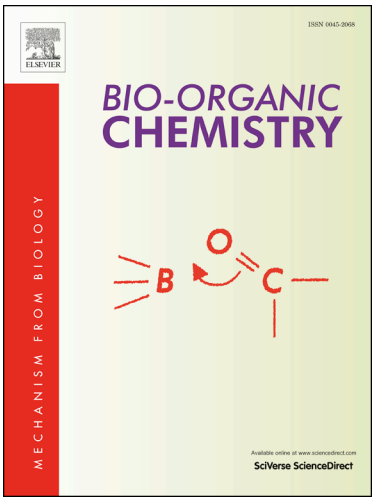

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Hydrolases-mediated transformation of oleuropein into demethyloleuropein

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KEYWORDS. Oleuropein, demethyloleuropein, hydrolases, biocatalysis, enzyme promiscuity.

[1]

[1] ABBREVIATIONS

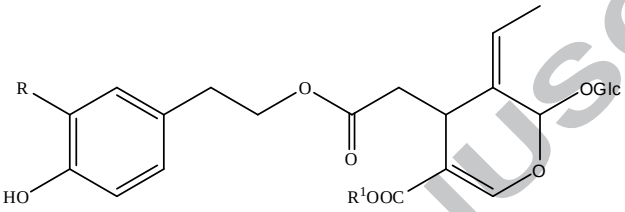
3,4-DHPEA-EDA, oleacein; 4-HPEA-EDA, oleochantal; VOO, Virgin olive oil; EC 3, hydrolases; EC 3.4.21.1, Chymotrypsin; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry.

ABSTRACT. Phenolic compounds present in extra virgin olive oil have recently attracted considerable attention due to their pharmacological activities. Among them oleacein (3,4-DHPEA-EDA), structurally related to oleochantal (4-HPEA-EDA), is one of the most studied. 3,4-DHPEA-EDA has been synthesized through decarboxylation of demethyloleuropein catalyzed by $Er(OTf)_3$. Demethyloleuropein is extracted from black olives drupes in very limited amounts and only in particular periods of the year. The availability of demethyloleuropein could be increased by a selective hydrolysis of the methyl ester moiety of oleuropein, a secoiridoid present in large amount in olive leaves. In this work we describe a new enzymatic method for carrying out a selective hydrolysis of oleuropein via the screening of a panel of hydrolases (lipases, esterases and proteases). Among all the enzymes tested the best results was obtained using α-chymotrypsyn from bovine pancreas as biocatalyst, thus revealing a classic example of catalytic enzyme promiscuity.

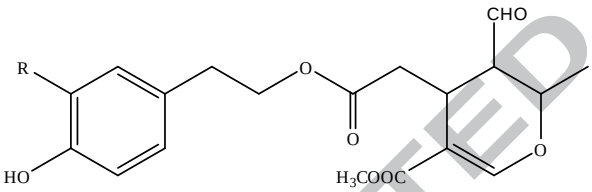
1. INTRODUCTION.

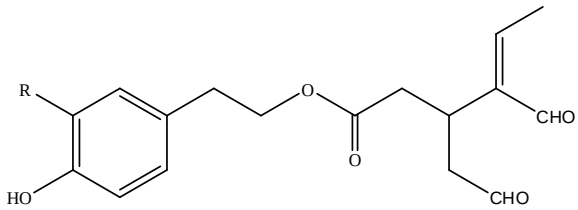
Virgin olive oil (VOO) plays a primary role in the Mediterranean diet and it represents a very important agricultural product not only for economic reasons but also for health care [1]. Numerous evidences suggest that the high consumption of VOO, rich in phenol and flavonoids, is responsible of the lowered incidence of pathology such as cardiovascular disease or diabetes [2]. Recently, it has also been demonstrated his benefic role in the incidence of Alzheimer disease and Parkinson disease [3]. The main phenolic compounds present in VOO are secoiridoids derivates of hydroxytyrosol (3,4-DHPEA) and tyrosol (*p*-HPEA) that occurs as either simple phenols or esterified with elenolic acid to form, respectively, oleuropein (**1**), its derived demethyloleuropein (**2**), ligstroside (**3**), together with their aglicones 3,4-DHPEA-EA (**4**) and *p*-HPEA-EA (**5**) (Figure 1) [4]. Furthermore, recently, intensive studies carried out in order to clarify the phenol composition of VOO highlighted the presence of two other important compounds characterized by a dialdehyde moiety linked with 3,4-DHPEA and *p*-HPEA, named 3,4-DHPEA-EDA (**6**) and *p*-HPEA-EDA (**7**), respectively [5], that show peculiar sensory and healthy properties [6].

