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PEG-immobilization of cardol and soluble polymer-supported synthesis of some cardol–coumarin derivatives: Preliminary evaluation of their inhibitory activity on mushroom tyrosinase

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ABSTRACT

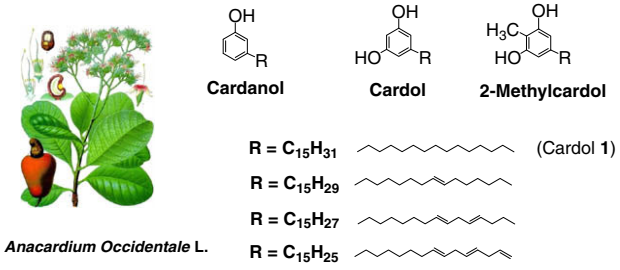
In this work, the PEG-immobilization and the liquid phase synthesis of some coumarins derived from cardol are presented. Some preliminary results on their tyrosinase inhibitory activity are also included.

Cardol, 2-methyl cardol and cardanol (Scheme 1) are the main phenolic components present in the cashew nut shell liquid (CNSL), an oil obtained as a by-product of the cashew (*Anacardium occidentale* L.) nut processing. As renewable raw materials, some of these could be useful for applications in fine chemical industry.[1–4]

Chemically, cardols are 5-*n*-pentadecylresorcinols with saturated, monoolefinic (8), diolefinic (8, 11) or triolefinic (8, 11, 14) hydrocarbon long side chain.[5] Due to their structure, similar to those of tocopherols, cardols are also known as the most prominent members of the resorcinolic lipids, so called because of their high lipophilic nature.[6] Resorcinolic lipids are of great interest from many points of view: (a) biotechnological, for the preparation of biodegradable polyethoxylate surfactants[7] or to synthesize soft materials based on self-assembling aryl glycolipids and used as nanomaterials (lipid nanotubes, nanofibers, liquid crystals or hydro/organogels as new drug delivery vehicles);[8] (b) biopharmaceutical and biomedical, as antimicrobial[9–11] and antitumor agents,[12] molluscicides[13] and prostaglandin synthetase inhibitors.[14]

More recently, some naturally occurring compounds such as coumarins[15–17] and cardol[18] itself, have gained attention as interesting inhibitors of tyrosinase (EC 1.14.18.1), a multifunctional copper-containing enzyme involved in melanin biosynthesis. Tyrosinase catalyzes, as a polyphenol oxidase, the *orto*-hydroxylation of tyrosine to DOPA and the oxidation of L-DOPA to dopaquinone, which further polymerizes spontaneously into melanin. Melanogenesis inhibitors are useful as skin-whitening agents in the treatment of pigmentation disorders associated with overproduction of melanin, including melasma, solar and senile lentigines, ocular *retinitis pigmentosa*[19] and Addison's disease.[20]

Despite the significant in vitro activity of cardols, especially those with unsaturated side chain, they, like hydroquinone or other active compounds,[16] can not be used for clinical application, since they suffer from citoxicity. Furthermore, they could cause



Scheme 1. Polyphenols isolated from *Anacardium occidentale* L.

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severe contact dermatitis[21] and skin irritation.[22] To avoid some of these problems, some β-glycosides of hydroquinone, such as arbutin and deoxyarbutin,[16] have been synthesized.

In an attempt to develop an effective and safer tyrosinase inhibitor, we have supported the more available cardol **1** on a non-toxic and versatile polymer such as the poly (ethylene glycol) (PEG), evaluating, as preliminary results, the activity of the PEG polymer-bound cardol on mushroom tyrosinase.

