SYNTHESIS AND CHARACTERIZATION OF PRECURSOR'S FOR BIOTRANSFORMATION

A

Thesis Submitted

in partial fulfillments of requirements for the

Degree of

Master of Science in Chemistry

Submitted By

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DEDICATED TO MY PARENTS

Acknowledgements

It's said that life is a carnival of experiences and a journey with various goals. So in my journey where I experienced this project I want to thank the supreme almighty for his presence in my soul and in my mind.

I take this opportunity to thank my guide Dr. Manmohan Chhibber, Lecturer, School of Chemistry & Biochemistry without whose presence the whole project just would have been a dream. I am extremely indebted to him for the scientific attitude and utmost patience he has installed in me which will definitely stand in all future endeavour and it was because of him that I was able to learn so much in this short period.

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Last but not least I owe my thesis to my parents and my elder brother Sandeep who are the building pillars of my life.

Dated: June 15, 2009

ANKUSH KANWAR

Candidate's Declaration

I hereby declare that the work being presented in the dissertation entitled **"Synthesis and Characterization of Precursors for Biotransformation"**, in partial fulfillment of the requirements for the award of the degree of Masters of science (Chemistry), School of Chemistry and Biochemistry (SCBC), Thapar University, Patiala, is my own work during the period of Jan 2009 to May 2009, under the supervision of Dr. Manmohan Chhibber, Lecturer, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree.

Patiala

Date: 16-06-09

Ankush Kanwar

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

Dr. Manmohan Chhibber
Project Supervisor,
Lecturer (SCBC),
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Dr. Susheel Mittal 29/6/09
Head, SCBC
Thapar University

Certificate

This is to certify that the project entitled **"Synthesis and Characterization of Precursors for Biotransformation"**, being submitted by Mr. Ankush Kanwar in partial fulfillment of the requirement for the award of degree of Master of Science (Chemistry), Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Manmohan Chhibber and that no part of this project has been submitted for the award of any other degree.

Dr. Manmohan Chhibber,
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Abbreviations

d	Doublet
dd	Double doublet
DPE	Diphenyl phenyl ethers
m	Multiplet
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
s	Singlet
S_NAr	Aromatic nucleophilic substitution reaction
LR	Laboratory reagent
t	Triplet
TLC	Thin layer chromatography

List of contents

INTRODUCTION

Every science at molecular level constitutes chemistry .Since the synthesis of urea[1] , the art of organic synthesis has come a long way .Whereas on one side it has given unique molecules, which not only mimic nature but also out perform them, there on other side the hazardous chemicals have posed a threat to health , environment and earth. Perhaps the time has come when such hazardous chemicals have to be replaced by safe and effective reagents. Biotransformation is mimicking the nature's way of doing chemistry. By definition the chemical conversion of a substance into a desired product with the aid of a living whole cell or an enzyme is termed as biotransformation [2]. Microorganisms, with a multiplicity of constitutive enzymes are capable of performing a vast number of chemical reactions which are essential for maintaining the life functions of the cell, including growth and reproduction [3].

Enzymes accept a certain substrate and catalyze particular reactions which usually represent one step in a metabolic pathway. Besides their natural substrates, many enzymes also accept foreign, but structurally related compounds and thus catalyze "unnatural" reactions with substrates supplied to the medium. Reaction products which are not further degraded accumulate in the medium, from where they can be isolated.

There is no denying the fact that biotransformation has several advantages over conventional synthesis[4,5] . Reaction specificity [6], regio specificity[7] and stereo specificity [8] are main advantages over conventional synthesis, that whole cell or enzymes offer. This is due to three-dimensional and asymmetric environment which enables the enzyme to display high selectivity with respect to its substrate and even to distinguish between different stereo chemical configurations of substrate molecule. Unlike chemical reactions where high temperature and sometimes high pressures are required, biotransformation display high catalytic activities even under mild reaction conditions [9]. The activation energy of chemical reactions is significantly lowered by enzyme/substrate complex thus offering reactions in aqueous media at temperatures below 40° C, neutral pH and normal pressure. Labile molecules can be converted without undesired decomposition or other side reactions using such mild conditions. Some unique transformations that are not possible or economically feasible by traditional chemical synthesis can be affected using

biotransformations. For example the hydroxylation of aromatic hydrocarbons to their corresponding *cis*-glycols is only possible by a biological route [10].

Despite several advantages this field has not matured sufficiently to be applied on an industrial scale. Long reaction times, low yield, non availability of sufficient strains to perform desired reactions are some of the challenges that need to be taken up. Also sometimes the reactants used or the products formed inhibit the enzymes thus poisoning the system [11]. Another difficulty while setting up such reactions is solubility of substrate in culture media. Most of the organic compounds being insoluble in water are unavailable to microorganisms or enzymes to be taken up. Therefore, it becomes necessary to make the compound available to organism in its biotransformable form. Therefore, even while working on biotransformation it becomes necessary to use synthetic organic chemistry to prepare substrates that inhibit or activate enzymes and to convert them into bioavailable organic molecules, either directly or by solubilizing them in aqueous culture media.

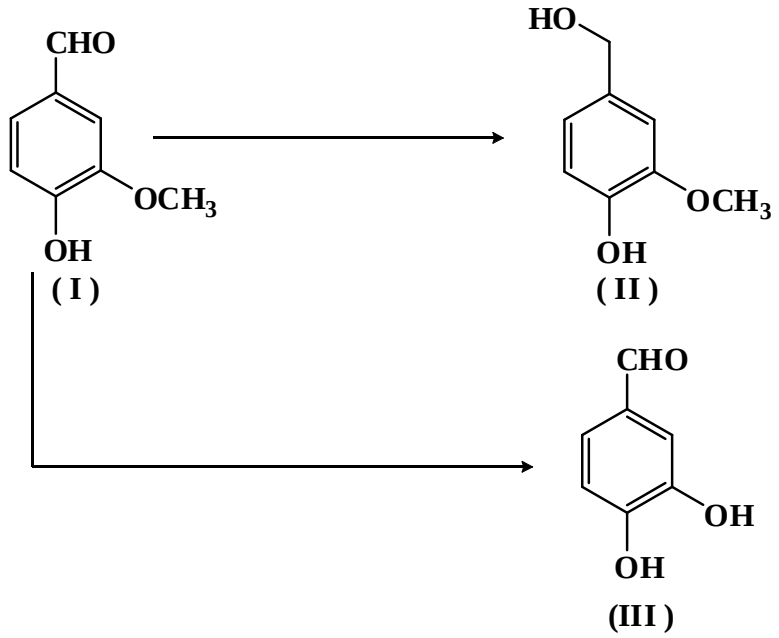
Present work reports certain synthetic organic reactions where organic molecules have been converted to their products to make them water soluble or bioavailable. The need of synthetic standards was also one of the motives so that biotransformed products can be compared with it.

All the prepared compounds were analysed by ^{1}H, ^{13}C NMR and by mass spectroscopy techniques. The reaction monitoring was done by was done by thin layer chromatography and purification done with the help of column chromatography

Aim and Objective

Biotransformation uses natural reagents for affecting a chemical reaction. For a substrate to be successfully biotransformed, it should be easily soluble in water in which the microorganism is growing. As most of the organic compounds are insoluble in water, so the microorganism is unable to use the given compound as its substrates. To solve this problem, this work was initiated with an objective to synthesize and characterize various compounds that are easily soluble in water so that they can be easily biotransformed into products. The solubility and usage of these compounds for chemical transformation will be further taken up**.**

