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Isolation and extraction of lucidin primeveroside from *Rubia tinctorum* L. and crystal structure elucidation

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Abstract

Madder (*Rubia tinctorum* L.) has been used as a dye for over 2000 years with alizarin and purpurin the major natural dyes analysed from extractions undertaken. The use of ethanol as the solvent in the extraction process produced an extract that yielded four anthraquinone compounds lucidin primeveroside, ruberythric acid, alizarin and lucidin- ω -ethyl ether. Gravitational separation of the extract was used to record the first crystal structure of lucidin primeveroside and the first ever known crystal structure of a glycoside containing anthraquinone moiety. The crystal structure along with ¹H and ¹³C NMR helped elucidate and confirm the structure of this overlooked natural dye which has been shown to be a major compound in *Rubia tinctorum* L.

1. Introduction

Examples of historical textile artefacts exist in most museums, particularly in the UK, and they reveal important information about our cultural past and heritage. From the perspective of the textile conservator, understanding of dyes used is important in terms of date,

geographical origin, colour change and dye degradation processes that may occur, and planning both interventive treatments and preventive conservation strategies.

One of the oldest dyes used throughout history is the mixture of compounds extracted from the European madder plant (*Rubia tinctorum* L.), which gives an orange-red dye; a red dye can also be obtained from the Indian madder plant (*Rubia cordifolia* L.) (Chenciner, 2000; Cardon, 2007). Madder plants contain an impressive number of anthraquinone derivatives; of the thirty-six compounds now identified in madder's roots, fifteen play an important role in dyeing, and are grouped together in the Colour Index as C. I. Natural Red 8. The main colouring species extracted from *R. tinctorum* and *R. cordifolia* are alizarin (**1**) and purpurin (**2**); other colorants present include xanthopurpurin (**3**), rubiadin (**4**), pseudopurpurin (**5**), munjistin (**6**), and lucidin (**7**) (Chenciner, 2000).

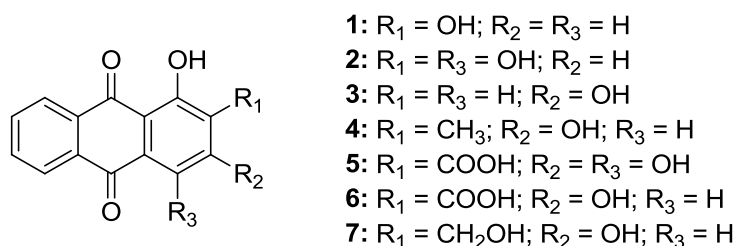


Figure 1. Anthraquinone structures found in *Rubia tinctorum*

There are many literature references that have separated the compounds in madder using an HPLC apparatus and several others that have used an older method of DCCC (droplet counter current chromatography) (Hostettmann, 1980; Derksen et al., 1998; Derksen and van Beek, 2002; Rafaëly et al., 2008; Bechtold, 2009; Slyshova et al., 2010). The amount of pure dye compounds recovered by these processes is in the magnitude of several milligrams; with this recovery being so low, limited analysis work has been performed on anthraquinone glycosides.

The aim of the work herein is to understand the structure and properties of dye molecules present in *Rubia tinctorum* and to isolate any colorants to comprehensively understand where the colour comes from on subsequent dyeings.

2. Results and Discussion

The use of ethanol as the only solvent in the extraction of *Rubia tinctorum* L. is a well-documented procedure, but not one commonly used as ethoxy derivative compounds can be synthesised (Derksen and van Beek, 2002; Bechtold, 2009; Slyshova et al., 2010). Ethanol

extraction of anthraquinones from *Rubia tinctorum* L. led to the identification of four compounds by HPLC (Figure 2): (a) lucidin primeveroside; (b) ruberythric acid; (c) alizarin; and (d) lucidin- ω -ethyl ether. The extract after filtration was an orange liquid; after being evaporated to dryness an orange powder was produced. This material was not moisture-sensitive and could be handled very easily.

