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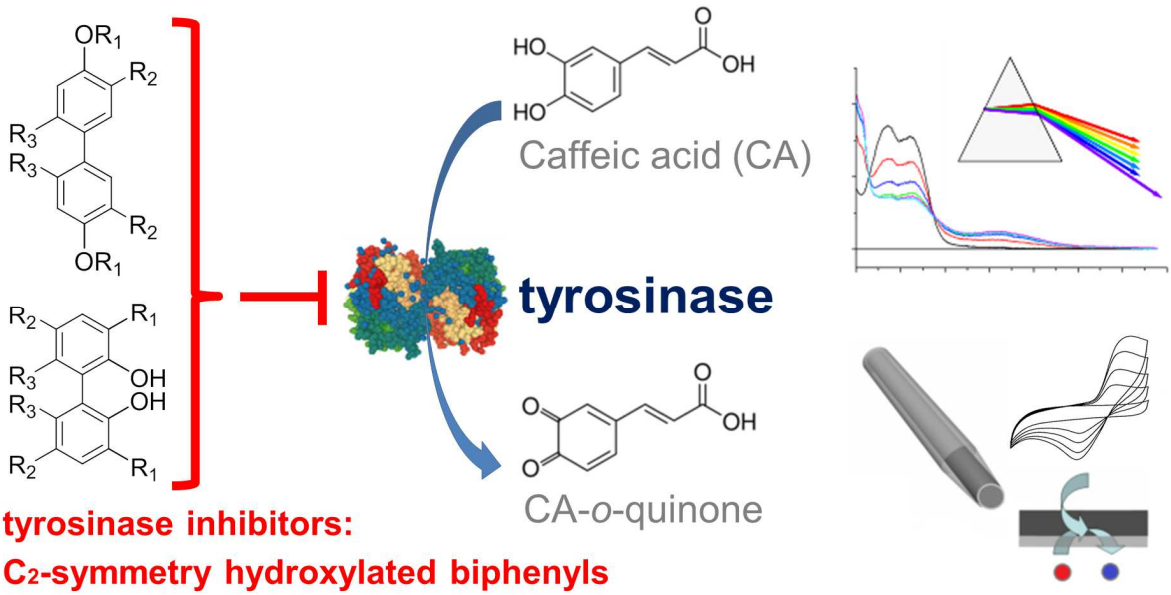

tyrosinase inhibitors:

C_2-symmetry hydroxylated biphenyls

Hydroxylated biphenyls as tyrosinase inhibitor: a spectrophotometric and electrochemical study

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Abstract: A small collection of C_2-symmetry hydroxylated biphenyls was prepared by straightforward methods and the capability to act as inhibitors of tyrosinase has been evaluated by both spectrophotometric and electrochemical assays. Our attention was focused on the diphenolase activity of this enzyme characterized by the absence of the characteristic lag time of enzymatic reaction of its monophenolase activity. To this purpose, we evaluated the capability of tyrosinase to oxidize a natural *o*-diphenol substrate to *o*-quinone analyzing the changes in the UV-Vis spectrum of a solution of caffeic acid and the reduction of the cathodic current in a tyrosinase-biosensor, respectively. Results of both the methods were comparable. Most of the compounds possessed higher inhibitory activity compared to compound **1**, a known hydroxylated biphenyl inhibitor of tyrosinase.

Keywords: Tyrosinase, spectrophotometric assay, biosensor, hydroxylated biphenyls, synthesis

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1. Introduction

Tyrosinase [EC 1.14.18.1] is a copper-containing enzyme involved in melanin biosynthesis, in unfavourable enzymatic browning of plant-derived foods, in pathological melanogenesis and in insects moulting process [1]. It catalyses the oxidation of both monophenols (monophenolase or cresolase activity) and *o*-diphenols (diphenolase or catecholase activity) to *o*-quinones (Figure 1A).

Figure 1.

Three forms of this enzyme (*oxy*-, *met*-, and *deoxy*-tyrosinase) with different copper structures of the active site are identified [2]. The monophenolase activity is catalysed by the *oxy* form (less than 15%) that is transformed in the *deoxy* form in the successive oxidation to *o*-quinone, while in the diphenolase cycle both the *oxy* and *met* forms are involved. The *oxy* form oxidizes *o*-diphenol to o-quinone, yielding the *met* form, and this latter form transforms another *o*-diphenol molecule into *o*-quinone and is reduced to the *deoxy* form, the only one capable of reacting with molecular oxygen. For this reason, the monophenolase activity presents a characteristic lag time that exists until a sufficient amount of catechol is produced [3]. The length of the lag time is strictly connected to the enzyme source and concentration, the concentration of monophenol and the presence of catalytic amounts of *o*-diphenol or transition metal ions, which completely abolish the lag period [4].

