

ORIGINAL ARTICLE

Paleic acid, a fatty acid from *Paenibacillus* sp.: taxonomy, fermentation, isolation, structure determination, and anti-*Mannheimia* and *Pasteurella* activity

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Paleic acid (**1**), an antibiotic, was obtained from a fermentation broth of *Paenibacillus* sp. BMK771-AF3. The compound is a fatty acid (9*Z*,16*R*)-16-hydroxy-9-octadecenoic acid ((*R*)-16-hydroxyoleic acid), whose isolation required protection of its polar functional groups. Mosher esters of paleic acid yielded information on the absolute configuration of secondary alcohol, and well-resolved ¹H NMR peaks around the double bond suggested that olefin adopted a *Z* geometry. Paleic acid showed potent antibacterial activity and narrow spectrum against *Mannheimia haemolytica* with MIC values ranging between 0.78 and 1.56 μg ml⁻¹.

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INTRODUCTION

Isolates of *Mannheimia haemolytica*¹ and *Pasteurella multocida*² are representative pathogens that cause a wide range of diseases in food-producing animals, such as cattle, poultry, pigs and rabbits. Especially, *M. haemolytica* is known as the primary bacterial pathogen of bovine respiratory disease, and antimicrobial agents are powerful tools for controlling infection. In fact, Tilmicosin,^{3,4} a derivative of desmycosin, was found to be effective against bovine respiratory disease and was introduced into the US market in 1990. Afterwards, a variety of antibiotics including β-lactams, macrolides, tetracycline and sulfonamides were shown to be effective against infectious diseases caused by these pathogens.^{5,6} One of the most successful veterinary drugs is a triamilide class of macrolide, tulathromycin⁷ (the active ingredient of Draxxin Injectable Solution launched from Pfizer Animal Health, New York, NY, USA), which has been approved for use in the treatment and prevention of bovine respiratory disease and the treatment of swine respiratory disease in the European Union and the United States of America. However, the prevalence of antimicrobial resistance among the pathogens⁸ requires the development of a new class of antibiotics with unique structure and/or mode of action.

Herein we describe the isolation and structural elucidation of oxygenated unsaturated C18-fatty acid **1** (paleic acid, (9*Z*,16*R*)-16-

hydroxy-9-octadecenoic acid, (*R*)-16-hydroxyoleic acid), which is produced by these microorganisms and is depicted in Figure 1. Paleic acid displays antibacterial activities against a number of strains of *M. haemolytica* and *P. multocida*. This is the first report of a fatty acid derivative being effective toward these pathogens.

Moreover, (*R*)-16-hydroxylinoleic acid (**2**) was also isolated along with **1**, albeit modest biological activity. Compounds **1** and **2** already reported as the components of products of biotransformation using oleic acid and linoleic acid as substrates.^{9,10} However, spectroscopic characterization and investigation on biological activity of these compounds has not been carried out.

RESULTS AND DISCUSSION

Isolation of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**)

The fermentation broth contained **1** and **2** along with other fatty acids of high structural similarity. With numerous efforts using reversed-phase HPLC, centrifugal partition chromatography (CPC), silica-gel chromatography and gel filtration, the fatty acids could not be separated completely. The NMR and MS analyses of the partially purified sample suggested, by bioassay, that many of the antibacterial substances had aliphatic chains, carboxyl groups, and hydroxyl or keto groups. The compounds of interest had no potent UV absorption,

which made their detection during the chromatographic procedure troublesome.

Therefore, we decided to convert the mixture of fatty acids to less polar derivatives that are easily purified on silica-gel and by HPLC. As carboxyl groups tend to give tailing peaks in various chromatographic techniques, the functional group was masked as diphenylmethyl ester. To mask the hydroxyl group, another polar functional group, the crude material was treated with 4-nitrobenzoyl chloride to give the corresponding 4-nitrobenzoate (Scheme 1). The derivatives were also expected to be easily detectable because of intense UV absorption of the newly introduced aromatic moieties.

