Discovery of Benzylidenebenzofuran-3(2*H*)-one (Aurones) as Inhibitors of Tyrosinase Derived from Human Melanocytes

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Tyrosinase is a copper-dependent enzyme which converts L- tyrosine to dopaquinone and is involved in different biological processes such as melanogenesis and skin hyperpigmentation. The purpose of this study was to investigate naturally occurring aurones (*Z*-benzylidenebenzofuran-3(2*H*)-one) and analogues as human tyrosinase inhibitors. Several aurones bearing hydroxyl groups on A-ring and different substituents on B-ring were synthesized and evaluated as inhibitors of human melanocyte-tyrosinase by an assay which measures tyrosinase-catalyzed L-Dopa oxidation. We found that unsubstituted aurones were weak inhibitors; however, derivatives with two or three hydroxyl groups preferably at 4,6 and 4′ positions are able to induce significant tyrosinase inhibition. The most potent aurone was found to be the naturally occurring 4,6,4′-trihydroxyaurone which induces 75% inhibition at 0.1 mM concentration and is highly effective when compared to kojic acid, one of the best tyrosinase inhibitors known so far (the latter is completely inactive at such concentrations). Active aurones are devoid of toxic effects as shown by in vivo studies.

Introduction

Tyrosinase, is a copper-containing enzyme, widely present in mammals, plants, and fungi and accepts many phenols and catechols as substrates.[1,2] In mammals, it is implied in the transformation of L-tyrosine to dopaquinone which occurs through two steps: hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and then oxidation of the latter to an *o*-quinone (dopaquinone). Dopaquinone is further transformed through several reactions to yield brown to black melanin which is responsible for color of mammals' skin.[3,4] Melanin pigments are also found in the mammalian brain where tyrosinase plays a key role in the synthesis of neuromelanin and is linked to dopamine neurotoxicity.[5] In the food industry, tyrosinase is a target enzyme in controlling the stability of fruits and vegetables.[6,7]

The tyrosinase-mediated transformation of tyrosine to melanin is of great importance since melanin has many key functions such light absorption and scattering. Compounds inhibiting tyrosinase are desired as therapeutics in treatment of some dermatological disorders and as cosmetic agents, in relation to hyperpigmentation. In cosmetology, tyrosinase inhibitors are used as skin whitening agents. In this regard, the well-known hydroquinone-*O*-*β*-glucopyranoside (arbutin)[8−10] and kojic acid[11] are considered as reference compounds (Figure 1). Due to undesirable side effects, their use as depigmenting agents is being compromised,[12] and arbutin use is now prohibited in several countries. Tropolone, a heptacyclic ketone has been reported as a powerful inhibitor.[13] Benzaldehyde derivatives and especially hydroxylated analogues have been extensively investigated.[14] Recently, Ley and Bertram have described benzaldoximes as tyrosinase inhibitors.[15] Finally, flavonoids and their biosynthetic precursors have been reported as tyrosinase inhibitors.[14,16−17]

In our continuing program aimed to search for natural products acting as skin protectors, we investigated benzylidenebenzofuran-3(2*H*)-one (aurones) (Figure 1) as this subclass of flavonoids have never been studied as tyrosinase inhibitors.

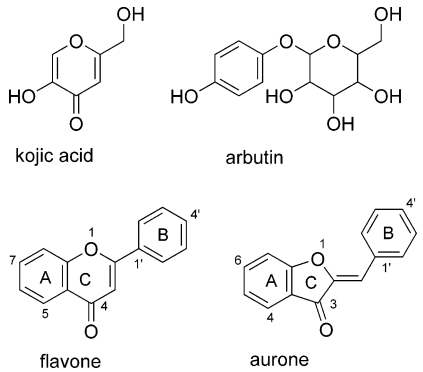

Figure 1.

Aurones, (2-benzylidenebenzofuran-3(2*H*)-ones) which are structurally isomeric of flavones, are met in vegetables, especially in flowers[18] where they are responsible for the gold-yellow color. Their occurrence in marine organisms has been recently reported.[19] They also have been described as phytoalexins, used by plants as defense agents against various infections.[20−22] Compared to flavones, the medicinal literature on aurones is in its infancy with trends and design just now emerging.[23,24]

Like all subclasses of flavonoids, aurones are mostly found in a hydroxylated form and especially at C-4, C-6, and C-4′ (in flavones, these positions correspond to C-5, C-7, and C-4′). On the basis of these remarks, we chose to target aurones possessing hydroxy groups at C-4 and/or at C-6 and bearing different substituents on B-ring, hoping to obtain highly active derivatives and to establish a structure−activity relationship (Figure 2).

