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A photoexcited porphyrin system as a biomimetic catalyst for D-limonene biotransformation

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A photoexcited porphyrin system has been found to be an efficient catalyst for D-limonene biotransformation. The catalyst showed high selectivity in hydroxylation of D-limonene. The best catalyst for limonene biotransformation was 5,10,15,20-Tetraphenylporphyrin (H_2TPP), which gave the highest accumulation of carvone and an unknown product with a verbenone-like mass spectrum. The highest conversion yield of these products was favoured at 1:2 molar ratio of H_2TPP to limonene. Some factors affecting the biotransformation yield were also investigated. Maximal yield of carvone was obtained in the medium containing 90% of the substrate, within the period of 18–36 h. Mechanism involved in limonene biotransformation catalysed by H_2TPP is also discussed.

KEY WORDS: biomimetic oxidation catalysts; biotransformation; D-limonene; porphyrin; uv–vis spectroscopy; photooxidation.

1. Introduction

The preparation, characterisation, and use of new catalysts as the analogues of the active sites in metalloenzymes (design, synthesis, structure, and catalytic activity) is a research area currently named “biomimetic catalysis”. The interest in the use of metalloporphyrins in biomimetic catalysis has been constantly growing during the last decades, as enzymology studies have been showing the role of these complexes as prosthetic groups of various transition metals containing enzymes (cytochromes P-450, peroxidases, and catalase) [1–3]. In particular, a number of research groups have focused their interest on the ability of metal porphyrins to catalyse redox processes in organic and inorganic compounds [4–7]

In recent years, a number of authors have reported some examples of catalytic systems based on the use of photoexcited Fe^{III}porphyrins that can induce hydrocarbon oxygenation under aerobic conditions [4,5]. In pure cyclohexane, high yields of cyclohexanone with small amounts of cyclohexanol as a by-product were obtained [6]. Other alkanes can also be successfully oxidised to the corresponding ketones. [4,7]. In the literature, only a few reports concerning the transformation of limonene into limonene epoxide by manganase porphyrins can be found [8,9].

Terpenes, widely distributed in nature, constitute suitable precursor substrates for potential production of valuable natural flavour and fragrance compounds. Monoterpenes, as substrates of microbial transformations, have led to a great variety of oxyfunctionalised compounds [10,11]. Among various monoterpenes, limonene is a cheap and readily available starting material, utilised for biotransformation into more valuable fragrance compounds like carvone, perillyl alcohol and α-terpineol. R-(+)-limonene, *p*-mentha-1,8-diene, can be obtained from orange peel oil in annual amounts of up to 50 million kg. They are widely distributed in many volatile oils, in some (e.g., citrus oils) as the main constituent, and widely applied in flavouring of cosmetics, soap, and many kinds of technical cosmetic substances [10].

A number of microorganisms have been reported to transform limonene to other monoterpenoid compounds [12–15] yet the amounts obtained (a few milligrams per litre range) were insufficient for industrial applications. In addition to the lack of effective biotransformation systems for the production of valuable natural flavour and fragrance compounds, terpene transformations generally suffer from volatility of the substrate and toxicity of terpenes towards microorganisms as well as their insolubility in water. In our study, we have utilised enhanced solubility of porphyrin in organic solvents for limonene transformation.

In our previous study, a novel method for enzymatic biotransformation of D-limonene to carvone was developed [16]. The present paper is the first report of a porphyrin-based photocatalytic system for D-limonene biotransformation. A photocatalytic system of this sort containing free porphyrin without metal for D-limonene biotransformation using dioxygen at atmospheric pressure, has not been previously reported. Our aim is to

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show that photochemical excitation is an appropriate and clean method of porphyrin-based biomimetic catalysis.

2. Experimental

2.1. Chemicals

(−)-Linalool (>97%), R-(+)-limonene, (−)-carveol and (−)-carvone were purchased in purity (>99%) from Fluka. 5,10,15,20-Tetraphenylporphyrin, 5,10,15, 20-Tetra(4-pyridyl) porphyrin, 5,10,15,20-Tetra-*p*-totyl-porphine, 2,3,7,8,12,13,17,18-octaethyl-porphine, Hematoporphyrin IX were purchased from Aldrich and used without any additional purification. Tetraphenylporphyrin zinc(II) and tetraphenylporphyrin cobalt(II) complexes were synthesised according to the procedure described earlier in the literature [17]. All reagents and solvents were of analytical grade.

