Unusual Fatty Acids in the Fat Body of the Early Nesting Bumblebee, *Bombus pratorum*

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Abstract Unusual fatty acids with 24, 26, and 28 carbon atoms were found in triacylglycerols (TAGs) isolated from fat body tissue of bumblebee *Bombus pratorum*. The most abundant one was (*Z,Z*)-9,19-hexacosadienoic acid. Its structure was determined by mass spectrometry after derivatization with dimethyl disulfide and by infrared spectroscopy. ECL (equivalent chain length) values of its methyl ester were determined on both DB-1 and DB-WAX capillary columns. (*Z,Z*)-9,19-Hexacosadienoic acid is quite rare in nature. So far it has been identified only in marine sponges, and this work is the first evidence of its occurrence in a terrestrial organism. HPLC/MS analysis of the bumblebee TAGs showed that (*Z,Z*)-9,19-hexacosadienoic acid is present in one third of all TAG molecular species. As it was found in all sn-TAG positions, it is likely that (*Z,Z*)-9,19-hexacosadienoic acid is transported to tissues. Interestingly, labial gland secretion of *B. pratorum* was found to contain (*Z,Z*)-7,17-pentacosadiene, a hydrocarbon with markedly similar double bond positions and geometry. Possible biosynthetic relationships between these two compounds are discussed.

Introduction

In insects, fat body is the major storage site for nutrients. Most lipid stores are triacylglycerols (TAGs). It is assumed that the fat body TAGs originate mainly from dietary fats absorbed by midgut epithelium during times of feeding. In addition, lipid reserves can be biosynthesized de novo from carbohydrates [1]. The fat body is also a key center of metabolism, which responds to the physiological and biochemical needs of insects. Lipids are mobilized in the form of 1,2-diacylglycerols and transported to tissues. Fatty acids (FAs) are known to be essential precursors in the biosynthesis of many important compounds, including eicosanoids, pheromones, cuticular hydrocarbons, and other semiochemicals [2]. It is hypothesized that FAs released from fat body of bumblebee males might serve as precursors of their marking pheromones [3]. The most common FAs found in insect fat bodies are palmitic, palmitoleic, stearic, oleic and linoleic acids [4–6]. The same FAs also prevail in bumblebees, and we have shown that the FA composition as well as the composition of TAGs in this genus are clearly species-specific [7]. Large differences exist among species, whereas variations within the species are substantially less significant. In our effort to prove connections between fat body TAGs and bumblebee male marking pheromones, several species were investigated, including the early nesting bumblebee *Bombus pratorum*. This is a relatively small bee with a bright yellow thorax and strikingly orange tail that is common all over Europe. The fat body of this species was found to

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contain rare FAs which have not been detected in any other insect species so far.

In this work we report on long-chain FAs with 24, 26 and 28 carbon atoms found in the fat body of the male bumblebee *B. pratorum*. Possible connections between the most abundant long-chain (Z,Z)-9,19-hexacosadienoic acid and labial gland secretion (marking pheromone) components are discussed.

Materials and Methods

Insect Material and Sample Preparation

The bumblebee males of *Bombus (Pyrobombus) pratorum* (Linnaeus, 1761) were collected during the summer season 2006 in the north region of the Czech Republic and transported to the laboratory. After immobilization of males in cold (−18 °C), their peripheral fat bodies were carefully dissected. The fat body tissue from each individual was transferred into a glass vial with 100 μL of CHCl₃/CH₃OH (1:1, *v/v*) containing di-*tert*-butyl-4-methylphenol (Fluka, Buchs, Switzerland) at a concentration of 25 mg/mL. Samples were sonicated for 15 min and stored at −18 °C.

Isolation of Triacylglycerols

The tissue was thoroughly crushed using a glass stick and extracted three times with 100 μL of CHCl₃/CH₃OH (1:1, *v/v*). The combined crude extract was separated on a pre-cleaned glass TLC plate (76 mm × 36 mm) coated with 0.2 mm of Adsorbosil Plus silica gel [Applied Science Laboratories, Alltech Associates, Inc., Deerfield, IL, USA; with gypsum (12%)] using a hexane/diethyl ether/formic acid (80:20:1) mobile phase. TLC zones were visualized by spraying Rhodamine 6G solution (0.05% in ethanol). The band containing TAGs ($R_F = 0.6$) was scraped off the plate and extracted with 7 mL of freshly distilled diethyl ether. The solvent was evaporated to dryness under argon stream. The samples were reconstituted in chloroform to a concentration of 1% and stored in sealed glass ampoules under argon at −18 °C until analyzed. The absolute amounts of TAGs isolated from samples 1–5 were 0.2, 0.4, 0.8, 0.9, and 0.1 mg, respectively.

Transesterification and DMDS Derivatization

TAG samples were transesterified using a method described earlier [8]. Briefly, TAGs were dissolved in CHCl₃/CH₃OH (2:3, *v/v*) in a small glass ampoule. After adding acetyl chloride (Fluka), the ampoule was sealed and placed in a water bath at 70 °C. This procedure prevents the loss of volatile FAMEs during transesterification. After 90 min the ampoule was opened and the reaction mixture was

neutralized with silver carbonate (Lachema, Brno, Czech Republic). To determine double bond position(s), FAMEs were derivatized with dimethyl disulfide (DMDS). The transesterified mixture was filtered through a column with a small amount of silica gel and evaporated to dryness under a stream of argon. The residues were dissolved in 50 μL of DMDS (Merck, Darmstadt, Germany), which was pre-cleaned by filtration through silica gel. A diethyl ether solution of iodine (Lachema, 12.5 μL, 60 μg/μL) was added and the reaction mixture was shaken for 24 h at laboratory temperature. Finally, hexane (60 μL) was added, the excess of iodine was removed by 5% solution of sodium thiosulfate, and the hexane layer was separated and directly injected onto the GC column.

