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CHEMICAL AND SPECTROSCOPIC STUDIES ON RETINOIDS MICROBIAL METABOLITES AND RETINOIDS/PROTEIN INTERACTIONS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By
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* * * * *

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Retinoids have recently been developed as useful agents in the treatment of skin disorders and as cancer chemopreventive agents. Etretinate, one of the synthetic retinoids, is used in the treatment of psoriasis. Etretinate has a long elimination half-life and is recognized as one of the most potent teratogens. Etretinate has been less well studied than the more common retinoids in terms of its metabolism and its mechanism of action is not fully known. Retinoids are believed to exert their biological actions by binding to nuclear receptors. A number of retinoid binding proteins have been discovered and it is generally believed that these binding proteins are involved in binding and transporting retinoids to their target sites of action. Some of the retinoid-binding proteins are also thought to be involved in retinoid metabolism, although no direct evidence has been provided yet in support of this belief.

Microbial fermentation methods are often used as models to study the mammalian metabolism of xenobiotic compounds. In this study we have used *Cunninghamella elegans* 9245 and *Syncephalastrum racemosum* 18192 for studying the biotransformation of etretinate model compound 11 and etretinate. We have developed methods to isolate and identify their major biotransformation products. Using these methods we have isolated 21 biotransformation products and have successfully characterized 9 of them.
We have also synthesized 4-oxo-β-ionone and $^{13}$C-labelled β-ionones and have used them as models or ligands for the protein β-lactoglobulin B, in our isotope-edited NMR studies to characterize the protein-bound conformation of retinoids.

The methods developed for the biotransformation studies of etretinate and its analog 11 are reported. Also, the syntheses of 4-oxo-β-ionone and $^{13}$C-labelled β-ionones are summarized. We predict a twisted 6-s-cis relationship between the side chain and the trimethylcyclohexenyl ring in the bound-conformation of these retinoids. The results of the experiments supporting our prediction are also reported.
DEDICATION

Dedicated to my grandparents and my parents
ACKNOWLEDGMENTS

I wish to thank my advisor Dr. Robert W. Curley, Jr. for his advice, guidance, and patience during my career as a graduate student. I also wish to thank Dr. Larry W. Robertson for his suggestions and input regarding my research.

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<td>Alcohol dehydrogenase</td>
<td>ADH</td>
</tr>
<tr>
<td>American type culture collection</td>
<td>ATCC</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>ATP</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>BLG</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>CNS</td>
</tr>
<tr>
<td>Correlation spectroscopy</td>
<td>COSY</td>
</tr>
<tr>
<td>Cellular retinoic acid binding protein</td>
<td>CRABP</td>
</tr>
<tr>
<td>Cellular retinol binding protein</td>
<td>CRBP</td>
</tr>
<tr>
<td>Cerebro spinal fluid</td>
<td>CSF</td>
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<tr>
<td>Decoupling in the presence of scalar interactions</td>
<td>DIPSI</td>
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<tr>
<td>9,10-Dimethyl-1,2-benz[a]-anthracene</td>
<td>DMBA</td>
</tr>
<tr>
<td>Globally optimized alternating-phase rectangular pulses</td>
<td>GARP</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>GC</td>
</tr>
<tr>
<td>Heteronuclear multiple quantum coherence</td>
<td>HMQC</td>
</tr>
<tr>
<td>High performance liquid chromatography</td>
<td>HPLC</td>
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<tr>
<td>N-(4-hydroxyphenyl)retinamide</td>
<td>4-HPR</td>
</tr>
<tr>
<td>Interphotoreceptor retinoid binding protein</td>
<td>IRBP</td>
</tr>
<tr>
<td>International Units</td>
<td>IU</td>
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<tr>
<td>International Union of Biochemists</td>
<td>IUB</td>
</tr>
<tr>
<td>Kilodalton</td>
<td>kD</td>
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<tr>
<td>Lecithin:retinol acetyl transferase</td>
<td>LRAT</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------</td>
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<tr>
<td>Magic angle spinning</td>
<td>MAS</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
<td>NAD⁺</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
<td>NADH</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
<td>NADP⁺</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
<td>NADPH</td>
</tr>
<tr>
<td>N-bromosuccinimide</td>
<td>NBS</td>
</tr>
<tr>
<td>Nuclear Overhauser enhancement</td>
<td>NOE</td>
</tr>
<tr>
<td>Pharmamedia-yeast</td>
<td>PY</td>
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<tr>
<td>Retinoic acid</td>
<td>RA</td>
</tr>
<tr>
<td>Retinoic acid receptor</td>
<td>RAR</td>
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<tr>
<td>Retinoic acid receptor response element</td>
<td>RARE</td>
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<tr>
<td>Retinol binding protein</td>
<td>RBP</td>
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<tr>
<td>Retinoyl β-glucuronide</td>
<td>RBG</td>
</tr>
<tr>
<td>Retinoyl β-glucose</td>
<td>RBGL</td>
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<tr>
<td>Retinol equivalents</td>
<td>RE</td>
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<tr>
<td>Reverse insensitive nucleus enhancement by polarization transfer</td>
<td>REVINEPT</td>
</tr>
<tr>
<td>Rod outer segment</td>
<td>ROS</td>
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<tr>
<td>Retinal pigment epithelium</td>
<td>RPE</td>
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<tr>
<td>Retnoid X receptor</td>
<td>RXR</td>
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<tr>
<td>Serum retinol binding protein</td>
<td>SRBP</td>
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<tr>
<td>Thin-layer chromatography</td>
<td>TLC</td>
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<tr>
<td>Total correlation spectroscopy</td>
<td>TOCSY</td>
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<td>Transthyretin</td>
<td>TTR</td>
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CHAPTER 1

INTRODUCTION

1.1 HISTORICAL BACKGROUND

Retinoid is a generic term for both naturally occurring molecules and also synthetic compounds showing specific biological activities resembling those of vitamin A (retinol). Vitamin A and its metabolites, retinal and retinoic acid, are fat-soluble, unsaturated isoprenoids necessary for growth, vision, differentiation and maintenance of epithelial tissue and also for reproduction.

Night blindness was a recognized disease in ancient Egypt. It has been suggested since ancient times that there might be a substance in the diet necessary for night vision. In 1913 McCollum and Davis reported the existence in certain foods of an essential lipid-soluble substance capable of promoting growth in rats. They called this substance "Fat-Soluble A" to differentiate it from essential water-soluble nutrients, which they called "Water Soluble B." In the next decade it was shown that "Fat Soluble A" (later named vitamin A) not only maintained growth in rats but was capable of preventing xerophthalmia and night blindness. The relationship between vitamin A in animals and
the provitamin carotene in plants was also clarified after Karrer and his associates
determined the chemical structure of β-carotene in 1930 and of retinol (1) in 1931.\textsuperscript{1,2}
Arens and Van Dorp, and Isler and his associates succeeded in achieving the chemical
synthesis of pure retinoic acid and retinol.\textsuperscript{3,4} In the first half of this century studies
dealing with various aspects of the physiology and metabolism of vitamin A were
conducted. These studies led to the identification of retinal as the chromophore of the
visual pigment, besides providing considerable information about the role of vitamin A in
vision and the pathology and pathophysiology of vitamin A deficiency.\textsuperscript{5}

1.2 NOMENCLATURE

Vitamin A-active substances are compounds, other than carotenoids, that exhibit
qualitatively the biological activities of retinol. The IUPAC-IUB Commission drew up a
number of recommendations and rules for the nomenclature of vitamin A and its
derivatives.\textsuperscript{6} Retinoids are defined as a class of compounds consisting of four isoprenoid
units joined in a head-tail manner. Many synthetic analogs do not fit this definition of a
retinoid. Such compounds can exhibit their vitamin A-like activity without being vitamin
A analogs chemically, i.e. without showing “four isoprenoid units joined in a head-tail
fashion.” Also, not all biologically active synthetic retinoids are carried by cytosolic
binding proteins such as cellular retinol binding proteins (CRBP) or cytosolic retinoic
acid binding proteins (CRABP), and binding to or activation of nuclear retinoid receptors
may not be a necessary precondition for their action.\textsuperscript{7} The systematic name for retinol is
(2E, 4E, 6E, 8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol. Figure 1 shows the basic retinoid skeletal structure and its more common numbering scheme. Retinal (2) and retinoic acid (3) are oxidized metabolites of retinol. Derivatives of retinol are named as retinyl derivatives while those of retinal are named as either aldehyde derivatives or as compounds substituted by the bivalent retinylidene moiety. Derivatives of retinoic acid are named as carboxylic acid derivatives.

\[
\begin{align*}
1 \quad & R = \text{CH}_2\text{OH} \\
2 \quad & R = \text{CHO} \\
3 \quad & R = \text{COOH}
\end{align*}
\]

Figure 1. Structures of retinol and analogs.

1.3 NUTRITION AND TOXICITY

Animals are not capable of de novo synthesis of 1, or any other vitamin A-active substances or the carotenoid precursor forms. Carotenoids are red and yellow fat-soluble pigments composed of a class of hydrocarbons (carotenes) and their oxygenated derivatives (oxycarotenoids or xanthophylls). Their basic structure has eight isoprenoid units. The conjugated double bonds provide the characteristic chromophore. The major
Figure 2. Structures of carotenoids.

