Novel quinoxaline 1,4-di-*N*-oxide derivatives as new potential antichagasic agents

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ABSTRACT. As a continuation of our research and with the aim of obtaining new agents against Chagas disease, an extremely neglected disease which threatens 100 million people, eighteen new quinoxaline 1,4-di-*N*-oxide derivatives have been synthesized following the Beirut reaction. The synthesis of the new derivatives was optimized through the use of a new and more efficient microwave-assisted organic synthetic method. The new derivatives showed excellent *in vitro* biological activity against *Trypanosoma cruzi*. Compound **17**, which was substituted with

fluoro groups at the 6- and 7-positions of the quinoxaline ring, was the most active and selective in the cytotoxicity assay. The electrochemical study showed that the most active compounds, which were substituted by electron-withdrawing groups, possessed a greater ease of reduction of the *N*-oxide groups.

KEYWORDS: Chagas disease; *Trypanosoma cruzi*; quinoxaline 1,4-di-*N*-oxide; mutagenicity; cytotoxicity; reduction potential.

1. INTRODUCTION

Chagas disease or American trypanosomiasis was first described by Dr. Carlos Chagas in 1909. It is a chronic parasitosis caused by the haemoflagellated protozoan, *Trypanosoma cruzi* (*T. cruzi*), which is naturally transmitted by the triatomine hematophagous insect, *Triatoma infestans*. It is a major parasitic disease in Latin America, and it is endemic in 21 countries. [1-3] According to World Health Organization (WHO) data, Chagas disease affects about 8 million people and 100 million are at risk of infection, causing 13000 deaths annually. [4,5] In addition, it has an important socioeconomic impact on the endemic countries due to the fact that the chronic phase of the disease causes incapacity in infected people and due to the burden of high medical expenses placed upon the countries' governments. Moreover, in recent years, the incidence of the disease in non-endemic countries has increased due to new migration patterns. [6-8]

Despite the fact that it has been more than 100 years since the disease has been discovered, safe and effective treatments have yet to be found. The largest decrease in the number of people affected is due to the control strategies carried out against the disease vector. [3, 9] Only two drugs have been commercialized, Nifurtimox (Nfx) and Benznidazole (Bzn), two nitroaromatic

heterocycles, empirically discovered over three decades ago. Both drugs have a number of limitations, such as low activity in the chronic phase of the disease, emergence of resistance, and toxicity. Therefore, there is an urgent need to develop new, safe and effective therapeutic alternatives that are also cost-effective. [10-13]

Multiple anti-infective activities are well known for quinoxaline derivatives. [14, 15] Our group has vast experience in the synthesis and biological evaluation of multiple quinoxaline 1,4-di-*N*-oxide derivatives, having identified a broad spectrum of anti-infective activities. [16-26] The evaluation of several libraries of compounds led us to find a series with high *in vitro* activity against *T. cruzi*. [27] This finding allowed us to establish structural features for maintaining high *in vitro* activity against the parasite (**Scheme 1**).

Scheme 1

Continuing with the optimization of the structure-activity scheme planned by our working group, eighteen new quinoxaline 1,4-di-*N*-oxide derivatives, series 1 (**1-9**) and series 2 (**10-18**) (**Table 2**), were designed and synthesized. In order to carry out the synthesis, a traditional method and a new microwave-assisted method optimized by our group were used. The use of this new synthetic method was necessary due to the limitations encountered during the traditional synthesis. Microwave-assisted organic synthesis is an up-and-coming technique in medicinal chemistry and offers a number of important advantages. [28-31] The newly synthesized derivatives were modified at position 2 of the quinoxaline ring, possessing an ester group instead of a ketone group. These compounds were then evaluated against Tulahuen 2 strain of *T. cruzi*, using Nfx as the reference drug.

In previous works, the presence of the *N*-oxides significantly potentiated the activity against *T. cruzi*, which may indicate the importance of a bioreduction or metabolization process of these groups by specific enzymes within the activity of these types of derivatives. [18, 27, 32] However, the mechanism of action through which these derivatives carry out their activity is unclear. In previous studies, an inhibition of mitochondrial dehydrogenases was demonstrated and it was also observed that benzofuroxan derivatives containing *N*-oxide cause a mitochondrial membrane depolarization in *T. cruzi*. [27, 33, 34]

In order to shed some light on this subject, a study was conducted which involved the measuring of the reduction potentials of the *N*-oxide groups in different synthesized derivatives and selected analogues. This experiment allowed us to conduct a study regarding the relationship between structure, *N*-oxides potential reduction and anti-*T. cruzi* activity. The aim of this study was to corroborate the importance of *N*-oxide reduction in biological activity and select the best substituents so as to find new leaders with higher activity and selectivity.

To evaluate the preliminary toxicity profile of the new derivatives, the cytotoxicity, the selectivity against the parasites and the mutagenicity were studied. [35]

2. RESULTS AND DISCUSSION

2.1. CHEMISTRY

Eighteen new quinoxaline 1,4-di-*N*-oxide derivatives were prepared using a variation of the Beirut reaction, in which the corresponding benzofuroxan (BFX) reacted with the appropriate trifluoroacetoacetate (INT) so as to obtain the target compounds (**Scheme 2**). In the derivatives from series 1 (**1-9**), a –CO_2CH_3 group was introduced into the 2-position of the quinoxaline ring

which was unsubstituted or substituted by different electronic substituents in the 6- and/or 7-position of the heterocycle. Chloro, fluoro and trifluoromethyl were used as electron-withdrawing groups, whereas methyl and methoxy were used as electron-donating groups.

Scheme 2

Derivatives of series 2 (**10-18**) possessed a $-CO_2CH_2CH_3$ group in the 2-position and the same variety of substituents in the 6- and/or 7-positions of the heterocycle position as in series 1. The starting compounds, 5-substituted or 5,6-disubstituted BFXs, were obtained by previously described methods. [36] Commercial reagents were also used. The new derivatives were prepared using two different synthetic methods, as shown in **Scheme 2**. In the first, a traditional synthetic method normally used by our group in which the BFX reacts with INT using acetone as solvent and sodium carbonate as base at room temperature, was carried out (**METHOD I**). This synthetic method has several important limitations. Mainly low reaction yields, long reaction times and complex purification. We attempted to optimize the synthetic method by conducting the reaction at 40-50 ºC in an oil bath. However, with an increased temperature, the occurrence of by-products also increased, making purification difficult. Consequently, a new strategy of synthesis, microwave-assisted organic synthesis (MAOS), was attempted. A variation of Beirut reaction by microwaves irradiation, in which toluene was used as solvent and triethylamine (Et_3N) as base, was performed (**METHOD II**) (**Scheme 2**).

In order to determine if the microwave-assisted method offered advantages, the synthesis of nine of the new derivatives was performed in duplicate and two strategic parameters, reaction yield and reaction time, were compared. Compounds **6-8**, **10-12** and **14-17** were synthesized using both of the proposed synthetic methods. As shown in **Chart 1**, the use of the new microwave-

assisted synthesis method increased the reaction yields. The largest increase was observed in compound **16**, where the yield increased more than 20 times, from 1% to 22%. The results suggest that derivatives with halogen substituents at positions 6 and/or 7 of the quinoxaline ring react better using the microwave method.

The increase in yield was also influenced by easier purification method of the final product. Moreover, this resulted in a reduction of the quantity of solvent needed, with economic and environmental advantages.

The new optimized microwave-assisted synthesis method also permitted significantly reduced reaction times. As can be observed in **Table 1**, the data indicate a huge decrease in reaction times when using microwave irradiation. For example, compound **12** requires seven days using the traditional method while the new microwave method reduces the time to 52 minutes, representing a decrease of almost 200 times in the required reaction time. These observed reductions in reaction times are a key advantage of the optimized microwave method compared to the traditionally used method.

