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# Vanilloid Derivatives as Tyrosinase Inhibitors Driven by Virtual Screening-Based QSAR Models

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**A number of vanilloids have been tested as tyrosinase inhibitors using Ligand-Based Virtual Screening (LBVS) driven by QSAR (Quantitative Structure-Activity Relationship) models as the multi-agent classification system. A total of 81 models were used to screen this family. Then, a preliminary cluster analysis of the selected chemicals was carried out based on their bioactivity to detect possible similar substructural features among these compounds and the active database used in the QSAR model construction. The compounds identified were tested *in vitro* to corroborate the results obtained *in silico*. Among them, two chemicals, isovanillin ($K_M^{app}$ = 1.08 mM) near to kojic acid (reference drug) in one cluster and isovanillyl alcohol ($K_M^{app}$ = 0.88 mM) at the same distance as hydroquinone (reference drug) in another cluster showed inhibitory activity against tyrosinase. The algorithm proposed here could result in a suitable approach for faster and more effective identification of hit and/or lead compounds with tyrosinase inhibitory activity, helping to shorten the long pipeline in the research of novel depigmenting agents to treat skin disorders. Copyright © 2010 John Wiley & Sons, Ltd.**

*Supporting information may be found in the online version of this article.*

**Keywords:** Ligand-Based Virtual Screening (LBVS); Quantitative Structure-Activity Relationship (QSAR); tyrosinase inhibition

## Introduction

Tyrosinase (EC 1.14.18.1) is a common enzyme catalyzing the *ortho*-hydroxylation of monophenols to the corresponding *ortho*-diphenols (catechols) and the oxidation of catechols to the corresponding *ortho*-quinones.[1] The enzyme has a dicopper centre formed by two $Cu^{2+}$ ions coordinated by six histidine residues as its outstanding structural feature. The enzyme is deeply involved in melanogenesis, either physiological or pathological, in insect development and metamorphosis, and in vegetable browning,[2–4] so the understanding of its physiopathological expression, activity, regulation, and inhibition is the focus of a large number of studies. Many inhibitors have been found that are important for potential or actual practical applications [5–6] or interesting to help solve the catalytic cycle mechanism.[7] Therefore, our attention has focused on some simple 'vanilloid' compounds to assess their activity – if any – towards tyrosinase, in the light of their insertion in a purpose-built constructed database.

Vanilloids, by conventional definition – perhaps not quite correct, but widely accepted – indicate guaiacyl derivatives ideally based on the vanillin (4-hydroxy-3-methoxy-benzaldehyde) structure. As the isomeric aldehyde 3-hydroxy-4-methoxy-benzaldehyde is commonly known as isovanillin, its derivatives where the guaiacyl moiety is conserved could well be defined as 'isovanilloids'. Both classes of compounds are represented in nature where they exert a number of known functions. Some of them are important bioactive substances, with specific receptors also found in humans.[8]

Therefore, it seemed interesting to examine the behaviour of three vanilloids (vanillyl alcohol, vanillin, and vanillic acid) and the corresponding isovanilloid counterparts (isovanillyl alcohol, isovanillin, isovanillic acid) as putative tyrosinase inhibitors with the aid of advanced computational methods.

Recent advances in medicinal chemistry involve the combination of several disciplines for the identification of promising leads. This crucial pathway is of primary importance in the drug discovery process. Among them, high throughput screening (HTS) in chemical data mining can be of valid help in the biological evaluation of very large sets of chemicals. Indeed, a biological approach alone could not give satisfactory results due to the increasing tests and costs associated with the evaluation of a huge number of compounds.[9]

Therefore, the computer-aided structure-activity relationship analysis and molecular modelling are currently widely used by pharmaceutical chemists to discover new lead compounds.[10–12]

