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Synthesis of Novel Agrochemicals as Potential Plant Immunization Agents.

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Synthesis of Novel Agrochemicals as Potential Plant Immunization Agents

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by

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ABSTRACT

Synthesis of Novel Agrochemicals as Potential Plant Immunization Agents

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The world's population is expected to grow from 6 billion to about 10 billion by 2050. The greatest population increase is expected to occur in Africa, Latin America, and Asia. To feed a world with huge increases in population and to sustain the well-being of humans, a large increase in food production must be achieved. The projected increase in food production must be accomplished on the existing cultivated areas because the expansion of new land is limited by environmental concerns, urbanization and increasing water scarcity.

Different compounds have been developed for the "immunization" of plants against several pathogens. These compounds induce systemic acquired resistance (SAR) in plants, leading to broad-based, long-lasting resistance to a wide range of pathogens. The salicylic acid binding protein 2 (SABP 2) has been identified as a key enzyme in the salicylic acid mediated pathogen resistance pathway, converting methyl salicylate (MeSA) to salicylic acid (SA), a key compound responsible for SAR . S-methyl benzo [1, 2, 3,] thiadiazole-7-carbothiate (BTH) was the first commercial compound used for plant immunization. We have synthesized and characterized some new salicylic acid derivatives [methyl-2-(2-hydroxy benzoyl thio) acetate and derivatives], and we have studied the in-vitro activity with SABP2 of BTH by HPLC analysis.

DEDICATION

To my brother (R.I.P), my sisters and mom and dad. Love you all.

ACKNOWLEDGEMENTS

I would like to say thanks to the Department of Chemistry here at East Tennessee State University for giving me the opportunity to pursue a graduate degree. This department has been like a door opening to many things to come.

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CHAPTER 1

INTRODUCTION

Global Population and Food Demands

The current world population stands at approximately 6 billion people and it is expected to grow from 6 billion to 10 billion by 2050. This is a median projection representing the addition of an extra 4 billion people onto the present global population [1]. Figure 1 by the United Nations Population Division shows population estimates on the basis of fertility by 2050 [2].

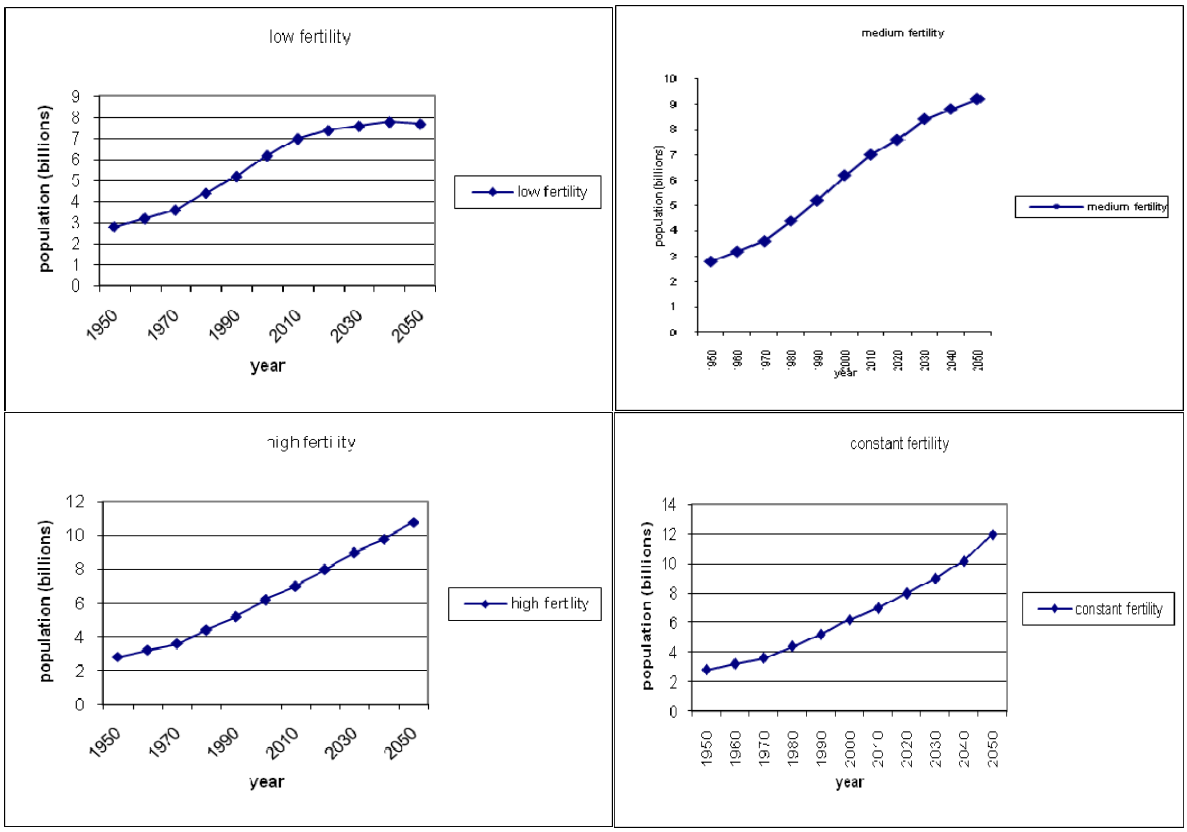


Figure 1. Population of the world, estimates and projection variants, 1950-2050 (redrawn from Cakmak [1])

Virtually all of the anticipated increase in the world population will occur in only 3 of the 7 major continents-Africa, Latin America and Asia [1]. Table 1 below shows projection in population figures in these continents by 2050[3].

Table 1. Population Projection in some Major Continents by 2050

Population (thousands)								
Medium variant								
1950-2050								
AFRICA			ASIA			LATIN AMERICA & CARRIBEAN		
Year	Population		Year	Population		Year	Population	
1950	224 202		1950	1 410 649		1950	167 626	
1955	250 633		1955	1 550 986		1955	192 022	
1960	282 241		1960	1 704 289		1960	220 167	
1965	319 574		1965	1 898 591		1965	252 850	
1970	364 132		1970	2 138 765		1970	287 543	
1975	416 446		1975	2 393 643		1975	324 834	
1980	479 786		1980	2 635 738		1980	364 379	
1985	554 294		1985	2 896 192		1985	404 492	
1990	637 421		1990	3 181 211		1990	444 271	
1995	726 334		1995	3 451 674		1995	483 860	
2000	820 959		2000	3 704 838		2000	523 048	
2005	922 011		2005	3 938 020		2005	557 979	
2010	1 032 013		2010	4 166 308		2010	593 697	
2015	1 149 117		2015	4 389 000		2015	627 958	
2020	1 270 528		2020	4 596 189		2020	659 562	
2025	1 393 871		2025	4 778 988		2025	688 030	
2030	1 518 310		2030	4 930 983		2030	712 841	
2035	1 642 679		2035	5 051 850		2035	733 378	
2040	1 765 372		2040	5 147 894		2040	749 670	
2045	1 884 446		2045	5 219 778		2045	761 599	
2050	1 997 935		2050	5 265 895		2050	769 229	

Presently, these regions are facing major life-threatening problems concerning food production, access to food, water scarcity, and nutritional disorders/malnutrition. It was reported that more than 800 million people in the developing countries are undernourished caused by inadequate food availability. In Asia, where the problem is particularly widespread, many people live below the poverty line (less than a dollar a day). About 70% of the undernourished people globally live in Asia, predominantly in India and China [1]. Another critical region of the world in which malnutrition is a major concern is in Sub-Saharan Africa, where about 200 million undernourished people reside [1]. The Food and Agricultural Organization (FAO) has projected that the number of undernourished people will decline only to 680 million by 2010, and this decrease will mainly take place in Asia, while in Sub-Saharan Africa food insecurity will likely continue with increasing numbers of people affected [1].

In order to feed a world with staggering increases in population and to sustain the well-being of humans, a large increase in food production must be achieved. This cannot be achieved without careful and meticulous planning, as the expected increases in world population will result in a serious pressure on the existing agricultural land via urbanization and intensification of crop production [1].

Moreso, the projected increase in food production must be accomplished on the existing cultivated areas because the expansion of new land is limited due to environmental concerns, urbanization, and increasing water scarcity. Global plant-based food production is expected to increase by more than 60%, while the area used for crop production will only grow by 10% [1].

However, recent trends indicate that the growth rate (based on ton ha-1) in crop production has begun to decline in the last 10 years, and possibly, cannot keep pace with the projected increase in the global food demand in the following decades [1].

Erosion, nutrient depletion, water scarcity, acidity, salinization, depletion of organic matter, and poor drainage have been the causes of reductions in soil productivity in many parts of the world. Nearly 40% of the agricultural land has been affected by soil degradation, in particularly in Sub-Saharan Africa and Central America [1].

Different methods have been applied to improve productivity while trying to keep the best quality of produce. Some of these methods have been:

a) The use of fertilizers to improve on the availability of depleted plant nutrients:

   Generally, improving the nutritional status of plants by applying fertilizers and maintaining fertility has been the critical step in the doubling of food production both in developed and developing countries since the beginning of 'Green Revolution'. From Figure 2, increases in cereal production in the past 40 years were well associated with corresponding increases in fertilizer consumption in developing countries [1].

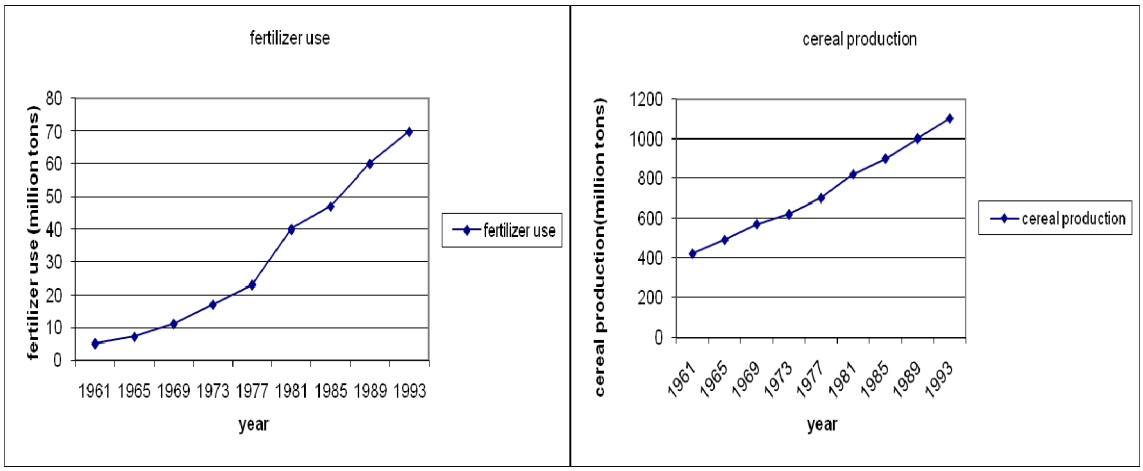


Figure 2. Growth in fertilizer use and cereal production in developing countries during 1961-1995(redrawn from Cakmak [1])

Enhanced use of fertilizers does not come without its own environmental concerns and problems. Adverse effects on the environment in terms of eutrophication of surface waters, pollution of drinking water, and gaseous emission causing global warming have been some of the results this enhanced used of these chemicals [1].

b) The use of pesticides (fungicides, insecticides, herbicides) to protect crops against invading insects, plant diseases, etc. The use of these agrochemicals has helped improve crop yields in many countries because they help the plant fight against invading insects and pathogens, thus giving the plant a chance to thrive. Despite their usefulness, pesticide use has been associated with several environmental hazards. These compounds can seep into groundwater and can also be washed of as runoff into seas and oceans.

c) Biotechnological advances have also aided in improved crop yields and also resistance to invading pathogens and insects.

Even though the use of fertilizers and pesticides has been very useful in the improvement of crop yields, a lot is to be desired as a result of the environmental impact of these chemicals. We therefore need to find a way to minimize the use of these compounds while maintaining the same levels of crop output to meet our global food demands.

Our research focuses on the development of a series of novel agrochemicals that could be used as resistance inducing chemicals to improve the plant's own resistance to a wide range of pathogens. These compounds themselves do not show any direct anti-microbial properties but could stimulate pathogen resistance genes in plants. Application of small amounts of these compounds could help "immunize" crops against pathogens for longer time periods, thus minimizing exposure of agricultural soils to large amounts of chemicals. We also seek to develop simple synthetic strategies for these compounds and also test their activity against plant pathogens.

Disease Resistance In Plants

Following pathogen attack, various plant defense responses are activated. These responses include the induction of local and systemic resistance, the potentiation of cell death, and the containment of pathogen spread.

Subsequent to initial pathogen attack, many surviving plants develop heightened resistance to a broad spectrum of pathogens. This phenomenon is referred to as systemic acquired resistance (SAR). Such systemic, long-lasting, and broad-based resistance can be

developed by many plant species in response to a wide variety of viral, bacterial, and fungal pathogens [4, 11].

One of the earliest responses observed following pathogen attack is a rapid increase in reactive oxygen species (ROS), known as the oxidative burst. Other responses that frequently occur after pathogen attack include ion fluxes and the activation of *G* proteins and protein kinases. In addition, enzymes associated with the phenyl propanoid pathway that are involved in the synthesis of anti-microbial compounds termed phytoalexins are activated. Salicylic acid (SA) biosynthesis may also be initiated at this time, as phenylalanine ammonia-lyase (PAL), which is the first enzyme of the phenylpropanoid pathway, synthesizes trans-cinnamic acid, a precursor of SA [4].

After several hours to days following these rapid responses, additional defense responses are activated. These responses include development of a hypersensitive response (HR) that is characterized by formation of necrotic lesions at the site(s) of pathogen entry. In addition, the pathogen is restricted to the cells within or immediately surrounding these lesions. Just prior to or concurrent with the development of HR of a wide variety of proteins are synthesized in the inoculated leaf, including various hydrolytic enzymes, proteinase inhibitors, and several families of pathogenesis-related (PR) proteins. Activation of PR expression has been used as a convenient marker for the induction of SAR [4].

