Synthesis and Biological Activity Evaluation of Differently Substituted 1,4-Dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamides and -carbothioamides

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Differently substituted phthalic anhydrides can react either with semicarbazide or thiosemicarbazide to give respectively 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamides or 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamides under mild conditions and generally with good yields. These compounds have been tested in order to evaluate their anti-microbial activity. Furthermore a new synthetic pathway to phthalazino[2,3-*b*]phthalazine-5,7,12,14-tetraone has been devised.

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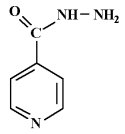
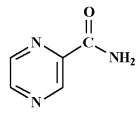
The dramatic resurgence of tuberculosis (TB), both for developed and poor countries, is becoming a public health emergency. The social and economic toll is enormous, especially in the case of immunocompromised individuals, such as those infected by HIV, those undergoing antitumor chemotherapy, and transplant recipients using antirejection therapy.

Since the discovery of streptomycin several anti-TB drugs, such as isoniazid, pyrazinamide, ethambutol, and the newer quinolones resembling derivatives [1], have been introduced in therapy.



ISONIAZID



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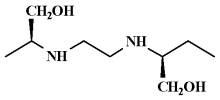
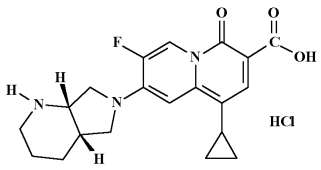


ETHAMBUTOL



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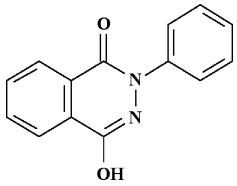
Unfortunately, together with the new molecules, the growth of resistant mutants is observed. By using a multiple drug therapy for a few months, the growth of resistant strains is highly reduced. However, in the poorer countries, patients, both for inconvenience and economic problems, interrupt the therapy as soon as they start to feel better, which happens before the TB infection is completely eradicated.

On this basis, the urgency for new and more potent molecules to fight against tuberculosis is becoming higher day by day.

It has been reported that 4-hydroxy-2-phenyl-2*H*-phthalazin-1-one and related compounds are active against *M.tuberculosis* [2,3].

Desai and coworkers devised a new method for the synthesis of 4-hydroxy-2-phenyl-2*H*-phthalazin-1-one [4].



The synthesis of related compounds, based on the 2,3-dihydro-1,4-phthalazinedione ring, starting from phthalic anhydride [5-7], 3-nitrophthalic acid [8], or phthalimide [6-9], has been reported.

Later, a general method for the synthesis of 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide or –carbothioamide was devised [10]: an equimolar mixture of phthalic anhydride and either semicarbazide or thiosemicarbazide is heated at 160 °C for 30 minutes in the presence of polyphosphoric acid (PPA). After cooling, the mixture is decomposed with ice water and crystallized from acetic acid.

Pursuing our research in the field of antibacterial compounds [11-13], we attempted to repeat this procedure several times, in order to obtain derivatives of 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide or –carbothioamide as potential antimycobacterial agents.

In spite of the fact that we carefully followed the reported conditions, we have not obtained the reported product.

Furthermore, only when thiosemicarbazide was used, an emission of H_2S, from the reaction mixture, was observed together with the obtained product which was purified as described by the authors. Although the measured melting point is similar to the reported one (mp = 310 °C dec.,

reported 307 °C), it exhibits a mass spectrum with a molecular ion at m/z 292. This ion exhibits an m/z value 71 units higher than that of the expected product.

The product was then washed in boiling methanol and then in diethyl ether and the melting point raised to 343-344 °C. In particular decomposition with reduction of the sample volume is observed starting from 312 °C.

In addition to the mass spectrum, ^{1}H-NMR does not support the proposed structure. Only aromatic protons are observed, with no evidence of exchangeable proton signals.

Based on analytical (elemental analysis) and spectroscopic data, the product which is obtained corresponds to the tetracyclic structure **1**, phthalazino[2,3-*b*]phthalazine-5,7,12,14-tetraone (Figure 1, formula **a**) and not to the expected 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide or –carbothioamide (Figure 1, formula **b**).

