# SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF DIPHENYL ETHERS/AMINES

A thesis submitted

By

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In partial fulfilment of the requirement
for the degree of

**Doctor of Philosophy**

Under the supervision of
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## Candidate's Declaration

I, hereby declare that work presented in the thesis entitled **"Synthesis and antibacterial activity of diphenyl ethers/amines"**, in partial fulfilment of the requirement for the award of degree of Doctor of Philosophy at School of Chemistry and Biochemistry, Thapar University, Patiala, is an authentic record of my own work under the supervision of Dr. Manmohan Chhibber, Associate Professor, School of Chemistry and Biochemistry, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

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## Certificate

This is to certify that thesis entitled **"Synthesis and antibacterial activity of diphenyl ethers/amines"**, being submitted by Ms. Ramandeep Kaur in the partial fulfillment of the requirement for the award of degree of Doctor of Philosophy to the School of Chemistry and Biochemistry, Thapar University, Patiala, is a record of candidate's own work carried out by her under my supervision and guidance. The matter presented in this thesis has not been submitted in part or full for the award of any degree in any other university or institute.

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Dedicated to

My

Beloved family

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# Summary

Multidrug resistance is a burgeoning phenomenon across the world which has attained alarming levels in many countries. Antibacterial agents are frequently misused leading to development of resistance in bacteria. This situation has posed a serious challenge to the medical community for the development of effective therapy against infectious multidrug resistant bugs. In recent years, there has been a persistent increase in the occurrence of antibiotic resistance to many common bacterial pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae* and *Enterococcus faecalis*. Methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *pneumococcus* and vancomycin-resistant *Enterococcus faecalis* (VRE) are now commonplace pathogens that are proving difficult to be treated effectively.  Also malaria and tuberculosis constitute major causes of mortality in humans. Therefore, it is imperative to design and synthesize new antibacterial agents to continue the fight against such microbes.Many new targets have been identified that are not shared with the host organisms and can be targeted specifically to bacteria. Fatty acid biosynthesis is one of the important target for the development of new bioactive molecules. Triclosan is a diphenyl ether which inhibits enoyl-ACP-reductase (FabI) enzyme responsible for bacterial fatty acid biosynthesis. Despite diphenyl ethers being so widely studied, the potential of this lead molecule has not been explored completely for FAS-II. Although, literature describes a number of diphenyl ethers with improved antibacterial properties, there is still scope to explore different substituents and their properties on the phenyl rings. This along with poor solubility of these compounds in water and difficulty to cross biological membranes is also one of the challenges that need to be explored. Thus, on the basis of gaps following research objectives were identified and have been met. The work done on all the above objectives has been divided into Five Chapters. Chapter 1 describes the preface of problem which is taken up for the research work and available literature regarding that problem. Chapter 2 and Chapter 3 deal with synthesis of diphenyl ethers whereas Chapter 4 describes the synthesis of diphenyl amines. Chapter 5 illustrates the use of different surfactants to enhance the solubility of diphenyl ethers taking triclosan as a model molecule. All the synthesized compounds were evaluated for their antibacterial activity.

**General**

Purified reagents were procured from SD Fine Chemicals, Bombay. Solvents were distilled before doing the column chromatography. $^{1}H$ and $^{13}C$ NMR spectral analyses were performed on 400.1 and 100.6 MHz spectrometer (Bruker) with tetramethylsilane and $CDCl_3$ as the internal standard (δ, ppm) respectively. Antibacterial activity was done to evaluate Minimal inhibitory concentration using In vitro Microbroth dilution Assay. All the docking simulation were done using AutoDock Vina program in PyRx.

**Chapter 2**

**Derivatives of 4-(2,4-dichlorophenoxy)-3-nitro benzoic acid**

**2.1 Synthesis**

Nucleophilic aromatic substitution of fluoro in 4-fluoro-3-nitro benzoic acid **(2.4)** with 2,4-dichlorophenol **(2.3)** in the presence of mild base ($K_2CO_3$) and phase transfer catalyst (18-Crown-6) in aprotic solvent gave compound **(2.5)** in 88 % yeild. This was converted to its corresponding methyl ester, compound **2.6**, by heating in the presence of methanol in acidic medium. The nitro group of compound **2.6** was reduced to its corresponding amine, compound **2.7**, by reductions in water using Fe/ $FeSO_4.7H_2O$ in 51% yield. After making parent compound **(2.5)** and its methyl ester **(2.6)** and amino ester **(2.7)**, scheme of synthesis was directed toward preparing amides through acid chloride route. Using chlorinating agent ($PCl_3$) acid group in compound **2.5** was converted to its corresponding chloride that reacted at low temperature ($0^{o}$ C) with amines to give amides aliphatic amines with chain length C-2 to C-6 were used. In addition to above chain lengths methoxypropan-1-amine was also used.

Reduction of nitro to amine group in compound **2.8** to compound **2.14** was afforded in presence of $Fe/FeSO_4.7H_2O$ in water under refluxing conditions. This process afforded compound **2.15** to compound **2.21 (Figure Sa)**.

**R'=O/H**
**R''= -$(CH_2)$n-$CH_3$**

**Figure Sa. General structure of compounds 2.8-2.14 and 2.15-2.21**

## 2.2 Antibacterial Activity

In case of *E. coli* compound **2.10** have MIC value (0.98 μg/ml) for better than the control triclosan (MIC= 15.6 μg/ml) other control, Streptomycin, did not show any activity against *E. coli*. The antibacterial activity of other molecules (compounds **2.5**, **2.9**, **2.11**, and **2.13**) against the organism was also impressive MIC values were 1.95 μg/ml, 1.95 μg/ml, 1.95 μg/ml, 7.81 μg/ml and 62.5 μg/ml for compounds **2.5**, **2.9**, **2.11** and **2.13** respectively. The exceptions to this observation are compounds **2.6**, **2.8** and **2.12** which did not show any activity against *E. coli*. Although 2-position contains nitro group in all the 3 molecules, 4-position has methyl ester of carboxylic acid (compound **2.6**), methyl ether chain terminal (compound **2.8**) and long hydrocarbon chain with add number of carbon (compound **2.12**) in these molecules which may be responsible for the in activity of these compounds against *E. coli* compound **2.14**, a higher analogue of compound **2.12** with odd number of carbons in the side chain at 4-position, has also shown increased MIC value for *E. coli* (62.50 μg/ml). None of the molecules with amino group at 2-position of ring B (compounds **2.7**, **2.15**, **2.16**, **2.17**, **2.18**, **2.19**, **2.20** and **2.21**) were active against *E. coli*. Unlike case of *E. coli*, against *Pseudomonas aeruginosa* all the compounds including amino derivatives have shown antibacterial activity. However, the trend of activity against the organism was similar to that observed for *E. coli* in case of nitro compound was an exception here as it was inactive. Against *Pseudomonas aeruginosa* compound **2.11** was the most potent with a MIC of 1.95 μg/ml. The molecule has even number of four carbon chain. Compounds **2.9** and **2.13** with 2 and 6 carbon chains were having a MIC of 3.90 μg/ml against *P. aeruginosa* 3541. Interestingly compound **2.9** with 2 carbon chain was having lowest MIC in this case of *P. aeruginosa* 647. Compounds **2.10**, **2.12** and **2.14** with odd number of carbons and increasing chain length gave increasing trend for their MIC values. Compound **2.8** with methyl ether chain terminal also gave higher MIC (62.5 μg/ml) against *P. aeruginosa* 3541.

All the amino derivatives were active against *Pseudomonas aeruginosa* but gave MIC on higher side (7.81 to 125.00 μg/ml) compounds **2.15**, **2.17** and **2.18** in this case gave lower MIC value (7.81 μg/ml) than compound 5b with 2 carbon chain (MIC 15.62 μg/ml). Higher carbon chain length molecules compound **2.20** and compound **2.21** gave comparatively higher MIC values in this case. For other two organisms *P. putida* and *S. epidermidis* the antibacterial activity, although observed for the most compounds in the series, was in higher range (15.62- 31.25

μg/mL). Only exception here was compound **2.9** for which MIC was 7.81 μg/mL. Controls triclosan (MIC 3.91 μg/mL and 1.95 μg/ml) streptomycin (MIC 1.95 μg/mL and 7.81 μg/ml) gave comparable MIC against *P. putida* & *S. epidermidis* respectively. Compound **2.5** and **2.21** did not show any antibacterial activity

***In silico* Docking Studies**

It has been reported the hydroxychlorophenyl ring (ring A) of triclosan stacks with the nicotinamide ring of the $NAD^+$ making Π-Π interactions with an interplanar distance of 3.4 Å. All the nitro derivatives showed a flipped conformation *In silico* docking studies for *E. coli* FabI as compared to triclosan. A new hydrogen bond can be seen between nitro group of ring A and phosphate group of $NAD^+$ (3.2 $A^o$) besides Π-Π stacking of ring B and nicotinamide ring of $NAD^+$ in compound **2.10**. The probable basis for the flipping of ring A and B at the active site could be the 4- substitution of a heavier amide group that makes analogs sterically impossible to acquire the triclosan-like conformations.

**2.2 Structure Activity Relationship**

(i)Nitro group at 2- position of ring A is preferred over amino group at the same position for the antibacterial activity.

(ii) Amide linkage at 4-position having chain length upto 4 carbon atoms is preferred for higher MIC values. As the length of chain beyond 4 carbon atom increases, MIC value starts decreasing.

(iii) For higher chain length odd number of carbons atoms in amide linkage give lower MIC value as compared to even number of carbon atoms.

**Chapter 3**

**Derivatives of 1-(2,4-dichlorophenoxy)-2,4-dinitrobenzene/ 1-(2,4-dichlorophenoxy)-2-nitrobenzene**

**3.1 Synthesis**

Parent Compounds **3.9** and **3.19** were synthesized by nucleophilic aromatic substitution of nitro-substituted flouro benzene **(3.8)** with 2,4-dichlorophenol **(3.7)** in presence of mild base, phase transfer catalyst and aprotic solvent at room temperature by simple stirring. Further compounds **3.9** and **3.19** were subjected to reduction using Fe/ $FeSO_4.7H_2O$ and water to get corresponding amines **(3.10** and **3.20)**. Nin-hydrin test was done to observe the progress and completion of the reaction. Appearance of new broad singlets at 3.4 and 3.6 ppm for the compounds **3.10** and **3.20**

confirmed presnec of two and one amino group respectively. Both the broad signals disappeared when $^{1}H$ NMR was again recorded in $D_2O$.

Amino groups of both the compounds **3.10** and **3.20** were exploited further to incorporate amide linkage with various acid chlorides. Acid chloride required for the purpose was made from corresponding acid using $PCl_3$ as chlorinating agent at low temperature ($0^oC$). Aliphatic acids with varying chain length (C-1 to C-4) were used. For all the amide compounds confirmation of the product formation was done $^{1}H$, $^{13}C$ NMR and mass spectroscopic techniques. Spectroscopic studies were done to ensure the synthesis of diamide diphenyl ethers **(3.11-3.18)** and monoamide diphenyl ethers **(3.21-3.24)** respectively.

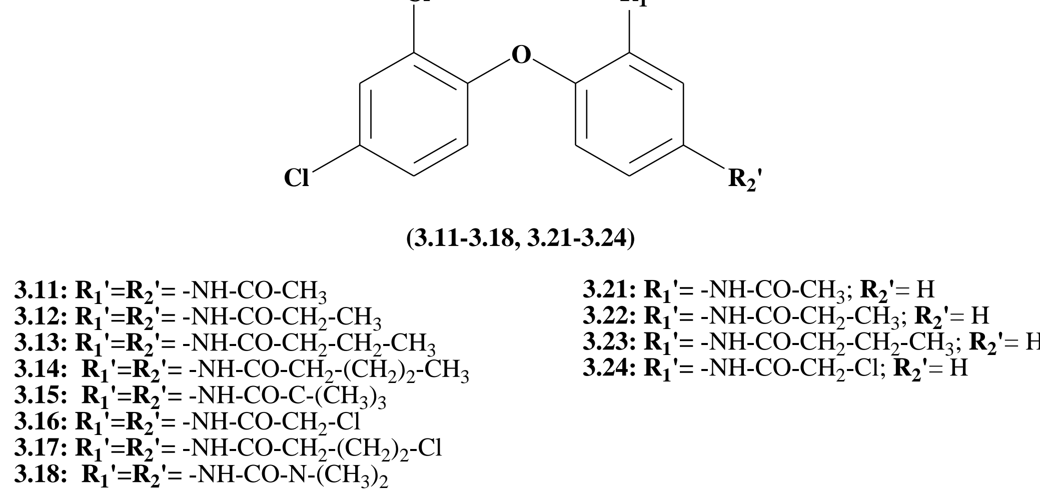



**Figure Sb. General structure of compounds 3.11-3.18 and 3.21-3.24.**

### 3.2 Antibacterial Activity

Compounds **3.9** and **3.10** were active against nearly all the selected strains. Compound **3.9** was more potent among the two with MIC value ranging from 7.81- 62.50 µg/mL and compound **3.10** with MIC value (15.6- 125.0 µg/mL) was in higher range. The most impressive value of compound **3.9** was against *E. coli* (7.81 µg/mL) where as standard has MIC value of 15.6 µg/mL. Compound **3.19** and **3.20** were comparatively less potent showing MIC against only two and five organisms respectively. The activity also ranged from 7.81- 125.00 µg/mL. From the structures of compounds (**3.9** and **3.10)** and compounds **(3.19** and **3.20)**, it is clear that nitro and amino groups at 4-position enhance the antibacterial activity though for nitro, enhancement is more than amino group. In case of compounds **(3.19** and **3.20)** higher MIC values can be attributed to missing nitro or amino substituents at position -4. All the amide made from compounds **3.10** and

**3.20** did not show any significant antibacterial activity against any of the selected strains except *P. aeruginosa* (MTCC 3541). Compound **3.11**, acetamide product of compound **3.10**, was an exception to this. It showed antibacterial activity against seven of the eight selected strains with MIC value ranging from 7.81- 62.50 μg/mL. Compound **3.12** propanamide of compound **3.10**, was also active but with higher MIC value (15.62- 62.50 μg/mL) against six strains only. Interestingly ten and thirteen of the sixteen synthesized compounds were active against *P. aeruginosa* and *P. putida* respectively. Compounds **3.9**, **3.11** and **3.20** gave MIC value of 7.8 μg/mL which was comparable to standard drug against *P. aeruginosa*. The most impressive activity against *P. aeruginosa* was from an unexpected compound **3.21** (MIC= 3.9 μg/mL) that was active against none of the selected stains. Against *P. putida* also, it showed an MIC value of 15.6 μg/mL. Rest of the compounds showed an MIC value ranging from 15.62 to 62.50 μg/mL against *P. aeruginosa*. Ten compounds active against *P. putida* had their MICs in the range of 15.62- 125.00 μg/mL.

### 3.3 *In silico* Docking Studies

*In silico* studies carried out with all the compounds against FabI of *P. aeruginosa* gave very encouraging results. The rings of the nitro and amide group of the compounds flipped as compared to triclosan but for amino compounds **(3.10** and **3.20)** the interaction was similar to that of reference compound. For compounds **3.10** the $NH_2$ substituent at the 2-position of ring A, interaction (2.7A$^o$) can be seen between hydroxyl (-OH) group of tyrosine (Tyr 159) and 2'-hydroxyl group of nicotinamide ribose (2.8 A$^o$).

### 3.4 Structure Activity Relationship

(i) 2,4-dichloro-1-(2,4-dinitrophenoxy)benzene **(3.9)** compound having nitro groups at ortho and para position to ether linkage in ring A of diphenyl ether moiety is more effective than the amino derivative 4-(2,4-dichlorophenoxy)benzene-1,3-diamine **(3.10)**.

(ii) The better activity of compound **3.9** might be due to the more topological surface area than compound **3.10**.

(iii) With the introduction of alkyl group through amide linkage decreased the biological activity. Amide linkage at ortho position having chain length upto 3 carbon atoms is preferred for better biological acivity.

(iv) Synthesized compounds were selectively more active against *P. aeruginosa species*. This property could be exploited for the exclusive anti-*Pseudomonas* activity.

**Chapter 4**

**Synthesis and Antibacterial Activity of 2,4-Disubstituted diphenylamines**

**4.1 Synthesis**

All the initial compounds (**4.12** to **4.15**) were synthesized by nucleophilic aromatic substitution of nitro-fluorobenzene with corresponding anilines. For the synthesis of compound **4.12** there was no need of base, metal salt or the ligand. Compounds **4.13**, **4.14** and **4.15** were synthesized by a known procedure using potassium carbonate, cuprous iodide and catalytic amount of *L*-Proline under heating conditions in aprotic solvent at 100 $^{o}$C. All the three compounds have also been synthesized by alternate routes.

**$R_1$**: $NO_2$, Cl, $NH_2$; **$R_2$**: H, $NO_2$, Cl,
**$R_3$**: $NO_2$, $NH_2$, -$OCH_3$, -OH, -NHCO-alkyl chain

**Figure Sc. General structure of Diphenyl amines**

The demethylation of compound **4.13** and **4.14** was done by heating in 48% aqueous hydrobromic acid and acetic acid to give corresponding 2-hydroxy diphenyl amines **4.16** and **4.17** respectively. The nitro groups on compounds **4.13**, **4.14** and **4.15** were reduced to corresponding amines **4.18**, **4.19** and **4.20** by heating with powdered iron and ferrous sulphate in aqueous media. The amine **4.20** obtained above was converted into novel compounds **4.21**, **4.22** and **4.23** by reacting with corresponding acid chlorides in the presence of potassium carbonate and methylene chloride. The acid chlorides used were freshly prepared by treating acetic acid, proponic acid and chloroacetic acid with phosphorous trichloride. Characterisation of synthesised compounds was done using NMR and Mass studies.

**4.2 Antibacterial Activity**

Antibacterial screening was carried out against eight organisms comprising of both Gram positive and Gram negative. Gram positive microorganisms included two strains of *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis* while Gram negative included two strains of *Pseudomonas aeruginosa*, *Escherichia coli* and *Pseudomonas putida*. Preliminary results showed that 7 of the 13 compounds were active against *Staphylococcus*

*aureus.* Against rest of the strains three compounds (**4.12**, **4.16** and **4.17**) gave encouraging results. Gram negative, *Pseudomonas putida* was an exception to this as it displayed activity against all the synthesized compounds, except compounds **4.18** and **4.19**, with a MIC ranging from 7.8 - 62.5 µg/mL.

Most notable among all, were the compounds **4.16** and **4.17** as both were active against all the selected stains. Compound **4.16** having MIC in the range 7.8- 15.6 µg/mL was the most effective as it gave comparable antibacterial activity to that of control against all the organisms. Compound **4.17**, athough had a higher MIC range, (7.8- 62.5 µg/mL) was also active against all the organisms. Triclosan and Streptomycin, used as control compounds, had MIC range of 1.9 – 7.8 µg/mL and 1.9- 15.6 µg/mL respectively in the preliminary studies. Whereas triclosan was active against all the starins Streptomycin did not give encouraging results against many stains including *Staphylococcus aureus.* Common feature of both the compounds **4.16** and **4.17** is presence of hydroxyl group (OH) at ortho position of ring A.

Similarly, compounds with 2, 4-dichlorosubstitution on ring A were also effective against *S. aureus* strains. MIC rage of compounds **4.15**, **4.20** and **4.23** were 7.8 – 15.6, 15.6 – 31.25 and 7.8 – 31.25 µg/mL respectively. Compound 4.1d, in addition to *S. aureus* strains was also active against three Gram negative strains having MIC range of 31.25-62.5 µg/mL. However, compounds **4.20** and **4.23** were active only against *Pseudomonas putida* in Gram negatives. While compound 4.1a showed activity against *S. aureus*, it was more effective against *Pseudomonas putida* with MIC value of 7.8 µg/mL.

**4.3 *In silico* Docking Studies**

Most of the compounds synthesized in this part of the work gave good antibacterial activity against *Staphylococcus aureus.* SaFabI (PDB identifier 4ALL) was chosen as the enzyme for the docking studies with the synthesized compounds. It has been reported that in the ternary SaFabI-$NAD^+$-triclosan (PDB identifier 4ALL) complex, ring A of triclosan, having OH- group at position 2 and chloro group at position 4, stacks over the nicotinamide ring of $NAD^+$. The ring A of triclosan stacks over the nicotinamide of $NAD^+$ and OH group forms two hydrogen bonds. Results in the docking studies are consistent with the antibacterial studies and have revealed a clear preference for phenolic (OH) group at 2 position of ring A.

### 4.4 Structure Activity Relationship

(i) Presence of hydroxyl group at 2-position of ring A is essential for the enhanced antibacterial activity.

(ii) This is evident from the fact that two compounds **4.16** and **4.17**, having hydroxyl group at the said position, have shown good activity against all the Gram positive and Gram negative strains taken in this study.

(iii) Presence of two nitro groups at position 2' and 4' of ring B enhances the antibacterial activity of diphenyl amines as compared to only one at position 2.

(iv) Compounds containing methoxy group at 2-position of ring A in diphenyl amines (**4.13**, **4.14**, **4.18**, **4.19**) did not yield any antibacterial activity.

(v) Amidation of amino group at 2- position of ring A further obliterates the antibacterial activity.

## Chapter 5

## Optimizing Surfactant and the Conditions to Enhance Aqueous Solubility of Triclosan

**5.1 Effect of surfactants on the solubility of Triclosan** Solubility of triclosan in LB broth was studied in the presence of the selected surfactants - Tween-80, Tween-20, Triton X-100, octylglucoside and sodium deoxycholate. First, a 10 mM stock solution of triclosan was made in DMSO which was serially diluted with DMSO. Surfactant stocks of the desired concentrations (around their CMC values) were made in LB broth. The serial dilutions of triclosan in DMSO mentioned above were dissolved in LB broth containing desired concentrations of surfactants so that the resulting concentration of triclosan ranged between 0-800 μM and that of DMSO was 0.1%. Solubility of triclosan in LB broth containing 0.1 % DMSO and various concentrations of surfactants was assessed by light scatter at 600 nm. Triclosan could be dissolved only up to 100 μM LB broth containing DMSO to a final concentration of 0.1 %. This was used as the baseline for comparison with solutions containing different concentrations of surfactants. The ability of five surfactants, Tween-80, Tween-20, Triton X-100, octyl glucoside and sodium deoxycholate to improve the aqueous solubility of triclosan was studied in relation to their effect on the $IC_{50}$ value of triclosan in *Escherichia coli* K12 (MTCC: 1302). All the surfactants studied enhanced the solubility of triclosan but to varying extents. The concentrations of surfactant effective for improving triclosan solubility were dissimilar and independent of CMC value.

## 5.2 Effect of surfactants on antibacterial activity of Triclosan

$IC_{50}$ values of triclosan in *E. coli* K12 were determined by broth dilution assay in the presence of various surfactants. A 10 mM stock solution of triclosan was made in DMSO and serially diluted in DMSO. *Escherichia coli* K-12 strain was freshly streaked on LB agar plates from a glycerol stock maintained at −70˚C. After 24 hours at 37 °C, a single colony was picked up from the plate, inoculated into 5 mL of LB broth and cultured at 37˚C with shaking for 12 hours. Fresh LB broth was then inoculated with one percent of this primary culture and cultured at 37˚C with shaking until the culture reached mid log phase. This mid log phase cell suspension (100 μL) was used to inoculate each tube containing 10 ml of LB broth along with different concentrations of triclosan (and a resulting DMSO concentration of 0.1%) as well as the required concentration of surfactant and incubated at 37˚C. Growth of *E. coli* K12 was measured after 8 hours by light scatter at 600 using a spectrophotometer. The absorbance values were normalized to control experiments performed with only triclosan or only surfactant added to LB broth and inoculum. Data were fitted to a sigmoidal curve by nonlinear curve fitting using Origin-8 software and the value of $IC_{50}$ determined by calculating the concentration of the compound which inhibited bacterial growth by 50%. The antibacterial efficacy of triclosan was inhibited by use of surfactants, Tween-80 and Tween-20 at concentrations around their CMC, which were effective in improving aqueous solubility of triclosan. Octyl glucoside and sodium deoxycholate could improve the aqueous solubility of triclosan but themselves killed *E. coli* K-12 at the concentrations required for improving triclosan solubility. Significantly, our study indicates that Triton X-100 at concentrations between 0.05 mM and 0.02 mM can effectively improve aqueous solubility of triclosan without affecting its $IC_{50}$ value.

## 6.0 Conclusion

Diphenyl ethers and diphenyl amines were successfully synthesized by nucleophilic aromatic substitution reaction of substituted phenol/ substituted amine with aryl halide. The synthesized compounds purified by chromatography techniques and characterized using $^{1}H$ NMR, $^{13}C$ NMR and mass spectral data. The structures were established on the basis of spectral studies. The synthesized compounds were screened *in vitro* for antibacterial potential against Gram positive strains included *Staphylococcus aureus* (NCTC 6571 and MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and *Bacillus subtilis* while Gram negative strains included *Escherichia coli* (MTCC 1302), *Pseudomonas aeruginosa* (MTCC 647 and MTCC 3541) and

*Pseudomonas putida* by microbroth dilution assay to determine minimal inhibitory concentration (MIC). The synthesized compounds possessed poor to promising activity against the test organisms. Diphenyl ethers **(2.5- 2.21** and **3.9- 3.24)** were exclusively active against *P. aeruginosa* species and diphenyl amines **(4.12- 4.23)** were found to be more active against *Staphylococcus aureus*. This property could be exploited for the development of narrow spectrum antibiotics.

Also the poor solubility of triclosan in the aqueous media was resolved by assessing the impact of surfactants used to enhance the solubility. It was found that Triton X-100 at concentrations between 0.05 mM and 0.02 mM effective to improve the aqueous solubility of triclosan without affecting its $IC_{50}$ value.

# Chapter 1
# Introduction and Review of Literature

## 1.1 Introduction

Infectious diseases remain the main cause of human premature deaths in developing countries. In recent years, there has been a persistent increase in the occurrence of antibiotic resistance to many common bacterial pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae* and *Enterococcus faecalis*. Methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *pneumococcus* and vancomycin-resistant *Enterococcus faecalis* (VRE) are now commonplace pathogens that are proving difficult to be treated effectively [1,2]. Probably more alarming is the emergence of multi-drug resistant strains [3]. Also malaria [4] and tuberculosis [5] constitute major causes of mortality in humans. Thus, there is an important demand for the discovery and development of new classes of antibiotics to add to our current arsenal of drugs.

OH Cl O Cl Cl

**Triclosan**

**1.1**

Many new targets have been identified that are not shared with the host organisms [6,7] and can be targeted specifically for the bacteria. Fatty acid biosynthesis is one such important target for the development of new bioactive molecules. Triclosan **(1.1)** first introduced in1960s [8a], is a diphenyl ether which inhibits enoyl-ACP-reductase (FabI) enzyme responsible for bacterial fatty acid biosynthesis [8b, 9]. A number of analogs of triclosan with different substituent on both the rings have been synthesized by various groups [10-12]. Despite diphenyl ethers being so widely studied, the potential of this lead molecule has not been explored completely for the fatty acid biosynthesis. Although, literature describes a number of diphenyl ethers with improved antibacterial properties, there is still scope to explore different substituents on the phenyl rings. This, along with poor solubility of these compounds in water and difficulty to cross biological membranes is also one of the challenges that need to be explored. Thus, on the basis of these

gaps in the research objectives were identified and have been met. The work in this thesis has tried to explore above mentioned gaps in the research by synthesising diphenyl ethers/ diphenyl amines and evaluating their antibacterial activity against different non-pathogenic organisms. The results have been supported with computational studies of the synthesised molecules with target enzymes.

## 1.2 Review of Literature

### 1.2.1 Natural existence of diphenyl ethers

The existence of diphenyl ethers and the molecules containing diphenyl ether moiety is well documented in the literature. Vancomycin, the antibiotic of last report, has two diphenyl ether linkages as shown in **Figure 1a** [13]. This glycopeptide antibiotic is used extensively for the cure of infections caused by Gram-positive bacteria especially those caused by methicillin-resistant *Staphylococcus aureus* and for patients sensitive to to β-lactam antibiotics. Bastadins **(Figure 1a)** are another group of molecules isolated from marine sponge, *Ianthella basta*, known to contain diphenyl ether linkages [14]. Their biological action includes cytotoxic activity and anti-inflammatory action.

**1.2**

1.3

**Figure 1a. 1.2 and 1.3 Structures of Vancomycin and Bastadin having diphenyl ether moiety**

Thyroid hormones, T3 and T4 **(Figure 1b)**, are essential diphenyl ethers synthesised in human body and are derived from amino acid tyrosine. These molecules are responsible for the development and the differentiation of cells in the human body. Besides circulation in the bound form with certain proteins (Globulin, 70 % and albumin 15-20 %), T4 is responsible for maintaining protein, fat and carbohydrates metabolism [15,16].

1.4 1.5

**Figure 1b. 1.4 and 1.5 are Thyroid hormone T4 and T3**

Marine life, in fact is a rich source of potential bioactive polybrominated diphenyl ethers that are subject of investigation for development of new pharmaceutical agents. For example new

polybrominated diphenyl ether and eight known compounds, **(Figure 1c)**, were isolated from the crude organic extract of the marine sponge *Dysidea sp*. The compounds inhibited a *Streptomyces* 85E in the hyphae formation inhibition (HFI) assay along with displayed antiproliferative activities against the human breast adenocarcinoma cancer cell line MCF-7 [17].

**1.6** $R_1$= $R_2$= H; $R_3$= Br; $R_1$= $R_3$= H; $R_2$= Br;
**1.7** $R_2$= $R_3$= Br; $R_1$= H; $R_1$= $R_3$= Br; $R_2$= H;
**1.8** $R_2$= $R_3$= H; $R_3$= Br; $R_1$= $R_3$= Br; $R_2$= H;
**1.9** $R_1$= $R_2$= $R_3$= Br.

**Figure 1c. Polybrominated Diphenyl ethers (1.6-1.9) isolated from marine sponge**

Cyprin **(Figure 1d)** is a natural diphenyl ether, produced by several fungal plant pathogens. Its mechanism of action is similar to that of synthetic molecules, triclosan , that inhibits enoyl (ACP) reductase (ENR) in plants. *Arabidopsis thaliana* is one such species of plants. Although the extent of inhibition of this enzyme is less as compared to its synthetic analogue triclosan. The binding of cyprin like triclosan is stabilized by the Π-Π stacking interaction between one of their phenyl rings and the nicotinamide ring of the $NAD^+$ [18].

**1.10**

**Figure 1d. Cyprin (1.10), inhibitor of enoyl (ACP) reductase in plants**

Besides natural diphenyl ethers many synthetic diphenyl ether find application in pharmaceutical industry due to their biological activity such as antimicrobial, antifungal, antimalarial, antituberculosis, anticancer and antidiabetic [19-24].

### 1.2.2 Synthesis of Diphenyl ethers

A brief review of methodologies discovered in last one and a half decade is presented in following paragraphs.

The synthesis of diphenyl ether is known for more than a century. Ullmann, in 1905, coupled the phenol with aryl halide to synthesise diaryl ether **(1.11)** using copper in stochiometric amounts in the presence of base and high temperature [25].

OH, $O_2N$ + Br — Cu, KOH, 160°C, 30 min. → O, $O_2N$

**1.11**

Alexander Williamson in 1850 proposed a reaction involving $SN^2$ reaction mechanism to make an ether linkage.[26]

Evans et al. and Chang et al. simultaneously reported coupling of aryl boronic acids and phenols in the presence of copper (II) promoted reaction at room temperature to give diaryl ethers **(1.12)** in high yields [27]. The reaction was tolerant to a wide range of substituents on both the rings. Pyridine or trimethyl amine was used as base in the same solvent. Chan et al. even extended the methodology to include amines, anilines, amides, imides, ureas, carbamates and sulfonamaindes along with diphenyl ethers **(1.13)** at room temperature [28].

Ar—OH + (HO)$_2$B—Ar' (1-3 eq.) — 1 eq. $Cu(OAc)_2$, 4 A°MS, 5 eq. pyridine or TEA; $CH_2Cl_2$, air, 25°C, 18 h → Ar–O–Ar'

**1.12**

1.13

Buchwald's group in 1999 synthesized a number of diphenyl ethers **(1.14)** using wide range of electron deficient, electrons rich and electronically neutral alkyl halides and sulfonates. The palladium catalysed reaction in the presence of biphenyl phosphine ligands gave desired product with a variety of phenols.[29]

1.14

Ligand

Peng et al. in 2002 eliminated the need for phase transfer catalyst for synthesis of diphenyl ethers **(1.15)** by introducing simultaneous microwave and ultrasound irradiation (SMUI) [30] This was improved upon the earlier Williamson ether synthesis promoted by only ultrasound in the presence of phase transfer catalyst [31]

1.15

Dawei Ma used *N, N*-dimethyl gylcine as a ligand to carry out synthesis of diphenyl ether **(1.16)** using aryl iodide and bromides in the presence of cesium carbonate at 90 °C. [32]

X: I; Br

CuI, *N,N*-dimethylglycine-HCl

$Cs_2CO_3$, dioxane, 90°C

**1.16**

Robet A Batey's group developed a protocol for the synthesis of ethers using organotrifluoroborate salts [33]. They also demonstrated that aryltrifluoroborate salts with acetate, 4-(dimethylamino) pyridine as ligand in the presence of 4 A° molecular sieves in oxygen atmosphere undergoes cross coupling with phenols to give diphenyl ethers **(1.17)**.

$BF_3K$ HO CN

2 eq.

$Cu(OAc)_2 \cdot H_2O$ (10 mol %)

DMAO (20 mol %) 4 A° MS

$CH_2Cl_2$, R.T., 5 min.

**1.17**

Cristau et al. demonstrated use of imine based ligands to accelerate Ullmann-type coupling of aryl bromides and iodides with phenols to synthesise diphenyl ethers **(1.18)** in the presence of cesium chloride, catalytic copper (I) oxide in acetonitrile [34].

X: I, Br

$Cu_2O$, Ligand

$Cs_2CO_3$, MS 3A°, $CH_3CN$

**1.18**

Ligand

Buchwald improved upon his synthesis by improvising the ligand and demonstrated that the new Ligand **(1.19)** due to its rigid frame work can couple electron deficient aryl halides, halopyridines and quinolines to phenols in the presence of catalytic palladium [35].

Flexible

Locked

**1.19**

Verkade and co-workers improved upon previous methodologies [36 and 37], of synthesizing diphenyl ethers **(1.20)** from electron deficient aryl fluorides and electron rich TBDMS- protected phenols in the presence of proazaphosphatranes, by use of microwave irradiation.[38]

Toluene or DMF
Microwave/ 180°

**1.20**

Ligand

K. Ding reported another (2-pyridyl) acetone promoted copper-catalysed coupling of aryl halides with various phenols in the good yield **(1.21)** using mild conditions [39].

Cl HO + CuBr (10 mol %) 20 mol % Ligand $Cs_2CO_3$, DMSO,120°C

O O

**1.21**

N O

Ligand

Bert Olofsson used phenol and diphenyl iodonium triflate to yield diphenyl ethers **(1.22)** eliminating the use of ligand or any metal. The reaction required strong base such as potassium t-butoxyoxide or sodium hydride. [40]

OH + $X^-$ $I^+$ + t-BuOK; THF O

$OCH_3$ X: OTf, $BF_4$ $OCH_3$ $H_3CO$ $OCH_3$

**1.22**

Pallapothulla's group developed an efficient protocol for the synthesis of diaryl ethers **(1.23)** using $CuFe_2O_4$ nano powder that could be used as recyclable catalyst by reaction of aryl halides and phenols in the presence of KOH as base in aprotic solvent at 120°C. The coupling did not require any ligand [41].

I HO + $CuFe_2O_4$ (6 mol %) KOH(2equiv.), DMSO(2mL) 120°; 2h O OH

**1.23**

Wang's group in 2003 reported a simple protocol to make diphenyl ethers **(1.24)** by nucleophilic aromatic substitution of aryl halides by corresponding phenols, without any ligand, In the presence of a mild base ($K_2CO_3$) under microwave irradiation. The reaction done in aprotic solvent, DMSO, required only 5-10 minutes in good yields [42].

The proposed mechanism of the reaction has been shown in the **Scheme 1.1**.

**Scheme 1.1.** Formation of diphenyl ether linkage by nucleophilic aromatic substitution reaction

Chhibber et al. and others modified this procedure by replacing microwave irradiation with phase transfer catalyst and carrying out reaction at room temperature. In the present work we have used the same procedure to make diphenyl ethers [10].

### 1.2.3 Synthesis of Diphenyl amines

The work included in this thesis also involves synthesis of diphenyl amines and their antibacterial activity. Therefore, following paragraphs briefly describe the literature relating to synthesis of diphenyl amines.

Fritz Ullmann and Irma Goldberg's promising work, at the beginning of last centuary, explored ways to form Aryl-N, Aryl –O and aryl-C bonds. K. Kunz et al. [43] have reported that both scientists worked on modern homogenous cross- coupling chemistry achieving respectable yields and selectivities with bidentate- coordinating substrate [44]. Although use of bidentate co-ordinating substrate was unintentional. Thus first catalytic aryl amination **(1.25)** and amidation **(1.26)** was done in Goldberg's lab.

NH$_2$ COOH + Br

Cat. Cu, $K_2CO_3$
3h; 210°C, Ph-NO$_2$, 99 %

COOH H N

**1.25**

OH H N O

Cat. Cu, NaOAc
3h; 210°C, Ph-NO$_2$, 99 %

**1.26**

More recently Buchwald et al. have developed palladium catalysed protocols to carry amination of aryl halides in the presence of various amines. They have reported use of different ligands and reaction conditions to carry out synthesis of diphenyl amines **(1.27)**.

R X + H—N R' R''

Ligand, Base
Solvent

R N R' R''

**1.27**

Besides Buchwald's extensive study on the synthesis of diphenyl amines therefore reports by other groups also where palladium catalysed coupling has been done in the presence of other ligands. L. Ackermann's group [49] utilised recemic BINAP and palladium acetate in the presence of cesium carbonate in toluene to afford diphenyl amines from imidazolyl sulfonates. Same manuscript also reported use of nickel catalyst and dppf to react with aryl sulfamates to afford diphenyl amines **(Table 1.1)** **(1.28)**.

**Table 1.1.** List of different ligands and reaction conditions used by Buchwald et al.

| Sr.No. | Ligand | Base | Conditions | References |
|---|---|---|---|---|
| 1. | Pd-P, AcO | NaOMe, N | Toluene, 60 $^{o}$C | 45 |
| 2. | $PCy_2$<br>$P(tBu)_2$ | $K_2CO_3$, $H_2O$<br>NaOtBu | tBuOH<br>1,4-dioxane | 46 |
| 3. | P, O | NaOtBu | 80 $^{o}$C | 47 |
| 4. | $NH_2$, Pd, L, Cl<br>$OCH_3$, $OCH_3$, $PCy_2$<br>O, O, O, $PCy_2$ | $Cs_2CO_3$ | tBuOH, 110$^{o}$C | 48 |

**1.28**

Similarly Verkade's group [50] made use of proaza phosphatranes, as ligand, in presence of sodium tert-butoxide to afford diphenyl amines **(1.29)** from alkyl halides and amines.

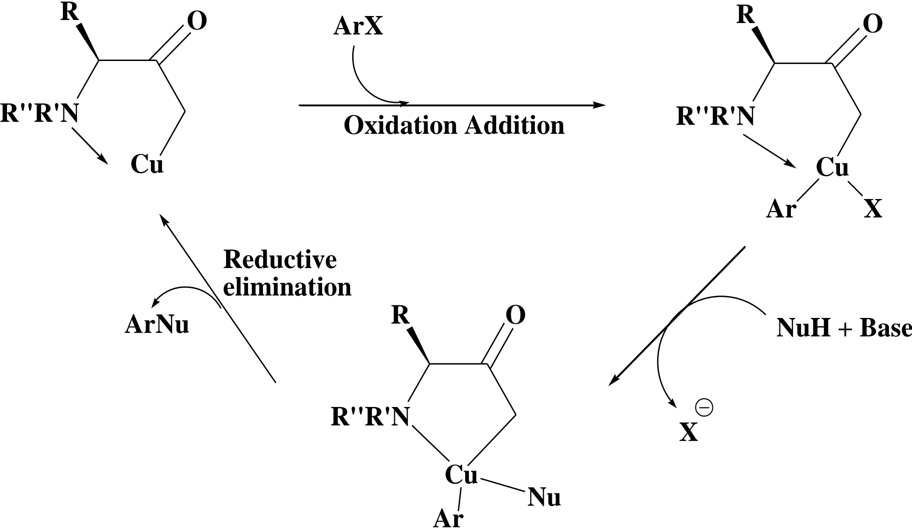
**1.29**

Ligand

Dawei Ma's group [51] developed a methodology to use amino acids for the Ullmann type chemistry (**1.30**). The highlight of their method was low temperature range (60-90 $^{o}$C) of the reaction and use of commercially available ligands like *L*-proline or *N*-methyl glycine in the presence of CuI and potassium carbonate.

**1.30**

Same methodology has been used to synthesize diphenyl amines for present work. Possible catalytic cyclic for amino acid a promoted reaction is given **Scheme 1.2**.




**Scheme 1.2.** Mechanism of copper catalysed coupling reaction

### 1.2.4 Antibacterial Activity of Diphenyl ether

Diphenyl ethers are known to have antibacterial activity. As discussed at the beginning of the introduction triclosan is a known to be broad spectrum antibacterial that is commonly used in toothpastes, oral mouth washes, deodorants and children toys [52]. It was thought to be a non specific antiseptic but was later found to target fatty acid biosynthesis (FAS I) [53]. Results of toxicology studies show that triclosan and its metabolites are well tolerated by a variety of species, including human beings [52, 54, 55, 56] and are not carcinogen, mutagen or teratogen.

(a) Fatty acid biosynthesis

Fatty acid biosynthesis (FAS) is an essential pathway responsible for the survival of living organisms. FAS II, which operates in bacteria and in the plastids of plants and algae, [57, 58] is different from [59] FAS I that operates in fungi and mammals [60]. Whereas FAS I uses a single polypeptide chain with various domains to catalyzes all the reactions, [61] FAS II requires several discrete enzymes to carry out fatty acid biosynthesis **(Figure 1e)**. This difference in organization makes bacterial fatty acid biosynthesis a potentially selective antibacterial target.

(b) Inhibitors of FAS-II

Natural as well as synthetic inhibitors for all the enzymes of FAS II have been reported. **Figure 1f** below summarizes all the known inhibitors as reviewed by Zhang et.al. [62]. Among these cerulenin **(1.31)**, thiolactomycin **(1.32)**, diazaborines **(1.33)**, isoniazid **(1.34)**, and triclosan **(1.1)** have been known for quite some time.

**Figure 1e. Enzymes and Inhibitors involved in type II fatty acid synthesis (FAS-II)**

These molecules have been in use as antibacterial though their specific targets were identified in the post genome era.

**Figure 1f. Known inhibitors of Fatty acid biosynthesis (FAS II)**

Purified *P. falciparum* enoyl-ACP reductase [8] has been shown to be inhibited by triclosan and the compound also hampers parasite growth in vitro and *in vivo* experiments [63, 64]. It also inhibits InhA, the FabI homologue from *M. smegmatisoi* [9] and *M. tuberculosis* [65].

A number of analogs of triclosan with different substituent on both the rings have been either synthesized or isolated from natural resources by different groups. **Figure 1g** below illustrates important diphenyl ethers that have been evaluated for different bacterial strains or Fab I enzymes and have shown superior results.

Heath et al. reported antibacterial activity of compounds **(1.35** and **1.36, Figure 1g)** against *E. coli* strains [66]. They have been found to possess promising activity against FabI with $IC_{50}$ value 0.4 µg/ml and 0.5 µg/ml respectively.

A series of synthesized dicationic diaryl ethers with piperidinyl and thiomorpholinyl groups have shown improved antimicrobial selectivity and are potent anti-MRSA and anti-VRE inhibitors. The most potent bis-indole diphenyl ether **(1.38, Figure 1g)** exhibited anti-MRSA MIC value of 0.06 µg/mL and enhanced antimicrobial selectivity [67].

