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Research Paper

Tyrosine-like condensed derivatives as tyrosinase inhibitors

Maria João Matos[a], Lourdes Santana[a], Eugenio Uriarte[a], Silvia Serra[a,b], Marcella Corda[c], Maria Benedetta Fadda[c], Benedetta Era[c] and Antonella Fais[c]

[a]Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain and [b]Dipartimento Farmaco Chimico Tecnologico, Facoltà di Farmacia and [c]Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Cagliari, Italy

Keywords
depigmenting agents; mixed-type inhibitor; tyrosinase inhibition; tyrosine-like compounds

Correspondence
Maria João Matos, Department of Organic Chemistry, University of Santiago de Compostela, Fac Farmacia, Campus Vida, Santiago de Compostela, 15782, Spain.
E-mail: mariajoao.correiapinto@rai.usc.es

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Abstract

Objectives We report the pharmacological evaluation of a new series of 3-aminocoumarins differently substituted with hydroxyl groups, which have been synthesized because they include in their structures the tyrosine fragment (tyrosine-like compounds), with the aim of discovering structural features necessary for tyrosinase inhibitory activity.

Methods The synthesized compounds **4** and **7–9** were evaluated *in vitro* as mushroom tyrosinase inhibitors.

Key findings Two of the described compounds showed lower IC50 (concentration giving 50% inhibition of tyrosinase activity) than umbelliferone, used as a reference compound.

Conclusions Compound **7** (IC50 = 53 μM) was the best tyrosinase inhibitor of this small series, having an IC50 value 10-fold lower than umbelliferone. Compound **7** (3-amino-7-hydroxycoumarin) had amino and hydroxyl groups precisely mimicking the same positions that both groups occupy on the tyrosine molecule.

Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional dinuclear copper centre metalloenzyme widely distributed in nature.[1] This enzyme catalyses two distinct reactions of conversion of the tyrosine: 3′-hydroxylation of L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) and oxidation of the resultant L-DOPA into DOPA quinone, which further polymerizes spontaneously into melanin.[2] Most melanin-biosynthesis inhibitors are phenol or catechol analogues, which are structurally similar to tyrosinase substrates: tyrosine or L-DOPA.[3] Therefore, tyrosine-like molecules could be an interesting scaffold to the tyrosinase inhibition process. Tyrosinase is mainly involved in the formation of pigments such as melanins and other polyphenolic compounds.[1] Tyrosinase oxidizes phenols and diphenols using a catalytic mechanism dependent on the presence of copper at its active site.[1,2] This enzyme is responsible for unwanted browning of fruits and vegetables.[4] Therefore, it is involved in the process of maintaining the appearance, flavour, texture and nutritional value of many fresh-cut products.[4] Tyrosinase is also responsible for the colouring of skin, hair and eyes in animals, including humans.[5] In fact, tyrosinase inhibitors have been used as depigmenting agents for the treatment or prevention of hyperpigmentation disorders.[6] In recent years, many tyrosinase inhibitors have been reported, including vitamin C (ascorbic acid), kojic acid, umbelliferone, resveratrol, hydroquinone and oxyresveratrol.[7–12] Because of the structural similarity, umbelliferone was used as a reference inhibitor (Figure 1).

Coumarins are a large family of compounds, of natural and synthetic origin, which present different pharmacological actions.[13] Chemically they are lactones of cinnamic acid. Their structural variety is responsible for the important place that they occupy in the natural product and synthetic organic chemistry realm.[14] Some important studies pay special attention to their antioxidative, anti-cancer anti-inflammatory, cardioprotective and enzymatic inhibitory properties.[15–21] In recent studies, some coumarins proved to be mushroom tyrosinase inhibitors.[22,23] In those studies, esculetin (6,7-dihydroxycoumarin) and umbelliferone (7-hydroxycoumarin) exhibited some of the strongest inhibitory activity of the tested series, with esculetin proving to be the strongest inhibitor of the series.[22] Recently, and in contrast to the initial findings, Sollai *et al.* showed that esculetin is a tyrosinase substrate rather than an inhibitor, whereas umbelliferone seems to be an inhibitor of tyrosinase.[24] These studies revealed that tyrosinase affinity

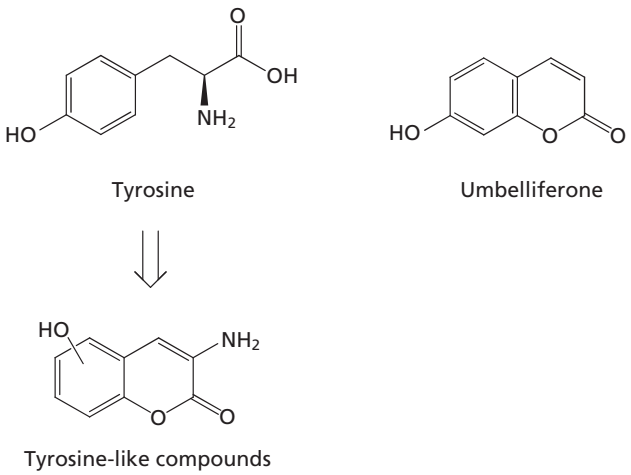

Figure 1 Chemical structures of tyrosine (tyrosinase substrate), tyrosine-like condensed molecules and umbelliferone (tyrosinase inhibitor).

