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Rare Hybrid Dimers with Anti-Acetylcholinesterase Activities from a Safflower (*Carthamus tinctorius* L.) Seed Oil Cake

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Supporting Information

ABSTRACT: Safflower (*Carthamus tinctorius*) is commercially cultivated for vegetable oil extracted from the seeds. However, during the production process of seed oil, a large amount of the oil cake is thrown away or fermented as fertilizer to improve the homing rate of pigeons. Therefore, to solve the ecological problem and develop its new function, we investigated the chemical constituents of a safflower seed oil cake, and six new hybrid dimers, (±)-carthatins A−F (**1**−**6**, respectively), with a phenylpropanoid and a feruloylserotonin fused via a dihydrofuran ring, together with four known compounds, including sinapyl alcohol (**7**), coniferyl alcohol (**8**), serotobenine (**9**), and feruloylserotonin (**10**), were isolated. The extensive nuclear magnetic resonance spectra, combined with electronic circular dichroism analysis and chiral high-performance liquid chromatography, allowed the complete structural assignments of (±)-carthatins A−F. Moreover, we evaluated their anti-acetylcholinesterase activities. Racemic carthatins A and B (**1** and **2**, respectively) showed anti-acetylcholinesterase effects with IC_{50} values of 17.96 and 66.83 μM, respectively. To some extent, our findings provide a new scaffold of acetylcholinesterase inhibitors, which could be beneficial for developing therapeutic molecules for the treatment of Alzheimer's disease and supporting folk application of a safflower seed oil cake.

KEYWORDS: *safflower seed oil cake, Carthamus tinctorius L., anti-AChE activity, ECD analysis, chiral HPLC*

INTRODUCTION

Safflower (*Carthamus tinctorius* L.), of the Asteraceae family, is one of the important aromatic and medicinal plants. Traditionally, the crop was grown for its flowers. Its flowers have occasionally been used as a cheaper substitute for saffron, as a natural dye source for orange-red pigment carthamin, and as a traditional Chinese medicine to treat angina pectoris, stroke, gynecological disease, coronary heart disease, and hypertension.[1−4]

Later, safflower was widely cultivated for the seed oil that is valuable for human consumption in the form of cooking, salad oil, margarine, and processed foods, because its nutritional qualities are similar to those of sunflower oil.[5] Moreover, safflower seed oil contains plentiful linoleic acid[6] and can been used to prevent cardiovascular and cerebrovascular diseases.[7,8] However, a large amount of the oil cake, as the waste residue, was thrown away during the production of seed oil, which resulted in environmental pressure and waste. To solve the ecological problem and develop a new function, phytochemists and pharmacologists paid close attention to the chemical constituents and biological activities of the oil cake. A series of serotonin derivatives were identified and showed antioxidant,[9,10] antiproliferation,[11] antitumor,[12] liver-protective,[13] and anti-inflammatory activities.[14]

Furthermore, our investigations found that the safflower oil cake is used as fodder to feed pigeons to improve their homing rate as it relates to memory. Thus, in this paper, we systematically investigated its serotonin constituents, and six novel hybrid dimers, (±)-carthatins A−F (**1**−**6**, respectively), possessing a phenylpropanoid and a feruloylserotonin fused via a dihydrofuran ring, along with four known compounds (Figure 1) were isolated. Subsequently, their anti-AChE (acetylcholinesterase) activities were evaluated.

MATERIALS AND METHODS

General Experimental Procedures. A Jasco P-1020 polarimeter (Jasco, Tokyo, Japan) was used to detect the optical rotations. A Shimadzu UV2401PC spectrophotometer (Shimadzu, Kyoto, Japan) was used to obtain ultraviolet (UV) spectra. The Bruker AV-400 and AV-600 instruments (Bruker, Zurich, Switzerland) with transcranial magnetic stimulation (TMS) were used to detect the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra. ESIMS and HRTOF-ESIMS data were recorded on an API QSTAR Pulsar spectrometer (Waters), and a Bruker Tensor-27 instrument using KBr pellets (Bruker) was used to record infrared spectra. Circular dichroism spectra were recorded on an Applied Photophysics spectropolarimeter (Agilent). Semipreparative high-performance liquid chromatography (HPLC) was performed on an Agilent 1100 or 1260 series instrument (Agilent Technologies, Foster City, CA) with a ZORBAX SB-C18 column (5 μm, 9.4 mm × 250 mm). Chiral compounds were analyzed with a chiral column (CHIRALCEL OJ-H, 5 μm, 4.6 mm × 250 mm). TLC plates (200−250 μm thickness, F254 Si gel 60, Qingdao Marine Chemical, Inc.) were used for TLC detection. The common column

