

STUDY OF KINETIC PARAMETERS AND POSSIBLE INHIBITORY EFFECT ON THE TYROSINASE OF THE HALOGENATED BOROXINE DIPOTASSIUM TRIOXOHYDROXYTETRAFLUOROTRIBORATE $K_2[B_3O_3F_4OH]$

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Abstract

INTRODUCTION: A number of biochemical and medical researchers have detected increased activity of tyrosinase in skin tumor cells. The most famous and available inhibitor, kojic acid, has several side effects and is not completely safe for use.

OBJECTIVES: This paper describes the study of inhibitory influence of halogen boroxine $K_2[B_3O_3F_4OH]$ on tyrosinase. The research was prompted by the ability of this compound to inhibit enzymes through metal ion chelation as well as its synthesis and application in cosmetic skin products that produce no serious side-effects.

METHOD: Tyrosinase activity was measured by spectrophotometric analysis for the appearance of dopachrome pigment at a wavelength of 475 nm. Tyrosinase exhibited typical Michaelis-Menten kinetics.

RESULTS: Tests of the proposed inhibition of the enzyme tyrosinase showed that $K_2[B_3O_3F_4OH]$ had weak inhibitory properties.

CONCLUSION: It will be necessary to search for new ways of antitumor mechanisms that differ from those of previous results.

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Introduction

According the World Health Organization, two to three million people are diagnosed with some form of malignant skin tumor each year, with 41,000 patients dying each year from melanoma. Worldwide, the number of new cases of skin cancer is growing at the rate of between three and seven per cent a year (Siegel, Miller & Jemal, 2015). Numerous people suffer from many form of skin disorders (www.skincancer.com). Most investigated is the enzyme, tyrosinase (EC number 1.14.18.1), which is thought to play a main role in skin disorders (Saran et al., 2004). Tyrosinase is present in many plants, fungi, and mammalian cells. Tyrosinase extracted from the champignon mushroom, *Agaricus bisporus* (J.E.Lange) Imbach (1946), is homologous with that of the mammalian. Almost all studies of tyrosinase inhibition have used mushroom tyrosinase, because this enzyme is commercially available. Tyrosinase was named because of the activity of the amino acid, tyrosine, which is found in nearly all animal cells and is very important in melanin synthesis (Uchida, Ishikawa, & Tomoda, 2014). Human tyrosinase is a single membrane-spanning transmembrane protein, and when tyrosine forms the substrate, it forms dopaquinone, an intermediate in the production of the pigment, melanin. Dopaquinone spontaneously forms an orange-red pigment called dopachrome, which undergoes a final reaction to form the blackish brown pigment, melanin.

In the earliest stage, Stage 1, of melanoma skin cancer, the enzyme is scarcely noticeable, but is widespread and evenly distributed in Stage 2, and then unevenly distributed in Stage 3 (metastases; Saewan, Koysoomboon, & Chantrapromm, 2011). Several polyphenols, including flavonoids and stilbenoids, substrate analogues, free radical scavengers, and copper chelators, have been known to inhibit tyrosinase. Well known tyrosinase inhibitors include kojic acid, tropolone, coumarins, vanillic

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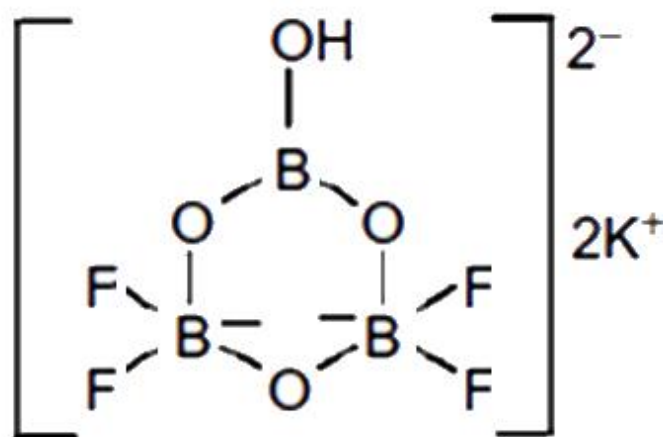
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acid, vanillin, and vanillic alcohol (Chen, Lin, Yang, Bordon, & Wang, 2015). The best known of the above-mentioned inhibitors is kojic acid, which is used as a positive standard in experimental measurements. Today, kojic acid is mainly used in cosmetic formulations (Balakrishna, Payili, Yennam, Uma Devi, & Behera, 2015). Kojic acid shows a competitive inhibitory effect on monophenolase activity and a mixed inhibitory effect on the diphenolase activity of the mushroom tyrosinase. Skin irritation is the most common kojic acid side-effect, although cell mutation in mammals was also detected from results of 165 studies of kojic acid toxicity presented by the nonprofit Environmental Working Group (Burnett et al. 2010). However, kojic acid is not expected to be a human carcinogen. Some studies of animals have shown that high amounts of kojic acid can cause liver, kidney, reproductive, cardiovascular, gastrointestinal, and respiratory side-effects and hence, detection of a less dangerous inhibitor has been the aim of several studies in the last five years and this type of research still continues (Chang, 2012).

