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Tetranorsesquiterpenoids as Attractants of Yucca Moths to Yucca Flowers

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Abstract
The obligate pollination mutualism between *Yucca* and yucca moths is a classical example of coevolution. Oviposition and active pollination by female yucca moths occur at night when *Yucca* flowers are open and strongly scented. Thus, floral volatiles have been suggested as key sensory signals attracting yucca moths to their host plants, but no bioactive compounds have yet been identified. In this study, we showed that both sexes of the pollinator moth *Tegeticula yuccasella* are attracted to the floral scent of the host *Yucca filamentosa*. Chemical analysis of the floral headspace from six *Yucca* species in sections Chaenocarpa and Sarcocarpa revealed a set of novel tetranorsesquiterpenoids putatively derived from (*E*)-4,8-dimethyl-1,3,7-nonatriene. Their structure elucidation was accomplished by NMR analysis of the crude floral scent sample of *Yucca treculeana* along with GC/MS analysis and confirmed by total synthesis. Since all these volatiles are included in the floral scent of *Y. filamentosa*, which has been an important model species for understanding the pollination mutualism, we name these compounds filamentolide, filamentol, filamental, and filamentone. Several of these compounds elicited antennal responses in pollinating (*Tegeticula*) and non-pollinating (*Prodoxus*) moth species upon stimulation in electrophysiological recordings. In addition, synthetic (*Z*)-filamentolide attracted significant numbers of both sexes of two associated *Prodoxus* species in a field trapping experiment. Highly specialized insect-plant interactions, such as obligate pollination mutualisms, are predicted to be maintained through "private channels" dictated by specific compounds. The identification of novel bioactive tetranorsesquiterpenoids is a first step in testing such a hypothesis in the *Yucca*-yucca moth interaction.

Keywords *Yucca* · Floral scent · Tetranorsesquiterpenoids · DMNT-derivatives · Structure elucidation · Total synthesis · Pollinator attraction

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Introduction

Since its discovery by Engelmann almost 150 years ago (Engelmann 1872), the obligate nursery pollination mutualism between *Yucca* (Asparagaceae) plants and *Tegeticula* and *Parategeticula* (Prodoxidae) moths has served as an important model system for understanding coevolution. Even Darwin was fascinated by this plant–insect interaction, which he considered 'the most remarkable example of fertilisation ever published' (Burkhardt and Smith 1994), as this mutualism was the first documented case of active pollination. Phylogenetic analyses suggest the overall mutualism between yuccas and yucca moths has persisted for at least 4 million years (Smith et al. 2008; 2021) and potentially for as many as 40 million years (Pellmyr and Leebens-Mack 1999). To pollinate the flower, the female yucca moth uses uniquely derived mouthparts called maxillary tentacles to gather pollen from host flowers (Pellmyr and Krenn 2002) (Fig. 1). She deposits eggs into the pistil of a host flower and then actively pollinates the flower by pushing pollen into the cup-shaped stigma, thereby securing developing seeds as a food source for her offspring. Because the larvae only consume a fraction of the available seeds, the plant gains reproductive success by interacting with these specialized, active pollinators. Active pollination is exceptionally rare in nature and has only been documented in five other plant–insect associations (Fleming and Holland 1998; Weiblen 2002; Kawakita 2010; Nunes et al. 2018; Milla 2019).

In addition to the pollinating yucca moths, the bogus yucca moths (genus *Prodoxus*) are also specialists on *Yucca*. Rather than pollinating the flowers, they instead lay their eggs within the plant tissue and their offspring consume plant parts other than seeds (Davis 1967; Pellmyr et al. 2006). Because *Prodoxus* are closely related to the pollinating yucca moths but have a parasitic relationship with *Yucca* plants, they have been used extensively to understand how mutualism and antagonism drive the processes underlying the formation of new species. During the last three decades, considerable progress has been made in elucidating the species richness, phylogenetic relationships, functional adaptations, and drivers of co-diversification in yucca plants and associated moths (Althoff et al. 2012; Godsoe et al. 2008; Pellmyr 1999; Pellmyr and Balcázar-Lara 2000; Pellmyr and Krenn 2002; Pellmyr and Leebens-Mack 1999; Pellmyr and Segraves 2003; Pellmyr et al. 2006; 2007) as well as mechanisms for stabilizing the plant–insect interaction (Huth and Pellmyr 1999; Pellmyr and Huth 1994; Segraves 2003; Marr and Pellmyr 2003).

One aspect of the *Yucca*-yucca moth interactions that has received little attention is the suite of sensory signals mediating attraction of both the pollinating and non-pollinating moths to their host plants. Mating, pollination, and oviposition of eggs by yucca moths typically occur at night, within flowers, when the flowers are open and fragrant, suggesting that floral scent may play a key role in host and mate location by the moths (Svensson et al. 2005), as has been reported for both fig wasps and *Epicephala* moths (Borges et al. 2008; Svensson et al. 2010). To the human nose, floral fragrance

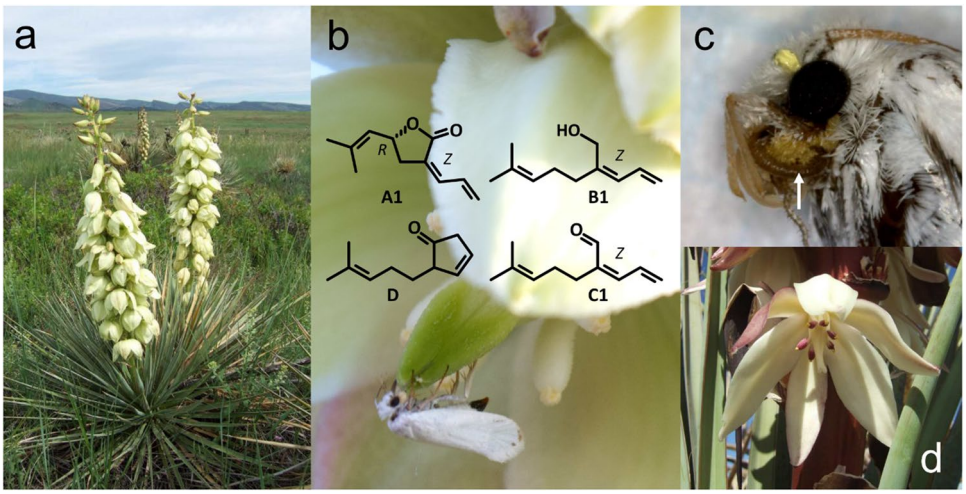

Fig. 1 Flowering *Yucca glauca*, Boulder Valley Ranch Trailhead (CO), June 2009 (**a**); female *Tegeticula yuccasella* pollinating *Y. glauca* flower with (*Z*)-filamentolide (**A1**), (*Z*)-filamentol (**B1**), (*Z*)-filamental (**C1**), and filamentone (**D**), all present in the floral scent of this host, Boulder Valley Ranch Trailhead (CO), June 2009 (**b**); female *Tegeticula mexicana* with yellow *Yucca treculeana* pollen collected using specialized mouthparts (arrow), Big Bend NP (TX), March 2013 (**c**); *Y. treculeana* flower, Big Bend NP (TX), March 2013 (**d**). Photos a, c, and d by Glenn P. Svensson, and photo b by Gabi Louisedotter

differs greatly between yucca species. Most species produce a pleasant soap-like scent, whereas the strong musky, over-ripe bleu cheese odor of Joshua trees (*Yucca brevifolia*, sensu lato) was described by Trelease as 'so oppressive as to render the flowers intolerable in a room' (Trelease 1893). So far, detailed analyses of the yucca odor bouquet have been restricted to three closely related but allopatric species within the capsular fruited section Chaenocarpa. These species emit the same set of volatiles with little intra- and interspecific variation (Gäbler et al 1991; Svensson et al. 2005; 2006; 2011), but their volatile compounds have not been previously described in floral bouquets and could not be identified solely by interpretation of their mass spectra. Subsequent studies of yucca floral scent added two species of the spongy fruited Joshua tree (*Yucca* section Clistocarpa), whose floral scent profiles combine C_8 alcohols and ketones (e.g. 1-octen-3-ol, 3-octanone) with the novel yucca volatiles described from section Chaenocarpa (Svensson et al. 2016). Finally, the most recent study identified the chain lengths and double bond configurations for unsaturated hydrocarbons common to many *Yucca* species by focusing on a single species (*Yucca reverchonii*) in Chaenocarpa series Rupicolae, whose floral scent was largely limited to medium chain length hydrocarbons (Tröger et al. 2019).

Binary choice behavioral assays provided the key evidence of attraction by a pollinator yucca moth species, *Tegeticula yuccasella*, to the scent of *Yucca glauca* host flowers in the absence of other stimuli (Svensson et al. 2011), justifying closer analysis of yucca volatiles and their behavioral importance in active pollination. Here, we combine chemical analysis, organic synthesis and electroantennographic and behavioral assays, to identify the volatile attractants and explore specific differences in the floral scent composition of additional *Yucca* species. Our findings suggest an important behavioral role for novel tetranorsesquiterpenoid floral volatiles, presumably derived from (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), in mediating the interaction between yucca moths and their host plants.

Methods and Materials

Biological Analysis of Floral Volatiles

Collection of Yucca floral scent

Floral headspace samples for chemical and electrophysiological analyses were collected from six *Yucca* species (Table S1) covering sections Sarcocarpa (fleshy fruited yuccas) and Chaenocarpa including series Rupicolae. All collections were performed on intact inflorescences during peak flowering and from native plant populations for most samples. Collections from *Yucca pallida* and *Y. reverchonii* were performed on plants transplanted from natural populations and growing in a common garden environment at Syracuse University, Onondaga Co., NY (Althoff et al. 2014). Scent collection was performed between 20:00 h and midnight, coinciding with the maximum release of floral scent and peak activity of the associated yucca moths. To collect the headspace samples, we enclosed an inflorescence in a Reynolds® nylon resin oven bag, and connected a glass cartridge (7 mm i.d.) filled with 100 mg of Super Q adsorbent (Alltech Associates, Waukeegan, IL, USA) to the bag. For collection, a PAS-500 personal air sampler was used (Supelco, Bellefonte, PA, USA) with the flow rate set to 200 ml min^{-1}. We used empty oven bags as controls to check for possible background contaminants. After collection, the filter was eluted with 3 ml of GC/MS purity *n*-hexane and the sample was stored at -18 °C. Before chemical analysis, scent samples were concentrated to 75 µl under a gentle flow of N_2.