- β-carotene
- α-carotene
- γ-carotene
- cryptoxanthin
carotenoids found in animal and human foods are α-, β-, and γ- carotenes and cryptoxanthin. Figure 2 shows the structures and numbering system for the carotenoids. All photosynthetic organisms contain carotenoids. In order to possess vitamin A-like activity, a compound must have at least the unaltered β-ionone ring with an attached polyene side chain containing 11 carbon atoms. β-carotene has the most provitamin A activity, while the α- and the γ- carotenes and cryptoxanthin have only about half the activity of the β form. Carotenoids are present in significant amounts in commonly eaten natural foods such as fruits and vegetables. They are also found in processed foods as added colorants. These compounds are also present in animal products such as egg, milk, and butter. The dietary source of vitamin A-active substances for herbivores and carnivores are carotenoids and retinol and its esters respectively. The vitamin A value of diets is evaluated by a biological assay and a chemical assay. Biological assay in animals measures the total physiologically available vitamin A activity of the diet and therefore accounts for variables like absorption, conversion, and utilization of preformed and precursor vitamin A sources. This method, however, is time consuming and expensive. Analytical techniques, on the other hand, provide quantitative data of the potential vitamin A in a diet on a weight basis and is quick and less expensive. Since the two approaches do not measure the same qualities the need arose for a means of expressing the potential dietary vitamin A value on a weight basis in terms of its physiological equivalence. The quantitative constitution of vitamin A in foods is usually expressed in international units (IU) or as retinol equivalents (RE). One IU is defined as equivalent to
0.3 μg retinol, 0.6 μg β-carotene, and 1.2 μg of other mixed carotenoids.\textsuperscript{10,11} One RE is equivalent to 1 μg of retinol or 6 μg of β-carotene.\textsuperscript{12}

As mentioned earlier, animals require an external source of vitamin A to maintain a number of normal functions like growth, differentiation and integrity of epithelial tissue, bone marrow development, vision and reproduction. Therefore deficiency of vitamin A in the diet will have different effects depending on at what stage of the life cycle the deficiency occurs and also on whether it is an acute or a chronic deficiency. Different approaches that have been used to study the effects of vitamin A deficiency in laboratory animals are: (1) conventional rearing on vitamin A deficient diets, (2) rearing in germ-free conditions on deficient diets, and (3) conventional rearing on diets that are retinol deficient but contain other vitamin A analogs such as retinoic acid. Rearing under germ-free conditions excludes the secondary symptoms resulting from infections. Use of retinol deficient diet supplemented with other analogs provides deficiency symptoms specific for retinol besides permitting studies of vitamin A-like activity of the particular analog that has been included in the diet.\textsuperscript{9} An elevation in cerebrospinal fluid pressure is one of the first signs specific for vitamin A deficiency.\textsuperscript{13} This elevation in CSF pressure was observed in animals as a result of chronic vitamin A deficiency, but not with acute deficiency.\textsuperscript{14} This is then followed by loss of appetite and loss of weight gain when the body stores of vitamin A becomes inadequate. Hence a decline in rate of weight gain is usually considered synonymous with a vitamin A deficient diet by many investigators.\textsuperscript{15} Vitamin A deficiency also leads to hyperkeratinization of epithelial cells and also affects
the rate of differentiation of a certain subclass of goblet cells. Growing animals fed with diets containing suboptimal levels of vitamin A had altered bone formation such as thickened and less-dense bones as a result of an altered balance in the number and activity of osteoblast and osteoclast cells. Vitamin A is also required for normal reproduction in both males and females. The developing fetus is dependent on the placental transfer of retinol for organogenesis and development. This placental transfer is believed to involve maternal holo-retinol-binding protein (holo-RBP).

Vitamin A levels in humans can be affected by various other nutrients and factors. Vitamin E has been found to increase the liver levels of vitamin A, possibly due to its antioxidant property. Zinc deficiency accompanied by an impaired hepatic mobilization of vitamin A has been reported in humans, resulting in lower levels of plasma retinol and its transport protein, retinol binding protein (RBP). Decreased levels of RBP have been suggested to be due to its decreased synthesis as a result of zinc deficiency.

Hypervitaminosis A, although rare at present, could be a serious problem in the future since it appears that large doses of vitamin A may be increasingly used to treat skin disorders and for cancer chemoprevention. Hypervitaminosis A occurs as a result of acute intoxication or as a result of chronic ingestion of excessive amounts. The symptoms include headache, nausea, anorexia, vomiting and bone and joint pain. The mucocutaneous symptoms include skin and mucosal dryness, skin fragility and dermatitis. Chronic vitamin A intoxication also leads to changes in bone formation.
which include hyperostosis, periostosis, demineralization and premature closure of epiphyses.24

All biologically active retinoids, at toxic or normal levels, are found to be highly teratogenic both in animals as well as in humans. Doses of retinol exceeding 25,000 IU/day have been reported to be associated with abnormal human pregnancy outcomes. The teratogenic nature of retinoids is therefore a major concern today for future retinoid research.7,25 The clinical abnormalities induced by retinoids are rather characteristic and include CNS and craniofacial abnormalities (with internal ear and eye malformations), bone abnormalities with skeletal malformations (occasionally leading to limb defects), and cardiovascular disorders. General retardation, thymus hormone abnormalities, and parathyroid hormone deficiency have also been described in some cases. Therefore, all known therapeutic schedules with retinoids are regarded as potentially teratogenic. Based on current pharmacokinetic data, use of contraceptives 1 month before initiation of oral treatment with isotretinoin (13-cis-retinoic acid) and etretinate (a synthetic retinoid described below) and continuation of contraception for 2 months after isotretinoin and at least 2 years after etretinate/acitretin (acitretin is the free acid form of etretinate) treatment has been recommended. The US recommended daily allowance for vitamin A during pregnancy is 8,000 IU.

Metabolism of retinoids seems to play an important role in their teratogenicity. Retinol exerts its teratogenic effect after it is converted to retinoic acid (RA). It has also been suggested that 13-cis-RA is metabolized to all trans-RA which then shows teratogenicity. Retinoyl β-glucuronide (RBG) has been found to be less teratogenic than
all *trans*-RA and retinoyl β-glucose (RBGL). The difference in the teratogenicity of RBG and RBGL has been attributed to the rate of absorption from the GI tract and also to the rate of hydrolysis of these retinoids in vivo. The rate of absorption of RBGL seems to be similar to that of RA, whereas RBG appears to be bound tightly to macromolecular components in the stomach contents for a long time (several hours).26

1.4 BIOLOGICAL FUNCTIONS OF VITAMIN A

As mentioned earlier, vitamin A is essential for a variety of normal biological functions such as growth, development, the visual cycle and epithelial cell differentiation. This complex set of biological functions are served by a relatively simple retinoid structure and is brought about by a single functional group and a set of conjugated double bonds. Retinol is required for reproduction and retinal for the visual cycle while retinoic acid and its derivatives appear to be essential for differentiation and growth and development. Also, the *cis*- and *trans*- isomers confer specificity for the actions of these molecules.

1.4.1 VISUAL CYCLE

Vitamin A has been associated with the eye and vision for thousands of years as evidenced by cures for night blindness in ancient Egypt.27 Two aspects of retinoid function are uniquely associated with the visual system. They are 1) the use of photoisomerization to trigger a response, and 2) the enzymatic production of 11-*cis*-

retinal (4) and associated metabolic processing. Thus, 11-\textit{cis}-retinal is considered the chromophore of all known visual pigments. The vertebrate retina is a specialized organ for the detection of light. It detects both the intensity and the wavelength of the stimulus since the photoreceptors are further specialized as cones, which are used for color vision at high levels of illumination, and rods, used for the perception of motion and monochromatic vision at low levels of illumination.\textsuperscript{28} The inner retina is nourished by retinal blood vessels whereas the outer retina, including the photoreceptors, depend on choroidal blood flow for its nourishment. Since Retinal Pigment Epithelium (RPE) is present in between the photoreceptors and choroidal vessels, they play an important role in photoreceptor nourishment, including the delivery of retinoids. Also, the isomerase enzyme that generates 11-\textit{cis}-retinoid as well as several other enzymes involved in the visual cycle are present in RPE. The extracellular compartment between the photoreceptors and RPE is referred to as the interphotoreceptor matrix (subretinal space). There is a considerable flow of retinoids between RPE and the photoreceptors during the visual cycle.

The absorption of a photon by rhodopsin triggers a process of signal transduction (phototransduction) i.e., the photoactivated rhodopsin starts a chain of sequential activation of other components of the rod photoreceptor outer segment (ROS), leading ultimately to a decrease in the conductance of the plasma membrane of the cell to cations.\textsuperscript{29} The degree of polarization of the plasma membrane determines the amount of neurotransmitter released by the photoreceptor cells and therefore the intensity of signal transmission to the secondary neurons.
Figure 3. Overview of photochemical reactions in visual cycle.
The term 'visual cycle' refers to the set of dark reactions that produce the 11-cis-retinoid needed for vision from the all-trans-retinoid precursors. Absorption of a photon by rhodopsin results in photoisomerization of 11-cis-retinal to all-trans-retinal. All-trans-retinol dehydrogenase of the ROS then catalyzes the formation of all-trans-retinol in an NADPH dependent reaction. All-trans-retinol then moves to RPE with the help of interphotoreceptor retinoid binding protein (IRBP). Within RPE it is then converted into all-trans-retinyl palmitate, which is the substrate for the isomerohydrolase enzyme that catalyzes the concerted isomerization and hydrolysis of the ester bond to form 11-cis-retinol. It is then either stored in RPE in the form of an ester or oxidized to 11-cis-retinal by 11-cis-retinol dehydrogenase in an NAD dependent reaction. Finally, 11-cis-retinal is released from RPE and moves to photoreceptor outer segments, where it associates with opsin to regenerate the visual pigment.

The purpose of the visual cycle is obviously to regenerate the bleached visual pigment. During this visual cycle the enzymatic processing of vitamin A occurs at a site removed from the photoreceptor membranes and involves transport between at least two different cell types and several enzymatic steps. It has been suggested that this aspect of the visual cycle is likely to occur in response to the toxic properties of aldehydes and retinoids in particular. The concentration of rhodopsin is found to be 2.5 mM and the 11-cis-retinal of rhodopsin is very tightly bound and the free retinoid in equilibrium with the bound retinoid is very low. The all-trans-retinal formed upon illumination has a very low affinity for opsin and therefore is converted into all-trans-retinol immediately. Since retinol itself can be toxic at concentrations reaching 3mM, the transport of retinol to RPE
where it is esterified, may occur to prevent retinol from reaching its toxic level. The dehydrogenases of the visual cycle catalyze the reduction of retinal in ROS and the oxidation of retinol in RPE while using different pyridine nucleotides, thus separating the reactions into two different compartments.

1.4.2 GROWTH

Vitamin A plays a major role in growth and development in animals and humans. Weanling rats when kept on vitamin A deficient diet continue to grow until their initial reserves of the vitamin are exhausted. Soon after this they lose weight rapidly and eventually die. However the actual cause of death has been attributed to widespread secondary infection. As compared to the animals on a vitamin A deficient diet under the conventional conditions, those raised in the germ-free environment took much longer to reach the weight-plateau stage, and once they reached such a stage, they continued to live for an appreciable length of time without further significant loss of weight. Also, these animals, when given a fresh retinol supplement resumed normal growth. Recent reports have conclusively demonstrated that vitamin A deprivation decreases the mitotic activities in various tissues of animals. Based on the composition of various tissues of rats at the weight-plateau stage of the deficiency, and on the basis of the changes in the DNA contents Zile and coworkers suggested that vitamin A stimulates growth of an animal by directly participating in the process of cell replication.
Retinoic acid has been suggested to be the first morphogen discovered. Thaller and Eichele have conclusively identified retinoic acid in the extracts of more than 500 limb buds of chick embryos and that it forms a concentration gradient, specifying the anterior-posterior digit pattern of the developing chick wings. The discovery of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) as retinoid binding nuclear receptors which influence transcription, in a ligand-dependent manner, of the expression of various target genes provides further evidence for the importance of vitamin A in the growth and development.

In 1929, Mellanby and Green observed that vitamin A deficient rats were very susceptible to infection especially of the alimentary tract, kidney, and bladder. Therefore they referred to vitamin A as "anti-infective vitamin". Subsequently it was also noted that the common cause of death of vitamin A deficient rats was secondary infection. Thus, the link between vitamin A and the immune system has been well known since the late 1920s. Recently it has been shown that cell-mediated immunity is markedly affected in rats during vitamin A deficiency. Humoral immunity has also been found to be decreased in vitamin A deficiency.

