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SYNTHESIS AND *IN VITRO* INVESTIGATION OF HALOGENATED 1,3-BIS(4-NITROPHENYL)TRIAZENIDE SALTS AS ANTITUBERCULAR COMPOUNDS

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Keywords: TB, *Mycobacterium tuberculosis*, triazenide salts, antibiotic, cytotoxicity, genotoxicity.

Abstract

The diverse pharmacological properties of the diaryltriazenes have sparked the interest to investigate their potential to be repurposed as antitubercular drug candidates. In an attempt to improve the antitubercular activity of a previously constructed diaryltriazene library, eight new halogenated nitroaromatic triazenides were synthesized and underwent biological evaluation. The potency of the series was confirmed against the *Mycobacterium tuberculosis* lab strain H37Ra and for the most potent derivative we observed a minimal inhibitory concentration of 0.85 µM. The potency of the triazenide derivatives against *Mycobacterium tuberculosis* was found to be highly dependent on the nature of the halogenated phenyl substituent and less dependent on cationic species used for the preparation of the salts. Although the inhibitory concentration against J774A.1 macrophages was observed at 3.08 µM, the cellular toxicity was not mediated by the generation of nitroxide intermediate as confirmed by electron paramagnetic resonance spectroscopy, whereas no *in vitro* mutagenicity could be observed for the new halogenated nitroaromatic triazenides when a trifluoromethyl substituent was present on both the aryl moieties.

1. Introduction

The global incidence of TB, caused by the weakly gram-positive bacteria, *Mycobacterium tuberculosis (M. tuberculosis),* is alarmingly high. Over 10.4 million persons developed active TB in 2015 and over 1.8 million people died because of the disease[1]. Consequently, TB is on the list of the leading ten causes of death worldwide and is now the most lethal disease caused by a single infectious agent, surpassing even the HIV/AIDS pandemic. The conventional chemotherapeutic treatment of TB includes the daily administration of four drugs: rifampicin (RIF), isoniazid (INH), ethambutol (ETH) and pyrazinamide (PZA) which needs to be maintained for six to nine months. Because the recommended TB treatment is very demanding on the patient, noncompliance has contributed to the emergence of strains of drug resistant *M. tuberculosis*. Multi drug-resistant (MDR) TB with resistance to at least two of the most powerful first-line anti-TB medications INH and RIF can still be treated with second-line drugs such as the fluoroquinolones and injectable aminoglycoside or polypeptide drugs. However, when classified as extensively drug-resistant tuberculosis (XDR-TB), the MDR *M. tuberculosis* strain has acquired additional resistance to one or more of the second-line drugs[2]. Therefore, there is an urgent demand for new chemotherapeutics for the treatment of tuberculosis (TB). One strategy to identify, new antitubercular drug candidates that has gained popularity in the past decade is the repurposing of existing pharmacophores. A major advantage of retooling existing pharmacophores is the *a priori* knowledge of their toxicological and pharmacological properties *in vivo* while the possibility to tailor the compound to the specific disease is still maintained[3]. Previously, structural derivatives of Berenil® were reported as potent growth inhibitors of *M. tuberculosis*[4]. Berenil® or diminazene aceturate (**Figure 1a**) been widely used as a chemotherapeutic agent for trypanosomiasis in livestock since 1955. In addition to direct antitrypanocidal activity, diminazene aceturate showed the ability to modulate the host cellular and inflammatory responses to *Trypanosoma congolense* infection[5]. The antiprotozoal activity of diminazene is based on both intercalation as well as minor groove binding to DNA and RNA duplexes[6–8]. However, by replacing the amidine moieties of diminazene with alternative electron-withdrawing groups, such as nitro substituents, the DNA binding properties are abrogated[9]. Halogenation of the 1,3-diaryltriazene scaffold was reported to increase both the specificity and activity against mycobacterial species. For the most potent derivative 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene a minimal inhibitory concentration (MIC) of 0.54 µM against *M. tuberculosis* H37Rv was observed[4]. In addition, Vajs *et al*. reported on the nitroaromatic triazenes as efficient antimicrobials with low levels of resistance (**Figure 1b**)[10]. Due to potential toxicity and mutagenic issues, nitro drugs are often avoided in drug discovery programs. The nitro moiety is a strong electron-withdrawing, small, polar group which can easily form hydrogen bonds with the target protein. However, alternatively, the nitro group can be bioactivated by enzymatic reduction to give reactive oxygen species (ROS) which in many cases are responsible for the biological effects of nitro group containing drugs. In

latter case, the ROS generated by the non-selective reduction of the nitro group within both host and pathogen can react with biomolecules to exert toxic and mutagenic effects to the host. However, routinely removing compounds containing nitro groups from screening collections may result in missed opportunities. In addition, several FDA licensed drugs are nitroaromatic drugs such as the broad-spectrum antibiotic and antiprotozoal medication metronidazole and the anti-trypanosomatid drug of choice for acute-stage Chagas disease, the 2-nitroimidazole benznidazole[11]. Because the mutagenic effect provoked by the generation of ROS is a direct risk, the mutagenic potential of the nitroaromatic triazene derivatives is investigated in the early stages of the drug bio-evaluation. In this work, 1,3-diaryltriazenide analogues were prepared and screened for their *in vitro* antibacterial activity and further evaluated for their *in vitro* antimycobacterial activity. Here, we report that the triazenide analogs that originate from the common 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene all exhibit a nanomolar MIC against the Gram-positive bacteria *S. aureus* and *M. tuberculosis*, while ineffective against Gram-negative bacterial species such as *E. coli*, *P. aeruginosa* and *S. typhimurium* and the yeast *C. albicans.* Although the compounds were only mildly selective against *M. tuberculosis* over the macrophage-like monocyte cell line J774A.1, no genotoxicity could be observed for the compounds based on the VITOTOX^{TM} results[12]. To dismiss that the observed cytotoxicity is mediated through ROS, paramagnetic resonance spectroscopy was performed on J774A.1 macrophage-like cells exposed to compound **3** or compound **5** but no increase in ROS could be observed.

