A Novel Synthesis and Molluscicidal Activity of some Functionally Substituted Pyridine, Pyrido-[3,2-c]pyridazine, and Pyrido[3,2-c]pyridazino-[2′,3′-a]quinazoline Derivatives

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Ethyl benzoylacetate reacts with the malononitrile dimer to afford 4-amino-5-benzoyl-2-dicyanomethyl-6-hydroxypyridine, which undergoes the coupling reaction with aromatic diazonium salts to afford azo derivatives. These azo derivatives could be cyclized into pyrido[3,2-c]pyridazine and pyrido[3,2-c]pyridazino[2′,3′-a]quinazoline derivatives upon reflux in ethanolic NaOH, presumably via their hydrazo tautomers. The molluscicidal activity of these compounds was evaluated.

Keywords: Ethyl benzoylacetate; Malononitrile dimer; Pyridine; Pyrido[3,2-c]pyridazine; Pyrido[3,2-c]pyridazino[2′,3′-a]quinazoline derivatives

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Introduction

In the last two decades, we have been involved in a program aiming at the synthesis of heterocyclic compounds of biological interest that may find applications as biodegradable agrochemicals [1−4]. *Schistosomiasis (bilharziasis)* is an irritating health problem in Egypt and great efforts are made to combat this disease by combating the water snail *Biomphalaria alexandrina*, the intermediate host of the infective phase of *Schistosoma mansoni,* through molluscicides. Intensive research work to prepare synthetic or extract naturally occurring molluscicides has evolved [5−7]. We have found it mandatory to share these efforts and directed a part of our research towards the synthesis of heterocyclic compounds that can be used as molluscicides [8−10]. In the context of this program, some new functionally substituted pyridopyridazine derivatives were required. Pyridopyridazines are reported to have pronounced biological activity [11, 12], however, their molluscicdal activity has not yet been evaluated. Therefore, it was planned to prepare these required derivatives and then to test their potential molluscicidal activity. The pyridine derivative **3** (Scheme 1) seemed a good candidate to fulfil this objective by coupling with the aromatic diazonium salts **5a**−**d** on the active methylene group to afford azo derivatives which can be cyclized to pyridopyridazine derivatives, similar to the previously reported work on related systems [13]. This urged us to investigate the reaction of ethyl benzoylacetate **1** with 2-amino-1,1,3-tricyanopropene (malononitrile dimer) **2** [14] aiming to obtain the cyanomethyl pyridine derivative **3** via the assumed reaction of **1** on the enaminonitrile site of **2**.

Malononitrile dimer **2** has been extensively utilized in the synthesis of substituted pyridines reacting with different reagents [15−21]. However to our knowledge, its reaction with **1** is hitherto not reported.

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Results and discussion

Synthesis

Ethyl benzoylacetate **1** was allowed to react with malononitrile dimer **2** in refluxing dimethylformamide (DMF) base catalyzed to afford a coffee-brown solid product with mp 325 °C. The mass spectrum of this product showed m/e 278. This mass is consistent with both the pyridine **3** and the tautomers **4a**−**c**. The IR spectrum however showed a single cyano absorption band at ν = 2199 cm^{-1} and only one carbonyl absorption band at ν = 1651 cm^{1}. These data suggest that our product is not the pyridine derivative **3** but most likely the dicyanomethyl pyridine **4a.** The ^{1}H-NMR spectrum of this product revealed two singlets (1H, 1H) at 4.4 and 6.8 ppm, respectively assignable to the methylene proton and the pyridine-3H, respectively. It showed also two singlets at 7.4 ppm (2H) and at 9.5 ppm (1H), which disappeared on D_2O exchange, beside two aromatic multiplets at 7.42−7.56 (3H) and 7.76−7.90 (2H). The two D_2O exchangeable signals may be attributed to NH_2 and OH, respectively. A pyridine like **3** should have revealed two cyano and two carbonyl absorption bands in the IR spectrum