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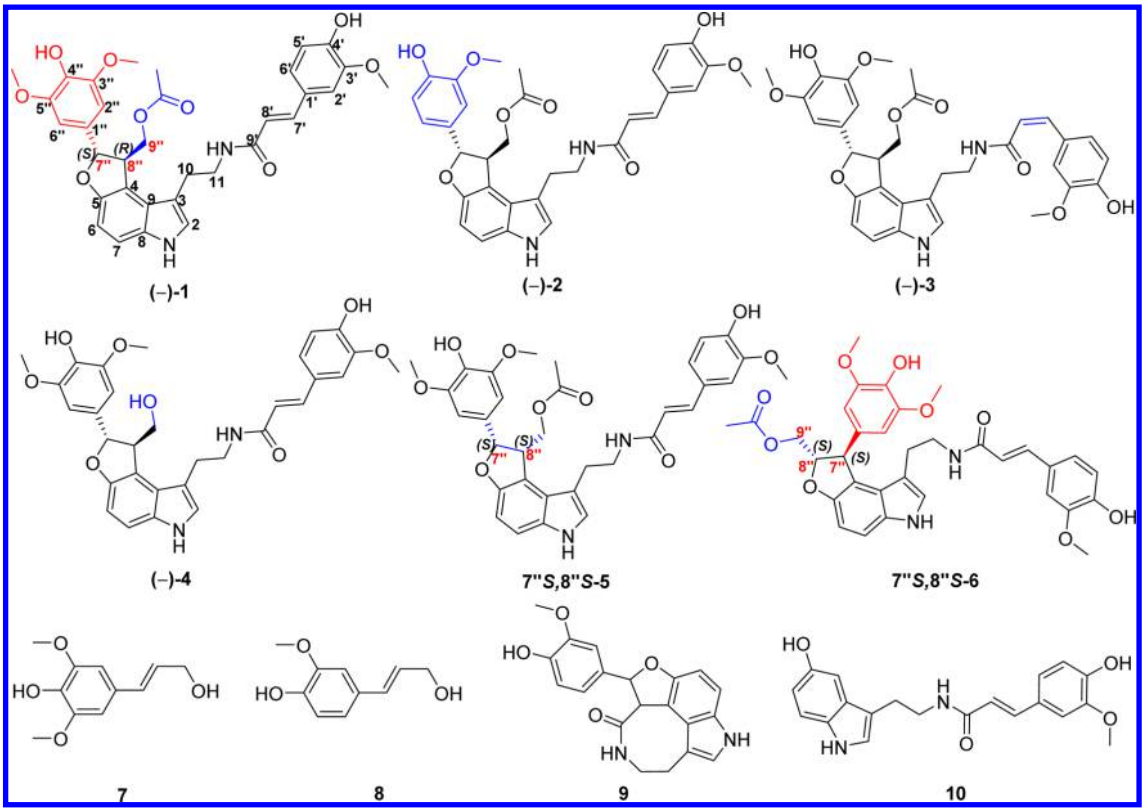

Figure 1. Structures of compounds from the seed oil cake of safflower.

chromatographic materials contain Sephadex LH-20 (20−150 μm, Pharmacia), Lichroprep RP-18 (40−63 μm, Merck), and silica gel (200−300 mesh, Qingdao Marine Chemical, Inc.). Chromatogram class methanol and acetonitrile were purchased from Shanghai Youshi Chemical Co., Ltd. (Shanghai, China). Industrial-grade methanol, chloromethane, ethyl acetate, acetone, petroleum ether, and *n*-butanol were purchased from Tianjing Chemical Reagents Co. (Tianjing, China).

Plant Materials. The safflower seed oil cake was a research sample provided by Lijiang Yongsheng Biantun Shishang Yangshengyuan Co. Ltd. in Yunnan Province in July 2015.

Extraction and Isolation. The seed oil cake (21 kg) was extracted with methanol (63 L, 95% MeOH/H_2O) under reflux three times at 60 °C, each for 3 h. The methanol extracts were concentrated under reduced pressure, and the residue (1.5 kg) was extracted using petroleum ether (3 × 5 L, PE) and ethyl acetate (3 × 5 L, EtOAc). The combined EtOAc extract (99.7 g) was reduced and fractionated by column charomatography (4.8 cm × 37 cm, CC, silica gel; 80:1, 70:1, 60:1, 50:1, 40:1, 30:1, 20:1, and 5:1 $CHCl_3$/MeOH, step gradients, 25 L) to obtain fractions I−VIII. Fraction II (7.3 g) was treated with LH20 (3 L, MeOH) to give three subfractions (Fr. II-1−Fr. II-3). Fr. II-2 (2.7 g) was purified by CC (silica gel, 10:1 PE/acetone, 3 L) to yield sinapyl acohol (**7**, 7.2 mg) and coniferyl acohol (**8**, 5.3 mg). Fr. II-4 (8.3 g) was separated by LH20 (2 L, MeOH) to yield five subfractions (Fr. II-4-1−Fr. II-4-5). Fr. II-4-4 (126 mg) was subjected to HPLC (40% → 45% CH_3CN/H_2O, 20 min) to yield compounds **1** (21.2 mg; t_R = 16.2 min), **2** (13.2 mg; t_R = 13.4 min), **3** (5.4 mg; t_R = 17.4 min), **4** (3.2 mg; t_R = 18.9 min), and **5** (6.3 mg; t_R = 15.2 min). Compounds **1** (3.2 mg; t_R = 16.3 min) and **6** (5.2 mg; t_R = 17.6 min) were obtained from Fr. II-4-5 (23 mg) by HPLC (40% → 45% CH_3CN/H_2O, 20 min). Fr. II-4-6 (18.5 g) was subjected to CC (1.4 cm × 18 cm, reversed-phase silica gel, 50% MeOH/H_2O, 3 L) to yield serotobenine (**9**, 11.0 mg). Feruloylserotonin (**10**, 121.0 mg) were obtained from Fr. III and purified by the recrystallization method.