Gas Chromatography/Mass Spectrometry

FAMEs and their DMDS derivatives were analyzed using a 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) coupled to a 5975B quadrupole mass spectrometer and equipped with a fused silica capillary column HP-5 ms (30 m × 0.25 mm, 0.25 μm, Agilent). The carrier gas was helium at 1 mL/min. The injector was operated in splitless mode at 230 °C. The temperature program for FAMEs: 40 °C (1 min), then 50 °C/min to 140 °C, then 3 °C/min to 320 °C (20 min). The temperature program for DMDS derivatives: 70 °C (1 min), then 10 °C/min to 320 °C (20 min). Standard 70 eV mass spectra were recorded in the mass range (m/z) of 25–600; a 4-min solvent delay was used. The temperatures of the transfer line, ion source and quadrupole were 250, 230 and 150 °C, respectively.

Gas Chromatography/Flame Ionization Detection

FAMEs were quantified and their ECL (equivalent chain length) values were determined using an HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). Two fused silica capillary columns were used: (a) DB-WAX (30 m × 0.25 mm, 0.25 μm, J&W Scientific, USA) and (b) DB-1 (30 m × 0.25 mm, 0.25 μm, J&W Scientific, Folsom, CA, USA). The injector and detector temperatures were 230 and 250 °C, respectively. The injector was operated in split mode with a split ratio of 20:1. The carrier gas was hydrogen (87 kPa, ū = 40 cm/s for DB-WAX and 95 kPa, ū = 41 cm/s for DB-1); nitrogen was used as make-up gas (25 mL/min). Temperature program: (a) 45 °C (2 min), then 15 °C/min to 150 °C, then 4 °C/min to 320 °C (5 min) (FAME quantification on DB-WAX column); (b) isothermally at 220 °C (60 min) (both columns, for determining ECL values). Data were collected with an HP 3393A integrator (Hewlett-Packard). ECL values were calculated from the retention times of FAMEs and

co-injected FAME standards (22:0–28:0, both odd and even) using a program written in GW-BASIC [9].

Gas Chromatography/Infrared Spectroscopy

Double-bond positions were determined using an Agilent 6850 gas chromatograph connected to an Equinox 55 FT-IR spectrometer (Bruker Optics Inc., Ettlingen, Germany). A DB-5 column (30 m × 0.32 mm, 0.25 μm, J&W Scientific) was used for the separations; the injector temperature was 220 °C (splitless mode), while the carrier gas was He at a flow rate of 2.5 mL/min. The temperature program: 50 °C (0 min), then 50 °C/min to 150 °C (0 min), then 1 °C/min to 240 °C (5 min). A liquid nitrogen-cooled photoconductive mercury-cadmium-telluride (MCT) detector was used with an FT-IR resolution of 8 cm^{-1}; the light pipe temperature was 200 °C. Methyl (E)-hexadec-9-enoate and methyl (Z)-hexadec-9-enoate (Applied Science Laboratories; 20 mg/mL in cyclohexane) were used as standards for infrared (IR) spectra of unsaturated methyl esters.

High-Performance Liquid Chromatography/Mass Spectrometry

HPLC was performed on a TSP liquid chromatograph (Thermo Separation Products, Piscataway, NJ, USA) with an ion-trap mass spectrometric detector (LCQ classic) controlled by Xcalibur software (Thermo Finnigan Corp., San Jose, CA, USA). TAGs were separated on two Nova-Pak C18 stainless steel columns (300 × 3.9 and 150 × 3.9 mm, 4 μm, Waters Corp., Milford, MA, USA) connected in series. The mobile phase was mixed from acetonitrile (A) and 2-propanol (B) [10]. A linear gradient from 0 to 70% of B in 108 min (1.0 mL/min) was followed by a linear gradient to 100% B (150 min, 0.7 mL/min). The columns were kept at 30 °C during analyses. The mobile phase was mixed post-column in a low dead volume T-piece with 100 mM ammonium acetate prepared in 2-propanol/water 1:1 (v/v), at a flow rate of 10 μL/min. The full scan mass spectra were recorded in the mass range (m/z) of 75–1,300, the temperatures of the vaporizer and heated capillary were set to 400 and 200 °C, respectively; the corona discharge current was 4.5 μA. TAGs were identified using the TriglyAPCI software [11] and quantified from reconstructed chromatograms calculated for [M+H]$^+$ and [M+NH$_4$]$^+$ molecular adducts [7].