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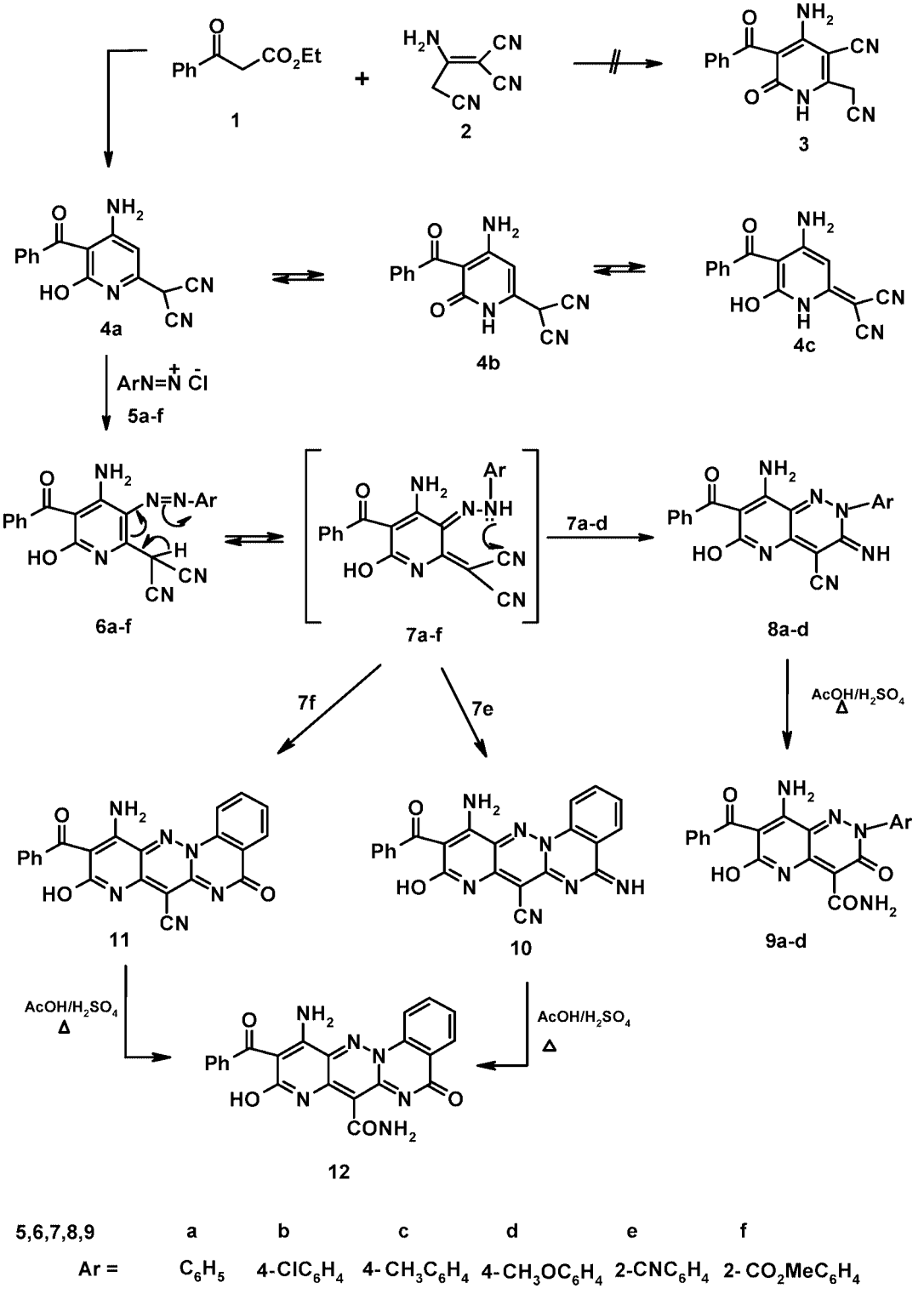

Scheme 1. Bio-active pyridine and pyrido[3,2-c]pyridazine derivatives.

