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# Type II Estrogen Binding-Site Agonist: The Synthesis and Biological Evaluation of the Enantiomers of Methyl-Para-Hydroxyphenyllactate (MeHPLA)

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# TYPE II ESTROGEN BINDING SITE AGONIST: THE SYNTHESIS AND BIOLOGICAL EVALUATION OF THE ENANTIOMERS OF METHYL-PARA-HYDROXYPHENYLLACTATE (MEHPLA)

by

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# ABSTRACT

## TYPE II ESTROGEN BINDING SITE AGONIST: THE SYNTHESIS AND BIOLOGICAL EVALUATION OF THE ENANTIOMERS OF METHYL-PARA-HYDROXYPHENYLLACTATE (MEHPLA)

Lester G. Pretlow
Old Dominion University, 1997
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The function of the type II estrogen binding site (EBS) has yet to be determined. However, a high affinity ligand for the binding site has been identified through HPLC and GC-MS. This ligand, MeHPLA, bears a structural relationship to a group of compounds called "phytoestrogens" which, along with MeHPLA, have been observed to suppress the cellular proliferation of estrogen sensitive MCF-7 breast cancer cells *in vitro*. Additionally, MeHPLA has been observed to suppress the growth of rat uteri *in vivo*. The high affinity of MeHPLA for the type II EBS suggests that this interaction is responsible for the observed suppression of cell growth. If this interaction is the mode of cell growth suppression, then the chiral center of MeHPLA might be expected to change the binding affinity of the ligand and the associated cellular activity. In this study, the enantiomers of MeHPLA were synthesized and separated by three methods. The methods included the use of the enantioselective catalyst, oxazoborolidine, the enzyme, lactate dehydrogenase, and the diastereomeric separation using a chiral amine. When the methods were compared, it was found that the method using the diastereomeric separation gave the superior yield for the two enantiomers. Binding studies for the

enantiomers to the type II EBS showed that the L-MeHPLA isomer has a higher affinity for the binding site. However, binding affinity did not translate into cell growth suppression. Both enantiomers had the equivalent ability to suppress cellular growth. The conclusion is that the interaction of MeHPLA with the type II binding site may not be the mode by which cell growth suppression is achieved. This was supported by the evaluation of MeHPLA against LnCap prostate cancer cells and HxGC3 colon cancer cells in culture. Type II binding sites have been observed in prostate and colon cancer cell lines. However, their existance in these particular cell lines have not been confirmed. Some cellular growth suppression was observed in these cell lines upon treatment with MeHPLA. The possible function of MeHPLA was also compared to other phytoestrogens that appear to act independantly of the type II EBS Their activity was compared to a group of compounds known as xenoestrogens. MeHPLA, phytoestrogens, and xenoestrogens have the ability to stimulate, inhibit, or compromise normal or malignant cells with in the reproductive tract.

**This dissertation is dedicated to Alice, Crystal, and Gabrielle.**

# ACKNOWLEDGMENTS

I wanted to write an acknowledgement that was beautiful and meaningful. However, as I began to write, I realized that my words could not adequately express the deep love and appreciation I feel in my heart. My God is a mighty God. He has loved me and blessed me far beyond anything that I have dared to dream. Yes, a doctorate is wonderful. Yet, God has taught me things through this experience that are imperative. Those things; love, patience, hope, faith, and endurance are life itself.

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Lastly, I thank all my aunts, uncles, family, and friends who loved me and prayed for me. Special thanks go out to the members of the my church for beseeching God on their knees for my benefit. This celebration belongs to them as much as it belongs to me.

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# INTRODUCTION

The differential binding nature of estrogen receptors have been observed in cytosolic and nuclear fractions derived from several cell lines. This binding is concentration dependent and is characterized by a biphasic binding curve (1). At physiological concentrations, estradiol binds the estrogen receptor. However, in cytosolic and nuclear cell-free preparations, if the concentration of estradiol is increased above physiological levels, a second binding site is observed and is eventually saturated (Fig. 1). Since the concentration of estradiol would not reach these levels under physiological conditions, it is unlikely that estradiol is the endogenous ligand for this second binding site. This differential binding has given rise to the present classification of estrogen receptors, designated as the type I estrogen receptor (ER) and the type II estrogen binding site (EBS).

The type II EBS has been labeled the "bioflavonoid receptor," because of its ability to bind a group of compounds known as phytoestrogens. Through this association, phytoestrogens appear to have a protective value against cancer (48). It is possible that phytoestrogens activate the type II EBS to interact with a cell's transcriptional machinery to inhibit the protein production associated with cell proliferation (48-49). Recently, unique endogenous flavonoid-like ligands have been observed to bind to the type II EBS and modulate the cellular proliferation that is associated with estradiol binding to the type I ER. Extraction and chromatography of these ligands from normal and malignant tissue

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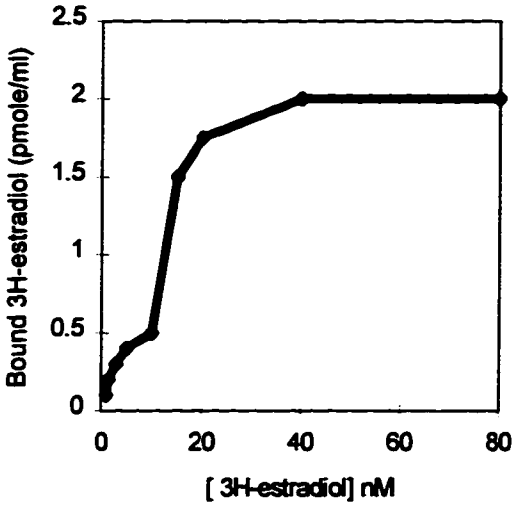



Fig. 1. **Biphasic Binding Curve for Estrogen Receptors with $^{3}$H-estradiol.** Distinct binding sites are exemplified at different concentrations of hormone. The first site becomes saturated between 1-10 nM and the second site between 10-30 nM (1).

have revealed two components. One is designated by an alpha binding activity and the other designated by beta (2). Even more compelling, the beta component is present in normal tissue but absent in malignant tissue and has been observed to inhibit the growth of MCF-7 breast cancer cells *in vitro* (2). This evidence suggests that the beta component may bind to the nuclear type II EBS and modulate cellular proliferation associated with estradiol binding at the type I ER (2). Using HPLC and GC-MS, the beta component has been identified as methyl-p-hydroxyphenyllactate (MeHPLA) (2) The chemical structure of MeHPLA is shown in Fig. 2.

An endogenous ligand for the type II EBS was first suspected when it was observed that cytosolic homogenates increased $^{3}$H-estradiol binding activity upon dilution (3). These homogenates were subsequently chromatographed and the alpha and beta components were collected and assessed for binding activity. This assessment confirmed that the beta peak, MeHPLA, had significant activity as the ligand for the type II EBS.

MeHPLA has subsequently been identified as a ubiquitous substance of all normal tissues. Its presence has been detected in uterine, serum, breast, and liver tissue (3). The origin of this endogenous ligand appears to be from the degradation of certain dietary bioflavonoids and/or tyrosine metabolism (4-5). Competitive binding analysis with $^{3}$H-estradiol has revealed that MeHPLA binds to nuclear type II EBS's with high affinity ($K_D$ = 4 nM), and physiological levels blocked estradiol stimulation of uterine growth *in vivo* and inhibited the growth of MCF-7 human breast cancer cells *in vitro* (2).

The alpha component was found to be the corresponding parent acid, para-hydroxyphenyllactic acid (HPLA). HPLA is a hydrolysis product of MeHPLA and is likely derived from specific esterase(s) activity in cancer cells (2). It is therefore possible

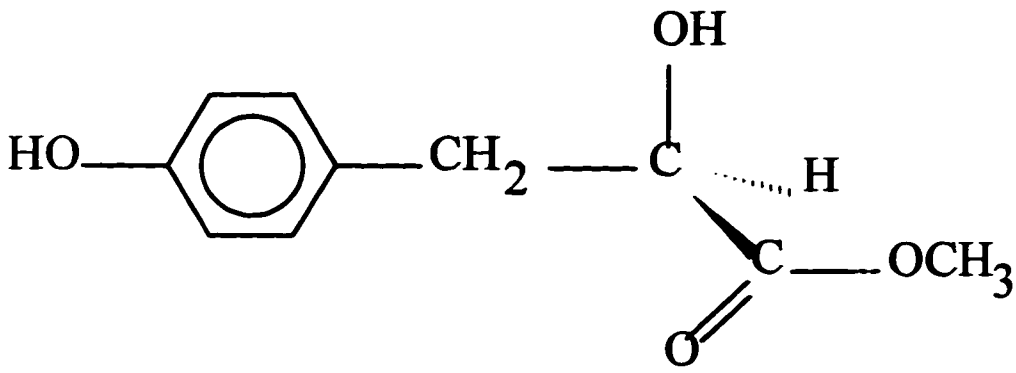



Fig. 2. **D-Methyl-para-hydroxyphenyllactate (MeHPLA).** Endogenous ligand observed to bind to the type II EBS (2).

that the absence of MeHPLA is a marker of malignant tissue. HPLA has a low affinity ($K_D$ is 30-40 times larger) for the type II EBS and does not block the uterotropic response to estradiol or inhibit MCF-7 cell growth (2). Such evidence seems to confirm the regulatory properties of MeHPLA.

One property of MeHPLA that has not been studied is the relative binding of the different stereoisomers (L,D) of MeHPLA. To date, binding and growth studies have used a racemic mixture of L (S) and D (R) isomers. It is therefore possible that one of the enantiomers may have a greater binding affinity for the type II receptor and a greater ability to inhibit cellular proliferation. HPLA found in the urine of patients with tyrosyluria is the L-isomer, therefore it is likely that the natural isomer of MeHPLA is "L" (2). The objective of this research was to synthesize the L and D isomers of MeHPLA, purify them, and test their binding affinity for nuclear type II EBS's and their ability to inhibit cellular proliferation in MCF-7 cells in culture. Since MeHPLA is a ubiquitious endogenous compound, the implications of its regulatory effects may be more far reaching than currently believed. Several cells lines were tested with the isomers of MeHPLA to ascertain possible regulatory effects beyond that of estrogen sensitive cell lines. Additionally, a correlation will be made between MeHPLA, phytoestrogens and a group of compounds known as xenoestrogens, which are synthetic componds that mimic the activity of estrogens, such as (DDT) and diethylstilbestrol (DES).

**Background and Significance**

Breast cancer is one of the most common forms of cancer. The American Cancer Society estimates that 183,000 new cases will be reported in 1997 and the number of

deaths attributed to breast cancer this year is estimated at 46,300 (6). Though it is primarily a disease of women, breast cancer can also occur in men. Contributing causes have been linked to cigarette smoking, alcohol consumption, diet, oral contraceptives, body size, radiation, and psychosocial influences (7). However, the preceding possible causes have not addressed specific answers or the etiology of breast cancer.

For many years, it has been known that estrogen stimulates the growth of some breast cancers, and the growth of these cancers is inhibited by antiestrogens (8). Classically, hormone action involves the synthesis and secretion of hormone by endocrine cells. The hormone then travels via the blood stream to its target tissue and enters the cell through simple or facilitated diffusion. Once within the cell, hormones activate many cellular processes by interacting with cellular proteins (Fig. 3). The hormone indicated as having a possible connection to some hormone-related breast cancers is estradiol (and its mimics).

Estradiol induces the synthesis of a large number of enzymes involved in protein and nucleic acid synthesis including DNA polymerase, thymidine and uridine kinases, thymidylate synthetase, carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydrofolate reductase (9-10). Physiological concentrations of estradiol, stimulate nucleotide synthesis by both scavenger and de novo biosynthetic pathways (8). Estradiol regulates many of these biosynthetic pathways at the mRNA transcriptional level by altering the cellular synthesis and/or secretion of several proteins (11). Synthesized proteins include plasminogen activators and collagenolytic enzymes that contribute to breast cancer progression by allowing the tumor to digest and traverse encapsulating

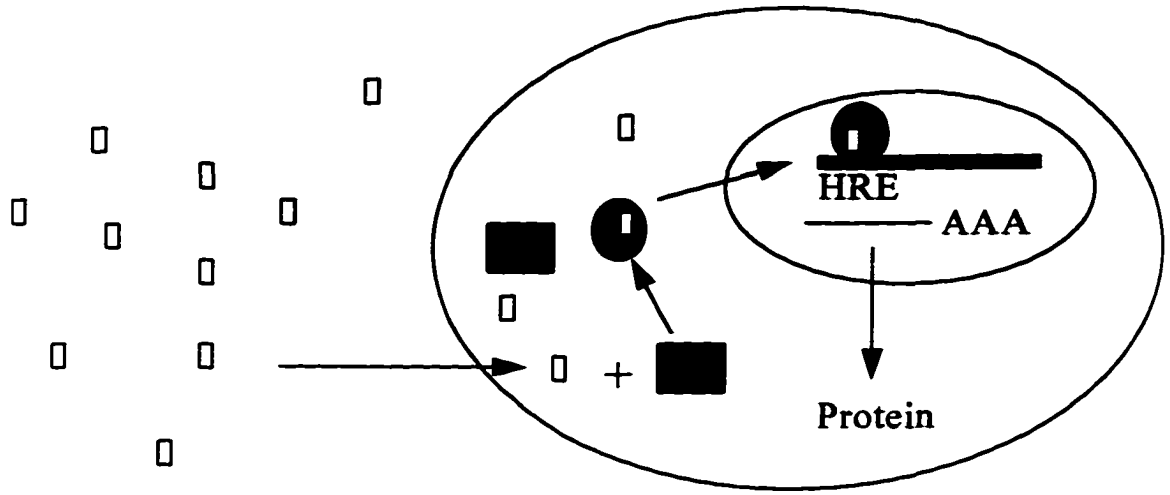



Fig. 3. **Classical Steroid Hormone Action.** Estradiol (white squares) moves through the blood stream to target cells and interacts with the estrogen receptor (black rectangle). This causes a conformational change in the receptor represented by the black rectangle changing into a black circle. This conformational change is known as activation. The activated receptor then enters the nucleus and binds to the hormone response element (HRE) located on the DNA to activate transcription. Gene transcription produces a messenger RNA with a poly A tail which is then translated by the ribosomes into a new protein in the cytoplasm (15).

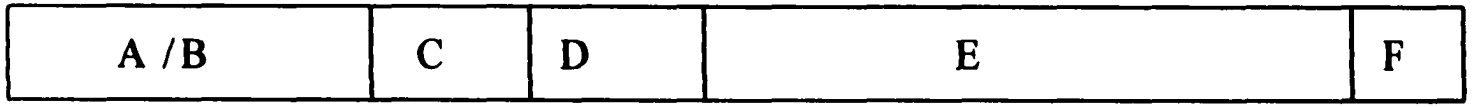
basement membrane (12). Additionally, these proteins may serve to release mitogenic factors like somatomedin C from carrier proteins, thus causing the processing of inactive precursor growth factors to active species (13). This kind of mitogenic activation by estradiol has also been shown to induce surface receptors or binding proteins for laminin in MCF-7 breast cancer cells (14). The laminin receptor mediates attachment of cells to basement membrane laminin to contribute to invasiveness of tumor cells and to promote colonization of new tissue areas. In vitro, estradiol treatment of MCF-7 cells increases laminin binding to artifically laminin-coated membranes and the migration of the same cells across an artificial membrane toward a diffusable source of laminin (14). Estradiol activates these physiological effects through its interaction with the type I ER.

The type I ER is part of the largest family of intracellular transcription factors in eukaryotes (15). The Steroid Hormone Receptor Superfamily includes receptors for estrogen, progesterone, glucocorticoids, mineralocorticoids, and androgens. In addition, it includes receptors for vitamin D, thyroid hormone, androgens, retinoic acid, 9-cis-retinoic acid, and ecdysone. The type I ER exists as an inactive apoprotein in the cytoplasm or nucleus. Known as a latent transcriptional activator, upon binding estradiol, the receptor goes through a series of conformational changes knows as transformation. This allows the type I ER to bind an enhancer region (estrogen response element or ERE) of DNA that regulates transcription.

The structure of the type I ER may be divided into several functional domains (Fig. 4) (15). From the N-terminal, the A/B domain is the most highly variable region for sequence and length. It contains a transactivation function that interacts with the

transcriptional machinery to activate transcription. The C domain is the DNA- binding domain (DBD). This domains contains two zinc fingers responsible for DNA recognition. It also contains sequences that are important for dimerization of the receptor. The D domain is a hinged region that is responsible for the conformational changes the receptor must undergo for activation. Within the D domain, there is also a nuclear localization region. The E domain is the ligand-binding domain (LBD). It is the largest domain, containing approximately 250 amino acid residues. Most of these residues are important for ligand binding, as confirmed by mutational analysis in which mutation compromises the ability of the altered receptor to bind its ligand. Within the LBD, small stretches of amino acid sequences serve as regions important for heat-shock protein association, dimerization, nuclear localization, and transactivation. Located at the C-terminal, one finds the F domain, which presently has no discernible function and deletion of the F domain has no effect on receptor function is unaffected.

The DBD is a globular structure that can be divided into two zinc fingers folded into a structural unit, known as Type II or Class II zinc fingers (16). One arm of the zinc finger unit recognizes a palindromic region within the 35 base pair ERE (16). Mutational analysis of the ERE has revealed that all 35 base pairs are necessary for the strongest hormonal response, however, as few as 18 base pairs will yield some response (17-18). A 13 base pair palindromic region is essential for receptor-DNA binding (18). The type I ER binds its response element as a homodimer with the residues of one arm of the zinc finger unit interacting with DNA and the other arm interacting to form the dimeric complex (19). The formation of the dimeric complex has been shown to be important for the binding of the type I ER to the ERE (15).




A /B = transactivation, transcription factor binding

C = DNA binding, dimerization

D = nuclear localization

E = ligand binding, heat-shock protein binding

F = function unkown

Fig. 4 **Functional Domains of the Estrogen Receptor**. The type I ER is a large globular protein that can be divided into five functional domains (15).

The importance of type I ER dimerization was recently analyzed. Formerly, it was observed that estrogen, progesterone, and glucocorticoid receptors bind to DNA as dimers (15). Monomers of the preceding receptors bind to DNA with only low affinity. Therefore, any lack of detectable DNA binding in the absence of ligand may be due to an inability of receptor to form a dimer. In an experiment, an antibody was used as the dimerization agent (one divalent antibody forces two receptor molecules to bind to DNA at adjacent sites) in the absence of ligand (15). The divalent antibody induced receptors to bind their response element with high affinity.

Thus the role of estrogen in DNA binding is likely to be the induction of a conformational change in the LBD which exposes a major dimerization function present in this region (10). The major dimerization region is also occluded by several heat-shock proteins, hsp90, hsp70, and hsp56 (20-21). Heat-shock proteins provide protection against elevated body temperatures associated with fever. If body temperature rises above a certain threshold, heat-shock proteins are activated to provide structural protection against tertiary damage of physiologically active proteins. In the case of hormone receptors, upon ligand binding, heat-shock proteins dissociate from the LBD to reveal the dimerization region (20-21) (Fig. 5). Heat-shock protein dissociation is caused by conformational changes in the hormone receptor upon ligand binding.

Conformational changes in the type I ER upon ligand binding have been confirmed using protease digestion procedures (22). Upon binding of the ligand, the receptor transforms to a conformation that is either more sensitive or less sensitive to protease digestion. It has been shown that the ligand-bound receptor for steroid

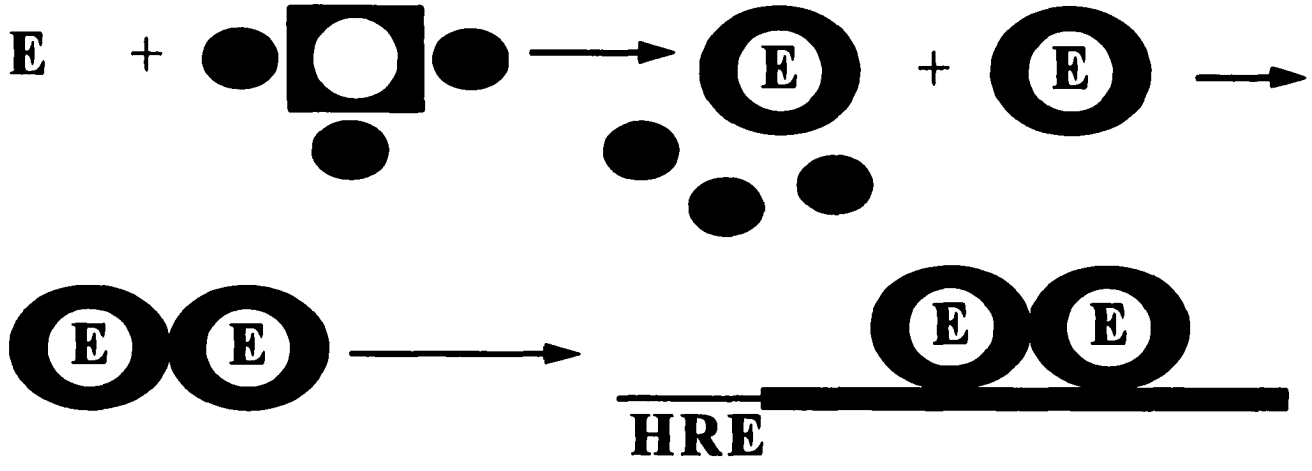



Fig. 5. **Heat-Shock protein (HSP's) dissociation and receptor dimerization.** Upon binding ligand, receptor conformational changes causes HSP's to dissociate, exposing dimerization domains and DNA binding domains for receptor interaction (15).

hormones has a more protease insensitive conformation (15). Therefore, one can deduce that binding of the ligand sequesters certain regions of the receptor so that a protease is unable to attack those regions. It is this sequestering of certain regions of the receptor that leads to another aspect of receptor-ligand interaction.