2.2. Measurements

Uv–vis measurements were carried out using a Carl Zeiss-Jena M42 spectrophotometer with 1 mm Hellma quartz cells. Spectra were recorded between 200 and 900 nm at a temperature of 21 ± 1 °C. Absorption spectra were recorded digitally and the SigmaPlot (Jandel Corp.) program was used in manipulation and plotting the data.

2.3. Biotransformations procedure and analysis

Catalytic oxidation of R-(+)-limonene with dioxygen was carried out at room temperature and atmospheric pressure (using oxygen dissolved in the system as oxygen-atom donor) in closed glass fials. In typical run, 1% of R-(+)-limonene (corresponding to 8.4 g/L and 123.3 μmol) and 8.1×10^{-4} mol catalyst were dissolved in 2 mL of $CHCl_3$ and excited (photoirradiated) with sunlight. After specified period of time 500 μL of a standard solution of 0.05% (v/v) linalool in hexane was added to the equal volume of reaction medium and mixtured. Subsequently, progress of reaction (in terms of the selectivity and conversion) was monitored using Varian GC system fitted with DB-5 capillary column. The reaction products were analysed using a Phinigan DSQ spectrometer coupled to Trace GC Ultra Chromatograph equipped with a RTX-5 column. The GC and GC/MS analyses conditions were the same as reported previously [16]. Substances were identified by comparison of their mass spectra and retention indexes with those of authentic sample to spectra in a standard library database system. Quantification of the volatile compounds was done by comparison with added standard.

Reference chemical blank experiments were performed in the same way, but in the darkness, to ensure the absence of not photochemical transformation reactions or without catalyst to exclusion possibility of chemical transformation reactions in the presence of light during specified period of time.

Biotransformations were performed in two replicate samples and analyses carried out in duplicate. The data given here are the average of measurements.

3. Results and discussion

The initial investigation was aimed at specifying the potential of different porphyrin catalysts for limonene biotransformation. Experiments confirmed that limonene oxidation by H_2TPP does not take place in the absence of light indicating, a photochemical process (both control reactions run in the absence of light and in the absence of catalyst do not give observable oxidation). GC-MS data showed that each porphyrin catalysed oxidation of limonene to two main products, identified as carvone and an unknown product with a verbenone-like mass spectrum **(1)**. The catalytical activities of the few porphyrins with different properties (listed in table 1) were checked. The best catalyst for limonene biotransformation was 5,10,15,20-Tetraphenylporphyrin (H_2TPP), which gave the highest yield

Table 1
The catalytical properties of the analysed porphyrins

Porphyrin $C_M = 8.1*10^{-4}$ mol/L	Yield of carvone [mg/L]	Carvone productivity [mg/L/h]	Yield of **(1)**[mg/L]	Yield of **(2)** [mg/L]	Limonene [mg/L]
I. 5,10,15,20-Tetraphenyl porphyrin	371.4	20.6	1340.3	198.4	3371
II. 5,10,15,20-Tetra(4-pyridyl) porphyrin	165.4	9.2	851.4	933.8	4365.5
III. 5,10,15,20-Tetra-*p*-totyl-21 H, 23 H-porphine	178.1	9.9	980.3	1602.6	2198
IV. 2,3,7,8,12,13,17,18-Octaethyl-21 H, 23 H-porphine	157.9	8.8	732.6	688.7	5218
V. Tetraphenylporphyrin zinc(II) complex	72.9	4.0	319.3	101.4	7900
VI. Tetraphenylporphyrin cobalt(II) complex	Traces	–	Traces	Traces	8400
VII Hematoporphyrin IX*	340	18.9	1057.5	4.0	3601.3

Conversion time: 18 h; solvent: $CHCl_3$.
*Experiment was done in acetone, as hematoporphyrin IX is not soluble in chloroform.

of carvone and **(1)** (over 0.37 g/L and 1.3 g/L respectively). Hematoporphyrin IX was not soluble in chloroform. When dissolved in acetone, an insignificantly lower yields of carvone (about 10%) and **(1)** (over 20%) were obtained. It is worth noting that the amount of an unidentified new product **(2)** (with a longer retention time – 17.2 min) was over 50-fold lower in comparison with the biotransformation using H_2TPP. Hematoporphyrin IX and H_2TPP were the cheapest catalysts among those used in the experiment. Other porphyrins gave a lower yield of biotransformation products. Pyridyl porphyrin (II) and aryl porphyrin (III) with relatively longer substituents had a two times lower efficiency of catalysis. Also Zn(II) and Co(II) tetraphenyl porphyrins had lower catalytical properties. Such a "negative" result was caused by the strong electron-donor properties of the pyridyl group and, on the other hand, by the growing electron withdrawing properties of the metalloporphyrins. The diminishing of the catalytical effect for *p*-totyl porphyrin can be explained by steric effects of the "bulky" methyl groups. However, the use of 5,10,15,20-Tetra-*p*-totyl-porphine as biocatalyst gave the highest concentration of the unidentified product **(2)**.