Chart 1

Table 1

2.2. *IN VITRO* ANTI-*T. CRUZI* ACTIVITY

The *in vitro* activity evaluation of 18 new quinoxaline 1,4-di-*N*-oxide derivatives was carried out on the epimastigote form of *T. cruzi*. The selection of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been re-evaluated and confirmed [37-39]. The results obtained are shown in **table 2**. The strain used was Tulahuen 2. The ability of each compound

(dose 25 μM) to inhibit the growth of the parasite (PGI) was evaluated and compared to the control (no drug added to the media) on day 5. For compounds showing high values of PGI, the 50% inhibitory growth concentrations (IC_{50}), were evaluated. In both assays, Nifurtimox (Nfx) was used as the reference drug. The PGI and the IC_{50} were calculated as indicated in the Experimental Section. **Table 2** shows the PGI and IC_{50} results obtained for the 18 newly synthesized derivatives and the values for the reference drug, Nfx. The new derivatives showed potent *in vitro* activity against the Tulahuen 2 strain of *T. cruzi*: all of them presenting PGI values higher than 85%. Fourteen out of 18 compounds presented PGI values of 100% and sixteen out of 18 were more potent than the reference drug Nfx. Compound **17** was identified as the most active, showing an IC_{50} value of 0.4 µM, 18 times more active than Nfx (7.7 µM).

The results obtained permitted us to establish a series of structure-activity relationships with regard to the idealness of the substituents used in positions C-2, C-3, C-6 and C-7 of the quinoxaline ring. In addition, there is a clearly observed relationship between the electronic characteristics of substituents in the C-6 and/or C-7 of the quinoxaline ring and the *in vitro* biological activity obtained. As shown in **Table 2**, when substituents in C-6 and/or C-7 possessed high electron-withdrawing characteristics, such as those in compounds **5**, **8**, **14-18**, i.e. by chlorine, fluorine and trifluoromethyl, the biological activity increased, resulting in lower IC_{50} values. However, when substituents in C-6 and/or C-7 had electron-releasing character, such as the CH_3 group in compounds **3** and **13**, the activity decreased.

Within each series of compounds (**1-9** and **10-18**), the best results were obtained from the derivatives substituted by fluorine in C-6 and C-7 of the quinoxaline ring (compounds **8** and **17**).

In order to carry out a study on the structure-activity relationships with respect to the substituents used in C-2 and C-3, **Table 5** has been included in the supporting information. This **Table 5** shows the *in vitro* activity results of 19 quinoxaline 1,4-di-*N*-oxide derivatives, previously evaluated by our research group [27]. With respect to position C-3 of the quinoxaline ring, the introduction of the trifluoromethyl group is decisive in the *in vitro* biological activity shown by the derivatives with with-drawing substituents in C-6 and C-7; this is clearly observed upon comparing the activity of the new derivatives with the activity shown by the analogs with a methyl group or phenyl group in C-3 (A,L vs 13, E vs 1, D vs 4, O vs 11 and N vs 12, **Tables 2** and **5**). However, this influence appears not to be as great in the derivatives substituted by chlorine, and especially by fluorine, in C-6 and C-7. We observed this in the results obtained from the analog compounds J, P and R, which show very good biological activity against *T. cruzi* and in C-3 they present methyl or phenyl, respectively. These data appear to indicate that the influence of the trifluoromethyl group on the activity depends on the electronic character of the substituents used in C-6 and C-7 of the quinoxaline ring.

A similar explanation could be given for the rationale involved in the replacement of the ketone at C-2 [27] of quinoxaline ring by a methyl or an ethyl ester. The ester derivatives improve the biological activity of the corresponding ketone in every case with the exception of when the substituents in C-3, C-6 and C-7 are CF_3, F and F respectively.

Finally, with regard to the C-2 substituent, the influence of a methyl or ethyl group in determining the biological activity is not clear. Comparing the activity of the two series of new derivatives presented in our work (**1-9** vs. **10-18**), the presence of the ethyl ester group (compounds **10-18**) slightly improves the *in vitro* activity results shown by the derivatives that possess the methyl ester group (compounds **1-9**) with the exception of the derivatives without

substitutions (**1** vs. **10**) and in those that are substituted with CH_3 at C-6 and C-7 of the quinoxaline ring (**4** vs. **13**).

As our group had observed in previous works that we had carried out [27], it can be confirmed that the most decisive structural characteristic, in terms of *in vitro* biological activity, is the presence of substituents with an with-drawing character in positions 3, 6 and 7 of the quinoxaline ring, especially fluorine atoms. It is well-known that the introduction of fluorine atoms can modify the electronic, lipophilic and pharmacokinetic properties of drugs considerably. [40-42]

Table 2

2.3. CYTOTOXICITY IN MAMMALIAN CELLS

Due to the excellent *in vitro* activity data against *T. cruzi* obtained for the 18 new compounds we decided to explore their *in vitro* cytotoxicity in mammalian cells using the MTT assay. The IC_{50} values obtained and selective indexes (SI) calculated for 13 out of 18 new derivatives after 48 h of incubation are shown in **Table 3**. The selective indexes were calculated as the ratio of IC_{50} in macrophages to IC_{50} in *T. cruzi* Tulahuen 2 *strain*. Nfx data were previously reported. [43] As shown in **Table 3**, the newly synthesized compounds showed lower selectivity against the parasite than Nfx. The compounds **1** and **17** showed the highest selectivity against the parasite. In the conducted assay, cytotoxicity was determined from the function of mitochondrial dehydrogenases enzymes activity. Analogous compounds to those showed inhibitory capacity on these kinds of enzymes. [27] The significant effect observed on the cytotoxicity in the MTT assay could be related to mitochondrial dehydrogenases inhibitory activity.

Table 3

2.4. AMES TEST: MUTAGENICITY

The results obtained from the Ames test in *S. typhimurium* TA98 strain for compounds **1-18** with and without S9 activation are shown in **Table 4**. The evaluated doses of each compound were determined in previous solubility and toxicity studies on the test system. We observed that four of the eighteen tested derivatives (**6**, **9**, **15** and **18**) were not mutagenic in any of the two conditions tested, with or without S9 activation. Compounds **6** and **9** belong to **the** methyl-ester series and **15** and **18** to the ethyl-ester series. All of these four compounds have electron-withdrawing substituents at C-6- and/or C-7 in the quinoxaline ring, with compounds **6** and **15** being the 6,7-diCl derivatives and compounds **9** and **18** being the 7-CF$_3$ derivatives.

The other derivatives with electron-withdrawing substituents and the unsubstituted derivatives (compounds **5**, **7**, **8**, **10**, **12**, **13**, **14**, **16** and **17**) showed mutagenicity in the assay without metabolic activation. However, this mutagenicity disappeared when the assay was performed with metabolic activation.

Derivatives **1-4** and **11**, all possessing electron-releasing substituents (CH$_3$O, CH$_3$ and diCH$_3$) at positions 6 and/or 7, exhibited mutagenicity in both conditions tested (-S9 and +S9).

Derivatives with electron-withdrawing groups at the 6- and/or 7-positions either showed no mutagenicity or the mutagenicity would disappear after the derivatives were metabolized. On the other hand, derivatives with electron-releasing substituents in these same positions showed high mutagenicity, especially derivative **2**, with a methoxy group at C-7. These results suggest a clear relationship between the mutagenicity observed in the Ames test and the electronic character of substituents at C-6- and/or C-7 of the quinoxaline ring.

Table 4

2.5. ELECTROCHEMISTRY

The redox properties of 16 quinoxaline derivatives between -0.4 and -2.6 V (vs Ag/AgNO$_3$) were investigated using cyclic voltammetry in DMF with TBAP as supporting electrolyte. These compounds fall into two different categories: derivatives with a 3-methyl group (**A**, **B** and **C**) and derivatives with a 3-trifluoromethyl group (**3-4**, **6-12** and **14-17**). All redox potentials are reported versus the ferrocene/ferrocinium (Fc/Fc$^+$) redox couple. All voltammetric reductions were found to be diffusion controlled, as indicated by fairly constant current functions at the different scan rates used in the study. [44, 45]

The electrochemical reductions of various quinoxaline di-*N*-oxide derivatives in aprotic solvent systems have been reported previously. [46-52]. In the current study, several voltammetric waves were observed during cyclic voltammetry of the quinoxaline di-*N*-oxide derivatives between -0.4 and -2.3 V. The first reduction process observed in the voltammograms may be attributed to reduction of the nitrone functionality, forming a radical anion (**Figure 1**). [46-52]

Figure 1

The influence of structure on reduction potential may be examined by comparison of the data found in **Table 2**. Substitution of an electron withdrawing group onto the quinoxaline ring at positions C-3 or C-7 causes a positive shift in reduction potential, making reduction more facile. For example, replacing the 7-hydrogen atom in compound **1** with the 7-fluoro group in compound **7** and the 7-trifluoromethyl group in compound **9** resulted in shifts in E$_{1/2}$ of +0.102 V and +0.232 V, respectively. Likewise, replacing the 3-methyl group in compound **C** with the 3-

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trifluoromethyl group in compounds **6** and **15** resulted in a positive shift in $E_{1/2}$ of +0.290 V and +0.285 V, respectively. Other examples may be observed by comparing compounds **B** vs. **12**, and **10** vs. **14** vs. **16**. In addition, substitution of a second electron-withdrawing group onto the quinoxaline ring at C-6 enhanced these effects (cf. **1** vs. **7** vs. **8**, **10** vs. **14** vs. **15**, **10** vs. **16** vs. **17**).

