Synthesis and antitumor activity evaluation of some novel pyrazolotriazine derivatives

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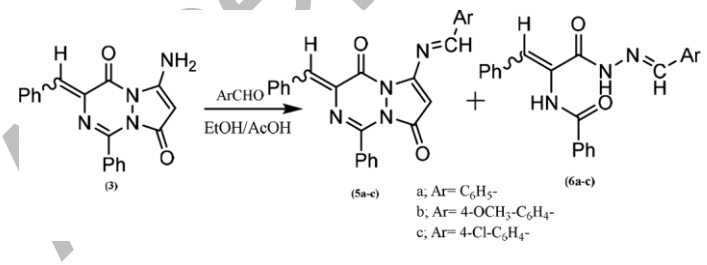
Abstract

6-Aminopyrazolo[1,2-a][1,2,4]triazine-4,8-dione derivative **3** was obtained upon the reaction of the acid hydrazide derivative **2a** with ethyl cyanoacetate. The reactions of **3** with several electrophiles such as aldehydes, isatin, acetic anhydride, phenyl isocyanate, benzoyl isothiocyanate and p-toluenesulphonyl chloride were studied. The structures of the newly synthesized compounds were established on the basis of IR, ^{1}H-NMR, mass spectra, and elemental analyses. The antitumor activities of some selective compounds were examined against two cell lines as liver carcinoma cell line (HEPG-2) and human breast cancer cell line (MCF7).

GRAPHICAL ABSTRACT



KEYWORDS: pyrazolotriazine, pyrazoline-3,5-dione, antitumor activities, HEPG-2, MCF7

INTRODUCTION

Among the wide variety of nitrogen heterocycles, 1,2,4-triazine and its fused derivatives were explored for developing pharmaceutical and agrochemical molecules[1-2].

The pyrazolotriazine ring system is similar to adenine analogues antagonists in their structure. Therefore, this ring has considerable biological and medicinal activities such as antischistosomal, antitumor agents[3-6] and antimicrobial activity[7]. Also, the Pyrazolotriazine derivatives were reported to have remarkable cytotoxic activity against colon, breast and lung carcinoma cells[8]. Some derivatives showed selective cytotoxicity in hypoxic and normoxic conditions [9]. The valuable biological and pharmaceutical activities of these ring systems, prompted us to synthesis new derivatives of pyrazolo[1,2-a][1,2,4] triazines for the evaluation of antitumor activities.

RESULTS AND DISCUSSION

The geometrical isomers of the acid hydrazide derivatives Z-(**2a**) & E-(**2b**) were previously prepared by our research group upon hydrazinolysis of 4-benzylidene-2-phenyl-1,3-oxazole-5(4*H*)-one **1** [10,11]. Treatment of the Z-(**2a**) with ethyl cyanoacetate in refluxing ethanol gave 6-amino pyrazolo[1,2-a][1,2,4]triazines-4,8-diones derivative **3**. Similar treatment of the E-(**2b**) with ethyl cyanoacetate under the same conditions, gave E-(**2b**) recovered unchanged. However, its treatment with ethyl cyanoacetate in the presence of a catalytic amount of sodium ethoxide in refluxing ethanol affored the pyrazoline-3, 5-dione derivative **4** (Scheme 1).

The difference in the chemical behavior of Z-(**2a**) and E-(**2b**) may be due to the steric crowding between benzylidene and benzamido groups that forces the compound to cyclize easily before or after the reaction with ethyl cyanoacetate to give compound **3**.

The structures of compounds **3** and **4** were substantiated from their analytical and spectral data. Their IR spectra exhibit bands corresponding to NH and C=O groups. The ^{1}H-NMR spectra are in accord with the proposed structures as they showed signals for protons of NH, olefinic and aromatic groups as well as the signals of the coupling pattern of CH_2-CH moiety of compound **4**. The higher δ value for the proton of NH pyrazolo suggests the existence of compound **4** as its chelated form shown (Scheme 1). Further evidence was gained from their mass spectra that showed the molecular ion peaks beside some of abundant peaks (cf. experimental).