Salicylic Acid and Induction of Resistance Responces

Salicylic acid has been identified as a key component in the induction of many plant responses mentioned above:

1. Activation of local and systemic acquired resistance.

   Many analyses have shown that exogenous SA treatment enhances resistance to many bacterial, fungal, and viral pathogens and induces PR gene expression in a wide variety of dicotyledonous species [4]. In tobacco and Arabidopsis, SA has also been shown to induce the same set of PR genes as those expressed during SAR. The first evidence that SA is an endogenous plant signal for the activation of defense responses came from the analyses of SA levels in pathogen-infected tobacco and cucumber plants. In the inoculated leaves of TMV-resistant tobacco, SA levels increased 20- to 50- fold, and these increases paralleled or preceded the activation of PR gene expression. A 10-fold increase in SA levels that correlates with accumulation of PR transcripts was also detected in upper, uninoculated leaves. Similarly, the phloem sap of cucumber infected with *Colletotrichum lagenarium*, *Pseudomonas syringae*, or tobacco necrosis virus exhibited a 10- to 100-fold increase in SA levels prior to the development of SAR and elevation of a defense-associated peroxidase activity [4, 13].

2. Initiation of Hypersensitive response(HR) and Cell death:

   The main defense response in gene-for-gene resistance is a programmed cell death (PCD), known as hypersensitive response. HR is always associated with an oxidative burst caused by rapid production of reactive oxygen species (ROS). An O_2^- generating membrane-bound NADPH- oxidase, presumably located on the outer surface

of the plasma membrane, was demonstrated, for the first time in plants, associated with potato protoplasts [5, 12]. It has been reported that, although O_2^- generation is necessary for PCD in many plant systems, it is not sufficient to trigger cell death in wild type plants on its own. This is reasonable as the sole O_2^- generation is a highly unspecific response of plants to stresses of different origin, e.g. environmental, chemical and pathogenic, that do not normally lead to cell death and tissue necrosis. Activation of NADPH- oxidase has been reported not only in response to pathogen attack, but also to a variety of abiotic stress. In wild type Arabidopsis, visible necrotic lesions developed 6-12 hr after treatment of the leaves with an O_2^- generating system (xanthine-xanthine oxidase) and SA; infiltration of each compound separately appeared symptomless. SA levels actually increase in response to PDC-inducing infection, and PDC development can be inhibited by expression of salicylate hydroxylase encoded by the bacterial *nahG* gene. SA is undoubtedly involved in lesion formation and the contribution of SA to HR-associated resistance has been demonstrated to operate through the potentiation of ROS generation and cell death. However, SA induces PCD only at very high concentrations [5, 15].

3. Containment of Pathogen:

A number of studies have suggested that SA is involved in restricting the invading pathogen to the area immediately surrounding the point of infection. This may be related, in part, to SA's ability to enhance host cell death. However, a growing body of evidence indicates that necrotic lesion formation is either insufficient or unnecessary for

the restriction of pathogen spread. Thus, SA may regulate the activation of other currently uncharacterized defenses that are responsible for pathogen localization. Support for this possibility has come from TMV-inoculated transgenic tobacco expressing salicylate hydroxylase (SH) either constitutively or early after infection prior to visible lesion formation. Transgenic plants expressing salicylic hydroxylase, hydrolyse SA into catechol which is inactive for inducing plant defences. The lesions developed by these plants are abnormally large and grow rapidly, eventually coalescing to form a spreading necrosis that moves into the stem and enters adjacent leaves [4]. Strikingly, TMV coat protein (and thus the virus) was detected in the asymptomatic tissues surrounding the spreading necrosis as well as in the necrotic regions. In contrast, plants that expresses SH at a later time after infection (concurrent with HR) did not exhibit spreading necrosis and TMV coat protein was detected only within the lesions. Based on these results, early accumulation of SA appears to be required for effective pathogen localization [4].

Plant Defense Pathways

Subsequent to pathogen attack, a cascade of biochemical reactions is triggered, leading to the production of vital signaling compounds such as salicylic acid, ethylene and jasmonic acid. The different plant defense pathways are named after these signaling compounds. These signaling compounds are responsible for triggering the various plant defense responses. This project focuses on the SA pathway shown in Figure 3 shown below.

From chorismate, SA can be produced from two different subpathways- the isochorisamte (ICS) and the phenylalanine ammonia lyase subpathways. ICS is the dominant pathway in Arabidopsis, while the PAL subpathway is dominant in tobacco. The end-product of each of these subpathways is salicylic acid. The salicylic acid produced is methylated by the enzyme salicylic acid methyl transferase (SAMT) to produce methyl salicylate (MeSA). Methylation of SA causes a change in the redox potential of the chloroplast cell wall causing MeSA to go across into the cytoplasm. In the cytoplasm, MeSA is hydrolyzed to SA by a key enzyme called salicylic acid binding protein (SABP2). The free SA thus produced activates the NPR proteins which in turn triggers defense resistance.

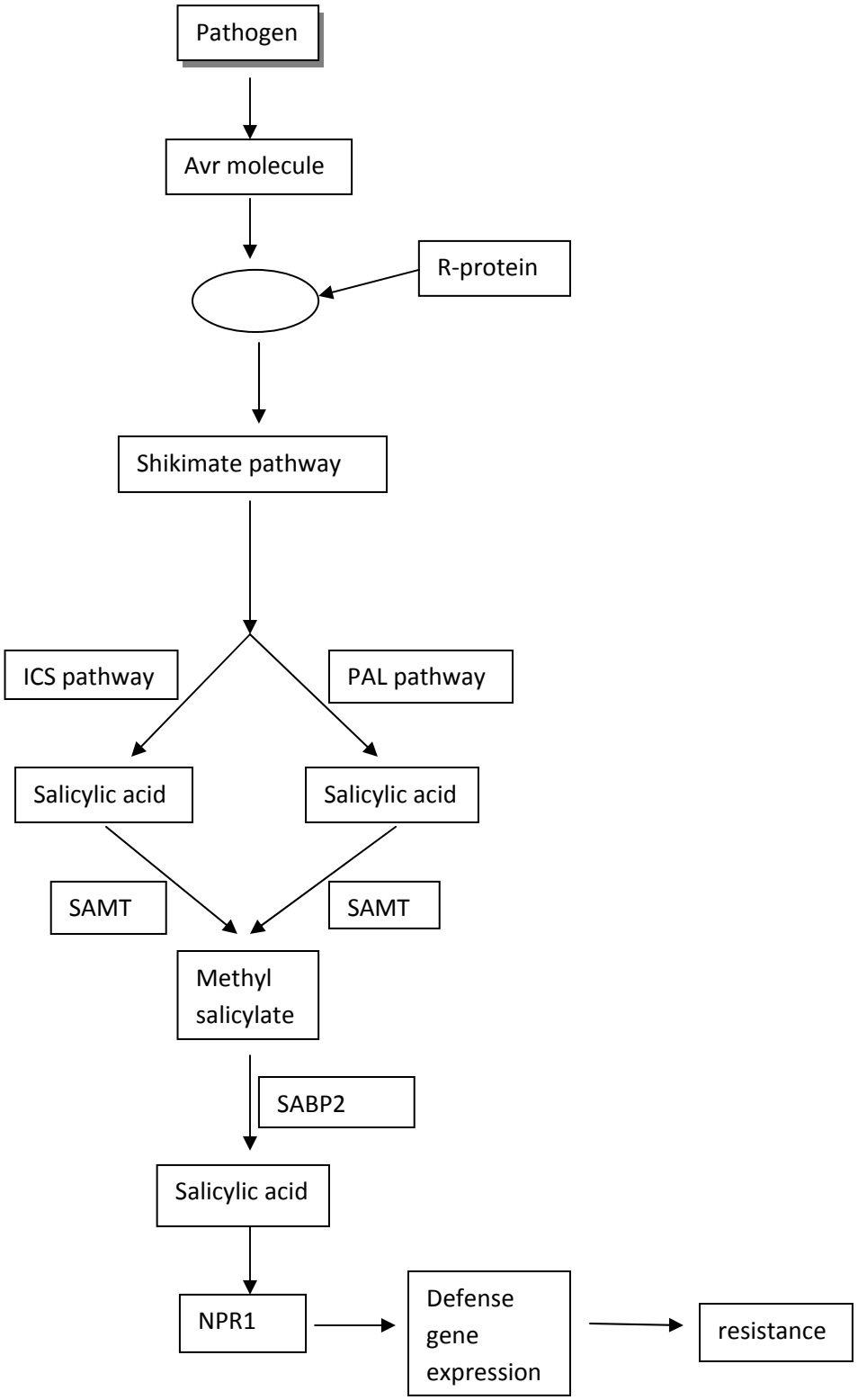
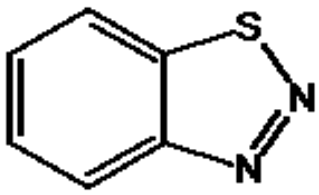


Figure 3. Schematic Representation of the SA-Biosynthetic Pathway. SAMT, Salicylic Acid Methyl Transferase; SABP2, Salicylic Acid Binding Protein; NPR, None-expressor of Pathogenesis-related Gene

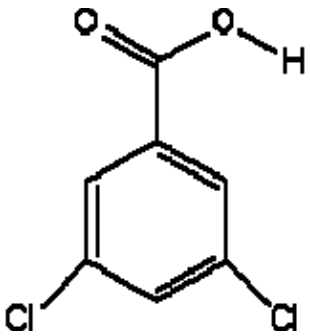
In the previous sections, we have covered the various plant responses to pathogen attack and also the involvement of SA in these responses. We have also been able to see an overview of the biosynthetic pathway through which SA defense response is expressed. A key enzyme in the SA signal transduction pathway is the salicylic acid binding protein (SABP2) believed to be found in the cytoplasm, which converts methyl salicylate (MeSA) to SA. The SA produced from this step in turn activates the PR-1 genes to elicit the pathogen resistance.

Bioactive Synthetic Analogues of Salicylic Acid

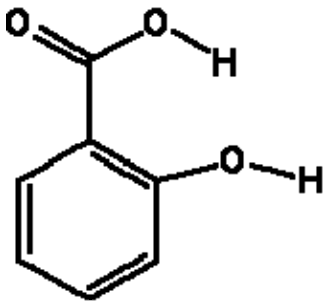
Upon identification of SA as a key chemical in the induction of pathogen resistance, various synthetic bioactive analogues of SA have been synthesized and tested for activity. Two classes of SA analogues are 2,6-dichloro isonicotinic acid (INA) and benzo [1,2,3] thiadiazole and their derivatives. Their chemical structures are given below:



Benzo [1,2,3] thiadiazole



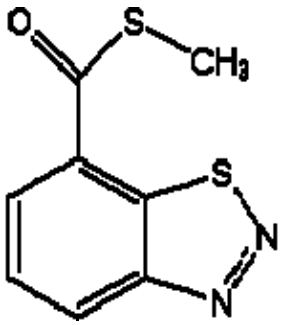
INA



SA

It can be seen that these compounds share similar features, particularly the benzoic acid-like backbone.

S-methyl benzo [1,2,3,] thiadiazole-7-carbothiate (acibenzolar-S-methyl; ASM) was the first commercial BTH derivative under the trade names BION, ACTIGARD, and BOOST.



BTH (ASM)

It is important to note that these chemicals do not show any antimicrobial activity *in vitro* and activate resistance against the same spectra of pathogens as the biological inducers of SAR on the plant species where this information is available. The same characteristic set of SAR genes that are induced by biological or SA inducers are also induced by these chemicals. From these findings and results with *NahG* (SH) plants and SAR deficient *nim 1* mutants (mutation of NPR1) of Arabidopsis, it was concluded that both chemicals act as functional analogues of SA in the SAR signaling pathway [6].

While none of the INA derivatives were commercialized (mainly due to insufficient crop tolerance and stability issues), they served as important research tools to investigate the biology and mode of action of SAR induced by chemicals. One important demonstration was the face that xenobiotic compounds could induce the same resistance spectrum and the same biochemical changes as previously described for the biological SAR induction on cucumber and tobacco. Also, it was also found that INA is fully systemic and does not require SA production for the activation of the SAR response [6].

Like INA, the spectrum of resistance activation and the induced biochemical changes by ASM matches that of the biological induction on those crops where such comparisons have been made. It is worth noting that in wild-type plants this activation by ASM takes place without the accumulation of SA. Moreso, ASM is still fully active on *NahG* tobacco and Arabidopsis plants that lack SA and do not respond to biological induction. Also, *nim 1* mutants of Arabidopsis selected for ASM insensitivity lost at the same time their responsiveness to SA and INA as well as to biological inducers of SAR. This is therefore evidence that ASM and INA are acting in the signal pathway of biological SAR induction at or downstream of the SA site of action [6]. These compounds are systemic themselves and therefore may not induce MeSA, the systemic signal of the biological SAR process, [11] nor do they induce the production of SA in the treated plants [6]. Figure 4 below shows the site of action of ASM in the signal transduction pathway.

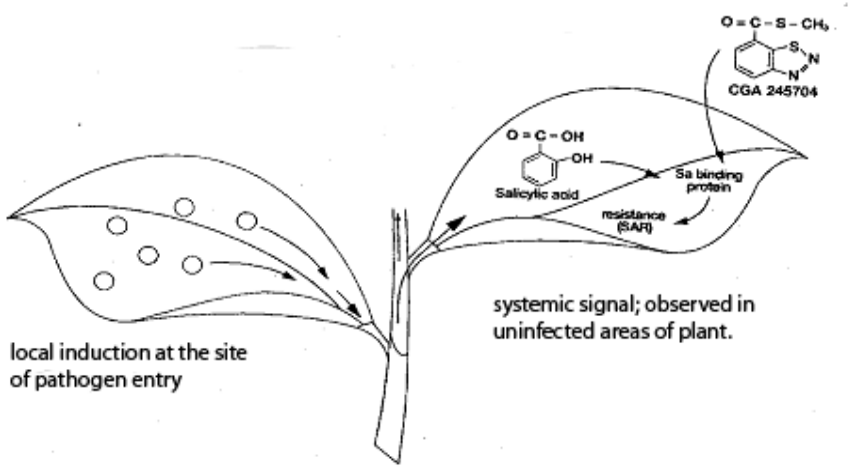


Figure 4. Site of action of ASM (CGA 245704) in the signal transduction pathway. (redrawn from Oostendorp [6] and Goerlach [7])

ASM activates a wide spectrum of resistance under practical field conditions that includes fungal, bacterial, or viral pathogens. This is illustrated by Figure 5.

