



## New norfloxacin/nitric oxide donor hybrids: Synthesis and nitric oxide release measurement using a modified Griess colorimetric method

Hossameldin Ali Aziz <sup>1</sup>, Gamal Abdeltawab Idris Moustafa <sup>1,\*</sup>, Samar Hafez Abbas <sup>1</sup>,  
Sayed Mohamed Derayea <sup>2</sup> and Gamal El-Din Ali Ahmed Abuo-Rahma <sup>1</sup>

<sup>1</sup> Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, Minia 61519, Egypt

<sup>2</sup> Department of Analytical Chemistry, Faculty of Pharmacy, Minia University, Minia 61519, Egypt

\* Corresponding author at: Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, Minia 61519, Egypt.  
Tel.: +2.086.2369075. Fax: +2.086.2369075. E-mail address: [gamal.moustafa@mu.edu.eg](mailto:gamal.moustafa@mu.edu.eg) (G.A.I. Moustafa).

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### ABSTRACT

Oximes and nitrate esters are considered as important nitric oxide (NO) donors with diverse biological activities. Herein, we report the synthesis and characterization of new oxime and nitrate ester derivatives of norfloxacin as potential NO donor hybrids with expected synergistic antimicrobial activity. The release of NO from those hybrids was measured by a modified Griess method in which *p*-nitroaniline was employed instead of sulfanilamide. The increased electrophilicity of the intermediate 4-nitroaniline diazonium salt accelerated the coupling process and shortened the overall assessment time. The improved detection limits and enhanced sensitivity would pave the way for the future application of this method in nitrite determination in biological or non-biological systems.

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### 1. Introduction

Nitric oxide is a key signalling molecule regulating various physiological functions such as platelet aggregation [1], vascular tone [2,3], immune response and neurotransmission [4-8]. Likewise, numerous reports demonstrated the significance of NO in mediating the antiproliferative, antibacterial, antiviral and antiprotozoal properties of activated macrophages [9,10].

Given the aforementioned diverse and important physiological roles of NO and considering the difficulty of handling NO as a gas, a great research interest has been directed to the modulation of NO in physiological systems by increasing its concentration through exogenous release by NO donors. Such NO progenitors have now received great attention as promising candidates for the treatment of various diseases associated with NO-based cellular activities [11,12]. In this regard, various classes of NO donors have been identified as organic nitrates and nitrites, *S*-nitrosothiols, sydnonimines, metal nitrosyls, furoxans, diazeniumdiolates and oximes. Of particular significance is the proven clinical implication of organic nitrates as glyceryl trinitrate, isosorbide dinitrate and

isosorbide mononitrate as primary antianginal medications [13].

It is generally accepted that the therapeutic potential of the NO donors largely depends on their ability to release NO in the biological systems. In order to correlate the NO release with biological effects, it is important to estimate NO concentrations generated from NO donors *in vitro* as well as the time course of the NO production. Generally, various methods have been utilized to analyze NO release from NO donors. NO can be estimated by gas chromatography-mass spectrometry [14], chemiluminescence [15], or by fluorescent probes such as 4,5-diaminofluorescein [16], laser magnetic resonance spectroscopy [17], electrochemical methods [18] and capillary electrophoresis with laser induced fluorescence detection [19]. In addition, Griess colorimetric assay [20,21] and its modifications [22,23] represent the most commonly used methods for the analysis of NO in view of the simplicity and low cost. The method relies on indirect determination of NO *via* spectrophotometric measurement of its stable decomposition product NO<sub>2</sub>. The reaction involves two steps; the first step is the reaction of dinitrogen trioxide, resulting from the auto oxidation of NO, with sulfanilamide to produce a diazonium ion. The second step is the coupling of diazonium

ion with *N*-(1-naphthyl)ethylenediamine (NED) to form a coloured azo dye that absorbs at  $\lambda_{\text{max}}$  546 nm. Despite the feasibility of the method, it sometimes suffers from poor sensitivity and inferior reproducibility at submicromolar concentrations of NO.

In continuation of our efforts to discover new NO-based drug candidates, the present work aims at the synthesis and characterization of new norfloxacin/NO donor hybrids with potential synergistic antimicrobial properties. The release of NO from the prepared hybrids is estimated by adapting a modification to the common Griess method with a view to enhancing the sensitivity and shortening the assessment time.