Moths

GC/EAD Electrophysiological Assays Most adult yucca moths used for electrophysiological screening were collected from host flowers in the field and used within a week (Table S2). The only exception was *T. cassandra*, for which some adults were obtained from a rearing experiment (Segraves 2003) where larvae had been placed into cylindrical metal cans filled with soil and buried in the ground at ≈ 60 cm depth at Lake Placid, FL, during the previous year. These cans (19 cm × 16 cm) had the top and bottom cut out and were covered with fine screening. All moths used in the GC/EAD analyses were stored in the refrigerator (4 °C) and were kept individually in glass vials with moist paper toweling until used.

Four-Way Olfactometer Behavioral Assays We collected adult *T. yuccasella* moths from *Yucca filamentosa* flowers during the early afternoon from three small populations across Ithaca, Tompkins Co., NY, July 5–15, 2019. Moths were collected singly in 25 ml glass scintillation vials, transported to the laboratory in a cooler with ice, and the vials were then capped with nylon mesh and held within plastic boxes humidified with wet paper towels. Captive moths were stored within a shaded laboratory room at 22 °C under a natural photoperiod until bioassays began during the evening of capture. We allowed the moths to acclimate for 15 min within a dark laboratory room after sunset (20:00 h) as the olfactometer was prepared for bioassays. After concluding the experiments, all moths were euthanized at -20 °C, pinned, and identified to sex. Females with pollen balls beneath their heads were assumed to have mated. Voucher specimens have been deposited in the Cornell University Insect Collection (accessions CUIC000002359-CUIC000002431).

Olfactometer Bioassays

Choice bioassays were conducted at Cornell University, USA, using a four-arm olfactometer with an air flow delivery system (OLFM-4C-ADS + V; Analytical Research Systems, Inc., Gainesville, FL, USA). Laminar flow of humidified air divides the arena into four sectors that converge upon a central well, from which the test subject enters the arena (Vet et al. 1983). Air flow was adjusted to 300 ml min^{-1} through all four arms and was evacuated through the loading well by a vacuum pump set to 1.2 l min^{-1}. The olfactometer was enclosed within a booth (90 × 90 × 115 cm) hung with dark cloth curtains that effectively eliminated ambient light from the surrounding laboratory (0.01–0.04 lx, as measured by a LX1102 light meter (Reed Instruments, Wilmington, NC). Incoming "house" air was pumped through the delivery system through water bubblers to standardize humidity and was evacuated from the olfactometer through the loading well and into an adjacent fume hood. An HM141 humidity and temperature indicator fitted with an HMP42 probe (Vaisala Corporation, Helsinki, Finland) was used to monitor relative humidity (RH) at different positions in the olfactometer, using a modified Plexiglas lid with six evenly spaced (20 mm) holes (4 mm d) bored from the center to each of four arms. A mean ± s.e. of 81.3 ± 0.1% RH was measured across the olfactometer and was consistent across all arms through 30 min of repeated measures.

Test Stimuli Flowers of *Y. filamentosa* growing in Ithaca, NY, provided olfactory stimuli for choice bioassays. Three freshly cut flowers were placed into the glass receptacle (350 ml volume) of one olfactometer arm, leaving the remaining three arms empty. Up to 12 moths were assayed each night, due to the narrow time window (90 min) of behavioral activity for *T. yuccasella* (Svensson et al. 2011) after sunset. The glass receptacle holding yucca flowers was rotated to a new position in the olfactometer after three moths had been assayed, and the Plexiglas lid was flipped or replaced. Spot checks of floral volatiles emitted by test flowers during the time period of the bioassays (using GC/MS) revealed a suite of scent compounds consistent with headspace volatiles from *Y. filamentosa* inflorescences.

Bioassays Moths were chosen randomly for bioassays based on their activity levels after dark acclimation. Only moths that walked vigorously within the holding vials were used for assays. Active moths were placed individually into the loading well of the olfactometer by inverting the holding vial over the well and tapping lightly. Each moth was given 5 min to enter the arena from the loading well and moths were removed if they did not respond. Moths entering the arena were given an additional 10 min to respond to test stimuli. Bioassays were terminated either when a moth entered one of the four ports or when the additional time had expired without a decision. In total, 24 female and 22 male *T. yuccasella* moths were used for bioassays. All moths were filmed under infrared illumination using a Panasonic, Inc. CCTV surveillance video camera. After completion of the bioassays each evening, the olfactometer was disassembled and washed with hot water, with plastic components air-dried and glassware baked at 80 °C in an odor-free oven.

Statistical Analyses Olfactometer assays with yucca moths resulted in three distinct outcomes: 1) remaining within the loading well for the duration of the assay, 2) emerging from the well and selecting an unscented (control) arm, or 3) emerging from the well and selecting the flower-scented arm. Assays resulting in outcomes 2 or 3 ended when a moth entered the terminal port of the selected olfactometer arm. Use of the four-way olfactometer for testing responses to an odorant (one scented arm vs. three unscented/control arms) adds statistical power in the case of modest sample sizes because the expected null response to the scented arm is 0.25 instead of 0.5 (Vet et al. 1983). Yucca moth responses were evaluated using a binomial test for females ($N = 24$), males ($N = 22$), and all moths combined ($N = 46$). Potential bias for a specific arm of the olfactometer was evaluated using X^2-statistics.

Electrophysiological Analysis

Coupled gas chromatography-flame ionization detection-electroantennographic detection (GC/FID-EAD) was used to identify which floral volatiles in headspace samples of *Y. filamentosa* and *Yucca treculeana* elicited antennal responses in associated *Tegeticula* and *Prodoxus* species. For all species, both sexes of moths were included in the analyses.

In 2004, floral headspace of *Y. filamentosa* was presented to the antennae of two pollinating moth species (*T. cassandra* and *T. yuccasella*) and a bogus yucca moth (*P. decipiens*) that use this plant as a host. To do this, we used a Shimadzu GC-17A gas chromatograph equipped with a non-polar DB-5 GC column, 30 m × 0.32 mm i.d. × 1 μm film thickness (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas at a velocity of 43 cm sec^{-1}, and the injector temperature was 270 °C. The oven temperature was programmed for 50 °C for 2 min after injection and then increased at 10 °C min^{-1} to 275 °C. A glass Y connector (J&W Scientific) at the end of the column allowed a 1:1 division of the GC effluent to the FID and to the antennal preparation. A moth antenna was cut at the base and at the tip, mounted between two glass electrodes filled with saline solution, and the preparation was placed 1 cm from the glass tube of the GC outlet. The air stream through the glass tube was charcoal-filtered and humidified before reaching the antenna. The GC effluent presented to

the antenna passed through a heated transfer line (Syntech, Kirchzarten, Germany) set at 275 °C. Data were analyzed using Shimadzu Class-VP version 7.2.1 software.

In 2009, floral headspace of *Y. treculeana* was presented to the antennae of a pollinating yucca moth (*Tegeticula mexicana*) and a bogus yucca moth (*Prodoxus tamaulipellus*) that use this plant as a host. An Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a polar HP-INNOWax column, 30 m × 0.25 mm i.d. × 0.25 µm film thickness (J&W Scientific) was used. Hydrogen was used as the carrier gas with a flow rate of 1 ml min^{-1}, and the injector temperature was 250 °C. The oven temperature was maintained at 60 °C for 1 min after injection, then increased by 5 °C min^{-1} or 10 °C min^{-1} to 220 °C, with a final hold of 10 min at this temperature. The column effluent was split 1:1 between the FID and the antennal preparation. The tip of the moth antenna was cut off, and the head with both antennae was mounted to a PRG-2 EAG probe (10 × gain) (Syntech, Kirchzarten, Germany) using Blågel conductive gel (Cefar, Malmö, Sweden). The column effluent passed through a heated transfer line set at 255 °C and was mixed with charcoal-filtered and humidified air before reaching the antennal preparation, which was placed 1 cm from the glass tube outlet. Simultaneous FID and EAD signals were recorded using the GC/EAD Pro Version 4.1 software (Syntech, Kirchzarten, Germany).

Field Trapping Experiments

The attractiveness of one of the novel floral volatiles, which we have named "filamentolide" (see 12), was tested in a field trapping experiment within a flowering population of the source species (*Y. treculeana*) at Laguna Atascosa National Wildlife Refuge, TX, March 12–16, 2012. All three yucca moth species using *Y. treculeana* as a host (pollinating *T. mexicana*, and bogus yucca moths *Prodoxus atascosanellus* and *P. tamaulipellus*) are known to occur at the study site (Pellmyr, 1999; Pellmyr et al., 2006). White plastic delta-traps (Pherotech, Delta, BC, Canada) with sticky inserts were hung on wooden poles at 1.5 m height and placed at least 5 m apart. To produce a dispenser for the synthetic compound, we mixed 10 mg of neat (*Z*)-filamentolide (**A1**) dissolved in 1 ml of *n*-pentane with 10 ml of SPLAT emulsion (Specialized Pheromone and Lure Application Technology, ISCA Technology, Riverside, CA, USA), and then placed 0.25–0.30 ml of the resulting mixture in a line across the sticky insert of a trap. Fresh dispensers were used each night except for the final night, when the previous night's lures were reused because no more SPLAT mixture was available. Five trap replicates were used in a randomized block design with the following treatments: (i) twelve freshly collected and strongly scented *Y. treculeana* flowers, (ii) (*Z*)-filamentolide (**A1**) in SPLAT, and (iii) blank control. Traps were baited between 20:00 and 21:00 h. Traps were checked and moth catches recorded the following morning. All trapped moths were removed from the glue and pinned for determination of species and sex. For each species, trap catch data were pooled for each trap and log(x + 1)-transformed before using one-way ANOVA, followed by multiple comparisons adjusted according to the Bonferroni *post-hoc* test, to compare catches among treatments.

