Journal of Chemical Ecology (2021) 47:243–247
https://doi.org/10.1007/s10886-021-01255-0

Cervidins A-D: Novel Glycine Conjugated Fatty Acids from the Tarsal Gland of Male Whitetail Deer, *Odocoileus virginianus*

Ke Li[1,2] · Michael J. Siefkes[3] · Weiming Li[1]

Received: 30 June 2020 / Revised: 30 June 2020 / Accepted: 5 February 2021 / Published online: 24 February 2021

Abstract

Sexually mature male deer are known to rub-urinate, a process where urine is deposited on the tarsal gland. The resulting mixture of compounds from urine and secretions from the tarsal gland are used to signal sex, age, maturation status, and other information at close distance. We examined the difference in metabolites of tarsal gland extracts from male and female whitetail deer, *Odocoileus virginianus,* harvested during the mating season. Using NMR spectroscopy and high-pressure liquid chromatography linked to high resolution mass spectrometry (HPLC/HR-MS) we identified a homologous series of four male-specific compounds. The compounds are novel glycine conjugates of 10-hydroxy-6,9-oxido fatty acids, which we term cervidins A-D. Cervidins were deemed to possess the absolute configuration 6*S*,9*R*,10*R* through comparison of their spectroscopic data with those of known compounds. In addition, cholesterol 3-sulfate and 3-(3-hydroxyphenyl)-propanoic acid were found to be present in the extracts. Our results clearly demonstrate the diversity of potential semiochemicals contained in the mammalian integument.

Keywords Metabolites · Deer · Oxygenated fatty acids · Glycine conjugates · Integument

Introduction

The mammalian integument is an important source of novel natural products (Nicolaides 1974). Comparative chemical studies have indicated the occurrence of sterols, wax esters, hydroxyacids, ceramides, glycolipids, and other compounds in mammalian skins (Birkby et al. 1982; Lindholm et al. 1981). Some of these chemicals have also been shown to occur in the domestic sheep (Itô et al. 1971), horse (Colton and Downing 1982), and guar (Albone et al. 1986; Ishii et al. 2004). In ruminants, skin metabolites, in particular those from specialized scent glands (Albone et al. 1986), are thought to function as chemical cues in various contexts (Müller-Schwarze 1971).

Deer urinate on their tarsal glands (rub-urinate) as a means to release skin metabolites that arouse a marking or agonistic behavior (Müller-Schwarze 1971). The tarsal organ consists of a scent gland and a hair tuft that carries the scent and plays a primary role in mutual recognition and other aspects of social behavior (Müller-Schwarze 1971). The primary component of tarsal scent of the male black-tailed deer, *Odocoileus columbianus,* was identified as *cis*-4-hydroxydodec-6-enoic acid lactone (Brownlee et al. 1969), which possesses s lactone motif compatible with it functioning as a semiochemical (Schulz and Hötling 2015). However, the isolated compound and similar synthetic analogues did not duplicate the original tarsal scent in inducing expected behaviors and are currently considered inactive (Müller-Schwarze 1969). Bioactivity driven fractionation experiments further demonstrated that ketones and aldehydes may not contribute significantly to the total activity of tarsal scent (Müller-Schwarze 1971).

We report chromatographic analysis of the tarsal gland extracts of whitetail deer, *Odocoileus virginianus*. Metabolite differences between male and female glands were investigated using HPLC/HR-MS analysis. The predominant components of male extracts were isolated and identified, as well as the known compounds cholesterol 3-sulfate (Hoye et al. 2007) and 3-(3-hydroxyphenyl)propanoic acid (Pouchert and Campbell 1974). Structures were determined by HR-MS and spectroscopic methods, including ^{1}H, ^{13}C, DFQ-COSY, HSQC, HMBC, and TOCSY. Relative configurations were supported with NOESY correlations, and absolute configurations deduced by comparing these spectroscopic data with those of identical substructures.

