Five New Maleic and Succinic Acid Derivatives from the Mycelium of *Antrodia camphorata* and Their Cytotoxic Effects on LLC Tumor Cell Line

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Five new maleic and succinic acid derivatives were isolated from the mycelium of *Antrodia camphorata*. Their structures were determined by various spectroscopic means. Maleimide derivatives **2** and **3** showed appreciable cytotoxic activity against LLC cells.

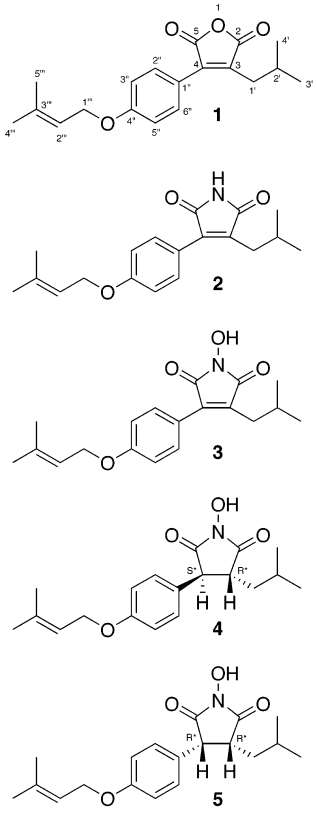
The fruiting body of *Antrodia camphorata* (Polyporaceae, Aphyllophorales) is well known in Taiwan as a traditional Chinese medicine. It grows only on the inner heartwood wall of the endemic evergreen *Cinnamomun kanehirai* (Hay) (Lauraceae) in Taiwan. It is rare and has not been cultivated. The fruiting bodies have been used for treating food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer.[1] Very few biological activity studies have been reported hitherto.

In this paper we describe the structural elucidation of five new maleic and succinic acid derivatives from the mycelium of *A. camphorata* and their cytotoxic effects on LLC tumor cell line.

Results and Discussion

The $CHCl_3$ extract of the mycelium of *A. camphorata* was repeatedly chromatographed on normal and reversed-phase silica gel to afford five new maleic and succinic acid derivatives (**1**−**5**) together with ergosterol peroxide (**6**).[2,3] The structures of the new compounds were determined as follows.

Compound **2** gave yellow needles, mp 110−111 °C, and the molecular formula $C_{19}H_{23}NO_3$ was assigned by HREIMS. The IR spectrum showed an imide carbonyl absorption at 1724 cm^{-1}. The ^{13}C NMR spectrum showed signals of four methyl carbons, two methylene carbons, and one methine carbon in the aliphatic region, as well as one benzene ring, one olefinic group, and two carbonyl carbons. The ^1H NMR spectrum showed the presence of an isobutyl moiety at δ 0.90, 2.06, and 2.51, a 3-methyl-2-butenyloxy moiety at δ 1.76, 1.81, 4.56, and 5.50, and a *para*-substituted benzene moiety at δ 6.95 and 7.50, which was further supported by $^1H-^1H$ COSY and HMQC experiments. Long-range correlations were observed by HMBC as shown in Figure 1. On the basis of the molecular formula and the ^{13}C NMR spectrum, this compound was deduced to contain further CHNO atoms, including one more carbonyl carbon. Thus, this ambiguous part was speculated to be a maleimide group. This structure was then established to be 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrole-2,5-dione by X-ray analysis (Figure 2).



The molecular formula of compound **1** was assigned as $C_{19}H_{22}O_4$ by HREIMS. The IR spectrum revealed a carbonyl absorption of acid anhydride at 1763 cm^{-1}. The ^1H NMR spectrum of **1** was similar to that of **2** and showed the presence of an isobutyl moiety, a 3-methyl-2-butenyloxy moiety, and a *para*-substituted benzene ring. From the HMBC spectrum, **1** was demonstrated to have the same partial structure as **2** (Figure 1), in which the presence of a maleic anhydride group was deduced on the basis of the molecular formula. Compound **1** was consequently defined as 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione.

