Anthraquinones, Naphthohydroquinones and Naphthohydroquinone Dimers from *Rubia cordifolia* and Their Cytotoxic Activity

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Further investigation of the roots of *Rubia cordifolia* resulted in the isolation of four new naphthohydroquinones and two naphthohydroquinone dimers, and one known naphthohydroquinone, one naphthoquinone, two anthraquinones and one naphthohydroquinone dimer. The structures of these compounds were established by various chemical and spectroscopic methods including two dimensional NMR techniques. Also, the isolated compounds were submitted to a bio-assay for cytotoxic and antitumor activity.

Keywords *Rubia cordifolia*; cytotoxicity; naphthoquinone; Rubiaceae; anthraquinone; naphthoquinone dimer

Anthraquinones, naphthoquinones, naphthohydroquinones, naphthohydroquinone dimers,[1-8] triterpenes[9,10] and iridoids[11,12] have been isolated from the genus *Rubia*. We wish to describe here the isolation and structure elucidation of anthraquinones, naphthohydroquinones, and naphthohydroquinone dimers from the chloroform extract (antitumor active fraction) of *Rubia cordifolia*, their further investigation and their cytotoxic and antitumor activities, because antineoplastic cyclic hexapeptides have been already isolated from the same fraction.[13-17]

The methanolic extract of the dried roots of *Rubia cordifolia* was partitioned between chloroform and water. The chloroform-soluble fraction was subjected to column chromatography on different adsorbents, including Diaion HP-20, silica gel and RP-18 to give compounds **1**—**11**.

Compounds **1**, **2**, **8**, **9** and **10** were identified as mollugin,[1] 2-carboxymethyl-3-prenyl-2,3-epoxy-1,4-naphthoquinone,[3] 1-hydroxy-2-hydroxymethyl-9,10-anthraquinone,[5] 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone[1] and rubioncolin B[4] by comparing their various physical and spectral data with those in the literature and/or by direct comparison with authentic samples.

Compound **3**: Fine pale yellowish needles, mp 135—137 °C. The mass spectrum (MS) showed a molecular ion peak at *m/z* 512 for $C_{30}H_{24}O_8$, positive fast-atom bombardment mass spectrum (FAB-MS) at *m/z* 512 (M^+) and 551 $[M+K]^+$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a pair of AA′BB′ type aromatic protons (Table I) which were assigned by ^{1}H–^{1}H shift correlation (COSY) spectrum and ^{1}H decoupling experiments. This was very important information for demonstrating that compound **3** consisted of two naphthohydroquinone moieties. The ^{1}H-NMR, broad-band proton decoupling ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra also showed the presence of four multiplet protons for two methylene groups, one methyl and two methoxyl groups, a singlet signal for one proton and two phenolic hydroxyl protons, two ester carbonyl groups, five oxygenated aromatic carbons in twenty-two aromatic carbons and one oxygenated sp^3 quarternary carbons (Tables I and II). The ^{13}C–^{1}H COSY, and two or three bond correlations in