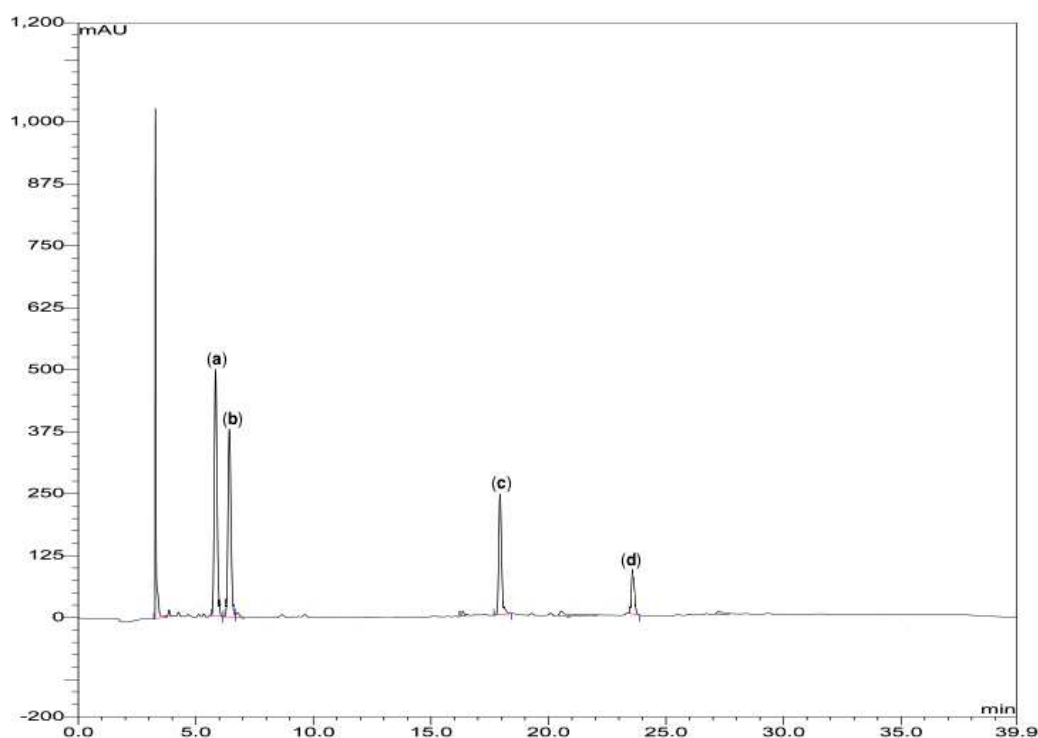


Figure 2. HPLC chromatogram of the madder extract at 254 nm showing four compounds analysed (a) lucidin primeveroside (b) ruberythric acid (c) alizarin (d) lucidin- ω -ethyl ether

UV-Vis chromatograms were obtained along with mass spectrometry to clearly identify the signals seen. The results were as follows: lucidin primeveroside ($\lambda_{\max} = 406$, [M-H]⁻; m/z = 563); ruberythric acid ($\lambda_{\max} = 415$, [M-H]⁻; m/z = 533); alizarin ($\lambda_{\max} = 430$, ([M-H]⁻, m/z = 239); and lucidin- ω -ethyl ether ($\lambda_{\max} = 412$, [M-H]⁻; m/z = 298). Lucidin- ω -ethyl ether had not been previously observed during any previous studies, and literature revealed that this compound was not very abundant in other extraction profiles of *Rubia tinctorum*. Nevertheless, Orban et al. (2008) reported detecting lucidin- ω -ethyl ether by HPLC methods when using ethanol as the solvent at reflux temperatures. Herein, many attempts were made to successfully isolate and fully analyze the lucidin- ω -ethyl ether using many analytical

techniques, but it proved unsuccessful, however, this compound has been previously isolated, characterized and tested by Westendorf et al. (1988).

After using an ultrasound bath to dissolve the orange powdered crude extract in methanol, yellow crystals were obtained after 4 days and analysis confirmed this product to be lucidin primeveroside. X-ray crystallography was conducted on the product and herein we present the first known example of a glycoside containing anthraquinone moiety crystal structure (Figure 3).

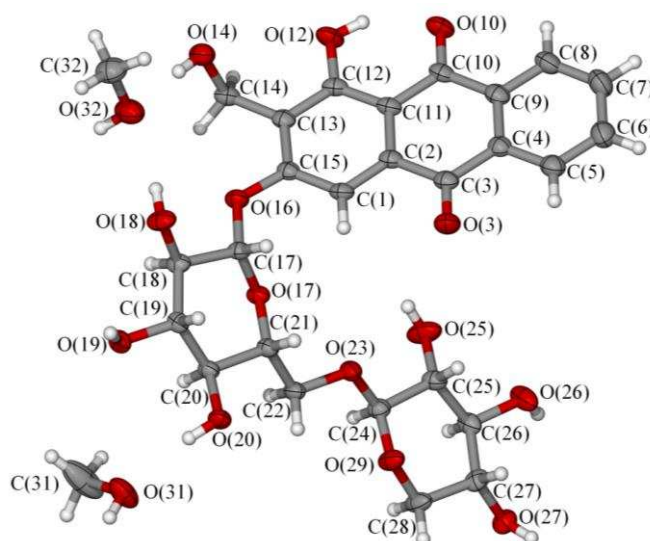


Figure 3. Ellipsoid plot from the crystal structure of lucidin primeveroside. Ellipsoids shown at 50% probability level.