Results and Discussions

Identification of Fatty Acids

TAGs from fat body of bumblebee males were transesterified and analyzed by GC/MS. Methyl esters of both saturated and unsaturated FAs were found. To determine the position(s) of double bond(s) in FA chains, DMDS derivatives were prepared [12]. DMDS derivatives of monoenic FAs provided molecular ions M$^{+\bullet}$ accompanied by less intense ions formed by the loss of methoxy (M$^{+\bullet}$-31) and methylthio (M$^{+\bullet}$-47) radicals from the molecular ions. The most abundant peaks resulted from α-cleavage between the two carbon atoms originally constituting the double bond, and these ions were denominated A$^+$ ([CH$_3$(CH$_2$)$_n$CH = S–CH$_3$]$^+$) and B$^+$ ([CH$_3$–S = CH(CH$_2$)$_m$COOCH$_3$]$^+$) in this work. Neutral losses of methanethiol from A$^+$ and methanol and methanethiol from B$^+$ were also observed ([A-48]$^+$, [B-32]$^+$ and [B-32-48]$^+$, respectively). In the low-mass region, fragment ions of the C$_n$H$_{2n-1}$ series, [CH$_2$=S–CH$_3$]$^+$ (m/z 61), and methyl ester-related ions at m/z 74 and 87 were found.

The most abundant dienoic acid found in *B. pratorum* contained 26 carbons, and the mass spectrum of its methyl ester (M = 406) is shown in Fig. 1a. The corresponding DMDS derivative provided a mass spectrum (Fig. 1b) with a molecular ion at m/z 594, which indicated the formation of a tetrasubstituted acyclic derivative. Therefore, at least
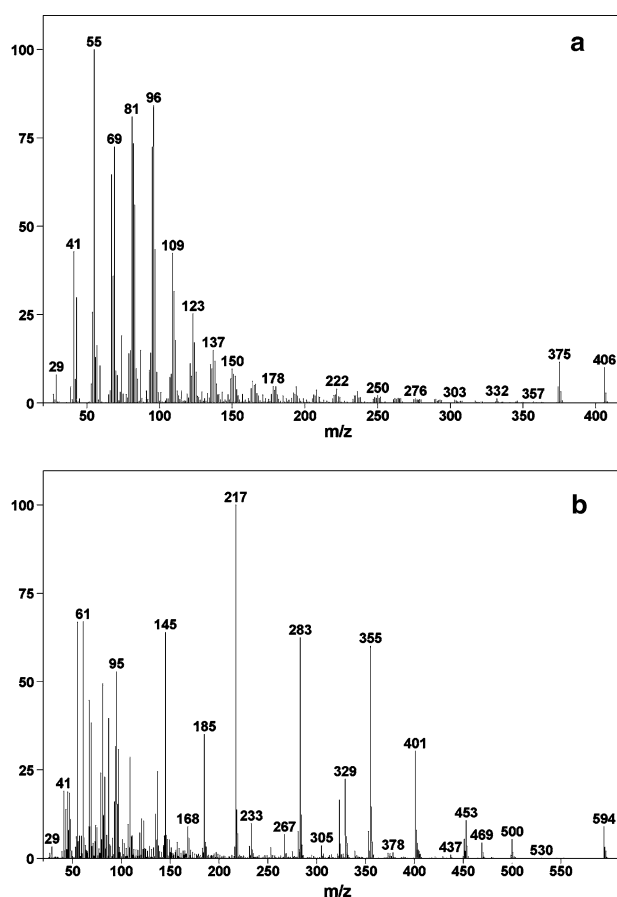
Fig. 1 Mass spectra of (Z,Z)-9,19-hexacosadienoic acid methyl ester (**a**) and its DMDS derivative (**b**)

four methylene groups separating the two double bonds were expected [13]. An ion at m/z 500 corresponded to the elimination of both methanethiol and thioformaldehyde. Further losses of methoxy or methylthio radicals resulted in ions at m/z 469 and 453, respectively. As two double bonds existed in the molecule, two pairs of diagnostic ions were expected. However, only two ions were found; the other two diagnostic ions lost neutral molecules giving the secondary fragments. Based on the fragmentation, the spectrum was interpreted as 9,19-hexacosadienoic acid methyl ester. Figure 2 indicates the expected fragments after α-cleavage between the carbons originally constituting the double bonds, which were labeled A+, B+, C+, and D+ here. The diagnostic ions A+ and D+ were found at m/z 145 and 217, respectively; D+ was the spectrum base peak. The B+ and C+ eliminated neutral molecules, either methanethiol, giving m/z 401 ([B-48]+) and m/z 329 ([C-48]+), or both methanethiol and thioformaldehyde, providing m/z 355 ([B-48-46]+) and 283 ([C-48-46]+), respectively. In addition, B+ eliminated methanol from the methyl ester moiety ([B-48-46-32]+; m/z 323). Ions A+ and D+ also provided additional fragments after the elimination of neutral molecules, giving m/z 97 ([A-48]+), m/z 185 ([D-32]+) and m/z 137 ([D-32-48]+). The low-mass region contained ions of the C_nH_{2n-1} series, the m/z 67, 81, 95, 109 series, $[CH_2 = S–CH_3]^+$ (m/z 61), and methyl ester-related ions at m/z 74 and m/z 87. The identities of all common FAMEs were also confirmed by determining ECL values and comparing them with published data [14].

