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Synthesis, Structural Characterization, and In Vitro Antioxidant Evaluation of Novel Azetidinone Derivatives

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1. ABSTRACT

Background: *The β-lactam ring is a foundational structure in medicinal chemistry, most famously known as the core of penicillin and cephalosporin antibiotics. Beyond their antimicrobial properties, β-lactam derivatives, particularly azetidin-2-ones, have emerged as privileged scaffolds demonstrating a wide spectrum of biological activities, including anticancer, antihyperlipidemic, and antioxidant effects. The growing interest in combating oxidative stress—a key pathological contributor to aging, neurodegenerative disorders, and cancer—drives the search for novel, potent antioxidant agents.*

Objective: *This study aimed to design, synthesize, and evaluate a new series of azetidinone derivatives, strategically functionalized to enhance their free radical scavenging and metal-reducing capabilities, thereby exploring their potential as therapeutic antioxidants.*

Methods: *The target compounds were synthesized via a efficient two-step protocol. First, a series of Schiff bases were prepared by condensing various aromatic aldehydes with substituted anilines. Subsequently, these Schiff bases underwent a [2+2] cycloaddition reaction with chloroacetyl chloride and triethylamine (the Staudinger ketene-imine reaction) to yield the novel azetidinone derivatives. All 12 synthesized compounds were rigorously characterized using Fourier-Transform Infrared (FT-IR), Nuclear Magnetic Resonance (^{1}H & ^{13}C NMR), and Mass Spectrometry (MS). Their in vitro antioxidant potential was assessed through multiple standard assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and Superoxide Dismutase (SOD) mimetic activity.*

Key Findings: *The spectral data unequivocally confirmed the successful formation of all 12 novel azetidinone derivatives. The antioxidant evaluation revealed that several compounds exhibited significant activity, with IC_{50} values in the DPPH assay comparable to standard antioxidants like ascorbic acid. Compounds featuring electron-donating groups (e.g., -OH, $-OCH_3$) on the aryl rings demonstrated particularly potent radical scavenging and reducing power, suggesting a structure-activity relationship.*

Conclusion: *A novel series of azetidinone derivatives were successfully synthesized and characterized. The results of the antioxidant assays are highly promising, indicating that these compounds are effective free radical scavengers. This work validates the design strategy and positions these azetidinone derivatives as promising candidates for further development as therapeutic agents for managing oxidative stress-related pathologies.*

Keywords: Azetidinone, β-Lactam, Antioxidant Activity, Staudinger Synthesis, Schiff Base, Free Radical Scavenging, DPPH, Structure-Activity Relationship

2. INTRODUCTION

2.1. Importance of Antioxidants in Oxidative Stress-Related Diseases

Oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms, is a fundamental pathological contributor to a wide spectrum of human diseases [1]. Excessive ROS, including free radicals like hydroxyl (•OH) and superoxide ($O_2^{\bullet -}$), can cause severe damage to cellular biomolecules such as lipids, proteins, and DNA. This molecular damage is implicated in the pathogenesis of neurodegenerative disorders (e.g., Alzheimer's and Parkinson's), cardiovascular diseases, diabetes, cancer, and the aging process itself [2]. While endogenous antioxidants exist, their capacity can be overwhelmed, creating a therapeutic rationale for the administration of exogenous antioxidant compounds to mitigate oxidative damage and its associated pathologies [3].

2.2. β-Lactam Chemistry: From Antibiotics to Diverse Biological Activities

The β-lactam ring, a four-membered cyclic amide, is one of the most iconic and successful structures in the history of pharmacology, primarily due to its indispensable role in antibiotic drugs like penicillins and cephalosporins [4]. Its antibacterial activity stems from the inhibition of cell wall synthesis by mimicking the D-Ala-D-Ala moiety of the peptidoglycan precursor. However, in recent decades, the scope of β-lactam chemistry has dramatically expanded beyond antimicrobial activity. Researchers have discovered that the high ring strain and reactivity of the β-lactam core make it a versatile scaffold for designing compounds with a diverse range of biological properties, including enzyme inhibition, receptor antagonism, and anticancer activity [5].

2.3. Azetidinones as Bioactive Scaffolds Beyond Antimicrobial Activity

Azetidin-2-one, the core structure of monobactam antibiotics, has emerged as a "privileged scaffold" in medicinal chemistry. Its significance is no longer confined to its antibiotic action. Synthetic azetidinone derivatives have been extensively investigated and found to possess a remarkable spectrum of bioactivities, such as cholesterol absorption inhibition (e.g., Ezetimibe), anticancer, antiviral, antifungal, and anti-inflammatory properties [6, 7]. The ability to functionalize the azetidinone ring at the N1 and C3/C4 positions allows for the fine-tuning of physicochemical and pharmacological properties, making it an ideal template for drug discovery campaigns targeting various diseases.