The crystal structure has up to 10 hydrogen bond interactions (Figure 4) which form the crystal lattice, several of these are intermolecular (some occurring via the methanol solvent molecule) and one is an intramolecular hydrogen bonding interaction between O10 and H12. The intramolecular hydrogen bond is evidenced by the fact that a pH greater than 13 is required to deprotonate the hydroxyl group in the β -position to the carbonyl in alizarin, having a pK_a of 12 (Drivas, et al., 2011). This interaction does contradict the crystal structure of alizarin; Guilhem (1967) states both carbonyl groups have intermolecular hydrogen bonding between molecules to form a ‘triple molecule complex’.

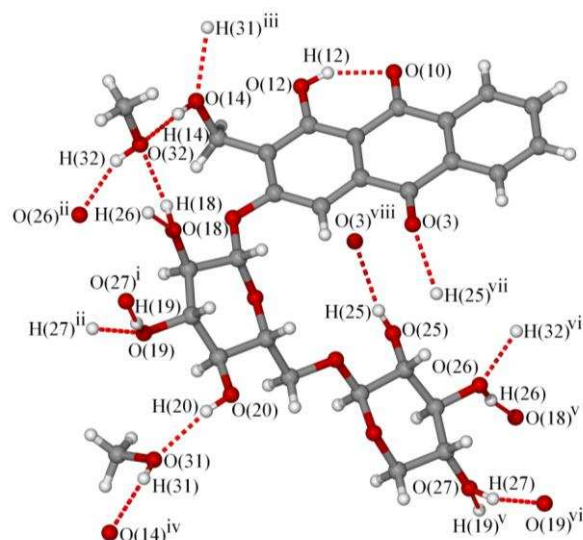


Figure 4. Hydrogen bonding interactions observed in the solid state in the single crystal structure of lucidin primeveroside. Symmetry operations for symmetry generated atoms i: $1+x, y, 1+z$; ii: $x, y, 1+z$; iii: $1-x, \frac{1}{2}+y, 1-z$; iv: $1-x, y-1/2, 1-z$; v: $x-1, y, z-1$; vi: $x, y, z-1$; vii: $1+x, y, z$; viii: $x-1, y, z$.

Each lucidin primeveroside molecule hydrogen bonds to two additional lucidin primeveroside molecules via three intermolecular hydrogen bonding interactions (O19-H19-O27, O25-H25-O3, O26-H26-O18). Hydrogen bond O25-H25-O3 connects lucidin primeveroside molecules stacked directly on top of one another. Hydrogen bonds O19-H19-O27 and O26-H26-O18 connects lucidin primeveroside stacked directly above a neighboring lucidin primeveroside molecule, as viewed in Figure 5a. Additional π - π stacking interactions are evident between the lucidin primeveroside molecules stacked directly on top of one another (ring centroid separation 4.601 Å). Two additional intermolecular hydrogen bonding interactions via the methanol solvent molecule O31-C31 connects parallel stacks of lucidin primeveroside molecules; one layer is shown in Figure 5b. As the lucidin primeveroside molecule is v-shaped, π - π stacking interactions can occur in two dimensions, which gives rise to the layer shown in Figure 5c that shows stacks of lucidin primeveroside molecules neatly arranged on top of each other as well as being hydrogen bonded across from one another.

