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Isolation and Identification of 2,4,6-Trihydroxyphenanthrene as a Byproduct of *trans*-Resveratrol Photochemical Isomerization and Electrocyclization

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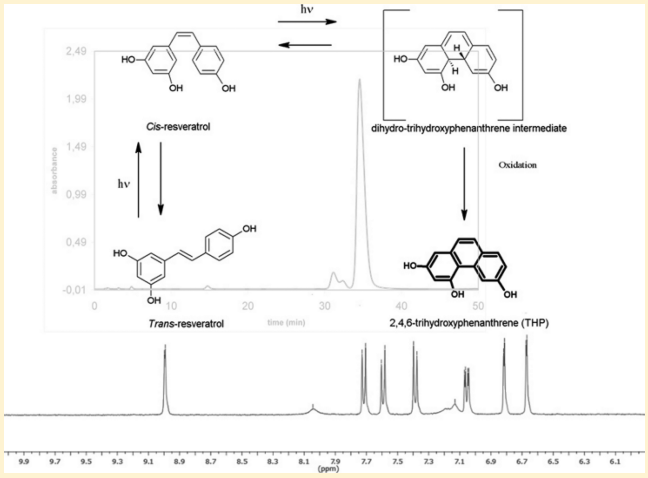
ABSTRACT: UV irradiation of *trans*-resveratrol leads to its photochemical isomerization and electrocyclization, giving rise to different byproducts. Preliminary attempts to purify and characterize these products were in the majority of cases unsuccessful. In the present work, the resveratrol photoreaction products were analyzed by HPLC, and one of these compounds, 2,4,6-trihydroxyphenanthrene (THP), was purified and unambiguously identified. The structure of THP was unequivocally characterized for the first time by combined GC−MS, ESI-MS/MS, NMR, and FT-IR analyses.



Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a bioactive polyphenol occurring in a variety of fruits and vegetables such as grapes, peanuts, and berries and in nonedible plants such as Japanese knotweed (*Poligonum cuspidatum*). Since its discovery, this molecule has attracted the interest of the scientific community because of its multiple biological and pharmacological activities. Indeed, resveratrol has strong antioxidant, antiinflammatory, cardioprotective, neuroprotective, chemopreventive, and antiaging properties.[1] Resveratrol occurs in *cis* and *trans* isomeric forms, the latter being obviously favored. The *trans* form can be converted into the *cis* form by exposure to sunlight for several hours or by UV irradiation.[2] The *cis*/*trans* isomerization, however, is not the only event that can occur after irradiation of *trans*-resveratrol. Other chemical species can be formed and have been reported to convey a strong biological impact.[3−5] As a matter of fact, the *trans*-resveratrol photodegradation pathway includes quinonoid and reactive radical species, whose identification is often elusive to spectrometric investigations.[6,7] Clues on the nature of resveratrol photooxidation intermediates have been inferred from previous studies on unsubstituted stilbene, which has been reported to undergo photoisomerization and oxidative photocyclization reactions similar to resveratrol.[8−10] The photocyclization process is a formal 6π electrocyclization and takes place when the triene system of *cis*-stilbene undergoes a $(4n + 2)$-electron conrotatory pericyclic ring closure. After ring closure, the formation of a dihydrophenanthrene species occurs. Dihydrophenanthrene is

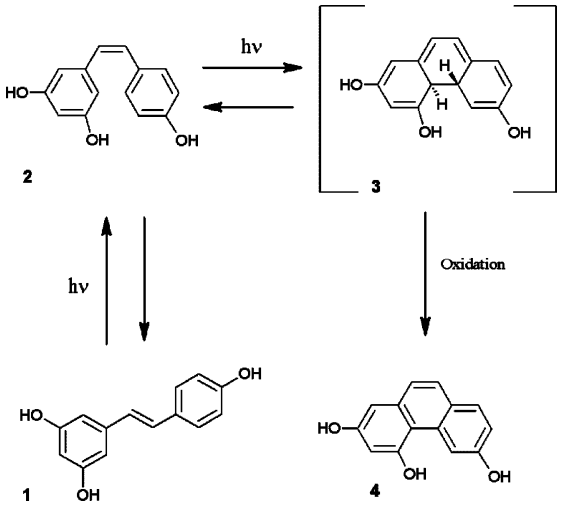


Figure 1. *trans*-Resveratrol photochemical isomerization and electrocyclization mechanism: (1) *trans*-resveratrol; (2) *cis*-resveratrol; (3) dihydrotrihydroxyphenanthrene (DHTHP); (4) 2,4,6-trihydroxyphenanthrene (THP).

