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This work is dedicated to the memory of Prof. Paolo Sanna

HIV-1 integrase (IN) is a very promising and validated target for the development of therapeutic agents against AIDS. In an effort to design and synthesize biological isosteric analogs of β -diketoacid-containing inhibitors of IN, we prepared a series of substituted isoxazole carboxylic acids. Several of these compounds inhibited catalytic activities of purified IN at micromolar concentration range. With an aim to prepare a large number of analogues based on the isoxazole pharmacophore we focused our study on a series of 3,5-disubstituted isoxazole isomers. For a rapid structural analysis we discovered a convenient ¹H-nmr method for distinguishing between isomeric structures based on their H-4 assignments. This "finger print" approach to isomer identification will be useful in combinatorial chemistry settings where a mixture can be further derivatized.

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Introduction.

HIV-1 integrase (IN) is an essential enzyme for effective viral replication. IN catalyses the integration of the viral DNA into the host genome through coordinated reactions of processing and joining. IN does not have a human homologue and is considered a promising target for the development of new antiretroviral drugs [1-3].

In recent years a plethora of IN inhibitors were identified by systematic screening of natural and synthetic products against purified enzyme [4-6]. In particular, a class of compounds bearing a diketo acid moiety (**1**) was independently discovered by the scientists from Shionogi & Co. and Merck as selective IN inhibitors [7-10].

The diketoacid functionality is not only responsible for antiviral activity, but it also, unfortunately, contributes to cytotoxicity. Therefore, replacement of the diketo group with a pertinent bioisostere, endowed with reduced cytotoxicity, is of paramount importance in drug discovery targeting IN. Our goal in this study was to take advantage of bioisosteric replacement strategy to develop compounds with desirable pharmaceutical properties. In this context, our current research project was aimed at developing novel inhibitors by incorporating the 1,3-diketo moiety into a constrained isoxazole ring (**2**) (Figure 1).

With this rational in mind we designed and synthesized a series of aryl and heteroaryl isoxazole-carboxylic acids of

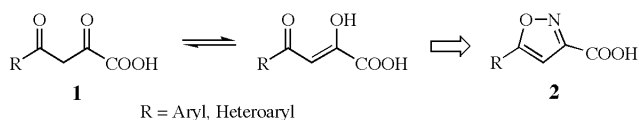


Figure 1

general structure **2** and observed that some of these compounds inhibited purified IN at micromolar concentration range. Details of these results will be published elsewhere [11].

Results and Discussion.

The preparation of these compounds can be envisaged using the so-called [3+2] atom fragments synthetic route, subtype CCC+NO [12-14], by condensation of an appropriate β -dicarbonyl intermediate (**3**) with hydroxylamine hydrochloride followed by alkaline hydrolysis (Figure 2). At this stage it was important to study the isoxazole-carboxylic acid moiety in order to establish the correct structure before further synthesis.

In conjunction with this project we extended our study to investigate some chemical aspects arising from the above synthetic approach. In general, preparation of isoxazoles from unsymmetrical disubstituted β -diketones (**3**) and hydroxylamine is less straightforward and can *a priori* lead to two isomeric 3,5-disubstituted isoxazoles **4** and **5** (Figure 2), [12-17].

A review of the literature on the structural assignments reveals that data are scanty and controversial [15,18-21]. For example, the oximes originated from 1-aryl-3-phenylpropane-1,3-diones have subsequently been demonstrated to give rise either to one of the two possible isoxazoles or to a mixture of both isomers [22]. From a survey of the cases reported in the literature, it became obvious that many factors can influence the yield and regioselectivity of the isomers. Also, it has been observed that the direction of enolization may be a governing factor in the ratio of the products obtained as well as the site-selectivity [15,19,23]. In fact this reaction proceeds through an attack of hydrox-

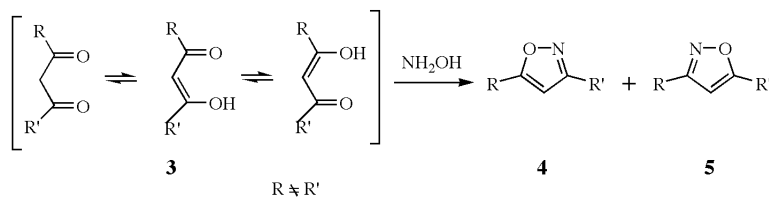


Figure 2. Formation of 3,5-disubstituted isoxazoles by reaction of β -diketones with hydroxylamine.

