Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 3888–3895
Bioorganic & Medicinal Chemistry

Preparation of yuanhuacine and relative daphne diterpene esters from *Daphne genkwa* and structure–activity relationship of potent inhibitory activity against DNA topoisomerase I

Shixuan Zhang,* Xiaona Li, Fenghong Zhang, Puwen Yang, Xiujuan Gao and Qiling Song

State Key Laboratory of Fine Chemicals, Dalian University of Technology, Zhongshan Road 158-90, Dalian 116012, China

Received 11 November 2005; revised 14 January 2006; accepted 18 January 2006
Available online 20 February 2006

Abstract—Two new daphne diterpene esters Yuanhuajine (**2**) and Yuanhuagine (**4**), together with three known daphne diterpene esters yuanhuacine (**1**), yuanhuadine (**3**), and yuanhuapine (**5**), were isolated and identified from *Daphne genkwa*, a traditional Chinese medicine. Their structures were elucidated by a combination of UV, IR, MS and NMR (^{1}H NMR, ^{13}C NMR, HSQC, and HMBC) spectra. In order to explore the structure–activity relationship, three compounds **6**, **7**, and **8** were prepared as three derivatives of **1**. Inhibitory activities against DNA topoisomerase I (topo I) were assessed for the compounds **1**–**8**. These compounds, except for **8**, exhibited potent inhibitory activities against DNA topo I at IC_{50} levels of 11.1–53.4 μM and they are new type of topo I inhibitors bearing different structures compared with the known topo I inhibitors. The agarose-gel electrophoresis experiments showed that the orthoester group of daphne diterpene esters was necessary for the inhibitory activity against DNA topo I, and the inhibition against DNA topo I is probably one of the anti-tumor mechanisms of daphne diterpene esters.

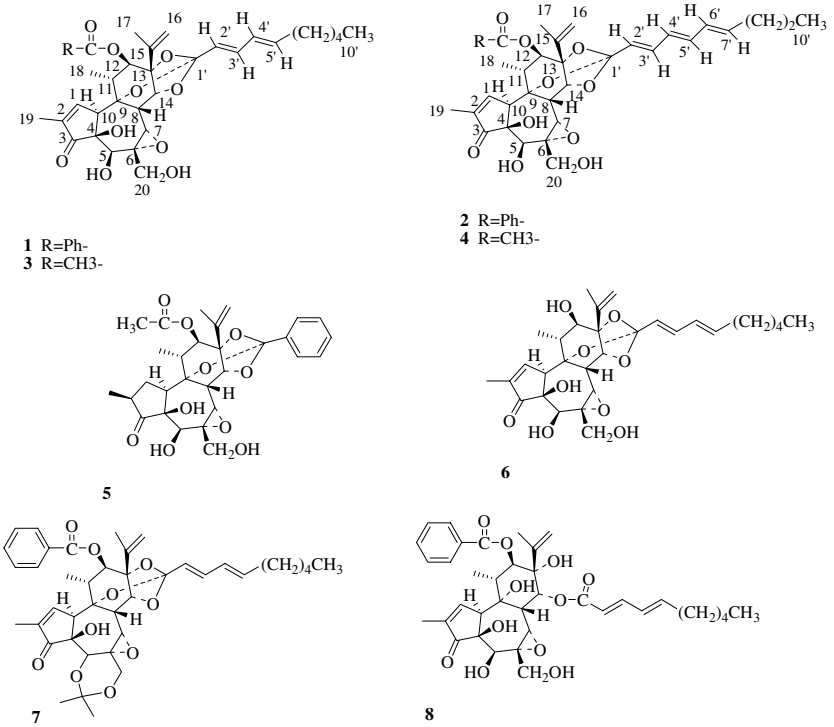
1. Introduction

Daphne genkwa Sieb. et Zucc. (Thymelaeaceae) is a traditional Chinese medicine. It was mainly used for dispelling retained water, abortion, and mammary cancer. The principal active components are daphne diterpene esters. Yuanhuacine (gnidilatidin) (**1**),[1] yuanhuadine (**3**), and yuanhuapine (**5**) have been isolated from *D. genkwa* by earlier research, and **1** has been used for abortion.[2–4] Compound **1** showed potent cytotoxic activity in vitro, against P-388 lymphocytic leukemia, L-1210 lymphoid leukemia, human KB carcinoma (IC_{50} 6.42, 10.1, and 1.95 μM),[5–7] P-388, A-549 tumor cell lines. and endothelial cell HMEC (IC_{50} 30.0, 0.15, and 14.0 μM).[8] In addition, **1** could inhibit DNA and protein synthesis without binding with DNA or interfering with the template activity of nucleic acid synthesis. Further research proved **1** was a special inhibitor toward receptor of phorbol esters which has the ability of activating protein kinase C (PKC), and had no effect on the activity of normal PKC.[9] And also, compound **1** had only weak inhibitory effect on tumor necrosis factor (TNF-α) and interleukin-1 (IL-1α, IL-1β) biosynthesis.[10,11] It has been reported that compound **5** showed potent inhibitory activity against P338, A-549 tumor cell lines at IC_{50} levels of 73.0 and 0.42 μM, respectively.[8]

In this paper, two new daphne diterpene esters yuanhuajine (**2**) and yuanhuagine (**4**) together with three known compounds **1**, **3** and **5** were isolated and identified from *D. genkwa*. The structures of **2** and **4** were identified as 6′, 7′-dehydro-yuanhuacine and 6′, 7′-dehydro-yuanhuadine, respectively, on the basis of IR, UV, MS, and NMR spectra. The purity of **1** was more than 99.7%, and its ^{13}C NMR spectrum was reported for the first time. Compounds **1**–**5** showed potent inhibitory activity against DNA topo I at IC_{50} levels of 38.0–53.4 μM. In order to explore the structure–activity relationship, three derivatives **6**–**8** of **1** were prepared according to reported methods.[4,12] The agarose-gel electrophoresis experiments showed that **1**–**7** have the inhibitory activity against DNA topo I, and **8** nearly has no inhibitory activity, which indicated that the orthoester group was necessary for the inhibitory activity against DNA topo I. The inhibition against DNA topo I is probably one of the anticancer mechanisms of these daphne diterpene esters.[13,14] The structures of **1**–**8** are given in Figure 1.

