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Original article

Synthesis and cytotoxicity study of alkannin derivatives

Zhi-Shu Huang [a,*], Hai-Qiang Wu [a,b], Zhi-Fang Duan [b], Bing-Fen Xie [c], Zong-Chao Liu [c], Gong-Kan Feng [c], Lian-Quan Gu [a,b,*], Albert S.C. Chan [d], Yue-Ming Li [d,*]

[a] *School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510080, China*
[b] *School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, China*
[c] *Cancer Center, Sun Yat-Sen University, Guangzhou 510060, China*
[d] *Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong*

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Abstract

Alkannin derivatives (**3–19**) were prepared through the reaction of β,β-dimethylacrylalkannin (**1**), the most abundant isohexenylnaphthazarin isolated from the roots of *Arnebia euchroma*, with different types of nucleophiles such as amines and thiols in the absence or presence of a reducing agent. The cytotoxicities of **1–8, 10–14** and **19** against four human carcinoma cell line (GLC-82, CNE2, Bel-7402, K-562) were found to be markedly higher than that of the naturally occurring β,β-dimethylacrylalkannin (**1**) and acetylalkannin (**2**). This study also shed light on the understanding of the biological activities in terms of the chemical reactivity of alkannins.

Keywords: β,β-Dimethylacrylalkannin; Alkannin derivatives; Reductive alkylation; Conjugated addition; Cytotoxicity

1. Introduction

Natural products like alkannin, shikonin and related naphthazarin compounds have shown significant biological activities including the inhibition of telomerase [1], DNA topoisomerase-I [2], and cytotoxicity [2–5]. From a chemist's viewpoint, the conjugated addition of cellular components (as nucleophiles) to quinones (as electrophiles) may be partly responsible for the cytotoxic behavior of quinones [6], and another possible mechanism for the cytotoxicity of quinone methides may involve bioreductive alkylation [7–9]. The mechanism of bioreductive alkylation involves an in vivo reduction of the quinone which leads to the elimination of a leaving group from a suitable position followed by the addition of the cellular nucleophiles, such as glutathione, proteins or DNA, to quinones to form cytotoxic nucleophile–quinone adducts and consequently causes the cell death. Since chemical evidence supporting these hypotheses is rare [10], it is of high interest to examine the chemical reactions that may occur between nucleophiles and natural alkannin, and the origin of cytotoxicity of the resulting alkannin derivatives.

In this paper we wish to report the reductive alkylation and oxidation-conjugated addition of natural β,β-dimethylacrylalkannin (**1**) [11] isolated from the roots of *Arnebia euchroma* [12], with a variety of nucleophiles, and the in vitro cytotoxic activity of the synthesized products. The results are consistent with the hypothesis that natural alkannins have a tendency to undergo bioreductive alkylation and conjugated addition reactions, and most of the reaction products showed higher cytotoxicity than the corresponding parent compounds. These results partially explain the biological activities of naphthazarin compounds and provide a good lead for the search of more potent anticancer agents.

2. Chemistry

Conjugated addition reactions between a variety of nucleophiles and β,β-dimethylacrylalkannin (**1**) were carried out either in the presence or in the absence of reducing agents (Fig. 1 and Table 1). Compound **1** is the most abundant isohexenylnaphthazarin isolated from the roots of *A. eu-*

* Corresponding authors. Tel.: +86-20-8411-5536; fax: +86-20-8411-0272.
E-mail addresses: huangzhishu@hotmail.com (Z.-S. Huang), cedc42@zsu.edu.cn (L.-Q. Gu), bcyli@polyu.edu.hk (Y.-M. Li).

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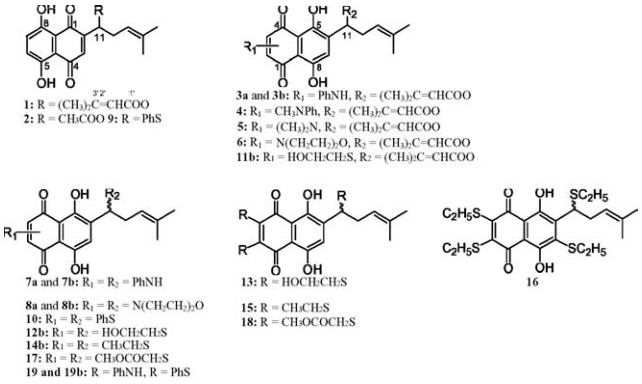


Fig. 1. Structures of natural alkannins (**1** and **2**) and semi-synthesized products (**3–19**); a: an isomer with nucleophilic group located at C-2; b: an isomer with nucleophilic group located at C-3.

chroma, and the addition of amines to **1** took place at C-6 or C-7 of the quinone ring (single-position reaction), respectively, producing positional isomers **3a** and **3b**, or mixtures (**4–6** and **11**) of positional isomers in the absence of a reducing reagent. For the purpose of clarity we denote the C-6 substituted product isomer "**a**" and the C-7 substituted product isomer "**b**" in this paper.

More complicated products were obtained in the presence of a reducing agent because of the possible involvement of the two-positions reaction at C-6 (or C-7) of the ring and C-11 of the side chain (**7a** and **7b, 8a** and **8b, 9, 10, 12, 14, 17, 19a** and **19b**), three-positions reaction at C-6, C-7, and C-11 (**13, 15**, and **18**), and the four-positions reaction at C-3, C-6, C-7, and C-11 (**16**). These results clearly showed that natural alkannin underwent a bioreductive alkylation at C-11. Meanwhile, a multipositional oxidation-conjugated addition also took place [13]. Some of the positional isomers could not be separated by rapid column chromatography and even HPLC; however, 2D-NMR studies including ^{1}H-^{13}C COSY and HMBC on separated isomers such as **3a** and **3b, 7a** and **7b**, and the mixed isomers **10a** and **10b** provided important information on the chemical shifts of these isomers and helped the analysis of the NMR spectra of the mixed isomers (Tables 2–4).

2.1. Reaction of β,β-dimethylacrylalkannin (1) with amine in the absence of $NaBH_4$

The reaction of β,β-dimethylacrylalkannin **1** with an amine in methanol or in a mixed solvent of methanol and chloroform led to the conjugate addition and autoxidation products **3a/3b, 4, 5**, and **6**. The nucleophilic attack took place at the C-6 or C-7 position of **1**.