In vivo, there are ligands that can act as antagonist or partial agonist to hormone receptors. It has been shown that these antagonists/partial agonists can induce heat-shock protein dissociation and dimerization, but they cannot produce transcriptional activation (23). It appears that these ligands are unable to cause the conformational changes that fully sequester the appropriate regions of the receptor necessary for complete transcriptional activation. Using protease digestion, it has been shown that the conformational changes caused by these other ligands, such as the antiestrogen, tamoxifen,

are different from those caused by the receptor's intended hormone (24-26). From these results, it was shown that the extreme C-terminus of the LBD acts as an intracellular repressor and must be completely sequestered into the receptor or it will block the transactivation region of the receptor (27). It is the transactivation region that acts with the transcriptional machinery to cause gene activation. Just how the type I ER interacts with the transcriptional machinery to facilitate gene activation and protein production is not fully known. It is possible that hormone binding to type I ER enhances or stabilizes the formation and activation of the transcriptional machinery (15).

The transcriptional machinery is made up of at least seven transcription factors (TFII A, B, D, J, F, E, and H) that must bind to the DNA-promoter to activate

transcription (15). These transcription factors bind in a sequential manner and it is possible that the type I ER may act anywhere within the sequence. However, it is plausible that the type I ER may bind to TFII B and assist it in binding to the preinitiation transcriptional machinery, since evidence has shown the TFII B interacts with the estrogen receptor, the thyroid receptor and the progesterone receptor (28). The addition of TFII B to the transcriptional machinery complex is a rate-limiting step in gene activation (15). However, it should be emphasized that the preceding mechanism has not been proven and that the type I ER may interact with other components of the transcriptional machinery.

Type I ER's also have other factors that may influence their activation. Many receptors are phosphorylated in the ligand unbound state, but become highly phosphorylated when bound to ligand (29). Functionally, phosphorylation appears to be redundant, and not a switch which turns on the receptor, but appears to enhance receptor activity synergistically. However, phosphorylation has recently been shown to activate receptors in the absence of ligand. In experiments by Denner et al (1990), chicken progesterone receptors were activated in the complete absence of progesterone by phosphorylation. Ligand-independent activation may also be seen in the presence of dopamine, cAMP inducers, and growth factors (30).

Other components in the nuclear millieu may also have an effect on receptor activity. Type I ER's are latent transcriptional activators and may be downregulated by interaction with certain nuclear components such as Fos and Jun (15). Also, the availability of the transcription factors that form the transcriptional machinery complex

may be regulatory (15). Lastly, chromatin structure must always be considered as a regulatory modulator (15). If chromatin is conformationally unavailable for gene transcription, receptors and other transcription factors will be unable to interact with their DNA elements.

Conformationally dependent, multi-level regulatory type I ER's may have arisen out of a need to control cellular proliferation which supports the idea that increased estrogen concentrations have a causal role in some forms of cancer. One function of estrogen is to promote cellular proliferation and growth of sex organ tissues and other tissues related to reproduction. If type I ER's were able to dimerize and bind to promoters in the absence of ligand, genes could be more easily transactivated and the net result might be an increase in cellular proliferation and cancer. Therefore, the various levels of conformational activation of type I ER's is another protective feature to prevent unwanted gene activation, cellular proliferation, and cancer.

Another protective mechanism of type I ER's may be related to the heterogeneity of estrogen-binding intracellular proteins. Multiple estrogen-binding proteins has been discovered in the immature and mature ovariectomized rat uterus (1). Type I ER's represent the classical steroid hormone receptor, and binds estradiol with high affinity ($K_D$ = 1 nM) (31). Type II estrogen-binding sites are intracellular nuclear proteins that bind estradiol with a lower affinity ($K_D$ = 33 nM) (31). Presently, it is not clear whether type II EBS's are distinctly different from the steroid hormone receptor family. Type II EBS's are stimulated 30-fold by estradiol, while type I ER's remain relatively constant (32). This suggests that they are different proteins. Conversely, type II EBS's and type I

ER's have the same apparent molecular weight, 37K (33). However, nuclear type II EBS's are not recognized by rat monoclonal antibodies which readily recognized type I ER's (33-34). Though monoclonal antibody studies are not conclusive, it is good evidence that the proteins are distinct (32).

Despite decrepancies in the identity of type II EBS's, one of the most promising approaches to an understanding of a more specific mechanism for cellular proliferation in breast cancer comes from the recent work of Markaverich (2) and Scambia (35). Their work demonstrates a positive correlation between the incidence of breast cancer and the role of type I ER's and nuclear type II EBS's. Both are effected by estrogen, phytoestrogens, and flavonoid-like ligands which have been demonstrated to have growth inhibitory activity. Urinary excretion of flavonoid-like ligands is usually high in areas with a low risk of breast cancer, and low in areas associated with a high risk of breast cancer (36). This strongly suggests that these compounds have cancer protective properties.

**Specific Aims**

The goal of the Enological Research Laboratory at Old Dominion University is to study the protective value of natural compounds against various cancer cell lines, such as MCF-7, MDA-231, and human fibroblast cells. Natural compounds found in wines are polyphenolic, and appear to interact favorably with many cancer cell types to decrease their growth. Of particular importance is the effect these phytoestrogens have on breast cancer cells which express type I or type II ER's or both. These polyphenols are

phytoestrogens because of their ability to bind to ER's and modulate cellular proliferation. MeHPLA is a likely metabolite of phytoestrogen catabolism.

The specific aims of the research were as follows:

1. Synthesis of racemic L/D-MeHPLA: A racemic mixture of HPLA will be methylated by diazomethane to yield L/D-MeHPLA.

2. Cytotoxicity of racemic L/D-MeHPLA on MCF-7 breast cancer cells: A racemic mixture of L/D-MeHPLA will be evaluated for toxic levels on MCF-7 breast cancer cells.

The binding affinity of racemic L/D-MeHPLA to type II EBS's will also be evaluated.

3. Methylation of HPPA: Hydroxyphenylpyruvic acid (HPPA) will be methylated by diazomethane to yield methyl-p-hydroxyphenylpyruvate (MeHPPA) in preparation for enantioselective reduction.

4. Synthesis of the Enantioselective Catalyst: An oxazoborolidine catalyst is synthesized following the protocol outlined by E.J. Corey in J. AM. Chem, 1987.

5. Enantioselective Reduction: MeHPPA will be enantioselectively reduced to L and D-MeHPLA by the enantioselective catalyst, oxazoborolidine.

6. Synthesis of L-HPLA: HPPA will be enzymatically reduced to L-HPLA by Lactate Dehydrogenase. Confirmation of the L-isomer will be accomplished by comparing authentic L and D-HPLA with the enzymatically synthesized L-HPLA upon the Sumichiral OA 5000 HPLC column which separates the enantiomers of carboxy acids. L-HPLA will be purified by HPLC on a semi-prep column. Synthesized L-HPLA will be methylated with diazomethane to yield L-MeHPLA.

7. Confirmation of L and D-isomers: The structure of the produced L and D-MeHPLA

from the enantioselective reductions will be confirmed by HPLC and GC-MS.

8. Type II EBS Binding Study: L and D-MeHPLA will be evaluated against racemic MeHPLA for binding to the type II EBS's.

9. Cell Growth Study: L and D-MeHPLA will be evaluated against a racemic mixture for growth effects on MCF-7 and MDA-231 breast cancer cells, HxGC-3 colon cancer cells and LNCAP prostate cancer cells.

10. Discuss and correlate the effects MeHPLA, phytoestrogens, and a group of compounds known as xenoestrogen and their relationship to malignant diseases. Xenoestrogens include a number of insecticides, industrial chemical, and synthetic hormone analogs that number in the hundreds and include dichloro-diphenyl-trichloroethane (DDT), dioxin, and diethylstilbestrol (DES).

# RESEARCH DESIGN AND METHOD

## Synthesis of racemic L/D-MeHPLA

L/D-HPLA was methylated with diazomethane (Fig. 6). All reagents were purchased from the Aldrich Chemical Company. Using a millimole-size N-methyl-N-nitro-N-nitrosoguanidine (MNNG) apparatus, 130 mg of MNNG was added to the inner chamber of the generator. To the outer chamber, 2.0 ml of terahydrofuran (THF) was added. The two chambers were connected, secured for air tightness, and placed in a zero degree ice bath. 600 μl of 20% NaOH was then added dropwise with an air-tight syringe at 5 second intervals to the inner chamber. Diazomethane gas was generated and dissolved in the THF giving a yellow color to the solution. The yellow solution was pipetted directly onto the L/D-HPLA to yield L/D-MeHPLA. The reaction was complete when the reaction solution began to retain the yellow color, indicating an excess of diazomethane. The reaction was followed with HPLC by monitoring the disappearance of starting material and the appearance of racemic MeHPLA. The Varian HPLC system used a C18-AQ analytical column with a 60% water / 40% acetonitrile solution and flow rate of 1.0 ml/min. The absorbance of the phenyl ring was detected by an Applied Biosystems 759A absorbance detector monitoring at 254 nm. Excess diazomethane and THF were evaporated under a stream of nitrogen gas to yield the product. L/D-MeHPLA was recrystalized in ether to yield the pure product.

## Cell growth study of racemic L/D-MeHPLA on MCF-7 breast cancer cells

The MCF-7 breast cancer cell growth study was carried out in the following manner. MCF-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM),

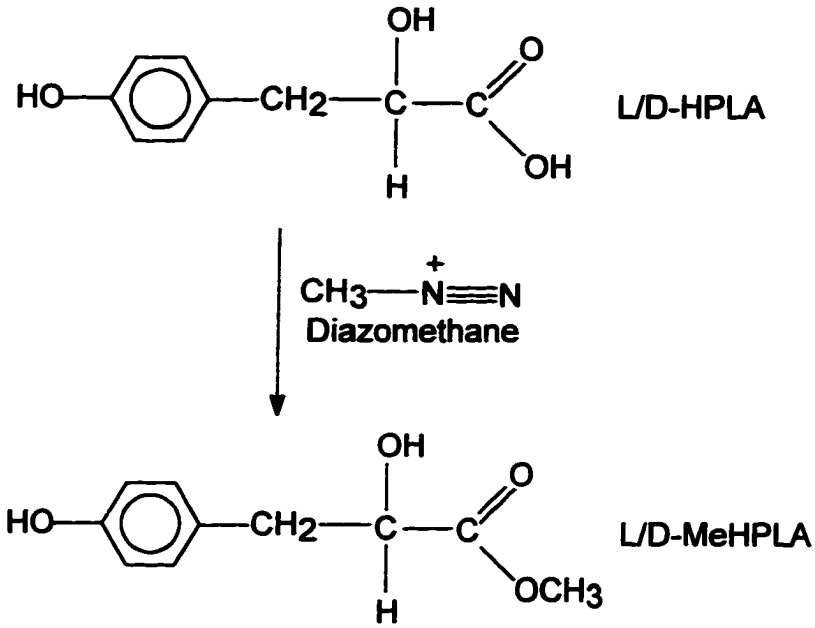



Fig. 6. **Methylation of Racemic HPLA.** L/D-HPLA is methylated using a solution of diazomethane dissolved in THF.

containing 10% fetal bovine serum, 1μg/ml ampicilin, and 1 μg/ml insulin. DMEM contains no phenol red which is an estrogenic agent and will contribute to the proliferation of estrogen receptor positive cells. Cells were subcultured by versene wash and trypsinization. Versene scavenges calcium from plated cells so that they can be enzymatically cleaved by trypsin from the culture surface. Calcium inhibits trypsin. Cells were subcultured into eighty-five 33 mm petri dishes and allowed to incubate 24-72 hours at 37 °C under a 4% $CO_2$ atmosphere. After allowing one to three days of growth, a control was counted by hemacytometry and was designated as the number of cells on Day 0. The growth media was changed on Day 0 and supplemented with L/D-MeHPLA in the concentration range of 2 - 10 μg/ml.

The cell population density was determined on Days 2, 4, and 6. Cell growth media was exchanged for fresh media and resupplemented with experimental compounds on Days 2 and 4.

**Synthesis of MeHPPA**

Following the procedure outlined for synthesis of L/D-MeHPLA, HPPA was successfully methylated using diazomethane.

**Synthesis of the Enantioselective Catalyst**

The synthesis of the enantioselective catalyst, oxazoborolodine, was carried out in the following manner (37) (Fig. 7). In a round bottom flask, one equivalent of diphenylhydroxymethyl-pyrrolidine (101 mg) was reacted with 1.1 equivalents of methylboronic acid in 5.0 ml THF. The solution was gently stirred and refluxed at 40 °C for three hours. The solvent was evaporated under a stream of nitrogen gas. The solid

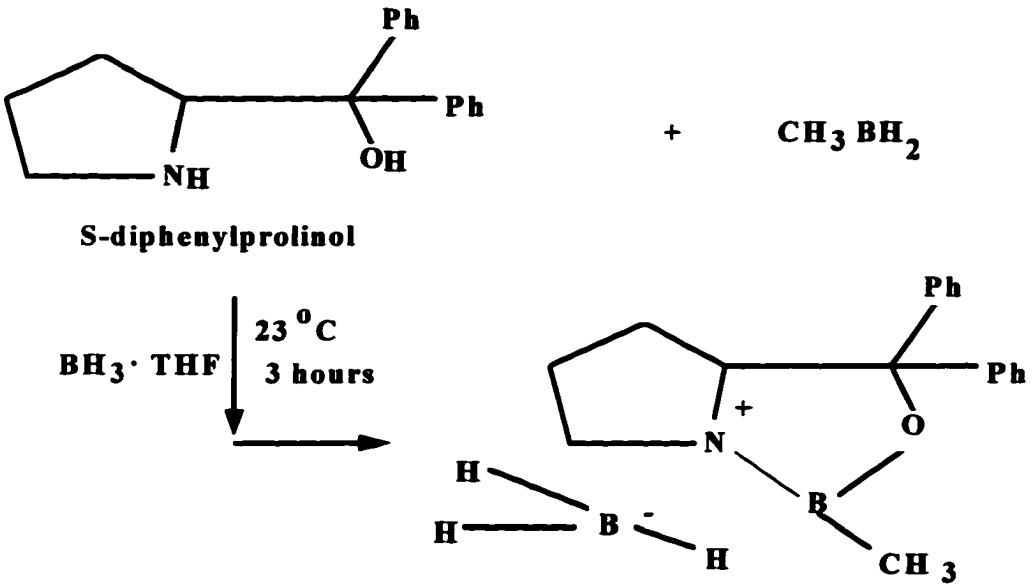



Fig. 7. **The Synthesis of the Enantioselective catalyst, Oxazoborolidine.**

was then recrystalized by adding a small amount of ether and allowed to crystalize in the freezer over a 48 hour period. The resulting product was confirmed by melting point verification (107-124 °C), GC-MS, and reduction of a model compound, acetophenone (Fig. 8).

The acetophenone verification of the enantioselective catalyst was done by combining 54μl of acetophenone and 6.95 mg of catalyst in 5 ml of THF. To this solution, 150μl aliquots of 1.0M $BH_3$-THF was added at approximately 15 minute intervals. Five minutes after each injection of $BH_3$-THF, a small amount of the reaction solution was taken, mixed with deionized water, and extracted with ethyl acetate. Twenty μl of the ethyl acetate extraction was injected on a C18-AQ HPLC column with solvent ratio of 40% acetonitrile, 60% water, and a flow of 1 ml/min.

The disappearance of acetophenone was monitored in this manner, along with the appearance of phenylethylalcohol.

**Enantioselective Reduction of MeHPPA**

MeHPPA, was redissolved in THF for enantioselective reduction to MeHPLA. Initially, one equivalent of oxaborolidine (6.95 mg) was added to 6 equivalents of $BH_3$-THF (150 μl) (37) (Fig. 9). As in the reduction of acetophenone, BH3-THF was added in intervals until the starting product disappeared. The enantioselective reduction took place in approximately one hour at 25 °C. The catalyst made from the R-pyrolidinemethanol is enantioselective for the S-isomer of ketones, while the S-catalyst is selective for the R-isomer. Additionally, the same enantioselective reduction was attempted on HPPA. However, the acidic proton of HPPA reacted with the catalyst and destroyed it.

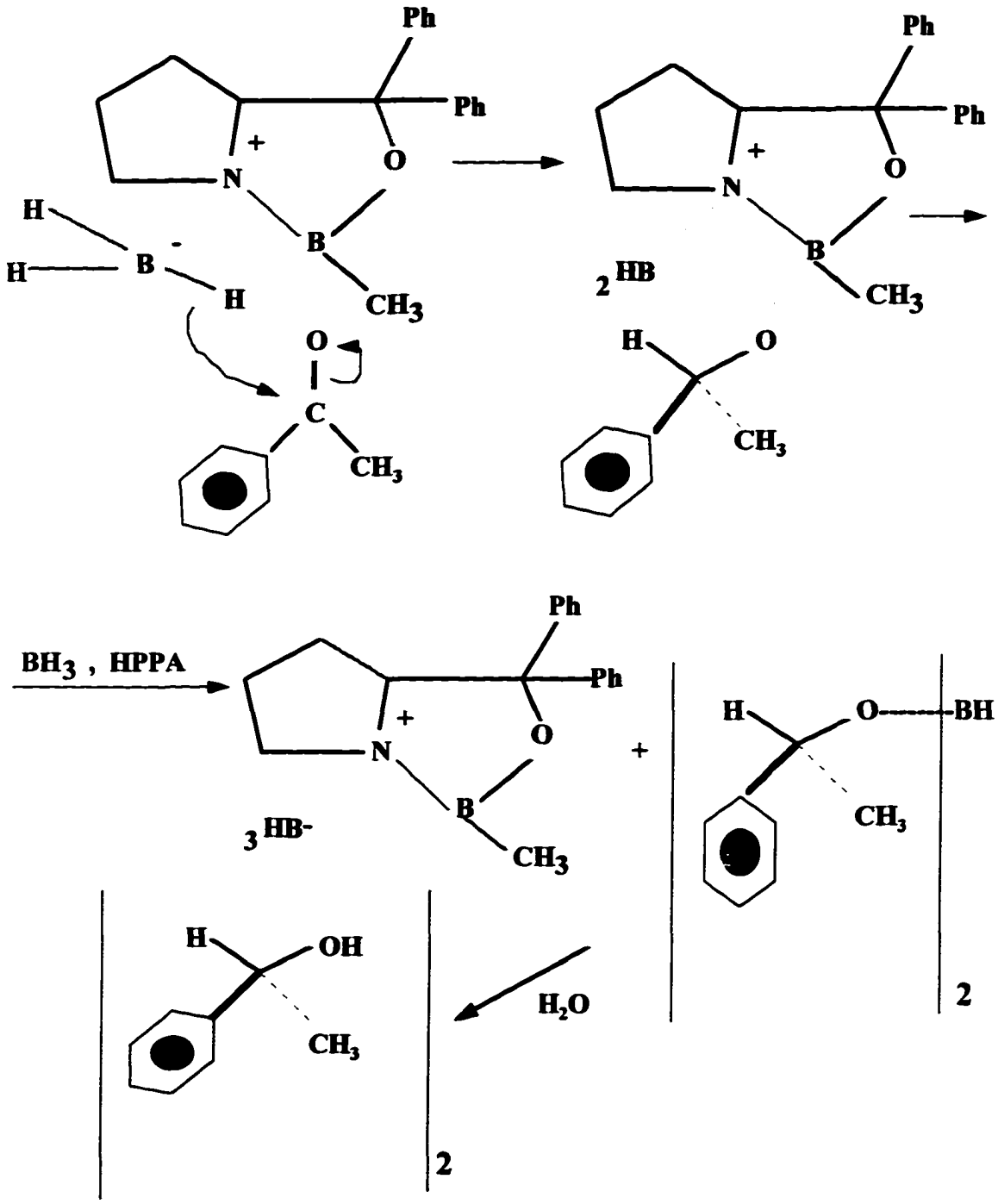



Fig. 8. **Enantioselective Reduction of Acetophenone.** To test the effectiveness of the catalyst, the novel compound, acetophenone was reduced. The successful reduction of the compound confirmed that the catalyst had been successfully synthesized.

