DETERMINATION OF ANTIOXIDANT CAPACITY OF SELECTED BOROXINES

Maja Marasović,[1] Antonio Roščić,[2] Borivoj Galić,[3] Mladen Miloš[4]

Abstract: Our previous *in vitro* and *in vivo* studies on standard tumor cell lines: mammary adenocarcinoma 4T1, melanoma B16F10, and squamous cell carcinoma SCCVII have demonstrated that dipotassium-trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$, affects the growth of cancer cells. Based on indicative results of its anticancer activity, that are comparable to the standard cytostatic 5-fluorouracil, we decided to analyze the antioxidant capacity of $K_2[B_3O_3F_4OH]$. In our research, we include two other simpler representatives of the boroxine family compounds: trimethoxyboroxine and trimethylboroxine, which are commercially available. The study objective is to explore the possibility of similar behavior within the same class of boron compounds, that is, to examine the activity of $K_2[B_3O_3F_4OH]$ compared to simpler representatives of the same family of compounds. On the one hand, $K_2[B_3O_3F_4OH]$, theoretically has the ability to exchange electrons in the extinction of reactive radicals, since two boron atoms are sp3-hybridized and use electrons from the inner shell. On the other hand, trimethoxyboroxine, and trimethylboroxine, in theory, should not exchange electrons. However, recent studies indicate the potential for the boron atom to act like carbon and participate in the exchange of protons. The study used the standard laboratory method of 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay. The selected boroxines were treated with a DPPH radical at a temperature of 35° C in various concentrations, and with a reaction time of one hour. Results of the DPPH test show an extremely weak antioxidant capacity exists for all investigated boroxines. When $K_2[B_3O_3F_4OH]$ was tested at high concentrations, instead of decreased color in the DPPH radicals, there was an increase in absorbance readings, which could mean that this compound acts as a pro-oxidant at higher concentrations. Future research is recommended to examine the length of reaction times needed, and whether a change in the reaction conditions would boost the antioxidant capacity of $K_2[B_3O_3F_4OH]$. Finally, future research could test the hypothesis that $K_2[B_3O_3F_4OH]$, in the absence of the expected antioxidant activity, acts as a pro-oxidant.

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Introduction

In the last 20 years, the chemistry of boron compounds has been progressively investigated because of boron's unusual characteristics. This element is at the boundary of metals and non-metals and thus, exhibits characteristics of both elemental types. Often characterized as highly toxic and as such, unacceptable in human medicine, boron compounds have been neglected and unexplored for some time. Within the family of boron compounds, boronic acids and boroxines are particularly interesting. Boroxines are compounds of the six-membered heterocyclic structure, anhydrides of boronic acids, which have a ring structure with three oxygen atoms and three boron atoms. Boroxines are prepared by dehydration of boronic acid. In some cases, the creation of a known six-membered heterocyclic ring can be achieved simply by heating the corresponding boronic acid in an anhydrous solvent, such as carbon tetrachloride or chloroform. Today, they are known as antimicrobials and enzyme inhibitors. Individual boroxines show potent antifungal activity, some anhydrides possess activity against a broad spectrum of gram-negative bacteria, which is based on inhibition of fatty acid synthesis.

Our primary interest is the halogenated boroxine (dipotassium-trioxohydroxytetrafluorotriborate), $K_2[B_3O_3F_4OH]$, originally synthesized in 1951 in the Union of Soviet Socialist Republics (USSR) by Ryss and Slutskaya (1951). Galic (2012, 2013) observed that halogenated boroxine, $K_2[B_3O_3F_4OH]$, acted on hyperkeratosis on the skin with dark-colored growths, exposed to small concentrations of $K_2[B_3O_3F_4OH]$, losing their color and irrevocably shedding from the surface of the skin. Halogenated boroxine shows high solubility and stability in water, and these features can facilitate its eventual use in medical, dermatological and cosmetic formulations. Toxicological studies have shown that $K_2[B_3O_3F_4OH]$ has low harmful effects on the health of humans and mammals. Haveric et al. (2011) investigated the antiproliferative, cytotoxic, and genotoxic potential of $K_2[B_3O_3F_4OH]$ using an alamarBlue® test on the basal cell carcinoma and lymphocyte. Islamovic et al. (2014) and Vullo et al.

