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Layer-by-Layer coated tyrosinase: An efficient and selective synthesis of catechols

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ABSTRACT

Agaricus bisporous tyrosinase was immobilized on commercial available epoxy-resin Eupergit®C250L and then coated by the Layer-by-Layer method (LbL). The two novel heterogeneous biocatalysts were characterized for their morphology, pH and storage stability, kinetic properties (K_m, V_{max}, V_{max}/K_m) and reusability. These biocatalysts were used for the efficient and selective synthesis of bioactive catechols under mild and environmental friendly experimental conditions. Ascorbic acid was added in the reaction medium to inhibit the formation of *ortho*-quinones, thus avoiding the known enzyme suicide inactivation process. Catechols were obtained mostly in quantitative yields and conversion of substrate. Tyrosinase immobilized on Eupergit®C250L and coated by the LbL method showed better catalytic activities, higher pH and storage stability, and reusability with respect to immobilized uncoated tyrosinase. Since chemical procedures to synthesize catechols are often expensive and with high environmental impact, the use of immobilized tyrosinase represents an efficient alternative for the preparation of this family of bioactive compounds.

1. Introduction

Tyrosinase (Tyro; EC 1.14.18.1), a polyphenol oxidase widely diffused in nature, catalyzes the oxidation of phenols to catechols (cresolase or monophenolase activity) and that of catechols to corresponding *ortho*-quinones (catecholase or diphenolase activity).[1] Both catechols and *ortho*-quinones derivatives are characterized by several biological activities, including antioxidant and antitumoral properties.[2] The transformation of phenols to catechols and quinones is usually difficult to perform by chemical methods under environmental friendly conditions.[3] For this reason, tyrosinase received great attention as useful green alternative to chemical treatments.[4] The application of tyrosinase in biocatalysis is partially limited due to the formation of reactive *ortho*-quinones that can covalently bond to the enzyme or autoxidize, producing brown pigments.[5] This drawback can be overcome working in reducing conditions in the presence of ascorbic acid or NADH.[6] Moreover, the possibility to immobilize tyrosinase on stable and low cost supports further increase the interest for this enzyme in industrial applications.[7] Examples of the immobilization of tyrosinase on carbon nanotube,[8] copolymer matrices,[9] chitosan,[10] gold nanoparticles,[11] alumina sol-gel,[12] membrane alginate, polyacrylamide, and gelatine gels[13] have been reported in the production of L-dopa,[14] in the removal of phenolic compounds from waste water,[15] and in other industrial applications.[16] However, some of these immobilization methods are rather complicated and do not give good enzyme stability or retention.[17] Recently, the Layer-by-Layer (LbL) technique was reported as a general and versatile tool for the controlled productions of multilayer surface coatings on a large variety of surfaces.[18] This method is based on the consecutive deposition of alternatively charged polyelectrolytes onto a surface.[19] The polyelectrolyte films have the ability to protect encapsulated protein from high-molecular-weight denaturing agents or bacteria and to allow regulation of the permeability towards small substrates, which can enter the multilayer and react with the catalytic site of the enzyme.[20] In this study we describe the synthesis of two novel tyrosinase biocatalysts: one based on the chemical immobilization of mushroom tyrosinase on the epoxy-resin Eupergit®C250L, and the other based on the chemo-physical procedure consisting, first, in the immobilization of tyrosinase on Eupergit®C250L and then in the coating of the biocatalyst by the LbL technique. The novel biocatalysts were applied for the selective synthesis of catechol derivatives by oxidation of a large panel of substituted phenols, including biologically active compounds, under friendly environmental conditions. The reactions were performed in buffer at room temperature with dioxygen as primary oxidant in the presence of ascorbic acid (AA) to avoid the formation of *ortho*-quinones.[6a] The role of AA in the process is still under discussion. It acts as an internal reducing agent to transform the

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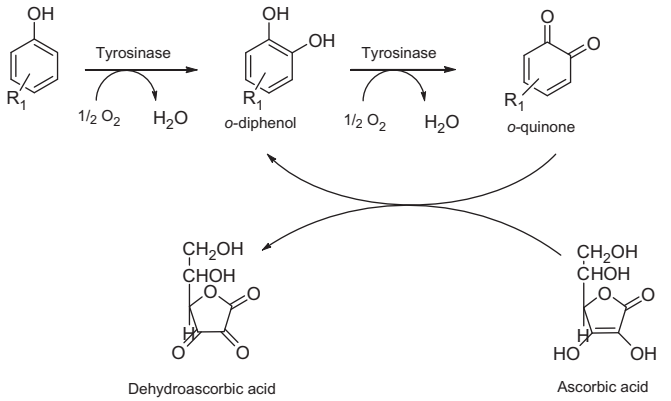


Figure 1. Role of ascorbic acid in the oxidation of phenols by tyrosinase.

ortho-quinone formed during the oxidation into the desired catechols (Fig. 1). It is also known that tyrosinase can be inhibited at relatively high concentrations of AA.[21]

For this reason, the AA concentration requires to be optimized for a good compromise between an high enzyme activity and high yield in catechols.[22] The comparison between the efficiency and selectivity of tyrosinase with and without the LbL procedure, as well as the possibility to recycle the biocatalysts for more runs, are also reported.

2. Results and discussion

2.1. Optimization of tyrosinase immobilization

The immobilization of tyrosinase was performed with the commercially available epoxy-acrylic resin Eupergit®C250L using a modification of previously reported procedures.[23] The ratio between the enzyme and the resin (mg/g), the incubation time and pH were investigated to optimize the yield of immobilization. As a general procedure, the enzyme (1.0–6.0 mg, 13900–83400 IU) was suspended in Na-phosphate buffer (pH 5.0–8.0) (7.0–9.0 mL) in the presence of a defined amount of resin (1.0 g) for 24–48 h at room temperature. The immobilized tyrosinase (Tyro/E) was washed with water to remove excess of protein and treated with glycine to block residual epoxy-groups (Fig. 2, panel A).

The effectiveness of the immobilization procedure was investigated in terms of immobilization yield (Eq. 1, where U_a is the total activity of enzyme added in the solution and U_r is the activity of the residual enzyme recovered in the washing solutions, and activity yield (Eq. 2, where U_x is the activity of the immobilized enzyme assayed by dopachrome method.[24]

$$\text{Imm. Yield } (\%) = [(U_a - U_r) \times U_a^{-1}] \times 100 \quad (1)$$

$$\text{Activity Yield } (\%) = [U_x \times (U_a - U_r)^{-1}] \times 100 \quad (2)$$

Table 1
Tyrosinase immobilization on Eupergit®C250L at different conditions

Entries	Ratio Tyro/support (mg/g)	Incubation time (h)	pH	%Immobilization yield (mg bounded Tyro)	Activity yield (%)
1	1	24	7	74 (0.74)	16
2	2	24	7	76 (1.52)	20
3	4	24	7	70 (2.80)	30
4	5	24	7	66 (3.30)	38
5	6	24	7	68 (4.08)	31
6	5	48	7	77 (3.85)	30
7	5	24	5	58 (2.90)	23
8	5	24	6	60 (3.00)	30
9	5	24	8	56 (2.80)	26