Longer reaction time and excess amount of base were necessary for the reaction of **1** with aniline, a relatively weak base. Flash chromatography of the reaction mixture gave two red-colored products **3a** and **3b** in a ratio of 1.2:1 and in a total yield of 73%. Compounds **3a** and **3b** were confirmed as a pair of positional isomers of each other and their structures have been determined by fast-atom bombardment mass spectra (FAB-MS) and NMR (^{1}H-^{13}C COSY, HMBC) experiments. The main differences in the spectra of **3a** and **3b** are the signals at C-1, C-4, C-5, C-7 and C-8 positions (Tables 2 and 4).

The reaction of **1** with secondary amines, such as *N*-methyl aniline, dimethyl amine, and morpholine produced

Table 1
Reaction of β,β-dimethylacrylalkannin **1** with nucleophiles

Entry	Nucleophile	Ratio [a]	Conditions	Products (% [b])
1	$PhNH_2$	1:10	MeOH, r.t., 6 d	**3a** (40), **3b** (33)
2	$PhNHCH_3$	1:2	MeOH, r.t., 3 d	**4** (55) [c]
3	$(CH_3)_2NH$	1:3	$CHCl_3$–MeOH, 0 °C, 3 h	**5** (42) [c]
4		1:2.5	MeOH, r.t., 1 h	**6** (17) [c]
5	$PhNH_2$	1:10	$NaBH_4$, MeOH, 0 °C→r.t., 12 h	**7a** (28), **7b** (13)
6		1:10	$NaBH_4$, MeOH, 0 °C, 4 h	**8a** (7.7), **8b** (3.3)
7	PhSH	1:2	MeOH, r.t., 8 h	**9** (12), **10** (52) [c]
8	PhSH	1:2	MeOH, r.t., 24 h	**10** (51) [c]
9	$HOCH_2CH_2SH$	1:50	MeOH, r.t., 0.5 h	**11b** (30), **12b** (20)
10	$HOCH_2CH_2SH$	1:50	MeOH, r.t., 3 d	**12b** (35), **13** (25)
11	CH_3CH_2SH	1:50	MeOH, r.t., 4.5 d	**14b** (18), **15** (27)
12	CH_3CH_2SH	1:50	MeOH, r.t., 7 d	**15** (33), **16** (2)
13	CH_3OOCCH_2SH	1:40	MeOH, r.t., 1 h	**17** (34) [c], **18** (8)
14 [d]	PhSH	1:10	MeOH, reflux, 72 h	**19a** (58)
15 [e]	PhSH	1:10	MeOH, reflux, 30 h	**19b** (71)

[a] Molar ratio of compound **1** to nucleophile.
[b] Isolated yield.
[c] Mixture of a pair of position isomers.
[d] **3a** as starting material.
[e] **3b** as starting material.

Table 2
^{1}H NMR data of compounds **3, 7, 8, 10** and **19** [a]

Number of H [b]	**3a**	**3b**	**7a**	**7b**	**8a**	**8b**	**10a**	**10b**	**19a**	**19b**
H-2		6.32 s		6.33 s		6.00 s		6.12 s		6.32 s
H-3	6.32 s		6.33 s		5.99 s		6.12 s		6.33 s	
H-7	7.11 s	7.28 s	7.19 s	7.37 s	7.26 s	7.36 s	7.14 s	7.21 s	7.18 s	7.28 s
H-11	6.14 dd (7.3, 4.1)	6.13 dd (7.3, 2.7)	4.81 dd (7.2, 4.4)	4.81 dd (7.2, 4.3)	4.01 d (5.2)	4.05 d (5.2)	4.76 dd (16.4, 7.2)	4.76 dd (16.4, 7.2)	4.82 t (7.2)	4.75 t (7.2)
H-12	2.55 m	2.55 m	2.56 m	2.56 m	2.53 m	2.53 m	2.60 m	2.60 m	2.62 t (7.2)	2.62 t (7.2)
H-13	5.16 t (7.2)	5.16 t (7.1)	5.17 t (7.2)	5.17 t (7.2)	5.03 m	4.99 m	5.12 m	5.12 m	5.15 t (6.8)	5.13 t (6.8)
H-15 [c]	1.57 s	1.57 s	1.66 s	1.66 s	1.45 s	1.46 s	1.56 s	1.56 s	1.57 s	1.57 s
H-16 [c]	1.68 s	1.69 s	1.74 s	1.74 s	1.60 s	1.60 s	1.66 s	1.66 s	1.66 s	1.66 s
OH-5	13.71 s	12.33 s	13.91 s	12.52 s	13.62 s	12.37 s	13.17 s	12.51 s	13.93 s	12.52 s
OH-8	11.89 s	13.19 s	11.89 s	13.14 s	12.38 s	13.00 s	12.26 s	12.85 s	11.87 s	13.14 s
Nu CH	2-PhNH	3-PhNH	2-PhNH	3-PhNH	2-morpholine	3-morpholine	2-PhS	3-PhS	2-PhNH	3-PhNH
	7.43 t, 7.27 d	7.44 t, 7.26 d	7.25–7.44 m	7.25–7.44 m	3.57 d 3.87 d	3.55 d 3.88 d	7.30–7.52 m	7.30–7.52 m	7.19–7.44 m	7.20–7.44 m
	7.24 t	7.24 t	11-PhNH	11-PhNH	11-morpholine	11-morpholine	11-PhS	11-PhS	11-PhS	11-PhS
			6.48–6.67 m	6.48–6.67	2.47 d 3.70 d	2.53 m 3.73 d	7.22–7.30 m	7.22–7.30 m	7.19–7.32 m	7.20–7.32 m
Nu NH	7.74 s	7.70 s	4.19 s, 7.71 s	4.15 s, 7.70 s					7.71 s	7.69 s
RCOO	5.81 m, 2.17 s, 1.94 s	5.79 s, 2.16 s, 1.93 s								

[a] The spectra were recorded on a 400 MHz NMR spectrometer in $CDCl_3$, with Me_4Si as an internal standard. Symbols are as follows: s, singlet; d, doublet; t, triplet; m, multiplet.
[b] The number of the hydrogen in each compound was shown in the footnote of Table 4.
[c] Exchangeable signals.