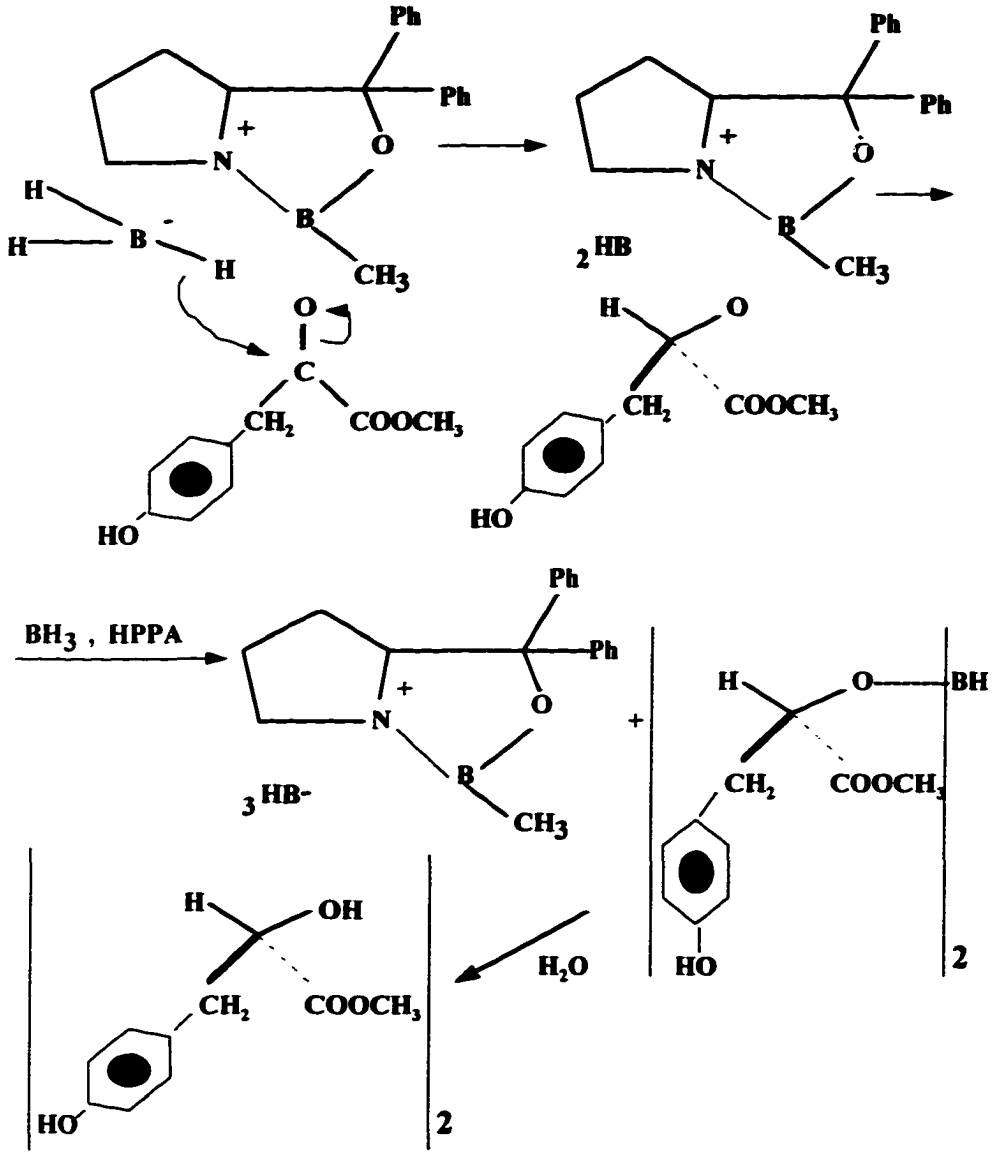



Fig. 9. **Enantioselective Reduction of MeHPPA.** Ketones are enantioselectively reduced by the oxazoborolidine catalyst. MeHPPA is not a ketone, but contains the functional groups of a ketone, thereby making the catalyst was an effective reduction agent.

**Confirmation of L and D-MeHPLA synthesized from Enantioselective Reduction**

L and D-MeHPLA synthesized from the enantioselective reduction of MeHPPA was confirmed by two methods. Reduction products were chromatographed on an analytical C18-AQ column in a 60% water / 40% acetonitrile solution with a flow rate of 1 ml/min. The retention time of a standard racemic L/D-MeHPLA was compared to the retention time of the reduction product. Reduction products were purified by HPLC upon a semi-prep C18-AQ column with solvent ratio of 40% acetonitrile/60% water and flow rate of 2.0 ml/min.

Reduction products were also confirmed by GC-MS. Using a DB-5 capillary column and a temperature gradient of 100 to 300 °C, a standard retention time and molecular cracking pattern was obtained for a standard of L/D-MeHPLA. This standard was compared to the reduction products.

**Enzymatic Synthesis of L-HPLA**

The enzymatic reduction of HPPA was accomplished using the procedure outlined in the Worthington Manual for the enzymatic reduction of pyruvic acid to yield L-lactic acid by the enzyme lactate dehydrogenase. Previously, it had been noted that HPPA differs from pyruvic acid by the presence of a phenyl group on carbon number 3. This similarity allowed for the possibility of enzymatic reduction of HPPA to L-HPLA. Subsequently, a reference was found for the endogenous conversion of L-tyrosine to HPPA and finally to L-HPLA, by an enzyme that was later identified as malate dehydrogenase (38) (Fig. 10). Such enzymatic reactions are stereospecific.

Reagents

0.2 MTris-HCL, pH 7.3

6.6 mM NADH in above 0.2 M Tris buffer, pH 7.3

30 mM HPPA in above 0.2 M Tris buffer, pH 7.3

Enzyme

Dissolve 1 mg of bovine heart LDH in 1 ml of 0.2 Tris buffer, pH 7.3

Procedure

Pipette into 25 ml round-bottom at room temperature as follows:

| | |
|---|---|
| Tris-HCl, 0.2 M pH 7.3 | 11.2 ml |
| 6.6 mM NADH | 0.4 ml |
| 30 mM HPPA | 1.8 ml |

Reactants were allowed to incubate at room temperature for 4-5 minutes to equilibrate.

50 µl of 1 mg/ml LDH in 0.2 M Tris buffer was added and the reaction was allowed to proceed for 30 minutes. The reaction was followed by monitoring the disappearance of NADH which absorbs at 340 nm. L-HPLA was detected pipetting off approximately 0.5 ml of reaction solution and filtering off the protein using centricon 3 centrifuge tubes. 20 µl of filtrate was injected onto Sumichiral 5000 column (2 mM $CuSO_4$ in 95% water / 5% acetonitrile (ACN) at 1 ml/min, 254 nm) to detect the presence of L-HPLA and residual HPPA.

**Confirmation of L and D isomers of MeHPLA by Hydrolysis and HPLC**

Confirmation of the L and D isomers of MeHPLA was attempted by converting enantioselectively synthesized MeHPLA into L or D HPLA by hydrolysis of the methyl group with a strong base (1M NaOH). Once the hydrolysis was accomplished, L and D-HPLA were injected on the Sumichiral OA 5000 column for enantiomeric quantification.

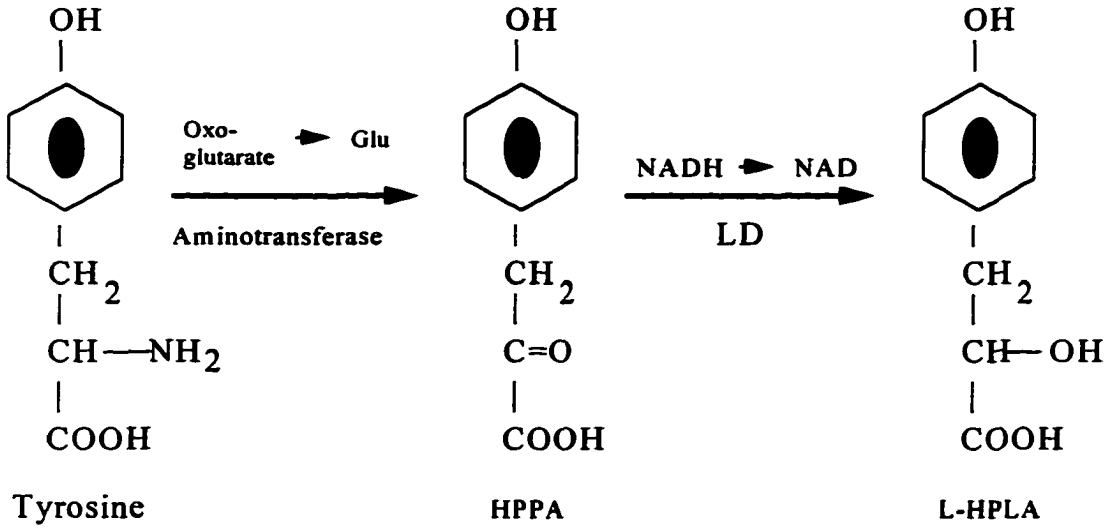



Fig. 10. **Endogenous Pathway for the enzymatic reduction of HPPA to L-HPLA** (39). MeHPLA could be a derived from this endogenous pathway for tyrosine.

**Diastereomeric separation of L/D-HPLA**

Racemic solutions of chiral carboxylic acids can be resolved into enantiomerically pure components by reacting the acid with an enantiomerically pure chiral amine (40) (Fig. 11). The reaction forms the diastereomeric salt of the two chiral components. Diastereomers are different compounds with different properties and can be separated by several different methods. Once the racemates have be separated, they can be retrieved by acidification of the solution and extraction of the chiral carboxylic acid. Upon heating, 72 mg of R/S-HPLA was reacted with 101 mg of S-diphenylpyrolidinemethanol in 10 ml of THF. After allowing the reaction solution to cool, the S-S diastereomer recrystalized from the solution and was subsequently filtered off by vacuum filtration. The R-S diastereomer, which remained in the THF, was retrieved by evaporating the THF under a stream of nitrogen. The purity of the R-S distereomer was further enriched by rinsing the crystals with ether and subsequent filtering.

The diastereomeric salts were then dissolved in water and acidified with 5% Hydrochloric acid (HCl). The solution was then extracted with ethylacetate to finally yield the R-HPLA and the S-HPLA. R and S-HPLA were chromatographed upon the Sumi Chiral OA-5000 Column to obtain their enantiomeric purity and confirm that the retention times corresponded to the enantiomers. Additionally, the Infra-Red spectra of a standard R/S-HPLA solution was compared to the products from the diastereomeric separation. Products were subsequently methylated with diazomethane.

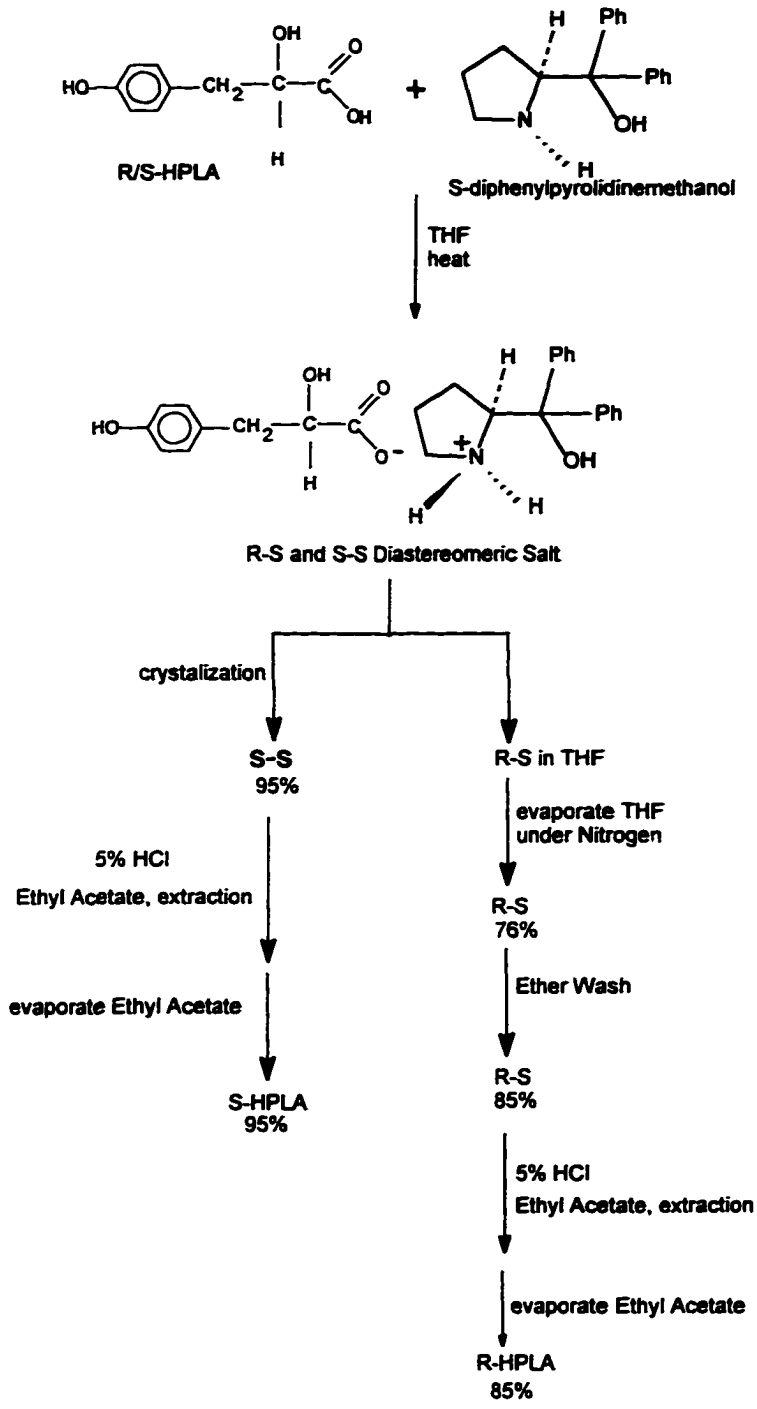



Fig. 11. **Flow Chart of Diastereomeric Separation of L/D-HPLA.** The diastereomers of racemic HPLA and a chiral amine were synthesized and then separated using recrystalization.

**Type II EBS Binding Study for Experimental Compounds**

$^{3}$H-estradiol binding studies were carried out on cytosolic preparations of MCF-7 and MDA-231 breast cancer cells. MCF-7 cells contain type I ER's and type II EBS's, while MDA-231 contain only the type II EBS. Cells were again cultured in 25 $cm^2$ flasks containing 5 ml of DMEM with 10% fetal bovine serum (FBS), and 1 μl/ml insulin. Cells

were allowed to grow for 48-72 hours at 37 °C. Cells were harvested by removing the media, washing in versene, and then trypsinization. Five ml of versene was added to the cells to resuspend them and the cells were centrifuged pouring off the versene layer after each spin. Approximately, 10 ml of versene was then added to the cells and they were homogenized using a dounce-homogenizer. After homogenization, the insoluble cellular components were removed by ultra-centrifugation (30,000 x g) and the cytosolic preparation was divided among 42 test tubes in 200 μl aliquots. Diethylstilbestrol (DES) (1 nM) was added to block all binding to type I ER's, so that the binding observed was only to type II EBS's. Binding agents (L-MeHPLA, D-MeHPLA, and L/D-MeHPLA) were added and allowed to react for 30 minutes at room temperature. Concentrations of binding agents were in the range of 100 μM to 1 nM.

$^{3}$H-estradiol was then added and allowed to incubate for 30 minutes at room temperature. Since saturation of type II EBS's is reported at 30 nM, a 30 nM solution of $^{3}$H-estradiol was used.

After incubation with experimental compounds, bound proteins were precipitated with 5% trichloroacetic acid (TCA) on ice and filtered on to glass fiber filter paper by

vacuum filtration and rinsed with 30 ml of 5% TCA. The glass filters were then placed in scintillation vials and 20 ml of scintillation cocktail was add to each vial for quantification of bound tritiated estradiol.

**Cell Growth Studies with the Enantiomers of MeHPLA**

MCF-7 and MDA-231 breast cancer cells, HxGC3 colon cancer cells, and LnCap prostate cancer cells were all tested for growth response to the enantiomers of MeHPLA. HxGC3 cells were cultured in modified eagle media (MEM) containing phenol red, 10% bovine serum and antibiotics. LNCap cells were cultured in RPMI 1640. Each cell line was subjected to a 7-10 day growth study depending on how rapidly cells proliferated. MDA-231 cells were strikingly more vigorous in their growth rate than the other cell lines. The cell study was carried out in 5 days, counting the cells on days 1, 2, and 5. The concentration range for experimental compounds was 10 μg/ml - 2 μg/ml and all cells were counted by hemacytometry.

# EXPERIMENTAL RESULTS

## The Chromatographic Separation of L/D-HPLA

Racemic HPLA was separated using the Sumichiral OA 5000 column (Fig. 12). The column separates enantiomers of carboxy acids by complexing analytes with $Cu^{2+}$ that is chelated to penicillamine bound to a stationary phase. Analytes react with the $Cu^{2+}$-penicillamine chealate in a manner which allows the enantiomers specific points of interaction with the stationary phase. D-enantiomers have three points of interaction and are therefore retained on the column longer (41-42). The L-enantiomers have only two points of interaction (41-42). By ligand-exchange with $Cu^{2+}$ and $SO_4^{2-}$ in the mobile phase, enantiomers are moved down the column to effect separation. Enantiomers elute as homodimers held together by ionic copper complexed between them (Fig. 13) .

The original experimental strategy was to resolve the enantiomers chromatographically and then methylate them in an acidic methanolic solution. However, the Cu-HPLA complex could not be separated due to the strength of the coordinate covalent bounds holding it together. Three approaches were attempted to break the bonds. The first involved stirring the complex with a polystyrene resin to chelate the copper away from HPLA. The second approach involved acidifying the complex with concentrated HCl. The third approach involved the acid catalyzed methylation of HPLA. HPLA can be esterified in an acidic methanolic solution. It was thought that copper could be removed from the complex by direct esterification. All of these methods were unsuccessful. Additionally, it was observed the acid-catalyzed methanolic approach to methylation was not the most efficient way to methylate HPLA.

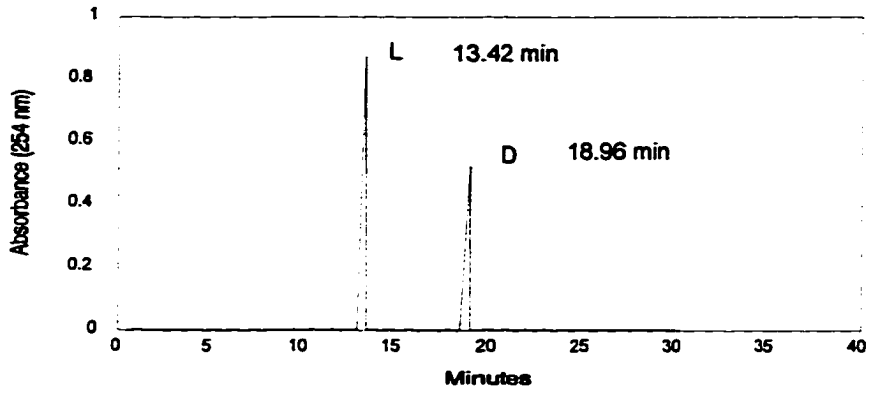



Fig. 12. **Separation of L/D-HPLA on the OA 5000 Column.** The first successful chromatographic separation of racemic HPLA.

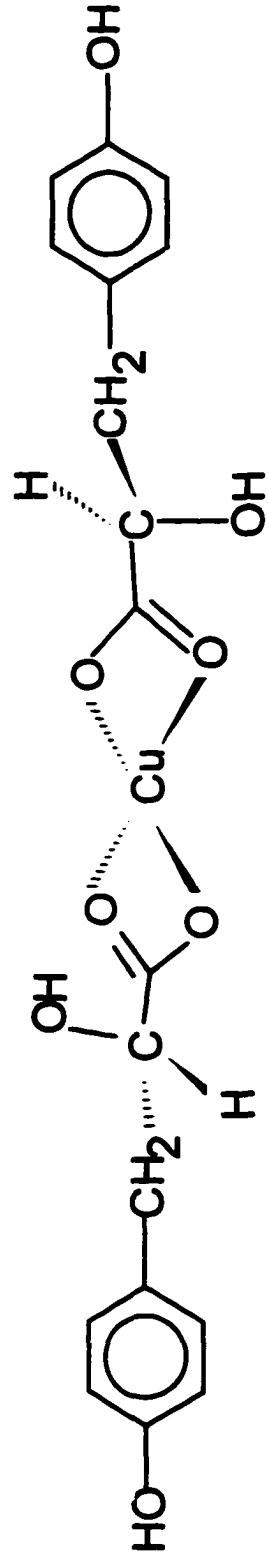



Fig. 13. **Cu-HPLA Complex.** The enantiomers of HPLA elute from the OA 5000 column as homodimers complexed with copper.

