The action of a specific white rot fungus, Polyporus anceps, on simple lignin model compounds

THE ACTION OF A SPECIFIC WHITE ROT FUNGUS, *POLYPORUS ANCEPS*, ON SIMPLE LIGNIN MODEL COMPOUNDS

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ABSTRACT

This work was done in an attempt to determine some of the characteristics of the action of Polyporus anceps, a white rot fungus, in its attack on the lignin component of wood. Simple lignin model compounds of both the guaiacyl and syringyl type were used for in vitro and in vivo tests. In the case of in vitro studies, extracellular extracts as well as purified horseradish peroxidase and laccase were used while in the case of in vivo studies model compounds were added to the growth medium of fungal cells. Different types of action, depending on whether in vitro or in vivo tests were performed, were noted. In vitro reactions were oxidative in nature whereas the principal reaction observed under in vivo conditions was reduction. In order for oxidative reactions to occur it was necessary for a free phenolic hydrogen to be present in the compound being attacked. When the phenolic oxygen was blocked in an ether linkage no reaction took place under in vitro conditions while reduction did take place in in vivo trials. Results lend support to the idea of a mechanism involving alkyl side chain cleavage at a position which is para to a free phenolic hydrogen. On the other hand, the fact that reactions did not take place when the phenolic position was blocked indicated the absence of a postulated "etherase"

activity for fungi which degrade wood, at least in the case of Polyporus anceps.

INTRODUCTION

Lignin occurs as a ubiquitous material in terrestrial plants. It is an integral structural element in these plants and serves to function either directly or indirectly in many supportive mechanisms pertaining to plant life.

Investigators have been interested in the biological degradation of lignin for a number of reasons. Among these reasons are the following:

1. The relationship of lignin to the metabolizing of plants by animals.
2. The decomposition of vegetable materials in soil to form materials such as humus, peat, and coal.
3. The decomposition of trees.
4. The problem that lignin containing wastes present as water pollutants and an attempt to eliminate it.
5. Related to 4, an economic means of dealing with lignin wastes and, in addition, a possible means of producing useful products at the same time.

However, because of the complexity of the lignin molecule, it has proven to be difficult to study. Although many studies had been carried out on the decomposition of lignin by microorganisms, it was not until the late 1950's that evidence was obtained on the relationship between the decomposition of lignin and the enzymes involved. Gottlieb

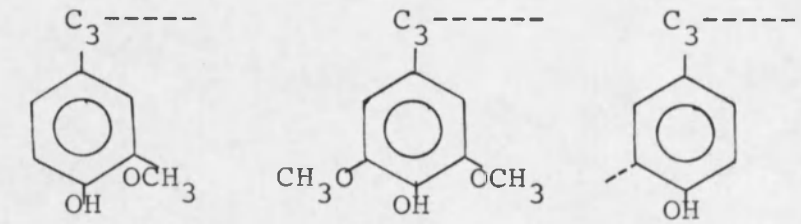
and Palczar (1) pointed out that a confusing array of data pertaining to the action of bacteria, fungi, and enzymes on lignin, which appeared in the literature before 1950, resulted from the lack of definite knowledge of the structure and properties of lignin.

For this reason and others, many workers have turned to studies involving the use of simpler model compounds related to the primary structural units of lignin. The literature on these studies is voluminous. It will be the purpose of this writer to cite only a relatively small number of the articles that have been published as an example of work that has been done. Of particular concern will be studies involving white rot fungi, which belong to the class Basidiomycetes. These fungi are aerobic, non-chlorophyll bearing organisms which have been shown to be capable of growth on lignin and lignin-like materials as their sole carbon source (1, 2).

The enzymes most commonly discussed in relation to the degradation of lignin by fungi are the phenoloxidases such as tyrosinase and laccase. A few fungi are also known to produce an enzyme which is similar to horseradish peroxidase. Workers have concerned themselves primarily with extracellular enzymes, which is reasonable when the size of the lignin macromolecule is taken into consideration. This size makes it imperative that the lignin must first be broken up, outside the fungal cells, into smaller pieces,

which could then be taken in and metabolized further within the cell. This external breakdown is a postulated role for the extracellular enzymes.

The lignin macromolecule is a polymer, which in recent years has been determined to be made up primarily of derivatives of the three monomers shown below.



guaiacyl syringyl p-hydroxyphenol

In most cases the phenolic oxygen of one monomer is also linked to another monomeric unit in an ether linkage. Guaiacyl units occur to a higher degree in soft wood lignins, while syringyl units predominate in hard wood lignins. In addition, the incidence of free phenolic hydrogens is higher in soft wood lignins than in hard wood lignins.

In 1955, Henderson (3) reported on a study in which he treated both softwood sawdust and hardwood sawdust, in vivo, with two different strains of white rot fungi, Polystictus versicolor and Trametis pina. He found that softwood lignins released vanillic acid alone, while hardwood

lignin released both guaiacyl and syringyl acids. In addition to the acids, he reported finding small amounts of corresponding aromatic aldehydes in each case.

In the same year, Higuchi, Kawamura, and Kawamura (4) reported similar results obtained upon extraction of white-rotted beachwood meal. It was suggested that the open vanillin or syringaldehyde portions of lignin were attacked preferentially during fungal degradation. In addition, they concluded that guaiacyl units were attacked more readily than syringyl moieties. Shortly after this, Fukuzumi (5) reported that he obtained 4-hydroxy-3 methoxyphenylpyruvic acid as an intermediate in the degradation of lignin. He hypothesized that this material was further metabolized to simpler molecules like vanillin and vanillic acid.

From this point on, the literature becomes increasingly contradictory, and the lack of consistency makes it difficult to hypothesize with any great confidence as to what is occurring during fungal degradation of lignin.

A number of papers describe experiments in which model compounds are mixed with fungal cells or extracellular medium in which they have been grown. Most of these papers are concerned with the oxidative nature of the enzymes present. Thus, although Farmer, Henderson, and Russell (6) did present some evidence for a reduction of models which was occurring in the presence of fungal cells, they were more concerned with oxidative processes which they attributed to extracellular

enzymes. Many authors have based the ability of a fungus to degrade lignin on its production of extracellular polyphenoloxidases (7, 8) and the bulk of material on the subject stands in support of this conclusion.

However, here too contradictions arise. It has been noted that there are strains of white rot fungi which are similar to brown rot fungi, in that they do not produce phenoloxidase type enzymes, but still maintain the ability to degrade lignin.

A question which has been of major importance to workers in the field is: how does depolymerization of the lignin macromolecules occur? Two major proposals are currently being examined. The work of Ishikawa, Schubert, and Nord (9, 10) has led them to hypothesize a mechanism of degradation whereby DL-glycerol β-aryl ether bonds, the most common linkages between monomeric units in the internal structure of the lignin polymer and those which account for almost half of the linkages present, are cleaved by a so-called β-etherase. A scheme postulated by Nord and his associates is shown in Figure 1.

Ishikawa et al. (9, 10) concluded that 4-hydroxyl-3 methoxyphenylpyruvic acid was therefore an important intermediate in the degradation of lignin which arose from cleavage of guaiacylglycerol-β-coniferyl ether units. Further side chain oxidation of this compound led to vanillin and particularly vanillic acid which could then be

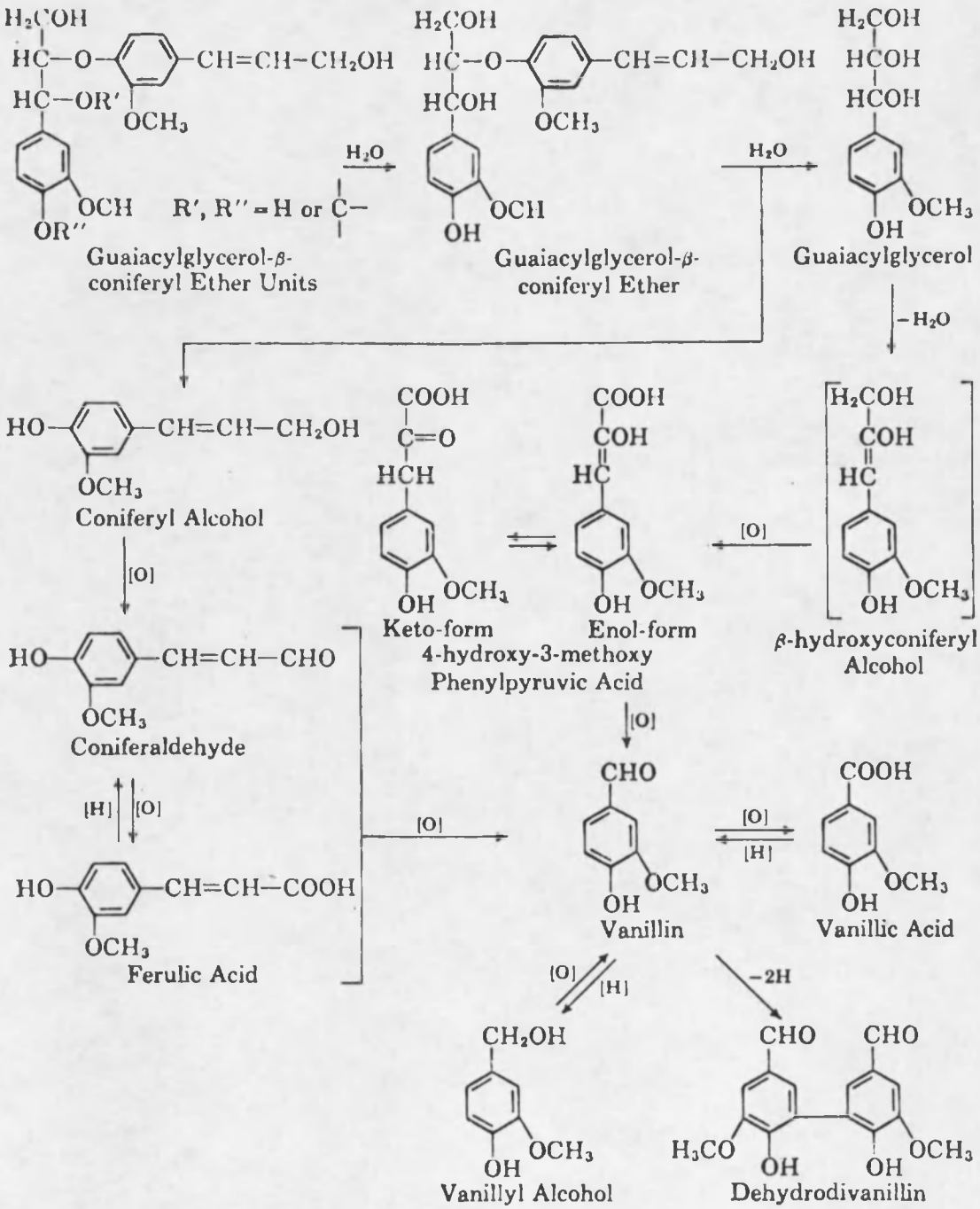


Figure 1. Postulated degradation mechanism involving β-aryl ether bond cleavage -- Source: Ishikawa et al. (9).

further metabolized to protocatechuic acid. Although the β-etherase theory is appealing, many workers have reported negative results in an attempt to find etherase activity (11, 12).

Another theory of depolymerization of lignin is that oxidative cleavage is taking place within the molecule. Kirk, Harkin, and Cowling (11, 12) presented evidence that tended to counter the "β-etherase" theory for lignin depolymerization and supported that for oxidative cleavage of the macromolecule. From results obtained from model compound studies, these workers hypothesized a mechanism for cleavage in which alkyl aryl bonds were being split. This theory is appealing when one considers the predominance of phenylpropanoid monomeric units in the polymeric structure of lignin. It is important to note here that free radical formation was assumed to be a key step in the initiation of the depolymerization process. Free radical formation is initiated at a free phenolic hydrogen as has been confirmed by a number of studies (13, 14, 15, 16). Many reports (11, 12), including the work to be discussed in the following sections of this thesis, indicate that if this phenolic position is etherified, microbiological attack does not occur.

In recent years Caldwell and Steelink (14), Fitzpatrick and Steelink (15), and Young and Steelink (16) have reported many studies concerning free radical formation

of stable phenoxy intermediates from starting materials such as whole and rotted wood, assorted lignins, and various model compounds related to the structure of lignin. In addition, much has been said about subsequent demethylation and quinone formation in materials treated with phenoloxidative enzymes (13, 17, 18). Steelink proposed a series of steps involving one electron loss, leading to the formation of these quinones, which are known to be present in larger amounts in rotting wood, as opposed to whole wood samples. These compounds have also received much attention with regard to their role in color formation, particularly in wood samples which have been treated for use in the paper industry.

Brunow (19) reported in 1969 on model compound studies in which he investigated the mechanism of oxidative side chain cleavage. He reported on the ability of phenol oxidases to cleave a side chain containing an α carbonyl group from the phenolic ring structure.

More recently, the formation of α-carbinols during oxidative degradation of lignin model compounds has been recognized as a major reaction pathway (16). Further oxidation of these intermediates leads to formation of o-quinones, through demethylation, and p-quinone, through side chain displacement, in addition to other products.

Work which will be described in this thesis was undertaken in an attempt to observe the effects of a

particular white rot fungus, Polyporus anceps, on simple lignin model compounds. In vitro studies were performed using purified enzymes and crude extracellular extracts. In vivo work was done using cultured Polyporus anceps. It was found that under the experimental conditions used, major differences existed between the nature of the reaction occurring in vitro and that taking place in vivo.

EXPERIMENTAL

General

Starting materials were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, and all the chemicals used were A.R. grade.

Thin-layer chromatography was carried out on pre-coated plates of silica gel F-254, layer thickness 0.25 mm, purchased from E. Merck, Darmstadt, Germany.