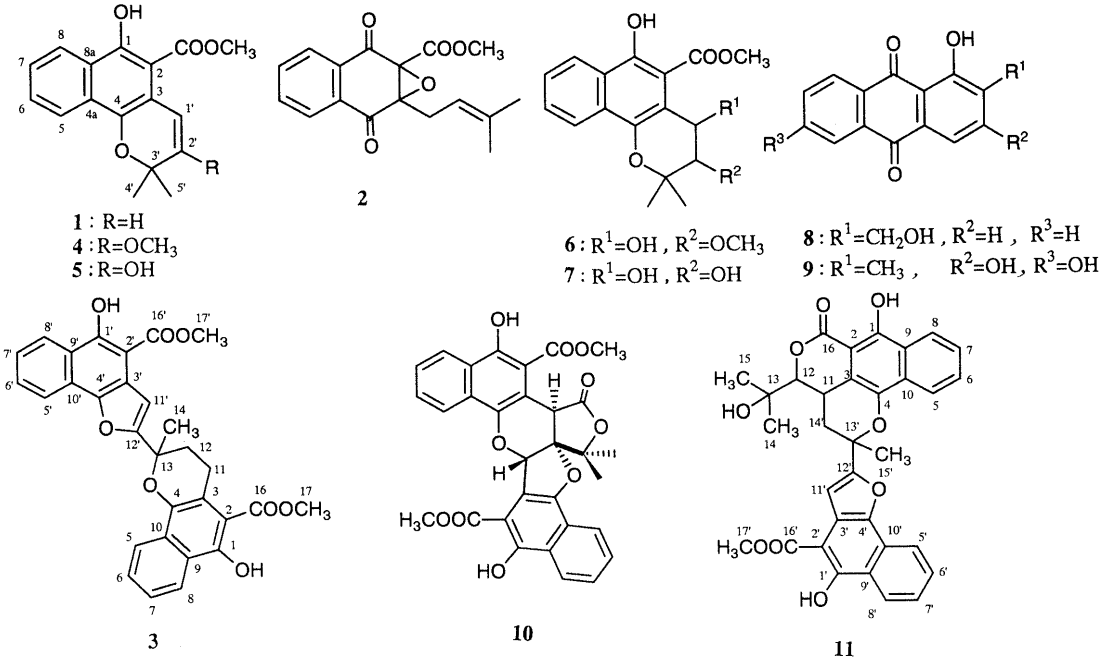
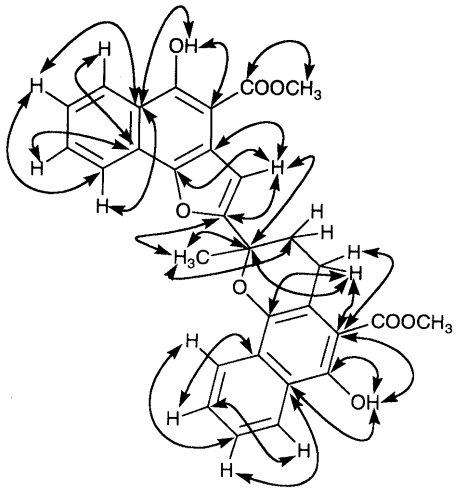


Fig. 1. Isolated Compounds from *Rubia cordifolia*



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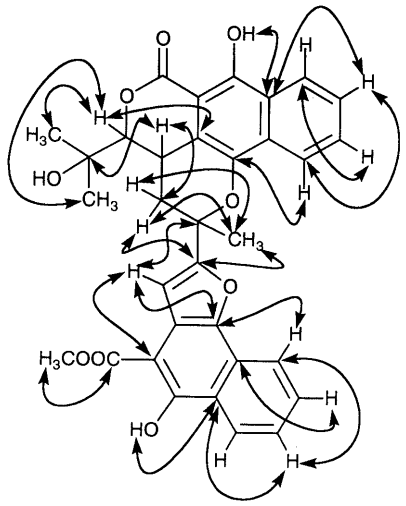

11

Fig. 2. ^{13}C–^{1}H Long Range Correlations of Compounds **3** and **11**

TABLE I. ^{1}H-NMR Spectral Data of Compounds **3** and **11** ($CDCl_3$, δ, J=Hz)

Compd.	HO-1 HO-1′	H-5 H-5′	H-6 H-6′	H-7 H-7′	H-8 H-8′	H-11 H-11′	H-12 —	H-14 H-14′	H-15 H-15′	H-17 H-17′
3	12.24 (s)	8.36 (br d, 8.0)	7.68 (br t, 8.0)	7.55 (br t, 8.0)	8.40 (br d, 8.0)	2.90, 3.16 (m)	2.19, 2.72 (m)	1.91 (s)	—	3.87 (s)
	12.20 (s)	8.18 (br d, 8.0)	7.96 (br t, 8.0)	7.50 (br t, 8.0)	8.44 (br d, 8.0)	6.91 (s)	—	—	—	3.89 (s)
11	11.56 (s)	8.21 (br d, 8.0)	7.63 (br t, 8.0)	7.57 (br t, 8.0)	8.39 (br d, 8.0)	3.44 (dt, 11.0, 6.0)	4.13 (d, 11.0)	1.51 (s)	1.47 (s)	—
	12.27 (s)	8.23 (br d, 8.0)	7.67 (br t, 8.0)	7.52 (br t, 8.0)	8.46 (br t, 8.0)	7.26 (s)	—	2.74 (dd, 13, 6), 2.37 (dd, 13, 11)	1.86 (s)	4.10 (s)