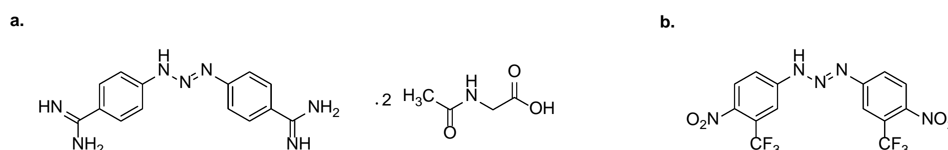


Figure 1: Structures of a. diminazene aceturate and b. 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene.

2. Materials and Methods

2.1. Chemistry

2.1.1. Reagents and materials

Starting materials and solvents for the synthesis of the examined compounds were used as obtained without further purification, from Sigma-Aldrich, Fluka, and Alfa Aesar. Melting points were determined on a Kofler micro hot stage and were uncorrected. NMR spectra were recorded with a Bruker Avance DPX 300 spectrometer operating at 300 MHz (^{1}H), 75.5 MHz (^{13}C) at 302 K. Proton spectra were referenced to $Si(CH_3)_4$ as the internal standard (δ = 0.00 ppm) and carbon chemical shifts were given against the central line of the solvent signal (DMSO-d_6 septet at δ = 39.5 ppm). IR spectra were obtained with Bio-Rad FTS 3000MX (KBr pellets for all compounds). Elemental analyses (C, H, N) were performed with Perkin–Elmer

2400 CHN Analyzer. The progress of all reactions was monitored on Fluka silica-gel TLC-plates (with fluorescence indicator UV254), using ethyl acetate/petroleum ether as the solvent system. All the compounds tested were of ≥95% purity, as verified by ^{1}H NMR and ^{13}C NMR spectroscopy as well as with elemental microanalysis. The values obtained for C, H, N analyses were within ±0.40% of the calculated values.

2.1.2. Synthesis

Precursor 1,3-diaryltriazenes, i.e., 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene, 1,3-bis(2-bromo-4-nitrophenyl)triazene, 1,3-bis(2-chloro-4-nitrophenyl)triazene, and 1,3-bis(2-fluoro-4-nitrophenyl)triazene, were synthesized as described in the literature[9]. Triazenide salts **1**, **2**, **3**, **4** and **6** were prepared according to known procedures[10,13]. Synthesis and characterization of triazenide salts **5**, **7** and **8** is given below.

Piperidin-1-ium 1,3-bis(2-bromo-4-nitrophenyl)triaz-2-en-1-ide (5)

To the suspension of 1,3-bis(2-bromo-4-nitrophenyl)triazene (1 mmol) in acetone (25 mL) piperidine (2 mmol) was added. After 30 min of stirring at room temperature, precipitated **5** was removed by filtration.
Yield: 85 %; MP: 206–208 °C; IR (KBr): 1567, 1497, 1326, 1281, 1212, 1180, 1165, 1099, 879 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.50-1.70 (6H, m, piperidine), 2.98-3.07 (4H, m, piperidine), 7.71 (2H, d, J = 9.2 Hz, ArH), 8.05 (2H, dd, J_1 = 2.6 Hz, J_2 = 9.2 Hz, ArH), 8.32 (2H, d, J = 2.6 Hz, ArH); ^{13}C NMR (DMSO-d_6): δ 21.6 (s, piperidine), 22.2 (s, piperidine), 43.7 (s, piperidine), 115.9 (s, Ar), 116.5 (s, Ar), 123.5 (s, Ar), 128.5 (s, Ar), 140.8 (s, Ar), 157.7 (s, Ar); elemental analysis for $C_{17}H_{18}Br_2N_6O_4$: calculated: C, 38.51; H, 3.42; N, 15.85; found: C, 38.76; H, 3.52; N, 15.82.

Triethylamonium 1,3-bis(2-chloro-4-nitrophenyl)triaz-2-en-1-ide (7)

The suspension of 1,3-bis(2-chloro-4-nitrophenyl)triazene (10 mmol) in acetone (500 mL) was treated with trimethylamine gas until triazene was completely dissolved. After 24 h stirring at -19 °C precipitated **7** was removed by filtration.
Yield: 54 %; MP: 193–195 °C; IR (KBr): 1572, 1514, 1341, 1263, 1163, 1099, 890 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 2.70 (9H, s, CH_3), 7.77 (2H, d, J = 9.2 Hz, ArH), 8.03 (2H, dd, J_1 = 2.6 Hz, J_2 = 9.2 Hz, ArH), 8.19 (2H, d, J = 2.6 Hz, ArH); ^{13}C NMR (DMSO-d_6): δ 44.5 (s, CH_3), 116.5 (s, Ar), 123.1 (s, Ar), 125.5 (s, Ar), 125.8 (s, Ar), 141.2 (s, Ar), 155.1 (s, Ar); elemental analysis for $C_{15}H_{16}Cl_2N_6O_4$: calculated: C, 43.39; H, 3.88; N, 20.24; found: C, 43.21; H, 3.99; N, 20.16.