The interest in boron compounds has grown in the last 20 years. Boronic acids are a class of boron compounds that first appeared in the literature in the 1860 (Yang, Gao, & Wang, 2003). These compounds are interesting to study because of their stability, low toxicity, and potential for producing hydrogen and covalent bonds at the active site of the enzyme; this means they can be used as inhibitors of enzymes. Boronic acid forms several derivatives that remain relatively unexplored. Boroxines are one of these, which involves 6-membered heterocyclic compounds with a unique electronic configuration (Hall, 2005). Preliminary tests using halogen boroxine (dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$; Ryssi & Slutskaya, 1951) revealed that it displays anticancer effects (Ivankovic et al., 2015). This compound can react with the Lewis bases and, as the ion $[B_3O_3F_4OH]^{2-}$, is potentially a selective inhibitor of enzymes. It has the ability to bind to active sites of enzymes and thus prevent the reaction of catalyzation. For now, a small number of publications about this compound suggests that, possibly, a halogenated derivative of this compound can be used for the prevention and treatment of benign and malignant lesions in the epidermis of the skin (skin cancer). Furthermore, halogen boroxine displayed properties that demonstrated its potential in future conventional, medical, dermatological, or cosmetic formulations. The compound is highly soluble in water and thus, could facilitate the production of pharmaceutical formations. This solubility contributes to its high bioavailability with effective absorption at the site of administration to the skin. Haverić, Haveric, Bajrovic, Galic, and Maksimovic (2001), at the Institute for Genetic Engineering and Biotechnology in Sarajevo, examined the anti-proliferation, cytotoxic, and genotoxic potential of the halogen derivatives in toxicological studies, and these indicated the halogen derivative has no damaging effect on human health or mammals.

Figure 1: Structure of dipotassium trioxohydroxytetrafluorotriborate $K_2[B_3O_3F_4OH]$



Source: Authors

Preliminary results from in-vitro and in-vivo antitumor activity on cell lines, 4T1 adenocarcinoma, B16F10 melanoma, and squamous cell carcinoma SCCVII, at the Institute Ruđer Bošković in Zagreb

revealed a strong expression of anti-tumor activity, comparable to the well-known anti-cancer drug, 5-fluorouracil.

Studies in enzyme inhibition of catalase (EC number 1.11.1.6) have shown that the enzyme follows Michaelis-Menten kinetics in the absence and presence of inhibitors (Islamovic, Galic & Milos., 2014). Recent studies focused on the possibility of inhibition of carbonic anhydrases (EC number 4.2.1.1) isoforms, which are associated with tumorigenesis and metastasis of some tumors. It was assumed that the mechanism of action of $K_2 [B_3O_3F_4OH]$ is associated in its binding with the zinc ion in the active site of the enzyme. Inhibitory activity of halogen boroxine on carbon anhydrases, in other species (bacteria and fungi), suggests that this compound could also have anti-microbial and anti-infectious properties (Vullo, Milos, Galic, Scozzafava, & Supuran, 2015).

As the halogenated boroxine derivative has shown effects on human molecules and mechanisms of carbon anhydrases inhibition through chelation of zinc atoms (similar to that of Kojic acid on copper in the active site of tyrosinase; Ghani & Ullah, 2010.), it is reasonable to hypothesize that a halogen derivative thereof may have an inhibitory action on the enzyme, tyrosinase. That theory encourages the notion that enzyme tyrosinase is also found in skin tumor cells in a "bewildering" state that should be inhibited. With this information one could easily assume that boroxine would have inhibitory effects on this same enzyme.

