CHEMISTRY

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 (^{1}H, ^{13}C, 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-2*H*-pyran-2-yl]oxy}-[3,3′-bipyridine]-2,2′,5,6(1*H*,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 reproduction 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) [^{1}H, ^{13}C, 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

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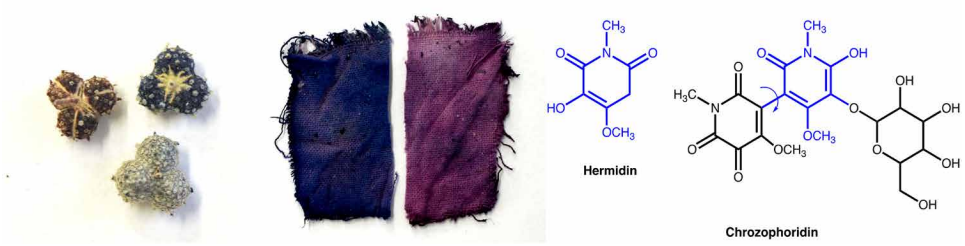

Fig. 1. The molecule of this study, chrozophoridin. Left: Close-up of *C. tinctoria* fruits (collected in Alentejo, Portugal) and clothlets prepared with the juice of the fruits following the instructions in the *Book of all color paints*. Light green fruits were used in this study shortly after collection. Right: Molecular structures of the blue colorants, hermidin (from *M. perennis*), and chrozophoridin (from *C. tinctoria*). Photo credit: Paula Nabais, Universidade NOVA de Lisboa.

lichens (e.g., *Roccella tinctoria* and *Lasallia pustulata*), and consequently, *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 folium in manuscript illuminations based on molecular fluorescence. Fortunately, we were able to select some medieval recipes to produce *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 *C. tinctoria* (and not of lichens). We also have 19th century documental 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), "*vers la fin de septembre 1838, et dans les derniers jours du mois d'août 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*):

"*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*): "*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 bleuie par les chiffons, 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 *Chrozophora* species, but also *Mercurialis perennis* (dog's mercury) and *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, "*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 *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 *Mercurialis* species and molecular structures for its blue colors

In 1985, Swan proposed the first molecular structure for the "blue chromogen" isolated from *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 suggested that the chromogen present in the colorless aqueous extract of *Mercurialis perennis* (...) is compound hermidin (2,3,6-trihydroxy-4-methoxy-1-methyl-pyridinium) and that on oxidation by air or $K_3[Fe(CN)_6]$ the blue transient color is due to a semiquinone-like (**17**), and that at the moment when the solution turns yellow it contains compounds (**16**), (**22**), and (**23**)." (fig. S1).

On drying, *M. perennis* plants take on a dark blue color. As Swan could only obtain a temporary blue in solution, this made him doubt 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 *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

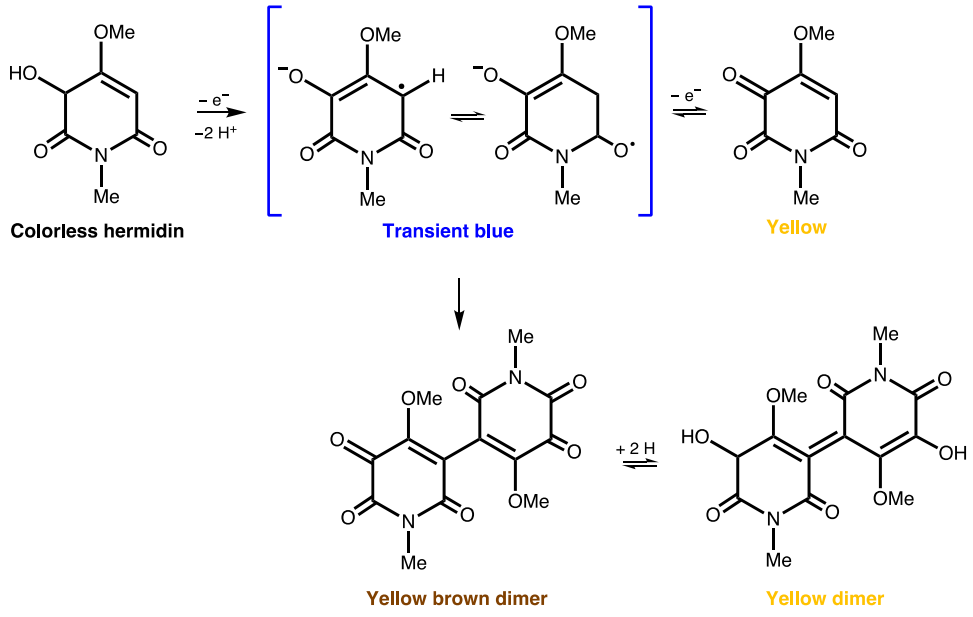

Fig. 2. Causes of color in hermidin extracted from *M. perennis*. Conversion of colorless hermidin into the blue hermidin quinone and formation of dimeric structures as proposed by Lorenz *et al.* (*23*, *24*).

