Research Article

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Study of the reactivity of aminocyanopyrazoles and evaluation of the mitochondrial reductive function of some products

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Abstract: This research investigated the general high-throughput synthetic protocol for the accelerated synthesis of functionalized trifluoromethylpyrazolopyrimidines **3** and *N*-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) benzamide **4** from aminocyanopyrazole **1** precursors. The action of chlorosulfonyl isocyanate (CSI) with aminopyrazolo[3,4-*d*]pyrimidines **2** was found to produce triazolopyrimidinones **5**. The MTT test that quantifies mitochondrial reductive function demonstrated that in both cell lines tested (PE/CA-PJ41 and HePG2 cells), the benzamide compounds **4** are moderately toxic with PE/CA-PJ41 cells and more sensitive than HePG2 cells.

Keywords: aminocyanopyrazole, trifluoromethyl pyrimidines, benzamide, MTT test

1 Introduction

The pyrazole ring has been of great interest [1–7], thanks to its accessibility and various properties. The importance of these heterocyclic compounds lies in their potential biological antitumor [8] and antiviral [9] activities. It also lies in their efficient use as starting materials for the synthesis of other fused heterocyclic pyrazolopyrimidine derivatives of substantial chemical and pharmacological significance [10–16]. The synthesis of fused bicycles has been the subject of several works [17,18]. In the present research work, and for the purpose of preparing aminocyanopyrazoles **1**, a classical method of pyrazole synthesis was applied. Compounds **1** possess two reactive sites, namely a cyano group and amino group, which react with trifluoroacetic acid and phenylisothiocyanate to offer a novel class of condensed heterocycles **3** and **4**.

Furthermore, fluorinated compounds have drawn the attention of researchers in the agrochemical and medicinal fields. Overall, the combination of a fluoro or trifluoromethyl group produces compounds with enhanced biological activity, thanks to the boosted pharmacokinetic and physicochemical properties compared to their non-fluorinated analogues [19,20].

The reactivity of di- and trifunctionalized compounds containing heteroatoms like oxygen, nitrogen, and sulfur toward heterocumulenes, such as benzoylisothiocyanates [21], have been proven to depend on the structure of reactive intermediates along with the other substrates in the reaction, solvents, and reaction conditions. It has been reported that the action of benzoylisothiocyanates with 1,2-phenylenediamines [22] generates 2-arylbenzimidazole via the formation of bis-thiourea derivatives. The reaction of salicylamide with benzoylisothiocyanates through its hydroxyl group leads to the formation of benzoxazine derivatives [23].

As an extension to the studies of the reactivity of pyrazoles **1**, the investigation of the reaction of benzoylisothiocyanates with 4-amino-5-cyanopyrazole (Scheme 2), which leads to *N*-(5-cyano-1-phenyl-1*H*-pyrazol-4-yl)benzamide **4,** is pertinent. Another method of synthesizing benzamides from aminocyanopyrazoles has been described in the literature [24] as well as the study of their reactivities leading to polyheterocycle compounds.

The second part of this article reports the reaction of chlorosulfonyl isocyanate (CSI) with aminopyrazolo[3,4-*d*]pyrimidines **2** that has an amidine motif in its structure. CSI, which was discovered by Graf in 1966 [25], has two electrophilic sites, namely the carbonyl carbon (the isocyanate part) and the sulfur of the sulfonyl chloride group. Hence, this substrate is likely to be utilized in

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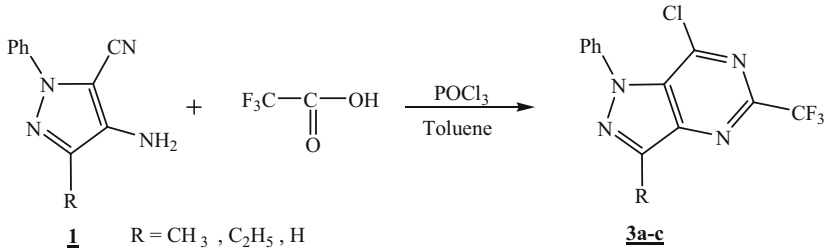


Scheme 1: Synthesis of benzamide trifluoromethyl pyrazolopyrimidine.

organic synthesis reactions. Owing to its excellent reactivity toward mobile hydrogen reactants such as alcohols, thiols, phenols, and amines [19,20], it has been given an important place in chemical synthesis. With respect to the previously realized studies dealing with CSI reactivity, Dhar and Murthy [26] have introduced a review comprising more than 120 references pertaining to this reagent's use in organic and heterocyclic synthesis.