and a methylene singlet in the field of $\delta = 3-4$ ppm in the ^{1}H-NMR spectrum. Furthermore, the singlet at 6.8 ppm assignable to the pyridine-3H would have not appeared. Thus structure **3** was ruled out. The tautomeric structures **4b** and **4c** were also ruled out, since both should have revealed two exchangeable singlets (1H) for OH and NH; **4b**

should have revealed an amide carbonyl absorption band in the IR and in the ^{13}C-NMR spectrum; **4c** was expected to reveal the aliphatic carbon at $\delta > 50$ ppm in the ^{13}C-NMR spectrum, however, it appeared at $\delta = 22.5$ ppm and splitted into a doublet in the off-resonance spectrum (see Experimental). Thus structure **4a** was assigned to our product. It is assumed that the reaction took place via the attack of **1** on the amino-cyanomethyl site of **2** to afford **4a**. It should be mentioned that the difference in the pyridine structure in the present case from that obtained by Wakefield et al. [22] is attributed presumably to the presence of the OH group in the 6-position which enables an amide-iminol tautomerism **4a/4b** in which the hydrogen is resonating between the O and N thus preventing the formation of the dicyanomethylene structure **4c**.

Despite not having obtained our envisaged pyridine, the obtained pyridine **4a** seemed also a good precursor. The free 3-position was found to be active toward the diazotized aromatic amines **5a**−**f** and couples readily with them to afford the azo derivatives **6a**−**f**, respectively as highly colored solid compounds with relatively high melting points. The structures of these azo compounds were established on the basis of their elemental analyses and spectral data (see Experimental).

The azo derivatives of similar pyridines have found wide applications in the dyeing of synthetic fibers [23] and the azo derivatives described in the present work may find similar applications.

Refluxing the azo derivatives **6a**−**d** in ethanolic sodium hydroxide (20% aqueous solution) accomplished their cyclization to the desired pyrido[3,2-c]pyridazine derivatives **8a**−**d**, presumably via the hydrazo derivatives **7a**−**d** which are formed *in situ* through a 1,5-hydrogen shift under the effect of the base [24].

Compounds **8a**−**d** could be transformed into the amide derivatives **9a**−**d** upon reflux in acetic/sulfuric acid mixture [1].

Refluxing the azo derivatives **6e** and **6f** under the same basic conditions led to the pyrido[3,2-c]pyridazino[2′,3′-a]quinazoline derivatives **10** and **11,** respectively, presumably via the hydrazo derivatives **7e** and **7f**, which undergo two successive cyclizations either through a Michael addition to the CN of anthranilonitrile or through elimination of methanol in case of methyl anthranilate. The elemental analyses and spectral data of compounds **8a**−**d**, **9a**−**d**, **10,** and **11** are in agreement with their proposed structures (see Experimental).

Both compounds **10** and **11** afforded the amide **12** upon reflux in acetic/sulphuric acid mixture. The identity of the two products obtained from **10** and **11** was inferred from their melting points and TLC analysis. The IR spectrum of **12** did not show any cyano absorption bands and instead three carbonyl absorption bands are revealed.

Table 1. The mean number of snails killed ±1 after an exposure time of 24 h at different concentrations.

Compound No.	4 ppm	6 ppm	8 ppm	10 ppm	12 ppm	15 ppm
				[nM]		
6a	1	3	5	8	10	10
6b	4	6	10	10	10	10
6c	1	2	4	7	9	10
6d	2	2	4	6	7	9
6e	6	10	10	10	10	10
6f	1	2	3	6	7	9
8a	–	–	3	5	10	10
8b	–	3	7	10	10	10
8c	–	–	2	3	6	9
8d	–	–	2	3	6	9
9a	–	–	4	6	8	10
9b	–	2	6	10	10	10
9c	–	1	3	4	7	8
9d	–	1	2	4	6	8
10	–	1	2	3	5	7
11	–	1	1	2	4	6
12	–	1	1	3	4	5

Molluscicidal activity

The toxicity of compounds **6a**−**f, 8a**−**d, 9a**−**d, 10, 11, and 12** to *Biomphalaria alexandrina* snails was evaluated as shown in Table 1. The full lethal dose (LC_{100} in ppm/ nM) for each compound was determined and is shown in Table 2. An insight inspection of the results listed in Table 2 shows that all compounds have generally moderate to low effect on the snails, and they all showed no effect below 4 ppm (cf. Table 1). The most effective of them are **6b**, **6e**, **8b,** and **9b** (LC_{100} = 8, 6, 10, and 10 ppm, respectively). The activity of **6b, 8b,** and **9b** is presumably due to the 4-chlorophenyl

Table 2. Molluscicidal activity of compounds **6a**−**f**, **8a**−**d**, **9a**−**d**, **10**, **11**, and **12** expressed as LC_{100} in ppm [nM].