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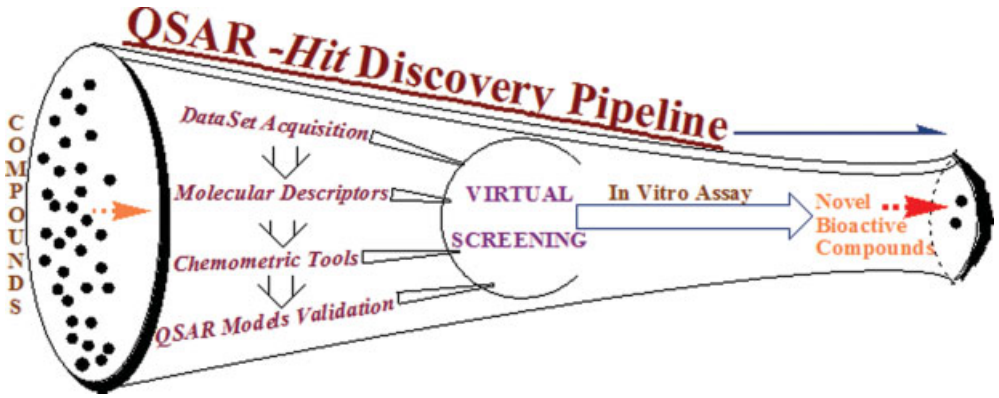



**Figure 1.** Scheme for the virtual screening followed in this work.

The *in silico* screening of combinatorial databases is one of the techniques often used to predict potential lead structures.[13] In this way, the ligand-based virtual screening (LBVS) has emerged as a theoretical tool for the development of novel drug-like compounds or for the identification of new lead-chemical compounds.[14] One of the approaches related to LBVS includes the development of empirical quantitative structure-activity relationships (QSARs) via the application of statistical methods to sets of biological data and structural descriptors.[15–17] LBVS encompasses the relationship of the specific activities and the chemical structures of known bioactive compounds and could result in a suitable approach.

The main impact of QSAR is the identification of a specific endpoint that is referred to as a well-defined biological activity. This is the first goal of the five OECD (Organization for Economic Cooperation and Development) Principles for (Q)SARs Validation to make up QSAR models for regulatory purposes.[18] In the case of tyrosinase inhibitory activity, a large number of tyrosinases isolated from different sources are used in the experimental assays to confirm the biological activity of compounds, resulting in a great variability in experimental measurements. In this sense, tyrosinase inhibition could be defined as the specific endpoint. Nevertheless, in the last few years, some chemoinformatic studies have been carried out related to tyrosinase, most of them using only one type of chemical class or one type of enzyme.[19–25] These QSAR models are considered *local* models as opposed to *general* models encompassing a wider range of both tyrosinases and (putative) inhibitors.[18]

On the other hand, one of our research groups has developed QSAR models using 2D atom- and bond-based *TOMOCOMD-CARDD* (TOpological MOlecular COMputational Design – Computer Aided 'Rational' Drug Design) descriptors and linear discriminant analysis (LDA), employing a database with a wide coverage of molecular structures of compounds reported as tyrosinase inhibitors.[26] In this way, a large set of QSAR-LDA models for discrimination between true tyrosinase inhibitors and inactive compounds were proposed.[26–30] All these QSAR-LDA models have been used in the identification of novel tyrosinase inhibitors. For example:

1) Natural compounds (cycloartanes, lignans, dipertenoid alkaloids, and ethylsteroids)[26–28,30]
2) Synthetic compounds (tetraketones and dicoumarins)[29,31]

This wide spectrum of models could be used as a multiclassification system for lead-QSAR identification of promising compounds as tyrosinase inhibitors. To this purpose, several classes of other enzyme inhibitors with different biological profiles have been discovered by using algorithms based on LBVS approaches.[32–33]

Following this aim, the objective of this study was to perform both an LBVS of organic chemicals based in QSAR models and *in vitro* assays of the compounds as novel tyrosinase inhibitors. The present results could constitute a step towards the translation of *in silico* methods to facilitate the faster discovery of lead compounds.

