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# Synthesis and evaluation of 3-methyl-4-nitro-5-(substitutedstyryl) isoxazoles for antioxidant and anti-inflammatory activities

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**ABSTRACT**

A series of 3-methyl-4nitro-5-(substitutedstyryl)isoxazoles were synthesized and evaluated for antioxidant, anti-inflammatory and analgesic activities with a view to evaluate effect of nitro substitution on styrylisoxazoles. Compounds with sterically hindered phenolic groups exhibited good anti-inflammatory activity with better antioxidant properties and are devoid of toxicity as well as ulcerogenic potential.

**Keywords:** Styrylisoxazole, Antioxidant properties, Anti-inflammatory agents

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## INTRODUCTION

Isoxazoles are a class of heterocyclic compounds having a wide a range of pharmacological activities such as hypothermic, analgesic, anti-inflammatory, antitussive [1], antibacterial [2], antiviral [3] and inhibition of p38α mitogen-activated protein kinase [4]. A careful literature survey revealed that various substituted styrylisoxazoles were synthesized through molecular modeling studies, as analogs of curcumin, with potential anti-inflammatory activity, devoid of ulcerogenicity [5]. Recently isoxazole analogs of curcuminoids were synthesized and evaluated for antioxidant, COX-1/COX-2 (Cyclooxygenage) inhibitory activity and anti-inflammatory activity [6]. Later, curcumin derived isoxazole was prepared and evaluated for antitumor activity [7, 8]. From the above study it was found that isoxazole analog of curcumin exhibited significant anti-inflammatory activity with higher antioxidant properties. Further, the literature survey on styryl compounds revealed that ß-nitro styrenes were reported as inhibitors of free radical polymerization [9] and inhibitors of human telomerase [10]. Keeping these facts in view, it was planned to synthesize substituted styrylisoxazoles possessing a nitro group on isoxazole ring and to evaluate them for antioxidant, anti-inflammatory activities as well as ulcerogenicity and toxicity.

## MATERIALS AND METHODS

All the chemicals for synthesis of title compounds and for the biological evaluations are of Analar grade, they were procured from sd fine chemicals and/or Sigma Aldrich. The title compounds were prepared according to the available literature [11]. Rat paw edema was induced by using type IV lambda carrageenan procured from Sigma chemicals.

### Animals

Male Wistar albino rats (150-200g) and Swiss albino mice (18-25 g) of either sex were used for the study, obtained from Venkateshwara Enterprises, Bangalore, and Karnataka. After ten days of acclimatization, the animals were used for further experiments as per the CPCSEA guidelines and approval was obtained from Institutional Animal Ethics Committee (Regd No. 1016/ a/ 06/ CPCSEA/ 15/ 2009).

### Biological activities

***In-vitro*** **Antioxidant activity:** The title compounds were evaluated for *in vitro* antioxidant activity in three different models viz DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging [12], NO (Nitric Oxide) free radical scavenging [13] and iron induced lipid peroxidation using rat brain homogenate [14].

**Assay of DPPH radical scavenging:** The test compounds at 100 µM concentration were added to 100 µM DPPH in 95% ethanol and tubes were kept at ambient temperature for 20 minutes. The absorbance of the incubated solutions was measured at 517 nm. The absorbance of test

compounds were compared with the absorbance of vehicle control and the results were expressed as mean of triplicate.

**Assay of NO radical scavenging:** Sodium nitroprusside (10 µM) in phosphate buffer pH 7.4 was incubated with 100 µM concentration of drug dissolved in a suitable solvent (dioxan/methanol) and tubes were incubated at 25°C for 150 minutes. Control experiment was kept without test compound but equal amount of solvent was added in an identical manner. 2 ml of incubation solution was removed and diluted with 2 ml of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent naphthylethylenediamine was read at 546 nm.