Substitution of an electron donating group, i.e. methyl and methoxy, in these same positions on the quinoxaline ring causes a negative shift in reduction potential, i.e. reduction becomes more difficult. For example, replacing the 7-hydrogen atom in compound **10** with the 7-methyl group in derivative **12** and the 7-methoxy group in **11** resulted in shifts in $E_{1/2}$ of -0.043 V and -0.076 V, respectively. The results of the present study agree with previous electrochemical studies dealing with the effects of structure on the reduction potentials of quinoxaline di-*N*-oxide derivatives, [46-48, 50-52] and indicate that reduction is facilitated by a positive charge at the reaction site. [53]

The influence of the substituent at R_2, i.e. $-CO_2Me$ vs. $-CO_2Et$, on potential appeared to be negligible, in agreement with a previous study. [51]

The voltammetric results provide some pieces of information that may be important for understanding the mechanism of anti- *T. cruzi* activity for these compounds. The data suggest a possible relationship between ease of reduction and activity (**Table 2**). A plot of $E_{1/2}$ versus PGI % is shown in **Chart 2**. The chart shows that there is not a direct correlation between reduction potential and activity. However, examination of the data shows that the compounds with reduction potentials more negative than -1.3 V tend to be generally less active. In fact, of the

seven compounds with $E_{1/2}$ values lower than -1.3 V, five exhibit activities below a PGI of 100%.

The quinoxalines 1,4-di-*N*-oxide functionality could be activated through a bioreduction process which leads to the production of OH radicals or other oxy radical species and anionic active forms in the biological medium. If this is the case, bioreductive activation would generally be expected to be more facile for more easily reduced derivatives. In a previous study, we demonstrated that mitochondrial dehydrogenases are involved in the anti-*T. cruzi* activity of active quinoxaline-1,4-di-*N*-oxide-2-ketone derivatives, [27] indicating that redox behavior is important to the mechanism of action of the latter quinoxaline di-*N*-oxides. It is conceivable that a similar mechanism of action is involved with the quinoxaline 1,4-di-*N*-oxide-2-alkyl carboxylate derivatives. However, exceptions in the data, represented by derivatives **11** and **12**, indicate that factors besides bioreduction must also be considered in the mechanism of anti-*T. cruzi* action, i.e. absorption, membrane permeability, metabolism, solubility, and site binding, just to name a few examples.

Chart 2

3. CONCLUSIONS

Eighteen new 1,4-di-*N*-oxide derivatives were synthesized using a variation of the Beirut reaction. A new synthetic method was optimized by microwave-assisted synthesis, providing an increase in reaction yields and a dramatic decline in the reaction times. The new derivatives showed high biological *in vitro* activity against *T. cruzi*, sixteen of which had greater *in vitro* efficacy against Tulahuen 2 strain than that of Nifurtimox. The importance of the presence of a trifluoromethyl group at the C-3 of the quinoxaline ring in the *in vitro* biological activity was

confirmed. Introduction of electron-withdrawing substituents at C-6 and/or C-7 of the quinoxaline ring enhanced *in vitro* biological activity against *T. cruzi*. In addition, this led to the obtainment of non-mutagenic derivatives in both the Ames assays that were performed or their mutagenicity disappeared when performing the assay using metabolic activation. In general, the selectivity shown by the new derivatives against the parasite could be improved. However, due to the inhibition exerted on mitochondrial dehydrogenases by related compounds and the characteristics of the MTT trial used, this issue will be discussed in depth in future trials.

Furthermore, it was found that the presence of electron-withdrawing substituents facilitated the reduction process of the *N*-oxides: and compounds with less negative values of reduction potential generally showed greater efficacy against the parasite. These results suggested that a bioreduction process of the *N*-oxides might be taking place in the mechanism of action of these types of derivatives. Compound **17** was identified as the most active and showed the highest selectivity against the parasite. In addition, it was not mutagenic in the Ames test conducted with metabolic activation.

The information obtained will be useful for the design of future derivatives with greater biological activities against *T. cruzi* and better toxicity profiles.

4. EXPERIMENTAL SECTION

4.1. SYNTHESIS

All of the synthesized compounds were chemically characterized by melting point, thin layer chromatography (TLC), infrared spectroscopy (IR), proton nuclear magnetic resonance (^{1}H NMR) and elemental microanalyses (CHN).

Melting points were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland) and have not been corrected. The IR spectra were recorded on a Nicolet Nexus FTIR (Thermo, Madison, USA) using KBr pellets. The ^{1}H NMR spectra were recorded on a Bruker 400 Ultrashield instrument (400 MHz), using TMS as the internal standard and with $CDCl_3$ as the solvent; the chemical shifts are reported in ppm (δ) and coupling constant (J) values are given in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), dd (double doublet), ddd (doublet of double doublet), t (triplet), q (quadruplet) and m (multiplet). Elemental microanalyses were obtained on a CHN-900 Elemental Analyzer (Leco, Tres Cantos, Spain) from vacuum-dried samples. The analytical results for C, H and N, were within 0.4 of the theoretical values.

Microwave-assisted synthesis was carried out with a Discover SP System reactor (CEM Corporation), using the Synergy[TM] software.

Alugram SIL G/UV254 (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG., Düren, Germany) was used for TLC. Column chromatography was developed by Silica gel 60, with 0.040-0.063 mm particle size (Merck, Darmstadt, Alemania).

Chemicals were purchased from Panreac Química S.A. (Barcelona, Spain), Sigma-Aldrich Química, S.A. (Alcobendas, Spain), Acros Organics (Janssen Pharmaceuticalaan, Geel, Belgium) and Lancaster (Bischheim-Strasbourg, France).

4.1.1. General procedure of the synthesis of 2-alkylcarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide derivatives using K₂CO₃ (METHOD I).

The corresponding BFX (10 mmol) was dissolved in acetone (25 mL) and then cooled by placing it on ice. Next, 4,4,4-trifluoroacetoacetate (15 mmol) was added dropwise and K_2CO_3 (20 mmol) was added as the base. The reaction mixture was stirred at room temperature for 1-7 days, depending on the BFX substituents that were used. The product formation was checked out using TLC. Then the solvent was evaporated under reduced pressure. The obtained solid was dissolved in dichloromethane and washed with water in order to eliminate the catalyst salt. The organic phase was dried with anhydrous K_2SO_4 and then the solvent was evaporated under reduced pressure. The obtained oil was purified by column chromatography using dichloromethane as the eluent. The pure oil obtained was treated with diethyl ether and precipitated as a yellow solid.

4.1.2. General procedure of the synthesis of 2-alkylcarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide-derivatives using microwave-assisted method (METHOD II).

The appropriate BFX (10 mmol) was dissolved in 15 mL of toluene in a microwave vessel (35 mL). The mixture was cooled with ice. Next, 4,4,4-trifluoroacetoacetate (15 mmol) was added dropwise and finally Et$_3$N (1.5 mL) was added dropwise as the base. The mixture reaction was inserted in the microwave reactor and then subjected to an optimized method: microwave irradiation at 60 W for 40-60 minutes, depending on the BFX substituents used, keeping the temperature at 70 ºC. Once the reaction time finished, formation of the product was observed by TLC. The solvent was then eliminated under reduced pressure. Brown oil was obtained and it was purified by column chromatography, using dichloromethane as eluent. The obtained pure oil was precipitated with diethyl ether and then filtered off.

4.1.3. 2- Methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (1)

Yield: 3%. IR (KBr): 3108 (w, vC-H Ar), 1748 (s, vC=O), 1364 (s, vN-oxide), 1163 (m, vC-F).
^{1}H NMR (400 MHz, CDCl$_3$) δ ppm: 8.63-8.57 (m, 2H, H$_5$ + H$_8$); 7.97-8.04 (m, 2H, H$_6$ + H$_7$);
4.12 (s, 3H, CH$_3$). Calc. Anal. for C$_{11}$H$_7$F$_3$N$_2$O$_4$: C, 45.85%; H, 2.45%; N, 9.72%. Found: C,
45.98%; H, 2.34%; N, 9.55%.

