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Article

A Novel Class of Defensive Compounds in Harvestmen: Hydroxy-γ-Lactones from the Phalangiid *Egaenus convexus*

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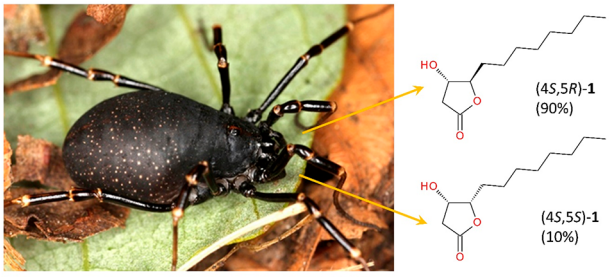
ABSTRACT: When threatened, the harvestman *Egaenus convexus* (Opiliones: Phalangiidae) ejects a secretion against offenders. The secretion originates from large prosomal scent glands and is mainly composed of two isomers of 4-hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (**1**), a β-hydroxy-γ-lactone. The compounds were characterized by GC-MS of their microreaction derivatives, HRMS, and NMR. After the synthesis of all four possible stereoisomers of **1**, followed by their separation by chiral-phase GC, the absolute configurations of the lactones in the *Egaenus* secretion was found to be (4*S*,5*R*)-**1** (90%) and (4*S*,5*S*)-**1** (10%). Hydroxy-γ-lactones represent a new class of exocrine defense compounds in harvestmen.



Chemical defense in harvestmen (arachnid order Opiliones) is associated with large prosomal scent glands[1] from which these arachnids discharge repellent secretions against predators.[2] Exudates from these glands have been studied for more than 50 years,[3] representing a rich source of rare, unusual, and new natural products. Current knowledge indicates that scent gland exudates comprise compounds such as naphthoquinones, chloro-naphthoquinones, and aliphatic methyl ketones in the harvestman suborder Cyphophthalmi, nitrogen-containing substances, terpenes, aliphatic ketones, and phenolics in the suborder Insidiatores, alkylated phenolics and benzoquinones in the suborder Grassatores, and secretions of rather miscellaneous chemistry in the Palpatores.[2,4−6] The latter group, the Palpatores, is subdivided into suborders Eupnoi and Dyspnoi[7] and includes more than 2100 species of commonly occurring and conspicuous harvestmen. Despite this species richness, the chemistry of eupnoan and dyspnoan secretions has remained largely enigmatic.

In Dyspnoi, the secretions of only four species have hitherto been analyzed, showing naphthoquinones along with methyl- and ethyl-ketones.[5,8] For Eupnoi, representatives of Sclerosomatidae appear to rely on acyclic compounds such as ethyl ketones (and derivatives),[9] whereas initial investigations of the other large eupnoan family, the Phalangiidae (with only two species of the subfamily Phalangiinae studied), indicate naphthoquinones (*Phalangium*, *Rilaena*), 1,4-benzoquinone, and medium chain fatty acids (*Rilaena*).[6,10] However, dyspnoan and eupnoan secretions appear to be key elements in a comprehensive, phylogenetically founded picture of harvestmen scent gland chemistry.[4]

We focus here on the secretions of a first representative of the phalangiid subfamily Opilioninae, *Egaenus convexus*, a massively built species that is widespread in Central and Southeastern Europe. When threatened, individuals of *E. convexus* eject a "jet" from scent glands toward offenders (Figure 1).

Individual secretions directly dabbed from gland openings of adults of both sexes constantly showed two major peaks (A, B) by GC-MS. The peaks exhibited nearly identical EI-mass spectra, indicating isomeric compounds, with an earlier-eluting major isomer or mixture of isomers (peak A: RI = 1878 on a ZB-5MS column) and a later-eluting minor isomer or mixture of isomers (peak B: RI = 1901). Peaks A and B were present in a ratio of about 9:1 (Figure 2).

A molecular ion for these compounds was very weak in EI-MS but confirmed to be at *m*/*z* 214 by PCI-MS (positive ion chemical ionization; using methane as reagent gas: MH^+ at *m*/*z* 215; M + $C_2H_5^+$ at *m*/*z* 243; M + $C_3H_5^+$ at *m*/*z* 255). HRESI-MS revealed an exact monoisotopic mass for $[M + H]^+$ at *m*/*z* 215.1642, corresponding to a molecular formula of $C_{12}H_{22}O_3$ and thus two sites of unsaturation/rings, respectively.

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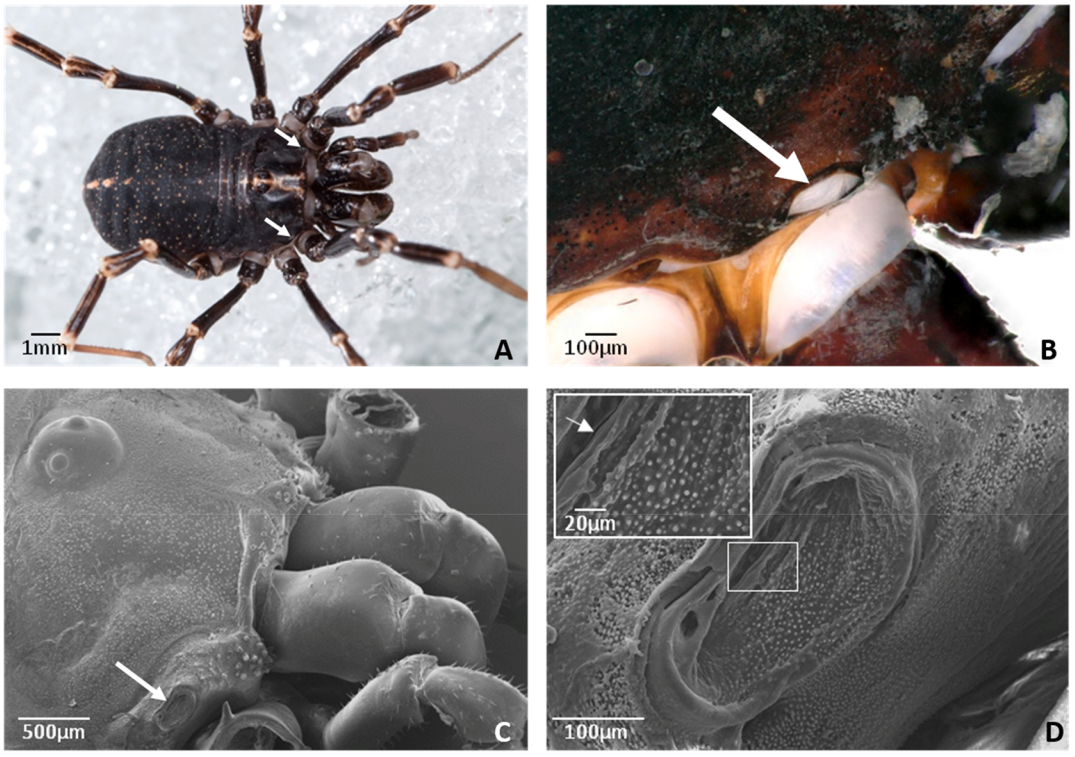

Figure 1. (A) A female individual with the position of the ozopores (gland openings) indicated (arrows). (B) Ozopores (right ozopore is shown: arrow) are located near-dorsal to the coxa of leg I, are oval-shaped with a dimension of about 340 μm × 150 μm, and are surrounded by a cuticular rim (C, D). The flat central bottom is of smooth cuticle, whitish, thin, and membrane-like in appearance. This bottom is movable and trapdoor-like, and secretion can be released through a slit (D). Details of the right ozopore: (B) light microscopic photograph; note the whitish and membranous structure of the bottom of the pore; (C, D) scanning electron micrographs; note that the pore is slightly opened (arrow in (D)).

