Research Article

Phenylisoxazole-3/5-Carbaldehyde Isonicotinylhydrazone Derivatives: Synthesis, Characterization, and Antitubercular Activity

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Eight new phenylisoxazole isoniazid derivatives, 3-(2′-fluorophenyl)isoxazole-5-carbaldehyde isonicotinylhydrazone (**1**), 3-(2′-methoxyphenyl)isoxazole-5-carbaldehyde isonicotinylhydrazone (**2**), 3-(2′-chlorophenyl)isoxazole-5-carbaldehyde isonicotinylhydrazone (**3**), 3-(3′-chlorophenyl)isoxazole-5-carbaldehyde isonicotinylhydrazone (**4**), 3-(4′-bromophenyl)isoxazole-5-carbaldehyde isonicotinylhydrazone (**5**), 5-(4′-methoxiphenyl)isoxazole-3-carbaldehyde isonicotinylhydrazone (**6**), 5-(4′-methylphenyl)isoxazole-3-carbaldehyde isonicotinylhydrazone (**7**), and 5-(4′-clorophenyl)isoxazole-3-carbaldehyde isonicotinylhydrazone (**8**), have been synthesized and characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectral data. The 2D NMR (^{1}H-^{1}H NOESY) analysis of **1** and **2** confirmed that these compounds in acetone-d_6 are in the *trans(E)* isomeric form. This evidence is supported by computational calculations which were performed for compounds **1–8**, using DFT/B3LYP level with the 6-311++G(d,p) basis set. The *in vitro* antituberculous activity of all the synthesized compounds was determined against the *Mycobacterium tuberculosis* standard strains: sensitive H37Rv (ATCC-27294) and resistant TB DM97. All the compounds exhibited moderate bioactivity (MIC = 0.34–0.41 μM) with respect to the isoniazid drug (MIC = 0.91 μM) against the H37Rv sensitive strain. Compounds **6** (X = 4′-OCH$_3$) and **7** (X = 4′-CH$_3$) with MIC values of 12.41 and 13.06 μM, respectively, were about two times more cytotoxic, compared with isoniazid, against the resistant strain TB DM97.

1. Introduction

Tuberculosis (TB) is a major public health problem in the world; it is an infectious disease caused by *Mycobacterium tuberculosis*. The presence of this bacterium in the body, as well as the inappropriate treatment of this disease, is one of the causes of millions of deaths in the world. In 2019, the World Health Organization (WHO) reported 10 million new TB cases and more than a million deaths [1]. The emergence of multidrug resistant (MDR), extensively drug resistant (XDR), and totally drug resistant (TDR) tuberculosis has attracted considerable attention from researchers to develop novel anti-TB agents with fewer side effects and shorter duration of treatment [2–4].

The isoniazid, pyridine-4-carbohydrazide, $C_5H_4NCONHNH_2$, is a prodrug clinically used for the treatment of *Mycobacterium tuberculosis* infection [5, 6]. There is evidence that its mechanism of action requires *in vivo* activation of this prodrug (INH). The active form produces various reactive species, which have as an intracellular target the reductase of the fatty acid transporter protein, in order to inhibit the synthesis of mycolic acid, from the *M. tuberculosis* cell wall [6, 7].

It is well known that the N-acetyltransferases (NATs) have been implicated in the resistance developed by "isoniazid, one of the first-line drugs in TB treatment" [1], due to the enzymatic acetylation of its primary amino group to form N-acetyl INH [2, 8–12]. In this sense, one of the benefits of isoniazid is obtaining a broad spectrum of novel isoniazid derivatives by condensing the terminal amine group (-NH$_2$) with the carbonyl group (-C=O) from aldehydes or ketones to produce new compounds with functional groups of the acylhydrazone type (~CH=NH-NH-C=O~) [13, 14] that can act as bidentate ligands (N,O) and coordinate to transition metals to obtain bis-chelate complexes [15, 16]. These acylhydrazone derivatives emerge as an alternative to block the acetylation of INH [9, 10].

In recent years, the isoniazid derivatives with phenyl, pyridyl, amidoether, dibenzofuran, and carvone fragments have been investigated since they exhibit wide spectrum of pharmacological applications, such us antitubercular [17–20], antimicrobial [21, 22], antileishmanial [23], antifungal [24], and anticancer [23, 25–27] activities.

The isoniazid-derived Schiff base compounds, with aromatic or aliphatic moiety, were tested for their antitubercular activity against *M. tuberculosis* H37Rv. It was found that the compound N^2-3,5,5-trimethylcyclohexylidenyl isonicotinic acid hydrazide was more active (MIC$_{90}$ value of 0.025 μg/mL) than the other prepared compounds against *M. tuberculosis* Erdman strain. Besides, the compound N^2-(2-benzyloxy)benzylidenyl isonicotinic acid hydrazide showed acceptable activity (MIC$_{90}$ = 0.06 μg/mL) assayed in this same strain compared with the MIC value found for INH (0.03–0.06 μg/mL) [10]. On the other hand, the compound N$'$-cyclohexylidenepyridine-4-carbohydrazide was slightly more active (MIC = 0.03 μg/mL) than isoniazid (MIC = 0.03–0.05 μg/mL) against *M. tuberculosis* H37Rv. These results indicate that these derivatives from isoniazid can be considered as potential antitubercular

agents [9, 28]. In this sense, the compound (*E*)-4-(4-((2-isonicotinoylhydrazono)methyl)phenoxy)-3-(phenyl-sulfonyl)-1,2,5-oxadiazole 2-oxide containing heterocyclic fragments (O,N) bound to the pyridine-4-carboxyhydrazide group was found to be very active against the *Mycobacterium tuberculosis* H37Rv and MDR-TB strains with MIC$_{90}$ values of 1.03 and 7.0 μM, respectively [29]. Moreover, the compound (*E*)-6-((2-isonicotinoylhydrazono)-methyl)benzo[c]-oxadiazole-1-oxide [1, 2, 5] containing the benzofuroxan derivative bound to the pyridine-4-carbohydrazide group showed remarkable inhibitory activity against the active and nonreplicating *M. tuberculosis* strains with MIC$_{90}$ values of 1.10 and 6.62 μM, respectively [30]. Furthermore, it has been shown that compounds ethyl 4-methyl-2-[(*E*)-2-[1-(pyridin-2-yl)ethylidene]hydrazin-1-yl]-1,3-thiazole-5-carboxylate and ethyl 2-[(*E*)-2-[(2-hydroxyphenyl)methylidene]hydrazin-1-yl]-4-methyl-1,3-thiazole-5-carboxylate display noticeable inhibitory activity against *M. tuberculosis* H$_{37}$Rv with MIC$_{80}$ values of 12.50 and 25 μM, respectively [31].

Recently, we have reported *in vitro* antimicrobial studies of indole-3-carbaldehyde semicarbazone derivatives against various Gram-positive and Gram-negative bacteria. The results demonstrated that compounds 2-((5-bromo-1H-indol-3-yl)methylene)hydrazine carboxamide and 2-((5-chloro-1H-indol-3-yl) methylene)hydrazine carboxamide were moderately active against *Staphylococcus aureus* (MIC = 100 and 150 μg/mL, respectively) and *Bacillus subtilis* (MIC = 100 and 150 μg/mL, respectively) relative to tetracycline (antimicrobial drug) [32]. Continuing with our research on new heterocyclic compounds with potential biological activity, we herein describe the synthesis and spectral characterization of eight new phenylisoxazole isoniazid derivatives. All the prepared compounds were tested for their *in vitro* antitubercular activity against the *Mycobacterium tuberculosis* strains: H37Rv (ATCC-27294) and TB DM97.

2. Materials and Methods

2.1. Chemicals and Instrumentation. All the chemicals used in this work were purchased from Merck and Sigma-Aldrich. The *M. tuberculosis* strains, susceptible H37Rv (ATCC 27294) and resistant wild type MDR (DM97), were obtained from Universidad Peruana Cayetano Heredia. Susceptibility tests were performed in duplicate (two plates) using the tetrazolium microplate assay (TEMA), whose salt is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide [20, 33–35]. Isoniazid was used as a reference drug [8, 19, 33].

