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Original article

Comparison of the cytotoxic activities of naturally occurring hydroxyanthraquinones and hydroxynaphthoquinones

Xing-Ri Cui [a], Maiko Tsukada [a], Nao Suzuki [a], Takeshi Shimamura [a], Li Gao [b], Jyunichi Koyanagi [c], Fusao Komada [d], Setsuo Saito [a,*]

[a] *Faculty of Pharmaceutical Sciences, Josai University, Keyakidai 1-1, Sakado, Saitama 350-0295, Japan*
[b] *College of Pharmacy, Yanbian University, Yanji 133000, China*
[c] *Faculty of Pharmaceutical Sciences, Josai International University, Gumyo 1, Togane, Chiba 281-8555, Japan*
[d] *Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, Kami Ohono 7-2-1, Himeji, Hyogo 670-8524, Japan*

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Abstract

Seven hydroxyanthraquinone derivatives, **1**–**7**, were isolated from the root of *Rheum palmatum* (Polygonaceae). Two propionated anthraquinone derivatives, **8** and **9**, were synthesized. Four hydroxynaphthoquinone derivatives, **13**, **14**, **16** and **21**, were isolated from the root of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) and also three naphthoquinone derivatives, **19**, **22** and **23**, were isolated from the root of *Macrotomia euchroma* (Royle) Pauls. (Boraginaceae). The cytotoxicity of the anthraquinone and naphthoquinone derivatives on P-gp-underexpressing HCT 116 cells and P-gp-overexpressing Hep G2 cells was examined by MTT assay. Among the anthraquinone derivatives, compounds **3**–**5** which had OH, CH_2OH and COOH substituent groups on the anthraquinone skeletons, respectively, showed potent growth inhibitory activities against both types of cancer cells (IC_{50} values: 5.7 ± 0.9 to 13.0 ± 0.7 μM in the case of HCT 116 cells and 5.2 ± 0.7 to 12.3 ± 0.9 μM in the case of Hep G2 cells). All hydroxynaphthoquinone derivatives isolated in this study exhibited extremely potent growth inhibitory activities against both types of cancer cells (IC_{50} values: 0.3 ± 0.09 to 0.46 ± 1.0 μM in the case of HCT 116 cells and 0.22 ± 0.03 to 0.59 ± 0.06 μM in the case of Hep G2 cells) as well as shikonin **10** (IC_{50} values: 0.32 ± 0.02 μM in the case of HCT 116 cells and 0.24 ± 0.03 μM in the case of Hep G2 cells).

Keywords: Hydroxyanthraquinone; Hydroxynaphthoquinone; Cytotoxic activity; HCT 116 cells; Hep G2 cells; P-Glycoprotein

1. Introduction

Several papers have been published on the cytotoxic activities of hydroxyanthraquinone derivatives using cancer cell lines such as L1210 [1], HL-60 [1], LNCap [2], PC3 [3] and A431 [3] and those of hydroxynaphthoquinone derivatives using cancer cell lines such as Sarcoma 180 [4], HL-60 [5] and A431 [6]. However, there are no published studies involving a comparison of the cytotoxic activities between hydroxyanthraquinone and hydroxynaphthoquinone derivatives. The present study deals with a comparison of the cytotoxic activities between hydroxyanthraquinone derivatives isolated from the root of Rhubarb (*Rheum palmatum* (Polygonaceae)) and hydroxynaphthoquinone derivatives isolated from the roots of *Lithospermum erythrorhizon* Sieb. et Zucc. and *Macrotomia euchroma* (Royle) Pauls. (Boraginaceae) using human colorectal (HCT 116) [7] and human hepatoma (Hep G2) [8] cancer cell lines.

2. Chemical results and discussion

Hydroxyanthraquinone derivatives **1**–**7** (Fig. 1) were isolated according to the extraction and isolation process (shown in Fig. 2) which was a modification of the method reported by Zhou et al. [3]. Dried Rhubarb (1.0 kg) was extracted with

* Corresponding author. Tel.: +81 49 271 7667; fax: +81 49 271 7984.
E-mail address: saitoset@josai.ac.jp (S. Saito).

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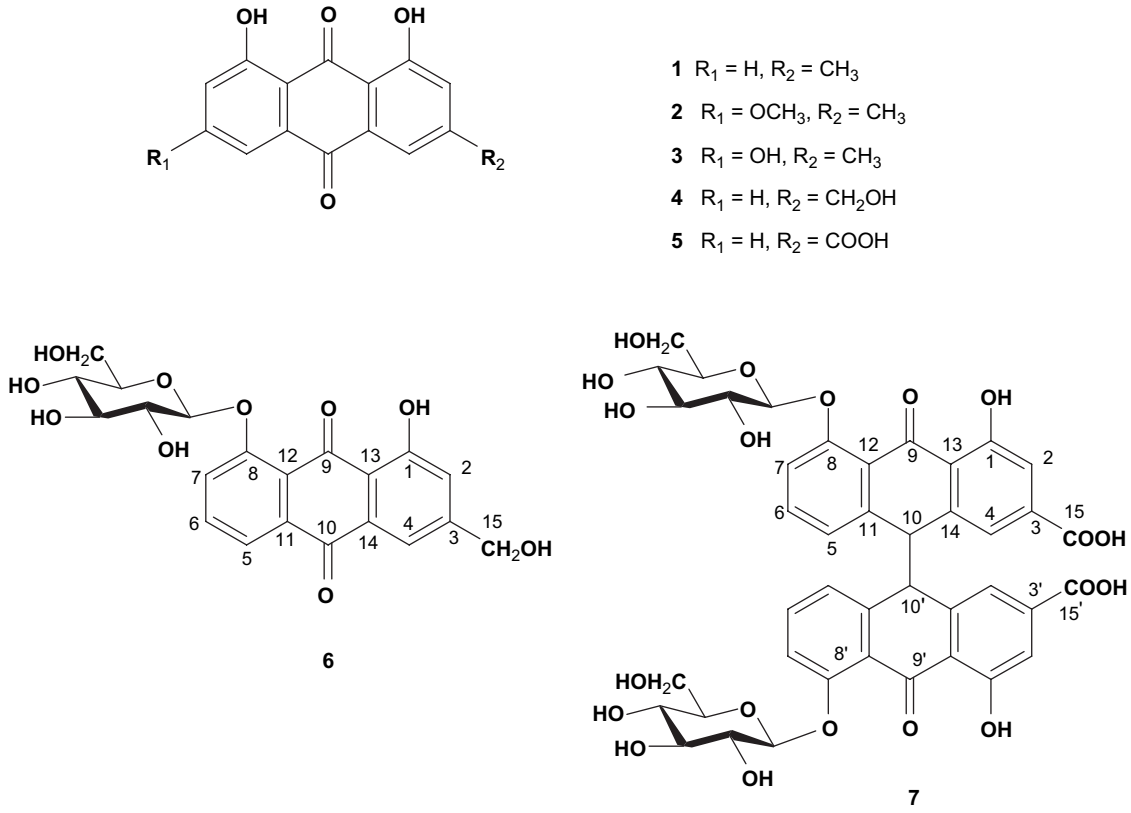


Fig. 1. Structures of compounds **1**–**7**.

MeOH (2.0 l × 2). The extracts were evaporated to about half their volume and suspended in H_2O. The suspension was extracted with AcOEt to give AcOEt and H_2O phases. A residue was obtained after evaporation of the AcOEt layer and following the addition of acetone gave a precipitate (ppt, 8.9 g) and a supernatant (sup). The ppt was subjected to column chromatography to give rhein (**5**, 2.1 g). The sup was evaporated to give a residue (9.7 g) which was subjected to column chromatography to provide three separate fractions, Fr.1–Fr.3. Repeated recrystallization of Fr.1 from acetone gave chrysophanol (**1**, 2.4 g). Chromatography of the Fr.2 and Fr.3 gave emodin (**3**, 1.1 g) and aloe-emodin (**4**, 0.9 g), respectively. The mother liquor of **1** contained chrysophanol **1** and an another component. Separation of the two components from the mother liquor by preparative high performance liquid chromatography (HPLC) was unsuccessful because of the low solubility of the mixture in the solvent used for the preparative HPLC. So a separation of the acyl derivatives obtained from the mixture was tried. Although acetyl derivatives were not isolated because they were insufficiently soluble for the preparative HPLC, chromatography (column: ODS-4251-D, 10 × 250 mm; solvent system, H_2O–CH_3COCH_3–AcOH (225:271:4); flow rate: 1.0 ml/min; column temperature: 40 °C) of the propionylated mixture gave 1,8-di-*O*-propionyl physcion (**8**, 180 mg) and 1,8-di-*O*-propionyl chrysophanol (**9**, 70 mg) (Fig. 3). After alkaline hydrolysis of **8**, physcion **2** (90 mg, 83.3%) was obtained.