Nuclear magnetic resonance (NMR) studies were done on a Varian T-60 spectrometer using tetramethylsilane as an internal standard. Chemical shift values are given in δ (ppm) units.

Infrared spectra were recorded on a Perkin-Elmer 137 spectrophotometer in $CHCl_3$ solution unless otherwise noted. Mass spectra were recorded on a Hitachi-Perkin-Elmer double-focusing RMU-6E mass spectrometer. UV spectra were obtained on a Beckman DB-GT double beam spectrometer, at a scanning rate of either 10 nm/min or 50 nm/min, over the range 360 nm to 200 nm. These spectra were generally run on solutions of the compound being tested, in 95% ethanol.

Melting points were taken on a Fischer Meltemp and are given as the uncorrected values.

Original fungal cultures of Polyporus anceps, #8239, were obtained from Dr. R. L. Gilbertson of The University of Arizona Department of Plant Pathology. Whenever experimental flasks were inoculated, sub-culture tubes consisting of a small amount of fungal material on solid agar support were also made up. This was done both as a check on sterility of transfer techniques and in order to ensure a continuing supply of virile fungus.

Synthesis

Synthesis of Veratryl Alcohol (3,4-Dimethoxybenzyl alcohol)

A solution of 4.45 gms. (.027 moles) of veratraldehyde (3,4-dimethoxybenzaldehyde) in 25 mls. MeOH was poured into a 500 ml. three necked flask, which was equipped with a mechanical stirrer, thermometer, and a dropping funnel. From the dropping funnel, a solution of 2 gms. of KBH_4 in 2 mls. of NaOH, which had been diluted with 18 mls. of distilled H_2O, was added at a rate of .5 mls./min. with continuous stirring of the reaction mixture. During this addition, the temperature tended to rise, and an ice bath was used to maintain the temperature of the reaction mixture between 22°C and 23°C. After about 3.75 mls. of the KBH_4 solution had been added, there was no further tendency for the temperature to rise and the reaction was stopped. The reaction mixture was next put into a distillation apparatus

and the MeOH was removed by distillation (B.P. 70-72°). The viscous oil remaining in the distillation flask was diluted with 25 mls. of distilled H_2O and then extracted with three portions of anhydrous ethyl ether (30, 20, and 10 mls., respectively). The ether fractions were pooled, washed with a small amount of distilled H_2O, filtered through Whatman #2 paper, and dried for five minutes over anhydrous $MgSO_4$. The light yellow liquid remaining was filtered and then concentrated on a Roto-vap. The oil remaining was next placed in a vacuum distillation apparatus. The neck of the distillation flask was wrapped with glass wool in order to facilitate distillation. At a pressure of approximately 1 mm Hg the liquid, which boiled off at a temperature of between 140 and 150° (lit. value: 135° at 1 mm Hg, 157 - 160° at 5 mm Hg), was collected. The liquid in the collection flask was a clear light yellow oil. A dark brown residue remained in the distillation flask. An I.R. spectrum was run on this product on a Perkin-Elmer Model 137 double beam spectrometer in order to confirm its identity. The spectrum was run on a null of the product and A.R. grade CCl_4 between NaCl plates over the range 2.5-15 microns. The spectrum obtained was in complete agreement with that given for veratryl alcohol in the Sadtler files.

Synthesis of 4-Ethoxy-3,5-dimethoxybenzaldehyde

Syringaldehyde (5 gms., .027 moles, Aldrich Chemical Co.) was dissolved in 77 mls. of 2:1 dioxane; distilled H_2O mixture and 4 drops of 50% HaOH were added, which brought the pH to approximately 10. Upon addition of the base, the solution turned to a pale greenish to turquoise color. This could possibly be accounted for by the formation of some o-quinone from the starting material. The solution was poured into a 200 ml. three necked flask which was equipped with a small dropping funnel and a reflux condenser. Three mls. of ethyl iodide was added from the dropping funnel over a period of about 15 minutes, and the mixture was stirred well upon each addition. Heat was gradually increased by means of a heating mantle attached to a Variac, until a gentle reflux was occurring. The greenish tint was gradually replaced by a yellowish one as the reaction proceeded. It became necessary at one time during the one hour reflux period to add 3 drops of the 50% NaOH to maintain alkaline pH. After refluxing, the flask was allowed to cool and its contents were then extracted with 30 mls. of A.R. $CHCl_3$. A second extraction was performed using 20 additional mls. of $CHCl_3$. The pooled $CHCl_3$ phases were then washed with 10 mls. of 1 M HCl. The $CHCl_3$ fraction was dried over anhydrous $MgSO_4$, filtered through Whatman #1 filter paper, and concentrated on a Roto-vap. About 5 mls. of gelatinous brown material remained. The product was recrystallized

from ligroin. Upon cooling, a white solid formed which was collected on a Buchner funnel on #1 Whatman filter paper. The crystals were washed with a small amount of cold distilled water. This step was later repeated to yield 2.44 gms. of light flaky crystals (M.P. 110-121°; lit. value 122°). The product was also confirmed to be that desired by NMR spectroscopy (see spectrum, Appendix B).

Synthesis of 4-O-Benzylsyringaldehyde

Syringaldehyde, 2 gms., was put into 13.5 mls. MeOH, but did not completely dissolve. To this was added .5325 gms. of NaOH (pellet form) which had also not completely dissolved. However, it was hoped that this problem would be alleviated during reflux. This mixture was then put into a 50 ml. three necked flask equipped with a small separatory funnel and a reflux condenser. This setup was placed in a heating mantle which was in turn connected to a Variac. Heating was begun and 1.37 mls. of benzyl bromide in 6 mls. of MeOH was poured into the separatory funnel. This aryl halide was added over a 15 min. period to the reaction flask.

The reaction mixture had a pale yellow color throughout the reaction period, and it became necessary at one point during reflux to add 3 drops of concentrated (50%) NaOH to maintain alkaline pH. Upon the addition of this base, the mixture darkened a bit to an orange yellow color.

After one hour and ten minutes, heating was stopped and the mixture was allowed to cool. It was next poured into 20 mls. of dilute $NaHCO_3$ and a yellowish oil resulted. Extraction with 50 mls. of $CHCl_3$ was then carried out. The organic layer was dried, filtered, and flash evaporated to yield about 20 mls. of a light yellow oil of medium viscosity. This oil did not crystallize upon standing for several days, so it was mixed with the minimum amount of pet ether necessary to dissolve it. Excess pentane was then added. Fine white needle-like crystals formed, which were collected on a Buchner funnel, washed with a few mls. of pentane, and dried by suction. NMR spectroscopy identified these crystals as the desired product (m.p. 61.5-63°, see spectrum, Appendix B).

Synthesis of 4-Ethoxy-3-methoxybenzaldehyde

Vanillin (15.2 gms., .1 mole) was dissolved in approximately 150 mls. of 2:1 dioxane:H_2O mixture, and 1 M NaOH was used to make the mixture slightly alkaline. This solution was then poured into a three necked 500 ml. flask, equipped with a dropping funnel and a reflux condenser. Excess ethyl bromide (8 mls.) was poured into the dropping funnel and heating of the reaction flask was begun. The 8 mls. of ethyl bromide were added gradually, with stirring after each addition. After about 45 minutes reflux, 10 more mls. of NaOH were added to maintain alkaline pH. Reflux was

continued for two hours and the reaction flask was allowed to cool before its contents were extracted with $CHCl_3$. However, when extraction was attempted, a gelatinous yellow solid formed. This material was collected on a Buchner funnel and, when dried, it was a granular yellow brown solid. Tests indicated that this solid was probably the sodium salt of the starting phenol. It was subsequently decided to use this material in an attempt to synthesize the desired derivative of vanillin. It was found that the desired product could be obtained in the following manner. The brown-yellow solid was dissolved in 1:1 MeOH:H_2O and reacted with a large excess of ethyl bromide. This could be done either by reflux or by allowing the mixture to stand overnight at room temperature. Upon collection of the bromoethane layer and its subsequent evaporation, a light orange-yellow oil would remain. If this oil was then treated with H_2O, a solid would precipitate out. This solid, when isolated and dried, would give TLC and NMR results which indicated that it was the desired product. (m.p. 57-58°; lit. 59-60°; see spectrum, Appendix B).

Synthesis of 4-O-Benzylvanillin (4-O-Benzyl-3-methoxybenzaldehyde)

Vanillin (15.2 gms., 1 mole) was dissolved in 200 mls. of 2:1 dioxane:H_2O and this solution was poured into a 500 ml. three necked flask equipped in the same manner as the one described in the preceding synthesis. The mixture

was made slightly alkaline to pH paper by using a combination of small amounts of 1 M NaOH and 15% NaOH. Reflux was initiated and when it was proceeding gently, 13 mls. of benzyl bromide was added over a period of about 20 minutes. It was necessary to add small amounts of base a total of three times during the two hour reflux period in order to maintain alkaline pH. After cooling, the reaction mixture was extracted with 50 mls. of $CHCl_3$. The organic material was then shaken with 25 mls. of 1 M NaOH to remove unreacted starting material. The lower phase was collected, dried over anhydrous $MgSO_4$, filtered, and evaporated down on a Roto-vap to yield a thin golden yellow oil. When this oil did not solidify upon standing at room temperature, it was placed in a refrigerator in an attempt to bring about crystallization. This still did not occur. The oil was now treated with distilled H_2O to yield a light yellow solid. The solid was collected on a Buchner funnel and dried by suction. After drying, a large amount of bright yellow plates remained on the filter. TLC of this material yielded a single spot which was well above that for vanillin, and NMR spectroscopy confirmed that it was the desired product (see spectrum, Appendix B).

Synthesis of 4-O-Acetylsyringaldehyde

Syringaldehyde (1.82 gms., .01 mole) was dissolved in about 30 mls. pyridine. To this solution was added an

excess of acetic anhydride. This mixture was then warmed on a hot plate for a period of one hour. It was allowed to cool and MeOH/H_2O was added to bring about crystallization. The white solid which formed was filtered onto Whatman #1 filter paper to yield a fine white powdery material (m.p. 111-112.8°; lit. 112-113°).

Enzyme Activity

Relative activities of purified enzyme solutions and crude extracellular enzyme extracts were ascertained by the relative rates at which these aqueous solutions formed o-quinone, which has a deep cherry red color in aqueous solution, from catechol. This test was also useful in determining whether or not extracellular enzyme oxidative activity was present in solutions extracted from fungal culture flasks. In a typical test, .2 gms. of catechol was added to 15 mls. of the solution being tested in a 25 ml. Erlenmeyer flask. Color was then noted and comparisons made at regular intervals following this addition. Controls were never observed to undergo any color change.

Enzyme Experiments

Experiments Involving the Use of Purified Laccase and Peroxidase

In vitro work was performed to determine the effects of purified preparations of peroxidase and laccase on various model compounds. In the case of peroxidase, a stock

solution of horseradish peroxidase (M.W. 44,100), Sigma Chemical Co., St. Louis, Mo., prepared from 3.8×10^{-3} gms. of crystalline enzyme dissolved in 10 ml. of glass distilled water was used. The enzyme was approximately 1.86×10^{-6} M and was stored in a cold room at -10°C when not in use.

Prior to mixing with substrate model compounds, the stock solution of HRP was thawed and 1-2 ml. was added to 10 ml. distilled H_2O. To this solution was added .3 ml. of 3% H_2O_2. An equal volume of 10^{-2} M model compound was then mixed with the enzyme solution.

In the case of reactions involving the use of laccase, a small amount of purified crystalline laccase was dissolved in 10 ml. of 10^{-2} M substrate. Reactions with laccase were carried out both with O_2 being bubbled through the reaction mixture, and without additional O_2.

The products of these enzymatic reactions were recovered as follows. The aqueous solution was extracted with 30, 30, and 15 ml. portions of A.R. chloroform respectively in a separatory funnel. The organic phases were collected and pooled. At this point, one of two methods was used to further isolate reaction products. In one type of work-up, the pooled $CHCl_3$ layers were dried over anhydrous $MgSO_4$, filtered through Whatman #2 filter paper, and then concentrated on a rotary evaporator. In the second extraction scheme, the pooled $CHCl_3$ layers from above were

first shaken with 20 mls. of 8% $NaHCO_3$ (aq.) solution. The $CHCl_3$ layer from this extraction was then taken and treated as described above. The remaining $NaHCO_3$ layer was next taken and made acidic to pH paper with 1 M HCl. The acidified solution was then extracted with 20 mls. $CHCl_3$. The lower layer was collected, dried, and concentrated. TLC was performed on the concentrated extracts and appropriate standards in an attempt to identify reaction products. Developing solvents used are listed in Table 1.

Table 1. Chromatographic Solvents Used for TLC Work

A.	Butanol/NH_3	97:3
B.	Benzene/95% ETOH	8:2
C.	Benzene/Dioxane/Glacial acetic acid	90:25:4
D.	5% Glacial acetic acid in H_2O	
E.	Isooctane/Chloroform/Methanol	60:20:20

Following development, the plates were allowed to dry and then examined under UV light. Any aromatic compounds present showed up as blue spots, which were outlined with a pencil. Plates were then usually sprayed with $Fe(NO_3)_3$ or acidic 2,4-DNPH. (Duplicate plates were run on each trial.) In some cases, an additional plate was prepared and subjected to sulfuric acid (8%) charring.

Ultraviolet spectra were measured on a Beckman DB-GT grating spectrophotometer, against solvent as a blank, in quartz cells, over the range 360-200nm at a scanning rate of either 50 nm/min. or 10 nm/min. Scans of appropriate standards were also made in order to aid in identification of reaction products.

Studies Involving the Use of Extracellular Enzyme Extracts

Reactions of various model compounds with extracellular growth medium in which cells had been allowed to grow were studied. Cells were grown and the medium prepared in the following manner.

