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Synthesis, spectral characterization and *in vitro* antimicrobial activity of some new azopyridine derivatives

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ABSTRACT

A series of arylpicolino and/or isonicotinohydrazonyl cyanide **2a–d** and **4a–f** were prepared by coupling the approprite aryl diazonium salt with 2-cyanomethyl and/or 4-cyanomethyl-pyridine, respectively. These compounds were characterized by analytical and spectral analyses and screened for their antibacterial activity against Gram-positive bacteria, Gram-negative bacteria and antifungal activity. Among the synthesized compounds, *N′*-(4-phenyldiazenyl)phenylisonicotinohydrazonyl cyanide **4f** showed a significant activity toward both Gram-positive, Gram-negative bacteria and exhibit the most potent *in vitro* antifungal with MIC's (625 μg/mL) against *Aspergillus nieger*.

1. Introduction

The rich chemistry of azo compounds is associated with several important biological reactions such as protein synthesis, carcinogenesis, azo reduction monoamine oxidase inhibition mutagenic, immunochemical affinity labeling, nitrogen fixation, important medical and industrial uses [1,2]. Fadda et al. [3–19] published a series of papers to throw light on the chemistry of azo dyes. In our laboratory, some (*E*)-2-(aryl diazenyl)-2-(pyridine-2-yl)acetonitrile and its isomer 2-(pyridine-4-yl)acetonitrile derivatives have been prepared and characterized by elemental and spectral analyses. The electronic absorption spectra of these aryl azo derivatives were studied.

In a sequel of continuation, the present work is undertaken to study the structural chemistry of these aryl azo derivatives through (i) discussing IR, ^{1}H NMR, mass and UV spectra to support the tautomeric behavior of these compounds. (ii) These compounds were assayed against different Gram-positive bacteria, Gram-negative bacteria and antifungal activity in order to test their biological activity.

2. Results and discussion

2.1. Chemistry

In a sodium acetate buffered solution of ethanol 2- and 4-cyanomethylpyridine (**1**) and (**3**) reacts with diazotized aryl amines to form the corresponding (*Z*)-*N′*-arylpicolinohydrazonyl cyanide (**2**) and its isonicotino-hydrazonyl derivatives **4** in overall good yields (Fig. 1). However, no details of the dyeing behavior or their antimicrobial activity are reported.

The possible tautomeric forms of arylazo derivatives could be set out as outlined in Fig. 2.

There are referred to as the CH azo form (**A**), the NH azo form (**B**) and the cyanohydrazone form (**C**). The presently available data indicate that the tautomeric structure **2** or **4** is the chelated hydrazone form (**C**). It was therefore considered worthwhile preparing arylazo compounds containing the pyridine ring to evaluate their biological activities. In this investigation, ten *N′*-arylpicolinohydrazonyl cyanides **2a–d** and *N′*-arylisonicotinohydrazonyl cyanides **4a–f** were prepared by coupling of **2** and/or 4-cyanomethylpyridines **1** and/or **3** with the appropriate diazonium salt in ethanol containing sodium acetate. The newly prepared dyes were characterized by elemental analyses, as well as by spectral analysis.

On the basis of the IR spectra of arylhydrazones **2** and **4**, it was possible to assign the absorption bands of the diazonium coupling products **2** and **4**. Each of the compounds investigated possessed a weak and broad band in the region 3100–3300 cm^{-1}. This was assigned to NH stretching of the hydrazone moiety. The large shift and broadening of this band, as reported by Ramirez and Kirby [15], for simple hydrazones, can result only from intramolecular hydrogen bonding as in (**C**). The fact that the compounds **2** and **4** show evidence for intramolecular hydrogen bonding is in favor of the hydrazone structure. The IR spectra of all compounds showed absorption bands in the region 2220–2222 cm^{-1}. These bands assigned to C≡N vibration. So, on the basis of these data, structure (**B**) can be ruled out. The IR spectra of almost all

