A new male sex-pheromone and novel cuticular cues for chemical communication in *Drosophila*

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Supplemental experimental procedures

UV-LDI MS-guided laser marking of the cuticle

In order to physically mark the fly, a second UV laser producing a high-intensity Gaussian beam profile with a $1/e^2$-diameter of ca. 100 μm was used in parallel with the standard flat-top UV-laser beam. On the cuticle, both laser beams generated a fluorescence signal that is visible on the observation monitor. The flat-top beam was first used to establish a site of high intensity hydrocarbon signal in the anal-genital region of male flies. The Gaussian laser beam then was positioned in the center of that laser spot and its intensity increased until the laser produced carbonization. Optical images before and after irradiation were taken with an Olympus E-330 camera mounted on an Olympus microscope.

Electrospray ionization (ESI) mass spectrometry parameters

Exact mass measurements were performed using the micrOTOF I mass spectrometer (Bruker Daltonik, Bremen, Germany) using a sample loop mode and Sodium formate for mass referencing. Pure methanol was used as ESI solvent. The capillary voltage was set to 4.5 kV and the end plate offset to -500 V.

Nano-ESI low energy CID experiments were performed with both the QTOF (Waters/ Micromass, Manchester, UK) and the triple-quadrupole instrument (QuattroLC; Waters/ Micromass, Manchester, UK) using Argon as a collision gas. Capillary voltages were applied on an internal wire electrode and were set to values of about 1.1 kV (QTOF) and 1.2 kV (triple Quad), respectively; the cone voltages used were 60 V (QTOF), and 25 V (triple quad); The collision energies were varied from 15 – 40 eV (QTOF) and 12 – 25 eV (triple quad), respectively.

Gas chromatography (GC) MS analysis

Cuticular extracts were prepared by placing 30 – 40 flies in hexane for 1 h at room temperature, after which the solvent was transferred into a clean glass vial. GC/MS analysis was performed with a Quattromicro-GC (Waters, Manchester, UK) equipped with a HP-5 (5%-Phenyl)-methylpolysiloxane column (30 m length, 0.32 ID, 0.25 μm film thickness (Agilent)). A splitless injector was used. The Helium flow was set at 1.3 ml/min. The column temperature program started at 50°C for 2 min, then increased to 300°C at a rate of 15°C/min. The quadrupole mass spectrometer was set to unit mass resolution and 3 scans/min, from *m/z* 37 to 700.

Preparation of CH503 doses for courtship behavior assays

For dose-response experiments, a dilution series using chloroform/ methanol (2:1, v/v) as the solvent was made to provide dosages at 50%, 25%, 20%, 10%, 5%, 2%, and 0% of the original concentration from the full strength aliquot. All solutions were made in a final volume of 200 μl to control for solvent effects and evaporated to dryness at room temperature in a vacuum oven.

The concentration of the full strength aliquot was estimated as follows: The CH content of approximately 8000 male and female flies was extracted from the cuticle. Half of this extract was used for perfuming experiments. We estimate 10% loss during the purification process. This amount was divided into 6 individual aliquots. The aliquot was used at 50% concentration. Taking into account that 8 flies at a time were placed in each vial during the perfuming process and that the estimated transfer efficiency of this perfuming method is approximately 25% of the vial contents to the flies (personal communication, Jean-Christophe Billeter and Joel Levine), each perfumed fly is calculated to be

perfumed with the equivalent of CH503 from 7-8 males. This estimate represents an upper limit since each vial can be used for 3-4 different perfuming experiments.

Chemical derivatization of the HPTLC-purified CH503 fraction

Trans-esterification: An aliquot of the CH503-containing HPTLC fraction (prepared as described in the Experimental procedures) was mixed with 200 µl of methanol containing 2 mg NaOH, sonicated and heated to 60°C for 20 min. The reaction mixture then was acidified with formic acid. Solvent and excess acid were removed under a stream of N_2. The residue was extracted with 200 µl of iso-octane. The solvent was evaporated to dryness under a stream of N_2 prior to silylation and analysis with GC/ MS.

Silylation: Silylation was performed on both an unmodified aliquot of the CH503-containing HPTLC fraction and an aliquot that had been subjected to trans-esterification (see above). As a reference, the reaction also was carried out on 1,3-nonanediol acetate, a synthetic standard consisting of a mixture of esters (Sigma-Aldrich, Munich, Germany, catalog no. W278300). The samples were silylated with 50 µl N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) for 10 min at 60ºC then diluted with iso-octane (analytical grade; Fisher) to 200 µl. The products of the reaction were analyzed using GC/MS.

Ozonolysis: An aliquot of the CH503-containing HPTLC fraction was dissolved in 0.5 ml of chloroform/ methanol (2:1, v/v) followed by treatment at room temperature with ozone from an ozone generator. A reductive workup of the products was performed by adding 100 µl of MeOH containing 10 µg/µl dimethylsulfide. The products of the reaction were analyzed using GC/MS.

Nuclear magnetic resonance (NMR) spectroscopy

Approximately 1 mg of CH503 purified by HPTLC was dissolved in 700 µl of deuterated chloroform. Tetramethylsilane was used as the internal standard. NMR experiments were performed on a Unity Plus 600 (^{1}H: 600 MHz; Varian) and on an INOVA 500 (^{1}H: 500 MHz; Varian) spectrometer at 298 K. ^{1}H- and ^{13}C-NMR spectra were recorded. One-dimensional (gCOSY, 1D-TOCSY) and two-dimensional (gHSQC, and gHMBC) NMR experiments were performed using standard Varian pulse sequences.

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Table S1. Averaged, normalized values for the relative abundance of the major male and female CHs shown in Figure S1.