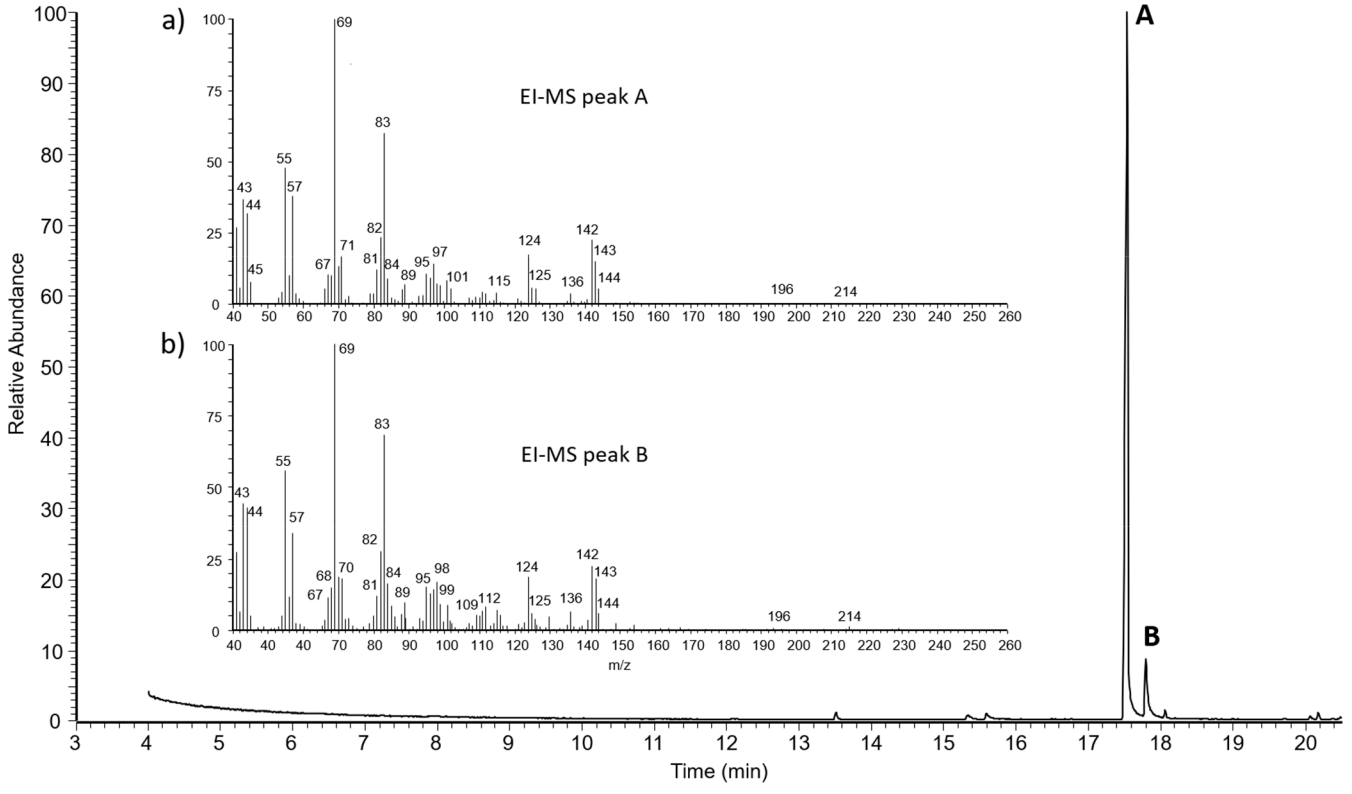

Figure 2. Total ion chromatogram of scent gland secretion of an individual of *Egaenus convexus*, showing main peaks A and B, and their EI-mass spectra (a, b). The secretion was dabbed from ozopores in the moment of extrusion (compare Figure S23).

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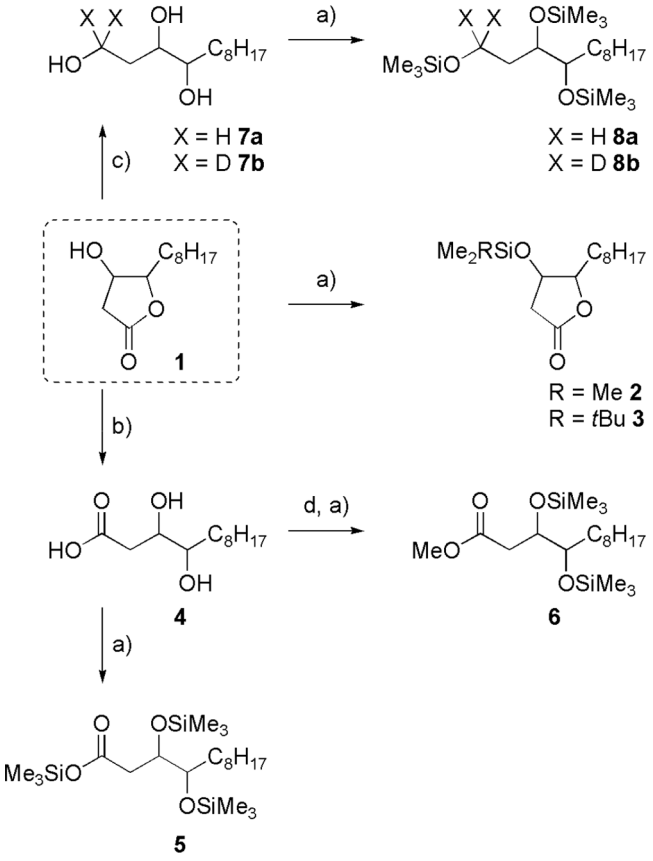
Silylation of the extract with MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoracetamide) led to the TMS (trimethylsilyl)-derivatives **2** of the original compounds (Scheme 1). EI-

Scheme 1. Procedure of the Identification of 4-Hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (1) by GC-MS of Derivatives Obtained by Microderivatization[a]



[a](a) Silylation (MSTFA, MTBSTFA); (b) saponification ($NaOCH_3$); (c) reduction ($LiAlH_4$); (d) methylation (diazomethane). For details, see the Supporting Information.

