Steroids 74 (2009) 424–434

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Structural analysis and antitussive evaluation of five novel esters of verticinone and bile acids

Jiu-liang Zhang, Hui Wang, Hui-fang Pi*, Han-li Ruan, Peng Zhang, Ji-zhou Wu

Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Tongji School of Pharmacy, Huazhong University of Science and Technology, Wuhan 430030, PR China

ARTICLE INFO

Article history:
Received 1 September 2008
Received in revised form 10 December 2008
Accepted 17 December 2008
Available online 30 December 2008

Keywords:
Bile acids
Cholic acid
Verticinone
Antitussive activity
Cholic acid–verticinone ester

ABSTRACT

Shedan-Chuanbei powder, a complex of traditional Chinese medicine preparation, which consists of Snake Bile (Chinese name "Shedan") and *Fritillariae Cirrhosae* (Chinese name "Chuanbei"), is the most popular antitussive and expectorant formulation in Chinese communities. However, the clinical application of Shedan-Chuanbei powder is now stringently limited because of the shortage of the two crude medicinal materials, especially for the sake of animal protection. In addition, the inherent defects of the most of the complex of traditional Chinese medicine such as the indistinct basal pharmacodynamic materials and the difficulties in quality control had blocked them heading into the international medicinal market. So we attempted to seek new substitute for Shedan-Chuanbei powder for antitussive drugs. In order to gain some new compounds with better bioactivity and attenuated toxicity, we tried to combine two kinds of drugs through ester bond. Enlightened with "combination principle" in drug discovery, we synthesized five novel esters of verticinone and bile acids, both of which are the major bioactive components in Shedan-Chuanbei powder. We then evaluated the antitussive activity and the acute toxicity of the five ester-linked compounds. The five ester-linked compounds had much more potent antitussive activity and expectorant activity than single bile acids at the same doses, and had equivalent antitussive activity and expectorant activity in comparison with about double moles dose of the monomer verticinone. Especially, cholic acid–verticinone ester had much more potent antitussive effects than the monomer verticinone or cholic acid at the same dose. A further acute toxicity study showed that the LD_{50} values of the five ester-linked compounds exceeded 3.5 g/kg by intraperitoneal injection in mice. Based on the studies of pharmacology and acute toxicity, the five ester-linked compounds have synergic pharmacodynamic action and attenuated toxicity compared with single verticinone and single bile acids.

1. Introduction

Cough remains among the most common complaints for which patients seek medical attention. Presently available therapies to treat cough are limited for lack of effective medications. Moreover, most existed antitussive drugs could bring inevitable or intolerable side effects. The most pressing and unmet current need is for safe and more effective cough suppression [1]. Complex preparations of traditional Chinese medicine are the major means to cure diseases in traditional medicine. At present traditional Chinese medicines represent a promising new trend for developing new antitussive drugs.

Shedan-Chuanbei powder, consisted of Snake Bile (Chinese name "Shedan") and *Fritillariae Cirrhosae* (Chinese name "Chuanbei"), is the most popular antitussive and expectorant formulation in Chinese communities. It has been used in clinic for thousands years in China due to the positive potent therapeutic effects, low toxicity and minimal side effects. Therefore, it has been officially listed in the Chinese Pharmacopoeia (1995, 2000, 2005). But the clinical application of Shedan-Chuanbei powder is now stringently limited because of the shortage of the two crude medicinal materials, especially for the sake of animal protection. In addition, the inherent defects of the most of the complex of traditional Chinese medicine such as the indistinct basal pharmacodynamic materials and the difficulties in quality control had blocked them heading into the international medicinal market. Therefore, it prompts us to search for new

Abbreviations: CA-Ver, cholic acid–verticinone ester; CDCA-Ver, chenodeoxycholic acid–verticinone ester; UDCA-Ver, ursodeoxycholic acid–verticinone ester; HDCA-Ver, hyodeoxycholic acid–verticinone ester; DCA-Ver, deoxycholic acid–verticinone ester; m.p., melting point; IR, infrared; ^{1}H NMR, proton nuclear magnetic resonance spectrum; ^{13}C NMR, Carbon-13 nuclear magnetic resonance spectrum; EI-MS, electron ionization mass spectra; HRESIMS, high-resolution ESI mass spectra; TLC, thin layer chromatography; TBDMSiCl, tert-butyl dimethyl chlorosilane; DCC, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; MeOH, methanol; THF, tetrahydrofuran; CH_2Cl_2, dichloromethane; PE, petroleum ether; EtOAc, ethyl acetate; Et_2NH, diethylamine.

* Corresponding author. Tel.: +86 27 83692739; fax: +86 27 83692739.
E-mail address: huifangpi@gmail.com (H.-f. Pi).

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doi:10.1016/j.steroids.2008.12.007

bioactive substitutes for Shedan-Chuanbei powder for antitussive drugs.

Bile acids are amphiphilic compounds in the steroid family and serve as emulsifiers in the gastro-intestinal tract. They are stored in the gallbladder and released during meals for the digestion of fat in food. They are reabsorbed in the intestine to complete the enterohepatic circulation with very little loss. They are a group of compounds consisting of a steroid backbone, an extended carboxylic acid group and different numbers of hydroxyl groups. Compounds derived from bile acids are expected to be safe and nontoxic when used in biomedical and pharmaceutical fields. In recent years, bile acids have been used in the development of new polymeric materials used in drug delivery system, asymmetric synthesis, molecular recognition, molecular umbrella and polymeric biomaterial [2–5].

Fritillaria hupehensis Hsiao *et* K.C. Hsia is a well known antitussive Traditional Chinese Medicine used for a long history, especially in the folk of Hubei province. Its bulbs have been recorded in the Pharmacopoeia of the People's Republic of China (2000 version and 2005 version) named "Hubeibeimu" [6]. The pharmacological studies indicate that the alkaloids of the bulbs have good antitussive, expectorant and antiasthmatic activities as *Fritillariae Cirrhosae* [7]. The prior studies indicate that verticinone's antitussive, expectorant and antiasthmatic activity is very good. On the basis of the pharmacodynamics experiments of verticinone, we examined the acute toxicity of verticinone [8]. We can draw the conclusion that the toxicity of verticinone is very large. In order to gain some new antitussive with better bioactivity and attenuated toxicity compared with verticinone, we tried to combine two kinds of drugs through ester bond. Enlightened with "combination principle" in drug discovery, we synthesized five novel esters of verticinone and bile acids. Herein we report synthetic procedure, structural analysis and antitussive evaluation of five novel esters of verticinone and bile acids.

Many of the complex traditional Chinese medicine formulations have sound scientific basis through modern pharmacological evaluation. For example, many combination formulations showed significantly better pharmacological results than individual herbal medicines participated in the formulation, through synergistic interaction and attenuation of their toxicity as such. In order to gain some new compounds with better bioactivity and attenuated toxicity, we attempted to combine two kinds of drugs through ester bond. Many famous drugs in clinic use such as sultamicillin and benorilate were discovered through this way. Based on nearly 20 years studies on the chemical constituents and the bioactivities of the *Fritillaria hupehensis* and more than 10 years study on the bioactive constituents of Snake bile, verticinone and bile acids were clearly elucidated as major bioactive components respectively in bulbs of *Fritillariae* and snake bile. We synthesized five novel esters of verticinone and bile acids, named as cholic acid–verticinone ester (simplified as CA-Ver in the paper), chenodeoxycholic acid–verticinone ester (simplified as CDCA-Ver in the paper), ursodeoxycholic acid–verticinone ester (simplified as UDCA-Ver in the paper), hyodeoxycholic acid–verticinone ester (simplified as HDCA-Ver in the paper), deoxycholic acid–verticinone ester (simplified as DCA-Ver in the paper). We then evaluated the antitussive activity and the acute toxicity of the five ester-linked compounds.

2. Experimental

2.1. Materials and instruments

Verticinone was isolated from *Fritillariae hupehensis* Hsiao *et* K.C. Hsia, which is commercially available from the Hubei Institute of Chinese Materia Medica (Wuhan, China) and was identified by Prof. De-tai Peng (Lichuan Institute of Chinese Materia Medica, Lichuan, Hubei province, China). A voucher specimen (F030823) was deposited at the Faculty of Pharmaceutical Science, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Cholic acid, chenodeoxycholic acid, hyodeoxycholic acid and deoxycholic acid were purchased from Asia Talent Enterprise (Shenzhen, Guangdong province, China). Ursodeoxycholic acid was purchased from Leawell International Limited (Guangzhou, Guangdong province, China). Their identities were confirmed with IR, ^{1}H NMR, ^{13}C NMR, and MS analyses. Codeine phosphate was purchased from Qinghai Pharmaceutical Factory (Qinghai, China). Guaifenesin was purchased from Shanxi Yuanjingkangye Pharmaceutical Factory (Shanxi, China).

