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Short communication

A duplicated nitrotienyl derivative with antimycobacterial activity: synthesis, X-ray crystallography, biological and mutagenic activity tests

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Abstract

A duplicated nitrotienyl derivative was obtained as a by-product from the synthesis of a proposed molecular hybrid of a nitrotienyl derivative and isoniazid with an expected dual antimycobacteria mechanism. The structure was shown to be the 5,5′-dinitro-2-(2,3-diaza-4-(2′-tienyl)buta-1,3-dienyl)tiophene by X-ray crystallography. The minimal inhibitory concentration (MIC) determination of this compound proved to be promising against *Mycobacterium* pathogenic strains such as *M. avium* and *M. kansasei*, although it had a high level of mutagenicity, as observed in mutagenic activity tests.

Keywords: Nitrotienyl derivative; Potential antimycobacterial agent; X-ray structure determination; Antimycobacterial activity; Mutagenicity

1. Introduction

Tuberculosis (TB) has reemerged as one of the leading causes of death in the world, reaching a million deaths annually [1]. The estimated 8.8 million new cases every year correspond to 52,000 deaths per week or more than 7000 a day, which translates into more than 1000 new cases every hour [2]. The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* and co-infections with AIDS has been responsible for this serious situation, and since resistance decreases the effectiveness of most antituberculous agents, the search for new chemotherapeutic agents against the infection is highly necessary.

Based on our previous work [3] and with the purpose of obtaining a new tuberculostatic candidate, we designed a new molecular hybrid of a nitrotienyl moiety and isoniazid, one of the drugs most used in tuberculosis [1], with a potential dual mechanism of action. However, an unexpected duplicated nitrotienyl derivative was obtained instead. This paper presents the synthesis of this compound, its crystal structure, and its antimycobacterial and mutagenic activities.

2. Chemistry

We synthesized benzoic acid-[(5-nitro-thiophen-2-yl-) methylene]-hydrazides] using Topliss' decision tree method [3] in the search for new tuberculostatic candidates. Fig. 1 shows the general synthesis of these compounds. A substituted benzydrazide is an intermediary of the synthesis of the designed nitroheterocyclic compounds. Since isoniazid (**1**) is the hydrazide of isonicotinic acid, the rational approach was to synthesize a nitrothiophilidene derivative using the drug as the intermediary. Combining these two pharmacophoric groups, a new compound with a mixed mechanism of action

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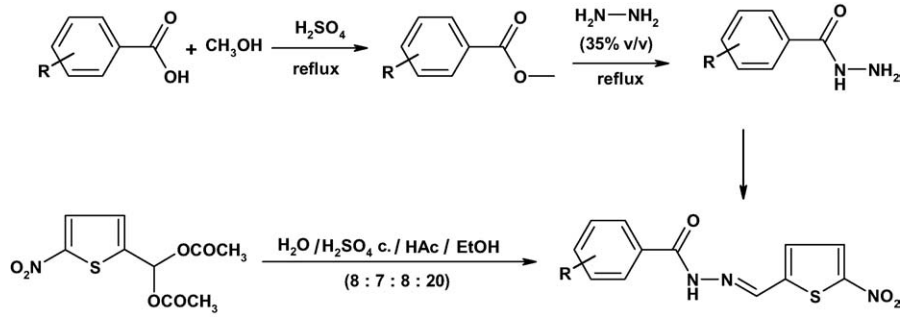

Fig. 1. General synthesis of benzoic acid-[(5-nitro-thiophen-2-yl-)methylene]-hydrazides.