## Materials and Methods

### QSAR Models Driven by 2D atom- and bond-based *TOMOCOMD-CARDD* Descriptors

The wide range of QSAR models applied in this work was developed by our research group as reported in previous papers.[26–30] These mathematical models related the whole set of atom and bond-based 2D molecular descriptors, implemented in the *TOMOCOMD-CARDD* software,[34] to experimental data derived from the tyrosinase inhibition. The broad range of 81 models used in this report, was obtained by using the LDA as a statistical algorithm to build the classification models, owing to its simplicity and extensive use in the field of drug design and QSAR modelling.[15,35–37] This set of models was chosen to include several chemical compounds from both natural and synthetic sources such as kojic acid, tripeptides, Vitamin B derivatives, flavonoids, N-benzyl-N-nitrosohydroxylamines, hydroxychalcones, azobenzene derivatives, catechins, hydroxystilbenes, benzaldoximes and so on, ensuring a wide structural diversity and domain applicability. The database was readily accessible and could provide an estimate of likely potency of novel chemicals as tyrosinase inhibitors. The details of the QSAR-LDA protocol and its development are described elsewhere.[26–30] Figure 1 illustrates the algorithm used in this work.

The QSAR models classified the compounds according to their inhibitory activity against the enzyme. The inhibitory activity was codified by a dummy variable '***Class***'. This variable indicates the presence of either an active compound [(***Class***) = 1] or an inactive compound [(***Class***) = −1]. The classification of cases was performed by means of the posterior probabilities classification. By using these models, one compound can then be classified as active, if $\Delta P\% > 0$, being $\Delta P\% = [P(\text{Active}) - P(\text{Inactive})] \times 100$, otherwise as inactive. $P$(Active) and $P$(Inactive) are the probabilities where the equations classify a compound as active or inactive, respectively. In our case, we make use of a rule



for the classification of tyrosinase inhibitors as a multiclassification system. The compounds were selected as potential tyrosinase inhibitors (virtual hits) if they adhered to the following criteria: Compounds evaluated as active by more than 90% of the models with a percent of classification above zero ($\Delta P\% > 0$ by at least 73 models).

### Cluster analysis

In our case, *k*-MCA (*k*-means cluster analysis) algorithms were used to group the compounds according to their chemical similarity encoded by the molecular descriptors used as variables.[38–39] The statistical software package [40] was used to develop the *k*-MCA. The algorithm was set to define 10 clusters. The number of members in each cluster, and the standard deviation of the variables in the cluster (kept as low as possible) were taken into account, to have an acceptable statistical quality of data partitions into the clusters. The values of the standard deviation (SS) among and within clusters, of the respective Fisher ratio and their *p* level of significance, were also examined.[41–42]

### Evaluation of tyrosinase inhibitory activity

*Enzymatic assay*

One tyrosinase unit corresponds to the amount of enzyme that causes an absorbance increase of 0.001 per minute in KPs buffer at pH 6.5 and 25 °C (L-tyrosine as substrate, 3.0 mL reaction volume). Since tyrosinase catalyzes a reaction between two substrates – molecular oxygen and a phenolic – the assay was carried out in air-saturated solutions. Mushroom tyrosinase used in these experiments was partially purified as described previously.[43] Tyrosinase activity was estimated in the presence of 722 U enzyme, 50 mM sodium phosphate buffer pH 6.5 and tyrosine (ranging from 0.125 to 1.26 mM), in a final volume of 10 mL. A stock solution of 2 mM tyrosine was prepared in 5 mM HCl. When used, the chosen vanilloid was present at a final concentration of 0.3 or 0.6 mM. As some vanilloids are substrates of the enzyme laccase, a partially purified tyrosinase preparation was carefully inspected to rule out any laccase contamination that could alter experimental results.[44] All samples were incubated at 25 °C under gentle stirring. Then, 0.998 mL of the reaction mixture was removed at established times and the reaction was stopped with concomitant protein precipitation by adding 2 μL of 85% phosphoric acid. Samples were centrifuged at 8000 g for 10′ and the supernatants were immediately injected into the HPLC system.