Melting points were determined on a Fisher Scientific instrument (NJ, USA) and were uncorrected. IR spectra were obtained as KBr pellets with a Bruker FT-IR Vertex 70 spectrometer (Bruker, Germany). The NMR spectra were recorded on a Bruker-400 spectrometer (Bruker, Germany) with $CDCl_3$ or CD_3OD containing 0.1% TMS as the solvent and chemical shifts are expressed in δ values using TMS as the internal standard. NMR assignments were made using DEPT, H–H COSY, HSQC, and HMBC experiments. Electron ionization (EI) mass spectra (EI-MS) were determined on a Finnigan Trace mass spectrometer at 70 eV. HRESIMS were determined on a Bruker-micrOTOF-Q II mass spectrometer (electrospray: 1 μl/min-1 ml/min) (Bruker, Germany). Optical rotations were measured on

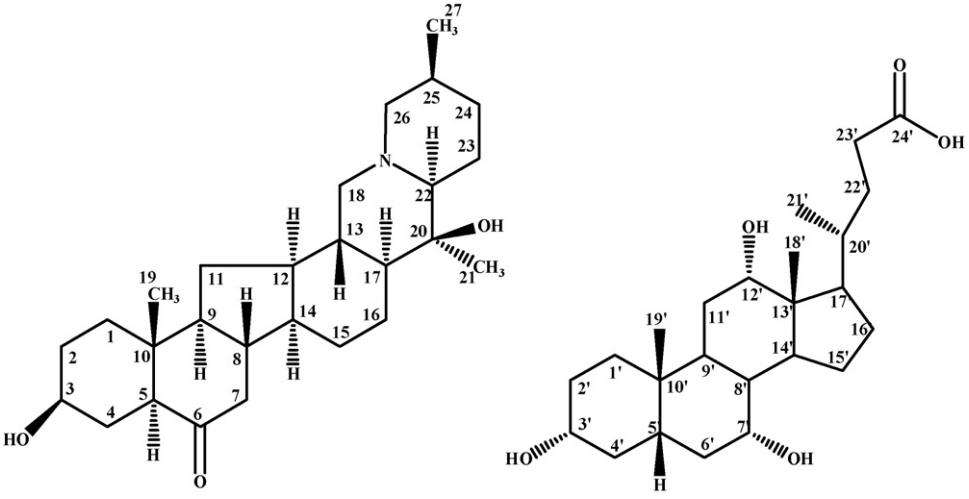


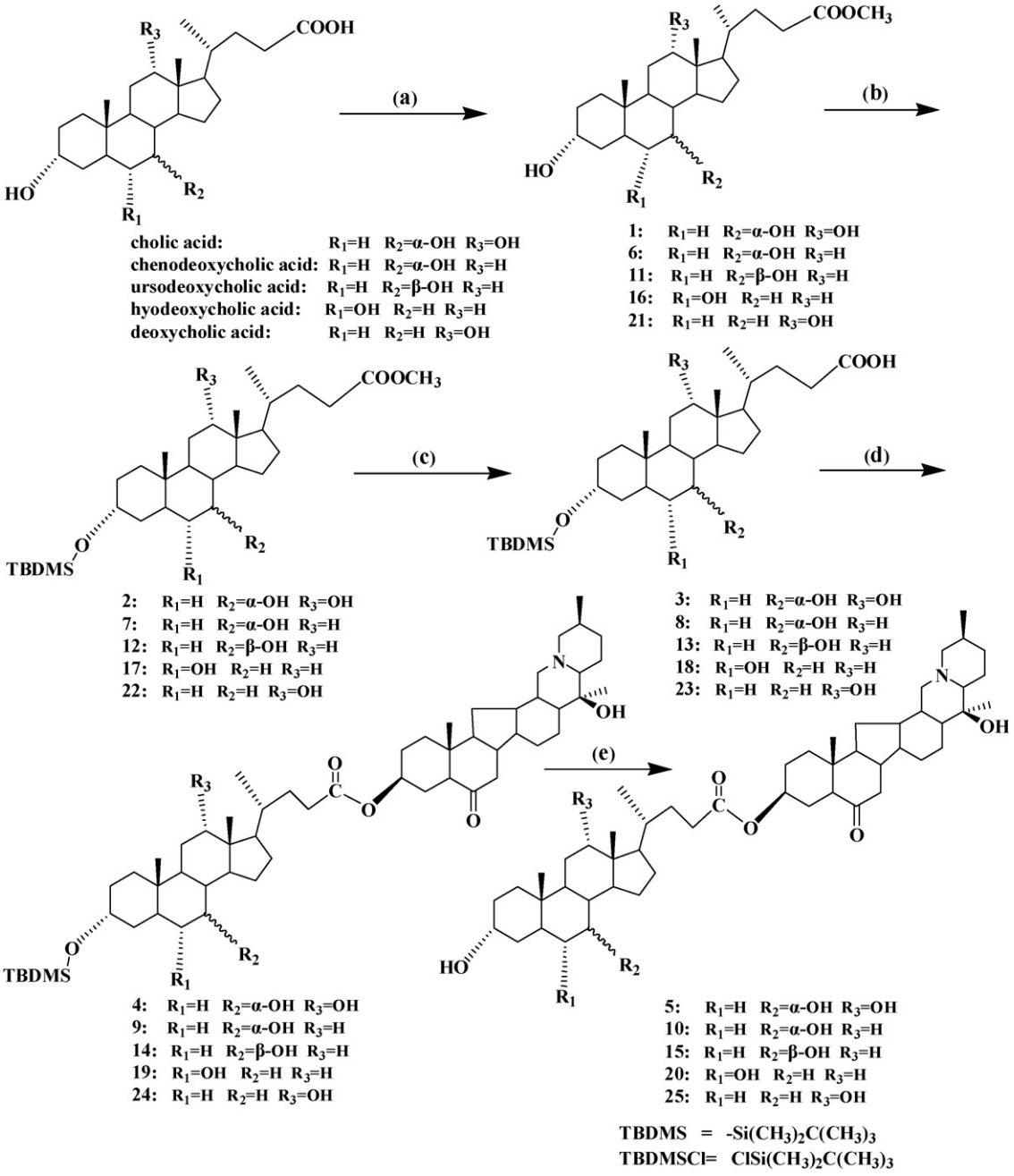
Fig. 1. Structures of verticinone and cholic acid.

a PerkinElmer Polarimeter (Model 341, USA). The compressor nebulizer (Model ap112U, Polo di Torrile, Italy) was used to produce an aerosol with a particle median diameter of 1.87–3.54 μm. Normal-phase (NP) thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (0.25 mm layer thickness; Qingdao Haiyang Chemical Co., Ltd, China) and visualized under UV illumination and/or by I_2 vapour using PE–EtOAc–Et_2NH mixtures as the developing solvent. All chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (SCRC) and all redistilled prior to use. Some of the solvents were treated before distillation: THF was refluxed with sodium; CH_2Cl_2 was dried with CaH_2 for 8 h; MeOH was dried with anhydrous $CaCl_2$.

2.2. Synthesis

2.2.1. Preparation of verticinone

The powdered bulbs of *Fritillariae hupehensis* Hsiao *et* K.C. Hsia (10 kg) were extracted with 95% EtOH (ethanol) and the solvent was removed to give an EtOH extract. Then the extract was dissolved in 2% HCl and filtered. After filtration, the water layer was basified with



Scheme 1. Reagents and conditions: (a) MeOH/concentrated sulfuric acid, r.t., 1 day. (b) TBDMSCl/imidazole/THF, low temperature, 48 h. (c) (1.) 10% NaOH/THF, r.t., 12 h; (2.) H2O/H+, r.t., 30 min. (d) Verticinone/DCC/DMAP/CH2Cl2, r.t., 48 h. (e) 5% HF(aq)/THF, r.t., 24 h.

Table 1
^{1}H NMR data of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver (Fig. 2). Spectra obtained in $CDCl_3$, 400 MHz.

Proton	δ (ppm)					Proton	δ (ppm)				
	5	10	15	20	25		5	10	15	20	25
3α-CH	4.69	4.68	4.68	4.67	4.67	3′β-CH	3.47	3.46	3.58	3.62	3.60
19-CH_3	0.80	0.79	0.79	0.79	0.80	6′β-CH	–	–	–	4.06	–
21-CH_3	1.03	1.02	1.03	1.04	1.03	7′α-CH	–	–	3.68	–	–
27-CH_3	1.08	1.07	1.07	1.09	1.08	7′β-CH	3.84	3.85	–	–	–
						12′β-CH	3.97	–	–	–	3.98
						18′-CH_3	0.68	0.66	0.68	0.64	0.68
						19′-CH_3	0.88	0.90	0.88	0.90	0.90
						21′-CH_3	0.98	0.93	0.96	0.93	0.98

ammonia (pH 11) and continuously extracted with chloroform to give a crude alkaloid fraction (60 g, 0.6% yield). The crude alkaloid fraction was then subjected to silica gel column chromatography using PE–EtOAc–Et_2NH mixtures (8:1:0.1, v/v/v) as the developing solvent. The elution was monitored by TLC and the fractions only containing verticinone were combined and dried. Then the residue was recrystallized from EtOAc to give verticinone (Fig. 1), which was in the form of colorless needles: yield, 30 g (0.3%); m.p., 213–214 °C. $[\alpha]_D^{20} = -62.5$ (*c* 0.5, MeOH). IR, υ_{max} cm^{-1}: 3410, 1055 (O–H), 1705 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.75 (3H, s, 19-CH_3), 1.01(3H, s, 21-CH_3), 1.06 (3H, d, *J* = 5.3 Hz, 27-CH_3), 3.58 (1H, m, $W_{1/2}$ = 24 Hz, 3-H). ^{13}C NMR ($CDCl_3$, 100 MHz), δ: 211.0 (C-6), 71.0 (C-20), 70.9 (C-3), 70.3 (C-22), 62.3 (C-26), 61.8 (C-18), 56.7 (C-9), 56.5 (C-5). EI-MS, *m*/*z*: 430 $[M+H]^+$, 428 $[M-H]^+$.

