitrogen at room temperature. For each

373 solvent, 10 µL of each extract were suspended on a pin. In addition to testing the individual

374 solvent extracts, the combination of all four solvents on a single pin was tested (3 µL of each

375 solvent). In total eight trials were conducted over two days.

376 In the experiment evaluating SPE fractions (Figure 1), 10 µL of each fraction (500 µL)

377 from 15 flowers were suspended on a pin (representing ~ a 1/50 flower extract equivalent per

378 pin). In the experiment testing GC fractions, 10 µL of each fraction (20 µL eluted, from 25

379 µL injected of 500 µL extract) from 48 flowers were suspended on a pin (representing ~ one

380 flower extract equivalent per pin).

381 In experiments testing synthetic compounds, doses of 2-50 µg were used (see the

382 results for individual experiments). These doses were based on preliminary dose-response

383 experiences (<mark>Supporting Information, Table S1</mark>), where doses from 0.4 ng to 100 µg were

384 tested, confirming that doses from 20 ng to 100 µg elicited close approaches to the pins.

385   Throughout the study, trials where no responses were observed, were not included in

386 analyses. Across all experiments, neither lands on, nor attempted copulation with the map pin

387 was observed. Therefore, the number of wasp approaches to within 5 cm of the map pin was

388 recorded as our response variable. The outcome of each trial was recorded by the same

389 researcher. The number of wasps attracted to each treatment was compared using Mann

390 Whitney *U*-tests (two treatments) and the Kruskal-Wallis Rank Sum test (three treatments) in

391 Rv3.4.0 as the data were non-normally distributed (Shapiro-Wilk Normality test, $p < 0.01$).

392

393 **ASSOCIATED CONTENT**

394

395 **Supporting Information.**

396   NMR-spectra of **1-3**, <mark>E</mark>CD spectrum of **1,** GC-MS traces of floral extracts and
397 enantiomeric separation of (*R*)-**1** and (*S*)-**1,** and results of additional field bioassays are
398 provided.

399

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412   **Notes**

413   The authors declare no competing financial interest.

414

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416

425   **REFERENCES**

426   (1) Peakall, R. *Funct. Ecol.* **1990,** *4*, 159-167.
427   (2) Bohman, B.; Flematti, G. R.; Barrow, R. A.; Pichersky, E.; Peakall, R. *Curr. Opin. Plant*
428   *Biol.* **2016,** *32*, 37-46.
429   (3) Phillips, R. D.; Faast, R.; Bower, C. C.; Brown, G. R.; Peakall, R. *Aus. J. Bot.* **2009,** *57*,
430   287-306.
431   (4) Peakall, R.; Ebert, D.; Poldy, J.; Barrow, R. A.; Francke, W.; Bower, C. C.; Schiestl, F. P.
432   *New Phytol.* **2010,** *188*, 437-450.
433   (5) Gaskett, A. C. *Biol. Rev.* **2011,** *86*, 33-75.
434   (6) Peakall, R.; Beattie, A.; James, S. *Oecologia* **1987,** *73*, 522-524.
435   (7) Kullenberg, B. *Zoologiska Bidrag från Uppsala* **1961,** *34*, 1-340.
436   (8) Blanco, M. A.; Barboza, G. *Ann. Bot.* **2005,** *95*, 763-772.
437   (9) Phillips, R. D.; Scaccabarozzi, D.; Retter, B. A.; Hayes, C.; Brown, G. R.; Dixon, K. W.;
438   Peakall, R. *Ann. Bot.* **2014,** *113*, 629-641.
439   (10) Weinstein, A. M.; Davis, B. J.; Menz, M. H. M.; Dixon, K. W.; Phillips, R. D. *Biol. J.*
440   *Linn. Soc.* **2016,** *119*, 283-298.
441   (11) Nicholls, W. H. *Victorian Nat.* **1938,** *54*, 182-183.
442   (12) Coleman, E. *Victorian Nat.* **1927,** *44*, 333-340.
443   (13) Coleman, E. *Victorian Nat.* **1930,** *46*, 203-206.
444   (14) Coleman, E. *Victorian Nat.* **1929,** *46*, 62-66.
445   (15) Coleman, E. *Victorian Nat.* **1930,** *46*, 236-238.
446   (16) Coleman, E. *Proc. Entomol. Soc. London* **1931,** *6*, 22-24.
447   (17) Erickson, R., *Orchids of the West*. Paterson Brokensha: Perth, 1951.