**Iron induced lipid peroxidation in rat brain homogenate:** A 10% w/v rat brain homogenate was prepared in 0.15 M KCl and centrifuged at 800 *g* for 10 minutes and the supernatant was used immediately for the study. The incubation mixture contained in a final volume of 1.5 ml, brain homogenate (0.5 ml, 10% w/v), KCl(0.15M) and ethanol (10 µl) or test compound dissolved in ethanol. Peroxidation was initiated by adding to give the final concentrations stated, ferric chloride (100 µM). After incubating for 20 minutes at 37°C, reactions were stopped by adding 2 ml of ice cold 0.25 M HCl containing15% trichloroacetic acid(TCA), 0.38% thiobarbituric acid(TBA) and 0.05% butylated hydroxyl toluene (BHT). Following heating at 80°C for 15 minutes samples were cooled and centrifuged at 1000 *g* for 10 minutes. The absorbance of supernatant was measured at 532 nm. Percentage inhibition of thiobarbituric acid reactive substances formed by test compounds was calculated by comparing with vehicle control experiments.

**Anti-inflammatory Activity:** Carrageenan induced rat foot paw edema assay of Winter et al was used to study the anti-inflammatory activity of the title compounds [15].

**Carrageenan Foot paw edema Assay:** The male Wistar rats weighing 150-180 gm were divided into groups of five or six animals. One group consisting of six animals served as vehicle control, while the other groups of five animals received the test compound or standard drug. The rats were dosed with compounds (100 mg/kg in 0.5% sodium carboxy methyl cellulose) one hour before injection of 0.05 ml of 1% suspension of carrageenan into the subplantar region of the rat hind paw. Additional groups were similarly treated with 100 mg/kg ibuprofen (positive control) or 10 ml/kg 0.5% sodium carboxy methyl cellulose (vehicle control). Initial paw volumes were measured immediately following carrageenan challenge by water displacement in a plethysmograph. The paw volumes were again measured after 3 hours. The average edema volumes for test group animals and positive control group were compared statistically with those of the vehicle control animals and expressed as the percent edema inhibition.

**Gastric Ulcerogenicity:** Male wistar rats weighing 180-200 gm were fasted for 24 hours. After fasting, test compounds as well as standard drugs were administered orally at a dose of 100mg/kg and the rats were denied access to food for six more hours. The rats were then sacrificed; stomachs were removed, opened along the greater curvature and observed for the presence of gastric lesions on the mucosa [16].

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**Acute Toxicity** Acute toxicity of the test compounds was determined in mice (18-25gm) by administering the compounds at doses of 1000mg/kg and 750mg/kg *p.o.* The animals were observed for their death over a period of seven days.

## EXPERIMENTAL

The melting points were determined in open capillaries and were uncorrected. The purity of compounds was checked by TLC. UV spectra of the compounds were obtained from systronic UV-VIS spectrophotometer. IR spectra were run in KBr pellet on a Thermo Nicolet Nexus 670 spectrophotometer. The $^1$H NMR spectra of the compounds in $CDCl_3$ were measured in Avance 300 MHz spectrophotometer. The Mass spectra were recorded using VG-Autospec-M Mass spectrophotometer.

**General procedure for the synthesis of 3-methyl-4-nitro-5-(substituted)styryl isoxazole:**

To pure dry powdered 3,5-dimethyl-4-nitro isoxazole (4 gm; 0.028 mol) dissolved in alcohol, an equimolar amount of substituted benzaldehyde (0.028 mol) was added, the reactants were thoroughly mixed and heated for 0.5 to 4 hr, on boiling water bath. The solution was cooled to room temperature and separated product was filtered, washed with cold alcohol. The crude compound was recrystallized from suitable solvent.

**3-Methyl-4-nitro-5-styrylisoxazole (5a):** $C_{12}H_{10}N_2O_3$, yield 84%, m.p 156°C (lit 155°C) [17]; IR (KBr) $cm^{-1}$: 1630 (C=C stretching), 1579 (C=N stretching), 1505 ($NO_2$ asymmetric stretching), 1357 ($NO_2$ symmetric stretching), 971 (H-C=C- H bending); Mass: m/z230$M^+$.