The ^{1}H NMR (400 MHz, Pyr) data of cholic acid (Fig. 1), δ: 0.79 (3H, s, 18′-CH_3), 0.98 (3H, s, 19′-CH_3), 1.23 (3H, d, 21′-CH_3), 3.72 (1H, m, 3′β-CH), 4.07 (1H, m, 7′β-CH), 4.23 (1H, m, 12′β-CH) and the ^{13}C NMR (100 MHz, Pyr) data of cholic acid, δ: 176.7 (C-24′), 72.5 (C-12′), 72.0 (C-3′), 67.8 (C-7′).

2.2.2. Cholic acid–verticinone ester (CA-Ver)

The synthetic route for the CA-Ver is shown in Scheme 1. The ^{1}H NMR chemical shifts of CA-Ver are listed in Table 1 and the ^{1}H NMR chemical shifts of intermediate products are listed in Table 2. The ^{13}C NMR chemical shifts of CA-Ver are listed in Table 3.

*2.2.2.1. Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (**1**).* A 500-ml, round-bottomed flask was charged with 24.5 g (0.060 mol) of cholic acid and 180 ml of absolute MeOH. The flask was equipped with a reflux condenser, and the mixture was heated at reflux for 1 h. Then the solution was acidified with 1.5 ml concentrated sulfuric acid and the mixture was stirred at room temperature for 1 day. 100 ml of saturated sodium bicarbonate solution was added to the reaction mixture, which was stirred for 10 min before the precipitated solid was filtered, washed with absolute methanol for three times. The combined solution was dried and removed in a rotary evaporator; a residue of 26.5 g was isolated. This crude product was dried under vacuum and recrystallized from benzene to give the title compound 1 in the form of colorless crystals: yield, 22.28 g (88%); m.p., 153–154 °C (lit. 155–156 °C). IR (cm^{-1}): 3417, 2938, 2869, 1739, 1465, 1441, 1376, 1254, 1173, 1079, 1012, 981. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.88 (3H, s, 19′-CH_3), 0.98 (3H, d, 21′-CH_3), 3.43 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 3.84 (1H, m, 7′β-CH), 3.96 (1H, m, 12′β-CH).

*2.2.2.2. Methyl 3α-tert-butyldimethylsilyloxy-7α,12α-dihydroxy-5β-cholan-24-oate (**2**).* Imidazole (1.8 g, 0.0265 mol) was added to a solution of **1** (6.3 g, 0.015 mol) in anhydrous THF(100 ml) in a 250-ml, two-necked, round-bottomed flask with a coil condenser. Then a solution of tert-butyldimethylsilyl chloride (TBDMSiCl; 2.4 g, 0.016 mol) in anhydrous THF(30 ml) was added dropwise over a period of 2 h with ice-bath cooling at 10 °C with a 100-ml separatory funnel. After that the mixture was left to stir at room temperature for 48 h. The reaction mixture was quenched with water (50 ml). The reaction product was extracted with CH_2Cl_2 (100 ml), and the combined extract was washed with water, dried over anhydrous magnesium sulfate and evaporated, a residue of 6.9 g was isolated. The residue was dried under vacuum and recrystallized from methanol to give the title compound **2** in the form of colorless amorphous solids: yield, 5.80 g (72.1%); m.p., 172–175 °C. IR (cm^{-1}): 3520, 3333, 2934, 2862, 1736, 1464, 1440, 1375, 1253, 1171, 1071, 1043, 982. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.87 (3H, s, 19′-CH_3), 0.97 (3H, d, 21′-CH_3), 3.42 (1H, m, 3′β-CH), 3.66 (3H, s, –OCH_3), 3.84 (1H, m, 7′β-CH), 3.97 (1H, m, 12′β-CH).

*2.2.2.3. 3α-tert-butyldimethylsilyloxy-7α,12α-dihydroxy-5β-cholan-24-oic acid (**3**).* 30 ml of 5% sodium hydroxide aqueous solution was added to a solution of **2** (5.4 g, 0.01 mol) in THF (100 ml) in a 250-ml, two-necked, round-bottomed flask with a coil condenser. Then the mixture was stirred overnight at room temperature. After 12 h, the resulting solution was adjusted to pH 3 with 10% hydrochloric acid. The reaction product was extracted with EtOAc (100 ml), and the combined extract was washed with water (100 ml × 2), dried over anhydrous magnesium sulfate and evaporated. The solids were dried under vacuum for 12 h and purified by recrystallization from EtOAc to give pure **3** in the form of colorless crystals: yield, 4.90 g (93.8%); m.p., 158–161 °C. IR (cm^{-1}): 3418, 2933, 2861, 1710, 1467, 1410, 1378, 1254, 1197, 1081, 1043, 981, 874, 837, 776. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.70 (3H, s, 18′-CH_3), 0.88 (3H, s, 19′-CH_3), 1.00 (3H, d, 21′-CH_3), 3.48 (1H, m, 3′β-CH), 3.83 (1H, m, 7′β-CH), 3.96 (1H, m, 12′β-CH).

*2.2.2.4. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-7α,12α-dihydroxy-5β-cholan-24-oate (**4**).* To a stirred solution of **3** (3.1 g, 0.006 mol) in dry CH_2Cl_2 (100 ml) was added DMAP (79 mg, 0.00065 mol) and then 5α, 14α-cevanin-3β, 20β-dihydroxy-6-one (verticinone, 2.6 g, 0.006 mol) at room temperature in a 250-ml, two-necked, round-bottomed flask with a coil condenser. After cooling to 4 °C, a solution of DCC (1.34 g, 0.0065 mol) in dry CH_2Cl_2 (20 ml) was added slowly to the reaction mixture and stirred continued for another 30 min at 4 °C, then 48 h at room temperature. Finally, a precipitate (dicyclohexylurea) was formed. The precipitated solid was filtered and the filtrate evaporated down in vacuum. The residue was taken up in CH_2Cl_2 and washed subsequently twice with 0.5N HCl and saturated sodium bicarbonate solution. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was purified by column chromatography (silica gel, EtOAc/PE/Et_2NH = 1/10/0.1 as the eluent). The title compound **4** was found as a colorless solid: yield: 3.30 g (58.9%); m.p., 171–173 °C. IR (cm^{-1}): 3478, 3327, 2932, 2855, 2775, 1693, 1634, 1453, 1380, 1253, 1193, 1092, 986, 878, 838, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.68 (3H, s, 18′-CH_3), 0.88 (3H, s, 19′-CH_3), 0.99 (3H, d, 21′-CH_3), 3.47 (1H, m, 3′β-CH), 3.84 (1H, m, 7′β-CH), 3.96 (1H, m, 12′β-CH), 4.69 (1H, m, 3α-CH), 0.79 (3H, s, 19-CH_3), 1.03 (3H, s, 21-CH_3), 1.08 (3H, d, 27-CH_3).

Table 2
^{1}H NMR data of the intermediate products. Spectra obtained in $CDCl_3$, 400 MHz.

Proton	δ (ppm)					Proton	δ (ppm)				
	1	6	11	16	21		1	6	11	16	21
3α-CH	–	–	–	–	–	3′β-CH	3.43	3.46	3.51	3.62	3.61
19-CH_3	–	–	–	–	–	6′β-CH	–	–	–	4.05	–
21-CH_3	–	–	–	–	–	7′α-CH	–	–	3.60	–	–
27-CH_3	–	–	–	–	–	7′β-CH	3.84	3.86	–	–	–
						12′β-CH	3.96	–	–	–	3.98
						18′-CH_3	0.67	0.66	0.67	0.64	0.67
						19′-CH_3	0.88	0.91	0.92	0.90	0.90
						21′-CH_3	0.98	0.92	0.94	0.93	0.96
						MeO	3.67	3.67	3.67	3.67	3.67

Proton	δ (ppm)					Proton	δ (ppm)				
	2	7	12	17	22		2	7	12	17	22
3α-CH	–	–	–	–	–	3′β-CH	3.42	3.45	3.51	3.59	3.58
19-CH_3	–	–	–	–	–	6′β-CH	–	–	–	4.03	–
21-CH_3	–	–	–	–	–	7′α-CH	–	–	3.61	–	–
27-CH_3	–	–	–	–	–	7′β-CH	3.84	3.84	–	–	–
						12′β-CH	3.97	–	–	–	3.97
						18′-CH_3	0.67	0.65	0.67	0.63	0.67
						19′-CH_3	0.87	0.89	0.92	0.90	0.89
						21′-CH_3	0.97	0.92	0.94	0.92	0.97
						MeO	3.66	3.67	3.67	3.67	3.67