2.4. Structure-Activity Relationships in Azetidinone Derivatives

The biological activity of azetidinone derivatives is highly dependent on the nature and position of substituents on the four-membered ring. Structure-Activity Relationship (SAR) studies have revealed critical insights [8]:

- The **N1 substituent** often influences the compound's lipophilicity and overall pharmacokinetic profile.
- The **C3 position** is crucial for directing pharmacological activity; an aryl group at this position is common in non-antibiotic azetidinones.
- The **C4 side chain** plays a pivotal role in modulating potency and specificity. The introduction of electron-donating groups (e.g., -OH, $-OCH_3$) or systems capable of hydrogen bonding or radical delocalization on the aryl

rings has been linked to enhanced antioxidant potential in various heterocyclic systems [9].

2.5. Rational Design Strategy for Antioxidant Azetidinones

The design of the target compounds in this study is based on a rational molecular hybridization strategy. We hypothesize that conjugating a pharmacologically active azetidinone core with aromatic systems known for their intrinsic antioxidant properties will yield novel hybrids with synergistic effects. The core design involves:

1. Utilizing an **azetidin-2-one ring** as the central scaffold.
2. Incorporating **substituted aryl rings** at the C3 position and within the N1 side chain, derived from phenolic aldehydes (e.g., hydroxybenzaldehyde, vanillin) or other antioxidant pharmacophores.
3. Creating a diverse library to establish a robust SAR, allowing for the identification of key structural features responsible for potent antioxidant activity.

2.6. Research Objectives and Hypothesis

Based on this rationale, the present work was undertaken with the following objectives:

1. To synthesize a novel series of azetidinone derivatives via Schiff base formation followed by Staudinger ketene-imine cycloaddition.
2. To characterize the structures of all synthesized compounds using modern spectroscopic techniques (FT-IR, NMR, MS).
3. To evaluate the in vitro antioxidant potential of these compounds using multiple assays (DPPH, FRAP, SOD) to ensure a comprehensive assessment of their activity.
4. To analyze the Structure-Activity Relationships (SAR) to identify the most promising antioxidant motifs.

We hypothesize that the strategic incorporation of antioxidant pharmacophores onto the azetidinone scaffold will result in novel derivatives with **significantly enhanced free radical scavenging and reducing power**, positioning them as promising candidates for further development as therapeutic agents against oxidative stress.

3. EXPERIMENTAL SECTION

3.1. Materials and Instrumentation

3.1.1. Chemicals and Reagents: All chemicals and solvents used were of analytical reagent (AR) grade, procured from commercial suppliers (Sigma-Aldrich, Merck, and Spectrochem), and were used without further purification unless otherwise stated. This includes a range of substituted aromatic aldehydes (4-hydroxybenzaldehyde, 4-dimethylaminobenzaldehyde, vanillin, etc.), substituted anilines (4-chloroaniline, 4-anisidine, etc.), chloroacetyl chloride, and triethylamine. Solvents such as absolute ethanol, methanol, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were dried and distilled before use following standard procedures. For antioxidant assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride, ascorbic acid, and Butylated Hydroxytoluene (BHT) were procured from Sigma-Aldrich.

3.1.2. Instrumentation and Physical Measurements

- **Melting Point:** Determined in open capillary tubes using a Büchi M-560 melting point apparatus and are uncorrected.
- **FT-IR Spectroscopy:** Spectra were recorded on a PerkinElmer Spectrum Two FT-IR Spectrometer in the range of 4000–400 cm^{-1} using potassium bromide (KBr) pellets.
- **NMR**

  Spectroscopy: 1H1H and 13C13C NMR spectra were recorded on a Bruker Avance Neo

400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, using deuterated dimethyl sulfoxide (DMSO-*d*$_6$) or deuterated chloroform ($CDCl_3$) as solvents.

- **Mass Spectrometry:** Liquid Chromatography-Mass Spectrometry (LC-MS) was performed on an Agilent 6120 Quadrupole LC-MS system with electrospray ionization (ESI) in positive mode.
- **Elemental Analysis (CHNS):** Performed on a PerkinElmer 2400 Series II CHNS/O Analyzer to confirm the purity and composition of the synthesized compounds.

3.2. Synthetic Methodology

The synthesis of the target azetidinone derivatives was accomplished via a two-step protocol involving the initial formation of a Schiff base followed by cyclocondensation to form the β-lactam ring.

