University of Nevada, Reno

Characterization of Ipsdienol Dehydrogenase in an Eastern Population of *Ips pini*.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
By

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ERIC Y. CHANG

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Characterization Of Ipsdienol Dehydrogenase In An Eastern Population Of Ips Pini.

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Dr. Gary J. Blomquist, Advisor

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Abstract

Bark beetles are a major group of pest insects in North America. Each year, they destroy millions of acres of trees in the United States resulting in a huge economic loss. The objective of this study is to understand pheromone production in *Ips* spp.; specifically, how the stereochemistry of ipsdienol is achieved in an eastern population of *Ips pini*, which will be referred to as eastern *I. pini* The pheromone of eastern *I. pini* consists of an approximately 35:65 *(R)*-(-):*(S)*-(+). We hypothesized that the ipsdienol dehydrogenase in eastern *I. pini*, which will be referred to as eastern IDOL-DH, catalyzes the final step that determines ipsdienol stereochemistry. Eastern IDOL-DH was cloned and expressed in a baculovirus system. The enzyme was assayed by measuring the change in absorption at 340 nm, and its products analyzed by GC-MS on a chiral column to determine its role in achieving the final *(R)*-(-)/*(S)*-(+) ratio of ipsdienol. The results showed that recombinant eastern IDOL-DH interconverts *(R)*-(-) ipsdienol and ipsdienone and only produces the *(R)*-(-) ipsdienol from ipsdienone. The data suggest the final step to the natural occurring ratio of ipsdienol enantiomers was not carried out by eastern IDOL-DH. However, eastern IDOL-DH may play a role in the production of anti-aggregation pheromone, ipsenol, as it readily converted ipsenone to *(S)*-(-) ipsenol.

Acknowledgments

I thank Dr. Gary Blomquist and Dr. Claus Tittiger for giving me an opportunity to work on this project. I would like to express my gratitude to Ruby Figueroa-Teran for her tremendous support, and the time she spent on helping me with this project. I want to thank Mory Aw, Minmin Song, Andrew Gorzalski, Nicole Abbot, Sharon Young, Sally Qui, and Ana Gilg for all the helps through the years I have been in the lab. A special thanks to Dr. William Welch and Heidi Pak for performing enzyme assays. In addition, I want to thank Craig Osborne from Nevada Genomic Center, Dr. Kathleen Schegg, Dr. David Quilici, and Rebekah Woosley from Nevada Proteomics Center for their help in DNA sequencing and GC-MS, respectively.

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Introduction

Aggregation pheromone in bark beetles

Bark beetles (Coleoptera: Curculionidae, Scolytinae) comprise a large group of insects with great diversity. There are more than 550 species in North America. Many are important pest insects in the United States. Bark beetles are one of the most important factors in coniferous forest tree mortality (Fettig et al., 2007). The pine engraver, *Ips pini* (Say), is one of the most common bark beetle species in North America. They usually attack weak and stressed pine trees or downed trees or branches with primary hosts being ponderosa pine, lodgepole pine, and Jeffrey pine. Occasionally, they can also infest other pine species. During outbreak conditions, they can kill healthy trees (http://www.barkbeetles.org/ips/f122.htm). Because bark beetles spend the majority of their life cycle under the bark of the tree, traditional management methods with pesticides do not work well. In order to develop a better strategy for bark beetle population control, it is important to understand the mechanism of their communication through pheromones and the origination of pheromone components.

Pheromone communication in Coleoptera is a complex system that is not yet fully understood. There are a variety of pheromones that carry out different tasks. They include sex pheromones, anti-aphrodisiac pheromones, long-range and short-range aggregation pheromones, anti-aggregation pheromones, and epideictic (spacing) pheromones. Aggregation pheromones are chemical compounds produced by the beetles to attract other individuals. Bark beetles use aggregation pheromones to coordinate mass attacks on trees. The first pheromone isolated from a beetle was a mixture of (4*S*)-(-)-ipsenol (2-methyl-6-methylene-7-octen-4-ol), (4*S*)-(+)-ipsdienol (2-Methyl-6-methylene-

2, 7-octadien-4-ol), and (1*S*, 2*S*)-(+)-cis-verbenol [4,6,6-trimethylbicyclo{3.1.1}hept-3-en-2ol]. This multi-component pheromone blend was discovered in *I. paraconfusus* (Silverstein et al., 1966). It was the first multi-component pheromone found, which was different than the concept at that time that each insect species only used a single chemical compound for its pheromone (Silverstein and Young 1976). Indeed, single component pheromones are now extremely rare.

In the past several decades, our understanding of the mechanism of pheromone production in the *Ips* species has seen huge strides forward. Hughes (1974) showed that several *Ips* species produced more ipsdienol when they were exposed to myrcene, and suggested ipsdienol was derived from host tree-derived myrcene. Byers et al., (1979) demonstrated that increasing the concentration of myrcene vapor resulted in increasing amount of ipsenol and ipsdienol production in *I. paraconfusus.* Hendry et al. (1980) showed that deuterium labeled myrcene was readily converted to ipsenol and ipsdienol in *I. paraconfusus*. These results supported Hughes's hypothesis that unlike other insects, bark beetles use host tree-derived monoterpenes as the precursor to pheromone production. By 1990, it had become firmly established that bark beetle pheromones were derived from host precursors. When the beetles attack a tree, the tree would react by producing resin. The resin not only creates a physical barrier to drive the beetles out of the boring holes, but the monoterpenes are toxic and can kill the beetles. Bark beetles possess mechanisms to detoxify these compounds and some of the products of these processes evolved to be used as pheromone components (Raffa and Smalley 1995).

Most other insect species produce pheromone components de novo. This led to the question of whether bark beetles can produce the carbon skeleton that serves as the

precursors to pheromone components or if they depend on the tree to provide the precursors. Due to the similarity of the structure of pheromone components and tree produced monoterpene, pheromone components were thought to be produced from host precursors (Vanderwel, 1994). A paradigm shift occurred in the 1990s after evidence began accumulating that most bark beetle pheromone components are produced de novo, and the data from earlier studies were reinterpreted. When *I. paraconfusus* was treated with JH III, the beetles were able to produce ipsdienol in the absence of added myrcene (Borden et al., 1969). Another experiment suggested that the amount of ipsdienol produced by fed beetles is more than the amount of myrcene that the host phloem can provide (Byers, 1984; Byers and Birgersson, 1990a). These observations indicated that bark beetles may have the ability to make the precursor de novo. Later, more evidence added support that pheromone components were produced *de novo*. Direct evidence that bark beetles produced pheromone components de novo was obtained by the demonstration of the incorporation of labeled acetate into ipsdienol and ipsenol in *I. pini* and *I. paraconfusus* (Seybold et al., 1995a). A later study using [1-^{14}C]acetate (Tillman et al., 1998) demonstrated that *I. pini* uses the mevalonate pathway to synthesize ipsdienol *de novo*. This study not only provided conclusive evidence on the *de novo* synthesis of pheromone production, it also established a better understanding on the mechanism of pheromone biosynthesis.

Within *Ips*, different species produce different pheromone components. The principal pheromone component for *I. pini* is ipsdienol while the main pheromone component for *I. grandicollis* and *I. paraconfusus* is ipsenol. Previous studies have shown *de novo* pheromone synthesis of these compounds via the mevalonate pathway in

both *I. pini* and *I. grandicollis* (Seybold et al., 1995a; Ivarsson et al., 1997; Tillman et al., 1998). Radio labeling studies with ^{14}C-acetate also demonstrated that phloem feeding stimulated pheromone biosynthesis in *I. pini*, *I. grandicollis*, and *I. paraconfusus* (Tillman et al., 2004). However, JH III treatment only stimulated pheromone production in *I. pini* and not in the other two species. Interestingly, the same report also showed that the transcript level of a key enzyme in the mevalonate pathway, hydroxymethylglutaryl-CoA reductase (HMG-R), increase in both *I. pini* and *I. paraconfusus* when treated with JH. The enzyme activity of HMG-R remains low while the transcript level increases in *I. paraconfusus*. This result suggested different HMG-R posttranslational regulation by JH in *I. paraconfusus* and *I. pini*. Since *I. grandicollis*, and *I. paraconfusus* are both from *grandicollis* group, Tillman et al., (2004) suggested that other species in the group would likely behave in the same manner. This difference between *I. pini* and the *grandicollis* group appears to indicate a phylogenic difference in pheromone regulation in the *Ips* genus.

Male *I. pini* produces pheromone that contains an enantimeric mixture of *(R)*-(-)-ipsdienol and *(S)*-(+)-ipsdienol. The western population of *I. pini*, which will be referred to as western *I. pini*, produces more than 95% *(R)*-(-) ipsdienol, while eastern populations produce about 35% *(R)*-(-). Seybold et al. (1995) analyzed the enantiomeric composition of ipsdienol from *I. pini* across the United States, and divided them into three phenotypes. There is the New York type, California type, and a third type near southwestern British Columbia which is a possible hybridization zone. Cognato et al. (1999) used mitochondrial DNA variation to study the gene flow of *I. pini*, and the results corroborate the study from Seybold et al. (1995b). Based on the gene flow of cytochrome oxidase I

gene, Cognato et al. (1999) concluded there are different mtDNA haplotype lineages in the eastern, western, and Rocky mountain regions. Eastern and western populations of *I. pini* were originally categorized as different species. *Ips pini* (Say, 1826) is from New York, and *I. oregonis* (Eichhoff, 1869) is from California. These two groups showed distinctive difference in the pheromone composition. Dominigue et al. (2006) studied the genetic nature of ipsdienol enantiomeric composition between the eastern and western *I. pini*. Beetles were collected from California, New York and British Columbia (hybridization zone). The investigators split the beetles from hybridization zone into two groups according to the level of *(R)*-(-)/*(S)*-(+)-ipsdienol enantiomeric ratios. The hybrid beetles were crossed with the western and eastern beetles. The amount of *(S)*-(+) ipsdienol from the offspring was then measured. The study suggested the possibility that the *(S)*-(+) ipsdienol enantiomeric variation in the hybrid zone is controlled by a single recessive allele. The same group later performed another experiment that crossed western and eastern *I. pini*. They confirmed the X-linked effect, and suggested a possible dominant epistatic interaction with an autosomal locus (Domingue and Teale, 2008).

Juvenile hormone III is one of the most widely studied stimuli that activate the biosynthesis of pheromone production in bark beetles. The first report regarding JH stimulation was from Borden et al. (1969). They applied JH to beetles that had emerged from ponderosa pine logs, and compared pheromone production to beetles that were introduced into fresh pine logs. The experiment showed the JH treatment increased pheromone production in *I. paraconfusus*. With a higher amount of JH, the investigators found that the male gut tissues were more attractive to females than males fed on logs.

Later, Tillman et al. (1998) reported evidence that showed that male *I. pini* produced higher amounts of JH III in the corpora allata when feeding than non-feeding controls.

As with some other bark beetle species, JH upregulates pheromone biosynthesis in *I. duplicatus*. Ivarsson and Birgersson (1995) showed that a JH analog increased ipsdienol and *E*-myrcenol production, which are the main aggregation pheromone components for *I. duplicatus.* However, exposure to the precursor of ipsdienol, myrcene, didn't significantly increase pheromone production. The same report also showed Hez-PBAN has no effect on *I. duplicatus*. This finding supports the idea that bark beetle ipsdienol and *E*-myrcenol are produced *de novo*. *De novo* synthesis makes the beetles less dependent on host-tree produced myrcene. It is especially true for *I. duplicatus* because they attack weakened trees which might not contain enough myrcene in the resin. This study again strengthens the argument of *de novo* pheromone synthesis in *Ips* spp., and indeed, it is now clear that most bark beetle pheromone components are produced de novo (Blomquist et al., 2010).

A functional genomics experiment on how JH III affects *I. pini* pheromone production was performed by Keeling et al. (2006). In this study, male beetles were treated with JH III and microarrays were used to investigate the expression pattern of the major genes involved in the mevalonate pathway. Real time polymerase chain reaction was used to confirm the results. One of the genes that showed upregulation was *IPG012D04* which encodes a dehydrogenase. This gene was later named IDOL-DH (Figueroa-Teran, unpublished data) and was shown to play a still not completely defined role in pheromonal ipsdienol production. It oxidizes *(R)*-(-) ipsdienol to ipsdienone, and reduces ipsdienone to *(R)*-(-) ipsdienol (R. Figueroa Teran, unpublished data) (Fig. 1).

The goal of this study was to clone and express *ipsdienol dehyrogenase* from the eastern *I. pini*, and compare its function to the IDOL-DH from the western *I. pini*. We hypothesize that IDOL-DH is the key step that controls the stereochemistry of the final product, ipsdienol. The result of this experiment will allow us to gain more insight about the genetic variation among the *Ips* species, especially between eastern and western *I. pini*.