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an unstable compound that rapidly relaxes back to *cis*-stilbene unless it is trapped by oxidation to form phenanthrene.[11,12] On the basis of structural analogies between stilbene and resveratrol, similar photochemical oxidation processes can be hypothesized for these two molecules (Figure 1).

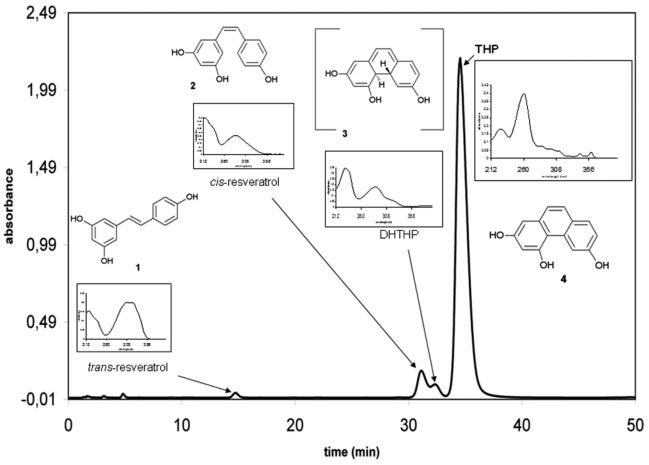

Figure 2. HPLC chromatogram (at 260 nm) and UV−vis spectra of main peaks after irradiation of *trans*-resveratrol for 24 h.

However, because of the presence of the three hydroxyl groups, photochemical reactions of resveratrol are more complex than those of stilbene. Indeed, the tendency of resveratrol to form quinonoid species may lead to the formation of polymers arising from the condensation of quinones, thus diverting substrates from the production of phenanthrenoid compounds. Therefore, the yield of 2,4,6-trihydroxyphenanthrene (THP) is low because of the formation of other byproducts. To date, THP formation has only been inferred on the basis of HPLC and LC−MS data, but no complete chemical analysis has been carried out to identify this compound unequivocally.[13] The aim of this work was to provide a method for the isolation and analysis of THP as a byproduct of resveratrol photooxidation and to carry out a complete spectrometric characterization of this compound.

Photochemical oxidation of stilbene has been reported to occur in cyclohexane as a solvent and using iodine as an oxidizing agent.[11,12] In our experimental setup, the reported method was optimized to the more hydrophilic nature of resveratrol. Resveratrol was dissolved in isopropanol and subjected to photoirradiation in the presence of I_2. After 30 h, the solution was centrifuged for 10 min at 1000*g* to separate the insoluble polymeric fraction, and then the supernatant was analyzed and purified by HPLC. As shown in Figure 2, the compounds that elute at 15 and 31.3 min are *trans*- and *cis*-resveratrol, respectively, as shown from their characteristic UV−vis spectra. *cis*-Resveratrol and the coeluting peak show similar absorbance spectra, namely, a maximum at 285 nm and a shoulder at 315 nm. In our experimental system, this coeluting peak disappeared when the irradiation was stopped. It can be hypothesized that this transient species is dihydrotrihydroxyphenanthrene (DHTHP), in agreement with the finding of several authors for dihydrophenanthrene.[14,15] The compound with a retention time of 35 min has a UV−vis spectrum closely related to that of phenanthrenes, with a maximum at 260 nm, a small shoulder at 315 nm, and two other absorption peaks at 360 and 366 nm. This feature suggests that this molecule is THP.

To confirm its structure, the compound was purified by HPLC and analyzed by GC−MS, ESI-MS/MS, ^{1}H NMR, ^{13}C NMR, and FT-IR. GC−MS analysis as the *tert*-butyldimethylsilyl ether derivative showed the presence of a single chemical species with a molecular mass of 568 Da, i.e. 2 Da less than that of resveratrol (Figure 3). These data also indicate that the hydroxyl moieties on the molecule were not oxidized to quinones. The ESI-MS/MS spectrum operating in positive-ion mode shows the $[M + H]^+$ ion signal at m/z 227.1 (Figure 4). The product ion spectrum, obtained by collision-induced dissociation of the parent ion, indicates major fragment ions at m/z 209.1 and 199.0 attributable to the $[M + H - H_2O]^+$ and $[M + H - 28]^+$ fragments, respectively. The neutral loss of 28 Da in CID experiments on phenanthrene derivatives has been observed by other authors and can be attributed to a loss of CO from the parent ion.[16]

The ^{1}H and ^{13}C NMR spectra in CD_3CN (Figure 5) display an array of signals in agreement with the hypothesized structure and

