Med Chem Res
DOI 10.1007/s00044-013-0645-x

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Pharmacophore combination as a useful strategy to discover new antitubercular agents

Dharmarajsinh N. Rana · Mahesh T. Chhabria · Nisha K. Shah · Pathik S. Brahmkshatriya

Received: 4 March 2013 / Accepted: 22 May 2013

Abstract The present study is aimed at combining two well-known pharmacophores (pyrazoline and benzoxazole nucleus) to design and synthesize a series of substituted pyrazoline-based benzoxazole derivatives. In vitro antitubercular evaluation against *Mycobacterium tuberculosis* H37Rv, multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) strains showed that most of the target compounds displayed potent activity (MIC ~1.25–25 μg/mL) where few compounds were found to be better than isoniazid against MDR-TB (MIC = 3.25 μg/mL) and XDR-TB (MIC = 12.5 μg/mL). Cytotoxicity assay of these active compounds in VERO cell lines displayed good selectivity index. In order to gain insights into the plausible binding motifs, the target compounds were docked into enoyl-acyl carrier protein reductase, a molecular target of isoniazid. All the docked compounds occupied the same hydrophobic binding pocket and interacted mostly by dispersion interactions. Contribution of the three pharmacophoric fragments (pyrazoline, benzoxazole and aryl ring) toward protein–ligand binding was evaluated at semi empirical quantum mechanics level. The interaction energies suggested that most of the binding was governed by the benzoxaxole moiety followed by pyrazoline and aryl rings.

Keywords Antitubercular · Benzoxazole · Interaction energy · Molecular docking · Pharmacophore · Pyrazoline

Introduction

The designation of tuberculosis as a global public health crisis by the World Health Organization (WHO) in the mid-1990s has underscored the severe challenges facing the research community globally. It has been estimated by the WHO that almost one-third of the world's population, around 2 billion people, are infected with the disease (Dye *et al*., 2009). In 2002, the WHO estimated that if the worldwide spread of tuberculosis was left unchecked, then the disease would be responsible for approximately 36 million more deaths by the year 2020. Further, the emergence of multidrug-resistant TB (MDR-TB), a form of TB that does not respond to the first-line TB drugs and extensively drug-resistant TB (XDR-TB), an MDR-TB with resistance to fluoroquinolones and aminoglycosides, have become a serious threat to TB control and its treatment. On top of this, the recent cases of totally drug-resistant tuberculosis has raised alarming concerns on the existing drug regimen implying urgent need to discover newer antitubercular agents with newer molecular mechanisms (Udwadia *et al*., 2012).

Electronic supplementary material The online version of this article (doi:10.1007/s00044-013-0645-x) contains supplementary material, which is available to authorized users.

D. N. Rana · N. K. Shah (✉)
Department of Chemistry, Gujarat University, Ahmedabad 380 009, Gujarat, India
e-mail: nishchem2004@yahoo.co.in

M. T. Chhabria
Department of Pharmaceutical Chemistry, L. M. College of Pharmacy, Ahmedabad 380 009, Gujarat, India

P. S. Brahmkshatriya (✉)
Academy of Sciences of the Czech Republic and Center for Biomolecules and Complex Systems, Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic
e-mail: pathik26@yahoo.com

Published online: 02 June 2013

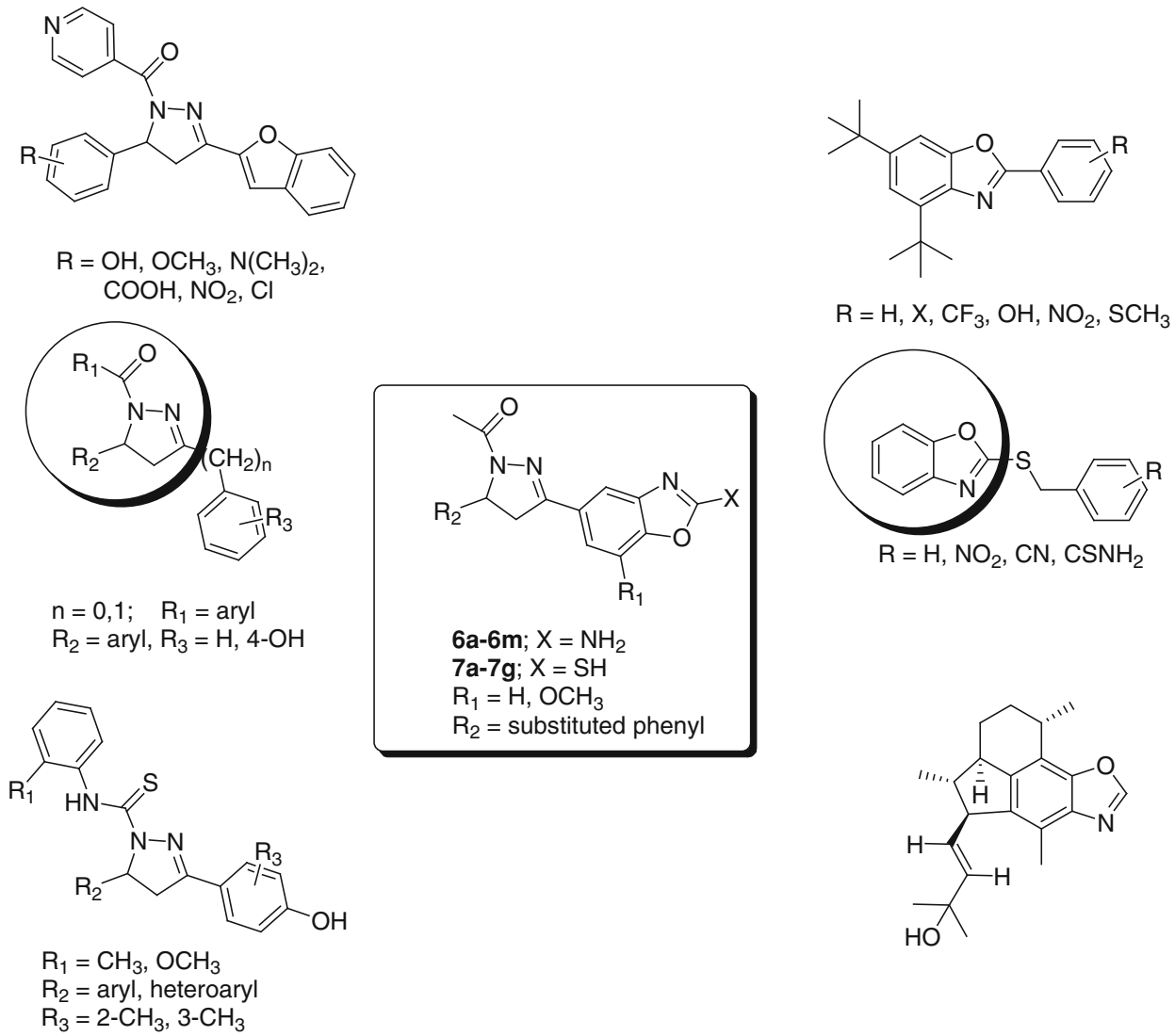


Fig. 1 Structures of some representative pyrazoline, benzoxazole derivatives, and designed compounds

The benzoxazole scaffold has provided the basis for the design of biologically relevant molecules with broad therapeutic importance. Several benzoxazole derivatives have been reported to possess antitubercular activity (Fig. 1, right pane) (Vinsova *et al*., 2006; Özlem *et al*., 2008; Klimesova *et al*., 2009; Ileana *et al*., 2006). Compounds with N–N bond are of importance primarily due to the known fact that living organism finds it difficult to construct N–N bonds which limits the natural abundance of compounds having such bonds. Pyrazoline and their derivatives, a class of compounds containing the N–N bond, exhibit a wide range of biological activities including antimycobacterial activity (Fig. 1, left pane) (Kuntal and Agrawal, 2010; Mohammad *et al*., 2006; Sharma *et al*., 2011). In light of the these facts, we thought of incorporating the two scaffolds to come out with chemically new antimycobacterial agents (Fig. 1, compound **6a**–**6m**, **7a**–**7g**).