A 2% malt extract solution in water was prepared by adding 20 gms. of commercial malt extract (Difco Laboratories) to 980 mls. of tap water. This mixture was dissolved using both heat and stirring. Culture flasks were then prepared by pouring 125 to 150 mls. of this mixture into each 250 ml. Erlenmeyer flask. A cotton plug was fitted into the top of each flask and all flasks to be used

were autoclaved at 15 lbs/sq. in. at 252°F for at least 15 minutes. After sterilization, the flasks were set aside to cool. When they were at least cool enough to hold in the hand, they were ready for culturing. Inoculation of culture flasks was carried out in a transfer room, using sterile technique. A small piece of fungal material which came from mycelia grown in a test tube on agar was transferred to each flask containing liquid culture medium which was to be used in growing fungus. Control flasks were also set aside in each experiment. These flasks were treated in the same manner as those described above, except for inoculation with fungal material. Both control and treated flasks were then put on a shaker (60 cycles/min.) and shaken at low speed for a period of from one to two weeks. Temperature was not controlled strictly in any of the experiments. Progress of growth was noted periodically. Good growth was usually indicated by the formation of furry, ball-like colonies, which increased rapidly in number from about the third day of the growth period until about the middle of the second week. In addition, a marked lightening of the growth medium was observed in those flasks where healthy colonies were present. After about the tenth day of the growth period, the most noticeable change in the culture flasks was an increase in size of many of the ball-like colonies. If shaking was discontinued at any time prior to harvesting of the fungi, the mycelia would form mats on the surface of

the growth medium, as would be expected since the fungi being grown were aerobic.

Unhealthy conditions or growth were discernible in the following ways:

1. The medium would not become lighter with the passage of time.
2. Ball-like fungal colonies would form, but would be much fewer in number. These were not furry, but were covered by a silky looking material.
3. Black mats of bacterial cells would indicate contamination of the medium.
4. No growth would occur in an inoculated flask.
5. The control flask would contain growth. In this case, all flasks would be discarded.

When it was desired to isolate some extracellular material, the flasks were removed from the shaker, opened, and filtered through a Buchner filter containing Whatman #2 filter paper. In most cases, the filtrate, which contained the extracellular enzyme(s), was used immediately. In no case was material from any flask used in an experiment unless it had been isolated immediately after opening of the flask from which it came. In those cases where it was desired to store extracellular material, filtrate was poured into a plastic screw-capped bottle and stored in a coldroom at -10°C. If the isolate was allowed to stand at room

temperature, mycelial mats would usually begin to develop on its surface after three or four days. This growth could be stopped by the addition of a few drops of A.R. $CHCl_3$ at the time of storage. About 100 mls. of liquid were obtained from each culture flask.

The effect of the extracellular filtrate on model compounds as substrates was determined in the following manner. To 30 mls. of the extracellular fluid was added .3 to .5 gms. of the compound to be tested. The mixture was then stirred vigorously to dissolve as much of the model compound as possible. In some cases, the model was first dissolved in the minimum necessary amount of absolute ethanol. At other times, undissolved solid was left in the flasks. It was hoped that equilibrium conditions would bring about dissolution of this solid as material in solution was metabolized by the enzyme(s) believed to be present. Whenever extract was reacted with the models, it was also reacted with catechol. The formation of o-quinone, which was cherry red, served as a good indicator of whether or not enzyme activity was present, and if so, its relative intensity. In most cases the extracellular preparation used would begin to show color with catechol within an hour after mixing, and was black by the following morning. All mixtures involving the use of guaiacyl or syringyl model compounds were allowed to stand for at least 24 hours. Controls were run in all cases and were made up by mixing

.3 to .5 gms. of the models which were being tested with enzyme with 30 mls. of 2% malt extract solution. Isolation and identification of materials present in the reacted mixtures was carried out as has been previously described. The first portion of $CHCl_3$ was added directly to the reaction flask in an attempt to dissolve any organic compounds which were not already in solution.

In Vivo Studies Involving the Use of Model Compounds Added to the Culture Medium of Growing Fungi

The preparation and growth of fungus in these experiments was the same as that described in the preceding section with the following exceptions.

1. Prior to autoclaving of the flasks, .3 to .5 gms. of the model compound to be tested was added to the 2% malt extract growth medium.
2. The growth period of these experiments was extended to a minimum of three weeks (except where otherwise noted).
3. At the time of extraction of any unreacted starting materials and any products present, the first portion of $CHCl_3$ was added directly to the flask being treated, in an attempt to dissolve any solid organic materials which were not in solution.

The growth medium was then filtered through #2 Whatman filter paper in a Buchner funnel, in order to remove

fungal cells. During these extractions, emulsions, which cleared very slowly, often formed. These emulsions were usually treated with about 10 mls. of saturated KCl (aqueous) solution in an attempt at "salting out" of organic materials. Pooled $CHCl_3$ layers were dried over anhydrous $MgSO_4$, filtered, and evaporated down on a rotary evaporator. Concentrated material was used for TLC analysis. Diluted samples were used in UV analysis whenever it was desired to obtain UV spectral data. Concentrated solutions were then allowed to evaporate to dryness and any solid material obtained was redissolved in TMS 1% $CDCl_3$, or $CDCl_3$ with one drop of TMS added as internal standard. These solutions were then used to obtain NMR spectra. Whenever possible, spectra of standards were run in order to serve as standards for comparison.

In another set of experiments designed to test the ability of fungi which had first been allowed to grow for a period of two weeks to resist the toxic effects of free phenols, all procedures were the same with the following exceptions:

Fungal material was used to inoculate 2% malt extract growth medium which had been prepared for culturing as has already been described. Cultured flasks and controls were then placed on the shaker for a period of two weeks. At the end of this time period, those flasks designated for use as test flasks had either .3 gms. of vanillin or

syringaldehyde added to them, using as sterile a technique as was practical. In these cases the phenols were not subjected to autoclaving, but this had already been shown to have no effect on added model compounds. Flasks were then returned to the shaker for a period of one week. From this point on, all procedures used were identical to those described previously for in vivo experiments.

RESULTS

Reactions of Peroxidase, Laccase, and Extracellular Medium with Vanillin (4-Hydroxy-3-methoxybenzaldehyde)

When 10^{-2} M solutions of vanillin were allowed to react with the above at pH 4-5, formation of a milky-brown suspension was observed. This occurred most rapidly with the purified horseradish peroxidase and most slowly with crude extracellular extracts. This solid, which fluoresced under UV light when wet, was found to be a mixture of materials with dehydrodivanillyl alcohol as a major component, as confirmed by mass spectroscopy. In addition, small amounts of unreacted starting material were present. In the case of the extracellular extracts, a small amount of vanillic acid was also formed. This reaction did not occur in the presence of purified enzyme preparations.

Growth of fungal cells could not be initiated in culture flasks containing vanillin as a starting material. At the end of the three week shaking period, analysis indicated that no reaction had occurred. (See Appendices A and B for spectral and TLC results.)

Reactions with Syringaldehyde (3,5-Dimethoxy-4-hydroxybenzaldehyde)

When 10^{-2} M syringaldehyde was reacted with purified peroxidase, laccase, or crude extracellular extracts, the

only differences noted were in the times required to form reaction products. Whereas observable color formation took several hours in the cases of the fungal extracts, it was noticeable within 15 minutes when purified peroxidase was used, and within 30 minutes when purified laccase was used. Any reaction mixture treated when its color was a deep cherry red contained the same products. These products were identified as:

1. Starting material (syring.).
2. 3,5-Dimethoxybenzoquinone.
3. 3-methoxy-o-benzoquinone-5-carboxaldehyde.
4. 3,4-Dihydroxy-5-methoxybenzaldehyde.

No other products were found.

All attempts to initiate growth of the fungus in the presence of syringaldehyde as starting material failed. Analysis of the contents of both test and control flasks after three weeks of shaking revealed that no materials, other than starting materials, were present.

Reactions with Veratraldehyde (3,4-Dimethoxybenzaldehyde)

When purified peroxidase or purified laccase was allowed to stand with 10^{-2} M veratraldehyde, the only material isolated after periods of time varying from one hour to overnight was unreacted starting material. In the case of crude extracellular enzyme preparations, small

amounts of veratric acid (3,5-dimethoxybenzoic acid) were also formed.

Fungi grew well in the presence of this compound. At the end of three weeks, extraction yielded unreacted starting material in most but not all cases, and reduced starting material or veratryl alcohol (3,5-dimethoxybenzyl alcohol).

Reactions with Vanillyl Alcohol (4-Hydroxy-3-methoxybenzyl alcohol)

Reactions with purified peroxidase, purified laccase, or crude extracellular extract gave a milky-brown suspension. Analysis of products indicated the presence of starting material, vanillin, vanillic acid, and dehydrodivanillynal as well as one or two additional unidentified products. Fungal growth could not be initiated in the presence of this compound.

Reactions with 3,4,5-Trimethoxybenzaldehyde

When TMB was allowed to stand with purified peroxidase, purified laccase, or crude extracellular extract, the only compound found was starting material. The color of these reaction mixtures remained the same throughout, indicating the absence of any quinones at all times.

Fungal growth in the presence of this material was good. In trials with this compound, shaking was carried out for periods of one, two, or three weeks and results varied

depending on the length of this period. Materials isolated after one week of shaking included starting material and 3,4,5-trimethoxybenzyl alcohol. The only material found after a shaking period of two weeks was 3,4,5-trimethoxybenzyl alcohol. However, when shaking was continued for three weeks, both 3,4,5-trimethoxybenzaldehyde and 3,4,5-trimethoxybenzyl alcohol were found to be present.

Reactions with 4-Ethoxy-3,5-dimethoxybenzaldehyde

In vitro tests with this compound in the presence of purified peroxidase, purified laccase, or crude extracellular enzyme preparation yielded only starting material, indicating that no reaction had occurred.

In vivo tests were carried out for one, two, or three weeks. Results were similar to those found with 3,4,5-trimethoxybenzaldehyde. After either one or three weeks of shaking, both 4-ethoxy-3,5-dimethoxybenzaldehyde and 4-ethoxy-3,5-dimethoxybenzyl alcohol were found, while only 4-ethoxy-3,5-dimethoxybenzyl alcohol was found after a period of shaking of two weeks.

Reactions with 4-O-Benzyl-3,5-dimethoxybenzaldehyde

In all cases of in vitro tests involving the use of this compound, nothing other than starting material was ever found. Fungi grew well in the presence of this aldehyde and analysis at the end of the three week shaking period

revealed the presence of starting material and 4-O-benzyl-3,5-dimethoxybenzyl alcohol.

Reactions with 4-Ethoxy-3-methoxybenzaldehyde

In vitro solutions of this material plus purified peroxidase, purified laccase, or crude extracellular enzyme underwent no color change, and suspensions similar to those observed to form in the cases of free phenolic guaiacyl compounds were never obtained. The only compound ever found on analysis of the test flasks was starting material. Growth of fungi in in vivo tests proceeded well and materials found after three weeks of shaking included starting material and 4-ethoxy-3-methoxybenzyl alcohol.

Reactions with 4-O-Benzyl-3-methoxybenzaldehyde

Results obtained in the case of this material correspond to those obtained with 4-ethoxy-3-methoxybenzaldehyde, with no reaction taking place in in vitro tests and reduction occurring in in vivo trials. Here too, growth of the fungus was excellent.

Reactions with Vanillyl Acetate (4-O-Acetylvanillin)

This compound was not tested in in vitro experiments. When used in in vivo tests, fungal growth was poor and death resulted within a few days. However, a milky-brown suspension did form and analysis of the contents of

these flasks revealed the presence of starting material, vanillin, vanillic acid, dehydrodivanillin, dehydrodivanillyl alcohol, vanillyl alcohol, and three additional unidentified compounds.

Reaction with Syringyl Acetate (4-O-Acetylsyringaldehyde)

This compound was not tested with purified enzymes or with crude extracellular extracts. Fungal growth was excellent in its presence and concentrated extracts obtained after the three week shaking period were a cherry red color, indicating the presence of o-quinone. Analysis of these extracts revealed the presence of o-quinone, p-quinone, starting material, syringyl alcohol, and small amounts of syringic acid.

Reactions with Syringyl Alcohol (4-Hydroxy-3,5-dimethoxybenzyl alcohol)

When purified peroxidase or laccase, or crude extracellular enzyme preparations were mixed with this compound, it turned cherry red in about two days. Extraction and analysis of the products formed indicated the presence of syringaldehyde, o-quinone, p-quinone, and 3,4-dihydroxy-5-methoxysyringaldehyde. No starting material or syringic acid was detected. Fungal growth could not be initiated in the presence of the material.

Reactions with 1-Methoxy-1(3,5-dimethoxy-4-hydroxy)-phenylpropane

Products found when purified or crude extracellular enzymes were allowed to stand with this material were o-quinone, p-quinone, syringaldehyde, the reduced form of the o-quinone corresponding to syringaldehyde, and starting material. No in vivo studies were attempted with this material.

It was found that if the fungi were first allowed to grow for a period of two weeks before addition of free phenol to their growth medium, that good growth continued as indicated by increases in the number of colonies present and continued size increase of colonies which were present at the time free phenol was added. In flasks containing fungi and to which vanillin had been added, a milky-white suspension formed. Isolation and analysis of materials present at the end of the total three week shaking period revealed the presence of starting material, vanillic acid, dehydrodivanillyl alcohol, vanillyl alcohol, and three unidentified products. In flasks which were used as controls, the suspension did not form and only starting material was isolated at the end of the three weeks. When syringaldehyde was added to pre-inoculated flasks, the culture medium gradually assumed a more reddish tint. Analysis at the end of three weeks revealed the presence of starting material, syringyl alcohol, syringic acid, o-quinone, and p-quinone. Controls

did not become more deeply colored and only syringaldehyde was found in these flasks.

