Antioxidant Activity of Ecdysteroids from *Serratula strangulata*

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One new ecdysteroid, (24*R*)-24-(2-hydroxyethyl)-20-hydroxyecdysone (3), as well as three known ecdysteroids, has been isolated from Chinese herb *Serratula strangulata* and these compounds 1—4 showed effective antioxidant activity on AAPH-induced hemolysis of human RBC and Fe^{2+} + cysteine-induced lipid peroxidation of liver microsome.

Keyword *Serratula strangulata*, ecdysteroid, antioxidant activity

Introduction

Ecdysteroids (insect molting hormones) are also present in many plant species[1-4] where they are thought to provide some degree of protection against phytophagous insects.[1] Besides this, ecdysteroids have interesting pharmacological effects on mammals, including a stimulation of protein synthesis, a reduction of blood glucose and cholesterol levels.[4,5] Phytoecdysteroids have been reported in many species of the family Compositae, including the *Serratula* genus which was earlier shown to contain 20-hydroxyecdysone,[6-12] sogdisterone,[13] integristerone A,[10] viticosterone E[8,10,11] and ecdysone.[11,14] Preliminary studies with *Serratula strangulata* have shown the presence of 20-hydroxyecdysone (**1**) and 25-deoxy-11,20-dihydroxyecdysone (**2**) at high concentrations, and 20-hydroxyecdysone-20,22-monoacetonide (**4**)[15] and a new ecdysteroid, (24*R*)-24-(2-hydroxyethyl)-20-hydroxyecdysone (**3**) (Fig. 1).

A large body of clinical and experimental evidence has showed that free radical induced peroxidation of membrane lipids is associated with a variety of chronic health problems, such as cancer, aging and atherosclerosis. Natural antioxidants are becoming increasingly important in applied science, including food and medicine. In searching for new natural antioxidants, which would be one of the sources of weapons to combat some diseases, we found that these compounds possess antioxidant activity, which may be useful to treat certain diseases.

Results and discussion

The chopped dry whole plant of *Serratula strangulata* was extracted by alcohol followed by carefully column chromatographic separation giving compounds **1**—**4** respectively. Compound **3** was obtained as a white solid. The molecular formula $C_{29}H_{48}O_8$ was determined by FABMS *m/z* 525 $[M+H]^+$ and HREIMS *m/z* 524.3341, calcd for 524.3349). Bands at 3387 and 1642 cm^{-1} in the IR spectrum are indicative of a hydroxyl group and a conjugated carbonyl group, respectively. Its ^1H NMR spectrum showed an olefinic proton as a singlet at δ 5.61 and two hydroxymethylene protons [δ: 3.20—3.26 (m, 2H, H-29)] and three hydroxymethine proton [δ: 3.81—3.85 (m, 1H, H-2), 3.92—3.96 (m, 1H, H-3), 3.41—3.44 (m, 1H, H-22)], as well as five methyl singlets at δ 0.89, 0.96, 1.10, 1.20, 1.21. A comparison with **1**, a known ecdysterone, suggested that both compounds were very similar except that an additional hydroxyl ethyl group was present. The ^1H-^1H COSY and HMQC spectram revealed a partial structure: -CH(22)-CH_2(23)-CH(24)-CH_2(28)-CH_2(29)-. The locations were further confirmed by H-24, H-26 and H-27 correlating to C-25 (δ 68.7) in the HMBC spectrum of **3**. The configuration of the C-24 hydroxyethyl group in **3**

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was deduced by comparison of the ^{13}C chemical shift of the side chain carbons with those of a series of sterols having *R*- and *S*-configurations at C-24, particularly (24*S*)-24-ethyl-cholest-5-en-3β-ol and (24*R*)-24-ethyl-cholest-5-en-3β-ol[16-19] showing a close proximity of the chemical shifts to the 24*R*-series. Based on the above information, the structure of compound **3** was elucidated as (24*R*)-24-(2-hydroxyethyl)-20-hydroxyecdysone.