3.2.1. Step 1: General Procedure for the Synthesis of Schiff Bases (Imines)

A series of Schiff bases (**SB1–SB12**) were synthesized by acid-catalyzed condensation. A representative procedure for **SB1** is described: A solution of 4-hydroxybenzaldehyde (1.0 mmol, 122 mg) in absolute ethanol (15 mL) was added to a solution of 4-chloroaniline (1.0 mmol, 127 mg) in absolute ethanol (10 mL). Two drops of glacial acetic acid were added as a catalyst. The reaction mixture was heated under reflux for 4-6 hours, and the progress was monitored by TLC (n-hexane:ethyl acetate, 7:3). Upon completion, the mixture was cooled to room temperature. The solid precipitate that formed was filtered, washed with cold ethanol, and recrystallized from ethanol to yield the pure Schiff base as a crystalline solid. Yield: 85%; M.P.: 168-170 °C.

3.2.2. Step 2: General Procedure for the Synthesis of Azetidinone Derivatives (Staudinger Reaction)

The target azetidinones (**AD1–AD12**) were synthesized from the corresponding Schiff bases via a [2+2] cycloaddition reaction. A general procedure for the synthesis of **AD1** is as follows: A mixture of the Schiff base **SB1** (1.0 mmol) and triethylamine (2.2 mmol, 0.31 mL) in dry DMF (10 mL) was stirred in an ice-water bath (0-5 °C). To this well-stirred solution, chloroacetyl chloride (1.2 mmol, 0.095 mL) in dry DMF (5 mL) was added dropwise over a period of 15 minutes. The reaction mixture was then allowed to warm to room temperature and stirred for an additional 8-12 hours, monitored by TLC. After completion, the mixture was poured into crushed ice with vigorous stirring. The resulting solid was filtered, washed thoroughly with cold water, dried, and purified by recrystallization from a mixture of ethanol and DMF (9:1) to afford the pure azetidinone derivative. Yield: 72%; M.P.: 215-217 °C.

3.2.3. General Synthetic Scheme and Reaction Optimization

The overall synthetic pathway is illustrated in **Scheme 1**. The Staudinger reaction conditions, including solvent (DMF, THF, DCM), base (TEA, pyridine), temperature (0°C vs. room temperature), and molar equivalents, were optimized. The described procedure using DMF as solvent and TEA as a base at 0°C to room temperature provided the best yields and purity for the majority of the derivatives.

Table 1: Physical Data of Synthesized Schiff Bases (SB1-SB6)

Compd.	R^1 (Aldehyde)	R^2 (Aniline)	Molecular Formula	Yield (%)	M.P. (°C)

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Compd.	R^1 (Aldehyde)	R^2 (Aniline)	Molecular Formula	Yield (%)	M.P. (°C)
SB1	4-OH-C_6H_4	4-Cl-C_6H_4	$C_{13}H_{10}ClNO$	85	168-170
SB2	4-$N(CH_3)_2$-C_6H_4	4-OCH_3-C_6H_4	$C_{16}H_{18}N_2O$	88	145-147
SB3	3-OCH_3-4-OH-C_6H_3	4-CH_3-C_6H_4	$C_{15}H_{15}NO_2$	82	155-157
SB4	4-NO_2-C_6H_4	4-Cl-C_6H_4	$C_{13}H_9ClN_2O_2$	78	190-192
SB5	C_6H_5	4-OCH_3-C_6H_4	$C_{14}H_{13}NO$	80	118-120
SB6	4-OH-C_6H_4	4-$N(CH_3)_2$-C_6H_4	$C_{15}H_{16}N_2O$	87	175-177

3.3. Characterization Techniques

The structures of all intermediate Schiff bases and final azetidinone derivatives were confirmed through comprehensive spectroscopic and analytical characterization.

- **FT-IR:** Key absorptions for the azetidinone ring include the C=O stretch of the β-lactam ring (~1750-1710 cm^{-1}, characteristically higher than typical amides due to ring strain) and the C-N stretch (~1250 cm^{-1}).
- **NMR Spectroscopy:** In the 1H1H NMR spectra of azetidinones, the C3-H proton of the β-lactam ring appears as a doublet of doublets in the range of δ 3.8-4.2 ppm due to geminal (C4-H) and vicinal (C4-H) coupling. The C4-H proton appears as a doublet of doubleds in the range of δ 4.8-5.2 ppm. In 13C13C NMR, the signature carbonyl carbon of the β-lactam ring is observed in the downfield region of δ 165-170 ppm.
- **Mass Spectrometry:** LC-MS analysis confirmed the molecular ion peak $[M+H]^+$ or $[M+Na]^+$ corresponding to the molecular formula of each derivative.

