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Synthesis and Evaluation of 1,4-Dihydropyridine-Based Urea Derivatives as Polyphenol Oxidase Inhibitors

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Abstract: This study investigated the potential inhibitory effects of nine novel synthesized urea-substituted 1,4-dihydropyridine derivatives (DT-DEN-1-9) on polyphenol oxidase (PPO) activity. The compounds were synthesized via the Hantzsch reaction, providing a series of structurally diverse urea and thiourea-modified 1,4-dihydropyridines. Polyphenol oxidase enzyme was extracted from banana (*Musa cavendishii*) and purified using affinity chromatography with a Sepharose 4B-L-tyrosine-p-aminobenzoic acid affinity gel. The purified enzyme's activity was measured spectrophotometrically using catechol as the substrate, monitoring the increase in absorbance at 420 nm. The inhibitory effects of the synthesized compounds on PPO activity were evaluated through *in vitro* assays. Various concentrations of each compound were incorporated into the enzyme reaction mixture, and the residual PPO activity was determined. The percentage of PPO activity was calculated relative to a control reaction without inhibitors. IC_{50} values, representing the concentration of inhibitor required to reduce enzyme activity by 50%, were determined using Lineweaver-Burk plots. Among the tested compounds, DT-DEN-6, featuring a phenyl thiourea substituent, exhibited the most potent inhibition with an IC_{50} value of 100.14 µM. DT-DEN-8, containing a 2,5-dichlorophenyl thiourea moiety, also showed strong inhibitory activity with an IC_{50} below 150 µM. Structure-activity relationships were observed, with electron-withdrawing substituents generally enhancing inhibitory potency. Conversely, DT-DEN-5, bearing a 4-(trifluoromethyl)phenyl thiourea substituent, exhibited the weakest inhibition profile (IC_{50}: 233.33 µM). Our findings provide valuable insights for the design of next-generation PPO inhibitors, potentially leading to the development of novel anti-browning agents for applications in food preservation and other industries where control of enzymatic browning is crucial.

Keywords: 1,4-dihydropyridine, urea derivatives, polyphenol oxidase, inhibitory effect, Hantzsch reaction, prediction of activity spectra for substances

1. Introduction

Compounds derived from the 1,4-dihydropyridine (DHP) core possess attractive properties for drug discovery and development. Their unique chemical structure allows the DHP scaffold to serve as the foundation for a wide range of pharmaceutical agents. Through facile synthesis routes involving 1,2- and 1,4-dihydropyridine intermediates, medicinally relevant derivatives spanning therapeutic compounds and natural products such as alkaloids can be synthesized. Notably, the DHP motif underpins several approved drugs, highlighting its significance as a platform for designing bioactive molecules. Organic chemists