Synthesized compounds (**6a**–**6m** and **7a**–**7g**) were screened for their antitubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$, MDR-TB and XDR-TB strains. To gain insights into the plausible binding interactions, we carried out docking of the designed compounds into crystal structure of *M. tuberculosis* enoyl reductase (InhA) (PDB Code 2H7I) (He *et al*., 2006). In consistent with our earlier work (Mohan *et al*., 2012), we chose Glide (Friesner *et al*., 2006) for the molecular docking as it performed better in finding the docked geometry closer to experimental geometry (root mean square deviation = 0.47 Å for the native ligand of 2H7I).

Materials and methods

Chemistry

*General procedure for the synthesis of 4′-hydroxy-3′-nitroacetophenone (**2a**)/4′-hydroxy-3′methoxy-5′-nitroacetophenone (**2b**)*

Respective acetophenone derivatives (**1a/1b**, 146 mmol) were dissolved in 200 mL of glacial acetic acid. To this reaction mixture, conc. H_2SO_4 (2 mL) was added drop wise and the reaction mixture was cooled to 5–10 °C. After 10 min, fuming nitric acid (7.5 mL) in glacial acetic acid (20 mL) was added dropwise to the reaction mixture. After completion of addition, cooling bath was removed and the reaction mixture was further stirred for 2 h at room temperature. Completion of the reaction is monitored on TLC using hexane: ethyl acetate (6:4) as eluent. After completion of the reaction, reaction mixture was poured in ice-water and the pale yellow residues were filtered and dried to get the crude product which was further purified by recrystallization from ethanol to give respective nitro derivatives (**2a/2b**) as pure product (Yoshida *et al*., 2007; Kiss *et al*., 2010).

*General procedure for the synthesis of 3-(aryl)-1-(4-hydroxy-3-nitrophenyl)prop-2-en-1-one (**3a–3e**)/3-(aryl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-one (**3f–3m**)*

4′-Hydroxy-3′-nitroacetophenone (**2a**)/4′-hydroxy-3′methoxy-5′-nitroacetophenone (**2b**) (27.6 mmol) and various substituted benzaldehydes were dissolved in 40 mL of methanol. To the reaction mixture, 25 % aqueous NaOH solution (40 mL) was added slowly not to increase the temperature above room temperature. After completion of the reaction, the resulting reaction mixture was stirred overnight at room temperature. Completion of the reaction was monitored on TLC using chloroform: methanol (9.5:0.5) as eluent. After completion of the reaction, the reaction mixture was poured in 5 N HCl solution with constant stirring. Yellow mass thus obtained was filtered at pump and dried to afford 3-(aryl)-1-(4-hydroxy-3-nitrophenyl)prop-2-en-1-ones (**3a–3e**)/3-(aryl)-1-(4-hydroxy-3-methoxy-5-nitrophenyl)prop-2-en-1-ones (**3f–3m**) in good practical yields (58–70 %) (Szell *et al*., 1964).

*General procedure for the synthesis of 4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-nitrophenol (**4a–4e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-methoxy-6-nitrophenol (**4f–4m**)*

The above synthesized chalcone derivatives (**3a–3m**, 6.1 mmol) were dissolved in 25 mL of glacial acetic acid. To the resulting reaction mixture, hydrazine hydrate solution (80 % w/v, 7.3 mmol) was added dropwise to avoid frothing. After completion of the addition, the reaction mixture was refluxed for the 3 h. Completion of the reaction was monitored on TLC using chloroform: methanol (9.5:0.5) as eluent. After completion of the reaction, the reaction mixture was poured in ice-water to afford yellow precipitates which were filtered at pump and dried to get corresponding 4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-nitrophenols (**4a–4e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-methoxy-5-nitrophenols (**4f–4m**) in good practical yields (see Supplementary information for their characterization data).

*General procedure for the synthesis of 4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminophenol (**5a–5e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-amino-6-methoxyphenol (**5f–5m**)*

4-(1-Acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-nitrophenol (**4a–4e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-methoxy-5-nitrophenols (**4f–4m**, 2.6 mmol) were dissolved in 25 mL of methanol. The reaction mixture was warmed up to 55–60 °C after which solid sodium dithionite (13.0 mmol) was added portion wise at an interval of 2–3 min to avoid vigorous frothing. The color of the reaction slowly turned orange to colorless with the progress of the reaction. The resulted reaction mixture was then further refluxed for an additional half an hour to insure the complete reduction of the nitro group. Completion of the reaction was monitored on TLC using hexane: ethyl acetate (6:4) as eluent. After completion of the reaction, the reaction mixture was poured in ice-water with constant stirring to afford off-white residues which were filtered at pump and dried to afford respective 4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminophenol (**5a–5e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-amino-5-methoxyphenol (**5f–5m**) in good practical yields (see Supplementary information for their characterization data).

*General procedure for the synthesis of 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**6a–6e**)/5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazoles (**6f–6m**)*

Potassium hydroxide (1.45 mmol) was dissolved in 15.0 mL of methanol and to the resulting solution was added carbon disulfide (1.45 mmol) drop wise. The reaction mixture slowly turned light yellow upon the addition of carbon disulfide. To this clear yellow solution, 4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminophenol derivatives (**5a–5e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-amino-5-methoxyphenol derivatives (**5f–5m**) (1.26 mmol) was added and the resulting reaction mixture was then stirred

for 15 min to bring about the complete dissolution of the starting material. This clear solution was then refluxed for next 3 h. Hydrogen sulfide gas evaluates during the course of the reaction. Completion of the reaction was monitored on TLC using hexane: ethyl acetate (5:5) as eluent. After completion of the reaction, the reaction mixture was poured in 2 N HCl solution with constant stirring. Off-white residues thus obtained were filtered at pump and dried to get the crud product which was further purified by recrystallization from ethanol to afford corresponding 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**6a**–**6e**)/5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazoles (**6f**–**6m**).

*5-(1-Acetyl-5-(3,4-dimethoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**6a**)* Yield: 75.6 %; MP: 141–144 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.782 (s, 1H), 7.611-7.589 (d, 2H, $J = 8.52$ Hz), 7.375–7.354 (d, 1H, $J = 8.32$ Hz), 6.835–6.815 (d, 1H, $J = 8.08$ Hz), 6.754–6.726 (d, 2H, $J = 9.32$ Hz), 5.559–5.518 (dd, 1H, $J_{HH''} = 4.56$ Hz, $J_{HH'} = 11.72$ Hz), 3.825 (s, 3H), 3.813 (s, 3H), 3.463–3.428 (quartet, 1H, $J_{H'H''} = 17.74$ Hz, $J_{HH'} = 11.51$ Hz), 3.195–3.139 (dd, 1H, $J_{H'H''} = 17.84$ Hz, $J_{HH''} = 4.68$ Hz), 2.391 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 22.41, 40.15, 60.12, 73.81, 104.26, 113.42, 116.49, 122.78, 122.97, 124.04, 126.42, 147.11, 149.54, 152.26, 153.44, 155.06, 156.10, 167.34, 180.67; ESI–MS: 398.2 (M + 1).

*5-(1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**6b**)* Yield: 80.1 %; MP: 128–130 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.829 (s, 1H), 7.625–7.599 (dd, 1H, $J = 8.44$ Hz, $J = 1.6$ Hz), 7.578–7.575 (d, 1H, $J = 1.3$ Hz), 7.413–7.392 (d, 2H, $J = 8.48$ Hz), 7.134–7.112 (d, 2H, $J = 8.68$ Hz), 6.845–6.823 (d, 1H, $J = 8.72$ Hz), 5.545–5.505 (dd, 1H, $J_{HH''} = 4.44$ Hz, $J_{HH'} = 11.72$ Hz), 3.857–3.783 (quartet, 1H, $J_{H'H''} = 17.84$ Hz, $J_{HH'} = 11.80$ Hz), 3.751 (s, 3H), 3.169–3.113 (dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 4.60$ Hz), 2.347 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.17, 40.28, 60.19, 74.20, 104.28, 113.49, 116.12, 123.01, 124.35, 126.29, 127.65, 147.11, 150.92, 153.86, 156.18, 167.41, 180.62; ESI–MS: 368.1 (M + 1).