The molecular formula of compound **3** was assigned as $C_{19}H_{23}NO_4$ by HREIMS. The IR spectrum showed a carbonyl absorption at 1717 cm^{-1}, assignable to a hydroxy

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Table 1. ^{1}H NMR Spectral Data for Compounds **1**−**5** (δ ppm, J = Hz) (500 MHz, $CDCl_3$)

H	1	2	3	4	5
3				2.87 (1H, m)	3.08 (1H, m)
4				3.52 (1H, d, J = 4.0)	4.07 (1H, d, J = 8.0)
1′	2.59 (2H, d, J = 7.0)	2.51 (2H, d, J = 7.0)	2.50 (2H, d, J = 7.0)	1.51 (1H, m)	1.02 (1H, m)
				1.72−1,84 (1H)	1.42−1.48 (1H)
2′	2.12 (1H, sep, J = 7.0)	2.06 (1H, sep, J = 7.0)	2.05 (1H, sep, J = 7.0)	1.72−1.84 (1H)	1.42−1.48 (1H)
3′	0.94 (6H, d, J = 7.0)	0.90 (6H, d, J = 7.0)	0.88 (6H, d, J = 7.0)	0.70 (3H, d, J = 6.5)	0.66 (3H, d, J = 6.5)
4′				0.89 (3H, d, J = 6.5)	0.80 (3H, d, J = 6.5)
2″, 6″	7.50 (2H, d, J = 9.0)	7.50 (2H, d,J = 9.0)	7.50 (2H, d, J = 9.0)	7.07 (2H, d, J = 8.5)	6.96 (2H, d, J = 9.0)
3″, 5″	7.02 (2H, d, J = 9.0)	6.95 (2H, d, J = 9.0)	6.98 (2H, d, J = 9.0)	6.87 (2H, d, J = 8.5)	6.84 (2H, d, J = 9.0)
1‴	4.57 (2H, d, J = 6.6)	4.56 (2H, d, J = 6.5)	4.55 (2H, d, J = 6.9)	4.47 (2H, d, J = 6.5)	4.47 (2H, d, J = 6.5)
2‴	5.50 (1H, brt, J = 6.6)	5.50 (1H, brt, J = 6.5)	5.49 (1H, brt, J = 6.9)	5.47 (1H, brt, J = 6.5)	5.47 (1H, brt, J = 6.5)
4‴	1.81 (3H, s)	1.81 (3H, s)	1.81 (3H, s)	1.79 (3H, s)	1.79 (3H, s)
5‴	1.76 (3H, s)	1.76 (3H, s)	1.76 (3H, s)	1.73 (3H, s)	1.73 (3H, s)

Table 2. ^{13}C NMR Spectral Data for Compound **1**−**5** (δ ppm) (125 MHz, $CDCl_3$)

C	1	2	3	4	5
2	166.4 (s)	171.7 (s)	168.8 (s)	174.8 (s)	175.1 (s)
3	139.8 (s)	138.8 (s)[a])	135.9 (s)[a])	44.6 (d)	40.3 (d)
4	140.2 (s)	139.2 (s)[a])	136.0 (s)[a])	49.8 (d)	47.5 (d)
5	165.4 (s)	171.1 (s)	168.1 (s)	173.2 (s)	173.6 (s)
1′	33.6 (t)	32.8 (t)	33.2 (t)	40.4 (t)	35.3 (t)
2′	27.9 (d)	28.1 (d)	28.4 (d)	25.3 (d)	25.2 (d)
3′	22.7 (q)	22.7 (q)	23.0 (q)	21.3 (q)	21.8 (q)
4′				23.0 (q)	22.4 (q)
1″	119.9 (s)	121.2 (s)	120.8 (s)	127.9 (s)	125.1 (s)
2″, 6″	131.1 (d)	130.9 (d)	131.0 (d)	128.8 (d)	130.2 (d)
3″, 5″	115.1 (d)	114.9 (d)	115.0 (d)	115.4 (d)	115.0 (d)
4″	160.9 (s)	160.1 (s)	160.2 (s)	158.7 (s)	158.7 (s)
1‴	65.0 (t)	64.9 (t)	65.1 (t)	64.1 (t)	64.8 (t)
2‴	118.7 (d)	119.3 (d)	119.2 (d)	119.4 (d)	119.3 (d)
3‴	139.1 (s)	138.6 (s)[a]	138.9 (s)	138.3 (s)	138.4 (s)
4‴	25.2 (q)	25.8 (q)	26.1 (q)	25.8 (q)	25.8 (q)
5‴	18.2 (q)	18.2 (q)	18.5 (q)	18.1 (q)	18.2 (q)

[a] Assignments may be interchangeable.

