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Sweritranslactone D, a hepatoprotective novel secoiridoid dimer with tetracyclic lactone skeleton from heat-transformed swertiamarin

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ABSTRACT

Swertia mileensis, known as Qing-Ye-Dan (QYD), has been documented in Chinese Pharmacopoeia to cure hepatitis. Interestingly, its announced main active component, swertiamarin, could not be detected in the decoction, which indicated that the efficacy of QYD might be attributed to heat-transformed products of swertiamarin (HTPS). Further investigation on HTPS led to the isolation of sweritranslactone D (**1**), a novel secoiridoid dimer possessing a tetracyclic lactone skeleton, with better hepatoprotective activity than *N*-acetyl-L-cysteine in vitro.

1. Introduction

Swertia mileensis, well-known in China under the name "Qing-Ye-Dan" and documented in Chinese Pharmacopoeia (1977–2020 editions) for curing viral hepatitis, belongs to the genus *Swertia* of family Gentianaceae [1]. Clinical reports revealed that Qing-Ye-Dan tablets, prepared from the boiling water extract of *S. mileensis*, showed curing rate up to 95.3% for 422 patients and 96.8% for 93 patients, respectively, to cure acute hepatitis with high alanine transaminase (ALT) and aspartate transaminase (AST) levels [2]. Swertiamarin, an iridoid glycoside, is generally considered to be the main active component of *S. mileensis* to treat hepatitis [3]; its content in the whole plant of *S. mileensis* is up to 12%, and beyond 50% in its cold-water extract [4–9]. Owing to structural instability of iridoid glycosides, swertiamarin was not detected 6 h later, in both Qing-Ye-Dan (QYD) decoction and heat-transformed products from swertiamarin (HTPS), by TLC method after heat treatment in water. Hence, the actual active components of QYD might be HTPS. Based on this hypothesis, we evaluated the hepatoprotective activity of swertiamarin, QYD tablets, and HTPS via serum ALT and AST analyses in mice model of acute liver injury by carbon tetrachloride (CCl_4). Results showed that both the low- and high-dose groups of HTPS significantly alleviated liver injury by decreasing the levels of serum ALT and AST. Further investigations on HTPS led to the isolation of a novel tetracyclic lactone iridoid dimer, sweritranslactone D (**1**) (Fig. 1), along with a new analogue (**2**) and 7 known compounds. Their structures were established on the basis of extensive spectroscopic methods and X-ray crystallographic diffraction analysis. The plausible transformation pathways of these products are also proposed. Furthermore, these compounds were evaluated for hepatoprotective activity in vitro in L-O2 cell model induced by 4-acetamidophenol (AP), with N-Acetyl-L-cysteine (NAC) as positive control. All the isolates exhibited hepatoprotective activity, and interestingly, compound **1** showed a potent activity compared to the positive control.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. All the 1D and 2D NMR spectra were recorded on Bruker DRX-600 spectrometer. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. ESI-MS and HR-ESI-MS analyses were carried out on Waters Xevo TQS and Waters AutoSpec Premier P776 mass spectrometers, respectively. Silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Co., Ltd., China), and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) were used for column chromatography. Fractions were monitored by TLC (GF 254, Qingdao Marine Chemical Co., Ltd.), and the spots were visualized by 10% H_2SO_4/EtOH reagent.

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2.2. Preparation and isolation