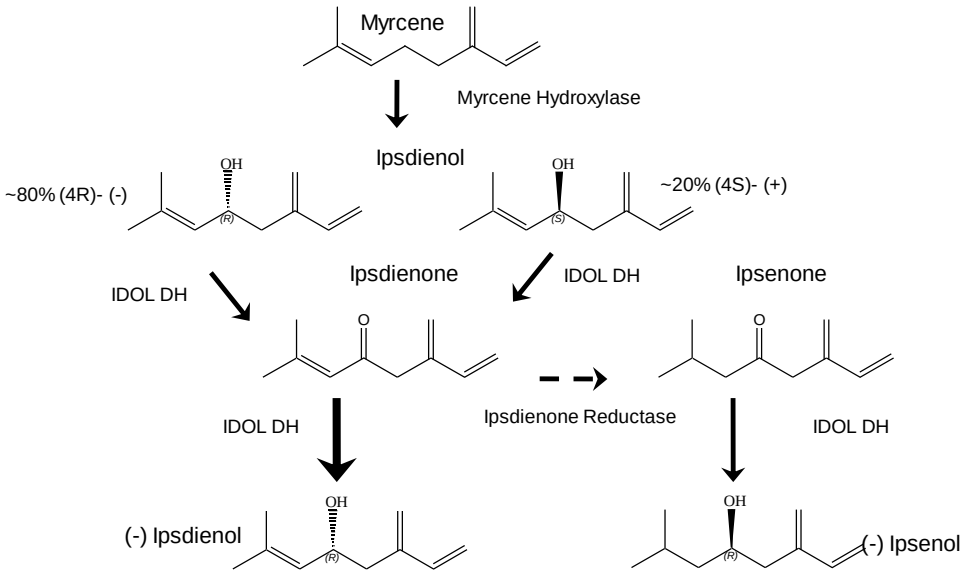

Figure 1. Proposed terminal steps in the pathway for ipsdienol production in western *I. pini* (Figueroa-Teran, unpublished). Solid arrows indicate characterized reactions. The gapped arrow indicates the uncharacterized reaction. Myrcene is converted to ipsdienol by CYP9T2 in an approximate 80:20 (-/+) ratio (Sandstrom et al., 2006). *(R)*-(-) ipsdienol is oxidized to ipsdienone, and reduced back to ipsdienol by IDOL-DH. Ipsdienone can also be converted to ipsenone and reduced to *(S)*-(-) ipsenol.

Materials and Methods

Cloning eastern IDOL-DH into pST-Blue vector

PCR was used to amplify eastern IDOL-DH cDNA. The cDNA was isolated and sequenced by Jordan Naviaux, and Jessica Moskwa. The beetles were provided by Matt Ginzel from Purdue University. The PCR reaction mixture included 10μl water, 4μl 5X Colorless GoTaq® Flexi buffer, 1.2 μl 25mM $MgCl_2$, 0.4 μl 10mM dNTP mix, 1μl of 10υμM Eip12D04F7 forward primer (CAGGTGACCTAAATGATGGT), 1μl of 10μM Eip12D04R3 reverse primer (GACCTTAAAAATTGATTTAA), 0.4 μl of 5U/μl GoTaq® Flexi DNA Polymerase from Promega, and 1-5 μg of eastern IDOL-DH cDNA. MJ Research PTC-200 Peltier thermal cycler was used for the PCR reaction. The parameters of PCR were: 95°C for 1 minute, 35 cycles of 95°C for 40 sec, 45°C for 1 minute, 72°C for 2.5 minute, and the final extension of 72°C for 5 minute. The size of PCR product was checked by running the sample on a 1% argrose gel.

Ligation and transformation

The DNA fragment was ligated into Novagen® pST-blue-1 AccepTor™ vector by standard methods and transformed into NovaBlue Singles™ Competent Cells. Recombinant colonies were picked, replica plated and queried for insert by PCR using U19 (GTTTTCCCAGTCACGACG), and T7 (TAATACGACTCACTATAGGG) vector primers. The PCR profile was: 95°C for 1 minute, 35 cycles of 94°C for 30 sec, 45°C for 0.5 minute, 72°C for 1.5 minute, and the final extension of 72°C for 10 minute. The size of colony PCR product was checked by running the sample on a 1% agarose gel. A clone containing the correct insert was selected for plasmid preparation using QIAprep Spin

Miniprep Kit and sequenced at the Nevada Genomic Center (NGC) to confirm the integrity of the insert. Complete sequencing included reactions primed from two internal primers, Eip12D04F1 (CAATATGGACACTCAACAACC) and Eip12D04F4 (GATTTAACTAATAATAAATA).

Cloning eastern IDOL-DH into pENTR^{TM}4 vector

PCR was used to amplify the eastern IDOL-DH cDNA from the pST-Blue construct. The reaction included 1μl of the pST-Blue construct plasmid, 5μl of 10× *PfuUltra* HF reaction buffer, 1μl of 10mM dNTP mix, 2.5μl of Eip12D04F8 primer (GCGAATTCAGATTCAGGT), 2.5μl of Eip12D04R5 primer (GCGCTCTCAGACCTTAAGAATTCGC), 0.4μl of 2.5U/μl *PfuUltra* HF DNA polymerase from Stratagene. Both primers were designed to include the restriction digestion site of EcoR1. The cycling profile was: 95°C for 1 minute, 35 cycles of 95°C for 40 sec, 55°C for 1 minute, 72°C for 2.5 minute, and the final extension of 72°C for 5 minute. The size of the PCR product was confirmed by using 1% agarose gel.

The PCR product and pENTR^{TM}4 vector were digested with EcoR1. The digested vector was treated with Calf Intestinal Alkaline Phosphatase (CIP) from New England BioLabs. Both fragments were recovered from a 0.8% gel using the Wizard® SV Gel and PCR Clean-Up System.

Purified insert gene and the vector were mixed in 3:1 ratio and ligated with T4 DNA Ligase from Promega. Recombinant plasmid was transformed into DH5α cells by standard method and verified by PCR amplification of transformed cells using

Pentr4F2 (GCGTTTCTACAAACTCTTCC), and Pentr4R2 (GCAATGTAACATCAGAGATT) primers. The insert was confirmed by sequencing at the NGC

Baculovirus system

After the sequencing result confirmed the gene was successfully cloned into the pENTR4, we could use the BaculoDirect™ Baculovirus Expression System from Invitrogen to express the gene. The cell line used in the baculovirus system was Sf9 cell, also from Invitrogen. LR reaction, transfection, and infection of the cells were performed using manufacture's protocol with BaculoDirect Linear DNA C-Terminus.

Protein expression

Five culture plates were used in this experiment, including one negative control without virus. 10ml of 1×10^6 cells/ml Sf9 cells were seeded on each plate for 1 hour. 4 different amount of virus were added in different plates. The amounts of P3 virus added were 0.1ml, 0.5ml, 1ml, and 2ml. 5% of FBS was added to each well. The plates were stored at 27°C for 72 hours.

Western blot

Western blot was used to detect the presence of the protein, and confirm the success of viral infection in P1, P2, and P3 stock. The protein was detected using 1:1000 primary rabbit-anti-IDOL-DH antibody (a gift from Rubi Figueroa-Teran) and 1:5000 goat-anti-rabbit IgG antibody from BioRad and imaged using Kodak X-ray film exposed to the Supersignal West Pico Chemiluminescent substrate. The film was developed in the dark room.

Time course study for protein expression

Ten milliliter of 1×10^6 cells/ml of Sf9 cell was placed in the 125ml TRIFOREST Polycarbonate Erlenmeyer Flasks. 0.5ml of P3 viral stock was added to infect the cells, followed by the addition of 0.5ml of FBS. The flask was kept at 27℃ on a shaker for 6 days total. 500μl of infected cells was removed each day, and run on the western blot.

Protein expression for enzymatic reaction

To produce recombinant IDOL-DH, 30ml of 1×10^6 cell/ml of Sf9 cell was placed in the 125ml TRIFOREST Polycarbonate Erlenmeyer Flasks. 0.5ml of P3 viral stock and 0.15ml of FBS was then added. The flask was incubated at 27°C on a shaker for 3 days.

Protein extraction

After 3 days, the infected cell was transferred to a 50ml conical tube, and centrifuged with Beckman CS-6R at 3000 rpm for 10 minutes at 4°C. The supernatant was discarded. The pallet was suspended with 1/10 volume of ice cold lysis buffer. The suspended cells were sonicated 3 times, and aliquoted into microcentrifuge tube. The cells were centrifuged at 13000rpm at 4°C for 20 minutes with Sorvall Biofuge Pico table top centrifuge. The pallet was discarded, and the supernatant was combined and kept at 4°C.

Alcohol dehydrogenase assay

The assay for (*R*)-(-) ipsdienol oxidation was set up by mixing 0.066M NaH_2PO_4 buffer at pH 7.52 with 0.1% gelatin, 0.008M $NADP^+$, 0.017mM (*R*)-(-) ipsdienol, and 148μg of eastern IDOL-DH enzyme. The ratio of $NDAP^+$ to (*R*)-(-) ipsdienol is about 470:1. The reduction of ipsdienone was setup the same way except $NADP^+$ was replaced with NADPH and *(R)*-(-) ipsdienol was replaced with ipsdienone. A control experiment was included using Sf9 cell lysate instead of eastern IDOL DH enzyme. The mixtures

were incubated at room temperature for 5-10 minutes, and the absorbance at 340nm was recorded with HP 452A Diode Array Spectrometer.

Enzyme reactions

Five types of reactions were prepared. Each type of reaction included 2 separately expressed enzymes and 2 negative controls (enzyme absent and Sf9 cell lysate). A total of 20 reactions were performed. Reactions 1 to 4 were the oxidation of *(R)*-(-) ipsdienol to ipsdienone using $NADP^+$ as coenzyme. Reactions 5 to 8 were also the oxidation, but using *(S)*-(+) ipsdienol as the substrate. Reactions 9 to 12 were the reduction of ipsdienone to ipsdenol using NADPH as coenzyme. Reactions 13 to 16 were the reduction of ipsenone to *(S)*-(-) ispenol using NADPH as coenzyme. Reactions 17 to 20 are the oxidation of ipsenol to ispenone with $NADP^+$ as coenzyme. The reactions were mixed in the assay buffer, which is made of 0.1M Sodium Phosphate buffer pH 7.8 and 1.1mM EDTA. 976.5µl of assay buffer was first placed in the glass vial. 0.225mM of coenzyme ($NADP^+$ or NADPH) was added, followed by 100ug of enzyme, and 0.0675mM substrate to make the total volume of 1000µl. The reactions were incubated at room temperature for 30 minutes.

Pentane:ether extraction

The enzyme reactions were stopped by extraction with 1:1 pentane:ether containing 200ng/ml of *n*-octanol for standard. One milliliter of the pentane ether mixture was added to the vial contained the reaction mixture. The vial was vortexed and the top layer was transferred to the new glass vial. The extraction was repeated for 3 times. The combined top layers were dried to about 100µl with nitrogen, and stored in -20°C.

GC-MS

The extracted samples were sent to Nevada Proteomic Center for GC-MS analysis.

Results

Eastern IDOL-DH cDNA isolated by Jessica Moskwa was successfully amplified and cloned into pST-Blue cloning vectors (Fig. 2A, B). The pST-Blue construct sequence was aligned to the western and eastern IDOL-DH sequences (Fig. 3A, B). Even with a few unmatched nucleotides, the construct was verified to have the same amino acid sequence as eastern IDOL-DH. This construct was amplified again, and cloned into pENTR4 cloning vector (Fig. 2C, D). The gene sequence in pENTR4 vector is identical to the sequence in pST-Blue. The gene was expressed in a baculovirus system, and confirmed by western blot. IDOL-DH recombinant proteins were found in all viral stocks (Fig. 4A, B). Protein expression studies showed positive correlation between the protein production, and the amount of virus used to infect the cells (Fig. 4C). A time course study indicated that proteins were present in the pelleted cells on the second day of incubation, while the proteins in the supernatant started to appear on the fourth day. In both cases the amount of protein increased over time. The intensity of the bands also suggested the protein was more abundant in the pelleted cells than in the media (Fig. 4D).