have devoted significant research efforts toward establishing methods for synthesizing 1,4-dihydropyridines (DHPs). Hantzsch reaction allows the preparation of dihydropyridine derivatives by multicomponent organic reaction of an aldehyde with an α,β-keto ester and a nitrogen carrier such as ammonium acetate. Some approaches utilize asymmetric synthesis or differences in chiral solubility to generate enantiopure forms. Additional methods explored include the use of nanocatalysts like graphene oxide (PdRuNi@GO), silica coated magnetite (Fe_3O_4@SiO_2), and nickel ferrite to facilitate the reactions. Investigations have also involved citric acid and various sulfuric acid derivatives as catalysts. A diversity of synthetic pathways has thus been uncovered for efficiently assembling these compounds (Ziarani et al., 2015; Demirci et al., 2016). Beyond their role as organic catalysts, DHPs produced using these synthetic methods are well-known as bioactive compounds in medicinal chemistry. These 1,4-dihydropyridine-containing medicines efficiently block the entrance of calcium ions into cells by selectively inhibiting L-type calcium channels, providing therapeutic benefits and preclinical studies have shown that 1,4-dihydropyridine-based structures have the potential to treat a variety of neurological illnesses, including Alzheimer's disease and Parkinson's disease, as well as cancer indications (Stout and Meyers, 1982). The versatile pharmacological profile of these derivatives, which can offer treatment options for a diverse range of diseases and conditions, underscores their significant pharmacological importance. Furthermore, they have been shown to possess a wide range of additional pharmacological effects such as antiarrhythmic (Bryzgalov et al., 2006), antioxidant (Viveka et al., 2015), anti-tubercular, anti-convulsant (Khoshneviszadeh et al., 2009; Trivedi et al., 2011), anti-inflammatory (Tale et al., 2013; Ulloora et al., 2013), cytotoxic (Marín-Prida et al., 2017), anti-protozoal (Nava-Zuazo et al., 2010), anti-hypertensive (Datar and Auti, 2012) and vasorelaxation properties as well as relevance to tumor-related pathways (Huang et al., 2012). These diverse bioactivities underscore the polypharmacological promise of chemical modifications targeting the DHP scaffold. Furthermore, these compounds can also function as modulators of L-type calcium channels (Teleb et al., 2017). The flexible ring system of the DHP scaffold reveals crucial structure-activity correlations. The mobile amine (NH) group in the DHP ring exerts strong pharmacological effects. Substitutions with methyl groups at positions 2 and 6 are also significant for various biological functions. Some of the main structural motifs associated with the biological activities of 1,4-DHP derivatives include ester substitutions at the 3 and 5 positions, as well as an aryl substituent at the 4 position (Triggle, 2003; Pedemonte et al., 2007). On the other hand, polyphenol oxidase (PPO), a copper-containing metalloenzyme, is present in various plant tissues, bacteria, mammals, and fungi (Diwakar et al., 2015a). It is also known as tyrosinase, catechol oxidase, or laccase, depending on its substrate selectivity and structural characteristics (Mishra and Gautam, 2016). Polyphenol oxidase, an oxidoreductase, may be found in a variety of cellular compartments, including thylakoid membranes, chloroplast lumen, mitochondria, and peroxisomes. Its extensive distribution emphasizes the importance of this metalloprotein in various species (Batista et al., 2014). Its uses include removing phenols from wastewater in industries and maintaining the quality of bread, dried fruits, tea, cocoa, canola meal, and coffee manufacturing (Lacki and Duvnjak, 1999; Diwakar et al., 2015b). Polyphenol oxidase's high substrate specificity makes it ideal for constructing sensitive biosensors with low substrate concentrations; thus, its medicinal applications include treating Parkinson's disease, vitiligo, cancer, and oral infections caused by Streptococcus sobrinus (Kamal et al., 2015). Keeping in mind that PPO produces catechols, which are important intermediates in the production of many chemicals and medicines (Vaidya et al., 2006). Polyphenol oxidase is a widely distributed enzyme that plays a crucial role in enzymatic browning, making its careful extraction and purification an essential step for further characterization and inhibition studies. Affinity-based chromatographic techniques have proven effective in obtaining high-purity PPO samples from various plant sources. For instance, Erzengin (2009) reported the purification of PPO from Jerusalem artichoke tubers using a special acid affinity gel, which resulted in a significant increase in specific activity and a high yield.

Electrophoretic examination of the purified PPO confirmed its high purity and homogeneity, demonstrating only a single protein band with a molecular weight of approximately 65 kDa (Erzengin, 2009). These affinity-based purification strategies provide a robust approach for obtaining well-characterized PPO samples suitable for evaluating the inhibitory effects of synthetic compounds. However, enzymatic browning induced by PPO poses a key challenge for the food industry because it can cause undesirable quality changes in fruits, vegetables, and other plant-based foods during handling, processing, and storage. The undesired browning affects organoleptic properties. Polyphenol oxidase catalyzes the oxidation of phenolic substrates to generate highly reactive quinones, which subsequently undergo

polymerization reactions to form brown pigments. This catalytic action of PPO underlies the enzymatic browning process (Mayer, 2006; Queiroz et al., 2008). Controlling activity is crucial for maintaining the sensory, nutritional, and economic value of these products.