Proton	δ (ppm)					Proton	δ (ppm)				
	3	8	13	18	23		3	8	13	18	23
3α-CH	–	–	–	–	–	3′β-CH	3.48	3.45	3.52	3.59	3.62
19-CH_3	–	–	–	–	–	6′β-CH	–	–	–	4.05	–
21-CH_3	–	–	–	–	–	7′α-CH	–	–	3.62	–	–
27-CH_3	–	–	–	–	–	7′β-CH	3.83	3.84	–	–	–
						12′β-CH	3.96	–	–	–	3.94
						18′-CH_3	0.70	0.66	0.67	0.64	0.68
						19′-CH_3	0.88	0.89	0.92	0.90	0.91
						21′-CH_3	1.00	0.94	0.95	0.93	1.00

Proton	δ (ppm)					Proton	δ (ppm)				
	4	9	14	19	24		4	9	14	19	24
3α-CH	4.69	4.68	4.69	4.68	4.68	3′β-CH	3.47	3.45	3.51	3.58	3.58
19-CH_3	0.79	0.79	0.79	0.80	0.79	6′β-CH	–	–	–	4.05	–
21-CH_3	1.03	1.03	1.03	1.04	1.03	7′α-CH	–	–	3.60	–	–
27-CH_3	1.08	1.07	1.09	1.09	1.07	7′β-CH	3.84	3.84	–	–	–
						12′β-CH	3.97	–	–	–	3.97
						18′-CH_3	0.68	0.65	0.67	0.64	0.67
						19′-CH_3	0.88	0.91	0.92	0.90	0.89
						21′-CH_3	0.99	0.93	0.93	0.93	0.98

*2.2.2.5. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α,7α,12α-trihydroxy-5β-cholan-24-oate (**5**).* To a solution of 4 (2.5 g, 0.0027 mol) in THF (50 ml) in a 100-ml, two-necked, round-bottomed flask with a coil condenser and 20 ml of 5% HF aqueous solution was added slowly to the reaction mixture and stirred for 24 h at 0 °C with ice bath. Then the resulting solution was adjusted to pH 8 with 10% sodium bicarbonate solution. The reaction product was extracted with EtOAc (100 ml), and the combined extract was washed with water (100 ml × 2) and saturated sodium bicarbonate solution. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude product was purified by column chromatography (silica gel, EtOAc/PE/Et_2NH = 1/1/0.1 as the eluent). The title compound **5** was found as a colorless amorphous solid: yield: 2.10 g (95%); m.p., 148–150 °C. $[\alpha]_D^{20} = -45.5$ (*c* 0.001, $CHCl_3$). IR (cm^{-1}): 3441, 2939, 2870, 2774, 1713, 1466, 1450, 1380, 1259, 1174, 1078, 1042, 982. EI-MS *m*/*z*: 820 ($[M+H]^+$, 5.19%), 819 (M^+, 4.78%). HRESIMS calculated for $C_{51}H_{82}NO_7$ $[M+H]^+$:820.6086; found:820.6088. Its analysis led to the molecular formula $C_{51}H_{81}NO_7$, supported by the ^{13}C NMR, H–H COSY, HSQC and DEPT data. The ^{1}H NMR spectrum (Table 1) showed a set of downfield resonances at δ 4.69 (1H, m, 3α-CH), 3.97 (1H, s, 12′β-CH), 3.84 (1H, s, 7′β-CH), 3.47 (1H, s, 3′β-CH) and a set of typical upfield resonances at δ 1.08 (3H, d, 27-CH_3), 1.03 (3H, s, 21-CH_3), 0.98 (3H, d, 21′-CH_3), 0.88 (3H, s, 19′-CH_3), 0.80 (3H, s, 19-CH_3), 0.68 (3H, s, 18′-CH_3). The ^{13}C NMR spectrum (Table 3) showed a set of typical resonances at δ 210.4 (C-6), 173.8 (C-24′), 73.0 (C-12′), 72.8 (C-3), 71.9 (C-3′), 70.9 (C-20), 70.5 (C-22), 68.4 (C-7′), 62.2 (C-26), 61.8 (C-18), 56.4 (C-9), 56.4 (C-5). Further 2D NMR analyses of **5** indicated that in the HMBC spectrum the C-3 α-H showed three-bond correlations between C-24′.

2.2.3. Chenodeoxycholic acid–verticinone ester (CDCA-Ver)

The synthetic route for the CDCA-Ver is shown in Scheme 1. The ^{1}H NMR chemical shifts of CDCA-Ver are listed in Table 1 and the ^{1}H NMR chemical shifts of intermediate products are listed in Table 2. The ^{13}C NMR chemical shifts of CDCA-Ver are listed in Table 3.

*2.2.3.1. Methyl 3α,7α-dihydroxy-5β-cholan-24-oate (**6**).* This compound was synthesized with the same procedure as described for **1**, with different reactants. The title compound **6** was in the form of colorless crystals: yield, 20.71 g (85%); m.p., 144–146 °C. IR (cm^{-1}):

Table 3
^{13}C NMR data of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver (Fig. 2). Spectra obtained in $CDCl_3$, 100 MHz.

Carbon	δ (ppm)					Carbon	δ (ppm)				
	5	10	15	20	25		5	10	15	20	25
1	36.75	36.73	36.59	36.74	36.67	1′	35.19	35.34	35.23	35.58	35.06
2	30.94	30.64	30.83	30.66	30.97	2′	29.67	29.69	29.68	29.70	30.46
3	72.83	72.77	72.81	72.80	72.89	3′	71.91	71.99	71.51	71.60	71.83
4	30.40	30.61	30.36	30.28	30.52	4′	39.73	39.98	37.34	28.18	36.52
5	56.38	56.39	55.81	56.22	56.43	5′	41.52	41.51	42.48	48.79	42.11
6	210.44	210.46	210.17	210.43	210.44	6′	34.73	34.60	36.87	68.06	27.14
7	45.91	45.93	45.74	45.93	45.78	7′	68.42	68.51	71.38	35.32	26.15
8	41.92	41.91	41.48	41.82	41.50	8′	39.58	39.65	43.60	35.09	36.08
9	56.43	56.42	56.45	56.44	56.50	9′	26.57	32.89	39.30	39.89	33.70
10	38.34	38.34	38.33	38.36	38.33	10′	34.62	35.05	34.10	35.98	34.14
11	29.33	29.35	28.67	29.32	29.69	11′	28.24	20.59	21.44	21.05	28.69
12	40.89	40.87	40.40	40.86	40.68	12′	72.99	39.44	40.16	39.98	73.16
13	39.12	39.23	39.20	39.83	38.35	13′	46.51	42.72	43.80	42.89	46.57
14	43.95	43.96	43.80	43.98	43.90	14′	41.81	50.48	55.73	56.01	48.26
15	24.55	24.57	24.19	24.55	24.72	15′	23.18	23.70	26.89	24.73	23.61
16	21.00	21.03	21.19	20.77	21.30	16′	27.54	28.21	28.30	30.98	27.40
17	48.75	48.77	48.12	48.42	48.37	17′	46.95	55.65	54.99	55.77	47.13
18	61.79	61.80	61.10	61.80	61.60	18′	12.45	11.74	12.15	12.06	12.65
19	12.67	12.71	12.47	12.72	12.73	19′	22.50	22.79	23.37	24.21	23.15
20	70.94	70.92	71.46	70.94	71.02	20′	35.31	35.51	35.42	35.51	35.23
21	20.47	20.49	20.17	20.50	20.20	21′	17.25	18.25	18.35	18.52	17.30
22	70.47	70.39	71.40	70.60	70.47	22′	31.53	31.46	31.04	31.64	31.50
23	19.00	19.01	18.64	19.00	18.67	23′	31.53	31.01	30.97	31.48	31.45
24	29.03	29.05	28.47	29.02	28.85	24′	173.82	173.79	173.79	173.82	173.79
25	27.59	27.61	27.77	27.58	27.51						
26	62.20	62.26	62.30	62.09	61.65						
27	17.30	17.25	16.41	17.26	17.58						

3399, 2934, 2866, 1744, 1464, 1441, 1375, 1249, 1167, 1077, 1046, 979. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.66 (3H, s, 18′-CH_3), 0.91 (3H, s, 19′-CH_3), 0.92 (3H, d, 21′-CH_3), 3.46 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 3.86 (1H, m, 7′β-CH).

*2.2.3.2. Methyl 3α-tert-butyldimethylsilyloxy-7α-hydroxy-5β-cholan-24-oate (**7**).* This compound was synthesized with the same procedure as described for **2**, with different reactants. The title compound **7** was in the form of colorless amorphous solids: yield, 5.48 g (70.3%); m.p., 190–193 °C. IR (cm^{-1}): 3521, 2945, 2866, 1727, 1468, 1444, 1378, 1358, 1256, 1225, 1157, 1088, 1008, 981, 874, 836, 774. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.65 (3H, s, 18′-CH_3), 0.89 (3H, s, 19′-CH_3), 0.92 (3H, d, 21′-CH_3), 3.45 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 3.84 (1H, m, 7′β-CH).