All melting points were measured on a Büchi melting point B-545 apparatus. The infrared spectra were recorded using a Nicolet iS10 FT IR spectrometer, Mass spectra were recorded on the high-resolution mass spectrometers: Waters-Quattro Premier XE^{TM} tandem quadrupole and VG Micromass ZAB-2F, using methanol as the sample dissolution medium. The values of molecular ions are reported as ESI$^+$ ([M+H]$^+$, [M+Na]$^+$) or ESI$^-$ ([M-H]$^-$). The NMR spectra were recorded in deuterated dimethyl sulphoxide (DMSO-d$_6$) or deuterated acetone (CD$_3$COCD$_3$) with an

Agilent NMR spectrometer (500 MHz for ^{1}H and 126 MHz for ^{13}C) or a Bruker AVANCE^{TM} NMR spectrometer (400 or 600 MHz for ^{1}H and 100 or 150 MHz for ^{13}C) using tetramethylsilane (TMS) as an internal reference. The chemical shifts values were reported in parts per million (δ, ppm) from internal standard TMS.

2.2. Experimental Procedures

2.2.1. Synthesis of the Phenylisoxazole-3/5-Carbaldehyde Isonicotinylhydrazone Derivatives (1–8)

(1) General Method. Isoniazid (0.137 g, 1 mmol) dissolved in 24 mL of a hot methanol-water mixture (v/v, 10 : 2) was added dropwise to a solution of the phenylisoxazole 3/5-carbaldehyde derivative (1 mmol) in 20 mL of methanol during 20 minutes. Then 0.2 mL of glacial acetic acid (catalyst) was added to the solution. The reaction mixture was heated under reflux condition for 3 h. The reaction mixture was concentrated in vacuum. The solid product obtained after a slow evaporation was filtered and recrystallized from a methanol-DMSO mixture (v/v, 1 : 1) to obtain the corresponding phenylisoxazole-3/5-carbaldehyde isonicotinylhydrazone derivatives in pure form.

(2) 3-(2′-Fluorophenyl)Isoxazole-5-Carbaldehyde Isonicotinylhydrazone (1). White solid. Yield: 0.225 g (73%). m. p. 230–232°C. FT-IR (ν, cm^{-1}): 3211.45 (N-H), 3052.61(C$_{ar}$-H), 1653.36 (C = O), 1531.94 (C = N). ESI-MS: m/z 309.105 [M − H]$^-$ (Cal. for C$_{16}$H$_{11}$FN$_4$O$_2$: 310.283 g/mol). ^{1}H-NMR (500 MHz, Acetone-d$_6$, ppm): δ 11.61 (s, 1H, =N-NH), 8.80 (d, 2H, J = 5.1 Hz, H-2′′, H-6′′), 8.72 (s, 1H, CH = N), 8.02 (s, 1H, H-6′), 7.84 (br, 2H, H-3′′, H-5′′), 7.60 (d, 1H, J = 6.4 Hz, H-4′), 7.41–7.34 (m, 2H, H-3, H-5), 7.26 (s, 1H, H-4). ^{13}C-NMR (126 MHz, Acetone-d$_6$, ppm): δ 150.61, 121.20 (C-2′′, C-6′′; C-3′′, C-5′′, Pyr), 132.40, 129.15/129.17, 124.94/124.97, 116.36/116.53 (C-4′, C-6′, C-5′, C-3′, Ph), 104.31/104.24 (C-4, isoxazole ring), 135.62 (CH = N).

(3) 3-(2′-Methoxyphenyl)Isoxazole-5-Carbaldehyde Isonicotinylhydrazone (2). White solid. Yield: 0.252 g (78,3%). m. p. 202–204°C. FT-IR (ν, cm^{-1}): 3201.32 (N-H), 3042.47 (C$_{ar}$-H), 1663.09 (C = O), 1540.73 (C = N). ESI-MS: m/z 321.084 [M − H]$^-$ (Cal. for C$_{17}$H$_{14}$N$_4$O$_3$: 322.318 g/mol). ^{1}H-NMR (500 MHz, Acetone-d$_6$, ppm): δ 11.55 (s, 1H, =N-NH), 8.80 (d d, 2H, J = 5.8 Hz, H-2′′, H-6′′), 8.68 (s, 1H, CH = N), 7.91 (d, 1H, J = 6.8 Hz, H-6′), 7.86 (br, 2H, H-3′′, H-5′′), 7.50 (t, 1H, J = 7.8 Hz, H-4′), 7.32 (s, 1H, H-4), 7.21 (d, 1H, J = 7.8 Hz, H-3′), 7.08 (t, 1H, J = 6.8 Hz, H-5′), 3.43 (s, 3H, -OCH$_3$). ^{13}C-NMR (126 MHz, Acetone-d$_6$, ppm): δ 150.59, 121.23 (C-2′′, C-6′′; C-3′′, C-5′′, Pyr), 157.46, 131.67, 128.92, 120.71, 117.24, 111.91 (C-2′, C-4′, C-6′, C-5′, C-1′, C-3′, Ph), 160.24, 105.45 (C-3, C-4, isoxazole ring), 136.07 (CH = N), 55.19 (-OCH$_3$).

(4) 3-(2′-Chlorophenyl)Isoxazole-5-Carbaldehyde Isonicotinylhydrazone (3). White solid. Yield: 0.212 g (65%). m. p.

212–214°C. FT-IR (ν, cm^{-1}): 3159.74 (N-H), 3020.30 (C$_{ar}$-H), 1654.74 (C = O), 1541.09 (C = N). ESI-MS: m/z 327.065 [M + H]$^+$, 349.046 [M + Na]$^+$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{35}Cl: 326.253 g/mol), m/z 329.063 [M + H]$^+$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{37}Cl: 328.250 g/mol). ^{1}H-NMR (600 MHz, DMSO-d$_6$, ppm): δ 12.48 (s, 1H, =N-NH), 8.82 (d, 2H, J = 5.0 Hz, H-2′′, H-6′′), 8.57 (s, 1H, CH = N), 7.84 (d, 2H, J = 5 Hz, H-3′′, H-5′′), 7.76 (d, 1H, J = 7.3 Hz, H-6′), 7.67 (d, 1H, J = 6.6 Hz, H-3′), 7.57 (t, 1H, J = 7.4 Hz, H-5′), 7.51 (t, 1H, J = 7.4 Hz, H-4′), 7.40 (s, 1H, H-4). ^{13}C-NMR (150 MHz, DMSO-d$_6$, ppm): δ 150.94, 140.33, 122.03 (C-2′′, C-6′′; C-4′′; C-3′′, C-5′′, Pyr), 132.34, 131.61, 130.92, 128.24, 127.71 (C-1′, C-5′; C-6′, C-3′, C-4′, C-2′, Ph), 165.55, 161.52, 106.85 (C-5, C-3, C-4, isoxazole ring), 136.12 (CH = N).

(5) 3-(3′-Chlorophenyl)Isoxazole-5-Carbaldehyde Isonicotinylhydrazone (4). White solid. Yield: 0.268 g (65%). m. p. 200–202°C. FT-IR (ν, cm^{-1}): 3506.43, 3118.28 (N-H), 2996.75 (C$_{ar}$-H), 1673.85 (C = O), 1546.15 (C=N). ESI-MS: m/z 325.04 [M − H]$^-$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{35}Cl: 326.25 g/mol), m/z 326.97 [M − H]$^-$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{37}Cl: 328.25 g/mol). ^{1}H-NMR (500 MHz, DMSO-d$_6$, ppm): δ 12.46 (br, 1H, =N-NH), 8.82 (d, 2H, J = 6.0 Hz, H-2′′, H-6′′), 8.56 (s, 1H, CH = N), 8.03 (s, 1H, H-2′), 7.95 (d, 1H, J = 7.5 Hz, H-6′), 7.85 (d, 2H, J = 6.0 Hz, H-3′′, H-5′′), 7.68 (s, 1H, H-4), 7.62–7.57 (m, 2H, H-4′, H-5′). ^{13}C-NMR (126 MHz, DMSO-d$_6$, ppm): δ 150.95, 140.34, 122.02 (C-2′′, C-6′′; C-4′′; C-3′′, C-5′′, Pyr), 134.45, 131.63, 130.86, 130.48, 126.94, 125.79 (C-5′, C-1′, C-3′, C-6′, C-4′, C-2′, Ph), 166.41, 161.85, 104.03 (C-5, C-3, C-4, isoxazole ring), 162.58 (C = O), 136.07 (CH = N).