## 2. Experimental

### 2.1. Instrumentations

All chemicals and solvents used for the preparation of the intermediates and target compounds were purchased from commercial resources and used without purification. The reaction progress was monitored using TLC (Kieselgel 60 F254 pre-coated plates, E. Merck, Darmstadt, Germany) and the spots were detected by exposure to UV lamp at  $\lambda$  254 and 365 nm. Melting points were determined on Stuart electrothermal melting point apparatus and were uncorrected. IR spectra were recorded as KBr disks on SHIMADZU IR-470 spectrophotometer, at the Central Laboratory, Faculty of Science, Sohag University. NMR spectra (400 MHz for  $^1\text{H}$ , 100 MHz for  $^{13}\text{C}$ ) were recorded on a BRUKER Avance III spectrometer at the Faculty of Science, Sohag University, using TMS as an internal standard and  $\text{CDCl}_3$  or  $\text{DMSO-}d_6$  as solvents. Chemical shifts ( $\delta$ ) values are expressed in parts per million (ppm) and coupling constants ( $J$ ) in Hertz (Hz). The signals are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; brs, broad singlet. Elemental microanalysis was carried out in Al-Azhar University at the Regional Centre for Mycology and Biotechnology. High resolution mass spectra were recorded on Thermo Scientific Q Exactive Orbitrap mass spectrometer, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada.

### 2.2. Synthesis

#### 2.2.1. Synthesis of 7-(4-(2-bromoacetyl) piperazin-1-yl)-1-ethyl-6-fluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (2)

To a stirred solution of norfloxacin (0.319 g, 1.0 mmol) in dichloromethane (50 mL) was added a solution of potassium carbonate (0.152 g, 1.1 mmol) in distilled water (50 mL) at 0-5 °C. Then, bromoacetyl bromide (0.22 g, 1.1 mmol) in dichloromethane (25 mL) was slowly added over a period of 30 min. Stirring was continued for 2 h at 0-5 °C, then at room temperature for additional 12 h. The whole mixture was then transferred to a separatory funnel where it was extracted with dichloromethane and washed successively with 1.0 N HCl (2×25 mL) and water (2×25 mL). The organic layer was separated, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure to give compound **2** in a sufficiently pure form suitable for use in the next reaction without further purification (Scheme 1). Color: White powder. Yield: 0.370 g (84%). M.p.: 247-249 °C. FT-IR (KBr,  $\nu$ ,  $\text{cm}^{-1}$ ): 1713 (carboxylic C=O), 1666 (amidic C=O), 1620 (4-keto C=O).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 1.45 (t, 3H,  $J = 7.6$  Hz,  $\text{NCH}_2\text{CH}_3$ ), 3.38-3.42 (m, 4H, piperazinyl-H), 3.70-3.74 (m, 4H, piperazinyl-H), 4.20 (s, 2H, BrCH<sub>2</sub>), 4.59 (q, 2H,  $J = 7.6$  Hz,  $\text{NCH}_2\text{CH}_3$ ), 7.22 (d, 1H,  $J_{\text{H-F}} = 7.6$  Hz, H8), 7.96 (d, 1H,  $J_{\text{H-F}} = 13.6$  Hz, H5), 8.94 (s, 1H, H2), 15.22 (s, 1H, COOH).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 14.86, 28.29, 41.76, 46.11, 49.56, 106.86, 107.58, 111.73 (d,  $J = 23$  Hz), 120.06 (d,  $J = 8$  Hz), 137.62, 145.56 (d,  $J = 11$  Hz), 149.14, 153.29 (d,  $J = 247$  Hz), 165.49,

166.57, 176.67. Anal. calcd. for  $\text{C}_{18}\text{H}_{19}\text{BrFN}_3\text{O}_4$ : C, 49.11; H, 4.35; N, 9.54. Found: C, 49.34; H, 4.31; N, 9.78%.

