SYNTHESIS AND CHARACTERIZATION OF 2,5-DI(4-ARYLOYLARYL OXYMETHYL)-1,3,4-OXADIAZOLES

This work has been published in European Journal of Medicinal Chemistry 63 (2013) 536-543

Men love to wonder, and that is the seed of science- Ralph Waldo Emerson.
2.1.0 INTRODUCTION

Oxadiazole and their analogues can be considered as simple five membered heterocycles possessing one oxygen and two nitrogen atoms. The oxadiazole exists in different isomeric forms such as 1,3,4-, 1,2,5-, 1,2,4-, and 1,2,3-oxadiazoles (Figure - 2.1.1) out of which thermally stable 1,3,4-oxadiazole (a) is the only isomer not containing a nitrogen-oxygen bond.

1,3,4-Oxadiazole is a thermally stable neutral aromatic molecule\(^1\) and its estimated resonance energy is 167.4 kJ/mol. Particularly, aryl group at position 2 increases the thermal stability of 1,3,4-oxadiazole. The ring is stable to heat, a property which has been exploited in the production of heat-resistant poly-1,3,4-oxadiazoles. UV spectra of substituted 1,3,4-oxadiazoles are similar to those of similarly substituted benzenes, particularly in the case of 2-phenyl-1,3,4-oxadiazoles (\(\lambda_{\text{max}}\) in ethanol = 247.5 nm, \(\log \varepsilon 4.26\)). Studies on 1,3,4-oxadiazoles and its cation indicate a maximum positive charge is on the second position. Alkyl and aryl-1,3,4-oxadiazoles are neutral compounds and 2-amino-1,3,4-oxadiazoles are weak bases.\(^2\)

1,3,4-Oxadiazoles have a relatively low electron density at carbon (position 2 and 5) and relatively high electron density at nitrogen (position 3 and 4). Consequently the major reactions performed by nucleophilic attack at carbon, followed by ring cleavage and electrophilic attack at nitrogen atom. The attack of a nucleophile at carbon 2 leads either to nucleophilic displacement (path A) or ring cleavage (path B), the latter being the most common result. For instance, the most frequently encountered result of the reaction
of a 1,3,4-oxadiazole with a nucleophile is ring opening reaction which leads to a hydrazine derivative, as shown below (Figure 2.1.2).

\[
\begin{align*}
\text{N-N} & \xrightarrow{\text{Nu}} \text{N-N} \xrightarrow{A} \text{N-N} \\
\text{N-N} & \xrightarrow{B} \text{N-N} \\
\text{N-N} & \xrightarrow{H} \text{RCONHN=C} \\
\end{align*}
\]

Figure 2.1.2: RING OPENING REACTIONS OF OXADIAZOLE

The relatively low electron density of carbon, coupled with the possibility of protonation of nitrogen, makes electrophilic substitution at carbon difficult. No examples of nitration or sulphonation of the oxadiazole ring have been reported and the attempted bromination reaction was unsuccessful. 2-Aryl-1,3,4-oxadiazoles undergo reactions similar to those of benzene derivatives, such as electrophilic substitution in an aryl group.

2.1.1. GENERAL ASPECTS OF THE CHEMICAL OXADIAZOLE SYNTHESSES

1,3,4-Oxadiazole is associated with potent pharmacological activity due to the presence of toxophoric -N=C-O- linkage. 1,3,4-Oxadiazoles are of considerable pharmaceutical and material interest, which is documented by a steadily increasing number of publications and patents. Consequently, a number of synthetic approaches to the 1,3,4-oxadiazole systems have been developed and most of these involve the use of acetohydrazides or its derivative, as a source of two contiguous nitrogen atoms, and a variety of cyclizing agents. Typically, the reaction is promoted by heat and anhydrous reagents including thionyl chloride, phosphorous pentoxide, triphenylphoshine, triflic anhydride, and phosphorous oxychloride. Alternative synthetic methods comprise reaction of aceto hydrazides with keteneylidene triphenylphosphorane or base promoted cyclization reaction of trichloroacetic acid hydrazones. Kagthara et al have
synthesized 2-aryl-5-[2-(benzimidazol-2-yl)-phenyl]-1,3,4-oxadiazoles by refluxing equimolar mixture of 2-(benzimidazol-2-yl)benzoyl hydrazides and aromatic acids in phosphorous oxychloride. In this chapter, based on the literature survey on a synthesis of 1,3,4-oxadiazoles and by modifying the earlier procedure the synthesis of 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles (9a-j) have been carried out from N,N-di(2-(4-aryloylaryloxy)acetyl)hydrazines (8a-j) using 2,6 lutidine and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU).

2.1.2. PLAN OF THE SYNTHESIS OF 2,5-DI (4-ARYLOYLARYL OXYMETHYL) - 1,3,4-OXADIAZOLES (9a-j)

The synthesis of the hitherto unreported title compounds is as outlined in SCHEME - 2.1.1. (4-Hydroxyaryl)aryl methanones commonly known as hydroxy benzophenones 4a-e were achieved in excellent yield using benzyolation of compound 1 with benzooyl chloride derivatives 2a-e followed by Fries rearrangement of substituted arylbenzoates 3a-e. Compounds 4a-e on reaction with ethyl bromoacetate afforded ethyl 4-aryloylaryloxyacetates 5a-e which on treatment with sodium hydroxide in the presence of THF gave 4-aryloylaryloxyethanoic acids 6a-e.

Further, compounds 5a-e on treatment with hydrazine hydrate in the presence of ethanol yield 4-aryloylaryloxyacetohydrazides 7a-e. Condensation of 6a-e with 7a-e in the presence of 2,6 lutidine, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) and dichloromethane (DCM) afforded N,N-di(2-(4-aryloylaryloxy)acetyl)hydrazines 8a-j.

Finally, title compounds 9a-j were achieved by intramolecular cyclization of 8a-j in the presence of triflic anhydride, pyridine and DCM.
2.1.3. RESULTS AND DISCUSSION

Benzoylation of 2-chloro-6-fluoro phenol (1) was achieved with benzoyl chlorides (2a-e) using triethylamine solution as a base by Schotten-Baumann method\textsuperscript{13,14} in the presence of dichloromethane. The benzoyl compounds frequently occlude traces of
unchanged benzoyl chloride, which thus escapes hydrolysis by the alkali. Therefore the crude product was recrystallized with ethanol, which esterified the unchanged benzoyl chloride to afford arylbenzoates 3a-e in excellent yield. Further 3a-e were confirmed by IR and $^1$H NMR data and compound 3a is taken as a representative example of this series. The IR spectrum of 3a showed absorption in the region 1738 cm$^{-1}$ assigned to the ester carbonyl stretching frequency. In $^1$H NMR spectrum compound 3a showed the aromatic signals as two multiplets for seven protons in the range $\delta$ 7.42-7.53 and 8.25-8.28. The compound 3a gave significant stable M$^+$ peak at m/z 268.5 with relative abundance of 75%.

In spite of the fact that the Fries rearrangement requires two steps, the preparation of aryl benzoate and the rearrangement to the (hydroxy aryl)aryl methanone, commonly known as hydroxybenzophenone, as compared to the single step in the Friedel-Crafts synthesis. Fries method was preferred as a useful route for the preparation of (hydroxy aryl) aryl methanones, since the yield in this method is better and the experimental procedure needs no modification to adopt it to a variety of aryl benzoates. Extensive study has been carried out on the reaction using Lewis acids or Bronsted acids. Catalytic acids used were anhydrous aluminum chloride, mercuric chloride, stannic chloride, ferric chloride, TsOH, orthophosphoric acid, hydrogen fluoride, borontrifluoride etc. Among the wide variety of catalysts, aluminum chloride has been most extensively used. Based on these initial reports, (4-hydroxy aryl) methanones (4a-e) containing halo groups in the phenolic ring were synthesized from 3a-e. Some of the known compounds gave sharp melting point in perfect agreement with literature value. Furthermore, the assignment of the structures of compounds 4a-e follows from the analysis of their IR, $^1$H NMR and mass spectral data. As a specific example IR spectrum of the compound 4a showed the presence of absorption peaks at 1671 and in between 3545-3635 cm$^{-1}$ due to
aromatic carbonyl and phenolic hydroxyl groups, respectively. The $^1$H NMR spectrum of 4a shows multiplet in the range $\delta$ 7.36-7.82 for six aromatic protons. In addition, a broad singlet at $\delta$ 11.64 for phenolic hydroxy proton was also observed and the downfield absorption of phenolic hydroxyl proton is attributed to the hydrogen bonding with the aromatic carbonyl group. The compound 4a gave significant stable $M^+$ peak at m/z 268.5 with relative abundance of 83%.

Preparation of phenoxy acetates is an important synthetic reaction for which a wide variety of procedures have been developed. Generally they were synthesized by the condensation of the phenolic hydroxyl group with halo acetate in dry acetone along with anhydrous potassium carbonate. By adopting reported methods thereby ethyl 4-aryloylaryloxy acetates (5a-e) were prepared from 4a-e. The compounds 5a-e were confirmed by IR, $^1$H NMR and mass spectral data. For example, IR spectrum of 5a showed absorptions in the region 1660 and 1730 cm$^{-1}$ corresponding to aromatic carbonyl and ester carbonyl stretching frequencies, respectively. The $^1$H NMR spectrum of 5a showed a triplet in the range $\delta$ 1.16-1.22 corresponding to methyl protons of ethyl group, a quartet in the range $\delta$ 4.14-4.21 and a singlet at $\delta$ 5.02 corresponding to CH$_2$ of ethyl and OCH$_2$ groups, respectively. In addition, it showed one multiplet in the range $\delta$ 7.64-7.8 corresponding to six aromatic protons. The mass spectrum of compound 5a gave significant stable $M^+$ peak at m/z 354.5 with relative abundance of 59%.