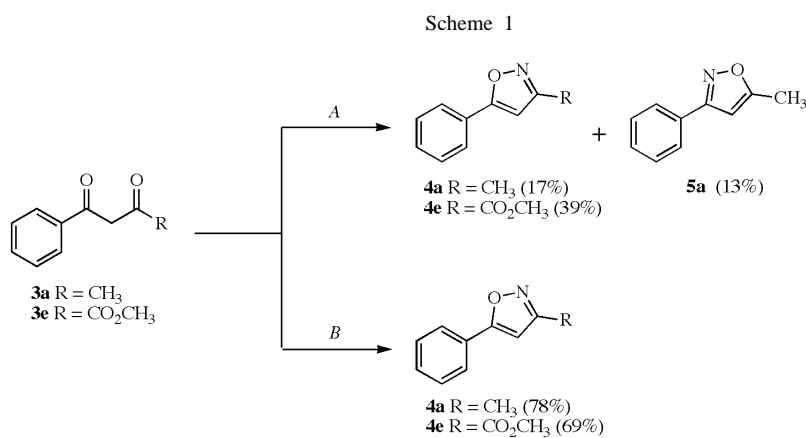
ylamine on the electrophilic center, and this is important for the subsequent ring closure to isoxazole. Since this reaction depends on the nature of R and R', all the variables which affect enolization (such as acidity or alkalinity of the medium) may influence the ratio in which the two isomers are formed [12]. According to Barnes *et al.* [19], in the highly enolized diketones, which possess alternative H-bonded (tautomeric structures), the structure of the principal reaction product can be predicted on the basis of the more or less electrophilic character of the two carbon atoms bearing R and R'. Nevertheless, although a rigorous study on the direction of enolization of disubstituted β -diketones was reported [24], in many cases it is not easy to predict if such a reaction can be regioselective. Also, when the substituents are not electronically very different, the separation of the 3,5-disubstituted isomers is difficult.

In the course of our combinatorial drug design efforts targeted against HIV-1 IN we investigated the structure of several isoxazoles. Interestingly, when we generated compounds **4a**, **4e** and **5a** (Scheme 1) by reaction of β -diketones and hydroxylamine using different conditions, we obtained different results in accordance with the previously reported observations. For example, the reaction of

the regioselective product **4a** was obtained on treating the starting **3a** with hydroxylamine hydrochloride (1.1 mole eq) in pyridine at 50 °C (Method B [26]) (Scheme 1). This was in contrast with previous reports where regioselectivity was observed for Method A but not for Method B [15,26]. The ester **4e** was instead obtained regioselectively using both methods.

Although the spectral data for compounds **4a** [27] and **4e** [25] were previously reported, we unambiguously assigned their structures on the basis of NOE-difference and NOESY data of corresponding *N*-Methyl isoxazolium salts **6a** and **6e**. In fact, we thought that the position of the *N*-methyl group could be detectable by NOE experiments. Both experiments showed NOE correlations between $\text{N}^+\text{-CH}_3$ and the methyl in position-3 of isoxazole ring (**6a**) and the methyl ester group in position-3 of **6e** (Figure 3). According to our prediction no NOE interactions between the *N*-methyl isoxazolium group and the 2',6'-aromatic protons could be observed for compounds **6a** and **6e**.

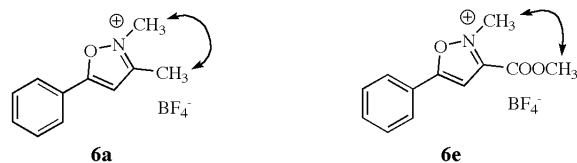
The isoxazolium salts **6a** and **6e**, necessary for NOE experiments, were prepared by heating in toluene the corresponding isoxazole derivatives **4a** and **4e** with dimethyl-



