Isolation and Characterization of Luteoskyrin and Rugulosin, Two Hepatotoxic Anthraquinonoids from *Penicillium islandicum* Sopp. and *Penicillium rugulosum* Thom

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Luteoskyrin and its stereoisomer rugulosin, two hepatotoxic and carcinogenic hydroxyanthraquinonoids, were extracted from their respective fungus strains, i.e. *Penicillium islandicum* and *Penicillium rugulosum*, cultivated on synthetic Czapek-Dox medium. An improved procedure for purification of luteoskyrin (several grams) and a new method for rapid isolation of large amounts of rugulosin using Sephadex LH 20 as chromatographic adsorbent are related in detail. From the yields obtained, it is concluded that both noxious mycotoxins are present at high concentration inside the respective strain cells (i.e., about 5% with respect to the total dry mycelium). Luteoskyrin and rugulosin are characterized by their spectroscopic properties; in the second part of this work, ir, ^{1}H NMR, uv–visible light absorption, circular dichroism, and mass spectrometric data are reported and discussed from the structural point of view.

Luteoskyrin (Ls) and rugulosin (Rg), two yellow anthraquinone-like pigments (Figure 1), are synthesized by Penicillia contaminating rice, maize, and other cereals, especially in eastern countries, where human malignant and nonmalignant hepatomas are more frequent. These noxious compounds have been the subject of extensive chemical, structural, and biological investigations (Uraguchi et al., 1961a–c; Shibata et al., 1968; Sankawa et al., 1968; Kobayashi et al., 1958a,b, 1968, 1970; Pham Van Chuong et al., 1973, 1974; Toma et al., 1975). Both pigments are known to be highly toxic to mice and rats (Ueno et al., 1971) inducing chronic liver injuries, such as malignant and nonmalignant hepatomas (Enomoto, 1959; Kobayashi et al., 1959; Uraguchi et al., 1961a–c). Luteoskyrin is also toxic to Chang's liver cells, to HeLa cells, and to protozoa such as *Paramecium caudatum* (Kawamoto et al., 1964) and *Tetrahymena pyriformis* (Ueno and Saheki, 1968; Mouton and Fromageot, 1971). This pigment induces chromosome aberrations in cultures of *Ehrlich ascites* (Schachtschabel et al., 1969), and chromosome breakage in cultured human leukocytes (Keutel and Möckel, 1969). Moreover, Ls and Rg have been proved to inhibit both in vitro and in vivo, essential biological functions such as replication, transcription, and DNA repair (Umeda et al., 1963; Umeda, 1964; Schachtschabel et al., 1969; Mouton and Fromageot, 1971; Sentenac et al., 1967; Ruet et al., 1973). In relation to these inhibitory effects, both pigments have been found to form, respectively, two types of complexes with DNA (or synthetic polynucleotides) in the presence of divalent cations. One complex is formed specifically with single-stranded purine polynucleotides, the other one being unspecifically formed with double-stranded polynucleotides or monostranded polypyrimidines (Ueno et al., 1967; Ohba and Fromageot, 1967, 1968; Pham Van Chuong et al., 1976). Such complexes formed in vivo can account at least in part for the toxicity of these pigments.

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The physico- and biochemical investigations carried out in our laboratory required large quantities of both pure pigments and analogues. Since literature sources gave only scanty details on the extraction methods, we had to investigate each step of purification. We describe below the progress made in the extraction of luteoskyrin from *Penicillium islandicum* Sopp., and report an efficient and reliable technique for nearly quantitative extraction of rugulosin from *Penicillium rugulosum* Thom.

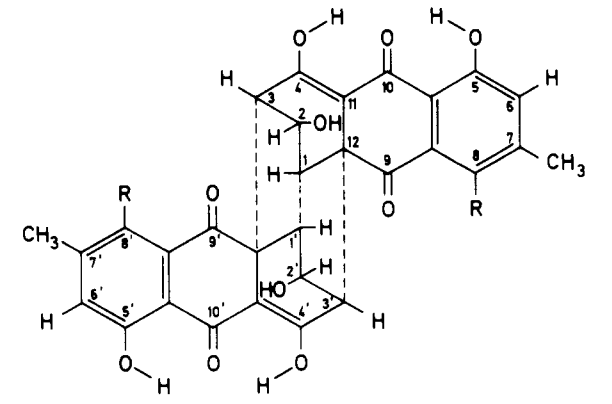
EXPERIMENTAL SECTION

Materials. Spectroscopic grade solvents from Merck were used as such or after further purification as already reported (Pham Van Chuong et al., 1973). Deuterated solvents (99.5% D content) were provided by C.E.N. Saclay.

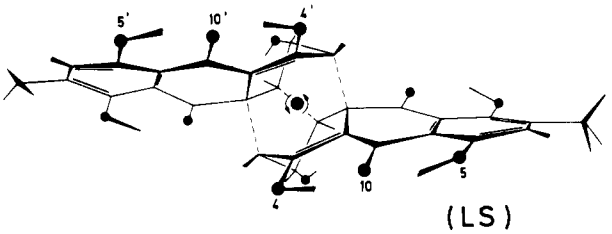
Methods: (1) Isolation of Luteoskyrin. *Penicillium islandicum* Sopp., strain E, kindly supplied by Dr. Tatsuno, was transferred every 3 months on solid Czapek-Dox agar medium (saccharose, 30 g; $NaNO_3$, 2.0 g; KH_2PO_4, 10 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; Difco malt extract, 2 g; agar, 15 g; per liter of distilled water), incubated in the dark at 30 °C for 1 month, and then stored at 4 °C. The mold initially grew colorless on the surface of the slanted tube, then became deeply reddish-brown, and sporulated. When needed, the spores were collected, washed with sterile 0.6% NaCl solution, and shaken with glass beads to make a homogeneous suspension. The suspension was filtered over glass wool and stored at 0 °C before use.

Preparative Culture. Roux flasks (100) containing 250 ml of liquid Czapek medium, i.e. without agar, were sterilized at 120 °C for 20 min; each flask was inoculated with 0.5 ml of the saline sporal suspension. The flasks were stirred and placed in the dark at 30 °C. After a few days mycelium began to develop on the surface. Maximum growth was reached over 40 days. The deeply colored fungus mat was then carefully recovered by decantation, washed with distilled water, and dried at 60 °C for 3–4 days. The yield was 100 g of dry mycelium.

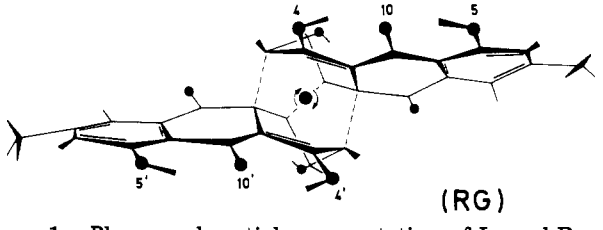
Extraction and Purification. Since luteoskyrin is highly photosensitive (Seo et al., 1969), each step of extraction and purification was performed in a dark room, using an artificial yellow filtered light. The dry mycelium (100 g) was finely powdered, then extracted twice with *n*-hexane or *n*-heptane (500 ml) under reflux for 8 h to remove the



LS : R = OH
RG : R = H



(LS)



(RG)

Figure 1. Planar and spatial representation of Ls and Rg molecules. Dashed lines represent covalent C–C bonds between the two moieties. (•) represents a C_2 symmetry axis.

lipids. Two similar extractions with acetone allowed a nearly quantitative extraction of the pigments from the mold (yield 12 g). The crude extract obtained was further purified by fractionate dissolution in cold (20 °C, fraction 1), warm (40 °C, fraction 2), and hot (70 °C, fraction 3) benzene (1 l. per fraction). An insoluble residue was further obtained (~3 g, fraction 4). Each fraction was analyzed by thin-layer chromatography (TLC) on silica gel (G nach Stahl) acidified by oxalic acid as previously reported (Platel et al., 1968). The plates were developed with the upper phase of the acetone–*n*-hexane–water (6:3:1, v/v/v) mixture. The R_f values were: islandicin (0.88), iridoskyrin (0.85), luteoskyrin (0.46), skyrin (0.37), rubroskyrin (0.27), and lumiluteoskyrin (0.03). Using this procedure, fractions 1 and 2 (total 8 g) were revealed to have approximately the same composition and to contain together almost all the luteoskyrin of the crude extract, whereas fractions 3 and 4 contained mainly skyrin mixed with traces of luteoskyrin and the other pigments of low R_f (<0.4).