The mixture of swertiamarin (5.8 kg) in water (17.5 kg) was heated under reflux conditions for 6 h to obtain an aqueous mixture (15 kg). A portion of the mixture (5 kg) was subjected to medium pressure liquid chromatography (MPLC) column (5.6 × 48.0 cm) with gradient elution ($MeOH/H_2O$, v/v, from 5 to 100%) to get 5 fractions A-E on the basis of TLC detection. Fr. B (250 g) was chromatographed on macroreticular resin column (5.0 × 100.0 cm), eluted with gradient solvent system of $MeOH/H_2O$ (0–100%, v/v) to yield 9 subfractions (Fr. B1–B9). Fr. B4 (206 g) was chromatographed on silica gel column (5.0 × 65.0 cm) with gradient elution (petroleum ether/ethyl acetate, 8:1–2:1, v/v) to get 12 fractions (Fr. B4-1–B4-12). Compound **4** (17.8 g) was obtained by recrystallization from Fr. B4-2. Compounds **5** (3.1 g) and **3** (700 mg) were isolated through MCI column, eluted with $MeOH/H_2O$ (30–100%, v/v) and $MeOH/H_2O$ (20–100%, v/v), from Fr. B-4 and Fr. B-5, respectively. Compounds **2** (100 mg) and **6** (1.5 g) were obtained by recrystallization from Fr. B4-5 and Fr. C (65 g), respectively. The rest of Fr. C was subjected to macroreticular resin column (5.0 × 76.0 cm), eluted with gradient solvent system of $MeOH/H_2O$ (0–100%, v/v) to yield 8 subfractions (Fr. C1–C8). Fr. C6 was chromatographed over MCI column ($MeOH/H_2O$, 0–100%, v/v) and silica gel column (4.8 × 26.0 cm, petroleum ether/ethyl acetate, 8:1–2:1, v/v) to get compound **8** (105 mg) and **9** (45 mg). Fr. D (48.3 g) was separated into 15 subfractions by using silica gel column (10.0 × 60.0 cm). Compounds **1** (10 mg) and **7** (2.7 g) were obtained from Fr. D13 and Fr. D3 through MPLC ($MeOH/H_2O$, 75:25, v/v) and silica gel column chromatography (petroleum ether/acetone, 95:5, v/v).

2.2.1. Sweritranslactone D (1)

Colorless cubic crystals (MeOH); $[\alpha]_D^{23}$ −5.2 (*c* 0.10, $CHCl_3$/MeOH, v/v = 1:1); ^1H and ^{13}C NMR data: see Table 1; HR-ESI-MS *m/z* 369.1308 $[M + Na]^+$ (Calcd for $C_{19}H_{22}O_6Na$, 369.1309).

2.2.2. Sweritranslactone E (2)

Colorless cubic crystals (MeOH); $[\alpha]_D^{23}$ −4.3 (*c* 0.10, $CHCl_3$/MeOH, v/v = 1:1); ^1H and ^{13}C NMR data: see Table 1; HR-ESI-MS *m/z* 333.1349

Table 1
^{13}C (150 MHz) and ^1H (600 MHz) NMR spectroscopic data of **1** (Chloroform-d_1) and **2** (DMSO-d_6).

Position	1		2	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
3	94.2, CH	5.28, s	69.3, CH	3.84, overlap
4	125.5, C		52.1, C	
5	154.4, C		141.0, C	
6	31.4, CH_2	2.83, m; 2.20, m	124.4, CH	6.10, dd (6.1, 2.9)
7	65.6, CH_2	4.32, m	66.8, CH_2	4.73, dd (15.6, 6.0); 5.08, d (15.6)
8	31.3, CH	2.39, m	71.1, CH	3.97, dd (6.0, 6.2)
9	40.6, CH	2.03, dd (2.9, 11.4)	47.3, CH	2.32, br s
10	17.3, CH_3	1.03, d (7.1)	20.3, CH_3	0.86, d (6.0)
11	162.5, C		171.2, C	
12	56.2, CH_3	3.59, s		
3′	60.6, CH	5.00, d (3.0)	72.8, CH	3.84, overlap
4′	123.7, CH		44.7, C	
5′	157.1, CH		127.5, C	
6′	28.3, CH_2	2.64, m; 2.23, m	36.6, CH_2	3.21, br s; 3.16, br s
7′	65.3, CH_2	4.39, m	171.6, C	
8′	120.9, CH_2	5.34, dd (1.1, 10.2); 5.16, dd (1.1, 17.0)	36.5, CH	2.65, br s
9′	52.0, CH	2.88, dd (4.9, 8.7)	126.5, CH	5.33, br s
10′	132.3, CH	5.63, ddd (8.7, 10.1, 17.0)	21.6, CH_3	0.97, d (7.2)
11′	163.5, C		72.6, CH_2	4.36, d (11.0); 4.26, d (11.0)