An alcohol dehydrogenase assay using *(R)*-(-) ipsdienol as substrate verified the enzymatic activity of expressed eastern IDOL-DH. The assay measures the amount of NADPH produced in the oxidation of ipsdienol to ipsdienone. The absorbance at 340 nm increased as more NADPH was formed. In the ipsdienol oxidation, the assay showed a positive slope (Fig. 5A) corresponding to the conversion of $NADP^+$ to NADPH. The rate of ipsdienone produced in this reaction is 0.0132ng/sec. In ipsdienone reduction, the NADPH level decreased as expected (Fig. 5B). The rate of ipsdienol produced is 0.00836ng/sec. The oxidation of NADPH reduced ipsidenone to ipsdienol. Sf9 cells

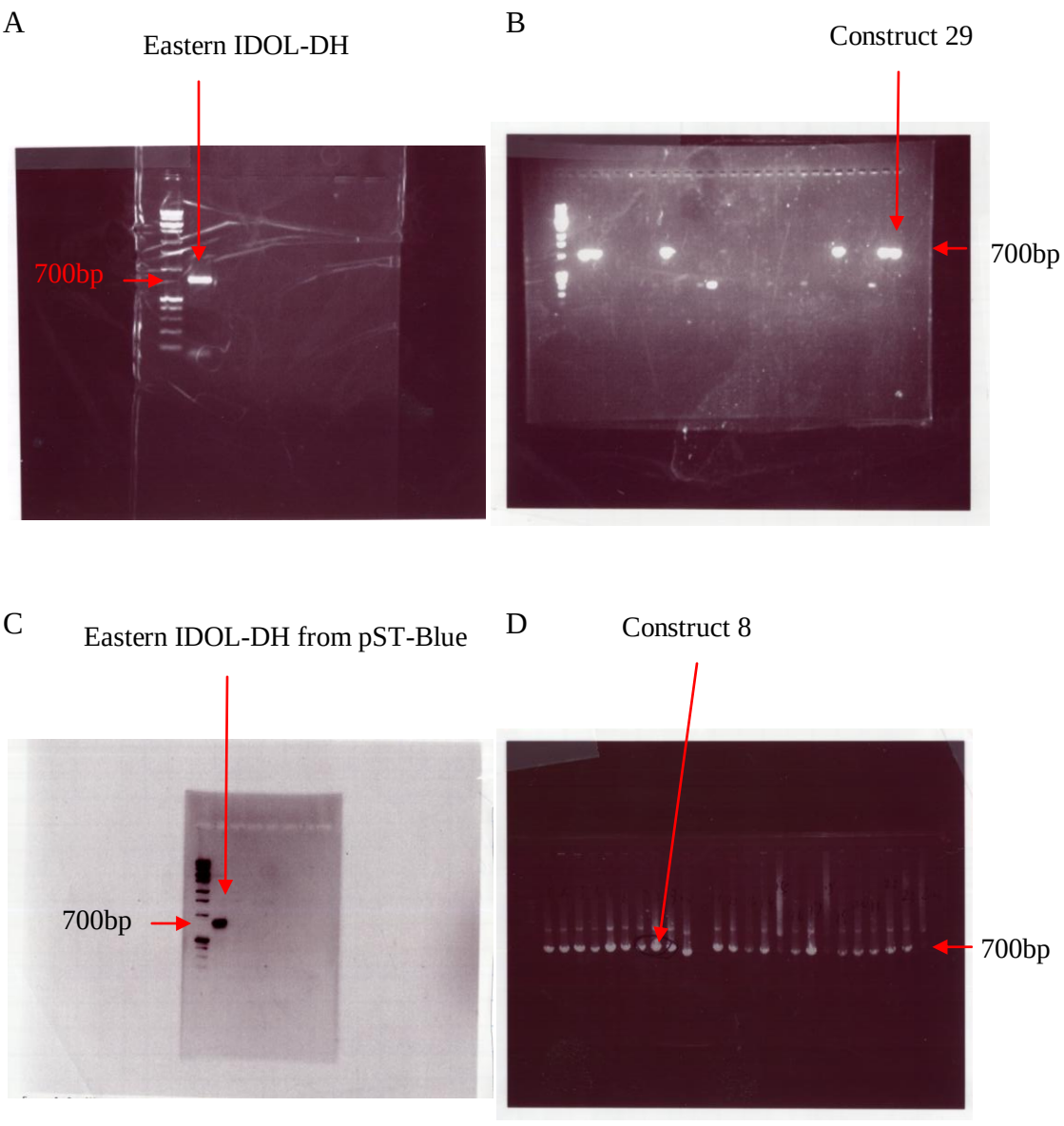

Figure 2. PCR product from eastern *I. pini* cDNA was shown at around 700bp on the gel (A). The colony screen confirmed eastern IDOL-DH gene was cloned into pST-Blue vector in construct 29 (B). PCR product amplified from construct 29 was shown at around 700bp (C). The colony screen confirmed eastern IDOL-DH was cloned into pENTR4 vector in construct 8 (D).

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construct29      ATGATGGTTAAAATCCAGGATTCCGTTTATCTGGTGACTGGTGGTGGATCAGGTCTGGGT 60
East IDOLDH      ATGATGGTTAAAATCCAGGATTCCGTTTATCTGGTGACTGGGGGTGGATCAGGTCTGGGT 60
West IDOLDH      ATGATGGTTAAAATCCAGGATTCCGTTTATCTGGTGACTGGTGGTGGATCAGGTCTGGGT 60
                 ************************************** ******************

construct29      GAAGCCACCGCTAAGCTACTATTGACCGAAGGTGCTCGAGTGACCATTTTCAGTCGAAAC 120
East IDOLDH      GAAGCCACCGCCAAGCTACTATTGACCGAAGGTGCTCGAGTGACCATTTTCAGTCGAAAC 120
West IDOLDH      GAAGCCACCGCCAAGTTATTATTGACCGAAGGTGCTCGAGTGACCATTTTCAGTCGAAAC 120
                 *********** *** ** ****************************************

construct29      GAATACAACAATGAATTTCCTCATGATCGAGTGTTGTCCCTTAGAGGAGATGTGCGTTCG 180
East IDOLDH      GAATACAACAATGAATTTCCTCATGATCGAGTGTTGTCCCTTAGAGGAGATGTGCGTTCG 180
West IDOLDH      GAATACAAGAATGAATTTCCTCATGATCAAGTGTTGTCCGTTAAGGGAGATGTGCGTTCG 180
                 ******** ****************** ********** ***  ***************

construct29      GAAAGTGATGTGAAAAGGGCTTTAGAAGCTACGATCCAGAAGTTTGGAAAGTTGGACGGT 240
East IDOLDH      GAAAGTGATGTGAAAAGGGCTTTAGAAGCTACGATCCAGAAGTTTGGAAAGTTGGACGGT 240
West IDOLDH      GAAAGTGATGTGAAAAGGGCTTTAGAAGCTACGATCCAGAAGTTTGGAAAGTTGGACGGT 240
                 ************************************************************

construct29      GTCATGCATTGTGCTGGCGTTTTTCAAAATGGCGACGAACTTTTCAATATGGACACTCAA 300
East IDOLDH      GTCATGCATTGTGCTGGCGTTTTTCAAAATGGCGACGAACTTTTCAATATGGACACTCAA 300
West IDOLDH      GTCATGCATTGTGCTGGCGTTTTTCAAAATGGCGACGAACTTTTCAATATGGACACTCAA 300
                 ************************************************************

construct29      CAACCTGGCGACTATACAGTTTTAACCGATATTGTTACTACTAACCTCCTAGGGACTTTT 360
East IDOLDH      CAACCTGGCGACTATACAGTTTTAACCGATATTGTTACTACTAACCTCCTAGGGACTTTT 360
West IDOLDH      CAACCTGGCGACTATACAGTTTTAACCGATATTGTTACTACTAACCTCCTAGGGACTTTT 360
                 ************************************************************

construct29      AACGTTAACAGACTGGCTATTCCGTATTTCTTGAACAATCAACCAGACGAAGAGGGACAA 420
East IDOLDH      AACGTTAACAGACTGGCTATTCCGTATTTCTTGAACAATCAACCAGACGAAGAGGGACAA 420
West IDOLDH      AACGTTAACAGACTGGCTATTCCGTATTTTTTGACCAATCAACCGGACGAAGAGGGACAA 420
                 ***************************** **** ********* ***************

construct29      AAAGGGATAATCATCAACTGCTCAAGTACTTCAGGGCACAGCCCTATGTCTTCGGCGGTA 480
East IDOLDH      AAAGGGATAATCATCAATTGCTCAAGTACTTCAGGGCACAGCCCTATGTCTTCGGCGGTA 480
West IDOLDH      AAAGGGATAATCATCAATTGCTCAAGTACTTCAGGGCACAGCCCTATGTCTTCGGCGGTA 480
                 ***************** ******************************************

construct29      GCTTACAGTACCAGCAAAGCTGCTATTATAGGTTTGACTTATGCTTTAGCCAAACAACTT 540
East IDOLDH      GCTTACAGTACCAGCAAAGCTGCCATTATAGGTTTGACTTATGCTTTAGCCAAACAACTT 540
West IDOLDH      GCTTACAGTACCAGCAAAGCTGCTATTATAGGTTTGAGTTATGCTTTAGCCAAACAACTT 540
                 ********************** ************* **********************

construct29      AGTACTCTAGGTATTCGGGTAATGGATATTGCTCCAGCCCTCTGTGATACGCCAATGTTT 600
East IDOLDH      AGTACTCTAGGTATTCGGGTAATGGATATTGCTCCAGCCCTCTGTGATACGCCAATGTTT 600
West IDOLDH      AGTACTCTAGGTATTCGGGTAATGGATATTGCTCCAGCCCTTTGTGATACGCCAATGTTT 600
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construct29      CGTCGTGCAATCGGTTTTAATCAGGACATAGCAAATTTCCGTAATTTGTTCCCATCGAGA 660
East IDOLDH      CGTCGTGCAATCGGTTTTAATCAGGACATAGCAAATTTCCGTAATTTGTTCCCATCGAGA 660
West IDOLDH      CGTCGTGCAGTCGGTTTTAATCAGGACATAGCAAATTTCCGTAATTTGTTCCCAGCGAGA 660
                 ********* ****************************************** *****

construct29      CTGATTCAACCCATTGAATACGCGAACGCAGTCAAACATATCATAGAGACACCCATGTTG 720
East IDOLDH      CTGATTCAACCCATTGAATACGCGAACGCAGTCAAACATATCATAGAGACACCCATGTTG 720
West IDOLDH      CTGATTCAACCCATCGAATACGCGAACGCAGTCAAACATATCATAGAAACACCCATGTTG 720
                 ************** ******************************** ************

construct29      AATGGTTCGTCCTATCAATTAGATGGCGCTCTCAGACCTTAA 762
East IDOLDH      AATGGTTCGTCCTATCAATTAGATGGCGCTCTCAGACCTTAA 762
West IDOLDH      AATGGTTCGTCCTATCAATTAGATGGCGCTCTCAGACCTTAA 762
                 *****************************************
```

Figure 3A. Sequence of eastern IDOL-DH from Jordan Naviaux compared to the sequence of western *I. pini* IDOL-DH and the sequence of the construct.

```
Construct29     MMVKIQDSVYLVTGGGSGLGEATAKLLLTEGARVTIFSRNEYNNEFPHDRVLSLRGDVRS 60
East IDOLDH     MMVKIQDSVYLVTGGGSGLGEATAKLLLTEGARVTIFSRNEYNNEFPHDRVLSLRGDVRS 60
West IDOLDH     MMVKIQDSVYLVTGGGSGLGEATAKLLLTEGARVTIFSRNEYKNEFPHDQVLSVKGDVRS 60
                ****************************************:******:***::*****

Construct29     ESDVKRALEATIQKFGKLDGVMHCAGVFQNGDELFNMDTQQPGDYTVLTDIVTTNLLGTF 120
East IDOLDH     ESDVKRALEATIQKFGKLDGVMHCAGVFQNGDELFNMDTQQPGDYTVLTDIVTTNLLGTF 120
West IDOLDH     ESDVKRALEATIQKFGKLDGVMHCAGVFQNGDELFNMDTQQPGDYTVLTDIVTTNLLGTF 120
                ************************************************************

Construct29     NVNRLAIPYFLNNQPDEEGQKGIIINCSSTSGHSPMSSAVAYSTSKAAIIGLTYALAKQL 180
East IDOLDH     NVNRLAIPYFLNNQPDEEGQKGIIINCSSTSGHSPMSSAVAYSTSKAAIIGLTYALAKQL 180
West IDOLDH     NVNRLAIPYFLTNQPDEEGQKGIIINCSSTSGHSPMSSAVAYSTSKAAIIGLSYALAKQL 180
                ***********.****************************************:*******

Construct29     STLGIRVMDIAPALCDTPMFRRAIGFNQDIANFRNLFPSRLIQPIEYANAVKHIIETPML 240
East IDOLDH     STLGIRVMDIAPALCDTPMFRRAIGFNQDIANFRNLFPSRLIQPIEYANAVKHIIETPML 240
West IDOLDH     STLGIRVMDIAPALCDTPMFRRAVGFNQDIANFRNLFPARLIQPIEYANAVKHIIETPML 240
                ***********************:**************:********************

