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RESEARCH ARTICLE

Synthesis and Antibacterial Screening of Some Novel Isoxazole Derivatives

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Abstract: The emergence of antimicrobial resistance necessitates the development of novel antibacterial agents. This study focuses on the synthesis, characterization, and antibacterial evaluation of a series of N3, N5-di(substituted)isoxazole-3,5-diamine derivatives (178a-f) as potential antibacterial agents. The compounds were synthesized using a two-step process involving the formation of N1, N3-di(substituted)malonamide intermediates followed by cyclization with hydroxylamine hydrochloride under microwave irradiation. All synthesized compounds were thoroughly characterized using spectroscopic techniques including IR, 1H-NMR, and mass spectrometry to confirm their structural integrity. The antibacterial activity of the synthesized compounds was evaluated using the Mueller Hinton broth turbidometric method against both Gram-negative (*Escherichia coli* MTCC 443) and Gram-positive (*Staphylococcus aureus* MTCC 96) bacterial strains. The minimum inhibitory concentration (MIC) was determined for each compound and compared with the standard drug cloxacillin. Among the synthesized derivatives, compounds 178d, 178e, and 178f exhibited the most potent antibacterial activity. Against *E. coli*, these compounds showed MIC values of 117 µg/mL, 110 µg/mL, and 95 µg/mL, respectively, surpassing the activity of cloxacillin (MIC 120 µg/mL). Similarly, against *S. aureus*, compounds 178d and 178e demonstrated superior activity with MIC values of 100 µg/mL and 95 µg/mL, respectively, compared to cloxacillin (MIC 100 µg/mL). Structure-activity relationship analysis revealed that the presence of electron-withdrawing groups (F, Cl) and para-substitution on the phenyl rings generally enhanced antibacterial activity. In conclusion, this study presents a series of novel N3, N5-di(substituted)isoxazole-3,5-diamine derivatives with promising antibacterial activity against both Gram-negative and Gram-positive bacteria.

Keywords: N1, N3-di(substituted)malonamide; N3, N5-di(substituted)isoxazole-3,5-diamine; Microwave irradiation; Anti-bacterial activity; Turbidometric method.

1. Introduction

An infection is the invasion of the body's natural barriers by microscopic organisms like bacteria, fungi, virus etc. which multiply to create symptoms of infectious disease. Microbes which cause disease are known as pathogens [1-2]. In infectious diseases, the pathogenic microorganism penetrates the body surface and gains access to the internal tissues [3]. The treatment of many infectious diseases is challenging due to development of resistance to existing antimicrobial agents. The emergence of resistance among bacteria to a wide variety of structurally unrelated antibacterial agents such as β-lactams, macrolides, tetracyclines and fluoroquinolones as well as selected dyes and disinfectants has become a serious public health concern so makes it necessary to continue the search for new antibacterial agents [4].

From the literature point of view Isoxazole derivative have exhibited various biological activities [5-8]. such as antibacterial (sulfamethoxazole, oxacillin), anticonvulsant(zonisamide), anticholestermic, anticancer, anthelmintic, anti-inflammatory(valdecoxib), adenosine antagonist, fungicidal(drazoxalone), herbicidal, hypoglycemic, muscle relaxant, nematocidal, insecticidal, antiviral(acivicin), antimicrobial(acetylisoxazole), GABAA antagonist, antinociceptive agents, antithrombotic agents. As a part of our research, we herein reporting synthesis and evaluation of series of novel isoxazole derivative as anti-bacterial activity [9].

The development of novel antimicrobial agents is crucial due to the increasing prevalence of multidrug-resistant bacterial strains. Isoxazole derivatives have shown promise as potential antibacterial agents due to their diverse biological activities and synthetic versatility. [10, 11] The isoxazole ring system is present in various natural products and synthetic drugs, making it an attractive scaffold for drug discovery efforts

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2. Material and methods

2.1. Chemicals

All the chemicals were purchased from SD Fine Chemicals and Alfa aesar Mumbai, Thin layer chromatography was carried out using aluminum plates 20 × 20 cm coated with silica gel 60 (Merck). Anti-bacterial activity was carried out using Muller Hinton Broth media.