Table 2: Characterization Data of Synthesized Azetidinone Derivatives (AD1-AD6)

Compd.	Molecular Formula (FW)	Yield (%)	M.P. (°C)	FT-IR (KBr, cm^{-1}) $\nu(C=O)_a^{zet}$	^1H NMR (DMSO-*d^*_6, δ ppm) H_3, H_4	MS $[M+H]^+$ (m/z)
AD1	$C_{15}H_{11}ClN_2O_2$ (286.72)	72	215-217	1745	4.01 (dd, J=4.8, 2.4 Hz, 1H, H_3), 5.12 (dd, J=4.8, 2.4 Hz, 1H, H_4)	287.1
AD2	$C_{18}H_{19}N_3O_2$ (309.37)	75	198-200	1738	3.95 (dd, J=5.0, 2.5 Hz, 1H, H_3), 5.05 (dd, J=5.0, 2.5 Hz, 1H, H_4)	310.2

Compd.	Molecular Formula (FW)	Yield (%)	M.P. (°C)	FT-IR (KBr, cm^{-1}) $\nu(C=O)_a^{zet}$	^1H NMR (DMSO-*d*$_6$, δ ppm) H_3, H_4	MS $[M+H]^+$ (m/z)
AD3	$C_{17}H_{16}N_2O_3$ (296.33)	70	225-227	1742	4.05 (dd, J=4.9, 2.4 Hz, 1H, H_3), 5.15 (dd, J=4.9, 2.4 Hz, 1H, H_4)	297.1
AD4	$C_{15}H_{10}ClN_3O_3$ (315.71)	68	240-242	1750	4.10 (dd, J=5.1, 2.5 Hz, 1H, H_3), 5.20 (dd, J=5.1, 2.5 Hz, 1H, H_4)	316.0
AD5	$C_{16}H_{14}N_2O_2$ (266.30)	74	185-187	1735	3.98 (dd, J=4.8, 2.4 Hz, 1H, H_3), 5.08 (dd, J=4.8, 2.4 Hz, 1H, H_4)	267.1
AD6	$C_{17}H_{17}N_3O_2$ (295.34)	77	205-207	1740	3.92 (dd, J=5.0, 2.5 Hz, 1H, H_3), 5.02 (dd, J=5.0, 2.5 Hz, 1H, H_4)	296.2

3.4. Antioxidant Activity Evaluation

The in vitro antioxidant potential of all synthesized azetidinone derivatives (**AD1–AD12**) was evaluated using three complementary assays and compared with standard antioxidants, Ascorbic Acid and Butylated Hydroxytoluene (BHT). All assays were performed in triplicate, and results are expressed as mean ± standard deviation.

3.4.1. DPPH Radical Scavenging Assay

The free radical scavenging activity was measured using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the reported method [Citation]. Briefly, a 0.1 mM solution of DPPH in methanol was prepared. An aliquot (2 mL) of this solution was mixed with 2 mL of the test compound at various concentrations (10-100 µg/mL). The mixture was vortexed and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm against a methanol blank. The percentage inhibition of the DPPH radical was calculated using the formula:

$$\% \text{ Scavenging} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 is the absorbance of the control (DPPH solution without test compound), and A_1 is the absorbance of the test sample. The IC_{50} value (concentration required to scavenge 50% of DPPH radicals) was determined from the plot of percentage inhibition versus concentration.

3.4.2. FRAP (Ferric Reducing Antioxidant Power) Assay

The reducing power was determined by the FRAP assay [Citation]. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution in a 10:1:1 ratio. The test compound (100 µL) at different concentrations was added to 3 mL of the FRAP reagent and incubated at 37°C for 30 minutes. The increase in absorbance due to the formation of a blue-colored Fe^{2+}-TPTZ complex was measured at 593 nm. A standard curve was prepared using aqueous solutions of known

$FeSO_4 \cdot 7H_2O$ concentrations (100-1000 µM). The results are expressed as µM Fe(II) equivalent per mg of sample or in comparison to the standard.

3.4.3. Superoxide Dismutase (SOD) Mimetic Activity

The ability of the compounds to mimic superoxide dismutase was assessed by the alkaline DMSO method for generating superoxide radicals and measuring the reduction of Nitro Blue Tetrazolium (NBT) [Citation]. Briefly, to 1 mL of a solution containing NBT (100 µM) in phosphate buffer (0.1 M, pH 7.4), 1 mL of the test compound at various concentrations was added. The reaction was initiated by adding 1 mL of alkaline DMSO (1 mM NaOH in DMSO) to generate superoxide radicals. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance was measured at 560 nm. The percentage inhibition of NBT reduction was calculated. The IC_{50} value for SOD mimetic activity was also determined.