**Synthesis of racemic L/D-MeHPLA**

The synthesis of racemic MeHPLA was followed by HPLC. Using an ODS-AQ C18 column (4.6 x 250 mm) with previously described solvent ratio and flow rate, L/D-HPLA, purchased from the Aldrich Chemical Company, had a retention time of 2.16 - 2.21 minutes. Upon reaction with diazomethane, synthesized L/D-MeHPLA had a retention time of 4.43 - 4.44 minutes (Fig 14). The yield of MeHPLA was approximately 95.6%.

**Methylation of HPPA**

Following the same procedure outlined for the methylation of L/D-HPLA, HPPA was methylated using diazomethane. The reaction was followed with HPLC and the starting material had a retention time of 2.17 - 2.18 minutes. The product, Methyl-p-hydroxyphenylpyruvate (MeHPPA), proved to be unstable in the solid state. White crystals of MeHPPA rapidly discolored to a yellow oil within 24 hours. The methylation of HPPA had a yield of approximately 98%.

A unique property of MeHPPA was revealed from its chromatogram where two distinctive peaks suggested the presence of a second product from the methylation reaction (Fig. 15-16). Upon further investigation, it was discovered that HPPA exists in a ketonic and an enolic form (43). Keto-enol tautomerization is driven by resonance stabilization of the enolic form when compared to the ketonic form. The enolic form is resonance stabilized by a conjugated system of double bonds.

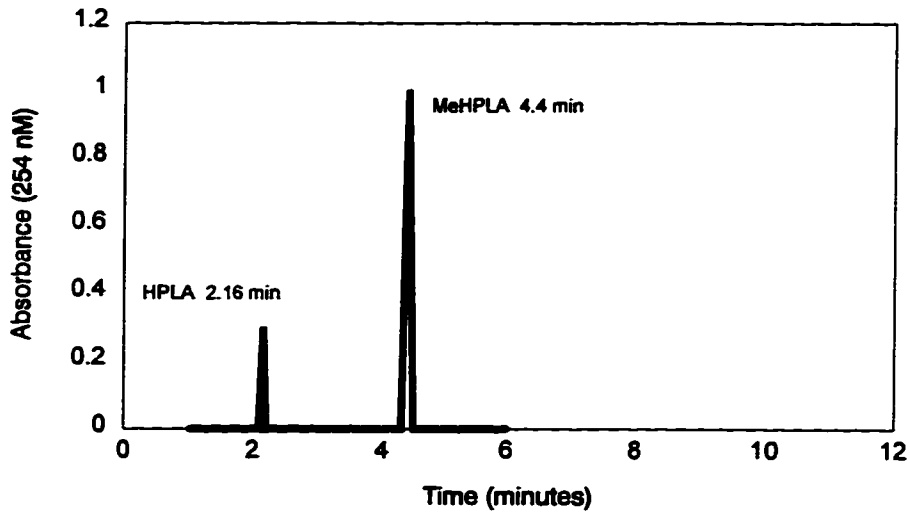



Fig. 14. **Methylation of HPLA.** The methylation of HPLA was followed by HPLC. HPLA elutes at 2.16 minutes followed by MeHPLA at 4.4 minutes. The chromatogram shows the disappearance of HPLA and the appearance of MeHPLA.

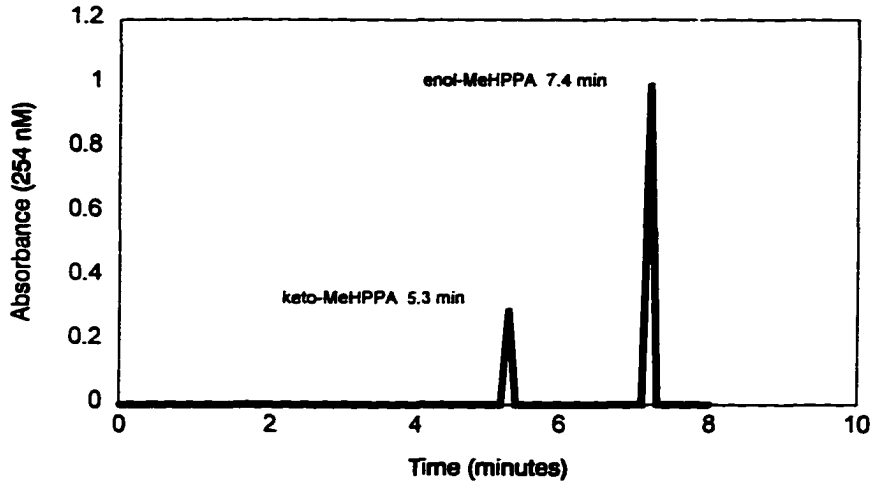



Fig. 15. **Chromatogram of methylated-HPPA.** Distinctive peaks of the ketonic and enolic form were separated on the ODS-AQ C18 HPLC column.

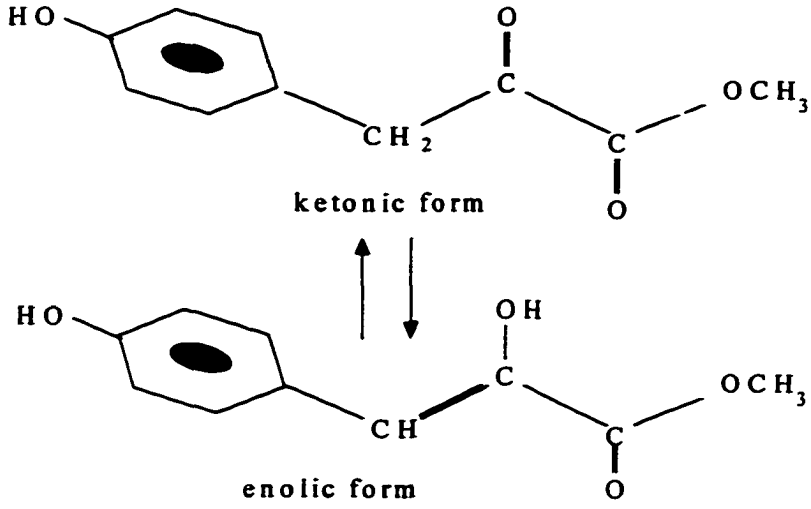



Fig. 16 **Keto and Enolic form of methylated HPPA.** MeHPPA and HPPA exists in an equilibrium with the enolic form predominating.

Additionally, the influence of an intramolecular hydrogen bond contributes to the stability of the enolic system. The methylated ketonic form had a retention time of approximately 5.35 minutes and the enolic form had a retention time of 7.47 minutes. The chemistry of HPPA and MeHPPA is such that it exists predominately in the enolic form regardless of the solvent and HPPA exists in the enolic form even in the solid state (43). The equilibrium can be enhanced toward the ketonic form by increasing the polarity of the solvent, however, it was observed that the enolic form predominated even when the solvent system was mostly water.

The NMR of HPPA and MeHPPA confirmed the presence of the enolic structures. For HPPA a single peak at 6.5 ppm corresponds to a single proton on the C-3 carbon of the enol form (Fig. 17). There is no peak that corresponds to the two protons on the C-3 carbon when it is in the ketonic form.

After methylation of HPPA, the NMR of MeHPPA (Fig. 18) shows only the presence of the enolic form in a solution of deuterated acetonitrile and $D_2O$. The enolic proton on the C-3 carbon absorbs at 7 ppm. The methyl peak is at 4.4 ppm. The peak at 4.8 ppm corresponds to the presence of HOD.

**Synthesis of the Enantioselective Catalyst**

The enantioselective catalyst, oxazoborolidine, was synthesized and confirmed by its melting point, GC-MS, and the reduction of a model compound, acetophenone. The melting point of the oxazoborolidine catalyst was reported in the range of 107 - 124 °C (37). Such a large melting point range is due to the ability of the

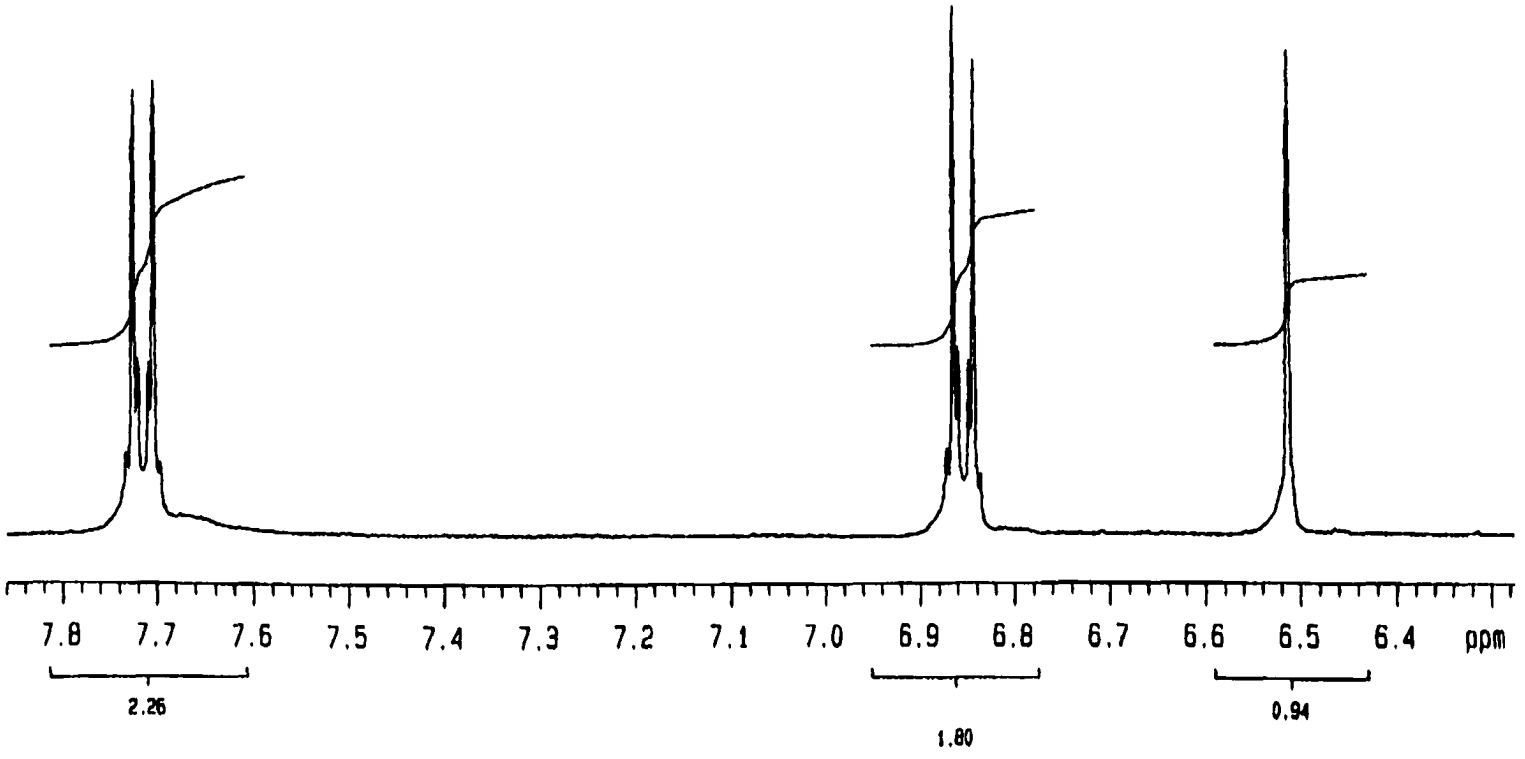



Fig. 17. **NMR of HPPA.** The NMR of HPPA shows the presence of the enolic proton on carbon 3. A peak at 6.5 ppm integrates to one proton and not two protons, as would be expected if the ketone were present.

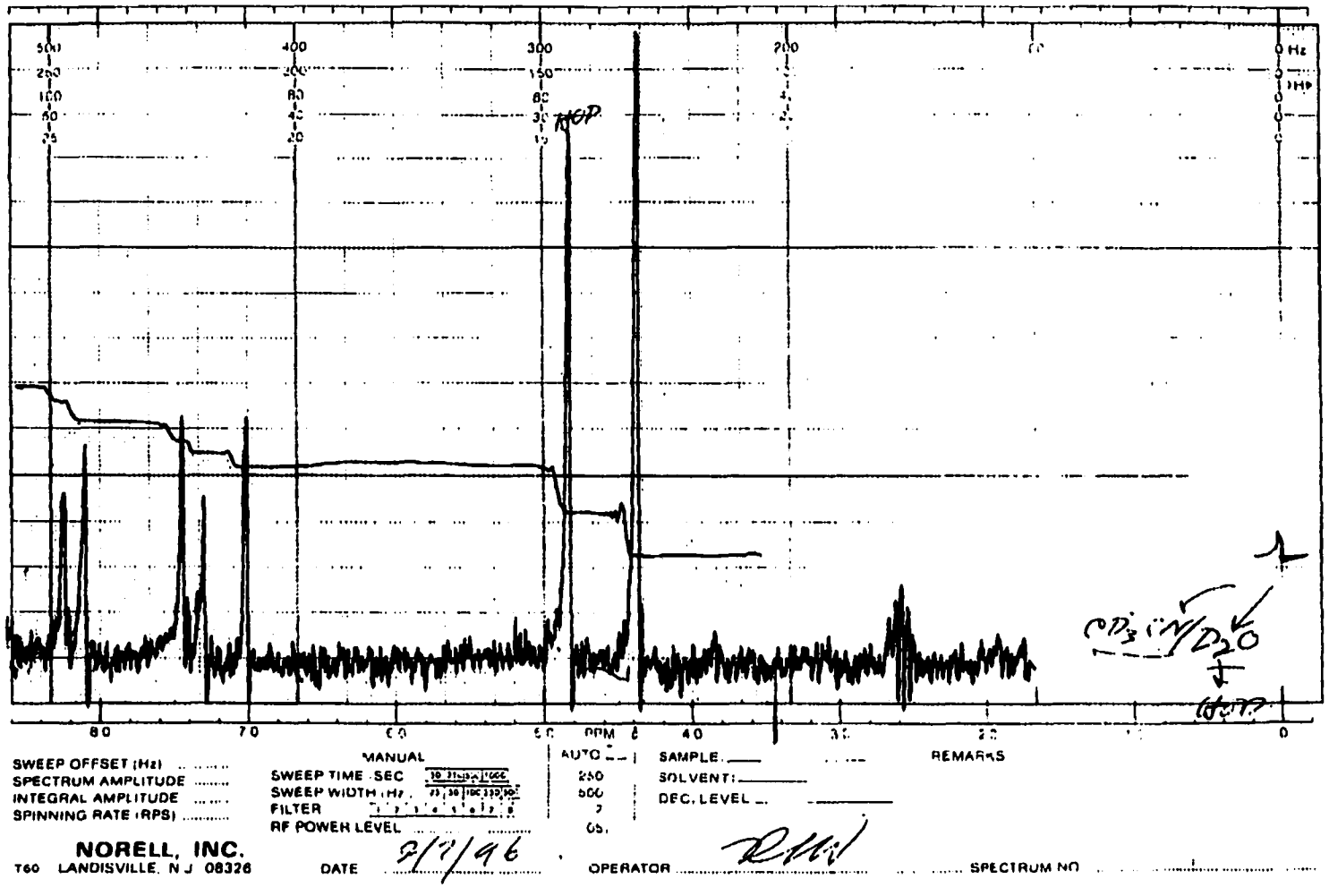



Fig. 18. **NMR of MeHPPA.** The MeNMR of HPPA shows the presence of the enolic proton on carbon 3. A peak at 7.0 ppm integrates to one proton and not two protons, as would be expected if the ketone were present.

catalyst to dimerize, and even trimerize. The more dimerization or trimerization present in the product the higher the melting point of the catalyst. The crystals of the R-catalyst melted at 110 - 115 °C, while crystals of the S-catalyst melted at 115 - 117 °C. Both melting points were in the appropriate range to conclude that the catalyst had been successfully synthesized.

Further confirmation of the enantioselective catalyst was accomplished using GC-MS. The calculated molecular weight of the oxazoborolidine catalyst is 277 grams/mole.

The mass spectra of the synthesized catalyst (Fig. 19) shows that the molecular ion peak has a molecular weight corresponding to 277 grams/mole.

The synthesized catalyst was then used to reduce a model compound. Acetophenone was chosen from the literature (37) and the reduction was carried out as described previously. Using HPLC with previously described solvent ratio and flow rate, the reaction was followed and the ketone was detected by a peak at 8.10 minutes. As the reduction proceeded, this peak at 8.10 minutes disappeared and a peak began to appear at 6.05 minutes (Fig. 20a). After several minutes, the peak at 8.10 minutes had almost completely disappeared to be replaced by the peak at 6.0 minutes (Fig. 20b). Additionally, a known reductant, sodium borohydride ($NaBH_4$), was used to reduce acetophenone for chromatographic comparison against the product made by the enantioselective catalyst. $NaBH_4$ reduces ketones to their corresponding alcohols, however not enantioselectively. Again, the HPLC chromatogram began with a peak at approximately 8.10 minutes which was replaced by a peak at 6.0 minutes as the reduction

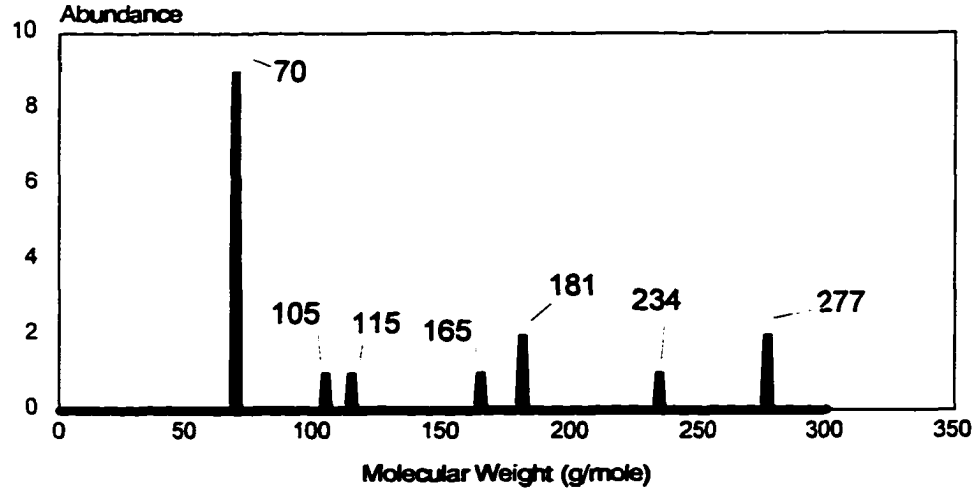



Fig 19. **Mass Spectra of Synthesized Oxazoborolidine Catalyst.** Mass Spectra showing the molecular ion peak of 277 g/mole.

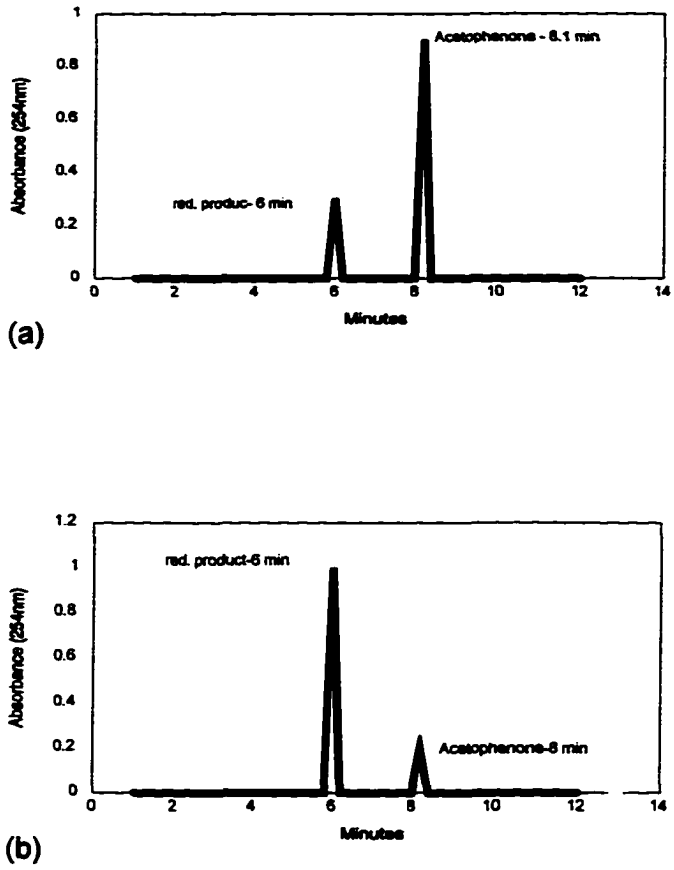



Fig. 20. **Reduction of acetophenone by enantioselective catalyst.** (a) Initial reaction mixture shows the presence of acetophenone (8.1 min) and beginning appearance of phenylethylalcohol (6.0 min). (b) As the reaction proceeds to completion, the alcohol replaces the ketone.

of the ketone proceeded. The 6.0 minute peak was analyzed by infrared and found to be identical to phenylethylalcohol.