TABLE II. ^{13}C-NMR Spectral Data of Compounds **3** and **11** ($CDCl_3$, δ)

Compd.	1 1′	2 2′	3 3′	4 4′	5 5′	6 6′	7 7′	8 8′	9 9′	10 10′	11 11′	12 12′	13 13′	14 14′	15 15′	16 16′	17 17′
3	156.99	112.79	125.00	141.14	119.81	129.52	126.03	124.09	124.67	129.37	31.87	23.44	74.82	27.08	—	172.03	52.20
	159.30	99.48	120.83	144.08	121.63	130.13	125.21	125.02	122.86	124.96	105.25	159.88	—	—	—	172.98	52.25
11	156.09	99.91	110.16	138.96	122.08	129.95	126.65	124.15	124.99	130.01	29.78	88.71	72.69	24.56	28.56	170.27	—
	159.55	99.40	120.50	144.25	120.07	130.26	125.39	125.21	123.22	124.99	105.41	159.58	74.58	33.75	22.34	172.08	52.54

^{13}C–^{1}H long range COSY (COLOC) experiments were invaluable in assigning the structure and allowing complete proton and carbon signal assignments. The linkage between the two naphthohydroquinone derivatives was determined from the COLOC spectrum as follow; H-11′ was correlated with C-3′, C-4′ and C-13, and H_3-14 was correlated with C-13 and C-12′. The other correlations are exhibited in Fig. 2. From the above description, compound **3** was determined to be that shown in Fig. 1.

Compound **4**: Pale yellowish needles: mp 121—123 °C. The infrared (IR) spectrum showed the presence of hydroxyl (3350 cm^{-1}, br s) and carbonyl groups (1660 cm^{-1}), and the MS at m/z 314 (M^+). The ^{1}H-NMR spectrum ($CDCl_3$) showed an AA′BB′ pattern, phenolic hydroxyl group, carbomethoxyl group, two singlet methyls in addition to one methoxyl and one olefinic proton. The ^{13}C-NMR spectrum (Table IV) showed the same pattern as compound **1** except for the appearance of a methoxyl signal and only one singlet olefinic carbon. The COLOC experiments were used in order to assign the position of the methoxyl group as follow; H-1′ was correlated with C-3, C-4 and C-3′, and the methoxyl protons were correlated with C-2′ and C-3′. The ^{1}H-, ^{13}C- and COLOC spectra showed that the skeleton was in agreement with structure **4**, which was established as 2′-methoxymollugin.

Compound **5**: Pale yellowish needles, mp 143—145 °C, MS at m/z 300 (M^+). It showed the same ^{1}H- and ^{13}C-NMR spectra as compound **4** except for the disappearance of a methoxyl signal at δ 3.18 in the ^{1}H- and at δ 51.31 in the ^{13}C-NMR spectra (Tables III and IV). Therefore, compound **5** was determined to be 2′-hydroxymollugin.