Investigation of tyrosinase inhibitors may lead to development of novel skin whitening agents, medicinal products, antibrowning substances or insect control compounds [5]. Many natural occurring compounds have been identified for those targets whose structure has inspired the preparation of several collections of synthetic tyrosinase inhibitors [6]. The most studied chemical scaffolds of tyrosinase inhibitors belong to the polyphenols group. In spite of a large number of tyrosinase inhibitors only a few of these are generally used.

4,4'-Dihydroxy-biphenyl **1**, a natural biphenol, was found to be a potent tyrosinase inhibitor, more effective than kojic acid and arbutin [7]. Hydroxylated biphenyls are a class of polyphenols widely present in nature [8], some of them manifest high biological activity like ellagitannins, vancomicin, biphenomicins, others, structurally less sophisticated, are natural occurring dimers of 2-methoxy phenols and phenols. Compared to phenols, hydroxylated biphenyls manifest higher antioxidant activity and generally they are less toxic than the corresponding phenolic monomer [9, 10]. There is an increasing interest in using the hydroxylated biphenyl unit as building block to prepare bioactive molecules involved in human pathologies and disorders because most of them manifest interesting pharmacological and biological activities such as neuroprotective and antiproliferative properties [11].

In our continuing search on synthesis of hydroxylated biphenyls with interesting stereochemical and biological features [12-15]. we have prepared a small collection of C_2-

symmetry biphenols and evaluated their capability to act as inhibitors of tyrosinase by both spectrophotometric and electrochemical assays.

2. Results and discussion

2.1. Chemistry

Taking in account the high level of specificity of hydroxylated biphenyl structure in protein binding in comparison to other aromatic compounds [16], we have considered C_2-symmetry isomer of biphenyl **1** (eg. compound **2**) and its derivatives by introducing hydroxylated functionalities in key positions (eg. compounds **3**, **4**) as depicted in Figure 2A.

Figure 2

It is generally demonstrated that the presence of hydroxylated groups in a phenol structure improves tyrosinase activity [17-19]. According to those considerations, a small collection of biphenyls was selected. Different hydroxylated functionalities are present in compounds **6** and **7** whereas compounds **5** and **8** are lipophylic derivatives of biphenol **1**. Compound **6** possesses a benzodioxole moiety in a biphenylic structure that reminds sesamol structure, recently studied for the effective activity as tyrosinase inhibitor [20].

Except compound **7**, all compounds studied in this work are known in literature, though some of them were achieved in scarce yields or as side-products. Yields of compounds **5** and **8** were increased and new straightforward synthetic procedures improved the sustainability of the process compared to those described in literature (Supplementary Data). Claisen rearrangement of the corresponding 2-(prop-2-en-1-yl)phenol derivative gave compound **5** in high yield following two microwave procedures carried out in aqueous solvents. Compounds **3**, **4**, **6**-**8** were obtained by coupling reactions of the corresponding monomer with different reaction conditions according to substituents in the phenolic ring. Synthesis and spectroscopic data of compounds **3** – **6** and **8** have been reported in the Supplementary Data. Compound **7** was obtained by oxidation of veratraldehyde C_2-symmetry dimer **9** in the presence of *m*-chloroperbenzoic acid (mCPBA) (Figure 2B). Due to substitutions in *ortho-ortho´* and *ortho-meta* positions, biphenyls **3** and **6** - **8** are conformationally hindered. Biphenyls **6** - **8** were tested in racemic form.

2.2 *Biological evaluation*

With the aim to explore the biological properties of compounds **1**-**8**, we evaluated the capability of these molecules to inhibit the tyrosinase activity. In particular, our attention was focused on the diphenolase activity of this enzyme [21]. To this purpose, we evaluated the capability of tyrosinase to oxidize a natural *o*-diphenol substrate to *o*-quinone analyzing the changes in the UV-Vis spectrum of a solution of caffeic acid. The spectrum recorded before mixing the substrate and enzyme solutions, strongly immediately changes after the mixing and the absorbance at 280 and 311 nm, characteristic of caffeic acid, decrease in time-dependent mode, while a band at 413 nm attributable at the *o*-quinone appeared [22]. The time-course of caffeic acid oxidation, obtaining monitoring the absorbance values at 311 nm at different time, has been reported in Figure 3 and is characterized by the absence of the characteristic lag time of enzymatic reaction catalyzed by tyrosinase due to its monophenolase activity.