Chemical Analysis of Floral Volatiles

GC/EI-mass spectrometry

Headspace samples from yucca flowers were analyzed by using a 7890A gas chromatograph coupled to a quadrupole mass spectrometer 5975C inert XL MSD (both Agilent Technologies, Santa Clara, CA, USA) operated at 70 eV electron impact ionization (EI), helium as the carrier gas at a velocity of 25 cm min^{-1}, and un injector temperature of 250 °C, or a Fisons GC 8060 linked to an MD 800 quadrupole mass spectrometer (Fisons Instruments, Mainz-Kastel, Germany) using 70 eV EI, helium carrier gas, and an injector temperature of 250 °C. Separation of volatiles was achieved using fused silica capillary columns with coatings of different polarities applying the following conditions: 1) HP-5 ms 50 m × 0.25 mm i.d. × 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA), splitless injection (30 s), temperature program: 50 °C for 3 min, heating to 80 °C at 3 °C min^{-1}, then 5 °C min^{-1} to 150 °C and 7.5 °C min^{-1} to 300 °C; 2) VF-WAXms, 60 m × 0.25 mm i.d. × 0.25 µm film thickness (J&W Scientific): 50 °C for 3 min, ramped to 80 °C at 5 °C min^{-1}, to 180 °C or to 250 °C at 8 °C min^{-1}; 3) VF-1 ms, 30 m × 0.25 mm i.d. × 0.25 µm film thickness (J&W Scientific): 60 °C for 5 min, then 10 °C min^{-1} to 310 °C. GC/MS data were analyzed using ChemStation E.02.02.1431 (Agilent) or MassLynx 3.2 (Micromass Ltd., Manchester, UK) software respectively.

GC/High Resolution Mass Spectrometry

High resolution GC/MS analyses (GC/HR-EI-MS) were carried out on a 6890 gas chromatograph (Agilent) equipped with an Optic 3-PTV injector (ATAS GL International B.V., Veldhoven, NL) coupled to a GCT TOF mass spectrometer (Micromass Ltd., Manchester, UK). Separations were achieved using an EC-5 ms capillary column, 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Alltech Associates, Inc., Deerfield, IL, USA) and helium as the carrier gas at a constant flow of 1 ml min^{-1} and an injector temperature of 260 °C. The oven was programmed from 50 °C to 120 °C at 10 °C min^{-1}, then to 190 °C at 3 °C min^{-1} and

finally to 275 °C at 30 °C min^{-1}. For HR-MS measurements perfluorotributylamine served as the internal reference.

Enantioselective Gas Chromatography

Enantioselective gas chromatography was performed using an AS GC800 gas chromatograph (Fisons Instruments, Manchester, UK) equipped with a flame ionization detector. Hydrogen served as the carrier gas. The injector temperature was 250 °C. A fused silica capillary column, coated with *heptakis*-(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin, 25 m × 0.25 mm i.d. × 0.25 µm film thickness (FS-Hydrodex® β-6TBDM, Macherey–Nagel, Düren, Germany), was used as the chiral selector. The oven temperature was held at 120 °C for 30 min and then programmed to 150 °C at 10 °C min^{-1}.

GC/Fourier Transform Infrared Spectroscopy

GC/FT-IR analyses were performed using a Shimadzu GC 2010 coupled to a DiscovIR infrared detector (4000–750 cm^{-1}, 8 cm^{-1} resolution; Spectra Analysis, Marlborough, MS, USA). Compounds were separated using an RTX-5 capillary column, 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Restek Bellefonte, PA, USA). The initial temperature was 50 °C for 1 min, increasing to 250 °C at 7 °C min^{-1}, and held at this temperature for 10 min. Helium was used as carrier gas, and the injector was operated in splitless mode at 250 °C.

Chemicals

The total syntheses of filamentolides **A1** and **A2**, filamentols **B1** and **B2**, filamentals **C1** and **C2**, filamentone **D**, and reference standards **12**–**15** used in the attempted identification of unknown **E1** are described in detail in the online Supplementary Information.

Results

Yucca Moths Respond to their Host's Floral Scent

Choice bioassays were performed with a four-arm olfactometer to evaluate the responses of a yucca pollinator to the floral scent of its host. Of the 46 *T. yuccasella* moths tested, 63% ($N = 29$) emerged from the holding well into the test arena, and 21 of these 29 moths (72%) selected the arm containing floral scent of *Y. filamentosa* (binomial test, $P < 0.001$; Fig. 2). When considered by sex, half of the 22 males entered the test arena, and 7 of these 11 males selected the arm with yucca floral scent ($P < 0.01$). Three quarters of the 24 females entered the test arena, and 14 of these 18 females selected the scented arm ($P < 0.001$). Responses of females were not strongly biased by the presence of a pollen ball (remaining in well [3 with, 3 without pollen]; choice of control [1 with, 3 without pollen]; choice of scented arm [8 with, 6 without pollen]). After leaving the well, the mean time to decision for females was 1:58 ± 0:33 min to the scented arm ($N = 14$) and 3:27 ± 0:53 min to an empty arm ($N = 4$), whereas males took 2:27 ± 0:40 min to select the scented arm ($N = 7$) and 0:39 ± 0:11 min to select an empty arm ($N = 4$). No significant bias was observed to any arm of the olfactometer, either in the choice of the scented arm (X^2 [3, $N = 21$] = 0.91, $P = 0.83$) or in all combined choices (X^2 [3, $N = 29$] = 1.61, $P = 0.66$). Both adult female and male yucca moths are attracted to the scent of yucca flowers in the absence of visual, tactile, or contact chemoreceptive cues, thus making floral scent a key attractant mediating their obligate mutualistic relationship. These behavioral results, combined with those of earlier Y-tube experiments using the same moth species (*T. yuccasella*) in response to a nearly identical floral scent blend from *Y. glauca* (Svensson et al. 2011), justified detailed characterization of yucca floral scent using chemical analysis and organic synthesis, electroantennography, and field trapping with specific compounds.

Fig. 2 Behavioral responses of *Tegeticula yuccasella* moths to floral scent from *Yucca filamentosa* in four-arm olfactometer assays. Responses indicate moths having entered either the scented or the unscented (control) arms of the olfactometer during a 5 min assay, and n indicates sample size including non-responsive moths (Binomial test: ** $P < 0.01$; *** $P < 0.001$)

Identification of *Yucca* Floral Volatiles

Chemical composition of the floral scent from six *Yucca* species (*Y. filamentosa*, *Y. glauca*, *Y. pallida, Y. reverchonii, Y. schidigera*, and *Y. treculeana*) was compared using GC/MS techniques to reveal a diversity of volatile components, the majority of which were assigned to two groups, acetogenin hydrocarbons, and terpenoids. The comparative GC/MS-analysis of floral headspace samples of the four Chaenocarpa species *Y. filamentosa*, *Y. glauca*, *Y. pallida,* and *Y. reverchonii* is shown in Fig. 3.

The identification of a variety of homologous medium chain length acetogenin hydrocarbons, which comprised the majority of floral fragrance components in *Y. reverchonii*, has been described previously (Tröger et al. 2019). Similar to *Y. reverchonii*, the headspace samples of *Y. filamentosa*, *Y. glauca,* and *Y. pallida* contained homologous series of unbranched alkanes and alkenes, predominantly from C_{15} to C_{21}, with those of 17 and 19 carbon atoms as the most abundant representatives. In addition to the monoenes, dienes with chain lengths of 17 and 19 carbons were detected. For *Y. glauca* the double bond positions were determined by DMDS derivatization (Dunkelblum et al. 1985; Francis and Veland 1981) of the headspace samples and subsequent GC/MS analysis as described previously (Tröger et al. 2019). This method also enabled the identification of additional positional isomers (8-hexadecene, 7-heptadecene, and 8-octadecene) as trace components (Table S1). The respective configurations were annotated for the main acetogenin compounds, (*Z*)-7-hexadecene (7*Z*-C_{16}ene), (6*Z*,9*Z*)-6,9-heptadecadiene (6*Z*,9*Z*-C_{17}diene), (*Z*)-8-heptadecene (8*Z*-C_{17}ene), (*Z*)-9-octadecene (9*Z*-C_{18}ene), and (*Z*)-9-nonadecene (9*Z*-C_{19}ene) (Fig. 3) by GC/MS comparison with synthetic reference samples. These medium chain length hydrocarbons are also present in the floral headspace of other yucca species, including species in *Yucca* section Chaenocarpa (*Y. filamentosa*, Svensson et al. 2005; *Yucca elata*, Svensson et al. 2006; *Y. glauca*, Svensson et al. 2011) and Clistocarpa (*Yucca brevifolia* and *Yucca jaegeriana*, Svensson et al. 2016).