In fact, fully protected derivatives of paleic acid (**5**) and (*R*)-16-hydroxylinoleic acid (**6**) could be isolated in pure form after successive silica-gel column chromatography and HPLC. Each derivative was independently deprotected under alkaline conditions and subse-

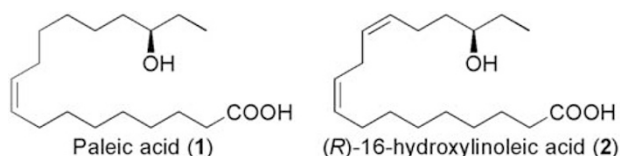
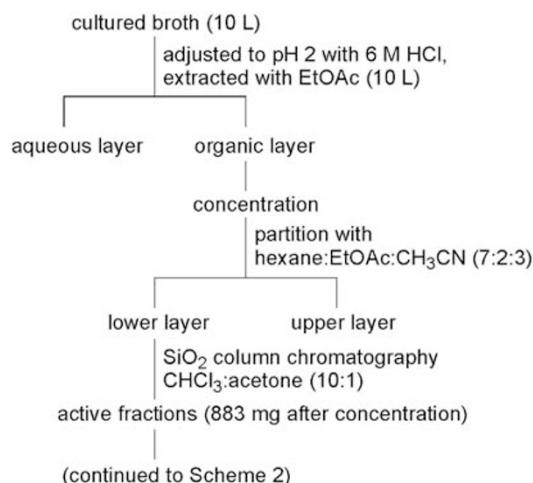


Figure 1 Structure of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**).



Scheme 1 Isolation of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**).

quently purified with CPC to give paleic acid (**1**, 15 mg) and (*R*)-16-hydroxylinoleic acid (**2**, 15 mg).

Although many other fatty acids (judged by mass spectrum) were found in the bioactive fractions obtained by the first silica-gel column chromatography, these compounds except for **1** and **2** could not be separated as analytically pure samples. Further purification of the rest of the fatty acids is currently ongoing.

Structure determination

The physicochemical data of the paleic acid (**1**) are summarized in Table 1. The compound was obtained as a colorless oil ($[\alpha]_D^{28} - 5.00^\circ$, c 0.2, CHCl_3), and its molecular formula was determined to be $\text{C}_{18}\text{H}_{34}\text{O}_3$ by HRFABMS ($(M-H)^-$, m/z 297.2426, $\Delta 0.2$ mDa), revealing a degree of unsaturation of 2. The compound displayed end absorption in its UV spectrum and a characteristic absorption at 1712 cm^{-1} in its IR spectrum.

As shown in Table 2, the ^1H , ^{13}C , DEPT, HMQC, ^1H - ^1H COSY, TOCSY and HMBC spectra of **1** suggested that the structure of the compound is an unsaturated linear fatty acid composed of 18 carbons, with one hydroxyl group at the C16 position and one olefin moiety (Figure 2).

The UV absorption data indicated that paleic acid has no aromatic moiety within its structure. The ^{13}C signal at 178.3 p.p.m., IR absorption signal at 1712 cm^{-1} , and the result of treatment with trimethylsilyldiazomethane (a synthetic intermediate of the Mosher derivatives of paleic acid) strongly suggested that the compound contains a carboxyl group.

Compound **1** displayed the characteristic DEPT charts with 13 of 18 carbon signals corresponding to CH_2 . The other carbon signals correspond to one quaternary carbon of a COOH group, two CH and one CH_3 . Two protons correlated to one methine carbon peak (130.0 p.p.m.) in the HMQC experiment, suggesting that the two carbon signals arising from the disubstituted olefin completely overlap.

Figure 2 summarizes the multidimensional NMR experiments of **1**. The HMQC and HMBC experiments revealed that the CH_3 was separated by one CH_2 from the CH carbon at 73.5 p.p.m. The proton connected to the methine has a chemical shift of 3.54 p.p.m. On the basis of the molecular formula of **1**, the substituent must be OH.

