The Mutagenic Constituents of *Rubia tinctorum*

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Twenty compounds were isolated from the roots of *Rubia tinctorum* which are used as a commercial source of madder color. Among these compounds, mollugin (1), 1-hydroxy-2-methylanthraquinone (2), 2-ethoxymethylanthraquinone (11), rubiadin (13), 1,3-dihydroxyanthraqunone (14), 7-hydroxy-2-methylanthraquinone (16), lucidin (17), 1-methoxymethylanthraquinone (18) and lucidin-3-*O*-primeveroside (19) showed mutagenicity with *Salmonella typhimurium* TA 100 and/or TA 98. Since the muntagenic compounds isolated are anthraquinone derivatives with the exception of compound 1, structure–mutagenicity relationships of the anthraquinones were also studied. The results suggested that the greatest activity is exhibited by 1,3-dihydroxyanthraquinones possessing methyl or hydroxylmethyl group on carbon 2.

Keywords Rubiaceae; *Rubia tinctorum*; madder color; anthraquinone; natural food colorant; mutagenicity; lucidin; rubiadin; mollugin

Introduction

In recent years natural food colorants have been widely used because of consumer preference for natural products. It is widely believed that natural colorants are safer than synthetic colorants because the long history of use provides proof of safety. But it is also true that some of them are derived from natural sources containing biologically active constituents, that might be harmful. Therefore, we initiated studies to obtain more information on the safety of natural food colorants.

In the course of the studies,[1] mutation tests using *Salmonella typhimurium* TA 98 and TA 100 were performed on various kinds of commercially available natural food colorants. The commercial madder color produced from *Rubia tinctorum* (Rubiaceae) was found to be mutagenic.[2] Yasui *et al.*[3] and Westendorf *et al.*[4] described the mutagenicity of *R. tinctorum* extracts. They reported that lucidin (**17**; 1,3-dihydroxy-2-hydroxymethylanthraquinone), which was first isolated by Barnett and Thomson[5] from this plant and found to be mutagenic by Brown and Dietrich,[6] was the active substance. However, we postulated that the mutagenicity of the madder color may be due to a number of compounds, because some studies[7] on chemical constituents in *R. tinctorum* have revealed the presence of various quinonyl compounds in addition to lucidin. Other studies[8] have revealed that some quinonyl compounds are genotoxic in both prokaryotic and mammalian systems. Therefore we separated the mutagenic constituents in *R. tinctorum* with the aid of mutagenicity testing of chromatographic fractions. Structural identification of active compounds was done by spectroscopic techniques.

Materials and Methods

Melting points were determined on a Yanagimoto micro hot-stage melting point apparatus and are uncorrected. Ultraviolet-visible (UV-VIS) spectra were taken on a Shimadzu UV-240 spectrometer. Infrared (IR) spectra were taken on a JASCO A-102 or a JEOL JIR-3510 spectrometer. Proton or carbon-13 nuclear magnetic resonance (^{1}H- or ^{13}C-NMR) spectra were measured with tetramethylsilane as internal standard with one or more of the following instruments: JEOL FX-200, JEOL GSX-500, Varian Gemini-300, or Varian VXR-400S spectrometer. The following abbreviations are used: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; br, broad; Ar, aromatic group. Mass spectra (MS) were measured on a JEOL JMS-DX-300 spectrometer. High performance liquid chromatography (HPLC) was performed with a JASCO 800 LC system with a Shimadzu C-R3A chromatopac integrator or a Shimadzu LC-6A system with a Shimadzu C-R4AD chromatopac integrator. Photodiode-array detection on HPLC was performed with the JASCO 800 LC system equipped with a Shimadzu SPD M6A detector. All materials, unless otherwise specified, were of reagent grade or the highest grade available from commercial sources.

Plant Materials The roots of *R. tinctorum* imported from Iran were kindly supplied by San-Ei Co. (Osaka).

Mutation Test *Salmonella typhimurium* TA 98 and TA 100 used as tester strains were kindly supplied by Dr. T. Noumi of this Institute. Polychlorinated biphenyl induced rat liver S9 mixture was provided by Kikkoman Co. (Noda in Chiba Prefecture). The test sample was dissolved in 0.1 ml dimethyl sulfoxide (DMSO). The assays were carried out according to the pre-incubation method described by Yahagi *et al.*[9] In the absence of other notations, the mutagenicity was ranked as "+++", "++" and "+" for activities of ten or more, five or more, and two or more times the spontaneous mutation frequency, respectively. A test level of 300 μg/plate was used unless otherwise specified. If a cytotoxic effect was observed at the level of 300 μg/plate or inadequate quantities were available for the level of 300 μg/plate, then 30 μg/plate was used for the evaluation of mutagenicity. The results of mutation tests utilizing TA 98, TA 98 with the S9 mixture, TA 100, and TA 100 with the S9 mixture are shown in brackets from the left. The dose dependency studies were done with compounds that were available in sufficient quantities *i.e.*, compounds **1**, **2**, **11**, **13**, **17**, **18**, and **19**.