Fractions 1 and 2 were thus pooled and chromatographed on columns of unpretreated commercial $CaHPO_4$ (precipitated and for analysis grade, provided by Rhone Poulenc). The glass columns (internal diameter (D) = 7.4 cm; height (H) = 40 cm) were half-filled with benzene and then loaded with the adsorbent (250 g of $CaHPO_4$ per column). Benzene was passed through the columns for 2 h at a flow rate of 1.5 ml/min to obtain a rapid sedimentation of the adsorbent (final height 7.5 cm). Benzene-soluble fractions 1 and 2 (4 g of total pigment per column) were carefully poured on top of the columns. Elution was started at a flow rate of 1.5–1.7 ml/min, using benzene as solvent. After 2 h several colored rings began to separate from bottom to top: orange (islandicin), purple (iridoskyrin), orange, pinkish, yellowish, orange (i.e. four minor unidentified pigments), yellow (luteoskyrin), brownish red (rubroskyrin), brownish red (skyrin), and four other rings (minor and unidentified pigments). Islandicin and iridoskyrin were completely eluted within the first 6 h of elution. Luteoskyrin appeared as a well-separated gold yellow ring measuring up to 4 cm in height after 24 h and began to flow out after 30 h. Almost all the luteoskyrin of the column was eluted after 11 days of such continuous elution (20 l. of benzene; yield of crude luteoskyrin, ~6 g; purity, ~80%). Then skyrin began to flow out mixed with rubroskyrin and traces of luteoskyrin. Elution of these pigments could then be accelerated by using an acetone–benzene (95:5, v/v; 5 l.) mixture to obtain crude skyrin (0.5 g). The column could be decolorized in a few hours by final elution with 1 l. of acetone. Each eluate collected from the columns was analyzed by TLC as already mentioned.

Fractions rich in luteoskyrin of relatively high purity (≥80%) obtained after a single chromatography on $CaHPO_4$ were collected and evaporated to dryness under vacuum. The residue (6 g) was dissolved in a minimum volume of hot acetone and filtered through about 500 mg of Norite active charcoal on a Mitex Millipore LSWP 2 500 filter. Aromatic impurities remained adsorbed on the charcoal. Luteoskyrin recovered from the filtrate was then crystallized in acetone–hexane (2:1, v/v) mixture or in pure acetone. Slow evaporation of the solvent under N_2 atmosphere was first performed to prime the crystallization; the solution was then stored at 4 °C for crystal growth. Yellow rhombohedric crystals were obtained which measured up to 0.05 cm^3 (0.5 × 0.5 × 0.2 cm) after 1 month. They were washed with cold acetone (minimum volume, 1 ml/g of crystal) and *n*-hexane successively, then gently dried under N_2 atmosphere to eliminate excess of solvent. Strong desiccation (over P_2O_5 or under vacuum) was avoided as crystals turned into a dark-yellow highly hygroscopic powder, though no degradation could be observed. Dry crystals were stored in the dark at room temperature. The purity of luteoskyrin was checked by TLC, mass spectrometry, ir, ^1H NMR, uv–visible absorption, and CD spectroscopy. The yield of the pure luteoskyrin was 4% of the initial dry mycelium and about 40% of the crude pigment extract.

(2) Isolation of Rugulosin. Rugulosin and skyrin are the two major pigments synthesized by *Penicillium rugulosum* Thom. Some other minor pigments are also present. Purification is facilitated due to the small number of these pigments and the relatively high concentration of rugulosin. The strain was periodically renewed by transferring on solid Czapek-Dox medium as previously described for *Penicillium islandicum*. For maximum pigment yield, *preparative cultures* were performed on solid medium as follows.

Ninety Roux flasks were filled (at 50 °C) each with 200 ml of a Czapek-Dox solution containing malt extract (2 g/l.) and agar (15 g/l.). The medium was sterilized at 120 °C for 20 min, then cooled to room temperature for solidification, and finally inoculated with the spore suspension

as already reported. The flasks were kept at 30 °C in the dark. After 15 days, the mycelium developed on the surface of the medium and began to color in yellow-green and brown. Maximum growth was reached after 2 months. To recover the thick fungus mat covering the surface of the solid medium, the flasks were heated up to 80 °C in a water bath. The content was carefully poured into a large beaker (10 l.) half-filled with warm distilled water. The supernatant mycelium was recovered, warmed, and washed again to remove traces of agar, and finally dried (yield, 115 g of dry mycelium).

Extraction from the Mycelium. The pigments were tightly complexed to divalent metal ions and cell material, as none could be extracted directly, even under reflux, from the finely powdered mycelium either by ethyl ether or any other solvent. The pigments could be extracted, nearly quantitatively, by simple contact with ether (20 l.) and acetone (12 l.) successively only after strong acidification of the mycelium with 2 N HCl (200 ml). The ether fraction was neutralized first by 1 N NaOH and then washed and concentrated to dryness. The acetone fraction was concentrated to 50 ml and then neutralized and reextracted by ether. Dry residues of pigments were washed again with *n*-hexane to remove lipids and weighed (pigments from ether, 5.3 g; pigments from acetone, 2.1 g). The greenish residual mycelium further extracted twice under reflux by acetone (600 ml) for 4 h gave an additional quantity of 1.2 g of crude pigment (total yield at this step, 7.5%). Each crude extract was analyzed by TLC on silica gel G as previously described. The yellow-brown stain (R_f 0.32) contained skyrin mixed with Rg but well separated from the other minor unidentified pigments (R_f 0.17, 0.25; stained-tail, 0.35–0.50; all orange revealed by magnesium acetate in methanol). The pure Rg control stain gave a slight yellow fluorescence under uv light at 396 nm. However, when skyrin was present with rugulosin, no fluorescence was observed. To confirm the presence of skyrin in rugulosin, TLC was carried out on silica gel (Merck F 254 or G nach Stahl without acidification). Skyrin migrated with the R_f already mentioned (0.32), while Rg remained on the starting line complexed to the adsorbent.