*HPLC analysis*

The HPLC apparatus was a Beckman System Gold equipped with an UV-Vis detector module. The column used was a Phenomenex, Luna, RP-C18 (250 mm × 3 mm i.d., 5 μm) purchased from Chemtek Analytica (Bologna, Italy). Separation was achieved with 0.085% phosphoric acid in water v/v (solvent A) and 95% acetonitrile in solvent A (solvent B). Chromatographic conditions: initial isocratic elution, 5% B for 5′, followed by gradient phase, 5 → 90% B in 10′ at 0.6 mL $min^{-1}$ flow rate. The detector was set at 280 nm. Reported values represent the mean of two injections from duplicate experiments. To determine $K_M$ values, kinetic data were examined using a Lineweaver-Burk plot

*Statistical analysis and software*

All experiments were run at least in triplicate. Origin 7.0 (Origin Corporation, Northampton, MA, USA) was used for statistical analysis. Lineweaver-Burk data were analyzed with GraFit 4.0.21, Erithacus Software, Horley, UK.

## Results and Discussion

### QSAR models and multiclassification system for guiding compound selection

Innovative approaches for computer-aided molecular design such as LVBS have gained popularity and utility over the last several years.[45] Some of these methodologies are fundamental in QSAR analysis of heterogeneous data sets, including thousands of compounds from different chemical series.[46]

These tools are very useful in achieving substantial savings in both time and money when carrying out interdisciplinary projects for experimental studies of new pharmaceutical agents. *In silico* predictions could reduce the number of compounds that have to be synthesized and experimentally studied using *in vitro* or *in vivo* tests. For this purpose, and encouraged by the good results from the QSAR modelling of different bioactivities,[36,47–48] one of our research teams focused on theoretical studies and developed a series of QSAR models related to the prediction of tyrosinase inhibition through LDA as statistical technique.[26–30] These models, developed in earlier works, proved to be useful in detecting compounds from natural and synthetic sources as novel tyrosinase inhibitors. In addition, drugs with other pharmacological uses were identified from simulated LBVS based on results reported in the literature for these drugs.[7,49–52] The good results of accuracy, high sensitivity and specificity for these 81 QSAR models as provided by the OECD Principles, permit their use in a multiclassificatory system to enhance the reliability of predictions by the use of information derived from more than one model.[18,53]

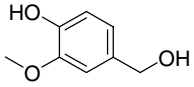
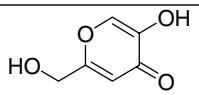
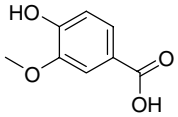
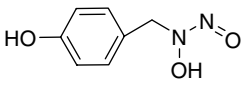
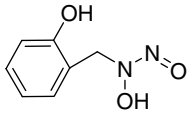
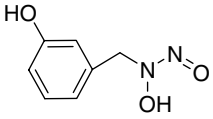
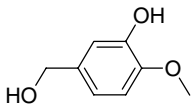
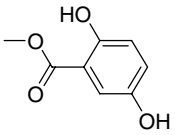
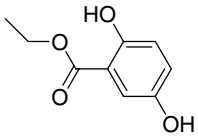
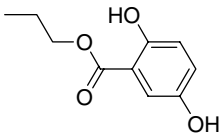
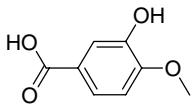
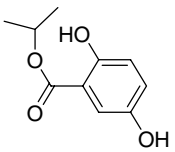
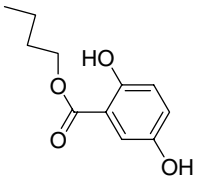
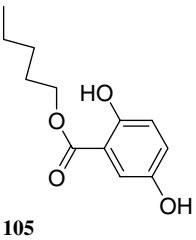
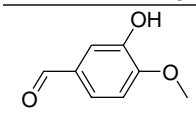
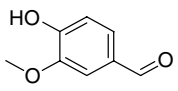
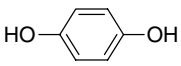
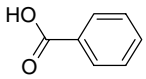
Prediction of tyrosinase inhibition was carried out for a family of vanilloid derivatives using the 81 LDA-QSAR models developed earlier,[26–30] with the goal of discriminating tyrosinase inhibitors from inactive compounds. The compounds were selected as potential tyrosinase inhibitors (virtual hits) when meeting the following criteria: *Compounds evaluated as active by more than 90% of the models with a percent of classification above zero*. Six vanilloid derivatives were therefore selected by this battery selection method proposed above. The values of posterior probabilities classification are depicted in Table 1 of the Supplementary Material.