Compound	LC_{100}
6a	12 [31.4]
6b	8 [19.2]
6c	> 12 [> 30.3]
6d	> 15 [> 36.4]
6e	6 [14.7]
6f	> 15 [> 34.1]
8a	12 [31.4]
8b	10 [24.0]
8c	> 15 [> 37.9]
8d	> 15 [> 36.4]
9a	15 [37.4]
9b	10 [23.0]
9c	> 15 [> 36.1]
9d	> 15 [> 34.8]
10	> 15 [> 36.9]
11	> 15 [> 36.8]
12	> 15 [> 35.2]

residue. The azo compounds **6a**−**f** are generally more active than their cyclized analogues of the same concentration perhaps due to their marked toxicity. Compound **6e** shows relatively better activity than the other azo series [despite not having a 4-chlorophenyl moiety] apparently due to the presence of an extra cyano group in its structure which renders it more toxic. A comparison of the molluscicidal activity of our compounds with an international standard: 2,5-dichloro-4-nitrosalicylanilide which is reported to possess LC_{100} = 1 ppm [25, 26] showed that our compounds are far inferior as molluscicidal agents. Compounds **6b**, **8b,** and **9b** seem promising after some modification by replacing the OH groups by halogen and using polyhalogenated aniline in the diazotization process which will be considered in a future study.

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Experimental

Chemistry

General

Melting points were determined on an electrothermal (9100) apparatus and are uncorrected. IR spectra were recorded as KBr pellets on a Perkin Elmer 1430 spectrophotometer (Perkin Elmer, Norwalk, CT, USA). The ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Varian Gemini 300 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) in DMSO-d_6 using TMS as internal standard and chemical shifts are expressed in δ (ppm) values. Assignments were made by correlation of the off-resonance decoupled ^{13}C NMR spectra and determination of the ^{1}H chemical shifts. Mass spectra were taken on a Shimadzu GCMS-GB 1000 PX (70 eV). Elemental analyses were carried out by the Microanalytical Center at Cairo University. Satisfactory elemental analysis results (± 0.4%) have been obtained for all compounds. Molluscicidal activity tests were conducted in the Medicinal Chemical Department, Laboratory of Parasitology, National Research Center of Egypt.

Although all the final products are structurally related, they show slight differences in their colors (from bright red to brownish red) and this may be attributed to two reasons: First, the different crystallization solvents and the volume of the solvent used (dioxan and acetic acid) and accordingly to the crystalline shape, however, when powdered, they have almost the same color. Second, it may be attributed also to the different substituents (phenyl, *p*-chlorophenyl, *p*-tolyl, *p*-anisyl, 2-cyano phenyl, 2-methoxycarbonyl phenyl, imino, carobonyl, or amide groups). These chromophores cause a slight shift of the wave length λ to longer or shorter sides which may cause this difference [28].

*4-Amino-5-benzoyl-2-dicyanomethyl-6-hydroxypyridine **4a***

To a mixture of ethyl benzoylacetate **1** (19.2 g, 100 mmol) and malononitrile dimer **2** (13.2 g, 100 mmol) in 75 mL of DMF 1 mL of piperidine was added. The reaction mixture was refluxed for 3 h, then left to cool to room temperature, poured on crushed ice, and neutralized with HCl (just neutral). The precipitated solid that appeared was filtered off, washed thoroughly with cold water, dried in an oven at 120 °C, and recrystallized from acetic acid to afford **4a** (yield 18 g, 64.75 mmol) as a coffee-brown crystalline solid, mp 325 °C; $C_{15}H_{10}N_4O_2$ (m/e = 278); ν_{max} (KBr) 3741 and 3355−3100 (OH and NH_2), 2199 (CN), 1651(C=O) cm^{-1}. δ_H (300 MHz, DMSO-d_6) 4.4 (s, 1H, CH), 6.8 (s, 1H, pyridine-3H), 7.4 (s, 2H, NH_2), 7.42−7.56 & 7.76-7.90 (m, 5H, ar. H), 9.5 (s, 1H, OH). ^{13}C NMR δ C2, 22.5(d); C4, 91.5 (d); C6, 112.7 (s); C1, 114.5 (s); [ar. C's, 128.8 (d), 129.7 (d), 132.5 (d), 133.4 (s)]; C3, 157.2 (s); C5, 157.9 (s); C8, 167.2 (s); C7, 187.5 (s).