Construct29     NGSSYQLDGALRP- 253
East IDOLDH     NGSSYQLDGALRP- 253
West IDOLDH     NGSSYQLDGALRP- 253
                *************
```

Figure 3B. Amino acid sequence of eastern IDOL-DH from Jordan Naviaux compared to the sequence of western *I. pini* IDOL-DH and the sequence of the construct.

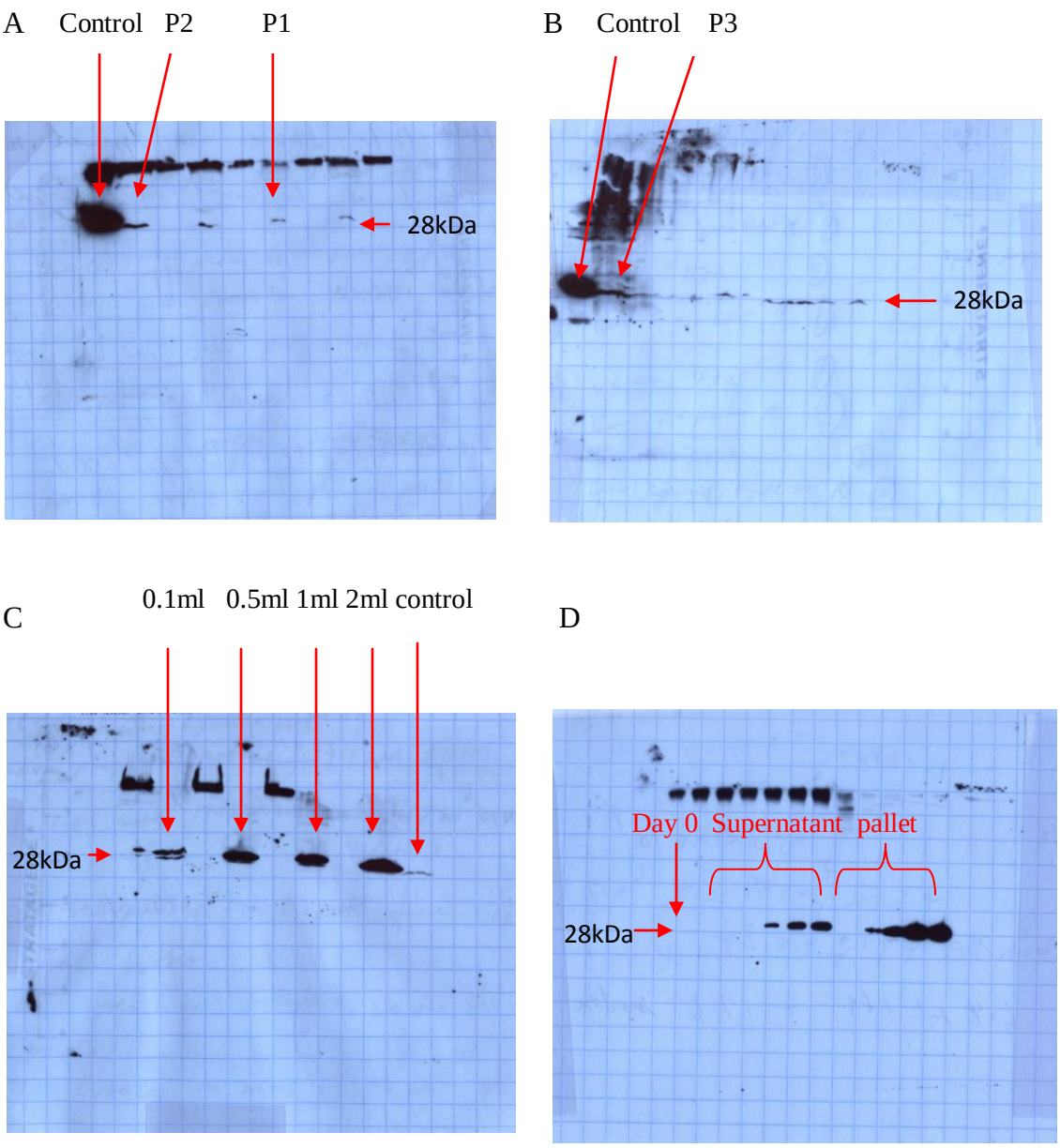
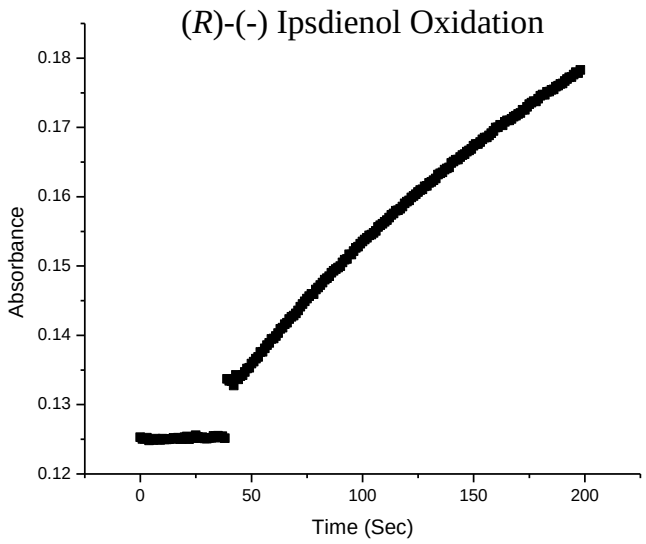


Figure 4. Western blot analysis was used to detect eastern IDOL-DH protein in P1, P2, and P3 viral stock (A, B), protein production with four different amounts of P3 viral stock (C), and the time course study for the proteins in supernatant and pellet (D). The molecular weight of the protein is approximately 28kDa.

were used as a negative control to ensure the assay was not affected by endogenous proteins. In the forward reaction (Fig. 5C), the slope remained constant after the addition of the enzyme at around 50 seconds. The gap was due to the addition of the enzyme. For the reverse reaction, the initial slope appeared to be negative (Fig. 5D). Because the slope remained the same after adding the enzyme at about 50 seconds, the NADPH level in Sf9 cells was determined to be unchanged. The control experiment verified the alcohol dehydrogenase activity was from the enzyme, and not by endogenous proteins.

GC-MS analyses were used to verify the products from all reactions. Eastern IDOL-DH carried out all 5 reactions. The amount of products was estimated based on comparison to an added *n*-octanol standard. Both *(S)*-(+) and *(R)*-(-) ipsdienol were oxidized to ipsdienone (Fig.7A-D). The amount of ipsdienone produced from *(R)*-(-) ipsdienol was about 348ng, which is about 3.4% of the amount of substrate added. The amount of ipsdienone produced from *(S)*-(+) ipsdienol was much smaller, only about 0.3 percent of substrate recovered. The reduction from ipsdienone to ipsdienol had about 2% recovery (Fig. 7E, F). With the chiral column, we distinguished (*R*)-(-) and (*S*)-(+) ipsdienol by GC-MS analysis. The (*R*)-(-) isomer eluted earlier than the (*S*)-(-) isomer (Fig. 7G). The chiral column demonstrated that the reaction produced only *(R)*-(-) ipsdienol (Fig. 7H). The reduction of ipsenone (Fig. 7I, J) resulted in relatively large amounts of product, which had about 5.8% recovery. The amount of ipsenone produced by the oxidation of ipsenol had about 2.6% recovery (Fig. 7K, L).

A



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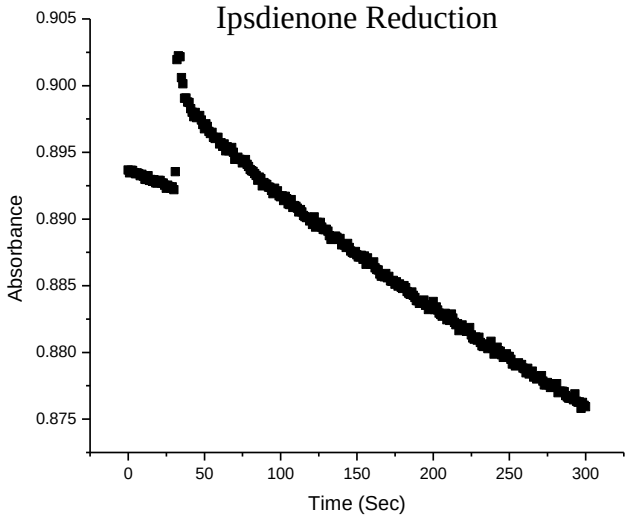
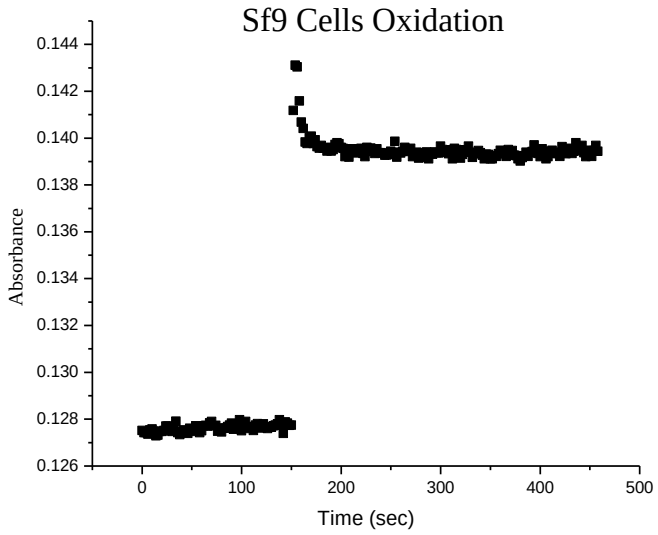