**Enantioselective Reduction of MeHPPA**

Even though the enantioselective catalyst effectivity reduced MeHPPA to MeHPLA, the major product of this reduction was not MeHPLA. The chromatogram of the reaction mixture (Fig. 21b) using the ODS-AQ C18 column contained two major products. The peak at time 4.44 minutes corresponded to MeHPLA and constituted 20% of the reaction mixture. Residual enol of MeHPPA corresponding to 5% of the reaction mixture and had a retention time of 7.26 minutes. Unreacted ketone was present in about 3% and had a retention time of 5.33 minutes. The predominant peak of the reaction mixture (72%) of had a retention time of approximately 3.46 minutes.

The origin of this product was traced back to the starting material and the presence of keto-enol tautomerization (21a). The chromatogram of the starting material reveals that approximately 20% percent of MeHPPA is in the ketonic state at the beginning of the reaction and 80% is in the enolic state. Since the oxazoborolidine catalyst reacts effectively with the carbonyl group of ketones to reduce them to alcohols, it was assumed that the catalyst would not react with the enolic form MeHPPA. The expectation was that the catalyst would react with the ketone carbonyl exclusively, and as the ketone was consumed, the equilibrium would shift more of the enol to the ketone. In this manner, the equilibrium would continuously shift from the enol to the ketone of a MeHPPA. This appears not to happen. Since most of the starting material was in the enolic form, it appears that the catalyst reacted with the carbonyl of the ester.

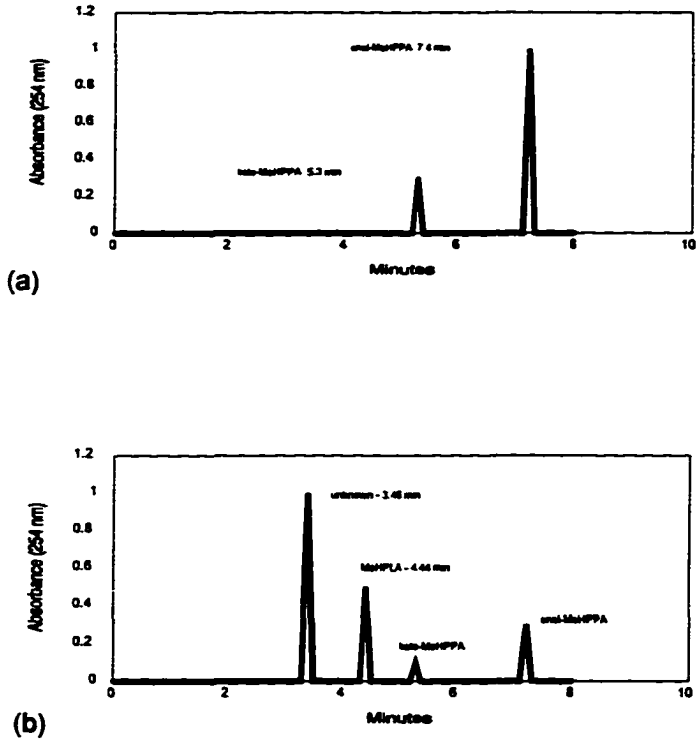



Fig 21. **Starting and Final Reduction Product.** (a) The starting product (MeHPPA) shows the presence of the ketonic form and the enolic form of MeHPPA. (b) The final reaction mixture shows the presence of residual starting material, MeHPLA, and a major product at 3.46 minutes.

Therefore, the predominant peak at 3.46 minutes corresponds to the product of the reduction of the enolic form of MeHPPA. MeHPLA would be formed from the reduction of the ketonic form of MeHPPA. Since only 20% of the starting material was in the ketonic form, only a fraction of the starting material is actually in the appropriate state to be reduced to MeHPLA. The majority of the starting material was reduced to the major product peak at 3.46 minutes. When the reduction reaction was allowed to continue without quenching, the MeHPLA peak at 4.44 minutes eventually disappeared and only the peak at 3.46 minutes remained. Therefore, the peak at 3.46 minutes is likely the double reduction product of both carbonyls in MeHPPA. This means that the peak at 7.26 minutes is most likely the enolic form of MeHPPA while the peak at 5.2 minutes is the ketonic form.

**Enzymatic Synthesis of L-HPLA**

The similarity between the structure of pyruvic acid and HPPA indicated that HPPA might undergo enzymatic reduction to L-HPLA using the enzyme Lactate Dehydrogenase (LDH). The major structural difference was the addition of a phenol group on carbon 3 of pyruvate acid. The question was whether HPPA, with its phenyl group on C-3, would fit into the enzyme active site of LDH. Molecular modeling on a UNIX SGI was used to obtain preliminary results on the feasibility of the LDH reduction. Molecular modeling showed that the phenol group could fit into the active sight of the enzyme. A reference was subsequently discovered for the enzymatic reduction of HPPA by an enzyme originally called α-Keto-Acid Reductace, later identified as Malate

Dehydrogenase (38). However, since LDH had already been purchased, it was appropriate to attempt the reduction using LDH.

The integrity of LDH was tested by subjecting the enzyme to a model reduction. Pyruvic acid was combined with NADH to see if reduction occurred in the absence of LDH. The enzymatic reduction of pyruvic acid to L-lactic acid requires NADH as the proton donator. The reaction was followed by a decrease in absorbance at 340 nm corresponding to the consumption of NADH to NAD. Figure 22a shows that when pyruvic acid and NADH were combined in the absence of LDH, no oxidation of NADH occurred. However, when LDH was added, there was an immediate decrease in the absorbance at 340 nm (Fig. 22b). This indicated that the enzyme was functioning properly.

Next, pyruvic was replaced with HPPA. Figure 23 shows the linear decrease in the absorbance for NADH. This indicated that the enzyme was oxidizing NADH to NAD and possible conversion of HPPA to L-HPLA. However, this was not conclusive for the synthesis of L-HPLA.

To further confirm that L-HPLA was the product of the enzymatic reduction of HPPA, the reaction mixture was analyzed on the Sumi Chiral OA-5000 column was used . The reaction mixture was filtered and the filtrate was injected on the chiral column to look for the presence of L-HPLA. Previously, it had been confirmed that no L-HPLA was detected in the reaction mixture prior to the LDH reduction. Analysis of the LDH reduction mixture produced a peak that corresponded to L-HPLA (Fig. 24a). When the filtrate was supplemented with authentic racemic HPLA, there was a corresponding

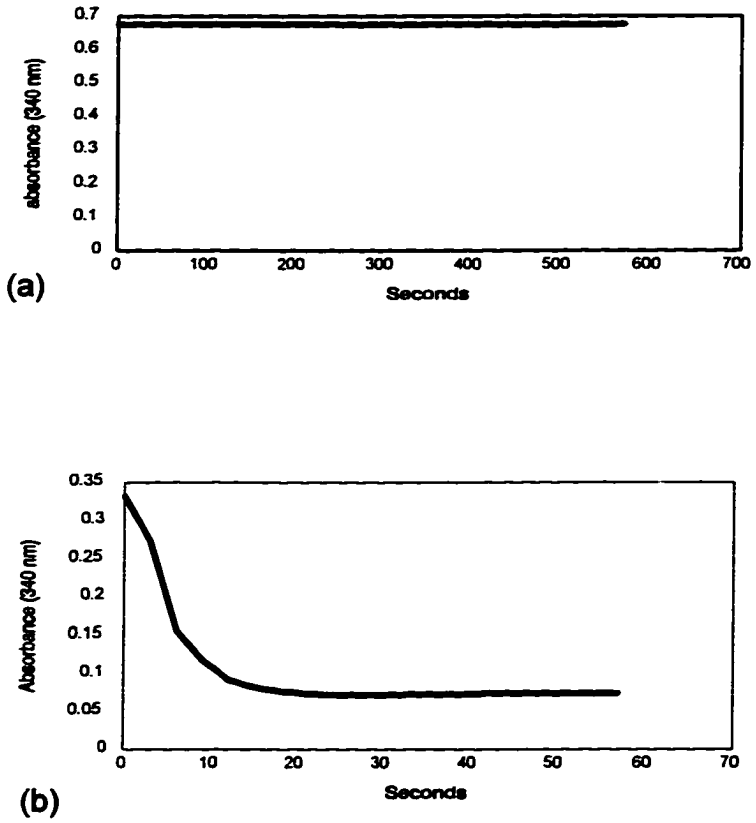



Fig. 22. **LDH Reduction of Pyruvic Acid.** (a) NADH combined with pyruvic acid is not oxidized in the absence of LDH. NADH maintains its absorbance at 340 nm. (b) NADH combined with pyruvic acid and LDH is oxidized as accompanied by a decrease in absorbance.

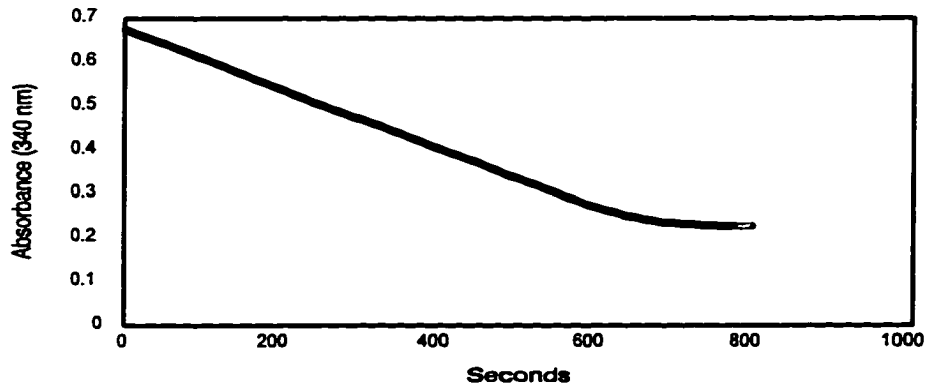



Fig. 23. **The Reduction of HPPA by LDH.** HPPA is enzymatically reduced to L-HPPA. The reaction is followed by monitoring the decrease in absorbance as NADH is oxidized to NAD.

increase in the size of the L-HPLA peak (Fig. 24b). This was proof that the reduction had produced L-HPLA.

LDH was more effective at reducing pyruvate acid than HPPA. The addition of the hydroxyphenyl group on carbon-3 of HPPA caused a structural hinderance at the active site of the enzyme. Therefore, the reduction of HPPA proceeded slowler than the reduction of pyruvate acid. The slowness of the enzymatic reduction of HPPA was not a problem to the further use of the LDH method as the choosen method to produce the enantiomers of MeHPLA. However, there were two objections to the further pursuit of this procedure.

First, milligram quantities of L-HPLA were needed for the biological study. The feasibility of scaling-up an enzymatic reaction to obtain milligram quantities of product seemed unfavorable. Enzymes are designed to react with nanogram quantities of substrates, therefore the amount of enzyme needed to synthesize milligram quantities of L-HPLA was considered to be prohibitive. Second, the procedure only produces the L-enantiomer of HPLA. For the biological study to be comprehensive, both the L and D-enantiomer were needed. Because of these limitations, it was decided that the enzymatic procedure would no longer be pursued as a viable method for the synthesis of the enantiomers.

**Confirmation of MeHPLA from Enantioselective Reduction**

MeHPLA synthesized from the enantioselective reduction was confirmed by GC-MS. Authentic MeHPLA, synthesized from racemic HPLA with diazomethane, was analyzed and compared to enantioselectively produced MeHPLA. Figure 25 shows the mass spectra for authentic and enantioselectively produced MeHPLA. The mass spectra

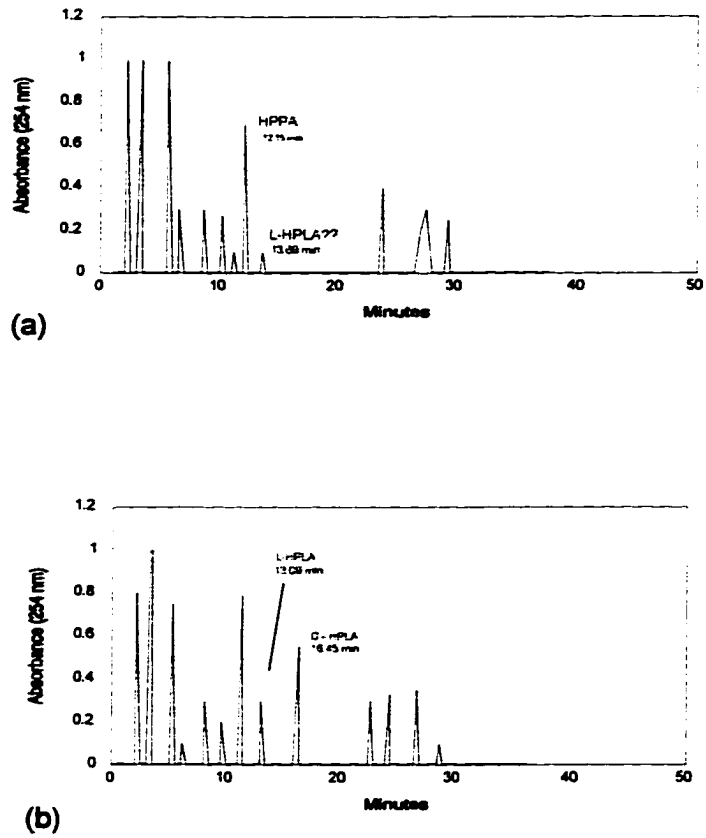



Fig. 24. **The Detection of L-HPLA in the Enzymatic Reduction Mixture.** (a) Enzymatically synthesized L-HPLA is detected on the Sumichiral OA 5000 column. A peak at 13.69 minutes is indicative L-HPLA. (b) When authentic crystals of L/D-HPLA is added to the reaction mixture the peak at 13.85 minutes is proportionally increased.

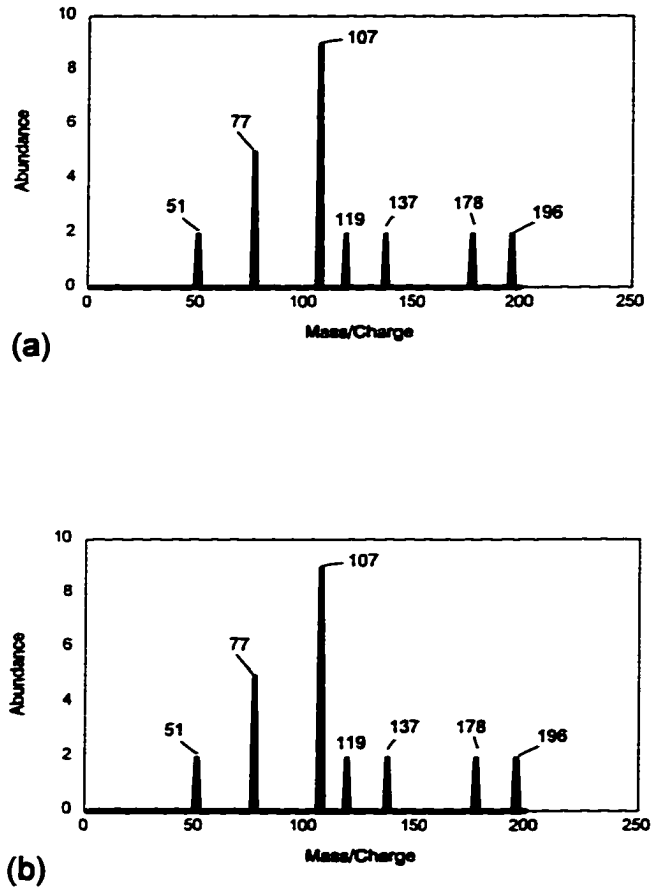



Fig. 25. **Mass Spectral of Authentic and Enantioselectively Produced MeHPLA.** The mass spectras of authentic (a) and enantioslectively synthesized (b) MeHPLA are identical.

were identical. Mass spectral data, along with HPLC data, confirmed that the enantioselective reduction had successfully produced MeHPLA.

**Diastereomeric Separation of L/D-HPLA**

The diastereomers of L/D-HPLA and the secondary amine, R-diphenylprolinol, had distinctive different solubilities in THF. Within minutes after L/D-HPLA and the amine had dissolved, crystals began to appear in the solution. These crystals were filtered and then chromatographed on the OA 5000 column and were found to be 95% of the L-enantiomer of HPLA. These crystals corresponded to the L-R acid-amine diastereomer. After work up of the diastereomer with HCl and extraction of the L-HPLA with ethylacetate, the yield of the L-isomer remained 95%.

Having now isolated the L-isomer, it was assumed that the D-R diastereomer was the predominant compound left in solution. The THF was evaporated and the residual crystals were chromatographed on the OA 5000 column to and found to be the crystals were 76% of the D-R diastereomer. The D-R diastereomer was then stirred with ether and filtered three times in an effort to increase the percentage of the D-enantiomer. The final yield of D-enantiomer, after work up with HCl and ethylacetate extraction was 85%. Figure 26 shows the chromatographic results of the separated L and D-HPLA from the diastereomic resolution.

The retention times of separated L and D-HPLA qualitatively demonstrated that the diastereomeric resolution had yielded HPLA. However, to insure that the separated HPLA was indeed authentic, the IR of L/D-HPLA was compared to the IR of the separated products. Figure 27 shows that the IR's were identical. Therefore, the products from the diastereomeric resolution were the separated enantiomers of HPLA.

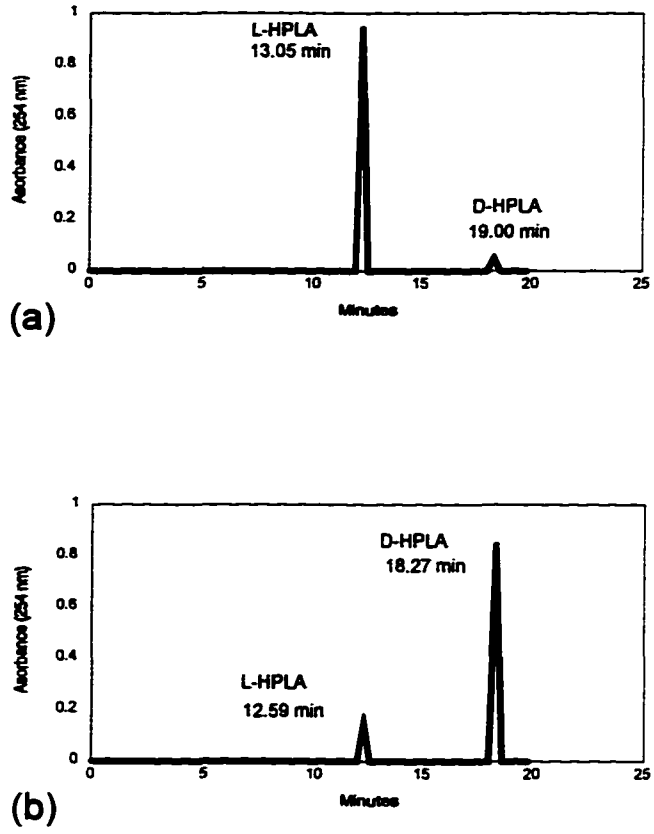
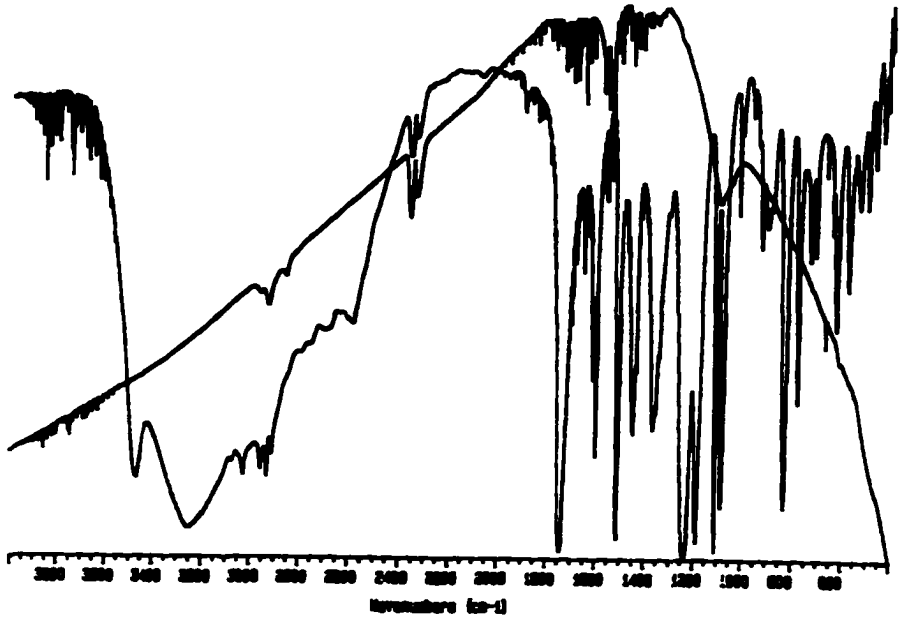
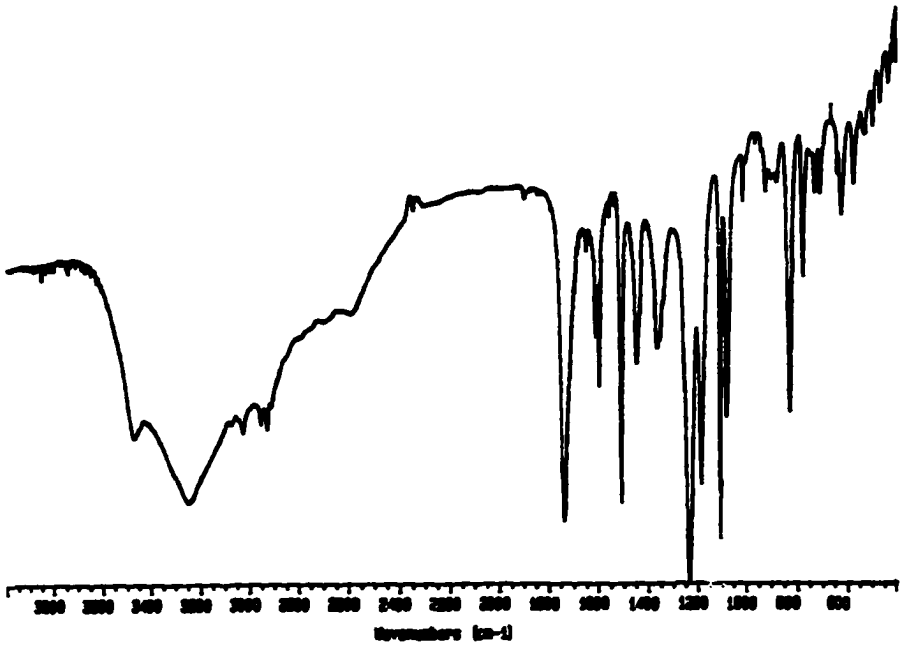



Fig. 26. **Diastereomeric Separation of L/D-HPLA.** (a) The final results of diastereomeric separation of L-HPLA. Chiral HPLC shows that the product is 95% L-enantiomer. (b) The final results of diastereomeric separation of D-HPLA. Chiral HPLC shows that the product is 85% D-enantiomer.