The geometric configuration of double bonds in 9,19-hexacosadienoic acid methyl ester was determined by IR spectroscopy. Unsaturated dienoic compounds with at least one (E)-double bond provide a characteristic strong band at 990–965 cm^{-1} assigned to the =C–H out-of-plane deformation vibration [15]. Compounds with a (Z)-double bond do not provide a signal in this area, but show a typical band from =C–H stretch absorption at 3,028–3,011 cm^{-1} [16]. The IR spectrum of 9,19-hexacosadienoic acid methyl ester (Fig. 3) did not show any signal that could be attributed to (E)-double bond(s). By contrast, a well-resolved band at 3,012 cm^{-1} was found, which indicated the presence of double bond(s) with a (Z)-configuration. Other bands were those expected for methyl esters (1,758 cm^{-1} (C=O stretching), 1,176 cm^{-1} (C–O stretching), 1,651 cm^{-1} (C=C stretching), and 718 cm^{-1} (–(CH_2)_n-skeletal overlapping with =CH deformation).

Based on the MS and IR data, we have concluded that structure of the most abundant dienoic acid is (Z,Z)-9,19-hexacosadienoic acid [(Z,Z)-9,19-26:2].

ECL values of (Z,Z)-9,19-Hexacosadienoic Acid Methyl Ester

To characterize the retention behavior of the (Z,Z)-9,19-26:2 methyl ester for future reference, ECL values at 220 °C were determined. The measurements were done in quadruplicate on both nonpolar (DB-1) and polar (DB-WAX) stationary phases, and the values obtained are listed in Table 1.

Quantification of Fatty Acids

The compositions of the FAs were determined by GC/FID, because this technique provides almost identical response factors for FAMEs [17]. FAs identified in the fat body of *B. pratorum* and their relative abundances are summarized in

Fig. 2 Labels of the main fragments expected in the EI mass spectrum of the DMDS derivative of (Z,Z)-9,19-hexacosadienoic acid methyl ester
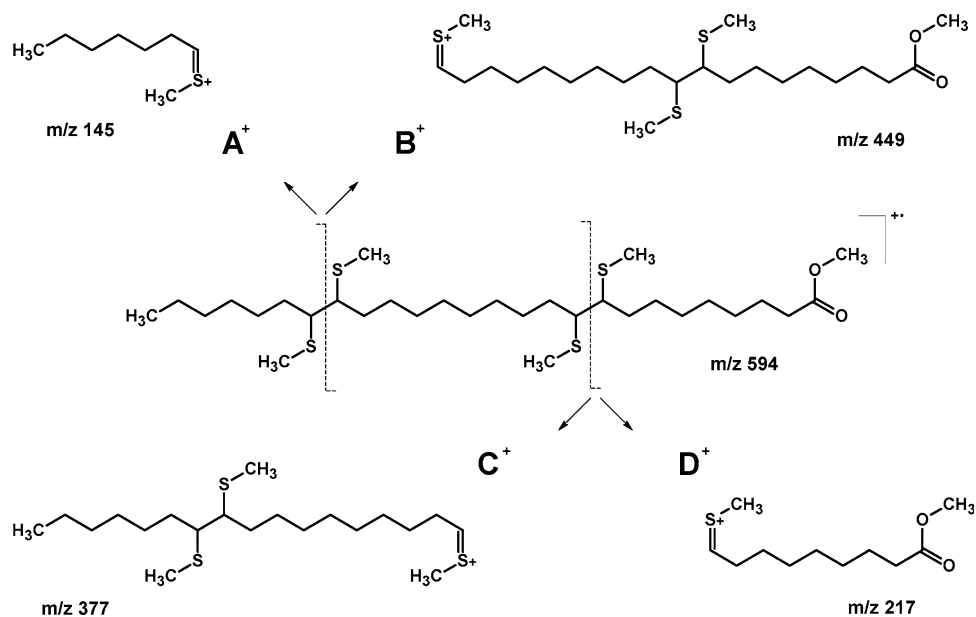
Fig. 3 Infrared spectrum of (Z,Z)-9,19-hexacosadienoic acid methyl ester

Table 1 ECL values of (Z,Z)-9,19-hexacosadienoic acid methyl ester

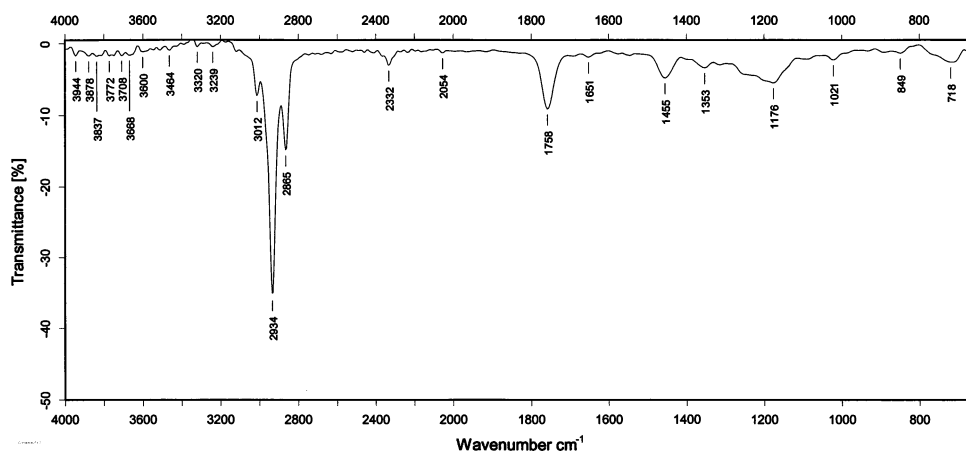| Column	ECL ± SD
DB-WAX	26.461 ± 0.002
DB-1	25.393 ± 0.003

Table 2. To compare variations between individuals, results for five bumblebees collected in different localities and at different times during the season are given.