mass spectra showed the addition of a single TMS moiety to the original molecules (plus 72 mass units), with diagnostic ions at *m/z* 271 (M-15) and a very weak molecular ion at *m/z* 286 (intensity < 1%). The TMS products were subsequently analyzed by PCI-MS, confirming the molecular ion at *m/z* 286 (MH^+ at *m/z* 287, M + $C_2H_5^+$ at *m/z* 315). Accordingly, the derivatization with MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluotoacetamide) indicated the addition of one butyldimethylsilyl group, leading to adduct **3**, with M^+ at *m/z* 328 (plus 114 mass units), as evidenced by PCI-MS (MH^+ at *m/z* 329, along with $C_2H_5^+$ and $C_3H_5^+$ adducts at *m/z* 357 and 369, respectively). Upon saponification (NaOMe), an addition of 18 mass units to the original compounds was observed (**4**: M^+ at *m/z* 232). Subsequent derivatization with MSTFA led to compound **5** with M^+ at *m/z* 448 (as confirmed by PCIMS), now showing the addition of three TMS moieties (232 plus 3 × 72 = 448). These data are consistent with three hydroxy groups or two hydroxy groups and one carboxyl group after saponification. Treatment with diazomethane following saponification and subsequent derivatization with MSTFA produced compound **6** with M^+ at *m/z* 390, suggesting (i) the addition of a methyl group (to a carboxyl group) plus (ii) the addition of two TMS moieties to two hydroxy groups. This is consistent with a methyl-ester bis-TMS-ether structure or a carboxyl group plus two hydroxy groups after saponification. Reduction of the original compounds using $LiAlH_4$ led to compound **7** with M^+ at *m/z* 218, indicating the addition of four hydrogens, as confirmed by subsequent MSTFA derivatization (**8**: M^+ at *m/z* 434; *m/z* 218 plus 3 × 72). By using $LiAlD_4$, two hydrogens and two deuterium atoms were added, resulting in a TMS product **8b** of molecular weight at *M* = 436 g/mol (Scheme 1). Thus, the parent structure was indicated to carry two ketone/aldehyde functions with no ring or was a lactone.

EI-MS of the original compound **1** as well as the fragmentation of the TMS-ether **2** were consistent with the structure of isomeric *β*-hydroxy-*γ*-alkyl-lactones.[11] In detail, a *β*-hydroxy-*γ*-octyl lactone was supported by (i) characteristic fragment ions from the rearrangement of the lactone leading to *m/z* 143/142 (= furan oxygen plus an octyl group: $Me(CH_2)_7CHO^+$ and $Me(CH_2)_7CH_2O^+$, respectively); (ii) ions at *m/z* 124/125 (elimination of H_2O from the latter), together with (iii) *m/z* 44 (= OH-bearing moiety of the lactone ring: $C_2H_4O^+$). In the TMS derivative **2**, the corresponding fragment ions were recorded at *m/z* 116 (44 + 72: $C_5H_{12}OSi$) and *m/z* 215 (143 + 72: $Me(CH_2)_7CHO\text{-}SiMe_3^+$). The latter ion at *m/z* 215 arises by rearrangement of 3-O-TMS-alkano-4-lactones, supporting the octyl group and the OH group in the *γ*- and *β*-positions on the lactone ring, respectively. With this information given, the mass spectra of the remaining microderivatives **3**–**8** were interpreted (Scheme 1), finding full accordance with a 4-hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one structure: saponification of **1** led to the opening of the lactone ring and to the generation of a 3,4-dihydroxydodecanoic acid (**4**), which could be converted into trimethylsilyl 3,4-bis((trimethylsilyl)oxy)dodecanoate (**5**) by methylation and silylation. On the other hand, the reduction of **1** led to lactone-ring opening and to the generation of 1,3,4-dodecanetriol (**7**), which could be converted into 1,3,4-tris((trimethylsilyl)oxy)dodecane (**8**) by silylation. Thus, peaks A and B were proposed to be isomers or mixtures of isomers of 4-hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (**1**).

Because the quantity of **1** in extracts of single individuals was too low to perform NMR analyses, a pooled extract containing the secretions of 400 individuals of both sexes was prepared. The major isomer of **1** from the pooled extract (corresponding to peak A in the chromatograms) was purified by column chromatography, followed by preparative gas chromatography. One dimensional ^1H NMR of **1** (Figure 3; Table 1) fully supported the proposed hydroxy-lactone structure, revealing the presence of a linear alkyl (octyl) residue, two protons located on carbon atoms carrying oxygen substituents, and two diastereotopic protons present next to a carbonyl group.

A COSY spectrum indicated that these protons where located on adjacent carbon atoms (Figure S2). This is realized within a 5-membered lactone. Moreover, the observed chemical shifts and coupling constants were remarkably similar to the reported values for 4-hydroxy-5-methylbutyrolactone and 4-hydroxy-5-octylbutyrolactone in the literature.[12,13] Additionally, a comparison to these *γ*-butyrolactones of defined configuration tentatively suggested that the major isomer of **1** was a 4,5-anti-diastereomer, as indicated by coupling constants of the isolated H-3a and H-3b resonances (Figure 4).

Because 4-hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (**1**) possesses two stereogenic centers, four stereoisomers are

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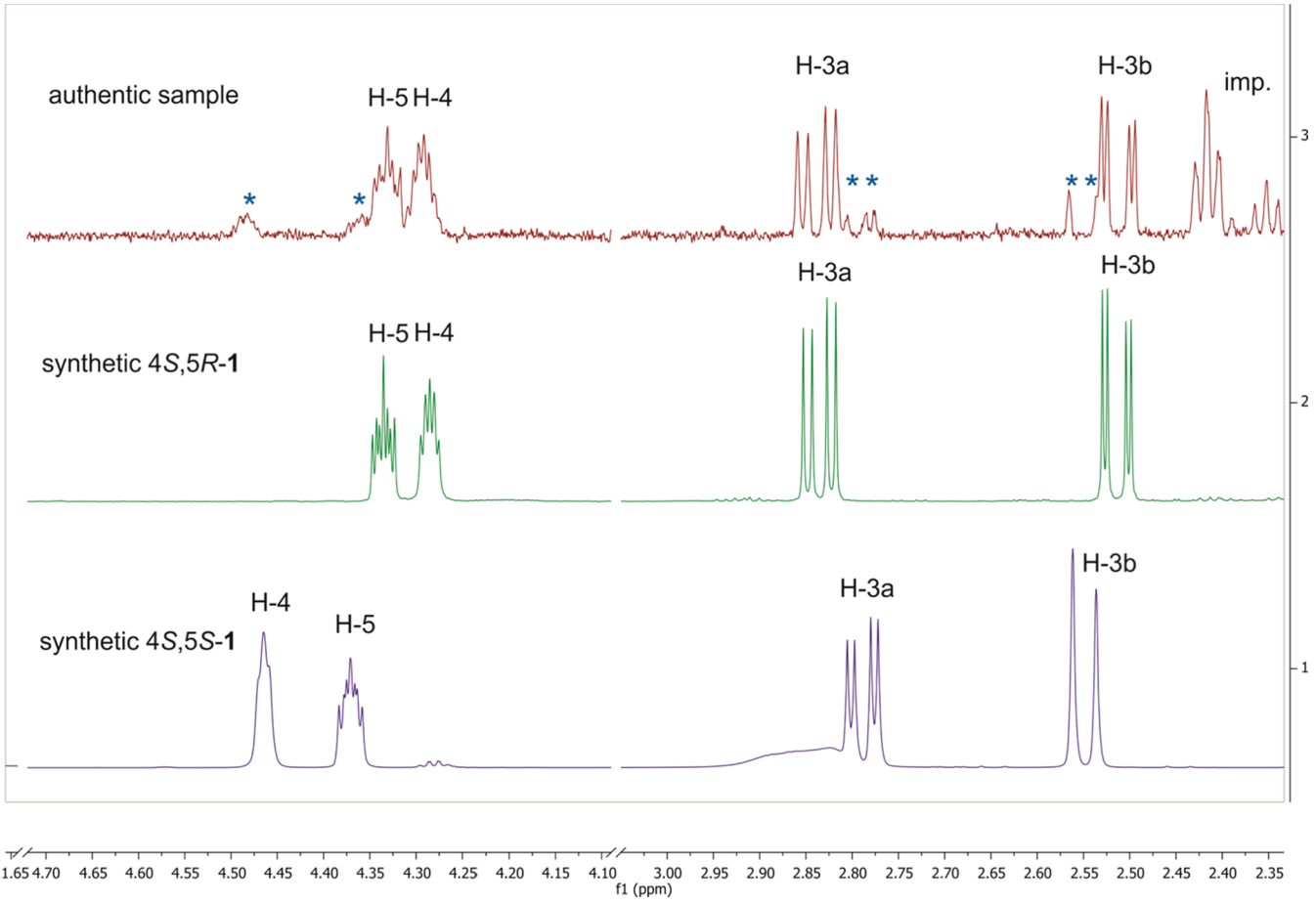