Isolation and Identification of Substances Dried roots of *Rubia tinctorum* (9.2 kg) were successively extracted with the chloroform, methanol and water to afford dried material weighing 126, 900, and 1853 g, respectively. The preliminary mutation tests for these mixtures were carried out at a level of 500 μg/plate with TA 98 and TA 100 strains with and without the S9 mixture. The results of the mutation tests were as follows: the chloroform extract was ranked as +++ except no activity was observed in the case of TA 100 without the S9 mixture. The methanol extract was ranked as + except no activity was observed with TA 98 without the S9 mixture. The water extract exhibited no mutagenic activity.

Since the chloroform and methanol extracts showed appreciable mutagenicity, both extracts subjected to initial fractionation by chromatography with Wako gel C200 or Diaion HP-20. The fractions were further separated by Merck Kiselgel 60 Art. 9385 (SiO_2), Merck LiChroprep Si 60 Art. 9390 (SiO_2), oxalate treated Merck Kieselgel 60 Art. 9385 (SiO_2 [H^+]), Pharmacia Sephadex LH-20 (LH-20), and/or HPLC as shown in Charts 1—3. The HPLC columns used in the separations were a Shim-pack CLC-ODS (5 μm, 6 × 150 mm) (ODS-HPLC), a Shim-pack Prep-ODS (15 μm, 20 × 250 mm) (ODS-HPLC), a Shim-pack CLC-SIL (5 μm, 6 × 150 mm) (Si-HPLC) and a Dynamax Macro Si (8 μm, 21.4 × 250 mm) (Si-HPLC).

Authentic samples of mollugin (**1**) 1-hydroxy-2-methylanthraquinone (**2**) and 1,3-dihydroxy-2-ethoxymethylanthraquinone (**11**) were kindly supplied by Prof. H. Itokawa of Tokyo College of Pharmacy. Authentic tectoquinone (**3**) and alizarin (**12**) were purchased from Wako Co. (Osaka). Authentic nordamnacanthal (**5**) and lucidin (**17**) were kindly supplied by Prof. Y. Hirose of Kumamoto University. Authentic scopoletin (**15**) was kindly supplied by Prof. Y. Ebizuka of the University of Tokyo. Authentic lucidin-3-*O*-primeveroside (**19**) was the kind gift of Prof. H. Inoue of

Kyoto University. Authentic ruberythric acid (**20**) was purchased from Funakoshi Co. (Tokyo).

Lapachol methylether (**4**): Yellow oil. UV λ_{max}^{MeOH} nm: 252, 278, 333, 381. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.70 (3H, s, $-CH_3$), 1.73 (3H, s, $-CH_3$), 3.31 (2H, d, J=7.7 Hz, $-CH_2-$), 3.95 (3H, s, $-OCH_3$), 5.09 (1H, t, J=7.7 Hz, $-CH_2-CH=$) 7.62—7.84 (2H, m, C6-H, C7-H), 7.99—8.22 (2H, m, C5-H, C8-H). IR ν_{max}^{KBr} cm^{-1}: 1669, 1645, 1598.

Hexadecyl, octadecyl and icosyl ferulate (**7**—**9**): The fraction D in Chart 1 afforded a mixture of long chain alkyl esters of ferulic acid (**7**—**9**) after separation by repeated Sephadex LH-20 chromatographies. The ^{1}H-NMR (200 MHz, $CDCl_3$) data for the mixture is as follows. δ: 0.88 (3H, br t, J=4.3 Hz, $-CH_3$), 1.1—1.4 (about 30H, s, $-CH_2-$), 1.5—1.8 (2H, m, $-O-CH_2-CH_2-$), 3.93 (3H, s, $-OCH_3$), 4.19 (2H, t, J=6.6 Hz, $-O-CH_2-$), 5.85 (1H, s, -OH), 6.28 (1H, d, J=17.0 Hz, $=CH_2-CO-$), 6.91 (1H, d, J=8.7 Hz, C5(Ar)-H), 7.04 (1H, d, J=2.9 Hz, C2(Ar)-H), 7.61 (1H, d, J=17.0 Hz, $Ar-CH_2=$), 7.70 (1H, dd, J=8.7, 2.9 Hz, C6(Ar)-H). Part of the mixture was further separated by HPLC with a YMC-5 RP8 (5 μm, 20 × 250 mm) column, with detection at 254 nm. A solvent consisting of acetonitrile–acetic acid (97 : 3) at a flow rate of 1.4 ml was used to give three fractions. Electron impact MS (EI-MS) of each fraction showed molecular ion (M^+, m/z) of 418, 446 and 474, corresponding to hexadecyl ferulate (**7**), octadecyl ferulate (**8**) and icosyl ferulate (**9**), respectively. In addition, the EI-MS of the earliest fraction showed trace m/z=444 ion peak corresponding to octadecenyl ferulate.