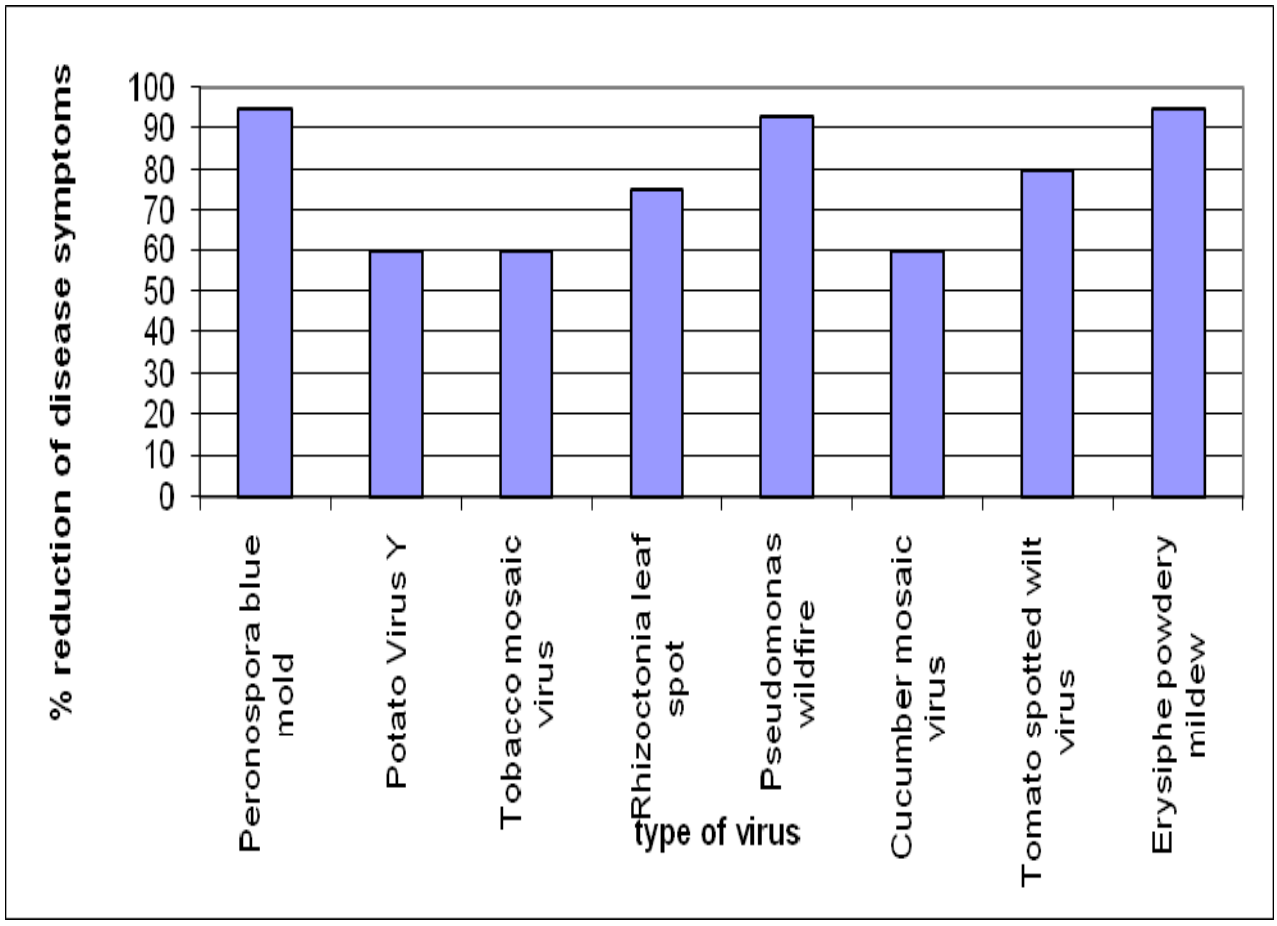


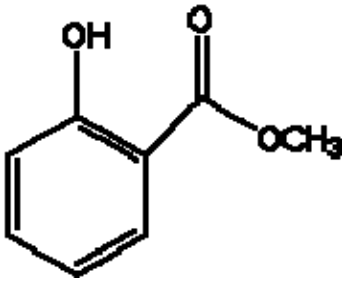
Figure 5. Broad-spectrum SAR activation by ASM against diseases of tobacco: summary of fields trial results with BION®/ ACTIGARD™ used at 12-37g per ha. (redrawn from Oostendorp [6])

On tobacco, one interesting difference between the chemical and biological induction is the duration of protection. Chemical treatments need to be applied at intervals of 1-2 weeks, while the biological induction lasts much longer [6].

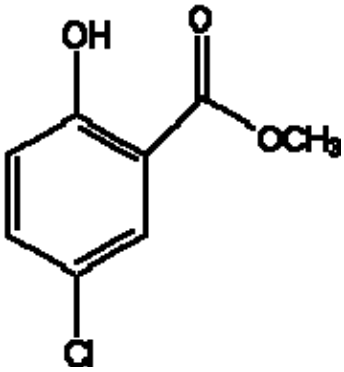
Structure-Activity Studies of Some Salicylic Acid Analogues

In this study, a two-tiered approach was used to screen several salicylic acid derivatives in which the primary screen qualified the induction and accumulation of PR-1 protein and the second tier tested the ability to increase resistance of tobacco to tobacco mosaic virus (TMV).

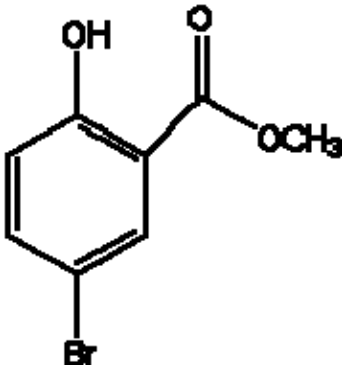
The 36 derivatives studied here were mono-, di-, and tri-substituted methyl salicylate derivatives. The compounds shown below bear a direct structural relevance to the compounds we intend to synthesize in that they have the same set of substituent at the 5-position on the benzene ring[10].



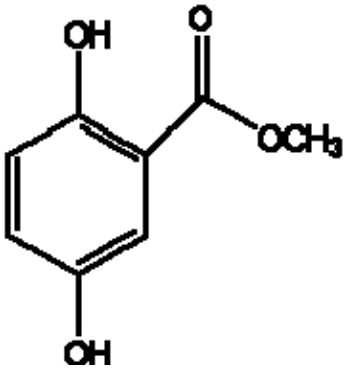
Methyl salicylate



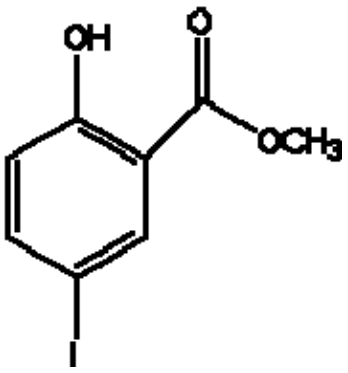
Methyl-5-chloro salicylate



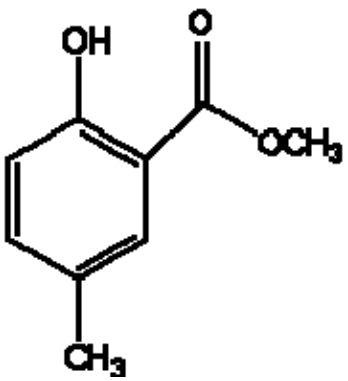
Methyl-5-bromo salicylate



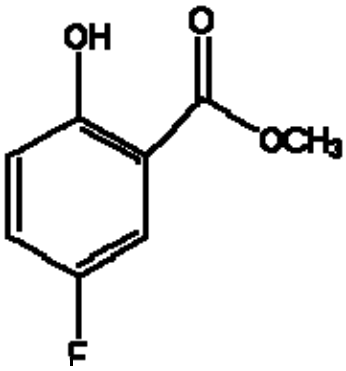
Methly-5-hydroxy salicylate



Methyl-5-iodo salicylate



Methyl-5-methyl salicylate



Methyl-5-fluoro salicylate

Among the 36 monosubstituted salicylates tested, 3 had greater PR-1a induction than SA (3-chlorosalicylate, 3-fuorosalicylate and 5-fluorosalicylate with 167%, 113%, and 60% greater induction respectively).

It was also found that within a family of derivatives, the 3- or 5-position substitutions were more active than 4- or 6-position substitutions. The 5-substituted halosalicylates showed decreasing activity with increase in atomic weight and steric bulk of the substituent group (i. e., activity of 5-fluorosalicylate > 5-chlorosalicylate > 5-bromosalicylate > 5-iodosalicylate) [10]. From the PR-1a-inducing activity of the monosubstituted and multiply substituted salicylates, it was concluded that enhanced activity was observed only when specific conditions were met:

1. Enhanced activity was observed only with substitutions at the 3- and 5-postions. Substitutions at other ring positions resulted in decreased activity.
2. Substituents must be electron-withdrawing to enhance activity.
3. Substituents had to contain no more than one atom and have a van der Waals radius no greater than that of chlorine. Also, position 3 was more tolerant to steric bulk than position 5. [10]

This study also found that except for 6-fluorosalicylate, all fluoro- and chlorosalicylates tested were more active resistance inducers than SA against TMV (3-chlorosalicylate > 3,5-difluorosalicylate > 3,5-dichloro-6-hydroxysalicylate > 3,5,6-trichlorosalicylate > 5-chlorosalicylate > 5-fluorosalicylate > 3,5-dichlorosalicylate > 4-fluorosalicylate > 3-fluorosalicylate > 3-chloro-5-fluorosalicylate > 4-chlorosalicylate > SA) [10]

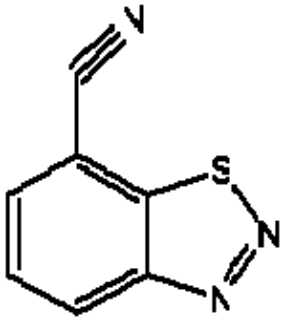
This study shows the effects of varying the substituent at the 5-position of the ring but the effects of the changing the length of the side chain while varying the substituents at the same time has not really been investigated.

BTH-Derived Agrochemicals

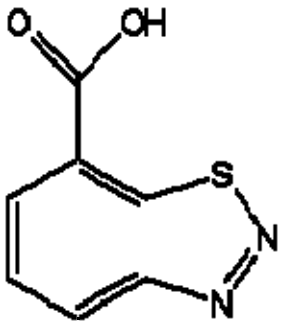
Considering the high binding affinity of BTH for SABP2 [17] and also its high potency for induction of PR-1 genes, BTH derivatives have been the centre of research in the development of new agrochemicals.

Several patents have been submitted on various methods used for the synthesis and testing of several BTH derivatives used for plant immunization. It is important to point out that these compounds below are illustrative of the many compounds that were described in the patents and detailed information can be obtained by consulting the actual patent.

One of these patents shows a method and composition for the immunization of healthy useful plants against plants diseases containing as active various BTH derivatives. The invention describes the application of small amounts of 7-cyano-1, 2, 3-benzothiadiazole derivatives or derivatives of 1, 2, 3-benzothiadiazole-7-carboxylic acid [8].

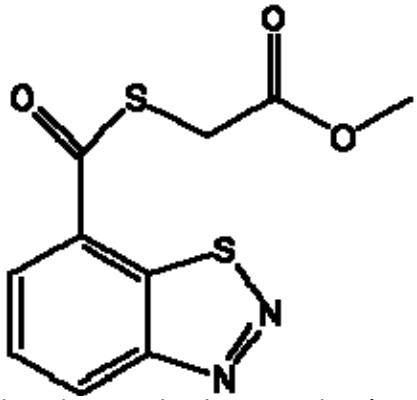


7-cyano-1,2,3-benzothiadiazole



1,2,3-benzothiadiazole-7-carboxylic acid (BTH-acid)

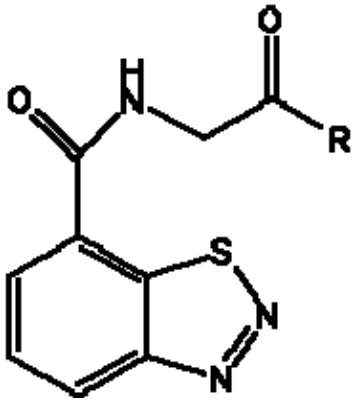
One of the BTH-acid derivatives 1,2,3-Benzothiadiazole-7-carbothioic acid, S-(2-methoxy-2-oxoethyl) ester is shown below.



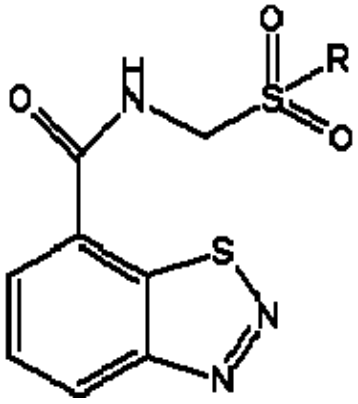
1,2,3-Benzothiadiazole-7-carbothioic acid, S-(2-methoxy-2-oxoethyl) ester

Immunization was done against various pathogens on various plants. The application of the derivatives was either foliar or to the soil.

