

Enzymatic Oligomerization of Ampyrone- and Phenylhydrazine-Based Monomers: Structural, Thermal, and Antioxidant Functional Analysis

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Abstract

In this study, 2,4-dihydroxybenzaldehyde was subjected to separate condensation reactions with phenylhydrazine and ampyrone (4-amino antipyrine), yielding a hydrazine-based monomer (M-PHy) and an azomethine monomer (M-Amp), respectively. To address the structural and functional limitations of conventional Schiff base derivatives, both monomers were subsequently polymerized via horseradish peroxidase/hydrogen peroxide (HRP/H₂O₂)–mediated enzymatic oxidative oligomerization, affording two novel oligomeric products, O-PHy and O-Amp. The chemical structures of all synthesized compounds were confirmed by FT-IR, UV-Vis, and ¹H NMR spectroscopic analyses. Molecular weight distributions of the oligomers were determined using gel permeation chromatography (GPC). Thermal properties were evaluated by thermogravimetric analysis (TGA), revealing initial decomposition temperatures of 178 °C for O-PHy and 192 °C for O-Amp, with residual masses of 16.5% and 15.6% at 1000 °C, respectively. Surface morphologies of the oligomers were examined by scanning electron microscopy (SEM), demonstrating distinct microstructural features associated with their molecular architectures. These findings highlight the successful synthesis, structural verification, and physicochemical characterization of two new enzymatically derived oligomers. Crucially, the antioxidant performance evaluated by DPPH assay demonstrated a remarkable functional enhancement upon enzymatic polymerization; the radical scavenging activity escalated from 8.83% ± 0.51% (M-PHy) to 54.65% ± 1.22% (O-PHy), and from 39.74% ± 2.28% (M-Amp) to 62.82% ± 0.91% (O-Amp). These results highlight that the HRP-mediated oligomerization effectively extends the conjugated system, significantly boosting both thermal stability and antioxidant potential for advanced material applications.

Keywords

HRP/H₂O₂,
Enzymatic
reaction,
Oligomerization,
Hydrazine,
Ampyrone,
Macromolecules.

Ampiyon ve Fenilhidrazin Tabanlı Monomerlerin Enzimatik Oligomerizasyonu: Yapısal, Termal ve Antioksidan Fonksiyonel Analizi

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Özet

Bu çalışmada, 2,4-dihidroksibenzaldehit, fenilhidrazin ve ampiron (4-amino antipirin) ile ayrı ayrı kondenzasyon reaksiyonlarına tabi tutularak sırasıyla hidrazin-türevli bir monomer (M-PHy) ve bir azometin monomeri (M-Amp) elde edilmiştir. Geleneksel Schiff bazı türevlerinin yapısal ve işlevsel sınırlamalarını gidermek için, her iki monomer daha sonra yaban turpu peroksidazi/hidrojen peroksit (HRP/H₂O₂) aracılı enzimatik oksidatif oligomerizasyon ile polimerleştirilmiş ve iki yeni oligomerik ürün, O-PHy ve O-Amp, sentezlenmiştir. Tüm sentezlenen bileşiklerin kimyasal yapıları FT-IR, UV-Vis ve ¹H NMR spektroskopik analizleri ile doğrulanmıştır. Oligomerlerin moleküler ağırlık dağılımları jel geçirgenlik kromatografisi (GPC) kullanılarak belirlenmiştir. Termal özellikler termogravimetrik analiz (TGA) ile değerlendirilmiş; O-PHy için 178 °C ve O-Amp için 192 °C başlangıç bozunma sıcaklıkları ile sırasıyla 1000 °C'de %16,5 ve %15,6 artış kütle değerleri elde edilmiştir. Oligomerlerin yüzey morfolojileri taramalı elektron mikroskobu (SEM) ile incelenmiş ve moleküler yapılarıyla ilişkili belirgin mikroyapısal özellikler gözlemlenmiştir. Bu bulgular, iki yeni enzimatik olarak üretilmiş oligomerin başarılı sentezini, yapısal doğrulamasını ve fizikokimyasal karakterizasyonunu ortaya koymaktadır. Önemli bir bulgu olarak, DPPH analiziyle değerlendirilen antioksidan performans, enzimatik polimerizasyonun ardından dikkat çekici bir fonksiyonel artış göstermiştir; radikal giderme aktivitesi M-PHy için %8,83 ± %0,51'den O-PHy için %54,65 ± %1,22'ye, M-Amp için ise %39,74 ± %2,28'den O-Amp için %62,82 ± %0,91'e yükselmiştir. Bu sonuçlar, HRP aracılı oligomerizasyonun konjuge sistemi etkili bir şekilde genişlettiğini; böylece ileri malzeme uygulamaları için hem termal kararlılığı hem de antioksidan potansiyeli önemli ölçüde artırdığını vurgulamaktadır.

Anahtar kelimeler

HRP/H₂O₂,
Enzimatik
reaksiyon,
Oligomerizasyon,
Hidrazin,
Ampiron,
Makromoleküller

