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# Synthesis, mass spectroscopic studies, cytotoxicity evaluation and quantitative structure activity relationship of novel isoindolin-1,3-dione derivatives

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**Abstract**

3-Amino-4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine **(5)** was synthesized as starting material and investigated as cytotoxic and antitumor agent. Compound **5** reacted with different acid anhydrides to afford the corresponding imides and bisimide derivatives **6-12.** Imides **6-12** were tested for their *in vitro* cytotoxicity against four different cancer cell lines and structure activity relationship's (SAR's) was studied.

**Keywords:** 2-Thiomethyl-4,6-diphenyl-3-carbonitrilpyridine, Acid Anhydrides, Cytotoxicity.

## 1. Introduction

1*H*-pyrazolo[3,4-*b*]pyridines comprise a very interesting class of compounds because of their significant and divers biological and pharmacological activities, such as antimalarial (Menezes *et al.* 2002), antiproliferative (Poreba *et al.* 2002), antimicrobial (Goda *et al.* 2004), cyclin-dependent kinase-inhibiting (Misra *et al.* 2003), cardiovascular (Stasch *et al.* 2002), antiviral (Azevedo *et al.* 2002), and antileishmanial activities (Mello *et al.* 2004).

Pyrazole-fused pyridines and pyrimidines are known to possess a wide range of biological activity. Specifically, pyrazolopyridines exhibit antitubercular and anxiolytic effects (Sekikawa *et al.* 1973).

In view of the above mentioned findings and as continuation of our effort from our laboratory (Fadda *et al.* 2013; Fadda *et al.* 2012; Fadda *et al.* 2013; Fadda *et al.* 2012; Fadda *et al.* 2013; Fadda *et al.* 2012; Fadda 2012; Fadda & El-Mekawy 2012; Fadda *et al.* 2011) to identify new candidates that may be of value in designing new potent, selective and less toxic antimicrobial agents, we report herein the synthesis of some new heterocycles incorporated pyrazolopyridine moiety starting from 3-Amino-4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine (5) in order to investigate their antimicrobial activity. It has been reported that pyrroles are important analgesic and anti-inflammatory agents (Otmar *et al.* 2004). In view of these facts, and in continuation of our interest in the chemistry of *N*-phthalimide (Fadda *et al.* 1999), we undertook the synthesis and cytotoxicity effects against different carcinoma cells of some new isoindolin-1,3-dione (*N*-phthalimides) containing pyrazolopyridine moiety. Hence, it was thought that the molecules containing both pyrazolopyridine and phthalimide moieties would have a more pronounced biological activity. With this in mind and with ingoing efforts in our laboratory, this paper reports a variety of synthetic routes leading to the synthesis of some novel pyrazolopyridine-isoindolin-1,3-dione analogues bearing different functional groups, some of which resemble that 5-fluorouracil (5-Fu). Novel analogues were evaluated *in vitro* against HepG2 (human heptacellular liver carcinoma cell lines), WI-38 (skin carcinoma cell lines), VERO (cell line was initiated from the kidney of a normal adult African green monkey) and MCF-7 (breast cancer cell lines).

## 2. Results and discussion

### *2.1. Chemistry*

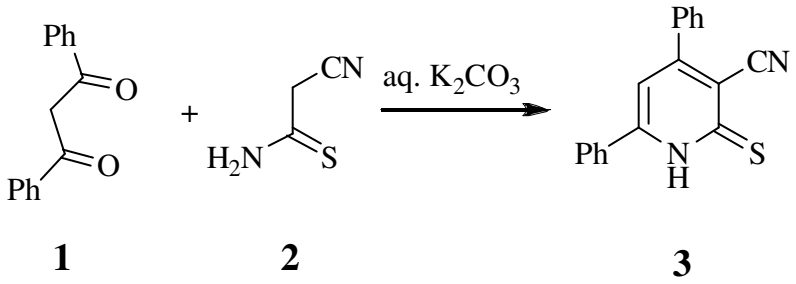
In view of these observations and in continuation of previous work in phthalimide chemistry (Fadda *et al.* 2012), we synthesized some new heterocyclic compounds containing *N*-phthalimide and tested their biological activities. The synthetic procedures adopted to obtain the target compounds are depicted in Scheme 1-3.

The starting material 3-cyano-4,6-diphenyl-2-pyridinethione **(3)** was prepared in 60% yield by reaction of dibenzoylmethane **(1)** with thiocyanoacetamide **(2)** under Guareschi-Thorpe reaction condition.

On the other hand, water at room temperature is an effective medium for simple condensations leading to pyrimidine, pyrrole, and pyridine derivatives etc, and such reactions are usually termed "synthesis under

physiological (or cell-possible) conditions" (Haley & Maitland 1951). Therefore, dibenzoylmethane **(1)** and thiocyanoacetamide **(2)** condense together in the presence of aqueous potassium carbonate to yield **3** in 90% yield.

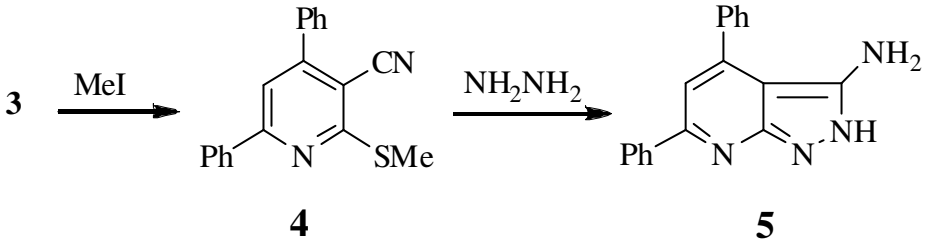



**1** **2** **3**

Scheme 1.