Particularly, the structural similarity between 3,4-DHPEA-EDA and *p*-HPEA-EDA, a phenol owning an anti-inflammatory activity similar to that reported of ibuprofen [5a], and the presence of a catechol moiety that potentially gives greater antioxidant activity, has triggered an intensive study of the pharmacological properties of 3,4-DHPEA-EDA. The powerful antioxidant activity of 3,4-DHPEA-EDA against reactive oxygen and nitrogen species was proved, using *in vitro* cellular and non cellular screening systems [7]. Furthermore, the anti-inflammatory action of compound **6** and its ability to inhibit 5-lipoxigenase was demonstrated as well [8].



1 R=OH, R^1=Me; oleuropein (OL)
2 R= OH, R^1=H; demethyloleuropein
3 R=H; R^1=Me; ligstroside





4 R=OH; 3,4-DHPEA-EA (oleuropein aglycone)
5 R=H; p-HPEA-EA (ligstroside aglycone)

6 R=OH; 3,4-DHPEA-EDA (oleacein)
7 R=H, p-HPEA-EDA (oleochantal)

Figure 1. Chemical structure of main phenolic compounds present in VOO

Additionally to these important pharmacological properties, it has to be mentioned that compound **6** is the predominant secoiridoid in the crushed paste and in VOO. 3,4-DHPEA-EDA content in VOO ranges from 111 to 285 mg/kg, depending on the irrigation condition [9], while the amount of other secoiridoids is much lower compared to 3,4-DHPEA-EDA [10]. It is presumed that demethyloleuropein (**2**) act as a precursor for the formation of 3,4-DHPEA-EDA during crushing due to an increase in the esterases and β-glucosidases activity [11]. Demethyloleuropein is an indicator of the maturation of the

olives. Indeed, his relative amount increases as soon as the maturation proceeds, while the content of oleuropein decreases [12]. In the past ten years, we invested numerous efforts for the development of new green methods aimed at realizing an efficient manipulation of oleuropein and its derivatives in order to obtain more powerful pharmacologically active compounds [13]. In particular, we recently developed a new method for the synthesis of compound **6** using demethyloleuropein (**2**) as substrate and therefore mimicking the natural formation of 3,4-DHPEA-EDA in olive fruits [14]. The transformation was carried out in water, under reflux, in the presence of $Er(OTf)_3$ as very low toxic Lewis acid. This synthetic protocol allowed to obtain a contemporary deglucosylation and decarboxylation affording the desired product in only 4 hours. Despite the process accomplishes many green chemistry tasks, the use of demethyloleuropein (**2**) as starting material, usually extracted from black olive fruits only in particular periods of the year, makes this process difficult to replicate. Therefore, we decided to carry out a synthesis of demethyloleuropein *via* screening of a panel of hydrolases (EC 3), using oleuropein (**1**) as starting material. We demonstrate that as oleuropein is indeed the secoiridoid present in larger amount in olive leaves, the method allows for increasing the availability of the precursor demethyloleuropein and consequently increasing the availability of 3,4-DHPEA-EDA as well.

2. MATERIAL AND METHODS.

2.1. General

All chemicals were obtained from Aldrich Chem. Co. The HPLC were performed by RP-HPLC analysis on a Thermo Scientific Ultimate Dionex 3000 UHPLC equipped with an Hypersil gold column (C18) (dim.mm 250x4.6, particle size 5μm) and a mobile phase consisted of the following solvents: A (H_20 + TFA pH 2,46), B (methanol). The gradient was as follows: 0-1 min, 95% A, 5% B, 1-17 min 40% A - 60% B, 19-25 min 5% A-95% B, 27-30 min 95% A-5% B, 30-35 min 95% A-5% B Absorbance were obtained at the wavelength of 230 nm, 254 nm and 280 nm. The instrumentation performance, chromatograms, and initial data processing were carried out with Chromelion software. TLC were performed using silica plates 60-F264 on alumina, commercially available from Merk. Liquid flash chromatography was performed on a Supelco VERSA FLASH HTFP station using octadecyl-functionalized silica gel commercially available from Sigma-Aldrich. The qualitative identification was performed a Thermo Scientific Q Exactive (Thermo Fisher, Milan, Italy) mass spectrometer working both in positive and negative mode at 35,000 resolving power, operating in SIM mode by flow injection