A convenient synthesis for the valuable building blocks, aryloylaryloxy ethanoic acids was achieved by a simple laboratory procedure which derives from the reaction of aryloylaryloxy acetates in ethanol and sodium hydroxide solution. For the present work the desired 4-aryloylaryloxyethanoic acids (6a-e) were gratifyingly synthesized by the alkaline hydrolysis of 4-aryloylaryloxy acetates (5a-e) in excellent yield. Compound 6a is taken as a representative of 6a-e series, to explain the IR, $^1$H NMR and mass spectral
data. In IR spectrum it showed three bands at 1660, 1738 and 3470-3575 cm\(^{-1}\) assigned to aromatic carbonyl, acid carbonyl and acid OH stretching frequencies, respectively. In \(^1\)H NMR spectrum, \(\text{6a}\) showed one singlet at \(\delta 4.9\) assigned to OCH\(_2\) protons. It also showed one multiplet in the range \(\delta 7.3-7.87\) for six aromatic protons. In addition, it showed a broad singlet at \(\delta 13.1\) (D\(_2\)O exchangeable) assigned to acid OH proton. Finally, the observance of M\(^+\) peak at m/z 326.5, with relative abundance of 55% confirms the structure as \(\text{6a}\).

Acetohydrazides are important building blocks in many ring transformation reactions\(^{23}\) and the standard method for preparing acetohydrazides is hydrazinolysis of esters in ethanol at reflux temperature.\(^{24-26}\) For the present work, the desired 4-aryloylaryloxyacetohydrazides (7a-e) were synthesized in 75-80\% yield, by adding 80\% hydrazine to compounds 5a-e in ethanol with constant stirring at cold condition. This coupling procedure is rapid, convenient, proceeds under mild conditions and is suitable for large scale preparations. The compounds 7a-e were characterized by IR, \(^1\)H NMR and mass spectral studies. In a typical example, IR spectrum of 7a showed absorption peaks at 1610, 1645 and in between 3100-3205 cm\(^{-1}\) assigned to aromatic carbonyl, amide carbonyl and NH-NH\(_2\) stretching frequencies respectively. The \(^1\)H NMR spectrum of 7a revealed one singlet at \(\delta 4.69\) assigned OCH\(_2\) proton. It also revealed two broad singlets at \(\delta 4.35\) and 9.32 assigned to amino and amide protons. In addition, it revealed one multiplet in the range \(\delta 7.2-7.86\) for six aromatic protons. The mass spectrum of compound 7a gave significant stable M\(^+\) peak at m/z 340.5 with relative abundance of 42%.

In many research plans, (aryloylaryloxy) acetyl hydrazines has been prepared by the union of carboxylic acid and amine.\(^{27}\) However, the unification of these two functional groups does not occur spontaneously at ambient temperature, with the
necessary elimination of water only taking place at high temperatures (>200°C), conditions typically detrimental to the integrity of the substrates.\(^\text{28}\) For this reason, it is usually necessary to first activate the carboxylic acid, a process that usually takes place by converting the OH of the acid into a good leaving group prior to treatment with the amine. In order to activate carboxylic acids, one can use coupling reagents, like EDCI, DCC, HOBT etc. which act as stand-alone reagents to generate compounds such as acid chlorides, anhydrides, carbonic anhydrides or active esters. The choice of coupling reagent is however critical. A coupling reagent needs to be able to cope with the whole portfolio of reactivity. Many reviews on coupling reagents have been published, illustrating their importance in the synthetic armory of the synthetic chemist.\(^\text{29,30}\) Based on the previous reports, N,N-di(2-(4-aryloylaryloxy)acetyl hydrazines (8a-j) were synthesized by the condensation of compounds 6a-e with 7a-e in the presence of 2,6 lutidine and TBTU. Interestingly, the synthesized compounds by this method were found to be within the expectation which was confirmed by IR, \(^1\)H NMR and mass spectral studies. The IR spectrum of 8a as a representative example of this series, showed absorption at 1690, 1610 and 3700-3500 cm\(^{-1}\) due to aromatic C=O, amide C=O and NH-NH stretching frequencies respectively. Compound 8a showed in its \(^1\)H NMR spectrum one singlet at \(\delta\) 4.9 assigned to OCH\(_2\) proton and one broad singlet at \(\delta\) 10.36 assigned to two NH protons. It also showed one multiplet in the range \(\delta\) 7.1-7.87 for twelve aromatic protons. Besides, the mass spectrum of compound 8a gave significant stable M\(^+\) peak at m/z 649 with relative abundance of 61%.

Oxadiazole moiety and its various derivatives studied frequently in the past time and found potent in various pharmacological and pathological conditions.\(^\text{31}\) By the intramolecular cyclization of N,N-di(2-(4-aryloylaryloxy)acetyl hydrazines (8a-j) using in particularly strong electrophile like triflic anhydride 2,5-di(4-aryloylaryloxy methyl)-
1,3,4-oxadiazoles (9a-j) were synthesized. The products 9a-j synthesized by the above method were confirmed by IR, $^1$H NMR and mass spectral studies. The IR spectrum of 9a as a representative example of this series, showed absorption at 1153, 1658 and 1683, 3124 cm$^{-1}$ due to C-O-C, C=O and C=N stretching frequencies respectively. Compound 9a showed in its $^1$H NMR spectrum one singlet at δ 5.6 assigned to OCH$_2$ protons. Since in 9a the OCH$_2$ protons are adjacent to the oxadiazole ring, in which one oxygen and two nitrogen atoms are present, they are more deshielded and their signal observed at downfield compared to 8a. In addition, it showed one multiplet in the range δ 7.05-7.81 for twelve aromatic protons. Besides, the mass spectrum of compound 9a gave significant stable M$^+$ peak at m/z 631 with relative abundance of 70%.

2.1.4. EXPERIMENTAL SECTION

- All reagents were of commercial quality and were purified before use and some of the starting materials were synthesized according to standard procedure.
- The organic solvents were of analytical grade and purified by standard procedure. The chemicals used for the synthesis of intermediates and end products were purchased from Sigma Aldrich, Merck, Ranbaxy, Spectrochem and S D fine chemicals.
- Analytical thin layer chromatography (TLC) was performed on Merck precoated silica gel-G F254 aluminum plates. Visualization of the spots on TLC plates was achieved either by exposure to iodine vapor or UV light.
- The purity of the compounds was checked by using these plates, varying the solvent proportions and by the multiple irrigations.
- Column chromatography was performed on silica gel (high purity grade 60-120 mesh) using suitable eluent, which are given in the respective experimental procedure.
- All synthesized products were crystallized by suitable solvents, which are given in the respective experimental procedure.
All evaporation of solvents was carried out under reduced pressure on IKA rotary evaporator. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer.

- IR spectra were recorded on FT IR Shimadzu 8300 spectrophotometer using nujol mull or potassium bromide wafer.
- $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer in DMSO-$d_6$/CD$_3$OD/CDCl$_3$ and the chemical shifts were recorded in parts per million down field from tetramethylsilane.
- Mass spectra were obtained with a VG70-70H mass spectrometer and important fragments are given with the relative intensities in the brackets.
- Microanalyses were performed by the Regional Sophisticated Instrumentation Centre, C.D.R.I, Lucknow and the results are within 0.5% of the calculated value.

### 2.1.4.1. GENERAL PROCEDURE FOR THE SYNTHESIS OF ARYL BENZOATES (3a-e)

2-Chloro-6-fluoro phenol (1, 0.2054 mol) was dissolved in DCM, triethylamine (TEA, 0.4519 mol) was added and the reaction mixture was cooled to 0 °C. A solution of benzoyl chloride derivatives (2a-e, 0.2157 mol) in DCM was added slowly to the above mixture and stirred for 3 h. Then the reaction mass was diluted with DCM (200 mL), washed with 10% sodium hydroxide solution (3×30 mL), water (3×30 mL), brine (2×60 mL), and again with water (3×30 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated to achieve compounds 3a-e.

A typical procedure is described for the synthesis of 2-chloro-6-fluorophenyl-4-fluorobenzoate (3a): 2-Chloro-6-fluoro phenol (1, 30 g, 0.2054 mol) was dissolved in DCM, triethylamine (TEA, 45.73 g, 0.4519 mol) was added and the reaction mixture was cooled to 0 °C. A solution of 4-fluorobenzoyl chloride (2a, 33.9 g, 0.2157 mol) in DCM
was added slowly to the above mixture and internal temperature was maintained to 0-10 °C. Finally the reaction mixture was stirred at ambient temperature for 3 h. Then the reaction mass was diluted with DCM (200 mL), washed with 10% sodium hydroxide solution (3×30 mL), water (3×30 mL), brine (2×60 mL), and again with water (3×30 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated to achieve compound 3a as a white solid. Yield: 94%; m. p.: 52.6-54.1 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1738 (ester, C=O); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 7.42-7.53 (m, 4H, Ar-H), 8.25-8.28 (m, 3H, Ar-H); MS (EI): m/z (75%) M\(^+\) 268.5; Anal. Calcd. for C\(_{13}\)H\(_7\)ClF\(_2\)O\(_2\) (268.5): C, 58.12; H, 2.63; Cl, 13.20; F, 14.14. Found: C, 58.22; H, 2.43; Cl, 13.30; F, 14.29%.

Compounds 3b-e were synthesized analogously starting with 2b-e respectively.

2-Chloro-6-fluorophenyl-4-chlororobenzoate (3b): Yield: 95%; m. p.: 52.1-53.5 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1750 (ester, C=O); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 7.39-7.53 (m, 4H, Ar-H), 7.69-7.71 (d, 2H, Ar-H), 8.16–8.17 (d, 2H, Ar-H). MS (EI): m/z (72%) M\(^+\) 285; Anal. Calcd. for C\(_{13}\)H\(_7\)BrClFO\(_2\) (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66. Found: C, 54.57; H, 2.33; Cl, 24.64; F, 6.42%.

