Lipids (2011) 46:263–270
DOI 10.1007/s11745-010-3497-x

ORIGINAL ARTICLE

The Absolute Configurations of Hydroxy Fatty Acids from the Royal Jelly of Honeybees (*Apis mellifera*)

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Received: 20 June 2010 / Accepted: 22 October 2010 / Published online: 17 November 2010

Abstract 9-Hydroxy-2*E*-decenoic acid (9-HDA) is a precursor of the queen-produced substance, 9-oxo-2*E*-decenoic acid (9-ODA), which has various important functions and roles for caste maintenance in honeybee colonies (*Apis mellifera*). 9-HDA in royal jelly is considered to be a metabolite of 9-ODA produced by worker bees, and it is fed back to the queen who then transforms it into 9-ODA. Recently we found that 9-HDA is present in royal jelly as a mixture of optical isomers (*R*:*S*, 2:1). The finding leads us to suspect that chiral fatty acids in royal jelly are precursors of semiochemicals. Rather than looking for semiochemicals in the mandibular glands of the queen bee, this study involves the search for precursors of pheromones from large quantities of royal jelly. Seven chiral hydroxy fatty acids, 9,10-dihydroxy-2*E*-decenoic, 4,10-dihydroxy-2*E*-decenoic, 4,9-dihydroxy-2*E*-decenoic, 3-hydroxydecanoic, 3,9-dihydroxydecanoic, 3,11-dihydroxydodecanoic, and 3,10-dihydroxydecanoic acids were isolated. The absolute configurations of these acids were determined using the modified Mosher's method, and it was revealed that, similar to 9-HDA, five acids are present in royal jelly as mixtures of optical isomers.

Keywords Absolute configuration · *Apis mellifera* · Honeybees · Hydroxy fatty acid · Modified Mosher's method · Royal jelly

Abbreviations

COSY Two-dimensional ^{1}H-^{1}H shift correlation
HMBC Two-dimensional heteronuclear multiple-bond connectivity
MTPA 2-Methoxy-2-(trifluoromethyl)phenylacetic acid
TMS Tetramethylsilane

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Introduction

The so-called "queen substance," 9-oxo-2-decenoic acid (9-ODA), was isolated from the honeybee queen mandibular glands by Butler et al. [1]. This compound is a well-known semiochemical which has various important functions, such as queen recognition and inhibition of ovary development in worker bees for caste maintenance in honeybee colonies [2].

In 1965, Johnston and co-workers reported that 9-ODA was rapidly metabolized to 9-hydroxy-2*E*-decenoic (9-HDA) or 9-hydroxydecanoic acids [3]. They further postulated a "pheromone cycle," in which worker bees would receive 9-ODA from the queen and convert it into 9-HDA. The metabolite would then be transported to the worker mandibular glands where it would be passed back to the queen through royal jelly. The queen would then convert it back into the active oxo-form, 9-ODA. In fact, significant amounts of 9-HDA have been found in royal jelly, but 9-ODA has not yet been detected. Furthermore, our previous study revealed that 9-HDA is present as a mixture of optical isomers (*R*:*S*, 2:1) in royal jelly [4]. This interesting finding is important with regard to searching for precursors of unknown semiochemicals in royal jelly.

The present study was undertaken to examine the constituents of royal jelly in the hope of discovering precursors of unknown queen substances. We conducted a more

detailed survey of the components, particularly the fatty acid fraction, and isolated seven chiral fatty acids in the pure state. The absolute configurations of the chiral acids were determined using the modified Mosher's method [5–7]. Similar to 9-HDA found in royal jelly, five acids are present as mixtures of optical isomers.

Experimental Procedure

Materials

Lyophilized royal jelly powder was supplied by Yamada Apiculture Center Inc. (Okayama, Japan). A voucher specimen was deposited in the Faculty of Pharmaceutical Sciences, Setsunan University.

Instrumentation

Optical rotations were measured at 25 °C with a JASCO DIP-140 polarimeter (JASCO, Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL JMN GX400 or ECA 600SN spectrometers (JEOL, Tokyo, Japan), using tetramethylsilane (TMS) as an internal reference. All samples were measured at a probe temperature of 35 °C. The two-dimensional heteronuclear multiple bond connectivity (HMBC) spectrum was recorded at 600 MHz with 64 scans ($^{2,3}J_{CH} = 8$ Hz). Fast atom bombardment mass spectrometry (FAB-MS) including high-resolution mass was recorded on a JEOL JMS-700T spectrometer (JEOL). (Accelerating voltage, 5 kV; matrix, glycerin or triethanolamine; collision gas, He). Column chromatography was carried out on Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), silica gel (Kiesergel 60, Merck), and Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc., Tokyo, Japan). Preparative HPLC was conducted over Mightysil RP-18 GP (5 μm, 4.6 × 250 mm, Kanto Chemical Co. Inc. Tokyo, Japan) and Inertsil ODS-3 (4 μm, 4.6 × 100 mm, GL Sciences Inc., Tokyo, Japan) columns with a JASCO 980-PU (JASCO, Tokyo, Japan). The elution profile was monitored by a refractive index detector, RI504R (GL Sciences Inc., Tokyo, Japan).