*4-Amino-5-benzoyl-2-dicyanomethyl-3-arylazo-6-hydroxypyridine derivatives **6a−f***

To a cold solution of **4** (2.78 g, 10 mmol) and sodium acetate 1.5 g in 40 mL of pyridine a cold solution of the diazotized aromatic amines **5a**−**f** (aniline, 4-chloroaniline, 4-toluidine, 4-anisidine, anthranilonitrile or methyl anthranilate, 10 mmol) was added dropwise, while stirring over a period of 30 min, after which stirring was continued for another 1 h. The dark-colored solution was then diluted with cold water and the solid precipitates that appeared were filtered off, washed several times with cold water, and recrystallized from dioxan to give the azo derivatives **6a**−**f**:

6a Red powder (yield 3.25 g, 8.5 mmol) mp 209 °C; $C_{21}H_{14}N_6O_2$; ν_{max} (KBr) 3417−3200 (OH NH_2), 2210 (CN), 1665 (CO); δ_H (300 MHz, DMSO-d_6) 4.45 (s, 1H), 7.4 (s, 2H), 7.43−7.88 (two groups m, 10H, ar. H), 9.6 (s, 1H).

6b Crimson red powder (yield 3.3 g, 7.9 mmol) mp 273 °C; $C_{21}H_{13}ClN_6O_2$; ν_{max} (KBr) 3420−3210 (OH and NH_2), 2205 (CN), 1661 (CO); δ_H (300 MHz, DMSO-d_6) 4.42 (s, 1H), 7.4 (s, 2H), 7.42−7.85 (two groups m, 9H, ar. H), 9.45 (s, 1H).

6c Dark red powder (yield 2.65 g, 6.69 mmol) mp 268−270 °C; $C_{22}H_{16}N_6O_2$ (m/e = 396); ν_{max} (KBr) 3397−3155 (br. OH and NH_2), 2212 (CN), 1660 (CO); δ_H (300 MHz, DMSO-d_6) 2.4 (s, 3H), 4.45 (s, 1H), 7.39 (s, 2H), 7.42-7.84 (two groups m, 9H, ar. H), 9.65 (s, 1H).

6d Reddish brown powder (yield 2.7 g, 6.55 mmol) mp 305 °C; $C_{22}H_{16}N_6O_3$ (m/e = 412); ν_{max} (KBr) 3412-3200 (br. OH and NH_2), 2213 (CN), 1663 (CO); δ_H (300 MHz, DMSO-d_6) 3.8 (s, 3H), 4.47 (s, 1H), 7.4 (s, 2H), 7.23−7.68 (two groups m, 9H, ar. H), 9.55 (s, 1H).

6e Pink colored crystals (yield 2.9 g, 7.12 mmol) mp 257 °C; $C_{22}H_{13}N_7O_2$ (m/e = 407); ν_{max} (KBr) 3420−3150 (br. OH and NH_2), 2207 and 2218 (CN), 1670 (CO); δ_H (300 MHz, DMSO-d_6) 4.42 (s, 1H), 7.39 (s,2H), 7.20−7.80 (two groups m, 9H, ar. H), 9.55 (s, 1H).

6f Dark crimson red crystals (yield 3.3 g, 7.5 mmol) mp 210 °C; $C_{23}H_{16}N_6O_4$ (m/e = 440); ν_{max} (KBr) 3397−3155 (br. OH and NH_2), 2208 (CN), 1712 and 1665 (2 CO); δ_H (300 MHz, DMSO-d_6) 3.85 (s, 3H, CH_3), 4.43 (s, 1H), 7.15−7.85 (two groups m, 11H, ar. H + NH_2), 9.54 (s, 1H).