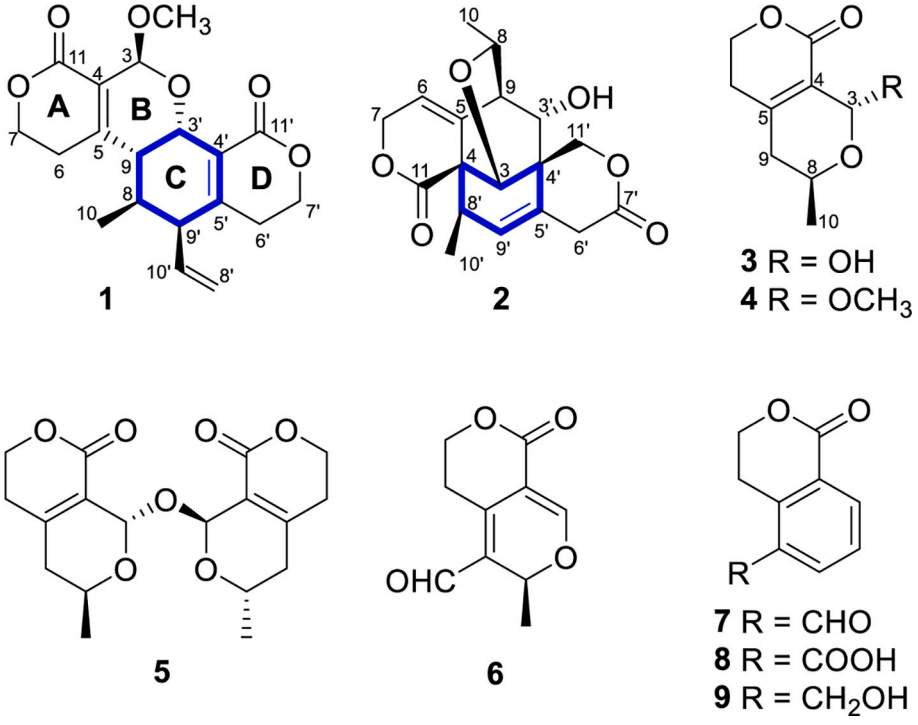


Fig. 1. Chemical structures of compounds **1**–**9**.

$[M + H]^+$ (Calcd for $C_{18}H_{21}O_6$, 333.1333).

2.2.3. Crystal data

Crystal data for sweritranslactone D (**1**): $C_{19}H_{22}O_6$, M = 346.37, monoclinic, a = 16.603(3) Å, b = 7.2973(11) Å, c = 14.317(2) Å, α = 90.00°, β = 104.624(2)°, γ = 90.00°, V = 1678.3(4) Å^3, T = 100(2) K, space group $P21/c$, Z = 4, μ(Mo Kα) = 0.102 mm^{-1}, 15,656 reflections measured, 4038 independent reflections (R_{int} = 0.0351). The final R_1 values were 0.0372 ($I > 2\sigma(I)$). The final w$R(F^2)$ values were 0.0920 ($I > 2\sigma(I)$). The final R_1 values were 0.0512 (all data). The final $wR(F^2)$ values were 0.1062 (all data). The goodness of fit on F^2 was 1.087. (CCDC 1500816).

Crystal data for sweritranslactone E (**2**): $C_{18}H_{20}O_6$, M = 332.34, monoclinic, a = 12.076(3) Å, b = 15.221(3) Å, c = 8.4687(17) Å, α = 90.00°, β = 106.346(3)°, γ = 90.00°, V = 1493.6(5) Å^3, T = 100(2) K, space group $P21/c$, Z = 4, μ(Mo Kα) = 0.111 mm^{-1}, 13,632 reflections measured, 3577 independent reflections (R_{int} = 0.0472). The final R_1 values were 0.0449 ($I > 2\sigma(I)$). The final w$R(F^2)$ values were 0.1234 ($I > 2\sigma(I)$). The final R_1 values were 0.0639 (all data). The final $wR(F^2)$ values were 0.1369 (all data). The goodness of fit on F^2 was 1.043. (CCDC 978382).

Crystal data for Swerimilegenin E (**4**): $C_{10}H_{14}O_4$, M = 198.21, triclinic, a = 7.5840(11) Å, b = 8.2096(11) Å, c = 8.3996(12) Å, α = 82.695(2)°, β = 73.440(2)°, γ = 72.745(2)°, V = 478.20(12) Å^3, T = 100 (2) K, space group P-1, Z = 2, μ(Mo Kα) = 0.106 mm^{-1}, 5038 reflections measured, 2608 independent reflections (R_{int} = 0.0234). The final R_1 values were 0.0389 ($I > 2\sigma(I)$). The final w$R(F^2)$ values were 0.1077 ($I > 2\sigma(I)$). The final R_1 values were 0.0405 (all data). The final $wR(F^2)$ values were 0.1093 (all data). The goodness of fit on F^2 was 1.110. (CCDC 978476).