#### 2.2.2. Synthesis of 1-ethyl-6-fluoro-7-((4-(2-nitrooxy)acetyl) piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (3)

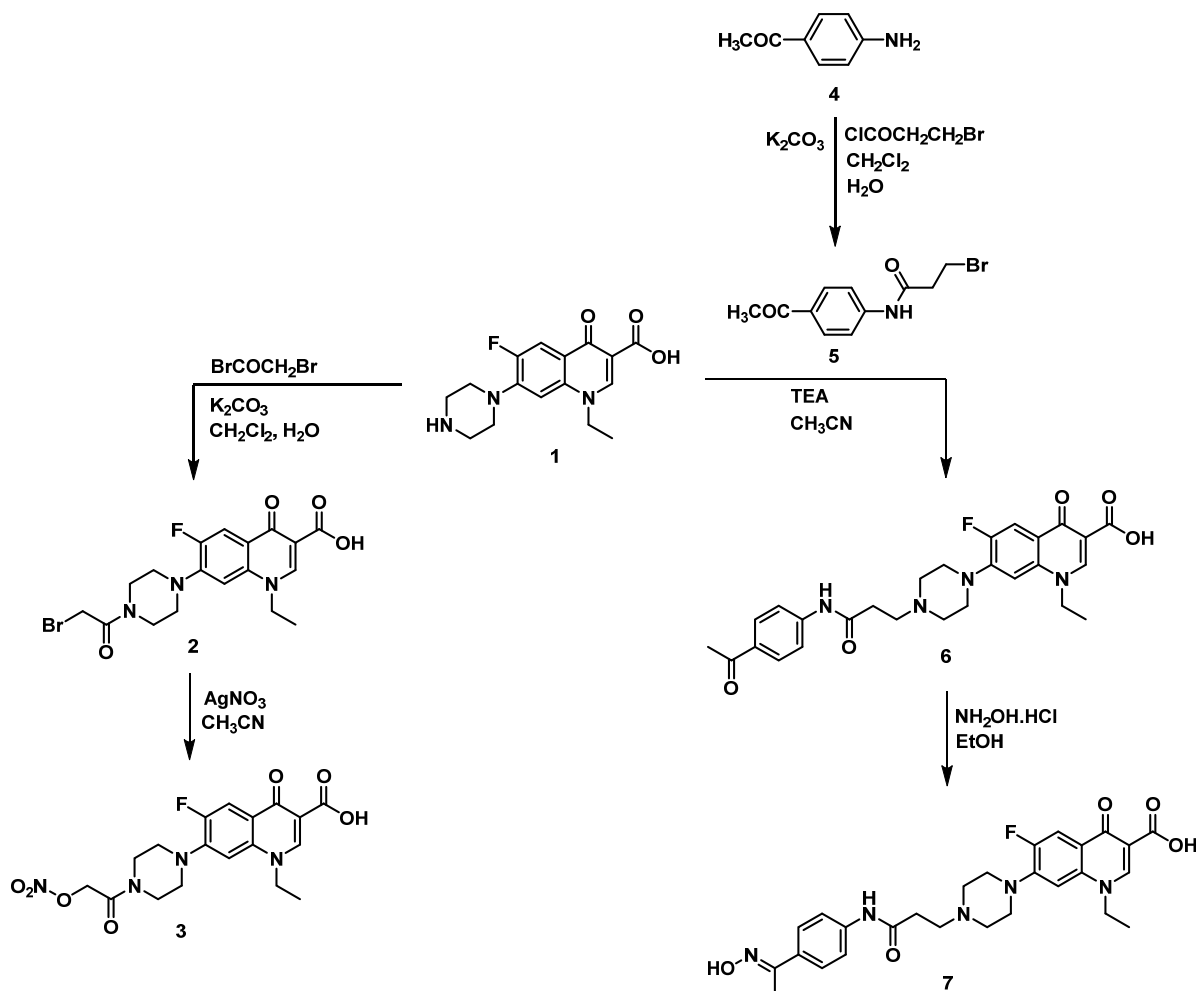
To a stirred solution of bromide **2** (0.44 g, 1.0 mmol) in acetonitrile (10 mL), silver nitrate (4 mmol) in acetonitrile (10 mL) was added portion wise and the mixture was heated for 16 h at 80 °C. The formed precipitate of silver bromide was filtered off, and the filtrate was evaporated till dryness, dissolved in dichloromethane (30 mL) and washed with distilled water (2×25 mL) and brine (2×25 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was crystallized from acetonitrile to give nitrate ester **3** (Scheme 1). Color: Yellow crystals. Yield: 0.177 g (41.9%). M.p.: 201-203 °C. FT-IR (KBr,  $\nu$ ,  $\text{cm}^{-1}$ ): 1715 (carboxylic C=O), 1668 (amidic C=O), 1623 (4-keto C=O).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 1.45 (t, 3H,  $J = 7.6$  Hz,  $\text{CH}_2\text{CH}_3$ ), 3.35-3.45 (m, 4H, piperazinyl-H), 3.61-3.71 (m, 4H, piperazinyl-H), 4.59 (q, 2H,  $J = 7.6$  Hz,  $\text{CH}_2\text{CH}_3$ ), 5.46 (s, 2H,  $\text{CH}_2\text{ONO}_2$ ), 7.22 (d, 1H,  $J_{\text{H-F}} = 6.8$  Hz, H8), 7.96 (d, 1H,  $J_{\text{H-F}} = 13.6$  Hz, H5), 8.94 (s, 1H, H2), 15.23 (s, 1H, COOH).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 14.76, 41.61, 44.00, 49.54, 50.03, 69.32, 106.70, 107.76, 111.79 (d,  $J = 23$  Hz), 120.09, 137.70, 145.54, 148.98, 153.29 (d,  $J = 248$  Hz), 163.97, 166.44, 167.71. HRMS (ESI,  $m/z$ ) calcd. for  $\text{C}_{18}\text{H}_{18}\text{FN}_4\text{O}_7$  [M-H]<sup>-</sup>: 421.11650, found: 421.11700.