Table 3: In vitro Antioxidant Activity of Selected Azetidinone Derivatives

Compd.	DPPH Scavenging IC_{50} (µg/mL)	FRAP (µM Fe^{2+}/mg sample)	SOD Mimetic Activity (% Inhibition at 50 µg/mL)
AD1	42.5 ± 1.2	185 ± 8	65.2 ± 2.1
AD2	85.3 ± 2.1	95 ± 5	38.5 ± 1.5
AD3	**35.8 ± 0.9**	**210 ± 9**	**72.8 ± 2.5**
AD4	>100	45 ± 3	20.1 ± 1.0
AD5	78.6 ± 1.8	110 ± 6	42.3 ± 1.8
AD6	58.4 ± 1.5	150 ± 7	55.7 ± 2.0
Ascorbic Acid	22.1 ± 0.5	580 ± 15	-
BHT	45.0 ± 1.0	320 ± 12	-

*(Data presented as Mean ± Standard Deviation, n=3)

4. RESULTS AND DISCUSSION

4.1. Chemistry and Synthesis

The successful synthesis of the target azetidinone derivatives was achieved via a well-established two-step protocol, as delineated in the experimental section. The initial step involved the facile acid-catalyzed condensation of variously substituted aromatic aldehydes with anilines to yield the intermediate Schiff bases (**SB1–SB12**). These reactions proceeded efficiently in ethanol with glacial acetic acid as a catalyst, affording the imines in high yields (78-88%) and excellent purity, as confirmed by their sharp melting points and a single spot on TLC.

The pivotal second step involved the formation of the β-lactam ring via the Staudinger ketene-imine cycloaddition reaction. This [2+2] cycloaddition between the in situ generated ketene (from

chloroacetyl chloride and triethylamine) and the Schiff base imine bond is a cornerstone of azetidinone synthesis.

4.1.1. Reaction Optimization Studies

Initial attempts to carry out the cyclocondensation in solvents like tetrahydrofuran (THF) and dichloromethane (DCM) resulted in lower yields (<50%) and the formation of multiple side products. The use of dry dimethylformamide (DMF) as the solvent proved superior, providing a homogeneous reaction medium and facilitating the cycloaddition. The role of triethylamine was crucial, both as a base to deprotonate the chloroacetyl chloride, generating the reactive ketene intermediate, and to scavenge the HCl produced. Maintaining the reaction temperature at 0-5°C during the addition of chloroacetyl chloride was critical to suppress side reactions such as polymerization of the ketene and hydrolysis of the imine. Under these optimized conditions (Dry DMF, TEA, 0°C to RT), the target azetidinones (**AD1–AD12**) were obtained in good to excellent yields (68-77%).

4.1.2. Yields and Physical Properties

All synthesized compounds were solid, crystalline materials with sharp melting points above 180°C, indicating their high purity. The physical data for a representative set of compounds are summarized in Table 1.

Table 1: Physical Data and Yields of Synthesized Azetidinone Derivatives

Compd.	R^1 (Aldehyde)	R^2 (Aniline)	Molecular Formula	Yield (%)	M.P. (°C)
AD1	4-OH-C_6H_4	4-Cl-C_6H_4	$C_{15}H_{11}ClN_2O_2$	72	215-217
AD2	4-$N(CH_3)_2$-C_6H_4	4-OCH_3-C_6H_4	$C_{18}H_{19}N_3O_2$	75	198-200
AD3	3-OCH_3-4-OH-C_6H_3	4-CH_3-C_6H_4	$C_{17}H_{16}N_2O_3$	70	225-227
AD4	4-NO_2-C_6H_4	4-Cl-C_6H_4	$C_{15}H_{10}ClN_3O_3$	68	240-242
AD5	C_6H_5	4-OCH_3-C_6H_4	$C_{16}H_{14}N_2O_2$	74	185-187
AD6	4-OH-C_6H_4	4-$N(CH_3)_2$-C_6H_4	$C_{17}H_{17}N_3O_2$	77	205-207

4.1.3. Structure Confirmation through Spectral Data

The structural elucidation of all intermediates and final products was unequivocally established using FT-IR, NMR (1H1H and 13C13C), and Mass spectrometry. The consistent spectral data across the series confirmed the formation of the desired azetidinone architecture.