Freundlich et al. have synthesised a series of 4-substituted triclosan derivatives and tested their potency against purified InhA and two isoniazid resistant *M. tuberculosis* strains. The most efficacious inhibitor **(1.37, Figure 1g)** displayed an $IC_{50}$ value of 21 nM, against enzyme and a different compound had an MIC value of 4.7 microg $mL^{-1}$. This enzyme inhibition and bactericidal activity was respectively 50-fold and 10 fold more potent than triclosan [68]. Earlier the group had synthesized and evaluated activity of 2'-substituted, 4'-substituted and 4-substituted derivatives against enoyl-ACP-reductase (Fab I) enzyme of *Plasmodium falciparum*. Most potent compounds among them were **(1.39, Figure 1g)** [72,21], **(1.40, Figure 1g)** [69] and **(1.41, Figure 1g)** [70] with an $IC_{50}$ value of 7±2 µM, 57 ± 24 nM and 49±20 nM respectively. A similar study by Chhibber et al. have shown compound **(1.42, Figure 1g)** to have comparable result with that of triclosan for both *P. falciparum and E. coli* FabI enzyme and culture [10]. Substitution of -Cl at position 2' in triclosan with amino, nitro and acetyl amino groups was carried out and their inhibitory potencies against *Pf* ENR were determined. Among them, compounds **(1.43, Figure 1g)** and **(1.44, Figure 1g)** exhibited good potencies both against enzyme and *P. falciparum* culture [11].

Alkyl diphenyl ethers with up to eight carbon chain long substituents at 4'-position of triclosan have been developed that have shown uncompetitive inhibition of InhA, the enoyl reductase enzyme in the MTB fatty acid biosynthesis. The most potent compound **(1.45, Figure 1g)** had an Ki' value of 1 nM for InhA and $MIC_{99}$ values of 2-3 microg $mL^{-1}$ (6-10 microM) for both drug-sensitive and drug-resistant strains of MTB [71]. Studies by same group also showed that compound named **SBPT04 (1.46, Figure 1g)** is more potent in vitro than current drugs (streptomycin and gentamicin fall into a range of 2–4 and 1–2 mg/L) to treat *F. tularensis* infections [72].

Bromo diphenyl ether **(1.47, Figure 1g)** isolated off the coast of Lakshadweep islands, Indian Ocean, exhibited potent and broad spectrum *in-vitro* antibacterial activity against many resistant pathogens including methicillin resistant *Staphylococcus aureus* (MRSA) [73]. The observed MIC range was 0.117-2.5 µg/mL against all the Gram positive bacteria and 0.5-2 µg/mL against Gram negative bacteria. The *in-vitro* antibacterial activity observed was better than that of the standard antibiotic linezolid, a marketed anti-MRSA drug.

Use of acrylic ester derivative of triclosan **(1.48, Figure 1g)** has been suggested by copolymerising it with 2-hydroxy methylacrylate (HEMA) in cyclohexane at 70$^{o}$C. The synthesized fibre was tested against *Staphylococcus aureus* for its antibacterial properties. Results were excellent compared to control polymer such as poly(HEMA) and poly(ethylene-co-vinyl acetate)[74].

Mishra et al. synthesized a series of triclosan analogs bearing biodegradable ester with quaternized linkage so as to increase their cellular uptake have been synthesized, characterized and evaluated for their antimalarial and antibacterial activities. Many of these compounds exhibit good inhibition against *Plasmodium falciparum* and *Escherichia coli*. Among them tertiary amine containing triclosan-conjugated prodrug **(1.49, Figure 1g)** inhibited both *P. falciparum* ($IC_{50}$; 0.62 microM) and *E. coli* ($IC_{50}$; 0.26 microM) at lower concentrations as compared to triclosan [75].

Further the modifications were done at either positions 5 or 4′ of the diaryl ether skeleton. These compounds were evaluated against wild-type *Mycobacterium* culture, and seven derivatives were found to be more active than the reference compound. The best analog was compound **(1.50, Figure 1g)** was approximately 29-fold more potent than triclosan in the whole-cell anti-Mtb assay [12].

Kozikowski et al. proposed two compounds **(1.51, Figure 1g)** and **(1.52, Figure 1g)** exhibit improved activity against both the BaENR and Sterne strain of *B. anthracis* as well as methicillin–resistant *Staphylococcus aureus* [76]. Also these two compounds were tested against *Toxoplasma gondii* and showed better activity than triclosan [77]. After rational design new TgENR inhibitors were synthesized by Kozikowski et al. Compound **(1.53 and 1.54 Figure 1g)** had antiparasitic $MIC_{50}$ values of 250 nM, which is approximately ten-fold better than that of the lead compound, triclosan. Both the compounds were same except an alkyl chain present on the heterocyclic ring [78].

OH O O HO 1.35

OH O HO 1.36

OH Cl O Ph($H_2$C) Cl 1.37

NH R N O N NH H HN R 1.38

OH $NH_2$ O Cl 1.39

OH Cl O Cl Ac C O 1.40

OH Cl O NC Cl 1.41

Cl OH O Cl CHO 1.42

$NH_2$ O Cl OH Cl 1.43

$O{=}N^{+}{-}O^{-}$ O Cl HO Cl 1.44

O OH 1.45

OH O 1.46

Continue…

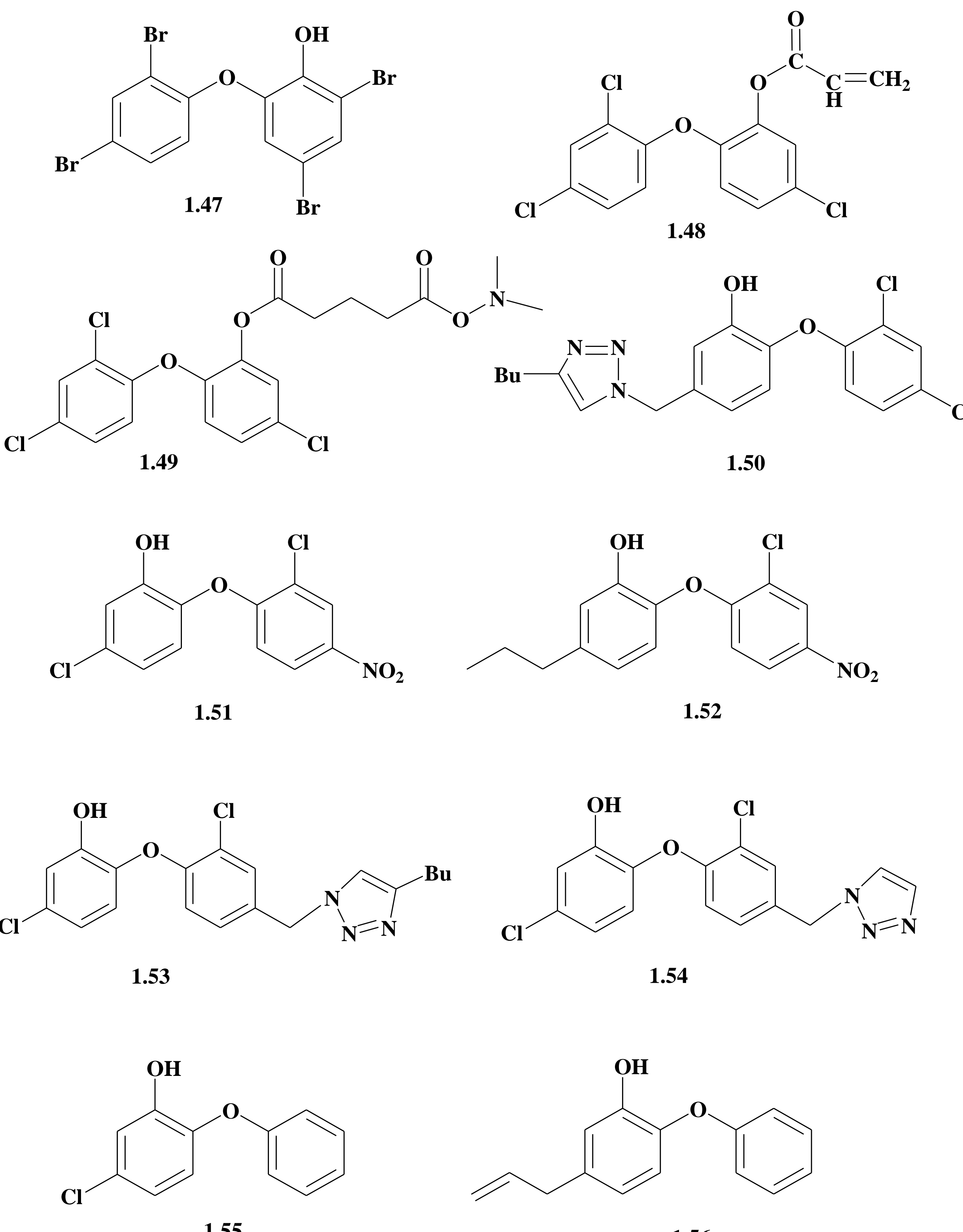



**Figure 1g. Diphenyl ether derivatives**

Compounds **(1.55, Figure 1g)** and **(1.56, Figure 1g)** were found to be very effective in reducing the *Burkholderia pseudomallei* bacterial culture in the spleen. *Ex vivo* activities ($MIC_{90}$) of Compounds **1.55** and **1.56** against *B. pseudomallei* were 2.5±2.5 mg/liter 2.0±2.0 mg/liter. Compound **(1.55, Figure 1g)** was also effective against lung infection for untreated mice. The slow-onset inhibitor mechanism of **(1.55, Figure 1g)** and **(1.56, Figure 1g)** against FabI may arise from A-ring substituent and from the bigger van der Waals radius of chlorine **(1.55, Figure 1g)** [79].

### 1.2.5 Antibacterial Activity of Diphenyl amines

Although diphenyl amines are synthesised extensively but these molecules are not that much exploited for taken up as successful bioactive molecules. During the last few years the potential of diphenyl amine and its derivatives in molecular recognition [80] and medicinal properties [81, 82] have been subjected to investigation. Substituted Diphenyl amine derivatives are associated with number of apparent antibacterial activities against Gram positive and Gram negative organism.

Bhatt et al. [83] emphasized the importance of incorporating bulky groups as trisubstituted *N*-[4-(4-substitutedphenyl)-1,3-thiazol-2-yl]-1,3,5-triazin-2-amine (MIC= 4- 64 μg/ml) are more efficient than disubstituted, diamino-s-triazine (MIC= 16-128 μg/ml) against three gram positive (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus)* and three gram negative microorganism (*Salmonella typhi, Escherichia coli, Klebsiella aerogenes*). Recently thorium(IV) nitrato, dioxouranium(VI) nitrato and cerium(IV) sulphato, metal complexes of *N*-[(Diphenylamino)methyl]acetamide had been synthesized and evaluated for their in vitro antibacterial activity against against six bacterial species (*E. coli, P. aeruginosa, S. typhi, B. subtilis, S. pyogenes, S. aureus*) and it was found that all the metal complexes were more potent then the ligand [84]. A similar result was also been reported earlier where there was a considerable increase in the biocidal activity of diphenylamine-2,2'-dicarboxylic acid and its derivatives on being coordinated with the zinc ion has been reported [85]. The activity increased with increasing lability of the metal complexes. *N*-(2-methoxybenzoyl)-*N*'-(4-diphenylamine)thiourea was also found to be most active compound against *Staphylococcus aureus* with 9.5 mm zone of inhibition due to its small molecular size. The diarylamine skeleton and the different substituents (-H, -OMe or -F) proved to be important for activity, changing selectivities and MICs, when compared with the functionalized (Br and $NH_2$) benzo[b]thiophene

precursors [86]. Similarly Estivinoh et al. [87] had also prepared *ortho*-Chlorodiarylamines in the 2,3,7-trimethylbenzo[*b*]thiophene series by palladium-catalyzed C–N cross-coupling. Various groups present on *ortho*-chlorodiarylamine plays important role in determing the efficacy of the molecule. The minimal bacteriostatic concentration of the 3, 3’, 5, 5'-tetrachlorotetranitrodiphenylamine for *S. aureus* and *B. subtilis* was 0.156 ug/ml. The bactericidal concentration was 0.312 ug/ml. The compound was bacteriostatic for *Streptococcus species* at 5.0 ug/ml and bactericidal above this level [88].

### 1.2.6 Enhancement of Aqueous Solubility of Triclosan

Bioavailability and solubility are two important factors for remedial effectiveness of any drug molecule. It is necessary to improve solubility of drug by some methods like pH adjustment [89] salt formation [90], or chemical modification of the substrate (formation of pro-drugs). If these methods are not applicable, complexing agents or cosolvents can be added to aid in dissolution. Some examples include cyclodextrins [91], dendrimers [92], low-molecular-weight polyethylene glycols [93] (PEGs, e.g., PEG 400) and solvents such as glycerin [94] and *N*-methyl pyrrolidone (NMP) [95].

Triclosan **(1.1)**, a non-ionic antibacterial agent, is widely used in personal care products like toothpastes, deodorants, soaps, skin-care, hand washing solutions and cosmetic products. Its primary target is enoyl-ACP-reductase, an enzyme of the type II fatty acid biosynthesis pathway in bacteria [96,53]. Triclosan has been demonstrated to inhibit fatty acid biosynthesis in *Plasmodium falciparum* [64] and *Mycobacterium smegmatis* [9], making it a promising scaffold, around which a number of newer compounds have been synthesized and tested for possible therapeutic use against malarial [10], tuberculosis [22] and other bacterial infections [97].

A limitation of triclosan and other diphenyl ethers is their poor aqueous solubility (0.002 mg/mL at 30 °C) [98]. Consequently, the general procedure for use in biochemical assays entails the preparation of a stock solution in DMSO prior to dilution in the aqueous solution of interest. Even so, triclosan is soluble only up to 100.0 μM in 0.1 % DMSO. Alternative methods to increase the bioavailability of triclosan include making use of surfactants [99], lysozyme [100], water-dispersible nanoparticles [101], salt formation [98] or vegetable oils [102].

For instance, Hoq et al. have explored the complexation of the bacteriolytic enzyme, lysozyme with triclosan to improve its aqueous solubility. The triclosan-lysozyme complex, as compared to triclosan alone, demonstrated significantly enhanced bactericidal activity against

several strains of Gram-positive and Gram-negative bacteria [100]. In another study, Zhang et al. have demonstrated the formation of nano dispersions by freeze-drying emulsions containing triclosan.

The antibacterial activity of the nano dispersion containing triclosan was significantly better than that of triclosan in organic or aqueous solutions [101]. Chiappetta et al. demonstrated that at low pH, triclosan incorporation into polymeric micelles of poloxamine [103] enhanced the aqueous solubility for triclosan by up to four orders of magnitude. Their study also established that there was significant improvement in the antibacterial activity of this formulation as compared to triclosan alone. Lu et al. synthesized antibiotic biodegradable polymers with demonstrable resistance against *E. coli* by processing poly $\varepsilon$-caprolactone with an inclusion compound of triclosan and $\beta$-cyclodextrin [99]. Apart from these formulations, the aqueous solubility of triclosan is significantly improved by oils and surfactants. However, recent reports in literature indicate that the antibacterial potency of triclosan is severely compromised by the presence of organic solvents and oils [102], surfactants like tween-80 [104], and exogenous fatty acids [105], of which serum is a rich source, thus making triclosan a poor choice for the treatment of systemic diseases. These studies also have implications on the use of oils and surfactants as additives in topical personal care product formulations containing triclosan.

It is therefore essential that antimicrobial studies are conducted to assess the impact of additives which are employed in formulations on the efficacy of triclosan. Here, we report results of a study conducted in order to assess the aqueous solubility and antibacterial potency of triclosan in solutions containing 0.1 % DMSO and surfactants. The surfactants selected for this study included tween-80, tween-20 and triton X-100, octylglucoside and sodium deoxycholate. While tween-80, tween-20 and triton X-100 contain long or alkoxy chains, octyl glucoside and sodium deoxycholate are carbohydrate and steroid skeleton based surfactants, respectively.

# Chapter 2

# Derivatives of 4-(2, 4-dichlorophenoxy)-3-nitro benzoic acid

## 2.1 Introduction

Diphenyl ethers with alkyl chain in ring A, para to ether linkage **(Figure 2a)**, have shown promising results in case of sensitive and resistant strains of MTB [71]. These compounds **(Figure 2a)** showed very good anti- MTB [71] activity and anti-*Francisella tularensis* [106] with only hydroxyl group in ring B and six to eight carbon chain of diphenyl ethers. The ring B in these compounds did not contain any chloro substituents unlike most of reported compounds till date.

**Figure 2a. Compound 2.1 and Compound 2.2 show good antibacterial activity**

We therefore envisioned benzoic acid derivatives of the diphenyl ethers which could be converted to corresponding amides by reaction with amines of varying chain length. Hydroxyl group at ring A is an important moiety which forms a ternary complex with phenol of Tyr-156 enoyl-ACP-reductase, FabI and 2-hydroxyl of the $NAD^+$ ribose for enzyme's inhibition **(Figure 2b)** [107]. This work explores replacement of hydroxyl group with other polar groups such as nitro and amine to examine the effect on biological activity of diphenyl ethers. The dichloro substituents on ring B as in case of triclosan have been retained as such with above parameters in place, synthesis of parent compound 4-(2,4-dichlorophenoxy)-3-nitrobenzoic acid **(2.5)** was taken up.

## 2.2 Synthesis of diphenyl ethers

**Scheme 2.1** represents synthesis of compound **(2.5)** and its various derivatives. Nucleophilic aromatic substitution of fluoro in 4-fluoro-3-nitrobenzoic acid **(2.4)** with 2, 4-dichlorophenol **(2.3)** in the presence of mild base ($K_2CO_3$) and phase transfer catalyst (18-Crown-6) in aprotic solvent gave compound **(2.5)**. Appearance of corresponding signals in the aromatic region

representing six protons in $^{1}$H NMR and m/z signal at 368 for the potasiated peak of compound **2.5** confirmed its synthesis.

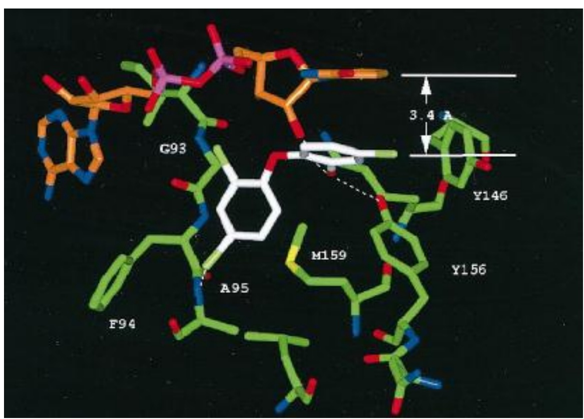



**Figure 2b. Structure of FabI-NAD$^{+}$-inhibitor ternary complex**

$^{13}$C NMR of the compound **(2.5)** also showed presence of aromatic carbons, six of which were quartnary and rest six are primary. The acidic carbon appeared at 164.94 ppm.

**Figure 2A (a and b) (Refer Page No. 91-92)** represent $^{1}$H and $^{13}$C NMR for the compound 2.5. This was converted to its corresponding methyl ester, compound **2.6**, by refluxing in the presence of methanol in acidic medium. Appearance of a prominent singlet at 3.9 ppm in $^{1}$H NMR and at 52.76 ppm in $^{13}$C NMR confirmed the synthesis of compound **2.6**. The electrospray mass spectrometer gave m/z value at 342 corresponding to its actual mass. Compound **2.7** was synthesised from compound **2.6** by reduction of nitro group in presence of Fe/ $FeSO_4.7H_2O$ and water under refluxing conditions. Besides nin-hydrin test, appearance of a broad singlet in $^{1}$H NMR at 3.8 ppm that disappeared on addition of $D_2O$ confirmed the reduction of nitro to corresponding amino group. Simultaneous appearance of the prominent signal at 3.9 ppm (-$OCH_3$) in $^{1}$H NMR and at 166.75 ppm (ester's carbonyl) in $^{13}$C NMR along with amino protons confirmed that ester group remained intact while refluxing in water **(Scheme 2.1)**.

After making parent compound **(2.5)** and its methyl ester **(2.6)** and amino ester **(2.7)**, scheme of synthesis was directed toward preparing amides through acid chloride route. Using chlorinating agent ($PCl_3$) acid group in compound **2.5** was converted to its corresponding chloride that reacted at low temperature (0 $^{o}$C) with amines to give amides. Aliphatic amines with chain length C-2 to C-6 were used **(Scheme 2.2)**. In addition to above chain lengths 3-methoxypropan-1-amine was also used. For all the seven compounds (Compound **2.8** to

Compound **2.14**) confirmation of the product formation was done by $^{1}H$, $^{13}C$ NMR and mass spectroscopic techniques. **Table 2.1** below summarizes prominent parameters in the spectroscopic studies of compound **2.8** to **2.14** that were observed before taking up biological activity of compounds and proceeding further in the reaction scheme.

**Scheme 2.1.** (i) 18-Crown-6, $K_2CO_3$, DMF, Room temperature (ii) Refluxing in Methanol, HCl, 60-70 $^{o}C$ (iii) Fe, $FeSO_4$. $7H_2O$, Reflux

For compound **2.8** no change in the procedure was required for making acid chloride. The methoxy protons and carbon in this case appeared at 3.4 ppm and 72.80 ppm respectively in $^{1}H$ and $^{13}C$ NMR spectra. The pattern for aromatic protons and carbon in the series of compounds from compound **2.8** to compound **2.14** was similar. **Figure 2B (a and b), 2C (a and b)** and **2D (a and b) (Refer Page No. 93-98)** show NMR spectroscopic data for compounds **2.8**, **2.9**, **2.12** respectively. Reduction of nitro to amine group in compound **2.15** to compound **2.21** were afforded in presence of Fe/$FeSO_4$. $7H_2O$ in water under refluxing conditions. These afforded compound **2.15** to compound **2.21** were characterised with NMR and Mass studies. Protons due to amine group appeared merged with two methylene protons in the range of 3.4 to 4.0 ppm. The appearance of protons and carbon due to amide (-CO-NH-) linkage as shown in **Table 2.2** confirmed that amide group formed in the previous step was intact under refluxing conditions of reduction. Rest of aliphatic and aromatic for compounds **2.8** to **2.14** as per prediction and matched with expected structure.

**Figure 2E(a and b), 2F(a and b)** and **2G(a and b) (Refer Page No. 99-104)** shows NMR spectroscopic data for compounds **2.15**, **2.16** and **2.19** respectively. Purification of all the compounds mentioned in **Scheme 2.2** was done using silica gel column chromatography and solvent mixture of polar and non polar gradient.

**Table 2.1. Summary of prominent signals in spectroscopic data for compounds 2.8 to 2.14.**

| Sr. No. | Compound | $^{1}$H NMR | | $^{13}$C NMR | Mass |
|---|---|---|---|---|---|
| | | -CO-NH-C**H**$_2$ | -CO-N**H**- | -**C**O-NH- | m/z |
| 1. | 2.8 | 3.6, m, 4H | 8.4, app. br. s,1H | 163.98 | 421 $[M+23]^+$ |
| 2. | 2.9 | 3.5, m, 4H | 6.1, app. br. s,1H | 164.54 | 355 $[M]^+$ |
| 3. | 2.10 | 3.4, q, 4H | 6.2, app. br. s,1H | 164.46 | 369 $[M]^+$ |
| 4. | 2.11 | 3.5, m, 2H | 6.3, app. br. s,1H | 164.44 | 383 $[M]^+$ |
| 5. | 2.12 | 3.5, q, 2H | 6.3, app. br. s,1H | 164.42 | 397 $[M]^+$ |
| 6. | 2.13 | 3.4, q, 2H | 6.4, app. br. s,1H | 164.41 | 411 $[M]^+$ |
| 7. | 2.14 | 3.4, q, 2H | 6.4, t, 1H | 164.45 | 425 $[M]^+$ |

**2.8: R**= $-CH_2-CH_2-CH_2-OCH_3$
**2.9: R**= $-CH_2-CH_3$
**2.10: R**= $-CH_2-CH_2-CH_3$
**2.11: R**= $-CH_2-(CH_2)_2-CH_3$
**2.12: R**= $-CH_2-(CH_2)_3-CH_3$
**2.13: R**= $-CH_2-(CH_2)_4-CH_3$
**2.14: R**= $-CH_2-(CH_2)_5-CH_3$

**2.15: R**= $-CH_2-CH_2-CH_2-OCH_3$
**2.16: R**= $-CH_2-CH_3$
**2.17: R**= $-CH_2-CH_2-CH_3$
**2.18: R**= $-CH_2-(CH_2)_2-CH_3$
**2.19: R**= $-CH_2-(CH_2)_3-CH_3$
**2.20: R**= $-CH_2-(CH_2)_4-CH_3$
**2.21: R**= $-CH_2-(CH_2)_5-CH_3$

**Scheme 2.2.** (i) $PCl_3$, $K_2CO_3$, DCM, aliphatic amine (ii) Fe, $FeSO_4.7H_2O$, Reflux,

**Table 2.2. Summary of prominent signals in spectroscopic data for compounds 2.15 to 2.21.**

| Sr. No. | Compound | $^{1}$H NMR | | $^{13}$C NMR | Mass |
|---|---|---|---|---|---|
| | | -N**H**$_2$ | -CO-N**H**- | -**C**O-NH- | m/z |
| 1. | 2.15 | 4.0, s, 2H | 6.9, br-t, 1H | 166.86 | 369 $[M]^+$ |
| 2. | 2.16 | 3.4, m, 2H | 6.1, app. br. s, 1H | 167.11 | 325 $[M]^+$ |
| 3. | 2.17 | 3.6, s, 2H | 6.1, app. br. s, 1H | 167.21 | 339 $[M]^+$ |
| 4. | 2.18 | 4.0, br-s, 2H | 6.0, app. br. s, 1H | 167.18 | 353 $[M]^+$ |
| 5. | 2.19 | 3.5, br-s, 2H | 6.1, app. br. s, 1H | 167.13 | 367 $[M]^+$ |
| 6. | 2.20 | 3.6, br-s, 2H | 6.1, app. br. s, 1H | 167.11 | 381 $[M]^+$ |
| 7. | 2.21 | 3.7, br-s, 2H | 6.1, app. br. s, 1H | 167.08 | 395 $[M]^+$ |

### 2.3 Antibacterial Activity

All the compounds synthesized **Scheme 2.1 and 2.2** were screened by Microbroth dilution assay for their antibacterial activity against four non-pathogenic organisms, Gram negative strains included *Pseudomonas aeruginosa* (MTCC-3541), *Pseudomonas putida* and *Escherichia coli* while Gram positive included *Staphylococcus epidermis* (MTCC 2639) **Table 2.3** shows that compound 5 was the most potent in the whole series having MIC value in the range of 0.98 µg/ml to 7.81 µg/ml against all the four organisms.

#### 2.3.1 Activity against *Escherichia coli*

In case of *E. coli* compound **2.10** have MIC value (0.98 µg/ml) far better than the control triclosan (MIC= 15.6 µg/ml) other control, Streptomycin, did not show any activity against *E. coli*. The antibacterial activity of other molecules (compounds **2.5**, **2.9**, **2.11**, and **2.13)** against the organism was also notable; MIC values were 1.95 µg/ml, 1.95 µg/ml, 1.95 µg/ml and 7.81 µg/ml for compounds **2.5**, **2.9**, **2.11**, and **2.13** respectively. These MIC values were better than the control triclosan (MIC- 15.6 µg/ml). All the above mentioned molecules including compound **2.9**, have nitro group at 2- position and a carbonyl of carboxylic acid or amide at 4- position of ring A. as shown in **Figure 2c**. The exceptions to this observation are compounds **2.6, 2.8** and **2.12** which did not show any activity against *E. coli*. Although 2-position contains nitro group in all the 4 molecules, 4-position has methyl ester of carboxylic acid (compound **2.6**), methyl ether

R=OH
R= NH-$(CH_2)$n-$CH_3$
R'= O/H

**Figure 2c. General structure of the compounds (2.5-2.21)**

**Table 2.3. Screening of diphenyl ethers against Gram +ve and Gram –ve bacterial strains**

| Sr. No. | Compound | *E. coli*[a] | *P. aeru. 647*[b] | *P. aeru. 3541*[c] | *P. putida* | *S. epidermidis*[d] |
|---|---|---|---|---|---|---|
| | | MIC (µg/mL) | | | | |
| 1. | 2.5 | 1.95 | - | - | - | - |
| 2. | 2.6 | - | 31.25 | 7.81 | 31.25 | 15.62 |
| 3. | 2.7 | - | 15.62 | 15.62 | 15.62 | 15.62 |
| 4. | 2.8 | - | 125.00 | 62.50 | 125.00 | 125.00 |
| 5. | 2.9 | 1.95 | 7.81 | 3.90 | 7.81 | 7.81 |
| 6. | 2.10 | 0.98 | 15.62 | 7.81 | 15.62 | 15.62 |
| 7. | 2.11 | 1.95 | 15.62 | 1.95 | 15.62 | 15.62 |
| 8. | 2.12 | - | 31.25 | 15.62 | 31.25 | 31.25 |
| 9. | 2.13 | 7.81 | 15.62 | 3.90 | 31.25 | 15.62 |
| 10. | 2.14 | 62.50 | 31.25 | 62.50 | 31.25 | 62.50 |
| 11. | 2.15 | - | 15.62 | 7.81 | 15.62 | 15.62 |
| 12. | 2.16 | - | 31.25 | 15.62 | 15.62 | 15.62 |
| 13. | 2.17 | - | 15.62 | 7.81 | 15.62 | 31.25 |
| 14. | 2.18 | - | 31.25 | 7.81 | 31.25 | 31.25 |
| 15. | 2.19 | - | 31.25 | 125.00 | 15.62 | 15.62 |
| 16. | 2.20 | - | 31.25 | 15.62 | 31.25 | 31.25 |
| 17. | 2.21 | - | - | 125.00 | - | - |
| 18. | Triclosan* | 15.62 | 1.95 | 7.81 | 3.90 | 1.95 |
| 19. | Streptomycin* | - | 15.62 | 7.81 | 1.95 | 7.81 |

*Triclosan, a diphenyl ether, and Streptomycin were used as reference compounds.

***a***- MTCC 1302, ***b*** - MTCC 647, ***c*** - MTCC 3541, ***d*** -MTCC 2639

chain terminal (compound- **2.8**) and long hydrocarbon chain with odd number of carbon (compound **2.12**) in these molecules which may be responsible for the in activity of these compounds against *E. coli* compound **2.14**, a higher analogue of compound **2.12** with odd number of carbons in the side chain at 4-position, has also shown increased MIC value for *E. coli* (162.5 µg/ml). None of the molecules with amino group at 2-position of ring A (compounds **2.7**, and **2.15 - 2.21**) were active against *E. coli*.

### 2.3.2 Activity against *Pseudomonas aeruginosa*

Unlike case of *E. coli*, against *Pseudomonas aeruginosa* all the compounds including amino derivatives have shown antibacterial activity. However, the trend of minimum inhibitory concentration (MIC) against the organism was similar to that observed for *E. coli* in case of nitro compound was an exception here as it was inactive.

Against *Pseudomonas aeruginosa* compound **2.11** was the most potent with a MIC of 1.95 µg/ml. The molecule has even number of four carbon chain. Compounds **2.9** and **2.13** with 2 and 6 carbon chains were having a MIC of 3.90 µg/ml against *P. aeruginosa* 3541. Interestingly compound **2.9** with 2 carbon chain was having lowest MIC in this case of *P. aeruginosa* 647. Compounds **2.10**, **2.12** and **2.14** with odd number of carbons and increasing chain length gave increasing trend for their MIC values **(Table 2.3).** Compound **2.8** with methyl ether chain terminal also gave higher MIC (62.5 µg/ml) against *P. aeruginosa* 3541.

All the amino derivatives listed in **Scheme 2.2** were active against *Pseudomonas aeruginosa* but gave MIC on higher side (7.81 to 125.00 µg/ml) compounds **2.15**, **2.17** and **2.18** in this case gave lower MIC value (7.81 µg/ml) than compound **2.16** with 2 carbon chain (MIC 15.62g/ml). Higher carbon chain length molecules compound **2.20** and compound **2.21** gave comparatively higher MIC values in this case.

### 2.3.3 Activity against other organisms

For other two organisms *P. putida* and *S. epidermidis* the antibacterial activity, although observed for the most compounds in the series, was in higher range (15.62- 31.25 µg/mL) as shown in **Table 2.3**. Only exception here was compound **2.9** for which MIC was 7.81 µg/mL. Controls triclosan (MIC 3.91 µg/mL and 1.95 µg/ml) streptomycin (MIC 1.95 µg/mL and 7.81

μg/ml) gave comparable MIC against *P. putida* & *S. epidermidis* respectively. Compound **2.5** and **2.21** did not show any antibacterial activity

### 2.4 *In silico* docking studies

It has been reported that formation of a FabI-NAD-triclosan ternary complex accounts for the effectiveness of triclosan as an antibacterial agent. The hydroxychlorophenyl ring (ring A) stacks with the nicotinamide ring of the $NAD^+$ making Π-Π interactions with an interplanar distance of 3.4 Å. The hydroxyl group of the triclosan forms hydrogen bonds with phenol of Tyr-156 and with the 2'-hydroxyl of the $NAD^+$ ribose. The 2',4'-dichlorophenyl ring of triclosan (ring B) sits in a hydrophobic pocket in contact with Met-159. The 4'-chloro substituent accepts a hydrogen bond from the amide backbone nitrogen of Ala-95. Ring B is closed in a pocket surrounded by nicotinamide ribose, the phosphates of $NAD^+$, Met159 and the substrate binding loop residues. The 2' chloro group of ring B resides near the pyrophosphate moiety of $NAD^+$ while the 4' chloro group is in van der Waals contact with the hydrophobic side chains of Leu100 and Met159 **(Figure 2.2)** [107].

Interestingly, all the nitro compounds as synthesised in **Scheme 2.2 (Figure 2c above)** showed a flipped conformation *In silico* docking studies for *E. coli* FabI as compared to triclosan. **Figure 2e** and **2f** shows flipped conformation for triclosan and compound **2.10**. A new hydrogen bond can be seen between nitro group of ring A and phosphate group of $NAD^+$ (3.2 $A^o$) besides Π-Π stacking of ring B and nicotinamide ring of $NAD^+$. Literature has also cited some examples where $NO_2$ in ring A forms hydrogen bond with –OH group **(Figure 2d)** of tyrosine due to inability of the hydroxyl phenyl ring to fit in the cavity of the enzyme. The results in this case were supported by good antibacterial anti *P. falciparum* activity ($IC_{50}$= 0.23 μM) [11]. The probable rational for the flipping of the molecule as compared to triclosan at the active site could be presence of bulky amide group that makes sterically impossible for the molecule triclosan-like conformations.

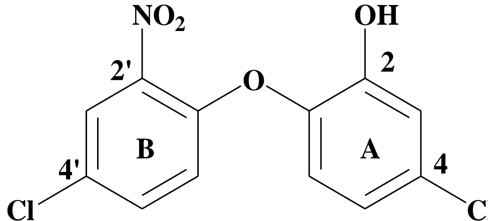



**Figure 2d. Triclosan analogue**

**Table 2.4. Antibacterial activity and binding affinity of the compounds against *E. coli* strains and with the EcFabI protein respectively**

| Sr. No. | Compound | *E. coli* | Binding Affinity |
|---|---|---|---|
| | | **MIC (µg/mL)** | **(kcal/mol)** |
| 1. | 2.5 | 1.95 | -7.9 |
| 2. | 2.6 | - | -6.7 |
| 3. | 2.7 | - | -8.1 |
| 4. | 2.8 | - | -8.0 |
| 5. | 2.9 | 1.95 | -8.1 |
| 6. | 2.10 | 0.98 | -8.2 |
| 7. | 2.11 | 1.95 | -7.9 |
| 8. | 2.12 | - | -8.0 |
| 9. | 2.13 | 7.81 | -7.6 |
| 10. | 2.14 | 62.50 | -8.0 |
| 11. | 2.15 | - | -8.1 |
| 12. | 2.16 | - | -8.3 |
| 13. | 2.17 | - | -8.4 |
| 14. | 2.18 | - | -8.5 |
| 15. | 2.19 | - | -8.4 |
| 16. | 2.20 | - | -8.2 |
| 17. | 2.21 | - | -8.1 |
| 18. | Triclosan | 15.62 | -9.5 |

As the result of flipping, the chloro groups at 2' and 4' position of analogs are now found to be surrounded by the hydrophobic residues Pro191, Ile200, Phe203 and Met206 of the pocket. Similarly, the organic amide substitutions at position 4 of ring A occupies the position of the 4-chloro group of triclosan and is observed in weak van der Waals contact/hydrophobic interactions with the hydrophobic side chain residues of Phe94, Ala95, Gly97, Leu100, Met159, Ala196 and Gly199. However when nitro compounds synthesised in **Scheme 2.2** were replaced

by amino compounds the H-bonding is lost due to replacement of electronegative oxygen atoms by hydrogen **(Figure 2g)**. As a result no binding takes place. This is complimented by biological activity of compounds where none of amino compounds gave activity against *E. coli*.

### 2.5 Structure activity Relationship

From compounds mentioned in **Scheme 2.1 and 2.2**, **Table 2.3** and above discussion following conclusions can be made about structural activity relationship. Nitro group at 2- position of ring A is preferred over amino group at the same position for the antibacterial activity.

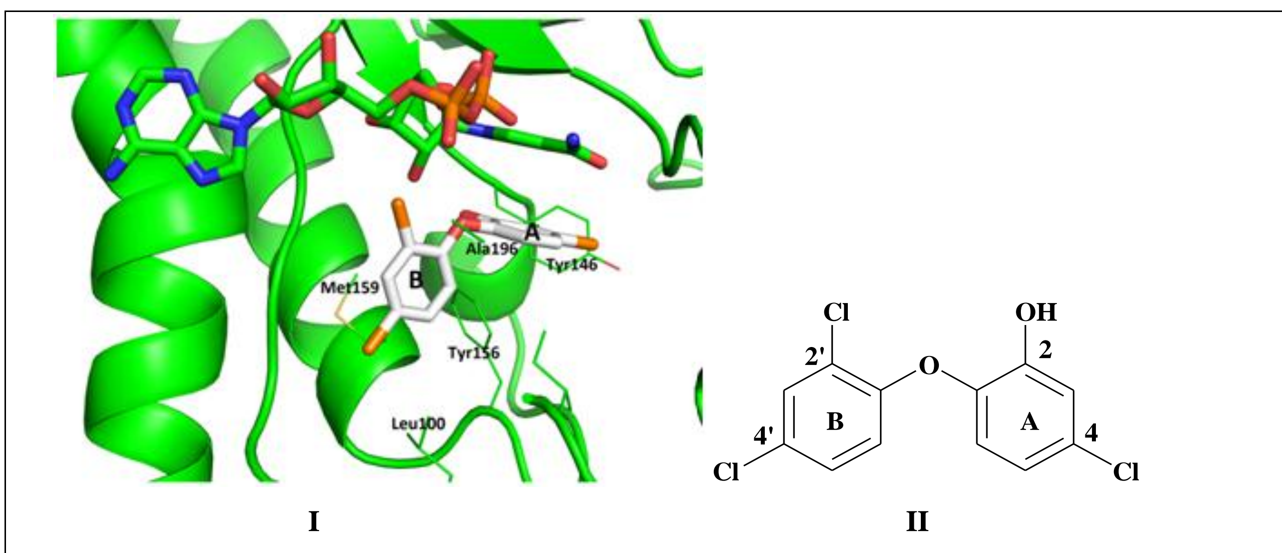



**Figure 2e. I: EcFabI-NAD$^+$-Triclosan ternary complex, II: Triclosan molecule**

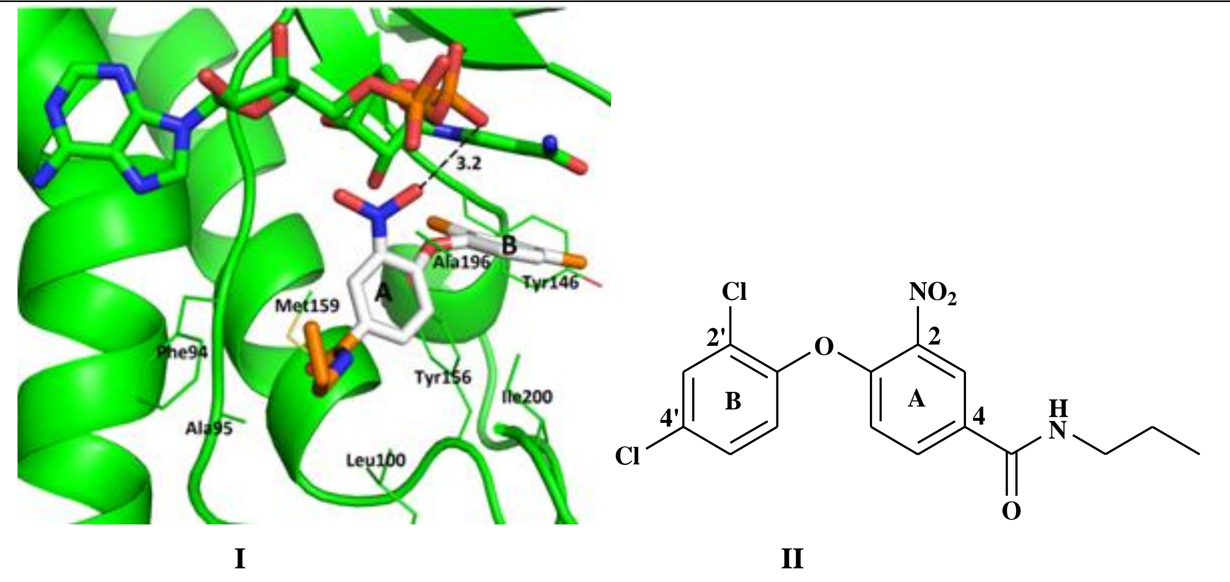



**Figure 2f. I: EcFabI-NAD$^+$-Test molecule 2.10 ternary complex, II: Test molecule 2.10**

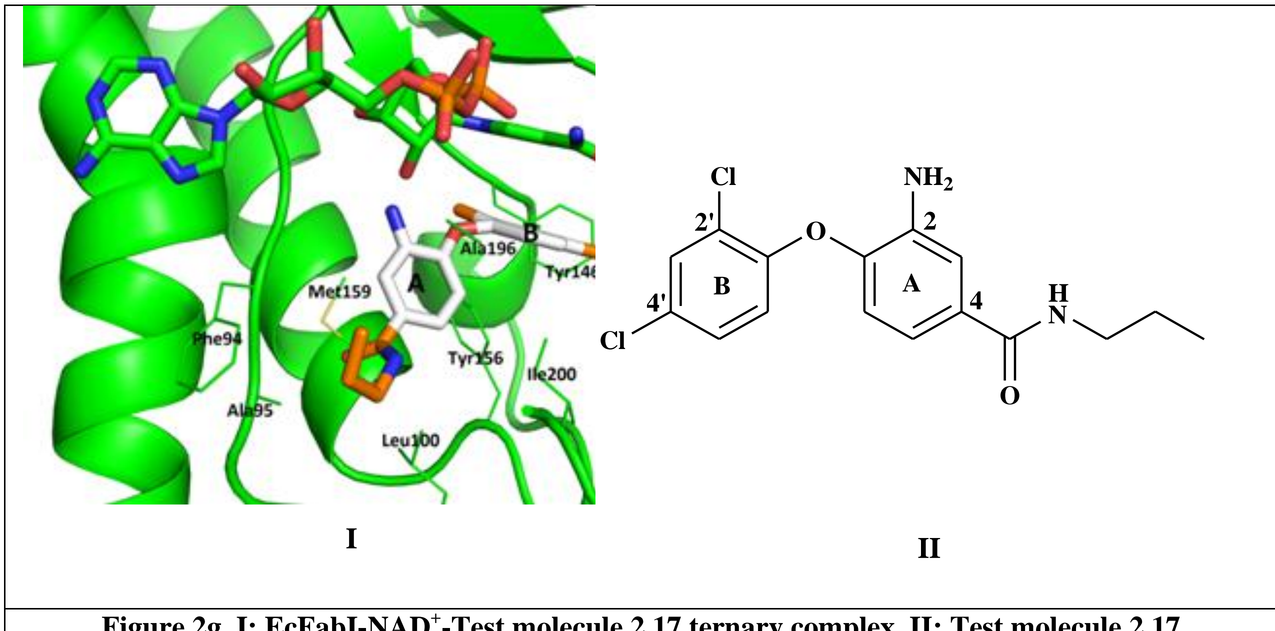
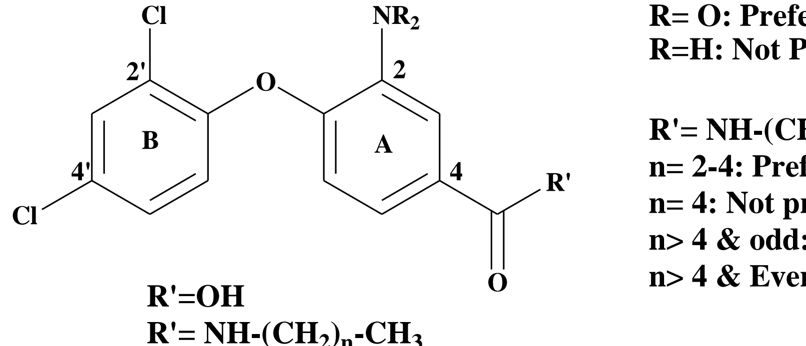



**Figure 2g. I: EcFabI-NAD$^+$-Test molecule 2.17 ternary complex, II: Test molecule 2.17**

Amide linkage at 4-position having chain length upto four carbon atoms is preferred for higher MIC values. As the length of chain beyond four carbon atom increases, MIC value starts decreasing. For higher chain length odd number of carbons atoms in amide linkage give lower MIC value as compared to even number of carbon atoms. **Figure 2h** below summarize antibacterial activity.