2.2. Instrumentation

Melting points were determined using open capillaries on a VEP-D VEEGO melting point apparatus and are reported uncorrected. Thin layer chromatography (TLC) was performed on aluminum plates precoated with silica gel GF254 (Merck). TLC spots were visualized under UV light (254 nm and 365 nm) and by exposure to iodine vapor. Infrared (IR) spectra were recorded on a Shimadzu FT-IR 8400S spectrophotometer using potassium bromide (KBr) pellets. The spectra were collected in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per sample. Proton Nuclear Magnetic Resonance (1H-NMR) spectra were obtained on a BRUKER Avance-II 400 MHz spectrometer using DMSO-d6 as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Coupling constants (J) are reported in Hertz (Hz).

Mass spectra were recorded on a Shimadzu 2010EV LCMS instrument operating at an ionization potential of 70 eV. The mass spectrometer was operated in both positive and negative ionization modes, with a scan range of m/z 50-1000. Microwave-assisted reactions were carried out using a CEM Discover microwave reactor. The reaction parameters, including temperature, pressure, and power, were carefully controlled and monitored throughout the synthesis process. All reagents and solvents used in this study were of analytical grade and used without further purification unless otherwise specified. Anhydrous reactions, when necessary, were performed under a nitrogen atmosphere using oven-dried glassware.

2.3. Method of synthesis

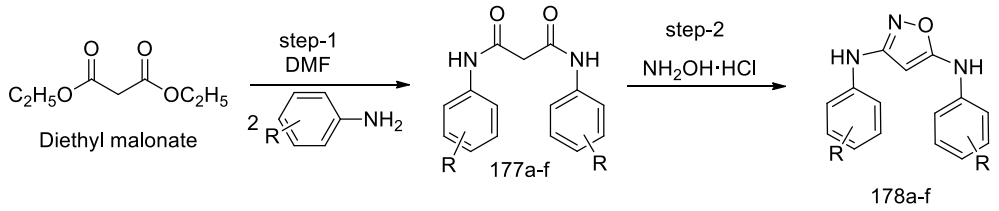

Figure 1. Scheme of synthesis for series of N3, N5-di(substituted)isoxazole-3,5-diamine derivatives