4.1.4. 7-Methoxy-2-methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (2)

Yield: 4%. IR (KBr): 3089 (w, vC-H Ar), 1739 (s, vC=O), 1354 (s, vN-oxide), 1261 (m, vC-O),
1145 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ ppm: 8.54 (d, 1H, H$_5$, J_{5-6} = 9.3 Hz); 7.88 (d, 1H,
H$_8$, J_{8-6} = 2.3 Hz); 7.54 (dd, 1H, H$_6$, J_{6-8} = 2.6 Hz, J_{6-5} = 9.3 Hz); 4.11 (d, 3H, CH$_3$, $J_{CH3-CF3}$ = 1.5
Hz); 4.06 (s, 3H, CH$_3$O). Calc. Anal. for C$_{12}$H$_9$F$_3$N$_2$O$_5$: C, 45.28%; H, 2.83%; N, 8.80%. Found:
C, 45.18%; H, 2.90%; N, 8.86%.

4.1.5. 2-Methoxycarbonyl-7-methyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (3)

Traditional yield: 8%. IR (KBr): 3105 (w, vC-H Ar), 1735 (s, vC=O), 1355 (s, vN-oxide), 1274
(m, vC-O), 1152 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ ppm: 8.54 (d, 1H, H$_5$, J_{5-6} = 8.6 Hz);
8.40 (s, 1H, H$_8$); 7.79 (d, 1H, H$_6$, J_{6-5} = 8.6 Hz); 4.10 (s, 3H, CH$_3$); 2.67 (s, 3H, CH$_3$-C$_7$). Calc.
Anal. for C$_{12}$H$_9$F$_3$N$_2$O$_4$: C, 47.69%; H, 3.00%; N, 9.27%. Found: C, 47.49%; H, 3.07%; N,
9.13%.

4.1.6. 2-Methoxycarbonyl-6,7-dimethyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (4)

Traditional yield: 4%. IR (KBr): 3090 (w, vC-H Ar), 1756 (s, vC=O), 1343 (s, vN-oxide), 1268
(m, vC-O), 1174 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.38 (s, 1H, H$_5$); 8.35 (s, 1H,
H$_8$); 4.10 (s, 3H, CH$_3$); 2.57 (s, 6H, CH$_3$-C$_6$ + CH$_3$-C$_7$). Calc. Anal. for C$_{13}$H$_{11}$F$_3$N$_2$O$_4$: C,
49.38%; H, 3.51%; N, 8.86%. Found: C, 48.99%; H, 3.20%; N, 8.17%.

4.1.7. 7-Chloro-2-methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (5)

Yield: 22%. IR (KBr): (w, vC-H Ar), (s, vC=O), (s, vN-oxide), (m, vC-O), (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.58 (d, 1H, H$_5$, $J_{5\text{-}6}$ = 9.0 Hz); 8.58 (d, 1H, H$_8$, $J_{8\text{-}6}$ = 2.0 Hz); 7.90 (dd, 1H, H$_6$, $J_{6\text{-}5}$ = 9.3 Hz, $J_{6\text{-}8}$ = 2.0 Hz); 4.0 (d, 3H, CH$_3$, $J_{CH3\text{-}CF3}$ = 1.5 Hz). Calc. Anal. for C$_{11}$H$_6$F$_3$N$_2$O$_4$Cl: C, 40.95%; H, 1.87%; N, 8,68%. Found: C, 41.03%; H, 1.88%; N, 8.58%.

4.1.8. 6,7-Dichloro-2-methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (6)

Yield: 4%. IR (KBr): 3177 (w, vC-H Ar), 1756 (s, vC=O), 1339 (s, vN-oxide), 1278 (m, vC-O), 1169 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.74 (s, 1H, H$_5$); 8.71 (s, 1H, H$_8$); 4.10 (s, 3H, CH$_3$). Calc. Anal. for C$_{11}$H$_5$Cl$_2$F$_3$N$_2$O$_4$: C, 36.97%; H, 1.4%; N, 7.84%. Found: C, 36.66%; H, 1.38%; N, 7.72%.

4.1.9. 7-Fluoro-2-methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (7)

Yield: 25%. IR (KBr): 3077 (w, vC-H Ar), 1756 (s, vC=O), 1340 (s, vN-oxide), 1278 (m, vC-O), 1168 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.69 (dd, 1H, H$_5$, $J_{5\text{-}6}$ = 9.6 Hz, $J_{5\text{-}F}$ = 4.9 Hz); 8.28 (dd, 1H, H$_8$, $J_{8\text{-}F}$ = 8.0 Hz, $J_{8\text{-}6}$ = 2.7 Hz); 7.72 (ddd, 1H, H$_6$, $J_{6\text{-}5}$ = 9.7 Hz, $J_{6\text{-}F}$ = 7.2 Hz, $J_{6\text{-}8}$ = 2.7 Hz); 4.12 (s, 3H, CH$_3$). Calc. Anal. for C$_{11}$H$_6$F$_4$N$_2$O$_4$: C, 43.15%; H, 1.98%; N, 9.15%. Found: C, 43.18%; H, 1.84%; N, 8.85%.

4.1.10. 6,7-Difluoro-2-methoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (8)

Yield: 4%. IR (KBr): 3066 (w, vC-H Ar), 1750 (s, vC=O), 1356 (s, vN-oxide), 1252 (m, vC-O), 1187 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.47-8.39 (m, 2H, H$_5$ + H$_8$; 4.12 (d, 3H,

CH$_3$). Calc. Anal. for C$_{11}$H$_5$F$_5$N$_2$O$_4$: C, 40.70%; H, 1.55%; N, 8.64%. Found: C, 40.79%; H, 1.35%; N, 8.54%.

4.1.11. 2-Methoxycarbonyl-3,7-bis (trifluoromethyl) quinoxaline-1,4-di-*N*-oxide (9)

Yield: 2%. IR (KBr): 3108 (w, vC-H Ar), 1755 (s, vC=O), 1351 (s, vN-oxide), 1267 (m, vC-O), 1170 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.92 (d, 1H, H$_8$, J_{8-6} = 1.8 Hz); 8.80 (d, 1H, H$_5$, J_{5-6} = 9.0 Hz); 8.18 (dd, 1H, H$_6$, J_{6-5} = 9.0 Hz, J_{6-8} = 1.7 Hz); 4.12 (s, 3H, CH$_3$). Calc. Anal. for C$_{12}$H$_6$F$_6$N$_2$O$_4$: C, 40.45%; H, 1.68%; N, 7.86%. Found: C, 40.82%; H, 1.75%; N, 7.73%.

4.1.12. 2-Ethoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (10)

Yield: 1%. Microwave yield: 8%. IR (KBr): 3016 (w, vC-H Ar), 1744 (s, vC=O), 1360 (s, vN-oxide), 1264 (m, vC-O), 1154 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.66-8.62 (m, 2H, H$_5$ + H$_8$); 7.97-8.03 (m, 2H, H$_6$ + H$_7$); 4.59 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{12}$H$_9$F$_3$N$_2$O$_4$: C, 47.69%; H, 3.00%; N, 9.27%. Found: C, 47.49%; H, 3.07%; N, 9.13%.

4.1.13. 2-Ethoxycarbonyl-7-methoxy-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (11)

Yield: 1%. Microwave yield: 5%. IR (KBr): 3087 (w, vC-H Ar), 1742 (s, vC=O), 1356 (s, vN-oxide), 1263 (m, vC-O), 1143 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.54 (d, 1H, H$_5$, J_{5-6} = 9.5 Hz); 7.89 (d, 1H, H$_8$, J_{8-6} = 2.6 Hz); 7.53 (dd, 1H, H$_6$, J_{6-8} = 2.7 Hz, J_{6-5} = 9.5 Hz); 4.59 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 4.05 (s, 3H, CH$_3$O); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{13}$H$_{11}$F$_3$N$_2$O$_5$: C, 46,98%; H, 3.31%; N, 8.43%. Found: C, 46.83%; H, 3.24%; N, 8.14%.