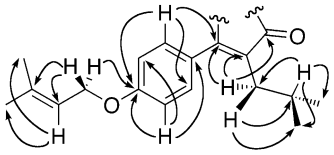

Figure 1. HMBC correlations of compound **2**.

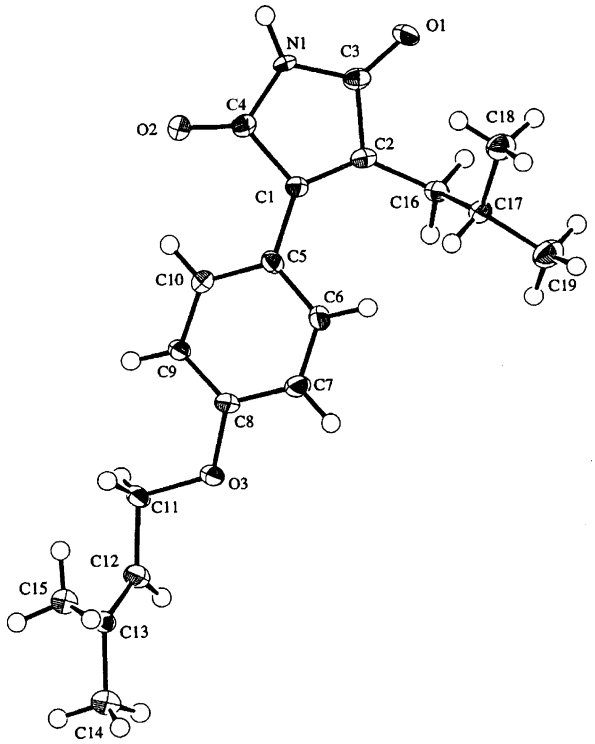

Figure 2. ORTEP drawing of compound **2**.

imide. The ^{1}H and ^{13}C NMR spectra were also similar to those of **1** and **2** and showed the presence of an isobutyl moiety, a 3-methyl-2-butenyloxy moiety, and a *para*-substituted benzene ring. In the HMBC experiment, **3** was shown to have the same partial structure as **2** (Figure 1). Compound **3** contains one more oxygen atom than **2**; therefore, the structure of this compound was defined as 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ol-2,5-dione.

Compounds **4** and **5** had the same R_f values on silica gel in $CHCl_3$−MeOH (10:1) and the same molecular formula by HREIMS ($C_{19}H_{25}NO_4$, found 331.1747 and 331.1766, respectively); however, they could be separated by preparative HPLC. The IR spectrum of both compounds showed a hydroxy imide carbonyl absorption at 1715 cm^{-1}. In the ^{1}H and ^{13}C NMR spectra, both compounds showed the presence of an isobutyl moiety, a 3-methyl-2-butenyloxy moiety, and a *para*-substituted benzene ring, but the isobutyl methylene protons displayed a multiplet and not a doublet as for **1**−**3**. The ^{1}H−^{1}H COSY spectrum indicated that this methylene group is attached to a −CH−CH− unit. The ^{13}C NMR spectra of **4** and **5** exhibited two additional sp^3 carbon signals, replacing two sp^2 carbon signals observed for **1**−**3**. Therefore, **4** and **5** were not *N*-hydroxy maleimides, but rather *N*-hydroxy succinimides, with stereocenters at positions C-3 and C-4 in the succinimide ring. Compounds **4** and **5** were determined to be *trans* and *cis* isomers, respectively, from the coupling constant between H-3 and H-4 (4.0 and 8.0 Hz for **4** and **5**, respectively). No NOE was observed between H-3 and H-4 in the NOESY spectrum of **4**, while appreciable NOE was observed in that of **5**. The optical rotations of **4** and **5** showed +2.5° and +3.0°, respectively, while their CD spectra showed no Cotton effects, suggesting that both **4** and **5** are racemic mixtures. Resolution of these racemic mixtures by HPLC using a chiral column with several solvent systems was unsuccessful. At present, we cannot definitely conclude whether these compounds are optically active compounds or racemic mixtures. Thus, their relative structures were determined as 3*R**,4*S**- and 3*R**,4*R**-1-hydroxy-3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione, respectively.