Crystal data for Swerimilegenin G (**5**): $C_{18}H_{22}O_7$, M = 350.36, monoclinic, a = 9.4346(6) Å, b = 8.3218(5) Å, c = 21.8117(14) Å, α = 90.00°, β = 101.3520(10)°, γ = 90.00°, V = 1679.00(18) Å^3, T = 100(2) K, space group $P21/c$, Z = 4, μ(Mo Kα) = 0.107 mm^{-1}, 17,390 reflections measured, 4761 independent reflections (R_{int} = 0.0224). The final R_1 values were 0.0363 ($I > 2\sigma(I)$). The final w$R(F^2)$ values were 0.0937 ($I > 2\sigma(I)$). The final R_1 values were 0.0414 (all data). The final $wR(F^2)$ values were 0.0973 (all data). The goodness of fit on F^2 was 1.044. (CCDC 950258).

2.3. Detailed description of hepatoprotective studies in CCl_4-induced liver injury mice model

2.3.1. Model of CCl_4-induced acute liver injury in mice

After 5 days of adaptive feeding with food and water ad libitum, 96 mice were randomly divided into eight groups (n = 12): Normal group (Normal, olive oil, 0.1 mL/kg/day by intragastrical route); CCl_4 group (Model, olive oil, 0.1 mL/kg/day by intragastrical route); the positive group (DBB, dimethyl diphenyl bicarboxylate, 150 mg/kg/day); Qing-Ye-Dan tablet group (QYD, 10.5 g/kg/day); low dose of swertiamarin group (L-SW, 0.52 g/kg/day); high dose of swertiamarin (H-SW, 1.05 g/kg/day); low dose of HTPS (L-HTPS, 0.42 g/kg/day); high dose of HTPS (H-HTPS, 0.84 g/kg/day). Animal treatment was continued for 7 consecutive days. After 1 h of the last administration, all mice were intraperitoneally injected with CCl_4 (0.1 mL of 0.12% CCl_4 in olive oil/10 g body weight) except normal group. Mice were executed after 24 h of injection and the blood was collected from the orbit in sodium heparin tubes [10].

2.3.2. Detection of serum transaminases ALT and AST

As described previously [11], the serum transaminases ALT and AST levels were determined by using assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

2.4. Detailed description of hepatoprotective studies in AP-induced L-O2 cell model

2.4.1. Model of AP-induced L-O2 cells

The L-O2 cells were cultured in the exponential growth phase; the model was established successfully under the conditions of AP at a concentration of 5 mmol/mL for 48 h, and then the cells were treated with different concentrations of the isolates from HTPS (0.8 μg/mL, 1.6 μg/mL, 3.1 μg/mL, 6.3 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL) at a density of 5 × 10^4 cells/100 μL per well and incubated for 24 h at 37 °C in 5% CO_2 incubator [12].

2.4.2. Detection of the survival rate of AP-induced L-O2 cells

The L-O2 cell survival rate was measured by an assay based on the cleavage of yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to form purple formazan crystals in viable cells. The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The cells were then treated with 10% (v/v) MTT dye solution (5 mg/mL) for 4 h. The medium of MTT solution was replaced with DMSO (100 μL). The 96-well culture plates were then gently shaken in the dark for 30 min, and the absorbance at 570 and 630 nm (background) was measured with a microtiter plate reader. The positive (cells treated with different concentrations of NAC at 0.25–4 mg/mL) controls were run in parallel. All the assays were carried out in triplicate.

3. Results and discussion

Sweritranslactone D (**1**) was obtained as colorless cubic crystals (MeOH). Its molecular formula $C_{19}H_{22}O_6$ was established by ^{13}C NMR and HR-ESI-MS data (m/z 369.1308, $[M + Na]^+$, calcd 369.1309) with nine double-bond equivalents. The ^{13}C NMR spectroscopic data (Table 1) exhibited 19 carbon resonance signals due to two methyls (including one methoxy), five methylenes (one with terminal double bond), six tertiary carbons (one with an olefinic carbon), and six quaternary carbons (two ester carbonyls and four olefinic carbons). Thus, a tetracyclic ring structure was proposed for compound **1** to satisfy nine degrees of unsaturation.

