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Symmetrical and unsymmetrical analogues of isoxyl; active agents against *Mycobacterium tuberculosis*

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Abstract—Symmetrical and unsymmetrical analogues of the antimycobacterial agent isoxyl-have been synthesized and tested against *Mycobacterium tuberculosis H37Rv* and *Mycobacterium bovis* BCG, some showing an increased bactericidal effect. In particular, compounds 1-(*p*-*n*-butylphenyl)-3-(4-propoxy-phenyl) thiourea (**10**) and 1-(*p*-*n*-butylphenyl)-3-(4-*n*-butoxy-phenyl) thiourea (**11**) showed an approximate 10-fold increase in in vitro potency compared to isoxyl, paralleled by increased inhibition of mycolic acid biosynthesis in *M. bovis* BCG. Interestingly, these isoxyl analogues showed relatively poor inhibition of oleate production, suggesting that the modifications have changed the spectrum of biological activity.

Mycobacterium tuberculosis continues to be one of the most prevalent causes of morbidity and mortality worldwide.[1] The impact of this infection on nearly one-third of the world's population justifies extensive research into new chemotherapeutic reagents.[1] Eisman et al. first published in vitro and in vivo data on the anti-tuberculosis activity of substituted thioureas tested in mice and guinea pigs.[2] Subsequent modification and biological testing produced a library of compounds, in which the thiourea derivative 4,4′-diisoamythio-carbanilide (isoxyl, thiocarlide, ISO [**1**]) was shown to have considerable antimycobacterial activity in mice, guinea pigs and rabbits.[3,4] Clinical use of ISO began in the 1960s to treat tuberculosis.[5–8] Furthermore, ISO was shown to be effective against various multi-drug resistant *M. tuberculosis* isolates.[9] In an attempt to understand the biological basis for the activity of ISO, Phetsuksiri et al. investigated its mode of action and reported that ISO, like isoniazid and ethanionamide, strongly inhibited the synthesis of mycolic acids.[10]

The fatty acid synthase I (FAS-I) in mycobacteria synthesizes long-chain fatty acids, including stearic acid, which are subsequently desaturated to oleic acid, a ubiquitous constituent of mycobacterial membrane phospholipids.[11,12] Over-expression of the *M. tuberculosis* putative fatty acid desaturases in *Mycobacterium bovis* BCG identified *desA3*, which encodes the Δ9-acyl-CoA desaturase responsible for the biosynthesis of oleic acid. Over-expression of *desA3* also resulted in increased resistance of *M. bovis* BCG to ISO, identifying *desA3* as a novel target.[10]

The crystal structure of the related Δ9-stearoyl-acyl carrier protein desaturase has been resolved to 2.4 Å.[13] By using docking experiments, it has become possible to suggest modifications to the ISO skeleton that may improve the affinity of the inhibitor for the active site. As a result an initial series of symmetrical and unsymmetrical ISO analogues have been developed as potential antimycobacterial agents.

Symmetrical ISO analogues were prepared using a four-step sequence.[14,15] 4-Acetomidophenol is alkylated upon base treatment with an appropriate halide substituent. The acetamide functionality is deprotected to form the

Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DTT, dithiothreitol; FAS, fatty acid synthase; FAMEs, fatty acid methyl ester; MAMEs, mycolic acid methyl ester; MIC, minimum inhibition concentration; SI, selectivity index.

Keywords: *Mycobacterium tuberculosis*; Mt; *Mycobacterium bovis*; Mb; Mycolic acids; Isoxyl; Inhibitor.