1.4.3 REPRODUCTION

Vitamin A and its derivatives are essential for normal reproduction of both males and females. In males the effect is pronounced in the testes. During spermatogenesis the stem cells of the seminiferous tubules undergo several steps of mitotic division eventually
yielding spermatogonia. Spermatogonia undergo further division and differentiation to give rise to primary spermatocytes, which in turn undergo meiotic division to form spermatids. Spermatids contain haploid DNA and do not divide any further, but instead differentiate into mature spermatozoa. The testes of the vitamin A deficient rats were found to be about half the normal size with marked atrophy and the germinal epithelium revealed degenerative changes with the sperm developing only up to the spermatid stage. Recent studies show that vitamin A deprivation affects the division of stem cells like spermatogonia or primary spermatocytes. Earlier it was shown that rats maintained on vitamin A-free diet but supplemented with retinoic acid still had noticeable testicular atrophy and similar degenerative changes in the germinal epithelium as seen in the classical vitamin A deficient rats. However it was recently shown that in cultured cryptorchid testes addition of retinol, retinyl acetate, or RA reinitiated spermatogenesis. This suggests that retinoic acid does not easily reach the functional sites of the testes and that once it gets there it is also active in maintaining normal testicular function.

The effects of vitamin A deprivation on females are rather more subtle than in males. However, it has been observed that the ovaries of vitamin A deficient rats are small in size. Vitamin A deficiency also leads to changes in the cycling patterns of vaginal epithelial cells of rats, i.e., a constant state of cornification of vaginal epithelial cells occurs, giving an impression that the animals are in constant estrus. Vitamin A deficient rats often become pregnant when mated to normal males but the fetuses seldom
survive and their death is generally followed by resorption of products of degeneration into the bloodstream. When the deficient rats were supplemented with RA, they appeared normal and conceived when mated with normal males, but the pregnancy did not go to completion and was terminated around day 14 of pregnancy. Again, RA seems to have a problem getting to the functional sites of action. Since retinoic acid seems to have a transport problem, vitamin A is needed to fully support reproductive functions both in males and females.

1.4.4 DIFFERENTIATION

Retinoids have been shown to exert effects on differentiation of both epithelial cells and mesenchymal cells. As early as in 1925 it was observed that the first symptoms of vitamin A deprivation are noticed in the epithelial cells of an animal. Early histological changes in xerophthalmia in rats were observed to be keratinization of conjunctival epithelium. It has also been repeatedly demonstrated that vitamin A deficiency leads to keratinization of normally nonkeratinizing cells of the epithelia as in paraocular and salivary glands, larynx, trachea, bronchi, and the urogenital tract. Wolbach and Howe observed that the columnar epithelia of the trachea of vitamin A deprived rats undergo progressive degenerative changes leading to squamous metaplasia. De Luca et al. had reported that the number of goblet cells in the intestinal epithelium of vitamin A deficient rats fell to about 50% of the control values. Keratinization of tracheal epithelium upon vitamin A deficiency and its reversal by vitamin A supplementation have been well
established from earlier work. Later on, all-trans-RA, 13-cis-RA, and retinyl acetate were found to be active. Although most of the retinoids were found to be less active than the parent compound, some retinoid derivatives containing two aromatic rings in the side chain showed activity equal to or greater than that of all-trans- or 13-cis-RA.48

Noticeable effects of retinoids on the growth and differentiation of many neoplastic cell types in cultures have been observed. In some cases retinoids appear to make these cells responsive to control mechanisms operative on the untransformed cell types.49 Differentiation of mouse embryonal carcinoma cells, which can differentiate in vivo and in vitro into a variety of cell types, has been considered to be a good model system for study of normal embryogenesis and early mammalian development. Several cell lines have been successfully isolated from and propagated from these cell lines and F9 is one of them. The F9 cell line is quite stable and does not normally differentiate. RA was found to induce differentiation of F9 cell lines into endoderm.50 Only retinoic acid was found to be active in this system with retinol or retinal being about 1000-times less active than the acid. It was later noted that RA promotes differentiation but does not influence the direction of differentiation of these cells.

Various leukemia cell lines have been shown to be induced to differentiate in vitro in response to a variety of agents including butyrate and dimethyl sulfoxide. For suspension cultures of the human promyelocytic leukemia cell line HL-60, RA was reported to be 500-160,000 times as potent as the other agents in inducing terminal differentiation to morphologically and functionally mature granulocytes51,52 All-trans-
and 13-cis-RA are found to be equally effective, while retinal, retinol and retinyl acetate had less than 1/1000 the activity of RA.

1.4.5 CHEMOPREVENTION OF EXPERIMENTAL CANCER

Retinoids are primary agents for cancer chemoprevention since they regulate cell proliferation and differentiation, and cancer is an abnormal growth with the loss of differentiation. Vitamin A deficient animals are also more susceptible to chemical carcinogens than are nondeficient animals.\textsuperscript{53,54} It has been shown that exogenously administered retinoids can inhibit tumor formation \textit{in vivo} under a variety of experimental conditions.\textsuperscript{55} In the last ten years, the role of retinoids as chemopreventive agents has been studied in a number of \textit{in vivo} and \textit{in vitro} model systems. The results of these studies suggest that retinoid action is probably tissue-dependent, and may result from differences in the tissue distribution and retinoid metabolism in various organs.

The most convincing evidence for cancer chemoprevention by retinoids is from studies of chemical carcinogenesis in the mammary glands in rats. Chemopreventive efficacy in these models were determined as indicated by: 1) an increase in the latency of first tumor appearance, 2) a decrease in the number of cancers per animal, and 3) a decrease in the number of animals with cancer. Moon \textit{et al.} reported that a 52% reduction in the incidence of mammary cancer in rats treated with DMBA (9,10-dimethyl-1,2-benz[a]-anthracene) and 2.5 mg of retinyl acetate per day as compared to the incidence in rats receiving DMBA and a placebo diet.\textsuperscript{56} Since then, a series of synthetic retinoids
have been evaluated for activity against chemically-induced mammary cancer, of which retinyl acetate and N-(4-hydroxyphenyl)retinamide (4-HPR) have been found to be the most efficacious. However, retinyl acetate accumulates in the liver in the form of retinyl ester and is very hepatotoxic. On the other hand, 4-HPR accumulates in the mammary gland in a dose-dependent manner and is metabolized by the mammary epithelial cells in both rodents and humans.\(^{57,58}\)

It has been demonstrated that several natural and synthetic retinoids are highly effective in inhibiting carcinogenesis induced by chemical carcinogens. Further, the synthetic retinoids have been found to be more efficacious than the natural retinoids and are less toxic.\(^{59}\) Further research in this area is currently aimed towards identifying additional retinoids with increased chemopreventive activity, and towards identifying the mechanism by which the retinoids inhibit carcinogenesis.

1.5 ABSORPTION, TRANSPORT AND STORAGE

Extensive hydrolysis of fed retinyl esters in the intestine of rats was first demonstrated by Gray \textit{et al.}\(^{60}\) It was also observed that of the total vitamin A recovered in the gut wall, 59\% was in the alcohol form at 220 minutes after dosing, and it increased to 82\% after 400 minutes. Later, it was demonstrated that the fed retinyl esters undergo extensive hydrolysis and the fed retinol is esterified in the lumen of the intestine of the rats.\(^ {61,62}\) The results of further experiments revealed that the dietary retinyl esters are hydrolyzed before absorption into the enterocyte and the absorbed retinol is then
reesterified in the mucosal cells, indicating that only retinol crosses the mucosal cell membranes. The dietary carotenoids and preformed retinoids undergo a series of metabolic conversions, extracellularly in the lumen of the intestine and intracellularly in the intestinal mucosa, which results in the conversion of a majority of the dietary retinoids to retinol for absorption. It was postulated that carotenoids can undergo one of the two following pathways to be converted to vitamin A: (1) central oxidative cleavage to retinal, followed by reduction to retinol, and (2) excentric oxidative cleavage via a series of \( \beta \)-apocarotenals to retinal, followed by reduction to retinol.\(^{63}\) Recently, it has been shown that \( \beta \)-carotene is primarily converted to retinal by the intestinal enzyme, \( \beta \)-carotene-15,15' dioxygenase.\(^{64,65}\) In intestinal homogenates of several species, including humans, \( \beta \)-carotene has been shown to be converted aerobically to several \( \beta \)-apocarotenals, retinal, and RA.\(^{66}\) Two of the identified products were \( \beta \)-apo-14'-carotenal and \( \beta \)-apo-13-carotene, which would be the excentric oxidative cleavage products of \( \beta \)-carotene at the C-13,C-14 double bond. Therefore it appears that both excentric and central oxidative cleavage of \( \beta \)-carotenones do occur in mammalian tissues, although the relative rates of each pathway have not been worked out yet.

The discovery and characterization of cellular retinol binding protein type II (CRBP-II) in the rat intestinal mucosa, and the subsequent studies of physiological roles of this CRBP has helped greatly in understanding the intestinal uptake and processing of retinoids.\(^{67,68}\) Kakkad and Ong have shown that when bound to CRBP-II, retinal is readily reduced to retinol by mucosal retinaldehyde reductase.\(^{69}\) Retinol thus formed by
reduction of retinal as well as the retinol absorbed as such by the intestinal mucosa is then reesterified and packaged into chylomicrons. It has been conclusively demonstrated that for the human intestinal Caco-2 cell line, lecithin:retinol acetyl transferase (LRAT) is important in the intestinal esterification of retinol.\textsuperscript{70} In the mucosal cell, retinol bound to CRBP-II is the substrate for LRAT.

Once secreted into the lymphatic system, the nascent chylomicrons undergo lipolysis, catalyzed by lipoprotein lipase, resulting in the formation of chylomicron remnants. Chylomicron remnants are removed from circulation mostly by the liver.\textsuperscript{71,72} Chylomicron uptake by the liver in healthy humans has been found to be a dose-dependent process that is saturated by fat intakes of 70-100 g.\textsuperscript{73}

In the liver, parenchymal cells contain 90% of the total liver proteins and are directly involved in the uptake of chylomicron remnants and in the synthesis and secretion of RBP.\textsuperscript{74-76} The smaller and less abundant stellate cells are the major storage cells for retinyl esters in the liver.\textsuperscript{75} The retinyl esters of chylomicron remnants are first taken up by the parenchymal cells and then transferred to the stellate cells for storage. It was also shown that the dietary retinyl ester must first undergo hydrolysis to retinol soon after its uptake by the parenchymal cells and this hydrolyzed retinol is then transferred to the stellate cells. The parenchymal cells are the major cellular site for the synthesis of RBP.\textsuperscript{76} The stellate cells have been reported to contain only trace amounts of RBP and no RBP mRNA.\textsuperscript{75,76} Therefore it has been suggested that some of the dietary retinoids taken up by the parenchymal cells may be directly secreted into the circulation to meet the
body needs, bound to newly synthesized RBP, and the remainder is then stored in the stellate cells.