Another patent on the preparation and testing of novel N-acyl- and N-sulfonylbenzo-1,2,3-thiadiazole-7-carboxylic acid amides was submitted by Kunz et al. [9] . These compounds also have the benzo [1,2,3] thiadiazole structure as the backbone



N-acylbenzo-1,2,3-thiadiazole-7-carboxylic acid amides

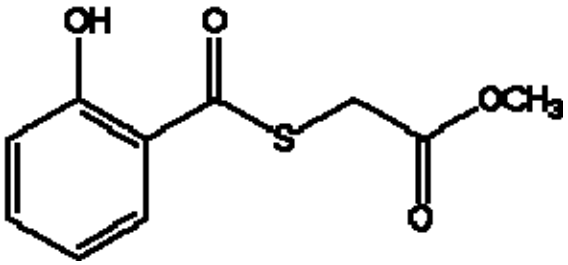


N-sulfonylbenzo-1,2,3-thiadiazole-7-carboxylic acid amide

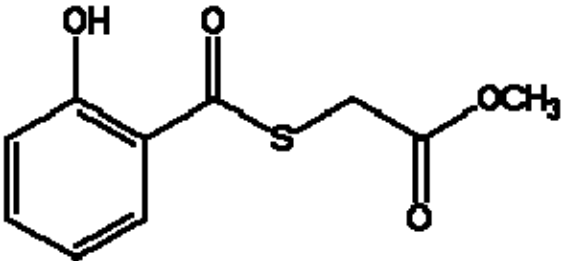
Even though BTH has shown many useful effects in plant protection against pathogens, a lot is yet to be discovered about its effects on the plant and its effects on the environment and even on humans. Some studies have been conducted to investigate the effects of BTH on the plant.

In one study on the effect of BTH treatment of " Gala" apples against fire blight, it was shown that BTH could modify some important market quality and organoleptic characteristics of the fruit, such as causing a less pronounced red colour and reduced total soluble solids (TSS) content. Apple skin colour is a key criterion for harvest maturity and also corresponds to an important quality trait that is clearly related to consumer preferences. [16]

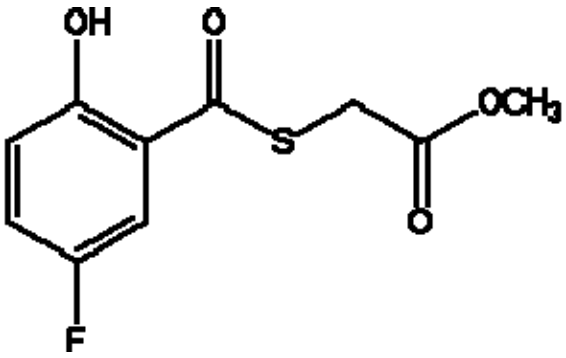
In view of some of the effects of BTH we have sought to try to develop new compounds that would show as much activity and effectiveness as BTH, while trying to minimize any unwanted side effects. The compounds we are seeking to produce incorporate properties of both sets of active compounds described previously- a long side chain and a substituent at the 5-position of the ring. The S-(2-methoxy-2-oxoethyl) salicylic acid ester is the backbone of our design. 5-fluoro, -bromo, -chloro, -iodo, -hydroxy, -methyl, and –H compounds will be synthesized and used for in-vitro studies. It is important to note here that upon SABP 2 hydrolysis of these compounds, salicylic acid and 5-substituted salicylic acid derivatives are produced. SA is the natural resistance inducing compounds and is shown to have no unwanted side effects if present in the acceptable amounts in plants. The 5-substituted salicylic acid derivatives that result for the hydrolysis of the 5-substituted compound also do not pose any threat to the plant. The compounds to be synthesized are shown below.



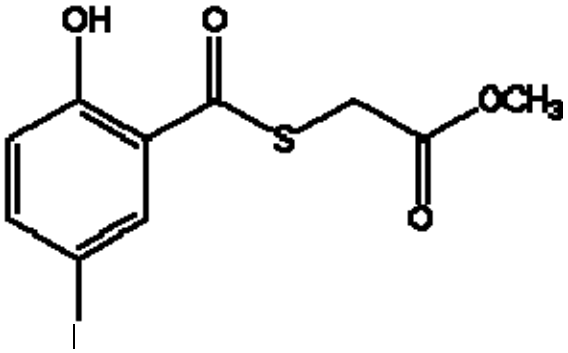
S-(2-methoxy-2-oxoethyl) salicylic acid



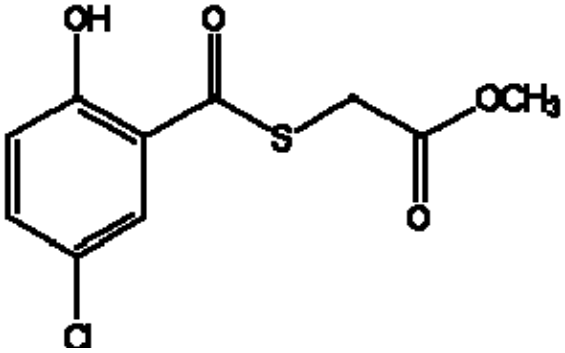
methyl-2-(2-hydroxy benzoyl thio) acetate (YAL 01)



methyl-2-(4-fluoro2-hydroxy benzoyl thio) acetate (YAL 02)

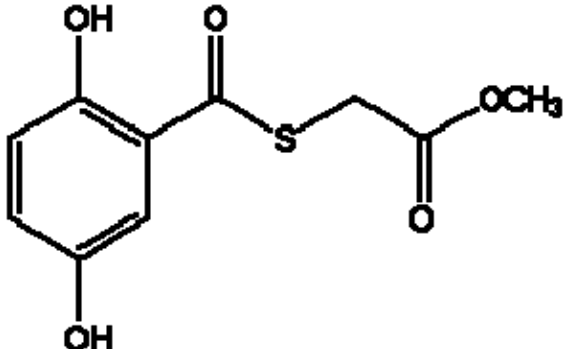


methyl-2-(2-hydroxy-4-iodo benzoyl thio) acetate (YAL 03)



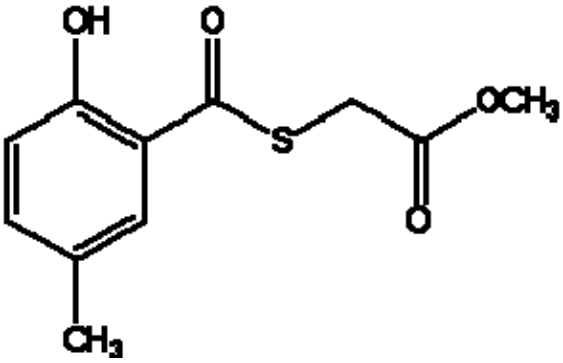
methyl-2-(4-chloro2-hydroxy benzoyl thio) acetate

(YAL 04)



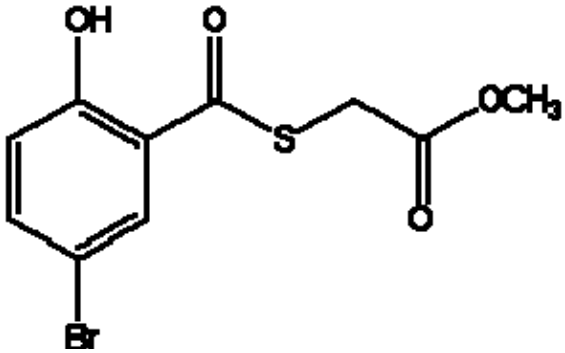
methyl-2-(2,4-dihydroxy benzoyl thio) acetate

(YAL 05)



methyl-2-(2-hydroxy-4-methyl benzoyl thio) acetate

(YAL 06)



methyl-2-(4-bromo-2-hydroxy benzoyl thio acetate)

(YAL O7)

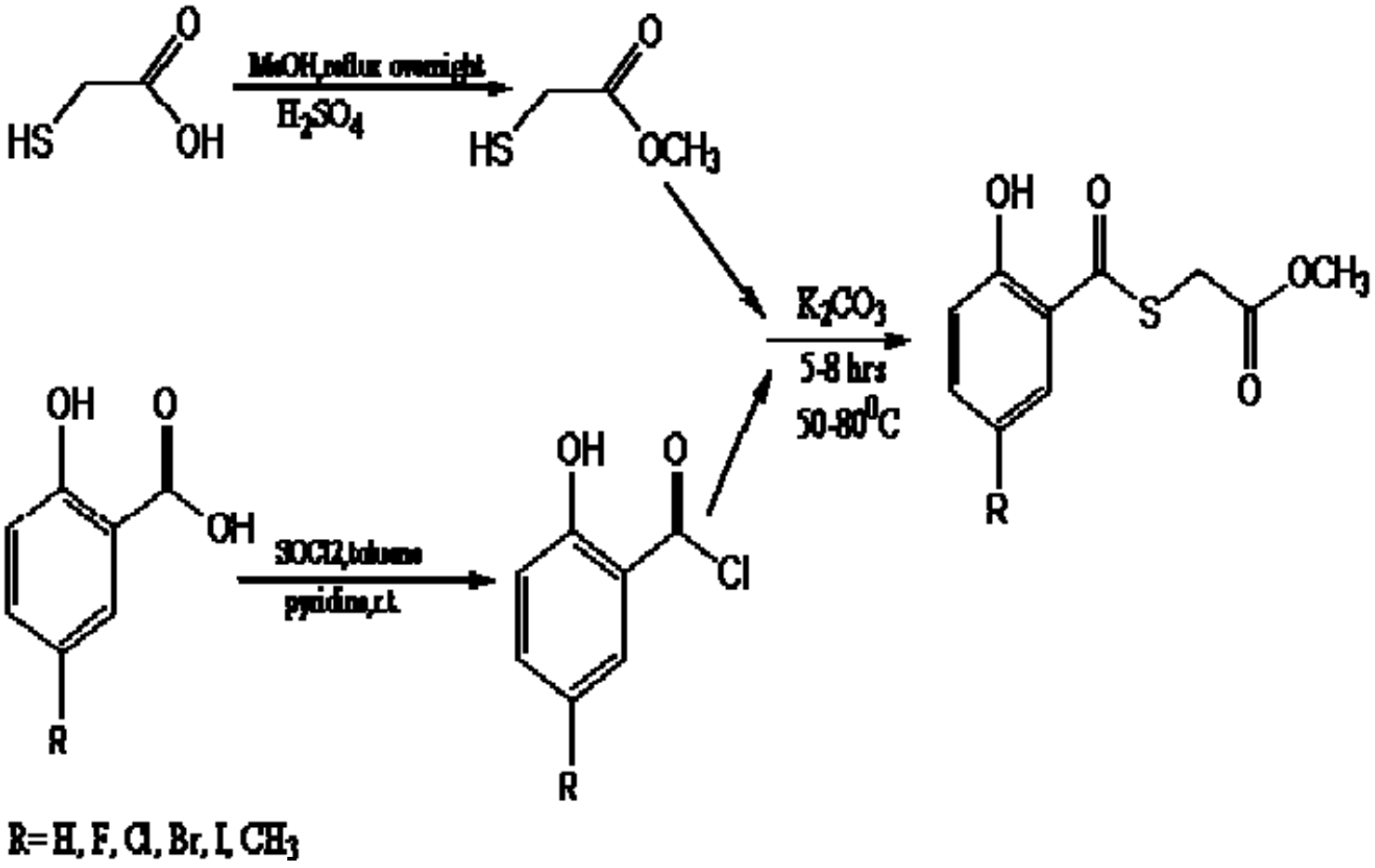
CHAPTER 2

RESULTS AND DISCUSSION

This project is directed towards the synthesis and in-vitro activity studies of novel synthetic agrochemicals. All the compounds share a similar structural characteristic i.e., they all have a long thio-, alkyl-diester side chain. The major structural difference is the presence of a different atom or group of atoms on the 5-position if the benzene ring. One part of this project is putting the side chain on the ring. The second part of the project is the in-vitro activity studies using the salicylic acid binding protein 2 (SABP2). The enzyme is stored in a pH 7 phosphate buffer.

The synthetic methodology involved a convergent reaction scheme. The first step involved the esterification of one of the starting materials-thioglycolic acid using methanol as reagent and concentrated sulfuric acid as catalyst. It is important to note here that the product from this step was not isolated and characterized. The second reaction in this procedure involved the conversion of the second starting material, the 5-substituted salicylic acid, into the acid chloride. This step makes the carboxylic acid more reactive towards esterification. It is also worth noting that the product from this step was not isolated and characterized. The third reaction in this synthetic procedure involved the mixing of the reaction mixtures from the two reactions above in the presence of potassium carbonate to obtain the final product.

The general reaction scheme for this synthetic procedure is given in Scheme 1.



Scheme 1. Synthetic Reaction Scheme for YAL Derivatives

Synthesis of YAL 01

YAL 01 was synthesized successfully through the process described above. The low yield of the reaction was probably due to the formation of side products and also due to some of the starting materials not reacting. Purification was done using column chromatography with the solvent system being-ethyl acetate/hexane in a gradient fashion, starting with 100% hexanes. The product was characterized using NMR (Appendix 1,2) and infrared spectroscopy (Appendix 3). The proton NMR is consistent with the compound structure, as well as the C-13 NMR. The peak at δ 1.5ppm is from water and should be disregarded. The IR spectrum also confirms the structure, showing peaks for the two carbonyl functions, the ester, and the thioester bonds.

Synthesis of YAL 03

YAL 03 was also synthesized successfully through the procedure described above. Some difficulty was encountered in the purification of the product due the formation of other side-products in significant amounts with R_f values very close to the R_f of the product. This could explain the low yield of the reaction. The proton and carbon-13 NMR's also show the presence of some impurities. Gradient elution was done using hexanes and ethyl acetate, beginning with 100% hexanes and slowly increasing the percentage of ethyl acetate. The product structure was confirmed by NMR spectroscopy (Appendix 4,5). The proton NMR was consistent with the structure as well as C-13 NMR.

Synthesis of YAL 04

YAL 04 was synthesized through the same procedure described above. In this procedure, gradient elution was also used for purification of the compound. The presence of other side products with R_f values close to the R_f of the product is a possible reason for low yield of the reaction because purification was difficult. The product structure was determined through NMR spectroscopy (Appendix 6,7). The proton NMR was consistent with the structure as well as the C-13 NMR. The NMR spectra show the presence of some impurity peaks.

Synthesis of YAL 05

The procedure for the synthesis of YAL 05 was followed the same way as for the other derivatives. The proton and carcon-13 NMRs were inconclusive though. The spectra were taken using acetone-D6 as NMR solvent. Two expected singlets for the two OH-groups on the ring were absent. The broad peak on the proton NMR could not be accounted for (Appendix 8). Even though the carbon-13 confirmed the presence of the methoxy group, the diester methylene proton was not found. This could be because it carbon-13 chemical shift occurs very close to the acetone-D6 multiplet(Appendix 9).