The fat body was found to contain only even FAs, with the exception of pentadecanoic acid, which was present at trace levels. Palmitic acid was the most abundant saturated acid; stearic, myristic, and lauric acids were also detected, but at lower concentrations. FAs with one double bond prevailed in the samples. Oleic acid itself formed about one-third and together with *cis*-vaccenic acid more than half of all acids. The typical position of the double bond in the monoenoic FAs was 9, but FAs with other double bond positions (11 or 13) also occurred. Among polyenoic acids, (Z,Z)-9,19-26:2, linoleic and α-linolenic acid were detected.

Whereas most of the FAs found in *B. pratorum* are common in bumblebees [7], those with 24 and 26 carbon atoms have not been detected in this genus and they are quite rare in nature. Males of six bumblebee species (*Bombus lucorum*, *B. terrestris*, *B. lapidarius*, *B. hypnorum*, *B. hortorum*, and *B. confusus*) underwent analyses of TAGs in their pool lipids, but none of the species contained fatty acids longer than 22 carbon atoms [7]. 9,19-26:2 has so far been identified only in marine sponges [18–22]. Even there, its concentrations are substantially lower than those of the more common isomer 5,9-26:2. Morales and Lichtfield [18] showed in labeling experiments with *Microciona prolifera* that 9,19-26:2 is biosynthesized from 9-16:1 by chain elongation followed by Δ^9 desaturation. The reaction intermediate 19-26:1 was found in high quantities in the sponge. By contrast, *B. pratorum* produces 9-26:1 as the only monoenic FA with 26 carbons, which might be indicative of a different

biosynthetic pathway (e.g., Δ^9 desaturation of 26:0 followed by Δ^{19} desaturation). However, no data on the biosynthesis of 9,19-26:2 in bumblebees are available at the moment. 9,19-26:2 has also been found in the marine sponges *Dysidea fragilis* [19], *Halichondria panicea* [20], and *Hymeniacidon sanguinea* [21, 22].

Monounsaturated 9-26:1 and 9-24:1 were detected in *B. pratorum* at trace levels, but they are more common in nature than 9,19-26:2. Several papers reported their presence in both marine [19–26] and freshwater [27, 28] sponges. Their abundances are also low, at percent or subpercent levels. Marine fish have been shown to contain 9-24:1 in substantially lower quantities than isomeric 15-24:1 [29]. Higher levels of monoenic 9-24:1 and 9-26:1 were only found in the bacteria *Mycobacterium tuberculosis* [30] and *Francisella tularensis* [31]. So far there has been no evidence of 9,19-26:2, 9-26:1 or 9-24:1 in insects or any other higher organism.

Composition of Triacylglycerols

HPLC/MS was employed to elucidate the structures of intact TAGs and thus to reveal how the rare long-chain FAs are bonded to the glycerol backbone [32]. Fat body of *B. pratorum* was found to contain more than 80 different TAG molecular species (Table 3). The most abundant TAGs were those which are common in bumblebees ([16:0, 18:1, 18:1] and [18:1, 18:1, 18:1]). Note that 18:1 might represent oleic and/or *cis*-vaccenic acid, as both of them were detected by GC/MS. Equivalent carbon number (ECN) values of TAGs from *B. pratorum* ranged from 38 to 66, i.e., up to unusually high values for bumblebees [7]. In accordance with GC data, rare long-chain FAs with 24 and 26 carbons were detected. In addition, FAs with 28 carbons and two or one double bonds were found, although at sub-percent levels. 9,19-26:2 occupied at least one position in one-third of all TAG molecular species. Not surprisingly, the most abundant TAGs with this acid also

Table 2 Fatty acids identified in triacylglycerols from the fat body of male *B. pratorum*

Fatty acid	Relative peak area (%)					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD
12:0	1.3	3.7	4.3	13.1	2.2	3.7 ± 4.2
14:0	3.1	5.4	6.0	8.5	2.8	5.4 ± 2.1
9-14:1	0.2	0.2	0.2	1.0	0.2	0.2 ± 0.3
15:0	<0.1	0.1	<0.1	<0.1	0.2	<0.1
16:0	21.9	18.5	19.1	14.5	17.7	18.5 ± 2.4
9-16:1	9.0	6.2	7.7	11.7	7.2	7.7 ± 1.9
11-16:1	0.4	1.4	1.4	0.7	0.8	0.8 ± 0.4
18:0	1.2	0.7	0.7	0.4	1.1	0.7 ± 0.3
9-18:1	40.5	35.1	32.9	32.5	38.6	35.1 ± 3.2
11-18:1	9.9	19.5	17.3	12.6	17.5	17.3 ± 3.6
9,12-18:2	0.6	1.3	2.2	0.8	0.7	0.8 ± 0.6
9,12,15-18:3	2.8	2.6	3.4	2.5	2.7	2.7 ± 0.3
20:0	0.2	0.1	0.2	0.2	0.3	0.2 ± 0.1
9-20:1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
13-20:1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
22:0	0.1	0.1	0.3	0.3	0.5	0.3 ± 0.1
24:0	0.2	<0.1	<0.1	<0.1	0.2	<0.1
9-24:1	0.2	<0.1	<0.1	<0.1	0.1	<0.1
9-26:1	0.2	0.2	0.3	<0.1	0.4	0.2 ± 0.1
9,19-26:2	8.1	5.0	4.0	1.1	6.7	5.0 ± 2.4