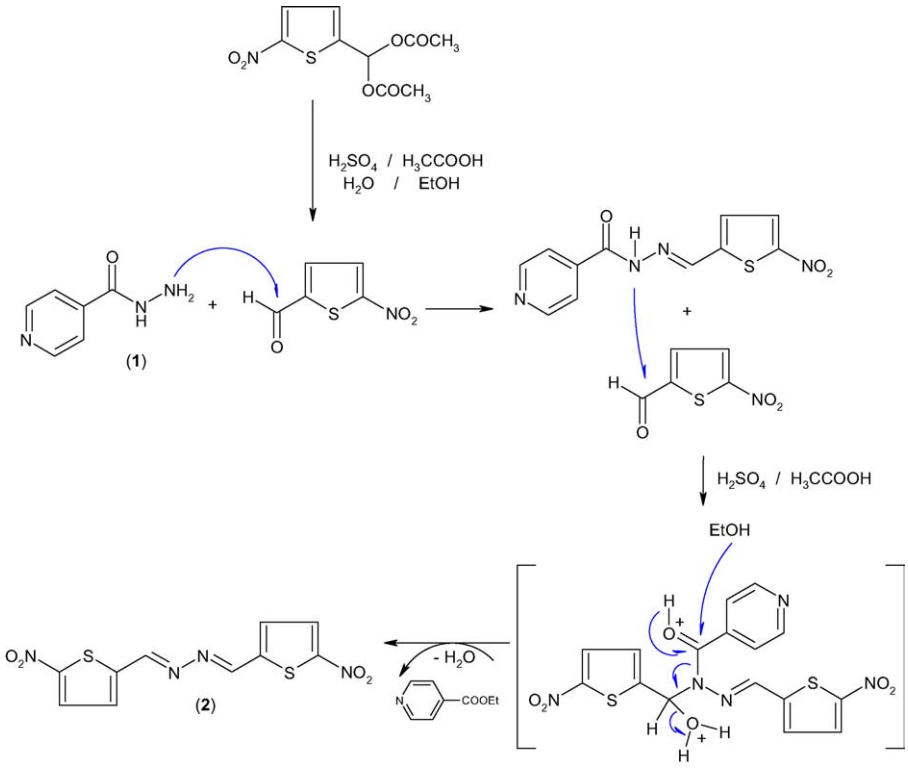

Fig. 2. Hypothesis advanced for the mechanism of 5,5′-dinitro-2-(2,3-diaza-4-(2′-tienyl)buta-1,3-dienyl)tiophene (**2**) formation.

could arise. However, a molecular duplication of the nitrothiophilidene portion, as confirmed by crystallographic studies, was obtained. A possible explanation for the formation of this compound is depicted in Fig. 2. The excess of the aldehyde formed in situ could react with the nucleophilic nitrogen of the hybrid in the acidic conditions, favoring the reaction with EtOH and the release of nicotinyl ethyl ester after rearrangement.

3. Results and discussion

The crystal structure of the 5,5′-dinitro-2-(2,3-diaza-4-(2′-tienyl)buta-1,3-dienyl)tiophene (**2**), was determined in the $Pna2_1$ space group. Fig. 3 shows its ORTEP-3 [4] view. Crystal data, data collection procedures, structure determination methods and refinement results can be found in the supplemen-

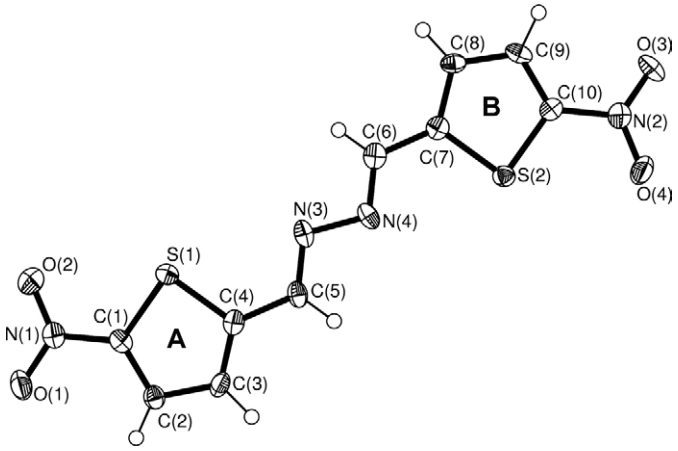

Fig. 3. ORTEP-3 (Farrugia, 1997) view of 5,5′-dinitro-2-(2,3-diaza-4-(2′-tienyl)buta-1,3-dienyl)tiophene, showing the atom and ring labeling and 50% probability ellipsoids.

Table 1
Mutagenic activity of compound **2** in *S. typhimurium* in assays with and without metabolic activation

Treatment (μg per plate)	Revertants his+/plate(M ± S.D.) in *S. typhimurium* strains (MI)			
	TA 98		YG1024	
	–S9	+S9	–S9	+S9
0	25 ± 8	49 ± 1.4	37 ± 8	79 ± 36
0.23	154 ± 17** (6.2)	56 ± 12 (1.1)	788 ± 161** (21.3)	531 ± 59* (6.7)
0.45	308 ± 53** (12.3)	95 ± 15* (1.9)	1498 ± 1 9** (40.5)	1153 ± 115** (14.6)
0.90	590 ± 14** (23.6)	172 ± 29* (3.5)	1450 ± 291* (39.2)	1329 ± 105** (16.8)
1.35	1142 ± 195** (45.7)	270 ± 6** (5.5)	394 ± 6** (10.7)	Not determined
1.80	1397 ± 5** (55.8)	375 ± 6** (7.7)	257 ± 41* (6.9)	1147 ± 86** (14.5)
Control +	770 ± 1 (30.8)	1463 ± 120 (29.9)	2045 ± 33 (55.3)	1130 ± 65 (14.3)
Potency	703	129	3255	2239