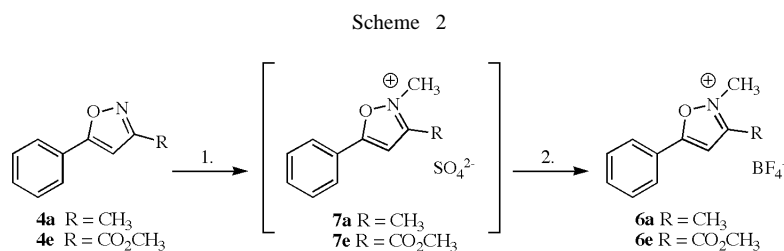
Reagents and Conditions: A) $\text{NH}_2\text{OH HCl}$ (3 eq), MeOH, reflux, 8h for **4a**, **5a** or 1h for **4e**; B) $\text{NH}_2\text{OH HCl}$ (1.1 eq), Pyridine, 50 °C, 1.5h.

benzoylacetone (**3a**) with hydroxylamine hydrochloride (3 mole eq) in methanol under reflux (Method A [25]) afforded both isoxazole isomers **4a** and **5a**. However only

sulfate. The isoxazolium methansulfate intermediates **7a** and **7e** were then converted to the respective tetrafluoroborates [28] according to the reaction of Scheme 2.

Figure 3. NOE correlations for compounds **6a** and **6e**.

An explanation of these results may be due to the nature of the “aromaticity” of isoxazole nucleus, which is considerably less aromatic than other five-membered heterocycles [39-41], and its low resonance energy [42]. This aspect favours a stabilization of the double bond at position-4,5 thus causing a direct interaction between H-4 with the substituents at position-5. This turns out to influence the chemical shift of H-4 as also documented by Battaglia *et al.* [31].



Reagents and Conditions: 1. Dimethylsulfate, anhydrous toluene, reflux, 46h for **6a** or 70h for **6e**; 2. NaBF₄, 0-5 °C.

Previously ¹³C-nmr studies were used to distinguish between isoxazole isomers [29,30]. Moreover, ¹H-nmr was used to compare the electronic effects of the substituents based on evaluation of their Hammett σ -constant parameters [31]. Unfortunately, the 60-MHz instrument used was not sufficient to unequivocally discern the splitting patterns for each isomer. In this context we have now discovered a robust and convenient ¹H-nmr method to identify 3,5-disubstituted isoxazole isomers.

We examined a series of unsymmetrical 3(5)-substituted-3(5)-phenyl-isoxazole derivatives (**4** and **5** series), where R is an electron-donating or electron-withdrawing substituent in order to study the influence of these groups on H-4 chemical shift of isoxazole ring (Table 1). Although the interatomic distances between substituents and H-4 are the same in both series, signals were dependent on the nature of the substituents.

In the case of isoxazole isomers bearing two different substituents, one of which being more electron-donating than the other one (*e.g.* CH₃, NH₂, OCH₃, 4-CH₃-phenyl vs Phenyl), the H-4 resonance is shifted upfield for the isomers where these electron-donating groups are located on position-5. In fact, the H-4 signal for compounds **5a-d** was shifted by 0.03 – 0.80 ppm to higher fields compared with the corresponding isomers **4a-d**. Opposite results were obtained for those compounds containing stronger electron-withdrawing groups (such CO₂CH₃, CF₃, CN, 4-NO₂-Phenyl vs Phenyl). In these cases the H-4 signal shifted downfield by 0.11 – 0.31 ppm for compounds bearing an electron-withdrawing substituent on position-5 of isoxazolic ring (**5e-h**) compared with those of the corresponding isomers **4e-h**. These results correlated with the electronic properties of the substituents.

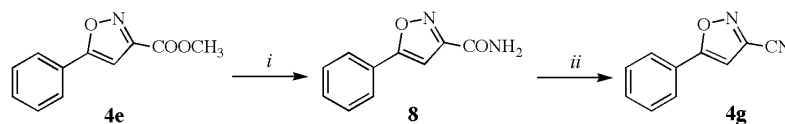
Table 1
Assignments of ¹H-NMR Chemical Shifts for H-4