In addition, a second class of compounds was detected whose mass spectra indicated a terpenoid origin with a C_{11}-skeleton. Moreover, GC/MS analysis suggested the presence of isomeric pairs and GC/HR-MS confirmed that all of

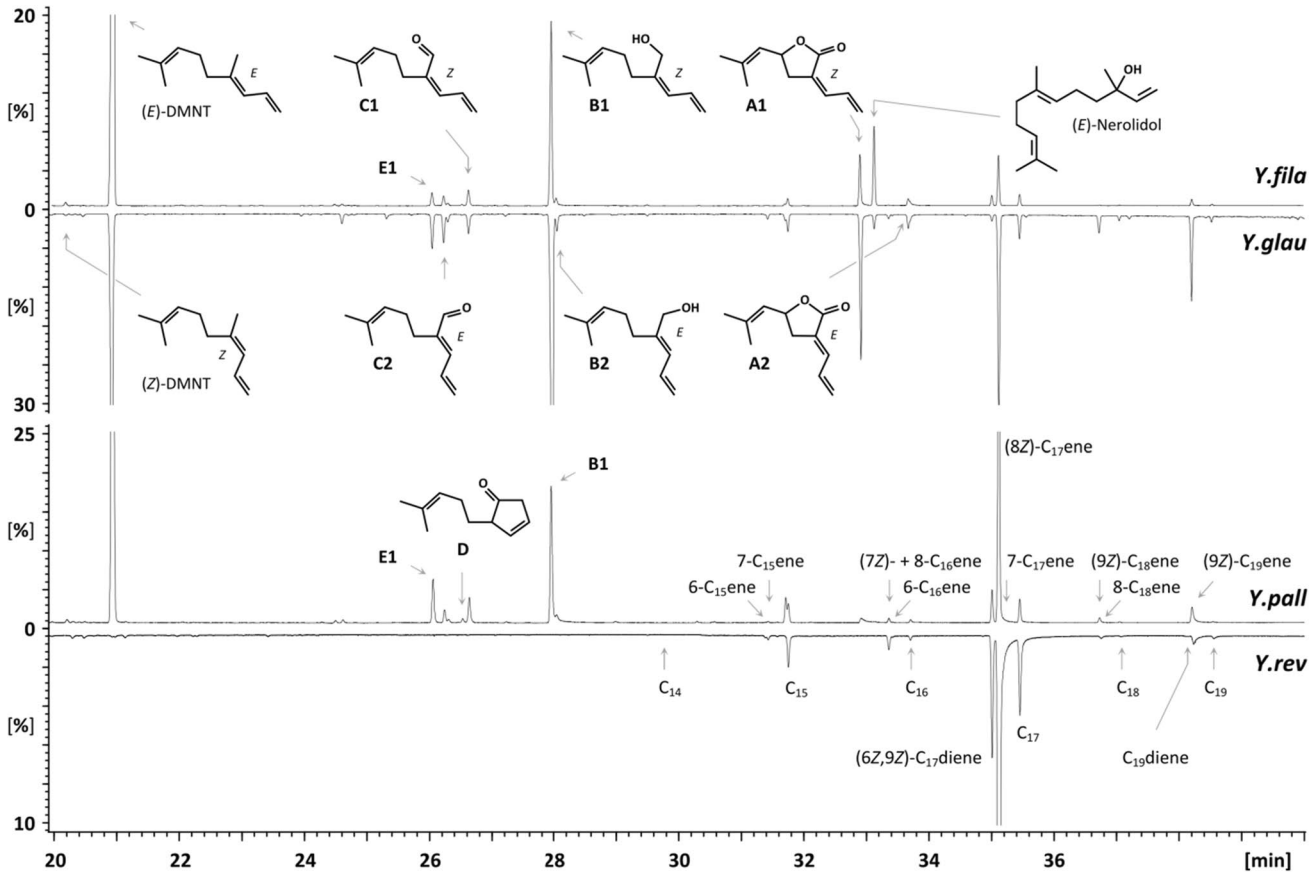

Fig. 3 Comparative GC/MS-analysis of floral headspace samples of the four Chaenocarpa species *Yucca filamentosa*, *Yucca glauca*, *Yucca pallida,* and *Yucca reverchonii*. Conditions: HP-5 ms × 60 m × 0.25 mm i.d., 0.25 µm film; temperature program 50/3–3-80–5-150–7-300/8

them were oxygenated C_{11}-compounds, whose EI-MS fragmentation patterns were indicative of putative derivatives of the C_{11}-tetranorsesquiterpene DMNT, a major component of the *Yucca* volatiles. While absent in *Y. reverchonii*, these C_{11}-terpenoids were abundant in *Y. filamentosa*, *Y. glauca,* and *Y. pallida* volatiles (Fig. 3 and Table 1). In contrast to the complex mixtures of these species (a detailed analysis of *Y. filamentosa* is provided in Fig. S1) the floral scent of *Y. treculeana* lacked the medium chain length hydrocarbons and was dominated by one of the unknown C_{11}-terpenoids, the dioxygenated **A1** (M^+ 178, BP 66), constituting about 85% of the total volatiles (along with 15% of a putative isomer **A2**) (Table 1). Furthermore, the headspace from *Y. schidigera* consisted predominantly of a C_{11}-terpenoid alcohol **B1** (M^+ 166, BP 69) (Table 1). Nevertheless, their structures could not be identified from their mass spectra alone.

Identification of Filamentolides (A1, A2)

Using GC/EI-HR-MS, both isomeric structures **A1** and **A2** exhibited a molecular ion signal at $M^+ = 178.1360$ corresponding to $C_{11}H_{16}O_2$, with four double bond equivalents. Because microreactions with the floral scent extract were inconclusive and no further indications of the structure could be derived from their EI-mass spectra (Fig. 4), the crude headspace sample of *Y. treculeana* was analyzed by NMR spectroscopy (Twele 2009). Inspection of the ^{1}H NMR spectrum revealed an allylidene moiety (δ_H 6.00, *d*, $J = 11$ Hz, 1H; 8.17, *dt*, $J = 17.3$ Hz, $J = 10.6$ Hz, 1H; 5.11–5.18, *m*, 2H), two anisochronous methylene protons (δ_H 2.01, *dd*, $J = 17.2$ Hz, $J = 6.6$ Hz, 1H; 2.27, *dd*, $J = 16.5$ Hz, $J = 7.0$ Hz, 1H) next to a chiral methine group (δ_H 4.62, *dt*, $J = 7.9$ Hz, $J = 7.2$ Hz, 1H) that is linked to an isopropenyl moiety (δ_H 4.94, *d*, $J = 8.8$ Hz, 1H; 1.32, *s*, 3H; 1.42, *s*, 3H). Analysis of ^{13}C NMR data confirmed the presence of an isopropenyl group (δ_C 18.0 *q*, 25.4 *q*, 138.4 s, 124.5 *d*) and an allylidene moiety (δ_C 123.7 *t*, 132.4 *d*, 138.0 *d*, 125.8 s), along with a methylene group (δ_C 36.3 *t*) connected to an oxygen-linked methine group (δ_C 73.5 *d*). The connectivity between these building blocks was deduced from two-dimensional H,H-COSY, HSQC, and HMC spectra, which enabled the identification of the dominant compound **A1** as 3-allylidene-5-(2-methylprop-1-enyl)dihydro(3*H*)furan-2-one. The stereochemistry of the allylidene moiety was subsequently established as (*Z*) based on H,H-interactions in the NOESY spectrum (Fig. S2).

The structural assignment of **A1** was unambiguously established by synthesis (Fig. 5a), which also enabled the elucidation of its absolute configuration. Thus, commercially available (*R*)- and (*S*)-5-(hydroxymethyl)dihydrofuran-2(3*H*)-one ((*R*)- and (*S*)-**1**) were oxidized using Dess-Martin periodinane (DMP) (Dess and Martin 1983, 1991) and crude solution of the resulting aldehydes (**2**) was converted in situ into the isopropylidene derivatives (**3**) by Wittig olefination (Wittig and Geissler 1953; Maryanoff and Reitz 1989). In these lactones ((*R*)- and (*S*)-**3**) the allylidene group was introduced by Peterson olefination (Larson and Betancourt de Perez 1985). Thus, (*R*)- and (*S*)-**3** were deprotonated with lithium diisopropylamide (LDA) and *C*-silylated with diphenylmethysilyl chloride to give the diastereoisomeric α-substituted derivatives (**4**). After a second deprotonation with LDA they were reacted with acrolein to furnish (*R*)- and (*S*)-filamentolides as a 2:1 mixture of the (*Z*)- and (*E*)-double bond isomers (**A1** and **A2**) that could be separated by column chromatography. Detailed analysis suggested that the final elimination step of this sequence initially formed the (*Z*) isomer, which subsequently isomerized (at least in part) to the (*E*)-isomer during purification. The allylidene lactones, (*Z*)- and (*E*)-filamentolide (**A1** and **A2**) proved to be sensitive towards polymerization even when stored at -20 °C. An alternative reaction sequence (data not shown) starting with Peterson olefination of the respective 5-(*tert*-butyldimethylsilyloxymethyl)-dihydrofuran-2(3*H*)-ones, furnished the allylidene derivatives that were deprotected with

Table 1 Relative quantities ([rel%]: ●●●●● > 50, ●●●● > 20, ●●● > 5, ●● > 1, ● < 1) of C_{11}-terpenoids, DMNT isomers, and (*E*)-nerolidol in the floral scents of *Yucca treculeana*, *Yucca schidigera*, *Yucca filamentosa*, *Yucca glauca*, *Yucca pallida*, and *Yucca reverchonii*. Elution order using HP-5 as the GC stationary phase

compound	#	*Y.tre*	*Y.schi*	*Y.fila*	*Y.glau*	*Y.pall*	*Y.rev*
(*Z*)-DMNT				●	●	●	
(*E*)-DMNT		●	●●●	●●●●●	●●●●●	●●●●●	●
Unknown 67–164	E1			●●●	●●●	●●●	
(*E*)-Filamental	C2			●●	●●●	●●	
Filamentone	D			●	●	●	
Unknown 67–164	E2			●		●	
(*Z*)-Filamental	C1			●●●	●●	●●	
(Z)-Filamentol	B1		●●●●●	●●●●●	●●●●●	●●●	
(*E*)-Filamentol	B2		●	●●	●●●	●●	
(*Z*)-Filamentolide	A1	●●●●●		●●●●	●●●●	●●	
(*E*)-Nerolidol				●	●●	●	
(*E*)-Filamentolide	A2			●●	●●	●	