Figure 3

Any significant contribute of compounds **1**-**8** in the examined UV-Vis region of caffeic acid, in presence or not of tyrosinase, in the experimental conditions was detected (data not shown). With the exclusion of compounds **2** and **8**, all tested hydroxylated biphenyl showed either a comparable (compounds **3** and **5**) or a superior efficiency to inhibit the tyrosinase activity respect to biphenyl **1**. It should be taken in account that compounds **5**, structurally related to magnolol and honokiol, showed no cytoxicity in normal human lymphocytes (IC_{50} < 100 µM) making this compound a good candidate for further evaluation in vivo model [23].

As show in Figure 4A, compound **4** is the most effective inhibitor of tyrosinase activity, while compounds **6** and **7** have a lower ability to inhibit the caffeic acid oxidation by tyrosinase compared to **4**, even if it is significantly higher to that of biphenyl **1**.

Figure 4

Thereafter, tyrosinase inhibition activity of compounds **1**-**8** were determined by a tyrosinase-biosensor as schematically represented in Figure 1B. A significant inhibition of tyrosinase enzyme by all studied compounds was recorded in the range of 20 (compound **2**) and 82 % (compound **6**) (Figure 4B). Taking as reference the inhibitor **1**, only compound **2** showed a lower inhibition effect while compounds **4**, **7** and **8** had a similar inhibition power. A significant progressive increase of tyrosinase inhibition was observed by exposing the biosensor to the compounds **3**, **5** and **6,** respectively. Electrochemical detection of caffeic acid and inhibitor clearly demonstrate the absence of the lag time of enzymatic reaction catalyzed by tyrosinase (Figure 4D). In a few minutes, tyrosinase inhibition reached the steady-state. The diphenolase activity of tyrosinase have been observed also in spectrophotometric assay where UV-Vis spectra of a solution of caffeic acid

and inhibitor before and after addition of tyrosinase has been depicted in Figure 4C. After six minutes a reduction of almost 60% of the curve has been observed.

Taking together, the results obtained with the two different analytical assays suggest that compound **6** acts as inhibitor of the diphenolase activity of tyrosinase more efficiently than biphenyl **1**. The main reason of this increased activity might be due to the high nucleophilic character of phenolic-OH groups in virtue of the electron-donating effect of groups bonded in *para*-position. Generally, the presence of hydroxyl groups in a phenol structure and the ability of substituents to donate electrons increase the electron density of the aromatic ring through a resonance donating effect that improves the potency of tyrosinase [17, 24, 25]. Nevertheless, in compound **8**, steric hindrance of methoxyl groups in *ortho*-position to phenolic-OH group would negatively influence the interaction with the enzyme and, as results, a decrease of tyrosinase inhibition was evidenced specially by spectrophotometric assay.

Significantly higher inhibitory activity was observed for biphenyl **4** with spectrophotometric assay respect to biosensor detection, this seems due to the low lipophilicity of the compound (LogP 2.15), more soluble in aqueous solution. Analogously to biphenyl **4**, the difference in inhibition activity of compounds **3, 5** and **7** observed in the two analytical assays might be due to the environment of the experiment that could influence, albeit limited, tyrosinase activity as documented in literature [26]. Infact, in spectrophotometric assay, tyrosinase forms an aqueous solution whereas in biosensor probe, tyrosinase has been adsorbed in two lipophilic layers.

Compounds **2** showed a low efficiency of about 10% in both the experimental assays, suggesting that the possible hydrogen bond between the two phenolic-OH groups would strongly interfere with tyrosinase interaction. Surprisingly, compounds **6** possesses a high capability to inhibit the oxidation of caffeic acid to *o*-quinone when detected by tyrosinase biosensor, while not so marked activity was observed in spectrophotometric tyrosinase oxidation assay although it is significantly different from **1**. Compared to the other studied biphenyls, compound **6** possesses two benzodioxole moieties that remind sesamol structure. It is acknowledged that sesamol is an effective inhibitor that interact with tyrosinase by a mechanism influenced by the hindered position of the benzodioxole ring [20]. Probably, the biphenyl structure enhances the conformational rigidity of the benzodioxole moiety evidencing different response in the two analytical assays.

Recently, it has been observed that biphenyl **4** inhibited amyloid-β (Aβ) aggregation by 50% in Congo red assays [27]. Although biphenyl **4** lacks in structural features generally required for an efficient Aβ inhibitor [28], compound **4** appears to be a promising building block to develop a new series of inhibitors of pathogenic Aβ peptide involved in neurodegenerative diseases. Taking in account that amyloid formation has a critical role in melanin formation in humans [29] and different form of metallo-Aβ peptide can damage neurotransmitters via reactive oxygen species (ROS) [30], investigation of tyrosinase inhibitors having a biphenilic structure would be a useful start point to identify efficient pathogenic Aβ peptide inhibitors. Our interest in this field is high [12] and our work

on tyrosinase inhibitors would like to give insights also into targeting agents for neurodegenerative disorders.

