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Design, synthesis and evaluation of novel diphenyl ether derivatives against drug susceptible and resistant strains of *Mycobacterium tuberculosis*

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ABSTRACT

In our efforts to develop druggable diphenyl ethers as potential antitubercular agents, a series of novel diphenyl ether derivatives (**5a-f**, **6a-f**) were designed and synthesized. The representative compounds showed promising *in vitro* activity against drug susceptible, isoniazid resistant and multi drug resistant strains of *Mycobacterium tuberculosis* with MIC values of 1.56 µg/mL (**6b**), 6.25 µg/mL (**6a-d**) and 3.125 µg/mL (**6b-c**) respectively. All the synthesized compounds exhibited satisfactory safety profile (CC_{50} > 300 µg/mL) against Vero and HepG2 cells. Reverse phase HPLC method was used to probe the physicochemical properties of the synthesized compounds. This series of compounds demonstrated comparatively low logP values. p*K*a values of representative compounds indicated that they

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were weak acids. Additionally, *in vitro* human liver microsomal stability assay confirmed that the synthesized compounds possessed acceptable stability under study conditions. The present study thus establishes compound **6b** as the most promising antitubercular agent with acceptable drug-likeness.

KEY WORDS
Diphenyl ether; anti-tubercular agents; safety profile; physicochemical studies; human liver microsomal stability.

Tuberculosis (TB) is a global pandemic and was responsible for the death of 1.3 million HIV-negative patients and 374,000 HIV positive people in the year 2016. Recent WHO report has highlighted that TB ranks alongside HIV/AIDS as the world's leading infectious disease killer, while shining a spot light on progress made in several countries, including India.[1] Campaign to reduce TB infections is seriously jeopardized by multi drug resistant tuberculosis(MDR-TB), extremely drug resistant tuberculosis (XDR-TB) and total drug resistant (TDR) tuberculosis.[2] There is an urgent need to develop safe and effective drugs for the treatment of TB infection.

Mycobacterium tuberculosis (MTb) synthesizes fatty acid via the type II pathway, which is absent in human beings. Enoyl-ACP Reductase (ENR) is a key regulatory enzyme of the type II FAS pathway and it catalyzes the final step of type II fatty acid elongation cycle. Inhibition of the fatty acid synthesis pathway is an attractive option since the drugs, which inhibit this pathway, can be selectively toxic to MTb. Triclosan (**1**) (Figure 1), a diphenyl ether derivative, targets mycobacterial ENR and exhibits MIC of 12.5 µg/ mL against *Mycobacterium tuberculosis* H37Rv.[3]

Unlike isoniazid (INH), triclosan does not require prior activation to bind with MtbENR.[4] From the literature reports on existing inhibitors of Mtb ENR, it can be proposed that blocking the entry of natural substrate (acyl carrier protein) by the inhibitors like triclosan, could be a plausible approach to disturb the essential reduction step in the mycobacterial mycolic acid biosynthesis.[5] Thus, the attempts to find diphenyl ether based antiTB agents have geared up in the recent past.[6-9] Although triclosan carries the promise to combat resistant strains of mycobacterium, its utility for human treatment is limited due to its poor solubility and sub-optimal bioavailability.[10] Hence, transforming triclosan in to an efficinet antitubercular agent is a major challenge.

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Encouraged with our previous experience with diphenyl ether scaffold [11,12] and subsequent literature reports of antitubercular potential of related compounds, we decided to carry out further studies and develop novel diphenyl ether derivatives as potential antitubercular agents with improved druggability. Another purpose of this research work is to get relevant information on the structural requirement of diphenyl ether scaffold to improve its antitubercular potential.

1. EXPERIMENTAL SECTION

1.1. Methods and Materials

All moisture-sensitive reactions were carried out under nitrogen atmosphere in anhydrous solvents. Column chromatography was carried out on 100-200 mesh silica gel. Progress of the reactions was monitored by TLC using Aluminum backed sheets of silica gel 60 F24 (Merck). Melting points were recorded with a laboratory melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a NMR Spectrometer (AV400-400 MHz High Resolution Multinuclear FT-NMR Spectrometer, Bruker) using DMSO-d_6 as the solvent. Mass spectrometry (MS) data were obtained on a LC-MS (Agilent 6520 series, Q-TOF LC/MS) and GC-MS (Shimadzu GC-17A, GCMS-QP5050A). IR spectra were recorded on Shimadzu Affinity-1 FT IR spectrophotometer. UV-Visible spectra were recorded on Shimadzu UV-2450 spectrophotometer and λmax was recorded. The purity of the final compounds was checked by reverse phase HPLC (UFLC, Shimadzu) using C-18 column in isocratic mode solvent systems (methanol and buffer, pH = 7.4) and found to be ≥ 95%.

