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Caulobacter segnis Dioxygenase CsO2: A Practical Biocatalyst for Stilbenoid Ozonolysis

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Ozonolysis is a useful as well as dangerous reaction for performing alkene cleavage. On the other hand, enzymes are considered a more sustainable and safer alternative. Among them, *Caulobacter segnis* dioxygenase (CsO2) known so far for its ability to catalyze the coenzyme-free oxidation of vinyl-guaiacol into vanillin, was selected and its substrate scope evaluated towards diverse natural and synthetic stilbenoids. Under optimized conditions, CsO2 catalyzed the oxidative cleavage of the C=C double bonds of various *trans*-stilbenes, providing that a hydroxyl moiety was necessary in *para*-position of the phenyl group (e.g., resveratrol and its derivatives) for the reaction to take place, which was confirmed by modelling studies. The reactions occurred rapidly (0.5–3 h) with high conversions (95–99%) and without formation of by-products. The resveratrol biotransformation was carried out on 50–mL scale thus confirming the feasibility of the biocatalytic system as a preparative method.

Introduction

Oxidative alkene cleavage is a widely employed synthetic tool for introducing oxygen-functionalities (e.g., alcohols, acids, aldehydes, ketones) into molecules, removing protecting groups, or degrading large compounds.[1–3] Furthermore the synthesis of a large quantity of bioactives involves the cleavage of alkenes as a key step to obtain the corresponding carbonyl derivatives. Among the arsenal of oxidation methods available to date, ozonolysis is the most employed as it is considered an efficient reaction. However, it requires drastic conditions such as very low temperature (−78 °C), the use of special equipment (ozoniser) as well as the presence of reducing agents during the work-up procedures.[1,4] Moreover safety hazards due to the formation of explosive ozonide intermediates, complicate the employment of this reaction on preparative scale.[5] Alternative chemical procedures mainly rely on heavy metals such as Cr, Ru, Os as catalysts; these reactions are typically characterized by mediocre yields and low selectivity.[4] In the view of a more sustainable manner to perform alkene cleavage *via* oxidation, enzymes have been considered due to their safe and benign reaction conditions (e.g., ambient temperature, atmospheric pressure, aqueous environment), as well as the possibility to functionalize complex molecules in a regio-, chemo- and stereospecific fashion, allowing biocatalysis to compete with conventional chemistry.[6,7] Additionally, enzymes can efficiently activate the most innocuous oxidant, molecular oxygen. The rising popularity of natural products during the last decade has triggered off significant efforts in the development of biocatalytic processes especially for the preparation of ketones and aldehydes employed as aroma-compounds.[8–10] In fact, bioprocessing natural substrates through the use of whole-cell microorganisms or pure enzymes allow for the obtainment of products claimed as natural following European and US regulation. The tag "natural" was one of the main reasons for seeking biotechnological routes to obtain high-value natural flavours such as vanillin or cinnamaldehyde,[11–13] and nowadays biocatalysis represents a convenient alternative for their green preparation. The enzymes able to perform alkene cleaving are divided in heme and non-heme iron-dependent proteins.[4] The first group includes peroxidases and oxygenases requiring either hydrogen peroxide or molecular oxygen as oxidants. In the case of peroxidases the alkene cleavage is observed just as a side reaction.[1,4] Non-heme iron-dependent oxygenases constitute a heterogeneous group of enzymes able to activate molecular oxygen in a different manner; it was also hypothesized that the same enzyme can cleave olefinic functionalities through different mechanisms depending on the starting material. This is the case of carotenoid cleavage dioxygenases (CCDs)[14] which are among the most studied dioxygenases, being involved in the catabolism of carotenoids in many microorganisms and plants. The search for CCD homologues led to the discovery of enzymes that, albeit not able to cleave