The selected support was the mono methyl ether of poly (ethylene glycol) with M_w = 5000 Da, which demonstrated to be easily soluble in a wide range of solvents, non-toxic, inexpensive, commercially available, easy to functionalize and also resistant to drastic operative conditions.[23] Due to these features, PEG chemistry has shown broad-based application, which may be in large part ascribed to the use of PEG-conjugates to deliver drugs, oligonucleotides or enzymes.[24]

The synthetic procedure for the preparation of PEG polymer-bound cardol[25,26] started by anchoring the mono allylated cardol **1** to PEG-mesylate **3** giving the intermediate **4**, which was rapidly deprotected to obtain the final product **5** (Scheme 2).[27]

Tyrosinase enzyme activity was estimated by measuring the rate of oxidation of L-DOPA to dopaquinone in a modification[28] of a previously described method.[18] The compound **5** was examined for its inhibitory activity on mushroom tyrosinase at a concentration of 0.8 mM, using L-DOPA 0.5 mM as substrate. In these experimental conditions, the PEG-cardol **5** showed an inhibition of 25.37% and an IC_{50} of 1.52 mM.

A comparison on the activity of compound **5** over cardol **1** was not possible because of the limited solubility of the last compound. In fact, in contrast to previous reports,[18] cardol **1** started to exhibit its insolubility at a concentration of 0.2 mM.

Encouraged by these preliminary results and on the basis of our previous experiences in PEG-supported synthesis,[25] we decided to utilize compound **5** as starting material for the liquid-phase synthesis of some new PEG-cardol coumarins **6**–**8**[25,26,29] (Scheme 3).

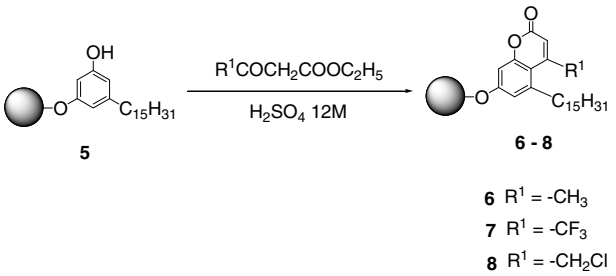
To evaluate the possible influence of the side alkyl chain on mushroom tyrosinase, similar compounds have been synthesized[25,26,30] starting from 5-methylresorcinol **9** (orcinol) instead of cardol **1** (Scheme 4).

All PEG-coumarins were examined for their tyrosinase inhibitory activity, in the same experimental conditions reported above.[28]

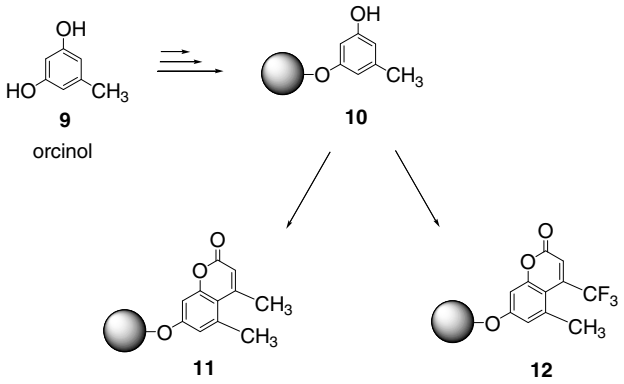
The results reported in Table 1 suggest that R groups in the C4 and C5 position of the coumarin skeleton could have a role on the inhibition of the tyrosinase. In fact, 4-methyl-5-pentadecyl-7-O-PEG-coumarin **6** showed a weaker inhibitory effect than 4,5-dimethyl-7-O-PEG- coumarin **11**. Similarly, 5-pentadecyl-4-trifluoromethyl-7-O-PEG-coumarin **7** revealed a decrease in inhibitory activity when compared with 5-methyl-4-trifluoromethyl-7-O-PEG-coumarin **12**.

Furthermore, the presence of a strong electron withdrawing group in compounds **7** and **12**, as well as in 4-chloromethyl-5-methyl-7-O-PEG-coumarin **8**, could be responsible of their reduced inhibitory activity.