Materials and Methods

Enzyme kinetics provide methods for quantification of the parameters of enzyme activity. Data obtained from enzyme kinetics provided K_M values (substrate concentration at which the reaction velocity is half-maximal) and V_{max} values (maximal velocity of a reaction, which occurs at the saturation of an enzyme). The Michaelis-Menten's constant is a measure of affinity of an enzyme for its substrate. Each enzyme-substrate reaction provides a unique K_M .

Tyrosinase activity can be measured by monitoring the appearance of the dopachrome pigment at a wavelength of 475 nm. The absorbance allows spectrophotometric analysis of tyrosinase activity by determining the rate of dopachrome formation from the substrate, L-DOPA. Tyrosinase exhibits typical Michaelis-Menten kinetics. The potential inhibitor was examined in the presence of tyrosine and L-DOPA, as the enzyme substrate, and activity was assessed in terms of dopachrome formation.

All measurements were made in the laboratory of Organic Chemistry and Biochemistry at the Faculty of Chemical Technology, at the University of Split, using a spectrophotometer Specord 200plus (Edition 2010).

The source of enzyme used was the tyrosinase from *Agaricus bisporus* (T3824 SIGMA, Tyrosinase from mushroom, lyophilized powder, ≥ 1000 unit/mg solid). We prepared a solution of tyrosinase, wherein every 1 ml of the solution had 104.17 units of enzyme solution. This solution was kept on ice throughout the experiment. Also, a sample solution boroxine was prepared using $K_2 [B_3O_3F_4OH]$ of 0.198 mM (1 mg/mL), 0.596 mM (3 mg/mL), and 0.994 mM (5 mg/mL) aliquots; and substrate solution of L-DOPA of 0.198 mM (1 mg/mL), 0.596 mM (3 mg/mL), and 0.994 mM (5 mg/mL) in phosphate buffer of pH 6.5. Two "blind" probes were used to monitoring the course of the reaction without enzymes. Before the addition of inhibitor of various concentrations, a series of measurements of kinetics of non-inhibited chemical reaction between L-DOPA and the enzyme tyrosinase were performed to facilitate a comparison between potentially inhibited and noninhibited conditions.

The reaction was initiated with 0.4 mL of tyrosinase and 0.05 mL of boroxine, placed in a vial to react for five minutes. The total volume of solution in the cuvette was 1 ml. The substrate L-DOPA and a buffer were then added, and the changes in absorbance at a wavelength of 475 nm measured. Measurements were adjusted to the three-minute duration.

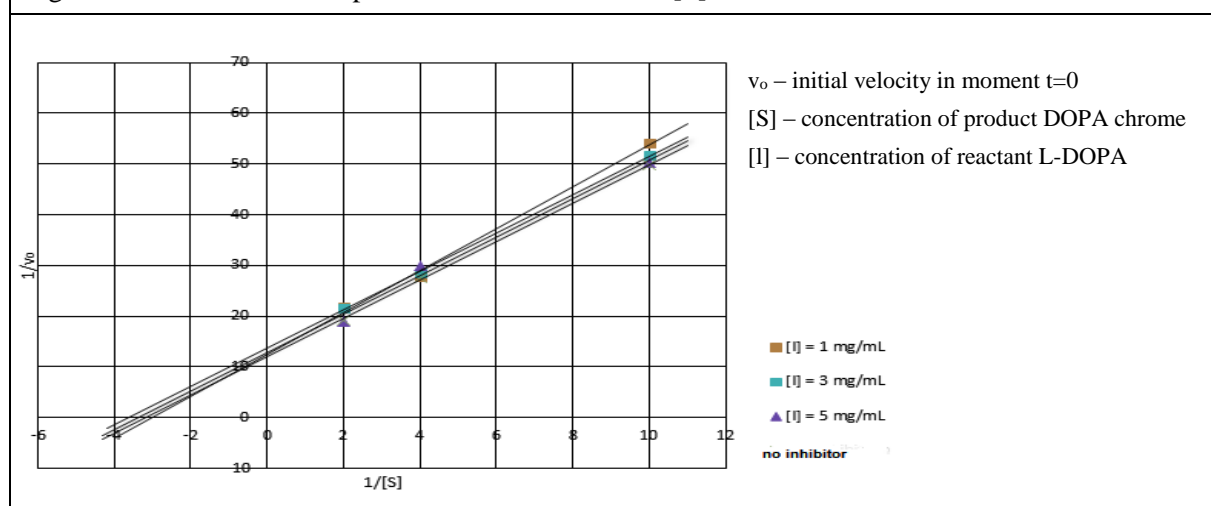
From our data, we calculated and graphed rates of reaction as $\Delta A/\Delta \text{min}$ to establish initial velocity. Absorbance, divided by time (min), was converted to volume (μmol), divided by time (/min), using Beer's law and the extinction coefficient for dopachrome ($3600 \text{ m}^{-1}\text{cm}^{-1}$), as follows:

$$\mu\text{mol}/\text{min} = \frac{\Delta A/\text{min}}{3600M^{-1}} \times (10^6 \mu\text{M}/M) \times 0,001L \quad (1)$$