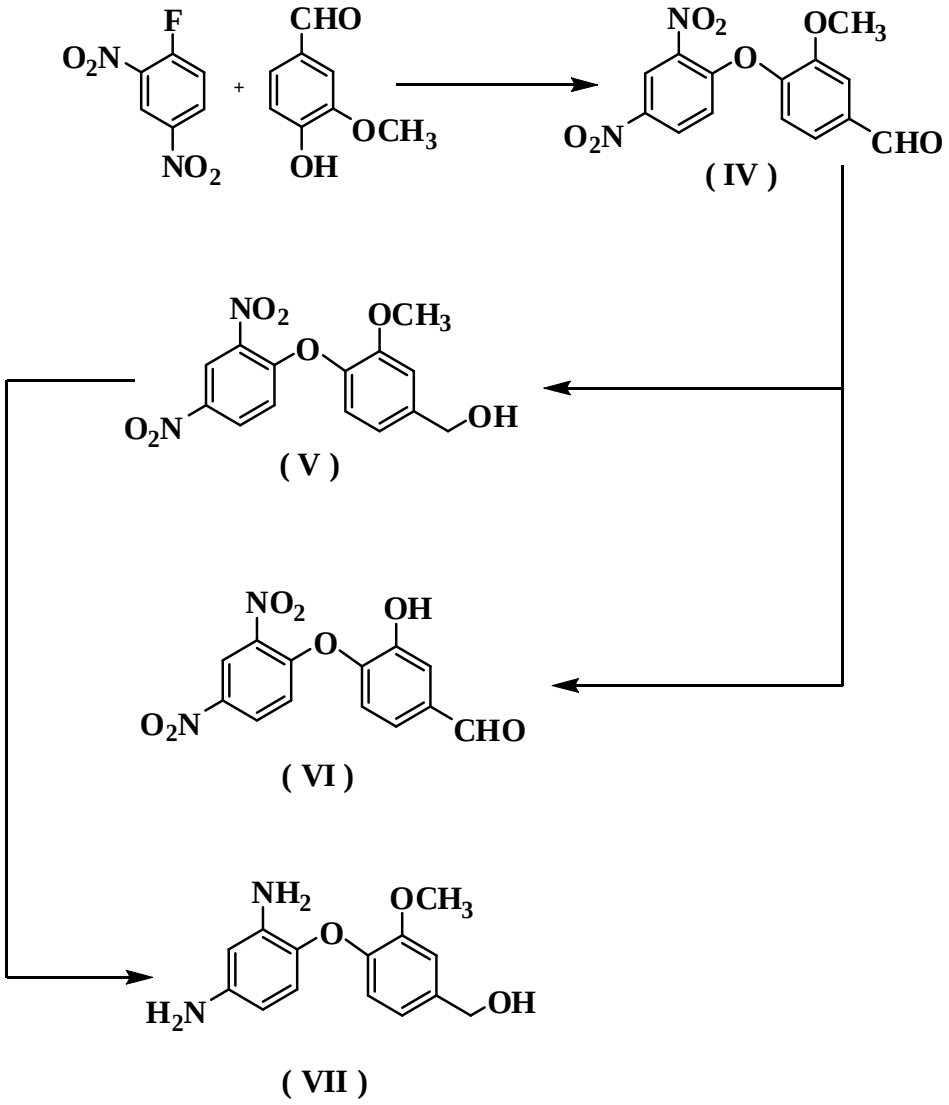
Starting with Vanillin **(I)** first plan to prepare the reduced vaniyl alcohol **(II)** and demethylate **(III)** it using $NaBH_4$ and 48 % aqueous HBr respectively. **Scheme I** shows the schematic representation of the reactions planned**.**



Scheme- I. Schematic representation of synthesis of reduced and dealkylated forms of vanillin.

We further plan to prepare a diphenyl ether **(IV)** using 2,4-dinitrofluorobenzene and vanillin using S_NAr reaction. The aldehyde group of DPE **(IV)** will be reduced to corresponding alcohol **(V)** and its nitro groups will be converted into corresponding

amines **(VII)**. The DPE **(IV)** will be also be dealkylated with HBr to to give dealkylated DPE **(VI)**. **(Scheme II)** shows the schematic representation of the reactions planned.



Scheme- II. Schematic representation of synthesis of various diphenyl ethers **.**

Experimental work

All the chemicals and solvents used for synthesis were of LR grade and procured from S.D. Fine-chem Limited, Mumbai, India. ^{1}H and ^{13}C NMR spectral analysis were performed on BRUKER AVANCE II 400 MHz spectrometer and mass analysis was done on MS instrument using turbo spray.

Preparation of 4-(hydroxymethyl)-2-methoxy-phenol. (II)

To an ice cold suspension of 4-hydroxy-3-methoxy-benzaldehyde (1 g, 6.5 mmol) in methanol (20 ml) added $NaBH_4$ (0.27 g , 7.1 mmol) in small amounts over a period of 15 min. After the reaction is complete (TLC monitoring), the solvent was evaporated in vacuo and reaction quenched with water. It was then extracted with ethyl acetate (3x 10 ml) and the combined organic layers were washed with water (2x25 ml) 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 = 50:50) to afford a yellow solid in 69.1 % (700 mg) yield. ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO): δ 3.7 (s, 3H), 4.3 (s, 2H), 4.9 (br s, 1H), 6.7 (m, 2H), 6.8(s, 1H), 8.7 (s, 1H):); ^{13}C NMR ($CDCl_3$ and DMSO): 55.5, 63.0, 111.0, 115.0, 119.1, 133.4, 145.2, 147.3: GC- MS: m/z 154.2 $[M]^+$.

Preparation of 3,4- dihydroxy-benzaldehyde [12]. (III)

To 4-hydroxy-3-methoxy-benzaldehyde (1g, 6.57 mmol) in acetic acid (10 ml) was added 48% aqueous HBr (2ml) and heated under reflux for 6 h.After cooling to room temperature and confirming completion of the reaction (TLC monitoring),acetic acid was evaporated in vacuo. Water (5 ml) was added to dissolve the contents and the organic 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 SiO_2 column chromatography and solvent (hexane/ethyl acetate=75:25) to afford a yellow solid in 33 % (300 mg) yield. . ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO δ 6.9(d, J = 8.4 Hz 1H),7.2 (m,1H), 7.7 (s, 1H), 9.3 (br s, 2H), 9.7 (s, 1H); ^{13}C NMR ($CDCl_3$ and DMSO): 114.4, 115.5, 124.5, 128.9, 145.9, 152.1, 191.1: GC- MS: m/z 138.2 $[M]^+$.

Preparation of 4-(2',4'- dinitrophenoxy)-3-methoxybenzaldehyde. (IV)

To a solution of 1-fluoro-2, 4-dinitrobenzene(0.65ml, 5.22 mmol) in DMF (10 ml) were added K_2CO_3 (2.875 g, 20.9 mmol), 3-methoxy-4-hydroxybenzaldehyde (1g, 6.57 mmol) and 18-crown-6 in catalytic amount.The mixture was stirred at room temperature for 12 hrs.After the reaction is complete (TLC monitoring),the reaction mixture was diluted with CH_2Cl_2 (100 ml), washed with water (50 ml), 1 N NaOH (3x10 ml),water (until neutral to litmus paper) and dried over Na_2SO_4. Evaporation of the organic solvent gave product which was purified using SiO_2 column chromatography and solvent (pet.ether/ethyl acetate=70:30) to afford a yellow solid in 60 % (1.25 g, mp 132-133°C) yield. ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO): δ 3.8 (s ,3H), 7.1 (d, J = 9.6 Hz, 1H), 7.5 (d, J = 8.0 Hz 1H), 7.6(m, 2H), 8.3 (dd, J = 2.8 Hz 1H), 8.8 (d, J = 2.8 Hz 1H), 10.0 (s,1H); ^{13}C NMR ($CDCl_3$ and DMSO): δ 56.3, 113.0, 118.3, 121.8, 122.6, 124.5, 129.5, 135.4, 138.6,141.5, 145.9, 151.2, 154.1, 191.9: GC- MS: m/z 318.1 $[M]^+$.