1. INTRODUCTION

Hydrazine-based compounds constitute an important class of organic molecules due to their versatile reactivity, strong nucleophilic character, and ability to form stable hydrazine linkages with carbonyl-containing substrates. These structural features have enabled hydrazine derivatives to play key roles in medicinal chemistry, coordination chemistry, sensing applications, and the design of functional materials [1], [2], [3]. Among carbonyl-amine condensation products, Schiff bases represent a particularly valuable family of compounds, widely recognized for their structural tunability, metal-binding capacity, and broad spectrum of physicochemical properties. Their imine ($-C=N-$) functionality not only imparts electronic conjugation but also provides a robust platform for constructing supramolecular assemblies, catalysts, and polymeric architectures [4]. Among various phenolic aldehydes, 2,4-dihydroxybenzaldehyde stands out as a highly promising building block due to its unique structural features, possessing multiple active hydroxyl groups that impart outstanding potential for bioactivity and antioxidant capacity [5]. The literature reports that hydroxybenzaldehydes and their derivatives exhibit potent radical-scavenging and enzyme-inhibitory activities that are highly effective at preventing oxidative stress [6]. This high antioxidant efficiency is primarily attributed to the presence of the phenolic hydroxyl groups, which readily donate hydrogen atoms to stabilize free radicals. However, the direct use of 2,4-dihydroxybenzaldehyde in its monomeric form is often limited by its low thermal stability and poor long-term performance under demanding conditions. To overcome these limitations, converting this dihydroxy-substituted unit into polymeric or oligomeric architectures via green chemistry pathways—such as enzymatic oxidative polymerization—has emerged as a highly effective strategy [7]. This approach not only preserves but significantly enhances the baseline antioxidant potential of 2,4-dihydroxybenzaldehyde by extending the conjugated system, while simultaneously boosting its thermal stability and radical-stabilizing performance [8]. Ampyrone (4-amino antipyrine), a heterocyclic pyrazolone derivative, has long been utilized in analytical chemistry, pharmaceutical formulations, and chromogenic reactions due to its electron-rich aromatic system and reactive amino group [9], [10]. Its ability to participate in condensation and oxidative coupling reactions makes it an attractive monomeric precursor for the synthesis of functional azomethine-based materials [11]. In recent years, enzymatic oxidative polymerization has emerged as a sustainable and biologically inspired alternative to conventional chemical polymerization methods. Horseradish peroxidase (HRP), in the presence of hydrogen peroxide (H_2O_2), catalyzes the formation of phenoxy radicals under mild, aqueous, and environmentally benign conditions [12]. This HRP/ H_2O_2 system has been successfully applied to the polymerization of various phenolic and aromatic substrates, offering advantages such as high selectivity, low energy demand, and avoidance of toxic oxidants [13]. The approach mimics natural oxidative coupling pathways found in lignin biosynthesis and other

biological polymer formation processes, underscoring its relevance as a green synthetic strategy [12], [14]. Within this context, inspired by the well-documented antioxidant and inhibitory profiles of natural phenolic aldehydes such as vanillin, the design of new hydrazine- and azomethine-based monomers and their transformation into oligomeric materials via enzymatic oxidative pathways presents a promising route to generating functional macromolecules. The present study focuses on the synthesis of two novel monomers derived from 2,4-dihydroxybenzaldehyde—one obtained via condensation with phenylhydrazine and the other with ampyrone—and their subsequent HRP/ H_2O_2 mediated oligomerization. By evaluating their structural, thermal, and morphological properties, we elucidate the relationship between monomer architecture and enzymatically driven polymer formation. Crucially, this work also aims to demonstrate how eco-friendly enzymatic polymerization can significantly enhance the baseline antioxidant potential of these benzaldehyde-derived systems, offering a highly efficient strategy for developing advanced materials with enhanced thermal stability and radical-scavenging performance.

2. MATERIAL AND METHOD

2.1. Materials

All chemicals used in this study were commercially obtained and used without any further purification. As the primary components for the reaction system, 2,4-dihydroxybenzaldehyde (Thermo Scientific), phenylhydrazine, and 4-aminoantipyrine (ampyrone) (Sigma-Aldrich) were employed. To establish oxidative conditions, hydrogen peroxide (H_2O_2 , ISOLAB) was used together with horseradish peroxidase (HRP) enzyme (Beyotime Biotech). Phosphate buffer solution (Merck) was utilized for pH control and buffering of the reaction medium. As organic solvents, ethanol and dioxane (Merck) were used throughout the experiments.

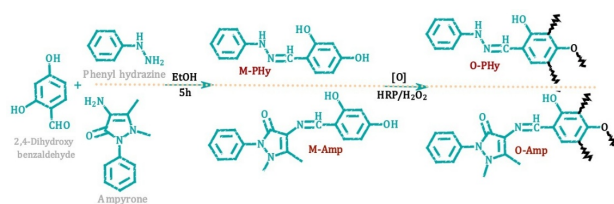
2.2. Synthesis of the Hydrazine and Ampyrone-Based Monomers (M-PHy, M-Amp)

In a two-necked round-bottom flask equipped with a reflux condenser, 0.001 mol of phenylhydrazine was dissolved in 20 mL of ethanol and heated to 80 °C for 10 minutes. A separate solution containing 0.001 mol of 2,4-dihydroxybenzaldehyde in 15 mL of ethanol was then added dropwise to the reaction flask under continuous stirring. Upon addition, an immediate color change was observed, indicating the formation of the hydrazine structure. The reaction mixture was refluxed for 5 hours, after which it was transferred into a Petri dish, and the solvent was allowed to evaporate slowly. The resulting solid was washed sequentially with distilled water and hexane to remove unreacted starting materials and impurities. The purified hydrazine-derived monomer (M-PHy) was dried overnight in an oven at 45 °C to obtain the product in solid form [15]. In a similar procedure, 0.001 mol (1.8921 g) of ampyrone (4-aminoantipyrine) was dissolved in 20 mL of ethanol in a two-necked round-bottom flask fitted with a reflux condenser and heated at 80 °C for 10 minutes. A solution of 0.001 mol of

2,4-dihydroxybenzaldehyde prepared in 15 mL of ethanol was added dropwise from a separate beaker. A visible color change occurred upon addition, yielding a yellowish reaction mixture characteristic of azomethine formation. The mixture was refluxed for 5 hours, then poured into a Petri dish to allow solvent evaporation. The obtained solid was washed with distilled water followed by hexane to remove residual impurities. The product was dried overnight at 45 °C, affording the azomethine monomer (M-Amp) in pure form [15].