**R= O: Preferred Higher MIC value**
**R=H: Not Preferred  Lower MIC value**

**R'= NH-$(CH_2)_n$-$CH_3$**
**n= 2-4: Preffered Higher MIC value**
**n= 4: Not preffered Lower MIC value**
**n> 4 & odd: Not preffered Lower MIC value**
**n> 4 & Even: Relative Lower MIC value**

**Figure 2h. Structure-activity relationship**

## 2.6 Materials and Methods

### 2.6.1 Synthesis of substituted diphenyl ethers and their derivatives

Purified reagents were procured from SD Fine Chemicals, Bombay. Solvents were distilled before doing the column chromatography. Melting points are reported in $^oC$ and are uncorrected. $^1H$ and $^{13}C$ NMR spectral analyses were performed on 400.1 and 100.6 MHz spectrometer

(Bruker) with tetramethylsilane and $CDCl_3$ as the internal standard (δ, ppm) respectively. The following abbreviations were used to explain the multiplicities: s, singlet; app. br. s, apparent broad singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet, br, broad. Mass analysis was done using Waters Micromass mass spectrometer. **Schemes 2.1 and 2.2** summarize all the synthetic procedures.

**Procedure for synthesis of 4-(2,4-dichlorophenoxy)-3-nitrobenzoic acid (2.5):** To a solution of 4-fluoro-3-nitrobenzoic acid **(2.4)** (3.9g, 20.9 mmol) in DMF (25 ml) were added $K_2CO_3$ (11.5 g, 83.9 mmol), 2,4-dichlorophenol **(2.3)** (4.3 g, 26.3 mmol) and 18-crown-6 (50 mg, 0.2 mmol). The mixture was stirred at room temperature for 12 h. After the reaction is complete (TLC monitoring), the reaction mixture was diluted with DCM (100 ml), washed with water (50 ml), 1 N NaOH (3× 10 ml), water (until neutral to litmus paper), brine and dried over $Na_2SO_4$. Evaporation of the organic solvent gave crude compound **(2.5)**. The product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ethyl acetate = 85:15) to afford yellow solid in 88 % yield (7.6 gm). $^1$H NMR (400.1 MHz, $CDCl_3$) δ 6.8 (d, 1H, *J* 8.76, Ar-H), 7.2 (d, 1H, *J* 8.72, Ar-H), 7.4 (dd, 1H, *J* 2.48, 8.68, Ar-H), 7.5 (t, 1H, *J* 9.44, Ar-H), 7.6 (d, 1H, *J* 2.48, 8.72, Ar-H), 7.8 (br-s, 1H, COOH), 7.4 (dd, 1H, *J* 2.12, 8.76, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) δ 117.57, 123.02, 126.34, 126.50, 126.95, 128.73, 130.38, 131.02, 135.19, 139.07, 148.26, 152.27, 164.94; Electrospray-MS *m/z*, calcd. for $C_{13}H_7Cl_2NO_5$ $[M]^+$: 328, found: 368 $[M+K^+]$.

**Procedure for synthesis of methyl 4-(2,4-dichlorophenoxy)-3-nitrobenzoate (2.6):** To the solution of compound **(2.5)** (500 gm, 1.5 mmol) in methanol (10 ml), HCl (1 mL) was added drop wise and the reaction mixture refluxed at 60–70 $^o$C. After the reaction is complete (TLC monitoring) the solvent was evaporated in vacuo and reaction was quenched with water. It was extracted with DCM (3 x 30 ml) and the combined organic layers were washed with water (2 x 25 ml), brine and dried over $Na_2SO_4$. Evaporation of the organic solvent gave crude product which was purified using $SiO_2$ column chromatography and solvent (pet ether/ethyl acetate = 75:25) to afford a light yellow solid **(2.6)** in 85.0 % yield (442 mg) $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.9 (s, 3H, $CH_3$), 6.7 (d, 1H, *J* 8.76, Ar-H), 7.1 (d, 1H, *J* 8.72, Ar-H), 7.3 (dd, 1H, *J* 2.48, 8.72, Ar-H), 7.5 (d, 1H, *J* 2.44, Ar-H), 8.1 (dd, 1H, *J* 2.08, 8.72, Ar-H), 8.6 (d, 1H, *J* 2.12, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 52.76, 117.34, 123.35, 125.30, 127.65, 127.71, 128.83,

131.12, 132.15, 135.19, 139.61, 148.47, 153.45, 164.53; Electrospray-MS *m/z*, calcd. for $C_{14}H_9Cl_2NO_5$ $[M]^+$: 342, found: 342$[M]^+$.

**Procedure for synthesis of methyl 3-amino-4-(2,4-dichlorophenoxy)benzoate (2.7):**

To a suspension of compound **(2.6)** (3.6 g, 10.6 mmol) in refluxing $H_2O$ (100 ml) were added Fe (5.95 g, 106.2mmol) and $FeSO_4.7H_2O$ (2.9 g, 10.4 mmol). The reaction mixture was refluxed for 8 h. After cooling to room temperature and confirming the completion of reaction (TLC monitoring), it was filtered through celite, washed thoroughly with ethyl acetate (2 x 75 ml). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave corresponding amine. This was confirmed by spotting the product on alumina plate which upon dipping in ninhydrin solution (2% in ethanol) and further heating turned black. The compound was isolated and purified using $SiO_2$ column chromatography and solvent (pet ether/EtOAc = 80:20) to afford a dark brown solid in 51% yield. $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.8 (br-s, 2H, $NH_2$), 3.9 (s, 3H, $CH_3$), 6.7 (d, 1H, *J* 8.4 Hz, Ar-H), 6.9 (d, 1H, *J* 8.72, Ar-H), 7.2 (dd, 1H, *J* 2.52, 8.76, Ar-H), 7.4 (dd, 1H, *J* 2.0, 8.44, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 7.5 (d, 1H, *J* 2.08, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 52.12, 116.63, 117.47, 120.53, 121.18, 126.33, 126.39, 128.25, 129.82, 130.63, 137.25, 147.16, 150.33, 166.75; Electrospray-MS *m/z*, calcd. for $C_{14}H_{11}Cl_2NO_3$ $[M]^+$: 312, found: 312$[M]^+$.

**General procedure of preparation amide derivatives of (2.8-2.14):** To aliphatic carboxylic acid (0.5 mmol) was added $PCl_3$ (0.5 mmol) and the reaction mixture stirred at 60–70°C. After 45 min, the reaction mixture was poured in-situ to another reaction mixture of compound **(2.5)** (0.5 mmol), $K_2CO_3$ (0.3 mmol) and DCM (10 mL) already stirred at 0°C. The reaction mixture was allowed to stir for another 2 hrs. The expected ester amide product was extracted with DCM (3*60 ml), dried over $Na_2SO_4$, and concentrated in vacuo. Crude product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ ethyl acetate= 70:30) to afford light brown solids **(2.8-2.14)**.

**4-(2,4-dichlorophenoxy)-*N*-(3-methoxypropyl)-3-nitrobenzamide (2.8):** White solid in 56 % yield; mp 129-131 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.9 (m, 2H, $CH_2$), 3.4 (s, 3H, $OCH_3$), 3.6 (m, 4H, $2CH_2$), 6.8 (d, 1H, *J* 8.3, Ar-H), 7.1 (d, 1H, *J* 8.28, Ar-H), 7.3 (dd, 2H, *J* 2.12, J 8. 62, Ar-H), 7.5 (d, 1H, *J* 2.08, Ar-H), 7.9 (dd, 1H, *J* 1.76, 8.76, Ar-H), 8.4 (app. br. s, 1H, 1NHCO); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 28.55, 39.86, 59.09, 72.80, 118.02, 122.97, 124.71, 127.45,

128.73, 129.98, 131.05, 131.79, 133.10, 139.48, 148.85, 152.18, 163.98; Electrospray-MS *m/z*, calcd. for $C_{17}H_{16}Cl_2N_2O_5$ $[M]^+$: 399, found: 421$[M+23]^+$.

**4-(2,4-dichlorophenoxy)-*N*-ethyl-3-nitrobenzamide (2.9):** White in 52 % yield; mp 122-126 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.3 (t, *J* 7.28, 3H, $CH_3$), 3.5 (m, 2H, $CH_2$), 6.1 (app. br. s, 1H, 1NHCO), 6.8 (d, 1H, *J* 8.68, Ar-H), 7.1 (d, 1H, *J* 8.72 Hz, Ar-H), 7.3 (dd, 1H, *J* 2.4, 8. 6, Ar-H), 7.5 (d, 1H, *J* 2. 48, Ar-H), 7.9 (dd, 1H, *J* 2.2, 8.72, Ar-H), 8.4 (d, 1H, *J* 2.24, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 11.45, 42.10, 118.02, 123.02, 124.59, 127.45, 128.77, 129.92, 131.07, 131.89, 133.32, 139.39, 148.74, 152.26, 164.54; Electrospray-MS *m/z*, calcd. for $C_{15}H_{12}Cl_2N_2O_4$ $[M]^+$: 355, found: 355$[M]^+$.

**4-(2,4-dichlorophenoxy)-3-nitro-*N*-propylbenzamide (2.10):** Off white solid in 48 % yield; mp 132-135 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 7.36, $CH_3$), 1.7 (m, 2H, $CH_2$), 3.4 (q, 2H, *J* 6.6, 13.52, $CH_2$), 6.2 (app. br. s, 1H, 1NHCO), 6.8 (d, 1H, *J* 8.72, Ar-H), 7.1 (d, 1H, *J* 8.72, Ar-H), 7.3 (dd, 1H, *J* 2.4, 8.68, Ar-H), 7.5 (d, 1H, *J* 2.4, Ar-H), 8.0 (dd, 1H, *J* 2.12, 8.72, Ar-H), 8.4 (d, 1H, *J* 2.08, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 11.45, 22.86, 42.09, 118.05, 123.03, 124.52, 127.49, 128.76, 129.93, 131.09, 131.90, 133.26, 139.44, 148.76, 152.29, 164.46; Electrospray-MS *m/z*, calcd. for $C_{16}H_{14}Cl_2N_2O_4$ $[M]^+$: 369, found: 369$[M]^+$.

***N*-butyl-4-(2,4-dichlorophenoxy)-3-nitrobenzamide (2.11):** Off white solid in 67 % yield; mp 120-122 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.0 (t, 3H, *J* 7.36, $CH_3$), 1.4 (m, 2H, $CH_2$), 1.6 (m, 2H, $CH_2$), 3.5 (m, 2H, $CH_2$), 6.3 (app. br. s, 1H, 1NHCO), 6.8 (d, 1H, *J* 8. 72, Ar-H), 7.1 (d, 1H, *J* 8.68, Ar-H), 7.3 (dd, 1H, *J* 2.48, 8.68, Ar-H), 7.5 (d, 1H, *J* 2.44, Ar-H), 8.0 (dd, 1H, *J* 2.24, 8.72, Ar-H), 8.4 (d, 1H, *J* 2.2, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.73, 20.13, 31.62, 40.15, 118.08, 122.95, 124.51, 127.45, 128.73, 130.01, 131.07, 131.87, 133.21, 139.52, 148.83, 152.21, 164.44; Electrospray-MS *m/z*, calcd. for $C_{17}H_{16}Cl_2N_2O_4$ $[M]^+$: 383, found: 383$[M]^+$.

**4-(2,4-dichlorophenoxy)-3-nitro-*N*-pentylbenzamide (2.12):** Yellow solid in 59 % yield; mp 112-116 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 5.8, $CH_3$), 1.4 (q, 4H, *J* 3.56, 7.24, $2CH_2$), 1.6 (m, 2H, $CH_2$), 3.5 (q, 2H, *J* 6.84, 13.16, $CH_2$), 6.3 (app. br. s, 1H, 1NHCO), 6.8 (d, 1H, *J* 8.72, Ar-H), 7.1 (d, 1H, *J* 8.68, Ar-H), 7.3 (dd, 1H, *J* 2.44, 8.68, Ar-H), 7.5 (d, 1H, *J* 2.4, Ar-H), 8.0 (dd, 1H, *J* 2.12, 8.68, Ar-H), 8.4 (d, 1H, *J* 2.12, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.98, 22.36, 29.10, 29.26, 40.43, 118.06, 122.98, 124.52, 127.47, 128.75, 129.98,

131.08, 131.88, 133.24, 139.48, 148.80, 152.24, 164.42; Electrospray-MS *m/z*, calcd. for $C_{18}H_{18}Cl_2N_2O_4$ $[M]^+$: 397, found: 397$[M]^+$.

**4-(2,4-dichlorophenoxy)-*N*-hexyl-3-nitrobenzamide (2.13):** Dark brown solid in 53 % yield; mp 144-147 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 6.88, $CH_3$), 1.4 (m, 6H, $3CH_2$), 1.6 (m, 2H, $CH_2$), 3.4 (q, 2H, *J* 7.04, 13.08, $CH_2$), 6.4 (app. br. s, 1H, 1NHCO), 6.8 (d, 1H, *J* 8.76, Ar-H), 7.1 (d, 1H, *J* 8.72, Ar-H), 7.3 (dd, 1H, *J* 2.4, 8.64, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 8.0 (dd, 1H, *J* 2.24, 8.68, Ar-H), 8.4 (d, 1H, *J* 2.16, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.02, 22.55, 26.65, 29.54, 29.71, 31.47, 40.46, 118.07, 122.98, 124.51, 127.48, 128.74, 130.00, 131.08, 131.88, 133.23, 139.50, 148.82, 152.24, 164.41; Electrospray-MS *m/z*, calcd. for $C_{19}H_{20}Cl_2N_2O_4$ $[M]^+$: 411, found: 411$[M+1]^+$.

**4-(2,4-dichlorophenoxy)-*N*-heptyl-3-nitrobenzamide (2.14):** Dark brown solid in 57 % yield; mp 99-103 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 6.88, $CH_3$), 1.3 (m, 8H, $4CH_2$), 1.6 (m, 2H, $CH_2$), 3.5 (q, 2H, *J* 7.04, 13.04, $CH_2$), 6.4 (t, 1H, *J* 5.28, 1NHCO), 6.8 (d, 1H, *J* 8.72, Ar-H), 7.1 (d, 1H, *J* 8.72, Ar-H), 7.3 (dd, 1H, *J* 2.44, 8.68, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 8.0 (dd, 1H, *J* 2.24, 8.68, Ar-H), 8.4 (d, 1H, *J* 2.2 Hz, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.09, 22.60, 26.96, 28.98, 29.57, 31.74, 40.47, 118.03, 123.01, 124.56, 127.46, 128.76, 129.95, 131.07, 131.88, 133.30, 139.42, 148.77, 152.25, 164.45; Electrospray-MS *m/z*, calcd. for $C_{20}H_{22}Cl_2N_2O_4$ $[M]^+$: 425, found: 425$[M]^+$.

**General procedure for synthesis of 3-amino-4-(2,4-dichlorophenoxy)-*N*-alkylbenzamide (2.15-2.21):** To a suspension of compound **(2.8-2.14)** (10.6 mmol) in refluxing $H_2O$ (100 ml) were added Fe (5.95 g, 106.2mmol) and $FeSO_4.7H_2O$ (2.9 g, 10.4 mmol). The reaction mixture was refluxed for 8 h. After cooling to room temperature and confirming the completion of reaction (TLC monitoring), it was filtered through celite, washed thoroughly with ethyl acetate (2 x 75 ml). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave corresponding amine. This was confirmed by spotting the product on alumina plate which upon dipping in ninhydrin solution (2% in ethanol) and further heating turned black. The compound was isolated and purified using $SiO_2$ column chromatography and solvent (pet ether/EtOAc = 80:20) to afford a light to dark brown solids **(2.15-2.21)**.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-(3-methoxypropyl)benzamide (2.15):** Brown solid in 63 % yield; mp 96-99 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.9 (m, 2H, $CH_2$), 3.4 (s, 3H, $OCH_3$), 3.5

(q, 4H, *J* 5.68, 11.4, $2CH_2$), 4.0 (s, 2H, $NH_2$), 6.7 (d, 1H, *J* 8.32, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 6.9 (br-t, 1H, 1NHCO), 7.0 (dd, 1H, *J* 2.12, 8.34, Ar-H), 7.2 (dd, 1H, *J* 2.52, 8.78, Ar-H), 7.3 (d, 1H, *J* 2.08, Ar-H), 7.5 (d, 1H, *J* 2.52, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 28.90, 39.01, 58.91, 72.24, 115.53, 116.64, 117.54, 120.37, 125.89, 128.13, 129.29, 130.51, 131.50, 137.98, 145.39, 150.80, 166.86; Electrospray-MS *m/z*, calcd. for $C_{17}H_{18}Cl_2N_2O_3$ $[M]^+$: 369, found: 369$[M]^+$.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-ethylbenzamide (2.16):** White solid in 47 % yield; mp 87-91 °C; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.2 (t, 3H, *J* 7.32,$CH_3$), 3.4 (m, 4H, $CH_2$, NH), 6.1 (app. br. s, 1H, 1NHCO), 6.6 (d, 1H, *J* 8.28, Ar-H), 6.8 (d, 1H, *J* 8.76, Ar-H), 6.9 (dd, 1H, *J* 1.68, 8.32, Ar-H), 7.1 (dd, 1H, *J* 2.44, 8.76, Ar-H) 7.2 (d, 1H, *J* 12.36, Ar-H), 7.4 (d, 1H, *J* 2.4, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.90, 34.96, 115.45, 116.70, 117.50, 120.40, 125.91, 128.16, 129.37, 130.54, 131.47, 137.96, 145.46, 150.73, 167.11; Electrospray-MS *m/z*, calcd. for $C_{15}H_{14}Cl_2N_2O_2$ $[M]^+$: 325, found: 325$[M+1]^+$.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-propylbenzamide (2.17):** Off white solid in 42 % yield; mp 85-89 °C; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 7.36, $CH_3$), 1.5 (m, 2H, $CH_2$), 3.3 (q, 2H, *J* 6.6, 13.38, $CH_2$), 3.6 (s, 2H, $NH_2$), 6.1 (app. br. s, 1H, 1NHCO), 6.6 (d, 1H, *J* 8.28, Ar-H), 6.8 (d, 1H, *J* 8.76, Ar-H), 6.9 (dd, 1H, *J* 1.8, 8.3, Ar-H), 7.1 (dd, 1H, *J* 2.44, 8.74, Ar-H), 7.2 (d, 1H, *J* 1.68, Ar-H), 7.4 (d, 1H, *J* 2.48, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 11.47, 22.93, 41.78, 115.52, 116.79, 117.51, 120.39, 125.90, 128.16, 129.36, 130.53, 131.55, 137.87, 145.47, 150.73, 167.21; Electrospray-MS *m/z*, calcd. for $C_{16}H_{16}Cl_2N_2O_2$ $[M]^+$: 339, found: 339$[M]^+$.

**3-amino-*N*-butyl-4-(2,4-dichlorophenoxy)benzamide (2.18):** Off white solid in 56 % yield; mp 100-101 °C; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 7.32, $CH_3$), 1.3 (m, 2H, $CH_2$), 1.5 (m, 2H, $CH_2$), 3.3 (q, 2H, *J* 6.76, 13.02, $CH_2$), 4.0 (br-s, 2H, $NH_2$), 6.0 (app. br. s, 1H, 1NHCO), 6.6 (d, 1H, *J* 8.28, Ar-H), 6.8 (d, 1H, *J* 8.72, Ar-H), 6.9 (dd, 1H, *J* 1.08, 8.22, Ar-H), 7.1 (dd, 1H, *J* 2.36, 8.76, Ar-H), 7.2 (d, 1H, *J* 10.2, Ar-H), 7.4 (d, 1H, *J* 2.36, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.82, 20.17, 31.73, 39.83, 115.43, 116.67, 117.53, 120.36, 125.89, 128.15, 129.34, 130.54, 131.56, 138.00, 145.41, 150.75, 167.18; Electrospray-MS *m/z*, calcd. for $C_{17}H_{18}Cl_2N_2O_2$ $[M]^+$: 353, found: 353$[M]^+$.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-pentylbenzamide (2.19):** Yellow solid in 61 % yield; mp 124-126 °C; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.85 (t, 3H, *J* 4.48, $CH_3$), 1.3 (m, 4H, $2CH_2$), 1.5

(m, 2H, $CH_2$), 3.3 (q, 2H, *J* 6.88, 13.16, $CH_2$), 3.5 (br-s, 2H, $NH_2$), 6.1 (app. br. s, 1H, 1NHCO), 6.6 (d, 1H, *J* 8.28, Ar-H), 6.8 (d, 1H, *J* 4.32, Ar-H), 6.9 (dd, 1H, *J* 2, 8.3, Ar-H), 7.1 (dd, 1H, *J* 2.44, 8.74, Ar-H), 7.2 (d, 1H, *J* 1.92, Ar-H), 7.4 (d, 1H, *J* 2.48, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.03, 22.41, 29.15, 29.37, 40.10, 115.46, 116.70, 117.52, 120.38, 125.91, 128.15, 129.36, 130.54, 131.57, 137.94, 145.44, 150.74, 167.13; Electrospray-MS *m/z*, calcd. for $C_{18}H_{20}Cl_2N_2O_2$ $[M]^+$: 367, found: 367$[M]^+$.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-hexylbenzamide (2.20):** Brown solid in 57 % yield; mp 111-113 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 6.32, $CH_3$), 1.3 (m, 6H, $3CH_2$), 1.6 (m, 2H, $CH_2$), 3.4 (q, 2H, *J* 6.8, 12.96, $CH_2$), 3.6 (br-s, 2H, $NH_2$), 6.1 (app. br. s, 1H, 1NHCO), 6.7 (d, 1H, *J* 8.32, Ar-H), 6.9 (d, 1H, *J* 8.72, Ar-H), 7.0 (d, 1H, *J* 8.16, Ar-H), 7.2 (dd, 1H, *J* 2.32, 8.72, Ar-H), 7.3 (s, 1H, Ar-H), 7.5 (d, 1H, *J* 2.36, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.06, 22.59, 26.69, 29.65, 31.53, 40.14, 115.58, 116.85, 117.49, 120.43, 125.94, 128.16, 129.40, 130.54, 131.57, 137.72, 145.54, 150.71, 167.11; Electrospray-MS *m/z*, calcd. for $C_{19}H_{22}Cl_2N_2O_2$ $[M]^+$: 381, found: 381$[M]^+$.

**3-amino-4-(2,4-dichlorophenoxy)-*N*-heptylbenzamide (2.21):** White solid in 64 % yield; mp 109-111 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (t, 3H, *J* 6.6, $CH_3$), 1.3 (m, 8H, $4CH_2$), 1.6 (m, 2H, $CH_2$), 3.4 (q, 2H, *J* 6.92, 13.16, $CH_2$), 3.7 (br-s, 2H, $NH_2$), 6.1 (app. br. s, 1H, 1NHCO), 6.7 (d, 1H, *J* 8.32, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.0 (dd, 1H, *J* 2.0, 8.32, Ar-H), 7.2 (dd, 1H, *J* 2.48, 8.76, Ar-H), 7.3 (d, 1H, *J* 1.96, Ar-H), 7.5 (d, 1H, *J* 2.44, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.08, 22.61, 26.97, 29.01, 29.69, 31.76, 40.13, 115.48, 116.71, 117.54, 120.37, 125.92, 128.15, 129.36, 130.54, 131.64, 137.92, 145.44, 150.76, 167.08; Electrospray-MS *m/z*, calcd. for $C_{20}H_{24}Cl_2N_2O_2$ $[M]^+$: 395, found: 395$[M]^+$.

**2.6.2 *In vitro* Antimicrobial Assay**

**2.6.2.1 Test isolates:** For screening, the test spectrum consist of standard surrogate Gram Positive and Gram negative bacteria. Gram positive strain included *Staphylococcus epidermidis* (MTCC 2639) while Gram negative strains included *Escherichia coli* (MTCC 1302), *Pseudomonas aeruginosa* (MTCC 647 and MTCC 3541) and *Pseudomonas putida*

**2.6.2.2 *In vitro* Microbroth Dilution Assay to determine Minimal Inhibitory Concentration (MIC):** Stock solutions of the compounds were prepared and tested against a spectrum of standard and clinical isolates of *Staphylococcus aureus* by *in vitro* microbroth dilution assay to

ascertain antimicrobial potential of the compounds as per Clinical and Laboratory Standards Institute (CLSI) M7-A4 [108].

MIC is considered to be the gold standard technique to evaluate the susceptibility of a microorganism to a particular antibiotic. Briefly 50 µL of the 18 hrs old 0.5 McFarland adjusted bacterial suspension in physiological saline was dispensed in each well. 125 µL of MH broth was added in the wells to achieve a final bacterial cell concentration of $10^6$cells in the well. Plates were then incubated at 37°C for 2.5 hours. After which 25 µL of the test compound at different concentrations (i.e two fold serial dilutions in range between 125.00- 0.48 µg/mL) was added into the well and incubated for 24 hrs at 37°C. Subsequently 20 µL of 0.02% of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into each well and incubated at 37°C for 1 hr. The color change due to formation of formazan was visually observed. All the tests were performed in triplicates [109].

### 2.6.3 Docking of inhibitors with EcENR

All the docking simulations were done using AutoDock Vina program in PyRx [110].

#### 2.6.3.1 Preparation of the receptor and ligand molecules

The crystal structure of EcENR (PDBid: 1QSG) by Stewart et al. [111] was used as receptor molecule for docking studies. The structure co-ordinates of receptor and triclosan ligand molecule were retrieved from the RCSB Protein Data Bank [112]. Energy-minimized co-ordinates of inhibitors were generated using PRODRG server [113].

#### 2.6.3.2 Docking simulations and docking analysis

Using PyRx 0.8, a grid box (size_x = 25, size_y = 25 and size_y =25) was centered in the active site of the receptor with center_x = 9.8, center_y = 13.9 and center_z = 10.6. The same parameters were used for all docking experiments. The minimum energy conformations of docked inhibitors were collected and further analysed by comparing with the Triclosan-bound *Ec*ENR structure.

# Chapter 3

## Derivatives of 1-(2, 4-dichlorophenoxy)-2,4-dinitrobenzene/ 1-(2, 4-dichlorophenoxy)-2-nitrobenzene

### 3.1 Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy)phenol is well known broad spectrum antibacterial agent that is commonly used in toothpastes, mouth washes, deodorants and children toys. It has been been reported to be one of most frequently detected pollutants in river and lake waters [114]. In aqueous solutions buffered at pH= 8 or above it has been observed to undergo ring closure to make dioxin, 2,8-dichlorobenzo-p-dioxin (2,8-DCDD) as a result of photolysis as shown below [115] **(Figure 3.1)**.

hν, $Cl^-$

3.2 3.3

**Figure 3a. Formation of Dioxin**

Dioxin is a probable human carcinogen that long time to degrade in environment. Similarly methyl triclosan **(Figure 3b)** is believed to be the microbial methylated product of triclosan that gets further halogenated and accumulates in life forms. Methyl triclosan is known to disrupt thyroids endocrine systems in amphibians [116].

Microbes

3.4 3.5

3.5 3.6

**Figure 3b. Formation of methylated triclosan**

We hypothesize replacement of hydroxyl group by nitro group to prevent these undesired products in to environment. This will be of use if nitro compound show antimicrobial activity similar to triclosan. Present work explores replacement of hydroxyl group with other functional groups such as nitro, amino and amide to study the effect on biological activity. In all the compounds synthesized in this chapter, 2,4- dichlorophenyl ring was retained as such.

### 3.2 Synthesis of Diphenyl ethers

Synthesis of all the compounds has been illustrated in **Scheme 3.1**. Parent Compounds **3.9** and **3.19** were synthesized by nucleophilic aromatic substitution of nitro-substituted flouro benzene **(3.8)** with 2,4-dichlorophenol **(3.7)** in presence of mild base, phase transfer catalyst and aprotic solvent at room temperature by simple stirring. Formation of parent compounds **3.9** and **3.19** was confirmed by the presence of six and seven aromatic protons in $^{1}H$ NMR spectra respectively. Further compounds **3.9** and **3.19** were subjected to reduction using Fe/ $FeSO_4.7H_2O$ solution in water to get corresponding amines. Nin-hydrin test was done to observe the progress and completion of the reaction. Appearance of new broad singlets at 3.4 and 3.6 ppm for the compounds **3.10** and **3.20** confirmed presnec of two and one amino group respectively. Both the broad signals disappeared when $^{1}H$ NMR was again recorded in $D_2O$.

Amino groups of both the compounds **3.10** and **3.20** were exploited further to incorporate amide linkage with various acid chlorides. Acid chloride required for the purpose was made from corresponding acid using $PCl_3$ as chlorinating agent at low temperature ($0^oC$). Aliphatic acids with varying chain length (C-1 to C-4) were used **(Scheme 3.1)**. For all the amide compounds confirmation of the product formation was done $^{1}H$, $^{13}C$ NMR and mass spectroscopic techniques. **Table 3.1** below summarizes prominent parameters observed in the spectroscopic studies for diamide diphenyl ethers **(3.11-3.18)** and monoamide diphenyl ethers **(3.21-3.24)** respectively. All the terminal methyl protons and carbon for compound **3.11** to **3.15** and **3.21** to **3.23** appeared in the range of 0.9- 2.2 ppm and 9.5- 27.4 pp respectively. The value of each methyl proton shifted downfield with the decrease in chain length. Methylene protons and carbons also appeared appropriately in the $^{1}H$ and $^{13}C$ NMR respectively. For compounds **3.16**, **3.17** and **3.24** there was no terminal methyl group but a terminal chloro substituted. The methylene protons and carbon in these cases also appeared downfield as expected.

3.7

3.8
$R_1$= $NO_2$
$R_2$= $NO_2$/ H

(i)

3.9: $R_1$= $NO_2$, $R_2$= $NO_2$
3.19: $R_1$= $NO_2$, $R_2$= H

(ii)

3.10: R'= R''= $NH_2$
3.20: R'= $NH_2$, R''= H

(iii)

(3.11-3.18, 3.21-3.24)

**3.11:** **$R_1$'=$R_2$'**= -NH-CO-$CH_3$
**3.12:** **$R_1$'=$R_2$'**= -NH-CO-$CH_2$-$CH_3$
**3.13:** **$R_1$'=$R_2$'**= -NH-CO-$CH_2$-$CH_2$-$CH_3$
**3.14:** **$R_1$'=$R_2$'**= -NH-CO-$CH_2$-$(CH_2)_2$-$CH_3$
**3.15:** **$R_1$'=$R_2$'**= -NH-CO-C-$(CH_3)_3$
**3.16:** **$R_1$'=$R_2$'**= -NH-CO-$CH_2$-Cl
**3.17:** **$R_1$'=$R_2$'**= -NH-CO-$CH_2$-$(CH_2)_2$-Cl
**3.18:** **$R_1$'=$R_2$'**= -NH-CO-N-$(CH_3)_2$

**3.21:** **$R_1$'**= -NH-CO-$CH_3$; **$R_2$'**= H
**3.22:** **$R_1$'**= -NH-CO-$CH_2$-$CH_3$; **$R_2$'**= H
**3.23:** **$R_1$'**= -NH-CO-$CH_2$-$CH_2$-$CH_3$; **$R_2$'**= H
**3.24:** **$R_1$'**= -NH-CO-$CH_2$-Cl; **$R_2$'**= H

**Scheme 3.1.** (i) 18-Crown-6 , $K_2CO_3$, DMF, Room temperature (ii) Fe, $FeSO_4.7H_2O$, Reflux (iii) $PCl_3$, $K_2CO_3$, DCM, aliphatic acids

All the compounds having amide linkage, a singlet due to one proton linked to nitrogen atom appeared in range of 6.2-10.3 ppm (-NH-CO-). Carbonyl carbon atom of both the amide linkages gave signal in the range of 154.7- 177.1 ppm and 163.9- 172.1 ppm in compopunds **3.11-3.18** and **3.21-3.24** respectively. All the signals have been listed in **Table 3.1**. $^1$H and $^{13}$C NMR spectra for compounds **3.9, 3.11, 3.13, 3.19, 3.21, and 3.23** are given in **Figures 3A, 3B, 3C, 3D, 3E and 3F (a: $^1$H NMR and b $^{13}$C NMR) (Refer Page No. 105-116).**

### 3.3 Antibacterial Activity

All the synthesized compounds were tested *in vitro* for their antibacterial activity against eight Gram positive and negative strains. This included *Staphylococcus aureus* (NCTC: 6571) and *Staphylococcus epidermidis* (MTCC: 2639), *Bacillus subtilis*, *Escherichia coli* (MTCC: 1302), *Pseudomonas aeruginosa* (MTCC: 647) and *Pseudomonas* putida. Micro dilution assay method was used and the results have been expressed in terms of MIC value.

**Table 3.2** shows that compounds **3.9** and **3.10** were active against nearly all the selected strains. Compound **3.9** was more potent among the two with MIC value ranging from 7.81- 62.50 µg/mL and compound **3.10** with MIC value (15.62- 125.00 µg/mL) was in higher range. The most impressive value of compound **3.9** was against *E. coli* (7.81 µg/mL) where as standard has MIC value of 15.62 µg/mL. Compound **3.19** and **3.20** were comparatively less potent showing MIC against only two and five organisms respectively. The activity also ranged from 7.81- 125.00 µg/mL. From the structures of compounds **(3.9** and **3.10)** and compounds **(3.19** and **3.20)**, it is clear that nitro and amino groups at 4-position enhance the antibacterial activity though for nitro, enhancement is more than amino group. In case of compounds **3.19** and **3.20** higher MIC values can be attributed to missing nitro or amino substituents at position -4. All the amide made from compounds **3.10** and **3.20** did not show any significant antibacterial activity against any of the selected strains except *P. aeruginosa* (MTCC 3541). Compound **3.11**, acetamide product of compound **3.10**, was an exception to this. It showed antibacterial activity against seven of the eight selected strains with MIC value ranging from 7.81- 62.50 µg/mL. Compound **3.12** propanamide of compound **3.10**, was also active but with higher MIC value (15.62- 62.50 µg/mL) against six strains only. Interestingly ten and thirteen of the sixteen synthesized compounds were active against *P. aeruginosa* and *P. putida* respectively. **Table 3.3** shows MIC value of all the compounds against *Pseudomonas* bacterial strains. However the most impressive MIC values against this strain was not different from that reported in **Table 3.2**. Compounds **3.9**, **3.11** and **3.20** gave MIC value of 7.81µg/mL which was comparable to standard drug. The most impressive activity against *P. aeruginosa* was from an unexpected compound **3.21** (MIC= 3.90 µg/mL) that was active against none of the selected stains. Against *P. putida* also, it showed an MIC value of 15.62 µg/mL. Rest of the compounds showed an MIC value ranging from 15.62 to 62.50 µg/mL against *P. aeruginosa*. Ten compounds active against *P. putida* had their MICs in the range of 15.62- 125.00 µg/mL. From **Table 3.3** and structures given in **Scheme 3.1.** It can be

**Table 3.1. Summary of prominent signals in spectroscopic data for compounds 3.9- 3.24**

| Sr. No. | Compound | $^1$H NMR | | $^{13}$C NMR | Mass |
|---|---|---|---|---|---|
| | | -CO-NH-C**H**$_n$ | -CO-N**H**- | -**C**O-NH- | m/z |
| 1. | 3.9 | - | - | - | 332[M+3]$^+$ |
| 2. | 3.10 | 3.4,br-s,<br>4H($NH_2$) | - | - | 269 [M]$^+$ |
| 3. | 3.11 | 2.1,s,3H<br>2.2,s,3H | 7.8, s, 1H<br>8.2, s, 1H | 168.86 (intense) | 376 [M+Na]$^+$ |
| 4. | 3.12 | 2.3,m,4H | 9.0, s, 1H<br>9.8, s, 1H | 171.95, 172.16 | 382 [M+1]$^+$ |
| 5. | 3.13 | 2.3,m,4H | 7.6, s, 1H<br>7.7, s, 1H | 171.50,171.61 | 409[M]$^+$ |
| 6. | 3.14 | 2.4,m,4H | 7.7, s, 1H<br>8.2, d, 1H | Not found | 437 [M]$^+$ |
| 7. | 3.15 | 1.2,s,9H<br>1.3,s,9H | 7.5, s, 1H<br>8.0, 2, 1H | 176.76, 177.08 | 437[M]$^+$ |
| 8. | 3.16 | 4.1,s,1H<br>4.3,s,1H | 9.7, s, 1H<br>10.3, s, 1H | 164.37, 167.84 | 424[M+2]$^+$ |
| 9. | 3.17 | 2.2,m,4H<br>2.5,m,4H<br>3.6,m,4H | 7.8, s, 1H<br>8.3, s, 1H | 170.21,170.28 | 479[M+1]$^+$ |
| 10. | 3.18 | 1.2,m,6H<br>3.3,m,6H | 6.2,s,1H<br>6.8, s, 1H | 154.68(intense) | 413[M+2]$^+$ |
| 13. | 3.19 | - | - | - | 284[M]$^+$ |
| 14. | 3.20 | 3.6,br-S,2H<br>($NH_2$) | - | - | 254 [M]$^+$ |
| 15. | 3.21 | 2.3,s,3H | 7.8,br-S,1H | 168.40(-CO) | 296 [M]$^+$ |
| 17. | 3.22 | 2.4,q,2H | 7.7,br-s,1H | 172.06(-CO) | 310[M]$^+$ |
| 18. | 3.23 | 2.4,t,2H | 7.7,br-s,1H | 171.35 (-CO) | 324[M]$^+$ |
| 16. | 3.24 | 4.2,s,2H | 9.0, br-s, 1H | 163.89(-CO) | 331[M]$^+$ |

concluded that substitution in the ring A at 2 and 4 positions is responsible for antibacterial activity. Amidation of amino groups with two or three carbon chain is responsible for antibacterial activity but if the chain length is increased beyond three, the activity is lost.

**Table 3.2. Screening of diphenyl ethers against Gram +ve and Gram –ve bacterial strains**

| Compound | *S. aureus* [a] | *S. aureus* [b] | *B. subtilis* | *E. coli* [c] | *P. aeru.* [d] | *P. aeru.* [e] | *P. putida* | *S. epidermidis* [f] |
|---|---|---|---|---|---|---|---|---|
| | **MIC (µg/mL)** | | | | | | | |
| 3.9 | 31.25 | 15.62 | 15.62 | 7.81 | 62.50 | 7.81 | 31.25 | - |
| 3.10 | 125.00 | 125.00 | 62.50 | 62.50 | 62.50 | 31.25 | 31.25 | 15.62 |
| 3.11 | 62.50 | 15.62 | 62.50 | 62.50 | 62.50 | 7.81 | 31.25 | - |
| 3.12 | - | 62.50 | 62.50 | 62.50 | 62.50 | 15.62 | 62.50 | - |
| 3.19 | - | - | - | - | | 15.62 | 15.62 | - |
| 3.20 | 62.50 | 125.00 | - | - | | 7.81 | 15.62 | 62.50 |
| 3.21 | - | - | - | - | | 3.90 | 15.62 | - |
| Triclosan* | 7.81 | 3.90 | 3.90 | 15.62 | 1.95 | 7.81 | 3.90 | 1.95 |
| Streptomycin* | - | - | - | - | 15.62 | 7.81 | 1.95 | 7.81 |

* Triclosan, a diphenyl ether, and Streptomycin were used as reference compounds.
***a***- NCTC 6571, ***b***- MTCC 737, ***c*** – MTCC 1302, ***d***- MTCC 647, ***e*** - MTCC 3541. ***f***- MTCC-2639.

### 3.4 *In silico* Docking Studies

As reported in previous chapter triclosan forms a ternary complex with active site of FabI with $NAD^+$ and this complex is responsible for the inhibition of FabI enzyme. It has been observed in the previous chapter that all the compounds both nitro and amino formed the ternary complex with FabI and $NAD^+$ but in flipped conformations. The flipping of diphenyl ether rings was attributed to the presence of bulky amide substituent in the ring A at 4-position. Among all the compounds synthesized in this chapter only four molecules (**3.9**, **3.19**, **3.10** and **3.20**) did not carry any amide linkage at 2 and 4-position of ring A. In fact the series of compounds can be classified as:

(a) Nitro **(3.9** and **3.19)**
(b) Amino **(3.10** and **3.20)**
(c) Amide

**Table 3.3. Screening of diphenyl ethers against *Pseudomonas* bacterial strains and binding affinity of test molecules with PaFabI**

| Compound | *P. aeruginosa 3541* | *P.putida* | Binding Affinity |
|---|---|---|---|
| | MIC (µg/mL) | | (kcal/mol) |
| 3.9 | 7.81 | 31.25 | -7.3 |
| 3.10 | 31.25 | 31.25 | -8.7 |
| 3.11 | 7.81 | 31.25 | -7.1 |
| 3.12 | 15.62 | 62.50 | -7.4 |
| 3.13 | - | - | -7.2 |
| 3.14 | - | - | -7.4 |
| 3.15 | - | - | -7.7 |
| 3.16 | 15.62 | 31.25 | -7.0 |
| 3.17 | 62.50 | - | -7.5 |
| 3.18 | 62.50 | 125.00 | -7.7 |
| 3.19 | 15.62 | 15.62 | -7.0 |
| 3.20 | 7.81 | 15.62 | -8.7 |
| 3.21 | 3.90 | 15.62 | -7.2 |
| 3.22 | 31.25 | - | -6.4 |
| 3.23 | 15.62 | - | -7.2 |
| 3.24 | 31.25 | - | -7.0 |
| Triclosan | 7.81 | 3.90 | -9.3 |
| Streptomycin | 7.81 | 1.95 | |

*In silico* studies carried out with all the compounds against FabI of *P. aeruginosa* gave very interesting results. The rings of the nitro and amide group of the compounds flipped as compared to triclosan but for amino compounds (**3.10** and **3.20**) the interaction was similar to that of reference compound. **Figure 3c, 3d** and **3e** shows interaction of compound **3.10**, **3.20** and triclosan respectively with FabI of *P. aeruginosa.* For compounds **3.10** and **3.20** the $NH_2$ substituent at the 2-position of ring A, interaction (2.7A$^o$) can be seen between hydroxyl (-OH) group of tyrosine (Tyr 159) and 2'-hydroxyl group of nicotinamide ribose (2.8 A$^o$).