Triethylamonium 1,3-bis(2-fluoro-4-nitrophenyl)triaz-2-en-1-ide (8)

To the refluxing suspension of 1,3-bis(2-fluoro-4-nitrophenyl)triazene (1 mmol) in acetone (25 mL), triethylamine (3 mmol) was added. Solution was then left at room temperature. After 24 h precipitated **8** was removed by filtration.

Yield: 38 %; MP: 218–219 °C; IR (KBr): 1598, 1509, 31330, 1286, 1265, 1232, 1196, 1064, 932 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.12 (9H, t, J = 7.4 Hz, CH_3), 2.97 (6H, q, J = 7.4 Hz, CH_2), 7.76-7.84 (2H, m, ArH), 7.95-8.05 (4H, m, ArH); ^{13}C NMR (DMSO-d_6): δ 9.2 (s, CH_3), 45.7 (s, CH_2), 111.9 (d, J = 25 Hz, Ar), 116.6 (d, J = 4 Hz, Ar), 120.7 (d, J = 3 Hz, Ar), 140.8 (d, J = 8 Hz, Ar), 147.7 (s, Ar), 153.0 (d, J = 250 Hz, Ar); elemental analysis for $C_{18}H_{22}F_2N_6O_4$: calculated: C, 50.94; H, 5.22; N, 19.80; found: C, 51.10; H, 5.33; N, 19.87.

2.2. *Biology*

2.2.1. *In vitro* antimicrobial screening

In vitro antimicrobial screening against *E. coli* (ATCC® 8739™), *S. typhimurium* (ATCC® 14028™), *P. aeruginosa* (ATCC® 9027™), *S. aureus* (ATCC® 6538™), *M. tuberculosis* H37Ra (ATCC® 25177™) and *C. albicans* (ATCC® 10231™) was evaluated by a resazurin assay. All compounds were solubilized in 100% DMSO at a stock concentration of 10 mM. The 1,3-diaryltriazenides were diluted in LB medium, Middlebrook 7H9 broth + 10% OADC (complete 7H9 broth) or Roswell Park Memorial Institute 1640 (RPMI 1640) medium, depending on the micro-organism tested, to obtain the final test concentrations of 100 µM, 10 µM and 1 µM. Compounds were tested in triplicate in transparent, flat-bottomed 96-well plates. As a positive control, norfloxacin (for *E. coli*), doxycylin (for *S. typhimurium*, *P. aeruginosa* and *S. aureus*), isoniazid (for *M. tuberculosis* H37Ra) and flycytosin (for *C. albicans*) was included. For each strain, a microbial suspension was made by thawing a frozen glycerol stock and, subsequently, diluting it in LB medium, complete 7H9 broth or RPMI 1650 medium to obtain a suspension with an appropriate inoculum size. From this microbial suspension, 100 µl was added to each well, containing 100 µl of the compound dilution. The outer-perimeter wells were filled with 200 µl of deionized water. Test plates were incubated at 37°C for 24 hours (for *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus* and *C. albicans*) or 7 days (for *M. tuberculosis* H37Ra). After this exposure time, 20 µl of 0.01% resazurin was added to each well and test plates were incubated again at 37°C until a color change from blue to pink occurred. Fluorescence was measured at $\lambda_{excitation}$ = 550 nm and $\lambda_{emission}$ = 590 nm using a spectrophotometer (Promega Discover).

2.2.2. *In vitro* antimycobacterial activity

In vitro antimycobacterial activity of the 1,3-diaryltriazenides was further evaluated by a luminometric assay based on a *M. tuberculosis* H37Ra laboratory strain transformed with a pSMT1 luciferase reporter plasmid (H37Ra-*lux*). A two-fold serial dilution of each compound was made in Middlebrook 7H9 broth + 10% OADC (complete 7H9 broth) with final concentrations ranging from 128 µM to 0.5 µM. Volumes of 100 µl of the serial dilutions were added in triplicate to black, flat-bottomed 96-well plates. As a positive control, isoniazid, a first-line antimycobacterial drug, was included. The mycobacterial suspension was

made by thawing a frozen glycerol-stock of H37Ra-*lux* and, subsequently, diluting it in complete 7H9 broth to obtain a suspension with 10.000 relative light units (RLU)/ml. A volume of 100 µl of bacteria was added to each well. All of the outer-perimeter wells were filled with 200 µl of sterile deionized water to minimize evaporation of the medium in the test wells during incubation. After 7 days, the bacterial replication was analyzed by luminometry. To evoke a luminescent signal, 25 µl of 1% n-decanal in ethanol was added to each well, where after light emission was measured using a luminometer (Promega Discover).

2.2.3. *In vitro* cytotoxicity assay

In vitro cytotoxicity on the J774A.1 murine monocyte-like macrophages cell line (ATCC® TIB-67™) was assessed for each analogue by a NRU assay. The J774A.1 cells were cultured in 75 cm^2 sterile Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum in a 5% CO_2 atmosphere at 37°C. When a semi-confluent layer of cells was formed, the cells were trypsinized, washed with sterile PBS, seeded into a transparent, flat-bottomed 96-well plate at a density of 4 x 10^4 cells per well and left for recovery at 37°C, 5% CO_2. For each compound, a two-fold serial dilution was made in complete DMEM with final concentrations ranging from 128 µM to 0.5 µM. Subsequently, the J774A.1 cells were exposed to the compounds (in triplicate) by adding 100 µl of the serial dilutions to the wells. Test plates were incubated for 24 hours in an atmosphere of 5% CO_2 at 37°C. For the NRU assay, the cells were washed 2 times with 200 µL PBS and 100 µL neutral red working solution was added per well. Subsequently, the plates were left for incubation at 37°C, 5% CO_2 for 3 hours. After incubation, the wells were washed 2 times with 200 µL PBS and 150 µL of an ethanol/acetic acid mixture was added per well. The plates were left shaking until the color became homogeneous purple and the optical density was measured at 530 and 620 nm (reference wavelength) using a plate reader (Promega Discover).

