Unidad de Investigación y Desarrollo de Nuevos Medicamentos[1], Facultad de Farmacia, CIFA, Universidad de Navarra, Pamplona, España, y Facultad de Farmacia y Bioquímica[2], Universidad Nacional Mayor de San Marcos, Lima, Perú

Antimycobacterial activity of new quinoxaline-2-carbonitrile and quinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives

M. A. Ortega[1], M. E. Montoya[2], A. Jaso[1], B. Zarranz[1], I. Tirapu[1], I. Aldana[1] and A. Monge[1]

The compounds being reported in this paper have all been evaluated within the TAACF Antituberculosis Screen Program, and some of them have been shown to possess high growth inhibition activity against *Mycobacterium tuberculosis* and *Mycobacterium avium* in the run of the first and second level *in vitro* screenings. The three compounds which have shown a good SI (Selectivity Index) are **2b**, **4b** and **4d**; in addition**,** 6,7-dimethyl-3-[4-(4′-nitrophenyl)piperazin-1-yl]quinoxaline-2-carbonitrilo 1,4-di-*N*-oxide (**4b**) is currently being tested within the *in vivo* antituberculosis screening in view of its very good *in vitro* activity.

1. Introduction

The development of drug-resistant strains of *Mycobacterium* species, produced by spontaneous genetic mutation and administration of a single drug, has contributed to the inefficiency of the conventional antituberculosis therapy [1, 2]. Hence, new drugs are urgently needed to improve the up-to-date antimycobacterial treatments.

With this idea, for several years our group has been investigated the synthesis and biological assessment of new antimycobacterial agents derived from the heterocycle quinoxaline, with some hopeful results. In previous papers we have demonstrated that some quinoxaline 1,4-di-*N*-oxide derivatives could be useful against tuberculosis. For example, 7-chloroquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives bearing different anilines in position 3 showed growth inhibition values of 99% [3], and 6,7-dichloro-2-ethoxycarbonyl-3-methylquinoxaline 1,4-di-*N*-oxide and 3-acetamide-6,7-dichloroquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives produced growth inhibition values of 100% [4–6]. On the other hand, we observed that the lack of the two *N*-oxide groups generally led to the loss of the antimycobacterial activity [5, 7].

As a continuation of this research, we have synthesized and evaluated several 3-[4-(4'-nitrophenyl)piperazin-1-yl]quinoxaline-2-carbonitrile derivatives and their *N*-oxides, bearing different groups in positions 6 and 7. Some chemical intermediates have also been evaluated.

2. Investigations, results and discussion

2-Quinoxalinecarbonitrile 1,4-di-*N*-oxide derivatives were prepared according to the procedures shown in the Scheme. From the adequately 5,6-disubstituted benzofuroxane **1**, 3-amino-6,7-disubstituted-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **2** were obtained, as described previously [8]. The amino group of **2** was replaced by chlorine using *tert*-butyl nitrite in dry acetonitrile, in the presence of copper(II) chloride as chlorine donor affording compounds **3** [9]. Reaction of the latter with 1-(4-nitrophenyl)piperazine was carried out in dichloromethane, in the presence of anhydrous sodium carbonate and protected from light, and the derivatives **4** were obtained. The compounds **7** were synthesized according to previously described methods [10].

