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Article

Dialkylphosphorylhydrazones as Potent Tyrosinase Inhibitors

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Dezenove dialquilfosforilidrazonas com diferentes substituintes no anel aromático foram avaliadas como inibidores potenciais de tirosinase, que poderiam ser usadas como agentes eficientes no controle de desordens de pigmentação. A atividade inibitória foi medida através de uma modificação do método de UV-Vis de Patil e Zucker. Os ensaios foram realizados com soluções contendo tampão fosfato, L-3,4-diidroxifenilalanina (L-DOPA), EDTA, tirosinase e diferentes concentrações de compostos organofosforados e a formação de dopacromona foi determinada através do monitoramento da absorvância a 475 nm. Três compostos foram os mais ativos da série (CI_{50} = 105 ± 20 μmol L^{-1}), (CI_{50} = 127 ± 16 μmol L^{-1}) e (CI_{50} = 188 ± 27 μmol L^{-1}), sendo de 3 a 7 vezes mais ativos do que o ácido ascórbico (CI_{50} = 730 μmol L^{-1}), utilizado como controle. O composto mais ativo é apenas 1,5 vezes menos ativo do que o ácido kójico (IC_{50} = 69,4 μmol L^{-1}), que é utilizado comercialmente. Esta investigação pode levar à descoberta de agentes potentes contra importantes desordens de pigmentação inclusive a hiperpigmentação.

Nineteen dialkylphosphorylhydrazones with different substituents at the aromatic ring were evaluated as potential tyrosinase inhibitors, which could then be used as efficient agents in the control of pigmentation disorders. The inhibition activity was measured by a modified Patil and Zucker UV-Vis method. Briefly, the assays were carried out with solutions containing phosphate buffer, L-3,4-dihidroxyphenylalanine (L-DOPA), (EDTA), tyrosinase and varying concentrations of organophosphorus compounds. The formation of dopachromone was determined by monitoring the absorbance at 475 nm. Three compounds were found to be the most active compounds of the series (IC_{50} = 105 ± 20 μmol L^{-1}), (IC_{50} = 127 ± 16 μmol L^{-1}) and (IC_{50} = 188 ± 27 μmol L^{-1}), being three to seven times more active than ascorbic acid (IC_{50} = 730 μmol L^{-1}), used as a standard. The most active compound is only 1.5 times less potent than the commercial kojic acid (IC_{50} = 69.4 μmol L^{-1}). This study may lead to the discovery of potent agents against very important pigmentation disorders including hyperpigmentation.

Keywords: dialkylphosphorylhydrazones, tyrosinase inhibitor, organophosphorus compounds

Introduction

Tyrosinase (systematic name, monophenol, L-dopa:oxygen oxidoreductase, recommended name, monophenol monooxygenase, EC 1.14.18.1), is a widespread enzyme, which is present in animals, plants and microorganisms, being essential for the biosynthesis of melanins.[1-4]

Tyrosinase belongs to a type-3 copper protein family harboring a catalytic center formed by dinuclear copper and catalyzes the *o*-hydroxylation of phenol and the subsequent oxidation of catechol to the corresponding quinone (Scheme 1).[5] The quinone product is a reactive precursor for the synthesis of melanin pigments. In mammals, this enzyme is responsible for skin pigmentation abnormalities, such as flecks and albinism.[6] The development and screening of potent inhibitors of tyrosinase is therefore of particular interest to the cosmetics industry.