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C=C of carotenoids, cleave α-β double bond of stilbene derivatives (lignostilbene dioxygenases, LSDs).[15] One of the first LSDs was found in *Sphingomonas paucimobilis*,[16] and subsequently an LSD from *Novosphingobium aromaticivorans* (NOV1) was characterized.[17] NOV1 is able to catalyze the cleavage of double bonds of a wide range of stilbene-like compounds with a 4′-OH group. Its structure showed that during the oxidation reaction the 4′-OH group is coordinated by Y101 and K134 residues,[17,18] while O_2 is bound to Fe^{2+}. Among the CCDs, *Caulobacter segnis* dioxygenase (CsO2) was firstly identified by Furuya and co-workers with the aim of designing a 2-step cofactor-free biocatalytic cascade to convert ferulic acid to 4-vinylguaiacol *via* decarboxylation, and the latter to vanillin through oxidative alkene cleavage (Figure 1).[19–21]

Among the 3 different proteins identified and expressed from *Caulobacter segnis*, CsO2 demonstrated higher catalytic efficiency and represented a typical case of dioxygenase originally classified as CCD, but working also on substrates different from carotenoids (e.g., 4-vinylguaiacol, isoeugenol).[19,21] The performances of CsO2 were subsequently improved by site directed mutagenesis with the obtainment of mutants with enhanced activity (Q390 A) or improved thermostability (A49P).[20]

A dioxygenase from the thermophilic fungus *Thermothelomyces thermophila* (Ado) was found after a BLAST search based on CsO2 sequence and it was also used for the transformation of vinylguaiacol into vanillin.[21]

Due to the interesting properties of CsO2 as biocatalyst, an in-depth study, especially focused on stilbenoids, was here performed to better investigate its substrate scope. Particular attention has been paid to the potential of CsO2 as preparative biocatalyst. The high substrate-to-catalyst ratio (5 10^3 ($mmol_{substrate}/mmol_{enzyme}$) and the cofactor independence make this process an efficient and cost-effective procedure leading to a sustainable and selective ozonolysis-like reaction. To further assess enzymatic selectivity and substrate recognition computational studies have been carried out.

Results and Discussion

Gene cloning and CsO2 preparation

Expression of CsO2 was firstly studied using a pET21(a) vector to obtain a protein with a C-terminal His-tag. The resulting plasmid was then inserted in *E. coli* BL21 (DE3) cells, for a classic expression mediated by isopropyl-β-D-thiogalactopiranoside (IPTG) under the control of T7 promoter, producing a largely insoluble fraction.[19] Improvements have been observed through co-expression with the chaperonin gene *groEL* and the co-chaperonin gene *groES* (plasmid pGRO, Takara Bio), although better results were obtained using pETDuet-1 to yield a gene product without the His-tag, completely losing the possibility for a rapid purification *via* conventional ion affinity chromatography.[17] A change of the vector system (pET26b(+)), with consequent change of the position of the His-tag (from C- to N-terminus), gave a stable and soluble CsO2 in good yields (50 mg starting from 3.0 g of wet cell paste) (Figure S1). Although protein purification is often considered a cost and time-consuming technique, the advantages of using purified enzymes with respect to recombinant whole cells often overcome its limitations. Firstly, a close contact between the substrate and the catalyst is guaranteed as no physical barrier (i.e., cellular membrane) is present, thus allowing fast reactions and high substrate loading. Moreover, all unnecessary pathways typical of whole cell systems deeply impacting on the overall final yield are avoided, giving rise to more selective and highly productive biotransformations. Furthermore, CsO2 does not need any cofactor addition, which is one of the main reasons for using microbial cell systems.

Detection of the CsO2 specific activity by a fast and sensitive colorimetric assay

The detection of CsO2 enzymatic activity with a sensitive colorimetric assay avoiding HPLC-based labor-intensive and time-consuming methods so far utilized for dioxigenases has been developed.[22–24] The spectrophotometric assay is based on the oxidation of 2,6-dimethoxyphenol (2,6-DMP) a canonical substrate for the activity determination of oxidoreductases such as laccases,[25] aromatic peroxygenases,[26] and manganese peroxidase[27] with the formation of the orange product coerulignone absorbing at 469 nm (Figure 2, and Figure S2). The activity assay consists of a 2-step reaction: the formation of 2,6-DMP radicals which dimerize into hydrocoerulignone in the presence of H_2O_2, and its subsequent oxidation into coerulignone.[27] The rapidity and simplicity of this method make it a valuable tool for the quick assessment of CsO2 activity, stability as well as characterization.