A comment is deserved on the biological evaluation of tyrosinase inhibitors. Although, the mushroom tyrosinase is a commercially available enzyme and is frequently used for biological evaluation, it is obvious that this model is not sufficient for evaluating molecules destined for human use.[14] In this study, we have chosen to work on human melanocytes which express tyrosinase.

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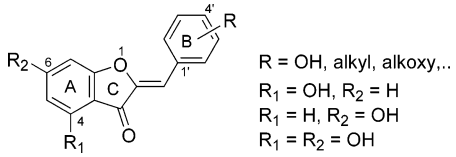
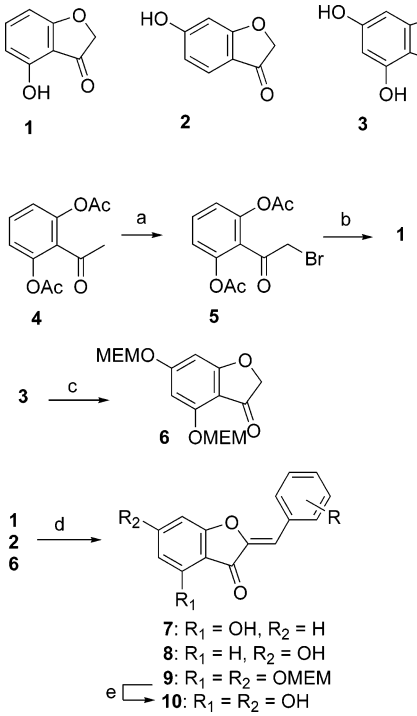


Figure 2.

Scheme 1[a]

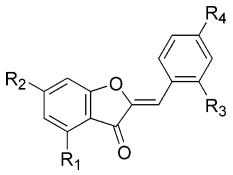


[a] (a) $(Me)_3N(Br_3)Ph$, THF, 6 h; (b) KOH, $MeOH/H_2O$, 60 °C; (c) MEM-Cl, $(i\text{-Pr})_2NEt$, DMF (R = MEM); (d) benzaldehyde derivative, KOH, $MeOH/H_2O$, 60 °C; (e) HCl (1 M in Et_2O), 60 °C, 2 h.

Chemistry. Aurones disclosed in this study were synthesized by means of an aldol condensation of the appropriate benzofuranone with the selected benzaldehyde derivative.[25,26] The synthesis required the preparation of benzofuranones **1**, **2**, and **3** (Scheme 1) which are needed for the synthesis of 4-hydroxyaurones, 6-hydroxyaurones, and 4,6-dihydroxyaurones, respectively. The synthesis of **2** and **3** has been already reported.[25] The synthesis of 4-hydroxybenzofuranone **1** was accomplished starting from 2′,6′-diacetyloxyacetophenone **4**. The latter was brominated with trimethylphenylammonium tribromide to yield bromoacetophenone **5**.[27] Subsequent base hydrolysis and cyclization afforded the desired compound **1**. Prior to aldol condensation, a protection step of **3** was needed, otherwise poor yields and purification problems were faced. The protection of the hydroxyl groups with a methoxyethyloxymethyl (MEM) group affords benzofuranone **6**. Having **1**, **2**, and **6** in hand, the final step was a condensation with benzaldehyde derivatives in the presence of an excess of KOH in $H_2O/MeOH$ to provide aurones **7**, **8**, and **9**. Aurones **9** were submitted to a deprotection step by using molar HCl in diethyl ether for removing MEM groups and affording dihydroxyaurones **10**.