The purpose of the present study is to report evaluation of potential and novel PPO inhibitors to reduce browning reactions, which are significant in industrial applications such as food processing and wastewater treatment, as well as in health-related applications. For this reason, DHP-modified urea/thiourea derivatives were studied on PPO under *in vitro* conditions. In addition to the chemical composition of the synthesized compounds, and also aimed to present computational datas. The effects of DHP modified urea/thiourea derivatives on PPO could be a significant step toward discovery of new agents in the context of industrial and health-related applications. The industrial and health-related features of these substances will serve as the foundation for future study and guide future investigations.

2. Materials and Methods

2.1. Materials

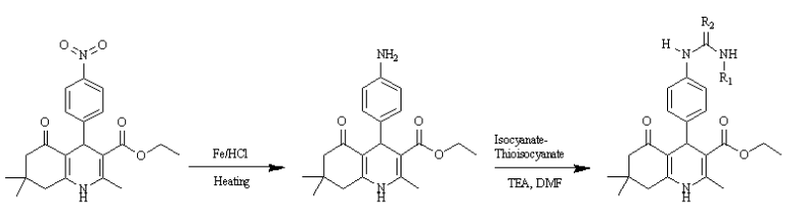
The infrared spectra have been collected using a SHIMADZU Prestige-21 (Shimadzu Corporation, Kyoto, Japan) spectrophotometer with an attenuated total reflectance (ATR). The analysis of elements was carried out utilizing a Thermo Scientific Flash 2000 (Thermo Scientific Corporation, USA) equipment with a tin pan & standard 2,5-Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT). Proton and carbon-13 Nuclear magnetic resonance spectra have been collected using VARIAN infinity plus (Varian, Inc. Agilent Technologies) 300 MHz and 75 MHz, accordingly, applying internal deuterated solvent. All of the reagents were purchased from Sigma-Aldrich as commercial company with high grade.

2.2. The synthesis method

The synthesis of novel DHP derivatives functionalized with urea/thiourea moieties (DT-DEN-1-9) was accomplished through a multi-step process (Figure 1). The synthetic method encompassed an initial Michael addition-Knoevenagel condensation sequence, followed by reduction of the nitro group, and culminating in the incorporation of urea/thiourea functionalities. This methodology yielded a series of nine structurally diverse urea-substituted DHP analogs. The molecular structures of the synthesized compounds were elucidated and confirmed through a combination of spectroscopic techniques, including proton nuclear magnetic resonance (^{1}H NMR), carbon-13 nuclear magnetic resonance (^{13}C NMR), fourier-transform infrared spectroscopy (FT-IR), and elemental analysis. The spectral data corroborated the desired structural features and functional groups. The comprehensive synthetic procedures and full spectroscopic characterization data for all compounds have been previously reported (Kaya et al., 2024).

2.3. Purification of polyphenol oxidase

Under carefully controlled conditions at 25 °C, a refined extraction procedure, inspired by the works of Wesche-Ebeling and Montgomery (Wesche-Ebeling and Montgomery, 1990), was executed. Following a thorough triple-wash with distilled water, 50 grams of banana were rapidly transformed into thin slices. These obtained slices underwent vigorous homogenization in a waring blender for precisely two minutes, utilizing 100 mL of a (0.1 M) phosphate buffer solution (pH= 7.3) supplemented with 5% polyethylene glycol and ascorbic acid (10 mM). The resulting homogenate was filtered through muslin cloth, followed by centrifugation at 15,000 g for 30 minutes to obtain the supernatant. To isolate crude proteins, a precipitation method was employed using ammonium sulfate [$(NH_4)_2SO_4$] to achieve 80% saturation. The obtained precipitate was