As reported in Table 1, the activity yield increased with the amount of the enzyme in contact with the resin (entries 1–5), reaching the maximum value of 38% (that corresponds to an immobilization yield of 66%) at 5:1 enzyme/support ratio (entry 4). When the amount of enzyme was further increased, the recovered activity yield decreased to 31%; nevertheless, the bound protein was slightly higher (entry 5 vs entry 4). Similar results were obtained for the immobilization of cyclodextrin glucosyltransferase[25] and lipase[26] on Eupergit®, probably because the close packing of the enzymes on the support surface limits the access of substrate. Once defined the optimum value of the enzyme/support ratio, the immobilization was performed at different times (24 and 48 h). Data reported in Table 1 show the highest immobilization yield at 48 h (77% and 66%), even if a longer incubation time led to a reduction of the activity yield (30% and 38%, respectively) (entry 6 vs entry 4). The influence of pH on the immobilization procedure was also studied in the range of 5.0–8.0. The optimum binding was achieved with sodium phosphate buffer at pH 7.0 (Table 1 entries 5 vs 7–9). With the aim to further increase the stability of Tyro/E, the LbL technique was applied by coating Tyro/E through a sequential deposition of charged polyelectrolytes. Briefly, Tyro/E was first suspended in

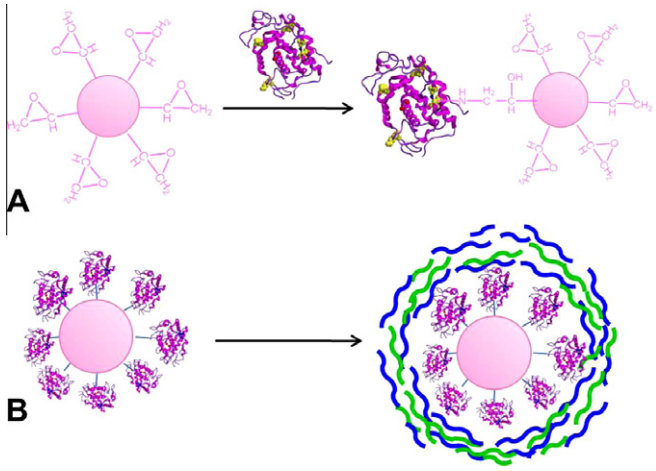


Figure 2. Schematic representation of Tyro/E (panel A) and Tyro/E-LbL (panel B) preparation.

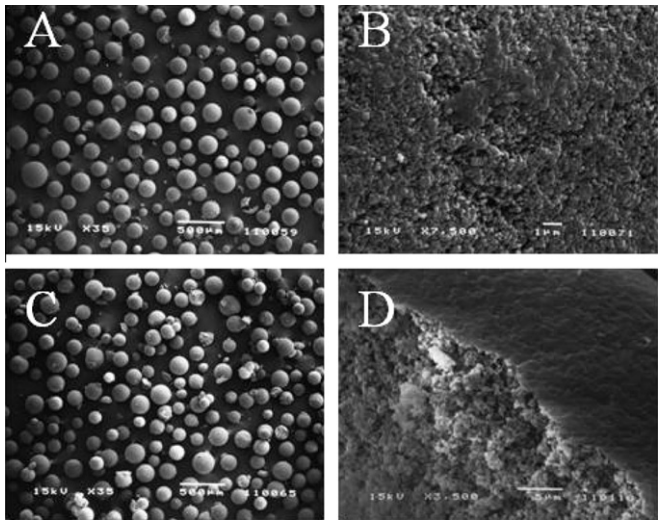


Figure 3. SEM images of Tyro/E (panel A and B) and Tyro/E-LbL (panel C and D) at different magnification.

positively charged polyallylamine hydrochloride (PAH) (2 mg/mL in 0.5 M NaCl), and then treated with negatively charged polystyrene sulfonate (PSS) (2 mg/mL in 0.5 M NaCl). The procedure was repeated until the formation of three layers (Fig. 2, panel B).

The immobilized LbL enzyme (Tyro/E-LbL) retained about 87% of the activity with reference to Tyro/E. A set of scanning electron microscopy (SEM) photographs showing the morphology of the surface of Tyro/E and Tyro/E-LbL particles are reported in Figure 3. Tyro/E shows particles with a regular shape and an average diameter value of 100–150 μm (Fig. 3, panel A). A low number of irregular fragments was observed, which are probably formed by a mechanical damage of particles during the sample preparation. At larger magnification the particles show an irregular surface characterized by grumes of different dimension (Fig. 3, panel B). The SEM photographs of a group of particles (average diameter of 100–150 μm) and a magnification of the cross-section of a single particle of Tyro/E-LbL are shown in Fig. 3, panel C and D. In this latter case the ultrathin coating layers cover the surface of the particle. Both immobilized tyrosinases were characterized for their pH and storage stability, kinetic properties and reusability.

2.2. Effect of pH on tyrosinase activity

The pH/activity curves related to free (Tyro) and immobilized tyrosinase (Tyro/E and Tyro/E-LbL) at 25 °C are shown in Figure 4. Irrespective to immobilization procedure, tyrosinase showed the optimum pH 7.0 that is the same value selected for the immobilization of the enzyme.

Noteworthy, Tyro/E and Tyro/E-LbL were more active than the free enzyme in the range of pH studied. Changes in pH-activity profile after immobilization, eventually involving the shift of the optimal pH value, have been reported during immobilization of tyrosinase on others support.[14,27]

2.3. Storage stability

The storage stability of free and immobilized tyrosinase was evaluated by storing the enzyme in Na-phosphate buffer at −20, 4 and 25 °C for 25 days (Fig. 5, panels A, B and C, respectively). The activity was then measured at specific times at room temperature by the dopachrome method. At each of the temperature studied, Tyro/E and Tyro/E-LbL were more stable than free enzyme, being the Tyro/E-LbL the most stable biocatalyst. To further confirm the integrity of Tyro/E-LbL over time, a novel SEM analysis of particles at time zero (just prepared) and after two months upon storage at 4 °C was carried out at a higher magnification (×15,000). As reported in Figure 6, the surface morphology of the particles (panels A and B) is not significantly altered during the storage period. In addition, the stability of the system was confirmed by Transmission Electron Microscopy (TEM) analysis of a section of the particles after two months of storage, which shows a substantial integrity of the layers (Fig. 6, panel C). This fact, coupled with the maintenance of the catalytic activity for multiple subsequent reactions (see below) confirms a substantial stability of the system. The enhanced stability of enzyme activities within LbL assembly was previously discussed in details by Onda et al.[28] These authors reported that glucose oxidase assembled alternately with polyions retained about 80% of native enzyme activity, thus suggesting that the LbL procedures did not cause significant denaturation.

Also in that case, after the LbL coating, the enzyme maintained its activity for several days.

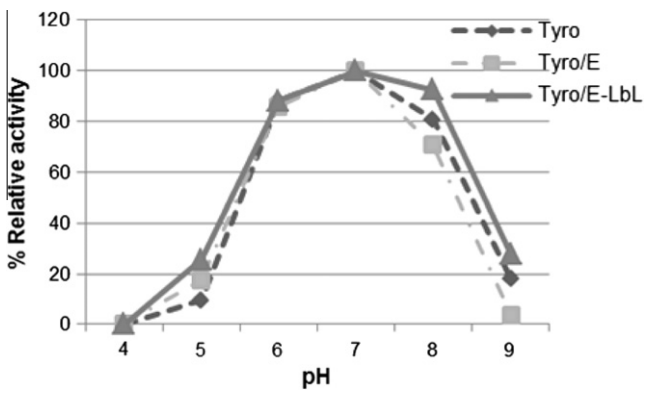


Figure 4. pH optima of free (Tyro) and immobilized tyrosinase (Tyro/E and Tyro/E-LbL). Tyrosinase activity was determined using L-Tyrosine as substrate, pH 4.0–9.0.