2-Chloro-6-fluorophenyl-4-bromobenzoate (3c): Yield: 97%; m. p.: 67.2-68.7 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1710 (ester, C=O); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 7.40-7.54 (m, 3H, Ar-H), 7.85-7.87 (d, 2H, Ar-H), 8.03-8.06 (d, 2H, Ar-H). MS (EI): m/z (70%) M\(^+\) 329.5; Anal. Calcd. for C\(_{13}\)H\(_7\)BrClFO\(_2\) (329.5): C, 47.38; H, 2.14; Br, 24.25; Cl, 10.76; F, 5.76. Found: C, 47.18; H, 2.29; Br, 24.36; Cl, 10.51; F, 5.58%.

2-Chloro-6-fluorophenyl-4-iodobenzoate (3d): Yield: 93%; m. p.: 78.4-79.2 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1765 (ester, C=O); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 7.39-7.54 (m, 3H, Ar-H), 7.66-7.97 (d, 2H, Ar-H), 8.03-8.06 (d, 2H, Ar-H). MS (EI): m/z
(74%) M⁺ 376.5; Anal. Calcd. for C₁₃H₇ClFIO₂ (376.5): C, 41.47; H, 1.87; Cl, 9.42; F, 5.05; I, 33.70. Found: C, 41.29; H, 1.68; Cl, 9.31; F, 5.23; I, 33.53%.

2-chloro-6-fluorophenyl-4-methylbenzoate (3e): Yield: 96%; m. p.: 62.0-63.1 °C; IR (KBr) ν_max (cm⁻¹): 1780 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 7.38-7.51 (m, 3H, Ar-H), 7.6-7.9 (d, 2H, Ar-H), 8.05-8.07 (d, 2H, Ar-H). MS (EI): m/z (78%) M⁺ 264.5; Anal. Calcd. for C₁₄H₁₀ClFO₂ (264.5): C, 63.53; H, 3.81; Cl, 13.39; F, 7.18. Found: C, 63.69; H, 3.71; Cl, 13.09; F, 7.30%.

2.1.4.2. GENERAL PROCEDURE FOR THE SYNTHESIS OF (4-HYDROXY ARYL) ARYL METHANONES (4a-e)

Compounds 3a-e (0.1903 mol) and aluminum chloride (0.5388 mol) were blended and the mixture was heated to 150 °C and this temperature was maintained for 1 h. Then the reaction mixture was cooled to 0°C and quenched with 6N hydrochloric acid (200 mL) and extracted with DCM (3×100 mL). The organic layer was washed with water (3×40 mL), brine (3×30 mL) and again with water (3×40 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford compounds 4a-e.

A typical procedure is described for the synthesis of (3-chloro-5-fluoro-4-hydroxyphenyl)-4-fluorophenyl methanone (4a): Compound 3a (51 g, 0.1903 mol) and aluminum chloride (71.05 g, 0.5388 mol) were blended and the mixture was heated to 150 °C and this temperature was maintained for 1 h. Then the reaction mixture was cooled to 0 °C and quenched with 6N hydrochloric acid (200 mL) and extracted with DCM (3×100 mL). The combined organic layer was washed with water (3×40 mL), brine (3×30 mL) and again with water (3×40 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford compound 4a as a pale yellow solid.
Yield: 61%; m. p.: 146.3-147.7 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 1671 (C=O), 3545-3635 (OH);

$^1$H NMR (400 MHz) (DMSO-d$_6$) $\delta$ (ppm): 7.36-7.82 (m, 6H, Ar-H), 11.64 (bs, 1H, OH).

MS (EI): m/z (83%): M$^+$ 268.5; Anal. Calcd. for C$_{13}$H$_7$ClF$_2$O$_2$ (268.5): C, 58.12; H, 2.63; Cl, 13.20; F, 14.14.

Found: C, 58.21; H, 2.52; Cl, 13.20; F, 14.25%.

Compounds 4b-e were synthesized analogously starting with 3b-e respectively.

(3-Chloro-5-fluoro-4-hydroxyphenyl)4-chlorophenyl methanone (4b): Yield: 68.6%; m. p.: 167.8-169.1 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 1660 (C=O), 3525-3625 (OH);

$^1$H NMR (400 MHz) (DMSO-d$_6$) $\delta$ (ppm): 7.52-7.94 (m, 6H, Ar-H), 11.60 (bs, 1H, OH).

MS (EI): m/z (80%): M$^+$ 285; Anal. Calcd. for C$_{13}$H$_7$ClFO$_2$ (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66.

Found: C, 54.65; H, 2.32; Cl, 24.71; F, 6.53%.

(3-Chloro-5-fluoro-4-hydroxyphenyl)4-bromophenyl methanone (4c): Yield: 71%; m. p.: 172.1-173.3 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 1610 (C=O), 3495-3580 (OH);

$^1$H NMR (400 MHz) (DMSO-d$_6$) $\delta$ (ppm): 7.54-7.79 (m, 6H, Ar-H), 11.63 (bs, 1H, OH).

MS (EI): m/z (81%): M$^+$ 329.5; Anal. Calcd. for C$_{13}$H$_7$BrClFO$_2$ (329.5): C, 47.38; H, 2.14; Br, 24.25; Cl, 10.76; F, 5.76. Found: C, 47.46; H, 2.29; Br, 24.41; Cl, 10.59; F, 5.62%.

(3-Chloro-5-fluoro-4-hydroxyphenyl)4-iodophenyl methanone (4d): Yield: 65%; m. p.: 182.1-183.2 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 1635 (C=O), 3495-3590 (OH);

$^1$H NMR (400 MHz) (DMSO-d$_6$) $\delta$ (ppm): 7.46-7.8 (m, 6H, Ar-H), 11.60 (bs, 1H, OH).

MS (EI): m/z (79%): M$^+$ 376.5; Anal. Calcd. for C$_{13}$H$_7$ClFO$_2$ (376.5): C, 41.47; H, 1.87; Cl, 9.42; F, 5.05; I, 33.70. Found: C, 41.32; H, 1.71; Cl, 9.58; F, 5.21; I, 33.61%.

(3-Chloro-5-fluoro-4-hydroxyphenyl)4-methylphenyl methanone (4e): Yield: 68%; m. p.: 198.1-199.5 °C; IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 1640 (C=O), 3580-3685 (OH);

$^1$H NMR (400 MHz) (DMSO-d$_6$) $\delta$ (ppm): 3.03 (s, 3H, CH$_3$), 7.2-7.6 (m, 6H, Ar-H), 11.52 (bs, 1H, OH).

MS (EI): m/z (85%): M$^+$ 264.5; Anal. Calcd. for C$_{14}$H$_{10}$ClFO$_2$ (264.5): C, 63.53; H, 3.81; Cl, 13.39; F, 7.18. Found: C, 63.41; H, 3.72; Cl, 13.22; F, 7.29%.
2.1.4.3. GENERAL PROCEDURE FOR THE SYNTHESIS OF ETHYL 4-ARYLOYLARYLOXYACETATES (5a-e)

To a solution of compounds 4a-e (0.1156 mol) in dry DMF (175 mL), potassium carbonate (0.3468 mol) and ethyl bromoacetate (0.1273 mol) were added and the reaction mass was heated to 60 °C for 3 h. The reaction mass was diluted with ethyl acetate (200 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (100 mL). The organic layer was washed with water (3×30 mL), brine (2×40 mL), dried over sodium sulfate and concentrated to yield compounds 5a-e.

A typical procedure is described for the synthesis of ethyl [2-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acetate (5a): To a solution of compound 4a (31 g, 0.1156 mol) in dry DMF (175 mL), potassium carbonate (47.83 g, 0.3468 mol) and ethyl bromoacetate (21.11 g, 0.1273 mol) were added and the reaction mass was heated to 60 °C and maintained for 3 h. The reaction mass was diluted with ethyl acetate (200 mL), potassium carbonate was filtered off and the bed was washed with ethyl acetate (100 mL). The organic layer was washed with water (3×30 mL), brine (2×40 mL), dried over sodium sulfate and concentrated to yield compound 5a as brown pasty mass.

Yield: 97%; IR (KBr) ν_max (cm⁻¹): 1660 (C=O), 1730 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 1.16-1.22 (t, 3H, CH₃), 4.14-4.21 (q, 2H, CH₂), 5.02 (s, 2H, OCH₂), 7.64-7.8 (m, 6H, Ar-H). MS (EI): m/z (59%): M⁺ 354.5; Anal. Calcd. for C₁₇H₁₃ClF₂O₄ (354.5): C, 57.56; H, 3.69; Cl, 9.99; F, 10.71. Found: C, 57.41; H, 3.52; Cl, 9.79; F, 10.88%.

Compounds 5b-e were synthesized analogously starting with 4b-e respectively.

Ethyl [2-(4-chlorobenzoyl)-2-chloro-6-fluorophenoxy]acetate (5b): Yield: 92%; IR (KBr) ν_max (cm⁻¹): 1650 (C=O), 1740 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 1.18-1.22 (t, 3H, CH₃), 4.12-4.19 (q, 2H, CH₂), 5.02 (s, 2H, OCH₂), 7.59-7.75 (m,
6H, Ar-H). MS (EI): m/z (58%): M⁺ 371; Anal. Calcd. for C₁₇H₁₃Cl₂FO₄ (371): C, 55.01; H, 3.53; Cl, 19.10; F, 5.12. Found: C, 55.19; H, 3.41; Cl, 19.18; F, 5.23%.

