Identification of Cuticular And Web Lipids of The Spider *Argiope Bruennichi*

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Abstract

Emerging evidence shows that the cuticular and silk lipids of spiders are structurally more diverse than those of insects, although only a relatively low number of species have been investigated so far. As in insects, such lipids might play a role as signals in various context. The wasp spider *Argiope bruennichi* has probably the best investigated chemical communication system within spiders, including the known structure of the female sex pheromone. Recently we showed that kin-recognition in *A. bruennichi* is mediated through the cuticular compounds consisting of hydrocarbons and to a much larger proportion of wax esters. By use of MS and various derivatization methods these esters were identified here to be esters of 2,4-dimethylalkanoates with varying chain length and 1-alkanols, such as tetradecyl 2,4-dimethylheptadecanoate. A representative enantioselectively synthesis to this compound was performed which proved the identifications and allowed to postulate the natural enantiomer to have (2*R*,4*R*)-configuration. Cuticular profiles of the silk and cuticula of females were similar, while male cuticular profiles differed quantitatively from those of females. In addition, minor female specific 4-methylalkyl esters were detected.

Introduction

Chemical communication is the predominant form of communication in arthropods, as their often small size limits the efficacy of, for example, visual or acoustic communication (Greenfield 2002). Often, cuticular waxes, besides their role as water barrier (Gibbs and Rajpurohit 2010), have important communicative functions, as is well documented for insects (Howard and Blomquist 2005, Blomquist and Bagnères 2010). In arthropods outside the insects, however, cuticular chemistry and its role in communication is much less investigated.

In spiders, for instance, the occurrence of volatile pheromones that function in long-range mate attraction is well known (Schulz 2004; Gaskett 2007; Schulz 2013; Fischer 2019), but the chemistry of external lipids of the cuticle and especially the silk have been investigated in only few species. Generally, the composition of cuticular lipids seems to be more divers in spiders than in insects. In a couple of previous studies it was shown that next to alkanes, the dominating compound class within insects (Blomquist and Bagnères 2010), major components can be long-chain, methyl-branched methyl ethers, as found in linyphiids (Schulz and Toft 1993), *Nephila clavipes* (Schulz 2001), and *Tetragnatha* (Adams et al. 2021), fatty acids and aliphatic saturated alcohols in various other species (Trabalon et al. 1996; Trabalon et al. 1997; Prouvost et al. 1999; Trabalon and Assi-Bessekon 2008; Trabalon 2011), and esters. Such esters can be simple methyl esters of fatty acids (Prouvost et al. 1999; Trabalon 2011), but some species have evolved more elaborate compounds. The social spider *Anelosimus eximus* produces a complex array of long-chain methyl branched *n*-propyl esters, e. g. propyl 4,20-dimethylhentriacontanoate, as epicuticular wax (Bagnères et al. 1997). Another type of esters is present on the cuticle of the spider *Argyrodes elevatus* (Theridiidae), short, partly branched acids conjugated to longer acids, occurring in completely different sex-specific mixtures of few compounds. These include undecyl 2-methyltridecanoate in males and 2,8-dimethylundecyl 2,8-dimethylundecanoate and heptadecyl 4-methylheptanoate in females. Other

spiders exclusively use insect-type hydrocarbons (Trabalon and Assi-Bessekon 2008; Grinsted et al. 2011). As in insects, these cuticular constituents were discussed to transmit intraspecific information in various ways (Witte et al. 2009; Trabalon and Bagnères 2010; Xiao et al. 2010; Grinsted et al. 2011; Beeren et al. 2012; Schulz 2013; Ruhland et al. 2019; Adams et al. 2021).

Among spiders, the chemical communication system of the European wasp spider *Argiope bruennichi* is probably one of the best studied. As entelegyne spiders genital structures are paired in both sexes, but only one can be used during a copulation (Foelix 2011). After sperm transfer, males effectively plug the genital opening of the female with parts of their genitalia that are useless thereafter (Nessler et al. 2007; Uhl et al. 2010). Most males fall victim to sexual cannibalism after their first copulation but those that copulate twice can monopolize paternity with a female. As a consequence *A. bruennichi* is restricted to a maximum of two copulations. Females are known to produce a volatile pheromone, trimethyl methylcitrate, that attracts males in the field and elicit courtship behavior (Chinta et al. 2010). Behavioral experiments indicate, however, that males also use the pheromone to assess a female`s condition, age and mating status. For example, field observations (Welke et al. 2012) and laboratory experiments (Cory and Schneider 2016) demonstrated that males are more likely to be monogynous when the female is relatively heavy and old. This is likely due to females modulating pheromone release according to their state (Cory and Schneider 2016). Interestingly, mating experiments with siblings and non-siblings indicate that males are also capable of kin recognition. Male *A. bruennichi* readily copulate with sisters in laboratory mating trials, but terminate copulation earlier as compared to unrelated females (Welke and Schneider 2010). Consequently, they survive copulation more often (Welke and Schneider 2010), allowing them to leave and search for another female. Recently some of us showed that kin-recognition might be mediated by cuticular compounds occurring in family-specific bouquets and suggested an important role of unusual long-chain esters in kin recognition (Weiss and Schneider 2021). We present here the identification and synthesis of the dominating group of compounds of these bouquets, long chain esters of 2,4-dimethyl-branched acids with mostly unbranched fatty alcohols and clarify their distribution.

Methods And Materials

Spider Extracts. Subadult male and female *Argiope bruennichi* (Scopoli, 1772) (Araneae, Araneidae) were collected from natural meadows in Northern Germany (Buxtehude, Harmstorf, Pevestorf, Lower Saxony; Wedel, Schleswig-Holstein), between 24 June and 5 July 2019. *A. bruennichi* is common throughout Europe and its collection requires no permits. Spiders were transferred to the laboratory at the University of Hamburg, Germany, where they were individually housed in upturned plastic cups (250 or 500 mL depending on the spider’s size) with a hole in the bottom stuffed with cotton wool. Spiders were kept under natural light conditions at a constant temperature of 25°C and a relative humidity of 45 %. Twice a week, subadult spiders were provided with approximately 15 *Drosophila* spp. and adult females with three *Calliphora* sp. houseflies. Adult males were fed with approximately 10 *Drosophila* spp. once a week. All spiders were provided with water from a sprayer at least six days a week. Twenty virgin male (mean age ± SD: 13.5 ± 1.2 days) and 30 virgin female *A. bruennichi* (mean age ± SD: 11.8 ± 0.9 days) were used for chemical analysis.

Analysis by GC/MS. Spiders were cold anesthetized and stored at − 25° C until analysis. Cuticular extracts were prepared by individually placing females in 3 ml of dichloromethane (DCM; GC/MS grade, Merck, Darmstadt, Germany) and males in 1 ml DCM for one hour. Twenty virgin male (mean age ± SD: 13.5 ± 1.2 days) and 30 virgin female *A. bruennichi* (Mean age ± SD: 11.8 ± 0.9 days) were used for chemical analysis. To obtain silk samples, the females were placed into clean Perspex frames (35 x 35 x 6 cm) and allowed to build a web. After 24 h, females were removed from their webs and the silk was collected by slowly winding it around a glass Pasteur-pipet washed with ethanol. The tip of the pipet holding the silk was then snapped off into a small glass vial. Males and females were placed in clean glass vials and cold anaesthetized. All samples were stored at − 25°C until analysis. Cuticular extracts were prepared by individually placing females in 3 ml of dichloromethane (DCM; GC/MS grade, Merck, Darmstadt, Germany) and the smaller males in 1 ml DCM for one hour. Silk samples were extracted in 1 ml DCM. Extracts were concentrated by evaporation at room temperature to approximately 90 µl (females) and 50 µl (males and silk), respectively. An aliquot of 1 µl of each sample was analyzed by GC/MS on a Shimadzu GCMS-QP2010S system (Shimadzu Corporation, Kyoto, Japan). After individual analyses, the female silk and body extracts and male samples were pooled separately for further chemical identification. For the analysis of samples of individuals, the gas chromatograph was equipped with a SH-Rtx-5MS fused silica capillary column (30 x 0.25 mm ID, 0.25 µm film thickness; Shimadzu Corporation, Kyoto, Japan). The oven temperature raised from 80 to 260°C at a constant heating rate of 30°/min and from 260 to 300°C at a constant rate of 1°/min, with a 1-minute initial isothermal and a 10-minute final isothermal hold. A split-splitless injector was operated at 250°C in the splitless mode. Carrier gas was helium at a constant flow rate of 1 ml/min. The ionization voltage of the electron ionization mass spectrometer was 70 eV. Source temperature was 200°C and interface temperature was 280°C. Data acquisition and storage were performed with the software GCMSsolution (Version 4.45; Shimadzu Corporation, Kyoto, Japan). Peak areas were obtained by manual integration using the GCMSsolution software. Linear retention indices of all substances were calculated according to van den Dool and Kratz (1963). *n*-Alkanes were identified by comparing their mass spectra with those of authentic reference compounds. Alkenes were identified by their typical mass spectra. Methyl-branched hydrocarbons were identified by diagnostic ions resulting from their typical α-cleavage at the position of the methyl-branch and by a fragment at M-15 if the molecular ion was not detected. Moreover, their linear retention indices were compared to those published by (Carlson et al. 1998; El-Sayed 2019). Other compounds were tentatively identified by comparing their mass spectra and linear retention indices with those of a databases (NIST 08 mass spectral library 2008). Mean relative peak areas were calculated by standardizing total mean peak areas to 100%. GC/MS analysis of pooled samples were performed on a GC 7890A coupled to a MSD 5975C (Agilent Technologies, Germany). The gas chromatograph was equipped with a HP-5 MS column (Agilent, 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness) with helium as the carrier gas. The combined samples were analyzed with a temperature program of 50°C for 5 min that increased by 3°C/min to 320°C with a final hold time of 10 min. Derivatized samples were analyzed with a temperature program of 50°C for 5 min, increased by 5°C/min to 320°C with a final hold time of 10 min. To compare the chemical composition between samples, relative peak areas were calculated for each of the 20 male and 30 female and web silk extracts

separately by standardizing the total peak area of each extract to 100%. Values given in Table 1 are means ± standard deviation (SD). GC on chiral phases was performed using BetaDex™ 225 (Sigma, 30.0 m x 0.25 mm) or Hydrodex β-6TBDM (Machery and Nagel, 30.0 m x 0.25 mm) phases with a flow of 1.5 ml min^{-1} hydrogen as the carrier gas and a flame ionization detector. Individual time programs are given at the appropriate Fig. 6 and Fig. S 5 in the Supporting Information.