(flow rate 15 μL/min for each stock solution). Data were evaluated by Xcalibur 2.2.SP1 (Thermo Fisher Scientific, Bremen, Germany). The mass accuracy, directly calculated from Xcalibur, is defined by the formula Δ(ppm) = [(theoretical mass measured mass)/theoretical mass] × 1.000.000. The synthesized compound is known, its ^{1}H-NMR and ^{13}C-NMR were compared with that reported in the literature [14]. Spectra were recorded on a Bruker WM 300 instrument, at 300 MHz on samples dissolved in $CDCl_3$. Chemical shifts are given in parts per million (ppm) from tetramethylsilane as the internal standard for ^{1}H-NMR spectra (0.0 ppm).

2.2. Oleuropein Extraction

The MW-assisted extraction of oleuropein (**1**) [13a] was performed on several samples of olive leaves from *Coratina* cultivar of *Olea europea L., Oleaceae*, dried for 48 h at 50 °C, milled, and kept at r.t. until use. Oleuropein was purified by flash chromatography in a SUPELCO VERSA FLASH station (eluent: $CHCl_3$/MeOH 8:2) and characterized by ^{1}H-NMR, ^{13}C-NMR and HRMS, by comparison with data reported in the literature [13a]. Pure oleuropein was used as reagent for the hydrolysis reaction.

2.3. Enzymes- mediated synthesis of demethyloleuropein

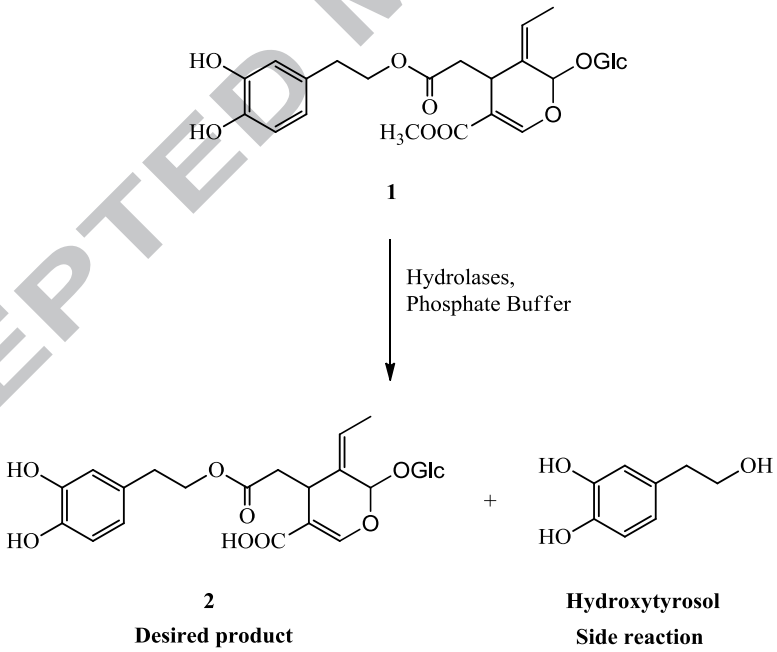
Oleuropein (**1**), 0.55 mmol, and α-chymotripsin from bovine pancreas, 26 units, were added to a solution (50 mL) of buffer potassium phosphate at pH 7. The reaction was shacked for 72 h, 130 rpm at 4°C. Afterwards the reaction was stopped, the aqueous buffer was removed via lyophilization and the reaction mixture was solubilized in methanol until reaching a concentration of 50 μg/mL and analyzed by HPLC/MS. (See Supporting Information) The product was purified by flash chromatography and characterized by HRMS, while ^{1}H-NMR and ^{13}C-NMR were compared with those reported into the literature [14] (See Supporting Information).