Comprehensive interpretation of 2D NMR spectroscopic data, including ^{1}H—^{1}H COSY, HSQC, and HMBC, allowed the establishment of planar structure of **1**. In ^{1}H—^{1}H COSY spectrum, cross peaks of H_2-6 (δ_H 2.20, 2.83)/H_2-7 (δ_H 4.32), H-3′ (δ_H 5.00)/H-9 (δ_H 2.03)/H-8 (δ_H 2.39) (H_3-10, δ_H 1.03)/H-9′ (δ_H 2.88)/H-10′ (δ_H 5.63)/H_2-8′ (δ_H 5.16, 5.34), and H_2-6′ (δ_H 2.23, 2.64)/H_2-7′ (δ_H 4.39) suggested the presence of three key fragments (C-6/C-7), (C-3′/C-9/C-8(C-10)/C-9′/C-10′/C-8), and (C-6′/C-7′) (Fig. 2). In the HMBC spectrum of **1**, correlations of H_2-7 with C-11 (δ_C 162.5) and C-5 (δ_C 154.4), of H_2-6 with C-4 (δ_C 125.5), of H_2-7′ with C-11′ (δ_C 163.5) and C-5′ (δ_C 157.1), and of H_2-6′ with C-4′ (δ_C 123.7) indicated two separately constructed α,β-unsaturated δ-lactone rings (A and D) (Fig. 2). The HMBC correlations of H-3 (δ_H 5.28)/C-5 and C-3′ (δ_C 60.6), and of H-9/C-4 led to B ring. The presence of ring C was identified with cross peaks of H-3′/C-5′ and H-9′/C-4′ in the HMBC spectrum of **1**. Rings A, B, C, and D were fused successively on the basis of HMBC correlations from H-3 to C-11, from H-6 to C-9, from H-3′ to C-11′, from H-9′ to C-6′ and from H-10′ to C-5′. Owing to the HMBC correlation of H_3-12 (δ_H 3.59)/C-3, the methoxy group (C-12, δ_C 56.2) was placed at C-3. Thus, the planar structure of compound **1** was established as an iridoid dimer derivative having 6/6/6/6 tetracyclic architecture together with two α,β-unsaturated δ-lactone rings, which is unprecedented.

The relative configuration of **1** was deduced from its ROESY spectrum. As depicted in 3D molecular model (Fig. 2), the correlations of H-9/H_3-10 and H-10′, and of H-3′/H_3-12 implied that the orientations of H-9, H_3-10 and H-10′, and of H-3′ and H_3-12, were at the same side, respectively. Furthermore, a small coupling constant (3.0 Hz) between H-3′ and H-9 demonstrated a cofacial orientation of H-3′ and H-9.

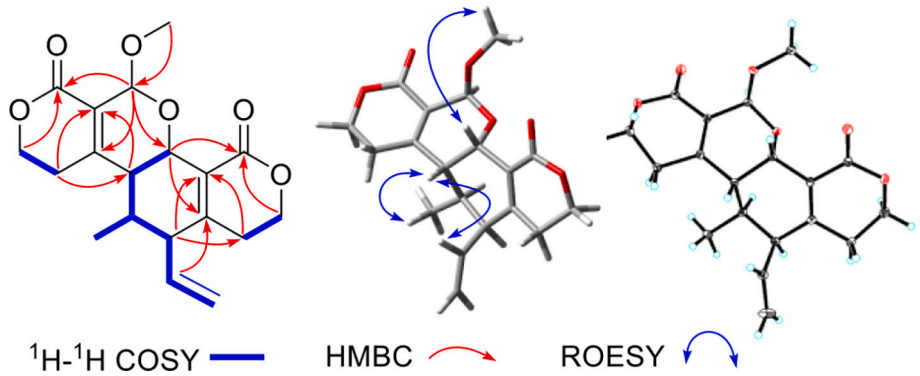


Fig. 2. Key 2D NMR correlations and X-ray ORTEP drawing of **1**.

Colorless cubic crystals of **1** were obtained in MeOH, which allowed a successful performance of X-ray crystallographic analysis (Fig. 2).

The X-ray crystallographic data (CCDC 1500816) corroborated the planar structure and the relative configuration of **1** elucidated via NMR data. Thus, the relative configuration of **1** was determined as 3S*,3′S*,8R*,9R*,9′S*.