**3-Methyl-4-nitro-5-(4-chlorostyryl)isoxazole(5b):** $C_{12}H_9ClN_2O_3$, yield 82%, m.p 176°C (lit 172°C) [17]

**3-Methyl-4-nitro-5-(4-methylstyryl)isoxazole (5c):** $C_{13}H_{12}N_2O_3$, yield 88%, m.p 160°C (lit 155°C) [17]

**3-Methyl-4-nitro-5-(4-methoxystyryl)isoxazole (5d):** $C_{13}H_{12}N_2O_4$, yield 87%, m.p 152°C (lit 148°C) [17]; IR (KBr) $cm^{-1}$:1633 (C=C stretching), 1597 (C=N stretching), 1508 ($NO_2$ asymmetric stretching), 1372 ($NO_2$ symmetric stretching), 1024(Ar-O stretching), 970 (H-C=C-H bending); Mass: m/z 260$M^+$.

**3-Methyl-4-nitro-5-(4-hydroxystyryl)isoxazole (5e):** $C_{12}H_{10}N_2O_4$, yield 76%, m.p 229°C; IR (KBr) $cm^{-1}$: 3273(O-H stretching), 1629 (C=C stretching), 1575 (C=N stretching), 1507 ($NO_2$ asymmetric stretching), 1354 ($NO_2$ symmetric stretching), 972 (H-C=C-H bending).

**3-Methyl-4-nitro-5-(2-hydroxystyryl)isoxazole (5f):** $C_{12}H_{10}N_2O_4$, yield 78%, m.p 232°C(lit 230°C) [17]; IR (KBr) $cm^{-1}$: 3232 (O-H stretching), 1600 (C=C stretching), 1572 (C=N stretching), 1500 ($NO_2$ asymmetric stretching), 1363($NO_2$ symmetric stretching), 977 (H-C=C-H bending); Mass:

m/z 246$M^+$; $^1$H NMR ($CDCl_3$) δ: 2.59 ( s, 3H, $CH_3$), 6.70-7.58 ( m, 4H, Ar-H), 7.85 ( d,1H, H-C=C-H; J = 16.6 Hz ), 8.05 ( d,1H, H-C=C-H; J = 16.6 Hz), 9.95 (br. s, 1H, OH).

**3-Methyl-4-nitro-5-(4-dimethylaminostyryl)isoxazole (5g):** $C_{14}H_{15}N_3O_3$, yield 85%, m.p 198°C; IR (KBr) $cm^{-1}$: 1566 (C=C stretching), 1485 (C=N stretching), 1435 ($NO_2$ asymmetric stretching), 1352($NO_2$ symmetric stretching), 964 (H-C=C-H bending); Mass: m/z 273$M^+$

**3-Methyl-4-nitro-5-(4-isopropylstyryl)isoxazole (5h):** $C_{15}H_{16}N_2O_3$, yield 69%, m.p 272°C; IR (KBr) $cm^{-1}$: 1630(C=C stretching), 1604 (C=N stretching), 1581 ($NO_2$ asymmetric stretching), 1363 ($NO_2$ symmetric stretching), 969 (H-C=C-H bending); Mass: m/z 272$M^+$

**3-Methyl-4-nitro-5-(3,4-dimethoxystyryl)isoxazole (5i):** $C_{14}H_{14}N_2O_5$, yield 86%, m.p 181°C; IR (KBr) $cm^{-1}$: 1631(C=C stretching), 1593 (C=N stretching), 1516 ($NO_2$ asymmetric stretching), 1357 ($NO_2$ symmetric stretching), 965 (H-C=C-H bending); Mass: m/z 290$M^+$; $^1$H NMR ($CDCl_3$) δ: 2.60 ( s, 3H, $CH_3$), 3.97 (d, 6H, $OCH_3$), 6.91-7.29( m, 3H, Ar-H), 7.55( d,1H, H-C=C-H J = 16.6 Hz; ), 7.77 ( d,1H, H-C=C-H; J = 16.6 Hz).