*5-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**6c**)* Yield: 80.0 %; MP: 111–114 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.841 (s, 1H), 7.638–7.614 (dd, 1H, $J = 8.48$ Hz, $J = 1.48$ Hz), 7.572 (s, 1H), 7.448–7.418 (m, 2H), 7.274–7.251 (m, 2H), 7.076–7.053 (m, 1H), 5.848–5.806 (dd, 1H, $J_{HH''} = 4.96$ Hz, $J_{HH'} = 11.92$ Hz), 3.991–3.916 (quartet, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH'} = 11.96$ Hz), 3.109–3.052 (dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 4.96$ Hz), 2.406 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.25, 40.37, 74.17, 104.39, 116.48, 117.48, 125.07, 125.26, 125.83, 126.48, 135.33, 136.00, 136.67, 139.82, 149.76, 156.35, 167.77, 180.43; ESI–MS: 372.1 (M + 1, 100 %), 374.1 (30 %).

*5-(1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**6d**)* Yield: 65.0 %; MP: 119-121 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.617 (s, 1H), 7.623–7.598 (dd, 1H, $J = 8.44$ Hz, $J = 1.56$ Hz), 7.586 (s, 1H), 7.396–7.375 (d, 1H, $J = 8.44$ Hz), 7.325–7.286 (t, 1H), 7.245–7.225 (dd, 1H, $J = 1.2$ Hz, $J = 6.28$ Hz), 7.201–7.193 (t, 1H), 7.144–7.125 (d, 1H, $J = 7.6$ Hz), 5.589–5.548 (dd, 1H, $J_{HH''} = 4.8$ Hz, $J_{HH'} = 11.88$ Hz), 3.905–3.830 (quartet, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH'} = 11.96$ Hz), 3.201–3.144 (dd, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH''} = 4.88$ Hz), 2.388 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 22.14, 42.62, 78.85, 106.83, 115.58, 128.81, 128.87, 129.69, 136.60, 137.30, 140.65, 142.43, 144.09, 156.45, 167.25, 180.61; ESI–MS: 372.1 (M + 1, 100 %), 374.4 (35 %).

*5-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**6e**)* Yield: 70.7 %; MP: 127-129 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.722 (s, 1H), 7.616–7.591 (dd, 1H, $J = 8.48$ Hz, $J = 1.56$ Hz), 7.577 (s, 1H), 7.395–7.374 (d, 1H, $J = 8.48$ Hz), 7.312–7.291 (d, 2H, $J = 8.44$ Hz), 7.205–7.183 (d, 2H, $J = 8.48$ Hz), 5.589–5.548 (dd, 1H, $J_{HH''} = 4.72$ Hz, $J_{HH'} = 11.88$ Hz), 3.895–3.821 (quartet, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH'} = 11.96$ Hz), 3.177–3.121 (dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 4.84$ Hz), 2.372 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 22.19, 44.01, 78.72, 106.89, 115.11, 129.09, 129.31, 136.91, 137.87, 140.26, 143.12, 151.63, 156.39, 167.46, 180.74; ESI–MS: 372.2 (M + 1, 100 %), 374.2 (35 %).

*5-(1-Acetyl-5-(3,4-dimethoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6f**)* Yield: 82.4 %; MP: 165–167 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.825 (s, 1H), 7.251–7.249 (d, 1H, $J = 0.96$ Hz), 7.137–7.135 (d, 1H, $J = 1.04$ Hz), 6.844–6.823 (d, 1H, $J = 8.32$ Hz), 6.768–6.763 (d, 1H, $J = 1.88$ Hz), 6.729–6.704 (dd, 1H, $J = 8.48$ Hz, $J = 1.92$ Hz), 5.544–5.503 (dd, 1H, $J_{HH''} = 4.56$ Hz, $J_{HH'} = 11.72$ Hz), 4.026 (s, 3H), 3.798 (s, 3H), 3.783 (s, 3H), 3.551–3.498 (quartet, 1H, $J_{H'H''} = 14.0$ Hz, $J_{HH'} = 7.0$ Hz), 3.211–3.155 (dd, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH''} = 4.68$ Hz), 2.369 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.16, 44.74, 59.70, 60.87, 78.82, 106.54, 112.22, 112.87, 115.76, 122.05, 125.41, 137.34, 140.11, 141.38, 141.49, 147.71, 149.39, 156.45, 167.23, 180.60; ESI–MS: 428.2 (M + 1).

*5-(1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6g**)* Yield: 75.9 %; MP: 150–154 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.803 (s, 1H), 7.249 (s, 1H), 7.134–7.114 (d, 3H, $J = 8.12$ Hz), 6.847–6.826 (d, 2H, $J = 8.60$ Hz), 5.547–5.507 (dd, 1H, $J_{HH''} = 4.44$ Hz, $J_{HH'} = 11.8$ Hz), 4.030 (s, 3H), 3.849–3.775 (quartet, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH'} = 11.88$ Hz), 3.754 (s, 3H), 3.184–3.123 (dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 4.56$ Hz), 2.356 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.16, 43.91, 59.74, 61.17, 78.39, 106.31, 115.48, 116.26, 129.83, 133.06, 134.48, 141.04, 143.00, 143.36, 156.67, 167.43, 180.77; ESI–MS: 398.4 (M + 1).

*5-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6h**)* Yield: 70.5 %; MP: 126–129 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.795 (s, 1H), 7.447–7.405 (m, 1H), 7.274–7.224 (m, 3H), 7.117–7.115 (d, 1H, $J = 0.92$ Hz), 7.071–7.048 (m, 1H), 5.863–5.821 (dd, 1H, $J_{HH''} = 4.96$ Hz, $J_{HH'} = 11.96$ Hz), 4.019 (s, 3H), 3.964–3.889 (quartet, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH'} = 12.0$ Hz), 3.120–3.063 (dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 5.0$ Hz), 2.423 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.16, 44.74, 59.17, 77.39, 106.31, 115.48, 127.26, 129.73, 129.86, 131.48, 133.06, 136.33, 137.67, 137.82, 143.78, 145.35, 149.93, 156.49, 167.51, 180.65; ESI–MS: 402.3 (M + 1, 100 %), 404.1(35 %).

*5-(1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6i**)* Yield: 83.9 %; MP: 123–125 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.785 (s, 1H), 7.325–7.289 (m, 1H), 7.244–7.196 (m, 3H), 7.143–7.120 (m, 2H), 5.589–5.548 (dd, 1H, $J_{HH''} = 4.6$ Hz, $J_{HH'} = 11.88$ Hz), 4.032 (s, 3H), 3.893–3.819 (quartet, 1H, $J_{H'H''} = 17.96$ Hz, $J_{HH'} = 12.0$ Hz), 3.222–3.165 (dd, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH''} = 4.7$ Hz), 2.392 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.14, 44.85, 59.91, 78.66, 106.38, 115.71, 126.17, 126.24, 128.89, 128.20, 133.09, 134.84, 142.13, 144.93, 149.60, 156.47, 167.75, 180.73; ESI–MS: 402.1 (M + 1, 100 %), 404.1 (20 %).

*5-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6j**)* Yield: 78.1 %; MP: 136–138 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.840 (s, 1H), 7.323–7.301 (d, 2H, $J = 8.44$ Hz), 7.238–7.236 (d, 1H, $J = 0.96$ Hz), 7.210–7.189 (d, 2H, $J = 8.44$ Hz), 7.121–7.119 (d, 1H, $J = 0.88$ Hz), 5.587–5.545 (dd, 1H, $J_{HH''} = 4.76$ Hz, $J_{HH'} = 11.88$ Hz), 4.022 (s, 3H), 3.895–3.819 (quartet, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH'} = 11.96$ Hz), 3.206–3.149 (dd, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH''} = 4.84$ Hz), 2.363 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.21, 45.55, 60.90, 78.63, 106.41, 115.66, 131.37, 133.15, 136.99, 138.36, 138.87, 141.27, 144.05, 149.67, 156.51, 167.43, 180.72; ESI–MS: 402.2 (M + 1, 100 %), 404.1 (35 %).