3. Conclusions

A small collection of hydroxylated biphenyls was prepared by straightforward methods and the inhibitory activity toward diphenolase tyrosinase oxidation was detected by spectrophotometry and amperometric biosensor using caffeic acid as enzyme substrate. Results of both the methods were comparable. Most of the compounds possessed higher inhibitory activity compared to compound **1**, a known hydroxylated biphenyl inhibitor of tyrosinase. Substituents in the aromatic ring as well as steric hindrance of functional groups close to the phenolic-OH group influenced conformational features of the biphenyl structure that affect tyrosinase interaction. Our results suggest that hydroxylated biphenyls are suitable building block to develop effective tyrosinase inhibitors, further studies are currently in progress.

4. Experimental

4.1. Material and general remarks

Compounds **1**-**2** and all starting materials were purchased by Sigma-Aldrich (Milan, Italy) and used without purification. NMR spectra of compound **7** were recorded on Varian Mercury Plus spectrometer at rt in acetone-d_6. Chemical shifts are given in ppm (δ) and coupling costants in Herz; multiplicities are indicated by s (singlet), bs (broad singlet), d (doublet). Elemental analyses were performed using an elemental analyser Perkin-Elmer model 240 C.

*4.2. Synthesis of 5,5',6,6'-tetramethoxy-[1,1'-biphenyl]-3,3´-diol **7**.*

To a solution of *5,*5',6,6'-tetramethoxybiphenyl-3,3'-dicarbaldehyde (veratraldehyde dimer) **9** [13] (2 g, 6.11 mmol) in dry dichloromethane (100 mL) was added m-chloroperbenzoic acid 77% (MCPBA) (2.98 g 13.32 mmol). The mixture was stirred at rt for 12h under N_2. The reaction mixture was quenched with a saturated sodium persulfate solution (10 mL) to remove mCPBA excess, washed with brine (10 mL) and extracted with dichoromethane (3 x 30 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The residue was dissolved in MeOH (30 mL), and then treated with 6N NaOH aqueous solution (62 mL). The mixture was stirred at room temperature for 15 min. After removal of MeOH, the residue was dissolved in ethyl acetate (30 mL), washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), and dried over Na_2SO_4. The organic phase was filtered and concentrated, to provide compound **7** as a white solid (1.12 g, 60%): mp 212-213°C: ^{1}H NMR (acetone-d_6) δ 3.61 (s, 6H), 3.94 (s, 6H), 6.23 (d, *J* = 2.8 Hz, Ar, 2H), 6.45 (d, *J* = 2.8 Hz, Ar, 2H), 8.21 (bs, 2 OH);^{13}C-NMR **(**acetone-d_6) δ 55.05, 59.66, 99.90, 108.44, 133.35, 139.92, 152.87, 153.45; Anal. Calcd for $C_{16}H_{18}O_6$: C, 62;74 H, 5.92; Found: C, 62.77; H, 5.95.

4.3. *Spectrophotometric enzyme activity assay.*

The capability of compounds **1**-**8** to inhibit oxidation of caffeic acid by tyrosinase/O2 oxidizing system was monitored by means of a UV-VIS spectroscopy method, carried out in a Shimadzu UV-Visible UV-2501 spectrophotometer using a Helma (Mülheim, Germany) duple chambers UV-cell (2×4.375 mm). Briefly, 980 µL of a caffeic acid solution (180 µM) and 1000 µL of a mushroom tyrosinase solution (166 U/mL, Sigma-Aldrich) both dissolved in 50 mM phosphate buffer (pH 6.8) were separately introduced in the separated UV-cell chambers. 20 µL of an appropriate DMSO stock solution of inhibitors (166 µM) were added to the chamber containing caffeic acid solution. The tyrosinase activity in absence of inhibitors was detected adding 20 µL of DMSO. Ground UV-Vis spectra were recorded in the 250-600 nm wavelength range. Thereafter, the solutions were mixed and the spectra were registered again at fixed times (from 1 to 25 min). The inhibition activity was determined as percentage of the caffeic acid consumption at 6 min.

The efficiency of compounds **1-8** to inhibit the diphenolase activity of tyrosinase was determined analyzing the absorbance values at 311 nm using equation (1) [22, 31]:

$$\% \text{ inhibition} = (1-((D-C)/(B-A))*100 \quad (1)$$

where C and D are the absorbance values at 311 nm in presence of inhibitor before the mixing and after 6 min, respectively, while A and B are the corresponding values at 311 nm in absence of inhibitor. The measurement was performed in triplicate and averaged before calculation.

