Antimicrobial Activities of Naphthazarins from *Arnebia euchroma*

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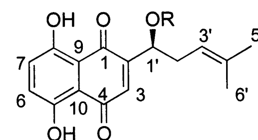
Bioassay-directed fractionation of extract of *Arnebia euchroma* led to the isolation of alkannin (**1**), shikonin (**2**), and their derivatives (**3**−**8**) as the active principles against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). The stereochemistry of α-methylbutyryl alkannin (**8**) is revealed for the first time, and the antimicrobial activity of **8** was compared with its corresponding diastereomer (**9**). The derivatives **3**−**9** showed stronger anti-MRSA activity [minimum inhibitory concentrations (MICs) ranged from 1.56 to 3.13 μg/mL] than alkannin or shikonin (MIC = 6.25 μg/mL). Anti-MRSA activity of derivatives was bactericidal with minimum bactericidal concentration (MBC)/MIC ≤ 2. In a time-kill assay, the bactericidal activity against MRSA was achieved as rapidly as 2 h. The derivatives **3**−**9** were also active against vancomycin-resistant *Enterococcus faecium* (F935) and vancomycin-resistant *Enterococcus faecalis* (CKU-17) with MICs similar to those with MRSA. Aromatic ester derivatives were also synthesized for antimicrobial activity comparison. None of these compounds were active against Gram-negative bacteria tested. Their cytotoxicity was also evaluated on selected cancer cell lines, and they expressed their activity in the range 0.6−5.4 μg/mL (CD_{50}). Our results indicate that the ester derivatives of alkannin are potential candidates of anti-MRSA and anti-VRE agents with antitumor activity.

Due to the emergence of increasing drug resistance, in particular methicillin resistance in staphylococci[1,2] and vancomycin resistance in enterococci,[3,4] much attention is drawn to the search for new antimicrobials from medicinal plants. Plants are known to synthesize various antimicrobial agents, which contain different types of structures.

In search for new antimicrobials against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) from medicinal plants, the extract of *Arnebia euchroma* (Royle) Johnst. (Boraginaceae) was screened and led to the isolation of eight active naphthazarins: alkannin (**1**), its enantiomer shikonin (**2**), and their analogues (**3**−**8**). Although the absolute configurations of alkannin and shikonin have been defined as *S* and *R*,[5] respectively, in many of the previous boraginaceous plant studies, the absolute stereochemistry of naphthoquinone pigments however has not been assigned.[6] It is also known that the ratio between the enantiomers from the same plant may vary depending on the locations.[7] In this study, using HPLC with a chiral phase column,[8] CD, and NMR spectroscopic analyses, we defined the stereochemistry of naphthoquinone compounds where necessary. The stereochemistry of isolated α-methylbutyryl alkannin (**8**) was also revealed for the first time by comparison with the synthesized (*S*)-α-methylbutyryl alkannin (**8**) and (*R*)-α-methylbutyryl alkannin (**9**).

A. euchroma has sweet and bitter tastes and a cold property, acting on the liver and heart channels.[9] It has the functions of promoting blood circulation and counteracting toxicity that is associated with measles, inflammation, and sores. It has traditionally been used in jaundice, burns, eczema, and constipation.[10] It is the major ingredient of the ointment named Tzu-Yun-Kao for the treatment of burns and wounds in Asia.[11] Previous studies indicate



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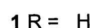
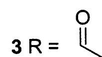
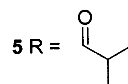
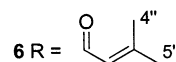
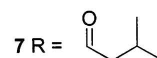
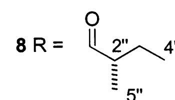


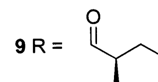
3 R = 

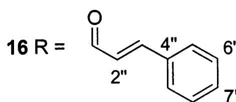
5 R = 

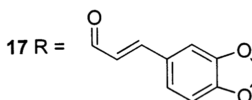
6 R = 

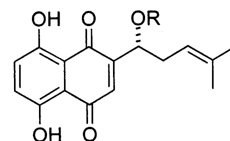
7 R = 

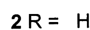
8 R = 

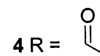
9 R = 

16 R = 

17 R = 



2 R = H 

4 R = 

that alkannin (**1**), shikonin (**2**), and their derivatives possess wound healing,[12,13] antimicrobial,[14−16] antiinflam-

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matory,[17,18] and cytotoxic[19−21] activities. In this paper, we report the isolation and synthesis of naphthazarins that act against MRSA and VRE. The antimicrobial activity of naphthazarins was also compared to other synthesized aromatic ester derivatives (**16**, **17**) and plumbagin, another plant-derived naphthoquinone representative. The cytotoxicity of the alkannin analogues against selected cancer cell lines was also evaluated.