In plants, tyrosinase catalyzes the initial reactions in the formation of melanin from tyrosine but it also oxidizes various phenolic compounds, aminoacids and proteins that promote darkening of plant tissues when damaged.[7]

In insects, tyrosinase also has a fundamental role, both in their development and for defense, in processes such as melanogenesis, tissue healing, parasite encapsulation and sclerotization.[8,9]

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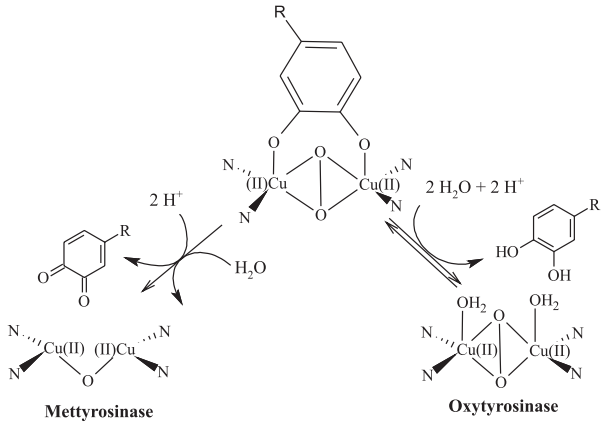


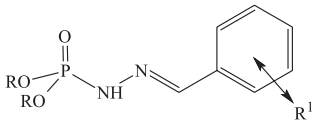
Scheme 1. Catalytic oxidation of *o*-diphenol to *o*-quinone by tyrosinase; R = alkyl group (adapted from reference 5).

Although tyrosinase plays important roles in the metabolism of animals, plants and microorganisms, some disorders are also associated with this enzyme. Alterations on the distribution of melanins give rise to a series of dermatological disorders, among which there are those resulting from excessive accumulation of skin pigmentation, such as melasma, age related spots and actinic damages. Investigations are being carried out to determine the possible association of melanin biosynthesis with malign melanomes, in the same way that pigmentation deficiency in cases of oculo cutaneous albinism is correlated to various mutations of the human tyrosinase gene.[8-10] The excessive accumulation of dopamine, which is produced in the initial steps, leads to the formation of reactive oxygen species, such as *o*-quinones, which bring about cell death.[11] The neurodegenaration associated with Parkinson's disease may be related to this effect, together with an excessive number of non pigmented neurons, *i.e.*, which do not possess melanins, which in their turn, stop to be produced due to oxidation of dopamine to reactive oxygen species.[11-13] Dopamine and its oxidation metabolites are also associated with disorders that cause vitiligo.[11]

Because of the undesirable effects either caused by or associated with tyrosinase, there has been a growing interest in compounds which may inhibit this enzyme, thus efficiently acting on pigmentation disorders. Also, cosmetic industries have shown their interest in the search for skin whiteners, food industries are interested in preventing darkening of foods and finally pesticide industries search for tyrosinase inhibitors as alternatives in the control of insect infestations.[8,14]

The interaction of organophosphorus compounds with tyrosinase has been investigated by some researchers who found that aminophosphonates are good inhibitors of that enzyme. Among these, (3,4-dihydroxyphenyl)-methylamino phosphonic acid has shown the higher inhibitory potential.[15,16]

Thus, in continuation of our research dealing with the synthesis of new organophosphorus compounds and their potential biological activities, we have screened nineteen new dialkylphosphorylhydrazones, whose syntheses are described elsewhere,[17] (Figure 1) for their tyrosinase inhibitory potential.



(**1**) R = propyl; R^1 = *o*-NO_2
(**2**) R = propyl; R^1 = *m*-NO_2
(**3**) R = propyl; R^1 = *p*-NO_2
(**4**) R = propyl; R^1 = *p*-CN
(**5**) R = propyl; R^1 = *p*-CO_2H
(**6**) R = propyl; R^1 = 3.4-OCH_2O
(**7**) R = isopropyl; R^1 = *o*-NO_2
(**8**) R = isopropyl; R^1 = *m*-NO_2
(**9**) R = isopropyl; R^1 = *p*-NO_2
(**10**) R= isopropyl; R^1 = *p*-CN
(**11**) R = isopropyl; R^1 = 3.4-OCH_2O
(**12**) R = isopropyl; R^1 = H
(**13**) R = isopropyl; R^1 = *o*-CH_3
(**14**) R = isopropyl; R^1 = *p*-OCH_3
(**15**) R = isopropyl; R^1 = *p*-$N(CH_3)_2$
(**16**) R = isopropyl; R^1 = *m*-OH
(**17**) R = isopropyl; R^1 = *p*-OH
(**18**) R = isopropyl; R^1 = *m*-OH; *p*-OCH_3
(**19**) R = isopropyl; R^1 = *p*-OH; *m*-CH_2CH_3

Figure 1. Dialkylphosphorylhydrazones assayed for tyrosinase inhibition.