The plausible transformation pathways of **1** from swertiamarin in boiling water are proposed in Scheme 1. Under the boiling conditions, swertiamarin might undergo a series of chemical reactions, from dehydration to intramolecular nucleophilic substitution (Route I), to form **6** [13]. Then, via decarboxylation and reduction, **6** could produce **3** [14–15]. The key intermediate (**X1**) could be obtained by selective ring opening and dehydration of **3**. Finally, a [4 + 2] cyclization reaction [16–17] of two **X1** moieties, followed by intermolecular nucleophilic addition and methylation reaction may lead to sweritranslactone D.

Sweritranslactone E (**2**), colorless cubic crystals (MeOH), was assigned a molecular formula $C_{18}H_{20}O_6$, based on HR-ESI-MS quasi-molecular ion peak at *m/z* 333.1349 ([M + H]$^+$, calcd 333.1333). The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) of **2** were similar to those of swerilactone A [18], except for the location of a double bond in **2**. The assignment was supported by detailed analysis of 2D NMR data of **2** (Fig. 3). The correlations in the ROESY spectrum could not provide

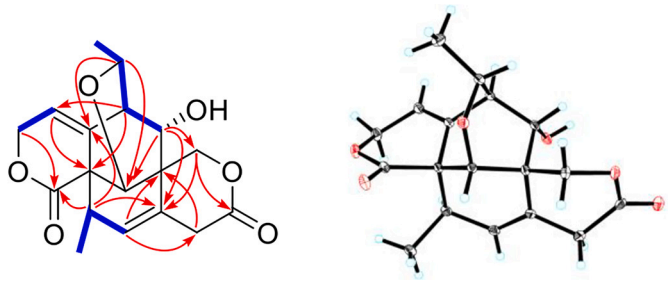
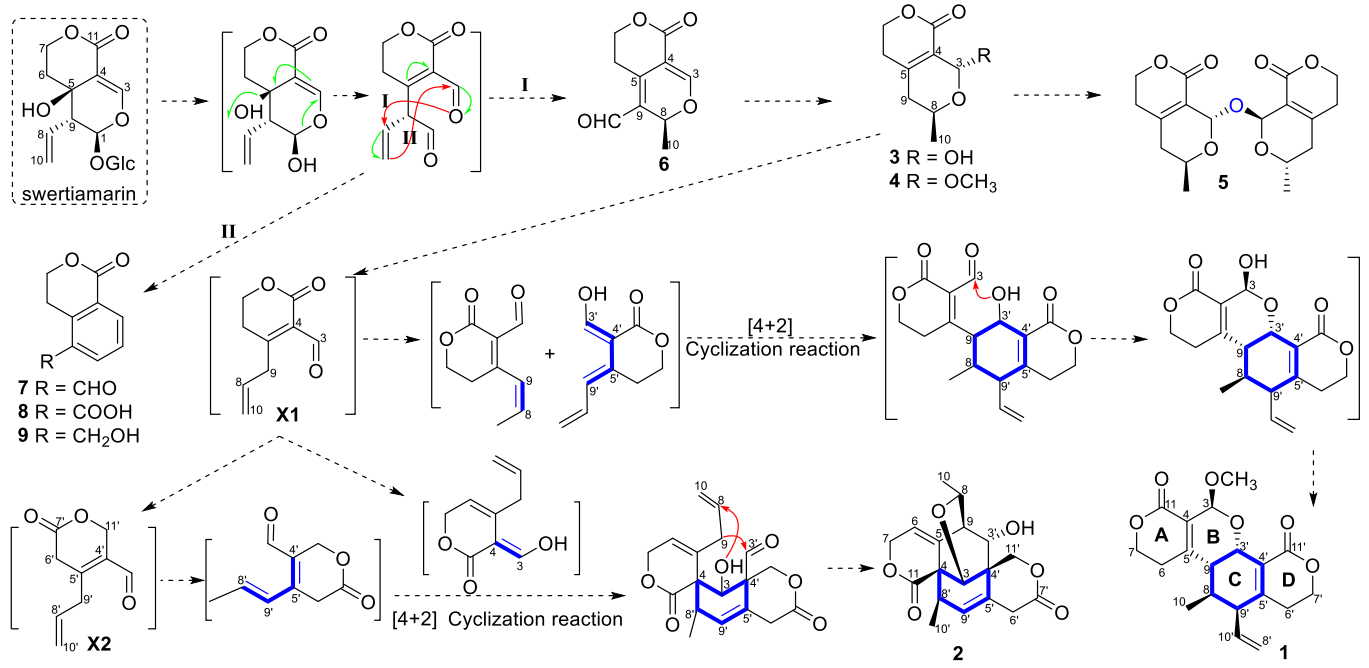