1-Hydroxy-2-methoxyanthraquinone (**10**): Yellow needles from methanol (mp 233—234 °C). UV λ_{max}^{EtOH} nm: 223, 250, 425. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 4.02 (3H, s, $-OCH_3$), 7.18 (1H, d, J=8.4 Hz, C3-H), 7.60—7.85 (2H, m, C6-H, C7-H), 7.88 (1H, d, J=8.4 Hz, C4-H), 8.20—8.35 (2H, m, C5-H, C8-H), 13.06 (1H, s, -OH). EI-MS m/z (rel. int., %): 254 (M^+, 100), 236 (10), 225 (46), 211 (21), 208 (14). The authentic compound **10** was prepared by methylation of alizarin with diazomethane, in the absence of catalysts at room temperature.

Rubiadin (**13**): Yellow needles from ethanol (mp 300—303 °C). UV λ_{max}^{EtOH} nm: 245, 278, 412. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.10 (3H, s, $-CH_3$), 7.28 (1H, s, C4-H), 7.86—7.94 (2H, m, C6-H, C7-H), 8.12—8.22 (2H, m, C5-H, C8-H), 11.25 (1H, br s, C3-OH), 13.12 (1H, s, C1-OH). EI-MS m/z (rel. int., %): 254 (M^+, 100), 226 (10). IR ν_{max}^{KBr}cm^{-1}: 3390, 1655, 1618, 1580.

Xanthopurpurin (**14**): Yellow needles from methanol (mp 269—270 °C). UV λ_{max}^{EtOH} nm: 246, 284, 415. ^{1}H-NMR (300 MHz, acetone-d_6) δ: 6.67 (1H, d, J=2.3 Hz, C2-H), 7.28 (1H, d, J=2.3 Hz, C4-H), 7.88—7.94 (2H, m, C6-H, C7-H), 8.22—8.34 (2H, m, C5-H, C8-H), 12.90 (1H, br s, C3-OH), 13.25 (1H, s, C1-OH). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 185.9, 181.9, 165.5, 164.7, 135.0, 134.7, 134.5, 133.0, 132.9, 126.8, 126.3, 109.4, 108.4, 107.7. EI-MS m/z (rel. int., %): 240 (M^+, 100). IR ν_{max}^{KBr} cm^{-1}: 3380, 1672, 1637, 1589.

1,3-Dihydroxy-2-methoxymethylanthraquinone (**18**): Yellow needles from methanol (mp 170—171 °C). UV λ_{max}^{EtOH} nm: 246, 282, 417. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.58 (3H, s, $-OCH_3$), 4.93 (2H, s, $-CH_2-$), 7.30 (1H, s, C4-H), 7.74—7.81 (2H, m, C6-H, C7-H), 8.24—8.23 (2H, m, C5-H, C8-H), 9.39 (1H, s, C3-OH), 13.29 (1H, s, C1-OH). EI-MS m/z (rel. int., %): 284 (M^+, 9), 252 (100), 196 (22), 168 (13), 139 (20). IR ν_{max}^{KBr} cm^{-1}: 3180, 1673, 1625, 1592.

HPLC Analysis of Extracts HPLC analysis of the chloroform extract for the quantitation of the main constituents was carried out with the solvent system of methanol : 5% aqueous acetic acid (7 : 3) with a Shim-pack CLC-ODS (5 μm, 6 × 150 mm) column at 40 °C with a detection at 280 nm. A flow rate of 1.2 ml/min was used. The chloroform extract contained compounds **1**, **5**, **11**, **12**, **13**, **14**, **16** and **17** at levels of 0.92%, 3.21%, 0.93%, 7.06%, 0.39%, 0.22%, 0.03% and 0.63%, respectively. Quantitative analyses could not be carried out for the other compounds because they were present in trace amounts only or poor peak separation was realized.

HPLC analysis of the mathanol extract for the quantitation of the main constituents was carried out with the same HPLC conditions as those used for the chloroform extract, except the solvent system consisted of methanol : 5% aqueous acetic acid (6 : 4). The methanol extract contained compounds **17**, **18**, **19** and **20** in amounts of 0.30%, 2.26%, 8.43% and 1.93%, respectively.

Structure–Mutagenicity Relationships of the Anthraquinones All of the isolated anthraquinones (**2**, **3**, **5**, **6**, **10**—**14** and **16**—**20**), with the exception of munjistin methylester (**6**) which was present in trace amounts only, were subjected to mutagenicity studies. Purpurin (**21**) and quinizarin (**22**) were purchased from Wako Co. 1,4-Dihydroxy-2-methylanthraquinone (**23**), chrysophanol (**24**), 1,3,5-trihydroxy-2-methylanthraquinone (**25**), damnacantol (**26**), 2-hydroxymethyl-1,3-dimethoxyanthraquinone (**27**), 2-acetoxymethyl-1,3-dihydroxyanthraquinone (**28**), aloeemodin (**30**), damnacantal (**31**) and munjistin (**32**) were kindly provided by Prof. Y. Hirose of Kumamoto University. 2-Hydroxymethylanthraquinone (**29**) was purchased from Aldrich Co. (Milwaukee).