The IR spectrum of **3** showed the presence of absorption bands at 3150, 2220, and 1300 $cm^{-1}$ for NH, CN and C=S functions, respectively. The mass spectrum of **3** showed the molecular ion peak at *m/z* 288 ($M^+$) corresponding to the molecular formula $C_{18}H_{12}N_2S$.

Alkylation of pyridinethione derivative **3** was performed by its boiling with methyl iodide in alkaline medium resulted in the formation of the S-alkylated product **4**. A chemical confirmation of the structure of **4** was obtained through the cyclization with hydrazine hydrate, forming the key intermediate 3-amino-4,6-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridine **(5)**. *N*-alkylation was excluded, since the sole product obtained was the amino pyrazolopyridine derivative.




**4** **5**

Scheme 2.

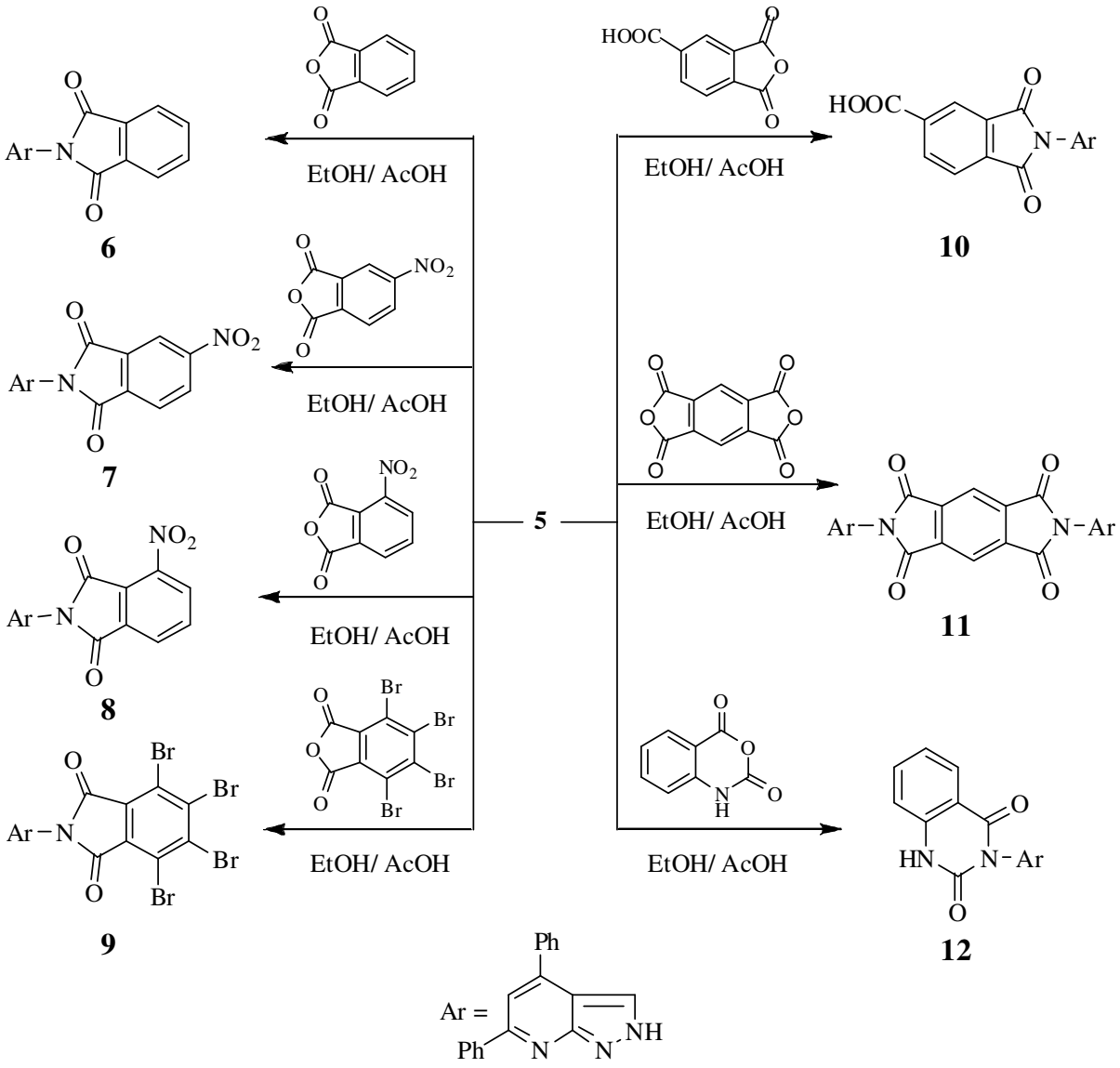
$^1$H-NMR spectrum of the S-methyl derivative **4** showed two singlet signals at $\delta$ 2.47 and 8.56 ppm attributable to S-methyl and $C_5$-H protons, respectively besides the aromatic protons at $\delta$ 7.67-8.30 ppm as multiplet. While $^1$H-NMR spectrum of compound **5** showed the disappearance of singlet signal at $\delta$ 2.5 ppm and instead appeared $D_2O$ exchangeable signals at $\delta$ 6.51 and 12.8 ppm due to $NH_2$ and NH protons. Moreover, the IR spectrum of **5** showed absorption bands at 3350 and 3225 $cm^{-1}$ corresponding to $NH_2$ and NH function groups.

Moreover, the mass spectrum gave an additional evidence for the structure **5** which showed its molecular ion peak at *m/z* 286 corresponding to a molecular formula $C_{18}H_{14}N_4$. $^{13}$C-NMR spectrum showed signals at $\delta$ 155 $C_6$-py, 154 C-$NH_2$, 150 $C_4$-py, 127-136 ppm for aromatic carbons, 91 and 132 for $C_3$ and $C_4$ of pyrazole ring and 121 $C_5$-pyridine.