Keywords: Yuanhuacine; *Daphne genkwa*; Daphne diterpene esters; Inhibitory activity against DNA topoisomerase I.
* Corresponding author. Tel.: +86041188993891; fax: +86041188993892; e-mail: zsx56@yahoo.com.cn

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.01.055



1 R=Ph-
3 R=CH3-

2 R=Ph-
4 R=CH3-

5

6

7

8

Figure 1. Structures of **1**–**8**.

2. Result and discussion

2.1. Structural chemistry

Yuanhuacine (**1**) was recrystallized from absolute alcohol as a white needle crystal. Two ion peaks at *m/z* 649.3031 $[M+H]^+$ and 671.2817 $[M+Na]^+$ were shown by HR-ESI-MS and the molecular formula $C_{37}H_{44}O_{10}$ was determined at *m/z* 648.2952 (calcd 648.2935), indicating the presence of 16° unsaturation. The UV–vis spectrum (EtOH) gave a band at λ_{max} 232 nm, $\varepsilon = 52800$ (Ref. $\varepsilon = 52{,}800\ M^{-1}\ cm^{-1}$).[1] The IR (KBr) spectrum showed features at 3465 cm^{-1} (OH), 1706 cm^{-1} (C=O), 1720, 1270, 1067 cm^{-1} (O–C=O), 1664, 1632 cm^{-1} (C=C), 1170 cm^{-1} (C–O–C), 908 cm^{-1} (epoxy), and 733, 712 cm^{-1} (phenyl). The ^1H NMR spectrum was in correspondence with the reported data[1,2] and revealed 1-substituted phenyl groups at δ_H 7.894 (2H, d, $J = 7.6$, H-3″, 7″), δ_H 7.441 (2H, t, $J = 7.6$, H-4″, 6″), and δ_H 7.58 (1H, s, H-5″), four methyl groups at δ_H 1.871 (3H, s, CH_3-17), δ_H 1.387 (3H, d, $J = 7.2$, CH_3-18), δ_H 1.764 (3H, s, CH_3-19), and δ_H 0.887 (3H, t, CH_3-10′), four aliphatic methylene protons at δ_H 2.106 (2H, d, CH_2-6′), δ_H 1.387 (2H, m, CH_2-7′), δ_H 1.289 (2H, m, CH_2-8′), and δ_H 1.257 (2H, m, CH_2-9′). According to APT and HSQC spectra, seventeen positive carbon signals afforded 10 quaternary carbons and 7 methylene carbons, and 18 negative carbon signals represented 4 methyl carbons and 14 methine carbons; the proton signal of H-7 (δ_H 4.193), the carbon signals C-7 (δ_C 71.9) and C-6 (δ_C 72.4) were upfield shifted, suggesting the presence of a 6,7-epoxy. The ^{13}C NMR and HSQC spectra revealed a quaternary carbon signal at δ_C 117.2, which is the most notable structural feature for orthoester group in daphne diterpene esters. Three oxygenated methine carbons (δ_C 80.6, C-14, δ_C 71.9, C-7, and δ_C 64.2, C-5), four oxygenated quaternary carbons (δ_C 60.8, C-4, δ_C 72.4, C-6, δ_C 78.4, C-9, δ_C 84.0, C-13), and one oxygenated methylene (δ_C 64.9, C-20); two carbonyl carbons (δ_C 209.6, C-3, δ_C 165.6, C-1″) and one terminal double bond (δ_C 143.2, C-15; δ_C 113.8, C-16) were distinguished; C-4″ and C-4″/C-6″ were concurrent at δ_C 128.8. Except for these characteristic signals, the olefin protons as a typical AB system at δ_H 5.016 (1H, s, H-16) and 4.999 (1H, s, H-16) are a feature of terminal double bond; HSQC spectrum also showed the methylene protons of the hydroxylmethyl group resolved at δ_H 3.854 (1H, s, H-20) and δ_H 3.923 (1H, s, H-20) which displays a typical AB system too.

Analysis of the ^1H NMR, ^{13}C NMR, and HMBC spectra enabled us to allot the protons to their bonded carbons, and further demonstrated that **1** was a daphne diterpene ester. In the HMBC spectrum, the carbon signal at δ_C 117.2 (C-1′) correlated with the proton signals at δ_H 4.904 (H-14), δ_H 5.682 (H-2′), and δ_H 6.698 (H-3′), confirming the presence of an orthoester group in **1**. The H-7 (δ_H 4.193) correlated with C-4 (δ_C 60.8), C-5 (δ_C 64.2), and C-6 (δ_C 72.4), H-17 (δ_H 1.871) correlated with C-13 (δ_C 84.0), C-16 (δ_C 113.8), and C-15 (δ_C 143.2) showed the linkage and oxygenated patterns of these carbons.

^1H–^1H COSY spectrum showed the correlations between δ_H 5.682 (H-2′, $J = 15.6$), δ_H 6.698 (H-3′, dd, $J = 15.6$, 10.8), δ_H 6.065 (H-4′, dd, $J = 10.8$, 15.0), and δ_H 5.873 (H-5′, m, $J = 15.0$, 7.2), which represented trans-conjugated-diene. The protons coupling at δ_H

7.551, δ_H 3.833, and δ_H 1.764 assigned to H-1, H-10, and H-19, respectively. The stereochemistry was deduced on the basis of ^1H–^1H COSY spectra. No crossing peaks between δ_H 2.550 (H-11) and δ_H 5.215 (H-12) indicated H-11 and H-12 were situated in trans-orientation; the correlation pair of H-5/H-10 permitted the assignment of H-5 and H-10. H-7 (δ_H 4.193) was correlated with H-8 (δ_H 3.627) and H-14 (δ_H 4.902), which showed that H-7, H-8, and H-14 were all in the β-configuration. H-5 showed correlation with one of the H-20 protons at δ_H 3.93, suggesting that they were located on the same side and the epoxy at C_6–C_7 was of α-configuration. Therefore, the structure of **1** was elucidated. Ascriptions of protons and carbons are shown in Table 1.