(18) Xu, H.; Bohman, B.; Wong, D. C.; Rodriguez-Delgado, C.; Scaffidi, A.; Flematti, G. R.; Phillips, R. D.; Pichersky, E.; Peakall, R. *Curr. Biol.* **2017,** *27*, 1867-1877. e5.

(19) Bohman, B.; Berntsson, B.; Dixon, R. C.; Stewart, C. D.; Barrow, R. A. *Org. Lett.* **2014,** *16*, 2787-2789.

(20) Bohman, B.; Jeffares, L.; Flematti, G. R.; Byrne, L. T.; Skelton, B. W.; Phillips, R. D.; Dixon, K., W; Peakall, R.; Barrow, R. A. *J. Nat. Prod.* **2012,** *75*, 1589-1594.

(21) Bohman, B.; Jeffares, L.; Flematti, G. R.; Phillips, R. D.; Dixon, K. W.; Peakall, R.; Barrow, R. A. *Org. Lett.* **2012,** *14*, 2576-2578.

(22) Bohman, B.; Peakall, R. *Insects* **2014,** *5*, 474-487.

(23) Bohman, B.; Phillips, R. D.; Flematti, G. R.; Barrow, R. A.; Peakall, R. *Angew. Chem. Int. Ed.* **2017,** *129*, 8575-8578.

(24) Bohman, B.; Phillips, R. D.; Flematti, G. R.; Peakall, R. *Fitoterapia* **2018**, *126*, 78-82.

(25) Gaskett, A. C. *Biol. J. Linn. Soc.* **2012,** *106*, 469-481.

(26) Gaskett, A. C.; Herberstein, M. E. *Victorian Nat.* **2006,** *123* (3), 128.

(27) Gaskett, A. C.; Herberstein, M. E. *Naturwissenschaften* **2010,** *97*, 97-102.

(28) Gaskett, A. C.; Winnick, C. G.; Herberstein, M. E. *Am. Nat.* **2008,** *171*, E206-E212.

(29) Wallace, B. *The* Orchadian **1978,** *5*, 168-169.

(30) Whitehead, B. *The Orchadian* **1964,** *1*, 104-104.

(31) Bell, S. A. *Cunninghamia* **2001,** *7*, 195-204.

(32) Schiestl, F. P.; Peakall, R.; Mant, J. G. *Bot. J. Linn. Soc.* **2004,** *144*, 199-205.

(33) Agurell, S.; Granelli, I.; Leander, K.; Lüning, B.; Rosenblom, J. *Acta Chem. Scand. B.* **1974,** *28*, 239-243.

(34) Leander, K.; Lüning, B. *Tetradedron Lett.* **1968,** *9*, 1393-1394.

(35) Leander, K.; Luning, B.; Ruusa, E. *Acta Chem. Scand.* **1969,** *23*, 244-248.

(36) Shu, S.; Jones, R. L. *J. Chem. Ecol.* **1993,** *19*, 2563-2576.

(37) Davies, N.; Madden, J. *J. Chem. Ecol.* **1985,** *11*, 1115-1127.

(38) Robacker, D.; Hendry, L. *J. Chem. Ecol.* **1977,** *3*, 563-577.

(39) Eller, F. J.; Bartelt, R. J.; Jones, R. L.; Kulman, H. M. *J. Chem. Ecol.* **1984,** *10*, 291-300.

(40) Hrabar, M.; Danci, A.; Schaefer, P. W.; Gries, G. *J. Chem. Ecol.* **2012,** *38*, 253-261.

(41) Bellur, E.; Freifeld, I.; Böttcher, D.; Bornscheuer, U. T.; Langer, P. *Tetrahedron* **2006,** *62*, 7132-7139.

(42) Laxmi, Y. R. S.; Iyengar, D. S. *Synthesis* **1996,** *1996*, 594-596.

(43) Červinka, O.; Bajanzulyn, O.; Fábryová, A.; Šačkus, A., *Coll. Czech. Chem. Comm.* **1986,** *51*, 404-407.

(44) Lee, S.; Kaib, P. S.; List, B. *J. Am. Chem. Soc.* **2017,** *139*, 2156-2159.