**3-Methyl-4-nitro-5-(3,4,5-trimethoxystyryl)isoxazole (5j):** $C_{15}H_{16}N_2O_6$, yield 87%, m.p 179°C; IR (KBr) $cm^{-1}$: 1626 (C=C stretching), 1558 (C=N stretching), 1508 ($NO_2$ asymmetric stretching), 1354 ($NO_2$ symmetric stretching), 965 (H-C=C-H bending); Mass: m/z 320 $M^+$; $^1$H NMR ($CDCl_3$) δ: 2.60 ( s, 3H, $CH_3$), 3.93 (s, 9H, $OCH_3$), 6.87( s, 2H, Ar-H), 7.55( d,1H, H-C=C-H J = 16.6 Hz; ), 7.73 ( d,1H, H-C=C-H; J = 16.6 Hz).

**3-Methyl-4-nitro-5-(4-hydroxy-3-methoxystyryl)isoxazole (5k):** $C_{13}H_{12}N_2O_5$, yield 78%, m.p 196°C; IR (KBr) $cm^{-1}$: 3216 (O-H stretching), 1625 (C=C stretching), 1592 (C=N stretching), 1511 ($NO_2$ asymmetric stretching), 1355 ($NO_2$ symmetric stretching), 1272 (Ar-O stretching), 963 (H-C=C-H bending); Mass: m/z 275 $M^+$; $^1$H NMR ($CDCl_3$) δ: 2.60 ( s, 3H, $CH_3$), 3.99 (s, 3H, $OCH_3$), 5.99 (s, 1H, OH), 6.97-7.22( m, 3H, Ar-H), 7.51( d,1H, H-C=C-H J = 16.6 Hz; ), 7.73 ( d,1H, H-C=C-H; J = 16.6 Hz).

**3-Methyl-4-nitro-5-(5-bromo-4-hydroxy-3-methoxy styryl) isoxazole (5l):** $C_{13}H_{11}BrN_2O_5$, yield 88%, m.p 168°C; IR (KBr) $cm^{-1}$: 3447 (O-H stretching), 1630 (C=C stretching), 1597 (C=N stretching), 1508 ($NO_2$ asymmetric stretching), 1352 ($NO_2$ symmetric stretching), 966 (H-C=C-H bending).

**3-Methyl-4-nitro-5-(4-hydroxy-3,5-dimethoxystyryl) isoxazole (5m):** $C_{14}H_{14}N_2O_6$, yield 71%, m.p 181°C; IR (KBr) $cm^{-1}$: 3523 (O-H stretching), 1624 (C=C stretching), 1579 (C=N stretching); 1514 ($NO_2$ asymmetric stretching), 1353 ($NO_2$ symmetric stretching), 1115 (Ar-O stretching), 958 (H-C=C-H bending); Mass: m/z 306 $M^+$; $^1$H NMR ($CDCl_3$) δ: 2.25 ( s, 3H, $CH_3$), 3.50 (s,6H, $OCH_3$), 5.99 (s, 1H, OH), 6.55 ( s, 2H, Ar-H), 7.10( d,1H, H-C=C-H J = 16.6 Hz; ), 7.37 ( d,1H, H-C=C-H; J = 16.6 Hz).

**3-Methyl-4-nitro-5-(4-hydroxy-3,5-di-tert-butylstyryl) isoxazole (5n):** $C_{20}H_{26}N_2O_4$, yield 73%, m.p 200°C; IR (KBr) $cm^{-1}$: 3526 (O-H stretching), 1624 (C=C stretching), 1577 (C=N stretching) ,

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1509 ($NO_2$ asymmetric stretching), 1355 ($NO_2$ symmetric stretching), 970 (H-C=C-H bending); Mass: m/z 306 $M^+$; $^1H$ NMR ($CDCl_3$) δ: 1.45 (s, 18H, di-tert-butyl), 2.50 ( s, 3H, $CH_3$), 5.60 (s, 1H, OH), 6.89-7.19 ( m, 2H, Ar-H), 7.41 ( d,1H, H-C=C-H; J = 16.6 Hz), 7.70 (d,1H, H-C=C-H; J = 16.6 Hz).