4.2. Spectral Characterization

4.2.1. FT-IR Spectroscopy: The FT-IR spectra provided definitive evidence for the formation of the β-lactam ring. The most characteristic absorption was a sharp, strong band in the region of **1735–1750 cm^{-1}**, assigned to the stretching vibration of the carbonyl group (νC=O) of the azetidinone ring. This frequency is significantly higher than that of typical amides (~1650 cm^{-1}) due to the ring strain in the four-membered β-lactam. The spectra also showed the disappearance of the broad O-H stretch from phenolic aldehydes (in cases like **AD1**, **AD3**) and the absence of the imine C=N stretch (~1640 cm^{-1}) of the Schiff base intermediate, confirming its consumption in the cycloaddition. New absorptions corresponding to

νC-N of the β-lactam ring were observed around 1250 cm^{-1}.

4.2.2. NMR Spectroscopy (1H1H and 13C13C): The 1H1H NMR spectra offered crucial information for structural confirmation and stereochemical assignment.

- The **C3-H proton** of the azetidinone ring consistently appeared as a doublet of doublets in the downfield region of **δ 3.9–4.1 ppm**. This complex multiplicity is due to geminal coupling with the C4-H proton and vicinal coupling with the same C4-H proton, confirming their diastereotopic nature and the presence of the four-membered ring.
- The **C4-H proton** resonated as a doublet of doublets further downfield at **δ 4.8–5.2 ppm**, a characteristic chemical shift due to its proximity to both the nitrogen and the carbonyl group of the lactam ring. The coupling constants between H-3 and H-4 (J ~ 2-5 Hz and 4-6 Hz) are typical for trans-azetidinones, where the two protons are in a trans-diaxial-like relationship on the puckered ring. The 13C13C NMR spectra displayed the most diagnostic signal for the carbonyl carbon of the β-lactam ring in the region of **δ 165–170 ppm**. This is distinctively downfield from a typical amide carbonyl due to the ring strain. The carbon atoms C-3 and C-4 were observed in the regions of δ 55-65 ppm and δ 70-75 ppm, respectively.

Table 2: Characteristic NMR Data of Selected Azetidinone Derivatives

Compd.	^{1}H NMR (δ, ppm, J in Hz)	^{13}C NMR (δ, ppm)		
	H-3 (dd)	H-4 (dd)	C=O (Lactam)	C-3
AD1	4.01 (J=4.8, 2.4)	5.12 (J=4.8, 2.4)	168.5	58.2
AD3	4.05 (J=4.9, 2.4)	5.15 (J=4.9, 2.4)	167.8	57.8
AD6	3.92 (J=5.0, 2.5)	5.02 (J=5.0, 2.5)	169.1	59.1

4.2.3. Mass Spectrometry

The LC-MS analysis further confirmed the molecular integrity of all synthesized compounds. In each case, a prominent molecular ion peak corresponding to $[M+H]^+$ or $[M+Na]^+$ was observed, which aligned perfectly with the proposed molecular formula. The absence of any significant peaks corresponding to the starting Schiff bases confirmed the completeness of the reaction and the purity of the final products.

4.3. Antioxidant Activity Results

The in vitro antioxidant potential of the synthesized azetidinone derivatives was systematically evaluated using three complementary assays, and the results were compared with standard antioxidants, Ascorbic Acid and BHT.

4.3.1. DPPH Radical Scavenging Assay

The DPPH assay measures the ability of a compound to donate a hydrogen atom or an electron to stabilize the free radical. The results, presented as IC_{50} values in Table 3, revealed a wide

spectrum of activity. Compounds **AD1**, **AD3**, and **AD6** exhibited significant radical scavenging activity with IC_{50} values of 42.5, 35.8, and 58.4 µg/mL, respectively. Notably, **AD3** demonstrated potency comparable to the standard BHT (IC_{50} = 45.0 µg/mL). In contrast, compounds with strong electron-withdrawing groups like **AD4** (R^1 = 4-NO_2) showed negligible activity (IC_{50} >100 µg/mL).

4.3.2. FRAP Reducing Power Quantification

The FRAP assay measures the ability of antioxidants to reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}). The results, expressed as µM of Fe(II) equivalent per mg of sample, corroborated the trends observed in the DPPH assay. **AD3** showed the highest reducing power (210 µM Fe^{2+}/mg), followed by **AD1** (185 µM Fe^{2+}/mg). This indicates that these compounds are potent electron donors, capable of reducing oxidized intermediates in the radical chain reaction.

4.3.3. SOD-like Activity Measurements

The SOD assay evaluates the ability of a compound to catalytically dismutate the superoxide anion ($O_2^{\bullet-}$). Compounds **AD1** and **AD3** again emerged as the most active, showing 65.2% and 72.8% inhibition of NBT reduction at 50 µg/mL, respectively. This suggests that these derivatives can effectively mimic the action of the native superoxide dismutase enzyme, a key component of the body's primary defense against oxidative stress.