Results and Discussion

The crude ethanolic extract of *A. euchroma* was initially evaluated with the disk diffusion assay and exhibited inhibitory activity against MRSA and VRE. Bioassay-directed fractionation of the extract led to the isolation of a mixture of alkannin (**1**) and its enantiomer shikonin (**2**), a mixture of acetyl alkannin (**3**) and its enantiomer acetyl shikonin (**4**), isobutyryl alkannin (**5**), and a mixture of three alkannin ester derivatives (**6**−**8**). The mixtures of **1** and **2**, and **3** and **4**, were separated by HPLC with a chiral phase column.[8] On the basis of HPLC data, the ratios of **1**:**2** and **3**:**4** were 55%:45% and 42%:58%, respectively. The mixture of **6**−**8** was separated into individual components of β,β-dimethylacryloyl alkannin (**6**), isovaleryl alkannin (**7**), and α-methylbutyryl alkannin (**8**) from repeated silica gel column chromatography. The identification of these bioactive naphthazarins was made by means of spectroscopic methods and comparison with literature data.[22−24] The stereochemistry of C-1′ of the above compounds was confirmed by a chiral column attached on a HPLC and CD spectral methods.[8,25]

Compounds **7** and **8** were isolated for the first time from *A. euchroma*, and the configuration of the α-methylbutyryl moiety of **8** had not been determined in the previous studies. The CD spectrum of **8** showed a positive maximum at 307 nm ($[\theta] = +2334$) and a negative maximum at 356 nm ($[\theta] = -3640$). The CD data of **8** are consistent with the literature report of alkannin, which exhibits $[\theta]_{300\ nm} = +808$ and $[\theta]_{350\ nm} = -3585$.[25] This result indicates that compound **8** is an alkannin analogue. To study the stereochemistry of the α-methylbutyryl moiety of **8**, both (*S*)-α-methylbutyryl alkannin (**8**) and (*R*)-α-methylbutyryl alkannin (**9**) were synthesized. The syntheses were unambiguously carried out by acylating 1′-OH of alkannin with appropriate carboxylic acids in the presence of DCC and DMAP.[26] (*S*)-(+)-2-Methylbutyric acid was commercially available, while (*R*)-(−)-2-methylbutyric acid was prepared from the purchased DL-2-methylbutyryl chloride as described in the Experimental Section.

Diastereomers **8** and **9** showed a difference in the chemical shifts of the two methyl protons on the α-methylbutyryl moiety in their ^{1}H NMR spectral data (Table 1). H-4″ and H-5″ resonate at δ 0.89 and 1.17, respectively ($\Delta\delta = 0.28$), for **8**, and at δ 0.91 and 1.14 ($\Delta\delta = 0.23$) for **9** (Figure 1). In addition, the ^{13}C NMR spectra showed chemical shifts at δ 41.2 (C-2″), 26.6 (C-3″), 11.6 (C-4″), and 16.6 (C-5″) for **8**, whereas the corresponding carbons for **9** appeared at δ 41.0, 26.7, 11.5, and 16.4, respectively (Table 2). Comparison of both ^{1}H and ^{13}C NMR spectra of the natural product and the synthetic analogue showed that the structure of compound **8** could be assigned as (*S*)-α-methylbutyryl alkannin.

Structurally unrelated to naphthoquinone compounds, hopenone-I (**10**),[27] ergosta-4,6,8(14),22-tetraen-3-one (**11**),[28] stigmast-4-ene-3,6-dione (**12**),[29] tetracosyl ferulate (**13**),[30] hexacosyl ferulate (**14**),[31] and octacosyl ferulate (**15**)[32] were also isolated. These compounds were isolated for the first time from this plant; however, they did not exhibit antimicrobial activity. The structures of these compounds were determined by 2D NMR spectroscopic methods. However, in the ^{1}H NMR spectral data of **10**, the chemical shifts of isopropyl methyl protons H-29 and H-30 appeared at δ 0.90 and 0.97, respectively, in contrast to the literature report (both H-29 and H-30 at δ 1.01).[28] ^{1}H and ^{13}C NMR of **10** and **12** are reported in the Experimental Section since they were not reported or the previous data were different from our assignments.

Table 1. ^{1}H NMR Assignments (δ_H (*J*, Hz)) of **8**, **9**, **16**, and **17** in $CDCl_3$[a]

position	**8**	**9**	**16**	**17**
3	6.96 s	6.96 s	7.03 s	7.04 s
6	7.16 s	7.16 s	7.17 s	7.17 s
7	7.16 s	7.16 s	7.17 s	7.17 s
1′	6.01 dd (4.5, 7.5)	6.01 dd (4.5, 7.5)	6.13 dd (4.0, 6.5)	6.10 dd (4.5, 7.5)
2′	2.45 m 2.58 m	2.45 m 2.58 m	2.54 m 2.68 m	2.54 m 2.68 m
3′	5.10 t (7.5)	5.10 t (7.5)	5.16 t (7.5)	5.16 t (7.5)
5′	1.66 s	1.66 s	1.68 s	1.69 s
6′	1.57 s	1.57 s	1.59 s	1.59 s
2″	2.43 m	2.44 m	6.49 d (15.5)	6.31 d (15.5)
3″	1.49 m 1.69 m	1.49 m 1.69 m	7.72 d (15.5)	7.61 d (15.5)
4″	0.89 t (7.5)	0.91 t (7.5)		
5″	1.17 d (7.0)	1.14 d (7.0)	7.54 m	7.01 d (1.5)
6″			7.39 m	
7″			7.39 m	
8″			7.39 m	6.81 d (8.0)
9″			7.54 m	7.01 dd (1.5, 8.0)
OH-5	12.40 s	12.40 s	12.41 s	12.40 s
OH-8	12.57 s	12.57 s	12.60 s	12.58 s
−OCH₂O−				6.01 s

[a] Recorded at 500 MHz.