Yuanhuajine (**2**) was obtained as a white needle crystal. Its HR-ESI-MS spectrum displayed pseudomolecular ion at 647.2874 $[M+H]^+$, 669.2735 $[M+Na]^+$, and the molecular formula $C_{37}H_{42}O_{10}$ was provided at *m/z* 646.2795 (calcd 646.2778), indicating the presence of 17° unsaturation. The UV–vis spectrum (EtOH) showed two bands at 232 nm (ε = 15116 M^{-1} cm^{-1}), 270 nm. The IR spectrum (KBr) gave absorption at 3437 cm^{-1} (OH), 1706 cm^{-1} (C=O), 1720, 1270, 1069 cm^{-1} (O–C=O), 1664, 1634, 850 cm^{-1} (C=C), 1107 cm^{-1} (C–O–C), 908 cm^{-1} (epoxy), 733, 713 cm^{-1} (phenyl). Compound **2** lacks two protons compared with **1**, and it was decided as 6′,7′-dehydro-yuanhuacine. They have the same basic backbone, and the positions of proton in basic backbone are nearly correspondingly in NMR spectra. In ^1H–^1H COSY spectrum, the correlations between δ_H 5.745 (1H, d, J = 15.2) and δ_H 6.733 (1H, dd, J = 15.4, 11.2), δ_H 6.131 (1H, m, J = 11.2, 14.8) and δ_H 6.342 (1H, dd, J = 14.8, 10.4), δ_H 6.093 (1H, m, J = 10.4, 14.8) and δ_H 5.777 (1H, m, J = 14.8, 7.6) proved the existence of total trans-conjugate the triolefin. Except for these characteristic signals, the methylene protons of the hydroxylmethyl group were resolved at δ_H 3.930 (1H, s, H-20) and δ_H 3.836 (1H, s, H-20) by the ^1H–^1H COSY spectrum as a typical AB system.

The molecular formula and molecular weight of **2** were the same with gniditrin,[15] but NMR spectrum (see Table 2) and UV spectrum (λ_{max} 232, 270 nm, gniditrin: λ_{max}

Table 1. NMR spectral data of **1** (400 M Hz, in $CDCl_3$, J in Hz)

No.	δ_C	δ_H (J)	^1H–^1H COSY	HMBC
1	160.5	7.55 (1H, s)	H-10, 19	H-10, 19
2	137.1	—	—	H-1, 19
3	209.6	—	—	—
4	60.8	—	—	H-5, 7, 10, 20
5	64.2	3.63 (1H, s)	H-7	H-7, 10, 20
6	72.4	—	—	H-1, 4, 7
7	71.9	4.19 (1H, s)	H-5	H-10, 20
8	35.9	3.63 (1H, s)	H-14	H-10
9	78.4	—	—	H-11, 12, 18
10	47.6	3.82 (1H, m)	H-1, 19	H-1, 11
11	44.3	2.55 (1H, d, 7.2)	H-18	H-8, 12, 18
12	79.1	5.22 (1H, s)	—	H-14, 16, 18
13	84	—	—	H-11, 16, 17
14	80.6	4.90 (1H, d)	H-8	H-8, 12
15	143.2	—	—	H-12, 14, 17
16	113.8	5.02 (2H, s)	H-17	H-12, 17
17	18.9	1.87 (3H, s)	H-16	H-16
18	18.5	1.39 (3H, d, 7.2)	H-11	H-1, 12, 19
19	10.0	1.76 (3H, s)	H-1, 10	H-1, 10
20	64.8	3.84 (1H, s), 3.93 (1H, s)	—	—
1′	117.2	—	—	H-14,3′
2′	122.4	5.68 (1H, d, 15.6)	H-3′	H- 4′,5′
3′	135.3	6.70 (1H, dd, 15.6, 10.8)	H-2′,4′	H-5′,6′
4′	128.8	6.07 (1H, dd, 10.8, 15.0)	H-3′,5′	H-2′,6′
5′	139.6	5.87 (1H, m, 5.0, 7.2)	H-4′,6′	H-2′,3′,7′
6′	32.8	2.11 (2H, d)	H-5′,7′	H-4′,10′
7′	28.9	1.39 (2H, m)	H-6′,8′	H-5′,6′
8′	31.5	1.29 (2H, m)	H-7′,9′	H-6′,10′
9′	22.7	1.26 (2H, m)	H-8′,10′	H-7′,8′,1
10′	14.2	0.89 (3H, t)	H-9′	H-8′,9′
1″	165.6	—	—	H-12, 3″,4″
2″	129.9	—	—	H-7″,4″,5″
3″	129.6	7.89 (1H, d, 7.6)	—	H-7″,5″
4″	128.8	7.44 (1H, t, 7.6)	—	H-6″,5″
5″	133.5	7.58 (1H, s)	—	H-3″,7″
6″	128.8	7.44 (1H, t, 7.6)	—	H-4″,5″
7″	129.6	7.89 (1H, d, 7.6)	—	H-3″,5″

All assignments were confirmed by HSQC and HMBC spectra.

