A 1000-year-old mystery solved: Unlocking the molecular structure for the medieval blue from *Chrozophora tinctoria*, also known as folium

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The molecular structure of the medieval watercolor known as folium has finally been solved in the 21st century. The interdisciplinary approach taken was the key to producing extracts that had been prepared following medieval instructions, and shows the blue/purple chromophore as the major dye in *Chrozophora tinctoria* fruits (shell). A multi-analytical characterization of its structure was made using HPLC-DAD-MS, GC-MS, NMR (1H, 13C, COSY, HSQC, HMBC, INADEQUATE), and computational studies. The results demonstrate that the blue compound corresponds to 6′-hydroxy-4,4′-dimethoxy-1,1′-dimethyl-5′-[(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy]-2,2′,5,6(1H,1′H)-tetraone, a hermidin derivative, which we named chrozophoridin. Experimental data and computational modeling studies show that this mono-glycosylated dimer is represented by two stable isomers (atropisomers). This is an indispensable piece of knowledge for the characterization of this medieval dye in works of art such as medieval manuscript illuminations and for testing its stability and contributes to the preservation of our cultural heritage.

**INTRODUCTION**

*Chrozophora tinctoria* in medieval and 19th century written sources

The use of the plant *Chrozophora tinctoria* (L.) A.Juss. to produce colors for illuminated manuscripts is extensively described in medieval written sources (1–4). What distinguishes *C. tinctoria* from other medieval natural sources to dye or produce paints is that, until now, the blue color structure remained elusive (5–7), despite the efforts by many groups in the last decades of the 20th century and into the 21st century (8–11). To tackle this mystery, our interdisciplinary group assembled a team of chemists who have expertise in natural products identification; conservation scientists, working in the re-production of medieval colors; and a biologist with a great deal of botanical and field knowledge of the Portuguese flora, who oversees plant sourcing. This interdisciplinary approach proved essential to solving the complex structure of the blue dye.

On the basis of the detailed descriptions that were selected from three medieval treatises, we planned the field expeditions and the sampling methods for collecting plant materials. Fruits were collected during July, August, and September 2017 and 2018 (unripe and ripe) in southern Portugal (Granja/Mourão). *C. tinctoria* extracts were prepared following the treatises’ instructions, and the main colorant was isolated, purified, and characterized through a multi-analytical approach: high-performance liquid chromatography–high-resolution mass spectrometry–diode array detector (HPLC-HRMS-DAD), gas chromatography–mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) [1H, 13C, correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), INADEQUATE], and electron paramagnetic resonance (EPR) (12, 13). On the basis of the experimental results, the molecular structure for the blue color compound has finally been solved and will be discussed in this paper. Theoretical calculations supported this assignment, and the predicted ultraviolet-visible (UV-VIS) absorption spectra overlapped well the experimental spectra. Other families of chromophores, present in minor amounts, were also detected and characterized by HPLC-HRMS.

*C. tinctoria* and its uses were well known in antiquity and medieval times. However, the practice of creating the blue color fell out of use and it was lost in the 19th century. Its medicinal properties were first described by Dioscorides (*De Materia Medica*, 1st century) and were also mentioned in medieval pharmacopoeia texts, and studies focusing on its anti-inflammatory properties have been published recently (14–16). The dyeing properties of this species and their applications are a fascinating subject that will be revisited, as it is relevant to this research.