2.4. Kinetic assay

Kinetic parameters of free and immobilized tyrosinases were examined by measuring the enzyme activity at different concentrations of L-tyrosine (L-Tyr; range 330–1000 μM) and plotting data to a double reciprocal plot (Lineweaver–Burk plot).[29] Irrespective to procedures used for the immobilization, V_{max} decreased and K_m increased for supported tyrosinases, leading to a partial reduction of the catalytic efficiency (V_{max}/K_m) with respect to free enzyme (Table 2). Similar trends in K_m values were reported for tyrosinase immobilized on other carriers and are attributed to alteration of three-dimensional structure and mass transfer limitations.[30]

2.5. Recycle and reusability

Recycle and reusability assay was performed using L-Tyr as substrate. The oxidations were followed spectrophotometrically at 475 nm. After reaching absorbance plateau, the immobilized biocatalyst was recovered, washed and reused with fresh added substrate. One unit of enzyme activity was defined as the increase in absorbance of 0.001 at defined wavelength, temperature and pH. For successive runs, the enzyme activity measured in the first oxidation was used as the reference value. As shown in Table 3, Tyro/E-LbL was more stable than Tyro/E, retaining 75% of activity after 5 runs.

This behavior suggest that the LbL coating process effectively stabilizes the enzyme from inactivating agents. Different examples of the stabilization effect of LbL are reported.[31]

2.6. Oxidation of phenols

With the aim to evaluate the synthetic relevance of immobilized tyrosinases, a large panel of phenols (Figure 7) was oxidized, including *para*-cresol **1**, 4-ethyl phenol **2**, 4-*tert*-butyl phenol **3**, 4-*sec*-butyl phenol **4**, 2,4-di-*tert*-butyl phenol **5**, *meta*-cresol **6**, 3,4-dimethyl phenol **7**, 4-chloro phenol **8,** 4-chloro-2-methyl phenol **9**, 2-methoxy-4-methyl phenol **10**, 2-methoxy phenol **11**, 3-(4-hydroxyphenyl)propionic acid **12**, 4-hydroxyphenylacetic acid **13**, bis(4-hydroxyphenyl)methane **14** and tyrosol **15**. The oxidation of **1** (0.05 mmol) with free tyrosinase (Tyro; 263 IU) in buffer (5.0 mL) was performed in the presence of AA in a previously optimized concentration (1.5 equiv).[32] For low soluble phenols **3, 5, 14**, the substrates were dissolved in CH_3CN (1.0 mL) and then added to the reaction mixture (see next). Reactions were performed at room temperature for 24 h. Under these experimental conditions catechol **1a** was obtained as the only recovered product in quantitative yield and conversion of substrate (Scheme 1, Table 4, entry 1)

Tyro/E and Tyro/E-LbL performed in a similar way affording **1a** in quantitative yield and conversion of substrate (Scheme 1, Table 4, entries 2 and 3). Thus, the reactivity and selectivity of tyrosinase

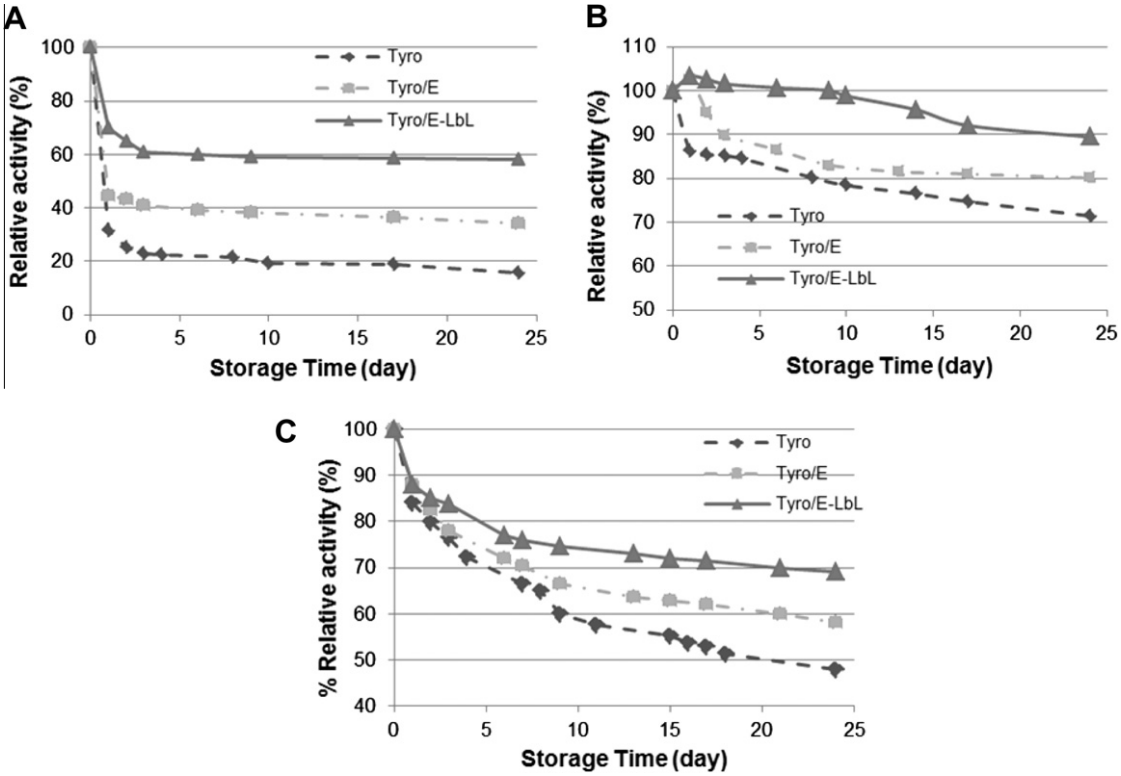


Figure 5. Storage stability of free (Tyro) and immobilized tyrosinase (Tyro/E and Tyro/E-LbL) at (A) −20 °C, (B) 4 °C and (C) 25 °C in Na-phosphate buffer 0.1 M, pH 7.

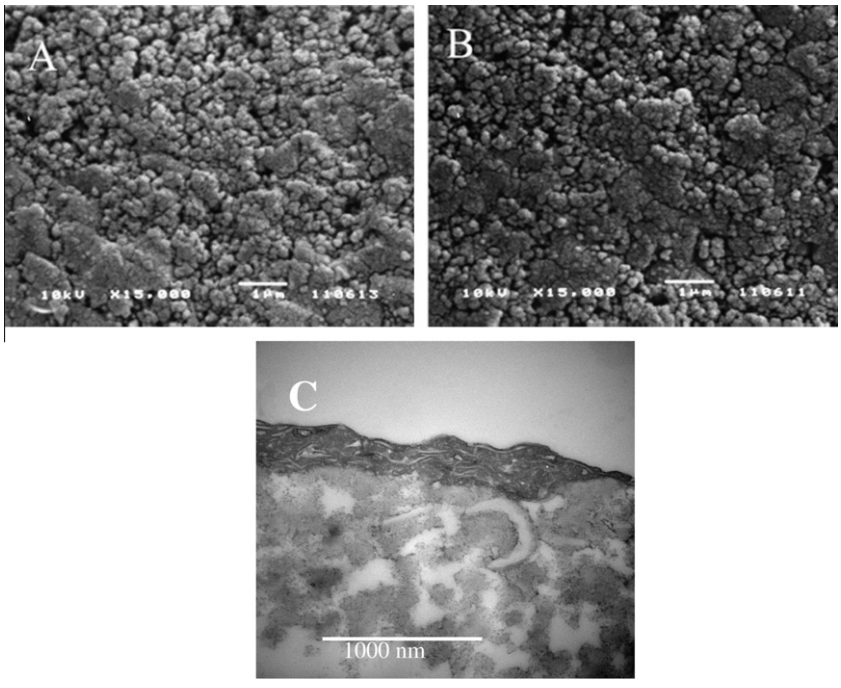


Figure 6. Panels A and B: SEM images of Tyro/E-LbL at a higher magnification (×15,000) at time zero (panel A) and after two months (panel B) upon storage at 4 °C. Panel C: TEM image of a section of Tyr/E-LbL particle upon storage for two months at 4 °C. LBL layers are clearly visible in the upper part of the picture.