4.4. *Biosensor construction and immobilized tyrosinase inhibition assay.*

Tyrosinase biosensors [32] were made using silver wires insulated with Teflon™ modifying procedures previously described [33, 34]. Approximately 3 mm of the wire were exposed and inserted into a glass capillary tube (20 mm in length; I.D. Ø = 1 mm) partly filled with graphite loaded with epoxy resin (55% w/w) (Araldite-M®, Sigma-Aldrich, Milan, Italy) obtained by mixing 850 mg of graphite with 500 mg of Araldite-M and 200 mg of hardener. After 24 h at 40 °C, the surface of the transducers where smoothed by using a high speed drill (Dremel® 300) equipped with an aluminum oxide grinding wheel. Finally, the carbon transducer was immersed five times into a solution of tyrosinase enzyme and let it dry for 5 min after each dip. A final net that entrapped the enzyme was deposited on the top of enzyme layers by quick dipping of the biosensors in a polyurethane solution (0.25%) as previously described [35].

Constant potential amperometry (CPA) was used for caffeic acid calibrations and *in-vitro* inhibition experiments by applying a potential of -100 mV against an Ag/AgCl reference electrode (See caffeic acid cyclic voltammetry in the Supplementary Data). Calibrations and experiments were performed at room temperature and in a 10 ml electrochemical cell filled with fresh PBS (pH 7.40) 24 h after sensors' fabrication. The percent inhibition of diphenolase activity of tyrosinase by compounds **1** - **8** was assessed by recording the baseline-subtracted signal (Delta-I) of 500 µM of

caffeic acid (100%) and the stabilized current after the introduction of the inhibitor (50 µM, dissolved in 100 µL of DMSO) in the electrochemical cell. The measurement was performed in triplicate and averaged before calculation. A control experiment was performed for each biosensor by adding the same amount of DMSO alone.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http:// These data include synthesis and characterization of the compounds described in this article and supplementary electrochemical data.

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Caption of Figures

Figure 1. (A) Schematic representation of the reactions catalyzed by tyrosinase. (B) Caffeic acid is oxidized to the correspondinq quinone by the enzyme and reduced-back at carbon surface by applying a reducing potential of -100 mV vs Ag/AgCl reference electrode. The first reaction, inhibited by the compounds synthetized in this study, resulted in a significant reduction of the cathodic current.

Figure 2. (A) Hydroxylated biphenyl structures. (B) Preparation scheme of biphenyl **7**.

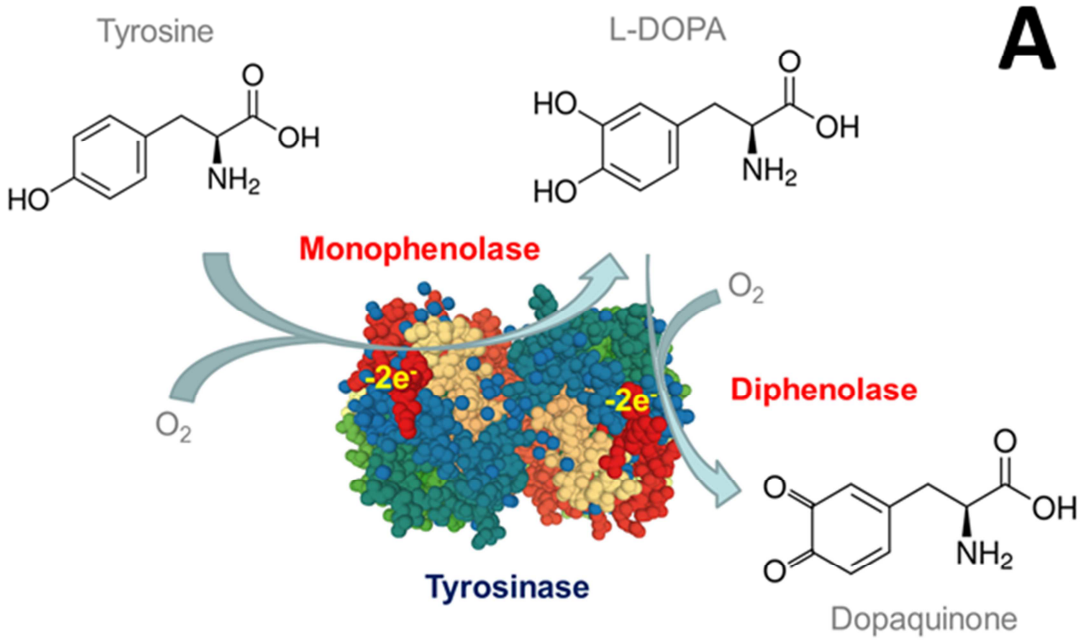
Figure 3. UV-Vis spectra of caffeic acid (88.2 µM) in 50 mM phosphate buffer, pH 6.8, before and after addition of tyrosinase (83 U/mL). In the insert the time-course of caffeic acid oxidation detected at three different wavelength.