Compound	R_1	R_2	R_1	R_2	R_2
DT-DEN-1	Phenyl	O	DT-DEN-6	Phenyl	S
DT-DEN-2	4-F Phenyl	O	DT-DEN-7	3-OCH_3- Phenyl	S
DT-DEN-3	3-OCH_3- Phenyl	O	DT-DEN-8	2,5-Dichloro- Phenyl	S
DT-DEN-4	4-NO_2- Phenyl	S	DT-DEN-9	NH_2	O
DT-DEN-5	4-CF_3- Phenyl	S			

Figure 1. The synthesis of novel compounds (DT-DEN-1-9)

resuspended in a minimal volume of phosphate buffer (5 mM) solution before undergoing overnight dialysis against the same buffer. The resulting enzyme solution was then introduced onto a pre-equilibrated Sepharose 4B-L-tyrosine-p-aminobenzoic acid affinity column (Arslan et al., 2004) using a phosphate buffer (5 mM) solution (pH= 5.0). After thoroughly washing the affinity gel with the aforementioned buffer, the protein was successfully eluted using a solution of (1 M) NaCl and phosphate buffer (5 mM) (pH= 7.0).

2.4. Determination and quantification of enzyme activity

To quantitatively assess PPO enzyme activity, catechol was employed as the substrate. The enzymatic reaction was monitored using a Biotek automated recording spectrophotometer, which tracked the increase in absorbance at a wavelength of 420 nm, following the protocol established by Chilaka et al. (2002). Enzyme activity was calculated by analyzing the linear region of the absorbance curve. In accordance with the methodology reported by Arslan et al. (2004), one unit of activity was defined as the amount of enzyme required to cause an increase in absorbance of 0.001 units per min per mL of enzyme solution, under controlled conditions at 25 °C. This standardized approach allows for precise quantification of PPO activity and facilitates comparison with other studies in the field. The spectrophotometric measurements were conducted in triplicate to ensure reproducibility, and the mean values were used for subsequent calculations. This method provides a reliable and sensitive means of assessing PPO activity, which is crucial for understanding the enzyme's kinetics and its potential applications in various biological and industrial processes.

2.5. The inhibition studies on activity

The investigation into the activation profile of PPO involved incorporating various concentrations of synthesized urea derivatives into the enzyme reaction medium. Polyphenol oxidase activity in the absence of these test molecules was established as the baseline and designated as 100%. Subsequently, regression analysis was rigorously employed to quantify the percentage activity values of PPO across a spectrum of concentrations for each (DT-DEN-1-9) urea compound. To estimate IC_{50} values (the concentration that inhibits enzyme velocity by 50%), Lineweaver-Burk plots were constructed, adhering to the methodology outlined by Lineweaver and Burk (1934).

2.6. The prediction of activity spectra for substances

The prediction of activity spectra for substances (PASS) tool employs a Bayesian approach to predict the biological activity of novel compounds by comparing their structures to known active substrates in its database. The tool generates Pa:Pi (probability of active : probability of inactive) ratios at prediction thresholds of Pa > 30%, Pa > 50%, and Pa > 70%. The average prediction accuracy is approximately 95%, as determined by leave-one-out cross-validation (LOO CV). The prediction of activity spectra for substances can rapidly analyze the biological activity spectra for up to 1000 compounds, based solely on their 2D structures. However, it's important to note that these predictions are not definitive and do not account for molecular energy levels. The accuracy of predictions relies heavily on the comprehensive biological activity data available in the PASS training set. The 2D structures of newly synthesized molecules were drawn using ChemDraw version 16, saved as Mol files (*.mol), and uploaded to the PASS prediction website.