The cinnamoyl alkannin (**16**) and 3,4-(methylenedioxy)-cinnamoyl alkannin (**17**) were also synthesized for the first time for the antimicrobial activity comparison. ^{1}H and ^{13}C NMR assignments of alkannin derivatives were made by analysis of their COSY, DEPT, HMQC, and HMBC data, and they are shown in Table 1 and Table 2, respectively.

In broth microdilution assays, the ester derivatives (**3**−**9**) (MIC = 1.56−6.25 μg/mL) exhibited considerably more potent anti-MRSA and anti-VRE activities than alkannin or shikonin (MIC = 6.25−50 μg/mL) (Table 3). Noticeably, to act against VRE, a much higher MIC (50 μg/mL) was needed than that against MRSA (MIC = 6.25 μg/mL) for alkannin or shikonin. This may have resulted from the intrinsic resistant nature of *Enterococcus* species. There was no significant difference in antimicrobial activity between the enantiomers **1** and **2** or between **3** and **4**. The antimicrobial activity of (*S*)-α-methylbutyryl alkannin (**8**) was similar to its diastereoisomer (**9**) in that there was usually a 2-fold or less difference in MIC values. The MIC values for **3**−**9** against MRSA are comparable to those of vancomycin, which is often a drug of choice for serious MRSA infections. Therefore, the ester derivatives (**3**−**9**) are potentially useful candidates of anti-MRSA and anti-VRE agents.

Shikonin derivatives were shown to be active against some soil Gram-negative bacteria.[33] However, our results indicated that alkannin/shikonin compounds were not active against *Escherichia coli* and *Pseudomonas aeruginosa* up to the testing concentration of 100 μg/mL. The apparent inactivity against *E. coli* and *P. aeruginosa* is due to the presence of the outer membrane that acts as a selective barrier to antibiotics or toxins. The plant extracts[34,35] and high molecular weight tannins[36] are, in

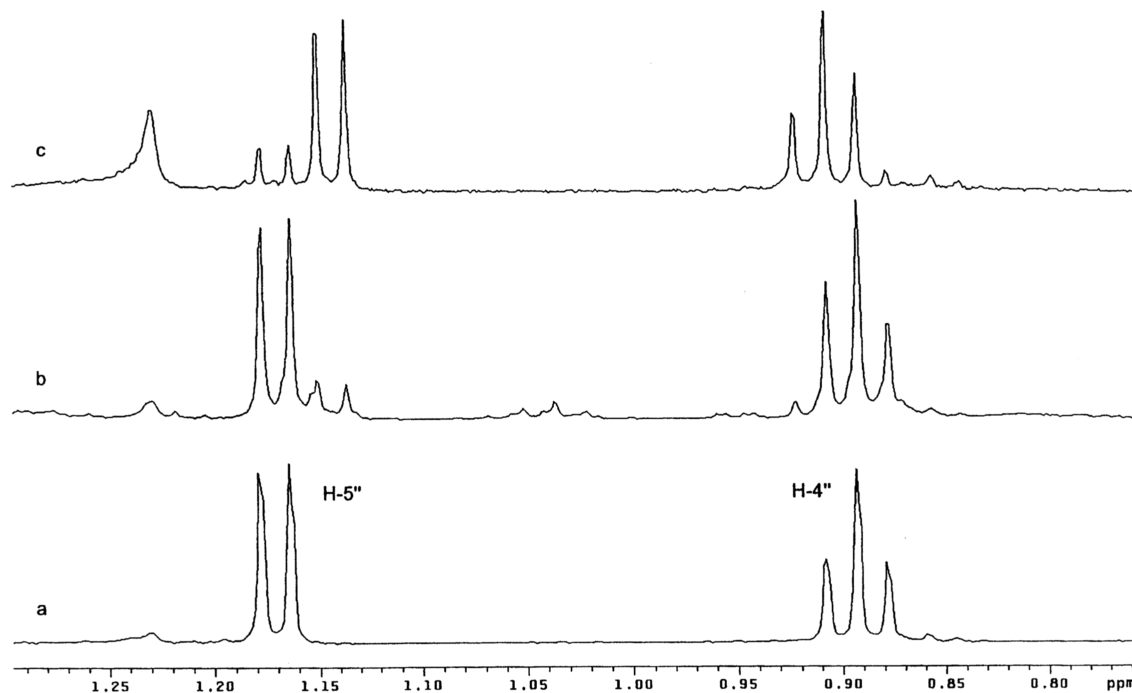

Figure 1. Comparison of ^{1}H NMR spectra of the two methyl protons on the α-methylbutyryl moiety of (a) naturally occurring α-methylbutyryl alkannin (**8**) and with (b) synthetic (*S*)-α-methylbutyryl alkannin (**8**) (with addition of 10% **9** as reference) and (c) synthetic (*R*)-α-methylbutyryl alkannin (**9**) (with addition of 10% **8** as reference).