#### 2.2.3. Synthesis of *N*-(4-acetylphenyl)-3-bromopropan amide (5)

To a stirred solution of *p*-aminoacetophenone (**4**) (0.675 g, 5.0 mmol) in dichloromethane (30 mL) was added a solution of potassium carbonate (0.76 g, 5.5 mmol) in water (30 mL) at 0-5 °C. Then, 3-bromopropionyl chloride (0.94 g, 5.5 mmol) in dichloromethane (30 mL) was slowly added over a period of 30 min. Stirring was continued for 2 h at 0-5 °C, then at room temperature for additional 12 h. The whole mixture was then transferred to a separatory funnel where it was extracted with dichloromethane and washed with water (2×25 mL). The organic layer was separated, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure. The residue was crystallized from 95% ethanol to give bromide **5** in a sufficiently pure form suitable for use in the next reaction without further purification (Scheme 1). Color: White crystals. Yield: 1.25 g (93%). M.p.: 175-176 °C. FT-IR (KBr,  $\nu$ ,  $\text{cm}^{-1}$ ): 3323 (NH), 1692 ( $\text{NHCOCH}_2$ ), 1654 ( $\text{COCH}_3$ ).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ , ppm): 2.61 (s, 3H,  $\text{COCH}_3$ ), 3.00 (t, 2H,  $J = 8.0$  Hz,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 3.73 (t, 2H,  $J = 8.0$  Hz,  $\text{CH}_2\text{CH}_2\text{Br}$ ), 7.64 (d, 2H,  $J = 8.8$  Hz, Ar-H), 7.96 (d, 2H,  $J = 8.8$  Hz, Ar-H), 10.70 (s, 1H, NHC=O).

#### 2.2.4. Synthesis of 7-(4-(3-((4-acetylphenyl)amino)-3-oxopropyl) piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (6)

To a stirred solution of compound **5** (0.297 g, 1.1 mmol) in acetonitrile (10 mL) was added norfloxacin (0.319 g, 1.0 mmol). Then, triethylamine (0.202 g, 2.0 mmol) was added and the mixture was heated under reflux for 18 h. The formed precipitate was filtered while hot, washed with acetonitrile and dried under vacuum to give compound **6** (Scheme 1). Color: White powder. Yield: 0.274 g (54%). M.p.: 275-276 °C. FT-IR (KBr,  $\nu$ ,  $\text{cm}^{-1}$ ): 3271(NH), 1725 (carboxylic C=O), 1675 (amidic C=O), 1652 ( $\text{CH}_3\text{C=O}$ ), 1617 (4-keto C=O).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 1.44 (t, 3H,  $J = 7.6$  Hz,  $\text{NCH}_2\text{CH}_3$ ), 2.52 (s, 3H,  $\text{COCH}_3$ ), 2.59 (t, 2H,  $J = 7.6$  Hz,  $\text{COCH}_2\text{CH}_2$ ), 2.65-2.68 (m, 4H, piperazinyl-H), 2.78 (t, 2H,  $J = 7.6$  Hz,  $\text{COCH}_2\text{CH}_2$ ), 3.34-3.42 (m, 4H, piperazinyl-H), 4.58 (q, 2H,  $J = 7.6$ ,  $\text{NCH}_2\text{CH}_3$ ),