Fig. 3. Key ^{1}H—^{1}H COSY and HMBC correlations, and X-ray ORTEP drawing of **2**.

sufficient information to establish the stereochemistry of compound **2**. The crystallographic data (CCDC 978382) of **2** was obtained, which verified the planar structure, and also clarified the stereochemistry of **2** (Fig. 3) as 3*R**,3′*S**,4*S**,4′*R**,8*R**,8′*R**,9*R**.

Thus, the differences between **2** and swerilactone A were the epimeric C-8′ and double bond migration from C-5′/C-6′ to C-5′/C-9′ in **2**. Sweritranslactone E, iridoid dimer derivative, is another example of HTPS. Its conversion process is also proposed in Scheme 1. Intermediate



Scheme 1. Putative transformation pathways of compounds **1**–**8** from swertiamarin in boiling water.

X1 via hydrolysis, reduction, and intramolecular nucleophilic substitution, might produce intermediate (**X2**). Then, a [4 + 2] cyclization reaction might occur between **X2** and **X1** via enolization. Finally, intramolecular nucleophilic addition reaction may produce sweritranslactone E. Transformation pathways of other known compounds **4**–**8** [14,19], isolated from HPTS, are proposed in Scheme 1.

The hepatoprotective activity of swertiamarin, QYD tablets and HTPS was determined, using a mouse model of CCl_4-induced liver injury, based on the analyses of serum ALT and AST activities. The results (Fig. 4) revealed that the serum levels of liver injury markers ALT and AST increased in the mice treated with CCl_4 and significantly inhibited the CCl_4-treated over-expressing mice in experimental groups. Furthermore, both the low- and high-dose groups of HTPS showed equal activity, compared with positive control (DBB), indicating that HTPS were mainly responsible for hepatoprotective effect, rather than swertiamarin.

For further elucidation of the hepatoprotective activity of HTPS, all the isolates were tested in vitro in L-O2 AP-induced cell model, with NAC as positive control. The results (Table 2) displayed that all the isolates exhibited hepatoprotective activity, while the novel compound **1** showed a more potent activity than the positive control.

Table 2
Effect of the isolates from HTPS on the survival rate of L-O2 AP-induced liver injury cells.

Compounds	Dose/μg/mL	Survival rate/%	e_{max}[a]/%	c_{max}[b]/μg/mL
NAC	0.25–4 mg/mL	−2.16–15.84	15.84	1000
1	0.8–100	−4.38–20.28	20.28	25
2	0.8–100	−9.78–10.55	10.55	1.6
3	0.8–100	0.72–13.10	13.10	25
4	0.8–100	−3.26–4.19	4.19	50
5	0.8–100	−6.85–5.56	5.56	6.3
6	0.8–100	−4.01–0.61	0.61	1.6
7	0.8–100	−13.79–3.89	3.89	6.3
8	0.8–100	−4.11–4.75	4.75	0.8
9	0.8–100	−0.55-4.43	4.43	25

[a] e_{max}: Maximal Effect.
[b] c_{max}: Concentration at Maximal Effect.

4. Conclusion

Swertiamarin is the main active component in QYD tablets recorded in Chinese Pharmacopoeia, but after the decoction (one step for QYD in the manufacturing process) for 6 h, swertiamarin could not be detected. Further research on the material base on HTPS led to the isolation of sweritranslactone D (**1**), a novel secoiridoid dimer possessing a tetracyclic lactone skeleton with better hepatoprotective activity than *N*-acetyl-L-cysteine in vitro.