✉ Weiming Li
liweim@msu.edu

[1] Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, USA

[2] Present address: Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China

[3] Great Lakes Fishery Commission, Ann Arbor, MI 48105, USA

Methods and Materials

Equipment and Reagents

Mass spectra were recorded on a TQ-S TOF LC mass spectrometer (Waters Corporation, Milford, Massachusetts, USA). Silica gel (70–230 and 230–400 mesh, Merck, Darmstadt, Germany), RP-18 reverse-phase silica gel (Merck), and Sephadex LH-20 (Merck) were used for open column chromatography. Thin layer chromatography (TLC) was conducted on glass plates pre-coated with GF254 silica gel (Merck). Spots were first visualized under UV light at 254 nm and then stained with sprays of an acidic methanol solution of 5% anisaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). The standard *N*-octanoylglycine was purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

Collection and Extraction of Tarsal Glands

Tarsal gland scents were extracted with ethanol from the hair tuft of tarsal glands excised from female and male whitetail deer harvested during the firearm hunting season in Richland County, Wisconsin during November 2015. Tarsal glands were excised immediately after harvest and preserved in 95% ethanol at −20 °C. All deer were harvested legally by licensed hunters.

Metabolite Analysis

Metabolites were fractionated using an Acquity ultra-high performance liquid chromatography (UPLC) system (Waters), and detected at high resolution using a Xevo G2-S QToF mass spectrometer equipped with an electrospray ionization (ESI) source (Waters). An ACQUITY C18 BEH UPLC column (2.1 × 100 mm, 1.7 μm particle size; Waters) was used to profile polar metabolites, and the column temperature was set at 30 °C. The mobile phase consisted of water (A) and acetonitrile (B) with a gradient program at a flow rate of 250 μL min^{-1} for 10 min: 80% A for 1 min; 0% A from 1 to 9 min; and 80% A from 9 to 10 min for column equilibrium. To avoid cross-contamination of samples during the analysis, the needle was washed twice with 80% methanol after each injection. Analyte residues were also reduced by injecting 10 μL methanol as a 'rinsing solution' on the column after each sample injection using the elution gradient program described above.

High-resolution mass spectrometry was performed in negative electrospray ionization mode. Full-scan spectra were recorded in the range of *m/z* 100–1000. Nitrogen gas was used as both desolvation gas (600 L h^{-1}) and cone gas (50 L h^{-1}). Argon was used as the collision gas at a pressure of 5.3×10^{-5} Torr. Source and desolvation temperatures were 100 and 400 °C. The cone voltage and capillary voltage were set to 30 V and 2.8 kV. For MS spectrum and MS/MS spectrum, the collision energies for collision-induced dissociation were 5 and 40 eV. Scan time was set at 0.2 s, with an interscan delay of 0.5 s. The LockSpray™ dual electrospray ion source with internal references used for these experiments was leucine enkephalin at a concentration of 100 ng mL^{-1}. Lock-mass calibration data at *m/z* 554.2615 in negative ion mode were acquired for 1 s at every 10 s interval and the flow rate was set at 5 μL min^{-1}. Data treatment, alignment, peak picking, normalization, deconvolution, and multivariate analysis were performed using Massylynx 4.1 (Waters).

NMR Spectra

1D and 2D NMR spectra were recorded on a Bruker Avance III 900 NMR (Bruker; Billerica, Massachusetts, USA) and Varian 500 NMR spectrometer equipped with 5-mm probe (Agilent Technologies, Santa Clara, CA 95051) in CD_3OD and DMSO at 298 K. Chemical shifts (δ) in ppm were referenced to tetramethylsilane (TMS) at 0.00 ppm for 1H and 13C. Coupling constants (J) were given in Hertz. The pulse conditions were: for 1H, spectrometer frequency (SF) = 899.01 MHz, spectral width (SWH) =18,518.5 Hz, pulse 90° width (P1) = 9.35 μs, acquisition time (AQ) = 1.99 s, relaxation delay (D1) = 1.00 s, number of dummy scans (DS) = 4; for 13C, SF = 226.08 MHz, SWH = 54,200.25 Hz, P1 = 25.00 μs, AQ = 0.99 s, D1 = 1.5 s, DS = 36,000; for 1H–1H COSY, SF = 899.00 and 899.00 Hz, SWH = 10,776 and 10,788 Hz, AQ = 0.26 s, D1 = 3.0 s, DS = 16; for HSQC, SF = 899.00 (1H), 226.07 Hz (13C), SWH = 14,367.8, 37,450.2 Hz, AQ = 0.035 s, D1 = 1.5 s, DS = 32; for HMBC, SF = 899.00 (1H), 226.07 Hz (13C), SWH = 10,775.9, 54,152.9 Hz, AQ = 0.19 s, D1 = 1.5 s, DS = 32; for NOESY, SF = 499.91, 499.91 Hz, SWH = 2490, 2490 Hz, AQ = 0.15 s, D1 = 1.0 s, DS = 16, mixing time (D8) = 0.86 s.