Scheme 1

7.18 (d, 1H,  $J_{H-F} = 7.6$  Hz, *H8*), 7.73 (d, 2H,  $J = 8.8$  Hz, Ar-*H*), 7.91-7.93 (m, 3H, Ar-2*H* + *H5*), 8.91 (s, 1H, *H2*), 10.28 (s, 1H, *NHCO*), 15.22 (s, 1H, *COOH*).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 14.74, 26.76, 34.80, 49.49, 50.01, 52.63, 53.92, 106.25, 107.69, 111.65 (d,  $J = 23$ Hz), 118.85, 119.78, 129.85, 132.23, 137.75, 143.99, 145.95, 148.84, 153.37 (d,  $J = 247$  Hz), 166.48, 171.14, 176.67, 196.81. HRMS (ESI,  $m/z$ ) calcd. for  $\text{C}_{27}\text{H}_{28}\text{FN}_4\text{O}_5$  [ $\text{M-H}$ ] $^-$ : 507.20492, found: 507.20514.

### 2.2.5. Synthesis of (*E*)-1-cyclopropyl-6-fluoro-7-(4-(3-((4-(1-(hydroxyimino)ethyl)phenyl)amino)-3-oxopropyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7)

To a stirred suspension of the ketone **6** (0.508 g, 1.0 mmol) in absolute ethanol (10 mL) were added hydroxylamine hydro-chloride (0.278 g, 4.0 mmol) and anhydrous sodium acetate (0.328 g, 4.0 mmol). The mixture was heated under reflux for 12 h. The formed precipitate was filtered while hot, washed with ethanol, dried and crystallized from acetonitrile to afford oxime **7** (Scheme 1). Color: White crystals. Yield: 0.334 g (65.8 %). M.p.: 290-292 °C. FT-IR (KBr,  $\nu$ ,  $\text{cm}^{-1}$ ): 1679 (carboxylic C=O), 1627 (4-keto C=O), 1601 (C=N).  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 1.43 (t, 3H,  $J = 7.6$  Hz,  $\text{NCH}_2\text{CH}_3$ ), 2.13 (s, 3H,  $\text{CH}_3\text{C}=\text{NOH}$ ), 2.55 (t, 2H,  $J = 7.6$  Hz,  $\text{NHCOC}_2\text{H}_5$ ), 2.63-2.71 (m, 4H, piperazinyl-*H*), 2.76 (t, 2H,  $J = 7.6$  Hz,  $\text{NHCOC}_2\text{H}_5$ ), 3.33-3.41 (m, 4H, piperazinyl-*H*), 4.58 (q, 2H,

$J = 7.6$  Hz,  $\text{NCH}_2\text{CH}_3$ ), 7.18 (d, 1H,  $J_{H-F} = 7.6$  Hz, *H8*), 7.60 (m, 4H, Ar-*H*), 7.91 (d, 1H,  $J_{H-F} = 13.6$  Hz, *H5*), 8.92 (s, 1H, *H2*), 10.06 (s, 1H, *NHCO*), 10.94 (s, 1H,  $\text{C}=\text{NOH}$ ), 15.27 (s, 1H, *COOH*).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ ,  $\delta$ , ppm): 11.80, 14.76, 34.69, 49.50, 50.01, 52.63, 54.06, 106.27, 107.65, 111.64 (d,  $J = 23$ Hz), 119.51 (d,  $J = 49$  Hz), 126.43, 129.87, 132.20, 137.73, 140.06, 145.96, 148.85, 153.38 (d,  $J = 246$  Hz), 166.50, 170.61, 176.65. HRMS (ESI,  $m/z$ ) calcd. for  $\text{C}_{27}\text{H}_{29}\text{FN}_5\text{O}_5$  [ $\text{M-H}$ ] $^-$ : 522.21582, found: 522.21606.