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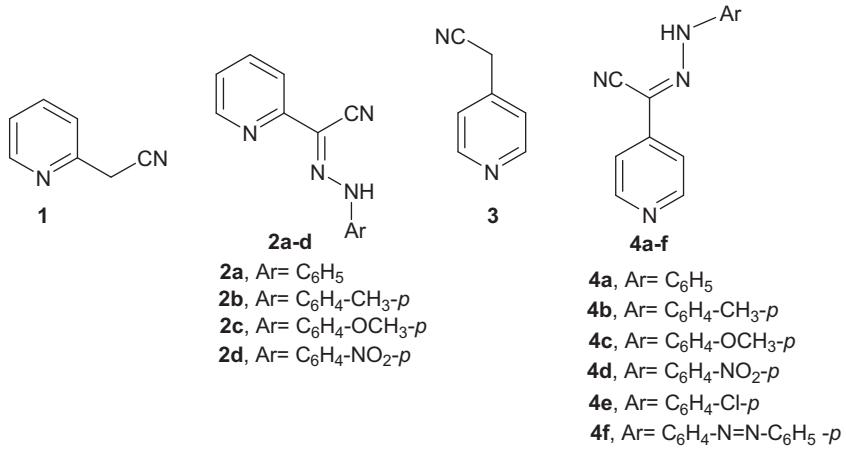

Fig. 1. Structures of the investigated arylpicolino and isonicotinohydrazonyl cyanide derivatives **2a–d** and **4a–f**.

compounds showed absorption in the region 1400–1450 cm^{-1}. These bands, however, cannot be assigned to N=N vibration according to LeFèvre [16]. Thus, the absence of bands characteristic for the N=N group might be taken as an indication that structure (A) is not present in the series studied.

Since, it has been deduced that compounds **2a–d** and **4a–f** exist as arylhydrazone (**C**), a band due to C=N would be expected in the double bond region. Bellamy [20], quoted a range of 1690–1640 cm^{-1} for the C=N stretching vibration falling slightly to 1680–1630 cm^{-1} for α,β-unsaturated compounds. The IR spectral data revealed that none of the compounds under investigation exhibited absorption bands above 1600 cm^{-1}. However, each compound showed strong absorption between the aromatic 1500 cm^{-1} band and NH deformation band near 1550 cm^{-1}, this band is relatively strong and in some cases, it could not be resolved from the 1500 cm^{-1} band. Such a band was not observed in the spectra of the corresponding starting compound. This new band may be due to the C=N vibration. Fadda suggested that, the downward shift of the C=N band of the nearly prepared compounds may be attributed to its conjugation with the nitrile group.

The UV spectra of the diazonium coupling products of **1** and **3** provide additional evidence that such compounds have the hydrazone structure (**C**) rather than (**A**) or (**B**). Most of the dyes show three main absorption bands in the region 240–390 nm (2445–2455 nm "π–π*", 2710–2950 "*n*–π*" related to pyridine moiety and 3445–390 "*n*–π*" assigned to hydrazo center). These bands are referred to as **A**, **B** and **C**, starting from the long wavelength side. The data indicate that absorption maxima in the solvents examined almost coincide. This suggests that such a compound is not in a tautomeric equilibrium, but exists in one form. The relatively small differences in λ_{max} may be due to the general solvent effect [18].

It has been reported that, the UV spectra of monophenylazo compounds differ from those of monophenylhydrazones The azo compounds generally show two absorption bands at 410–400 and 290–300 nm corresponding to *n*–π* and π–π* transitions, respectively [17]. The monophenylhydrazones, on the other hand, show three intense bands in the 220–228, 250–280 and 330–390 nm regions [18].