Microreactions of Extracts. The extracts were derivatized in microreactions to obtain more structural information about methyl-branch positions of the acid and alcohol components of the long chain esters. Extracts were transesterified with trimethylsulfonium hydroxide (TMSH) (Müller et al. 1990) to form the corresponding methyl esters and free alcohols (Fig. 1). Subsequent transesterification of the methyl esters with sodium 3-pyridinylmethylate to form the corresponding pyridin-3-ylmethyl esters was performed (Harvey 1982) as well as esterification of the free alcohols with nicotinic acid to form the corresponding esters (Vetter and Meister 1981; Harvey 1991).

Transesterification with Trimethylsulfonium Hydroxide. Trimethylsulfonium hydroxide (TMSH, 100 µl, 0.25 m in methanol) was added to the natural sample (20 µl in dichloromethane) in a GC-vial (2 ml). The reaction mixture was placed in a heating block at 90°C for 6 h and regularly shaken vigorously. The solvents and reagents were removed with a stream of nitrogen and the residue was dissolved in DCM (20 µl) (Müller et al. 1990).

Transesterification with 3-Pyridinylmethanol. 3-Pyridinylmethanol (50 µl) was added to freshly cut sodium (0.2 mg) in a GC-vial (2 ml). The reaction mixture was heated to 80°C in a heating block until the sodium was dissolved. Sodium 3-pyridinylmethoxide (2 % in 3-pyridinylmethanol) was obtained as a syrupy yellow liquid. The thus prepared fresh sodium 3-pyridinylmethoxide (2 drops, 2 % in 3-pyridinylmethanol was added to the methyl ester sample (20 µl in dichloromethane) in a GC-vial (2 ml). The reaction was placed in a heating block at 80°C for 3 h and regularly shaken vigorously. Methanol (200 µl) and water (3 drops) was added to the solution and the mixture was extracted with pentane (3 × 200 µl). The pentane phases were combined, the solvent was removed in a stream of nitrogen and the residue was dissolved in DCM (20 µl).

Preparation of Nicotinates. DCM (50 µl), nicotinic acid (1 mg), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 1 mg) and 4-dimethylaminopyridine (DMAP, catalytic amount) were added to the sample of the methyl esters and free alcohols (50 µl in dichloromethane) in a GC-vial (2 ml). The reaction mixture was kept at room temperature for 2 h and regularly shaken vigorously. The solvents were removed in a stream of nitrogen and the residue was extracted with pentane (3 × 100 µl). The combined pentane phases were again concentrated in a stream of nitrogen and the residue was dissolved in DCM (50 µl).

Synthesis of 2,4-Dimethylheptadecanoic Acid. The synthesis is based on a general procedure developed by Feringa et al. for the stereoselective synthesis of homo-vicinal oligo-methyl-branched acids (Des Mazery et al. 2005; Horst et al. 2007; Ruiz et al. 2007). The synthesis of 2,4-dimethylheptadecanoic acid

(**9**) started with compound **1** (synthesis described in the SI) which underwent an enantioselective 1,4-addition with methyl magnesium bromide using Josiphos (**11**) as ligand for CuBr (Fig. 2). The resulting thioester **2** was then reduced by Pd with Et_3SiH as a hydrogen source, giving the corresponding aldehyde that underwent a Wittig reaction with $Ph_3PCHCOSEt$ (synthesis described in the SI) forming the α,βunsaturated thioester **3**. Ester **3** was used as a substrate for the second enantioselective 1,4-addition with methylmagnesium bromide to form compound **4**. Reduction with diisobutylaluminium hydride delivered the corresponding alcohol **5**. Tosylate **6** was formed by reaction with tosyl chloride that undernwent substitution by $C_{11}H_{23}MgBr$ to give compound **7**. The cleavage of the silyl ether with tetra-*n*-butylammonium fluoride (TBAF) delivered alcohol **8** that was oxidized with RuO_4 to finally furnish carboxylic acid **9**. Esterification yielded tetradecyl (2*S*,4*S*)-2,4-dimethylheptadecanoate (**10**) as final product.

General Experimental Procedures. All reactions were performed in oven dried glassware under a nitrogen atmosphere. Solvents were dried according to standard procedures. Column chromatography: silica 60 (0.063–0.200 mm, 70–230 mesh ASTM). Thin layer chromatography (TLC): Polygram® SIL G/UV silica 60, 0.20 mm. Compounds were stained with potassium permanganate solution. NMR spectra were recorded either on Avance III HD 300N (^{1}HNMR: 300 MHz, ^{13}CNMR: 76 MHz), DRX 400 (^{1}HNMR: 400 MHz, ^{13}CNMR: 101 MHz), AVII 400 (^{1}HNMR: 400 MHz, ^{13}CNMR: 101 MHz) or AVII 600 (^{1}HNMR: 600 MHz) instruments. Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (Hz). IR spectra were measured on a Bruker Tensor 27 (diamond-ATR). Mass spectra were recorded with a combination of an Agilent Technologies 5977B gas chromatograph connected to an Agilent Technologies 8860 Series MSD. Optical rotation was determined with the help of an MCP 150 polarimeter (Anton Paar) with a cell length of 1 cm (*c* given in mg/mL).

*Preparation of S-Ethyl (S)-4-((tert-Butyldiphenylsilyl)oxy)-3-methylbutanethioate (*2 *).* The synthesis was performed according to Horst et al. (2007) using (*R*)-1-[(S_P)-2-(diphenylphosphino)ferrocenyl]ethyldicyclohexylphosphine (Josiphos, **11**, 50 mg, 0.08 mmol, 0.012 eq.) and CuBr·SMe_2 (13 mg, 0.065 mmol, 0.01 eq.), methyl *tert*-butyl ether (MTBE, 50 mL), MeMgBr (7.78 mmol, solution in diethyl ether), and *S*-ethyl (*E*)-4-((*tert*-butyldiphenylsilyl)oxy)but-2-enethioate (6.48 mmol, 2493 mg, 1 eq.) to afford **2** as a colorless oil (2199 mg, 85%). $[\alpha]_D^{20}$ = −4.60 (10 mg/mL; CH_2Cl_2). FT-IR: $\tilde{\nu}$ / cm^{-1} = 2960, 2895, 2859, 1688, 1466, 1427, 1389, 1261, 1150, 1108, 1040, 1005, 941, 821, 802, 763, 741, 703, 615. ^{1}H-NMR: (400 MHz, $CDCl_3$) δ / ppm = 7.71–7.66 (m, 4H), 7.47–7.37 (m, 6H), 3.53 (dddd, *J* = 16.2, 9.9, 5.7 Hz, 2H), 2.93–2.82 (m, 3H), 2.40 (dd, *J* = 14.4, 8.4 Hz, 1H), 2.36–2.25 (m, 1H), 1.26 (t, *J* = 7.4 Hz, 3H), 1.08 (d, *J* = 2.8 Hz, 9H), 0.98 (d, *J* = 6.6 Hz, 3H). ^{13}C-NMR, DEPT: (101 MHz, $CDCl_3$) δ / ppm = 199.2 (C = O), 135.7 (CH_{Ar}), 135.7 (CH_{Ar}), 133.8 (CH_{Ar}), 133.8 (C_{Ar}), 129.7 (CH_{Ar}), 127.7 (CH_{Ar}), 68.0 (CH_2), 47.7 (CH_2), 33.9 (CH), 27.0 (CH_3), 23.4 (CH_2), 19.4 (C), 16.6 (CH_3), 14.9 (CH_3). EI-MS (70 eV): *m/z* = 344 (23), 343 ([*M* − ^{t}Bu]$^+$, 76), 244 (26), 243 (100), 197 (13), 183 (39), 181 (22), 135 (25), 137 (23), 105 (16). The enantiomeric excess (ee) was determined by cleavage of the silyl ether using tetrabutylammonium fluoride (TBAF) (Horst et al. 2007). The formed lactone was separated by GC on a

chiral phase (30.0 m x 0.25 mm, initial temp. 50°C then 10°C min^{-1} to final temp. 160°C, Fig. S 5). Retention time, 13.75 min (minor), 13.85 (major) showed 94% ee (Lit.: 98% ee Horst et al. 2007).

*Preparation of S-Ethyl (S,E)-6-((tert-Butyldiphenylsilyl)oxy)-5-methylhex-2-enethioate (*3*).* The synthesis was performed as described by Horst et al. (2007) using Et_3SiH (36.69 mmol, 4266 mg, 3 eq.), **2** (12.23 mmol, 4900 mg, 1 eq.) and 10% Pd/C (5 mol%, 650 mg) in CH_2Cl_2 (20 mL) to afford the crude aldehyde that underwent a Wittig-reaction with *S*-ethyl 2-(triphenyl-λ^5-phosphaneylidene)ethanethioate (3.221 mmol, 1174 mg) in CH_2Cl_2 (40 mL) to afford **3** as a colorless oil (2010 mg, 39% over two steps). $[\alpha]_D^{20}$ = −6.2 (10 mg/mL; $CHCl_3$). ^{1}H-NMR: (300 MHz, $CDCl_3$) δ / ppm = 7.70–7.60 (m, 4H), 7.46–7.33 (m, 6H), 6.93–6.79 (m, 1H), 6.11 (d, *J* = 15.5 Hz, 1H), 3.49 (ddd, *J* = 16.3, 10.0, 5.9 Hz, 2H), 2.94 (q, *J* = 7.4 Hz, 2H), 2.50–2.36 (m, 1H), 2.04 (ddd, *J* = 15.4, 8.4, 7.3 Hz, 1H), 1.94–1.77 (m, 1H), 1.28 (dd, *J* = 7.7, 7.2 Hz, 3H), 1.06 (s, 9H), 0.91 (d, *J* = 6.8 Hz, 3H). ^{13}C-NMR, DEPT: (76 MHz, $CDCl_3$) δ / ppm = 190.1 (C), 144.0 (CH), 135.7 (CH), 135.7 (CH), 133.9 (CH), 133.8 (CH), 130.1, 129.8 (CH), 127.8 (CH), 68.2 (CH_2), 36.1 (CH_2), 35.6 (CH), 27.0 (CH_3), 23.2 (CH_2), 19.4 (C), 16.6 (CH_3), 15.0 (CH_3). EI-MS (70 eV): *m/z* = 283 ([*M* − ^{t}Bu]$^+$, 31), 200 (19), 199 (100), 181 (24), 175 (16), 139 (38), 105 (18), 83 (23), 77 (19), 41 (16).