Iridoids are famous for their unstable structures with many reaction points. Hence, traditional phytochemical research methods may result in the generation of artifacts during the process of extraction, isolation, and storage of plants. Our study uncovered that the isolates from HTPS contained some compounds previously considered to be natural products. This phenomenon will cause the thinking for the process of traditional Chinese medicine, especially for those containing unstable active components. The work might spark controversy on the current experimental results. Meanwhile, this study also provides an effective method

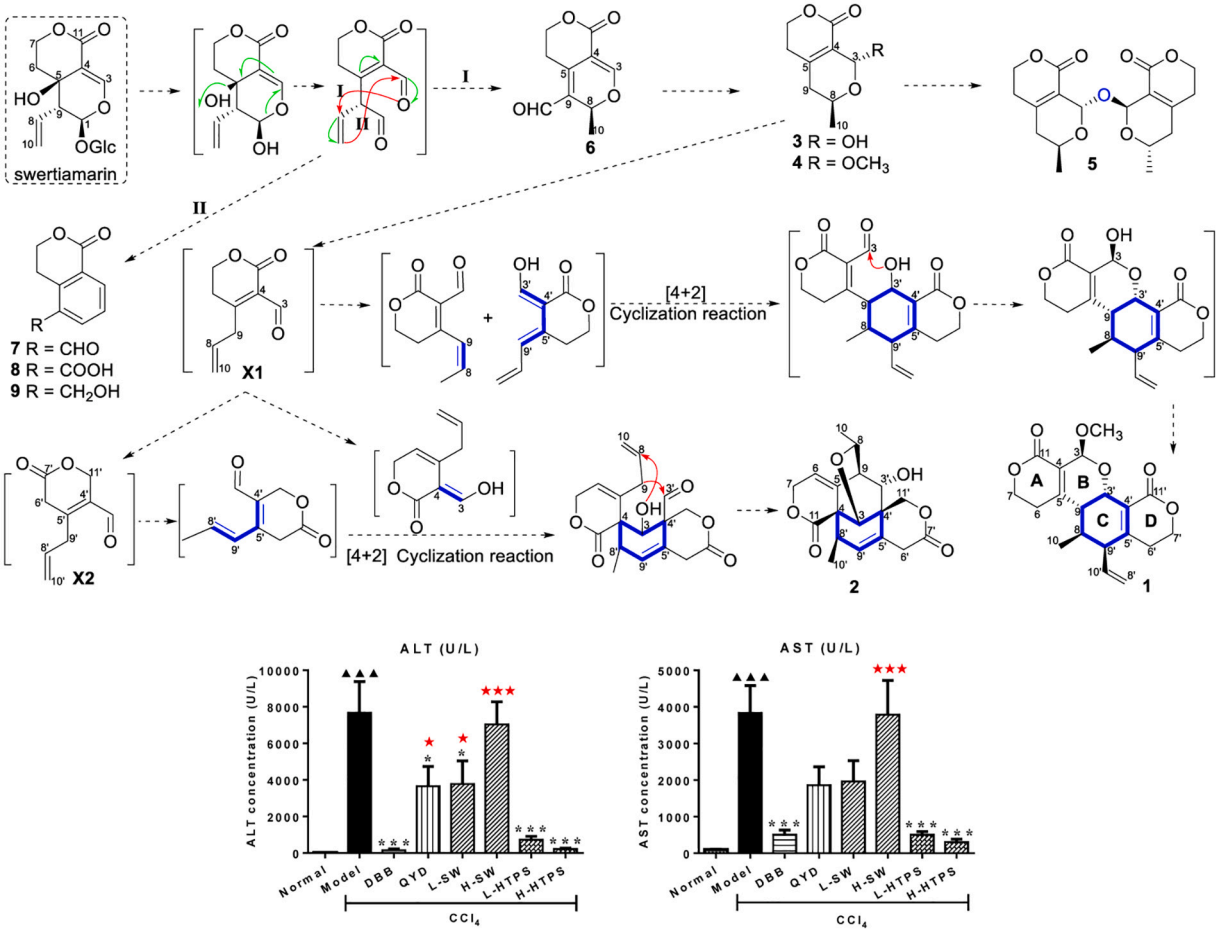


Fig. 4. Effect of QYD, SW and HTPS on the serum levels of ALT, AST after CCl_4- induced acute liver injury in mice. Bar graphs with error bars represent mean ± SEM ($n = 12$). ▲▲▲$P < 0.001$ versus Normal; ***$P < 0.001$, *$P < 0.05$ versus Model; ★★★$p < 0.001$, ★$p < 0.05$ versus DBB.

to obtain novel compounds from natural products.

Declaration of competing interest

The authors declared that there is no conflict of interest.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.104879.

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