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ISSN: 2278-1862

Journal of Applicable Chemistry

2023, 12 (2): 67-72

(International Peer Reviewed Journal)

Synthesis and anti-tuberculosis activity of 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1, 3-dione

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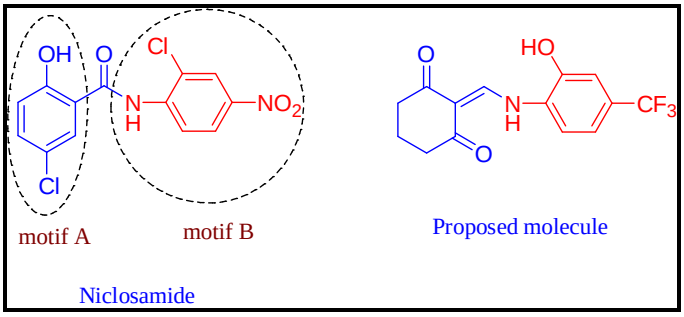
Accepted on 08th February, 2023

ABSTRACT

In the present study 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1,3-dione was synthesized and screened for anti-tuberculosis activity against Mycobacterium tuberculosis (H37Rv). The condensation reaction of cyclohexan-1,3-dione with 2-hydroxy-4- trifloromethyl aniline and triethylorthformate led to the formation of 2-[(2-Hydroxy-4- trifluoromethyl-phenylamino)-methylene]-cyclohexane-1,3-dione in descent yield. The compound significantly inhibited the growth of Mycobacterium tuberculosis H37Ra in dose-dependent manner. The minimum inhibitory concentration of 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1,3-dione against Mycobacterium tuberculosis H37Ra was found to be 1.25 µg/ml. In summary, the present study demonstrates a simple method for the synthesis of 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1,3-dione. The compound effectively inhibits the growth of Mycobacterium tuberculosis H37Ra and therefore can be developed for the treatment of tuberculosis.

Graphical Abstract:



Structure of niclosamide and schematic theme of our proposed library.

Keywords: Tuberculosis treatment, Condensation, Diones, Minimum inhibitory concentration.

INTRODUCTION

Tuberculosis, a deadly infectious disease has infected one-third of the world's population and is common in the developing countries like India and China. Currently TB is treated using first line

chemotherapeutic drug regimen consisting of isoniazid (INH, **1**), rifampicin (RIF, **2**), pyrazinamide (PZA, **3**) and ethambutol (EMB, **4**), Figure 1, for first 2-months (intensive phase) followed by 4 months (continuation phase) of INH and RIF [**1**]. However, drug-resistant TB is treated with more toxic and expensive second line anti-TB drug regimens. It is to be noted that the duration for drug resistant-TB, treatment is more than 2 years [**2**]. This necessity for prolonged TB treatment arises because the *Mycobacterium tuberculosis* (*M.tb*) bacilli escape stringent microenvironments present in the host and enter a non-replicating (NR) and phenotypically drug-tolerant state [**3-5**]. The first-and second-line TB drugs are active only against replicating (R) *M.tb* with no effect on the NR *Mtb* [**6, 7**] Furthermore, the prolonged TB treatment is associated with the development of drug-resistance resulting in worsening of the disease [**8**] Another limitation of the lengthy treatment regime is frequent patient noncompliance leading to drug-resistant TB. Therefore, development of a novel, efficient and short term TB treatment regimen is urgently desired [**9**].

The two approaches for TB-drug discovery include: (a) development of the chemical entities which target a specific biosynthetic pathway and (b)screening of synthetic or natural product libraries phenotypically. The former approach involves development of the inhibitors for proteasome, fatty acid biosynthesis, dihydrolipoamideacetyltransferase, N-acetyltransferase, and the mycobacterial topoisomerase. For example, D157070 (**5**), [**10**] an inhibitor of rhodaninedihydrolipoamide acetyltransferase (DlaT) has been found to kill the nonreplicating mycobacteria selectively. Screening of a library of 15000 compounds (Chemical Diversity Inc.), led to the establishment of the SAR followed by synthesis of new molecular leads and identification of a potent DlaT inhibitor [**7**].

Currently emphasis is laid on the phenotypic screening strategies rather than development of inhibitor for a specific target [**11**]. The S (BTZ04364, **6**) and R (BTZ04464 **7**) enantiomers of benzothiazinones (BTZ) were found to possess bactericidal activity with an MIC of 1 ng mL^{-1} against M. tuberculosis H37RV [**12**]. Similarly, 2-aminothiazole-4-carboxylates (ATC) compounds also displayed promising in vitro activity against M. tuberculosis H37Rv [**13**].

