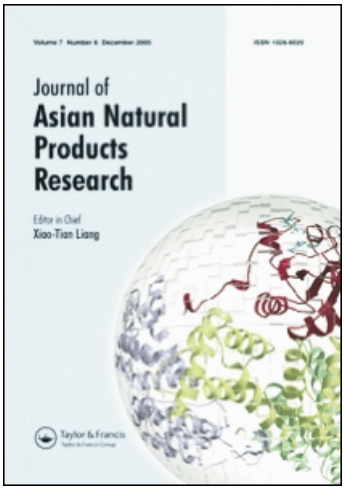

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Nian-Yun Yang[a]; Kun Li[a]; Yi-Fang Yang[a]; Yong-Hui Li[a]
[a] Department of Traditional Chinese Medicine, Shanghai Institute of Pharmaceutical Industry, Shanghai, China

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Aromatase inhibitory fatty acid derivatives from the pollen of *Brassica campestris* L. var. *oleifera* DC.

Nian-Yun Yang, Kun Li, Yi-Fang Yang* and Yong-Hui Li

Department of Traditional Chinese Medicine, Shanghai Institute of Pharmaceutical Industry, Shanghai, China

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Two new fatty acid derivatives, 9*Z*,12*Z*,15*Z*-octadecatrienoic acid sorbitol ester (**1**) and (10,11,12)-trihydroxy-(7*Z*,14*Z*)-heptadecadienoic acid (**2**), were isolated from the pollen of *Brassica campestris* L. var. *oleifera* DC., along with the four known fatty acid derivatives, 9*Z*,12*Z*,15*Z*-octadecatrienamide, *N*-(2-hydroxyethyl) (**3**), hexadecanoic acid sorbitol ester (**4**), 15,16-dihydroxy-9*Z*,12*Z*-octadecadienoic acid (**5**), and 9*Z*,12*Z*,15*Z*-octadecatrienoic acid 2,3-dihydroxypropyl ester (**6**). Their structures were elucidated by extensive spectroscopic analysis, including 1D- and 2D-NMR as well as HR-ESI-MS experiments. All compounds were tested using a noncellular aromatase assay, and the results showed that some compounds possessed strong inhibitory activity.

Keywords: *Brassica campestris* L. var. *oleifera* DC.; fatty acid derivatives; aromatase inhibitory activity

1. Introduction

Rape pollen is a natural product that has great nutritional value to human beings because of the medical properties attributed to pollen loads. Fatty acids and their derivatives are commonly found in natural product extracts, especially from rape pollen, and they play an important role in the regulation of trace levels of a variety of physiological and biological functions. Fatty acids have been shown to interfere with some of these assays including COX-1/COX-2 [1,2], adenosine A_1 receptor binding [3], and 5α-reductase assays [4,5], and fatty acids and their derivatives could interfere with other noncellular screening assays, such as aromatase inhibitory activity, which are proving to affect the clinical development and progression of hormone-responsive breast cancers. Oilseed rape (*Brassica campestris* L. var. *oleifera* DC.) has been cultivated in northwest China for centuries. This investigation sought to determine whether fatty acid derivatives from the pollen of *Brassica campestris* L. var. *oleifera* DC. interfere with the results from a noncellular microsomal radiometric aromatase assay. The effects of the supercritical fluid extract and its residue of the pollen were compared so as to clarify its active constituents, its supercritical fluid extract demonstrated remarkable effects of aromatase inhibition through *in vitro* experiments, and the chemical constituents of the supercritical fluid extract were analyzed by GC–MS and six fatty acid derivatives were isolated by manifold chromatographic methods.

In this paper, we deal with the structural elucidation of six fatty acid derivatives, 9*Z*,12*Z*,15*Z*-octadecatrienoic acid sorbitol ester (**1**), (10,11,12)-trihydroxy-(7*Z*,14*Z*)-hep-

*Corresponding author. Email: yangyf4912@163.com

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tadecadienoic acid (**2**), 9*Z*,12*Z*,15*Z*-octadecatrienamide, *N*-(2-hydroxyethyl) (**3**), hexadecanoic acid sorbitol ester (**4**), 15,16-dihydroxy-9*Z*,12*Z*-octadecadienoic acid (**5**), and 9*Z*,12*Z*,15*Z*-octadecatrienoic acid 2,3-dihydroxypropyl ester (**6**) (Figure 1), and report some of their aromatase inhibition activities. Among these, compounds **1** and **2** are new fatty acid derivatives. Compounds **3**–**5** were isolated from this genus for the first time. Their structures were elucidated by spectral and chemical means.