Biology. As indicated in the Introduction, we decided to use a cellular assay which measure the tyrosinase activity of human melanocytes obtained from healthy individuals. The inhibitory potencies against tyrosinase were evaluated by the transformation of L-Dopa to L-dopaquinone. The latter can be trapped by a chromophore MBTH (3-methyl-2-benzothiazolinone hydrazone), easily quantifiable at 490 nm.[28] Therefore, the use of a compound able to modify tyrosinase activity is accompanied with a decrease in absorbance at 490 nm compared to the negative test (without molecule).[28]

Table 1. Tyrosinase Inhibition Results



compd	R_1	R_2	R_3	R_4	inhibition % at 0.1 mM	IC_{50} (μM)
7a	H	H	H	OH	39 ± 6	
7b	OH	H	H	OH	71 ± 7	31.7 ± 2.6
8a	H	OH	H	H	0 ± 23	
8b	H	OH	H	OH	69 ± 3	38.4 ± 2.6
8c	H	OH	H	Et	0 ± 29	
10a	OH	OH	H	H	3 ± 3	
10b	OH	OH	H	OH	75 ± 8	38.0 ± 2.9
10c	OH	OH	H	Et	nd	
10d	OH	OH	Et	H	0 ± 34	
10e	OH	OH	OH	H	4 ± 9	
10f	OH	OH	H	OMe	11 ± 4	
10g	OH	OH	H	OEt	5 ± 8	
10h	OH	OH	H	*n*-Pr	5 ± 3	
10i	OH	OH	H	*t*-Bu	0 ± 14	
10j	OH	OH	H	3′-OMe, 4′-OH	4 ± 4	
apigenin (5,7,4′-trihydroxyflavone)						0 ± 2
kojic acid (evaluated at 7 mM)						20 ± 5

Percent Inhibition of Tyrosinase from Different Melanocyte Sources

concentration of **10b** (IC_{50} = 38 μM)	source of melanocytes (skin color)		
	white	dark	black
0.1 mM	75 ± 5	67 ± 3	66 ± 3
0.01 mM	38 ± 10	32 ± 3	39 ± 5

Results and Discussion

The strategy adopted in this study was to submit all synthesized compounds to a screening test aimed to evaluate the cytotoxic effect. This step is needed prior measuring tyrosinase inhibition because it allows us to rule out cytotoxic compounds. Cytotoxicity was assessed by measuring cell viability after incubation for 24 h with a molecule at 0.1 mM. Compounds which do not alter cell viability (viability remains higher than 75%) were retained for inhibitory study. In this study, our reference inhibitor was kojic acid, which was used at its effective concentration (7 mM).

A preliminary screening on a set of representative compounds ruled out any tyrosinase inhibition with aurones without hydroxyl groups on A or/and B-rings (results not shown).

With regards to hydroxylated aurones, it emerged that the presence of hydroxy groups at 4,4′ (compound **7b**), 6,4′ (compound **8b**), or 4,6,4′ (compound **10b**) positions was vital for good tyrosinase inhibitory potencies. Interestingly, deletion of the hydroxyl group on the B-ring (**10a**) led to complete loss of activity. However, maintaining the 4′-hydroxy group and removing those on the A-ring led to aurone (**7a**) which induces 39% of inhibition. With regard to these results, it emerged that a hydroxyl group at the B-ring plays a more vital role than those on the A-ring. Attempts to identify other important substituents on the B-ring was unsuccessful as indicated by weak inhibitory activity shown in Table 1. The crucial importance of C-4′

hydroxyl group was checked by protection of the latter as a methoxy group, which led to inactive aurone **10f**. The same observation was made when either or both hydroxyls at C-4 and C-6 were methylated (data not shown). Interestingly, when hydroxyl groups at C-4 and C-4′ are maintained and 6-OH is methylated (data not shown), the inhibitory activity is completely lost, indicating probably that steric hindrance in this region is not tolerated. It is worth noting that antityrosinase activity was dramatically decreased when the most active aurone **10b** was substituted at C-3′ (**10j**). This may indicate an unfavorable sterical hindrance at this region of molecule. To confirm that the C-4′ is the most suitable position for B-ring hydroxylation, we prepared and tested 4,6,2′-trihydroxyaurone **10e** which led to this order of potency: 4′-position ≫ 2′-position. It is important to mention that the tyrosinase inhibitory activity is specific to aurones among flavonoids subclasses. For example, apigenin (5,7,4′-trihydroxyflavone), which is the structurally closest flavone (Figure 1) to aurone **10b**, was inactive at 0.1 mM.

On the basis of these results, we selected compounds **7**, **8b**, and **10b** and evaluated IC_{50} and found them (Table 1) very much stronger than arbutin (IC_{50}, 8.4 mM)[29] and kojic acid (IC_{50}, 280 μM).[11] As outlined before, this comparison is not highly significant since arbutin and kojic acid are evaluated on mushroom tyrosinase.

