Abstract

Cyclohex-3-enyl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol (MSDRT 12) is a novel triazole-based antitubercular compound with two chiral centers. To evaluate the enantiospecific antitubercular activity, the four stereoisomers were isolated using preparative chiral chromatography and the individual stereoisomers were evaluated using the resazurin microtiter assay method (REMA) and a microbroth dilution technique against the Mycobacterium tuberculosis H37Rv strain. Isomer III of MSDRT 12 was found to be the most potent with a minimum inhibitory concentration (MIC) of 0.78 µg/mL, Isomer II had a MIC of 12.5 µg/mL, and isomers I and IV showed no activity. The diastereomeric mixture of MSDRT 12 showed a MIC of 3.125 µg/mL and isoniazid, used as the standard drug, showed a MIC of 0.4 µg/mL. This confirms the necessity of screening individual enantiomers for their pharmacological activity early in the discovery phase to identify the most potent isomer for further development efforts.

Keywords

Chiral Separation • Preparative Chiral Chromatography • Triazoles • Antitubercular Compounds • REMA testing
Introduction
Molecular chirality is a fundamental consideration in drug discovery to understand and describe biological targets as well as to design effective pharmaceutical agents [1]. The regulatory guidance for chiral drugs published by the United Stated Food and Drug Administration (USFDA) in 1992 requires that the manufacture and control of a chiral drug should ensure its stereoisomeric composition with respect to identity, strength, quality, and purity [2]. Racemates are considered mixtures of compounds with different pharmacological activities, and the estimation of pharmacokinetic parameters based on 'total' drug concentrations following administration of a racemate is therefore of limited value [3]. It is necessary to develop quantitative assays for individual enantiomers and to determine the main pharmacologic activities of each enantiomer.

Enantioselective chromatography is now playing an increasing role not only as an analytical tool for chiral analysis, but also as a preparative technique to obtain pure enantiomers from racemates quickly from a wide diversity of chemical structures [4, 5]. Asymmetric synthesis in early phases of drug discovery is not preferred as it is expensive, time-consuming, and can generate only one of the enantiomers. Preparative chiral chromatography on chiral stationary phases (CSPs), however, is widely used as it is efficient, easily scalable, and can generate all the stereoisomers with high purity [6–10].

Tuberculosis is a major health problem worldwide, with approximately two million people dying annually from the disease. The long-standing current drug regimen, the emergence of drug-resistant strains, and HIV co-infection have resulted in a resurgence in research efforts to address the urgent need for new anti-tuberculosis drugs [11]. A number of new potential anti-tuberculosis drug candidates with novel modes of action are being developed and the shikimate pathway represents an attractive target for the development of new antitubercular agents since it is present and essential in bacteria, but absent in mammals [12]. Hybrid compounds containing an alicyclic scaffold which is a known dehydroquinase inhibitor, and a substituted heterocyclic moiety like triazole which is a known shikimate kinase inhibitor, were synthesised as novel antitubercular agents [13].

![Structure of Cyclohex-3-enyl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol (MSDRT 12)](image)

Cyclohex-3-enyl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol (MSDRT 12) (Figure 1), a novel synthetic triazole compound, has been characterized using $^1$H-NMR and IR spectroscopy and mass spectroscopy [13]. The compound MSDRT 12 showed good in vitro antitubercular activity in the resazurin microtiter assay [14–17]. MSDRT 12 has two chiral centers with four isomers as shown in Figure 2.
Fig. 2. Stereoisomers of MSDRT 12
2A: (S)-(1R)-Cyclohex-3-en-1-yl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol
2B: (S)-(1S)-Cyclohex-3-en-1-yl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol
2C: (R)-(1S)-Cyclohex-3-en-1-yl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol
2D: (R)-(1R)-Cyclohex-3-en-1-yl(5-phenyl-4H-1,2,4-triazol-3-yl)methanol

This paper describes the separation of the four stereoisomers by preparative chiral chromatography and the evaluation of the enantiospecific antitubercular activity of each stereoisomer.