3. Results and Discussion

The chemical structure and structural characteristics of these urea/thiourea-substituted DHP derivatives have been extensively documented in the academic literature. The DHP-modified urea/thiourea derivatives used in this study showed quite strong inhibitory effects as seen in Table 1. In fact, the molecules whose *in vitro* effects were investigated in this study showed almost all better inhibitory effects than the 3,4-dihydroxy-N-(3,4,5-trihydroxybenzyl)benzamide (Cho et al., 2006) and salicylaldoxime (Ben-Shalom et al., 1977) molecules, which are the best inhibitors known in the literature, as clearly stated in Table 1. Interestingly, beyond their significant PPO inhibitory capabilities, these compounds demonstrated remarkable efficacy as rabbit muscle pyruvate kinase activators in our previous study (Kaya et al., 2024). The PASS is a widely used tool for predicting various aspects of chemical compounds, including physical, chemical, biological, and toxicological properties. It utilizes the quantitative structure-activity relationship (QSAR) method, generating predictive models from an extensive database of chemicals and their attributes (Filimonov et al., 2014). In PASS, "Pa" represents the probability of a compound being active, while "Pi" denotes the probability of it being inactive. Table 2 presents the PASS predictions for DT-DEN-1-9 compounds, encompassing a range of

Table 1. The obtained IC_{50} values of the DT-DEN-1-9 compounds

Compound	IC_{50} (μM)
DT-DEN-1	171.33±9.66
DT-DEN-2	288.80±19.97
DT-DEN-3	239.60±6.45
DT-DEN-4	183.87±6.86
DT-DEN-5	419.13±15.62
DT-DEN-6	100.14±3.14
DT-DEN-7	212.80±0.82
DT-DEN-8	144.90±0.82
DT-DEN-9	233.33±2.50
3,4-dihydroxy-N-(3,4,5-trihydroxybenzyl)benzamide	280
Salicylaldoxime	430

biological activities. While our previous discussions centered on PPO inhibition, the table illustrates the compounds' potential for diverse therapeutic applications. Antihypertensive (blood pressure-lowering) activity is the most frequently predicted, with most compounds showing Pa values above 0.45, indicating a moderate to high likelihood of this action.

To examine the inhibitory activity against banana-purified PPO, nine synthetic DHP-modified urea/thiourea derivatives (DT-DEN-1-9) were systematically assessed. Lineweaver-Burk plots were carefully constructed to determine the IC_{50} values, which are comprehensively reported in Table 1. Among this intriguing compounds, DT-DEN-6, with a phenyl thiourea substituent, emerged as the most potent inhibitor, boasting an impressive IC_{50} value of 100.14 μM as seen in Table 1. Notably, DT-DEN-8, featuring a 2,5-dichlorophenyl thiourea moiety, also displayed remarkable activity, achieving an IC_{50} value below 150 μM (Table 1). These observations suggest a propensity for enhanced inhibitory activity by electron-withdrawing substituents, such as the chlorine atoms present in DT-DEN-8 derivative. DT-DEN-3, bearing a 3-methoxyphenyl urea substituent, had an IC_{50} of 183.87 μM as shown in Table 1, indicating that electron-donating groups may also contribute to potency. In contrast, DT-DEN-5, with a 4-(trifluoromethyl)phenyl thiourea substituent, exhibited the weakest inhibitory effect (IC_{50}: 233.33 μM, Table 1), suggesting that strongly electron-withdrawing trifluoromethyl substituents may be detrimental to activity. DT-DEN-1, DT-DEN-2, and DT-DEN-7, containing un/mono-substituted phenyl urea/thiourea scaffolds, displayed moderate activities. Overall, the present results highlight the importance of substituents in modulating the potency. The structure-activity relationships observed provide valuable insights for designing second-generation DHP inhibitors, with DT-DEN-6 and DT-DEN-8 emerging as promising lead candidates. Further optimization of the substituents, linkers, and core scaffold may yield

Table 2. Potential activity type predictions of synthesized (DT-DEN-1-9) candidates via PASS

