ACCEPTED MANUSCRIPT – The published paper in the Journal of Chemical Ecology can be found online at: http://link.springer.com/article/10.1007/s10886-015-0582-x

N-3-Methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester – Pheromone Component of Western Black Widow Females

Catherine Scott (✉) · **Sean McCann** · **Regine Gries** · **Grigori Khaskin** · **Gerhard Gries**

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada.

Abstract–Chemical communication is common in spiders but few pheromones have been identified. Female widow spiders in the genus *Latrodectus* spin webs that disseminate an attractive sex pheromone, and contact pheromone on the silk elicits courtship behaviour by males. The methyl ester of *N*-3-methylbutanoyl-*O*-(*S*)-2-methylbutanoyl-*L*-serine is such a contact pheromone of the Australian redback spider *Latrodectus hasselti.* We predicted that the contact pheromone of congeneric *L. hesperus* resembles that of *L. hasselti*. We extracted the silk of virgin *L. hesperus* females with methanol, analyzed aliquots of silk extract by gas chromatography-mass spectrometry (GC-MS), and found evidence for *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S), a lower homologue of the *L. hasselti* contact pheromone. We tested behavioural responses of *L. hesperus* males to test stimuli on T-shaped rods with the end sections of the horizontal arm enveloped in filter paper. Males spent 40% longer in contact with paper bearing female silk than with blank paper, and 39% longer in contact with paper treated with silk extract than with solvent controls. Contact with silk and silk extract induced courtship behaviour by 96% and 80% of males, respectively, indicating that there is a methanol-soluble courtship-eliciting contact pheromone on the silk. Males responded less strongly to synthetic MB-MP-S than to silk or silk extract. Paper impregnated with synthetic MB-MP-S (10 or 100 µg) induced courtship behaviour in 3-16% of males, and prompted males to stay 10-16% longer than on control paper. Our data support the conclusion that MB-MP-S is part of a multi-component contact pheromone of *L. hesperus*.

Keywords *Latrodectus*, Chemical communication, Pheromone, Amino acid, Silk, Courtship.

Introduction

Chemical communication is common in spiders, but only few spider pheromones have been identified (Gaskett 2007; Schulz 2013). Spider sex pheromones on the silk or cuticle mediate attraction, mate recognition, and courtship behaviour (Uhl and Elias 2011). Generally, volatile airborne pheromones attract mates at long range, whereas contact pheromones elicit courtship, and may reveal information about the identity, quality, or mating history of the signaler, which is usually the female (Gaskett 2007). To date, female sex pheromones have been identified for only seven spider species in six families (Schulz 2013). Three of these pheromones are volatile and attract males. For example, (2*R*,3*S*)- and (2*S*,3*S*)-trimethyl methyl citrate at ratios of 6:1 to 25:1 comprise the attractive pheromone of virgin female orb-weaving wasp spiders, *Argiope bruennichi* (Araneidae)(Chinta et al. 2010). *N*-3-Methylbutanoyl-*O*-(*S*)-2-methylbutanoyl-*L*-serine methyl ester represents one of four known silk-borne contact pheromones; it is produced by virgin females of the tangle-web weaving Australian redback spider *Latrodectus hasselti* (Theridiidae) and elicits searching and courtship behaviours by males (Jerhot et al. 2010). 8-Methyl-2-nonanone exemplifies a bi-functional pheromone; it is produced by sexually receptive females of *Agelenopsis aperta* (Agelenidae) and both attracts males and induces courtship behaviour (Papke et al. 2001).

The attractiveness of volatile spider pheromones to conspecific males is typically tested in still-air olfactometers in the laboratory (e.g., Xiao et al. 2009) or in field-trapping experiments (e.g., Chinta et al. 2010). Both choice and no-choice bioassays allowing males to contact silk or pheromone-impregnated filter paper have been used to determine the behavioural responses of males to contact pheromones. For example, in no-choice experiments, *L. hasselti* males were placed on filter paper impregnated with synthetic pheromone or a solvent control and their behaviour was video recorded (Stoltz et al. 2007; Jerhot et al. 2010). Because specific courtship behaviours are difficult to observe in this type of bioassay, movement instead of courtship behaviour of males was used to assess pheromone activity.

Here we investigate the function and molecular structure of the sex pheromone of female western black widows, *Latrodectus hesperus.* As shown in field studies, volatile pheromone emanating from webs of conspecific females attracts *L. hesperus* males and allows them to discriminate between virgin and mated females (Kasumovic and Andrade 2004; MacLeod and Andrade 2014). When a male contacts a virgin

female's silk, he engages in courtship behaviour that includes vibratory signaling and web reduction behaviour, during which he cuts the female's web and wraps sections of it with his own silk (Ross and Smith 1979; Scott et al. 2012). We do not yet know whether the same pheromone acts as both an airborne attractant and a contact courtship-releaser in this species or in congeners. In *Linyphia triangulosa* (Linyphiidae), the volatile breakdown products of the web reduction-eliciting contact pheromone (3*R*,3*R*′)-3-hydroxybutyryloxybutyric acid on the silk are airborne attractants (Schulz and Toft 1993; Schulz 2013).

The sex pheromones of *Latrodectus* females may not be entirely species-specific. The webs of *L. mactans* females elicit courtship behaviour not only of conspecific males but also of *L. hesperus* males (Ross and Smith 1979). Furthermore, in a field experiment in British Columbia, *L. hesperus* males were attracted not only to empty webs of local conspecific females, but also to webs of *L. hasselti* females, even more so than to the webs of conspecific females from Arizona (Kasumovic and Andrade 2004). Several species in the genus *Linyphia* share the same contact pheromone that elicits web reduction behaviour but discriminate between the silk of con- and hetero-specifics, presumably based on additional silk-borne compounds (Schulz and Toft 1993; Schulz 2013).

We tested the hypotheses (1) that a silk-borne pheromone of female *L. hesperus* elicits short-range attraction of conspecific males and/or courtship behaviour, and (2) that the molecular structure of the pheromone resembles that of the *L. hasselti* pheromone.

Methods and Materials

Experimental Animals All spiders in this study were offspring from mated females collected at Island View Beach, on the Saanich peninsula of Vancouver Island, British Columbia, Canada (48° 35' N, 123° 22' W, elevation 3–4 m). We reared spiders in the laboratory on a diet of house crickets (*Acheta domesticus*) and blow flies (*Lucilia sericata*), and kept spiders at 20–25 °C on a reversed 12L:12D photo regime to facilitate experimentation during their nocturnal activity period.