Purification. Skyrin and rugulosin were separated by chromatography of the crude extract on a column of Sephadex LH20 in acid acetone (0.01 N HCl). A set of glass columns (D = 4.2 cm; H = 60 cm) were filled each with 225 g of LH20 gel previously swollen for 2 h in acetone. Gel inside the column was packed by passing slowly (within 2 h) 150 ml of acetone through the column. Final height of the gel at equilibrium was 47.5 cm. The column exit was equipped with a siphon device (Teflon catheter of 2 mm diameter) to prevent further excessive packing of the gel. The horizontal top surface of the gel was preserved by a disk of filter paper. Then 1.8 g of crude extract was solubilized in 75 ml of warm acetone (50 °C). The solution was cooled to room temperature without any precipitation of the pigments and carefully poured on top of the gel. Elution was performed first by using pure acetone as solvent (flow rate, 100 ml/h) until the front of the colored zone reached the third upper part of the column. At this step no definite separate rings could be observed. Elution was then accelerated by using acid acetone as solvent at the same flow rate, and separation in many colored rings began to appear along the column. Four principal bands could be seen from bottom to top, respectively, colored light yellowish green, orange, yellow (Rg), and red (skyrin). Rugulosin started to elute 8 h after the begining of the elution. The most important fraction (1.1 g pigment content) of almost pure Rg (90%) was eluted within 4.5 h by only 450 ml of solvent. An additional amount (125 mg) of impure Rg (20% by TLC) was further eluted over 2.5 h by another 200-ml volume of the same solvent. (The yield of impure Rg was then 68% of the crude extract.)

Fractions containing Rg were evaporated to dryness under vacuum. Hexane-washed residues at this step hardly crystallized as brownish-yellow microcrystals in methanol or acetone, due to the presence of some impurities. Repeated fractional precipitations of the rugulosin were performed by evaporating the liquid phase to the minimum volume (~1 ml) and then discarding the impure supernatant. After five or six similar fractionations microcrystals appeared bright yellow colored. Rg was finally recrystallized in methanol or in an acetone–hexane (2:1, v/v) mixture. Priming was done by slow evaporation of the solvent under N_2 atmosphere. Crystals grew from the solution by further long storing at 4 °C for at least 1 month. The pale yellow needles obtained were washed with hexane and then gently dried and stored at room temperature in the dark. As Ls, Rg must be prevented from strong desiccation, to keep its crystal structure.

Five such columns of LH 20 packed with a total amount of 9.2 g of crude pigments yielded 5 g of pure Rg. Since a maximum loss of 10% is expected during repeated crystallizations, we may conclude that *Penicillium rugulosum* synthesizes about 5% of rugulosin relative to dry mycelium; the Rg content of the mold is about 60% of the whole pigments.

The purity of Rg crystals was checked by mass spectrometry, ir, ^{1}H NMR, uv, and visible absorption and CD spectroscopy.

(3) Conditions of Solubility. Maximum solubility of the dyes was reached by weighing an excess of the crystals and then by finely powdering and dissolving the crystals in the given solvent. When necessary for better solubilization, solutions were warmed (40–50 °C) for at least 30 min and then filtered or centrifuged (15 min, 1500 rpm). All these operations were performed in a glove box under dry N_2 atmosphere. The concentration of the solution was finally checked by uv–visible absorption spectroscopy under standard conditions (see below) using the predetermined molar absorbance. Aqueous solutions useful for biological assays were prepared by direct dissolution of the previously weighed and then finely powdered crystals in a minimum volume of 0.1 M NaOH aqueous solution. Commonly, 1.7 mg of Ls and 1.8 mg of Rg were completely soluble in only 0.2 ml of alkaline solution, giving respective molar ratios of NaOH/Ls $\simeq$6.8 and NaOH/Rg $\simeq$5.8. The alkaline solution was then rapidly diluted with suitable buffer (0.001 to 0.1 M ionic strength Tris, glycine phosphate or cacodylate buffer). Oxalate, citrate, and borate buffers which complex either Ls and Rg were avoided. The final concentration of the solution was checked by uv–visible absorption spectroscopy, using the known ϵ value.

(4) Spectroscopy. *Uv–visible light absorption and CD spectra* were recorded on a Cary 14 (or a Cary 15) spectrophotometer and on a Jouan CD II dichrograph, respectively. The dye crystals were weighed on a CAHN G 2 microelectrobalance (precision better than 1 μg) and then dissolved completely in the given solvent. Various concentrations (10^{-5} to 10^{-4} M) and appropriate path lengths (0.1 to 2 cm) were used in each case. Molar absorbances (ϵ) were then determined with an accuracy better than 97% in the best solvents (such as acetone, dioxane, tetrahydrofuran (THF), and dimethyl sulfoxide (Me_2SO)) and 95% otherwise, by repetitive separate experiments in

each solvent. The error in wavelength was about 0.2 nm. Standard conditions for optical absolute measurements were defined as follows: neutral solvent meant pure and dry solvent; acid solvent was obtained by adding to the dye solution microvolumes (dilution effect smaller than 1‰) of 0.1–1.0 N HCl or concentrated $HClO_4$, until no more spectral change was observed (final acid concentration did not exceed 10^{-2} M); basic solvent was obtained similarly by adding microvolumes of aqueous 0.1 N NaOH to have a molar ratio NaOH/dye approximately equal to 10. Under these conditions both dyes were in the dye^{4-} deprotonated form.

Ir spectra were recorded in the 4000–200 cm^{-1} range on a PE 180 spectrometer with a resolution of 2 cm^{-1}. Ls and Rg suspensions in Nujol or voltalef between CsI plates were placed in a cryostat to permit studies at either room (300 K) or liquid nitrogen (77 K) temperature.

Preparation of LS-d_8 and Rg-d_6. Simple repetitive dissolution of Ls-h_8 or Rg-h_6 in D_2O or in any deuterated organic solvent led to only partial H–D exchange essentially of the hydroxyl protons at the 2,2′ position. Successive and repetitive deprotonation (in basic deuterated solvent) and protonation (in acidified deuterated solvent) were required to obtain a complete exchange of all the hydroxyl groups of the molecule: 150 mg of Ls-h_8 or Rg-h_6 was first strongly dehydrated over P_2O_5 for 12 h or more under vacuum, then dissolved in 2.1 to 2.5 ml of 1 M NaOD. After 1 h, 3 ml of molar DCl was added to precipitate the dye. The residue was washed back to neutral pH with D_2O. After two similar operations, the deuterium content of the dye reached about 85–90% as deduced from the ir spectrum.

The proton NMR spectra were recorded either at 100 MHz using a Jeol PSN-100 spectrometer or at 60 MHz on a Perkin-Elmer R 12 spectrometer. The latter was equipped with a digital signal averager to improve the signal-to-noise ratio when studying diluted samples. Tetramethylsilane (Me_4Si) was used as an internal reference.

Mass analysis was performed on a MS 902 S spectrometer using heptacosafluorobutylamine as reference. The source temperature was about 150–200 °C.

RESULTS AND DISCUSSION

Isolation of Luteoskyrin. Many procedures of purification have been tested by us during the last years to obtain high yields of luteoskyrin in the shortest time. Among them preparative chromatography on columns of $CaHPO_4$ finally appeared to be the best, yet under definite conditions. First, the choice of the parameters H and D of the columns was determinant. The H value should not exceed 7.5 cm in order to prevent prohibitive duration of elution and excessive decrease of the flow rate. The value of H/D = 40 cm/7.5 cm allowed an optimal separation. Indeed the time of elution (3 weeks) was very long but was required to separate the 13 mixed pigments of close structure which were present in the crude extract even after the fractionate dissolution in benzene. Secondly, this time disadvantage was counterbalanced by suppression of the previously recommended activation of the $CaHPO_4$ with H_3PO_4 (Platel et al., 1968). We found that despite a slower elution, direct use of commercial $CaHPO_4$ was the best to keep proper reproducibility, homogeneity, porosity, and dryness of the adsorbent particles, all conditions required for a good chromatographic process. The third important condition was the necessity of loading the columns only with soluble fractions of crude pigments in benzene to avoid initial precipitation on top of the adsorbent.