*5-(1-Acetyl-5-(2,5-Dimethoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6k**)* Yield: 62.0 %; MP: 156–160 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.812 (s, 1H), 7.206–7.204 (d, 1H, $J = 1.0$ Hz), 7.103–7.100 (d, 1H, $J = 1.12$ Hz), 6.894–6.872 (d, 1H, $J = 8.88$ Hz), 6.764–6.734 (dd, 1H, $J = 3.0$ Hz, $J = 8.84$ Hz), 6.472–6.464 (d, 1H, $J = 2.96$ Hz), 5.718–5.676 (dd, 1H, $J_{HH''} = 4.56$ Hz, $J_{HH'} = 11.76$ Hz), 4.008 (s, 3H), 3.807 (s, 3H), 3.807–3.743 (quartet, 1H, $J_{H'H''} = 17.76$ Hz, $J_{HH'} = 11.84$ Hz), 3.676 (s, 3H), 3.050–2.994 (dd, 1H, $J_{H'H''} = 18.0$ Hz, $J_{HH''} = 4.8$ Hz), 2.391 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.26, 43.33, 59.38, 60.66, 60.90, 78.34, 106.52, 115.08, 117.62, 129.47, 133.38, 142.63, 144.60, 145.32, 145.53, 149.37, 156.70, 168.57, 180.61; ESI–MS: 428.1 (M + 1).

*5-(1-Acetyl-5-(3-bromophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6l**)* Yield: 78.3 %; MP: 110–112 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.774 (s, 1H), 7.398–7.375 (d, 1H, $J = 8.84$ Hz), 7.353 (s, 1H), 7.263–7.224 (t, 2H), 7.183–7.164 (d, 1H, $J = 7.8$ Hz), 7.115–7.112 (d, 1H, $J = 1.24$ Hz), 5.579–5.537 (dd, 1H, $J_{HH''} = 4.8$ Hz, $J_{HH'} = 11.92$ Hz), 4.033 (s, 3H), 3.888–3.813 (quartet, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH'} = 12.0$ Hz), 3.218–3.161 (dd, 1H, $J_{H'H''} = 17.92$ Hz, $J_{HH''} = 4.88$ Hz), 2.394 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.14, 44.63, 59.41, 79.54, 106.09, 115.83, 124.78, 126.66, 126.30, 131.10, 133.99, 134.17, 137.81, 142.12, 143.49, 149.76, 156.62, 168.43, 180.79; ESI–MS: 446.1 (M + 1, 100 %), 448.0 (90 %).

*5-(1-Acetyl-5-(4-bromophenyl)-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**6m**)* Yield: 76.7 %; MP: 122–125 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.768(s, 1H), 7.461–7.440 (d, 2H, $J = 8.4$ Hz), 7.232–7.229 (d, 1H, $J = 1.2$ Hz), 7.146–7.125 (d, 2H, $J = 8.44$ Hz), 7.102–7.099 (d, 1H, $J = 1.16$ Hz), 5.573–5.531 (dd, 1H, $J_{HH''} = 4.72$ Hz, $J_{HH'} = 11.88$ Hz), 4.031 (s, 3H), 3.881–3.806 (quartet, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH'} = 12.0$ Hz), 3.188–3.132(dd, 1H, $J_{H'H''} = 17.88$ Hz, $J_{HH''} = 4.84$ Hz), 2.377 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.21, 45.76, 60.68, 78.95, 106.16, 115.37, 124.79, 133.16, 134.58, 137.95, 141.65, 141.77, 149.72, 156.41, 167.55, 180.77; ESI–MS: 446.0 (M + 1, 100 %), 447.9 (100 %).

*General procedure for the synthesis of 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole (**7a–7e**)/ 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenzoxazole (**7f–7g**)*

4-(1-Acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminophenol derivatives (**5a–5e**)/4-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-amino-5-methoxyphenol derivatives (**5f–5m**)

(1.26 mmol) was suspended in 20 mL of methanol–water (1:1). To the reaction mixture, a solution of cyanogen bromide (1.30 mmol) in 5 mL of methanol was added dropwise. The resulting solution was then stirred for next 30 min after which solid sodium bicarbonate was added to the reaction mixture and the reaction mixture was stirred for more 30 min. Completion of the reaction was monitored on TLC using hexane: ethyl acetate (2:8) as eluent. After completion of the reaction, the reaction mixture was poured in ice-water. Off-white residues thus obtained were filtered at pump and dried to yield the crude product which was further purified by recrystallization from ethanol to afford the corresponding 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**7a**–**7e**)/5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercapto-7-methoxybenz oxazoles (**7f**–**7g**).

*5-(1-Acetyl-5-(3,4-dimethoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazole (**7a**)* Yield: 93.0 %; MP: 171–173 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.608–7.604 (d, 1H, J = 1.0 Hz), 7.411–7.387 (dd, 1H, J = 8.24 Hz, J = 1.4 Hz), 7.311 (s, 2H), 7.267–7.246 (d, 1H, J = 8.28 Hz), 6.840–6.819 (d, 1H, J = 8.32 Hz), 6.778–6.773 (d, 1H, J = 1.88 Hz), 6.739–6.714 (dd, 1H, J = 1.92 Hz, J = 8.24 Hz), 5.518–5.478 (dd, 1H, $J_{HH''}$ = 4.36 Hz, $J_{HH'}$ = 11.48 Hz), 3.852–3.785 (quartet, 1H), 3.799 (s, 3H), 3.785 (s, 3H), 3.180–3.124 (dd, 1H, $J_{H'H''}$ = 17.8 Hz, $J_{HH''}$ = 4.6 Hz), 2.361 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.46, 42.08, 56.95, 56.49, 74.41, 108.48, 112.46, 112.38, 115.05, 121.61, 126.79, 127.00, 139.85, 145.52, 149.27, 149.49, 152.13, 154.33, 165.52, 169.35; ESI–MS: 381.0 (M + 1).

*5-(1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazole (**7b**)* Yield: 79.3 %; MP: 162–165 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.611 (s, 1H), 7.406–7.386 (d, 1H, J = 8.04 Hz), 7.263–7.241 (d, 2H), 7.143–7.123 (d, 2H, J = 8.0 Hz), 6.841–6.821 (d, 2H, J = 7.96 Hz), 5.511–5.490 (dd, 1H), 3.843–3.751 (quartet, 1H), 3.751 (s, 3H), 3.153–3.109 (broad, 1H), 2.347 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.07, 41.49, 55.09, 76.38, 114.97, 117.77, 123.45, 126.17, 131.39, 133.22, 139.59, 143.59, 144.37, 147.18, 154.39, 165.66, 169.41; ESI–MS: 350.9 (M + 1).

*5-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazole (**7c**)* Yield: 84.5 %; MP: 168–171 °C; 1H NMR (400 MHz, DMSO-d_6): δ 7.608 (s, 1H), 7.411–7.347 (m, 3H), 7.244 (broad hump, 4H), 7.146–7.072 (m, 2H), 5.844–5.827 (dd, 1H), 3.962–3.886 (quartet, 1H), 3.140–3.028 (dd, 1H), 2.428 (s, 3H); 13C NMR (400 MHz, DMSO-d_6): δ 23.44, 42.83, 76.46, 115.71, 117.34, 125.26, 129.16, 129.82, 129.90, 139.52, 142.79, 144.72, 149.08, 152.35, 155.27, 165.61, 169.55; ESI–MS 355.2 (M + 1, 100 %), 357.2 (35 %).

*5-(1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazole (**7d**)* Yield: 80.0 %; MP: 174–176 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.604–7.601 (d, 1H, J = 1.24 Hz), 7.414–7.383 (m, 3H), 7.336–7.211 (m, 4H), 7.157–7.137 (d, 1H, J = 7.64 Hz), 5.559–5.518 (dd, 1H, $J_{HH''}$ = 4.68 Hz, $J_{HH'}$ = 11.8 Hz), 3.909–3.834 (quartet, 1H, $J_{H'H''}$ = 17.92 Hz, $J_{HH'}$ = 11.92 Hz), 3.192–3.136 (dd, 1H, $J_{H'H''}$ = 17.92 Hz, $J_{HH''}$ = 4.84 Hz), 2.361 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.51, 42.29, 76.90, 116.18, 121.59, 123.48, 126.54, 126.65, 127.99, 129.53, 133.07, 139.47, 142.45, 147.76, 149.41, 156.38, 165.67, 169.53; ESI–MS: 355.1 (M + 1, 80 %), 357.3 (10 %).