The intermolecular distance between the other $NH_2$ group, at 4-position of ring A in compound **3.10**, and residues of FabI, does not indicate any interaction. **Table 3.3** shows same binding energy for compound **3.10** and **3.20** (8.7 kcal/mol) proves this claim. This is further strengthened by the fact that biological acivity of compound **3.20** was better as compared to **3.10**. Although Π-Π staking for ring A and nicotinamide ring of $NAD^+$ can be seen both for compounds **3.10** and **3.20**.

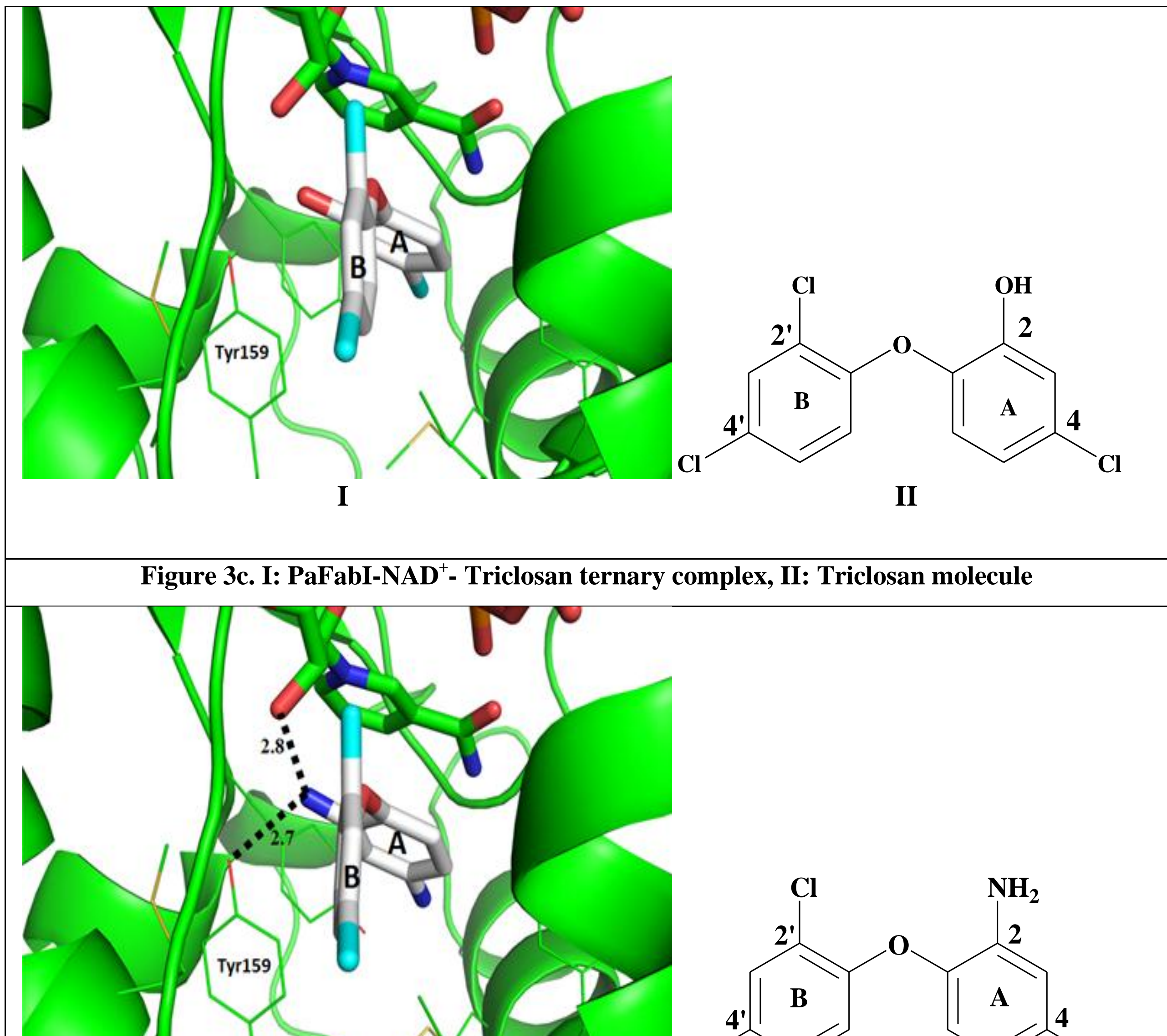



**Figure 3c. I: PaFabI-NAD$^+$- Triclosan ternary complex, II: Triclosan molecule**



**Figure 3d. I: PaFabI-NAD$^+$-Test molecule 3.10 ternary complex, II: Test molecule 3.10**

Although nitro compounds **3.9** and **3.19** showed good antibacterial activity 7.8 and 15.6 μg/mL, their binding affinity was not comparable to amino compounds. Similarly the binding affinity of amide derivatives have energy ranging between 6.4-7.7 kcal/mol but their biological activity was not impressive except compound **3.11**. It can be concluded from previous series and this series that nitro and amide group at 2 or 4-position of ring A activated ring flipping.

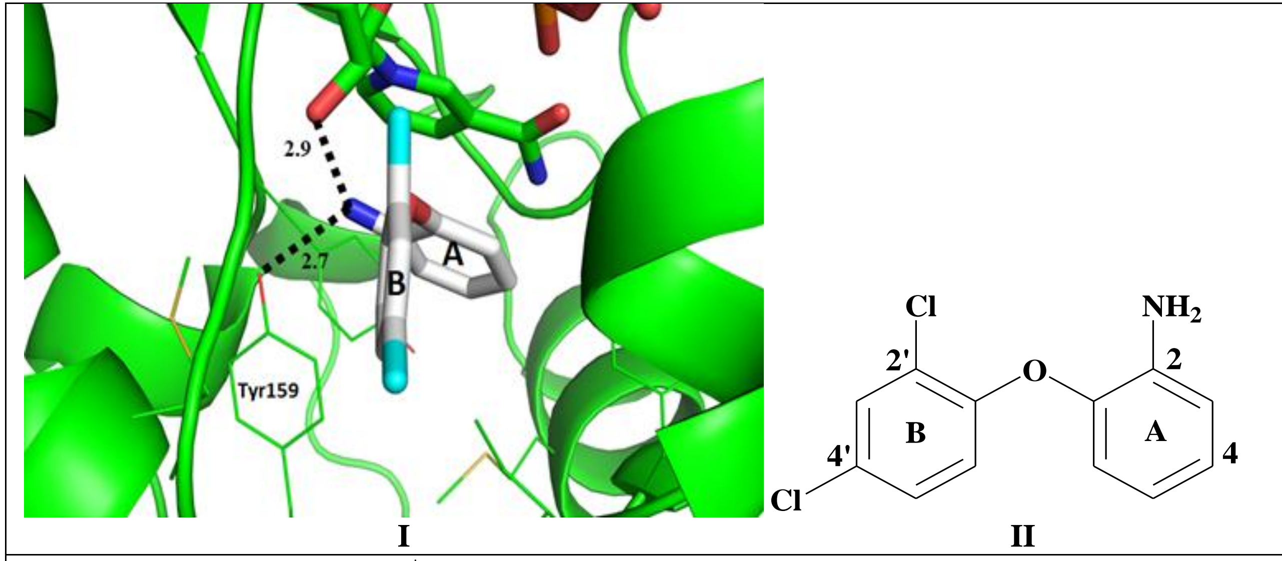


**Figure 3e. I: PaFabI-NAD$^{+}$- Test molecule 3.20 ternary complex, II: Test molecule 3.20**

### 3.5 Structure activity Relationship

From compounds mentioned in **Scheme 3.1 and Table 3.1. 3.2 and 3.3** and above discussion following conclusions can be made about structural activity relationship. Nitro group at 2-position of ring A is preferred over amino group at the same position for the antibacterial activity. Although amino derivatives have more binding affinity with protein (*In silico* studies) but for bacterial cell culture nitro substituted diphenyl ethers molecules **(3.9** and **3.19)** are more active. Also compound **3.20**, having only one amino group at 2-position, is more active than **3.10** which is having two amino groups at 2, 4-position. Amide linkage at 2, 4-position having chain length upto 3 carbon atoms is preferred for good antibacterial activity. As the length of chain beyond 4 carbon atom increases, MIC value starts increasing.

### 3.6 Materials and Methods

#### 3.6.1 Synthesis of substituted diphenyl ethers and their derivatives

Purified reagents were procured from SD Fine Chemicals, Bombay. Solvents were distilled before doing the column chromatography. Melting points are reported in $^{o}$C and are uncorrected. $^{1}$H and $^{13}$C NMR spectral analyses were performed on 400 and 100 MHz spectrometer (Bruker) with tetramethylsilane and $CDCl_3$ as the internal standard (δ, ppm) respectively. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet, br, broad. Mass analysis was done using Waters Micromass mass spectrometer.

**Procedure for synthesis of 2,4-dichloro-1-(2,4-dinitrophenoxy)benzene (3.9):** To a solution of 1-fluoro-2,4-dinitrobenzene **(3.8)** (2.6 ml, 20.9 mmol) in DMF (25 ml) were added $K_2CO_3$ (11.5 g, 83.9 mmol), 2,4-dichlorophenol **(3.7)** (4.3 g, 26.3 mmol) and 18-crown-6 (50 mg, 0.2 mmol). The mixture was stirred at room temperature for 12 h. After the reaction is complete (TLC monitoring), the reaction mixture was diluted with DCM (100 ml), washed with water (50 ml), 1 N NaOH (3× 10 ml), water (until neutral to litmus paper), brine and dried over $Na_2SO_4$. Evaporation of the organic solvent gave crude compound **(3.9)**. The product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ethyl acetate = 85:15) to afford yellow solid in 84 % yields (7.6 gms) m.p.: 96- 98 $^oC$. $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 6.9 (d, 1H, *J* 9.2, Ar-H), 7.2 (d, 1H, *J* 8.64, Ar-H), 7.4 (dd, 1H, *J* 2.48, 8.64, Ar-H), 7.6 (d, 1H, *J* 2.48, Ar-H), 8.3 (dd, 1H, *J* 2.68, 9.22, Ar-H), 8.9 (d, 1H, *J* 2.72, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 117.35, 122.34, 123.75, 76, 128.93, 129.15, 131.35, 133.09, 139.01, 142.00, 147.70, 154.73; Electrospray-MS *m/z*, calcd. for $C_{12}H_6Cl_2N_2O_5$ $[M]^+$: 329, found: 332 $[M+3]^+$.

**Procedure for synthesis of 4-(2,4-dichlorophenoxy)benzene-1,3-diamine (3.10):** To a suspension of compound **(3.9)** (3.5 g, 10.6 mmol) in refluxing $H_2O$ (100 ml) were added Fe (5.95 g, 106.2mmol) and $FeSO_4.7H_2O$ (2.9 g, 10.4 mmol). The reaction mixture was refluxed for 8 h. After cooling to room temperature and confirming the completion of reaction (TLC monitoring), it was filtered through celite, washed thoroughly with ethyl acetate (2 x 75 ml). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave corresponding amine. This was confirmed by spotting the product on alumina plate which upon dipping in ninhydrin solution (2% in ethanol) and further heating turned black. The compound was isolated and purified using $SiO_2$ column chromatography and solvent (pet ether/EtOAc = 70:30) to afford a dark brown solid (mp 142-144 $^oC$) in 51% yield. $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.5 (br-s, 4H, $2NH_2$), 6.1 (d, 1H, *J* 7.88, Ar-H), 6.2 (s, 1H, Ar-H), 6.7 (t, 2H, *J* 8.08, Ar-H) , 7.1 (dd, 1H, *J* 2.48, 8.84, Ar-H), 7.4 (d, 1H, *J* 2.48, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 117.00 (intense), 121.99, 123.69, 127.11, 127.76 (intense), 130.06 (intense), 134.65, 139.41, 152.89; Electrospray-MS *m/z*, calcd. for $C_{12}H_{10}Cl_2N_2O$ $[M]^+$: 269, found: 269$[M]^+$.

**General procedure of preparation amide derivatives of *N*-[5-Acetylamino-2-(2,4-dichloro-phenoxy)-phenyl]-acetamide and its derivatives (3.11- 3.18):** To aliphatic carboxylic acid (0.5 mmol) was added $PCl_3$ (0.5 mmol) and the reaction mixture stirred at 60–70 $^oC$. After 45 min, the reaction mixture was poured in-situ to another reaction mixture of compound **(3.10)** (0.5

mmol), $K_2CO_3$ (0.3 mmol) and DCM (10 mL) already stirred at 0 $^oC$. The reaction mixture was allowed to stir for another 2 hrs. The expected amide product was extracted with DCM (3· 60 ml), dried over $Na_2SO_4$, and concentrated in vacuo. Crude product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ ethyl acetate= 70:30) to afford light brown solids (III - XII).

***N*-[5-Acetylamino-2-(2,4-dichloro-phenoxy)-phenyl]-acetamide (3.11):** Light Brown solid in 70 % yield; mp 131-133 $^oC$ ; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 2.1 (s, 3H, $CH_3$), 2.2 (s, 3H, $CH_3$), 6.7 (d, 1H, *J* 8.88, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.2 (dd, 1H, *J* 2.4, 8.76, Ar-H), 7.5 (d, 1H, *J* 2.4, Ar-H), 7.7 (dd, 1H, *J* 2.2, 8.88, Ar-H), 7.8 (s, 1H, NH), 8.2 (s, 1H, NH), 8.2 (d, 1H, *J* 1.27, 2.16, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 24.40, 24.89, 112.83, 116.30, 117.45, 120.84, 125.98, 128.33, 129.16, 129.81, 130.59 (intense), 134.72, 141.23, 150.71, 168.75, 168.86 ; Electrospray-MS *m/z*, calcd. for $C_{16}H_{14}Cl_2N_2O_3$ $[M]^+$: 353, found: 376$[M+Na]^+$.

***N*-[2-(2,4-Dichloro-phenoxy)-5-propionylamino-phenyl]-propionamide (3.12):** Light Brown solid in 68 % yield; mp 129-130 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.1 (m, 6H, 2$CH_3$), 2.3 (m, 4H, 2$CH_2$), 6.7 (d, 1H, *J* 8.88, Ar-H), 6.9 (d, 1H, *J* 8.8, Ar-H) , 7.2 (dd, 1H, *J* 2.4, 8.8, Ar-H), 7.5 (m, 2H, Ar-H), 8.2 (s, 1H, NH), 9.0 (s, 1H, NH), 9.8 (s, 1H, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 9.49 (intense), 29.39, 29.52, 114.52 115.47, 118.13, 119.85, 124.67, 127.72, 127.82, 129.58 (intense), 135.73, 141.52, 151.52, 171.95, 172.16; Electrospray-MS *m/z*, calcd. for $C_{18}H_{18}Cl_2N_2O_3$ $[M]^+$: 381, found: 382$[M+1]^+$.

***N*-[5-Butyrylamino-2-(2,4-dichloro-phenoxy)-phenyl]-butyramide (3.13):** Light Brown solid in 67 % yield; mp 112-115 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.0 (m, 6H, 2$CH_3$), 1.7 (m, 4H, 2$CH_3$), 2.3 (m, 4H, 2$CH_2$), 6.7 (d, 1H, *J* 8.88, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.2 (dd, 1H, *J* 2.48z, 8.78, Ar-H), 7.5 (d, 2H, *J* 2.48, Ar-H), 7.6 (s, 1H, NH), 7.7 (s, 1H, NH), 7.7 (d, 2H, *J* 2.12, Ar-H), 8.2 (d, 1H, *J* 1.88, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.62, 13.76, 19.00 (intense-2-$CH_2$), 39.52, 39.84, 112.53, 115.93, 117.77, 120.48, 125.80, 128.28, 129.42, 129.69, 130.58, 134.79, 140.92, 150.84, 171.50, 171.61; Electrospray-MS *m/z*, calcd. for $C_{20}H_{22}Cl_2N_2O_3$ $[M]^+$: 409, found: 409$[M]^+$.

**Pentanoic acid [2-(2,4-dichloro-phenoxy)-5-pentanoylamino-phenyl]-amide (3.14):** Light Brown solid in 70 % yield; mp 139-141 $^oC$; $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 0.9 (m, 6H, 2$CH_3$), 1.4 (m, 4H, 2$CH_2$), 1.7 (m, 4H, 2$CH_2$), 2.4 (m, 4H, 2$CH_2$), 6.8 (d, 1H, *J* 8.88, Ar-H), 6.9 (d, 1H,

*J* 8.76, Ar-H), 7.2 (dd, 1H, *J* 2.4, 8.76, Ar-H), 7.5 (d, 2H, *J* 2.44, Ar-H), 7.7 (s, 1H, NH), 7.7 (dd, 1H, *J* 1.92, 8.8, Ar-H), 8.2 (d, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 27.67, 27.02, 29.26, 29.35, 29.52, 29.69, 31.86, 40.13, 115.44, 116.65, 117.55, 120.35, 125.91, 128.14, 129.35, 130.54, 131.64, 137.99, 145.41, 150.77, 166.89, 167.09 ; Electrospray-MS *m/z*, calcd. for $C_{22}H_{26}Cl_2N_2O_3$ $[M]^+$: 437, found: 437$[M]^+$.

***N*-[2-(2,4-Dichloro-phenoxy)-5-(2,2-dimethyl-propionylamino)-phenyl]-2,2-dimethyl-propionamide (3.15):** Light Brown solid in 59 % yield; mp 132-133 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.2 (s, 9H, $3CH_3$), 1.3 (s, 9H, $3CH_3$), 6.9 (q, 2H, *J* 6.8, 8.84, Ar-H), 7.2 (dd, 1H, *J* 2.52, 8.8, Ar-H), 7.4 (s, 1H, NH), 7.5 (d, 1H, *J* 2.44, Ar-H), 7.8 (dd, 1H, *J* 2.64, 8.94, Ar-H), 8.0 (s, 1H, NH), 8.3 (d, 1H, *J* 2.6, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 27.40 (intense peak for 3-$CH_3$), 27.59 (intense peak for 3-$CH_3$), 39.64, 40.03, 112.50, 115.78, 118.37, 119.36, 125.17, 128.17, 129.34, 129.79, 130.59, 135.17, 140.42, 150.97, 176.76, 177.08; Electrospray-MS *m/z*, calcd. for $C_{22}H_{26}Cl_2N_2O_3$ $[M]^+$: 437, found: 437 $[M]^+$.

**2-Chloro-N-[5-(2-chloro-acetylamino)-2-(2,4-dichloro-phenoxy)-phenyl]-acetamide (3.16) :** Light Brown solid in 75 % yield; mp 99-102 °C;$^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 4.1 (s, 2H, $CH_2$), 4.3 (s, 2H, $CH_2$), 6.7 (d, 1H, *J* 8.72, Ar-H), 6.9 (d, 1H, *J* 8.68, Ar-H) , 7.3 (d, 1H, *J* 7.92, Ar-H) , 7.5 (m, 2H, Ar-H), 8.3 (s, 1H, Ar-H), 9.7 (s, 1H, NH), 10.3 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 42.99, 43.26, 114.06, 116.24, 117.72, 120.92, 125.26, 128.22, 128.40, 128.72, 129.80, 134.67, 142.40, 151.04, 164.37, 167.84; Electrospray-MS *m/z*, calcd. for $C_{16}H_{12}Cl_4N_2O_3$ $[M]^+$: 422, found: 424 $[M+2]^+$.

**4-Chloro-N-[5-(4-chloro-butyrylamino)-2-(2,4-dichloro-phenoxy)-phenyl]-butyramide (3.17):** Light Brown solid in 78 % yield; mp 122-125 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 2.2 (m, 4H, $2CH_2$), 2.5 (m, 4H, $2CH_2$), 3.6 (m, 4H, $2CH_2$), 6.7 (d, 1H, *J* 8.88, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.2 (dd, 1H, *J* 2.44, 8.74, Ar-H), 7.5 (d, 1H, *J* 2.4, Ar-H), 7.6 (dd, 1H, *J* 1.84, 8.74, Ar-H), 7.7 (s, 1H, Ar-H), 7.8 (s, 1H, NH), 8.3 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 27.82, 27.89, 33.98, 34.38, 44.24, 44.47, 112.70, 116.03, 117.38, 120.93, 126.10, 128.35, 129.14, 129.98, 130.68, 134.33, 141.34, 150.58, 170.21, 170.28; Electrospray-MS *m/z*, calcd. for $C_{20}H_{20}Cl_4N_2O_3$ $[M]^+$: 478, found: 479 $[M+1]^+$.

**3-[4-(2,4-Dichloro-phenoxy)-3-(3,3-dimethyl-ureido)-phenyl]-1,1-dimethyl-urea (3.18):** Light Brown solid in 54 % yield; mp 98-99 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.2 (m, 6H, $2CH_3$), 3.3 (m, 6H, $2CH_3$), 6.2 (s, 1H, NH), 6.4 (s, 1H, NH), 6.5 (dd, 1H, *J* 8.58, 2.52, Ar-H),

6.7 (t, 2H, *J* 7.84, Ar-H, NH), 7.1 (d, 2H, *J* 8.2, 2.48, Ar-H), 7.2 (d, 1H, *J* 2.52, Ar-H), 7.4 (d, 1H, *J* 3.36, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.73, 13.94, 41.70, 41.86, 108.53, 110.08, 117.77, 120.60(intense) , 124.15, 127.62, 127.84, 130.15, 136.95, 137.62, 138.97, 152.27, 154.68; Electrospray-MS *m/z*, calcd. for $C_{18}H_{20}Cl_2N_4O_3$ $[M]^+$: 411, found: 413 $[M+2]^+$.

**Procedure for synthesis of 1-(2,4-dichlorophenoxy)-2-nitrobenzene (3.19):** To a solution of 1-fluoro-2-nitrobenzene **(3.8)** (2.2 mL, 20.9 mmol) in DMF (25 ml) were added $K_2CO_3$ (11.5 g, 83.9 mmol), 2,4-dichlorophenol **(3.7)** (4.3 g, 26.3 mmol) and 18-crown-6 (50 mg, 0.2 mmol). The mixture was stirred at room temperature for 12 h. After the reaction is complete (TLC monitoring), the reaction mixture was diluted with DCM (100 ml), washed with water (50 ml), 1 N NaOH (3× 10 ml), water (until neutral to litmus paper), brine and dried over $Na_2SO_4$. Evaporation of the organic solvent gave crude compound **(3.19)**. The product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ethyl acetate = 85:15) to afford yellow solid in 77% yields.

**1-(2,4-dichlorophenoxy)-2-nitrobenzene (3.19):** $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 6.9 (dd, 1H, *J* 1, 8.4, Ar-H), 7.0 (d, 1H, *J* 4.24, Ar-H), 7.2 (m, 2H, Ar-H), 7.5 (m, 2H, Ar-H), 8.0 (dd, 1H, *J* 1.6, 8.14, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 119.21, 121.84, 123.78, 126.08, 126.92, 128.43, 130.74, 130.84, 134.46, 140.61, 149.81, 149.92; Electrospray-MS *m/z*, calcd. for $C_{12}H_7Cl_2NO_3$ $[M]^+$: 284, found: 284 $[M]^+$.

**Procedure for synthesis of 2-(2,4-dichlorophenoxy)aniline (3.20):** To a suspension of compound (**3.19)** (2.9 g, 10.6 mmol) in refluxing $H_2O$ (100 ml) were added Fe (2.97 g, 106.2mmol) and $FeSO_4.7H_2O$ (1.4 g, 5.2 mmol). The reaction mixture was refluxed for 8 h. After cooling to room temperature and confirming the completion of reaction (TLC monitoring), it was filtered through celite, washed thoroughly with ethyl acetate (2 x 75 ml). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave corresponding amine. This was confirmed by spotting the product on alumina plate which upon dipping in ninhydrin solution (2% in ethanol) and further heating turned black. The compound was isolated and purified using $SiO_2$ column chromatography and solvent (pet ether/EtOAc = 70:30) to afford a dark brown solid in 55% yield.

**2-(2,4-dichlorophenoxy)benzenamine (3.20):** $^1H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.6 (br-s, 2H, $NH_2$), 6.7 (t, 1H, *J* 7.9, Ar-H), 6.8 (d, 1H, *J* 1.4, Ar-H), 6.8 (d, 1H, *J* 2.2, Ar-H), 6.8 (dd, 1H, *J* 1.44, 7.88, Ar-H), 7.0 (t, 1H, *J* 7.68, Ar-H), 7.1 (dd, 1H, *J* 2.58, 8.8, Ar-H), 7.4 (d, 1H, *J* 2.48,

Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 116.73, 118.86 (intense), 119.49, 124.98, 125.47, 127.94, 128.22, 130.31, 138.21, 142.63, 151.74; Electrospray-MS *m/z*, calcd. for $C_{12}H_9Cl_2NO$ $[M]^+$: 254, found: 254$[M]^+$.

**General procedure of preparation amide derivatives of *N*-(2-(2,4-dichlorophenoxy)phenyl)acetamide and its derivatives (3.21- 3.24):** To aliphatic carboxylic acid (0.5 mmol) was added $PCl_3$ (0.5 mmol) and the reaction mixture stirred at 60–70 °C. After 45 min, the reaction mixture was poured in-situ to another reaction mixture of compound **(3.20)** (0.5 mmol), $K_2CO_3$ (0.3 mmol) and DCM (10 mL) already stirred at 0 °C. The reaction mixture was allowed to stir for another 2 hrs. The expected amide product was extracted with DCM (3* 60 ml), dried over $Na_2SO_4$, and concentrated in vacuo. Crude product obtained was purified using $SiO_2$ column chromatography and solvent (pet ether/ ethyl acetate= 70:30) to afford light brown solids **(3.21-3.24)**.

***N*-(2-(2,4-dichlorophenoxy)phenyl)acetamide (3.21):** Light Brown solid in 76 % yield; mp 119-120 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 2.2 (s, 3H, $CH_3$), 6.7 (dd, 1H, *J* 1.04, 8.18, Ar-H), 7.0 (m, 2H, Ar-H), 7.1 (t, 1H, *J* 7.82, Ar-H), 7.2 (dd, 1H, *J* 2.5, 8.74, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 7.8 (br-s, 1H, Ar-H), 8.4 (d, 1H, *J* 8.04); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 24.89, 116.52, 121.37, 121.46, 124.01, 124.52, 126.45, 128.35, 129.20, 130.03, 130.63, 145.09, 150.47, 168.40; Electrospray-MS *m/z*, calcd. for $C_{14}H_{11}Cl_2NO_2$ $[M]^+$: 296, found: 296$[M]^+$.

***N*-(2-(2,4-dichlorophenoxy)phenyl)propionamide (3.22):** Light Brown solid in 71 % yield; mp 98-99 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.2 (t, 3H, *J* 7.52, $CH_3$), 2.4 (q, 2H, *J* 7.56, 15.08, $CH_2$), 6.7 (dd, 1H, *J* 1.24, 8.16, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.0 (t, 1H, *J* 7.9, Ar-H), 7.1 (t, 1H, *J* 7.96, Ar-H), 7.2 (dd, 1H, *J* 2.52, 8.76, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 7.7 (br-s, 1H, NH), 8.4 (d, 1H, *J* 8.04, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 9.60, 30.98, 116.73, 121.16, 121.33, 123.86, 124.65, 126.28, 128.31, 129.34, 129.90, 130.62, 144.93, 150.55, 172.06; Electrospray-MS *m/z*, calcd. for $C_{15}H_{13}Cl_2NO_2$ $[M]^+$: 310, found: 310$[M]^+$.

***N*-(2-(2,4-dichlorophenoxy)phenyl)butyramide (3.23):** Light Brown solid in 69 % yield; mp 105-107 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.0 (t, 3H, *J* 7.4, $CH_3$), 1.7 (m, 2H, $CH_2$), 2.4 (t, 2H, *J* 7.28, $CH_2$), 6.7 (dd, 1H, *J* 1.32, 8.14, Ar-H), 6.9 (d, 1H, *J* 8.76, Ar-H), 7.0 (t, 1H, *J* 7.92, Ar-H), 7.1 (t, 1H, *J* 7.64, Ar-H), 7.2 (dd, 1H, *J* 2.52, 8.76, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 7.7 (br-s, 1H, NH), 8.4 (d, 1H, *J* 7.88, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 13.68, 19.01, 39.86,

116.75, 121.17, 121.41, 123.91, 124.64, 126.28, 128.31, 129.31, 129.90, 130.61, 144.96, 150.57, 171.35; Electrospray-MS *m/z*, calcd. for $C_{16}H_{15}Cl_2NO_2$ $[M]^+$: 324, found: 324$[M]^+$.

**2-Chloro-*N*-(2-(2,4-dichlorophenoxy)phenyl)acetamide (3.24):** Light Brown solid in 70 % yield; mp 121-123 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 4.2 (s, 2H, $CH_2$), 6.8 (dd, 1H, *J* 1.4, 8.14, Ar-H), 7.0 (d, 1H, *J* 8.72, Ar-H), 7.1 (t, 1H, *J* 8.04, Ar-H), 7.2 (t, 1H, *J* 7.66, Ar-H), 7.2 (dd, 1H, *J* 2.56, 8.78, Ar-H), 7.5 (d, 1H, *J* 2.48, Ar-H), 8.4 (dd, 1H, *J* 1.56, 8.12, Ar-H), 9.0 (br-s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 43.04, 116.66, 121.06, 121.11, 124.54, 125.00, 126.50, 128.12, 128.22, 130.08, 130.73, 145.40, 150.30, 163.89; Electrospray-MS *m/z*, calcd. for $C_{14}H_{10}Cl_3NO_2$ $[M]^+$: 331, found: 331$[M]^+$.

### 3.6.2 *In vitro* antimicrobial assay

**3.6.2.1 Test isolates:** The test panel comprised of eight bacterial strains namely *Staphylococcus aureus* (NCTC: 6571), *Staphylococcus aureus* (MTCC: 737) *Staphylococcus aureus* (MTCC: 2639), *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruganosa* (MTCC: 647), *Pseudomonas aeruganosa* (MTCC:3541), *Pseudomonas putida*. Trypticase Soy Agar (TSA) slants were the maintenance medium and the cultures were activated in cation adjusted Mueller Hinton (MH) broth 18- 24 hours prior to the test.

**3.6.2.2 *In vitro* Microbroth Dilution Assay to determine Minimal Inhibitory Concentration (MIC):** Stock solutions of the compounds were prepared and tested against a spectrum of standard and clinical isolates of Staphylococcus aureus by *in vitro* microbroth dilution assay to ascertain antimicrobial potential of the compounds as per Clinical and Laboratory Standards Institute (CLSI) M7-A4 [108]. MIC is considered to be the gold standard technique to evaluate the susceptibility of a microorganism to a particular antibiotic. Briefly 50 µL of the 18 hrs old 0.5 McFarland adjusted bacterial suspension in physiological saline was dispensed in each well. 125 µL of MH broth was added in the wells to achieve a final bacterial cell concentration of $10^6$cells in the well. Plates were then incubated at 37°C for 2.5 hours. After which 25 µL of the test compound at different concentrations (i.e two fold serial dilutions in range between 125.00- 0.48 µg/mL) was added into the well and incubated for 24 hrs at 37°C. Subsequently 20 µL of 0.02% of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into each well and incubated at 37°C for 1 hr. The color change due to formation of formazan was visually observed. All the tests were performed in triplicates [109].

### 3.6.3 Docking of inhibitors with PaFabI

All the docking simulations were done using AutoDock Vina program in PyRx [110].

#### 3.6.3.1 Preparation of the receptor and lignad molecules

The crystal structure of PaFabI (PDB identifier 4NR0) was used as receptor molecule for docking studies [117]. The structure co-ordinates of receptor and triclosan ligand molecule were retrieved from the RCSB Protein Data Bank [112]. Energy-minimized co-ordinates of inhibitors were generated using PRODRG server [113].

#### 3.6.3.2. Docking simulations and docking analysis

Using PyRx 0.8, a grid box (size_x = 25, size_y = 25 and size_y =25) was centered in the active site of the receptor with center_x = 3.16, center_y = 33.51 and center_z =40.75. The same parameters were used for all docking experiments. The minimum energy conformations of docked inhibitors were collected and further analyzed by comparing with the triclosan-bound PaFabI structure.

# Chapter 4

## Synthesis and Antibacterial Activity of 2, 4-Disubstituted diphenylamines

### 4.1 Introduction

There have been continuous efforts to advance the antibacterial activity of diphenyl ethers by substituting different functional groups on both the rings by a number of groups [10,70], including work done in previous chapters. To the best of our knowledge replacement of ether oxygen in diphenyl ethers by corresponding NH group for improving antibacterial activity has not been attempted so far. Work done here for the first time shifts focus from diphenyl ethers to corresponding amines. Here the synthesis, characterization and *in silco* docking studies of a series of diphenyl amines has been described, that we contemplate have enoyl-ACP- reductases as its target. There are earlier but limited reports on the antimicrobial activity of *N*-phenyanthranilic acid based diphenyl amines **(4.1, Figure 4a)** [118]. The antibacterial and anti fungal activities of benzo[b]thiophene or pyridine containing diarylamines has been reported **(4.2 and 4.3, Figure 4a)** [86, 119].

**Figure 4a. Structures of active diphenyl amines and diphenyl ethers**

The synthesized diphenyl amines were screened for their antibacterial activity against eight strains that included both gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*) and gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcusepidermidis*). Most of the synthesized compounds demonstrated antibacterial activity against *Staphylococcus aureus.* Therefore the biological activity was extended further to evaluate the compounds against resistant strains of *Staphylococcus aureus.*

This is important because *Staphylococcus aureus* causes life threatening infections and a serious public health issue **(4.4, Figure 4a)** [120]. Penicillin was one of the effective treatments in the past for the infections caused by the organism but evolution of penicillinase by bacteria made the drug resistant.

## 4.2 Synthesis of diphenyl amines

**Scheme 4.1** illustrates the synthesis of all the diphenyl amines. Compounds **4.12** to **4.15** were synthesized starting nitro fluorobenzene **(4.6/4.8)** and corresponding anilines by nucleophilic aromatic substitution reaction. Compound **4.12** was synthesised without any base, metal salt or the ligand using 2,4-dinitrofluorobenzene and aniline. For the synthesis of compounds **4.13**, **4.14** and **4.15** a known procedure was employed that required use of potassium carbonate, cuprous iodide and catalytic amount of amino acid, *L*-Proline. The mixture on heating at 100 $^{o}$C in aprotic solvent gave corresponding product [121]. Literature shows synthesis of all the above compounds by alternate routes [122-124].

R1, R2, X + Y, R3 —(i) or (ii)→ R1, R2, B, H N, A, R3

**4.6:** $R_1$= $R_2$= $NO_2$; X= F
**4.8:** $R_1$= $NO_2$; $R_2$= H; X= F
**4.10:** $R_1$= $R_2$= Cl; X= $NH_2$

**4.7:** $R_3$= H; Y= $NH_2$
**4.9:** $R_3$= $OCH_3$; Y= $NH_2$
**4.11:** $R_3$= $NO_2$; Y= F

**4.12:** $R_1$= $R_2$= $NO_2$; $R_3$= H
**4.13:** $R_1$= $R_2$= $NO_2$; $R_3$= $OCH_3$
**4.14:** $R_1$= $NO_2$; $R_2$= H; $R_3$= $OCH_3$
**4.15:** $R_1$=$R_2$=Cl; $R_3$= $NO_2$

**Scheme 4.1.** (i) CuI, L-Proline, $K_2CO_3$, DMF, 100$^{o}$C (yield 82-84%) (ii) Heating in DMSO at 100$^{o}$C (yield 85 %)

Compounds **4.13** and **4.14** were demethylated in acetic acid using 48% aqueous hydrobromic acid and under refluxing conditions to afford corresponding 2-hydroxy diphenyl amines **4.16** and

**4.17** respectively **(Scheme 4.2)**. This was confirmed by the absence of signals due to $OCH_3$ group in $^{1}H$ (3.8-3.9 ppm) and $^{13}C$ (55.7 ppm) NMR as compared to their starting materials. Earlier, such compounds have been synthesized by Smiles rearrangement [126].

**4.13:** $R_1 = R_2 = NO_2$
**4.14:** $R_1 = NO_2$; $R_2 = H$

**4.16:** $R_1 = R_2 = NO_2$
**4.17:** $R_1 = NO_2$; $R_2 = H$

**Scheme 4.2.** (iii) 48 % Aq. HBr, Acetic acid, Reflux (yield 37-45%)

Reduction of nitro groups on compounds **4.13**, **4.14** and **4.15** to corresponding amines **4.18**, **4.19** and **4.20** was done by refluxing in water in the presence of powdered iron and ferrous sulphate **(Scheme 4.3)**[127]. $^{1}H$ NMR corroborated the presence of amine after reaction due to appearance of broad singlet in the range of 3.6-3.9 ppm.

;

**4.13:** $R_1 = R_2 = NO_2$; $R_3 = OCH_3$
**4.14:** $R_1 = NO_2$; $R_2 = H$; $R_3 = OCH_3$
**4.15:** $R_1 = R_2 = Cl$; $R_3 = NO_2$

**4.18:** $R_1 = R_2 = NH_2$; $R_3 = OCH_3$
**4.19:** $R_1 = NH_2$; $R_2 = H$; $R_3 = OCH_3$
**4.20:** $R_1 = R_2 = Cl$; $R_3 = NH_2$

**Scheme 4.3.** (iv) Fe, $FeSO_4.7H_2O$, Reflux, (yield 62-65%)

The amine **4.20** obtained above was reacted corresponding acid chlorides in the presence of potassium carbonate and methylene chloride to afford novel compounds **4.21**, **4.22** and **4.23** **(Scheme 4.4)**. Appearances of $^{13}C$ NMR signal in the range of 164-173 ppm confirmed the presence of amide groups due to carbonyl carbon. The acid chlorides used for the purpose were freshly prepared by treating acetic acid, proponic acid and chloroacetic acid with phosphorous trichloride.

**Table 4.1** below summarizes prominent parameters in the spectroscopic studies of compound **4.12** to **4.23**. $^{1}H$ and $^{13}C$ NMR spectra for compounds **4.14**, **4.16**, **4.18** and **4.21** are given in **Figures 4A, 4B, 4C and 4D (a: $^{1}$H NMR and b: $^{13}$ C NMR) (Refer Page No. 117-124).**

(v)

4.20

**4.21:** $R_1$= -NH-CO-$CH_3$
**4.22:** $R_1$= -NH-CO-$CH_2$-$CH_3$
**4.23:** $R_1$= -NH-CO-$CH_2$-Cl

**Scheme 4.4.** (v) $K_2CO_3$, DCM (yield 64-69 %) Acid Chloride ($CH_3COCl$ for **4.21**, $CH_3CH_2COCl$ for **4.22**, $ClCH_2COCl$ for **4.23**)

**Table 4.1. Summary of prominent spectroscopic data for synthesized diphenyl amines**

| S.No. | Compound | $^1$H NMR | | Observed Mass |
|---|---|---|---|---|
| | | Position | Proton | |
| 1. | 4.12 | - | - | 261[M+2]$^+$ |
| 2. | 4.13 | 3.8, s, 3H | -$OCH_3$ | 289 [M] $^+$ |
| 3. | 4.14 | 3.9, s, 3H | -$OCH_3$ | 245 [M+1] $^+$ |
| 4. | 4.15 | - | - | 283 [M]$^+$ |
| 5. | 4.16 | 5.7, s, 1H | -OH | 275[M]$^+$ |
| 6. | 4.17 | 5.7,br-s, 1H | -OH | 230[M]$^+$ |
| 7. | 4.18 | 3.6-3.7,br s, 4H | -$NH_2$ | 230[M+1]$^+$ |
| 8. | 4.19 | 3.8, br s, 2H | -$NH_2$ | 215[M+1]$^+$ |
| 9. | 4.20 | 3.9, br-s, 2H | -$NH_2$ | 253[M]$^+$ |
| 10. | 4.21 | 7.7, s, 1H | -CONH- | 318[M+23]$^+$ |
| 11. | 4.22 | 7.6, s, 1H | -CONH- | 332 [M+23]$^+$ |
| 12. | 4.23 | 8.7, s, 1H | -CONH- | 330 [M]$^+$ |

### 4.3 Antibacterial Activity

To evaluate the antibacterial activity of the synthesized diphenyl amines against both Gram positive and Gram negative strains, eight organisms were short listed. Gram positive included two strains of *Bacillus subtilis, Staphylococcus epidermidis* and *Staphylococcus aureus* each while Gram negative included two strains of *Escherichia coli, Pseudomonas aeruginosa* and *Pseudomonas putida* each. Initial results **(Table 4.2)** illustrate that 6 of the 13 compounds were active against *Staphylococcus aureus.* These compounds were **4.12**, **4.15**, **4.16**, **4.17**, **4.20** and **4.23**. Three among these six compounds (**4.12**, **4.16** and **4.17**) gave encouraging results for most of the strains **(Table 4.2).** *Pseudomonas putida,* the Gram negative strain was an

exception as it displayed activity against all the synthesized compounds, except compounds **4.18** and **4.19** with a MIC ranging from 7.81 - 62.50 µg/mL

**Table 4.2** shows that most notable compounds were **4.16** and **4.17** as these were active against all the selected stains. Compound **4.16** having MIC in the range 7.81 – 15.6 µg/mL was the most potent as it gave comparable antibacterial activity to that of control against all the organisms. Compound **4.17**, had a comparatively higher MIC range, (7.81 – 62.5 µg/mL) but was also active against all the organisms. Triclosan and Streptomycin, used as controls that gave MIC range of 1.95 – 7.81 µg/mL and 1.95 – 15.62 µg/mL respectively in the preliminary studies. Whereas triclosan was active against all the organisms, Streptomycin did not give encouraging results against many stains including *Staphylococcus aureus.* **Scheme 4.1** shows that both compounds **4.16** and **4.17** have hydroxyl group (OH) at ortho position of ring A.

**Table 4.2. Screening of Diphenyl amines against other Gram positive and Gram negative strains**

| Compound | *S. aureus*[a] | *S. aureus*[b] | *B. subtilis* | *S. epidermidis*[c] | *E. coli* [d] | *P. aeru.*[e] | *P.aeru.*[f] | *P.putida* |
|---|---|---|---|---|---|---|---|---|
| | **MIC$_{90}$ (µg/mL)** | | | | | | | |
| 4.12 | 15.62 | 15.62 | - | 15.62 | 31.25 | - | - | 7.81 |
| 4.13 | - | - | - | - | - | - | - | 31.25 |
| 4.14 | - | - | - | - | - | - | - | 15.62 |
| 4.15 | 15.62 | 7.81 | - | - | 62.50 | - | 62.50 | 31.25 |
| 4.16 | 7.81 | 7.81 | 15.62 | 15.62 | 7.81 | 7.81 | 7.81 | 7.81 |
| 4.17 | 31.25 | 31.25 | 62.5 | 31.25 | 31.25 | 31.25 | 7.81 | 31.25 |
| 4.18 | - | - | 62.5 | - | - | - | - | - |
| 4.20 | 15.62 | 31.25 | - | - | - | - | - | 15.62 |
| 4.21 | - | - | - | - | - | - | - | 31.25 |
| 4.22 | - | - | - | 62.5 | - | - | - | 62.50 |
| 4.23 | 31.25 | 7.81 | - | - | - | - | - | 15.62 |
| **Triclosan*** | 7.81 | 3.90 | 3.90 | 1.95 | 15.62 | 1.95 | 7.81 | 3.90 |
| **Streptomycin*** | - | - | - | 7.81 | - | 15.62 | 7.81 | 1.95 |

* Triclosan, a diphenyl ether, and Streptomycin were used as reference compounds.