1.1.1 Synthesis of 1-(3-methoxy-4-phenoxyphenyl) ethanone (2)

The 1-(3-methoxy-4-phenoxyphenyl) ethanone **(2)** was prepared as per the previously reported method [12] and purified by column chromatography over silica 100-200 with hexane: ethyl acetate (8:2) as the mobile phase.

1.1.2 Synthesis of 1-(3-hydroxy-4-phenoxyphenyl) ethanone (3)

The 1-(3-hydroxy-4-phenoxyphenyl) ethanone **(3)** was prepared as per the previously reported method [12] and purified by column chromatography over silica 100-200 with hexane: ethyl acetate (6:4) as the mobile phase.

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1.1.3 General method for synthesis of 1-(3-hydroxy-4-phenoxyphenyl)-3-aryl prop-2-en-1-one (4a-f)

The 1-(3-hydroxy-4-phenoxyphenyl)-3-aryl prop-2-en-1-one **(4a-f)** were prepared as per the previously reported method [12] and purified by column chromatography over silica 100-200 with hexane: ethyl acetate (6:4) as the mobile phase.

1.1.4 General method for synthesis of 1-(3-hydroxy-4-phenoxyphenyl)-3-aryl propan-1-one (5a-f)

To a solution of compounds **4a-f** (2 mmol) in absolute alcohol (50 mL) and THF (10 mL) was added with 15 mL saturated aqueous solution of NH_4Cl (80 mmol) at 25-27 °C. Then the reaction mixture was added with Zn (12 mmol) in four portions at 10 min. interval and stirred for 6h at ambient temperature. Progress of the reaction was monitored by TLC, using hexane: ethyl acetate (8:2) as the mobile phase. After the completion of reaction (6h), the reaction mixture was filtered and the filtrate was evaporated under vacuum to remove the volatiles. The residue obtained was added with ice cold water and extracted with ethyl acetate (3x25 mL). The organic layers were separated, pooled, washed with water, brine, dried over anhydrous $MgSO_4$ and evaporated under vacuum. The crude compound was purified by column chromatography over silica 100-200 with hexane: ethyl acetate (8:2) as the mobile phase to afford the target compound.

1.1.5 General method for synthesis of 5-(1-hydroxy-3-phenylpropyl)-2-phenoxyphenol (6a-f)

To the solution of compounds 1-(3-hydroxy-4-phenoxyphenyl)-3-aryl propan-1-one (**5a-f**) (0.75 mmol) in methanol (4 mL) and THF (2 mL), $NaBH_4$ (3.0 mmol) dissolved in aq. NaOH (4N, 1 mL) was added in three portions and stirred at ambient temperature. Progress of the reaction was monitored by TLC, using hexane: ethyl acetate (6:4) as the mobile phase. After the completion of reaction (2h), the reaction mixture was quenched with saturated NH_4Cl solution (5 mL) and evaporated under vacuum to remove the volatiles. The residue obtained was added into ice cold water, neutralized to pH 7 (by adding dil. HCl) and extracted with ethyl acetate (3x25 mL). The organic layers were separated, pooled, washed with water, brine, dried over anhydrous $MgSO_4$ and evaporated under vacuum. The crude compound was purified by column chromatography over silica 100-200 with hexane: ethyl acetate (6:4) as the mobile phase to afford the target compound.

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1.2 *In vitro* antitubercular screening against drug susceptible strains of *Mycobacterium tuberculosis* H37Rv and *In vitro* cell cytotoxicity screening

Procedure for MIC determination (micro plate alamar blue assay) against drug susceptible strains of *Mycobacterium tuberculosis* H37Rv has been reported previously.[12,13] Methods for the determination of CC_{50} (MTT assay) against Vero and HepG2 cells have been reported previously.[12,14,15]

1.3. *In vitro* antitubercular screening against drug resistant strains of *Mycobacterium tuberculosis*