2.3. Synthesis of the Hydrazine and Ampyrone-Based Oligomers (O-PHY, O-Amp)

For the enzymatic oxidative oligomerization, predetermined amounts of the synthesized monomers (M-PHY and M-Amp) were weighed and transferred into separate beakers. Each monomer sample was dissolved in 30 mL of dioxane and magnetically stirred for 15 minutes to ensure complete dissolution. In a separate container, 24 mg of horseradish peroxidase (HRP) was dissolved in 60 mL of phosphate buffer to prepare an enzyme stock solution. From this stock, 15 mL of the HRP solution was added to each monomer mixture, followed by an additional 15 minutes of stirring to allow proper enzyme–substrate interaction [16]. After this pre-activation period, 10 mL of 30% hydrogen peroxide (H_2O_2) was introduced into each reaction mixture to initiate oxidative coupling. The oligomerization reactions were carried out for different durations to monitor the effect of reaction time: the first mixture was stirred for 4 hours. In each case, the formation of dark-colored precipitates indicated successful oxidative oligomer formation [17]. The solid products were collected by filtration using filter paper and washed thoroughly with hexane to remove residual impurities and unreacted components. The resulting oligomers (O-PHY and O-Amp, obtained at different reaction times) were dried overnight in an oven at 45 °C, yielding the final purified materials.



Scheme 1. Synthesis of Hydrazine and Ampyrone-Based Monomers and Oligomers

2.4. Characterization Techniques

The structural features of the synthesized monomers and oligomers were examined using a range of spectroscopic and analytical techniques. FT-IR spectra were collected on a PerkinElmer Spectrum Two spectrometer over the 400–4000 cm^{-1} region to identify characteristic functional groups. UV-Vis measurements were carried out at ambient temperature using sample solutions prepared in THF at a concentration of 6×10^{-4} M, and recorded with an Agilent 8453 Diode Array Spectrophotometer within the 200–700 nm wavelength range. Proton nuclear magnetic resonance analyses were performed using an Agilent Premium Compact 14.1 T (600 MHz) NMR

spectrometer, employing DMSO-d_6 as the solvent to confirm the chemical structures of the monomers. The molecular weight distributions of the oligomeric products were determined by gel permeation chromatography (GPC) using a Malvern Viscotek GPCmax system. Thermal behavior was evaluated through thermogravimetric and differential thermogravimetric analyses (TG/DTA) using a PerkinElmer TGA 8000 instrument under a nitrogen atmosphere. Measurements were conducted from 30 to 1000 °C at a heating rate of 10 °C/min to assess decomposition profiles and residual mass. Surface morphology and microstructural features were investigated using a Carl Zeiss Sigma 300 VP scanning electron microscope (SEM) after sputter-coating the samples with a thin Au/Pd layer to enhance conductivity.

3. RESULTS AND DISCUSSION

3.1. Structural Characterization

The FT-IR spectrum of O-PHY exhibits several characteristic absorption bands confirming the successful formation of the hydrazine-based oligomer. A distinct N–H stretching vibration appears at 3540 cm^{-1} , consistent with the presence of hydrazine-derived functional groups. A broad absorption band spanning 3500–3100 cm^{-1} corresponds to O–H stretching vibrations [18], indicating phenolic hydroxyl groups retained within the oligomeric structure. The imine functionality formed through condensation is evidenced by the $\text{CH}=\text{N}$ stretching band at 1672 cm^{-1} , a key marker of hydrazine formation [19]. Additionally, the band observed at 1274 cm^{-1} is attributed to C–O–C stretching, suggesting the presence of ether-type linkages generated during oxidative coupling [20]. These spectral features collectively confirm the successful enzymatic oligomerization of the M-PHY monomer. The FT-IR spectrum of O-Amp displays a broad O–H stretching band in the 3440–3100 cm^{-1} region, characteristic of phenolic hydroxyl groups and hydrogen-bonded structures within the oligomer. A strong absorption at 1695 cm^{-1} corresponds to the $\text{C}=\text{O}$ stretching vibration of the pyrazolone ring, confirming the retention of the heterocyclic core after oligomerization [21]. The imine group formed during condensation is indicated by the $\text{CH}=\text{N}$ stretching band at 1621 cm^{-1} [22]. Aromatic skeletal vibrations appear at 1470 and 1420 cm^{-1} , consistent with the conjugated aromatic framework of the ampyrone-derived structure. The C–O–C stretching band at 1250 cm^{-1} further supports the formation of ether linkages during HRP/ H_2O_2 -mediated oxidative coupling. Additional bands at 1010 cm^{-1} (C–N stretching) and 805 cm^{-1} (N–N vibration) provide further evidence of the azomethine and pyrazolone functionalities within the oligomeric backbone [16]. Together, these spectral features (Figure 1) confirm the structural integrity of the azomethine monomer and its successful transformation into the O-Amp oligomer through enzymatic oxidation.

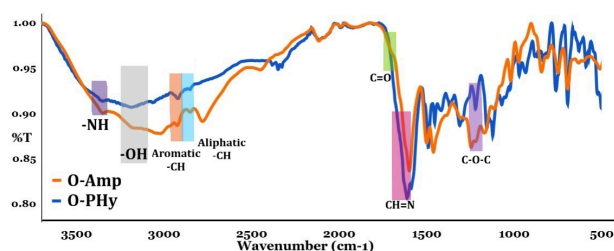


Figure 1. FT-IR spectra of M-PHy monomer and O-PHy oligomer synthesized via HRP-mediated oxidation ($4000\text{--}400\text{ cm}^{-1}$)