Table 3
^{1}H NMR data of compounds **1, 3–6, 9, 11–15** and **17–18** [a]

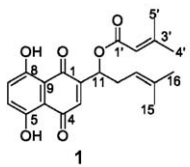
Number of H [b]	**1**	**4 (a + b)** [e]	**5 (a + b)** [e]	**6 (a + b)** [e]	**9**	**11b**	**12b**	**13**	**14b**	**15**	**17 (a + b)** [e]	**18**
H-2						6.72 s	6.71 s		7.07 s			
		6.33 s [f]	5.79 s [f]	5.98 s [f]							7.33 s [f]	
H-3	6.97 s				6.77 s							
H-6	7.18 s				7.18 s							
H-7	7.18 s	7.10/7.25 s	7.12/7.23 s	7.11/7.23 s	7.18 s	7.18 s	7.40	7.35 s	7.20 s	7.37 s	7.21/7.41 s	6.73 s
H-11	6.00 dd (7.2, 4.8)	6.11 dd (7.6, 4.4)	6.11/6.13 dd (7.6, 4.4)	6.11 dd (7.6, 4.4)	4.61 m	6.11 dd (7.2, 4.0)	4.38 t (5.0)	4.37 t (5.1)	4.23 t (7.5)	4.39 t (7.5)	4.51 t (7.5)	4.51 t (7.5)
H-12	2.51 m	2.55 m	2.52 m	2.55 m	2.60 m	2.60 m	2.55 m	2.69 m	2.55 t (7.0)	2.56 t (7.0)	2.60 t (7.5)	2.60 t (7.5)
H-13	5.14 t (6.8)	5.16 t (6.6)	5.17 t (6.8)	5.16 t (6.8)	5.12 m	5.14 t (7.1)	5.09 t (6.5)	5.09 t (6.4)	5.11 m	5.10 m	5.07 t (7.5)	5.07 t (7.5)
H-15 [c]	1.58 s	1.57 s	1.58 s	1.56 s	1.56 s	1.55 s	1.59 s	1.57 s	1.60 s	1.58 s	1.57 s	1.57 s
H-16 [c]	1.68 s	1.69 s	1.68 s	1.68 s	1.66 s	1.68 s	1.68 s	1.67 s	1.67 s	1.66 s	1.66 s	1.66 s
OH-5 [d]	12.44 s	13.57/12.34 s	13.81/12.62 s	13.48/12.74 s	12.43 s	12.20 s	12.55 s	12.61 s	12.47 s	12.62 s	13.13/12.53 s	12.17
OH-8 [d]	12.60 s	11.95/13.08 s	12.23/13.28 s	12.33/12.99 s	12.62 s	13.04 s	12.79 s	13.23 s [c]	12.63 s	13.23 s	12.20/12.74 s	13.18
Ester group	2.15 s, 1.94 s	5.79 m, 2.15 s, 1.93 s	5.78 s, 2.18 s, 1.92 s	5.78 m, 2.15 s, 1.92 s		5.79 s, 2.15 s, 1.92 s						
Nu CH		7.44–7.27 m	3.26 s	3.87 t (4.4)	7.22–7.30 m	3.97 t (6.0)	2.68 t (6.5)	2.59 t (6.5)	1.23 t (7.5)	1.20 t (7.5)	3.18 s	3.18 s
		2.26 s		3.55 t (4.4)		3.12 t (6.0)	3.11 t (6.0)	3.46 t (6.3)	2.50 q (7.5)	1.23 t (7.5)	3.67 s	3.67 s
							3.71 t (6.5)	3.49 t (6.4)		2.45 t (7.5)	3.80 s	3.71 s
							3.97 t (6.0)	3.72 t (6.5)				3.80 s
								3.80 t (6.3)				
								3.97 t (6.4)				

[a] The spectra were recorded with a 400 MHz NMR spectrometer in $CDCl_3$, with Me_4Si as an internal standard. Symbols are as follows: s, singlet; d, doublet; t, triplet; m, multiplet.
[b] The numbering of hydrogen of each compound was shown in the footnote of Table 4.
[c] Exchangeable signals.
[d] Exchangeable signals.
[e] Mixture of positional isomers.
[f] Signal of H-2 or H-3.

Table 4
^{13}C NMR data of compounds **1, 3–8a, 9–10, 12b** and **19** [a]