Selected compounds were screened against isoniazid resistant and multi drug resistant (MDR) strains of *Mycobacterium tuberculosis* H37Rv using mycobacterial growth indicator tube (MGIT) assay method. Isoniazid resistant/ multi drug resistant *Mycobacterium tuberculosis* strain (clinical isolates) suspension was prepared as per the literature method. [16] It was diluted to afford a turbidity of 0.5 McFarland standard. 1 mL of the adjusted suspension was diluted in 4 mL of sterile saline (1:5 dilution). About 0.1 mL of the isoniazid resistant/multi drug resistant *Mycobacterium tuberculosis* (clinical isolates) strain suspension was added into 10 mL of sterile saline to prepare the 1:100 growth control suspension and mixed thoroughly. Test compounds were dissolved in DMSO and sterilized by filtering through syringe filters (0.22 µm) to prepare stock solutions of concentration 10,000 µg/mL. The stock solutions were diluted with saline water to afford working solutions of 80X strength. Isoniazid and rifampicin were used as standard. Mycobacterium Growth Indicator Tubes (BBL™ MGIT™, 7 mL, BD Bioscience, catalog No.245122), labeled with compound codes and concentrations were added with 0.8 mL OADC, 0.1 mL test compounds and 0.5 mL of mycobacterial strain. Growth control tubes contained only OADC and Mycobacterial culture. Each test sample was added to four MGITs at different concentrations and placed in a tube holder with one growth control tube. Contents of tubes were mixed well. Then the tubes were placed inside the BACTEC MGIT-900 instrument at 37 °C for 14 days. The BACTEC MGIT-960 instrument monitored the fluorescence of the MGITs and the results were reported. The results were analyzed by comparing the fluorescence of test sample tube and growth control tube. The MIC was defined as the lowest concentration of drug that inhibited bacterial growth.

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1.4. Determination of logP, pKa and human liver microsomal stability assay

LogP, p*K*a and human liver microsomal stability of the compounds **5a-f** and **6a-f** were determined by previously reported methods. [12,17,18,19]

2. RESULTS AND DISCUSSION

2.1 Design and chemistry

Phenolic –OH at 2nd position of the A-ring of triclosan (**1**, Fig. 1) plays a crucial role in binding to the catalytic site of Mtb ENR. The presence of three chloro groups makes this compound highly lipophilic and nonselective to InhA. Sivaraman *et al*. reported that ring-B chloro substitutions in triclosan were involved in unfavorable steric interactions with the enzyme and their deletion from the scaffold increased the affinity 7-fold.[7] In addition to this, diphenyl ethers substituted with long alkyl chains at 4th position of ring-A have been reported to display better affinity towards Mtb ENR than triclosan.[6] But those compounds were highly lipophilic and showed low aqueous solubility. Hence design of structurally similar diphenyl ether derivatives focused mainly on incorporating substituents at the 4th position and one –OH group at 2nd position of ring-A. α, β- unsaturated ketone was introduced at the 4th position of ring-A of diphenyl ether moiety, forecasting that it would mimic the reactivity of trans-2-enoyl acyl part of enoyl acyl carrier protein (Fig. 1) at the catalytic site of Mtb ENR. Although the antitubercular activity of α, β- unsaturated ketones (chalcones) is already reported, their carcinogenicity and metabolic instability make them unsuitable for lead optimization.[20] Thus the metabolically labile reactive conjugated system in the chalcones was modified to lesser reactive corresponding non-conjugated ketones and alcohols.

Synthesis of this series of compounds is described in scheme-1. Acetovanillone was taken as starting material and condensed with phenyl boronic acid by Chan-Lam *O*-arylation reaction. Obtained ketone (compound **2**) was subjected to BBr_3 assisted demethylation reaction as described by Gillmore *et al*.[21] The Claisen-Schimidt condensation reaction of compound **3** with different benzaldehydes afforded respective chalcones (compounds **4a-f**). Selective reduction of conjugated double bond of compounds **4a-f** was optimized using Zn/NH_4Cl, where, Zn and NH_4Cl provided electron and proton.[22] Due to the weak polarization, conjugated double bond of the chalcones was not affected by this mild reducing agent and corresponding dihydro-chalcones **5a-f** were obtained with 63.70% yield. In order to increase the polar surface area, obtained dihydrochalcones (**5a-f**) were reduced further by $NaBH_4$ to give compounds **6a-f** with 82.93% yield. Notably, $NaBH_4$ assisted reduction of

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carbonyl group of compounds **5a-f** to corresponding alcohols (**6a-f**) observed to be fastened in presence of alkali (NaOH). Spectral data of all the synthesized compounds are included in the supporting information.