Collection of Silk for Behavioural Bioassays We allowed female spiders to build webs for 72 h in wood-frame boxes (30 × 30 × 20 cm). These boxes were re-used for housing virgin females only, and between uses all silk and prey remains were removed and the boxes were wiped out with a damp cloth. We did not

feed the spiders used for silk collection after placing them in boxes, so that no prey cues would be present on the silk. We then removed the silk from the box using glass pipettes and wrapped it around a filter paper envelope (see below; Fig. 1a), distributing it as evenly as possible along the length of the paper.

Collection of Silk for Chemical Analyses We provided virgin female spiders (1–3 months post maturity) with equilateral, triangular prism glass frames (Fig. 1b) for web construction. To facilitate grip on substrate and to prevent spiders from leaving, we sandblasted the frames and placed them on a glass base surrounded by a moat of water. Following 72 h of web building, we collected the silk with clean glass pipettes, placed it in a 4-mL glass vial, and extracted the silk with methanol for at least 24 h. For chemical analyses, we combined extracts of 21 webs and concentrated the sample to a volume of ~200 µL. For behavioural tests, we combined extracts of at least eight webs for each batch, and diluted the silk extract to a volume of 40 µL per web.

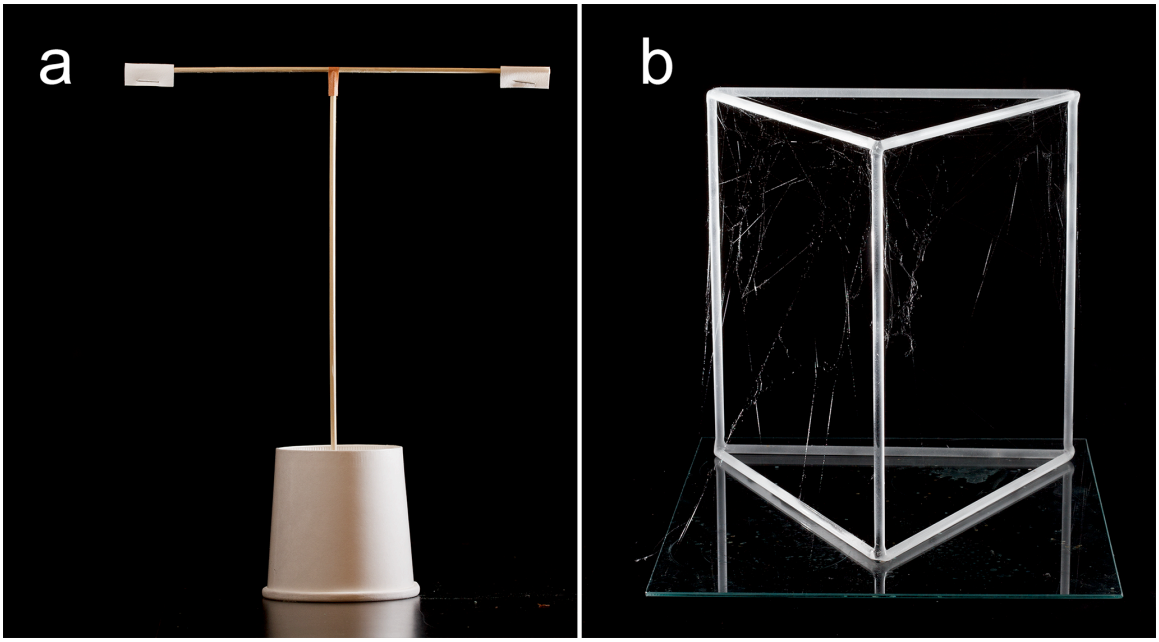

Fig. 1 (a) T-rod used for testing behavioural responses of *Latrodectus hesperus* males to test stimuli applied to filter paper envelopes on each end of the horizontal rod; (b) Glass frame with web of a female *L. hesperus*; after 72 h of web building, we collected the silk and extracted it with methanol

Analyses of Silk Extracts We analyzed aliquots of silk extract using a Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 GC-MS column (50 m × 0.25 mm i.d.), setting temperatures of the injector port and ion trap to 250 °C and 260 °C, respectively. We used helium as the carrier gas (35 cm s^{-1}) with the following temperature program: 100 °C for 5 min, 20 °C min^{-1} increase until 280 °C, 280 °C for 20 min.

Syntheses General methods and instrumentation for syntheses, a representative synthesis of ester-amide mixtures, and synthesis of *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S) are reported in Online Resource 1.

General Design of Behavioural Experiments We examined the responses of virgin male spiders (5–30 days post maturity) to test stimuli in two-choice experiments. We constructed T-shaped rods (henceforth 'T-rods'; Fig. 1a) from pairs of bamboo skewers (25.5 cm; Bradshaw International Inc., Rancho Cucamonga CA, USA) joined with a piece of labeling tape (3 × 0.5 cm; Fisher Scientific, Ottawa ON, Canada). We secured the vertical arm of the T-rod to an inverted paper cup (Solo Cup Company, Lake Forest, IL, USA) filled with floral foam, and placed a piece of filter paper (2.5 × 2.5 cm; Whatman No. 1, Whatman International Ltd., Maidstone, Kent, UK) folded in half to form an envelope over each end section of the horizontal arm and stapled it ca. 3 mm from the edge. We used new T-rods and filter papers for each bioassay.

We applied a test stimulus to one filter paper and a control stimulus to the other (see Table 1), alternating the treatment side of the T-rod between tests. When we tested extract or synthetic MB-MP-S, we allowed the methanol solvent to evaporate before we introduced a male spider to the base of the T-rod. We scored each spider's behaviour in real time using Jwatcher v1.0 (Blumstein et al. 2012), beginning when he reached the intersection of the 'T'. We ran bioassays for 30 min (experiments 1 and 5) or 15 min (experiments 2-4), or until the spider descended from the T-rod on his dragline; whichever came first. We used a cutoff of 15 min in experiments 2-4 because the results from experiments 1 and 5 were almost identical whether we analysed only the first 15 min or the entire 30 min. For each test, we recorded three

behavioral response criteria: (1) first choice; (2) percent time spent on treatment and control stimuli; and (3) courtship behaviour.

For criterion 1 (first choice), we recorded the filter paper a male contacted first, predicting that it would be the paper treated with silk, silk extract, or synthetic MB-MP-S if the response was based on airborne cues.

For criterion 2 (percent time spent on treatment and control stimuli), we calculated the percentage of total time on the T-rod that spiders spent on each filter paper, predicting that males would spend more time on the paper treated with silk, silk extract, or synthetic MB-MP-S if silk has a courtship-eliciting contact pheromone.