Table 2. ^{1}H NMR and ^{13}C NMR spectral data of **2**, **3**, and **4** (400 M Hz, in $CDCl_3$, *J* in Hz)

No.	2		3		4	
	δ_C	δ_H (*J*)	δ_C	δ_H (*J*)	δ_C	δ_H (*J*)
1	160.5	7.57 (1H, s)	160.4	7.54 (1H, s)	160.1	7.54 (1H, s)
2	137.1	—	137.0	—	136.9	—
3	209.6	—	209.5	—	209.6	—
4	60.8	—	60.9	—	61.0	—
5	64.2	3.65 (1H, s)	64.3	3.49 (1H, s)	64.2	3.49 (1H, s)
6	72.4	—	72.5	—	72.6	—
7	72.1	4.23 (1H, s)	71.6	4.23 (1H, s)	71.4	4.24 (1H, s)
8	36	3.63 (1H, d)	35.4	3.49 (1H, d)	35.4	3.49 (1H, d)
9	78.5	—	78.3	—	78.4	—
10	47.7	3.85 (1H, m)	47.6	3.83 (1H, m)	47.5	3.85 (1H, m)
11	44.3	2.38 (1H, d, 7.2)	44.1	2.37 (1H, d, 7.2)	44.0	2.40 (1H, d, 7.6)
12	79.2	5.22 (1H, s)	79.0	4.75 (1H, s)	79.4	4.75 (1H, s)
13	84.1	—	83.8	—	83.8	—
14	80.7	4.90 (1H, d)	80.5	4.95 (1H, s)	80.6	4.96 (1H, s)
15	143.2	—	143.2	—	143.2	—
16	113.8	5.01 (1H, s)	113.8	4.99 (1H, s)	113.8	5.01 (1H, s)
		5.01 (1H, s)		4.95 (1H, s)		4.97 (1H, s)
17	18.9	1.87 (3H, s)	18.8	1.81 (3H, s)	18.8	1.83 (3H, s)
18	18.5	1.31 (3H, d, 7.2)	18.4	1.36 (3H, d, 6.8)	18.3	1.29 (3H, m)
19	10.1	1.78 (3H, s)	10.0	1.76 (3H, s)	10.0	1.79 (3H, s)
20	64.9	3.93 (1H, s), 3.84 (1H, s)	65.2	3.84 (2H, s)	65.3	3.86 (2H, s)
1′	117.3	—	117.1	—	117.0	—
2′	122.6	5.75 (1H, d, 15.2)	122.4	5.63 (1H, d, 15.6)	123.7	5.71 (1H, d, 15.6)
3′	135.1	6.73 (1H, dd, 15.2, 11.2)	135.2	6.64 (1H, dd, 15.4, 10.8)	134.9	6.69 (1H, dd, 15.4,11.2)
4′	128.6	6.13 (1H, m, 11.2, 14.8)	128.7	6.03 (1H, dd, 11.2, 15.0)	128.5	6.11 (1H, m, 11.2, 14.8)
5′	137.5	6.34 (1H, dd, 14.8, 10.4)	139.5	5.84 (1H, m, 15.2, 6.8)	137.4	6.31 (1H, dd, 14.8,10.4)
6′	130.3	6.09 (1H, m, 10.4, 14.8)	32.8	2.08 (2H, d)	130.3	6.07 (1H, m, 10.4,15.2)
7′	137.1	5.78 (1H, m, 14.8, 7.6)	28.8	1.28 (2H, m)	137.0	5.76 (1H, m, 15.2, 7.2)
8′	31.5	2.09 (2H, d)	31.4	1.27 (2H, m)	32.0	2.09 (2H, d)
9′	22.5	1.43 (2H, m)	22.6	1.26 (2H, m)	22.4	1.42 (2H, m)
10′	13.9	0.92 (3H, t)	14.2	0.86 (3H, t)	14.2	0.86 (3H, t)
1″	165.6	—	169.9	—	169.8	—
2″	129.9	—	21.3	1.97 (3H, s)	21.3	2.00 (3H, s)
3″,7″	129.7	7.90 (1H, d, 7.2)	—	—	—	—
4″,6″	128.8	7.46 (1H, t, 7.6)	—	—	—	—
5″	133.5	7.59 (1H, s)	—	—	—	—

245, 306 nm) demonstrated that **2** and gniditrin are isomeric compounds with exchanging the positions of R and the long-chain fatty group upon orthoester. Compared with **1**, compound **2** lacks two protons and was determined as 6′,7′-dehydro-yuanhuacine, namely yuanhuajine.

Yuanhuadine (**3**) is a white-like needle crystal. The HR-ESI-MS spectrum revealed pseudomolecular ion peaks of 587.2872 $[M+H]^+$ and 609.2667 $[M+Na]^+$, and the molecular formula $C_{32}H_{42}O_{10}$ was determined at *m/z* 586.2793 (calcd 586.2777), implying the presence of 12° unsaturation. The UV–vis spectrum (EtOH) absorption λ_{max} = 232 nm, ε = 38700 (Ref. ε = 38000 M^{-1} cm^{-1}).[3] The IR spectrum (KBr) showed features at 3466 cm^{-1} (OH), 1701 cm^{-1} (C=O), 1741, 1232, 1081 cm^{-1} (O–C=O), 1664, 1632, 850 cm^{-1} (C=C), 1154 cm^{-1} (C–O–C), and 913 cm^{-1} (epoxy). Main distinction between **3** and **1** is that methyl group substituted phenyl group of R. The ^{1}H NMR and ^{13}C NMR spectra exhibit signals of methyl group in acyloxyl moiety at δ_H 1.969 (3H, s), δ_C 21.3 (C-2′), and δ_C 169.9 (C-1′). The characteristic signals of 1-substituted phenyl groups at δ_H 7.894 (2H, d, *J* = 7.6), δ_H 7.441 (2H, t, *J* = 7.6) and δ_H 7.58 (1H, s) can be distinguished, respectively. Ascriptions of other protons and carbons atoms are shown in Table 2.

