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Article:

Isolation and extraction of ruberythric acid from Rubia tinctorum L. and crystal structure elucidation

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Abstract

Madder (Rubia tinctorum L.) has been exploited as a dye throughout history. The roots of the plant are very rich in the highly coloured glycosidic compounds ruberythric acid and lucidin primeveroside, alongside the corresponding aglycons which can be readily formed by deglycosylation, particularly during extraction. Supported by ^{1}H and ^{13}C NMR data, the conclusive X-ray crystal structure of the natural dye ruberythric acid is presented for the first time. The solid state structure revealed extensive intermolecular hydrogen bonding interactions between the sugar moieties in the unit cell, but only intramolecular hydrogen bonding through the hydroxyquinone groups. There is also some additional π-π stacking from the anthraquinone moiety.

1. Introduction

Before the advent of synthetic dyes, all textiles were dyed using naturally-derived colorants. Rubia tinctorum L. (Dyer's madder) has been exploited as a natural red dye for many centuries, the oldest dating back to 1350 BC (Derksen & van Beek, 2002). The colorants of R. tinctorum are found in the roots of the plant, which historically were dried, ground and beaten and then extracted in an aqueous dyebath for textile coloration. Aqueous extraction for textile dyebaths accumulated high amounts of unused madder pulp which was hard to dispose of and in the late 19th century it was found that this unused pulp contained one third of the colouring matter; simple treatment with hot acid released this remaining colorant and hence could be sold as a lower grade dye (Desrochers, 2008).

Detailed analysis of dyes in artefacts can reveal important cultural and heritage information concerning historical textiles; another key aspect is one of preservation and the aim is to fully understand the nature of these dyestuffs in order to determine degradation patterns and structure-efficient conservation techniques. Understanding the precise molecular structure of these natural dyes and their chemical reactivity (e.g. during extraction, or the dyeing process) is important to provide knowledge of their interactions with physical substrates, such as textile fibres, which could be used to develop superior techniques for analysis of artefacts; many current analysis and extraction techniques damage the dye molecule, so 'softer' methods are becoming increasingly important, through the preservation of valuable chemical information embedded in the dye structure (Zhang & Laursen, 2005).

The dye obtained from the madder roots is an orange-red dye that is applied on to the textile fibres using a mordant metal ion. Aluminium (Al^{3+}) is the most commonly used metal ion for complexation between the fibre and dye moieties, however, iron sulphate ($FeSO_4 \cdot 7H_2O$) and tin chloride ($SnCl_2$) were also used (Hofenk de Graaff, 2004; Zhang & Laursen, 2005). The colorants in madder roots belong to the chemical family of the anthraquinones, and occur as a characteristic fingerprint of anthraquinone derivatives depending on origin of the natural material; there have been 36 anthraquinone derivatives detected in Rubia spp. to date, 15 of these adding to the colouring properties of the plant (Baghalian et al., 2010; Henderson et al., 2013). The two most abundant

colorants in untreated madder roots are the glycosides lucidin primeveroside (**1**) and ruberythric acid (**2**) (Derksen et al., 2003; Henderson et al., 2013). These glycoside compounds are often overlooked as colorant compounds in madder roots as they are acid sensitive, and many extraction and analytical techniques hydrolyse them to the corresponding aglycons. However the presence of the sugar moieties provides ample potential for binding to substrates, including via metal mordants. The colorants present in madder and madder-dyed artefacts have largely been concluded to be the aglycons alizarin and purpurin, with very little reference to the glycosides, which may be a gross oversimplification of the actual dyeing system.

Literature examples of madder analysis by HPLC-DAD (Derksen et al., 1998; Derksen et al., 2004; Karapanagiotis et al., 2005) and more recently HPLC-DAD-MS (Derksen et al., 2002; Balakina et al., 2006; Lech & Jarosz, 2011) provide quantitative information of the dyestuffs present, but do not provide any information on the structure of these molecules. The small quantities of anthraquinones present in the roots of the plant make isolation of the individual components difficult and hence there is limited information and spectroscopic data on the structures of these natural compounds especially for the glycosidic components.

2. Results and Discussion

Roots of Iranian madder which had previously been dried and ground, were extracted with ethanol to give an orange solution. Ethanol was chosen as the extraction solvent as the yield of glycosides from ethanol was higher than when using water (Figure 1 and 2) possibly due to water causing some hydrolysis to the glycosides in the high temperatures used for extraction. The glycoside to alizarin ratio was found to be 236.8:1 mg cm^{-3} in ethanol, and 21.7:1 mg cm^{-3} in water, hence ethanol was used for further experimentation.