The ^1H NMR spectrum of M-Amp displays all proton environments expected for the target structure. The phenolic --OH proton appears as a distinct singlet at 9.53 ppm, while the imine proton (CH=N) is observed at 9.40 ppm, consistent with the deshielding effect of the azomethine functionality[23]. The N-methyl group resonates as a singlet at 3.15 ppm, and the aliphatic C-CH_3 protons are detected at 2.46 ppm. Aromatic protons are distributed between 7.50–6.82 ppm as multiplets, reflecting the substituted aromatic ring system[24]. The integral values correspond well to the expected proton count, confirming the structural integrity and purity of the compound. The ^1H NMR spectrum of O-Amp exhibits characteristic resonances consistent with the proposed structure. The phenolic --OH proton appears as a singlet at 9.89 ppm, and the azomethine proton (CH=N) is observed at 9.52 ppm in the downfield region. The N-methyl group gives a singlet at 2.47 ppm, while the C-CH_3 protons resonate at 2.31 ppm. Aromatic protons appear as multiplets between 7.47–6.29 ppm, in agreement with the substitution pattern of the aromatic ring. The integral values match the theoretical proton distribution, supporting the successful synthesis and high purity of the compound [25], [26]. The ^1H NMR spectrum of M-PHy exhibits all characteristic resonances consistent with the proposed structure. The --NH proton appears as a downfield singlet at 10.24 ppm, while the phenolic --OH proton is observed at 10.19 ppm, both reflecting strong deshielding effects due to hydrogen bonding and the electron-withdrawing environment. The azomethine proton (CH=N) resonates at 9.72 ppm as a distinct singlet. Aromatic protons are distributed between 7.78–6.40 ppm as multiplets, corresponding to the substituted phenyl ring system. The overall chemical shift pattern and integrals confirm the successful formation and structural integrity of M-PHy [27], [28]. The ^1H NMR spectrum of O-PHy clearly supports the expected molecular structure. The --NH proton resonates at 10.21 ppm, and the phenolic --OH proton appears at 9.89 ppm, both showing characteristic downfield shifts. The azomethine proton (CH=N) is detected at 8.90 ppm, consistent with the imine functionality. Aromatic protons appear as multiplets in the 7.88–6.19 ppm region, in agreement with the substitution pattern of the aromatic ring. The observed chemical shifts and integrals collectively verify the formation and purity of O-PHy [26].

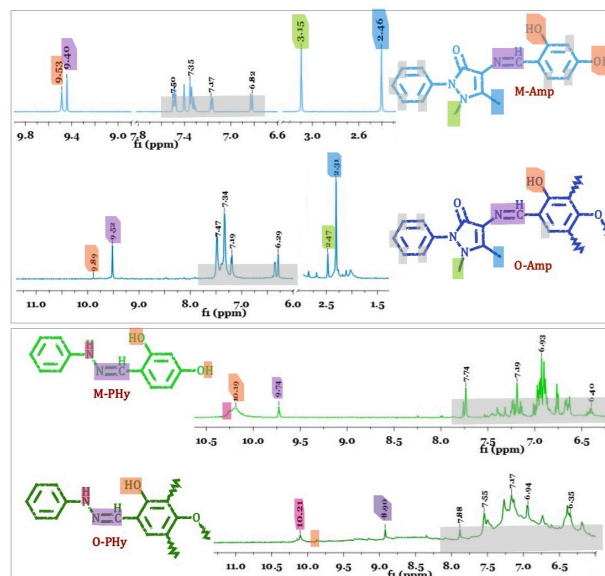


Figure 2. Comparative ^1H NMR profiles of the synthesized hydrazine/azomethine monomers and HRP-mediated oligomeric products (measured in DMSO-d_6 solution using a 600 MHz spectrometer; chemical shifts reported in ppm).

The UV–Vis spectrum in Figure 3 of M-Amp exhibits four characteristic absorption regions. The band observed at 250–325 nm corresponds to a $\sigma\rightarrow\sigma^*$ transition associated with the N-CH_3 and C-CH_3 groups. The absorption band in the 325–425 nm region is attributed to $\pi\rightarrow\pi^*$ transitions of the benzene and ampyron ring systems. A second $\pi\rightarrow\pi^*$ transition appears at 425–465 nm, arising from the carbonyl and imine functionalities. The weak band detected between 465–485 nm is assigned to an $n\rightarrow\pi^*$ transition of the carbonyl and imine groups. These transitions collectively confirm the conjugated electronic structure of M-Amp [29]. The UV–Vis spectrum of O-Amp shows a $\sigma\rightarrow\sigma^*$ transition at 250–323 nm, originating from the N-CH_3 and C-CH_3 groups. The $\pi\rightarrow\pi^*$ transition associated with the benzene and ampyron rings appears in the 323–428 nm region. A second $\pi\rightarrow\pi^*$ band is observed at 428–469 nm, corresponding to the carbonyl and imine chromophores. The broad absorption extending from 469–690 nm is attributed to an $n\rightarrow\pi^*$ transition of the carbonyl and imine groups, indicating enhanced conjugation and intramolecular charge-transfer character in O-Amp [30]. The UV–Vis spectrum of M-PHy displays a $\sigma\rightarrow\sigma^*$ transition at 250–355 nm, assigned to the NH-N moiety. The $\pi\rightarrow\pi^*$ transition of the benzene ring appears in the 355–500 nm region, reflecting the extended aromatic conjugation. A second $\pi\rightarrow\pi^*$ band is observed at 500–585 nm, arising from the imine group. The weak absorption at 585–605 nm corresponds to an $n\rightarrow\pi^*$ transition of the imine functionality. These features confirm the presence of a highly conjugated imine–aromatic system in M-PHy [31]. The UV–Vis spectrum of O-PHy shows a $\sigma\rightarrow\sigma^*$ transition at 250–505 nm, attributed to the NH-N group and the extended conjugation within the molecule. The $\pi\rightarrow\pi^*$ transition of the benzene ring appears in the 505–585 nm region. A second $\pi\rightarrow\pi^*$ band at 585–705 nm is associated with the imine chromophore. The broad $n\rightarrow\pi^*$ transition observed between 705–824 nm indicates strong electron delocalization and a pronounced charge-transfer character within the imine system. These transitions

collectively support the highly conjugated electronic structure of O-PHy [32].