In further studies, H_2TPP was used to improve the yield of carvone and **(1)**. Among the products analysed after transformation with H_2TPP were also *cis*- and *trans*-limonene-oxide, *trans*- and *cis*-carveol, perilla aldehyde, and perilla alcohol, which was accumulated in a lower concentration (figure 1). Accumulation of different biotransformation products may indicate that the examined porphyrins utilise more than one pathway of limonene conversion. The GC/MS analysis revealed two main peaks, which were correlated with the appearance of carvone and **(1)**. MS data of the unindentified products are summarised in table 2. Product **(1)** obtained from limonene was probably an isomer of verbenone or 6,6-dimethylcycloocta-2,4-dienone (figure 1) while **(2)** could be a mentha-derivated hydroperoxide. The allylic hydroperoxide products of the reactions are the important building blocks for synthetic organic chemistry, allowing an efficient introduction of oxygen into a variety of organic substrates [18]. The two main bioconversion products [carvone and (probably) verbenone **(1)**] are of particular importance due to their high bulk prices, which for carvone are at least in the range of 30–60 U.S. $/kg [11]; the cost of verbenone can reach 3000 U.S. $/kg [19].

Generally, high concentrations of organic solvents are toxic to biological systems [20]. To determine the optimum concentration of limonene for maximum bioconversion, the products formation was measured over a range of 0.05–100% (v/v) limonene (corresponding to 6.2×10^{-2} mol/L – 6.17 mol/L). The optimum substrate concentration for carvone and **(1)** production was 90% (v/v). An increase in limonene concentrations (to 100%) brought a decrease in carvone and **(1)** production (over 2-fold) (table 3). With a longer photoirradiation time (about 72 h), we obtained carvone and **(1)** in concentrations of over 3.4 and 6 g/L respectively from a solution of 90% (v/v) limonene (data not shown). It is worth noting that a large excess of an easily oxidised substrate (e.g., alkene) provides kinetic protection against oxidative destruction of the catalyst [21].

Absorption spectra of H_2TPP-limonene systems in chloroform (1 mm spectrophotometric cell) before and after exposition to sunlight are presented in figure 2. In every case, two main bands were found in the region of 400–450 and 500–650 nm which are known as the porphyrin Soret and Q bands, corresponding to the absorption transition of $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_1$, respectively. The Soret band arises from allowed electronic transitions and is about 15 times more intensive than the Q band, which occurs as a result of forbidden electronic transitions. A comparison of the porphyrin spectra before sunlight irradiation shows that they are essentially the same for the solutions containing various amounts of limonene (1, 5, 20, 50 and 100%) and neither band position changes, nor changes in peak intensity are observed. This is an additional proof that without photoirradiation, the reaction of limonene with porphyrin does not take place. However, after irradiation, important changes in peak intensity and wavelengths are observed. The Soret bands are red shifted from 418 to 445 nm, and their intensity is lower after irradiation than before it. The Q-bands undergo changes that are more complex – the appearance of new 670 nm bands can be observed. Such a change in the spectrum occurs only for the systems containing 1, 5 and 20% of limonene. For the systems with a constant amount of porphyrin and 50, 90 and 100% of limonene, a serious diminishing of the Soret and Q bands is observed. This suggests that two different mechanisms of limonene reaction take place, and perhaps both of them have an influence on the transformation. For the systems with a lower limonene concentration, a proton accepting mechanism is more important. It is well known that porphyrins undergo the following protonation equilibria:

$$H_4P^{2+} \rightarrow H_3P^+ + H^+ \tag{1}$$

$$H_3P^+ \rightarrow H_2P + H^+ \tag{2}$$

$$H_2P \rightarrow HP^- + H^+ \tag{3}$$

$$HP^- \rightarrow P^{2-} + H^+ \tag{4}$$

At room temperature and in acidic conditions, the central nitrogen atoms of the N_4 porphyrin moiety are protonated in two steps according to the equations (1) and (2). In the spectrum of H_2TPP-low concentration limonene, two Soret subbands can be observed. Such spectral changes suggest that in low-limonene-concen-