*Preparation of S-Ethyl (3R,5S)-6-((tert-Butyldiphenylsilyl)oxy)-3,5-dimethylhexanethioate (*4*).* The synthesis was performed according to Horst et al. (2007) using **11** (75 mg, 0.12 mmol, 0.012 eq.), $CuBr{\cdot}SMe_2$ (20 mg, 0.097 mmol, 0.01 eq.) dissolved in MTBE (65 mL), MeMgBr (11.64 mmol, solution in diethyl ether), and **3** (9.70 mmol, 3730 mg, 1 eq.) to afford **4** as a colorless oil (3533 mg, 82%). $[\alpha]_D^{20}$ = −4.3 (10 mg/mL; $CHCl_3$). FT-IR: ν / cm^{-1} = 2959, 2930, 2860, 1689, 1463, 1427, 1384, 1262, 1108, 1083, 1002, 821, 741, 702, 615. ^{1}H-NMR: (300 MHz, $CDCl_3$) δ / ppm = 7.72–7.66 (m, 4H), 7.47–7.36 (m, 6H), 3.48 (ddd, *J* = 24.5, 9.8, 5.9 Hz, 2H), 2.88 (q, *J* = 7.5 Hz, 2H), 2.53 (dd, *J* = 14.3, 5.0 Hz, 1H), 2.26 (dd, *J* = 14.3, 8.7 Hz, 1H), 2.18–2.02 (m, 1H), 1.81–1.65 (m, 1H), 1.48–1.35 (m, 1H), 1.30–1.21 (m, 3H), 1.12–1.05 (m, 9H), 1.05–0.98 (m, 1H), 0.93 (dt, *J* = 6.7, 4.5 Hz, 6H). ^{13}C-NMR, DEPT: (76 MHz, $CDCl_3$) δ / ppm = 199.3 (C = O), 135.8 (CH_{Ar}), 134.1 (C_{Ar}), 134.1 (C_{Ar}), 129.7 (CH_{Ar}), 127.7 (CH_{Ar}), 68.9 (CH_2), 51.3 (CH_2), 40.9 (CH_2), 33.3 (CH), 28.8 (CH), 27.0 (CH_3), 23.4 (CH_2), 20.4 (CH_3), 19.4 (C_q), 17.6 (CH_3), 14.9 (CH_3). EI-MS (70 eV): *m/z* = 386 (30), 385 ([*M* − ^{t}Bu]$^+$, 100), 323 (31), 243 (44), 199 (92), 183 (50), 181 (35), 135 (41), 83 (35), 55 (36).

*Preparation of (3R,5S)-6-((tert-Butyldiphenylsilyl)oxy)-3,5-dimethylhexan-1-ol (*5*).* To a solution of **4** (0.90 mmol, 400 mg, 1 eq.) in CH_2Cl_2 (10 mL) was added diisobutylaluminium hydride (1.17 mmol, 1.3 eq., solution in cyclohexane) at − 50°C under a nitrogen atmosphere. After stirring for 17 h the reaction mixture was allowed to warm up to room temperature. The reaction was quenched by addition of saturated Rochelle solution (10 mL) and stirred for 30 min at room temperature. The phases were separated and the aqueous phase was extracted three times with CH_2Cl_2 (30 mL). The combined organic phases were dried over Na_2SO_4 and the solvent removed under reduced pressure to yield the crude aldehyde. The reduction procedure was repeated and the obtained residue was purified by column

chromatography (pentane/diethyl ether; 1:1) to afford alcohol **13** as a slightly yellow oil (267 mg, 77%). In contrast to the published procedure (Horst et al. 2007) a two-step reduction proved to be necessary to obtain good yields. $[\alpha]_D^{20}$ = −3.7 (10 mg/mL; $CHCl_3$). FT-IR: ν / cm^{-1} = 2928, 2859, 1466, 1428, 1385, 1107, 1007, 821, 739, 700, 614. ^{1}H-NMR: (300 MHz, $CDCl_3$) δ / ppm = 7.69 (m, 4H), 7.48–7.36 (m, 6H), 3.74–3.58 (m, 2H), 3.57–3.40 (m, 2H), 1.86–1.68 (d, J = 6.6 Hz, 1H), 1.68–1.52 (m, 2H), 1.49–1.22 (m, 3H). ^{13}C-NMR, DEPT: (76 MHz, $CDCl_3$) δ / ppm = 135.8 (CH), 135.8 (C), 134.2 (CH), 129.6 (CH), 127.7 (CH), 68.9 (CH_2), 61.2 (CH_2), 41.3 (CH_2), 39.9 (CH_2), 33.2 (CH_3), 27.1 (CH_3), 27.0 (CH_3), 20.4 (CH_3), 19.4 (C_q), 17.8 (CH_3). EI-MS (70 eV): m/z = 327 ([M − ^{t}Bu]$^+$, 2), 200 (11), 199 (63), 181 (14), 139 (11), 135 (10), 111 (37), 69 (100), 57 (10), 55 (39), 41 (19).

*Preparation of (3R,5S)-6-((tert-Butyldiphenylsilyl)oxy)-3,5-dimethylhexyl 4-Methylbenzenesulfonate (*6 *).* Tosyl chloride (1.35 mmol, 257 mg, 2 eq.) was added to a solution of **5** (0.67 mmol, 259 mg, 1 eq.) and pyridine (1.35 mmol, 109 µL, 2 eq.) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at room temperature for 19 h under a nitrogen atmosphere. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (pentane/diethyl ether; 20:1) to afford **6** as a colorless oil (289 mg, 80%). $[\alpha]_D^{20}$ = −4.2 (10 mg/mL; $CHCl_3$). FT-IR: ν / cm^{-1} = 2955, 2923, 2854, 1463, 1428, 1389, 1377, 1362, 1110, 1007, 999, 824, 795, 738, 700, 671, 665, 614, 528. ^{1}H-NMR: (300 MHz, $CDCl_3$) δ / ppm = 7.81–7.75 (m, 2H), 7.70–7.63 (m, 4H), 7.48–7.27 (m, 8H), 4.12–3.96 (m, 2H), 3.42 (ddd, J = 16.1, 9.8, 5.9 Hz, 2H), 2.43 (s, 3H), 1.77–1.44 (m, 3H), 1.32 (ddt, J = 13.5, 10.8, 6.6 Hz, 3H), 1.05 (s, J = 2.6 Hz, 9H), 0.99–0.81 (m, 4H), 0.77 (d, J = 6.5 Hz, 3H). ^{13}C-NMR, DEPT: (76 MHz, $CDCl_3$) δ / ppm = 144.7 (C_{Ar}), 135.7 (CH_{Ar}), 134.1 (C_{Ar}), 133.4 (C_{Ar}), 129.9 (CH_{Ar}), 129.7 (CH_{Ar}), 128.0 (CH_{Ar}), 127.7 (CH_{Ar}), 69.2 (CH_2), 68.8 (CH_2), 41.1 (CH_2), 35.7 (CH_2), 33.1 (CH), 27.0 (CH_3), 27.0 (CH), 21.7 (CH_3), 19.9 (C_q), 19.4 (CH_3), 17.7 (CH_3). EI-MS (70 eV): m/z = 353 (35), 293 (66), 199 (52), 181 (20), 135 (21), 111 (58), 69 (100), 91 (51), (48), 41 (28).

*Preparation of tert-Butyl(((2S,4S)-2,4-dimethylheptadecyl)oxy)diphenylsilane (*7 *).* 1-Bromoundecane was added (2.13 mmol, 500 mg) to a mixture of magnesium turnings (2.55 mmol, 62 mg) in THF (10 mL). The reaction mixture was heated to refluxed for 30 min and cooled down to room temperature. Compound **6** (0.51 mmol, 273 mg, 1 eq.) and CuBr·SMe_2 (0.10 mmol, 21 mg, 0.2 eq.) were dissolved in THF (6 mL). The freshly prepared Grignard solution was added at 0°C under a nitrogen atmosphere. After warming to room temperature, the reaction mixture was stirred for 1 h. After quenching with saturated NH_4Cl solution (6 mL) the phases were separated and the aqueous phase was extracted three times with diethyl ether (30 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (pentane/diethyl ether; 20:1) to afford **7** as a colorless oil (113 mg, 42%). $[\alpha]_D^{20}$ = −3.3 (10 mg/mL; $CHCl_3$). FT-IR: ν / cm^{-1} = 3067, 2957, 2929, 2860, 2323, 1596, 1465, 1429, 1361, 1300, 1258, 1213, 1180, 1103, 945, 892, 816, 744, 701, 663, 614, 578, 555. ^{1}H-NMR: (300 MHz, $CDCl_3$) δ / ppm = 7.73–7.63 (m, 4H), 7.50–7.31 (m, 6H), 3.47 (ddd, J =

16.2, 9.8, 5.9 Hz, 2H), 1.74 (dh, *J* = 13.3, 6.7 Hz, 1H), 1.50–1.19 (m, 25H), 1.10–1.03 (m, 9H), 0.96–0.85 (m, 7H), 0.82 (d, *J* = 6.4 Hz, 3H). ^{13}C-NMR, DEPT: (76 MHz, $CDCl_3$) δ / ppm = 135.8 (CH_{Ar}), 134.3 (C_{Ar}), 129.6 (CH_{Ar}), 127.7 (CH_{Ar}), 69.1 (CH_2), 41.3 (CH_2), 37.0 (CH_2), 33.3 (CH), 32.1 (CH_2), 30.2 (CH_2), 30.2 (CH), 29.9 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 27.0 (CH_3), 27.0 (CH_2), 22.9 (CH_2), 20.5 (CH_3), 19.5 (C_q), 17.9 (CH_3), 14.3 (CH_3). EI-MS (70 eV): *m/z* = 465 (34), 200 (19), 199 (100), 97 (23), 83 (25), 83 (19), 69 (28), 57 (40), 55 (20), 43 (37).