More than 75% of hepatic retinoid is stored in the stellate cells as retinyl esters. Stellate cells are rich in cellular retinol binding protein (CRBP), cellular retinoic acid binding protein (CRABP), retinyl ester hydrolase, and LRAT. Clearly stellate cells are highly specialized for retinoid metabolism. The most characteristic structural feature of stellate cells is the presence of large and abundant lipid droplets in their cytoplasm. The composition of these droplets was found to be retinyl ester, triglyceride, cholesterol (both free and esterified), and phospholipid. The amount of these molecules in the lipid droplets were found to vary under different conditions. It has been reported that the dietary retinoids markedly affect the lipid composition of the stellate cell lipid droplets. Since RA has a sparing effect on hepatic retinol levels, intracellular RA may influence the hepatic secretion of retinol. Retinoic acid receptors of the α, β, and γ types (RAR-α, RAR-β, and RAR-γ) are all reported to be expressed in the stellate cells. Therefore retinoids may also play a role in regulation of differentiation and metabolism of these cells.

The transport of retinol from liver to the target tissues is exclusively accomplished by the transport protein RBP. It was first isolated in 1968 and is a single polypeptide chain with a molecular weight of 21 kD. RBP has one retinol binding site, and has a high affinity for all-trans-retinol. RBP circulates in the blood as a 1:1 molar complex with another protein, transthyretin (TTR). Retinol bound to RBP in the plasma is
effectively taken up by tissue, especially the eye, skin and other epithelia. Soon after the
discovery of RBP, a specific cell-surface receptor for holo-RBP was postulated to exist
on target cells.\textsuperscript{83,84} The initial hypothesis was that holo-RBP binds to this receptor,
retinol dissociates and gets internalized and the resulting apo-RBP, in an altered state, is
released and subsequently metabolized in the kidney.\textsuperscript{85} Later, holo-RBP was thought to
be internalized by means of receptor mediated endocytosis, followed by the release of the
altered apoRBP.\textsuperscript{86} Recently it has been suggested that holo-RBP in the plasma
spontaneously and rapidly dissociates to apo-RBP and retinol. The dissociated retinol
then traverses membranes, and the flux of retinol from plasma into cells is determined by
the concentration of the unbound retinol in plasma.\textsuperscript{87,88} Noy and Blaner have postulated
that the cellular content of apo-CRBP will dictate the rate of retinol uptake by the cells.\textsuperscript{89}
The release of retinol from the cells may occur by the reversal of this process. Therefore
the direction and flux of retinol between the plasma and the target tissue cells depends on
the concentrations of free retinol and apo-RBP in the plasma, and of apo- and holo-
CRBP in the cells. RA, retinyl and retinoyl $\beta$-glucuronides and retinyl esters are also
present in plasma in smaller amounts. RA is present in plasma mostly in the albumin
bound form and its passage across the membranes is thought to be similar to that of
retinol. The $\beta$-glucuronides of retinol and RA are efficiently taken up by the cells. Their
secretion from the cells is also thought to be fairly efficient. Retinyl esters are
constituents of chylomicron remnants and are thought to be taken up by the cells through
interaction with cell surface receptors for apolipoprotein E.
1.6 METABOLISM OF RETINOIDS

The structure of retinol has three distinct features: 1) the β-ionone ring, 2) a conjugated isoprenoid side chain, and 3) a terminal hydroxyl group. The conjugated double bonds can undergo isomerization on treatment with heat, and light, while the hydroxyl group can form an ester with a fatty acid or can be oxidized to an aldehyde and further to an acid. Many of the earlier metabolic studies on retinoids were concerned with the question as to what was the final physiologically active form of retinol in carrying out its functions in vision, reproduction, and differentiation. Recently, in addition to the natural retinoids, the metabolism of synthetic retinoids has also become the focus of interest due to their uses clinically as dermatologic and chemopreventive agents.

1.6.1.1 OXIDATIVE METABOLISM OF RETINOL

Most cells and tissues seem to have the metabolic machinery to oxidize retinol to retinoic acid. One of the earliest works on metabolism of vitamin A was carried out by Wolf and Johnson. They used $^{14}$C-labeled vitamin A for their work. Following an i.p. injection of radioactive vitamin A, they isolated two fractions from the urinary metabolites. The water soluble fraction contained a carboxyl group and a nonconjugated keto group. The other fraction which was soluble in both water as well as ether appeared to be a conjugated aldehyde.

The oxidation of retinol to RA in tissues has been reported to occur through the formation of the intermediate aldehyde. It has long been known that the relatively
nonspecific alcohol dehydrogenase (ADH) of liver can catalyze the oxidation of retinol to retinal, and that aldehyde oxidase can further convert the retinal to RA. Napoli concluded that the enzymes involved in the formation of RA were distinct from ADH and aldehyde oxidase because inhibitors of alcohol and acetaldehyde metabolism do not block the synthesis of RA. Later it was observed that for the oxidation of retinol to retinal by microsomal preparations, holo-CRBP must be the substrate. The oxidized form of nicotinamide adenine dinucleotide phosphate (NADP⁺) was more active than the dinucleotide NAD⁺, as the electron acceptor for this reaction. It was also demonstrated that retinol when bound to CRBP, is a poor substrate for other NAD⁺ and NADP⁺ dependent dehydrogenases from the rat liver microsomes. Cytosols of rat kidney, testis, and lung were also found to contain enzyme activity that converted retinal to RA. Therefore retinol oxidation occurs mainly via the CRBP-bound form with NADP⁺ as the electron acceptor. It is still not clear if the oxidation of retinal to RA is also CRBP-dependent or is catalyzed by a soluble aldehyde dehydrogenase or aldehyde oxidase. It has also been shown by radiolabelling that retinol undergoes decarboxylation, most likely by forming RA as an intermediate.

1.6.1.2 NONOXIDATIVE METABOLISM OF RETINOL

As we have seen earlier, dietary retinol is first esterified in the intestinal mucosa with a long chain fatty acid, and is then transported to the liver. In the liver, the retinyl
esters are hydrolyzed and then reesterified for storage. In addition to the intestine and liver, retinol esterification occurs also in the eye and tracheal epithelium.

\[ \text{Retinyl phosphate} \]

\[ \text{Retinyl-} \beta\text{-mannosyl phosphate} \]

Figure 4. Nonoxidative metabolites of retinol.

Retinol has been suggested to be involved in glycoprotein synthesis. *In vitro* synthesis of retinyl phosphate using either cell extracts or cells from liver, intestinal epithelium, and epidermis has been demonstrated in the 1970s.\(^8\)\(^9\)\(^9\) In addition to retinyl phosphate, formation of retinyl mannosyl phosphate, and retinyl galactosyl phosphate. *in vitro* has been reported.\(^1\)\(^0\)\(^0\)-\(^1\)\(^0\)\(^2\)
1.6.1.3 OTHER METABOLITES

Recently, 14-hydroxy-4,14-retro-retinol has been isolated from cells of the lymphoblastoid line 5/2, which were grown in the presence of retinol-RBP complex. It was also shown that 14-hydroxy-4,14-retro-retinol is a direct biosynthetic product in this system.\(^{103}\) It was also found that liver microsome preparations from some human subjects could metabolize retinol to 4-hydroxyretinol. Cytochrome P<sub>450</sub> isoforms from rabbit liver microsomes were found to catalyze the 4-hydroxylation of retinol.\(^{104}\) Several isomers of retinol including 13-cis-, 9-cis-, and 9,13-di-cis-retinol have been detected in fish liver oil.\(^{105,106}\) 3,4-Didehydroretinol has also been identified as one of the naturally occurring forms of retinol in freshwater fish. The major biosynthetic pathway for this compound appears to be through conversion of lutein to anhydrolutein followed by cleavage to 3-hydroxyretinol and 3,4-didehydroretinol.\(^{107,108}\) 3,4-Didehydroretinol is not physiologically significant in mammals.

It has been reported that cytosolic preparations from rat tissues could catalyze the conversion β-carotene into retinoic acid.\(^{109}\) In this process, however, retinal was not found to be an intermediate and hence the retinol generated during β-carotene metabolism is not considered to be the major substrate for retinoic acid synthesis.

1.6.2 METABOLISM OF RETINOIC ACID

Some of the metabolites of all-trans-retinoic acid generated \textit{in vivo} are 13-cis-RA, 9-cis-RA, retinoyl β-glucuronide, 5,6-epoxyretinoic acid, 4-hydroxyretinoic acid.
oxoretinoic acid, and 3,4-didehydroretinoic acid. Some of these metabolites show activity while others may just be catabolic products. Roberts et al. reported that the formation of polar metabolites of RA was catalyzed by NADPH dependent cytochrome P450 enzymes. Microsomal enzymes of rat testes have been shown to catalyze the formation of polar metabolites from CRABP-bound all-trans-retinoic acid. Binding of all-trans-RA to CRABP may provide the metabolic discrimination between all-trans- and 13-cis-RA and therefore may play an important role in the metabolism of all-trans-RA. Thaller and Eichele reported in 1990 that 3,4-didehydroretinoic acid was generated in vivo in chick limb bud system from retinol, through a 3,4-didehydroretinol intermediate. A decarboxylated metabolite of RA has also been isolated from the intestine of retinoid-deficient rats that were administered RA. Much of the research into the metabolism of retinoids has centered around the search for more active metabolites of retinol or RA.

1.6.2.1 ISOMERIZATION

It has been reported that following an intrajugular administration of all-trans-RA into vitamin-A depleted rats, 13-cis-RA was found in the plasma and the intestine within 2 minutes after injection. 13-cis-RA is widely accepted as a naturally occurring metabolite of all-trans-RA. The 13-cis-RA is found to be equipotent in biological activity to all-trans-RA, both in vivo and in vitro. The existence of 9-cis-RA in cells was reported first in 1992. Further it was shown that 9-cis-RA is an endogenous component
Figure 5. Structures of isomers of retinoic acid.

and is not formed from all-trans-RA during extraction and analysis. It was also demonstrated that 9-cis-RA is an activating ligand for the nuclear retinoid receptor, RXR-α.\textsuperscript{116} Although all-trans RA can bind to and activate RXR-α, RXR-β, and RXR-γ, 9-cis RA was found to be 40-fold more potent for activating these receptors.\textsuperscript{117} Hence 9-cis RA may be an important metabolite of RA.