N-Boc Protection of (±)-1, (±)-2, (±)-5, and (±)-6.[15] (±)-**1** (10 mg, 0.017 mmol) was dissolved in dry THF (tetrahydrofuran, 1 mL), and then DMAP (dimethylaminopyridine, 2 mg, 0.017 mmol), Boc_2O (di-*tert*-butyl dicarbonate ester, 37 mg, 0.17 mmol), and Et_3N (triethylamine, 0.01 mL, 0.06 mmol) were added to the solution described above. The mixture was stirred at reflux for 2 h. The solvent was evaporated until the material disappeared as determined by TLC detection. Water (10 mL) was added to the residue, and EtOAc (3 × 20 mL) was added to the mixture for extraction. The combined EtOAc layer was then dried over anhydrous Na_2SO_4. The solution was concentrated and purified by using RP-C18 HPLC with a CH_3CN/H_2O [96% (v/v)] eluent to give (±)-**1a** (5 mg, 21.807 min) as white powder. The structure of compound (±)-**1a** was confirmed by analyzing its molecular weight (ESI-MS *m*/*z* 1025 $[M + Na]^+$) (see the Supporting Information). Similarly, (±)-**2a**, (±)-**5a**, and (±)-**6a** were also derived from (±)-**2**, (±)-**5**, and (±)-**6**, respectively, using the method described above. Their structures were determined by ESI-MS: *m*/*z* 975 [M + Na] for (±)-**2a**, *m*/*z* 1025 $[M + Na]^+$ for (±)-**5a**, and *m*/*z* 1025 $[M + Na]^+$ for (±)-**6a**.

Chiral Separation of (±)-1a, (±)-2a, (±)-5a, and (±)-6a. Compounds (±)-**1a**, (±)-**2a**, (±)-**5a**, and (±)-**6a** were separated by chiral HPLC, which yielded (−)-**1a** (t_R = 18.4 min; 1.00 mg) and (+)-**1a** (t_R = 21.1 min; 1.00 mg) [90:10 (v/v) *n*-hexane/isopropanol], (−)-**2a** (t_R = 20.6 min; 0.61 mg) and (+)-**2a** (t_R = 28.8 min; 0.83 mg) [93:7 (v/v) *n*-hexane/isopropanol], **5a-1** (t_R = 11.6 min; 0.30 mg) and **5a-2** (t_R = 20.8 min; 0.22 mg) [90:10 (v/v) *n*-hexane/isopropanol], and **6a-1** (t_R = 9.0 min; 0.12 mg) and **6a-2** (t_R = 11.0 min; 0.10 mg) [92:8 (v/v) *n*-hexane/isopropanol], respectively.

ECD Computational Method. The absolute configuration of compound (±)-**1a** was confirmed by using a quantum chemical method with time-dependent density functional theory (TDDFT). First, Discovery Studio 4.1 Client conformational searching and molecular mechanics methods (MMFF94) were used for the conformational analysis, and the optimal conformers were selected. Second, the selected conformers were optimized at the B3LYP/6-31+G(d,p) level in the gas phase (Gaussian09).[16] Third, further ECD calculations were performed at the PCM-B3LYP/6-31+G(d,p) level in a MeOH solution. Finally, via comparison of the experimental and calculated ECD spectra, we can obtain the absolute configuration of (±)-**1a**.

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data (600 and 150 MHz, CD_3OD) of Compounds 1−6[a]