Number of compounds [c]	**1**	**3a**	**3b**	**4 (a + b)** [b]	**5 (a + b)** [b]	**6 (a + b)** [b]	**7a**	**7b**	**8a**	**9**	**10a**	**10b**	**12b**	**19a**	**19b**
1	179.0	182.9	187.3	184.1/186.7	185.7/186.3	184.6/186.4[e]	182.8	187.4	186.9[h]	181.2	182.0	181.2	182.1[j]	182.8	187.2
2	131.6	145.6	103.1	147.9/112.2	144.2/112.1	144.3/112.5	145.9	103.3	153.7	127.8	156.3	127.8	127.2	145.8	103.2
3	149.0	103.0	145.5	112.3/147.9	112.1/144.2	112.7/144.3	102.9	145.8	112.4	155.7	127.4	155.7	153.8	103.0	145.5
4	177.5	187.7	183.7	187.1/184.6	186.3/186.0	186.8/185.2[e]	188.1	183.9	184.5[h]	181.0	180.3	181.0	181.6[j]	187.9	183.6
5	165.2	153.6	155.2	153.6/155.8	152.2/157.8	153.6/153.9[f]	154.8	156.3	155.9[i]	160.4	159.3	160.4	159.7[k]	154.8	155.8
6	132.6[d]	145.6	139.9	147.9/140.7	144.2/140.0	140.9	148.4	142.1	144.9	143.8	146.1	143.8	144.5	146.7	140.5
7	132.4[d]	122.2	127.7	125.0/129.6	126.2/126.3	123.1/126.4	123.3	128.7	125.7	129.2	126.9	129.2	129.0	124.2	129.5
8	166.3	157.7	155.8	157.8/155.8	158.0/152.3	156.0/155.8[f]	158.3	156.3	158.3[i]	160.7	161.5	160.7	160.3[k]	157.8	156.3
9[e]	111.6	110.5	110.1	110.4	110.9/110.4	110.1	110.4	109.9	110.3	110.1	110.4	110.1	110.2	110.4	109.8
10[e]	111.9	110.5	111.1	110.8	112.0/112.6	110.5	110.7	111.4	111.7	111.0	110.7	111.0	111.3	110.4	111.0
11	68.6	69.4	68.7	69.6/69.3	69.2/69.0	69.3/68.9	52.7	52.1	60.8	44.9	45.1	44.9	41.4	45.2	44.9
12	32.9	33.1	33.2	33.0/33.1	33.2	33.2/33.1	34.0	34.1	29.7	33.2	33.3	33.2	33.4	33.4	33.3
13	118.0	118.3	118.2	118.3/118.5	118.6	118.3	119.2	119.2	119.0	119.8	119.8	119.8	119.9	120.0	119.9
14	135.8	135.2	135.3	135.3/135.2	135.3/135.2	135.2	136.0	136.0	133.8	134.9	134.9	134.9	135.1	134.8	134.7
15[f]	17.9	18.0	18.0	17.8/17.9	17.9/18.0	18.0	18.1	18.1	17.9	18.1	18.1	18.1	18.0	18.1	18.0
16[f]	25.7	25.9	25.6	25.7	25.7/25.5	25.8	26.0	26.0	25.7	25.8	25.8	25.8	25.7	25.8	25.7
1′	166.8	165.3	165.3	165.4	165.5/165.4	165.2/165.3	146.6	146.5	51.0	133.8	133.8	133.8	33.7[l]	134.3	134.0
2′	115.2	115.5	115.5	115.6	115.7	115.5	113.3	113.3	67.2	128.8	128.8	128.8	59.7[m]	128.7	128.7
3′	159.0	158.1	158.2	158.1	158.0/158.1	158.1158.3	129.1	129.1		132.0	132.0	132.0		131.5	131.9
4′	20.4[g]	20.4[g]	20.4[g]	20.3[g]	20.3[g]	20.4[g]	117.8	117.8		127.3	127.3	127.3		127.0	127.1
5′	27.6[g]	27.6[g]	27.6[g]	27.5[g]	27.5[g]	27.6[g]	129.1	129.1		132.0	132.0	132.0		131.5	131.9
6′							113.3	113.3		128.8	128.8	128.8		128.7	128.7
1″		136.9	136.9	140.7		49.8/49.7	136.9	137.0	49.7	127.0	127.0	127.0	35.1[l]	136.9	136.9
2″		122.8	122.8	122.8/122.6		66.4	122.8	122.8	66.4	130.4	130.6	130.4	60.6[m]	122.8	122.7
3″, 5″		129.6	129.6	129.6			129.7	129.7		135.6	135.6	135.6		129.7	129.6
4″		125.9	126.0	126.1/126.5			126.0	126.0		133.0	133.0	133.0		126.0	125.9
6″		122.8	122.8	122.8			122.8	122.8		130.4	130.6	130.4		122.8	122.7
NCH_3				43.4/43.8	43.3/43.5										

[a] The spectra were recorded on a 400 MHz NMR spectrometer in $CDCl_3$, with Me_4Si as an internal standard. Symbols are as follows: s, C; d, CH; t, CH_2; q, CH_3.
[b] Mixture of positional isomers.
[c] The numbering of the carbons of the representative compounds.



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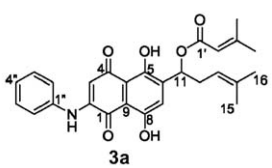

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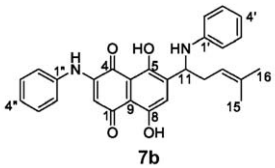

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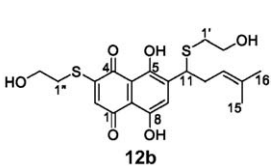

12b

[d–m] Values with the same superscript may be exchanged.

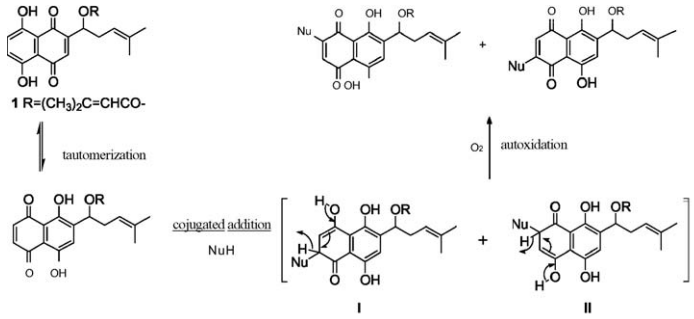

Fig. 2. A reaction mechanism involving oxidation-conjugated addition.

the mixtures **4–6**, respectively, which were positional isomers at C-6 and C-7 like **3a** and **3b**. The ^{1}H and ^{13}C NMR spectra of these mixtures displayed characteristic signals at C-1, C-4, C-5, C-7 and C-8 positions. Based on these results and the information obtained from a mass spectrometric study, it was concluded that the reaction of **1** with secondary amines is similar to that with aniline. However, the reaction with secondary amines showed higher reactivity and produced complicated mixtures compared to the reaction with aniline, and consequently led to lower yields. It was quite possible that the stronger nucleophility of secondary amines led to higher reactivity and their stronger alkalinity caused more byproducts because of the instability of naphthazarines towards base.

A possible reaction mechanism may be proposed as shown in Fig. 2. Tautomerization [14] of the starting material followed by nucleophilic addition to the α,β-unsaturated carbonyl group gave two unstable intermediates, **I** and **II**. This in turn was followed by an autoxidation step to re-form the naphthazarin moiety to afford the final products. Electron-donating substituents rendered the hydroquinones more susceptible to oxidation than the corresponding unsubstitued hydroquinones [15], and this might be responsible for the auto-oxidation of **I** and **II** to the final products in the presence of atmospheric oxygen.

*2.2. Reaction of β,β-dimethylacrylalkannin (**1**) with aniline in the presence of $NaBH_4$*

In order to confirm the hypothesis [7] of bioreductive-alkylating function of isohexenylnaphthazarin, the reaction of compound **1** with aniline was also carried out in the presence of a reducing agent $NaBH_4$. The color of the solution changed immediately from red to yellow, then slowly to reddish violet upon addition of $NaBH_4$. The products were purified by flash column chromatography followed by semi-preparative HPLC to give a pair of positional (C-6 and C-7) isomers **7a** and **7b** with a ratio of 2:1 and in a total yield of 41%. The structures of compounds **7a** and **7b** were determined on the basis of NMR (1D and 2D) and FAB-MS.

On the other hand, the reaction of morpholine under reducing condition gave positional isomers **8a** and **8b** in a total yield of 11%. It was possible that the higher alkalinity of morpholine caused the formation of more byproducts and consequently led to the low yield.

