Pheromones (2)

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Acylated Serine Derivatives: A Unique Class of Arthropod Pheromones of the Australian Redback Spider, *Latrodectus hasselti***

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Spiders are of great ecological importance, as they are the major predators of terrestrial insects. Many species use chemical communication along with other cues during courtship, but these systems are not well investigated, especially compared to other arthropods, such as insects or mites. Spiders commonly make use of silk; mate attraction, initiation of courtship, and other critical aspects of spider reproduction are apparently triggered by pheromones on the silk or the body of the female.[1,2] Most of the handful of spider pheromones that have been identified are structurally different from those known from insects. Herein, we report the identification of the unusual methyl ester of *N*-3-methylbutyryl-*O*-(*S*)-2-methylbutyryl-L-serine **3** as a female sex pheromone of the Australian redback spider, *Latrodectus hasselti*. This compound represents a new class of secondary metabolites, N,O-diacylated serine derivatives.

Despite widespread reports of the importance of chemical communication in spiders,[1] only five spider pheromones have been identified to date. The estolide of two (*R*)-3-hydroxybutyric acid units, (*R*,*R*)-3-(3-hydroxybutyryloxy)butyric acid, induces web reduction behavior in males of the linyphiid *Linyphia triangularis*.[3] The *S*-enantiomer of asymmetric dimethyl citrate releases courtship in males of the American wandering spider, *Cupiennius salei*.[4] 8-Methyl-2-nonanone, which is emitted by females, attracts males and induces courtship response in the desert spider *Agenelopsis aperta*.[5] A mixture of fatty acids has been reported to act as courtship pheromone in the agenelid spider, *Tegenaria atrica*,[6,7] whilst (*E*,*E*)-farnesyl acetate and hexadecyl acetate were identified as pheromone of *Pholcus beijingensis*.[8]

Black widow spiders (*Latrodectus*, Theridiidae) are of particular interest for humans because of their neurotoxic venom[9] and the invasive nature of several species, which tend to live in close association with humans. Behavioral investigations have shown that webs of female black widows contain pheromones that attract males, trigger courtship, and allow discrimination of female reproductive status, population of origin, and species.[10–14] For example, males of *L. hesperus* and *L. hasselti* are attracted to webs of females by volatile cues.[11,12]

L. hasselti males display courtship on webs of adult unmated females, whilst webs rebuilt by females after mating and those of juveniles or males cause no response.[14] Experiments have shown that a pheromone is deposited on the web by unmated adult females. This compound can be extracted with methanol, and it elicited searching and courtship behavior in males when applied to filter paper.[14] Therefore we became interested in the identification of this courtship-inducing pheromone.

Silk from unmated (active) and mated (inactive) females were collected during a period of several weeks and extracted with methanol. Both extracts were then analyzed to identify compounds specific for active silk; that is, putative pheromone candidates. After a derivatization step, the extracts were analyzed by GC-MS with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) to volatilize polar components (Figure 1). This procedure ensures a relatively broad overview of the compounds present on the silk. Whilst several lipids, carbohydrates, amino acids, and acids were present in both types of silk, two compounds, **A** and **B**, proved to be specific for the extract of active silk. The analysis of the mass

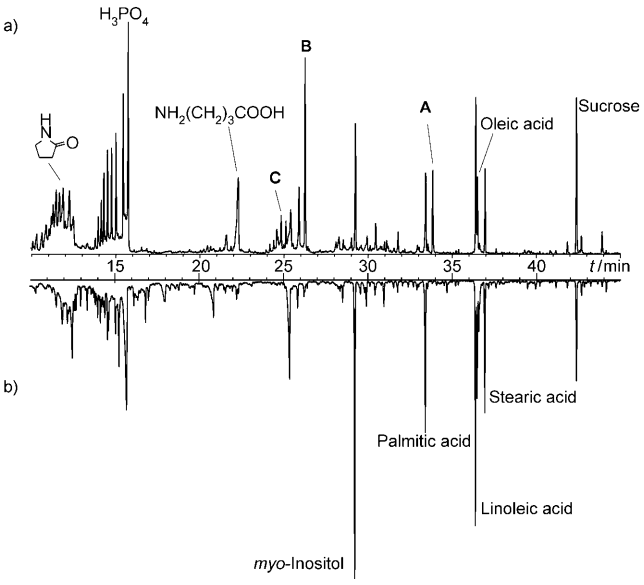

Figure 1. Gas chromatograms of methanol extracts of *Latrodectus hasselti* silk after trimethylsilylation with MSTFA. a) Silk of unmated females; b) silk of mated females.