***a-*** NCTC 6571, ***b*** - MTCC 96, ***c-*** MTCC 2639, ***d*** - MTCC 1302, ***e*** - MTCC 647, **f** - MTCC 3541.

Compounds containing 2,4-dichloro substitution on ring B of diphenyl amines were also effective against *S. aureus* strains. **Table 4.2** shows MIC rage of compounds **4.15** (7.81 – 15.62 µg/ mL), **4.20** (15.62 – 31.25 µg/mL) and **4.23** (MIC = 7.81 – 31.25 µg/mL). Compound **4.15**, in addition to *S. aureus* strains was also active against three Gram negative strains having MIC ange of 31.25- 62.50 µg $mL^{-1}$ **(Table 4.2)**. However, compounds **4.20** and **4.23** were active only against *Pseudomonas putida* in Gram negatives. While compound **4.12** showed activity against *S. aureus*, it was more effective against *Pseudomonas putida* with MIC value of 7.81 µg/mL.

## 4.4 Structure Activity Relationship

Results in **Table 4.2** indicate that hydroxyl group at 2-position of ring A is enhances the antibacterial activity of diphenyl amines. This is apparent from the two compounds **4.16** and **4.17**, having hydroxyl group at the said position. Other compounds containing methoxy functional group at 2-position of ring A (**4.13**, **4.14**, **4.18**, **4.19**) did not yield any antibacterial activity. Both the compounds have shown good activity against all the organisms taken in this study. MIC range for compound **4.16** was 7.81 to 15.62 µg/mL and for compound **4.17** was 7.81 to 62.50 µg/mL. However, compound **4.16** gave MIC value of 7.81 µg/mL against all the strains except *Bacillus subtilis* and *S. epidermidis,* compound **4.17** was comparatively less active having giving MIC values in the range of 7.81 - 62.50 µg/mL. Therefore, presence of nitro groups at positions 2' and 4' of ring B increases the antibacterial activity of diphenyl amines as compared to only one at position 2'. Literature reports have ascertained that 2-hydroxy group is crucial for the inhibition of enoyl-ACP reductases (FabI) [8]. Studies carried out with *N*-(2,4-dichlorophenyl)-2-methoxyaniline and *N*-(2,4-dichlorophenyl)-2-hydroxyaniline shows inhibition of enoyl-ACP reductases (FabI) of *P. falciparum* at the active site of enzyme due to later compound only. We also propose the possible the target for compounds **4.16** and **4.17** as FabI based on reported results. *In silico* docking studies described later in this chapter also confirm this.

The fact, that methoxy group at 2 position of ring A is unbearable as compared to hydrogen or hydroxyl for the antibacterial activity, is also supported by compound **4.12**. The compound has neither hydroxyl nor methoxy substituent at position-2 gave comparatively better results than compounds having methoxy group at the said position as discussed above. The MIC values of **4.12** against *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas putida* were in the range of 7.81- 31.25 µg/mL.

Presence of chloro groups at positions 2' and 4' of ring B and nitro and amino groups, both polar, at 2-position of diphenyl amine in the ring A in compounds **4.15** and **4.20** respectively were not broad spectrum like compounds **4.16** and **4.17**. Nevertheless, both demonstrated antibacterial activity against *Staphylococcus aureus* having MIC range 7.81- 15.62 μg/mL **(4.15)** and 3.90- 31.25 μg/mL (**4.20**). Compound **4.15** was also active against *Pseudomonas aeruginosa* and *Escherichia coli* but at higher MIC values. Therefore 2, 4-dichloro diphenyl amines do not show promising antibacterial activity but need to be explored further for broad spectrum antibacterial activity.

Derivatization of amino group in compound **4.20** to corresponding amides gave compounds **4.21**, **4.22** and **4.23**. However, except for **4.23**, these were found to be futile against all the strains other than *Pseudomonas putida.* Thus, amidation of amino group at 2-position of ring A further destroys the antibacterial activity. However, use of chloro acetic acid group for amidation retains the activity back as in case of compound **4.23**. *P. putida* was the most sensitive among all the strains that were selected for the study. Compounds **4.18** and **4.19** contained all the non required features as discussed above. These were presence of amino group and the presence of methoxy group at ortho positions. Therefore, these compounds were ineffective against all the strains including sensitive *P. putida*.

### 4.5 *In silico* docking studies

Most of the compounds synthesized in this part of the work gave good antibacterial activity against *Staphylococcus aureus.* Therefore, SaFabI (PDB identifier 4ALL) was chosen as the enzyme for the docking studies with the synthesized compounds. **Table 4.3** shows comparison of the results from the docking studies for all the diphenyl amines (DPA) with the *Sa*Fab I -$NAD^+$ complex and MIC value.

It has been reported that in the ternary SaFabI - $NAD^+$- triclosan (PDB identifier 4ALL) complex, ring A of triclosan, having OH- group at position 2 and chloro group at position 4, stacks over the nicotinamide ring of $NAD^+$ [120]. The ring A of triclosan stacks over the nicotinamide of $NAD^+$ and OH group forms two hydrogen bonds. One of them is oxygen (O) of the hydroxyl group of triclosan with H of OH in Tyr 157 and other H of the triclosan's OH with 2'oxygen of the 2'OH of the nicotinamide. The chloro group at position 4 of Ring A of triclosan is surrounded by conserved Val 201, Phe 204 and Met 207. Ring B containing both the chloro groups is closed in a pocket surrounded by nicotinamide ribose, $NAD^+$ and the substrate binding

loop residues containing residues Ser 197, Leu 102 and Met 160. The chloro group at 4' position forms a geometrically favourable halogen bond with carbonyl oxygen of Ala 97. The same chlorine is also weakly hydrogen bonded to the amide NH group of Ala 97 **(Figure 4b)**.

**Table 4.3. Results from the docking studies of SaFabI-NAD$^{+}$-DPA complex and MIC value**

| Compound | Binding Affinity (kcal/mol) | *S. aureus* MTCC 96 MIC (µg/mL) |
|---|---|---|
| 4.12 | -7.5 | 15.62 |
| 4.13 | -7.8 | - |
| 4.14 | -6.6 | - |
| 4.15 | -7.0 | 7.81 |
| 4.16 | -8.1 | 7.81 |
| 4.17 | -7.7 | 31.25 |
| 4.18 | -7.2 | - |
| 4.19 | -7.4 | - |
| 4.20 | -7.8 | 31.25 |
| 4.21 | -7.0 | - |
| 4.22 | -7.2 | - |
| 4.23 | -6.3 | 7.81 |
| Triclosan | -8.9 | 3.90 |

Docking studies with the diphenyl amines presented interesting results. In case of compound **4.15**, where ring B is similar to that triclosan's ring B and ring A has only $NO_2$ group at position- 2 instead of OH, hydrogen bond interactions were observed between oxygens of $NO_2$ with H of the phenol in Tyr157 (3.0Å) and with H of the hydroxyl group of nicotinamide ribose (2.5Å) **(Figure 4c)**.

Interestingly, when two chloro groups in ring B of triclosan were replaced in case of diphenyl amine by nitro groups and ring A's OH was retained as such no flipping of the rings was observed, as discussed in the earlier cases, at the active site of the Fab I. This can be seen in the docking studies of compound **4.17 (Figure 4d)** where OH group of ring A forms same interaction with Tyr157, as in case of triclosan, and with 2'oxygen of the 2'OH in the nicotinamide. In this case oxygens of the $NO_2$ in ring B interact with the pyrophosphate of NAD$^{+}$

(3.0 Å) and also of the hydroxyl group of Ser 197 (3.3Å) thus making stronger interactions as compared to chlorine. Results in the docking studies are consistent with the antibacterial studies and have revealed a clear preference for phenolic (OH) group at 2 position of ring A. Results from previous chapters also demonstrate that in the absence of phenolic OH group $NO_2$ can be tolerated but at the cost of antibacterial activity.

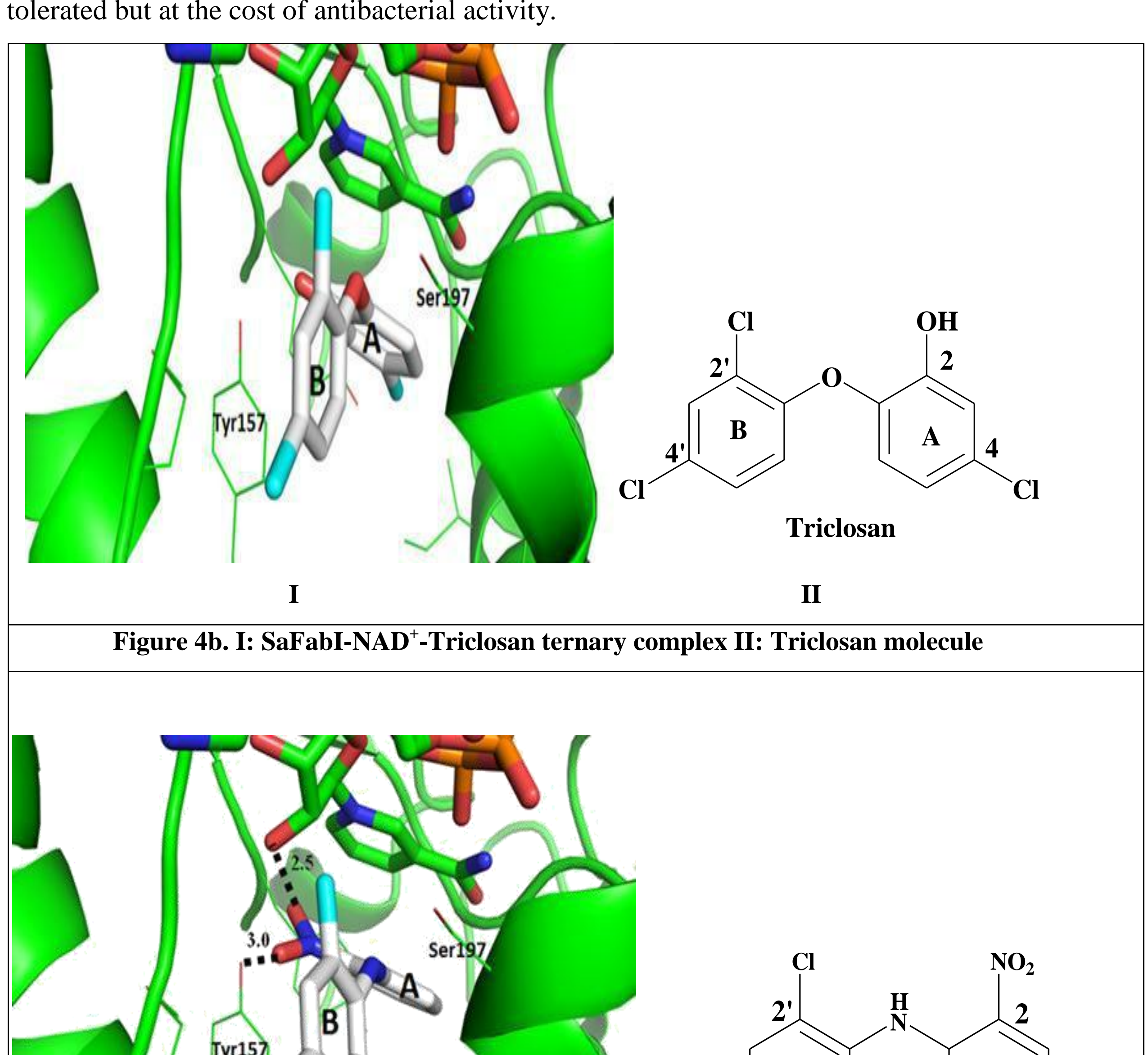



**Figure 4b. I: SaFabI-NAD+-Triclosan ternary complex II: Triclosan molecule**

**Figure 4c. I: SaFabI-NAD+- Test molecule 4.15 ternary complex, II: Test molecule 4.15**

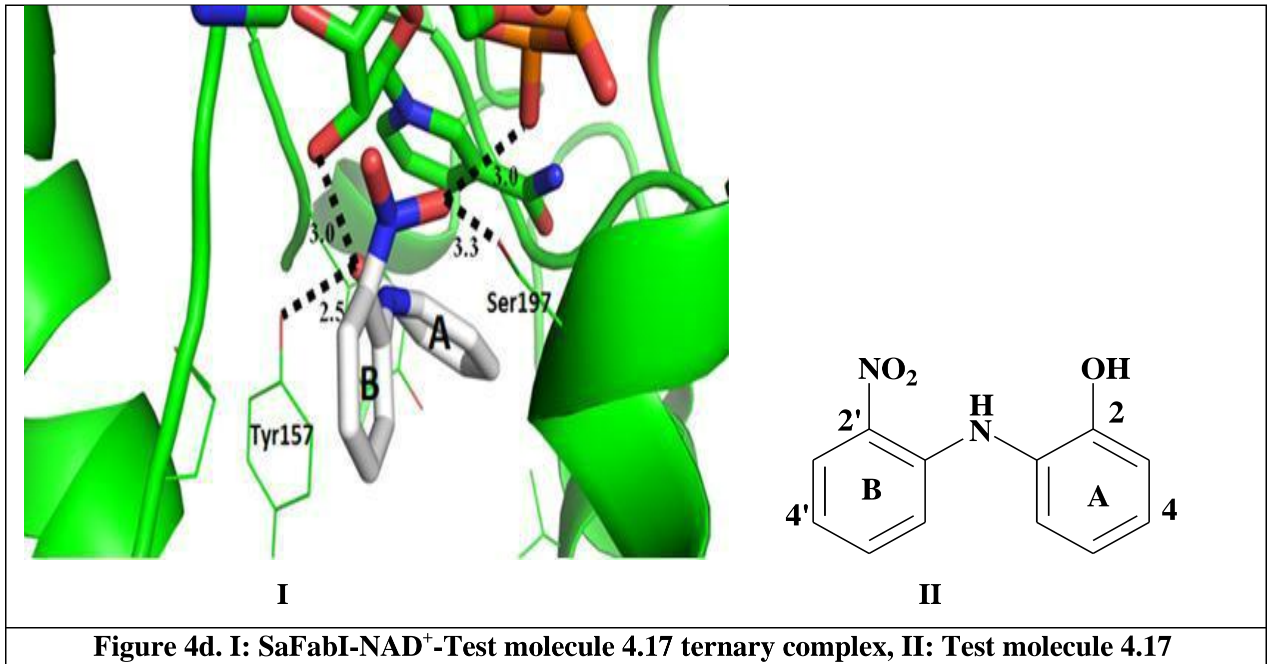



**Figure 4d. I: SaFabI-NAD$^+$-Test molecule 4.17 ternary complex, II: Test molecule 4.17**

## 4.6 Materials and Methods

### 4.6.1 Synthesis of substituted diphenyl amines and their derivatives

Purified reagents were procured from SD Fine Chemicals, Bombay. Solvents were distilled before doing the column chromatography. Melting points are reported in $^{o}$C and are uncorrected. $^{1}$H and $^{13}$C NMR spectral analyses were performed on 400.1 and 100.6 MHz spectrometer (Bruker) with tetramethylsilane and $CDCl_3$ as the internal standard ($\delta$, ppm) respectively. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet, br, broad. Mass analysis was done using Waters Micromass mass spectrometer. **Scheme 4.1** summarizes all the synthetic procedures.

**Synthesis of 2,4-dinitro-*N*-phenylaniline (4.12):** Although commercially available, we synthesized the compound using 1-fluoro-2,4-dinitrobenzene **(4.6)** (186 mg, 1 mmol) and aniline **(4.7)** (91 μL, 1 mmol) in dimethyl sulfoxide (DMSO) (1 mL) by heating in oil bath at 100 $^{o}$C for 16 h under inert atmosphere. On cooling the reaction mixture was partitioned between ethyl acetate (15 mL) and water (15 mL) and extracted again with ethyl acetate (5 mL). The combined organic layers were dried over $Na_2SO_4$, filtered and evaporated *in vacuo* to yield crude product that was purified by silica gel column chromatography and (petroleum ether/ethyl acetate = 85/15) solvent system to get orange solid in 85% yield; m.p.: 163-165 $^{o}$C; $^{1}$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 7.2 (d, 1H, *J* 9.56, Ar-H), 7.3 (d, 2H , *J* 7.56, Ar-H), 7.4 (dd, 1H, *J* 7.48, 1.00, Ar-H),

7.5 (m, 2H, Ar-H), 8.2 (m, 1H, Ar-H), 9.2 (d, 1H, *J* 2.64, Ar-H), 9.9 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 116.07, 124.13, 125.55 (Intense), 127.78, 129.96, 130.29 (Intense), 131.10, 136.68, 137.40, 147.15; Electrospray-MS *m/z*, calcd. for $C_{12}H_9N_3O_4$ $[M]^+$: 259, found: 261$[M+2]^+$.

**Synthesis of compounds 4.13, 4.14, and 4.15:** To a solution of nitro fluoro benzene **(4.8)** (7.5 mmol) in dimethylformamide (DMF) (3 mL) was added relevent aryl amine **(4.9/4.10)** (5 mmol), potassium carbonate (10 mmol), cuprous iodide (0.5 mmol) and *L*-proline (1.0 mmol). The reaction mixture was heated at 100 $^o$C for 24 hrs. After completion of the reaction (TLC monitoring) it was allowed to cool. Work up was done by adding water (7 mL) and organic components extracted using ethyl acetate (3 x 10mL). Combined organic solvent was washed with water till it gets neutral (pH paper) and dried over brine and $Na_2SO_4$. Evaporation of the organic solvent gave the crude product that was purified using silica column and (petroleum ether/ethyl acetate = 75/25) solvent system. Evaporation of the solvent gave pure products that were characterized using $^1$H, $^{13}$C NMR and mass spectroscopy.

**2-Methoxy-*N*-(2,4-dinitrophenyl)-aniline (4.13):** Orange solid in 84 % yield; m.p.: 161-163 $^o$C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.8 (s, 3H, $CH_3$)¸ 6.98 (m, 2H , Ar-H), 7.0 (d, 1H, *J* 9.52, Ar-H), 7.3 (m, 2H, Ar-H), 8.1 (dd, 1H, *J* 2.52, 9.54, Ar-H), 9.1 (d, 1H, *J* 2.64, Ar-H), 9.8 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 55.75, 112.05, 116.29, 121.05, 124.06, 125.39, 125.48, 128.49, 129.68, 131.37, 137.24, 146.81, 153.45; Electrospray-MS *m/z*, calcd. for $C_{13}H_{11}N_3O_5$ $[M]^+$: 289, found: 289 $[M]^+$.

**2-Methoxy-*N*-(2-nitrophenyl)-aniline (4.14):** Orange solid in 80 % yield; mp 86-88 $^o$C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.9 (s, 3H, $CH_3$), 6.8 (m, 1H, Ar-H), 6.89 (m, 2H, Ar-H), 7.2 (m, 1H , Ar-H), 7.3 (m, 1H, Ar-H), 7.4 (m, 2H, Ar-H), 8.2 (dd, 1H, *J* 1.6, 8.56, Ar-H), 9.4 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 55.71, 111.60, 116.21, 117.44, 120.67, 123.29, 125.77, 126.68, 127.84, 133.76, 135.46, 142.50, 152.53; Electrospray-MS *m/z*, calcd. for $C_{13}H_{12}N_2O_3$ $[M]^+$: 244, found: 245 $[M+1]^+$.

**2,4-Dichloro-*N*-(2-nitrophenyl)-aniline (4.15):** Orange solid in 82% yield; mp 114-116 $^o$C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 6.9 (t, 1H, *J* 7.84, Ar-H), 7.1 (dd, 1H, *J* 1.2, 8.6, Ar-H), 7.3 (d, 1H, *J* 2.32, Ar-H), 7.4 (d, 1H, *J* 8.6, Ar-H), 7.4 (m, 1H, Ar-H), 7.5 (d, 1H, *J* 2.36, Ar-H), 8.2 (dd, 1H,

*J* 1.52, 8.54, Ar-H), 9.4 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 112.06, 116.30, 121.05, 124.06, 125.37, 125.48, 128.49, 129.69, 131.37, 137.22, 150.01, 153.45; Electrospray-MS *m/z*, calcd. for $C_{12}H_8Cl_2N_2O_2$ $[M]^+$: 283, found: 283 $[M]^+$.

**Synthesis of compounds 4.16 and 4.17:** To the compound obtained above, **4.13** and **4.14** (0.62 mmol) in acetic acid (10 mL) was added 48% aqueous HBr (1 mL) and heated under reflux for 2 h. After confirming completion of the reaction (TLC monitoring) it was cooled to room temperature and acetic acid evaporated *in vacuo*. Water (5 mL) was added to dissolve the contents and the aqueous layer extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave product which was purified using silica column chromatography and solvent (pet ether/ethyl acetate = 65/35) to afford desired compounds.

**2-(2,4-Dinitrophenylamino)-phenol (4.16):** Dark brown solid in 45% yield; mp 140-143 $^oC$; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 5.7 (s, 1H , OH), 6.75 (dd, 1H , *J* 1.12, 8.6, Ar-H), 6.86 (m, 1H, Ar-H), 7.1 (m, 1H, Ar-H), 7.3 (dd, 1H, *J* 3.88, 5.88, Ar-H ), 7.4 (m, 1H, Ar-H), 8.2 (dd, 1H, *J* 8.56, 1.56, Ar-H), 8.9 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 116.14, 118.55, 119.72, 121.46, 124.33, 124.64, 126.68, 128.91, 136.24, 143.43, 152.97; Electrospray-MS *m/z*, calcd. for $C_{12}H_9N_3O_5$ $[M]^+$: 275, found: 275$[M]^+$.

**2-(2-Nitrophenylamino)-phenol (4.17):** Dark brown solid in 37% yield; mp 140-142 $^oC$; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 5.7 (br-s, 1H, OH), 6.7 (d, 1H, *J* 8.48, Ar-H), 6.9 (m, 1H), 7.1 (m, 2H, Ar-H), 7.3 (d, 1H, *J* 4.6, Ar-H), 7.4 (m, 1H, Ar-H), 8.2 (dd, 1H, *J* 1.8, 6.88, Ar-H), 8.4 (d, 1H, *J* 2.32, Ar-H), 8.9 (s, 1H, NH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 116.10, 118.52, 119.67, 121.46, 124.37, 124.73, 126.66, 128.92, 136.25, 138.91,143.41,152.70; Electrospray-MS *m/z*, calcd. for $C_{12}H_{10}N_2O_3$ $[M]^+$: 230, found: 230$[M]^+$.

**Synthesis of compounds 4.18, 4.19 and 4.20:** To a suspension of nitro diphenyl amines **4.13**, **4.14** and **4.15** (10.6 mmol) in $H_2O$ (50 mL) was added Fe (106.2 mmol) and $FeSO_4.7H_2O$ (10.4 mmol). The reaction mixture was refluxed for 8 hrs. After confirming the completion of reaction (TLC monitoring), it was cooled and filtered through celite. The organic components were extracted with ethyl acetate (2 x 75 mL). The combined organic layers were dried over $Na_2SO_4$. Evaporation of the organic solvent gave crude amino product that was purified using silica

column and (pet ether/ethyl acetate = 80/20) solvent system. Evaporation of the solvent gave pure product that was characterized using $^{1}H$, $^{13}C$ NMR and mass spectroscopy.

***N*^1^-(2-methoxyphenyl)-benzene-1,2,4-triamine (4.18):** Dark brown solid in 65 % yield; mp 78-80 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.6-3.7 (br s, 4H, $2NH_2$), 3.8 (s, 3H, $OCH_3$), 5.4 (s, 1H, NH), 6.1 (m, 2H, Ar-H), 6.6 (dd, 1H, *J* 1.52, 7.72, Ar-H), 6.7-6.8 (m, 4H, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 55.51, 102.19, 106.14, 109.78, 111.89, 117.40, 118.81, 121.23, 129.07, 136.94, 144.90, 145.51, 146.83; Electrospray-MS *m/z*, calcd. for $C_{13}H_{15}N_3O$ $[M]^+$: 229, found: $230[M+1]^+$.

***N*^1^-(2-methoxyphenyl)-benzene-1,2-diamine (4.19):** Dark brown solid in 63% yield; mp 118-121 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.8 (br s, 2H, $NH_2$), 3.9 (s, 3H, $OCH_3$), 5.7 (s, 1H, NH), 6.6 (dd, 1H, *J* 1.88, 7.48, Ar-H), 6.8 (m, 4H, Ar-H), 6.9 (dd, 1H, *J* 1.72, 7.56, Ar-H), 7.0 (m, 1H, Ar-H), 7.1 (dd, 1H, *J* 1.36, 7.8, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 55.56, 110.05, 113.03, 115.97, 118.44, 119.01, 121.09, 125.35, 125.73, 128.18, 135.13, 142.43, 147.33; Electrospray-MS *m/z*, calcd. for $C_{13}H_{14}N_2O$ $[M]^+$: 214, found: $215[M+1]^+$.

***N*^1^-(2,4-dichlorophenyl)benzene-1,2-diamine (4.20):** Black solid in 62 % yield; mp 83-85 °C; $^{1}H$ NMR (400.1 MHz, $CDCl_3$) $\delta$ 3.9 (br-s, 2H, $NH_2$)¸ 5.7 (s, 1H, NH), 6.5 (d, 1H, *J* 8.76, Ar-H), 6.8 (m, 1H, Ar-H), 7.0 (dd, 1H, *J* 2.4, 8.8, Ar-H), 7.1 (m, 2H, Ar-H), 7.3 (d, 1H, *J* 8.48, Ar-H); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 114.79, 116.18, 119.12, 120.06, 123.01, 126.01, 127.00, 127.37, 127.73, 128.89, 140.83, 143.10; Electrospray-MS *m/z*, calcd. for $C_{12}H_{10}Cl_2$ $N_2$ $[M]^+$: 253, found: $253[M]^+$.

**Synthesis of compounds 4.21, 4.22 and 4.23:** The acid chloride was prepared from corresponding acid (0.5 mmol) and $PCl_3$ (0.5 mmol) by heating and stirring at 60–70°C till the evolution of hydrochloric acid gas ceased. The acid chloride thus prepared was added to a stirred solution of compound **4.20** (0.5 mmol), $K_2CO_3$ (0.3 mmol) in DCM (10 mL) at 0°C. The reaction mixture was stirred for 2 h and quenched with water. The organic product was extracted with DCM (3 x 50 mL), dried over $Na_2SO_4$, and concentrated in vacuo. Crude product obtained was purified using silica column and solvent (pet ether/ ethyl acetate = 70:30) to afford light brown solids.

***N*-(2-(2,4-dichlorophenylamino)phenyl)acetamide (4.21):** Light Brown solid in 66 % yield; mp 118-120 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 2.1 (s, 3H, $CH_3$), 6.2 (s, 1H, NH), 6.6 (d, 1H, *J* 8.76, Ar-H), 7.0 (dd, 1H, *J* 2.24, 8.76, Ar-H), 7.2 (m, 3H, Ar-H), 7.3 (d, 1H, *J* 2.36, Ar-H), 7.7 (s, 1H, 1NHCO), 7.9 (d, 1H, *J* 7.54, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 24.34, 115.58, 121.31, 123.02, 124.12, 125.27, 125.76, 126.15, 127.71, 129.22, 132.16, 133.01, 140.53, 169.14; Electrospray-MS *m/z*, calcd. for $C_{14}H_{12}Cl_2N_2O$ $[M]^+$: 295, found: 318 $[M+23]^+$.

***N*-(2-(2,4-dichlorophenylamino)phenyl)propionamide (4.22):** Light brown solid in 69 % yield; mp 104-106 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 1.2 (t, 3H, *J* 7.6, $CH_3$), 2.3 (q, 2H, *J* 15.14, 7.56, $CH_2$), 6.0 (s, 1H, NH), 6.5 (d, 1H, *J* 8.76, Ar-H), 7.0 (dd, 1H, *J* 2.24, 8.8, Ar-H), 7.1 (m, 1H, Ar-H), 7.2 (m, 2H, Ar-H), 7.4 (d, 1H, *J* 2.36, Ar-H), 7.6 (s, 1H, 1NHCO), 8.0 (d, *J* 7.8, Ar-H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 9.68, 30.62, 115.35, 119.76, 121.10, 122.69, 124.08, 125.60, 126.45, 127.71, 129.17, 131.56, 133.40, 140.54, 172.59; Electrospray-MS *m/z*, calcd. for $C_{15}H_{14}Cl_2N_2O$ $[M]^+$: 309, found: 332 $[M+23]^+$.

**2-Chloro-*N*-(2-(2,4-dichlorophenylamino)phenyl)acetamide (4.23):** Light brown solid in 64 % yield; mp 79-82 °C; $^1$H NMR (400.1 MHz, $CDCl_3$) $\delta$ 4.1 (s, 2H, $CH_2$), 6.0 (s, 1H, NH), 6.5 (d, 1H, *J* 8.76, Ar-H), 7.0 (dd, 1H, *J* 2.32, 8.76, Ar-H), 7.3 (m, 3H, Ar-H), 7.4 (d, 1H, *J* 2.36, Ar-H), 8.0 (d, 1H, *J* 7.52, Ar-H), 8.7 (s, 1H, NHCO); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 42.96, 115.37, 121.31, 122.14, 124.45, 125.98, 126.23, 126.77, 127.77, 129.24, 131.51, 132.57, 140.25, 164.31; Electrospray-MS *m/z*, calcd. for $C_{14}H_{11}Cl_3N_2O$ $[M]^+$: 330, found: 330 $[M]^+$.

### 4.6.2 *In vitro* Antimicrobial Assay

**4.6.2.1 Test Isolates:** For preliminary screening, the test spectrum consist of standard surrogate Gram Positive and Gram negative bacteria **(Table 4.2)**. Gram positive strains included *Staphylococcus aureus* (NCTC 6571 and MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and *Bacillus subtilis* while Gram negative strains included *Escherichia coli* (MTCC 1302), *Pseudomonas aeruginosa* (MTCC 647 and MTCC 3541) and *Pseudomonas putida*. Triclosan, diphenyl ether known to have antibacterial activity and Streptomycin were used as reference compounds.

**4.6.2.2 *In vitro* microbroth dilution assay to determine Minimal Inhibitory Concentration (MIC):** Stock solutions of the compounds were prepared and tested against a spectrum of

standard strains and the clinical isolates of *Staphylococcus aureus* by *in vitro* microbroth dilution assay to ascertain antimicrobial potential of the compounds as per Clinical and Laboratory Standards Institute (CLSI) M7-A4 [108]. MIC is considered to be the gold standard technique to evaluate the susceptibility of a microorganism to a particular antibiotic and performed as reported earlier. Briefly 50 µL of the 18 hrs old 0.5 McFarland adjusted bacterial suspension in physiological saline was dispensed in each well. 125 µL of MH broth was added in the wells to achieve a final bacterial cell concentration $10^6$cells in the well. Plates were then incubated at 37 $^oC$ for 2.5 hours. After which 25 µL of the test compound at different concentrations (i.e two fold serial dilutions in range between 125.00- 0.48 µg/mL) was added into the well and incubated for 24 hrs at $37^oC$. Subsequently 20 µL of 0.02% of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into each well and incubated at $37^oC$ for 1 hr. The color change due to formation of formazan was visually observed. All the tests were performed in triplicates [109].

### 4.6.3 Docking of inhibitors with SaENR

All the docking simulations were done using AutoDock Vina program in PyRx [110]

#### 4.6.3.1 Preparation of the receptor and lignad molecules

The crystal structure of SaFabI (PDB identifier 4ALL) was used as receptor molecule for docking studies [120]. The structure co-ordinates of receptor and triclosan ligand molecule were retrieved from the RCSB Protein Data Bank [112]. Energy-minimized co-ordinates of inhibitors were generated using PRODRG server [113].

#### 4.6.3.2 Docking simulations and docking analysis

Using PyRx 0.8, a grid box (size_x = 25, size_y = 25 and size_y =25) was centered in the active site of the receptor with center_x = 0.23, center_y = -23.56 and center_z = -23.81. The same parameters were used for all docking experiments. The minimum energy conformations of docked inhibitors were collected and further analysed by comparing with the triclosan-bound SaFabI structure.

# Chapter 5

# Optimizing Surfactant and the Conditions to Enhance Aqueous Solubility of Triclosan

## 5.1 Introduction

Aqueous solubility of a drug is one of the important parameters besides its potency. Unfortunately most of the drug molecules are organic compounds that are poorly soluble in water. Therefore, it becomes important to enhance the solubility of any molecule at initial stage of evaluation to determine its actual biological activity. Many of the molecules synthesized in this work (Chapter-2) have a long hydrophobic chain due to which aqueous solubility of the compounds was a challenge. Interestingly triclosan, the reference compound used for all the studies in this work, has also very poor solubility in water (0.002 mg/mL at 30 $^{o}$C; low partion coefficient (log $P_{ow}$= 4.7) [98]. Therefore generally biochemical assays requires preparation of a stock in DMSO prior to its dilution in the aqueous solution of interest. Even then, solubility enhancement for triclosan is only up to 100.0 μM in 0.1 % DMSO. Recent literature reports have mentioned that acyl chain based surfactants act as fatty acid retrieve sources and compromise triclosan efficacy. Thus, there is a need to formally evaluate the effect of different surfactants on the efficacy of this molecule. Therefore, this chapter presents studies in the presence of different surfactants to enhance the aqueous solubility of triclosan while still maintaining its antibacterial potency in 0.1 % DMSO.

## 5.2 Effect of surfactants on the solubility and antibacterial activity of Triclosan

Five surfactants were chosen for the study based on their molecular structure and critical miceller concentration (CMC). The surfactants selected for the study included octylglucoside, sodium deoxycholate, triton X-100, tween-20 and tween-80. Structurally tween-80, tween-20 and triton X-100 contain long alkyl or alkoxy chains while octylglucoside and sodium deoxycholate have carbohydrate and bile acid skeleton respectively. **Figure 5a** provide the details of structure and CMC values of each surfactant.

In this study, solubility of triclosan was measured by UV-Visible spectrophotometer (600nm) in LB broth containing 0.1 % DMSO at different concentrations of the surfactant at 25˚C. Concentration of the triclosan in aqueous solution of LB broth containing 0.1 % of DMSO was only 100 μM. This was well in agreement with the literature reports. This value of

absorbance was used as the baseline for comparing solutions of different surfactants at varied concentrations. Based on the concentrations obtained in these experiments $IC_{50}$ values of triclosan in 0.1% DMSO for *E. coli* K12 strain were determined.

**5.1**
**CMC: 0.2-0.9 mM**

**5.3**
**CMC: 2.0-6.0 mM**

**5.4**
**CMC: 23.0-25.0 mM**

**5.2**
**CMC: 0.012 mM (T-80)**
**CMC: 0.06 mM (T-20)**

**Figure 5a. 5.1:** is structure of TritonX-100 where n = 9 or 10, **5.2:** Tween-20 when W+X+Y+Z =20 and Tween-80 when W+X+Y+Z=80 (Hydroxy group of triclosan interacts with polyoxyethylene groups of these surfactants), **5.3:** Sodium deoxycholate, **5.4:** Octyl glucoside

**Tween-80:** A final solution of 400.0 µM of triclosan in 0.1% DMSO could be made by using Tween 80 at 0.1 mM concentration. Increasing concentration of the surfactant higher further had no effect on increasing the solubility of triclosan. Moving to lower towards its CMC further decreased the solubility of triclosan (**Figure 5c**) but at lowest of the surfactant concentrations used (0.01mM) the solubility of antibacterial in 0.1% DMSO was still higher 200.0 µM than without any surfactant.

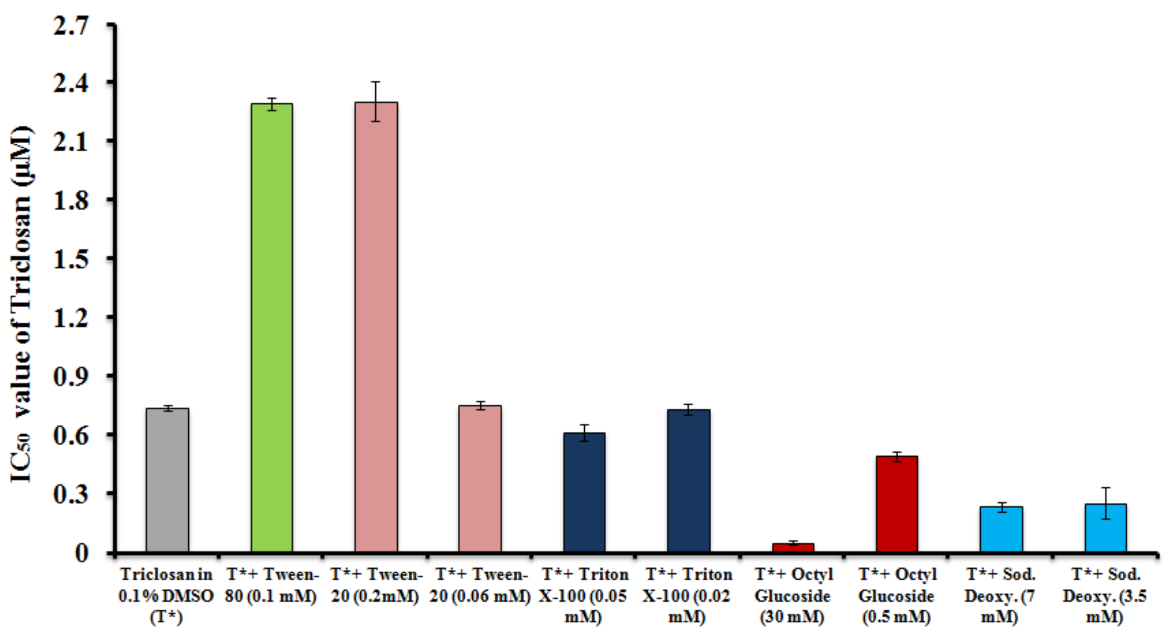



**Figure 5b.** $IC_{50}$ (µM) value of triclosan against *E. coli* culture (MTCC: 1302) in the presence of different surfactants

Interestingly, Tween 80 has adverse effect on the antibacterial activity of triclosan. $IC_{50}$ value increased from 0.7µM (without surfactant) to 2.3 µM when that concentration of surfactant was used which gave highest solubility of triclosan (**Figure 5b**). This confirmed the inhibitory effect of surfactant on triclosan that was inline with literature reports [128].

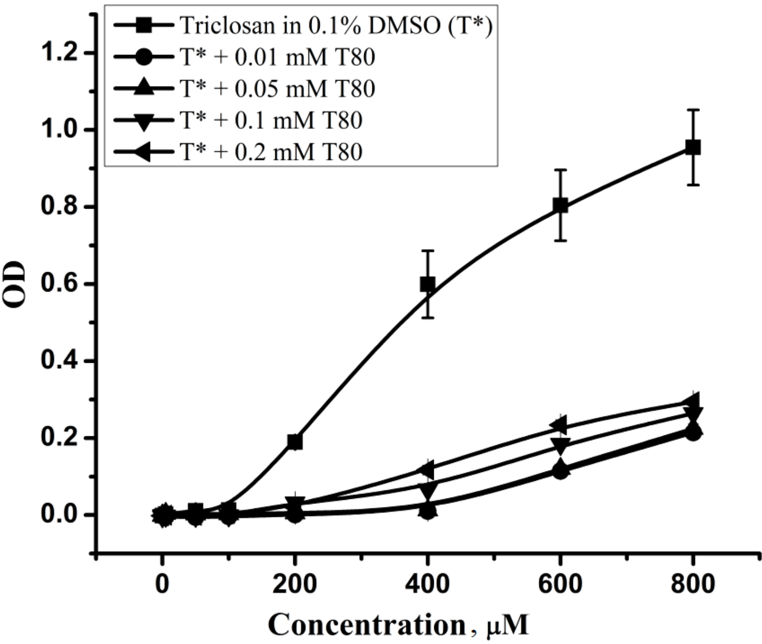



**Figure 5c.** Solubility of triclosan at different concentrations of Tween 80 (T80). X-axis: Intended concentartion of triclosan (µM); Y-axis: OD: Optical density taken at 600 nm

**Tween-20:** Tween 20 and 80 have same molecular skeleton and differ from each other in the molecular weight due to reduced number of ethoxy units (-$CH_2CH_2O$-) in former. As a result it has higher CMC value, 0.06 mM, as compared to Tween80. Maximum amount of triclosan in 0.1% DMSO that this surfactant could dissolve was ~200.0 µM. A wide range of concentrations of the surfactant (0.03 mM to 0.2 mM and 0.2 mM to 0.6mM) were used but amount of triclosan that could be solubilised in LB was only double the concentration (200.0 µM) to that without surfactant. **Figure 5d** shows the selected concentrations s from both the ranges.

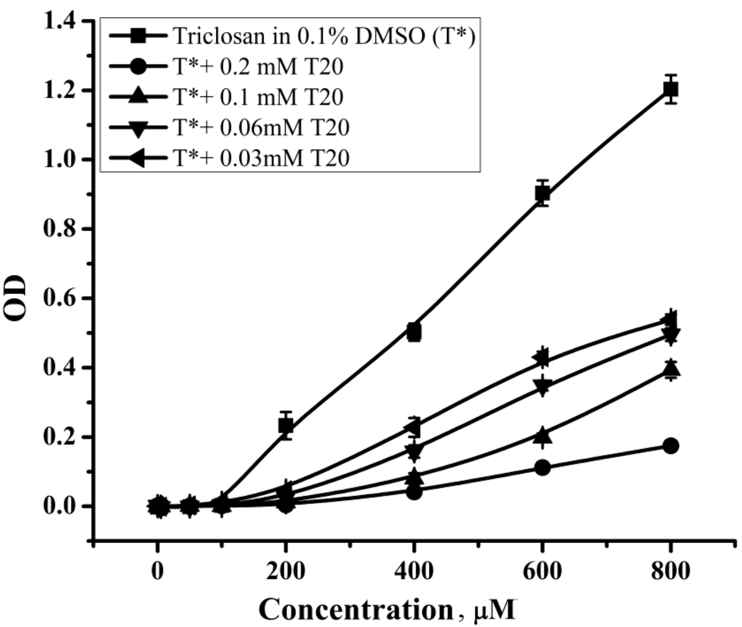



**Figure 5d.** Solubility of triclosan at different concentrations of Tween-20 (T20). X-axis: Intended concentartion of triclosan (µM); Y-axis: OD: Optical density taken at 600 nm

Antibacterial activity of Tween 20 demonstrated that at its lower concentrations, 0.06 mM also its CMC, the $IC_{50}$ value is comparable to that of its blank **(Figure 5b)**. At higher surfactant concentrations of Tween 20, the antibacterial activity of triclosan was compromised again as in case of Tween 80. Above results indicate that although tween-20 can be used for solubilising triclosan (0.03% by weight) in commercial antiplaque applications but it has long been substituted by formulations containing cetyl pyridinium chloride that co-act with triclosan to be more effective give better results [129]. However, above results of Tween-20 may be useful to prepare dilute solutions of triclosan.

**Triton-X100:** This surfactant has 9 to 10 ethoxy units attached to benzene ring, para position of which has four carbon long hydrophobic chain. This is one of very widely used surfactant for the biological work. Mostly used for the separation of proteins from cell membranes [130].

The aqueous solubility of triclosan 0.1% in DMSO improved significantly when Triton-X100 was used at concentrations much lower than its CMC of 0.2-0.9 mM. Only 0.02 mM of surfactant was used to solubilise 400.0 μM of triclosan in LB broth with 0.1 % DMSO. Solubility remained same when surfactant concentration was raised to 0.05 mM of Triton-X100. Decreasing the concentration of surfactant below 0.02 mM decreased the solubility of antibacterial also. **Figure 5e** shows the optimized concentration of Tween-80 as 0.02 to 0.05 mM. Increasing the surfactant concentration (0.2mM) also precipitated the triclosan.