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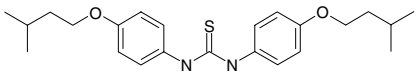
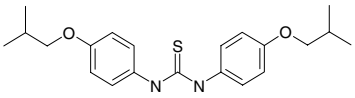
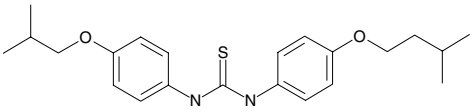
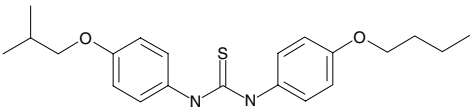
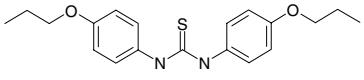
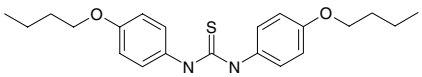
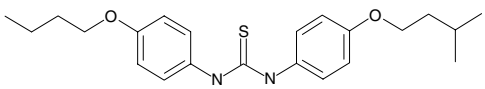
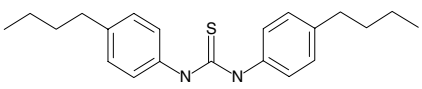
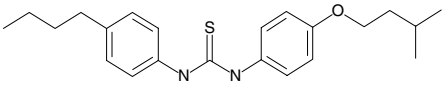
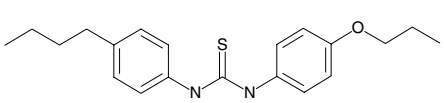
Table 1. Structures, in vitro and in vivo results of isoxyl analogues[20–22]

Compound	Structure	Yield	*M. tb* MIC_{99}[a] (μg/ml)[18]	*M. bovis* BCG MIC_{99}[a] (μg/ml)[18]	FAMEs and MAMEs			Oleate inhibition[e,19]	Cytoxicity SI[23]
					α[b]	Keto[c]	FA[d]		
1		53%	2.0	1.1	60.45	58.63	114.12	61.02	82
2		30%	ND[f]	0.9	46.37	45.83	110.36	51.62	ND[f]
3		55%	0.39	0.9	66.45	42.64	168.36	19.24	72
4		47%	0.1	0.6	56.65	36.50	132.26	63.99	79.9
5		35%	0.2	0.5	49.50	41.95	114.40	90.76	44
6		35%	ND[f]	0.5	43.73	33.92	123.10	94.25	ND[f]
7		55%	<0.1	0.4	61.27	44.05	136.04	79.96	89
8		52%	ND[f]	0.075	62.66	33.98	132.86	NI[g]	ND[f]
9		65%	ND[f]	0.6	38.78	30.51	121.47	14.82	ND[f]
10		85%	<0.1	0.06	53.79	37.40	130.65	NI[g]	100

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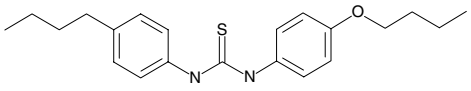
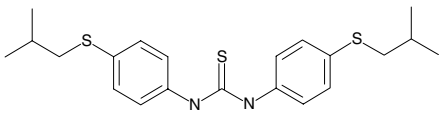
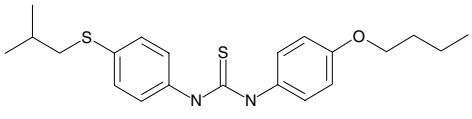
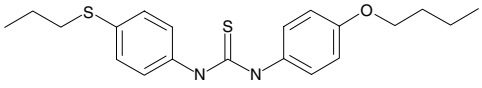
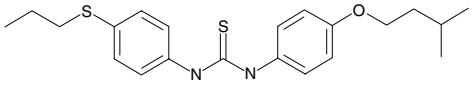
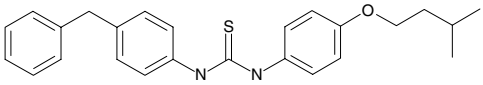
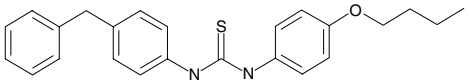
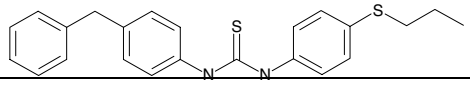
11		84%	<0.1	0.7	45.51	26.90	118.61	NI[g]	87
12		70%	0.78	0.6	62.25	27.39	104.41	NI[g]	12.4

Table 1 (*continued*)

Compound	Structure	Yield	*M. tb* MIC_{99}[a] (μg/ml)[18]	*M. bovis* BCG MIC_{99}[a] (μg/ml)[18]	FAMEs and MAMEs			Oleate inhibition[e,19]	Cytotoxicity SI[23]
					α[b]	Keto[c]	FA[d]		
13		59%	>6.25	0.7	54.42	35.76	131.38	17.75	ND[f]
14		48%	0.2	0.06	46.50	34.60	142.38	NI[g]	45.5
15		45%	ND[f]	0.5	88.84	66.09	183.79	53.32	ND[f]
16		50%	0.2	1.1	50.79	32.56	147.92	48.83	48
17		57%	0.39	1.5	54.94	39.12	140.70	66.15	22.1
18		48%	1.56	1.75	62.54	41.69	158.83	NI[g]	5.28