*2.2.3.3. 3α-tert-butyldimethylsilyloxy-7α-hydroxy-5β-cholan-24-oic acid (**8**).* This compound was synthesized with the same procedure as described for **3**, with different reactants. The title compound **8** was in the form of colorless crystals: yield, 4.86 g (96%); m.p., 149–151 °C. IR (cm^{-1}): 3384, 2930, 2859, 1706, 1468, 1381, 1253, 1178, 1077, 1017, 961, 873, 835, 776. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.66 (3H, s, 18′-CH_3), 0.89 (3H, s, 19′-CH_3), 0.94 (3H, d, 21′-CH_3), 3.45 (1H, m, 3′β-CH), 3.84 (1H, m, 7′β-CH).

*2.2.3.4. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-7α-hydroxy-5β-cholan-24-oate (**9**).* This compound was synthesized with the same procedure as described for **4**, with different reactants. The title compound **9** was found as a colorless solid: yield: 3.38 g (61.5%); m.p., 181–183 °C. IR (cm^{-1}): 3507, 2932, 2856, 2775, 1712, 1467, 1379, 1254, 1170, 1091, 1004, 985, 873, 837, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.65 (3H, s, 18′-CH_3), 0.91 (3H, s, 19′-CH_3), 0.93 (3H, d, 21′-CH_3), 3.45 (1H, m, 3′β-CH), 3.84 (1H, m, 7′β-CH), 4.68 (1H, m, 3α-CH), 0.79 (3H, s, 19-CH_3), 1.03 (3H, s, 21-CH_3), 1.07 (3H, d, 27-CH_3).

*2.2.3.5. 5α, 14α-cevanin-6-O-20β-hydroxy-3β-yl-3α,7α-dihydroxy-5β-cholan-24-oate (**10**).* This compound was synthesized with the same procedure as described for **5**, with different reactants. The title compound **10** was found as a colorless amorphous solid: yield: 2.02 g (93%); m.p., 133.5–137.5 °C. $[\alpha]_D^{20} = -90.0$ (*c* 0.001, $CHCl_3$). IR (cm^{-1}): 3516, 3439, 2930, 2867, 2753, 1742, 1711, 1693, 1465, 1448, 1376, 1249, 1164, 1080, 1001, 978. EI-MS *m/z*: 804 ($[M+H]^+$, 12.84%), 803 (M^+, 19.30%). HRESIMS calculated for $C_{51}H_{82}NO_6$ $[M+H]^+$:804.6137; found: 804.6126. Its analysis led to the molecular formula $C_{51}H_{81}NO_6$, supported by the ^{13}C NMR, H–H COSY, HSQC and DEPT data. The ^{1}H NMR spectrum (Table 1) showed a set of downfield resonances at δ 4.68 (1H, m, 3α-CH), 3.85 (1H, s, 7′β-CH), 3.46 (1H, m, 3′β-CH) and a set of typical upfield resonances at δ 1.07 (3H, d, 27-CH_3), 1.02 (3H, s, 21-CH_3), 0.93 (3H, d, 21′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.79 (3H, s, 19-CH_3), 0.66 (3H, s, 18′-CH_3). The ^{13}C NMR spectrum (Table 3) showed a set of typical resonances at δ 210.5 (C-6), 173.8 (C-24′), 72.8 (C-3), 72.0 (C-3′), 70.9 (C-20), 70.4 (C-22), 68.5 (C-7′), 62.3 (C-26), 61.8 (C-18), 56.4 (C-9), 56.4 (C-5).

2.2.3.6. Ursodeoxycholic acid–verticinone ester (UDCA-Ver). The synthetic route for the UDCA-Ver is shown in Scheme 1. The ^{1}H NMR chemical shifts of UDCA-Ver are listed in Table 1 and the ^{1}H NMR chemical shifts of intermediate products are listed in Table 2. The ^{13}C NMR chemical shifts of UDCA-Ver are listed in Table 3.

*2.2.3.7. Methyl 3α,7β-dihydroxy-5β-cholan-24-oate (**11**).* This compound was synthesized with the same procedure as described for **1**, with different reactants. The title compound **11** was in the form of colorless crystals: yield, 22.17 g (91%); m.p., 210–212 °C. IR (cm^{-1}): 3468, 2939, 2861, 1709, 1453, 1429, 1384, 1366, 1301, 1269, 1241, 1105, 1077, 1048, 1010, 948. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.92 (3H, s, 19′-CH_3), 0.94 (3H, d, 21′-CH_3), 3.51 (1H, m, 3′β-CH), 3.60 (1H, m, 7′α-CH), 3.67 (3H, s, –OCH_3).

*2.2.3.8. Methyl 3α-tert-butyldimethylsilyloxy-7β-hydroxy-5β-cholan-24-oate (**12**).* This compound was synthesized with the same procedure as described for **2**, with different reactants. The title compound **12** was in the form of colorless amorphous solids: yield, 5.60 g (71.8%); m.p., 155–158 °C. IR (cm^{-1}): 3547, 2933, 2860,

1729, 1451, 1379, 1252, 1169, 1102, 1021, 988, 875, 837, 773. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.92 (3H, s, 19′-CH_3), 0.94 (3H, d, 21′-CH_3), 3.51 (1H, m, 3′β-CH), 3.61 (1H, m, 7′α-CH), 3.67 (3H, s, –OCH_3).

*2.2.3.9. 3α-tert-butyldimethylsilyloxy-7β-hydroxy-5β-cholan-24-oic acid (**13**).* This compound was synthesized with the same procedure as described for **3**, with different reactants. The title compound **13** was in the form of colorless crystals: yield, 4.76 g (94%); m.p., 218–221 °C. IR (cm^{-1}): 3546, 3384, 2931, 2859, 1702, 1467, 1381, 1255, 1099, 1018, 952, 873, 836, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.92 (3H, s, 19′-CH_3), 0.95 (3H, d, 21′-CH_3), 3.52 (1H, m, 3′β-CH), 3.62 (1H, m, 7′α-CH).

*2.2.3.10. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-7β-hydroxy-5β-cholan-24-oate (**14**).* This compound was synthesized with the same procedure as described for **4**, with different reactants. The title compound **14** was found as a colorless solid: yield: 3.06 g (55.6%); m.p., 160–162 °C. IR (cm^{-1}): 3456, 2932, 2857, 2775, 1734, 1713, 1690, 1466, 1451, 1379, 1254, 1233, 1170, 1079, 956, 872, 836, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.92 (3H, s, 19′-CH_3), 0.93 (3H, d, 21′-CH_3), 3.51 (1H, m, 3′β-CH), 3.60 (1H, m, 7′ α-CH), 4.69 (1H, m, 3α-CH), 0.79 (3H, s, 19-CH_3), 1.03 (3H, s, 21-CH_3), 1.09 (3H, d, 27-CH_3).

*2.2.3.11. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α,7β-dihydroxy-5β-cholan-24-oate (**15**).* This compound was synthesized with the same procedure as described for **5**, with different reactants. The title compound 15 was found as a colorless amorphous solid: yield: 2.06 g (95%); m.p., 126.5–128.5 °C. $[\alpha]_D^{20} = 34.5$ (c 0.001, $CHCl_3$). IR (cm^{-1}): 3411, 2933, 2859, 1709, 1452, 1383, 1255, 1237, 1171, 1080, 1053, 959. EI-MS m/z: 804 ($[M+H]^+$, 4.26%), 803 (M^+, 2.26%). HRESIMS calculated for $C_{51}H_{82}NO_6$ $[M+H]^+$:804.6137; found: 804.6124. Its analysis led to the molecular formula $C_{51}H_{81}NO_6$, supported by the ^{13}C NMR, H–H COSY, HSQC and DEPT data. The ^{1}H NMR spectrum (Table 1) showed a set of downfield resonances at δ 4.68 (1H, m, 3α-CH), 3.68 (1H, m, 7′α-CH), 3.58 (1H, m, 3′β-CH) and a set of typical upfield resonances at δ 1.07 (3H, d, 27-CH_3), 1.03 (3H, s, 21-CH_3), 0.96 (3H, d, 21′-CH_3), 0.88 (3H, s, 19′-CH_3), 0.79 (3H, s, 19-CH_3), 0.68 (3H, s, 18′-CH_3). The ^{13}C NMR spectrum (Table 3) showed a set of typical resonances at δ 210.2 (C-6), 173.8 (C-24′), 72.8 (C-3), 71.5 (C-3′), 71.5 (C-20), 71.4 (C-22), 71.4 (C-7′), 62.3 (C-26), 61.1 (C-18), 56.5 (C-9), 55.8 (C-5).

2.2.4. Hyodeoxycholic acid–verticinone ester (HDCA-Ver)

The synthetic route for the HDCA-Ver is shown in Scheme 1. The ^{1}H NMR chemical shifts of HDCA-Ver are listed in Table 1 and the ^{1}H NMR chemical shifts of intermediate products are listed in Table 2. The ^{13}C NMR chemical shifts of HDCA-Ver are listed in Table 3.