	1		2		3		4		5		6	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2	7.11, br s	125.5, CH	7.12, br s	125.5, CH	7.11, br s	125.6, CH	7.10, br s	125.3, CH	7.15, br s	125.8, CH	6.99, br s	125.4, CH
3		111.4, C		111.4, C		111.5, C		111.4, C		112.0, C		117.8, C
4		113.2, C		113.2, C		113.2, C		114.3, C		116.8, C		112.4, C
5		155.0, C		155.0, C		155.0, C		154.7, C		154.5, C		154.6, C
6	7.22, d (8.6)	112.9, CH	7.22, d (8.6)	112.9, CH	7.22, d (8.6)	112.9, CH	7.19, d (8.6)	112.4, CH	7.22, d (8.6)	112.6, CH	7.21, d (8.6)	112.6, CH
7	6.76, d (8.6)	105.6, CH	6.76, d (8.6)	105.6, CH	6.76, d (8.6)	105.6, CH	6.75, d (8.6)	105.6, CH	6.76, d (8.6)	105.9, CH	6.71, d (8.6)	105.7, CH
8		134.7, C		134.7, C		134.7, C		134.7, C		134.7, C		134.9, C
9		125.6, C		125.6, C		125.7, C		125.6, C		125.6, C		125.5, C
10	3.04, m	27.3, CH_2	3.04, m	27.3, CH_2	3.04, m	27.1, CH_2	3.01, m	27.5, CH_2	3.12, m; 2.99, m	27.3, CH_2	2.42, m	27.3, CH_2
11	3.61, m; 3.42, m	42.2, CH_2	3.59, m; 3.45, m	42.2, CH_2	3.61, m; 3.42, m	42.5, CH_2	3.61, m; 3.42, m	42.1, CH_2	3.59, m	42.5, CH_2	3.40, m; 3.12, m	42.9, CH_2
1′		128.2, C		128.2, C		128.3, C		128.1, C		128.2, C		128.2, C
2′	7.08, br s	111.4, CH	7.09, br s	111.4, CH	7.08, br s	113.8, CH	7.09, br s	111.4, CH	7.11, br s	111.4, CH	7.11, br s	111.4, CH
3′		149.8, C		149.8, C		149.3, C		149.7, C		149.9, C		149.9, C
4′		149.8, C		149.2, C		149.3, C		149.7, C		149.3, C		149.3, C
5′	6.77, d (8.2)	116.4, CH	6.78, d (8.2)	116.4, CH	6.68, d (8.2)	116.1, CH	6.78, d (8.2)	116.4, CH	6.78, d (8.2)	116.4, CH	6.79, d (8.2)	116.4, CH
6′	6.99, d (8.2)	123.1, CH	7.00, d (8.2)	123.2, CH	6.89, d (8.2)	124.8, CH	7.00, d (8.2)	123.2, CH	7.02, d (8.2)	123.2, CH	7.01, d (8.2)	123.2, CH
7′	7.40, d (15.7)	141.9, CH	7.40, d (15.7)	141.9, CH	6.55, d (12.7)	138.0, CH	7.41, d (15.7)	142.0, CH	7.47, d (15.7)	142.0, CH	7.44, d (15.6)	142.0, CH
8′	6.38, d (15.7)	118.8, CH	6.38, d (15.7)	118.7, CH	5.70, d (12.7)	121.5, CH	6.38, d (15.7)	118.6, CH	6.43, d (15.7)	118.8, CH	6.39, d (15.6)	118.8, CH
9′		169.1, C		169.1, C		170.1, C		169.2, C		169.1, C		168.8, C
1″		136.0, C		135.5, C		136.0, C		136.0, C		135.9, C		136.6, C
2″	6.60, s	103.4, CH	6.86, s	109.8, CH	6.63, s	103.4, CH	6.65, s	103.4, CH	6.80, s	104.7, CH	6.42, s	105.7, CH
3″		149.2, C		148.9, C		149.2, C		149.2, C		149.1, C		148.9, C
4″		134.7, C		147.2, C		134.7, C		134.7, C		135.9, C		134.7, C
5″		149.2, C	6.72, d (8.2)	116.2, CH		149.2, C		149.2, C		149.1, C		149.2, C
6″	6.60, s	103.4, CH	6.76, d (1.4)	119.0, CH	6.63, s	103.4, CH	6.65, s	103.4, CH	6.80, s	104.7, CH	6.42, s	105.7, CH
7″	5.62, d (1.6)	87.5, CH	5.59, s	87.5, CH	5.61, s	87.5, CH	5.69, s	87.4, CH	5.74, d (6.6)	88.0, CH	4.74, d (5.2)	52.3, CH
8″	4.06, m	52.0, CH	4.01, m	51.9, CH	4.08, m	51.9, CH	3.87, br s	55.8, CH	4.09, m	47.1, CH	4.61, m	89.9, CH
9″	4.70, dd (10.9, 3.5); 4.11, m	68.2, CH_2	4.66, dd (10.9, 3.5); 4.08, m	68.1, CH_2	4.64, dd (10.9, 3.5); 4.13, m	68.1, CH_2	4.05, m	66.5, CH_2	4.07, m; 3.82, m	64.2, CH_2	4.33, m	66.7, CH_2
$\underline{CH_3}CO$	2.11, s	20.8, CH_3	2.08, s	20.8, CH_3	2.07, s	20.8, CH_3			1.58, s	20.4, CH_3	2.00, s	20.6, CH_3
$CH_3\underline{CO}$		172.8, C		172.8, C		172.8, C				172.3, C		172.7, C
3′-OMe	3.88, s	56.3, CH_3	3.86, s	56.3, CH_3	3.74, s	56.3, CH_3	3.88, s	56.3, CH_3	3.85, s	56.3, CH_3	3.77, s	56.3, CH_3
3″-OMe	3.74, s	56.6, CH_3	3.72, s	56.2, CH_3	3.72, s	56.6, CH_3	3.74, s	56.6, CH_3	3.84, s	56.7, CH_3	3.54, s	56.6, CH_3
5″-OMe	3.74, s	56.6, CH_3			3.72, s	56.6, CH_3	3.74, s	56.6, CH_3	3.84, s	56.7, CH_3	3.54, s	56.6, CH_3

[a]The assignments were based on COSY, HSQC, and HMBC experiments.

(±)-Carthatin A (**1**), obtained as white powder: $[\alpha]^{20}_D$ −0.38 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 318 (4.22), 285 (4.15), 227 (4.43), 206 (4.65) nm; IR (KBr) ν_{max} 3424, 2935, 1734, 1650, 1616, 1516, 1461, 1220 cm^{-1}; one-dimensional NMR data in Table 1; HRESIMS *m*/*z* 625.2165 $[M + Na]^+$, $C_{33}H_{34}N_2NaO_9$ (calcd 625.2162).

(±)-Carthatin B (**2**), obtained as white powder: $[\alpha]^{20}_D$ −1.50 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 319 (4.39), 285 (4.39), 225 (4.64), 202 (4.83) nm; IR (KBr) ν_{max} 3424, 2938, 1735, 1660, 1620, 1518, 1460, 1222 cm^{-1}; 1D NMR data in Table 1; HRESIMS *m*/*z* 585.2051 $[M + Na]^+$, $C_{32}H_{32}N_2NaO_8$ (calcd 595.2056).

(±)-Carthatin C (**3**), obtained as white powder: $[\alpha]^{20}_D$ −0.23 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 315 (4.08), 283 (4.08), 227 (4.41), 206 (4.62) nm; IR (KBr) ν_{max} 3423, 2940, 1733, 1657, 1617, 1520, 1461, 1219 cm^{-1}; 1D NMR data in Table 1; HRESIMS *m*/*z* 625.2160 $[M + Na]^+$, $C_{33}H_{34}N_2NaO_9$ (calcd 625.2162).