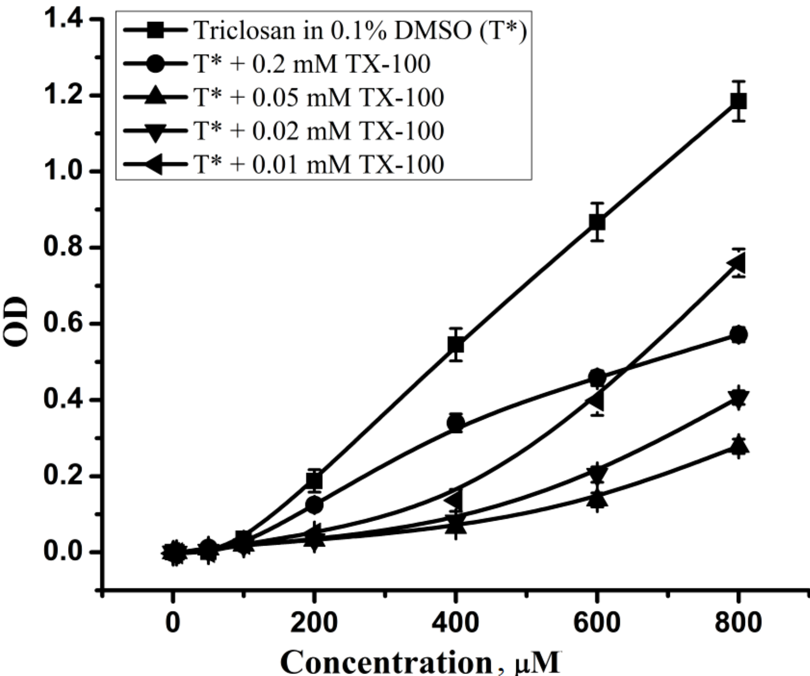



**Figure 5e.** Solubility of triclosan at different concentrations of Triton X-100 (TX-100). X-axis: Intended concentartion of triclosan (μM); Y-axis: OD: Optical density taken at 600 nm

The antibacterial activity of triclosan with 0.02 and 0.05 mM of Triton X-100 used in 0.1% DMSO were comparable to that without any surfactant (**Figure 5b**). $IC_{50}$ value with both concentrations of Triton X-100 was 0.7 μM for triclosan solutions dissolved in LB. $IC_{50}$ value used without surfactant was also same. Higher concentrations of Triton X-100, 0.1 mM to 0.2 mM, were toxic to *E. coli*, in agreement with earlier reports [131, 132]. Thus, Triton-X100, in a

concentration range of 0.05 mM to 0.02 mM, can be used to improve the aqueous solubility of triclosan without inhibiting its antibacterial effect.

**Octyl Glucoside:** Octyl glucoside is the surfactant that has one of the highest CMC values (24.0 mM) chosen for this study. Therefore solubility studies were carried out over a wide concentration range (0.1 mM to 30 mM). Concentrations range of 0.1 mM to 10.0 mM marginally improved the solubility of triclosan in 0.1 % DMSO to 200.0 µM **(Figure 5f)**. Interestingly, high solubility of triclosan in LB medium was obtained when the surfactant was used in the range of its CMC value. A 600 µM solution in 0.1 % DMSO of triclosan was obtained with 30 mM concentration of octyl glucoside.

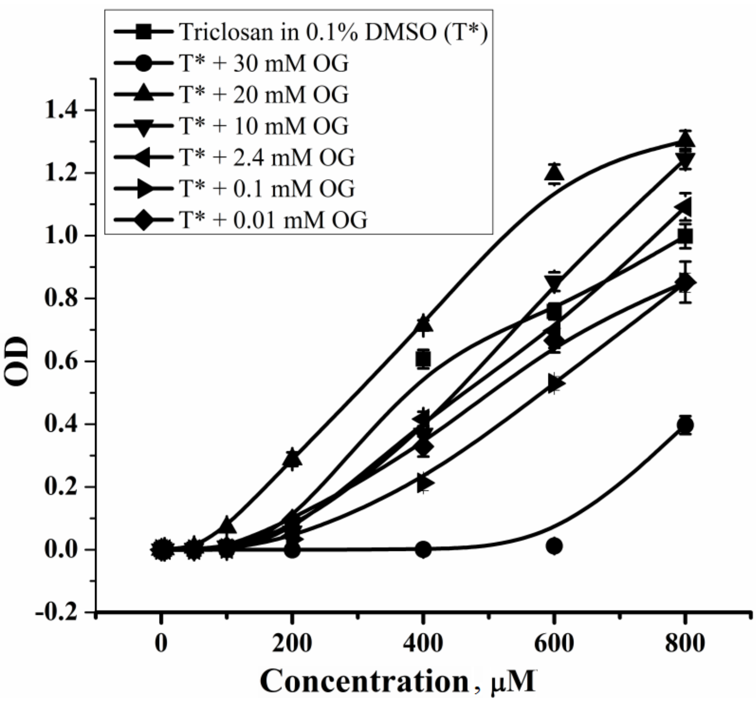



**Figure 5f.** Solubility of triclosan at different concentartions of Octyl Glucoside (OG). X-axis: Intended concentartion of triclosan (µM); Y-axis: OD: Optical density taken at 600 nm

Experiments for the antibacterial activity of triclosan in 0.1 % DMSO against *E. coli* in the presence of 30.0 and 0.5 mM octyl glucoside gave lower $IC_{50}$ values, 67.0 nM and 0.5 µM, respectively, without any surfactant . However blank experiment, without any triclosan, showed that the surfactant was itself toxic to *E. coli*. and therefore not suitable for use in procedures used to assess antibacterial activity of triclosan or its derivatives.

**Sodium deoxycholate :** Sodium deoxycholate, an ionic surfactant with CMC of 2.0-6.0 mM, gave very encouraging results for the solubility studies. It dissolved triclosan up to ~ 800.0 µM

in LB broth with 0.1 % DMSO at 7.0 mM of the surfactant. A 400.0 µM solution of triclosan with0.1 % DMSO was available when 3.5 mM of sodium deoxycholate was used **(Figure 5g)**.

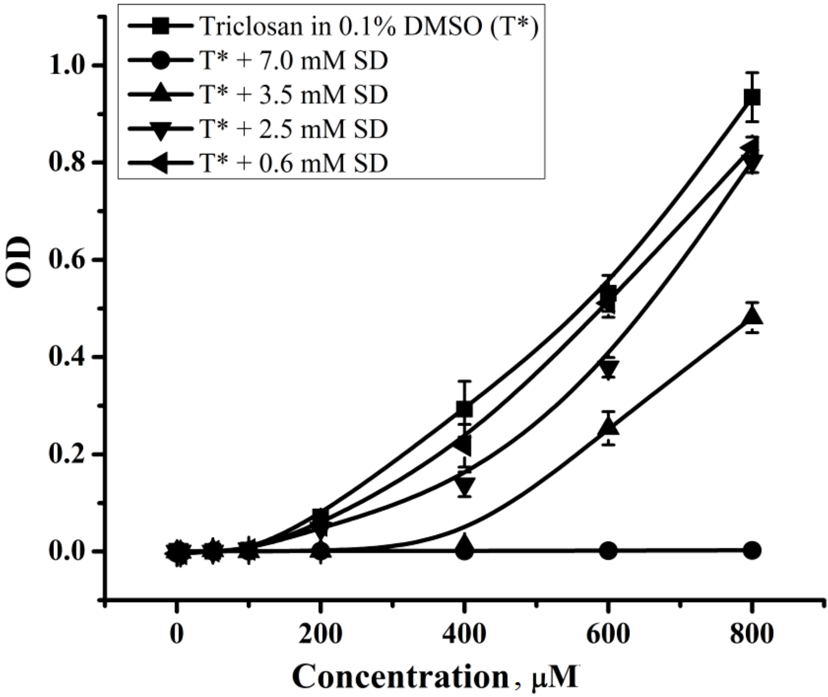



**Figure 5g.** Solubility of triclosan at different concentrations of Sodium Deoxycholate (SD). X-axis: Intended concentartion of triclosan (µM); Y-axis: OD: Optical density taken at 600 nm

However, the surfactant due to its ionic nature was expected to be toxic. *E. coli* cultures at concentrations 0.3 mM and 3.5 mM of sodium deoxycholate with triclosan in 0.1 % DMSO gave $IC_{50}$ values of 0.1 µM and 0.2 µM respectively. **Figure 5b** shows $IC_{50}$ values at 3.5 mM and 7.0 mM concentrations of the surfactants which is lower than that without surfacatnt. Thus, although this surfactant significantly improved the solubility of triclosan, its toxicity at these concentrations prevents its use for assessing the antibacterial potency of triclosan or its analogs.

Results of the studies carried out different surfactant studies to enhance the solubility of triclosan have been summarized in **Table 5.1**. Use of DMSO was necessitated due to its poor aqueous solubility. Literature reports have indicated use of DMSO in different formulations [133,134].

**Table 5.1. Concentrations of surfactants optimized to increases the solubility of triclosan**

| S.No. | Surfactant | Surfactant conc. (mM) to dissolve triclosan (µM) | Effect on $IC_{50}$ of *E. coli* [a] |
|---|---|---|---|
| 1. | Tween-80 | 0.1 (400.0) | 2.3 µM (Increases) |
| 2. | Tween-20 | 0.2 (200.0) | 2.3 µM (Increases) |
| 3. | Triton-X100 | 0.02-0.05 (400.0) | 0.7 µM (No change) |
| 4. | Octyl glucoside | 30.0 (600.0) | 67.0 nM (Decreases) |
| 5. | Sodium deoxycholate | 7.0 (800.0) | 0.2 µM[b] (Decreases) |

a. $IC_{50}$ value of only triclosan dissolved in 0.1% DMSO = 0.7 µM,
b. $IC_{50}$ value at 3.5 mM of surfactant.

In conclusion, Triton X-100 solubilised 400.0 µM of triclosan in 0.1% DMSO at very low surfactant concentrations without inhibiting antibacterial activity. Rest of the chosen surfactants although augmented the solubility of triclosan up to 800.0 µM but obstructed its activity.

**Figure 5a** shows 80 and 20 oxyethylene group in case of tween-80 and tween-20 respectively. Strong interactions between large number of polyoxyethylene groups and hydroxyl group of triclosan inhibited the activity of triclosan due to unavailability of the antibacterial to microorganisms [102,135]. Very less number of oxyethylene groups, only 9 to 10, in case of Triton X-100, interact weakly with triclosan and make triclosan available in aqueous solution to exert effect on the microorganisms. Unfortunately, octyl glucoside and sodium deoxycholate solublized triclosan at very high concentrations. These concentrations are known to kill microorganisms[136].

## 5.3 Materials and Methods

### 5.3.1 Surfactants

Surfactants tween-80, tween-20, triton X-100, octylglucoside, sodium deoxycholate and solvent dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich. Antibacterial agent triclosan was procured from S.D Fine Chemicals (Mumbai, India). Luria Bertani broth, Miller (LB broth) for the cultivation of bacterial strains was obtained from Himedia Laboratories (New Delhi, India). The *Escherichia coli* K-12 bacterial strain (catalogue number MTCC:1302), used in this study was obtained from the Microbial Type Culture Collection and Gene Bank facility housed at the Institute of Microbial Technology (IMTECH), Chandigarh, India. All spectroscopic

measurements were made on a double-beam UV-Vis spectrophotometer (Specord-205). All experiments were performed in triplicate.

### 5.3.2 Determination of extent of solubility using surfactants

Solubility of triclosan in LB broth was studied in the presence of the selected surfactants- tween-80, tween-20, triton X-100, octylglucoside and sodium deoxycholate **(Figure 5g)** at 25$^{o}$C. First, a 10 mM stock solution of triclosan was made in DMSO which was serially diluted with the same solvent. Surfactant stocks of the desired concentrations (around their CMC values) were made in LB broth. The serial dilutions of triclosan made above were dissolved in LB broth containing desired concentrations of surfactants so that the resulting concentration of triclosan ranged between 0.0-800.0 μM and that of DMSO was 0.1%. The solubility was assessed by monitoring light scatter at 600 nm using a spectrophotometer. The optical density at 600 nm was plotted against concentration of triclosan and increase in the absorption value above baseline was attributed to increase in turbidity due to precipitation of triclosan in LB broth. Appropriate controls (solution without surfactant, solution without triclosan) were employed for each surfactant.

### 5.3.3 Determination of $IC_{50}$ Value of Triclosan

$IC_{50}$ values of triclosan in *Escherichia coli* K12 were determined by broth dilution assay in the presence of various surfactants. A 10 mM stock solution of triclosan was made in DMSO and serially diluted in DMSO. *E. coli* K12 strain was freshly streaked on LB agar plates from a glycerol stock maintained at −70˚C. After 24 hours at 37 °C, a single colony was picked up from the plate, inoculated into 5.0 mL of LB broth and cultured at 37˚C with shaking for 12 hours. Fresh LB broth was then inoculated with one percent of this primary culture and cultured at 37˚C with shaking until the culture reached mid log phase. This mid log phase cell suspension (100.0 μL) was used to inoculate each tube containing 10 ml of LB broth along with different concentrations of triclosan (and a resulting DMSO concentration of 0.1%) as well as the required concentration of surfactant and incubated at 37˚C. Growth of *E. coli* K12 was measured after 8 hours by light scatter at 600 nm using a spectrophotometer. The absorbance values were normalized to control experiments performed with only triclosan or only surfactant added to LB broth and inoculum. Data were fitted to a sigmoidal curve by nonlinear curve fitting using Origin-8 software and the value of $IC_{50}$ determined by calculating the concentration of the compound which inhibited bacterial growth by 50%.

## Future Prospectives

Diphenyl ethers have emerged as potential inhibitors of Fab I and thus future antibacterials. Their scope for application in pharmaceutics has been narrowed down by recent discovery that effectiveness of triclosan is severely compromised by the presence of exogenous fatty acids of the host. Nevertheless their scope as non-pharmaceuticals antibacterial agents is unquestionable. Now a day there is a lot of demand for antibacterial agents for surface disinfection. For example remotes, mobiles, laptop surfaces, tiles, mirrors etc. The compound synthesised in the thesis, including diphenyl amines can be used to develop solutions to disinfect the surfaces.

## Clarifications during viva-voce examination

### i) Difference between absorbance and optical density

**Absorbance:** Physical process of absorbing light is termed as absorption. Mathematically absorbance is the ratio of transmitted light over incident light.

$$A_\lambda = \log_{10} (I_o / I)$$

Where $I_o$ is the intensity of incident light and I is the intensity of transmitted light. When light is irradiated on a sample some part of incident light is absorbed (absorption) by the dispersed medium and rest of the incident light is transmitted. Since absorbance is the ratio of two quantities it does not have any units. Therefore it is reported as absolute units or AU.

**Optical density:** It is the absorbance per unit length. Mathematically

$$OD_\lambda = A_\lambda / l$$

Where $A_\lambda$ is the absorbance at a particular wavelength ($\lambda$) and $l$ is the path length. So the units of optical density are AU $cm^{-1}$. The higher the optical density lower will be the transmittance as absorbance will be increased.

### ii) Reason for not writing the standard deviation in biological activity

The method for the determination of MIC (Minimum inhibitory concentration) value was based on visible color change due to formation of formazan of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) with dead cells. All though all the experiments (section 2.6.2, 3.6.2, 4.6.2) were done in triplicate the visible color change was observed (transparent to pink) at same concentrations of the drug molecules. Therefore no standard deviation was mentioned.