**Ethyl [2-(4-bromobenzoyl)-2-chloro-6-fluorophenoxy]acetate (5c):** Yield: 91%; IR (KBr) νmax (cm⁻¹): 1660 (C=O), 1765 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 1.18-1.22 (t, 3H, CH₃), 4.21-4.12 (q, 2H, CH₂), 5.0 (s, 2H, OCH₂), 7.38-7.85 (m, 6H, Ar-H). MS (EI): m/z (57%): M⁺ 415.5; Anal. Calcd. for C₁₇H₁₃BrClFO₄ (415.5): C, 49.12; H, 3.15; Br, 19.22; Cl, 8.53; F, 4.57. Found: C, 49.28; H, 3.01; Br, 19.13; Cl, 8.62; F, 4.47%.

**Ethyl [2-(4-iodobenzoyl)-2-chloro-6-fluorophenoxy]acetate (5d):** Yield: 94%; IR (KBr) νmax (cm⁻¹): 1605 (C=O), 1750 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 1.16-1.22 (t, 3H, CH₃), 4.14-4.21 (q, 2H, CH₂), 5.0 (s, 2H, OCH₂), 7.21-7.9 (m, 6H, Ar-H). MS (EI): m/z (57%): M⁺ 462.5; Anal. Calcd. for C₁₇H₁₃ClIFO₄ (462.5): C, 44.13; H, 2.83; Cl, 7.66; F, 4.11; I, 27.43. Found: C, 44.23; H, 2.72; Cl, 7.57; F, 4.23; I, 27.31%.

**Ethyl [2-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acetate (5e):** Yield: 96%; IR (KBr) νmax (cm⁻¹): 1610 (C=O), 17665 (ester, C=O); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 1.19-1.23 (t, 3H, CH₃), 2.4 (s, 3H, Ar- CH₃), 4.2-4.3 (q, 2H, CH₂), 5.1 (s, 2H, OCH₂), 7.1-7.55 (m, 6H, Ar-H). MS (EI): m/z (57%): M⁺ 350.5; Anal. Calcd. for C₁₈H₁₆ClIFO₄ (350.5): C, 61.63; H, 4.60; Cl, 10.11; F, 5.42. Found: C, 61.51; H, 4.52; Cl, 10.19; F, 5.29%.

### 2.1.4.4. GENERAL PROCEDURE FOR THE SYNTHESIS OF 4-ARYLOYL ARYLOXY ETHANOIC ACIDS (6a-e)

A mixture of compounds 5a-e (0.0532 mol), 10% aqueous sodium hydroxide solution (100 mL) and THF (100 mL) was stirred at room temperature for 1 h. The reaction mass was acidified with 6N hydrochloric acid (150 mL) and the aqueous layer
was extracted with ethyl acetate (3x100 mL). The organic layer was washed with brine (3x60 mL), dried over anhydrous sodium sulfate and concentrated to achieve compounds 6a–e.

A typical procedure is described for the synthesis of 4-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]ethanoic acid (6a): A mixture of compound 5a (18 g, 0.0532 mol), 10% aqueous sodium hydroxide solution (100 mL) and THF (100 mL) was stirred at room temperature for 1 h. The reaction mass was acidified with 6N hydrochloric acid (150 mL) and the aqueous layer was extracted with ethyl acetate (3x100 mL). The organic layer was washed with brine (3x60 mL), dried over anhydrous sodium sulfate and concentrated to achieve compound 6a as a white solid.

Yield: 92%; m. p.: 127.3-128.6 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1660 (C=O), 1738 (acid C=O), 3470-3575 (acid OH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.9 (s, 2H, OCH\(_2\)), 7.3-7.87 (m, 6H, Ar-H), 13.1 (s, 1H, COOH). MS (EI): m/z (55%): M\(^+\) 326.5; Anal. Calcd. for C\(_{15}\)H\(_9\)ClF\(_2\)O\(_4\) (326.5): C, 55.15; H, 2.78; Cl, 10.85; F, 11.63. Found: C, 55.25; H, 2.61; Cl, 10.72; F, 11.49%.

Compounds 6b–e were synthesized analogously starting with 5b–e respectively.

[4-(4-Chlorobenzoyl)-2-chloro-6-fluorophenoxy]ethanoic acid (6b): Yield: 90.6%; m. p.: 137.7-139.1 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1640 (C=O), 1750 (acid C=O), 3480-3590 (acid OH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.85 (s, 2H, OCH\(_2\)), 7.15-7.77 (m, 6H, Ar-H), 12.9 (s, 1H, COOH). MS (EI): m/z (53%): M\(^+\) 343; Anal. Calcd. for C\(_{15}\)H\(_9\)Cl\(_2\)FO\(_4\) (343): C, 52.50; H, 2.64; Cl, 20.66; F, 5.54. Found: C, 52.59; H, 2.54; Cl, 20.75; F, 5.39%.

[4-(4-Bromobenzoyl)-2-chloro-6-fluorophenoxy]ethanoic acid (6c): Yield: 92%; m. p.: 153.8-155.3 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1655 (C=O), 1760 (acid C=O), 3490-3595 (acid OH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.9 (s, 2H, OCH\(_2\)), 7.25-7.88 (m, 6H, Ar-
H), 12.8 (s, 1H, COOH). MS (EI): m/z (51%): M+ 387.5; Anal. Calcd. for C15H9BrClF4O4 (387.5): C, 46.48; H, 2.34; Br, 20.62; Cl, 9.15; F, 4.90. Found: C, 46.33; H, 2.46; Br, 20.52; Cl, 9.33; F, 4.79%.

[4-(4-Iodobenzoyl)-2-chloro-6-fluorophenoxy]ethanoic acid (6d): Yield: 88%; m. p.: 139.7-140.8 °C; IR (KBr) ν max (cm⁻¹): 1615 (C=O), 1770 (acid C=O), 3465-3575 (acid OH); ¹H NMR (400 MHz) (DMSO-d6) δ (ppm): 4.8 (s, 2H, OCH₂), 7.1-7.65 (m, 6H, Ar-H), 11.9 (s, 1H, COOH). MS (EI): m/z (54%): M+ 434.5; Anal. Calcd. for C15H9ClF4O4 (434.5): C, 41.46; H, 2.09; Cl, 8.16; F, 4.37; I, 29.20. Found: C, 41.33; H, 2.19; Cl, 8.29; F, 4.21; I, 29.41%.

[4-(4-Fluorobenzoyl)-2-chloro-6-fluorophenoxy]ethanoic acid (6e): Yield: 89%; m. p.: 119.2-120.5 °C; IR (KBr) ν max (cm⁻¹): 1630 (C=O), 1765 (acid C=O), 3415-3545 (acid OH); ¹H NMR (400 MHz) (DMSO-d6) δ (ppm): 2.4 (s, 3H, CH₃), 4.96 (s, 2H, OCH₂), 7.16-7.77 (m, 6H, Ar-H), 12.65 (s, 1H, COOH). MS (EI): m/z (53%): M+ 322.5; Anal. Calcd. for C16H12ClF4O4 (322.5): C, 59.55; H, 3.75; Cl, 10.99; F, 5.89. Found: C, 59.63; H, 3.61; Cl, 10.78; F, 5.72%.

2.1.4.5. GENERAL PROCEDURE FOR THE SYNTHESIS OF 4-ARYLOYL ARYLOXY ACETHYDRAZIDES (7a-e)

Hydrazine hydrate (0.3372 mol) was added to a solution of compounds 6a-e (0.0562 mol) in ethanol (100 mL) at 0 °C and stirred the reaction mixture at the same temperature for 1 h. A white solid was separated out, which was quenched with water (100 mL), filtered and washed with water (50 mL). Finally, the solid was dried under vacuum to obtain compounds 7a-e.

A typical procedure is described for the synthesis of 2-[4-(4-fluorobenzoyl)-2-chloro-6-fluorophenoxy]acethydrazone (7a): Hydrazine hydrate (16.90 g, 0.3372 mol) was added to a solution of compound 6a (19 g, 0.0562 mol) in ethanol (100 mL) at 0 °C
and stirred the reaction mixture at the same temperature for 1 h. A white solid was separated out, which was quenched with water (100 mL), filtered and washed with water (50 mL). Finally, the solid was dried under vacuum to obtain compound 7a as white needle.

Yield: 79%; m. p.: 107.5-109.1 °C; IR (KBr) νmax (cm⁻¹): 1610 (C=O), 1645 (amide, C=O), 3100-3205 (NH-NH₂); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.35 (bs, 2H, NH₂), 4.69 (s, 2H, OCH₂), 7.2-7.86 (m, 6H, Ar-H), 9.32 (bs, 1H, CONH). MS (EI): m/z (42%): M⁺ 340.5; Anal. Calcd. for C₁₅H₁₁ClF₂N₂O₃ (340.5): C, 52.88; H, 3.25; Cl, 10.41; F, 11.15; N, 8.22. Found: C, 52.75; H, 3.38; Cl, 10.29; F, 11.24; N, 8.11%.

Compounds 7b-e were synthesized analogously starting with 6b-e respectively.

2-[4-(4-Chlorobenzoyl)-2-chloro-6-fluorophenoxy]acethydrazide (7b): Yield: 75.66%; m. p.: 141.4-142.9 °C; IR (KBr) νmax (cm⁻¹): 1625 (C=O), 1655 (amide, C=O), 3150-3255 (NH-NH₂); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.25 (bs, 2H, NH₂), 4.70 (s, 2H, OCH₂), 7.1-7.75 (m, 6H, Ar-H), 9.30 (bs, 1H, CONH). MS (EI): m/z (43%): M⁺ 357; Anal. Calcd. for C₁₅H₁₁ClFN₂O₃ (357): C, 50.44; H, 3.10; Cl, 19.85; F, 5.32; N, 7.84. Found: C, 50.31; H, 3.22; Cl, 19.71; F, 5.21; N, 7.72%.