Although the structure of **1** was determined as 16-hydroxyoctadecenoic acid, the position of double bond could not be defined. To this end, an EI-MS fragmentation analysis of the pyrrolidine amide derivative of **1** was conducted according to reported procedures.¹¹ The

Table 1 Physicochemical data of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**)

	Paleic acid (1)	(<i>R</i>)-16-hydroxylinoleic acid (2)
Appearance	Colorless oil	Colorless oil
Molecular formula	$\text{C}_{18}\text{H}_{34}\text{O}_3$	$\text{C}_{18}\text{H}_{32}\text{O}_3$
<i>HRFABMS</i> (neg, m/z)		
Found:	297.2426 ($M-H$) ⁻	295.2270 ($M-H$) ⁻
Calcd:	297.2424 (as $\text{C}_{18}\text{H}_{33}\text{O}_3$)	295.2268 (as $\text{C}_{18}\text{H}_{31}\text{O}_3$)
$[\alpha]$	-5.00° (c 0.2, CHCl_3)	-0.48° (c 0.14, acetone) ^a
UV λ_{max} (CHCl_3)	End absorption	End absorption
IR ν_{max} (KBr) cm^{-1} ^a	3448, 2929, 2854, 1712, 1629, 1463, 1409, 1118, 966, 757	3415, 2929, 2856, 1712, 1461, 1411, 1116, 970, 725
TLC (R_f value) ^b	0.42	0.42
Color reaction: positive	Molybdophosphoric acid-sulfuric acid	

^a $[\alpha]_D^{28}$ was recorded.

^bPlate: Silica gel 60 F₂₅₄ (MERCK), $\text{CHCl}_3/\text{EtOH}=20:1$.

EIMS experiments of the pyrrolidine amide derivatives of the fatty acids showed characteristic series of fragments resulting from cleavage between each carbon-carbon single bond but not at a double bond. The observed mass numbers of the positive fragmentation ions containing the pyrrolidine moiety, when the methodology was applied to **1**, is detailed in Supplementary information (See Supplementary Figure S1). As a result, only the fragment ions bisected between C17 and C18 and between C9 and C10 were not detected. Because a C=C double bond at C17/C18 was ruled out, on the basis of the extensive NMR study described above, the mass experiment clearly demonstrated that the olefin is located between the C9 and C10 positions. However, the geometry of the double bond could not be identified at this point because the chemical shifts of the two olefinic protons of **1** in the ¹H NMR spectrum were too close and the coupling constant between these protons could not be determined.

The absolute configuration of the secondary alcohol was unambiguously determined as *R* by a modified Mosher method. Supplementary Figure S2 in Supplementary information summarizes the differences of the chemical shifts of (*S*)- α -methyl- α -trifluoromethylphenylacetyl-paleic acid methyl ester and the (*R*)- α -methyl- α -trifluoromethylphenylacetyl-congener.

The peaks corresponding to H9 and H10 (5.32 and 5.35 p.p.m.) of the (*S*)- α -methyl- α -trifluoromethylphenylacetyl ester were separated

presumably because of the magnetic anisotropic effect of the benzene ring of the Mosher ester moiety. A ¹H NMR decoupling experiment of the (*S*)- α -methyl- α -trifluoromethylphenylacetyl ester with irradiation at 1.31 and 1.98 p.p.m. (corresponding to H7 and H12, and H8 and H11, respectively) clearly showed a coupling constant of 10.6 Hz between H9 and H10, which is a typical value for the *Z* isomer. Eventually, the structure of **1** was unequivocally determined as (9*Z*,16*R*)-16-hydroxy-9-octadecenoic acid. The structure of **1** was also confirmed by chemical synthesis.¹²