The Schiff bases were exhibited many biological activities such as antifungal, antibacterial, antitumor, anti-inflammatory and antipyretic activities [12-15]. The presence of free amino group in compound **3** initiated our interest to synthesis a new series of Schiff bases in order to achieve heterocycles of anticipated biological activity.

Thus, the treatment of an ethanolic solution of compound **3** with different aromatic aldehydes such as benzaldehyde, *p*-methoxybenzaldehyde and *p*-chlorobenzaldehyde in the presence of a catalytic amount of acetic acid, afforded the expected Schiff bases **5a-c** beside small amounts of the unexpected product **6a-c**, respectively (Scheme 2).

The structure of compounds **5a-c** was established from their analytical and spectral data. Thus, their IR spectra were devoid of any absorptions due to NH_2 group. Further support for the suggested structure was gained from their ^{1}H-NMR and mass spectra (cf. experimental). The structure of compounds **6a-c** was identical in all respects (m.p., mixed m.p., TLC) with authentic samples prepared by reacting the acid hydrazides **2a** and/or **2b** with benzaldehyde, *p*-methoxybenzaldehyde and *p*-chlorobenzaldehyde, respectively. The mechanistic pathway for the formation of **6a-c** is depicted in (Scheme 3).

On the other hand, when compound **3** was treated with istain in refluxing ethanol and a catalytic amount of acetic acid, it afforded the Schiff base derivative **7** (Scheme 2). The structure of **7** was substantiated from its microanalytical and spectral data. The IR spectrum of compound **7** showed the appearance of bands for NH group at 3200, 3169 cm^{-1}. Its ^{1}H-NMR spectrum showed the appearance of a broad singlet signal for NH proton as well as signals for olefinic and aromatic protons. Further support for the assigned structure of compound **7** was gained from its mass spectrum that showed its correct molecular ion peak beside some of important peaks.

Refluxing of compound **3** in acetic anhydride afforded the diacetyl derivative **8a**. However, its heating on water bath with acetic anhydride yielded a mixture of diacetyl and monoacetyl derivatives **8a** & **8b** (Scheme 4).

Their structures were elucidated from their microanalytical and spectral data. The IR, ^{1}H-NMR and mass spectra were in accord with the suggested structures (cf. experimental).

Treatment of compound **3** with phenyl isocyanate in dry benzene gave the urea derivative **9**. However, its refluxing with benzoyl isothiocyanate in dry acetonitrile yielded the thiourea derivative **10**. The structures of compounds **9** & **10** were substantiated from their analytical and spectral data. Their IR spectra show absorptions characteristic of NH and C=O groups, as well as C=S group for compound **10**. Their ^{1}H-NMR spectra exhibit signals for protons of NH, olefinic and aromatic groups. Further support for the assigned structures of compounds **9** & **10** was found in their mass spectra. They revealed the molecular ion peaks and other abundant peaks.

Heating of the thiourea derivative **10** with phenylhydrazine in ethanol/dioxane (1:1) mixture in the presence of a catalytic amount of acetic acid afforded a mixture of two compounds one of them was identical in all respects (m.p., mixed m.p., TLC) with compound **3** and the other was proved to be the 1,2,4-triazole derivative **11** (Scheme 5).

The structure of compound **11** was evidenced by studying its spectral data that were in accord with the proposed structure. A chemical proof for its structure was gained by reaction of benzoyl isothiocyanate with phenylhydrazine under the same condition. The product obtained was identical in all respects (m.p., mixed m.p., TLC) with **11**. The mechanistic pathway for the conversion of **10** to compounds **3** and **11** could be visualized to proceed as shown in (Scheme 6).

Refluxing compound **3** with *p*-toluenesulphonyl chloride in dry benzene afforded *p*-toluene sulfonamide derivative **12** (Scheme 7). The structure of **12** was established from

its analytical and spectroscopic data. The IR spectrum showed the appearance of an absorption bands for NH, CH_3 and S=O group. Inspection of the ^{1}H-NMR spectrum of compound **12** revealed the existence of a singlet signal for CH_3 at 2.28 ppm, a broad singlet signal corresponds to proton of NH group at 7.18 ppm, multiplet signals for aromatic protons and singlet signals for olefinic protons. Furthermore, The EI-MS of compound **12** showed the correct molecular ion peak beside some of abundant peaks.