Purification of Extracts

The organic solvents were removed under reduced pressure at 40 °C by a rotary evaporator (Buchi Co., New Castle, Delaware, USA). This residue was suspended in methanol and filtered through 2 μm filter paper. The filtrate was concentrated again under reduced pressure at 40 °C to yield 0.5 g of a dark residue.

The residue was subjected to liquid chromatography over silica gel (50 g; gradient elution from 95% $CHCl_3$/MeOH to 100% MeOH, 0.5 L total volume). TLC analysis stained by spraying with an acidic methanol solution of 5% anisaldehyde was used to guide the pooling of the eluents into 6 fractions. Fraction 4 was concentrated and left a residue (12 mg) that was further purified using Sephadex LH-20, first on a $CHCl_3$-MeOH (1:1) column and then on a MeOH (100%) column.

Results and Discussion

Four major components were detected in the extracts of tarsal organs of male deer using UPLC/MS-MS (Fig. 1). These were termed cervidin A, B, C and D, in reference to the family Cervidae to which the whitetail deer belongs. The mass difference between each compound were *m/z* 14, indicating a series of analogues that differed by a single methylene group.

Purification by liquid chromatography provided samples of cervidin A (**1**, 0.23 mg), cervidin B (**2**, 0.15 mg), cervidin C (**3**, 0.25 mg), cervidin D (**4**, 0.10 mg), cholesterol 3-sulfate (**5**, 0.86 mg), and 3-(3-hydroxyphenyl)-propanoic acid (**6,** 0.25 mg). The structures of these compounds are shown in Fig. 2.

Cervidin B (**2**) was obtained as a pale oil. The molecular formula was determined to be $C_{19}H_{34}NO_5$ ($[M-H]^-$, calcd 356.2437) by HR-ESI-MS, suggesting three degrees of unsaturation (Supplementary Information: Table S1 and Figs. S1-S4). The 1D NMR and HMBC data (Table 1 and Fig. 3a) were used to establish the following features: two carbonyl (δ_C 176.4, 175.5), three *O*-methines (δ_H 3.37, δ_C 75.1; δ_H 3.93, δ_C 80.9; δ_H 3.82, δ_C 83.4), one methyl (δ_H 0.90, δ_C 14.6), and 13 other methylene groups. The connectivity between CH-CH-CH_2-CH_2-CH was supported by correlations in the COSY spectrum. The resonances in ^{1}H NMR spectrum at 3.37 ppm (H-10), 3.82 ppm (H-9), and 3.93 ppm (H-6) and long-range correlations from H-6 to C-9 and from H-9 to C-6 in the HMBC spectra suggested an 2,5-disubstituted tetrahydrofuranoid ring in which one of the substituent chains carries an adjacent sec-hydroxyl group, leading the construction of a scaffold in **2**. Furthermore, the COSY correlations between H_2–5 and H_2–4, H_2–4 and H_2–3, H_2–3 and H_2–2, respectively, extended the side chain of core moiety of tetrahydrofuranoid ring. The long-range correlations from H_2–3 to C-1 (δ_C 176.4) in HMBC spectrum further extended the side chain with an acid/amide group. The resonances and multiplicity of methylene [δ_H 3.80 (s), δ_C 43.6] and correlations from H_2–1′ to both carbonyl carbons in HMBC spectrum indicated a connectivity of acetic group. These NMR spectra and experiments are shown in Supplementary Material, Figs. S5-S10).

As a reference compound, *N*-octanoylglycine, which possesses similar connectivity sequences indicated for **2**, was used to record proton and carbon resonances in the same deuterated solvents and conditions specified by Pouchert and Campbell (1974). The glycine conjugated moiety in **2** was similar to that in *N*-octanoylglycine. The chemical shifts of methylene-1′ and carbonyl further confirmed the HOOC-CH_2-NH-CO- moiety and excluded the possibility of HOOC-NH-CH_2-CO- group. The remaining methylene and methyl groups accounted for a C-7 alkyl chain on the other side of the tetrahydrofuranoid ring moiety.