*5-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazole (**7e**)* Yield: 78.8 %; MP: °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.605 (s, 1H), 7.384–7.212 (m, 8H), 5.542 (broad, 1H), 3.833 (broad, 1H), 3.160–3.122 (dd, 1H), 2.361 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.49, 43.24, 76.63, 116.51, 121.12, 122.73, 129.50, 131.38, 133.75, 139.30, 142.35, 149.28, 151.23, 156.54, 166.76, 169.75; ESI–MS: 355.0 (M + 1, 100 %), 357.0 (35 %).

*5-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-3-pyrazolyl)-2-amino-7-methoxybenzoxazole (**7f**)* Yield: 87.1 %; MP: 140–143 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.493–7.428 (m, 3H), 7.316–7.262 (m, 3H), 7.161–7.159 (d, 1H, J = 1.1 Hz), 7.105–7.077 (m, 1H), 5.876–5.834 (dd, 1H, $J_{HH''}$ = 4.81 Hz, $J_{HH'}$ = 11.73 Hz), 4.049 (s, 3H), 3.927–3.852 (quartet, 1H, $J_{H'H''}$ = 17.76 Hz, $J_{HH'}$ = 11.77 Hz), 3.144–3.080 (dd, 1H, $J_{H'H''}$ = 17.76 Hz, $J_{HH''}$ = 4.85 Hz), 2.441 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.40, 43.87, 55.74, 75.23, 114.44, 116.59, 129.17, 133.31, 133.70, 134.46, 134.81, 139.36, 142.69, 144.71, 149.58, 155.63, 169.67; ESI–MS: 385.4 (M + 1, 100 %), 387.2 (30 %).

*5-(1-Acetyl-5-(4-bromophenyl)-4,5-dihydro-3-pyrazolyl)-2-amino-7-methoxybenzoxazole (**7g**)* Yield: 79.9 %; MP: 147–150 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.493–7.476 (d, 2H, J = 8.42 Hz), 7.256–7.253 (d, 1H, J = 1.08 Hz), 7.188–7.169 (d, 2H, J = 8.41 Hz), 7.119–7.116 (d, 1H, J = 1.1 Hz), 5.658–5.613 (dd, 1H, $J_{HH''}$ = 4.83 Hz, $J_{HH'}$ = 12.04 Hz), 4.028 (s, 3H), 3.914–3.847 (quartet, 1H, $J_{H'H''}$ = 17.73 Hz, $J_{HH'}$ = 11.99 Hz), 3.202–3.152 (dd, 1H, $J_{H'H''}$ = 17.73 Hz, $J_{HH''}$ = 4.81 Hz), 2.416 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 23.46, 44.15, 58.05, 78.21, 112.47, 116.96, 129.26, 131.10, 134.61, 137.63, 139.85, 143.31, 144.83, 147.44, 149.73, 152.11, 156.01, 166.59, 169.72; ESI–MS: 429.2 (M + 1, 100 %), 431.1 (100 %).

Antitubercular activity

The in vitro antitubercular activity was carried out by measuring the growth of *M. tuberculosis* ($H_{37}R_V$) using Lowensteine Jensen medium (L. J. medium) (Stover *et al*., 2000). In brief, eggs were broken aseptically to obtain 200 mL of egg solution. The solution was filtered through a sterile muslin cloth into a sterile conical flask containing glass beads. Sterilized mineral salt solution (120 mL) (consisting of 4.0 g potassium phosphate, 0.4 g of magnesium sulfate, 1.6 g magnesium citrate, 6.0 g of asparagine, 20 mL of glycerol, distilled water makeup up to 1,000 mL) and 4 mL of sterilized malachite green solution (2.0 %) were added to the 200 mL of egg solution. The contents were mixed well to form a uniform medium. Compounds (10 mg) were dissolved in 10.0 mL of dimethyl sulfoxide (DMSO) and were diluted with DMSO to make 250 and 10 mg/mL stock solutions. An aliquot (0.8 mL) of each concentration was transferred into different McCartney bottles. To this, 7.2 mL of L. J. medium was added and mixed well. Isoniazid was considered as a reference standard for the comparison of antitubercular activity. The drug was dissolved in DMSO and diluted as described above. The bottles were incubated at 75–80 °C for 3 days for solidification and sterilization.

Procedure for inoculation A sweep from MDR H_{37}Rv/extensively resistant strains of *M. tuberculosis* culture was transferred with the help of 22 S.W. nichrome wire loop of 3 mm external diameter into a sterile bijou bottle containing six 3 mm glass beads and 4 mL of sterile distilled water. Each loop of culture delivered approximately 4 mg of bacilli cells. The bottle was shaken with the help of vertex mixture for 2 min. The suspension was inoculated on the surface of each L. J. medium containing test compounds using 27 S. W. G nichrome wire loop of 3 mm external diameter and L. J. medium containing isoniazid. The medium containing DMSO (control) was inoculated with the test organism for positive and negative controls. Medium without any test compound/DMSO was also inoculated with the test organism to check whether the media supports the growth of the tubercle bacilli or not. The inoculated bottles were incubated at 37 °C for 6 weeks, at the end of which readings were taken. Bacterial counts were measured and compared with the standard drugs and controls (vehicle-treated).

Docking studies

The complex was subjected to preparation steps using the Protein Preparation Wizard in Maestro using the default settings. First, the waters beyond 5 Å from the ligand were removed, bond orders assigned and hydrogens added. Next, the orientation of amide (Asn and Gln), hydroxyl (Ser, Thr, and Tyr), and thiol groups (Cys), and the protonation and tautomeric state of the His residues were optimized using the exhaustive sampling option. A grid box of 20 × 20 × 20 $Å^3$ for the receptor was generated with a default inner box of 10 × 10 × 10 $Å^3$, which was centered on the corresponding ligand. The default parameters were used, and no constraints were included. Docking calculations were performed using the Glide Extra Precision (XP) algorithm (Friesner *et al*., 2006). In the protocol, Glide was set to determine the five best poses per ligand.

Interaction energies of the ligand fragments with enoyl-ACP reductase

The inhibitor structures were fragmented into three segments: pyrazoline ring, benzoxazole ring, and aryl ring. The separated side chains and the main chains were capped by hydrogens. PM6-D3H4X method developed in our laboratory (Brahmkshatriya *et al*., 2013) in implicit solvent model was used to calculate interaction energies.

Results and discussion

Chemistry

The title compounds, 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**6a**–**6m**) and 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**7a**–**7g**), were synthesized via multistep reactions from substituted acetophenones (**1a/1b**) as outlined in Fig. 2.

Nitration of acetophenone/acetovanilone (**1a/1b**) using fuming nitric acid in glacial acetic acid afforded respective nitro derivatives (**2a**–**2b**). Subsequent aldol condensation of the nitro derivatives (**2a**–**2b**) with various substituted benzaldehydes in the presence of sodium hydroxide yielded respective 3-aryl-1-(4-nitro-3-hydroxyphenyl)prop-2-en-1-ones (**3a**–**3m**). Cyclization of **3a**–**3m** with hydrazine hydrate in glacial acetic acid afforded corresponding 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-nitrophenols (**4a**–**4m**) in good yields. The nitro group was reduced by sodium dithionite to afford corresponding amino derivatives (**5a**–**5m**). Finally, cyclization of these o-aminophenol derivatives was carried out by two different ways: (a) treatment with carbon disulfide and potassium hydroxide at reflux temperature to afford 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazoles (**6a**–**6m**); and (b) treatment with cyanogen bromide in THF-water mixture at room temperature to yield corresponding 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazoles (**7a**–**7g**) (Table 1).