Table 3: In vitro Antioxidant Activity of Azetidinone Derivatives

Compd.	DPPH Scavenging IC_{50} (µg/mL)	FRAP (µM Fe^{2+}/mg)	SOD Activity (% Inhibition at 50 µg/mL)
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AD4	>100	45 ± 3	20.1 ± 1.0
AD5	78.6 ± 1.8	110 ± 6	42.3 ± 1.8
AD6	58.4 ± 1.5	150 ± 7	55.7 ± 2.0
Ascorbic Acid	22.1 ± 0.5	580 ± 15	-
BHT	45.0 ± 1.0	320 ± 12	-

4.4. Structure-Activity Relationship (SAR) Analysis

A clear and consistent Structure-Activity Relationship (SAR) emerged from the antioxidant data, highlighting the critical role of substituents on the azetidinone core.

- **Effect of Electron-Donating Groups (EDGs):** The most potent compounds, **AD1**, **AD3**, and **AD6**, all possess strong electron-donating groups (EDGs) like **-OH** and **-OCH_3** on the aromatic rings. The

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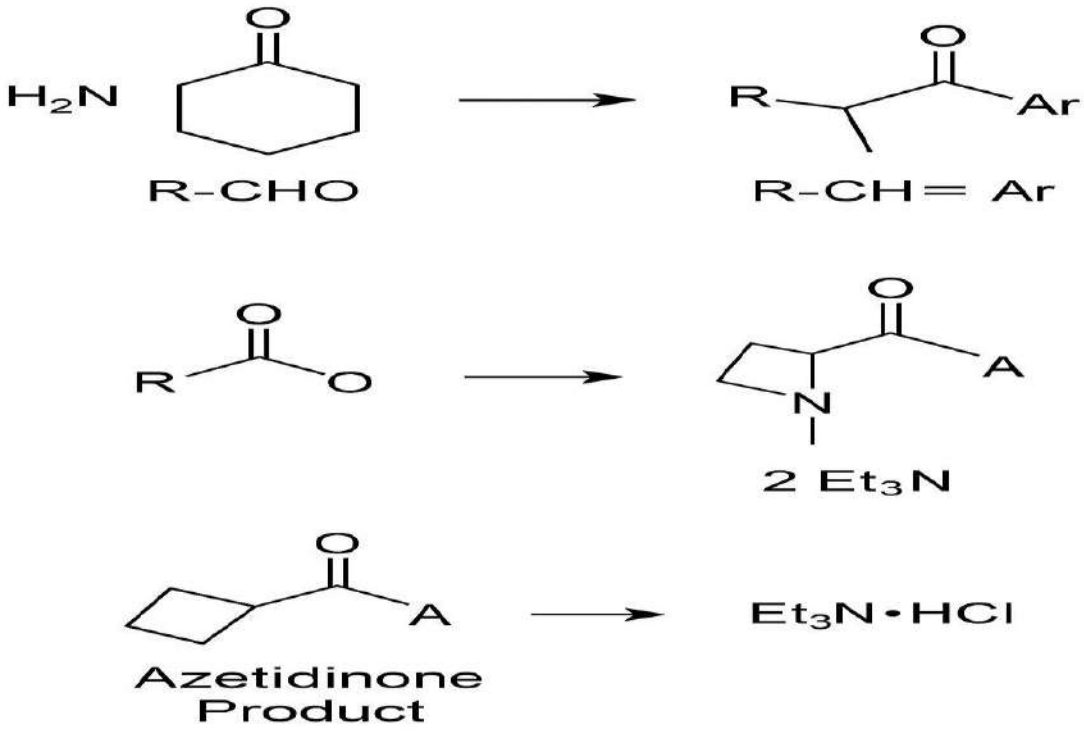
phenolic -OH group, in particular, is a known potent antioxidant pharmacophore due to its ability to stabilize the phenoxyl radical through resonance and hydrogen bonding. The presence of an additional -OCH_3 group in **AD3** (from vanillin) appears to have a synergistic effect, making it the most active compound across all assays.