Results and Discussion

The results of the Lineweaver-Burk, Eadie Hofstee, Hanes-Woolf, and Dixon's plots show that K_2 [$B_3O_3F_4OH$] exhibited little inhibition of tyrosinase. From the equations and sequences on the X and Y axes, the kinetic parameters (V_{max} and K_M) were represented in all used plots. However, the Dixon results were somewhat different from other results and in this case were disregarded because they were unsuitable for determination of graphical presentation and calculation of kinetic parameters. Thus, the Dixon's plot is not displayed.

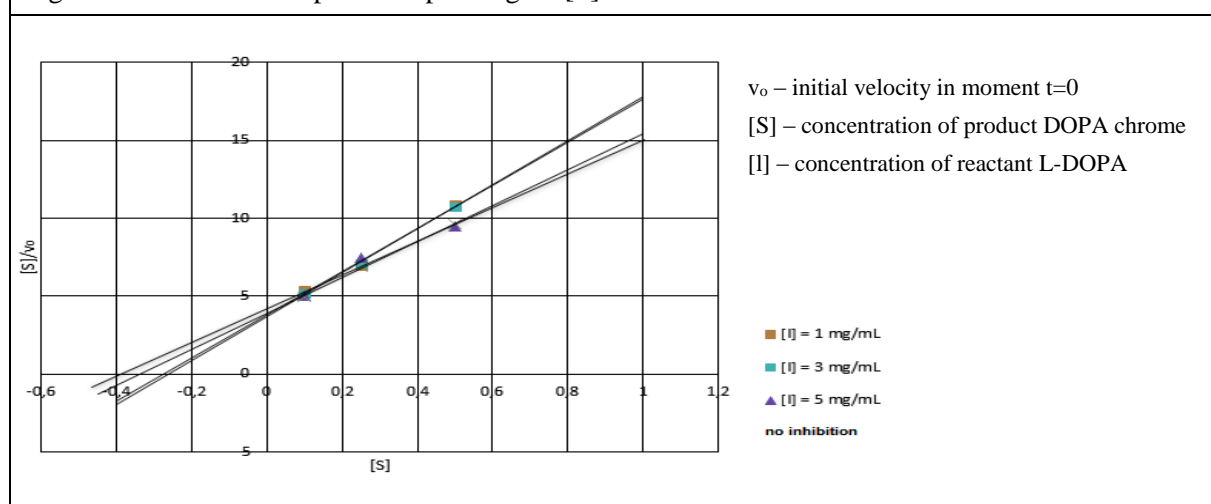
Figure 1: Lineweaver-Burk plot addition $1/v_0$ about $1/[S]$



Source: Authors

All values of V_{max} were reduced with the addition of the inhibitor in comparison to those without the inhibitor. The values of K_M were lower for inhibitor concentrations of 0.198 mM (1 mg/mL) and 0.596 mM (3 mg/mL) in all views. The Lineweaver-Burk, Hanes Woolf, and Eadie-Hofste plots show that the parameters V_{max} and K_M were reduced by the concentration of 0.596 mM (3 mg/mL), suggesting an acompetitive type of inhibition in this case.

Figure 2: Eadie-Hofstee plot v_0 depending $v_0/[S]$



Source: Authors

For all views, the concentration of 0.994 mM (5 mg/mL) associated with parameter values of V_{max} that were almost the same and K_M that increased.

Results suggest future investigations in the area of concentrations higher than 0.994 mM (5 mg/mL) of halogen boroxine derivative $K_2 [B_3O_3F_4OH]$.