contained 18:1, 16:1 or 16:0, i.e., the main FAs occurring in this bumblebee. 9,19-26:2 occupied one (e.g., [18:1, 26:2, 18:1], [16:0, 18:1, 26:2]), two (e.g., [26:2, 18:1, 26:2], [26:2, 16:1, 26:2]), or even all three positions ([26:2, 26:2, 26:2]), which is unusual. Other long-chain FAs, 24:1, 24:2, 26:1, 28:1 and 28:2, were found in a small number of TAGs, mostly at very low concentrations. It is important to note that the assignment of FAs to a particular sn position (distinguishing sn-2 from sn-1/3) is based on the intensities of diacyglycerol fragments observed in the mass spectra. Besides the position, the fragment intensities are to some extent also affected by the structures of FAs. Also, unlike GC/FID, TAG response factors in HPLC/APCI-MS are generally not equal. Therefore, the FA positions and quantities of individual TAGs shown in Table 3 are burdened with some uncertainty, which cannot be avoided without using authentic TAG standards. Despite these limitations, HPLC/APCI-MS provided valuable information on TAGs. An example chromatogram showing the separation of TAGs from *B. pratorum* is given in Fig. 4.

9,19-Hexacosadienoic Acid and Labial Gland Composition

FAs identified in the fat body were compared with the composition of the cephalic labial gland secretion of

B. pratorum males. Bumblebees use the secretions as scent marks for denoting prominent objects along the patrolling routes. The scent—which contains marking pheromone—attracts females for mating [33]. The composition of labial gland secretions is strictly species-specific. The biosynthesis of the marking pheromone components has not been completely clarified yet [34]. We attempt to explore the idea that FAs stored in the fat body might serve as the precursors of non-terpenic components of the labial gland secretions. The marking pheromone blend of *B. pratorum* contains isoprenoids (farnesol, geranylgeranyl acetate, farnesyl acetate), alcohols (11-octadecenol, hexadecanol [35]), and both saturated and unsaturated hydrocarbons [36, 37]. The hydrocarbon (*Z,Z*)-7,17-pentacosadiene is a medium-abundance component; it forms 9% of the labial gland secretion, which represents about 80% of all hydrocarbons. This rare diene has not been detected in the marking pheromone of any other bumblebee species. One can speculate about its biosynthesis from 9,19-26:2 by decarboxylation [38], as shown in Fig. 5. 9,19-26:2 is bonded in several relatively abundant TAGs in two or even all three positions, which ensures the presence of this acid in the transport of 1,2-diacylglycerols. Thus, the acid might be available to the labial gland for pheromone synthesis. Similar biosynthetic transformations to that proposed for 9,19-26:2 might also apply for 9-24:1 and 9-26:1. The