Figure. 5A, B. Alcohol dehydrogenase assay for the oxidation of (-) ipsdienol (A), and the reduction of ipsdienone (B) with eastern IDOL-DH enzyme. The absorbance represents the formation of NADPH in the reaction. The gap indicates the time when the enzymes were added.

C



D

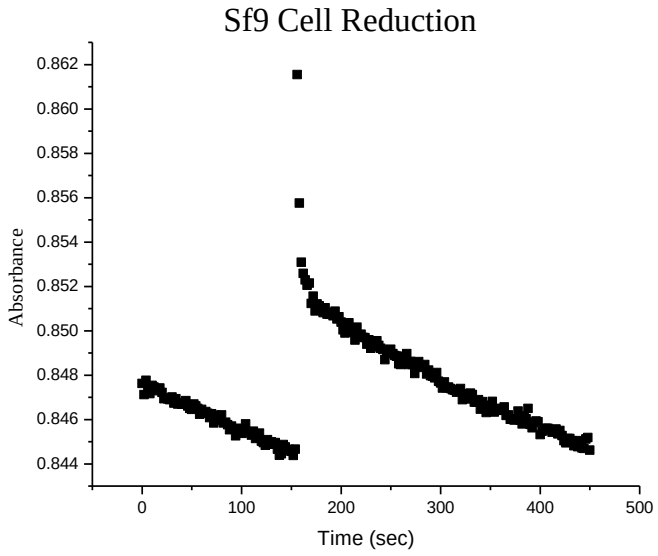


Figure 5C, D. Alcohol dehydrogenase assay for the oxidation of (-) ipsdienol (C), and the reduction of ipsdienone (D) with Sf9 cell lysate. The absorbance represents the formation of NADPH in the reaction. The gap is due to the addition of the cell lysate.

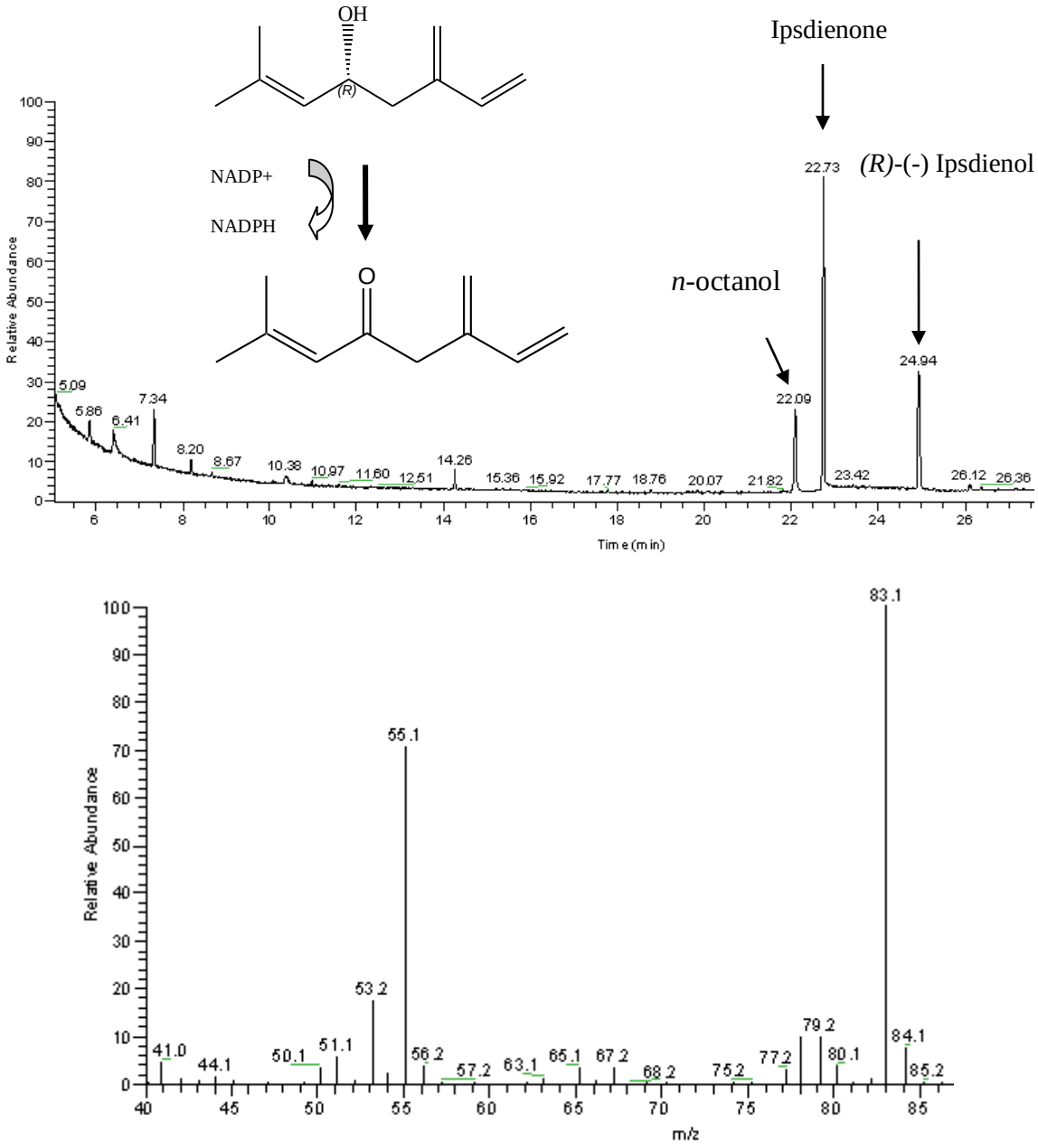


Figure. 6A. GC-MS analysis of ipsdienone produced from *(R)*-(-) ipsdienol oxidation by eastern IDOL-DH. *(R)*- (-) ipsdienol eluted at 24.94 min in the chromatogram. Ipsdienone and *n*-octanol eluted at 22.73 min, and 22.09 min. The mass spectrum was compared to a standard, and used to identify ipsdienone.

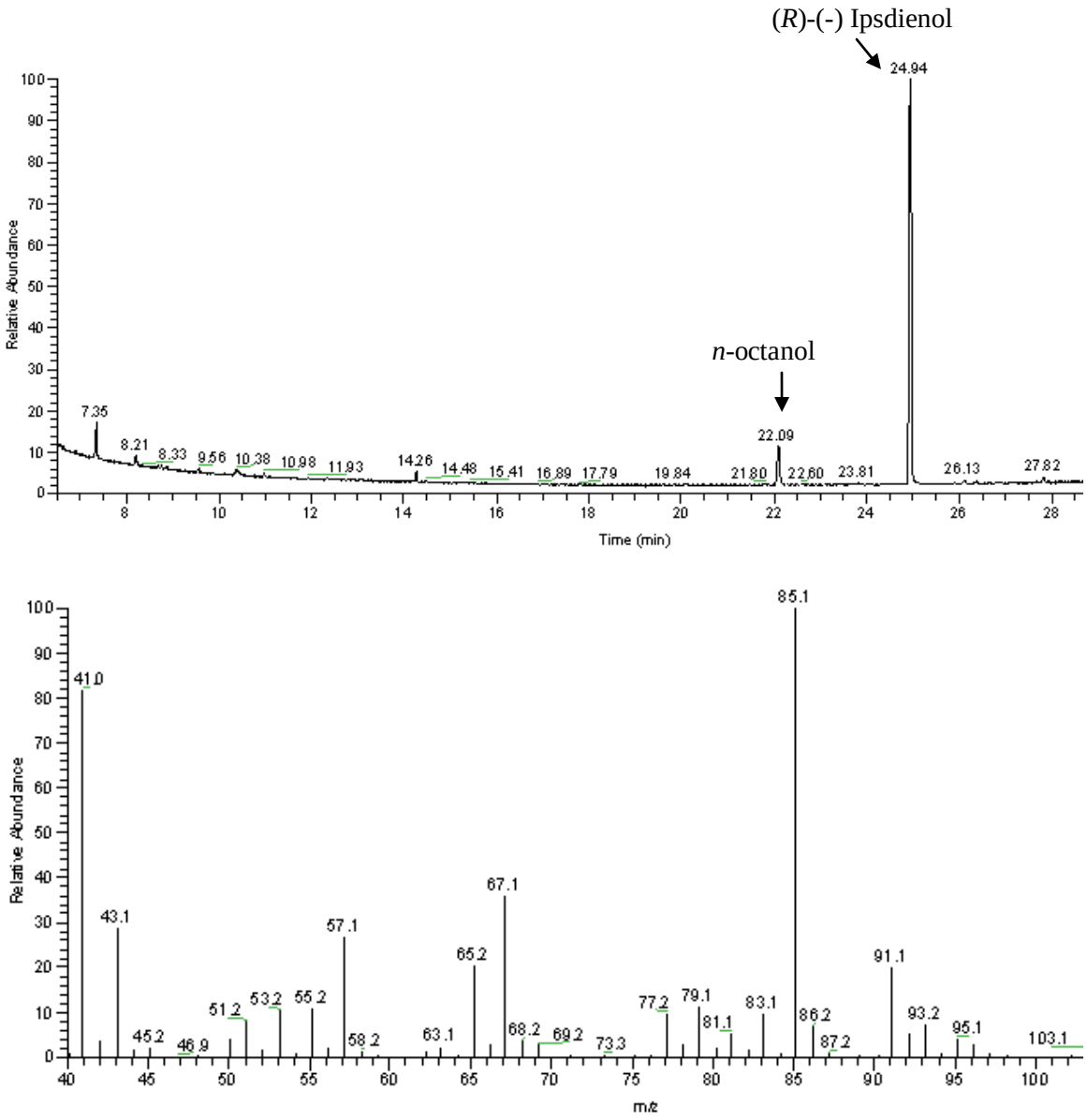


Figure. 6B. GC-MS analysis for the negative control of *(R)*-(-) ipsdienol oxidation. The control used Sf9 cell lysate instead of eastern IDOL-DH. The chromatogram showed ipsdienone was not produced in this reaction. The mass spectrum was compared to a standard, and used to identify ipsdienol.

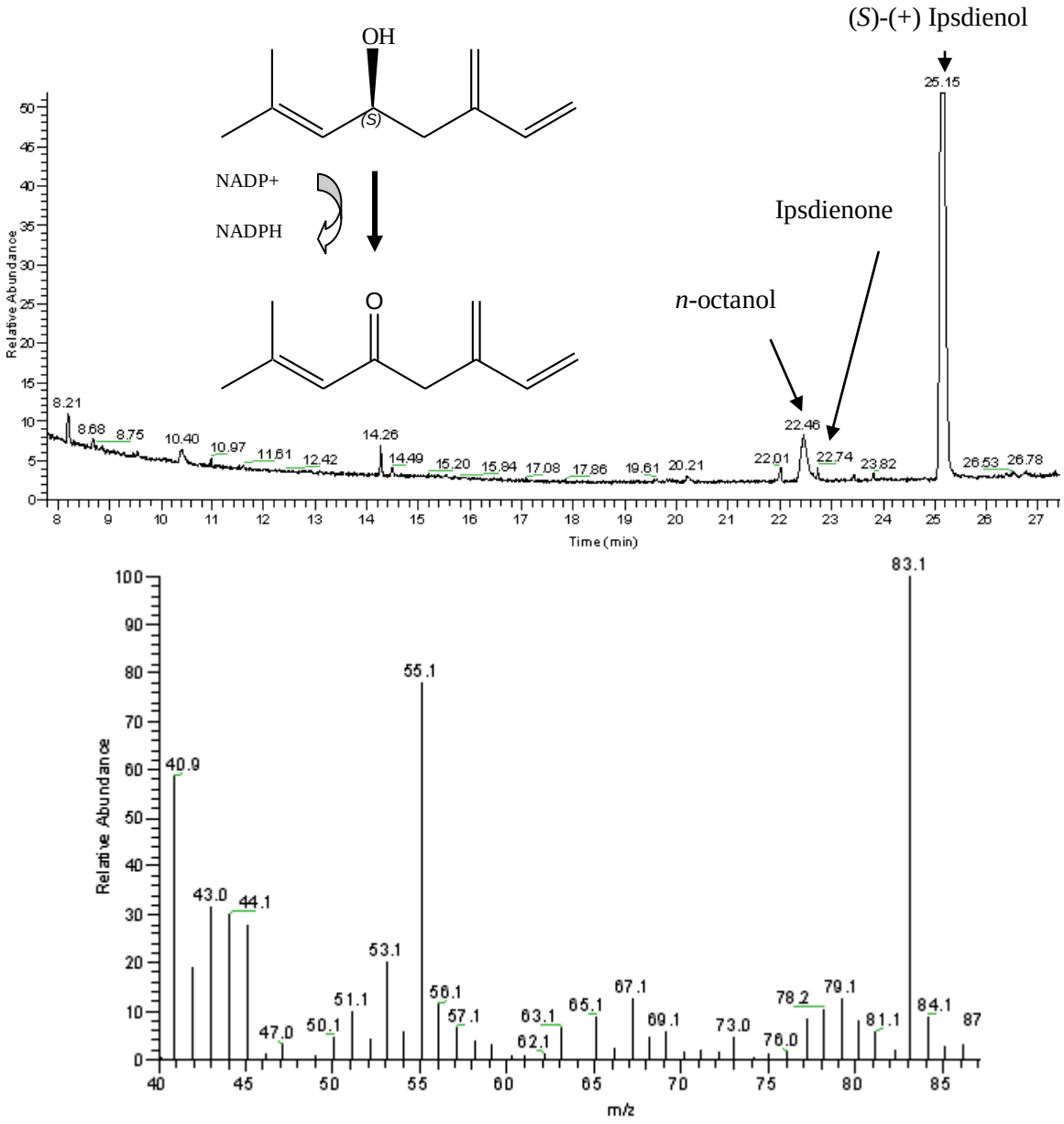


Figure 6C. GC-MS analysis of ipsdienone produced from *(S)*-(+) ipsdienol oxidation by eastern IDOL-DH. *(S)*-(+) ipsdienol eluted at 25.15 min in the chromatogram. Ipsdienone and *n*-octanol eluted at 22.74 min, and 22.46 min. The mass spectrum was compared to the standard, and used to identify ipsdienone.

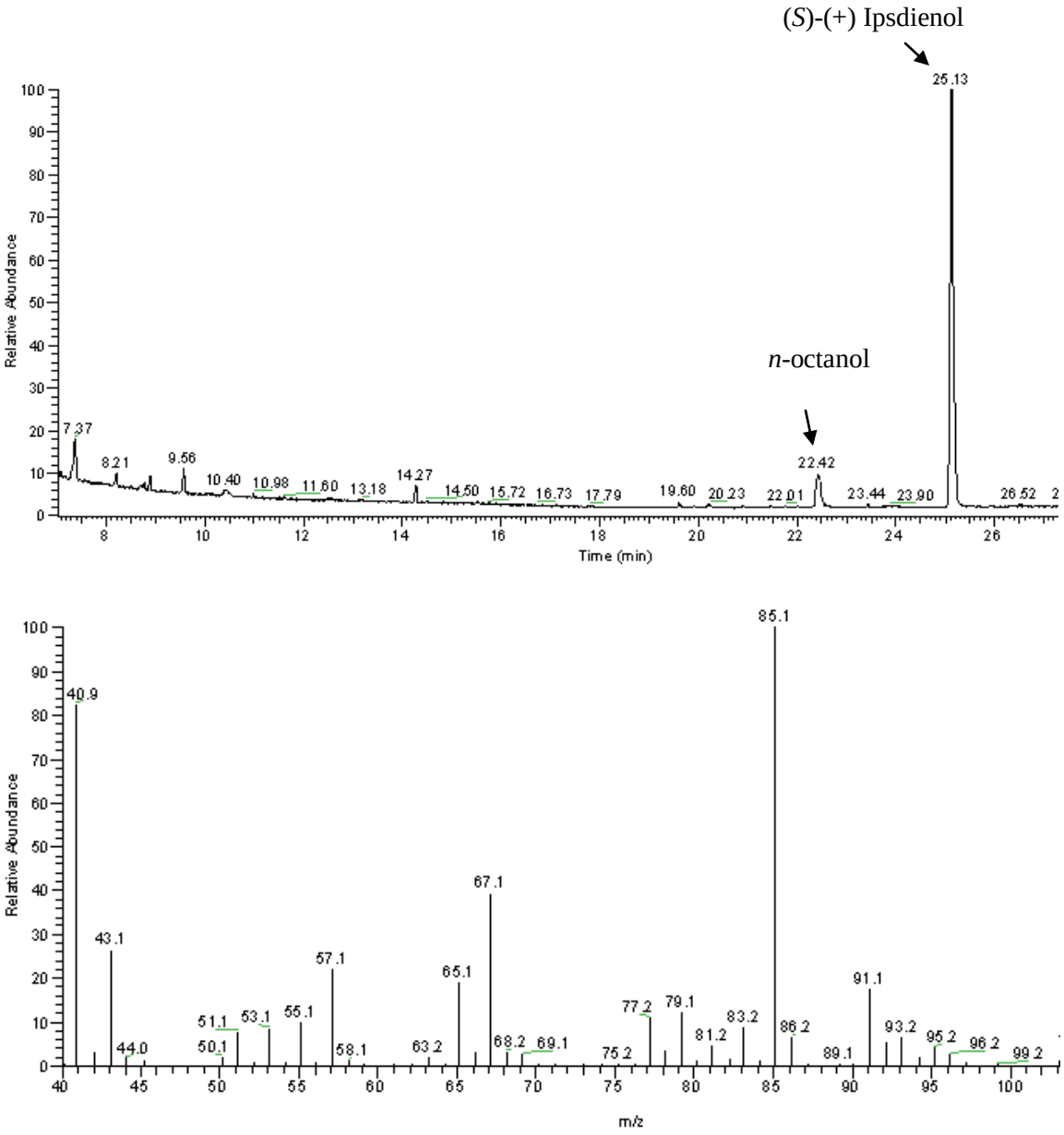


Figure 6D. GC-MS analysis for the negative control of (*S*)-(+) ipsdienol oxidation. The control used Sf9 cell lysate instead of eastern IDOL-DH. The chromatogram showed ipsdienone was not produced in this reaction. The mass spectrum was compared to a standard, and used to identify ipsdienol.

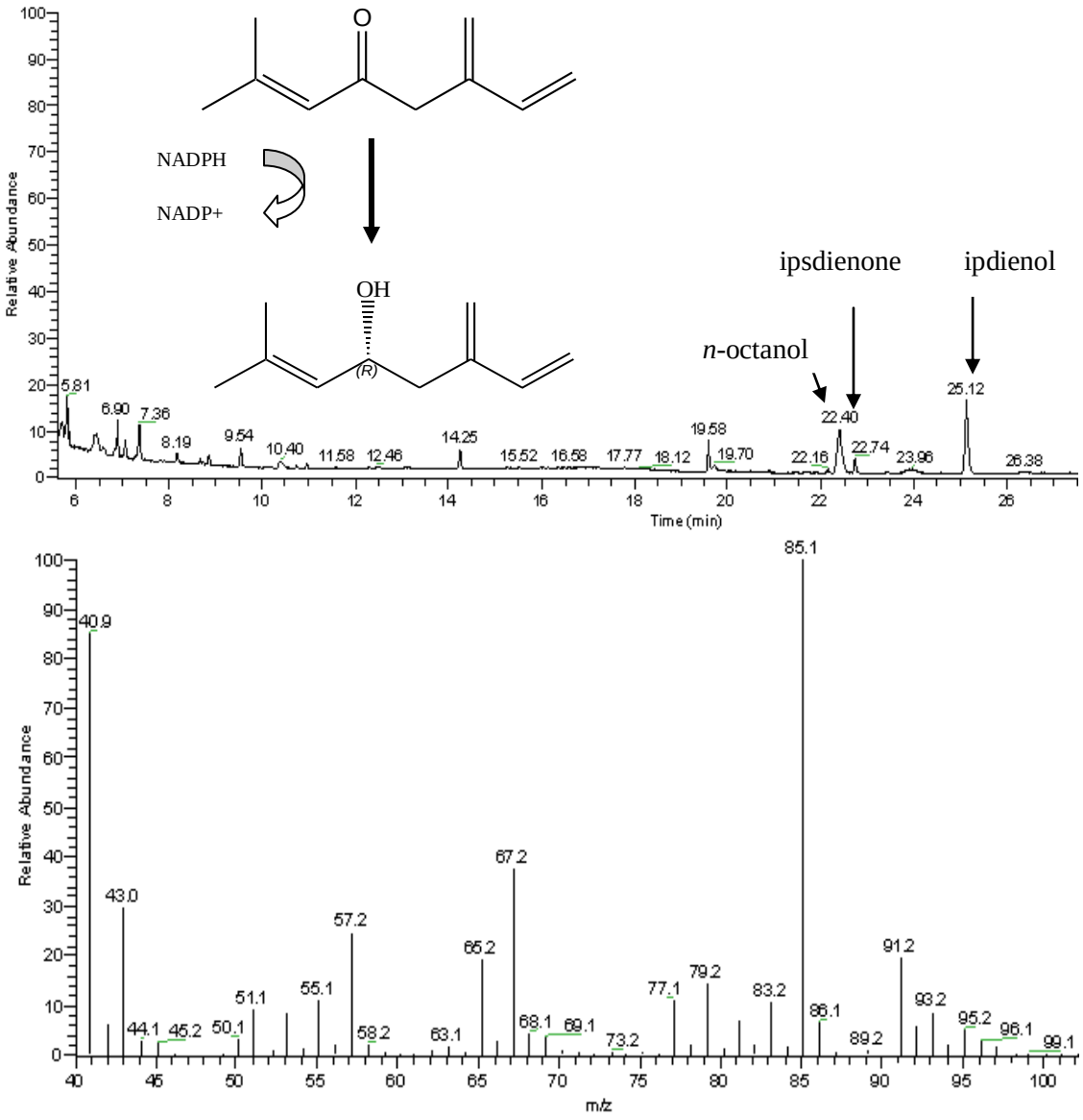


Figure 6E. GC-MS analysis of ipsdienol produced from ipsdienone reduction by eastern IDOL-DH. Almost all of the ipsdienone was converted to ipsdienol. Ipsdienone eluted at 22.74 min in the chromatogram. Ipsdienol and *n*-octanol eluted at 26.38 min, and 22.40 min. The mass spectrum was compared to the standard, and used to identify ipsdienol.

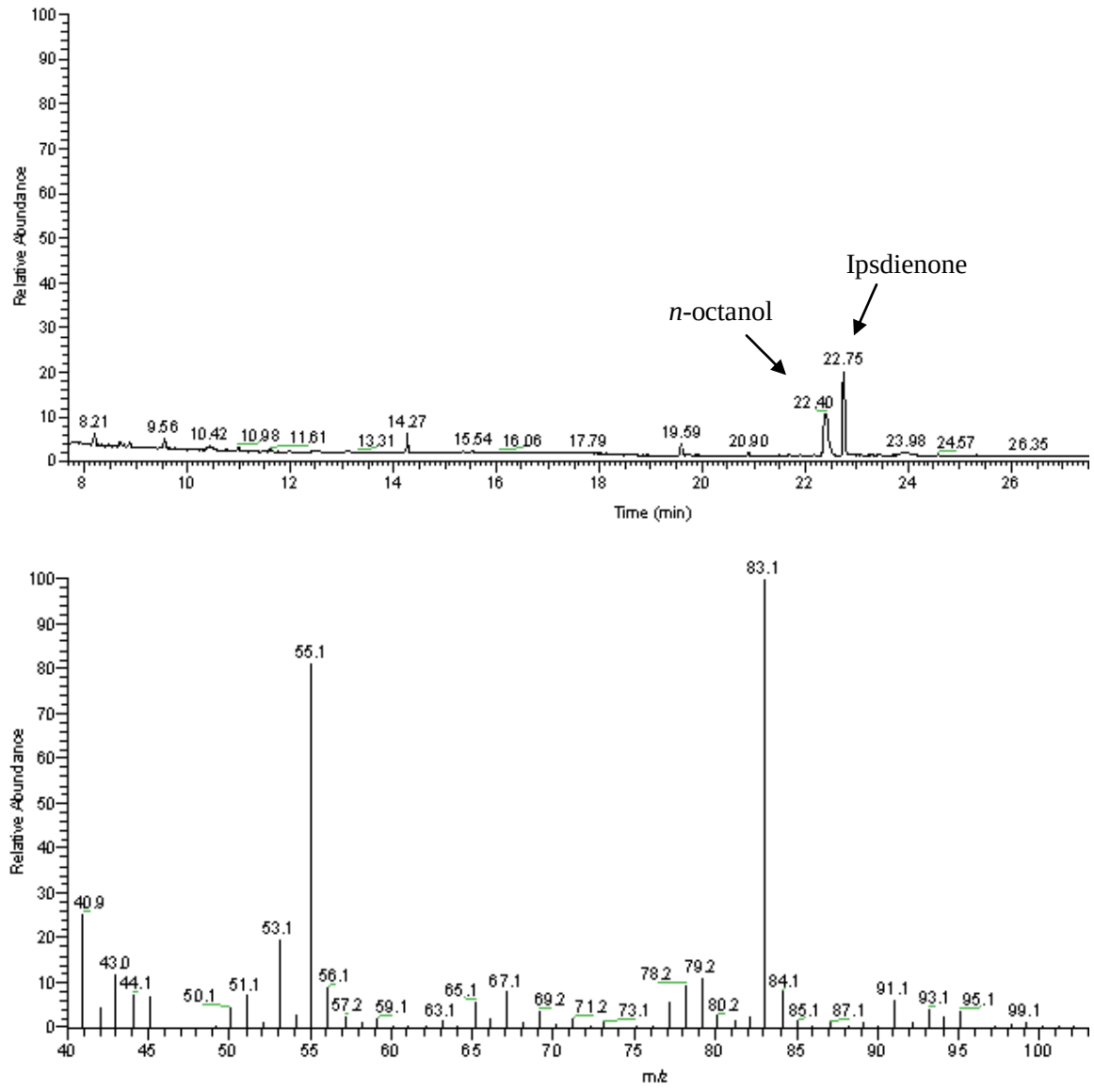