For criterion 3 (courtship behaviour), we recorded whether or not the spider wrapped the treatment filter paper with silk, predicting that males would engage in silk-wrapping on filter papers treated with silk, silk extract, or synthetic MB-MP-S. 'Silk-wrapping' refers to the male pulling silk from his spinnerets with his last pair of legs and depositing it onto the female's web (Fig. 2a). During silk wrapping, the male adopts a distinctive posture with his abdomen raised relative to his cephalothorax, and with his last pair of legs he describes wide arcs as he pulls silk out of his spinnerets (Fig. 2b and Online Resource 2). This behaviour is a part of normal courtship sequences in this species, wherein the male wraps silk around a section of the female's web (web reduction behaviour; Ross & Smith 1979; Scott et al, 2012). We selected silk-wrapping as the response criterion for courtship behaviour because it is unmistakable in the context of our bioassay.

Specific Behavioural Experiments In experiment 1, we tested the effect of female silk, silk extract, and synthetic MB-MP-S on responses of males (see Table 1 for summary). We tested the responses of the same 25 male spiders in each of four 2-choice tests presented in random order. Test stimuli were as follows: (*i*) the silk of a single virgin female's web (1 FW = the entire web of a single female) wrapped around filter paper vs. a blank paper control; (*ii*) methanol extract of 1 female web equivalent (1 FWE = total silk extract of one web) vs. methanol control, (*iii*) synthetic MB-MP-S (10 μg) vs. methanol control, and (*iv*) methanol control vs. methanol control. We ran the control vs. control test (*iv*) to ensure that there was no directional bias in the set-up.

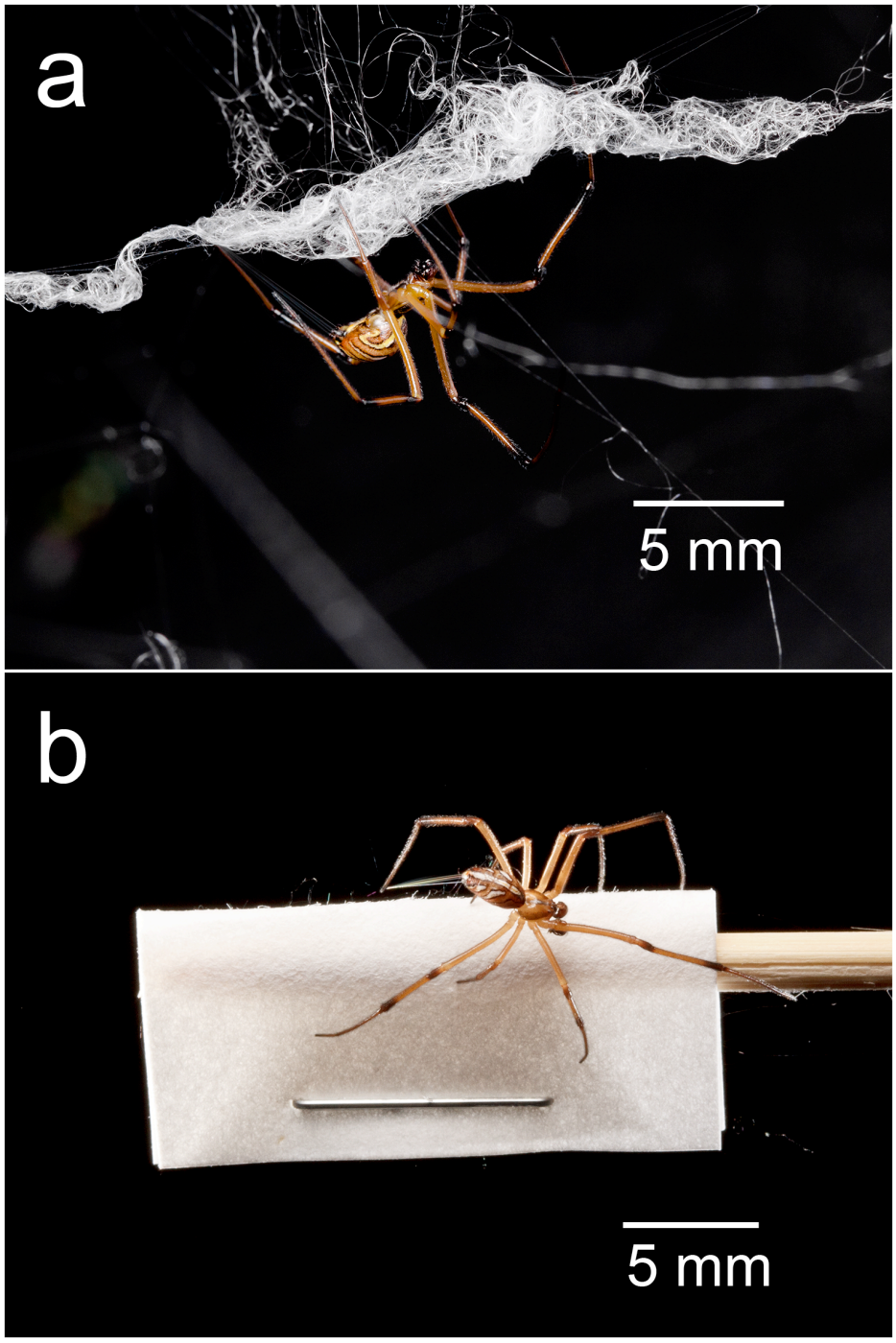

Fig. 2 (a) *Latrodectus hesperus* male silk-wrapping as part of his courtship behavior on a female's web; (b) *L. hesperus* male silk-wrapping on a filter paper treated with methanol silk extract. Note in (a) the male's raised abdomen (relative to his cephalothorax), and in (a) and (b) the silk being pulled from his spinnerets with his last pair of legs

Table 1 Details of T-rod choice experiments 1 to 5, testing the behavioural responses of male *Latrodectus hesperus* to various stimuli

Exp.	*n*	Treatment[a]	Control[a]	Max. time[b]
1	25[c]	Silk (1 FW)	Blank paper	30 min
		Silk extract (1 FWE)	Methanol	30 min
		Synthetic MB-MP-S (10 μg)	Methanol	30 min
		Methanol (right side)	Methanol (left side)	30 min
2	39	Silk extract (1 FWE)	Methanol	15 min
3	39	Synthetic MB-MP-S (10 μg)	Methanol	15 min
4	20	Synthetic MB-MP-S (100 μg)	Methanol	15 min
5	36	Silk extract (0.5 FWE) plus synthetic MB-MP-S (10 μg)	Silk extract (0.5 FWE)	30 min

FW = female's web; FWE = female's web equivalent; MB-MP-S = *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester

[a]Silk extract, synthetic MB-MP-S or corresponding methanol control stimuli were each applied to filter paper at 40-μL aliquots;

[b]Trials were terminated when the maximum time had elapsed or the male had dropped down to the substrate and walked away, whichever came first;

[c]Each of 25 males was tested with all four treatments in random order, with one test per day on consecutive days; males in all other experiments were tested only once

To verify that the results for experiment 1 were not affected by males being repeatedly exposed to silk pheromone components, we ran additional tests. In each of experiments 2 and 3, we used the same treatments and controls as in (*ii*) and (*iii*) above, testing 39 naïve males that had never courted a female or been exposed to female silk extract or synthetic pheromone.

