# Chrysophanol Glycosides from Callus Cultures of Monocotyledonous *Kniphofia* spp. (Asphodelaceae)

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**We established callus cultures of the monocotyledonous plants *Kniphofia foliosa* and *K. tuckii* (Asphodelaceae), which produce the anthraquinone derivatives chrysophanol and its glycosides. The minor product chrysophanol 8-*O*-$\beta$-gentiobioside was fully characterized by spectroscopic analysis and synthesis.**

**Key words** *Kniphofia foliosa*; *Kniphofia tuckii*; Asphodelaceae; cultured cell; chrysophanol 8-*O*-$\beta$-gentiobioside; chrysophanol 1,8-di-*O*-$\beta$-gentiobioside

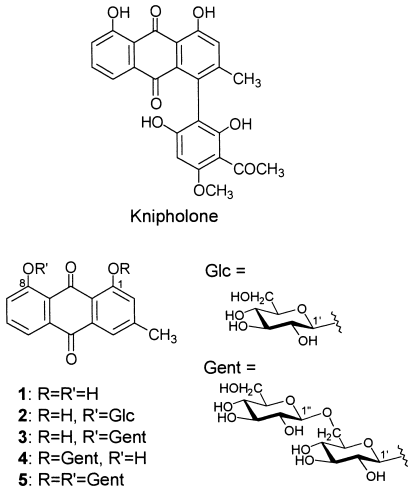
Anthraquinone pigments, including their glycosides, occur widely in several plant families, such as Polygonaceae, Rhamnaceae, Fabaceae, Asphodelaceae and Rubiaceae. They are recognized as important active constituents of many medicinal plants belonging to these families. Among the anthraquinone-rich plants are those of the genus *Kniphofia* (Asphodelaceae), most of which are restricted to tropical areas. A unique constituent, knipholone,[1] which is a conjugate of chrysophanol with an acetylphloroglucinol unit, was first isolated from the roots of *K. foliosa*, which is indigenous to Ethiopia; it was later suggested to be a chemotaxonomic marker of this genus.[2] The occurrence of novel anthraquinone constituents, including knipholone and its anthrone congener,[3] in the genus *Kniphofia*, prompted us to establish tissue cultures of the plant species to facilitate our ongoing biosynthetic study of anthraquinones, and to find new compounds. We report here the establishment of tissue cultures of two different *Kniphofia* species, *K. foliosa* and *K. tuckii*, and the characterization of their products.

## Results and Discussion

Callus cultures of *K. foliosa* and *K. tuckii* were induced, respectively, from germinated shoots on modified Murashige-Skoog (MS) agar medium[4] containing 3% sucrose, $10^{-5}$ M 2,4-dichlorophenyl acetic acid, and $10^{-5}$ M benzylaminopurine (BAP). Satisfactory cell growth under dark conditions was observed when developed callus cells were transferred to BDS[5] or MS medium.

HPLC profiles of acetone extracts from both species of cultured cells were similar to each other, and the products were characterized as follows. The acetone extract of the *K. tuckii* callus was subjected to a combination of chromatographies over silica gel and Toyopearl HW-40 followed by preparative HPLC, to give chrysophanol (**1**) as a major product, and two minor products (**2**, **3**) with higher polarity, one of which was identified as chrysophanein (**2**).[6]

Compound **3** showed the pseudomolecular ion $(M+Na)^+$ at *m*/*z* 601 in electrospray ionization (ESI)-MS; its molecular formula $C_{27}H_{30}O_{14}$ was determined by high-resolution (HR)-ESI-MS [*m*/*z* 596.1966 $(M+NH_4)^+$]. Comparison of its $^1$H- and $^{13}$C-NMR (Table 1) data with those of **2**, along with the MS data, indicated the structure of **3** to be a diglucoside of chrysophanol. One of the anomeric protons at $\delta$ 4.20 (1H, d, $J$=8 Hz, H-1″) displayed three-bond correlation with C-6 methylene carbon ($\delta$ 68.8, C-6′) of the other glucose residue in the heteronuclear multiple bond connectivity (HMBC) spectrum, which suggests that the sugar component of **3** is gentiobiose. The other anomeric proton signal at $\delta$ 5.10 exhibited an HMBC correlation with the carbon signal at $\delta$ 158.3, which was assigned to C-8 by two-bond correlation with H-7 ($\delta$ 7.81, dd, $J$=2.5, 7 Hz). In addition, a nuclear Overhauser effect (NOE) correlation was observed between this anomeric proton and H-7, proving the linkage of the gentiobiose residue at C-8 of the aglycone. $\beta$-Glucosidic linkage on the gentiobiosyl group was evidenced from a large coupling constant ($J$=7.5 Hz) of the anomeric proton signal at $\delta$ 5.10. Consequently, compound **3** was assumed to be chrysophanol 8-*O*-$\beta$-gentiobioside. This structure was confirmed by the identification with the product synthesized from **1** and $\alpha$-bromo-heptaacetylgentiobiose in the presence of $Ag_2CO_3$. The synthesis also yielded chrysophanol 1-*O*-$\beta$-gentiobioside (**4**)[7,8] and 1,8-di-*O*-$\beta$-gentiobioside (**5**), the latter of which has not been reported as natural product to our best knowledge. Chrysophanol 8-*O*-gentiobioside was reported to