In medieval times, the blue and purple solutions extracted from *C. tinctoria* were stored, after adsorption onto cloth and drying, as watercolors (clothlets), and were applied as paint by cutting a piece of cloth and extracting its color with the appropriate binding medium. Complete descriptions of the plant, when to collect it, and how it was processed are found in important medieval treatises such as The book on how to make all the colour paints for illuminating books (15th century), *Montpellier liber diversarum arcium* (14th century), and *Theophilus on divers arts* (12th century), hereafter referred to as *Book of all color paints* (1), *Montpellier* (2), and *Theophilus* (3). In these treatises, only the fruits were collected (Fig. 1). The paint thus obtained was named folium or tornasol (turnsole); this latter designation is common to the blue/purple watercolors obtained from...
lichen (e.g., \textit{Roccella tinctoria} and \textit{Lasallia pustulata}), and consequently, \textit{C. tinctoria} dyes remained forever interlinked to these lichens. Clarke, in his critical edition of texts written in the 14th and 15th centuries, goes further and suggests that the term tornasol was used generically to designate any water-based color stored in cloth (17). This had been already posited by Wallert (9) in his proposal to identify \textit{folium} in manuscript illuminations based on molecular fluorescence. Fortunately, we were able to select some medieval recipes to produce \textit{C. tinctoria} blues, because they are so detailed and offer accurate descriptions of the fruit and instructions to not break the fruits and free the seeds, thus pointing to the use of \textit{C. tinctoria} (and not of lichens). We also have 19th century documentary evidence that this medieval knowledge was preserved, until nearly the turn of that century, in the region of Grand-Gallargues (now, Gallargues-le-Montueux), in France. In 1842, Joly (18) published a clear, concise, and complete text on this subject, including references to earlier works such as Nissolle’s (19) publication that offers a precise description of the plant, accompanied with an equally accurate illustration. Joly’s text is remarkable in several aspects; first, he visits Grand-Gallargues to interview a priest by the name of Hugues and to gather information directly from the makers; precisely during the period when the plant should be collected (August/September), “\textit{vers la fin de septembre 1838, et dans les derniers jours du mois d’aôut 1839}.” According to Joly, in this region, this activity has been documented since 1600 (when records are available), but even so, according to Father Hugues, it is one of the most mysterious of crafts (18, 20):

“\textit{peu d’industries sont aussi mystérieuses: ceux qui l’exploitent n’en connaissent point la destination; ceux qui en profitent n’en connaissent point la préparation, et ceux qui l’ont décrite n’ont débité que des mensonges, parce qu’ils ne transcrivaient que de fausses indications}” [few industries are so mysterious: those who exploit it do not know its final applications; those who profit by it do not know how to prepare it, and those who have described it have only told lies, because they have only transcribed false directions] (18).

Joly describes the process using the entire plant to produce the dark blue color and the innovations that were introduced in the medieval process, and concludes that, at that time, the clothlets were mainly sold to dye cheese rinds red in the Netherlands (21): “\textit{mais il paraît que l’usage en est borné à donner aux croûtes du fromage de Hollande cette teinte rouge qui les distingue, (…) Il suffit de tremper les formages dans un baquet d’eau bleue de les chifrons, et de les en retirer presque aussitôt pour les faire sécher}” [but it seems that the use is limited to giving the rind of Dutch cheese that red hue that distinguishes it, (…) All you have to do is soak the cheese in a bucket of water dyed blue by the clothlets (rags), and to remove them almost immediately to left them dry] (18). Joly proposes that not only other species could be tested for dyeing more efficiently, such as other \textit{Chrozophora} species, but also \textit{Mercurialis perennis} (dog’s mercury) and \textit{Mercurialis tomentosa}. This is extraordinary, as now from this current project, we know that these dyes share a common molecular structure (Fig. 1). Joly also tested and concluded that the precursors for the blue may be found in all the parts of the plant and are more abundant in fruits. He concluded, “\textit{Sous l’influence de la vie, il existe dans ces organes à l’état incolore; après la mort du végétal, et sous l’influence de l’oxygène atmosphérique et d’une prompte dessiccation, il peut devenir bleu}” [Under the influence of life, it exists in these organisms in a colorless state; after the death of the plant, and under the influence of atmospheric oxygen and a rapid desiccation, it becomes blue] (18), which is precisely the way the appearance of the blue color in \textit{M. perennis} is described in more recent studies performed by Swan and Lorentz et al. (22–25). These authors have characterized the molecular structure for the blue chromophore as will be described below.

**Medicinal properties in \textit{Mercurialis} species and molecular structures for its blue colors**

In 1985, Swan proposed the first molecular structure for the “blue chromogen” isolated from \textit{M. perennis} (dog’s mercury), a perennial herb used in remedies for medicinal purposes (22, 23). Swan synthesized 2,3,6-trihydroxy-4-methoxy-1-methyl-pyridinium (fig. S1), a colorless compound in solution that he could oxidize to a blue transient color. In his own words: “To summarize, it is doubted that this chromogen could be the only source of the blue color. Later, in 2010 and 2014 (23, 24), the instability of the blue structure in solution was studied in detail by Lorenz et al. (Fig. 2). That research allowed this group to confirm the molecular structures proposed by Swan as the origin of the blue color in \textit{M. perennis} and to suggest interconversion mechanisms of the colorless hermidin into the blue hermidin quinone and the latter into yellow dimeric reduced forms, as described in detail in the Supplementary Materials and depicted in Fig. 2 (23, 24).