Treatment of a solution of compound **3** in acetic acid with sodium nitrite followed by aqueous solution of sodium azide and/or alkaline β-naphthol gave the hydroxy derivative **13** (Scheme 7). This is due to the unstability of the formed intermediate diazonium salt that underwent nucleophilic attack by H_2O to give the hydroxy derivative **13**.

The structure of compound **13** was devoid of any absorption corresponding to NH2 group; but shows bands for OH group. The ^{1}H-NMR spectrum was in accord with the proposed structure. The higher δ value for the signal of OH proton is in accord with its existence as its chelated form has shown (Scheme 7). On the other hand, when the compound **3** was treated with hydrazine hydrate in refluxing ethanol, it afforded the N-substituted pyrazole-5-one derivative **14** (Scheme 7).

The structure of compound **14** was substantiated from its spectral and analytical data. The IR spectrum exhibits bands corresponds to NH and C=O groups. Further evidence was ascertained from its ^{1}H-NMR spectrum that was consistent with the suggested structure (cf. experimental). Furthermore, the structure of compound **14** was supported

from its mass spectrum. The mechanistic pathway for the formation of **14** is presented in (Scheme 8).

BIOLOGICAL ACTIVITY

In Vitro Anticancer Screening (Antitumor Evaluation)

Compounds **2b**, **3**, **5a**, **5b**, **7**, **8a**, **10** and **12** were evaluated for their in vitro cytotoxic activity against two cell lines as liver carcinoma cell line (HEPG-2), human breast cancer cell line (MCF7). Doxorubicin which is one of the most effective anticancer agents was used as the reference drug in this study. The applied method is similar to that reported by Skehan, P. et al.[16] using Sulfo-Rhodamine-B stain (SRB) as shown in the experimental part. The results of in vitro cytotoxic activity against a liver carcinoma cell line (HEPG2) and a human breast carcinoma cell line (MCF7) were shown in tables 1 and 2, respectively. The response parameter calculated was the IC50 value, which corresponds to the concentration required for 50% inhibition of cell viability.

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From tables 1 and 2, we found that the better results were obtained with the five compounds (**3**, **7**, **8a**, **10** and **12**) only those showed a cytotoxic activity (IC50) against a liver carcinoma cell line (HEPG2) with a less than 50 µg/ml of these compounds. While the compounds (**2b**, **5a** and **5b**) against a liver carcinoma cell line (HEPG2) and all of the tested compounds against a human breast carcinoma cell line (MCF7) showed a cytotoxic activity (IC50) with a more than 50 µg/ml. The IC50 values of the compounds (**3**, **7**, **8a**, **10** and **12**) against a liver carcinoma cell line (HEPG2) compared to the reference drug were shown in table 1.

We can conclude that, Compound **12** was the most potent due to it showed a higher cytotoxic activity in this screening with IC50 equal to 6.8 µg/ml which can be attributed to the presence of the sulfonamide group. While, Compounds **3**, **8a** and **10** showed a moderate cytotoxic activity with IC50 equal to 18, 12 and 10 µg/ml which can be attributed to the presence of the amino group, two acetyl groups and one lactam and two thioamide groups, respectively. Compound **7** showed a poor cytotoxic activity with IC50 equal to 35.4 µg/ml.

EXPERIMENTAL

Melting points were uncorrected and were measured on a Gallen Kamp electric melting point apparatus. The infrared spectra were recorded using potassium bromide disks on FTIR Thermo Electron Nicolet 7600 (USA) infrared spectrometer at the Central laboratory of Faculty of Science, Ain Shams University. The ^{1}H-NMR spectra were run at 300 MHz on a GEMINI 300 BB NMR spectrometer using tetramethyl silane (TMS) as internal standard in deuterated dimethylsulphoxide (DMSO-d_6) at the main defense chemical laboratory. The mass spectra were recorded on a shimadzu GC-MS QP-1000EX mass spectrometer operating at 70 ev at the Regional Center for Mycology & Biotechnology of Al-Azhar University. The progress of all reactions was monitored by the thin layer chromatography using Merck Kiesel gel 60 F_{254} aluminum backed plates.