(6) 3-(4′-Bromophenyl)Isoxazole-5-Carbaldehyde Isonicotinylhydrazone (5). White solid. Yield: 0.267 g (72%). m. p. 261–263°C. FT-IR (ν, cm^{-1}): 3446.08, 3181.48 (N-H), 1677.65 (C = O), 1587.09 (C = N). ESI-MS: m/z 369.114 [M − H]$^-$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{79}Br: 370.203 g/mol), m/z 371.112 [M − H]$^-$ (Cal. for C$_{16}$H$_{11}$N$_4$O$_2$^{81}Br: 372.201 g/mol). ^{1}H-NMR (500 MHz, DMSO-d$_6$, ppm): δ 12.44 (br, 1H, =N-NH), 8.82 (d, 2H, J = 6.0 Hz, H-2′′, H-6′′), 8.55 (s, 1H, CH = N), 7.91 (d, 2H, J = 8.5 Hz, H-2′, H-6′), 7.84 (d, 2H, J = 6.0 Hz, H-3′′, H-5′′), 7.61 (s, 1H, H-4), 7.74 (d, 2H, J = 8.0 Hz, H-3′, H-5′).

(7) 5-(4′-Methoxyphenyl)Isoxazole-3-Carbaldehyde Isonicotinylhydrazone (6). White solid. Yield: 0.255 g (79%). m. p. 212–214°C. FT-IR (ν, cm^{-1}): 3308.24, 3108.83 (N-H), 1669.66 (C = O), 1544.86 (C = N). ESI-MS: m/z 323.113 [M + H]$^+$, 345.097 [M + Na]$^+$ (Cal. for C$_{17}$H$_{14}$N$_4$O$_3$: 322.318 g/mol). ^{1}H-NMR (400 MHz, DMSO-d$_6$, ppm): δ 12.36 (s, 1H, =N-NH), 8.81 (d, 2H, J = 5.5 Hz, H-2′′, H-6′′), 8.53 (s, 1H, CH = N), 7.91 (d, 2H, J = 9.2 Hz, H-2′, H-6′), 7.83 (d, 2H, J = 5.5 Hz, H3′′, H-5′′), 7.26 (s, 1H, H-4), 7.08 (d, 2H, J = 9.2 Hz, H-3′, H-5′), 3.81 (s, 3H, -OCH$_3$). ^{13}C-NMR (100 MHz, DMSO-d$_6$, ppm): δ 150.49, 140.0, 121.57 (C-2′′, C-6′′; C-4′′; C-3′′, C-5′′, Pyr), 161.08, 127.60, 119.06, 114.57 (C-4′; C-2′, C-6′; C-1′; C-3′, C-5′, Ph), 169.90, 160.74, 95.82 (C-5, C-3, C-4, isoxazole ring), 161.98 (C = O), 138.90 (CH = N), 55.40 (-OCH$_3$).

(8) 5-(4′-Methylphenyl)Isoxazole-3-Carbaldehyde Isonicotinylhydrazone (7). White solid. Yield: 0.279 g (91%). m. p. 209–211°C. FT-IR (ν, cm^{-1}): 3313.95, 3115.03 (N-H), 1670.61 (C=O), 1541.61 (C=N). ESI-MS: m/z 305.097 [M−H]$^-$ (Cal. for $C_{17}H_{14}N_4O_2$: 306.319 g/mol). ^{1}H-NMR (500 MHz, Acetone-d$_6$, ppm): δ 11.55 (s, 1H, =N-NH), 8.80 (d, 2H, $J = 5.1$ Hz, H-2′′, H-6′′), 8.61 (s, 1H, CH=N), 7.90–7.87 (m, 4H, H-2′, H-6′; H-3′′, H-5′′), 7.40 (d, 2H, $J = 9.0$ Hz, H-3′, H-5′), 7.19 (s, 1H, H-4), 2.42 (s, 3H, CH$_3$). ^{13}C-NMR (126 MHz, Acetone-d$_6$, ppm): δ 150.59, 140.93, 121.25 (C-2′′, C-6′′; C-4′′; C-3′′, C-5′′, Pyr), 140.93, 129.78, 125.79 (C-4′; C-3′, C-5′; C-2′, C-6′, Ph), 96.15 (C-4, isoxazole ring), 138.73 (CH=N), 20.51 (CH$_3$).

(9) 5-(4′-Chlorophenyl)Isoxazole-3-Carbaldehyde Isonicotinylhydrazone (8). White solid. Yield: 0.268 g (82%). m. p. 222–224°C. FT-IR (ν, cm^{-1}): 3447.15, 3224.99, 3112.73 (N-H), 1672.46 (C=O), 1554.16 (C=N). ESI-MS: m/z 349.046 [M+Na]$^+$ (Cal. for $C_{16}H_{11}N_4O_2{}^{35}$ClNa: 348.741 g/mol), m/z 351.045 [M+Na]$^+$ (Cal. for $C_{16}H_{11}N_4O_2{}^{37}$ClNa: 350.740 g/mol). ^{1}H-NMR (600 MHz, DMSO-d$_6$, ppm): δ 12.44 (s, 1H, =N-NH), 8.82 (d, 2H, $J = 5.3$ Hz, H-2′′, H-6′′), 8.57 (s, 1H, CH=N), 8.02 (d, 2H $J = 8.3$ Hz, H-2′, H-6′), 7.84 (d, 2H, $J = 5.3$ Hz, H-3′′, H-5′′), 7.62 (d, 2H, $J = 8.3$ Hz, H-3′, H-5′), 7.52 (s, 1H, H-4). ^{13}C-NMR (150 MHz, DMSO-d$_6$, ppm): δ 150.93, 140.47, 122.04 (C-2′′, C-6′′; C-4′′; C-3′′, C-5′′, Pyr), 135.91, 129.86, 128.15, 125.70 (C-4′; C-3′, C-5′; C-2′, C-6′, C-1′, Ph), 169.19, 161.43, 98.50 (C-5, C-3, C-4, isoxazole ring), 162.53 (C=O), 139.11 (CH=N).

2.2.2. Antimycobacterial Assay. The *in vitro* antituberculous activity of compounds **1**–**8** was tested against strains of *Mycobacterium tuberculosis*, H37Rv and DM97. The used method was tetrazolium microplate assay (TEMA). Drug concentration ranges were from 32 to 0.125 μg/mL dissolved in Middlebrook 7H9 medium supplemented with OADC medium. Plates were sealed with Ziploc bags and incubated at 37°C for five days. After addition of tetrazolium-Tween 80 solution and incubating for 24 h, the results were observed. A yellow color in the well was interpreted as no growth and a purple color was scored as growth. The detailed procedure is found in the reviewed literature [20]. The MIC value was defined as the lowest drug concentration, which prevents the visible growth of a bacterium or bacteria.

2.2.3. Computational Methodology. The quantum chemical calculations were carried out using the Gaussian 09 package [36]. The geometries of compounds **1**–**8** under investigation were optimized by using density functional theory (DFT), with the Becke 3-parameter and Lee-Yang-Parr (B3LYP) functional [37–40] and the 6-311++G(d,p) basis set without symmetric restrictions. The results of the harmonic vibrational frequency were also calculated at the same level to verify that the derived structures correspond to local minima of the potential energy surface (see Supplementary Figure 1 in Supplementary Materials). The computed energies and enthalpies for the most stable conformers were obtained in both gas and liquid (acetone and DMSO) phases. For the last

case, the continuous polarizable model (PCM) was used [40]. The population of conformers was determined from their Boltzmann distribution [37].

3. Results and Discussion

3.1. Synthesis and Characterization. The phenylisoxazole-3/5-carbaldehyde isonicotinylhydrazone derivatives **1**–**8** were obtained in satisfactory yields (65–91%) by refluxing isoniazid with the respective phenylisoxazole-3/5-carbaldehyde derivative in methanol, according to literature [41], as shown in Scheme 1. All the synthesized compounds were characterized by FT-IR, mass, 1D NMR (^{1}H- and ^{13}C-NMR), and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) spectral analyses. Spectroscopic data obtained for compounds **1**–**8** confirmed the proposed structures. All these compounds were purified by recrystallization using a methanol-DMSO (v/v, 1:1) mixture. In the ^{13}C-NMR spectra recorded in acetone-d$_6$ for **1**, **2**, and **7**, the signal of the carbonyl group (C=O) was not observed, due to the fact that these compounds were only slightly soluble in acetone-d$_6$. The signals of the ^{13}C-NMR spectrum of compound **5** were not recorded, due to its low solubility in DMSO-d$_6$

3.2. FT-IR Spectral Analysis. In the IR spectra of the studied compounds **1**–**8** (see Supplementary Figures 3, 10, 17, 21, 25, 27, 33, and 40 in Supplementary Materials), the less intense broad bands around 3108–3506 cm^{-1} are assigned to ν(N-H) vibrations of the NHCO group [2, 15, 21]. The intense absorption bands of the carbonyl group (CO) were observed in the 1653–1677 cm^{-1} region [17, 21]. These ν(CO) vibrations, found in all the compounds, confirm the presence of the keto tautomer in the solid state, in agreement with the stretching frequencies of the carbonyl group found for other compounds derived from semicarbazones with indole fragments [32]. The strong absorption bands corresponding to the imine C=N group appeared at 1531–1587 cm^{-1} [17, 30]. For **5** and **8**, the absorption bands of the C=N group were shifted to higher frequencies (45 and 12 cm^{-1}, respectively) with respect to (C=N) stretching vibrations of **7** (X = 4′-CH$_3$). These results indicate that the ν(C=N) vibrations of compounds **5** and **8** were affected by the presence of the *bromo* and *chloro* substituents in the *para* position, respectively, of the phenyl ring.