was completely retained after the immobilization procedures. The oxidation of **2** confirmed the high reactivity of immobilized tyrosinases, the catechol **2a** being again obtained as the only recovered product in quantitative yield and conversion of substrate (Scheme 1, Table 4, entries 4–6). Noteworthy, Tyro/E and Tyro/E-LbL were efficient and selective biocatalysts also in the oxidation of *para*-alkyl substituted phenols characterized by a high steric hindrance, as in the case of bulky substituted phenols **3** and **4**. As reported in Table 4, the oxidation of **3** required the addition a small amount of CH_3CN (0.1 mL) to increase the solubility of substrate. In these latter cases, irrespective to experimental conditions, catechols **3a** and **4a** were synthesized in yield higher than 90% (Scheme 1, Table 4, entries

Table 2
Kinetic parameters of free (Tyro) and immobilized (Tyro/E, Tyro/E-LbL) tyrosinase

Entry	Enzyme	K_m (μM)	V_{max}[a] ($\times 10^{-3}$)	V_{max}/K_m ($\times 10^{-6}$)
1	Tyro	180	6	33
2	Tyo/Eup	270	4	15
3	Tyro/E-LbL	300	3	10

[a] V_{max} was defined as δAbs/min μg_{enzyme}.

Table 3
Reusability of Tyro-immobilized systems

Run	Tyro/E[a]	Tyro/E-LbL[a]
1	90	91
2	76	86
3	68	80
4	63	79
5	56	75

[a] Reusability is expressed as percentage of activity in each runs respect to that measured in the first reference oxidation.

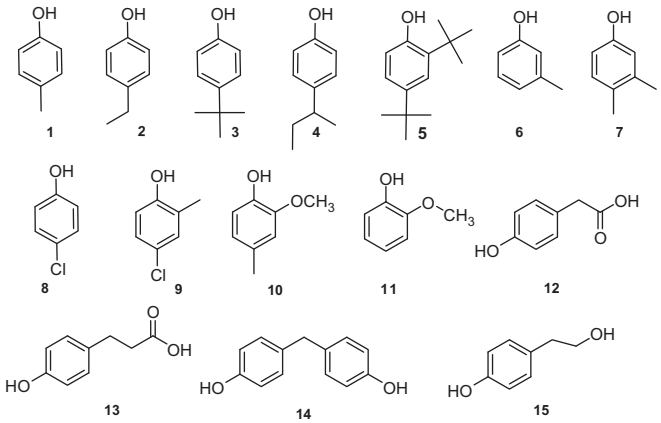
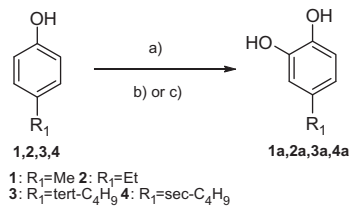


Figure 7. Phenols selected for the oxidation with free and immobilized tyrosinases.

7–12). On the other hand, 2,4-di-*tert*-butyl phenol **5** was stable under all of the conditions tested, probably due to known inhibition effect exerted by the steric encumbering of the *ortho*-substituent.[33] A substituent in the *meta*-position on the aromatic ring, as in the case of 3-methyl phenol **6** and 3,4-dimethylphenol **7**, showed a slightly inhibitory effect. In these latter cases, twice amount of enzyme was required (526 IU) to produce catechols **1a** and **7a** in 42–48% and 80–84% yield, respectively, Tyro/E-LbL being the best biocatalyst (Scheme 2, Table 5, entries 2, 3, 5 and 6).The catechols were obtained as the only recovered products.

The highest yield observed in the case of **7a** suggests that the inhibitory effect of the *meta*-substituent can be partially balanced by the presence of a *para*-substituent with an inductive electron-donor effect. Next we analyzed the oxidation of chloro phenol



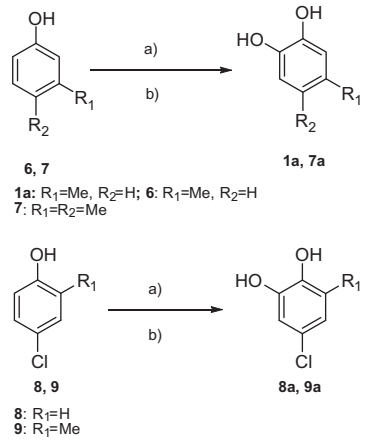
Scheme 1. Oxidation of phenols **1–4**. Reagents and conditions: (a) Tyro-based systems, O_2, AA; (b) Na-phosphate buffer; (c) Na-phosphate buffer/CH_3CN.

Table 4
Oxidation of *para*-alkyl substituted phenols **1–4**[a]

Entry	Substrate	Biocatalysts	Products	Conversion (%)	Yield (%)
1	1	Tyro	**1a**	>99	>99
2	1	Tyro/E	**1a**	94	94
3	1	Tyro/E-LbL	**1a**	97	97
4	2	Tyro	**2a**	>99	>99
5	2	Tyro/E	**2a**	95	95
6	2	Tyro/E-LbL	**2a**	98	98
7	3	Tyro	**3a**[b]	>99	>99
8	3	Tyro/E	**3a**[b]	92	92
9	3	Tyro/E-LbL	**3a**[b]	95	95
10	4	Tyro	**4a**	96	96
11	4	Tyro/E	**4a**	92	92
12	4	Tyro/E-LbL	**4a**	94	95

[a] Reaction conditions: substrate (0.05 mmol), AA (1.5 equiv) and tyrosinase (263 IU) were taken in 5.0 mL of phosphate buffer solution for 24 h.
[b] Oxidation performed in Na-phosphate buffer/CH_3CN.

derivatives, 4-chloro phenol **8** and 4-chloro-2-methyl phenol **9**. Treatment of **8** with Tyro/E and Tyro/E-LbL (263 IU) afforded catechol **8a** in quantitative yield and conversion of substrate (Scheme 2, Table 5, entries 8, 9). A similar result was obtained with the free enzyme (Table 5, entry 7). As expected, twice amount of tyrosinase (526 IU) was required for the oxidation of **9**, due to the presence of the *ortho*-substituent. Despite this request, the catechol **9a** was isolated in high yield (Scheme 2, Table 5, entries 11 and 12), confirming a beneficial role of the electron-donor substituent in the *para*-position of the aromatic ring. Note that, phenol derivatives characterized by an electron-donor *ortho*-substituent, as in the case of 2-methoxy-4-methyl phenol **10** and 2-methoxy phenol **11**, while requesting a twice amount of enzyme (526 IU), afforded the corresponding catechols **10a** and **11a** in significant yield (Scheme 3, Table 6, entries 2, 3, 5 and 6). Again, the Tyro/E-LbL was the best biocatalyst. Our attention was next focused on the synthesis of catechol derivatives characterized by a potential biological activity. Since acidic catechols shows antibacterial,[34] antimicrobial[35] and antioxidant[36] activities, we first evaluated the oxidation of two phenolic acid derivatives, 3-(4-hydroxyphenyl)propionic acid **12** and 4-hydroxyphenylacetic acid **13**. Irrespective to experimental conditions used, the catechols **12a** and **13a** were obtained in high yield as the only recovered products, confirming the generality of the procedure (Scheme 3, Table 6, entries 8, 9, 11 and 12). In a similar way, the oxidation of bis(4-hydroxyphenyl)methane **14** proceeded with high conversion of substrate to afford the mono-catechol and