Structures of compounds **1**–**5** were identified as chrysophanol, physcion, emodin, aloe-emodin and rhein, respectively, on the basis of a comparison with their spectral data in the literature [3]. The H_2O layer obtained after extraction with AcOEt (Fig. 2) was extracted with *n*-BuOH to afford an *n*-BuOH layer and an H_2O layer. A residue obtained after evaporation of the former organic layer was subjected to column chromatography to obtain compound **6** (0.4 g). In the ^1H NMR spectrum of **6**, signals due to a β-D-glucopyranosyl moiety were observed and signals due to the aglycon were superimposable on the corresponding signals due to the aloe-emodin moiety (see Section 5). In the heteronuclear multiple bond connection (HMBC) spectrum of **6**, a correlation between the anomeric proton signal at δ 5.79 and the carbon signal at δ 159.4 due to C-8 was observed (data not shown). Therefore, compound **6** is suggested to be 8-*O*-β-D-glucopyranosyl-aloe-emodin [9]. Trimethylamine was added to this aqueous layer to render it alkaline (pH 9.0) and then it was extracted with *n*-BuOH. After acidification with the addition of 1.0 M HCl and washing with H_2O, *n*-BuOH solution was evaporated to give a residue (120 g). Successive column chromatography and preparative HPLC (column: $N(CH_3)_2$-4251-N, 10 × 250 mm; solvent system: CH_3COCH_3–H_2O–AcOH (271:225:4); flow rate: 1.0 ml/min; column temperature: 40 °C) of a portion (7.0 g) of the residue gave sennoside A (**7**, 0.05 g), which was identified by comparison of its spectral data in the literature [10,11].

Hydroxynaphthoquinone derivatives were isolated from both the roots of *L. erythrorhizon* Sieb. et Zucc. and *M. euchroma* (Royle) Pauls. (Boraginaceae) called Ko-Shikon and Nan-Shikon, respectively, in Japan. It has been reported that hydroxynaphthoquinone derivatives obtained from Ko-Shikon

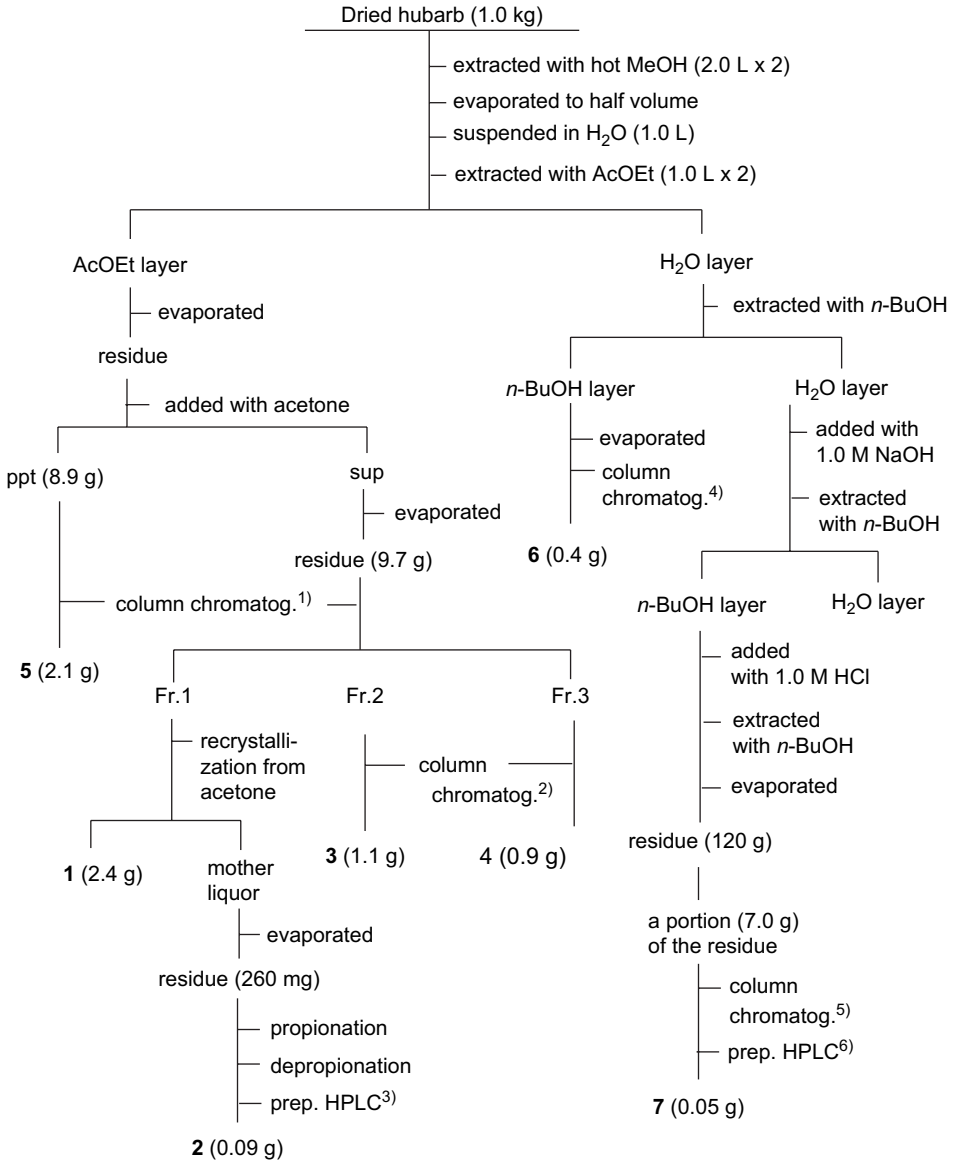


Fig. 2. The isolation process of hydroxyanthraquinone derivatives in Rhubarb. (1) Solvent system: a gradient of 0–5% MeOH in $CHCl_3$. (2) Solvent system: a gradient of 0–5% MeOH in $CHCl_3$. (3) Column: ODS-4251-D, 10 × 250 mm; solvent system: $H_2OCH_3COCH_3$–AcOH (225:271:4); flow rate: 1.0 mL/min; column temperature: 40 °C. (4) Solvent system: $CHCl_3$–MeOH–H_2O (65:35:10, upper layer). (5) Solvent system: AcOEt–propanol–H_2O–AcOH (40:10:30:0.5, upper layer). (6) Column: $N(CH_3)_2$-4251-N, 10 × 250 mm; solvent system: CH_3COCH_3–H_2O–AcOH (271:225:4); flow rate: 1.0 mL/min; column temperature: 40 °C.

are derivatives of shikonin **10** (absolute configuration at C-11; *S*) and those obtained from Nan-Shikon are derivatives of alkannin **11** (absolute configuration at C-11; *R*), an enantiomer of shikonin **10** [12–15].