In more recent times, a study conducted by Allouche et al. [27] was dedicated to the action of aminotriazoles with CSI. These authors have demonstrated that the behavior of CSI differs from the reaction's solvent and temperature.

2 Results and discussion

2.1 Chemistry

2.1.1 Reaction of 5-amino-4-cyanopyrazoles with trifluoroacetic acid

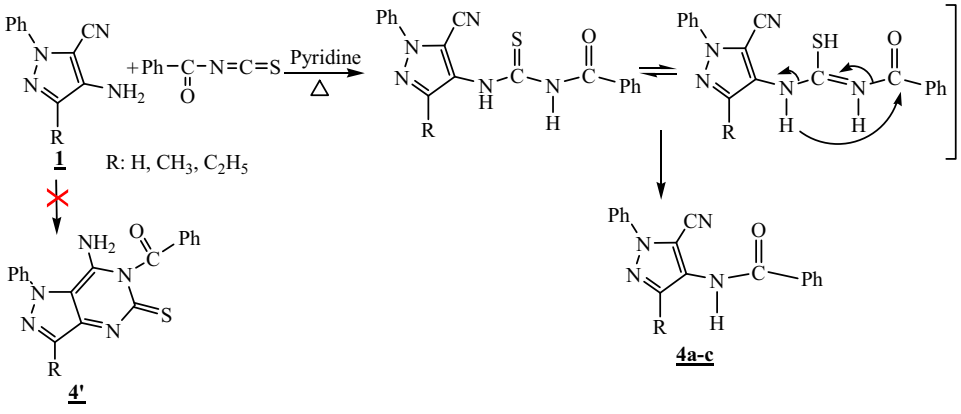
The action of trifluoroacetic acid with 5-amino-4-cyanopyrazoles generates 7-chloro-1-phenyl-5-(trifluoromethyl)-1*H*-pyrazolo[4,3-*d*] pyrimidine. Indeed, the reaction that occurs in toluene at 80°C with stirring for 24 h necessitates the presence of phosphorus oxychloride. The nitrogen of the amine group possessing a nucleophilic nature attacks the electrophilic carbon of trifluoroacetic acid and leads to the formation of the pyrazolopyrimidine group according to the reaction shown in Scheme 1.

2.1.2 Reaction of 5-amino-4-cyanopyrazoles with trifluoroacetic acid

Pyrazole is characterized by the presence of electrophilic and nucleophilic sites, which provide it with great reactivity. The action of benzoylisothiocyanates with 4-amino-5-cyanopyrazole **1** was realized to synthesize the corresponding pyrazolothioxopyrimidines **4′** (Scheme 2). However, this reaction produced the product of chemoselective *N*-benzoylation of pyrazole forming *N*-(5-cyano-1-phenyl-1*H*-pyrazol-4-yl)benzamide **4**. It also followed the same reaction mechanism as that of in the reaction of benzoylisothiocyanates with 2-aminophenol [22] illustrated in the literature.

2.1.3 Reaction of pyrazolopyrimidine with CSI

The synthesis of aminopyrazolo[3,4-*d*]pyrimidine derivatives **2a–c** was carried out according to the procedure



Scheme 2: Synthesis of benzamide.

described in the literature [28]. Actually, 5-amino-4-cyano-*N*1-phenyl pyrazole **1** treated with triethylorthoester and a catalytic amount of acetic acid leads to the formation of imidate. The latter reacts with ammonia and a catalytic amount of acetic acid to realize the pyrazolopyrimidines **2**.

The reaction of pyrazolo[3,4-*d*]pyrimidines **2** with thioisocyanate in acetonitrile and in the presence of triethylamine leads to heterotricyclic compound **5**.

The crude reaction products did not reveal any signs of the regioisomer based on the ^{1}H and ^{13}C NMR spectra. The identity of products **5a–c** was demonstrated by high-resolution mass spectrometry (HRMS). As observed in our earlier research work [29], in each case, it can be assumed that the formation of the involved triazolopyrimidinones **5a–c**. First, the selective attack of the sulfonyl group by the extracyclic NH_2 following the substitution of chlorine, intermediate (I) is formed. Indeed, the reason behind considering this hypothesis is that all previously realized studies have mentioned that elevated temperature favors this regioselectivity of the attack [30]. Besides, the role of triethylamine is to fix the formed HCl. Second, an intracyclization by attacking the nitrogen doublet on the CO of the isocyanate function leads to the tricyclic structure **5** (Scheme 3).