Table 2. ^{13}C NMR Data of Alkannin Derivatives in $CDCl_3$ (δ_C, ppm)[a]

position	3	5	6	7	8	9	16	17
1	176.6	176.8	177.5	176.7	176.8	176.8	176.8	177.0
2	148.2	148.4	149.0	148.5	148.6	148.6	148.4	148.5
3	131.4	131.3	131.6	131.5	131.4	131.4	131.6	131.6
4	178.2	178.3	179.0	178.2	178.3	178.3	178.3	178.5
5	167.0	166.8	166.2	166.9	166.9	166.9	166.9	166.7
6	132.9	132.8	132.6	132.9	132.8	132.8	132.9	132.8
7	132.7	132.7	132.4	132.7	132.7	132.7	132.7	132.6
8	167.5	167.4	166.8	167.5	167.4	167.4	167.5	167.2
9	111.8	111.8	111.8	111.8	111.8	111.8	111.9	111.9
10	111.5	111.6	111.6	111.6	111.6	111.6	111.6	111.6
1′	69.5	69.0	68.6	69.1	69.0	69.0	69.7	69.5
2′	32.8	32.9	32.9	33.0	33.0	33.0	32.9	32.9
3′	117.7	117.8	118.0	117.9	117.8	117.9	117.8	117.8
4′	136.1	136.0	135.8	136.0	136.0	135.9	136.1	136.1
5′	25.7	25.7	25.7	25.7	25.7	25.7	25.8	25.8
6′	17.9	17.9	17.9	17.9	17.9	17.9	18.0	18.0
1″	169.8	175.8	165.2	171.8	175.4	175.4	165.7	165.9
2″	20.9	34.2	115.3	43.3	41.2	41.0	117.3	115.1
3″		18.8	158.9	25.8	26.6	26.7	146.0	145.7
4″		18.9	20.3	22.3	11.6	11.5	134.1	128.6
5″			27.5	22.4	16.6	16.4	128.2	106.6
6″							129.0	148.4
7″							130.6	149.9
8″							129.0	108.6
9″							128.2	124.8
−OCH₂O−								101.6

[a] The assignments were based on the DEPT, HMQC, HMBC, and COSY experiments (125 MHz).

general, more inhibitory toward Gram-positive bacteria than Gram-negative bacteria. This may be attributed to the fact that Gram-positive bacteria lack the outer membrane but have a much thicker cell wall of peptidoglycan layer, which is not an effective permeability barrier.

The more potent antimicrobial activity of derivatives **3**−**9** may have resulted from acylating 1′-OH of alkannin/shikonin. When the vinylic methyl groups of **6** were replaced by an aromatic group, the antimicrobial activity however was reduced as seen with **16** and **17**. The antimicrobial activity for **1**−**9** was bactericidal with MBC/MIC ratios ≤2 against Gram-positive bacteria. The bactericidal effect of compounds **1**−**9** was achieved at a much lesser MBC/MIC ratio than that of plumbagin (MBC/MIC ≥ 4) (Table 3). Plumbagin is another naphthoquinone representative that is biogenetically derived from *Plumbago zeylanica*.

To study the dynamics of bactericidal effect, the time-kill assays were performed and two typical examples are shown in Figures 2 and 3. Bactericidal activity for compound **3** against MRSA was achieved rapidly at the MIC within 2 h. At half of the MIC, compound **3** appeared as a bactericidal (≥99.9% (3 log) reduction of the original inoculum) within 2 h; however, a gradual regrowth occurred thereafter (Figure 2). Therefore a small percentage of viable cells (≤0.1%) have recovered, and regrowth occurred after 2 h of incubation as previously observed.[37] On the other hand, the bactericidal action was shown to be concentration-dependent for VRE. Compound **7** against VRE CKU-17 (VanB) (Figure 3) and VRE F935 (VanA) expressed a similar bactericidal effect within 4 h at twice the MIC or greater. However, at the MIC, the number of bacteria gradually decreased until 6 h, but regrowth occurred. At half of the MIC, there was an initial bacteriostatic effect followed by a gradual regrowth. In the experiments, the detection threshold was 100 CFU/mL, which is 10 CFU per 0.1 mL aliquot spreading.

Since antibacterial activity of quinolone derivatives is attributed to the inhibition of a special protein, bacterial topoisomerase II (DNA gyrase),[38] the synergism between cell wall inhibitor antibiotics (oxacillin and vancomycin) and alkannin (or its derivatives) was studied using the broth checkerboard method.[39] The combination of antimicrobial agents with different modes of action may enhance and broaden the total biological activity and is less likely to develop resistant mutants. However, no synergism was observed in all the combinations studied, and they only showed an additive effect with the fractional inhibitory

Table 3. Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Naphthazarins (μg/mL)

	S. aureus		MRSA		*E. faecalis*		VRE (F935, VanA)		VRE (CKU-17, VanB)	
compound	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	6.25	12.5	6.25	12.5	50	50	50	50	25	50
2	6.25	12.5	6.25	12.5	50	50	50	50	25	50
3	1.56	1.56	1.56	1.56	3.13	3.13	6.25	6.25	6.25	6.25
4	1.56	1.56	1.56	1.56	3.13	3.13	6.25	6.25	6.25	6.25
5	1.56	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	6.25
6	1.56	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13
7	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13
8	0.78	0.78	1.56	1.56	1.56	3.13	1.56	3.13	1.56	3.13
9	1.56	1.56	3.13	3.13	3.13	6.25	3.13	6.25	3.13	6.25
16	50	100	50	>100	100	>100	25	100	25	100
17	50	100	50	50	100	>100	50	50	50	50
plumbagin	3.13	25	3.13	25	25	100	12.5	50	12.5	50
oxacillin	0.39	0.39	>100	NB[a]	25	50	>100	NB	>100	NB
vancomycin	1.56	1.56	1.56	1.56	3.13	NB	>100	NB	>100	NB
chloramphenicol	NT[b]		NT		6.25	NB	6.25	NB	6.25	NB

[a] NB = nonbactericidal. [b] NT = not tested.