	Elemental composition[a]	Male AG[b] (n=13)	Male leg (n=12)	Female AG (n=12)	Female leg (n=12)
1	$C_{20}H_{38}O_2$ (cVA)*	39.3 ± 5.2	3.9 ± 0.7	0.3 ± 0.1	0.3 ± 0.1
2	$C_{23}H_{44}$ (tricosadiene)*	0.8 ± 0.2	1.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
3	$C_{23}H_{44}O$	4.0 ± 0.7	9.3 ± 1.0	0.6 ± 0.1	1.2 ± 0.2
4	$C_{23}H_{44}O_2$	12.8 ± 3.2	25.7 ± 4.1	0.2 ± 0.1	0.5 ± 0.1
5	$C_{23}H_{44}O_3$	(+)	8.2 ± 2.5	(+)	(+)
6	$C_{23}H_{46}$ (tricosene)*	0.7 ± 0.2	2.3 ± 0.3	(+)	(+)
7	$C_{23}H_{46}O$	11.0 ± 1.9	20.6 ± 2.3	1.9 ± 0.4	2.5 ± 0.5
8	$C_{23}H_{46}O_3$	0.4 ± 0.1	2.7 ± 0.8	n.d.	(+)
9	$C_{25}H_{48}$ (pentacosadiene)*	5.2 ± 1.2	7.0 ± 0.7	4.4 ± 0.7	4.1 ± 0.6
10	$C_{25}H_{48}O$	(+)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
11	$C_{25}H_{50}$ (pentacosene)*	(+)	0.9 ± 0.3	-	-
12	$C_{25}H_{50}O$	0.5 ± 0.2	1.1 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
13	$C_{27}H_{50}$ (heptacosatriene)	0.3 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
14	$C_{27}H_{52}$ (heptacosadiene)*	0.5 ± 0.1	0.7 ± 0.1	43.0 ± 2.2	43.3 ± 2.7
15	$C_{27}H_{54}$ (heptacosene)*	0.2 ± 0.1	0.3 ± 0.1	-	-
16	$C_{27}H_{54}O$	0.8 ± 0.2	1.2 ± 0.2	1.9 ± 0.6	1.5 ± 0.3
17	$C_{27}H_{54}O_2$	(+)	4.0 ± 1.1	4.0 ± 0.7	0.7 ± 0.2
18	$C_{29}H_{54}$ (nonacosatriene)	n.d.	n.d.	0.8 ± 0.1	0.8 ± 0.1
19	$C_{29}H_{56}$ (nonacosadiene)*	0.5 ± 0.1	0.4 ± 0.1	35.2 ± 2.5	36.1 ± 2.8
20	$C_{29}H_{58}$ (nonacosene)*	0.3 ± 0.1	0.4 ± 0.1	-	-
21	$C_{29}H_{58}O$	(+)	(+)	0.9 ± 0.4	0.9 ± 0.3
22	$C_{30}H_{56}$ (triacontatriene)	n.d.	n.d.	(+)	(+)
23	$C_{30}H_{58}$ (triacontadiene)*	(+)	(+)	0.3 ± 0.1	0.4 ± 0.1
24	$C_{30}H_{56}O_3$ (CH503)	16.7 ± 3.3	1.0 ± 0.4	n.d.	n.d.
25	$C_{31}H_{60}$ (hentriacontadiene)	0.5 ± 0.1	0.7 ± 0.1	1.4 ± 0.2	1.6 ± 0.1
26	$C_{31}H_{62}$ (hentriacontene)	0.5 ± 0.1	0.8 ± 0.2	-	-
27	$C_{33}H_{64}$ (tritriacontadiene)	1.7 ± 0.3	2.3 ± 0.4	0.6 ± 0.1	0.5 ± 0.1
28	$C_{35}H_{68}$ (pentatriacontadiene)	1.3 ± 0.2	1.8 ± 0.2	0.5 ± 0.1	0.4 ± 0.1

[a]Elemental composition is based on exact mass measurements within a 20 ppm mass accuracy; previously unreported CH species are highlighted in gray; *indicates compounds that were also detected in extracts from males or females in previous GC/MS studies [Ref. 20]. [b]The averaged relative abundance of each CH species is calculated by dividing the area under the monoisotopic peak by the total area under the monoisotopic peaks for all CHs detected in the same experiment. The mean ± S.E.M. is indicated. Only those CH species that could be reliably detected in all animals are included in this analysis: ++++: >10% of the total area; +++: 5-10%; ++: 1=5%; +: <1%. (+): excluded from analysis since the signal is detected in some but not all flies; n.d.: not detected; - : unable to detect due to overlapping signals from other hydrocarbons. AG: anogenital region; cVA: 11-cis-vaccenyl acetate.

Table S2. Proposed elemental composition and detected *m/z* value for each of the major ion signals recorded from adult virgin male flies shown in Figure 1C, E, and comparison across different mass spectrometric desorption/ ionization methods.

Elemental composition	Calculated *m/z* of $[M+K]^+$ ion[a]	Experimental *m/z*[a, b]: AG (UV-LDI-MS)	Experimental *m/z*[a, b]: Leg (UV-LDI-MS)	Detected by GC/MS[c]	Detected by ESI-MS[d]	Detected by DART-MS[e]
$C_{20}H_{38}O_2$ (cVA)	349.25	349.25	349.26	+	+	+
$C_{23}H_{44}$ (tricosadiene)	359.31	359.32	359.33	+*	+	+
$C_{23}H_{44}O$	375.30	375.31	375.32	n/d	+	+
$C_{23}H_{44}O_2$	391.30	391.30	391.31	n/d	+	+
$C_{23}H_{44}O_3$	407.29	407.28	407.30	n/d	+	+
$C_{23}H_{46}$ (tricosene)	361.32	361.34	361.35	+*	+	+
$C_{23}H_{46}O$	377.32	377.32	377.33	n/d	+	+
$C_{23}H_{46}O_3$	409.31	409.29	409.31	n/d	+	+
$C_{25}H_{48}$ (pentacosadiene)	371.37 ($[M+Na]^+$)	371.36 ($[M+Na]^+$)	371.37 ($[M+Na]^+$)	n/d	+	n/d
$C_{25}H_{48}$ (pentacosadiene)	387.34	387.34	387.34	+*	+	+
$C_{25}H_{48}O$	403.33	403.35	403.34	n/d	+	+
$C_{25}H_{50}$ (pentacosene)	389.36	-	389.35	+*	n/d	+
$C_{25}H_{50}O$	405.35	405.36	405.35	n/d	+	+
$C_{29}H_{56}$ (nonacosatriene)	413.35	413.34	413.34	n/d	+	n/d
$C_{27}H_{52}$ (heptacosadiene)	415.37	415.38	415.37	n/d	+	+
$C_{27}H_{54}$ (heptacosene)	417.39	417.38	-	+*	+	+
$C_{27}H_{54}O$	433.38	433.38	433.39	n/d	+	+
$C_{27}H_{54}O_2$	449.38	449.36	449.36	n/d	+	n/d
$C_{29}H_{56}$ (nonacosadiene)	443.40	443.41	443.40	+*	+	+
$C_{29}H_{58}$ (nonacosene)	445.42	445.42	445.44	+*	+	+
$C_{29}H_{58}O$	461.41	461.41	461.41	n/d	+	+
$C_{30}H_{58}$ (triacontadiene)	457.42	457.43	457.40	n/d	n/d	n/d
$C_{30}H_{56}O_3$ (CH503)	503.39	503.39	503.38	n/d	+	+
$C_{31}H_{60}$ (hentriacontadiene)	471.43	471.44	471.44	n/d*	n/d	+
$C_{31}H_{62}$ (hentriacontene)	473.45	473.44	473.44	n/d	+	+
$C_{33}H_{64}$ (tritriacontadiene)	499.46	499.46	499.47	+	+	+
$C_{35}H_{68}$ (pentatriacontadiene)	527.50	527.49	527.51	+	+	+
$C_{12}H_{22}O_{11}$ (dihexose)	381.08	n/d	381.09	n/d	+	n/d

[a]monoisotopic mass; previously unreported CH species are highlighted in gray. [b]UV-LDI signals correspond to the potassiated molecule ($[M+K]^+$).[c]GC/MS was performed using cuticular extracts as described in Supplemental experimental procedures; radical molecules ($M^{\bullet+}$] form the base peaks in the mass spectrum; *indicates compounds that were also detected in extracts from males or females in previous GC/MS studies [Ref. 20]. [d]Nano-electrospray ionization (ESI)-MS was performed using cuticular extract as described in Experimental procedures; protonated $[M+H]^+$, sodiated $[M+Na]^+$, and potassiated $[M+K]^+$molecules are detected. [e]Direct Analysis in Real Time (DART)-MS was performed using single intact flies; protonated molecules $[M+H]^+$ form the base peaks in the mass spectrum. +: ion species successfully detected; n.d.: ion species not detected; AG: anogenital region.