Morimoto et al. [16] reported the isolation of the hydroxynaphthoquinone derivatives, β,β-dimethylacrylshikonin **12**, isobutylshikonin **13** and β-hydroxyisovalerylshikonin **14** from Ko-Shikon, and that of teracrylshikonin **15** from Nan-Shikon (Fig. 4). In addition, Kyogoku et al. [17] reported the isolation of other hydroxynaphthoquinones; shikonin **10**, acetylshikonin **16**, isovalerylshikonin **17** and deoxyshikonin **18** from Ko-Shikon, and acetylalkannin **19** and isovalerylalkannin **20** from Nan-Shikon. In this study, isolation of the hydroxynaphthoquinone derivatives was performed using the isolation process briefly described in Fig. 5. The extraction of dried Ko-Shikon (1.0 kg) with toluene (4.0 l × 2), followed by filtration and evaporation provided a residue (8.7 g) which was subjected to column chromatography to give a red colored fraction (1.9 g) and a blue colored fraction (3.8 g). The preparative HPLC (column: C18 CAPCELPAC, 10 × 250 mm; flow rate: 1.0 ml/min; solvent system: 10% H_2O–acetone; column temperature: 40 °C) of the red colored fraction gave compounds **13** (270 mg), **14** (410 mg), **16** (270 mg) and **21** (250 mg). On the basis of a comparison with their spectral data in the literature [17], compounds **13**, **14** and **16** were identified as isobutylshikonin, 3′-hydroxyisovalerylshikonin and acetylshikonin, respectively. Compound **21** showed a parent peak at *m*/*z* 316 $[M]^+$ in the EI mass spectrum. In the

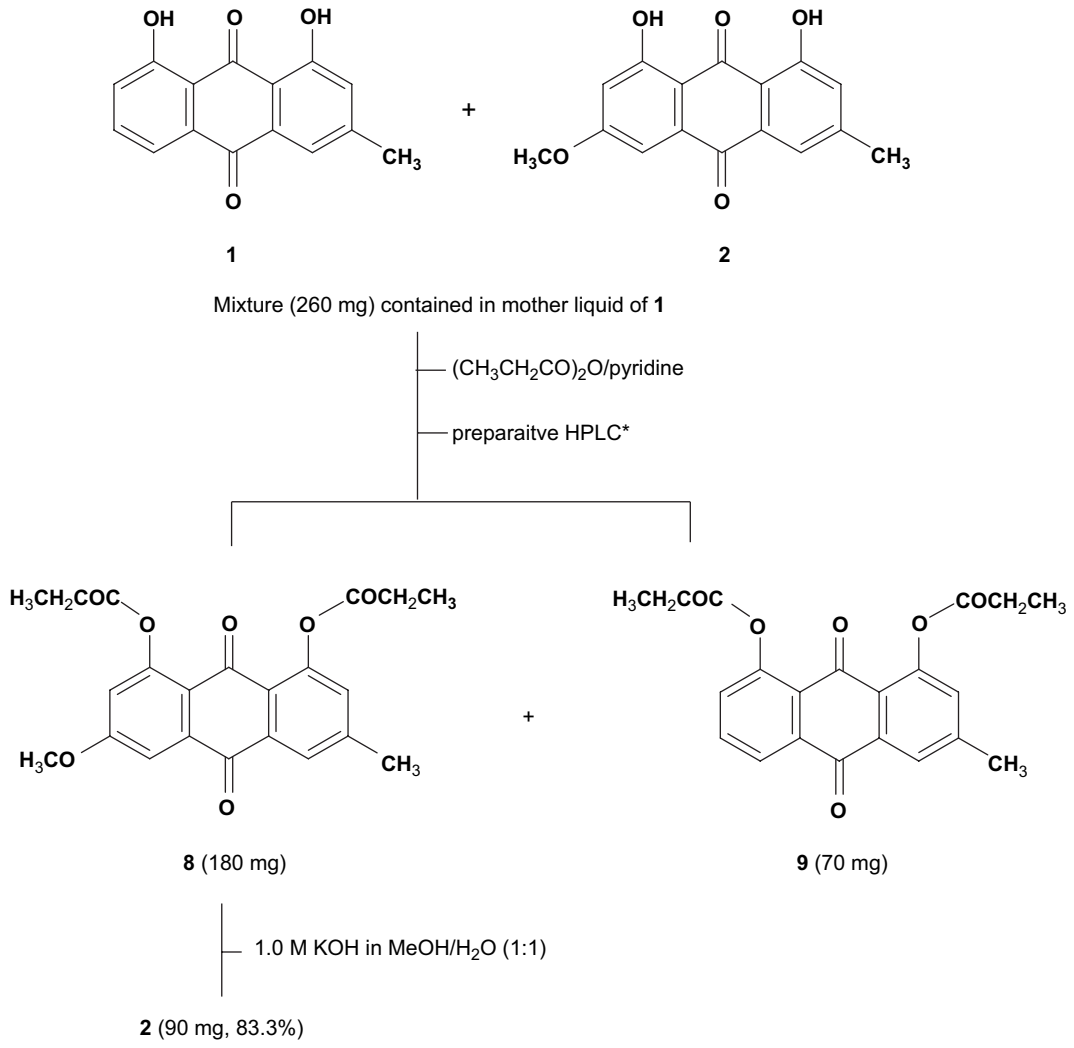
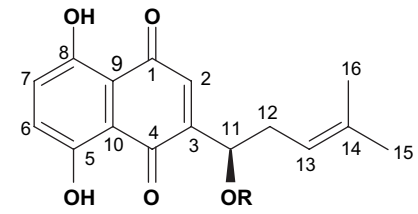
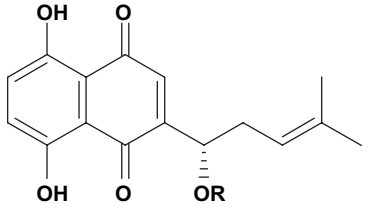


Fig. 3. The propionation of a mixture of **1** and **2**, and depropionation of **8**. *Column: ODS-4251-D, 10 × 250 mm; solvent system: H_2O–CH_3COCH_3–AcOH (225:271:4); flow rate: 1.0 ml/min; column temperature: 40 °C.

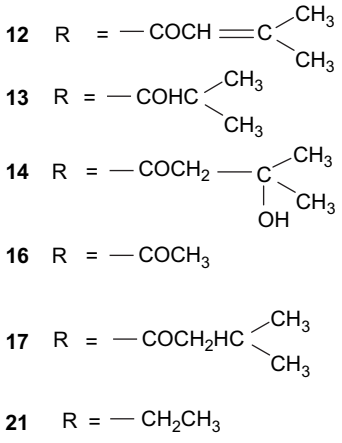
^{1}H NMR spectrum of **21**, the proton signals on the shikonin skeleton were superimposable on those of acetylshikonin **16**. Although the methyl proton signal of an ethyl group substituted at O-11 was observed at δ 1.23 as a triplet ($J = 7.0$ Hz), each ethylene proton of the ethyl group was observed at δ 3.48 and 3.46 as a double quartet ($J = 13.7$ and 7.0 Hz), which suggests that the rotation of the bond between O-11 and C-1′ is hindered. However, these spectral data suggest that compound **21** is ethylshikonin, which is a newly isolated naturally occurring hydroxynaphthoquinone. Dried Nan-Shikon (1.0 kg) was also extracted with toluene (4.0 l × 2) and then evaporated to dryness to give a residue (25.1 g) which was subjected to column chromatography to provide a red colored fraction (2.5 g) and a blue colored fraction (18.9 g). The preparative HPLC of the red colored fraction under the same conditions as those in the case of Ko-Shikon gave compounds **19** (660 mg), **22** (340 mg) and **23** (400 mg). Compound **19** had the same parent peak at *m*/*z* 330 $[M]^+$ as that of acetylshikonin **16**. The ^{1}H NMR spectrum of **19** had the same spectrum as that of **16**, which suggests that **19** is acetylalkannin. Compound **22** exhibited a parent peak at *m*/*z* 358 $[M]^+$ in the EI mass spectrum. In the ^{1}H NMR spectrum of **22**, all proton signals were coincided with those of isobutylshikonin **13** (see Section 5), which suggests that **22** is isobutylalkannin. Compound **22** is the first hydroxynaphthoquinone isolated from Nan-Shikon, although it has been isolated from *Alkanna tinctoria* (Boraginaceae) [18]. Although compound **23** showed a single spot on thin-layer chromatography (TLC) and a single peak on HPLC (column: C18 CAPCELL-PAC, 10 × 250 mm; flow rate: 1.0 ml/min; solvent system: 10% H_2O–acetone; column temperature: 40 °C), the ^{1}H NMR spectrum showed that it contained some impurities (7–10% from the ratio of the signal peak heights). However, the structure of compound **23** was proved by the mass spectrum and ^{1}H NMR, ^{13}C NMR and HMBC spectra. The EI mass spectrum of **23** had a parent peak at *m*/*z* 372 $[M]^+$. In the ^{1}H NMR spectrum of **23**, all proton signals on the alkannin skeleton were superimposable on those of **22** and furthermore signals of 2′-H (δ 2.15, d, $J = 1.2$ Hz), 3′-H (δ 2.18, m) and 4′-CH_3 (δ 0.98, dd, $J = 6.7$ and 2.1 Hz) due to the 2′,3′-epoxybutanoyl group were observed. The substituted position of the group was confirmed