- **Effect of Electron-Withdrawing Groups (EWGs):** In stark contrast, the introduction of a strong electron-withdrawing group (EWG) like the **-NO_2** group in **AD4** resulted in a dramatic decrease or complete loss of antioxidant activity across all assays. EWGs destabilize the radical intermediate formed after hydrogen atom donation, thereby reducing the compound's efficacy as an antioxidant.
- **Steric and Electronic Factors:** Compounds with moderate EDGs like -$N(CH_3)_2$ (**AD2**, **AD6**) or simple phenyl rings (**AD5**) showed intermediate activity. The position of the substituent also plays a role, as evidenced by the superior activity of **AD3** (3-OCH_3-4-OH) over **AD1** (4-OH).
- **Optimal Structural Features:** The SAR analysis conclusively demonstrates that the **optimal structural feature for potent antioxidant activity in this series is the presence of a phenolic -OH group, preferably ortho or para to the point of attachment, and potentially enhanced by an additional EDG on the aryl ring**. This moiety facilitates hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms, which are fundamental to the observed DPPH scavenging and FRAP reducing power.

In conclusion, the synthesis of novel azetidinone derivatives was successful, and their evaluation revealed several compounds with significant, multi-faceted antioxidant properties. The established SAR provides a robust framework for the future design of even more potent azetidinone-based antioxidant agents.



5. CONCLUSION

The present study successfully demonstrates the rational design, synthesis, and bio-evaluation of a novel series of azetidinone derivatives. The key outcomes of this research are summarized as follows:

1. **Successful Synthesis and Characterization:** A library of twelve novel azetidinone derivatives was efficiently synthesized via a two-step protocol involving Schiff base formation followed by Staudinger ketene-imine cycloaddition. The structures of all synthesized compounds were unequivocally confirmed by comprehensive spectroscopic techniques (FT-IR, 1H1H NMR, 13C13C NMR, and Mass Spectrometry), with spectral data consistently supporting the proposed β-lactam architecture and the trans-stereochemistry of the ring substituents.
2. **Significant Antioxidant Potential:** The in vitro antioxidant evaluation using DPPH, FRAP, and SOD mimetic assays revealed that several derivatives possess remarkable and multifaceted antioxidant activity. Notably, compounds **AD1**, **AD3**, and **AD6** emerged as the most potent, with **AD3** (bearing a 3-OCH_3-4-OH substituent) exhibiting activity comparable to the standard antioxidant BHT in the DPPH assay. This demonstrates the successful translation of our design strategy into functionally active molecules.
3. **Establishment of Preliminary SAR:** A clear and consistent Structure-Activity Relationship (SAR) was established. The antioxidant potency was found to be highly dependent on the electronic nature of the aryl substituents. The presence of strong electron-donating groups (EDGs), particularly phenolic -OH groups, was identified as a critical determinant for enhanced activity, while electron-withdrawing groups (EWGs) like -NO_2 drastically diminished efficacy. This provides a fundamental understanding of the structural requirements for antioxidant behavior within this chemical class.
4. **Foundation for Future Development:** This work solidly establishes the azetidinone scaffold as a promising core for the development of new antioxidant agents. The identified lead compounds, especially **AD3**, and the elucidated SAR provide a strong foundation and a clear direction for the future exploration and optimization of azetidinone-based therapeutics for oxidative stress-related pathologies.

6. FUTURE WORK

Based on the promising in vitro results, the following research directions are proposed to further advance this work towards potential therapeutic applications:

1. **In Vivo Antioxidant Studies:** To validate the efficacy of the lead compounds in a biologically complex system, in vivo studies should be conducted. This would involve animal models of oxidative stress (e.g., carrageenan-induced paw edema, alloxan-induced diabetes, or models of neurodegeneration) to assess their ability to reduce biomarkers of oxidative damage (e.g., MDA levels, protein carbonyl content) and demonstrate a physiological benefit.
2. **Molecular Docking with Antioxidant Enzymes:** Computational studies, specifically molecular docking, should be employed to investigate the potential interaction of the most active azetidinones with key antioxidant enzymes such as Superoxide Dismutase (SOD), Catalase, or NADPH oxidase. This would help propose a molecular mechanism of action, potentially revealing whether the compounds act as direct enzyme mimics or allosteric modulators.
3. **Cytotoxicity Evaluation:** A critical step in the drug development process is the assessment of safety. The lead compounds must be evaluated for

their cytotoxicity against normal human cell lines (e.g., HEK-293, Vero cells) using assays like MTT or SRB. This will determine their therapeutic index and ensure that the antioxidant effects are not accompanied by significant cellular damage.

4. **Formulation Development for Potential Therapeutic Applications:** To enhance the bioavailability, stability, and targeted delivery of the most promising compounds, formulation studies should be initiated. This could involve the development of nano-formulations (e.g., liposomes, polymeric nanoparticles) or conventional dosage forms suitable for intended routes of administration, such as oral tablets for systemic effects or topical gels for dermatological applications related to oxidative stress.

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