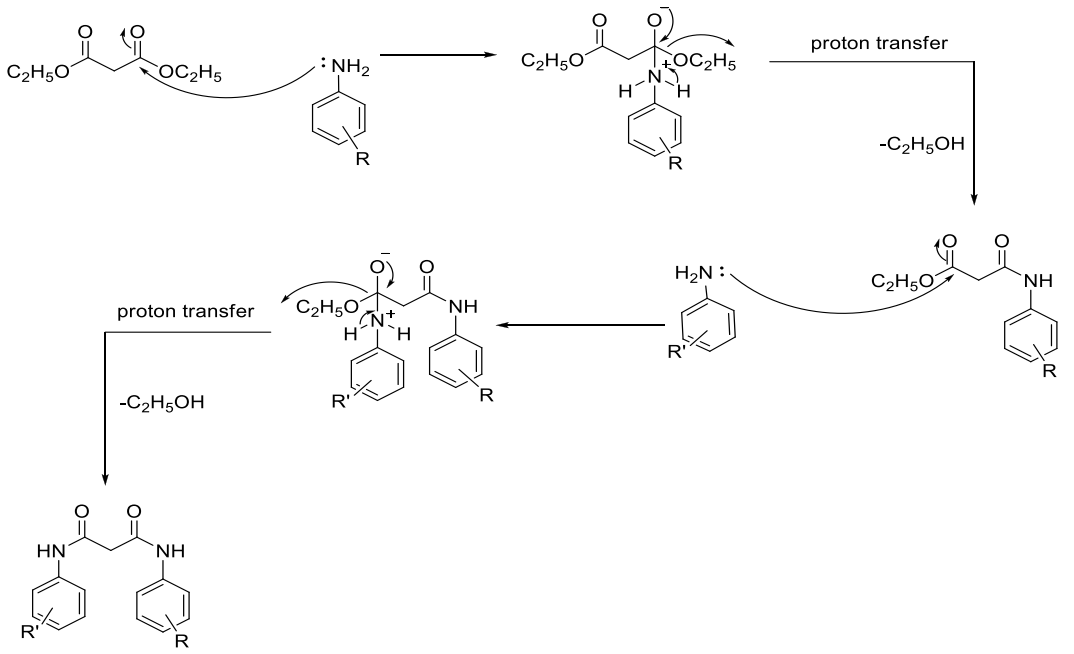

Figure 2. Mechanism of N1, N3-di(substituted)malonamide

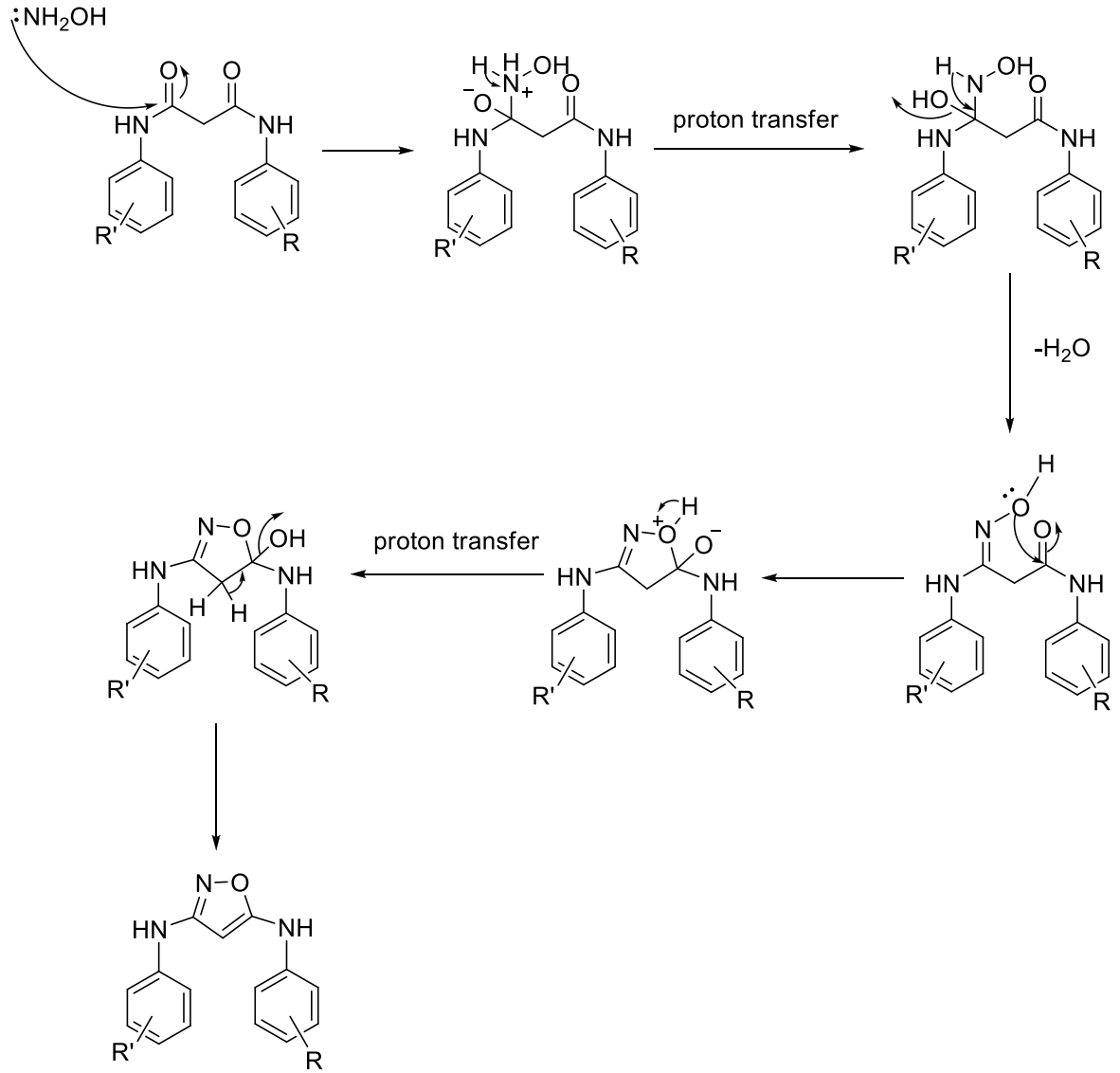

Figure 3. Mechanism of N3, N5-di(substituted)isoxazole-3,5-diamine

2.4. Synthesis of N^1, N^3-di(substituted) malonamide derivatives (177a-f)

In round bottom flask, diethylmalonate(1 mmol) and aromatic amine(2 mmol) were refluxed in DMF(2 mL) for 8-10h. The progress of reaction was monitored by TLC(n-hexane:ethylacetate::7:3V/V). After completion of reaction, the reaction mixture was poured in ice-cold water. [12-14] The solid obtained was filtered, dried and washed with n-hexane, to obtain pure product. [15] It was identified as N1, N3- di(substituted)malonamide. Scheme is given in Figure 1 and results are shown in Table 2.

2.5. Synthesis of N^3, N^5-di(substituted)isoxazole-3,5-diamine derivatives (178a-f)

In round bottom flask, N1, N3-di(substituted)malonamide(1mmol) and hydroxylamine hydrocholoride(1.1mmol) in nitrobenzene(2 mL) were refluxed under microwave irradiation for 1-1.5 h at 120 W. The progress of reaction was monitored by TLC. After completion of reaction, excess of solvent was distilled under vacuum distillation and residue was directly taken in ethylacetate or after adding water extracted with ethylacetate. [16-18] Solid was obtained and recrystallized it with n-hexane and it was characterized as N3, N5- di(substituted)isoxazole-3,5-diamine. Results are shown in Table 3.

2.6. Biological evaluation

Anti-bacterial activity was carried out by Turbidometric method using Mueller Hinton Broth nutrient media and it was prepared from below formula (Table 1).

Table 1. Composition of Mueller Hinton Broth nutrient agar media

Component	Quantity (for 1000 mL)
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	q.s.
pH (after sterilization)	6.5-6.6