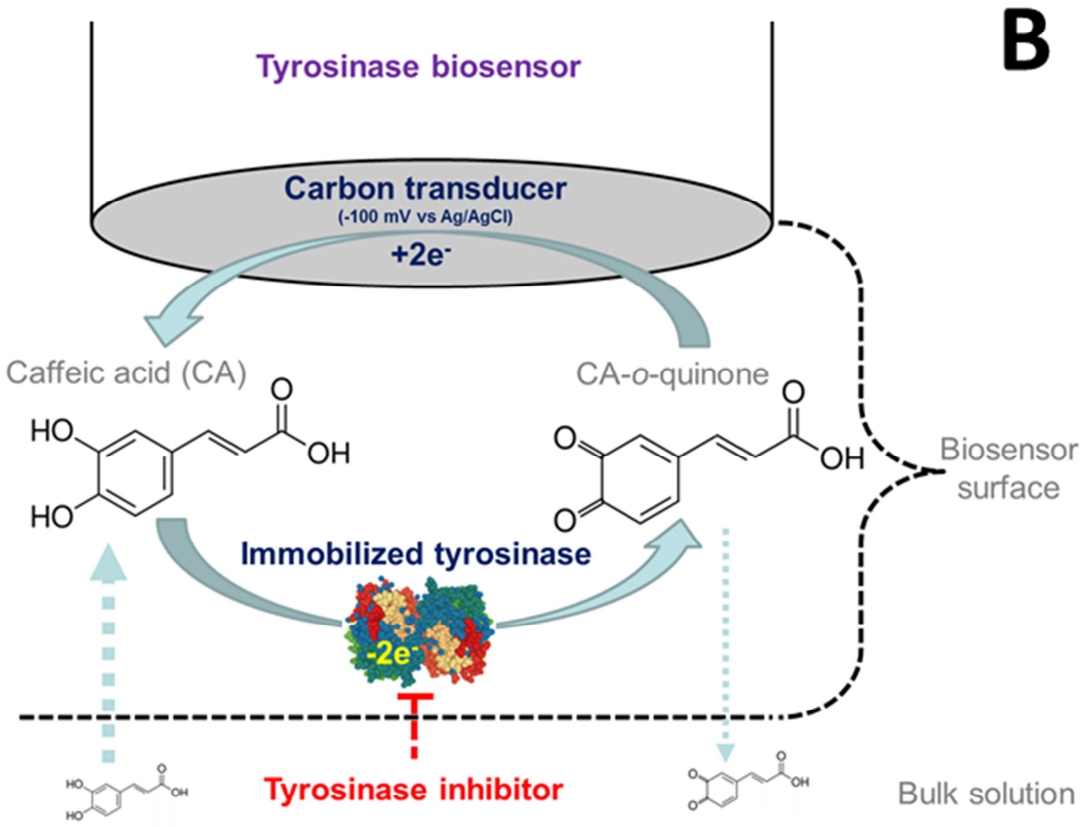
Figure 4. (A) Percentage of tyrosinase inhibition by caffeic acid oxidation with a unimmobilized tyrosinase enzyme determined by UV-VIS spectroscopy. *, ** = $p<0.05$ and <0.01 respectively *vs* compound **1**.

(B) Percentage of tyrosinase inhibition by caffeic acid oxidation with a tyrosinase enzyme immobilized on the surface of an amperometric biosensor monitored at a potential of -100 mV vs Ag/AgCl reference electrode. *, **, *** = $p<0.05$, <0.01, <0.001 respectively *vs* compound **1**.

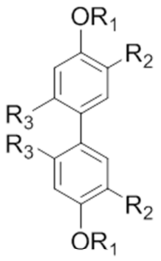
(C) Spectrophotometric curve of tyrosinase activity decay in the presence of inhibitor **4**.

(D) Amperometric plotting of tyrosinase activity decay in the presence of inhibitor **6**; *arrow a***:** addition of caffeic acid; *arrow b:* biphenyl **6** inhibition.