[1] Department of Biochemistry, Faculty of Chemistry and Technology, University of Split, Croatia, maja.marasovic@ktf-split.hr

[2] Faculty of Chemistry and Technology, University of Split, Croatia

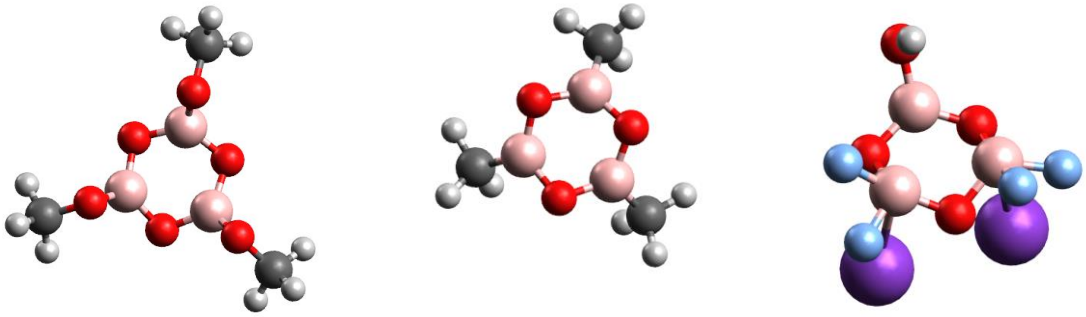
[3] Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina, borivoj.galic@gmail.com

[4] Department of Biochemistry, Faculty of Chemistry and Technology, University of Split, Croatia, mladen@ktf-split.hr

(2015) investigated the kinetic parameters and mechanism of inhibition of enzymes, catalase and human carbonic anhydrases, in the presence of $K_2[B_3O_3F_4OH]$. Ivankovic et al. (2015) showed the potent antitumor activity of $K_2[B_3O_3F_4OH]$ that is comparable to the well-known anticancer drug, 5-fluorouracil. The antitumor activity was tested *in vitro* on a number of tumor cell lines (adenocarcinoma 4T1, melanoma B16F10, and squamous cell carcinoma SCCVII) and *in vivo* on solid tumors of the same type in syngeneic mice. The study revealed high sensitivity of tumor cells with the $K_2[B_3O_3F_4OH]$, independent of the mode of application.

This study aims to determine the antioxidant capacity and antiradical activity of $K_2[B_3O_3F_4OH]$ using the standard 2,2-diphenyl-1-picrylhydrazyl (DPPH) method of antioxidant assay. The research includes two other simpler representatives of the boroxine family (Figure 1), trimethoxyboroxine ($C_3H_9B_3O_6$) and trimethylboroxine ($C_3H_9B_3O_3$), which are commercially available. The objective is to explore the possibility of similar actions within the same class of boron compounds, that is, to examine the activity of $K_2[B_3O_3F_4OH]$ compared to the simpler representatives of the same compound family.

Figure 1: 3-dimensional structures of selected boroxines



Trimethoxyboroxine ($C_3H_9B_3O_6$)

Trimethylboroxine ($C_3H_9B_3O_3$)

Halogenated boroxine ($K_2[B_3O_3F_4OH]$)

Source: Author

Data and Methodology

All chemicals used were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), trimethoxyboroxine, and trimethylboroxine were supplied by Sigma-Aldrich, Merck, Germany. The halogenated boroxine, $K_2[B_3O_3F_4OH]$, was prepared through a simple reaction between potassium hydrofluoride, KHF_2, and boric acid in the molar ratio of 2:3, at room temperature, as reported in the literature by Ryss and Slutskaya (1951).

The DPPH method was the standard method for the determination of antioxidant activity using a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$, Mw=394.32 g/mol). 2,2-diphenyl-1-picrylhydrazyl is characterized as a stable radical due to the existence of delocalized electrons that are not concentrated in one place. The electrons' influence is felt throughout the molecule, and therefore there is no dimerization. The radical system has a dark purple color. After mixing radicals with antioxidants, hydrazyl is reduced to hydrazine, donating hydrogen atoms by antioxidants to unpaired electrons of nitrogen. The reduction from hydrazyl to hydrazine occurs through the gradual loss of color, which can be observed by measuring absorbances at a wavelength of 520 nm.

$DPPH^\circ + AH \rightarrow DPPHH + A^\circ$

As discussed in more detail by Kedare and Singh (2011), while the DPPH radical readily accepts an electron or proton and becomes a stable diamagnetic molecule, its oxidation is complex and irreversible.