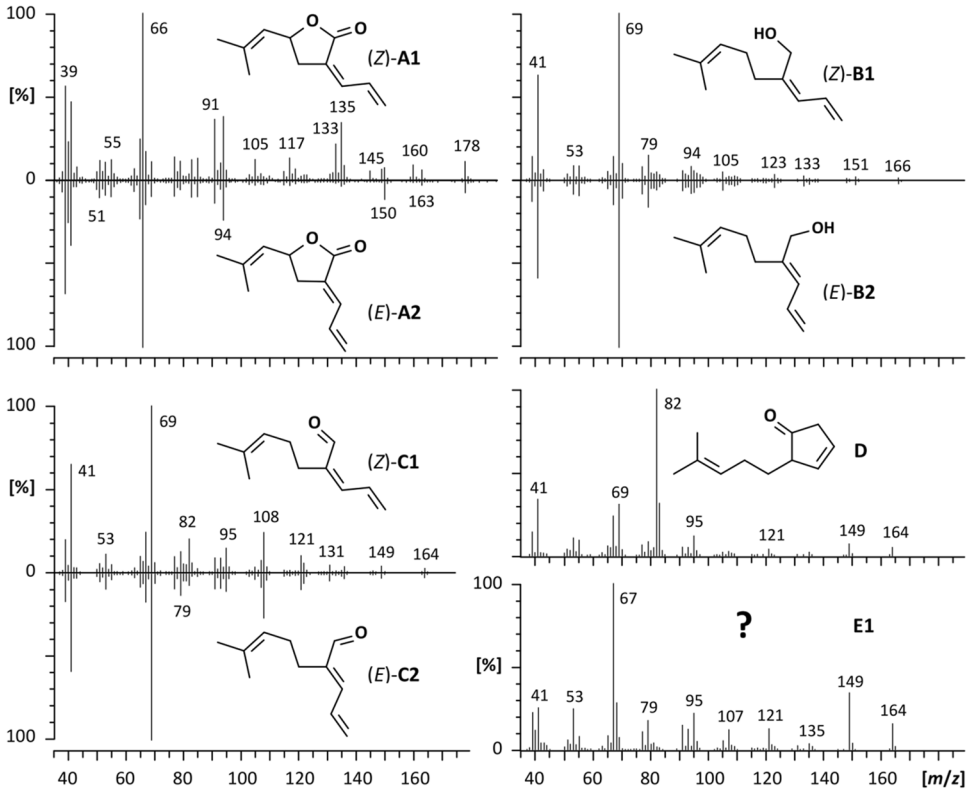

Fig. 4 MS spectra of (*Z*)- and (*E*)-filamentolide (**A1** and **A2**), (*Z*)- and (*E*)-filamentol (**B1** and **B2**), (*Z*)- and (*E*)-filamental (**C1** and **C2**), filamentone (**D**), and the unknown oxygenated C_{11}-compound (**E1**)

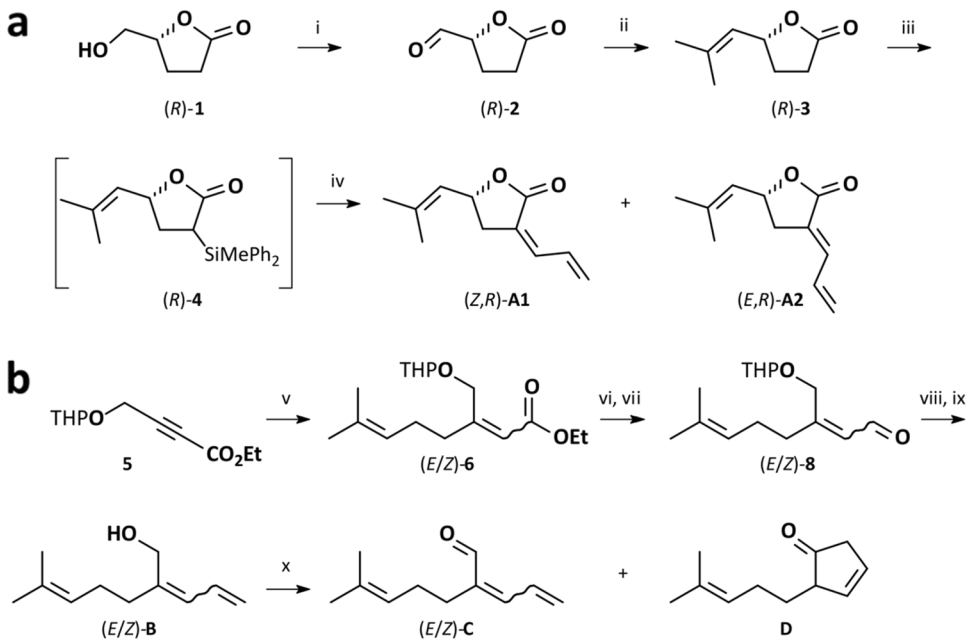

Fig. 5 **a** Synthesis of (*Z*)- and (*E*)-filamentolide (**A1** and **A2**) and **b** synthesis of filamentol (**B1** and **B2**), filamental (**C1** and **C2**), and filamentone (**D**). Conditions: **i**) DMP, py, DCM, -40 °C to RT, 1 h; **ii**) 1.: *i*PrPh$_3$PBr, *n*-BuLi, THF, -40 °C to RT, 1 h; 2.: (**2**), -90 °C to RT, 12 h; **iii**) 1.: LDA, THF, -80 °C, 1 h; 2.: MDPSCl, -80 °C to RT, 16 h; **iv**) 1.: LDA, THF, -80 °C, 45 min; 2.: acrolein, -80 °C to RT, 30 min, reflux, 5 min; **v**) $C_6H_{11}MgBr$, CuI, TMEDA, Et_2O, -78 °C, 1.5 h; **vi**) $LiAlH_4$, Et_2O, -80 °C to RT, 12 h; **vii**) DMP, py, DCM -40 °C to RT, 12 h; **viii**) H_2CPPh_3, THF, -78 °C to RT, 12 h; **ix**) *p*-TsOH, MeOH, RT, 1 h; **x**) PCC, DCM, -20 °C to RT, 2 h

hydrofluoric acid, but the subsequent DMP oxidation of the alcohols was not successful, probably also due to polymerization of the highly reactive allylidene structure.

Analysis of the NOESY spectra of the synthetic filamentolide isomers (**A1** and **A2**) confirmed the assignment of the (*Z*)- and (*E*)-configurations (Fig. S2). Comparison of the NMR and GC/EI-MS data confirmed the identity of synthetic (*Z*)-filamentolide ((*Z*)-**A1**) with the natural product from *Y. treculeana*, whereas the (*E*)-filamentolide ((*E*)-**A2**) was identified as a minor component. The absolute configuration was subsequently assigned by enantioselective gas chromatography (Fig. S3). Partial separation of the synthetic

(*S*)- and (*R*)-enantiomers was achieved using *heptakis*-(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin as the chiral phase. By co-injection, the absolute configuration of natural (*Z*)-filamentolide (*Z*)-**A1** was shown to be (*R*). In the same way, it was established that (*Z*)-filamentolide (*Z*)-**A1** is the primary geometric isomer present in the headspace samples of *Y. filamentosa*, *Y. glauca,* and *Y. pallida.*

Identification of Filamentols(B1, B2)

Having elucidated the structure of the dominant compound from *Y. treculeana* as (*Z*,*R*)-filamentolide ((*Z*,*R*)-**A1**) we turned to the identification of the main component **B1** of the *Y. schidigera* samples, which is also found in the *Yucca* section Chaenocarpa floral scents. In these, the very similar mass spectrum of another compound (**B2**) suggested the presence of two stereoisomers. Using GC/EI-HR-MS, both isomeric structures exhibited a molecular ion signal at $M^+ = 166.1360$ corresponding to $C_{11}H_{18}O$, with three double bond equivalents. The base peak of the EI-mass spectrum (Fig. 4) at *m/z* 69.0697 for the prenyl cation $[C_5H_9]^+$ suggested an open chain terpenoid moiety. Considering the structural relationship between the C_{11}-terpenoids, filamentolide (**A1**), and (*E*)-DMNT, we hypothesized **B1** to represent the closely related acyclic derivative 2-allylidene-6-methylhept-5-en-1-ol (4-hydroxymethyl-8-methyl-1,3,7-nonatriene).

The structural assignments for the natural products **B1** and **B2** were subsequently confirmed by synthesis as shown in Fig. 5b. Carboxylation of commercially available THP-protected propargyl alcohol with ethyl chloroformate furnished the 2-butynoic acid ester (**5**). Copper-(I) catalyzed conjugate addition of 4-methylpent-3-en-1-ylmagnesium bromide and **5** provided the C10-monoterpenoid backbone in **6** (Poulter et al. 1981; Kramp and Bohlmann 1986). Subsequently, reduction to the mono-THP-protected diol and its oxidation to the aldehyde (**8**) were followed by Wittig methylenation to provide the C_{11}-skeleton of **9**. Acid catalyzed deprotection afforded a 3:1 mixture of (*E*)- and (*Z*)-2-allylidene-6-methylhept-5-en-1-ol (**B1** and **B2**) in 19% yield over six steps. The isomers could be separated by chromatography on silver nitrate impregnated silica gel and their double bond configurations were determined by NOESY spectroscopy (Fig. S4).

The (*Z*)-alcohol was shown to be identical to the natural product **B1** from *Y. schidigera* by comparison of their GC/EI-MS data and was named (*Z*)-filamentol. Furthermore, the synthesis of (*E*)-filamentol (**B2**) enabled its identification as a trace component in the *Y. schidigera* floral scent. Comparative GC/MS analysis confirmed the assignment of **B1** and **B2** in the *Yucca* section Chaenocarpa headspace samples and demonstrated that in all these *Yucca* spp., the predominant filamentol isomer is (*Z*)-configured.

Identification of Filamentals (C1, C2) and Filamentone (D)

After the identification of filamentolide (**A**) and filamentol (**B**) as co-occurring C_{11}-terpenoids in all *Yucca* section Chaenocarpa samples, we aimed to identify some of the minor components of the floral volatiles from these species. GC/HR-MS analysis showed that several of these unknowns also possessed a C_{11}-skeleton. Therefore, we considered them, together with those now identified, as part of a complex of structurally closely related compounds. One pair of potentially stereoisomeric substances, **C1** and **C2**, exhibited a molecular ion signal at $M^+ = 164.1202$ corresponding to $C_{11}H_{16}O$ (with four double bond equivalents) and a base peak at *m/z* 69.0697 (Fig. 4), reminiscent of filamentol (**B**). Furthermore, a fragment ion at *m/z* 82.0415 for $[C_5H_6O]^+$ suggested a highly unsaturated fragment ion from a McLafferty rearrangement, in agreement with a 2-allylidene-6-methylhept-5-en-1-al (**C**) structure, the corresponding aldehyde of filamentol.