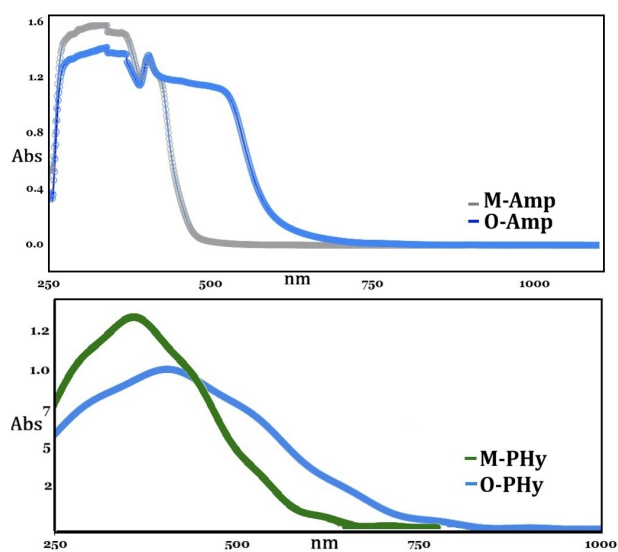
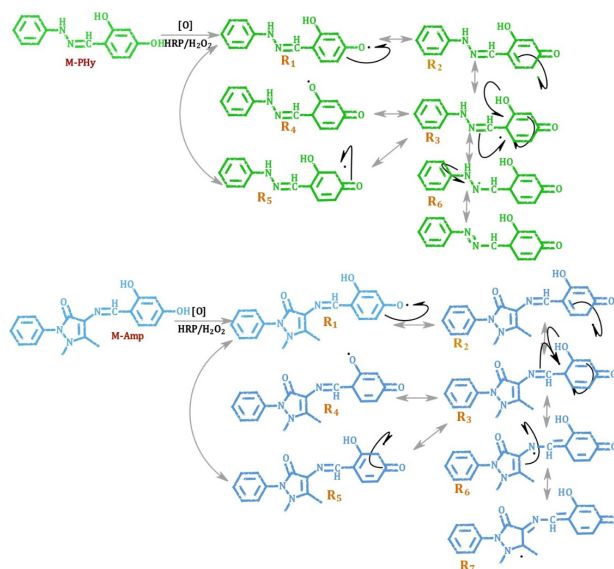


Figure 3. UV-Vis electronic absorption spectra of the hydrazine- and ampyrone-derived monomers and oligomers, monitored in DMF solvent (6×10^{-4} M) across the spectral region of 250–1000 nm.

The molecular weight distributions of the oligomers were evaluated by gel permeation chromatography (GPC), and the results demonstrate the successful formation of low-molecular-weight conjugated structures with relatively narrow dispersities. For O-PHy, the number-average molecular weight (M_n) was determined as 4298 Da, while the weight-average molecular weight (M_w) was 4408 Da, and the z-average molecular weight (M_z) was 4395 Da. The polymer exhibited a dispersity ($PDI = M_w/M_n$) of 1.25, indicating a moderately narrow molecular weight distribution. The degree of polymerization (D_p) was calculated as 14, consistent with the formation of short oligomeric chains [33]. For O-Amp, the number-average molecular weight (M_n) was 4104 Da, with a weight-average molecular weight (M_w) of 4603 Da and a z-average molecular weight (M_z) of 4415 Da. The dispersity was found to be 1.12, reflecting a narrower molecular weight distribution compared to O-PHy. The degree of polymerization (D_p) was determined as 18, suggesting slightly longer oligomer chains [33]. In the HRP/ H_2O_2 catalytic system, horseradish peroxidase (HRP) acts as an efficient oxidizing enzyme that initiates radical formation through a well-established redox cycle. Upon reaction with hydrogen peroxide, HRP is converted into its highly oxidizing Compound I species, which subsequently abstracts electrons from the monomers to generate reactive radical intermediates. These transient radicals represent the key propagating species responsible for the subsequent coupling and oligomerization steps [34], [35]. The stability of the generated radicals plays a central role in determining the preferred coupling pathways. Radicals stabilized through resonance delocalization—particularly those involving aromatic rings, imine groups, or heteroatom-adjacent positions—exhibit longer lifetimes and therefore a higher probability of participating in bond-forming reactions. However, due to the coexistence of multiple resonance-stabilized radical forms and the

dynamic nature of the HRP-mediated oxidation, it is not possible to definitively identify which specific radical species predominantly contribute to oligomer growth [35], [36]. As a result, the polymerization process is interpreted in terms of average structural contributions rather than radical-specific propagation routes. Since the repeating units formed from the various radical pathways are expected to possess comparable molecular weights, the resulting oligomers are described based on their overall average molecular weight distribution. The plausible radical intermediates proposed for each monomer, which may participate in the coupling reactions, are illustrated in Scheme 2 [37], [38].



Scheme 2. Plausible radical intermediates formed during the HRP/ H_2O_2 -mediated oxidation of the monomers.

3.2. Molecular Weight Distribution and Enzymatic Polymerization Kinetics

The molecular weight distributions of the synthesized oligomers were evaluated by gel permeation chromatography (GPC), demonstrating successful formation of low-molecular-weight conjugated structures with remarkably narrow dispersities. For O-PHy, the number-average molecular weight (M_n) was 4298 Da, while the weight-average molecular weight (M_w) was 4408 Da, yielding a dispersity ($PDI = M_w/M_n$) of 1.25 and a degree of polymerization (D_p) of 14. In contrast, O-Amp exhibited an M_n of 4104 Da and an M_w of 4603 Da, resulting in a noticeably narrower dispersity ($PDI = 1.12$) and a slightly higher degree of polymerization ($D_p = 18$). These values are highly consistent with previously reported HRP-mediated oxidative oligomerizations of phenolic Schiff bases, which typically yield short-chain oligomeric materials (M_n approximately 2000–6000 Da) due to the unique kinetics of enzymatic catalysis. The distinct variance in PDI values between the two oligomers can be explained by the structural influence of the monomer cores on radical stabilization kinetics. In the HRP/ H_2O_2 catalytic system, horseradish peroxidase (HRP) initiates oxidation through a redox cycle in which the enzyme is converted into Compound I, which subsequently abstracts electrons from the monomers to generate reactive radical intermediates (Scheme 2). The