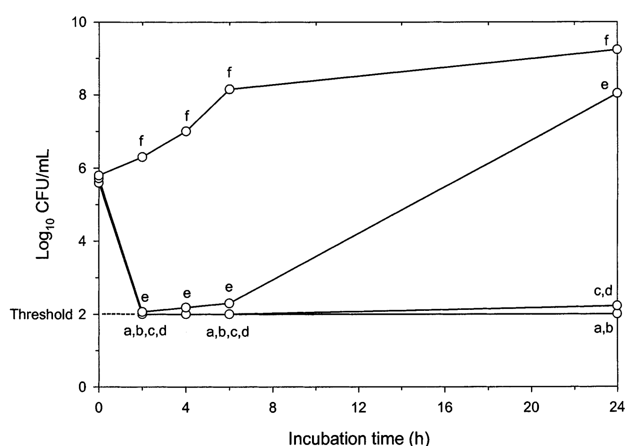

Figure 2. Time-kill assay of acetyl alkannin (**3**) against MRSA: (a) 8 MIC, (b) 4 MIC, (c) 2 MIC, (d) MIC, (e) 1/2 MIC, (f) growth control.

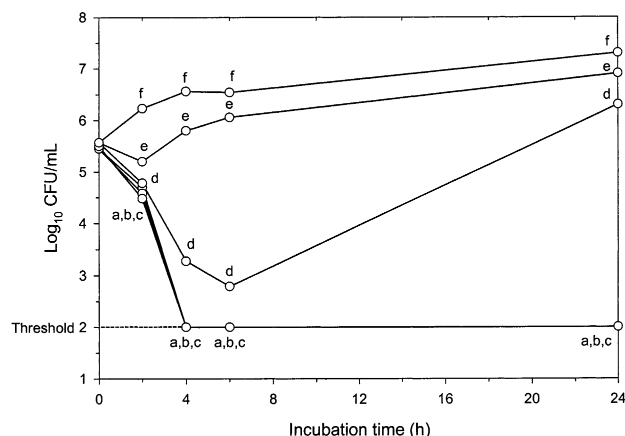

Figure 3. Time-kill assay of isovaleryl alkannin (**7**) against VRE CKU-17: (a) 8 MIC, (b) 4 MIC, (c) 2 MIC, (d) MIC, (e) 1/2 MIC, (f) growth control.

concentration (FIC) index of 1 as shown in Table 5 in the Supporting Information. Although shikonin, which could have been a mixture of alkannin and shikonin,[6] was reported to act synergistically with its analogues,[37] our results indicated that alkannin compounds do not act synergistically with the antibiotics studied.

Alkannin/shikonin compounds exhibit both antimicrobial[14−16] and antitumor[19−21,40] activities, which make them ideal antitumor agents with antibacterial activity. Therefore we evaluated cytotxicity against selected human cancer cell lines of HepG2, Hela, and OVCAR-3, which are not reported thus far. In MTT experiments, alkannin analogues showed moderate cytotoxicities and CD_{50} ranged from 0.6 to 5.4 μg/mL (Table 4). The ester derivatives had slightly higher CD_{50} values than alkannin except for compound **8**.

Table 4. Cytotoxicity of Alkannin and Its Analogues to Human Cancer Cell Lines

	CD_{50} (μg/mL)		
compound	HepG 2	HeLa	OVCAR-3
1	1.0	0.8	0.8
3	2.0	1.5	1.5
5	4.0	5.4	2.0
6	2.6	1.8	1.6
7	2.2	1.7	0.9
8	0.6	0.8	0.6
9	5.0	3.9	1.7
16	3.8	3.6	2.4
17	2.1	1.5	1.0

In summary, the antimicrobial activities of alkannin or shikonin compounds against MRSA and VRE are important since VRE are more commonly associated with patients of MRSA infections. The ester derivatives showed greater potency than alkannin or shikonin except for cinnamoyl esters **16** and **17**. The results of this study showed significant biological activities to warrant further pharmacological studies on alkannin compounds for the development of new antitumor agents with antimicrobial activity.