### Detecting novel tyrosinase inhibitors similarities inside the active database

A preliminary similarity study was carried out with the compounds identified as tyrosinase inhibitors by the *in silico* procedures. In this case, a *k-means* Cluster Analysis, using the descriptors included in the QSAR models as input, helped us to group these vanilloids according to their chemical similarity to the compounds in the active database. The new tyrosinase inhibitors (vanilloids) were placed in two clusters with equal distance between them, also sharing the main sub-structural features with the rest of compounds of both clusters in which they were included.

An exhaustive analysis of each cluster was carried out. At first, vanillyl alcohol, vanillic acid, isovanillyl alcohol, and isovanillic

**Table 1.** Structures of Tyrosinase Inhibitors Vanilloid Derivatives and Compounds of Active Database

| Vanilloids | Tyrosinase Inhibitors on Active Database | | |
|---|---|---|---|
| **CLUSTER 1** | | | |
|  **Vanillyl alcohol** |  **1** Kojic acid | | |
|  **Vanillic acid** |  **74** |  **75** |  **79** |
|  **Isovanillyl alcohol** |  **100** |  **101** |  **102** |
|  **Isovanillic acid** |  **103** |  **104** |  **105** |
| **CLUSTER 2** | | | |
|  **Isovanillin** | | | |
|  **Vanillin** |  **4** Hydroquinone |  **9** Benzoic acid | |

acid were included in one cluster. In this cluster, most of the compounds of the training set showed the presence of benzene rings with hydroxyl substituents at the C-3 or C-4 position as the common feature of these bioactive compounds. The majority of these tyrosinase inhibitors were at the same distance in the cluster from the included vanilloid derivatives. Examples of these compounds are kojic acid (reference drug), and compounds **74, 75, 79**, and **100–105 (**Table 1).

In the case of the second cluster, the remaining vanilloids (isovanillin and vanillin) were included together with hydroquinone and benzoic acid, two well-known tyrosinase inhibitors. Vanillin and isovanillin have some substructural features related to these tyrosinase inhibitors and other compounds inside the cluster. By this logic, they were nearby inside the cluster. The active database and molecular structures of tyrosinase inhibitors used to perform the preliminary Cluster Similarity Analysis, and the QSAR models described in previous works are depicted in Table 2 and 3 of the Supplementary Material, respectively. The structures of the six potential tyrosinase inhibitors selected on the basis of this criterion, are shown in Table 1 together with the compounds included in the preliminary cluster similarity analysis.

### Effect of the vanilloids on tyrosinase activity *in vitro*

All selected vanilloids were experimentally tested for their inhibitory activity when tyrosine was used as the enzyme substrate. Figures 2 and 3 show the experimental values of apparent $K_M$ and $V_{max}$ in the presence of these six compounds.