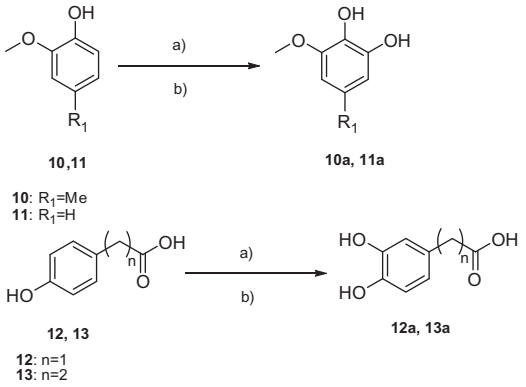


Scheme 2. Oxidation of phenols **6–9**. Reagents and conditions: (a) Tyro-based systems, O_2, AA; (b) Na-phosphate buffer.

Table 5
Oxidation of phenols **6**–**9**[a]

Entry	Substrate	Biocatalysts	Products	Conversion (%)	Yield (%)
1	**6**	Tyro	**1a**	52	52
2	**6**	Tyro/E	**1a**	42	42
3	**6**	Tyro/E-LbL	**1a**	48	48
4	**7**	Tyro	**7a**	88	88
5	**7**	Tyro/E	**7a**	80	80
6	**7**	Tyro/E-LbL	**7a**	84	84
7	**8**	Tyro	**8a**[b]	>99	>99
8	**8**	Tyro/E	**8a**[b]	94	>99
9	**8**	Tyro/E-LbL	**8a**[b]	97	>99
10	**9**	Tyro	**9a**	>99	>99
11	**9**	Tyro/E	**9a**	82	82
12	**9**	Tyro/E-LbL	**9a**	89	89

[a] Reaction conditions: substrate (0.05 mmol), AA (1.5 equiv) and tyrosinase (526 IU) were taken in 5.0 mL of phosphate buffer solution for 24 h.
[b] Oxidation performed with 263 IU of tyrosinase.



Scheme 3. Oxidation of phenols **10**–**13**. Reagents and conditions: (a) Tyro-based systems, O_2, AA; (b) Na-phosphate buffer.

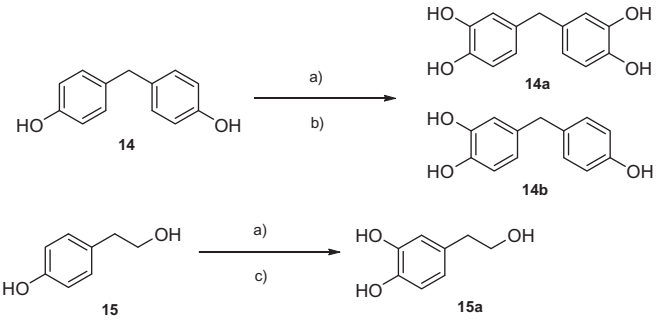
Table 6
Oxidation of phenols **10**–**13**[a]

Entry	Substrate	Biocatalysts	Products	Conversion (%)	Yield (%)
1	**10**	Tyro	**10a**[b]	80	80
2	**10**	Tyro/E	**10a**[b]	73	73
3	**10**	Tyro/E-LbL	**10a**[b]	78	78
4	**11**	Tyro	**11a**[b]	84	84
5	**11**	Tyro/E	**11a**[b]	73	73
6	**11**	Tyro/E-LbL	**11a**[b]	79	79
7	**12**	Tyro	**12a**	88	88
8	**12**	Tyro/E	**12a**	75	75
9	**12**	Tyro/E-LbL	**12a**	84	84
10	**13**	Tyro	**13a**	48	88
11	**13**	Tyro/E	**13a**	37	77
12	**13**	Tyro/E-LbL	**13a**	40	80

[a] Reaction conditions: substrate (0.05 mmol), AA (1.5 equiv) and tyrosinase (263 IU) were taken in 5.0 mL of phosphate buffer solution for 24 h.
[b] Oxidation performed with 526 IU of tyrosinase.

bis-catechol derivatives **14a** and **14b** in appreciable yield (Scheme 4, Table 7, entries 1–3).

In accordance with the selectivity of the free enzyme, immobilized tyrosinases afforded **14a** as the main reaction product, Tyro/E-LbL being the best biocatalys. Moreover, the reaction performed with twice amount of enzyme and for longer reaction time (48 h) produced **14a** as the only recovered product in quantitative yield (Table 7, entries 4 and 5). This transformation is of synthetic interest because polyhydroxylated diphenylmethane derivatives are characterized by antiviral,[37] antioxidant,[38] and antimicrobial activities.[39]



Scheme 4. Oxidation of phenols **14** and **15**. Reagents and conditions: (a) Tyro-based systems, O2, AA; (b) Na-phosphate buffer/CH3CN; (c) Na-phosphate buffer.

Table 7
Oxidation of phenols **14**–**15**[a]

Entry	Substrate	Biocatalysts	Products	Conversion (%)	Yield (%)
1	**14**	Tyro	**14a(14b)**[b]	>99	65(34)
2	**14**	Tyro/E	**14a(14b)**[b]	95	53(41)
3	**14**	Tyro/E-LbL	**14a(14b)**[b]	98	66(32)
4	**14**	Tyro/E	**14a**[b,c]	>99	>99
5	**14**	Tyro/E-LbL	**14a**[b,c]	>99	>99
6	**15**	Tyro	**15a**	85	85
7	**15**	Tyro/E	**15a**	70	70
8	**15**	Tyro/E-LbL	**15a**	77	77

[a] Reaction conditions: substrate (0.05 mmol), AA (1.5 equiv) and tyrosinase (263 IU) were taken in 5.0 mL of phosphate buffer solution for 24 h.
[b] Oxidation performed in Na-phosphate buffer/CH_3CN.
[c] Oxidation performed with 526 IU of tyrosinase for 48 h.

Finally, we studied the synthesis of 3,4-dihydroxyphenylethanol (hydroxytyrosol), a low molecular weight component in virgin olive oil and in mill wastes.[6a] This compound shows several biological activity, including antimicrobial,[40] hypoglycemic,[41] antioxidant,[41] cardiovascular properties,[42] inhibition of platelet aggregation[43] and inhibition of lipoxygenases,[44] or induction of apoptosis.[45] When 2-(4-hydroxy phenyl)ethanol (tyrosol) **15** was treated with Tyro/E and Tyro/E-LbL under previously reported conditions, catechol **15a** was obtained in 70% and 77% yield, respectively (Scheme 4, Table 7, entries 7 and 8). In this latter case, free enzyme showed a reactivity slightly higher than immobilized biocatalysts (Table 7, entry 6 vs entries 7 and 8).