3.3. Mass Spectra. The ESI mass spectra display molecular ion peaks corresponding to the fragments [M+H]$^+$, [M+Na]$^+$, or [M−H]$^-$ for the synthesized compounds **1**–**8** (see Supplementary Figures 2, 9, 16, 20, 24, and 39 in Supplementary Materials). The mass data obtained for all the synthesized compounds are shown in Table 1. Figures 1 and 2 show the molecular ion peaks found for compounds **6** (323.113 [M+H]$^+$) and **7** (305.097 [M−H]$^-$), respectively. Mass spectra of compounds **3**, **4**, and **8** displayed two molecular ion peaks m/z at 327.065/329.063 [M+H]$^+$, 325.039/326.977 [M−H]$^-$, and 349.046/351.045 [M+Na]$^+$, respectively. These results reveal the presence of the ^{35}Cl and

(a)

(b)
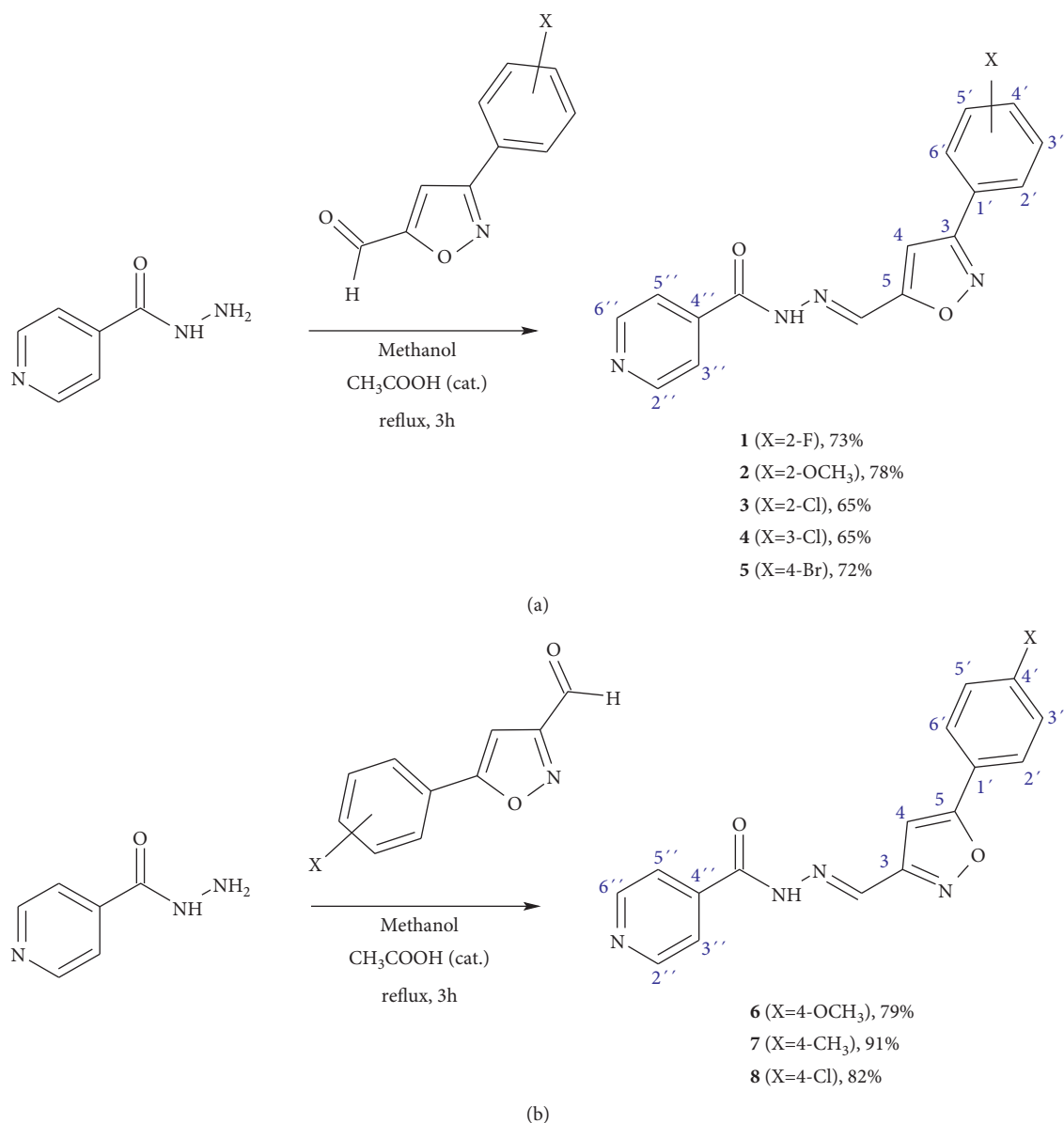
SCHEME 1: Synthesis of the isoniazid derivatives. (a) Phenylisoxazole-5-carbaldehyde isonicotinylhydrazone derivatives **1–5** and (b) phenylisoxazole-3-carbaldehyde isonicotinylhydrazone derivatives **6–8**.

TABLE 1: m/z values of compounds **1–8** obtained by ESI-MS.