Kinetic Studies for BTH

BTH is a widely used commercial agrochemical that is a much stronger inducer of disease resistance than salicylic acid. It is not known whether BTH is actually converted to BTH acid by SABP2 during the disease resistance process. The kinetic studies shown below, coupled with other data, shows SABP2 can convert BTH to BTH acid. Moreso, because we are trying to

make compounds that could be more effective than BTH, we decided to study the in-vitro SABP2 activity.

The kinetic study of BTH was carried out at room temperature (28^0C) in 0.5mL test tubes using SABP2 as enzyme. The enzyme used was in a pH 7 phosphate buffer. The final enzyme concentrations used for the kinetic study for BTH was determined on a trial and error basis. The reactions were carried out in a pH 7 buffer with 20% DMSO in order to allow for BTH to remain in solution. Trials with 100% phosphate buffer led to precipitation of BTH. The BTH standards were prepared in DMSO and its influence on the reaction has not been investigated. The reaction time for each measurement was taken from the time the enzyme was mixed with the compound to the time the reaction mixture was injected into the HPLC. The total reaction time was about 2 minutes. The HPLC loop used was a 50µL loop, thus injecting 10µL of the reaction mixture into the loop caused a 5-fold dilution hence stopping the reaction. The result for BTH is given below. A graphical picture is found in Appendix 10. Kinetic data for BTH is shown in Table 2.

Table 2. Kinetic Data for BTH

Compound	V_{max}	K_m
BTH	63.19	275.41

Table 2 shows some kinetic data from in-vitro kinetic studies for BTH. Michaelis-Menten enzyme-substrate kinetics is assumed. V is the reaction velocity, which is the number of moles of product formed per second. V_{max} is the maximum velocity. K_m is the Michaelis constant,

which is the substrate concentration at half V_{max}. From the table, we see that BTH shows a high in-vitro activity. From the data, we can also calculate the turnover number, k , which is the number of substrate molecules converted to product per unit time when the enzyme is fully saturated with substrate. Table 3 shows the calculated turnover number for BTH.

Table 3. Turnover Number for BTH

	BTH
$k(s^{-1})$	4269.675

CHAPTER 3

EXPERIMENTAL

Materials and Methods

All chemical reagents were bought and used without purification unless indicated otherwise. The following chemicals were bought from TCI America: 2,5-dihydroxy benzoic acid, 5-chlorosalicylic acid, 5-iodosalicylic acid, 5-bromosalicylic acid, 5-fluorosalicylic acid, 5-methylsalicylic acid. Thioglycolic acid, hexanes, ethyl acetate, toluene, pyridine, concentrated sulfuric acid, methanol, and salicylic acid were bought from Fisher Scientific chemical company. Thionyl chloride was bought from Alpha Aesar chemical company. DMSO was purchased from Aldrich chemical company and chloroform-D for NMR was bought from Acros organics.

All NMR spectra were recorded on the JEOL-NMR Eclipse spectrometer at 400MHz in $CDCl_3$, unless stated otherwise. Chemical shifts were recorded as delta values in parts per million (ppm), relative to the TMS. The multiplicity of the signals is reported as follows: s, singlet; d, doublet; dd, doublet of doublet; dt, doublet of triplet; t, triplet; tt, triplet of triplet; q, quartet; m, multiplet.

Column chromatography separation was done on silica gel purchased from SILICYCLE chemical company. The thin layer chromatography (TLC) was done using silica gel plates with a UV_{254} fluorescent indicator bought from Aldrich.The kinetic studies were done using the Varian Prostar HPLC-210. The mass spectra were obtained using the Shimadzu GCMS-QP 2010 Plus and the HP 5890 Series II GCMS. Purified SABP2 was provided by Dr. Dhrendra Kumar, Department of Biological Sciences, East Tennessee State University.

Synthesis of methyl-2-(2-hydroxy benzoyl thio) acetate (YAL 01)

To a 100mL round bottom flask was added 0.75mL (0.011mol) of thioglycolic acid, 6.48mL (0.16mol) methanol, and 5.9µL concentrated sulfuric acid. The mixture was refluxed overnight to give a clear liquid. The excess methanol was removed by bubbling under nitrogen.

To a 100mL round bottom flask was added 1.519g (0.011mol) of salicylic acid, then 1.2mL (0.017mol) of thionyl chloride, 20µL of pyridine, and 4mL of toluene. The mixture was heated to dissolve the solid, then left to stir at room temperature for 1hour to obtain a faint yellow liquid. The excess thionyl chloride was removed by blowing with nitrogen.

Both reaction mixtures were combined in a 100mL round bottom flask to which was added 2.04g K_2CO_3. The mixture was stirred at 60^{o}C for 5hours.

To the reaction mixture was added 10mL of water and extracted with ethyl acetate. The organic phase was dried with anhydrous calcium chloride and evaporated on the rotary vapor. The resulting liquid was purified by column chromatography using a gradient hexane/ ethyl acetate system, beginning with 100% hexanes, on a column with 20g of silica gel. 0.679g (27%) of a yellow liquid was obtained. ^{1}H NMR ($CDCl_3$, 400MHz, ppm): δ 10.76(s, 1H), δ 7.84(d, *J*=1.44, 1H), δ 7.48(t, 1H), δ 6.97(d, *J*= 8.44, 1H), δ 6.92(t, *J*= 0.76), δ 3.87(s, 2H), δ 3.78(s, 3H). ^{13}C NMR ($CDCl_3$, 100MHz, ppm): δ 194.50, δ 168.66, 165.56, 159.55, 136.54, 128.99, 119.63, 53.11, 30.91. IR: 1739.79cm^{-1}, 1625.99cm^{-1}, 1579cm^{-1}, 1481cm^{-1}.

Synthesis of methyl-2-(4-chloro-2-hydroxy benzoyl thio) acetate(YAL 04)

To a 100mL round bottom flask was added 0.75mL (0.011mol) of thioglycolic acid, 6.48mL (0.16mol) methanol and 5.9µL concentrated sulfuric acid. The mixture was refluxed overnight to give a clear liquid. The excess methanol was removed by bubbling with nitrogen.

To a 100mL round bottom flask was added 1.90g (0.011mol) of 5-chlorosalicylic acid, then 1.2mL (0.017mol) of thionyl chloride, 20µL of pyridine, and 4mL of toluene. The mixture was heated to dissolve the solid, then left to stir at room temperature for 1hour to obtain a faint yellow liquid. The excess thionyl chloride was removed by bubbling with nitrogen.

Both reaction mixtures were combined in a 100mL round bottom flask to which was added 2.04g K_2CO_3. The mixture was stirred at 80^{o}C for 8hours.

To the reaction mixture was added 10mL of water and extracted with ethyl acetate. The organic phase was dried with anhydrous calcium chloride and evaporated on the rotary vapor, and purified by column chromatography using hexane/ ethyl acetate solvent system on a column with 20g of silica gel. Gradient elution was used beginning with 100% hexanes to obtain 0.69g of product. ^{1}H NMR ($CDCl_3$, 400MHz, ppm): δ 10.68(s, 1H), δ 7.80(s, 1H), δ 7.39(d, *J*= 2.56, 1H), δ 6.92(d, *J*= 9.2, 1H), δ 3.87(s, 2H), δ 3.77(s, 3H). ^{13}C NMR ($CDCl_3$, 100MHz, ppm): δ 194.59, 168.58, 158.12, 136.44, 134.02, 128.12, 124.38, 120.06, 53.17, 31.05.

Synthesis of methyl-2-(4-iodo-2-hydroxy benzoyl thio) acetate(YAL 03)

To a 100mL round bottom flask was added 0.75mL (0.011mol) of thioglycolic acid, 6.48mL (0.16mol) methanol, and 5.9µL concentrated sulfuric acid. The mixture was refluxed overnight to give a clear liquid. The excess methanol was removed by bubbling with nitrogen.

To a 100mL round bottom flask was added 2.90g (0.011mol) of 5-iodosalicylic acid, then 1.2mL (0.017mol) of thionyl chloride, 20µL of pyridine, and 6mL of toluene. The mixture was heated to dissolve the solid, then left to stir at room temperature for 1hour to obtain a faint yellow liquid. The excess thionyl chloride was removed by bubbling with nitrogen.

Both reaction mixtures were combined in a 100mL round bottom flask to which was added 2.00g K_2CO_3. The mixture was stirred at 70^{o}C for 8hours.

To the reaction mixture was added 10mL of water and extracted with ethyl acetate. The organic phase was dried with anhydrous calcium chloride, evaporated on the rotary vapor, and purified by column chromatography using gradient elution starting with 100% hexanes and slowly increasing the percentage of ethyl acetate on a column with 20g of silica gel to obtain 0.35g of product.^{1}H NMR ($CDCl_3$, 400MHz, ppm): δ 10.68(s, 1H), δ 8.12(s, 1H), δ 7.68(d, *J*= 6.6, 1H), δ 6.77(d, *J*= 8.8, 1H), δ 3.87(s, 2H), δ 3.78(s, 3H). ^{13}C NMR ($CDCl_3$, 100MHz, ppm): δ 194.27, 168.60, 159.21, 144.83, 143.00, 137.21, 125.86, 120.78, 53.18, 31.03.

Synthesis of methyl-2-(2,4-dihydroxy benzoyl thio) acetate(YAL 05)

To a 100mL round bottom flask was added 0.45mL (0.0065mol) of thioglycolic acid, 3.89mL (0.096mol) methanol, and 3.54µL concentrated sulfuric acid. The mixture was refluxed overnight to give a clear liquid. The excess methanol was removed by bubbling with nitrogen.

To a 100mL round bottom flask was added 1.00g (0.0065mol) of 5-hydroxysalicylic acid, then 0.8mL (0.011mol) of thionyl chloride, 15µL of pyridine and 3mL of toluene. The mixture was heated to dissolve the solid, then left to stir at room temperature for 1hour to obtain a faint yellow liquid. The excess thionyl chloride was removed by bubbling with nitrogen.

Both reaction mixtures were combined in a 100mL round bottom flask to which was added 1.00g K_2CO_3. The mixture was stirred at 70^{o}C for 8hours.

To the reaction mixture was added 10mL of water and extracted with ethyl acetate. The organic phase was dried with anhydrous calcium chloride, evaporated on the rotary vapor, and purified by column chromatography using gradient elution starting with 100% hexanes and slowly increasing the percentage of ethyl acetate on a column with 20g of silica gel to obtain 0.365g of product. ^{1}H NMR (C_3D_6O, 400MHz, ppm): δ 7.30(s, 1H), δ 7.05(d, 1H), δ 6.79(d, *J*= 5.1, 1H), δ 3.79(s, 2H), δ 3.69(s, 3H). ^{13}C NMR ($CDCl_3$, 100MHz, ppm): δ 171.74, 155.45, 149.53, 124.09, 117.92, 114.79, 112.24, 51.95, 40.13.

Kinetic Studies for S-methyl benzo [1,2,3,] thiadiazole-7-carbothiate (BTH)

PART 1:

In a 0.5mL container was prepared 200µL of a 0.0148µM SABP2 in a pH 7 phosphate buffer solution to be used for kinetic studies. In other containers were prepared 100µL of 50µM, 100µM, 200µM, 400µM, 600µM, 800µM, 1mM and 100mM solutions of BTH. 10µL portions of the 50µM, 100µM, 200µM, 400µM, 600µM, 800µM solutions were run on the HPLC to obtain chromatograms to be used as standards. Measurements were done in duplicates.

PART 2:

A constant amount of the enzyme prepared was made to react in a 0.5mL test tube with BTH solutions at the different concentrations above.

- Reaction 800µM = 16µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 2µL buffer
- Reaction 600µM = 12µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 6µL buffer
- Reaction 400µM = 8µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 10µL buffer
- Reaction 200µM = 4µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 14µL buffer
- Reaction 100µM = 2µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 16µL buffer
- Reaction 50µM = 1µL of 1mM BTH+ 2µL 0.0148µM SABP2+ 17µL buffer

The reactions were carried out over a 2-minute period then 10µL portions of the reaction mixture were run on the HPLC to obtain a chromatogram. The data from Parts 1 and 2 were then processed to obtain an enzyme kinetics graph. The reactions were carried out over a 2-

minute period, and then 10µL portions of the reaction mixture were run on the HPLC to obtain a chromatogram. The data from Part 1 and Part 2 are then processed to obtain an enzyme kinetics graph.

CHAPTER 4

CONCLUSION

The quest for new compounds to be used for plant “immunization” has been and is still an uphill challenge. In order for humans to be able to meet the global food demands, new compounds would have to be made that will confer unto plants a long-lasting broad based resistance to a wide variety of pathogens. The use of BTH over the years has been very successful in plant immunization but more efficient compounds with fewer side effects are still desired. The discovery of the salicylic acid binding protein (SABP2) and the role of salicylic acid in disease resistance have also been important in the understanding of the disease pathway and also in the design of new resistance inducing compounds.

In our project we were able to successfully synthesize some compounds that we expect to be useful in plant immunization. We were unable to begin the in-vitro activity studies on these compounds using the salicylic acid binding protein (SABP2) due to difficulties in obtaining pure samples and also due to time constraints.

This project is yet to be completed and we remain hopeful to be able to develop new compounds that will hit the markets some day as useful plant immunization products.