Solvent was evaporated in vacuo to give an orange powder, which became darker and sticky if left open to the air overnight, consistent with being a hygroscopic material. This orange powder was soluble in water, methanol and ethanol. HPLC analysis showed three main peaks present in the madder sample: lucidin primeveroside (**1**); ruberythric acid (**2**); and alizarin (**3**). These compounds were detected using a photodiode array detector with UV/visible data consistent with their structures as follows: lucidin primeveroside (λ_{max} = 200, 246, 285 nm), ruberythric acid (λ_{max} = 224, 256 nm) and alizarin (λ_{max} = 198, 249, 279 nm) (Figure 1). These peaks were identified using commercial standards of the above compounds which gave the same UV data and retention times. Notably, the glycosidic compounds showed by far the largest peaks suggesting that they are much more abundant in the plant that the aglycon derivatives shown to be 236.8:1 mg cm^{-3} glycoside concentration to alizarin in the ethanol extract. Hence if there is no further treatment to the dyebath then the main colorant components are the glycosides, and not the aglycons which is often inferred in other publications (Liu et al., 2011, Novotna et al., 1999).

It is interesting to observe that in the ethanol extraction almost 100% of the colouring species recovered were in a glycosylated form. Schweppe (1993) states that in traditional dyeing hydrolysis to the aglycons occurred during the drying, storage and the dyeing process, and that hydrolysis was required to achieve colour depth. Traditional methods of drying included the use of underground pits called tandirs to steam the roots of the madder plant, which reduced the volume of the roots to a third. Steaming may have stimulated the enzymatic hydrolysis of the glycosides to increase the abundance of the aglycon colorants, which was said to increase the brightness of the root and produce a more

purple red (Cardon, 2007). However, in hotter climates the roots were usually left to dry in air, and in wet countries the use of stoves to dry the madder roots was employed; this may have denatured hydrolytic enzymes, inhibiting hydrolysis to aglycons. This may explain why the colorants in the Iranian madder used herein did not hydrolyse to the aglycon form during the drying process.

The dried extracts of the madder roots were dissolved in methanol and after 5 days a red amorphous solid had precipitated out of solution. The liquid was removed by decantation and the red solid was dried under reduced pressure. DMSO d_6 was then added which dissolved the solid, and after 4 days, a large yellow crystalline needle was obtained. Analysis by X-ray crystallography gave the structure shown (Figure 3), and a section of the crystal was also used to obtain NMR data, both of which showed it unambiguously to be ruberythric acid (**2**). The data clearly shows ruberythric acid as a derivative of alizarin, containing a primeveroside disaccharide moiety consisting of a glucose molecule and a terminal xylose, attached through the β-phenolic oxygen of the anthraquinone. Both sugar moieties are linked at the anomeric centre with the β stereochemistry, which is also consistent with the high coupling constants (J=7.5 Hz) of both sugar anomeric protons, having the trans-coplanar relationship with the proton on the adjacent carbon. This is only the second known example of a fully characterised glycoside-containing anthraquinone, including an X-ray crystal structure, following the recent structural elucidation of lucidin primeveroside by Henderson et al. (2013).

The pK_a of the hydroxyl group β to the carbonyl is 12 in alizarin (Derksen et al., 2002; Drivas et al., 2011), which is relatively high for a phenol, but arises from the intramolecular hydrogen bond between the C12 hydroxyl and the C14 carboxyl groups (numbering based on Figure 3) bond length 2.563(6) Å. This intramolecular hydrogen bonding pattern is evident in both the crystal structure of ruberythric acid shown in this paper and for lucidin primeveroside previously reported (Henderson et al., 2013). Interestingly, there is no evidence of this moiety playing a major role in intermolecular interactions, although this may be, at least in part, due to the packing within the crystal lattice.

The crystal packing (Figure 4) shows that the main intermolecular interactions are due to hydrogen bonding between the sugar moieties. Each ruberythric acid molecule forms hydrogen bonds to another two ruberythric acid molecules, which are through the terminal xylose, which forms two hydrogen bonds with the glucose of the primeveroside of another ruberythric acid molecule (Figure 5). The crystal structure shows significant intermolecular hydrogen bonding between sugars of ruberythric acid units: O26-H26…O37, 2.696 Å; O27-H27…O36, 2.766 Å; O37…H26-O26, 2.696 Å; O36…H27-O27, 2.766 Å (Figure 5). There is also evidence of π-π stacking between the anthraquinone rings with a plane separation of 3.438(7) Å (Figure 6). These intermolecular interactions lead to formation of helices along the c-axis, displaying the four-fold symmetry of the space group. Note the structure also shows significant disordered solvent (DMSO and water) incorporation into the lattice, with some variability in precise orientation. The solvent is modelled as one and a half molecules of water and one and a quarter molecules of DMSO in the asymmetric unit.