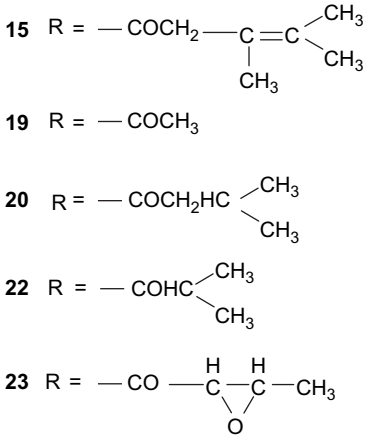


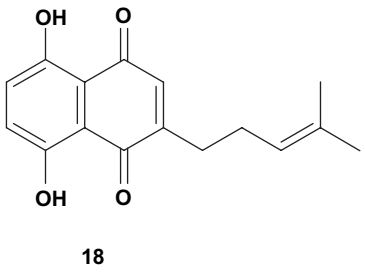


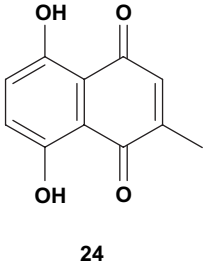
10 R= H shikonin

11 R = H alkannin









18 **24**

Fig. 4. Structures of compounds **10**–**24**.

by the HMBC spectrum as shown in Fig. 6; a correlation between the H-11 at δ 6.05 and carbonyl carbon (C-1′) at δ 171.8 due to the carbonyl group of the 2′,3′-epoxybutanoyl group was observed.

Shikonin **1** and alkannin **2** were not detected in the toluene extracts by TLC and analytical HPLC in the present study.

3. Pharmacological results and discussion

In this study, human colorectal (HCT 116) [7] and human hepatoma (Hep G2) [8] cancer cell lines were used. It is known that the former cell lines scarcely express MDR 1 (P-glycoprotein, P-gp) [7] and the latter cell lines overexpress it [8]. P-gp acts as an efflux pump to remove antitumor agents, Ca^{2+} antagonists, cyclosporine, digoxin and other compounds, from cells [19].

First, the cytotoxic effects of the isolated hydroxyanthraquinone derivatives (**1**–**7**) and synthetic propionylnaphthoquinone derivatives (**8** and **9**) were tested by MTT assay [20], and the IC_{50} values were calculated based on the percentage inhibition of cell growth and these are listed in Table 1. In the case of HCT 116 cells, compounds **2**–**5** showed potent cytotoxicity (IC_{50} values: 5.7 ± 0.9 to 19.0 ± 1.2 μM) among non-glycosidic derivatives, except for compound **1** (IC_{50} value: 47.4 ± 18.1 μM) which had only weak activity, which suggests that the presence of substituents such as OCH_3, OH, CH_2OH and COOH on the hydroxynaphthoquinone skeleton is required for enhancement of the inhibition of HCT 116 cells. 1,8-*O*-Di-propionated compounds **8** and **9** also exhibited potent cytotoxic effects (IC_{50} values: 9.2 ± 0.7 and 14.8 ± 0.5 μM, respectively). On the other hand, both glycosides **6** and **7** showed weak activities (IC_{50} values: 75.0 ± 11.5 and 68.2 ± 4.6 μM, respectively). In the case of Hep G2 cells, while compounds **3**–**5** exhibited almost the same activities (IC_{50} values: 5.2 ± 0.7, 10.0 ± 0.8 and 12.3 ± 0.9 μM, respectively) as those in the HCT 116 cells, activity of **1** was reduced (IC_{50} values: from 47.4 ± 18.1 to 64.4 ± 17.0 μM), as was those of **2** (IC_{50} values: from 19.0 ± 1.2 to 77.3 ± 4.5 μM), **6** (IC_{50} values: from 75.0 ± 11.5 to >100 μM), **8** (IC_{50} values: from $9.2 + 0.7$ to >100 μM) and **9** (IC_{50} values: from $14.8 + 0.5$ to >100 μM).

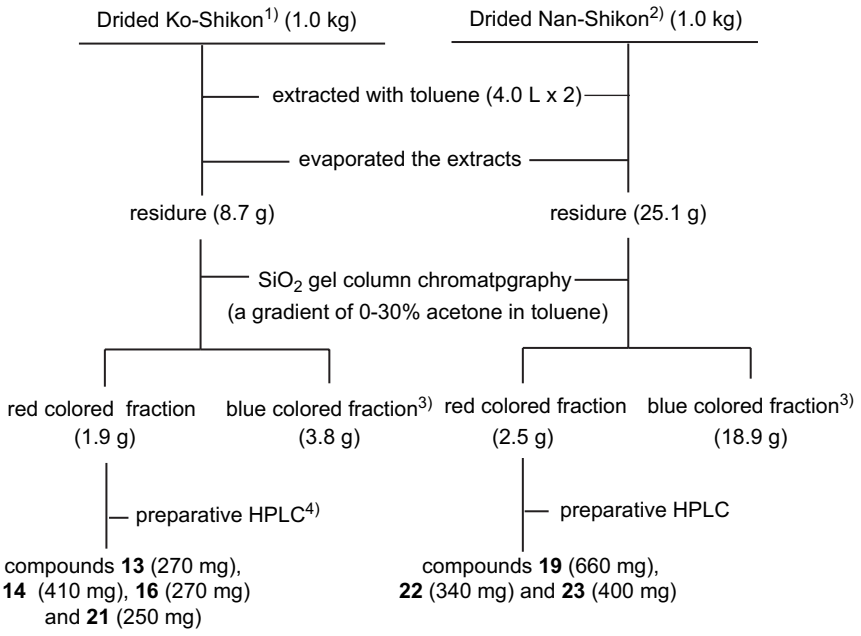


Fig. 5. The isolation process of hydroxynaphthoquinone derivatives in Ko-Shikon and Nan-Shikon. (1) Ko-Shikon: *Lithospermum erythrorhizon* Sieb. et zucc. (2) Nan-Shikon: *Macrotomia euchroma* (Royle) Pauls. (3) These blue colored fractions gave obscure spots on TLC and separation of compounds was impossible even by HPLC. (4) Preparative HPLC: column: C18-CAPCELPAC, 10 × 250 mm; flow rate: 1.0 mL/min; column temperature, 40 °C.

This indicates that compounds **3**–**5** together with **7** may be less sensitive substrates for P-gp transport compared with compounds **1**, **2**, **6**, **8** and **9**.

The cytotoxic effects of the isolated hydroxynaphthoquinone derivatives (**13**, **14**, **16**, **19** and **21**–**23**) as well as those of shikonin (**10**) and vitamin K (**24**) were then compared using the same cancer cells (HCT 116 and Hep G2 cells) (Table 2). Shikonin **10** showed markedly potent growth inhibitory activity (IC_{50} values: 0.23 ± 0.02 and 0.24 ± 0.03 μM, respectively) against HCT 116 and Hep G2 cells. All the isolated hydroxynaphthoquinone derivatives also exhibited potent inhibition of both cancer cells (IC_{50} values: 0.30 ± 0.09 to 0.45 ± 0.10 μM in the case of HCT 116 cells; 0.22 ± 0.03 to 0.59 ± 0.06 μM in the case of Hep G2 cells), which suggests that the presence of a substituent at the O-11 position of the naphthoquinone skeleton is not essential for their activity. However, vitamin K **24** had weaker activities (IC_{50} value: 1.19 ± 0.17 in the case of HCT 116 cells and 2.34 ± 0.38 μM in the case of Hep G2 cells) than **10** and all hydroxynaphthoquinone derivatives isolated in this study against HCT 116 and Hep G2 cells, although it still had a potent effect on both cells, which suggests that the presence of phenolic hydroxyl groups at the 5 and 8 positions of the naphthoquinone skeleton enhances the activity against both HCT 116 and Hep G2 cells.