*Preparation of (2S,4S)-2,4-Dimethylheptadecan-1-ol (*8 *)*. TBAF (0.57 mmol, 574 µL, 3 eq.) was added to a solution of **7** (0.19 mmol, 100 mg, 1 eq.) in THF (5 mL). The reaction mixture was stirred at room temperature for 24 h under a nitrogen atmosphere. The solvent was separated under reduced pressure and the residue purified by column chromatography (pentane/diethyl ether; 20:1) to afford **8** as colorless oil with traces of siloxanes as impurities (21 mg, crude). $[\alpha]_D^{20}$ = −37.6 (10 mg/mL; $CHCl_3$). FT-IR: *v* / cm^{-1} = 3341, 2955, 2922, 2853, 1462, 1377, 1112, 1036, 987, 865, 821, 704, 606. ^{1}H-NMR: (600 MHz, $CDCl_3$) δ 3.49–3.27 (m, 2H), 1.70–1.60 (m, 1H), 1.46–1.37 (m, 1H), 1.27–1.10 (m, 26H), 1.09–0.91 (m, 2H), 0.90–0.75 (m, 10H). ^{13}C-NMR, DEPT: (151 MHz, CDCl3) δ / ppm = 68.2 (CH_2), 40.9 (CH_2), 36.5 (CH_2), 32.9 (CH), 31.7 (CH_2), 29.9 (CH), 29.8 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.2 (CH_2), 26.7 (CH_2), 22.5 (CH_2), 20.2 (CH_3), 17.1 (CH_3), 13.9 (CH_3). EI-MS (70 eV): *m/z* = 283 (< 1), 266 (< 1), 224 (10), 209 (6), 196 (7), 168 (7), 111 (22), 97 (31), 83 (100), 71 (44), 70 (39), 69 (52), 57 (97), 56 (60), 55 (85), 43 (81), 41 (61).

*Preparation of (2S,4S)-2,4-Dimethylheptadecanoic acid (*9 *)*. Under a nitrogen atmosphere $RuCl_3$ (0.014 mmol, 3 mg, 0.3 eq.) and $NaIO_4$ (0.281 mmol, 60 mg, 5 eq.) were added to a mixture of crude **8** (0.056 mmol, 16 mg, 1 eq.), H_2O (1.2 mL), CH_3CN (1.2 mL) and CCl_4 (2.4 mL). The mixture was stirred at room temperature for 3.5 h. After addition of CH_2Cl_2 (4 mL) and H_2O (1 mL) the phases were separated and the aqueous phase was extracted three times with CH_2Cl_2 (10 mL). The combined organic phases were dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography (pentane/ethyl acetate/acetic acid; 90:10:1) to afford acid **9** as a colorless oil (11 mg, 66%). $[\alpha]_D^{20}$ = +7.0 (10 mg/mL; $CHCl_3$). FT-IR: *v* / cm^{-1} = 2956, 2923, 2853, 1815, 1707, 1464, 1416, 1379, 1290, 1235, 1091, 1018, 947, 810, 722, 529. ^{1}H-NMR: (600 MHz, $CDCl_3$) δ / ppm = 9.58 (d, *J* = 2.5 Hz, 1H), 2.67–2.51 (m, 1H), 1.80–1.66 (m, 1H), 1.53–1.41 (m, 1H), 1.40–1.02 (m, 31H), 0.95–0.79 (m, 7H). ^{13}C-NMR, DEPT: (151 MHz, $CDCl_3$) δ / ppm = 172.7 (C), 41.4 (CH_2), 37.2 (CH_2), 32.1 (CH_2), 30.9 (CH_2), 30.1 (CH), 29.9 (CH_2), 29.5 (CH_2), 26.9 (CH_2), 22.9 (CH_2), 19.7 (CH_3), 18.0 (CH_3), 14.27 (CH_3). A small portion was converted into the respective methyl ester by treatment with trimethylsilyldiazomethane. EI-MS (70 eV): *m/z* = 312 ([*M*]$^+$, 3), 241 (12), 129 (7), 101 (59), 88 (100), 71 (7), 69 (12), 57 (13), 55 (12), 43 (12), 41 (10).

*Preparation of tetradecyl (2S,4S)-2,4-dimethylheptadecanoate (*10 *)*. 1-Ttetradecanol (0.007 mmol, 0.53 mg, 1 eq., 1%wt in DCM), dicyclohexylcarbodiimide (0.007 mmol, 1.28 mg, 1 eq., 1%wt in DCM) and 4-dimethylaminopyridine (0.007 mmol, 1.38 mg, 1 eq., 1%wt in DCM) were added to a solution of

(2*S*,4*S*)-2,4-dimethylheptadecanoic acid (**9**) (0.007 mmol, 2 mg, 1 eq.) in DCM (5 mL). After stirring for 20 h at room temperature the solvent was removed under reduced pressure and the residue was purified by column chromatography (pentane/diethyl ether; 100:1) to afford **11** as a colorless oil (2 mg, 58%). $[\alpha]_D^{20}$ = +8.0 (2 mg/mL; $CHCl_3$). FT-IR: v / cm^{-1} = 2923, 2855, 2319, 1736, 1461, 1372, 1172, 673, 603, 565, 546. ^{1}H-NMR: (500 MHz, $CDCl_3$) δ / ppm = 4.11–3.99 (m, 2H), 2.58–2.46 (m, 1H), 1.74–1.66 (m, 1H), 1.65–1.57 (m, 3H), 1.42–1.17 (m, 38H), 1.16–1.05 (m, 5H), 0.91–0.78 (m, 10H). ^{13}C-NMR, DEPT: (126 MHz, $CDCl_3$) δ / ppm = 177.4 (C_q), 64.4 (CH_2), 41.8 (CH_2), 37.7 (CH), 37.3 (CH_2), 32.1 (CH_2), 31.0 (CH), 30.1 (CH_2), 29.9 (CH_2), 29.8 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 28.9 (CH_2), 27.0 (CH_2), 26.1 (CH_2), 22.9 (CH_2), 19.7 (CH_3), 18.2 (CH_3), 14.3 (CH_3). EI-MS (70 eV): m/z (%) = 299 (100), 241 (17), 196 (23), 111 (14), 97 (24), 87 (50), 83 (25), 75 (30), 74 (61), 71 (36), 69 (31), 57 (55), 55 (57), 43 (41), 41 (17).

Results

Dichloromethane extracts of female silk and of the cuticle of both sexes of *Argiope bruennichi* were individually analyzed by GC/MS and the average relative proportions of the compounds were determined (Table 1). The samples were then sex-specifically combined to allow identification of even minor components by GC/MS (**Fig. 3**). The female sex pheromone, trimethyl methylcitrate (*I* 1523) as well as 3-octanoyloxy-γ-butyrolactone, an unusual *A. bruennichi* specific compound of unknown function (Chinta et al. 2016) were the only compounds eluting early. Next to some hydrocarbons the dominant cuticular lipids showed mass spectra consistent with wax-type esters (McLafferty and Turecek 1993; Chinta et al. 2016). These spectra are dominated by ions formed in a characteristic McLafferty-rearrangement that cleaves the O-alkyl bond and together with hydrogen transfer leads to the protonated acid and alkene ions. Most of the esters were identified by interpretation of the mass spectra, derivatization procedures and synthesis. Because a large number of different esters were present, we will show the identification procedure by one example.

The mass spectrum of the peak with a retention index *I* of 3273 in both male and female samples (**Fig. 4**) showed a molecular ion of *m/z* 494 and a large peak at *m/z* 313, likely the protonated acid ion $C_{20}H_{41}O_2^+$. The ion *m/z* 182 (likely $C_{13}H_{26}^+$) corresponds to the alcohol part. Additionally, smaller amounts of *m/z* 299 and 327 were present, along with *m/z* 196 and 168. These peak pairs indicate that three esters elute together, consisting of C_{19}-acid and C_{14}-alcohol, C_{20}-acid and C_{13}-alcohol, as well as C_{21}-acid and C_{12}-alcohol. A characteristic ion at *m/z* 74 proved a methyl group at C-2 of the acid (Chinta et al. 2016).

To identify the number and position of methyl groups in the chains, microreactions with the extracts were performed. The natural samples were transesterified with TMSH to the corresponding methyl esters and free alcohols (Müller et al. 1990). The methyl esters allow determination of methyl group positions near the carbonyl group (Ryhage and Stenhagen 1960a). These esters were then again transesterified with sodium 3-pyridinylmethoxide to form the corresponding 3-pyridinylmethyl esters, while the free alcohols

were esterified under Steglich conditions with nicotinic acid to the corresponding nicotinates. All these derivatized extracts were analyzed by GC/MS and the data were used to identify the number and position of methyl branches in both acids and alcohols (Fig. 1).

Methyl esters display characteristic fragment ions formed by β-cleavage and McLafferty-rearrangement. Because C-2-substituents are included into the rearrangement, unsubstituted methyl esters have a base peak of *m/z* 74 while the base peak is *m/z* 88 for 2-methyl-substituted methyl esters (Ryhage and Stenhagen 1960a), as is the case for a major component of both derivatized samples (**Fig. 5**a). Ryhage and Stenhagen (1960b) showed that polymethyl substituted methyl esters display characteristic ratios of fragment ions. The base peak at *m/z* 88 together with higher intensities of *m/z* 101 and 129 as well as the ester specific loss of C-2/C-3 + H (*m/z* 269, $[M - 43]^+$) and C-2/C-3/C-4 + H (*m/z* 241, $[M - 71]^+$) indicate a 2,4-dimethyl substitution pattern (Ryhage and Stenhagen 1960b). Nevertheless, additional methyl groups along the chain cannot be reliably deduced from the methyl ester spectra. Therefore, the methyl esters were transformed into 3pyridinylmethyl esters. Such esters display a regular fragmentation pattern of successive losses of CH_2 units, with branches resulting in gaps in the pattern and increased intensity in the fragments next to the branching position because of the stability of the resulting secondary radical (Harvey 1991).

The mass spectrum of this derivative is shown in **Fig. 5**b. The ion *m/z* 165 together with the base peak *m/z* 178 and the gap in the regular fragmentation pattern between *m/z* 178 and *m/z* 206 support a 2,4-dimethylheptadecanoate structure of the acid part of the natural esters. This identification was verified through synthesis of 2,4-dimethylheptadecanoic acid (**9**) using the strategy of Feringa et al. (**Fig. 2**) for the stereoselective construction of bishomolog methyl-branched acids (Des Mazery et al. 2005; Horst et al. 2007; Ruiz et al. 2007). Esterification of **9** under the same conditions as performed with the natural samples led to methyl 2,4dimethylheptadecanoate and 3-pyridinylmethyl 2,4dimethylheptadecanoate and confirmed our identification (mass spectra see SI, Figs. S 1 and 2).