The assay performed at pH 7.5 gave the best results and was used for enzyme characterization (Figure S3–S5) showing

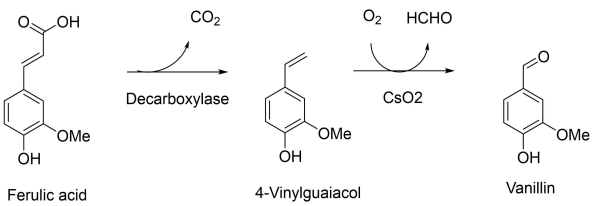


Figure 1. Vanillin synthesis from ferulic acid *via* 4-vinylguaiacol.

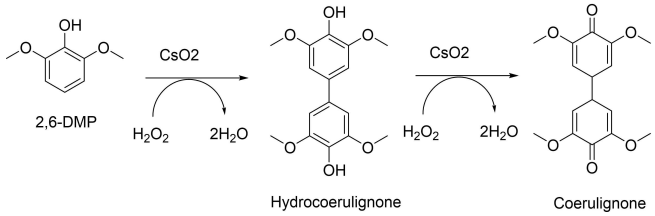


Figure 2. CsO2 activity assay at 469 nm employing 2,6-DMP in 50 mM Tris HCL buffer pH 7.5 with 20 μL CsO2 (6 mg/mL), 20 μL H_2O_2 (500 mM), 100 μL 2,6-DMP (100 mM). Negative control: colorless; CsO2 assay: orange.

that the optimal temperature was in the range of 20–30 °C. Storage conditions were also evaluated; since the enzyme lost around 80% of its initial activity after 72 h at 4 °C, freezing just after purification was preferred. Solubility of organic substrates is often a major issue in aqueous biotransformations; therefore, the influence of different co-solvents on CsO2 activity was studied (Table S1). Acetone maintained the same initial enzymatic activity, which was negatively affected by the other water-miscible solvents, including DMSO which was previously used for CsO2-catalyzed oxidation of 4-vinylguiacol.[17–20]

Investigation of CsO2 substrate specificity

Based on the previous experiments, purified CsO2 (1 mg/mL) was used at 25 °C, pH 7.5 in the presence of 10% acetone for the biotransformation of different unsaturated substrates (10 mM) (Table 1), including 4-vinylguiacol as standard substrate.

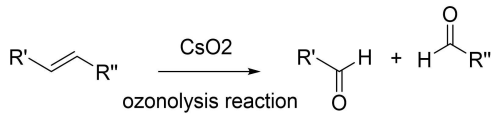
4-Vinylguiacol and isoeugenol (Entries 1, 2, Table 1), gave vanillin with almost complete conversion and rapid reactions (> 99% and 95% in 2 h and 3 h, respectively). This represents an improvement with respect to the previously reported CsO2 biotransformations characterized by long reaction times (24–32 h), high enzymatic loading (7 mg/mL), and lower productivity (8–8.4 mM starting from 10 mM substrates).[17–20] A crucial structural determinant seems to be the occurrence of a hydroxyl group in *para*-position of the aromatic ring, both for its recognition in the active site and the ability to stabilize the intermediate radical.[20] Although with ferulic acid derivatives (Entries 3, 4, Table 1),), allylglycine (Entry 5, Table 1), and 4-nitrochalcone (Entry 6, Table 1) CsO2 did not show any reaction, the enzyme demonstrated to cleave resveratrol with high performance (> 99%, 1 h).