The planar structure of **2** was identical to the 16-bovidic acid from the wool fat of domestic sheep, *Ovis aries* (Itô et al. 1971), and to the 18-bovidic acid from pelage extracts of banteng, *Bos javanicus* (Ishii et al. 2004). The COSY and HMBC correlations fully supported the skeleton of **2** within a hydroxyl adjacent to the tetrahydrofuranoid ring. A 2D TOCSY experiment was performed and the correlations supported the connectivity of hydroxylated tetrahydrofuranoid core moiety (Fig. 3a). Therefore, the planar structure of **2** was determined as glycine conjugated 10-hydroxy-6,9-oxidooctadecanoic acid (Ishii et al. 2004).

The stereochemistry of the hydroxylated 2,5-tetrahydrofuranoid ring has been widely studied due to its specific physiological function, which provides analogs for cervidin compounds. The relative configurations of **2** was established through analyses of the NOESY correlations, as well as comparisons of its NMR spectra with those of previously reported compounds. A detailed analysis of the NOESY spectrum of **2** revealed correlations between H-6/H-7b, H-6/H-8b, and H-8b/H-10 (Fig. 3b), placing these protons on the same face of the tetrahydrofuran ring. A complementary NOESY correlation was observed between H-9/H-7a, indicating their *cis* relationship to one another.

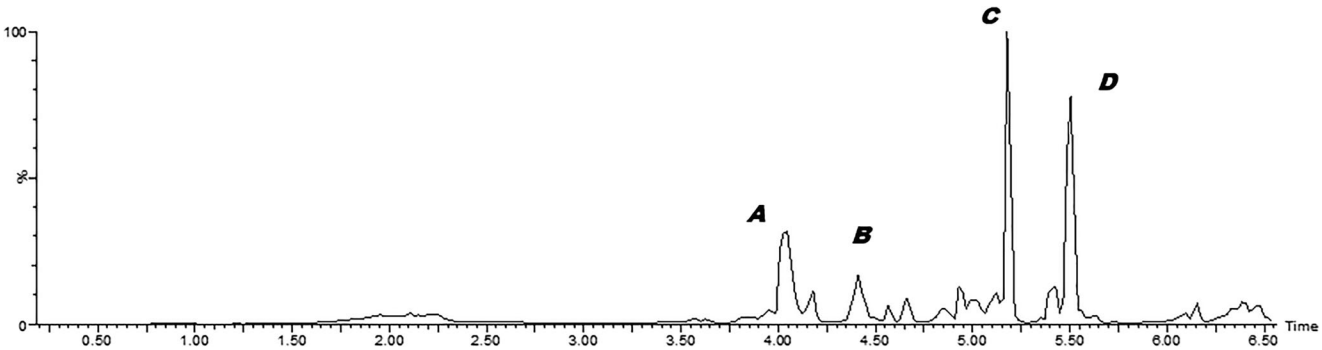

Fig. 1 UHPLC/HR-MS chromatogram of component analysis of extract of tarsal organ of male deer showing four major components **A-D**