Analysis of Rubiadin Metabolites by S9 Mixture Rubiadin (1.85 mg, **13**) was dissolved in DMSO (1 ml) and S9 mixture (1 ml) was added to the DMSO solution. After the incubation of the mixture at 37 °C for 18 h, the mixture was extracted with three 5 ml portion of chloroform. After the evaporation to dryness a portion of the extract was dissolved in methanol and subjected to analytical HPLC under the same conditions as those described above with a solvent system consisting of methanol : 5% aqueous acetic acid (8 : 2).

Results and Discussion

Isolation and Identification of the Constituents of *R. tincotorum* Commercial food colors typically contain a small amount of extract from a natural source[1c-e] along with much greater amounts of excipients, stabilizers, pH regulators and so forth. Thus, our extractions were limited to the roots of *R. tinctorum*, which are the source of

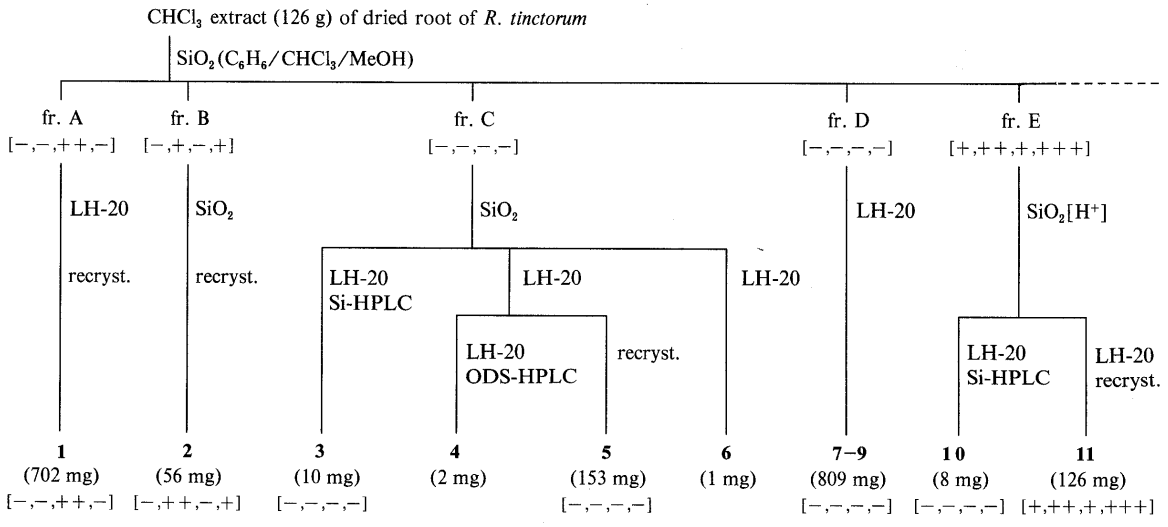


Chart 1. Procedure for Purification of Constituents from Chloroform Extract of *R. tinctorum*

The results of mutation tests utilizing TA98, TA98 with S9 mixture, TA100, TA100 with S9 mixture are shown in brackets reading from left to right. Mutagenicity is ranked as +++, ++ and + for activities of 10 or more, 5 or more and 2 or more times the spontaneous mutation frequency, respectively. A test level of 300 μg/plate is used.

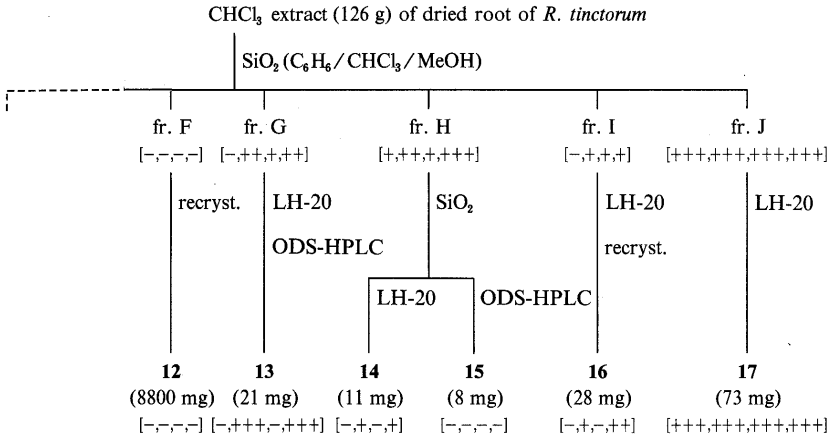


Chart 2. Procedure for Purification of Constituents from Chloroform Extract of *R. tinctorum* (Cont.)