In 1984, Forrester had already reported that aqueous solutions of hermidin readily give rise to a transient blue radical-anion on
exposure to air, the identity of which has been established by electron spin resonance spectroscopy (25).

**Plant description**

*C. tinctoria* (L.) A.Juss. (Euphorbiaceae) is an annual herb native to the Mediterranean region, north Africa, and central and southwestern Asia. This species can be found on dry and disturbed lands, ruderal habitats, fallows, and along the edges of cultivated fields, mostly in limestone. The plants are 10 to 40 cm tall, gray-green, and tomentous (densely covered with stellate hairs). Stems are erect and branched and leaves are alternate, rhombic to ovate, cuneate at the base, and with sinuate leaf margin (26).

*C. tinctoria* is monoecious, bearing male and female reproductive organs in different flowers but on the same plant. Flowers are grouped into spike-like racemes, with male flowers at the top and female flowers at the base, usually solitary. Male flowers are yellow and inconspicuous. Female flowers exhibit a spherical ovary and petals are not present. Flowering occurs from May to September and fruit maturation is between July and October. Mature fruits are dark green, inconspicuous. Female flowers exhibit a spherical ovary and petals are not present. Flowering occurs from May to September and fruit maturation is between July and October. Mature fruits are dark green, inconspicuous. Female flowers exhibit a spherical ovary and petals are not present. Flowering occurs from May to September and fruit maturation is between July and October. Mature fruits are dark green, inconspicuous.

**Molecular structures for colorants in C. tinctoria**

In contrast to the successful discovery of the molecular structure for the blue in *M. perennis* (dog’s mercury), the works published for *C. tinctoria* have been inconclusive or have suggested incorrect structures. In 1997, Guineau (8) proposed orcinol for the blue color based on time-of-flight secondary ion MS data, which supported the previous suggestions of the presence of lichen colorants in *C. tinctoria* that were reviewed by Wallert (9). As we will show in this paper, orcinol-based structures are not present in the extracts of the fruits of *C. tinctoria* (27). More recently, the dye was investigated by Aceto’s group and, although they did not propose any structure for the blue dye, they reported that “the mass spectrum is dominated by a peak at 266 m/z” (10). Anthocyanins have also been proposed, but never identified (9).

In the past 2 years, we have endeavored to unlock the chemical structure behind the blue/purple color in *C. tinctoria*. This is an indispensable piece of knowledge for the preservation of our European cultural heritage, in works of art such as medieval manuscript illuminations where this dye could have been used.

**RESULTS**

**Characterization of the blue dye extracted from the fruits (shell) of C. tinctoria**

The blue extract of *C. tinctoria*, obtained from the fruits as reported in Materials and Methods, was analyzed using HPLC-DAD and LC-MS (Table 1 and fig. S3). The extract essentially showed the presence of one chromatographic peak characterized by a maximum absorption wavelength, $\lambda_{max}$, at circa 540 nm, indicative of a purple/bluish color, and an ion mass mass/charge ratio (m/z) at 501, in positive ion mode. The following fragments were also detected, at m/z 339, compatible with the loss of a hexose moiety (shown to be an O-glucose linkage), and at m/z 307, 279, and 254, in the MS$^3$ spectrum, consistent with the loss of methoxyl and methyl groups. In addition, an ion mass at m/z 334 with a MS$^2$ spectrum at m/z 172 (loss of 162 unified atomic mass unit) and a MS$^3$ structure with two fragments at m/z 157 and 115 were also detected in the extract. The ion mass m/z 172 and the respective fragments at m/z 157 and 115 were described in the literature by Lorenz et al. (23) and correspond to hermidin present in *M. perennis*. In this case, the ion mass at m/z 334 should correspond to the glycosylated form of hermidin (Fig. 1).

We did not observe a peak at 266 m/z, contrary to what was observed by Aceto et al. (10) that reported that in their extracts of *C. tinctoria* “the mass spectrum is dominated by a peak at 266 m/z.”

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*Fig. 2. Causes of color in hermidin extracted from *M. perennis*. Conversion of colorless hermidin into the blue hermidin quinone and formation of dimeric structures as proposed by Lorenz et al. (23, 24).*
Adding to this, several anthocyanins were also identified as minor compounds in the fruits’ extract, peonidin-3-O-glucoside, and the coumarylated derivatives of delphinidin, cyanidin, pelargonidin, and peonidin (Table 1 and figs. S3 and S4). These anthocyanins were tentatively identified by mass spectra and the chromatographic retention time compared to standards.