can be efficiently modulated by appropriate substitutions in the coumarin moiety. The introduction of different numbers of hydroxyl groups in different positions of the coumarin is one of the most important modifications.[22,23] Also, until recently, polyphenols were the largest scaffold in tyrosinase inhibition.[25,26]

Tyrosinase inhibitors could have broad applications. As the ideal drug candidate has not been attained, an intensive search for new tyrosinase inhibitors is still needed. This effort has considerably increased in the recent years. In this context, and in an attempt to develop novel tyrosinase inhibitors, we had previously synthesized and described 3-arylcoumarin derivatives in which both the coumarin and the resveratrol templates were present.[23,27] Based on this work, and with the aim of finding new structure–activity features, we propose the study of a series of tyrosine-like condensed molecules (Figure 1). The similarity of these compounds to the tyrosinase substrate is the novelty of this study. The proposed compounds are structurally related to the amino acid tyrosine, the natural substrate of this enzyme. Tyrosinase inhibitors based on the aminocoumarin scaffold have not been previously studied.

Materials and Methods

We synthesized (Figure 2) and evaluated a small series of 3-aminocoumarins. We decided to explore the importance of the position of a hydroxyl group under the 3-aminocoumarin moiety, based on the idea of mimicking the molecular structure of the tyrosinase substrate.

The coumarin derivatives **4** and **7–9**[28–30] were efficiently synthesized according to the protocol outlined in Figure 2. Starting from different substituted commercially available salicylaldehydes and ethyl nitroacetate, we afforded 3-nitrocoumarins **1–3** in good yields (75–95%). They were synthesized in a dry Schlenk tube, with acetic anhydride as solvent, in the presence of sodium hydride, at room temperature, for 3 h. The reaction mixture was purified by flash chromatography, using hexane/AcOEt (9 : 1) as eluent. The 3-aminocoumarins **4–6** were prepared from previously synthesized 3-nitrocoumarins, in EtOH, with Pd/C as catalyst, under H_2 atmosphere. The obtained products were purified by crystallization in AcOEt to give the desired 3-aminocoumarins, in a yield of 95%. The hydroxy derivatives **7** and **8** were obtained from the methoxy derivatives (compounds **5** and **6**, respectively) by a hydrolysis reaction with hydriodic acid, in the presence of acetic acid and acetic anhydride, at 110°C, for 5 h. The residue was purified by crystallization of acetonitrile, and the hydroxy derivatives were obtained in a yield of 60%. Compound **9**[31] was obtained via reduction reaction, under the same conditions as described above, of the commercially available 4-hydroxy-3-nitrocoumarin, in a yield of 93%.

The biological assays were carried out using the following protocol. Pre-incubation with the enzyme was carried out: 1/15 M phosphoric acid buffer solution (pH 6.8, 1.8 ml), an aqueous solution of mushroom tyrosinase (1000 U/ml; Sigma Chemical Co., Milan, Italy, 0.1 ml) and dimethyl sulfoxide (DMSO) (0.1 ml) with or without the sample. The mixture was incubated at 25°C for 10 min. Then, 1.5 mM L-DOPA solution (1 ml) was added and the reaction was monitored at 475 nm for 5 min. The percent of tyrosinase activity inhibition was calculated as: inhibition (%) = $(A - B)/A \times 100$, where A represents the difference in the absorbance of control sample between 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between 0.5 and 1.0 min. The mushroom tyrosinase activity was determined by spectrophotometric assays (Varian Cary 50). Umbelliferone was used as a reference tyrosinase inhibitor.

Statistical methods

All experiments were carried out three times. Continuous variables were expressed as mean ± SD. The IC50 value (concentration giving 50% inhibition of tyrosinase activity) was determined by interpolation of dose–response curves and all data were statistically evaluated using Student's *t*-test or Mann–Whitney test (Statistica 6; Statsoft Tulsa, Tulsa, OK, USA). To assess the slopes of curves in Figure 3 the Kruskal–Wallis test followed by Dunn's post-hoc test (Statistica 6; Statsoft Tulsa) was used. The criterion for statistical significance was generally taken as $P < 0.05$.