General Procedure for the Reaction of the Acid Hydrazide 2a with Ethyl Cyanoacetate

To a solution of the acid hydrazide **2a** (10 mmol) in ethanol (40 ml), ethyl cyanoacetate (10 mmol) was added. The reaction mixture was refluxed for 6h., then left to cool at

room temperature. The precipitated solid obtained was filtered off and washed with small amount of ethanol, recrystallized from ethanol to give compound **3**.

6-Amino-3-Benzylidene-1-Phenyl-3h-Pyrazolo[1,2-a][1,2,4]Triazine-4,8-Dione (3)

Yellow crystals (34%), m.p. 188-189 ^{o}C, (ethanol); FTIR (KBr) cm^{1}: 3334, 3272 (NH_2), 3062, 3027 (aryl-H), 1704 (C=O amide), 1639 (C=N), 1609 (C=C), 766, 687 (δ_{5H}). ^{1}H-NMR (DMSO-d_6): δ 5.37 (br.s, 2H, NH_2 exchangeable), 7.18 (s, 1H, =CHPh), 7.44 - 8.39 (m, 11H, ArH+ =CH pyrazolone). MS (70 ev) m/z (%): 331 ($M^{\cdot+}$ +1, 2), 330 ($M^{\cdot+}$, 8), 328 (12), 285 (22), 263 (43), 248 (25), 233 (33), 178 (25), 161 (42), 151 (47), 122 (29), 89 (30), 81 (59), 74 (94), 61 (39), 49 (23), 41 (100). Anal. Calcd. for $C_{19}H_{14}N_4O_2$ (330.34): C, 69.08; H, 4.27; N, 16.96. Found: C, 69.23; H, 4.21; N, 17.09%.

BIOLOGICAL TESTING

Measurement of Potential Cytotoxicity by SRB Assay

Potential cytotoxicity of the compounds was tested using the method of Skehan et al. (1990). Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the test compound to allow attachment of cell to the wall of the plate. Different concentrations of the compound under test (0, 1, 2.5, 5, and 10 µg/ml) were added to the cell monolayer in triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37^{o}C and in atmosphere of 5 % CO_2. After 48 h, cells were fixed, washed and stained with SRB stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug

concentration is plotted to get the survival curve of each tumor cell line after the specified compound and the IC50 was calculated.

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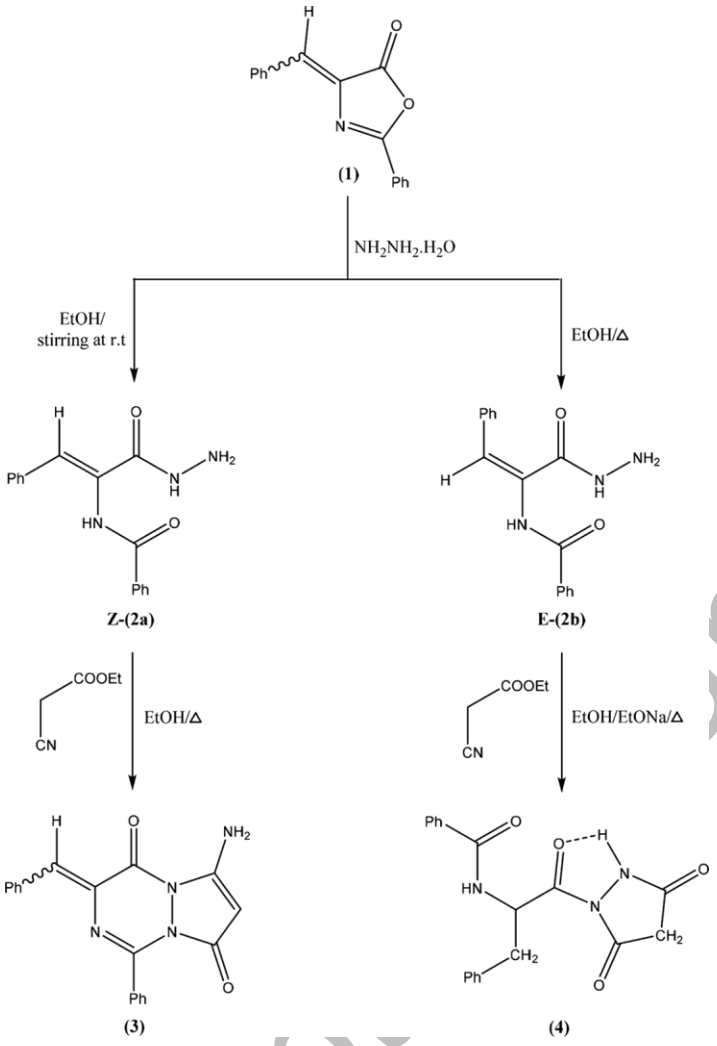
Table 1. Represents the results of in vitro cytotoxic activity against a liver carcinoma cell line (HEPG2).