BIBLIOGRAPHY

1. Cakmak, I; *Plant and Soil*. **2002,** *247*, pp 3-6
2. Population Division, Department of Social and Economic Affairs; Population Newsletter, **June 2007,** *83,* pp3-4
3. Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat, World Population Prospects: The 2006 Revision and World Urbanization Prospects: The 2005 Revision, http://esa.un.org/unpp, Saturday, February 23, 2008
4. Dempsey, A. D.; Shah, J.; Klessig, D. F.; *Critical Reviews in Plant Sciences.* **1999,** *18(4),* pp 547-558
5. Molinary, S.; *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*. **2007**, *2*, pp 5-6
6. Oostendorp, M.; Kunz, W.; Dietrich, B.; Staub, T.; *European Journal of Plant Pathology.* **2001,** 107, pp 21-23
7. Goerlach, J.; Freidrich, L.; Larton, K.; Ryals, J.; *Brighton Crop Protection Conference.* **1996**, pp 963-965
8. Schurter, R.; Kunz, W.; Nyfeler, R.; *United States Patent*, **June 5 1990,** US4931581
9. Kunz, W.; Schurter, R; *United Staes Patent,* **September 24 1991,** US5051436
10. Silverman, F. P.; Petracek, P. D.; Heiman D. F.; Fledderman C. M.; Warrior, P.; *J. Agric. Food Chem.* **2005**, *53*, pp 9775-9778
11. Park, S. W.; Kaimoyo, E.; kumar, D.; Mosher, S.; Klessig, D. F.; *Science*. **2007**, 318, pp 113-115
12. Bolwell, G. P.; Butt, V. S.; Davies, D. R.; Zimmerlin, A.; *Free Rad. Res. Comm.* **1995**, 23, pp 517-532

13. Klessig, D. F.; Malamy, J.; *Plant Mol. Biol.* **1994**, 26, 1439-1458

14. Wellets, M. G.; Ryals, J. A.; *Mol. Plant-Microbe Interact.* **1998**, 11, 795-800

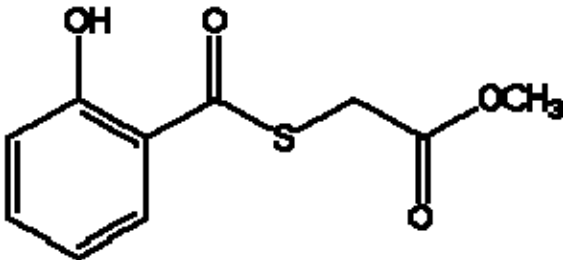
15. Alvarez, M. E.; *Plant Mol. Biol.* **2000**, 44, pp 429-431

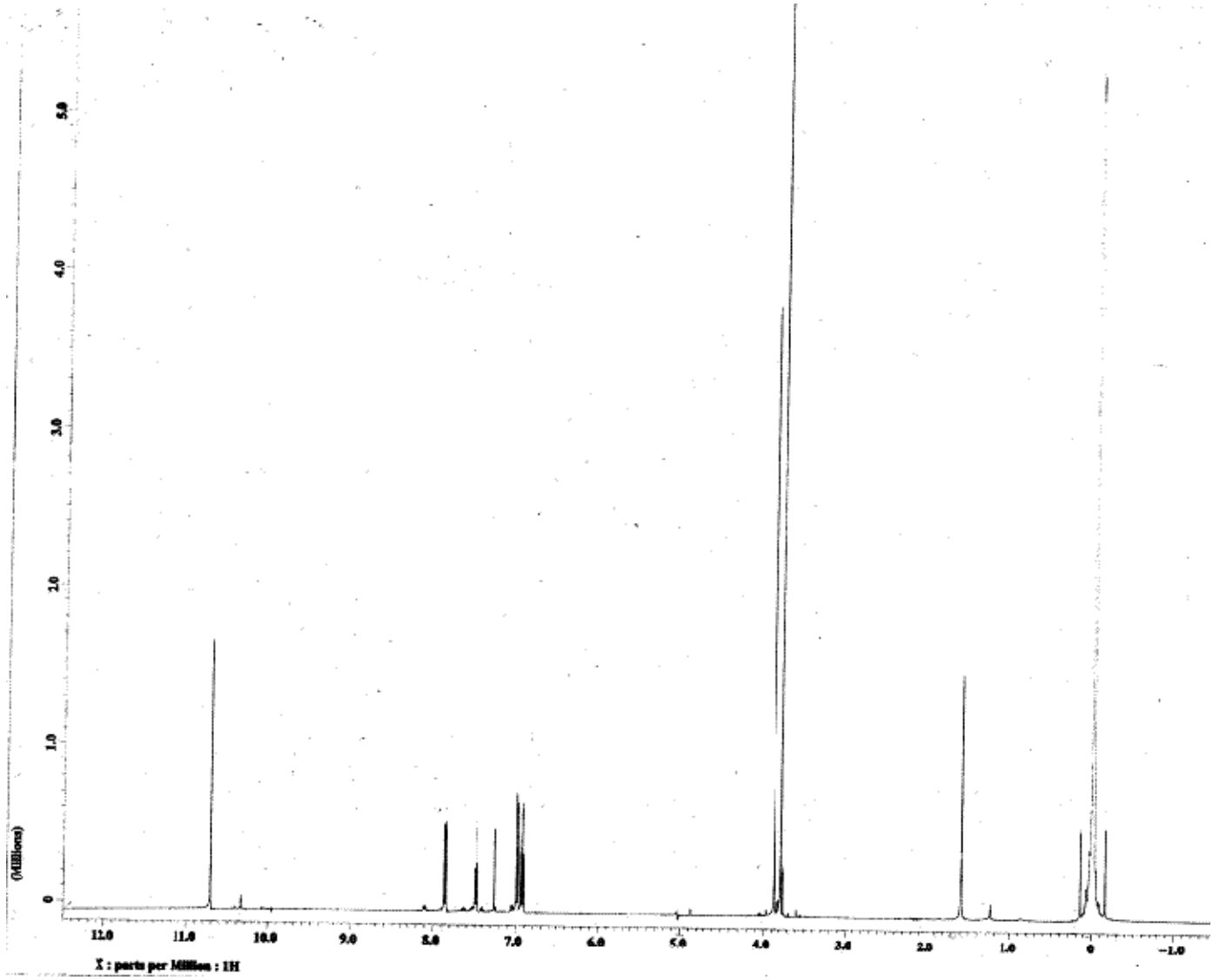
16. Roux, C.; Lemarquand, A.; Orain, G.; Canpion, C.; Simoneu, P.; Poupard, P.; *Journal of Horticultural Science and Biotechnology*, **2006**, 81 (1), pp 142-143