Experimental

Reagents employed in the inhibitory investigation were obtained from Sigma-Aldrich.

Tyrosinase inhibition activity was measured by a modified Patil and Zucker UV-Vis method.[18] The modifications consisted on the use of a different concentration of EDTA. Also, L-DOPA was employed as a substrate and finally, commercial tyrosinase was used whereas the authors isolated the enzyme.

All the assays were carried out with solutions containing phosphate buffer (50 mmol L^{-1}, pH 8), L-DOPA (0.17 mmol L^{-1}), EDTA (0.022 mmol L^{-1}), tyrosinase (50-100 units) and varying concentrations of organophosphorus compounds and were done in triplicate. The inhibitor solutions were prepared in DMSO with an initial concentration of 10 mmol L^{-1}.

Different aliquots were added to the solution containing buffer, L-DOPA and EDTA to reach a final concentration of 660 μmol L^{-1}, the enzyme being added in the end. Formation of dopachromone was determined by monitoring the absorbance at 475 nm with a Shimadzu (Kyoto, Japan) Mini UV-Vis 1240 spectrometer. Ascorbic acid was used as standard with an initial concentration of 1 mmol L^{-1}. Percent inhibition was calculated using the following equation,

$$\% \text{ of Inhibition} = \{[(B_{30} - B_0) - (A_{30} - A_0)] / (B_{30} - B_0)\} \times 100 \quad (1)$$

where B_0 = absorbance of L-DOPA + tyrosinase at t = 0 min, B_{30} = absorbance of L-DOPA + tyrosinase at t = 30 min, A_0 = absorbance of L-DOPA + tyrosinase + inhibitor at t = 0 min, and A_{30} = absorbance of L-DOPA + tyrosinase + inhibitor at t = 30 min.

The IC_{50} values were calculated from the equation generated by exponential fit of the experimental data.

Results and Discussion

The ability of organophosphorus compounds to inhibit the conversion of L-DOPA into dopachromone, which is catalyzed by tyrosinase, was evaluated by monitoring the intensity of the absorption at 475 nm, characteristic of dopachromone. If a tyrosinase inhibitor is present, the amount of dopachromone decreases and so does the intensity of the absorption at 475 nm. A preliminary screening was performed at a maximum concentration of 660 μmol L^{-1}, which was the highest concentration where the inhibitors were soluble (Table 1).

Table 1. Percent inhibition of tyrosinase

Compound at 660 mmol L^{-1}	% Inhibition
1	19
2	22
3	0
4	25
5	90
6	7
7	14
8	64
9	0
10	21
11	37
12	74
13	14
14	7
17	33

At that concentration, compounds **5**, **8** and **12** showed the highest inhibitory potencies and were screened at different concentrations in order to obtain their corresponding IC_{50} values (Figure 2).

The IC_{50} values for **5**, **8** and **12** suggest that these dialkylphosphorylhydrazones are potent inhibitors of tyrosinase, with **5** being the most active. Comparison of the IC_{50} value for **5** with those reported in the literature for commercial products shows that **5** (IC_{50} = 105 ± 20 μmol L^{-1}) is only less active than kojic acid (IC_{50} = 69.4 μmol L^{-1}),[19] but is seven times more active than ascorbic acid (IC_{50} = 730 μmol L^{-1}). Compounds **8**