Isolation of Compounds, 1–7

Lyophilized royal jelly powder (1 kg) was percolated with chloroform to remove a major component, 10-hydroxy-2*E*-decenoic acid. The residue was extracted with chloroform/acetone (1:1, vol/vol), and the solvent was removed in vacuo to give an extract (36.1 g). This was chromatographed on silica gel and eluted successively with chloroform/methanol/water (7:2:0.2 → 7:3:0.5 → 6:4:1 by vol) to give four fractions, fr. 1 (30.0 g), fr. 2 (1.8 g), fr. 3 (0.9 g), and fr. 4 (0.5 g). Fr. 2 was chromatographed on Sephadex LH-20 (methanol) and Cosmosil 75C_{18}-OPN (methanol/water, 1:1 vol/vol → methanol) columns to give three fractions, fr. 5 (115 mg), fr. 6 (314 mg), and fr. 7 (340 mg). Fr. 5 was subjected to preparative HPLC on a reversed-phase column (Inertsil ODS-3) using acetonitrile/water/trifluoroacetic acid, 30:70:0.25 by vol to give compound **1** (7.6 mg). Fr. 3 was chromatographed on Sephadex LH-20 (methanol) and Cosmosil 75C_{18}-OPN (methanol/water, 3:7 → 1:1 by vol → methanol) columns to give four fractions, fr. 8–11. Fr. 8 (31.2 mg) was subjected to preparative HPLC on a reversed-phase column (Mightysil RP-18 GP) using a mobile phase (methanol/water/trifluoroacetic acid, 25:75:0.5 by vol) to give compounds **2** (11.7 mg) and **3** (1.3 mg). Fr. 9 (499 mg) was separated by an Inertsil ODS-3 column using a mobile phase (methanol/water/trifluoroacetic acid, 25:75:0.5 by vol) to give compounds **5** (5.4 mg) and **7** (118 mg). Fr. 10 was subjected to HPLC on an Inertsil ODS-3 column using a solvent (methanol/water/trifluoroacetic acid, 37:63:0.5 by vol) to give compounds **4** (3.4 mg) and **6** (4.2 mg). Compound **7** was identified as 3*R*,10-dihydroxydecanoic acid by comparison of the NMR and FABMS spectroscopic data with those of an authentic sample [4].

1: High resolution negative ion FABMS *m/z*: 201.1120 $[M-H]^-$ (calcd. for $C_{10}H_{17}O_4$: 201.1127). $[\alpha]_D$ −5.5° at 0.8 g/100 mL in methanol. **2**: High resolution negative ion FABMS *m/z*: 201.1118 $[M-H]^-$ (calcd. for $C_{10}H_{17}O_4$: 201.1127). **3**: High resolution negative ion FABMS *m/z*: 201.1117 $[M-H]^-$ (calcd. for $C_{10}H_{17}O_4$: 201.1127). $[\alpha]_D$ −3.1° at 0.2 g/100 mL in methanol. **4**: High resolution negative ion FABMS *m/z*: 187.1339 $[M-H]^-$ (calcd. for $C_{10}H_{19}O_3$: 187.1334). $[\alpha]_D$ +1.1° at 0.3 g/100 mL in methanol. **5**: High resolution negative ion FABMS *m/z*: 203.1291 $[M-H]^-$ (calcd. for $C_{10}H_{19}O_4$: 203.1283). $[\alpha]_D$ +3.3° at 0.5 g/100 mL in methanol. **6**: High resolution negative ion FABMS *m/z*: 231.1588 $[M-H]^-$ (calcd. for $C_{12}H_{23}O_4$: 231.1596). $[\alpha]_D$ +3.4° at 0.4 g/100 mL in methanol. ^{1}H- and ^{13}C-NMR spectroscopic data of **1**–**6** are shown in Tables 1 and 2.

Preparation of 2-Methoxy-2-(trifluoromethyl)phenylacetyl (MTPA) Ester Derivatives

Compounds **1** through **6** (*ca.* 1 mg) were each treated with diazomethane in diethyl ether. (−)- and (+)-MTPA Chlorides (Tokyo Kasei Kogyo Co. Ltd., 20 mg each) were separately added to a solution of the above product in carbon tetrachloride (0.3 mL) and pyridine (1.0 mL), and the mixture was stirred at room temperature for 12 h. After removal of the solvent under a nitrogen stream, the reaction

Table 1 ^{1}H-NMR chemical shifts of **1–6** (CD_3OD)

	1	2	3	4	5	6
H-2	5.80 (1H, d, 15.6)	5.97 (1H, d, 15.6)	5.97 (1H, d, 15.6)	2.36 (1H, dd, 8.0, 15.2)	2.39 (1H, dd, 8.4, 15.0)	2.36 (1H, dd, 8.0, 15.6)
				2.44 (1H, dd, 4.8, 15.2)	2.48 (1H, dd, 4.8, 15.0)	2.44 (1H, dd, 4.8, 15.6)
H-3	6.89 (1H, dt, 15.6, 6.8)	6.92 (1H, dd, 4.8, 15.6)	6.89 (1H, dd, 4.8, 15.6)	3.97 (1H, m)	3.97 (1H, m)	3.97 (1H, m)
H-4	2.21 (2H, dt, 6.8, 6.8)	4.22 (1H, m)	4.22 (1H, m)			
H-9	3.57 (1H, m)		3.72 (1H, m)		3.71 (1H, m)	
H-10	3.41 (1H, dd, 6.8, 11.2)	3.54 (2H, t, 6.6)	1.14 (3H, d, 6.6)	0.90 (3H, t, 6.8)	1.14 (3H, d, 6.6)	
	3.47 (1H, dd, 4.4, 11.2)					
H-11	–	–	–	–	–	3.70 (1H, m)
H-12	–	–	–	–	–	1.14 (3H, d, 6.4)