*2.3. Reaction of β,β-dimethylacrylalkannin (**1**) with thiols*

Since a sulfanyl group possesses both nucleophilic and reductive properties. The reaction of **1** with thiols is anticipated to proceed in a similar manner as that of **1** with aniline in the presence of reducing reagents.

A reddish violet mixture **10** was isolated in 51% yield when **1** was allowed to react with thiophenol for 24 h at ambient temperature. The ^{1}H and ^{13}C NMR spectra of the products indicated that they were a pair of isomers (**10a** and **10b**) resulting from two-positions reaction (at C-6/C-11 or C-7/C-11) in an approximate ratio of 5:4. For a shorter reaction time of 8 h, a single-positional (only at C-11) product **9** was also obtained as a minor product, and the ratio of **9–10** was 19:81.

When 2-sulfanylmethanol was used as the nucleophile, the products were also influenced by the reaction time. Single-position reaction (only at C-7) product **11b** and two-positions reaction product **12b** (C-7/C-11) were obtained when the reaction was quenched in 0.5 h, while **12b** and three-positions (C-6/C-7/C-11) reaction product **13** were obtained when the reaction was carried out for 3 d. In the reaction of **1** with ethanethiol, in addition to the two-positions and three-positions reaction products **14b** and **15**, a four-positions reaction product **16** was also detected through FAB-MS experiment. It was found that ethanethiol groups have replaced three hydrogen atoms (at C-3, C-6, and C-7) on the aromatic rings and an ester group (C-11) on the side chain.

Methyl thioglycolate were also tested as nucleophiles. The results showed that the major reactions seemed to be the two-position one (**17**) and three-position one (**18**) according to ^{1}H NMR data, UV special absorption spectra, and mass spectra.

The substitution of the ester group by a nucleophile in the presence of a reducing agent supported the hypothesis that isohexenylnaphthazarin derivatives could function as bioreductive-alkylating agents. The study also showed the replacement of the aromatic protons by nucleophiles, which probably involved a two-step reaction, i.e. the conjugate addition of α,β-unsaturated carbonyl group followed by the autoxidation of phenol. The different intermediates obtained from the reactions of thiophenol and 2-mercaptoethanol might be due to the difference of nucleophilicity and reduc-

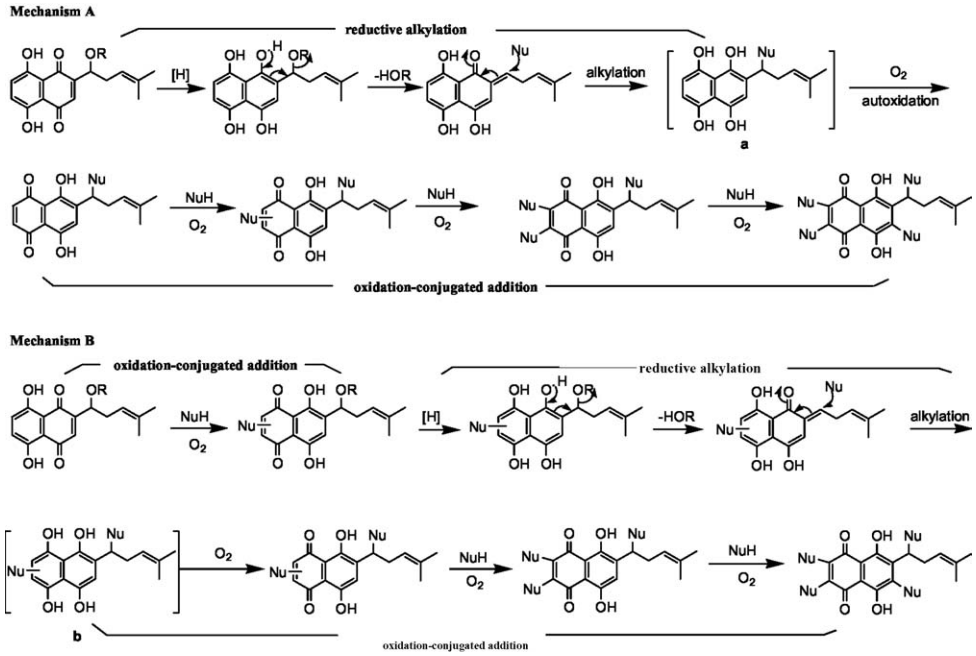

Fig. 3. Two possible mechanisms involving oxidation-conjugated addition and reductive alkylation.

ing power of the two nucleophiles. Accordingly, two possible mechanisms were proposed as shown in Fig. 3. In mechanism A, the reduction of **1** was followed by the leaving of the ester group and the attack of nucleophilic group to give hydroquinone intermediate **a**. A fast autoxidation followed by a conjugate addition on the naphthazarin ring gave the final products. In mechanism B, the first step was a conjugated addition, which was followed byreductive alkylation and oxidation-conjugated addition to give the final product. In the case of thiophenol with relatively strong reducing power and weak nucleophilicity, mechanism A might dominate. On the other hand, in the case of 2-sulfanylethanol with stronger nucleophilicity, the first step of the reaction might be conjugated addition of the naphthazarin ring (mechanism B). To examine these possibilities, the reactions of **3a** and **3b** with a large excess of thiophenol was studied and products **19a** (71% yield) and **19b** (58% yield) were obtained, respectively. The reaction rate was so slow that the reaction required refluxing condition and long reaction time. The slow rate might be due to the presence of the phenylamino group (PhNH-) on the naphthazarin ring that caused the steric hindrance to the reductive alkylation. This result and the formation of product **9** are consistent with mechanism A. For the reaction using 2-mercaptoethanol as the nucleophile, mechanism B was possible as evidenced by the formation of compound **11b**.

3. Cytotoxic activity of naphthazarin derivatives

The in vitro cytotoxicity of alkannin and derivatives was evaluated. The growth inhibition effect was assayed using MTT method. The concentration of alkannin derivatives for 50% inhibition (IC_{50}) on the human lung adenocarcinoma cell line (GLC-82), human nasopharyngeal carcinoma cell line (CNE2), human liver carcinoma cell line (Bel-7402) and human leukemia cell line (K-562) were determined and the results were summarized in Table 5. The cell line study clearly showed that most of the derivatives (except for compounds **5** and **13**) display substantially higher cytotoxicity than that of the natural parent compounds β,β-dimethylacrylalkannin (**1**) and acetylalkannin (**2**).

The derivatives **8, 11b, 12b**, and **14b** were found to be the most cytotoxic compounds for the four cell lines. It is interesting that all of them are products derived from non-aromatic nucleophiles such as 2-sulfanylethanol and morpholine.