2. Results and discussion

Compound **1** was obtained as colorless oil. The molecular formula of **1** was established as $C_{24}H_{42}O_7$ by HR-ESI-MS (*m*/*z* 465.2831 $[M+Na]^+$). The negative ESI-MS showed a quasi-molecular ion peak at *m*/*z* 441.2 $[M-H]^-$, 477.2 $[M+Cl]^-$, and 883.4 $[2M-H]^-$, the positive ESI-MS showed a quasi-molecular ion peak at *m*/*z* 465.3 $[M+Na]^+$ and 907.6 $[2M+Na]^+$. Comparison of the ^{13}C NMR spectral data of **1** with those of 9*Z*,12*Z*,15*Z*-octadecatrienoic acid 2,3-dihydroxypropyl ester (**6**) showed that they were very similar, except for the six carbon signals (δ 73.3, 71.8, 70.2, 70.0, 65.9, and 63.7) of sugar alcohol in the ^{13}C NMR spectrum of **1** instead of the three carbon signals of glycerine moiety in **6** (Figure 1). The ^{13}C NMR spectral data of sugar alcohol moiety of **1** were consistent with those of sorbitol [4], except that the chemical shift of C-1′ of **1** was shifted downfield by 3.5 ppm, while that of C-2′ of **1** was shifted upfield by 3.3 ppm, which suggested that compound **1** was a 1-*O*-substituted sorbitol. Sorbitol was identified after alkali hydrolysis of **1** by comparing with the authentic samples (Sigma-Aldrich, St. Louis, MO) on HPTLC. HMBC and $^1H—^1H$ COSY (Figure 2) experiments were run to support these assignments. The 2D-NMR including HMQC and HMBC experiments allowed us to assign all the protons and carbon signals for **1**. Thus, the structure of compound **1** was established as 9*Z*,12*Z*,15*Z*-octadecatrienoic acid sorbitol ester.

Compound **2** was obtained as colorless oil, $[\alpha]_D^{20}$ −21.00 ($c = 0.50$, $CHCl_3$). The molecular formula of **2** was established as $C_{17}H_{30}O_5$ by HR-ESI-MS (*m*/*z* 315.2178 $[M+H]^+$). The negative ESI-MS showed a quasi-molecular ion peak at *m*/*z* 313.2 $[M-H]^-$, 349.2 $[M+Cl]^-$, and 627.4 $[2M-H]^-$, the positive ESI-MS showed a quasi-molecular ion peak at *m*/*z* 337.2 $[M+Na]^+$ and 651.4 $[2M+Na]^+$. The ^1H NMR spectrum showed the olefinic protons at δ 5.56 (2H, m) and 5.40 (2H, m), the methenyl protons at δ 3.53 (2H, m) and 3.46 (1H, m), the methene protons at δ 2.30 (6H, m), 2.08 (2H, m), 2.04 (2H, m), and 1.60 (2H, m), and the methyl protons at δ 0.95 (3H, t, $J = 7.5$ Hz), and the ^{13}C NMR spectrum showed the carboxyl carbon at δ 178.7 (s), the olefinic carbons at δ 135.1 (d), 133.4 (d), 124.8 (d), and 124.1 (d), the methenyl carbons at δ 74.0 (d) and 73.3 (d), and the methyl carbon at δ 14.2 (q), which indicated that compound **2** was a trihydroxy-heptadecadienoic acid. In the HMBC spectrum (Figure 2), the methyl protons at δ 0.95 and the methene protons at δ 2.06 correlated with an olefinic carbon at δ 135.1, which suggested an ethylenic linkage at C-14 and C-15 (δ 124.1). HMQC of **2** showed the correlation between C-15 and an olefinic proton at δ 5.56, which was assigned to H-15. The assignments of H-14 (δ 5.40) and C-14 (δ 135.1) are likely. In the $^1H—^1H$ COSY spectrum (Figure 2), the olefinic protons at δ 5.40 correlated with the methene protons at δ 2.30, the methene protons at δ 2.30 correlated with the methenyl protons at δ 3.53, the methenyl protons at δ 3.53 correlated with the methenyl protons at δ 3.46, which suggested another ethylenic linkage at C-7 and C-8, and three hydroxyls at C-10, C-11, and C-12; HMBC experiment was run to support these assignments. The 2D-NMR including $^1H—^1H$ COSY, HMQC, and HMBC experiments allowed us to assign all the other proton and carbon signals for **2**. By comparison with the spectral data of C-8 and C-17 of compounds **1**, **3**, and **6**, the resonance at δ 27.3 (C-6) and 20.7 (C-16) confirms the 7*Z* and 14*Z* geometry of the olefinic bonds. The configuration of C-10,