Experimental
Materials
The compound MSDRT 12 has been synthesized at the Institute of Chemical Technology (ICT), Mumbai, India. The HPLC grade solvents, \( n \)-hexane, isopropyl alcohol, diethylamine, dimethylsulfoxide, and methanol, were procured from Merck, whereas ethanol was from Tedia. Middlebrook medium, albumin-dextrose catalase (ADC), and 96-well non-treated plates used for the REMA test were supplied by HiMedia. Resazurin was supplied by SRL, India. The \textit{Mycobacterium tuberculosis} H37Rv strain (Mtb) was maintained at the Radiation Medicine Centre, Mumbai.

Equipment
The K-Prep system with a flow range of 0–100 mL/minute manufactured by YMC was used for preparative chromatography. A Sanyo carbon dioxide incubator was used for incubating the plates for REMA testing.

Isolation of Four Stereoisomers of MSDRT 12
Compound MSDRT 12 (diastereomeric mixture) was supplied by ICT, Mumbai, India. Using the chiral analytical column Chiralpak ID (4.6 mm i.d. x 250 mm, particle size 5µ) and a mobile phase of \( n \)-hexane:isopropyl alcohol: ethanol: diethylamine (60:35:05:0.1 v/v/v/v) at a flow rate of 1 mL/min with detection at 230 nm, it was established that four stereoisomers (identified as Isomer I, Isomer II, Isomer III, and Isomer IV based on the
order of elution with retention times of 8.6, 9.6, 13.0, and 19.2 minutes, respectively) were in the ratio 37:13:37:13 in MSDRT 12. This analytical method could not be directly scaled up for preparative chromatography because of poor resolution between isomers I and II due to the increased loading in the preparative scale. Hence, a two-step procedure was developed for preparative scale chromatography.

In the first step, fraction 1 (consisting of isomers I and II) was separated from the second fraction (consisting of isomers III and IV) by injecting 10 mL of a solution of MSDRT 12 in methanol (2.5 mg/mL) on a Chiralpak IE (250 x 30mm, 5 µ) column at 25°C using the mobile phase n-hexane:isopropyl alcohol:diethylamine (70: 30: 0.1) at a flow rate of 42 mL/minute and detected at 250 nm (Figure 3A). Each fraction was evaporated to dryness using a roto-evaporator at a temperature of 30°C and reconstituted in methanol (2.5 mg/mL) for further separation.

![Fig. 3. Preparative loading chromatogram showing the three-step purification for separation of the four stereoisomers](image)

3A: MSDRT 12 (diastereomeric mixture)
3B: Isomers I and II
3C: Isomers III and IV
In the second step, separation of isomers I and II was achieved by injecting 6 mL of fraction 1 into a preparative column, Chiralpak IA (250 x 20mm, 5µ particle size) using n-hexane:isopropyl alcohol:diethylamine (70:30:0.1 v/v/v) as the mobile phase with a flow rate of 18 mL/minute. The column temperature was maintained at 25°C and each isomer was detected at 250 nm (Figure 3B).

Similarly, isomers III and IV were further separated by injecting 10 mL of fraction 2 into a preparative column, Chiralpak AY-H (250 x 30 mm, 5 µ) at 25°C using the mobile phase n-hexane: isopropyl alcohol:methanol:diethylamine (50:40:10.1 v/v/v/v) at a flow rate of 42 mL/min. The column temperature was maintained at 25°C and each isomer was detected at 250 nm (Figure 3C).

**Resazurin microtitre Assay (REMA)**

All five compounds (Isomers I, II, III, IV, and MSDRT 12) were tested against *Mycobacterium tuberculosis* H37Rv strain to determine the minimum inhibitory concentration (MIC) in the broth microdilution assay. The MIC is defined as the minimum concentration of a compound required for the complete inhibition of bacterial growth, as indicated by the colour change of resazurin (from blue to pink in case of growth). A serial dilution (from 50 µg/mL to 0.1 µg/mL) of each of the five compounds (Isomer I, Isomer II, Isomer III, Isomer IV, and MSDRT 12 (diastereomeric mixture)) was prepared in a volume of 200 µL containing a homogenous *Mycobacterium tuberculosis* H37Rv culture suspension 5x10⁴ Mtb in Middlebrook media. In case of the control, an equivalent amount of dimethyl sulfoxide was added to the plate. The plates were incubated at 37°C for 7 days in a carbon dioxide incubator. Resazurin dye (0.02% in Middlebrook medium) was added after 7 days and the plates were incubated for 48 hours. The MIC was determined by visual inspection of the dye colour (blue to pink). Isoniazid was used as the standard drug.