In experiment 4, we tested the effect of a higher (100 μg) dose of synthetic MB-MP-S on responses of naïve males (Table 1). We gave 20 male spiders a choice between synthetic MB-MP-S (100 μg) and a solvent control, predicting that the 100-μg dose would elicit stronger responses from males than the 10-μg dose in experiment 1 or 3. In spiders, 10-fold increases in pheromone dose are typically required to increase responses by a factor of two (e.g., Papke et al. 2001; Jerhot et al. 2010).

In experiment 5, we tested the effect of synthetic MB-MP-S admixed with silk extract on responses of a new group of naïve males (Table 1). We gave 34 male spiders a choice between silk extract (0.5 FWE) admixed with synthetic MB-MP-S (10 μg) and silk extract alone (0.5 FWE), predicting that the former

stimulus would elicit stronger responses from males because it contained a larger amount of the candidate pheromone in addition to other potential pheromone components present in the silk extract.

Statistical Analyses For each T-rod test, we analyzed data with 2-tailed paired *t*-tests to determine whether males spent a greater percentage of time on the treatment filter paper than the control filter paper. For experiment 1, we analyzed data with ANOVA, using individual spiders as blocks, to determine whether the difference between the percentage of time males spent on the treatment filter paper relative to the control filter paper differed between tests. We followed the ANOVA with post-hoc tests for differences among treatments, adjusting for multiple comparisons using the Tukey method. We ran all statistical tests in R 3.0.2 (R Core Team 2013).

Results

Pheromone Analysis

GC-MS analyses of silk extract of female *L. hesperus* revealed a quantitatively minor component (**A** in Fig. 3a) with a fragmentation pattern (Fig. 3b) closely resembling that of *N*-3-methylbutanoyl-*O*-2-methylbutanoyl-*L*-serine methyl ester (MB-MB-S), the contact pheromone of *L. hasselti* (Jerhot et al. 2010). Based on the retention index (RI) (Van den Dool and Kratz 1963) of **A** (1740) we concluded that it could be a lower homologue of the *L. hasselti* pheromone (RI: 1832). We therefore synthesized ester-amide mixtures, using *L*-serine hydrochloride, isobutyric and isovaleric acids as starting materials (Online Resource 1). One of eight resulting compounds had retention and mass spectral characteristics identical to **A**. Its mass spectrum was indicative of *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S), a lower homologue of the *L. hasselti* pheromone. Retention and mass spectral characteristics of synthetic MB-MP-S (synthesized according to Jerhot et al. 2010; Fig. 4 and Online Resource 1) were in complete agreement with those of **A**, confirming our structural assignment. We considered MB-MP-S a good candidate pheromone component for *L. hesperus* because it resembled the pheromone of female *L. hasselti* and it was present in minor amounts in extracts of *L. hasselti* silk (Jerhot et al. 2010), which might explain the attraction of *L. hesperus* males to female *L. hasselti* webs.

Table 2 Responses of *Latrodectus hesperus* males in behavioural bioassays (see Table 1 and methods for details of treatment and control stimuli) on T-rods (Fig. 1). For each experiment, the percentage of males that made contact with the filter paper on the treatment side of the T-rod first and the percentage of males that engaged in silk-wrapping behaviour in response to test stimuli are given with 95% Confidence Intervals (CIs) calculated using the Agresti-Coull method. For each experiment, the mean of the differences between the percentage of time each male spent on the treatment filter paper compared to the control filter paper are given with 95% CIs. Different capital letters indicate means that differ significantly within experiment 1 based on an ANOVA followed by Tukey's HSD (experiment-wise $P < 0.05$). Also shown are the results of individual *t*-tests for each experiment, indicating whether males spent a significantly greater percentage of time on the treatment filter paper than on the control filter paper

Exp.	*N*	Treatment (Control)	Chose treatment side first [% of males] (95% CI)	Time spent on treatment minus time on control [% of total time] (95% CI)	*t*	*df*	*P*	Silk-wrapped [% of males] (95% CI)
1	25[a]	Silk [1 FW] (Blank paper)	52 (33,70)	40 (26,55) A	5.85	24	<0.001	96 (78,100)[c]
		Silk extract [1 FWE] (Methanol)	40 (23,59)	39 (24,54) A	5.32	24	<0.001	80 (60,92)[c]
		MB-MP-S [10 µg] (Methanol)	52 (33,70)	10 (0,19) B	1.99	24	0.058	16 (6,35)[c]
		Methanol right side (Methanol left side)	56 (37,73)	6 (-1,14) B	1.72	24	0.099	0 (0,16)[d]
2	39[b]	Silk extract [1 FWE] (Methanol)	41 (27,57)	52 (38,65)	7.87	38	<0.001	92 (79,98)[c]
3	39[b]	MB-MP-S [10 µg] (Methanol)	54 (39,68)	14 (5,24)	2.99	38	0.005	3 (0,14)[c]
4	20[b]	MB-MP-S [100 µg] (Methanol)	55 (34,74)	16 (7,24)	3.92	19	0.001	15 (4,37)[c]
5	36[b]	Silk extract [0.5 FWE] + MB-MP-S [10 µg] (Silk extract [0.5 FWE])	58 (40,70)	10 (2,18)	2.46	35	0.019	89 (74,96)[e]

FW = female's web; FWE = female's web equivalent; MB-MP-S = *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester

[a]Each of 25 males was used in all four tests, in random order;

[b]Each male was used in only one test, and had not previously been exposed to female silk/pheromones;

[c]Males that silk-wrapped on the treatment (no males wrapped controls);

[d]Males that silk-wrapped on either side;

[e]Males that silk-wrapped on both sides (all other males did not wrap either side)

Behavioural Experiments

First Choice In experiments 1-5 (Table 1), males made first contact equally often with treatment and control stimuli (Table 2), indicating that males were not attracted to any volatile component of the treatment stimuli. When both test stimuli were solvent controls, males made first contact equally often with the stimulus on the right or left side, indicating that there was no directional bias in the bioassay.