Condensation of the key intermediate **5** with acid anhydrides, namely, phthalic anhydride, 4-nitrophthalic anhydride, and 3-nitro phthalic anhydride in refluxing ethanol containing few drops of acetic acid afforded the corresponding 2-(4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-isoindolin-1,3-dione derivatives **6-8**, respectively. The IR spectra of compounds **6-8**, in general, showed the absence of absorption frequency of $NH_2$ function at 3350 $cm^{-1}$ of compound **5**, and instead, the appearance of new band at 1697 $cm^{-1}$ corresponding to the amidic carbonyl function. The mass spectrum of compound **6** showed the molecular ion peak at *m/z* 416 ($M^+$). Similarly, the mass spectra of compounds **7** and **8** showed the molecular ion peaks at *m/z* 461 ($M^+$) corresponding to molecular formula $C_{26}H_{15}N_5O_4$.

Similarly, condensation of **5** with 3,4,5,6-tetrabromophthalic anhydride, and 1,2,4-benzene-tri-carboxylic anhydride (trimellitic anhydride) in refluxing ethanol containing few drops of acetic acid afforded the corresponding 2-(4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-4,5,6,7-tetrabromoisoindolin-1,3-dione **(9)** and 2-(4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine-3-yl)isoindolin-1,3-dione-5-carboxylic acid **(10)**.




Scheme 3.

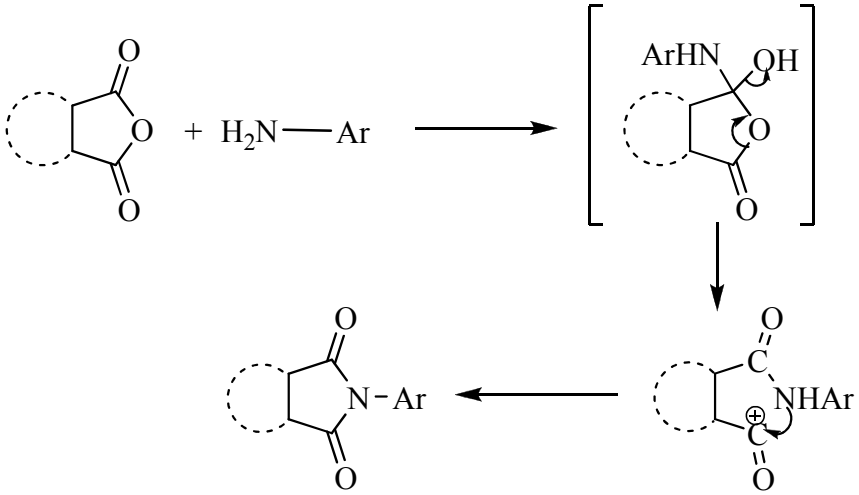
Structures **9** and **10** were confirmed by both elemental and spectral analyses. The IR spectra of compound **9** and **10** showed absorption bands at 3249 and 3234 $cm^{-1}$corresponding to NH functions, 1739-1695 $cm^{-1}$ corresponding to carbonyl groups. The mass spectra of compounds **9** and **10** showed the molecular ion peak at *m/z* 731 ($M^+$) and 460 ($M^+$), respectively.

In addition, refluxing of compound **5** with pyromellitic anhydride in boiling ethanol containing few drops of glacial acetic acid in a molar ratio 1:2 afforded the corresponding pyromellitimide **11**. The mass spectrum of **11** showed the molecular ion peaks at *m/z* 754 ($M^+$) and 710 ($M^+$-$CO_2$). Moreover, stirring of compound **5** with isatoic anhydride in ethanol containing few drops of acetic acid at room temperature afforded the corresponding 3-(4,6-diphenyl-2*H*-pyrazolo[3,4-*b*]pyridine-3-yl)-quinazoline-2,4-(1*H*,3*H*)dione **(12)**. The IR spectrum of compound **12** showed absorption bands at 3250 and 1677 $cm^{-1}$ corresponding to NH and CO function groups. The mass spectroscopic measurement gave an additional evidence for the correct structure of compound **12** which showed the molecular ion peak at *m/z* 431 ($M^+$, 100%).

However, no details reported regarding the synthesis of such compounds in literature. The reaction possibly takes place according to one of the following two mechanisms (Fig 1)

**Route A**




**Route B**

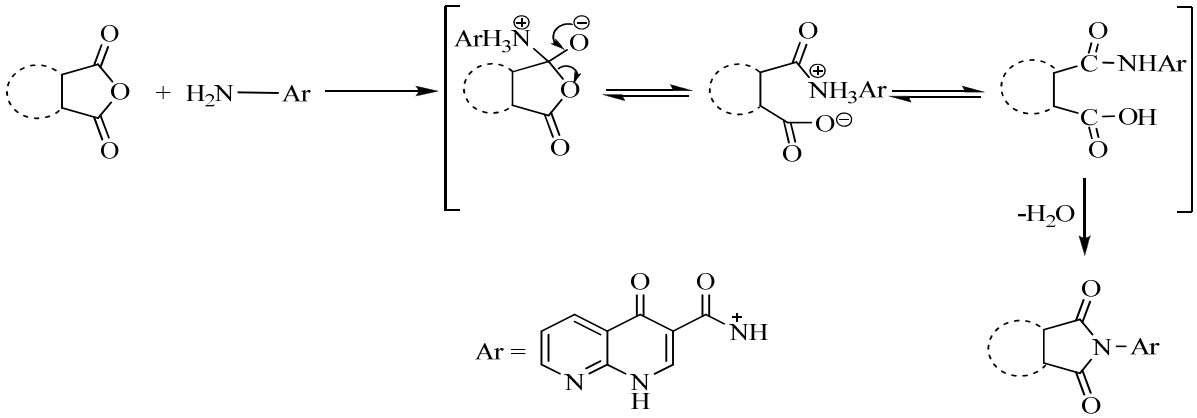



Fig 1.: Routes for the mechanism of formation of pyrazolopyridine-isoindolin-1,3-dione **6-12**