Results

The tyrosinase inhibitory activity of compounds **4** and **7–9** was evaluated *in vitro* by measuring the enzymatic activity of

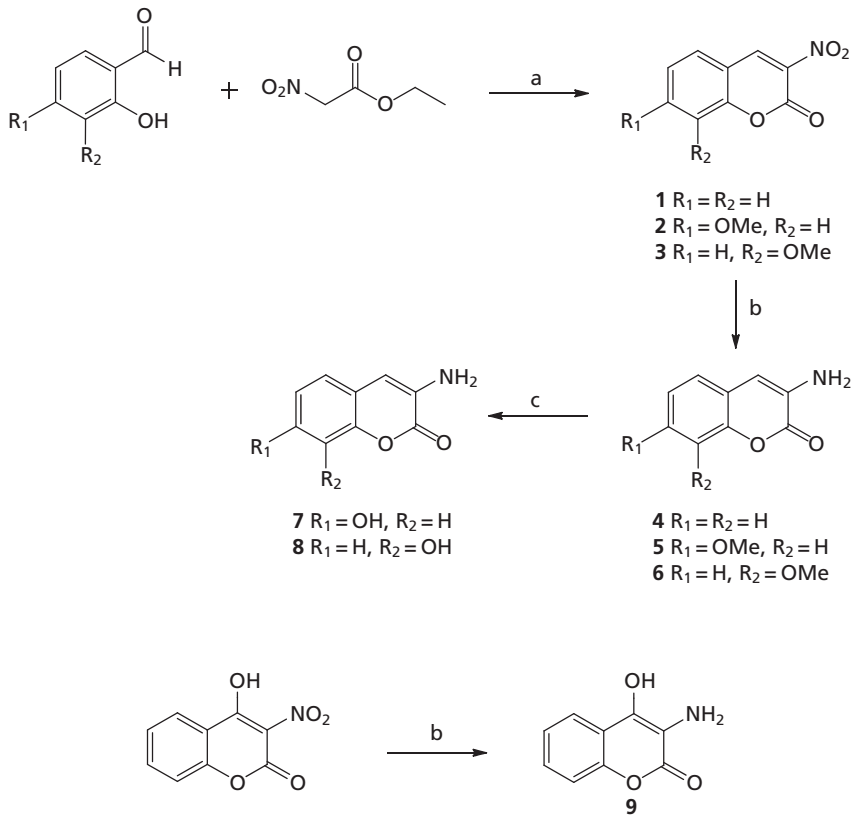

Figure 2 Protocols for synthesis of coumarin derivatives. *Reagents and conditions:* (a) acetic anhydride, NaH, r.t., 3 h; (b) H_2, EtOH, Pd/C, r.t., 5 h; (c) HI, AcOH, Ac_2O, 110°C, 5 h.

mushroom tyrosinase enzyme extracted from the mushroom *Agaricus bisporus*. Then, the IC50 values for the inhibitory effects of the new compounds were calculated (Table 1).

In the presence of compound **7**, kinetic studies on mushroom tyrosinase, using a Lineweaver–Burk double reciprocal plot (Figure 3), showed that compound **7** was a mixed-type inhibitor. Increasing the concentration of **7** resulted in a family of lines with different slope and intercept, which intersected in the second quadrant. This behaviour showed that compound **7** can bind not only with free enzyme but also with the enzyme–substrate complex, with different equilibrium constants. The inhibition constants for the inhibitor binding with free enzyme, K_I (2.14 mM), and with enzyme–substrate complex, K_{IS} (0.78 mM), were obtained from the linear secondary plots of $1/V_{max}$ and K_m/V_{max} versus the concentration of compound **7**, respectively.

Discussion

In this communication, the possible tyrosinase inhibitory effect of coumarins that incorporate a portion of tyrosine on their skeletons was described. The introduction of a hydroxyl substituent into different positions of the 3-aminocoumarin moiety was studied. In this way a small series of compounds that are both tyrosine (tyrosinase substrate) and umbelliferone (tyrosinase inhibitor) analogues was obtained.

It is known that umbelliferone (7-hydroxycumarin) has a tyrosinase inhibitor effect (IC50 = 0.42 mM), despite having no amino group in its position 3. The 3-aminocoumarin (compound **4**) was synthesized and evaluated and did not show tyrosinase inhibitory activity. Then, other coumarins were prepared, maintaining the amino group in the 3-position, also incorporating a hydroxyl group in different positions of the coumarin skeleton.