Experimental Section

General Experimental Procedures. Preparative thin-layer chromatography (PTLC) was carried out on precoated silica gel plates (Merck Art. 113895). Enantiomers of alkannin/shikonin compounds were separated by HPLC equipped with a 520 nm detector and a Chiralcel OD column[8] (4.6 mm i.d. × 250 mm, Daicel Chem. Ind., Ltd.) by a flow rate of 0.7 mL/min. CD spectra were measured on a Jasco J-715 spectropolarimeter. EIMS spectra were measured with the direct insertion probe on a Finnigan GCQ spectrometer at 30 eV. HREIMS data were taken on a Finnigan MAT 95S mass spectrometer. (*S*)-(+)-2-Methylbutyric acid, DL-2-methylbutyric acid, and DL-2-methylbutyryl chloride were purchased from Acros Organics. Other experimental methods were described previously.[41]

Plant Material. The stem bark of *Arnebia euchroma* was purchased from the Cheng-Chi Chinese herbal shop in Taipei in January 2000. A voucher specimen (NRICM 99003) is retained in the National Research Institute of Chinese Medicine, Taipei.

Extraction and Isolation. The whole air-dried plant (10 kg) was extracted with 95% ethanol (50 L) three times at 60 °C for 24 h. The ethanolic extracts were combined and concentrated in vacuo to 2 L. The concentrated extract was suspended in H_2O (10 L) and then extracted with EtOAc (3 × 10 L). After evaporation of EtOAc, the concentrated mixture was mixed with 150 g of silica gel (230−400 mesh). The air-dried mixture was subjected to a chromatographic column (4 × 100 cm) and then eluted with hexane and 20%, 40%, 60%, 80%, and 100% $CHCl_3$/hexane, followed by 5% MeOH/$CHCl_3$ (4 L each). Fractions (500 mL each) were collected, and similar fractions were combined. The combined fractions 15−19, 22−27, and 31−37 showed antimicrobial activity. Fractions 15−19 were rechromatographed by PTLC using 50% $CHCl_3$/hexane as the developing solvent to yield isobutyryl alkannin (**5**)[22] (19 mg) and a mixture of **6**, **7**, and **8** (55 mg). The mixture **6**−**8** was further chromatographed on a silica gel column (1 × 120 cm) with hexane and 3%, 5%, and 8% EtOAc/hexane (600 mL each) as the eluent to yield β,β-dimethylacryloyl alkannin (**6**)[23] (32 mg), isovaleryl alkannin (**7**)[24] (11 mg), and (*S*)-α-methylbutyryl alkannin (**8**) (4 mg). Fractions 22−27 were purified on a silica gel column (1 × 120 cm) with 10% and 20% $CHCl_3$/hexane (600 mL each) as the eluent to yield a mixture of acetyl alkannin (**3**)[22] and acetyl shikonin (**4**)[22] (33 mg). The mixture of **3** and **4** was further separated by HPLC with a chiral phase column using 2.5% 2-propanol/hexane (v/v) as mobile phase to obtain two peaks with retention time of 15.58 and 14.26 min, respectively. Fractions 31−37 were rechromatographed on a silica gel column (1 × 120 cm). Elution with 20%, 25%, 30%, 35%, and 40% $CHCl_3$/hexane (600 mL each) gave hopenone-I (**10**)[27] (10 mg), ergosta-4,6,8(14),22-tetraen-3-one (**11**)[28] (18 mg), stigmast-4-ene-3,6-dione (**12**)[29] (3 mg), tetracosyl ferulate (**13**)[30] (4 mg), hexacosyl ferulate (**14**)[31] (7 mg), octacosyl ferulate (**15**)[32] (5 mg), and a mixture of alkannin (**1**)[22,25] and shikonin (**2**)[22,25] (65 mg). The mixture of **1** and **2** was further separated by a chiral HPLC eluted with 10% 2-propanol/hexane (v/v) to result in two peaks with retention times of 19.92 and 14.80 min, respectively.

Preparation of (*R*)-(−)-2-Methylbutyric Acid. DL-2-Methylbutyryl chloride was esterified with (*S*)-2-hydroxy-3-phenylpropionic acid in the presence of triethylamine and 4-(dimethylamino)pyridine (DMAP). The resulting (*S*)-2-[2-methylbutyryloxy]-3-phenylpropionic acid was treated with oxalyl chloride in 1% DMF/CH_2Cl_2 and converted to (*S*)-*N*-methyl-2-[2-methylbutyryloxy]-3-phenylpropionamide with methylamine in THF. The amide diasteroisomers were separated by silica gel column chromatography to give (*S*)-*N*-methyl-2-[(*R*)-2-methylbutyryloxy]-3-phenylpropionamide, which was subsequently hydrolyzed to yield optically pure (*R*)-(−)-2-methylbutyric acid.[42]

General Syntheses of Alkannin Esters. To a solution of alkannin (20 mg) in anhydrous CH_2Cl_2 (10 mL) were added dicyclohexylcarbodiimide (30 mg), DMAP (4 mg), and the corresponding carboxylic acid (0.07 mmol) under nitrogen atmosphere in an ice bath. The reaction mixture was stirred in the ice bath for 3 h, and the stirring mixture was kept at room temperature overnight. The resulting mixture was diluted with hexane (20 mL) and filtered. The filtrate was concentrated in vacuo and purified by PTLC. The pure alkannin esters were obtained in 8−15% yield.