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# FIGURE: SPECTRAL DATA OF SYNTHESIED COMPOUNDS

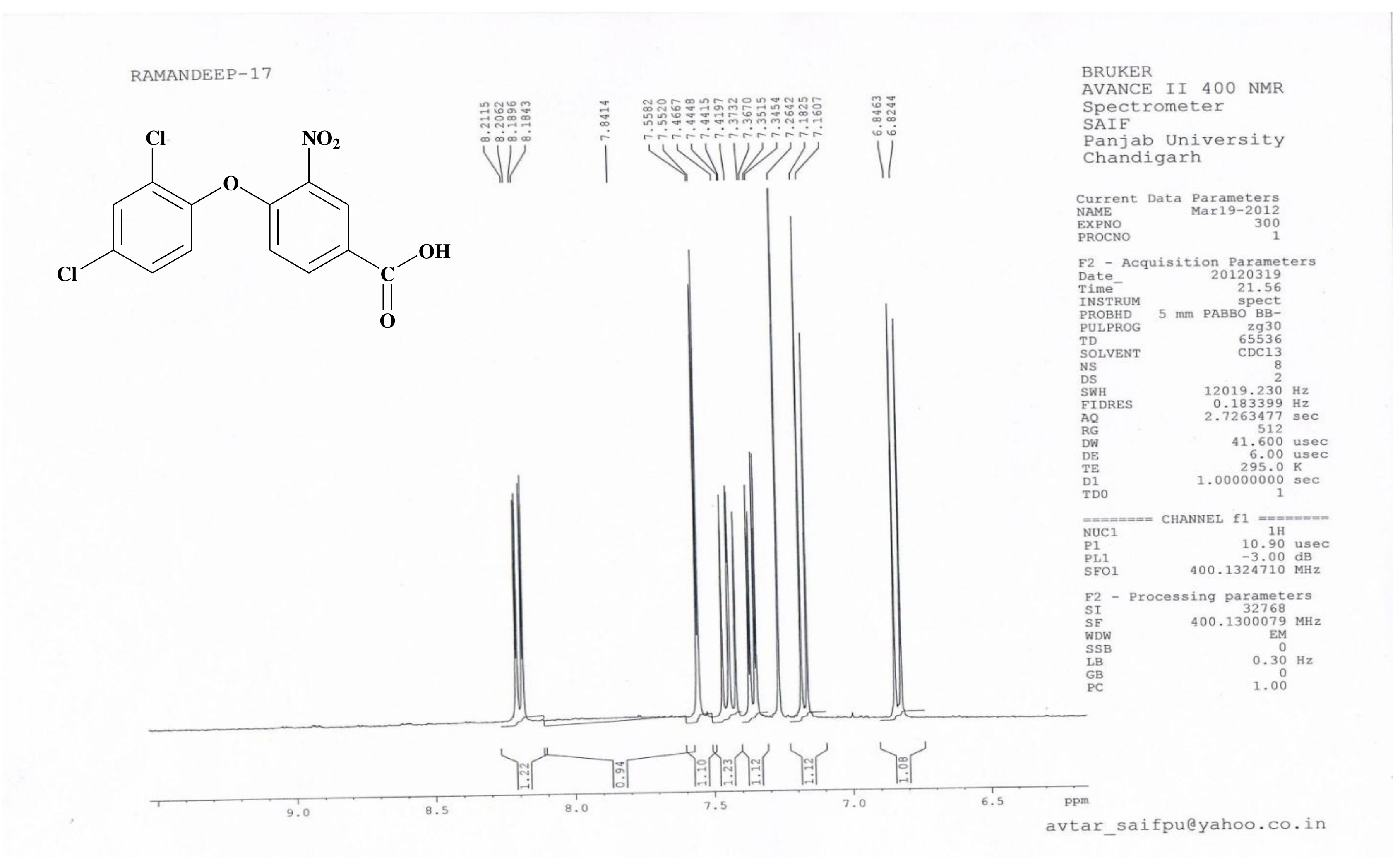


**Figure 2A (a): $^1$H NMR data of compound 2.5**

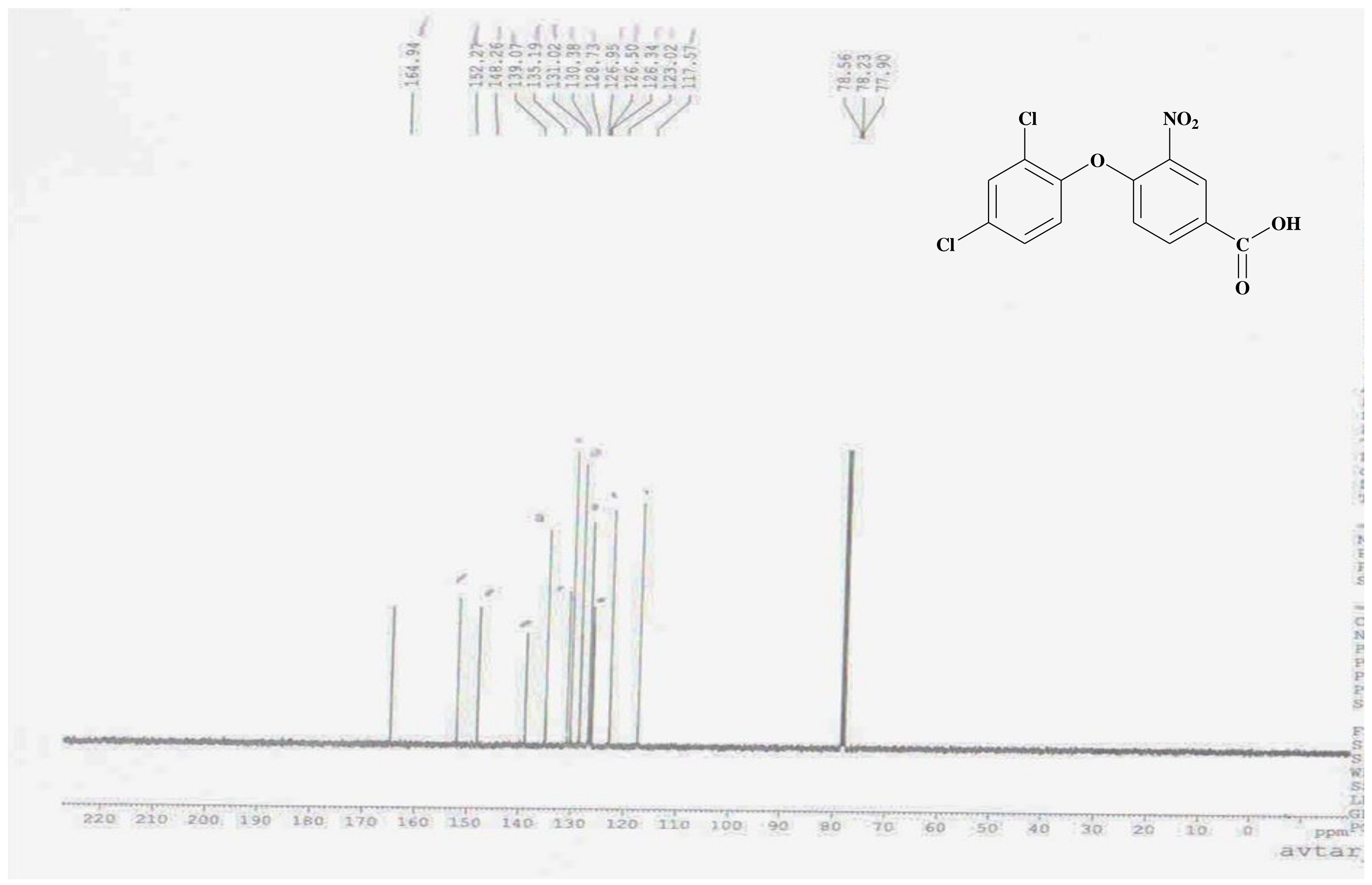


Figure 2A (b): $^{13}$C NMR data of compound 2.5

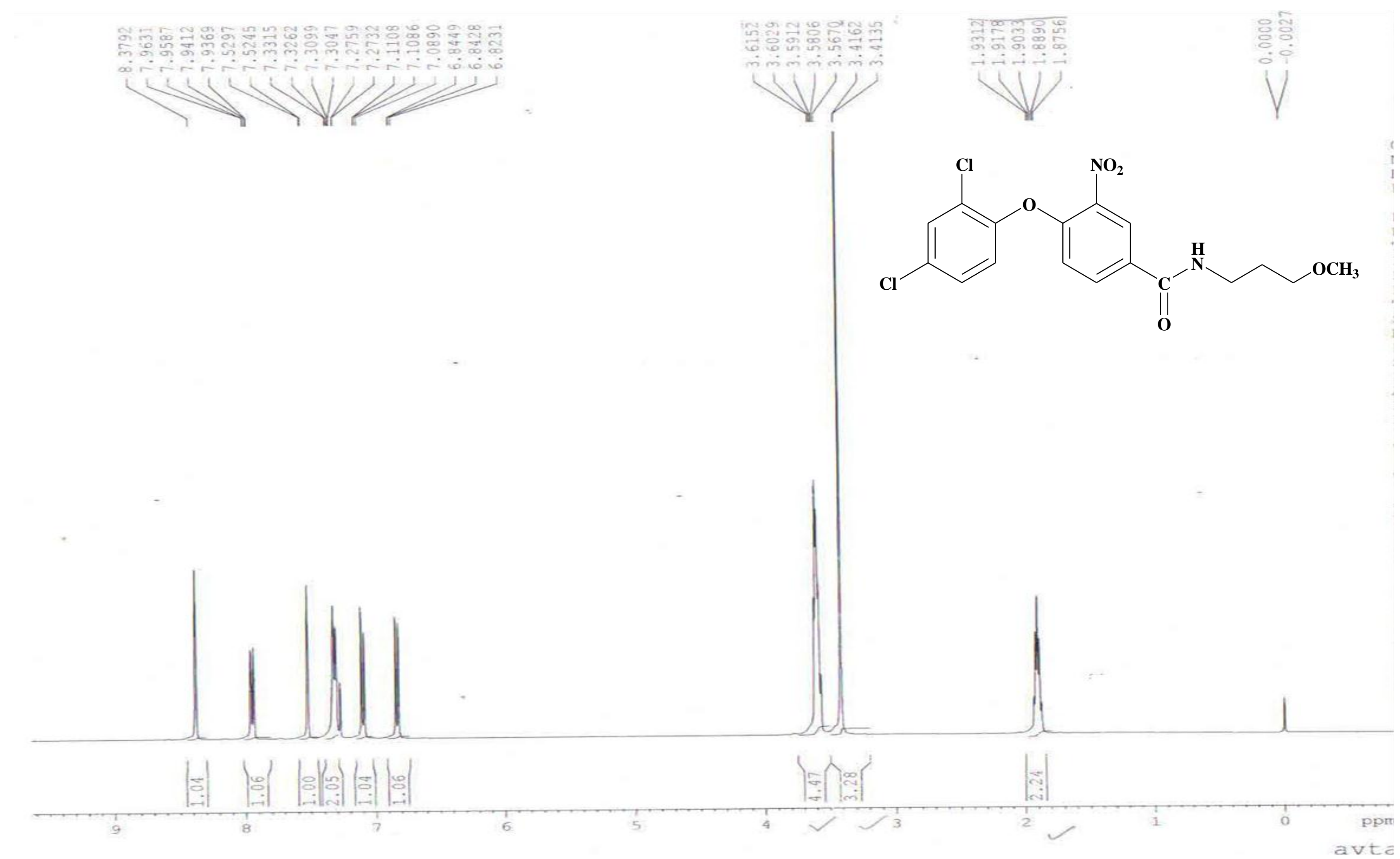


**Figure 2B (a): $^1$H NMR data of compound 2.8**

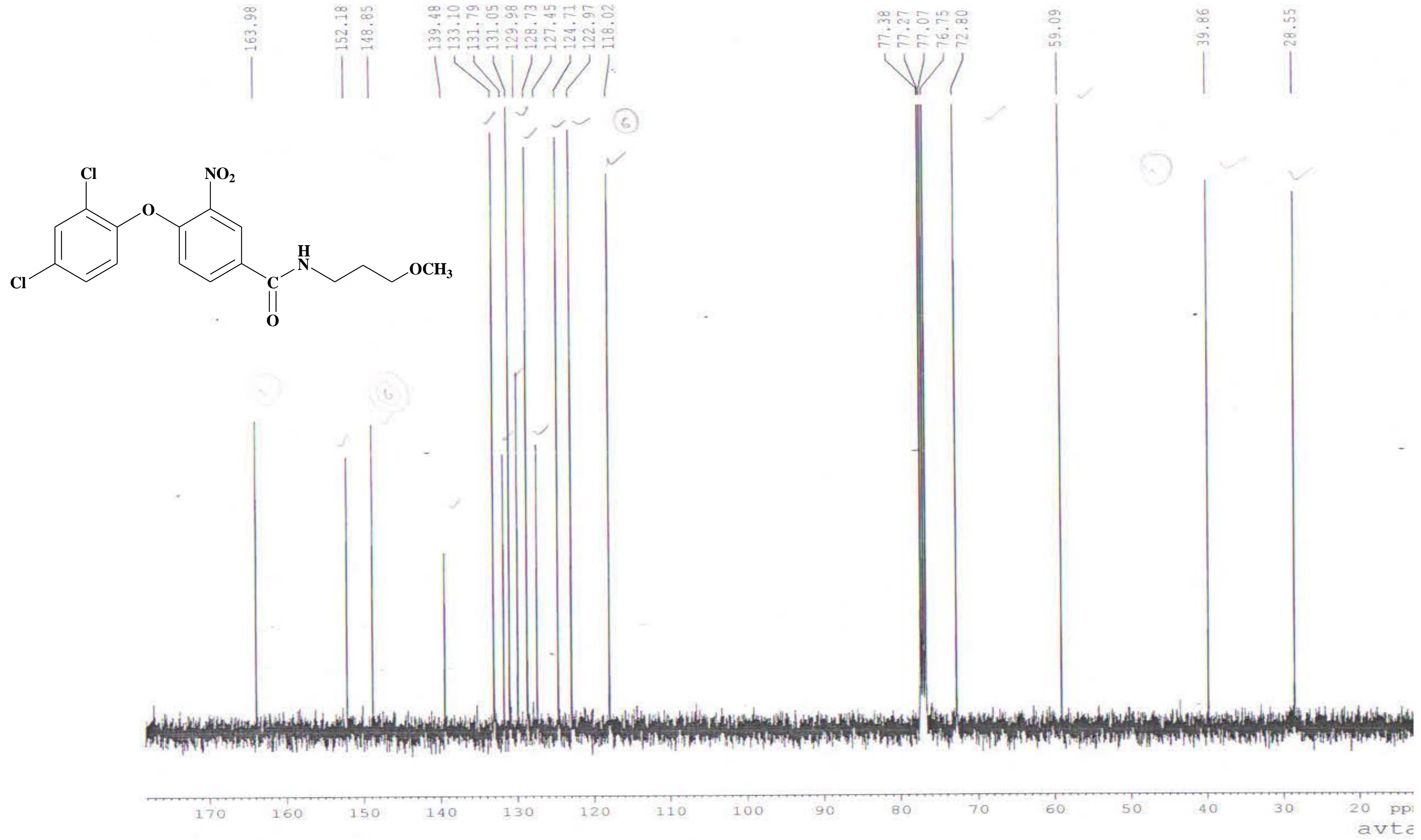


**Figure 2B (b): $^{13}$C NMR data of compound 2.8**

**Figure 2C (a): $^1$H NMR data of compound 2.9**

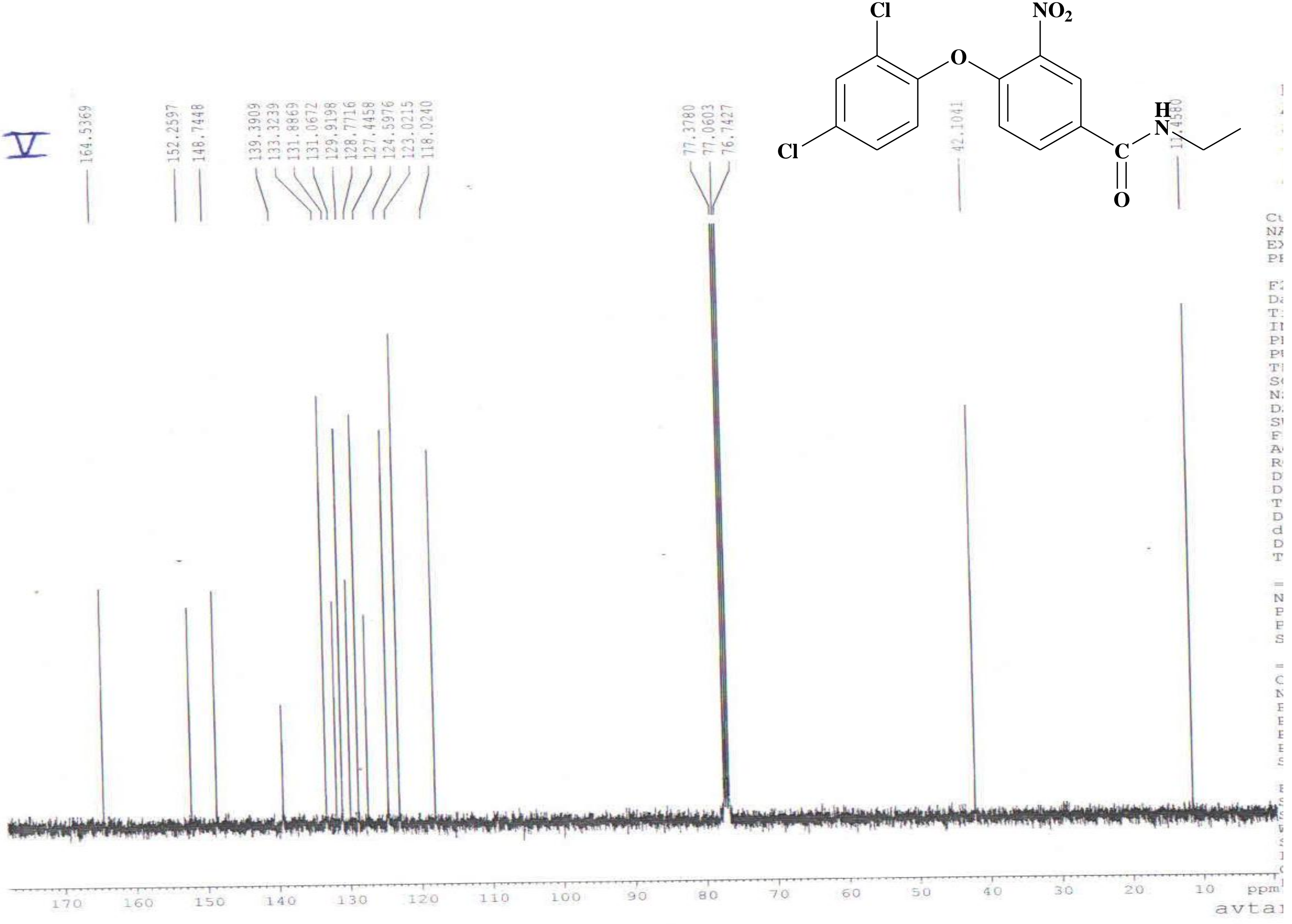


**Figure 2C (b):** $^{13}$**C NMR data of compound 2.9**

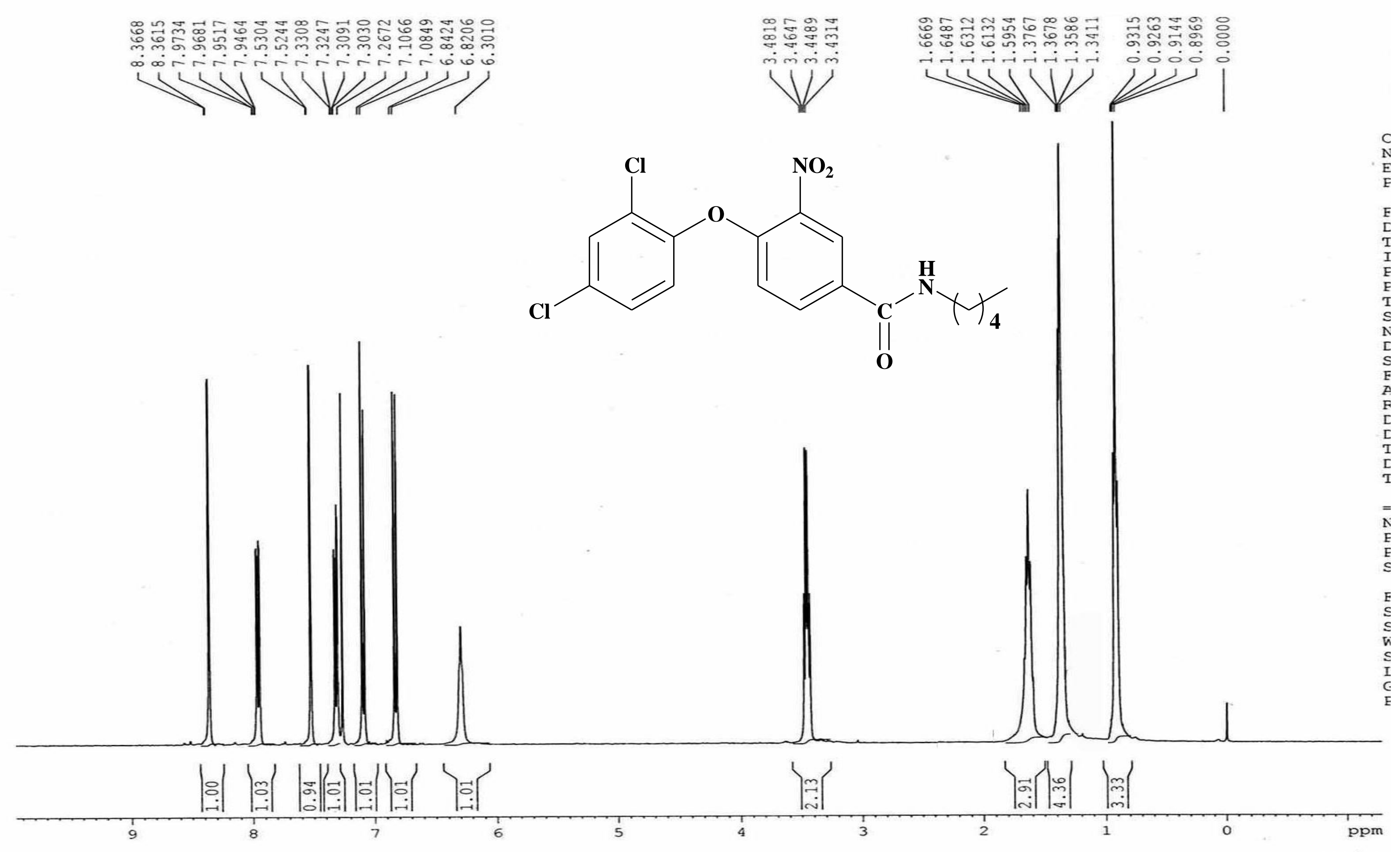


**Figure 2D (a): $^{1}$H NMR data of compound 2.12**

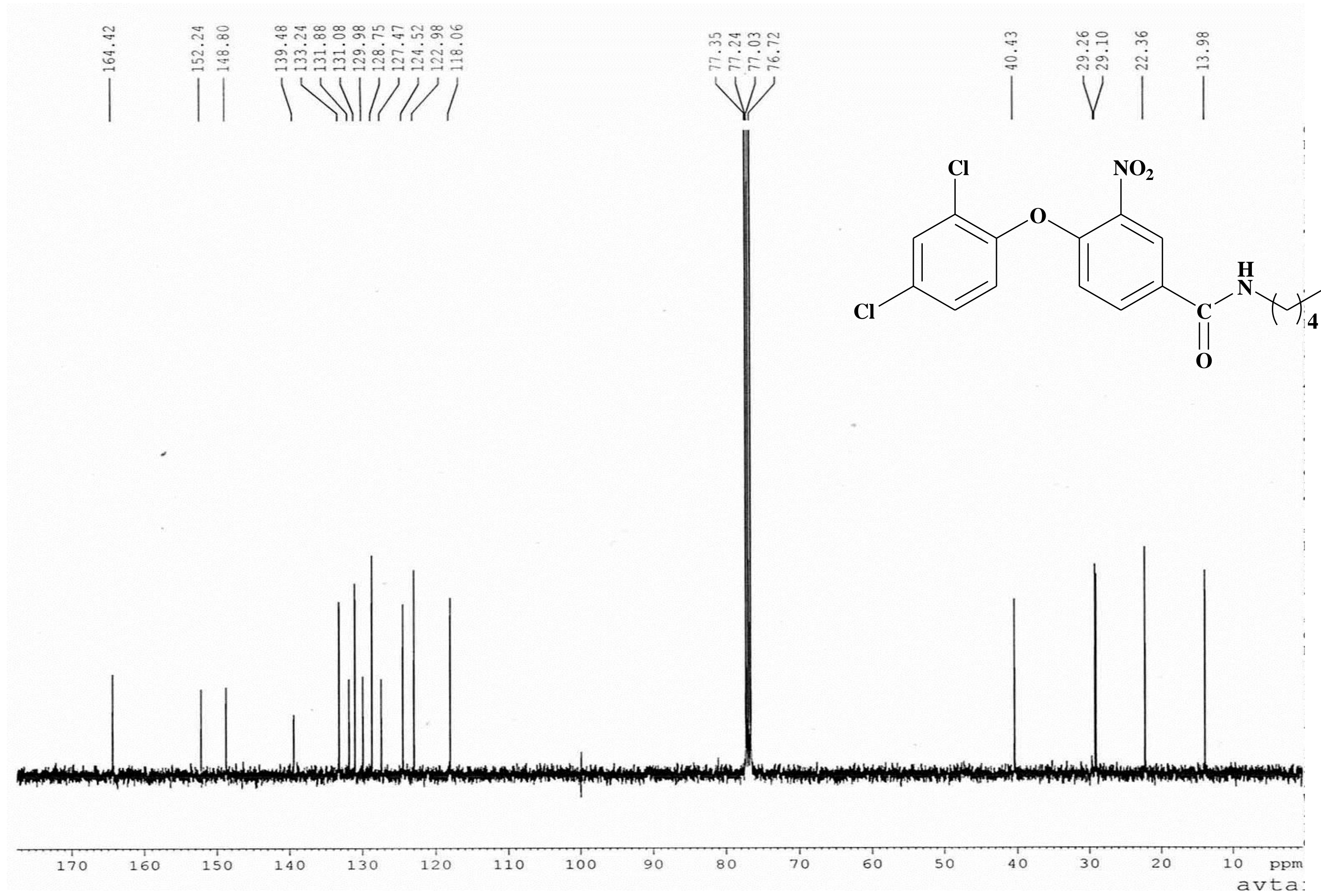


**Figure 2D (b): $^{13}$C NMR data of compound 2.12**

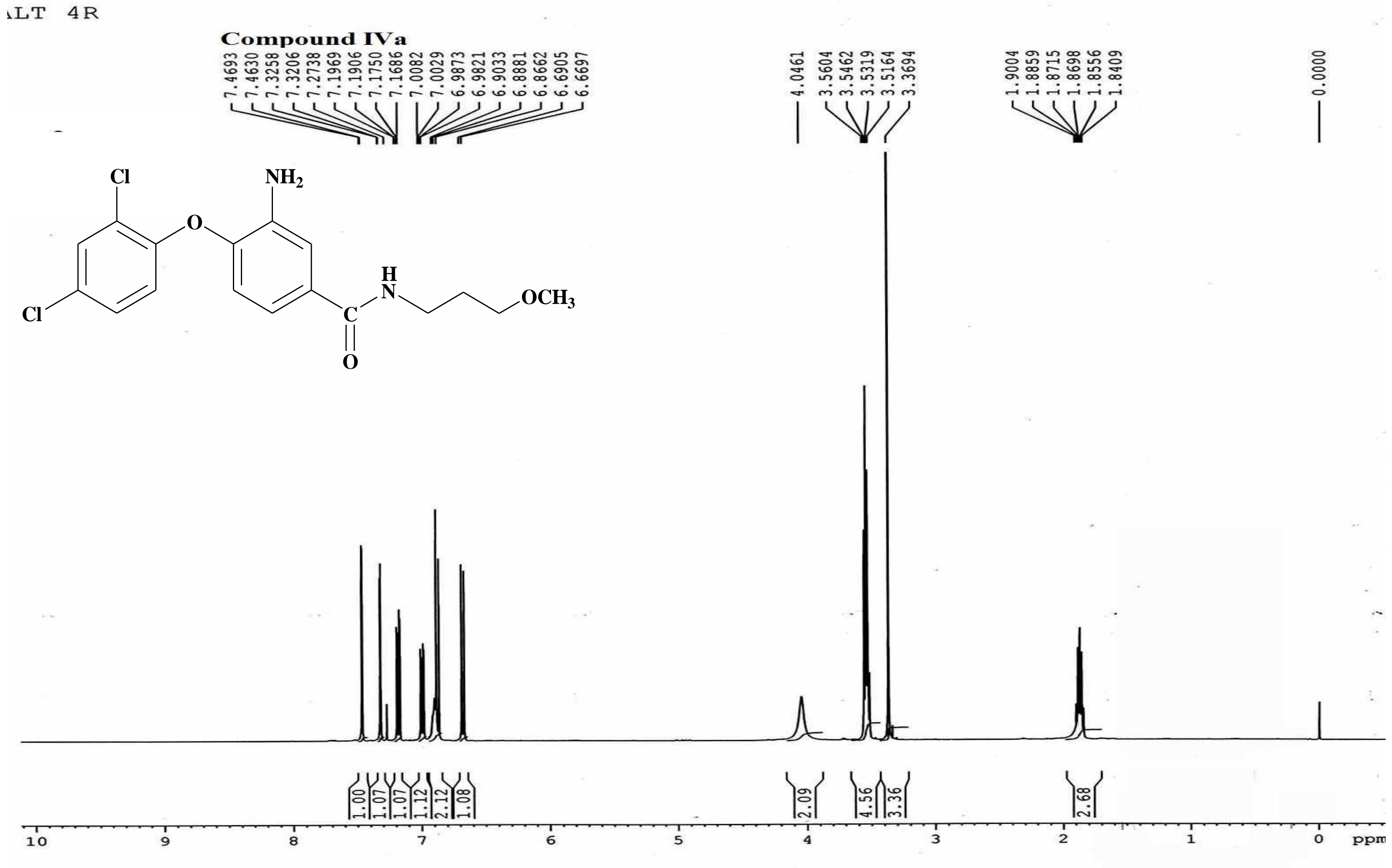


**Figure 2E (a): $^{1}$H NMR data of compound 2.15**

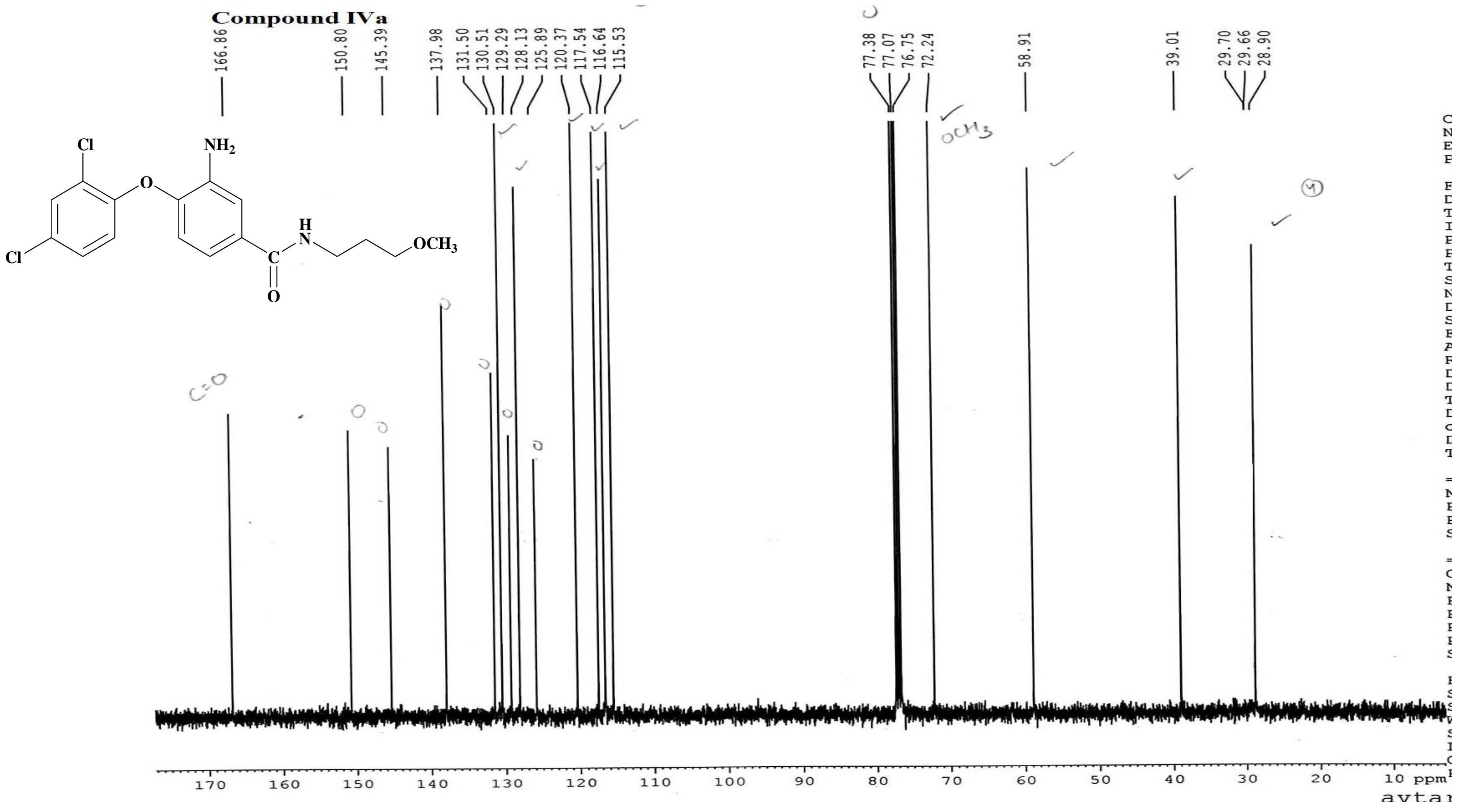


**Figure 2E (b): $^{13}$C NMR data of compound 2.15**

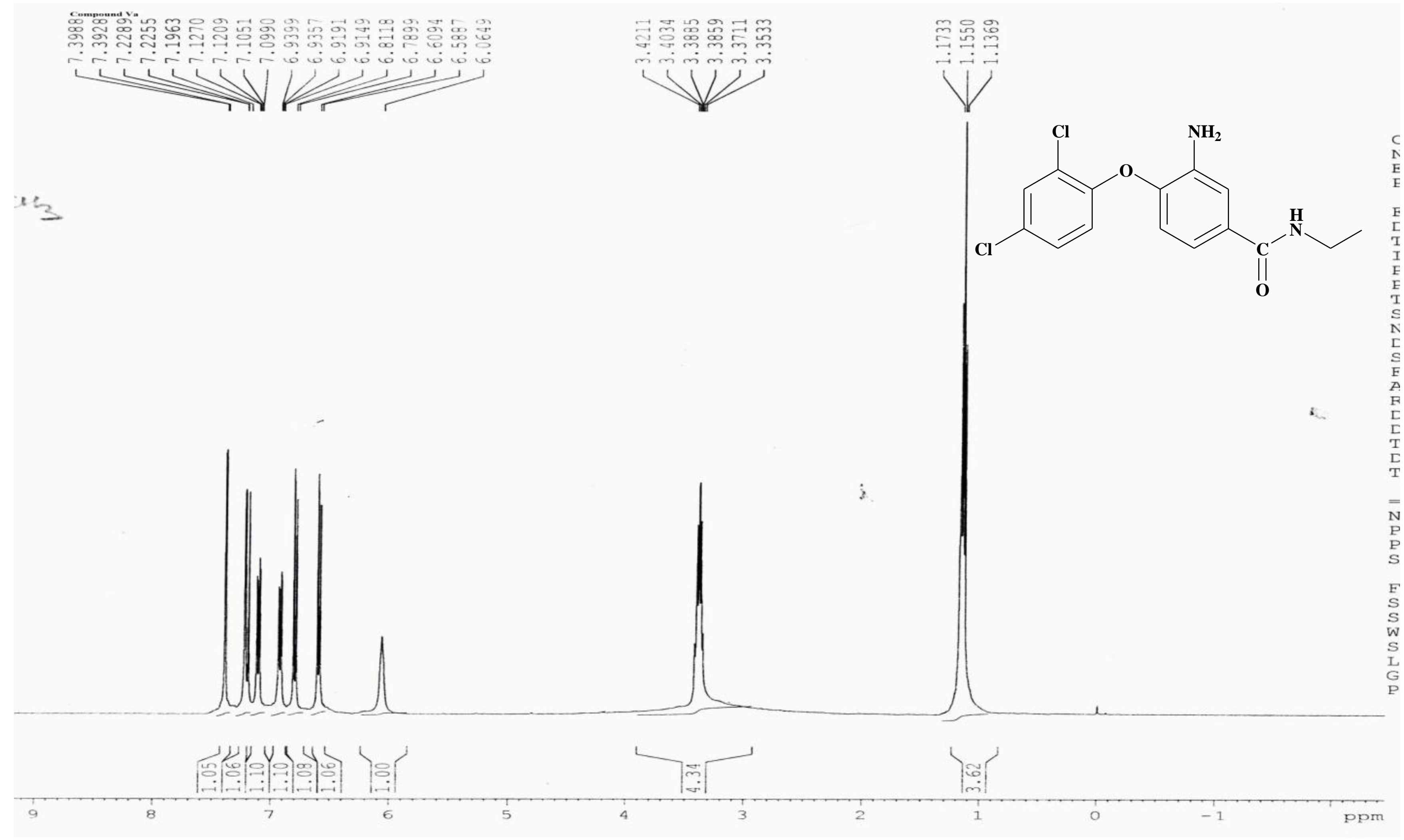


**Figure 2F (a): $^{1}$H NMR data of compound 2.16**

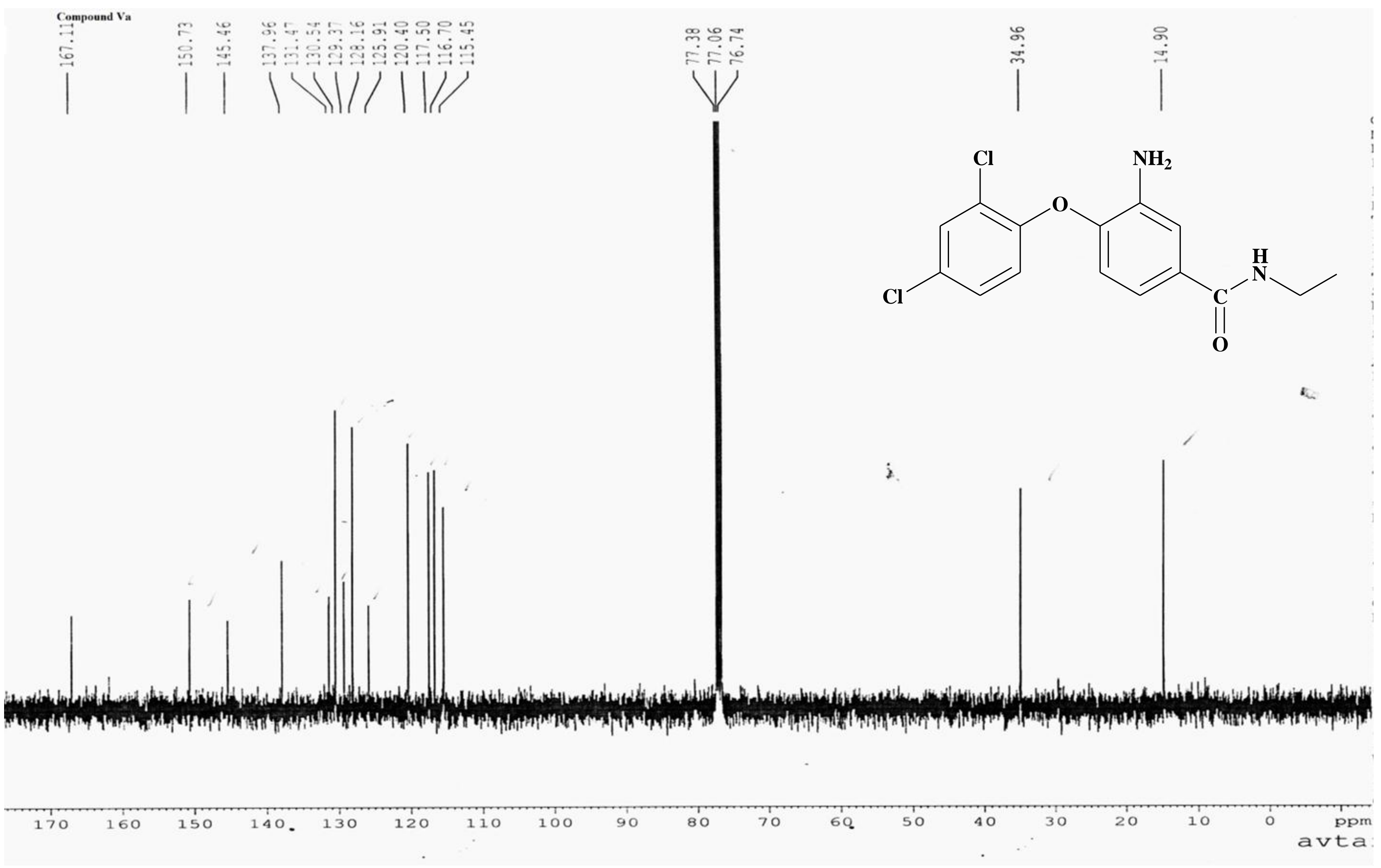


Figure 2F (b): $^{13}$C NMR data of compound 2.16

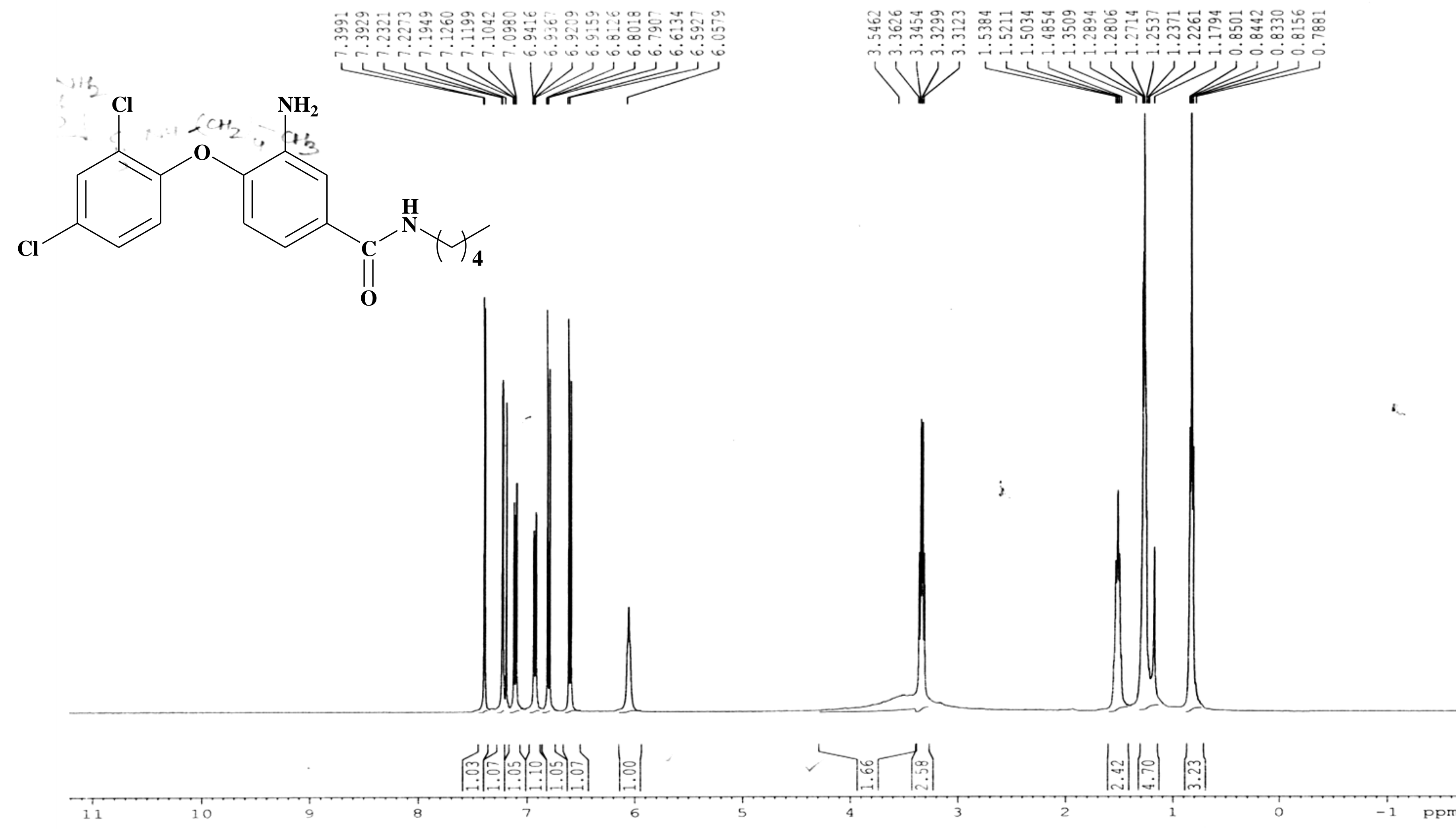


**Figure 2G (a): $^{13}$H NMR data of compound 2.19**

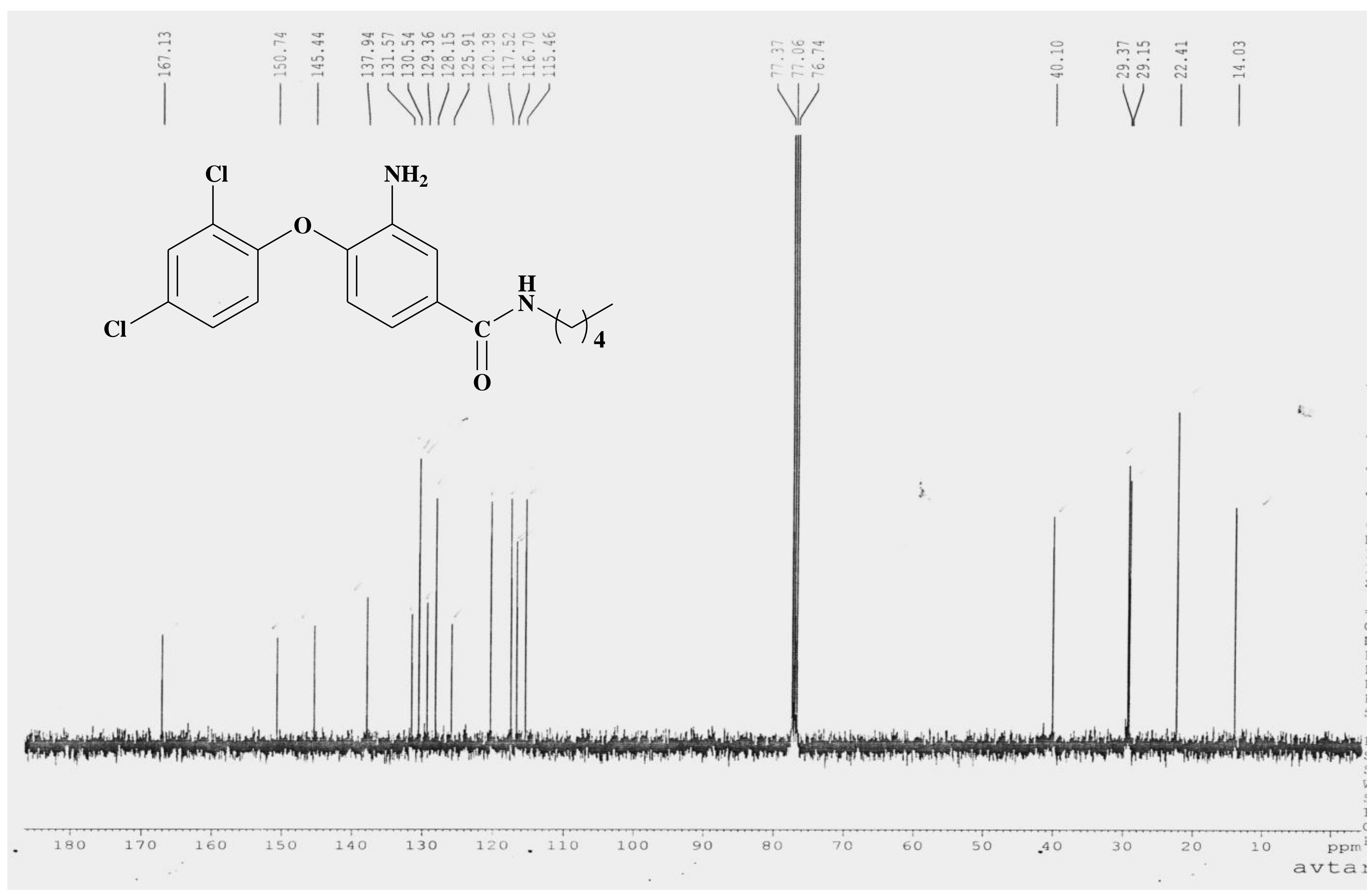


**Figure 2G (b): $^{13}$C NMR data of compound 2.19**

**Figure 3A (a): $^{1}$H NMR data of compound 3.9**

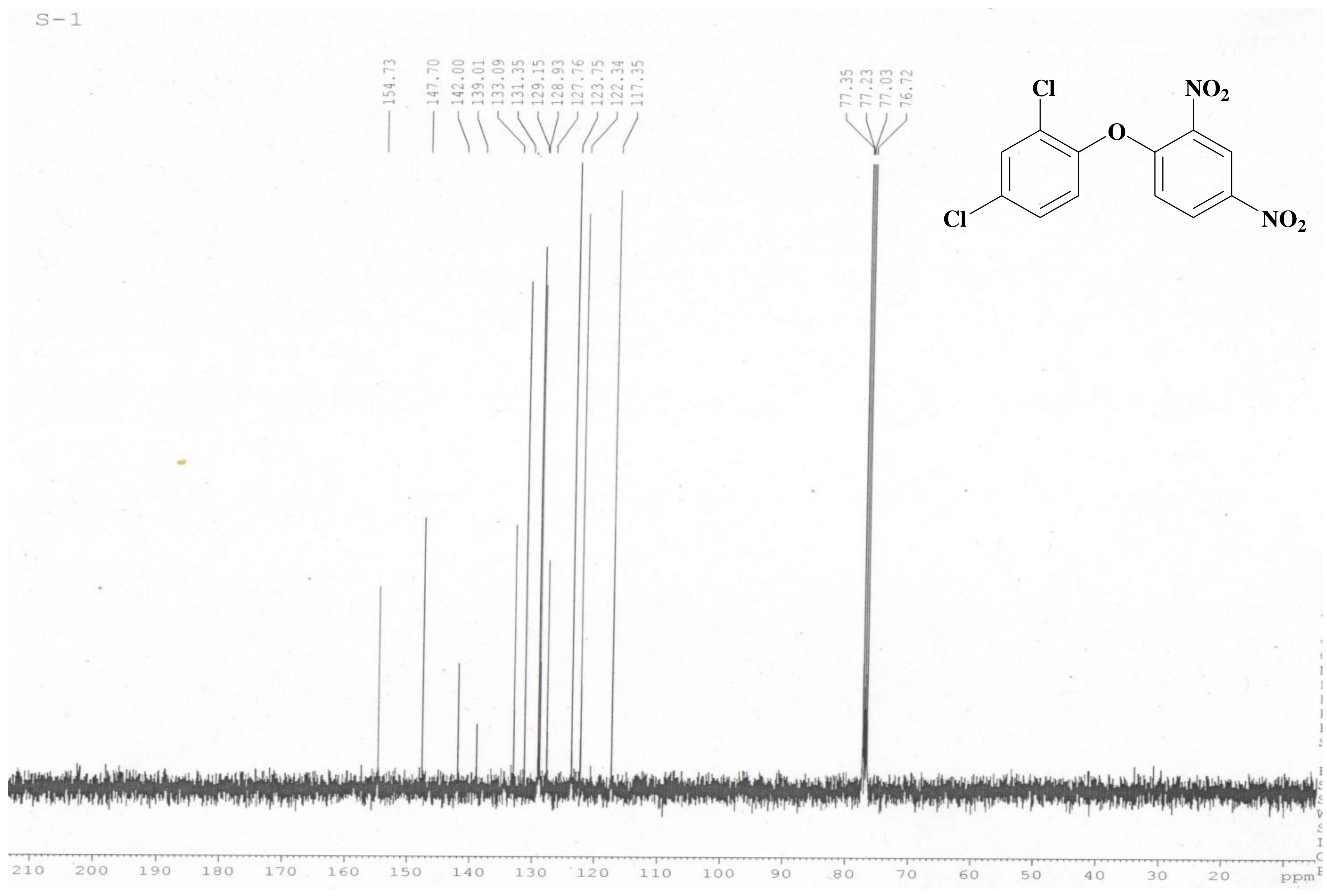


**Figure 3A (b): $^{13}$C NMR data of compound 3.9**

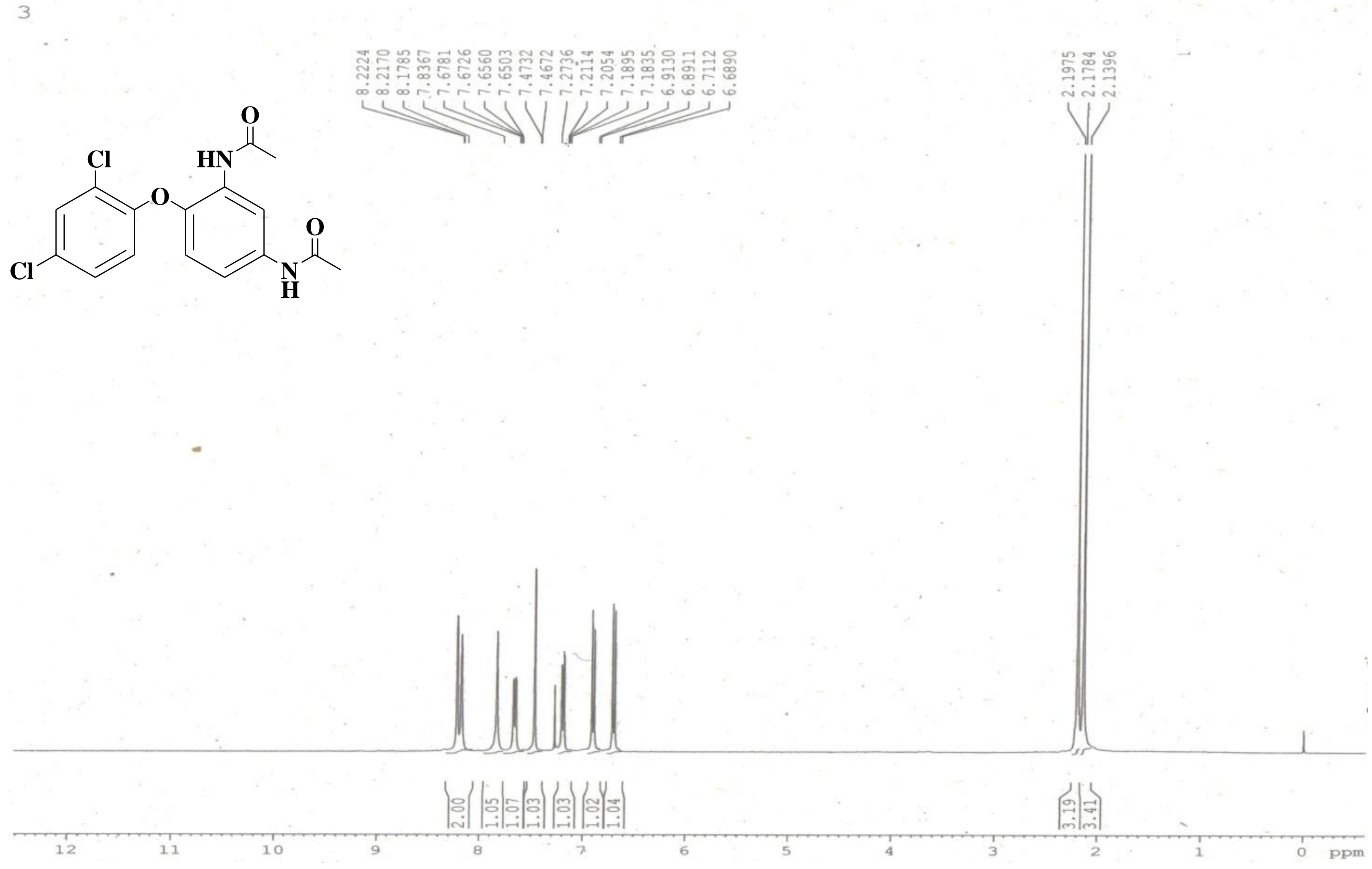


**Figure 3B (a): $^{1}$H NMR data of compound 3.11**

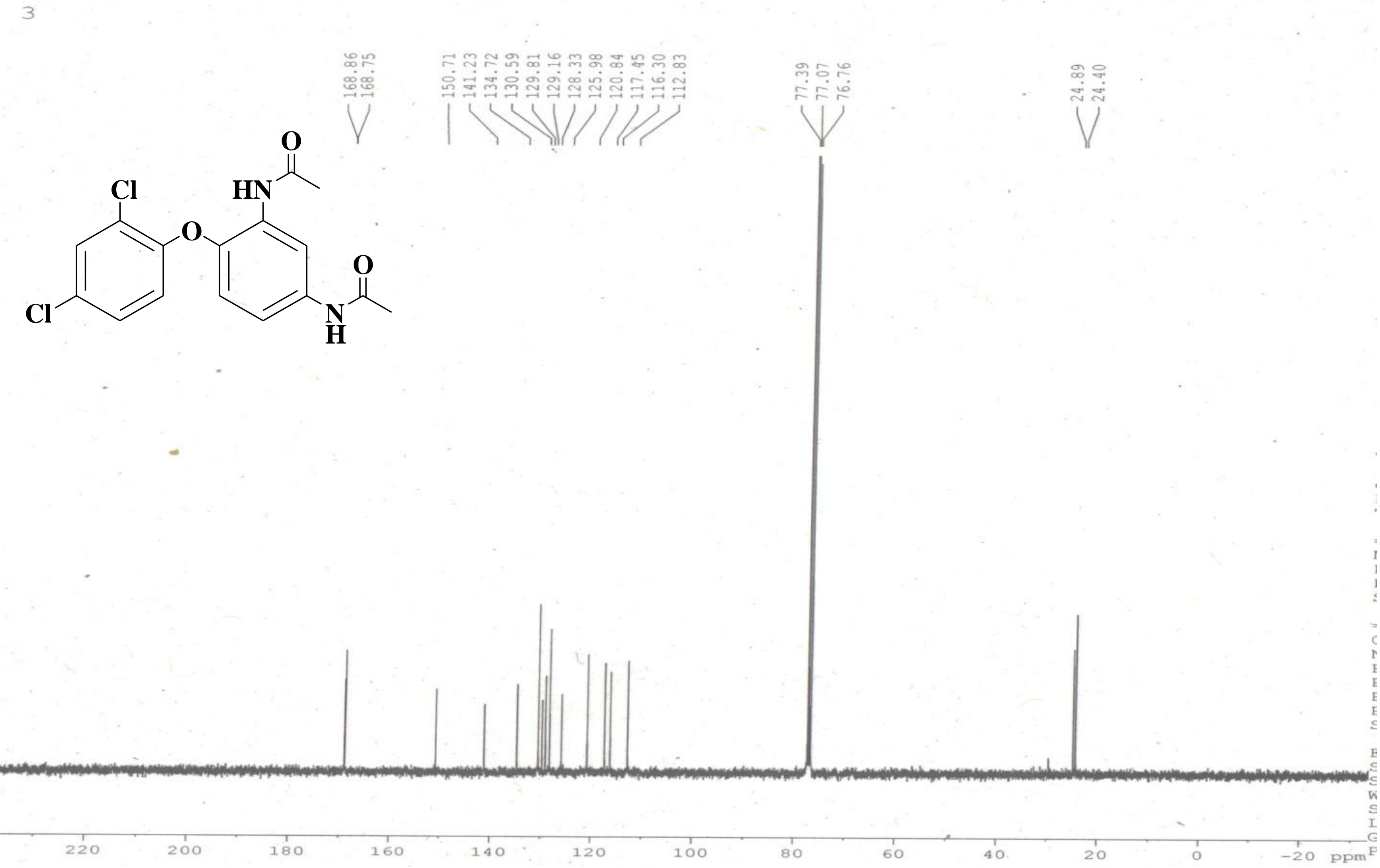


**Figure 3B (b): $^{13}$C NMR data of compound 3.11**

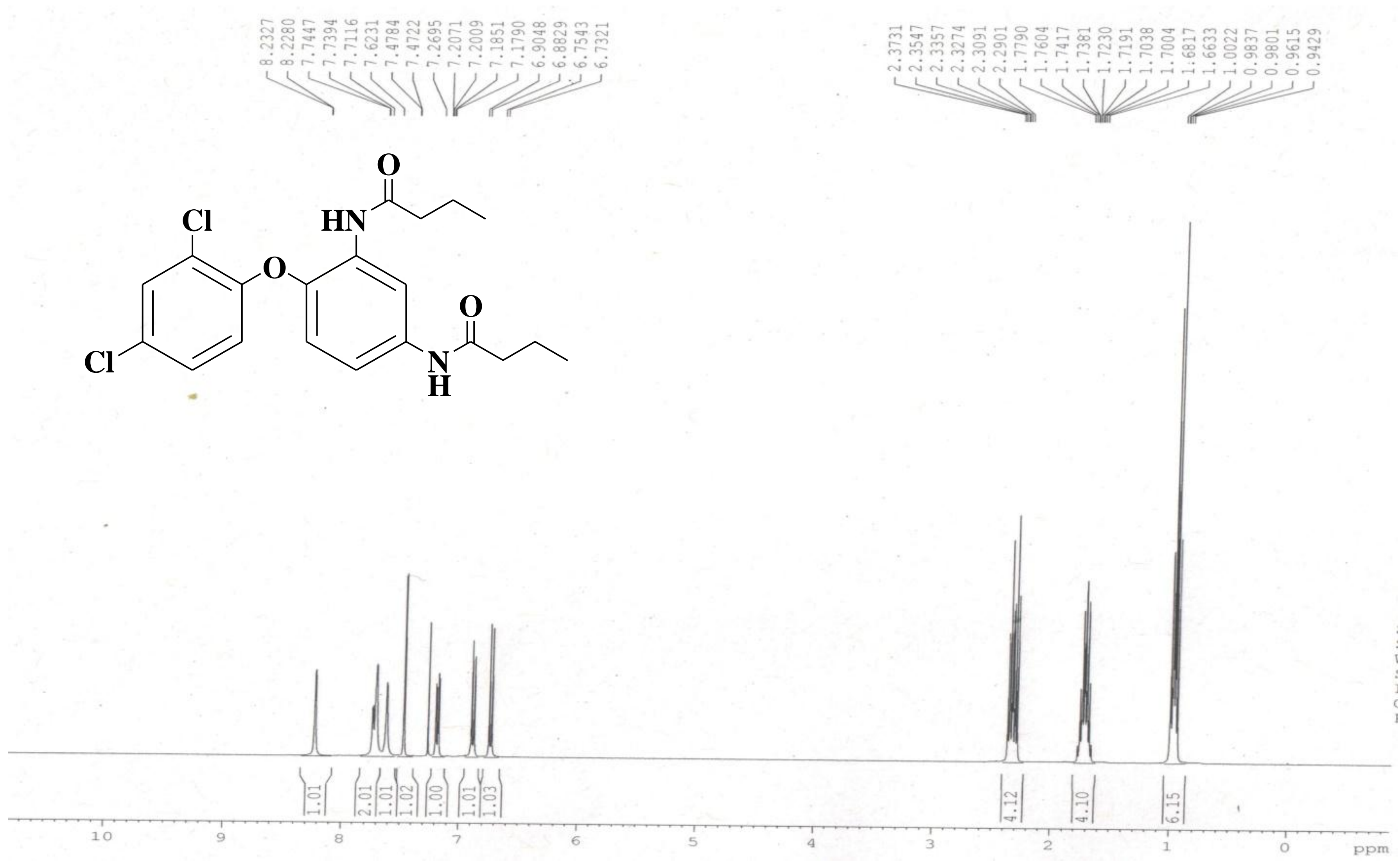


**Figure 3C (a): $^{1}$H NMR data of compound 3.13**

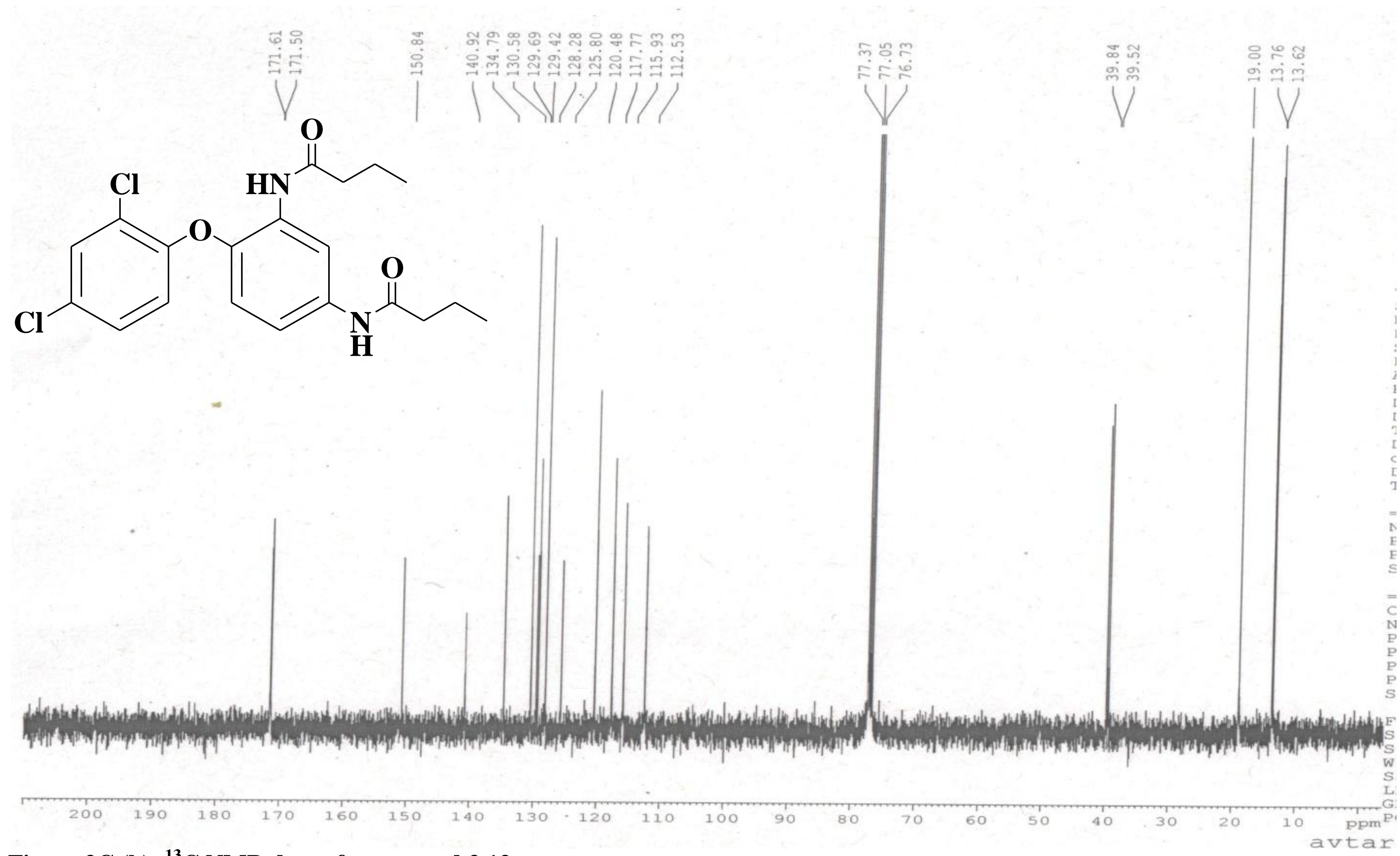


**Figure 3C (b): $^{13}$C NMR data of compound 3.13**

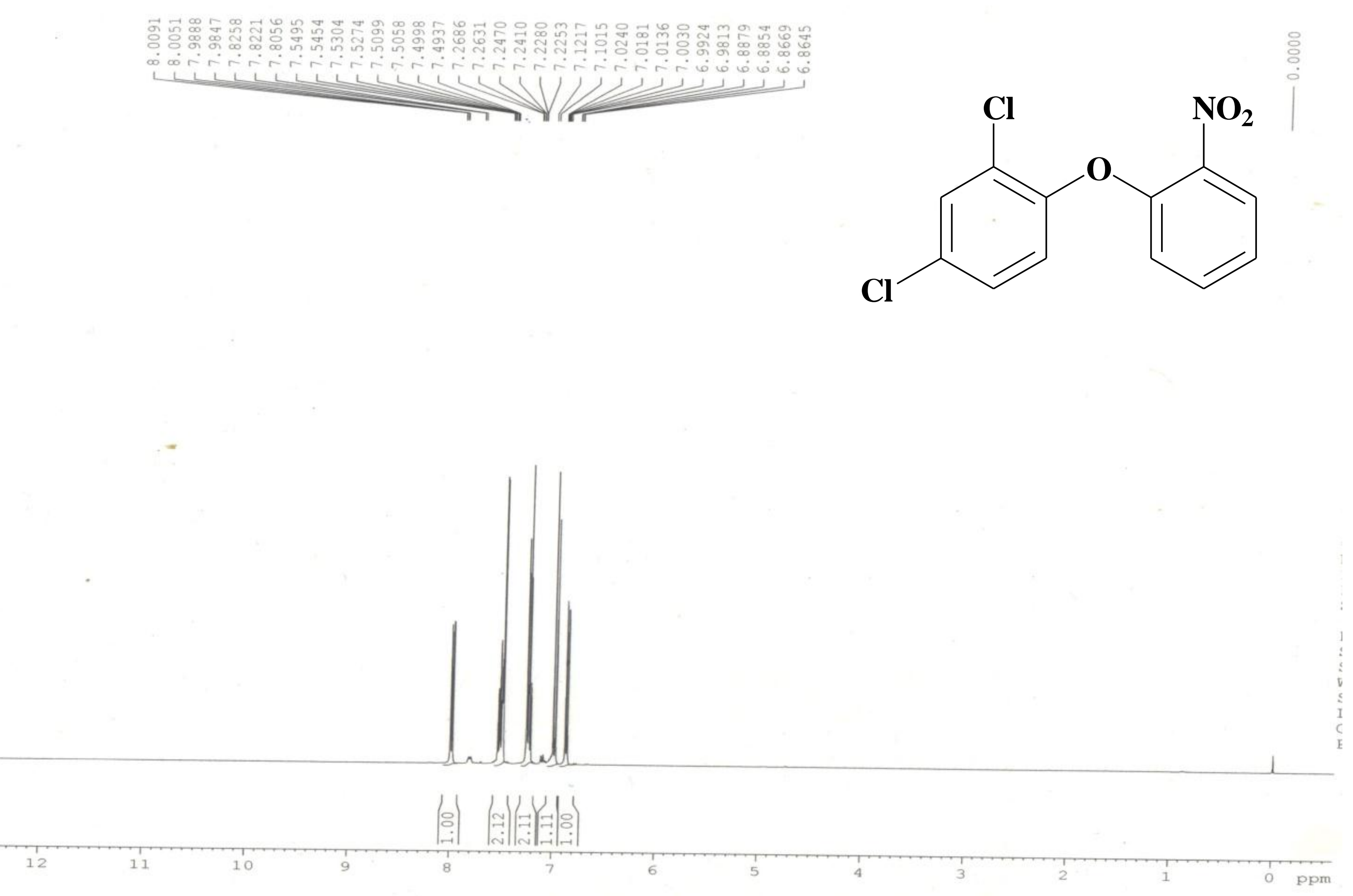


**Figure 3D (a): ^{1}H NMR data of compound 3.19**

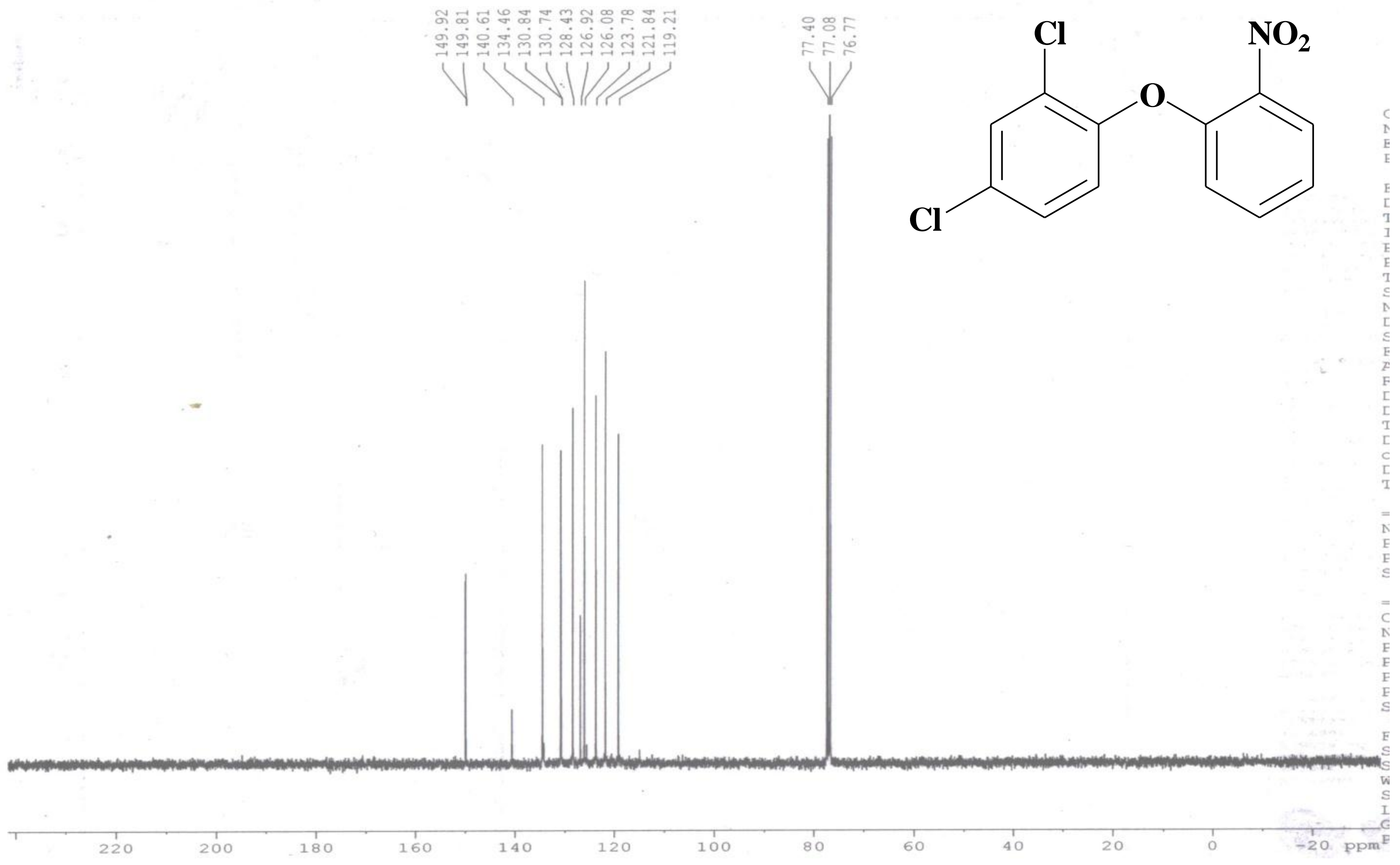


**Figure 3D (b): $^{13}$C NMR data of compound 3.19**

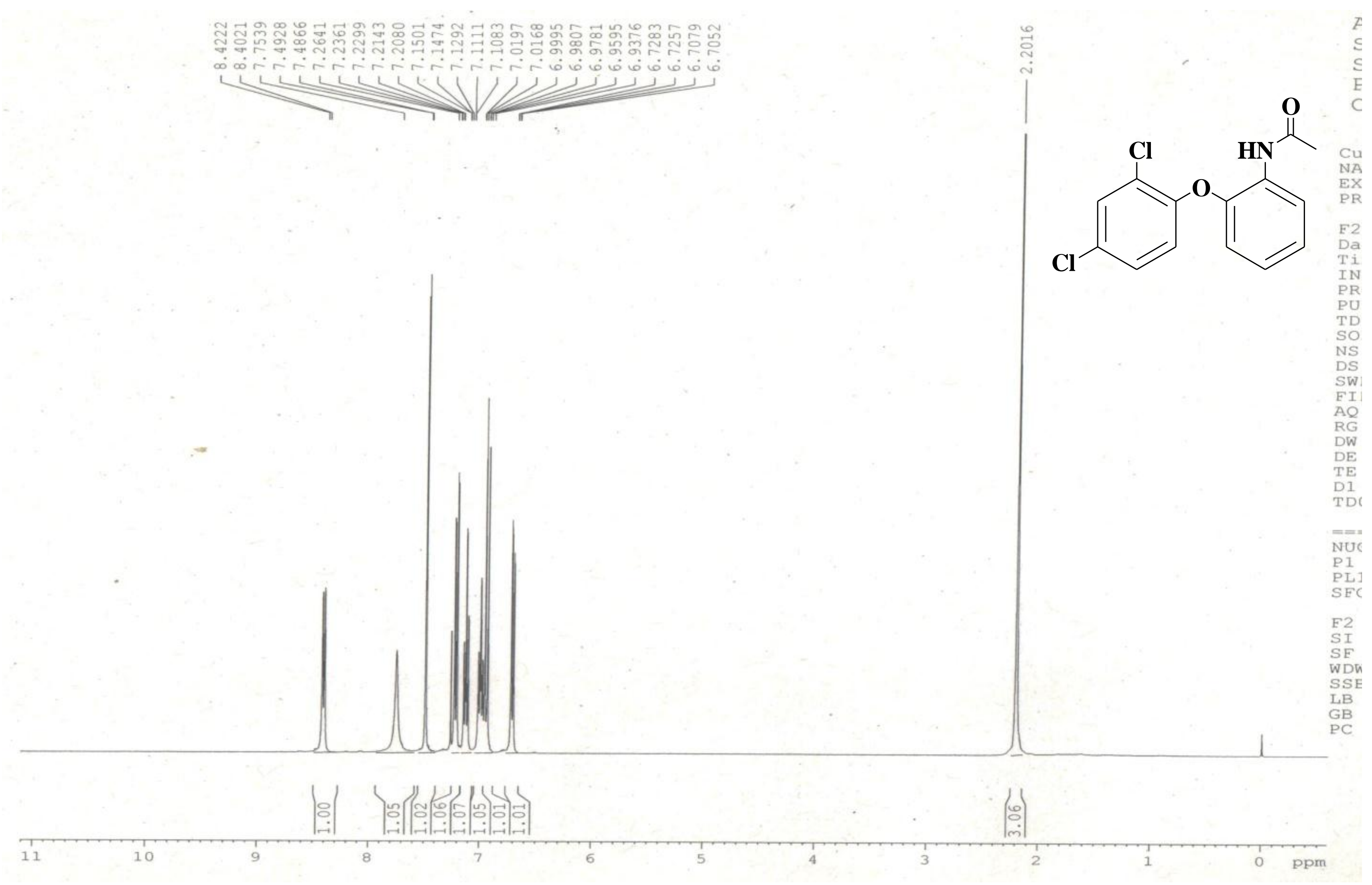


**Figure 3E (a): ^{1}H NMR data of compound 3.21**

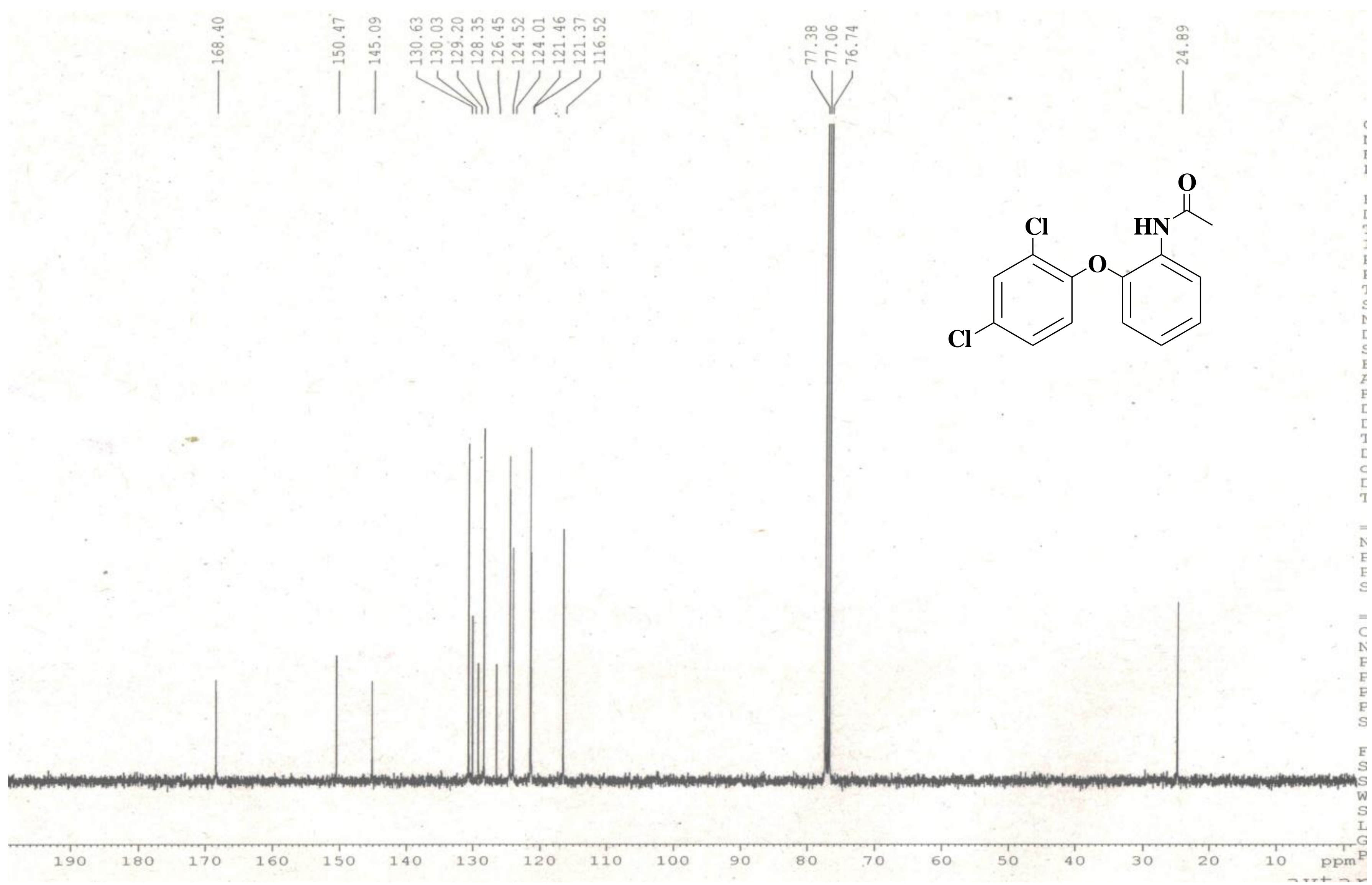


**Figure 3E (b): $^{13}$C NMR data of compound 3.21**

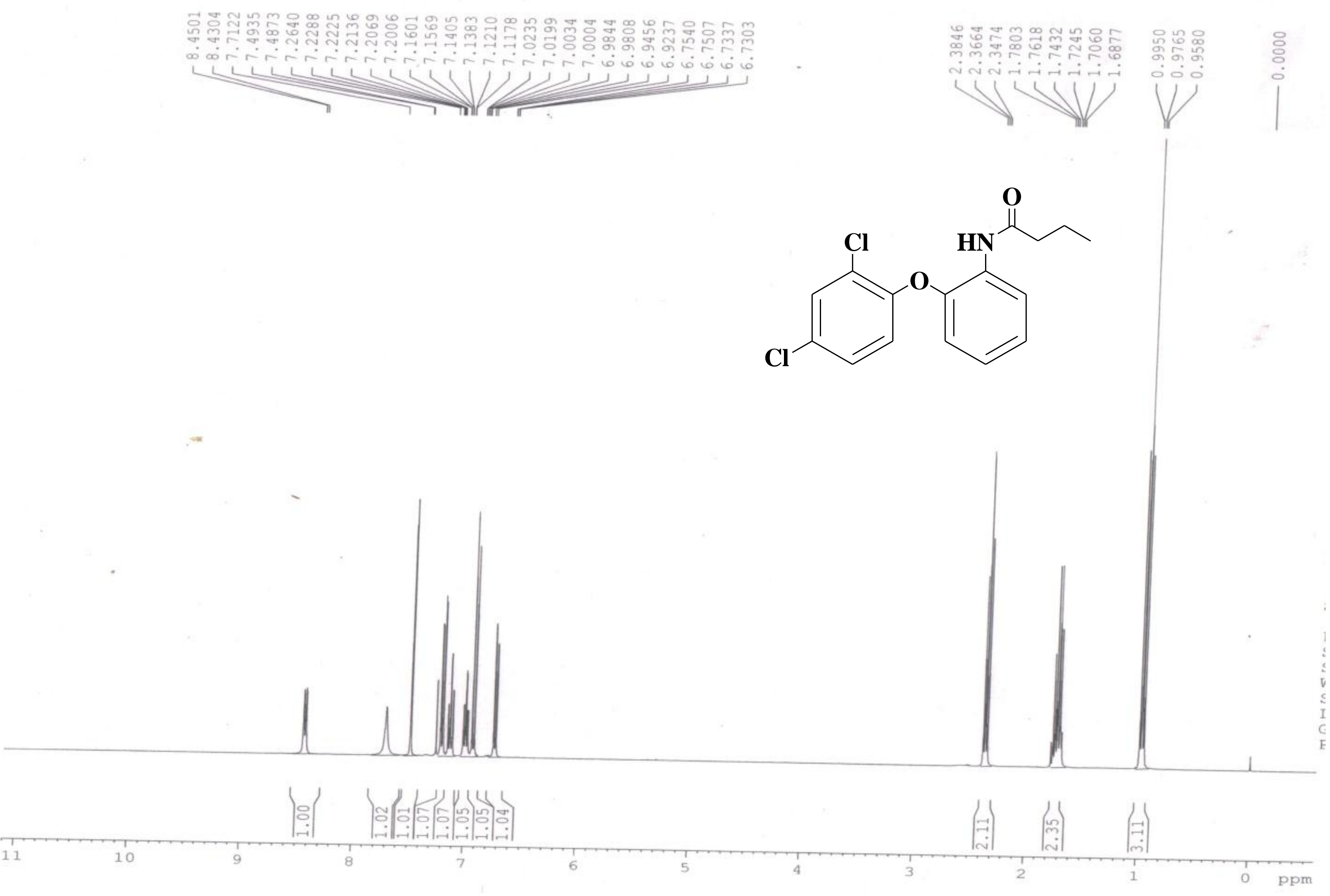