2.2 Biological activity

2.2.1 Cytotoxicity results

The results from the MTT assay that quantifies the mitochondrial reductive function demonstrated that, in both cell lines tested, these two series of compounds were only moderately toxic with PE/CA-PJ41 cells being more sensitive than HePG2 cells overall. The **4a–c** series was more toxic than the **1a–c** series, which was essentially nontoxic in HepG2 cells (IC_{50} > 100 μM) and only displayed limited toxicity in PE/CA-PJ41 cells. For the MJ series of compounds, the following order of potency was observed: **4c** > **4b** > **4a** in both cell lines tested (Figure 1).

3 Conclusion

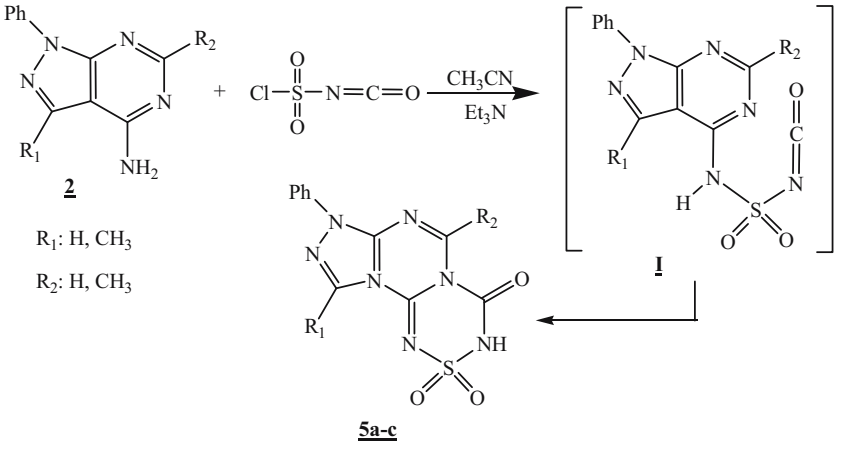
The aminocyanopyrazole condensation with trifluoroacetic acid has led to a new protocol for the synthesis of trifluoromethyl pyrazolopyrimidines. These compounds whose condensations have provided access to new families of trifluoromethylpyrazolopyrimidine compounds are of biological interest. The condensation of isothiocyanate with 5-amino-4-cyanopyrazoles allowed access to a novel family of pyrazolothioxopyrimidines.

The results from the MTT assay that quantifies mitochondrial reductive function demonstrated that benzamides were moderately toxic to PE/CA-PJ41 cells that were more sensitive than HePG2 cells, and the synthesized benzamide was more toxic than the starting aminocyanopyrazoles.

4 Experimental

4.1 Chemistry

The IR spectra were recorded for KBr on a Jasco Fourier transform (FT)–IR 420 spectrometer with a precision of 2 cm^{-1} covering the 400–4,000 cm^{-1} range. Furthermore, the ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$



Scheme 3: Synthesis of pyrazolopyrimidothiatriazinone.

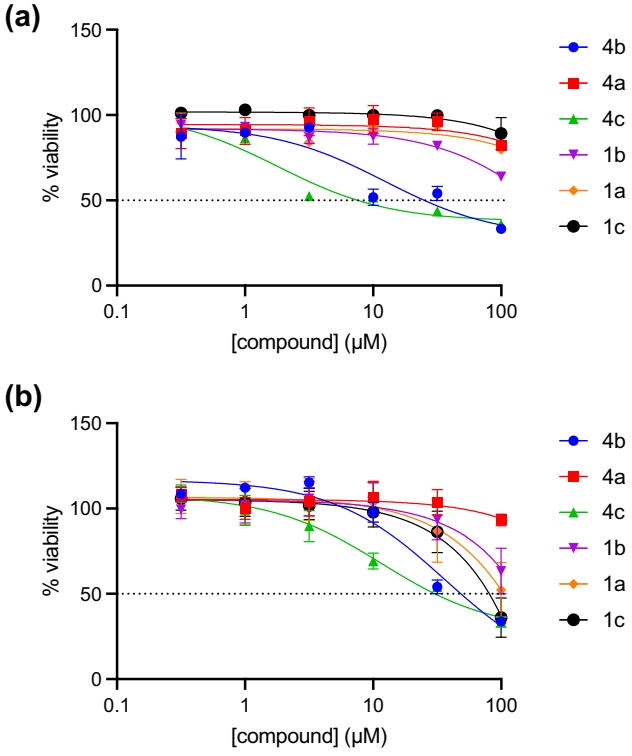


Figure 1: Cytotoxicity of **4a–c** and **1a–c** in (a) PE/CA-PJ41 and (b) HeG2/C3A cells as assessed by the MTT assay. The results represent the mean of three independent biological experiments ($n = 3$, ± SEM).