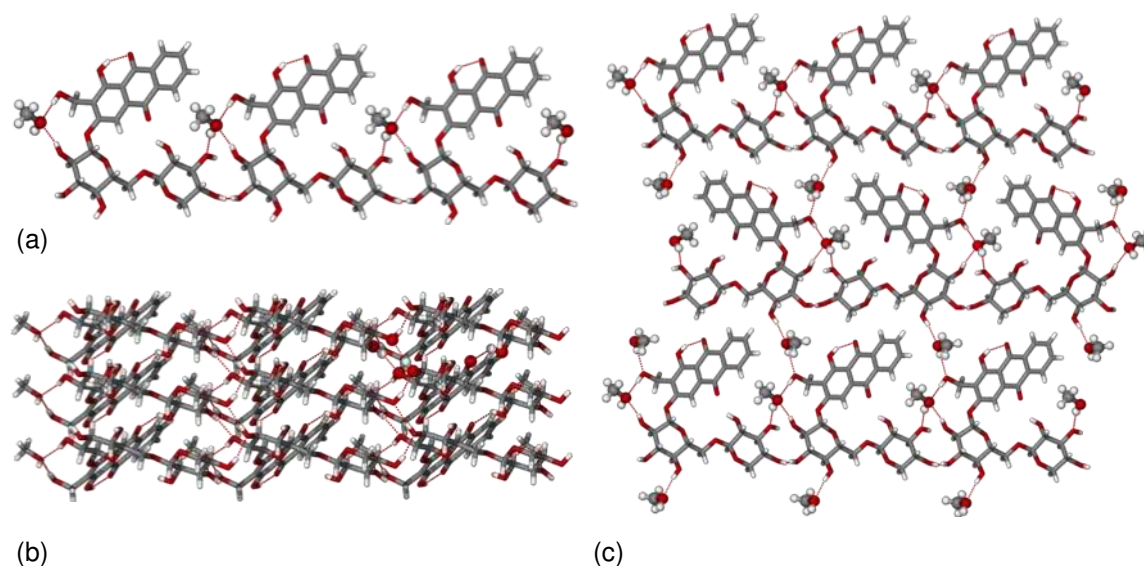


Figure 5. (a) 1D chain propagated through three intermolecular hydrogen bonds via MeOH solvent molecule (C32 O32) and one intermolecular hydrogen bond O27-H27-O19. MeOH solvent molecule (C32 O32) shown as ball and stick model. Hydrogen bonding interactions shown as dotted lines. (b) 2D hydrogen bonded sheet propagated through intermolecular hydrogen bonding interactions via methanol solvent molecule O31-C31. (c) Stacks of hydrogen bonded lucidin primeveroside. Donor, hydrogen and acceptor atoms involved in hydrogen bonding the lucidin primeveroside layers are shown as spheres. Hydrogen bonding interactions shown as dotted lines.

3. Conclusions

Four compounds were identified by HPLC when ethanol is used as the solvent, namely lucidin primeveroside, ruberythric acid, alizarin and lucidin- ω -ethyl ether. Lucidin- ω -ethyl ether is not a naturally occurring anthraquinone compound and is only formed by the use of ethanol as the solvent at refluxing temperatures. The advantage of using ethanol as the solvent is the dried extract recovered, which is not moisture sensitive and can be re-dissolved into methanol and many other solvents. The ability to do this led to the compound lucidin primeveroside being successfully separated from the three other anthraquinone compounds present and the crystal structure of this compound being discovered. This discovery will help in the understanding of how the molecule will pack and interact with itself, which could be useful for dyeing studies undertaken with the madder root extract.

4. Experimental

4.1 General Procedures and Instruments

Nuclear magnetic resonance spectra were recorded for ^1H at 500 MHz and ^{13}C at 125 MHz on a Bruker DPX500 spectrometer using deuterated DMSO respectively, as the solvent and residual proton signals of respective solvents as an internal standard. Chemical shifts are expressed in parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm) for proton resonance. The proton coupling constants are reported as correct values and are given in Hz (multiplicities, s singlet, bs broad singlet, d doublet, t triplet, q quartet). Mass spectra electron ion spray (EIS) was carried out on an Agilent technology 1200 series Bruker Daltonics HCT ultra. All solvents used in analytical procedures were purified before use using established procedures (Perrin et al., 1980). Solvents were removed at reduced pressure using a Buchi rotary evaporator at 20 mbar, followed by further drying under high vacuum at 0.5 mmHg.

HPLC conditions were carried out at room temperature on a Phenomenex Hyperclone C_{18} column, 5-mm particle size, 250 mm x 4.6 mm I.D. column equipped with a pre-column. Chromatography was carried out using two solvents: (A) water and 0.1% trifluoroacetic acid solution and (B) acetonitrile and 0.1% trifluoroacetic acid solution. A linear gradient programme was applied: 0 – 6 minutes 27% B; 6 – 20 minutes linear increase to 60% B; 20 – 23 minutes hold on 60% B; 23 – 25 minutes linear increase to 70% B; 25 – 35 minutes hold on 70% B; 35 – 40 minutes linear decrease to 27% B. The flow rate during the experiment was 1.0 ml/minute. Injections were made by a Basic Marathon autosampler equipped with a 20-ml loop. This method was carried out on an Agilent 1290 Infinity UHPLC using a Diode Array Detector. Peaks were detected at 254 nm. pH of the solutions measured through the HPLC machine was pH 6.8; all resultant UV spectra obtained were measure at pH 6.8.