## 2.3. In vitro determination of nitric oxide release

### 2.3.1. Materials and methods

All measurements were carried out using UV-visible spectrophotometer (Implen nanophotometer, Germany). Nitrite stock aqueous solution (0.1 M sodium nitrite in water) was prepared from which a dilute solution of 100  $\mu\text{M}$  nitrite solution was prepared by diluting 1 ml of the stock solution to 1000 ml with phosphate buffer of pH = 7.4. Working standard solutions containing different concentrations of the nitrite (100, 50, 25.00, 12.50, 6.25, 3.125, 1.56, 0.78 and 0.39  $\mu\text{M}$ ) were prepared by further dilution of the later solution with phosphate buffer. The tested compounds were dissolved in DMF and diluted with the buffer system to afford a concentration of 100  $\mu\text{M}$ . *N*-Acetyl cysteine solution was prepared in a concentration of 500  $\mu\text{M}$  in methanol.

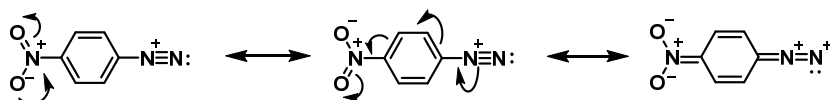


Figure 1. Resonance stabilization and increased diazonium cations' electrophilicity by electron-withdrawing substituents.

The modified Griess reagent consists of 0.1% (*w:v*) *N*-(1-naphthyl)ethylenediamine (NED) solution in water and 4-nitroaniline solution (0.5% *w:v* of 4-nitroaniline in 5% *w:v* phosphoric acid).

### 2.3.2. Preparation of nitrite standard curve

An aliquot of 100  $\mu\text{L}$  of 4-nitroaniline solution was added to the working standard nitrite solution. After 1 minute, 100  $\mu\text{L}$  of the NED solution was added and absorbance of the formed purple colour was measured after 10 minutes at  $\lambda_{\text{max}}$  539 nm. A blank experiment was performed under the same conditions. The procedure was repeated three times for each concentration of the nitrite and the average absorbance was calculated. The calibration curve was constructed by plotting the average absorbance value against the corresponding nitrite concentration.

### 2.3.3. NO release assay

Solutions of the tested compounds **3** and **7** in DMF were diluted with phosphate buffer of pH = 7.4 to give a final concentration of 100  $\mu\text{M}$  (test solutions). To 100  $\mu\text{L}$  of each test solutions, 100  $\mu\text{L}$  of *N*-acetyl cysteine solution was added and the obtained solution was kept in an incubator at 37  $^{\circ}\text{C}$  (treated solutions). The solutions were treated similarly; as for nitrite standard solution; with 100  $\mu\text{L}$  of 4-nitroaniline. Then, we just waited for one minute after adding 4-nitroaniline and for additional 10 minute after adding NED reagent and the absorbance were as recorded at  $\lambda_{\text{max}}$  539 nm every 10 minutes over a period of one hr. A blank experiment was performed under the same conditions, the procedure was repeated three times for each tested compound and the average absorbance was calculated. The corresponding concentration of nitrite was determined by comparison to the nitrite standard calibration curve and the amount of NO released (revealed by the corresponding nitrite concentration) was calculated as percentage of moles of NO released from 1 mole of tested compound.

## 3. Results and discussion

### 3.1. Synthesis of norfloxacin-NO donor hybrids

The synthesis of the target hybrids **3** and **7** (Scheme 1) commenced with the base-promoted acylation of norfloxacin (**1**) with bromoacetyl bromide followed by treating the resultant bromide **2** with silver nitrate to afford nitrate ester **3**. The IR spectrum of nitrate ester **3** showed asymmetric stretching band of  $\text{ONO}_2$  at 1544  $\text{cm}^{-1}$  and symmetric stretching at 1294  $\text{cm}^{-1}$ . Comparison of  $^1\text{H}$  NMR spectra of the prepared nitrate ester **3** with its corresponding bromide **2** showed that replacement of bromine in compound **2** by nitrate in compound **3** leads to a remarkable downfield shift of methylene  $\text{CH}_2\text{ONO}_2$  protons by  $\delta$  1.26 ppm,  $^{13}\text{C}$  NMR spectrum of compound **3** showed shift of carbon by  $\delta$  41.03 ppm due to replacement of bromine with  $\text{ONO}_2$ .