2.2.4. VITOTOX™ assay

Observations on early signs of genotoxicity were assessed with the VITOTOX™ test and the recommended protocol was followed. Briefly, both the Genox (RecN2-4) and the Cytox *pr1* strain were diluted 250 times and cultivated at 36°C for 16 hours in poor 869 media. After incubation, the bacterial cultures were diluted 10 times more and incubated for 1 hour at 36°C. To test the genotoxic properties of the metabolites of the compounds, S9-mix was added to the designated +S9 cultures. The bacterial suspensions were then incubated with shaking at 36°C. The luminescent signal was measured for 4 hours with a 5-minute interval.

2.2.5. Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectroscopy (EPR) was used to measure the formation of reactive oxygen species (ROS). The J774A.1 macrophage-like cells were exposed to compound **3** or compound **5**. J774A.1

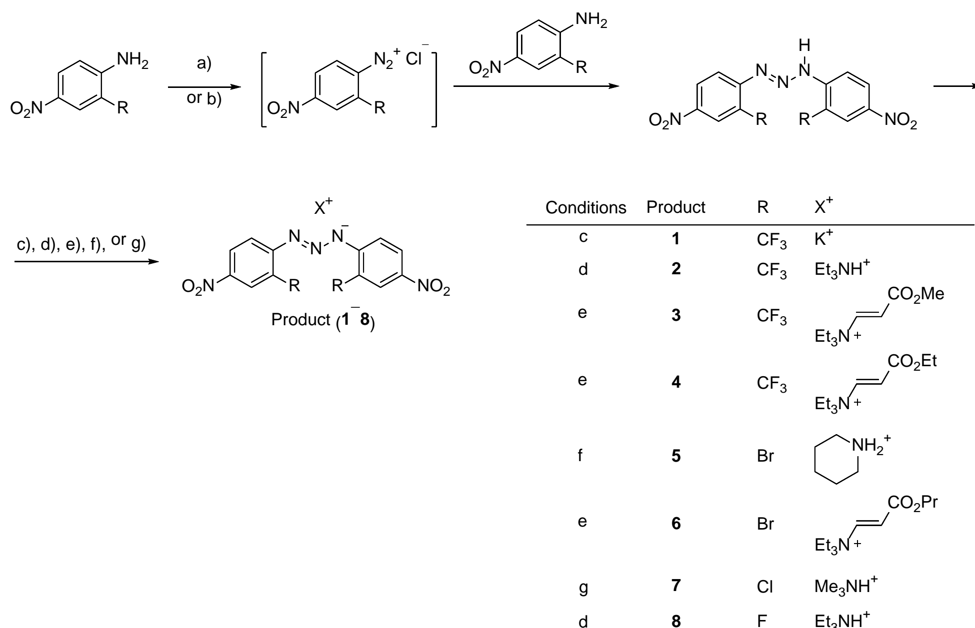
macrophage-like cells were grown in 24-well plates at 2 x 10^5 cells/well one day prior to the testing. After overnight incubation at 37°C and 5% CO_2, the cells were treated with compound **3** and compound **5** at a final concentration of 0.85 µM and 2.03 µM, respectively. Non-treated cells were used as a negative control. All EPR measurements were performed in duplicate in Krebs HEPES buffer (KHB, pH 7.4). After an exposure period of 3 and 24 hours to the compounds and 50 minutes of incubation with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CM-H) at 37°C and 5% CO_2, 50 µl of the supernatant was sampled into a capillary tube for measurement. As a positive control, the ROS stimulating agent phorbol myristate acetate (PMA) was added at a final concentration of 10 µM for 5 minutes before adding the spin probe. All EPR measurements were performed on a Magnettech MiniScope MS 200 spectrometer as follows: frequency 9.4 GHz, power 5 dBm (3.16 mW), modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep time 40 s, time constant 0.1, sweep width 10 mT, number of scans 3. For the measurements, the analyzed samples (50 µL) were contained in glass capillaries (Hirschmann). The signal intensity reported was obtained *via* double integration of the respective simulated spectra of the formed nitroxide radical ($a_N = 1.58$ mT). The simulations were performed using a NIEHS P.E.S.T. WinSIM ver. 0.96.

3. Results and discussion

2.1. Chemistry

Precursor 1,3-diaryltriazenes were prepared starting from *ortho* substituted 4-nitroanilines. The reactions proceeded *via* the nitrosation of the appropriate aromatic anilines by using sodium nitrite in hydrochloric acid or isoamyl nitrite to intermediately formed aryldiazonium salts. The resulting aryldiazonium salts were *in situ* coupled with unreacted portion of the same aromatic aniline. Subsequent treatment of the resulting 1,3-diaryltriazenes with a base such as potassium hydroxide, triethylamine, piperidine or trimethylamine yielded triazenide salts **1**, **2**, **5**, **7** and **8** whereas the treatment with triethylamine in the presence of methyl or ethyl propiolate gave alkoxycarbonylvinyltriethylammonium salts **3** and **4**. The addition of triethylamine to alkyl propiolate forming alkoxycarbonylvinyltriethylammonium cations has been reported. These compounds readily undergo transesterification in the presence of alcohols, which was employed for the synthesis of triazenide **6**[13] (**Scheme 1**).