SUMMARY OF RESULTS AND DISCUSSION

In the course of these experiments it became apparent that two different types of reactions dominated, depending on whether or not fungal cells were present in the reaction medium. In the absence of the cells, oxidative reactions were favored, and in the case of free phenolic substrates of the syringyl type, quinone formation appeared to be of major importance. When free phenolic compounds of the guaiacyl type were used, dimerization was the favored reaction. Oxidation of alcohols or aldehydes seems to have only been of minor import in any of the tests performed. When a syringyl or guaiacyl model which had previously been etherified at the phenolic position was used as a test substrate, reaction was severely limited. No quinones or dimerization products were produced in any of these experiments.

When fungal cells were present, reduction, in which aldehydic starting materials were converted to the corresponding alcohols, was favored. Whenever inoculation of cells was attempted in 10^{-2} M concentrations of free phenolic starting materials, growth did not occur and only unreacted starting material was ever isolated from these flasks. This was also true of the control flasks which received no inoculation. When etherified substrates were used, growth was healthy and vigorous.

Results of these experiments would appear to support a free radical side chain cleavage mechanism for lignin degradation. At the same time, no evidence which would support an "etherase" mechanism of cleavage was ever obtained. This seems reasonable when one considers the stability of phenolic ether linkages. It is tempting to look at sites within the lignin molecule where a side chain is attached to a phenyl ring para to a free phenolic hydrogen as potential areas for lignin depolymerization. A mechanism of this type also serves to explain nicely the formation of p-quinone as a degradation product in experiments utilizing syringyl models and accounts for the occurrence of free radicals and quinones in rotting wood.

Again, free phenol in relatively concentrated form will kill fungal inoculate, while lignin does not. I believe this is due to a relatively low concentration of free phenolic hydrogens in lignin, on a volume basis, compared to compounds like vanillin or syringaldehyde. Thus, if one envisions a fungal cell as a sphere which is in contact with a lignin macro-molecule, he can imagine that free hydroxyl groups are scattered and small in number, in comparison to what the situation would be if the same fungal cell were surrounded by small molecules of vanillin or syringaldehyde. In in vivo experiments with syringaldehyde, for example, no quinone is formed and death occurs; whereas, in in vitro tests using purified enzymes and extracellular extracts,

quinone formation does occur. In addition, fungi which have first been allowed to grow and then had free phenols added to their flasks appear to remain healthy, and formation of quinone occurs. Thus, quinone formation may not only serve in depolymerization of lignin, but may also be due to protective activity of the fungal enzymes which develop as growth proceeds. In relation to this also, should be considered the reduction of aldehyde groups para to phenolic positions which had been etherified, when fungal cells were present. This reaction was never observed when purified enzymes or extracellular extracts were tested with model compounds. Workers who have studied the effect of fungi on lignin samples report the presence of oxidation products after treatment, but not the products corresponding to reductive processes. It may be that reduction occurs as a transitory process during fungal degradation or that the reduction observed in the experiments described in this report was noted because of the relatively high concentration of the compounds being tested. It seems possible that concentrations are low enough in lignin that reduced products are not formed, or that any which are formed are quickly metabolized further. In this light, it is tempting to speculate that the reductive activity observed and described herein was also part of a protective mechanism. As the fungi grow, their environment tends to become quite acidic. Reduction of certain groups, such as aldehydic

groups, would allow for tying up excess hydrogens. Under natural conditions, groups which were originally reduced might later be re-oxidized or be present in the lignin in very small amounts. It may be that in wood, extracellular phenoloxidases gain access to portions of the lignin macromolecule that the fungal cells can not. These fungal cells appear to be necessary for reduction to occur. If this "buffering" system is indeed operative, it would be somewhat analogous to the human body's conversion of ammonia to urea and thence to urine in order to dispose of toxic wastes. The fact that reaction was seen to occur at an acid pH, but not at basic pH, would tend to lend support to this idea.

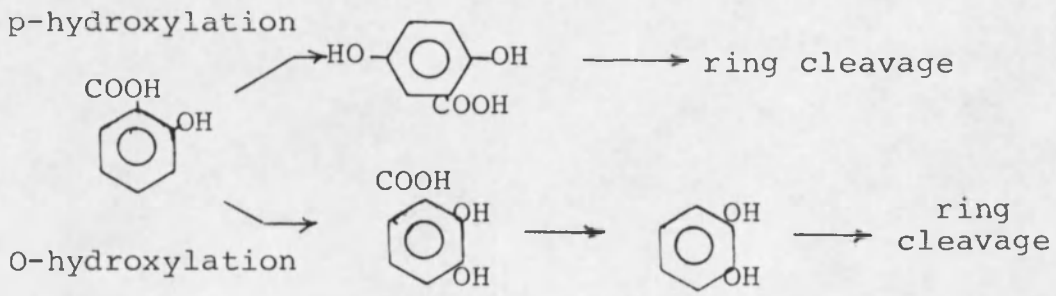
Differences in metabolism of syringyl and guaiacyl type compounds are possibly accounted for by susceptibility of the phenolic hydroxyl position to attack by fungal enzymes. Dimerization of syringyl type compounds can be assumed not to occur because of the presence of an extra blocking methoxyl group which is not present in guaiacyl compounds. In the case of the latter substrates, dimerization appears to be a relatively important reaction. The absence or presence of an additional methoxyl group may also be a determining factor in how fast attack occurs. It appears that in the case of 4-O-acetylsyringaldehyde, both reduction and quinone formation occurred, and growth was healthy. In similar tests with 4-O-acetylvanillin, some dimer formation took place, but the general rule was death

of the inoculate. One can speculate that these compounds are converted to free phenol prior to further metabolism. Other data indicate that the fungi can handle free phenol if first allowed to build up "protective" activity. It then seems plausible that the fungi did better in solutions containing 4-O-acetylsyringaldehyde than in those containing 4-O-acetylvanillin because the greater degree of steric hindrance of the former reduced the rate of formation of free phenolic hydrogen to a level where protective activity could be developed. In addition, if formation of quinones is protective in nature, than this too could be an explanation for survival in the case of the 4-O-acetylsyringaldehyde, but not with the 4-O-acetylvanillin.

No evidence was found for any 4-O-etherase activity. This fact, together with observed quinone formation, lends support to the idea that lignin degradation takes place by way of free radical formation followed by alkyl side chain cleavage and subsequent quinone formation.

Results obtained with one of the model compounds indicate that the fungi do possess the ability to hydrolyze α-ether linkages. This activity has been extensively investigated by Ciaramitaro (20), who worked with purified peroxidase and model compounds containing α-ether linkages. He reported that hydrolysis was definitely occurring and that up to 31% of the monomer to monomer linkages in lignin were susceptible to attack in this manner. At this point,

the possible reason for formation of quinone from syringyl type models will be considered. The mechanisms to be discussed may also be applicable to metabolic intermediates such as vanillic acid which results from degradation of guaiacyl structures. As noted by Sarkanen (21), it would seem that in order for white rot fungi to use benzyl intermediates, ring cleavage must be occurring. A large number of soil microorganisms are known to degrade aromatic compounds, although bacteria appear to be more capable in this respect than yeasts or fungi. Generally, ring cleavage can occur on a molecule which contains ortho or paradihydroxy groups. Resulting aliphatic carboxylic acids can then be used in various biochemical pathways. An example of such a process, using salicylic acid as substrate is shown below.



A number of other systems (22, 23) in which similar mechanisms are employed have been documented. Although little is known as to whether or not white rot fungi utilize a process of this nature, Sarkanen (21) felt that it was possible to postulate at least two mechanisms for white rot

metabolism of benzyl aromatic intermediates, which appear as shown in Figure 2.

Of course, this mechanism implies an "etherase" activity which was not indicated in experiments performed in our lab and by others. Kirk et al. (11, 12) proposed a mechanism based on the action of p-diphenyl oxidase in which side chain cleavage is an important step, as shown in Figure 3.

As noted previously in this report, recent evidence appears to favor this type of depolymerization reaction. In addition, Kirk found that substrate etherified in the 4 position was not depolymerized, but that the 4 methyl ether derivative of guaiacylglycerol-α-o-methoxyphenyl ether was oxidized to the α-carbonyl compound by Polyporus versicolor in the presence of wood meal or milled wood lignin. All of this is consistent with results obtained by this worker. In this light, quinones could then have arisen from further oxidation of ortho or para-dihydroxyphenyl intermediates which were not cleaved. Amounts of quinones and other aromatic compounds remaining after fungus growth make it appear that much of the starting material could have been further metabolized by conversion to aliphatic materials. Thus, quinone formation could be the result of a minor side reaction. The work described in this report was not oriented toward detection of aliphatic products. Much of what has been said here is highly speculative, as is most of

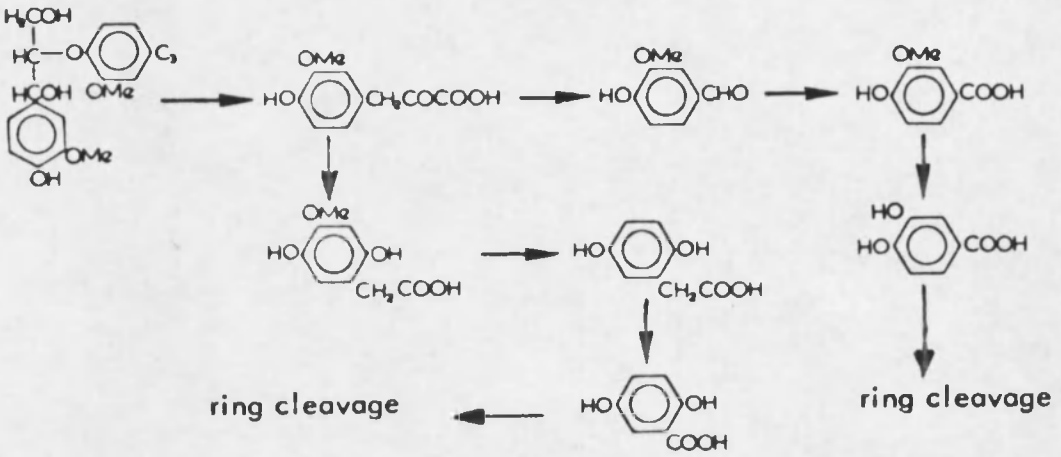


Figure 2. Postulated mechanism involving "etherase" activity in lignin degradation -- Source: Sarkanen (21).

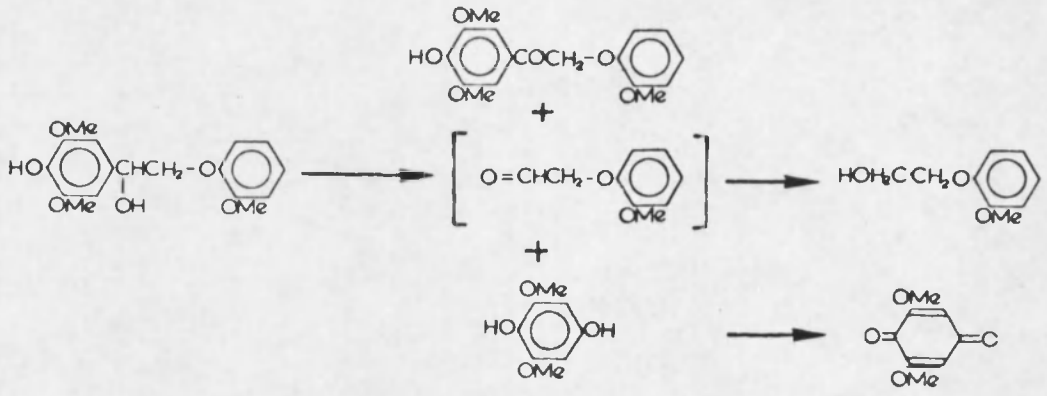


Figure 3. Postulated mechanism involving alkyl side chain cleavage in lignin degradation -- Source: Sarkanen (21).

the literature. It would seem that further work, possibly with radioactively labeled benzyl model compounds, is indicated.

Thus, aromatic lignin model compounds, labeled at a ring position, could be subjected to fungal action. Reaction products could then be analyzed for aliphatic labeled materials which, if present, would imply a mechanism for fungal degradation entailing ring cleavage. Work of this nature could be a major step in the attempt to elucidate the mechanism by which fungi degrade lignin.

APPENDIX A

Rf VALUES AND SPRAY COLORS FOR CHROMATOGRAPHIC STANDARDS AND PRODUCTS OF ENZYMATIC REACTIONS

Table A.1 Syringyl Compounds as Reference Standards and as Substrates in Enzyme Reactions--Solvent A

Compound	Rf	Color
Syringaldehyde	.19	DNPH--orange-red $Fe(NO_3)_3$--purple
Orthoquinone of syringaldehyde. 3-methoxy-o-benzoquinone-5-carboxaldehyde	.36	cherry red prior to spray DNPH--green $Fe(NO_3)_3$ no color
3,5-dimethoxy-p-benzoquinone	.36	DNPH--brownish yellow $Fe(NO_3)_3$--no color
Syringaldehyde + purified enzyme extracts	.19 .36	.19--DNPH--orange-red Fe(NO) --purple .36--DNPH--green slightly above yellow range $Fe(NO_3)_3$--no color
4-O-ethyl-3,5-dimethoxybenzaldehyde	.64	DNPH--orange-red $Fe(NO_3)_3$--no color
3,4,5-trimethoxybenzaldehyde	.61	DNPH--orange $Fe(NO_3)_3$--no color
3,4,5-trimethoxybenzaldehyde + all in vitro systems	.61	DNPH--orange $Fe(NO_3)_3$--no color

Table A.1--Continued Syringyl Compounds as Reference Standards and as Substrates in Enzyme Reactions--Solvent A

Compound	Rf	Color
4-O-ethyl-3,5-dimethoxy-benzaldehyde + all in vitro systems	.64	DNPH--orange-red $Fe(NO_3)_3$--no color
4-O-benzyl-3,5-dimethoxy-benzaldehyde	.66	DNPH--orange $Fe(NO_3)_3$--no color
4-O-benzyl-3,5-dimethoxy-benzaldehyde + all in vitro systems	.66	DNPH--orange $Fe(NO_3)_3$--no color

Note: Solvent A not used with products of any in vivo reactions.