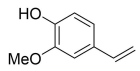
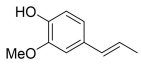
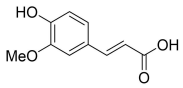
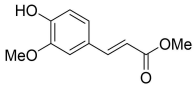
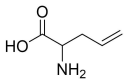
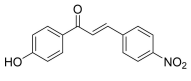
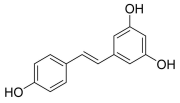
Considering these results, biotransformation conditions were evaluated using resveratrol as a model substrate. Optimal enzyme loading was firstly investigated (Figure S5) showing that even decreasing 10-times the enzyme concentration (0.1 mg/mL), CsO2 was able to completely cleave resveratrol (10 mM) in the corresponding aldehydes in only 1.5 h. This high catalytic activity further decreases the process-related costs and makes this strategy not only sustainable when compared with traditional chemistry, but also economically appealing. The reaction carried out in different buffers at different pH occurred with a total conversion between 5.5–8.5, showing the high reactivity of this enzyme even when neutral and mildly acidic conditions are employed. The biotransformation was then re-evaluated on 4-vinlyguaiacol and isoeugenol under optimized conditions showing conversions above 95% after 1.5 and 4 h, respectively.

The new conditions were used to check the reactivity of CsO2-mediated ozonolysis (Table 2) on a panel of differently decorated stilbenoids.

CsO2 showed high reactivity and selectivity towards *trans*-stilbenes presenting a hydroxyl moiety in *para*-position of the phenyl group such as resveratrol and its derivatives (Entries 1–4 Table 2), which were cleaved with complete conversion in short times (> 99% m.c., 1.5 h). The only exception was represented by 3,5-diacethyl-resveratrol (Entry 7, Table 2), wherewith no biotransformation was observed, although the prolonged reactions. Piceatannol and analogues (Entries 10, 11, 12, Table 2), whose aldehydes were obtained with 95% conversion in 2 h, demonstrated to be well accepted by the enzyme. To the best of our knowledge, this is the first time a CDD enzyme shows such a wide substrate scope, making CsO2 an interesting and versatile catalyst. Previously reported stilbene-α-β-oxygenase such as the ones from *Novosphingobium aromaticivorans* NOV1 and NOV2 displayed selectivity for a few stilbene molecules such as resveratrol and piceatannol or rhapontigenin and rhaponticin, respectively. To further establish the synthetic applicability of CsO2-mediated ozonolysis-like reaction a preparative scale biotransformation (50 mL) was carried out. By employing 10 mM resveratrol (114 mg), 0.1 mg/mL of the catalyst (5 mg) in Tris-HCl buffer pH 7.5 the corresponding 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde were obtained with excellent yields (62 and 60 mg, respectively) in 1.5 h.

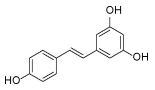
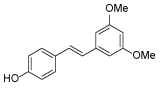
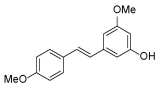
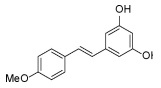
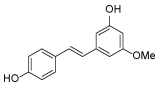
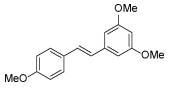
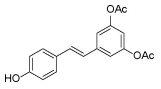
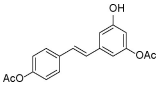
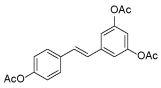
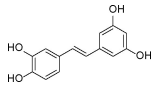
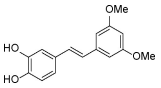
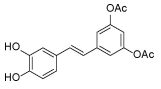
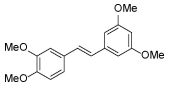
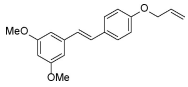
Table 1. CsO2 substrate scope.



Entry	Substrate	m.c.[a]	t (h)[b]
1		> 99%	2
2		95%	3
3		–	24
4		–	24
5		–	24
6		–	24
7		> 99%	1

Reactions conditions: 10 mM substrate, 1 mg/mL enzyme, 25 °C in Tris HCl (50 mM, pH 7.5). To increase the substrate solubility 10% acetone was employed as co-solvent. [a] m.c. = conversion; [b] Time corresponding to the maximum conversion. No reaction was observed by adding the substrates in the same reaction conditions without the catalyst.

Table 2. CsO2-mediated ozonolysis reaction with different stilbenes.