It is remarkable that all the synthesized compounds are completely soluble in water, suggesting the possibility of performing the inhibition test without organic solvents. To this intention, the most active compound **11** was examined in the absence of DMSO, showing a decrease of about 20% in inhibition, measured in the usual experimental conditions (DOPA 0.25 mM and 0.5 mM). Moreover, the effect of PEG on enzymatic activity was excluded. Indeed, the same experiment performed using PEG as inhibitor, did not show any decrease of tyrosinase activity. Noteworthy, PEG proved to be an important synthetic helper, playing a significant role in the reaction regiochemistry. In fact, compounds **5** and **10**



Scheme 3. Synthesis of PEG-cardol coumarins **6**–**8**.

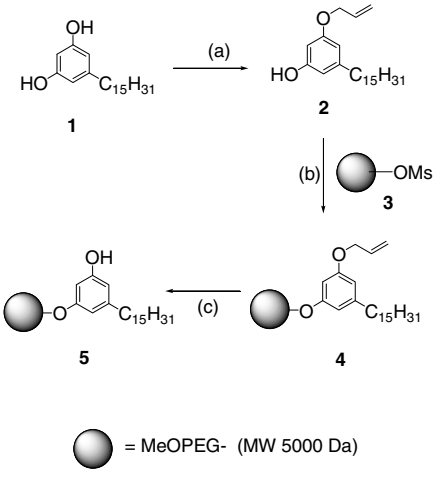


Scheme 4. Synthesis of PEG-orcinol coumarins **11** and **12**.

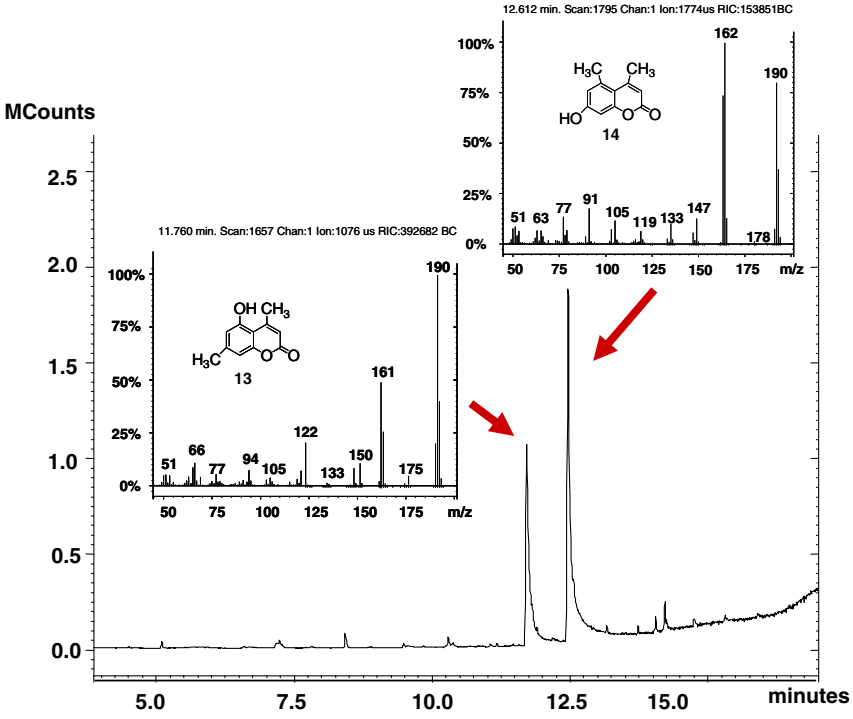
Table 1
Tyrosinase inhibitory activity of PEG-cardol coumarins[28]

Compounds	Inhibition% (0.8 mM)		IC_{50}(mM)
	[DOPA 0.25 mM]	[DOPA 0.5 mM]	[DOPA 0.5 mM]
6	21.5	12.3	1.68
11	36.19	33	1.73
7	20	0	–
12	28.5	15.3	3.81
8	0	0	–
Kojic acid	*	*	9.5×10^{-3}
4-Hexylresorcinol	*	*	0.98×10^{-3}
Resorcinol	*	*	1.44

* Not tested.