**Figure 3F (a): $^1$H NMR data of compound 3.23**

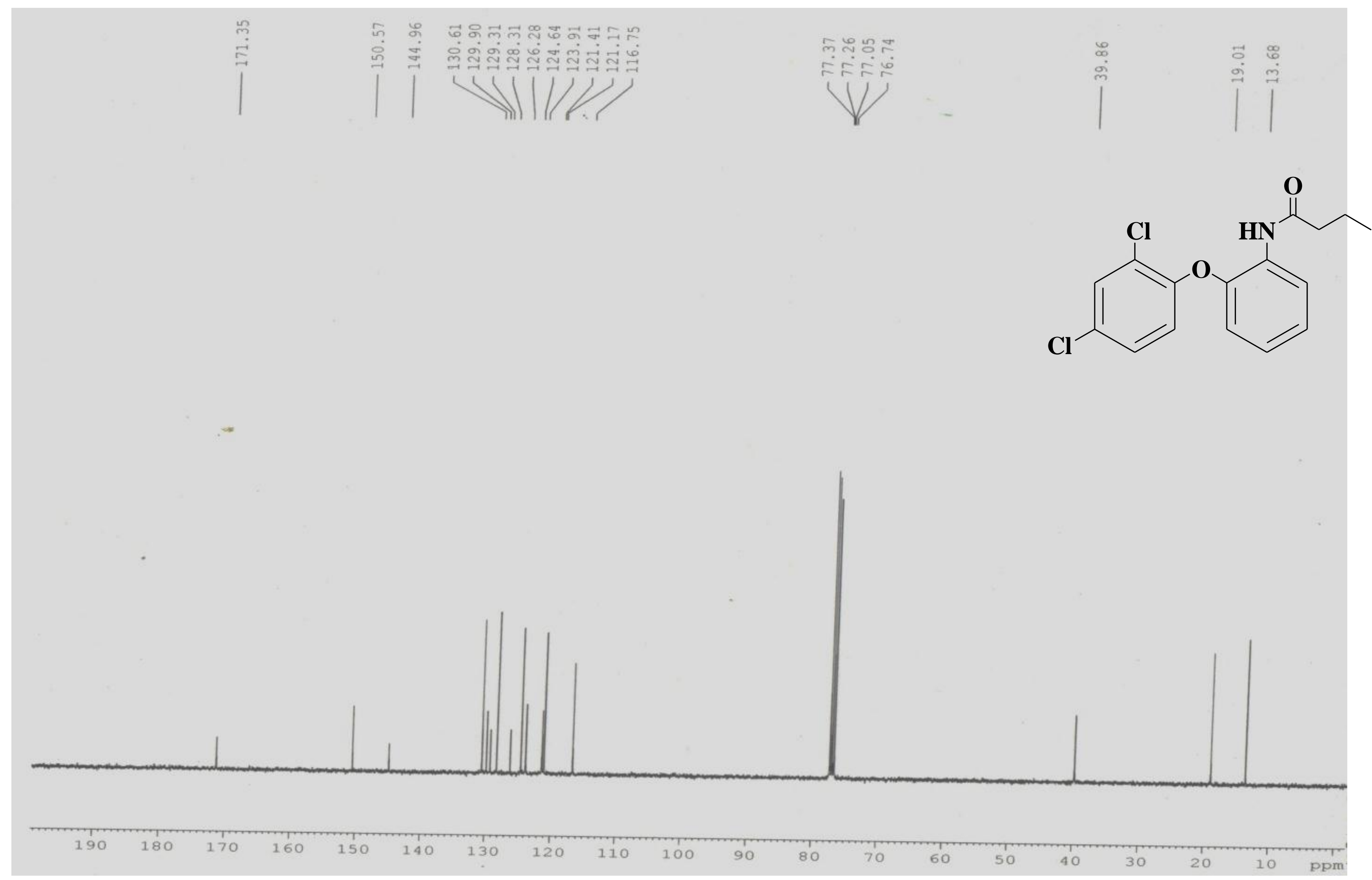


**Figure 3F (b): $^{13}$C NMR data of compound 3.23**

**Figure 4A (a): $^{1}$H NMR data of compound 4.14**

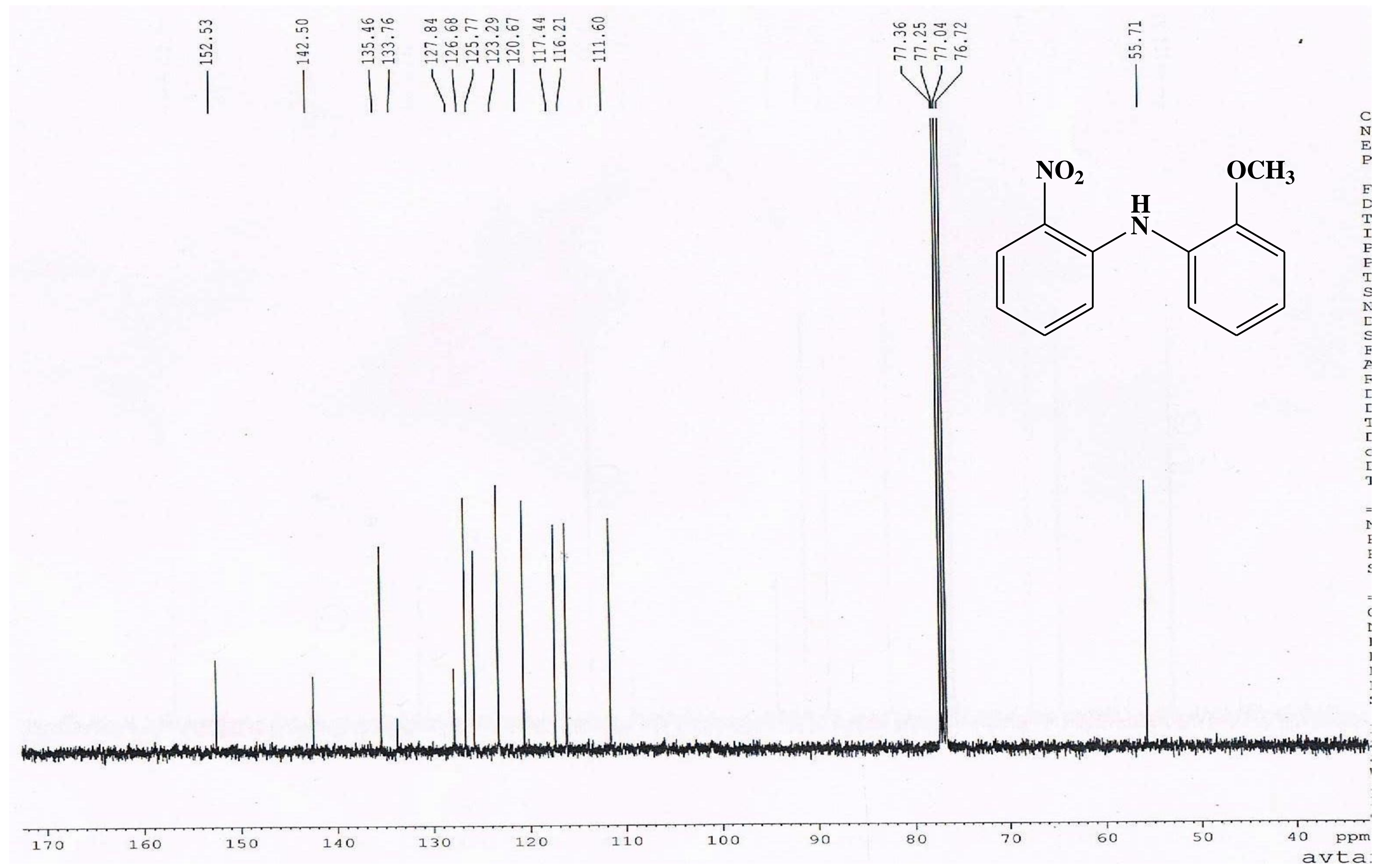


**Figure 4A (b): $^{13}$C NMR data of compound 4.14**

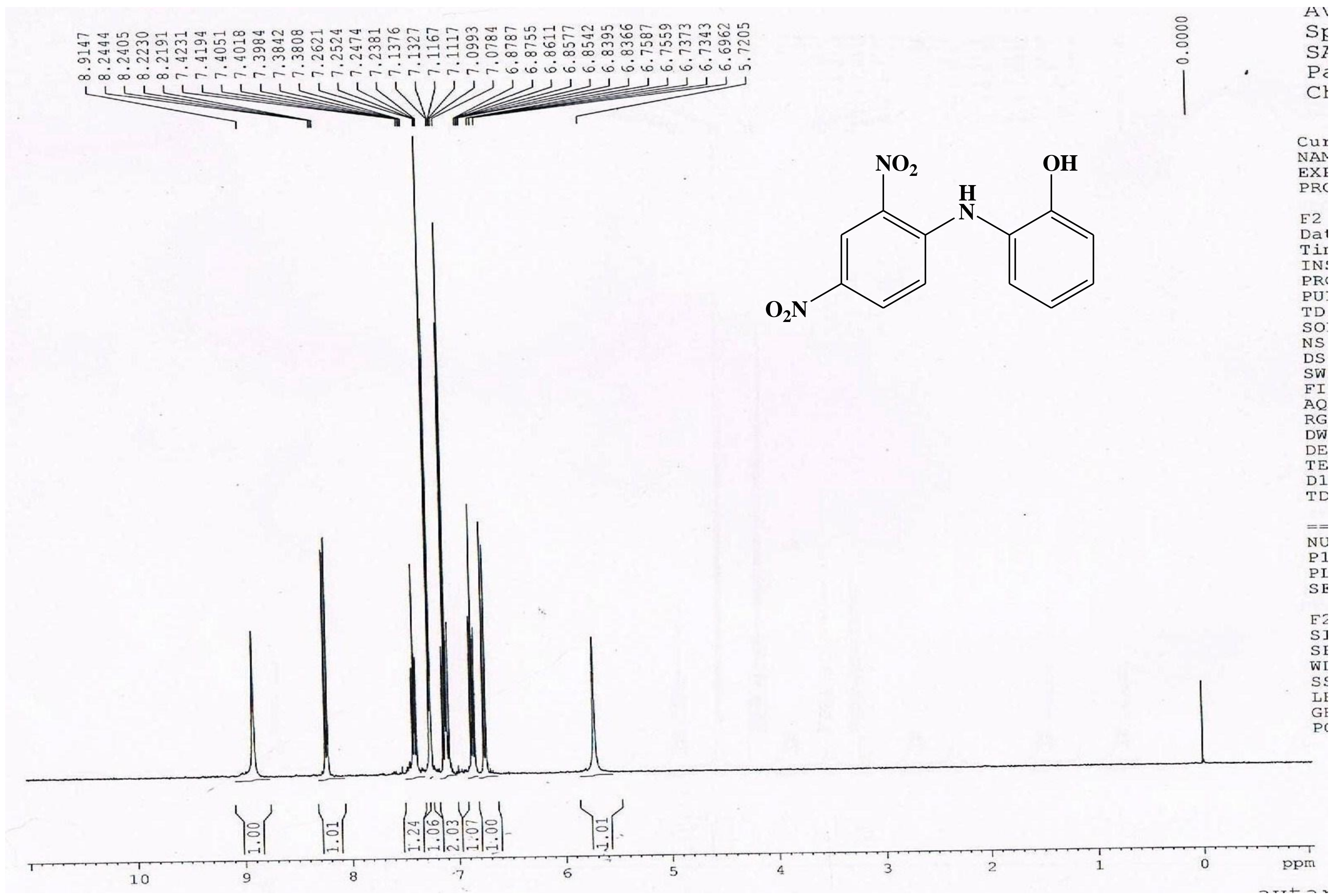


**Figure 4B (a): ^{1}H NMR data of compound 4.16**

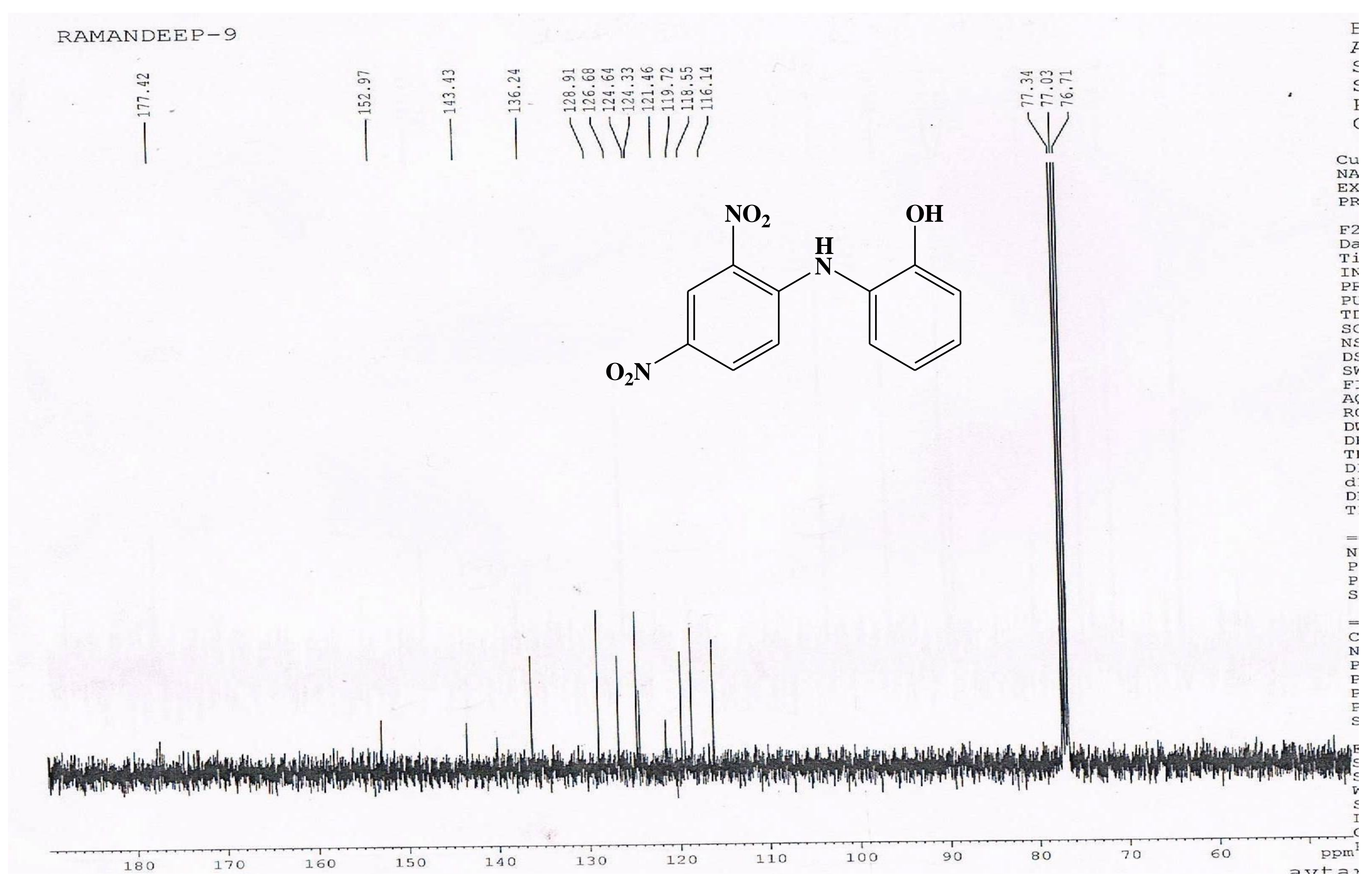


Figure 4B (b): $^{13}$C NMR data of compound 4.16

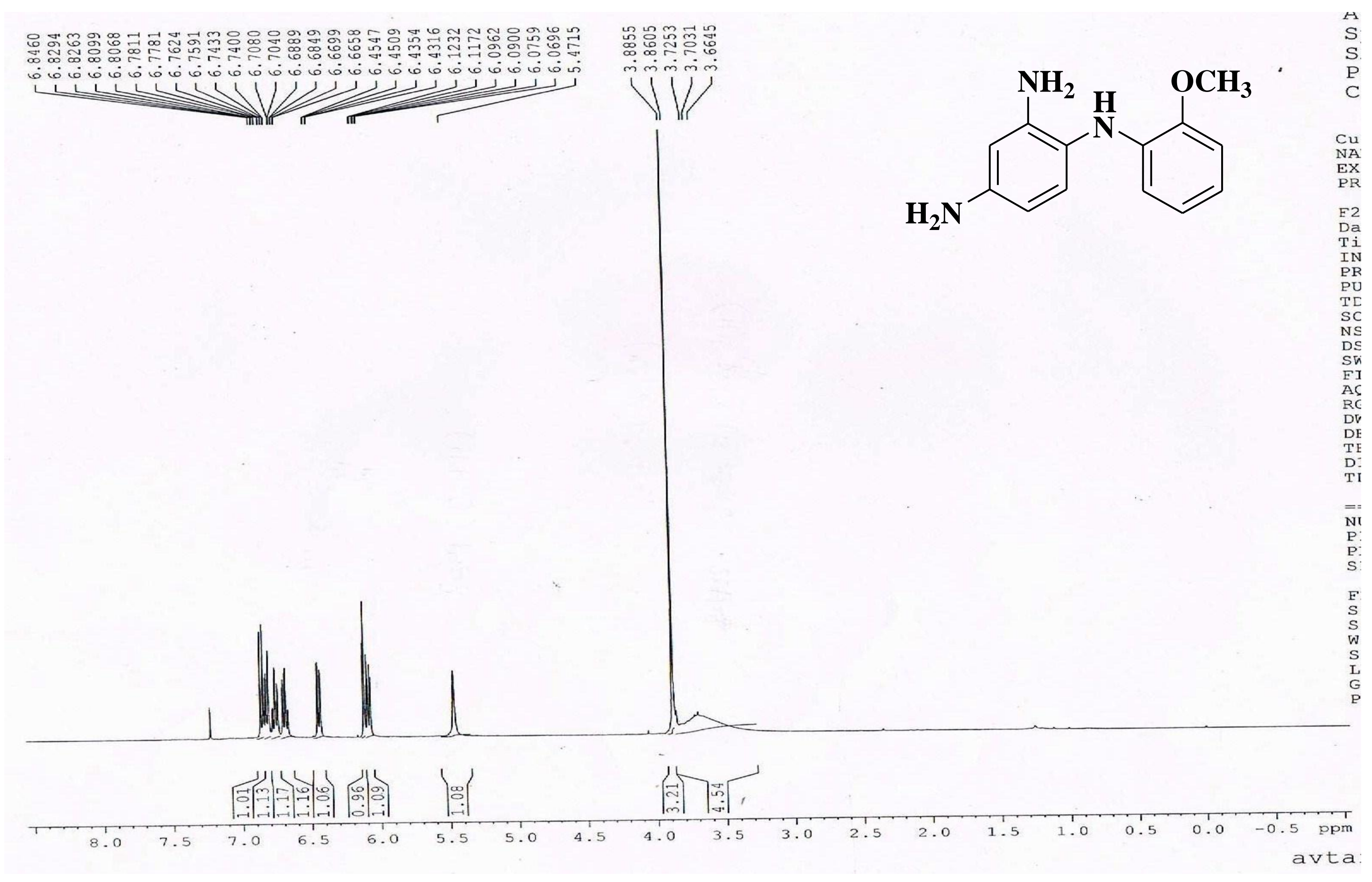


**Figure 4C (a): $^{1}$H NMR data of compound 4.18**

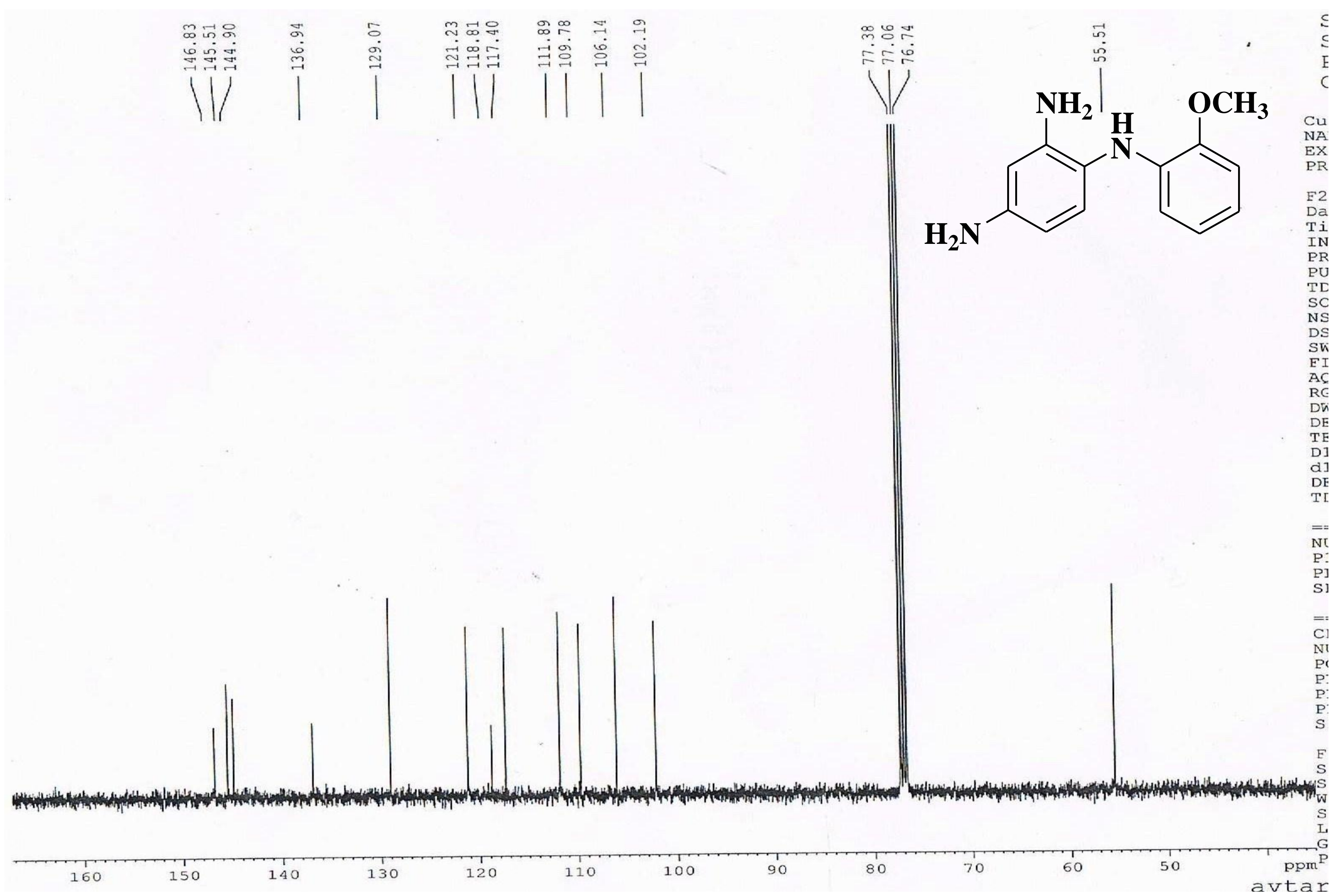


**Figure 4C (b): $^{13}$C NMR data of compound 4.18**

**Figure 4D (a): $^1$H NMR data of compound 4.21**

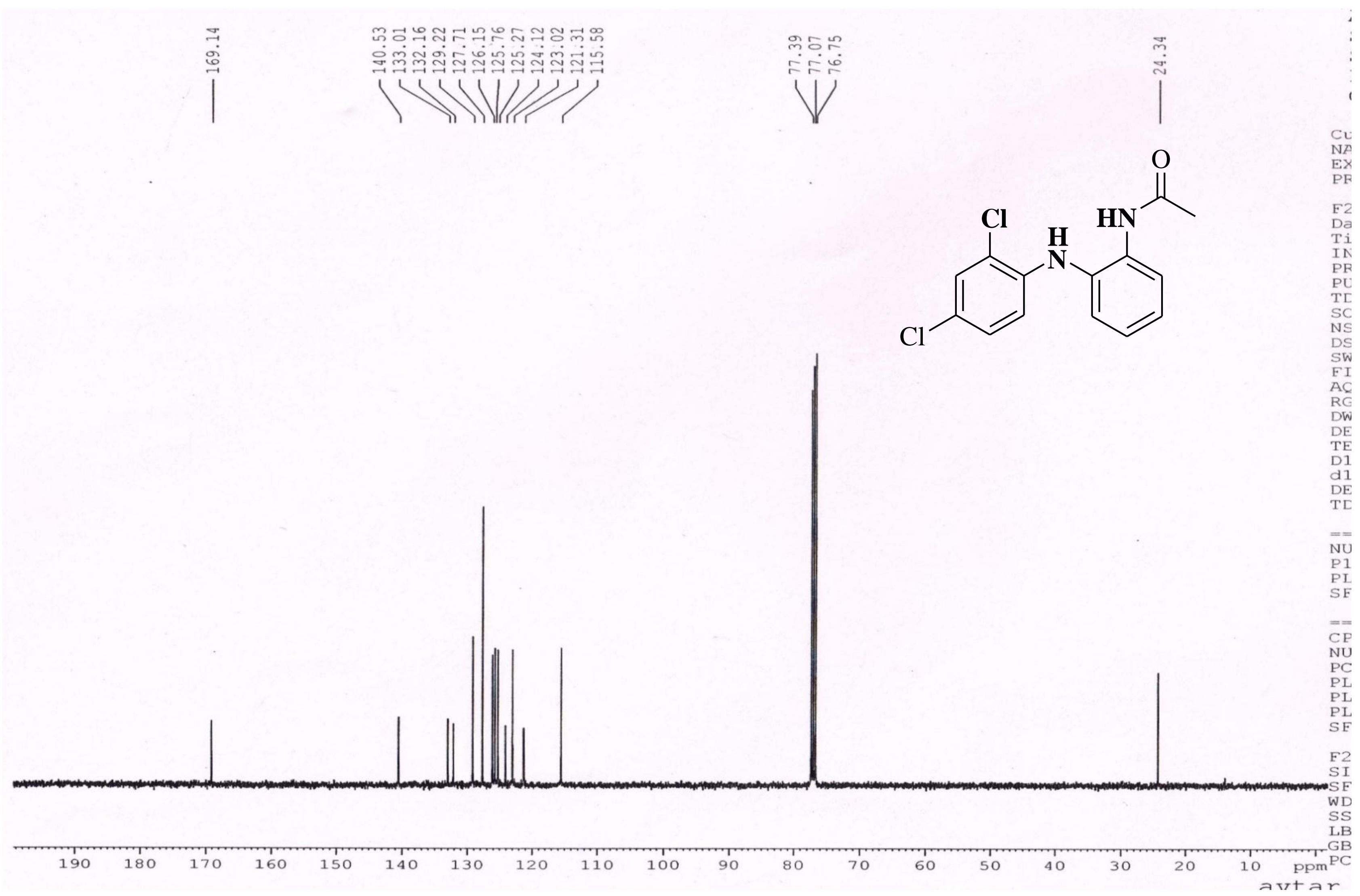


**Figure 4D (b): $^{13}$C NMR data of compound 4.21**

**Publications**

1. Mehton Ramandeep Kaur, Ramya T.N.C. and Chhibber Manmohan[1]. Enhancement in the solubility of triclosan with Triton X-100 without affecting its antibacterial activity in *Escherichia coli*. *Research Journal of Chemistry and Environment*. (2015) 19(11): 14-21.
2. Ramandeep K. Mehton, Vineet Meshram, Sanjai Saxena and Manmohan Chhibber. Synthesis and Anti-Staphylococcal Activity of 2,4-Disubstituted Diphenylamines. *Journal of the Brazilian Chemical Society* (2015) 27(7):1236-1244.

**Workshops and Conferences attended**

- 5th School on analytical chemistry, sponsored by BRNS-AEACI, held at I.I.T.Roorkee. Dec. 3rd to Dec. 10th, 2012.
- Ramandeep Kaur, Ramya T.N.C, Manmohan Chhibber, Effect of different surfactants on the efficacy of triclosan against *Escherchia coli*. National Conference on Recent Advances in Biosciences and Drug Discovery- 2014, Department of Pharmaceutical Sciences, Faculty of Ayurved & Medical Sciences. March 03-04, 2014, OP-36, Page 20.
- Ramandeep Kaur, Vineet Meshram, Sanjai Saxena, Manmohan Chhibber, Diphenylamines show in vitro anti-bacterial activity against *Staphylococcus aureus* , National Conference Science Colloquium, Emerging Trends in Basic & Applied Sciences. DAV College, Jalandhar, Punjab, March 6-7, 2014, PP-107, Page 14.
- Ramandeep Kaur, Ramya T.N.C, Manmohan Chhibber, Effect of different surfactants on the efficacy of triclosan against *Escherchia coli*. Global sustainability Transitions: Impacts and innovations, Krishi Sanskriti, New Delhi, ISBN: 978-93-83083-77-0, May 31-June 1, 2014, Page 292.