*2.2. Mass spectroscopic studies of isoindolin-1,3-dione (N-phthalimide) compounds*

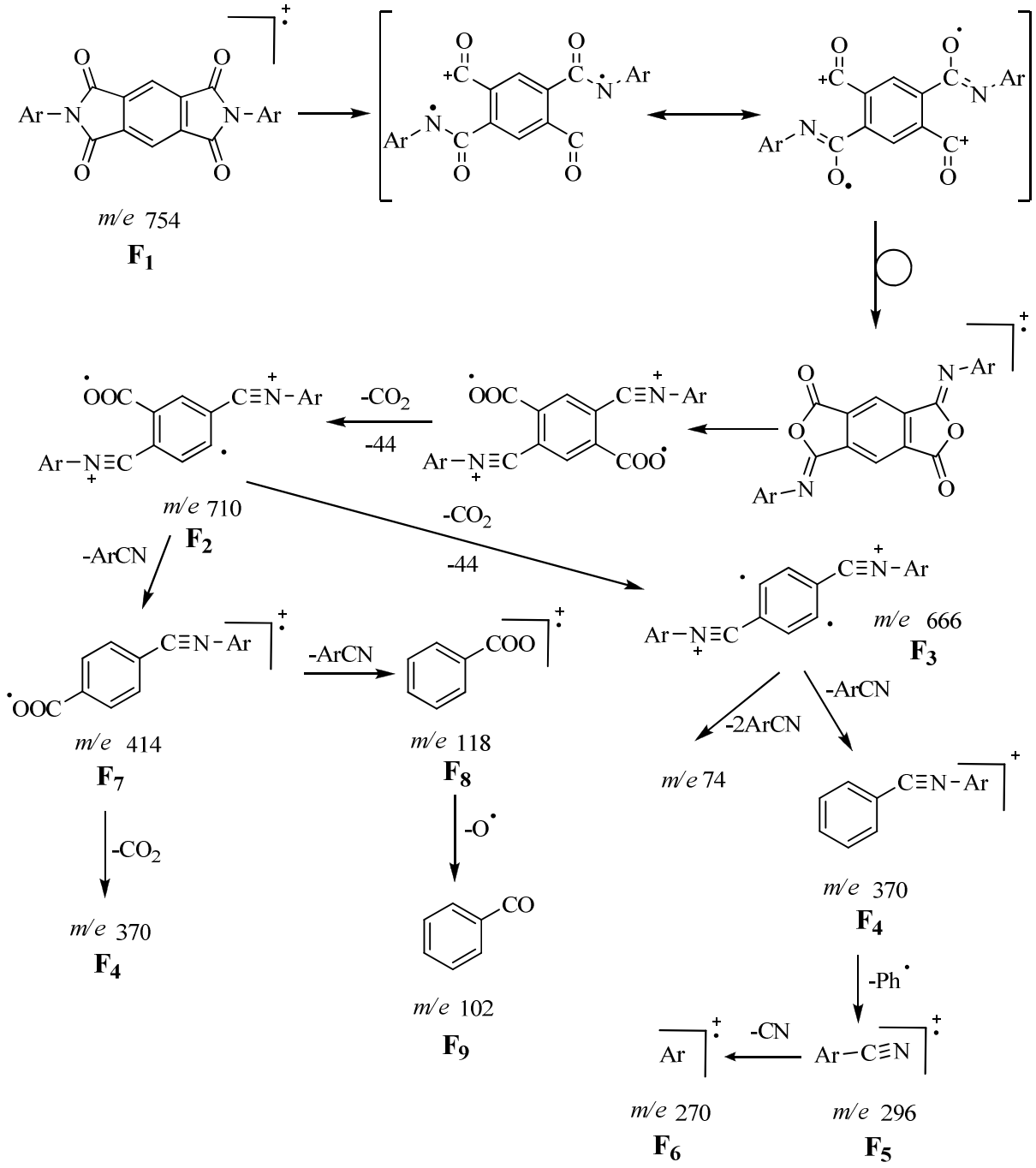
In recent years, mass spectroscopic studies have been used in various aspects of biochemistry (Garteiz & Walle 1972; Bosin & Sinnott 1974).

Nonavialability of the literature on phthalimide derivatives coupled with their use in the field of medicine and biochemistry, have promoted us to investigate the fragmentation pathways of some phthalimide derivatives. It appears that no report has been made to date on these compounds. In this type of compounds it may be expected that the initial charge is localized preferentially on either nitrogen, oxygen, or on aromatic ring.

The mass spectrum of the pyromellitimide **10**, for example, has been investigated. All the compounds **6-12** yielded easily recognizable molecular ions with characteristic isotopic contribution and weak (M-1) peaks. The principle general route of fragmentation peaks of compound **10** is given in Scheme 4.