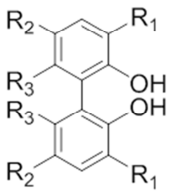

A
Tyrosine
L-DOPA
Monophenolase
Diphenolase
O_2
O_2
-2e⁻
-2e⁻
Tyrosinase
Dopaquinone


B
Tyrosinase biosensor
Carbon transducer
(-100 mV vs Ag/AgCl)
+2e⁻
Caffeic acid (CA)
CA-o-quinone
Biosensor surface
Immobilized tyrosinase
-2e⁻
Tyrosinase inhibitor
Bulk solution

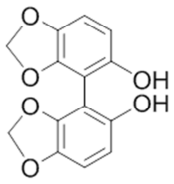
A



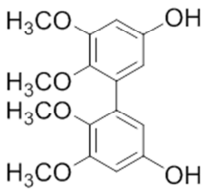
1 $R_1 = R_2 = R_3 = H$,
4 $R_1 = R_3 = H$, $R_2 = OH$,
5 $R_1 = R_3 = H$, R_2 = prop-2-en-1-yl,
8 $R_1 = R_3 = CH_3$, $R_2 = OH$,



2 $R_1 = R_2 = R_3 = H$,
3 $R_1 = R_2 = H$, $R_3 = OH$,

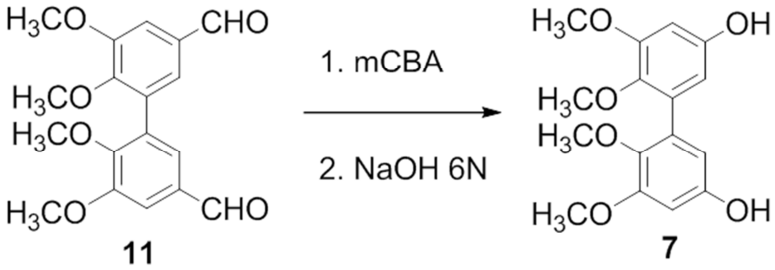


6