3. Conclusion

New heterogeneous tyrosinase biocatalysts were synthesized by immobilization on Eupergit®C250L and LbL coating with polyelectrolyte. With respect to the free enzyme used as reference, tyrosinase retained the catalytic activity and selectivity after immobilization on Eupergit®C250L and successive coating by LbL technique. It is interesting to note, that in all of the cases studied, Tyro/E-LbL was more efficient than Tyro/E, suggesting a stabilization effect exerted by the polyelectrolyte coating. Tyro/E and Tyro/E-LbL were stable enough to perform at least five recycling experiments with similar conversion and selectivity. The stability of tyrosinase under storage conditions at different temperatures was also found to be increased in the presence of the support. Again, Tyro/E-LbL was the most stable and reusable catalyst. The structure of the interaction between the Eupergit-supported enzyme and the polyelectrolyte coating has not been studied in detail, because of the greater complexity of this system compared to the previously described film in which the enzyme is immobilized directly within the LbL coating. However, the maintenance of the catalytic activity for multiple runs by Tyro/E-LbL,

suggests that the system does not undergo structural changes.[46] About the selectivity of the oxidations, *para*-substituted phenols were efficiently oxidized, even in the case of highly encumbering alkyl *para*-substituents. *Meta*-substituted and *ortho*-substituted phenols required a twice amount of enzyme to yield the corresponding catechols in high yield, probably due both to the effect exerted by the substituent on the electronic distribution of the aromatic ring[33] and the steric encumbering for the formation of the first intermediate with the Cu atom in the active site of the enzyme. Since catechols are biologically active compounds difficult to synthesize by traditional chemical procedure under environmental friendly conditions, the use of immobilized tyrosinases open a novel synthetic alternative for this interesting family of substances.

4. Experimental section

Mushroom tyrosinase from *Agaricus bisporus* (Tyro), Eupergit®C250L, poly(sodium 4-styrenesulfonate) (PSS, MW 70000), poly(allylamine hydrochloride) (PAH, MW 56000), L-tyrosine (L-Tyr), ascorbic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), bovine serum albumin (BSA), ethyl acetate (EtOAc), acetonitrile (CH_3CN), sodium sulfate anhydrous (Na_2SO_4), dodecane, pyridine, hesamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and phenols were purchased from Sigma–Aldrich. All spectrophotometric measurements were made with a Varian Cary50 UV–vis spectrophotometer equipped with a single cell peltier thermostatted cell holder. Spectrophotometric data were analyzed with Cary WinUV software. All experiments were carried out in triplicate using free and immobilized tyrosinase.

4.1. Tyrosinase immobilization on Eupergit®C250L

The immobilization of tyrosinase was performed by a modification of literature procedures.[23] Dry Eupergit®C250L (1.0 g) was added to different amount of buffer 0.1 M (pH 5.0–8.0) containing tyrosinase (Tyro, 1.0–5.0 mg, 13900 U/mg). The mixture was incubated for 24–48 h at room temperature with orbital shaking. At the end of the coupling period, the resin beads were filtered, washed (5 × 8 mL) with buffer until no activity was detected in the washing. The obtained beads were incubated with glycine (3.0 M) for 2 h to block residual epoxy groups,[47] then washed with buffer and finally air-dried and stored at 4 °C. The amount in milligrams and the units of coupled tyrosinase (Tyro/E) were calculated by the difference between the amount/units loaded and that recovered in the washings by conventional Bradford and activity assay.

4.2. Tyrosinase immobilization on Eupergit®C250L coated with Layer-by-Layer method

Tyro/E, synthesized using the optimal experimental conditions described above, was coated with the Layer-by-Layer method (LbL) in accordance to literature procedures.[48] Briefly, PAH and PSS solutions (2.0 mg/mL in 0.5 M NaCl) were alternately added to Tyro/E system: each polyelectrolyte layer was adsorbed for 20 min at room temperature with orbital shaking and then washed with 0.5 M NaCl to remove excess of polyelectrolytes. The deposition of polyelectrolytes started with PAH and was repeated to obtained three layers (PAH-PSS-PAH). Immobilized tyrosinase (Tyro/E-LbL) was air-dried and stored at 4 °C.

4.3. Determination of protein concentration

Protein concentration was determined spectrophotometrically at 595 nm according to Bradford using BSA as standard.[49]

4.4. Activity assay

Tyrosinase assay was performed by the dopachrome method as previously described.[24] Briefly, L-Tyr solution (1.0 mL, 2.5 mM), Na-phosphate buffer 0.1 M, pH 7.0 (1.9 mL) was incubated under vigorous stirring at 25 °C for 10 min. Then, an appropriate amount of free or immobilized enzyme in Na-phosphate buffer (100 μl) was added to the mixture and the initial rate was immediately measured as linear increase in optical density at 475 nm, due to dopachrome formation. One unit of enzyme activity was defined as the increase in absorbance of 0.001 per minute at pH 7, 25 °C in a 3.0 mL reaction mixture containing 0.83 mM of L-tyrosine and 67 mM of Na-phosphate buffer pH 7.0. The specific activity of biocatalysts was also analyzed in the pH range of 4.0–9.0.

4.5. Kinetic assay

Kinetic parameters, K_m and V_{max} and V_{max}/K_m, were determined by measuring enzyme activity at different concentrations of L-Tyr (330–1000 μM) and plotting data to a double reciprocal plot (Lineweaver–Burk plot).[29] Reactions were carried out by means of the same procedure as for activity assay, using Tyro (53 μg) and Tyro/E (70 μg) and Tyro/E-LbL (70 μg), and measuring absorbance at 475 nm as described above.

4.6. Stability assay

Tyrosinase (53 μg for Tyro and 70 μg for Tyro/E and Tyro/E-LbL) in Na-phosphate buffer 0.1 M (pH 7.0) was stored at three temperatures (−20, +4, 25 °C). At different times (0–25 days), aliquots were taken and the activity was determined at room temperature by the dopachrome method. For each sample, tyrosinase activity was expressed as relative percentage activity respect to that at time zero.

4.7. Transmission Electron Microscopy (TEM) measurements

Samples were prepared at the Interdepartmental Centre of Electron Microscopy, Tuscia University, Viterbo, Italy, using conventional procedures. For Transmission Electron Microscopy (TEM), samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 overnight at 4 °C. After rinsing in the same buffer, they were post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 h and then washed in distilled water. Specimens were dehydrated in a graded ethanol series and embedded in LRWhite resin. Thin sections were cut with Reichert Ultracut ultramicrotome using a diamond knife, collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

4.8. Scanning Electron Microscopy (SEM) measurements

Samples were prepared at the Interdepartmental Centre of Electron Microscopy, Tuscia University, Viterbo, Italy, using conventional procedures. For Scanning Electron Microscopy (SEM), samples were sputter-coated with gold in a Balzers MED 010 unit and observed with a JEOL JSM 5200 electron microscope. Micrographs were taken by a Mamiya camera applied to the microscope using TMAX 100 ASA films.

4.9. Enzyme recycling

Immobilized enzyme (Tyro/E and Tyro/E-LbL) was recycled as follow: L-tyrosine (0.83 mM), immobilized Tyro (70 μg) and Na-phosphate buffer 0.1 M, pH 7.0 (3.0 mL) were placed in vials

at 25 °C. At specific time, solutions were removed to measure absorbance at 475 nm and then returned to the vials as rapidly as possible. After reaching plateau, enzyme was washed with buffer, recycled and reused again. One unit of enzyme activity was defined as the increase in absorbance of 0.001 at defined wavelength, temperature and pH. For each run, tyrosinase activity was expressed as relative percentage activity respect to that at first run.