The ¹H NMR spectra obtained from **2** are similar to those of **1**. With molecular formula of C₁₈H₃₂O₃, the structure of **2** was predicted to be a C18 fatty acid with two olefinic double bonds and one hydroxyl group. In fact, the ¹³C NMR spectrum of **2** has four peaks corresponding to olefinic carbons (CH at 127.9, 128.7, 129.5 and 130.2 p.p.m. as confirmed by DEPT). As shown in Figure 2, the planar structure of **2** was determined to be 16-hydroxylinoleic acid by DEPT, HMQC and HMBC spectra. Extensive decoupling experiments with **6** clearly showed that each olefin adopts a *Z* geometry. Finally, the modified Mosher method was used to determine the *R* absolute configuration of the C16 center (Supplementary Figure S2). The two sets of two proton peaks adjacent to the olefins (2.72/1.97 and 2.72/2.15 p.p.m.) were irradiated to give simplified ¹H NMR spectra around the olefinic region. Both of the coupling constants between H9 and H10 and between H12 and H13 were 10.7 Hz, which is typical of *Z* olefin. Therefore, the structure of **2** was determined to be (*R*)-16-hydroxylinoleic acid.

Table 2 ¹H- and ¹³C-NMR data of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**)

Position	Paleic acid (1)			(<i>R</i>)-16-hydroxylinoleic acid (2)		
	¹³ C (δ)	¹ H (δ)	Multiplicity, J (Hz)	¹³ C (δ)	¹ H (δ)	Multiplicity, J (Hz)
1	178.2			178.2		
2	33.9	2.34	t, 7.4	33.8	2.34	t, 7.4
3	24.7	1.63	m	24.7	1.64	m
4	29.0 ^a	1.31	m	^b	1.34	m
5	29.1 ^a	1.31	m	^b	1.34	m
6	29.1 ^a	1.31	m	^b	1.34	m
7	29.4 ^a	1.31	m	^b	1.34	m
8	27.1	1.98	m	27.1	2.05	m
9	129.9	5.35	m	130.2	5.38	m
10	129.9	5.35	m	127.9	5.33	m
11	27.2	1.98	m	25.6	2.80	m
12	29.6 ^a	1.31	m	128.7	5.38	m
13	29.8 ^a	1.31	m	129.5	5.39	m
14	25.5	1.44	m	23.6	2.18	m
15	36.8	1.44	m	36.6	1.52	m
16	73.5	3.54	m	73.1	3.57	m
17	30.1	1.44, 1.53	m	30.2	1.50	m
18	9.9	0.93	t, 7.5	9.9	0.94	t, 7.5

^aThese assignments are interchangeable.
^bEither 28.9 or 29.4 (overlap).

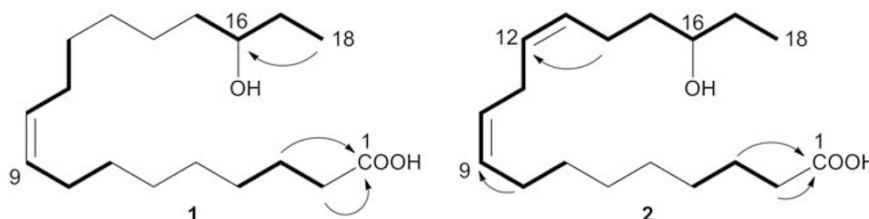


Figure 2 COSY (bold line) and selected HMBC correlation (arrows) of **1** and **2**.