The UV spectra of compounds **2** and **4** investigated cannot be interpreted in terms of the azo structure of the types (**A** and **B**) but evidently they appear compatible with the indicated hydrazone structure (**C**). The presence of diazonium coupling products of **1** and **3**, apparently exclusively, in the hydrazone form can be explained by a smaller degree of resonance stabilization of the azo forms (**A** and **B**). The six-membered hydrogen-bonded ring structure in the hydrazone form, as in (**C**), would also undoubtedly enhance its relative stability [21], since the color of an azodye depends on the structure of the diazotizable amines. It is clear that, these dyes exhibit three absorption bands, of these, the medium and high wavelength bands seem to be affected by the nature of the polar substituent in the arylazo group and the low wavelength band is unaffected. Table 1 shows that, both electron withdrawing and electron donating groups cause the absorption to occur at higher wavelengths A "C=N" linkage was reported to have properties especially analogous to those of an ethylenic linkage [22]. Hence, an electronic effect of a substituent on the aromatic nucleus of the arylazo moiety will be transmitted to the whole conjugate system through π–P conjugation, exerting a considerable influence upon the conjugation bands **A** and **B**. Table 1 also shows that, the presence of electron donating or electron withdrawing groups has not brought about any marked increase or decrease in λ_{max} in the visible region and log ε has nearly remained constant. This does point towards the hydrazone structure (**C**) where the resonance in the diazo components is minimal, owing to steric factors. The ^{1}H NMR spectra of arylazo derivatives **2a–d** and **4a–f** showed a

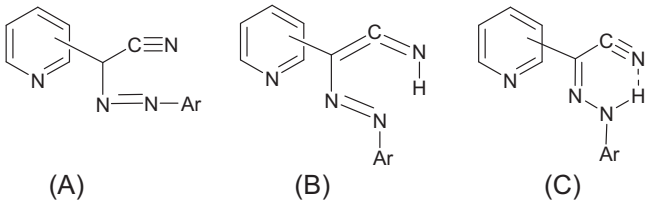

Fig. 2. The possible tautomeric forms of 2-, 4-[substituted-diazenyl]-2-[pyridin-2-yl](acetonitrile/ethenimine) and (*Z*)-*N*′-substituted-(picolino/nicotino)hydrazonoyl cyanide derivatives **2** and **4**.

Table 1
UV absorption bands of the newly synthesized compounds **2a–d** and **4a–f**.

Dye	λ_{max} (nm)[a]		
2a	395 (45)	275 (41)	248 (43)
2b	385 (45)	280 (41)	248 (43)
2c	390 (46)	275 (42)	248 (42)
2d	390 (46)	285 (40)	245 (41)
4a	390 (46)	285 (42)	245 (42)
4b	395 (47)	275 (42)	248 (41)
4c	385 (46)	275 (41)	248 (42)
4d	390 (47)	285 (41)	245 (42)
4e	385 (46)	280 (41)	248 (42)
4f	390 (46)	280 (41)	245 (41)

[a]Values in parentheses show log ε.

Table 2
Minimal inhibitory concentration (MIC, μg/mL) and inhibition zone (mm) of the newly synthesized compounds.

Compound no	MIC[a] in μg/mL and inhibition zone (mm)				
	Bacteria				Fungi
	Gram(+) bacteria		Gram(−) bacteria		
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. nieger*
1	100 (15)	100 (14)	100 (16)	100 (18)	100 (18)
2a	25 (20)	625 (38)	100 (14)	100 (15)	100 (15)
2b	50 (15)	50 (18)	625 (38)	100 (18)	100 (18)
2c	25 (30)	25 (21)	50 (24)	100 (15)	100 (14)
2d	3125 (38)	625 (34)	50 (23)	125 (32)	25 (25)
3	50 (25)	50 (30)	50 (18)	100 (15)	100 (16)
4a	125 (38)	625 (41)	50 (19)	50 (20)	100 (15)
4b	25 (30)	625 (39)	50 (16)	25 (35)	100 (18)
4c	125 (40)	625 (40)	50 (18)	50 (19)	50 (18)
4d	625 (44)	625 (41)	125 (37)	25 (33)	125 (31)
4e	3125 (45)	625 (42)	25 (35)	25 (39)	125 (35)
4f	3125 (43)	625 (44)	625 (38)	625 (38)	625 (37)
Chloramphenicol	3125 (42)	3125 (44)	625 (39)	625 (38)	NT
Cephalothin	625 (40)	625 (41)	625 (38)	625 (38)	NT
Cycloheximide	NT	NT	NT	NT	3125 (44)