δ in ppm from TMS. Splitting patterns and coupling constants (J) in Hz are given in *parentheses*

Table 2 ^{13}C-NMR chemical shifts of **1–3** and **5–6** (CD_3OD)

	1	2	3	5	6
C-1	171.4	170.0	170.6	174.1	173.8
C-2	123.7	120.9	121.7	43.3	43.3
C-3	149.5	152.9	152.2	69.4	69.3
C-4	33.0	71.6	71.6		
C-9	73.1		68.6	68.6	
C-10	67.3	63.0	23.5	23.5	
C-11	–	–	–	–	68.5
C-12	–	–	–	–	23.5

δ in ppm from TMS

mixture was subjected to silica gel column chromatography (chloroform) to give a MTPA derivative.

Bis[(−)-MTPA] Ester of Methyl Ester of **1** (**1a**) ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.18–1.17 (8H, m, $CH_2 \times 4$), 2.12 (0.5H, dt, $J = 7.2$, 7.2 Hz, H_2-4 of *S*-form), 2.16 (1.5H, dt, $J = 7.2$, 7.2 Hz, H_2-4 of *R*-form), 3.42 (2.25H, m, OCH_3 of *R*-form), 3.44 (2.25H, m, OCH_3 of *R*-form), 3.48 (0.75H, m, OCH_3 of *S*-form), 3.49 (0.75H, m, OCH_3 of *S*-form), 3.73 (3H, s, CO_2CH_3), 4.27 (0.75H, dd, $J = 4.8$, 12.0 Hz, Ha-10 of *R*-form), 4.30 (0.25H, dd, $J = 6.0$, 12.0 Hz, Ha-10 of *S*-form), 4.54 (0.75H, dd, $J = 3.0$, 12.0 Hz, Hb-10 of *R*-form), 4.62 (0.25H, dd, $J = 3.0$, 12.0 Hz, Hb-10 of *S*-form), 5.28–5.32 (1H, m, H-9), 5.79 (0.25H, d, $J = 15.6$ Hz, H-2 of *S*-form), 5.80 (0.75H, d, $J = 15.6$ Hz, H-2 of *R*-form), 6.92 (0.25H, dt, $J = 15.6$, 7.2 Hz, H-3 of *S*-form), 6.93 (0.75H, dt, $J = 15.6$, 7.2 Hz, H-3 of *R*-form), 7.33–7.40 (6H, m, H-ph), 7.43–7.50 (4H, m, H-ph).

Bis[(−)-MTPA] Ester of Methyl Ester of **2** (**2a**) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.22–1.72 (10H, m, $CH_2 \times 5$), 3.53–3.55 (6H, m, $OCH_3 \times 2$), 3.74 (1.5H, s, CO_2CH_3 of *R*-form), 3.76 (1.5H, s, CO_2CH_3 of *S*-form), 4.24–4.34 (2H, m, H_2-10), 5.57–5.60 (1H, m, H-4), 5.86 (0.5H, d, $J = 15.6$ Hz, H-2 of *R*-form), 5.98 (0.5H, d, $J = 15.6$ Hz, H-2 of *S*-form), 6.78 (0.5H, dd, $J = 6.0$, 15.6 Hz, H-3 of *R*-form), 6.84 (0.5H, dd, $J = 6.0$, 15.6 Hz, H-3 of *S*-form), 7.38–7.42 (6H, m, H-ph), 7.49–7.52 (4H, m, H-ph).

Bis[(−)-MTPA] Ester of Methyl Ester of **3** (**3a**) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.86-1.61 (11H, m, H_3-10 and $CH_2 \times 4$), 3.52–3.57 (6H, m, $OCH_3 \times 2$), 3.74–3.76 (3H, m, CO_2CH_3), 5.04–5.13 (1H, m, H-9), 5.51–5.60 (1H, m, H-4), 5.85 (0.29H, d, $J = 15.6$ Hz, H-2 of 4*R*9*R*-form), 5.86 (0.21H, d, $J = 15.6$ Hz, H-2 of 4*R*9*S*-form), 5.96 (0.37H, d, $J = 15.6$ Hz, H-2 of 4*S*9*R*-form), 5.98 (0.13H, d, $J = 15.6$ Hz, H-2 of 4*S*9*S*-form), 6.76 (0.29H, dd, $J = 5.6$, 15.6 Hz, H-3 of 4*R*9*R*-form), 6.78 (0.21H, dd, $J = 5.6$, 15.6 Hz, H-3 of 4*R*9*S*-form), 6.81 (0.37H, dd, $J = 5.6$,

15.6 Hz, H-3 of 4*S*9*R*-form), 6.83 (0.13H, dd, $J = 5.6$, 15.6 Hz, H-3 of 4*S*9*S*-form), 7.39–7.43 (6H, m, H-ph), 7.50–7.53 (4H, m, H-ph).