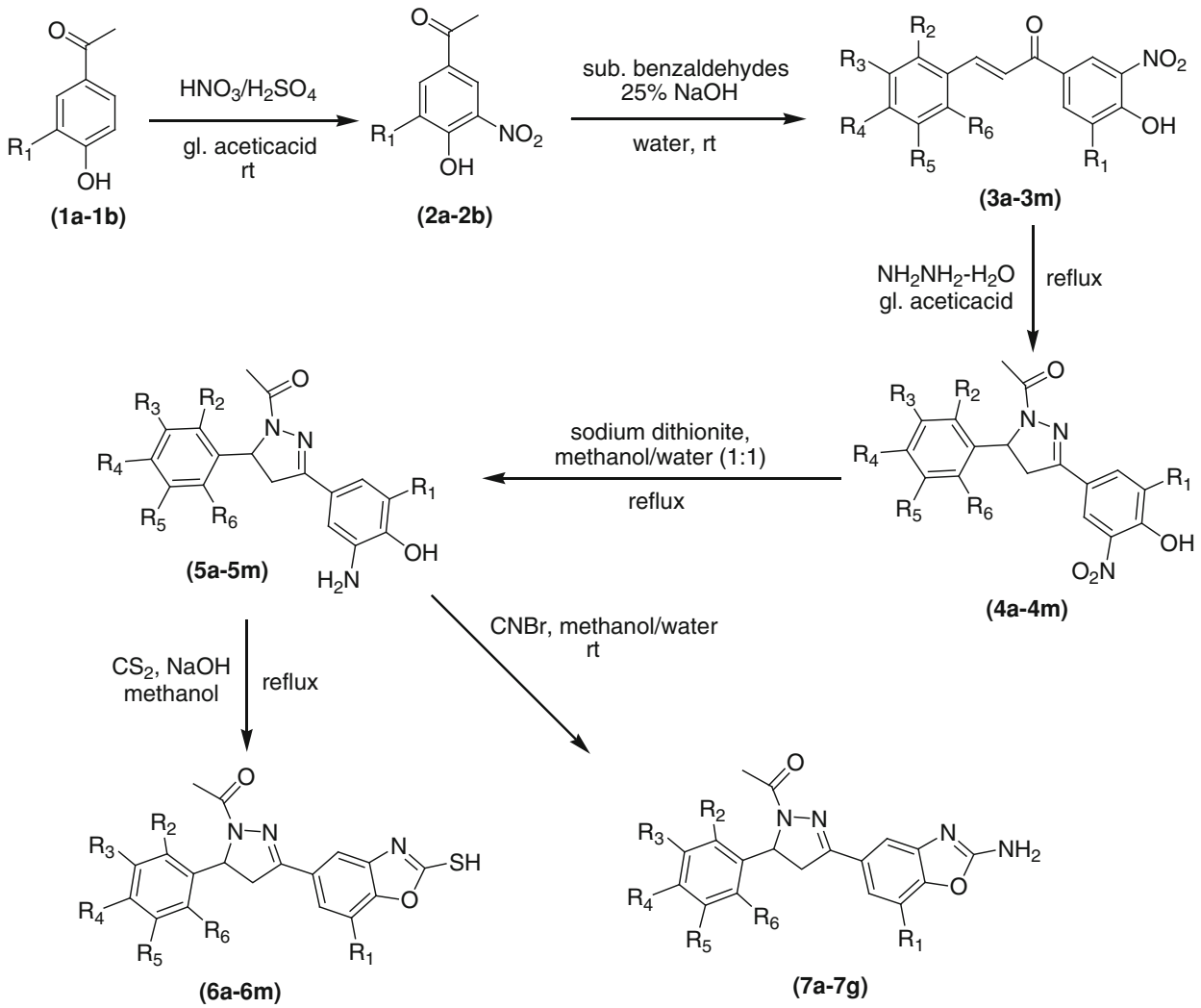
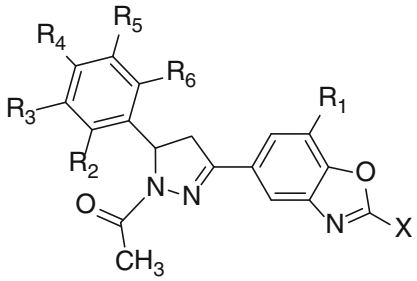


Fig. 2 Pathway for the synthesis of the title compounds

Antitubercular screening

Synthesized compounds (**6a**–**6m** and **7a**–**7g**) were screened for their antitubercular activity against *M. tuberculosis* $H_{37}Rv$, MDR-TB and XDR-TB strains. Table 1 shows the results of the biological screening. Isoniazid was also screened as a standard drug for its antitubercular activity against all $H_{37}Rv$, MDR-TB, and XDR-TB strains under similar experimental conditions. It was encouraging to see that majority of the compounds displayed satisfactory MIC values against *M. tuberculosis* $H_{37}Rv$, MDR-TB, and XDR-TB strains. Compounds **6d**, **6e**, **6j**, **6m,** and **6l** displayed potent activity against *M. tuberculosis* $H_{37}Rv$. However, none of the compounds was found to be potent than isoniazid against *M. tuberculosis* $H_{37}Rv$. Compounds **6b**, **6i**, **6l**, **7a**, **7e,** and **7g** were found to have potent activity against MDR-TB strain. Of these, **6b**, **6i**, and **7a** were found to be equipotent to isoniazid against MDR-TB strain (MIC = 6.25 μg/mL), and **6l**, **7e**, and **7g** were found to be more potent than isoniazid (MIC = 3.25 μg/mL). On the other hand compounds **6d**, **6l,** and **6m** were found to have potent activity against XDR-TB strain than isoniazid (MIC = 12.5 μg/mL). In order to rationalize the concept of "pharmacophore hybridization," it was important to compare the results with the literature activity of pyrazoline and benzoxazole derivatives. Indeed, we could obtain a satisfactory gain in the potency of the compounds when compared to some of the literature pyrazoline (Kuntal and Agrawal 2010; Mohammad *et al*., 2006) and benzoxazole (Vinsova *et al*., 2006; Klimesova *et al*., 2009) derivatives. Few selected compounds which showed potent activity against all *M. tuberculosis* H37Rv, MDR-TB and XDR-TB strains were also evaluated for their cytotoxicity in VERO cell lines (Cory *et al*., 1991). The results of cytotoxicity are

Table 1 Structures of the synthesized target compounds and their in vitro antitubercular activity and cytotoxicity in vero cell line



Comp. no.	X	R_1	R_2	R_3	R_4	R_5	R_6	MIC (μg/mL)			Cytotoxicity IC50 (μg/mL)
								$H_{37}Rv$	MDR-TB	XDR-TB	
6a	–SH	–H	–H	$–OCH_3$	$–OCH_3$	–H	–H	12.5	12.5	>100	ND
6b	–SH	–H	–H	–H	$–OCH_3$	–H	–H	6.25	6.25	50	ND
6c	–SH	–H	–Cl	–H	–H	–H	–H	6.25	12.5	>100	ND
6d	–SH	–H	–H	–Cl	–H	–H	–H	3.25	12.5	12.5	>62.5
6e	–SH	–H	–H	–H	–Cl	–H	–H	3.25	25	50	32.5
6f	–SH	$–OCH_3$	–H	$–OCH_3$	$–OCH_3$	–H	–H	6.25	12.5	50	ND
6g	–SH	$–OCH_3$	–H	–H	$–OCH_3$	–H	–H	12.5	12.5	50	ND
6h	–SH	$–OCH_3$	–Cl	–H	–H	–H	–H	6.25	50	25	ND
6i	–SH	$–OCH_3$	–H	–Cl	–H	–H	–H	25	6.25	25	ND
6j	–SH	$–OCH_3$	–H	–H	–Cl	–H	–H	3.25	25	50	>62.5
6k	–SH	$–OCH_3$	$–OCH_3$	–H	–H	–H	$–OCH_3$	12.5	50	50	>62.5
6l	–SH	$–OCH_3$	–H	–Br	–H	–H	–H	1.25	3.25	12.5	>62.5
6m	–SH	$–OCH_3$	–H	–H	–Br	–H	–H	3.25	25	12.5	32.5
7a	$–NH_2$	–H	–H	$–OCH_3$	$–OCH_3$	–H	–H	6.25	6.25	25	ND
7b	$–NH_2$	–H	–H	–H	$–OCH_3$	–H	–H	12.5	25	>100	ND
7c	$–NH_2$	–H	–Cl	–H	–H	–H	–H	25	25	50	ND
7d	$–NH_2$	–H	–H	–Cl	–H	–H	–H	25	50	50	ND
7e	$–NH_2$	–H	–H	–H	–Cl	–H	–H	6.25	3.25	>100	ND
7f	$–NH_2$	$–OCH_3$	–Cl	–H	–H	–H	–H	12.5	12.5	>100	ND
7g	$–NH_2$	$–OCH_3$	–H	–H	–Br	–H	–H	25	3.25	>100	ND
Isoniazid								0.5	6.25	50	ND

ND not determined

shown in Table 1. As seen from Table 1, most of the compounds displayed selectivity index (cytotoxicity IC50/MIC_{MTB}) >10 which implies that the compounds are safe and could be explored as potential lead for further development.