Scheme 1: Synthesis of compounds **1-8**[a].



[a] Reagents: a) $NaNO_2/HCl$;[9] b) isoamyl nitrite;[9] c) KOH;[13] d) Et_3N;[13] e) Et_3N, $HC\equiv CCO_2CH_3$ or $HC\equiv CCO_2C_2H_5$, in case of **6** followed by *n*-propanol;[13] f) piperidine; g) Me_3N.

2.1. Biology

The 1,3-diaryltriazenides **1**–**8** were screened *in vitro* for their antimicrobial activity. In order to study the selectivity of the compounds, a panel of test organisms was selected in addition to the *M. tuberculosis* lab strain H37Ra. This panel consisted of bacteria, *E. coli*, *S. typhimurium* and *P. aeruginosa*, *S. aureus*, the avirulent *M. tuberculosis* lab strain H37Ra and the yeast *C. albicans*. To determine the activity range of the compounds, the selected panel of microorganisms was exposed to 100, 10 and 1 µM of the compounds after which the viability was derived by measuring the reduction of resazurin to the fluorescent resorufin and compared with an untreated control culture. None of the derivatives showed activity against the Gram-negative bacteria *E. coli*, *S. typhimurium, P. aeruginosa, S. aureus* and the yeast *C. albicans.* However, an $IC_{50} < 1$ µM, against the Gram-positive bacterium *S. aureus*, was observed for compound **1**–**4** sharing the *ortho*-trifluoromethyl on both aryl moieties. Against the weakly Gram-positive *M. tuberculosis* labstrain H37Ra an $IC_{50} < 1$ µM was observed for derivatives **1**–**6**. There was no apparent influence by the cationic species used for the preparation of new compounds on the bioactivity. Activity seemed to be governed by the *ortho* substituent carried on both aryl moieties and the larger trifluoromethyl and the heavier halogen bromide were preferred over the lighter chlorine and fluorine substituents.

Table 1: *In vitro* antimicrobial activity of 1,3-diaryltriazenide **1**–**8**

Derivative	IC_{50} (μM)[a]					
	E. coli (ATCC® 8739™)	*S. typhimurium* (ATCC® 14028™)	*P. aeruginosa* (ATCC® 9027™)	*S. aureus* (ATCC® 6538™)	*M. tuberculosis* H37Ra (ATCC® 25177™)	*C. albicans* (ATCC® 10231™)
1	> 100	> 100	> 100	< 1	< 1	> 100
2	> 100	> 100	> 100	< 1	< 1	> 100
3	> 100	> 100	> 100	< 1	< 1	> 100
4	> 100	> 100	> 100	< 1	< 1	> 100
5	> 100	> 100	> 100	> 100	< 1	> 100
6	> 100	> 100	> 100	10 < x < 100	< 1	> 100
7	> 100	> 100	> 100	> 100	1 < x < 10	> 100
8	> 100	> 100	> 100	10 < x < 100	> 100	> 100
Norfloxacin	< 1	N.D.	N.D.	N.D.	N.D.	N.D.
Doxycyclin	N.D.	< 1	< 1	< 1	N.D.	N.D.
Isoniazid	N.D.	N.D.	N.D.	N.D.	< 1	N.D.
Flucytosine	N.D.	N.D.	N.D.	N.D.	N.D.	< 1

[a] The concentration of the compounds at which a growth inhibition of 50% of the strain investigated was observed as compared to the negative control. N.D.= Not done

The *in vitro* antimycobacterial activity of the 1,3-diaryltriazenides **1**–**8** was further evaluated by luminometry using a pSMT1-transformed *M. tuberculosis* H37Ra strain. As reported previously in the literature, this luminometric technique offers a rapid, cheap and reproducible tool to test the efficacy of potential novel antitubercular compounds, replacing the fastidious CFU enumeration on agar[14,15]. After 7 days of exposure, the IC_{50} and IC_{90} was evaluated by testing serial dilutions of the investigated 1,3-diaryltriazenides **1**–**8**. As shown in **Table 2**, compounds **1**, **2**, **3** and **4**, having both NO_2 and CF_3

substitutions, showed sub-micromolar IC_{90} values (0.88, 0.88, 0.85 and 0.88 µM respectively), excelling the IC_{90} value of the most potent compound of the previous library[4]. When the CF_3 group at the *ortho* position was replaced with bromine, the activity was reduced and the IC_{90} values increased to 4.95 and 2.03 µM for compound **5** and **6** respectively. For the compound **7** carrying the chlorine substituent, IC_{90} increased to 7.23 µM and the antimycobacterial activity was completely abrogated for derivative **8**, when trifluoromethyl was replaced by a fluorine substituent. To calculate the Selectivity Index (SI), the acute cytotoxicity against a J774.A1 macrophage cell line by the 1,3-diaryltriazenides **1**–**8** was studied using a neutral red dye uptake (NRU) assay[16]. The concentration at which the viability of the macrophages is reduced to 50% (CC_{50}) was similar for the derivatives **1**–**4** and was independent from the cationic species used during preparation of the nitro aromatic triazenides. However, toxicity decreased significantly by exchanging the trifluoromethyl substituent with the halogens bromine, fluorine and chlorine (**5**–**8**). By comparing compound **5** and compound **6**, it was observed that exchanging the piperidinium cation by (*E*)-*N*,*N*,*N*-triethyl-3-oxo-3-propoxyprop-1-en-1-aminium cation significantly increased toxicity. Based on the SI of 10.60 and despite, the reduced activity with an IC_{90} of 4.95, derivative **5** was selected as the provisional best derivative from this small compound series due to its decreased in vitro toxicity with a CC_{50} of 52.46 µM.