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spectrum and comparison with a synthetic sample showed that compound **A** was *N*-acetyldopamine (**4**). The mass spectrum of the second compound **B** was unusual (Figure 2), as it showed that no trimethylsilyl groups are present, which indicates the absence of hydroxy, amine, or

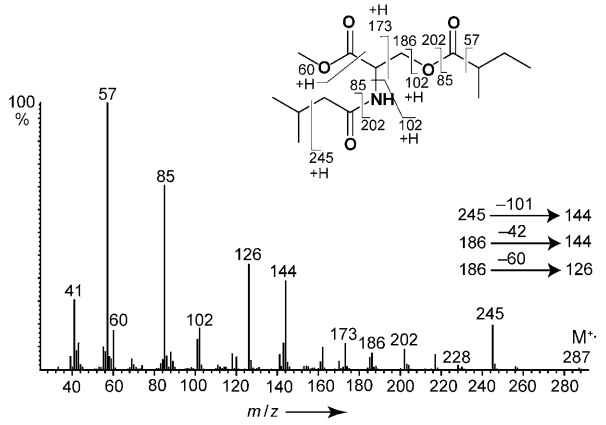


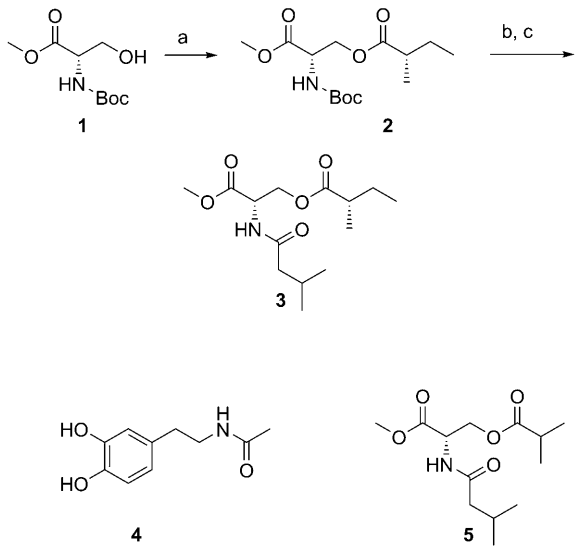
Figure 2. Mass spectrum and fragmentation pattern of *N*-3-methylbutyryl-*O*-2-methylbutyryl-L-serine (**3**), a silk constituent of juvenile female *Latrodectus hasselti*.

acid groups. Additional analyses by GC-CI-MS and high-resolution GC-MS indicated the molecular ion to be *m*/*z* 287.1754, which corresponds to a molecular formula of $C_{14}H_{25}N_1O_5$ (calc. HR-MS 287.1733) with three double-bond equivalents. The fragments *m*/*z* 57 (C_4H_9) and 85 ($C_5H_9O_1$) suggested the presence of a pentanoyl group. This group appears to be present twice, one being bound to an oxygen and the other to a nitrogen atom, because two ions at *m*/*z* 102 were found: 102.05658 corresponding to $C_5H_{10}O_2$ (calc. 102.06808) and 102.09259 corresponding to $C_5H_{12}N_1O_1$ (calc. 102.09188). The ion *m*/*z* 60 ($C_2H_4O_2$) as well as small ions at $[M-32]^+$ and $[M-59]^+$ suggested the presence of a methyl ester group.

Extracts of both types of silks were then analyzed by NMR spectroscopy. Although a wide range of signals was detected, a CO-CH(NH)-CH_2 system was present exclusively in the active silk (see the Supporting Information). Therefore, the mass spectral and also the NMR data seemed to be consistent with a serine methyl ester acylated at both the O- and N-atoms with a pentanoyl group.

To verify this proposal, serine methyl ester was acylated with a mixture of pentanoic acid, 2-methylbutyric acid, 3-methylbutyric acid, and pivalic acid, the four possible C_5 acid isomers. One of the compounds that was formed showed an identical mass spectrum and gas chromatographic retention time with the natural product, thus confirming the general proposal. After the synthesis of several structural isomers, it turned out that the natural compound was *N*-3-methylbutyryl-*O*-2-methylbutyrylserine methyl ester (**3**), which exhibited identical analytical data to the natural compound. This compound is an unusual unsymmetrically *N*,*O*-acylated serine derivative that has not been found in nature to date. Furthermore, minor amounts of the related *N*-3-methylbutyryl-*O*-methylpropanoylserine methyl ester (**5**; compound **C** in Figure 1) were also present in the active silk.

Several enantiomers of **3** were then synthesized for evaluation of their biological activity. The *tert*-butoxycarbonyl-protected L-serine methyl ester (**1**) was acylated with 2-methylbutyric acid. After removal of the protecting group, the (*S*)-3-methylbutyryl side chain was introduced using (*S*)-3-methylbutyric acid chloride (Scheme 1). Similarly, several other enantiomers and diastereomers were synthesized.



Scheme 1. Synthesis of **3** and structures of other female-specific web constituents. a) (*S*)-$CH_3CH_2CH(CH_3)COCl$, DMAP; b) CF_3COOH; c) $CH_3CH(CH_3)CH_2COCl$, DMAP. DMAP = 4-dimethylaminopyridine.

The absolute configuration of natural **3** produced by the spider was elucidated using chiral gas chromatography. All four enantiomers could be partly separated on a Lipodex phase. Only the L,*S* enantiomer is produced by the spider (Figure 3). This configuration was expected as it is the configuration of serine and naturally occurring 2-methylbutyric acid, which is commonly formed in nature from isoleucine.