2-[4-(4-Bromobenzoyl)-2-chloro-6-fluorophenoxy]acethydrazide (7c): Yield: 66%; m. p.: 137.2-138.6 °C; IR (KBr) νmax (cm⁻¹): 1635 (C=O), 1660 (amide, C=O), 3155-3270 (NH-NH₂); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.35 (bs, 2H, NH₂), 4.70 (s, 2H, OCH₂), 7.2-7.65 (m, 6H, Ar-H), 9.32 (bs, 1H, CONH). MS (EI): m/z (41%): M⁺ 401.5; Anal. Calcd. for C₁₅H₁₁BrClFN₂O₃ (401.5): C, 44.86; H, 2.76; Br, 19.90; Cl, 8.83; F, 4.73; N, 6.98. Found: C, 44.74; H, 2.62; Br, 19.78; Cl, 8.71; F, 4.61; N, 6.85%.

2-[4-(4-Iodobenzoyl)-2-chloro-6-fluorophenoxy]acethydrazide (7d): Yield: 78%; m. p.: 120.1-123.2 °C; IR (KBr) νmax (cm⁻¹): 1615 (C=O), 1615 (amide, C=O), 3120-3250 (NH-NH₂); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.38 (bs, 2H, NH₂), 4.72 (s, 2H,
OCH₂), 7.15-7.55 (m, 6H, Ar-H), 9.32 (bs, 1H, CONH). MS (EI): m/z (40%): M⁺ 448.5;
Anal. Calcd. for C₁₅H₁₁ClFIN₂O₃ (448.5): C, 40.16; H, 2.47; Cl, 7.90; F, 4.23; I, 28.29;
N, 6.24. Found: C, 40.03; H, 2.33; Cl, 7.79; F, 4.15; I, 28.18; N, 6.14%.

2-[4-(4-Methylbenzoyl)-2-chloro-6-fluorophenoxo]acethydrazide (7e): Yield: 80%;
m. p.: 78.3-79.7 °C; IR (KBr) νmax (cm⁻¹): 1605 (C=O), 1610 (amide, C=O), 3135-3270
(NH-NH₂); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 2.40 (s, 2H, CH₃), 4.37 (bs, 2H,
NH₂), 4.69 (s, 2H, OCH₂), 7.05–7.69 (m, 6H, Ar-H), 9.31 (bs, 1H, CONH). MS (EI): m/z
(43%): M⁺ 336.5; Anal. Calcd. for C₁₆H₁₄ClFIN₂O₃ (336.5): C, 57.07; H, 4.19; Cl, 10.53;
F, 5.64; N, 8.32. Found: C, 57.17; H, 4.11; Cl, 10.41; F, 5.78; N, 8.47%.

2.1.4.6. GENERAL PROCEDURE FOR THE SYNTHESIS OF N,N-di[2-(4-
ARYLOYLARYLOXY)ACETYL]HYDRAZINES (8a-j)

To a solution of compounds 6a-e (0.0032 mol) in DCM (20 mL), 2, 6-
dimethylpyridine (0.0107 mol) and TBTU (0.00323 mol) were added at room
temperature. Finally, compounds 7a-e (0.00294 mol) were added to the reaction mixture
and stirred at room temperature for 12 h. The reaction mixture was quenched with 10%
sodium bicarbonate solution (20 mL) and stirred for 30 min. The solid precipitate was
filtered, washed with water (20 mL) and dried to yield compounds 8a-j.

A typical procedure is described for the synthesis of N, N-di[2-chloro-6-
fluoro-4-(4-fluoro-benzoyl)phenoxy]acetyl hydrazide (8a): To a solution of
compound 6a (1.05 g, 0.0032 mol) in DCM (20 mL), 2, 6-dimethylpyridine (1 g, 0.0107
mol) and TBTU (1.04 g, 0.00323 mol) were added at room temperature. Finally,
compound 7a (1 g, 0.00294 mol) was added to the reaction mixture and stirred at room
temperature for 12 h. The reaction mixture was quenched with 10% sodium bicarbonate
solution (20 mL) and stirred for 30 min. The solid precipitate was filtered, washed with
water (20 mL) and dried to yield compound 8a as a white solid.
Yield: 81%; m. p.: 194.8-196.2 °C; IR (KBr) ν_max (cm⁻¹): 1690 (C=O), 1610 (amide, C=O), 3700-3500 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.90 (s, 4H, 2CH₂), 7.1-7.87 (m, 12H, Ar-H), 10.36 (bs, 2H, 2NH). MS (EI): m/z (61%): M⁺ 649; Anal. Calcd. for C₃₀H₁₈Cl₂F₄N₂O₆ (649): C, 55.49; H, 2.79; Cl, 10.92; F, 11.70; N, 4.31. Found: C, 55.37; H, 2.88; Cl, 10.83; F, 11.77; N, 4.43%.

Similarly compounds 8b-j were synthesized starting from compounds 6b-e and 7a-e.

2-(2-Chloro-4-(4-chlorobenzoyl)-6-fluorophenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-fluorobenzoyl)phenoxy)acetyl)acetohydrazide (8b): Yield: 78%; m. p.: 189.2-190.5 °C; IR (KBr) ν_max (cm⁻¹): 1680 (C=O), 1605 (amide, C=O), 3710-3510 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.85 (s, 4H, 2CH₂), 7.15-7.85 (m, 12H, Ar-H), 10.38 (bs, 2H, 2NH). MS (EI): m/z (60%): M⁺ 665.5; Anal. Calcd. for C₃₀H₁₈Cl₃F₃N₂O₆ (665.5): C, 54.12; H, 2.72; Cl, 15.97; F, 8.56; N, 4.21. Found: C, 54.21; H, 2.63; Cl, 15.84; F, 8.42; N, 4.13%.

2-(2-Chloro-4-(4-bromobenzoyl)-2-chloro-6-fluorophenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-fluorobenzoyl)phenoxy)acetyl)acetohydrazide (8c): Yield: 79%; m. p.: 209.1-210.4 °C; IR (KBr) ν_max (cm⁻¹): 1685 (C=O), 1610 (amide, C=O), 3720-3520 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.86 (s, 4H, 2CH₂), 7.0-7.79 (m, 12H, Ar-H), 10.4 (bs, 2H, 2NH). MS (EI): m/z (61%): M⁺ 710; Anal. Calcd. for C₃₀H₁₈BrCl₂F₃N₂O₆ (710): C, 50.73; H, 2.55; Br, 11.25; Cl, 9.98; F, 8.02; N, 3.94. Found: C, 50.61; H, 2.47; Br, 11.13; Cl, 9.86; F, 8.16; N, 3.82%.

2-(2-Chloro-6-fluoro-4-(4-fluorobenzoyl)phenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-iodobenzoyl)phenoxy)acetyl)acetohydrazide (8d): Yield: 78%; m. p.: 189.3-190.7 °C; IR (KBr) ν_max (cm⁻¹): 1675 (C=O), 1615 (amide, C=O), 3725-3525 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.85 (s, 4H, 2CH₂), 7.1-7.75 (m, 12H, Ar-H), 10.8 (bs,
2H, 2NH). MS (EI): m/z (59%): M+ 757; Anal. Calcd. for C30H18Cl2F3N2O6 (757): C, 47.58; H, 2.40; Cl, 9.36; F, 7.53; I, 16.76; N, 3.70. Found: C, 47.46; H, 2.49; Cl, 9.25; F, 7.61; I, 16.66; N, 3.79%.

2-(2-Chloro-6-fluoro-4-(4-fluorobenzoyl)phenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-methylbenzoyl)phenoxy)acetyl)acetohydrazide (8e): Yield: 84%; m. p.: 178.5-180.1 °C; IR (KBr) νmax (cm⁻¹): 1690 (C=O), 1620 (amide, C=O), 3735-3530 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 2.4 (s, 3H, CH₃), 4.8 (s, 4H, 2CH₂), 7.2-7.72 (m, 12H, Ar-H), 10.36 (bs, 2H, 2NH). MS (EI): m/z (58%): M+ 645; Anal. Calcd. for C31H21Cl2F3N2O6 (645): C, 57.69; H, 3.28; Cl, 10.99; F, 8.83; N, 4.34. Found: C, 57.58; H, 3.36; Cl, 10.88; F, 8.74; N, 4.25%.

N, N-di[di(2-chloro-6-fluoro-4-(4-chlorobenzoyl)phenoxy)]acetil hydrazide (8f): Yield: 79%; m. p.: 193.2-194.4 °C; IR (KBr) νmax (cm⁻¹): 1695 (C=O), 1605 (amide, C=O), 3720-3520 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 4.88 (s, 4H, 2CH₂), 7.12-7.73 (m, 12H, Ar-H), 10.38 (bs, 2H, 2NH). MS (EI): m/z (59%): M+ 682; Anal. Calcd. for C31H21Cl₂F₂N₂O₆ (682): C, 57.69; H, 3.28; Cl, 10.99; F, 8.74; N, 4.11. Found: C, 52.72; H, 2.55; Cl, 20.69; F, 5.66; N, 4.21%.

2-(2-Chloro-4-(4-chlorobenzoyl)-6-fluorophenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-methylbenzoyl)phenoxy)acetyl)acetohydrazide (8g): Yield: 84%; m. p.: 178.3-179.8 °C; IR (KBr) νmax (cm⁻¹): 1680 (C=O), 1615 (amide, C=O), 3735-3525 (NH-NH); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 2.3 (s, 3H, CH₃), 4.86 (s, 4H, 2CH₂), 7.05-7.75 (m, 12H, Ar-H), 10.35 (bs, 2H, 2NH). MS (EI): m/z (61%): M+ 661.5; Anal. Calcd. for C31H21Cl₂F₂N₂O₆ (661.5): C, 56.26; H, 3.20; Cl, 16.07; F, 5.74; N, 4.23. Found: C, 56.35; H, 3.28; Cl, 16.15; F, 5.65; N, 4.31%.