The long time of elution was also balanced by using a set of columns at the same time, providing a mean quantity of pure luteoskyrin of about 1 g/month. The other advantage was the suppression of repeated chromatography on $CaHPO_4$ which was previously proposed (Shibata and Kitagawa, 1956). After a single chromatography followed by treatment on active charcoal and only one crystallization, we isolated luteoskyrin with a yield of 4% with respect to dry mycelium. This yield seemed to be close enough to the total quantity which was synthesized by the mold. In fact the TLC analysis of the crude extract (obtained after delipidation) revealed, after recovering in a solution of the luteoskyrin spot and subsequent spectrophotometric titation, a luteoskyrin content of maximum 5% in the mold.

We also noted a relationship between the time of culture, which was very long (40 days) and gave rather a poor yield of mycelium (100 g of dry matter for 25 l. of culture broth), and the high content of luteoskyrin in the fungus cells.

Isolation of Rugulosin. A relationship between the time of culture, the rate of mycelium growth, and the high production of Rg was also observed. In fact, the mycelium of *Penicillium rugulosum* grew very slowly even on solid Czapek-Dox and gave after 2 months only 128 g of dry matter for 25 l. of culture broth. Nevertheless, the rugulosin fungus cell content was shown to be unexpectedly high (probably more than 5% of the dry mycelium). Such high concentrations of the pigment in the mold cultivated on synthetic medium were never reported before, probably because of the difficulties of complete extraction.

It would not be surprising to discover now that under natural conditions of culture, i.e. on contamined cereals, for instance, such concentrations of the noxious pigments could be reached.

Another point which is worth discussing is the chromatographic process of purification of rugulosin from the crude extract on Sephadex LH 20. It is well known that molecules containing polar functions such as phenolic or alcoholic OH groups are strongly *adsorbed* on the gel especially by H bonding effect (Brook and Munday, 1970), and consequently do not usually filter in the normal reciprocal order of their molecular weight (Horobin and Gardiner, 1969; Repas et al., 1969). Elution of such compounds occurs later than would be expected from the molecular size and the theory of gel filtration (Brooks and Keates, 1969; Joustra et al., 1967). Standard elution volume actually depends on the number and nature of the polar groups, on the structure, conformation, and bulkiness of the molecule to be separated, and on the nature and acidity of the eluent. Indeed, we observed here that polyhydroxyanthraquinones were strongly adsorbed on LH 20. Elution of such small molecules (mol wt $\simeq$500) is thus considerably delayed, which fact probably accounts for the good separation observed for the pigments in spite of their similar molecular weights.

When elution is made in acid acetone, rugulosin (a near biplanar anthraquinone molecule having a big middle cage; see Figure 1 and Pham Van Chuong et al., 1973) elutes before skyrin (a quasiplanar molecule with six strong intramolecular H bonds; see Figure 2 and Shibata et al., 1955). Because of the acidity of the gel and consequently of the enhanced strength of internal H bonding, the adsorption of the OH groups of the pigments onto the gel is presumably considerably weaker than in neutral acetone. Thus, the determinant factor should be here the structure itself of the Rg molecule, which presents a bulky "middle cage".

Table I. Solubility Values of Ls and Rg and Ls Stability in Various Solvents (at 20 °C)

Solvent	Ls (molar) solubility	Ls stability	Rg (molar) solubility
Benzene	4×10^{-3}	Stable in the dark	
Acetic acid	1.6×10^{-2}	Stable in the dark	
Pyridine	10^{-2}	Unstable	
Chloroform	2.8×10^{-3}	Unstable	6×10^{-3}
Acetone	1.8×10^{-2}	Highly photosensitive	2×10^{-3}
Me_2SO	10^{-1}	Unstable	1.5×10^{-1}
THF	3×10^{-1}	Unstable	5.5×10^{-2}
EtOH and other alcohols	5×10^{-3}	Stable in the dark	2×10^{-3}
CCl_4	10^{-4}	Stable	2.2×10^{-3} (saturated solution)
Dioxane	10^{-2}	Unstable	4×10^{-2}

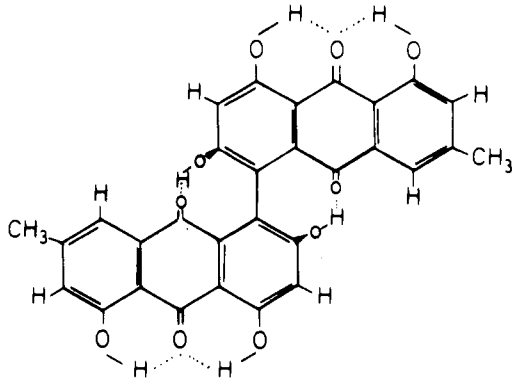


Figure 2. Developed formula of skyrin. Dotted lines represent H bonds.

Alternatively, when elution is made in neutral acetone, the same pigments elute more slowly and skyrin elutes before rugulosin. A rapid preparation of small amounts of pure skyrin is even possible by this method. The number and nature of OH groups play a more important role in this case. Electrostatic adsorption of skyrin which possesses six strong chelated OH groups should be weaker than adsorption of the rugulosin molecule which has two alcoholic free OH groups and four less strongly intramolecular bonded OH groups. Ls, which possesses two additional OH functions, is also in fact a little more strongly retained on gel than Rg.

Solubility. The question of solubility must be considered as it gives proper conditions for chemical and biochemical studies.

LS and Rg are poorly soluble in most of the organic solvents (Table I) or in weakly alkaline aqueous solutions (pH ~8.0). At higher pH (>12), the solubility of Ls is increased ($\sim 10^{-2}$ M) due to the full deprotonation of the molecule ($pK_1^{Ls} = 6.5$; $pK_2^{Ls} = 12$; $pK^{Rg} = 6.8$). However, in this case and in most of the organic solvents, both pigments are unstable; for instance, a highly concentrated (0.1 M) solution of luteoskyrin in Me_2SO begins to decompose after 2–3 days in the dark at room temperature, as shown by TLC chromatography. This pigment is also very photosensitive especially in acetone solution. Rugulosin is less photosensitive and decomposes also more slowly in any solvent. The highest solubilities used for ir and NMR studies are reported in Table I.

Ls and Rg are quite insoluble in aliphatic hydrocarbons such as isopentane, hexane, heptane, octane, or cyclohexane. The best solvents for ir and NMR studies are acetone, Me_2SO, and THF (protio or per deuterio solvents). From pH 8 to 10 in buffered solutions, Ls and Rg are soluble enough for biological experiments (10^{-4} M) and have a good stability. Buffer used must be free from any metallic divalent cation (concentration of the traces must be lower than 10^{-7} M) to prevent rapid and strong chelation with the dyes (Pham Van Chuong et al., 1974).

Identification: Mass Spectrometry. Molecular weight and precise (~5 ppm) mass values determined by high-resolution spectrometry were found to be m/e 574.15 and 542.12, respectively, for Ls and Rg. Characteristic values of m/e 288 for Ls and 272 for Rg were also observed which corresponded to the monomeric moieties ($C_{15}H_{12}O_6^+$ and $C_{15}H_{12}O_5^+$) of the respective dyes. Thus, it is confirmed that the dimer is easily dissociated by heat (150 °C) into its anthraquinonoid monomer. Another peak appeared at m/e 270 (Ls)) and 254 (Rg) which corresponded to the easy dehydration of the secondary alcoholic function leading to a planar true anthraquinone.