A bioassay[14] was used to test the activity of the synthesized compounds with male *L. hasselti*. *N*-Acetyldopamine (**4**) was inactive; it evoked no more interest from males than did solvent controls. In contrast, the L,*S* ester **3** triggered high levels of activity in the males when 100 μg were used. This activity was strongly dependent on the stereochemistry of **3**. Only the L,*S* compound initiated a response in the males, whilst all other isomers tested were ignored (Figure 4). The correct stereochemistry is obviously highly important in both the acyl and amino acid moieties to obtain a positive response. Even a mixture of two stereoisomers epimeric in the side chain, (L,*S*)-**3** and (L,*R*)-**3**, or (L,*S*/*R*)-**3**, is not active. This finding might indicate that the wrong stereoisomer inhibits the positive response of the males, which is a phenomenon not reported from spiders before but repeatedly found in insect pheromone systems.[15] The minimal amount necessary to

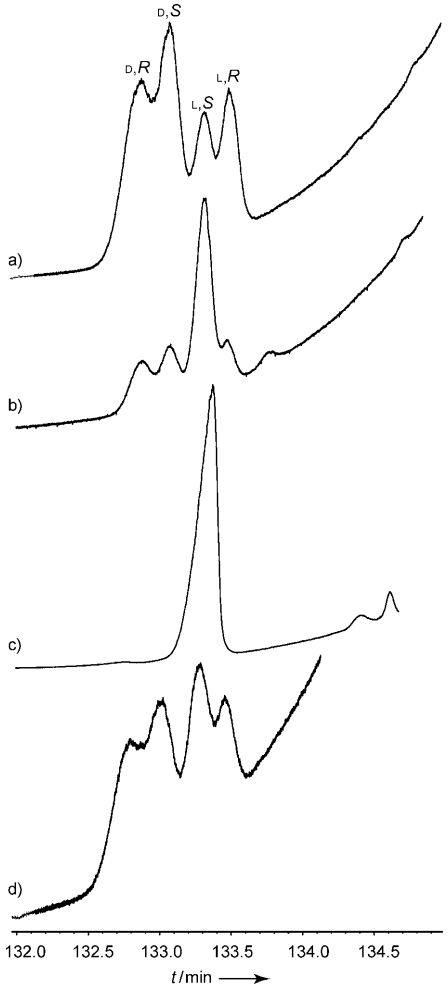


Figure 3. Gas chromatograms of naturally occurring **3** and synthetic reference compounds on a chiral Lipodex G phase. a) All stereoisomers of **3**; b) co-injection of (L,*S*)-**3** and all stereoisomers; c) natural sample of **3**; d) co-injection of natural sample and all stereoisomers.

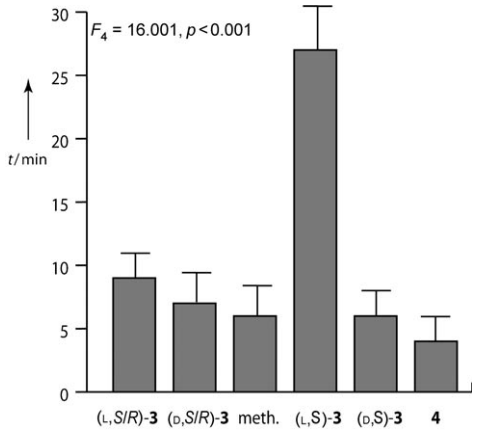


Figure 4. Bioassay results. Time that males spent searching or courting on filter papers treated with 100 µL of 1 µg mL^{-1} test compound. meth.: methanol, solvent.

evoke a response in our test was 25 µg (L,*S*)-**3**, and 1 µg proved to be inactive (Supporting Information, Figure S1).

In conclusion, we have identified and synthesized the first amino acid derived sex pheromone of spiders, *N*-3-methylbutyryl-*O*-(*S*)-2-methylbutyryl-L-serine methyl ester (**3**). Together with **5**, this pheromone defines a new class of natural products, *N*,*O*-bisacylated serine derivatives. The only other known amino acid pheromones are the methyl esters of valine, leucine, and isoleucine that are active in a single insect genus, the scarab beetles *Phyllophaga* spp.[16–18] The direct use of amino acids derivatives as sex pheromones may have important implications for the evolution of sexual communication in these species. Pheromone production was historically thought to entail relatively little physiological cost for most insects, although costs are critical to the evolutionary maintenance of a honest signal.[19] This view has been challenged recently, but there have been very few demonstrations of costly pheromone production.[20,21] Production costs of amino acid derived pheromones, such as that identified herein, may arise directly from trade-offs with other physiological processes. Thus future studies with a focus on production of this new class of pheromones may be fruitful.

Four of the seven now-known pheromones from spiders are biosynthetically closely related to primary metabolites as amino acids (**3**), citric acid (cupilure;[4] and trimethyl methylcitrate reported in the previous Communication),[22] or 3-hydroxybutyric acid (fatty acid metabolism), whilst one is a mixture of fatty acids and the rest more similar to typical insect pheromones. Although it is too early to establish as a general trait that spider pheromones are formed directly from primary metabolites, it remains important to identify additional spider pheromones and to investigate their biosynthesis.

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