2-(2-Chloro-4-(4-chlorobenzoyl)-6-fluorophenoxy)-N’-(2-(2-chloro-6-fluoro-4-(4-iodobenzoyl)phenoxy)acetyl)acetohydrazide (8h): Yield: 77%; m. p.: 180.3-181.6 °C;
IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1685 (C=O), 1610 (amide, C=O), 3745-3530 (NH-NH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.75 (s, 4H, 2CH\(_2\)), 7.05-7.8 (m, 12H, Ar-H), 10.6 (bs, 2H, 2NH). MS (EI): m/z (60%): \( \text{M}^+ \) 773.5; Anal. Calcd. for C\(_{30}\)H\(_{18}\)Cl\(_3\)F\(_2\)IN\(_2\)O\(_6\) (773.5): C, 46.57; H, 2.34; Cl, 13.75; F, 4.91; I, 16.40; N, 3.62. Found: C, 46.64; H, 2.26; Cl, 13.83; F, 4.84; I, 16.47; N, 3.55%.

2-(4-(4-Bromobenzoyl)-2-chloro-6-fluorophenoxy)-N'(2-(2-chloro-4-(4-chloro benzoyl)-6-fluorophenoxy)acetyl)acetohydrazide (8i): Yield: 80%; m. p.: 182.4-183.8 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1670 (C=O), 1605 (amide, C=O), 3715-3520 (NH-NH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.85 (s, 4H, 2CH\(_2\)), 7.0-7.79 (m, 12H, Ar-H), 11.0 (bs, 2H, 2NH). MS (EI): m/z (62%): \( \text{M}^+ \) 726.5; Anal. Calcd. for C\(_{30}\)H\(_{18}\)BrCl\(_3\)F\(_2\)N\(_2\)O\(_6\) (726.5): C, 49.58; H, 2.50; Br, 10.99; Cl, 14.64; F, 5.23; N, 3.85. Found: C, 49.49; H, 2.56; Br, 10.89; Cl, 14.77; F, 5.31; N, 3.78%.

N, N-di[di(2-chloro-6-fluoro-4-(4-chloro-benzoyl)phenoxy)]acetyl hydrazide (8j): Yield: 82%; m. p.: 201.7-203.1 °C; IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)): 1685 (C=O), 1605 (amide, C=O), 3720-3535 (NH-NH); \(^1\)H NMR (400 MHz) (DMSO-d\(_6\)) \( \delta \) (ppm): 4.88 (s, 4H, 2CH\(_2\)), 6.9-7.71 (m, 12H, Ar-H), 10.35 (bs, 2H, 2NH). MS (EI): m/z (61%): \( \text{M}^+ \) 771; Anal. Calcd. for C\(_{30}\)H\(_{18}\)BrCl\(_2\)F\(_2\)N\(_2\)O\(_6\) (771): C, 46.72; H, 2.35; Br, 20.72; Cl, 9.19; F, 4.93; N, 3.63. Found: C, 46.64; H, 2.42; Br, 20.65; Cl, 9.28; F, 4.82; N, 3.71%.

2.1.4.7. GENERAL PROCEDURE FOR THE SYNTHESIS OF 2,5-DI(ARYLOYL ARYLOXYMETHYL)-1,3,4-OXADIAZOLES (9a-j)

To a solution of compounds 8a-j (0.0023 mol) in DCM (20 mL), pyridine (0.0069 mol) and triflic anhydride (0.0051 mol) were added at 0 °C and the reaction mixture was stirred at 0 °C for 3 h. The reaction mass was diluted with DCM (20 mL), the organic layer was washed with 10% sodium bicarbonate (3×10 mL), water (3×10 mL) and brine
(3×10 mL). Finally, the organic layer was dried over sodium sulfate and concentrated to yield compounds 9a-j.

A typical procedure is described for the synthesis of 2,5-di[2-fluoro-4-(4-fluoro)benzoyl-6-chlorophenoxy methyl] 1,3,4-oxadiazole (9a): To a solution of compound 8a (1.5 g, 0.0023 mol) in DCM (20 mL), pyridine (0.56 g, 0.0069 mol) and triflic anhydride (1.44 g, 0.0051 mol) were added at 0 °C and the reaction mixture was stirred at 0 °C for 3 h. After the completion of the reaction monitored by TLC, the reaction mass was diluted with DCM (20 mL), the organic layer was washed with 10% sodium bicarbonate (3×10 mL), water (3×10 mL) and brine (3×10 mL). Finally, the organic layer was dried over sodium sulfate and concentrated to yield a brown gummy mass. The crude product was purified by column chromatography using silica gel as stationary phase and hexane:ethyl acetate as mobile phase to achieve compound 9a as a white solid.

Yield; 73%; m. p.: 129.5-130.2 °C; IR (Nujol) ν<sub>max</sub> (cm<sup>-1</sup>): 1153 (C=O-C linkage), 1658 (C=O), 1683 cm<sup>-1</sup> (C=N); <sup>1</sup>H NMR (400 MHz) (DMSO-d<sub>6</sub>) δ (ppm): 5.6 (s, 4H, 2CH<sub>2</sub>), 7.05-7.81 (m, 12H, Ar-H); <sup>13</sup>C NMR (400 MHz, DMSO-d6) δ: 64.32-64.37, 115.77-117.4, 126.93-127.78, 132.52-134.8, 144.48-144.61, 153.46, 155.95, 162.99-166.26 191.28; MS (EI): m/z (70%): M<sup>+</sup> 631; Anal. Calcd. for C<sub>30</sub>H<sub>16</sub>Cl<sub>2</sub>F<sub>4</sub>N<sub>2</sub>O<sub>5</sub> (631): C, 57.07; H, 2.55; Cl, 11.23; F, 12.04; N, 4.44. Found: C, 57.17; H, 2.47; Cl, 11.31; F, 12.13; N, 4.36%.

Similarly compounds 9b-j were synthesized starting from compounds 8b-j.

2-Fluoro-4-(4-fluoro)benzoyl-6-chlorophenoxy methyl-2-Fluoro-4-(4-chloro)benzoyl-6-chlorophenoxy methyl 1,3,4-oxadiazole (9b): Yield; 76%; m. p.: 88.9-90.2 °C; IR (Nujol) ν<sub>max</sub> (cm<sup>-1</sup>): 1158 (C=O-C linkage), 1660 (C=O), 1685 (C=N); <sup>1</sup>H NMR (400 MHz) (DMSO-d<sub>6</sub>) δ (ppm): 5.6 (s, 4H, 2CH<sub>2</sub>), 7.1-7.79 (m, 12H, Ar-H); <sup>13</sup>C NMR (400 MHz) (DMSO-d6) δ: 64.33-64.38, 115.78-117.4, 127.00-127.8, 132.54-134.8, 144.48-144.61, 153.46, 155.95, 162.99-166.26 191.28; MS (EI): m/z (70%): M<sup>+</sup> 631; Anal. Calcd. for C<sub>30</sub>H<sub>16</sub>Cl<sub>2</sub>F<sub>4</sub>N<sub>2</sub>O<sub>5</sub> (631): C, 57.07; H, 2.55; Cl, 11.23; F, 12.04; N, 4.44. Found: C, 57.17; H, 2.47; Cl, 11.31; F, 12.13; N, 4.36%.
MHz, DMSO-d6) δ: 64.80-64.86, 116.24-117.92, 127.36-129.35, 132.06-138.68, 144.97, 145.11-145.24, 153.93, 156.42, 163.45, 164.23, 166.73, 191.73-192.01; MS (EI): m/z (71%): M⁺ 647.5; Anal. Calcd. for C₃₀H₁₆Cl₃F₃N₂O₅ (647.5): C, 55.62; H, 2.49; Cl, 16.42; F, 8.80; N, 4.32. Found: C, 55.71; H, 2.41; Cl, 16.51; F, 8.72; N, 4.23%.

2-Fluoro-4-(4-fluorobenzoyl)-6-chlorophenoxymethyl-2-Fluoro-4-(4-bromo)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9c): Yield: 71%; m. p.: 88.9-90.2 °C; IR (Nujol) ν_max (cm⁻¹): 1155 (C=O-C linkage), 1665 (C=O), 1680 (C=N); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 5.55 (s, 4H, 2CH₂), 7.05-7.78 (m, 12H, Ar-H); ¹³C NMR (400 MHz, DMSO-d₆) δ: 64.80-64.86, 116.23-117.91, 127.38-128.26, 132.13-133.27, 134.35-135.43, 144.98-145.26, 153.93, 156.42, 163.45, 164.23, 166.73, 191.72-192.20; MS (EI): m/z (69%): M⁺ 692; Anal. Calcd. for C₃₀H₁₆BrCl₂F₃N₂O₅ (692): C, 52.05; H, 2.33; Br, 11.54; Cl, 10.24; F, 8.23; N, 4.05. Found: C, 52.15; H, 2.26; Br, 11.46; Cl, 10.17; F, 8.31; N, 4.11%.

2-Fluoro-4-(4-fluorobenzoyl)-6-chlorophenoxymethyl-2-Fluoro-4-(4-iodo)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9d): Yield: 75.2%; m. p.: 88.9-90.6 °C; IR (Nujol) ν_max (cm⁻¹): 1160 (C=O-C linkage), 1665 (C=O), 1680 (C=N); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 5.6 (s, 4H, 2CH₂), 7.05-7.78 (m, 12H, Ar-H); ¹³C NMR (400 MHz, DMSO-d₆) δ: 64.37-64.43, 102.07, 115.8-116.05, 117.24-117.49, 126.95-127.81, 131.43-137.72, 144.67, 153.5, 156.0, 163, 191.3-192.1; MS (EI): m/z (68%): M⁺ 739; Anal. Calcd. for C₃₀H₁₆BrCl₂F₃IN₂O₅ (739): C, 48.74; H, 2.18; Cl, 9.59; F, 7.71; I, 17.17; N, 3.79. Found: C, 48.66; H, 2.24; Cl, 9.51; F, 7.63; I, 17.27; N, 3.69%.