Demethyloleuropein (**2**). Pink powder. HRMS [M-H]= 525.1611 m/z (theoretic mass 525.1617 m/z)

3. RESULTS AND DISCUSSION.

We turned our attention in the use of the enzymes for carrying out the conversion of oleuropein (**1**) into demethyloleuropein (**2**) because common organic protocols for cleavage of esters involve strong basic

or acidic conditions. The presence of two ester groups in the oleuropein (**1**) structure and the poor reactivity of methyl group as leaving group make impossible to use these methods for carrying out a selective hydrolysis of oleuropein. Although some methods for a selective hydrolysis of methyl ester are present in the literature, they usually require either toxic solvents or expensive catalysts [15]. On the contrary, hydrolytic enzymes - usually employed to catalyze the formation or the cleavage of esters and amides - allow for carrying out these transformations in very green conditions [16]. Thereby, the chemoselective hydrolysis of the methyl ester moiety of oleuropein was carried out via the screening of a panel of hydrolases. Especially, 23 hydrolases commercially available were screened. As a first attempt the reactions were performed using 0.092 mmol of oleuropein, run at 30°C in an orbital shaker at 150 rpm, pH 8, in 0.5 mL of potassium phosphate buffer, for 24 hours in presence of 13 units of enzyme (1 unit is defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute) (Scheme 1).



Scheme 1. Enzymatic oleuropein hydrolysis.

The solvent was removed by lyophilization and the crude was solubilized in methanol until reaching a concentration of 1 mg/mL. The crude was analyzed by UHPLC and HRMS. Unfortunately, the hydrolysis carried out at pH 8 led only to the formation of hydroxytyrosol due to the basic pH (data not

shown). We thought that this side-reaction was due to the basicity of the reaction medium rather than the enzymatic activity because several reports present in the literature demonstrate that oleuropein is not directly converted in hydroxytyrosol through the action of esterases [17]. We decided to use a lower pH until to reach a neutral value in order to verify if under this condition the selective hydrolysis could be obtained. The results are shown in Table 1.

We observed that compound **2** was detected in few of the biotransformations performed (entries 16, 21, 22, 23, Table 1). Therefore some enzymes showed the ability to hydrolyze the methyl ester, but a low conversion was obtained in all of these cases. Also in this case the side reaction, namely the hydrolysis of oleuropein in hydroxytyrosol, was the predominant reaction (all entries, Table 2). In order to demonstrate the influence of the pH on the reaction profile, a reaction at pH 8 was carried out in absence of enzyme (entry 24, Table 1). A remarkably hydrolysis of oleuropein into hydroxytyrosol was noticed, confirming our previous assumption. In general, the best result for the desired enzymatic hydrolysis was obtained with α-chymotrypsin from bovine pancreas, also known as *Bos taurus* α-chymotrypsin (entry 21, Table 1).

Table 1. Screening of hydrolases for oleuropein hydrolysis carried out at pH 7[a].

Entry	Enzyme	Substrate (%)	Demethyloleuropein[b](%)	Hydroxytyrosol (%)
1	L. *Candida cylindracea*	79	-	21
2	L. porcine pancreas type2	78	-	22
3	L. *Pennicillium camemberti* AE014	75	-	25
4	L. C_1AE0 *Candida cylindracea*	79	-	21
5	L. AE77 *Pseudomonas stutzeri*	74	-	26
6	L. C_2AE02 *Candida cylindracea*	59	-	41
7	L. AE08 *Rizhopus* spp.	59	-	41
8	L. AE078 *Pseudomonas florescense*	78	-	22
9	L. AE010 *Aspergillus niger*	17	-	83
10	L. AE057 *Mucor miehei*	62	-	38
11	L. AE04 *Achromobacter* spp.	78	-	22
12	L. AE0156 *Candida antarctica B*	27	-	73
13	L. A_1AE05 *Alcaligenes* spp.	55	-	45

Entry	Enzyme	Substrate (%)	Demethyloleuropein[b](%)	Hydroxytyrosol (%)
14	L. A_2AEO11 *Alcaligenes* spp.	76	-	24
15	L. AE0158 *Candida antarctica A*	70	-	30
16	L. P2AE012 *Pseudomonas cepacia*	65	8	27
17	Pepsine porcine gastric mucosa	74	-	26
18	Papaine from *Papaia Latex*	70	-	30
19	Proteinase Bacterial	77	-	23
20	*Thermolysin bacillus (Thermoproteolyticus rokko)*	73	-	27
21	*α-chymotrypsin bovine pancreas*	41	20	39
22	L. P_1A06 *Pseudomonas cepacia*	52	6	42
23	*L. Rhizomucor miehei*	52	5	43
24	No enzyme[c]	30	-	70