The needle crystal of Yuanhuagine (**4**) showed pseudomolecular ion at 585.2713 $[M+H]^+$, 607.2536 $[M+Na]^+$ by HR-ESI-MS and the molecular formula $C_{32}H_{40}O_{10}$ was determined at *m/z* 584.2634 (calcd 584.2621), indicating the presence of 13° unsaturation. The UV (EtOH) gave the absorption at 235 nm (ε = 10840 M^{-1} cm^{-1}) and 268 nm. The IR spectrum (KBr) showed bands at 3390 cm^{-1} (OH), 1705 cm^{-1} (C=O), 1737, 1243, 1081 cm^{-1} (O–C=O), 1635, 823 cm^{-1} (C=C), 1142 cm^{-1} (C–O–C), and 912 cm^{-1} (epoxy). Compound **4** lacks two protons than **3**, and they have the same frame structures. Compounds **4** and **2** have the same side chain radicals of orthoester with similar chemical shifts in these positions. The ^{1}H NMR coupling constants of H-2′ (δ_H 5.705, d, *J* = 15.6), H-3′ (δ_H 6.691, dd, *J* = 15.6, 11.2), H-4′ (δ_H 6.112, m, *J* = 11.2, 14.8), H-5′ (δ_H 6.314, dd, *J* = 14.8, 10.4), H-6′ (δ_H 6.076, m, *J* = 10.4, 15.2), H-7′ (δ_H 5.765, m, *J* = 15.2, 7.2) indicate

the existence of trans-conjugated triolefin. Its acyloxyl group is assisted with δ_H 1.999 (3H, s), δ_C 21.3 (C-2″), and δ_C 169.8 (C-1″) by the HSQC spectrum. Ascriptions of other protons and carbons atoms are shown in Table 2. Compound **4** was identified as 6′,7′-dehydro-yuanhuadine, namely yuanhuagine. To our knowledge, there is no report on this structure until now.

Yuanhuapine (**5**) is a known compound. Its structure was presumed by a combination of MS and the other spectra.[16]

In order to compare the inhibitory activity against DNA topo I and investigate the structure–activity relationship, three derivatives (**6**, **7**, and **8**) of **1** were prepared by reported methods. [4,12]

Commonly, esters can easily hydrolyze in basic solution, compound **6** was prepared as hydrolysis product of **1** under basic condition. While orthoester cannot hydrolyze in basic solution, it can easily hydrolyze in acidic solution, compound **8** obtained as hydrolysis product of **1** under acidic condition. Compound **7** was prepared by the condensation reaction between C_4,C_{20}-hydroxyl groups with acetone.

2.2. Topoisomerase I inhibitory activities

DNA topo I is a kind of important enzyme in all of the living organisms and participates in many cellular metabolic processes, such as replication, transcription, recombination, and repair.[17] It has been established as an important target of anticarcinogen by NCI, and the inhibitors against DNA topo I offer hope for a new way of cancer chemotherapy. As far, camptothecin and its analogs are the only type of anticancer agents toward DNA topo I under clinical application, and the daphne diterpene esters are another kind of topo I inhibitors with completely different structures compared with camptothecin and its analogs.

The inhibitory activities against topo I of these compounds were evaluated by agarose-gel electrophoresis experiments. In order to verify the activities of these compounds, compound **1** was chosen for agarose-gel electrophoresis experiment and compared with the known topo I inhibitor hydroxycamptothecin (hCPT). As shown in Figure 2, compound **1** exhibited inhibitory activity against DNA topo I at IC_{50} level of 40.0 μM, which value can br compared to that displayed by hCPT (IC_{50} = 48.0 μM).

The photopictures of **1**–**8**'s agarose-gel electrophoresis experiment are presented in Figure 3. Take compound **1** as an example: lane 1 is DNA alone, existing in two kinds of shapes—supercoiled DNA and a little of loosened DNA; lane 2 is topo I together with DNA, and supercoiled DNA was relaxed by topo I completely; in lane 3, the system consisted of DNA and **1**. Lane 3 is similar to lane 1, which indicated that **1** could not combine with DNA; In lanes 4–11, compound **1**, DNA, and topo I existed in the same system. Lanes 4–11 clearly manifested that **1** is the inhibitor of topo I, which can

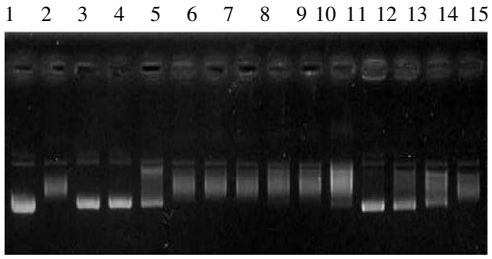


Figure 2. Compound **1** inhibited the relaxation activity of topo I compared with hCPT. Lane 1, DNA alone; lane 2, DNA + topo I; lane 3, DNA + **1**; lanes 4–11, **1** at concentrations of 0.78, 0.39, 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.006 mM + DNA + topo I, respectively; lanes 12–15, hCPT at concentrations of 1.0, 0.76, 0.38, and 0.2 mM + DNA + topo I, respectively.

inhibit the relaxation activity of topo I toward DNA. It was also found that as the concentration of **1** reduced, the inhibition weakened gradually, once less than the critical concentration, there is no inhibitory activity. Compounds **2**–**7** illustrated the similar situation as **1**. Their agarose-gel electrophoresis pictures are also given in Figure 3.

Through the agarose-gel electrophoresis experiments, these daphne diterpene esters indicated potent inhibitory activity against topo I, and the inhibitory activities were found to be structure related. The results are shown in Table 3, the IC_{50} levels of **1**–**5** being 40.0, 38.3, 52.7, 50.1, and 53.4 μM, respectively. Compounds **6** and **7** have inhibitory activity against topo I at IC_{50} levels of 11.1 and 28.1 μM, respectively, which indicate a stronger inhibitory activity than those of **1**–**5**. Most interestingly, **8** gave an IC_{50} level of 204.2 μM, nearly inactive (IC_{50} > 100 μM) (Fig. 4).