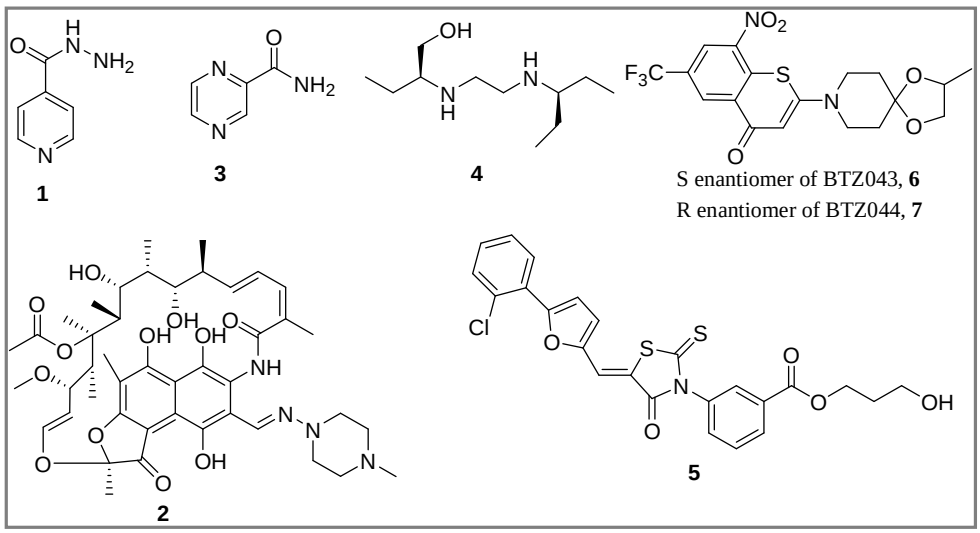


Figure 1. Structure of anti-tuberculosis drugs/agents.

Niclosamide, **8**, (Figure 2**)**, ananthelmintic drug exhibited promising *in-vitro* anti-TB activity and its MIC against M. tuberculosis H37Ra was found to be 0.5-1 mcg mL^{-1}. One of the interesting property of niclosamide is its bactericidal activity against M. tuberculosis in both active as well as stationary non-replicating state [**14**]. Despite its promising *in-vitro* anti-TB activity, niclosamide failed *in-vivo* because of its limited absorbance in intestine. In addition, niclosamide was found be mutagenic *in-vitro* and also showed harmful effect on the morphology of sperm in animals [**15**]. This poor pharmacokinetic profile and its mutagenic capability had considerably blighted its potential in

TB chemotherapy. While working in the field of synthetic organic and medicinal chemistry our group has developed several bioactive molecules [**16-24**]. However, keeping in minds its simple structural features and promise against MDR-TB strains, we initiated a research program in our laboratory to synthesize an analogue around this structural motif to find a better lead molecule/s for future research. In the present study change in the motif A and motif B of niclosamide was proposed and the resulting compound was screened for anti-tuberculosis activity against *Mtb.in-vitro* (Figure 2).

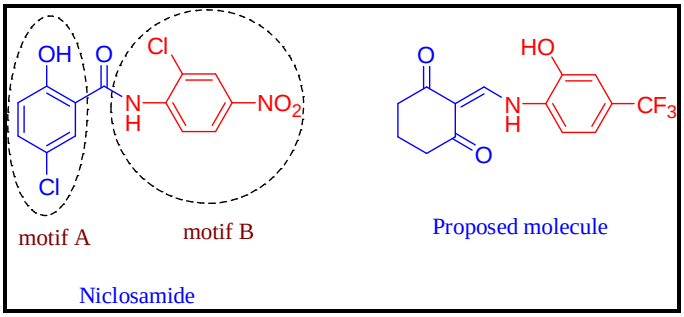


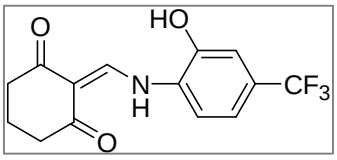
Figure 2. Structure of niclosamide and schematic theme of our proposed library.

MATERIALS AND METHODS

Shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants were measured in Hz. Mass spectra were recorded on Bruker Daltonics electro spray ionization apparatus. Column chromatography was carried out on silica gel (Qualigens, 60-120 mesh) and pre-coated silica gel thin layer chromatographic (TLC) plates were viewed with ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Ceric sulfate was used as visualizing agents. Retinoic acid was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA).

General procedure for the synthesis of compound: A clean and dry 50 mL round bottom flask fitted with reflux condenser was charged with 1,3-cyclohexanedione (500 mg, 4.46 mmol), 2-hydroxy-4-trifloromethyl aniline (487 mg, 4.46 mmol) and triethylorthoformate (1.1 mL, 6.69 mmol). The reaction mixture was stirred for 2 h at 120^{o}C. The crude solid material formed was then purified by silica gel column chromatography to obtain pure compound 1 in 20% ethyl acetate: hexane.