See footnotes for Chart 1.

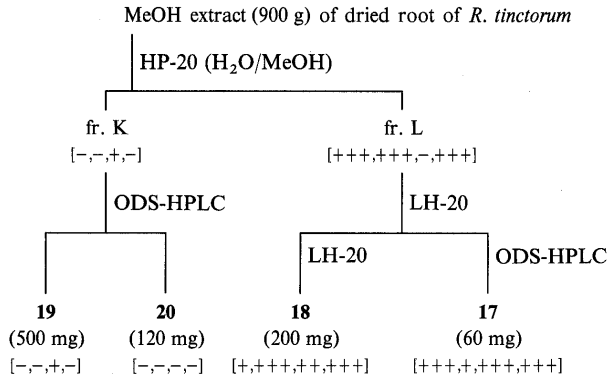


Chart 3. Procedure for Purification of Constituents from Methanol Extract of *R. tinctorum*

See footnotes for Chart 1.

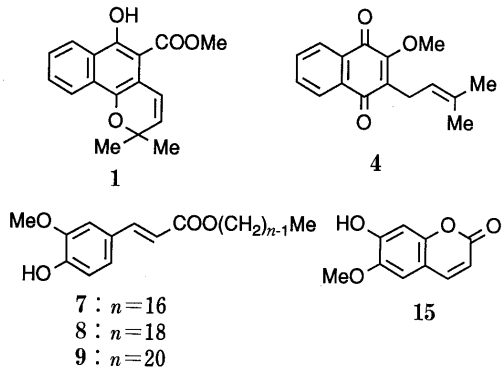


Chart 4. Structures of Isolated Compounds That Are Not Anthraquinones

commercial madder color.

In the preliminary mutation tests it was found that mutation activity was concentrated in the chloroform and methanol extracts. Therefore these extracts were further fractionated by chromatographies. Mutagenicity tests were used to determine the activity of the fractions. The separation schemes that yielded compounds **1**—**20** are shown in Charts 1—3.

Ten of these compounds were characterized by spectroscopy and identified by direct comparison with authentic samples as mollugin (**1**), 1-hydroxy-2-methylanthraquinone (**2**), tectoquinone (**3**), nordamnacantal (**5**), 1-hydroxy-2-methoxyanthraquinone (**10**), 1,3-dihydroxy-2-ethoxymethylanthraquinone (**11**), alizarin (**12**), scopoletin (**15**), lucidin (**17**), lucidin primeveroside (**19**) and ruberythric acid (**20**). Compounds **4**,[10] **13**,[11] **14**[12] and **18**[13] were identified as lapachol methylether, rubiadin, xanthopurpurin and 1,3-dihydroxy-2-methoxymethylanthraquinone by comparison of spectral data with that reported in the literature. Compounds **7**—**9** were first isolated as a mixture and ^{1}H-NMR of the mixture showed signals caused by a methoxy group (δ 3.93), a hydroxy proton that δ 5.85, disappeared on the addition of D_2O, a 1,3,4-trisubstituted benzene ring, a *trans*-substituted vinylic group and long chain alkyl protons. To confirm the position of the methoxy group the irradiation analysis of the signal at δ 3.93 was carried out and the irradiation furnished 10% nuclear

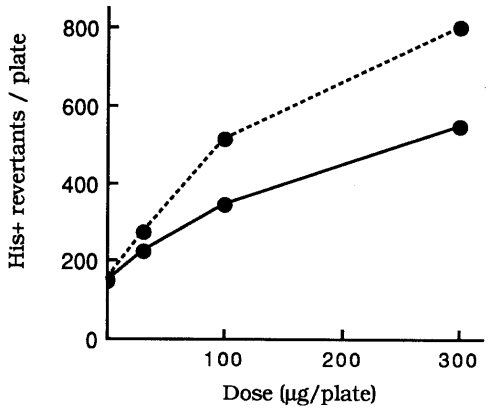
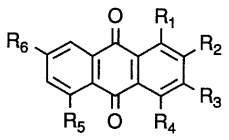


Fig. 1. Number of Revertant Colonies of *S. Typhimurium* TA100 Induced by Mollugin (**1**)

Circles with dotted and solid lines represent the results without and with the S9 mixture, respectively.

