Synthetic Analogues of Natural Product Disulphides from *Allium stipitatum* Regel Demonstrate Potent Anti-tubercular Activities and Inhibit Mycobacterial Drug Efflux Pumps

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BACKGROUND

The need to overcome the emergence of multidrug- and extensively-drug-resistant strains of *Mycobacterium tuberculosis* has triggered the exploration of novel and unconventional approaches to control microbial infections.[1] One major component of resistance to many classes of antimicrobials is multidrug efflux, and efflux mechanisms significantly contribute to antibiotic-resistance in mycobacteria. The activation of multidrug efflux pumps also plays a role in biofilm formation and it is suggested to be responsible for the enhanced antibiotic resistance of biofilms.[2] The identification of efflux pump inhibitors is therefore an attractive lead in designing new anti-tubercular therapy as well as reversing the resistance. Plants play a major role in drug discovery by providing bioactive scaffolds against a variety of targets. The genus *Allium* are well-known worldwide as spices, ornamental plants, but most importantly for their medicinal properties. [3] Synthetic analogues base on the structure of bioactive natural products *Allium stipitatum* were produced in a bid to optimize antibacterial activity.

OBJECTIVES

The objective of this research was to synthesize analogues of natural product disulphides isolated and characterized from *Allium stipitatum* Regel and evaluate their possible efflux pump inhibition and biofilm inhibition in mycobacteria, fungi and other Gram positive and negative microorganisms.

MATERIALS AND METHODS

Total synthesis

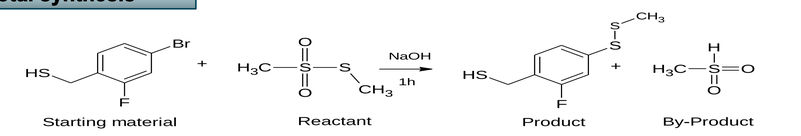
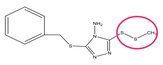
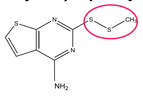


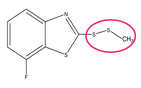
Fig 1: Synthesis procedure (a) Magnetic stirring of reactants (b) Separation of the dichloromethane layer (c) drying with rotary evaporator (d) Nuclear Magnetic Resonance Spectroscopy (e) Mass Spectroscopy



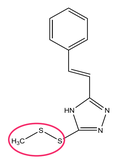
[1] 3-(benzylthio)-5-(methyldisulfanyl)-4H-1,2,4-triazol-4-amine



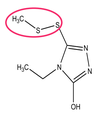
[2] 2-(methyldisulfanyl)thieno[2,3-d]pyrimidin-4-amine



[3] 7-fluoro-2-(methyldisulfanyl)benzo[d]thiazole



[4] (E)-3-(methyldisulfanyl)-5-styryl-4H-1,2,4-triazole



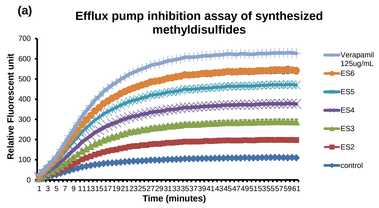
[5] 4-ethyl-5-(methyldisulfanyl)-4H-1,2,4-triazol-3-ol

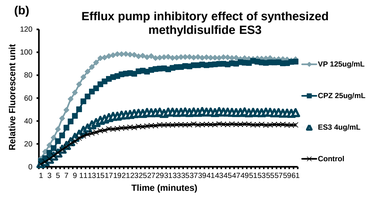
Fig 2: Synthesized methyl disulphides

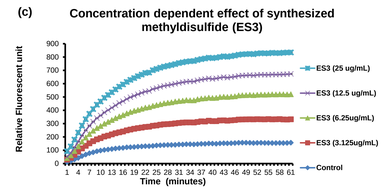
RESULTS

Biological evaluation

1. High throughput spot culture growth inhibition (HT-SPOTi) assay using *M. aurum.* [4]
2. Drug efflux pump inhibition in *M. aurum.*
3. Biofilm inhibition studies in *M. smegmatis.*







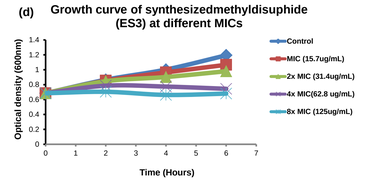
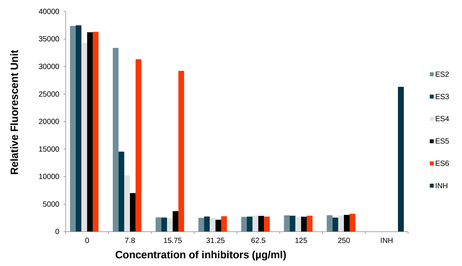


Fig 3: (a), (b), (c) Efflux pump inhibition assay of ES compounds using *M. aurum.* Ethidium bromide (EtBr) is ig an efflux pump substrate (used at a final concentration of 0.5 µg/mL). Verapamil and Chlorpromazine are known efflux pump inhibitors and are used as controls, (d) growth curve with optical density taken at 600nm