Potency = number of revertants per μg. The results are reported as means ± S.D. 0 = negative control (100 μl DMSO per plate). Control + (–S9): 4-nitro-*o*-phenylenediamine (10 μg per plate). Control + (+S9): 2-anthramine (1.25 μg per plate). MI = mutagenic index; $^{**}P < 0.01$ (ANOVA); $^{*}P < 0.001$ (ANOVA). ND = Not determined

Table 2
Mutagenic activity of nifuroxazide in *S. typhimurium* strains

Treatment (μg per plate)	Revertants his+/plate (M ± S.D.) in *S. typhimurium* strains (MI)			
	TA 98		YG1024	
	–S9	+S9	–S9	+S9
0	21 ± 5	24 ± 1.7	24 ± 6	28 ± 4.4
0.0017	159 ± 10** (7.6)	***ND***	91 ± 20* (3.8)	***ND***
0.0035	237 ± 35* (11.3)	264 ± 3.8** (11.0)	191 ± 11** (8.0)	288 ± 53.5** (10.3)
0.007	277 ± 1** (13.2)	245 ± 44.1** (10.2)	297 ± 33** (12.4)	355 ± 34.4** (12.7)
0.014	214 ± 3.5** (10.2)	201 ± 28.3** (8.4)	236 ± 24**(9.8)	305 ± 38.4**(10.9)
0.028	36 ± 0 (1.7)	25 ± 6.4 (1.0)	24 ± 3 (1.0)	0
Control+	756 ± 19 (36.0)	1048 ± 126 (43.7)	1120 ± 147 (46.7)	1514 ± 92 (54.1)
Potency	67,000	43,000	41,000	62,000

Potency = number of revertants per μg. The results are reported as means ± S.D. 0 = negative control (100 μl DMSO per plate). Control + (–S9): 4-nitro-*o*-phenylenediamine (10 μg per plate). Control + (+S9): 2-anthramine (1.25 μg per plate). MI = mutagenic index; $^{**}P < 0.01$(ANOVA); $^{*}P < 0.001$ (ANOVA).

tary material deposited at Cambridge Crystallographic Data Center (CCDC).

Submitted to in vitro tests against *Mycobacterium* sp., compound (**2**) showed a minimal inhibitory concentration (MIC) < 2.0 μg/ml (< 6.45 nmol) against *M. tuberculosis* H37Ra and H37Ry strains. The most interesting and promising finding is that this compound was shown to be highly active against *M. malmoense* (MIC = 16.0 μg/ml) and *M. avium* (MIC ≤ 8.0 μg/ml). A high activity was really expected, since the nitro group is responsible for the action against bacteria and other microorganisms and this compound has two of this group. It is also important to emphasize that the activity of this compound was higher than that observed for isoniazid in the same strains—MIC = 128 μg/ml, in *M. malmoense*, and MIC > 128.0 μg/ml, in *M. avium*.

Considering that nitro compounds are generally toxic, derivative (**2**) was also submitted to mutagenic assays (Table 1) and compared with nitrofuroxazide (**3**) (Table 2), a structure-related drug. While the presence of two nitro groups in nitro-heterocyclic rings favors this activity, it influences its interaction with DNA and its carcinogenic potential [5]. Although toxic, compound (**2**) showed to be less mutagenic than (**3**), and its biotransformation does not lead to higher toxicity.

The mutagenicity of nifuroxazide (**3**) (Table 2) was also observed by Dayan et al. [6], in a study in which the drug was described as mutagenic in *Salmonella typhimurium*, strains TA100 and TA100 Fr1. This drug also had positive results with and without metabolic activation in the SOS chromotest. The strains, TA98 and YG1024, involved in these experiments reverted the mutation for the frame shift mechanism. Therefore, derivative (**2**) could be considered to be mutagenic that induces insertion or deletion of pairs of bases of DNA, as also observed with R7000, a nitrofuran derivative studied by Quillardet et al. [7]. The reversion frequency was more accentuated for the YG1024 strain in assays without metabolic activation; this strain is derived from TA98 and it is characterized by overproduction of *O*-acetyltransferases [8]. In agreement with other studies [8–10], the cellular activities of the nitroredutases and O-acetyltransferases seem to influence the genotoxic activity of nitro compounds in *E. coli* and *S. typhimurium*. O-acetyltranferase influenced the mutagenic activity of compound **2**.

4. Conclusion

The crystal structure of the duplicated nitrotienylderivative (**3**), obtained as a by-product from the synthesis of hydrazide derived from the nitrotienyl group and isoniazid, was elucidated by single crystal X-ray diffraction analysis. This compound has shown to be promising, since it demonstrates antimycobacterial activity. The higher activity of compound (**2**) against *M. avium* and *M. maomoense* in comparison to isoniazid (**1**) is very promising, considering the high pathogenicity of these strains. Nevertheless, its mutagenicity has been demon-

strated by the Ames test. Although it is toxic, it is important to also take into account the chemotherapeutic index of this compound, i.e. the difference between toxic doses and effective doses. If this index is high enough, the compound could still be useful. Further studies are being undertaken in order to answer this question.