4.1.14. 2-Ethoxycarbonyl-7-methyl-3-trifluoromethylquinoxaline-1,4-di-N-oxide (12)

Yield: 1%. Microwave yield: 4%. IR (KBr): 3096 (w, vC-H Ar), 1743 (s, vC=O), 1352 (s, vN-oxide), 1271 (m, vC-O), 1153 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.53 (d, 1H, H$_5$, J_{5-6} = 8.8 Hz); 8.41 (s, 1H, H$_8$); 7.78 (dd, 1H, H$_6$, J_{6-5} = 8.9 Hz, J_{6-8} = 1.4 Hz); 4.58 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 2.67 (s, 3H, CH$_3$); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{13}$H$_{10}$F$_3$N$_2$O$_4$: C, 49,36%; H, 3.48%; N, 8.86%. Found: C, 49.22%; H, 3.38%; N, 8.79%.

4.1.15. 2-Ethoxycarbonyl-6,7-dimethyl-3-trifluoromethylquinoxaline-1,4-di-N-oxide (13)

Yield: 1%. IR (KBr): 3068 (w, vC-H Ar), 1749 (s, vC=O), 1356 (s, vN-oxide), 1270 (m, vC-O), 1175 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δppm: 8.38 (s, 1H, H$_5$); 8.36 (s, 1H, H$_8$); 4.58 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 2.57 (s, 6H, CH$_3$-C$_6$ + CH$_3$-C$_7$); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{14}$H$_{13}$F$_3$N$_2$O$_4$: C, 50.90%; H, 3.93%; N, 8.48%. Found: C, 51.09%; H, 3.91%; N, 8.24%.

4.1.16. 7-Chloro-2-ethoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-N-oxide (14)

Yield: 8%. IR (KBr): 3071 (w, vC-H Ar), 1749 (s, vC=O), 1355 (s, vN-oxide), 1267 (m, vC-O), 1161 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.61 (d, 1H, H$_8$, J_{8-6} = 2.1 Hz); 8.59 (d, 1H, H$_5$, J_{5-6} = 9.2 Hz); 7.90 (dd, 1H, H$_6$, J_{6-5} = 9.2 Hz, J_{6-8} = 2.1 Hz); 4.59 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{12}$H$_8$ClF$_3$N$_2$O$_4$: C, 42.79%; H, 2.37%; N, 8.32%. Found: C, 42.61%; H, 2.28%; N, 8.20%.

4.1.17. 6,7-Dichloro-2-ethoxycarbonyl-3-trifluoromethylquinoxaline-1,4-di-N-oxide (15)

Yield: 3%. Microwave yield: 31%. IR (KBr): 3081 (w, vC-H Ar), 1755 (s, vC=O), 1342 (s, vN-oxide), 1269 (m, vC-O), 1171 (m, vC-F), 1022 (w, $v$ar-Cl) . ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.74 (s, 1H, H$_5$); 8.72 (s, 1H, H$_8$); 4.58 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.46 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{12}$H$_7$Cl$_2$F$_3$N$_2$O$_4$: C, 38.81%; H, 1.88%; N, 7.54%. Found: C, 38.67%; H, 1.87%; N, 7.39%.

4.1.18. 2-Ethoxycarbonyl-7-fluoro-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (16)

Yield: 1%. Microwave yield: 22%. IR (KBr): 3119 (w, vC-H Ar), 1750 (s, vC=O), 1359 (s, vN-oxide), 1267 (m, vC-O), 1171 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.69 (dd, 1H, H$_5$, J_{5-6} = 9.4 Hz, J_{5-F} = 4.8 Hz); 8.28 (dd, 1H, H$_8$, J_{8-F} = 8.0 Hz, J_{8-6} = 2.6 Hz); 7.72 (ddd, 1H, H$_6$, J_{6-F} = 7.2 Hz, J_{6-8} = 2.6 Hz); 4.59 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{12}$H$_8$F$_4$N$_2$O$_4$: C, 45.00%; H, 2.50%; N, 8.75%. Found: C, 45.00%; H, 2.41%; N, 8.63%.

4.1.19. 2-Ethoxycarbonyl-6,7-difluoro-3-trifluoromethylquinoxaline-1,4-di-*N*-oxide (17)

Yield: 10%. IR (KBr): 3048 (w, vC-H Ar), 1745 (s, vC=O), 1354 (s, vN-oxide), 1253 (m, vC-O), 1182 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.46 (dd, 1H, H5, J_{5-F6} = 6.3 Hz, J_{5-F7} = 4.3 Hz,); 8.43 (dd, 1H, H8, J_{8-F7} = 6.4 Hz, J_{8-F6} = 4.3 Hz); 4.58 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{12}$H$_7$F$_5$N$_2$O$_4$: C, 42.60%; H, 2.07%; N, 8.28%. Found: C, 42.59%; H, 1.97%; N, 8.16%.

4.1.20. 2-Ethoxycarbonyl-3,7-bis (trifluoromethyl) quinoxaline-1,4-di-*N*-oxide (18)

Yield: 2%. IR (KBr): 3112 (w, vC-H Ar), 1750 (s, vC=O), 1350 (s, vN-oxide), 1268 (m, vC-O), 1159 (m, vC-F), 1118 (m, vC-F). ^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.93 (s, 1H, H$_8$); 8.79 (d,

1H, H$_5$, J_{5-6} = 9.0 Hz); 8.16 (dd, 1H, H$_6$, J_{6-5} = 9.0 Hz, J_{6-8} = 1.4 Hz,); 4.60 (q, 2H, CH$_2$, $J_{CH2-CH3}$ = 7.1 Hz); 1.47 (t, 3H, CH$_3$, $J_{CH3-CH2}$ = 7.1 Hz). Calc. Anal. for C$_{13}$H$_8$F$_6$N$_2$O$_4$: C, 42.16%; H, 2.16%; N, 7.56%. Found: C, 42.21%; H, 1.96%; N, 7.49%.

4.2. *IN VITRO* ANTI-*T. CRUZI* TULAHUEN 2 STRAIN ASSAY

T. cruzi Tulahuen 2 *strain* epimastigotes were grown at 28 °C in an axenic medium (BHI-tryptose) as previously described, [19-21, 54-57] complemented with 5% fetal bovine serum. Epimastigotes from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture medium in order to give an initial concentration of 1×10^6 cells/mL. Cell growth was followed by measuring the absorbance of the culture at 600 nm every day. Before inoculation, the media was supplemented with a given amount of the drug from a stock solution in DMSO (25 µM). The final concentration of DMSO in the culture medium never exceeded 0.4%, and the control was run in the presence of 0.4% DMSO and in the absence of drugs.

The percentage of inhibition (PGI) was calculated as follows: PGI (%) = {1-[(Ap - A0p)/(Ac - A0c)]} $\times$ 100, where Ap = A$_{600}$ of the culture containing the drug on day 5, A0p = A$_{600}$ of the culture containing the drug just after addition of the inocula (day 0), Ac = A$_{600}$ of the culture in the absence of drugs (control) on day 5, and A0c = A$_{600}$ in the absence of the drug on day 0.

In order to determine IC$_{50}$ values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and in the presence of a range of concentrations of the corresponding drug. On day 5, the absorbance of the culture was measured and related to the control. The IC$_{50}$ value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

4.3. CYTOTOXICITY IN MAMMALIAN CELLS

J-774 murine macrophage-like cells (ATCC, USA) were maintained by passage in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, and supplemented with 10% heatinactivated fetal calf serum and 1% of antibiotics (10,000 U/mL penicillin and 10,000 lg/mL streptomycin). J-774 cells were seeded (1×10^5 cells/ well) in 96 well microplates with 200 µL of RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum. Cells were allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 ºC and, then, exposed to compounds (100.0–400.0 µM) for 48 h. Afterwards, cell viability was assessed by measuring the mitochondrial-dependent reduction of MTT (Sigma) to formazan. For that purpose, MTT was added to cells to a final concentration 0.4 mg/ mL and cells were incubated at 37 ºC for 3 h. After removing the media, formazan crystals were dissolved in DMSO (180 µL), and the absorbance at 595 nm was read using a microplate spectrophotometer. Cytotoxicity percentages (% C) were determined as follows: % C = [100- (OD_d - OD_{dm})/(OD_c - OD_{cm})] x 100, where OD_d is the mean of OD_{595} of wells with macrophages and different concentrations of the compounds; OD_{dm} is the mean of OD_{595} of wells with different compound concentrations in the medium; OD_c is the growth control and OD_{cm} is the mean of OD_{595} of wells with medium only. Results are expressed as IC_{50} (compound concentration that reduce 50% control absorbance at 595 nm). Every IC_{50} is the average of three different experiments. The selectivity indexes, SI, were expressed as the ratio between IC_{50} in macrophages and IC_{50} in *T. cruzi* (Tulahuen 2 strain). [58, 59]