solution or in dimethylsulfoxide (DMSO-d_6) on a Bruker spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz). for the chemical shifts are expressed in parts per million (ppm) using tetramethylsilane as an internal reference. Besides, the multiplicities of the signals are denoted by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quadruplet; and m, multiplet, and the coupling constants are expressed in hertz. The melting points that were determined in an Electrothermal 9100 apparatus are not corrected. The reactions were monitored by thin-layer chromatography (TLC) using aluminum sheets with silica gel 60 F254 from Merck. The mass spectrometer was operated in electrospray ionization mode at 70 eV, and mass (MS) spectra were recorded from m = z 50 to 650.

4.1.1 General procedure for the synthesis of 7-chloro-3-alkyl-1-phenyl-5-(trifluoromethyl)-1*H*-pyrazolo [4,3-*d*]pyrimidine 3

One-pot synthesis of 7-chloro-3-alkyl-1-phenyl-5-(trifluoromethyl)-1*H*-pyrazolo[4,3-*d*] pyrimidine **3**.: A mixture of compound **1** (10.0 mmol), trifluoroacetic acid (TFA, 1.0 mL), toluene (10 mL), and phosphorus oxychloride (3.0 mL) was heated to 80°C with thorough stirring. The monitoring of the progress of the reactions was realized by TLC with petroleum ether/ethyl acetate (3:1, v/v) as a developing solvent. When the reaction was completed, toluene was eliminated by vacuum distillation. The residue was poured onto ice-cold water and neutralized with a saturated sodium bicarbonate solution. Moreover, the recrystallization from *n*-hexane produced a yellowish compound **3** in moderate yield (64–69%).

4.1.1.1 7-Chloro-1-phenyl-5-(trifluoromethyl)-1*H*-pyrazolo[4,3-*d*]pyrimidine 3a

Yield: 64%; mp: 198°C.

^{1}H NMR (DMSO-d_6, 300 MHz): 6.50–7.81 (m, 5H); 8.52 (s, 1H).

^{13}C NMR (DMSO-d_6, 75 MHz): C4 73.92; C2 108.09; C_{arom} 124.58–137.89;
C6 128.12; C1 130.03; C5 150.66; C3 151.66.

^{19}F NMR ($CDCl_3$): δ 68.51.

4.1.1.2 7-Chloro-3-methyl-1-phenyl-5-(trifluoromethyl)-1*H*-pyrazolo[4,3-*d*]pyrimidine 3b

Yield: 69%; mp: 181°C.

^{1}H NMR (DMSO-d_6, 300 MHz): 2.2 (s, 3H); 6.50–7.51 (m, 5H).

^{13}C NMR (DMSO-d_6, 75 MHz): C7 13.04; C4 74.20; C1 137.96; C2 115.50; C_{arom} 124.35–137.96; C6 127.99; C5 150.49; C3 151.68.

^{19}F NMR ($CDCl_3$): δ 68.73.

4.1.1.3 7-Chloro-3-ethyl-1-phenyl-5-(trifluorométhyl)-1*H*-pyrazolo[4,3-*d*]pyrimidine 3c

Yield: 65%; mp: 193°C.

^{1}H NMR (DMSO-d_6, 300 MHz): 1.2 (t, 3H); 2.5 (q, 2H); 6.50–7.51 (m, 5H).

^{13}C NMR (DMSO-d_6, 75 MHz): C8 12.87; C7 21.07, C4 73.02; C2115.47; C_{arom} 124.40–138.01; C6 127.99; C1 138.01; C5 152.07; C3 155.53.

^{19}F NMR ($CDCl_3$): δ 68.54.

4.1.2 General procedure for the synthesis of *N*-(5-cyano-3-alkyl-1-phenyl-1*H*-pyrazol-4-yl) benzamide 4a–c

The mixture of aminocyanopyrazole (0.01 mmol) in pyridine (10 mL) and benzoylisothiocyanate (0.012 mol) was

stirred with a magnetic stirrer at room temperature for 24 h. The solvent was evaporated and then the residue was treated with dry diethyl ether. The precipitated product **4** was filtered off and recrystallized from ethyl alcohol.