Biological activity

The antibacterial activities of the isolated compounds were examined toward a panel of bacteria covering various strains of *M. haemolytica* and *P. multocida*, and representative pathogens of human disease. The results from these experiments are summarized in Table 3. Paleic acid

Table 3 Antibacterial activity of paleic acid (**1**) and (*R*)-16-hydroxylinoleic acid (**2**)

Test organisms	MIC ($\mu\text{g ml}^{-1}$)		
	1	2	Tilmicosin
<i>M. haemolytica</i> N791	0.78	12.5	1.56
<i>M. haemolytica</i> N811	0.78	6.25	12.5
<i>M. haemolytica</i> S801	1.56	12.5	3.13
<i>P. multocida</i> No.6	12.5	50	3.13
<i>P. multocida</i> Kobe	>50	>50	3.13
<i>P. multocida</i> TS-8	6.25	50	3.13
<i>P. multocida</i> M-17	>50	>50	3.13
<i>H. somni</i> 23N2359	>50	50	6.25
<i>A. pleuroneumoniae</i> NB001	6.25	12.5	6.25
<i>E. coli</i> NIHJ	>50	>50	25
<i>K. pneumoniae</i> PCI 602	>50	>50	12.5

(1) showed antibacterial activity toward several strains from *M. haemolytica* and *P. multocida* with MIC value ranging from 0.78 to 1.56 $\mu\text{g ml}^{-1}$, and from 6.25 to $>50 \mu\text{g ml}^{-1}$, respectively. Notably, 1 displayed no activity against *Escherichia coli* and *Klebsiella pneumoniae*, suggesting that 1 is an antibiotic selectively effective against pathogens of bovine respiratory disease. On the other hand, 2 showed only moderate antibacterial activity against the tested organisms. In addition, 1 exhibited no activity toward representative strains of *Staphylococcus aureus* (Gram-positive bacteria).

A preliminary examination showed that 1 displayed practically no acute toxicity toward ICR mice by i.v. administration. Further studies, including *in vivo* tests and structure–activity relationships of synthetic analogs, are currently underway.

EXPERIMENTAL PROCEDURE

General

The purification steps before the treatment of diazodiphenylmethane were monitored with antimicrobial activity against *M. haemolytica* N811 using microbroth dilution method. The purification steps after the treatment of diazodiphenylmethane were monitored with analytical TLC using precoated (0.25 mm) silica-gel plates (Silica Gel 60 F₂₅₄, cat. no. 1.05715 Kieselgel 60 F₂₅₄, Merck KGaA, Darmstadt, Germany). Column chromatography was conducted with neutral silica-gel (Wakogel C-200, Wako Pure Chemical Industries, Osaka, Japan). Centrifugal liquid–liquid partition chromatography (CPC) was performed using a CPC240 system (Senshu Scientific, Tokyo, Japan). Optical rotations were obtained on a JASCO P1030 spectropolarimeter (JASCO, Hachioji, Japan) using a micro-cell (light path of 10 cm). A Hitachi High-Tech U-2800 spectrophotometer (Hitachi, Tokyo, Japan) was used to record UV spectra, and IR spectra were measured using a Horiba FT-210 spectrometer (Horiba, Kyoto, Japan). NMR spectra were recorded using a JEOL JMN ECA 600 (600 MHz) spectrometer (JEOL, Akishima, Japan). Chemical shifts (δ) are given in parts per million (p.p.m.), based on solvents as internal references, and coupling constants (*J*) are presented in Hz. The abbreviations t and m indicate triplet and multiplet, respectively. HR-MS were taken under electron