(*S*)-α-Methylbutyryl alkannin (8): red solid; UV (CH_3CN) λ_{max} (log ϵ) 192 (4.93), 216 (4.51), 277 (3.97), 488 (3.39), 520 (3.47), 559 (3.20) nm; IR (film) ν_{max} 2959, 2922, 1727, 1461, 1272, 1122, 1071 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; EIMS *m*/*z* 372 [M]$^+$ (2), 288 (5), 270 (100), 255 (83), 237 (10), 227 (8), 220 (18); HREIMS *m*/*z* 372.1550 (calcd for $C_{21}H_{24}O_6$, 372.1567); CD (*c* 0.144, CH_3OH) θ (nm) −680 (230), −3625 (250), 0 (288), +2334 (307), 0 (328), −3640 (356), −715 (398).

(*R*)-α-Methylbutyryl alkannin (9): red solid; 2.1 mg; UV (CH_3OH) λ_{max} (log ϵ) 214 (4.45), 275 (3.76), 487 (3.65), 518 (3.68), 559 (3.45) nm; IR (film) ν_{max} 2960, 2923, 1727, 1460, 1271, 1122, 1071 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; CD (*c* 0.130, CH_3OH) θ (nm) −1243 (234), −3503 (250), 0 (288), +2564 (307), 0 (329), −3687 (356), −402 (398).

Hopenone-I (10): colorless solids; mp 195−197 °C (lit.[27] 197−199 °C); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.83 (3H, s, H-28), 0.90 (3H, d, J = 7.0 Hz, H-29), 0.91 (3H, s, H-25), 0.96 (3H, s, H-26), 0.97 (3H, d, J = 7.0 Hz, H-30), 1.01 (3H, s, H-24), 1.03 (3H, s, H-27), 1.07 (3H, s, H-23), 1.28 (1H, m, H-19β), 1.65 (1H, dd, J = 7.0, 12.0 Hz, H-19α), 1.91 (1H, m, H-16ax), 1.93 (1H, m, H-1eq), 2.10 (1H, dd, J = 9.5, 15.5 Hz, H-20β), 2.18 (1H, m, H-20α), 2.26 (1H, dt, J = 14.0, 3.5 Hz, H-16eq), 2.41 (1H, ddd, J = 5.0, 8.0, 16.0 Hz, H-2eq), 2.48 (1H, ddd, J = 8.0, 10.0, 16.0 Hz, H-2ax), 2.62 (1H, sept, J = 7.0 Hz, H-22); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 218.6 (s, C-3), 140.0 (s, C-17), 136.5 (s, C-21), 55.1 (d, C-5), 50.4 (d, C-9), 50.0 (s, C-18), 49.7 (d, C-13), 47.6 (s, C-4), 42.3 (s, C-14), 42.0 (t, C-19), 41.9 (s, C-8), 40.0 (t, C-1), 37.1 (s, C-10), 34.4 (t, C-2), 33.0 (t, C-7), 32.1 (t, C-15), 27.7 (t, C-20), 26.9 (q, C-23), 26.6 (d, C-22), 24.3 (t, C-12), 22.2 (t, C-11), 22.1 (q, C-29), 21.5 (q, C-30), 21.3 (q, C-24), 20.0 (t, C-6, C-16), 19.3 (q, C-28), 16.4 (q, C-26), 16.3 (q, C-25), 15.1 (q, C-27); EIMS *m*/*z* 424 [M]$^+$ (17), 409 (14), 381 (100), 202 (9), 188 (10), 175 (11), 161 (31), 148 (12), 133 (23), 119 (16).

Stigmast-4-ene-3,6-dione (12): colorless solids; mp 57−59 °C (lit.[29] 60−64 °C); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.71 (3H, s, H-18), 0.81 (3H, d, J = 6.5 Hz, H-26), 0.83 (3H, d, J = 6.5 Hz, H-27), 0.85 (3H, t, J = 7.0 Hz, H-29), 0.92 (3H, d, J = 6.5 Hz, H-21), 1.15 (3H, s, H-19), 1.50 (1H, dt, J = 4.0, 13.0 Hz, H-11ax), 1.58 (1H, m, H-15), 1.64 (1H, m, H-11eq), 1.67 (1H, m, H-25), 1.90 (3H, m, H-1ax, H-8, H-16β), 2.02 (1H, dd, J = 12.5, 16.0 Hz, H-7β), 2.10 (1H, m, H-12eq), 1.91 (1H, m, H-16ax), 1.93 (1H, m, H-1eq), 2.44 (1H, m, H-2ax), 2.52 (1H, dt, J = 5.0, 16.0 Hz, H-2eq), 2.67 (1H, dd, J = 4.0, 16.0 Hz, H-7α); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.3 (s, C-6), 199.5 (s, C-3), 161.1 (s, C-5), 125.4 (d, C-4), 56.5 (d, C-14), 55.9 (d, C-17), 51.0 (d, C-9), 46.8 (t, C-7), 45.8 (d, C-24), 42.5 (s, C-13), 39.8 (s, C-10), 39.1 (t, C-12), 36.0 (d, C-20), 35.5 (t, C-1), 34.2 (d, C-8), 34.0 (t, C-2), 33.8 (t, C-22), 29.1 (d, C-25), 28.0 (t, C-16), 26.0 (t, C-23), 24.0 (t, C-15), 23.1 (t, C-28), 20.9 (t, C-11), 19.8 (q, C-27), 19.0 (q, C-26), 18.7 (q, C-21), 17.5 (q, C-19), 12.0 (q, C-29), 11.9 (q, C-18).