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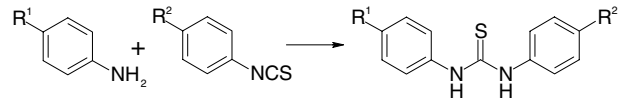
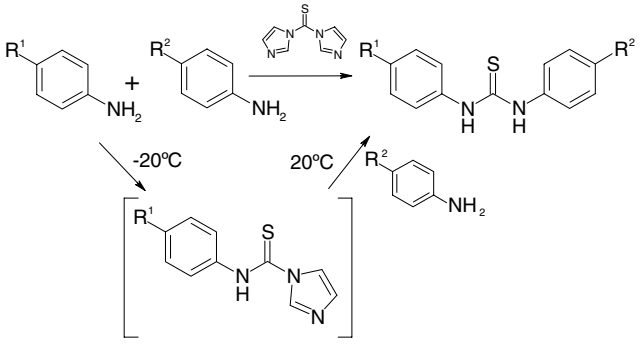
amine under acidic reflux conditions. The resulting product is coupled using ethanolic carbon disulfide, catalyzed by sulfur to generate the symmetrical ISO analogues. The products were recrystallized and fully characterized by NMR (^{1}H and ^{13}C) and mass spectrometry (**1**–**2** and **5**–**6**) (Table 1).

Unsymmetrical ISO analogues were synthesized by two methods. Isothiocyanate groups can be coupled with amines to generate the isothiourea subunit indicative of ISO analogues (Scheme 1). Commercially available 4-*n*-butylphenyl isothiocyanate was used to generate three novel unsymmetrical ISO analogues



Scheme 1.



Scheme 2.

(**9**–**11**) (Table 1). 4-*n*-Butylphenyl isothiocyanate is coupled with an amine substituent in ethanol at 50 °C and the products recrystallized from hot ethanol. These compounds (**9**–**11**) were fully characterized by NMR (^{1}H and ^{13}C) and mass spectrometry. More recently, Sriram et al. prepared a series of isonicotinyl hydrazones by reacting isonicotinyl hydrazide (INH) with thiourea units derived from various phenyl isothiocyanate substituents.[16]

Due to the lack of commercially available isothiocyanate substituents a second method was developed (Scheme 2). Perkins et al. coupled two different amine substituents with 1,1-thiocarbonyldiimidazole, by controlling the temperature of the reaction.[17] In a one-pot reaction the first equivalent of the amine substituent is reacted with 2 equiv of 1,1-thiocarbonyldiimidazole at −20 °C in acetonitrile. Once the amine has fully reacted, the second equivalent of a different amine is added and the temperature of the reaction is raised to 20 °C. The desired products were recrystallized and fully characterized by NMR (^{1}H and ^{13}C) and mass spectrometry (**3**, **4**, **7** and **13**–**21**) (Table 1).

As shown by Phetsuksiri et al. ISO is an anti-mycobacterial agent implicated in targeting the membrane-bound Δ9-desaturase, DesA3 and unknown targets in mycolic acid biosynthesis.[10] Therefore, aryl thioureas present themselves as anti-tuberculosis drugs worthy of further development.

The use of an ISO analogue without functional groups attached to the core thiourea unit (**22**) clearly showed that, without modification, poor in vivo and in vitro activity was observed; therefore the functional R^1 and R^2-groups of ISO play a key role in the effectiveness of the compound. The incorporation of R-group functions (see Table 1) leads to an increase in activity of the compounds and gives justification for functionality at both positions. The use of a biphenyl at one or both of the positions (**16**–**18**) was also tolerated in comparison to ISO in terms of in vivo activity. The incorporation of biphenyl to both positions (**19**) produced poorer in vivo activity in comparison to ISO (**1**), however inhibition was shown against, both mycolate and oleate production. Interestingly the unsymmetrical biphenyl ISO (**18**) failed to inhibit oleate production leading to the possible conclusion that this compound does not inhibit DesA3 desaturase activity but some other enzyme specifically involved in the production of mycolic acids.