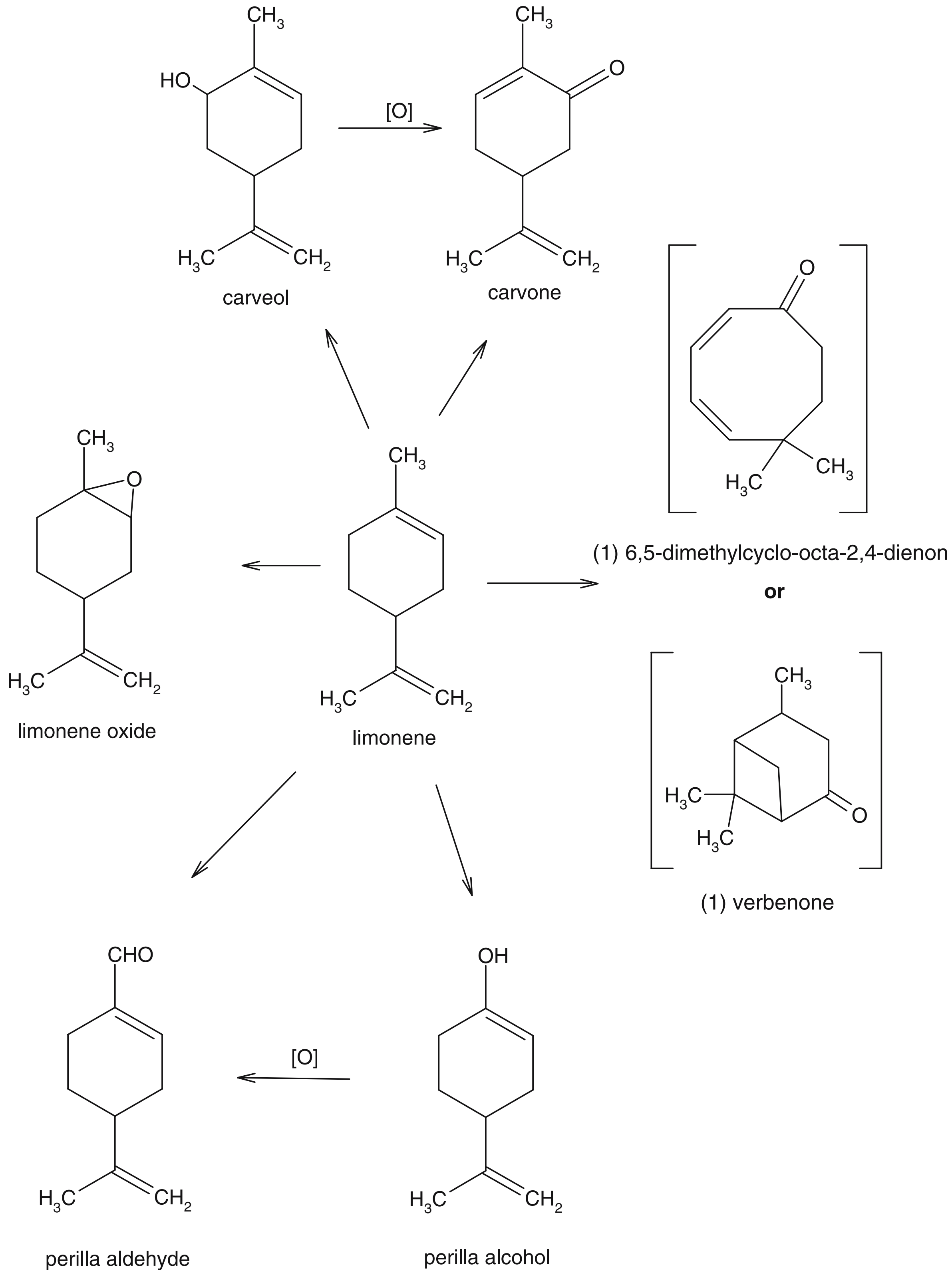

Figure 1. Products of limonene phototransformation in the presence of H_2TPP catalyst.

tration systems porphyrin exists in a protonated form of dication H_4P^{2+}. This mechanism is additionally proved by the fact that metalloporphyrin as a photocatalyst has a lower efficiency of limonene transformation. In the metalloporphyrins, all the protons from the N_4 moiety are removed when metal-nitrogen bonds are formed.