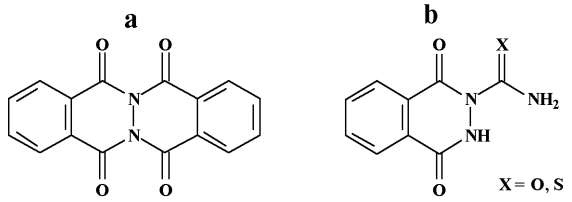


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Figure 1

A synthetic pathway to compound **1** has been previously reported [6,14].

In all the reported cases elemental analysis data support the proposed structures, but, while in case of Drew and co-workers [6] the melting point was 350-360 °C, in the case of T. J. Kealy [14] a gradual decomposition starting from 300 °C was observed, leading to a blunt melting point.

In this communication we describe a convenient and simple method to synthesize both 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide and 1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamide derivatives **2a-j**, under mild conditions and, in most cases, with good yields. The synthesized products, as well as their synthetic pathway are depicted in Scheme 1.

Differently substituted phthalic anhydrides and either semicarbazides or thiosemicarbazides are reacted for 1.5 hours in refluxing isopropyl alcohol in the presence of a catalytic amount of acetic acid.

The yields range from 60 to 75% with the exception of the nitro-derivatives, where the yields are 37% and 32% for compounds **2e** and **2j**, respectively.

All the synthesized compounds have been fully characterized by means of elemental analysis, mass spectrometry, ^{1}H-nmr and ^{13}C-nmr.

All the synthesized compounds exhibit a similar behavior, in the electron ionization obtained mass spectra: the formation of quite abundant molecular ions, with the exception of compounds **2e** and **2j**, is observed, together with two main fragmentation pathways, the first originating from the cleavage of the carbothioamide or carboxamide group which lead to the most abundant fragments in all the spectra, but for compounds **2e** and **2j**, and the second originating from a subsequent C=O loss. In the case of compounds **2e** and **2j** the most abundant ions in the spectra originate from the cleavage of the amide group, followed by the loss of two C=O fragments, leading to ions at m/z = 149.

Compounds **2a-j** have been tested in order to evaluate their anti-microbial activity against several microbial species.

In particular the antimicrobial activity of compounds **2a-j** was evaluated against five Gram positive species (*Staphilococcus aureus*, *S.epidermidis*, *Streptococcus agalactiae*, *S.faecalis* and *B.subtilis*), and Gram negative species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus mirabilis*, *Klebsiella pneumoniae*) isolated from clinical specimens. For the evaluation of the antifungal activity, *Candida albicans* ATCC E10931 was employed. The effects on the growth of mycobacteria was investigated against *M.tubercolosis* H37Rv ATCC 25548, *M.tubercolosis* resistant to isoniazid (INH-R) ATCC 35822, *M.tubercolosis* resistant to streptomycin (SM-R) ATCC 35820, *M.tubercolosis* resistant to rifampicin (RIF-R) ATCC 35838 and *M.tubercolosis* resistant to pyrazinamide (PZA-R) ATCC 35828, *M.avium* NC 08559.06, *M.phley* NC 08151.07, *M.fortuitum* NC 10394.02, *M.scrofulaceum* NC 10803.03, *M.kansasii* NC 10268.07, *M.intracellulare* NC 10425.05, *M.szulgai* NC 10831.03, *M.gordonae* NC 10267.05, *M.chelonae* subspecies *abscessus* NC 10882.02 and *M.bovis* NC 10772.02.

Unfortunately, none of the tested compounds exhibit inibition effects on the growth of both bacteria and mycobacteria, even at the highest concentrations (100μg/ml).

Nethertheless these compounds can constitute the basis for the development of newer and hopefully more active molecules.

This synthetic method is a convenient route to substituted phthalhydrazides, which can be useful intermediates in the synthesis of derivatives of pharmaceutical interest.