Most of the natural occurring esters were identified using the described procedure. As can be seen from Table 1, the 2,4-dimethyl motif dominates within the natural esters. In addition, small amounts of 2,4,6trimethylalkanoate esters and several other 2,4,xtrimethylalkanoates esters were identified using the approach described.

The alcohol parts of the esters were identified in similarly as *n*-alkan-1-ols using 3pyridinecarboxylate derivatives, nicotinates, that display a regular fragmentation pattern of successive losses of CH_2 units. In addition, the nicotinates obtained from the females showed small amounts of 4methyl alcohols. A typical mass spectrum is shown in **Fig. 5**c displaying the branch indicating gap between *m/z* 164 and 192.

With these results in hand we were able to identify most of the natural wax esters (Table 1). Both female silk and body contained acids with between 17 and 24 carbons, with mostly 2,4dimethyl substitution, as well as smaller amounts of 2,4,6trimethyl substitution and various other 2,4,xtrimethyl substitution patterns. The alcohol parts were found being mainly n-alkan-1-ols, along with small amounts of 4-methyl

alcohols with chain lengths between 13 and 24 carbons. The males contained the same acids, although the shorter acids were more prominent. The alcohols were identified as n-alkanols with chain lengths between 12 and 15 carbons. The identification of *n*-alkyl 2,4-dimethylalkanoates was verified through the synthesis of tetradecyl 2,4-dimethylheptadecanoate (Fig. 2).

The characterization of the acid and alcohol components was used to assign proposed structures to the wax esters of the natural samples basing on their mass spectra and *I* (Table 1). The largest signals in the derivatized as well as the natural samples were 2,4-dimethylalkanoates, indicating these to be the major lipid components. The remaining wax esters were assumed to have similar relative retention indices as the derivatives (Schulz 2001) and were identified as the respective 2,4,x-trimethylalkanoates. In the male samples a small amount of unbranched esters was present, while in female samples small amounts of 4-methylalkyl 2,4-dimethylalkanoates were found.

Table 1. List of compounds Detected in extracts of *A. bruennichi*.

Individual samples of 30 female bodies and webs, as well as 20 male bodies were analyzed. Often compounds were eluting together within one peak. The average percentage of each peak within the whole sample is reported. Peaks are separated with lines in the table. Major components of peak groups are marked bold. The three most concentrated compounds of each sample types are also marked in bold.

		Female body		Female web		Male body	
I	Substance	mean %	SD	mean %	SD	mean %	SD
1523	Trimethyl methylcitrate	0.84	0.77	0.95	1.17	—	
1803	3-Octanoyloxy-γ-butyrolactone	—		3.07	2.83	—	
1900	Nonadecane	tr		0.20	0.18	—	
1946	Hexadecenoic acid	0.08	0.30	—		—	
1959	Hexadecanoic acid	0.08	0.14	1.33	1.65	0.17	0.12
1992	Ethyl hexadecanoate	tr		0.65	0.29	—	
	Eicosane						
2090	Nonadecanal	0.10	0.13	tr		—	
2100	Heneicosane	0.10	0.07	1.25	0.44		
2142	Octadecadienoic acid	tr		—		tr	
	Octadecenoic acid						
2160	Octadecanoic acid	tr		0.68	1.02	0.22	0.54
	Ethyl octadecenoate						
2193	Ethyl octadecanoate	0.09	0.06	1.50	0.57	—	
	Docosane						
2209	Unknown	tr		—		—	
2259	2-Methyldocosane	0.07	0.04	0.91	0.47	—	
2276	Tricosene	0.07	0.14	0.07	0.12	—	
2283	Tricosene	tr		—		—	
2300	Tricosane	1.56	1.01	3.00	1.03	0.24	0.18
	1-Eicosanol						
2334	9-Methyltricosane	0.07	0.05	0.49	0.30	—	
2339	7-Methyltricosane						
2348	5-Methyltricosane	tr		tr		—	
2371	3-Methyltricosane	tr		0.23	0.18	—	
2400	Tetracosane	0.13	0.08	1.59	0.57	tr	
2459	2-Methyltetracosane	0.15	0.07	0.36	0.25	0.06	0.11

2470	3-Methyltetracosane	tr		0.18	0.18	—	
2474	Pentacosene	tr		—		—	
2483	Pentacosene	tr		—		—	
2500	Pentacosane	3.74	1.71	3.30	1.19	0.99	0.37
2527	11-Methylpentacosane + impurity			—		—	
2531	9-Methylpentacosane + impurity						
2538	7-Methylpentacosane	tr		—		tr	
2547	5-Methylpentacosane	tr		0.09	0.18	—	
2558	2-Methylpentacosane	tr		0.17	0.18	tr	
2571	3-Methylpentacosane	tr		0.28	0.23	—	
2592	Unknown	tr		—			
2600	Hexacosane	0.16	0.06	1.08	0.42	tr	
2628	Unknown	0.09	0.07	0.58	0.51	2.80	1.04
2658	2-Methylhexacosane	0.19	0.11	0.64	0.48	—	
2677	Heptacosene	tr		—		—	
2700	Heptacosane + impurity	3.76	1.32	5.99	2.04	1.13	0.45
2733	7-Methylheptacosane			—		—	
2748	5-Methylheptacosane	tr		—		—	
2758	Hydrocarbon	tr		0.47	0.59	—	
2771	3-Methylheptacosane	0.12	0.08	0.24	0.35	0.28	0.32
2830	Unknown	0.21	0.17	—		4.13	1.96
2842	Unknown	tr		—		—	
2860	2-Methyloctacosane	1.03	0.60	0.54	0.55	1.46	0.61
2871	3-Methyloctacosane	tr		0.17	0.21	—	
2900	Nonacosane	2.23	0.82	2.55	0.60	1.00	0.40
2905	Unknown	tr		0.30	0.98	—	
2929	15-Methylnonacosane	1.31	0.80	2.08	1.36	0.41	0.38
2933	13-Methylnonacosane						
	11-Methylnonacosane						

	9-Methylnonacosane						
2938	7-Methylnonacosane	0.69	0.39	0.44	0.37	tr	
2948	5-Methylnonacosane	0.17	0.10	0.10	0.20	—	
2955	Hydrocarbon	tr		—		—	
2960	Hydrocarbon	0.09	0.09	tr		0.12	0.25
2966	7,11-Dimethylnonacosane	tr		—		tr	
2971	3-Methylnonacosane	0.11	0.06	2.14	1.62	0.23	0.26
2978	5,9-Dimethylnonacosane	tr		—		0.15	0.19
3000	Triacontane	0.20	0.10	1.83	0.73	—	
3008	Hydrocarbon	tr		0.39	0.38	—	
3030	15-Methyltriacontane	0.51	0.35	1.83	2.72	0.70	0.39
	16-Methyltriacontane						
3042	6-Methyltriacontane	0.08	0.07	0.23	0.28	—	
3061	2-Methyltriacontane	1.82	0.90	1.11	0.65	1.72	0.70
3072	Hydrocarbon	0.10	0.12	0.32	0.36	—	
3076	Tridecyl stearate	—		—		1.59	0.54
	Dodecyl nonadecanoate						
3091	2,12-Dimethyltriacontane	0.42	0.38	0.14	0.30	—	
	2,14-Dimethyltriacontane						
3101	2,6-Dimethyltriacontane	1.70	0.88	2.69	0.80	0.48	0.37
	2,8-Dimethyltriacontane						
3114	Hydrocarbon	0.25	0.29	0.35	0.42	—	
3118	Hydrocarbon						
3122	Hydrocarbon	0.16	0.20	0.09	0.36	—	
3129	15-Methylhentriacontane	2.58	1.55	2.69	1.71	—	
3135	13-Methylhentriacontane						
	11-Methylhentriacontane						
	9-Methylhentriacontane						
3139	7-Methylhentriacontane	0.25	0.13	0.27	0.43	—	

3160	Hydrocarbon	0.53	0.40	0.48	0.44	—	
3166	Hydrocarbon	0.26	0.19	—		—	
3173	**Tridecyl *syn*-2,4-dimethylheptadecanoate**	0.29	0.24	0.22	0.41	**14.88**	2.44
	5,15-Dimethylhentriacontane						
3176	Tridecyl *anti*-2,4-dimethylheptadecanoate						
3190	Unknown	0.06	0.09	—		—	
3199	Tridecyl 2,4,8-trimethylheptadecanoate	0.45	0.36	1.72	0.88	0.70	0.39
3217	Tridecyl 2,4,14-trimethylheptadecanoate	—		—		0.21	0.25
	Tetradecyl 2,4,14-trimethylhexadecanoate						
3226	16-Methyldotriacontane	0.43	0.28	1.91	2.00	0.89	0.22
3230	14-Methyldotriacontane						
	Tridecyl 2,4,16-trimethylheptadecanoate						
	Tetradecyl 2,4,16-trimethylhexadecanoate						
3256	6-Methyldotriacontane	0.16	0.16	—		—	
3260	2-Methyldotriacontane	0.20	0.12	—		—	
3273	Tetradecyl 2,4-dimethylheptadecanoate	1.19	1.08	1.05	0.88	**16.03**	1.12
	Tridecyl 2,4-dimethyloctadecanoate						
	Dodecyl 2,4-dimethylnonadecanoate						
3289	Unknown	0.86	0.58	0.34	0.44	—	
3300	Tritriacontane	0.51	0.26	1.31	0.69	—	
3316	Unknown	0.33	0.33	0.24	0.31	—	
3323	17-Methyltritriacontane	2.45	0.92	2.41	1.12	—	
3329	11-Methyltritriacontane						
3336	9-Methyltritriacontane						
	Unknown						
3330	Tetradecyl 2,4,14-trimethylheptadecanoate	—		—		0.35	0.33
3352	7,11,15-Trimethyltritriacontane	1.36	1.05	0.78	0.75	0.29	0.28