(±)-Carthatin D (**4**), obtained as white powder: $[\alpha]^{20}_D$ −3.20 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 317 (4.41), 283 (4.35), 227 (4.67), 206 (4.88) nm; IR (KBr) ν_{max} 3422, 2938, 1743, 1654, 1616, 1510, 1460, 1220 cm^{-1}; 1D NMR data in Table 1; HRESIMS *m*/*z* 583.2058 $[M + Na]^+$, $C_{31}H_{32}N_2NaO_8$ (calcd 583.2056).

(±)-Carthatin E (**5**), obtained as white powder: $[\alpha]^{20}_D$ −8.33 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 316 (4.04), 283 (4.04), 228 (4.74), 207 (4.88) nm; IR (KBr) ν_{max} 3420, 2935, 1740, 1653, 1618, 1517, 1467, 1223 cm^{-1}; 1D NMR data in Table 1; HRESIMS *m*/*z* 625.2161 $[M + Na]^+$, $C_{33}H_{34}N_2NaO_9$ (calcd 625.2162).

(±)-Carthatin F (**6**), obtained as white powder: $[\alpha]^{20}_D$ −0.33 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 320 (4.46), 285 (4.39), 229 (4.66), 206 (4.93) nm; IR (KBr) ν_{max} 3422, 2925, 1732, 1650, 1617, 1527, 1467, 1222 cm^{-1}; 1D NMR data in Table 1; HRESIMS *m*/*z* 625.2160 $[M + Na]^+$, $C_{33}H_{34}N_2NaO_9$ (calcd 625.2162).

Acetylcholinesterase (AChE) Inhibitory Activity. An acetylthiocholine iodide substrate-based colorimetric method was used to assay the AChE activity of isolates.[17] Whole brains of mice were homogenized in a hand homogenizer with 10 volumes of homogenization buffer [12.5 mM sodium phosphate buffer (pH 7.0)

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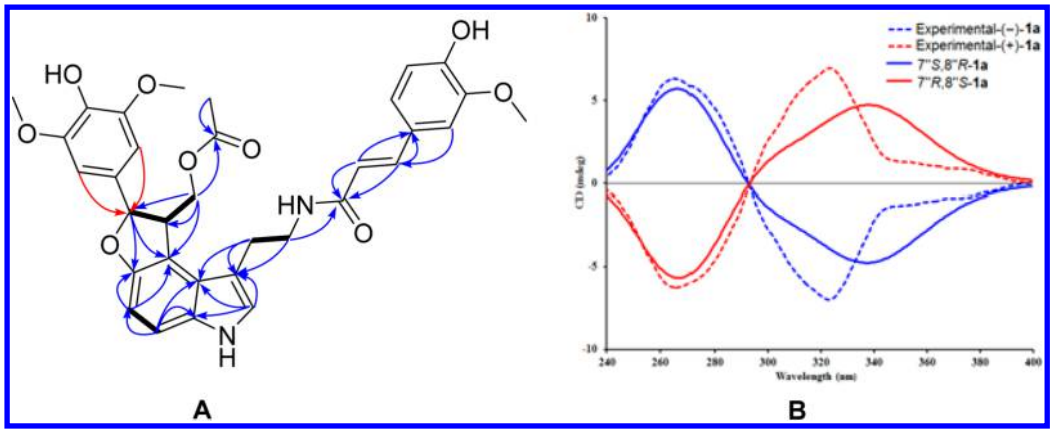

Figure 2. (A) Key HMBC (solid arrows) and $^{1}H-^{1}H$ COSY (thick bonds) correlations of (±)-carthatin A (**1**). (B) Calculated and experimental CD spectra of 7″(*S*),8″(*R*)-**1a** and 7″(*R*),8″(*S*)-**1a**.

and 400 mM NaCl] and then centrifuged at 1000*g* for 10 min at 4 °C to obtain the supernatant as the enzyme for a future assay. First, the isolates was dissolved in buffer A [100 mM sodium phosphate buffer (pH 8.0)] and then diluted to various concentrations in buffer A before use. An aliquot of the isolate solution diluted in buffer A (1.5 mL) was then mixed with buffer A (2.6 mL), an acetylthiocholine iodide solution (20 μL, 75 mM), and buffered Ellman's reagent [100 μL, 10 mM DTNB (dithiobisnitrobenzoic acid) and 15 mM sodium bicarbonate] and reacted at room temperature for 30 min. After the enzyme source (400 μL) had been added to the reaction mixtures (UV-1700 PharmaSpec, Shimadzu Co. Ltd.), the absorbance at 412 nm was immediately measured. Readings were taken at 30 s intervals for 5 min. An enzyme inhibition dose−response curve was used to calculate the half-inhibition rate against AChE activity (IC_{50}).