Table A.2 Guaiacyl Compounds as Reference Standards and as Substrates in Enzyme Reactions--Solvent A

Compound	Rf	Color
4-hydroxy-3-methoxybenzaldehyde (vanillin)	.40	DNPH--orange $Fe(NO_3)_3$--purple
3-methoxy-p-benzoquinone	.75	DNPH--yellow gray $Fe(NO_3)_3$--no color
3-methoxy-p-benzohydroquinone	.88	DNPH--red brown $Fe(NO_3)_3$--no color
3,4-dimethoxybenzaldehyde	.80	NDPH--dark orange $Fe(NO_3)_3$--no color
3,4-dimethoxybenzoic acid	.74	DNPH--no color $Fe(NO_3)_3$--no color

Table A.3 Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions--Solvent B

Compound	Rf	Color
Syringic acid	.33	DNPH--orange brown color slowly developed $Fe(NO_3)_3$--brown, purple
Syringyl alcohol	.39	DNPH--late appearing red-brown $Fe(NO_3)_3$--yellow, blue
4,5-dihydroxy-3-methoxy-benzaldehyde	.31	DNPH--red before spray red-brown turned green upon exposure to NH_3
3-methoxy-o-benzoquinone-5-α-methylcarbinol	.02	DNPH--red before spray,
Syringaldehyde	.44	DNPH--orange, red $Fe(NO_3)_3$--purple
3,4,5-trimethoxybenzaldehyde	.63	DNPH--orange, brown $Fe(NO_3)_3$--no color
3,4,5-trimethoxybenzyl alcohol	.41	DNPH--late developing orange $Fe(NO_3)_3$--no color
4-o-ethyl-3,5-dimethoxy-benzaldehyde	.66	DNPH--orange-red $Fe(NO_3)_3$--no color
3,5-dimethoxy-p-benzoquinone	.47	DNPH--yellow $Fe(NO_3)_3$--no color
Orthoquinone of syringaldehyde 3-methoxy-o-benzoquinone-5-carboxaldehyde	.49	No spray-red DNPH--green $Fe(NO_3)_3$--no color
4-o-benzyl-3,5-dimethoxy-benzaldehyde	.65	DNPH--orange $Fe(NO_3)_3$--no color
Syringaldehyde + purified peroxidase, laccase, or crude extracellular extracts	.31	DNPH--red then green on exposure to NH_3 $Fe(NO_3)_3$--did not develop

Table A.3--Continued Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent B

Compound	Rf	Color
	.43	DNPH--orange, red $Fe(NO_3)_3$--purple
	.47	DNPH--yellow $Fe(NO_3)_3$--no color
	.49	DNPH--green $Fe(NO_3)_3$--no color
3,4,5-trimethoxybenzaldehyde + purified peroxidase, laccase, or extracellular extracts	single spot at .63	DNPH--orange $Fe(NO_3)_3$--no color
4-o-ethyl-3,5-dimethoxybenzaldehyde + purified peroxidase, laccase, or crude extracellular extracts	single spot at .66	DNPH--orange-red $Fe(NO_3)_3$--no color
4-o-benzyl-3,5-dimethoxybenzaldehyde + all in vitro systems	single spot at .65	DNPH--orange $Fe(NO_3)_3$--no color
Syringaldehyde in vivo with Polyporus anceps		fungi dead--TLC revealed only spot at .43 for starting material
TMB in vivo with P.A.		
1 week	two spots .61 and .41	DNPH--orange, brown $Fe(NO_3)_3$--no color lower DNPH--late developing orange brown $Fe(NO_3)_3$--no color
2 weeks	single spot .41	DNPH--late developing orange brown spot $Fe(NO_3)_3$--no color

Table A.3--Continued Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent B

Compound	Rf	Color
3 weeks	two spots .49 and .70	Same as for 1 week in vivo
4-o-ethyl-3,5-dimethoxy-benzaldehyde	.69	DNPH--orange $Fe(NO_3)_3$--no color
4-o-ethyl-3,5-dimethoxy-benzaldehyde in vivo		
1 week	two spots .49 and .70	upper DNPH--orange $Fe(NO_3)_3$ --no color lower brown
2 weeks	.51	DNPH--slow developing orange $Fe(NO_3)_3$--no color
3 weeks 4- -ethyl-3,5-dimethoxy-benzaldehyde		Same as for 1 week in vivo
4-o-benzyl-3,5-dimethoxybenzaldehyde	.62	DNPH--yellow, orange $Fe(NO_3)_3$--no color
4-o-benzyl-3,5-dimethoxy-benzaldehyde + all in vitro systems	.62	Same as for starting material
4-o-benzyl-3,5-dimethoxy-benzaldehyde + P.A. in vivo	.66	DNPH--yellow orange $Fe(NO_3)_3$--no color
	.42	DNPH--late appearing red brown $Fe(NO_3)_3$--no color
4-o-acetate derivative of syringaldehyde	.64	DNPH--orange-red $Fe(NO_3)_3$--no color

Table A.3--Continued Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent B

Compound	Rf	Color
4-o-acetate derivative of syringaldehyde in vivo	.67	DNPH--orange-red $Fe(NO_3)_3$--no color
	.30	DNPH--slow developing red brown
4-o-acetate derivative of syringaldehyde was not tested in any in vitro system		

Table A.4 Guaiacyl Compounds as Reference Standards and as Substrates for Enzyme Reactions--Solvent B

Compound	Rf	Color
Vanillyl alcohol	.47	DNPH--yellow-gray $Fe(NO_3)_3$--blue
Vanillic acid	.46	DNPH--no color $Fe(NO_3)_3$--gray
Vanillin	.60	DNPH--orange $Fe(NO_3)_3$--purple
4-o-aceto-3-methoxy-benzaldehyde	.59	DNPH--orange, yellow $Fe(NO_3)_3$--no color
Veratraldehyde	.81	DNPH--dark orange $Fe(NO_3)_3$--no color
Veratric acid	.49	DNPH-- $Fe(NO_3)_3$--no color
Veratryl alcohol	.52	DNPH--late developing brown $Fe(NO_3)_3$--no color
Vanillin + all in vitro systems (solution turned to a cloudy suspension)	Spots at van. .6 van. acid .46 + 3 unidentified brown spots in range .08 to .35	$Fe(NO_3)_3$-- all spots were gray
Vanillyl alcohol + all in vitro systems		Same results as listed for vanillin
3,4-dimethoxybenzaldehyde + purified in vitro systems	.81	DNPH--dark orange $Fe(NO_3)_3$--no color
3,4-dimethoxybenzaldehyde + crude extracellular extract in vitro	.81	DNPH--dark orange $Fe(NO_3)_3$--no color
	.49	DNPH-- $Fe(NO_3)_3$--no color

Table A.4--Continued Guaiacyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent B

Compound	Rf	Color
3,4-dimethoxybenzaldehyde in vivo	.81	DNPH--dark orange $Fe(NO_3)_3$--no color
	.52	DNPH--late developing brown $Fe(NO_3)_3$--no color
3-methoxy-p-benzoquinone	.70	DNPH--yellow-purple $Fe(NO_3)_3$--no color
4-o-acetate derivative of vanillin in vivo	.46 .47 .6	spots for van., van. acid, and alcohol + 3 unidentified spots DNPH--not tested $Fe(NO_3)_3$--all spots were gray
4-o-ethyl-3-methoxybenzaldehyde	.65	DNPH--light orange $Fe(NO_3)_3$--no color
4-o-ethyl-3-methoxybenzaldehyde + all in vitro systems	single spot at .65	Same as starting material
4-o-ethyl-3-methoxybenzaldehyde in vivo	.52	Same as starting material
	.65	DNPH--slow brown, red $Fe(NO_3)_3$--no color
4-o-benzyl-3-methoxybenzaldehyde	.63	DNPH--Orange $Fe(NO_3)_3$--no color
4-o-benzyl-3-methoxybenzaldehyde + all in vitro systems	single spot at .62	DNPH--orange $Fe(NO_3)_3$--no color
4-o-benzyl-3-methoxybenzaldehyde in vivo	.65	DNPH--orange $Fe(NO_3)_3$--no color
	.52	DNPH--late developing brown $Fe(NO_3)_3$--no color

Table A.4--Continued Guaiacyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent B

Compound	Rf	Color
Dehydrodivanillin	.28	DNPH--yellow $Fe(NO_3)_3$--brown, gray
Dehydrodivanillyl alcohol	.10	DNPH--brown $Fe(NO_3)_3$--grey

Table A.5 Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions--Solvent C

Compound	Rf	Color
4-hydroxy-3,5-dimethoxy-benzaldehyde	.63	DNPH--orange-red $Fe(NO_3)_3$--purple
	.54	DNPH--brown developing orange $Fe(NO_3)_3$--
4-hydroxy-3,5-dimethoxy-benzaldehyde alcohol	.37	DNPH--slow developing orange $Fe(NO_3)_3$--yellow
3-methoxy- -benzoquinone-5-carboxaldehyde	.63	DNPH--cherry red prior to spray, green after $Fe(NO_3)_3$--no color
Hydroquinone form of o-quinone	.47	DNPH--red brown, green upo upon exposure to NH_3 $Fe(NO_3)_3$--
3,5-dimethoxy-p-benzo-quinone	.63	DNPH--brown, yellow $Fe(NO_3)_3$--no color
4-hydroxy-3,5-dimethoxy-benzaldehyde + all in vitro systems	.63	DNPH--orange, yellow $Fe(NO_3)_3$--purple
	.50	DNPH--red, brown $Fe(NO_3)_3$--
4-hydroxy-3,5-dimethoxy-benzyl alcohol + all in vitro systems		Same results as for above.
4-o-ethyl-3,5-dimethoxy-benzaldehyde	.71	DNPH--orange, red $Fe(NO_3)_3$--no color
4-o-benzyl-3,5-dimethoxy-benzaldehyde	.70	DNPH--orange $Fe(NO_3)_3$-no color

Solvent C was generally not used in identification of the products of in vitro or in vivo reactions of the ether derivatives of syringaldehyde. The only exception was 3,4,5-trimethoxybenzaldehyde.

Table A.5--Continued Syringyl Compounds as Reference Standards and as Substrates for Enzyme Reactions --Solvent C

Compound	Rf	Color
3,4,5-trimethoxybenzaldehyde	.73	DNPH--brown $Fe(NO_3)_3$--no color
3,4,5-trimethoxybenzaldehyde + all in vitro systems		Results same as for starting material.

Note: No in vivo systems were tested.

Table A.6 Guaiacyl Compounds as Reference Standards and as Substrates for Enzyme Reactions--Solvent C

Compound	Rf	Color
4-hydroxy-3-methoxy-benzaldehyde (vanillin)	.73	DNPH--orange $Fe(NO_3)_3$--purple
4-hydroxy-3-methoxy-benzoic acid	.66	DNPH-- $Fe(NO_3)_3$--brown
4-hydroxy-3-methoxy-benzyl alcohol	.64	DNPH--yellow, gray $Fe(NO_3)_3$--blue
mono-methoxybenzo-p-quinone	.64	DNPH--yellow-purple $Fe(NO_3)_3$--
4-hydroxy-3-methoxy-benzaldehyde in vitro		Not run in Solvent C
4-hydroxy-3-methoxy-benzaldehyde in vivo		Fungus died
3,4-dimethoxybenzal-dehyde (veratral-dehyde)	.82	DNPH--orange, red $Fe(NO_3)_3$--no color
3,4-dimethoxybenzoic acid	,59	DNPH-- $Fe(NO_3)_3$--
3,4-dimethoxybenzyl alcohol	.53	DNPH--slow developing red-brown $Fe(NO_3)_3$--no color

APPENDIX B

NMR SPECTROSCOPY

s - singlet

d - doublet

t - triplet

m - multiplet

Figure B.1. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-ethyl-3,5-dimethoxybenzaldehyde (offset for aldehyde peak is 300 Hz) -- Control; δ 1.42 (t, 2), 3.86 (s, 6), 4.1 (m, 3), 7.12 (s, 2), 9.94 (s, 1).