4.4. MUTAGENICITY ASSAY

The preliminary *Salmonella* mutagenicity assay (Ames assay) was performed according to the method described earlier and following the OECD (Organisation for Economic Co-operation and Development) guideline 471 (Bacterial reverse mutation test) but only in *S. typhimurium* His⁻,

TA98 strain. [35, 60-62] Prior to starting the assay, the concentrations to be tested were selected in terms of solubility and toxicity results in the test system. For the mutagenicity test, a suspension of 2×10^9 S. typhimurium/mL was prepared and the treatment procedure with the different compounds was performed both with and without S9 mix as an external enzymatic metabolizing system. Rat liver S9, of commercial origin, was prepared as described earlier. [35] S9 fraction contains cytochrome P450 isoform enzymes, which are involved in drug metabolism and other enzyme activities. [63] In order to perform the assay without metabolic activation, the following was mixed in test tubes: 50 µL of each test compound solution, 500 µL of PBS (0.1 M, pH 7.4) and 100 µL of bacterial suspension. Once each compound was mixed, it was incubated for 1 hour at 37ºC and then 2 mL of molten top agar supplemented with histidine and biotin traces (0.05 mM each) were added. The mixture was vortexed immediately and then poured onto glucose minimal (GM) agar plates.

The assay with metabolic activation was performed following the same procedure but replacing the 500 µL of PBS by 500 µL of S9 mix (10%, v/v S9, 4.7 mM NADP, 6mM d-glucose-6-phosphate, 19 mM $MgCl_2$, 36 mM KCl, phosphate buffer 0.1 M, pH 7.4).

When the top agar had solidified, the plates were incubated in an inverted position in an incubator at 37ºC for 48 h. After this time had elapsed, the histidine revertant colonies were counted.

 The test always used a negative control (solvent control), in which the solvent (DMSO) was added without compound, giving us the basal spontaneous mutation rate and two positive controls, 4-O-nitrophenylendiamine (NPD) without metabolic activation and 2-aminofluorene (2-AF) with metabolic activation. In addition, a bacterial control was carried out in order to

verify correct growing, and a phenotype control was carried out in order to assure that work was actually being carried out with the TA98 strain.

For all the assays, the data were analyzed using the modified 2-fold rule in which a response was considered to be positive if the average response for at least two consecutive dose levels was more than twice the spontaneous frequencies and a dose-response relationship is observed. [64]

4.5. ELECTROCHEMICAL STUDIES

Cyclic voltammetric measurements were performed with a CHI Instruments 630 Voltammetric Analyzer using scan rates ranging from 50 mV/s to 1000 mV/s. Solution resistance was uncompensated. The electrochemical cell consisted of a Pt-disk (1.6 mm diameter) working electrode, a Pt-wire auxilliary electrode, and a $Ag/AgNO_3$ (0.1 M in acetonitrile) reference electrode. All test solutions contained 1 mM of the particular quinoxaline compound and 0.10 M tetrabutylammonium perchlorate (TBAP) as supporting electrolyte. Deoxygenation of test solutions was carried out by passing a gentle and constant stream of prepurified dinitrogen through the solution for 15 minutes. The quinoxaline compound was added to the solution during the deoxygenation and allowed to dissolve. A blanket of dinitrogen was maintained over the solution during all experiments. Half-wave potentials and the difference in peak potentials were calculated using the following equations, [44] respectively: $E_{1/2} = (E_{pa} + E_{pc})/2$ and $\Delta E_p = E_{pa} - E_{pc}$. Ferrocene (Fc) was used as an internal reference redox system to account for daily variations in the reference electrode and liquid junction potentials. [65] Fc (1 mM) was added to each solution after the voltammetric measurements of the test quinoxaline compound were complete. [66] All potentials are reported versus the ferrocene/ferrocinium (Fc/Fc^+) redox couple, i.e. $E_{pc, SRE} - E_{1/2, Fc/Fc}+$ or $E_{1/2, SRE} - E_{1/2, Fc/Fc}+$. Half-wave potentials ($E_{1/2}$) for ferrocene varied from

0.0050 V to 0.022 V during the course of this study. Dimethylformamide (DMF) (Fisher Scientific) was used as the solvent. TBAP and ferrocene were obtained from Aldrich Chemical Company. All reagents were obtained in the highest purity available and used without further purification.

Supporting information available: Cyclic voltammetry data obtained for the quinoxaline 1,4-di-*N*-oxide derivatives studied derivatives.

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ABBREVIATIONS

T. cruzi, *Trypanosoma cruzi*; Nfx, Nifurtimox; Bnz, benznidazole; BFX, Benzofuroxan; PGI, percentage of growth inhibition; IC_{50}, concentration for 50% growth inhibition; SI, selectivity index; GM, glucose minimal; 4-NPD, 4-nitro-o-phenylendiamine; AF, 2-aminofluorene; NR, number of revertants; TBAP, tetrabutylammonium perchlorate; Fc, ferrocene;

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Figure legends

Figure 1. One electron reduction of quinoxaline 1,4-di-*N*-oxides to form a radical anion.

Scheme 1. Structure-activity relationships observed and used for the design of novel derivatives.

Scheme 2. Synthetic procedures used to prepare compounds **1-18**: (**I**) K_2CO_3, acetone, room temperature. (**II**) Et_3N, toluene, microwave assisted method (60 W, 40-60 min, 70ºC).

Chart 1. Comparison of reaction yields, after purification, obtained by synthetic methods I and II.

Chart 2. Plot of reduction potentials ($E_{1/2}$ vs. Fc/Fc^+) for the first voltammetric wave of quinoxaline 1,4-di-*N*-oxide-2-alkyl carboxylates against the percentage of growth inhibition (PGI%).

Table 1. Comparison of reaction times obtained by synthetic methods I and II.

Table 2. Structure, anti-*T. cruzi* activity and cyclic voltammetry data of quinoxaline derivatives.

Table 3. Biological characterization of new Quinoxaline 1,4-di-*N*-oxide against Mammal Macrophages

Table 4. Revertants in *Salmonella typhimurium* TA98 strain treated with different doses of compounds **1**–**18** for the two different treatment procedures (-S9 and +S9).