What is notable about the crystal structures of ruberythric acid (herein) and lucidin primeveroside (Henderson et al., 2013) is that they both display significant interaction between the sugars in the lattice; the anthraquinone aglycon moiety does not provide any intermolecular hydrogen bonding interactions within the lattice. These observations lead to questions of how these dye molecules interact with textile fibre substrates. Hydrogen bonding interactions are cited as the main attractive force operating between natural anthraquinonoid dye molecules and textile polymer fibres, either inherently (e.g. proteinaceous fibres) or upon the addition of a mordant metal salt before dyeing (e.g. cellulosic fibres) (Cardon, 2007; Bechtold, 2009). Accordingly, understanding the structure and hydrogen bonding interactions of these natural dye molecules will lead to a more informed model for

dye-fibre binding and affinity. The importance of glycosides in binding interactions has previously been overlooked, and this insight is not only interesting in terms of fundamental sorption properties, but also aids the development of methods and analytical techniques for extraction of the dyes from artefacts.

3. Conclusions

HPLC analysis of extracts of Iranian madder identified three major components; lucidin primeveroside, ruberythric acid and alizarin when using ethanol as a solvent. The most abundant colorant components in the extracts were the glycosides lucidin primeveroside and ruberythric acid. The X-ray crystal structure was solved for ruberythric acid showing the hydrogen bonding interactions between the sugar moieties within the molecules, rather than the β-hydroxy ketone of the anthraquinone. This information provides a deeper understanding of the fundamental processes controlling dyeing with anthraquinonoid natural dyes, and also improved methods to extract and analyse dyed artefacts.

4. Experimental

4.1. General procedures and instrumentation

Nuclear magnetic resonance spectra were recorded for ^1H at 500.23 MHz and for ^{13}C at 75.67 MHz on a Bruker DRX500 and DPX300 spectrometer respectively using DMSO d_6 as a solvent. Chemical shifts are reported as parts per million (ppm) downfield of tetramethylsilane (singlet at 0 ppm) for proton NMR data. The proton coupling constants are reported as corrected values and are given in Hz (multiplicities; s singlet, d doublet, t triplet, q quartet and m multiplet).

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 analysis was carried out at 30 °C on a Phenomenex Hyperclone C_{18} column, 5-mm particle size, 250 mm x 4.6 mm I.D column equipped with a guard column. Two solvents were used in the elution of samples (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. A gradient programme was applied that follows a previous paper published by Henderson et al. (2013). 0-6 min 27% B; 6-20 min linear increase to 60% B; 20-23 min hold on 60% B; 23-25 min linear increase to 70% B; 25-35 min hold on 70% B; 35-40 min linear decrease to 27% B. The flow rate during the run was 1.0 ml/min. Injections were made by a Basic Marathon autosampler equipped with a 20 μl loop. This method was carried out on a Dionex Ultimate 3000 HPLC using a diode array detector. Peaks were detected at 254 nm.

A suitable single crystal was selected for X-ray analysis and immersed in an inert oil. Measurements were carried out at 120K on an Agilent SuperNova diffractometer equipped with an Atlas CCD detector and connected to an Oxford Cryostream low temperature device using mirror monochromated Cu Kα radiation (λ = 1.54184 Å) from a Microfocus Nova X-ray source. The structure was solved by direct methods using SHELXS and refined by a full matrix least squares technique based on F2 using SHELXL97.

High resolution electrospray (ESI+) mass spectrometry was performed on a Bruker MaXis Impact spectrometer, m/z values are reported in Daltons to four decimal places.

Infrared spectra were recorded on a Bruker Alpha Platinum ATR. Samples were analysed in the solid phase and absorption maxima (ν_{max}) are given in wave numbers (cm^{-1}) to the nearest whole wavenumber.

4.2. Materials

Alizarin (supplied as 90%) was purchased from Sigma-Aldrich. Ruberythric acid (supplied as 75%) was purchased from Apin chemicals was shown to be a mixture of ruberythric acid and lucidin primeveroside by NMR and HPLC. Deuterated DMSO was purchased from Euriso-Top. Methanol (HPLC grade) and ethanol (HPLC grade) were purchased from Sigma-Aldrich. HPLC water was distilled on site. Iranian madder root was purchased from George Weil.

4.3. Ethanol extraction

Dried and ground Iranian madder root (10 g) was immersed in ethanol (300 cm^3) and heated under reflux with stirring for 3 hours. Solids were removed by filtration and the filtrate was evaporated to dryness first on a rotary evaporator and then on the high vacuum system. This resulted in an orange powder (1.67 g). The sample for HPLC analysis was prepared by dissolving (0.75 g) in water (100 cm^3) and an aliquot taken, then filtering through a 0.45 μm syringe filter before being injected into the HPLC.

4.4. Water extraction

Dried and ground Iranian madder root (0.75 g) was immersed in water (100 cm^3) and heated under reflux with stirring for 3 hours. The solution was filtered and aliquot taken straight from the dyebath for HPLC analysis and was filtered through a 0.45 μm syringe filter before being injected into the HPLC.