(45) Maestre, L.; Ozkal, E.; Ayats, C.; Beltrán, Á.; Díaz-Requejo, M. M.; Pérez, P. J.; Pericàs, M. A. *Chem. Sci.* **2015,** *6*, 1510-1515.

(46) Francke, W.; Pan, M.-L.; König, W.; Mori, K.; Puapoomchareon, P.; Heuer, H.; Vité, J. *Naturwissenschaften* **1987,** *74*, 343-345.

(47) Birgersson, G.; Debarr, G. L.; de Groot, P.; Dalusky, M. J.; Pierce, H. D.; Borden, J. H.; Meyer, H.; Francke, W.; Espelie, K. E.; Berisford, C. W. *J. Chem. Ecol.* **1995,** *21*, 143-167.

(48) Pierce, H. D.; de Groot, P.; Borden, J. H.; Ramaswamy, S.; Oehlschlager, A. C. *J. Chem. Ecol.* **1995,** *21* (2), 169-185.

(49) Balao, F.; Herrera, J.; Talavera, S.; Dotterl, S. *Phytochem.* **2011,** *72*, 601-609.

(50) Okamoto, T.; Okuyama, Y.; Goto, R.; Tokoro, M.; Kato, M. *J. Evol. Biol.* **2015,** *28*, 590-600.

(51) Mant, J.; Brändli, C.; Vereecken, N. J.; Schulz, C. M.; Francke, W.; Schiestl, F. P. *J. Chem. Ecol.* **2005,** *31*, 1765-1787.

(52) Schiestl, F. P.; Ayasse, M.; Paulus, H. F.; Löfstedt, C.; Hansson, B. S.; Ibarra, F.; Francke, W. *J. Comp. Physiol. A* **2000,** *186*, 567-574.

498   (53) Schiestl, F. P.; Peakall, R.; Mant, J. G.; Ibarra, F.; Schulz, C.; Franke, S.; Francke, W.
499   *Science* **2003,** *302*, 437-438.

500   (54) Ayasse, M.; Schiestl, F. P.; Paulus, H. F.; Ibarra, F.; Francke, W. *Proc. R. Soc. Lond. B*
501   *Biol. Sci.* **2003,** *270*, 517-522.

502   (55) Bohman, B.; Phillips, R. D.; Menz, M. H. M.; Berntsson, B. W.; Flematti, G. R.;
503   Barrow, R. A.; Dixon, K. W.; Peakall, R. *New Phytol.* **2014,** *203*, 939-952.

504   (56) Bohman, B.; Karton, A.; Flematti, G. R.; Scaffidi, A.; Peakall, R. *J. Chem. Ecol.* **2018**,
505   *44*, 436-443.

506

507   (57) Tomlinson, S.; Phillips, R. D. *J. Zool.* **2012,** *287*, 81-90.

508   (58) Cesar, J.; Sollner Dolenc, M. *Tetrahedron Lett.* **2001,** *42*, 7099-7102.

509   (59) James, C. A.; DeRoy, P.; Duplessis, M.; Edwards, P. J.; Halmos, T.; Minville, J.;
510   Morency, L.; Morin, S.; Simoneau, B.; Tremblay, M.; Bethell, R.; Cordingley, M.; Duan, J.;
511   Lamorte, L.; Pelletier, A.; Rajotte, D.; Salois, P.; Tremblay, S.; Sturino, C. F. *Bioorg. Med.*
512   *Chem. Lett.* **2013,** *23*, 2781-2786.

513   (60) Uyanik, M.; Hayashi, H.; Iwata, H.; Ishihara, K. *Chem. Lett.* **2016,** *45*, 353-355.

514   (61) Bohman, B.; Nordlander, G.; Nordenhem, H.; Sunnerheim, K.; Borg-Karlson, A.-K.;
515   Unelius C.R. *J. Chem. Ecol.* **2008**, *34*, 339-352.

516   (62) R Core Team. R: A language and environment for statistical computing. Vienna,
517   Austria: R Foundation for Statistical Computing; **2017**.

518   (63) Bohman, B.; Karton, A.; Dixon, R. C. M.; Barrow, R. A.; Peakall, R. *J. Chem. Ecol.*
519   **2015,** *42*, 17-23.

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522   **TOC Graphic**