(a)



(b)

Fig. 27. **IR's of authentic L/D-HPLA and Diastereomeric Separation Product.** (a) The IR of authentic HPLA with unsubstracted background. (b) The IR of the product (HPLA) from the diastereomeric separation.

**Type II EBS Binding Study**

Figure 28 shows the results of the competitive binding $^{3}$H-estradiol binding study performed on MDA-231 breast cancer cells. MDA-231 are positive for type II EBS's, but negative for type I ER's. Therefore, all observed $^{3}$H-estradiol binding was due to interaction of analytes with the type II EBS. L-MeHPLA, D-MeHPLA, and racemic MeHPLA were observed to effectively compete with $^{3}$H-estradiol for type II EBS's. However, L-MeHPLA had approximately 30% greater binding affinity than D-MeHPLA. As might be expected, racemic MeHPLA had a binding activity that was between the activity of the two enantiomers.

**Cell Growth Study of MCF-7 and MDA-231 Breast Cancer Cells**

There was no observable difference in the growth effects of the enantiomers of MeHPLA on MCF-7 breast cancer cells. L-MeHPLA, D-MeHPLA, and racemic MeHPLA, all inhibited cell growth at concentrations between 6-10 μg/ml. However, statistically, there was not a significant difference in the suppression of cell growth between the two enantiomers. Figure 29 shows the growth inhibition on day 10 for MCF-7 breast cancer cells. All data points for the enantiomers have significant overlap.

In comparison to MCF-7 cells, MDA-231 cells were a more vigorous cell-line. The ten day cell study performed on MCF-7 cells was modified to six days for MDA-231 cells because of their rapid growth. Control cell numbers for MDA-231 cells reached 1.7 million per $cm^2$ as compared to only 300,000 per $cm^2$ for MCF-7 cells. Nevertheless, significant suppression of cell growth was still observed for higher concentrations of the enantiomers between 6-10 μg/ml. Figure 30 shows MDA-231 suppression of cell growth

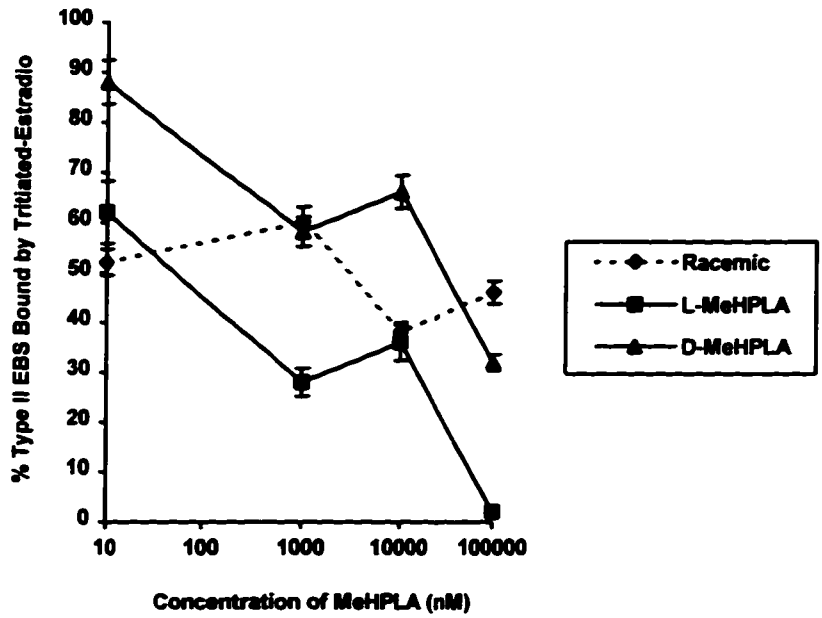



Fig. 28. **Competitive Binding Assay: MeHPLA versus Tritiated-Estradiol.** A concentration dependent decrease in the binding of tritiated-estradiol is observed as the concentration of L or D-MeHPLA is increased. L-MeHPLA appears to have a much better ability to bind the type II EBS.

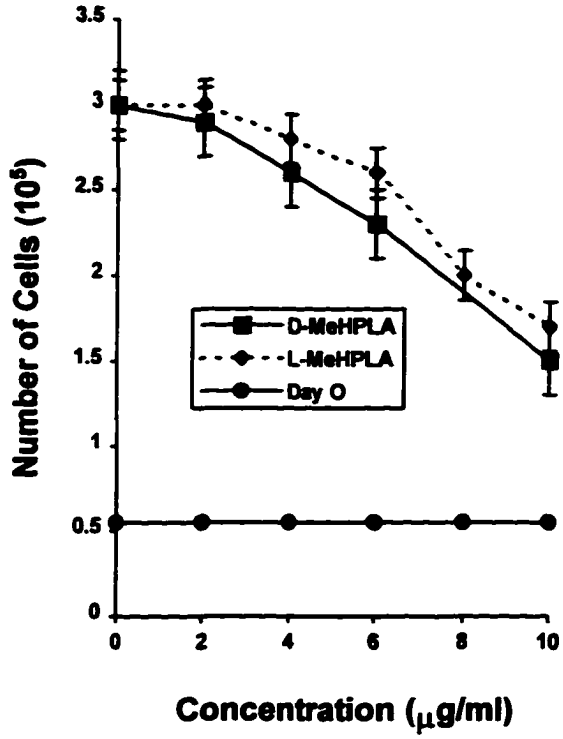



Fig. 29 **Effects of L and D-MeHPLA on MCF-7 human breast cancer cell growth in vitro.** Cell growth was evaluated by counting the number of cells per dish after 10 days.

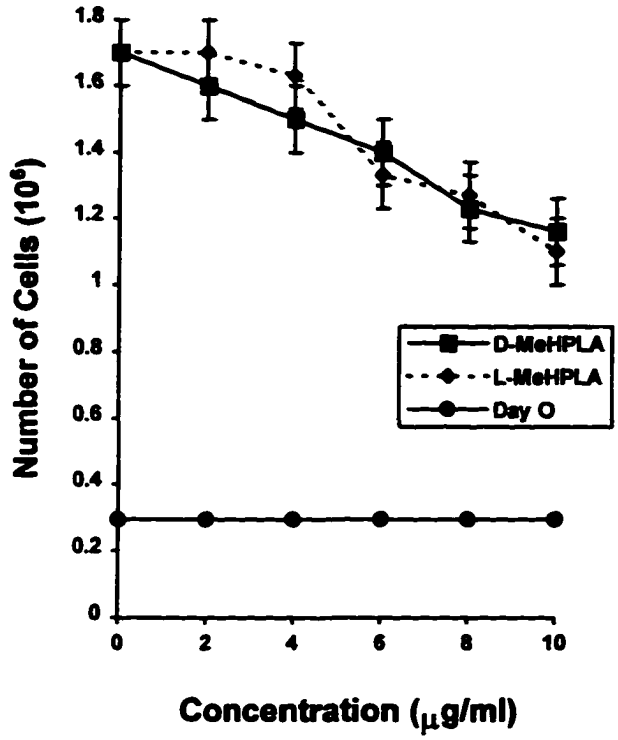



Fig. 30. **Effects of L and D-MeHPLA on MDA-231 human breast cancer cell growth in vitro.** Cell growth is evaluated by counting the number of cells per dish after 6 days.

after six days of treatment with the enantiomers of MeHPLA. Again, no significant difference is observed in the inhibitory activity for the enantiomers of MeHPLA.

To insure that cells did not die during the middle of the study, cells were counted every other day. The results of these counts were plotted to show the daily growth of the cells (Fig. 31-36) (appendix) versus the concentration of enantiomeric MeHPLA. Again, the most significant growth suppression was seen at high concentrations of MeHPLA (6-10 μg/ml), with both enantiomers showing equivalent activity.

**Cell Growth Study of HxGC3 human colon cancer cells and LNCap human prostate cancer cells.**

Because of the ubiquitous nature of MeHPLA, it was important to obtain data on the possible involvement of MeHPLA in the suppression of other cancer cell lines. The prevalence of the endogenous receptor for MeHPLA, the type II EBS, has not been determined. However, if the receptor exists in several other cell line, as it does in MDA-231 cells, it is possible that the function of MeHPLA may be broader than currently thought. The cell growth studies on HxGC3 colon cells and LNCap prostate cells were the first attempt at testing the validity of this hypothesis. Both cell groups were subjected to growth conditions that were identical to the study for MCF-7 breast cancer cells.

Figure 37 shows the growth suppression of HxGC3 colon cancer cells by the enantiomers of MeHPLA. Although the rate of cellular proliferation was approximately equivalent to MCF-7 cells, the suppression of cell growth was a third less. As was observed in both MCF-7 and MDA-231 cells, suppression of cell growth was seen at high

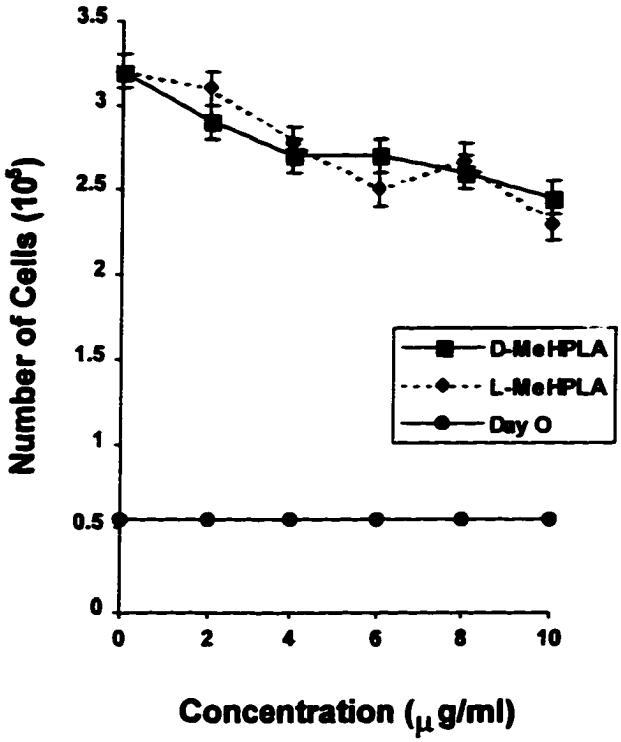



Fig. 37. **The Comparison of the effects of L and D-MeHPLA on HxGC3 colon cancer cell growth in vitro.** Cell growth was evaluated by determining the number of cells per dish after 10 days. No significant difference was observed in the effects of L and D-MeHPLA.

concentrations of MeHPLA for both enantiomers. However the suppression of cell growth was proportionally less than that of the MDA or MCF-7 cells.

LNCap prostate cancer cells were thought to be a likely target for cell growth suppression by MeHPLA. LNCap cells contain receptors for androgens, which are members of the steroid hormone receptor super family (15). As previously mentioned, this family of receptors includes the estrogen receptor and other receptors that have structural and functional similarities. Though the type II EBS cannot yet be classified as a member of the steroid hormone receptor super family, its association with the type I ER and its interaction with estradiol makes a plausible link to the receptor super family.

Figure 38 shows the effects of the enantiomers of MeHPLA on LnCap prostate cells in vitro. For both enantiomers, no significant suppression of cell growth was observed. The number of cells in the control sample was approximately equivalent to the number of cells per sample for the highest concentration of analyte. Also, there was no difference in suppression seen between the L and D enantiomer.

Figures 39-44 (appendix) shows the ten day growth of HxGC3 and LnCap cells treated with the enantiomers of MeHPLA. Suppression of cell growth was seen at higher concentrations of MeHPLA for both enantiomers, however, the amount of suppression was not significant when compared to the control.

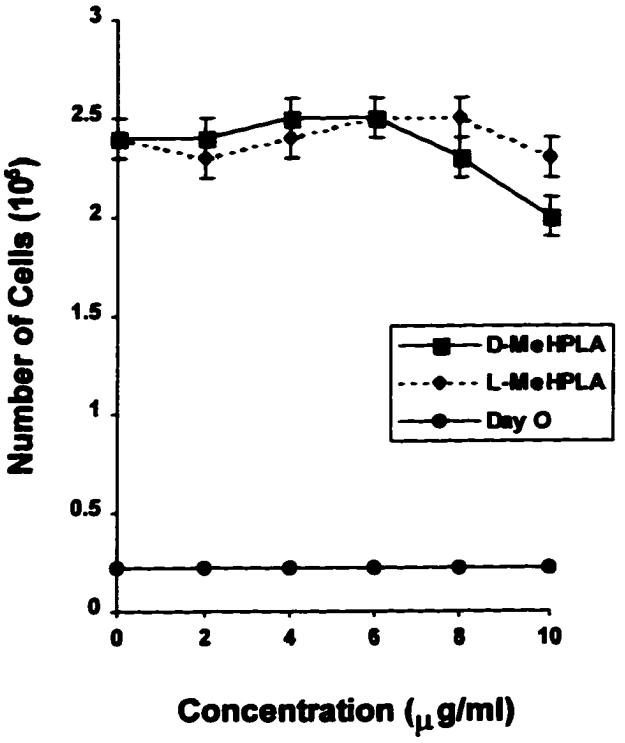



Fig. 38. **The Comparison of the effects of L and D MeHPLA on LNCap prostate cancer cell growth in vitro.** No significant suppression of growth was observed on day 10 when compared to the day 10 control.

# DISCUSSION

## Comparison of Synthetic Methods

The enantioselective reduction of MeHPPA required a large quantity of starting material (150-200 mg) to yield two to three milligrams of MeHPLA. Seventy percent of the starting product, ended up as a product other than MeHPLA due to keto-enol tautomerization. Keto-enol tautomerization caused low catalytic yield because most of the available starting product was in the wrong conformation to be reduced to L or D-MeHPLA. Additionally, both the catalytic and purification procedures for the enantioselective reduction were labor intensive. The reduction had to be carefully monitored because the catalyst would reduce any available carbonyl group. Once MeHPLA was made, it was not protected from further attack by the catalyst. HPLC injections were made at 15 minute intervals over 90 minutes to insure that the reaction was quenched at the peak of MeHPLA production.

Isolating the low yield of MeHPLA from the enantioselective reduction mixture was also labor intensive. The catalyst, MeHPLA, residual starting material, and major product were separated from the reaction mixture exclusively by HPLC on a semi-preparatory C18 Ag-column. This method yielded pure samples of MeHPLA, however, many hours were sacrificed collecting product as it eluted from the semi-prep column. The final recovery of MeHPLA from the catalytic reduction was approximately 2%.

The diastereomeric method for the synthesis of the enantiomers of MeHPLA was superior to the enantioselective reduction for several reasons. First, the yield of the enantiomers was approximately 23-fold greater. The final yield for the diastereomeric

separation was approximately 46%. The reaction needed no intense monitoring because there were no side reactions that could destroy the desired product. Shortly after the chiral amine and racemic HPLA dissolved in THF, crystals of the L-salt began to precipitate. Purification of the racemates of HPLA was done by harvesting the crystals, acidifying in an aqueous solution, and extracting of the HPLA with ethyl acetate. This yielded samples that were approximately 95% L-HPLA and 85% D-HPLA. The amount of enantiomeric purity was quantified by direct injection on the OA 5000 column.

The quantification of enantiomeric purity from the enantioselective reduction required the development of additional chemical procedures. Originally, quantification was attempted by hydrolyzing the methyl group of MeHPLA and then injecting the subsequent HPLA on the OA 5000 column. However, the hydrolysis of MeHPLA lead to the production of an enolate anion because of the acidity of the proton at the chiral center. The acid-base chemistry at the chiral center was faster than the hydrolytic chemistry of the ester group. The strong base quickly reacted with the acidic proton on the chiral carbon, destroying the chiral center. Upon neutralization of the reaction, the chiral center was reprotonated to yield equal amounts of the L and D-enantiomer. This was confirmed by injection of the hydrolysis product on to the OA 5000 column to find that the enantiomers were present in equal quantities.

Another method was discovered that could be used to quantify the enantioselectivity of the catalyst. Diastereomers of MeHPLA could be made by derivatizing the hydroxyl groups of MeHPLA with enantiomerically pure methyl chloroformate (44). This would create diastereomers of L and D-MeHPLA which could

then be quantified by HPLC or GC-MS. Although this method was very promising, it was still labor intensive in comparison the diasteremeric separation of HPLA with the chiral amine. Using the diastereomeric separation of HPLA, the product could be injected directly on the OA 5000 column after the recrystalization step or after the organic extraction step to quantitate the amount of each enantiomer present.

**Type II EBS Binding Study**

Binding studies with the L/D enantiomers of MeHPLA suggested that there may be stereospecificity associated with the ligand binding domain (LBD) of the type II EBS. Our study showed that L-MeHPLA had a 26% greater affinity for the type II EBS than D-MeHPLA. Asymmetry in the type II EBS LBD is consistent with what is known about the LBD of the type I ER and other members of the steroid receptor superfamily. Mutational analysis of the type I ER has mapped specific amino acids that are necessary for the binding of estradiol to the receptor. Presently a group at Baylor University, lead by B. Markaverich, is working on the isolation and purification of the type II EBS. This will allow for the mapping of the receptor and the eventual mutational analysis of the type II EBS LBD.

Because of the possible asymmetry of the type II EBS LBD, it is likely that the identity of the endogenous isomer of MeHPLA is the L-enantiomer. This projection idea is supported by several observations. First, only the L-isomer of HPLA is found in the urine of patients with tyrosyluria. Second, the endogenous pathway for the enzymatic synthesis of HPLA would only produce the L-isomer, because naturally occurring enzymes such as LDH and malate dehydrogenase are specific for the production of L-

enantiomers. If the L-HPLA is the endogenous starting material for the synthesis of MeHPLA, one would expect MeHPLA to be the L-isomer. Also, specific esterase activity is believed to cleave the methyl group from MeHPLA to produce HPLA. If HPLA from the enzymatic cleavage of MeHPLA were the D-isomer, it could be detected as such. However, all detectable HPLA has been the L-enantiomer. D-MeHPLA or D-HPLA have not been detected as a consequence of any anabolic or catabolic pathway.

**Cell Growth Studies on MCF-7 and MDA-231 Breast Cancer Cells**

Enantioselective binding of MeHPLA to the type II EBS did not translate into superior inhibitory activity for either MCF-7 or MDA-231 cells. Both L and D-MeHPLA suppressed the growth of breast cancer cells in vitro, however, no significant difference was found in their ability to suppress cellular proliferation in the 2 - 10 μg/ml range. A possible reason for this finding may be due to concentration effects that have been observed with other chiral molecules under physiological conditions.