| Compound | R | H ₄ (δ) | Lit. Ref. |
|-----------|---|--------------------|-----------|
| 4a | CH ₃ | 6.37 | [a, 27] |
| 5a | CH ₃ | 6.29 | [a, 27] |
| 4b | NH ₂ | 6.18 | [32] |
| 5b | NH ₂ | 5.38 | [32] |
| 4c | OCH ₃ | 6.12 | [33] |
| 5c | OCH ₃ | 5.51 | [34] |
| 4d | 4-CH ₃ C ₆ H ₄ | 6.80 | [22] |
| 5d | 4-CH ₃ C ₆ H ₄ | 6.77 | [22] |
| 4e | CO ₂ CH ₃ | 6.94 | [a, 25] |
| 5e | CO ₂ CH ₃ | 7.25 | [35] |
| 4f | CF ₃ | 6.67 | [36] |
| 5f | CF ₃ | 6.98 | [37] |
| 4g | CN | 6.83 | [a] |
| 5g | CN | 7.10 | [38] |
| 4h | 4-NO ₂ C ₆ H ₄ | 6.92 | [22] |
| 5h | 4-NO ₂ C ₆ H ₄ | 7.03 | [22] |

[a] This study.

Spectral data of model compounds of Table 1 were either previously reported (**4b-d,f,h** and **5b-h**) or obtained after chemical synthesis in this study (**4a,e,g** and **5a**). The only unknown compound **4g** was synthesized using the sequence of reactions depicted in the Scheme 3 that involves a classical transformation of an ester into a nitrile [43,44].

Scheme 3



Reagents and Conditions: *i*) 30% NH₃, r.t, 12h; *ii*) POCl₃, reflux, 24h.

Conclusion.

The ¹H-nmr analysis of two series of 3,5-disubstituted isoxazole isomers showed that the resonance values of H-4 on the isoxazole ring could be used as an important parameter to distinguish the isoxazole isomers. Analysis of spectral data showed that changes in H-4 chemical shifts on the isoxazole ring is due to electronic effects of the substituents through the heterocyclic system. Particularly, the electronic effect of a substituent is significant when it is directly connected to H-4 through a π bond on the 4- and 5-positions of the isoxazole ring.

EXPERIMENTAL

General.

Anhydrous solvents and all reagents were purchased from Aldrich, Merck or Carlo Erba. All reactions involving air- or moisture-sensitive compounds were performed under nitrogen atmosphere using oven-dried glassware and syringes to transfer solutions. Melting points (mp) were determined using an Electrothermal melting point or a Kofler apparatus and are uncorrected. Infrared (ir) spectra were recorded as thin films or nujol mulls on NaCl plates with a Perkin-Elmer 781 IR spectrophotometer and are expressed in ν (cm⁻¹). Nuclear magnetic resonance (¹H-nmr, NOE-difference and NOESY) spectra were determined in CDCl₃, DMSO or CDCl₃/DMSO (in the ratio 1:3) and were recorded on a Varian XL-200 (200 MHz). Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br s, broad singlet; dd, double doublet. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D₂O. Electron ionization mass spectra (70 eV) were recorded on a Hewlett-Packard 5989 Mass Engine Spectrometer. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel F-254 plates. Pure compounds showed a single spot on TLC. For flash chromatography Merck Silica gel 60 was used with a particle size 0.040-0.063 mm (230-400 mesh ASTM). Elemental analyses were performed on a Perkin-Elmer 2400 instrument at Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari (Italy), and the results were within $\pm 0.4\%$ of the theoretical values.

General Procedure for Preparation of Isoxazoles.

Method A.

Synthesis of 3-Methyl-5-phenylisoxazole (**4a**) and 5-Methyl-3-phenylisoxazole (**5a**).

A mixture of benzoylacetone (**3a**) (1 g, 6.17 mmol) and hydroxylamine hydrochloride 1.3 g, 3 mole eq) in methanol (25 mL) was refluxed for 8 h. After evaporation of the solvent, the solid obtained was purified by silica gel flash chromatography (petroleum ether:ethyl acetate = 9:1) to give a white solid that was a mixture of the isomers **4a** and **5a**. These were separated by silica gel flash chromatography (petroleum ether:ethyl acetate = 9.5:0.5) to give **4a** and **5a** in 17 and 13% yield, respectively.

3-Methyl-5-phenylisoxazole (**4a**).