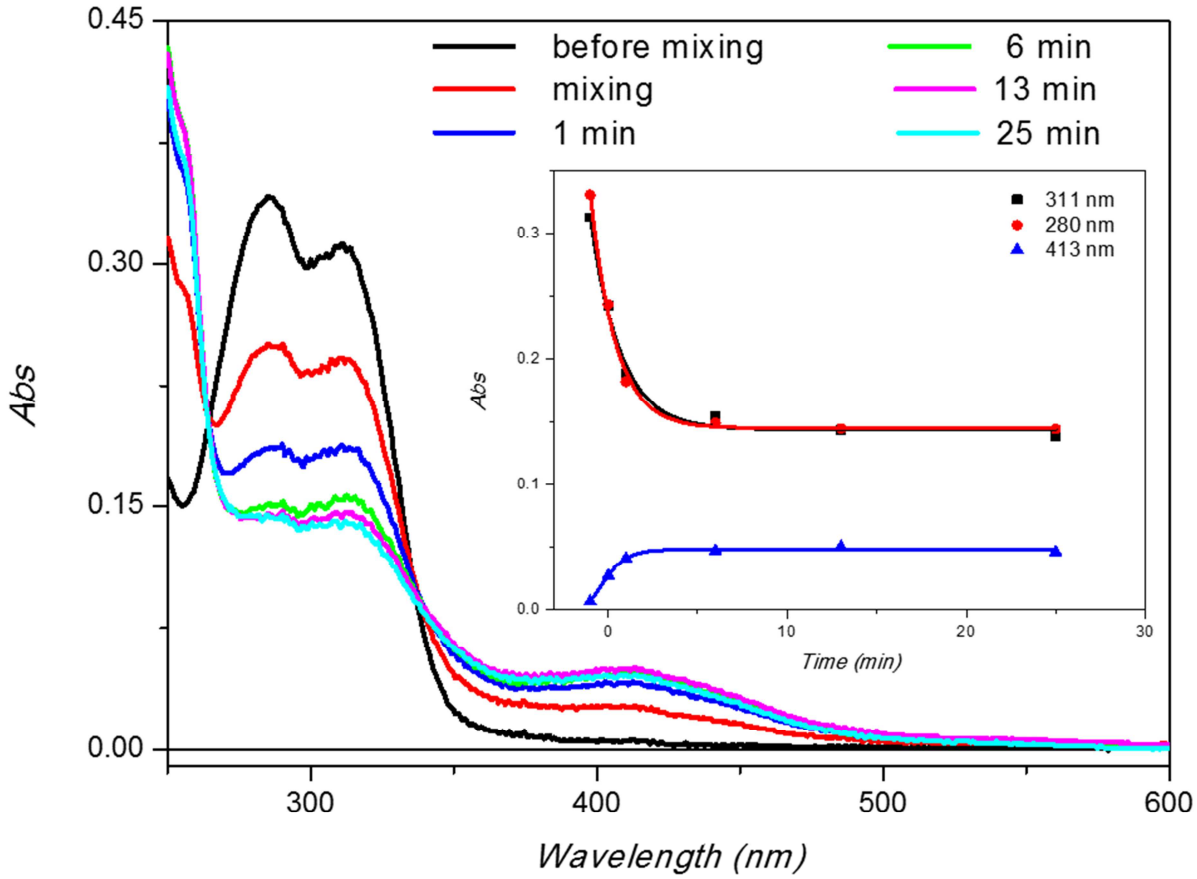


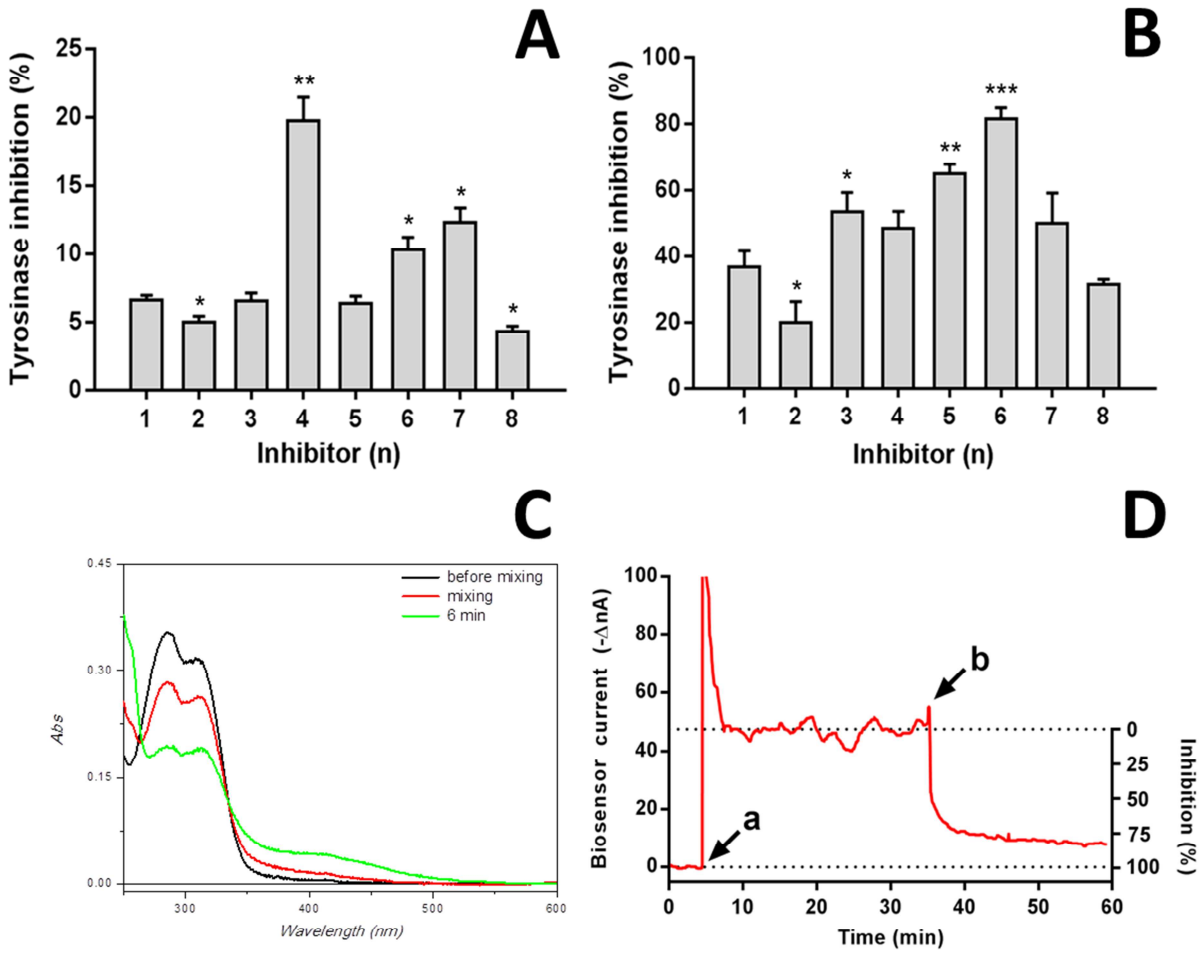
7

B



11 **7**




A
Tyrosinase inhibition (%)
Inhibitor (n)
B
Tyrosinase inhibition (%)
Inhibitor (n)
C
before mixing
mixing
6 min
Abs
Wavelength (nm)
D
Biosensor current (-ΔnA)
Inhibition (%)
Time (min)
a
b

Highlights

- A small collection of hydroxylated biphenyls was synthesized by straigtforward methods.
- Significant tyrosinase inhibitor activity was detected in most of the compounds.
- Selective diphenolase activity of tyrosinase enzyme have been detected by both spectrophotometric and electrochemical assays.
- Results of both the analytical methods were comparable.