EXPERIMENTAL

Melting points are uncorrected and were determined on a Reichert Kofler thermopan apparatus. ^{1}H-nmr spectra were recorded on a Bruker AMX (300 MHz) using tetramethylsilane (TMS) as internal standard (chemical shifts in δ values). Electron ionisation (EI) mass spectra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature

Scheme 1

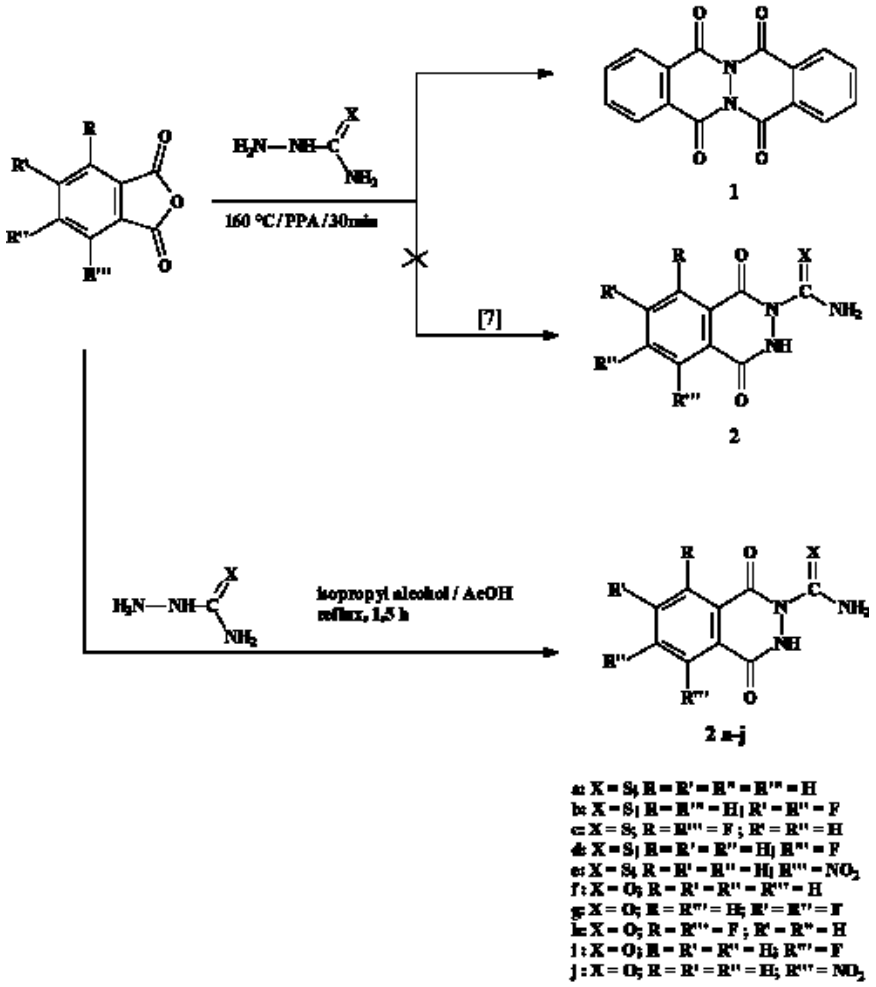

Table 1

Analytical Data of Compounds **2 a-j**

Compound	Formula	mp °C	Yield%	C%*	H%*	N%*
1,4-Dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothiomide **2a**	$C_9H_7N_3O_2S$	211-213	75	48.86 (49.06)	3.19 (3.21)	18.99 (19.07)
6,7-difluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamide **2b**	$C_9H_5F_2N_3O_2S$	215-216	69	42.03 (41.87)	1.96 (1.98)	16.34 (16.28)
5,8-difluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamide **2c**	$C_9H_5F_2N_3O_2S$	237-238	71	42.03 (42.17)	1.96 (1.99)	16.34 (16.40)
5-fluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamide **2d**	$C_9H_6FN_3O_2S$	220-222	74	45.19 (45.31)	2.53 (2.55)	17.57 (17.65)
5-nitro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carbothioamide **2e**	$C_9H_6N_4O_4S$	223-225	37	40.60 (40.49)	2.27 (2.26)	21.04 (20.98)
1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide **2f**	$C_9H_7N_3O_3$	285-287	61	52.69 (52.91)	3.44 (3.47)	20.48 (20.55)
6,7-difluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide **2g**	$C_9H_5F_2N_3O_3$	261-263	63	44.83 (45.05)	2.09 (2.11)	17.42 (17.36)
5,8-difluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide **2h**	$C_9H_5F_2N_3O_3$	278-279	62	44.83 (45.00)	2.09 (2.10)	17.42 (17.53)
5-fluoro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide **2i**	$C_9H_6FN_3O_3$	288-290	66	48.44 (48.62)	2.71 (2.69)	18.83 (18.91)
5-nitro-1,4-dioxo-3,4-dihydrophthalazine-2(1*H*)-carboxamide **2j**	$C_9H_6N_4O_5$	279-281	32	43.21 (43.46)	2.42 (2.40)	22.40 (22.36)

* Found values are in parentheses.