Structures of Knipholone, and Chrysophanol (**1**) and Its Glucosides

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Table 1. $^{13}$C-NMR Spectral Data for **3** and **4** [126 MHz, DMSO-$d_6$–$D_2O$ (20 : 1)]

| Carbon | 3 | 4 | Carbon | 3 | 4 |
|---|---|---|---|---|---|
| C-1 | 161.5 | 158.6 | C-1′ | 100.6 | 100.6 |
| C-1a | 115.0 | 118.6[a)] | C-2′ | 73.7 | 73.8 |
| C-2 | 123.3 | 123.1 | C-3′ | 76.5 | 76.6 |
| C-3 | 147.9 | 148.1 | C-4′ | 69.8 | 69.9 |
| C-4 | 120.8 | 121.8 | C-5′ | 76.4 | 76.1 |
| C-4a | 132.4 | 134.7[b)] | C-6′ | 68.8 | 69.4 |
| C-5 | 119.7 | 118.8 | C-1″ | 103.7 | 104.1 |
| C-5a | 134.9 | 132.8[b)] | C-2″ | 73.4 | 73.5 |
| C-6 | 136.6 | 136.6 | C-3″ | 76.7 | 76.8 |
| C-7 | 124.3 | 124.6 | C-4″ | 70.2 | 70.4 |
| C-8 | 158.3 | 161.4 | C-5″ | 77.4 | 77.2 |
| C-8a | 120.7 | 117.1[a)] | C-6″ | 61.1 | 61.3 |
| C-9 | 187.8 | 188.0 | | | |
| C-10 | 182.4 | 182.6 | | | |
| 3-$CH_3$ | 21.7 | 22.2 | | | |

*a*, *b*) Assignments may be interchanged.

be a constituent of *Rheum emodi*.[9] However, as no physicochemical data were available in the literature, the full assignments of $^1$H- and $^{13}$C-NMR spectra (Table 1), are given in this paper comparing with those of **4**.

In the present study, we could not detect knipholone or its related anthraquinones, nor, in particular any intermediary precursors, in the cultured cells. However, we found that the glucosidation of anthraquinone occurred in the established callus cultures of *K. foliosa* and *K. tuckii*; the intact plants, however, have not been reported to produce any glycosides. This study is also noteworthy as a rare example of natural product producing tissue cultures of a monocotyledonous plant, which have generally been recognized as rather difficult to establish.

## Experimental

**General** Melting points were measured on a Yanaco micro-melting point apparatus and are uncorrected. $^1$H- and $^{13}$C-NMR spectra were recorded in DMSO–$D_2O$ (20 : 1) on a Varian VXR-500 instrument (500 MHz for $^1$H and 126 MHz for $^{13}$C) and chemical shifts are given in $\delta$ (ppm) values relative to that of the solvent [DMSO–$D_2O$ (20 : 1) ($\delta_H$ 2.49; $\delta_C$ 39.7)] on a tetramethylsilane scale. The standard pulse sequences that were programmed into the instrument (VXR-500) were used for each two-dimensional measurement. The $J_{CH}$ value was set at 6 Hz in the HMBC spectra. Optical rotations were measured with a Jasco DIP-1000 polarimeter. UV spectra were measured with a Hitachi U-2000 spectrophotometer. ESI-MS including high-resolution mass spectra were recorded on a Micromass Auto Spec OA-TOF mass spectrometer (solvent: 50% aqueous MeOH containing 0.1% $AcONH_4$; flow rate: 0.02 ml/min).

**Source of Seeds** *Kniphofia foliosa* and *Kniphofia tuckii* were obtained from Dr. Ermias Dagne of the Department of Chemistry at the University of Adis Ababa, Adis Ababa, Ethiopia.