5. Experimental protocols

5.1. Synthesis

The 5-nitro-2-tiophilidene diacetate (10 mmol) was added to the water/concentrated sulfuric acid/glacial acetic acid/ethanol mixture (8:7:8:20). The reaction mixture was refluxed and isoniazid (10 mmol) was slowly added and maintained under stirring for 15 min. A yellow precipitate was obtained and washed with cold water. Yellow crystals were obtained from dimethylformamide. This compound was identified through RMN and mass spectrometry.

5.2. Crystallographic analysis

A yellow needle-shaped crystal of dimensions $0.01 \times 0.03 \times 0.2$ mm was used for data collection. The X-ray diffraction data were collected at low temperature (120 K) on an Enraf-Nonius Kappa-CCD diffractometer with graphite monochromated Mo Kα ($\lambda = 0.71073$ Å) radiation up to 50° in 2θ, with a redundancy of 4. The final unit cell parameters were based on all reflections. The temperature was controlled using an Oxford Cryosystem low temperature device. Data collections were made using the COLLECT program [11]; integration and scaling of the reflections were performed with the HKL Denzo-Scalepack system of programs [12]. Absorption corrections were carried out using the multi-scan method [13]. The structure was solved using direct methods with SHELXS-97 [14]. The models were refined by full-matrix least-squares procedures on F^2 using SHELXL-97 [15]. All H atoms of the structure were positioned stereochemically and were refined with fixed individual displacement parameters [$U_{iso}(H) = 1.2U_{eq}(C)$] using the SHELXL riding model. The program WINGX was used to analyze and prepare the data for publication [16]. Crystallographic data for the structure reported in this paper have been deposited at CCDC as supplementary publication no. 272189.

5.3. MIC test

A stock solution (10 mg/ml) prepared in sterilized DMSO and isoniazid was diluted in Middlebrook 7H9 broth to final concentrations of 2.0–128.0 μg/ml for the nitroderivatives and 0.06–4.0 μg/ml for isoniazid were obtained. *M. tuberculosis* $H_{37}Rv$ ATCC-25177, *M. avium* ATCC-15769 and *M. malmoense* ATCC-29571 were maintained in Lowenstein–Jensen medium. A culture suspension was prepared by subculturing in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC (BBL™) at 37 °C for 7–10 days, until a density corresponding to a McFarland standard no. 1 was obtained. This procedure yielded a suspension of actively growing culture containing 10^7 viable bacilli per ml, as confirmed by plate counts on Middlebrook 7H10 agar. The antimycobacterial activity was determined as the MIC against mycobacteria using the microplate alamar blue assay (MABA), following methodology previously described employing visual readings [17]. The visual MIC was defined as the lowest compound concentration that prevented a color change from blue to pink.

6. *Salmonella* mutagenicity test

The test was performed using the direct plate incorporation method [18] with *S. typhimurium* strains TA98 and YG1024 (derivative of TA98 that have higher levels of O-acetyltransferase), with and without metabolic activation. The S9-mix was freshly prepared before each test using an Aroclor-1254-induced rat liver fraction purchased (lyophilized) from Moltox - Molecular Toxicology Inc. *S. typhimurium* strain, TA98, was kindly provided by Dr. B. Ames, University of California, Berkeley, CA, USA and YG1024, by Dr. T. Nohmi and M. Watanabe, from National Institute of Hygienic Sciences, Tokyo, Japan. Compound **2** was tested in the following concentrations: 0.23; 0.45; 0.90; 1.35 and 1.80 μg per plate. Nifuroxazide was used in 0.0017; 0.0035; 0.007; 0.014 and 0.028 μg per plate concentrations. The assay was performed with and without metabolic activation. These doses were determined after the toxicity tests were carried out. In all subsequent assays, the upper limit of the dose range tested was either the highest non-toxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was apparent either as a reduction in the number of his+ revertants, or as an alteration in the auxotrophic background (i.e. background lawn). The positive controls were 4-nitro-*o*-phenylenediamine, in assays without S9, and 2-anthramine, in assays with metabolic activation. The statistical analysis was performed with the Salanal computer program, adopting the Bernstein et al. [19] model. The mutagenic index (MI)—the average number of revertants per plate divided by the average number of revertants per plate from the negative (solvent) control—was also calculated for each dose. A sample was considered positive when the MI was equal to or greater than 2 for at least one of the tested doses and if it had a reproducible dose–response curve [20,21].

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