4.5. Recrystallization of ethanol extract

Madder root extract (1.5 g) was re-dissolved into methanol (150 cm^3) and sonicated to ensure everything was re-dissolved; any remaining solid was removed by filtration. The solution was then left for 5 days after which an amorphous solid was observed in the bottom of the flask. The liquid was then decanted off and the remaining solid was dried on the high vac system. Deuterated DMSO (~3 ml) was then added to the flask and left open to the air for a further 4 days to give a large yellow crystalline needle. Deuterated solvent was used due to the small yields of the compound produced and the need for NMR analysis throughout the process. An X-ray crystal structure of the compound was recorded, and a segment of the crystal was analysed by ^{1}H and ^{13}C NMR spectroscopy in DMSO-d_6.

^{1}H NMR (500 MHz): δ 8.27 (d, 1H, J=9.2 Hz, H^2), δ 8.22 (d, 1H, J=9.2 Hz, H^5), δ 7.97 (m, 2H, H^3 and H^4), δ 7.77 (d, 1H, J=8.5 Hz, H^9), δ 7.65 (d, 1H, J=8.5 Hz, H^{10}) aromatic peaks, δ 5.47 (d, 1H, J=5.3 Hz, H^{25}), δ 5.22 (d, 1H, J=5.0 Hz, H^{27}), δ 5.20 (d, 1H, J=5.5 Hz, H^{26}), δ 5.09 (d, 1H, J=7.5 Hz, anomeric glucose H^{19}), δ 5.02 (d, 1H, J=4.8 Hz, H^{36}), δ 4.95 (d, 1H, J=4.7 Hz, H^{38}), δ 4.92 (d, 1H, J=5

Hz, H[37]), δ 4.17 (d, 1H, J=7.5 Hz, anomeric xylose H[30]). Other sugar hydrogens are seen as multiplets between δ 3.9-2.9. Hydrogen numbering is based on Figure 3.

^{13}C NMR carried out in d_6 DMSO (500 MHz): aromatic peaks δ 188.47 (C[14]), δ 180.92 (C[7]), δ 151.74 (C[12]), δ 151.12 (C[11]), δ 135.17 (C[13]), δ 134.31 (C[8]), δ 133.39 (C[10]), δ 132.96 (C[1]), δ 126.79 (C[6]), δ 126.58 (C[9]), δ 125.87 (C[2]), δ 120.79 (C[5]), δ 120.42 (C[3]), δ 116.23 (C[4]), anomeric carbons δ 103.96 (C[19]), δ 99.85 (C[30]), C-OH sugars δ 76.58 (C[20]), δ 76.44 (C[22]), δ 76.07 (C[21]), δ 73.40 (C[31]), δ 72.99 (C[33]), δ 69.57 (C[32]), δ 68.26 (C[23]), δ 65.55 (C[34]), CH$_2$ sugars δ 40.05 (C[28]), δ 38.94 (C[34]).

HRMS: m/z (ESI+) calculated for $C_{25}H_{26}O_{13}$ [M+ Na]$^+$:557.1373; found [M+ Na]$^+$: 557.1275.

IR spectrum shows characteristic peaks at 3255 cm^{-1} (OH stretching), 1667 cm^{-1} and 1632 cm^{-1} (ketones) (Silverstein et al., 1976).

4.6. X-ray crystallography

Ruberythric acid crystallised as orange needles in a tetragonal cell and was solved in the I4 space group, with one molecule and disordered solvent (modelled as one and a half molecules of water and one and a quarter molecules of DMSO d_6) in the asymmetric unit. There was significant disordered solvent in the asymmetric unit. This has been modelled as one and a half molecules of water and one and a quarter molecules of DMSO d_6. One molecule of DMSO d_6 has been modelled with sulfur disordered across two positions in a 50:50 ratio. The quarter molecule of DMSO d_6 is disordered across a four-fold rotational axis. The correct composition of this DMSO d_6 has been modelled, but molecular structure has not been refined. Disordered solvent was refined isotropically. No hydrogen atoms were added to the disordered solvent during refinement, but the correct number of atoms has been added to the molecular formula in the CIF.