ampyrone framework in M-Amp possesses a rich density of heteroatoms, including the polar pyrazolone carbonyl group (C=O) and tertiary amines. These functional groups exert powerful electronic and steric directing effects, thereby restricting the configuration of the propagating radical intermediates. These structural constraints direct the coupling process via a more uniform pathway, thereby suppressing random or hyperbranched side-reactions and leading to an exceptionally narrow molecular weight distribution (PDI=1.12). Conversely, the phenylhydrazine framework of M-PHY exhibits greater conformational flexibility and less localized electronic control, allowing the radical intermediates to couple through a slightly broader range of orientations, which manifests as a marginally broader dispersity (PDI=1.25)[39]. Furthermore, the enzymatic kinetics directly dictate the self-limiting chain growth observed in both materials. Initially, the propagation rate is rapid due to the high accessibility of the small monomer molecules to the deeply buried heme active site of HRP. However, as oligomerization proceeds and the chain length reaches a certain threshold ($D_p=14-18$), severe steric hindrance restricts these bulky macromolecular chains from effectively re-entering the enzyme's catalytic pocket for further activation. This mass-transfer limitation forces the reaction toward chain termination rather than continuous propagation[34]. The proposed termination pathways are closely linked to the specific radical profiles illustrated in Scheme 2. For the hydrazine-based system, the homolytic cleavage primarily generates phenoxy radicals located on the -OH groups (R_1 and R_4 , along with their resonance-stabilized carbon radical counterparts situated at the active ortho and para positions of the aromatic ring (R_2 and R_5). Chain termination predominantly occurs via radical-radical recombination (radical coupling) between these highly active propagating species[40]. When two growing oligomer chains bearing these terminal phenoxy or aromatic carbon radicals collide, they undergo irreversible covalent bonding, thereby neutralizing the radical centers and terminating chain growth. Additionally, disproportionation reactions involving proton transfer from the solvent or the surrounding matrix to localized radical sites serve as auxiliary termination mechanisms. Consequently, the interplay between sterically restricted enzymatic access and the targeted recombination of specific resonance-stabilized radicals successfully governs the controlled synthesis of these structurally tailored, low-dispersity conjugated oligomers [26].

3.3. Thermal Characterization of Synthesized Oligomers

The thermal behaviors of the synthesized oligomers were investigated using thermogravimetric (TG) and derivative thermogravimetric (DTG) analyses, and the corresponding curves are presented in Figure 4. Both oligomers exhibit multistep degradation patterns characteristic of conjugated structures containing heteroatoms and aromatic domains. The O-PHY oligomer displays an onset decomposition temperature of 178 °C. An initial mass loss of 7% is observed between 30–187 °C, which is attributed to the evaporation of

physically adsorbed moisture and low-molecular-weight volatiles. The material undergoes three distinct degradation steps. The first degradation stage occurs between 187–395 °C and is accompanied by a 20.6% mass loss, corresponding to the cleavage of less stable structural segments. The second stage, observed between 395–800 °C, results in a 25.7% mass loss and is associated with the decomposition of the conjugated backbone and heteroatom-containing units. The third degradation step takes place between 800–1000 °C, with a 30.2% mass loss, reflecting the breakdown of highly condensed aromatic structures. At 1000 °C, the residual char content is 16.5%, indicating the presence of thermally stable aromatic and heteroaromatic fragments [15]. The O-Amp oligomer exhibits a slightly higher onset decomposition temperature of 192 °C, suggesting improved initial thermal stability compared to O-PHY. A moisture-related mass loss of 7.5% is detected between 30–180 °C. The thermal decomposition proceeds through two major degradation steps. The first degradation stage occurs between 180–600 °C, with a 23.2% mass loss, corresponding to the breakdown of low-stability units and partial degradation of the oligomer backbone. The second stage, observed between 600–1000 °C, results in a substantial 53.7% mass loss, attributed to the decomposition of the conjugated aromatic-imine framework and subsequent carbonization processes. The residual mass at 1000 °C is 15.6%, consistent with the formation of a carbonaceous char typical of conjugated oligomeric systems [41]. Overall, the TGA results demonstrate that both oligomers possess multi-stage thermal degradation behavior, with O-Amp exhibiting a slightly higher initial thermal stability, while both materials retain a significant char residue at elevated temperatures due to their aromatic and heteroaromatic content.

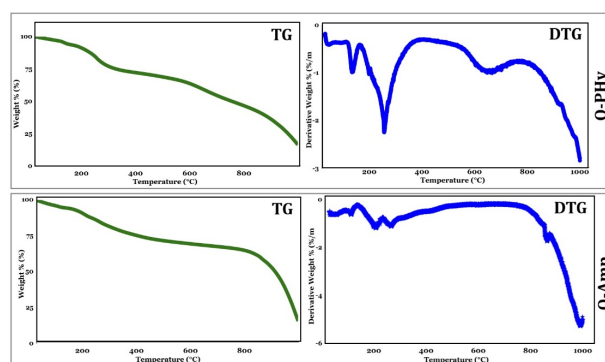


Figure 4. TGA analysis of hydrazine and ampyrone based monomers and oligomers.

A comparative assessment of the TGA results reveals that both oligomers exhibit multi-stage thermal degradation behavior, yet with notable differences in their initial and overall thermal stabilities. O-Amp shows a slightly higher onset decomposition temperature (192 °C) compared to O-PHY (178 °C), indicating that the ampyron-based structure provides improved resistance to the onset of thermal degradation. However, O-PHY undergoes three distinct degradation steps, reflecting a more complex decomposition pathway associated with its phenylhydrazine-derived framework. In contrast, O-Amp

degrades through two major steps, with a significantly larger mass loss occurring at higher temperatures (600–1000 °C), suggesting deeper fragmentation of the aromatic–imine backbone. Despite these differences, both oligomers retain comparable char residues at 1000 °C (16.5% for O-PHY and 15.6% for O-Amp), demonstrating the presence of thermally stable aromatic and heteroaromatic domains in both structures [42]. The DTG curves presented in Figure 4 provide further insight into the degradation kinetics of the oligomers. For O-PHY, three distinct DTG peaks correspond to the sequential degradation stages: the first peak in the 187–395 °C region reflects the cleavage of low-stability segments; the second, broader peak between 395–800 °C indicates progressive decomposition of the conjugated backbone; and the final peak in the 800–1000 °C region is associated with the breakdown of highly condensed aromatic structures. In contrast, the DTG profile of O-Amp displays two major peaks. The first peak, occurring between 180–600 °C, corresponds to the initial fragmentation of the oligomer chain, while the second, more intense peak in the 600–1000 °C region signifies extensive decomposition of the aromatic–imine framework and subsequent carbonization. The sharper and more intense high-temperature DTG peak of O-Amp suggests a more abrupt degradation of its conjugated structure compared to the more gradual, multi-step decomposition observed for O-PHY.