Compounds **1**–**7** have the same basic backbone with an orthoester group on it, they exhibited the inhibitory activity against topo I, and different substitute groups only influenced the inhibitory activity limitedly. As observed for **8**, once the orthoester group was broken, the inhibitory activity was lost. So it can be concluded that the orthoester group is necessary in the inhibition against DNA topo I for daphne diterpene esters. Although **1**–**7** have the same basic backbone, changes in the branch chain affected their inhibitory activity to some extent. The structure–activity relationship will be comparatively discussed in the following: (1) by transforming the electron-withdrawing hydroxyl groups at C-5 and C-20 to ketal, the inhibitory activity can be dramatically promoted, as for **7**, it manifested the strongest activity among these compounds. (2) Acyloxyl group at C-12 position substituted for less electron-withdrawing benzyloxyl group results in stronger inhibitory activity against topo I, for example, **1** showed stronger activity than **3**, **2** showed stronger activity than **4**. For compound **6**, the hydroxyl group at C-12 is a less electron-withdrawing group compared with the ester groups in **1**–**4**. This variation makes **6** have a much stronger activity than compounds **1**–**4**. (3) Changes at C-1′ position of orthoether group give a negligible effect on the inhibitory activity against topo I, for example, although bearing different substi-

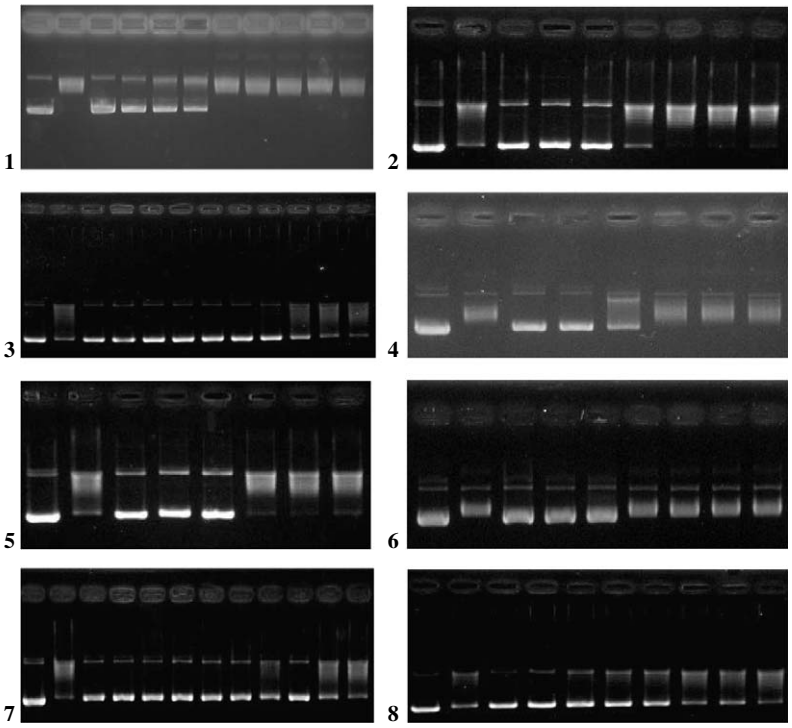


Figure 3. Compounds **1**–**8** inhibited the relaxation activity of topo I. Lane 1, DNA alone; lane 2, DNA + topo I. Compounds **1** lane 3, DNA + **1**; lanes 4–11, **1** at concentrations of 1.6, 0.78, 0.39, 0.32, 0.16, 0.05, 0.03, and 0.015 mM + DNA + topo I, respectively. Compounds **2** lane 3, DNA + **2**; lane 4–9, **2** at concentrations of 0.77, 0.39, 0.15, 0.08, 0.04, and 0.02 mM + DNA + topo I, respectively. Compounds **3** lane 3, DNA + **3**; lanes 4–12, **3** at concentrations of 8.53, 6.83, 5.12, 3.41, 1.70, 0.85, 0.43, 0.21, and 0.10 mM + DNA + topo I, respectively. Compounds **4** lane 3, DNA + **4**; lanes 4–8, **4** at concentrations of 0.85, 0.43, 0.21, 0.10, and 0.05 mM + DNA + topo I, respectively. Compounds **5** lane 3, DNA + **5**; lanes 4–8, **5** at concentrations of 1.0, 0.45, 0.23, 0.12, and 0.06 mM + DNA + topo I, respectively. Compounds **6** DNA + **6**; lanes 4–9, **6** at concentrations of 0.45, 0.23, 0.11, 0.06, 0.03, and 0.015 mM + DNA + topo I, respectively. Compounds **7**. lane 3, DNA + **7**; lanes 4–12, **7** at concentrations of 11.5, 5.8, 2.9, 1.5, 0.75, 0.36, 0.18, 0.09, and 0.05 mM + DNA + topo I, respectively. Compounds **8** lane 3, DNA + **8**; lanes 4–10, **8** at concentrations of 12.0, 6.0, 3.0, 1.5, 0.75, 0.38, and 0.19 mM + DNA + topo I, respectively.

Table 3. Inhibitory activity against DNA topo I of **1**–**8** compared with hCPT

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	hCPT
IC_{50} (μM)	40.0	38.0	53.0	50.0	53.4	28.1	11.1	204.2	48.0

tutions at C-1′, compounds **1** and **2** showed similar activities as well as the case of **3**, **4**, and **5**. Taking these influences into account comprehensively, the inhibitory activities of these compounds decreased in the order: **7** > **6** > **2** > **1** > **4** > **3** > **5**.

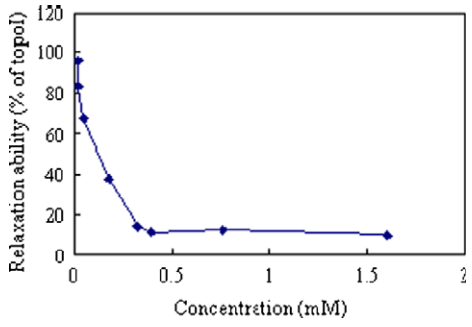


Figure 4. Concentration–response curve of compound **1** inhibiting the relaxation activity of topo I. Each point of the curve represents the relaxation ability of topo I toward DNA when compound 1 existed with different concentrations in agarose-gel electrophoresis experiment.

To conclude, our research indicated that the orthoester group is necessary in inhibition against DNA topo I for daphne diterpene esters and the activity is structure related. Compounds with less electron-withdrawing groups, in other words, more electron-donating groups especially at the positions of C-12, C-5, and C-20, performed better inhibitory activities. Since it has been reported that the linkage area of DNA topo I has the positive charge.[18] It is suggested that the orthoester group with more electronegativity facilitated the combination between the inhibitor and DNA topo I.