*2.2.4.1. Methyl 3α,6α-dihydroxy-5β-cholan-24-oate (**16**).* This compound was synthesized with the same procedure as described for **1**, with different reactants. The title compound **16** was in the form of colorless crystals: yield, 20.34 g (83.5%); m.p., 122–124 °C. IR (cm^{-1}): 3392, 2938, 2866, 1742, 1452, 1377, 1255, 1168, 1039, 957. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.64 (3H, s, 18′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.93 (3H, d, 21′-CH_3), 3.62 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 4.05 (1H, m, 6′β-CH).

*2.2.4.2. Methyl 3α-tert-butyldimethylsilyloxy-6α-hydroxy-5β-cholan-24-oate (**17**).* This compound was synthesized with the same procedure as described for **2**, with different reactants. The title compound **17** was in the form of colorless amorphous solids: yield, 5.62 g (72.1%); m.p., 151–153 °C. IR (cm^{-1}): 3602, 3418, 2941, 2858, 1733, 1467, 1440, 1377, 1311, 1251, 1168, 1073, 1033, 988, 871, 837, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.63 (3H, s, 18′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.92 (3H, d, 21′-CH_3), 3.59 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 4.03 (1H, m, 6′β-CH).

*2.2.4.3. 3α-tert-butyldimethylsilyloxy-6α-hydroxy-5β-cholan-24-oic acid (**18**).* This compound was synthesized with the same procedure as described for **3**, with different reactants. The title compound **18** was in the form of colorless crystals: yield, 4.86 g (96%); m.p., 206–208 °C. IR (cm^{-1}): 3395, 2942, 2858, 1730, 1467, 1444, 1392, 1252, 1173, 1096, 1029, 963, 873, 837, 774. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.64 (3H, s, 18′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.93 (3H, d, 21′-CH_3), 3.59 (1H, m, 3′β-CH), 4.05 (1H, m, 6′β-CH).

*2.2.4.4. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-6α-hydroxy-5β-cholan-24-oate (**19**).* This compound was synthesized with the same procedure as described for **4**, with different reactants. The title compound **19** was found as a colorless solid: yield: 2.83 g (51.5%); m.p., 179–182 °C. IR (cm^{-1}): 3528, 3311, 2931, 2856, 2757, 1699, 1673, 1658, 1453, 1381, 1252, 1168, 1087, 1004, 986, 875, 837, 774. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.64 (3H, s, 18′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.93 (3H, d, 21′-CH_3), 3.58 (1H, m, 3′β-CH), 4.05 (1H, m, 6′β-CH), 4.68 (1H, m, 3α-CH), 0.80 (3H, s, 19-CH_3), 1.04 (3H, s, 21-CH_3), 1.09 (3H, d, 27-CH_3).

*2.2.4.5. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α,6α-dihydroxy-5β-cholan-24-oate (**20**).* This compound was synthesized with the same procedure as described for **5**, with different reactants. The title compound **20** was found as a colorless amorphous solid: yield: 2.10 g (97%); m.p., 121.5–123.6 °C. $[\alpha]_D^{20} = -52.5$ (c 0.001, $CHCl_3$). IR (cm^{-1}): 3381, 2935, 2870, 2777, 1712, 1454, 1380, 1243, 1167, 1038, 957. EI-MS m/z: 804 ($[M+H]^+$, 12.84%), 803 (M^+, 3.36%). HRESIMS calculated for $C_{51}H_{82}NO_6$ $[M+H]^+$:804.6137; found: 804.6131. Its analysis led to the molecular formula $C_{51}H_{81}NO_6$, supported by the ^{13}C NMR, H–H COSY, HSQC and DEPT data. The ^{1}H NMR spectrum (Table 1) showed a set of downfield resonances at δ 4.67 (1H, m, 3α-CH), 4.06 (1H, m, 6′β-CH), 3.62 (1H, m, 3′β-CH) and a set of typical upfield resonances at δ 1.09 (3H, d, 27-CH_3), 1.04 (3H, s, 21-CH_3), 0.93 (3H, d, 21′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.79 (3H, s, 19-CH_3), 0.64 (3H, s, 18′-CH_3). The ^{13}C NMR spectrum (Table 3) showed a set of typical resonances at δ 210.4 (C-6), 173.8 (C-24′), 72.8 (C-3), 71.6 (C-3′), 70.9 (C-20), 70.6 (C-22), 68.1 (C-6′), 62.1 (C-26), 61.8 (C-18), 56.4 (C-9), 56.2 (C-5).

2.2.5. Deoxycholic acid–verticinone ester (DCA-Ver)

The synthetic route for the DCA-Ver is shown in Scheme 1. The ^{1}H NMR chemical shifts of DCA-Ver are listed in Table 1 and the ^{1}H NMR chemical shifts of intermediate products are listed in Table 2. The ^{13}C NMR chemical shifts of DCA-Ver are listed in Table 3.

*2.2.5.1. Methyl 3α,12α-dihydroxy-5β-cholan-24-oate (**21**).* This compound was synthesized with the same procedure as described for **1**, with different reactants. The title compound **21** was in the form of colorless crystals: yield, 20.6 g (84.5%); m.p., 148–150 °C. IR (cm^{-1}): 3630, 3511, 3332, 2943, 2867, 1744, 1449, 1372, 1249, 1196, 1164, 1039, 1017, 973. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.96 (3H, d, 21′-CH_3), 3.61 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 3.98 (1H, m, 12′β-CH).

*2.2.5.2. Methyl 3α-tert-butyldimethylsilyloxy-12α-hydroxy-5β-cholan-24-oate (**22**).* This compound was synthesized with the same procedure as described for **2**, with different reactants. The title compound **22** was in the form of colorless amorphous solids: yield, 5.73 g (73.5%); m.p., 165–167 °C. IR (cm^{-1}): 3623, 3453, 2940, 2862, 1737, 1465, 1444, 1377, 1252, 1211, 1175, 1100, 1030, 949, 876, 840, 774. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3),

0.89 (3H, s, 19′-CH_3), 0.97 (3H, d, 21′-CH_3), 3.58 (1H, m, 3′β-CH), 3.67 (3H, s, –OCH_3), 3.97 (1H, m, 12′β-CH).

*2.2.5.3. 3α-tert-butyldimethylsilyloxy-12α-hydroxy-5β-cholan-24-oic acid (**23**).* This compound was synthesized with the same procedure as described for **3**, with different reactants. The title compound **23** was in the form of colorless crystals: yield, 4.76 g (94%); m.p., 229–231 °C. IR (cm^{-1}): 3549, 3171, 2933, 2864, 1721, 1468, 1452, 1399, 1375, 1254, 1154, 1049, 949, 873, 837, 782. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.68 (3H, s, 18′-CH_3), 0.91 (3H, s, 19′-CH_3), 1.00 (3H, d, 21′-CH_3), 3.62 (1H, m, 3′β-CH), 3.94 (1H, m, 12′β-CH).

*2.2.5.4. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-12α-hydroxy-5β-cholan-24-oate (**24**).* This compound was synthesized with the same procedure as described for **4**, with different reactants. The title compound **24** was found as a colorless solid: yield: 3.1 g (55.6%); m.p., 170–172 °C. IR (cm^{-1}): 3504, 3350, 2934, 2858, 2775, 1710, 1467, 1452, 1379, 1256, 1174, 1097, 953, 875, 837, 801, 775. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.67 (3H, s, 18′-CH_3), 0.89 (3H, s, 19′-CH_3), 0.98 (3H, d, 21′-CH_3), 3.58 (1H, m, 3′β-CH), 3.97 (1H, m, 12′β-CH), 4.68 (1H, m, 3α-CH), 0.79 (3H, s, 19-CH_3), 1.03 (3H, s, 21-CH_3), 1.07 (3H, d, 27-CH_3).

*2.2.5.5. 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α, 12α-dihydroxy-5β-cholan-24-oate (**25**).* This compound was synthesized with the same procedure as described for **5**, with different reactants. The title compound **25** was found as a colorless amorphous solid: yield: 2.06 g (95%); m.p., 108.5–110.4 °C. $[\alpha]_D^{20} = +15.5$ (*c* 0.001, $CHCl_3$). IR (cm^{-1}): 3442, 2935, 2860, 2777, 1709, 1451, 1381, 1256, 1172, 1092, 1043, 945. EI-MS *m*/*z*: 804 ($[M+H]^+$, 2.11%), 803 (M^+, 0.60%). HRESIMS calculated for $C_{51}H_{82}NO_6$ $[M+H]^+$:804.6137; found: 804.6132. Its analysis led to the molecular formula $C_{51}H_{81}NO_6$, supported by the ^{13}C NMR, H–H COSY, HSQC and DEPT data. The ^{1}H NMR spectrum (Table 1) showed a set of downfield resonances at δ 4.67 (1H, m, 3α-CH), 3.98 (1H, s, 12′β-CH), 3.60 (1H, m, 3′β-CH) and a set of typical upfield resonances at δ 1.08 (3H, d, 27-CH_3), 1.03 (3H, s, 21-CH_3), 0.98 (3H, d, 21′-CH_3), 0.90 (3H, s, 19′-CH_3), 0.80 (3H, s, 19-CH_3), 0.68 (3H, s, 18′-CH_3). The ^{13}C NMR spectrum (Table 3) showed a set of typical resonances at δ 210.4 (C-6), 173.8 (C-24′), 73.2 (C-12′), 72.9 (C-3), 71.8 (C-3′), 71.0 (C-20), 70.5 (C-22), 61.7 (C-26), 61.6 (C-18), 56.5 (C-9), 56.4 (C-5).