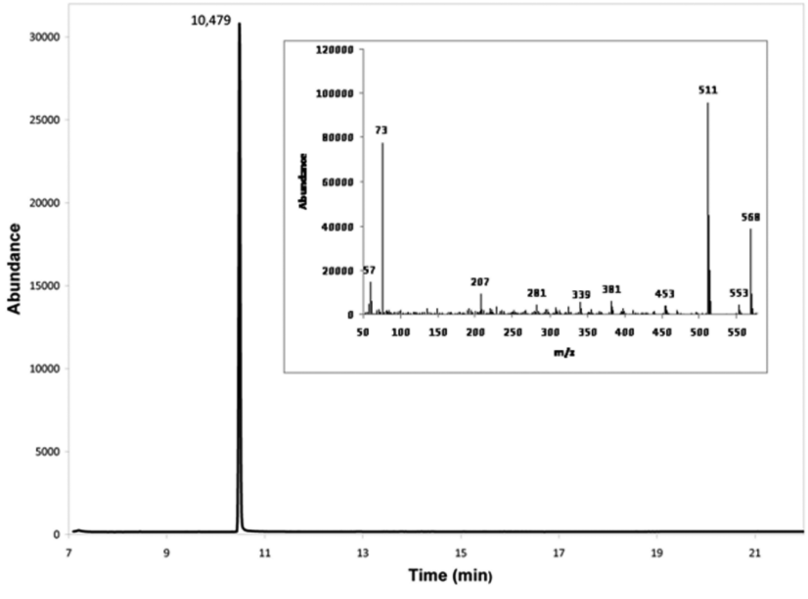

Figure 3. GC−MS chromatogram and EI-MS spectrum of purified THP (*tert*-butyldimethylsilyl ether).

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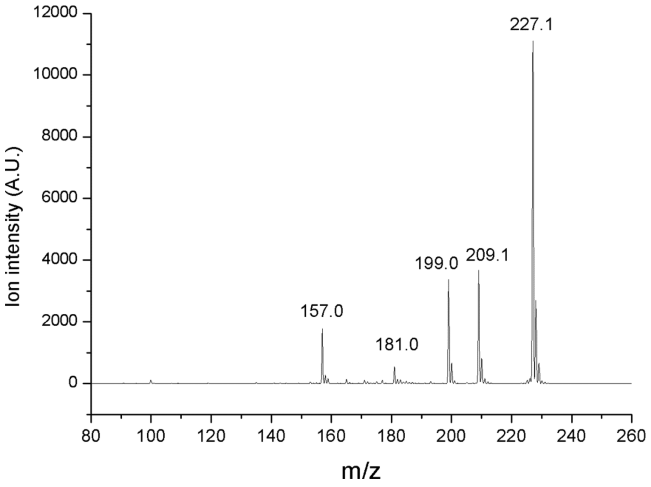

Figure 4. ESI-MS/MS fragmentation pattern.

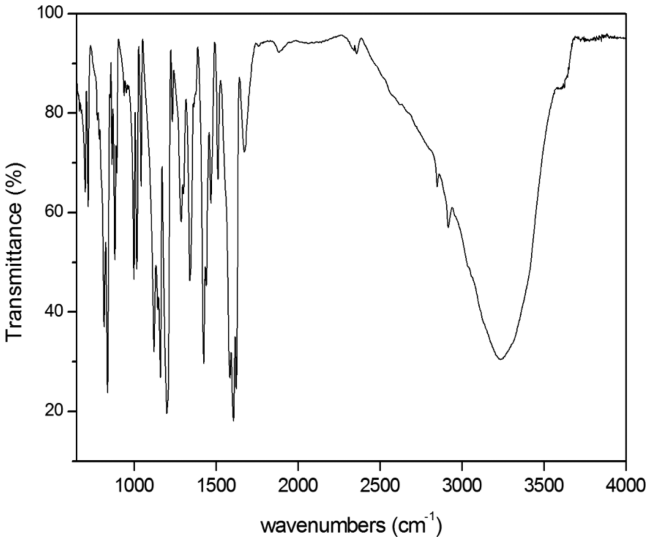

Figure 6. FT-IR (ATR) spectrum of dry THP.

consistent with literature data.[17] The broad signals at δ 8.04 and 7.13 in the ^{1}H NMR spectrum disappeared when D_2O was added to the sample, consistent with the presence of hydroxyl groups.