2.7. Methods used for primary and secondary screening

Each synthesized compound was diluted to obtain 1000µg/ml concentration as a stock solution. In primary screening 200µg/ml, 100 µg/ml, 50µg/ml, 25µg/ml and 12.5 µg/ml, concentrations of the synthesized compounds were taken. [19-21] The significantly active compound in primary screening was further tested in a second set of dilution against all microorganisms. The compounds found active in primary screening were similarly diluted to obtain 6.25µg/ml and 3.125µg/ml concentrations.

2.8. Structure-Activity Relationship (SAR) Analysis

To understand the relationship between chemical structure and antibacterial activity, we performed a preliminary SAR analysis. The effects of various substituents on the phenyl rings were examined, including electron-donating groups (methyl, methoxy) and electron-withdrawing groups (fluoro, chloro). [22-24] The position of these substituents (ortho, para) was also considered in relation to the observed antibacterial activity.

3. Results

The characteristics of N1, N3-di(substituted) malonamide derivatives are displayed in Table 2.

Table 2. Physical data of N1, N3-di(substituted) malonamide derivatives

Compound	-R	Molecular weight	Yield (%)	M. P. (^{0}C)	R_f value (*n*-hexane: ethylacetate ::7:3% v/v)
177a	-H	254.00	87	239-243	0.48
177b	p-CH_3	282.00	87	235-240	0.40
177c	p-OCH_3	314.34	80	226-229	0.48
177d	o-CH_3, p-OCH_3	310.39	75	215-219	0.44
177e	p-F	290.00	85	207-211	0.50
177f	p-Cl	323.17	80	260-262	0.41

3.1. N1, N3-bis(p-methoxyphenyl)malonamide (177c):

IR data (KBr, cm^{-1}): 3222.17 (-NH- stretch), 2883.38 (-CH stretch), 2758.19 (-OCH_3 stretch), 1681.93 (-C=O stretch); 1H NMR data (δ ppm, DMSO-d_6): 7.50-7.42 (d, 4H, Ar-H), 7.25 (s, 2H, -NH-), 6.96-6.88 (d, 4H, Ar-H), 3.89 (s, 6H, -OCH_3), 3.65 (s, 2H, -CH_2-); Mass Spectral Data m/z: 315 (M+1)