[a] General reaction conditions: the reactions were run at 30°C in an orbital shaker at 150 rpm, oleuropein 92 mM, phosphate buffer pH 7 (1 mL), for 24 hours in presence of 13 enzyme units. [b] Conversion determined by HPLC. [c]Reaction carried out at pH 8

Chymotrypsin (EC 3.4.21.1) is an enzyme that belongs to class of proteases, and it catalyzes therefore the hydrolysis of peptide bonds. It is active as homodimer and each monomer is constituted by 245 amino acid residues with MW of 25.7 kDa (PDB: 4CHA and 1YPH) [18]. The reactivity of α-chymotrypsin on an ester moiety is a classic example of catalytic enzyme promiscuity. Indeed, several studies have already demonstrated that proteases, which can cleave a much stronger amide bond, are capable of hydrolyzing the weaker carboxylic ester bond. In fact, the bonds broken in the two cases (C-N, C-O) differ, but the catalytic mechanism is very similar [19]. Therefore, chymotrypsin from bovine pancreas was selected as the lead enzyme for trying to improve the conversion and consequently the yield. The results of the reaction optimization study are depicted in Table 2.

No improvement was noticed prolonging the reaction time and increasing the concentration of the biocatalyst (entry 1, Table 2). The influence of water-miscible organic cosolvents or the use of biphasic aqueous/organic media were not investigated as oleuropein possesses an elevated solubility in aqueous buffer at neutral or slightly basic pH. Conversely, the use of common

organic cosolvents for biocatalytic application such as DMSO may reduce enzyme activity and complicate the isolation of the final product after the reaction.

Table 2. Oleuropein hydrolysis carried out with α-chymotrypsyn from bovine pancreas[a]

Entry	T (°C)	Enzyme units	Time(h)	**2**[(b)](%)
1	30	26	48	20[(c)]
2	4	13	24	10
3	20	13	24	15[(c)]
4	4	26	24	10
5	4	13+13[(d)]	48	20
6	4	13+13+13[(e)]	72	35
7	4	13+13	72	35
8	4	13+13	72	35[(f)]

[a]General reaction conditions: the reactions were run in an orbital shaker at 150 rpm, phosphate buffer pH 7 (1 mL), oleuropein 0,092 mmol. [b]Conversion determined by HPLC. [c]Hydroxytyrosol was detected as additional product in these samples to be 41% and 37%, respectively. [d]Another aliquot of the enzyme was added after 24 hours. [e]Two aliquots of the enzyme were added after 24 and 48 hours. [f]The reaction was performed on 0.55 mmol. The product was characterized by HRMS while ^{1}H-NMR and ^{13}C-NMR were compared with those reported into the literature [14]

A reduction in terms of conversion was noticed reducing the temperature (entries 2, 3, Table 2). However, it has to be mentioned that when the reaction was carried out at 4°C the formation of the side product was not observed. This phenomenon is very likely due to a complete depletion of the spontaneous chemical hydrolysis of oleuropein into hydroxytyrosol that can occur in aqueous buffers, even at pH 7. Therefore this temperature was used for carrying out further studies on the hydrolytic reaction of the methyl ester group. No improvement was noticed increasing the concentration of the biocatalysis (entry 4, Table 2). The best result was achieved in presence of 26 enzyme units portion-wise added, and running the reaction for 72 hours (entry 7, Table 2). The reason of this increased conversion is due to the fact that enzyme activity was completely lost after 48 h incubation time in the reaction buffer. Hence, the addition of small aliquots of enzyme during the time enabled to sustain a higher catalytic activity during the

overall course of the reaction (entries 1, 4, Table 2). However, a further increase in the enzyme concentration, while keeping the same total reaction time (72 h), did not ameliorate the conversion into the desired product (entry 6, Table 2). Once the optimized reaction conditions were found, the reaction was repeated using 300 mg of oleuropein (entry 8, Table 2). The purification of the crude mixture by flash chromatography allowed us to obtain the product with 25% of yield. Even if the yield is moderate, this procedure allowed us to obtain demethyloleuropein in very practical green and economic fashion, and without the need for extraction methods commonly used for its recovery. These additional extraction procedures are expensive and require high amount of solvents and several purification steps [20]. The present protocol combined with the catalytic decarboxylation of demethyloleuropein allows for obtaining 3,4-DHPEA-EDA in only two synthetic steps, therefore increasing the availability of this important biophenol and allowing detailed studies on its pharmacological properties.