Uv–Visible Absorption and Circular Dichroism. The spectra of Ls and Rg in protophilic or protogenic solvents depend upon the acidity of the solvent. Moreover, in neutral organic solvents or in neutral nonbuffered aqueous solutions, the spectra vary with the extent of oxidation of the dye–phenolic OH groups due to the oxygen content of the medium. For instance, Ls and Rg solutions in Me_2SO, dioxane, formamide, or dimethylformamide rapidly change in color with simple agitation of the sample under contact with air for 1 min or by gently bubbling air through the solution. Thus, spectroscopic data (reported in Table II) are available only under standard conditions (as defined in the Experimental Section), to identify the dye, to determine its concentration, as well as for any study in solution.

The knowledge of the characteristic molar absorbance ϵ value at a given wavelength is very useful for direct determination of the exact dye concentration and to know the oxido reduced state of the dye in any solution because it avoids a further acidification or alkalinization of the sample. From the opposite signals of the CD spectra (Figure 3) of Ls and Rg at any wavelength, it is obvious that these molecules present reciprocal stereoisomeric structures.

Ir Results. Detailed assignments of the whole spectrum of Ls and Rg will be published elsewhere. Here, we will just consider and discuss some particular aspects concerning the various $\nu_{C=O}$ and ν_{OH} frequencies of vibration.

The spectrum of Rg (Figure 4) presents two characteristic bands: the first one at 1687 cm^{-1} due to the $\nu_{C=O}$ free vibration, the other one at 1615 cm^{-1} corresponding to the $\nu_{C=O}$ chelated frequency. Accordingly, Ls, which lacks apparent free C=O (Figure 1), presents only one band at 1618 cm^{-1}, belonging clearly to both intramolecular chelated $C=O_{9,9'}$ and $C=O_{10,10'}$ groups. These assignments are in agreement with those previously given by Shibata et al. (1968).

We also may note that the frequency $\nu_{C=O}$ free of Rg at 1687 cm^{-1} is significantly shifted higher than the frequency $\nu_{C=O}$ of 1,4-naphthoquinone (1674 cm^{-1}), 9,10-anthraquinone (1671 cm^{-1}), or any of the hydroxy derivatives of naphtho- or anthraquinones provided that they

Table II. Characteristic Uv-Visible Molar Absorbance ($10^{-3} \times \epsilon$, M^{-1} cm^{-1}) of Ls and Rg at Various Wavelengths (λ, nm), in Organic and Aqueous Solutions (at 20 °C)

	λ, nm	ε	λ, nm	ε	λ, nm	ε	λ, nm	ε	λ	ε	Isosbestic points
					Luteoskyrin						
Acid acetone	446 (max)	31.8	429 (max)	31.5	355 (min)	7.6					$\epsilon_{458nm}^{Ls-Ls^{4-}}$ = 17.5
Neutral acetone	446 (max)	28.8	429	28.4	355 (min)	8.0					
Basic acetone	465 (max)	17.7			355 (max)	20.4					
Acid EtOH	445 (max)	29.0	429 (max)	29.3	351 (min)	7.3	275 (max)	22.6	230	20.0	$\epsilon_{455nm}^{Ls-Ls^{4-}}$ = 21.1
Neutral EtOH	445 (max)	26.8	429 (max) (sh)[a]	26.2	351 (max)	9.6	275 (max)	21.7	235	21.0	
Basic EtOH	460 (max)	21.2			351 (max)	20.4	265 (max)	20.6	230	27.3	
CCl_4	450 (max)	29.5	433 (max)	29.0	310 (sh)[a]	9.3	277 (max)	24.3			
Buffered aq solution	ϵ_{max}(pH 8.0) = $\epsilon_{452\ nm}$[isosbestic point (6.5 < pH < 8.5)] = 21.5										
					Rugulosin						
Acid acetone	400 (sh)	27.8	391 (max)	29.2							Two different series for Rg-Rg^{2-} and Rg^{2-}-Rg^{4-}, respectively
Neutral acetone	400 (sh)	27.0	391 (max)	28.0							
Basic acetone	430 (max)	15.8	345 (max)	21.5							
Acid EtOH	400 (sh)	23.7	391 (max)	24.9	345 (sh)	11.7	275 (sh)	18.1	248 (max)	26.5	No well separated in visible range, two series in uv
Neutral EtOH	400 (sh)	21.6	391 (max)	22.3	345 (sh)	12.1	275 (sh)	17.1	248 (max)	25.4	
Basic EtOH	430 (max)	15.4			345 (max)	18.0	270 (sh)	16.1	245 (max)	25.3	
CCl_4	400 (sh)	26.9	391 (max)	28.2	345 (sh)	12.9	278 (max)	22.9			
Buffered aq solution	$\epsilon_{414\ nm}$[isosbestic (6 < pH < 12)] = 17.2										

[a] sh = shoulder.

Table III. Ir; Assignment of the Principal Bands (in cm^{-1}) of OH (and OD) Vibrations of Rugulosine-h_6 (or -d_6) and Luteoskyrin-h_8 (or -d_8)

Rg-h_6	Rg-d_6	OH/OD	Ls-h_8	Ls-d_8	OH/OD	Assignment[a]
3420	2545	1.34	3380	2520	1.34	a
3000	2280	1.32	3000	2290	1.31	p $\nu_{OH/D}$
1452	1191	1.22	1432	1149	1.25	a
1228	1042	1.18	1238	1037	1.19	p
1177	1021	1.15	1184	1015	1.17	p $\delta_{OH/D}$
			1126	913	1.23	p
885	775	1.14	897	778	1.15	a
781	604	1.29	796			$\gamma_{OH/D}$
756	584	1.29	767	554	1.38	p

[a] a refers to alcoholic 2,2′-OH groups. [b] p refers to all phenolic OH groups.

have at least one free carbonyl. Thus, the free C=$O_{9,9'}$ group of Rg presents a more acetonic character than a quinonic one as is the case in a planar naphtho- or anthraquinone structure.

The deuteration of all the OH groups of Rg and Ls permits assignment of certain bands of the ir spectrum to the different modes of OH vibrations. As shown, in Table III, we observe in the 4000–2000-cm^{-1} range the bands of OH stretching vibrations (ν_{OH}), in the 1500–900-cm^{-1} range those of OH in-plane deformation vibrations (δ_{OH}), and in the 900–700-cm^{-1} range the bands of OH out-of-plane deformation vibrations (γ_{OH}). The numerous bands of OH in-plane deformation modes may be due to the multiple couplings of these vibrations with the C=C or C–H vibrations of Rg and Ls molecules (Bouhet et al., 1973).