Preparation of 4-(2',4'-dinitrophenoxy)-3-methoxybenzylalchol. (V)

To an ice cold suspension of 4-(2',4'- dinitrophenoxy)-3-methoxybenzaldehyde (800 mg , 2.5 mmol) in methanol (20 ml) added $NaBH_4$ (0.11 g , 3.0 mmol) in small amounts over a period of 15 min. The reaction mixture changes color to violet during addition.After the reaction is complete (TLC monitoring), the solvent was evaporated in vacuo and reaction quenched with water. It was then extracted with ethyl acetate (3x 10 ml) and the combined organic layers were washed with water (2x25 ml) 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 = 50:50) to afford a yellow solid in 56.6 % (453 mg, mp 109 -110°C) yield. ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO): δ 3.7 (s, 3H), 4.5 (s, 2H), 5.3 (br s, 1H), 6.9 (d, J = 9.2 Hz, 1H), 7.0 (m, 1H), 7.2 (m, 2H) 8.3 (m, 1H), 8.9 (d, J = 2.8); ^{13}C NMR ($CDCl_3$ and DMSO): δ 55.8, 62.4, 111.6, 117.2, 119.1, 121.7, 121.9, 129.4, 138.1, 139.3, 140.8, 142.8, 150.4, 155.3: MS: m/z 320.0 $[M]^+$.

Preparation of 4-(2',4'-dinitrophenoxy)-3-hydroxybenzaldehyde. (VI)

To 4-(2',4'- dinitrophenoxy)-3-methoxybenzaldehyde (700 mg, 2.2 mmol) in acetic acid (30 ml) was added 48% aqueous HBr (3.5 ml) and heated under reflux for 2 h.After cooling to room temperature and confirming completion of the reaction (TLC monitoring),acetic acid was evaporated in vacuo. Water (5 ml) was added to dissolve the contents and the organic 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 SiO_2 column chromatography and solvent (toluene/ethyl acetate=75:25) to afford a yellow solid in 45 % (304 mg, mp 209 -210°C) yield. ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO): δ 7.0 (d, J = 9.2 Hz 1H), 7.2 (d, J = 8.4 Hz 1H), 7.8 (m, 2H), 8.3- 8.4 (m ,1H), 8.8 (m, 1H), 9.8 (s, 1H), 11.9 (br s, 1H); ^{13}C NMR ($CDCl_3$ and DMSO): δ 116.9, 117.6, 118.0, 121.8, 123.9, 129.2, 129.4, 129.8, 138.4, 140.4.141.1, 154.8, 190.5: GC- MS: m/z 304.8 $[M]^+$.

Preparation of 4-(2',4'-diaminophenoxy)-3-methoxybenzylalchol . (VII)

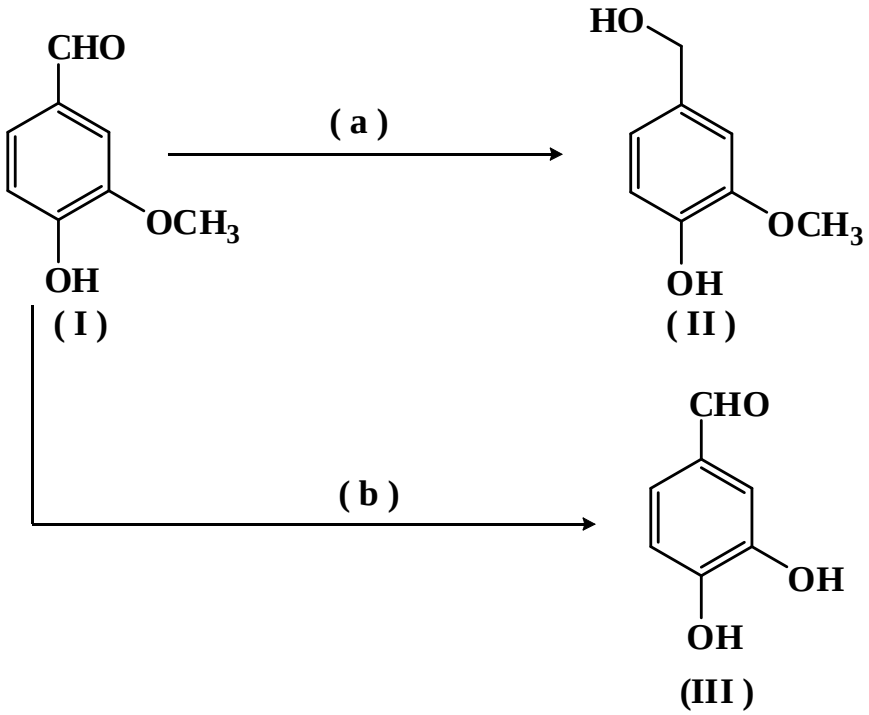
To a suspension of 4-(2',4'-dinitrophenoxy)-3-methoxybenzylalchol (180 mg , 0.56 mmol) in refluxing H_2O (6 ml) were added Fe (316.48 g, 5.64 mmol) and $FeSO_4 \cdot 7\ H_2O$ (154.2 mg, 0.55 mmol). The reaction mixture was refluxed for 8h. After cooling to room temperature and confirming the completion of reaction (TLC monitoring), it was filtered through Celite, washed thoroughly with CH_2Cl_2 (2 x 75 ml). The combined organic layers were dried over Na_2SO_4.Evaporation of the organic solvent gave corresponding crude 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. This crude product was purified using SiO_2 column chromatography and solvent (chlorofom/methanol = 90:10) to afford pure amine, 61 % (90 mg,)yield. ^{1}H NMR (400 MHz, $CDCl_3$ and DMSO): δ ^{13}C NMR ($CDCl_3$ and DMSO): δ 55.5, 62.7, 101.3, 103.0, 111.2, 115.8, 118.5, 120.5, 133.6, 136.7, 140.2, 145.7, 146.2, 149.1: MS: m/z 261 $[M]^+$.

Results and Discussion

Biotransformation, is one of the greener methods for affecting an organic reaction. However the challenge for biotransformation is to make the organic compound available to organisms or enzymes in such a form so that they can take it up. Present work describes some organic reactions for the synthesis of such precursors and their characterization.

Vanillin, **(I)** 4-hydroxy-3-methoxy-benzaldehyde, is an interesting molecule having three different (-OH, -CHO, -OCH_3) functional groups attached to the benzene ring. This compound is partially soluble in water. We undertook conversions depicted in **(Scheme- III)** to increase its solubility in water.

Scheme - III



Reagents : (a) $NaBH_4$; MeOH, **(b)** 48% Aq. HBr ; acetic acid, reflux.

Vanillin, **(I)** was reduced to corresponding benzyl alcohol**(II)** using sodium borohydride in methanol. Similarly, it was also demethylated using 48% aqueous HBr in acetic acid in reflux conditions. Both the reactions gave precursors for biotransformation with an additional hydroxyl group as compared to the starting material. In fact latter reaction remains one of the challenging reactions for biotransformations since the synthetic reactions requires harsh chemicals and produces low yield. Both the synthesized compounds **(I and II)** were analyzed by ^{1}H and ^{13}C NMR and by Mass Spectroscopy techniques.

^{1}H NMR of the compound **(II)** gave two singlets **(Appendix – I, Fig- 7)** at 3.7 and 4.3 ppm corresponding to three methoxy ($-OCH_3$) and two methylene ($-CH_2-$) protons respectively. Broad singlets at 4.9 ppm and 8.7 ppm corresponded to one hydroxyl each attached to methylene group and aromatic ring respectively. The intensity of the signals in aromatic region (6.9 – 7.2) was in proportion to three protons in the aromatic region. Similarly compound **(III)** gave sharp peak at 9.7 ppm for aldehydic proton .Missing singlet at 3.7 ppm indicated the conversion of methoxy group into corresponding hydroxyl group. Both the hydroxy in this case appeared as broad singlet at 9.3 ppm.

^{13}C NMR of both the compounds **(II and III)** was compatible with the proposed structure .While in case of **(II) (Appendix – I, Fig- 8)** all the aromatic carbons appeared at (114 - 147) ppm, whereas methylenic and methoxy carbons appeared at 63 and 55 ppm respectively. Similarly in case of compound **(III) (Appendix – I, Fig- 11)** the carbon for aldehyde was observed at 191 ppm. Rest all aromatic carbons appeared at (114 - 152) ppm.

Mass spectra of the compound **(II) (Appendix – I, Fig- 9)** gave molecular ion peak at m/z 154.2 and other peaks at m/z 139, 137 and 123 due to loss of methyl from methoxy , hydroxyl from benzyl alcohol moiety and methoxy from aromatic ring respectively. Similarly for compound **(III) (Appendix – I, Fig- 12)** molecular ion peak was observed at m/z 138 and at m/z 109 due to loss of aldehyde group.