Table S3. Proposed elemental composition and detected *m/z* value for each of the major UV-LDI MS ion signals shown in Figure 1D, F, recorded from adult virgin female flies.

Elemental Composition	Calculated *m/z* of $[M+K]^+$ ion[a]	Experimental *m/z*[a]: AG	Experimental *m/z*[a]: leg
$C_{20}H_{38}O_2$ (cVA)	349.25	349.23	349.25
$C_{23}H_{44}O_2$	391.30	n.d.	391.31
$C_{23}H_{46}O$	377.32	377.33	377.32
$C_{25}H_{48}$ (pentacosadiene)	387.34	387.35	387.35
$C_{25}H_{48}O$	403.33	403.34	403.35
$C_{25}H_{50}O$	405.35	405.36	405.35
$C_{27}H_{50}$ (heptacosatriene)	413.35	413.37	413.37
$C_{27}H_{52}$ (heptacosadiene)	399.40 ($[M+Na]^+$)	399.41 ($[M+Na]^+$)	399.40 ($[M+Na]^+$)
$C_{27}H_{52}$ (heptacosadiene)	415.37	415.38	415.38
$C_{27}H_{54}O$	433.38	433.38	433.38
$C_{27}H_{54}O_2$	449.38	449.37	449.38
$C_{29}H_{54}O_2$	441.39	441.39	441.39
$C_{29}H_{56}$ (nonacosadiene)	427.43 ($[M+Na]^+$)	427.44 ($[M+Na]^+$)	427.44 ($[M+Na]^+$)
$C_{29}H_{56}$ (nonacosadiene)	443.40	443.41	443.41
$C_{29}H_{54}$ (nonacosatriene)	461.41	461.41	461.41
$C_{33}H_{64}$ (tritriacontadiene)	457.42	457.42	457.42
$C_{31}H_{60}$ (hentriacontadiene)	471.43	471.44	471.44
$C_{33}H_{64}$ (tritriacontadiene)	499.46	499.47	499.48
$C_{35}H_{68}$ (pentatriacontadiene)	527.50	527.51	527.51
$C_{12}H_{22}O_{11}$ (dihexose)	381.08	377.33	381.09

[a]monoisotopic mass; unless noted, all listed values correspond to the potassiated molecule ($[M+K]^+$); previously unreported CH species are highlighted in gray; AG: anogenital region.

Table S4. Averaged, normalized values for the relative abundance of the major male CHs detected on female cuticles after successful mating (see Figure 2).

Time after mating	Anogenital region (AG)				Leg			
	$C_{20}H_{38}O_2$ (cVA)	$C_{23}H_{44}O$	$C_{23}H_{46}O$	$C_{30}H_{56}O_3$ (CH503)	$C_{20}H_{38}O_2$ (cVA)	$C_{23}H_{44}O$	$C_{23}H_{46}O$	$C_{30}H_{56}O_3$ (CH503)
Virgin (n=12)	0.4 ± 0.1	0.8 ± 0.1	2.3 ± 0.5	n.d.	0.4 ± 0.1	1.4 ± 0.2	3.0 ± 0.6	n.d.
15 min (n=12)	139.1 ± 44.7	1.2 ± 0.3	3.8 ± 1.0	101.2 ± 41.9	7.1 ± 2.5	1.1 ± 0.2	3.2 ± 0.6	1.8 ± 1.1
1 – 2 h (n=20)	41.7 ± 15.2	0.7 ± 0.2	2.4 ± 0.5	72.5 ± 21.7	3.1 ± 0.7	0.4 ± 0.04	1.3 ± 0.1	0.9 ± 0.2
24 h (n=17)	32.0 ± 6.7	9.5 ± 2.5	16.3 ± 3.9	185.5 ± 63.8	2.3 ± 0.4	7.0 ± 2.3	11.0 ± 3.1	3.6 ± 1.4
2 d (n=7)	9.7 ± 2.0	9.5 ± 0.7	29.1 ± 2.2	67.8 ± 11.1	1.4 ± 0.2	5.2 ± 0.8	19.2 ± 1.6	1.8 ± 0.4
3 d (n=15)	8.1 ± 1.4	4.2 ± 1.0	11.6 ± 3.3	32.3 ± 9.5	1.3 ± 0.3	3.8 ± 0.6	6.2 ± 1.3	0.8 ± 0.3
4 d (n_{AG}=13,n_{leg}=9)	4.5 ± 1.2	3.7 ± 1.0	10.2 ± 4.0	31.4 ± 11.5	0.7 ± 0.2	2.0 ± 0.5	2.4 ± 0.4	0.9 ± 0.3
6 d (n=10)	1.9 ± 0.6	1.7 ± 0.3	2.6 ± 0.4	15.8 ± 5.1	0.5 ± 0.1	2.2 ± 0.5	2.7 ± 0.6	0.4 ± 0.2
8 d (n=15)	0.4 ± 0.04	0.8 ± 0.1	1.8 ± 0.2	6.3 ± 0.9	0.4 ± 0.04	1.0 ± 0.1	1.7 ± 0.2	0.2 ± 0.1
10 d (n=9)	0.8 ± 0.2	0.8 ± 0.1	1.5 ± 0.2	2.9 ± 1.0	0.9 ± 0.2	1.7 ± 0.4	2.7 ± 0.4	0.1 ± 0.1
15 d (n=10)	0.5 ± 0.1	1.2 ± 0.2	2.0 ± 0.2	1.4 ± 0.5	0.4 ± 0.1	1.3 ± 0.2	2.0 ± 0.2	0.1 ± 0.04

The intensity value for each signal detected in the anogenital region (AG) and the leg is calculated by dividing the area under the monoisotopic peak by the total area under the monoisotopic peak of the signals for $C_{25}H_{48}$, $C_{27}H_{52}$, and $C_{29}H_{56}$, the three major female CHs found on female cuticles. The mean ± S.E.M. is indicated; see Fig. 2 for sample sizes. n.d.: not detected.

Table S5. Averaged, normalized values for the relative abundance of the major female CHs detected on male cuticles after successful mating (see Figure 3).

Time after mating	Anogenital region (AG)[a]		Leg[b]	
	$C_{27}H_{52}$ (heptacosadiene)	$C_{29}H_{56}$ (nonacosadiene)	$C_{27}H_{52}$ (heptacosadiene)	$C_{29}H_{56}$ (nonacosadiene)
Virgin (n_{AG}=13, n_{leg}=12)	0.8 ± 0.2	1.1 ± 0.3	1.5 ± 0.3	0.9 ± 0.2
1 – 2 h (n=24)	115.4 ± 36.4	96.6 ± 31.1	244.5 ± 37.7	359.7 ± 86.4
24 h (n=17)	1.8 ± 0.8	1.7 ± 0.6	10.4 ± 2.3	4.9 ± 1.0
48 h (n_{leg}=8)	n.a.	n.a.	1.6 ± 0.8	1.0 ± 0.4

[a]The relative intensity value for each signal detected in the anogenital region (AG) is calculated by dividing the area under the monoisotopic peak by the total area under the monoisotopic peak of the signals for cVA and CH503, the two major male CHs found in the AG. The mean ± S.E.M. is indicated. [b]The relative intensity value for each signal detected in the leg is calculated by dividing the area under the monoisotopic peak by the total area under the monoisotopic peak for $C_{23}H_{44}O$, $C_{23}H_{46}O$, and $C_{23}H_{44}O_2$. The mean ± S.E.M. is indicated. n.a.: not analyzed.