On the basis of its exact mass (501.1371), the elemental composition of the blue dye should correspond to C_{20}H_{24}O_{13}N_{2} (500.41 g mol^{-1}). On a first attempt, and due to the presence of a hermidin-glucoside in the extract, a hermidin-like structure was considered for it. Contrary to what was reported for the transient blue radical-anion of cyanohermidin in M. perennis (fig. S1), the blue extract from C. tinctoria was stable for several days, pointing to the absence of a radical species. Despite this indication, EPR experiments were performed and confirmed that no radical species were present in the aqueous solution of the purified compound.

To elucidate the structure of the blue dye present in C. tinctoria fruits, the purified compound was analyzed using one-dimensional (1D; 1H and 13C) and 2D (COSY, HSQC, HMBC and INADEQUATE) NMR in DMSO-d$_6$/D$_2$O (9:1). The assignment of the proton and carbon chemical shifts (Table 2) of the purified compound showed that no radical species were present in the aqueous solution of the purified compound.

The anomeric protons H-1’ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 3.13 and 3.20 parts per million (ppm), respectively. Protons H-2’, H-3’, H-4’, H-5’, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2). The anomeric protons H-1′ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, H-5′, H-6′a, and H-6′b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2).

The computational structure of the blue dye present in C. tinctoria fruits, the purified compound was analyzed using one-dimensional (1D; 1H and 13C) and 2D (COSY, HSQC, HMBC and INADEQUATE) NMR in DMSO-d$_6$/D$_2$O (9:1). The assignment of the proton and carbon chemical shifts (Table 2) of the purified compound showed the presence of two atropisomers (Fig. 3). The proposed structures correspond to two rotamers (atropisomer 1 and atropisomer 2), which were attributed to the signals present in the 13C experiment, with all the carbon signals appearing in duplicate and with the same intensity (Fig. 3). The anomeric protons H-1’ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 3.13 and 3.20 parts per million (ppm), respectively. Protons H-2’, H-3’, H-4’, H-5’, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2). The anomeric protons H-1′ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, H-5′, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2). The anomeric protons H-1′ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, H-5′, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2). The anomeric protons H-1′ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, H-5′, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2). The anomeric protons H-1′ from the two isomers were assigned to the two doublets (J = 7.7 and 7.5 Hz, respectively) present at 4.32 and 4.20 parts per million (ppm), respectively. Protons H-2′, H-3′, H-4′, H-5′, H-6’a, and H-6’b of glucose of both isomers were attributed to the signals present at 3.13, 3.10, 2.90, 3.16, 3.45, and 3.63 ppm, respectively, by their long-range correlations with each other in the COSY spectrum. Because of the superimposition of signals involving two similar compounds, it was not possible to accurately attribute the chemical shifts and coupling constants of all the protons for both isomers. The O-CH$_3$ protons present in carbon C-4 and C-10 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2).

The results are consistent with the structure of the blue compound corresponding to 6′-hydroxy-4,4′-dimethoxy-1,1′-dimethyl-5′-[[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy]-[3,3′-bipyridine]-2,2′,5,6(1H,1′H)-tetrone, a hermidin-mono-glycosylated dimer that we would hereafter designate as chrozophoridin (Fig. 3). This is a notable result, considering that previously, in M. perennis, the dimeric forms of hermidin were associated with yellow and yellow-brown solutions, whereas the blue color was based on a monomeric radical transient species. To get further insight into the proposed structure and as a means to predict its blue color, computational calculations were performed.