In general, the pyromellitimides contains the peaks corresponding to the loss of 44 mass units from the molecular ion (M-$CO_2$) (**$F_2$**). This fragmentation route was supported by corresponding metastable peaks. The pyromellitimide contains also an abundant peak due to the elimination of arylnitrile unit. This peak may be represented as (Ar-CN) $^+$, the elimination of $CO_2$ and the formation of the latter fragment may be explained in terms of Mclafferty rearrangement. A metastable peak at 241.4 verified the genesis of (Ar-CN) $^+$ ion from one step decomposition of the molecular ion (**$F_2$**) to give (**$F_7$**). The subsequent decomposition of arylnitrile ion (**$F_5$**) by the expulsion of CN afforded the substituted pyrazolopyridine ion (**$F_6$**). Similar elimination of CN and NCS from ($C_6H_5$-NCS) $^+$ has also been observed by Djhrassi et al (Kiahr *et al.* 1963). Ion **$F_2$** readily eliminates either mass 44 (-$CO_2$) or arylnitrile fragment to give more stable cation **$F_3$** or **$F_7$**, respectively. This observation is in harmony with the results of previous workers (Beynon & Williams 1960). Thus, further fragmentation of **$F_3$** by loss one or two




Scheme 4. General route of fragmentation of pyromellitimide derivative **10**

arylnitrile cation afforded an ion $\mathbf{F_4}$ or ion at *m/e* 74, respectively. Cation $\mathbf{F_7}$ on further decomposition yields cation $\mathbf{F_4}$ and $\mathbf{F_8}$.

Other lower fragmentation for the arylnitriles is in accordance with characteristic fragmentation of the corresponding hydrocarbons (Beynon *et al.* 1968).

In case of compounds **6** and **7** for example, the fragmentation of nitrophthalimide derivatives are unusual and distinctive. The ions $(P\text{-}O)^+$ or $(P\text{-}OH)^+$, $(P\text{-}NO)^+$ and $(P\text{-}NO_2)$ are almost always of relatively high abundance. The molecular ions are usually of high abundance also. The presence of the nitrogen atom is readily apparent by the application of the "nitrogen rule". Even if another substituent group contains a nitrogen atom, the nitro group can be quickly recognized by the characteristic fragmentation that is induces and hence the presence of a second nitrogen atom can be deduced by inference.

The molecular ion *m/z* = 461 loss firstly mass unit of 44 ($M^+$-$CO_2$), followed by loss the heteroaryl cation of

mass unit 270 to afford nitrobenzonitrile, of mass 148, which is readily detected. The fragments ions $(P\text{-}NO)^+$ and $(P\text{-}NO_2)^+$ are Permanent. The presence of a benzene ring is indicated by the ions of masses, 51 and 77 and also by the meta-stable transition.

$$77^+ \longrightarrow 51^+ + 26 \qquad \text{m.s. } 33.8$$

$$C_6H_5^+ \longrightarrow C_6H_5^+ + C_2H_2$$

This transition parallels the transition in benzene

$$78^+ \longrightarrow 52^+ + 26 \qquad \text{m.s. } 34.7$$

$$C_6H_6^+ \longrightarrow C_4H_4^+ + C_2H_2$$

A second meta-stable transition confirms the loss of CO from the $(p\text{-}NO)^+$

$$93^+ \longrightarrow 65^+ + 28 \qquad \text{m.s. } 45.4$$

$$C_6H_5O^+ \longrightarrow C_5H_5^+ + CO$$

The nitrobenzenonitrile ion (*m/e* 148) first of all loss a mass unit 26 or 27 from its molecular ion (148-CN) or HCN to give substituted phenyl ion (Ar = nitrobenzene), (*m/e* = 122, %RA 3.69, %RIC 0.95)

$$148^+ \longrightarrow 122^+ + 26 \qquad \text{m.s. } 101.5$$

$$NO_2\text{-}C_6H_4CN^+ \longrightarrow NO_2\text{-}C_6H_4^+ + CN$$

$$148^+ \longrightarrow 121^+ + 27 \qquad \text{m.s. } 99.9$$

$$p\text{-}NO_2\text{-}C_6H_4CN^+ \longrightarrow p\text{-}NO_2\text{-}C_6H_3^+ + HCN$$

The mass spectra of compounds **6-12** showed a similar fragmentation routes until the formation of the fragment $\mathbf{F_5}$, which further dissociated by individual routes in each compound according to the type of the heterocyclic moiety.

*2.3. Pharmacology*

*2.3.1. Cytotoxicity*

Cytotoxicity was expressed as the concentration that caused 50% loss of the cell monolayer ($IC_{50}$). The assay was used to examine the newly synthesized compounds. 5-Fluorouracil as a standard anticancer drug was used for comparison (Fadda *et al.* 2012; Fadda *et al.* 2009). The results of our preliminary screening indicated that compounds **8**, **9** and **11** showed moderate cytotoxicity activity. The other compounds showed weak cytotoxicity activity (Table 1).

Subsequently, we may conclude the structure activity relationship's (SAR's). 1) The presence of basic skeleton (phthalimide moiety) is necessary for the broad spectrum of cytotoxic activity towards different cell lines (HepG2, WI-38, VERO and MCF-7). 2) Introducing of a nitro group (electron withdrawing group, -ve inductive effect) in position 4 of benzene ring in phthalimide moiety increases the activity towards WI-38 and VERO also diminishes the activity towards HepG2 and MCF-7 (compound **8**). 3) Introducing four bromine atoms (electron withdrawing atoms, -ve inductive effect) in benzene ring of phthalimide moiety increases the activity against all cell lines (compound **9**). 4) According to the above findings the presence of two phthalimide moieties enhanced the cytotoxicity activity towards all cell lines (compound **11**). 5) In compound **12** the presence of NH group near

to carbonyl group in isatoic acid moiety act as electron withdrawing group and showed nearly moderate activity against HepG2 and WI-38. 6) The presence of electron withdrawing group ($NO_2$ and COOH) in position 5 of benzene ring in phthalimide moiety decreases the negative inductive effect on the reaction center of molecule and consequently decreases the cytotoxicity activity (compounds **7** and **10**). Compound **6** has phthalimide moiety with no substituents in benzene ring, showed weak activity. 8) Compound **5** has no phthalimide moiety, showed weak cytotoxicity activity.