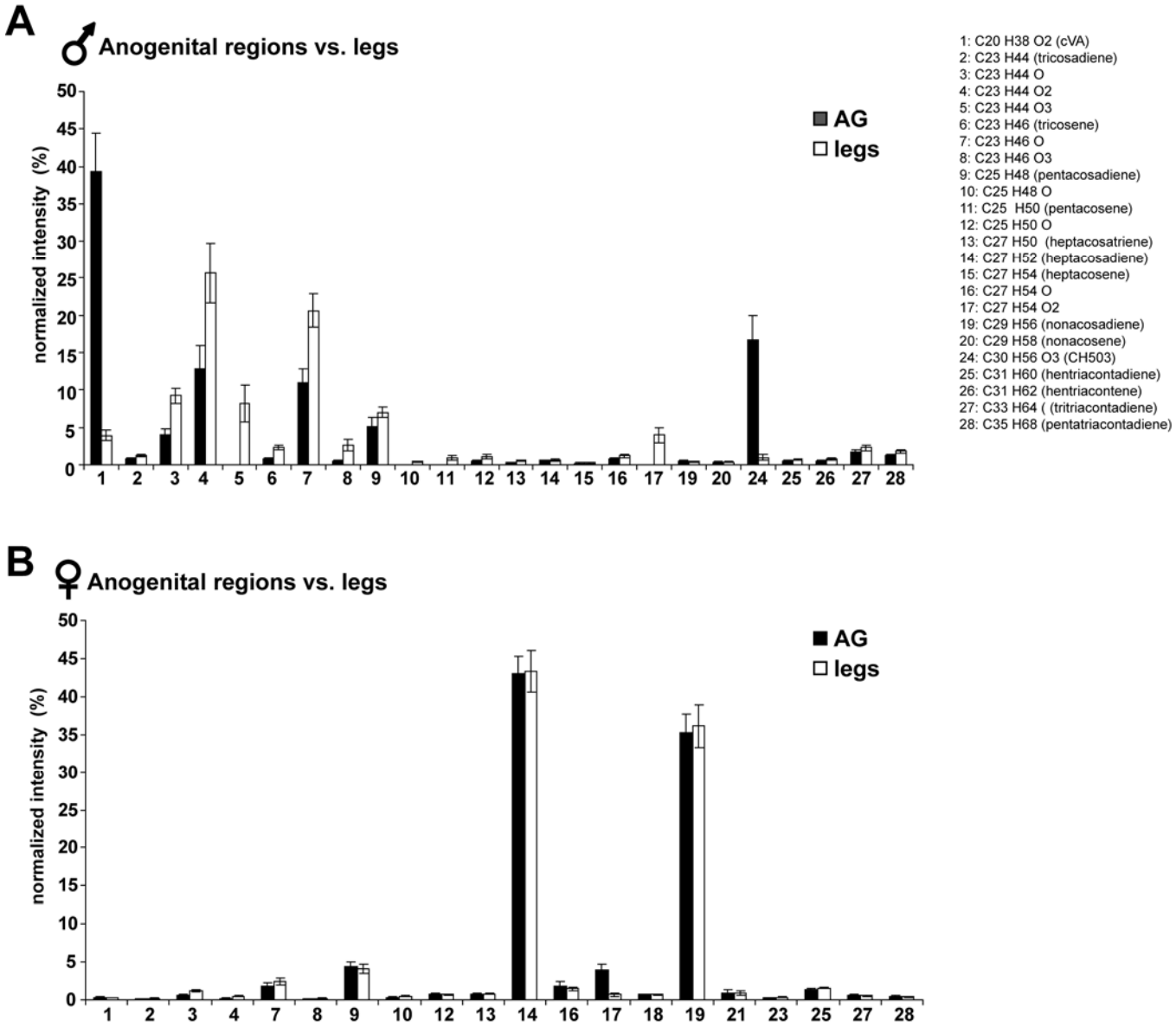

Figure S1. Semi-quantitative analysis of CH profiles in the anogenital regions (AG) and legs of socially naïve, virgin male and female flies. Each bar represents the averaged normalized signal intensity measured from individual, socially naïve virgin flies. The normalized intensity is calculated as the area under the monoisotopic peak of each CH species divided by the total area of the monoisotopic peak for all detected CHs in the same experiment. Relative intensities are not representative of absolute abundances since ionization efficiency can partly depend on chemical structure. See Table S1 for derived values.

(A) The AG and legs of males exhibit quantitatively different CH profiles. cVA and CH503 are the most abundant compounds in the AG. In the legs, hydroxylated tricosene, hydroxylated tricosadiene, and pentacosadiene are the most prominent CHs. Error bars indicate S.E.M; n=12 for legs and n=13 for AG.

(B) The AG and legs of females exhibit nearly identical CH profiles in terms of CH composition and the relative abundance of each species. Differences in relative intensities between the two regions are not statistically different ($p>0.05$, Student's t-test). Error bars indicate S.E.M; n=12.