Table 2: *In vitro* antimycobacterial activity and acute cytotoxicity of 1,3-diaryltriazenides **1**–**8**

Compound	*M. tuberculosis* H37Ra-lux		J774A.1 (ATCC® TIB-67™)	SI[d]
	IC_{50} (µM)[a]	IC_{90} (µM)[b]	CC_{50} (µM)[c]	
1	0.28	0.88	2.54	2.89
2	0.48	0.88	1.77	2.01
3	0.21	0.85	3.08	3.90
4	0.29	0.88	2.64	3.00
5	0.88	4.95	52.46	10.60
6	0.60	2.03	16.04	7.90
7	1.27	7.27	46.59	6.41
8	111.91	> 128	46.10	ND[e]
INH	0.11	0.31	ND	ND

[a, b] The concentration of the compounds at which respectively a growth inhibition of 50% and 90% of the *M. tuberculosis* H37Ra-lux laboratory strain was observed as compared to the negative control.
[c] The concentration of the compounds at which viability of the macrophages was reduced by 50%.
[d] The selectivity index (SI), calculated by dividing the IC_{90} by the CC_{50}.
[e] ND, Not Done.

Given the risk that the observed cellular toxicity is orchestrated by the generation of ROS through the non-specific reduction of the nitro group, the mutagenic potential of the nitro aromatic 1,3-diaryltriazenides was investigated by the VITOTOX™ test. This bacterial reporter model, that is closely correlated with the

AMES test, can detect early signs of genotoxicity by studying the induction of the regulatory SOS operon, a key to the repair of early cellular DNA damage. Transcription of the SOS operon is under control of the *recN* promoter, which is normally strongly repressed. In the presence of a genotoxic compound, the *RecA* regulator protein recognizes the damaged ssDNA fragments whereby the affinity for the *lexA* repressor protein increases and the strong *recN* promoter is de-repressed. In this assay two *S. typhimurium* reporter strains are used. The Genox strain TA104 (recN2-4) contains the *lux* operon of *Vibrio Harveyi* under the transcriptional control of the *recN* promoter. The Cytox *pr1* strain, used as an internal control, contains the integrated *lux* operon and is under the control of the strong constitutive *pr1* promoter. Since both recombinant reporter strains lack the oxidative machinery to metabolize the 1,3-diaryltriazenides, genotoxicity of the metabolites can be assessed by addition of S9 faction. Based on the signal to noise (S/N) ratio, being the luminescent signal produced by the bacterial suspension exposed to the compounds divided by the luminescent signal produced by the non-exposed bacterial suspension, genotoxicity can be assessed[17]. In this work, the 1,3-diaryltriazenides were tested at 1 µM, 0.5 µM and 0.25 µM in the absence and presence of S9 faction. Luminescence was recorded in real time every 5 minutes during a period of 4 hours. A compound or its metabolites are considered to have genotoxic properties if the S/N ratio for the Genox strain (recN2-4) is higher than 1.5. If the S/N value for the Cytox *pr1* strain is higher than 1.5 as well, the result is considered as a false positive since the product has a direct effect on the bacterial luciferase or the luminescent signal itself. Considering a direct cytotoxic effect is possible as well, a S/N ratio lower than 0.8 for the Cytox *pr1* strain indicates false negatives. As shown in **Figure 2**, compounds **1**, **2**, **3** and **4** showed to be non-genotoxic with and without S9 liver extract. After addition of the compounds, the S/N values of the Genox strain (recN2-4) did not exceed 1.5 in both the absence and presence of S9 metabolization, resulting in a negative result. For the 1,3-diaryltriazenides **5**, **6**, **7** and **8**, a negative result (S/N ratio < 1.5) was observed in the absence of S9 liver extract. However, when the Genox strain (RecN2-4) was incubated together with S9 liver extract, S/N values exceeded 1.5, indicating an activation of the SOS operon and, thus, a genotoxic effect caused by the metabolites of compounds **5**–**8**. Considering the structural differences between the 1,3-diaryltriazenides investigated, all members of the latter group did not contain a CF_3 side chain whereas compounds **1**–**4** did carry a CF_3 moiety. Based on these results, the derivatives **5**–**8** should be removed from further testing despite the favorable SI on the grounds of their mutagenic potential after their metabolism within the S9 liver fraction.