In addition, the blends of volatiles from *Y. filamentosa*, *Y. glauca*, and *Y. pallida* contained considerable quantities of an additional C_{11}-derivative **E1** (and small amount of a putative stereoisomer **E2**) with the molecular formula $C_{11}H_{16}O$ ($M^+ = 164.1066$) that exhibited a base peak at *m/z* 67.0564 for $[C_5H_7]^+$ and an intense fragment at *m/z* 149 $[M^+—CH_3]$ (Fig. 4). A third compound, **D**, of the same molecular formula ($C_{11}H_{16}O$) was characterized by a base peak at *m/z* 82, possibly from a McLafferty rearrangement (Fig. 4) along with the absence of a stereoisomeric partner. Moreover, about a dozen additional as-yet unknown substances were detected in the various headspace samples, some in very low concentrations, which, on the basis of their mass spectra, were considered to represent structurally related oxygenated C_{11}-derivatives.

Synthetic filamentol (**B**) served as the precursor for the synthesis of the corresponding aldehyde, 2-allylidene-6-methylhept-5-en-1-al (**C**), via pyridinium chlorochromate (PCC) mediated oxidation, in 67% yield (Fig. 5b). Even with the use of isomerically pure (*Z*)-alcohol (**B1**) as the starting material, variable amounts of the (*E*)-aldehyde were obtained and its relative proportion increased as a function of reaction time and temperature. The double bond configurations of the two isomers were determined by analysis of the NOESY spectra (Fig. S5). The identity of synthetic (*E/Z*)-2-allylidene-6-methylhept-5-en-1-als with the natural products **C1** and **C2** was finally confirmed by comparative GC/EI-MS analysis, and the aldehyde was named filamental. Both its isomers were present in the floral headspace samples from all three *Yucca* section Chaenocarpa spp., however in very different proportions, even when comparing various replicates of the same species.

Furthermore, GC/MS analysis of a crude reaction mixture revealed the presence of trace quantities of oxidation

by-products that were identical to the natural compounds **D** and **E** from *Yucca* spp., respectively. The oxidation product **D** could be enriched by column chromatography and inspection of its 1D and 2D NMR spectra enabled its identification as 2-(4-methylpent-3-en-1-yl)cyclopent-3-enone (Fig. 5b). The mass spectrum and the retention index were identical to those of natural **D,** which we have named filamentone.

Characterization of an Unknown C_{11}-terpenoid E

The second compound formed as a trace product during the PCC mediated oxidation of filamentol **B**, could be assigned to the natural product **E** by means of GC/MS comparisons. However, available amounts were too small to obtain any NMR data. The formation from filamentol (**B**) proves the structural relationship and GC/FT-IR suggested an allylidene substructure ($\tilde{\upsilon} = 1642\ cm^{-1}$) and possibly an (unsaturated) aldehyde or ketone moiety ($\tilde{\upsilon} = 1711\ cm^{-1}$) (Fig. S6). In addition to three acyclic oxo-DMNT derivatives, the known 5-oxo-DMNT (**10**) (Stamm et al. 2021) and 6-oxo-DMNT (**11**) (Wegener and Schulz 2002), as well as 9-oxo-DMNT (**12**), the cyclic carbonyl compounds 2-allylidene-5-isopropylcyclopentanone (**13**), 2-allylidene-6,6-dimethylcyclohexanone (**14**), and 3,3-dimethyl-2-vinylcyclohex-1-en-1-carbaldehyde (**15**) (Fig. 6) were considered as possible structures for this intriguing compound. Structures **10** and **11** were subsequently ruled out due to their mass spectrometric fragmentation patterns, whereas compounds **12** – **15** could all be excluded upon independent synthesis (Fig. 6) and comparison of the mass spectrum and retention time of the natural product **E** with those of the synthetic standards. Therefore, despite considerable efforts over the last decade, the molecular structure of this oxygenated DMNT derivative has remained elusive.

Bioactivity of *Yucca* Terpenoids in Field Trapping Assays

Having identified the molecular structures of the dominant C_{11}-terpenoids from yucca floral scent, their bioactivity was characterized by electrophysiological and field trapping experiments. In electroantennographic analyses using the floral headspace samples of *Y. treculeana*, reliable recordings were obtained from four females and ten males of the pollinating species *T. mexicana* and two individuals of each sex of the bogus yucca moth, *P. tamaulipellus*. In all but three of these analyses, antennal responses to

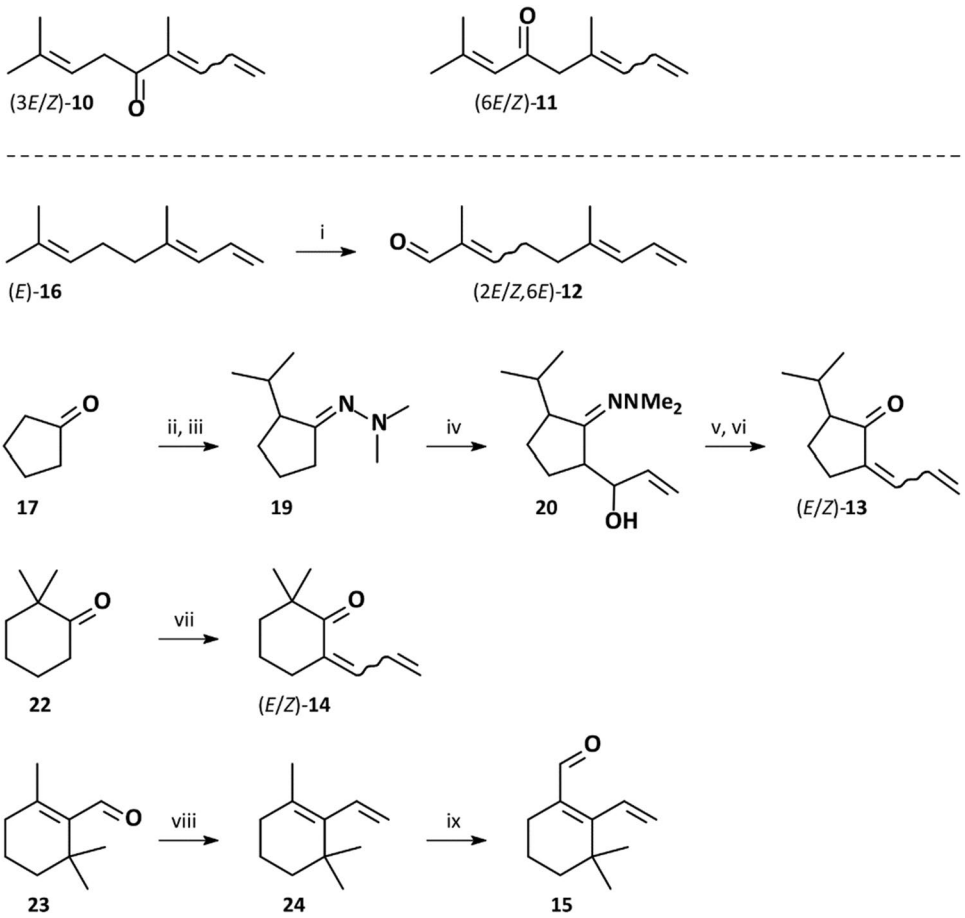

Fig. 6 Considered structures (**10**, **11**) and synthesis of the compounds **12**–**15** proposed for the unidentified C_{11}-terpenoid **E1**. Conditions: **i**) SeO_2, TBHP, DCM, RT, 24 h; **ii**) Me_2NNH_2, *p*-TsOH, CyHex, reflux, 4 h; **iii**) 1.: *n*-BuLi, THF, -70 °C, 40 min, 2.: *i*PrBr, -60 °C to RT, 14 h; **iv**) 1.: *n*-BuLi, THF, -70 °C, 45 min, 2.: acrolein, -60 °C to RT, 16 h; **v**) 1 N HCl, aq. sat. NH_4Cl, pentane, RT, 12 h; **vi**) MsCl, TEA, DCM, -40 °C to RT, 2 h; **vii**) LDA, THF, -40 °C, 30 min, acrolein, -20 °C, 90 min; **viii**) CH_2PPh_3, THF, -40 °C to RT, 14 h; **ix**) SeO_2, CyHex/H_2O, 5:1, RT, 48 h

(*Z*)-filamentolide (**A1**) were observed (Fig. 7a and 7b). In electroantennographic analyses using the floral scent from *Y. filamentosa*, reproducible antennal recordings were obtained from three females and four males of the pollinator *T. yuccasella*, six females, and eight males of the pollinator *T. cassandra*, and six females and seven males of the bogus yucca moth, *P. decipiens*. In all these recordings, strong antennal responses to (*Z*)-filamentol (**B1**) and (*Z*)-filamentolide (**A1**) were observed (Fig. 7c). Less frequent and smaller responses were observed for (*E*)-DMNT, (*Z*)-filamental (**C1**) and (*Z*)-9-nonadecene ((9Z)-C_{19}ene).