This compound was obtained as white solid, mp 65-67°; Rf 0.43 (petroleum ether:ethyl acetate = 9.5:0.5); ir (Nujol): 1610 (isoxazole), 1378 (CH₃ bending symm) cm⁻¹; ¹H-nmr (CDCl₃): δ 7.73–7.78 (m, 2H, Ar-H aromatic), 7.43–7.46 (m, 3H, Ar-H aromatic), 6.37 (s, 1H, H-4 isoxazole), 2.36 (s, 3H, CH₃); gc/ms: *m/z* 159 (M⁺).

Anal. Calcd. for C₁₀H₉NO (159.18). C, 75.45; H, 5.70; N, 8.80. Found: C, 75.21; H, 5.83; N, 8.87.

5-Methyl-3-phenylisoxazole (**5a**).

This compound was obtained as a white solid, mp 38-40°; Rf 0.52 (petroleum ether:ethyl acetate = 9.5:0.5); ir (Nujol): 1605 (isoxazole), 1373 (CH₃ bending symm) cm⁻¹; ¹H-nmr (CDCl₃): δ 7.75–7.81 (m, 2H, Ar-H aromatic), 7.42–7.47 (m, 3H, Ar-H aromatic), 6.29 (s, 1H, H-4 isoxazole), 2.48 (s, 3H, CH₃); gc/ms: *m/z* 159 (M⁺).

Anal. Calcd. for C₁₀H₉NO (159.18). C, 75.45; H, 5.70; N, 8.80. Found: C, 75.37; H, 5.74; N, 8.69.

Method A.

Synthesis of Methyl 5-phenylisoxazole-3-carboxylate (**4e**).

A solution of the methyl 2,4-dioxo-4-phenylbutanoate [25] (**3e**) (1 g, 4.85 mmol) and hydroxylamine hydrochloride (1.01 g, 3 mole eq) in methanol (20 mL) was refluxed for 1 h. After evaporation *in vacuo* a yellow solid was obtained that was purified by silica gel flash chromatography (hexane:ethyl acetate = 8:2). The crude product was recrystallized from water-ethanol to give **4e** as yellow crystals (39% yield), mp 81-82°; Rf 0.57 (petroleum ether:ethyl acetate = 8.5:1.5); ir (Nujol): 1728 (C=O, ester), 1610 (isoxazole) cm⁻¹; ¹H-nmr (CDCl₃): δ 7.77–7.85 (m, 2H, Ar-H aromatic), 7.47–7.55 (t, 3H, Ar-H aromatic), 6.94 (s, 1H, H-4 isoxazole), 4.01 (s, 3H, CO₂CH₃); gc/ms: *m/z* 203 (M⁺).

Anal. Calcd. for C₁₁H₉NO₃ (203.19). C, 65.02; H, 4.46; N, 6.89. Found: C, 65.26; H, 4.28; N, 6.72.

Method B.

Synthesis of 3-Methyl-5-phenylisoxazole (**4a**) and Methyl 5-Phenylisoxazole-3-carboxylate (**4e**).

To a solution of benzoylacetone (**3a**) (for **4a**) or methyl 2,4-dioxo-4-phenylbutanoate [25] (**3e**) (for **4e**) (10 mmol) in pyridine (10 mmol) was added a saturated aqueous solution of

hydroxylamine hydrochloride (11 mmol in 5 mL of deionized water). The mixture was stirred for 1.5 h at 50 °C. A solid was obtained that after purification by silica gel flash chromatography (petroleum ether:ethyl acetate = 9:1) gave compounds **4a** (78% yield) or **4e** (69% yield) identical (mixed mp, ir, ¹H-nmr) to the above samples.

General Procedure for the Synthesis of *N*-Methylisoxazolium Tetrafluoroborate Salts (**6a** and **6e**).

A mixture of the appropriate isoxazole (**4a** or **4e**) (2 mmol) and dimethylsulfate (1.1 mole eq) in anhydrous toluene (5 mL) was refluxed, under nitrogen atmosphere, for 46 h (for **6a**) or 70 h (for **6e**). Subsequently the toluene layer was decanted, and the oily residue was dissolved in water, and it was washed three times with ethyl ether (for **6a**) or ethyl acetate (for **6e**). To this aqueous solution was added a solution of sodium tetrafluoroborate (4 mole eq) in water and, after cooling with ice, a yellow solid was separated. Compounds **6a** or **6e** were collected in 31 and 11% yield, respectively.