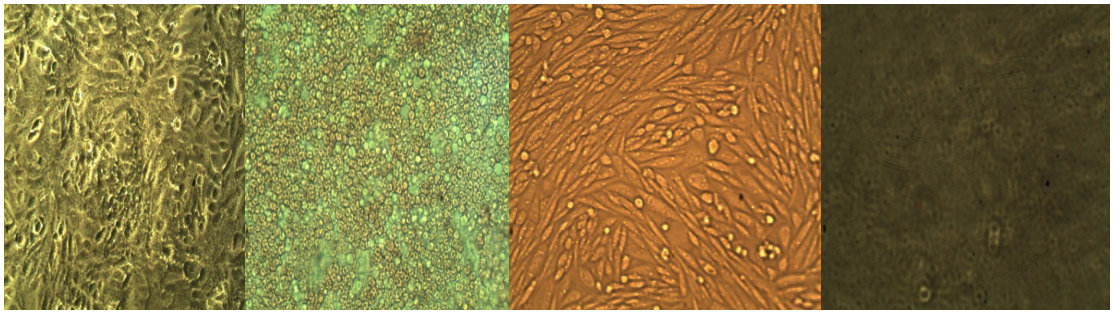
Table 1. Cytotoxicity ($IC_{50}$) of tested compounds on different cell lines

| Compound No. | $IC_{50}$ (µg/ml)[a] | | | |
|---|---|---|---|---|
| | **HepG2** | **WI-38** | **VERO** | **MCF-7** |
| **5-Fu** | 8 | 4 | 12 | 18 |
| **5** | 89 | 88 | 76 | 75 |
| **6** | 89 | 92 | 95 | 90 |
| **7** | 83 | 85 | 83 | 78 |
| **8** | 52 | 45 | 48 | 50 |
| **9** | 39 | 41 | 50 | 49 |
| **10** | 60 | 62 | 56 | 58 |
| **11** | 34 | 42 | 50 | 49 |
| **12** | 52 | 51 | 65 | 62 |

[a]**$IC_{50}$ (µg/ml):** 1-10 (very strong), 11-25 (strong), 26-50 (moderate), 51-100 (weak), 100-200 (very weak), above 200 (non cytotoxicity).

5-Flu = 5-Fluorouracil

Further, in *vivo* studies are warranted to confirm the biological activity of the newly synthesized pyrazolopyridineisoindolin-1,3-dione compounds and to investigate the molecular mechanisms responsible for the antitumor activity at the most compounds with a potential pharmaceutical used.



*Vero cells* *WI 38* *HEPG-2* *MCF-7*

Fig 2.: Confluent Monolayers of cell lines used for testing

## 3. Conclusion

In summary, modification of 3-amino-4,6-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridine **(5)** produced compounds with potential as anticancer agents. Based on these preliminary screening results, compounds **8**, **9** and **11** showed significant activity in certain cancer cell and have been targeted for further studies. Compounds **9** and **11** showed the highest cytotoxic activity. Meanwhile, compounds **5**, **6** and **7** proved to have the weakest activity.

## 4. Experimental

All melting points are recorded on Gallenkamp electric melting point apparatus and are uncorrected. The IR spectra $\nu$ $cm^{-1}$ (KBr) were recorded on Prerkin Elmer Infrared Spectrophotometer Model 157, Grating. The $^{13}C$ and $^{1}H$-NMR spectra were run on Varian Spectrophotometer at 400 MHz and 100 MHz using TMS as an internal reference and DMSO-$d_6$ as solvent. The mass spectra (EI) were recorded on 70 ev with Kratos MS equipment and/or a Varian MAT 311 A Spectrometer T Cairo Univ., Giza, Egypt, and at Assiut university central lab.

Elemental analyses (C, H, and N) were carried out at the Microanalytical Center of Cairo University, Giza, Egypt. The results were found to be in good agreement with the calculated values.

*4.1. Synthesis of 3-cyano-4,6-diphenyl-2-pyridine-thione* ***(3)***

It was prepared according to the previously reported method (Haley & Maitland 1951).

*4.2. Synthesis of 4,6-diphenyl-2-methylthiopyridin-3-carbonitrile* ***(4)***

A mixture of 3 (1 mmol) in 10 ml 40% sodium hydroxide was stirred on a water bath at 60°C until the reaction mixture became clear. Methyl iodide (1 mmol) was added dropwise to the stirred mixture and the immediately formed white precipitate was collected by filtration and crystallized from petroleum ether. Yield (85%); white powder; m.p. 120 °C; IR (KBr): ν/cm$^{-1}$= 2220 (CN); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 2.47 (s, 3H, $CH_3$-S), 7.67-8.30 (m, 10H, Ar-H), 8.56 (s, 1H, $C_5$-H pyridine); MS (EI, 70 eV): *m/z* (%) = 302 ($M^+$, 100). Anal. Calcd. for $C_{19}H_{14}N_2S$ (302.39): C, 75.47; H, 4.67; N, 9.26%. Found: C, 75.41; H, 4.62; N, 9.20%.