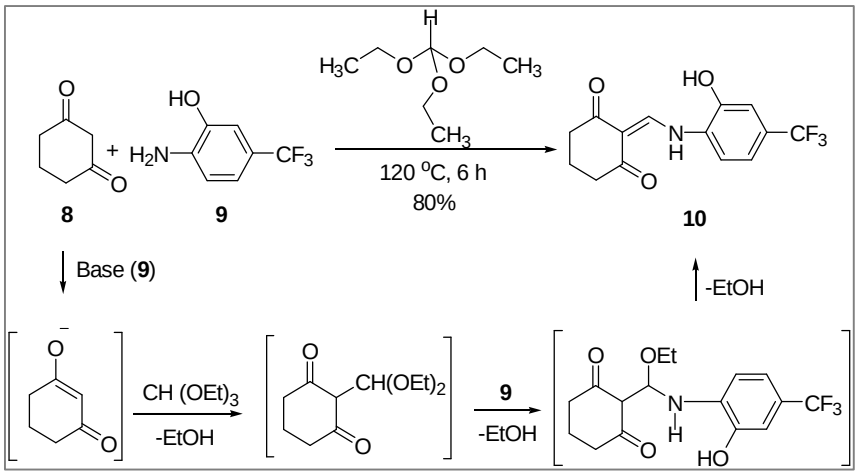
Spectral data of the synthesized compound 2-(((2-chloro-4-nitrophenyl)amino)methylene)cyclohexane-1,3-dione: **^{1}H NMR** (400 MHz, DMSO) δ 12.43 (1H, d, J = 12 Hz), 8.72 (1H, d, J = 12 Hz), 8.17 (1H, m), 7.89 (1H, s), 7.34 (1H, s), 2.72-2.27 (5H, m), 1.43-1.31 (2H, m); **^{13}C NMR** (101 MHz, DMSO) δ 200.6, 196.2, 152.1, 150.4, 148.5, 143.7, 140.5, 136.2, 118.1, 114.3, 111.8, 38.4, 36.5, 31.3.



RESULTS AND DISCUSSION

In the present study, our synthesis started from commercially availablecyclohexan-1,3-dione and 2-hydroxy-4-trifloromethyl aniline. Condensation of cyclohexan-1,3-dione with 2-hydroxy-4-trifloromethyl aniline in presence of triethylorthformate at 120^{o}C resulted the formation of corresponding aniline-dione conjugate in decent yield (Scheme 1). Mechanistic investigation of this reaction makes us to believe that this reaction might be happening initially with condensation of

cyclohexan-1,3-dione with triethylorthformate. The 2-hydroxy-4-trifloromethyl aniline is acting as a base and it subsequent undergoes condensation with the intermediate formed in first stepleading to elimination of ethanol to deliver the product quite uneventfully. Final product of the reaction was purified by column chromatography and characterized by ^{1}H NMR, ^{13}C NMR and MS analysis.



Scheme 1. Synthesis of niclosamide derivative and plausible reaction mechanism.

Determination of minimum inhibitory concentration (MIC) by Broth microdilution method: The MICs of the compound library and standard ATDs against *M.tuberculosis* (H37R*v*) strain were determined by broth microdilution method with slight modification. Briefly, a range of concentrations (0.5 to 256 µg m L^{-1}) of the synthesized compound and standard ATDs (INH, RIF and EMB) with serial two fold concentration (ranging from 0.039 to 10 µg mL^{-1}) were prepared in 96-well plates in 200 µL volume from well number 1 to well number 10. Two columns (column G and H) in all plate serve as drug free growth control and medium control columns respectively. Plates were inoculated with 50 µL of log phase growing *M.tuberculosis* suspension so that the final cell density in all wells (well No.1 to well No. 11) is 10^{5} CFUs m L^{-1}. The plates were incubated at 37°C and read after 12 days. The MIC was defined as the lowest concentration of an antimicrobial agent which prevented the visible growth of the microorganism. Each MIC test was carried out thrice in triplicate. The synthesized 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1,3-dione (10) was *in-vitro* screened against H37Ra strain. Screening of the compound **10** for anti-tuberculosis activity revealed its promising inhibitory activity against the growth of *Mycobacterium tuberculosis* H37Ra with an MIC value of 1.25 µg m L^{-1}.

APPLICATION

The present study demonstrates a methodology for the synthesis of new potential anti-TB agents from commercially available diones using condensation reaction. The synthesized most potential compound can be investigated further to develop the anti-tuberculosis agents.

CONCLUSION

The 2-[(2-Hydroxy-4-trifluoromethyl-phenylamino)-methylene]-cyclohexane-1, 3-dione, aniclos-amide analog was synthesized with an aim to develop potent anti-TB agents free from harmful effects. Screening of the compound against *Mycobacterium tuberculosis* H37Rv revealed that the synthesized compound exhibited significant anti-TB activity. The minimum inhibitory concentration of the compound was found to be 1.25 µg m L^{-1} against *Mycobacterium tuberculosis* H37Rv.

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