compound number	**Concentration:µg/ml**					**IC50 value**
	0.000	**5.000**	**12.500**	**25.000**	**50.000**	
2b	1.000	0.712	0.666	0.646	0.619	----
3	1.000	0.666	0.600	0.380	0.344	18
5a	1.000	0.835	0.822	0.699	0.709	----
5b	1.000	0.770	0.787	0.680	0.703	----
7	1.000	0.645	0.514	0.555	0.426	35.4
8a	1.000	0.877	0.475	0.270	0.283	12
10	1.000	0.755	0.404	0.381	0.373	10
12	1.000	0.528	0.423	0.350	0.315	6.8
Doxorubicin	1.000	0.396	0.245	0.222	0.183	3.9

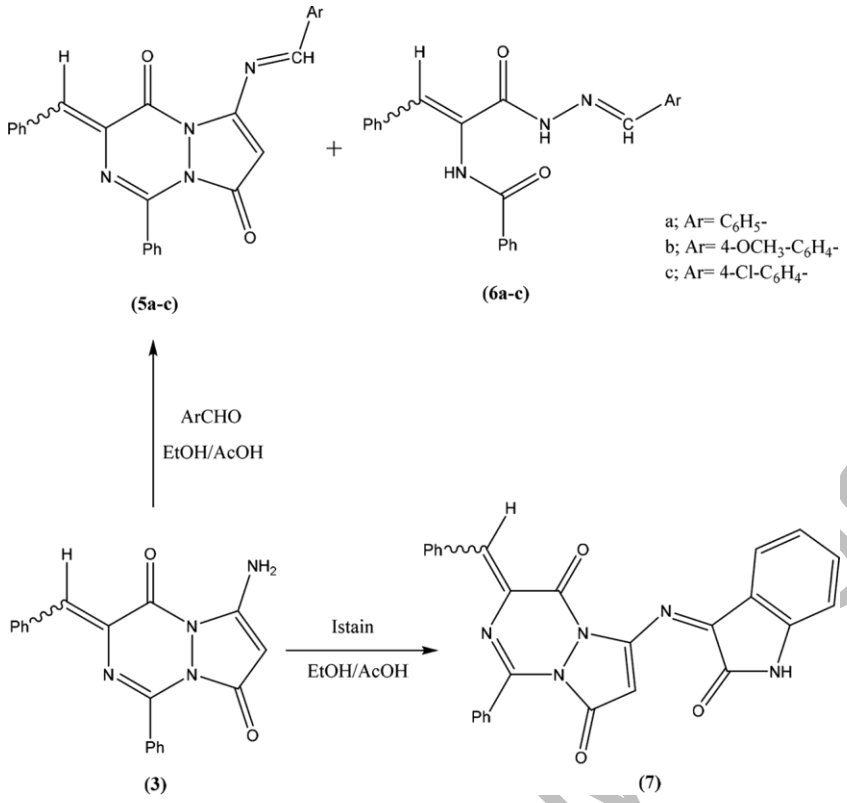
Table 2. Represents the results of in vitro cytotoxic activity against a human breast carcinoma cell line (MCF7).

compound number	**Concentration:µg/ml**					**IC50 value**
	0.000	5.000	12.500	25.000	50.000	
2b	1.000	0.882	0.812	0.708	0.718	----
3	1.000	0.988	0.943	0.939	0.939	----
5a	1.000	0.882	0.842	0.844	0.934	----
5b	1.000	0.988	0.998	0.988	0.988	----
7	1.000	0.797	0.753	0.766	0.638	----
8a	1.000	0.988	0.934	0.950	0.924	----
10	1.000	0.783	0.726	0.645	0.597	----
12	1.000	0.857	0.773	0.726	0.685	----
Doxorubicin	1.000	0.691	0.424	0.372	0.296	2.9