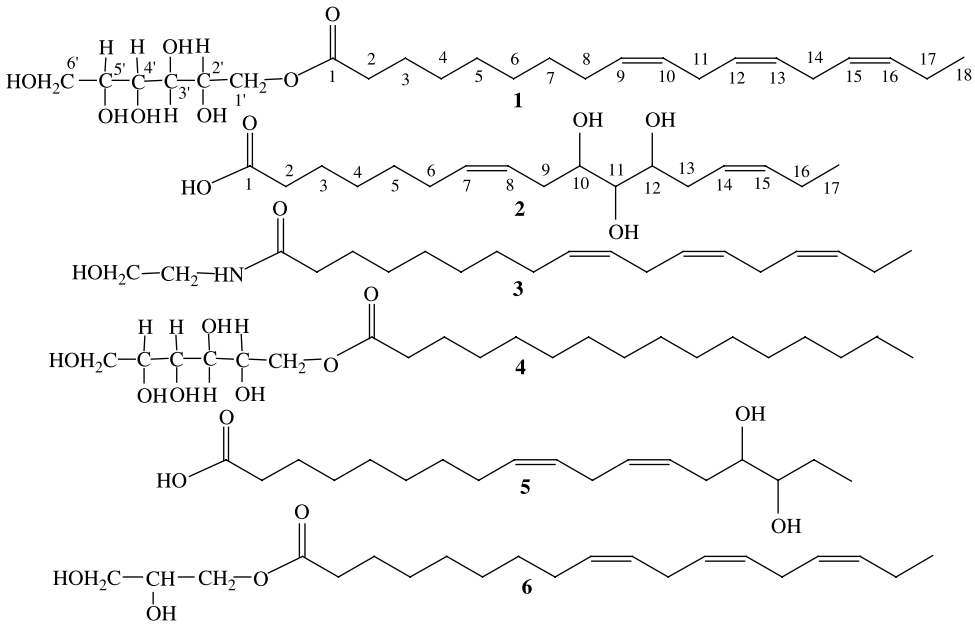

Figure 1. Structures of compounds **1**–**6**.

C-11, and C-12 remained undetermined. Thus, compound **2** was identified as (10,11,12)-trihydroxy-(7*Z*,14*Z*)-heptadecadienoic acid.

Four known fatty acid derivatives were identified as 9*Z*,12*Z*,15*Z*-octadecatrienamide, *N*-(2-hydroxyethyl) (**3**) [6], hexadecanoic acid sorbitol ester (**4**) [7,8], 15,16-dihydroxy-9*Z*,12*Z*-octadecadienoic acid (**5**) [9], and 9*Z*,12*Z*,15*Z*-octadecatrienoic acid 2,3-dihydroxypropyl ester (**6**) [10] by the comparison of their spectral data with those reported in the literatures. Some NMR spectral data of compounds **3**–**5** were reported in this paper for the first time.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter (Na filter, $\lambda = 589$ nm). The IR spectra were obtained on a Perkin-Elmer 577 spectrometer with KBr disk. The NMR spectra were recorded on a Bruker AV-400 spectrometer with TMS

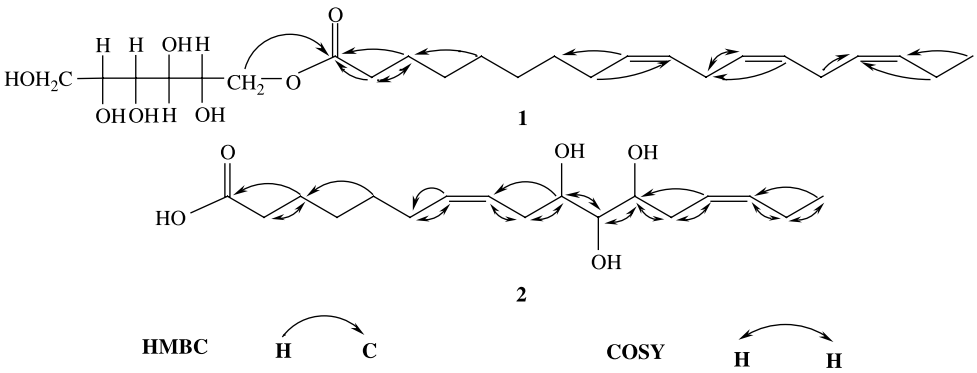

Figure 2. Key HMBC and $^{1}H—^{1}H$ COSY correlations for **1** and **2**.

as internal standard. ESI-MS was measured on a Finnigan MAT 95 instrument. HR-ESI-MS was measured on an Apex IV FTMS-7 instrument. Solvents used were of analytical grade (Shanghai Chemical Plant, Shanghai, China). Silica gel (300–400 mesh) was used for column chromatography, and precoated silica gel GF_{254} plates (Qingdao Haiyang Chemical Plant, Qingdao, China) for TLC.