Despite the strong chemical similarities among the six compounds examined, only isovanillyl alcohol and isovanillin showed significant inhibitory activity, whereas the other compounds were almost inactive (weak inhibitory activity). This finding is also interesting as the two inhibitors were included in two different clusters. It also opens a door to combinatorial guided chemical synthesis including structural changes aimed at searching for lead compounds with tyrosinase inhibitory activity. Further studies will explore the structural features needed for an inhibitory activity

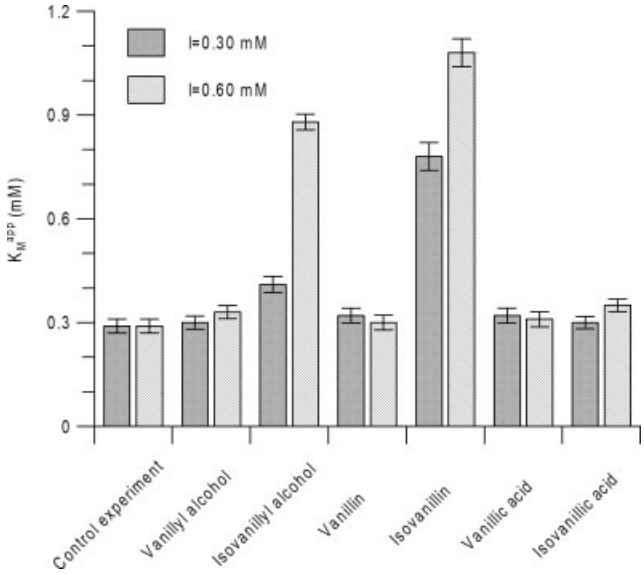



**Figure 2.** Graphic of experimental values of $K_M$ for the vanilloid derivatives.

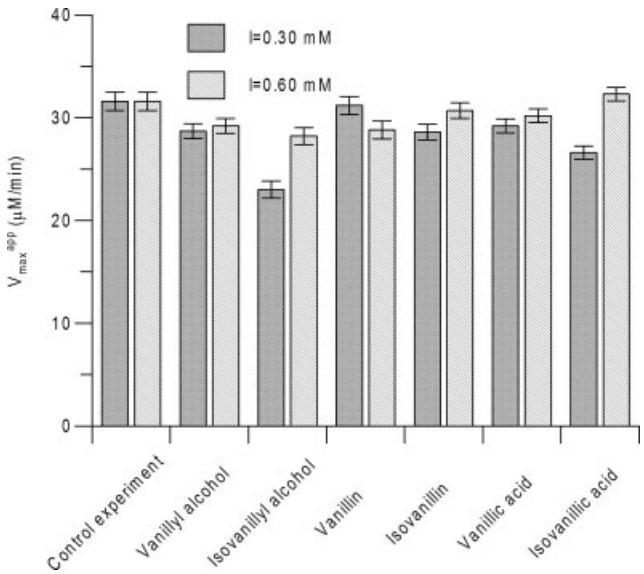



**Figure 3.** Graphic of experimental values of $V_{max}$ of the vanilloid derivatives.

by both vanilloids and isovanilloids in the light of the actual knowledge of tyrosinase active site structure.

In particular, the observed inhibition was clearly competitive, suggesting the effective entering of the compounds into the tyrosinase active site. As such, a promising future direction could be the study of the enzyme active site by means of purpose-built isovanilloids, whose design would be carried out with the aid of the calculation tools proposed.

In future studies, these models, which relate the chemical structure with a specific endpoint, could be programmed into expert systems capable of helping in the exhaustive search of bioactive molecules within huge chemical libraries, because *the integration of all available information into efficient screening protocols, although challenging, is perhaps the most fruitful way to discover potential new lead compounds.*

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**Supplementary material available**

The vanilloid data posterior probabilities classification according to LDA-based QSAR models analysis as well as database and structures of tyrosinase inhibitor compounds, is available free of charge via the Internet at. . . (this will be completed with the DOI of the article). . .

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