### Computational calculations

By exploring the conformational space of chrozophoridin, 10 energetically favored isomers (local minima) were found with ΔE < 1.0 kcal mol$^{-1}$ (table S1 reports their relative internal energy and both $\delta_1$ and $\delta_2$ values after optimization in an aqueous environment).
The various isomers of chrozophoridin can be assembled in two groups based on their dihedral values (group I composed of isomers 1, 3, 4, 5, 8, 9, and 10 that have similar dihedral values: $\omega_1 = -61.1 \pm 1.5^\circ$ and $\omega_2 = 83.0 \pm 0.9^\circ$, and group II composed by isomers 2, 6, and 7 that have similar dihedral values: $\omega_1 = 60.6 \pm 0.0^\circ$ and $\omega_2 = -80.9 \pm 0.3^\circ$). Each group can be represented by the most thermodynamically favored atropisomers 1 and 2. A mixture of both atropisomers is expected because of their very small energy difference (0.004 kcal mol$^{-1}$, which corresponds to 50.2% and 49.8% quantities of atropisomers 1 and 2, respectively, according to the Maxwell-Boltzmann distribution, and using a temperature of 298 K). The analysis of the molecular structures of both atropisomers in solution revealed that the electronic delocalization provided by the perpendicular orientation of both aromatic rings may be essential to their formation. However, atropisomer 2 has the B ring and the glucose group close to each other to make one hydrogen bond (as seen in Fig. 4). This stereochemical effect may cause the slight energy difference between the two isomers.

Time-dependent density functional theory (TD-DFT) calculations were performed to obtain the UV-VIS absorption spectra for the two atropisomers. $\lambda_{\text{max}}$ was evaluated using various density functionals (see table S4). It would appear that in comparison with experiments, the Boese-Martin for kinetics (BMK) density functional was the best-performing functional for both atropisomers (Fig. 4 compares their predicted UV-VIS spectra with an experimental spectrum at pH 7). Theoretical and experimental spectra overlap quite well. The small discrepancy might be the result of errors associated with the TD-DFT determination of $\lambda_{\text{max}}$ values (28) and from the use of an implicit solvent model approach instead of an explicit description of the solvent molecules. The latter may affect the intramolecular interactions and stability of the atropisomers (29). The spectral shift ($\Delta \lambda_{\text{max}}$) to experimental data obtained in water (pH 7) is $-23.8$ and $5.0$ nm for atropisomers 1 and 2, respectively (Fig. 4). Again, the hypsochromic shift observed in the maximum absorption wavelength for atropisomer 1 in relation to atropisomer 2 could be associated to the lower proximity between the B ring and the glucose unit. The $\lambda_{\text{max}}$ of absorption, in the visible, is essentially attributed to the electronic transition from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). In both conformers, the HOMO is spread through both A and B rings, while the LUMO is mainly distributed on B ring (fig. S5). The transition from HOMO to LUMO should have an intramolecular charge transfer character. Slight MO distribution differences are related with the marginally higher energy gap between HOMO and LUMO in isomer 1 in relation to isomer 2 (fig. S5). With respect to the stability of the blue color, it is interesting to note that the bond between the two aromatic rings may be viewed almost as a single bond (length of 1.464 Å in both conformers).

Overall, the UV-VIS spectra predicted for chrozophoridin corroborates that this dimer is the cause of the blue/purple colors (Fig. 4). Potentially, the blue color is the result of the abovementioned charge transfer.

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**Table 2. Essential NMR data for the identification of chrozophoridin.**

$^1$H and $^{13}$C chemical shifts of the blue dye present in *C. tinctoria* fruits, determined in DMSO-$d_6$:D$_2$O (9:1).

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta$ $^1$H (ppm); $J$ (Hz)</th>
<th>$\delta$ $^{13}$C (ppm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-N-CH$_3$</td>
<td>3.12; s</td>
<td>27.4/27.5</td>
</tr>
<tr>
<td>2 C=O</td>
<td>–</td>
<td>164.0/163.7</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>88.5/88.4</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>156.0/156.1</td>
</tr>
<tr>
<td>4-OCH$_3$</td>
<td>3.71; 3.78; s</td>
<td>59.2/59.4</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>122.0/122.3</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>159.0/158.8</td>
</tr>
<tr>
<td>B ring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-N-CH$_3$</td>
<td>3.12; s</td>
<td>27.3/27.5</td>
</tr>
<tr>
<td>8 C=O</td>
<td>–</td>
<td>161.7/161.6</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>128.4/127.7</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>161.1/161.0</td>
</tr>
<tr>
<td>10-OCH$_3$</td>
<td>3.75; 3.77; s</td>
<td>60.8/61.0</td>
</tr>
<tr>
<td>11 C=O</td>
<td>–</td>
<td>172.6/172.3</td>
</tr>
<tr>
<td>12 C=O</td>
<td>–</td>
<td>157.0/156.9</td>
</tr>
<tr>
<td>Glucose moiety</td>
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<td></td>
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<tr>
<td>1′</td>
<td>4.32; d, 7.7/4.20; d, 7.5</td>
<td>108.3/108.7</td>
</tr>
<tr>
<td>2′</td>
<td>3.13;*</td>
<td>74.1/74.1</td>
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<td>5′</td>
<td>3.16;†</td>
<td>76.9/76.9</td>
</tr>
<tr>
<td>6a′</td>
<td>3.45;†</td>
<td>61.3/61.5</td>
</tr>
<tr>
<td>6b′</td>
<td>3.63;†</td>
<td>61.3/61.5</td>
</tr>
</tbody>
</table>