To investigate the correlation between melanocyte source (skin color) and inhibitory activity, we selected aurone **10b** and tested it on melanocytes obtained from three individuals with white, dark, and black skin (Table 1). The inhibitory activity evaluated at two different concentrations (0.1 and 0.01 mM) showed no significant difference in inhibitory activity. Because our ultimate goal is to bring active aurones to human use, we evaluated its tolerance in animals and found that it is devoid of any toxicity after oral administration to rats at 5 g/kg dose. In rabbits, topical and eye application of aurone **10b** did not show any significant irritation.

In the literature, it is assumed that most inhibitors act by complexing the two copper atoms which are present in the active site of the enzyme.[30] Although, we do not have evidence regarding the mechanism of action of aurones, it is possible that aurones hydroxylated at the 4-position such as **7** and **10b** may act by the same mechanism. It is well established that 5-hydroxyflavones are good metal-complexing compounds, due to the proximity of 4-carbonyl group to the 5-hydroxyl. This hypothesis is challenged by **8b**. Indeed, the latter, which lacks the 4-hydroxy group and therefore its complexation capability and should be considerably weakened, is still as active as **7** and **10b**. Considering these data, we propose that copper complexation is not the only mechanism of action but that the aurones may bind to an another essential domain of tyrosinase.

In conclusion, we report for the first time the inhibition of human tyrosinase activity by aurones. The natural aspect of these molecules combined with absence of toxicity make them highly attractive. Currently, we are developing 4,6,4′-trihydroxyaurone as an active agent for treatment of hyperpigmentation-related anomalies. Further investigations are underway in our laboratory to clarify the possible mechanism of action.

Experimental Section

Chemistry. Melting point were measured on a Fisher micromelting point apparatus and are uncorrected. ^{1}H and ^{13} C NMR spectra were recorded on Bruker Avance 400 operating at 400 and 100 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are reported in ppm, in reference to TMS. Mass spectra were obtained on a JEOL HX-110 spectrometer and high-resolution mass spectra were obtained on a Spectrometer Varian MAT 311. Elemental analysis were performed by the analytical Department of CNRS, Vernaison, France. Thin-layer chromatography (TLC) was carried out using Machery-Nagel Polygram SIL/UV plates (thickness 0.20 mm). Chromatography was carried out using Merck silica gel 60, 200−400 mesh. Chemicals and reagents were obtained either from Aldrich or Acros companies.

The synthesis of 4,6-dihydroxybenzofuran-3(2*H*)-one (**1**) and 6-hydroxybenzofuran-3(2*H*)-one (**2**) have already been reported elsewhere.[25,26]

4-Hydroxybenzofuran-3(2*H*)one (1). A solution of 2,6-diacetoxyacetophenone **4** (1 g, 4.23 mmol) was dissolved in anhydrous THF, trimethylphenylammonium tribromide (1.2 equiv) was added under stirring, and the resulting mixture was stirred 6 h at room temperature. The reaction was quenched with water and extracted with ethyl acetate (2 × 10 mL). The ethyl acetate was dried over $MgSO_4$ and removed by evaporation to provide **5** as an amber oil which was used without purification. ^{1}H NMR ($CDCl_3$): δ 7.43 (t, 1H, J = 8.4 Hz, H_4); 7.05 (d, 2H, J = 8.4 Hz, H_3, H_5); 4.28 (s, 2H, CH_2Br); 2.24 (s, 6H, 2 × Ac). The crude material was dissolved in MeOH (10 mL) and treated with 0.5 mL of KOH (50% in H_2O). After refluxing for 2 h, the methanol was evaporated and the mixture was partitioned between HCl (1 N) and ethyl acetate. The ethyl acetate was separated, washed with water, brine, and concentrated, and the crude compound was purified by chromatography column eluted with ethyl acetate:hexane to provide **1** having spectral and physical data in accordance with literature.[29,30] ^{1}H NMR ($CDCl_3$): δ 7.42 (t, 1H, J = 8.4 Hz, H_6); 6.55 (d, 1H, J = 8.4 Hz, H_7); 6.42 (d, 1H, J = 8.4 Hz, H_5); 4.58 (s, 2H, H_2).