Numerous compounds in this study have similar if not better activities than that of ISO (see Table 1). Notably, the use of an aliphatic C_4 functionality to either or both R^1 and R^2-positions increased the potency of the inhibitor (**4**, **7**–**8**, **10**–**11** and **14**). Compound **10** has been shown to be the most effective inhibitor in this study possessing a significant 10-fold increase in potency against both *M. bovis* and *M. tuberculosis* H37Rv. It shows similar effects with regard to inhibition of mycolate production, but unusually does not inhibit oleate production in vitro. This leads to the possibility that compound **10** does not inhibit the Δ9-desaturase specifically, but inhibits other enzymes involved in the production of mycolic acids.

Interestingly, the oxygen containing compounds produced in this study indicate that oxygen is required for a more effective inhibitor of oleate biosynthesis. Nearly all the compounds that have one or no oxygens tend to be poor inhibitors of oleate biosynthesis, whereas compounds containing two oxygens (**2**–**7**) inhibit oleate biosynthesis, to the same degree if not better than ISO (**1**). Compounds **5**–**7** all showed a high degree of activity against the Δ9-desaturase inhibitory oleate biosynthesis in vitro 91%, 94% and 80%, respectively.

In conclusion, the analogues in this study have provided an insight into the functionalities and their roles in inhibition of ISO. The new analogues **10** and **11** have all show improved activities against *M. tuberculosis* to such a degree that the MIC values are nearly the same or better than those of other well-known anti-tuberculosis inhibitors, such as isoniazid and rifampin.