Appendix A. Supplementary data

CCDC 1043207 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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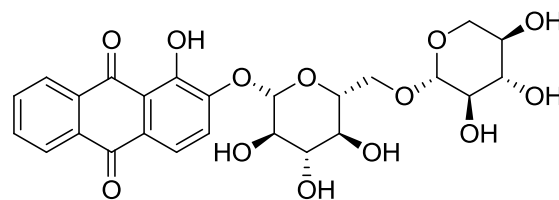
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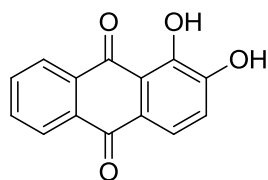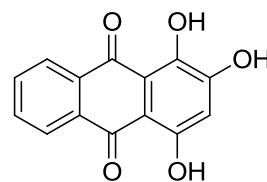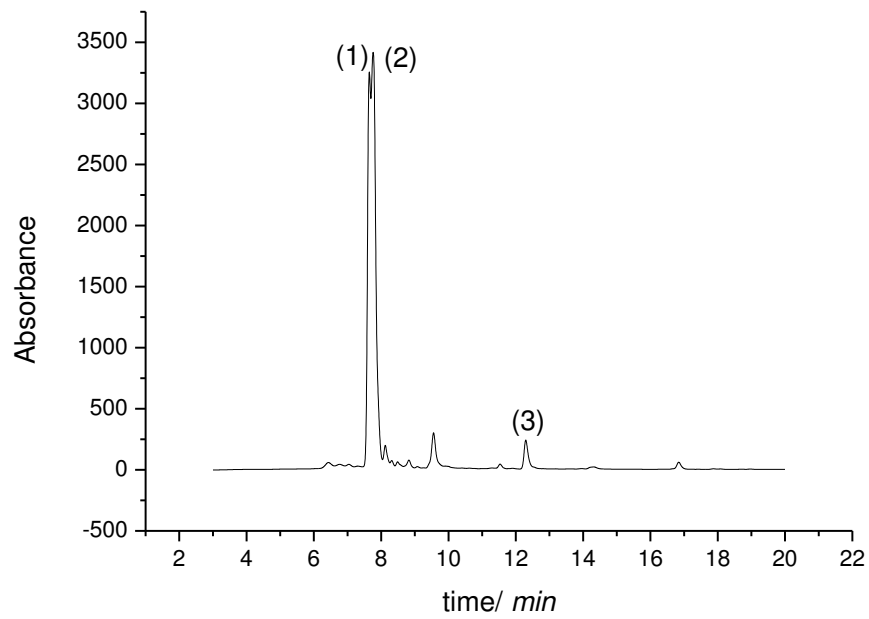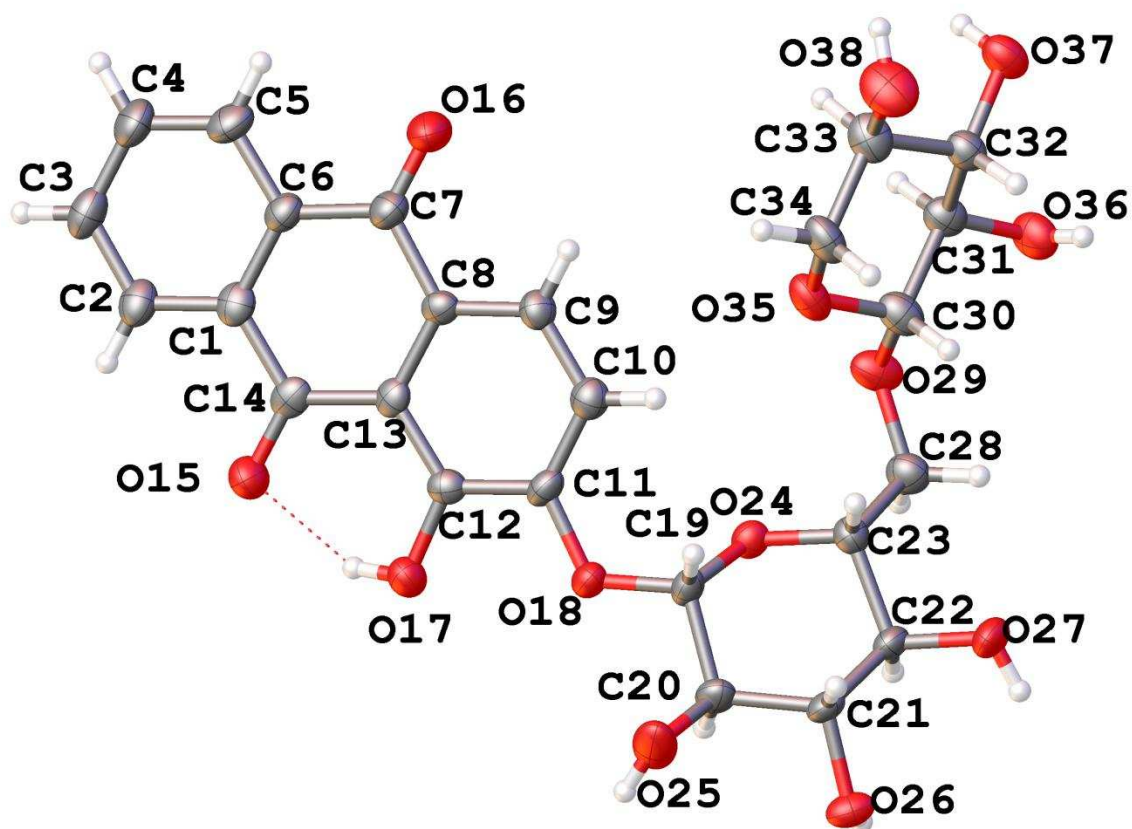
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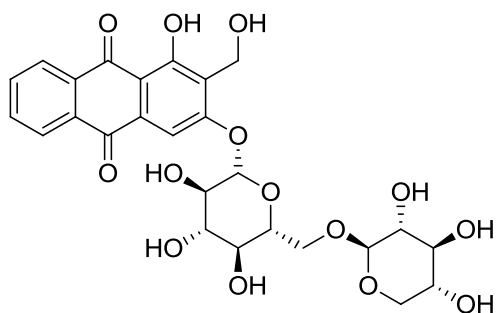
(1)