Figure 6F. GC-MS analysis for the negative control of ipsdienone reduction. The control used Sf9 cell lysate instead of eastern IDOL-DH. The chromatogram showed ipsdienol was not produced in this reaction. The mass spectrum was compared to a standard, and used to identify ipsdienone.

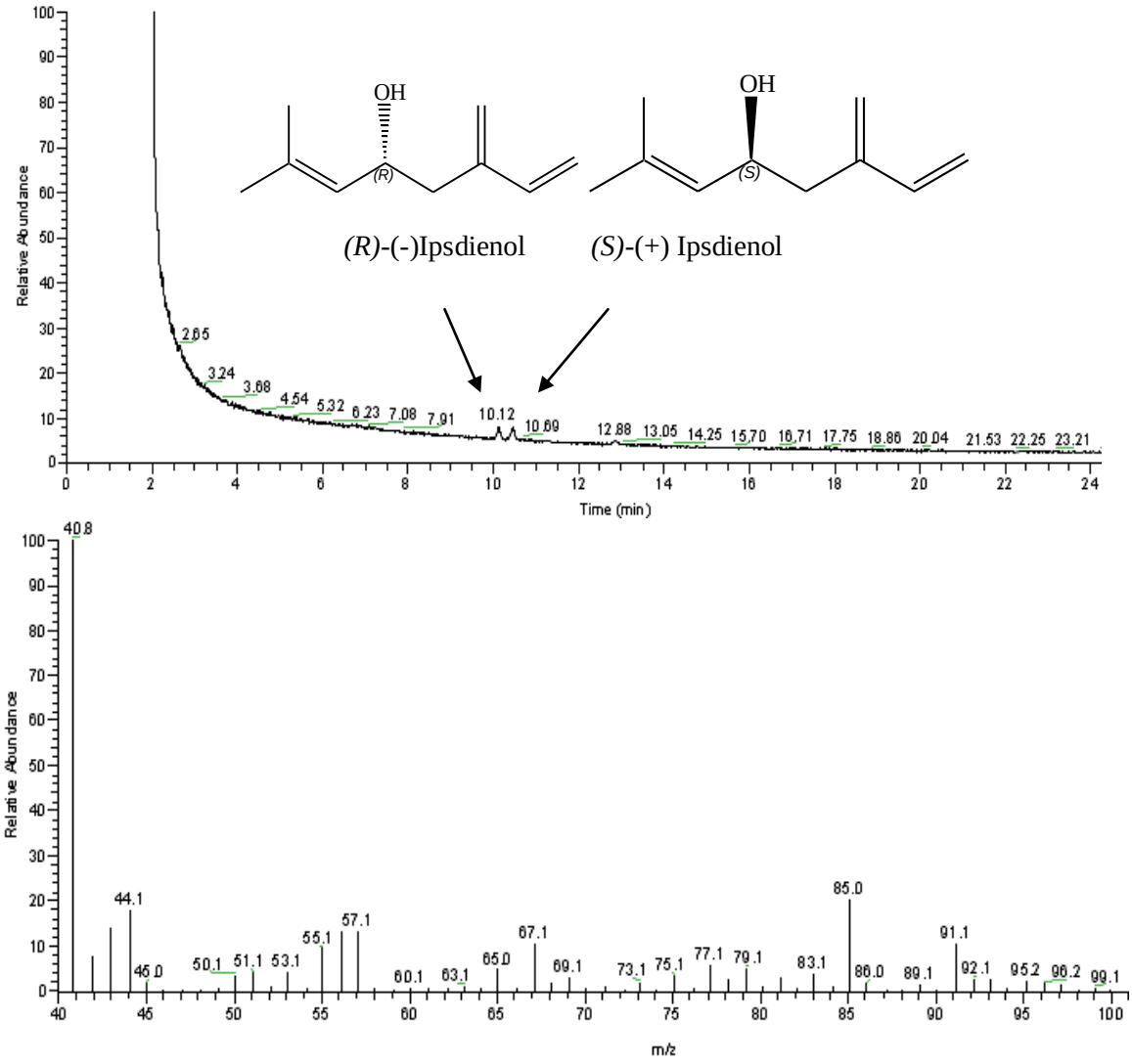


Figure 6G. Racemic ipsdienol standards analyzed by GC-MS with chiral column. *(R)*-(-) ipsdienol eluted at 10.12 min., and *(S)*-(+) ipsdienol eluted later at 10.44 min. in the chromatogram. This GC-MS result was used as the standard to identify ipsdienol stereoisomers.

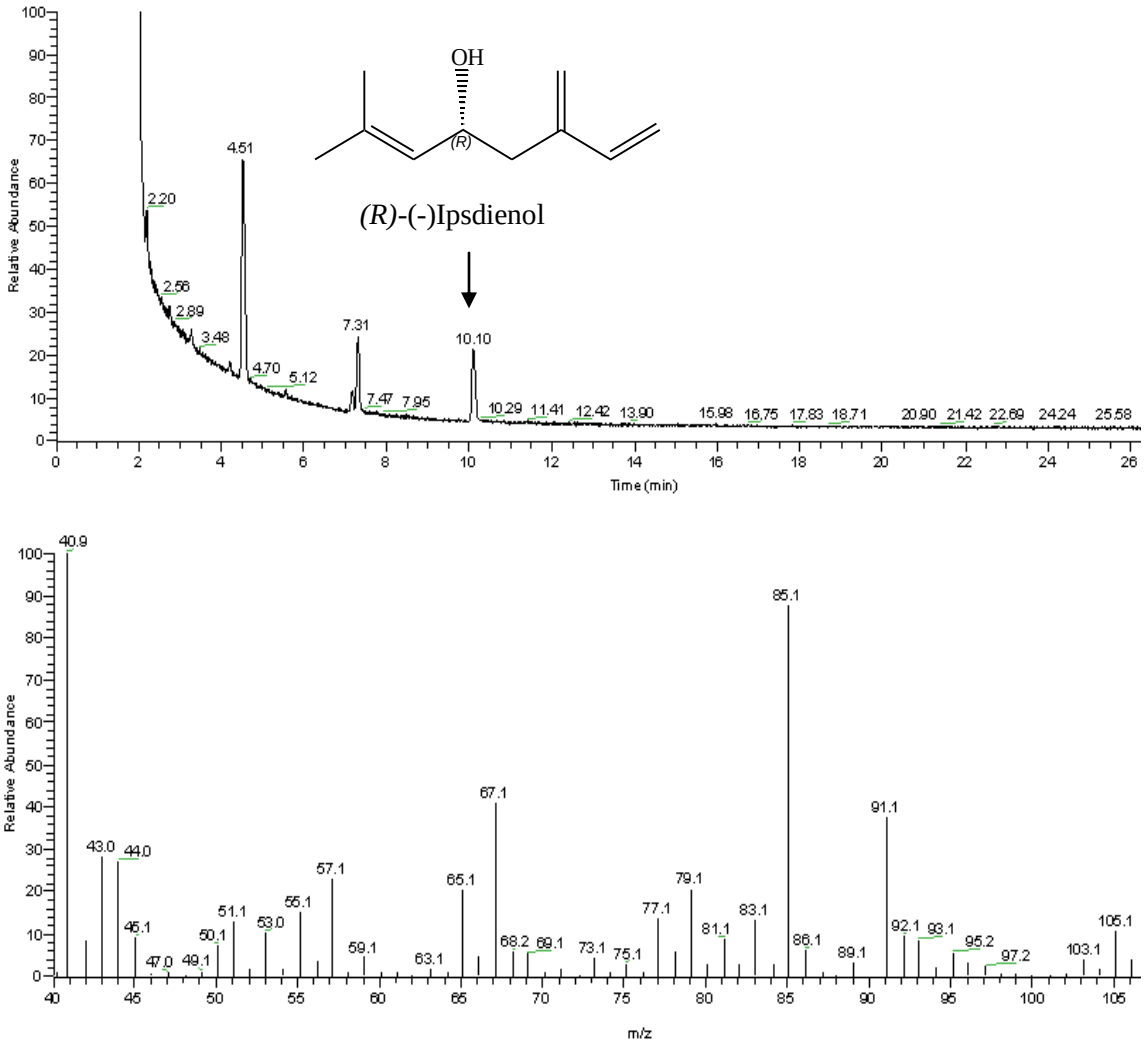


Figure 6H. Chiral column GC-MS analysis for *(R)*-(-) ipsdienol produced from ipsdienone reduction. *(R)*-(-) ipsdienol eluted at 10.10 min. in the chromatogram, and the mass spectrum was used to verify the identity of the compound.

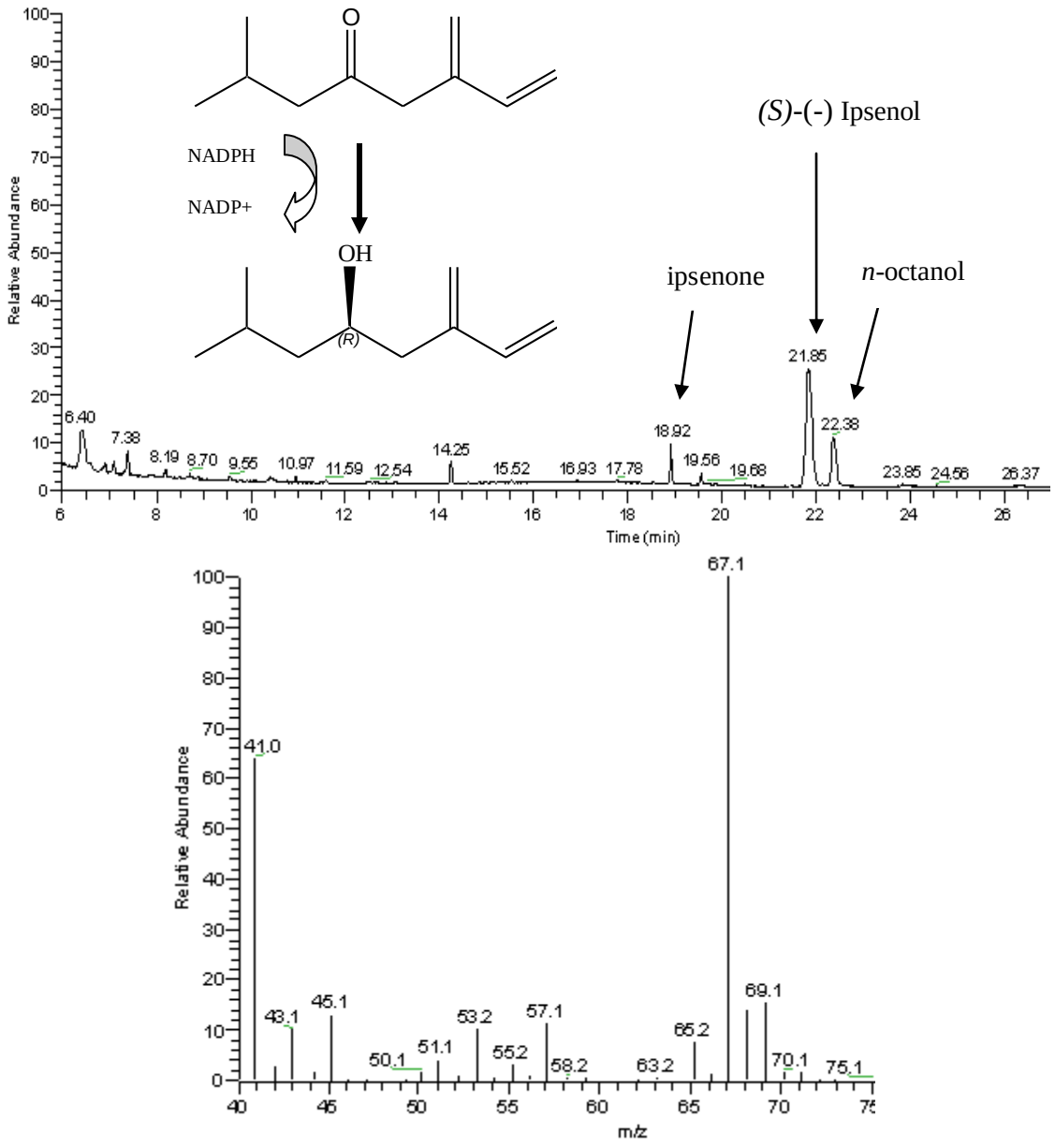


Figure 6I. GC-MS analysis for *(S)*-(-) ipsenol formed from ipsenone reduction by eastern IDOL-DH. Ipsenone was eluted at 18.92 min in the chromatogram. *(S)*-(-) ipsenol and *n*-octanol were eluted at 21.85 min, and at 22.38 min. The mass spectrum was compared to the standard, and used to identify *(S)*-(-) ipsenol.

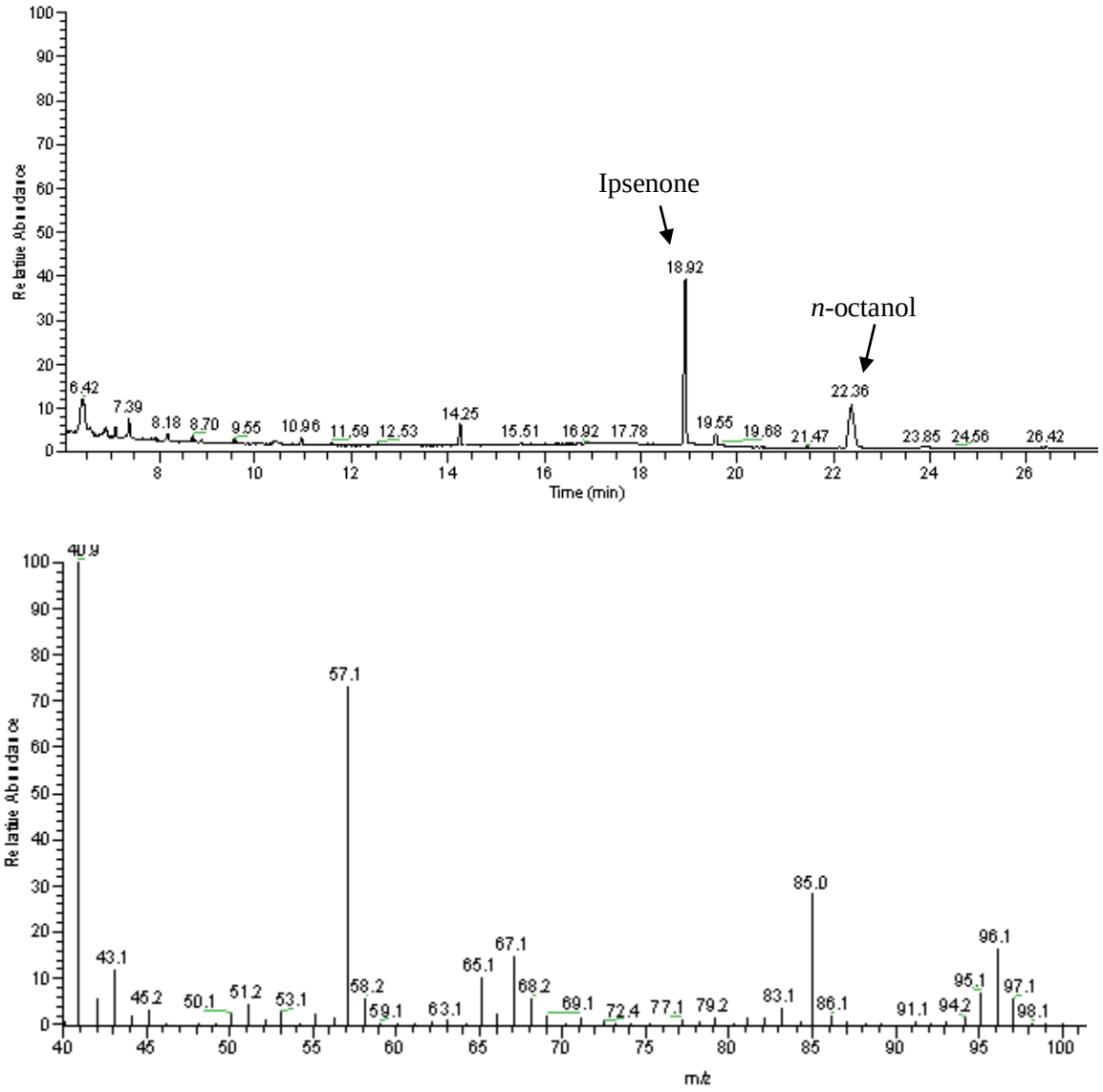


Figure 6J. GC-MS analysis for the negative control of ipsdenone reduction. The control used Sf9 cell lysate instead of eastern IDOL-DH. The chromatogram showed ipsenol was not produced in this reaction. The mass spectrum was compared to a standard, and used to identify ipsenone.

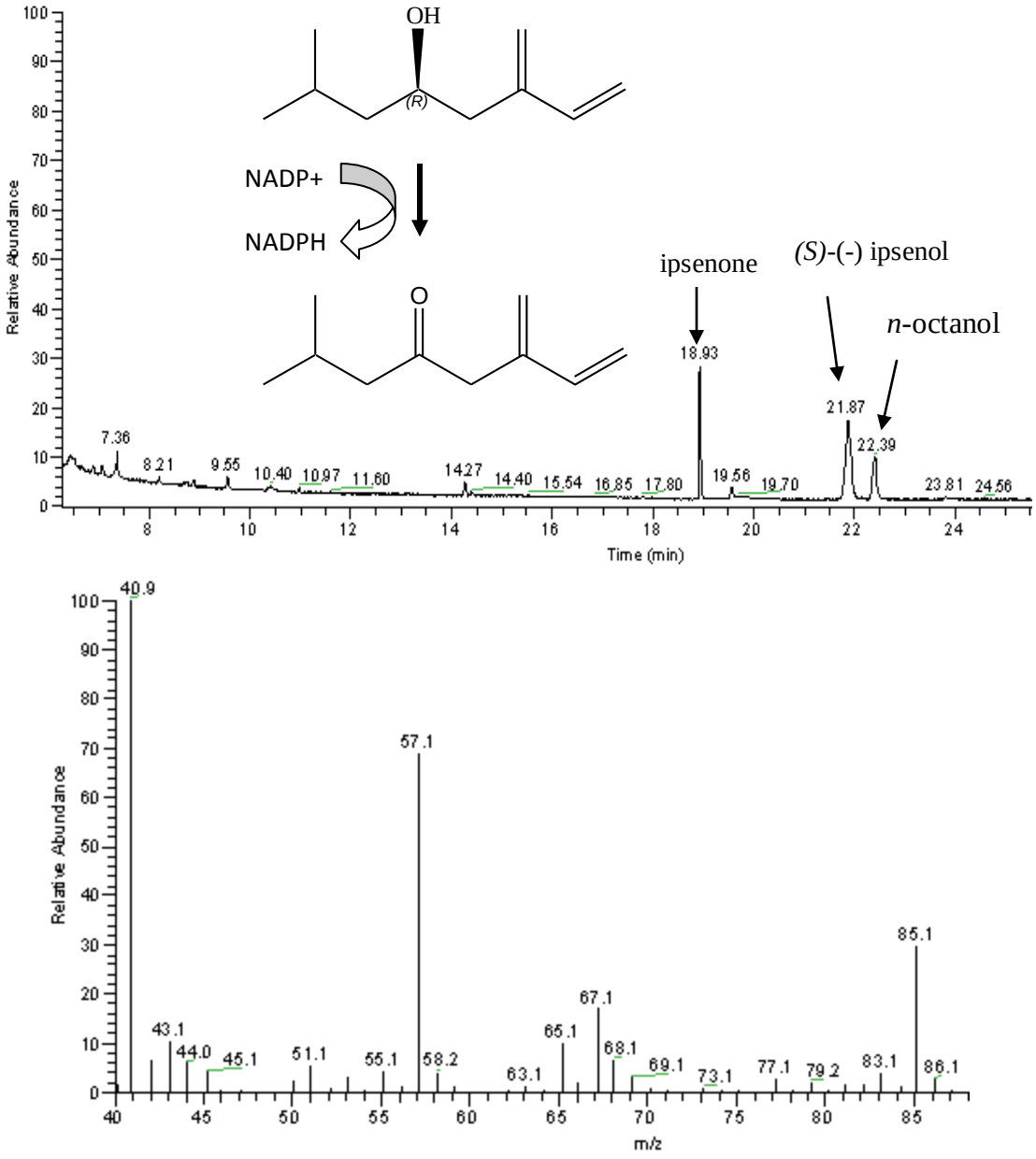


Figure 6K. GC-MS analysis for the ipsenone formed from *(S)*-(-) ipsenol oxidation by Eastern IDOL-DH. *(S)*-(-) ipsenol was eluted at 21.87 min in the chromatogram. Ipsenone and *n*-octanol were eluted at 18.93 min, and 22.39 min. The mass spectrum was compared to the standard, and used to identify ipsenone.

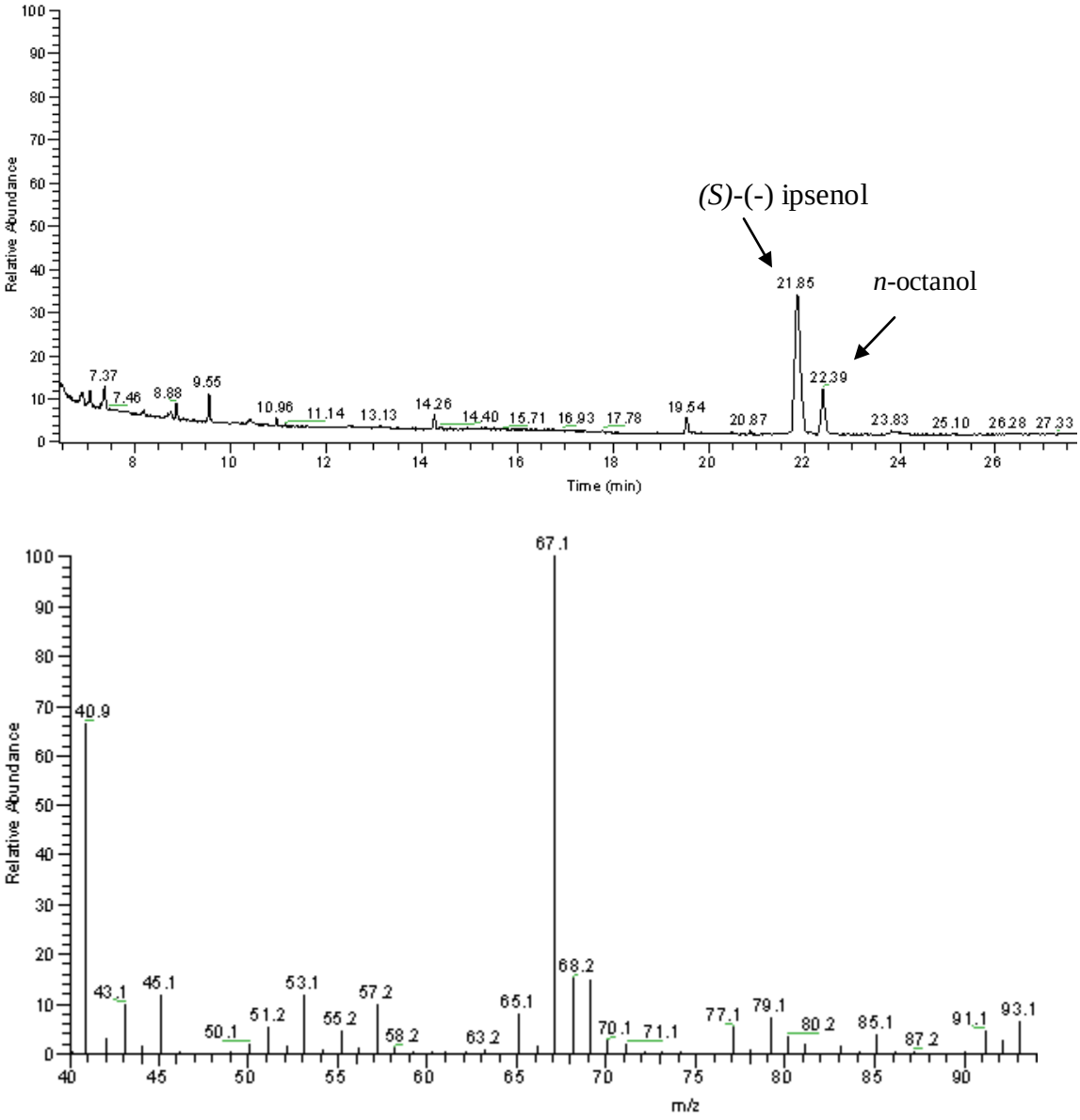


Figure 6L. GC-MS analysis for the negative control of *(S)*-(-) ipsenol oxidation. The control used Sf9 cell lysate instead of eastern IDOL-DH. The chromatogram showed ipsenone was not produced in this reaction. The mass spectrum was compared to a standard, and used to identify ipsenol.

Discussion

Members of *Ips* spp. have different pheromone production patterns. *Ips paraconfusus* produces about 87% *(S)*-(+) ipsdienol (Fish et al., 1984), and *I. duplicatus* produces about an equal ratio of (+) and (-) enantiomers (Byers et al., 1990b). For western *I. pini*, a close relative to eastern *I. pini*, myrcene is converted to ipsdienol by the cytochrome P450, CYP9T2. The ratio between the *(R)*-(-) and *(S)*-(+) ipsdienol is approximately 80:20 (Sandstrom et al., 2006). IDOL-DH from western *I. pini* oxidizes *(R)*-(-) ipsdienol to ipsdienone and also converts ipsdienone back to *(R)*-(-) ipsdienol, and may be involved in the process in achieving the final 95:5 *(R)*-(-):*(S)*-(+) ratio. For *I. confusus*, *(R)*-(-) and *(S)*-(+) ipsdienol are made in an 86:14 ratio by the cytochrome P450 CYP9T1 (Sandstrom et al., 2008), which is similar to the ratio produced by CYP9T2 in western *I. pini*. However, the final ipsdienol ratio in the pheromone *I. confusus* is 5:95 (-/+) (Young et al., 1973). This suggests that cytochromes P450 do not control the enantiomeric composition.

Our hypothesis was that the most likely way to achieve the *(R)*-(-):*(S)*-(+) ratio of 35:65 was for eastern IDOL-DH to function as an oxidoreductase, but produce more of the *(S)*-(+) stereoisomers. We expected eastern IDOL-DH would oxidize mostly *(R)*-(-) ipsdienol to ipsdienone, and converted it back to *(S)*-(+) ipsdienol to make the final 35:65 ratio between the *(R)*-(-) and *(S)*-(+) isomers. The result of this study indicated the pheromone production pattern of eastern *I. pini* is quite similar to western *I. pini*. Eastern IDOL-DH oxidized the *(R)*-(-) ipsdienol to ipsdienone, and did not oxidize *(S)*-(+) ipsdienol. Data using a chiral column showed the reduction of ipsdienone by eastern IDOL-DH produced almost exclusively the *(R)*-(-) ipsdienol. This data suggested

IDOL-DH alcohol dehydrogenase cannot be the final step to make the 35:65 (-/+) pheromone blend in eastern *I. pini*.

An alternative explanation for eastern *I. pini* pheromone production is that the ratio of ipsdienol isomers is determined by the cytochrome P450. Unlike western *I. pini*, which produces an approximate 80:20 (-/+) blend through P450, it is possible that the eastern *I. pini* produces a higher ratio of *(S)*-(+) ipsdienol. In other words, the composition of the pheromone blend may be decided by the stereochemistry of the hydroxylation of myrcene by the eastern CYP9T2. This speculation would also explain eastern IDOL-DH's poor catalytic activity in the oxidation with *(S)*-(+) ipsdienol. If the pheromone blend is regulated by the hydroxylation step, then eastern IDOL-DH would play a much lesser role in determining the final enatiomeric composition. Higher catalytic activity with the *(R)*-(-) isomer could be the mechanism to prevent *(S)*-(+) ispdienol from being consumed by eastern IDOL-DH to make Ipsdienone.