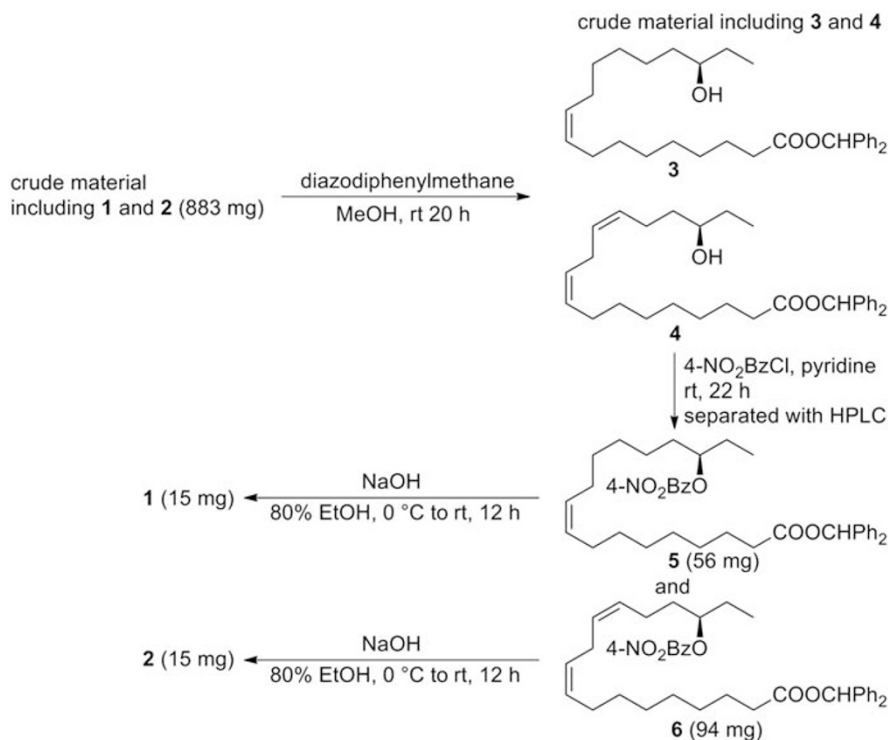
spray ionization conditions by a JEOL JMS-T100LC mass spectrometer (time of flight mass spectrometer) or a Thermo Fisher Scientific LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). EI mass spectrum was recorded using JEOL JMS-SX102 mass spectrometer.

Taxonomy

Strain BMK771-AF3 was isolated from a soil sample collected at Kitaazumi-gun, Nagano, Japan, after the following protocol; the sample was treated by dry heating (90 °C, 1 h), suspended in sterile water, and spread onto glycerol-arginine-salt agar,¹³ which is supplemented with cycloheximide and nystatin as the selective inhibitors for fungi. The strain is Gram-positive, spore-forming and rod-shaped bacterium (0.7 to 0.8 \times 1.6 to 2.5 μm). The cells of this strain are motile by means of peritrichous flagella. Central oval endospores (0.45 to 0.5 \times 1.0 μm) were observed in swollen sporangia. Colonies were colorless to light brownish gray, opaque and circular on nutrient agar. Growth occurred at 20–37 °C (optimally at 30 °C). The DNA G+C content of strain BMK771-AF3 was 44.9 mol%. The 16S ribosomal RNA gene sequence (1483 bp, positions 24–1497, *E. coli* numbering system) of the strain showed high identity with members of genus *Paenibacillus*,¹⁴ such as *P. taichungensis* (BCRC 17757T EU179327, 1469/1475 bp, 99%), *P. pabuli* (NRRL NRS-924T AB045094, 1473/1485 bp, 99%) and *P. xylanilyticus* (XIL14T AY427832, 1475/1484 bp, 98%). These morphological characteristics and genetic analysis of the strain BMK771-AF3 suggested that the strain belongs to genus *Paenibacillus*. Therefore, the strain was designated as *Paenibacillus* sp. BMK771-AF3. Strain BMK771-AF3 has been deposited in the National Institute of Advanced Industrial Science and Technology, the International Patent Organism Depository, Tsukuba, Japan, under the accession no. FERM P-16999.

Fermentation

A slant culture of the paleic acid-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of 2.0% galactose, 2.0% dextrin, 1.0% Bacto Soytone (enzymatic digest of soybean meal, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.5% corn steep liquor (byproduct of the corn wet-milling process, Oji Cornstarch, Tokyo,



Scheme 2 Partial purification of paleic acid (1) and (R)-16-hydroxylinoleic acid (2).