*8-Amino-2-aryl-7-benzoyl-4-cyano-6-hydroxypyrido[3,2-c]pyridazin-3-imines **8a−d**, 4-amino-3-benzoyl-12-cyano-2-hydroxypyrido[3,2-c]pyridazino[2′,3′-a]quinazolin-10-imine **10**, and 4-amino-3-benzoyl-12-cyano-2-hydroxypyrido[3,2-c]pyridazino[2′,3′-a]quinazolin-10-one **11**; General procedure*

To a solution of each of **6a**−**f** (10 mmol) in 20 mL ethanol 5 mL of 20% NaOH solution was added. The reaction mixture was re-

fluxed for 2 h in each case, left to cool to room temperature, poured on crushed ice, and neutralized with HCl. The precipitated solid products that appeared were filtered off, washed, dried, and recrystallized from dioxan to afford the title compounds:

8a Dark red powder (yield 3.3 g, 8.64 mmol) mp 245°C; $C_{21}H_{14}N_6O_2$ (m/e = 382); ν_{max} (KBr) 3450−3200 (br. NH_2, NH, OH), 2210 (CN), 1655 (CO); δ_H (300 MHz, DMSO-d_6) 7.1−8.0 (m, 12H, ar. H and NH_2), 8.25 (s, 1H, NH), 9.5 (s, 1H, OH).

8b Reddish green powder (yield 3.4 g, 8.19 mmol) mp 263°C; $C_{21}H_{13}ClN_6O_2$ (m/e = 415, 417); ν_{max} (KBr) 3430−3150 (br. NH_2, NH, OH), 2215 (CN), 1653 (CO); δ_H (300 MHz, DMSO-d_6) 7.15−8.0 (m, 11H, ar. H and NH_2), 8.27 (s, 1H, NH), 9.45 (s,1H, OH).

8c Dark red powder (yield 3.1 g, 7.8 mmol) mp >350°C; $C_{22}H_{16}N_6O_2$ (m/e = 396); ν_{max} (KBr) 3390−3100 (br. NH_2, NH, OH), 2208 (CN), 1655 (CO); δ_H (300 MHz, DMSO-d_6) 2.45 (s,3H,CH_3), 7.1−7.9 (m, 11H, ar. H and NH_2), 8.20 (s, 1H, NH), 9.4 (s, 1H, OH).

8d Crimson red crystalline solid (yield 3.3 g, 8.0 mmol) mp 216°C; $C_{22}H_{16}N_6O_3$ (m/e = 412); ν_{max} (KBr) 3420−3140 (br. NH_2, NH, OH), 2215 (CN), 1660 (CO); δ_H (300 MHz, DMSO-d_6) 3.75 (s, 3H, CH_3), 7.2−8.05 (m, 11H, ar. H and NH_2), 8.32 (s, 1H, NH), 9.55 (s, 1H, OH).

10 Dark brown solid (yield 3.5 g, 8.6 mmol) mp 210°C; $C_{22}H_{13}N_7O_2$ (m/e = 407); ν_{max} (KBr) 3370−3205 (br. NH_2, NH, OH), 2215 (CN), 1655 (CO); δ_H (300 MHz, DMSO-d_6) 7.25−7.90 (m, 11H, ar. H and NH_2), 8.35 (s, 1H, NH), 9.4 (s, 1H, OH).

11 Dark reddish brown solid (yield 3.5 g, 8.58 mmol) mp 280°C; $C_{22}H_{12}N_6O_3$ (m/e = 408); ν_{max} (KBr) 3450−3200 (br. NH_2 and OH), 2218 (CN), 1680 and 1655 (2 CO); δ_H (300 MHz, DMSO-d_6) 6.70−7.85 (m, 11H, ar. H and NH_2), 9.45(s, 1H, OH). ^{13}C NMR δ 94 (s), 104.3 (s), 114.3 (s), 115.7 (d), 119.0 (d), 123.5 (s), 129.2 (d), 129.8 (d), 130.5 (d), 134.6 (d), 135.0 (d), 137.1 (s), 148.0 (s), 155.8 (s), 160.2 (s), 161.0 (s), 162.5 (s), 187.1 (s), 189.0 (s).