Figure 3. Comparison of the characteristic regions of the ^{1}H NMR spectrum of the authentic sample with the same regions of the synthetic compounds. The resonances of the minor component in the authentic sample are marked with stars. The spectra were recorded in $CDCl_3$ at 600 MHz for the authentic sample and at 700 MHz for the synthetic compounds (imp. = impurities).

Table 1. NMR Chemical Shift Values of the Authentic Sample and Corresponding Synthetic Compounds (4*S*,5*R*)-1/(4*R*,5*S*)-1 and (4*S*,5*S*)-1/(4*R*,5*R*)-1 with the Same Relative Configuration[a]

		(4*S*,5*R*)-**1**/(4*R*,5*S*)-**1**		(4*S*,5*S*)-**1**/(4*R*,5*R*)-**1**	
atom	authentic sample major component δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2		174.8, C		176.6, C	
3	2.85 dd, (18.0, 6.7)	37.7, CH_2	2.84, dd (17.9, 6.7)	39.6, CH_2	2.80, dd (17.8, 5.6)
	2.52 dd (18.0, 3.8)		2.52, dd (17.9, 3.8)		2.54, d (17.8)
4	4.29, ddd (6.5, 4.0, 3.0)	71.8, CH	4.28, ddd (6.5, 4.0, 3.0)	69.0, CH	4.47, m
5	4.32, ddd (8.1, 5.4., 3.1)	87.7, CH	4.34, ddd (8.1, 5.4. 3.1)	85.2, CH	4.37, ddd (8.2, 5.7, 3.7)
1′	1.61−1.64, m	33.2, CH_2	1.64, m	28.3, CH_2	1.84, m
			1.61, m		1.72, m
2′	1.29−1.44	25.2, CH_2	1.49, m	25.6, CH_2	1.50, m
			1.41, m		1.38, m
3′	1.29−1.44	29.2, CH_2	1.28, m	29.2, CH_2	1.29, m
4′	1.29−1.44	29.3*, CH_2	1.28, m	29.5, CH_2	1.29, m
5′	1.29−1.44	29.4*, CH_2	1.29, m	29.5, CH_2	1.29, m
6′	1.29−1.44	31.8, CH_2	1.26, m	31.9, CH_2	1.27, m
7′	1.29−1.44	22.7, CH_2	1.29, m	22.7, CH_2	1.27, m
8′	0.88, t (7.5)	14.1, CH_3	0.88, t (7.2)	14.1, CH_3	0.88, t (7.0)

[a]Resonances marked with an "*" could be either C-4′ or C-5′. Data were recorded in $CDCl_3$; TMS was used as the internal standard. *J* values are in Hz.

possible, (4*R*,5*S*)-**1**, (4*S*,5*R*)-**1**, (4*R*,5*R*)-**1**, and (4*S*,5*S*)-**1** (Chart 1). The original extract showed two peaks (A, B) on an apolar nonchiral-phase column, indicating the presence of at least two (but potentially all four) stereoisomers of **1**. To determine which of the isomers actually were present in the *Egaenus* extract and to elucidate their absolute configuration, all four stereoisomers of **1** were synthesized in enantiopure

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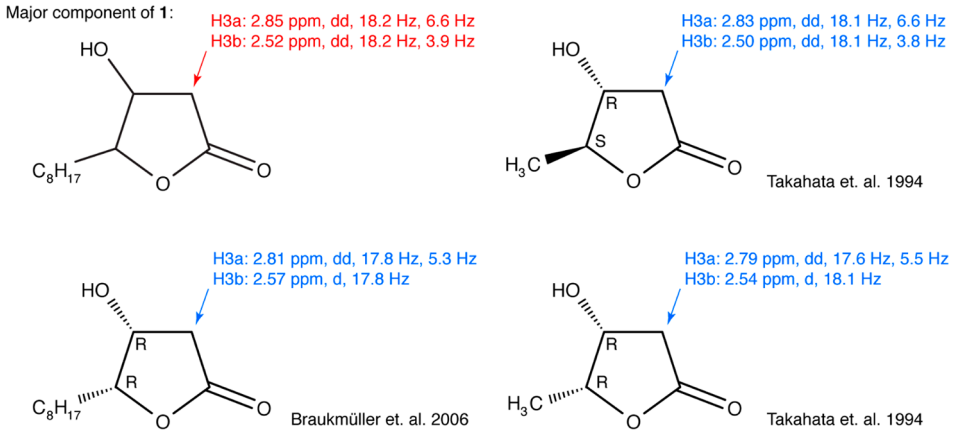
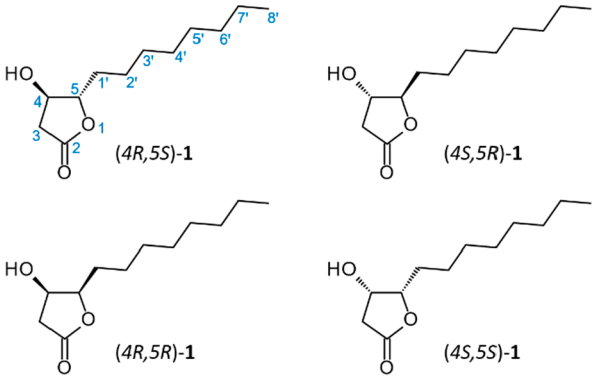