If we consider again the 4000–200-cm^{-1} range (Figure 4), we note a narrow peak of high intensity near 3450 cm^{-1}

Table IV. Solvent Effect on the Ir Shift of the Alcoholic ν_{OH} Valence Vibration of Ls and Rg (in cm^{-1})

Compd	Me_2SO-h_6 or -d_6	Solid state	THF-h_8 or -d_8	$CDCl_3$	CCl_4
Ls	3220	3400	3360	3600	3620
Rg	3210	3380	3360	3600	3620

and a broad band centered at 3000 cm^{-1}. These two bands disappear after deuteration of all the OH groups of the molecule, while two similar bands, having the same shape, appear at lower frequencies near 2500 and 2300 cm^{-1}, respectively. The calculated isotopic effect is 1.34–1.35, as expected. These bands clearly correspond to the respective ν_{OH} and ν_{OD} frequencies (stretching vibration). The first band centered near 3400 cm^{-1} is due to the $\nu_{alc.\ OH}$ frequency as it decreases by selective deuteration of this position and disappears as already mentioned by complete deuteration. More significantly this band also disappears by diacetylation of the $OH_{2,2'}$ group (Figure 5). Then, a new band corresponding to $\nu_{C=O}$ alkyl vibration appears at 1748 cm^{-1}.

Moreover, the $\nu_{alc.\ OH}$ band is more sensitive to solvent effects than the phenolic bands (Table IV), which appear near 2900–3000 cm^{-1} and are more or less confused with the ν_{C-H} vibrations.

From the results reported in Table IV, it can be seen that both alcoholic protons of Ls or Rg are strongly attracted and hydrogen bonded to protophilic solvents. These results are in agreement with those obtained by ^1H NMR studies (see below). In addition, when the dye is dispersed at the solid state in voltalef, the $OH_{2,2'}$ group is not fully free as it may be in CCl_4. This strongly suggests

Table V. Proton Chemical Shifts (ppm vs. Internal Me_4Si) and Coupling Constants of Ls and Rg in Different Solvents at 27 °C

	$OH_{4,4'}$	$OH_{8,8'}$	$OH_{5,5'}$	$H_{6,6'}$	$H_{8,8'}$	$OH_{2,2'}$	$H_{2,2'}$	$H_{1,1'}$	$H_{3,3'}$	CH_3
						Luteoskyrin				
$(CD_3)_2SO$	14.60, br s	12.43	11.37, br s	7.35		5.55 br d, ^3J = 3.8 Hz	4.53, m	3.36	2.96, d, ^3J = 5.4 Hz	2.29
$(CD_3)_2CO$	14.94, br s	12.58	11.65	7.32		4.68, d, ^3J = 4.7 Hz	4.81, m	3.53	3.13, d, ^3J = 5.4 Hz	2.33
$CDCl_3$	14.73	12.44	11.55	7.17		4.83[a]		3.51	3.10, d, ^3J = 5 Hz	2.33
						Rugulosin				
$(CD_3)_2SO$	14.57, br s		11.33	7.17	7.42	5.38, d, ^3J = 4.7 Hz	4.37, m	3.35	2.78, d, ^3J = 5.4 Hz	2.43
$(CD_3)_2CO$	14.84, br s		11.63	7.15	7.52	4.55, d, ^3J = 5 Hz	4.80	3.52	2.95, d, ^3J = 5.4 Hz	2.47
$CDCl_3$	14.76		11.64	7.10	7.48	4.63[a]		3.50	2.94, d, ^3J = 5.4 Hz	2.42

[a] Broad overlapped signal; br s, broad signal; m, multiplet; d, doublet. All the other nonabbreviated shift values correspond to sharp singlets.

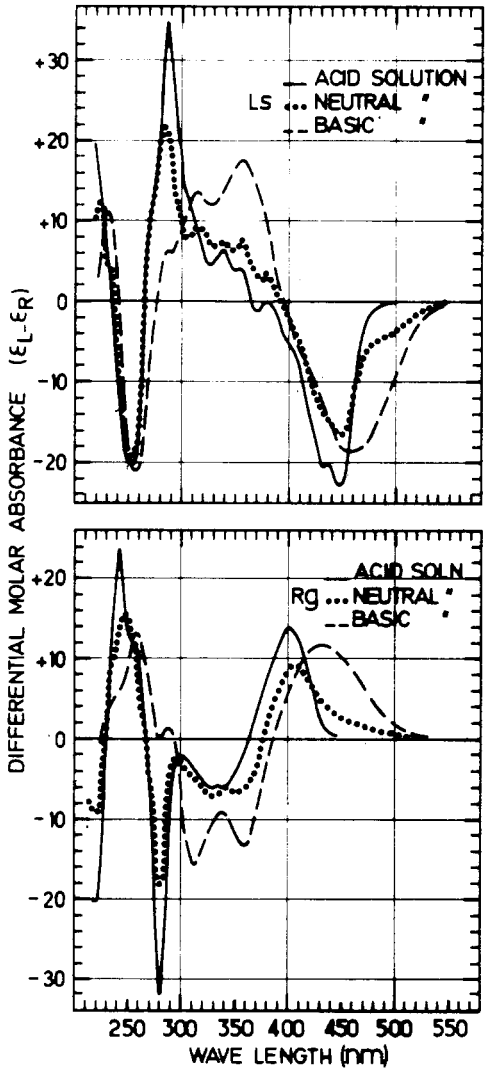


Figure 3. Circular dichroic spectra of Ls and Rg in ethanol (EtOH). Differential molar absorbance (in M^{-1} cm^{-1}) is expressed vs. wavelength (in nm).

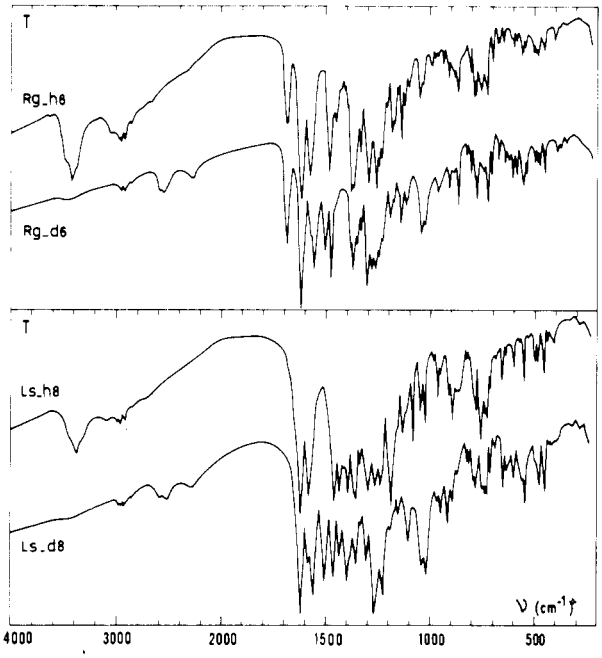


Figure 4. Ir spectra of Ls, Rg, and their perdeuteroxy derivatives in Nujol and voltalef mulls at 77 K.

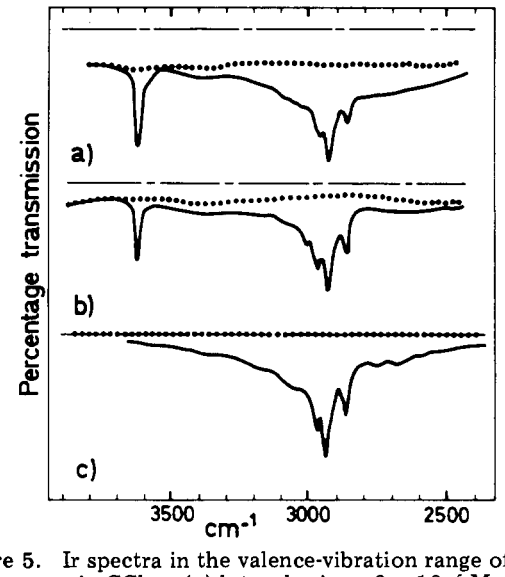


Figure 5. Ir spectra in the valence-vibration range of the -OH groups in CCl_4: (a) luteoskyrin, c 6 × 10^{-5} M, l = 5 cm; (b) rugulosin, c 5 × 10^{-5} M, l = 5 cm; (c) 2,2′-diacetylrugulosin, c 4.8 × 10^{-3} M, l = 1 mm.

that, even in the solid state, the $OH_{2,2'}$ groups may form intermolecular dye–dye H bonds.