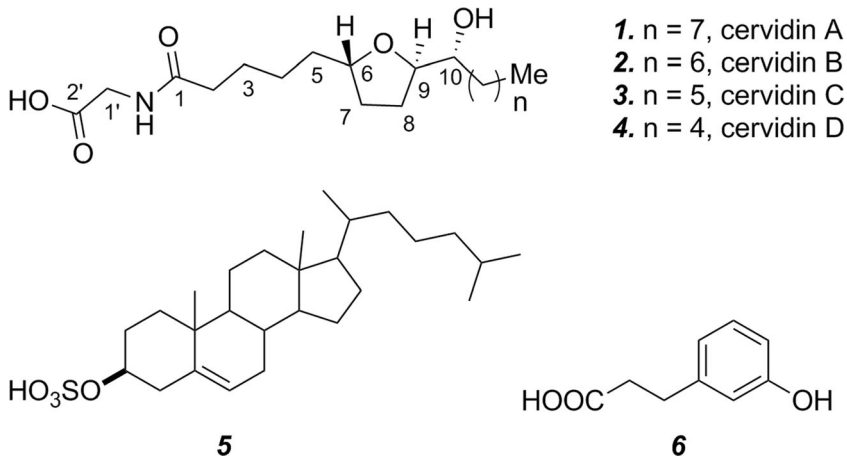

Fig. 2 Chemical structures of compounds **1**–**6**

An identical sub-structural analogue of compound **2** was the hydroxyfuranoid moiety in bovidic acid (Ishii et al. 2004), which was isolated from pelage extracts of banteng. Evans et al. (2004) synthesized the hydroxyfuranoid moiety with 6*S*, 9*R*, 10*R* configuration, and their spectroscopic data matched those of **2**. The comparison of the carbon and proton chemical shifts between **2** and each of the analogues (Supplementary Material Table S2) with possible configurations clearly suggested that **2** possesses a 6*S*, 9*R*, 10*R* absolute configuration. We combined the elemental composition information from mass spectra and NMR spectra to assign the remaining compounds as cervidins A, C, and D (Fig. 2). The similarity of NOESY correlations and proton and carbon resonances in cervidins A, C, and D indicated the same absolute configurations 6*S*, 9*R*, 10*R* as **2**.

Table 1 ^{1}H and ^{13}C NMR Data for natural cervidin B (**2**) recorded in MeOH-d_4 at 900 MHz for ^{1}H and 225 MHz for ^{13}C

No.	δ_H mult (*J* in Hz)	δ_C
1		176.4, C
2	2.26 t (7.6)	37.1, CH_2
3	1.65 m	27.1, CH_2 [b]
4	1.36 m, 1.42	27.1, CH_2 [b]
5	1.60 m, 1.44 m	36.6, CH_2
6	3.93 m	80.9, CH
7	2.05 m, 1.50 m	33.5, CH_2
8	1.95 m, 1.69 m	29.4, CH_2
9	3.82 m	83.4, CH
10	3.37 (ddd, 9.1, 5.5, 3.9)	75.1, CH
11	1.40 m	34.5, CH_2
12	1.32 m[a]	31.0, CH_2[a]
13	1.30 m[a]	30.6, CH_2[a]
14	1.36 m	27.0, CH_2[b]
15	1.29 m	33.2, CH_2
16	1.32 m	23.9, CH_2
17	0.90 t (7.1)	14.6, CH_3
1′	3.80 s	43.6, CH_2
2′		175.5, C

[a], [b] chemical shifts exchangeable, respectively

The biological function of an organic compound is determined by its properties, such as chain length, functional groups, and stereochemistry (Wyatt 2003). More specifically, chiral compounds have fixed spatial arrangements of atoms that contain no mirror plane, or centre of inversion, and thus occur in two non-superimposable mirror-image forms. Stereoisomers are characterized by differences in physicochemical properties and may trigger distinct biological responses in insects (Mori 1998) and fish (Li et al. 2018). Each cervidin compound possesses three chiral centers, potentially supporting eight stereoisomers. In addition to these chiral centers, the chain length on tetrahydrofuran ring further enriches the complexity of

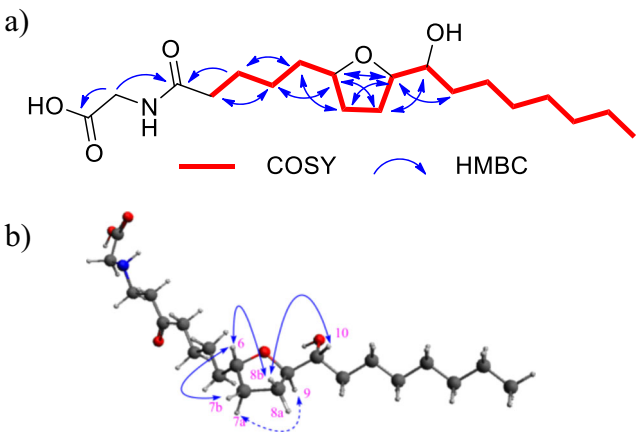

Fig. 3 (**a**) Key HMBC (blue arrows), COSY (red bold lines) and (**b**) NOESY correlations for cervidin B (**2**)

the cervidin mixture from male tarsal glands. The cervidins represent a new addition to the molecular diversity of deer metabolites.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10886-021-01255-0.