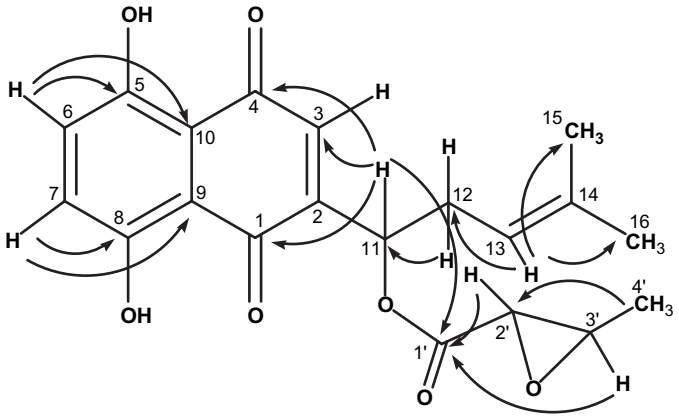


Fig. 6. $^{1}H-^{13}C$ Long-Range Correlations observed for compound **23**.

4. Conclusions

Hydroxyanthraquinone derivatives **1**, **3**, **4**, **5** and **7** were isolated from *R. palmatum*. Physcion **2** was purified by

Table 1
Cytotoxic effects of anthraquinone derivatives **1**–**9** on HCT 116 and Hep G2 cells

Compound	IC_{50} (μM)[a]	
	HCT 116	Hep G2
1	47.4 ± 18.1	64.4 ± 17.0
2	19.0 ± 1.2	77.3 ± 4.5*
3	5.7 ± 0.9	5.2 ± 0.7
4	8.7 ± 0.8	10.0 ± 0.8**
5	13.0 ± 0.7	12.3 ± 0.9
6	75.0 ± 11.5	>100[b]
7	68.2 ± 4.6	51.6 ± 8.0
8	9.2 ± 0.7	>100[b]*
9	14.8 ± 0.5	>100[b]*

*$P < 0.01$ and **$P < 0.05$, the significant difference between the activity on HCT 116 cells and that on Hep G2 cells. Each experiment was performed in duplicate wells, and drug treatments were performed separately three times.

[a] IC_{50} values (mean ± S.D.) are the concentrations at which 50% of the cells are inhibited from growing. S.D., standard deviation.

[b] IC_{50} values more than 100 μM were indicated as >100.

Table 2
Cytotoxic effects of anthraquinone derivatives **13**, **14**, **16**, **19** and **21**–**23** on HCT 116 and Hep G2 cells

Compound	IC_{50} (μM)[a]	
	HCT 116	Hep G2
10	0.23 ± 0.02	0.24 ± 0.03
13[b]	0.34 ± 0.05	0.22 ± 0.03*
14[b]	0.37 ± 0.06	0.59 ± 0.06
16[b]	0.45 ± 0.10	0.38 ± 0.07
21[b]	0.45 ± 0.12	0.42 ± 0.04
19[c]	0.34 ± 0.09	0.39 ± 0.07
22[c]	0.30 ± 0.09	0.42 ± 0.03
23[c]	0.39 ± 0.08	0.34 ± 0.04
24	1.19 ± 0.17	2.34 ± 0.38

*$P < 0.01$, the significant difference between the activity on HCT 116 cells and that on Hep G2 cells. Each experiment was performed in duplicate wells, and drug treatments were performed separately three times.

[a] IC_{50} values (mean ± SD) are the concentrations at which 50% of the cells are inhibited from growing. S.D., standard deviation.

[b] Derivatives isolated from Ko-Shikon.

[c] Derivatives isolated from Nan-Shikon.

propionation of the mixture followed by depropionation. 8-*O*-β-D-Glucopyranosyl-aloe-emodin **6** was purified by acetylation of the mixture followed by deacetylation. Furthermore, the new hydroxynaphthoquinone derivative **21** together with known derivatives **13**, **14** and **16** were isolated from Ko-Shikon (*L. erythrorhizon* Sieb. et Zucc.) and a new hydroxynaphthoquinone derivative **23** together with known derivatives **19** and **22** were isolated from Nan-Shikon (*M. euchroma* (Royle) Pauls.).

The cytotoxic activities of the hydroxyanthraquinone and hydroxynaphthoquinone derivatives were evaluated using HCT 116 and Hep G2 cancer cell lines. Glycosidic derivatives **6** and **7** had weak activity against both cells. Among hydroxyanthraquinone derivatives **1**–**5**, while compound **1** had weak activity, compounds **2**–**5**, which had OCH_3, OH, CH_2OH and COOH substituted groups on the hydroxyanthraquinone skeleton, exhibited potent growth inhibitory activities (IC_{50} values: 5.7 ± 0.9 to 19.0 ± 1.2 μM) against HCT 116 cells. The activity of **2** was reduced (IC_{50} values: from 19.0 ± 1.2 to 77.3 + 4.5 μM) against Hep G2 cells, whereas the activities of **3**–**5** on Hep G2 cells were almost similar to that on HCT 116 cells. These results suggest that the presence of polar substituents, OH, CH_2OH and COOH, on the hydroxynaphthoquinone skeleton may prevent the removal of compounds **3**–**5** by the P-gp in the Hep G2 cells. A similar reduction in activity was observed in the case of compounds **8** and **9** which contained weak polar and non-polar OCH_3 and CH_3 groups, respectively. Accordingly, the activities of 8-*O*-propionated derivatives **8** and **9** had potent activities (IC_{50} values: 9.2 ± 0.7 and 14.8 ± 0.5 μM, respectively) against HCT 116 cells, much more so than **2** and **1** (IC_{50} value: 19.0 ± 1.2 and 47.4 ± 18.1 μM), respectively. However, these activities were reduced in the case of Hep G2 cells (IC_{50} value: >100 μM).

All hydroxynaphthoquinone derivatives **13**, **14**, **16**, **19** and **21**–**23** isolated in this study showed markedly potent activities against both HCT 116 and Hep G2 cells compared with the activities of the hydroxyanthraquinone derivatives (compare Tables 1 and 2). This suggests that hydroxynaphthoquinone derivatives are more effective growth inhibitors of both HCT 116 and Hep G2 cells. The activities of these compounds (IC_{50} values: 0.30 ± 0.09 to 0.46 ± 0.10 μM in the case of HCT 116 cells and 0.22 ± 0.03 to 0.42 ± 0.04 μM in the case Hep G2 cells) were almost identical to those of shikonin **10** (IC_{50} value: 0.23 ± 0.02 μM in the case of HCT 116 cells and 0.24 ± 0.03 μM in the case Hep G2 cells) which has no substituent at the O-11 position. This indicates that the presence of substituent at the O-11 position is not essential for the inhibition of the growth of both HCT 116 and Hep G2 cells. However, the presence of phenolic OH groups on the naphthoquinone skeleton may be essential to enhance the activity because the activity of vitamin K **24** which has no phenolic OH group was weaker than that of hydroxynaphthoquinone derivatives. Compounds **13** and **16** are derivatives of shikonin (**10**, *S*-configuration at C-11) and compounds **19** and **22** are derivatives of alkannin (**11**, *R*-configuration at C-11). The activities of isobutylshikonin **13** and isobutylalkanin **22** (isomer of **13**) and those of acetylshikonin **16** and acetylalkannin **19** (isomer of **16**) were, however, almost identical against both HCT 116 and Hep G2 cells, which suggests that the difference in the stereochemistry at C-11 has no influence on the activity.