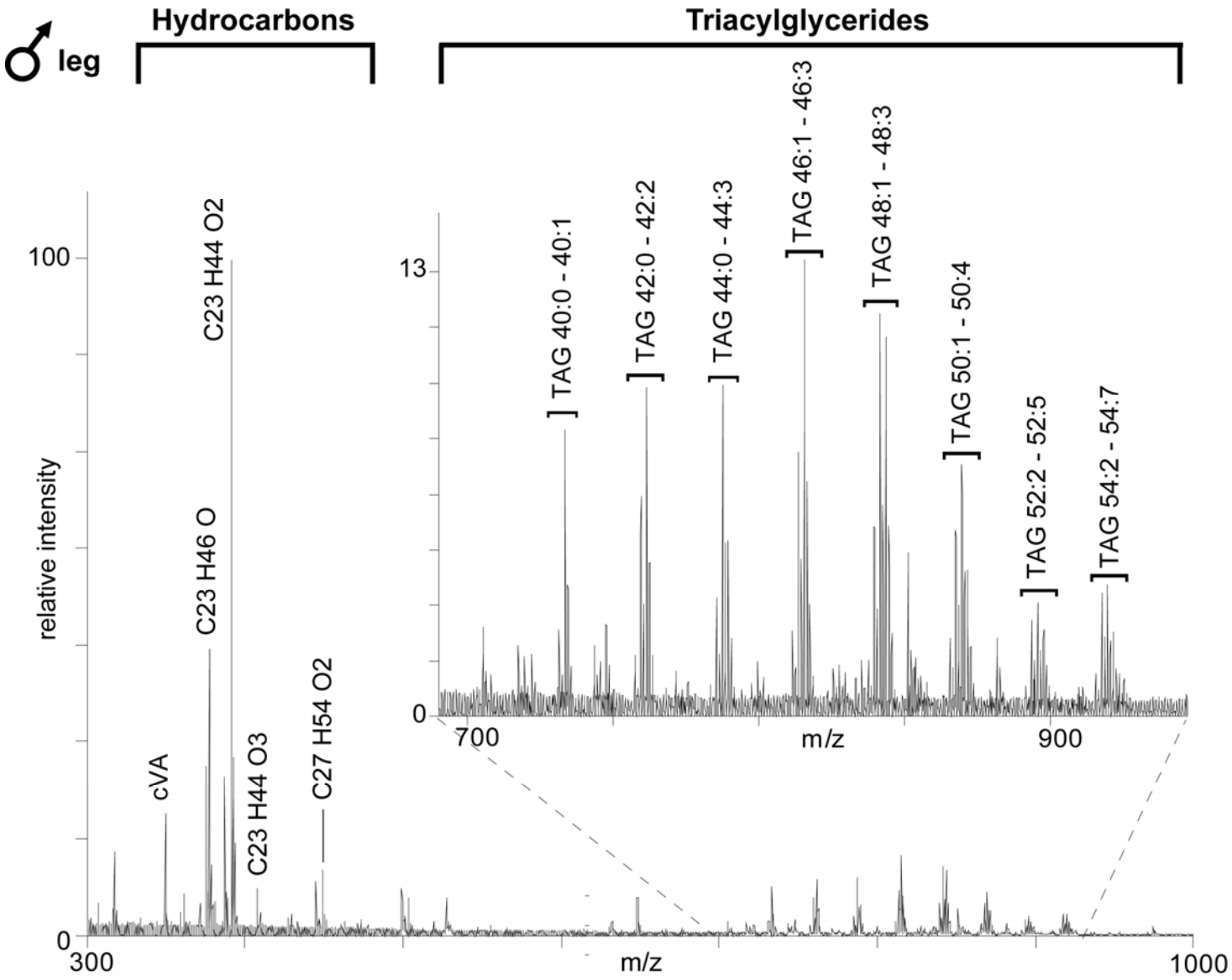

Figure S2. A representative UV-LDI mass spectrum taken from the leg of an adult male fly showing the *m/z* range from 300 – 1000. In addition to signals representing CHs, a series of ion signals with *m/z* values matching the calculated *m/z* of potassiated triacylglycerides (TAGs) is detected between *m/z* 700 – 950. The inset shows the cluster of signals corresponding to TAGs containing between 40 – 54 carbon atoms in the 3 fatty acid residues and up to 7 double bonds (number of double bonds is indicated after the colon). The degree of unsaturation increases with chain lengths. All labeled ions represent potassiated species $[M+K]^+$.

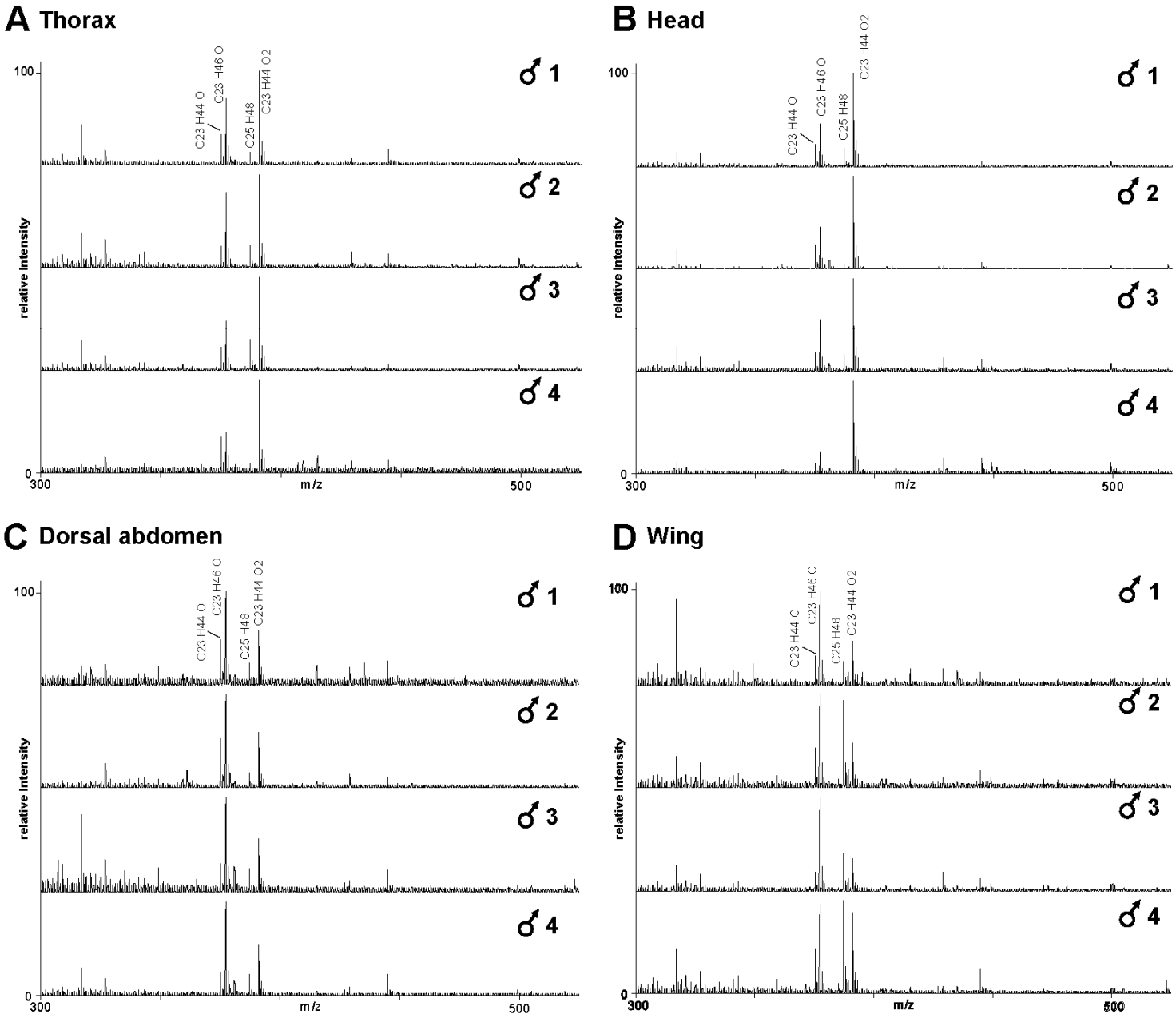

Figure S3. Representative UV-LDI mass spectra recorded from the thorax (**A**), head (**B**), dorsal abdomen (**C**), and wing (**D**) of four adult male flies. The different body parts show small differences in terms of CH composition. Some variation in the relative ratios of singly hydroxylated tricosene and tricosadiene ($C_{23}H_{44}O$ and $C_{23}H_{46}O$) relative to pentacosadiene ($C_{25}H_{48}$) and doubly hydroxylated tricosadiene ($C_{23}H_{44}O_2$) is evident when comparing the different regions to each other. For the most part, the same individual CH can be reliably compared from fly to fly within the same region. However, the wing appears to show more variation between individuals. Compounds other than CHs such as fatty acids and oligosaccharides are also detected particularly below *m/z* 400. All assigned signals correspond to potassiated molecules $[M+K]^+$. Peaks corresponding to sodiated species are not labeled.