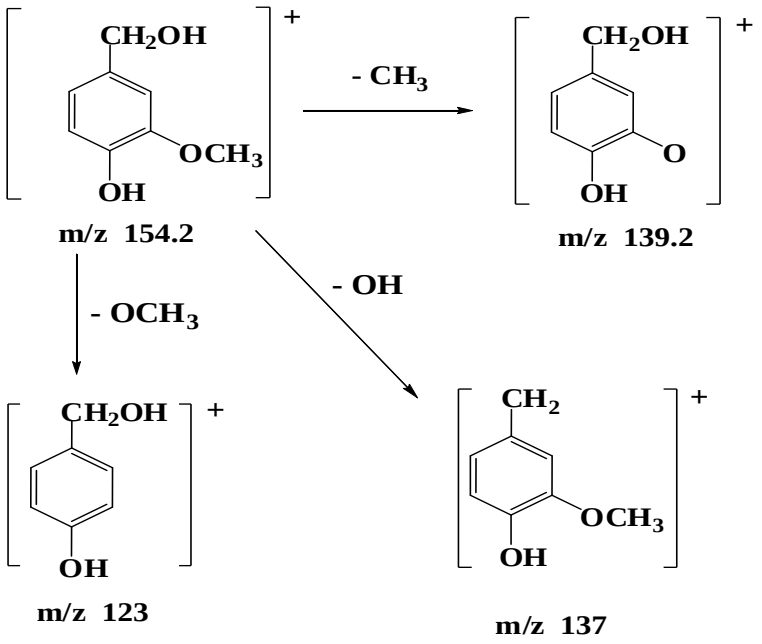


Fig-1 Mass fragmentation of compound **(II)** and its different m/z values**.**

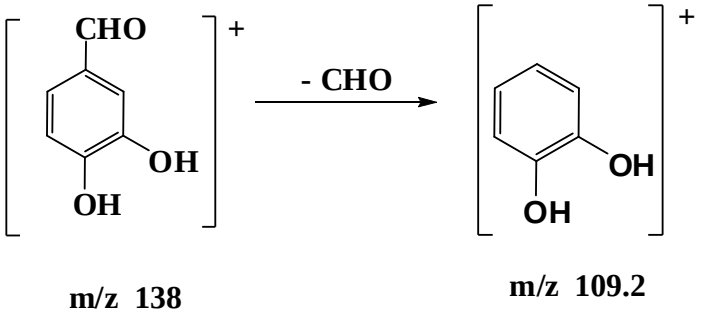


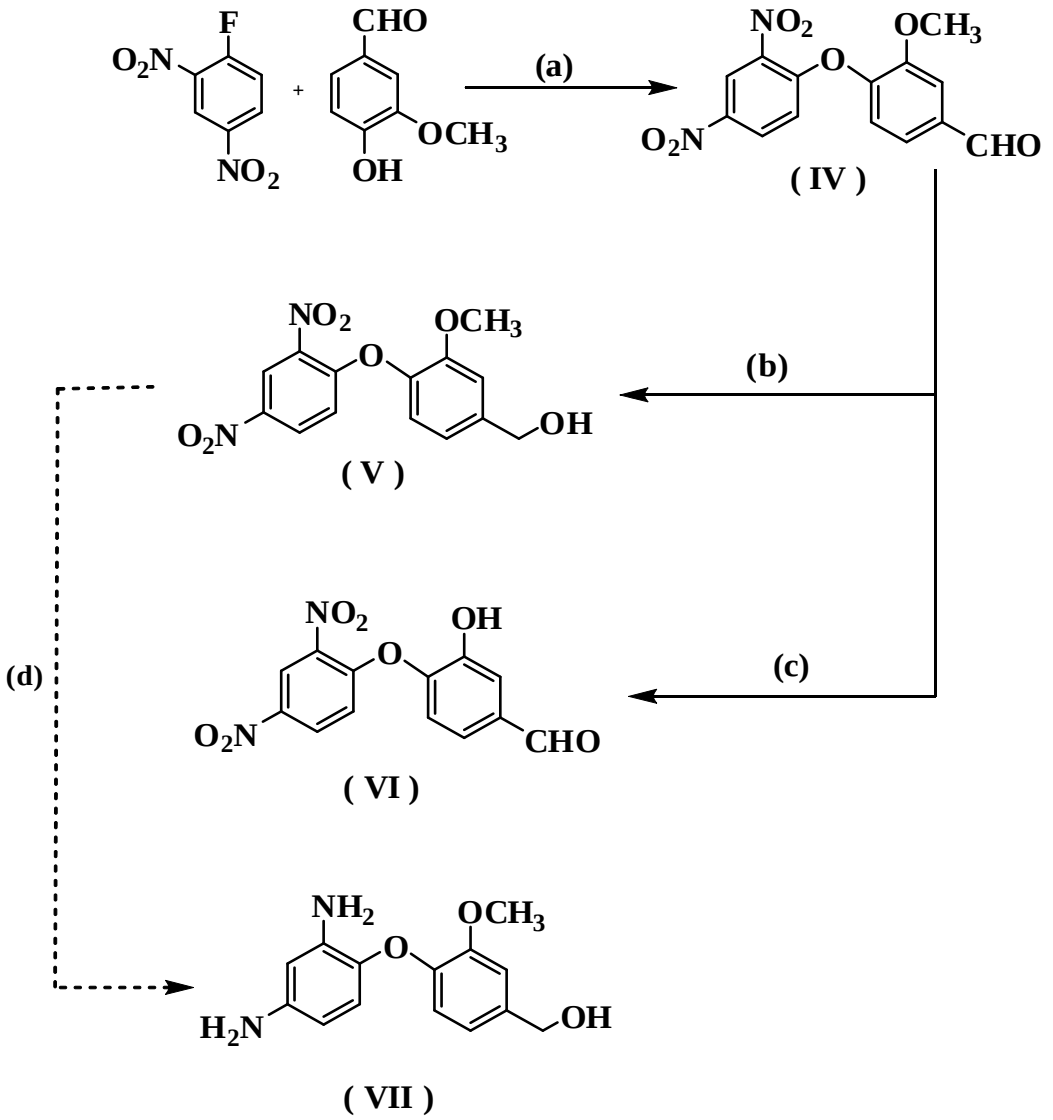
Fig-2 Mass fragmentation of compound **(III)** and its different m/z values**.**

Diphenyl ethers has two phenyl rings attached to oxygen, whereas aryl methyl ether has one aromatic ring and other aliphatic ring attached to oxygen. In order to study the selectivity of organism or enzyme for one such ether linkage, we undertook the synthesis of a compound [13] where both ether linkages are present simultaneously **(Scheme- IV)**.

Sanger's reagent (2,4-dinitrofluorobenzene) and vanillin were reacted together in the presence of potassium carbonate, 18-Crown-6 and DMF to afford a diphenyl ether **(IV)** . Unfortunately the compound was insoluble in water. To convert it in soluble form the benzaldehyde was reduced to corresponding alcohol **(V)** using sodium borohydride

in methanol.We also carried out demethylation of **(IV)** using 48% aqueous HBr repeating the procedure done for vanillin. The attempted reduction of nitro groups in **(V)** to corresponding amino groups resulted in separable mixture of products that could not be analyzed by ^{1}H NMR.

Scheme - IV



Reagents : (a) K_2CO_3, DMF, 18-Crown-6, **(b)** $NaBH_4$; MeOH, **(c)** 48% Aq. HBr **;** acetic acid, reflux **(d)** Fe, $FeSO_4 .7H_2O$.