(2)

(3)

(4)

Figures

Figure 1. HPLC of ethanol madder extracts: (1) lucidin primeveroside, (2) ruberythric acid and (3) alizarin. Ratio of glycosides to alizarin is 236.8:1 mg cm^{-3}.
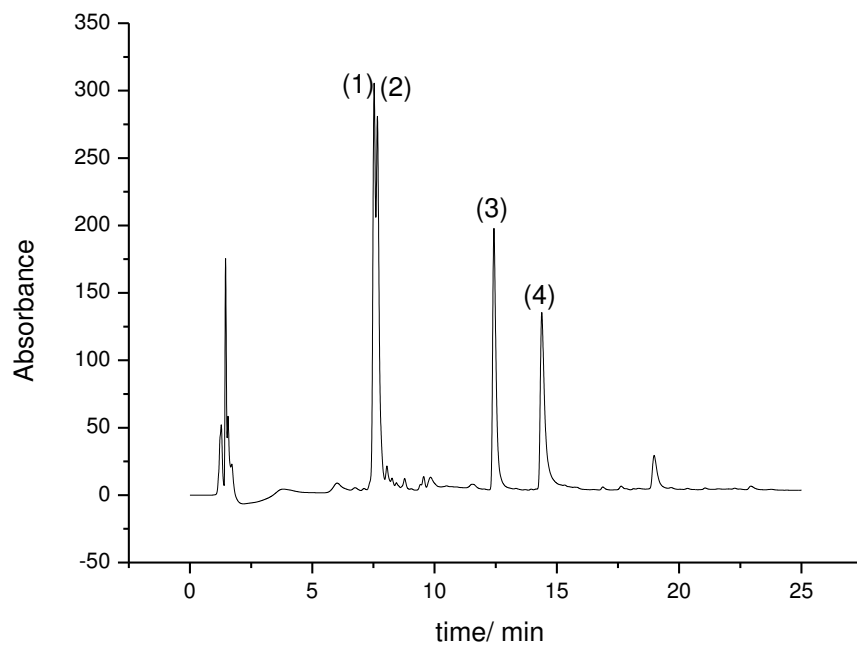
Figure 2. HPLC of water madder extracts: (1) lucidin primeveroside, (2) ruberythric acid, (3) alizarin and (4) purpurin. The ratio of glycosides to alizarin is 21.7:1 mg cm^{-3}.