2.3. Screening on the pharmacological action of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver

All protocols were approved by the institutional ethics committee.

2.3.1. Animals

Kunming mice of both sexes (Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China), weighing about 18–22 g, were used. The animals were placed in cages and kept in standard environmental conditions with a standard rodent diet and water ad libitum under a 12 h light–dark (07:00–19:00 h and 19:00–07:00 h) cycle. They were deprived of food for 24 h before experimentation but allowed free access to tap water throughout. This study was carried out in accordance with the "Regulation for the Administration of Affairs Concerning Experimental Animals" (State Council of China, 1988).

2.3.2. Drug administration

Codeine phosphate, guaifenesin, verticinone, bile acids, CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver were suspended in 0.5% carboxyl methylcellulose solution. Treatments were administered orally.

2.3.3. Antitussive activity

In the screening study of antitussive activity of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver, we employed the animal model such as cough induced by ammonia water in mice. The mice were divided into 13 groups of 10 each. Group 1 animals (Control) received the vehicle (0.5% carboxyl methylcellulose solution, 0.2 ml/20 g, p.o.); group 2 was treated with codeine phosphate as a standard antitussive agent (30 mg/kg, p.o.); group 3 was treated with verticinone (3 mg/kg, p.o.); groups 4–8 with bile acids (3 mg/kg, p.o., respectively) and groups 9–13 were treated with the five ester-linked compounds (3 mg/kg, p.o., respectively). The unanesthetized and unrestrained animals were placed in individually in a 500-ml beaker which was placed upside down and exposed to 25% ammonia water. Then the frequency of the cough for 3 min and latent period of cough were observed. During 3 min observation period, the animals were continuously watched by a trained observer and the frequency of coughs was recorded. The number of coughs produced 60 min after the administration of antitussive agents (Ct) was compared with the number of control coughs (Cc). The antitussive effect was expressed as the percentage of inhibition of the number of control coughs $[(Cc - Ct)/Cc \times 100\%]$.

2.3.4. Expectorant activity

In the screening study of expectorant activity of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver, we employed the mucus secretion experiment. Mucus secretion was evaluated in mice by measuring the tracheal output of phenol red. The procedures were performed as described previously. The mice were divided into 13 groups of 10 each. Group 1 animals (Control) received the vehicle (0.5% carboxyl methylcellulose solution, 0.2 ml/20 g, p.o.); group 2 was treated with guaifenesin as a standard expectorant agent (50 mg/kg, p.o.); group 3 was treated with verticinone (3 mg/kg, p.o.); groups 4–8 with bile acids (3 mg/kg, p.o., respectively) and groups 9–13 were treated with the five ester-linked compounds (3 mg/kg, p.o., respectively). Briefly, the test compounds were administered orally 30 min before intraperitoneal injection of phenol red solution (5% saline solution, 0.1 ml/10 g body weight). Then 30 min after application of phenol red, the mice were executed without damaging the tracheas. After dissected free from adjacent organs, the trachea was removed from the thyroid cartilage to the main stem bronchi and put into 2 ml normal saline immediately. In order to let the test compounds secreting to the tracheal lumen of mice resolve to the medium completely, the tracheas were vibrating for 30 min. Finally 0.20 ml NaOH (1 mol/l) was added to the saline and optical density of the mixture was measured at 558 nm using UC-756 PC spectrophotometer (Shanghai Spectrum Instrument Co., Ltd., China). The standard curve of phenol red was performed as Pharmacology Experiment Methodology.

2.3.5. Acute toxicity

Healthy Kunming mice of both sexes, weighing 18–22 g, were divided into 8 groups of 10 animals matched for weight and size. These animals were housed 5 mice per sex per cage in a well ventilated room with 12 h cycle of day and night light conditions and temperature maintained at around 25 °C. All animals had free access to tap water and the same type of food throughout the experiment, except for the short fasting period before the oral administration of the single doses of CA-Ver (or CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver). CA-Ver (or CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver) was aseptically suspended in 0.5% carboxyl methylcellulose solution and administered by the intraperitoneal (i.p.) route at doses of 0, 1500, 2000, 2500, 3000 and 3500 mg/kg or by gavage (p.o.) at doses of 0, 2, 4 and 6 g/kg. The general behavior of the mice

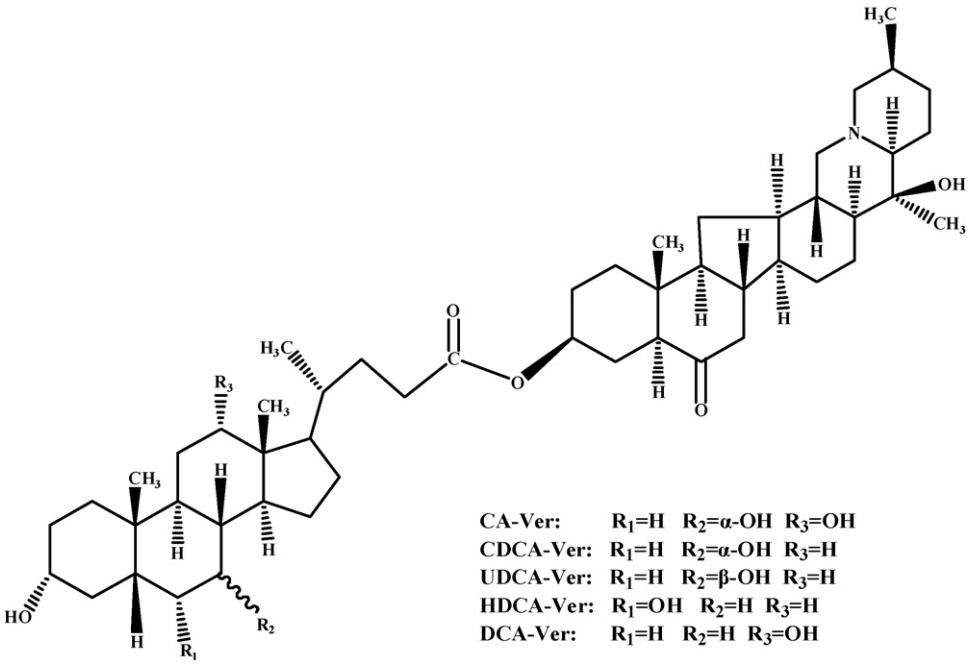


Fig. 2. Structures of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver.

was observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h. The mice were further observed for up to 14 days following treatment for any signs of toxicity and deaths, and the latency of death. Behavioral, toxic effects and mortality response were recorded.

2.3.6. Statistical analysis

In all studies the values were expressed as means ± s.e.m. of *n* observations. The results were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. $P < 0.05$ was considered significant.

3. Results and discussion

Shedan-Chuanbei powder, a complex traditional Chinese medicine formulation, is the most popular antitussive and expectorant formulation in Chinese communities. Its potent antitussive effect was previously reported in experimental models of coughs. Substitutes for Shedan-Chuanbei powder are already available in markets, such as an artificial snake bile substitute of natural snake bile or other *Fritillariae* species to substitute *Fritillariae cirrhosae*. But all these substitutes still confront many daunting challenges, such as standardization problems due to the natural variability of the crude materials and the chemical complexity of the preparation. However, CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver, five novel esters of verticinone and bile acids, would make standardization and preparation problems simpler because they are five single compounds.

Bile acids are a group of compounds consisting of a steroid backbone (with up to hydroxyl groups at positions C3 and C6 or C7 or C12) and an extended alkyl chain at position C17 terminating in a carboxylic acid group, which can be conjugated with verticinone. Cholic acid (Fig. 1), or 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, one of the most commonly occurring bile acids, consists of a steroid skeleton, three hydroxyl groups at positions C3, C7 and C12 and an extended alkyl chain at position C17 terminating in a carboxylic acid group, which can be conjugated with 3β hydroxyl of verticinone via ester bond. Verticinone, or 5α,14α-cevanin-3β, 20β-dihydroxy-6-one, has two hydroxyl groups that are all β configurations. For cholic acid, which has three α-hydroxyl groups, the equatorial hydroxyl group on the C3 position is more accessible and therefore more reactive than the other two axial hydroxyl groups at positions C7

Table 4
Effect of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver on cough induced by ammonia liquor in mice (Mean ± S.E, $n = 10$).