4,6-Di-MEM-benzofuran-3(2*H*)-one (6). A solution of **3** (3 g, 18 mmol) and *N*,*N*-diisopropylethylamine (2 equiv) in anhydrous DMF is cooled to 0 °C, and then 2-methoxyethoxymethyl chloride (MEM-Cl) was added dropwise (2 equiv). The resulting mixture was stirred at room temperature for 20 min. The reaction mixture was diluted with a large amount of water and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated to dryness to yield crude **6** which was used without purification. ^{1}H NMR ($CDCl_3$): δ 6.31 (m, 2H, H_5, H_7); 5.28 (s, 2H, OCH_2O); 5.19 (s, 2H, OCH_2O); 4.48 (s, 2H, H_2); 3.76 (m, 2H, CH_2O); 3.71 (m, 2H, CH_2O); 3.46 (m, 4H, 2 × OCH_2); 3.28 (s, 3H, OCH_3); 3.27 (s, 3H, OCH_3).

Condensation of benzaldehyde derivatives with benzofuran-3-ones 1, 2 or 6, general procedure: To a solution of **1**, **2**, **6**, or benzofuran-3(2*H*)-one (commercially available) in methanol (10 mL/mmol) was added an aqueous solution of potassium hydroxide (50%, 1.5 mL/mmol) followed by the addition of benzaldehyde derivative (1.5 equiv). The solution was heated at 60 °C for 1 h, and then methanol was evaporated. The residue was diluted in water, and the resulting mixture was extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and filtered, and solvent was removed by evaporation to yield crude products. Pure aurones **7** and **8** were obtained by purification on column chromatography (silica gel) eluted with EtOAc:cyclohexane (1:2). In the case of aurones **9**, a deprotection step was performed prior purification: to a methanolic solution (10 mL/mmol) of crude **9** was added a molar solution of HCl in Et_2O (5 mL/mmol). The reaction mixture was stirred at 60 °C for 2 h and cooled to room temperature, water (40 mL) was added, and the mixture was extracted with CH_2Cl_2. The organic layer was washed with water and brine, dried over $MgSO_4$, and purified by column chromatography using cyclohexane:ethyl acetate 6:4 to provide pure aurones **10** as orange or yellow powders, and no further recrystallization was needed.

(*Z*)-2-(4-Hydroxybenzylidene)benzofuran-3(2*H*)-one (7a): 23%, yellow powder, mp 256−258 °C; ^{1}H NMR (acetone-d_6): δ 7.86 (d, 2H, J = 6.8 Hz, $H_{2'}$, $H_{6'}$); 7.71 (m, 2H, H-4, H_6); 7.42 (d, 1H, J = 7.6 Hz, H_7); 7.24 (t, 1H, J = 7.6 Hz, H_5); 6.93 (d, 2H, J = 6.8 Hz, $H_{3'}$, $H_{5'}$); 6.76 (s, 1H, =CH). MS (EI) m/z 238 $[M]^{+\bullet}$; Anal. ($C_{15}H_{10}O_3$) C, H.

(*Z*)-2-(4-Hydroxybenzylidene)-4-hydroxybenzofuran-3(2*H*)-one (7b): 35%; orange powder; mp 275−277 °C; ^{1}H NMR (acetone-d_6): δ 7.81 (d, 2H, J = 8.8 Hz, $H_{2'}$, $H_{6'}$); 7.50 (t, 1H, J =

8 Hz, H_6); 6.91 (d, 2H, J = 8.8 Hz, $H_{3'}$, $H_{5'}$); 6,81 (d, 1H, J = 8.4 Hz, H_7); 6.66 (s, 1H, =CH); 6.59 (d, 1H, J = 8.4 Hz, H_5). MS (EI) m/z 254 $[M]^+$; Anal. ($C_{15}H_{10}O_3$) C, H.

(*Z*)-2-Benzylidene-6-hydroxybenzofuran-3(2*H*)-one (8a): 17%; yellow powder; mp 262 °C; ^{1}H NMR (acetone-d_6): δ 7.91 (d, 2H, J = 8.4 Hz, C_6H_5); 7.58 (d, 1H, J = 8.4 Hz, H_4); 7.42 (m, 3H, C_6H_5); 6.80 (d, 1H, J = 2 Hz, H_7); 6.74 (dd, J = 2 Hz, J = 8.4 Hz, H_5); 6.66 (s, 1H, =CH). MS (EI) m/z 238 $[M]^+$; Anal. ($C_{15}H_{10}O_3$) C, H.