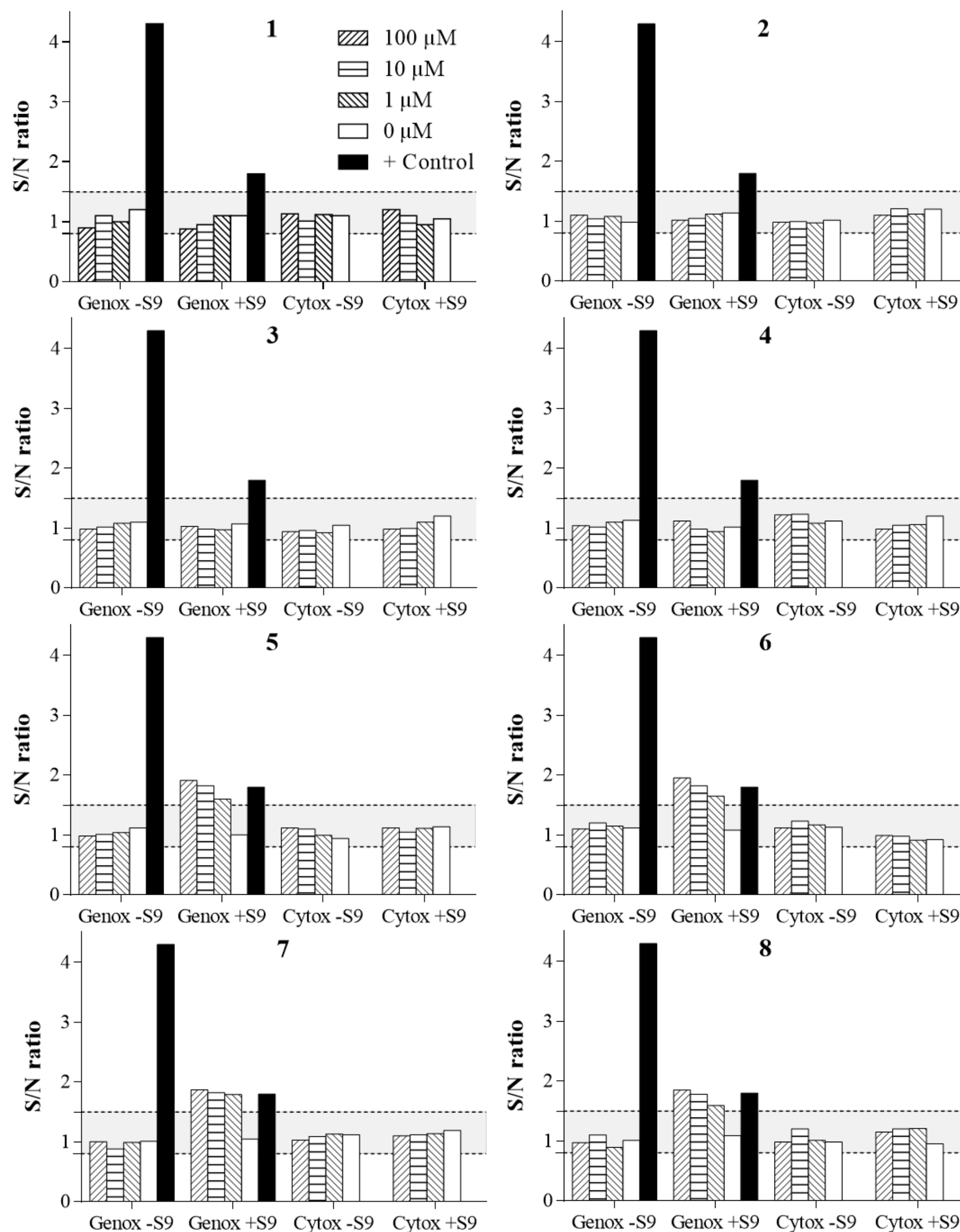


Figure 2: The VITOTOX™ assay for the detection of early signs of genotoxicity caused by the compounds. Maximum recorded S/N in a time span of 4 hours by the Genox (recN2-4) and Cytox (pr1) reporter strains. 4NQO; 4-nitroquinolone-1-oxide was used as a positive control in samples without S9 liver fraction (Genox-S9). Bap; benzo[a]pyrene, the positive control, only turned genotoxic after S9 metabolization (Genox+S9).

Compounds containing nitroaromatic groups have the potential risk to undergo enzymatic reduction in biological systems, which results in the production of ROS in the presence of molecular oxygen[11]. In turn, the produced ROS can react with various biomolecules such as fatty acids, proteins and DNA to exert toxic and mutagenic effects. To that extent we have selected the two compounds **3** and **5** to study if an increase in ROS can be observed by EPR in a J774A.1 macrophage-like monolayer when exposed to the compounds.

As shown in **Figure 3**, this was not the case for compounds **3** and **5** after 3 hours and 24 hours of exposure. This is in accordance to the VITOTOX^{TM} results and indicates that at least the toxicity and the genotoxicity is governed by the nitroaromatic moiety but rather the triazene backbone, in agreement with Cappoen *et al.* 2014[4].