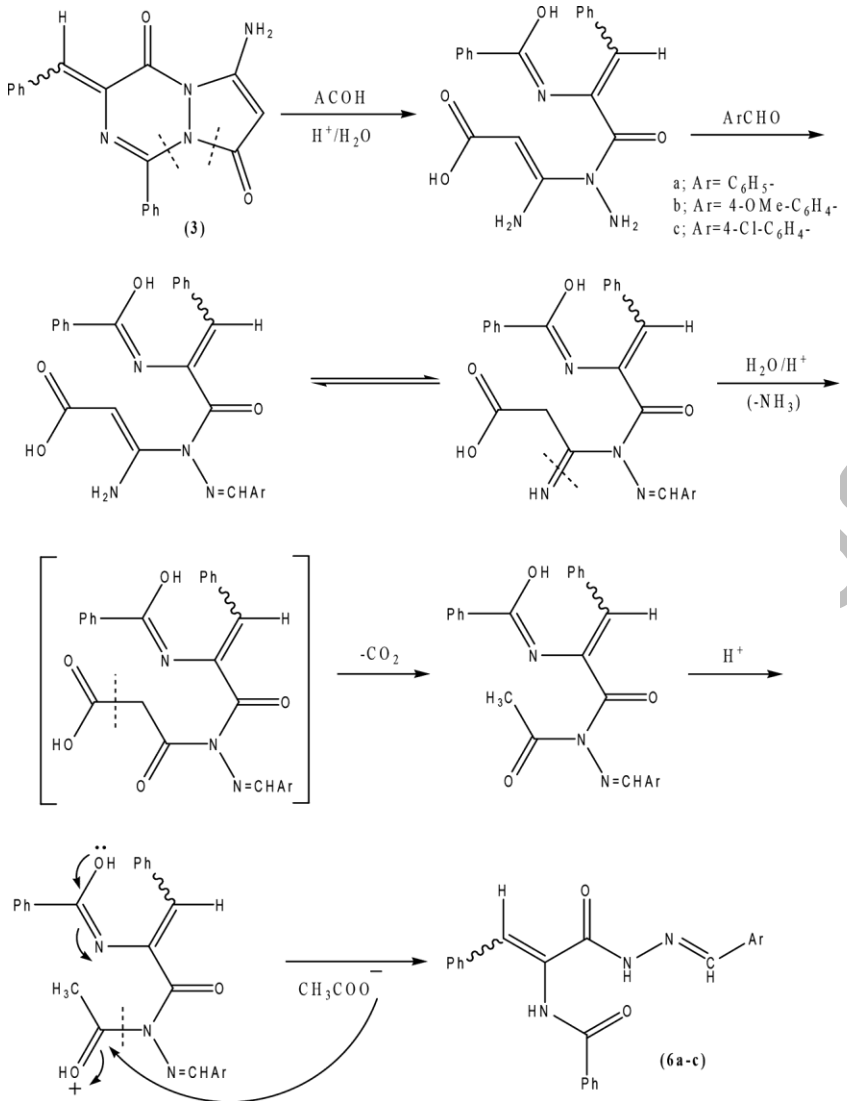
Scheme 1: reaction of **2a, b** with ethyl cyanoacetate



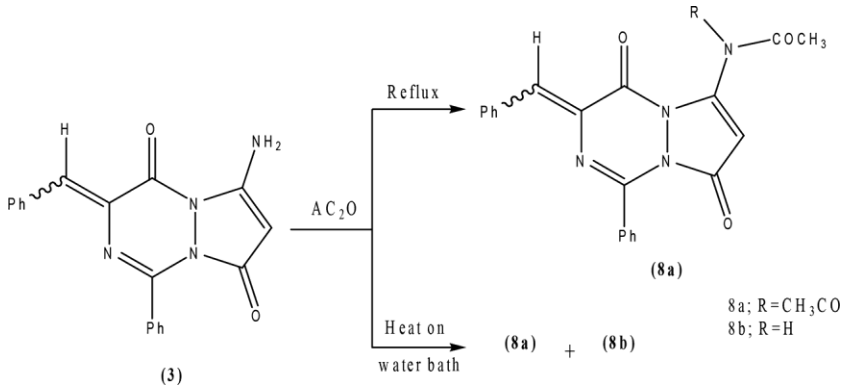
Scheme 2: Reactions of **3** with different aromatic aldehydes and istain