4.1.2.1 *N*-(5-Cyano-1-phenyl-1*H*-pyrazol-4-yl)benzamide

Rdt: 81%; mp: 168°C.

RMN ^{1}H (DMSO, 300 MHz): 0.90 (s, 3H); 2.28 (s,2H); 6.46–7.95 (m, 10H).

RMN^{13}C (DMSO, 75 MHz): C6 15.20; C5 101,24; C_{arom} 117.30–146.17; C1 148.75; C2 164.14; C7 166.88; C3 176.18.

HRMS calcd for $C_{17}H_{12}N_4O$ $[M + H]^+$ 288.1011; found: 288.1021.

4.1.2.2 *N*-(5-Cyano-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) benzamide

Rdt: 76%; mp: 201°C.

RMN ^{1}H (DMSO, 300 MHz): 2.47 (s, 3H); 7.28–8.30 (m, 10H); 12.22–12.34 (d, 1H).

RMN^{13}C (DMSO, 75 MHz): C6 15.20; C5 101.24; C_{arom} 117.30–146.17; C1 148.75; C2 164.14; C7 166.88; C3 176.18.

HRMS calcd for $C_{17}H_{12}N_4O$ $[M + H]^+$ 302.1168; found: 302.1175.

4.1.2.3 *N*-(5-Cyano-3-ethyl-1-phenyl-1*H*-pyrazol-4-yl) benzamide

Rdt: 90%; mp: 204°C.

RMN ^{1}H (DMSO, 300 MHz): 1.12 (s, 3H); 2.80 (s, 2H); 7.31–8.11 (m, 10H); 12.12–12.34 (d, 1H).

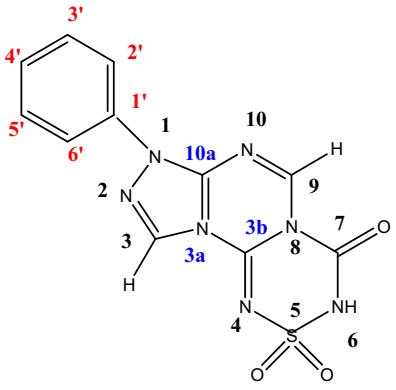
RMN^{13}C (DMSO, 75 MHz): C6 15.20; C5 101.24; C_{arom} 117.30–146.17; C1 148.75; C2 164.14; C7 166.88; C3 176.18.

HRMS calcd for $C_{17}H_{12}N_4O$ $[M + H]^+$ 316.1324; found: 316.1331.

4.1.3 General procedure for the synthesis of compounds 5a–c:

CSI (0.139 mL) was added to a stirred solution of 0.50 g of 4-aminopyrazolo[3,4-*d*]pyrimidine **2** in 1.89 mL of acetonitrile at 82°C for 20 min. Next, 0.310 mL of triethylamine was added. The mixture was heated under reflux for 3 h and the formed solid was filtered, washed with water, and then recrystallized from methanol to yield pyrazolo [3′, 4′: 4,5]pyrimido[6,1-*d*]thiatriazinone **5**.

4.1.3.1 N^1-Phényl-1*H*-pyrazolo[3′,4′:4,5]pyrimido[6,1-*d*] thiatriazinone (5a)



Structure of 5a

Yield: 87%; mp: 240°C.

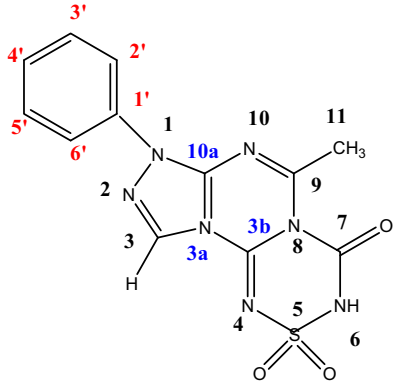
IR (cm^{-1}): νC═ N: 1,556, 1,561, 1,567; νC═0: 1,764; νNH: 3,320.

^{1}H NMR (DMSO-d_6, 400 MHz): 7.40 (1H, t, J = 7.3 Hz, ArH4′); 7.57 (2H, t, J = 7.3 Hz, ArH3′ and ArH5′); 8.13 (2H, d, J = 7.3 Hz, ArH2′ and ArH6′); 8.51 (1H, s, H9); 8.66 (1H, s, H3); 10.52 (1H, s, NH).