**Establishment of Callus Cultures** *K. foliosa* seeds were soaked in water (20 h) and sterilized in an aqueous solution of $HgCl_2$ (0.1%) for 18 min. The seeds were rinsed in sterile water, placed on Nitsch and Nitsch-medium,[10] and kept in darkness for 21 d. Shoots which developed were transferred to modified MS (MSK) agar medium[4] in which BAP (hormone) in MS medium is replaced by kinetin (2.0 mg per liter medium). After development of a callus, cells were transferred to BDS and to MS[4] medium, and subcultured in suspension at 25 °C at 1 month intervals in the dark. Both media gave good growth.

*K. tuckii* seeds were soaked in water (2 h), kept in ethanol for a short time, and subsequently kept in an aqueous solution of $HgCl_2$ (0.1%). The seeds were germinated in sterile petri dishes on moist filter paper. Shoots were placed on MSK[4] medium, and the callus propagated were transferred to either BDS or MS medium, both of which gave good growth. Subculturing was performed in a way similar to that for *K. foliosa*.

**Extraction and Isolation** The suspension-cultured cells of *K. tuckii* (40.4 g) or *K. foliosa* (25.1 g) were harvested by suction, washed with distilled water to remove media components, and freeze dried. The cells were soaked in acetone (600 and 300 ml, respectively) and filtered. Each filtrate was evaporated and submitted to column chromatography over silica gel, Sephadex LH-20, Toyopearl HW-40 (coarse grade), and/or YMC GEL ODS-AQ 120-50S with aqueous MeOH; the final purification was achieved by preparative HPLC [column: YMC-Pack ODS A-312 (10 mm i.d.×300 mm), solvent: 0.01 M $H_3PO_4$–0.01 M $KH_2PO_4$–MeOH (17.5 : 17.5 : 65), flow rate: 2.0 ml/min, column temp.: 40 °C] to yield chrysophanol (**1**) (9.1 mg), chrysophanein (**2**) (1.1 mg), and chrysophanol 8-*O*-gentiobioside (**3**) (0.3 mg) from *K. tuckii*, and **1** (2.2 mg) from *K. foliosa*.

The residue was then soaked in MeOH and filtered. The MeOH extract was similarly fractionated and purified to give **3** (0.3 mg) from *K. tuckii*, and **1** (14.4 mg), **2** (0.3 mg), and **3** (2.3 mg) from *K. foliosa*.

Chrysophanol 8-*O*-*β*-Gentiobioside (**3**): A yellow amorphous powder, $[\alpha]_D^{23}$ −21.4° (*c*=0.05, 50% aq. MeOH). UV $\lambda_{max}$ ($H_2O$) nm (log $\varepsilon$): 258 (3.23), 282 sh (2.94), 411 (2.68). ESI-MS *m/z*: 601 $[M+Na]^+$, 596 $[M+NH_4]^+$, 255 $[aglycone+H]^+$. HR-ESI-MS *m/z* 596.1966 $[M+NH_4]^+$ (Calcd for $C_{27}H_{30}O_{14}+NH_4$, 596.1979). $^1$H-NMR (DMSO-$d_6$+$D_2O$): $\delta$ 12.84 (1H, s, 1-OH, in not $D_2O$), 7.86 (1H, dd, *J*=2.5, 7 Hz, H-5), 7.85 (1H, t, *J*=7 Hz, H-6), 7.81 (1H, dd, *J*=2.5, 7 Hz, H-7), 7.50 (1H, br s, H-4), 7.19 (1H, br s, H-2), 5.10 (1H, d, *J*=7.5 Hz, H-1′), 4.20 (1H, d, *J*=8 Hz, H-1″), 4.01 (1H, d, *J*=11 Hz, H-6′), 3.70 (1H, br t, *J*=9 Hz, H-5′), 3.63 (1H, d, *J*=12 Hz, H-6″), 3.60 (1H, dd, *J*=7, 11 Hz, H-6′), 3.47 (1H, dd, *J*=6.5, 12 Hz, H-6″), 3.42 (1H, br t, *J*=8 Hz, H-2′), 3.31 (1H, t, *J*=9 Hz, H-3′), 3.18 (1H, t, *J*=9 Hz, H-3′), 3.07 (1H, br t, *J*=8 Hz, H-3″), 3.02 (1H, br t, *J*=9 Hz, H-4″), 3.00 (1H, m, H-5″), 2.96 (1H, br t, *J*=9 Hz, H-2″), 2.42 (3H, s, 3-$CH_3$). $^{13}$C-NMR (DMSO-$d_6$+$D_2O$): Table 1.