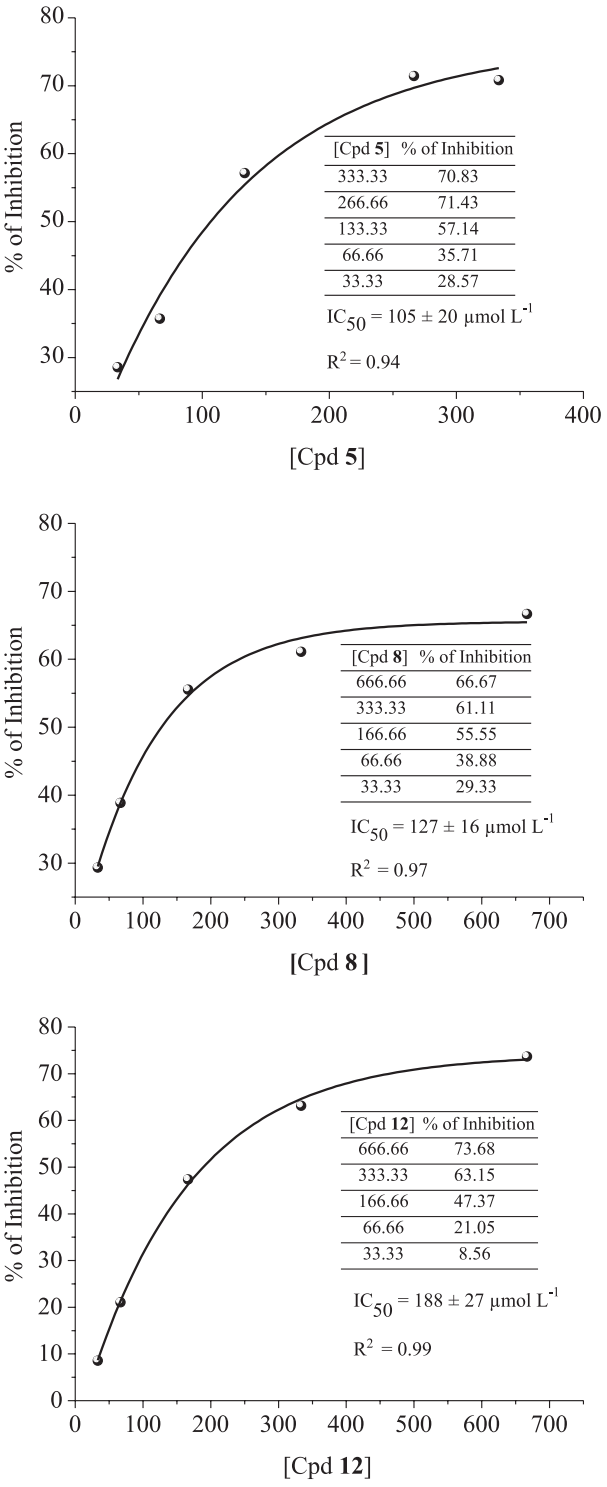


Figure 2. Tyrosinase inhibition by compounds **5**, **8** and **12**.

(IC_{50} = 127 ± 16 μmol L^{-1}) and **12** (IC_{50} = 188 ± 27 μmol L^{-1}) have also been found to be more active than ascorbic acid.

Since compound **5** was found to be the most active, the kinetics of dopachromone formation was investigated in the presence of that compound. Thus, the absorbance was monitored at 10 min intervals until 30 min at four different

concentrations. The results obtained were compared with those of solutions containing only L-DOPA and tyrosinase (Figure 3). It may be observed that, for all concentrations, after 30 min, the absorbance of the solution containing compound **5**, L-DOPA and tyrosinase is lower than the solution containing only L-DOPA and enzyme and also, as the concentration of compound **5** increases, there is a decrease in the absorbance of this solution. These results indicate an inhibition effect of compound **5** on tyrosinase.