Compound **6**: Fine colorless needles, mp 130—132 °C, MS at m/z 332 (M^+). The ^{1}H-NMR spectrum (Table III) showed an AA′BB′ splitting pattern, one phenolic hydroxyl group, carbomethoxyl group, methoxyl group, two singlet methyls in addition to two doublet protons at δ 3.94 and 4.97 (d, J=6.4 Hz, respectively). The ^{13}C-NMR spectrum showed, in addition to the naphthohydroquinone moiety, two doublet oxygenated carbons at δ 71.71 and 76.73, one singlet carbon at δ 76.92, two singlet methyls and one methoxyl group (Table IV). When mollugin was heated with perbenzoic acid in methanol, compound **6** was obtained as the main product. Therefore, the methoxyl group was presumed to be at C-1′ from knowledge of the reaction

TABLE III. ^{1}H-NMR Data of Compounds **4**, **5**, **6** and **7** ($CDCl_3$)

H	4	5	6	7
1-OH	12.25 (s)	12.25 (s)	12.19 (s)	11.44 (s)
8	8.45 (d, 8.1 Hz)	8.46 (d, 8.1 Hz)	8.35 (d, 8.0 Hz)	8.37 (d, 8.0 Hz)
7	7.51 (t, 8.1 Hz)	7.51 (t, 8.1 Hz)	7.56 (t, 8.0 Hz)	7.56 (t, 8.0 Hz)
6	7.70 (t, 8.1 Hz)	7.70 (t, 8.1 Hz)	7.61 (t, 8.0 Hz)	7.64 (t, 8.0 Hz)
5	8.19 (d, 8.1 Hz)	8.19 (d, 8.1 Hz)	8.19 (d, 8.0 Hz)	8.19 (d, 8.0 Hz)
$COOCH_3$	4.08 (s)	4.09 (s)	4.02 (s)	4.06 (s)
1′	7.01 (s)	7.05 (s)	4.97 (d, 6.4 Hz)	5.06 (d, 6.4 Hz)
2′			3.94 (d, 6.4 Hz)	3.80 (d, 6.4 Hz)
4′	1.76 (s)	1.71 (s)	1.46 (s)	1.40 (s)
5′	1.76 (s)	1.71 (s)	1.49 (s)	1.58 (s)
	3.18 (s)		3.40 (s)	

TABLE IV. ^{13}C-NMR Data of Compounds **4**, **5**, **6** and **7** ($CDCl_3$)

C	4	5	6	7
1	159.43	159.37	155.27	156.52
2	99.34	99.43	104.94	103.96
3	120.42	120.67	110.87	112.96
4	144.26	143.92	141.61	141.07
4a	123.06	122.97	128.62	129.09
5	125.14	124.98	123.80	124.04
6	130.19	130.18	129.14	129.66
7	125.18	125.17	126.24	127.05
8	120.10	119.85	122.36	122.58
8a	125.07	125.11	125.73	125.27
1′	106.63	103.53	76.73	76.18
2′	159.85	162.75	71.71	69.87
3′	73.77	69.69	76.92	a)
4′	25.73	29.23	20.82	19.77
5′	25.73	29.23	24.57	25.57
$\underline{C}OOCH_3$	172.17	172.15	171.98	171.35
$COO\underline{C}H_3$	52.24	52.24	52.50	52.86
OCH_3	51.31		55.69	

a) Overlaped with $CDCl_3$.

mechanism. From all the above mentioned spectral and chemical data, compound **6** was determined to be 1′-methoxy-2′-hydroxydihydromollugin.

Compound **7**: Colorless amorphous powder, MS at *m/z* 318 (M^+). Its IR spectrum is similar to compound **6**. The ^{1}H- and ^{13}C-NMR spectra showed the same pattern as compound **6**, except for the disappearance of the methoxyl signal at δ 3.40 in ^{1}H- and δ 55.69 in ^{13}C-NMR spectra (Tables III and IV). Consequently, compound **7** was confirmed to be 1′,2′-dihydroxydihydromollugin.