Cinnamoyl alkannin (16): red solid; UV (CH_3OH) λ_{max} (log ϵ) 215 (4.56), 278 (4.35), 487 (3.67), 518 (3.70), 558 (3.46) nm; IR (film) ν_{max} 2917, 1713, 1632, 1608, 1571, 1492, 1445, 1240, 1203, 1161, 1035 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; EIMS *m*/*z* 462 [M]$^+$ (1), 249 (3), 270 (98), 255 (100), 237 (12), 227 (17), 191 (23), 175 (66), 145 (42), 117 (19), 89 (19).

3,4-(Methylenedioxy)cinnamoyl alkannin (17): red solid; UV (CH_3OH) λ_{max} (log ϵ) 214 (4.68), 286 (4.18), 330 (4.24), 487 (3.80), 518 (3.83), 560 (3.61) nm; IR (film) ν_{max} 2917, 1715, 1632, 1609, 1575, 1453, 1333, 1263, 1231, 1200, 1157, 768 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; EIMS *m*/*z* 418 [M]$^+$ (2), 253 (5), 270 (98), 255 (100), 237 (17), 229 (23), 199 (20), 171 (13), 147 (56), 131 (21), 103 (18), 87 (24), 77 (8).

Bacteria. The methicillin-resistant *Staphylococcus aureus* (MRSA 8-8S), vancomycin-resistant *Enterococcus faecium* (VRE F935), and vancomycin-resistant *Enterococcus faecalis* (VRE CKU-17) were isolated from sputum, urine, and pus of patients, respectively. MRSA 8-8S and VRE F935 were collected from the clinical microbiology laboratory of the Tri-Service General Hospital, Taipei, while VRE CKU-17 was collected from National Cheng-Kung University Medical College, Taiwan.

Antibacterial Activity. Antibacterial activity of crude extracts or fractions against microorganisms were screened using the paper disk method on Luria-Bertani (LB) agar plates according to the National Committee for Clinical Laboratory Standards (NCCLS).[43] The MICs for the active components were then determined by the broth microdilution method.[44] Briefly, the test compounds were dissolved in DMSO, which was less than 5% for all the final tests. Subcultured microorganisms were diluted as recommended[44] and added to wells containing 0.1 mL of Mueller-Hinton (MH) (*Staphylococcus*) or LB (all other bacteria) broth with 2-fold serial dilutions of the test compounds, to yield the final inoculum of approximately 5 × 10^5 CFU/mL. The actual inoculum was quantitatively measured on an LB agar plate and served as MBC control. The MICs were determined after 24 h of incubation at 35 °C with ambient air. The MIC was defined as the lowest

concentration of the test compound at which no visible growth of the test microorganisms was observed. The experiments were repeated three times. For comparison, oxacillin, vancomycin, and chloramphenicol were included in the experiments as positive controls.

The MBCs were determined by plating out aliquots of 0.1 mL (undiluted) of the MIC testing mixtures showing no growth onto LB agar plates. The colonies were counted after incubating the recovery plates up to 48 h at 35 °C. The MBC was defined as the lowest concentration of the test compound that resulted in reduction of 99.9% (3 log) or more of the original bacterial counts.

Time-kill assays were performed for MRSA and VRE in MH or LB broth solutions containing multiples of the broth microdilution MIC of compounds **3** and **7**, respectively (Figures 2 and 3). The initial inoculum of approximately 5×10^5 CFU/mL was tested. The cultures were incubated at 35 °C, and samples were removed at 0, 2, 4, 6, and 24 h for dilution plating on the recovering LB agar plates. Viability counts were made after 24 h of incubation. The lower limit of detection of colony counts was set to 100 CFU/mL.

Antimicrobial combination studies were performed using the broth checkerboard technique in microtubes.[39] The serial 2-fold dilutions (four dilutions below and 2-fold above MIC) of antibiotics were tested in combination with 2-fold dilutions of alkannin or its derivatives. The MIC, MBC, and synergism were determined after 24 h of incubation.

Cytotoxicity Assay. The cell lines and culture conditions used were the same as previously described.[45] The growth inhibition was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay.[46] Briefly, cells seeded at a density of 5×10^3 cells per well (0.1 mL) were incubated for 24 h, and 2-fold dilutions (0.1 mL) of tested samples were added. At the end of 48 h incubation, the medium was replaced with a fresh medium without fetal calf serum. The cells were then labeled with 10 μL of MTT stock solution (5 mg/mL), and the incubation continued at 37 °C for 2 h. The medium was removed, and 0.2 mL of DMSO was added to each well to dissolve formazan crystals. Cytotoxic dose of 50% growth inhibition (CD_{50} was defined as the drug concentration causing 50% reduction in the net cell increase as compared to positive controls) was then estimated from optical density data at 550 nm. Negative control was also included, and the tests were done in quadruplicate and repeated at least twice.

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Supporting Information Available: Structures of compounds **10** and **12** and the results of antibiotic combination study (Table 5). This information is available free of charge via the Internet at http://pubs.acs.org.

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