(*Z*)-2-(4-Hydroxybenzylidene)-6-hydroxybenzofuran-3(2*H*)-one (8b): 92%; yellow powder; mp 286−288 °C; ^{1}H NMR (DMSO-d_6): δ 10.1 (br s, 1H, OH); 7.73 (d, 2H, J = 8.8 Hz, $H_{2'}$, $H_{6'}$); 7.52 (d, 1H, J = 8.8 Hz, H_4); 6.81 (d, 2H, J = 8.8 Hz, $H_{3'}$, $H_{5'}$); 6.71 (d, 1H, J = 1.6 Hz, H_7); 6.65 (s, 1H, =CH); 6.63 (dd, 1H, J = 1.6 Hz, J = 8.8 Hz, H_5). MS (DCI: NH_3 + isobutane) m/z 255 $[MH]^+$; Anal. ($C_{15}H_{10}O_4$) C, H.

(*Z*)-2-(4-Ethylbenzylidene)-6-hydroxybenzofuran-3(2*H*)-one (8c): 60%; yellow powder; mp 228 °C; ^{1}H NMR (acetone-d_6): δ 7.83 (d, 2H, J = 8 Hz, $H_{2'}$, $H_{6'}$); 7.56 (d, 1H, J = 8.4 Hz, H_4); 7.28 (d, 2H, J = 8 Hz, $H_{3'}$, $H_{5'}$); 6.78 (d, 1H, J = 2 Hz, H_7); 6.72 (dd, 1H, J = 2 Hz, J = 8.4 Hz, H_5); 6.65 (s, 1H, =CH-); 2.63 (q, 2H, CH_2); 1.19 (t, J = 7.8 Hz, CH_3). MS (EI) m/z 266 $[M]^+$; HRMS. Calcd for $C_{17}H_{14}O_3$: 266.09429. Found: 266.0919. Anal. ($C_{17}H_{14}O_3$) C, H.

(*Z*)-Benzylidene-4,6-dihydroxybenzofuran-3(2*H*)-one (10a): 40%; yellow powder; mp 252 °C; ^{1}H NMR (acetone-d_6): δ 6.09 (d, 1H, J = 1.6 Hz, H_7); 6.30 (d, 1H, J = 1.6 Hz, H_5); 6.57 (s, 1H, =CH-); 7.35 (m, 1H, $-C_6H_5$); 7.42 (m, 2H, $-C_6H_5$); 7.87 (2H, $-C_6H_5$). MS (EI) m/z $[M]^+$; Anal. ($C_{15}H_{10}O_4$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-hydroxybenzylidene)benzofuran-3(2*H*)-one (10b): 31%; yellow powder; 295 °C (decomposition); yellow powder; mp 295 °C; ^{1}H NMR (acetone-d_6): δ 7.82 (d, 2H, J = 7.8 Hz, $H_{3'}$, $H_{5'}$); 6.94 (d, 2H, J = 7.8 Hz, $H_{2'}$, $H_{6'}$); 6.59 (s, 1H, =CH); 6.33 (d, 1H, J = 1.7 Hz, H_7); 6.12 (d, 1H, J = H_5). MS (DCI) m/z 270 $[MH]^+$; Anal. ($C_{15}H_{15}O_5$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-ethylbenzylidene)benzofuran-3(2*H*)-one (10c): 65%; yellow powder; mp 236 °C; ^{1}H NMR (acetone-d_6): δ 7.80 (d, 2H, J = 8.4 Hz, $H_{2'}$, $H_{6'}$); 7.27 (d, 2H, J = 8.4 Hz, $H_{3'}$, $H_{5'}$); 6.56 (s, 1H, =CH-); 6.29 (d, 1H, J = 1.6 Hz, H_7); 6.09 (d, 1H, J = 1.6 Hz, H_5); 2.62 (q, 2H, J = 7.6 Hz, CH_2); 1.20 (t, 3H, J = 7.2 Hz, CH_3). MS (DCI) m/z 283 $[MH]^+$; Anal. ($C_{17}H_{17}O_4$) C, H.

(*Z*)-4,6-Dihydroxy-2-(2-ethylbenzylidene)benzofuran-3(2*H*)-one (10d): 84%; yellow powder; mp 208−210 °C; ^{1}H NMR (acetone-d_6): δ 8.09 (m, 1H, H4′); 7.25 (m, 3H, $H_{3'}$, $H_{5'}$, $H_{6'}$); 6.79 (s, 1H, =CH-); 6.26 (d, 1H, J = 2 Hz, H_7); 6.09 (d, 1H, J = 2 Hz, H_5); 2.80 (q, 2H, J = 7.6 Hz, CH_2); 1.22 (t, 3H, J = 7.6 Hz, CH_3). MS (EI) m/z 282 $[MH]^+$; Anal. ($C_{17}H_{14}O_4 \cdot H_2O$) C, H.