Compound **11**: Fine colorless needles, mp 139—141 °C. The FAB-MS showed $[M+K]^+$ at *m/z* 607 and $[M+1]^+$ at *m/z* 569 for $C_{33}H_{28}O_9$. The ^{1}H-NMR signals showed a pair of AA′BB′ type aromatic systems (Table I) which was confirmed by the combination of ^{1}H–^{1}H COSY and proton decoupling experiments. This was also very important information in confirming that compound **11** consisted of two naphthohydroquinone moieties. The ^{1}H-NMR spectrum also showed three singlet methyl, one methoxyl, two phenolic hydroxyl and four coupled aliphatic protons at δ 4.13 (1H, d, J=11.0 Hz), 3.44 (1H, dt, J=11.0, 6.0 Hz), 2.74 (1H, dd, J=13.0, 6.0 Hz) and 2.37 (1H, dd, J=13.0, 11.0 Hz). The broad-band proton decoupled ^{13}C-NMR and DEPT spectra showed the presence of two carbonyl, thirteen quarternary aromatic, nine doublet aromatic and four oxygenated (one methyl, one methine and two quarternary) sp^3 carbons and, in addition, an aliphatic moiety bearing three methyl, one methylene and one methine carbons. The lower field hydroxyl proton signals in the ^{1}H-NMR spectrum also suggested the presence of phenolic compounds whose hydroxyl groups were chelated with the *ortho*-positional carbonyl groups. It was established by the ^{13}C–^{1}H COSY and long range ^{13}C–^{1}H COSY experiments that compound **11** was made up of two naphthohydroquinone moieties, and this facilitated the identification of each proton and carbon signal (Table I and II). The linkage between the two moieties was confirmed from the two or three bond correlation peaks in the COLOC spectrum; from H-12 to C-13, C-14 and C-15, from H-11 to C-13 and C-14′, from H-14′ to C-13′ and C-12′, from H_3-15′ to C-12′, and from H-11′ to C-13′. From above results, compound **11** was assigned the structure shown in Fig. 1.

TABLE V. Cytotoxic and Antitumor Activities of the Isolated Compounds

Compound	Cytotoxic activity (IC_{50}, μg/ml)			Antitumor activity (dose/d)
	V-79	P388	KB cells	Sarcoma 180 ascites
2	1.7	0.12	0.7	+ (5 mg/kg)
3	>30	—	—	—
4	>30	—	—	—
5	>30	—	—	—
6	>30	—	—	− (30 mg/kg)
8	7.8	—	—	− (30 mg/kg)
9	9.7	—	—	− (30 mg/kg)
10	>30	—	—	+ (10 mg/kg)
11	4.7	2.9	1.2	++ (10 mg/kg)

—, the bio-assay was not performed. The effectiveness of Sarcoma 180 A in mice was evaluated by means of the total packed cell volume method. The assessment was carried out as follows, growth ratio (GR %)=(packed cell volume (PCV) of test groups/PCV of control group)×100, GR=0—10% (+++), 11—40% (++), 41—65% (+) and over 66% (−).

The isolated compounds **2**—**11** were submitted to bio-assays for cytotoxic activity against Chinese hamster lung (V-79), human nasopharynx carcinoma (KB) and P388 lymphocytic leukemia cells, and antitumor activity against Sarcoma 180 ascites in mice. From the results summarized in Table V, it is apparent that compounds **2** and **11** showed significant activities in *in vitro* and *in vivo* bio-assays, and compound **10** against Sarcoma 180A only *in vivo*.

Experimental

Melting points (uncorrected) were determined on a Yanagimoto MP-3 micro-melting point apparatus. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were measured with tetramethylsilane as an internal standard on a Bruker AM-400, MS on a Hitachi M-80 or a JEOL DX-300, IR on a JASCO A-302 or Perkin-Elmer 1710 FTIR, and UV on a Hitachi 557. Silica-gel column chromatography was carried out on Wakogel C-200 or Kieselgel 60 in amounts equivalent to 50—100 times the sample amount. Medium pressure liquid chromatography (MPLC) for final purification was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with 10 μm silica-gel as the stationary phase.