MIC, minimal inhibitory concentration values; NT, not tested.

singlet signal at δ 88 ppm corresponding to NH proton. The downfield shift of the former occurs because the nearby azo group has a deshielding effect [23]. Compound **2a–d** showed a doublet at δ 865 ppm corresponding to α-proton (C-6) in pyridine ring, while compounds **4a–f** showed two doublets (AB system) due to α- and β-protons of the pyridine ring at δ 855 and 721 ppm, respectively. Also, the ^{1}H NMR spectra of arylazo derivatives revealed a multiplet at δ 691–745 ppm corresponding to the aromatic protons.

Mass spectra of the newly synthesized arylazo derivatives, in general, confirm the proposed formula by detecting the following peaks. For compound **2a**, the observed peak at *m*/*z* 222 ($C_{13}H_{10}N_4$, calculated atomic mass 22,209) represents the molecular ion peak with 25% abundance, respectively The other molecular ion peaks (221, 195, 92, 91, 90, and 78) appeared in the mass spectra are attributed to the fragmentation of molecule obtained from the rupture of different bonds inside the molecule.

In general, from the data obtained for all compounds we conclude that the molecular weight was in good agreement with the calculated molecular weight of the investigated compounds.

2.2. Pharmacology

2.2.1. Antimicrobial evaluation

Ten of the newly synthesized target compounds besides the starting compounds were evaluated for their *in vitro* antibacterial activity against Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and fungi (*Aspergillus nieger*). Agar-diffusion method was used for the determination of the preliminary antibacterial and antifungal activity. Chloramphenicol, cephalothin and cycloheximide were used as reference drugs. The results were recorded for each tested compound as the average diameter if inhibition zone (IZ) of bacterial and fungal growth around the disks in mm, the minimum inhibitory concentration (MIC) measurement was determined for compounds showed significant growth inhibition zones (>14 mm) using two fold serial dilution method [24]. The MIC (μg/mL) and inhibition zone diameters values are recorded in Table 2. The inhibition zone diameters values cited in Table 2 between brackets are attributed to the tested original concentration (1 mg/mL) as preliminary test. The results depicted in Table 2 revealed that most of the tested compounds displayed variable inhibitory effects on the growth of the tested Gram-positive and Gram-negative bacterial strains, and also against fungal strains.

In general, most of the tested compounds revealed better activity against the Gram-positive rather than the Gram-negative bacteria. It would be also noticed that compounds belonging to the 4-cyanomethylpyridine exhibited better antibacterial potentials than members of the 2-cyanomethylpyridine.

Regarding the activity of the **2a–d** against Gram-positive bacteria, the results revealed that compound **2d** exhibited divergent antibacterial activity against the tested organisms. In this view, compound **2d** was equipotent to chloramphenicol in inhibiting the growth of *B. subtilis* (MIC 3125 μg/mL), while its activity was 50% lower than chloramphenicol against *S. aureus*. Compounds **2a** and **2d** showed 50% of the activity of chloramphenicol (MIC 625 μg/mL). On the other hand, compounds **1**, **2b** and **2c** exhibited weak to moderate growth inhibitory activity against Gram-positive bacteria revealed from their MIC values (25–100 μg/mL). Among the series of compounds **4a–f**, compounds **4a**, **4c**, **4d**, **4e** and **4f** showed relatively good growth inhibitory profiles against *B. subtilis* (MIC 3125–125 μg/mL) which were from equipotent to 25% of the activity of chloramphenicol and 50% of cephalothin against the same organism (**4c**).

Moreover, distinctive anti-Gram positive profile was displayed by compounds **4e**, **f** where it proved to be equipotent as chloramphenicol against both *B. subtilis* (MIC 3125 μg/mL) and *S. aureus* (MIC 3125 μg/mL). Concerning the antibacterial activity of the compounds **2c**, **3** and **4a–c** revealed weak growth inhibitory against the tested Gram-positive bacteria (MIC 50 μg/mL). On the other hand, compound **4f** showed equipotent activity as chloramphenicol and cephalothin (MIC 625 μg/mL) against *E. coli* and *P. aeruginosa*.