RESULTS AND DISCUSSION

Structure Elucidation. Compound **1** was isolated as white powder. Its HRESIMS molecular ion at *m/z* 625.2165 $[M + Na]^+$ and ^{13}C NMR data are consistent with the molecular formula $C_{33}H_{34}N_2O_9$ (calcd 625.2162). Its IR spectrum showed the absorption bands of the hydroxyl (3424 cm^{-1}), carbonyl (1734 cm^{-1}), and α,β-unsaturated carbonyl (1650 cm^{-1}) groups. The ^{1}H NMR spectroscopic data (Table 1) of **1** showed the presence of one singlet methyl (δ 2.11), three methoxyl (δ 3.88, 3.74, and 3.74), one oxymethylene [δ 4.70 (dd, *J* = 10.9, 3.5 Hz), 4.11 (m)], one oxymethine (δ 5.62, s), and 10 aromatic/olefinic proton signals. The ^{13}C DEPT spectra of **1** revealed 33 carbon resonances, belonging to three methoxyls, one acetyl, three methylenes (one oxygenated), 12 methines (including 10 aromatic/olefinic methines and one oxygenated), and 12 aromatic/olefinic quaternary carbons. Among them, three aromatic signals [δ 7.08 (s, H-2′), 6.99 (d, *J* = 8.2 Hz, H-6′), 6.77 (d, *J* = 8.2 Hz, H-5′)] and two olefinic signals [δ 7.40 (d, *J* = 15.7 Hz, H-7′), 6.38 (d, *J* = 15.7 Hz, H-8′)], along with the key HMBC (Figure 2) correlations of methoxyl proton signals (δ 3.88) with C-3′, of H-2′ and H-6′ with C-7′ (δ 141.9), and of H-7′ and H-8′ with C-1′ (128.2) and C-9′ (δ 169.1), indicated the presence of a 3-(4-hydroxy-3-methoxyphenyl)-2-acrylaminde.

Furthermore, a serotonin moiety was established from the $^{1}H-^{1}H$ COSY correlation (Figure 2) of two aromatic proton signals at δ 7.22 (d, *J* = 8.6 Hz, H-6) and δ 6.76 (d, *J* = 8.6 Hz, H-7), along with a series of HMBC correlations of H-6 and H-7 with C-4 (δ 113.2), C-5 (δ 155.0), C-8 (δ 134.7), and C-9 (δ 125.6), of H-7 with C-2 (δ 125.5), of H-2 [δ 7.11 (brs)] with C-8, C-9, and C-3 (δ 111.4), and of H_2-10 [δ 3.04 (m), 3.61 (m)] and H_2-11 [δ 3.42 (m)] with C-9′ (δ 169.1) and C-3. This information further suggested that the structure of **1** contains a feruloylserotonin (**10**[21]) fraction.

Additionally, two overlapped aromatic proton signals [δ 6.60 (2H, s, H-2″ and H-6″)] were characterized for the 1″,3″,4″,5″-tetra-substituted 3″,5″-dimethoxy-4″-hydroxybenzene moiety, which was consistent with their ^{13}C NMR data (Table 1) and the HMBC correlations (Figure 2) of H-2″ and H-6″ with C-1″ (δ 136.0), C-3″ (δ 149.2), C-4″ (δ 134.7), and C-5″ (δ 149.2) and of two methoxyl protons (δ 3.74) with C-3″ and C-5″. Then, the observed HMBC correlations of H-3″ and H-6″ with an oxymethine [δ 87.5 (C-7″)], of H-7″ with a methine [δ 52.0 (C-8″)] and an oxymethylene [δ 68.2 (C-9″)], of H-9″ [δ 4.70 (dd, *J* = 10.9, 3.5 Hz), 4.11 (m)] with the acetyl carbonyl (δ 172.8), and of H-8″ with C-4, as well as the $^{1}H-^{1}H$ COSY correlations (Figure 2) of H-7″, H-8″, and H-9″, confirmed that a 2-hydrobenzofuran ring existed in **1** and the acetoxyl was located at C-9″. Thus, the planar structure of **1** was unambiguously deduced.

Furthermore, coupling constants of the double bond [δ 7.40 (d, *J* = 15.7 Hz, H-7′), 6.38 (d, *J* = 15.7 Hz, H-8′)] led to the assignment of the *E* geometry to $\Delta^{7'}$. The ROESY cross-peak (Figure 3) of H-7″/H-9″ illustrated the opposite direction of H-7″ and H-8″. Furthermore, the optical rotation ($[\alpha]^{20}_{D}$ −0.38) suggested that compound **1** could be a racemic mixture [7″(*S*),8″(*R*)-carthamin A and 7″(*R*),8″(*S*)-carthamin A]. To further verify this deduction, the chiral HPLC method was used

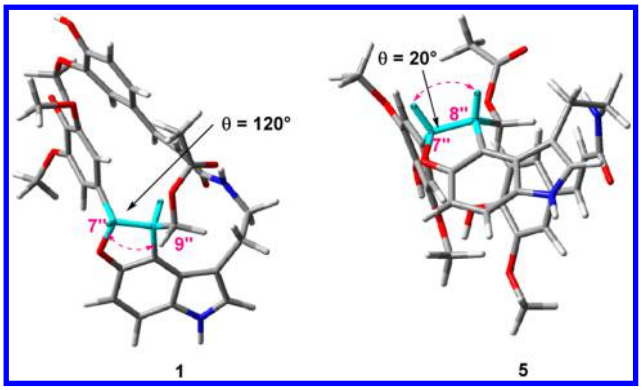

Figure 3. Three-dimensional molecular modeling, dihedral angle (θ), and selected ROESY (dashed arrows) correlation of (±)-carthatins A and E (**1** and **5**, respectively).

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to analyze **1**. Because of the presence of two nitrogen atoms in **1**, compound **1** was absorbed by the chiral HPLC column. Thus, **1a** was derived from **1** through an acylation with Boc_2O. Then, (+)-**1a** and (−)-**1a** were further created by chiral HPLC, which had the opposite optical rotation [$[\alpha]^{20}_D$ = −9.33 for (−)-**1a**; $[\alpha]^{20}_D$ = +14.00 for (+)-**1a**] and CD spectra (see the Supporting Information). The absolution configurations at stereogenic centers of (−)-**1a** and (+)-**1a** were assigned as (−)-7″*S*,8″*R* and (+)-7″*R*,8″*S*, respectively, using the computational methods (Figure 3).