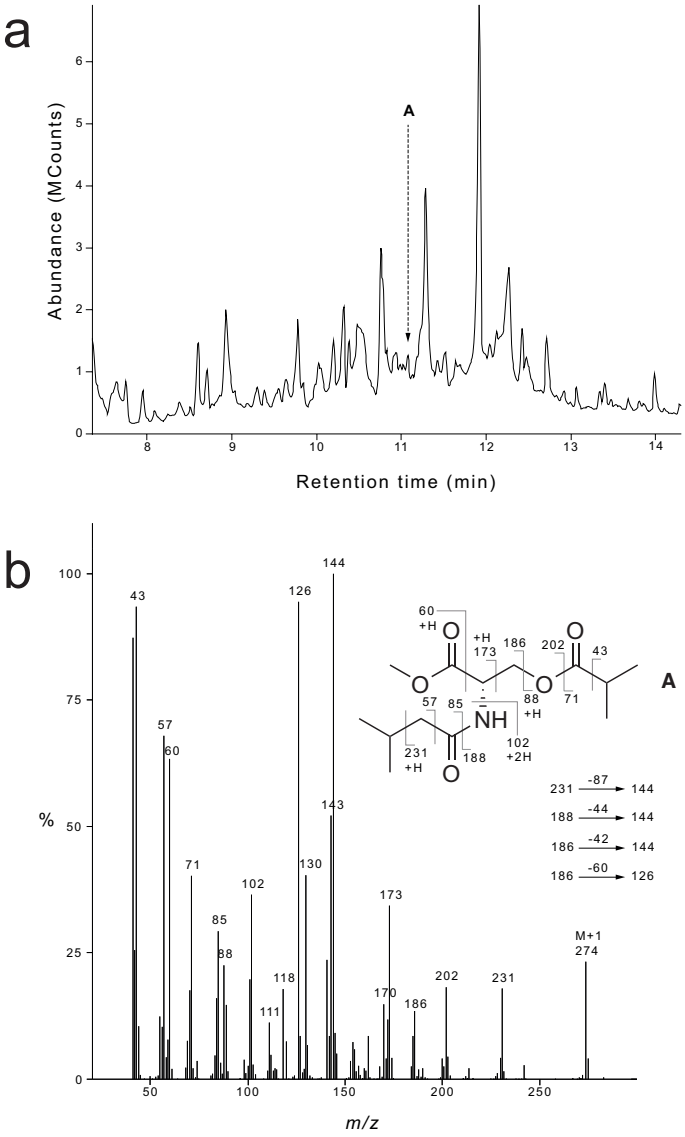

Fig. 3 (a) Total ion chromatogram (Saturn 2000 Ion Trap GC-MS) of a methanol extract of 21 webs of virgin *Latrodectus hesperus* females; **A** denotes the pheromone component *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S); (b) mass spectrum and fragmentation pattern of **A**

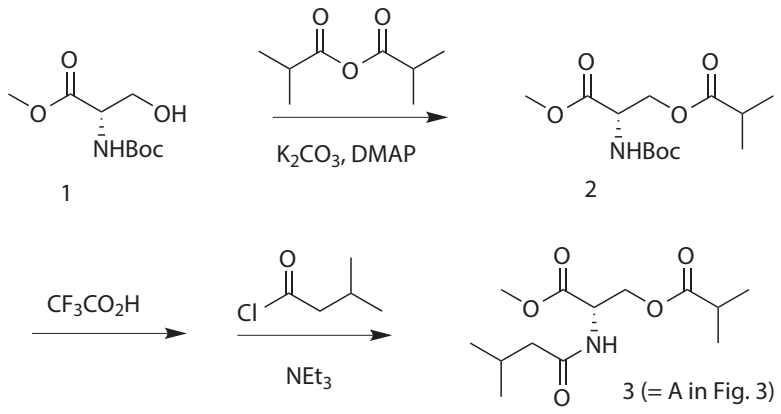

Fig. 4 Synthesis of *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester **3** (compound **A** in Fig. 3), a pheromone component of female *Latrodectus hesperus;* DMAP = 4- dimethylaminopyridine

Difference between Percentages of Time Spent on Treatment and Control Stimuli In experiment 1 (Table 1), males spent 40% more time on silk-bearing filter paper than on control paper (Table 2), and 39% more time on silk extract-treated paper than on MeOH-treated paper (Table 2). Males spent 10% more time on paper treated with synthetic MB-MP-S (10 µg) than on MeOH-treated paper (Table 2), and 6.2% more time on the right hand side paper than on the left hand side paper when both papers were MeOH-treated (Table 2). There was no difference between the percentage of time males spent on the treatment filter paper relative to the control filter paper (i.e. the effect sizes) when comparing responses to silk or silk extract (Tukey's HSD, $t = -0.15$, $df = 72$, $P = 1.0$). The time males spent on the filter paper treated with MP-MB-S relative to the control was also not significantly different from the time males spent on the right hand side filter paper relative to the left hand side filter paper when both were treated with methanol alone (Tukey's HSD, $t = -0.39$, $df = 72$, $P = 0.98$). All other pairwise comparisons were significantly different ($P < 0.001$ after adjusting for multiple comparisons within experiment 1, indicated by different letters in Table 2).

In experiments 2-5 (Table 2), naïve males spent 52% more time on paper treated with silk extract than on MeOH-treated paper (Experiment 2), 14% and 16% more time, respectively, on paper treated with synthetic MB-MP-S at 10 µg or 100 µg than on MeOH-treated paper (Experiments 3, 4), and 10% more time on paper treated with silk extract and synthetic MB-MP-S than on paper treated with silk extract alone (Experiment 5).

Courtship In experiment 1 (Table 1), none of the 25 males tested courted on controls but most responded with silk wrapping to silk and silk extract (96% and 80%, respectively; Table 2). Males also courted in response to synthetic MB-MP-S (10 µg), although at a lower rate (16% in experiment 1, 3% in experiment 3; Table 2). Silk-wrapping response rates (15%) were similar when males were exposed to a higher (100-µg) dose of synthetic MB-MP-S (Table 2). In experiment 5, 89% of the males bioassayed wrapped both test stimuli, the paper impregnated with silk extract alone and the paper impregnated with both silk extract and synthetic MB-MP-S, whereas 11% of males wrapped neither stimulus (Table 2). Two males responded with silk wrapping on the arm of the T-rod bearing the filter paper treated with the admixture of silk extract and synthetic MB-MP-S even before they made contact with either test stimulus.

Discussion

We have demonstrated that a courtship-eliciting contact pheromone on the silk of female *L. hesperus* can be extracted with methanol. Male *L. hesperus* prefer synthetic MB-MP-S over controls, but not to the same extent as they prefer silk or silk extract over controls. MB-MP-S occasionally elicits male courtship behaviour, whereas silk and silk extract consistently trigger silk-wrapping. Our results support the conclusion that MB-MP-S is one component of a multi-component pheromone. MB-MP-S is also present in minor amounts in extracts of *L. hasselti* silk (Jerhot et al. 2010), and thus might contribute to the attraction of *L. hesperus* males to female *L. hasselti* webs (Kasumovic and Andrade 2004). Below, we discuss the implications of our results for identifying the sex pheromone of *L. hesperus* and other spiders, and highlight the importance of using carefully designed bioassays for testing the various behavioural responses of spiders to chemical signals.