The FT-IR spectrum of THP (Figure 6) shows a large broad band between 3600 and 3200 cm^{-1}, corresponding to the O−H stretching of the hydroxyl groups. The spectral features in the region between 1600 and 1200 cm^{-1} are in agreement with those of phenanthrene, whereas the peaks at 1044, 1018, and 1000 cm^{-1} are due to the C−O stretches of the three phenolic moieties.[18]

The advantage of our HPLC elution system is the good separation of the *cis* isomer from its electrocyclization product, namely, THP formed via the nonaromatic intermediate DHTHP. Several authors have investigated the photochemistry of *trans*-resveratrol, but none of them gave a complete spectrometric pattern of THP produced from direct irradiation of *trans*-resveratrol.[5,7,13] In a recent paper, Rodríguez and co-workers aimed at purifying THP following photoirradiation of *trans*-resveratrol, but all of their efforts to isolate this compound in amounts large enough for complete characterization were unsuccessful.[19] The authors therefore relied on synthetic chemistry to obtain THP by protecting *trans*-resveratrol hydroxyl groups via methylation. Here, for the first time, THP has been produced by photoirradiation of *trans*-resveratrol in an amount high enough to allow a complete spectrometric characterization. Our work sheds light upon the photoisomerization and electrocyclization processes occurring during *trans*-resveratrol photoirradiation, which have been the subject of many studies with controversial results.[5] On the other hand, the unequivocal demonstration of the production of phenanthrenes arising from *trans*-resveratrol photooxidation raises some concerns regarding the increasing use of this compound in cosmetic products and medical devices. Nowadays, *trans*-resveratrol is widely used in a variety of products as an antiaging and antioxidant agent, many of which are exposed to solar UV radiation for a long time in direct contact with human skin. It is noteworthy that *trans*-resveratrol has been recently demonstrated to enhance solar-UV-induced responses in normal human epidermal keratinocytes by

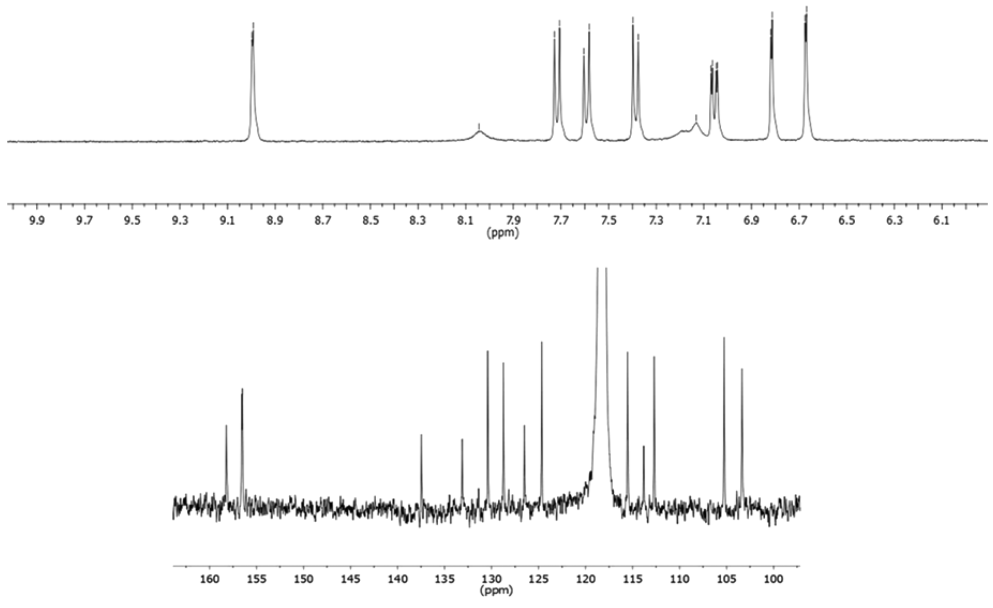

Figure 5. (top) ^{1}H and (down) ^{13}C NMR spectra in CD_3CN.

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activating transcription factors leading to deregulated inflammatory, metabolic, and proliferative responses in the cells.[20]

In conclusion, this work offers for the first time complete experimental evidence of the direct formation of THP after photochemical isomerization and ring-closure reactions of resveratrol. Because of the potential toxic effects of polycyclic aromatic hydrocarbons, this method represents a good tool to analyze the resveratrol derivative THP in biological, pharmaceutical, environmental, and food samples. This work also represents an important reference for further *in vivo* investigation of THP in view of its possible secondary biological effects on human health.

EXPERIMENTAL SECTION

Reagents. All reagents and solvents used for chromatography were analytical- and HPLC-grade products.