4.2 Materials

Alizarin (supplied as 97%) and purpurin (supplied as 90%) were purchased from Sigma-Aldrich. Ruberythric acid (supplied as 75%) was purchased from Apin Chemicals. Methanol (HPLC grade) and ethanol (analytical grade) were purchased from Fischer Scientific UK. Madder root was purchased from Aurorasilk (USA).

4.3 Extraction

4.3.1 Ethanol extraction

Madder root (17 g) was ground up in a blender until it was fine and of consistent particle size. This was dispersed into 500 ml of ethanol and heated to reflux with consistent stirring for 3 hours. It was then filtered and the liquor reduced to a solid. This was placed under high vacuum until dry. The result was an orange powder weighing 2.52 g (14 % yield). A HPLC chromatogram was recorded with the sample dissolved in ethanol. A ^1H NMR spectrum was taken in DMSO-d_6 and recorded. The ratio of anthraquinones to polysaccharides was 8%:92%.

4.3.2 Recrystallisation of Ethanol Extract

Madder root extract (2.2 g) was partially dissolved into 150 ml of methanol. This was then sonicated until all the extract had dissolved. The solution was then left in a freezer for 4 days. The resulting liquor was decanted and several crystals resided at the bottom of the flask. The yellow needles were collected by vacuum filtration and proven to be **9** (156 mg, yield: 7%). An X-ray crystal structure was recorded. The resulting pure compound was analysed by ^1H & ^{13}C NMR spectroscopy in $\text{DMSO-d}_6 + \text{D}_2\text{O}$. m.p. 300-301 °C; ^1H nmr, δ_{H} (500 MHz, $(\text{CD}_3)_2\text{SO}$); 8.19 – 8.17 (m, 1H, H-1 or H-2), 8.14 – 8.12 (m, 1H, H-1 or H-2), 7.92 – 7.86 (m, 2H, H-3 & H-4), 7.40 (s, 1H, H-5), 5.10 (d, 1H, H-1 glucose, $J = 7.25$ Hz), 4.61 (d, 1H, one of the CH_2OH protons, $J = 11.42$ Hz), 4.53 (d, 1H, one of the CH_2OH protons, $J = 11.43$ Hz), 4.12 (d, 1H, H-1 xylose, $J = 7.54$ Hz), 3.92 (d, 1H, sugar proton, $J = 9.85$ Hz), 3.69 – 3.59 (m, 3H, sugar protons), 3.42 – 3.26 (m, 4H, sugar protons), 3.07 (t, 1H, sugar proton, $J = 8.84$ Hz), 3.01 – 2.96 (m, 2H, sugar protons); ^{13}C nmr, δ_{C} (500 MHz, $(\text{CD}_3)_2\text{SO}$); 187.60 (s) (C=O), 182.29 (s) (C=O), 162.28 (s), 162.23 (s), 135.59 (d), 135.53 (d), 134.38 (s), 133.33 (s), 133.25 (s), 127.61 (s), 127.21 (s), 123.71 (s), 111.89 (s), 106.61 (s), 104.36 (s), 100.84 (s), 76.57 (s), 76.10 (s), 76.07 (s), 73.67 (s), 73.55 (s), 69.86 (s), 69.49 (s), 68.48 (s), 65.97 (s), 51.38 (s) ($\underline{\text{C}}\text{H}_2\text{OH}$)

4.4 X-ray crystallography

A suitable single crystal was selected and immersed in an inert oil. The crystal was then mounted on a glass capillary and attached to a goniometer head on a Bruker X8 Apex diffractometer using a graphite monochromated Mo-K_α radiation ($\lambda = 0.71703 \text{ \AA}$) using 1.0 ϕ -rotation frames. The crystal was cooled to 150 K by an Oxford cryostream low temperature device. The full data set was recorded and the images processed using DENZO and SCALEPACK programs. Mr. Colin Kilner solved the structure.

Structure solution by direct methods was achieved through the use of SHELXS86, SIR92 or SIR97 programs, and the structural model defined by full matrix least squares on F^2 using SHELX97. Molecular graphics were plotted using POV-RAY via the XSEED program. Editing of CIFs and construction of tables of bond lengths and angles was achieved using WC and Platon. Unless otherwise stated, hydrogen atoms were placed using idealised geometric positions (with free rotation for methyl groups), allowed to move in a “riding model” along with the atoms to which they were attached, and refined isotropically.

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Appendix A. Supplementary data

CCDC 932547 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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