1.6.2.2 CONJUGATION

After an oral administration of all-trans-RA to rats, all-trans-retinoyl β-glucuronide is secreted in the bile in significant amounts.\textsuperscript{99,118} Retinoyl β-glucuronide is synthesized from RA and UDP-glucuronic acid in the liver, intestine, kidney and other tissues by a microsomal glucuronosyl transferase. Of all these tissues intestinal mucosa is apparently the most active in synthesizing retinoyl β-glucuronide.\textsuperscript{114,119} When 13-cis-RA was administered, all-trans-retinoyl β-glucuronide was found to be a major metabolite in rats, indicating that isomerization to all-trans-RA occurs first.\textsuperscript{120} Later it
was reported that after administration of all-trans-RA to female *Cynomolgus* monkeys, all-trans-retinoyl β-glucuronide, as well as some 13-cis-retinoyl β-glucuronide were found in the plasma. Similarly the administration of 13-cis-RA also gave both all-trans- and 13-cis-retinoyl β-glucuronide, and the maximal plasma concentration of 13-cis-retinoyl β-glucuronide was 42 nM.\textsuperscript{121} It has been found that retinoyl β-glucuronide does not bind to any of the cellular retinoid-binding proteins or to nuclear retinoid receptors.\textsuperscript{110,122} Retinoyl β-glucuronide has been observed to have low toxicity with respect to skin, embryonic development, and cells in tissue culture. This low toxicity may be due to its water-solubility.

Retinol is also conjugated with glucuronic acid *in vivo* to form retinyl β-glucuronide. However, it is hydrolyzed *in vivo* to retinol, which is esterified and then
stored in the liver. Other retinoids, such as 5,6-epoxyretinoic acid, 4-hydroxyphenylretinamide, and 4-oxoretinoic acid also form retinoid β-glucuronides. It is also known that approximately one-third of the retinoid β-glucuronides excreted in the bile of rats is recycled back to the liver and therefore forms an enterohepatic circulation. A synthetic conjugate, retinoyl β-glucose is rapidly hydrolyzed in vivo and therefore is both cytotoxic and teratogenic. Another metabolite found in the bile has been identified as retinotaurine.

1.6.2.3 OXIDATION PRODUCTS

In 1980, Roberts et al. studied the formation of 4-hydroxy and 4-oxoretinoic acid from RA by hamster liver microsome preparations. First, RA is converted to 4-hydroxyretinoic acid which requires NADPH. The hydroxy metabolite then undergoes oxidation to form 4-oxoretinoic acid and this step requires NAD⁺. Later, it was shown that cytochrome P₄₅₀ isoforms in rat and human liver preparations promote the formation of 4-hydroxyretinoic acid from RA. Several urinary, biliary, and fecal metabolites of RA have been identified to contain the 4-oxo moiety as a common element. Therefore hydroxylation at C-4 of the cyclohexenyl ring is believed to be the first step in the elimination pathway of retinoic acid.

McCormick et al. showed that 5,6-epoxyretinoic acid is an in vivo metabolite of RA in the rat small intestine. Later it was shown that 5,6-epoxyretinoyl β-glucuronide was formed as a metabolite in the small intestinal mucosa of vitamin A deficient rats that
were given 5,6-epoxyretinoic acid intrajugularly. It was also shown that kidneys of vitamin A-replete rats given physiological doses of retinol also synthesized 5,6-epoxyretinoic acid. Therefore much like RA, 5,6-epoxyretinoic acid also seems to be formed endogenously from retinol in rat kidney under normal physiological conditions. In vitro studies have demonstrated the presence of a retinoic acid epoxidase in homogenates of rat kidney, liver, and small intestine. It was further observed that in the rat kidney, this enzyme activity is located in the particulate fraction, and requires NADPH, ATP, and oxygen. Further, this epoxidase activity is not affected by the vitamin A level, and the

![Chemical Structures]

**Figure 7. Oxidative metabolites of retinoic acid.**
vivo epoxidation occurs at a low level. Hence, it is concluded that epoxidation of RA is not one of the major metabolic reactions.

1.6.2.4 DECARBOXYLATION

Many of the biliary and urinary metabolites of RA have a shortened side chain compared to RA, indicating that RA can undergo decarboxylation as a metabolic reaction. It was shown that when [15-\(^{14}\)C]-retinoic acid was given to retinoid-deficient rats, 14% of the label appeared as \(^{14}\)CO\(_2\).\(^{133}\) Roberts and DeLuca also reported that 35% of the same \(^{14}\)C-labeled compound given to retinoic acid supplemented rats was recovered as \(^{14}\)CO\(_2\).\(^{97}\) The physiological significance of these metabolites with shorter side chains is still unclear.

1.6.3 SYNTHETIC RETINOIDS

The use of natural retinoids in dermatology and cancer chemoprevention has generated great interest among researchers in the past few years. Due to the toxic nature of the natural retinoids at pharmacological levels needed for these activities, less toxic analogs were sought. This search for more biologically active and less toxic analogs led to the development of some synthetic retinoids including 13-cis-RA, N-(4-hydroxyphenyl)retinamide, N-(2-hydroxyethyl)retinamide, etretinate, and arotinoids.\(^{134}\) It was found that alterations of the side chain may reduce their biological activity while modifications or esterification of the carboxylic group reduces the toxicity. Ring
substitutions were found to increase biological activity with reduced toxicity. Etretinate and 13-cis-RA are used for the treatment of psoriasis. They are also reported to be effective in the treatment of many cutaneous disorders of keratinization. There have been several reports that synthetic retinoids are effective in preventing skin cancer. In most of these diseases, etretinate is more effective than 13-cis-retinoic acid. Etretinate and 13-cis-RA differ not only in their clinical efficacy, but also in their observed toxicities and pharmacokinetics. Therefore each retinoid needs to be studied as a unique drug. The free acid form of etretinate, acitretin, is equally effective in the treatment of psoriasis, but has a much shorter elimination half-life. This advantage is, however, canceled out by the fact that re-esterification may occur in vivo. Polyaromatic compounds called arotinoids represent the third generation synthetic retinoids.

Metabolism of etretinate includes its hydrolysis to acitretin, followed by isomerization to 13-cis-acitretin, oxidation to more water soluble compounds and conjugation with glucuronic acid for biliary excretion. Most of the etretinate is, however, stored in the subcutaneous fat compartment, with slow elimination characteristics. The plasma concentration of etretinate during a long term washout period is extremely low, being most likely therapeutically ineffective, but potentially teratogenic. While etretinate has an elimination half-life of 100 days, trans-acitretin has an elimination half-life of 2-4 days. Therefore acitretin is less likely than etretinate to accumulate in subcutaneous tissue. Thus administration of trans-acitretin instead of etretinate is considered to be a preferable therapeutic option in psoriasis. However, partial in vivo conversion of trans-acitretin into etretinate has been described. Etretinate appears in
human epidermis shortly after oral administration. When treatment is discontinued, epidermal etretinate decreases rapidly, while it accumulates in the subcutaneous fat tissue.

![Etretinate](image)

![Acitretin](image)

![Ro 13-7410 (TTNPB)](image)

![Ro 15-0778 (Temarotene)](image)

Figure 8. Structures of some synthetic retinoids

The tissue distribution of etretinate is widespread. Adipose tissue contains almost exclusively etretinate, while the liver contains mainly trans-acitretin. The metabolites of etretinate present in human urine were divided into two groups, free metabolites and conjugated metabolites. Some of the urinary metabolites had shortened side chains.136,137 The water soluble metabolites were not identified. Similarly the metabolites in the feces were unidentified. After an intravenous injection of etretinate to rats, 70% of the dose was traced to the bile in the first 48 hours. The major metabolite in this study was found to be a conjugate of the free acid. About 10% of the metabolites were the free acid that had a hydroxyl group at position 4 in the aromatic ring instead of
Figure 9. Metabolites of etretinate and acitretin.
the methoxy group. Another compound which was a free acid hydroxylated at the 3-methyl group was also found to be a minor metabolite. In the case of acitretin, isomerization to the 13-cis-compound, isoacetretin, is considered to be the first metabolic step.

Some initial metabolic studies have also been carried out with N-(4-hydroxyphenyl)retinamide. When an intravenous dose of [\(^3\)H]-N-(4-hydroxyphenyl)retinamide was administered to rats, 64% of the dose appeared in the feces after 5 days and 13% in the urine. Although several polar metabolites of this compound were detected, only N-(4-hydroxyphenyl)retinamide-O-glucuronide has been identified. Two other tissue metabolites of N-(4-hydroxyphenyl)retinamide that were identified are N-(4-methoxyphenyl)retinamide and a fatty acid ester of N-(4-hydroxyphenyl)retinamide.

Etretinate and 13-cis-retinoic acid are potent teratogens. The characteristic birth defects include CNS abnormalities, external ear abnormalities, cardiovascular abnormalities, facial dysmorphia, eye abnormalities, and thymus gland abnormalities. Other adverse effects include premature births, parathyroid hormone deficiency, and cases of low IQ. No known safe dose or duration of therapy of these retinoids are available with respect to their teratogenicity. The current recommendation is to avoid pregnancy for at least two years after exposure to etretinate.
1.7 MECHANISMS OF ACTION

Retinoids regulate a variety of basic biological functions. The exact mechanisms through which these regulations are achieved by different retinoids are still not clearly understood. The involvement of vitamin A in the visual cycle, where 11-cis-retinal plays a major role, is by far the best understood retinoid-induced phenomena. Since the discovery of various retinoid-binding proteins and the recently identified retinoid nuclear receptors, it is believed that the actions of retinoids are mediated through binding to one or more of these proteins. Retinoids have been found to interact with two different kinds of proteins, i.e., the binding proteins and the retinoid-receptors.

1.7.1 BINDING PROTEINS

Retinol is transported from retinoid stores in the liver to the target tissues exclusively by means of a specific transport protein, serum retinol binding protein (SRBP).\textsuperscript{82} SRBP is a single polypeptide chain with a molecular weight of about 21 kD. and has one binding site for one molecule of all-trans-retinol. In the blood it is complexed with another serum protein, transthyretin (TTR), in a ratio of 1:1. The three-dimensional structure of human holo-SRBP has been studied to a great extent in the past few years.\textsuperscript{140,141} Human holo-SRBP is a single globular domain approximately 40 Å in diameter. SRBP is made of an N-terminal coil, a β-sheet core, an α-helix, and a C-terminal coil. There are eight antiparallel β-strands arranged in an up and down β-barrel. The β-barrel is arranged in two stacked orthogonal β-sheets. These orthogonal β-sheets
encapsulate the retinol in such a way that the β-ionylidene ring lies deep within the β-barrel, while the linear isoprene unit stretches out along the barrel axis to the surface of the protein. Based on the crystal structure of holo-SRBP it has been suggested that the removal of retinol from the holo-SRBP might trigger a collapse of the β-barrel and other conformational changes in the protein.142

The three-dimensional structure of SRBP is a prototype for a family of proteins all of which bind hydrophobic small molecules.143 Some of the proteins in this family are β-lactoglobulin, purpurin, apolipoprotein D, and bilin-binding protein. Most of these proteins have the function of binding and possibly transporting hydrophobic ligands. For example SRBP, β-lactoglobulin, purpurin, and androgen-dependent secretory protein, all bind retinoids. The structures of SRBP, β-lactoglobulin, and bilin-binding protein have been determined by X-ray crystallography and are remarkably similar.142,144-146 All three proteins have a β-barrel formed by two orthogonal β-sheets and an α-helix. Therefore SRBP represents a family of proteins that bind small hydrophobic ligands.