The DPPH method is fast, simple, inexpensive, and widely used as a reliable method of determining the ability of molecular systems that act as 'traps' for free radicals or hydrogen donors. The method is unique because samples react with the DPPH radical, which is dissolved in methanol, ethanol, or water. The measured values are comparable with the results of other methods: ferric ion reducing antioxidant power (FRAP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) etc. The advantage of this method is that the DPPH radical reacts with the entire studied molecular system. If the reaction time lasts long enough, the DPPH radical can react with weaker antioxidants, in polar and non-polar organic solvents. This allows the detection of hydrophilic and lipophilic antioxidant through the visible colorimetric change after 15–20 minutes of reaction, in a darkened room. The antioxidant efficiency is usually measured at room temperature, which reduces the risk of thermal degradation of the tested molecule. The limitation of the method is reflected in the DPPH radical reactivity towards other radicals, in that it loses the linear response curve of the standard state by varying the antioxidant:DPPH ratio. This radical is sensitive to some Lewis bases and the presence of oxygen. The method is generally accepted because the measurement results are easily reproducible and small volumes of chemicals in the slightly acidic pH range of 5.0 to 6.5 are used. The initial concentration of DPPH should give absorbance values less than one. The simplest approach to the interpretation of results is a graph showing the dependence of absorbance on the concentration of substrate.

The measurement of the DPPH radical scavenging activity was performed according to methodology described in Brand-Williams, Cuvelier and Berset (1995). The solution of 4 mg DPPH in 100 ml of H_2O was freshly prepared. The solution was diluted with H_2O until the absorbance readings were no longer detected in the area of 1.0 (± 0.2). All samples were made in the initial solution of 1 M ($K_2[B_3O_3F_4OH]$, Mw = 251.50 g mol^{-1}; trimethoxyboroxine, Mw = 173.53 g mol^{-1}, ρ = 1.195 g mL^{-1}; trimethylboroxine, Mw = 125.53 g mol^{-1}, ρ = 0.898 g/ml). Ethanol was used as the solvent for trimethoxyboroxine and trimethylboroxine, while $K_2[B_3O_3F_4OH]$ was dissolved in water. Lower sample concentrations (1 mM, 10 mM, and 100 mM) were prepared by serial dilutions of higher sample concentration. The cuvette microtiter plates were filled with 200 μL of DPPH and 10 μL of sample in various concentrations. The microtiter plate was allowed to stand in the dark, at a temperature of 35° C, for one hour, after which the absorbances were measured. The experiment was done in triplicate for each substance and each concentration. Radical scavenging activity was calculated using the following equation (1):

$$\text{Radical Scavenging Activity [\%]} = 100^* (A_{control} - A_{sample}) / A_{control} \quad (1)$$

where

$A_{control}$ is absorbance of control, and

A_{sample} is absorbance of sample

Measurements were obtained using a spectrophotometer Tecan Sunrise-Basic Tekan 2007 at the Department of Biochemistry at the Faculty of Chemical Technology, University of Split.

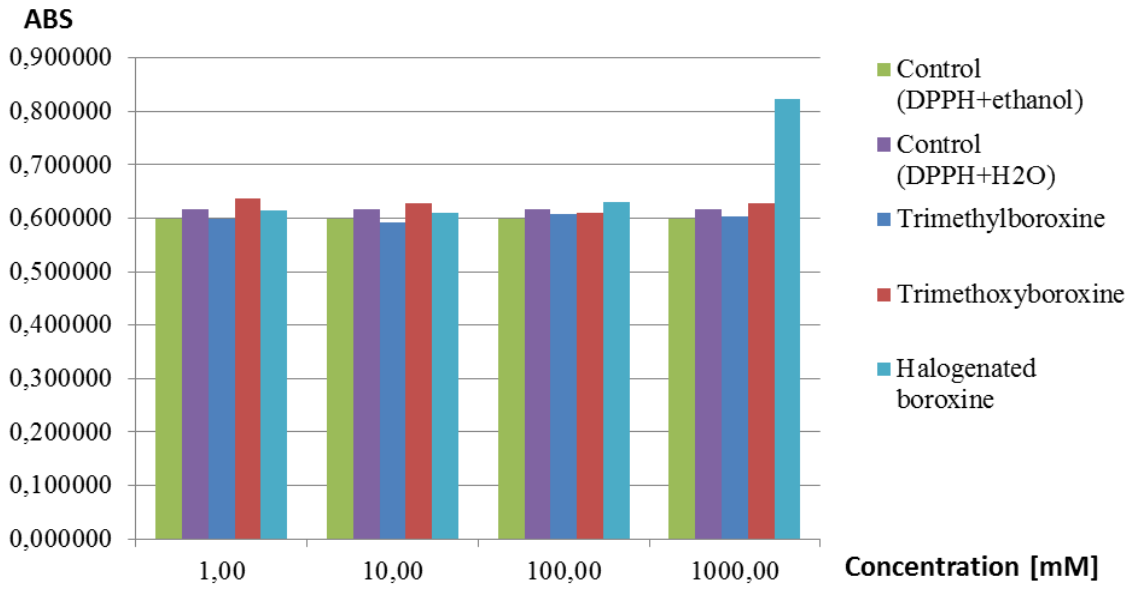
Results and Discussion

The results are shown as the dependence of absorbance readings on boroxine concentrations (Figure 2). The graph shows the mean absorbance value of samples and controls. The standard deviation expressed as a percentage of the mean value (for all measurements) was ±4.5%. Spectrophotometric test specimens themselves without DPPH radicals, showed no discoloration in a wide range around and at a wavelength of 520 nm, at which the measurements were made.