Table 3: Physical data of N^3, N^5-di(substituted)isoxazole-3,5-diamine derivatives

Compound	-R	Molecular weight	Yield (%)	M. P. (^{0}C)	R_f value (*n*-hexane :ethylacetate::3:7V/V)
178a	-H	251.00	25	82-86	0.83
178b	p-CH_3	279.00	23	135-140	0.81
178c	p-OCH_3	311.34	30	120-122	0.47
178d	o-CH_3, p-OCH_3	307.39	28	142-145	0.54
178e	p-F	287.00	40	143-146	0.87
178f	p-Cl	320.17	25	135-138	0.70

3.2. N3,N5-diphenylisoxazole-3,5-diamine (178a):

IR data (KBr, cm-1): 3294.19 (-NH- stretch), 1653.67 (-C=N- stretch), 3074.32 (Ar C–H stretch); 1H NMR data (δ ppm, DMSO-d6): 7.63-7.55 (d, 4H, Ar-H), 7.21-7.27 (t, 4H, Ar-H), 6.66-7.16 (m, 2H, Ar-H), 6.75 (s, 1H, Ar-H), 4.1 (s, 2H, -NH-); Mass Spectral Data m/z: 252 (M+1)

3.3. N3, N5-di-p-tolylisoxazole-3,5-diamine (178b):

IR data (KBr, cm-1): 3294.19 (-NH- stretch), 1650.95 (-C=N- stretch), 2866.02 (-CH3 stretch); 1H NMR data (δ ppm, DMSO-d6): 7.37-7.45 (d, 4H, Ar-H), 7.29-7.20 (d, 4H, Ar-H), 6.72 (s, 1H, Ar-H), 4.3 (s, 2H, -NH-), 2.35 (s, 6H, -CH3); Mass Spectral Data m/z: 280 (M+1)

3.4. N3,N5-di-p-methoxyisoxazole-3,5-diamine (178c):

IR data (KBr, cm-1): 3242.12 (-NH- stretch), 1606.59 (-C=N- stretch), 2836.16 (-OCH3 stretch); 1H NMR data (δ ppm, DMSO-d6): 7.52-7.44 (d, 4H, Ar-H), 7.00-7.08 (d, 4H, Ar-H), 6.74 (s, 1H, Ar-H), 3.99 (s, 2H, -NH-), 3.81 (s, 6H, -OCH3); Mass Spectral Data m/z: 252 (M+1)

3.5. N3,N5-bis(2,4-di-methyphenyl)lisoxazole-3,5-diamine (178d):

IR data (KBr, cm-1): 3250 (-NH- stretch), 1652 (-C=N- stretch), 2900 (-CH3 stretch); 1H NMR data (δ ppm, DMSO-d6): 6.87 (s, 2H, Ar-H), 6.81-6.73 (d, 2H, Ar-H), 6.59-6.51(d, 2H, Ar-H), 6.69 (s, 1H, Ar-H), 4.03 (s, 2H, -NH-), 2.36 (s, 6H, -CH3), 2.10 (s, 6H, -CH3); Mass Spectral Data m/z: 308 (M+1)

3.6. N3,N5-bis(p-fluorophenyl)lisoxazole-3,5-diamine (178e):

IR data (KBr, cm-1): 3288.40 (-NH- stretch), 1637.45 (-C=N- stretch), 1022.20 (-F stretch); 1H NMR data (δ ppm, DMSO-d6): 7.53-7.45 (d, 4H, Ar-H), 7.35-7.42 (d, 4H, Ar-H), 6.80 (s, 1H, Ar-H), 3.98 (s, 2H, -NH-); Mass Spectral Data m/z: 288 (M+1)