Table 2

Mass spectra of the identified unknown monoterpene products of limonene phototransformation by porphyrins in $CHCl_3$ solution

M/e with relative intensities in brackets (%)	
(1)	39(27); 40(12); 41(23); 42(4); 43(4); 50(3); 51(8); 52(5); 53(34); 54(17); 55(12); 56(2); 62(1); 63(3); 64(1); 65(12); 66(10); 67(42); 68(18); 69(14); 70(3); 71(1); 74(1); 76(1); 77(29); 78(11); 79(100); 80(29); 81(40); 82(9); 83(3); 84(1); 89(1); 90(3); 91(48); 92(12); 93(60); 94(22); 95(16); 96(5); 97(1); 103(3); 104(2); 105(16); 106(13); 107(89); 108(73); 109(22); 110(4); 111(1); 115(2); 116(1); 117(5); 119(8); 120(2); 121(11); 122(33); 123(6); 124(2); 131(1); 132(2); 133(1); 134(4); 135(51); 136(5); 137(2); 149(2); 150(34); 151(4)
(2)	38(1); 39(14); 40(3); 41(15); 42(2); 43(28); 44(1); 45(1); 50(2); 51(6); 52(4); 53(9); 54(2); 55(17); 56(1); 57(2); 58(2); 59(1); 62(1); 63(4); 64(2); 65(12); 66(5); 67(15); 68(4); 69(10); 70(1); 71(3); 74(1); 75(1); 76(1); 77(38); 78(11); 79(68); 80(15); 81(17); 82(3); 83(2); 84(4); 85(1); 89(2); 90(1); 91(70); 92(16); 93(100); 94(40); 95(15); 96(2); 97(1); 102(1); 103(5); 104(3); 105(25); 106(9); 107(99); 108(15); 109(31); 110(4); 111(1); 115(4); 116(1); 117(7); 118(2); 119(24); 120(6); 121(21); 122(4); 123(7); 124(2); 131(1); 132(1); 133(2); 134(17); 135(87); 136(14); 137(14); 138(1); 150(1); 152(1)

Table 3

Dependence of the catalytic properties of the H_2TPP from the limonene concentration. Reaction mixture was exposed for the sunlight for 18 h

Limonene concentration [%]	Yield of **carvone** [mg/L]	**Carvone** productivity z(mg/L/h)	Yield of **(1)**[mg/L]	Yield of **(2)**[mg/L]
0.05	34.2	1.9	143.2	–
0.1	66.4	3.7	281.4	22.4
0.5	157.4	8.7	828	374.5
0.75	239.4	13.3	1041.7	488.6
1	306.3	17.0	122	254.5
5	342.5	19.0	1245	326.7
20	390	21.7	1400	164.5
50	187.5	10.4	717.5	18.2
90	702	39	2407.5	66.8
100	290	16.1	970	23.5

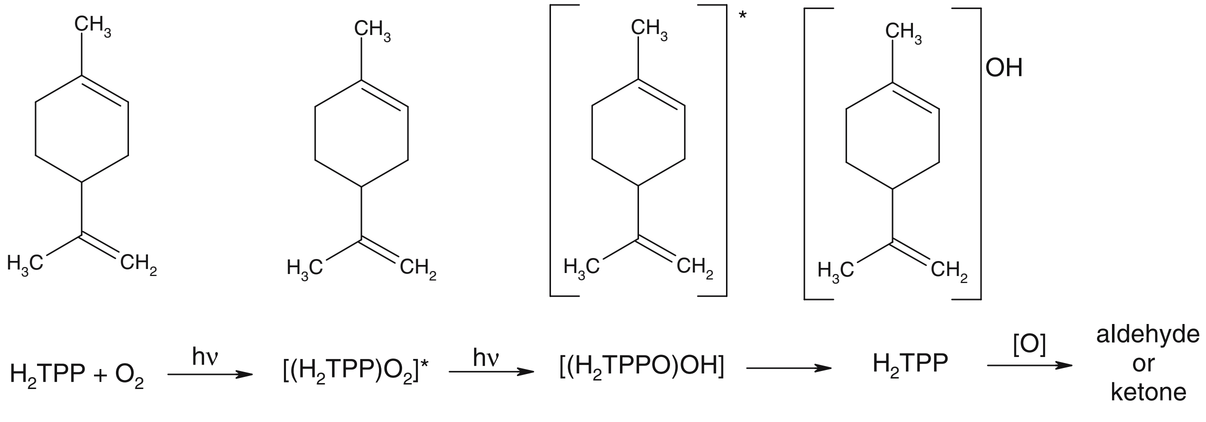

Figure 2. Absorption spectra of the H_2TPP – limonene system; before photoreaction (solid line), after 36 h (dotted line) and after 126 h (dash line) exposition for the sunlight. Solvent – chloroform; 1 mm spectrophotometric cell.