*4.3. Synthesis of 3-amino-4,6-diphenyl-2H-pyrazolo[3,4-b]pyridine* ***(5)***

A mixture of 3 (1 mmol) and hydrazine hydrate (1 mmol) was refluxed for 6h. The product separated after partial evaporation of the solution was collected by filtration and crystallized from ethanol. Yield (90%); white powder; m.p. 165 °C; IR (KBr): ν/cm$^{-1}$= 3350 ($NH_2$), 3225 (NH); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 6.51 (s, 2H, $NH_2$), 7.46-8.30 (m, 10H, Ar-H), 7.84 (s, 1H, $C_5$-H pyridine), 12.80 (s,1H, NH); $^{13}$C-NMR (DMSO-$d_6$) $\delta$ (ppm): 155.0, 154.0, 150.0, 132.0, 137.0-127.0 (aromatic carbons), 121.0, 91.5; MS (EI, 70 eV): *m/z* (%) = 288 ($M^+$+2, 15), 287 ($M^+$+1, 30), 286 ($M^+$, 100). Anal. Calcd. for $C_{18}H_{14}N_4$ (286.33): C, 75.50; H, 4.93; N, 19.57%. Found: C, 75.46; H, 4.88; N, 19.50%.

*4.4. General procedure for the synthesis of 2-(4,6-diphenyl-2H-pyrazolo[3,4-b]pyridine-3-yl-isoindolin-1,3-dione derivatives* ***6-11***

A mixture of compound 5 (1 mmol) and anhydride derivatives namely; phthalic anhydride (1 mmol), 4-nitrophthalic anhydride (1 mmol), 3-nitro-phthalic anhydride (1 mmol), 3,4,5,6-tetrabromophthalic anhydride (1 mmol), 1,2,4-benzenetricarboxylic anhydride (trimellitic anhydride) (1 mmol), and/ or 1,2,4,5-benzenetetracarboxylic dianhydride (0.5 mmol) in ethanol (15 ml) containing glacial acetic acid (5 drops) was refluxed for 2h. The reaction mixture was left to cool at room temperature; the precipitated solid product was filtered off, dried and recrystallized from ethanol to give compounds 6-11, respectively.

*4.4.1. 2-(4,6-Diphenyl-2H-pyrazolo[3,4-b]pyridine-3-yl)isoindolin-1,3-dione* ***(6)***

Yield (90%); pale yellow powder; m.p.> 300 °C; IR (KBr): ν/cm$^{-1}$= 3250 (NH), 1695 (C=O, imide), 1620 (C=N), 1579 (C=C); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 7.81 (s, 1H, $C_5$-H pyridine), 7.35-8.10 (m, 14H, Ar-H), 12.10 (s,1H, NH); $^{13}$C-NMR (DMSO-$d_6$) $\delta$ (ppm): 167.1 (C=O); 155.0, 154.0, 150.0, 131.0, 127.0-136.0 (aromatic carbons), 122.0, 91.5; MS (EI, 70 eV): *m/z* (%) = 417 ($M^+$+1, 28), 416 ($M^+$, 100), 415 ($M^+$-1, 12), 372 (27.5), 296 (30.1), 270 (15). Anal. Calcd. for $C_{26}H_{16}N_4O_2$ (416.43): C, 74.99; H, 3.87; N, 13.45%. Found: C, 74.91; H, 3.80; N, 13.39%.

*4.4.2. 2-(4,6-Diphenyl-2H-pyrazolo[3,4-b]pyridine-3-yl)-5-nitroisoindolin-1,3-dione* ***(7)***

Yield (80%); white powder; m.p.> 300 °C; IR (KBr): ν/cm$^{-1}$= 3235 (NH), 1684 (C=O), 1625 (C=N), 1580 (C=C), 1530,1350 ($NO_2$); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 7.62 (s, 1H, Ar-H), 7.40-8.10 (m,10H, Ar-H), 7.71 (d, 1H, Ar-H), 7.80 (s, 1H, $C_5$-H pyridine), 7.82 (d, 1H, Ar-H), 12.1 (s, 1H, NH); $^{13}$C-NMR (DMSO-$d_6$) $\delta$ (ppm): 173.0, 167.0, 155.0, 154.0, 150.0, 146.0 (C-$NO_2$), 131.0, 127.0-137.0 (aromatic carbons), 121.0, 91.0; MS (EI, 70 eV): *m/z* (%) = 462 ($M^+$+1, 28), 461 ($M^+$, 100), 417 (30), 295 (28), 269 (13), 118 (12), 102 (8). Anal. Calcd. for $C_{26}H_{15}N_5O_4$ (461.43): C, 67.68; H, 3.28; N, 15.18%. Found: C, 67.61; H, 3.76; N, 15.01%.

*4.4.3. 2-(4,6-Diphenyl-2H-pyrazolo[3,4-b]pyridine-3yl)-4-nitroisoindolin-1,3-dione* ***(8)***

Yield (89%); pale yellow powder; m.p.185 °C; IR (KBr): ν/cm$^{-1}$= 3189 (NH), 1680 (C=O), 1625 (C=N), 1575 (C=C), 1535,1345 ($NO_2$); MS (EI, 70 eV): *m/z* (%) = 462 ($M^+$+1, 18), 461 ($M^+$, 100), 417 (25), 296 (30), 269 (20), 118 (15), 102 (12). Anal. Calcd. for $C_{26}H_{15}N_5O_4$ (461.43): C, 67.68; H, 3.28; N, 15.18%. Found: C, 67.65; H, 3.22; N, 15.17%.

*4.4.4. 2-(4,6-Diphenyl-2H-pyrazolo[3,4-b]pyridine-3-yl)-4,5,6,7-tetrabromo-isoindolin-1,3-dione* ***(9)***

Yield (91%); pale yellow powder; m.p.> 300 °C; IR (KBr): ν/cm$^{-1}$= 3230 (NH), 1704 (C=O); $^{13}$C-NMR (DMSO-

$d_6$) $\delta$ (ppm): 173.0, 167.0, 155.0, 154.0, 151.0, 130.0, 126.0-135 (aromatic carbons), 121.0, 91.0; MS (EI, 70 eV): *m/z* (%) = 733 ($M^+$+2, 62.3), 731 ($M^+$, 100), 729 (66.5), 687 (35), 435 (41), 296 (30), 270 (15). Anal. Calcd. for $C_{26}H_{12}Br_4N_4O_2$ (732.02): C, 42.66; H, 1.65; N, 7.65%. Found: C, 42.62; H, 1.61; N, 7.60%.