Table 3 Triacylglycerols identified in the fat body of male *B. pratorum*

ECN	CN:DB	t_R (min)	Triacylglycerol(s)[a]	Peak area [rel. (%)]					
				Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD
38	46:4	52.6	[12:0, 16:1, 18:3]	–	0.1	0.1	0.3	–	0.1 ± 0.1
	44:3	53.7	[12:0, 14:0, 18:3]	–	–	0.1	0.3	–	<0.1 ± 0.1
40	54:7	58.6	[18:3, 18:1, 18:3], [18:3, 18:2, 18:2]	–	0.1	0.3	0.1	0.1	0.1 ± 0.1
	52:6	60.0	[16:0, 18:3, 18:3]	0.2	0.1	0.2	0.1	0.1	0.1 ± 0.1
	48:4	59.4	[12:0, 18:1, 18:3]	0.3	0.5	0.8	1.4	0.2	0.5 ± 0.4
	46:3	60.7	[12:0, 18:3, 16:0], [14:0, 14:0, 18:3]	0.2	0.3	0.4	0.7	0.1	0.3 ± 0.2
	42:1	60.5	[12:0, 18:1, 12:0], [12:0, 16:1, 14:0]	–	0.2	0.2	1.7	0.1	0.2 ± 0.6
42	54:6	64.3	[18:2, 18:2, 18:2]	–	0.2	0.4	0.2	0.2	0.2 ± 0.1
	52:5	64.6	[16:1, 18:1, 18:3]	1.1	1.0	1.4	1.3	1.0	1.1 ± 0.2
	50:4	66.0	[16:0, 16:1, 18:3], [14:0, 18:1, 18:3], [14:0, 18:2, 18:2]	1.4	1.1	1.9	1.7	0.8	1.4 ± 0.4
	48:3	67.3	[14:0, 14:0, 20:3]	–	0.3	0.5	0.5	0.1	0.3 ± 0.2
	48:3	65.4	[12:0, 18:1, 18:2]	0.3	0.4	0.4	0.9	0.1	0.4 ± 0.3
	46:2	65.7	[12:0, 16:1, 18:1]	0.4	1.0	0.9	4.0	0.5	0.9 ± 1.3
	44:1	67.1	[12:0, 18:1, 14:0], [12:0, 16:1, 16:0],	0.2	1.1	0.9	3.9	0.3	0.9 ± 1.3
	42:0	68.5	[12:0, 14:0, 16:0]	–	0.1	0.1	0.3	–	0.1 ± 0.1
44	54:5	70.3	**[18:1, 18:3, 18:1]**	2.3	3.5	4.3	2.7	3.2	**3.2 ± 0.7**
	52:4	71.9	**[16:0, 18:3, 18:1], [16:0, 18:2, 18:2]**	5.0	4.0	6.1	3.3	4.3	**4.3 ± 1.0**
	50:3	73.3	[16:0, 16:0, 18:3]	0.7	0.4	0.7	0.3	0.3	0.4 ± 0.2
	50:3	70.6	[16:1, 16:1, 18:1], [18:1, 14:1, 18:1]	1.1	1.3	1.5	2.5	1.1	1.3 ± 0.5
	48:2	71.7	**[12:0, 18:1, 18:1]**	1.9	4.3	3.4	9.7	2.5	**3.4 ± 2.8**
	46:1	73.3	**[12:0, 18:1, 16:0], [14:0, 14:0, 18:1]**	1.0	3.4	2.5	6.2	1.3	**2.5 ± 1.9**
	44:0	75.0	[14:0, 14:0, 16:0], [12:0, 16:0, 16:0]	–	0.2	0.2	0.2	–	0.2 ± 0.1
46	54:4	75.8	[18:2, 18:1, 18:1]	0.8	1.7	1.8	1.3	1.2	1.3 ± 0.3
	52:3	76.1	**[18:1, 16:1, 18:1]**	4.8	6.3	6.6	8.4	7.9	**6.6 ± 1.2**
	50:2	77.6	**[18:1, 14:0, 18:1], [16:0.16:1, 18:1]**	6.9	8.0	7.2	10.3	7.6	**7.6 ± 1.2**
	48:1	79.2	**[14:0, 18:1, 16:0], [16:0, 16:1, 16:0]**	1.8	3.0	2.4	3.0	1.3	**2.4 ± 0.7**
	46:0	81.0	[14:0, 16:0, 16:0]	–	0.1	0.1	0.1	–	0.1 ± 0.1
48	54:3	81.6	**[18:1, 18:1, 18:1]**	7.4	11.9	8.8	9.4	15.2	**9.4 ± 2.8**
	52:2	83.2	**[16:0, 18:1, 18:1]**	12.3	14.6	11.6	10.4	17.8	**12.3 ± 2.6**
	50:1	85.0	**[16:0, 18:1, 16:0]**	2.5	2.3	2.1	1.3	1.9	**2.1 ± 0.4**
	48:0	87.1	[16:0, 16:0, 16:0]	–	0.1	0.1	–	–	<0.1 ± 0.1
50	62:6	85.2	[18:2, 18:2, 26:2]	–	–	0.6	0.2	0.3	0.2 ± 0.2
	60:5	86.7	[16:1, 18:2, 26:2]	0.3	0.2	0.4	0.1	0.2	0.2 ± 0.1
	58:4	86.1	[16:1, 16:1, 26:2]	0.3	0.2	0.2	0.2	0.2	0.2 ± 0.1
	56:3	86.9	[18:1, 18:1, 20:1], [12:0, 26:2, 18:1]	1.0	1.1	1.0	1.0	1.5	1.0 ± 0.2
	54:2	88.9	[18:1, 16:0, 20:1], [18:1, 18:1, 18:0]	0.8	0.8	0.9	0.8	1.3	0.8 ± 0.2
	52:1	90.6	[16:0, 18:1, 18:0]	0.3	0.2	0.2	0.1	0.3	0.2 ± 0.1
52	62:5	90.4	[18:2, 26:2, 18:1], [18:1, 16:1, 26:2]	0.5	0.4	0.5	0.2	0.3	0.4 ± 0.1
	60:4	90.7	[16:1, 26:2, 18:1]	2.1	1.3	1.5	0.8	1.4	1.4 ± 0.4
	58:3	92.2	[16:0, 16:1, 26:2], [18:1, 14:0, 26:2]	1.3	0.6	1.1	0.6	1.0	1.0 ± 0.3
	56:2	94.2	[18:1, 18:1, 20:0]	0.4	0.2	0.4	0.2	0.3	0.3 ± 0.1
54	62:4	95.7	**[18:1, 26:2, 18:1]**	8.3	4.3	4.1	1.9	5.3	**4.3 ± 2.1**
	60:3	97.1	**[16:0, 18:1, 26:2]**	3.7	2.2	3.2	1.4	3.4	**3.2 ± 0.9**
	58:2	98.7	[16:0, 26:2, 16:0]	0.4	0.2	0.5	0.2	0.3	0.3 ± 0.1