Table 5
Cytotoxic activity of alkannin derivatives (IC_{50} (μM))

Compound	GLC-82	CNE2	Bel-7402	K-562
1	–	–	–	–
2	–	–	–	–
3a	5.51	12.2	nd	nd
3b	18.8	22.6	nd	nd
4	2.00	9.37	22.9	–
5	13.8	–	–	–
6	5.63	18.5	16.0	14.1
7a	13.6	17.0	nd	nd
7b	12.7	18.9	nd	nd
8	2.31	4.50	6.99	2.58
10	4.41	–	15.9	–
11b	2.17	8.23	3.57	5.70
12b	1.58	5.05	9.60	5.57
13	–	–	–	–
14b	5.49	8.76	11.3	9.25
19a	–	22.3	nd	nd
19b	21.7	–	nd	nd

–: no significant cytotoxicity and the IC_{50} value is higher than 25 μM. nd: not determined.

Comparing the cytotoxic potency of the single isomers **3a/3b, 7a/7b**, and **19a/19b** against GLC-82 and CNE2, no significant differences were observed; only **3a** is about 3.4 times as potent as **3b** against GLC-82.

In conclusion, we have carried out the conjugate addition and reductive alkylation of β,β-dimethylacrylalkannin with a variety of nucleophiles, and all of the addition products showed higher in vitro cytotoxicity against four human carcinoma cells than the parent compounds β,β-dimethylacrylalkannin (**1**) and acetylalkannin (**2**). The results are consistent with the hypothesis that the conjugate addition and reductive alkylation of cellular components (as nucleophiles) with quinones (as electrophiles) might be responsible for the cytotoxic behavior of quinines. Furthermore, the fact that the reaction products showing higher cytotoxicity than the parent compounds also provides a good lead for finding more potent anticancer agents.

4. Experimental

4.1. General

UV/visible absorbance spectra were measured on a MILTON ROY 3000 ARRAY spectrophotometer. All NMR spectra were collected on a JEOL JNM-EX 400 NMR spectrometer and Varian UNITYINOVA 500 NMR spectrometer in $CDCl_3$. The chemical shifts (δ) are expressed in ppm relative to tetramethylsilane and coupling constant (J) in Hertz. All FAB-MS were determined in NBA matrix in the positive ion mode on a Finnigan MAT TSQ 7000 spectrometer and a VG ZAB-HS spectrometer. HPLC was performed on a Waters 600E-996 PAD, the preparative column used was a Waters Nova-Pak, silica 6 μm, 19 × 300 mm column. Flash chromatography was performed on Merck silica gel (0.015 mm). Melting points were determined with a Thomas Hoover melting (capillary method) apparatus and were uncorrected. Elemental analyses were carried out in the Central Laboratory of Sun Yat-Sen University.

4.2. Isolation of β,β-dimethylacrylalkannin

Dry root powder of *A. euchroma* [12] was extracted with petroleum ether (30–60 °C). The extract was concentrated and the residue was chromatographed on silica gel (ethyl acetate/petroleum ether, 5:95), yielding crude β,β-dimethylacrylalkannin (**1**) (R_f = 0.64, hexane/ethyl acetate, 5:1). The crude sample was recrystallized in hexane to give pure β,β-dimethylacrylalkannin.

4.3. Semi-synthesis of isohexenylnaphthazarin analogs

*4.3.1. 2-Phenylamino-6-(11-β,β-dimethylacryl)isohexenylnaphthazarin (**3a**) and 3-phenylamino-6-(11-β,β-dimethylacryl)isohexenylnaphthazarin (**3b**)*

A solution of β,β-dimethylacrylalkannin (**1**, 74 mg, 0.2 mmol) and aniline (187 μl, 2 mmol) in methanol (8 ml) was stirred for 6 d (or 1 h if catalyzed by 2 mg of H_3BO_3 [16]) at ambient temperature. The solvent was removed and the mixture was purified by flash chromatography (hexane/ethyl acetate, 96:4) to afford compounds **3a** (37 mg, 40%) and **3b** (30.5 mg, 33%). **3a**: UV λ_{max} (lg ε) (MeOH): 212 (4.2), 268 (4.1), 321 (3.4), 507 (3.7) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 462 (M^+ + 1), 391, 362, 83. Anal. Calc. for $C_{27}H_{27}NO_6$: C, 70.25; H, 5.90; N, 3.04. Found: C, 70.20; H, 5.92; N, 2.99. **3b**: UV λ_{max} (lg ε) (MeOH): 212 (4.2), 269 (4.1), 515 (3.7) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 462 (M^+ + 1), 391, 362. Anal. Calc. for $C_{27}H_{27}NO_6$: C, 70.25; H, 5.90; N, 3.04. Found: C, 70.28; H, 5.93; N, 3.07.

*4.3.2. 2- or 3-Methylphenylamino-6-(11-β,β-dimethylacryl) isohexenylnaphthazarin (**4**)*

A mixture of **1** (74 mg, 0.2 mmol) and *N*-methyl aniline (0.4 mmol) in methanol (10 ml) was stirred for 3 d (or 1 h if catalyzed by 2 mg of H_3BO_3) at ambient temperature. TLC analysis showed complete conversion. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (hexane/ethyl acetate, 96:4) to afford a mixture of compounds **4a** and **4b** (1:1 ratio; 52 mg, 55%). UV λ_{max} (lg ε) (MeOH): 213 (4.3), 278 (4.0), 520 (3.7) nm; ^{1}H NMR (Table 3); ^{13}C NMR (Table 4); FAB-MS *m/z* 476 (M^+ + 1), 464, 392, 376, 324, 91, 83, 55. Anal. Calc. for $C_{28}H_{29}NO_6$: C, 70.74; H, 6.11; N, 2.95. Found: C, 71.22; H, 6.04; N, 3.05.