Electron-donating groups can increase the electronegativity of the orthoester group, which is helpful for the inhibitory activity against DNA topo I. Studies on specific binding site of these compounds to the topo I–DNA complex are in progress to understand the details of the inhibition mechanism.

3. Conclusions

Five daphne diterpene esters **1**–**5** were isolated and identified from *D. genkwa*, a traditional Chinese medicine. Among them, high-purity compounds, yuanhuajine (**2**) and yuanhuagine (**4**),were isolated with C_{18} preparation HPLC for the first time. Their structures were established as 6′,7′-dehydro-yuanhuacine and 6′,7′-dehydro-yuanhuadine, respectively. Compounds **1**–**5** exhibited potent inhibitory activities against DNA topo I (IC_{50}: 38–53.4 μM). Three derivatives **6**, **7**, and **8** of **1** were also prepared to explore the structure–activity relationship and gave IC_{50} levels of 11.1, 28.1, and 204.2 μM, respectively. The agarose-gel electrophoresis experiments showed that orthoester group was necessary for the inhibition against DNA topo I, and the activities of these compounds were structure related. The inhibition against DNA topo I is probably one of the anticancer mechanisms of daphne diterpene esters. These daphne diterpene esters maybe provide new lead structures for the development of potential anticancer agents.

4. Experimental

4.1. General experimental procedures

UV spectra were recorded on a HP-UV-8453 spectrophotometer. IR spectra were obtained as KBr pellets on a Nicolet 50X FT-IR spectrophotometer. HR-ESI-MS was determined with a HPLC-Q-Tof-MS spectrometer. NMR spectra were measured on a Varian INOVA-400 spectrometer, with chemical shifts reported as parts per million (in $CDCl_3$, TMS as an internal standard, *J* values were given in Hz). ^1H and ^{13}C NMR assignments were supported by ^1H–^1H COSY, HSQC, and HMBC. Preparation HPLC chromatograms were recorded by a JASCO-LC-1500 HPLC instrument. Chromatographic grade methyl alcohol, distilled water, and other analytical grade chemicals were used as received. Silica gel (50–75 μm, used for column chromatography) and silica gel-60-GF_{254nm} (used for TLC) were received from Qingdao Haiyang Chemical Group Co., China. The chromatograms were detected with a UV lamp at 254 nm and successively sprayed with ammonium metavanadate–sulfuric acid as developer. HCPT was purchased from Guanghan Biochemical Product Co., Led, China. The photopictures of agarose-gel electrophoresis experiments were imaged by Tanon GIS 2010 instrument.

4.2. Plant material

The root and flower of *D. genkwa* were collected from the Songshan Henan province of China.

4.3. Extraction and isolation

A sample of the dried root powder of *D. genkwa* (10 kg) was extracted with petroleum ether under reflux condition at 85 °C to give a brownish-black crude extract (188 g), which was then dissolved in water (1 L) and extracted with $CHCl_3$ for three times. The combined organic layers were concentrated in vacuum to give a residue (126 g), which was subjected to silica-gel column chromatography eluted with petroleum containing increasing amount of ether-ethyl acetate to offer a mixture (15 g). The further purification was performed by preparation C_{-18} HPLC using an elution system of 85% methyl alcohol solution to give four fractions. The fraction was extracted into $CHCl_3$. Removal of solvent under reduced pressure afforded **1**–**4** as needle microcrystallines, **1**: 1.69 g, **2**: 50 mg, **3**: 150 mg, and **4**: 50 mg, respectively.

The dried flower of *D. genkwa* (4.5 kg) was extracted with 95% ethanol (20 L) at the room temperature for 15 days to give a brownish-black crude extract (156 g), which was isolated and purified according to above-mentioned methods to afford compound **5** (50 mg).

4.3.1. Yuanhuajine (2). A white-like needle crystal, UV (EtOH) λ_{max} 232 ($\varepsilon = 15116\ M^{-1}\ cm^{-1}$), 270 nm. IR (KBr): 3437, 1720, 1706, 1634, 1107, 908; 733, 713 cm^{-1}. HR-ESI-MS: *m/z* 647.2874 $[M+H]^+$, 669.2735 $[M+Na]^+$ ($C_{37}H_{42}O_{10}$ calcd 647.2778 $[M+H]^+$). ^1H NMR and ^{13}C NMR data are shown in Table 2.

4.3.2. Yuanhuagine (4). A white-like needle crystal, UV (EtOH) λ_{max} 235 ($\varepsilon = 10840\ M^{-1}\ cm^{-1}$), 268 nm. IR (KBr): 3390, 1737, 1705, 1635, 1243, 1081, 1142, 912 cm^{-1}. HR-ESI-MS: *m/z*: 585.2713 $[M+H]^+$, 607.2536 $[M+Na]^+$ ($C_{32}H_{40}O_{10}$ calcd 584.2621). ^1H NMR and ^{13}C NMR data are shown in Table 2.

4.4. Derivative synthesis

4.4.1. Synthesis of compound 6. To a methanol solution (5 mL) of sodium methoxide (10 mg) was added **1** (10.0 mg) at room temperature, upon stirring overnight a yellow solution was formed. The mixture was concentrated under reduced pressure and dissolved in ether (5 mL), washed with water, dried with anhydrous sodium sulfate, filtered, and dried in vacuum to get the yellow solid compound **6**. ESI-MS: *m/z* 567.0 $[M+H]^+$, 1112.1 $[2M+Na]^+$; UV (EtOH) λ_{max} 237 nm.

4.4.2. Synthesis of compound 7. An anhydrous acetone solution (5 mL) containing **1** (10.2 mg) and *p*-toluenesulfonic acid which acted as a catalyst was stirred at room temperature for 18 h to result in a brownish solution. To this solution was added, drop by drop, 5% sodium carbonate (5 mL). After extraction with ether, the organic phase was dried with anhydrous sodium sulfate overnight, filtered, and dried in vacuum to give a brown powder **7**. ESI-MS: *m/z*: 689.4 $[M+H]^+$, 623.4 $[M+Cl]^+$; UV (EtOH) λ_{max} 263 nm.