Alkylation of norfloxacin (**1**) with alkyl halide **5**; generated from the reaction of *p*-aminoacetophenone (**4**) with 3-bromopropionyl chloride; afforded the corresponding ketone intermediate **6** that was treated with hydroxylamine hydrochloride to deliver the target oxime **7**. The structures of the target oxime **7** and its corresponding ketone intermediate

**6** were unambiguously confirmed by various spectroscopic tools. In addition to the basic skeleton of norfloxacin, the ketone intermediate **6** showed additional pair of doublets in the aromatic region at  $\delta$  7.73 ppm and at 7.92 ppm related to *p*-aminoacetophenone and two singlet peaks at  $\delta$  10.28 ppm (related to NH proton) and at  $\delta$  2.52 ppm (related to  $\text{COCH}_3$ ). Moreover, the appearance of two triplet signals at  $\delta$  2.59 ppm and at  $\delta$  2.78 ppm related to  $(\text{NHCOCH}_2\text{CH}_2\text{N})$  further confirmed the formation of ketone intermediate **6**.  $^{13}\text{C}$  NMR and HRMS were additional evidences for the formation of compounds **6**. The conversion of the ketone intermediate **6** into oxime **7** was confirmed by IR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and HRMS. The IR spectrum of compound **7** showed disappearance of ketonic carbonyl ( $\text{COCH}_3$ ) due to its conversion to ketoxime group ( $\text{C=N-OH}$ ).  $^1\text{H}$  NMR spectrum of compound **7** was characterized by the appearance of new singlet signal at about  $\delta$  10.94 ppm assigned to OH of the oxime, and a singlet signal for  $\text{CH}_3\text{C=NOH}$  that was up-field shifted by  $\delta$  0.38 ppm from the original  $\text{CH}_3$  of the ketone precursor **6**.  $^{13}\text{C}$  NMR spectrum of compound **7** showed the disappearance of the ketonic carbonyl ( $\text{COCH}_3$ ) due its conversion to ketoxime group ( $\text{C=N-OH}$ ) which appeared at  $\delta$  152.91 ppm. Moreover, the methyl of ( $\text{CH}_3\text{C=NOH}$ ) appeared at  $\delta$  11.80 ppm with up-field shifting by  $\delta$  15 ppm from the original  $\text{CH}_3\text{C=O}$  in the ketone precursor **6**.

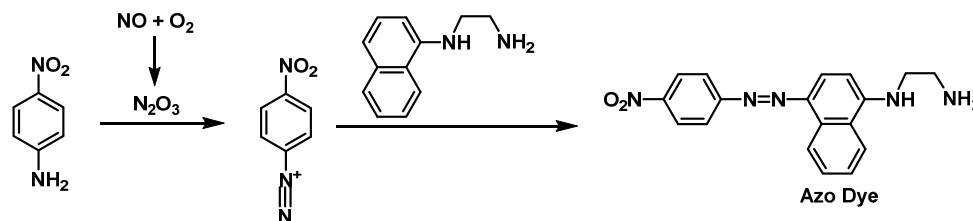
### 3.2. NO release measurement

Our next task was to estimate the ability of NO donor hybrids **3** and **7** to release NO. Disappointingly, we could not detect measurable amounts of NO from our compounds by the conventional Griess method. In an effort to overcome the problem, we adapted a modification to the Griess method by replacing sulphanilamide with 4-nitroaniline aiming to enhance the sensitivity of NO measurement. We envisioned that the transiently generated  $\text{NO}_2^-$  requires immediate capturing by an appropriate aromatic amine that would generate a highly reactive diazonium salt before the nitrous acid undergoes decomposition. The presence of nitro group in *para* position to the diazonium group, by virtue of its powerful electron-withdrawing and resonance properties, would potentially enhance the electrophilicity of the diazonium salt [24] (Figure 1) and accelerate the coupling with NED (Scheme 2) leading to enhanced sensitivity and improved detection limits. Moreover, the relative resonance stabilization of the diazonium salt is predicted to hasten the diazotization step via the intermediacy of a resonance-stabilized species (Scheme 2).