(−)-MTPA Ester of Methyl Ester of **4** (**4a**) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.88 (3H, t, $J = 7.2$ Hz, H_3-10), 1.18–1.58 (12H, m, $CH_2 \times 6$), 2.58 (1H, dd, $J = 4.8$, 16.0 Hz, Ha-2), 2.65 (1H, dd, $J = 8.0$, 16.0 Hz, Hb-2), 3.56 (3H, m, OCH_3), 3.59 (3H, s, CO_2CH_3), 5.48 (1H, m, H-3), 7.37–7.43 (3H, m, H-ph), 7.52–7.54 (2H, m, H-ph).

(+)-MTPA Ester of Methyl Ester of **4** (**4b**) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.87 (3H, t, $J = 7.2$ Hz, H_3-10), 1.20–1.67 (12H, m, $CH_2 \times 6$), 2.61 (1H, dd, $J = 4.8$, 16.0 Hz, Ha-2), 2.70 (1H, dd, $J = 8.0$, 16.0 Hz, Hb-2), 3.55 (3H, m, OCH_3), 3.66 (3H, s, CO_2CH_3), 5.48 (1H, m, H-3), 7.37–7.41 (3H, m, H-ph), 7.52–7.54 (2H, m, H-ph).

Bis[(−)-MTPA] Ester of Methyl Ester of **5** (**5a**) ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.25 (2H, d, $J = 6.6$ Hz, H_3-10 of 3*R*9*S*-form), 1.33 (1H, d, $J = 6.6$ Hz, H_3-10 of 3*R*9*R*-form), 1.31–1.66 (10H, m, $CH_2 \times 5$), 2.55 (0.33H, dd, $J = 4.8$, 16.2 Hz, Ha-2 of 3*R*9*R*-form), 2.56 (0.67H, dd, $J = 4.8$, 16.2 Hz, Ha-2 of 3*R*9*S*-form), 2.63 (0.33H, dd, $J = 7.8$, 16.2 Hz, Hb-2 of 3*R*9*R*-form), 2.64 (0.67H, dd, $J = 7.8$, 16.2 Hz, Hb-2 of 3*R*9*S*-form), 3.51–3.56 (6H, m, $OCH_3 \times 2$), 3.59 (3H, m, CO_2CH_3), 5.10–5.14 (1H, m, H-9), 5.43–5.48 (1H, m, H-3), 7.37–7.42 (6H, m, H-ph), 7.50–7.53 (4H, m, H-ph).

Bis[(+)-MTPA] Ester of Methyl Ester of **5** (**5b**) ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.24 (1H, d, $J = 6.6$ Hz, H_3-10 of 3*R*9*R*-form), 1.32 (2H, d, $J = 6.6$ Hz, H_3-10 of 3*R*9*S*-form), 1.11–1.65 (10H, m, $CH_2 \times 5$), 2.58 (0.67H, dd, $J = 4.8$, 16.2 Hz, Ha-2 of 3*R*9*S*-form), 2.59 (0.33H, dd, $J = 4.8$, 16.2 Hz, Ha-2 of 3*R*9*R*-form), 2.68 (0.67H, dd, $J = 7.8$, 16.2 Hz, Hb-2 of 3*R*9*S*-form), 2.69 (0.33H, dd, $J = 7.8$, 16.2 Hz, Hb-2 of 3*R*9*R*-form), 3.53–3.56 (6H, m, $OCH_3 \times 2$), 3.66 (3H, m, CO_2CH_3), 5.08–5.13 (1H, m, H-9), 5.42–5.48 (1H, m, H-3), 7.36–7.40 (6H, m, H-ph), 7.51–7.54 (4H, m, H-ph).

Bis[(−)-MTPA] Ester of Methyl Ester of **6** (**6a**) ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 1.18–1.68 (14H, m, $CH_2 \times 7$), 1.25 (2.25H, d, $J = 6.0$ Hz, H_3-12 of 3*R*11*S*-form), 1.33 (0.75H, d, $J = 6.0$ Hz, H_3-12 of 3*R*11*R*-form), 2.57 (1H, dd, $J = 4.8$, 15.6 Hz, Ha-2), 2.64 (1H, dd, $J = 7.8$, 15.6 Hz, Hb-2), 3.52 (3H, m, OCH_3), 3.54 (3H, m, OCH_3), 3.59 (3H, s, CO_2CH_3), 5.11–5.16 (1H, m, H-11), 5.45–5.49 (1H, m, H-3), 7.38–7.41 (6H, m, H-ph), 7.52–7.53 (4H, m, H-ph).