## RESULTS AND DISCUSSION

### Chemistry

The starting material 3,5-dimethylisoxazole was prepared by the cyclization of acetylacetone with hydroxylamine hydrochloride in aqueous alcohol. Nitration of 3, 5-dimethylisoxazole has been carried out with fuming nitric acid and concentrated sulphuric acid at 0°C to give 3,5-dimethyl-4-nitroisoxazole. This compound was condensed with various substituted benzaldehydes in absolute alcohol in presence of catalytic amounts of piperidine. The reaction was carried out on hot water bath at reflux temperature for half an hour to four hours to yield respective 5-styrylisoxazole but not 3-styrylisoxazole. The reason was well documented that 5-methyl group of 3,5-dimethylisoxazole was in conjugation with the nitro group and hence the carbanion was stabilized. On the other hand, the 3-methyl group which cannot be activated by the nitro group does not take part in the reaction [18].

Fourteen compounds were synthesized and characterized by their melting points, IR, $^1H$ NMR and Mass spectra. Among the synthesized compounds, few compounds (**5a, 5b**, **5c**, **5d** and **5f**) were reported in literature [17].

The IR spectra of the synthesized compound exhibited an absorption band at a region 1600 - 1633 $cm^{-1}$ and 1566 - 1604 $cm^{-1}$ indicative of C=C and C=N bonds respectively. The spectra exhibited characteristic absorption bands at 1485 – 1516 $cm^{-1}$ and 1352 - 1377 $cm^{-1}$ assignable to asymmetric and symmetric stretching of nitro group. The spectra also revealed the presence of an intense band at 958-977 $cm^{-1}$ characteristic of bending vibrations of styryl group, thus indicating E-configuration. The spectra of compounds **5e**, **5f, 5k**, **5l**, **5m** and **5n** showed absorption bands at 3216 - 3529 $cm^{-1}$ assigned to OH stretching of phenolic group.

The $^1H$ NMR spectrum of compounds **5f, 5i**, **5j**, **5k**, **5m** and **5n** revealed the presence of methyl protons as singlet at a region of δ 2.25-2.60 and two trans-olefinic protons as doublets at δ 7.10-7.85 and 7.37-8.05 with J value 16.6 Hz. The aryl protons appeared in the region of δ 6.55 to 7.58. The spectra of compounds **5i**, **5j**, **5k** and **5m** exhibited peaks at a region of δ 3.50 to 3.99 due to methoxy protons. Further, the spectra of phenolic compounds **5k**, **5m** and **5n** showed a singlet at δ 5.60 to 5.99 assignable to phenolic hydroxyl group, where as compound **5f** exhibited its phenolic hydroxyl proton at δ 9.95. The mass spectra of compounds (**5a**, **5d**, **5f**, **5g**, **5h**, **5i, 5j**, **5k**, **5m**,and **5n**) showed their characteristic molecular ion peaks.

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**Biological activities**

3-methyl-4-nitro-5-(substitutedstyryl)isoxazoles (**5a-5n**) were examined for antioxidant activity by different *in vitro* models such as DPPH free radical scavenging, NO free radical scavenging and iron induced lipid peroxidation in rat brain homogenate. All the compounds were also screened for the *in vivo* anti-inflammatory activity by carrageenan rat paw edema assay and the most active compounds were further evaluated for gastric ulcerogenicity in rats and acute toxicity in mice.