4.4.3. Synthesis of compound 8. To an ethanol (5 mL) solution of **1** (11 mg) was added water (5 mL) and hydrochloric acid used as catalyst under stirring. The mixture was reacted at 90 °C for 1 h, then extracted with chloroform, dried with anhydrous sodium sulfate, filtered, and dried in vacuum to get the compound **8**. ESI-MS: *m/z* 667.3 $[M+H]^+$, 689.3 $[M+Na]^+$, 701.2 $[M+Cl]^-$. The structure (Fig. 1) was deduced according to a former report.[12]

4.5. Biochemical material

Supercoiled plasmid DNA pBR322, DNA topo I (from calf thymus), and other reagents for agarose-gel electrophoresis were purchased from Takara Co., Ltd. One unit (1 U) of topo I activity was defined as the amount of enzyme necessary to completely relax 0.4 μg of supercoiled plasmid DNA pBR322 at 37 °C for 30 min.

4.6. Biological assays of inhibiting DNA topo I

The inhibitory activities of the **1**–**8** with DNA topo I were measured using supercoiled plasmid DNA pBR322. Briefly, each reaction mixture has a total volume of 20 μL containing 1 U of topo I (0.5 μL) and plasmid pBR322 (0.4 μg). First, 10× buffer (2 μL) (350 mM Tris–HCl, pH 8.0, 720 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, and 50 mM spermidine), 0.1% BSA (2 μL), compounds **1**–**8** (2 μL) with different concentrations, 1 U of topo I (0.5 μL), and plasmid pBR322 (1 μL) were diluted with ultrapure water to keep the total volume for 20 μL. Then, this mixture was incubated at 37 °C for 30 min and terminated by adding 10× loading buffer (10 mM EDTA, 50% glycerol, 0.25% xylene lyanol FF, and 0.25% bromophenol blue). Electrophoresis experiment was carried out on a 1% agarose-gel (85 V, 105 A) for 1 h and dyed in EB solution (0.5% μg/mL ethidium bromide) for 30 min.

4.7. Method of calculating IC_{50}

The inhibition of compounds **1**–**8** toward topo I was tested at 5–9 concentration levels, respectively, in separate experiments. IC_{50} values, defined as the concentration resulting in 50% inhibition toward the relaxation activity of topo I, were estimated from nonlinear regression analysis of concentration–response curves. Topo I activity unit and the inhibition of compounds towards topo I were corrected following the formulas below. Curve fitting was performed using the GraphPad Prism computer program (version 3.0). Taking compound **1** as an example, in Figure 1 concentration–response curve of compound **1** is presented.

Topo I activity unit by correction (U′) = (A–B)/A
Inhibition ratio by correction = (A–C)/A/U′
A—ratio of supercoiled DNA in lane 1,
B—ratio of supercoiled DNA in lane 2,
C—ratio of supercoiled DNA in lane 4 or the other lanes.

Acknowledgments

We sincerely thank Mr. Yuan Xiaodong and Ms. Yin Mengmeng of Takara Co., Ltd for the help in assessing of inhibitory activity against DNA topo I.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.01.055.

References and notes

1. Kupchan, S. M.; Shizuri, Y.; Sumner, W. C., Jr.; Haynes, H. R.; Leighton, A. P.; Sickles, B. R. *J. Org. Chem.* **1976**, *41*, 3850.
2. Ying, B. P.; Wang, C. R.; Zhou, B. N.; Pan, B. C.; Liu, J. C. *Acta Chim. Sinica* **1977**, *35*, 103.
3. The cooperation group of *Daphne genkw* root, *Yiyao Gongye*. **1978**, 6.
4. Wang, C. R.; Chen, Z. X.; Ying, B. P.; Zhou, B. N.; Liu, J. S.; Pan, B. C. *Acta Chim. Sinica* **1981**, *39*, 421.
5. Hall, I. H.; Kasai, R.; Wu, R. Y.; Wu, K.; Tagahara, Lee K. H. *Pharm. Sci.* **1982**, *71*, 1263.
6. Liou, Y. F.; Hall, I. H.; Lee, K. H. *Pharm. Sci.* **1982**, *71*, 1340.
7. Hall, I. H.; Liou, Y. F.; Qswald, C. B.; Lee, K. H. *Eur. J. Cancer Clin. Oncol.* **1986**, *22*, 45.
8. Zhan, Z. J.; Fan, C. Q.; Ding, J.; Yue, J. M. *Bioorg. Med. Chem.* **2005**, *13*, 645.
9. Zhang, D. C.; Ci, C. X.; Zhang, C. *J. China Sci. B* **1992**, 172.
10. Yesilada, E.; Ka, H. T.; Takaishi, Y. *Cytockine* **2001**, *13*, 359.
11. Taninaka, H.; Takaishi, Y.; Honda, G. *Phytochemistry* **1999**, *52*, 1525.
12. Yuan, S. T.; Zhang, B. X.; Xia, K. *China J. Chinese Materia Medica* **1996**, *21*, 469.
13. Redinbo, R. M.; Lance, S.; Peter, K.; James, J. C.; Wim, G. J. Hol. *Science* **1998**, *279*, 1504.
14. Stewart, L.; Redinbo, M. R.; Qiu, X. Y. *Science* **1998**, *279*, 1534.
15. Kupchan, S. M.; Sweeny, G. J.; Baxter, R. L.; Murae, T. S.; Zimmerly, V. A.; Sickles, B. R. *J. Am. Chem. Soc.* **1975**, *97*, 672.
16. Hu, H. B.; Sha, H.; He, Z. W.; Wu, X. C. *Acta Chim. Sinica* **1986**, *44*, 843.
17. Pommier, Y. *Biochimie* **1998**, *80*, 255.
18. Zhen, Y. S. *Study and Development of Antineoplastic Drug*; Chemistry Industry Press: Beijing, 2004, pp 248–249.