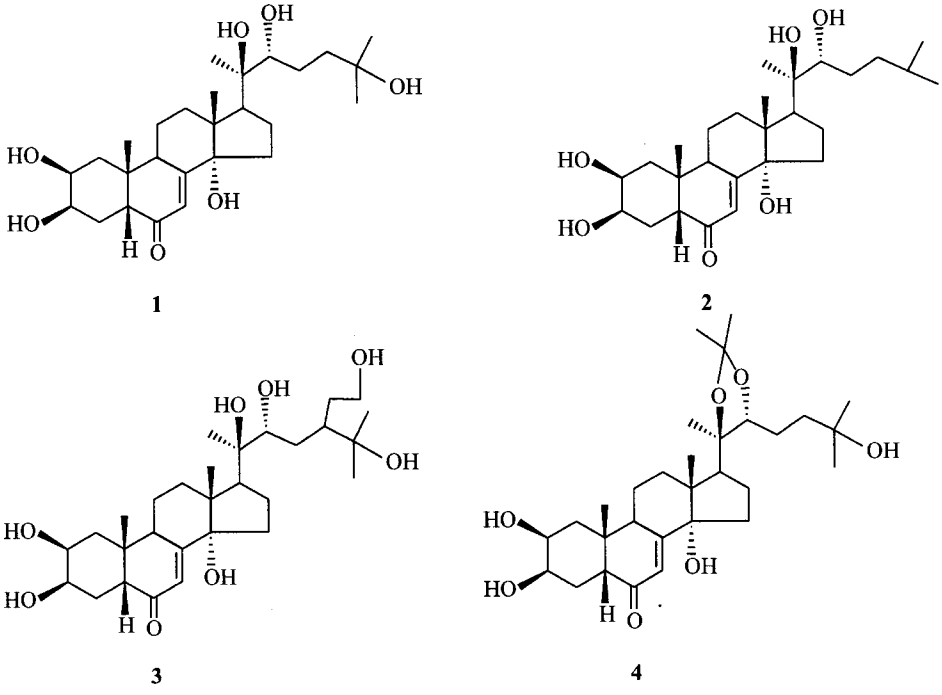

Fig. 1 Structure of compounds **1**—**4**.

The identity of **1**, **2** and **4** was established by comparison of spectroscopic data with those of the authentic compound previously isolated from other species.[16]

The occurrence of ecdysterone is of considerable biogenetic interest. Compounds **1**—**4** showed effective antioxidant activity on AAPH-induced hemolysis of human RBC[20] and Fe^{2+} + cysteine-induced lipid peroxidation of liver microsome.[21]

2,2′-Azobis(2-amidinopopane hydrochloride) (AAPH) has often been used as a water-soluble radical initiator at ambient temperature, and it generates carbon radical primarily and constantly through thermal decomposition, and the radicals thus formed react with oxygen rapidly to give peroxyl radicals. Thus the azo compound is therefore a useful tool for studying the cell damage induced by free radicals. Presented here is the study on **1**, **2**, **3** or **4** on AAPH initiated peroxidation of human red blood cells (RBC) in PBS (10 mol/L of phosphate-buffered saline) (pH 7.4) at 37 ℃. It is found that these compounds have distinctive antioxidative activity (Table 1), when hemolysis is initiated with 51.6 mmol/L of AAPH. The lag time caused by 40 μmol/L of **1**—**4** and GSH (glutathione) are 162.0 ± 2.0, 144.0 ± 4.0, 171.6 ± 5.0, 137.8 ± 6.5 and 114.7 ± 3.5 min, respectively, which gives an anti-hemolysis efficiency sequence of **3** > **1** > **2** > **4** > GSH.

Table 1 Effect of **1**—**4** on lag phase of human RBC oxidation hemolysis induced by AAPH[a]

Group	Lag Phase (min)
Control	99.3 ± 2.5
1	162.0 ± 2.0^{+++}
2	144.3 ± 4.0^{+++}
3	171.6 ± 5.0^{+++}
4	137.8 ± 6.5^{+++}
GSH	114.7 ± 3.5^{+++}

[a] The incubation mixture contained 5% human RBC in PBS (pH 7.4). Oxidation was initiated by addition of 51.6 mmol/L AAPH, **1**, **2**, **3** or **4** was added in 20 μL of DMSO and final concentration was 40 μmol/L. Each reaction was carried out after preincubation at 37 ℃ with gentle shaking. Values represent mean ± S. E of three experiments. $^{+++}p < 0.01$, as compared to control.