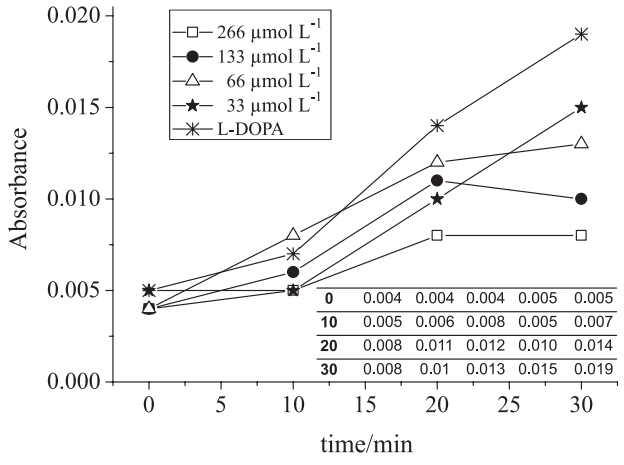


Figure 3. Kinetics of dopachromone formation.

In order to evaluate the interaction between the dialkylphosphorylhydrazones **5**, **8** and **12** with tyrosinase in the absence of L-DOPA, a set of UV-Vis curves were obtained for solutions containing only the organophosphorus compounds and another one for solutions containing both the enzyme and the organophosphorus compounds. Figure 4 shows these curves together with an experiment for L-DOPA.

As expected, the curve shows that L-DOPA interacts with tyrosinase, since the absorbance of the solution of L-DOPA plus enzyme is higher than that for only L-DOPA, evidencing a hyperchromic effect. Two new absorptions can be seen in the spectrum: one at 326 nm that can be attributed to the L-DOPA/tyrosinase complex[5] (Scheme 1) and another absorption that can be seen at 470 nm which is attributed to dopachromone that is being formed. It can be seen that both dialkylphosphorylhydrazones **5** and **8**, which possess the CO_2H and NO_2 groups respectively, interact with the enzyme in a similar way as L-DOPA, as shown in Figure 5.

On the other hand, compound **12**, which has no substituents at the aromatic ring, and a lower inhibitory