2.2. Biological evaluation

Compounds **5a-f**, and **6a-f** were screened against *Mycobacterium tuberculosis* H37Rv. Their MIC results were given in Table 1. Safety profile (CC_{50}) of this series of compounds was established from MTT assay against Vero and HepG2 cells (Table 1).

Antitubercular potential of diphenyl ether derivatives was improved upon extension of alkyl chain with bulky substituents at 4^{th} position of ring A. Alkyl substituents of dihydrochalcones bearing 4-F-Phenyl (**5d**) and furan ring (**5f**) demonstrated significant antitubercular activity (MIC 10 µg/ mL). All dihydrochalcone derivatives (Compounds **5a-f**) showed acceptable safety profile against normal and human hepatoma cells (CC_{50} >300 µg/mL). Dramatic improvement in antiTB activity was observed when keto functionality of dihydrochalcones (**5a-f**) was reduced to corresponding racemic alcohol derivatives (**6a-f**). Interestingly, compounds **6a** and **6c-f** exhibited MIC of 4 µg/mL and compounds **6b** and **6c** exhibited significant antitubercular activity with MIC of 1.56 µg/mL and 3.125 µg/mL respectively. In this case, electron donating groups on phenyl substituent (**6b-c**) was observed to be more favorable for antitubercular activity. A closer comparison between the MIC of triclosan (MIC 12.5 µg/mL) and the compounds of the series, shows that, presence of hydroxy substituent adjacent to ring A is critical for improving the antiTB activity. All the compounds of this series achieved adequate safety profile (CC_{50} >300 µg/mL) against Vero and HepG2 cells and demonstrated selective index greater than 10.

Most promising compounds **6a-f** having MIC < 10 µg/mL against drug susceptible strain of *Mycobacterial tuberculosis* H_{37}Rv were selected for antitubercular screening against INH resistant and multi drug resistant (MDR) strains of Mtb (clinical isolates) (Table 2). Compound **6b** exhibited MIC of 6.125 µg/mL and 3.125 µg/mL against INH resistant and multi drug resistant strains of *Mycobacterium tuberculosis* respectively. On the other hand, compounds **6c** and **6f** retained their antitubercular potential against MDR strains. Interestingly, compound **6e** indicated antiTB activity at MIC of 4 µg/mL against both drug susceptible and INH resistant strains of *Mycobacterial tuberculosis*.

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2.3. Physicochemical studies

In order to probe the lipophilicity, experimental logP of synthesized was determined by reverse phase HPLC method. In this method, the stationary phase simulated the biological lipophilic membrane, and retention of the compounds was correlated with its lipophilicity. Most of the synthesized compounds demonstrated moderate logP (Table 3). In-depth analysis of logP of compounds indicated that there was no relationship between lipophilicity, and observed antitubercular activity.

p*K*a of representative compounds was determined using Reverse phase HPLC method. p*K*a was calculated from the retention factor (*k*') are shown in Table 3. Compound **5b** showed p*K*a 9.72. When the carbonyl group of **5b** was replaced with an electron donating secondary alcohol group in case of compounds **6b** and **6e**, the acidity of phenolic-OH was shot up and resulted in p*K*a 6-6.24. With increased acidity, compounds **6b** (MIC 1.56 µg/mL) and **6e** (MIC 4 µg/mL) showed substantial improvement in antitubercular activity. This may be due to the fact that compounds having p*K*a near to 6 are capable of producing optimum proportion of ionized and unionized species at biological pH (pH 7.4) to solubilize and penetrate the membrane of the mycobacterium. This observation was totally in agreement with the fact that, acidity of the drugs influenced their antitubercular activity.

Human liver microsome (HLM) stability (%) of selected compounds with promising antitubercular activity was determined at different time points (1h and 2h). Presence of a phenolic –OH group in triclosan moiety makes it susceptible for metabolism.[10] Hence, phenolic –OH present in our diphenyl ether derivatives are theoretically susceptible to HLM assisted metabolism. Nevertheless, in contrast to literature reports, HLM stability assay results (Table 3) of our synthesized compounds indicated that they were stable when incubated with human liver microsomes (retention > 50% after 1h).