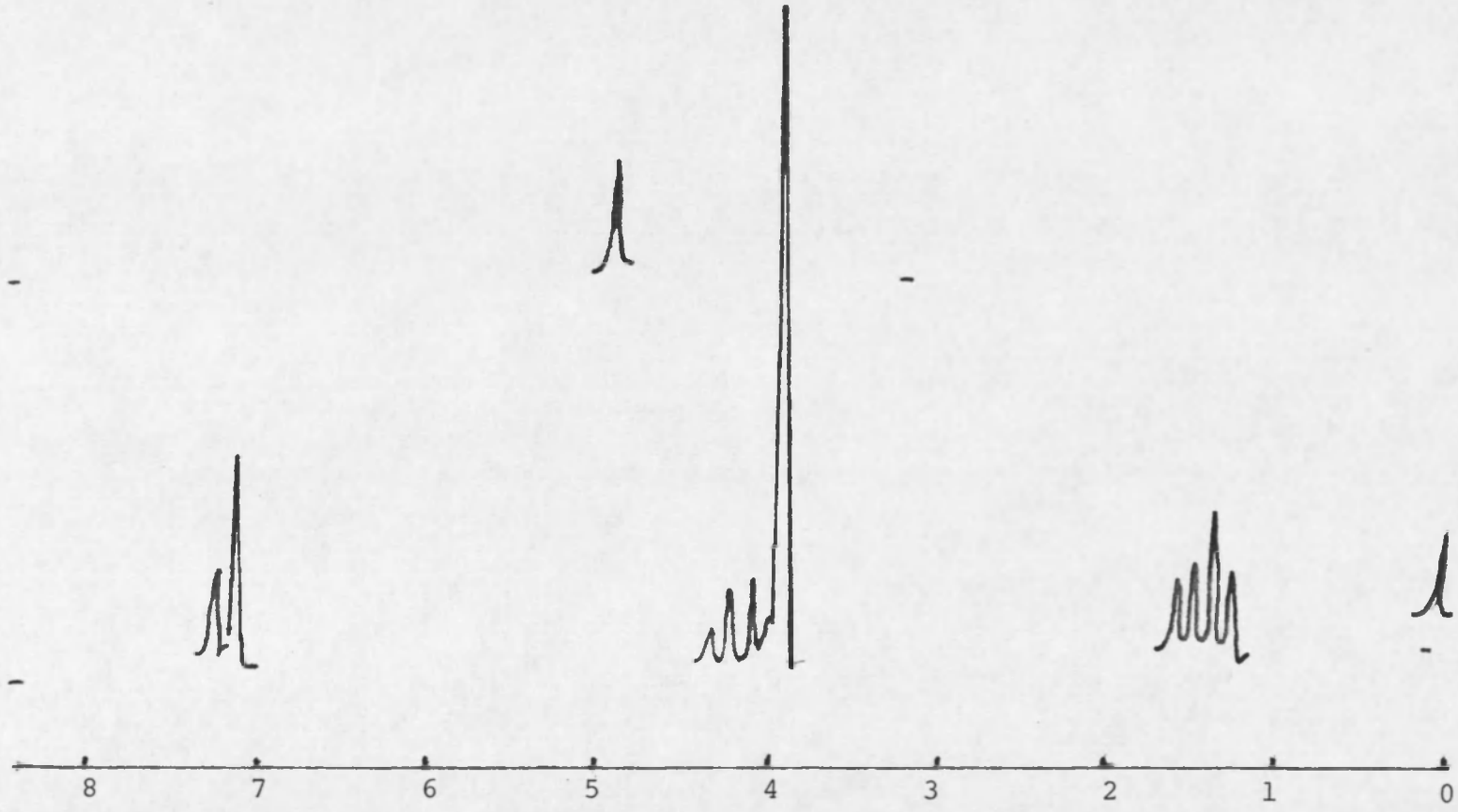


Figure B.1. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-ethyl-3,5-dimethoxybenzaldehyde (offset for aldehyde peak is 300 Hz).

Figure B.2. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-ethyl-3,5-dimethoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps -- In vivo 3 weeks; δ 1.40 (t, 2), 3.92 (s, 6), 4.14 (m, 3), 4.65 (s, 2), 6.62 (s, 2).

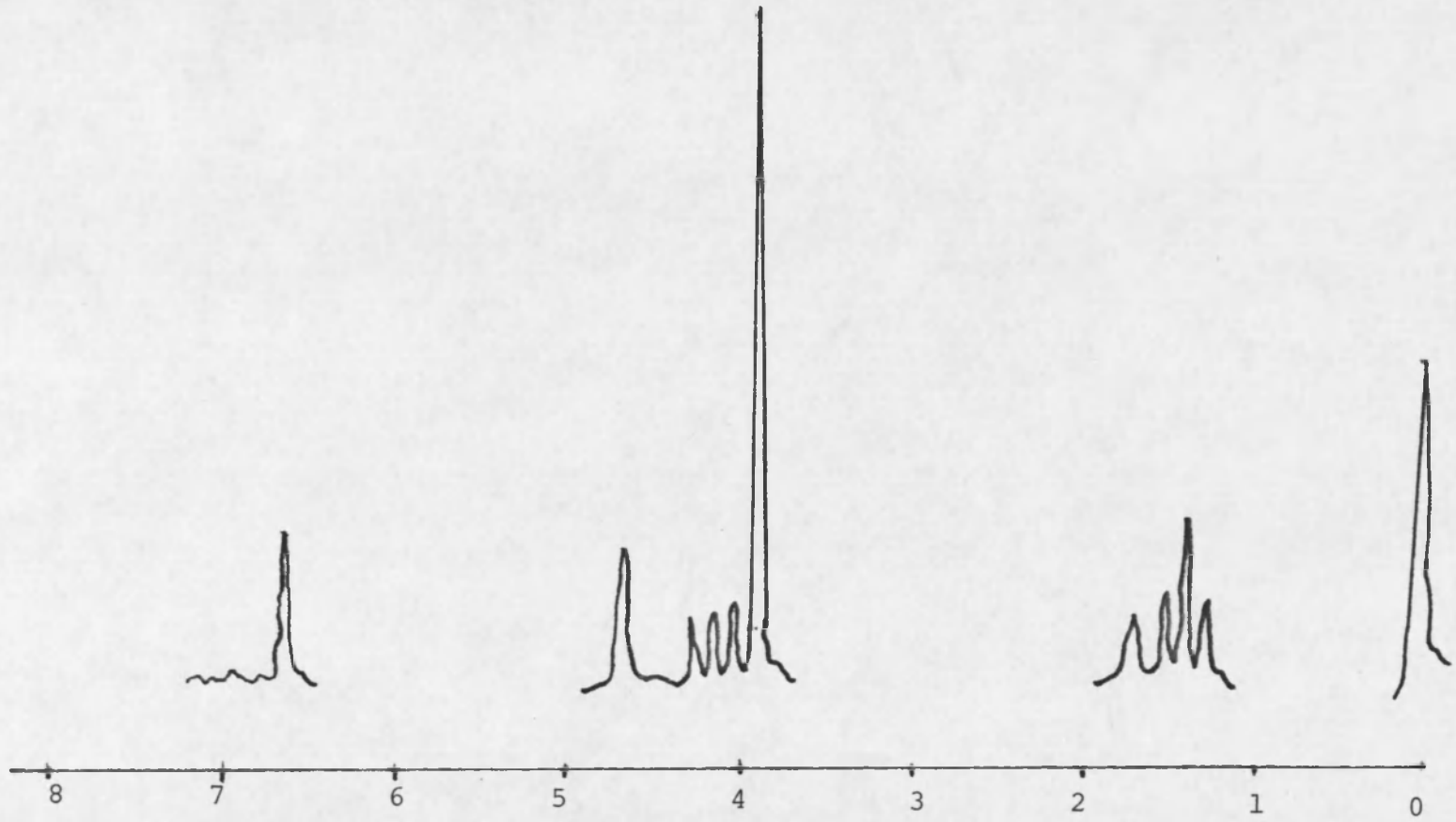


Figure B.2. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-ethyl-3,5-dimethoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps.

Figure B.3. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-O-benzyl-3,5-dimethoxybenzaldehyde (offset for aldehyde peak is 300 Hz) -- Control; δ 3.85 (s, 6), 5.15 (s, 2), 7.14 (s, 2), 7.39 (s, 5), 9.90 (s, 1).

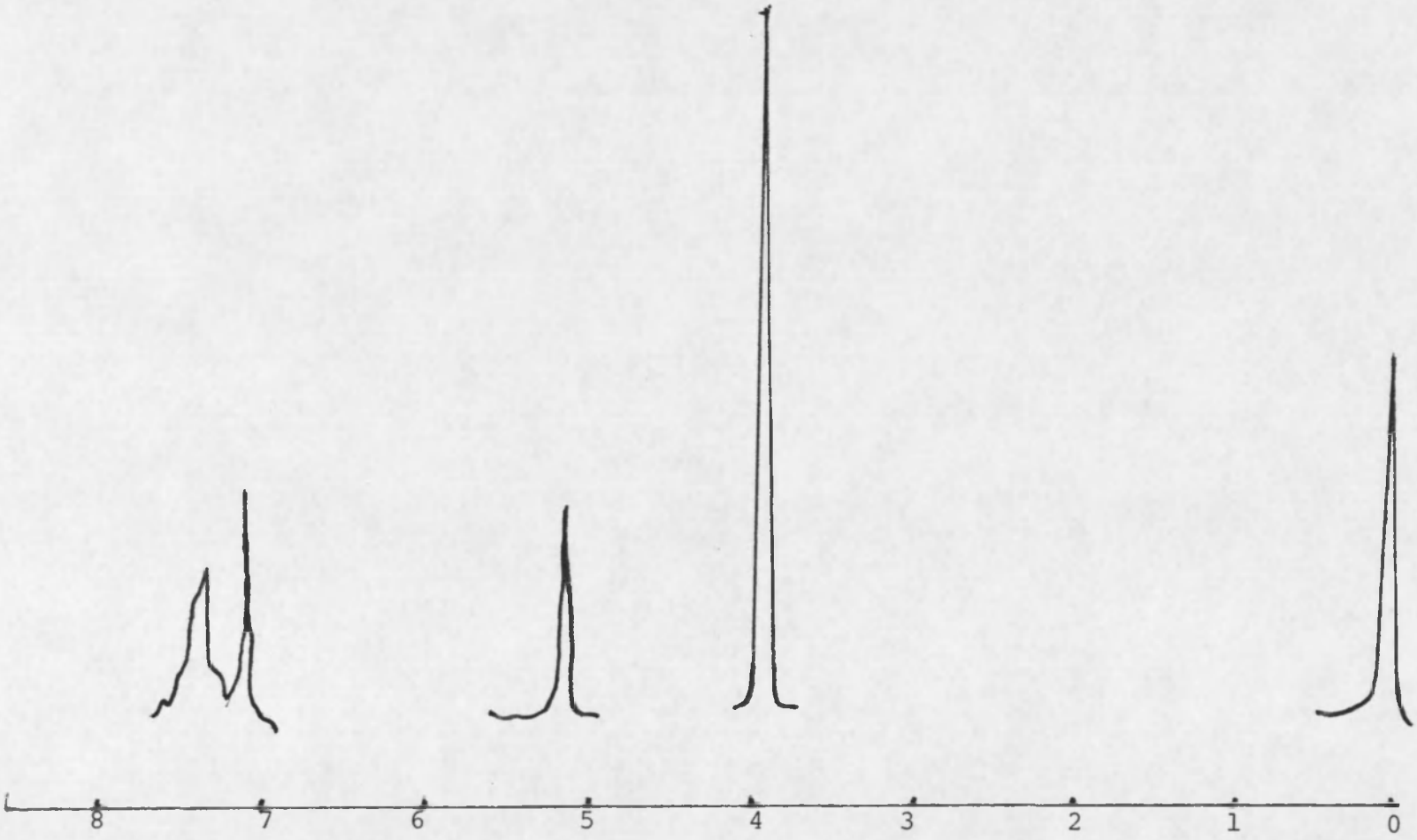


Figure B.3. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-benzyl-3,5-dimethoxybenzaldehyde (offset for aldehyde peak is 300 Hz).

Figure B.4. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-O-benzyl-3,5-dimethoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps -- In vivo 3 weeks; δ 3.88 (s, 6), 4.66 (s, 2), 5.04 (s, 2), 6.64 (s, 2), 7.40 (s, 5).

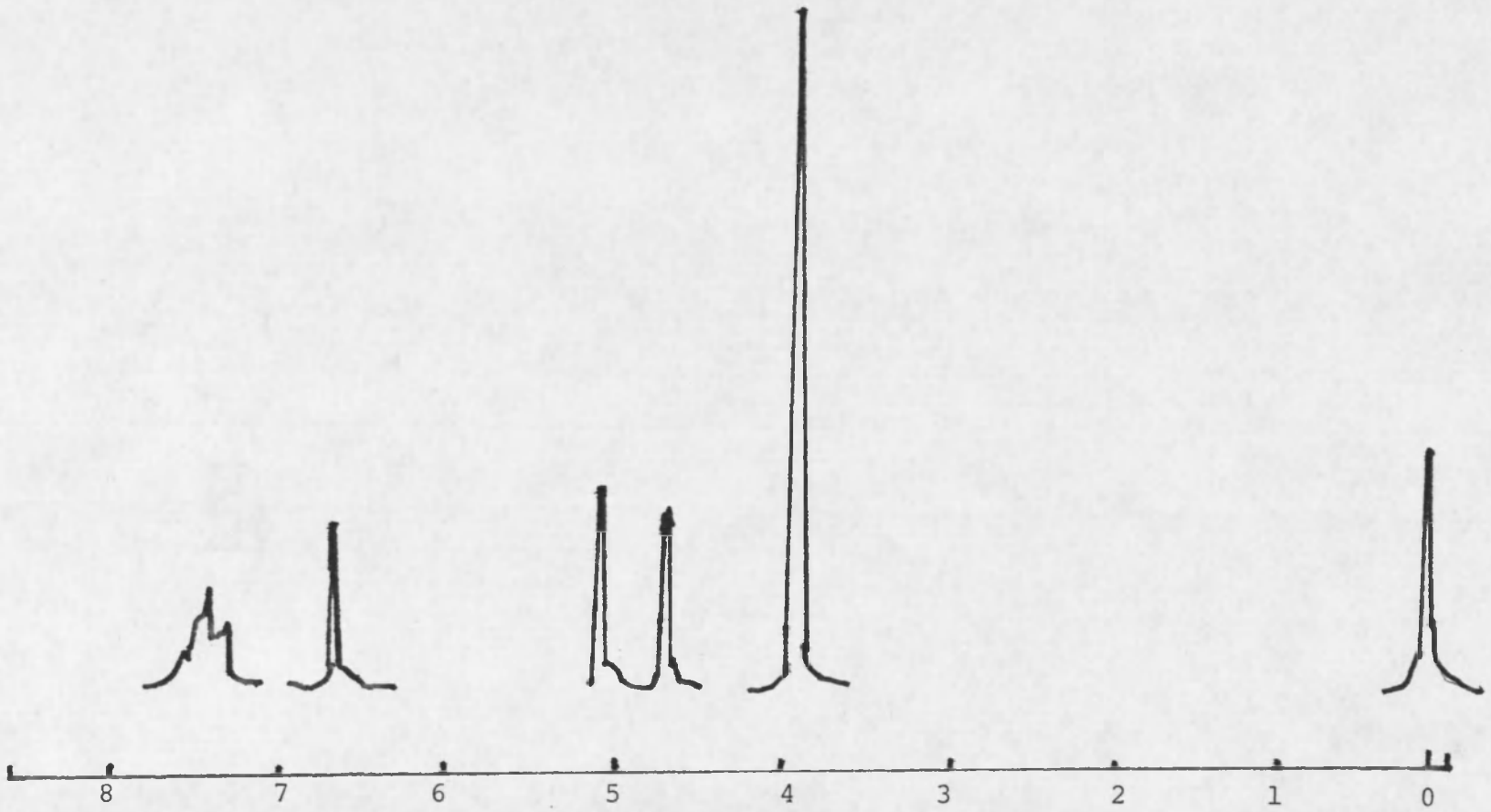


Figure B.4. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-benzyl-3,5-dimethoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps.