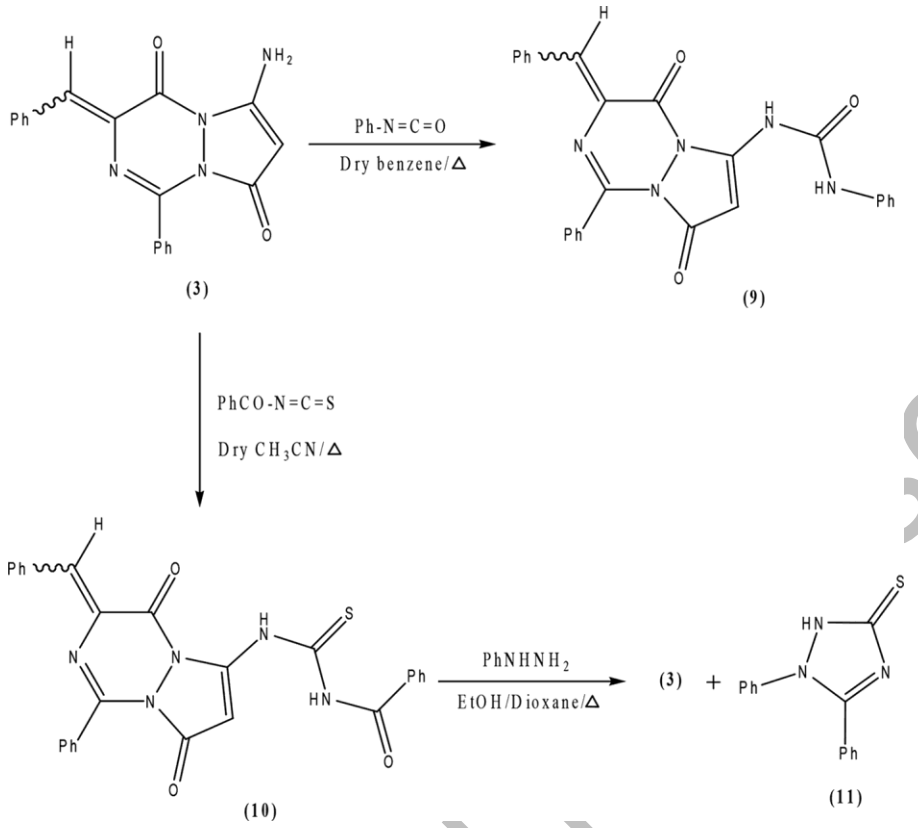
Scheme 3: Mechanistic pathway for the conversion of **3** to compounds **6a-c**



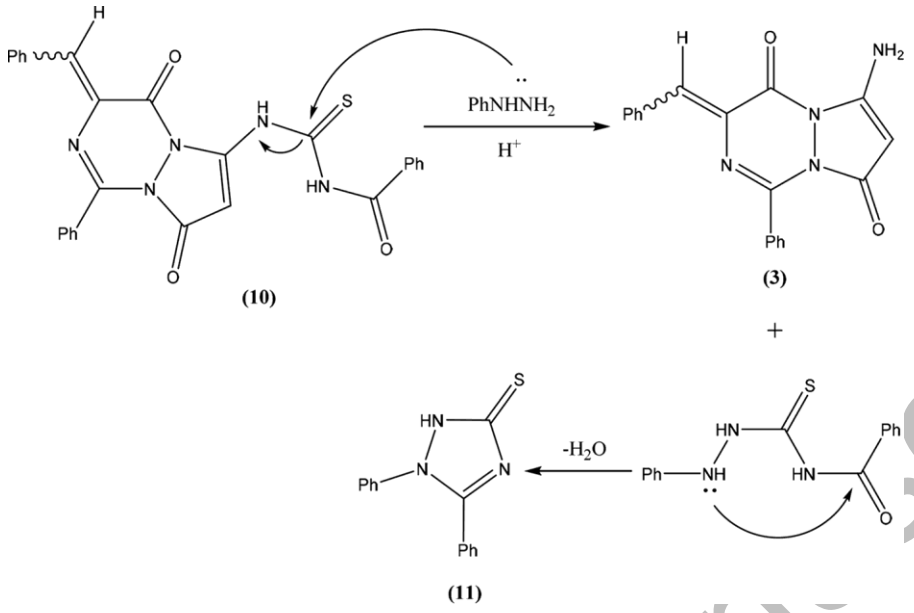
Scheme 4: Reaction of **3** with acetic anhydride under different conditions