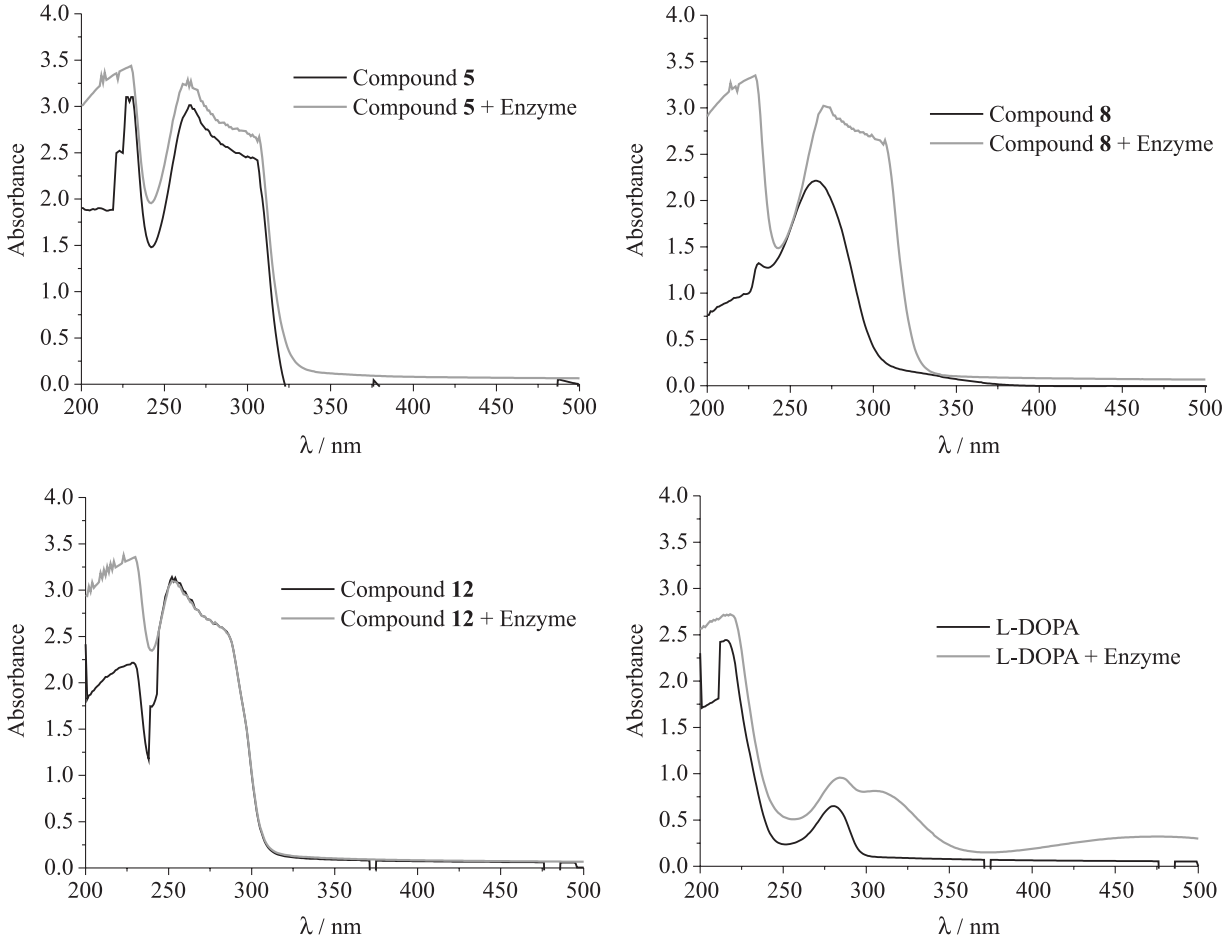


Figure 4. Evaluation of the interaction between the dialkylphosphorylhydrazones **5**, **8** and **12** with tyrosinase in the absence of L-DOPA.

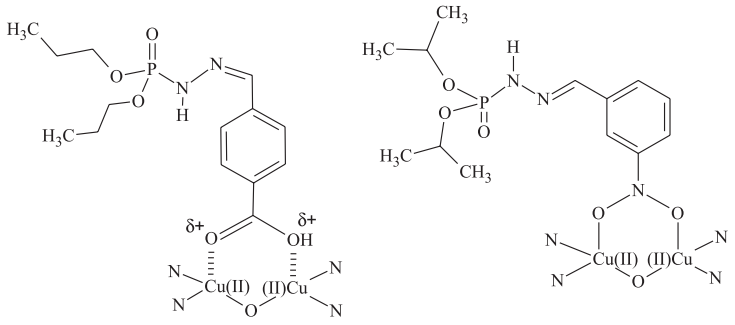


Figure 5. Suggested interaction between inhibitors (**5** and **8**) and tyrosinase.

ability, may interact with tyrosinase through both the phosphoryl moiety and the imidic nitrogen atom. Another possible way for inhibition to occur could be the interaction between the dialkylphosphorylhydrazones with L-DOPA, thus preventing the interaction between the oxidizing active site of the enzyme and the substrate. In order to investigate this possibility, the absorbance of solutions containing L-DOPA and the organophosphorus compounds **5**, **8** and **12** was monitored at 10 min intervals for 30 min (Figure 6), using ascorbic acid as standard. No significant changes in absorbance were observed, indicating that these compounds do not interact with L-DOPA.

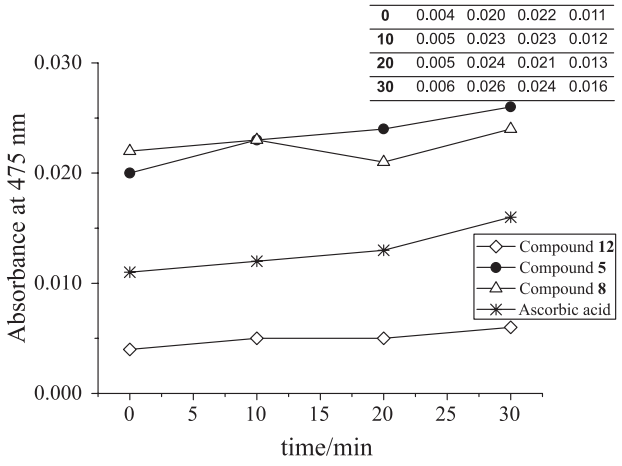


Figure 6. Absorbance of organophosphorus compounds **5**, **8**, **12** and L-DOPA (ascorbic acid as control).