CONCLUSION

In conclusion, novel diphenyl ether derivatives have been synthesized and evaluated for their *in vitro* antitubercular activity. **6b** was identified as the most promising compound which exhibited MIC of 1.56 µg/mL, 6.25 µg/mL and 3.125 µg/mL respectively against drug susceptible, isoniazid resistant and multi drug resistant (MDR) strains of *Mycobacterium tuberculosis*. Safety profile of all synthesized compounds against Vero and HepG2 cells (CC_{50} >300µg/mL) was satisfactory. In the present study, we were able to decrease the

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intrinsic lipophilicity of diphenyl ether scaffold to an acceptable limit with improved antitubercular potency against both drug susceptible and resistant strain. All the synthesized diphenyl ether derivatives showed acceptable metabolic stability (retention > 50% after 1h) against human liver microsomes. The present study indicates that there is further scope to explore diphenyl ethers as antitubercular agents. To this end, work is in progress to do the Mtb ENR enzyme assay of promising compounds and to elucidate the mechanism of action on a molecular level.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

FIGURE 1 Design of compounds 5a-f and 6a-f.

SCHEME 1 Synthesis of compounds 5a-f and 6a-f.

Suppl. Fig. 1. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 3

Suppl. Fig. 2. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 3

Suppl. Fig. 3. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 4a

Suppl. Fig. 4. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 4b

Suppl. Fig. 5. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 5a

Suppl. Fig. 6. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 5b

Suppl. Fig. 7. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 6a

Suppl. Fig. 8. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound 6b

Suppl. Fig. 9. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 3

Suppl. Fig. 10. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 4a

Suppl. Fig. 11. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 4b

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Suppl. Fig. 12. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 5a

Suppl. Fig. 13. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 5b

Suppl. Fig. 14. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 6a

Suppl. Fig. 15. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound 6b

Suppl. Fig. 16. ESI spectrum of compound 3

Suppl. Fig. 17. ESI spectrum of compound 4a

Suppl. Fig. 18. ESI spectrum of compound 4b

Suppl. Fig. 19. ESI spectrum of compound 5a

Suppl. Fig. 20. ESI spectrum of compound 5b

Suppl. Fig. 21. ESI spectrum of compound 6a

Suppl. Fig. 22. ESI spectrum of compound 6b

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Table 1: The *in vitro* antitubercular activity and cytotoxicity of compounds **5a-f** and **6a-f**.

Compounds	MIC (µg/ mL)[a]	CC_{50} (µg/ mL)[b]		SI[e]
	Drug susceptible strain	Vero[c]	HepG2[d]	
5a	15	>300	>300	>10
5b	15	>300	>300	>10
5c	12.5	>300	>300	>10
5d	10	>300	>300	>10
5e	15	>300	>300	>10
5f	10	>300	>300	>10
6a	4	101.06	>300	>10
6b	1.56	>300	>300	>10
6c	3.125	>300	>300	>10
6d	4	>300	>300	>10
6e	4	>300	>300	>10
6f	4	>300	>300	>10
1	12.5	>300	>300	>10
Isoniazid	0.125	-	-	-
Rifampicin	0.003	-	-	-

[a]MIC:minimal drug concentration required to stop the growth of *Mycobacterial tuberculosis* H37Rv;

[b]CC_{50}: minimal drug concentration required for 50% death of viable cells;

[c]VERO: African green monkey kidney cell line;

[d]HepG2: human liver cells. [e]SI (selective index) = CC_{50}/MIC;

The dash (-) denotes not tested.

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TABLE 2 Antitubercular activity of compounds **6a-f** against drug-resistant Mtb strains.

Compounds	MIC (μg/ mL)[a]	
	Isoniazid resistant strain	Multi drug resistant strain
6a	6.25	>12.5
6b	6.25	3.125
6c	6.25	3.125
6d	6.25	>12.5
6e	4	>12.5
6f	12.5	4
1	>12.5	>12.5
Isoniazid	>12.5	>12.5
Rifampicin	0.007	>12.5

[a]MIC = minimal drug concentration required to stop the growth of drug resistant strains of *Mycobacterial tuberculosis*

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TABLE 3 Physicochemical properties of compounds **5a-f** and **6a-f**.