Different synthetic methodologies were carried out to obtain compounds **7** and **8**, which have the hydroxyl group in positions 7 and 8, respectively, of the coumarin nucleus (benzene ring) and compound **9**, with the hydroxyl group in position 4 of the coumarin (pyrone ring). As shown in Table 1, compound **7** was the most active compound of this series, with an IC50 value in the micromolar range (53 μM). This compound was more than 10 times more active than umbelliferone, the reference compound. Compound **7** had the amino and hydroxyl groups that precisely mimicked the same positions that both groups occupy on the tyrosine molecule. Compound **8** was inactive against tyrosinase whereas

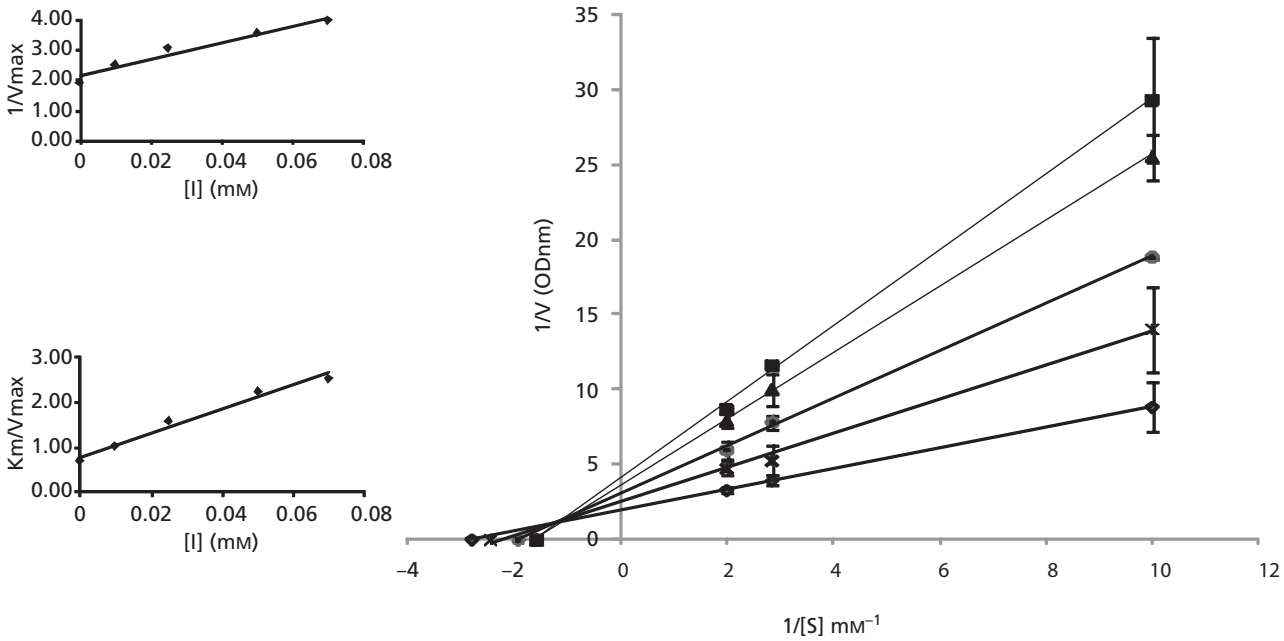

Figure 3 Lineweaver–Burk plots for inhibition of compound **7** on mushroom tyrosinase for catalysis of L-DOPA. Inhibitor concentrations were 0 (◆), 0.010 mM (●), 0.025 mM (x), 0.050 mM (▲), 0.070 mM (■). The insets are the secondary plots of $1/V_{max}$ and K_m/V_{max} versus concentration of compound **7**, respectively.

Table 1 Inhibitory effect of compounds **4** and **7**–**9** and umbelliferone on mushroom tyrosinase activity

Compounds	IC50 (mM) (L-DOPA 0.5 mM)
4	>5.0
7	0.05 ± 0.01
8	>5.0
9	0.25 ± 0.003
Umbelliferone	0.42[a]

[a]Obtained from Fais *et al*.[23]. These results are average results of three experiments.

compound **9** was active against this enzyme (IC50 = 0.26 mM). Compound **9** presented only slightly higher tyrosinase inhibitory activity than umbelliferone.

Therefore, it can be inferred that the tyrosinase inhibitory activity depends on the position of the hydroxyl group in the coumarin moiety. Also, the presence of the hydroxyl group at the same position that of tyrosine and umbelliferone is important to the inhibitory activity.

Conclusions

This study showed that some of the synthesized tyrosine-like condensed derivatives have inhibitory activity against mushroom tyrosinase. The two active compounds present tyrosinase inhibitory activity in the micromolar range. The presence of a hydroxyl group in the seven position of the 3-aminocoumarin, the same position as in tyrosine and umbelliferone, improves the inhibitory activity with respect to the other synthesized derivatives and the reference compound. So, the introduction of hydroxyl groups improves the pharmacological potential of these 3-aminocoumarins, confirming that this lead could be effectively optimized in a candidate for the treatment of some hyperpigmentation skin diseases. These findings have encouraged us to continue the effort towards the optimization of the pharmacological profile of these coumarins.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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