200 °C). The samples were introduced directly into the ion source. Elemental analyses were obtained on a Perkin-Elmer 240 B microanalyser.

Phthalazino[2,3-*b*]phthalazine-5,7,12,14-tetraone (**1**).

A mixture of phthalic anhydride (0.44 g, 3 mmol) and thiosemicarbazide (0.27 g, 3 mmol) is heated at 160 °C under vigorous stirring for 30 min. in PPA (35 g) (poly-phoshoric acid). The mixture is allowed to cool down, poured on 50 g of crushed ice, and the obtained suspension stirred for 1 h. Filtration of the suspension gave a crude whitish product, which is crystallised from acetic acid. Yield 55%, mp = 310 °C dec. The product is further purified by washing in boiling methanol and then in diethyl ether. Mp = 343-344 °C. M/z = 292; ^{1}H nmr (DMSO-d_6): δ 7.85 (4H, t, Ar, J = 7.1, 2.9); 8.00 (4H, d, Ar, J = 7.1, 2.9); ^{13}C nmr (DMSO-d_6): δ 123.8, 12 9.8, 135.3, 169.0

Anal. Calcd. for $C_{16}H_8N_2O_4$ C, 65.76; H, 2.76; N, 9.58. Found C, 65.00; H, 2.62; N, 9.43.

General Method for the Synthesis of Compounds **2 a-j**.

A mixture of appropriate phthalic anhydride (0.01 mole), thiosemicarbazide (0.01 mole), and 0.5 mL of acetic acid are reacted in refluxing isopropyl alcohol (200 mL), under vigorous stirring. After a period of 0.5 h a whitish foaming precipitate is formed and the stirring prolonged for further 0.5 h. The reaction mixture is allowed to cool down and the precipitate is collected by filtration and crystallized from ethanol, giving the required compound. With this procedure compounds **2a-j** have been synthesized. Their analytical and spectral data are listed respectively in Table 1 and Table 2.