Isolation of these maleic and succinic acid derivatives from nature is precedented by the earlier report of Aqueveque et al.[4]

The cytotoxic activities of the chloroform extract and isolated compounds were investigated using the LLC (Lewis lung carcinoma) cell line (Table 3). The chloroform extract showed moderate cytotoxic effects with an ED_{50}

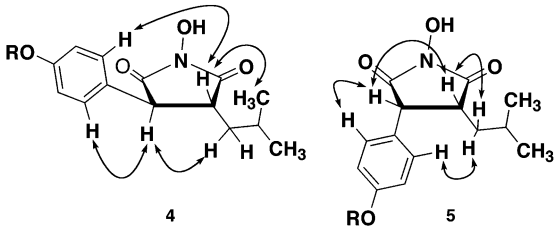

Figure 3. NOE correlations of compounds **4** and **5**.

Table 3. 50% Growth Inhibition (ED_{50}) Values of the $CHCl_3$ Extract and Compounds **1**−**4** from the Mycelia of *A. camphorata* against LLC Cell Line

	ED_{50} (μg/mL)
$CHCl_3$ extract	26.7
1	>20
2	3.6
3	7.5
4	>10
adriamycin[a]	0.14

[a] Positive control.

value of 26.7 μg/mL. Maleic compounds **1** and **4** had no cytotoxic activity, whereas **2** and **3** were found to be cytotoxic to the LLC cell line with ED_{50} values lower than that of the chloroform extract.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanagimoto micro hot-stage melting point apparatus and were uncorrected. Optical rotations were measured with a Jasco DIP-360 automatic polarimeter. UV spectra were measured with a Shimadzu UV-2200 recording spectrophotometer. IR spectra were measured with a Jasco FT/IR-230 infrared spectrometer. ^1H and ^{13}C NMR spectra were measured with a Varian Unity Plus 500 spectrometer. EIMS and HREIMS were measured with a JEOL JMS-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV. Column chromatography was carried out on silica gel BW-820MH (normal phase) and Chromatorex-ODS DM1020T (reversed phase) (Fuji Silysia).

Extraction and Isolation. Powdered mycelia of *Antrodia camphorata* (60 g), from Simpson Biotech Co. Ltd., Taiwan, October 2001, were extracted with $CHCl_3$ (× 3) for 3 h under reflux. The $CHCl_3$ extract (5.3 g) was chromatographed on silica gel eluted with *n*-hexane−acetone (19:1−14:6) and $CHCl_3$−MeOH (1:1) to give nine fractions (Fr. 1−9). Fraction 2 was chromatographed on silica gel to give **1** (8.7 mg). Fraction 4 was chromatographed on normal and reversed-phase silica gel to give **2** (13.6 mg). Fraction 5 was chromatographed on silica gel eluted with *n*-hexane−acetone (8: 2) to give **6** (35.8 mg). Fraction 6 gave **3** (14.6 mg) by combination of normal and reversed-phase silica gel column chromatography. Fraction 7 yielded a mixture of **4** and **5** (4:1) by column chromatography. The mixture of **4** and **5** was subsequently separated by preparative HPLC [column: Tosoh TSK-gel ODS-80T_M (21.5 × 300 mm), mobile phase: CH_3OH−H_2O containing 0.1% TFA (70:30)].