Ipsdienone can be converted to ipsenone by a carbon-carbon double bond reductase, and further oxidized to *(S)*-(-) ipsenol (Fig. 1). *(S)*-(-) Ipsenol is the anti-aggregation pheromone that suppresses the attraction of beetles to colonize trees (Miller et al., 2005). GC-MS showed eastern IDOL-DH was able to carry out the reduction of ipsenone to *(S)*-(-) ipsenol. In fact, it produced a large amount of *(S)*-(-) ipsenol. Based on our results, eastern IDOL-DH may not be as involved in the pheromone production as we originally thought, but it could play an important role in the production of the anti-aggregation pheromone, ipsenol.

In order to obtain more information on the pheromone production in eastern *I. pini*, two future studies could provide better understanding. The first study would be to

homogenize eastern *I. pini* midgut tissue and assay CYP9T2. Using GC-MS on a chrial column, we could determine the ratio of ipsdienol stereoisomers. The result of this study would confirm or deny the role of P450 in achieving the natural occurring stereochemistry in eastern *I. pini*. The second study that could answer the question as to how the stereochemistry in the east *I. pini* is achieved would be the expression and assay of the CYP9T2, to see if the final ipsdienol stereoisomeric ratio is determined in this step. This study would also allow us to find out if P450 is the key step of pheromone production that controls stereochemistry.

The formation of ipsdienol could be carried out by an as yet unknown oxioreductase instead of eastern IDOL-DH. It could change the stereochemical ratio in a different manner than eastern IDOL-DH to achieve the natural occurring ratio. In the *I. pini* microarray (Keeling et al., 2004, 2006, Eigenheer et al., 2003), there are other oxidoreductases that could be characterized and we might be able to identify another oxioreductase gene that is upregulated by JH treatment.

Domingue and Teale (2008) suggested epistatic interaction between the genes that control the production of (*R*)-(-) and *(S)*-(+) ipsdienol. The most likely gene to be involved in the regulation of the (-) to (+) ratio, IDOL-DH, clearly does not regulate the stereochemical ratio in eastern *I. pini*, and we are not sure of its role in western *I. pini*. Future work is needed to characterize the eastern CYP9T2 to determine if it plays a major role in achieving the final stereochemistry of ipsdienol, or if other genes, likely oxidoreductases, are involved.

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