Bis[(+)-MTPA] Ester of Methyl Ester of **6** (**6b**) ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H: 1.38–1.65 (14H, m, $CH_2 \times 7$), 1.25 (0.75H, d, $J = 6.0$ Hz, H_3-12 of 3*R*,11*R*-form), 1.33 (2.25H, d, $J = 6.0$ Hz, H_3-12 of 3*R*11*S*-form), 2.61 (1H, dd, $J = 4.8$, 15.6 Hz, Ha-2), 2.70 (1H, dd, $J = 8.4$, 15.6 Hz, Hb-2), 3.54 (3H, m, OCH_3), 3.57 (3H, m, OCH_3), 3.66 (3H, s, CO_2CH_3), 5.11–5.17 (1H, m, H-11), 5.44–5.49 (1H, m, H-3), 7.38–7.40 (6H, m, H-ph), 7.53–7.54 (4H, m, H-ph).

The ratio of *R* to *S* was determined by the intensity of the signals arising from both enantiomers.

Results

The total lipid fraction obtained from lyophilized royal jelly powder (1 kg) was separated repeatedly by silica gel column chromatography with various solvents to give the crude fatty acid fraction. This fraction was further separated with HPLC, and seven compounds **1**–**7** were isolated in pure form (see "Experimental Procedure").

The molecular formula of **1**, $C_{10}H_{18}O_4$, was established using high resolution FAB-MS. The ^{1}H-NMR spectrum of **1** showed *trans* olefinic (δ_H 5.80 and 6.89), oxymethine (δ_H 3.57), and allylic (δ_H 2.21) groups together with non-equivalent methylene signals. The ^{13}C-NMR spectrum showed one oxy methine (δ_C 73.1) and one oxy methylene (δ_C 67.3) units together with carboxyl (δ_C 171.4) and olefinic (δ_C 123.7 and 149.5) carbons. The COSY and HMBC spectra showed significant correlation peaks between H_2-10/H-9, H-2/C-1, and H-3/C-1, respectively. These findings revealed that **1** was 9,10-dihydroxy-2*E*-decenoic acid. The absolute configuration of C-9 was determined using the modified Mosher's method.[1]

Methylation of **1** with diazomethane, followed by treatment with (−)-MTPA chloride gave the (−)-MTPA ester (**1a**). The ^{1}H-NMR spectrum of **1a** showed two sets of signals in a ratio of *ca* 3:1 due to optical isomers. The $\Delta\delta$ values [δ (−)-MTPA ester – δ (+)-MTPA ester] of the major characteristic H-2 and H-3 protons were +0.011 and +0.013 ppm, respectively, whereas those of Ha-10 and Hb-10 were −0.025 and −0.076 ppm, respectively, indicating the 9*R* configuration. Hence, **1** was revealed to be a mixture of 9*R*,10- and 9*S*,10-dihydroxy-2*E*-decenoic acids in a ratio of *ca* 3:1 (Fig. 1).

The high resolution FAB-MS of **2** showed an $[M-H]^-$ ion peak at *m*/*z* 201.1118, consistent with the same molecular formula, $C_{10}H_{18}O_4$, as that of **1**, and indicating that **2** is an isomer of **1**. The ^{1}H-NMR spectrum of **2** showed similar *trans* olefinic (δ_H 5.97 and 6.92) signals due to protons H-2 and H-3, and equivalent H_2-10 (δ_H

[1] To establish whether the modified Mosher's method is applicable to compounds possessing a 1,2-diol, we firstly examined this method using 3-(octadecyloxy)-1,2*S*-propanediol. The $\Delta\delta$ values of the corresponding (−)- and (+)-MTPA esters were consistent with those predicted for *S* configuration.