3360	11,21-Dimethyltritriacontane	0.35	0.33	0.13	0.29	—	
3380	**Tridecyl 2,4-dimethylnonadecanoate**	**15.77**	7.93	**10.30**	7.15	**32.94**	2.79
	Tetradecyl 2,4-dimethyloctadecanoate						
3384	Tridecyl 2,4,6-trimethylnonadecanoate	0.72	0.72	0.17	0.39	—	
3397	**Tridecyl 2,4,8-trimethylnonadecanoate**	0.52	0.50	0.93	0.64	0.15	0.31
3405	Tridecyl 2,4,10-trimethylnonadecanoate						
3409	Tridecyl 2,4,12-trimethylnonadecanoate	0.09	0.15	—		—	
3416	**Tetradecyl 2,4,14-trimethyloctadecanoate**	0.33	0.13	—		0.21	0.24
	Tridecyl 2,4,14-trimethylnonadecanoate						
3430	Tetradecyl 2,4,16-trimethylocatadecanoate	0.79	0.27	1.91	1.07	0.75	0.29
	Tridecyl 2,4,16-trimethylnonadecanoate						
3445	Tetradecyl trimethyloctadecanoate	tr		—		—	
	Pentadecyl trimethylheptadecanoate						
3476	Tetradecyl *syn*-2,4-dimethylnonadecanoate	**12.89**	4.51	**7.38**	4.00	11.25	1.65
	Tridecyl *syn*-2,4-dimethylicosanoate						
	Dodecyl *syn*-2,4-dimethylhenicosanoate						
3481	Tetradecyl *anti*-2,4-dimethylnonadecanoate	0.67	0.64	0.28	0.81	0.22	0.29
	Tridecyl *anti*-2,4-dimethylicosanoate						
	Dodecyl *anti*-2,4-dimethylhenicosanoate						
3495	Pentadecyl 2,4,6-trimethyloctadecanoate	0.14	0.18	—		—	
	Tetradecyl 2,4,6-trimethylnonadecanoate						
	Tridecyl 2,4,6-trimethylicosanoate						
3500	Wax ester	0.13	0.13	0.71	0.51	—	
3504	Wax ester	tr		—		—	
3528	Tetradecyl 2,4,14-trimethylnonadecanoate	0.96	0.29	1.93	0.96	0.06	0.20
	Pentadecyl 2,4,14-trimethyloctadecanoate						

	Hexadecyl 2,4,14-trimethylheptadecanoate						
3544	**Pentadecyl 2,4,16-trimethyloctadecanoate**	0.33	0.12	—		—	
	Tridecyl 2,4,16-trimethylicosanoate						
3557	Hydrocarbon	1.07	0.88	0.41	0.76	0.29	0.76
3575	**Tridecyl *syn*-2,4-dimethylhenicosanoate**	**11.97**	5.03	**6.51**	4.88	2.74	0.83
	Tetradecyl *syn*-2,4-dimethylicosanoate						
	Pentadecyl *syn*-2,4-dimethylnonadecanoate						
3579	**Tridecyl *anti*-2,4-dimethylhenicosanoate**	0.73	0.75	0.24	0.89	—	
	Tetradecyl *anti*-2,4-dimethylicosanoate						
	Pentadecyl *anti*-2,4-dimethylnonadecanoate						
3596	Tridecyl 2,4,6-trimethylhenicosanoate	0.17	0.29	—		—	
3601	**Octadecyl 2,4,6-trimethylhexadecanoate**	2.46	1.32	1.43	1.23	—	
	Hexadecyl 2,4,6-trimethyloctadecanoate						
3627	Pentadecyl 2,4,16-trimethyloctadecanoate	0.62	0.17	1.35	0.85	—	
3656	4-Methyloctadecyl 2,4-dimethylheptadecanoate	0.17	0.16	—		—	
	4-Methylheptadecyl 2,4-dimethyloctadecanoate						
	4-Methylhexadecyl 2,4-dimethylheptadecanoate						
3670	Ooctadecyl 2,4-dimethylheptadecanoate	2.49	0.96	1.03	0.87	—	
	Heptadecyl 2,4-dimethyloctadecanoate						
	Hexadecyl 2,4-dimethylnonadecanoate						
	Pentadecyl 2,4-dimethylicosanoate						
	Tetradecyl 2,4-dimethylhenicosanoate						
	Tridecyl 2,4-dimethyldocosanoate						
3676	Wax ester	0.10	0.20	—		—	
3687	Wax ester	tr		—		—	
3699	Nonadecyl 2,4,6-trimethylhexadecanoate	1.63	1.01	0.87	0.76	—	

	Octadecyl 2,4,6-trimethylheptadecanoate					
	Heptadecyl 2,4,6-trimethyloctadecanoate					
3728	Heptadecyl 2,4,14-trimethylheptadecanoate	0.32	0.18	0.93	0.81	–
3755	Unknown	0.43	0.24	–		–
3771	Nonadecyl 2,4-dimethylheptadecanoate	2.57	1.50	0.62	0.83	–
	Heptadecyl 2,4-dimethylnonadecanoate					
	Pentadecyl 2,4-dimethylhenicosanoate					
3802	**Icosyl 2,4,6-trimethylhexadecanoate**	5.10	3.28	2.22	1.73	–
	Octadecyl 2,4,6-trimethyloctadecanoate					
3826	**Octadecyl 2,4,14-trimethyloctadecanoate**	0.21	0.25	0.59	0.64	–
	Nonadecyl 2,4,14-trimethylheptadecanoate					
3855	4-Methylnonadecyl 2,4-dimethyloctadecanoate	0.08	0.12	–		–
3869	Icosyl 2,4-dimethylheptadecanoate	0.31	0.27	–		–
	Nonadecyl 2,4-dimethyloctadecanoate					
	Octadecyl 2,4-dimethylnonadecanoate					
	Heptadecyl 2,4-dimethylicosanoate					
3897	Octadecyl 2,4,6-trimethyloctadecanoate	0.89	0.70	–		–

Stereochemistry of wax esters. The synthetic material was also used to determine the configuration of the natural esters, choosing 2,4-dimethylheptadecanoic acid (**9**) as target compound. The synthesis uses an enantioselective conjugate addition of methylmagnesium bromide onto α,β-unsaturated thioesters using the enantiomers of josiphos (**11**) as ligand (**Fig. 2**). The configuration of each stereogenic center can thus be controlled (Horst et al. 2007). Usually multiple synthesis of different stereoisomers are required for elucidation of the absolute configuration of compounds with multiple stereogenic centers (see e. g. Schulz et al. 2004). We tried to solve this problem here with only one stereoselective synthesis taking advantage of the controlled stepwise introduction of the stereogenic centers.

Thus, the first stereogenic center was introduced into building block **1** with an *S*/*R* ratio of 97:3 (see SI, Fig. S 5). Reduction and elongation led to compound **3**, on which the second stereogenic center was introduced similarly to form compound **4** with an *R*/*S* ratio of 93:7 (calculated back from the final product). Reduction of the esters to alcohol **5** and conventional Grignard-elongation delivers compound **7** that after deprotection and oxidation finally gives (2*S*,4*S*)-acid **9** as major product that after esterification with 1-tetradecanol furnished ester **10** (Fig. 2). GC showed two separated peaks in the final product in a

ratio of 91:9 (Fig. S 3). The major, first eluting peak was the *syn*-diastereomer (2*S*,4*S*/2*R*,4*R*), while the smaller, second one was the *anti*-diasteromer (2*S*,4*R*/2*R*,4*S*). The methyl ester of **9** showed similar separation. The stereoisomeric mixture of the methyl esters of **9** was separated on a Hydrodex β-6TBDM phase (Fig. 6). Peak assignment was performed using the *er* data from the stereoselective addition. Therefore, the largest peak was the (2*S*,4*S*)-enantiomer (0.93 × 0.97 = 0.9021 relative peak area, ee 99.8 %), while the smallest had a (2*R*,4*R*)-configuration (0.07 × 0.03 = 0.0021), likely not detectable due to its low abundance. The (2*R*,4*S*)-enantiomer (0.07 × 0.97 = 0.0679, ee 41.8 %) was slightly more concentrated than the (2*S*,4*R*)-enantiomer (0.93 × 0.03 = 0.0279). The ee data nicely showed that the consecutive introduction of chiral centers leads to a *syn*-product of very high ee due to the inherent ee amplification, while it is the opposite for the *anti*-diastereomer. The *anti*-enantiomers are well separated, while separation of the *syn*-enantiomers remained unclear. Comparison with the trnasesterified natural extracts revealed the natural *anti*-diastereomer to be (2*S*,4*R*)-configured. This indicates, that the natural *syn*-diastereomer can be assigned the (2*R*,4*R*)-stereochemistry because the (4*R*)-stereochemistry is fixed during biosynthesis, in contrast to the configuration at C-2. For a detailed discussion see the Supporting Information. The formation of the *anti*-diastereomer might be explained by partly epimerization of C-2 during biosynthesis of the esters. Epimerization during transesterification with TMSH is unlikely because of the reaction mechanism and the occurrence of diastereomers of some esters even in the original samples (Table 1).

In summary, over 180 cuticular compounds were detected and most of them identified. While the number of hydrocarbons (72) and wax esters (75) was almost equal, the esters dominated in both female (ratio amounts esters/hydrocarbons 64:28) and male (68:13) cuticular extracts, while they occurred in equal amounts in webs of females (44:41).

Discussion

In this study we identified for the first time wax esters with a homomethyl-branched acid head group that constitute the major portion of lipids on the cuticle and web of both sexes of *A. bruennichi*. These cuticular compounds likely play a role in kin-recognition, because family differences were observed within the wax esters, while cuticular hydrocarbons showed lower variation (Weiss and Schneider 2021). We developed an analytical procedure that can reliably be used to analyze and characterize these esters, basing on previous work (Chinta et al. 2016). Furthermore, a synthetic procedure was established that allows synthetic access to the enantiomers of each ester and enabled determination of the absolute configuration of the esters. Esters have been prviously described as cuticular compounds of spiders. Propyl esters of long-chain multiply methyl-branched acids occur in complex mixtures on *Anelosimus eximus* (Bagnères et al. 1997), while few shorter sex-specific esters such as 2,8-dimethylundecyl 2,8-dimethylundecanoate or 14-methylheptadecyl 4-methylheptanoate dominate the cuticular wax of *Argyrodes elevatus* (Chinta et al. 2016). In contrast, the bishomomethyl-branched esters reported here represent a unique group of compounds that have not been reported from other arthropods. Nevertheless, they do not seem to be specific to *Argiope*, because we also detected such compounds in another spider *Pholcus phalangoides* (S. Schulz, unpublished).