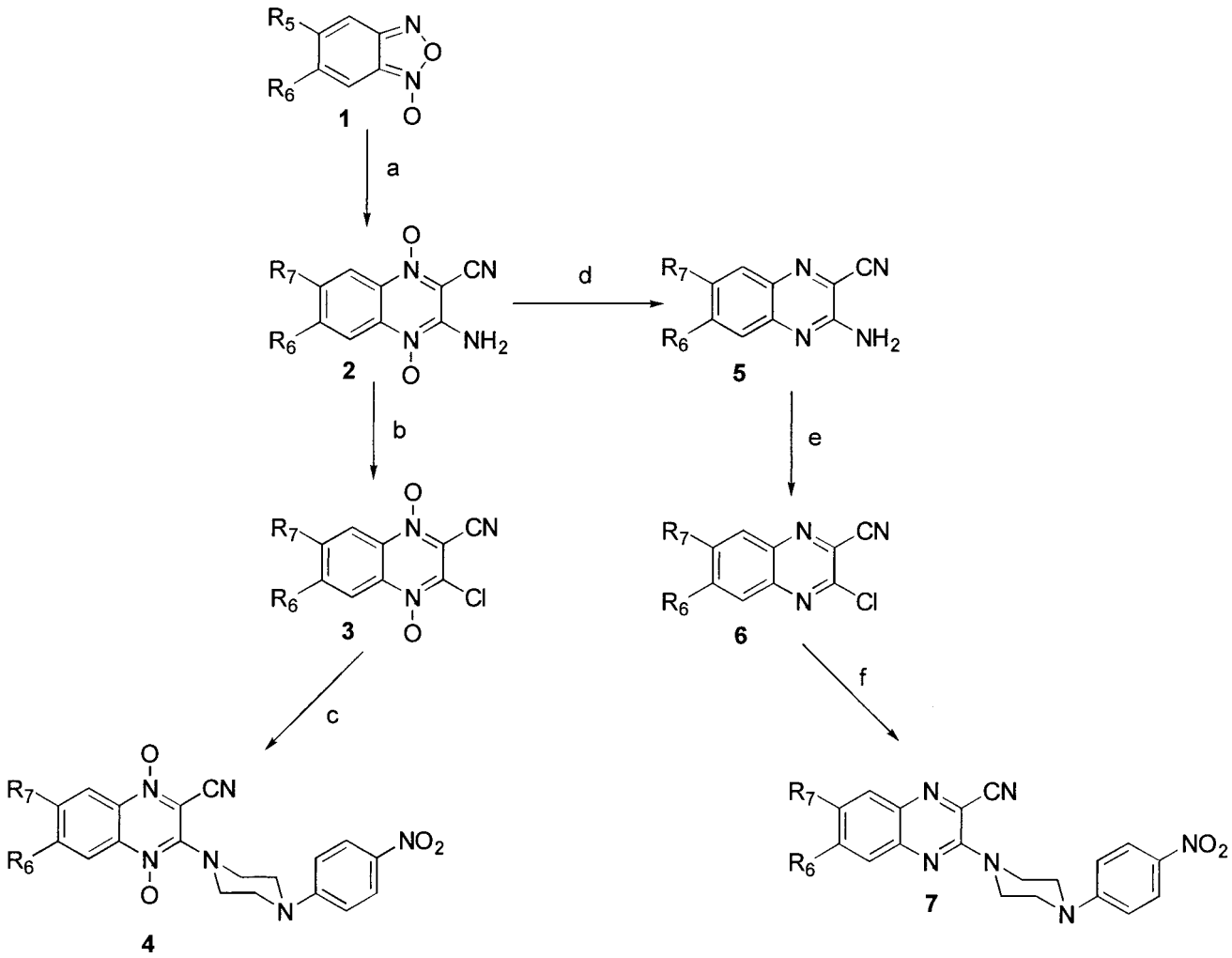
As far as biological activity is concerned, in general, compounds bearing two *N*-oxides have proven to be active (Table 1) whereas those without *N*-oxides have shown less or no activity (Table 2) in the run of the first level antituberculosis screening. This coincides with previously reported results [3, 5, 7]. Among all of the 1,4-di-*N*-oxide derivatives, compounds with a 4-(4′-nitrophenyl)piperazin-1-yl group in position 3, **4a**–**4f**, showed the best activity. The quinoxaline derivatives have shown to be notably less active with the exception of the compound **6a**. The decrease in activity is more marked for the quinoxalines substituted in position 3 by 4-(4′-nitrophenyl)piperazin-1-yl, (**7**) against the analogs 1,4-di-*N*-oxides, **4a**–**f**. Second level and cytotoxicity results are summarized in Table 3.

The MIC is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls in the Microplate Alamar Blue Assay (MABA) [11]. According to the protocols, a MIC $\leq$ 1 μg/ml in a novel compound means it is an excellent lead, so compounds **4a**–**e** should supposedly be moved further on within the biological evaluation, thus confirming what was stated before with regard to the influence of the 4-(4′-nitrophenyl)piperazin-1-yl exerting on the antituberculosis activity. Concurrent with the determination of MICs, compounds have been tested for cytotoxicity (IC_{50}) in VERO cells. Thus, the most potent compound was **6a** with a IC_{50} value of 0.9 μM. With regard to compound **2e**, calculation of the IC_{50} was impossible due to the poor compound solubility in the culture media. The selectivity index (SI), which is defined as the ratio of the measured IC_{50} in VERO cells to the MIC, was also calculated for compounds **2b**, **2f**, **4b**, **4d** and **6a**. For compounds having a SI $\geq$ 10, further advanced screenings will be carried out; compounds **2b** (> 16) and **4b** (> 125) are good candidates.

Compounds **2b**, **2e** and **6a** were also assessed against *Mycobacterium avium* and showed good activity in the run of the first level assay (GI between 95 and 100%).