Entry	Substrate	m.c.[a]	t (h)[b]
1		>99%	1.5
2		>99%	1.5
3		–	24
4		–	24
5		>99%	1.5
6		–	24
7		–	24
8		–	24
9		–	24
10		>99%	2
11		95%	2
12		95%	2
13		–	24
14		–	24

Reactions conditions: 10 mM substrate, 0.1 mg/mL enzyme, 25 °C Tris HCl (50 mM, pH 7.5). To increase the substrate solubility 10% acetone was employed as co-solvent. [a] m.c. = molar conversion; [b] Time corresponding to the maximum conversion. No reaction was observed by adding the substrates in the same reaction conditions without the catalyst.

Analysis of stilbenes binding modes by molecular docking

To gain deeper insights into CsO2 substrate specificity, we analyzed the binding mode of the tested stilbenes reported in Table 2 through molecular docking calculations. As shown in Figure 3A, compounds 1, 2, 5, 10, 11, and 12, Table 2, which were subject to the catalytic reaction, bind within CsO2 site with a mean distance of approx. 4.3 Å from the *sp2* carbons to the reactive Fe ion, confirming the feasibility of their oxidation. Substrates are stabilized by aromatic π-π interactions with His220 (part of the non-heme group that coordinates the catalytic iron), Phe307 (part of the lid-like loop that covers the catalytic site), and Phe61; multiple hydrogen bonds, involving Tyr103, Lys136 and Glu137, further stabilize oxygen atoms of the substituted aromatic rings. These latter electronic interactions may also be necessary to create an electronegative gradient stabilizing the reaction intermediates. Conversely, non-substrates (Figure 3B) including 3,5-diacethyl-resveratrol could not be docked within CsO2 catalytic site due to steric hindrance, and were shown to bind upwards in the substrate channel in a pose that is unlikely to be subject to the oxidation reaction. The only exception to this behaviour is represented by compounds 3 and 4, Table 2, which could instead bind in a similar fashion to substrates; however, their lack of reactivity may be explained by electronic effects, as a *para*-hydroxyl group is missing in these molecules.

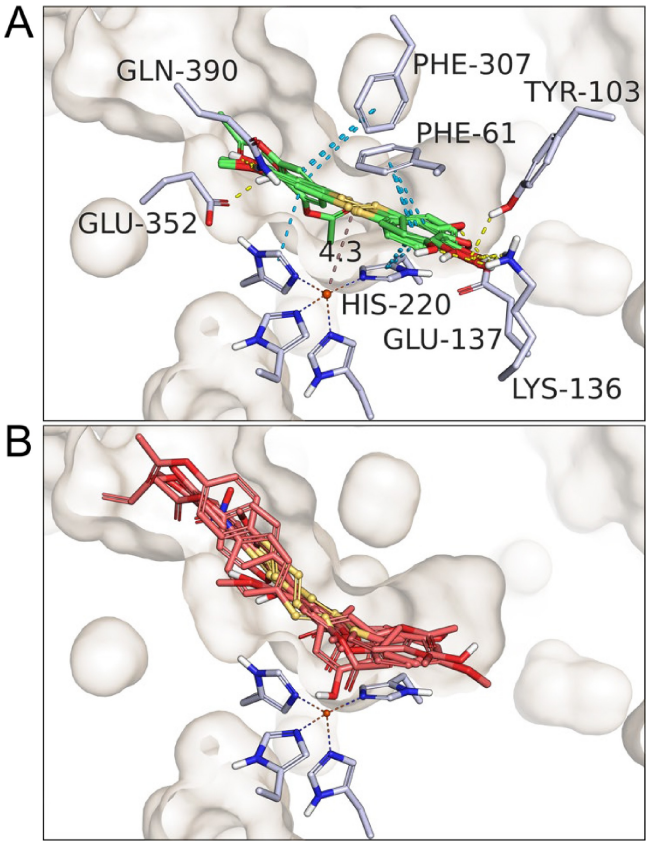


Figure 3. Binding modes of substrates *vs* non-substrates. A) Superposition of identified substrates (green) within CsO2 catalytic site. Stabilizing π-π interactions (cyan dashes) and hydrogen bonds (yellow dashes) are shown. Reactive *sp2* carbons (yellow spheres) are shown in proximity of the catalytic non-heme iron. B) Superposition of non-substrates binding modes; most of the non-reactive molecules appeared to wedge in the substrate channel (grey surface) due to steric hindrance, and were unable to correctly position themselves within CsO2 catalytic site.