Table 1 summarizes the antioxidant activity evaluated in three different *in vitro* models at 100 µM concentration of test compounds. From the activity data it is evident that, compounds **5n, 5m** and **5k** displayed potent inhibition of lipid peroxidation. This result was also consistent with other two models, DPPH and NO scavenging. The greater activity of above compounds was attributed to the presence of sterically hindered phenolic group. The anti-inflammatory activity data was also presented in table 1. The data showed statistically significant anti-inflammatory activity with compounds **5n, 5k** and **5m** in increasing order, at a dose 100 mg/kg, p.o. The anti-inflammatory activity of compound **5k** was better explained by the presence of 4-hydroxy, 3-methoxy groups on styrene structure similar to that of styryl carbonyl moiety of curcumin.

Introduction of alkyl or alkoxy substituents at both ortho position of phenolic hydroxyl group results in sterically hindered phenolic compounds. A number of compounds possessing sterically hindered phenolic moiety were reported to possess potent antioxidant and anti-inflammatory activities [5]. In view of potential biological activities of these molecules, compounds **5l, 5m** and **5n** were synthesized and evaluated. Compound **5m** possesses additional methoxy substitution at $5^{th}$ position when compared to **5k**. This resulted in significance increase in anti-inflammatory and antioxidant activities. Introduction of bromo substitution at $5^{th}$ position of **5k** slightly decreases the antioxidant and anti-inflammatory activities, thus indicating the influence of the nature of the substituents on biological activity. The presence of bulky alkyl groups at both ortho positions of phenolic hydroxyl group as seen in compound **5n** enhances the antioxidant activity. However, a decrease in anti-inflammatory activity was observed which may be due to the lipophilicity of di-tert-butyl groups at 3 and 5 positions. The active compounds **5k, 5m** and **5n** were evaluated for ulcerogenicity and acute toxicity. Interestingly, these compounds exhibited low ulcerogenicity at a dose of 100mg/kg, p.o. and found to be less toxic.

On observation of the results obtained, compounds with simple alkyl substitution such as 4-methyl and 4-isopropyl derivatives (**5c** and **5h**) possess insignificant antioxidant and anti-inflammatory activities, whereas N,N-di-alkyl substitution increases the activity. Compound **5g** containing 4-N,N-di-methyl substituent exhibited moderate activity**.** Compounds possessing only phenolic substitution such as **5e** and **5f** also exhibited moderate activity. Replacement of phenolic hydroxyl substitution at $4^{th}$ position of **5e** to methoxy group, as in compound **5d** substantially lowers the antioxidant and anti-inflammatory activities. Similar results were found for compounds **5i** and **5j**. This clearly indicates that the phenolic moiety of these

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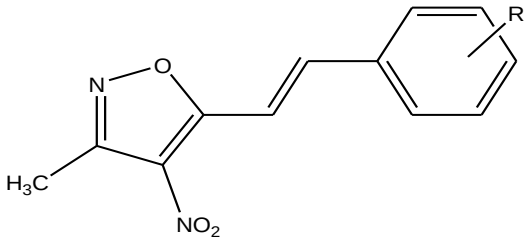
compounds confer antioxidant and radical scavenging properties which have been proposed to be relevant to their anti-inflammatory efficacy and low ulcerogenicity.