Finally, the behavioral activity of synthetic (*Z*)-filamentolide (**A1**) was established in field trapping experiments. Surveys of *Y. treculeana* inflorescences at the trapping site in 2012 revealed much higher densities of the bogus yucca moths *P. tamaulipellus* and *P. atascosanellus*, as compared with the pollinator *T. mexicana*, and this disparity was also reflected in the trap catch data. Significantly higher catches of both *Prodoxus* species were observed in traps baited with synthetic filamentolide (**A1**) versus unbaited control traps (*P. tamaulipellus*: $P = 0.028$; *P. atascosanellus*: $P = 0.007$; Fig. 6d and 6e). In addition, there was a trend for higher catches of both *Prodoxus* species in traps baited with fresh *Y. treculeana* flowers versus those baited with filamentolide (**A1**) (*P. tamaulipellus*: $P = 0.057$; *P. atascosanellus*: $P = 0.107$; Fig. 6d and 6e). For *T. mexicana*, five moths were captured in traps baited with host flowers, and no catches were observed for the other two treatments. For all three moth species, both sexes were trapped, and for the *Prodoxus* species, male captures dominated. In sum, these results indicate that (*Z*)-filamentolide (**A1**), the most distinctive and antennally active oxygenated DMNT derivative in *Yucca* spp., acts as a potent attractant for various *Yucca*-associated moth species.

Discussion

Floral Volatiles as Partner Encounter Signals in Nursery Pollination

The obligate mutualism between yuccas and yucca moths is called a "nursery pollination" system because the pollinated flower provides a host-specific resource for the offspring of the adult pollinators (Dufaÿ and Anstett 2003). Unlike vertically transmitted symbioses, participants in nursery pollination must renew their acquaintance each generation, requiring both the synchronization of reproductive timing and a sensory mechanism ensuring partner encounter and choice (Hossaert-McKey et al. 2010). Yuccas have served as an important model system for the study of obligate mutualism, particularly the discovery that selective abortion can occur when yucca moths lay too many eggs into the pistil of a pollinated flower, thereby using partner sanctions to

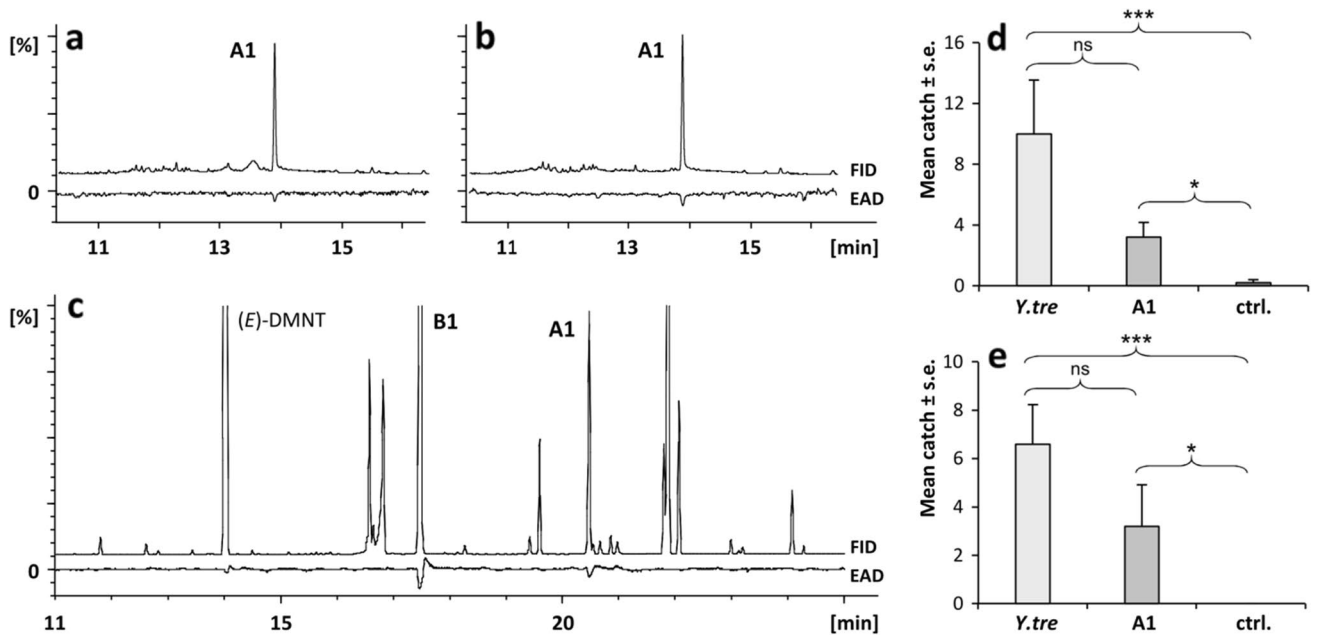

Fig. 7 GC/FID-EAD analyses of *Yucca treculeana* floral scent using antennae from (**a**) a female *Tegeticula mexicana*, and (**b**) a male *Prodoxus tamaulipellus*, respectively. GC/FID-EAD analyses of a *Yucca filamentosa* headspace sample using an antenna from a female *Tegeticula cassandra* (**c**). GC conditions (**a** and **b**) HP-INNOWax and (**c**) DB-5, see Methods. Field trapping experiments: Mean catches of *P. tamaulipellus* (**d**) and *Prodoxus atascosanellus* (**e**) in traps baited with flowers of *Y. treculeana*, synthetic (*Z*)-filamentolide (**A1**), and empty controls (* $P < 0.05$; *** $P < 0.001$)

stabilize the mutualism (Pellmyr and Huth 1994). However, our understanding of partner encounter in yucca-yucca moth interactions remains rudimentary. Yucca plants can produce enormous inflorescences, with tens to hundreds of greenish-white flowers that provide striking visual displays in the desert or grassland habitats where they grow (Fig. 1a). Nevertheless, the distinctive fragrances emitted by blooming yuccas (Svensson et al. 2005, 2006), combined with previous (Svensson et al. 2011) and current (Fig. 2) behavioral evidence for scent-mediated yucca moth orientation, suggested that their nursery pollination mutualism is mediated, at least in part, by floral volatiles. The highly specialized nature of nursery pollination led to the prediction that partner encounter attractants should be unusual or unique chemical compounds, thereby constituting a "private channel" of communication between mutualists (Raguso 2008; Soler et al. 2010). Therefore, we anticipated that yucca-yucca moth interactions might be mediated by novel floral volatiles.

Identification of a Novel Series of Tetranorsesquiterpenoid Volatiles

Our previous studies suggested a conservative scenario for chemical communication in yucca-yucca moth interactions, in which three allopatric species in *Yucca* section Chaenocarpa (*Y. filamentosa, Y. glauca*, *Y. elata*) produce nearly identical floral scent blends characterized by large amounts of (*E*)-DMNT, along with a series of structurally related but hitherto unidentifiable compounds (Svensson et al. 2005, 2006, 2011). (*E*)-DMNT is present in the floral volatiles of *Y. filamentosa* (Gäbler et al. 1991) and other night-blooming plants (Kaiser 1994). This substance also serves as an herbivore-induced plant volatile (HIPV) emitted systemically as a synomone from wounded plants to attract enemies of their attackers (Paré and Tumlinson 1999).

Our genus-wide survey of yucca volatiles revealed that two species in *Yucca* section Sarcocarpa, *Y. treculeana* (from the Rio Grande and adjacent regions of the Texas-Mexico border) and *Y. schidigera* (from the Mohave Desert of California south into Baja California), produce floral scents representing simplified subsets of the complex terpenoid blends produced by members of the section Chaenocarpa (Table 1), while lacking the medium chain length *n*-alkanes and alkenes characteristic of those species (Fig. 3). This allowed us to analyze crude headspace samples from *Y. treculeana* by NMR spectroscopy, providing key insights for the structural elucidation of the lactone (*Z*)-filamentolide (**A1**). The independent synthesis of all four stereoisomers ((*E*,*R*)-, (*Z*,*R*)-, (*E*,*S*)- and (*Z*,*S*)-filamentolide) (Fig. 5) confirmed this structural assignment and enabled the determination of the absolute configuration of the naturally dominant isomer as (*Z*,*R*) (Fig. S3), as well as the identification of small amounts of its (*E*)-isomer (Figs. 3 and 4). These novel lactones have a carbon skeleton identical to that of DMNT and carry the same allylidene and isopropenyl substructures (Fig. 8). Studies in *Y. filamentosa*, *Magnolia liliiflora nigra*, and *Phaseolus lunatus* established the biosynthesis of the C_{11}-tetranorsesquiterpene (*E*)-DMNT by degradation of the sesquiterpene alcohol (*E*)-nerolidol (Boland et al 1998, Gäbler et al. 1991). Since both (*E*)-nerolidol and (*E*)-DMNT were observed in all samples from *Yucca* section Chaenocarpa, we hypothesized that the remaining unknown yucca C_{11}-compounds all share the same biogenetic origin and exhibit the same carbon skeleton.

In particular, we predicted that the remaining yucca terpenoids might represent different derivatives of (*E*)-DMNT oxygenated at the allylic methyl group C10 rather than at C8 (as in *Selenicereus hamatus*) (Kaiser 1994) or at C6 (as in *Cyclanthus bipartitus*) (Schultz et al. 1999). The consideration of a common biosynthetic origin was decisive for their identification, in particular for the development of structure proposals for filamentol (**B**) and filamental (**C**) (Fig. 8), whose mass spectra, apart from the molecular ion and the typical terpenoid fragment *m/z* 69, allow only limited conclusions to be drawn about the molecular structure. Furthermore, the structure elucidation of NMR-characterized filamentone (**D**) was facilitated by taking into account the probable structural relationship.