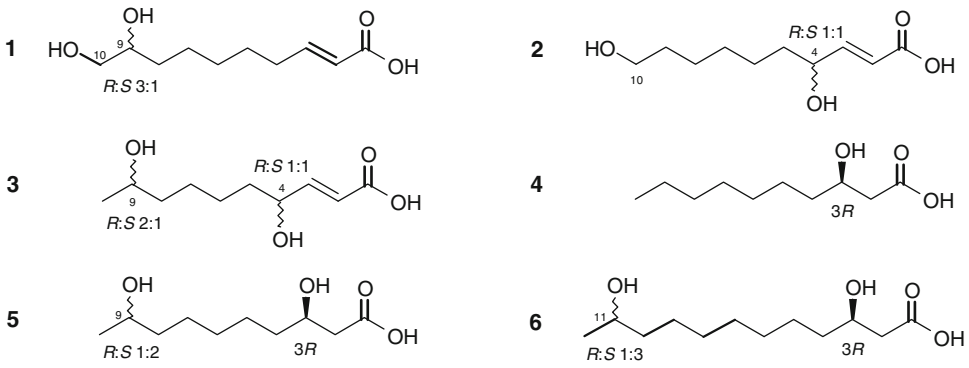


Fig. 1 Structures of chiral fatty acids **1–6**

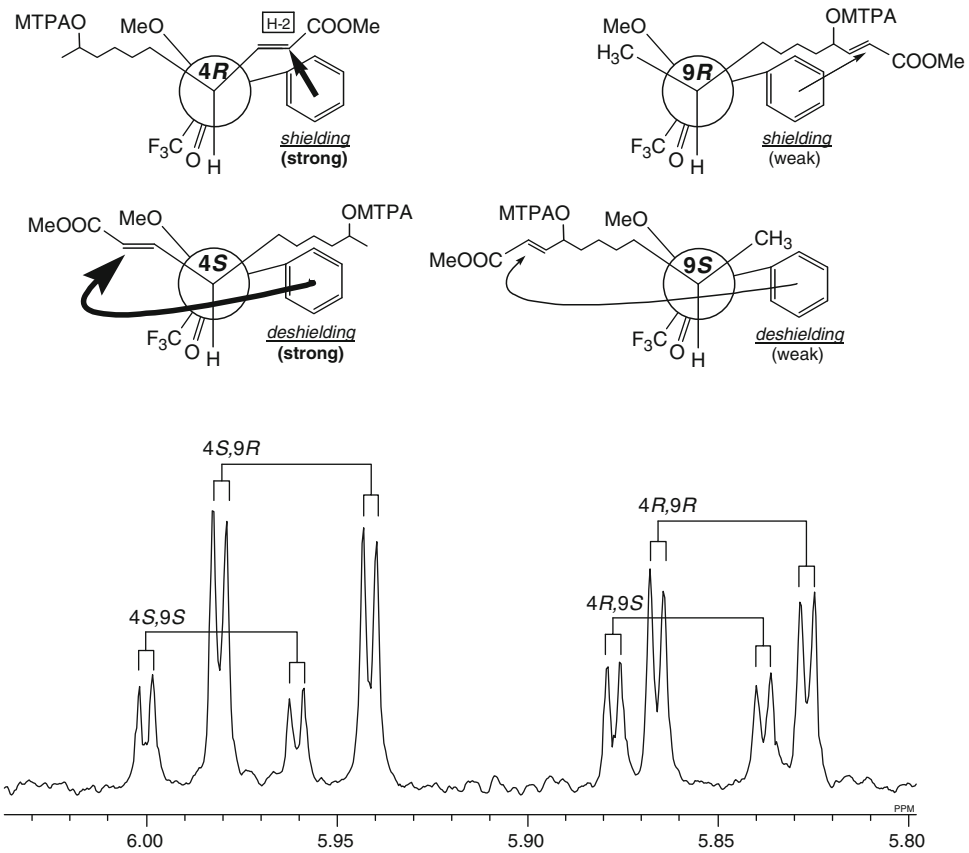
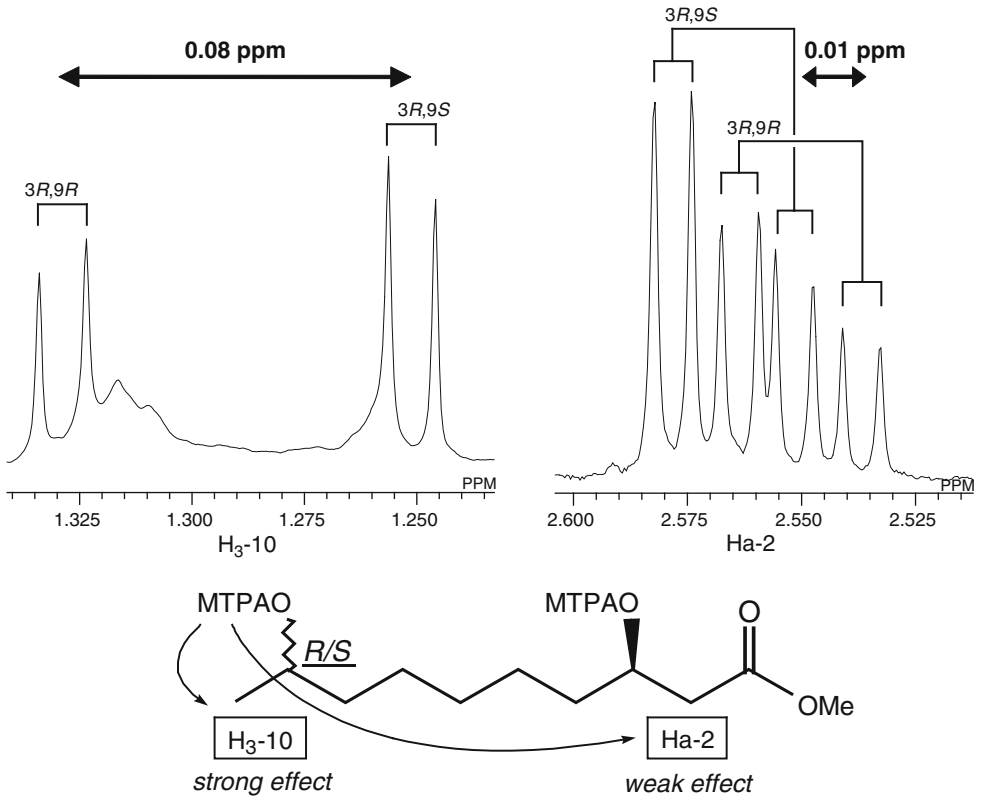


Fig. 2 ^{1}H-NMR spectrum of H-2 of **3a** and anisotropic effects of two MTPA groups

3.54) signals together with an oxy methine (δ_H 4.22) group coupled with H-3. These findings revealed that **2** was a positional isomer of **1**; that is, **2** differed in the position of the secondary hydroxyl group placed at C-4 instead of C-9 as in **1**, and hence the structure was determined to be 4, 10-dihydroxy-2*E*-decenoic acid. Methylation of **2** with diazomethane followed by esterification with (−)-MTPA chloride gave the (−)-MTPA ester (**2a**). The ^{1}H-NMR spectrum of **2a** showed, similar to that of **1a**, two sets of signals assignable to optical isomers, but, in a ratio of ca. 1:1. From these findings, it was determined that **2** was a mixture of 4*R*,10- and 4*S*,10-dihydroxy-2*E*-decenoic acids, in a ratio of ca. 1:1 (Fig. 1).