In this study, we found that naturally occurring hydroxynaphthoquinone derivatives are more cytotoxic than naturally occurring hydroxyanthraquinone derivatives. Furthermore, all hydroxynaphthoquinone derivatives **10**, **13**, **14**, **16**, **19** and **21**–**23** were markedly cytotoxic activities to P-gp overexpressed cancer cells. Generally, plane structural molecule and cationic molecule indicated the substrate for P-gp. Both hydroxyanthraquinone and hydroxynaphthoquinone derivatives in this study were plane structural molecules. The cytotoxic activities against HCT 116 of almost all hydroxyanthraquinone derivatives (**1**, **2**, **4**, **6**, **8**, and **9**) were greater than that against Hep G2 (P-gp overexpressing cell line). This phenomenon can be explained by P-gp mediated transport [21,22]. However, Ca antagonists, itraconazole, spironolactone, quinidine, propanolol, lidocaine, cyclosporine A, digoxin, erythromycin, doxorubicin, and vinblastine were well-documented substrates for P-gP. Molecular weights, physicochemical properties, lipophilicities and pharmacological effects of these drugs were completely different. Hence properties of the substrate for P-gp have not ever become clear. The cytotoxic activities against both cell lines of all hydroxynaphthoquinone derivatives investigated in this study were approximately same levels. Therefore, this demonstrated that these hydroxynaphthoquinone derivatives may not be transported by P-gp. That these hydroxynaphthoquinone derivatives were markedly cytotoxic to P-gp overexpressing cancer cells may give a new approach to overcome the obstacle posed by multidrug resistance in cancer.

5. Experimental

5.1. General procedures

Rhubarb, Ko-Shikon and Nan-Shikon, were purchased from Tochimoto Amamido Co. Ltd., Japan. Sennoside A and shikonin were purchased from Wako Pure Chemical Industries,

Ltd., Japan. Other chemicals and solvents were of reagent grade and obtained from commercial suppliers. Melting points (m.p.) were determined using a Yanagimoto micromelting point apparatus and are uncorrected. Kieselgel 60 F_{254} (E. Merck) was used for thin-layer chromatography (TLC). Spots were detected by spraying plates with 1:9 $Ce(SO_4)_2$–10% H_2SO_4 reagent followed by heating the plate at 250 °C for 4–5 min. Column chromatography was carried out using Silica gel 60 (E. Merck) and then the eluates were monitored using TLC. An SSC-6300 HPLC instrument (Senshu Co. Ltd.) was employed for analytical HPLC using columns ODS-4251-D and $N(CH_3)_2$-4251-N (Senshu Co. Ltd.) and C18 CAPCELL-PAC (Shiseido Co. Ltd.) attached to an SSC autoinjector 6310 and an SSC fraction collector 6320 for preparative HPLC. ^1H and ^{13}C NMR experiments were carried out at 500 and 125 MHz, respectively, as well as ^1H–^1H and ^1H–^{13}C COSY and HMBC spectra were obtained with a JEOL JNM-A500 FTNMR spectrometer. Tetramethylsilane was used as an internal standard. Chemical shifts are given in parts per million. Multiplicities of the ^1H NMR signals are indicated as s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), q (quartet) and m (multiplet). Electron impact mass spectra (EIMS) and fast-atom-bombardment mass spectra (FABMS) were recorded on a JEOL JMS-DX 300 mass spectrometer.

5.1.1. Chemistry

5.1.1.1. Isolation of hydroxyanthraquinone. Hydroxyanthraquinone derivatives **1** and **3**–**7** were isolated according to the procedure described in this text and the scheme shown in Fig. 2.

*5.1.1.2. Chrysophanol (**1**).* EIMS: *m/z* 254 $[M]^+$; m.p. 194–196 °C (after recrystallization from acetone); ^1H NMR ($CDCl_3$) δ 12.15 and 12.04 (each 1H, s, 1- and 8-OH), 7.85 (1H, dd, $J = 7.6$, 1.2 Hz, H-5), 7.69 (1H, dd, $J = 8.6$, 7.6 Hz, H-6), 7.68 (1H, br s, H-4), 7.31 (1H, dd, $J = 8.6$, 1.2 Hz, H-7), 7.13 (1H, br s, H-2), 2.49 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 192.5 (C-9), 190.7 (C-10), 162.7 (C-8), 162.4 (C-1), 149.3 (C-3), 149.3 (C-3), 133.6 (C-11), 124.3 (C-14), 121.2 (C-6), 119.9 (C-4), 115.8 (C-5), 113.7 (C-12), 113.6 (C-7), 108.2 (C-13), 106.7 (C-2), 22.1 (C-15).

*5.1.1.3. Emodin (**3**).* EIMS: *m/z* 270 $[M]^+$; m.p. 255–256 °C (after recrystallization from acetone); ^1H NMR (pyridine-d_5) δ 12.48 (2H, br s, 1- and 8-OH), 7.72 (1H, d, $J = 2.4$ Hz, H-4), 7.70 (1H, dd, $J = 0.9$, 0.6 Hz, H-5), 7.14 (1H, dd, $J = 0.9$, 0.6 Hz, H-7), 7.02 (1H, d, $J = 2.4$ Hz, H-2), 5.23 (1H, s, 3-OH), 2.24 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 182.2 (C-9), 179.1 (C-10), 167.7 (C-1), 165.2 (C-1), 165.9 (C-3), 162.6 (C-8), 148.3 (C-6), 136.1 (C-14), 136.0 (C-11), 133.7 (C-5), 124.5 (C-12), 121.1 (C-7), 114.1 (C-13), 110.2 (C-2), 108.8 (C-4), 21.7 (C-15).

*5.1.1.4. Aloe-emodin (**4**).* EIMS: *m/z* 270 $[M]^+$; m.p. 220–221 °C (after recrystallization from acetone); ^1H NMR (DMSO-d_6) δ 11.85 (2H, s, 1- and 8-OH), 7.75 (1H, t, $J = 8.2$ Hz, H-5), 7.63 (1H, dd, $J = 8.2$, 0.9 Hz), 7.59 (1H, d, $J = 1.5$ Hz, H-4), 7.31 (1H, dd, $J = 8.2$, 0.9 Hz, H-7), 7.21 (1H, d, $J = 1.5$ Hz, H-2), 5.06 (1H, s, CH_2OH), 4.60 (2H, s, CH_2OH); ^{13}C NMR (DMSO-d_6) δ 191.5 (C-9), 181.2 (C-10), 161.6 (C-8), 161.3 (C-1), 153.7 (C-3), 137.3 (C-14), 133.1 (C-11), 132.9 (C-6), 124.3 (C-5), 120.6 (C-12), 119.3 (C-13), 117.1 (C-4), 115.6 (C-7), 114.2 (C-2), 62.1 (C-15).

*5.1.1.5. Rhein (**5**).* EIMS: *m/z* 284 $[M]^+$; m.p. <300 °C; ^1H NMR (pyridine-d_5) δ 11.87 (2H, s, 1- and 8-OH), 8.90 (1H, t, $J = 1.5$ Hz, H-4), 8.35 (1H, d, $J = 1.5$ Hz, H-2), 7.94 (1H, dd, $J = 7.3$, 1.2 Hz, H-5), 7.67 (1H, dd, $J = 8.5$, 7.3 Hz, H-6), 7.42 (1H, dd, $J = 8.5$, 1.2 Hz, H-7); ^{13}C NMR (pyridine-d_5) δ 191.5 (C-9), 181.2 (C-10), 161.6 (C-8), 161.3 (C-1), 153.7 (C-3), 137.3 (C-14), 133.1 (C-11), 132.9 (C-6), 124.3 (C-5), 120.6 (C-12), 119.3 (C-13), 117.1 (C-4), 115.6 (C-7), 114.2 (C-2), 62.1 (C-15).