Overhauser effect (NOE) on the signal at δ 7.04. These data suggested that the mixture consisted of long chain alkyl esters of ferulic acid. Part of the mixture was further separated by a preparative HPLC to afford hexadecyl ferulate (**7**), octadecyl ferulate (**8**) and icosyl ferulate (**9**)—all of which were identified by EI-MS analyses. Compounds **6** and **16** were munjistin methylester and 7-hydroxy-2-methylanthraquinone, respectively. Structural elucidations of these compounds by spectral and chemical data

TABLE I. Structures and Mutagenicity of Anthraquinones



Compd. No.	Trivial name	R_1	R_2	R_3	R_4	R_5	R_6	Mutagenecity[b] TA 98 −S9	TA 98 +S9	TA 100 −S9	TA 100 +S9	Dose (μg) for evaluation
12	Alizarin	OH	OH	H	H	H	H	−	−	−	−	300
10		OH	OMe	H	H	H	H	−	−	−	−	300
20	Ruberytric acid	OH	G-X[a]	H	H	H	H	−	−	−	−	300
14	Xanthopurpurin	OH	H	OH	H	H	H	−	+	−	+	300
21	Purpurin	OH	OH	H	OH	H	H	−	−	−	−	300
22	Quinizarin	OH	H	H	OH	H	H	−	−	−	−	30
3	Tectoquinone	H	Me	H	H	H	H	−	−	−	−	300
2		OH	Me	H	H	H	H	−	++	−	+	300
16		H	Me	H	H	H	OH	−	+	+	++	300
13	Rubiadin	OH	Me	OH	H	H	H	−	+++	−	+++	30
23		OH	Me	H	OH	H	H	−	+	−	++	30
24	Chrysophanol	H	Me	H	OH	OH	H	−	−	−	−	30
25		OH	Me	OH	H	OH	H	−	++	−	++	30
17	Lucidin	OH	CH_2OH	OH	H	H	H	++	+	+++[c]	+++	30
11		OH	CH_2OEt	OH	H	H	H	+[d]	+++	+++	+++	30
18		OH	CH_2OMe	OH	H	H	H	+	+++	++	+++	30
26	Damnacanthol	OMe	CH_2OH	OH	H	H	H	−	−	+	+	300
27		OMe	CH_2OH	OMe	H	H	H	−	−	−	−	300
28		OH	CH_2OAc	OH	H	H	H	+++	+	+++	++	300
19		OH	CH_2OH	G-X[a]	H	H	H	−	−	+	−	300
29		H	CH_2OH	H	H	H	H	−	−	−	−	300
30	Aloeemodin	H	CH_2OH	H	OH	OH	H	−	−	−	+	300
5	Nordamnacantal	OH	CHO	OH	H	H	H	−	−	−	−	300
31	Damnacantal	OMe	CHO	OH	H	H	H	−	−	−	−	300
32	Munjistin	OH	COOH	OH	H	H	H	−	−	−	−	300
6	Munjistin methylester	OH	COOMe	OH	H	H	H			ND[e]		

a) *O*-Glc^{6}–^{1}Xyl. *b*) See footnotes for Chart 1. *c*) A cytotoxic effect was observed at the dose of 30 μg/plate, so the evaluation of mutagenicity was performed at the dose of 5 μg/plate. *d*) The evaluation of mutagenicity was performed at the dose of 100 μg/plate. *e*) The assay was not performed because inadequate quantities were available.

were previously reported.[1a,b] Our studies have provided the first identification of compounds **16** as a natural product and identified compounds **1**, **3**—**9**, **15**, **18** as constituents of *R. tinctorum*.

Mutagenicity of Isolated Compounds The extracts of *R. tinctorum* yield compounds **1**, **2**, **11**, **13**, **14**, **16**—**19** which are mutagenic. All are quinonyl compounds except compound **1**. Furthermore, HPLC analyses revealed that mutagenic compounds other than lucidin are present in quantities comparable to the amount of lucidin. These data suggested that mutagenicity of the extracts of *R. tinctorum* is not due exclusively to lucidin but is also the result of the contributions of several mutagenic compounds. Mutagenicity of **4** and **6** could not be determined because of the limited availability of these compounds.

Compound **1** is a non-quinonyl mutagenic compound and its mutagenic activity was observed only with TA 100 without the S9 mixture. The dose dependency is shown in Fig. 1. Compound **1** is a direct mutagen, so that the mechanism of mutagenicity by **1** is of interest. It is believed that direct or indirect modification of deoxyribonucleic acid (DNA) by the mutagenic compound triggers the mutation in most cases. Considering the structure of **1**, direct nucleophilic attack by a nucleic base seems to be unlikely. It is conceivable that the phenolic hydroxyl group of **1** would readily react with oxygen and form a phenoxyl radical and a superoxide anion—both of which are a source of oxygen radicals. It is well known that oxygen radicals react on the guanine base at the C-8 position. Further studies are needed to clarify the mechanism.

Leinster reported[14] that treatment of madder with boiling ethanol results in the conversion of **17** to **11**. In our experiments reagent grade chloroform was utilized without distillation; therefore, the isolation of **11** might be due to artifact formation by chemical condensation of **17** with the ethanol in the chloroform. In addition, **18** also might be an artifact since it was only found in the methanol extract.