*4.4.5. 2-(4,6-Diphenyl-2H-pyrazolo[3,4-b]pyridin-3-yl)isoindolin-1,3-dione-5-carboxylic acid* ***(10)***

Yield (85%); yellow powder; m.p.> 300 $^oC$; IR (KBr): ν/$cm^{-1}$= 3355 (NH), 1739 (C=O), 1700 (C=O), 1620 (C=N); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 7.40-8.30 (m, 10H, Ar-H), 7.65 (s, 1H, Ar-H), 7.70 (d, 1H, Ar-H), 7.76 (s, 1H, $C_5$-H pyridine), 7.82 (d,1H, Ar-H), 12.21 (s, 1H, NH), 13.10 (s, 1H, COOH); $^{13}$C-NMR (DMSO-$d_6$) $\delta$ (ppm): 173.0, 170 (COOH), 167.0, 155.0, 154.0, 151.0, 130.0, 126.0-135.0 (aromatic carbons), 121.0, 90.0; MS (EI, 70 eV): *m/z* (%) = 461 ($M^+$+1, 30), 460 ($M^+$, 100), 416 (35), 296 (38), 270 (40), 165 (12), 121 (31). Anal. Calcd. for $C_{27}H_{16}N_4O_4$ (460.44): C, 70.43; H, 3.50; N, 12.17%. Found: C, 70.38; H, 3.47; N, 12.10%.

*4.4.6.2,6-bis(4,6-diphenyl-2H-pyrazolo[3,4-b]pyridin-3-yl)pyrrolo[3,4-f]isoindole-1,3,5,7(2H,6H)-tetraone* ***(11)***

Yield (91%); pale yellow powder; m.p.> 330 $^oC$; IR (KBr): ν/$cm^{-1}$= 3380 (NH), 1689 (C=O); MS (EI, 70 eV): *m/z* (%) = 756 ($M^+$+2, 14), 755 ($M^+$+1, 50), 754 ($M^+$, 100), 710 (30), 666 (55), 414 (60), 370 (30), 296 (15), 270 (22), 118 (20), 102 (10), 74 (11). Anal. Calcd. for $C_{46}H_{26}N_8O_4$ (754.75): C, 73.20; H, 3.47; N, 14.85%. Found: C, 73.10; H, 3.37; N, 14.80%.

*4.5. Synthesis of 3-(4,6-diphenyl-2H-pyrazolo[3,4-b]pyridine-3-yl)-quinazoline-2,4-(1H,3H)-dione* ***(12)***

A mixture of compound 5 (1 mmol) and isatoic anhydride (1 mmol) in ethanol (50 ml) containing few drops of glacial acetic acid was stirred for 3h. at room temperature. The resulting crystals were filtered off, dried, and recrystallized from ethanol to give compound 12. Yield (80%); yellowish crystals; m.p 240 $^oC$; IR (KBr): ν/$cm^{-1}$= 3330, 3250 (2NH), 1677 (C=O), 1620 (C=N); $^1$H-NMR (DMSO-$d_6$) $\delta$ (ppm): 7.40-8.30 (m,14H, Ar-H), 7.79 (s, 1H, $C_5$-H pyridine), 12.30 (s, 1H, NH), 12.76 (s, 1H, NH); $^{13}$C-NMR (DMSO-$d_6$) $\delta$ (ppm): 158.0, 155.0, 154.0, 150.0, 132.0, 127.0-137.0 (aromatic carbons), 121.0, 91.5; MS (EI, 70 eV): *m/z* (%) = 432 ($M^+$+1, 28), 431 ($M^+$, 100), 387 (30), 336 (28), 286 (13), 118 (12), 102 (8). Anal. Calcd. for $C_{26}H_{17}N_5O_2$ (431.45): C, 72.38; H, 3.97; N, 16.23%. Found: C, 72.28; H, 3.90; N, 16.10%.

*4.6. Cytotoxicity Activity*

RPMI-1640 medium (Sigma Co., St. Louis, USA), Foetal Bovine serum (GIBCO, UK), and the cell lines from ATCC were used.

The cytotoxic activity of the synthesized compounds was targeted against human heptacellular liver carcinoma cell line (HepG2), human lung fibroblast cell line (WI-38), human causcasian breast adenocarcinoma cell line (MCF-7), and normal adult African green monkey kidney cell line (VERO). The stock samples of the compounds were diluted with RPMI-1640 medium to desired concentrations ranging from 10 to 1000 µg/mL. The final concentration of DMSO in each sample did not exceed 1% v/v.

The cells were bath-cultured for 10 d, then seeded in 96 well plates of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 $^\circ$C for 24 h. under 5% $CO_2$ using a water jacketed carbon dioxide incubator (Shedon. TC2323. Cornelius, OR, USA). The medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentrations of (1000, 500, 200, 100, 50, 20, 10 µg/mL). Cells were suspended in RPMI-1640 medium, 1% antibiotic-antimycotic mixture ($10^4$ u/mL) potassium penicillin, $10^4$ µg/mL streptomycin sulfate and 25 µg/mL A mphotericin B) and 1% L-glutamin in 96-well flat bottom microplates at 37 $^\circ$C under 5% $CO_2$. After 96 h. of incubation, the medium was again aspirated, trays were inverted onto a pad of paper towels, the remaining cells rinsed carefully with medium, and fixed with 3.7% (v/v) formaldehyde in saline for at least 20 min. The fixed cells were rinsed with water. The %viability of cells was examined visually as described previously [28].

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