Figure 4. Observed chemical shifts, multiplicities, and coupling constants of the major component **1** of the authentic sample (red) in comparison with previously reported values of synthesized γ-butyrolactones with a defined configuration (blue).[12,13]

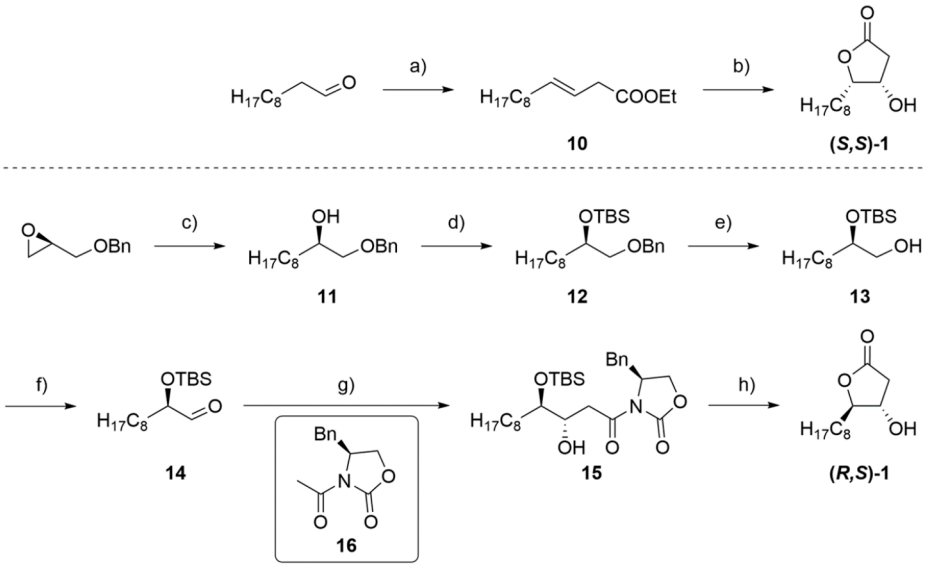
Chart 1. Stereogenic Centers and Possible Stereoisomers of 4-Hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (1)



form (Scheme 2; Supporting Information) and analyzed by NMR as well as by chiral-phase gas chromatography.

Both enantiomers of the *syn* diastereomer of **1** [(4*R*,5*R*)-**1** and (4*S*,5*S*)-**1**] were prepared in two steps from *n*-decanal according to an already described procedure.[13] Knoevenagl-type condensation of *n*-decanal and monoethyl malonate yielded skipped ester **10**. The Sharpless asymmetric dihydroxylation of this intermediate delivered both enantiomers of **1** in good yield and excellent optical purity. As the analogous route was not applicable to the corresponding anti-isomers [(4*S*,5*R*)-**1** and (4*R*,5*S*)-**1**], these were achieved in a different manner. Commercial enantiopure benzyl glycidyl ether was treated with *n*-heptylmagnesium bromide in the presence of catalytic amounts of copper salts to yield benzyl ether **11**. TBS protection of the resulting hydroxy group led to compound **12**, and the subsequent hydrogenolysis of the primary benzyl ether delivered monoprotected diol **13**. TEMPO-catalyzed oxidation of the said alcohol provided aldehyde **14**, which was subjected directly to an Evans aldol reaction with the boron enolate derived from **16**. The ensuing product **15** was isolated as a single diastereomer in fair yield. Finally, the fluoride-mediated

Scheme 2. Synthesis of (4*S*,5*S*)-1 and (4*S*,5*R*)-1[a]



[a]Conditions: (a) monoethyl malonate, Et_3N, 42%; (b) AD-mix α, *t*BuOH/H_2O, 70%, (c) n-$C_7H_{15}MgBr$, CuCN (5 mol %), THF, 75%; (d) TBSCl, imidazole, CH_2Cl_2, 89%; (e) H_2, Pd/C, EtOAc, quant.; (f) TEMPO (20 mol %), $PhI(OAc)_2$; (g) 4-(*S*)-3-acetyl-4-benzyl-oxazolidin-2-one, Bu_2BOTf, Et_3N, CH_2Cl_2, 50% over 2 steps; (h) Bu_4NF, THF, 71%. TBS: *tert*-butyldimethylsilyl; TEMPO: tetramethylpiperidineoxy radical.

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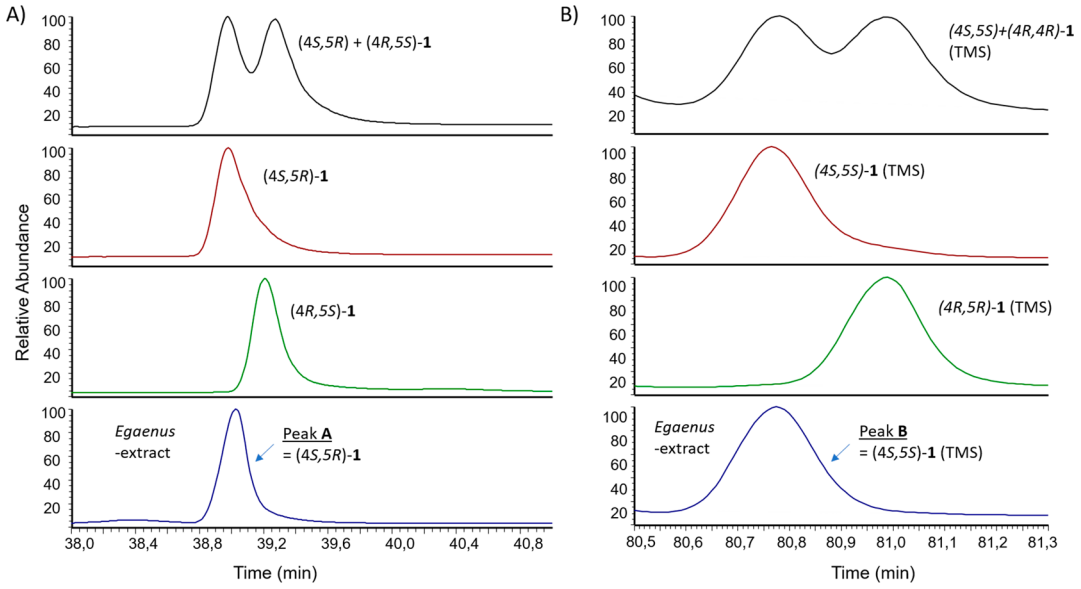

Figure 5. Identification of peaks A and B of the *Egaenus* extract as (4*S*,5*R*)-**1** and (4*S*,5*S*)-**1**. Chromatography was performed on a chiral ß-cyclodextrin phase. (A) Chromatographic comparison of synthetic (4*R*,5*S*)- and (4*S*,5*R*)-isomers of 4-hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (**1**) to *Egaenus* compounds A and B. (4*R*,5*R*)- and (4*S*,5*S*)-isomers of **1** remained inseparable under these conditions. (B) Comparison of MSTFA-derivatized (4*R*,5*R*)- and (4*S*,5*S*)-isomers of **1** to derivatized *Egaenus* extract.

removal of the TBS group resulted in spontaneous lactonization with the expulsion of the auxiliary, thus completing the synthesis of (4*S*,5*R*)-**1**. The opposite antipode (4*R*,5*S*)-**1** was prepared analogously in comparable yields and optical purities. Therefore, (*S*)-glycidyl benzyl ether and (*R*)-3-acetyl-4-benzyl-2-oxazolidinone were used instead of their respective optical antipodes. Moreover, in the first step of the sequence, $Cu(OAc)_2$ was substituted for CuCN as the catalyst for the epoxide opening reaction.