Conclusions

Although chemical methods for alkene cleavage are well established, more efficient strategies requiring less hazardous reagents are demanded. Besides safer and greener processes, more selective methodologies are required. In this work, we presented a highly stable carotenoid cleavage dioxygenase (CCD) from *Caulobacter segnis*, so far utilized just for the synthesis of vanillin from 4-vinylguaiacol or isoeugenol. By expanding its substrate scope, we discover its ability to perform ozonolysis-like reaction towards stilbenes, being extremely selective for *para*-hydroxy-substituted ones. Due to the mild reaction conditions (water as medium, atmospheric pressure, room temperature) as well as the high enzymatic performance, CsO2 can be considered an environmentally benign alternative to conventional chemistry for the obtainment of carbonyl compounds from stilbene-type substrates. Its activity and characterization have been here deeply explored, thanks to the optimization of a quick and simple colorimetric assay based on 2,6-DMP, thus facilitating the investigation of the optimal reaction conditions. By optimizing its purification yield and stability as pure protein through simple strategies (*E. coli* codon optimization, cloning vector, his-tag position) and considering the low enzymatic concentration (0.1 mg/mL) employed in the reactions, we made CsO2 an appealing catalyst not only for its sustainability but also from an economic point of view. As a proof of concept to further demonstrate the potential of this enzyme a 50–mL scale biotransformation towards resveratrol was carried out giving the corresponding aldehydes with excellent yields in short reaction time (98% yield, 1.5 h).

Experimental Section

Chemicals and reagents

Cell growing and strain maintaining media as well as commercially available reagents were purchased from Thermo Fischer Scientific or Merck (Sigma Aldrich). Organic solvents and chemical standards were bought from Merck (Sigma Aldrich). Merck Silica gel 60 F254 (aluminum foil) plates were used for TLC analysis; flash column chromatography was performed on Merck Silica gel (230–400 mesh). Detection of TLC analyses have been performed under UV light at 254 and 365 nm or revealed by a solution of anisaldehyde (2%) and H_2SO_4 (1%) in EtOH.

Synthesys of stilbenoid substrates

Resveratrol and pterostilbene are commercially available (Merck Life Science, Milano, Italy); Differently substituted stilbenoids (Table 2, substrates 1–14) were synthesized according to reported procedures.[29–31]

CsO2 cloning, expression, and purification

The gene coding for *Caulobacter segnis* dioxygenase (CsO2) was codon optimized for expression in *E. coli*, synthetized with N-terminal his-tag by BaseClear into vector pET-26b(+). Recombinant expression of CsO2 was achieved by employing BL21 (DE3) as host strain. For overexpression pET-26b-CsO2 plasmid together with pGro7 plasmid (Takara, Otsu, Japan) were transformed into chemically competent *E. coli* BL21 (DE3) and incubated overnight at 37 °C in Luria–Bertani (LB) agar medium supplemented with 25 μg/mL kanamycin and 35 μg/mL chloramphenicol. Precultures (20 mL) of LB medium (25 μg/mL kanamycin and 35 μg/mL chloramphenicol) were inoculated overnight from single colonies at 37 °C, 110 rpm. CsO2 expression was carried out inoculating 0.1 OD of the precultures in 200 mL LB medium supplemented with L-arabinose (20 g/L) kanamycin (25 μg/mL) and chloramphenicol (35 μg/mL). Protein expression was induced at 0.6–0.8 OD by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM and the culture was incubated overnight at 28 °C, 160 rpm. Bacterial cells were centrifuged at 5000 rpm for 30 min, washed with deionized water and stored at −20 °C until use. For purification, cell pellets were resuspended ice-cold Tris-HCl buffer 50 mM, NaCl 100 mM, imidazole 6 mM pH 8.0, and sonicated for 10 min (1 min on, 1 min off) using a Soniprep150 ultrasonicator (MSE, London, UK). Cell debris were removed by centrifugation (15 min, 4 °C, 8000 g), clear supernatant was filtered using 0.45 μm cellulose acetate filters. CsO2 purification was carried out employing ÄKTA Purifier (GE Healthcare) equipped with a 1 mL column HisTrapTM FF (GE Healthcare) equilibrated with $CuSO_4$. Pure protein was eluted with elution buffer (Tris-HCl buffer 50 mM, NaCl 100 mM imidazole, 250 mM pH 8.00) and dialyzed overnight against Tris-HCl buffer 100 mM, pH 8.00. Purity of dialyzed CsO2 was analyzed by SDS-PAGE 11% acrylamide gel (Figure S1).