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17. Perkins, J. J.; Zartman, A. E.; Meissner, R. S. *Tetrahedron Lett.* **1999**, *40*, 1103.
18. *Minimum inhibition concentration (MIC$_{99}$) and whole cell radiolabelling*. MIC_{99} of ISO analogues against *M. bovis* BCG were calculated by growth on solid media and whole cell radiolabelling are as described previously.[9,10]
19. *In vitro effect of ISO on oleic acid synthesis*. The wild type *M. bovis* BCG strain was grown in Sauton medium supplemented with 0.025% tyloxapol. Cells were harvested by centrifugation, resuspended in 0.25 M sucrose, pulse-disrupted by probe sonication and centrifugation at 27,000*g* for 30 min at 4 °C. The Δ9-stearoyl-CoA desaturase activity was assayed as described.[10]
20. *1,3-Bis-(p-2-methylpropoxyphenyl)thiourea*. A solution of 4-(2-methylpropoxy)aniline (500 mg, 3.2 mmol, 1 equiv) in ethanol (50 ml) was treated with carbon disulfide (0.18 ml, 3.2 mmol, 1 equiv) and sulfur (28 mg, 0.8 mmol, 0.25 equiv), and heated under reflux for 16 h. The ethanol was removed in vacuo to yield the crude product as an off-white solid that was recrystallized from methanol, to yield the title compound in 30% yield (100 mg). ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H: 0.98 (d, 12H, J = 6.6 Hz, $CH(CH_3)_2$), 2.12 (m, 2H, $CH(CH_3)_2$), 3.72 (d, 4H, J = 6.5 Hz, OCH_2CH), 6.90 (d, 4H, J = 8.8 Hz, H-3, H-5), 7.37 (d, 4H, J = 8.8 Hz, H-2, H-6), 7.71 (s, 2H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_c: 21.30 ($CH(CH_3)_2$), 29.40 ($CH(CH_3)_2$), 75.9 (OCH_2CH_3), 116.41 (C-3, C-5), 128.5 (C-2, C-6), 135.2 (C-1), 158 (C-4), 181 (NHCSNH) *m/z* (EI) 372 (M^+ 33%), 109 (MH^{2+}–$CH_2CH(CH_3)_2$ and $CSNHPhOCH_2CH(CH_3)_2$, 100%); HRMS calcd for $C_{21}H_{28}N_2O_2S$ (MH^+): 395.1769, found 395.1783; mp 156–158 °C.
21. *1-(p-n-Butylphenyl)-3-(4-n-butoxy-phenyl) thiourea*. 4-*n*-Butylphenyl isothiourea (1 ml, 5.8 mmol, 1 equiv) was added to ethanol (30 ml) at room temperature before addition of 4-butoxyaniline (0.96 g, 5.8 mmol, 1 equiv). After stirring at 50 °C for 2 h, the reaction mixture is cooled to room temperature and the crude product crashed out. The title compound was recrystallized using hot ethanol to give 84% yield (1.75 g). ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H: 0.85 (t, 3H, J = 7.4 Hz, $OCH_2CH_2CH_2CH_3$), 1.05 (t, 3H, J = 7.1 Hz, $CH_2CH_2CH_2CH_3$), 1.25 (m, 2H, CH_2CH_3), 1.30–1.50 (m, 2H, $CH_2CH_2CH_3$), 1.70–1.85 (m, 2H, $OCH_2CH_2CH_2$), 2.60(m, 2H, $CH_2CH_2CH_2CH_3$), 3.85 (t, 2H, J = 6.5 Hz, OCH_2), 6.75 (d, 2H, J = 8.4 Hz, H-3′, H-5′), 7.15–7.25 (m, 6H, H-2, H-6, H-3, H-5, H-2′, H-6′), 7.75 (s, 2H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_c: 10.2 (CH_2CH_3), 13.6 (CH_2CH_3), 22.0 (CH_2CH_3), 22.2 (CH_2CH_3), 33.2 ($CH_2CH_2CH_2$), 34.9 ($OCH_2CH_2CH_3$), 69.5 (OCH_2), 115.0 (C-3′, C-5′), 125.1 (C-3, C-5), 127.3 (C-2, C-6), 129.2 (C-2′, C-6′, C-4), 135.5 (C-1), 141.1 (C-4), 159.2 (C-1′), 180.3 (NHCSNH); *m/z* (EI) 379.4 [M^+ Na^+] (75%); HRMS calcd for $C_{21}H_{28}N_2OS$ (MH^+): 354.1653, found 354.1645; mp 137–140 °C.
22. *1-(p-n-Butoxyphenyl)-3-(p-methylbutoxy-phenyl)thiourea*. 1,1-Thiocarbonyldiimidazole (434 mg, 2.42 mmol, 2 equiv) is dissolved in anhydrous acetonitrile and left at −20 °C, while 4-(3-methylbutoxy)aniline (200 mg, 1.2 mmol, 1 equiv) was added dropwise to the solution. The reaction is monitored by TLC until all the aniline substituent is consumed. The second substituent is added, 4-butoxyaniline (200 mg, 1.2 mmol, 1.1 equiv) is then added dropwise and left to come to room temperature gradually. The acetonitrile is reduced in vacuo and the organic layer is extracted with ethyl acetate. This is acidified to pH 2 with 1 M HCl. The organic layer is then washed with water, brine, dried and reduced in vacuo. The compound is recrystallized cold in ethyl acetate in petrol to give the title compound in 55% yield (232 mg). ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H: 0.95–1.05 (m, 9 H, CH_3), 1.40 (m, 2H, CH_2CH_3), 1.65 (m, 2H, $CH_2CH(CH_3)_2$), 1.70–1.80 (m, 3H, $CH(CH_3)_2$ and $CH_2CH_2CH_3$), 3.95 (m, 4H, OCH_2), 6.40 (d, 4H, J = 6.5 Hz, H-3, H-5), 6.60 (d, 4H,J = 6.7 Hz, H-2, H-6), 7.65 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_c: 14.0 (CH_3), 20.1 (CH_2CH_3), 24.5 ($CH(CH_3)_2$), 25.7 ($CH(CH_3)_2$), 35.6 ($CH_2CH_2CH_3$ and $CH_2CH(CH_3)_2$), 70.9 (OCH_2), 115.6 (C-3, C-5), 127.8 (C-2, C-6), 131.2 (C-4), 155.6 (C-1), 180.4 (NHCSNH); *m/z* (EI) 409.4 [M^+ Na^+] (75%); HRMS calcd for $C_{22}H_{30}N_2O_2S$ (MH^+): 386.5514, found 386.5569; mp 145–147 °C.
23. Compounds were screened by serial dilution to assess toxicity to a VERO cell line, generally beginning at 10× the MIC. Selectivity index (SI) has defined as the ratio of the measured IC_{50} in VERO cells to the MIC value.