| Compound	Chemical formula	Ion mode	Calculated molecular weight (M, g/mol)	(m/z) found
1	$C_{16}H_{11}FN_4O_2$	$[M-H]^-$	310.283	309.105
2	$C_{17}H_{14}N_4O_3$	$[M-H]^-$	322.318	321.084
3	$C_{16}H_{11}N_4O_2{}^{35}Cl/C_{16}H_{11}N_4O_2{}^{37}Cl$	$[M+H]^+$	326.253/328.250	327.065/329.063
4	$C_{16}H_{11}N_4O_2{}^{35}Cl/C_{16}H_{11}N_4O_2{}^{37}Cl$	$[M-H]^-$	326.253/328.250	325.039/326.977
5	$C_{16}H_{11}N_4O_2{}^{79}Br/C_{16}H_{11}N_4O_2{}^{81}Br$	$[M-H]^-$	370.203/372.201	369.114/371.112
6	$C_{17}H_{14}N_4O_3$	$[M+H]^+$	322.318	323.113
7	$C_{17}H_{14}N_4O_2$	$[M-H]^-$	306.319	305.097
8	$C_{16}H_{11}N_4O_2{}^{35}Cl/C_{16}H_{11}N_4O_2{}^{37}Cl$	$[M+Na]^+$	326.253/328.250	349.046/351.045
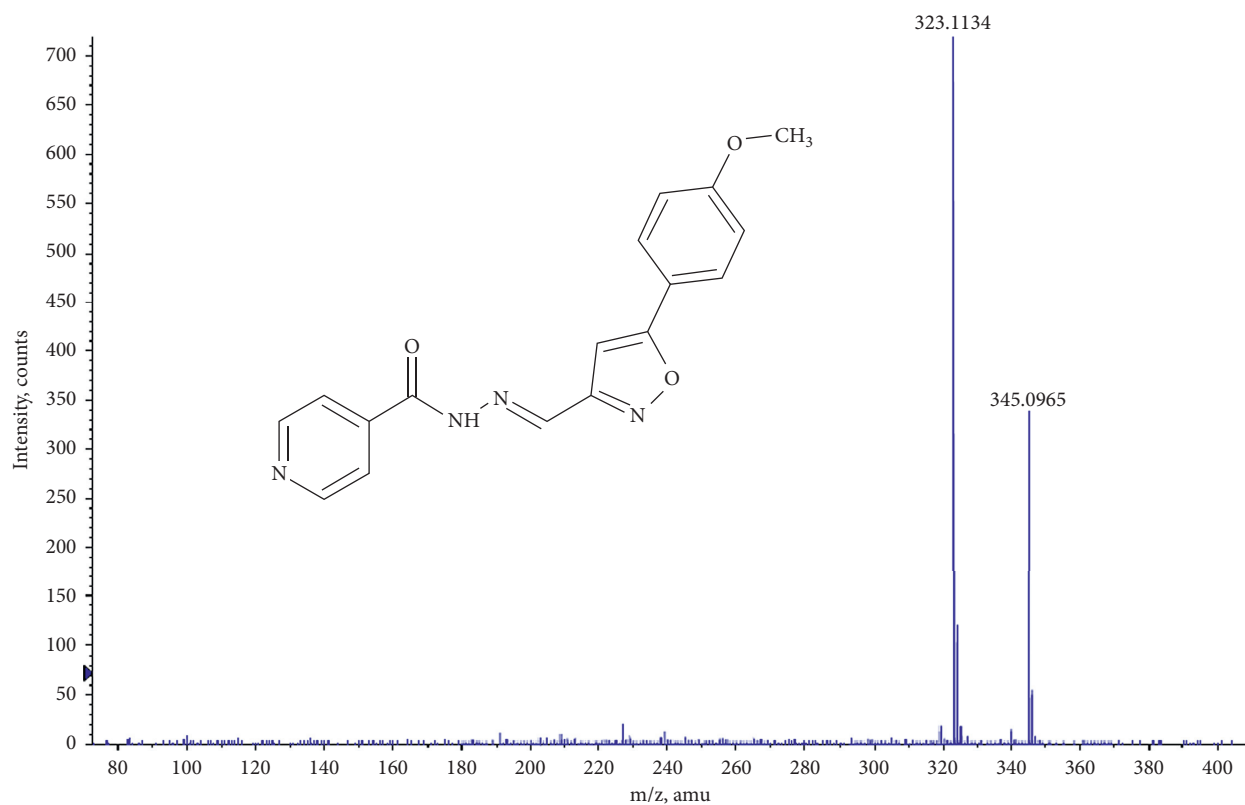
Figure 1: ESI-mass spectrum of compound 6.
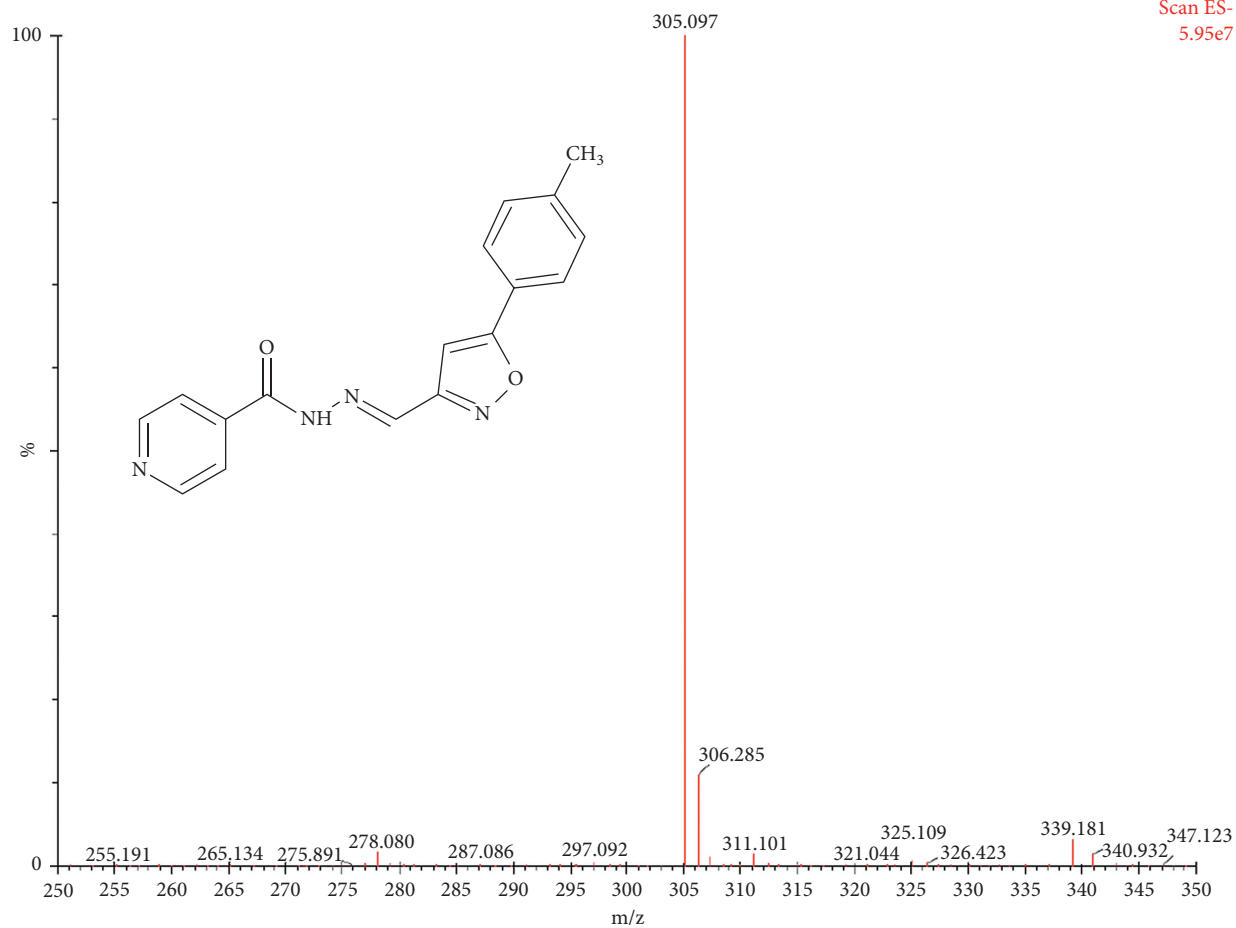

Figure 2: ESI-mass spectrum of compound 7.

^{37}Cl isotopes. On the other hand, **5** gives two molecular ion peaks at 369.114/371.112 [M−H]$^{-}$ due to the presence of the ^{79}Br and ^{81}Br isotopes [42].

3.4. NMR Spectra. The characterization of a novel series of the phenylisoxazole-3/5-carbaldehyde isonicotinylhydrazone derivatives **1–8** was performed by using the 1D NMR (^{1}H- and ^{13}C-NMR) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC) spectra, recorded in acetone-d$_{6}$/ DMSO-d$_{6}$ (see Supplementary Figures 4–8, 11–15, 18, 19, 22, 23, 26, 28–32, 34–38, 41, and 42 in Supplementary Materials).

The ^{1}H-NMR spectra of all the synthesized compounds showed a singlet in the region $\delta = 12.48–11.55$ and $8.72–8.53$ ppm, for the amide (-CONH-) and imine (–HC = N-) protons, respectively. These results are similar to the chemical shifts found for other isoniazid derivatives [15, 17, 30]. The signal of the -CH isoxazole aromatic proton was observed as a singlet at $\delta = 7.68–7.19$ [41, 43]. Moreover, the signals of the pyridine C-H proton appeared at $\delta = 8.82–7.83$, and these chemical shifts are in agreement with those found for other isoniazid derivatives [44].

For compounds **1** and **3**, the CH=N proton signals bound to the isoxazole phenyl group were affected by the presence of the *fluoro* and *chloro* substituents in the C-2′ positions of the aromatic ring, respectively. These signals are deshielded 0.4 and 0.25 ppm, for **1** and **3**, respectively, with respect to the imine proton of the compound N′-(4-(dimethylamino)benzylidene)isonicotinoylhydrazone monohydrate [45]. For compounds **1** (X = 2′-F), **2** (X = 2′-OCH$_{3}$), and **3** (X = 2′-Cl), the CONH proton signals are shielded 0.15 ppm and 0.21 ppm for **1** and **2**, respectively, and deshielded 0.72 ppm (for **3**) with respect to the chemical shift of the compound N′-(4-(dimethylamino)benzylidene) isonicotinoylhydrazone monohydrate [45].

The signals of the CH protons of the isoxazole ring of compounds **1** (X = 2′-F), **2** (X = 2′-OCH$_{3}$), **3** (X = 2′-Cl), **4** (X = 3′-Cl), and **5** (X = 4′-Br) are deshielded 0.53, 0.59, 0.67, 0.95, and 0.88 ppm, respectively, with respect to the compound 3-bromo-N-(3-phenyl-5-isoxazolyl)propanamide [46].