*4.3.3. 2- or 3-Dimethylamino-6-(11-β,β-dimethylacryl)isohexenylnaphthazarin (**5**)*

To a solution of **1** (74 mg, 0.2 mmol) in 10 ml of chloroform was added drop-wise a solution of dimethyl amine (0.6 mmol) in 2 ml of methanol in a period of 2 h. After another hour of stirring, the reaction mixture was washed with water (4 × 10 ml) and dried over Na_2SO_4. The solvent was removed and the residue was purified by flash chromatography and preparative TLC to give a mixture of **5a** and **5b** in a ratio of 1:0.9 (34 mg, 42%). UV λ_{max} (lg ε) (MeOH): 216 (4.4), 266 (3.9), 306 (3.4), 514 (3.6) nm; ^{1}H NMR (Table 3); ^{13}C NMR (Table 4); FAB-MS *m/z* 410 (M^+ + 1), 314, 262, 171, 83, 55. Anal. Calc. for $C_{23}H_{27}NO_6$: C, 67.48; H, 6.60; N, 3.42. Found: C, 66.87; H, 6.50; N, 3.34.

*4.3.4. 2- or 3-Morpholino-6-(11-β,β-dimethylacryl)isohexenylnaphthazarin (**6**)*

To a solution of **1** (56 mg, 0.15 mmol) in 6 ml of methanol was added a solution of morpholine (0.4 mmol) in 1 ml of methanol at ambient temperature. After another hour of stirring, the solvent was removed and the residue was purified by flash chromatography (hexane/ethyl acetate, 4:1) to give **6** (12 mg, 17%, a mixture of **6a** and **6b** in a ratio of 44:56). UV λ_{max} (lg ε) (MeOH): 214 (4.3), 260 (4.1), 321 (3.5), 509 (3.8) nm; ^{1}H NMR (Table 3); ^{13}C NMR (Table 4); FAB-MS *m/z* (%): 456 (M^+ + 1), 356. Anal. Calc. for $C_{25}H_{29}NO_7$: C, 65.93; H, 6.37; N, 3.08. Found: C, 66.17; H, 6.42; N, 3.14.

*4.3.5. 2,11-Bis(phenylamino)-6-isohexenylnaphthazarin (**7a**) and 3,11-bis(phenylamino)-6-isohexenylnaphthazarin (**7b**)*

To a solution of red mixture of **1** (56 mg, 0.15 mmol) and aniline (140 μl, 1.5 mmol) in methanol (6 ml) was added sodium borohydride (17 mg, 0.45 mmol) under ice-bath. A light yellow color solution was obtained. The stirring was continued for 30 min at 0 °C and 12 h at room temperature (r.t.). The color of the solution changed slowly from yellow to reddish violet. The solvent was removed and the residue was purified by flash chromatography to give a mixture of **7a** and **7b**. This mixture was further isolated by preparative HPLC (eluent: hexane/ethyl acetate/acetic acid = 40:58:2, flow rate: 5 ml/min) to yield **7a** (19 mg, 28%) and **7b** (9 mg, 13%).

7a: UV λ_{max} (lg ε) (MeOH): 212 (4.3), 251 (4.3), 266 (4.3), 325 (3.7), 508 (4.0) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 455 (M^+ + 1), 362. Anal. Calc. for $C_{28}H_{26}N_2O_4$: C, 73.98; H, 5.77; N, 6.17. Found: C, 73.88; H, 5.70; N, 6.28. **7b**: UV λ_{max} (lg ε) (MeOH): 208 (4.4), 250 (4.3), 517 (3.9) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 455 (M^+ + 1), 391. Anal. Calc. for $C_{28}H_{26}N_2O_4$: C, 73.98; H, 5.77; N, 6.17. Found: C, 73.91; H, 5.83; N, 6.10.

*4.3.6. 2,11-Bis(morpholino)-6-isohexenylnaphthazarin (**8a**) and 3,11-bis(morpholino)-6-isohexenylnaphthazarin (**8b**)*

To a solution of **1** (74 mg, 0.2 mmol) in 35 ml of methanol was added drop-wise a solution of morpholine (2 mmol) in 2 ml of methanol followed by the addition of sodium borohydride (38 mg, 1 mmol). The reaction mixture was stirred for 4 h at 0 °C. Ice-water (30 ml) was added into the reaction mixture and the solution was extracted four times with a mixture of hexane and ethyl acetate (1:1). The organic phase was combined, washed with water and was dried over sodium sulfate. The solvent was removed and the residue was purified by flash chromatography (hexane/ethyl acetate, 3:1) to give **8a** (7 mg, 7.7%) and **8b** (3 mg, 3.3%). **8a**: UV λ_{max} (lg ε) (MeOH): 212 (4.3), 250 (4.2), 322 (3.5), 506 (3.9) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 356 [M^+ – $O(CH_2CH_2)_2N$], 149, 113. Anal. Calc. for $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.11; H, 6.88; N, 6.25. **8b**: UV λ_{max} (lg ε) (MeOH): 208 (4.2), 250 (4.2), 515 (3.8) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 356 [M^+ – $O(CH_2CH_2)_2N$], 149, 113. Anal. Calc. for $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.13; H, 6.86; N, 6.27.

*4.3.7. 11-Phenylsulfanyl-6-isohexenylnaphthazarin (**9**) and 2,11-, or 3,11-bis(phenylsulfanyl)-6-isohexenylnaphthazarin (**10**)*

A mixture of thiophenol (0.33 mmol) and compound **1** (0.15 mmol) in methanol (6 ml) was stirred for 8 h at r.t. The solvent was removed by blowing cold air and the residue was purified by flash chromatography to give **9** and **10** (45 mg, 64%, a mixture of **9, 10a**, and **10b** in a ratio of 19:51:30). When the reaction was carried out for 24 h, a mixture of **10a** and **10b** in a ratio of 11:9 (37 mg of, 51% yield) was obtained. **9**: ^{1}H NMR (Table 3); ^{13}C NMR (Table 4). **10**: UV λ_{max} (lg ε) (MeOH): 208 (4.3), 247 (4.1), 526 (3.6) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 489 (M^+ + 1), 419, 381, 380, 379, 337, 271.