Figure 1

Scheme 1

Scheme 2

(1-18)
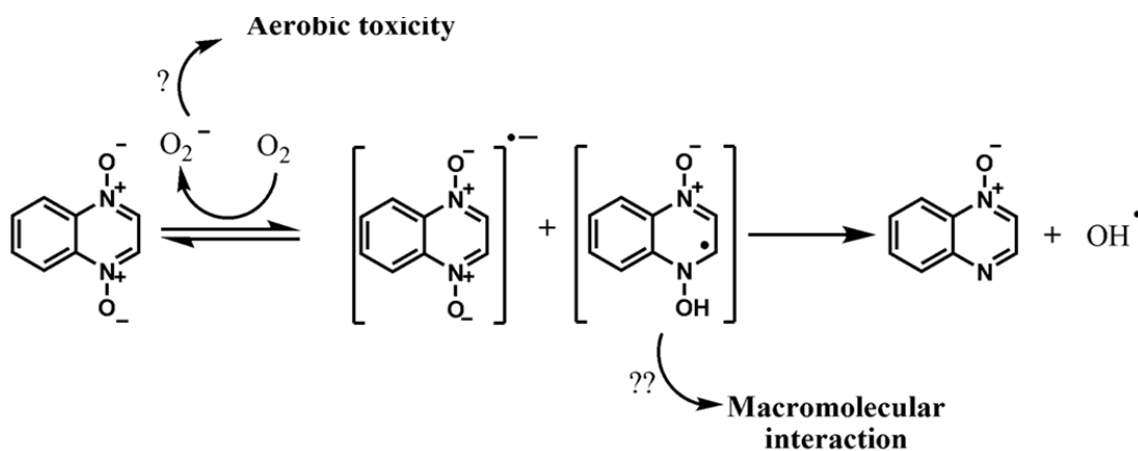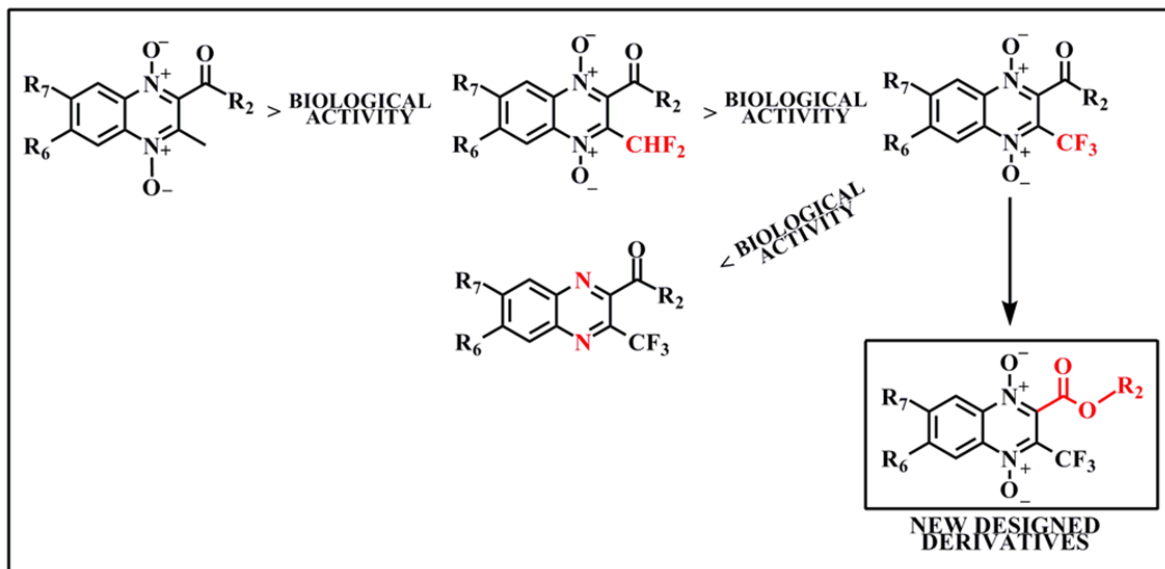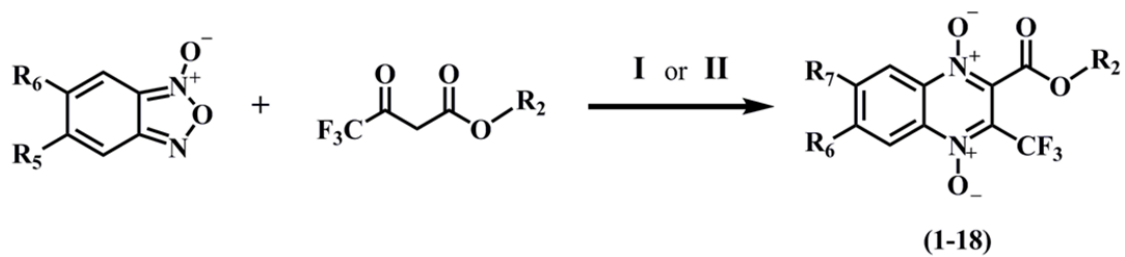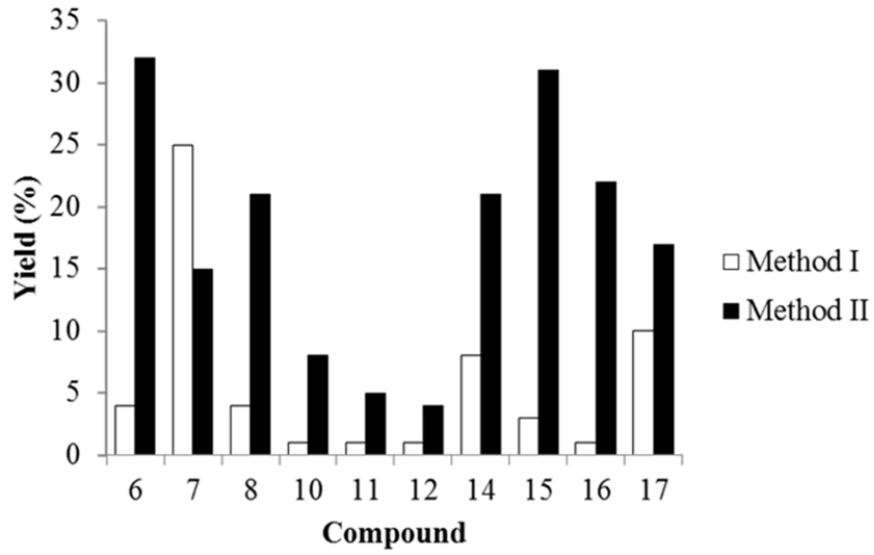
Chart 1

Chart 2
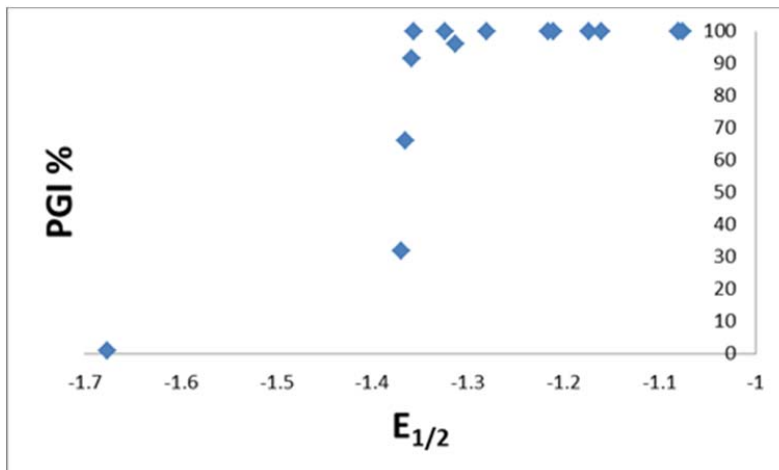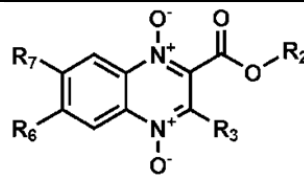
Table 1

	REACTION TIMES	
COMP.	**METHOD I**	**METHOD II**
6	1 day	50 min
7	2 day	50 min
8	1 day	60 min
10	3 day	50 min
11	4 day	70 min
12	7 day	52 min
14	1 day	65 min
15	1 day	65 min
16	15 h	50 min
17	1 day	50 min

Table 2

Code	-R$_2$	-R$_3$	-R$_6$	-R$_7$	Anti-*T. cruzi* activity PGI[a] (%)	IC$_{50}$ (μM)[b]	Cyclic voltammetry E$_{1/2}$ (V)
A[d]	-CH$_2$CH$_3$	-CH$_3$	-CH$_3$	-CH$_3$	1	-	-1.677
B[d]	-CH$_2$CH$_3$	-CH$_3$	-Cl	-Cl	32	-	-1.370
C[d]	-CH$_3$	-CH$_3$	-Cl	-Cl	66	-	-1.366
1	-CH$_3$	-CF$_3$	-H	-H	100.0	2.6	-1.313
2	-CH$_3$	-CF$_3$	-H	-CH$_3$O	86.6	4.6	N. D.[c]
3	-CH$_3$	-CF$_3$	-H	-CH$_3$	96.1	13.7	N. D.[c]
4	-CH$_3$	-CF$_3$	-CH$_3$	-CH$_3$	91.6	5.0	-1.359
5	-CH$_3$	-CF$_3$	-H	-Cl	100.0	1.8	N. D.[c]
6	-CH$_3$	-CF$_3$	-Cl	-Cl	100.0	10.8	-1.076
7	-CH$_3$	-CF$_3$	-H	-F	100.0	3.5	-1.211
8	-CH$_3$	-CF$_3$	-F	-F	100.0	1.5	-1.160
9	-CH$_3$	-CF$_3$	-H	-CF$_3$	100.0	5.0	-1.081
10	-CH$_2$CH$_3$	-CF$_3$	-H	-H	100.0	4.0	-1.281
11	-CH$_2$CH$_3$	-CF$_3$	-H	-CH$_3$O	100.0	4.8	-1.357
12	-CH$_2$CH$_3$	-CF$_3$	-H	-CH$_3$	100.0	4.8	-1.324
13	-CH$_2$CH$_3$	-CF$_3$	-CH$_3$	-CH$_3$	87.5	7.6	N. D.[c]
14	-CH$_2$CH$_3$	-CF$_3$	-H	-Cl	100.0	1.8	-1.174
15	-CH$_2$CH$_3$	-CF$_3$	-Cl	-Cl	100.0	2.9	-1.081

16	-CH$_2$CH$_3$	-CF$_3$	-H	-F	100.0	3.3	-1.216
17	-CH$_2$CH$_3$	-CF$_3$	-F	-F	100.0	0.4	-1.161
18	-CH$_2$CH$_3$	-CF$_3$	-H	-CF$_3$	100.0	1.0	N .D.[c]
Nifurtimox					100.0	7.7	
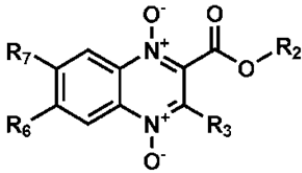
[a]Percentage of growth inhibition. Inhibition of epimastigote growth of Tulahuen 2 strain, dose = 25 µM. The results are the mean of three independent experiments with a SD less than 10% in all cases.