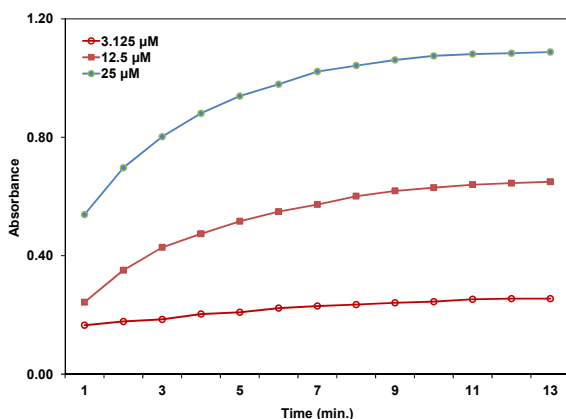
The release of NO from the prepared compounds was measured on the basis of the produced nitrite, which is a convenient index of nitric oxide production. In this context, the newly proposed Griess modification was first validated by constructing a standard sodium nitrite curve obtained by measuring the absorbance of various concentrations of sodium nitrite solution. Initially, sodium nitrite solutions were treated with a solution of 4-nitroaniline to form the diazonium salt followed by treating the resultant diazonium salt with NED to afford the purple azo dye that was measured at  $\lambda_{\text{max}}$  539 nm. We were delighted to find the short time needed for the diazocoupling process; the overall reaction time was 10 minutes beyond which a stable plateau absorbance was achieved (Figure 2). Conversely, the classical Griess method usually requires longer time (30 minutes) for completing the reaction.

**Table 1.** Comparison of the analytical parameters to validate the proposed method.

Parameters	Conventional Griess method (using sulfanilamide)	Newly modified method (using 4-nitroaniline)
Concentration range ( $\mu\text{g/mL}$ )	100-6.25	25-0.39
Intercept	0.1495	0.145
Correlation coefficient ( $r^2$ )	0.999	0.998
Limit of detection ( $\mu\text{g/mL}$ )	3.125	0.39
Limit of quantitation ( $\mu\text{g/mL}$ )	6.25	0.78
Reaction time (minutes)	30	10

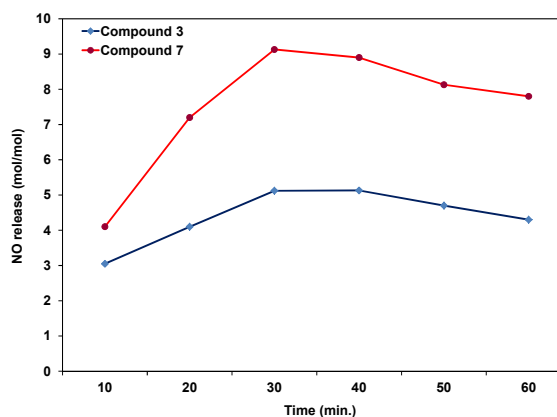


Scheme 2

**Figure 2.** Effect of time on the absorbance of reaction product of nitrite with the modified Griess reagent.

Moreover, the newly modified method showed an excellent reproducibility and linearity of the standard nitrite curve particularly at submicromolar concentrations (Table 1). A detection limit of  $0.39 \mu\text{M}$  was realized suggesting the reliable sensitivity of the new method in comparison to the classical one in which the sensitivity for the nitrite concentrations below  $3.125 \mu\text{M}$  was markedly reduced (Table 1). These outcomes suggest that the newly developed method would pave the way for potential applications in the precise determination of nitrite levels in water or biological fluids or even foodstuffs.

Motivated by the above-mentioned results, we applied the new method to estimate the NO release from NO donor hybrids **3** and **7**. The reaction was carried out in the presence of *N*-acetylcysteine as a source of thiol to facilitate the release of NO from the compounds. The NO release was measured at  $100 \mu\text{M}$  concentration of the tested compounds and assessed in the stable nitrite form relative to that of standard sodium nitrite solution and calculated as amount of NO released (mol/mol) as shown in Figure 3. The results indicated that oxime **7** and nitrate ester **3** had the ability to release NO, albeit in low amounts; 9 and  $5 \mu\text{M}$ , respectively, as maximum release after 30 minutes. In contrast, the classical Griess method did not allow the detection of measurable amounts of NO released from our compounds. This could probably be explained by considering a faster coupling rate between NED and the highly electrophilic 4-nitroaniline diazonium salt in our new method in comparison to the relatively slower reaction between NED and sulphanilamide diazonium salt in the conventional method.

**Figure 3.** NO release from compounds **3** and **7** by the newly developed Griess method.

#### 4. Conclusion

New norfloxacin/NO donor hybrids were synthesized and characterized by various spectroscopic tools. The NO release from the target compounds was estimated by a modified Griess method in which 4-nitroaniline was used instead of sulfanilamide. The modified method led to a faster reaction and improved sensitivity for nitrite determination in comparison to the classical method. Despite the modest NO release from our synthesized hybrids, it is anticipated that the new protocol would find great potential for the analysis of nitrites in various systems.

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