Liver is the major site of synthesis and secretion of SRBP. In vitamin A deficiency, SRBP accumulates in the liver to levels about four-to ten-fold higher than the normal level.75,85 Retinol deficiency, therefore, inhibits the secretion of SRBP by the liver. It has been shown that this is due to the blockage of movement of newly synthesized SRBP from endoplasmic reticulum to the golgi apparatus. As mentioned earlier, it is still not clear as to how cells take up retinol from SRBP. There are two differing views on this topic. One view is that the SRBP binds to the cell-surface receptor
leading to the uptake of retinol by the cell. The second view is that internalization of retinol from SRBP into the cells is achieved by a non-receptor-mediated process.

Once retinol is delivered to the cells, it then binds to the intracellular binding protein, cellular retinol binding protein (CRBP). The existence of CRBP was discovered in 1973. Early binding studies on CRBP indicated a preference for retinol over the other retinoids including retinoic acid, retinal, and retinyl esters. Further, it has also been observed that CRBP prefers to bind all-trans- and 13-cis-retinol over 9-cis- or 9, 13-di-cis-retinol. In humans, liver, ovary and testis are the richest sources of CRBP. Within the liver, parenchymal cells appear to have about twice as much CRBP as do the stellate cells. In the testis, the peritubular cells and the Sertoli cells are found to be enriched in CRBP as compared to whole testis. The Sertoli cells have been shown to be able to internalize retinol from the retinol-SRBP-TTR complex without the apparent internalization or retention of SRBP. Therefore CRBP may play an important role in the delivery of vitamin A to the developing sperm.

The existence of a second intracellular retinol-binding protein was recognized when whole rat pups were used as a source for the preparation of pure CRBP and CRABP. A small retinol-binding protein that resolved into two equally abundant forms during its preparation was isolated. Its physical properties were clearly different from but highly related to CRBP. Further, the amino acid sequencing revealed that this new protein's sequence was 56% identical to the CRBP. This recently isolated protein was later named cellular retinol-binding protein type two (CRBP II). The difference
between the two forms of CRBP II was the acetylation or non-acetylation of the N-terminal threonine. Functionally, no difference between the two forms has been observed. Determination of binding properties confirmed that retinol is the preferred ligand with all-trans-retinal showing a slightly lowered affinity. CRBP II also binds 13-cis-retinol but not RA, retinyl esters, 9-cis-, or 11-cis-retinol. Therefore the binding specificity of CRBP II is identical to that of CRBP, although the binding affinity differs. It has been observed that the affinity of CRBP for retinol is about 100-fold greater than that of CRBP II. In the adult rat, CRBP II has been found in the small intestine, adrenal gland, testis, and brain. Intestinal CRBP II has been identified in humans as well. CRBP II was localized in the mucosal epithelium, where it was restricted to the mature enterocytes on the villi. Therefore within the small intestine, CRBP II is present in cell that are actively involved in vitamin A absorption. This indicates that it may be involved in the uptake of luminal retinol as well as retinal generated from the dietary β-carotene.

The X-ray structures of rat holo-CRBP complexed with all-trans-retinol and of apo- and holo- forms of CRBP II have been determined. These proteins also have a β-barrel similar to the one observed in the SRBP. Therefore, CRBP and CRBP II seem to be similar to the SRBP in their 3-dimensional structure. The bound retinol has been found to be present in the β-barrel of CRBP and CRBP II, much like binding with SRBP. Unlike in the case of SRBP-bound retinol, the bound retinol in the case of CRBP and CRBP II has been found to be bound in such a way that the alcohol moiety and the
isoprene side chain are situated innermost in the β-barrel while the cyclohexenyl ring is extended towards the exterior of the binding pocket. These differences in the orientation of bound retinol may play an important role in the fate of the bound retinol.

Presence of cellular proteins that had a high affinity for RA and no affinity for retinol was first observed in chick embryo skin. The RA-binding protein was also observed in the rat testis, and bovine retina. This RA-binding protein from the cytosol of rat testis was shown to be different from the CRBP by ion-exchange chromatography. This protein was named cellular retinoic acid binding protein (CRABP). Since then, CRABP has been purified from rat testis, bovine retina, and the skin of the chick embryo. CRABP was found to be highly conserved between species. It was later noted that there exists two separate binding proteins for RA (CRABP I and CRABP II). Binding studies have revealed that CRABP specifically binds all-trans-RA and does not bind the other retinoids including retinol, retinal, 13-cis-RA, and retinoyl esters. The different synthetic retinoids have also been examined for their abilities to bind to CRABP. Results of these studies conclude that the synthetic retinoids that have a high potency in biological assays are often but not always found to have high affinity for CRABP, while inactive compounds do not bind. In adult rats the major organs like kidney, liver, lung, muscle and small intestine had little if any CRABP. However, the brain, eye, ovary, testis, and uterus had appreciable amounts of CRABP.

As mentioned earlier, the presence of CRABP II was detected and studied as early as 1974. CRABP and CRABP II differ from each other mainly in the N-terminal
sequence. It appears that CRABP II behaves much like CRABP as far as its ligand-binding properties are concerned, the only difference being the lower affinity of CRABP II for RA in comparison to that of CRABP. Very much less is known about the organ and cellular distribution of CRABP II. It appears that CRABP II is expressed most strongly during embryogenesis. In the case of adults noticeable amounts are present in the skin.

These intracellular retinoid binding proteins appear to be important to cells and organisms, as no species that relies on vitamin A has been found to be lacking such proteins. Their specificity and affinity imply that they have essential roles in the cellular trafficking of retinoids. The natural retinoids are hydrophobic and therefore have very limited water solubility. Due to their poor water solubility very little unbound retinoid is present in the cell. By providing a specific carrier protein with high affinity, the cell appears to maintain a pool of soluble, diffusible retinoid-protein complex where the retinoid is stable but sequestered from nonspecific partitioning into membranes or organelles. It has been suggested that the cellular retinoid binding proteins also play an essential role in directing the metabolism of bound retinoids. This is thought to be accomplished by making the retinoids available for reaction with specific enzymes.

Even though there has been a considerable advance in the knowledge about the intracellular retinoid-binding proteins in the last decade, much remains to be investigated. Although these binding proteins have been suggested to be interacting with specific enzymes in directing the metabolism of retinoids, the specifics or the exact mechanism has not yet been studied. It has also been postulated that these retinoid-binding proteins
may play an important role in delivering retinoids to and from the nuclear receptors. The validity of this postulation needs to be investigated.

1.7.2 RETINOID RECEPTORS

The mechanism of action of retinoids was a mystery until the discovery of nuclear retinoic acid receptors in 1987. Since then, two families of nuclear retinoid receptors have been characterized. The two families are the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), and each family has three subtypes α, β, and γ. It has further been recognized that they are both members of the steroid/thyroid hormone-receptor superfamily, thereby suggesting that the mechanisms of action of retinoids are similar to those of steroid and thyroid hormones. They act as ligand-dependent transcriptional factors. RARs can bind both all-trans-retinoic acid and 9-cis-retinoic acid with high affinity. On the other hand, RXRs can selectively bind 9-cis-retinoic acid. Retinoid receptors regulate the transcription of genes containing short DNA sequences in their promoter regions, called retinoid-responsive elements (RAREs and RXREs). Further, it has been found that RXR activated pathways are mediated by RXR homodimers while the RAR activated pathways are mediated by RAR-RXR heterodimers. Recently it has been shown that RAR-α, RAR-β, and RAR-γ are all encoded by different genes.

Expression of RAR has been found to be tissue-specific. Studies of RAR expression by in situ hybridization techniques indicate that each of the RAR subtypes
may have a role in embryonic development and limb formation. In the developing mouse embryo, RAR-α is ubiquitously expressed, whereas the expression of RAR-β and RAR-γ are temporally and spatially restricted. Regions where RAR-β are expressed include the interdigital mesenchyme, mesenchyme surrounding the epithelium of the

inner ear, endoderm derived tissues (epithelia of lungs, intestine and genital tract), and the developing nervous system. It has been suggested that RAR-β may be involved in regulating programmed cell death. RAR-γ has been found to be expressed in all mesenchyme giving rise to bone and facial cartilage and in differentiating squamous

Figure 10. Retinoid signal transduction pathway.
keratinizing epithelia. Also, in both the embryonic and adult skin RAR-γ is the predominant isoform.

The RXRs are widely expressed in the adult organisms. One of the interesting patterns in the expression of these receptors is that both RXR-β and RAR-α are found in almost all tissues, while RXR-α and -γ and RAR-β and -γ are very restricted. RXR-α is expressed abundantly in the liver, kidney, spleen, and a variety of visceral tissues. It has also been observed that in these sites the expression of RARs was very low. It has also been shown that the CRBP II gene is responsive to RXR but not RAR. Based on these studies it was proposed that RXR-α may be involved in the metabolism of retinoids. The abundant expression of both RXR-α and RAR-γ in the epidermis suggests that these two receptors may be involved in the dermatologic actions of retinoids. RXR-γ is abundantly expressed in muscle and brain. Also, selective co-expression of RXR-γ and RAR-β in the motor neurons of the spinal cord has been observed, besides the prominent expression of RXR-γ in the pituitary gland. All these studies suggest that the RARs and RXRs play important roles not only during embryogenesis and organogenesis but also in the adult physiology and metabolism.

Like the other nuclear receptors, RARs and RXRs also have a conserved modular structure, with five distinct regions for RXRs, and six distinct region for RARs. The DNA-binding domain and the ligand-binding domain are highly conserved in these receptors. The three-dimensional structure of the RXR-α ligand-binding domain has been solved and has been found to be similar to the prototypic fold that appears to be
conserved within the nuclear receptor superfamily. In this study, it has also been found that a number of amino acid residues, identified to be important for ligand binding in other nuclear receptors, have similar locations in the ligand binding domain of RXR-α. The ligand binding-pocket in RXR-α has been suggested to be similar to that of epididymis retinoic acid binding protein. The structure of the ligand binding domain of RAR-γ with the bound ligand has been obtained by X-ray crystallography, and has been found to be closely related to the structure of human RXR-α apo ligand binding domain. Further, the sequence alignment of RAR-α, β and γ shows that only three residues in the ligand-binding pocket are variable. Therefore the presence of these divergent amino acid residues in the ligand binding domain of RARs may play a significant role in the development of ligands with receptor-subtype specificity.