For example, L and D-thalidamide show a concentration dependent suppression of the release of Tumor Necrosis Factor-alpha (TNF-alpha). At low concentrations (5-12.5 μg/ml), both enantiomers inhibit the release of TNF-alpha equally (45). However, at high concentrations (25-50 μg/ml), there was a weak but statistically significant selectivity toward L-thalidomide (45).

In a related experiment, the opposite concentration effect was observed in the ability of the enantiomers of leucine to replace each other in an acid-extractable pool of mammalian cells. At high concentrations (millimolar range), L and D-leucine had similar cellular uptake and displacement properties (46). However, below the millimolar

concentration range, there was a distinct difference in the ability of L and D-leucine to be incorporated into the acid-extractable cell pool (46). Unlabeled L-leucine displaced an equivalent amount of labeled D-leucine from a preloaded acid-extractable pool (46). Similar differences were observed with other amino acids, including histidine, isoleucine, and phenylalanine (46).

Both of the preceding examples show that concentration dependent differences found in the utilization of chiral molecules can be found under physiological conditions. For this study, equivalent concentrations of L and D-MeHPLA had the same effect on cellular proliferation, despite their unequivalent ability of bind the type II EBS. Data from tritiated-MeHPLA studies showed that 1% of MeHPLA is incorporated into cells dosed with a concentration range of 1 to 10 mg/ml (3). Therefore, the intracellular concentration of MeHPLA incorporated into MCF-7 and MDA-231 cells was actually 1 to 10 ng/ml or 102 to 510 nM. This concentration range corresponds well with the binding studies which had a concentration range of 10 to 100000 nM. Thus, it appears that for equivalent concentrations of the enantiomers of MeHPLA, binding affinity is not related to cell growth inhibition. As observed with the enantiomers of thalidamide, low concentrations have an equivalent effect on TNF-alpha release, while high concentrations, show significant enantiomeric specificity. Though, MCF-7 and MDA-231 cells were treated with microgram analyte quantities, the intracellular concentration was significantly lower, in the nanogram range.

It is therefore conceivable that this concentration dependent effect observed with the enantiomers of thalidamide may also be in operation with the enantiomers of

MeHPLA. The next step in this study would be to increase the concentration of MeHPLA. It is possible that at higher concentrations of MeHPLA a significant difference may be found in the ability of the enantiomers to suppress cell growth.

Discrepancies in our findings may possibly be related to mutations within the type II EBS itself. Cell growth studies performed by B. Markaverich showed significantly better cellular suppression for the same concentration range as used in our study. It is conceivable that genetic changes have occurred in the type II EBS, since the cells have gone through a number of passages. This observation and the concentration differences discussed previously could possibly explain the discrepancies in cellular growth suppression seen for the two studies.

**Molecular Opposition to Estrogen**

Unopposed estrogen, described as "estrogen dominance", is the main cause of most uterine cancers and at least one third of all breast cancers (47). Molecular opposition to estrogen stimulated cellular proliferation is a recurring treatment in the battle against estrogen dependent cancers. The best example of this style of therapy is the drug tamoxifen which binds effectively with the type I ER and interrupts its ability to activate transcription. Interestingly, nature has provided mechanisms that have the same ability to oppose estrogen stimulation.

Natural compounds like MeHPLA and compounds known as "phytoestrogens," also appear to work in opposition to estrogen and provide a protective value against hormone dependent cancers. In this study, MeHPLA has been demonstrated once again to suppress the growth of breast cancer cells in vitro. Because of its strong interaction

with the type II EBS, the current thinking is that MeHPLA is a mediator of cellular suppression by its interaction with this receptor. A plausible mechanism of interaction involves MeHPLA binding the type II EBS. This complex then interacts with an enhancer region of the DNA to suppress transcription. This mechanism is supported by the observation that when estrogen is present the binding affinity of MeHPLA for the type II EBS is decreased at low concentrations of MeHPLA and cell inhibition is reversed. In this scenario, an increase in type II EBS's is observed in the cellular matrix upon estrogen stimulation. Estrogen's ability to change the binding affinity of the type II EBS for MeHPLA may account for this sudden increase in type II EBS concentration (2). However, at increased concentrations of MeHPLA, inhibition is restored (2).

Our study showed that for the enantiomers of MeHPLA, in the absence of estrogen, there was no significant difference in their ability to suppress cellular proliferation, even though there was a significant difference in the enantiomers' ability to bind the type II EBS. This indicates that a more complicated mechanism may exist for the interaction of MeHPLA in which multiple mechanisms of suppression may be responsible for effecting cell growth. This type of mechanism is not uncommon and has been noted in the interaction of dietary phytoestrogens with normal and malignant cells.

For example, dietary phytoestrogens have been demonstrated as effective binders to the type II EBS. Though designated the "bioflavanoid receptor", the mechanism of phytoestrgoen-type II EBS cellular suppression is unclear and in some cases, data suggests that interaction with the type II EBS is not the main effector of cellular suppression (48). In fact, it appears that phytoestrogens compete with endogenous

estrogens for the active site of estrogen biosynthesizing and metabolizing enzymes and thus reduce the concentration of biologically active endogenous estrogen (48).

Phytoestrogens, like genistein, have been demonstrated to inhibit protein tyrosine kinases (49). Oncogenes produce mutated cellular proteins that catalyze the autophosphorylation of tyrosine kinases. The phytoestrogen, genistein, is able to block this auto-catalysis. Also, in vitro experiments, genistein inhibited the phosphorylation of natural and artificial substrates of protein tyrosine kinases (49).

Other phytoestrogens interfere with the biosynthesis of estrogen. Compounds such as coumestrol, coumarin, and quercetin inhibit the reduction of estrone to active estradiol by the estrogen specific enzyme, 17β-hydroxysteroid oxidoreductase type I (17β-HSD type I) (48). These compounds are found in leguminous plants, beans, fruits, and vegetables. 17β-HSD type I is expressed in steroidgenic cells and placental cells, as well as in normal and malignant breast and endometrial tissue.

Phytoestrogens have been demonstrated to be inhibitors of DNA topoisomerases (49). The structure of genistein and other phytoestrogens allows them to intercalate between DNA strands. Geinistein inhibits the DNA:DNA topoisomerase complex by stabilizing the complex causing strand breaks during replication. This fragmentation of DNA may result in the programmed cell death (apoptosis) of rapidly growing malignant tissue.

Phytoestrogens may also help to prevent cancer as a result of their biological antioxidant properties (50). Reactive oxygen species may interact with electron rich DNA and play an important role in mutagenesis and carcinogenesis. Phytoestrogens have

been shown to inhibit the production of peroxide in response to phorbol ester TPA in HL-60 cells and human polymorphonuclear cells (49). Evidence indicates that the inhibition is not simply caused by a chemical reaction between phytoestrogens and hydrogen peroxide, but at a biochemical level.

Since all of the ramifications concerning the specific biological mechanisms for the interaction of MeHPLA is not known, to suggest that its ability to suppress cellular proliferation may only be due to its interaction with the type II EBS is premature. Since, there is a strong possibility that MeHPLA is a metabolite of bioflavonoid/phytoestrogen degradation, it may retain many of the properties of its' precursors and have several mechanisms for the suppression of cell growth. It is also possible that all bioflavonoids degrade to MeHPLA and that it is MeHPLA that is doing all these things. This would correlate to the observations seen with the enantiomers of MeHPLA.

**A Link Between MeHPLA, Phytoestrogens, Xenoestrogens and Cancer**

The endogenous compound MeHPLA and phytoestrogens have been observed to be disrupters of endocrine and reproductive function. Extreme cases of phytoestrogen interaction have been documented in quail, deer mice, and livestock (51). In these cases, phytoestrogen accumulation has lead to infertility in the adults of these species. However, phytoestrogen accumulation is rare because most of these compounds are metabolized and excreted from the host (51). For example, if MeHPLA is a metabolite of phytoestrogens, it is further metabolized by specific esterases and the resulting HPLA is excreted in urine. It has been hypothesized that the ability of certain plants to render predators infertile is an evolutionary response of the plant to protect its' species (51).

However, in most cases, evolution has a double edged sword, in which, predators are also able to adapt to the toxin and therefore extinguish its' effects. Such is the case for insects and bacteria, which have many generations in a short period. In this case, insects are able to adapt to poisons quickly and bacteria can quickly adapt to antibiotic therapies.

But, in the adaptation of animals to phytoestrogen, the result has been more than just a means of metabolizing toxic agents. Phytoestrogens have retained enough of their endocrine disruptive properties so that they are therapeutic for the prevention of hormone dependent malignancies, instead of being harmful agents. However, an interesting correlation has been noted in recent years between phytoestrogens and a group of compounds now known as xenoestrogens.

Xenoestrogens are foreign compounds that act as endocrine disrupters (51). These compounds number in the hundreds and include plastics, pesticides, and organics, such as PCB's, DDT, and Furan. The toxicity of these compounds have been most notably documented in the alligator population of Lake Apopka, FL (52-53). Alligator death and infertility has been linked to the accumulation of toxic chemicals in the animals reproductive system, leading to follicle damage in the female and sertoli cell damage in the male (52-53). Lipid soluble xenoestrogens from chemical contamination had become part of the ecosystem of Lake Apopka. Ultra-high concentration of xenoestrogens were found in plants and the fatty tissue of animals which used that lake as a resource. However, no xenoestrogens were found in the lake. All of the contaminates had been moved from the waters to the tissues of species inhabiting the area.

Like phytoestrogens, xenestrogens disrupt the endocrine and reproductive powers of the host. However, unlike phytoestrogens, xenoestrogens are nonbiodegradable and bioaccumulate in plants and animals. Subsequently, they are passed from host to host along the food chain (51). The effects of these xenoestrogens have shown up in salmon, trout, turtles, gulls, porpoises, and the Bald eagle (51). Sexual abnormalities and embryonic deformities were noted for all the previously mentioned animals. The evolutionary ability of these animals to adapt to these toxins was diminished, because the number of pollutants were excessive and higher animals do not have the ability to produce several generations in short periods as their insect and bacterial counterparts (51). Therefore, it appears that both phytoestrogens and xenoestrogens have caused reproductive anomalies in animals. However, the preponderance of toxic chemicals and their tendency to bioaccumlate in the fatty tissue of animals make them particularly toxic.

Recently, it has been suggested that the kind of follicular damage seen in female alligators may be a contributing factor to breast and uterine cancer in women (47). Unopposed estrogen is the major cause of most uterine cancers and at least one-third of all breast cancers. It has been suggested that xenoestrogen damaged follicles in humans have led to a condition in which the natural hormones of menstruating women have been adversely effected, so that estrogen is produced in preponderance without any natural opposition (47). It has been suggested that the natural opposer to estrogen is the hormone, progesterone (47).

Progesterone is one of the two main hormones made by the ovaries of women. Studies have shown that progesterone decreases the growth of human breast epithelial

cells in vivo in the presence or absence of estradiol (54). While unopposed estrogen leads to the proliferation of human breast epithelial cells (54). During the menstrual cycle, the release of estrogen is followed by the subsequent secretion of progesterone from the corpus luteum after ovulation. If follicles are damaged, as is the case with animals that have bioaccumlations of xenoestrogens, the corpus luteum is abnormal, and progesterone may not be produced or released. In a recent study, is was discovered that seven out of eighteen women age 24 to 42 did not ovulate (55). Without ovulation, no egg is released, the corpus luteum is not formed, and no progesterone is produced. This absence of progesterone in woman creates this condition in which estrogen is unopposed. Therefore, the lack of progesterone has been linked to uterine and breast cancer, luteal phase failure and miscarriage, PMS, osteoporosis, and autoimmune disorders such as Lupus erythematosis and Hashimoto's thyroiditis (47).

A classic example of follicular damage was seen with the use of the xenoestrogen, diethylstilbesterol (DES). One of the most susceptible times for xenoestrogen virulence is in the developing embryo. In the 1950's, DES was given to women who had difficulties maintaining pregnancies. It was subsequently discovered that the female children of patients who received DES during their pregnancies had a greater chance of developing cancer of the uterus and cervix. In male children, DES therapy lead to undescended testes, lower sperm counts, penis abnormalities, and increase incidence of prostate cancer. In these cases, the xenoestrogen damage effected the reproductive systems of the offspring of the patients that received DES therapy.

The same was true for the offspring of Florida alligators. Studies have looked at younger alligators and it was discovered that follicular and sertoli cell damage was already present even in the juvenile alligators. At 6 months of age, female alligators of Lake Apopka were found to have plasma levels of 17β-estradiol twice as high as alligators in control lakes (52). Juvenile male alligators were found to have low testosterone and undeveloped penises (53). Similar abnormalities were found in the Florida panther, in which male panthers had the same estradiol level as female panthers (56). Therefore, it appears that it is the offspring of affected species that show the greatest abnormalities in their sexual development. Wildlife studies demonstrate that both sexes are affected and experience modifications of gonadal and reproductive tract development. The ultimate effect is the abnormal functioning, synthesis, and metabolism of reproductive hormones. Data from wildlife studies represent models for the possible interaction of xenoestrogen in humans and may help to explain the increasing number of malignant diseases.

## Summary

MeHPLA, phytoestrogens, xenoestrogens and cancer are linked. Phytoestrogens are plant compounds that have been demonstrated to have cancer protective properties through various biological mechanisms. Phytoestrogens and xenoestrogens have been documented as causing infertility in several animal species. However, phytoestrogens are biodegradable and do not bioaccumlate in the animals, therefore adaptive evolution has provided animals with the means of metabolizing phytoestrogens, while harnessing their power for protection against disease. Unlike phytoestrogens, xenoestrogen are not biodegradable and bioaccumulate in the fatty tissue of animals. In the last few years, hundreds of xenoestrogen have been discovered in animals that have fertility abnormalities and other reproductive tract anomalies. The preponderance of xenoestrogens have impaired the adaptive ability of affected species to develop physiological mechanism to metabolize and excrete xenoestrogens.

A specific consequence of xenoestrogen poisoning is the development of the condition known as estrogen dominance. This excess of estrogen has been linked to breast and uterine cancer in humans. It appears that phytoestrogens play a role in providing protection against estrogen dominance in several ways. They inhibit specific enzymes that are involved in the biosynthesis of estrogen. They inhibit DNA topoisomerases that are necessary for DNA replication and cellular proliferation. Phytoestrogens can intercalate between DNA stands in rapidly growing cancer cells causing strand breaks and the subsequent programmed death of the cell. Phytoestrogens

may also act as antioxidants by scavenging reactive oxygen species that may also contribute to genetic abnormalities.

Since there are several mechanisms of biological action for phytoestrogens, it is likely that MeHPLA may also have several points of physiological interaction. Data from the study of the enantiomers of MeHPLA supports this conclusion. Both phytoestrogens and MeHPLA bind to the type II EBS. Phytoestrogen binding is not the major mechanism of cellular suppression. Since the L-MeHPLA has a greater affinity for the type II EBS than D-MeHPLA, it would be expected to have a greater biological activity. However, ER binding study data was obtained in cell free homogenates. Growth studies were done on living cells. It is perhaps possible that cellular methylases neutralized exogenously supplied MeHPLA (57). This would reduce the concentration of L and D MeHPLA. If L-MeHPLA is the endogenous isomer, it is possible that methylases could metabolize it in preference to the D-isomer. Such a situation would possibly give the observed biological activity for the enantiomers. Since, this research has shown that L and D-MeHPLA have equivalent biological activity, it is also possible that the mechanism of MeHPLA interaction is as varied as phytoestrogen interaction. This evidence supports the theory that MeHPLA is a metabolite of bioflavonoid /phytoestrogen degradation.

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# APPENDIX

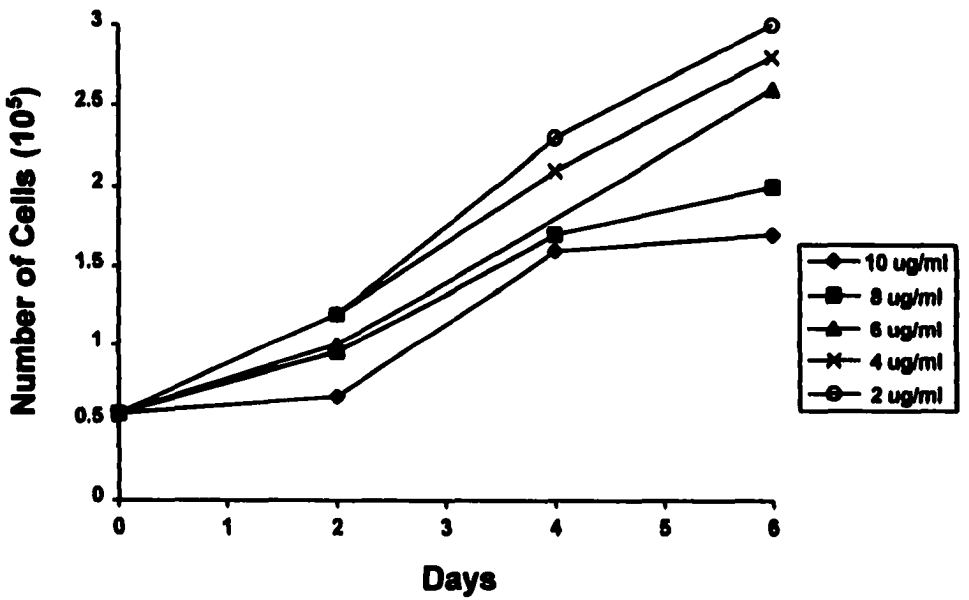


Fig. 31. **The Effects of L-MeHPLA on MCF-7 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

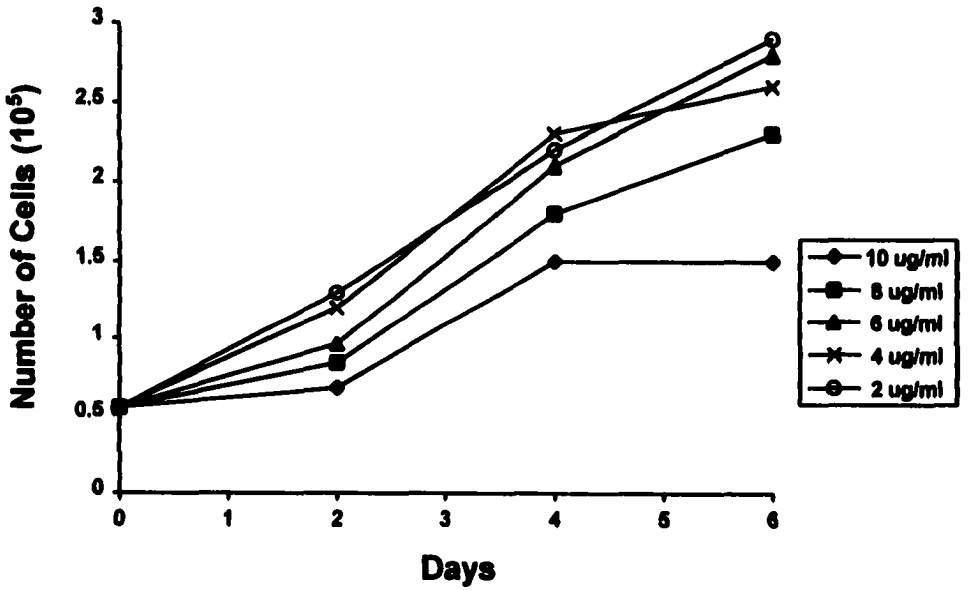


Fig. 32. **The Effects of D-MeHPLA on MCF-7 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

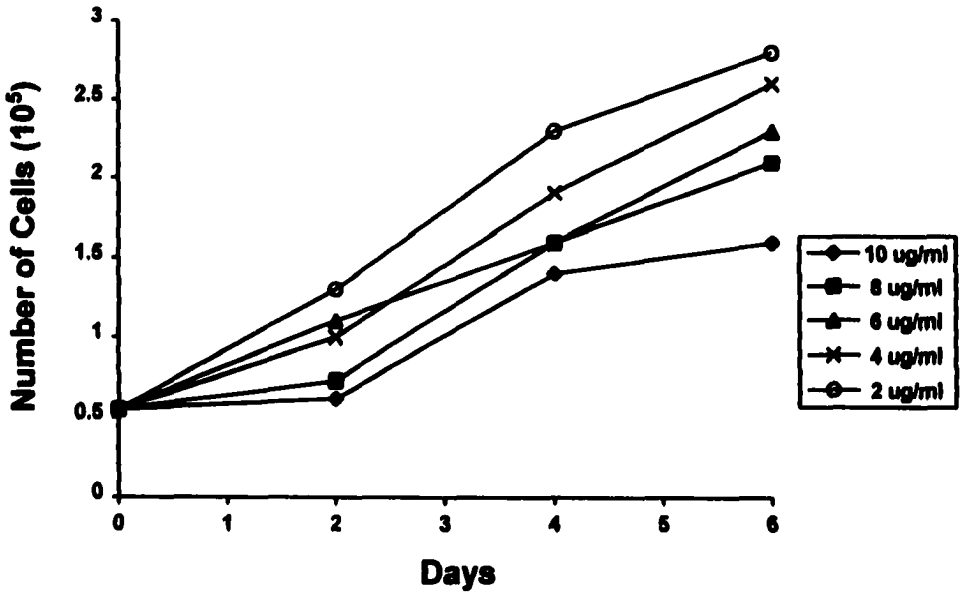


Fig. 33. **The Effects of Racemic MeHPLA on MCF-7 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