Table 2

Spectral Data of Compounds **2 b-j**

Compound	m/z	^{1}H nmr DMSO-d_6/TMS δ. J (Hz)	^{13}C nmr DMSO-d_6/TMS δ
2a	221	7.43 (1H, d, Ar, J = 8.0); 7.58 (1H, t, Ar, J = 7.3); 7.68 (1H, t, Ar, J = 6.9); 7.93 (1H, d, Ar, J = 7.3); 8.21 (1H, s, NH, D-exch.); 9.42 (1H, s, NH, D-exch.); 10.35 (1H, s, NH, D-exch.)	128.4, 129.9, 132.6, 137.0, 168.1, 182.5.
2b	257	7.66 (1H, s, Ar,); 7.94 (1H, s, Ar,); 8.20 (1H, s, NH, D-exch.); 9.45 (1H, s, NH, D-exch.); 10.45 (1H, s, NH, D-exch.).	16.9, 134.3, 156.5, 166.2, 182.9.
2c	257	7.54 (1H, d, Ar, J = 7.7); 7.57 (1H, d, Ar, J = 8.0); 8.29 (1H, s, NH, D-exch.); 9.73 (1H, s, NH, D-exch.); 10.72 (1H, s, NH, D-exch.).	120.4, 125.1, 161.4, 166.2, 182.6.
2d	239	7.58 (1H, t, Ar, J = 7.3); 7.61 (1H, d, Ar, J = 7.3); 7.82 (1H, d, Ar, J = 7.3); 8.11 (1H, s, NH, D-exch); 9.62 (1H, s, NH, D-exch.); 10.55 (1H, s, NH, D-exch.).	120.0, 120.3, 125.1, 131.3, 131.4, 160.2, 166.2, 166.4, 183.6.
2e	266	7.84 (1H, t, Ar, J = 8.1); 8.24 (1H, d, Ar, J = 7.6); 8.26 (1H, s, NH, D-exch.); 8.31 (1H, d, Ar, J = 7.6); 9.69 (1H, s, NH, D-exch.); 10.61 (1H, s, NH, D-exch.).	128.0, 130.9, 131.2, 131.7, 135.5, 146.8, 166.2, 166.5, 186.7
2f	205	7.49 (1H, d, Ar, J = 7.9); 7.53 (1H, t, Ar, J = 7.6); 7.7 (1H, t, Ar, J = 7.9); 7.95 (1H, d, Ar, J = 7.3); 8.20 (1H, s, NH, D-exch.); 9.37 (1H, s, NH, D-exch.); 10.32 (1H, s, NH, D-exch.).	128.1, 129.3, 134.2, 136.5, 163.7, 167.9
2g	241	7.52 (1H, s, Ar,); 7.99 (1H, s, Ar,); 8.24 (1H, s, NH, D-exch.); 9.57 (1H, s, NH, D-exch.); 10.87 (1H, s, NH, D-exch.).	118.9, 135.8, 156.6, 165.4, 170.3.
2h	241	7.51 (1H, d, Ar, J = 8.0); 7.83 (1H, d, Ar, J = 7.3); 8.33 (1H, s, NH, D-exch.); 9.54 (1H, s, NH, D-exch.); 10.43 (1H, s, NH, D-exch.).	119.6, 125.5, 161.0, 165.8, 168.3.
2i	223	7.76 (1H, t, Ar, J = 7.1); 7.93 (1H, d, Ar, J = 7.3); 7.96 (1H, d, Ar, J = 7.3); 8.26 (1H, s, NH, D-exch); 9.11 (1H, s, NH, D-exch.); 10.21 (1H, s, NH, D-exch.).	19.6, 120.1, 124.8, 131.6, 132.0, 159.7, 165.9, 166.3, 168.4.
2j	250	7.82 (1H, t, Ar, J = 8.1); 8.09 (1H, s, NH, D-exch.); 8.24 (1H, d, Ar, J = 7.7); 8.32 (1H, d, Ar, J = 7.6); 9.85 (1H, s, NH, D-exch.); 10.33 (1H, s, NH, D-exch.).	124.8, 130.6, 131.5, 132.2, 134.7, 146.7, 166.1, 166.3, 169.5.

Determination of MICs.

The MICs of the compounds against Gram positive and Gram negative were determined by a standard broth macro dilution method [15,16]. Tests with Gram positive and Gram negative bacteria were carried out in Mueller Hinton broth (Difco Laboratories, Detroit, MI, USA). The compounds were diluted in the test medium to obtain final concentration ranging between 100 and 0.19 μg/ml. Tubes containing 1 ml of the diluted compounds were inoculated with 1x10^5 bacteria and were incubated at 37 °C for 18 or 24 hours. Antifungal activity, against *C.albicans* ATCC E10231, was evaluated in yeast extract peptone dextrose medium (Difco Laboratories) [17]. The determination of MIC against Mycobacteria were carried out by the two fold agar dilution method [18] in 24-multiwell plates (Nunc, Naperville, H, USA) using 7H11 agar (Difco Laboratories) containing compounds 1-7 at concentrations that ranged between 100 and 0.19 μg/ml on which 100 μl of the test bacterial suspension were spotted.

Suspensions to be used for drug susceptibility testing were prepared from 7H9 broth cultures containing 0.05% Tween 80, washed, suspended in 0.1% Tween 80-saline to yield a turbidity no 1 McFarland and then diluted in saline to obtain inocula of 3x10^5- 1.5x10^4 cells/100μl of bacterial suspension. After a 21-day (slow growers) or 7-day (rapid growers) cultivation in a CO_2 (5% CO_2-95% humidified air) incubator at 37° C (33 °C for *M.chelonae*) the growth of organisms was scored. The MIC was defined as the minimum concentration causing complete growth inhibition of organisms or allowing no more than five colonies to growth.

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