Figure B.5. NMR spectrum ($CDCl_3$) of synthesized 4-0-benzyl-3-methoxybenzaldehyde (offset for aldehyde peak is 100 Hz) -- Synthesized product and control; δ 3.92 (s, 3), 5.2 (s, 2), 7.04 (s, 2), 7.38 (s, 5), 9.81 (s, 1).

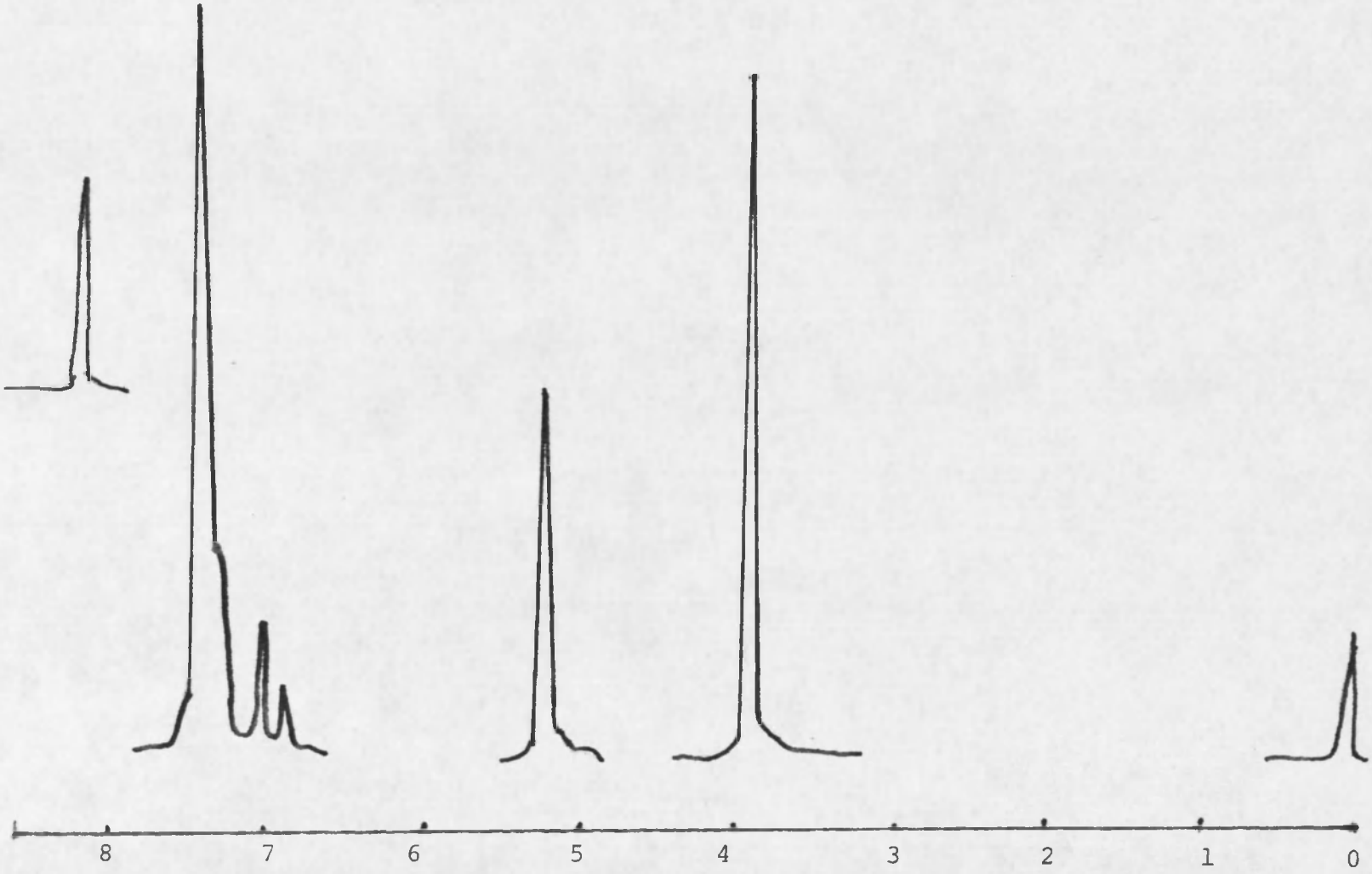


Figure B.5. NMR spectrum ($CDCl_3$) of synthesized 4-0-benzyl-3-methoxybenzaldehyde (offset for aldehyde peak is 100 Hz).

Figure B.6. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-benzyl-3-methoxybenzaldehyde (offset for aldehyde peak is 300 Hz) -- Synthesized product and control; δ 3.92 (s, 3), 5.2 (s, 2), 7.04 (s, 2), 7.38 (s, 5), 9.81 (s, 1).

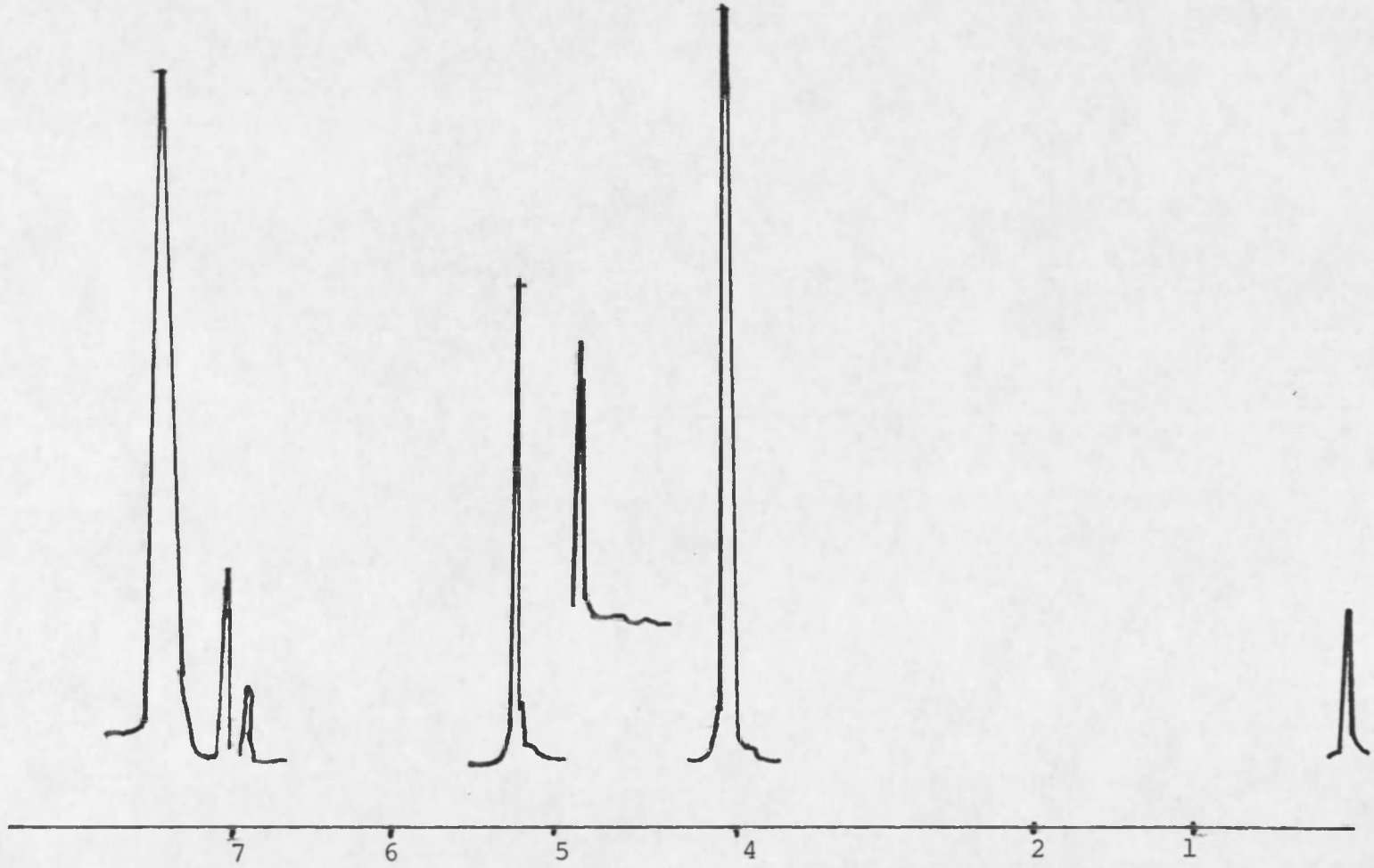


Figure B.6. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-benzyl-3-methoxybenzaldehyde (offset for aldehyde peak is 300 Hz).

Figure B.7. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-benzyl-3-methoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps -- In vivo 3 weeks; δ 3.86 (s, 3), 4.61 (s, 2), 5.18 (s, 2), 6.86 (s, 2), 7.19 (s, 5).

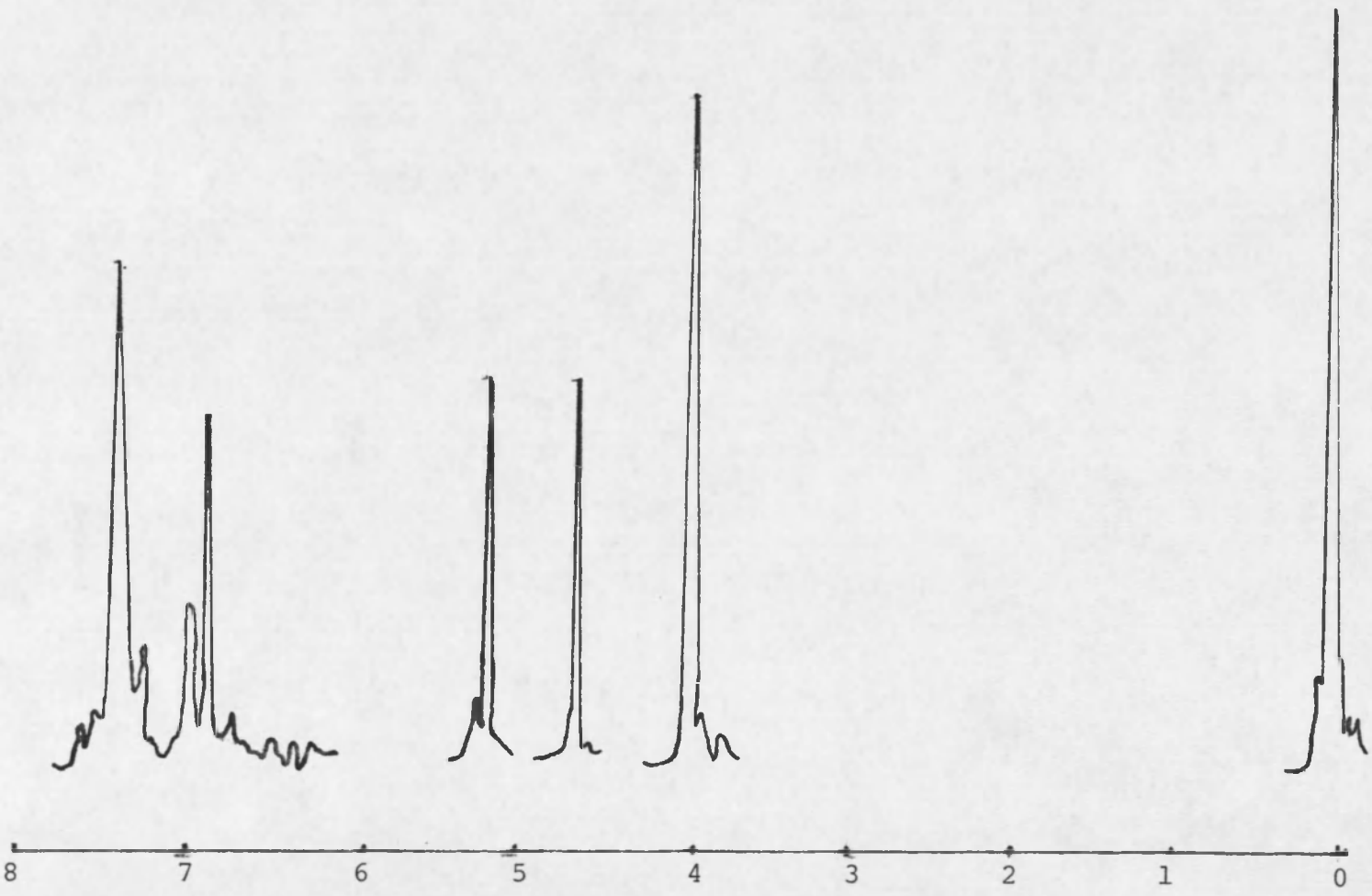


Figure B.7. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-benzyl-3-methoxybenzaldehyde kept 3 weeks in vivo with Polyporus anceps.