4.10. Phenols oxidation

A panel of phenols (Fig. 7) were oxidized, including *para*-cresol **1**, 4-ethyl phenol **2**, 4-*tert*-butyl phenol **3**, 4-*sec*-butyl phenol **4**, 2,4-di-*tert*-butyl phenol **5**, *meta*-cresol **6**, 3,4-dimethyl phenol **7**, 4-chloro phenol **8**, 4-chloro-2-methyl phenol **9**, 2-methoxy-4-methyl phenol **10**, 2-methoxy phenol **11**, 3-(4-hydroxyphenyl)propionic acid **12**, 4-hydroxyphenylacetic acid **13**, bis(4-hydroxyphenyl)methane **14** and tyrosol **15**. As a general procedure phenol (0.05 mmol), tyrosinases (263–526 IU) and AA (1.5 equiv) were placed in 0.1 M Na-phosphate buffer pH 7.0 (5.0 mL) in vigorous stirring at room temperature. For insoluble aqueous phenols **3, 5, 14** substrates were dissolved in CH_3CN (1.0 mL) and then added to the buffer solutions. Oxidations were performed using homogeneous and heterogeneous conditions. Reactions were monitored by thin layer chromatography (TLC). After the disappearance of the substrate, the reaction mixture was acidified with a solution of HCl 1.0 N and extracted twice with EtOAc. The organic extracts were treated with a saturated solution of NaCl and dried over anhydrous Na_2SO_4, then filtered and concentrated under vacuum to yield a colored crude. In the case of immobilized enzyme, biocatalyst was first recovered by filtration and the solution was subjected to the same work up described above. The obtained colored residue was treated with pyridine, HMDS and TMCS (HMDS–TMCS, 2:1 v/v) under vigorous stirring at room temperature for 30 min, then allowed to stand for 5 min.[50] All products were identified by ^{1}H NMR, ^{13}C NMR and GC–MS. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 200 MHz spectrometer using $CDCl_3$ as solvent. All chemical shift are expressed in parts per million (δ scale). GC–MS analysis were performed on a GCMS-QP5050 Shimadzu apparatus using a SPB column (25 m × 0.25 mm and 0.25 mm film thickness) and an isothermal temperature profile of 100 °C for 2 min, followed by a 10 °C/min temperature gradient to 280 °C for 25 min. The injector temperature was 280 °C. Chromatography-grade helium was used as the carrier gas with a flow of 2.7 mL/min. Mass spectra were recorded with an electron beam of 70 eV.

4.10.1. 4-Methylcatechol (4-methyl-1,2-benzenediol) (1a)

Oil. ^{1}H NMR[51] (200 MHz, $CDCl_3$) δ_H (ppm) 2.24 (3H, s, CH_3), 5.04 (1H, br s, OH), 5.18 (1H, br s, OH), 6.61–6.76 (3H, m, Ph-H). ^{13}C NMR[51] (50 MHz, $CDCl_3$) δ_C (ppm) 20.8 (CH_3), 115.3 (CH), 116.2 (CH), 121.5 (CH), 131.1 (C), 141.0 (C), 143.3 (C). MS, (*m/z*): 268 (M^+), 253 ($M-CH_3$), 238 [$M-(CH_3)_2$], 223 [$M-(CH_3)_3$], 195 [$M-Si(CH_3)_3$], 179 [$M-OSi(CH_3)_3$], 164 [$M-OSi(CH_3)_4$], 149 [$M-OSi(CH_3)_5$], 134 [$M-OSi(CH_3)_6$], 106 [$M-OSi_2(CH_3)_6$], 90 [$M-O_2Si_2(CH_3)_6$].

4.10.2. 4-Ethylcatechol (4-ethyl-1,2-benzenediol) (2a)

Oil. ^{1}H NMR[52] (200 MHz, $CDCl_3$) δ_H (ppm) 1.04 (3H, m, CH_3), 2.36 (2H, m, CH_2), 6.00–7.25 (3H, m, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 15.2 (CH_3), 28.1 (CH_2), 116.5 (CH), 117.4 (CH), 124.2 (CH), 139.3 (C), 145.7 (C), 148.4 (C). MS, *m/z:* 282 (M^+), 267 [$M-CH_3$], 252 [$M-(CH_3)_2$], 237 [$M-(CH_3)_3$], 209 [$M-Si(CH_3)_3$], 193 [$M-OSi(CH_3)_3$], 179 [$M-OSi(CH_3)_4$], 164 [$M-OSi(CH_3)_5$], 148 [$M-OSi(CH_3)_6$], 120 [$M-OSi_2(CH_3)_6$].

4.10.3. 4-*tert*-Butylcatechol (4-*tert*-butylbenzene-1,2-diol) (3a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 1.33 (9H, s, CH_3), 6.63–7.11 (3H, m, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 31.2 (3 × CH_3), 34.5 (C), 116.5 (CH), 116.9 (CH), 122 (CH), 144.3 (C), 146.2 (C), 147.1 (C). *MS, m/z:* 310 (M^+), 295 ($M-CH_3$), 280 [$M-(CH_3)_2$], 265 [$M-(CH_3)_3$], 237 [$M-Si(CH_3)_3$], 222 [$M-OSi(CH_3)_3$], 207 [$M-OSi(CH_3)_4$], 192 [$M-OSi(CH_3)_5$], 176 [$M-OSi(CH_3)_6$], 148 [$M-OSi_2(CH_3)_6$].

4.10.4. 4-*sec*-Butylcatechol (4-(1-methylpropyl)-1,2-benzenediol) (4a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 1.10 (3H, m, CH_3), 1.22 (3H, m, CH_3), 1.53 (2H, m, CH_2), 3.23 (1H, m, CH), 6.52–6.84 (3H, m, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 11.2 (CH_3), 22.3 (CH_3), 31.2 (CH_2), 43.1 (CH), 113.3 (CH), 114.1 (CH), 124.4 (CH), 136.2 (C), 145.1 (C), 147.0 (C). *MS, m/z:* 310 (M^+), 295 ($M-CH_3$), 280 [$M-(CH_3)_2$], 237 [$M-Si(CH_3)_3$], 222 [$M-OSi(CH_3)_3$], 207 [$M-OSi(CH_3)_4$], 192 [$M-OSi(CH_3)_5$], 149 [$M-OSi_2(CH_3)_6$], 133 [$M-O_2Si_2(CH_3)_6$].

4.10.5. 4,5-Dimethylcatechol (4,5-dimethyl-1,2,-benzenediol) (7a)

Oil. ^{1}H NMR[53] (200 MHz, $CDCl_3$) δ_H (ppm) 2.20 (s, 6H,CH_3), 6.51 (s, 2H, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 19.7 (2 × CH_3), 118.9 (2 × CH), 130.1 (2 × C), 143.9 (2xC). *MS, m/z:* 282 (M^+), 267 ($M-CH_3$), 252 [$M-(CH_3)_2$], 237 [$M-(CH_3)_3$], 210 [$M-Si(CH_3)_3$], 194 [$M-OSi(CH_3)_3$], 179 [$M-OSi(CH_3)_4$], 164 [$M-OSi(CH_3)_5$], 149 [$M-OSi(CH_3)_6$], 105 [$M-O_2Si_2(CH_3)_6$].