The presence of different retinoid receptors along with their complex nature of expression in various tissues have made it very difficult to study the exact role of each of the receptor subtypes in mediating various biological effects of retinoids. The possible metabolic interconversions of the natural retinoids complicates these studies even more. Studies on these interactions are further complicated due to the discovery of inverse agonists and reverse agonists. Thus, a number of synthetic analogs that can specifically bind to only one of these receptors and which can not be converted into another form that would bind to a different receptor or lead to a different pathway are currently being synthesized and studied, in order to understand the precise roles of each of these receptors.
CHAPTER 2

MICROBIAL MODELS OF MAMMALIAN RETINOID METABOLISM

2.1 STATEMENT OF THE PROBLEM

As mentioned earlier in Chapter 1, a series of natural and synthetic retinoids have been found to be biologically active in terms of epithelial cell and epidermal differentiation, leading to the introduction of retinoids into dermatology.\textsuperscript{186-188} In the recent past, retinoids have been used in the treatment of: 1) hyperkeratotic and parakeratotic skin diseases, and 2) severe acne-related dermatoses.\textsuperscript{7} The role of retinoids in the treatment/chemoprevention of various forms of cancer has also been recognized in the last decade. Etretinate (8), acitretin (9), and isotretinoin (13-cis-RA) are currently being used for treating psoriasis. Etretinate and acitretin are found to be more potent antipsoriatic agents than isotretinoin. Studies comparing the antipsoriatic potential of etretinate with acitretin showed only very little difference between the two.\textsuperscript{189-191} Retinoids in general are believed to exert their biological activity by binding to the two families of nuclear receptors, RARs and RXRs. Since the discovery of 9-cis-RA as a high-affinity ligand for the RXRs, the focus of research has shifted from all-trans-RA to its metabolites as well, suggesting one or more of these metabolites may also be
biologically active. RA has been studied extensively in terms of its metabolism. In the recent past RA and its metabolites and some synthetic retinoids have been studied in terms of their ability to bind to and/or activate these nuclear retinoid receptors. On the other hand, etretinate has been less well studied both in terms of metabolism as well as its mechanism of action. It is now believed that acitretin, the free acid form of etretinate, may be the active form of etretinate. Ligand-binding studies have revealed that neither etretinate nor acitretin binds to the nuclear retinoid receptors. Therefore, etretinate/acitretin may bring about their biological effect through some other, yet unidentified mechanism. Alternatively, one of the yet to be identified metabolites of etretinate may actually be the pharmacologically active form. In addition, etretinate is recognized as one of the most potent teratogens known. Again, it is not known yet as to whether etretinate itself or one of its metabolites is the actual teratogen. Etretinate is stored in body-fat depots, and is slowly released into the blood stream over a very long period. Its plasma half-life has been found to be about 100 days. Etretinate has been detected in the serum in small amounts for as long as 3 years after discontinuation of therapy. Acitretin is equipotent with etretinate, in terms of antipsoriatic activity, but has a plasma half-life of only 2 days. This very short plasma half-life of acitretin was believed to be advantageous for choosing acitretin over etretinate due to the reduced risk of delayed teratogenicity. Contrary to all expectations, however, isolation of etretinate from the plasma, possibly as a metabolite, after treatment with acitretin proved this belief to be not entirely correct. Although the origin of etretinate in the plasma of patients
Figure 11. Structures of etretinate and analogs.
treated with acitretin is still under investigation, the possibility of formation of etretinate from acitretin as an artifact during sample analysis has already been eliminated. Therefore, further studies of metabolism of etretinate will be of great use in identification of possible metabolites with favorable biological activity and may also be important in achieving an understanding of its mechanism of action.

We have used microbial models to study the metabolism of etretinate. In the past, microbial metabolism has been used to successfully model the mammalian metabolism of numerous classes of xenobiotics including such varied compounds as steroids, cannabinoids and alkaloids. Similar metabolic reactions have been observed in both microorganisms and mammals. Microbial fermentation methods have earlier been used to study the mammalian metabolism of RA and its analog β-ionone. In this investigation, we have used methyl 5-(4-methoxy-2,3,6-trimethyl-phenyl)-3-methyl-2,4-pentadienoate (11) as a model to develop methods to study the biotransformation of etretinate. The results of the microbial biotransformation experiments on compound 11, as well as etretinate are discussed in this chapter.

2.2 MICROBIAL MODELS OF MAMMALIAN METABOLISM

The use of microbial biotransformations as a model to systematically study and predict the mammalian metabolism was first proposed by Smith and Rosazza in 1974. In mammals, drugs and xenobiotics undergo phase I and phase II biotransformation reactions. Many of these mammalian type-reactions have been observed in microbial
systems as well.\textsuperscript{201} It has been found that bacteria degrade aromatic compounds by action of dioxygenases.\textsuperscript{205,206} It has also been demonstrated that fungi degrade aromatic substances by monooxygenase systems.\textsuperscript{207,208} The latter system of enzymes have been found to be similar to the mammalian cytochrome P-450 mixed-function oxygenases.\textsuperscript{204} It is now possible to achieve specific biotransformation products of a large variety of substrates by varying the culture selection, medium, and incubation conditions.\textsuperscript{197,209} The initial culture selection is generally done based on previous work. Microorganisms which have been reported to bring about the type of biotransformation reaction of interest, and if possible on compounds of similar composition, size, and functionality, are used for screening experiments. A literature search is of great importance in selecting the initial cultures for screening. Pure cultures from established culture collections are usually preferred to mixed cultures since the pure cultures show greater reproducibility.

There are several advantages in using microbial biotransformation as a model for mammalian metabolism studies. The technology is readily available, and a carefully selected group of about 50 microorganisms will normally be enough for most laboratories to pursue this work.\textsuperscript{201} It is also significantly less expensive compared to using the mammalian systems themselves. In general, microbial transformation products of xenobiotics are produced in higher yields and in less complex mixtures than metabolites formed by \textit{in vivo} or \textit{in vitro} mammalian metabolism.\textsuperscript{201} Also, useful milligram quantities of pure biotransformation products can often be obtained which would be of

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great value in terms of structural elucidation and conducting other analytical experiments and bioassays for the complete characterization of such metabolites.

2.3 BIOTRANSFORMATION OF AN ETRETINATE MODEL

We used compound 11 to develop methods to study the microbial metabolism of etretinate. Compound 11 is structurally similar to etretinate but is more readily available. It is also more soluble and stable than etretinate. Therefore we used 11 for our screening and methods development studies.

2.3.1 SYNTHESIS OF 11

We synthesized 11 from the plant growth hormone, abscisic acid (12). We serendipitously discovered that abscisic acid rearranges to 11 in 35% yield, when exposed to refluxing methanol and a catalytic amount of H$_2$SO$_4$. Compound 11 was formed in 1:1 ratio of cis, trans and trans, trans isomers. The rearrangement probably occurs via a series of carbocation intermediates, as shown in figure 13. Compound 11 is structurally

![Figure 12. Synthesis of 11](image-url)
Figure 13. Proposed mechanism for the formation of 11 from abscisic acid.
similar to etretinate, the differences being a shorter side chain in 11 which is also a methyl ester while etretinate is an ethyl ester.

2.3.2 MICROBIAL BIOTRANSFORMATION OF COMPOUND 11

First, we screened twenty-one microorganisms, shown in Table I, in a two-stage culture method to identify the strains that looked promising in terms of biotransformation of 11.197 To each of the Stage II shake flask cultures of the twenty-one microorganisms, 10 mg of compound 11 was added. The cultures were harvested after 7 days by treating with 50% ethyl acetate in hexanes. The organic phase was separated from the aqueous phase and the solvent was evaporated to give crude extracts. The thin-layer chromatograms of the extracts of control cultures were compared with those of cultures fed with compound 11 to identify the microorganisms that appeared to contain the biotransformation products of 11 in appreciable quantities. Comparisons of TLC plates of cultures fed with the model compound were made by semiquantitative inspection of the TLC plates by observing both the partial disappearance of the starting material and the appearance of more polar, potential metabolites as compared to control cultures. Based on those comparisons, two microorganisms Cunninghamella elegans ATCC 9245 and Syncephalastrum racemosum ATCC 18192 were selected for further biotransformation studies of 11.
Table 1. Microorganisms used for initial screening studies.

<table>
<thead>
<tr>
<th>Aspergillus aculeatus ATCC 1034</th>
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<td>A. terreus OSU</td>
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<tr>
<td>A. flavipes ATCC 1030</td>
<td>Cunninghamella elegans ATCC 9245</td>
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<td>Streptomyces species ATCC 15077</td>
</tr>
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<td>A. niger ATCC 11394</td>
<td>Syncphalastrum racemosum ATCC 18192</td>
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<tr>
<td>A. niger ATCC 16888</td>
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We then conducted a time course study with both these organisms. Stage II cultures of both the microorganisms were started and compound 11 was added to each of the stage II cultures. A culture of each organism was extracted at different time intervals i.e., 4 days, 6 days, 8 days and 10 days after addition of the substrate to the cultures. The extracts from different days were then compared using TLC as described previously, and we found that 6 days of incubation was optimum for the biotransformation. Therefore, for all our further studies we harvested the cultures 6 days after adding the substrate to the cultures.

We then did a scale-up of the biotransformation of 11. A total of 200 mg of compound 11 was added to the stage II cultures of each of the two microorganisms. The cultures were then incubated in a rotary shaker for six days and were then harvested by treating with 1:1 ethyl acetate/hexanes mixture. The suspensions were then filtered to remove the mycelia. The methods used in the isolation of the biotransformation products of 11 are shown in scheme 1. The organic layers were separated from the aqueous layers. The organic layers from cultures of the same organism were pooled together as were the aqueous layers from the same organism. The organic phase was dried, and concentrated to give the crude organic extract. Thin layer chromatograms of the organic extracts from cultures fed with 11 were then compared with those of the controls. This comparison indicated the presence of a few biotransformation products of 11. The concentrated organic extracts were then purified by column chromatography on silica gel using 30% ethyl acetate in hexanes as the mobile phase. By this purification method five different retinoid products were obtained from the organic extracts of each of the organism.
The different isolated retinoid metabolites purified from the organic extracts of *C. elegans* 9245 were analyzed spectroscopically. Ten percent of the substrate 11 was recovered unchanged in fraction 1. The spectroscopic data recorded for this compound were identical to those of compound 11 itself. One of the retinoids isolated as fraction 3 was identified as compound 13, which is formed as a result of phenol O-demethylation and ester hydrolysis, as evidenced by the disappearance of the signals for the methoxy group and the ethyl group in the $^1$H NMR spectrum. The mass spectrum obtained on this compound was consistent with the loss of both the methyl and the ethyl groups from compound 11. The yield of formation of 13 from 11 was found to be 1.7%. Another biotransformation product obtained in fraction 4 was identified as compound 14, which is formed from 11 by ester hydrolysis and hydroxylation of one of the methyl groups on the
aromatic ring. Phenols and alcohols have been shown to undergo methylation by treatment with sodium hydride and methyl iodide in tetrahydrofuran. A small fraction of compound 14 was dissolved in tetrahydrofuran and reacted with sodium hydride and methyl iodide at room temperature in order to methylate the hydroxyl group. The product obtained from this reaction was present in insufficient quantity to do all the possible spectroscopic experiments. Therefore we recorded a mass spectrum of this product. The mass spectrum showed that the molecular weight of the product was 28 units higher than that of 14, indicating that the hydroxyl group as well as the carboxyl group of compound 14 have been methylated, as expected from the above mentioned reaction. The hydroxymethyl group location on compound 14 was tentatively identified by conducting nuclear Overhauser enhancement (NOE) experiments with 14. Irradiation of the proton on the aromatic ring did not show any NOE to the hydroxymethyl group. Next, the vinylic protons on the side chain were irradiated. Irradiation of these vinylic protons also did not show any NOE to the hydroxymethyl group. Irradiation the methyl group at position 2' on the aromatic ring gave results which were complex and difficult to interpret since the signals for all the methyl groups resonate too close to each other to selectively irradiate one over the others. These NOE studies, however, suggest that the methyl groups on the carbon atoms adjacent to the one that has the pentadienoate side chain and the methyl group on the side chain are not hydroxylated. Hence, we tentatively identified this metabolite as compound 14. Similarly, a hydroxy derivative of acitretin, compound 15, has been isolated as a metabolite from the blood of patients treated with
etretinate.\textsuperscript{193,196,211} Two other metabolites isolated from \textit{C. elegans} 9245 were obtained in insufficient quantities to be structurally identified.