*4.3.8. 3-(2-Hydroxyethanesulfanyl)-6-(11-β,β-dimethylacryl) isohexenylnaphthazarin (**11b**), 3,11-bis-(2-hydroxyethanesulfanyl)-6-isohexenylnaphthazarin (**12b**), 2,3,11-tri-(2-hydroxyethanesulfanyl)-6-isohexenylnaphthazarin (**13**)*

A mixture of **1** (100 mg, 0.27 mmol) with 1-sulfanylethanol (1 ml, 14.3 mmol) in methanol (10 ml) was stirred for 30 min at ambient temperature. The solvent was removed and the residue was purified by flash chromatography (petroleum ether/ethyl acetate, 2:1 v/v) and preparative TLC to give **11b** (36 mg, 30%) and **12b** (23 mg, 20%). When the reaction was carried out for 3 d at ambient temperature, 40 mg (35%) of **12b** and 34 mg (25%) of **13** were obtained. **11b**: UV λ_{max} (lg ε) (MeOH): 214 (4.4), 245 (4.3), 320 (3.7), 508 (3.8) nm; ^{1}H NMR (Table 3); FAB-MS *m/z* 447 (M^+ + 1), 435, 379, 347, 309, 301, 83, 55. **12b**: UV λ_{max} (lg ε) (MeOH): 214 (4.3), 246 (4.4), 318 (3.8), 520 (4.0) nm; ^{1}H NMR (Table 3); ^{13}C NMR (Table 4); FAB-MS *m/z* 425 (M^+ + 1), 347, 149, 91, 55. Anal. Calc. for $C_{20}H_{24}O_6S_2$: C, 56.60; H, 5.66. Found: C, 56.63; H, 5.70. **13**: UV λ_{max} (lg ε) (MeOH): 217 (4.4), 265 (4.3), 368 (3.5), 509 (4.0) nm; ^{1}H NMR (Table 3).

*4.3.9. 3,11-Diethanesulfanyl-6-isohexenylnaphthazarin (**14b**), 2,3,11-triethanesulfanyl-6-isohexenylnaphthazarin (**15**) and 2,3,7,11-tetraethanesulfanyl-6-isohexanylnaphthazarin (**16**)*

A mixture of **1** (100 mg, 0.27 mmol) and ethanethiol (1 ml, 13.5 mmol) in 10 ml of methanol was stirred for 4.5 d at ambient temperature. The solvent was removed and the residue was purified by flash chromatography and preparative TLC to give **14b** (19 mg, 18%) and **15** (33 mg, 27%). When the reaction was continued for 7 d at ambient temperature, **15** (40 mg, 33%) and **16** (2.8 mg, 2%) were obtained. **14b**: ^{1}H NMR (Table 3); ESI-MS *m/z* 391 (%) (M^+ – 1, 23). **15**: UV λ_{max} (lg ε) (MeOH): 215 (4.4), 268 (4.1), 323 (3.5), 506 (3.9) nm; ^{1}H NMR (Table 3); FAB-MS *m/z* (%) 453 (M^+ + 1, 26), 391 (100), 383 (80), 361 (12), 91 (28), 69 (46), 55 (45). **16**: UV λ_{max} (lg ε) (MeOH): 214 (4.4), 272 (4.3), 349 (3.4), 536 (3.9) nm; FAB-MS *m/z* (%) 513 (M^+ + 1, 22), 451 (73), 390 (14), 329 (4), 268 (44), 130 (68), 69 (78), 55 (100).

*4.3.10. 2,11- or 3,11-Bis-methoxycarbonylmethanesulfanyl-6-isohexenylnaphthazarin (**17**), 2,3,11-trimethoxycarbonylmethanesulfanyl-6-isohexenylnaphthazarin (**18**)*

A mixture of **1** (100 mg, 0.27 mmol) and methyl mercaptoacetate (1 ml, 11.2 mmol) in 10 ml of methanol was stirred for 1 h at r.t. The solvent was removed and the residue was purified by flash chromatography and preparative TLC to give **17** (44 mg, 34%) and **18** (18 mg, 8%). **17**: UV λ_{max} (MeOH): 240, 269, 330, 496, 522, 561 nm; ^{1}H NMR (Table 3); FAB-MS *m/z* (%) 481 (M^+ + 1, 8), 375 (9), 219 (5), 167 (5). **18**: UV λ_{max} (MeOH): 234, 259, 333, 497, 528, 554;

^{1}H NMR (Table 3); FAB-MS *m/z* (%) 585 (M^+ + 1, 4), 479 (5), 341 (2), 257 (2), 205 (6), 156 (6).

*4.3.11. 2-Phenylamino-11-phenylsulfanyl-6-isohexenylnaphthazarin (**19a**) and 3-phenylamino-11-phenylsulfanyl-6-isohexenylnaphthazarin (**19b**)*

A solution of thiophenol (41 μl, 0.4 mmol) and **3a** or **3b** (18 mg, 0.04 mmol) in methanol (5 ml) was stirred for 30 h (for **3b**) or 72 h (for **3a**), respectively, under refluxing condition. After the removal of the solvent, the mixture was purified by flash chromatography to afford the compound **19a** (11 mg, 58%) or **19b** (13 mg, 71%), respectively. **19a**: UV λ_{max} (lg ε) (MeOH): 212 (4.4), 269 (4.4), 509 (4.0) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 472 (M^+ + 1), 402, 362. Anal. Calc. for $C_{28}H_{25}NO_4S$: C, 71.31; H, 5.35; N, 2.97. Found: C, 71.38; H, 5.40; N 2.92. **19b**: UV λ_{max} (lg ε) (MeOH): 211 (4.5), 269 (4.4), 522 (4.0) nm; ^{1}H NMR (Table 2); ^{13}C NMR (Table 4); FAB-MS *m/z* 472 (M^+ + 1), 402, 362. Anal. Calc. for $C_{28}H_{25}NO_4S$: C, 71.31; H, 5.35; N, 2.97. Found: C, 71.40; H, 5.38; N, 2.90.

4.4. Cytotoxic activity assay

4.4.1. Cancer cell lines and culture

The cell lines used for the tests were human lung adenocarcinoma line GLC-82, human nasopharyngeal carcinoma cell line CNE2, human liver carcinoma cell line Bel-7402, and human leukemia cell line K-562. They were, respectively, incubated at 37 °C in an incubator with 5% of CO_2. The media used was RPMI-1640 with 10% newborn calf serum (product of New Zealand).

4.4.2. Cytotoxic effect assay using MTT method

The cancer cells in exponential phase were placed in a 96-well plate, containing 1×10^4–2×10^4 per well. After incubating for 6 h the sample solutions were added to each well, respectively, making final concentrations of 0.1–25 μg/ml. The cells were continually incubated for 72 h. The groups treated by RPMI-1640 were used as control groups. To each well was added 10 μl (5 mg/ml) of MTT solution, and the incubation was continued for 4 h. The formazane crystals thus formed were dissolved by 100 μl DMSO. The absorbance was detected in the microplate reader Bio-red 550 model at 540 nm wave length. The IC_{50} values were determined using a Litchfield & Wilcoxon I: confidence limits of ED_{50} pharmcologic calculation system.

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