The molecular formula of racemic carthatin B (**2**) was established to be $C_{32}H_{32}N_2O_8$ by analysis of its HRESIMS, ^{13}C NMR, and DEPT spectra. The one-dimensionak (1D) NMR spectra of **2** are similar to those of (±)-carthatin A (**1**), except for the replacement of the quaternary carbon [δ 134.7 (C)] in **1** by an aromatic methine [δ 116.2 (CH, C-5″)] in **2**, and the downfield shift of C-2″ (δ 103.4 for **1**, δ 109.8 for **2**) and C-6″ (δ 103.4 for **1**, δ 119.0 for **2**). Furthermore, the HMBC correlations from H-5″ to C-4″, C-6″, and C-1″ and from H-2″ and H-6″ to C-7″ (δ 87.5) indicated that **2** had the same structure as **1**, except for the absence of a methoxyl group at C-5″ in **2**. The ROESY spectrum of **2** displayed a correlation between H-7″ and H_2-9″, suggesting that H-7″ and CH_2-9″ were oriented on the same side. The low optical rotation of **2** ($[\alpha]^{20}_D$ = −1.50) illustrated that it could be racemic. Separation of **2a** (acylated derivative of **2**) by chiral HPLC yielded two enantiomers. The CD spectrum and optical rotation of (+)-**2a** are in good agreement with those of (+)-**1a** (see the Supporting Information), revealing that the absolute configuration of (+)-**2a** is 7″*R*,8″*S*. Therefore, the structure of **2** was deduced and named (±)-carthatin B (**2**).

Racemic carthatin C (**3**) had the same molecular formula ($C_{33}H_{34}N_2O_9$) and 1D NMR data as **1**. However, the only difference between **1** and **3** was the coupling constant of $\Delta^{7'}$. The *Z* configuration of $\Delta^{7'}$ in **3** was established by the coupling constant at δ 6.55 (d, *J* = 12.7 Hz, H-7′) and δ 5.70 (d, *J* = 12.7 Hz, H-8′), while the configuration of $\Delta^{7'}$ in **1** was *E* according to the coupling constant [δ 7.40 (d, *J* = 15.7 Hz, H-7′), 6.38 (d, *J* = 15.7 Hz, H-8′)] (see the Supporting Information), which indicated that compounds **1** and **3** were a pair of *cis*−*trans* $\Delta^{7'}$ isomers. Compound **3** was also racemic on the basis of its optical rotation and CD spectrum. Similarly, H-7″ and H-8″ have the opposite relative configurations based on the ROESY correlation of H-7″/H_2-9″. Thus, the same 1D and 2D NMR data and identical CD spectra between **3** and **1** further indicated that the stereogenic centers of **3** should be also assigned as (−)-7″*S*,8″*R* and (+)-7″*R*,8″*S*.

Carthatin D (**4**) has the molecular formula $C_{31}H_{32}N_2O_8$ as determined by analysis of its HRESIMS data. **4** has the same 1D NMR spectroscopic data as **1** except that an acetyl group was not observed in **4**. Its 2D NMR spectra further indicated that they have the same planar structure. Moreover, according to the ROESY cross-peak of H-7″/H_2-9″, the lack of optical rotation, and its CD spectrum that is identical with that of **1**, the absolute configurations of chrial centers at C-7″ and C-8″ were therefore determined to be (−)-7″*S*,8″*R* and (+)-7″*R*,8″*S*.

Carthatin E (**5**) has the same molecular formula and 1D NMR spectroscopic data as **1**. Moreover, an interpretation of the HMBC and $^1H-^1H$ COSY spectra indicated that the planar structure of compound **5** resembles that of **1**. However, a detailed analysis of their ^{13}C NMR (Table 1) spectra showed the upfield shift of C-8″ and C-9″ in **5**. Meanwhile, the ROESY spectra of **5** displayed the H-7″/H-8″ correlation, but the spectra of **1** displayed the H-7″/H-9″ correlation. Furthermore, the three-dimensional (3D) molecular structures of **1** and **5** were built using ChemBio 3D (version 14.0), refined by geometry optimization, and subjected to conformational analysis using molecular dynamics simulations and minimize energy calculation (Figure 4). A comprehensive visualization of

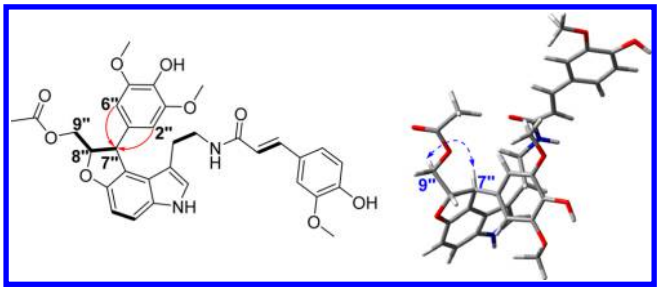

Figure 4. Key HMBC (solid arrows), $^1H-^1H$ COSY (thick bonds), and ROESY (dashed arrows) correlations of (±)-carthatin F (**6**).

the *J* coupling relationship was achieved, allowing for a ready distinction between the *J* patterns of **1** and **5**. The *J* value of 6.6 Hz representing the coupling of H-7″ with H-8″ in **5** was in particular agreement with the conformationally averaged 20° dihedral angle calculated from a molecular model. Similarly, the observed *J* value of 1.9 Hz between H-7″ and H-8″ in **1** was consistent with the 120° H-7″−C-7″−C-8″−H-8″ dihedral angle calculated for **1**. Thus, compounds **1** and **5** were diastereomers, and **5** was also a pair of racemes on the basis of its low optical rotation and chiral HPLC spectrum (see the Supporting Information). The structure of **5** was finally determined, and the absolute configurations at the stereogenic centers in **5** were 7″*R*,8″*R* or 7″*S*,8″*S*.