Compound ID	MIC_{H37Rv}	GIC_{50}	$SI=GIC_{50}/MIC_{H37Rv}$
ES2	62.50	40.93	0.65
ES3	4.00	18.23	4.56
ES4	31.30	9.92	3.16
ES5	31.30	>250	-
ES6	31.30	>250	-

SI - Selectivity Index
GIC – Growth Inhibitory Concentration
MIC – Minimum Inhibitory Concentration

Fig 4: Cytotoxicity testing using rezasurin assay with murine macrophage RAW 264.7 cell line

CONCLUSIONS

The synthetic analogues inhibited the growth of different *Mycobacterium* species more than Gram-negative, Gram-positive and fungal species and showed inhibitory effect in the EPI assay and also dose dependently inhibited biofilm formation, revealing their possible endogenous mechanisms of action although further studies is required.

They also did not significantly affect the viability of the murine macrophage cells.

These findings indicate that compounds structurally related to naturally-occurring disulphides can serve as leads for the identification of novel scaffolds for the development of effective new antimycobacterials.

Minimum Inhibitory Concentration (MIC) (µg/mL)

Compound ID	*M. smegmatis*	*M. aurum*	*M. bovis BCG*	*M. tuberculosis H37Rv* (clinical isolate)	MDR strain 1 *M. tb* H37Rv (clinical isolate)	MDR strain 2 *M. tb* H37Rv (clinical isolate)	Gram positive *S. aureus* SA 1199B	Gram-positive *S. aureus* EMRSA-15	Gram-negative *E. coli* (NCTC 10418)	Fungi *Tricho-phyton rubrum*	Fungi *Tricho -phyton equinum*
Compound 1	62.5	31.3	31.3	62.5	125.0	250	32	64	128	250	125
Compound 2	15.7	15.7	15.7	**4.0**	15.7	31.3	16	16	128	125	125
Compound 3	125.0	62.5	62.5	31.3	>500	>500	16	16	128	>500	>500
Compound 4	31.3	15.7	15.7	31.3	15.7	62.5	16	8	32	125	125
Compound 5	31.3	15.7	15.7	31.3	31.3	125.0	16	16	16	125	125
Isoniazid	0.2	0.1	0.1	0.1	0.1	0.1	-	-	-	-	-
Norfloxacin	-	-	-	-	-	-	64	8	-	-	-
Terbinafine	-	-	-	-	-	-	-	-	-	0.006	0.0012

Fig 5: Antibacterial activity of the synthesized methydisulphides tested using the whole cell phenotypic assay, SPOTi

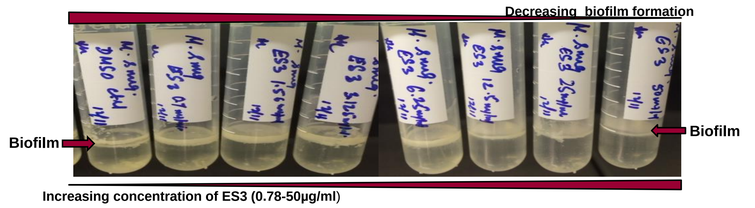


Fig 6: Concentration dependent inhibition of biofilm formation by synthesized methyldisulphide ES3 (OD_{600} 3.5)

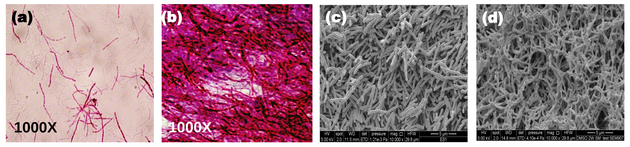


Fig 7: (a and b) Image of *M. smegmatis* cells (planktonic form) at early stationary phase (OD_{600} 3.5) and (biofilm form) at early stationary phase (OD 3.5) taken with light microscope at 1000X magnification, (c) and (d) images of *M. smegmatis* cells (planktonic form) at early stationary phase (OD_{600} 3.5) and image of *M. smegmatis* (biofilm form) at early stationary phase (OD 3.5) taken with scanning electron microscope (SEM) respectively.

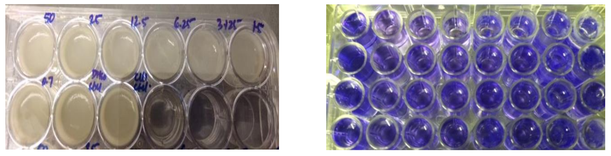


Fig 8: Quantification of biofilm using Crystal violet staining technique followed by measurement of absorbance.

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[1] World Health Organization, Global tuberculosis report, 2013. [2] Baugh, S. *et al. JAC Advance access,* 2014,69(3):673-81. [3] O'Donnell, G. *et al. J Nat Prod.,* 2009, 72, 360-5. [4] Guzman J.D., *et al. BMJ Open.,* 2013, 3(6). [5] Louw G.E. *et al. Antimicrob. Ag. Chem.* 2009 53:8 3181-89.

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