The independent synthesis of (*E*)- and (*Z*)-filamentols (**B1** and **B2**) and filamentals (**C1** and **C2**) confirmed our structural hypotheses and showed that the main compound of the

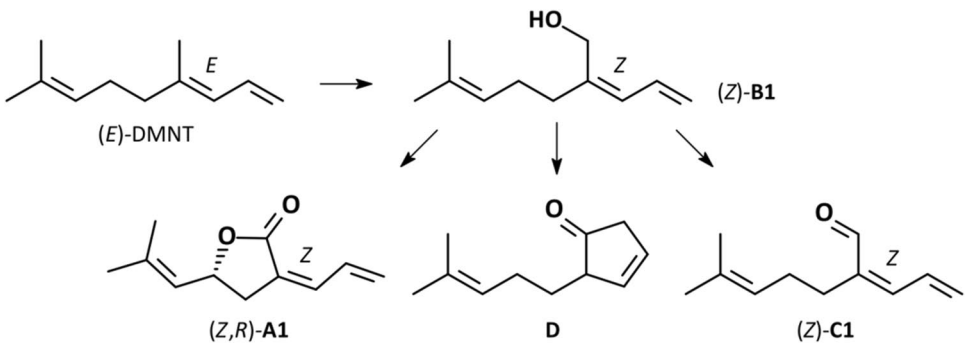

Fig. 8 Proposal for the biogenesis of the tetranorsesquiterpenoids in *Yucca* spp

Y. schidigera scent is (*Z*)-filamentol (**B1**), which is also the main isomer in all analyzed Chaenocarpa samples (Figs. 3 and 4). The two possible isomers **E1** and **E2** ($M^+ = 164$ and base peak at *m/z* 67) remain unidentified (Fig. 4). Indeed, concerning the carbon skeleton, even the naturally occurring (*E*)-configuration of DMNT is retained in the naturally dominating (*Z*)-isomers of filamentolide (**A1**) and filamentol (**B1**). This confirms our assumption of a DMNT-related biosynthesis, characterized by oxidation of the 10-methyl group (Fig. 8). The resulting (*Z*)-filamentol (**B1**) serves as a potential precursor to all the other oxygenated C_{11}-terpenoids identified so far, which involves oxidation to the corresponding open chain aldehyde (*Z*)-filamental (**C1**) and the cyclized ketone filamentone (**D**), as well as additional allylic oxidation to furnish the dioxygenated lactone filamentolide (**A1**) via as-yet unidentified intermediates. The isomerization of synthetic filamental in favor of the (*E*)-dienal substructure suggests that this tendency might also account for the observed variable *E*/*Z*-ratios in the natural headspace samples. Because all these tetranorsesquiterpenoids are present in the scent of *Y. filamentosa*, and in recognition of the foundational role played by this species in the study of obligate mutualism (e.g. Pellmyr and Huth 1994; Huth and Pellmyr 1999) and its contributions to the historical exploration of yucca volatiles (Wang and Kameoka 1978; Gäbler et al. 1991; Svensson et al. 2005), we have used the suffix "filament—" to name these novel compounds.

Finally, our characterization of floral scent emitted by living yucca flowers is largely consistent with earlier studies of essential oils from *Y. filamentosa* (Wang and Kameoka 1978) and two forms of the hybrid species *Yucca gloriosa* (Wang and Kameoka 1977, 1980), collected from cultivated plants in Japan. For example, nerolidol was identified with heptadecane and nonadecane in the essential oil of *Y. filamentosa* (Wang and Kameoka 1978), whereas (*Z*)-8-heptadecene and (*Z*)-9-nonadecene were more abundant in the essential oil of *Y. gloriosa* (Wang and Kameoka 1977), in accord with our own findings from floral headspace samples (Svensson et al. 2005; Tröger et al. 2019). *Yucca gloriosa* is a homoploid neospecies from southeastern coastal USA, resulting from hybridization between *Y. filamentosa* and *Yucca aloifolia* (Rentsch and Leebens-Mack 2012), with a floral scent profile nearly identical to that of *Y. filamentosa* (Svensson and Raguso, unpublished data). However, essential oil data need to be interpreted with caution due to the potential for chemical modification during the extraction process, as well as the presence of compounds that may not be volatilized under natural conditions. Nevertheless, our results confirm the identification of (*E*)-filamentol (**B2**) (4-hydroxymethyl-8-methyl-1,3,7-nonatriene) by Wang and Kameoka (1980) in the essential oil of *Yucca recurvifolia* (= *Y. gloriosa* var. *pendula*). However, many of the 79 other compounds reported (e.g. cresols, eugenol, carvacrol, decyl acetate) were absent in our headspace samples from living yucca flowers. Similarly, these authors described an (*E*)-filamentol acetate (8-methyl-4-acetoxy-1,3,7-nonatriene) and a sesquiterpene epoxide (2,6,10-trimethyl-6,10-epoxy-2,7,11-dodecatrien-1-ol) that we did not find in any of our samples.

The identified yucca volatiles filamentolide (**A**), filamental (**C**), and filamentone (**D**) are novel natural products, which, together with filamentol (**B**), supplement the family of known oxygenated DMNT-derivatives. Kaiser described oxygenation products at positions 7 and 8 ((6*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene and (3*E*,6*E*)-2,6-dimethyl-3,6,8-nonatrien-2-ol) from the fragrance of a night-blooming cactus, *Selenicereus hamatus* (Kaiser 1994). Similarly, Schultz et al. (1999) described C6-oxygenated DMNT-derivatives from the floral headspace of *Cyclanthus bipartitus*, a neotropical plant pollinated by dynastine scarab beetles. These volatiles included (6*E*)-2,6-dimethylnona-6,8-dien-4-one ((*E*)-cyclanthone) and its (*Z*)-isomer, their epoxy-derivatives (*cis*- and *trans*-6,7-epoxy-2,6-dimethylnon-8-ene-4-one), and the respective alcohol of (*E*)-cyclanthone ((*E*)-2,6-dimethylnona-6,8-dien-4-ol). Finally, Wegener and Schulz (2002) described DMNT-derived oxygenated compounds emitted by leaves of the European elm, *Ulmus minor*, in response to oviposition by elm leaf beetles. These compounds included (*E*)-cyclanthone, (*E*)-2,6-dimethyl-2,6,8-nonatriene-4-one (**11**), in which the DMNT-double bond at position C7 is conserved, and (*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene described by Kaiser (1994) from *S. hamatus* flowers. Finally, (*E*)-4,8-dimethyl-1,3,7-nonatriene-5-yl acetate was recently described from the night-blooming aroid, *Philodendron squamiferum* (Stamm et al. 2021). Based on their molecular masses and EI-MS fragmentation patterns, all these compounds could be excluded as components of the yucca floral fragrances.

Bioactivity of *Yucca* Terpenoids and their Potential to Mediate Mutualisms

The great value of structural elucidation and total synthesis of bioactive substances mediating plant-pollinator interactions is the creation of novel tools to test hypotheses about interaction specificity and diversification. For example, Schiestl et al. (2003) identified 2-ethyl-5-propylcyclohexan-1,3-dione (chiloglottone) as the sex pheromone of *Neozeleboria cryptoides* wasps and the behaviorally active compound in the volatiles of *Chiloglottis trapeziformis* orchids, which enlist male wasps as pollinators through sexual deception. The synthesis of chiloglottone enabled subsequent studies of niche partitioning, diversification (via related volatile signals), and reproductive isolation across the genus *Chiloglottis* (Schiestl and Peakall 2005; Peakall et al. 2010; Whitehead and Peakall 2014).

The present study represents a first step toward realizing similar goals in documenting the diversification of yucca-yucca moth interactions across North America. The simplistic floral scent of *Y. treculeana* provided an opportunity for proof-of-concept, as it is dominated by a single volatile ((*Z*)-filamentolide, **A1**) (Table 1) that elicits antennal responses in pollinating (*T. mexicana*) and bogus yucca moths (*P. tamaulipellus*) (Fig. 7a), and attracts *P. atascosanellus* and *P. tamaulipellus* in natural populations when used as a lure in pheromone traps (Fig. 7c). Similar bioassays can now be performed using synthetic (*Z*)-filamentol (**B1**) to test interactions between *Y. schidigera* and its pollinator moths (*Tegeticula mojavella, Tegeticula californica*) across the California-Mexico border (de la Rosa-Conroy et al. 2019). Interestingly, synthetic (*Z*)-filamentolide (**A1**) was highly unstable when applied (neat) to rubber septa, leading us to adopt a modified formulation using emulsified wax preparations (SPLAT; see Patt et al. 2011).

Experimental dissection of more complex floral blends, such as those found in *Yucca* section Chaenocarpa, will be more challenging. For example, flowers of *Y. filamentosa* emit DMNT and all of its novel oxygenated derivatives. Several of these compounds elicit antennal responses from *T. cassandra*, a host-specialized pollinator moth restricted to peninsular Florida and southern Georgia (Fig. 7b), and from *T. yuccasella*, a more widespread pollinator species that feeds on a broad range of *Yucca* host plant species (see 12). A survey across the range of *Y. filamentosa* found no evidence for geographic differences in floral scent composition between populations pollinated by these different moth species (Svensson et al. 2005). Bioassays using factorial combinations of EAG-active compounds will be needed to test whether partner encounter and attraction is initiated by additive, synergistic, or redundant volatiles for *Y. filamentosa, Y. glauca, Y. pallida*, and other yucca species that share *T. yuccasella* as a pollinator.

In summary, our analyses of the floral scent of *Yucca* revealed a series of novel tetranorsesquiterpenoid floral volatiles, which were identified using a combination of NMR and MS techniques along with total synthesis. The dominant component (*Z*)-filamentolide (**A1**) showed both electrophysiological and behavioral activity in associated *Tegeticula* and *Prodoxus* moths, and can thus be considered as a key signal mediating this insect-plant interaction. The identification of these compounds is a first step in elucidating the importance of floral scent in host finding and host discrimination in yucca moths, analysis of potential divergence in the scent signal among sympatric *Yucca* species with specialized pollinators, and phylogenetic analysis of floral scent evolution within this plant genus.

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Authors' Contributions Headspace samples were collected by GPS, KAS, DMA, JMP, and RAR. Field trapping, behavioral and electrophysiological assays were performed by GPS, JMP, and RAR. Chemical analyses, organic synthesis, and structural determinations were accomplished by AT, H-MG, RT, SB, PHGZ, SvR, and WF. The manuscript was written by AT, GPS, SvR, and RAR and was edited by all authors.

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Data Availability Voucher moth specimens have been deposited at the Cornell University Insect Collection (accessions CUIC000002359-CUIC000002431).

Code Availability not applicable.

Declarations

Conflicts of Interest/Competing Interests The authors disclose no competing interests.

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