*5.1.1.6. 8-O-β-D-glucopyranosyl-aloe-emodin (**6**).* FABMS: *m/z* 433 $[M + 1]^+$; m.p. 232–234 °C (after recrystallization from methanol); ^1H NMR (pyridine-d_5) δ 8.08 (1H, s, H-4), 8.03 (1H, dd, $J = 7.6$, 0.9 Hz, H-5), 7.99 (1H, dd, $J = 8.2$, 0.9 Hz, H-7), 7.61 (1H, dd, $J = 8.2$, 7.6 Hz, H-6), 4.97 (2H, s, 3-CH_2OH), 5.79 (1H, d, $J = 7.6$ Hz, H-1′), 4.61 (1H, dd, $J = 12.2$, 2.4 Hz, H-6′a), 4.55 (1H, dd, $J = 8.9$, 7.6 Hz, H-2′), 4.41 (1H, t, $J = 8.9$ Hz, H-3′), 4.41 (1H, dd, $J = 12.2$, 5.2 Hz, H-6′b), 4.36 (1H, t, dd, $J = 9.2$, 8.9 Hz, H-4), 4.23 (1H, ddd, $J = 9.2$, 5.2, 2.4 Hz, H-5′); ^{13}C NMR (pyridine-d_5) δ 188.6 (C-9), 182.6 (C-10), 163.2 (C-3), 159.4 (C-8), 153.2 (C-1), 135.9 (C-6), 133.2 (C-13), 123.9 (C-12), 122.9 (C-7), 121.7 (C-11), 121.5 (C-2), 121.3 (C-5), 116.8 (C-4), 116.2 (C-14), 63.3 (C-15), 102.8 (C-1′), 79.3 (C-5′), 78.3 (C-3′), 74.9 (C-2′), 62.4 (C-6′).

*5.1.1.7. Sennoside A (**7**).* FABMS: *m/z* 885 $[M + Na]^+$; ^{13}C NMR (pyridine-d_5) δ 186.5 (C-9 and 9′), 168.8 (C-15 and 15′), 159.1 (C-1 and 1′), 157.7 (C-8 and 8′), 142.3 (C-13 and 13′), 138.2 (C-12 and 12′), 135.6 (C-3 and 3′), 135.2 (C-6 and 6′), 123.9 (C-4 and 4′), 122.4 (C-11 and 11′), 121.5 (C-14 and 14′), 120.5 (C-5 and 5′), 118.2 (C-2 and 2′), 116.6 (C-7 and 7′), 54.0 (C-10 and 10′), 103.7 (C-1″ an 1‴), 77.5 (C-5″ and 5‴), 75.2 (C-3″ and 3‴), 73.5 (C-2″ and 2‴), 69.7 (C-4″ and 4‴), 60.7 (C-6″ and 6‴). The ^{13}C NMR signals agreed with those reported by Nakajima et al. [11]. The retention time (40 min) of this compound agreed with that of authentic sennoside A on analytical HPLC (column: $N(CH_3)_2$-4251-N, 10 × 250 mm; solvent system: THF–H_2O–AcOH (160:60:7); flow rate: 1.0 ml/min; column temperature: 40 °C).

*5.1.1.8. 1,8-O-Dipropionylphyscion (**8**) and 1,8-O-dipropionylchrysophanol (**9**).* A solution of a mixture (260 mg) of **1** and **2** obtained from the mother liquor of **1** in pyridine (5 ml) was mixed with propionyl anhydride (5 ml) and then stirred for 24 h at room temperature. The reaction mixture was poured into ice-water (20 ml) and extracted with AcOEt (20 ml × 2). The extracts were successively washed with 5% HCl, 10% $NaHCO_3$ and H_2O, dried over absolute $MgSO_4$ and filtered.

The filtrate was evaporated to give a residue which was subjected to preparative HPLC (column: ODS-4251-D, 10 × 250 mm; solvent system: H_2O–CH_3COCH_3–AcOH (225:271:4); flow rate: 1.0 ml/min; column temperature: 40 °C) to obtain 1,8-*O*-dipropionylphyscion (**8**, 180 mg) and 1,8-*O*-dipropionylchrysophanol (**9**, 70 mg). *Compound* ***8***: EIMS: *m/z* 396 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 7.99 (1H, dd, $J = 1.8$, 0.6 Hz, H-5), 7.66 (1H, d, $J = 2.7$ Hz, H-4), 7.18 (1H, dd, $J = 1.8$, 0.6 Hz, H-7), 6.86 (1H, d, $J = 2.7$ Hz, H-2), 3.95 (3H, s, OCH_3), 2.74 (2H, q, $J = 7.6$ Hz, $COCH_2CH_3$), 2.73 (2H, q, $J = 7.6$ Hz, $COCH_2CH_3$), 2.48 (3H, s, 3-CH_3), 1.34 (6H, t, $J = 7.6$ Hz, $COCH_2CH_3 \times 2$); ^{13}C NMR ($CDCl_3$) δ 182.3 (C-10), 179.5 (C-9), 172.8 and 172.6 ($COCH_2CH_3 \times 2$), 163.9 (C-6), 152.4 (C-8), 150.3 (C-1), 145.6 (C-3), 136.0 (C-11), 134.2 (C-14), 130.8 (C-4), 125.8 (C-13), 123.3 (C-2), 119.4 (C-12), 116.4 (C-5), 116.4 (C-7), 56.1 (6-OCH_3), 27.7 ($COCH_2CH_3 \times 2$), 21.6 (C-15), 8.7 ($COCH_2CH_3 \times 2$). Anal. Calcd for $C_{22}H_{20}O_7$: C, 66.66; H, 5.09. Found: C, 66.53; H, 4.95. *Compound* ***9***: EIMS: *m/z* 366 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 8.19 (1H, dd, $J = 7.6$, 1.2 Hz, H-5), 8.00 (1H, dd, $J = 1.8$, 0.6 Hz, H-4), 7.73 (1H, t, $J = 7.9$ Hz, H-6), 7.20 (1H, dd, $J = 1.8$, 0.6 Hz, H-2), 2.74 (2H, q, $J = 7.6$ Hz, $COCH_2CH_3$), 2.73 (2H, q, $J = 7.6$ Hz, $COCH_2CH_3$), 2.49 (3H, s, 3-CH_3), 1.35 (3H, t, $J = 7.6$ Hz, $COCH_2CH_3$), 1.34 (3H, t, $J = 7.6$ Hz, $COCH_2CH_3$); ^{13}C NMR ($CDCl_3$) δ 182.3 and 180.5 (C-9 an C-10), 172.8 ($COCH_2CH_3$), 172.8 ($COCH_2CH_3$), 150.3 (C-8), 150.2 (C-1), 146.2 (C-3), 134.6 (C-6), 134.3 (C-11), 134.2 (C-14), 130.7 (C-12), 130.2 (C-4), 125.8 (C-5), 125.8 (C-7), 125.3 (C-13), 123.4 (C-2), 27.8 ($COCH_2CH_3 \times 2$), 21.7 (C-15), 8.8 ($COCH_2CH_3 \times 2$). Anal. Calcd for $C_{21}H_{18}O_6$: C, 68.85; H, 4.95. Found: C, 66.52; H, 5.06.

*5.1.1.9. Physcion (**2**).* A solution of compound **8** (150 mg) in 1.0 M KOH (5 ml, MeOH–H_2O = 1:1) was stirred for 7 h at room temperature. The reaction mixture was poured into H_2O (20 ml) and then extracted with AcOEt (20 ml × 2). The organic extracts were successively washed with 5% HCl, 10% $NaHCO_3$ and H_2O, then dried over absolute $MgSO_4$ and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography to obtain physcion (**2**, 90 mg, 83.3%). EIMS: *m/z* 284 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 12.31 and 12.11 (each 1H, s, 1- and 8-OH), 7.62 (1H, d, $J = 1.2$ Hz, H-5), 7.36 (1H, d, $J = 2.4$ Hz, H-4), 7.07 (1H, dd, $J = 1.5$, 0.9 Hz, H-7), 6.68 (1H, d, $J = 2.4$ Hz, H-2), 3.94 (3H, s, OCH_3), 2.45 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ 190.8 (C-9), 182.0 (C-10), 166.5 (C-3), 165.2 (C-1), 162.5 (C-8), 145.4 (C-6), 135.2 (C-14), 133.2 (C-11), 124.5 (C-7), 121.3 (C-5), 113.7 (C-12), 110.2 (C-13), 108.2 (C-4), 106.8 (C-2), 56.1 (OCH_3), 22.2 (C-15).