Figure 3: Hanes-Woolf plot addiction $[S] / v_0$ of $[S]$

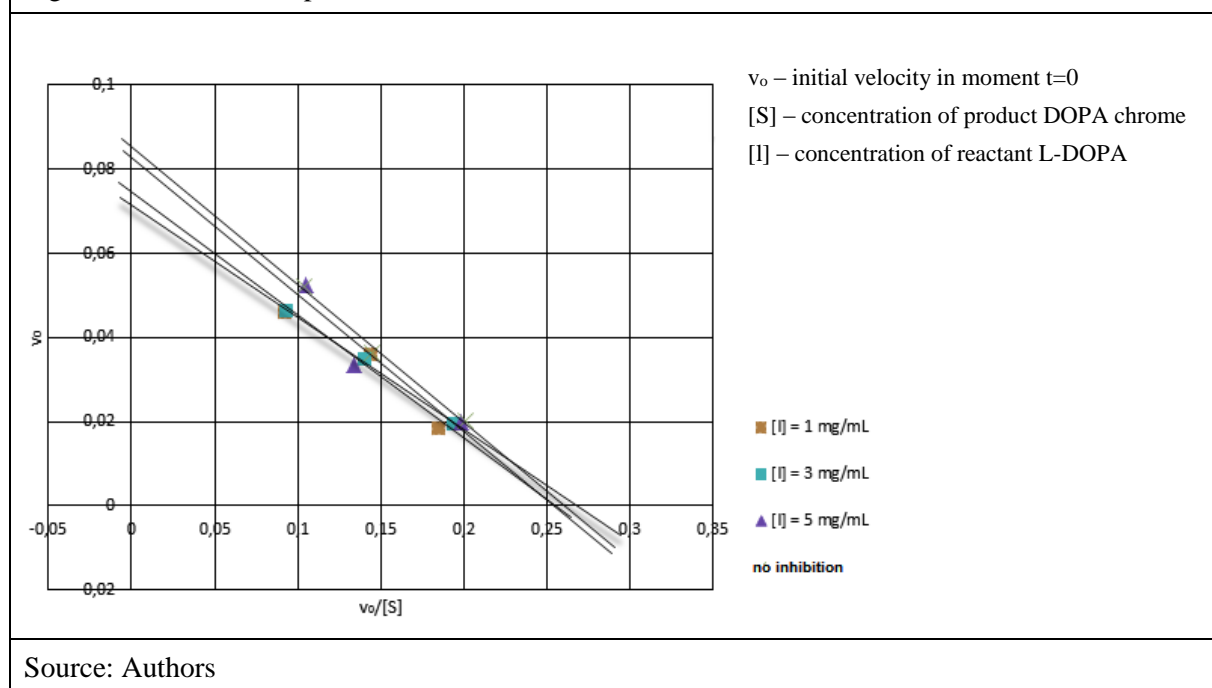


Table 1: The values V_{max} and K_M determined from graphic plots (total volume 1 mL)

	V_{max}	K_M	
1 mg/mL	0.0800	0.334	Lineweaver-Burk plot
3 mg/mL	0.0725	0.273	
5 mg/mL	0.0780	0.3053	
No inhibitor	0.0836	0.3175	
1 mg/mL	0.0744	0.2989	Eadie-Hofstee plot
3 mg/mL	0.0716	0.2679	
5 mg/mL	0.0829	0.3410	
No inhibitor	0.0852	0.3283	
1 mg/mL	0.0706	0.2686	Hanes-Woolf plot
3 mg/mL	0.0708	0.2617	
5 mg/mL	0.0860	0.3669	
No inhibitor	0.0866	0.3374	

Source: Authors

Conclusion

Tests of the proposed inhibition of the enzyme tyrosinase showed that $K_2[B_3O_3F_4OH]$ has weak inhibitory properties. This was confirmed by Lineweaver-Burk, Hanes-Woolf, and Eadie-Hofstee plots of the reaction of tyrosinase with $K_2 [B_3O_3F_4OH]$. All values for maximum speed, V_{max} , were less, with the addition of $K_2 [B_3O_3F_4OH]$, than the value without inhibitor. It was evident that added concentrations of $K_2 [B_3O_3F_4 OH]$ reduced the V_{max} and K_M , suggesting an acompetitive type of inhibition, except for the inhibitor concentration of 0.994 mM (5 mg/mL). Halogenated boroxine

derivatives should show inhibitory effects on the enzyme, tyrosinase, according to expectations that are based on human visible stains and the inhibitory action of chelation of metal ions in the active site of carbon anhydrases. Although study results failed to show such an effect, based on the selected concentrations, the importance of this research is in regard to directing future research towards the study of halogen boroxine derivative $K_2 [B_3O_3F_4OH]$ with concentrations higher than 0.994 mM (5 mg/mL), although it is more likely that a change of perspective and search for new ways of anticancer mechanisms that are different from that expected is needed.

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