[b]IC$_{50}$: concentration (in µM) that inhibits 50% of epimastigote form of *T. cruzi* growth. The results are the mean of three independent experiments with a SD less than 10% in all cases.

[c]Not determined.

[d]Compounds previously published (*Journal of medicinal chemistry* **2011,** *54* (10), 3624-36).

Table 3

Code	-R$_2$	-R$_3$	-R$_6$	-R$_7$	Cytotoxicity IC$_{50}$ (μM)[a]	SI[b]
1	-CH$_3$	-CF$_3$	-H	-H	19.0	7.3
3	-CH$_3$	-CF$_3$	-H	-CH$_3$	4.0	0.3
4	-CH$_3$	-CF$_3$	-CH$_3$	-CH$_3$	4.0	0.8
6	-CH$_3$	-CF$_3$	-Cl	-Cl	0.5	< 0.1
7	-CH$_3$	-CF$_3$	-H	-F	2.5	0.7
9	-CH$_3$	-CF$_3$	-H	-CF$_3$	4.0	0.8
10	-CH$_2$CH$_3$	-CF$_3$	-H	-H	4.0	1.0
11	-CH$_2$CH$_3$	-CF$_3$	-H	-CH$_3$O	6.0	1.2
12	-CH$_2$CH$_3$	-CF$_3$	-H	-CH$_3$	7.0	1.4
13	-CH$_2$CH$_3$	-CF$_3$	-CH$_3$	-CH$_3$	4.0	0.5
16	-CH$_2$CH$_3$	-CF$_3$	-H	-F	0.5	0.1
17	-CH$_2$CH$_3$	-CF$_3$	-F	-F	4.0	10
18	-CH$_2$CH$_3$	-CF$_3$	-H	-CF$_3$	4.2	4.2
Nifurtimox[c]					316.0	41.0

[a]IC$_{50}$: concentration (in μM) that inhibits 50% of J-774 murine macrophage-like cells growth after 48 h of incubation. The results are the mean of three independent experiments with a SD less than 10% in all cases.

[b]Selectivity index :expressed as the ratio of IC$_{50}$ in macrophages to IC$_{50}$ in *T. cruzi* Tulahuen 2 strain.

[c]Data from *Bioorganic & medicinal chemistry* **2007,** *15* (7), 2768-81.

Table 4

| | | N. R.[b] | | | | N. R.[b] | | | | | |
Comp	D^a	-S9	+S9	Comp	D^a	-S9	+S9	Comp	D^a	-S9	+S9
1	0.0	27±7	28±7	**7**	0.0	27±7	28±7	**13**	0.0	29±1	30±7
	0.06	17±4	17±3		0.3	15±7	22±7		0.06	25±4	32±3
	0.18	25±5	25±5		0.92	20±4	22±9		0.18	40±14	37±9
(+)	0.5	33±8	30±9	(+)*	2.7	39±15	27±7	(+)*	0.5	57±2	45±5
	1.6	118±11	45±2		8.3	115±5	27±4		1.6	174±12	38±1
	5	227±79	84±15		25	158±28	41±11		12.5	352±11	71±10
2	0.0	29±1	28±4	**8**	0.0	29±1	28±4	**14**	0.0	13±2	30±7
	0.3	29±4	31±4		0.06	30±9	31±3		0.06	14±4	32±3
	0.92	47±8	41±2		0.18	33±2	34±9		0.18	11±3	37±9
(+)	2.7	70±6	66±6	(+)*	0.5	45±1	30±5	(+)*	0.5	20±1	45±5
	8.3	285±10	245±15		1.6	118±17	29±7		1.6	31±4	38±1
	25	379±31	680±17		5	247±34	33±3		5	55±11	71±10
3	0.0	13±2	19±3	**9**	0.0	27±7	28±8	**15**	0.0	29±1	29±1
	0.06	15±1	15±4		0.06	25±4	25±2		0.06	29±1	30±7
	0.18	21±2	13±1		0.18	23±3	26±11		0.18	27±5	31±13
(+)	0.5	26±11	16±1	(-)	0.5	24±2	25±9	(-)	0.5	29±1	27±3
	1.6	79±9	29±5		1.6	24±5	20±6		1.6	32±4	25±4
	5	136±24	100±8		5	25±1	41±15		5	37±1	34±7
4	0.0	13±2	19±3	**10**	0.0	29±1	28±4	**16**	0.0	27±7	28±8
	0.61	10±1	18±2		0.06	32±1	31±7		0.06	16±1	24±1
	1.85	18±12	19±4		0.18	31±12	36±4		0.18	24±7	23±5
(+)	5.5	22±6	16±1	(+)*	0.5	45±4	29±5	(+)*	0.5	42±2	28±5
	16.6	53±8	74±11		1.6	109±8	27±2		1.6	80±9	30±5
	50	73±12	260±23		5	237±6	44±5		5	152±43	25±5

5	0.0	27±7	28±8	11	0.0	27±7	28±8	17	0.0	13±2	19±3
	1.2 x10⁻³	19±1	22±2		0.3	15±1	24±3		0.06	13±3	17±2
	3.7 x10⁻³	21±5	20±1		0.92	25±2	25±10		0.18	10±5	17±2
(+)*	0.011	20±1	24±3	(+)	2.7	40±9	30±5	(+)*	0.5	16±7	21±1
	0.033	40±8	24±4		8.3	133±20	36±1		1.6	33±2	18±3
	5	109±17	33±4		25	188±51	169±24		5	64±6	21±4
6	0.0	13±2	19±3	12	0.0	29±1	28±4	18	0.0	29±1	30±7
	0.012	17±7	17±1		0.3	33±12	38±7		0.06	25±3	39±2
	0.037	27±1	22±9		0.92	56±5	36±4		0.18	33±5	35±3
(-)	0.11	14±7	29±14	(+)*	2.7	90±1	41±3	(-)	0.5	31±8	32±7
	0.33	26±2	17±4		8.3	287±28	42±6		1.6	39±10	37±4
	1	39±11	19±4		25	352±3	71±18		12.5	39±7	32±7
Nfx[c]	0.0	21±4	31±10								
	0.5	29±6	37±5								
	1.0	43±17	39±18								
(+)	3.0	62±2	53±9								
	10.0	144±11	64±6								
	30.0	117±17	139±11								

[a]D: Doses in µg/plate. [b]N.R.: Number of revertants. [c]Data from *Toxicology letters* **2009,** *190* (2), 140-9.

(+): Mutagenic. (-): Not mutagenic. *Mutagenic without metabolic activation and not mutagenic with metabolic activation.

Table of Contents Graphic

Novel quinoxaline 1,4-di-*N*-oxide derivatives as new potential antichagasic agents

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Eighteen new quinoxaline 1,4-di-*N*-oxide derivatives have been synthesized and they have shown excellent *in vitro* biological activity against *Trypanosoma cruzi*, identifying one of the compounds as the most active and selective in the cytotoxicity assay.

PGI (25 μM) = 100.0 %
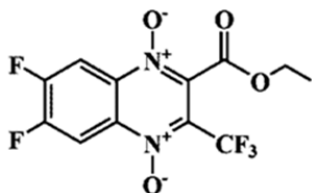
IC$_{50}$ = 0.4 μM

SI = 10

PGI: % Inhibition of epimastigote growth of Tulahuen 2 strain. IC$_{50}$: concentration that inhibits 50% of epimastigote form of *T. cruzi* growth. SI: Selectivity Index.