**Table 1: Antioxidant and anti-inflammatory activity data**




| Compound | R | % inhibition of DPPH | % inhibition of NO | % inhibition of LP | % inhibition of Edema* |
|---|---|---|---|---|---|
| 5a | 4-H | NA | 05.18 | 14.95 | 33.3 |
| 5b | 4-Cl | NA | NA | 12.24 | 21.4 |
| 5c | 4-$CH_3$ | NA | NA | 13.74 | 27.5 |
| 5d | 4-$OCH_3$ | 18.30 | 11.82 | 26.58 | 23.8 |
| 5e | 4-OH | 23.81 | 23.43 | 31.95 | 44.2 |
| 5f | 2-OH | 21.46 | 13.26 | 29.87 | 37.5 |
| 5g | 4-N $(CH_3)_2$ | 20.95 | 31.24 | 45.56 | 45.0 |
| 5h | 4-CH $(CH_3)_2$ | NA | NA | 13.76 | 27.5 |
| 5i | 3,4-$(OCH_3)_2$ | 23.50 | 15.80 | 30.76 | 32.5 |
| 5j | 3,4,5-$(OCH_3)_3$ | 31.93 | 22.07 | 42.56 | 30.9 |
| 5k | 3-$OCH_3$, 4-OH | 47.50 | 32.46 | 60.38 | 65.1 |
| 5l | 3-$OCH_3$, 4-OH, 5-Br | 39.46 | 20.08 | 47.16 | 47.6 |
| 5m | 3,5-$(OCH_3)_2$, 4-OH | 49.44 | 40.82 | 71.76 | 74.4 |
| 5n | 3,5-$\{[C(CH_3)_3]\}_2$, 4-OH | 53.42 | 44.80 | 89.78 | 48.8 |
| Standard | α- tocopherol | 56.8 | - | 61.4 | - |
| | Ibuprofen | | | | 88.4 |

NA: Not Active
* Statistically significant ($P < 0.05$ Mann – Whitney)

## CONCLUSION

A series of styryl isoxazoles were reported to possess good anti-inflammatory activity with low ulcerogenic potential. In the present study, 4-nitro containing styrylisoxazoles were

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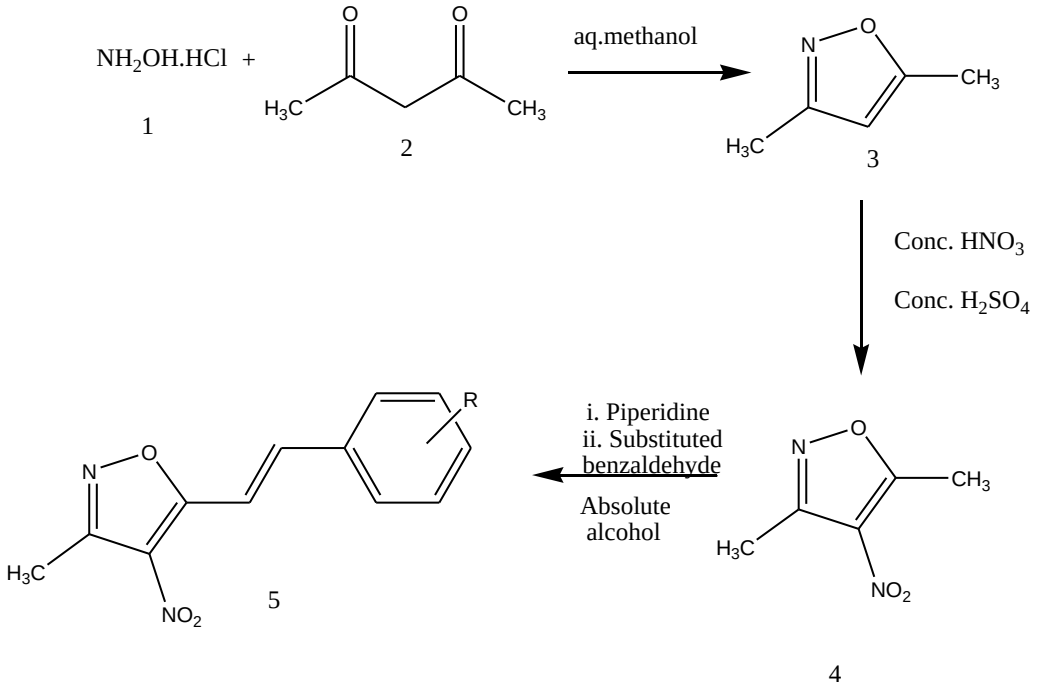
also found to possess similar trend of activity and low ulcerogenicity. However, the anti-inflammatory activity at molecular level has to be evaluated and there is scope for further investigations.




Scheme-I

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