3-Isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]furan-2,5-dione (1): yellow oil; UV (MeOH) λ_{max} (log ϵ) 227 (4.1), 258 (3.9), 275 (3.8), 355 (3.4) nm; IR ($CHCl_3$) ν_{max} 1763 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 314 $[M]^+$ (100), 246 (100), 131 (100); HREIMS *m/z* 314.1523 (calcd for $C_{19}H_{22}O_4$, 314.1518).

3-Isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrole-2,5-dione (2): yellow needles (*n*-hexane−AcOEt); mp 110−111 °C; UV (MeOH) λ_{max} (log ϵ) 230 (4.3), 272 (3.5), 355 (3.7) nm; IR ($CHCl_3$) ν_{max} 1724 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 313 $[M]^+$ (8), 245 (100), 203 (77), 131 (28); HREIMS *m/z* 313.1681 (calcd for $C_{19}H_{23}NO_3$, 313.1678).

X-ray Crystallography of 2.[5] Yellow needles were obtained by crystallization from *n*-hexane−AcOEt and selected for data collection. Crystal data: $C_{19}H_{23}NO_3$; M_r = 313.40; dimensions 0.15 × 0.02 × 0.02 mm; triclinic, space group *P*1 (#2), a = 6.3505(5) Å, b = 12.205(1) Å, c = 12.560(2) Å, α = 64.623(7)°, β = 75.358(4)°, γ = 84.681(5)°, V = 850.9(2) Å^3, Z = 2, D_{calc} = 1.223 g/cm^3, μ(Mo Kα) = 0.82 cm^{-1}, F_{000} = 336.00. Measurement was made on a Rigaku RAXIS-RAPID imaging plate diffractometer with graphite-monochromated Mo Kα (λ = 0.71069 Å) radiation at 93 K. Of the 8950 reflections that were collected, 4745 were unique (R_{int} = 0.108); equivalent reflections were merged. The crystal structure was solved by direct methods (SHELXS86)[6] and refined by full-matrix least-squares. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final indices were R = 0.074, R_w = 0.099, with GOF = 1.06. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.83 and −0.89 e$^-$/Å^3, respectively.

3-Isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1*H*-pyrrol-1-ol-2,5-dione (3): yellow oil; UV (MeOH) λ_{max} (log ϵ) 232.5 (4.3), 296 (3.7), 374 (3.7) nm; IR ($CHCl_3$) ν_{max} 1717 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 329 $[M]^+$ (12), 261 (100), 131 (50); HREIMS *m/z*: 329.1637 (calcd for $C_{19}H_{23}NO_4$, 329.1627).

3*R,4*S**-1-Hydroxy-3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione (4):** colorless oil; $[\alpha]^{23}_D$ +2.5° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 225 (4.3), 275 (3.3), 283 (3.2) nm; IR ($CHCl_3$) ν_{max} 1715 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 331 $[M]^+$ (2), 263 (67), 207 (66), 191 (30), 179 (40), 133 (64), 69 (100); HREIMS *m/z* 331.1747 (calcd for $C_{19}H_{25}NO_4$, 331.1783).

3*R,4*R**-1-Hydroxy-3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]pyrrolidine-2,5-dione (5):** colorless oil; $[\alpha]^{23}_D$ +3.0° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 227 (4.3), 275 (3.4), 283 (3.3) nm; IR ($CHCl_3$) ν_{max} 1715 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 2; EIMS *m/z* 331 $[M]^+$ (1), 263 (45), 207 (50), 191 (75), 179 (30), 133 (100), 69 (92); HREIMS *m/z* 331.1766 (calcd for $C_{19}H_{25}NO_4$, 331.1783).

Ergosterol peroxide (6): colorless needles (*n*-hexane−acetone); mp 165−169 °C (lit.[2] mp 171−174 °C).

Cytotoxic Assays. The in vitro LLC tumor cell assay was carried out by the sulforhodamin B (SRB) method.[7] The 50% growth inhibition (ED_{50}) was calculated by Probit method.[8]

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) Crystallographic data (excluding structure factors) for compound **2** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC205778. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].
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