Docking study

In order to gain better insights into the plausible binding motifs of the target compounds, we carried out molecular docking. One of the attractive targets for the design of new antitubercular agents is synthesis of mycolic acids, the major components of the cell wall of *M. tuberculosis*. Mycolic acids, unique to mycobacteria and related species, are high molecular weight fatty acids and represent the major lipid components of the mycobacterial cell wall (Asselineau and Lederer 1950). Enzymes that comprise the fatty acid synthetase (FAS) complex responsible for fatty acid biosynthesis are considered ideal target for designing new antibacterial agents. Enoyl-acyl carrier protein (ACP) reductase is a key regulatory enzyme in fatty acid elongation, and it catalyzes the NADH-dependent stereospecific reduction of α,β-unsaturated fatty acids bound to the ACP (Quemard *et al*., 1995; Dessen *et al*., 1995). Several substituted pyrazole derivatives have been reported to possess potent activity against enoyl-ACP reductase (Kuo *et al*., 2003; Julia *et al*., 2012; Andrew *et al*., 2002). Structural similarity of the designed compounds to these

reported compounds was taken into consideration to rationalize the docking efforts. Hence, we chose crystal structure of enoyl-ACP reductase as a target for docking the target compounds. In consistent with our earlier study (Mohan *et al*., 2012), we chose the X-ray structure of *M. tuberculosis* enoyl-ACP reductase (PDB Code 2H7I) (He *et al*., 2006).

The docking studies revealed that most of the target compounds occupied the hydrophobic cavity of enoyl-ACP reductase. A visual analysis of the crystal structure of enoyl-ACP reductase (PDB Code 2H7I) suggests that chief interactions are of dispersion type. The ligand is less exposed to solvent and buried deep inside a hydrophobic pocket made up of Tyr158, Ile215, Met103, and Met199 residues. Most of the target compounds showed consistent orientations of the pyrazoline, aryl, and benzoxazole moieties within the active site. Figure S1a (Supplementary information) shows detailed interactions of the most potent compound **6l** with the neighboring residues. It was encouraging to see that most of the ligands retained all the dispersion interactions found in the crystal structure (PDB Code 2H7I) with some additional interactions with residues Ile202, Met155and Leu218. However, it should also be noted that for some compounds, we could not obtain satisfactory docking poses.

Interaction energies of the ligand fragments with enoyl-ACP reductase

In order to investigate the pharmacophoric contribution of the target compounds, we investigated contribution of the three fragments/pharmacophores of the target compounds (pyrazoline core, benzoxazole moiety and aryl ring) toward protein binding. We have shown earlier that the semi empirical quantum mechanical scoring method with corrections for hydrogen bonding, dispersion, and halogen bond, called PM6-D3H4X, developed in our laboratory, accurately describes all protein–ligand noncovalent interactions (Brahmkshatriya *et al*., 2013). Thus, in consistent with our earlier study, we carried out fragmentation of the ligands and measured the interaction of energy of these ligand subsystems with the neighboring residues using PM6-D3H4X method. Results clearly showed that benzoxazole moiety is found to be a critical pharmacophore as interacted favorably with Met103, Phe149, Ala157, Tyr158, Ile202, and Ile215 (blue region, Fig. S1b). Pyrazoline ring interacted with Ala198 and Met199 by dispersion interactions (yellow region, Fig. S1b). However, the interaction energies were much lower than those for benzoxazole moiety. Finally, to our surprise, we found that the aryl ring faces toward solvent and does not find any interacting partner (Figure S1b) and hence, the interaction energies were found to be against the binding. This indicates that the 5th position on the pyrazoline ring need not be substituted with aryl ring and can be replaced with smaller substituents such as alkyl. Glide score values of the ligands and the interaction energies of the ligand fragments are summarized in Table 2 and Fig. 3, respectively. It was very encouraging to see that the total interaction energy (sum of all contributing interaction energies of the fragments) well correlated with the pMIC values of the compounds ($r^2 = 0.75$, Fig. 4). A very good predictive index (PI) (Pearlman and Charifson, 2001) of 0.93 indicates good rank ordering of the compounds based on the predicted interaction energy. Comparison of the docking pose of isoniazid was quite surprising. The designed compounds interacted favorably with the active site residues than isoniazid which is reflected by their better score values. We

Table 2 Glide_score of the target compounds and their interaction energies using PM6-D3H4X method (in kcal/mol)

Comp. no.	pMIC	Glide score	Interaction energy	Substituted phenyl	Acetyl pyrazoline	(Mercapto/amino) benzoxazole
6c	7.77	−7.39	−2.91	5.56	−3.54	−4.93
6d	8.06	−7.73	−6.99	2.56	−1.54	−8.01
6e	8.06	−7.37	−8.27	1.56	−3.49	−6.33
6h	7.81	−5.86	4.04	7.53	−0.26	−3.23
6i	7.21	−8.08	4.27	9.47	1.25	−6.44
6j	8.09	−7.75	−1.24	9.01	−3.56	−6.69
6l	8.55	−7.85	−9.36	1.11	−4.60	−5.87
7a	7.78	−5.97	0.57	−0.56	−1.25	2.38
7c	7.15	−5.12	7.36	5.64	5.25	−3.53
7f	7.49	−6.46	1.71	3.84	2.55	−4.68
Isoniazid	8.44	−3.59	–	–	–	–
1-Cyclohexyl-5-oxo-*N*-phenypyrrolidine-3-carboxamide (2H7I-ligand)	–	−8.01	–	–	–	–

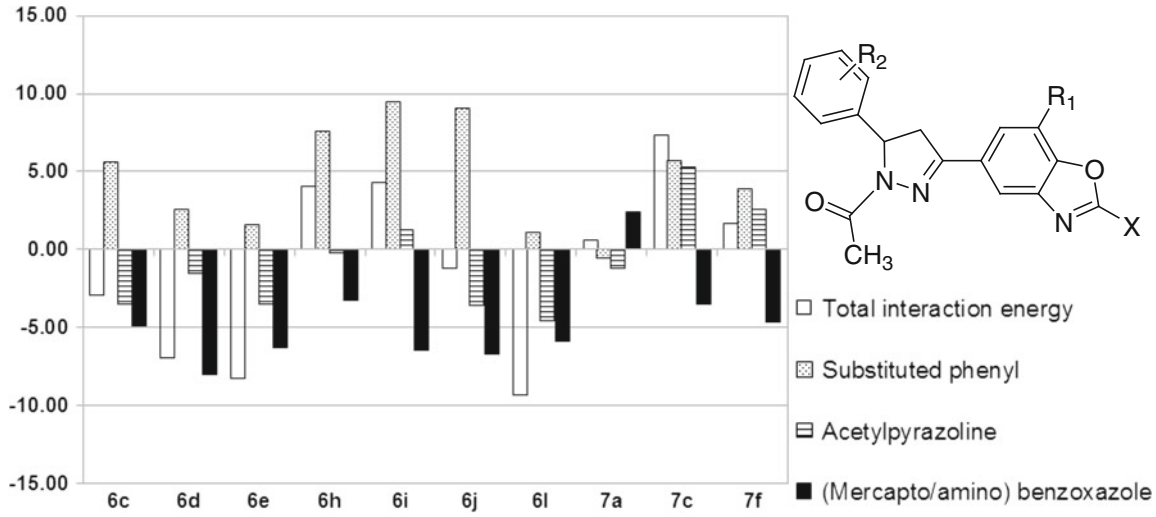


Fig. 3 Interaction energies of the fragments of the target compounds with enoyl-ACP reductase