The absorbance readings of trimethylboroxine and trimethoxyboroxine show a slight color increase of DPPH radical-sample complex almost in all tested concentrations. If the formula that calculates the percentage of radical inhibition is applied to calculate the percent of increase in color of DPPH radical-sample complex, the trimethylboroxine is 0.06% to 1.45%, and the trimethoxyboroxine 1.78% to 6.51%. Results show that trimethylboroxine and trimethoxyboroxine do not act as antioxidants by the principle of giving electrons to free radicals to 'turn off' their radical activity.

Similar results were measured with halogenated boroxine $K_2[B_3O_3F_4OH]$. At lower concentrations, it showed weak antioxidant activity (from 0.38% to 0.92%). The slight color increase in the DPPH radical-sample complex occurred in the sample of 100 mM (2.33%), while at a concentration of 1 M a strong color increase (33.55%) was observed. The results indicate that the halogenated boroxine acts as a highly weak antioxidant according to the principle of surrender electrons to free radicals at lower concentrations, whereas at higher concentrations shows no antioxidant activity.

Figure 2: Measured dependence of absorbance (ABS) and the concentration of tested samples



Source: Author

The results suggest that boron atoms in the ring of trimethylboroxine and trimethoxyboroxine connect with three valence electrons from the outer shell (sp^2 hybridized form). These compounds do not include OH groups, which could indirectly release electrons to the free radicals. Carbon in a CH_3 group is less electronegative than oxygen, and there is a small possibility that the CH_3 group releases a proton, and then an electron. It was expected that trimethylboroxine and trimethoxyboroxine would exhibit insignificant antioxidant activity, although according to the results of recent studies, boron, like carbon, has the ability to create individual ions and possibly exchange rings with radicals and halt their most reactive form.

The halogenated boroxine has two boron atoms in the ring that form four links. This configuration allows the boron atoms to connect through electrons of the inner shell, which are strongly affected by the attracting force of the core, especially in small atoms such as boron. Thus, these atoms convert to sp^3 hybridized forms. Both forms of boron atoms are strongly influenced by electronegative fluorine atoms, and excitation of electrons from the inner shell provides a theoretical possibility of giving but also receiving electrons. The halogenated boroxine molecule has one OH group, which could free an electron. It was expected that the halogenated boroxine would show some antioxidant activity at lower concentrations than observed. The readings of increased color may indicate that the atoms of boron, especially in halogenated boroxine, are 'hungry' for electrons, and that instead of terminating free radicals, they continue their propagation.

The experiment with a sample of the 1-M concentration of halogenated boroxine was repeated many times with results that consistently showed a pronounced standard deviation relative to the mean of ±1.9%. We conclude that at the high concentration, the halogenated boroxine reacted with the DPPH radical in an unusual way. As the solvent for the DPPH radical and halogenated boroxine is water, it was not expected to contribute to the observed reaction. If the reaction occurred at a high concentration, then there is no reason for a weaker form repeated at lower concentrations.

In the assay, at higher concentrations the ratio between the quantity of DPPH radical and that of the sample was affected, as compared to similar tests at lower concentrations, and it is possible that this

ratio affects the results. Future studies could first determine the sensitivity of the results on reaction parameters, such as temperature, reaction time, type of solvent, and the ratio of DPPH radicals to sample, and then determine how to lessen these effects on the results.

Where the reaction parameters show no substantial effect, and since a reduction in color intensity means antioxidant activity, an increase of color intensity could indeed be interpreted as a pro-oxidant effect. Future research, in such a case, would be needed to confirm the thesis that halogenated boroxine are pro-oxidative.

According to recent literature, a pro-oxidant activity is not insignificant in the fight against cancer, because tumor cells have a particularly high sensitivity to the accumulation of free radicals in the cytoplasm. Thus, pro-oxidants are cytotoxic for the tumor cells, though not for healthy cells. Nevertheless, research into pro-oxidative properties is in the early stages, and there remains a lack of substantial evidence supporting this case.

Conclusion

The tested boroxines showed an exceedingly weak antioxidant (halogenated boroxine at lower concentrations) or no antioxidant activity. In most samples, the color absorbance increased in the DPPH radical-sample complex, especially at the 1-M concentration of halogenated boroxine. Future research is needed to investigate whether the parameters of the test reaction (reaction time, temperature, the ratio of DPPH radicals, and sample) influence the results and how this might occur, and whether the tested boroxines behave as pro-oxidants.

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