In the systems with higher limonene concentration, H_2TPP plays the role of a photosensitiser and transformation is based on the photosensitised singlet oxygen. This phenomenon is well known from the chemistry of the porphyrins [18], and possible pathways of limonene transformation are shown in figure 1. It must be emphasised that in parallel experiments, in which the solution of porphyrin in chloroform without limonene was exposed to sunlight, for the same time period, no important changes in the spectrum were observed, except small decrease in the Soret band (about 5% in intensity), however tetraphenylporphyrin exposed to intense photoirradiation in the presence of strong electron donors or acceptors (as triethylamine or CCl_4) can undergo critical changes in the absorption spectra due to the photoreactions [22].

In the photochemical oxidation of limonene with H_2TPP, carvone and **(1)** could be formed either through a dioxygen – H_2TPP complex-mediated limonene intermediate or through a free radical chain pathway. In the first case, photoexcitation would form a highly reactive complex between dioxygen and H_2TPP, which would play a distinct role in the reaction, perhaps via a complex-mediated limonene intermediate. In the second case, photoexcitation would liberate high reactive hydroxy species, which would then go on to react with the limonene radical (figure 3). The second possibility is more probable. As abundant evidence shows,

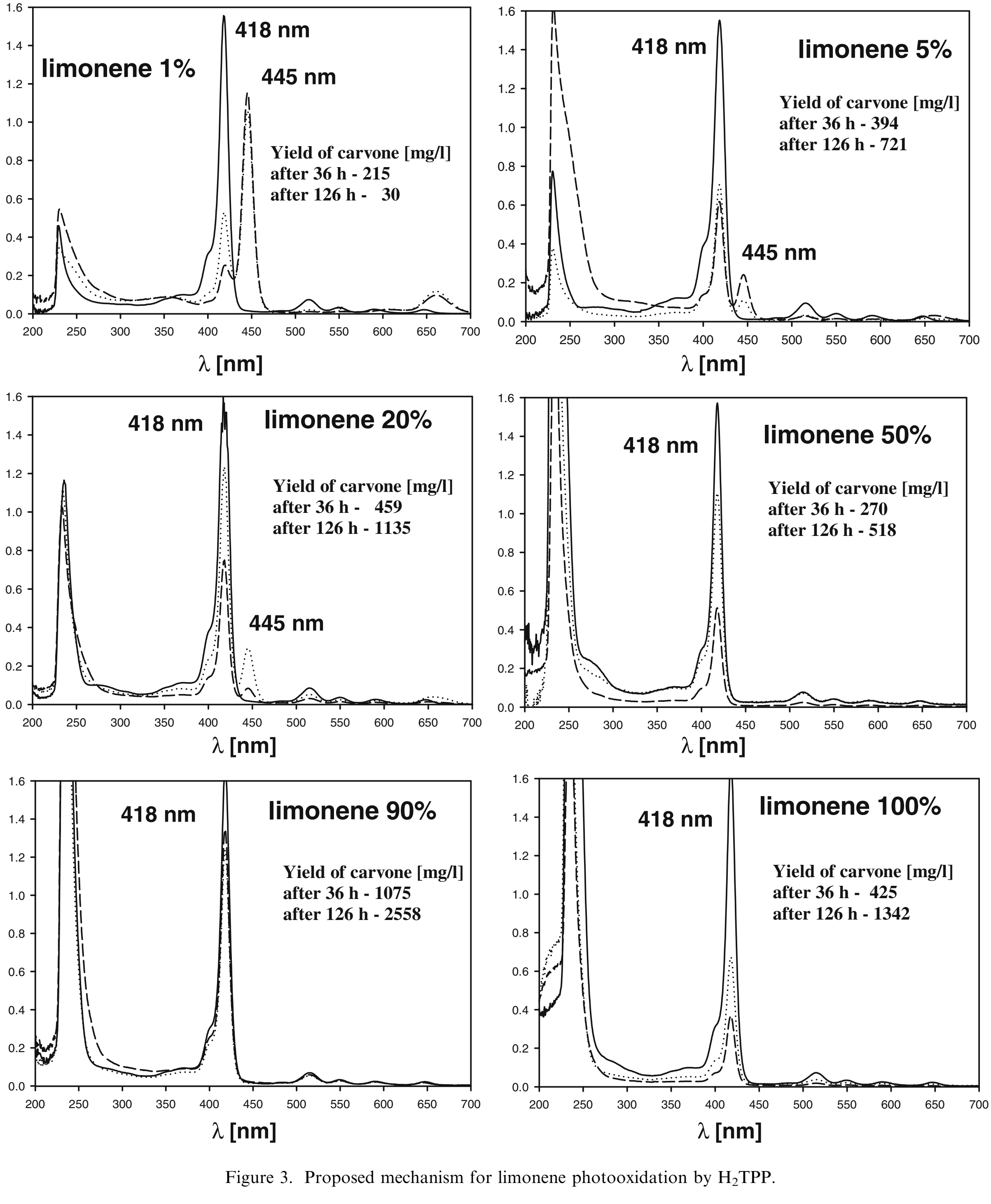

Figure 3. Proposed mechanism for limonene photooxidation by H_2TPP.

Table 4
Dependence of the H_2TPP catalytic properties from the catalyst/substrat molar ratio

Moles TPP:moles limonene	Photo-conversion time	Yield of **carvone** [mg/L]	Yield of **(1)** [mg/L]	Productivity of **(1)**[%]	Productivity of **carvone** [%]
1:1	18 h	13.2	52.1	38.3	9.7
	36 h	15.0	57.4	42.2	11.0
	54 h	8.7	47.1	34.6	6.4
1:2	18 h	27.0	96.0	35.7	10.0
	36 h	25.8	103.8	38.6	9.6
	54 h	12.4	82.5	30.7	4.6
1:0.5	18 h	10.1	42.1	50.1	12.0
	36 h	11.0	43.5	51.8	13.1
	54 h	7.3	41.1	48.9	8.7
1:0.1	18 h	1.3	5.2	38.2	9.5
	36 h	1.3	5.5	40.4	9.5
	54 h	0.86	4.6	33.8	6.3

hydroxylation of hydrocarbons catalysed by cytochrome P-450 occurs via a mechanism involving hydrogen atom abstraction from the substrate (R–H) followed by a rapid transfer of the metal-bound hydroxy radical to an intermediate alkyl radical ($R^{\cdot}$) [23]. In contrast with metalloporphyrins, oxidations with H_2TPP do not take place at the metal center; rather, they are due to a free radical chain pathway initiated by H_2TPP producing singlet oxygen (an energetically rich form of molecular oxygen). Furthermore, a low regioselectivity is observed in that reaction. Since radical based oxidations show little or no regioselectivity beyond that based on bond strengths [21], the supposition seems to be confirmed. However, singlet oxygen has