*The carbon signals are duplicated, which indicates that at least two isomers are present. †Unresolved (superposition).
DISCUSSION

Knowledge was lost on how to extract the blue color from *C. tinctoria*, but it was recovered through research on medieval written sources on the art of painting, in particular, the art of illuminating books (30, 31). Following medieval instructions, only the fruits were collected between July and September, and care was taken to not grind the seeds that are found inside. The extracts thus obtained presented a blue dye as a major chromophore (fig. S3). Last, in the 21st century, an interdisciplinary approach led to the discovery of a molecular structure for the blue colorant extracted from the fruits of *C. tinctoria*, namely, a hermidin-based mono-glycosylated dimer (Fig. 3), named chrozophoridin. Experimental data and computational quantum mechanics studies show that this dimer is represented by two isomers, atropisomers 1 and 2 (Fig. 4). Together with the blue hermidin-based dimer, several anthocyanins were identified for the first time (Table 1 and figs. S3 and S4) and are present as minor compounds. Chrozophoridin was compared to the blue found in a species from the same family, *M. perennis* (dog’s mercury), an old medicinal plant known mostly through ethnomedicine (22). The blue chromophore in both species includes the hermidin ring (Fig. 1), but in contrast to the transient radical species in *M. perennis*, the blue in *C. tinctoria* has a stable glycosylated dimeric structure and is therefore water soluble. To summarize, this molecular structure is key to identifying folium in works of art and to studying the structural, electronic, and reactive properties of this complex dye (32, 33). This will pave the way for evaluating conservation conditions and the determination and planning of the best preservation strategies.

In conclusion, chrozophoridin was used in ancient times to make a beautiful blue dye for painting, and it is neither an anthocyanin—found in many blue flowers and fruits—nor indigo, the most stable natural blue dye. It turns out to be in a class of its own. Thus, we believe that this will be not our final word on this amazing plant and its story and that further discoveries will follow soon.

MATERIALS AND METHODS

The fruits of *C. tinctoria* were collected between July and September of 2016, 2017, and 2018, in Alentejo (Granja/Mourão), Portugal (by A.C., F.P., M.J.M., and P.N.). The coordinates were provided by (34). Fresh fruits (160 g) were extracted with 4 liters of methanol:water (70:30, v/v) during 2 hours under stirring, giving rise to a deep blue–colored solution. Methanol was evaporated under vacuum, and the obtained crude blue extract was analyzed by HPLC-DAD and LC-MS.

For the characterization of the blue dye, the extract was further purified. In a first step, by applying the full extract to a reversed-phase C18 silica gel in a Büchner funnel under vacuum (G3 porosity) washed with deionized water, it was possible to observe the elution of yellow compounds and then the blue fraction was recovered slowly with deionized water. This blue fraction was concentrated in a rotoevaporator and then was purified, twice, by low-pressure column chromatography using the same C18-RP gel as stationary phase (250 mm × 16 mm inside diameter) and deionized water as a mobile phase. The purified blue fraction was lyophilized, and the blue powder obtained was analyzed by NMR, HPLC-DAD, HPLC-DAD-MS, GC-MS, and EPR.
The initial geometry of chrozophoridin was built with the GaussView software (35–37). Geometry optimizations were carried out, and the most energetically favored isomers (local minima with ΔE < 1.0 kcal mol⁻¹) were further optimized in solvent. TD-DFT single-point calculations were applied to determine the UV-VIS absorption spectra for the two most energetically stable tautomers of chrozophoridin. All calculations were performed by the Gaussian 09 package (38). For comprehensive details, please see the Supplementary Materials.

**SUPPLEMENTARY MATERIALS**

Supplementary Materials for this article is available at http://advances.sciencemag.org/cgi/content/full/6/16/eaa7772/DC1

**REFERENCES AND NOTES**


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**Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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