17. Du, H.; Klessig, D. F.; *Plant Physiol.* **1997,** *113*, pp 1319-1320

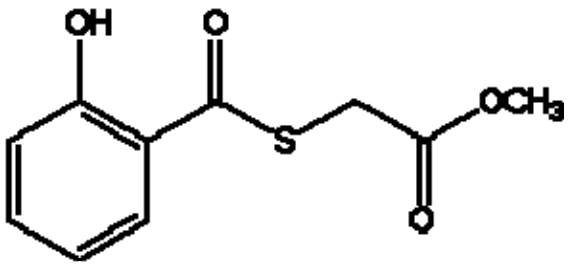
APPENDIXES

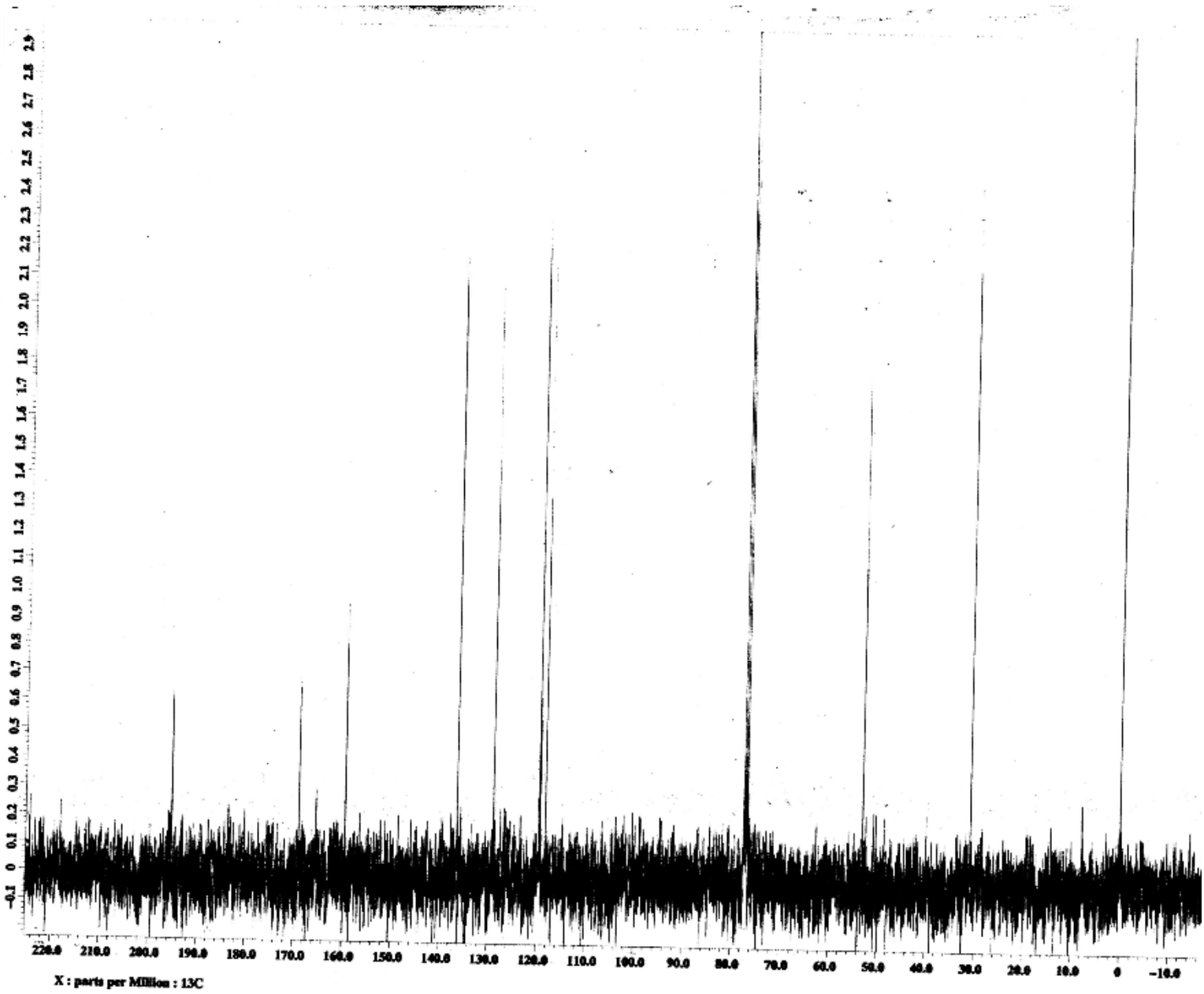
Appendix A. Proton NMR for YAL 01



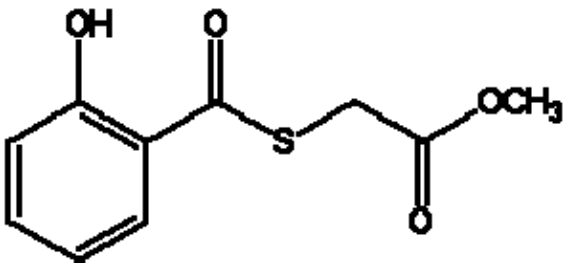


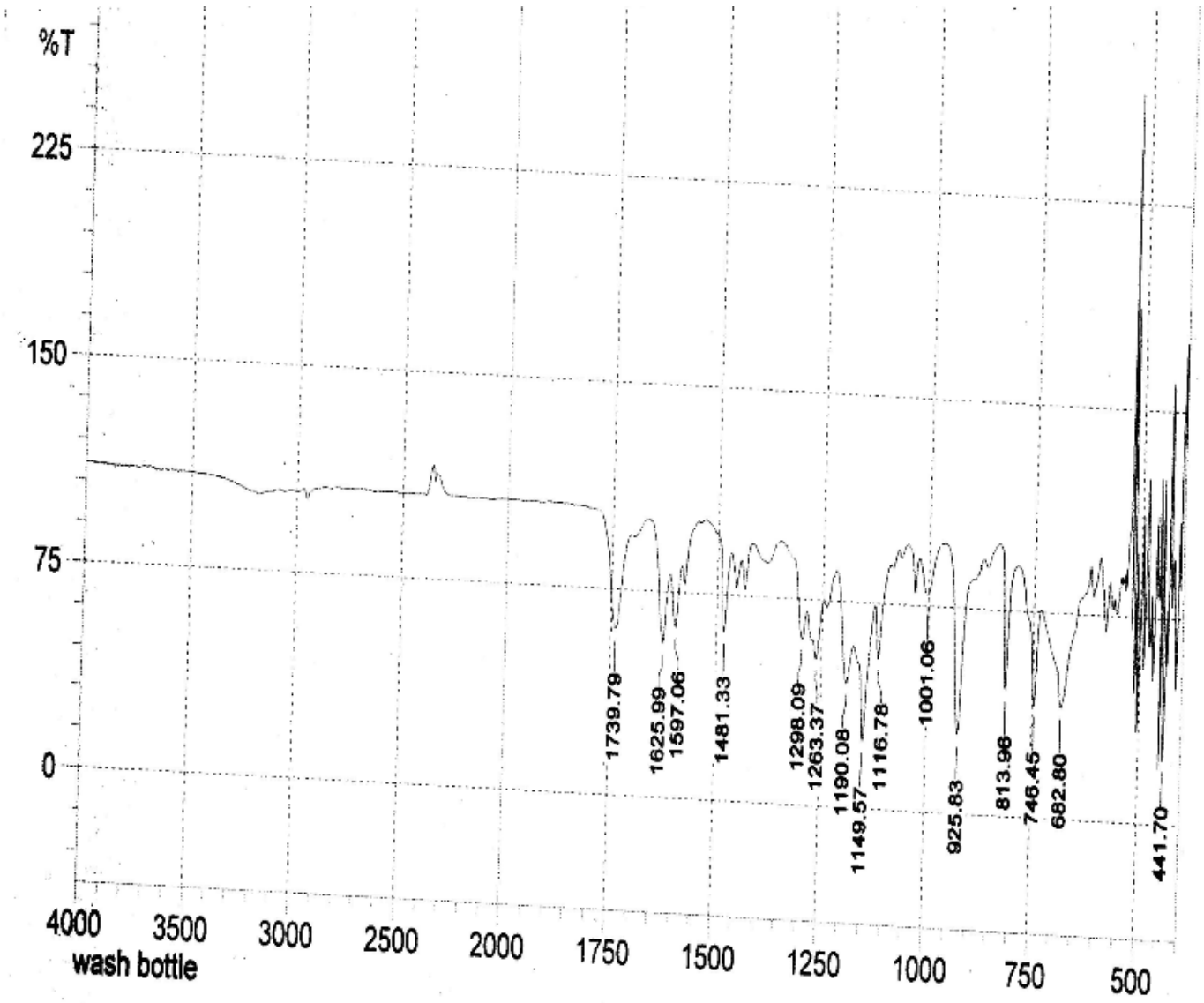
Appendix B. Carbon-13 NMR for YAL 01



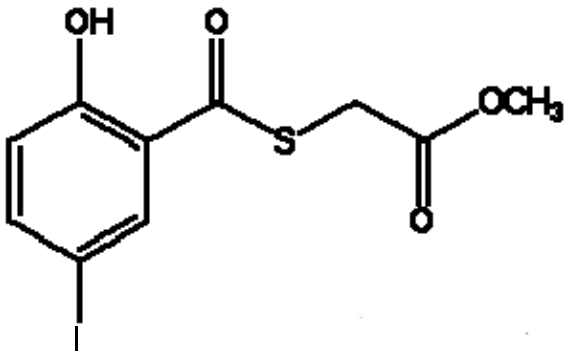


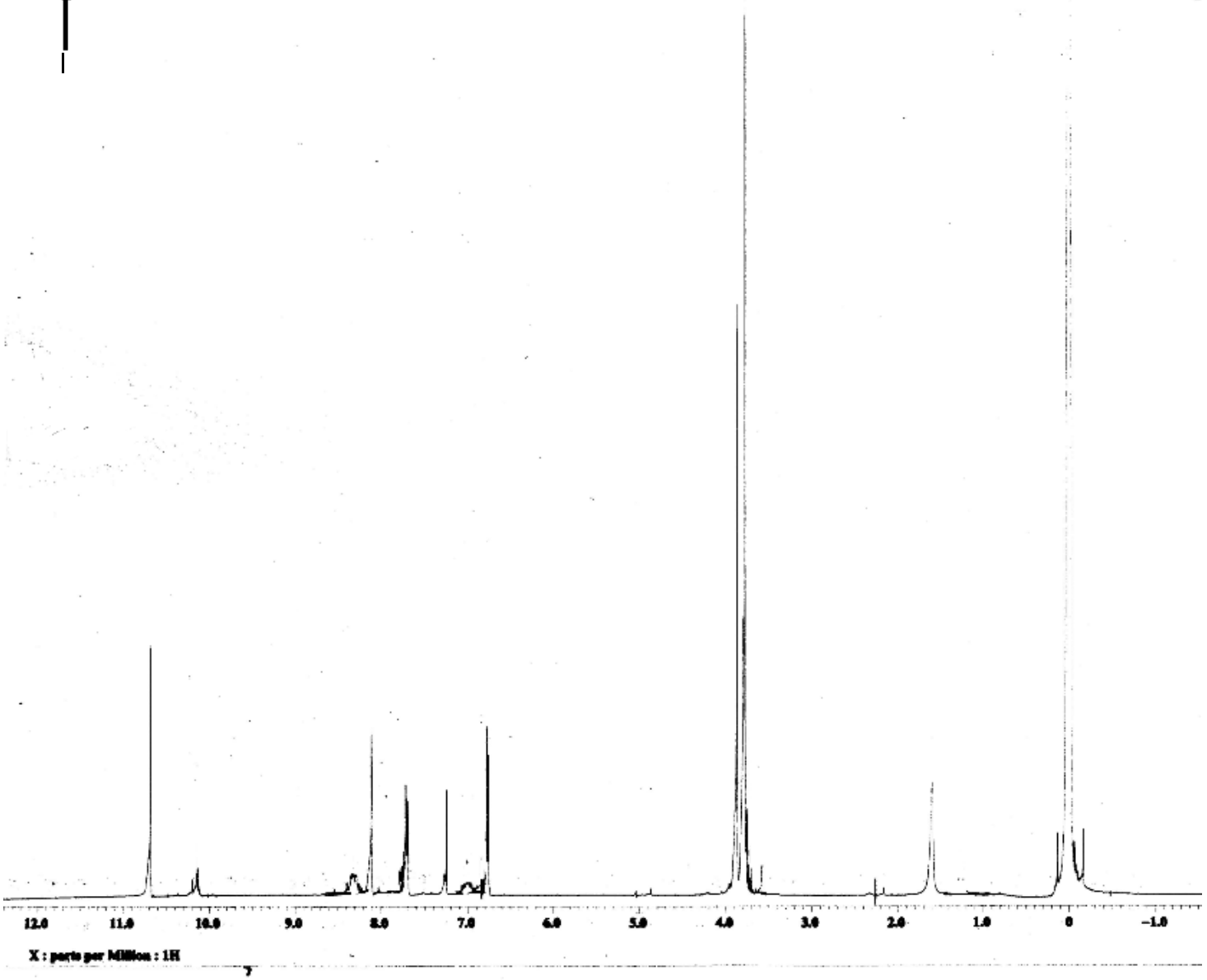
Appendix C. Infrared Spectrum for YAL 01