Contact pheromone is extractable with methanol from the silk of virgin female *L. hesperus* and elicits courtship behaviour by males. Males responded just as strongly to methanol extract of silk as they did to silk itself, spending most of their time in contact with silk- or silk extract-treated paper, and wrapping both stimuli with silk. These results indicate that there is a methanol-soluble sex pheromone on the silk that triggers silk-wrapping behaviour. Naïve males spent significantly more time on paper treated with synthetic MB-MP-S (10 or 100 µg) than on MeOH-treated paper but the preference was not as strong as for silk extract. That some males did respond to MB-MP-S with courtship, although less strongly than to extract,

suggests that MB-MP-S is a component of the courtship-eliciting sex pheromone. Males also slightly preferred silk extract admixed with synthetic MB-MP-S to silk extract alone, and most males responded with courtship to both stimuli, suggesting that MB-MP-S contributes to the pheromone signal but that other pheromone components in silk extract are required to consistently trigger courtship. Intriguingly, two males began silk-wrapping on the horizontal arm of the T-rod on the side treated with MB-MP-S admixed with silk extract before making contact with the filter paper, indicating that courtship behaviour was induced by one or more volatile pheromone components. In the spider *Agelenopsis aperta* (Agelenidae), the airborne pheromone 8-methyl-2-nonanone, which emanates from the female's body, functions not only as an attractant but also elicits male courtship behaviour (Papke et al. 2001).

The first-choice response of male spiders did not indicate attraction to treatment stimuli in any experiment. Because *L. hesperus* males are attracted to females' webs in the field (Kasumovic and Andrade 2004), we had predicted that males would orient first toward silk or silk extract in T-rod experiments. That they did not is not likely due a flight response after being introduced onto the test apparatus. Males typically paused at the intersection of the 'T' and briefly tapped each side of the horizontal arm with their first pair of legs before they walked on, sometimes reversing direction before making contact with a filter paper. We conclude that male *L. hesperus* did not orient toward volatile silk cues in the context of our bioassay. Males may be attracted to females only at a relatively long range (possibly exceeding the 13-cm length of each of the T-rod's horizontal arms), and they may rely primarily on contact pheromone at closer range. Alternatively, wrapping silk around filter papers and thus reducing the surface area exposed to air may have limited dissemination of male-attractant volatile pheromone components. Also, the volatile pheromone components may not be soluble in methanol.

When designing pheromone bioassays for spiders, it is important to consider the context in which the behavioural response being tested normally occurs. Our T-rod bioassay allows male black widows to approach, and engage with, test stimuli from a climbing/hanging position, which is akin to how they would engage with stimuli on a female's web. The silk-wrapping behaviour in this context is easy to see and to interpret as courtship behaviour. Our T-rod bioassay design allowed us to test whether and how male black widows respond to contact chemical stimuli, but it did not reveal any attraction to test stimuli, despite strong field evidence for attraction of male *L. hesperus* to females' webs. In the field, mate-searching males

traverse the ground, a context that was not provided in our bioassay. Experiments in the field or in field enclosures remain the only suitable method for testing attraction of male black widows (Kasumovic and Andrade 2004; MacLeod and Andrade 2014).

Of the seven female spider pheromones identified to date, several appear to be single compounds. However, *Pholcus beijingensis* (Pholcidae) females produce a two-component pheromone blend consisting of (*E*,*E*)-farnesyl acetate and hexadecyl acetate at a 2:1 ratio (Xiao et al. 2009). Even in species where a single compound has been found to have strong pheromonal activity, it is possible that other components contribute to chemical signaling. The contact pheromone of *Tegenaria atrica* (Agelenidae) is a complex mixture of fatty acids on the silk and cuticle of females, including four compounds, each of which when tested alone elicits courtship by males (Prouvost et al. 1999; Trabalon et al. 2005).

Some spider pheromones appear to have multiple functions. In *A. aperta,* a single compound both attracts males and induces courtship (Papke et al. 2001). In *Linyphia triangulosa,* a single compound elicits courtship, but its breakdown products function as airborne attractants (Schulz and Toft 1993; Schulz 2013). However, different pheromone components or combinations of components may have distinct functions. In studies of spider pheromones, commonly only one behavioural response, or type of bioassay for pheromonal activity, is reported. For example, the contact pheromone of *L. hasselti* elicits strong overall activity of males, but specific courtship behaviours were not recorded, and the pheromone was not tested as an attractant (Jerhot et al. 2010). Perhaps the *L. hasselti* contact pheromone primarily stimulates male activity on the web, but other components are necessary to elicit specific courtship behaviours like silk-wrapping, as we found for MB-MP-S in our study with *L. hesperus*. In the genus *Latrodectus*, pheromones on the silk of females not only attract males and elicit courtship behaviour, but also provide information about the age, reproductive status, and body condition of the female (Andrade and Kasumovic 2005; Stoltz et al. 2007; MacLeod and Andrade 2014). Possibly, each of several components, or complex blends thereof, have distinct functions or carry specific information about the signaler. As more spider pheromones are identified, and tested for a variety of functions, we will discover whether multi-component pheromones and/or multiple pheromones with distinct functions are common.

One challenge of studying the pheromones of spiders is that numerous and varied compounds are present on the silk and cuticle (Schulz 2013). Behavioural responses of males may be subtle or occur only

under certain conditions, and thus may not always be the best indicators of pheromonal activity. In studies of insect semiochemicals, samples are routinely subjected to gas chromatographic-electroantennographic detection (GC-EAD) analyses (Arn et al. 1975). This method allows researchers to determine the chemicals in the gas chromatogram that elicit responses from the insect antenna, thus limiting the number of compounds to be tested for pheromonal activity to only those that the insect can sense. Analogous electrophysiological screening for spider pheromones would be highly advantageous, but spider olfaction and contact chemoreception are still poorly understood, and only few examples are reported in the literature. In *Cupiennius salei* (Ctenidae), contact chemoreceptors on the dorsal surface of the male's pedipalps detect the female sex pheromone dimethyl citrate (Tichy et al. 2001). Similarly, results of 'electrotarsograms' with the tarsi of female *Pholcus beijingensis* implied the presence of receptors that detected the volatile male aphrodisiac pheromone (*Z*)-9-tricosene (Xiao et al. 2010). 'Electrolegograms' have already been developed for whip spiders (Amblypigi; Hebets and Chapman 2000). Analogous work focused on spider neurophysiology should supplement and corroborate the results of behavioural biossays of spider pheromones, and lead to a more nuanced understanding of spider chemical signaling.