CsO2 quantification and its activity assay

Protein concentration was determined by Bradford assay by measuring the absorbance at 595 nm of a solution containing the correct amount of protein sample opportunely diluted in water with 1 mL of Bradford reagent. A standard curve was built with the BSA protein. Activity measurements were performed spectrophotometrically at 469 nm by determining the formation of coerulignone at 30 °C in a half-microcuvette (total volume 1 mL) for 5 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 μmol of 2,6-DMP per minute under reference conditions, namely 20 μL CsO2 (6 mg/mL), 20 μL H_2O_2 (500 mM), 100 μL (100 mM) 2,6, DMP in 860 μL of 50 mM Tris HCl buffer, pH 7.5. Specific activity was 50 mU/mg.

CsO2 characterization

For CsO2 characterization please check Supplementary information.

CsO2 biotransformations

Batch reactions using CsO2 were performed in 10 mL screw cap tubes; 1 mL reaction mixture in 50 mM Tris-HCl pH 7.5, containing 10 mM substrate, 1 mg/mL or 0.1 mg/mL enzyme were left under magnetic stirring at 25 °C. 100 μL aliquots were extracted at different times (0.5, 1. 2, 4, 6, 24 h) with 100 μL of EtOAc for TLC analysis (n-hexane/EtOAc 7:3). Samples, after evaporation, were resuspended in the mobile phase for HPLC analysis.

Analytical methods

HPLC analysis

Biotransformations of stilbenes mediated by CsO2 were analyzed using a liquid chromatographer equipped with a pump system, autosampler, UV-VIS detector (Merck-Hitachi L-7400) and RP C18 (LichroCART 250×4.6 mm) as a column. Analyses were carried out

by employing the following gradient: 30/70 (*v/v*) H_2O milliQ/ACN for 15 min ($t_{0\ min} \rightarrow t_{15\ min}$) and increasing the ACN percentage till 100% ($t_{15\ min} \rightarrow t_{25\ min}$). Flow rate: 0.8 mL/min, $\lambda = 250$ nm. Molar conversions were calculated integrating the peak areas of substrates and products (See supplementary information for details).

NMR spectroscopy

NMR spectra were recorded on a Brucker Avance 300 MHz spectrometer or JEOL ECZ400R/S3 400 MHz spectrometer, employing the residual signal of the deuterated solvent as internal standard. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in Hertz (Hz) (See supplementary information for further details).

Molecular modelling

A model of CsO2 was generated *via* homology modelling, using the crystallographic structure of *P. brassicacearum* dioxygenase (PDB: 5V2D) [10.1186/s12858-018-0098-4] as template, which displayed a 73% sequence similarity. Energy-based homology modelling was carried out using Prime (Prime, Schrödinger, LLC, New York, NY, 2021). Stilbenes were docked in the cavity around the catalytic iron using Glide (Glide, Schrödinger, LLC, New York, NY, 2021) with standard-precision (SP) setting. NOE restraints with a radius of 6 Å were applied between the enzyme Fe and the reactive alkene carbons of the compounds to enrich for binding poses that could be representative of the first step of the oxidative reaction.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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