2-Fluoro-4-(4-fluorobenzoyl)-6-chlorophenoxymethyl-2-Fluoro-4-(4-methyl)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9e): Yield: 68.5%; m. p.: 72.0-73.4 °C; IR (Nujol) ν_max (cm⁻¹): 1150 (C=O-C linkage), 1635 (C=O), 1690 (C=N); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 2.40 (s, 3H, CH₃), 5.59 (s, 4H, 2CH₂), 7.05-7.8 (m, 12H, Ar-
$\text{H)}; ^{13}\text{C NMR (400 MHz, DMSO-d6) } \delta: 64.77-64.82, 116.21-117.83, 127.24-130.38, 132.97-135.12, 144.33-145.09, 153.91, 156.41, 163.46, 191.7-192.61; \text{ MS (EI): m/z (69%): M}^+ 627; \text{ Anal. Calcd. for C}_3\text{H}_{19}\text{Cl}_2\text{F}_3\text{N}_2\text{O}_5 (627): C, 59.35; H, 3.05; Cl, 11.30; F, 9.08; N, 4.47. \text{ Found: C, 59.43; H, 3.14; Cl, 11.21; F, 9.17; N, 4.55%}.

\text{2,5-di[2-fluoro-4-(4-chloro)benzoyl-6-chlorophenoxymethyl] 1,3,4-oxadiazole (9f):} \text{ Yield: 75.3%; m. p.}: 110.4-111.7 °C; \text{ IR (Nujol) } v_{\text{max}} (\text{cm}^{-1}): 1152 (\text{C-O-C linkage}), 1627 (\text{C=O}), 1685 (\text{C=N}); ^1\text{H NMR (400 MHz) (DMSO-d}_6) \delta (\text{ppm}): 5.6 (s, 4H, 2\text{CH}_2), 7.1-7.77 (m, 12H, Ar-H); ^{13}\text{C NMR (400 MHz, DMSO-d6) } \delta: 64.31-64.36, 117.23-117.44, 126.99, 127.76-127.79, 128.87, 131.59, 133.91-134.59, 138.20, 144.61-144.74, 153.44, 155.93, 162.96, 191.55; \text{ MS (EI): m/z (66%): M}^+ 664; \text{ Anal. Calcd. for C}_{30}\text{H}_{16}\text{Cl}_4\text{F}_2\text{N}_2\text{O}_5 (664): C, 54.24; H, 2.43; Cl, 21.35; F, 5.72; N, 4.22. \text{ Found: C, 54.31; H, 2.35; Cl, 21.26; F, 5.61; N, 4.32%}.

\text{2-Fluoro-4-(4-chloro)benzoyl-6-chlorophenoxymethyl-2-Fluoro-4-(4-methyl)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9g):} \text{ Yield: 72%; m. p.}: 87.6-89.9 °C; \text{ IR (Nujol) } v_{\text{max}} (\text{cm}^{-1}): 1100 (\text{C-O-C linkage}), 1600 (\text{C=O}), 1675 (\text{C=N}); ^1\text{H NMR (400 MHz) (DMSO-d}_6) \delta (\text{ppm}): 2.3 (s, 3H, CH}_3), 5.6 (s, 4H, 2\text{CH}_2), 7.05-7.79 (m, 12H, Ar-H); ^{13}\text{C NMR (400 MHz, DMSO-d6) } \delta: 64.37-64.41, 117.11-117.51, 126.85-127.86, 128.93-130.00, 131.66, 133.29-134.71, 138.26, 143.94-144.81, 153.50, 155.99, 163.03, 191.60; \text{ MS (EI): m/z (67%): M}^+ 643.5; \text{ Anal. Calcd. for C}_{31}\text{H}_{19}\text{Cl}_3\text{F}_2\text{N}_2\text{O}_5 (643.5): C, 57.83; H, 2.97; Cl, 16.52; F, 5.90; N, 4.35. \text{ Found: C, 57.74; H, 2.88; Cl, 16.41; F, 5.81; N, 4.26%}.

\text{2-Fluoro-4-(4-chloro)benzoyl-6-chlorophenoxymethyl-2-Fluoro-4-(4-iodo)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9h):} \text{ Yield: 71%; m. p.}: 118.3-119.6 °C; \text{ IR (Nujol) } v_{\text{max}} (\text{cm}^{-1}): 1105 (\text{C-O-C linkage}), 1610 (\text{C=O}), 1690 (\text{C=N}); ^1\text{H NMR (400 MHz) (DMSO-d}_6) \delta (\text{ppm}): 5.55 (s, 4H, 2\text{CH}_2), 7.12-7.72 (m, 12H, Ar-H); ^{13}\text{C NMR}
(400 MHz, DMSO-d6) δ: 64.37-64.43, 102.05, 117.30-117.51, 127.04-128.94, 131.43-131.66, 134.05-135.24, 137.72-138.27, 144.67-144.804, 153.50, 155.99, 163.03, 191.61-192.1; MS (EI): m/z (69%): M+ 755.5; Anal. Calcd. for C_{30}H_{16}Cl_{3}F_{2}IN_{2}O_{5} (755.5): C, 47.68; H, 2.13; Cl, 14.07; F, 5.03; I, 16.79; N, 3.71. Found: C, 47.61; H, 2.22; Cl, 14.15; F, 5.14; I, 16.68; N, 3.63%.

2-Fluoro-4-(4-chloro)benzoyl-6-chlorophenoxymethyl-2-Fluoro-4-(4-bromo)benzoyl-6-chlorophenoxymethyl 1,3,4-oxadiazole (9i): Yield: 73%; m. p.: 130.6-131.8 °C; IR (Nujol) v_{max} (cm⁻¹): 1110 (C=O-C linkage), 1615 (C=O), 1685 (C=N); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 5.6 (s, 4H, 2CH₂), 7.12-7.79 (m, 12H, Ar-H); ¹³C NMR (400 MHz, DMSO-d₆) δ: 64.86, 117.71-117.92, 127.46, 129.36, 132.07-132.30, 135.44, 165.44; MS (EI): m/z (67%): M⁺ 708.5; Anal. Calcd. for C_{30}H_{16}BrCl_{3}F_{2}N_{2}O_{5} (708.5): C, 50.84; H, 2.28; Br, 11.27; Cl, 15.01; F, 5.36; N, 3.95. Found: C, 50.77; H, 2.35; Br, 11.37; Cl, 15.11; F, 5.27; N, 3.88%.

2,5-di[2-fluoro-4-(4-bromo)benzoyl-6-chlorophenoxymethyl] 1,3,4-oxadiazole (9j): Yield: 71%; m. p.: 132.3-133.6 °C; IR (Nujol) v_{max} (cm⁻¹): 1106 (C-O-C linkage), 1610 (C=O), 1675 (C=N); ¹H NMR (400 MHz) (DMSO-d₆) δ (ppm): 5.57 (s, 4H, 2CH₂), 7.05-7.78 (m, 12H, Ar-H); ¹³C NMR (400 MHz, DMSO-d₆) δ: 64.30-64.36, 117.24-117.45, 126.97-127.79, 131.67-131.81, 133.87-134.92, 138.20, 144.61-144.75, 153.43, 155.92, 162.96, 191.72; MS (EI): m/z (68%): M⁺ 753; Anal. Calcd. for C_{30}H_{16}BrCl_{2}F_{2}N_{2}O_{5} (753): C, 47.84; H, 2.14; Br, 21.22; Cl, 9.41; F, 5.04; N, 3.72. Found: C, 47.75; H, 2.22; Br, 21.31; Cl, 9.48; F, 5.12; N, 3.65%.

2.1.5. BIBLIOGRAPHY

126.


2.1.6. SPECTRA
(3-chloro-5-fluoro-4-hydroxyphenyl) 4-fluorophenyl methanone (4a).
2-[4-(4-fluorobenzoyl)phenyl]-2-chloro-6-fluorophenoxy acetohydrazide (7a)
CHAPTER - 2
PART-II

EVALUATION OF 2,5-DI(4-ARYLOYLARYL OXYMETHYL)-1,3,4-OXADIAZOLES AS ANTI-CANCER AGENTS

"Two things are infinite: the universe and human stupidity; and I'm not sure about the universe." - Albert Einstein.
2.2.0 INTRODUCTION

Cancer is a disease in which a group of cells display uncontrolled growth in a body. Under normal conditions, cell death and cell proliferation are balanced throughout the life of multicellular organisms.\(^1\) The various types of mature cells in the body have a specific life span. After the death of these cells, new cells are generated by the proliferation and differentiation into different types of cells. Sometimes cells begin to divide in an uncontrolled manner and they no longer respond to the growth regulatory mechanisms. These cells divide and form a clump of cells producing a tumor or neoplasm.\(^2\) About 200 types of cancers affecting major organs like lungs, brain, kidneys, colon, breasts and stomach have been identified. According to the National Cancer Institute, most can fit into categories of carcinoma, sarcoma, leukemia, lymphoma, and central nervous system cancers. The development of efficient, selective and less toxic anticancer agents is a challenge in cancer research.\(^3\)

Studies have shown that modification in DNA and RNA through mutation leads to altered protein production which is the main cause for the development of cancer.\(^4^-^7\) Nowadays the field of drug discovery for cancer has expanded in such a way as to target the disease in regulating DNA replication by using nucleotide analogues like 5-fluorouracil and enzyme inhibitors like methotrexate.\(^8,\) \(^9\) Other than that imatinib, a drug developed against the activated tyrosine kinases in chronic myelogenous leukemia is yet another development in the field of cancer.\(^10\)

Heterocyclic compounds play an important role as anticancer agents because of their excellent inhibitory activity against receptor tyrosine kinases,\(^11\) raf kinases,\(^12\) protein tyrosine kinases\(^13\) and NADH oxidase,\(^14\) which plays critical roles in many aspects of tumorigenesis. Among heterocyclic compounds, nitrogen containing compounds like 1,3,4 oxadiazoles have exhibited broad spectrum antitumor activity against the HeLa
cancer cell lines\textsuperscript{15} and with an IC\textsubscript{50} of 9.3 μM against the DU145 cancer cell lines.\textsuperscript{16}

Oxadiazole analogues also inhibit bacterial growth by inhibiting DNA replication or DNA transcription.\textsuperscript{17}

\begin{align*}
\text{REACTION SCHEME - 2.1.1 FROM CHAPTER - 2 PART-I}
\end{align*}
In continuation of our research work on anticancer agents\textsuperscript{18,19} and with the goal of discovering new anticancer agents, this chapter is focused on the novel 2,5-di(4-aryloylaryloxymethyl)-1,3,4-oxadiazoles \textit{9a-j} which possess the antiproliferative property by inhibiting cell cycle and promoting apoptosis.