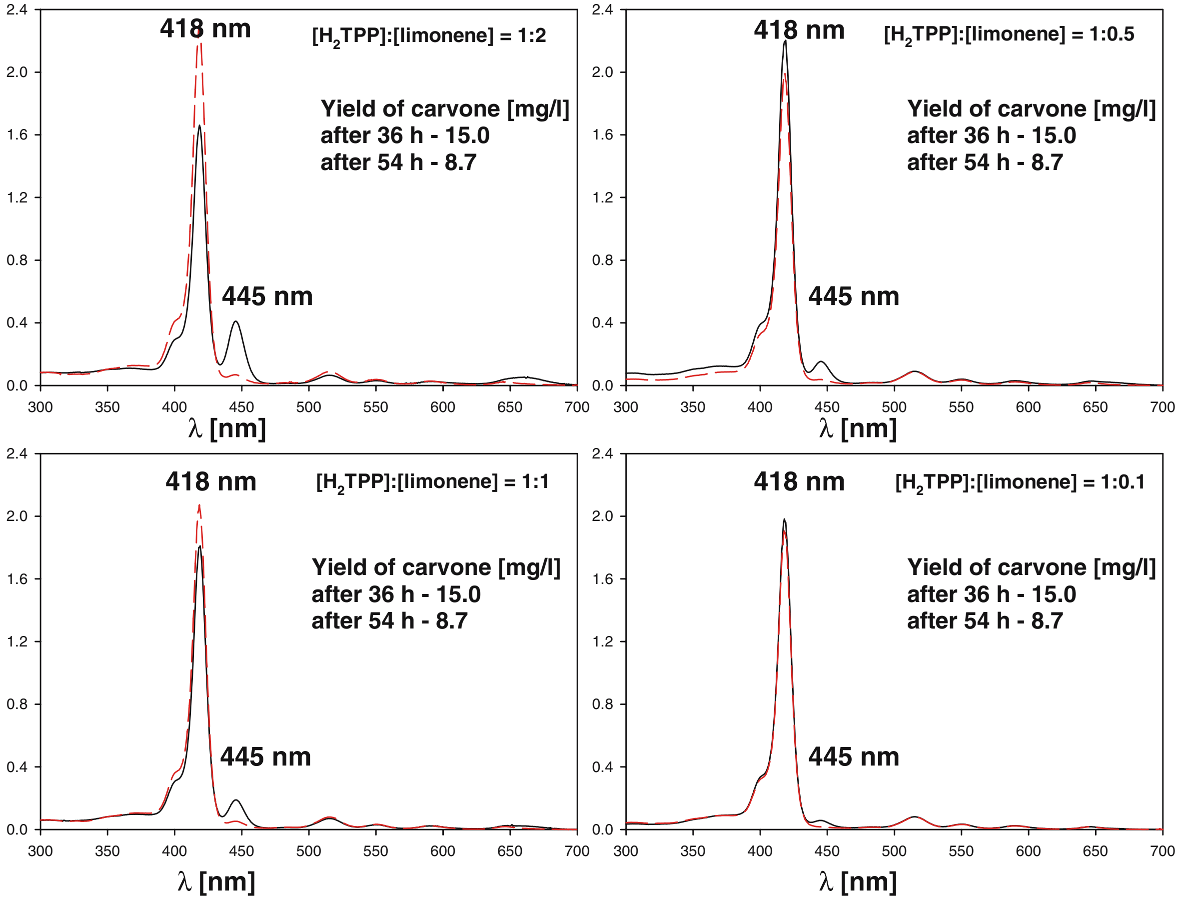

Figure 4. Absorption spectra of the H_2TPP – limonene system in chloroform (with various molar H_2P/limonene ratio); after 36 h (solid line) and after 54 h (dash line) of photoreaction.

a high degree of stereoselectivity properties. Basing on that hypothesis, we proposed a reaction mechanism of limonene oxidation catalysed by H_2TPP (figure 3). Early work with singlet oxygen demonstrated that this active species could oxidise substrates which were unaffected by oxygen in its normal energy state [18]. Singlet oxygen can also react with olefins having two or more allylic substituents causing a double bond shift and formation of an allylic hydroperoxide. The probable formation of the mentha-derivated hydroperoxide can be additional an evidence of the radical mechanism. It has also been suggested earlier in literature [18]. It is possible that some singlet oxygen reactions may proceed by electron transfer from the electron rich compound (limonene) to the electrophilic singlet oxygen.

In a subsequent experiment, different molar ratios of porphyrin to substrate (limonene) were examined. It is worth notice that the highest accumulation of carvone and **(1)** were favoured at 1:2 molar ratio of H_2TPP to limonene (table 4). The changes in the porphyrin–limonene system with various catalyst/substrate ratios, are shown in the spectra in figure 4. It must be noted that in this experiment the concentration of limonene was very low, and in consequence the UV bands of limonene and its transformation products were not observed (low molar absorbance coefficients in relation to those of the porphyrins). The highest concentration of carvone was observed when the catalyst/substrate ratio was 1:2. Such results support the hypothesis about the molecular mechanism of carvone formation (see also figure 1):

$$\text{limonene} \rightarrow 2\ \text{carveol-OH} + H_2TPP \rightarrow \text{carvone-O} + H_4TPP^{2+} + 2X^-$$

where X is a counter anion for porphyrin dication (445 and 670 nm in the spectra). It is very difficult to identify a counter anion in such a complicated biomimetic system. A possible explanation is that it comes from the chloroform or from one kind of the by-products.

The results reported here point to the possibility of using H_2TPP as an active oxygen photosensitiser which is able to transform limonene to carvone and other terpenoids. The advantages gained from utilising H_2TPP, as compared to traditional biocatalysts with microorganisms, are a lack of substrate toxicity towards catalyst and a higher (about 10-fold [24]) yield of biotransformation. In order to any catalyst to be used for oxidation of hydrocarbons, the catalyst should be oxidatively robust compared to the substrate. However, the instability of metalloporphyrins as regards oxidative degradation and the difficulty of recovering the expensive catalyst limit the practical applications of porphyrins in both synthetic chemistry and industrial processes. On the other hand, immobilisation of metalloporphyrin complexes on solid supports can provide catalysts that are easier to handle and may exhibit improved selectivities and activities because of the environment support. It also minimises contamination and lowers the expenses toward power consumed by aeration thanks to higher solubility of oxygen at lower temperatures.

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