UV Irradiation and HPLC Separation. *trans*-Resveratrol (200 mg) was dissolved in 40 mL of isopropanol containing 5 mg of I_2 in a glass Petri dish. Irradiation was carried out in air for 30 h at a distance of 20 cm from the irradiation source, a 14.7 W UV-B fluorescent tube emitting at wavelengths of 270−320 nm with a peak at 313 nm. Chromatographic analysis of the photoreaction products was performed by an HPLC system consisting of a Waters apparatus equipped with a 600 pump and pump controller, a Symmetry C18 column (reversed-phase, 3.9 mm × 150 mm, 5 μm particle size, with a 10 mm guard column of the same material), and a model 2996 UV−vis photodiode array detector. The separation method was set up by modifying our previously described HPLC method.[21] Elution was performed at a flow rate of 1 mL/min in isocratic mode with 80% solvent A (0.1% trifluoroacetic acid in water) and 20% solvent B (acetonitrile). The putative THP peak was directly analyzed via GC−MS and then collected and lyophilized for MS, NMR, and FT-IR experiments.

GC−MS. A 0.5 mL aliquot of the purified HPLC peak was saturated with NaCl and extracted with ethyl acetate (2 mL). The organic extract was dried under reduced pressure and derivatized with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (70 °C, 0.5 h). GC−MS analyses were performed with a gas chromatograph coupled to a quadrupole mass-selective detector. Chromatographic analyses were carried out with a fused-silica capillary column (30 m × 0.25 mm i.d.) coated with 5%-phenyl/95%-dimethylpolysiloxane (film thickness 0.25 μm) as the stationary phase. Injection mode: splitless at a temperature of 260 °C. Column temperature program: 70 °C for 1 min, then to 300 °C at a rate of 15 °C/min, and held at 300 °C for 5 min. The carrier gas was helium at a constant flow rate of 1.0 mL/min. The spectra were obtained in electron impact mode at an ionization energy of 70 eV with an ion source temperature of 280 °C and an ion source vacuum of 10^{-5} Torr. MS analysis was performed simultaneously in TIC mode (mass range scan from m/z 50 to 650 at a rate of 0.42 scans s^{-1}) and SIM mode (selected ions: m/z 511, 568, and 381).

ESI-MS/MS and HRMS. The CID MS experiments were performed on a linear ion trap mass spectrometer equipped with an ESI source and a syringe pump. Operating conditions of the ESI source were as follows: ion spray voltage = +4.0 kV; sheath gas = 5 (arbitrary scale); sweep gas = 5 (arbitrary scale); capillary temperature = 275 °C. Methanolic solutions of THP (1×10^{-4} M) were infused via a syringe pump into the ESI source at a flow rate of 5 μL/min. ESI of the solutions led to the formation of appreciable amounts of the corresponding proton-bound species $[THP\text{-}H]^+$, which was isolated and submitted to CID (normalized collision energy between 14% and 17%) by collisions with He gas into the trap (nominal pressure, 1.4×10^{-5} Torr; activation time = 30s; activation Q = 0.20). In each acquisition, the final spectrum was the average of about 40 scans, each consisting of two microscans. High-resolution MS spectra were obtained with an ESI-LTQ Orbitrap mass spectrometer. ESI was employed at a flow rate of 10 μL/min. MS (ESI): m/z 227 $[M + H]^+$, high-resolution ESI-MS orbitrap, calculated for $C_{14}H_{11}O_3^+$, 227.0698, 227.0703 found.

^{1}H/^{13}C NMR. THP NMR spectra were recorded at 400 and 100.6 MHz. Chemical shifts are reported in parts per million from TMS with the solvent resonance as the internal standard (deuterochloroform: δ 7.27 for ^{1}H and δ 77.0 for ^{13}C).

A white solid, 14 mg (yield ∼7%). ^{1}H NMR (400 MHz, CD_3CN): δ 8.99 (d, J = 2.1 Hz, 1H), 8.04 (br s, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.59 (d, J = 8.8 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 7.13 (br s, 2H), 7.06 (dd, J = 8.8, 2.4 Hz, 1H), 6.82 (d, J = 2.4 Hz, 1H), 6.67 (d, J = 2.1 Hz, 1H). ^{13}C NMR (100 MHz, CD_3CN): δ 158.2, 156.7, 156.6, 137.5, 133.3, 130.6, 128.9, 126.7, 124.8, 115.6, 113.9, 112.9, 105.3, 103.5.

FT-IR. The infrared spectrum was measured on an infrared spectrometer equipped with an ARK attenuated total reflectance device. The internal reflection element was ZnSe. Spectra were recorded at 4 cm^{-1} resolution with a DTGS detector.

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Notes

The authors declare no competing financial interest.

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