Compounds **1**, **2**, **4**, **6**, **7**, **10**, **11**, **13**, **14** and **17** showed low levels of inhibition while compounds **3** and **9** do not inhibit the enzyme. Compounds **15**, **16**, **18** and **19** potentiate the oxidative power of tyrosinase since the absorbance increased for these compounds. The relative increase for these compounds at a concentration of 660 μmol L^{-1} was calculated with equation 1 and the corresponding values of 59, 22, 32 and 75% were found. A possible explanation for this result may lie in the fact that these compounds have OH, OR and NR_2 groups and it has been reported that these compounds may be oxidized leading to dpachromone-like derivatives.[20] In order to investigate if the introduction of the R^1-phenylhydrazone moiety alters the enzyme activity, dipropylphosphorylhydrazine and diisopropylphosphorylhydrazine (Figure 7), precursors of the hydrazones, were also tested for inhibitory activity against tyrosinase.

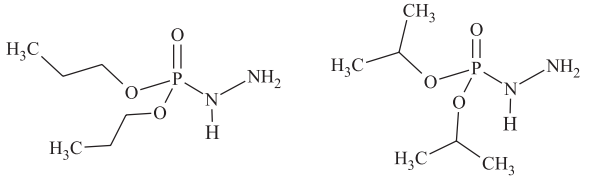


Figure 7. Dialkylphosphorylhydrazines structures.

Both hydrazines were initially tested at a concentration of 660 μmol L^{-1}. At this concentration, diisopropylphosphorylhydrazine showed an inhibition of 21% whereas dipropylphosphorylhydrazine showed 65% inhibition. In order to determine the IC_{50} value for dipropylphosphorylhydrazine, this compound was assayed at different concentrations. The relative inhibition was calculated with equation 1 for each concentration. Figure 8 shows the tyrosinase inhibition curve for dipropylphosphorylhydrazine. The higher IC_{50} value for

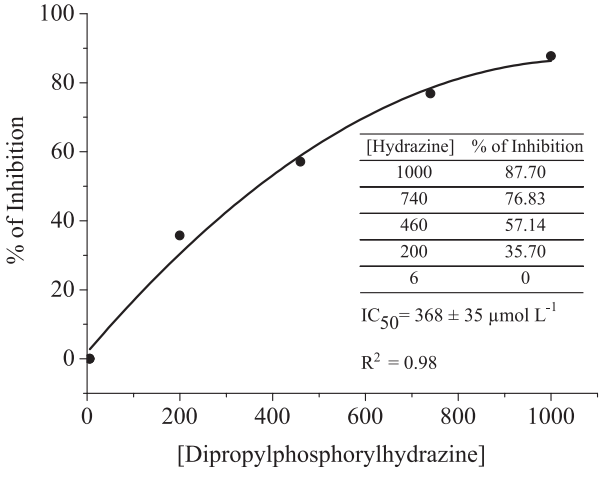


Figure 8. Inhibition of tyrosinase activity for dipropylphosphorylhydrazine.

dipropylphosphorylhydrazine when compared with those values for **5**, **8** and **12** suggests the R^1-phenylhydrazone moiety is important for inhibitory activity.

Compounds which possess the orto substituents, showed low inhibitory action, probably due to steric restrictions to binding to the enzyme.

Conclusions

Compounds **5**, **8** and **12** have been found to be more active than ascorbic acid. However, the results described above indicate that although these compounds are significantly active, unfortunately, the mechanism of inhibition of tyrosinase by the dialkylphosphorylhydrazones is dependent on more than one factor and compounds with more substitutions are needed in order to carry out a quantitative structure/activity study.

Acknowledgments

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