Table 3 continued

ECN	CN:DB	t_R (min)	Triacylglycerol(s)[a]	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD
				Peak area [rel. (%)]					
56	70:7	98.2	[18:3, 26:2, 26:2]	0.9	0.7	0.7	0.1	0.2	0.7 ± 0.3
	66:5	99.1	[16:1, 26:2, 24:2]	0.6	0.2	0.5	0.1	0.1	0.2 ± 0.2
	64:4	99.9	[18:1, 18:1, 28:2], [18:1, 26:2, 20:1]	1.8	0.7	0.8	0.4	0.9	0.8 ± 0.5
	62:3	101.9	[18:1, 18:1, 26:1], [18:1, 26:2, 18:0]	1.3	0.6	0.8	0.4	1.1	0.8 ± 0.3
	60:2	103.9	[16:0, 18:1, 26:1]	0.3	0.1	0.3	0.2	0.4	0.3 ± 0.1
58	68:5	103.0	**[26:2, 16:1, 26:1]**	4.3	2.5	2.4	0.8	1.6	**2.4 ± 1.2**
	66:4	104.3	[14:0, 26;2, 26:2], [22:1, 18:1, 26:2]	1.3	0.5	0.9	0.3	0.5	0.5 ± 0.4
	64:3	106.2	[18:1, 18:1, 28:1]	0.4	0.1	0.4	0.1	0.3	0.3 ± 0.1
	62:2	107.8	[18:0, 18:0, 26:2], [18:1, 18:1, 26:0]	0.1	–	0.1	0.1	0.1	0.1 ± 0.1
60	70:5	107.2	**[26:2, 18:1, 26:2]**	9.2	6.5	4.8	1.5	4.7	**4.8 ± 2.5**
	68:4	108.4	[16:0, 26:2, 26:2]	2.5	1.7	2.0	0.5	1.5	1.7 ± 0.6
	66:3	110.5	[16:0, 24:1, 26:2], [18:1, 22:0, 26:2]	0.2	0.1	0.2	0.1	0.2	0.2 ± 0.1
62	72:5	111.0	[26:2, 18:1, 28:2]	1.3	0.7	0.6	0.2	0.5	0.6 ± 0.4
	70:4	113.3	[26:2, 18:1, 26:1]	1.8	1.0	1.6	0.4	1.3	1.3 ± 0.5
	68:3	114.9	[16:0, 26:1, 26:2], [18:2, 26:1, 24:0]	0.3	0.2	0.4	0.1	0.3	0.3 ± 0.1
64	72:4	117.6	[20:0, 26:2, 26:2], [16:0, 28:2, 28:2]	0.4	0.2	0.2	0.1	0.3	0.2 ± 0.1
	70:3	119.1	[18:1, 26:1, 26:1]	0.1	0.1	–	0.1	0.1	0.1 ± 0.1
66	78:6	118.3	[26:2, 26:2, 26:2]	0.9	0.5	0.3	–	0.2	0.3 ± 0.3

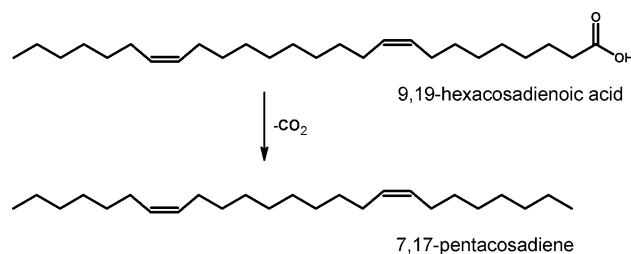The most abundant TAGs are shown in bold

CN number of carbon atoms in fatty acids in TAG; *DB* number of double bonds in the carbon chains of fatty acids

[a] TAG abbreviations reflect the assumed positions of FAs on the glycerol backbone. As APCI MS cannot distinguish between acyls in the sn-1 and sn-3 positions, the TAG abbreviations in the table do not differentiate between these two positions; FA residues in sn-1/3 were ordered by increasing molecular weight
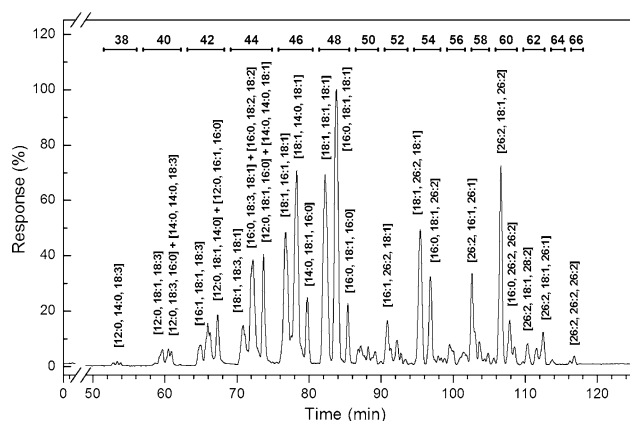
Fig. 4 HPLC/APCI-MS chromatogram of triacylglycerols isolated from the fat body of male bumblebee *B. pratorum*. The numbers in the upper part of the figure are ECN values

Fig. 5 Proposed biosynthesis of (Z,Z)-7,17-pentacosadiene in male marking pheromone of *B. pratorum* from (Z,Z)-9,19-hexacosadienoic acid

expected products, 8-tricosene and 8-pentacosene, were detected in the labial gland, though only at trace levels. Furthermore, tricosadiene and heptacosadiene are present in minor quantities in the pheromonal gland, which corresponds well with the presence of 24:2 and 28:2 acids in

TAGs. Clearly, our findings support the hypothesis about a connection between pheromone biosynthesis and FAs in the fat body, but more research is needed.

Concluding Remarks

In conclusion, unique FAs with 24, 26 and 28 carbon atoms were identified in the fat body of *B. pratorum*. Among them, (Z,Z)-9,19-hexadecadienoic acid was the most

abundant, comprising up to 8% of all FAs. In this work we have also shown remarkable similarities between the structure of this acid and the main hydrocarbon component of the male marking pheromone. Due to the uncommon structure of its acid and labial gland secretion component(s), *B. pratorum* represents a convenient model for studying marking pheromone biosynthesis.

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