Japan), 0.1% (NH₄)₂SO₄ and 0.2% CaCO₃ in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 r.p.m.) at 30 °C for 18 h. The seed culture (2.5 ml) was transferred into a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium consisting of 2.0% corn starch, 1.0% glucose, 0.5% corn steep liquor, 1.0% corn gluten meal (byproduct of the corn wet-milling process, Nihon Shokuhin Kako, Tokyo, Japan), 0.3% NaCl, 0.25% NH₄Cl and 0.6% CaCO₃ in deionized water. The fermentation was carried out on a rotary shaker (180 r.p.m.) at 27 °C for 3 days.

Isolation and purification

The fermentation broth (101, 91 flasks) was acidified with 6 M HCl (adjusted to pH 2) and was extracted with 101 of EtOAc. After centrifugation of the resulting mixture, the organic layer was concentrated to dryness. The resulting residue was partitioned with a solvent system of hexane/EtOAc/CH₃CN (7/2/3). The lower layer was concentrated and subjected to silica-gel column chromatography and then eluted with CHCl₃/acetone (10/1). The bioactive fractions were collected and evaporated to produce an oil (883 mg) containing **1** and **2** (Scheme 2).

The crude material was dissolved in 50 ml MeOH and treated with diazodiphenylmethane (860 mg, 4.43 mmol). The reaction system was stirred at ambient temperature for 20 h and concentrated *in vacuo*. The residue was purified with silica-gel column chromatography (200 g) eluted with toluene/EtOAc (30/1) to give diphenylmethyl esters of **1** and **2** (**3** and **4**, respectively). Evaporation of the solvents produced 423 mg of crude products.

4-Nitrobenzoyl chloride (500 mg, 2.69 mmol) was added to the residue in 25 ml of pyridine, and the resulting mixture was stirred at room temperature for 22 h under argon. After the reaction system was concentrated *in vacuo*, the residue was suspended in hexane/MeOH (6/1), and the insoluble material was filtered off. The filtrate was evaporated, purified successively with silica-gel (20 g) column chromatography (hexane/EtOAc=20/1) and HPLC (PEGASIL Silica (Senshu Scientific) 120–5, 20×250 mm; hexane/EtOAc=98/2; flow rate 8 ml min⁻¹) to give 4-nitrobenzoyl derivatives of paleic acid diphenylmethyl ester (**5**, 56 mg), and (*R*)-16-hydroxylinoleic acid diphenylmethyl ester (**6**, 94 mg).

Each sample was independently dissolved in a mixture of 4 ml of EtOH and 0.5 ml of H₂O, treated with 0.5 ml of 1 M NaOH at 0 °C, allowed to reach room temperature, and stirred for 12 h. The solutions were acidified to pH 3 by 1 M HCl, and then concentrated to dryness. The residues were purified with CPC (hexane/EtOAc/CH₃CN=5/1/4, ascending mode) to yield paleic acid (**1**, 15 mg) and (*R*)-16-hydroxylinoleic acid (**2**, 15 mg) (Scheme 1).

Structure determination

Extensive NMR experiments (¹H, ¹³C, DEPT, HMQC, ¹H-¹H COSY, TOCSY and HMBC) were performed on **1** and **2** to determine the planner structure of these compounds (Figure 2). Other physicochemical data of **1** and **2** were collected and summarized in Table 1.

Biological activity

The MIC determinations were performed by the serial agar dilution method recommended by the Japan Society of Chemotherapy.¹⁵ Brain Heart Infusion agar (Difco, Becton, Dickinson and Company) was used for *M. haemolytica* and *P. multocida*, *E. coli* and *K. pneumoniae*, and Mueller-Hinton agar (Difco) supplemented with 5% Fildes enrichment (BBL) was used for *Actinobacillus pleuropneumoniae* and *Histophilus somni*. The MIC values were observed

after incubation for 17 h at 37 °C, for *P. multocida* and *M. haemolytica*, and after incubation in air-5% CO₂ at 37 °C for 17 h, for *A. pleuropneumoniae* and *H. somni*. The MIC values are listed in Table 3. Paleic acid (**1**) showed MIC values of 100, 100 and >100 µg ml⁻¹ against *S. aureus* FDA209P, Smith and MS9610, respectively.