(*Z*)-4,6-Dihydroxy-2-(2-hydroxybenzylidene)benzofuran-3(2*H*)-one (10e): 61%; yellow powder; mp 262−264 °C; ^{1}H NMR (acetone-d_6): δ 8.12 (d, 1H, J = 7.2 Hz, $H_{6'}$); 7.17 (m, 1H, $H_{3'}$); 7.12 (s, 1H, =CH-); 6.90 (m, 2H, $H_{4'}$+$H_{5'}$); 6.29 (d, 1H, J = 2 Hz, H_5); 6.08 (d, 1H, J = 2 Hz, H_7). MS (DCI) m/z 270 $[MH]^{+\bullet}$; Anal. ($C_{15}H_{10}O_5$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-methoxybenzylidene)benzofuran-3(2*H*)-one (10f): 20%; yellow powder; mp 208−210 °C; ^{1}H NMR (acetone-d_6): δ 7.83 (d, 2H, J = 8.8 Hz, $H_{2'}$, $H_{6'}$); 6.97 (d, 2H, J = 8.8 Hz, $H_{3'}$, $H_{5'}$); 6.55 (s, 1H, =CH); 6.28 (d, 1H, J = 1.6 Hz, H_7); 6.07 (d, 1H, J = 1.6 Hz, H_5); 3.80 (s, 3H, OCH_3). MS (EI) m/z 284 $[M]^{+\bullet}$; Anal. ($C_{16}H_{12}O_5$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-ethyloxybenzylidene)benzofuran-3(2*H*)-one (10g): 29%; yellow powder; mp 215−217 °C; ^{1}H NMR (acetone-d_6): δ 7.82 (d, 2H, J = 6.8 Hz, $H_{2'}$, $H_{6'}$); 6.95 (d, 2H, J = 6.8 Hz, $H_{3'}$, $H_{5'}$); 6.55 (s, 1H, =CH); 6.28 (d, 1H, J = 1.6 Hz, H_5); 6.08 (d, 1H, J = 1.6 Hz, H_7); 4.05 (q, 2H, J = 7.2 Hz, CH_2); 1.32 (t, 3H, J = 7.2 Hz, CH_3). MS (EI) m/z 298 $[M]^+$; Anal. ($C_{17}H_{14}O_5 \cdot H_2O$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-propylbenzylidene)benzofuran-3(2*H*)-one (10h): 89%; yellow powder; mp 232−234 °C; ^{1}H NMR (acetone-d_6): δ 7.87 (d, 2H, J = 8.4 Hz, $H_{2'}$, $H_{6'}$); 7.25 (d, 2H, J = 8.4 Hz, $H_{3'}$, $H_{5'}$); 6.56 (s, 1H, =CH); 6.29 (d, 1H, J = 2 Hz, H_7); 6.09 (d, 1H, J = 2 Hz, H_5); 2.59 (t, 2H, J = 7.2 Hz, Ph-CH_2); 1.60 (m, 2H, CH_2); 0.91 (t, 3H, 7.6 Hz, CH_3). MS (EI) m/z 296 $[M]^+$; Anal. ($C_{18}H_{16}O_4 \cdot 0.5H_2O$) C, H.

(*Z*)-2-(4-*tert*-Butylbenzylidene)-4,6-dihydroxybenzofuran-3(2*H*)-one (10i): 93%; yellow powder; mp 218 °C; ^{1}H NMR (acetone-d_6): δ 7.82 (d, 2H, J = 8.8 Hz, $H_{2'}$, $H_{6'}$); 7.46 (d, 2H, J = 8.8 Hz, $H_{3'}$, $H_{5'}$); 6.56 (s, 1H, =CH); 6.29 (br s, 1H, H_7); 6.10 (br s, 1H, H_5); 1.27 (s, 9H, *t*-Bu). MS (EI): m/z 296 $[M]^+$; Anal. ($C_{19}H_{18}O_4$) C, H.