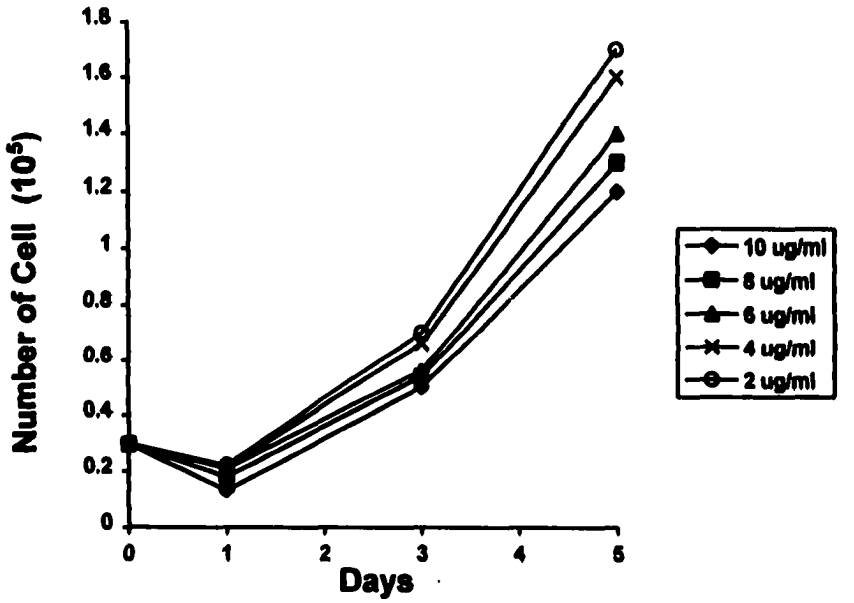


Fig. 34. **The Effects of L-MeHPLA on MDA-231 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

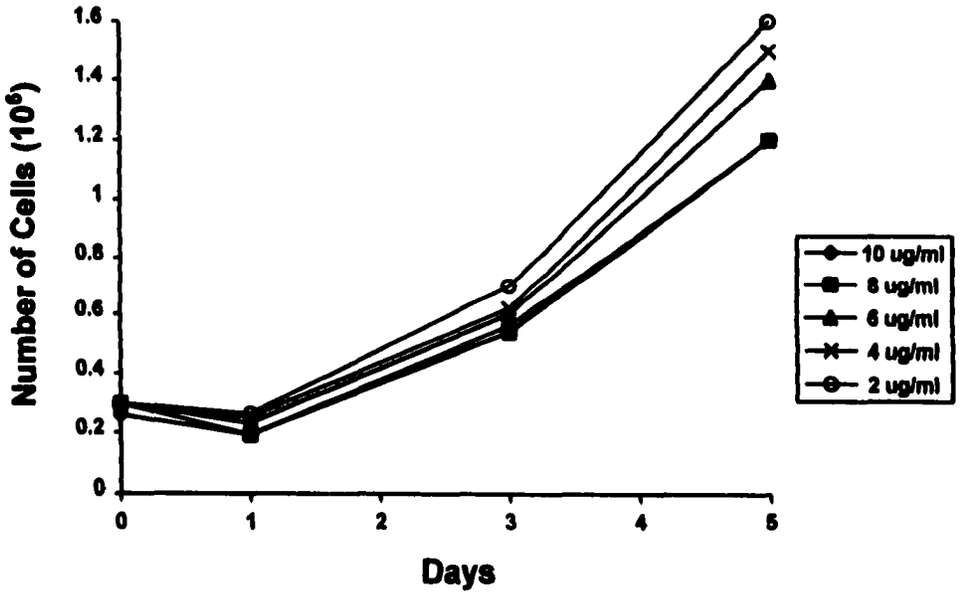


Fig. 35. **The Effects of D-MeHPLA on MDA-231 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

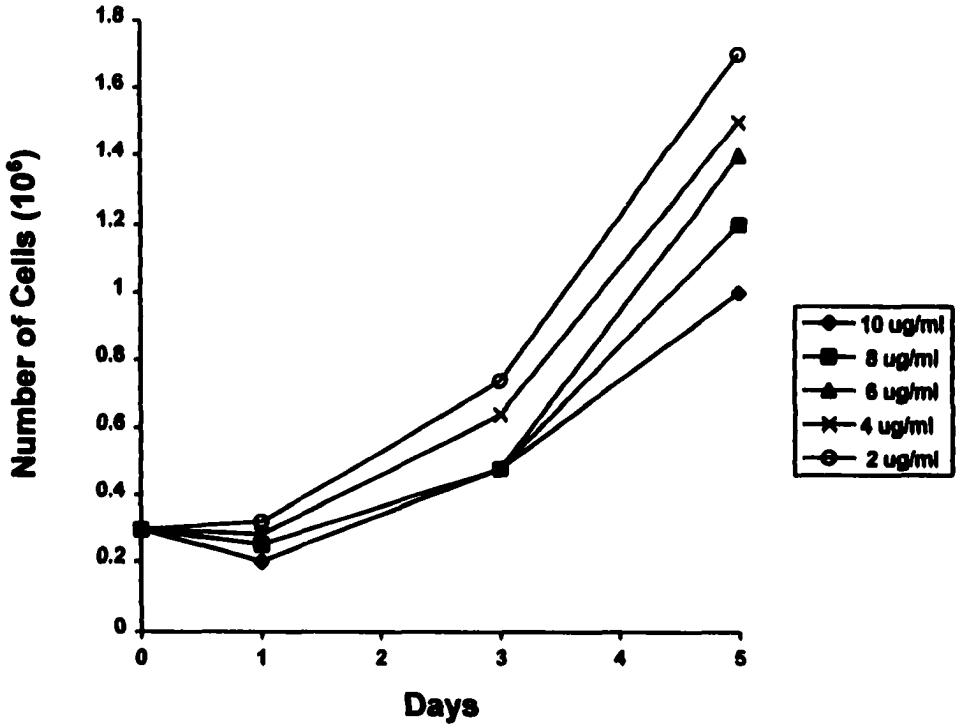


Fig. 36. **The Effects of Racemic MeHPLA on MDA-231 human breast cancer cell growth in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

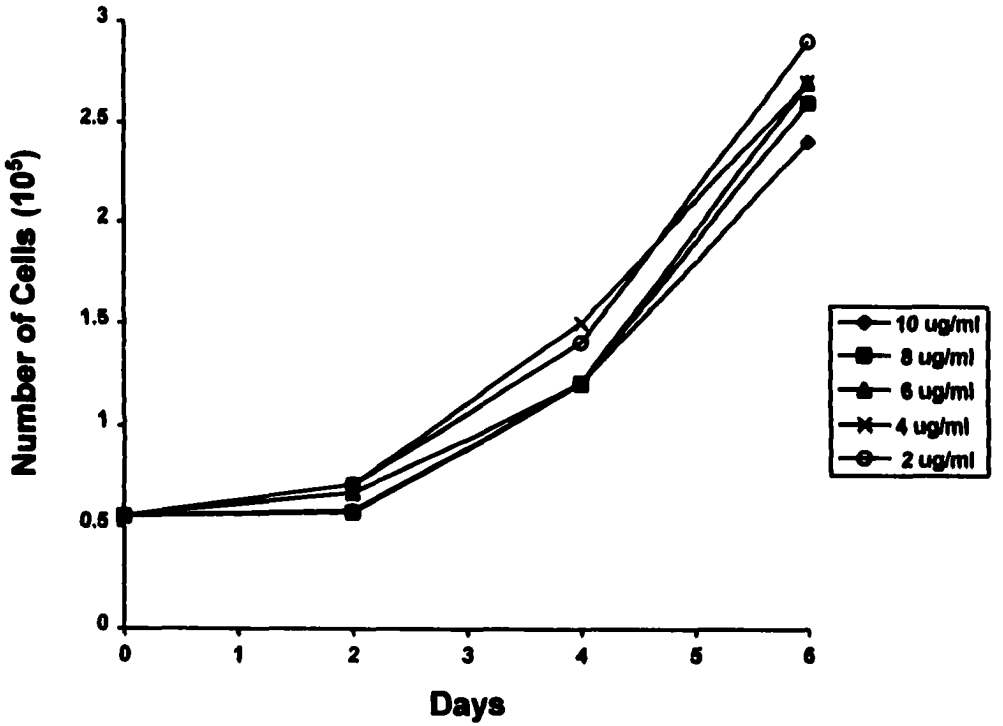


Fig. 39. **The Effects of D-MeHPLA on the growth of HxGC3 colon cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

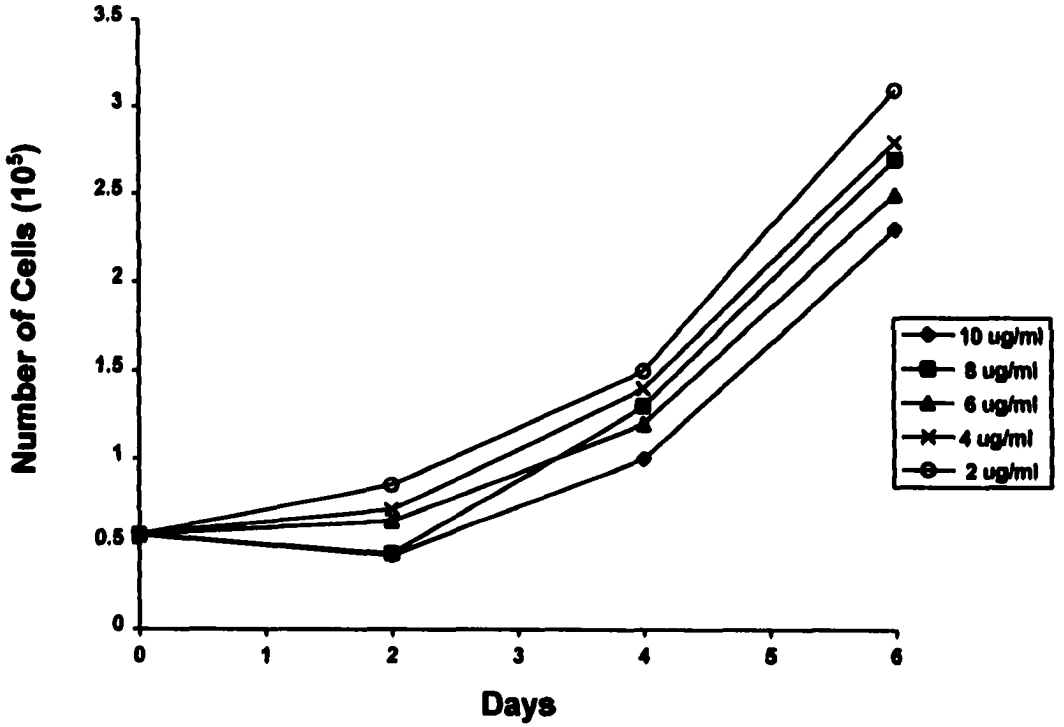


Fig 40. **The effects of L-MeHPLA on the growth of HxGC3 colon cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cell growth is seen at high concentrations.

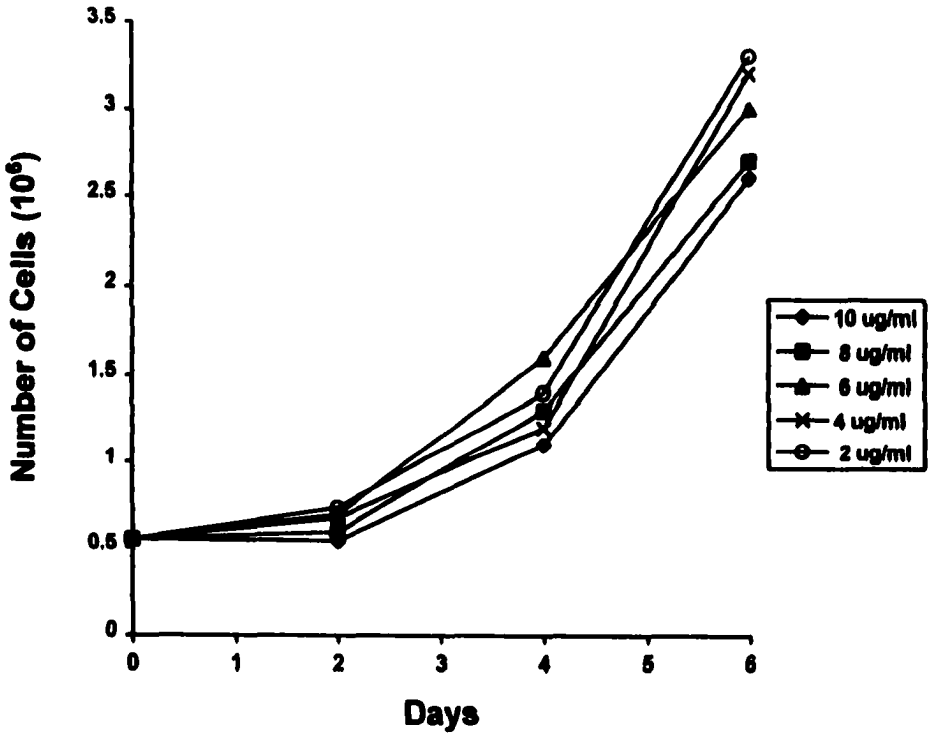


Fig. 41. **The effects of racemic MeHPLA on the growth of HxGC3 colon cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cell growth is seen at high concentrations.

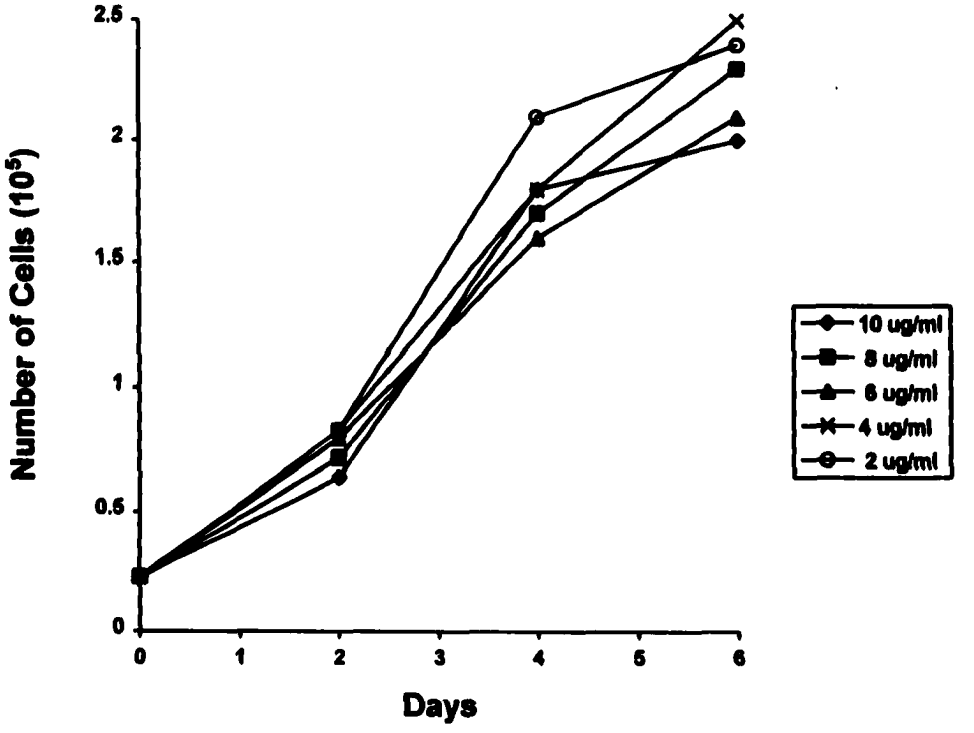


Fig. 42. **The effects of D-MeHPLA on the growth of LNCap prostate cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

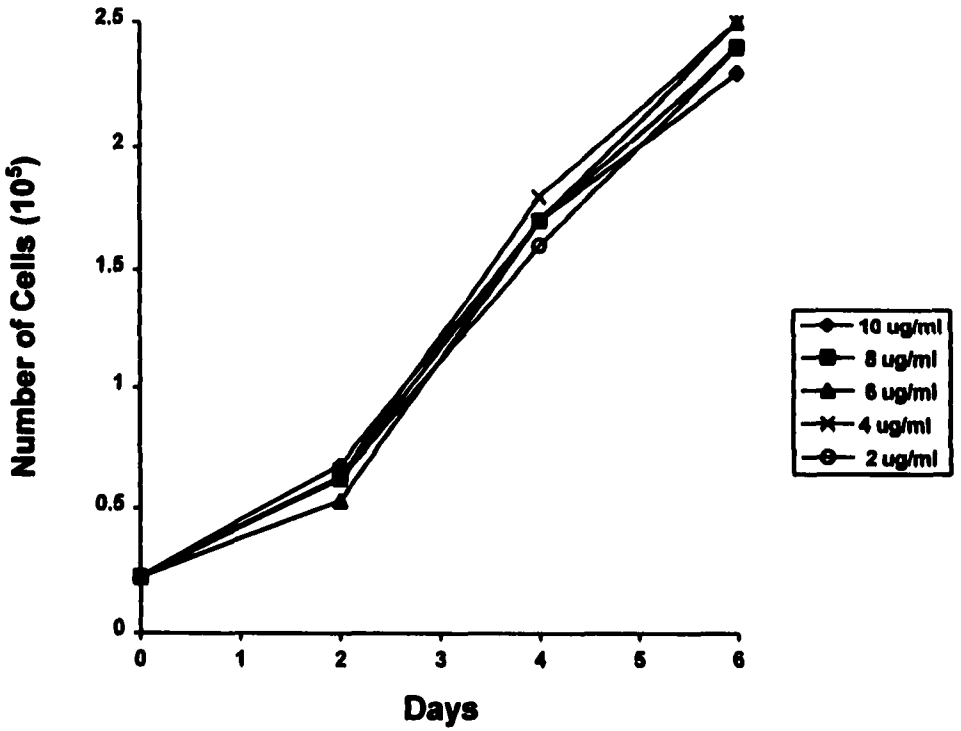


Fig 43. **The effects of L-MeHPLA on the growth of LNCap prostate cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

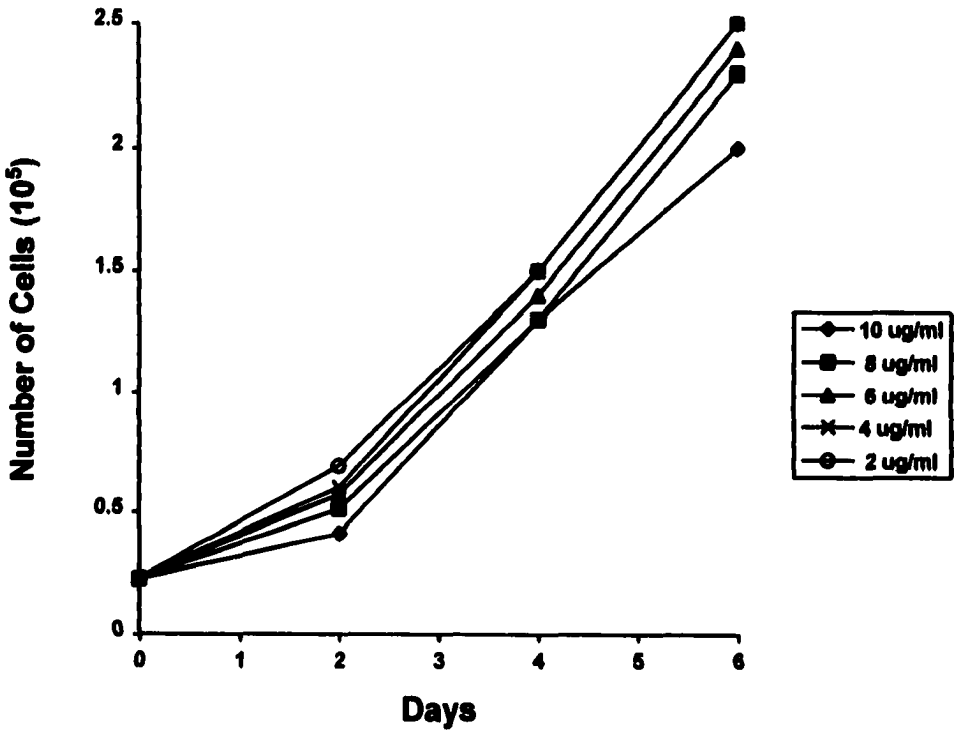


Fig 44. **The effects of racemic MeHPLA on the growth of LNCap prostate cancer cells in vitro.** Cell growth is determined by counting the number of cell per dish at various concentrations over several days. Suppression of cancer cell growth is seen at high concentrations of MeHPLA.

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