3.2 Plant material

The pollen of *Brassica campestris* L. var. *oleifera* DC. was collected from Inner Mongolia Autonomous Region of China in March 2004, and was identified by Prof. Xu Feng of Jiangsu Botanic Institute. A voucher specimen (No. Bc-2004-03) is deposited in the Herbarium of Shanghai Institute of Pharmaceutical Industry.

3.3 Extraction and isolation

The dried powder (10 kg) of the rape pollen, of which the cell wall was broken by zymolysis, was extracted with supercritical fluid CO_2, a dark brown residue (523 g) was obtained, which was chromatographed over macroporous resin column eluting with C_2H_5OH—H_2O (in gradient, 60:40–0:100, v/v) to yield five major fractions. Fraction 1 (C_2H_5OH—H_2O, 60:40) was separated by chromatography over silica gel column eluting with $CHCl_3$—CH_3OH (in gradient, 100:0–0:100, v/v) to yield eight major subfractions, on the basis of TLC analysis. Subfraction 2 ($CHCl_3$—CH_3OH, 100:5, v/v) was separated by chromatography over silica gel column eluting with petroleum ether–EtOAc (2:1, v/v) to give compounds **5** (125 mg), **3** (150 mg), and **6** (160 mg). Subfraction 3 ($CHCl_3$—CH_3OH, 100:10, v/v) was separated by chromatography over silica gel column eluting with petroleum ether–EtOAc (3:2, v/v) to give compounds **4** (9 mg), **1** (3 mg), and **2** (11 mg).

3.3.1 Compound *1*

Colorless oil, IR ν_{max} (KBr) (cm^{-1}): 3396, 2928, 2855, 1739 (C═O), 1653, 1462, 1391, 1175, 1055, 932, 866, and 720; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.40–5.30 (6H, m, H-9, 10, 12, 13, 15, 16), 4.19–3.67 (8H, m, H-1′–6′), 2.80 (4H, m, H-11, 14), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.07 (4H, m, H-8, 17), 1.60 (2H, m, H-3), 1.27–1.31 (H-4, 5, 6, 7), and 0.97 (3H, t, J = 7.5 Hz, H-18); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.9 (s, C-1), 34.2 (t, C-2), 25.4 (t, C-3), 29.2–29.7 (t, C-4, 5, 6, 7), 27.2 (t, C-8), 132.0 (d, C-9), 128.2 (d, C-10, 15), 25.6 (t, C-11), 127.8 (d, C-12), 127.2 (d, C-13), 25.5 (t, C-14), 128.3 (d, C-16), 20.4 (t, C-17), 14.3 (q, C-18), 65.9 (t, C-1′), 70.2 (d, C-2′), 70.0 (d, C-3′), 73.3 (d, C-4′), 71.8 (d, C-5′), and 63.7 (t, C-6′); HR-ESI-MS: *m/z* 465.2831 $[M+Na]^+$ (calcd for $C_{24}H_{42}O_7Na$, 465.2828); ESI-MS (positive + negative): *m/z* 465 $[M+Na]^+$, 443 $[M+H]^+$, 477 $[M+Cl]^-$, and 441 $[M-H]^-$.

3.3.2 Compound *2*

Colorless oil, $[\alpha]_D^{20}$ −21.00 (c = 0.50, $CHCl_3$); IR ν_{max} (KBr) (cm^{-1}): 3399, 2929, 2855, 1712 (C═O), 1459, 1403, 1217, 1130, 1046, 976, and 724; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.56 (2H, m, H-7, 15), 5.40 (2H, m, H-8, 14), 3.53 (2H, m, H-10, 12), 3.46 (1H, m, H-11), 2.30 (6H, m, H-2, 9, 13), 2.08 (2H, m, H-16), 2.04 (2H, m, H-6), 1.60 (2H, m, H-3), and 0.95 (3H, t, J = 7.5 Hz, H-17); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 178.7 (s, C-1), 33.9 (t, C-2), 25.3 (t, C-3), 28.9–29.3 (t, C-4, 5), 27.3 (t, C-6), 124.8 (d, C-7), 133.4 (d, C-8), 31.7 (C-9, 13), 73.3 (d, C-10, 12), 74.0 (d, C-11), 135.1 (d, C-14), 124.1 (d, C-15), 20.7 (d, C-16), and 14.2 (q, C-17); HR-ESI-MS: *m/z* 315.2178 $[M+H]^+$ (calcd for $C_{17}H_{31}O_5$, 315.2171); ESI-MS (positive + negative): *m/z* 337 $[M+Na]^+$, 651 $[2M+Na]^+$, 313 $[M-H]^-$, 349 $[M+Cl]^-$, and 627 $[2M-H]^-$.