Acknowledgements We thank the Tsawout First Nation for permission to do field work on their lands, the Capital Regional District for granting permits to collect spiders from Island View Beach; Maydianne Andrade, Bernard Roitberg, Tanya Stemberger, Samantha Vibert, and two anonymous reviewers for comments that improved the manuscript; Huimin Zhai for comments on the chemistry section; and Susan Chen and Elaine Wu for assistance with spider rearing and maintenance. Funding was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Discovery Grant and an NSERC-Industrial Research Chair to G.G., with Contech Enterprises Inc. and Global Forest Science as industrial sponsors.

References

Andrade M, Kasumovic M (2005) Terminal investment strategies and male mate choice: extreme tests of Bateman. Integr Comp Biol 45:838–47. doi: 10.1093/icb/45.5.838

Arn H, Städler E, Rauscher S (1975) The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Z Naturforsch 30c:722–725

Blumstein DT, Daniel JC, Evans CS (2012) Jwatcher. Available online: http://www.jwatcher.ucla.edu

Chinta SP, Goller S, Lux J, Funke S, Uhl G, Schulz S (2010) The sex pheromone of the wasp spider *Argiope bruennichi*. Angew Chemie Int Ed 49:2033–2036. doi: 10.1002/anie.200906311

Gaskett AC (2007) Spider sex pheromones: emission, reception, structures, and functions. Biol Rev Camb Philos Soc 82:27–48. doi: 10.1111/j.1469-185X.2006.00002.x

Hebets EA, Chapman RF (2000) Electrophysiological studies of olfaction in the whip spider *Phrynus parvulus* (Arachnida, Amblypygi). J Insect Physiol 46:1441–1448. doi:10.1016/S0022-1910(00)00068-8

Jerhot E, Stoltz JA, Andrade MCB, Schulz S (2010) Acylated serine derivatives: A unique class of arthropod pheromones of the Australian redback spider, *Latrodectus hasselti*. Angew Chemie Int Ed 49:2037–2040. doi:10.1002/anie.200906312

Kasumovic M, Andrade MCB (2004) Discrimination of airborne pheromones by mate-searching male western black widow spiders (*Latrodectus hesperus*): species- and population-specific responses. Can J Zool 82:1027–1034. doi:10.1139/z04-081

MacLeod EC, Andrade MCB (2014) Strong, convergent male mate choice along two preference axes in field populations of black widow spiders. Anim Behav 89:163–169. doi:10.1016/j.anbehav.2013.12.023

Papke MD, Riechert SE, Schulz S (2001) An airborne female pheromone associated with male attraction and courtship in a desert spider. Anim Behav 61:877–886. doi:10.1006/anbe.2000.1675

Prouvost O, Trabalon M, Papke M, Schulz S (1999) Contact sex signals on web and cuticle of *Tegenaria atrica* (Araneae, Agelenidae). Arch Insect Biochem Physiol 40:194–202. doi:10.1002/(SICI)1520-6327(1999)40:4<194::AID-ARCH4>3.0.CO;2-P

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, URL http://www.r-project.org

Ross K, Smith RL (1979) Aspects of the courtship behavior of the black widow spider, *Latrodectus hesperus* (Araneae: Theridiidae), with evidence for the existence of a contact sex pheromone. J Arachnol 7:69–77

Schulz S (2013) Spider pheromones - a structural perspective. J Chem Ecol 39:1–14. doi:10.1007/s10886-012-0231-6

Schulz S, Toft S (1993) Identification of a sex pheromone from a spider. Science 260:1635–1637. doi:10.1126/science.260.5114.1635

Scott C, Vibert S, Gries G (2012) Evidence that web reduction by western black widow males functions in sexual communication. Can Entomol 144:672–678. doi:10.4039/tce.2012.56

Stoltz JA, McNeil JN, Andrade MCB (2007) Males assess chemical signals to discriminate just-mated females from virgins in redback spiders. Anim Behav 74:1669–1674. doi:10.1016/j.anbehav.2007.03.011

Tichy H, Gingl E, Ehn R, Papke M, Schulz S (2001) Female sex pheromone of a wandering spider (*Cupiennius salei*): identification and sensory reception. J Comp Physiol A 187:75–78. doi:10.1007/s003590000175

Trabalon M, Niogret J, Legrand-Frossi C (2005) Effect of 20-hydroxyecdysone on cannibalism, sexual behavior, and contact sex pheromone in the solitary female spider, *Tegenaria atrica*. Gen Comp Endocrinol 144:60–6. doi:10.1016/j.ygcen.2005.04.011

Uhl G, Elias DO (2011) Communication. In: Herberstein ME (ed) Spider Behaviour: Flexibility and Versatility. Cambridge University Press, Cambridge, pp 127–189

Van den Dool H, Kratz P (1963) A generalization of retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr A 11:463–471. doi:10.1016/S0021-9673(01)80947-X

Xiao Y, Zhang J, Li S (2010) Male-specific (Z)-9-tricosene stimulates female mating behaviour in the spider *Pholcus beijingensis*. Proc R Soc B Biol Sci 277:3009–3018. doi:10.1098/rspb.2010.0763

Xiao Y, Zhang J, Li S (2009) A two-component female-produced pheromone of the spider *Pholcus beijingensis*. J Chem Ecol 35:769–778. doi:10.1007/s10886-009-9660-2

Electronic Supplementary Material

File ESM1 General methods and instrumentation for syntheses, a representative synthesis of ester-amide mixtures, and synthesis of *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester

Video ESM2 A *Latrodectus hesperus* male silk-wrapping a filter paper treated with methanol extract of a virgin female's web, first at normal speed, then slowed to half-speed

For Journal of Chemical Ecology

Supplementary Material

N-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester – Pheromone Component of Western Black Widow Females?

Catherine Scott (✉) · Sean McCann · Regine Gries · Grigori Khaskin · Gerhard Gries

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada.
email: ces14@sfu.ca

General Methods and Instrumentation for Syntheses Oven-dried glassware was assembled hot under Ar flow, and maintained under Ar. Liquids were transferred by cannula under Ar pressure. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Bruker BioSpin-400 spectrometer (Bruker, Rheinstetten, Germany) (at 400 MHz for ^{1}H, 100 MHz for ^{13}C) with $CDCl_3$ as a solvent; chemical shifts are reported in ppm relative to TMS (^{1}H, δ 0.00) and $CDCl_3$ (^{13}C, δ 77.00).