Synthetic (4*R*,5*S*)-**1** and (4*S*,5*R*)-**1** appeared to be well separable on a chiral ß-cyclodextrin phase, whereas (4*R*,5*R*)-**1** and (4*S*,5*S*)-**1** could only be separated as their TMS derivatives (Figure 5). Consequently, an *Egaenus* extract (underivatized and MSTFA-derivatized) was chromatographed on the chiral phase, clearly showing the presence of only two naturally occurring stereoisomers of **1**. A chromatographic comparison of all four synthetic stereoisomers to the *Egaenus* compounds proved the identities of peaks A and B as (4*S*,5*R*)-**1** and (4*S*,5*S*)-**1**, respectively (Figure 5).

Lactones are quite frequent in nature and are well-known as scent/aroma compounds in fruits and milk and as flavoring compounds in alcoholic beverages (i.e., whiskey and cognac lactones[14,15]). However, among arthropods, the γ-lactones found in the scent gland secretion of *Egaenus* are exceptional. First, they represent a novel class of compounds for the chemical inventory of scent gland secretions in harvestmen. Regarding the chemistry of Eupnoi, the compounds are similar neither to the acyclic compounds found in sclerosomatid Eupnoi ("sclerosomatid compounds" [sensu[4]]) nor to the aromatic/quinonic compounds from Phalangiinae.[6,10] Second, the particular β-hydroxy-γ-lactones as seen in *Egaenus* have not been found in any other arthropod yet. While lactones with OH groups on the side chains appear to be present in at least some insect species,[16] only one example for an exocrine lactone carrying a hydroxy group in the β-position (a 3-hydroxy-γ-decalactone = 5-hexyl-4-hydroxy-dihydro-furan-2-one from a tephritid fly) has recently been reported.[17]

In contrast, the lactone motif itself is frequently present in exocrine exudates of arthropods as well as vertebrates.[16,18,19] γ-Lactones, for instance, are known from butterflies and beetles where they may serve as sex pheromones.[16] Similar to large-ringed macrolides,[20,21] γ- and δ-lactones have also been described as antimicrobial agents,[22] and a few additionally possess a role in predator defense.[23−25]

Regarding harvestmen, scent gland exudates have generally been considered defensive,[1,2] even though additional functions may have evolved in particular taxa.[26] In some harvestmen species, chemical defense is indeed obvious: secretions are readily expelled as sprays or jets, reaching an offender at a distance of several centimeters,[2] deterring invertebrates as well as small vertebrates.[27] Concurringly, specimens of *E. convexus* forcefully eject secretion upon mechanical disturbance. This mode of secretion transfer is known from other harvestmen species and is called "jetting",[2] addressing the discharge of a fine, directed splash against offenders. So far, jetting has been mainly described for certain Laniatores whereas "spraying", defined as a fine, vaporized spray, is known from a group of sclerosomatid Eupnoi.[2] On the basis of our observations, the secretion of *Egaenus* also spreads over the body surface of jetting individuals, hence impregnating the body surface. Lactone amounts per individual were found to be highly variable, obviously depending on the filling status of the scent glands. In specimens extracted immediately after collection, we found 12.5 ± 7.9 μg/per individual with no obvious differences between the sexes. These amounts appear rather low, possibly indicating that the lactones are dissolved in a carrier matrix of currently unknown chemistry. In acyclic compound-producing leiobunines, this matrix is aqueous.[28]

Considering secretion discharge following a disturbance, the mode of secretion application (i.e., a directed jet against offenders), and self-wetting of the body−surface, a defensive and antimicrobial role of the *Egaenus* secretion appears to be likely. These newly discovered lactones add an unexpected component to the overall picture of harvestmen-secretion chemistry. It will be a logical next step to investigate their

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evolutionary origin and taxonomic distribution across the Opiliones.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations of compounds were measured using a Jasco P-2000 polarimeter at 25 °C in chloroform. IR spectra were recorded on an Alpha-ATR FTIR spectrometer (Bruker Biospin). NMR spectroscopy was performed on either a Bruker Avance III HD 600 spectrometer or Bruker Avance III HD 700, respectively. Frozen traps from pcGC (preparative capillary gas chromatography, see below) containing the purified authentic compound **1** were eluted with 500 μL of $CDCl_3$ containing 0.03% TMS for reference (99.8 atom %D, Armar, Germany). Spectra were measured with either a cryoprobe at 298 K or with a quadruple resonance probe (QXI $^{1}H/^{13}C/^{15}N/^{31}P$) at 293 K. Chemical shift assignment was achieved with COSY and TOCSY spectra (120 ms mixing time). Raw data were processed in Topspin 3.2 (Bruker Biospin), and 2D data was analyzed using Sparky (T. D. Goddard and D. G. Kneller, SPARKY3, University of California, San Francisco). COSY, HSQC, and HMBC spectra of the synthetic compounds were recorded in 720 μL of $CDCl_3$ at 25 °C with TMS as the internal standard on a Bruker Avance III HD 700 spectrometer. Data were processed with the MestReNova software package. Analytical GC-MS was performed on a Trace GC-DSQ I system (electron impact spectra; EI) and an ISQ Single Quadrupole mass spectrometer (positive ion chemical ionization (PCI), using methane as reagent gas); both systems were from Thermo Fisher. Aliquots of extracts (1.5 μL) or accordingly diluted compounds were directly subject to GC-MS. First, we used an apolar ZB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Phenomenex) and a temperature program (50 °C for 1 min with 10 to 300 °C for 5 min isotherm). EI and CI spectra were taken at 70 eV; detailed MS conditions are described elsewhere.[29] Retention indices (RIs) were calculated according to Van den Dool and Kratz.[30] Compounds of interest (i.e., peaks A and B) eluted at t_R = 17.54−17.61 min and t_R = 17.80−17.85 min, respectively ($RI_{peak\ A}$ = 1878; $RI_{peak\ B}$= 1901). For the separation of stereoisomers and enantiomers in particular, we used a chiral CycloSil-B capillary column (30m × 0.25 mm × 0.25 μm), coated with 30% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (DIME-β-CD) in DB-101 (Agilent J&W) and two different temperature programs: (1) For separation of the *R*,*S*- and *S*,*R*-isomers, the oven was programmed from 160 °C (1 min) with 1 °C/min to 200 °C and with 10 °C/min to 230 °C (5 min isotherm). (4*S*,5*R*)-**1** and (4*R*,5*S*)-**1** eluted at 38.97 and 39.27 min, respectively (Figure 5a). Both the *R*,*R*- and *S*,*S*-isomers eluted in one peak at 41.26 min. (2) For the separation of the *R*,*R*- and *S*,*S*-isomers, TMS derivatives were prepared (see below) and analyzed using the following temperature program: 100 °C (1 min) followed by an increase of 1 °C/min to 190 °C and then with 15 °C/min to 230 °C (5 min isotherm). (4*S*,5*S*)-**1**-TMS and (4*R*,5*R*)-**1**-TMS eluted at t_R = 80.83 and 81.06 min, respectively (Figure 5b). HRMS spectra were recorded on a Q-exactive high-resolution Orbitrap MS with a heated electrospray source coupled to an Accela 1250 HPLC-pump (Thermo Fisher). Analytical thin layer chromatography (TLC) was carried out on precoated 0.25 mm silica gel 60 (F254) plates from Macherey-Nagel. The visualization of substances was performed under UV light (254 nm) and/or by staining with either aqueous potassium permanganate/potassium carbonate solution ($KMnO_4$ stain) or 5% (w/v) phosphomolybdic acid solution in ethanol (PMA stain).