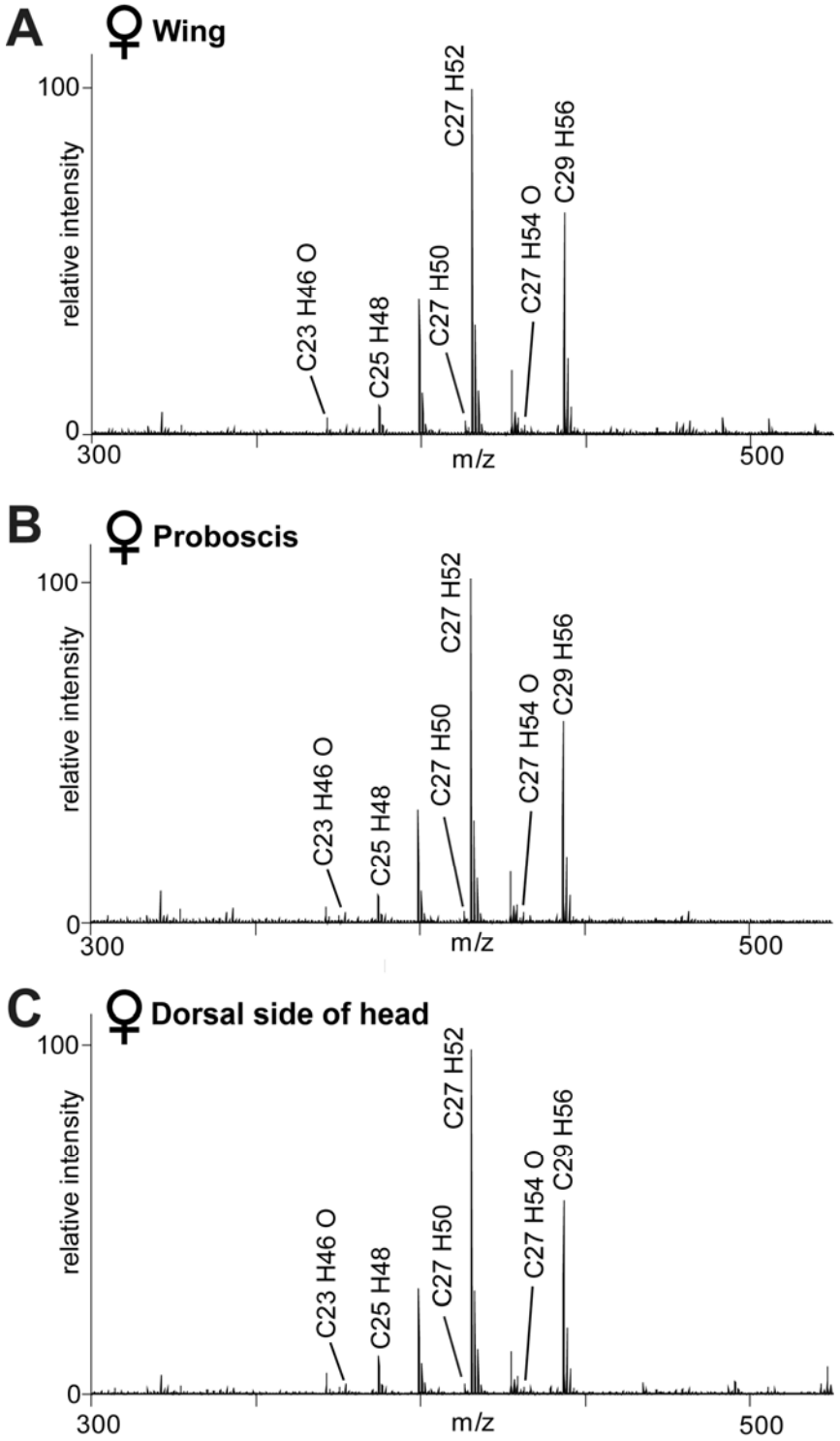

Figure S4. Representative UV-LDI mass spectra recorded from the wing (**A**), proboscis (**B**), and head (**C**) of an adult female fly. The different body parts show small differences in terms of CH composition and the relative intensity of the individual compounds. All assigned signals correspond to potassiated molecules $[M+K]^+$. Peaks corresponding to sodiated species are not labeled.

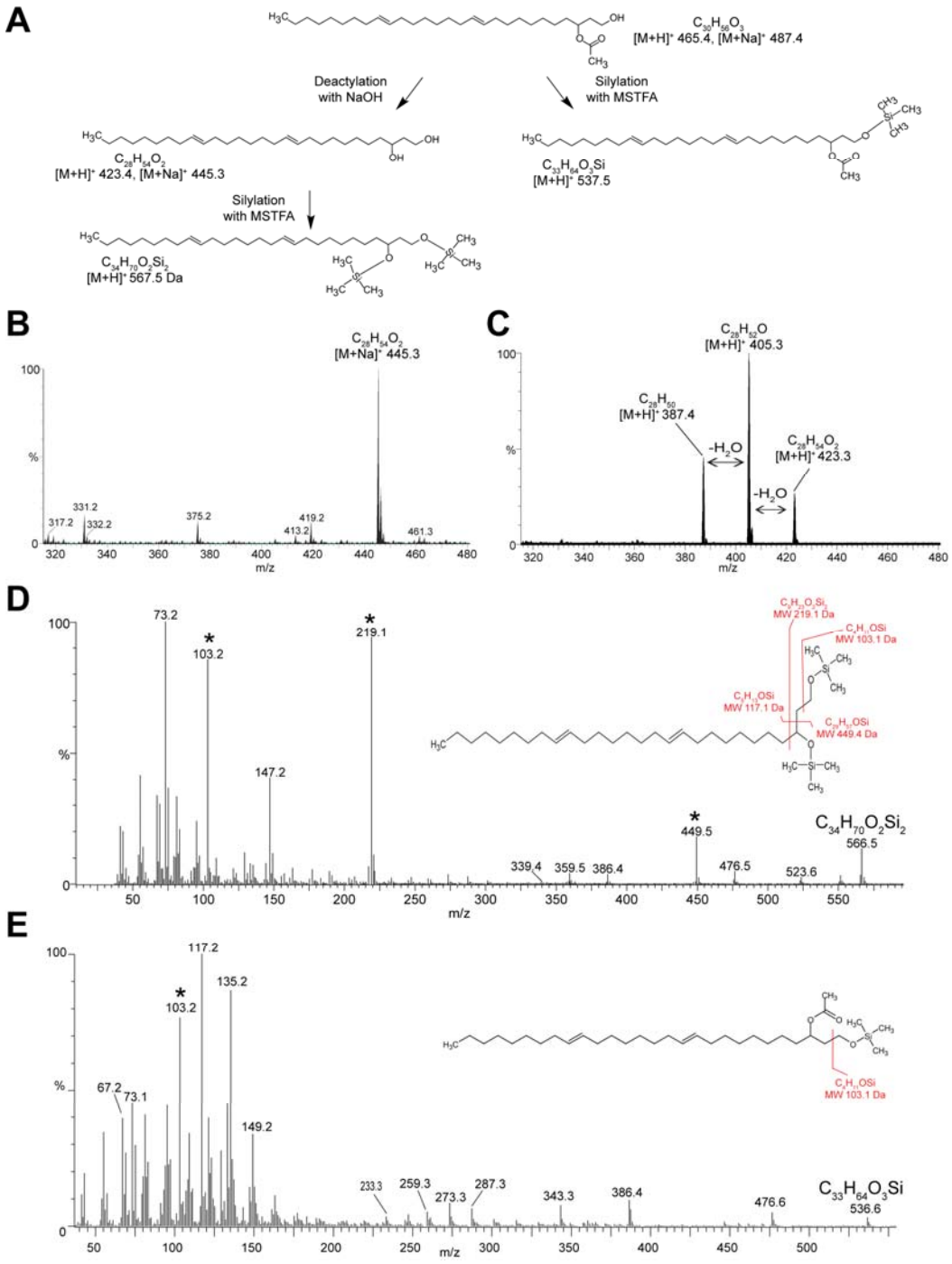

Figure S5. Chemical derivatization of HPTLC-purified CH503 followed by nano-ESI and GC/MS analysis determined the positions of the hydroxyl and the acetate groups to be at C1 and C3, respectively.