Regarding the activity of **2a–d** and **4a–f** against fungal strains, the results revealed that compound **4f** was 50% lower than cycloheximide in inhibitory the growth of *A. nieger* (MIC 3125 μg/mL), while the reactivity of compounds **4d** and **4e** was 25% lower than cycloheximide against *A. nieger* (MIC 125 μg/mL).

The results of antimicrobial screening demonstrated the following assumptions about the structural activity relationships (SAR's): (1) it is interesting to point out that all compounds having electron withdrawing groups such as NO_2, Cl, aminoazobenzene recorded higher antibacterial activity. (2) The substitution at position 4 of the pyridine ring produced a higher antimicrobial activity. (3) Conversion of compounds **1** and **3** to their corresponding arylhydrazone derivatives **2a–d** and **4a–f** enhanced also antimicrobial activity. (4) The tested compounds were more active against Gram-positive than Gram-negative bacteria, it may be concluded that the

antimicrobial activity of the compounds is related to cell wall structure of the bacteria. It is possible because the cell wall is essential to the survival of bacteria and some antibiotics are able to kill bacteria by inhibiting a step in the synthesis of peptidoglycan. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acid, but in contrast, Gram negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. These differences in cell wall structure can produce differences in antibacterial susceptibility and some antibiotics can kill only Gram positive bacteria and is infective against Gram negative bacteria pathogen [25]. (5) The importance of such work lies in the possibility that the new compounds might be more effective drugs against bacteria for which a through investigation regarding the structural activity relationships, toxicity and in their biological effects which could be helpful in designing more potent antibacterial agents for therapeutic use.

3. Conclusion

In conclusion, the objective of the present study was to synthesize and investigate the antimicrobial activities of some new functionalized 2- and 4-pyridyl arylhydrazone derivatives with the hope of discovering new structure leads serving as potent antimicrobial agents. Our aim has been verified by the synthesis of different groups of structure hybrids comprising basically the pyridine moiety attached at 2- and 4-positions. The obtained results clearly revealed that compounds derived from 4-pyridyl derivatives exhibited better antimicrobial activity than their 2-isomers.

4. Experimental

All melting points are recorded on Gallenkamp electrothermal melting point apparatus. The IR spectra were recorded for KBr disc on a Mattson 5000 FTIR spectrophotometer. The ^{1}H NMR spectra were measured on a Bruker AC 300 (300 MHz) in DMSO-d_6 as solvent, using TMS as an internal standard, and chemical shifts are expressed as δ_{ppm}. The mass spectra were determined on Finnigan Incos 500 (70 ev). Elemental analyses were carried out at the Microanalytical Unit of the Faculty of Science, Cairo University, Giza, Egypt.

4.1. Synthesis of arylhydrazonyl cyanide **2a–d** and **4a–f**

General procedure: a well stirred solution of the base aromatic amine (0.1 mol) in 2 N hydrochloric acid (125 mL) was cooled in an ice-salt bath and diazotized with 1 N sodium nitrite solution (100 mL). The mixture was then tested for complete diazotization using starch iodide paper which gives a weak blue test. If the mixture does not give the test, more sodium nitrite was added dropwise until a positive test is obtained and the color is stable for few minutes. If, on the other hand, strong test for nitrite is obtained, a few drops of a dilute solution of the base hydrochloride are added until the nitrite test is nearly negative. The above cold diazonium solution was added slowly to a well stirred solution to cyanomethyl pyridine (0.1 mol) in ethanol (150 mL) containing 10% sodium acetate solution (50 mL) and the mixture was cooled in an ice-salt bath. After the addition of the diazonium salt solution, the reaction was tested for coupling reaction. A drop of the reaction mixture was placed on a filter paper and the colorless ring surrounding the spot dye was treated with a drop of an alkaline solution of a reactive coupler, such as a sodium salt of 3-hydroxy-2-naphthanilide. If unreacted diazonium salt is present, a dye is formed. The presence of unreacted coupler can be determined in a similar manner using a diazonium salt solution to test the colorless ring. After the coupling reaction was complete, the reaction mixture was stirred for 15 min at room temperature to coagulate the dye particles. The crude product was filtered, dried and recrystallized from ethanol to give *N*-arylpicolino and/or isonicotinohydrazonyl cyanide derivatives **2a–d** and **4a–f** (Table 2).