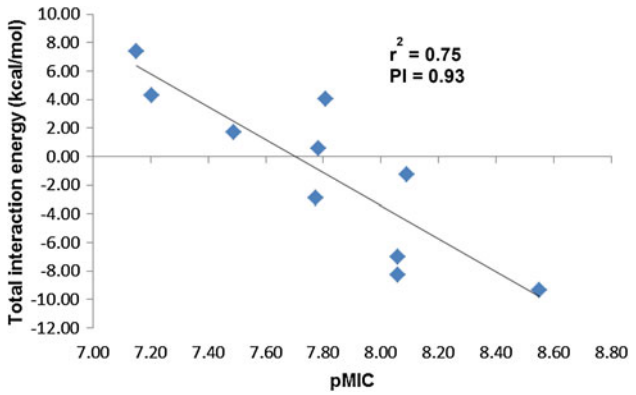


Fig. 4 The correlation of total interaction energy (in kcal/mol) with pMIC

think that this might be due to better fit of the target compounds in the active site cavity as compared to isoniazid (Figure S1c, d). However, it should also be noted that the poor score of isoniazid might be due to two well-known issues with the docking: *sampling* and *force field-based scoring function*. We understand that the Glide_Score is a crude estimate of the binding interactions as it depends on the underlying force field (OPLS in Glide) and better chemical description (e.g., quantum mechanics based scoring function) is necessary.

Conclusion

In summary, we designed a series of 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-mercaptobenzoxazole and 5-(1-acetyl-5-aryl-4,5-dihydro-3-pyrazolyl)-2-aminobenzoxazoles derivatives by combining the two pharmacophoric motifs (pyrazoline and benzoxazole) for antitubercular activity. The designed compounds were synthesized and evaluated for their ability to inhibit *M. tuberculosis* H37Rv, MDR-TB and XDR-TB strains in vitro. The biological screening provided interesting results where some of the compounds displayed significant antitubercular activity with a few analogs showing better activity than isoniazid against MDR-TB and XDR-TB strains. In order to have insights into the nature of binding of the target compounds with the molecular target of tuberculosis (enoyl-ACP reductase), molecular docking was carried out. Docking results suggest that the compounds chiefly interact with the protein by dispersion interactions. The interaction energies of the ligand fragments (the three key cores) provided useful information about the key pharmacophores suggesting importance of benzoxazole core over the other two ring nucleuses. The present study provides a useful protocol to combine two pharmacophoric motifs in one compound to design potent antitubercular agents.

Acknowledgments One of the authors (D. N. Rana) is thankful to UGC-BSR for Research fellowship.

References

Andrew J, Sneddon S, Staveski M, Yee C (2002) Inha inhibitors and methods of use thereof. US patent US6372752B1

Asselineau J, Lederer E (1950) Structure of the mycolic acids of mycobacteria. Nature 166:782–783

Brahmkshatriya PS, Dobeš P, Fanfrlík J, Rezáč J, Paruch K, Bronowska A, Lepšík M, Hobza P (2013) Quantum mechanical scoring: structural and energetic insights into cyclin-dependent kinase 2 inhibition by pyrazolo[1,5-a]pyrimidines. Curr Comput Aided Drug Des 9:118–129

Cory AH, Owen TC, Barltrop JA, Cory TG (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun 3:207–212

Dessen A, Quémard A, Blanchard JS, Jacobs WR Jr, Sacchettini JC (1995) Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science 267:1638–1641

Dye C, Lönnroth K, Jaramillo E, Williams BG, Raviglione M (2009) Trends in tuberculosis incidence and their determinants in 134 countries. Bull World Health Organ 87:683–691

Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC, Mainz DT (2006) Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J Med Chem 49: 6177–6196

He X, Alian A, Stroud R, Ortiz de Montellano PR (2006) Pyrrolidine carboxamides as a novel class of inhibitors of enoyl acyl carrier protein reductase from *Mycobacterium tuberculosis*. J Med Chem 49:6308–6323

Ileana IR, Rodríguez AD, Wang Y, Franzblau SG (2006) Ileabethoxazole: a novel benzoxazole alkaloid with antimycobacterial activity. Tetrahedron Lett 47:3229–3232

Julia CP, Raquel FM, Velando Esther Pilar F, Valle Silvia GD, Araceli MR (2012) 3-Amino-pyrazole derivatives useful against tuberculosis. WO2012049161A1

Kiss LE, Ferreira HS, Learmonth DA, Torrao L, Bonifacio MJ, Palma PN, Soares-Da-Silva P (2010) Discovery of a long-acting, peripherally selective inhibitor of catechol-O-methyltransferase. J Med Chem 53:3396–3411

Klimesova V, Koci J, Waisser K, Kaustova J, Mollmann U (2009) Preparation and in vitro evaluation of benzylsulfanyl benzoxazole derivatives as potential antituberculosis agents. Eur J Med Chem 44:2286–2293

Kuntal M, Agrawal YK (2010) Design, synthesis, and antitubercular evaluation of novel series of 3-benzofuran-5-aryl-1-pyrazolylpyridylmethanone and 3-benzofuran-5-aryl-1-pyrazolylcarbonyl-4-oxo-naphthyridin analogs. Eur J Med Chem 45:3831–3839

Kuo MR, Morbidoni HR, Alland D, Sneddon SF, Gourlie BG, Staveski MM, Leonard M (2003) Targeting tuberculosis and malaria through inhibition of enoyl reductase: compound activity and structural data. J Biol Chem 278:20851–20859

Mohammad S, Siddiqui AA, Mohamed AA (2006) Synthesis and evaluation of phenoxy acetic acid derivatives as anti-mycobacterial agents. Bioorg Med Chem Lett 16:4571–4574

Mohan SB, Ravi Kumar RVV, Dinda SC, Naik D, Seenivasan SP, Kumar V, Rana DN, Brahmkshatriya PS (2012) Microwave-assisted synthesis, molecular docking and antitubercular activity of 1,2,3,4-tetrahydropyrimidine-5-carbonitrile derivatives. Bioorg Med Chem Lett 22:7539–7542

Özlem T, Yıldız I, Özkan S, Kaynak F, Akı-Şener E, Yalçın I (2008) Synthesis and biological activity of some new benzoxazoles. Eur J Med Chem 43:1423–1431

Pearlman DA, Charifson PS (2001) Are free energy calculations useful in practice? A comparison with rapid scoring functions for the p38 MAP kinase protein system. J Med Chem 44:3417–3423

Quemard A, Sacchettini JC, Dessen A, Vilcheze C, Bittman R, Jacobs WR, Blanchard JS (1995) Enzymic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. Biochemistry 34:8235–8241

Sharma S, Sharma PK, Kumar N, Dudhe R (2011) A review on various heterocyclic moieties and their antitubercular activity. Biomed Pharmacother 65:244–251

Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, Anderson SW (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 405:962–966

Szell T, Eastham AM, Sipos GY (1964) New nitrochalcones VII. Can J Chem 42:2417–2423

Udwadia ZF, Amale RA, Ajbani KK, Rodrigues C (2012) Totally drug-resistant tuberculosis in India. Clin Infect Dis 54:579–581

Vinsova J, Cermakova K, Tomeckova A, Ceckova M, Jampilek J, Cermak P, Kunes J, Dolezal M, Staud F (2006) Synthesis and antimicrobial evaluation of new 2-substituted 5,7-di-*tert*-butylbenzoxazoles. Bioorg Med Chem 14:5850–5865

Yoshida S, Watanabe T, Sato Y (2007) Regulatory molecules for 5-HT3 receptor ion channel. Gating system. Bioorg Med Chem 15:3515–3523