CONCLUSIONS. In conclusion a new alternative synthesis of demethyloleuropein via screening of a panel of hydrolases is presented. Among all the enzymes α-chymotrypsyn from bovine pancreas has been found as the best hydrolase, thus revealing a classic example of enzyme promiscuity. Even if the yield is moderate the use of enzymes allowed to carry out a controlled hydrolysis in very green conditions hence critically improving the availability of demethyloleuropein from an agricultural waste. Moreover, the enzymatic reaction proceeded with complete chemoselectivity at pH 7 and 4 °C. The present protocol combined with the catalytic decarboxylation of demethyloleuropein mediated by $Er(OTf)_3$ [14], represents the first mixed catalytic/biosynthetic pathway for 3,4-DHPEA-EDA. Further optimization of the first biocatalytic step through the use of immobilized α-chymotrypsyn can enhance the stability of the enzyme in solution and consequently increase the overall yield and decrease the catalyst loading in order to meet the requirements for the application on a large scale [21].

ASSOCIATED CONTENT

Supporting Information., HPLC analysis of reaction mixtures and HRMS, ^{1}H-NMR and ^{13}C-NMR spectra of demethyl oleuropein are reported into a word file.

AUTHOR INFORMATION

Corresponding Author

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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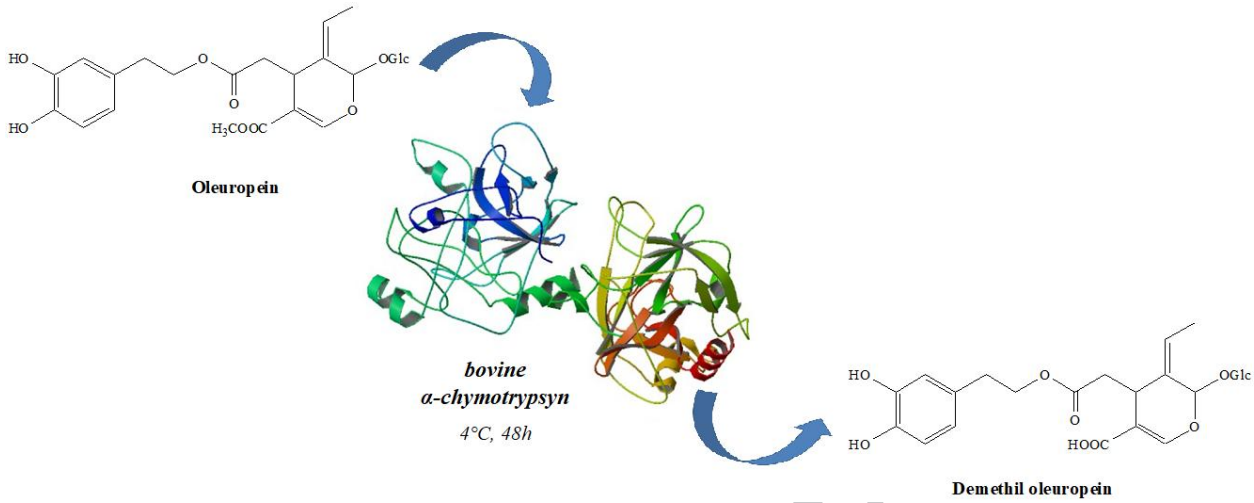
Graphical abstract



Highlights

- Demethyloleuropein was synthesized from oleuropein via the screening of a panel of hydrolases (EC 3).
- α-chymotrypsin was the best biocatalyst, thus revealing a classical example of enzyme promiscuity.
- The enzymatic reaction proceeded with complete chemoselectivity at pH 7 and 4°C.