4.1.1. (Z)-N′-Phenylpicolinohydrazonyl cyanide (**2a**)

This compound was prepared from coupling of benzene diazonium chloride with 2-cyanomethylpyridine.

Yield (70%); brown crystals; mp 160 °C; IR (KBr): ν/cm^{-1} = 3310 (NH), 1550 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 68–73 (m, 5H, Ar–H), 76–796 (m, 3H, Py–H), 86 (d, 1H, C-6,-Py), 88 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 222 (M$^+$, 286), 195 (96), 92 (44), 91 (100), 90 (40), 78 (19), 76 (38), 65 (34), 57 (30), 50 (57), 30 (51), 49 (30). Anal Calcd for $C_{13}H_{10}N_4$ (22,225): C, 70.26; H, 454; N, 2521%. Found: C, 70.11; H, 441; N, 2510%.

4.1.2. (Z)-N′-p-Tolylpicolinohydrazonyl cyanide (**2b**)

This compound was prepared from coupling of *p*-tolyl diazonium chloride with 2-cyanomethylpyridine.

Yield (75%); brown crystals; mp 139 °C; IR (KBr): ν/cm^{-1} = 3300 (NH), 1552 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 234 (s, 3H, CH_3), 65 (d, 2H, Ar–H), 696 (d, 2H, Ar–H), 764–796 (m, 3H, Py–H), 861 (d, 1H, C-6,-Py), 88 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 236 (M$^+$, 557), 207 (18), 182 (4), 180 (5), 117 (23), 90 (100), 78 (17), 77 (21), 76 (24), 65 (37), 63 (16), 51 (20), 50 (12). Anal Calcd for $C_{14}H_{12}N_4$ (23,627): C, 7117; H, 512; N, 2371%. Found: C, 710; H, 510; N, 2368%.

4.1.3. (Z)-N′-(4-Methoxyphenyl)picolinohydrazonyl cyanide (**2c**)

This compound was prepared from coupling of 4-methoxyphenyl diazonium chloride with 2-cyanomethylpyridine.

Yield (80%); brown crystals; mp 154 °C; IR (KBr): ν/cm^{-1} = 3320 (NH), 1552 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 284 (s, 3H, OCH_3), 703 (d, 2H, Ar–H), 753 (d, 2H, Ar–H), 761–793 (m, 3H, Py–H), 866 (d, 1H, C-6,-Py); MS (EI, 70 eV) *m*/*z* (%) = 252 (M$^+$, 50), 236 (52), 235 (26), 221 (18), 220 (20), 207 (20), 149 (23), 121 (20), 118 (21), 117 (21), 106 (36), 104 (23), 92 (23), 91 (100), 89 (32), 80 (25). Anal Calcd for $C_{14}H_{12}N_4O$ (25,227): C, 6665; H, 479; N, 2221%. Found: C, 6661; H, 473; N, 2220%.

4.1.4. (Z)-N′-(4-Nitrophenyl)picolinohydrazonyl cyanide (**2d**)

This compound was prepared from coupling of 4-nitrophenyl diazonium chloride with 2-cyanomethylpyridine.

Yield (85%); yellowish brown crystals; mp 180 °C; IR (KBr): ν/cm^{-1} = 3310 (NH), 1555 (C=N), 1530, 1350 (NO_2); ^{1}H NMR(DMSO-d_6) δ (ppm): 72 (d, 2H, Ar–H), 764–795 (m, 3H, Py–H), 80 (d, 2H, Ar–H), 861 (d, 1H, C-6-Py), 882 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 267 (M$^+$, 91), 266 (72), 241 (22), 240 (27), 136 (45), 123 (18), 122 (22), 117 (18), 104 (45), 91 (60), 90 (77), 89 (31), 80 (45), 78 (41), 65 (36), 64 (91), 63 (100), 62 (45), 58 (36), 57 (54), 51 (60), 50 (54). Anal Calcd for $C_{13}H_9N_5O_2$ (26,724): C, 5843; H, 339; N, 2621%. Found: C, 5840; H, 336; N, 2620%.