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, subspheric, and slightly lobed capsules, covered with white scales and 5 to 8 mm in diameter (Fig. 1 and fig. S2). During dehiscence, each of the three single-seeded loculus opens into two valves, releasing three gray to light brown, rough-textured obovate seeds approximately 4 mm in size (*26*).

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 proposed 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, λ_{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 spectrum 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*."

Table 1. API-LC-ESI-MS/MS data for the major and minor compounds extracted from *C. tinctoria* fruits. Molecular ion and respective fragments MS^2 and MS^3 obtained by atmospheric pressure ionization (API)–LC–electrospray ionization (ESI)–MS/MS (positive ion mode) found in the blue extract of *C. tinctoria* fruits.

R_t (min)	Identity	$[M^+]$	$[MS^2]$	$[MS^3]$
8.29	Hermidin-glycoside	334	172	157
15.63	Peonidin-3-glucoside	463	301	
20.33	Hermidin-derivative (blue dye)	501	339	307; 279; 254
52.36	Delphinidin-3-coumaroylglucoside	611	303	
52.67	Cyanidin-3-coumaroylglucoside	595	449	287
60.07	Pelargonidin-3-coumaroylglucoside	579	433	271
64.23	Peonidin-3-coumaroylglucoside	609	463	301

Adding to this, several anthocyanins were also identified as minor compounds in the fruits' extract, peonidin-3-*O*-glucoside, and the coumaroylated 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 ^{13}C) and 2D (COSY, HSQC, HMBC and INADEQUATE) NMR in DMSO-d_6/D_2O (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 perceived in the ^{13}C 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 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 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 the singlets present at 3.75/3.77 and 3.71/3.78 ppm, respectively, and the protons *N*-CH_3 present in rings A and B of both isomers appeared at 3.12 ppm as a singlet.

The assignment of the carbon resonances was made possible using 2D NMR techniques (*g*HSQC, *g*HMBC, and INADEQUATE). Carbons C-1′, C-2′, C-3′, C-4′, C-5′, and C-6′ from the glucose moiety of atropisomers 1 and 2 were assigned to 108.3/108.7, 74.1/74.1, 77.4/77.5, 75.0/75.0, 76.9/76.9, and 61.3/61.5 ppm through their direct correlations with the respective protons. Carbons *O*-CH_3 (rings A and B) and *N*-CH_3 (rings A and B) from isomers 1/2 were assigned to 59.2/59.4, 60.8/60.1, 27.4/27.5, and 27.3/27.5 ppm (Table 2).

Carbons C-5 from both atropisomers were assigned to 122.0 and 122.3 ppm, by the long-distance correlation (HMBC) with the anomeric protons H-1′. Carbons C-4 and C-10 from both atropisomers were attributed to 161.1/161.0 and 156.0/156.1 ppm, respectively, by their long-range correlations with methyl groups. Carbons C-2, C-6, C-8, and C-12 from atropisomers 1 and 2 were attributed to 164.0/163.7, 159.0/158.8, 161.7/161.6, and 157.0/156.9 ppm, respectively, and by their long-distance correlation (HMBC spectrum) with the protons present at the *N*-methyl group.

We could not make an unequivocal assignment of carbons C-3 and C-9 through NMR, because these two carbons do not present any proton in their vicinity (Fig. 3). Moreover, the chemical shift of carbon C-11 could only be determined using a 2D ^{13}C-^{13}C INADEQUATE experiment, where the correlation between this carbon with carbon C-12 was observed. The appearance of carbon C-11 at high chemical shifts concurs with a carbonyl group in that position.

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-2*H*-pyran-2-yl]oxy}-[3,3′-bipyridine]-2,2′,5,6(1*H*,1′*H*)-tetraone, a hermidin-monoglycosylated 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 $\Delta E <$ 1.0 kcal mol^{-1} (table S1 reports their relative internal energy and both ω1 and ω2 values after optimization in an aqueous environment).

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_2O (9:1).

Position	δ ^{1}H (ppm); *J* (Hz)	δ ^{13}C (ppm)*
A ring		
1-N-CH_3	3.12; s	27.4/27.5
2 C=O	–	164.0/163.7
3	–	88.5/88.4
4	–	156.0/156.1
4-OCH_3	3.71; 3.78; s	59.2/59.4
5	–	122.0/122.3
6	–	159.0/158.8
B ring		
7-N-CH_3	3.12; s	27.3/27.5
8 C=O	–	161.7/161.6
9	–	128.4/127.7
10	–	161.1/161.0
10-OCH_3	3.75; 3.77; s	60.8/61.0
11 C=O	–	172.6/172.3
12 C=O	–	157.0/156.9
Glucose moiety		
1′	4.32; d, 7.7/4.20; d, 7.5	108.3/108.7
2′	3.13;†	74.1/74.1
3′	3.10;†	77.4/77.5
4′	2.90;†	75.0/75.0
5′	3.16;†	76.9/76.9
6a′	3.45;	61.3/61.5
6b′	3.63;	61.3/61.5