3.3.3 Compound *3*

Colorless oil, ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 174.5 (s, C-1), 36.5 (t, C-2), 25.4 (t, C-3), 29.2–29.7 (t, C-4, 5, 6, 7), 27.2 (t, C-8), 131.8 (d, C-9), 128.2 (d, C-10, 15), 25.6 (t, C-11), 127.7 (d, C-12), 127.0 (d, C-13), 25.5 (t, C-14),

130.1 (d, C-16), 20.4 (t, C-17), 14.1 (q, C-18), 42.3 (t, C-1′), and 61.9 (t, C-2′).

3.3.4 Compound **4**

Colorless oil, ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 173.2 (s, C-1), 33.7 (t, C-2), 24.5 (t, C-3), 26.7–29.1 (t, C-4–16), 22.2 (t, C-17), 14.1 (q, C-18), 65.8 (t, C-1′), 70.8 (d, C-2′), 69.5 (d, C-3′), 73.7 (d, C-4′), 72.3 (d, C-5′), and 63.5 (t, C-6′).

3.3.5 Compound **5**

Colorless oil, ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.44 (2H, m, H-10, 12), 5.38 (2H, m, H-9, 11), 3.52 (1H, m, H-15), 3.40 (1H, m, H-16), 2.80 (2H, m, H-11), 2.32 (2H, t, $J = 7.2$ Hz, H-2), 2.05 (2H, m, H-8), 2.01 (2H, m, H-14), 1.63 (2H, m, H-3), 1.48 (2H, m, H-17), 1.27–1.31 (H-4, 5, 6, 7), and 0.95 (3H, t, $J = 7.5$ Hz, H-18); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 178.6 (s, C-1), 33.9 (t, C-2), 24.6 (t, C-3), 28.8–29.5 (t, C-4, 5, 6, 7), 27.1 (t, C-8), 131.2 (d, C-9), 130.4 (d, C-10), 25.7 (t, C-11), 127.4 (d, C-12), 125.1 (d, C-13), 31.9 (t, C-14), 75.2 (d, C-15), 73.6 (d, C-16), 26.3 (t, C-17), and 9.9 (q, C-18).

3.4 Placental microsome aromatase assay

The aromatase assay was done by previous method [11]. The substrate, [1β-^{3}H] androstenedione (specific activity, 25.3 Ci/mmol), was dissolved in serum-free cell culture medium. Placental microsomes were prepared as 0.1 g/l in a potassium phosphate buffer (67 mmol/l, pH 7.4) containing 20% glycerol, 0.5 mmol/l dithiothreitol, and 0.25 mol/l sucrose. The assay reaction mixture (225 μl), containing placental microsomes (2.5 μg), [^{3}H]androstenedione (50 nmol/l), progesterone (10 μmol/l), and bovine serum albumin (0.1%) in potassium phosphate (67 mmol/l, pH 7.4), with sample solution in DMSO, was introduced in wells of a 96-well plate and preincubated at room temperature for 10 min; then 25 μl of NADPH (3 mmol/l) were added and the mixture was incubated at 37°C for 15 min. The reaction was terminated by an addition of 50 μl of 20% trichloroacetic acid, and 250 μl of the solution were transferred to another well containing the charcoal–dextran pellet. The solution was thoroughly mixed and centrifuged (1000 *g*, 5 min) to remove the nonreacted substrate; an aliquot of the supernatant containing [^{3}H]H_2O as reaction product was counted in a Beckman Coulter LS 6500 multi-purpose scintillation counter.

Table 1. Inhibition ratio of the noncellular enzyme-based aromatase bioassays for compounds **1**–**6**.

Compound	Inhibition ratio (100 μg/ml)	IC_{50} (μg/ml)
1	79%	7.80
2	38%	–
3	79%	14.00
4	51%	–
5	51%	–
6	73%	–
AG	58%[a]	0.88

[a] Determined at 1 μg/ml.

Compounds **1**–**6** were tested using a noncellular aromatase assay. Aromatase inhibition activity was calculated as the percentage of remaining activity from the reaction without sample. Analyses were carried out in triplicate and data were expressed as the inhibition rate and IC_{50} with aminogluteth-imide as the positive control, and compounds **1** and **3** showed strong inhibitory activity with IC_{50} values of 7.80 and 14.00 μg/ml, respectively (Table 1).

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