4.1.5. (E)-N′-Phenylisopicolinohydrazonyl cyanide (**4a**)

This compound was prepared from coupling of benzene diazonium chloride with 4-cyanomethylpyridine.

Yield (76%); yellow crystals; mp 179 °C; IR (KBr): ν/cm^{-1} = 3300 (NH), 1551 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 681–735 (m, 5H, Ar–H), 798 (d, 2H, β-Py–H), 866 (d, 2H, α-Py–H), 882 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 222 (M$^+$, 54), 92 (78), 91 (100), 89 (76), 65 (31). Anal Calcd for $C_{13}H_{10}N_4$ (22,225): C, 70.26; H, 454; N, 2521%. Found: C, 70.19; H, 449; N, 2520%.

*4.1.6. (E)-N′-(p-Tolyl)isopicolinohydrazonyl cyanide (**4b**)*

This compound was prepared from coupling of *p*-tolyl diazonium chloride with 4-cyanomethylpyridine.

Yield (76%); yellow crystals; mp 167 °C; IR (KBr): ν/cm^{-1} = 3280 (NH), 1553 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 231 (s, 3H, CH_3), 651 (d, 2H, Ar–H), 699 (d, 2H, Ar–H), 796 (d, 2H, β-Py–H), 865 (d, 2H, α-Py–H), 89 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 236 (M^+, 78), 207 (40), 117 (38), 91 (100), 90 (50), 65 (51). Anal Calcd for $C_{14}H_{12}N_4$ (23,627): C, 7117; H, 512; N, 2371%. Found: C, 710; H, 510; N, 2369%.

*4.1.7. (E)-N′-(p-Tolyl)isopicolinohydrazonyl cyanide (**4c**)*

This compound was prepared from coupling of *p*-methoxyphenyl diazonium chloride with 4-cyanomethylpyridine.

Yield (81%); yellow crystals; mp 155 °C; IR (KBr): ν/cm^{-1} = 3310 (NH), 1556 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 391 (s, 3H, OCH_3), 701 (d, 2H, Ar–H), 753 (d, 2H, Ar–H), 795 (d, 2H, β-Py–H), 863 (d, 2H, α-Py–H), 879 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 252 (M^+, 50), 236 (65), 207 (38), 149 (30), 107 (32), 91 (100), 52 (50). Anal Calcd for $C_{14}H_{12}N_4O$ (25,227): C, 6665; H, 479; N, 2221%. Found: C, 6662; H, 477; N, 2220%.

*4.1.8. (E)-N′-(p-Nitrophenyl)isopicolinohydrazonyl cyanide (**4d**)*

This compound was prepared from coupling of *p*-nitrophenyl diazonium chloride with 4-cyanomethylpyridine.

Yield (80%); yellowish brown crystals; mp 289 °C; IR (KBr): ν/cm^{-1} = 3315 (NH), 1550 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 791 (d, 2H, Ar–H), 798–701 (m, 4H, Ar–H + 2β-Py–H), 865 (d, 2H, α-Py–H), 885 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 267 (M^+, 54), 266 (50), 122 (30), 117 (20), 104 (50), 91 (66), 90 (80), 65 (100). Anal Calcd for $C_{13}H_9N_5O_2$ (26,724): C, 5843; H, 339; N, 2621%. Found: C, 5839; H, 335; N, 2620%.

*4.1.9. (E)-N′-(p-Chlorophenyl)isopicolinohydrazonyl cyanide (**4e**)*

This compound was prepared from coupling of *p*-chlorophenyl diazonium chloride with 4-cyanomethylpyridine.