\subsection*{2.2.1 GENERAL PROCEDURE FOR ANTICANCER SCREENING}

The human leukemia cells, K562 and CEM were selected for the purpose of preliminary anticancer screening of newly synthesized compounds \textit{9a-j}. To assess the cytotoxicity, trypan blue dye exclusion assay, MTT assay and LDH assay were employed. For this, cells growing in log phase were treated with different concentrations (10, 50, 100 and 250 μM) of the title compounds \textit{9a-j}. Besides, cytotoxicity of compounds \textit{9a-j} on the growth of normal cells was assessed using MTT assay. A DNA fragmentation assay, which is an indicator of apoptosis, was also performed.

\subsection*{2.2.1.1. CELL LINES AND CULTURE}

Human cell lines, K562 and CEM (T- cell leukemia) were purchased from National Center for Cell Science, Pune, India. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 μg of streptomycin/mL and incubated at 37 °C in a humidified atmosphere containing 5% CO\textsubscript{2}.

\subsection*{2.2.1.2. IN VITRO CELL VIABILITY AND CELL PROLIFERATION ASSAY: TRYPSAN BLUE EXCLUSION ASSAY}

Cell viability was monitored by the trypan blue exclusion assay as reported earlier.\textsuperscript{20} To determine the effect of compounds \textit{9a-j} on the viability of K562 or CEM cells, approximately 0.75×10\textsuperscript{5} cells/mL were seeded in a 6-well tissue culture plate for 24 h and compounds \textit{9a-j} were added at a concentration of 10, 50, 100 and 250 μM.
5-Fluorouracil treated cells were used as positive control. Cells were collected at intervals of 24 h and resuspended in 0.4% Trypan blue (viable-unstained and non viable-blue). The number of viable cells were counted using haemocytometer chamber.

2.2.1.3. MTT ASSAY

Cell proliferation was further assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the ability of viable cells to metabolize a yellow tetrazolium salt to violet formazan. Exponentially growing K562, or CEM cells (1×10^4 cells /well) were plated in triplicates and incubated with 10, 50, 100 and 250 μM of compounds 9a-j. Cells were harvested after 48 and 72 h of treatment and incubated with MTT (0.5 mg/ mL) at 37 °C. The blue MTT formazan precipitate was then solubilized in detergent (50% final concentration of N,N-dimethylformamide and 10% of sodium dodecyl sulfate). Absorbance was measured at 570 nm using ELISA plate reader. The mean absorbance of culture medium was used as the blank and was subtracted. IC_{50} values (concentration of compound causing 50% inhibition of cell growth) were estimated after 72 h of title compounds treatment (Table - 2.2.1).

2.2.1.4. LDH RELEASE ASSAY

Lactate dehydrogenase (LDH) release is an indicator of membrane integrity and hence cell injury. LDH assay was performed as per standard protocols to assess the LDH release in the culture media following the treatment with compounds 9b, 9e, 9i and 9j (10, 50 and 100 μM) on K562 cells for 24 and 48 h. The intracellular LDH was determined after lysing the cells by freezing at -80°C and rapid thawing. The LDH release was measured at an absorbance of 490 nm.
The percentage of LDH release was calculated as: \( \frac{\text{LDH activity in the media}}{\text{LDH activity in media} + \text{LDH activity in total cells}} \times 100\% \). The LDH release was plotted as graph as shown in Figure - 2.3.1.

### 2.2.1.5. DNA FRAGMENTATION ASSAY

DNA fragmentation was performed for elucidating the mode of action of the investigated compounds, especially with respect to induction of oligonucleosomal DNA fragmentation (DNA ladder), which is a characteristic feature of the programmed cell death or apoptosis. During the apoptotic process, activated nucleases degrade the higher order chromatin structure of DNA into mono and oligonucleosomal DNA-fragments. Apoptotic degradation of DNA was analyzed by agarose gel electrophoresis.\(^{20}\) Briefly, K562 cells were cultured in the presence of \(9b\) or \(9i\) at 10, 50 and 100 μM for 72 h. Cells were harvested and genomic DNA was extracted using standard protocol. DNA was resuspended in 250 μl of TE buffer. The DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining and photographed.

### 2.2.2 RESULTS AND DISCUSSION

Newly synthesized 1,3,4-oxadiazole analogues \(9a-j\) were assessed for cytotoxicity against two human leukemia cell lines, K562 (Chronic myelogenous leukemia) and CEM (T-cell leukemia). The effective concentrations of compounds \(9a-j\) required to inhibit K562 and CEM cell growth and survival were determined by carrying out dose response experiments using a trypan blue dye exclusion assay. The cell viability was further assessed by MTT assay and 5-fluorouracil treated cells were used as a positive control. The cells with DMSO (equivalent to DMSO used in 250 μM) were used as vehicle control, since the compounds \(9a-j\) were dissolved in DMSO. The number of viable cells decreased at different points of time and concentrations on exposure to compounds \(9a-j\).
Compounds 9a-j did not exhibit any significant inhibition of cell proliferation at a concentration of 10 μM against both cell lines. The cell viability at both 48 and 72 h were affected at 50 and 100 μM concentrations of compounds 9b-f and 9h-j. Compounds 9b with fluoro and chloro groups, 9e with fluoro and methyl groups, 9i with chloro and bromo groups and 9j with two bromo groups in the two different benzophenone moieties showed complete inhibition at 48 and 72 h treatment at a 250 μM concentration against the CEM cell line. Compound 9i was most effective at 50 μM exhibiting complete inhibition. However the DMSO control did not show any significant toxic effect. Compounds 9a with two fluoro groups in the two different benzophenone moieties and 9g with chloro and methyl groups in the two different benzophenone moieties showed moderate inhibitory activity. The electron withdrawing halo groups at the para position in the benzophenone moieties are important for enhancing the inhibitory activity while the electron releasing methyl group at the para position decreases the activity as seen in 9e and 9g (Table - 2.2.1).

Table - 2.2.1: IC<sub>50</sub> values of compounds 9a-j as determined based on MTT assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K562</td>
</tr>
<tr>
<td>9a</td>
<td>190 ± 10.0</td>
</tr>
<tr>
<td>9b</td>
<td>16 ± 2.8</td>
</tr>
<tr>
<td>9c</td>
<td>60 ± 5.5</td>
</tr>
<tr>
<td>9d</td>
<td>75 ± 7.2</td>
</tr>
<tr>
<td>9e</td>
<td>22 ± 4.4</td>
</tr>
<tr>
<td>9f</td>
<td>73 ± 7.0</td>
</tr>
<tr>
<td>9g</td>
<td>122 ± 10.0</td>
</tr>
<tr>
<td>9h</td>
<td>73 ± 6.0</td>
</tr>
<tr>
<td>9i</td>
<td>10 ± 2.1</td>
</tr>
<tr>
<td>9j</td>
<td>20 ± 2.8</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>28 ± 3.8</td>
</tr>
</tbody>
</table>
Release of LDH serves as a marker for membrane integrity. Hence, LDH release assay was performed to test the cell damage by compounds 9b, 9e, 9i and 9j. Results showed a dose- and time-dependent increase in LDH (Figure - 2.2.1), which further confirmed our results.

Figure - 2.2.1: Time-and dose dependent LDH release in K562 cells treated with compounds 9b, 9e, 9i and 9j.

Oligonucleosomal DNA fragmentation and nuclear condensation are the criteria to analyze the DNA damage consequent upon treatment with compounds 9b and 9i. From the K562 cells treated with increasing concentrations of compounds 9b and 9i chromosomal DNA was extracted and used for agarose gel electrophoresis. The result of this assay showed that DNA fragmentation leads to a smear formation in gel lanes in which cells treated with compounds 9b and 9i (Figure - 2.2.2).

Number of breaks in the chromosomal DNA of K562 cells resulted in smearing. The intensity of smear increased with the dose, 50 μM showing moderate and 100 μM showing maximum smearing (Figure - 2.2.2). This suggests that compounds 9b and 9i induce fragmentation of chromosomal DNA leading to apoptosis.

In summary, a series of 1,3,4 oxadiazoles 9a-j were synthesized and evaluated for antiproliferative activity against human leukemic cell lines. From the current investigation, structural activity relationship of these compounds suggests that the position and the type of substituent on the aromatic ring in 9a-j are important for activity. Compounds 9b and 9i with chloro group play a dominant role in inhibiting the leukemic
cell proliferation. Further, a detailed investigation on the structural activity relationship should entail structural modification on the aromatic ring for the discovery of more potent cytotoxic compounds. Studies on the mechanism of action of the title compounds and modification are under progress.

Figure 2.2.2. Detection of DNA damage induced by 9b and 9i in K562 cells.

2.2.3 BIBLIOGRAPHY