Analysis of the HRESIMS, ^{13}C NMR, and DEPT spectra showed that (±)-carthatin F (**6**) has the molecular formula $C_{33}H_{34}N_2O_9$. The IR, UV absorption, and 1D NMR data of **6** were similar to those of **1**, with differences in the chemical shifts of C-2″, C-6″, C-7″, C-8″, and C-9″. The key HMBC correlations (Figure 4) from H-2″ and H-6″ to C-7″ (δ 52.3), from H-7″ [δ 4.76 (d, *J* = 5.2 Hz)] to C-4, and from H-8″ [δ 4.61 (m)] to C-5, combined with the $^1H-^1H$ COSY cross-peaks of H-7″/H-8″/H-9″, indicated the presence of a carbon−carbon single bond between C-7″ and C-4 and an ether bond between C-8″ and C-5 rather than the single bond between C-7″ and C-5 and the ether bond between C-8″ and C-4 in **1**. In the ROESY spectrum (Figure 4) of **6**, a H-7″/H_2-9″ correlation illustrated that H-7″ and CH_2-9″ were cofacial. Compound **6** is also racemic on the basis of its low optical rotation and chiral HPLC analysis. Thus, absolute configurations at the stereogenic centers of **6** were assigned to be 7″*S*,8″*S* or 7″*R*,8″*R*.

Four known compounds were isolated and identified as sinapyl alcohol (**7**),[18] coniferyl alcohol (**8**),[19] serotobenine (**9**),[20] and feruloylserotonin (**10**)[21] by comparing their spectroscopic properties with those previously reported for these substances.

Hypothetical Biogenetic Pathway of (±)-Carthatins A−F. (±)-Carthatins A−F (**1**−**6**, respectively) were a series of dimers involving a sinapyl alcohol (**7**) or a coniferyl alcohol (**8**) and a feruloylserotonin (**10**) connected via a dihydrofuran ring. Serotobenine (**9**), which was first isolated in 1985 and later in 1997,[12] was co-isolated with compounds (±)-**1**−**6** from the oil cake of safflower seed. Many synthetic chemists have confirmed

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that the dihydrobenzofuran fraction in **9** was facilitated by the key oxidative [3+2] cycloaddition,[15,22,23] which also resulted in the formation of racemic serotobenine (**9**). Thus, we deduced that compounds **1**−**6** were also formed between sinapyl alcohol (**7**) or coniferyl alcohol (**8**) and feruloylserotonin (**10**) through the oxidative [3+2] cycloaddition in the light (Scheme S1).

AChE Inhibitory Activity. The anti-AchE activities of compounds **1**−**10** were evaluated, and the results (Table 2) showed that compound (±)-**1** displayed significant inhibitory activity with an IC_{50} value of 17.96 μM and (±)-**2** had weak inhibition (IC_{50} = 66.83 μM). By comparison of the structural characteristics between active compounds (±)-**1** and (±)-**2** and inactive compounds (±)-**3**−(±)-**6**, a preliminary structure−activity relationship is discussed. (1) When two hydrogen atoms at C-7″ and C-8″ are on the opposite side, (2) when the geometry of the double bond between C-7′ and C-8′ is *E*, or (3) when the acetyl is located at the hydroxyl of C-9″, compounds (±)-**1** and (±)-**2** show inhibitory activities. However, compared to that of (±)-**1**, the level of inhibition of (±)-**2** is decreased because of the absence of a methoxyl of phenylpropanoid fraction in (±)-**2**. Additionally, compared to that of (±)-**1**, (±)-**6** has a different way of forming the dihydrofuran ring between the phenylpropanoid fraction and the feruloylserotonin moiety, which may be the key factor that affects their activity.

Table 2. Anti-AChE Activities of (±)-Carthatins A and B (1 and 2, respectively)

	IC_{50} (μM)
(±)-**1**	17.96
(±)-**2**	66.83
TA[a]	0.35

[a]Tacrine, positive control for anti-AChE activity.

In conclusion, a series of hybrid dimers, (±)-carthatins A−F (**1**−**6**, respectively), which had a phenylpropanoid fraction and a feruloylserotonin moiety fused via a dihydrofuran ring, were isolated from the safflower oil cake. Moreover, compounds (±)-**1** and (±)-**2** showed anti-AChE activities relating to Alzheimer's disease, suggesting that these compounds play a role in improving memory and cognitive ability. In recent years, studies have found that Alzheimer's disease patients exhibit depression and memory impairment; dual inhibitors of AChE and serotonin transporter (SERT) would be a better therapeutic method.[24] Therefore, in the future, we will test the inhibitory activities of compounds (±)-**1** and (±)-**2** against SERT and study their molecular docking with AChE to search for the key active molecules that could provide a cure for Alzheimer's disease.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03431.

NMR spectra of compounds (±)-**1**−**6**, CD spectra of (±)-**1a** and (±)-**2a**, chiral HPLC spectra of (±)-**5a** and (±)-**6a**, and a plausible biosynthetic pathway of compounds (±)-**1**−**6** (PDF)

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Notes

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