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

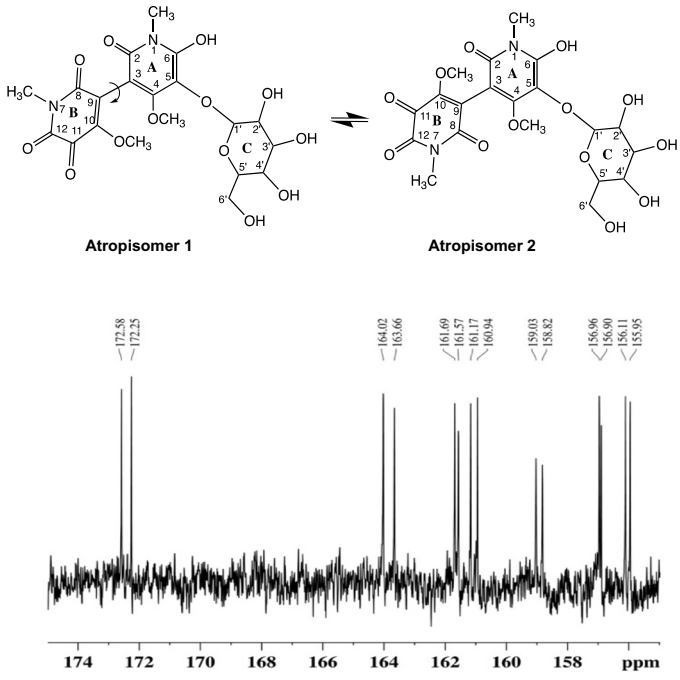

Fig. 3. Results of NMR (^{13}C). Structure for the blue dye present in *C. tinctoria* fruits (shell), chrozophoridin, and ^{13}C zoomed spectra (bottom).

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: ω1 = −61.1 ± 1.5° and ω2 = 83.0 ± 0.9°, and group II composed by isomers 2, 6, and 7 that have similar dihedral values: ω1 = 60.6 ± 0.0° and ω2 = −80.9 ± 0.3°). 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. λ_{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 λ_{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 ($\Delta\lambda_{max}$) 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 λ_{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.

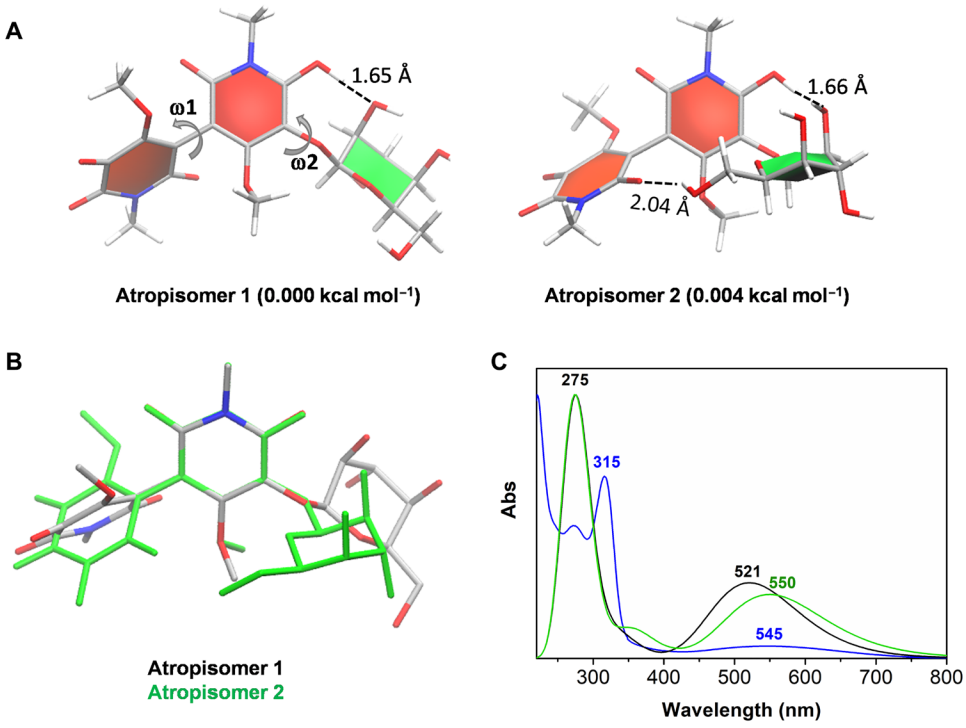

Fig. 4. Analysis of the thermodynamically favored atropoisomers of chrozophoridin, in solution. Molecules are depicted as sticks and colored by atom type. (**A**) A ring, B ring, and glucose ring are colored in red, orange, and green, respectively. Both ω1 and ω2 dihedrals and hydrogen bonds are also indicated. (**B**) Superposition of the A ring of the two molecules. Colored by element (atropisomer 1) and green (atropisomer 2). (**C**) UV-VIS spectra (water, pH 7, blue) for chrozophoridin compared with the predicted spectra [Boese-Martin for kinetics (BMK) functional] for atropisomers 1 (black) and 2 (green).

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 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 $\Delta E < 1.0$ 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 atropisomers 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/eaaz7772/DC1

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