3.4. Surface Properties of Synthesized Compounds

The surface morphologies of the oligomers were examined using SEM at magnifications corresponding to 20 µm and 3 µm, and the resulting micrographs are presented in Figure 5. The O-PHY sample exhibits a relatively smooth yet finely textured surface, characterized by soft, rounded features and a homogeneous distribution across the examined regions. This morphology suggests a more uniform packing and a less rigid microstructural organization within the O-PHY oligomer. In contrast, the O-Amp oligomer displays a markedly sharper and more irregular surface topology. The micrographs reveal pronounced roughness with needle-like or angular protrusions, indicating a more heterogeneous and rigid microstructural arrangement. Additionally, localized agglomeration is observed in certain regions of the O-Amp surface, suggesting partial clustering of oligomeric domains during formation or drying. These morphological differences highlight the influence of monomer structure on the resulting oligomer microtexture. A comparative evaluation of the SEM micrographs further highlights the influence of monomer structure on the resulting oligomer morphologies. The smoother and more rounded surface features observed for O-PHY suggest a more uniform microstructural organization, likely arising from a relatively homogeneous radical coupling pattern during HRP/H₂O₂-mediated polymerization. This softer, less angular texture may also indicate reduced internal stress and a more flexible packing arrangement within the oligomer matrix. In contrast, the O-Amp oligomer exhibits a distinctly sharper and more irregular surface topology, characterized by pronounced roughness and

angular protrusions. These features imply a more heterogeneous growth process, potentially driven by the formation of multiple radical species with differing reactivities. The presence of localized agglomerates in O-Amp further supports the notion of uneven nucleation and growth, which may lead to micro-domain clustering during oligomer formation or drying. From a structure–property perspective, these morphological differences may influence the mechanical, thermal, or interfacial behavior of the materials. The smoother O-PHY surface could facilitate more uniform film formation or improved flexibility, whereas the rougher and more agglomerated O-Amp morphology may enhance surface area or rigidity but reduce homogeneity. Overall, the SEM observations align well with the radical-based polymerization mechanism proposed for these systems and underscore the structural sensitivity of HRP/H₂O₂-mediated oligomerization. Overall, the GPC results indicate that both oligomers possess relatively low molecular weights and narrow dispersities, consistent with the enzymatic oxidative coupling mechanism. The slightly higher D_p and lower PDI of O-Amp suggest a more uniform chain-growth process relative to O-PHY, in agreement with the structural and morphological differences observed in the SEM and TGA analyses.

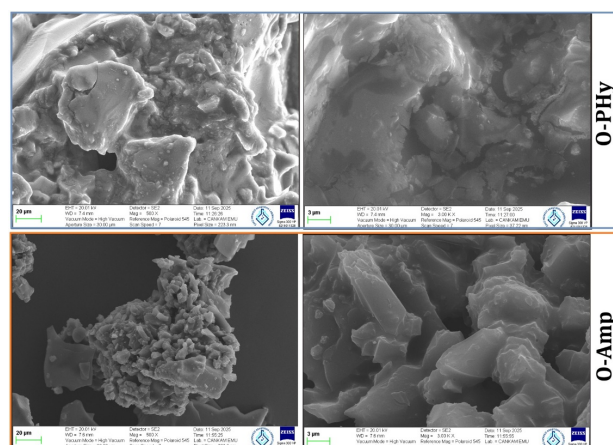


Figure 5. SEM images of hydrazine and ampyrone based monomers and oligomers.

3.5. Antioxidant Evaluation and Radical Scavenging Mechanism

The radical scavenging abilities of the target Schiff bases and oligomers were assessed via the standard DPPH method. Sample solutions were treated with 1.0 mL of 0.01% DPPH dissolved in methanol, followed by a 30-min incubation period in the dark. Afterward, the absorbance values were measured at 517 nm with a UV–Vis spectrophotometer. All experiments were performed in triplicate to calculate the mean inhibition percentage along with the standard deviation[43]. The radical scavenging efficiency was determined based on Equation 3.1:

$$\text{DPPH Scavenging Activity/Inhibition (\%)} = \left(\frac{C_0 - C_A}{C_0} \right) \times 100$$

Here, C_0 and C_A signify the absorbance values of the control and the sample at 517 nm, respectively. Detailed

quantitative findings and graphical analyses are summarized in Table 1 and Figure 6.

The antioxidant efficiencies of the synthesized Schiff base monomers (M-PHy and M-Amp) and their enzymatically derived oligomers (O-PHy and O-Amp) were quantitatively evaluated using the DPPH radical-scavenging assay. As summarized in Table 1, a pronounced change in antioxidant performance was observed upon transitioning from the monomeric states to the oligomeric architectures. Specifically, the inhibition percentage increased sharply from $8.83\% \pm 0.51\%$ for M-PHy to $54.65\% \pm 1.22\%$ for O-PHy, and from $39.74\% \pm 2.28\%$ for M-Amp to an impressive $62.82\% \pm 0.91\%$ for O-Amp.

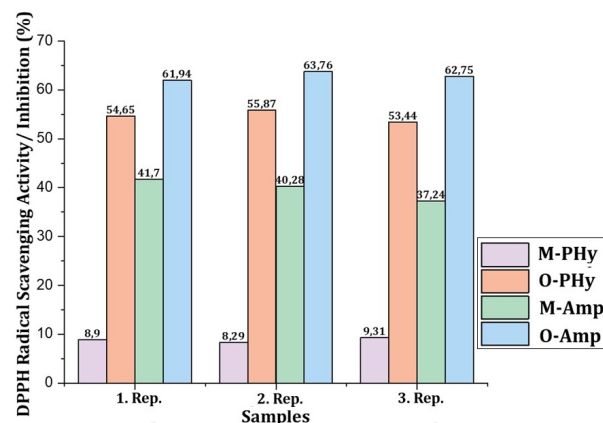


Figure 6. Inhibition percentage of Schiff bases and Oligomers

Table 1. Absorbance measurements and DPPH radical scavenging activity of control and Schiff base and oligomer samples.