(*Z*)-4,6-Dihydroxy-2-(4-hydroxy-3-methoxybenzylidene)benzofuran-3(2*H*)-one (10j): 17%; yellow powder; mp 262 °C; ^{1}H NMR (acetone-d_6): δ 7.50 (d, 1H, J = 2 Hz, $H_{2'}$); 7.42 (dd, 1H, J = 2 Hz, J = 8.4 Hz, $H_{6'}$); 6.87 (d, 1H, J = 8.4 Hz, $H_{5'}$); 6.54 (s, 1H, =CH); 6.29 (d, 1H, J = 2 Hz, H_7), 6.08 (d, 1H, J = 2 Hz, H_5); 3.86 (OCH_3). MS (EI) m/z 300 $[M]^+$; Anal. ($C_{16}H_{12}O_6 \cdot 0.5H_2O$) C, H.

Tyrosinase Inhibition. Human melanocytes were obtained from an abdominal plastic surgery. Melanocytes were seeded in 24 well plates (80000 cells per well) and were cultivated until confluence. Samples for evaluation were applied in cell culture medium during 24 h at 37 °C. After this time, cell culture medium was taken off and human tyrosinase was extracted from melanocytes by thermal choc lysis. After centrifugation (to eliminate cell membrane), supernatants were incubated with MBTH and L-Dopa during 30 min and then analyzed by spectrophotometry at 490 nm. Inhibition of tyrosinase activity was expressed in percentage of inhibition, compared with negative control (medium without tested molecule). A protein assay was performed on each extracellular medium to correlate the enzymatic activity to a protein content (BCA assay). Inhibition was calculated using the following formula: inhibition (%) = 100 − [(ODsample/protein content sample)/(ODnegative control/protein content negative control)] × 100.

Cytotoxic Assay. Cellular viability was assessed using a "pNPP assay as described by Yang et al.[31] The pNPP (*p*-nitrophenyl phosphate) was used for measuring AP (alkaline phosphatase) activity. In the presence of AP, pNPP is hydrolyzed rapidly to *p*-nitrophenol and inorganic phosphate. The released *p*-nitrophenol (yellow) was measured at 405 nm. The absorbance at 405 nm is directly proportional to the cell viability.

Melanocytes were seeded in 96 well plates with 50000 cells/cm^2, and cells were cultivated until confluence. Molecules were added at different concentrations (10^{-4} M and 10^{-5} M) to the cell culture medium at 37 °C in the presence of CO_2 (5%). After 24 h, the culture cell medium was taken off, a solution of *p*-nitrophenyl phosphate in sodium acetate buffer (pH 8) was added for 2 h, and the reaction was stopped by adding NaOH (1 N). The absorbance at 405 nm was measured with a multiplate reader, and results were expressed in percentage of viability compared to negative control (without molecule).

In Vivo Toxicity. The toxicological studies were realized on compound **10b** incorporated at 10% in a xanthane gel.

Oral Toxicity in Rats. Acute oral toxicity test in the rat acute toxic class method, OECD Guideline 423 (12-17-2001). The aim of the study was to assess qualitatively and quantitatively the toxic effects and the delay of appearance after single oral administration of a predefined dose of 5 g/kg of body weight, of test preparation as such, in six female rats, using a stepwise procedure. Similarly, six control rats (six females) received 2000 mg/kg of distilled water. The animals were daily observed for 14 days after administration, and the signs of toxicity (mortality, etc.) were noted. This test provided results allowing the test preparation to be classified according to the Globally Harmonized System (GHS) for the classification of chemicals which cause acute toxicity (OECD 1998). In this study, no mortality at the dose of 2 g/kg was observed. Therefore, the compounds were classified in the hazard category 5 or unclassified with a LD50 higher than 2 g/kg.

Primary Eye Irritation and Acute Dermal Irritation/Corrosion Test in the Rabbit. After single application of 0.1 mL (eye) or 0.5 mL (skin) of test preparation in three rabbits, the ocular reactions (redness, chemosis of conjunctiva, discharge, iris and cornea lesions) and the skin reactions (erythema and edema) were

scored at 1, 24, 48, and 72 h. On the basis of the observation that no alteration nor irritation occurred, the compounds were not classified among the preparation irritant to the eyes or to the skin in accordance with the criteria defined in the European Communities decree of 04-20-1994.

Supporting Information Available: Elemental analysis and high-resolution mass spectrometry (HRMS). These materials are available free of charge via the Internet at http://pubs.acs.org.

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