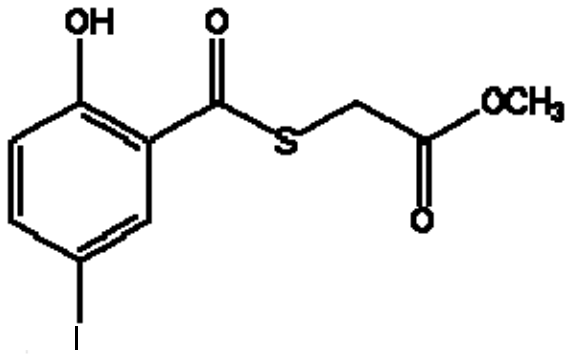


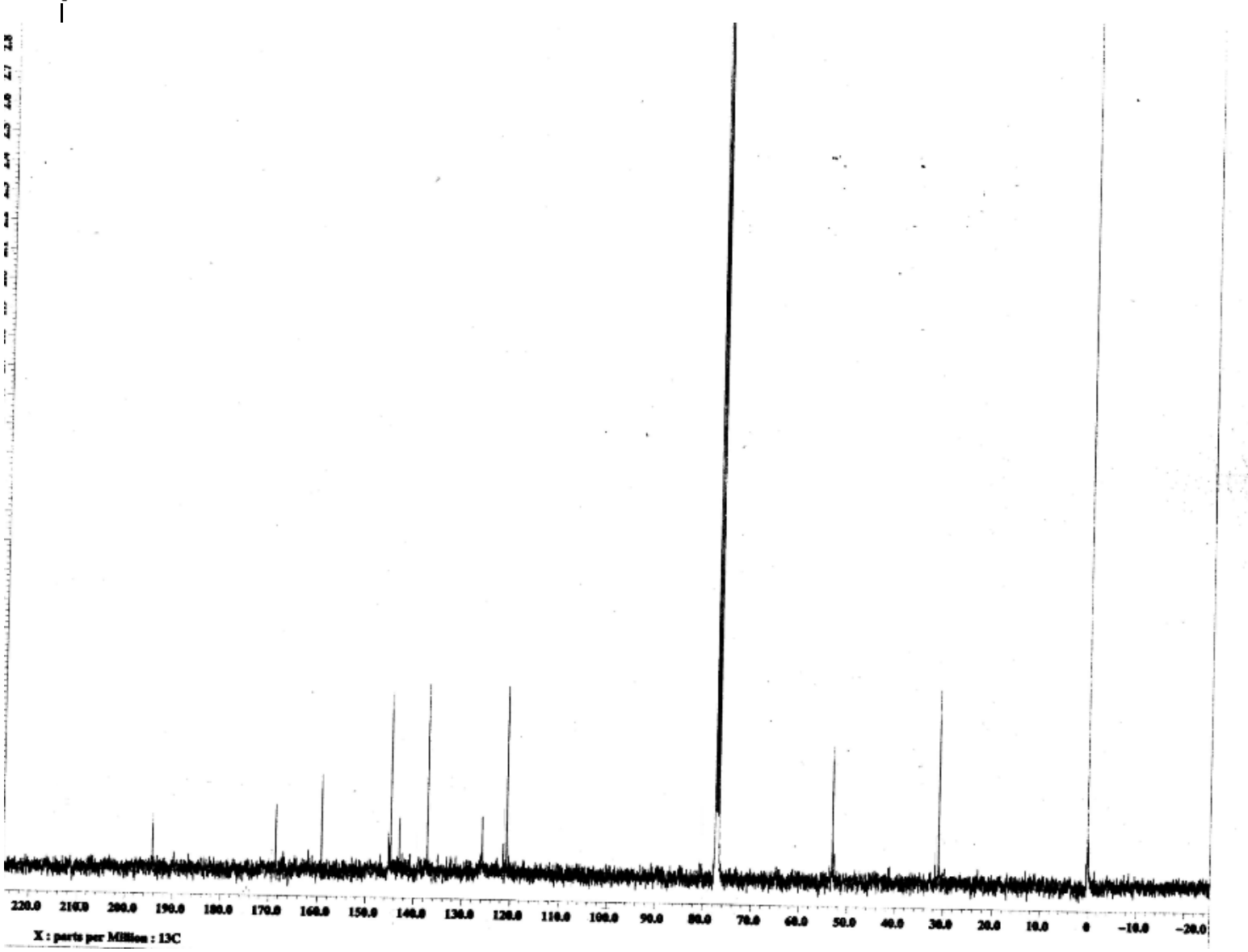
Appendix D. Proton NMR for YAL 03



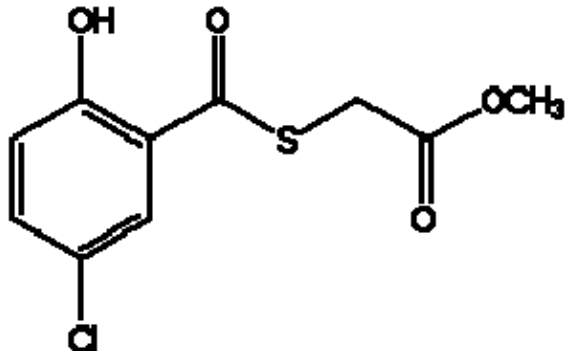


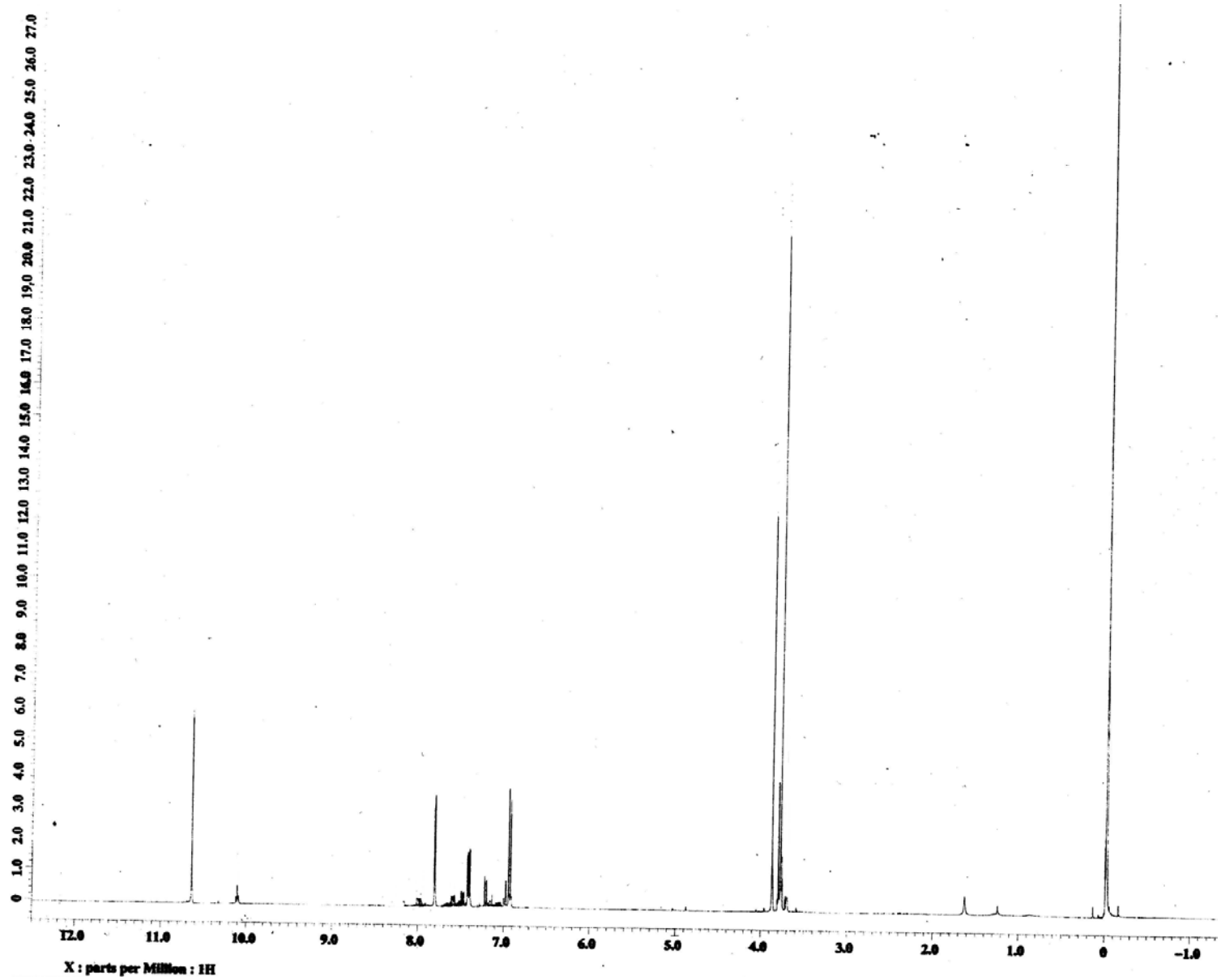
Appendix E. Carbon-13 NMR for YAL 03



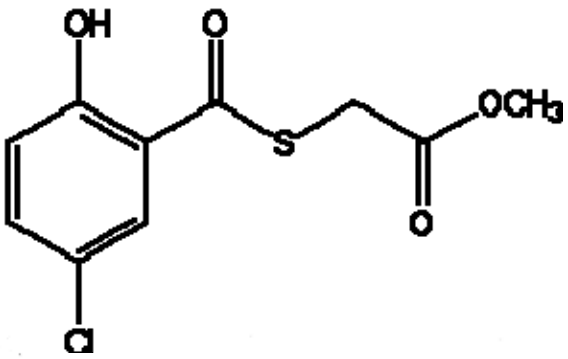


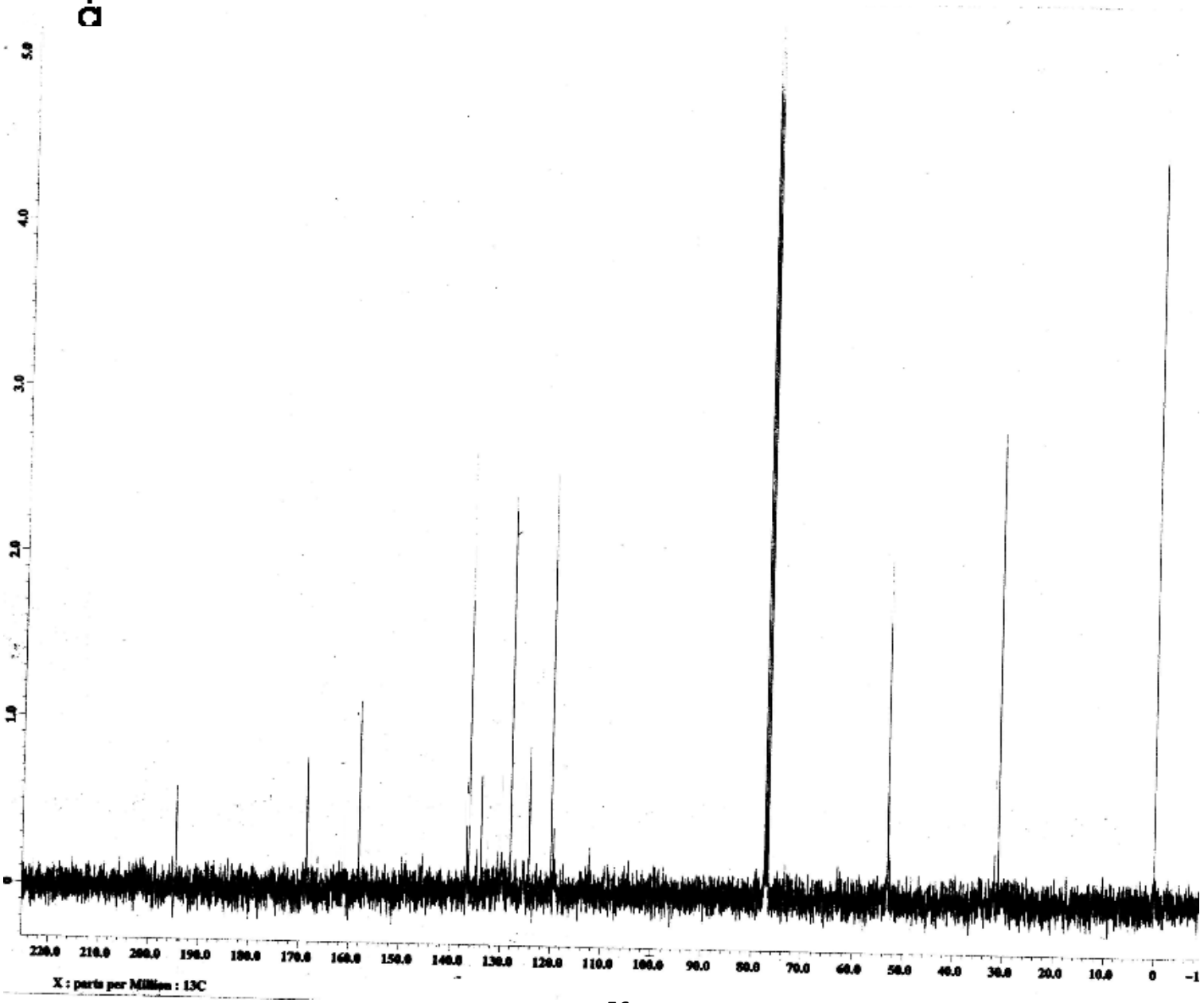
Appendix F. Proton NMR for YAL 04



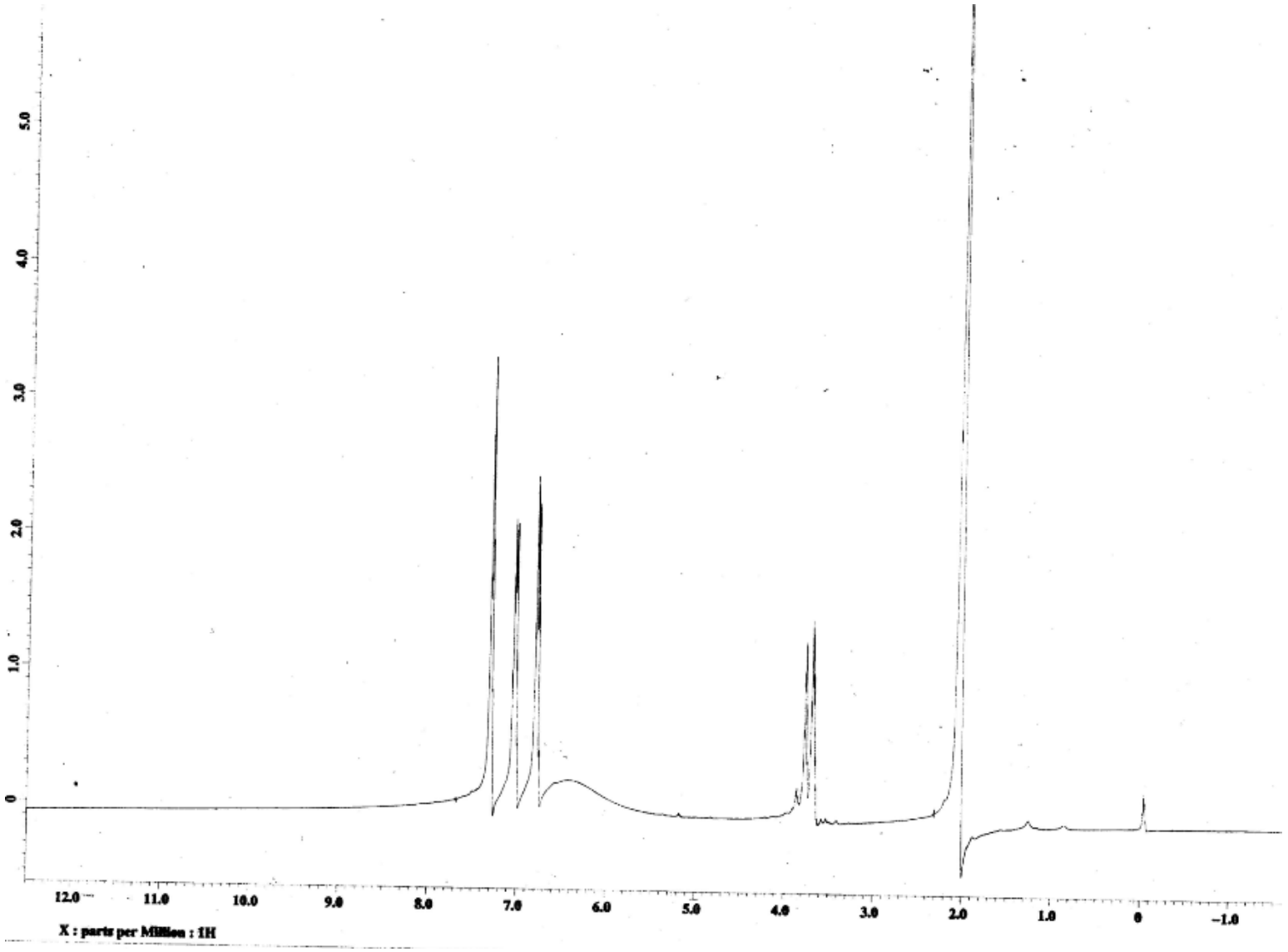


Appendix G. Carbon-13 NMR for YAL 04

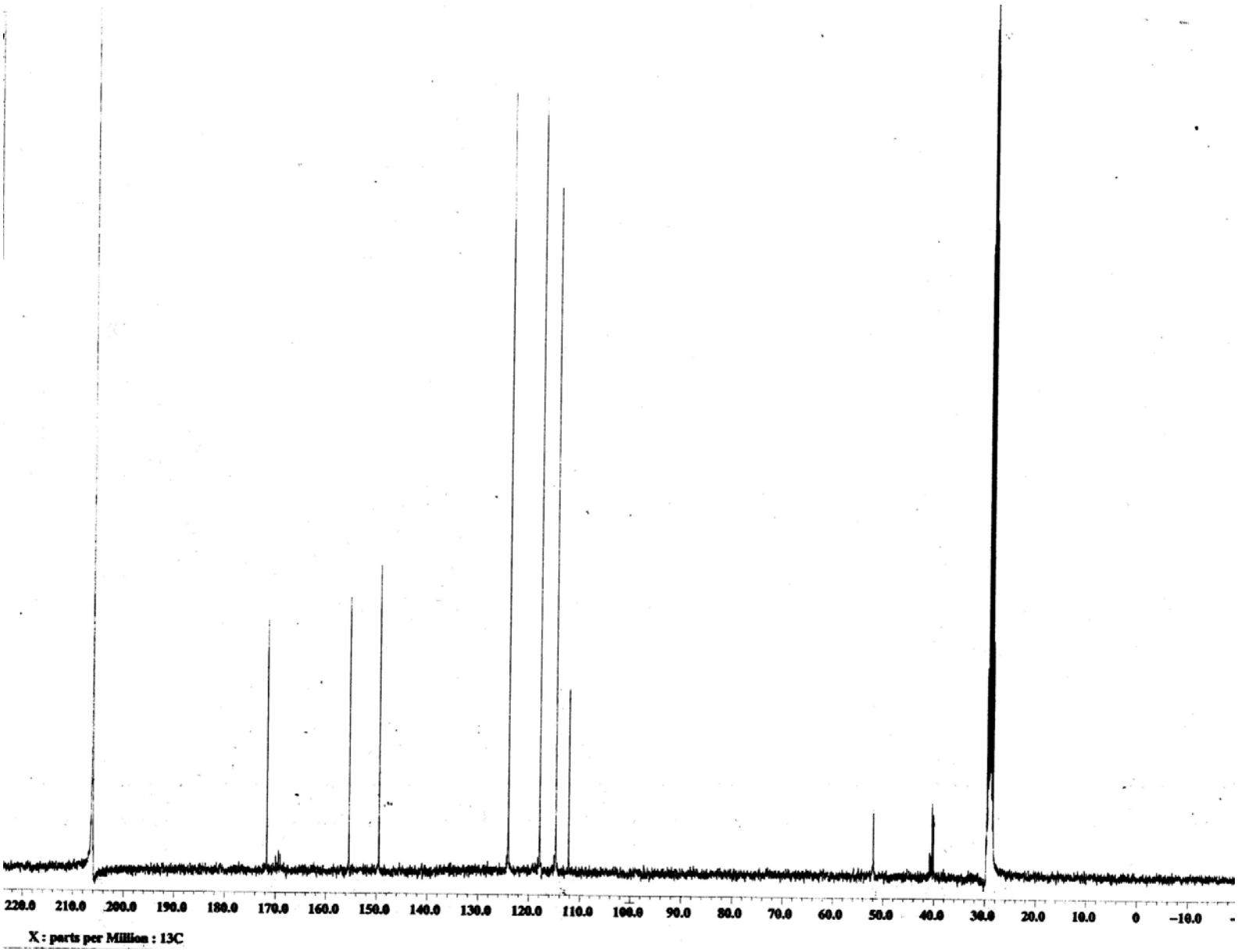




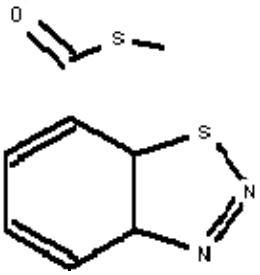
Appendix H. Proton NMR for supposed YAL 05 Product



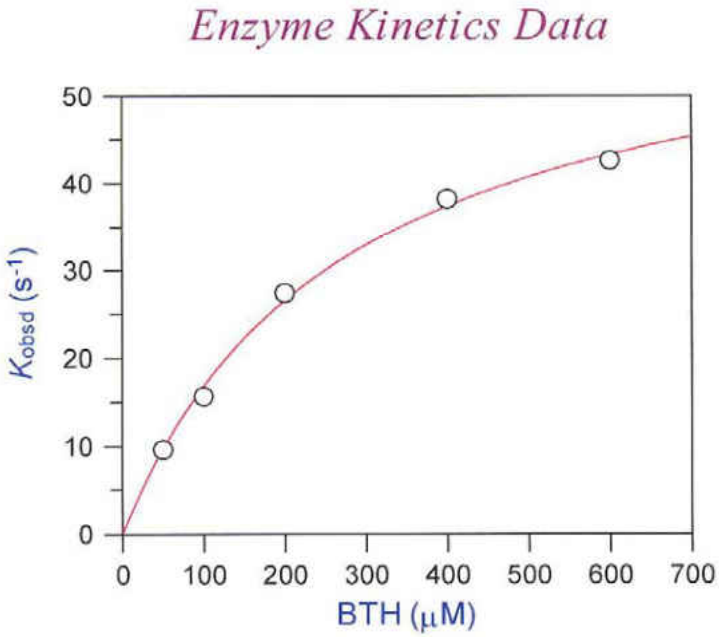
Appendix I. Carbon-13 NMR, for Supposed YAL 05 Product

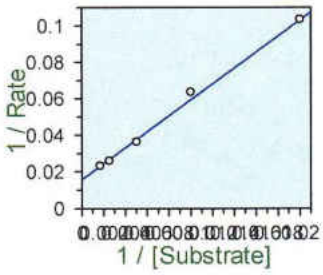


Appendix J. Kinetic Data for BTH-SABP2



BTH-SABP2 [Graph]





VITA

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