**Purity of the Stereoisomer**

The purity of each stereoisomer was confirmed using a validated chiral analytical method. Enantiopurities were 98.7, 99.8, 99.1, 99.5% for isomers I, II, III, and IV, respectively. To ensure the stability of the purified isomers, each isomer was analysed initially and also after evaporation in the roto evaporator at 30°C followed by reconstitution in methanol. Lack of interconversion and no significant increase (less than 0.3%) in impurity levels confirmed the stability of each pure stereoisomer (Figure 4). The four stereoisomers of MSDRT 12 were further characterised by ¹H NMR (Figure 5), IR Spectroscopy (Figure 6), and mass spectra (Figure 7).

**Results and Discussion**

Thus by using preparative chiral chromatography, it was possible to rapidly isolate all four stereoisomers with the high degree of enantiopurity required for the evaluation of the antitubercular activity.

The MIC of the pure isomers and the synthesized MSDRT 12 (diastereomeric mixture) is detailed in Table 1. Isomer III exhibited good activity with an MIC of 0.78 µg/mL. Isomers I and IV did not show any activity, while Isomer II had a MIC of 12.5 µg/mL, and isoniazid used as the standard drug showed a MIC 0.4 µg/mL. Isomer III was found to be four times...
more potent when compared to MSDRT 12 (diastereomeric mixture containing the four isomers in a ratio 37:13:37:13).

Fig. 4. Representative chromatograms of Isomer I (A), Isomer II (B), Isomer III (C), Isomer IV (D), Racemic mixture of MSDRT 12 (E)
Fig. 5. $^1$H NMR of Isomer I (5A), Isomer II (5B), Isomer III (5C), and Isomer IV (5D) of MSDRT 12
Fig. 6. IR spectra of Isomer I (6A), Isomer II (6B), Isomer III (6C), and Isomer IV (6D) of MSDRT 12
Fig. 7. Mass spectra of Isomer I (7A), Isomer II (7B), Isomer III (7C), and Isomer IV (7D) of MSDRT 12

Tab. 1. Biological evaluation data for the pure stereoisomers I, II, III, IV, and MSDRT 12

| Compound                | MIC µg/ml | Enantiopurity/Ratios |
|-------------------------|-----------|-----------------------|
| Isomer I                | No activity | 98.7                 |
| Isomer II               | 12.5       | 99.8                  |
| Isomer III              | 0.78       | 99.1                  |
| Isomer IV               | No activity | 99.5                 |
| MSDRT 12 (Diastereomeric mixture) | 3.125 | 37:13:37:13 |
| Isoniazid               | 0.4        | –                     |

Conclusion

Chiral separation and enantiospecific pharmacological activity in early discovery stages help in making a decision whether the chiral compound under investigation should be developed as a racemate or as a single enantiomer. Development of a single pharmacologically active isomer will eliminate/reduce the toxicity due to the unwanted isomers. MSDRT 12 is a newly synthesised, novel, alicyclic triazole compound with promising antitubercular activity against the *Mycobacterium tuberculosis* H37Rv strain. Four stereoisomers of MSDRT 12 were separated and tested individually against the
Mycobacterium tuberculosis H37Rv strain. Isomer III showed it was significantly more potent when compared to other isomers or the racemate MSDRT 12. Isomer III should therefore be considered as a potential lead compound for further development as an antitubercular drug.

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Authors’ Statement
Competing Interests
The authors declare no conflict of interest.