All solvents and reagents were obtained from ABCR, Carl Roth, and Sigma-Aldrich and were used as received unless stated otherwise.

Preparation of Extracts. 400 Adult individuals of both sexes of *Egaenus convexus* were collected by hand from May to July 2013 at the "Rosenhain", Graz, Austria (N47.084838; E15.449741). An additional 55 individuals (from the same location) were from collections in July 2019 and July 2020, respectively. All specimens were deposited in the collection of the Institute of Biology, Division of Zoology, University of Graz, Austria (voucher numbers RG 4240−4261, 4266−4275, 4277, 4288, 4325, 4349, 4356, 4365−4383, 4387, 4408−4411, 4416−4418, 4455, 4456, 4458, 4459, 5402−5411, 5414). A freshly emitted secretion was collected by dabbing the secretion on filter paper pieces (2 × 2 mm) directly from the gland openings (ozopores). "Loaded" filter papers were extracted in hexane (100 μL) for 15 min and gave extracts of pure secretion (Figure 2). Alternatively, individual whole-body extracts were prepared (500 μL of hexane; 15 min). The latter method was more feasible with respect to handling and resulted in equal or higher quantities of secretion per extract but showed additional, nonsecretion compounds in the extracts since some cuticular hydrocarbons were coextracted (Figures S24 and S25).

Prepurification by Flash Chromatography. Initial fractionation was performed on silica gel (40−63 μm) using solvents of >99% purity or p.a. grade. Hexane extracts, each containing the secretions of 40 individuals (in ∼10 mL), were concentrated in a stream of nitrogen to a volume of ∼300 μL. Concentrated extracts were purified on silica gel columns packed with 500 mg of unmodified SiOH (Chromabond, 3 mL, Machery-Nagel) using solvents of >99% purity or p.a. grade. Purification of these solutions by flash chromatography (100% hexane → 100% CH_2Cl_2) provided the compounds of interest in the CH_2Cl_2 fractions. In detail, columns were washed with six column equivalents (CE) of hexane before adding the extracts. Subsequently, the columns were eluted with 10 CE of hexane to remove the cuticular hydrocarbons. Finally, the purified polar fraction was eluted with 10 CE CH_2Cl_2. Fractions of 10 columns (corresponding to the secretion of 400 individuals) were combined and carefully concentrated in a stream of nitrogen. The residue was redissolved in hexane and subjected to preparative gas chromatography.

Preparative Capillary Gas Chromatography (pcGC). The final purification of the major compound of the prepurified extracts ("peak A") was accomplished by pcGC using a preparative fraction collector (PFC). The GC-PFC system consisted of a gas chromatograph equipped with a flame ionization detector (FID: Agilent 7890A), a PFC device (Gerstel), and a ZB-5 fused silica capillary column (30 m × 0.32 mm ID, 0.25 μm) from Phenomenex. Hydrogen was used as carrier gas with a flow rate of 3 mL/min. The column was split at the end by a μFlow splitter (Gerstel) into two columns leading to the FID (2 m × 0.15 mm ID) and the PFC (1 m × 0.2 mm ID), respectively. Nitrogen makeup gas with a flow rate of 25 mL/min was applied to the splitter. The PFC was connected with the GC oven via a heated transfer line, which was connected to seven transfer capillaries with an eight port zero-dead volume valve via the deactivated column (for further information about the setup, see refs 31 and 32). Four μL sample aliquots were injected via a multimode inlet (MMI) (Agilent) and heated to 320 °C. The temperature of the GC oven was raised from 40 to 270 °C with a heating rate of 25 °C per minute. The sampling time was 1 min, and the transfer line of the PFC was heated to 270 °C. The volatile traps were self-made microliter glass tubes filled with 50 mg of Carbotrap B (mesh 20−40, Supelco) and deactivated glass wool. The traps were fixed in a handmade closed cylindrical glass pipe with a screw coupling with a sealing ring (SciLabware Ltd. Stone). The glass pipe with the trap used for fraction collection was placed in a self-made cooling block and chilled to −20 °C. After the preparative fractionated collection of the main compound (from 9.5 to 9.9 min), the traps were frozen at −20 °C until further processing.

Compounds in Extracts. *(4S,5R)-4-Hydroxy-5-octyl-4,5-dihydro-3H-furan-2-one (4S,5R-1).* ^{1}H NMR, Table 1. EIMS (70 eV): *m*/*z* 196 [M − 18] (1), 143 (27), 142 (39), 125 (11), 124 (30), 115 (6), 111 (5), 102 (7), 101 (10), 98 (10), 97 (15), 96 (11), 95 (11), 89 (7), 88 (6), 84 (10), 83 (56), 82 (23), 81 (11), 71 (13), 69 (100), 68 (12), 67 (10), 57 (41), 55 (47), 44 (31), 43 (37), 41 (24). PCIMS *m*/*z* 255 [M + C_3H_5] (17), 243 [M + C_2H_5] (13), 215 [MH] (100), 197 (20), 195 (10), 179 (36), 161 (16), 155 (68), 137 (23). HRESIMS *m*/*z* 215.1642 $[M + H]^+$ (calcd for $C_{12}H_{23}O_3$, 215.1647); gas chromatographic retention index (ZB-5) 1878.

(4S,5S)-4-Hydroxy-5-octyl-4,5-dihydro-3H-furan-2-one (4S,5S-1). EIMS (70 eV): *m*/*z* 196 [M − 18] (3), 143 (23), 142 (41), 136 (11), 131 (7), 125 (11), 124 (28), 115 (11), 112 (11), 111 (13), 110 (12), 102 (15), 101 (8), 98 (15), 97 (18), 96 (13), 95 (15), 89 (11), 84

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(12), 83 (74), 82 (35), 71 (23), 70 (28), 69 (100), 57 (53), 55 (68), 44 (39), 43 (37), 41 (26). HRESIMS *m/z* 215.1642 $[M + H]^+$ (calcd for $C_{12}H_{23}O_3$, 215.1647); gas chromatographic retention index (ZB-5) 1901.