Proton NMR Results. Proton NMR spectra of Ls and Rg were carried out in hexadeuterioacetone, hexadeuteriodimethyl sulfoxide, and deuteriochloroform. The corresponding chemical shifts are reported in Table V. The peak assignment of the aliphatic cage protons is in agreement with that given in Me_2SO by Shibata et al. (1968).

For both Ls and Rg in all the solvents studied, the $H_{1,1'}$ signal is a singlet and the $H_{3,3'}$ signal is a doublet. The coupling constant $^3J_{2,3}$ value is almost independent of the solvent and temperature. The results confirm the high rigidity of the central cage of these dyes and are in agreement with the dihedral angles H–C(2)–C(3)–H (~35°) and H–C(1)–C(2)–H (~70°) obtained by model building.

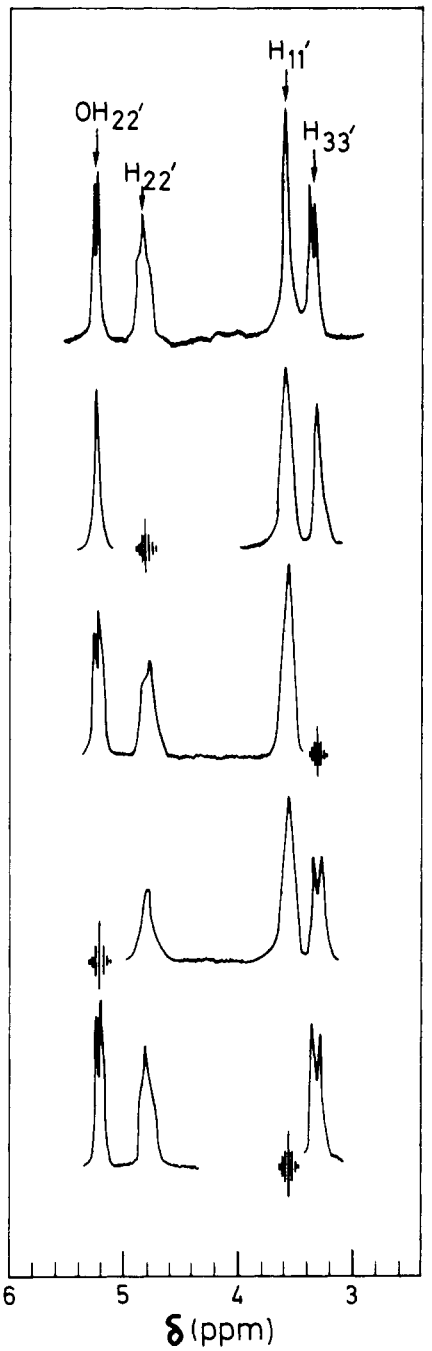


Figure 6. Proton NMR decoupling of Ls in acetone at −40 °C.

The $H_{2,2'}$ signal appears as a multiplet due to the splitting by both $OH_{2,2'}$ and $H_{3,3'}$ (Figure 6). This signal either is broad (as in Me_2SO or $CDCl_3$) or may overlap with the $OH_{2,2'}$ doublet (i.e. in $CDCl_3$ at 27 °C or in acetone around 15 °C; see Table IV and Figure 7).

In Me_2SO, as the temperature is increased, the $OH_{2,2'}$ doublet progressively broadens and collapses whereas simultaneously the $H_{2,2'}$ broad signal gives above 60 °C a well-defined doublet ($^3J_{2,3}$ = 5.5 Hz) due to a chemical exchange spin-decoupling process.

The aromatic protons of Rg show long-range couplings in acetone solution ($^4J_{6,8}$ = 1.6 Hz; $^4J_{6,Me} \simeq$ 0.8 Hz; $^4J_{8,Me}$ < 0.5 Hz). Similar values are observed in this solvent for the corresponding protons of emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone), namely H_5 (7.56 ppm) and H_7 (7.13 ppm) whose signals are easily distinguished from the two other doublets ($^4J_{2,4}$ = 2.5 Hz) belonging to the H_2(6.66 ppm) and H_4 (7.25 ppm) protons.

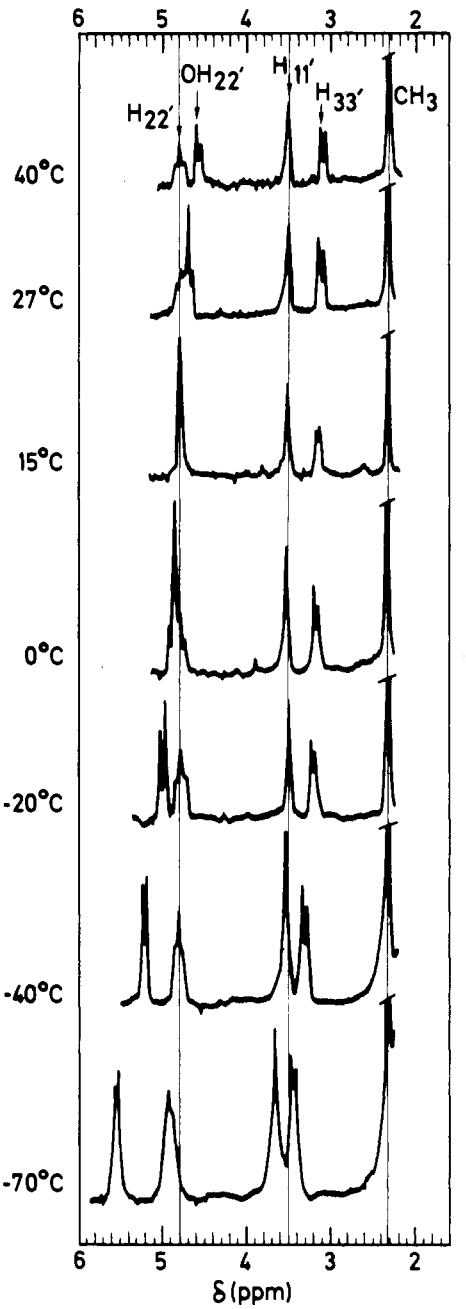


Figure 7. Proton NMR-temperature effect on $OH_{2,2'}$ proton chemical shift of Ls in acetone.

The chemical shifts of the true phenolic $OH_{5,5'}$ and $OH_{8,8'}$ protons of Ls and $OH_{5,5'}$ of Rg are close to those of the corresponding hydroxyl groups of the planar hydroxyanthraquinones, i.e., 1,4-dihydroxyanthraquinone (12.84 and 12.72 ppm, respectively, in acetone and Me_2SO), 1,8-dihydroxyanthraquinone (11.95 ppm in acetone and 11.35 ppm in Me_2SO), and emodin (12.19 and 12.08 ppm in acetone). On the contrary, the chemical-shift values of the $OH_{4,4'}$ protons of Ls and Rg confirm the enolic character of this group, as further demonstrated by ^{13}C NMR studies (Toma et al., 1975).