^{1}H, ^{13}C NMR and mass spectroscopic data for rest of compounds were compatible with the proposed structure. Three methoxy protons at 3.7 ppm were observed in case of compounds **(IV)** and **(V)**. The singlet due to aldehyde appeared at 9.6 – 10.7 ppm in case of compounds **(IV)** and **(VI)** . However in case of compounds **(V) (Appendix – I, Fig 13)** this singlet is missing and instead another appeared at 4.3 ppm due to two methylene protons. Aromatic protons in all cases appeared at 6.0 – 9.0 ppm. In ^{13}C NMR of all the compounds ,aldehydic carbon 191 and 190 ppm for compounds **(IV)** and**(VI)**,methylene carbon 62 ppm for compounds **(V) (Appendix – I, Fig- 14)** and **(VII)** and methoxy carbon 55 ppm for **(IV)** and **(VI)**,were clearly distinguishable from aromatic carbons **(**111 to 155 ppm).

Mass spectra of compound **(IV) (Fig-3)** gave molecular ion peak at m/z=318.0. The other two prominent peaks appeared at m/z 317.0 and at 303.0 due to loss of hydrogen from aldehyde and CH_3 group from methoxy moiety respectively.

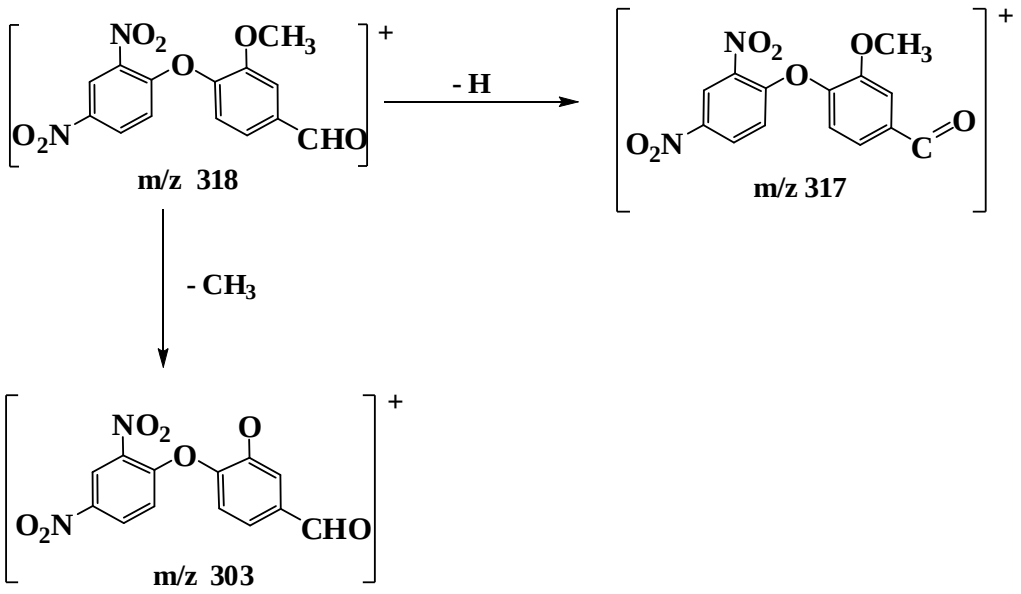


Fig-3 Mass fragmentation of 4-(2',4'- dinitrophenoxy)-3-methoxybenzaldehyde **(IV)** and its different m/z values.

Mass spectra of compound **(V) (Fig-4)** gave molecular ion peak at m/z 320.0 with $[M+1]^+$ and $[M+3]^+$ peaks at m/z=321.2 and 323.0 **(Appendix I Fig –10)**. Loss of

methyl from methoxy, hydroxyl from benzyl alcohol , and OCH_3 from aromatic group gave peak at m/z = 305.1, 303.0 and 288.9 respectively.

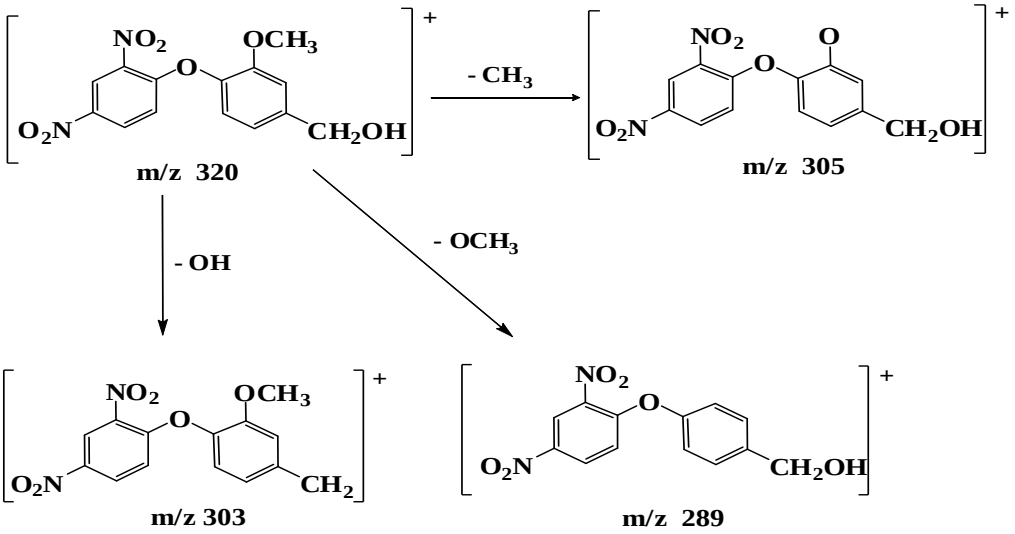


Fig-4 Mass fragmentation of 4-(2',4'-dinitrophenoxy)-3-methoxybenzylalchol **(V)** and its different m/z value

Mass spectra of compound **(VI) (Fig-5)** gave peaks at m/z 275 and 258 due loss of CHO and one of NO_2 groups from aromatic ring respectively along with molecular ion peak at m/z 304.

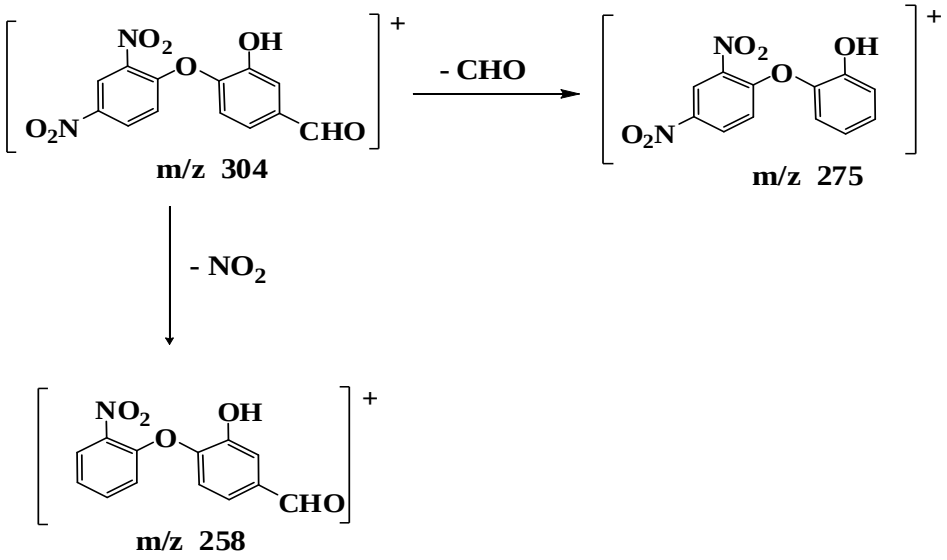
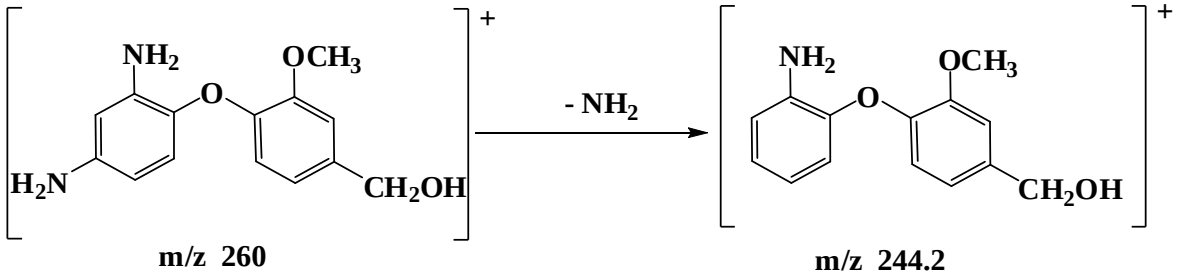


Fig-5 Mass fragmentation of 4-(2',4'-dinitrophenoxy)-3-hydroxybenzaldehyde **(VI)** and its different m/z values.