Table 2 Effect of **1**—**4** on rat liver microsomal lipid peroxidation induced by $FeSO_4$ + cysteine system in *vitro*[a]

Group	MDA (nmol/mg protein)	Inhibition rate (%)
$FeSO_4$ + cysteine	3.622 ± 0.085	100
$FeSO_4$ + cysteine + **1** (mmol/L)		
3.2	1.037 ± 0.049^{+++}	71.4
1.6	2.083 ± 0.085^{+++}	42.5
0.8	2.564 ± 0.130^{+++}	29.2
0.4	2.724 ± 0.085^{+++}	24.8
$FeSO_4$ + cysteine + **2** (mmol/L)		
3.2	1.378 ± 0.085^{+++}	62.0
1.6	2.404 ± 0.085^{+++}	33.6
0.8	3.013 ± 0.032^{+++}	16.8
0.4	3.109 ± 0.115^{+++}	14.2
$FeSO_4$ + cysteine + **3** (mmol/L)		
3.2	0.671 ± 0.168^{+++}	81.5
1.6	1.464 ± 1.193^{+++}	59.6
0.8	2.030 ± 0.103^{+++}	44.0
0.4	2.528 ± 0.409^{+++}	36.5
$FeSO_4$ + cysteine + **4** (mmol/L)		
3.2	1.334 ± 0.174^{+++}	63.2
1.6	2.553 ± 0.161^{+++}	29.5
0.8	3.173 ± 0.210^{+++}	12.4
0.4	3.575 ± 0.130^{+++}	1.3

[a] System compositon and incubation condition are described in the text. The values represent mean ± S.E of three experiments. $^{+++}p < 0.01$, as compared to control.

Iron (Fe^{2+} plus a reducing reagent) is one commonly used system for generating ROS (reactive oxygen species), causing lipid peroxidation in biomembranes. Using this system, the effect of **1**, **2**, **3** or **4** on lipid peroxidation of rat liver microsomes was first tested. It was found that compounds **1**—**4** inhibited MDA (malondialdehyde) production in a dose-dependent manner (Table 2).

Because the compounds **1**—**4** possess close antioxidant activity, we conclude that their antioxidant functions probably happen on enone functions.

Experimental

Optical rotation was measured on a Perkin-Elmer 241 polarimeter. The IR spectrum was taken on a Nicolet 170SX IR spectrometer. The UV spectrum was run on a Shimadzu UV-260 visible recording spectrometer. ^1H, ^{13}C and 2D NMR spectra were recorded on a Bruker AM 400 NMR spectrometer with TMS as internal standard. HREIMS spectrum was obtained on a VG ZAB-HS mass spectrometer.

Extraction and isolation procedure

The whole plant of *S. strangulata* was collected in Zhuanglang county, Gansu, China. The chopped whole plant material (3.0 kg) was extracted repeatedly (3 times, 7 days each time) with alcohol at room temperature to give a residue (90 g) after evaporation. This residue was suspended in H_2O and extracted with petroleum ether, EtOAC and BuOH, respectively. The EtOAC extract (40 g) was obtained and chromatographed on a silica gel column (200—300 mesh, 800 g) with a gradient (2000 mL each eluant) of petroleum ether-acetone (30:1, 20:1, 15:1, 10:1, 5:1, 3:1, 1:1, 0:1). From the fraction of petroleum ether-acetone (5:1, 10 g), a crude material was obtained and separated on CC (columu chromatography) over 200 g silica gel (200—300 mesh) with $CHCl_3$-MeOH (25:1, 1000 mL) to give two subfractions A and B. Fraction A (5.0 g) was placed on CC over 50 g silica gel (200—300 mesh) with petroleum ether-AcOEt (4:1, 350 mL) to give crude **1** and **2**. Fraction B (3.5 g) was separated on CC over 40 g silica gel (200—300 mesh) with petroleum ether-AcOEt (4:1, 250 mL) to give crude **3** and **4**. Subsequently, each of them was purified by rechromatograph on a silica gel column (300—400 mesh) with petroleum ether-Me_2CO (6:1) to give compounds **1** (500 mg), **2** (350 mg), **3** (25 mg) and **4** (20 mg).