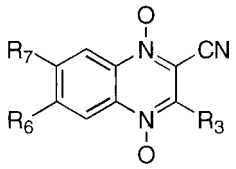
In conclusion, we can claim that together with the presence of the two *N*-oxide groups in the quinoxaline ring, the 4-(4′-nitrophenyl)piperazin-1-yl group in position 3 exerts a strong influence on the *in vitro* antituberculosis activity. In addition, the results obtained have proven these quinoxaline 1,4-di-*N*-oxides to represent a new class of molecules showing a promising preliminary activity

Scheme



a: $(CN)_2CH_2$, $(CH_3CH_2)_3N$, N,N-DMF, 0 °C; **b:** $CuCl_2$ anhydrous, $(CH_3)_3CONO$, CH_3CN, $N_2(g)$, 80–85 °C;
c: 1-(4-nitrophenyl)piperazine, Na_2CO_3, CH_2Cl_2; **d:** $Na_2S_2O_4$, CH_3OH; **e:** CH_3COOH, HCl, Na_2NO_2, 0 °C; **f:** 1-(4-nitrophenyl)piperazine, Na_2CO_3, $CHCl_3$

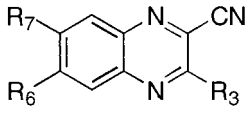
Table 1: Results of the antituberculosis first screening (Compounds 2–4)



Compd.	R_3	R_6	R_7	GI(%)*
2b	NH_2	CH_3	CH_3	97
2d	NH_2	H	OCH_3	97
2e	NH_2	OCH_3	Cl	99
2f	NH_2	H	Cl	100
3b	Cl	CH_3	CH_3	99
3c	Cl	H	C_6H_4-p-NO_2	94
3e	Cl	OCH_3	Cl	78
4a	4-(4′-nitrophenyl)piperazin-1-yl	H	H	100
4b	4-(4′-nitrophenyl)piperazin-1-yl	CH_3	CH_3	99
4c	4-(4′-nitrophenyl)piperazin-1-yl	H	C_6H_4-p-NO_2	94
4d	4-(4′-nitrophenyl)piperazin-1-yl	H	OCH_3	100
4e	4-(4’’-nitrophenyl)piperazin-1-yl	OCH_3	Cl	100
4f	4-(4′-nitrophenyl)piperazin-1-yl	H	Cl	100

* Growth Inhibition of virulent $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. According to the TAACF Program, compounds effecting <90% inhibition are considered to be inactive

Table 2: Results of the antituberculosis first screening (compunds 6, 7)



Compd.	R_3	R_6	R_7	GI(%)*
6a	Cl	H	H	94
6b	Cl	CH_3	CH_3	14
6d	Cl	H	OCH_3	22
6f	Cl	H	Cl	58
7a	4-(4′-nitrophenyl)piperazin-1-yl	H	H	0
7b	4-(4′-nitrophenyl)piperazin-1-yl	CH_3	CH_3	0
7f	4-(4′-nitrophenyl)piperazin-1-yl	H	Cl	0

Growth Inhibition of virulent $H_{37}Rv$ strain of *Mycobacterium tuberculosis*
According to the TAACF Program, compounds effecting <90% inhibition are considered to be inactive

Table 3: Results of second level and cytotoxicity antituberculosis assays

Compd.	MIC (μM)*	IC_{50} (μM)	SI (IC_{50}/MIC)**
2b	1.56	>25	>16
2d	>6.25		
2e	3.13***		
2f	6.25	5.9	0.94
3b	1.56		
4a	>0.1		
4b	>0.2	>25	>125
4c	0.78		
4d	0.78	7.3	9.4
4e	0.39		
6a	12.5	0.9	<0.07

* Minimal Inhibitory Concentration. ** Selectivity Index. *** Unable to determine IC_{50}: a precipitate was observed in the culture media

against *Mycobacterium tuberculosis* and *Mycobacterium avium*.

3. Experimental

Chemical identification of the structures was carried out as stated in a previous paper [3]. Results of elemental analyses were in an acceptable error range.

In vitro evaluation of antituberculosis activity was carried out at the GWL Hansen's Disease Center. Primary screening was conducted at 6.25 μg/ml against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using the MABA assay. Compounds exhibiting fluorescence were tested in the BACTEC 460 radiometric system. Compounds demonstrating at least 90% inhibition were retested (MABA assay) at lower concentrations in order to determine the actual MIC.

Cytotoxicity (IC_{50}) was determined in VERO cells for *M. tuberculosis* $H_{37}Rv$. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product. Compounds with a $SI \geq 10$ will have *in vitro* activity confirmed in the BACTEC 460 at 6.25 μM/ml. Compounds will then be tested for killing *M. tuberculosis* Erdman in monolayers of mouse bone marrow macrophages [12].

Further information regarding biological screening protocols is given elsewhere [11–13].

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Prof. Dr. A. Monge Vega
Centro de Investigación
en Farmacobiología Aplicada
C/ Iruñlarrea s/n
Universidad de Navarra
Pamplona, E-31080
Spain
amonge@unav.es