Microderivatization. Aliquots of extracts were subjected to silylation, saponification, reduction, and methylation, respectively. For silylation, we used (i) MSTFA (*N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide in pyridine 2:1 containing 1% trimethylchlorosilane) and (ii) MTBSTFA (*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide containing 1% *tert*-butyldimethylchlorosilane), respectively. Saponification was performed using $NaOCH_3$ in MeOH; reduction was performed with lithium aluminum hydride and methylation, with diazomethane.

Microderivatization of 4-Hydroxy-5-octyl-4,5-dihydro-3*H*-furan-2-one (1). *Preparation of 5-Octyl-4-(trimethylsilyloxy)-dihydro-3H-furan-2-one (**2**).* MSTFA (50 μL) was added to an aliquot of the scent gland extract in hexane (50 μL). The reaction mixture was incubated at 55 °C. After 30 min, an aliquot of the mixture (1.5 μL) was directly used for GC-MS analysis. EIMS (70 eV) *m/z* 286 (<1), 271 (6), 229 (78), 227 (7), 215 (100), 143 (9), 129 (11), 117 (42), 116 (95), 101 (75), 75 (25), 73 (45), 69 (5), 59 (12). 55 (12), 43 (18), 41 (17). PCIMS *m/z* 327 $[M + C_3H_5]$ (6), 315 $[M + C_2H_5]$ (14), 287 [MH] (100), 269 (10), 229 (20), 215 (17), 197 (19), 179 (42), 161 (8), 137 (12).

*Preparation of 4-(tert-Butyldimethylsilyloxy)-5-octyldihydro-3H-furan-2-one (**3**).* MTBSTFA (50 μL) was added to an aliquot of the secretion extract in hexane (50 μL). The reaction mixture was incubated at 55 °C. After 30 min, an aliquot of the mixture (1.5 μL) was directly used for GC-MS analysis: EIMS (70 eV) *m/z* 271 $[M - C_4H_9]$ (5), 230 (15), 229 (78), 192 (2), 143 (5), 129 (15), 117 (14), 111 (8), 101 (20), 97 (13), 84 (8), 81 (12), 75 (100), 73 (14), 55 (89), 41 (11). PCIMS *m/z* 369 $[M + C_3H_5]$ (13), 357 $[M + C_2H_5]$ (28), 329 [MH] (100), 327 (13), 311 (8), 271 (15), 229 (43), 225 (7), 203 (8), 197 (20), 179 (23), 161 (3), 179 (23), 161 (3), 137 (7).

*Preparation of Trimethylsilyl 3,4-Bis((trimethylsilyl)oxy)-dodecanoate (**5**).* 300 μL of a solution of $NaOCH_3$ in MeOH (25%) was added to an aliquot of hexane extract (50 μL), and the resulting mixture was incubated for 16 h at 75 °C. The reaction was stopped by the careful addition of 500 μL of H_2O, followed by acidification to pH 2−3 with 1 N HCl and extraction with 1 mL of EtOAc. An aliquot was analyzed by GC-MS for 3,4-dihydroxydodecanoic acid (**4**). The addition of H_2O during the workup leads to warming of the solution and complete saponification of the intermediately formed methyl ester by emerging NaOH. The remaining solution was dried under a stream of nitrogen for further derivatization. For silylation, MSTFA reagent (60 μL; containing 1% TMCS (trimethylchlorosilane); 2:1 in pyridine)) was added, and the resulting mixture was incubated for 40 min at 60 °C, resulting in **5**. An aliquot of the reaction mixture **5** was used directly for GC-MS analysis: EIMS (70 eV) *m/z* 433 $[M - CH_3]$ (1), 343 (6), 306 (50), 215 (54), 190 (11), 147 (34), 133 (11), 116 (7), 103 (17), 83 (14), 75 (28), 73 (100), 69 (20), 44 (16). PCIMS *m/z* 477 $[M + C_2H_5]$ (4), 449 [MH] (21), 447 (17), 433 (100), 387 (5), 359 (72), 343 (48), 335 (12), 306 (62), 269 (18), 215 (41), 147 (3), 73 (3).

*Preparation of Methyl 3,4-Bis((trimethylsilyl)oxy)dodecanoate (**6**).* Diazomethane was prepared from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; Sigma),[33] and a solution of diazomethane (in Et_2O/MeOH 9:1, saturated, 1 mL) was added to **4**. After 20 min at ambient temperature, the reaction mixture was concentrated to dryness in a stream of nitrogen. MSTFA reagent (60 μL; containing 1% TMCS, 2:1 in pyridine) was added, and the resulting mixture was incubated for 40 min at 60 °C, resulting in **6**. An aliquot of the reaction mixture **6** was used directly for GC-MS analysis: EIMS (70 eV) *m/z* 375 $[M - CH_3]$ (2), 359 $[M - OCH_3]$ (3), 343 (1), 285 (11), 248 (64), 215 (51), 175 (5), 159 (8), 147 (22), 133 (16), 116 (21), 103 (21), 89 (18), 83 (23), 75 (40), 73 (100). PCIMS *m/z* 419 $[M + C_2H_5]$ (7), 391 [MH] (31), 375 (100), 359 (22), 343 (5), 329 (12) 301 (58), 285 (38), 248 (15), 215 (18), 211 (15).

*Preparation of 1,3,4-Tris((trimethylsilyl)oxy)dodecane (**8**).* A solution of lithium aluminum hydride (10 mg/mL in Et_2O, 200 μL) was added to an aliquot of extract containing **1** (50 μL in hexane) at ambient temperature. After 30 min, H_2O (2 mL) was added carefully. After the exothermic reaction has subsided, the resulting slurry was extracted with Et_2O (3 × 1 mL). An aliquot was checked for 1,3,4-dodecanetriol (**7**). The combined organic layers were concentrated in a stream of nitrogen before adding MSTFA reagent (60 μL). The resulting mixture was incubated for 40 min at 60 °C, resulting in **8**. An aliquot of **8** was used directly for GC-MS analysis: EIMS (70 eV) *m/z* 419 $[M - CH_3]$ (<1), 344 (1), 329 (2), 219 (23), 215 (39), 147 (28), 115 (18), 103 (80), 75 (32), 73 (100). PCIMS *m/z* 435 [MH] (16), 433 (13), 420 (40), 419 (97), 391 (6), 346 (23), 345 (76), 343 (51), 329 (91), 321 (16), 289 (7), 256 (25), 255 (199), 219 (29), 215 (38), 165 (6).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00277.

Synthesis of reference materials; NMR spectra (^1H, COSY, and TOCSY spectra for authentic material, ^1H and ^{13}C spectra for (4*R*,5*S*)-**1**, (4*R*,5*R*)-**1**, **11**, **12**, **13**, **15**; COSY, HSQC, and HMBC spectra for (4*R*,5*S*)-**1**); EI-mass spectra of microderivatives (compounds **2**, **3**, **5**, **6**, **8a**, **8b**); comparison of sampling techniques (direct sampling of secretion vs whole body extraction: total ion chromatograms and ^1H spectrum of whole-body extract) (PDF)

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Notes

The authors declare no competing financial interest.

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