Sample	Absorbance Rep. 1	Absorbance Rep 2.	Absorbance Rep. 3	Inhibition(%) Rep.1	Inhibition(%) Rep. 2	Inhibition(%) Rep. 3	Inhibition%±SD
Control	0.494	-	-	-	-	-	-
M-PHy	0.450	0.453	0.448	8.90	8.29	9.31	8.83±0.51
O-PHy	0.224	0.218	0.230	54.65	55.87	53.44	54.65±1.22
M-Amp	0.288	0.295	0.310	41.70	40.28	37.24	39.74±2.28
O-Amp	0.188	0.179	0.184	61.94	63.76	62.75	62.82±0.91

This remarkable enhancement in radical-scavenging capacity can be attributed to the substantial increase in molecular weight and the expansion of conjugated systems resulting from HRP-mediated oxidative oligomerization. In the monomeric forms, the ability to stabilize a trapped radical is limited to localized resonance within a single aromatic unit. However, in the macromolecular oligomeric frameworks, the propagation of carbon-carbon (C-C) and carbon-heteroatom (C-X) bonds creates an extended π -conjugated backbone. Upon abstracting a hydrogen atom or donating an electron to the DPPH radical, the resulting phenoxy or nitrogen-centered radicals can undergo extensive delocalization across the entire oligomeric chain. This highly extended resonance stabilization reduces the overall energy of the radical intermediate, thereby shifting the thermodynamic equilibrium to favor radical entrapment[44], [45]. Furthermore, the comparative analysis of the two oligomeric systems shows that O-Amp has a higher antioxidant capacity (62.82%) than O-PHy (54.65%). This difference underscores the critical role of the structural and functional-group diversity inherent in the ampyrone (4-aminoantipyrine) moiety. Unlike the relatively straightforward phenylhydrazine framework of O-PHy, the ampyrone core in O-Amp introduces a rich variety of heteroatom-containing functional groups, including a highly polar pyrazolone carbonyl group (C=O), tertiary amine centers, and an adjacent phenyl ring. These electron-rich heteroatoms systematically alter the electron density across the azomethine linkage, lowering the bond dissociation energy (BDE) of the active homolytic cleavage sites (such as the phenolic OH groups derived from the 2,4-dihydroxybenzaldehyde core). The synergistic effect of these diverse functional groups in O-Amp facilitates more rapid hydrogen atom transfer (HAT) or sequential proton-loss electron transfer (SPLET) mechanisms to neutralize the DPPH radical[45],[46][47]. Consequently, the structural synergy between the

extended molecular weight—which provides the necessary platform for radical delocalization—and the chemical versatility of the ampyrone ring establishes these green-synthesized oligomers as highly effective, multifunctional radical scavengers.

4. CONCLUSION

In this work, two new conjugated oligomers, O-PHy and O-Amp, were successfully synthesized through HRP/H₂O₂-mediated enzymatic oxidative oligomerization of hydrazine- and azomethine-based monomers derived from 2,4-dihydroxybenzaldehyde. Comprehensive spectroscopic analyses (FT-IR, UV-Vis, and ¹H NMR) confirmed the formation of the expected imine-containing structures and revealed characteristic $\sigma \rightarrow \sigma^*$, $\pi \rightarrow \pi^*$, and $n \rightarrow \pi^*$ transitions associated with aromatic, carbonyl, and heteroatom-based chromophores. These findings, together with the plausible radical intermediates illustrated in Scheme 2, support a radical-mediated oxidative coupling mechanism in which multiple resonance-stabilized radical species may contribute to oligomer growth. GPC analysis demonstrated that both oligomers possess relatively low molecular weights and narrow dispersities, consistent with controlled enzymatic oxidation. The slightly higher degree of polymerization and lower PDI observed for O-Amp suggest a more uniform chain-growth process compared to O-PHy. Thermal analysis further highlighted structural differences between the two materials: O-Amp exhibited a higher onset decomposition temperature, whereas both oligomers retained significant char residues at elevated temperatures, reflecting the presence of thermally robust aromatic and heteroaromatic domains. SEM micrographs revealed distinct morphological features that correlate with the structural and thermal properties of the oligomers. O-PHy displayed a smoother and more homogeneous surface, while O-Amp exhibited

sharper, more irregular textures with localized agglomeration, indicating differences in microstructural organization arising from monomer structure and radical stability. Overall, the combined spectroscopic, thermal, morphological, and mechanistic findings demonstrate that HRP/H₂O₂-mediated oxidation is an effective and environmentally benign route for producing conjugated oligomers with tunable physicochemical properties. The distinct behaviors of O-PHY and O-Amp underscore the structural sensitivity of enzymatic oxidative polymerization and provide valuable insights for the future design of bio-derived functional materials. Most significantly, the functional performance of the synthesized oligomers was evaluated via DPPH radical scavenging assays, establishing a profound correlation between their extended macromolecular architecture, thermal robustness, and antioxidant efficiency. The enzymatic polymerization induced a remarkable surge in antioxidant activity; the inhibition percentage escalated from 8.83% ± 0.51% (M-PHY) to 54.65% ± 1.22% for O-PHY, and from 39.74% ± 2.28% (M-Amp) to an impressive 62.82% ± 0.91% for O-Amp. This dramatic functional enhancement directly mirrors the TGA and DTG findings; the extended π -conjugation and highly condensed aromatic/heteroaromatic domains that provide excellent thermal resistance up to 1000 °C also serve as highly efficient resonance-stabilized frameworks capable of effectively trapping and delocalizing free radicals. The higher overall antioxidant capacity of O-Amp over O-PHY further aligns with its superior thermal onset temperature (192 °C), highlighting the pivotal role of the ampyrone-based framework in stabilizing the macromolecular system. Consequently, these findings prove that "HRP"-mediated green synthesis not only enhances the physical durability of Schiff base derivatives but also unlocks their high-potential application as eco-friendly, thermally stable antioxidant additives for advanced biotechnological, polymeric, and industrial materials.

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