Acknowledgements We wish to thank Matt Otto and Scott Siefkes for excising and preserving the tarsal glands from whitetail deer legally harvested during the 2015 Wisconsin firearm deer season. Without their cooperation these analyses would not have been possible. This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

References

Albone ES, Blazquez NB, French J, Long SE, Perry GC (1986) Mammalian semiochemistry: issues and futures, with some examples from a study of chemical signalling in cattle. Chemical signals in vertebrates 4. Springer, Boston, pp 27–36

Birkby CS, Wertz PW, Downing DT (1982) The polar lipids from keratinized tissues of some vertebrates. Comp Biochem Physiol B 73:239–242. https://doi.org/10.1016/0305-0491(82)90278-4

Brownlee RG, Silverstein RM, Muller-Schwarze D, Singer AG (1969) Isolation, identification and function of the chief component of the male tarsal scent in black-tailed deer. Nature 221:284–285. https://doi.org/10.1038/221284a0

Colton SW, Downing DT (1982) Variation in skin surface lipid composition among the Equidae. Comp Biochem Physiol B 75:429–433. https://doi.org/10.1016/0305-0491(83)90353-x

Evans PA, Leahy DK, Andrews WJ, Uraguchi D (2004) Stereodivergent construction of cyclic ethers by a regioselective and enantiospecific rhodium-catalyzed allylic etherification: total synthesis of gaur acid. Angew Chem Int Ed Engl 43:4788–4791. https://doi.org/10.1002/anie.200460612

Hoye TR, Dvornikovs V, Fine JM, Anderson KR, Jeffrey CS, Muddiman DC, Shao F, Sorensen PW, Wang J (2007) Details of the structure determination of the sulfated steroids PSDS and PADS: new components of the sea lamprey (*Petromyzon marinus*) migratory pheromone. J Org Chem 72:7544–7550. https://doi.org/10.1021/jo070957l

Ishii H, Krane S, Itagaki Y, Berova N, Nakanishi K, Weldon PJ (2004) Absolute configuration of a hydroxyfuranoid acid from the pelage of the genus bos, 18-(6*S*, 9*R*, 10*R*)-bovidic acid. J Nat Prod 67:1426–1430. https://doi.org/10.1021/np049937u

Itô S, Endo K, Inoue S, Nozoe T (1971) 10(*S*)-hydroxy-6(*R*), 9(*S*)-oxidohexadecanoic acid, a new acid in wool fat. Tetrahedron Lett 12:4011–4014. https://doi.org/10.1016/S0040-4039(01)97347-2

Li K, Brant CO, Huertas M, Hessler EJ, Mezei G, Scott AM, Hoye TR, Li W (2018) Fatty-acid derivative acts as a sea lamprey migratory pheromone. Proc Natl Acad Sci U S A 115:8603–8608. https://doi.org/10.1073/pnas.1803169115

Lindholm JS, McCormick JM, Colton SW, Downing DT (1981) Variation of skin surface lipid composition among mammals. Comp Biochem Physiol B 69:75–78. https://doi.org/10.1016/0305-0491(81)90211-X

Mori K (1998) Chirality and insect pheromones. Chirality 10:578–586. https://doi.org/10.1002/(SICI)1520-636X(1998)10:7<578::AID-CHIR5>3.0.CO;2-Z

Müller-Schwarze D (1969) Complexity and relative specificity in a mammalian pheromone. Nature 223:525–526. https://doi.org/10.1038/223525a0

Müller-Schwarze D (1971) Pheromones in black-tailed deer (*Odocoileus hemionus columbianus*). Anim Behav 19:141–152. https://doi.org/10.1016/S0003-3472(71)80149-5

Nicolaides N (1974) Skin lipids: their biochemical uniqueness. Science 186:19–26. https://doi.org/10.1126/science.186.4158.19

Pouchert CJ, Campbell JR (1974) Aldrich library of NMR spectra. Aldrich Chemical Co., Milwaukee

Schulz S, Hötling S (2015) The use of the lactone motif in chemical communication. Nat Prod Rep 32:1042–1066. https://doi.org/10.1039/C5NP00006H

Wyatt TD (2003) Pheromones and animal behaviour: communication by smell and taste. Cambridge University Press, Cambridge