Acute toxicity

To 4-week-old female ICR mice, **1** (2.0, 1.0, 0.5, 0.25 and 0.125 mg per mouse, two mice were tested for each dosage) dissolved in 10% DMSO/saline (colorless, transparent) was injected i.v. Mortality of the mice was observed for 2 weeks. All the mice survived during the examination, which means LD₅₀ of **1** is >100 mg kg⁻¹.

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- 1 Rice, J. A., Carrasco-Medina, L., Hodgins, D. C. & Shewen, P. E. *Mannheimia haemolytica* and bovine respiratory disease. *Anim. Health Res. Rev.* **8**, 117–128 (2007).
- 2 Dabo, S. M., Taylor, J. D. & Confer, A. W. *Pasteurella multocida* and bovine respiratory disease. *Anim. Health Res. Rev.* **8**, 129–150 (2007).
- 3 Ose, E. E. & Tonkinson, L. V. Single-dose treatment of neonatal calf pneumonia with the new macrolide antibiotic tilmicosin. *Vet. Res.* **123**, 367–369 (1988).
- 4 Gorham, P. E. *et al.* Tilmicosin as a single injection treatment for respiratory disease of feedlot cattle. *Can. Vet. J.* **31**, 826–829 (1990).
- 5 Yoshimura, H., Ishimaru, M., Endoh, Y. S. & Kojima, A. Antimicrobial susceptibility of *Pasteurella multocida* isolated from cattle and pigs. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **48**, 555–560 (2001).
- 6 Norcia, L. J. L. *et al.* *In vitro* microbiological characterization of a novel azalide, two triamides and an azalide ketal against bovine and porcine respiratory pathogens. *J. Antibiotics* **57**, 280–288 (2004).
- 7 Evans, N. A. Tulathromycin: an overview of a new triamidine antimicrobial for livestock respiratory disease. *Vet. Ther.* **6**, 83–95 (2005) and references cited therein.
- 8 Hendriksen, R.S. *et al.* Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. *Acta. Vet. Scand.* **50**, 28–37 (2008).
- 9 Lanser, A.C., Plattner, R. D. & Bagby, M. O. Production of 15-, 16-, 17-hydroxy-9-octadecenoic acids by bioconversion of oleic acid with *Bacillus pumilus*. *J. Am. Oil Chem. Soc.* **69**, 363–366 (1992).
- 10 Brodowsky, I. D. & Oliw, E. H. Metabolism of 18:2(*n*-6), 18:3(*n*-3), 20:4(*n*-6) and 20:5(*n*-3) by the fungus *Gaeumannomyces graminis*: identification of metabolites formed by 8-hydroxylation and by w2 and w3 oxydation. *Biochim. Biophys. Acta.* **1124**, 59–65 (1992).
- 11 Andersson, B.Å. & Holman, R. T. Pyrrolidides for mass spectrometric determination of the position of the double bond in monounsaturated fatty acids. *Lipids* **9**, 185–190 (1974).
- 12 Watanabe, T. *et al.* Synthesis of paleic acid, antimicrobial agent effective against *Pasteurella* and *Mannheimia*, and its structurally related derivatives. Paper in preparation.
- 13 El-Nakeeb, M. A. & Lechevalier, H. A. Selective isolation of aerobic actinomycetes. *Appl. Microbiol.* **11**, 75–77 (1963).
- 14 Ash, C., Priest, F. G. & Collins, M. D. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie. Van. Leeuwenhoek.* **64**, 253–260 (1993).
- 15 Japan Society of Chemotherapy. Method of MIC determination. Final report from the committee on antimicrobial susceptibility testing, Japanese Society of chemotherapy, on the agar dilution method. *Chemother.* **56**, 49–57 (2008).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)