Structure–Mutagenicity Relationships of the Anthraquinones Anthraquinone compounds are the largest class of naturally occurring quinones and are widely distributed in lower and higher plants; therefore, several research groups are interested in the mutagenicity of these compounds and have studied the structure–mutagenicity relationships.[6,8,15] In their papers, most of the tested compounds were only derived from synthesized reagents and fungal metabolites which were biosynthesized *via* the acetate-malonate pathway. Consequently, systematic studies of alizarin-

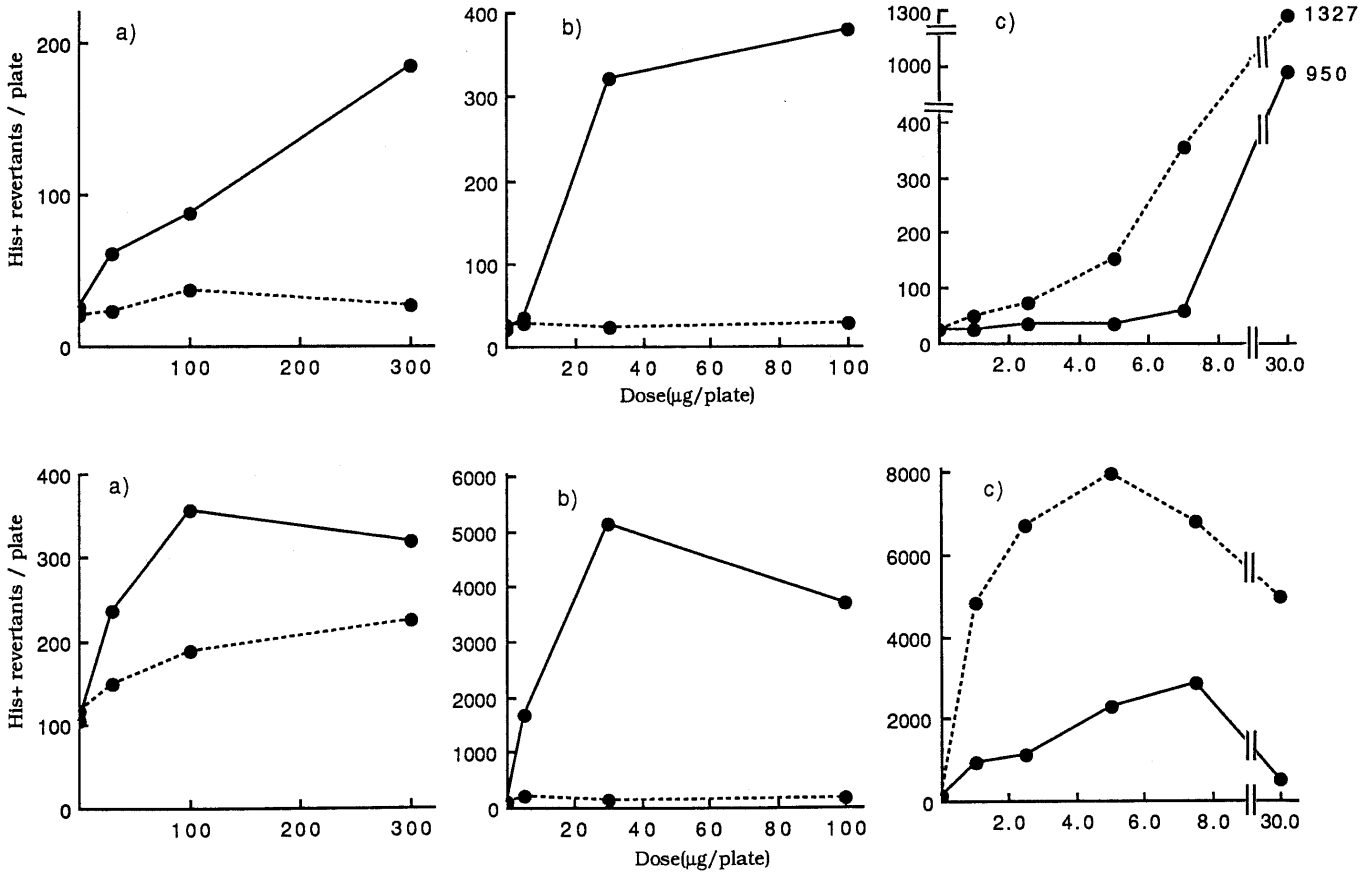


Fig. 2. Number of Revertant Colonies of *S. Typhimurium* TA98 (Upper) and TA100 (Lower) Induced by Anthraquinones

a) 1-Hydroxy-2-methylanthraquinone (**2**), b) rubiadin (**13**), c) lucidin (**17**). Circles with dotted and solid lines represent the results without and with the S9 mixture, respectively.