(**A**) Two derivatizations were used: (i) deacetylation of CH503 followed by silylation of the two hydroxyl groups in the product and (ii) silylation of singly hydroxylated CH503.

(**B**) Nano-ESI-MS analysis of the HPTLC-purified fraction following deacetylation shows that the acetyl group is removed from CH503, resulting in a mass shift of 42.1 Da. The molecular ion of the deacetylated product is detected at *m/z* 445.3, corresponding to the sodiated molecule $[M+Na]^+$.

(**C**) Nano-ESI tandem MS analysis of deacetylated CH503, detected as a protonated molecule $[M+H]^+$ at *m/z* 423.3, results in the successive loss of two water molecules. This observation indicates the presence of two hydroxyl groups in the deacetylated compound.

(**D**) GC/MS analysis of deacetylated and silylated CH503 determines that the positions of the hydroxyl groups are at C1 and C3. The presence of the silyoxy groups induce α-fragmentation, producing diagnostic fragments ions (labeled with *) at *m/z* 103.2 (indicative of a primary silyloxy group) and at *m/z* 219.1 and 449.4 (indicative of a silyloxy group at C3).

(**E**) GC/MS analysis of silylated CH503 (that has *not* been deacetylated) determines that the position of the siloxy group is at C1 and the acetate is at C3. The primary silyloxyl group produces a diagnostic fragment ion at *m/z* 103.2 (labeled with *). In contrast, the acetate group does not induce α-fragmentation. If the silyloxyl group were at the C3 position instead, ions at *m/z* 189.1 and 449.4 would be observed. The absence of these diagnostic ions indicates that the hydroxyl group is at C1 and the acetate group is at C3.

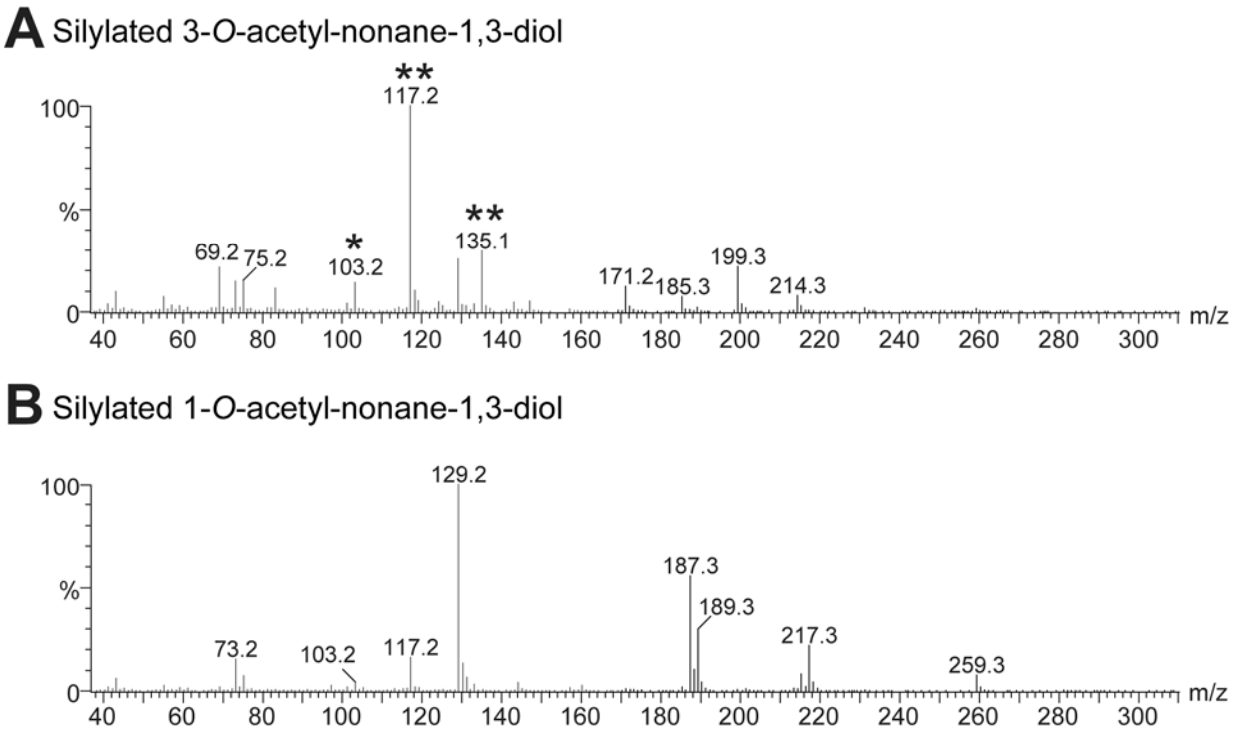

Figure S6. Additional structural confirmation was obtained by GC/MS analyses of silylated synthetic reference compounds.

(**A**) GC/MS of silylated 3-*O*-acetyl-nonane-1,3-diol (bearing the acetate group in the C3 position) produced a similar pattern of diagnostic fragment ions when compared to silylated CH503 (Supplemental figure 4E). Both spectra contain a diagnostic fragment at *m/z* 103.2 corresponding to the primary silyloxyl group (indicated by *), and characteristic rearrangement ions at *m/z* 117.2 and 135.2 (indicated by **).

(**B**) GC/MS analysis of the silylated 1-*O*-acetyl-nonane-1,3-diol (bearing the acetate group in C1 position) produced a different pattern of fragmentation.