For compounds **2** and **3**, the aromatic proton signals in positions C-3′ are shifted upfield (0.12 ppm for **2**) and downfield (0.34 ppm for **3**), while the aromatic proton signals in positions C-4′ are shifted 0.34 and 0.35 ppm downfield for **2** and **3**, respectively, with respect to the unsubstituted phenyl moiety [47]. Besides, for **4**, the aromatic proton signals in positions C-2′ and C-4′ are shifted downfield 0.42 and 0.46–0.41 ppm, respectively, while for **5** they are shifted downfield for the protons in positions C-2′ and C-6′ (0.30 ppm) and C-3′ and C-5′ (0.41 ppm), with respect to the unsubstituted phenyl moiety [47]. With respect to compounds **6** and **8**, the aromatic proton signals of the phenyl fragment were affected by the presence of the *methoxy* and *chloro* substituents in the C-4′ positions. These signals are shifted upfield (0.25 ppm for **6**) and downfield (0.29 ppm for **8**) for the protons in positions C-3′ and C-5′, while the protons in positions C-2′ and C-6′ are shifted

downfield (0.30 ppm and 0.41 ppm for **6** and **8**, respectively), with respect to the unsubstituted phenyl moiety [47].

On the other hand, for compounds **6** (X = 4′-OCH$_{3}$), **7** (X = 4′-CH$_{3}$), and **8** (X = 4′-Cl), the CH=N proton signals are deshielded 0.21, 0.29, and 0.25 ppm, respectively, while the CONH proton signals are deshielded (0.6 and 0.68 ppm for **6** and **8**, respectively) and shielded (0.21 ppm for **7**) with respect the compound N′-(4-(dimethylamino)benzylidene) isonicotinoylhydrazone monohydrate [45]. Besides, the signals of the CH proton of the isoxazole ring are deshielded 0.53, 0.46, and 0.79 ppm, respectively, with respect to the compound 3-bromo-N-(3-phenyl-5-isoxazolyl)prop-anamide [46].

In the ^{13}C-NMR spectra of compounds **1–4** and **6–8**, the pyridine aromatic carbons appeared at $\delta = 150.95–150.49$ (C-2′′, C-6′′) and 122.04–121.20 (C-3′′, C-5′′). For compounds **3**, **4**, **6**, and **8**, the chemical shifts of the C-4′′ carbon appeared in the range of 140.47–140.0 ppm. These results are in agreement with the resonance lines of the pyridine carbons found for other derivatives from isoniazid and isoniazid hydrazones [48, 49]. For compounds **1–4** and **6–8**, the resonance lines observed at 139.11–135.62 ppm were assigned to the azomethine carbons. This evidence confirms the formation of the hydrazones in these compounds [45]. For compounds **1**, **2**, and **3**, the signals of the C-4 isoxazole aromatic carbons observed at $\delta = 106.85–104.31$ were affected by the presence of the *fluoro, methoxy*, and *chloro* substituents in the C-2′ positions of the phenyl fragment bound to the isoxazole ring. These signals shifted downfield 3.61, 4.75, and 6.15 ppm, respectively, relative to dihydrocurcumin isoxazole, with the unsubstituted isoxazole moiety [50]. However, the signals of the C-4 isoxazole carbon of **6** (X = 4′-OCH$_{3}$), **7** (X = 4′-CH$_{3}$), and **8** (X = 4′-Cl), found at the range of 98.50–95.82 ppm, were shifted upfield 4.88, 4.55, and 2.2 ppm with respect to the unsubstituted isoxazole moiety found for other isoxazole derivatives [50].

The signals of the C-3′ phenyl aromatic carbons observed at 116.45, 111.91, and 130.92 ppm for **1**, **2**, and **3**, respectively, were affected by the presence of the *fluoro, methoxy*, and *chloro* substituents in the C-2′ positions of the phenyl fragments. These signals shifted upfield 10.63 and 15.17 ppm for **1** and **2**, respectively, and downfield 3.84 ppm for **3** with respect to the compound 3-[4-(methylthio) phenyl]-5-phenylisoxazole with the *meta* carbon of the unsubstituted phenyl group [51]. Besides, the signals of the C-3′ and C-5′ aromatic carbons of the phenyl fragment in **6** (X = 4′-OCH$_{3}$) and **8** (X = 4′-Cl) found at 114.57 and 129.86 ppm were shifted upfield 12.51 ppm and downfield 2.78 ppm with respect to the unsubstituted phenyl group found for other isoxazole derivatives [51].

The two-dimensional ^{1}H-^{1}H NOESY spectra recorded in acetone-d$_{6}$ for compounds **1** and **2** (Figure 3) show coupling between the amide proton (-CONH) and the imine proton (-CH=N) and also between the H-3′′ pyridine proton and the amide proton (-CONH). These results are in agreement with the *trans(E)* configurational isomer found for other acylhydrazone derivatives [17, 37].
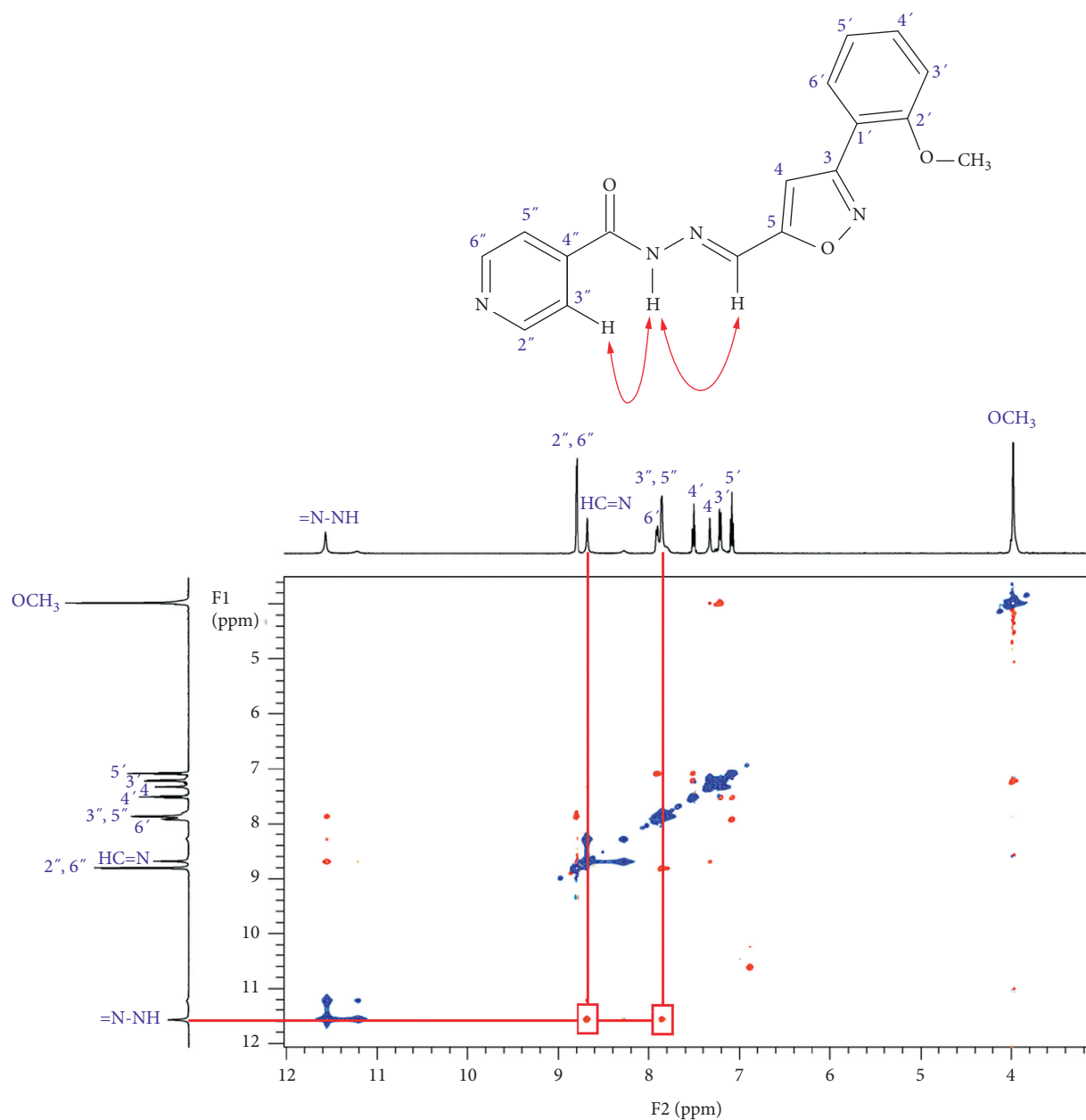
FIGURE 3: Two-dimensional ^{1}H-^{1}H NOESY NMR spectrum (500 MHz) recorded in acetone-d$_6$ for **2**.