3.7. N3,N5-di-p-chloroisoxazole-3,5-diamine (178f):

IR data (KBr, cm-1): 3269.12 (-NH- stretch), 1668.30 (-C=N- stretch), 752.19 (-Cl stretch); 1H NMR data (δ ppm, DMSO-d6): 7.66-7.58 (d, 4H, Ar-H), 7.32-7.25 (d, 4H, Ar-H), 6.69 (s, 1H, Ar-H), 3.99 (s, 2H, -NH-); Mass Spectral Data m/z: 319 (M-1)

Table 4. Minimum Inhibitory Concentrations (µg\mL) of different N3,N5-di(substituted)isoxazole-3,5-diamine

Compound Code	Minimum Inhibitory Concentrations (µg/mL)	
	***E. coli* MTCC 443**	***S. aureus* MTCC 96**
178a	140	120
178b	125	110
178c	125	110
178d	117	100
178e	110	95
178f	95	115
Cloxacillin	**120**	**100**

3.8. Structure-Activity Relationship Analysis

Electron-withdrawing groups (F, Cl) generally enhanced antibacterial activity compared to unsubstituted analogs. Para-substitution appeared more favorable than ortho-substitution for improving potency. [25] The presence of multiple substituents (e.g., compound 178d with o-CH_3 and p-OCH_3) resulted in improved activity, suggesting potential synergistic effects. Compounds with para-fluoro (178e) and para-chloro (178f) substituents showed the most promising antibacterial activity, possibly due to increased lipophilicity and membrane permeability [26,27]

3.9. Discussion

Based on an extensive literature survey revealing the antibacterial potential of various substituted isoxazole derivatives, we designed and synthesized a series of novel N3, N5-diarylisoxazole-3,5-diamines. The synthetic route involved a two-step process, starting with the formation of N1, N3-di(substituted)malonamide intermediates, followed by cyclization with hydroxylamine hydrochloride under microwave irradiation. The physical and spectroscopic data of all synthesized compounds are presented in Tables 2 and 3, respectively. All synthesized compounds were subjected to antibacterial screening using the Mueller Hinton broth turbidometric

method. The evaluation was conducted against both Gram-negative (*Escherichia coli* ATCC 25922) and Gram-positive (*Staphylococcus aureus* ATCC 29213) bacterial strains. The minimum inhibitory concentration (MIC) values for each compound are summarized in Table 4.

The synthesized compounds exhibited varying degrees of antibacterial activity against both tested strains. Against E. coli, the MIC values ranged from 95 µg/mL to 140 µg/mL, while for S. aureus, the range was 95 µg/mL to 120 µg/mL. These results were compared with the standard drug cloxacillin, which showed MIC values of 120 µg/mL and 100 µg/mL against *E. coli* and *S. aureus*, respectively.

Among the synthesized derivatives, compounds 178d, 178e, and 178f emerged as the most potent against E. coli, with MIC values of 117 µg/mL, 110 µg/mL, and 95 µg/mL, respectively. Notably, compound 178f demonstrated superior activity compared to the standard drug cloxacillin. Against S. aureus, compounds 178d and 178e showed the highest potency, with MIC values of 100 µg/mL and 95 µg/mL, respectively. Compound 178e exhibited activity comparable to cloxacillin against S. aureus. A preliminary structure-activity relationship (SAR) analysis revealed that the presence of electron-withdrawing groups (such as fluorine and chlorine) at the para position of the phenyl rings generally enhanced antibacterial activity. [28] This observation was particularly evident in compounds 178e and 178f, which showed the highest potency against both bacterial strains. The other synthesized compounds displayed moderate to weak antibacterial activity compared to the standard drug.

4. Conclusion

In this study, we successfully synthesized and characterized a series of novel N3,N5-di(substituted)isoxazole-3,5-diamine derivatives. The antibacterial screening revealed that several compounds exhibited promising activity against both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacterial strains. Notably, compounds 178d, 178e, and 178f demonstrated superior or comparable activity to the standard drug cloxacillin. The structure-activity relationship analysis provided valuable insights into the impact of various substituents on antibacterial activity. The enhanced potency observed with electron-withdrawing groups and para-substitution offers a rational basis for further structural optimization

Compliance with ethical standards (WJS-I-Heading no numbering)

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Conflict of interest statement (optional) (WJS-I-sub heading no numbering)

The authors declare that there is no conflict of interest.

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