24-(2-Hydroxyethyl)-20-hydroxyecdysone (**3**)

White solid, m.p. 242—244 ℃ ($CHCl_3$-MeOH); $[\alpha]_D^{24}$ +65.2 (*c* 0.04, MeOH); IR (KBr) ν_{max}: 3387 (OH), 1642 (C=C—C=O) cm^{-1}; UV-vis (MeOH): λ_{max}: 252 nm (TM 10000) (C=C—C=O); FABMS: 525 $[M+1]^+$, 510, 507, 492,389, 371, 189, 171, 145, 127. HREIMS (found 524.3341, calcd for $C_{29}H_{48}O_8$ 524.3349); ^1H NMR (DMSO-d_6, 400 MHz) δ: 5.61 (s, 1H, H-7), 3.92—3.96 (m, 1H, H-3α), 3.81—3.85 (m, 1H, H-2α), 3.41—3.44 (m, 1H, H-22β), 3.20—3.26 (m, 2H, H-29), 3.14—3.18 (m, 1H, H-9), 2.27—2.41 (m, 1H, H-17), 2.37 (dd, *J* = 12, 5 Hz, 1H, H-5), 2.10—2.16 (m, 1H, H-12_a), 1.97—2.03 (m, 2H, H-15_a, 16_a), 1.84—1.90 (m, 1H, H-12_e), 1.70—1.84 (m, 4H, H-1_e, H-4_e, H-11_e, H-16_e), 1.61—1.68 (m, 2H, H-4_a, H-11_a), 1.52—1.58 (m, 2H, H-15_e, 23_e), 1.45—1.55 (m, 1H, H-24), 1.41—1.45 (m, 1H, H-23_a), 1.37—1.40 (m, 2H, H-28), 1.30—1.35 (m, 1H, H-1_a), 1.21 (s, 3H, H-27), 1.20 (s, 3H, H-26), 1.19 (s, 3H, H-21), 0.96 (s, 3H, H-19), 0.89 (s, 3H, H-18); (DMSO-d_6, 100 MHz, TMS) (C-1 to C-29) δ: 36.59, 66.56, 66.74, 31.49, 50.50, 202.59, 120.41, 165.15, 33.15, 37.58, 20.04, 30.26, 46.62, 82.95, 30.82, 20.22, 48.65, 17.07, 23.81, 76.18, 20.92, 75.60, 26.06, 36.55, 68.66, 28.97, 29.92, 41.34, 66.06.

Antioxidant assay

Effect on AAPH-*induced hemolysis of human* RBC

Human red blood cells (RBC) were separated from heparinized blood that was draw from a healthy donor, then was washed three times with PBS (pH 7.4). 5% suspension of RBC was preincubated at 37 ℃ for 5 min, to which an water-soluble initiator 2,2′-azobis (2-amidinopopane hydrochloride) (AAPH) was added to initiate hemolysis. At specific intervals, aliquots of the reaction mixture was taken out, diluted with 10 vol. 0.15 mol/L NaCl, and centrifuged at 1000 × g for 10 min, the absorbance *A* of the supernatant at 540 nm was measured. Similarly, the reaction mixture was distilled in water to make complete hemolysis and the absorbance B of the supernatant after centrifugation was measured at 540 nm, too. Percentage hemolysis was calculated from the ratio of the measurements, $(A/B) \times 100$. The experiments were repeated at least three times. In the case of antioxidation experiments, **1** or **2** was added and preincubated before addition of AAPH. The lag phase of human RBC oxidation hemolysis was calculated as described by Esterbauer.[22]

Effect on Fe^{2+} + *cysteine-induced lipid peroxidation of liver microsome*

Female Wister rats weighing 250 ± 12 g were starved overnight before decapitation. The livers were perfused with ice-cold STE buffer (0.25 mol/L sucrose-0.01 mol/L Tris buffer, pH 7.4—10 mmol/L EDTA). Liver microsomes were prepared and microsomal protein was determined by the method of Lowry.[17] Incubation mixture (1 mL) contained 1 mg of microsomal protein in PBS (pH 7.4), $FeSO_4$ (50 μmol/L), cysteine (200 μmol/L), **1** (10 μL) or **2** (10 μL) in DMSO or only DMSO. After incubation for 30 min at 37 ℃, a stock reagent (TCA-TBA-HCl) was added to the reaction mixture. MDA in the supernatant after centrifugation was determined at 535 nm.

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