Representative Synthesis of Ester-Amide Mixtures Anhydrous K_2CO_3 (20 mg; 1.5 mmol) was mixed with *L*-serine methyl ester hydrochloride (100 mg; 0.65 mmol) (Sigma, St. Louis, MO 63103, USA) in dichloromethane (3 ml). Then 2-methylbutyric acid (60 ml; 0.65 mmol) (Aldrich, Milwaukee, WI, 53201, USA) and isobutyric acid (50 ml; 0.65 mmol) (Aldrich) were added, followed by addition of *N,N′*-dicyclohexylcarbodiimide (0.54 g; 4 equivalents) (Aldrich) and 4- dimethylaminopyridine (DMAP; 2 mg). After stirring the mixture for 6 h, products were separated from urea and unreacted materials by flash chromatography using ethyl acetate as eluent. The ethyl acetate solution was concentrated *in vacuo*, and ether was added. Insoluble precipitate was removed by filtration, and filtrate was concentrated, yielding 90 mg of a mixture containing di-acylated compounds (70%, GC).

Synthesis of N-*3-Methylbutanoyl*-O-*methylpropanoyl*-L-*serine methyl ester (MB-MP-S; Fig. 1)* MB-MP-S was synthesized according to Jerhot et al. (2010). Isobutyric anhydride (9.12 mmol; 1.51 ml, Aldrich) was added to *N*-(*tert*-butoxycarbonyl)-*L*-serine methyl ester (0.5 g; 2.28 mmol) (**1**) (Sigma). While stirring the reaction mixture, anhydrous potassium carbonate (1.26 g; 9.12 mmol) and then DMAP (2 mg) were added. Subsequently, the mixture was stirred for 48 h at ambient temperature. Products were extracted with ether (2 × 50 ml). Extracts were washed with a saturated aqueous sodium bicarbonate solution, water, and brine, and were dried ($MgSO_4$, anh.) and concentrated *in vacuo*. *N*-(Boc-)-*O*-isobutyryl-*L*-serine methyl ester (**2**) was purified by flash chromatography with 25% ether in hexane as eluent; quantitative yield (99% pure, GC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.27 (d, J = 7.8 Hz, 1H), 4.58 (m, 1H), 4.45 (dd, J = 11.2, 4.0 Hz, 1H), 4.29 (dd, J = 11.3, 3.6 Hz, 1H), 3.75 (s, 3H), 2.54 (hept, J = 7.0 Hz,1H), 1.45 (s, 9H), 1.14 (dd, J = 7.0, 3.1 Hz, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz): 176.5, 170.3, 155.1, 80.3, 64.0, 53.02, 52.6, 33.8, 28.3, 18.9, 18.8. The protective carbamate group was removed from **2** by treating it with excess of trifluoric acid (5 ml) in dichloromethane (25 ml) at room temperature for 1 h. Solvents were removed *in vacuo*, and the crude mixture was used without purification for N-acylation. To this effect, 0.95 ml (7.80 mmol) of isovaleryl chloride (Aldrich) was added dropwise at 0 °C to a stirred solution of *O*-isobutyryl-*L*-serine methyl ester and triethylamine (2.0 ml; 14.3 mmol) in dichloromethane (10 ml). The reaction mixture was warmed to room temperature and extracted with ether (3 × 25 ml). The extract was washed with 2N aqueous HCl, a saturated sodium bicarbonate solution, and brine. After drying the extract with anh. $MgSO_4$, it was filtered and concentrated, and the final product was purified by column chromatography with hexane, and hexane/ether (5:95 and 50:50%) as consecutive eluents. Yield of the pure di-acylated serine ester **3** was 0.55 g (2.01 mmol, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.22 (d, J = 7.2 Hz, 1H), 4.86 (dt, J = 7.6, 3.7 Hz, 1H), 4.47 (dd, J = 11.4, 4.0 Hz, 1H), 4.34 (dd, J = 11.4, 3.4 Hz, 1H), 3.76 (s, 3H), 2.53 (hept, J = 7.0 Hz,1H), 2.09-2.12 (m. 3H), 1.13 (dd, J = 7.0, 2.4 Hz, 6H), 0.93-0.98 (m, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz): 176.6, 172.2, 170.1, 63.7, 52.7, 51.8, 45.7, 33.8, 26.1, 22.4, 22.3, 18.9, 18.8. HREIMS: *m*/*z* calcd. for $C_{13}H_{24}NO_5$ $[M+H]^+$ 274.1649, found 274.1641; calcd. for $C_{13}H_{23}NNaO_5$ $[M+Na]^+$ 296.1468, found 296.1460; calcd. for $C_{13}H_{23}KNO_5$ $[M+K]^+$ 312.1208, found 312.1203.

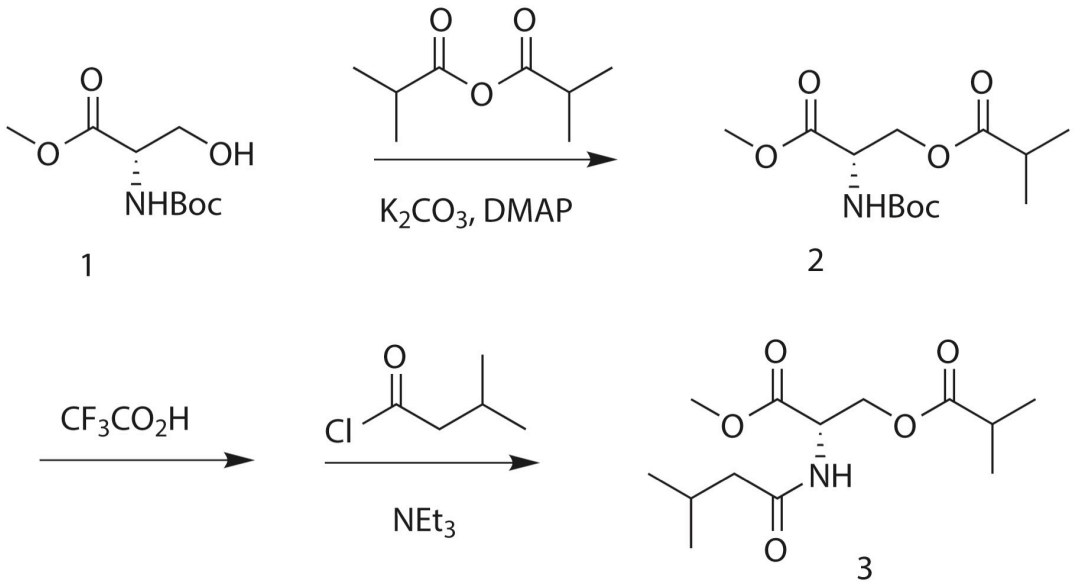

Fig. 1 Synthesis of *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (**3**), a pheromone component of female *Latrodectus hesperus;* DMAP = 4-dimethylaminopyridine

References

Jerhot E, Stoltz JA, Andrade MCB, Schulz S (2010) Acylated serine derivatives: A unique class of arthropod pheromones of the Australian redback spider, *Latrodectus hasselti*. Angew Chemie Int Ed 49:2037–2040