*8-Amino-2-aryl-7-benzoyl-4-carboxamido-6-hydroxypyrido[3,2-c]-pyridazin-3-ones **9a−d**, and 4-amino-3-benzoyl-12-carboxamido-2-hydroxy[3,2-c]pyridazino[2′,3′-a]quinazolin-10-one **12**; General procedure*

To a solution of each of **8a−d**, **10** or **11** (10 mmol) in 25 mL acetic acid 2 mL of conc. H_2SO_4 and 2 mL of water were added and the mixture was refluxed for 1 h. After being cooled to room temperature, it was poured on crushed ice, and neutralized cautiously with ammonia solution. The solid precipitates were collected by filtration, washed with water, and recrystallized from acetic acid to afford the title compounds:

9a Red crystalline solid (yield 3.2 g, 7.98 mmol) mp 285°C; $C_{21}H_{15}N_5O_4$ (m/e = 401); ν_{max} (KBr) 3395−3200 (br., NH_2 and OH), 1650, 1665, and1680 (3CO); δ_H (300 MHz, DMSO-d_6) 6.4 (s, 2H), 7.1−8.0 (m, 12H, ar. H and NH_2), 9.5 (s, 1H). ^{13}C NMR δ 109 (s), 121.5 (d), 124.3 (d), 128.7 (d), 129.1 (d), 129.8 (d), 130.1 (s), 134.3 (d), 136.9 (s), 138.5 (s), 155.5 (s), 156.5 (s), 161.0 (s), 164.5 (s), 166 (s), 168.7 (s), 187.5 (s).

9b Reddish powder (yield 3.6 g, 8.27 mmol) mp 276°C; $C_{21}H_{14}ClN_5O_4$; ν_{max} (KBr) 3400−3200 (br. NH_2 and OH), 1655, 1663, and 1670 (3 CO); δ_H (300 MHz, DMSO-d_6) 6.42 (s, 2H), 7.15−7.85 (m, 11H, ar. H and NH_2), 9.35 (s, 1H).

9c Reddish brown powder (yield 3.4 g, 8.19 mmol) mp 291°C; $C_{22}H_{17}N_5O_4$ (m/e = 415); ν_{max} (KBr) 3425−3210 (br. NH_2 and OH), 1655, 1660, and 1675 (3 CO); δ_H (300 MHz, DMSO-d_6) 2.55 (s, 3H, CH_3), 6.3 (s, 2H), 7.1−8.0 (m, 12H, ar. H and NH_2), 9.4 (s, 1H).

9d Crimson red solid (yield 3.7 g, 8.58 mmol) mp 237°C; $C_{22}H_{17}N_5O_5$; ν_{max} (KBr) 3385−3185 (br. NH_2 and OH), 1653, 1660, and 1685 (3 CO); δ_H (300 MHz, DMSO-d_6) 3.85 (s, 3H, CH_3), 6.45 (s, 2H), 7.28−8.05 (m, 11H, ar. H and NH_2), 9.55 (s, 1H).

12 Brick red powder (yield 3.7 g, 8.69 mmol) mp >340°C; $C_{22}H_{14}N_6O_4$ (m/e = 426); ν_{max} (KBr) 3390−3210 (br. NH_2 and OH), 1655, 1669, and 1684 (3 CO); δ_H (300 MHz, DMSO-d_6) 6.42 (s, 2H), 7.1−8.1 (m, 11H, ar. H and NH_2), 9.45 (s, 1H).

Biology

Molluscicidal activity tests

The molluscicidal activity tests were carried out by determination of the full lethal dose LC_{100} of each compound under investigation. *Biomphalaria alexandrina* snails were collected from the field (water canals) and maintained under laboratory conditions for a period of 30 days before use and fed daily by lettuce leaves. The snails were then examined to ensure that they were free of parasitic infection. A series of concentrations (seven) ranging from 2 ppm to 15 ppm of each compound under investigation was prepared. The required weight of the compound under investigation was mixed thoroughly with a few drops of Tween 20 and 2 mL of DMSO to render the compounds completely soluble, followed by addition of the appropriate volume of nontreated raw water (taken directly from the River Nile or its branches/canals) to get a homogeneous suspension (turbid) with the requisite concentration and placed in glass jar vessels 15 × 25 × 20 cm dimensions fitted with air bubblers. Ten snails having the same size and diameter (ca. 7 mm) were used in each experiment and maintained in the test solution under laboratory conditions at ambient temperature for 24 h. Each experiment was repeated three times and the mean number of killed snails was taken for each concentration as shown in Table 1. A control group was taken by placing 10 snails in water containing few drops of Tween 20 and 2 mL of DMSO. These bioassays are in accordance with the W.H.O. guidelines [27] .

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