The hydrocarbons identified are not very different from those observed in other spiders, with the typical spider specific feature of an enhanced concentration of 2-methyl-branched alkanes with an even number of carbons (Schulz 2001, 2013). Qualitatively, there is a large overlap between female and male samples, but female samples showed a much wider range of trimethyl substituted esters and a wider range in molecular weight, ranging from 480 to 564 u in males, while those in males from 466 u to 536 u. The major component of both female and males is tridecyl *syn*-2,4dimethylnonadecanoate but the next two most concentrated compounds in females are tridecyl *syn*-2,4-dimethylicosanoate and tridecyl *syn*-2,4-dimethylhenicosanoate, while in males they are tridecyl 2,4-dimethyloctadecanoate and tridecyl *syn*-2,4-dimethylheptadecanoate. In addition, some unique male wax esters were tridecyl and tetradecyl esters of octadecanoic and some 2,4,14trimethylalkanoic acids. The wax ester composition of webs of females is quite similar to that of the female cuticula.

In summary, we have clarified the complex cuticular chemistry of *A. bruennichi*. With these data and synthetic material, behavioral assays can be planned to elucidate which signals are used for kin-recognition. Although synthesis of a complete array of esters seems out of reach, addition of individual esters to manipulate cuticular chemistry might be a promising approach for further research.

Declarations

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Conflicts of interest/Competing interests (include appropriate disclosures)

The authors have no financial or proprietary interests in any material discussed in this article.

Availability of data and material (data transparency)

Not applicable

Code availability (software application or custom code)

Not applicable

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Figures

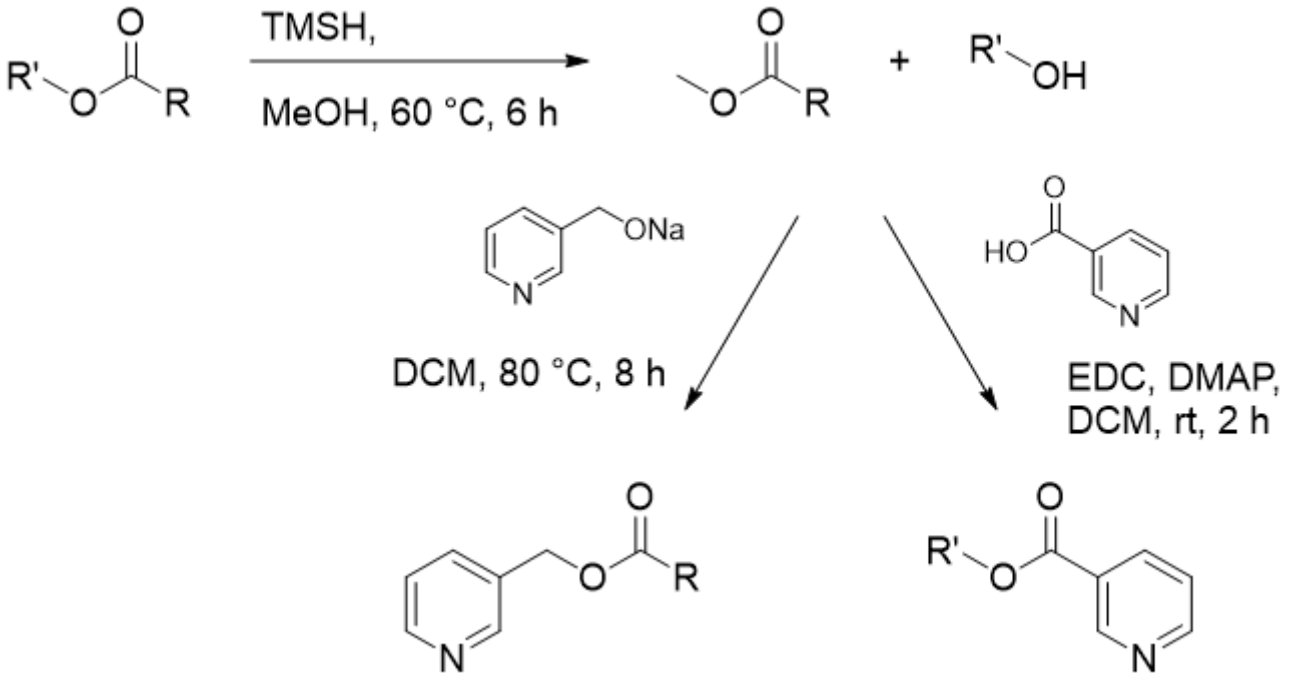

Figure 1

Microreactions performed with A. bruennichi extracts. Transesterification with TMSH transformed the wax esters into methyl esters that were again transesterified with 3-pyridinylmethanol or transformed into nicotinates with nicotinic acid.

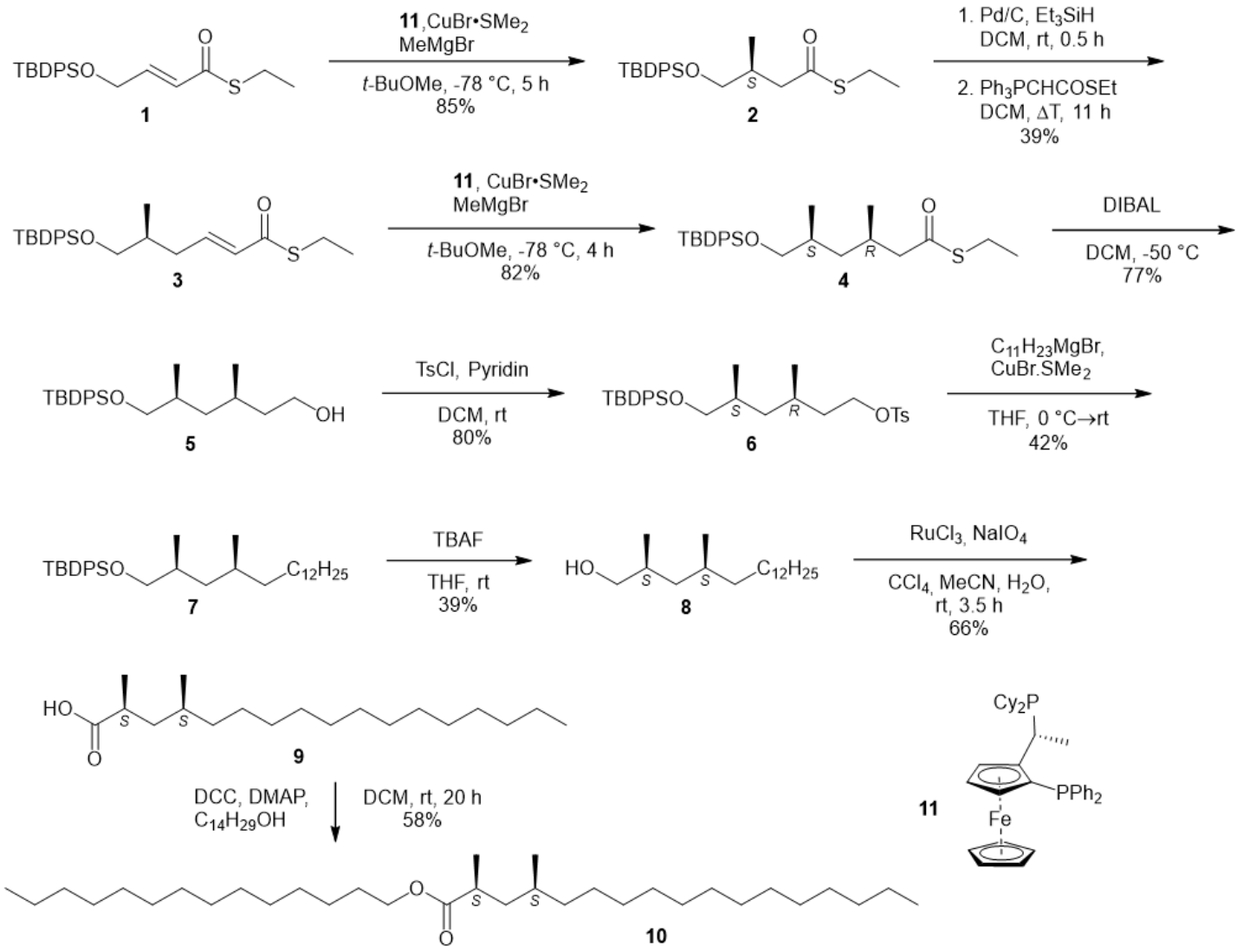

Figure 2

Synthesis of tetradecyl (2S,4S)-2,4-dimethylheptadecanoate (10). Please note that the configurational prefixes change during the synthesis due to the CIP-rules.

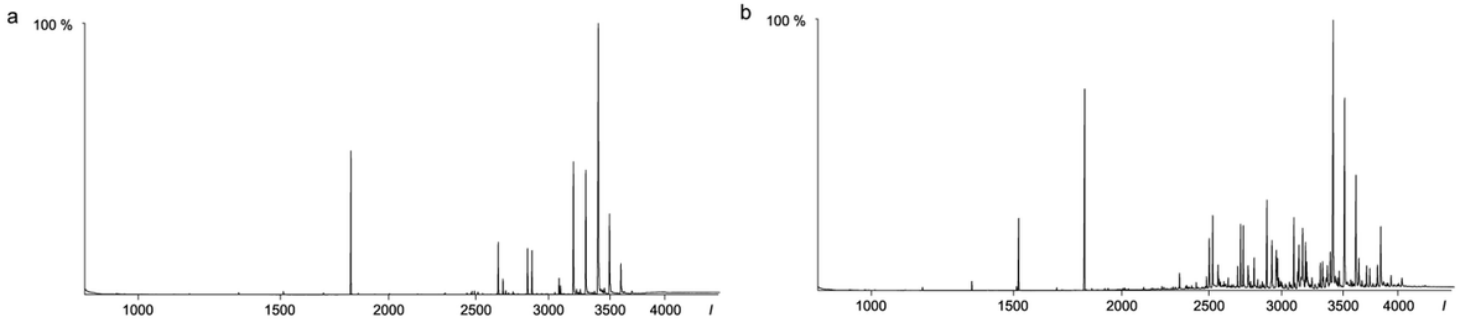

Figure 3

Total ion chromatogram of the combined cuticular extracts from male (a) and female (b) A. bruennichi.