Figure 3. Ellipsoid plot of the crystal structure of ruberythric acid. Ellipsoids are shown at the 50% probability level.
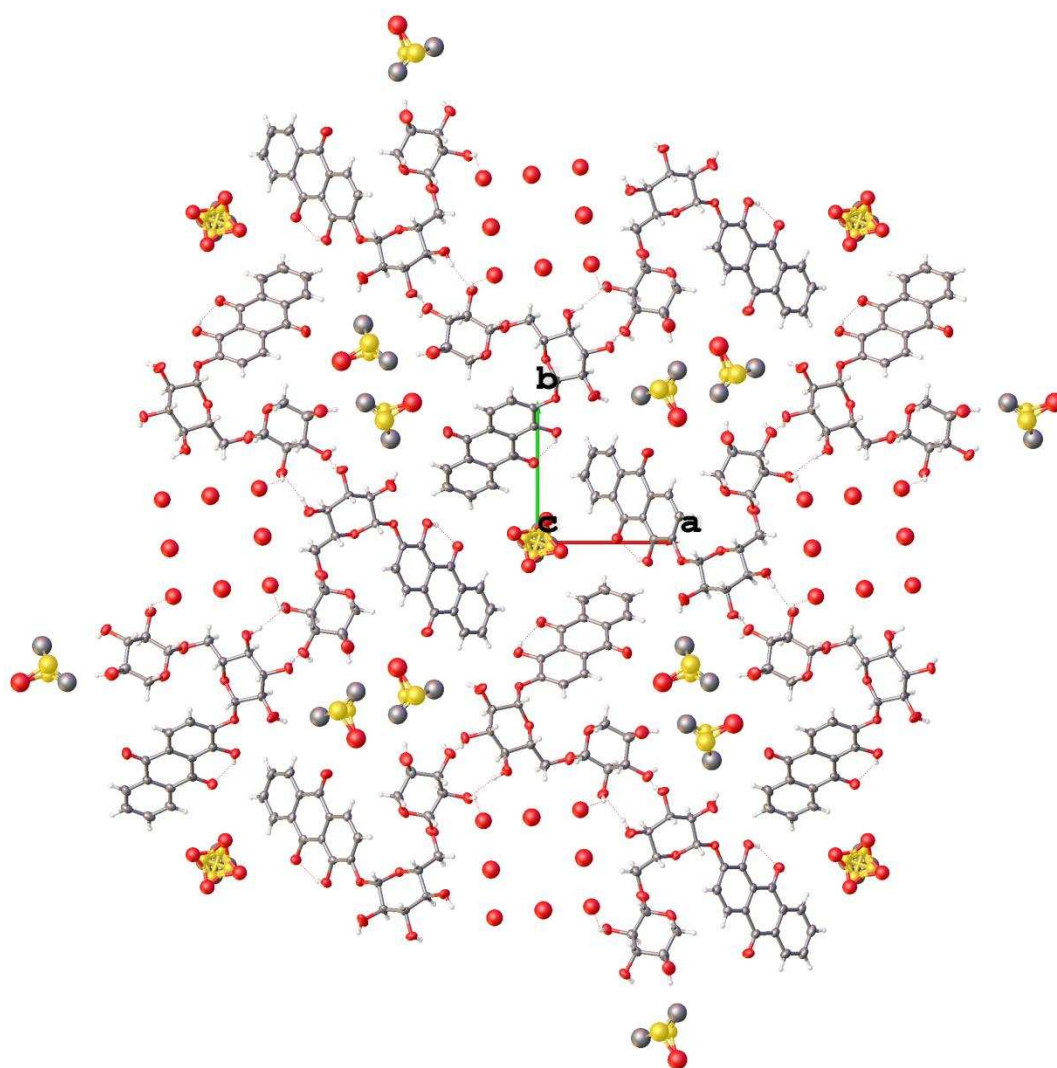
Figure 4. Crystal packing diagram of ruberythric acid with water and DMSO d$_6$ viewed down the c-axis. The diagram also shows hydrogen bonding to the unit square of the next layer of the crystal structure. Ellipsoids are shown at the 50% probability level.