**Synthesis of Chrysophanol Gentiobiosides** *α*-Bromo-heptaacetylgentiobiose was prepared by treatment of *β*-gentiobiose octaacetate (Extrasynthese (France)) with a saturated solution of hydrogen bromide according to the method described by Brauns.[11] To a solution of chrysophanol (**1**) (20 mg) in pyridine (1 ml) was added, first, Drierite (50 mg) and $Ag_2CO_3$ (36 mg), and then a solution of *α*-bromo-heptaacetylgentiobiose (50 mg) in pyridine (0.5 ml). After stirring for 16 h at room temperature with the exclusion of moisture and light, the mixture was diluted with $CHCl_3$ (5 ml), filtered, extracted with 5% HCl, and washed with $H_2O$. After evaporation, the crude product was deacetylated with 1 M NaOMe (0.2 ml) in MeOH (3 ml) at room temperature for 24 h. The solution was acidified to pH 6 with AcOH, and the products were purified by preparative HPLC [column: YMC-Pack J'sphere H80 (10 mm i.d.×300 mm), solvent: MeOH–$H_2O$–acetic acid (45 : 55 : 1), flow rate: 1.5 ml/min, column temperature: 40 °C] to yield chrysophanol 8-*O*-*β*-gentiobioside (**3**) (8.0 mg), 1-*O*-*β*-gentiobioside (**4**) (7.6 mg), and 1,8-di-*O*-*β*-gentiobioside (**5**) (1.0 mg).

Chrysophanol 1-*O*-*β*-Gentiobioside (**4**): Yellow needles, mp 275—277 °C (from MeOH), $[\alpha]_D^{23}$ −23.1° (*c*=0.05, 50% aq. MeOH). UV $\lambda_{max}$ ($H_2O$) nm (log $\varepsilon$): 259 (4.46), 283 sh (3.07), 410 (2.89). ESI-MS *m/z*: 601 $[M+Na]^+$, 596 $[M+NH_4]^+$, 255 $[aglycone+H]^+$. HR-ESI-MS *m/z* 596.1988 $[M+NH_4]^+$ (Calcd for $C_{27}H_{30}O_{14}+NH_4$, 596.1979). $^1$H-NMR (DMSO-$d_6$+$D_2O$): $\delta$ 12.95 (1H, s, 1-OH, in not $D_2O$), 7.73 (1H, t, *J*=7 Hz, H-6), 7.69 (1H, br s, H-4), 7.65 (1H, dd, *J*=1.5, 7 Hz, H-5), 7.58 (1H, br s, H-2), 7.34 (1H, dd, *J*=1.5, 7 Hz, H-7), 5.16 (1H, d, *J*=7.5 Hz, H-1′), 4.19 (1H, d, *J*=8 Hz, H-1″), 3.99 (1H, d, *J*=10.5 Hz, H-6′), 3.76 (1H, br t, *J*=10 Hz, H-5′), 3.63 (1H, dd, *J*=8.5, 10.5 Hz, H-6′), 3.63 (1H, d, *J*=11 Hz, H-6″), 3.38 (1H, dd, *J*=5.5, 11 Hz, H-6″), 3.45 (1H, dd, *J*=7.5, 9 Hz, H-2′), 3.32 (1H, t, *J*=9 Hz, H-3′), 3.19 (1H, t, *J*=9 Hz, H-3′), 3.09 (1H, br t, *J*=8.5 Hz, H-3″), 3.05 (1H, m, H-5″), 3.03 (1H, br t, *J*=8 Hz, H-4″), 2.97 (1H, dd, *J*=8, 8.5 Hz, H-2″), 2.48 (3H, s, 3-$CH_3$). $^{13}$C-NMR (DMSO-$d_6$+$D_2O$): Table 1.

Chrysophanol 1,8-di-*O*-*β*-Gentiobioside (**5**): A yellow amorphous powder, $[\alpha]_D^{23}$ −28.8° (*c*=0.14, 50% aq. MeOH). UV $\lambda_{max}$ ($H_2O$) nm (log $\varepsilon$): 259 (4.58), 375.5 (3.94). ESI-MS *m/z*: 925 $[M+Na]^+$, 920 $[M+NH_4]^+$, 255 $[aglycone+H]^+$. HR-ESI-MS *m/z* 925.2536 $[M+Na]^+$ (Calcd for $C_{39}H_{50}O_{24}$+Na, 925.2590). $^1$H-NMR (DMSO-$d_6$+$D_2O$): $\delta$ 7.74—7.79 (3H, m, H-5—7), 7.62 (1H, br s, H-4), 7.56 (1H, br s, H-2), 5.05, 5.01 (each 1H, d, *J*=8 Hz, H-1′), 4.21 (2H, d, *J*=8 Hz, H-1″), 2.96—4.00 (gentiobiose-H), 2.45 (3H, s, 3-$CH_3$).

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