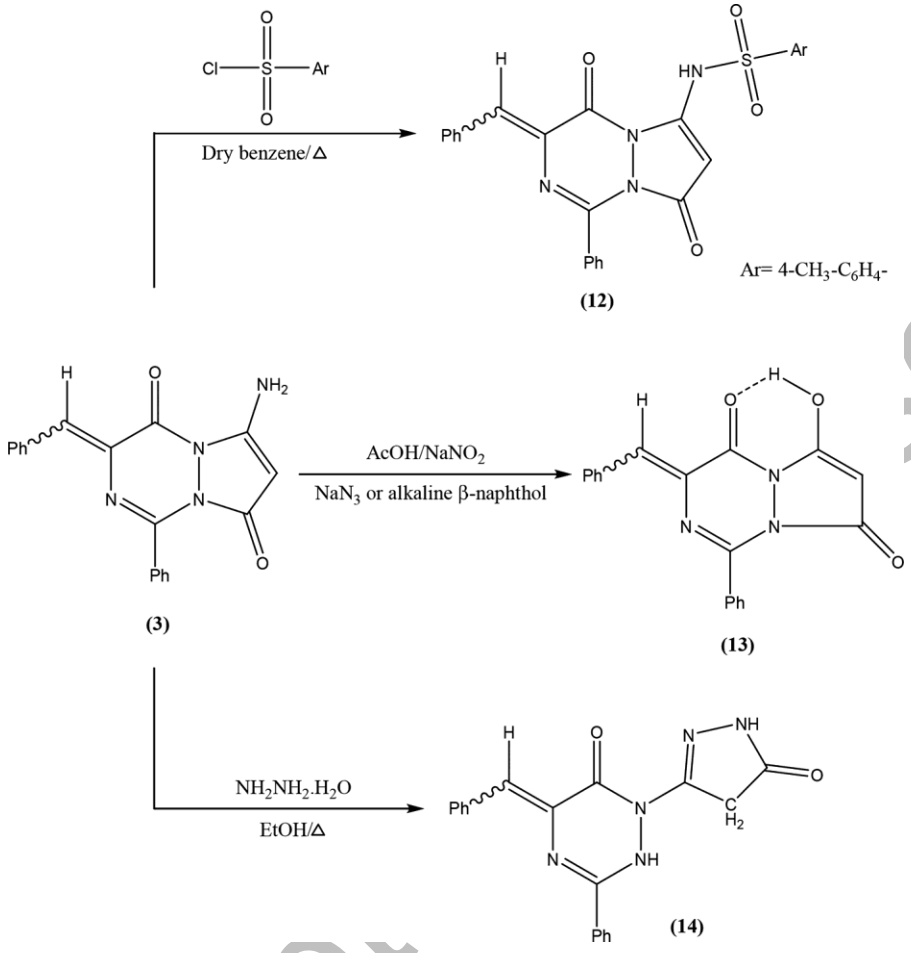
Scheme 5: Reactions of **3** with phenyl isocyanate and benzoyl isothiocyanate



Scheme 6: Mechanistic pathway for the conversion of **10** to compounds **3** and **11**



Scheme 7: Reactions of **3** with *p*-toluenesulphonyl chloride, sodium azide and/or alkaline β-naphthol and hydrazine monohydrate.



Scheme 8: Mechanistic pathway for the conversion of **3** to compound **14**