Compound	Pa	Pi	Activity
DT-DEN-1	0.507	0.020	Antihypertensive
	0.535	0.118	Antieczematic
	0.504	0.065	Gastrin inhibitor
DT-DEN-2	0.469	0.026	Antihypertensive
	0.398	0.004	Follicle-stimulating hormone agonist
	0.378	0.013	Urologic disorders treatment
DT-DEN-3	0.479	0.024	Antihypertensive
	0.424	0.003	Follicle-stimulating hormone agonist
	0.564	0.149	Gluconate 2-dehydrogenase (acceptor) inhibitor
DT-DEN-4	0.527	0.017	Antihypertensive
	0.476	0.041	Polarisation stimulant
	0.389	0.004	Follicle-stimulating hormone agonist
DT-DEN-5	0.457	0.028	Antihypertensive
	0.386	0.004	Follicle-stimulating hormone agonist
	0.362	0.030	Hepatic disorders treatment
DT-DEN-6	0.498	0.021	Antihypertensive
	0.431	0.003	Follicle-stimulating hormone agonist
	0.377	0.023	Antifertility, female
DT-DEN-7	0.470	0.025	Antihypertensive
	0.410	0.004	Follicle-stimulating hormone agonist
	0.547	0.163	Gluconate 2-dehydrogenase (acceptor) inhibitor
DT-DEN-8	0.449	0.029	Antihypertensive
	0.377	0.004	Follicle-stimulating hormone agonist
	0.327	0.019	Urologic disorders treatment
DT-DEN-9	0.500	0.006	Antineoplastic (melanoma)
	0.423	0.003	Follicle-stimulating hormone agonist
	0.441	0.030	Antihypertensive

highly potent and selective inhibitors of PPO. In conclusion, these findings provide valuable insights for the development of next-generation DHP inhibitors of PPO. Compounds DT-DEN-6 and DT-DEN-8 emerge as promising lead candidates due to their notable inhibitory potency. Further optimization of substituents, linkers, and the core DHP scaffold may yield highly potent and selective PPO inhibitors. Such optimized compounds could potentially surpass the efficacy of current alternatives, paving the way for improved applications in various fields where PPO inhibition is crucial.

4. Conclusions

In conclusion, this study explored the potential of novel DHP compounds substituted with urea or thiourea as PPO inhibitors. DT-DEN-6 emerged as the most effective inhibitor, demonstrating a significant IC_{50} value, due to its phenyl thiourea substituent. The effects of DT-DEN-6 on PPO could be a crucial step toward the discovery to reduce browning reactions in industrial applications, wastewater treatment and in health-related applications. The PPO- enhancing properties of this chemical may encourage the development of new industrial candidates for applications. Simultaneously, the features of DT-DEN-6 may warrant more investigation, indicating possibilities for future research. In conclusion, the potential significance and applications of the molecular interactions demonstrated by DT-DEN-6 with PPO suggest that it has exciting potential in the field of industry, environmental impact, health-related research and development. These findings may contribute to the discovery of new avenues in areas such as health, environment and especially food industry research and provide the basis for future studies.

Ethical Statement

The authors declare that ethical approval is not required for this research.

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Declaration of Author Contributions

Conceptualization, Material, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, *M.O. KAYA*; Material, Investigation, Data Curation, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing, *T. DEMİRCİ*; Investigation, Data Curation, Formal Analysis, *H.İ. TAŞ*; Investigation, Data Curation, Formal Analysis, *Ş. KARAYAĞIZ*; Investigation, Data Curation, Formal Analysis, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, *A.B. MUSATAT*; Investigation, Data Curation, Formal Analysis, Writing-Original Draft Preparation, *Y. KAYA*; Investigation, Data Curation, Formal Analysis, *M.N. KERİMAK ÖNER*; Investigation, Data Curation, Formal Analysis, *O. ÖZDEMİR*; Methodology, Data Curation, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing, *M. ARSLAN*. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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