2,3-Dimethyl-5-phenylisoxazolium Tetrafluoroborate (**6a**) [28].

This compound was obtained as a white solid, mp 165–166°; ir (Nujol): 1610 (isoxazole), 1375 (CH₃ bending symm.), 1060 (BF₄⁻) cm⁻¹; ¹H-nmr (DMSO): δ 8.00 (d, 2H, Ar-H aromatic), 7.78 (s, 1H, H-4 isoxazole), 7.60–7.74 (m, 3H, Ar-H aromatic), 4.32 (s, 3H, N-CH₃), 2.68 (s, 3H, CH₃); gc/ms: *m/z* 174 (M⁺) (cation).

Anal. Calcd. for C₁₁H₁₂NO•BF₄ (261.03). C, 50.62; H, 4.63; N, 5.37. Found: C, 50.77; H, 4.42; N, 5.41.

3-(Methoxycarbonyl)-2-methyl-5-phenylisoxazol-2-ium Tetrafluoroborate (**6e**).

This compound was obtained as a white solid, mp 175–176°; ir (Nujol): 1742 (C=O ester), 1605 (isoxazole), 1370 (CH₃ bending symm.), 1060 (BF₄⁻) cm⁻¹; ¹H-nmr (DMSO): δ 8.45 (s, 1H, H-4 isoxazole), 8.15 (d, 2H, Ar-H aromatic), 7.63–7.84 (m, 3H, Ar-H aromatic), 4.60 (s, 3H, N-CH₃), 4.08 (s, 3H, COOCH₃); gc/ms: *m/z* 218 (M⁺) (cation).

Anal. Calcd. for C₁₂H₁₂NO₃•BF₄ (305.04). C, 47.25; H, 3.97; N, 4.59. Found: C, 47.44; H, 3.68; N, 4.33.

Synthesis of 5-Phenylisoxazole-3-carboxamide (**8**).

A suspension of methyl 5-phenylisoxazole-3-carboxylate (**4e**) (0.30 g, 1.48 mmol) and 30% ammonia aqueous solution (15 mL) was stirred at room temperature overnight. The solid obtained was triturated with a mixture of petroleum ether and ethyl ether to give **8** as a white solid (65% yield), mp 200–201°; Rf 0.38 (petroleum ether:ethyl acetate = 6:4); ir (Nujol): 3400 (NH₂), 3230 (NH₂), 1660 (C=O, amide), 1610 (isoxazole) cm⁻¹; ¹H-nmr (CDCl₃): δ 7.78–7.85 (d, 2H, Ar-H aromatic), 7.46–7.55 (m, 3H, Ar-H aromatic), 6.97 (s, 1H, H-4 isoxazole), 6.79 (br s, 1H, NH₂, exchangeable with D₂O), 5.76 (br s, 1H, NH₂, exchangeable with D₂O); gc/ms: *m/z* 188 (M⁺).

Anal. Calcd. for C₁₀H₈N₂O₂ (188.18). C, 63.82; H, 4.28; N, 14.89. Found: C, 63.63; H, 4.39; N, 14.98.

Synthesis of 5-Phenylisoxazole-3-carbonitrile (**4g**).

A mixture of 5-phenylisoxazole-3-carboxamide (**8**) (0.15 g, 0.80 mmol) in 2.55 mL of POCl₃ (27.0 mmol) was heated at reflux for 24 h. After cooling the solution was poured on ice and made alkaline with aqueous sodium carbonate. The orange solid

obtained was purified by silica gel flash chromatography (hexane:ethyl acetate = 8:2) to give **4g** as a pale orange solid (37% yield), mp 87–89°; Rf 0.57 (Petroleum ether:Ethyl acetate = 9:1); ir (Nujol): 2260 (CN), 1610 (isoxazole) cm⁻¹; ¹H-nmr (CDCl₃): δ 7.76–7.83 (m, 2H, Ar-H aromatic), 7.50–7.56 (m, 3H, Ar-H aromatic), 6.83 (s, 1H, H-4 isoxazole); gc/ms: *m/z* 170 (M⁺).

Anal. Calcd. for C₁₀H₆N₂O (170.17). C, 70.58; H, 3.55; N, 16.46. Found: C, 70.77; H, 3.22; N, 16.50.

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