Similarly, mass spectra of compound **(VII) (Fig-6)** gave molecular ion peak at m/z 260.3 with [M+1]+ [M+2]+ and [M+3]+ peaks at m/z = 261.4, 262.0 and 263.1. Loss of NH_2 group from aromatic ring gave peak at 244.2.



m/z 260 **m/z 244.2**

Fig-6 Mass fragmentation of 4-(2',4'-diaminophenoxy)-3-methoxybenzylalchol **(VII)** and its different m/z values.

We were able to prepare some of the precursors for biotransformations with partial success.Vanillin **(I)** was converted to its corresponding alcohol **(II)** and demethylated product **(III)** . Similarly the 4-(2',4'- dinitrodiphenyl ether **(IV)** was prepared and then was converted to its corresponding amine **(VII)** and the aldehyde and methoxy groups were converted to their corresponding alcohols **(V)** and phenolic **(VI)** moiety.

Appendix-1

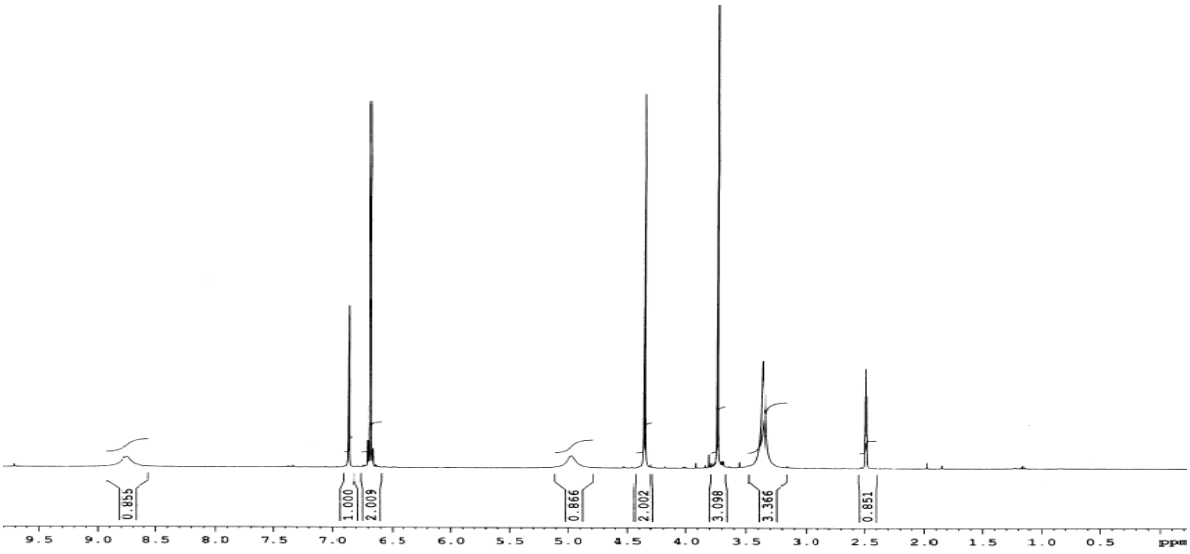


Fig-7 ^{1}H NMR of 4-(hydroxymethyl)-2-methoxy-phenol. **(II)**

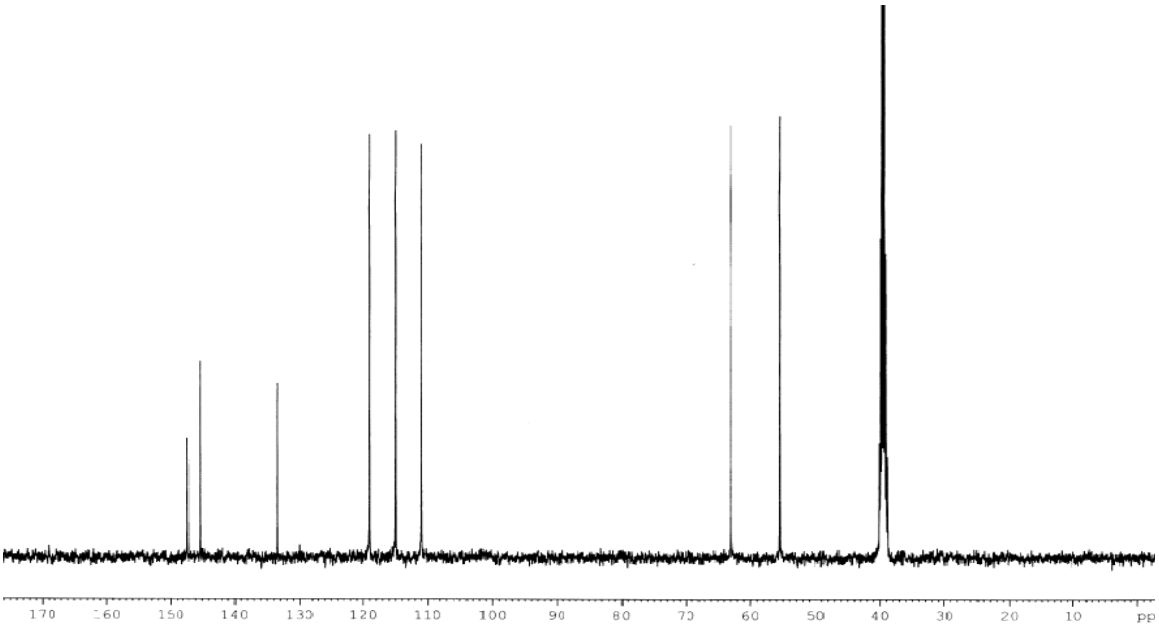


Fig-8 ^{13}C NMR of 4-(hydroxymethyl)-2-methoxy-phenol. **(II)**

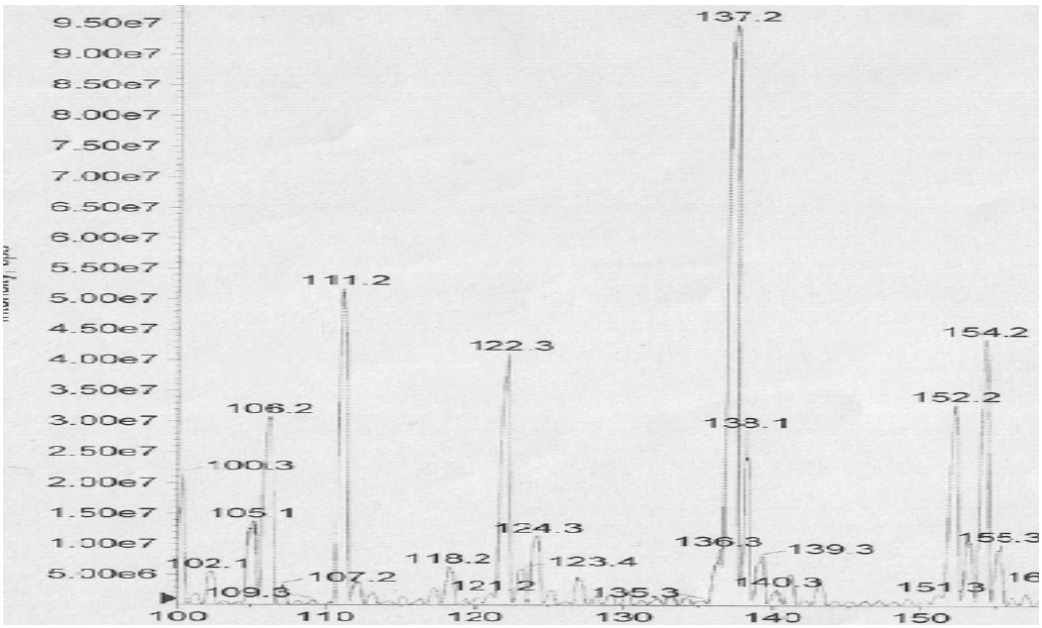


Fig-9 Mass spectra of 4-(hydroxymethyl)-2-methoxy-phenol. **(II)**

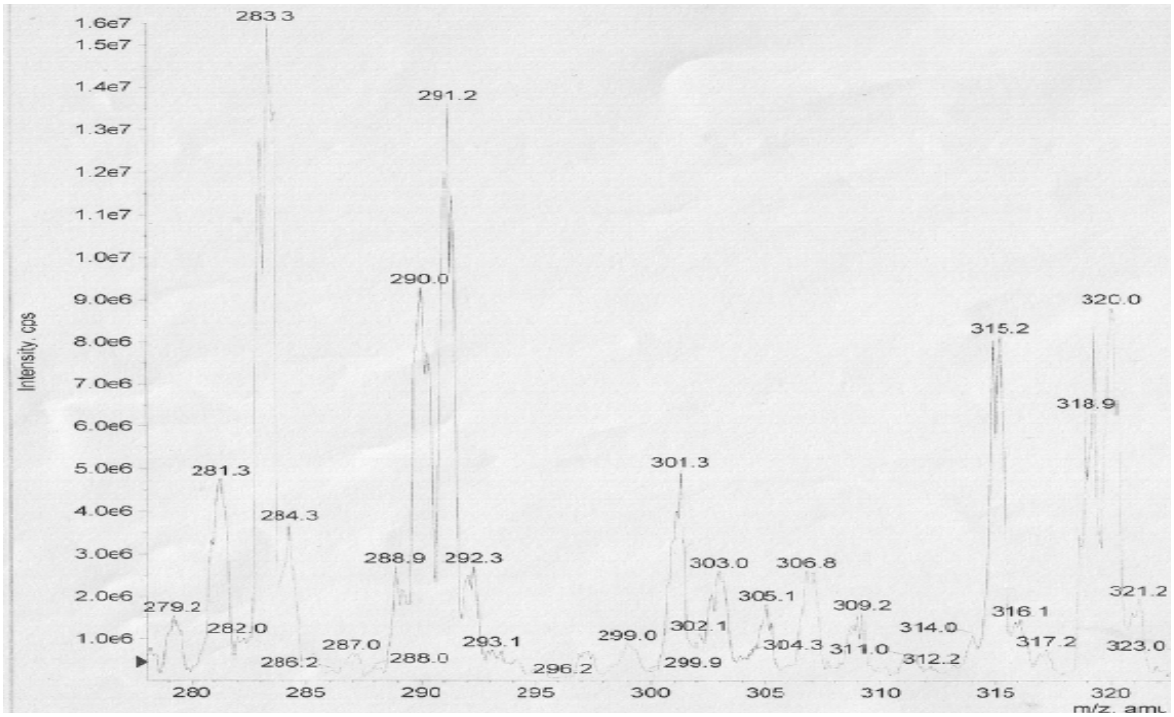