Scheme 2. Synthesis of PEG-cardol **5**. Reagents and conditions[25]: (a) allyl bromide, K_2CO_3, KI, acetone, reflux, 4 h. (b) Cs_2CO_3, DMF, 60 °C, overnight. (c) $Pd(OAC)_2$, PPh_3, EtOH, reflux, overnight.



Scheme 5. Gas-chromatogram and mass spectra of 4,7-dimethyl-5-hydroxycoumarin **13** and 4,5-dimethyl-7-hydroxy coumarin **14**.

underwent regioselective cyclization with all the employed β-keto-esters, just leading to 4,5-dialkyl-7-O-PEG-coumarin derivatives, independently of the length of the alkyl chain. Conversely, when we carried out the reaction in solution, under the classical von Pechmann conditions, it was observed, by means of GC–MS analysis, the formation of the both possible isomers **13** and **14**,[31] and not of only one, as previously reported.[32–34] (Scheme 5).

We can conclude that PEG-conjugates could be valid candidates as innovative tyrosinase-active compounds, since they are photostable,[35] widely soluble in water and in many other solvents and, last but not least, PEG did not show to affect the activity of anchored molecules.

In addition, due to PEG features,[23] we think that PEG-cardol and PEG-cardol coumarins, could lack skin irritation and some other undesirable side effects.

Finally, PEG confirmed again to be a fundamental tool for the synthesis of small organic molecules.

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26. *General Procedure*. All PEG samples (Aldrich) were melted in vacuum at 90 °C for about 45 min before use, to remove any trace of moisture. After reaction, the crude mixture was concentrated in vacuum to eliminate the solvent, and than it was added by 6–7 ml of CH_2Cl_2 to completely dissolve the residue. The obtained mixture was added to Et_2O (50 ml *per* g of polymer) cooled at 0 °C. The obtained suspension was filtered through a sintered glass filter and the solid obtained was repeatedly washed on the filter with pure Et_2O. All the samples have been crystallized from isopropyl alcohol, to eventually eliminate the excess of the polar reagents or the by-products. It is well known, in fact, that PEGs, as a result of their helical structure, show a strong propensity to crystallize.[25] The yields of PEG-supported compounds were determined by weight. The indicated yields were for pure products after crystallization from isopropyl alcohol. Their purity was confirmed by 300 MHz ^{1}H NMR analysis in $CDCl_3$ with a pre-saturation of the methylene signals of the polymeric support at 3.60 ppm. In recording the NMR spectra, a relaxation delay of 6 s and an acquisition time of 4 s were used to ensure complete relaxation and accuracy of integration. The integrals of the signals of the PEG CH_2OCH_3 fragment at $\delta = 3.30$ and 3.36, were used as internal standards.
27. *1-(Allyloxy)-3-O-PEG-5-pentadecylbenzene (4):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.28 (s, 2H), 6.25 (s, 1H), 5.97 (m, 1H, $^3J = 5.4$ Hz, ^3J *cis* = 10.5 Hz), 5.34 (d, 1H, $^3J = 17.3$ Hz), 5.20 (d, 1H, $^3J = 10.5$ Hz), 4.50 (d, 2H, $^3J = 5.4$ Hz), 3.36 (s, 3H), 2.45 (t, 2H, $^3J = 7.5$ Hz), 1.21 (m, 26H), 0.88 (t, 3H, $^3J = 7.0$ Hz). *IR (NaCl) cm^{-1}*: ν 3100, 2850, 1820, 1650, 1460, 1260, 1050, 820. *3-O-PEG-5-pentadecylphenol (5):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.27 (s, 1H), 6.22 (s, 2H), 3.33 (s, 3H), 2.43 (t, 2H), 1.21 (m, 26H), 0.83 (t, 3H). *IR (NaCl) cm^{-1}*: ν 3550, 3120, 2830, 1610, 1470, 1270.
28. The pre-incubation with enzyme consisted of 1.8 ml of a 1/15 M phosphoric acid buffer solution (pH 6.8), 0.1 ml of an aqueous solution of mushroom tyrosinase (1000 U/ml, Sigma Chemical Co.) and 0.1 ml of dimethyl sulfoxide (DMSO) with or without a sample. The mixture was pre-incubated at 25 °C for 10 min. Then, 1 ml of a 1.5 mM L-3,4-dihydroxyphenylalanine (DOPA) solution was added and the reaction was monitored at 475 nm for 5 min. The percentage of inhibition of tyrosinase activity was calculated as inhibition (%) = (A − B)/A × 100, where A represents the difference in the absorbance of control sample between an incubation time of 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between an incubation time of 0.5 and 1.0 min. The activity of mushroom tyrosinase was determined using a Varian Cary 50 UV–vis spectrophotometer. DMSO inhibitory activity, in the same experimental conditions, was evaluated as 10.7%.