Extraction and Isolation The roots of *Rubia cordifolia* used in this experiment were purchased in India. They were identified by Dr. Sang Rae Lee (Institute of Oriental Botanical Resources of Korea). The dry roots (20 kg) were extracted with MeOH–$CHCl_3$ (1 : 1, 60 l×3). The extract was partitioned between H_2O and $CHCl_3$, and then between H_2O and *n*-BuOH. Each partition was repeated 3 times. 20 g of the $CHCl_3$ extract (1.89 kg) was subjected to column chromatography over silica-gel. Elution was started with hexane, then a hexane–AcOEt solvent system and this resulted in the isolation of compounds **1**—**11**. Compounds **1**, **2**, **8**, **9** and **10** were identified by comparing their various physical and spectral data with those in the literature[1-5] and/or by direct comparison with authentic samples.

Compound **3**: Fine pale needles, mp 135—137°C. UV $\lambda_{max}^{CHCl_3}$ nm: 243, 263, 279, 288, 375. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420, 1671, 1642, 1602. EIMS *m/z* (rel. int.): 512 (5, M^+), 282 (60), 250 (100), 198 (11), 165 (20). FAB-MS *m/z*: 512 (M^+), 551 $[M+K]^+$.

Compound **4**: Needle-shaped crystals, mp 121—123 °C. EIMS *m/z* (rel. int.): 314 (M^+, 40), 299 (8), 282 (31), 267 (54), 251 (100), 223 (5), 165 (15), 134 (7). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 1660, 1628.

Compound **5**: Needle-shaped crystals, mp 143—145 °C. EIMS *m/z* (rel. int.): 300 (M^+, 36), 282 (20), 268 (66), 253 (100), 250 (55), 226 (11), 165 (21), 126 (11). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3340 (br), 1658, 1604, 1580.

Compound **6**: Fine colorless needles, mp 130—132 °C. EIMS *m/z* (rel. int.): 332 (M^+, 42), 300 (26), 268 (28), 229 (100), 227 (94), 210 (26), 157 (23), 115 (18). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3455 (br), 1668, 1645, 1582, 1415.

Compound **7**: Colorless amorphous powder. EIMS *m/z* (rel. int.): 318 (M^+), 286, 215, 168, 158, 83 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 1660, 1642, 1582, 1415.

Compound **11**: Fine needle-shaped crystals, mp 139—141 °C. UV $\lambda_{max}^{CHCl_3}$ nm: 243, 262, 268, 278 (sh), 275. FAB-MS *m/z* 607 $[M+K]^+$, and 569 $[M+1]^+$. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3360, 1672, 1640, 1604, 1580.

Bio-assay of Cytotoxic Activity towards V-79, KB and P388 Cells V-79, KB and P388 cells supplied by Dr. S. Tsukagoshi, Japan Foundation for Cancer Research, were maintained in a medium containing kanamycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

V-79 cells (3×10^2 cells/well) were cultured in Corning Disposable 6-well plates containing, 2 ml per well, RPMI-1640 medium (Nissui Pharm. Co. Ltd.) supplemented with 10% fetal calf serum (Whittaker M. A. Bioproducts Inc.). Drug solutions of various concentrations (10 μl) in 0.3% EtOH were added to the cultures on day 1 after the cell-transplantation (day 0). On day 5, the colonies were fixed with 10% HCHO solution (1.5 ml) for 30 min and stained with 0.05% crystal violet (0.75 ml).

KB cells (2×10^4 cells/well) were cultured in Corning Disposable 6-well plates containing, 2 ml per well, Eagle's minimum essential medium (MEM, Nissui Pharm. Co., Ltd.) supplemented with 5% fetal calf serum. Drug solutions of various concentrations (10 μl) in 0.3% EtOH were added to the cultures on day 1 after cell-transplantation. On day 4, the cells were counted with a Coulter counter (Model ZM, Coulter Electronics Ltd.).

P388 cells (2×10^4 cells/tube) were cultured in Falcon tubes containing 2 ml RPMI-1640 medium supplemented with 5% fetal calf serum per tube. Various drug concentrations (10 μl) dissolved in 0.3% EtOH were added to the cultures about 3 h after cell-transplantation. On the 3rd day, the cells were counted with a Coulter counter.

The cytotoxic activity of each drug was assessed by determining its IC_{50} value *i.e.* the concentration of the drug which inhibits cell growth by 50%.

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