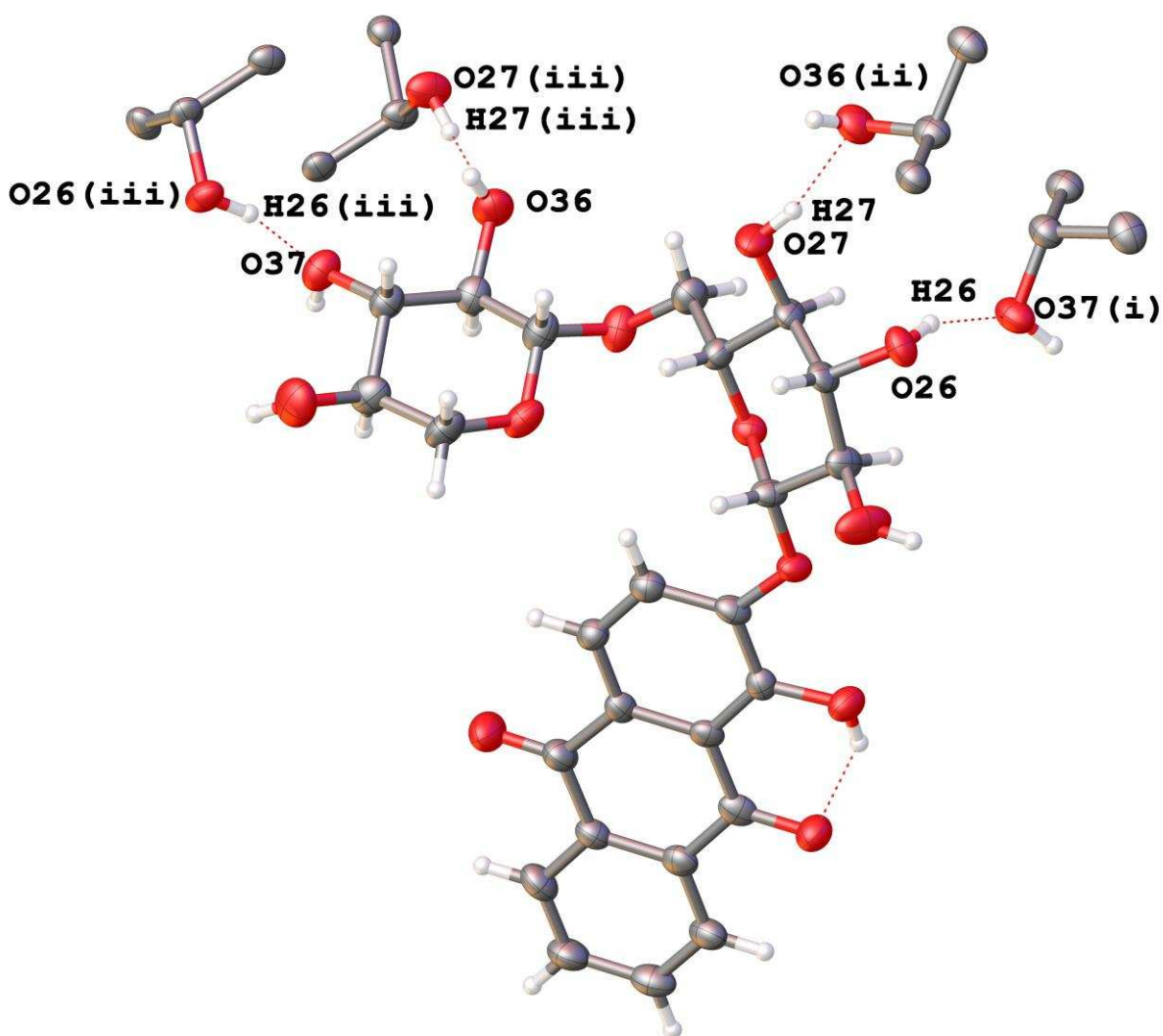
Figure 5. Intermolecular hydrogen bonding interactions between ruberythric acid units. Ellipsoids are shown at the 50% probability Symmetry operations for symmetry generated atoms i: 1.5-y, x-0.5, 0.5+z; ii: 1.5-y, x-0.5, 0.5+z; iii: 0.5+y, 1.5-x, z-0.5; iv: 0.5+y, 1.5-x, 0.5+z.
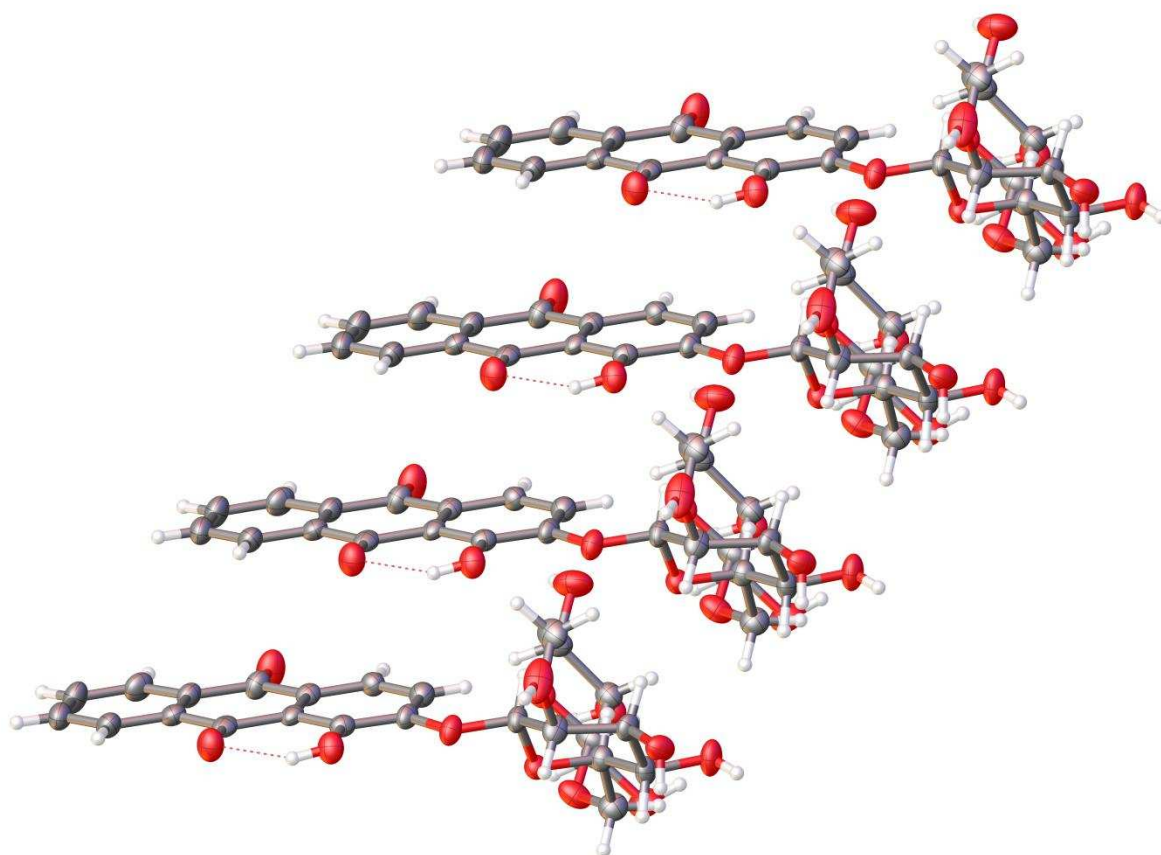
Figure 6. π-π stacking between anthraquinone rings with a plane separation of 3.438(7) Å.