29. *4-Methyl-5-pentadecyl-7-O-PEG-coumarin (6):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.69 (s, 1H), 6.45 (s, 1H), 5.98 (s, 1H), 3.40 (s, 3H), 2.54 (s, 3H), 2.43 (t, 2H, ^{3}J = 7.5 Hz), 1.21 (m, 26H), 0.83 (t, 3H, ^{3}J = 7.0 Hz). *IR (NaCl) cm^{-1}*: ν 3070, 2750, 1760, 1670, 1550, 1280, 1100. *5-pentadecyl-4-trifluoromethyl-7-O-PEG-coumarin (7):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.71 (s, 1H), 6.47 (s, 1H), 6.23 (s, 1H), 3.32 (s, 3H), 2.42 (t, 2H, ^{3}J= 7.4 Hz), 1.20 (m, 26H), 0.82 (t, 3H, ^{3}J= 7.1 Hz). *IR (NaCl) cm^{-1}*: ν 3020, 2840, 1770, 1630, 1550, 1280, 1220, 1100. *4-Chloromethyl-5-pentadecyl-7-O-PEG-coumarin (8):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.80 (s, 1H), 6.57 (s, 1H), 6.55 (s, 1H), 5.04 (s, 2H), 3.40 (s, 3H), 2.43 (t, 2H, ^{3}J = 7.5 Hz), 1.21 (m, 26H), 0.83 (t, 3H, ^{3}J= 7.0 Hz). *IR (NaCl) cm^{-1}*: ν 3045, 2800, 1750, 1610, 1550, 1280, 1110.
30. *4,5-Dimethyl-7-O-PEG-coumarin (11):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.71 (s, 1H), 6.48 (s, 1H), 6.01 (s, 1H), 3.33 (s, 3H), 2.56 (s, 3H), 1.06 (s, 3H). IR (NaCl) cm^{-1}: ν 3060, 2740, 1730, 1665, 1550, 1280, 1100. *5-Methyl-4-trifluoromethyl-7-O-PEG-coumarin (12):* 6.91 (s, 1H), 6.57 (s, 1H), 6.23 (s, 1H), 3.35 (s, 3H), 1.10 (s, 3H). IR (NaCl) cm^{-1}: ν 3060, 2835, 1720, 1600, 1550, 1280, 1110.
31. *4,7-Dimethyl-5-hydroxycoumarin (13):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.64 (s, 1H), 6.61 (s, 1H), 5.99 (s, 1H), 2.78 (s, 3H), 2.27 (s, 3H). IR (NaCl) cm^{-1}: ν 3200, 2965, 2880, 1770, 1670, 1456, 1210. *4,5-Dimethyl-7-hydroxycoumarin (14):* ^{1}H NMR (300 MHz, $CDCl_3$) ppm: 6.73 (s, 1H), 6.44 (s, 1H), 6.06 (s, 1H), 2.61 (s, 3H), 2.34 (s, 3H). IR (NaCl) cm^{-1}: ν 3195, 2920, 2830, 1770, 1670, 1456, 1210.
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