Fig-10 Mass spectra of 4-(2',4'-dinitrophenoxy)-3-methoxybenzylalchol. **(V)**

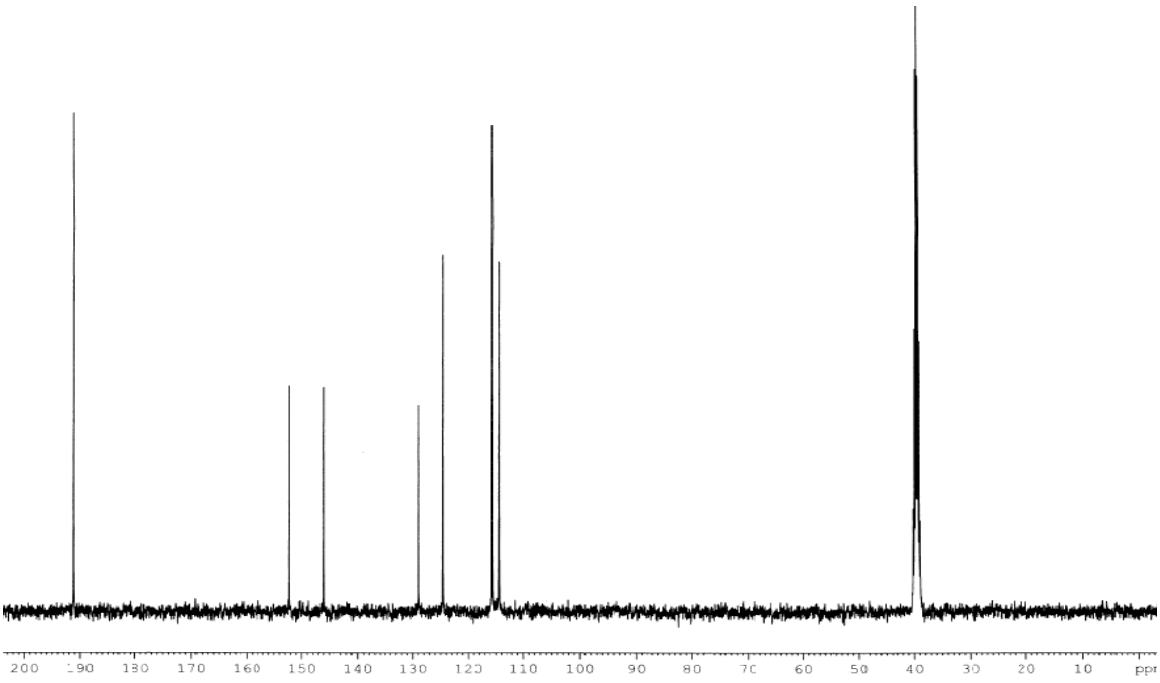


Fig-11 ^{13}C NMR of 3,4- dihydroxy-benzaldehyde. **(III)**

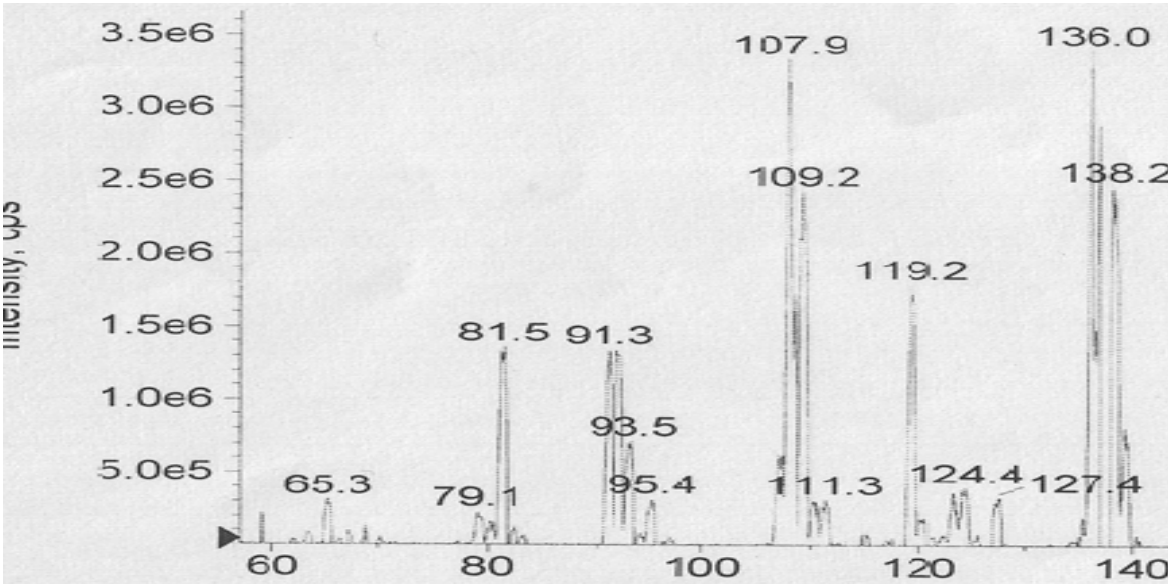


Fig-12 Mass spectra of 3,4- dihydroxy-benzaldehyde. **(III)**

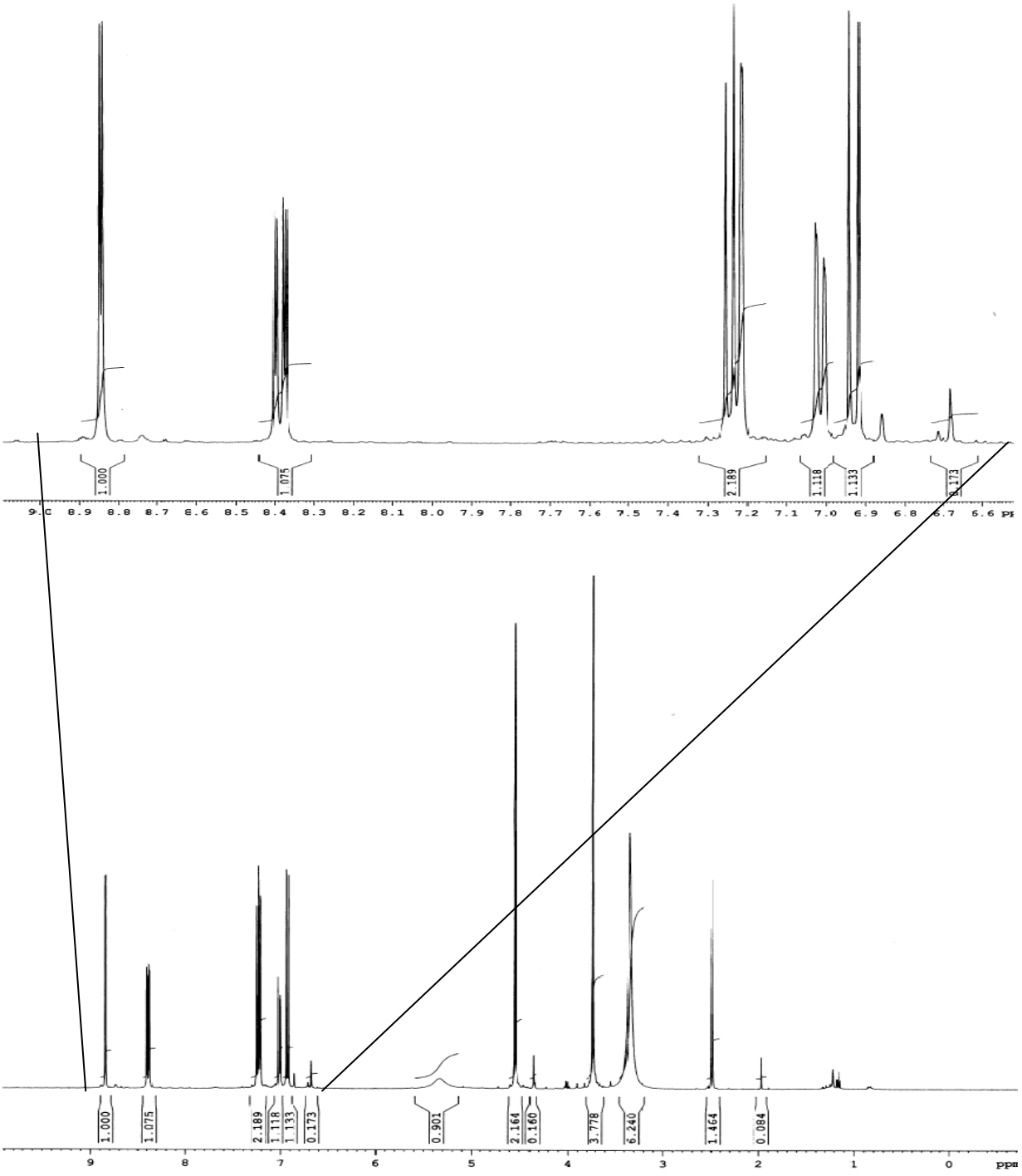


Fig-13 ^{1}H NMR of 4-(2',4'-dinitrophenoxy)-3-methoxybenzylalchol**. (V)**

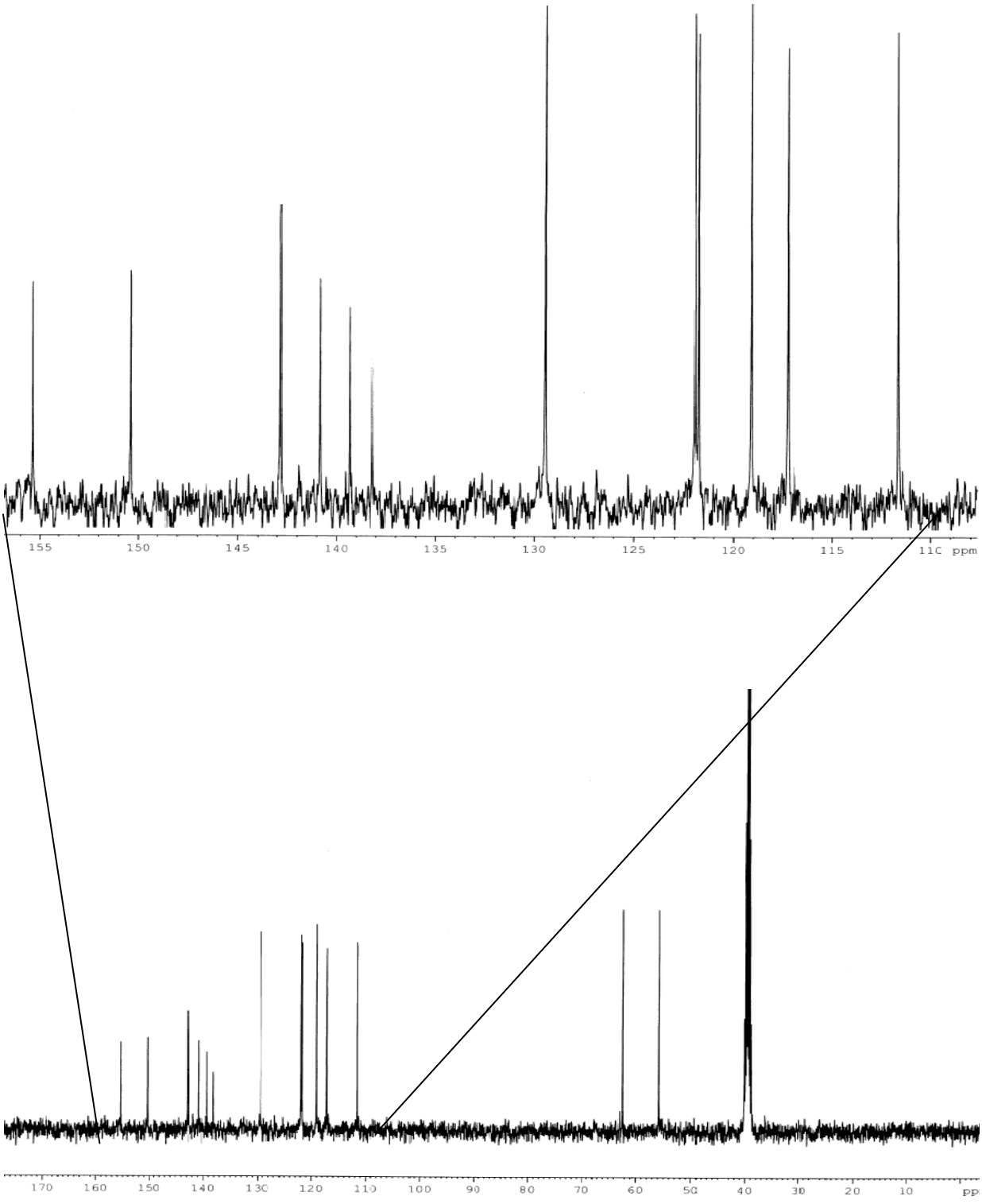


Fig-14 ^{13}C NMR of 4-(2',4'dinitrophenoxy)-3-methoxybenzylalchol. **(V)**

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