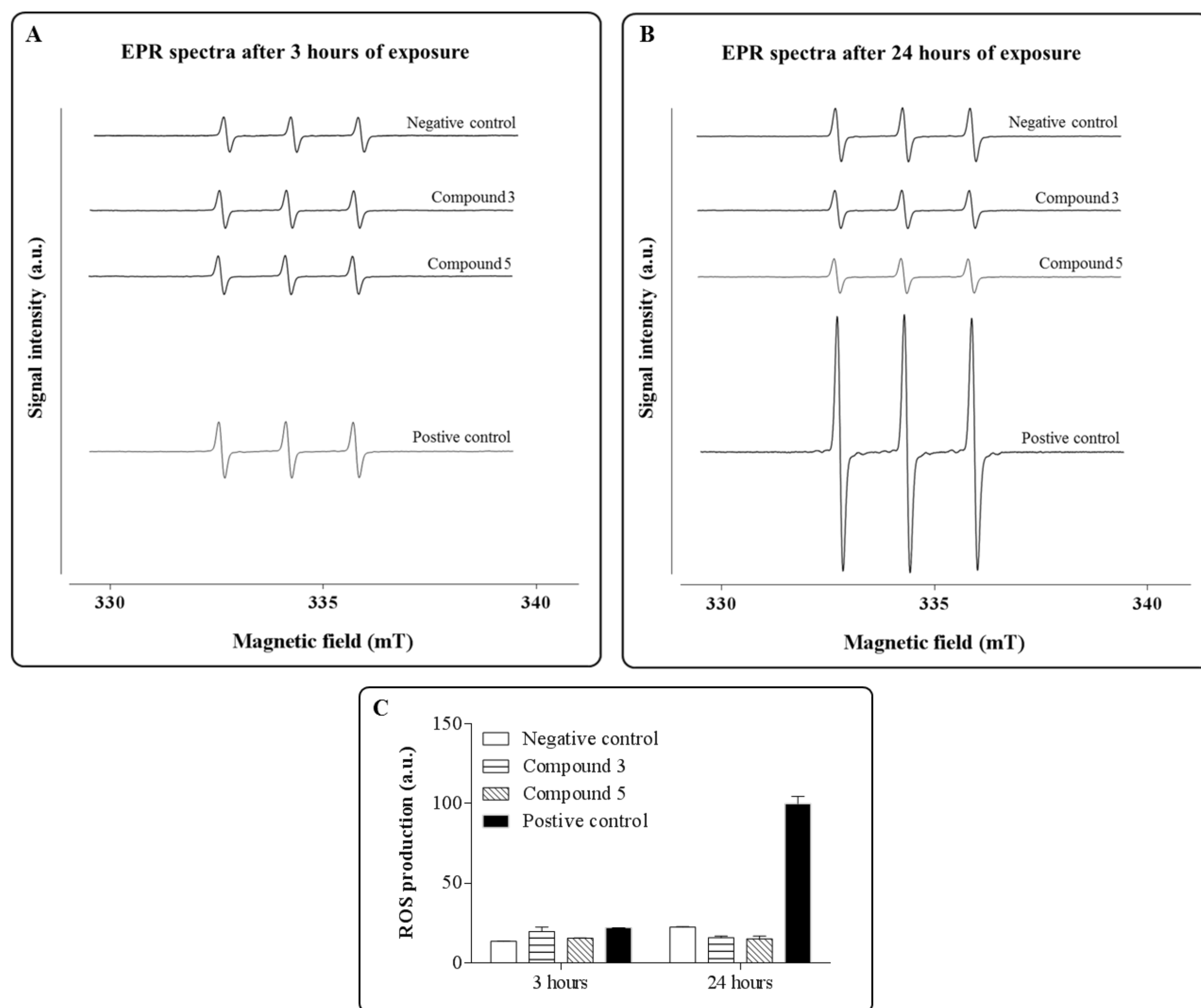


Figure 3: A) EPR spectra measured after 3 h and B) 24 h to compound **3** and compound **5**. Negative control includes the EPR spectra of unexposed cells, while the positive control contains the EPR spectra of cells exposed to phorbol myristate acetate (PMA). The signal intensity in function of ROS production is measured in arbitrary units. C) The simulated spectra were double integrated (aN = 1.58 mT). The numbers presented are averages of two measurements.

4. Conclusion

A small series of eight nitro functionalized 1,3-diaryltriazenides (**1–8**) was synthesized and screened against a panel of microorganisms but activity with IC_{50} values <1 µM was only observed against *S. aureus* and the *M. tuberculosis* lab strain H37Ra. Due to the limited size of the series, the structure activity relation is inconclusive. Nevertheless, it could be observed that derivatives **1–4** carrying a trifluoromethyl substituent at the *ortho* position of both the aryl moieties were significantly more potent with IC_{90} values below 1 µM

whereas the IC_{90} values for derivative **5** and **6** in which the trifluoromethyl substituent was exchanged with bromine where increased to 4.95 µM and 2.03 µM. In addition to a slightly decreased *in vitro* potency, the *in vitro toxicity* decreased significantly to 52.46 µM and 16.04 µM for derivative **5** and derivative **6** respectively. The derivatives **7** and **8** that carried the lighter halogen substituents chlorine and fluorine respectively showed least potency against *M. tuberculosis*. Although the potency was not directly governed by cationic species used in the preparation of the compounds, the toxicity between the otherwise identical derivative **5** and **6** was significantly different and exchanging the piperidinium cation by (*E*)-*N*,*N*,*N*-triethyl-3-oxo-3-propoxyprop-1-en-1-aminium cation, significantly increased toxicity. To address the risk of mutagenic potential of the derivatives due to the introduction of nitro-groups in the diaryltriazenide scaffold, the VITOTOX^{TM} was implemented in the investigation. No genotoxicity could be observed for the derivatives **1**–**4** or their metabolites. Although no mutagenic potential was detected for derivatives **5**–**7**, significant DNA damage was reported for their metabolites after incubation in S9 liver extract. Therefore, these compounds should be removed from further investigation. The observation that the metabolites are mutagenic is in agreement with previous observations and underlines the necessity of the trifluoromethyl substituent to prevent enzymatic metabolization of the diaryltriazenides into mutagenic species. Additionally, it might be an indication that the observed genotoxicity in this study is caused by the triazene backbone itself rather than the non-selective reduction of the nitro groups. Results acquired by EPR analysis were in line with the VITOTOX^{TM} results and no increase in ROS was observed in cases when J774A.1 cells were exposed to derivative **3** and derivative **5**. Despite the limited size of the series, this investigation has brought considerable insight in the optimization of triazenides as potential antitubercular compounds.

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