5.1.1.10. Isolation of hydroxynaphthoquinone derivatives. Hydroxynaphthoquinone derivatives were isolated according to the procedure described in this text and the scheme shown in Fig. 5.

*5.1.1.11. Isobutylshikonin (**13**) and isobutylalkannin (**22**).* EIMS: *m/z* 358 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 12.58 and 12.56 (each 1H, s, 5 and 8-OH), 7.18 (2H, s, H-6 and 7), 6.98 (1H, d, $J = 0.9$ Hz, H-3), 6.02 (1H, ddd, $J = 7.3$, 4.6, 0.9 Hz, H-11), 5.13 (1H, br t, $J = 7.3$ Hz, H-13), 2.48 (1H, m, H-2′), 1.69 (3H, s, 15-CH_3), 1.59 (3H, s, 16-CH_3), 1.22 (3H, d, $J = 7.0$ Hz, 3′-CH_3), 1.21 (3H, d, $J = 7.0$ Hz, 4′-CH_3). The ^{13}C NMR signals are listed in Table 3.

*5.1.1.12. 3′-Hydroxyisovalerylshikonin (**14**).* EIMS: *m/z* 388 (M^+); ^{1}H NMR ($CDCl_3$) δ 12.60 and 12.40 (each 1H, s, 5- and 8-OH), 7.20 (2H, s, H-6 and 7), 7.03 (1H, d, $J = 1.0$ Hz, H-3), 6.09 (1H, ddd, $J = 7.6$, 4.6, 1.0 Hz, H-11), 5.12 (1H, br t, $J = 7.6$ Hz, H-13), 2.63 (1H, m, H-12′a), 2.49 (1H, dt, $J = 15.3$, 7.6 Hz, H-2b′), 1.69 (3H, d, $J = 0.9$ Hz, 15-CH_3), 1.59 (3H, d, $J = 0.9$ Hz, 16-CH_3), 1.31 (3H, s, 4′-CH_3), 1.30 (3H, s, 5′-CH_3). The ^{13}C NMR signals are listed in Table 3.

*5.1.1.13. Acetylshikonin (**16**) and acetylalkannin (**19**).* EIMS: *m/z* 330 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 12.60 and 12.40 (each 1H, s, 5- and 8-OH), 7.18 (2H, s, H-6 and 7), 6.99 (1H, s, H-3), 6.02 (1H, ddd, $J = 7.4$, 4.6, 0.9 Hz, H-11), 5.12 (1H, br t, $J = 7.6$ Hz, H-13), 2.62 (1H, br dt, $J = 15.0$, 7.3 Hz, H-12′a), 2.48 (1H, dt, $J = 15.0$, 7.3 Hz, H-2b′), 2.14 (3H, s, $COCH_3$), 1.69 (3H, s, 15-CH_3), 1.57 (3H, s, 16-CH_3). The ^{13}C NMR signals are listed in Table 3.

*5.1.1.14. Ethylshikonin (**21**).* EIMS: *m/z* 316 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 12.60 and 12.51 (each 1H, s, 5- and 8-OH), 7.26 (2H, s, H-6 and 7), 7.20 (1H, s, H-2), 5.20 (1H, dt, $J = 7.6$, 1.2 Hz, H-13), 4.66 (1H, ddd, $J = 7.0$, 4.2, 0.9 Hz, H-11), 4.48 (1H, dq, $J = 13.7$, 7.0 Hz, H-1′a), 4.46 (1H, dq, $J = 13.7$, 7.0 Hz, H-1′b), 2.52 (1H, m, H-12′a), 2.30 (1H, dt, $J = 15.0$, 7.0 Hz, H-12b′), 1.70 (3H, s, 15-CH_3), 1.60 (3H, s,

Table 3
^{13}C NMR spectral data of hydroxynaphthoquinones **13**, **14**, **16**, **19**, **22**, **21** and **23** in $CDCl_3$

	13 and **22**	**14**	**16** and **19**	**21**	**23**
C-1	176.8	175.4	176.7	176.2	176.7
C-2	135.9	136.4	136.7	134.7	131.6
C-3	148.5	147.5	148.2	151.0	148.6
C-4	178.3	177.0	178.2	179.9	178.0
C-5	167.3	168.7	167.5	166.2	167.0
C-6	132.7[a]	133.3[a]	132.9[a]	132.4[a]	136.0
C-7	132.6[a]	133.1[a]	132.7[a]	132.3[a]	136.0
C-8	166.7	168.1	167.0	165.7	167.5
C-9	111.5[b]	111.6[b]	111.6[b]	111.6[b]	112.0
C-10	111.8[b]	111.8[b]	111.8[b]	112.0[b]	112.0
C-11	69.0	69.8	69.5	65.4	68.7
C-12	32.9	32.9	32.8	34.4	33.0
C-13	117.8	117.7	117.7	119.1	117.9
C-14	131.3	131.3	131.5	132.1	136.0
C-15	25.7	25.8	25.8	26.5	25.7
C-16	18.9	17.0	17.9	17.9	17.9
C-1′	175.7	171.7	169.8	65.3	171.8
C-2′	34.0	46.5	21.0	15.3	25.1
C-3′	17.9	69.2	–	–	43.2
C-4′	18.8	29.2[c]	–	–	22.4
C-5′	–	29.1[c]	–	–	–

a,b,c: These values may be interchangeable in each column.

16-CH_3), 1.19 (3H, t, $J = 7.0$ Hz, 2′-CH_3). The ^{13}C NMR signals are listed in Table 3.

*5.1.1.15. 2′,3′-Epoxyalkannin (**23**).* EIMS: *m/z* 396 $[M]^+$; ^1H NMR ($CDCl_3$) δ 12.60 and 12.58 (each 1H, s, 5- and 8-OH), 7.20 (2H, s, H-6 and 7), 6.99 (1H, d, $J = 0.9$ Hz, H-2), 6.05 (1H, ddd, $J = 7.6$, 4.6, 0.9 Hz, H-11), 5.14 (1H, m, H-13), 2.62 (1H, m, H-12′a), 2.47 (1H, m H-2b′), 2.18 (1H, m, H-3′), 1.69 (3H, s, 15-CH_3), 1.59 (3H, s, 16-CH_3), 0.98 (3H, dd, $J = 6.7$, 2.1 Hz, 4'-CH_3). The ^{13}C NMR signals are listed in Table 3.

5.1.2. Cytotoxic activities

5.1.2.1. Cell lines and culture. The human colorectal carcinoma cell line (HCT 116, ATCC No. CCL-247) and human hepatoma cell line (Hep G2 No. RCB0459) were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. Dulbecco's modified Eagle's medium (DMEM), McCoy's 5A medium, fetal bovine serum (FBS) and penicillin–streptomycin mixture (100 U/ml penicillin and 100 μg/ml streptomycin) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma (MO, USA), Biosource International (CA, USA) and Bio Whittaker (ND, USA), respectively. The HCT 116 cells were maintained in McCoy's 5A medium and Hep G2 cells were cultured in DMEM. Each medium was supplemented with 10% FBS and a penicillin–streptomycin mixture at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

5.1.2.2. Cytotoxicity test. Aliquots (200 μl) of 5×10^3 cells/ml of HCT 116 and Hep G2 cells were seeded in 96-well flat-bottomed plates (Microtest™ Tissue Culture Plate, 96 Well, Flat Bottom with Low Evaporation Ltd., Falcon, NJ, USA) and incubated in a medium containing 10% FBS and a penicillin–streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The test drugs were dissolved in dimethyl sulfoxide (DMSO). The incubation medium was replaced with each test medium giving a final concentration of 0.1–500 μmol/l of test compounds and no drug in 2 μl DMSO over 2 days.

The ability of the drug to inhibit cellular growth was determined using the MTT assay [20]. The cytotoxic activities of the test drugs were determined as previously described [23]. Each experiment was performed in duplicate wells, and all experiments involving a control (DMSO only) and the drug treatments were performed separately 3–5 times. Data represent mean ± S.D. values.

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