Experimental design	Dose (mg/kg)	No. of animals	Latent period of cough (s)	No. of coughs	% Inhibition
0.5%CMC-Na	–	10	36.82 ± 20.72	16.6 ± 3.37	
Codeine phosphate	30	10	64.20 ± 19.05**	8.7 ± 3.53***	47.59
Verticinone	3	10	78.03 ± 15.35***	7.4 ± 3.53***	55.42
CA	3	10	36.97 ± 10.89	12.4 ± 3.27	25.30
CDCA	3	10	41.75 ± 9.06	11.7 ± 1.42	29.52
UDCA	3	10	43.42 ± 20.18	11.9 ± 4.01	28.31
HDCA	3	10	42.59 ± 9.02	10.9 ± 2.38	34.34
DCA	3	10	40.40 ± 17.09	11.2 ± 2.15	32.53
CA-Ver	3	10	78.14 ± 25.47*	6.7 ± 2.67***	59.64
CDCA-Ver	3	10	76.51 ± 23.18***	9.4 ± 2.99***	43.37
UDCA-Ver	3	10	75.96 ± 28.18**	9.1 ± 2.85***	45.18
HDCA-Ver	3	10	77.72 ± 20.06***	7.8 ± 2.82***	53.01
DCA-Ver	3	10	78.53 ± 24.22***	7.9 ± 3.51***	52.41

** $P < 0.01$ (*vs.* 0.5%CMC-Na).
*** $P < 0.001$ (*vs.* 0.5%CMC-Na).

Table 5
Effect of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver on the amount of phenol red (PR) secreted in mice (Mean ± S.E, $n = 10$).

Experimental design	Dose (mg/kg)	No. of animals	Absorbance (A)	Phenol red secretion (μg/ml)
0.5%CMC-Na	–	10	0.1207 ± 0.0485	0.8670 ± 0.3505
Guaifenesin	50	10	0.2585 ± 0.0643	1.8526 ± 0.4635***
Verticinone	3	10	0.2494 ± 0.0742	1.7876 ± 0.5343***
CA	3	10	0.1279 ± 0.0704	0.9185 ± 0.5072
CDCA	3	10	0.1345 ± 0.0641	0.9657 ± 0.4621
UDCA	3	10	0.1229 ± 0.0380	0.8827 ± 0.2754
HDCA	3	10	0.1272 ± 0.0521	0.9134 ± 0.3763
DCA	3	10	0.1325 ± 0.0507	0.9514 ± 0.3662
CA-Ver	3	10	0.2370 ± 0.0714	1.6989 ± 0.5143***
CDCA-Ver	3	10	0.2175 ± 0.0670	1.5594 ± 0.4828**
UDCA-Ver	3	10	0.2219 ± 0.0722	1.5908 ± 0.5200**
HDCA-Ver	3	10	0.1957 ± 0.0793	1.4034 ± 0.5708*
DCA-Ver	3	10	0.1770 ± 0.0540	1.2697 ± 0.3920*

* $P < 0.05$ (*vs.* 0.5%CMC-Na).
** $P < 0.01$ (*vs.* 0.5%CMC-Na).
*** $P < 0.001$ (*vs.* 0.5%CMC-Na).

and C12 [9]. In general, the preparation of these 3α-conjugates by the formation of esters was relatively easy (especially when compared with the 3β-analogs) because of the accessibility of the equatorial OH group on C3, as well as its nucleophilic properties [10]. So attempts were made to attach the OH groups directly at C3 of verticinone molecule via an ester linkage using cholic acid molecule, without any success. Nor were we able to link directly chenodeoxycholic acid (or ursodeoxycholic acid, or hyodeoxycholic acid, or deoxycholic acid) molecule with verticinone molecule. The conjugates were not obtained in a reasonable yield. Therefore the 3α-OH functional group of bile acids was protected by reaction with tert-butyl dimethyl chlorosilane (TBDMSiCl) [11] via ether bond. Then the conjugates were achieved by linking verticinone and bile acids of the protected OH group. It seemed that this protecting group was necessary for a successful conjugation. In order to protect 3α-OH functional group of bile acids with TBDMSiCl, it was necessary to esterify the carboxyl groups of bile acids with MeOH, since the carboxyl group is more reactive than the hydroxyl groups. The methyl esters of bile acids were more soluble in organic solvents, such as THF, CH_2Cl_2, and EtOAc. Moreover, the methyl esters acted as a protecting group and facilitated the synthesis.

CA-Ver (Fig. 2) was synthesized from cholic acid and verticinone via esterification using DCC as the acid-activating agent, leading to the linkage of the carboxylic acid group on C24 position of cholic acid with the hydroxyl group on C3 position of verticinone. The synthesis of the CA-Ver **5** is illustrated in Scheme 1 (five steps, 33% overall yield). Reaction of cholic acid with absolute MeOH followed by esterification resulted in the formation of methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate **1**. Then reaction of **1** with TBDMSiCl [11] via ether bond afforded the intermediate **2**. Hydrolysis of the methyl ester groups of **2** with 5% sodium hydroxide aqueous solution furnished compound **3**. Then treatment of **3** with verticinone in CH_2Cl_2 followed by esterification gave the compound 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α-tert-butyldimethylsilyloxy-7α,12α-dihydroxy-5β-cholan-24-oate **4**. In this reaction, DMAP was used as a catalyst and DCC as a dehydration agent [12]. Finally, the deprotection reaction of **4** in 5% HF aqueous solution [13] gave the desired 5α,14α-cevanin-6-O-20β-hydroxy-3β-yl-3α,7α,12α-trihydroxy-5β-cholan-24-oate **5**. The chemoselective esterification reaction of **3** with verticinone in CH_2Cl_2 provided **4** in 58.9% yield. Consistent with previous reports [14], the reaction of cholic acid with verticinone did not favor the β-conjugate. The IR spectrum of CA-Ver, showed an absorption band at 1741 cm^{-1}, characteristic for an ester C=O. The EI-MS of CA-Ver showed M^+ and $[M-H]^+$ ions, respectively, at m/z 820 and 819 and thus confirmed the formation of conjugate. The ^1H and ^{13}C NMR spectra (Table 1 and 3) of CA-Ver showed signals similar to that of its component steroid monomers cholic acid and verticinone with the exception that the signals arising from the C-3 oxymethine proton (δ_H 4.69) and carbon (δ_C 72.8) were much deshielded compared to those of verticinone (δ_H 3.58 and δ_C 70.9). This downfield shift was due to esterification of verticinone at C-3 with the side-chain carbonyl of cholic acid. This fact was confirmed further by a ^1H–^{13}C long-range correlation (^3J) observed between the C-3 oxymethine proton (δ_H 4.69) of verticinone of and the C-24 carbonyl carbon of cholic acid (δ_C 173.82) in its HMBC spectrum. CDCA-Ver (or UDCA-Ver, or HDCA-Ver, or DCA-Ver) was synthesized from chenodeoxycholic acid (or ursodeoxycholic acid, or hyodeoxycholic acid, or deoxycholic acid) and verticinone with the same procedure as described for CA-Ver. They were readily identified by comparison of their m.p, $[\alpha]_D^{20}$, IR, MS, ^1H NMR and ^{13}C NMR data with their component steroid monomers.

We then evaluated the antitussive activity, expectorant activity and the acute toxicity of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver. Antitussive activity studies showed that the five ester-linked compounds had much more potent antitussive effects than single bile acids at the same doses, and had equivalent antitussive effects in comparison with about double moles dose of the monomer verticinone (Table 4). Especially, cholic acid–verticinone ester had much more potent antitussive effect than the monomer verticinone or cholic acid at the same dose. Further expectorant activity studies demonstrated that these compounds have much more potent expectorant activity than single bile acids at the same doses, and had equivalent expectorant activity compared with about double moles dose of the monomer verticinone (Table 5). The standard curve of phenol red was performed as Pharmacology Experiment Methodology and the regression equation was $Y = 0.1398X - 0.0005$ (Y = absorbance, X = the amount of phenol red secretion, $r = 0.9995$). Moreover, there were no deaths or any signs of toxicity observed after oral administration of single doses of the five ester-linked compounds at any dose level up to the highest dose tested (6 g/kg), which was the no-observed-adverse-effect level (NOAEL) [15]. Uniformly, there were no deaths after the intraperitoneal injection at any dose level up to the highest dose tested (3.5 g/kg). No mortalities had occurred during the study and all observations did not indicate evidence of substance-related toxicity. So the acute toxicity of CA-Ver, CDCA-Ver, UDCA-Ver, HDCA-Ver and DCA-Ver were considered as unclassified, since a dose of 6 g/kg (by gavage) or 3.5 g/kg (by intraperitoneal injection) did not induce deaths or toxic symptoms. Based on the studies of pharmacology and acute toxicity, the five novel esters of verticinone and bile acids have synergic pharmacodynamic action and attenuated toxicity compared with single verticinone and single bile acids.

Our present study not only synthesized the five novel esters of verticinone and bile acids, which will be used as antitussive and expectorant agents in future, it could also be an attempt at application structure combination idea to the research and development of TCM, which may exploit a novel field of new drug design from TCM. Cumulatively, this study highlights a conjugated extension of esterification with verticinone and single bile acid and illustrates a potential benefit to structure combination of natural products. Furthermore, our current project could build the foundation to further study structure–activity relationship of verticinone in the next step.

Acknowledgments

We thank the Huazhong University of Science and Technology Analytical and Testing Center for analytical support. This research was supported by the Fund of the Key Project of the Education Ministry, China, 2006 and the Fund of the International Cooperation Project of Science and Technology, the Ministry of Science and Technology, China (China and Korea, No. 2006DFA33210). We sincerely thank C.S. Pan and B.Q. Yan of the Bruker Daltonics Inc. for the micrOTOF-Q MS. The present study has been applied for patent in China (application no. 200810048722.9).

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