3.5. DFT Calculations. The geometry of the studied molecules was identified as *Z/E* isomers, with respect to the C=N bond, and *cis/trans* conformers compared to the CONH amide group [52]. The computed energies, enthalpies, and population of the thermodynamically stable conformers are described in Table 1. The most stable configurations in all the studied molecules **1**–**8**, in both gas and liquid phases, are considered as *E* isomers, with dihedral angles Φ (Figure 4) close to 180°. These results indicate the planar arrangement of these conformers. Φ is defined by O – C9 – C10 = N2 and N1 – C9 – C10 = N2 bonds for **1** to **5** and **6** to **8**, respectively.

In the gas phase, the most stable molecule is a *cis(E)* conformer with a population between 82 and 93%, while the population of the *trans(E)* conformers is between 7 and 17%. In the liquid phase (acetone and DMSO), the

conformational configuration changes, and the most stable molecule is the *trans(E)* conformer with a population between 54 and 96%, while the population of *cis(E)* conformers is from 4 to 46%. This behavior has been observed in the geometrical configurations of other hydrazone derivatives [37].

For all the compounds, in both the gas and liquid phases, the population of *cis* and *trans(Z)* conformers (described as "others" in Table 2) is minimal, since it is less than 2% of the total conformational population (see Supplementary Table 1 in Supplementary Materials). All of them display a considerable high enthalpy difference relative to the corresponding most stable one (greater than 2.5 kJmol^{-1}).

It is interesting to mention that DFT optimized geometries in all the synthesized compounds are in good
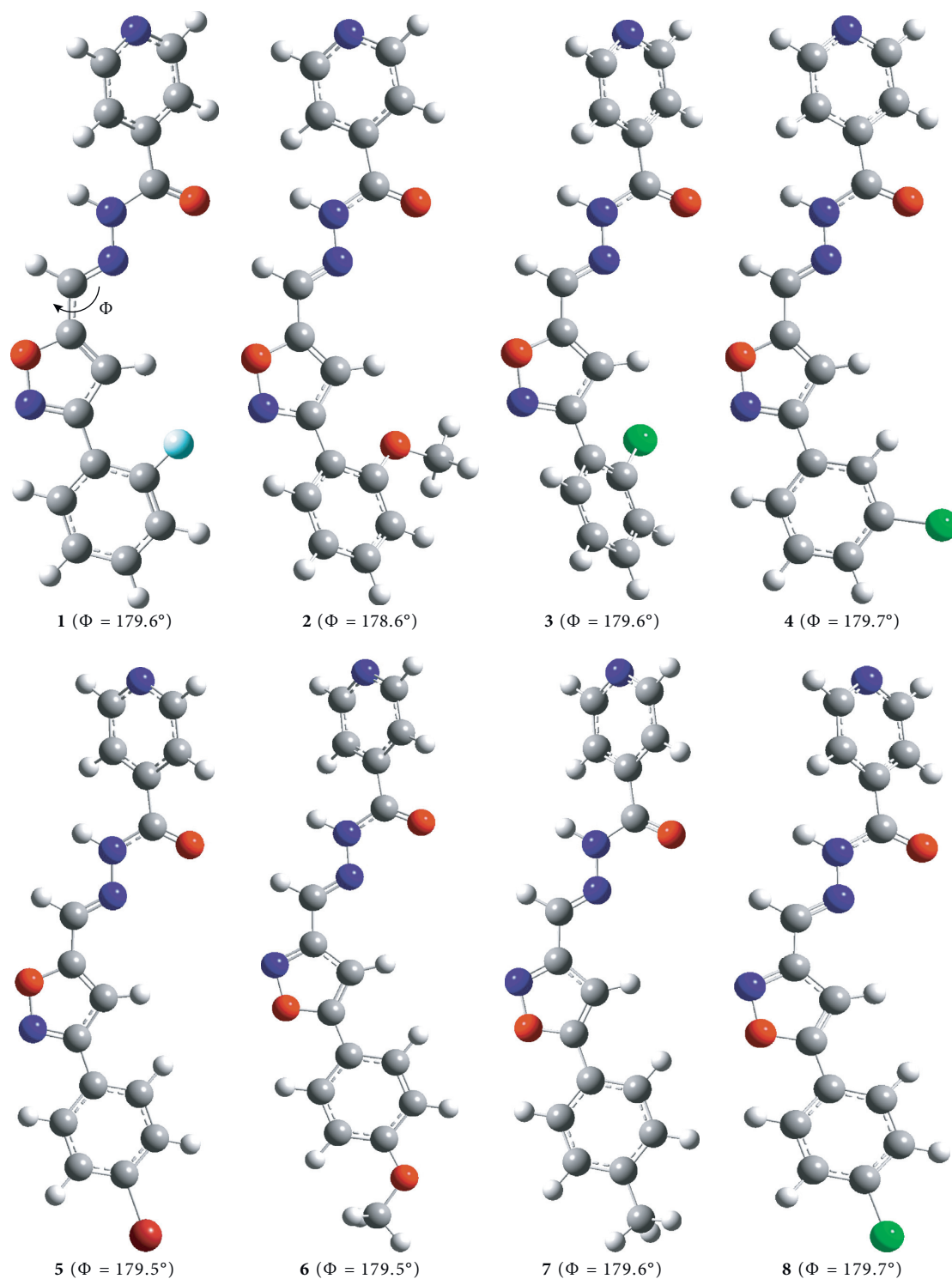
FIGURE 4: Molecular geometry of the more stable conformers (*trans(E)*) for compounds **1**–**8** optimized at the theory level B3LYP/6-311++G(d,p).

agreement with two-dimensional ^{1}H-^{1}H NOESY NMR results, particularly for **1** (see Supplementary Information) and **2** (Figure 3).

3.6. Antimycobacterial Assays. The *in vitro* anti-*Mycobacterium tuberculosis* activity of the isoniazid derivatives **1**–**8** was investigated against the standard strains of sensitive H37Rv (ATCC-27294) and resistant TB DM97. The values of minimum inhibitory concentration (MIC), expressed in μM, were determined, using the microplate susceptibility test (method TEMA). In these assays, the tetrazolium salt and the isoniazid antibiotic were used as growth indicator and reference drug, respectively [20, 34, 35]. These antitubercular tests were performed in duplicate (two plates),

TABLE 2: Computational results at B3LYP/6-311++G(d,p) for synthesized compounds **1**–**8**.

	Compounds																							
	1			**2**			**3**			**4**			**5**			**6**			**7**			**8**		
Conformers	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]	cis(E)	trans(E)	Others[a]
Gas phase																								
ΔH^b	0	6.6	>6.6	0	6.3	>6.3	0	6.2	>6.2	0	5.4	>5.4	0	5.2	>5.2	0	5.3	>5.3	0	5.2	>5.2	0	4.6	>4.6
μ^x	3.2	2.3	>3.1	3.1	1.2	>3.0	3	2.3	>3.0	2.3	2.9	>2.9	0.9	2.6	>1.5	5.2	3.7	>5.2	4.1	2.4	>4.1	1.7	1.5	>1.7
Population[d]	90.2	8.8	≤1.0	88.5	9.8	≤1.7	87.1	12.3	≤0.6	92.7	7.2	≤0.1	82.9	16.8	≤0.3	90.8	9.2	≤0.1	84	15.9	≤0.1	86	14	≤0.1
DMSO																								
ΔH^b	3.2	0	>3.2	3.3	0	>3.3	3.2	0	>3.2	3.4	0	>3.4	3.1	0	>3.1	3.2	0	>3.2	3.2	0	>3.2	3	0	>3.0
μ^x	4	3.5	>4.0	3.4	1.3	>3.4	4	3.6	>4.0	3.3	4.3	>4.2	1.5	3.5	>2.2	6.2	5	>6.2	4.9	3.2	>4.8	2.1	2.5	>2.5
Population[d]	24	76	≤0.1	8.3	91.6	≤0.1	19	80.9	≤0.1	26.4	73.5	≤0.1	29.1	70.9	≤0.1	21.8	78.2	≤0.1	19	81	≤0.1	35.5	64.5	≤0.1
Acetone																								
ΔH^b	2.5	0	>2.5	2.7	0	>2.7	2.6	0	>2.6	2.6	0	>2.6	2.5	0	>2.5	2.6	0	>2.6	2.6	0	>2.6	2.4	0	>2.4
μ^x	3.9	3.5	>3.9	3.4	1.3	>3.4	3.9	3.5	>3.9	3.3	4.2	>4.2	1.4	3.5	>2.2	6.2	4.9	>6.2	4.8	3.1	>4.8	2.1	2.5	>2.5
Population[d]	28.5	71.4	≤0.1	4.5	95.4	≤0.1	23.6	76.4	≤0.1	6.3	93.6	≤0.1	34.6	65.3	≤0.1	28.1	71.9	≤0.1	22.4	77.6	≤0.1	45.6	54.4	≤0.1

[a] cis(Z) and trans(Z) conformers. [b] Relative enthalpy of the stable conformer, in kJ·mol^{-1}. [c] Electric dipolar moment, in D (debye). [d] Equilibrium molar fractions or conformational population in %.