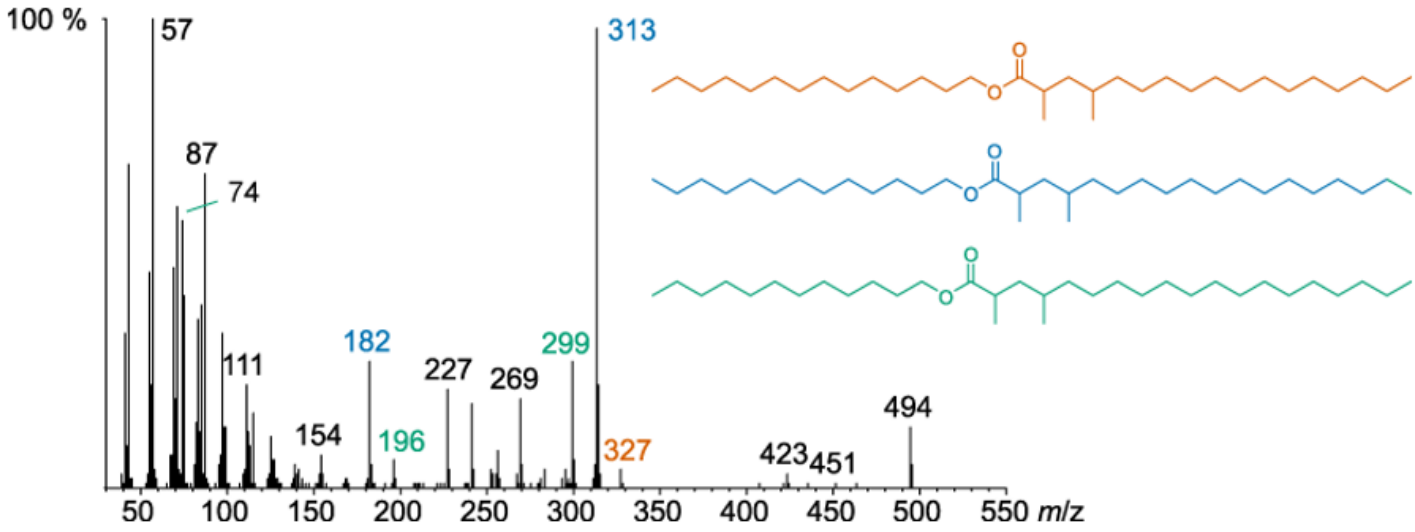

Figure 4

Mass spectrum and structures of 2,4-dimethylalkyl wax ester mixture with I 3237.

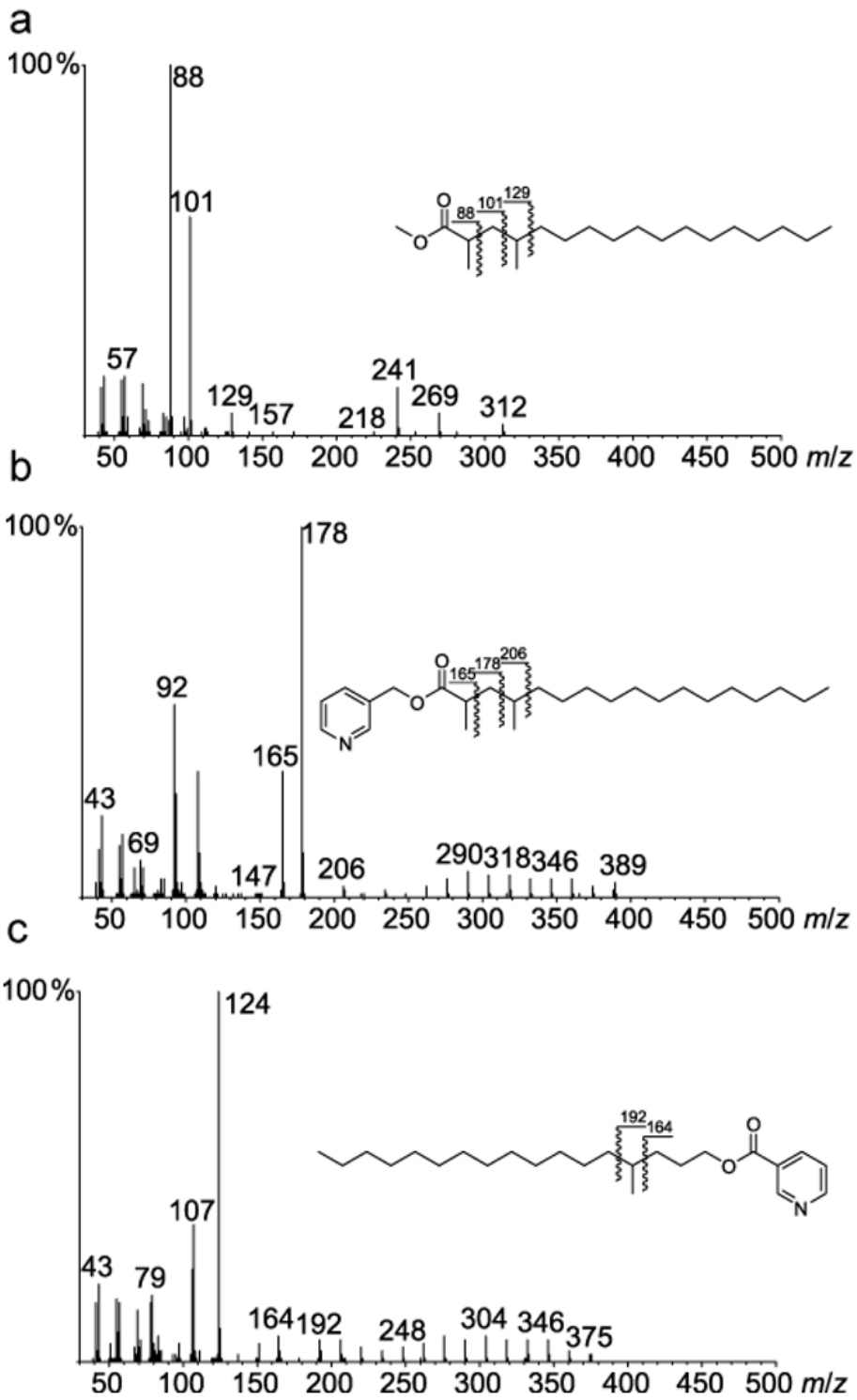

Figure 5

Mass spectra of methyl 2,4-dimethylheptadecanoate (a), 3-pyridinylmethyl 2,4- dimethylheptadecanoate (b), and 4-methylheptadecyl nicotinate (c).

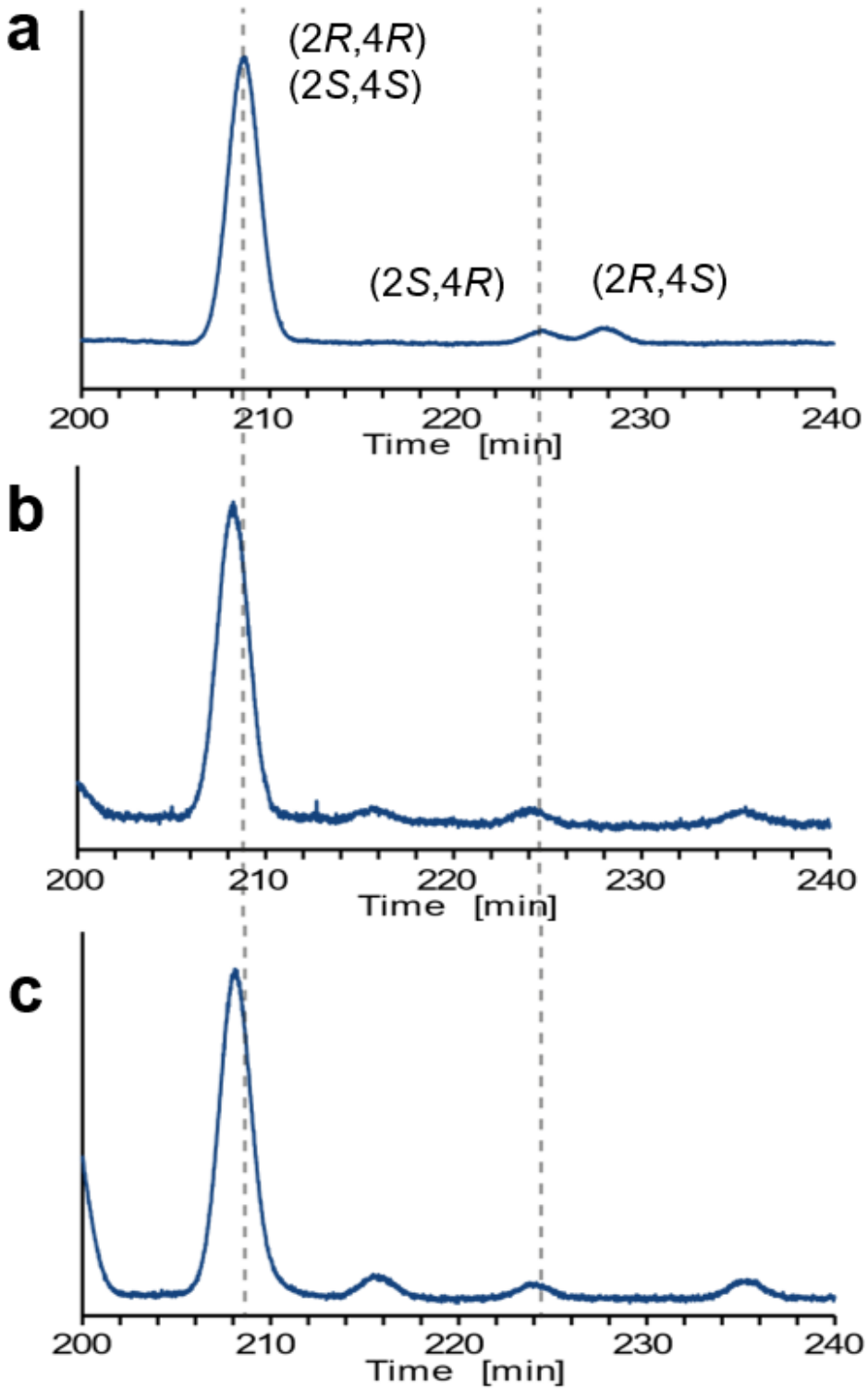

Figure 6

Separation of the methyl ester of 9 on a chiral GC phase. Separation was perfomred using a Hydrodex β-6TBDM phase (30.0 m x 0.25 mm, 1.5 mL/min H2, initial temp. 50 °C then 10 °C min-1 to 125 °C holding time for 240 min, then with 10 °C min-1 to final temp. 230 °C). A: methyl ester of 9. B: methyl ester of the transesterfied sample of a body extract of male Argiope bruenichii. C: same with female A. bruenichii.

Supplementary Files

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