Compound **3** ($C_{10}H_{18}O_4$) was found, using two dimensional ^{1}H-NMR (COSY and HMBC) spectroscopic analyses, to be another isomer, 4,9-dihydroxy-2*E*-decenoic acid. The ^{1}H-NMR spectrum of (−)-MTPA ester (**3a**) showed, in contrast to those of **1a** and **2a**, four sets each of signals due to protons H-2 and H-3, indicating that **3a** is a mixture of four isomers (4*R*9*S*, 4*S*9*R*, 4*R*9*R*, and 4*S*9*S*). As can be seen in Fig. 2, the olefinic signal for proton (H-2) of the 4*R*9*R* isomer should appear at the highest field because of the anisotropic effect of the two phenyl groups of the MTPA esters (strong shielding by the near 4*R*-MTPA+ weak shielding by the far 9*R*-MTPA). The signal observed at the second highest field could be assigned as proton H-2

Fig. 3 ^{1}H-NMR spectrum of **5a**



of the 4*R*9*S* isomer (strong shielding by the near 4*R*-MTPA + weak deshielding by the far 9*S*-MTPA). Again, employing the modified Mosher's method as described above, olefinic signals due to the four isomers were successively discriminated, and thus **3** was revealed to be a mixture of 4*S*9*R*-, 4*R*9*R*-, 4*R*9*S*-, and 4*S*9*S*-dihydroxy-2*E*-decenoic acids, in a ratio of ca. 9:7:5:3. On comparison with those of **2a**, the $\Delta\delta$ values for protons H-2 and H-3 were consistent with the assignment of configurations (Fig. 4).

Analysis of the NMR and FAB-MS spectra of **4** ($C_{10}H_{20}O_3$) revealed the structure to be 3-hydroxydecanoic acid. The ^{1}H-NMR spectrum of (−)-MTPA ester (**4a**) showed, unlike those of **1a**–**3a**, signals due to one optical isomer. We then prepared the (+)-MTPA ester (**4b**). The $\Delta\delta$ value for protons H_2-2 showed that the configuration at C-3 was *R* (Fig. 4).

The ^{1}H-NMR spectrum of **5** ($C_{10}H_{20}O_4$) showed two oxy methine signals (δ 3.71 and 3.97), and similar to that of **4**, no signals due to olefinic protons. One oxy methine was coupled with the terminal methyl, H_3-10, and the other was coupled with the nonequivalent protons, H_2-2. From this information, it was clear that the two hydroxyl groups were located at the C-3 and C-9 positions, and hence the structure of **5** was defined as 3,9-dihydroxydecanoic acid. To clarify the absolute configuration, **5** was converted into the (−)-MTPA derivative (**5a**). The ^{1}H-NMR spectrum of **5a**, in contrast to that of **3a**, showed two signals for protons H_2-2, and accordingly **5** was regarded as being a mixture of enantio- and/or diastereoisomers. We then prepared the (+)-MTPA ester (**5b**). If compound **5** was a mixture of enantiomers [(3*R*9*R* and 3*S*9*S*) or (3*R*9*S* and 3*S*9*R*)], the chemical shifts of all signals arising from the (+)- and (−)-MTPA esters would reverse each other, and similar to those of **2a**, the $\Delta\delta$ values would have the opposite sign (Fig. 4). However, the signals in the spectra of **5a** and **5b** gave different chemical shifts. Based on these findings, compound **5** was revealed to be a mixture of diastereomers. The anisotropic effect of the phenyl group of the MTPA ester decreases with increasing numbers of methylene groups between it and the chiral center. In the ^{1}H-NMR spectrum of **5a**, the difference in the chemical shifts for the Ha-2 group (0.01 ppm) was less than that for the H_3-10 group (0.08 ppm) (Fig. 3). Therefore it was determined that the C-9 position has two (*R* and *S*) configurations, while the C-3 carbon has only one. Since the $\Delta\delta$ values for the H_2-2 protons in both the (+)- and (−)-MTPA esters showed the same minus sign, the configuration of the C-3 position could be assigned as *R*. On the basis of the results obtained above, compound **5** was found to be a mixture of 3*R*9*R*- and 3*R*9*S*-dihydroxydecanoic acids, in a ratio of ca. 1:2.