References
[1] Zhang Y, Wu DR, Wang-IversonDB, Tymiak AA. Enantioselective chromatography in drug discovery. Drug Discov Today. 2005; 10: 571–577. http://dx.doi.org/10.1016/S1359-6446(05)03407-0
[2] FDA’s policy statements for the development of new stereoisomeric drugs. Chirality.1992; 4: 338–340. http://dx.doi.org/10.1002/chir.530040513
[3] Hutt AJ. Chirality and pharmacokinetics: an area of neglected dimensionality? Drug Metabol Drug Interaction. 2007; 22: 79–112. http://dx.doi.org/10.1515/DMDI.2007.22.2-3.79
[4] Aboul-Enein HY. High-performance liquid chromatographic enantioseparation of drugs containing multiple chiral centers on polysaccharide-type chiral stationary phases. J Chromatogr A. 2001; 906: 185–193. http://dx.doi.org/10.1016/S0021-9673(00)00950-X
[5] Francotte E. In: Ahuja S, ed. Chiral Separations Applications and Technology. American Chemical Society, Washington, 1997; Chapter 10.
[6] Francotte E. Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers. J Chromatogr A. 2001; 906: 379–397. http://dx.doi.org/10.1016/S0021-9673(00)00951-1
[7] Miller L, Orihuela C, Fronek R, Murphy J. 
Preparative chromatographic resolution of enantiomers using polar organic solvents with polysaccharide chiral stationary phases. 
J Chromatogr A. 1999; 865: 211–226. 
http://dx.doi.org/10.1016/S0021-9673(99)00743-8

[8] Kusters E, Nozulak J. 
The preparative chromatographic enantioseparation of a racemic morphanthridine analog on a chiral stationary phase. 
Chromatographia. 1998; 7: 440–442. 
http://dx.doi.org/10.1007/BF02466476

[9] Zhang T, Nguyen D, Franco p, Murakami T, Ohnishi A, Kurosawa H. 
Cellulose 3,5-dimethylphenylcarbamate immobilized on silica: A new chiral stationary phase for the analysis of enantiomers. 
Anal Chim Acta. 2006; 557: 221–228. 
http://dx.doi.org/10.1016/j.aca.2005.10.017

[10] Yashima E. 
Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation. 
J Chromatogr A. 2001; 906: 105–125. 
http://dx.doi.org/10.1016/S0021-9673(00)00501-X

[11] Rivers EC, Mancera RL. 
New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. 
Drug Discov Today. 2008; 13: 1090–1098. 
http://dx.doi.org/10.1016/j.drudis.2008.09.004

[12] Ducati RG, Basso LA, Santos DS. 
Mycobacterial shikimate pathway enzymes as targets for drug design. 
Curr Drug Targets. 2007; 8: 423–435. 
http://dx.doi.org/10.2174/138945007780059004

[13] Desai NHP, Bairwa R, Kakwani M, Tawari N, Ray M K, Rajan MG, Degani M. 
Novel 4H-1,2,4-triazol-3-yl cycloalkanols as potent antitubercular agents. 
Med Chem Res. 2013; 22, 401–408. 
http://dx.doi.org/10.1007/s00044-012-0043-9

[14] Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels FO. 
Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. 
Antimicrob Agents Chemother. 2002; 46: 2720–2722. 
http://dx.doi.org/10.1128/AAC.46.8.2720-2722.2002

[15] Martin A, Camacho M, Portaels FO, Palomino JC. 
Resazurin microtiter assay plate testing of Mycobacterium tuberculosis susceptibilities to second-line drugs: rapid, simple, and inexpensive method. 
Antimicrob Agents Chemother. 2003; 47: 3616–3619. 
http://dx.doi.org/10.1128/AAC.47.11.3616-3619.2003

[16] Taneja NK, Tyagi JS. 
Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing Mycobacterium tuberculosis, Mycobacterium bovis BCG and Mycobacterium smegmatis. 
J Antimicrob Chemother. 2007; 60: 288–293. 
http://dx.doi.org/10.1093/jac/dkm207

[17] Jadaun GP, Agarwal C, Sharma H, Ahmed Z, Upadhyay P, Faujdar J, Gupta AK, Das R, Gupta P, Chauhan DS, Sharma VD, Katoch VM. 
Determination of ethambutol MICs for Mycobacterium tuberculosis and Mycobacterium avium isolates by resazurin microtitre assay. 
J Antimicrob Chemother. 2007; 60: 152–155. 
http://dx.doi.org/10.1093/jac/dkm117