Compound	logP[a]	p*K*a[a]	Microsomal stability (% remaining)[b]	
			1h	2h
5a	4.78	-	59.53	40.50
5b	5.24	9.71	62.14	46.59
5c	5.04	-	62.82	48.46
5d	4.83	-	66.59	50.71
5e	4.78	-	55.35	42.34
5f	4.31	-	51.60	37.16
6a	4.50	-	67.59	65.89
6b	4.88	6.24	55.27	52.32
6c	4.24	-	62.37	52.23
6d	4.71	-	67.84	57.68
6e	5.04	-	70.73	69.76
6f	2.60	6.04	54.12	44.58

[a]logP,and p*K*a were estimated from reverse phase HPLC experiment;

[b]% compound remained at different time points during incubation with human liver microsomes (HLM).

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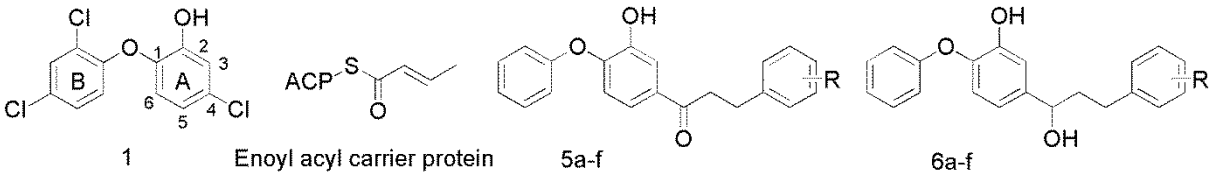

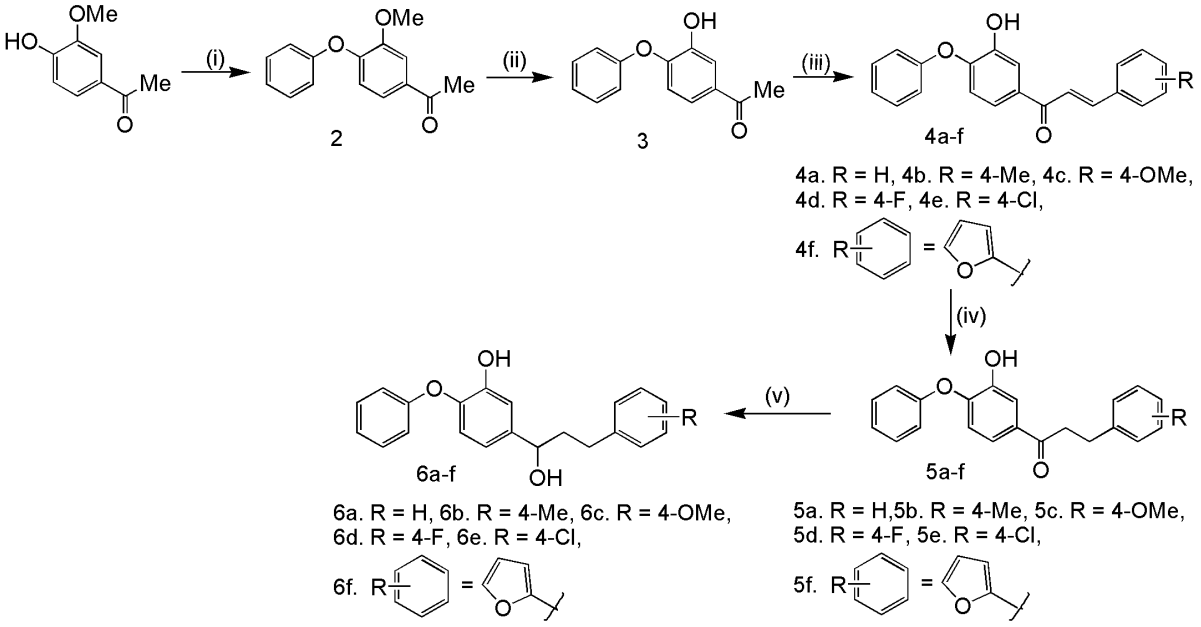

Reagents and conditions: (i). $PhB(OH)_2$, $Cu(OAc)_2$, C_5H_5N, CH_2Cl_2, 25-27 ^oC, 72h, 94%; (ii). BBr_3 (1M, CH_2Cl_2), CH_2Cl_2, -78 ^oC to 25-27 ^oC, 3h, 96%; (iii). ArCHO, KOH, EtOH, 25-27 ^oC, 24h, 55-73%; (iv). Zn, NH_4Cl, THF, EtOH, H_2O, 25-27 ^oC,6h, 63-70%; (v). $NaBH_4$, NaOH, MeOH, 25-27 ^oC, 5h, 82-93%.

Accepted Article

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