The ^{1}H-NMR spectrum of **6** was quite similar to that of **5**, including signals due to two methines and one terminal methyl group. Its negative ion FAB-MS showed

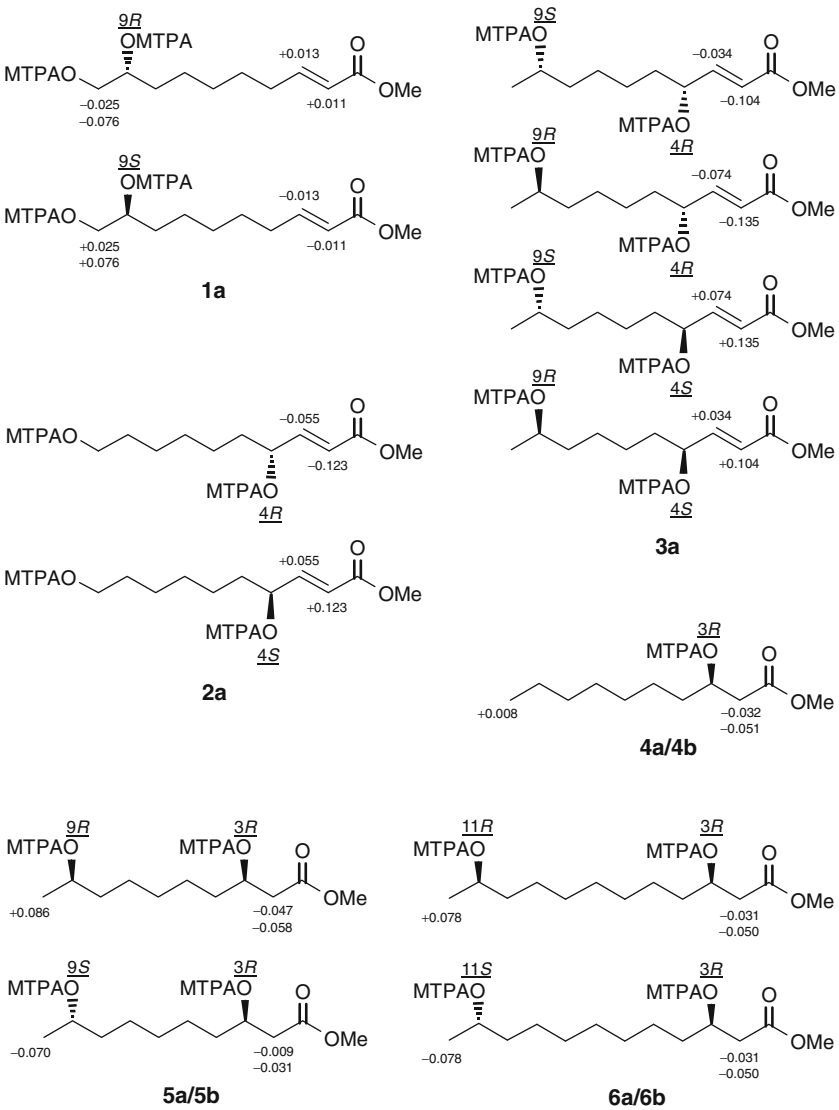


Fig. 4 $\Delta\delta$ values of ^{1}H-NMR chemical shift difference (ppm) for MTPA esters

an $[M-H]^-$ ion peak at *m/z* 231, which was 28 mass units more than that of **5**, while in the ^{13}C-NMR spectrum, two additional methylene signals were observed. From these findings, **6** was revealed to be 3,11-dihydroxydodecanoic acid. The absolute configurations of the two secondary hydroxy groups were determined in the same manner as described for **5**. With the $\Delta\delta$ values and signs of the diagnostic signals, similar to those of **5**, the C-3 position was determined to be the *R* configuration, while the C-11 carbon was found to have both forms (Fig. 4). Accordingly, **6** was identified as a mixture of the isomers, 3*R*11*R* and 3*R*11*S* in a ratio of 1:3.

Discussion

Seven hydroxy fatty acids were isolated in the pure state from the royal jelly of honeybees (*Apis mellifera*). Compounds **1**–**3** and **5** are the first hydroxy fatty acids isolated from royal jelly, although **5** has already been detected in human urine by Tserng et al. [8]. Compound **6**, was recently isolated from royal jelly, by Melliou and co-workers [9], however, the absolute configuration remains unclear. Similar to 9-HDA in royal jelly, the chiral acids obtained in this study are revealed to be mixtures of enantio- or diastereo-isomers (Fig. 4).

It should be noted that the absolute configurations of the secondary hydroxy groups located at C-3 of the chiral acids are all the *R* form, while those at positions C-4, C-9, and C-11 have both forms.

A recent study of the genome sequencing of the honeybee, *A. mellifera*, demonstrated that the species has more odorant receptors than other insects, such as *Drosophila melanogaster*, *Anopheles gambiae*, and *Bombyx mori*, indicating that the honeybee lifestyle involves enhanced pheromone communication [10].

Considering the above information together with the relationship between 9-ODA and 9-HDA, it is believed that the chiral acids isolated in the present study are precursors of unknown semiochemicals, and that their corresponding oxo-forms may play physiologically important functions and roles in the hierarchy of honeybee colonies. We are now synthesizing these oxo-derivatives in order to examine the biological activities of these compounds.

Acknowledgments The authors wish to thank Dr. K. Hashimoto, Institute for Bee Products & Health Science, for supplying the royal jelly. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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