Moreover, the chemical shifts of the hydroxyl groups of Ls and Rg present either no (i.e., $OH_{4,4'}$ and $OH_{8,8'}$ in Ls and $OH_{4,4'}$ in Rg) or only a small ($d\delta/dT \simeq -0.003$ ppm/deg for $OH_{5,5'}$) temperature dependence in both acetone and Me_2SO. Consequently, it is clear that analogously to the hydroxyl protons of the hydroxyanthraquinones ($d\delta/dT \simeq -0.0015$ ppm/deg) the $OH_{4,4'}$ and $OH_{8,8'}$ and to a less extent the $OH_{5,5'}$, of the considered

dyes are involved in stable intramolecular hydrogen bonds.

On the contrary and as expected, the alcoholic $OH_{2,2'}$ protons of both Ls and Rg ($d\delta/dT \simeq -0.008$ ppm/deg) are free and may form intermolecular hydrogen bonds with the solvent molecules.

In order to precisely determine the strength of these different hydrogen bonds, the proton–deuterium exchange rate on each OH group of Ls and Rg was measured in acetone solution at −40 °C. The decreasing order of the H–D exchange rate was: $OH_{2,2'} \simeq OH_{4,4'} > OH_{5,5'} \gg OH_{8,8'}$, revealing that the $OH_{8,8'}$ protons form the strongest hydrogen bonds, as compared to both more reactive $OH_{4,4'}$ and $OH_{5,5'}$ groups.

These results as well as those obtained by ir studies confirm the important role of the inter- and intramolecular hydrogen-bonded OH groups in the chromatographic procedure of purification. Moreover, the nature and strength of the hydrogen bonds are probably related to the pK values of the different OH groups. The fact that Ls and Rg, as opposed to ordinary hydroxyanthraquinones, have their p$K(OH_{4,4'})$ and p$K(OH_{5,5'})$ values near the neutral pH range suggests the explanation of chelation to magnesium and then the chelation by magnesium to nucleic acids, a phenomenon which is very likely connected to the molecular mechanism of toxicity.

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LITERATURE CITED

Bouhet, J. C., Pham Van Chuong, P., Henry, M., Leicknam, J. P., Rapport interne DRA/SRIR Ma. GB/73-15/JPL/FP, C.E.N., Saclay, 1973.
Brook, A. J. W., Munday, K. C., *J. Chromatogr.* **47**, 1–8 (1970).
Brooks, C. J. W., Keates, R. A. B., *J. Chromatogr.* **44**, 509–521 (1969).
Enomoto, M., *Acta Pathol. Jpn.* **9**, 189–215 (1959).
Horobin, R. W., Gardiner, J., *J. Chromatogr.* **43**, 545–546 (1969).
Joustra, M., Söderqvist, B., Fischer, L., *J. Chromatogr.* **28**, 21–25 (1967).
Kawamoto, A., Ueno, I., Tatsuno, T., 19th Annual Meeting of the Japanese Pharmacological Society (Tokyo), Abstracts, 1964, p 157.
Keutel, J., Möckel, H., *Hum. Genet.* **7**, 344 (1969).
Kobayashi, N., Iitaka, Y., Sankawa, U., Ogihara, Y., Shibata, S., *Tetrahedron Lett.* **58**, 6135–6138 (1968).
Kobayashi, N., Iitaka, Y., Shibata, S., *Acta Crystallogr., Sect. B*, **26**, 188–201 (1970).
Kobayashi, Y., Uraguchi, K., Sakai, F., Tatsuno, T., Tsukioka, M., et al., *Proc. Jpn. Acad.* **34**, 639–644 (1958a).
Kobayashi, Y., Uraguchi, K., Sakai, F., Tatsuno, T., Tsukioka, M., et al., *Proc. Jap. Acad.*, **35**, 501–506 (1959).
Kobayashi, Y., Uraguchi, K., Tatsuno, T., Sakai, F., Tsukioka, M., et al., *Proc. Jpn. Acad.* **34**, 736–741 (1958b).
Mouton, R. F., Fromageot, P., *FEBS Lett.* **15**, 45–48 (1971).
Ohba, Y., Fromageot, P., *Eur. J. Biochem.* 1, 147–151 (1967).
Ohba, Y., Fromageot, P., *Eur. J. Biochem.* **6**, 98–105 (1968).
Pham Van Chuong, P., Bouhet, J. C., Fromageot, P., manuscript to be published (1976).
Pham Van Chuong, P., Bouhet, J. C., Schneider, C., Fromageot, P., *J. Chim. Phys. Phys.-Chim. Biol.* **3**, 401–406 (1974).
Pham Van Chuong, P., Bouhet, J. C., Thiery, J., Fromageot, P., *Tetrahedron* **29**, 3533–3538 (1973).
Platel, A., Ueno, Y., Fromageot, P., *Bull. Soc. Chim. Biol.* **50**, 678–680 (1968).
Repaš, A., Nikolin, B., Dursun, K., *J. Chromatogr.* **44**, 184–187 (1969).
Ruet, A., Sentenac, A., Simon, E. J., Bouhet, J. C., Fromageot, P., *Biochemistry* **12**, 2318–2324 (1973).
Sankawa, U., Seo, S., Kobayashi, N., Ogihara, Y., Shibata, S., *Tetrahedron Lett.* **53**, 5557–5560 (1968).
Schachtschabel, D. O., Zilliken, F., Saito, M., Foley, G. E., *Exp. Cell Res.* **57**, 19–28 (1969).
Sentenac, A., Ruet, A., Fromageot, P., *Bull. Soc. Chim. Biol.* **49**, 247–252 (1967).
Seo, S., Sankawa, U., Ogihara, Y., Shibata, S., *Tetrahedron Lett.* **10**, 767–769 (1969).
Shibata, S., Kitagawa, I., *Pharm. Bull.* 4, 309–313 (1956).
Shibata, S., Ogihara, Y., Kobayashi, N., Seo, S., Kitagawa, I., *Tetrahedron Lett.* **27**, 3179–3184 (1968).
Shibata, S., Tanaka, O., Kitagawa, I., *Pharm. Bull.* **3**, 278–283 (1955).
Toma, F., Bouhet, J. C., Pham Van Chuong, P., Fromageot, P., Haar, W., Rüterjans, H., Maurer, W., *Org. Magn. Res.* **7**, 496–503 (1975).
Ueno, Y., Platel, A., Fromageot, P., *Biochim. Biophys. Acta* **134**, 27–36 (1967).
Ueno, Y., Saheki, M., *Jpn. J. Exp. Med.* **38**, 157–164 (1968).
Ueno, Y., Ueno, I., Sato, N., Iitoi, Y., Saito, M., et al., *Jpn. J. Exp. Med.* 41, 177–188 (1971).
Umeda, M., *Acta Pathol. Jpn.* **14**, 373–394 (1964).
Umeda, M., Takahashi, K., Saito, M., Proceedings 22nd Annual Meeting of the Japanese Cancer Association, Okayama, 1963, p 250.
Uraguchi, T., Sakai, F., Tsukioka, M., Noguchi, Y., Tatsuno, T., et al., *Jpn. J. Exp. Med.* **31**, 435–461 (1961a).
Uraguchi, T., Tatsuno, T., Sakai, F., Tsukioka, M., Sakai, Y., et al., *Jpn. J. Exp. Med.* **31**, 19–46 (1961b).
Uraguchi, T., Tatsuno, T., Tsukioka, M., Sakai, Y., Sakai, F., et al., *Jpn. J. Exp. Med.* **31**, 1–18 (1961c).

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