^{13}C NMR (DMSO-d_6, 100.6 MHz): C3a 101.89; (C3′′ , C5′′) 121.43; C4 -126.57; (C2′′, C6′′) 129.18; C1 137.28; C3 141.69; C10a 144.12; C9 152.07; C3b 152.80; C7155.45.

HRMS calcd for $C_{12}H_8N_6O_3S$ $[M + H]^+$ 317.0379; found: 317.0364.

4.1.3.2 9-Methyl-N^1-phenyl-1*H*-pyrazolo[3′,4′:4,5] pyrimido[6,1-*d*] thiatriazinone (5b)



Structure of 5b

Yield: 84%; mp: 227°C

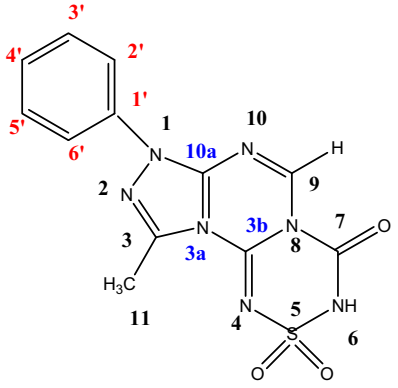
IR (cm^{-1}): νC═N: 1,558, 1,561, 1,573; νC═O: 1,761; νNH 3351.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.67 (3H, s, CH3); 7.37 (1H, t, J = 7.3 Hz, ArH4′); 7.55 (2H, t, J = 7.3 Hz, ArH3′ and ArH5′); 8.07 (2H, d, J = 7.3 Hz, ArH2′ and ArH6′); 8.42 (1H, s, H3); 10.27 (1H, s, NH).

^{13}C NMR (DMSO-d_6, 100.6 MHz): C11 14.40; C3a 101.08; (C3′, C5′) 120.39; C4′ 126.21; (C2′, C6′) 129.71; C1′ 138.34; C3 141.13; C10a 143.62; C9153.22; C3b 153.72; C7 156.43.

HRMS calculated for $C_{13}H_{10}N_6O_3S$ [M + H]$^+$ 331.0535; found: 331.0541.

4.1.3.3 3-Methyl-*N*^{1}-phenyl-1*H*-pyrazolo[3′,4′:4,5] pyrimido[6,1-*d*] thiatriazinone (5c)



Structure of 5c

Yield: 72%; mp: 220°C

IR (cm^{-1}): νC=N: 1,559, 1,562, 1,572; νC=0: 1,760; νNH: 3,318.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.65 (3H, s, CH3); 7.34 (1H, t, J = 7.3 Hz, ArH4′); 7.54 (2H, t, J = 7.3 Hz, ArH3′ and ArH5′); 8.12 (2H, d, J = 7.3 Hz, ArH2′ and ArH6′); 8.35 (1H, s, H9); 10.88 (1H, s, NH).

^{13}C NMR (DMSO-d_6, 100.6 MHz): C11 14.38; C3a 101.97; (C3″, C5″) 121.17; C4 -126.65; (C2″, C6″) 129.18; C1 »137.97; C3 141.83; C10a 144.16; C9 151.85; C3b 152.58; C7 155.06.

HRMS calcd for $C_{13}H_{10}N_6O_3S$ [M + H]$^+$ 331.0535; found: 331.0522.

4.2 Biological activity

4.2.1 Materials and methods

4.2.1.1 Cell culture

Hepatocellular carcinoma (HepG2/C3A) and oral squamous cell carcinoma (PE/CA-PJ41) cells were cultured in DMEM containing glucose (1 g L^{-1}), L-glutamine (3.9 mM), and sodium pyruvate (1 mM) further supplemented with FBS (10% v/v) and penicillin-streptomycin (100 units mL^{-1}, 100 mg mL^{-1}). Cells were maintained at 37°C, 5% CO_2 and passaged twice weekly. Cultures were routinely screened for *Mycoplasma* sp. using the EZ-PCR Mycoplasma test Kit (Biological Industries, Beit Haemek, Israel).

4.2.1.2 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell cytotoxicity as described previously [31]. Briefly, cells were seeded at a density of 10,000 cells per well in a 96-well plate, exposed to different concentrations of test compound, and incubated for 72 h. MTT was then added to a final concentration of 0.5 mg mL^{-1} followed by a 3 h incubation. DMSO was added to solubilize formazan and absorbance was read at 490 nm in Tecan Infinite F200 Pro (Tecan, Männedorf, Switzerland). Samples were analyzed in triplicate, and each experiment was repeated three times independently.

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Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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