Yield (70%); pale yellow crystals; mp 212 °C; IR (KBr): ν/cm^{-1} = 3310 (NH), 1555 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 710–726 (m, 4H, Ar–H), 801 (d, 2H, β-Py–H), 869 (d, 2H, α-Py–H), 889 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 258 (M^++2, 15), 257 (M^++1, 13), 256 (M^+, 78), 220 (60), 126 (413), 125 (100), 124 (13), 111 (17), 101 (13), 100 (7), 99 (45), 75 (21), 63 (27), 51 (33), 50 (15). Anal Calcd for $C_{13}H_9ClN_4$ (25,669): C, 60.83; H, 353; N, 1381%. Found: C, 60.77; H, 351; N, 1379%.

*4.1.10. (E)-N′-(4-((E)-Phenyldiazenyl)phenyl)isonicotinohydrazonoyl cyanide (**4f**)*

This compound was prepared from coupling of *p*-amino-azobenzene diazonium chloride with 4-cyanomethylpyridine.

Yield (85%); brown crystals; mp 223 °C; IR (KBr): ν/cm^{-1} = 3315 (NH), 1580 (N=N), 1555 (C=N); ^{1}H NMR(DMSO-d_6) δ (ppm): 714–798 (m, 11H, 9Ar–H + 2β-Py–H), 861 (d, 2H, α-Py–H), 889 (s, 1H, NH); MS (EI, 70 eV) *m*/*z* (%) = 328 (M^++2, 15), 327 (M^++1, 25), 326 (M^+, 70), 249 (25), 221 (78), 166 (50), 125 (51), 124 (30), 106 (25), 105 (30), 104 (43), 91 (78), 77 (100). Anal Calcd for $C_{19}H_{14}N_6$ (23,635): C, 6992; H, 432; N, 2575%. Found: C, 6989; H, 430; N, 2571%.

4.2. Antimicrobial evaluation

The disks of Whatman filter paper were prepared with standard size (50 mm diameter) and kept into 10 oz screw capped wide mouthed containers for sterilization. These bottles are kept into hot air oven at a temperature of 150 °C. Then, the standard sterilized filter paper disks impregnated with a solution of the test compound in DMF (1 mg/mL) were placed on nutrient agar plate seeded with the appropriate test organism in triplicates. Standard conditions of 10^6 CFU/mL (Colony Forming U/mL) and 10^4 CFU/mL were used for antibacterial and antifungal assay, respectively Pyrex glass petri dishes (9 cm in diameter) were used and two disks of filter paper were inoculated in each plate The utilized test organisms were *B. subtilis* and *S. aureus* as examples of Gram positive bacteria and *E. coli* and *P. aeruginosa* as examples of Gram negative bacteria They were also evaluated for their *in vitro* antifungal potential against *A. nieger* fungal strains chloramphenicol, cephalothin and cycloheximide were used as standard antibacterial and antifungal agents, respectively. DMF alone was used as control at the same above mentioned concentration and due this there was no visible change in bacterial growth. The plates were incubated at 37 °C for 24 h for bacteria and for 48 h for fungi. Compounds that showed significant growth inhibition zones (>14 mm) using the twofold serial dilution technique, were further evaluated for their minimal inhibitory concentrations (MICs).

4.3. Minimal inhibitory concentration (MIC) measurement

The microdilution susceptibility test in Muller–Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) was used for the determination of antibacterial and antifungal activity, respectively. Stock solutions of the tested compounds, chloramphenicol, cephalothin and cycloheximide were prepared in DMF at concentration of 1000 μg/mL. Each stock solution was diluted with standard method broth (Difco) to prepare serial twofold dilutions in the range of 500–3125 μg/mL 10 mL of the broth containing about 10^6 CFU/mL of test bacteria was added to each well of 96-well microtiter plate. The sealed microplates were incubated at 37 °C for 24 h for antibacterial activity and at 37 °C for 48 h for antifungal activity in a humid chamber. At the end of the incubation period, the minimal inhibitory concentrations (MICs) values were recorded as the lowest concentrations of the substance that had no visible turbidity. Control experiments with DMF and uninoculated media were run parallel to the test compounds under the same conditions.

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