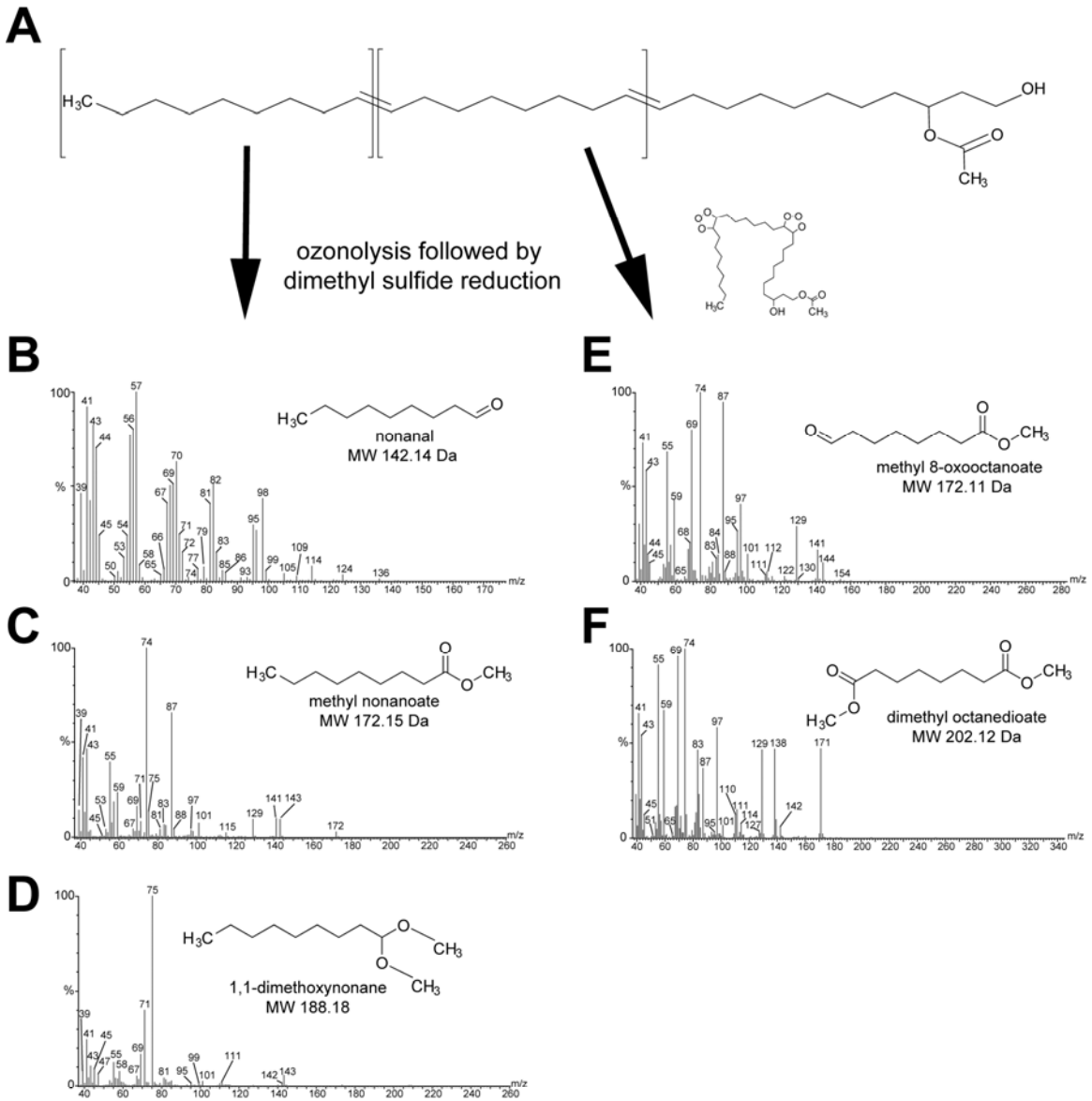

Figure S7. The locations of the two carbon-carbon double bonds were determined using chemical derivatization of the HPTLC-purified CH503 followed by ozonolysis and reductive workup with dimethyl sulfide (**A**). GC/MS analysis of the products placed the positions of the double bonds at C11 and C19 (B-F). Two different series of ions are apparent: a monofunctional group with a 9 Carbon atom chain length **(B-D**) and a bi-functional group of 8 Carbon atoms in length (**E, F**).

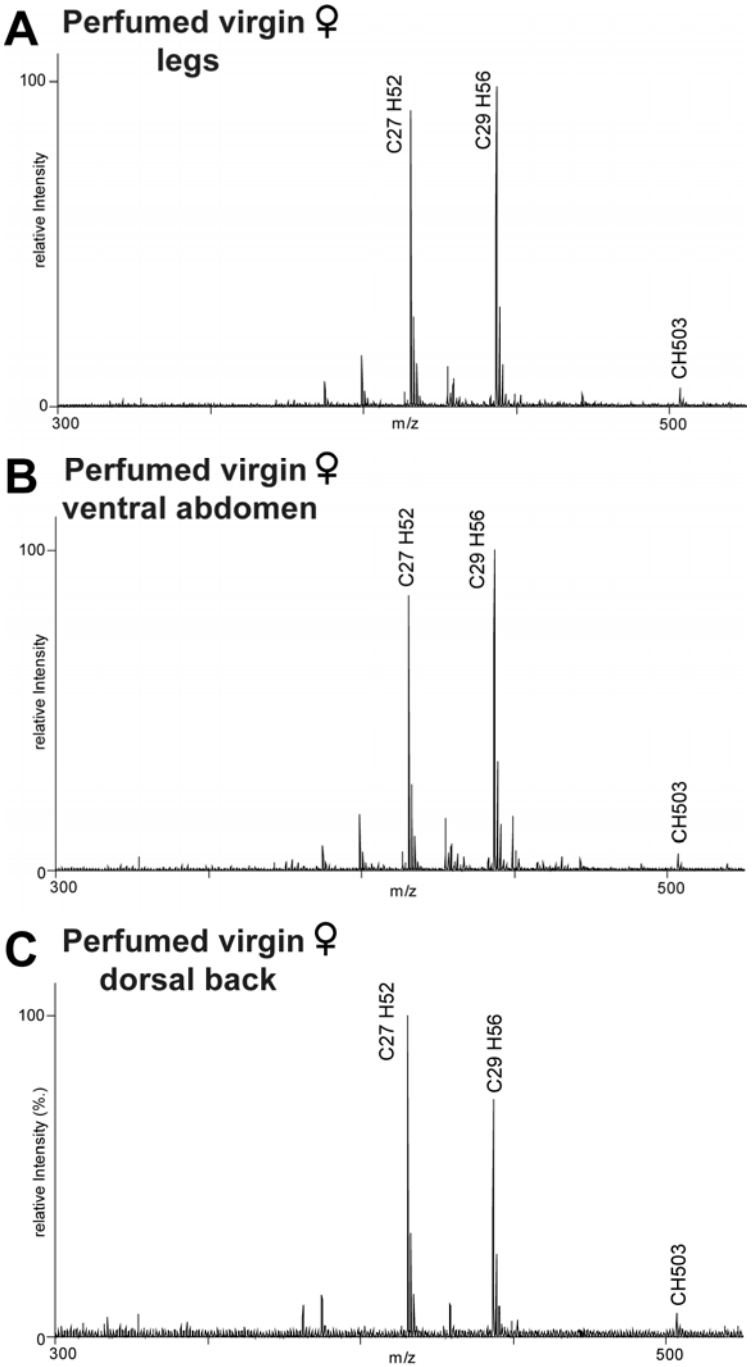

Figure S8. UV-LDI-MS profile of a 4 day old virgin female perfumed with CH503 at the 100% dose (see Experimental Procedures). Hydrocarbon profiles recorded from cuticle on the back (**A**), leg (**B**), and head (**C**) indicate relatively homogeneous perfuming of the whole animal. The signal intensity for CH503 is not greatly out of proportion with respect to the signals for labeled endogenous female CHs such as heptacosadiene ($C_{27}H_{52}$) and nonacosadiene ($C_{29}H_{56}$). All assigned peaks correspond to potassiated molecules $[M+K]^+$.