TABLE 3: MIC values (μM) of the isoniazid derivatives **1**–**8** and isoniazid (INH) against the standard strains: sensitive H37Rv (ATCC-27294) and resistant TB DM97.

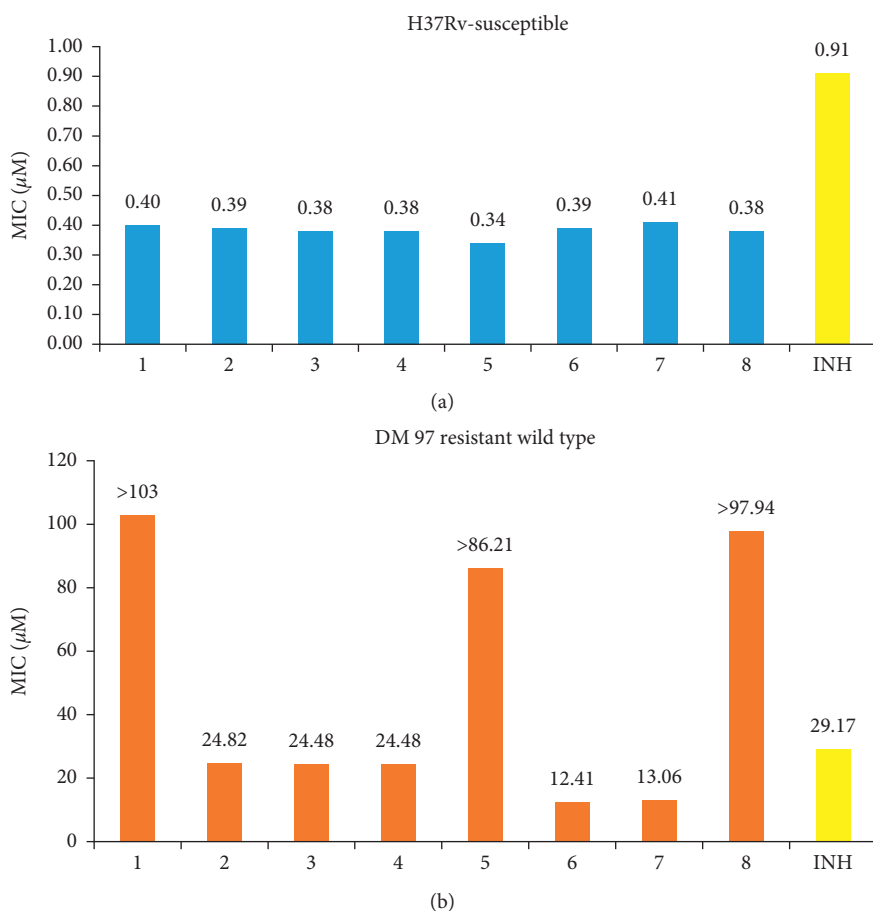| Compound	H37Rv-susceptible	DM97-resistant wild type
1	0.40	>103.13
2	0.39	24.82
3	0.38	24.48
4	0.38	24.48
5	0.34	>86.21
6	0.39	12.41
7	0.41	13.06
8	0.38	>97.94
INH	0.91	29.27

FIGURE 5: *In vitro* antitubercular activity expressed as MIC value (μM) for the isoniazid derivatives with isoxazole fragments **1**–**8** and isoniazid (INH) against the standard strains: (a) sensitive H37Rv (ATCC-27294) and (b) resistant TB DM97.

and the MIC values found in both plates yielded the same values.

As can be seen in Table 3, all the tested compounds **1**–**8** were demonstrated to be more active (MIC = 0.34–0.41 uM) than the isoniazid drug (INH, MIC = 0.91 μM) (Figure 5(a)) against the strain sensitive H37Rv [8, 33]. Compound **5** (MIC = 0.34 μM) was slightly more cytotoxic than the other tested compounds in this studied strain. Probably, the presence of the *bromo* substituent group at position C-4 of

the phenyl fragment plays an important role in the cytotoxicity of **5**. Thus, these results indicate that the presence of the isoxazole group bound to the phenyl ring with different substituent groups improves the antitubercular activity with respect to the cytotoxicity of the reference drug isoniazid.

Compounds **2** (X = 2′-OCH$_3$), **3** (X = 2′-Cl), and **4** (X = 3′-Cl) were slightly more cytotoxic (MIC = 24.82, 24.48, and 24.48 μM, respectively) than the reference drug (MIC = 29.17 μM) (Figure 5(b)) against the resistant TB

DM97 strain. However, **5** (the most active against the sensitive H37Rv strain) showed low cytotoxicity (MIC = >86.21 μM) in this studied strain. On the other hand, compounds **6** and **7** with MIC values of 12.41 and 13.06 μM, respectively, were about 2 times more cytotoxic than the reference drug. These results indicate that the cytotoxicity in this studied strain increased due to the presence of the OCH$_3$ and CH$_3$ substituent groups in the C-4′ position of phenyl fragment. With respect to **8**, the presence of the *chloro* substituent group at the C-4′ position of the phenyl ring was not relevant in the antitubercular activity (MIC = >97.94 μM).

4. Conclusions

In the present study, eight isoniazid derivatives with different substituents were synthesized, characterized, and evaluated for their antitubercular activity. The two-dimensional ^{1}H-^{1}H NOESY NMR (in acetone-d$_6$) spectra revealed that **1** and **2** in solution have a *trans(E)* isomeric form. This was confirmed by DFT calculations (at B3LYP/6-311++G(d,p) level of theory) carried out in both the gas and liquid (DMSO and acetone) phases. The results of the anti-*Mycobacterium tuberculosis* activity showed that compounds **1–8,** with different substituents on the phenyl fragment, exhibited moderate bioactivity (MIC = 0.34–0.41 μM) with respect to the isoniazid drug (MIC = 0.91 μM) against the sensitive H37Rv strain. On the other hand, compounds **6** (X = 4′-OCH$_3$) and **7** (X = 4′-CH$_3$), with MIC values of 12.41 and 13.06 uM, respectively, were about two times more cytotoxic compared to the isoniazid against the resistant TB DM97 strain. Among the tested derivatives, **6** and **7** could represent good candidates as antitubercular agents, since they exhibited good MIC values against the resistant TB DM97 strain.

Data Availability

The data used to support the findings of this study are included within the article, except for the computational results, which are found in Supplementary Materials (Table S1). These data will be available upon request to bona fide researchers.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Supplementary Materials

Table S1: computational results at B3LYP/6-311++G(d,p) for synthesized compounds **1** to **8**. Relative enthalpies (ΔH) of stable conformers and their equilibrium molar fractions (populations). Compounds **1–5** and **8**: mass spectra. Compounds **1–8**: linear correlation of vibrational frequencies—^{1}H-NMR spectra—infrared spectra. Compounds **1–4** and **6–8**: ^{13}C-NMR spectra. Compounds **1**, **2**, **6**, and **7**: two-dimensional NMR spectroscopy (^{1}H-^{1}H COSY)—two-dimensional NMR spectroscopy (^{1}H-^{13}C HSQC). Compound **1**: two-dimensional NMR spectroscopy (^{1}H-^{1}H NOESY). Compounds **2**, **6**, and **7**: two-dimensional NMR spectroscopy (^{1}H-^{13}C HMBC). Compound **6**:DEPT 45/90 spectrum. (*Supplementary Materials*)

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