4.10.6. 4-Chlorocatechol (4-chloro-1,2-benzenediol) (8a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 6.72–6.83 (3H, m, Ph-H), 8.10 (2H, br s, OH). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 113.0 (CH), 117.3 (CH), 125.4 (CH), 127.2 (C), 145.0 (C), 152.3 (C). MS, *m/z:* 288 (M^+), 273 ($M-CH_3$), 258 [$M-(CH_3)_2$], 243 [$M-(CH_3)_3$], 215 [$M-Si(CH_3)_3$], 199 [$M-OSi(CH_3)_3$], 184 [$M-OSi(CH_3)_4$], 169 [$M-OSi(CH_3)_5$], 126 [$M-OSi_2(CH_3)_6$].

4.10.7. 5-Chloro-3-methylcatechol (5-chloro-3-methyl-1,2-benzenediol) (9a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 2.10 (3H, s, CH_3), 6.53–6.74 (2H, s, Ph-H), 7.52 (2H, br s, OH). ^{13}C NMR (50 MHz $CDCl_3$) δ_C (ppm) 17.2 (CH_3), 116.3 (CH), 123.2 (C), 125.4 (C), 126.3 (CH), 140.2 (C), 151.0 (C). *MS, m/z*: 302 (M^+), 287 ($M-CH_3$), 272 [$M-(CH_3)_2$], 229 [$M-Si(CH_3)_3$], 213 [$M-OSi(CH_3)_3$], 198 [$M-OSi(CH_3)_4$], 168 [$M-OSi(CH_3)_6$].

4.10.8. 3-Methoxy-5-methyl-1,2-benzenediol (10a)

Oil. ^{1}H NMR[54] (200 MHz, $CDCl_3$) δ_H (ppm) 2.19 (3H, s, CH_3), 3.80 (3H, s, OCH_3), 6.27–6.29 (2H, m, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 22.0 (CH_3), 56.6 (CH_3), 106.2 (CH), 111.1 (CH), 131.2 (C), 133.6 (C), 145.4 (C), 145.8 (C). *MS, m/z*: 298 (M^+), 283 ($M-CH_3$), 268 [$M-(CH_3)_2$], 253 [$M-Si(CH_3)_3$], 225 [$M-Si(CH_3)_3$], 209 [$M-OSi(CH_3)_3$], 194 [$M-OSi(CH_3)_4$], 179 [$M-OSi(CH_3)_5$], 164 [$M-OSi(CH_3)_6$].

4.10.9. 3-Methoxy-1,2-benzenediol (11a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 3.79 (3H, s, CH_3), 6.54–6.80 (3H, m, Ph-H), 8.08 (2H, br s, OH). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 56.2 (CH_3), 105.4 (CH), 111.1 (CH), 122.8 (CH), 138.9 (C), 147.6 (C), 148.7 (C). *MS, m/z:* 284 (M^+), 269 ($M-CH_3$), 254 [$M-(CH_3)_2$], 239 [$M-(CH_3)_3$], 211 [$M-Si(CH_3)_3$], 195 [$M-OSi(CH_3)_3$], 180 [$M-OSi(CH_3)_4$], 165 [$M-OSi(CH_3)_5$], 106 [$M-O_2Si_2(CH_3)_6$].

4.10.10. 3,4-Dihydroxy-benzenepropanoic acid (12a)

Oil. ^{1}H NMR[55] (200 MHz, $CDCl_3$) δ_H (ppm) 2.47 (2H, m, CH_2), 2.70 (2H, m, CH_2), 6.50–6.65 (3H, m, Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 31.5 (CH_2), 37.2 (CH_2), 116.3 (CH), 116.4 (CH), 120.5 (CH), 133.8 (C), 144.1 (C), 146.2 (C), 177.1 (C). *MS, m/z:* 384 (M^+), 369 ($M-CH_3$), 354 [$M-(CH_3)_2$], 339 [$M-(CH_3)_3$], 311 [$M-Si(CH_3)_3$], 295 [$M-OSi(CH_3)_3$], 280 [$M-OSi(CH_3)_4$], 265 [$M-OSi(CH_3)_5$], 222 [$M-OSi_2(CH_3)_6$].

4.10.11. 3,4-dihydroxy-benzeneacetic acid (13a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 3.56 (2H, s, CH_2), 6.49–7.10 (3H, m Ph-H), 9.4 (3H, br s, OH). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 41.2 (CH_2), 114.5 (CH), 116.8 (CH), 120.6 (CH), 126.1 (C), 145.2 (C), 145.4 (C), 175.0 (C). *MS, m/z:* 384 (M^+), 369 ($M-CH_3$), 354 ($M-(CH_3)_2$), 339 [$M-(CH_3)_3$], 311 [$M-Si(CH_3)_3$], 295 [$M-OSi(CH_3)_3$], 280 [$M-OSi(CH_3)_4$], 265 [$M-OSi(CH_3)_5$], 222 [$M-OSi_2(CH_3)_6$].

4.10.12. 4-(*para*-Hydroxybenzyl)-pyrocatechol (4-[(4-hydroxyphenyl)methyl]-1,2-Benzenediol) (14a)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 4.10 (2H, m, CH_2), 6.51–7.22 (7H, m. Ph-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 42.2 (CH), 115.5 (CH), 116.1 (2 × CH), 116.5 (CH), 121.3 (CH), 130.2 (2 × C), 133.5 (C), 134.5 (C), 144.2 (C), 145.3 (C), 156.1 (C). *MS*, *m/z*: 432 (M^+), 417 (M-CH_3), 402 [M-$(CH_3)_2$], 359 [M-$Si(CH_3)_3$], 343 [M-OSi $(CH_3)_3$], 329 [M-$OSi(CH_3)_4$], 314 [M-$OSi(CH_3)_5$], 298 [M-$OSi(CH_3)_6$].

4.10.13. 4,4′-Methylenedi-pyrocatechol (4,4′-methylenebis-1,2-benzenediol) (14b)

Oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H (ppm) 4.12 (2H, m, CH_2), 6.62–7.10 (6H, m. Ph-H), 8.13 (4H, s, OH). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C (ppm) 40.2 (CH_2), 115.5 (2 × CH), 116.2 (2 × CH), 121.1 (2 × CH), 135.4 (2 × C), 144.3 (2 × C), 145.2 (2 × C). *MS*, *m/z*: 520 (M^+), 505 ($M-CH_3$), 447 [$M-Si(CH_3)_3$], 431 [$M-OSi(CH_3)_3$], 417 [$M-OSi\ (CH_3)_4$], 343 [$M-O_2Si_2(CH_3)_6$], 329 [$M-O_2Si_2(CH_3)_7$].

4.10.14. 3,4-Dihydroxyphenylethanol (hydroxytyrosol) (15a)

Oil. ^{1}H NMR[56] (200 MHz, acetone-d_6) δ_H (ppm) 2.65 (2H, m, CH_2), 3.67 (2H, mt, CH_2), 6.54–6.72 (3H, m, Ph-H), 7.67 (2H, br s, 2H); ^{13}C NMR (50 MHz, acetone-d_6) δ_C (ppm) 39.7 (CH_2), 64.2(CH_2), 115.9 (CH), 116.8 (CH), 121.0 (CH), 131.9 (C), 144.0 (C), 145.6 (C); *MS*, *m/z*: 386 (M^+), 371 ($M-CH_3$), 356 [$M-Si(CH_3)_2$], 341 [$M-Si(CH_3)_3$], 269 [$M-Si(CH_3)_3$], 253 [$M-OSi(CH_3)_3$], 238 [$M-OSi(CH_3)_4$], 223 [$M-OSi(CH_3)_5$], 208 [$M-OSi(CH_3)_6$].

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