Figure B.8. NMR spectrum ($CDCl_3$) of synthesized 4-0-ethyl-3-methoxybenzaldehyde (offset for aldehyde peak is 100 Hz) -- Synthesized product and control; 1.41 (t, 2), 3.96 (s, 3), 4.19 (m, 3), 7.4 (s, 2), 9.85 (s, 1).

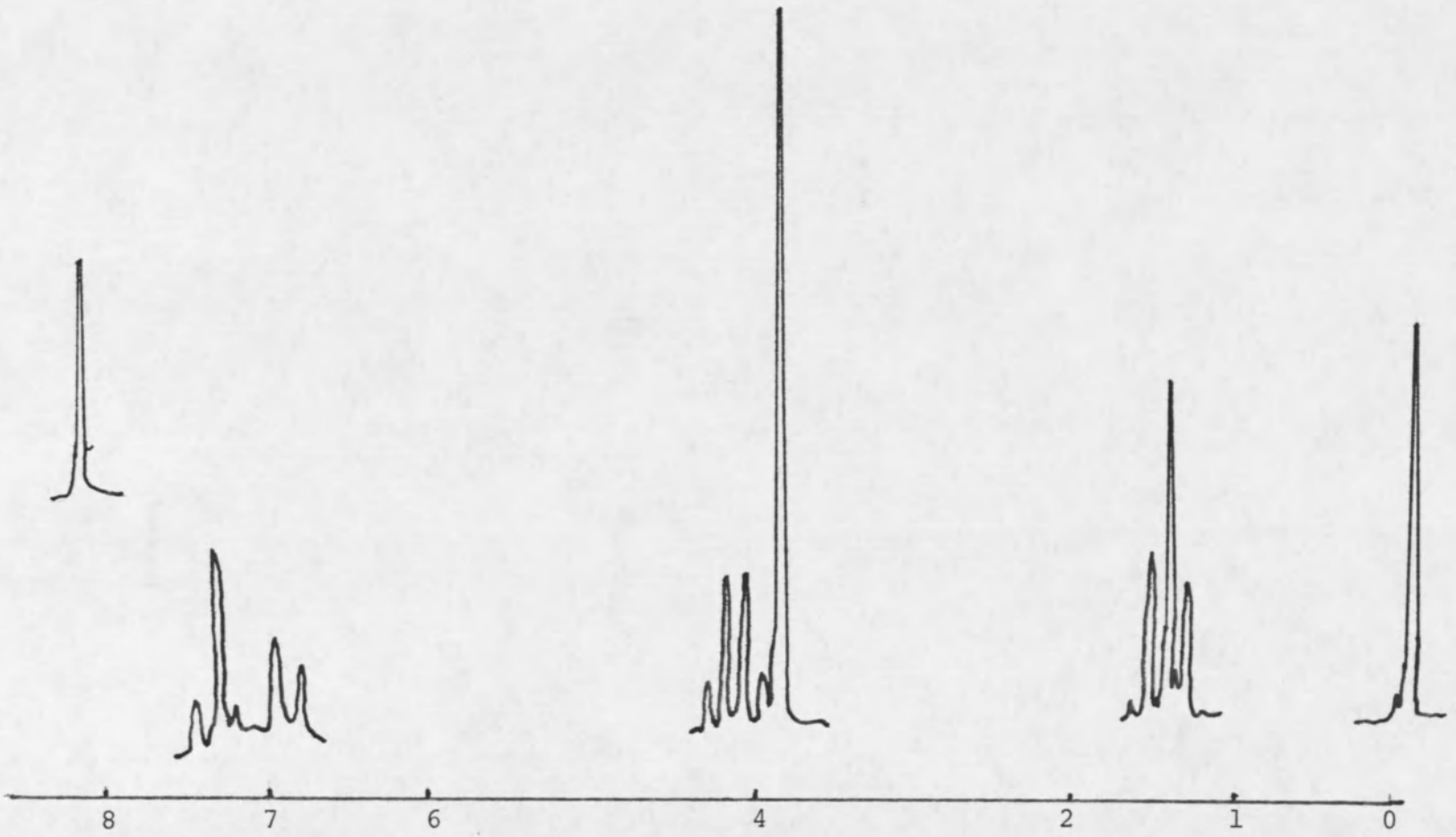


Figure B.8. NMR spectrum ($CDCl_3$) of synthesized 4-0-ethyl-3-methoxybenzaldehyde (offset for aldehyde peak is 100 Hz).

Figure B.9. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-ethyl-3-methoxybenzaldehyde (offset for aldehyde peak is 300 Hz) -- Synthesized product and control; δ 1.41 (t, 2), 3.96 (s, 3), 4.19 (m, 3), 7.4 (s, 2), 9.85 (s, 1).

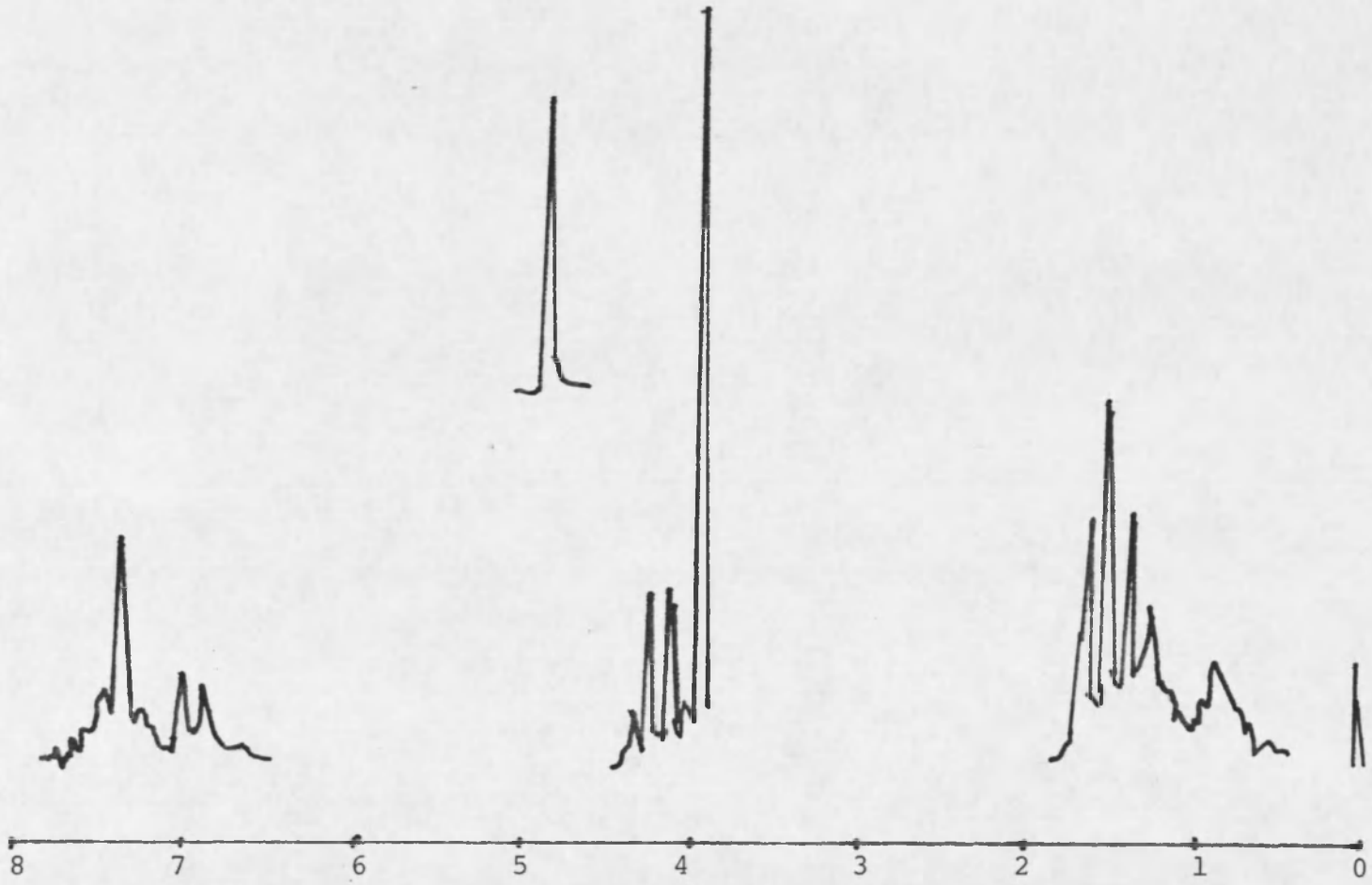


Figure B.9. NMR spectrum ($CDCl_3$) of isolate from control flask containing 4-0-ethyl-3-methoxybenzaldehyde (offset for aldehyde peak is 300 Hz).

Figure B.10. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-ethyl-3-methoxybenzaldehyde kept in vivo 3 weeks with Polyporus anceps -- In vivo 3 weeks; δ 1.40 (t, 2), 3.92 (s, 6), 4.17 (m, 3), 4.76 (s, 2), 6.78 (s, 2).

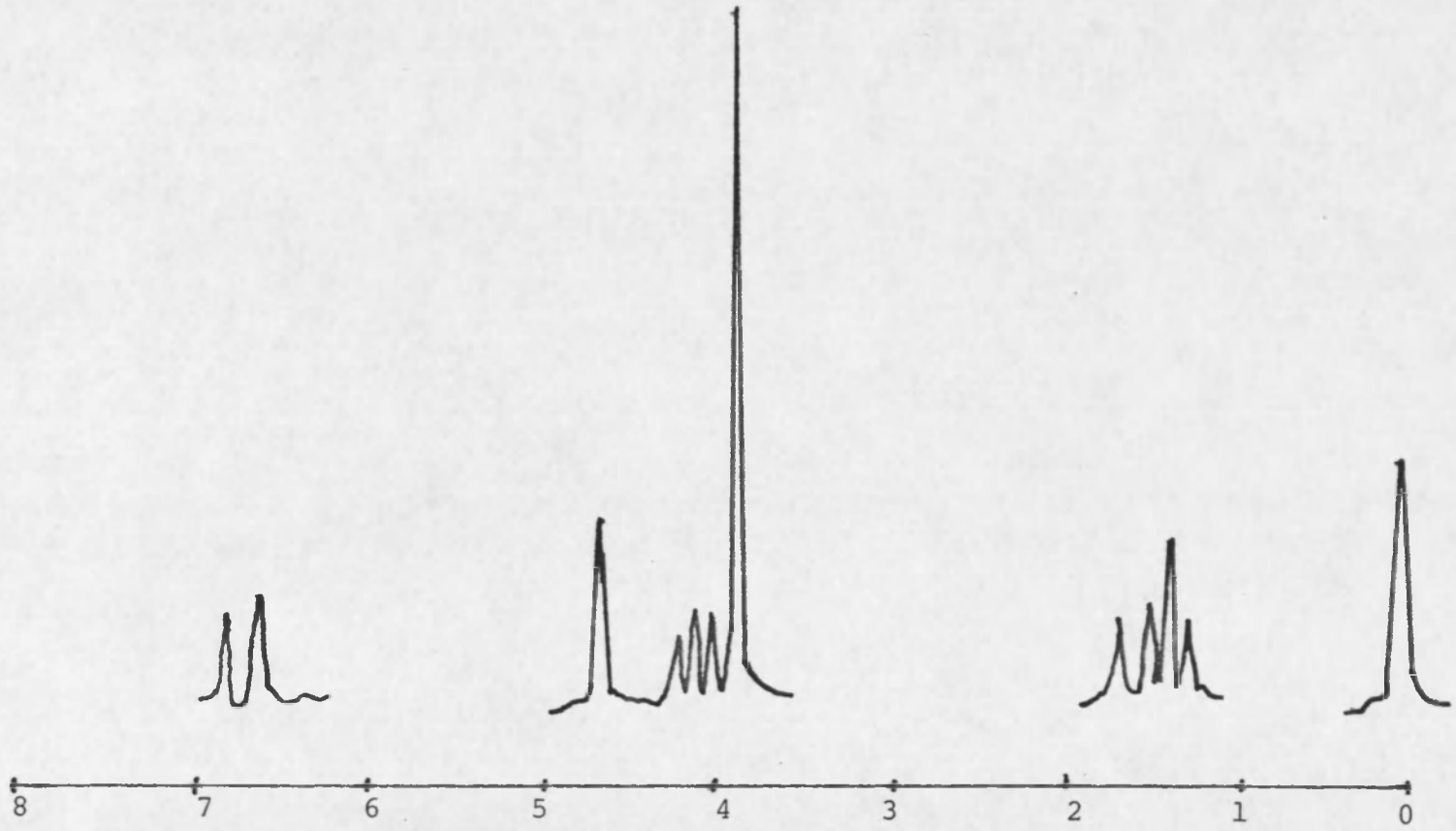


Figure B.10. NMR spectrum ($CDCl_3$) of isolate from flask containing 4-0-ethyl-3-methoxybenzaldehyde kept in vivo 3 weeks with Polyporus anceps.

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