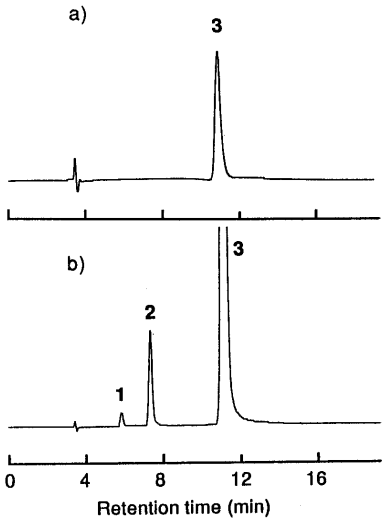


Fig. 3. HPLC Chromatograms of the Solution Containing Rubiadin (**13**) and the S9 Mixture

a) Before the incubation, b) after 18 h incubation. Peak 1, lucidin; peak 2, unknown; peak 3, rubiadin. Upper and lower chromatograms were recorded at 0.08 aufs and 0.01 aufs, respectively.

rubiadin type anthraquinones, which do not possess a substituent group in one benzene ring as is characteristic of Rubiaceae anthraquinone, had not been performed. Therefore, we attempted to define the structural features involved in the mutagenicity of anthraquinones isolated from Rubiaceae plants. The mutagenicity of Rubiaceae anthraquinones (mostly alizarin-rubiadin type) and some related derivatives (**24**, **25**, **27**, **28** and **30**) were summarized in Table I. In addition, the dose dependency of compounds **2**, **13** and **17** were shown in Fig. 2.

Among the 25 anthraquinones studied, lucidin (**17**) and its ω-ether derivatives (**11**, **18** and **28**) showed the most potent mutagenicity in our tests. In contrast, lucidin derivatives (**5**, **31** and **32**) that are oxygenated at hydroxymethyl to aldehyde and carboxylic acid did not exhibit mutagenicity and metabolic activation had no effect. Rubiadin (**13**), which can be regarded as lucidin reduced at the hydroxymethyl group, showed potent mutagenicity only after metabolic activation. In addition, none of hydroxy-2-methylanthraquinones, with the exception of compound **16**, showed mutagenicity without metabolic activation. These data clearly suggest that an oxygenated state of the benzyl position is very important for the mutagenicity of lucidin derivatives and that the oxymethylene group ($-CH_2-O-$) at C-2 position is essential for direct mutagenicity. Furthermore, it might be deduced that ultimate mutagenic compounds derived from metabolic activation of compounds **2**, **13**, **23** and **25** by the S9 mixture are the corresponding 2-hydroxymethylanthraquinones. In fact, we observed, upon HPLC analysis, a small peak at the same retention time of lucidin (**17**) after an 18 h incubation of rubiadin (**13**) with the S9 mixture (Fig. 3).

Substitution of the 1,3-dihydroxyl group of lucidin and rubiadin also had a profound effect on mutagenicity. Namely, modification of the hydroxyl group at 1- and/or

3-positions of lucidin drastically decreased or eliminated the mutagenicity (see compounds **19**, **26**, and **27**) and dehydroxylation of lucidin and rubiadin also led to the significant reduction or disappearance of the mutagenicity (see compounds **2**, **3** and **29**). In addition, xanthopurpurin (**14**) which is a 1,3-dihydroxyanthraquinone, showed weak mutagenicity with metabolic activation; although, other dihydroxyanthraquinones such as alizarin (**12**) and quinizarin (**22**) did not show any effect even with metabolic activation at the same dose.

In contrast to 1,3-dihydroxylation, 4,5-dihydroxylation of 2-methylanthraquinones or 2-hydroxymethylanthraquinones did not have an apparent effect on the mutagenicity (see compounds **3** and **24**, and compounds **29** and **30**). This suggested that not only the number of hydroxyl groups, but also the position of the hydroxyl groups are important for the mutagenicity of rubiadin (**13**) and lucidin (**17**).

We can conclude from the finding described above that 1,3-dihydroxyanthraquinones possessing a methyl or hydroxylmethyl group on carbon 2 show strong mutagenicity in the alizarin-rubiadin type anthraquinones and that the oxygenated state of the benzylic carbons and the number and positions of the hydroxyl groups at 1- and 3-position are important determinations of mutagenicity.

Although; it has been reported[3,4,6,8,15] that compounds **11**, **14**, **17** and **30** show mutagenicity with several strains of *Salmonella typhimurium*, the mutagenicity of compounds **2**, **13**, **16**, **18**, **19**, **23**, **25**, **26** and **28** has not been previously reported. Brown and Dietrich demonstrated[6] that a number of glycosides of mutagenic hydroxyquinones including **19** were nonmutagenic in the standard assay procedure; however, **19** is mutagenic with TA 100 without metabolic activation in our investigation. A detailed report of the mutagenicity of **19** will be presented in the near future.[16]

It is known that some Rubiaceae plants have been used for oriental medicines. Compounds **2**, **11**, **13**, **14**, **17**, **19**, **23** and **26** have been isolated[7,17] from oriental medicinal plants such as Rubiaceae (*Morinda umbellata*, *Rubia cordifolia*, *Hymenodictyon excelsum* and *Damnacanthus indicus*). Therefore, the medicinal use of these extracts, as well as madder colorant, should be re-examined in view of the carcinogenic risk that may exist.

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