\begin{center}
\begin{tikzpicture}
  \node (fig15) at (0,0) {
    \includegraphics[width=0.5\textwidth]{fig15.png}
  };
  \node[below=0.5cm] at (fig15) {15};
\end{tikzpicture}
\end{center}

Figure 15. Hydroxylated metabolite of 9 isolated from blood of patients treated with 9.

Five retinoids were isolated from the organic extract of \textit{Syncephalastrum racemosum} 18192. Of these five retinoids the most non-polar compound, obtained in fraction 1, was identified as unreacted substrate 11. Twelve and one-half percent of 11 was recovered unchanged from \textit{S. racemosum} 18192. Another retinoid compound isolated as fraction 4 from the organic extract of this organism was identified as compound 16, which is the ester hydrolysis product of 11. Conversion of 11 to compound 16 was calculated to be 2.12%. Another biotransformation product isolated as fraction 5 was identified as compound 17. Retinoid 17 is formed from 11 by hydroxylation of one of the methyl groups on the aromatic ring. This hydroxylated methyl group in compound 17 is again tentatively identified based on the NOE experiments. Irradiation of the aromatic proton did not show any NOE to the hydroxymethyl group. Irradiation of the vinylic protons also did not show any NOE to the hydroxymethyl group. Again our attempts to
irradiate the 2'-methyl group were not completely conclusive in confirming our assignment of the hydroxymethyl group. We also took a small portion of compound 17 and treated it with sodium hydride and methyl iodide in dry THF, in an attempt to methylate the benzylic alcohol. Although the product was obtained in insufficient amount to perform complete structural elucidation studies with it, we were able to get a mass spectrum of it. The mass of this product was found to be 14 units higher than that of 17. Also, two other metabolites obtained from the organic extracts of *S. racemosum* 18192 were present in insufficient quantities to be identified.

We also analyzed the aqueous layers for the presence of possible water-soluble metabolites, if any, formed as biotransformation products of compound 11. As in earlier extractions, after the mycelia were filtered off, the organic layer was separated from the

![Figure 16. Biotransformation products of 11 from *S. racemosum* 18192.](image-url)
aqueous phase. The separated aqueous layer was then stirred with XAD-2 resins for 10 minutes and the water was then filtered off. The resins were then washed with additional water, followed by stirring with methanol for 30 minutes. The methanol was then filtered to remove the resins and this methanol extract concentrated to give the crude water-soluble products. Chromatographic separation of this methanolic extract was performed on silica gel using 6:3:1 methanol/chloroform/acetic acid and it gave four different fractions. Of these four different fractions, only one from each organism was found to contain a potential biotransformation product of compound 11. Since these water-soluble products were also obtained in very small quantities, we were able to get only a very limited set of spectroscopic data for these compounds. Based on these very limited spectroscopic data available for these biotransformation products we speculate that these may be sugar conjugates of 11 and/or its metabolites (\(^1\)H NMR signals from 4 to 5 ppm). We could not prove this conclusively since they were available in insufficient quantities to do further experiments. In mammalian metabolic studies of acitretin, acitretin and its metabolites have been found to be eliminated as \(\beta\)-glucuronides.\(^{193}\)

In summary, through our screening studies, we have identified two microorganisms (from the set of twenty-one microorganisms that we employed in the initial screening studies) which biotransform compound 11. We have also developed methods to isolate and identify the organic-soluble biotransformation products of 11. Microbial biotransformation products of 11 that we have isolated and identified are structurally similar to some of the metabolites of 8 that have been characterized from the blood and bile extracts of patients treated with 8.\(^{193}\)
2.4 MICROBIAL BIOTRANSFORMATION OF ETRETINATE

2.4.1 PREPARATION OF ETRETINATE

Etretinate for our studies was obtained from Tegison® capsules, purchased from the pharmacy at The Ohio State University Hospitals. The contents of the Tegison® capsules were emptied into a round bottom flask and stirred with ethyl acetate to extract the etretinate. The ethyl acetate was then filtered and evaporated to give etretinate. The proton NMR spectrum and the mass spectrum obtained on a sample of the extract showed it to be apparently pure etretinate. The etretinate, thus extracted from Tegison® was used for our biotransformation studies.

2.4.2 BIOTRANSFORMATION OF ETRETINATE

After developing methods for the isolation and identification of microbial biotransformation products of 11, we extended our studies to microbial biotransformation of etretinate itself. Based on the results of the model compound 11, we selected two microorganisms Cunninghamella elegans 9245 and Syncephalastrum racemosum18192 for our further studies with etretinate. A two-stage culture method, similar to the one used for compound 11, was employed for the biotransformation studies of etretinate. To the stage II cultures of each of the organisms was added a total of 40 mg of etretinate and the cultures were left on the shaker for six days. One culture flask of each organism that did not have any etretinate added, was used as a control. After 6 days, the cultures were harvested by treating with 1:1 ethyl acetate/hexanes. The mycelia were then removed by
filtration and the organic layers from the same organism were pooled together, dried and concentrated to give the crude organic extracts. The control cultures were also treated similarly. Comparison of the thin layer chromatograms (20% ethyl acetate/hexanes) of the organic extracts of control cultures with those of cultures fed with etretinate was made by semiquantitative inspection of the TLC plates for the partial disappearance of the starting material and the appearance of more polar metabolites. These comparisons showed the possible presence of etretinate biotransformation products in the cultures of *C. elegans* 9245, although in apparently very small amounts. Etretinate did not appear to undergo any biotransformation by *S. racemosum* 18192.

Therefore, we decided to scale up the biotransformation of etretinate by *C. elegans* 9245. To one day old stage II cultures of *C. elegans*, a total of 400 mg of etretinate was added. After 7 days the cultures were harvested by extracting with 1:1 ethyl acetate/hexanes. The methods used for the isolation of biotransformation products of etretinate are shown in scheme 2. The organic layers were pooled together, washed with brine, dried, and concentrated to give the crude organic extract. Column chromatography on silica gel using 10% methanol/chloroform gave one fraction containing retinoids. This fraction was further purified by column chromatography on silica gel using 20% ethyl acetate/hexanes which gave two fractions containing retinoids. These two retinoid fractions were then analyzed spectroscopically.

One of the purified retinoids, obtained as fraction 1, was observed to be the unchanged etretinate. Six percent of etretinate was recovered unchanged. Although by $^1$H NMR analysis, the other product, obtained in fraction 2, appeared to be an etretinate
derivative, as evidenced by the presence of the signals for methyl protons (2-2.4 ppm) and vinyl protons (6-7 ppm) characteristic of the etretinate-derived compounds, it was present in insufficient quantity to be further characterized. The mass spectrum (m/e = 326.1849) obtained on this compound also suggested it to be an etretinate derivative, possibly the ester hydrolysis product, acitretin (with a calculated mass of 326.1882).

The aqueous layers obtained from the harvested cultures were pooled together and treated with XAD-2 resins. Water was then filtered off, and the resins were washed with additional water. The resins were then extracted with methanol and the methanol evaporated to give the crude aqueous extract. In our attempts to further analyze this aqueous extract, we tried to dissolve it in different solvents and we found that the aqueous extract did not go into solution except when excess methanol was added.

We also found that one of the components in this crude aqueous extract dissolved in pyridine. Therefore we partially purified this compound by extraction with pyridine. This pyridine-soluble compound appeared to be an etretinate derivative as determined by the characteristic resonances in the $^1$H NMR spectrum, possibly a sugar-conjugate, although we were not able to further characterize this compound since it was present in insufficient quantity and also is sparingly soluble.

Previously in our lab, microbial biotransformation of $\beta$-ionone was studied as a model for the microbial biotransformation of RA.$^{203}$ In that model study, it was found that $A. niger$ 16888 formed 4-hydroxy-$\beta$-ionone and 4-oxo-$\beta$-ionone in 61% and 8% yields respectively from $\beta$-ionone, while $C. blakesleean\,a$ 8688a formed 4-oxo-$\beta$-ionol in 28% yield.$^{203}$ When incubated with RA, however, $A. niger$ 16888 did not form any
biotransformation product of RA.\textsuperscript{202} \textit{C. blakesleeana} 8688a formed 1-hydroxymethyl-4-oxo-RA in 1.3\% yield and 2-hydroxy-RA in 2.2\% yield.\textsuperscript{202} Although microbial biotransformation of the model compound \(\beta\)-ionone was found to proceed reasonably well, the biotransformation of structurally related but less soluble, and less stable, RA seemed to be a more difficult process, at least in the case of the microorganisms we employed earlier in our lab.

It appears that some of the microorganisms that were able to biotransform the model compound \textbf{11}, may also biotransform the parent compound etretinate, although in very small yields. A much larger supply of both etretinate and compound \textbf{11} and a greatly scaled up fermentation study would be necessary to confirm this explanation of the results.
CHAPTER 3

NMR STUDIES OF RETINOID-PROTEIN INTERACTIONS.

3.1 STATEMENT OF THE PROBLEM

As mentioned earlier, retinol and its analogs have been developed to treat various dermatologic diseases. Retinoids are also currently being studied as cancer chemopreventive/chemotherapeutic agents. Although the exact mechanism of action of retinoids to cause these biological effects is not clearly known yet, it is now believed to be mediated through the association of retinoids with their binding proteins and/or the nuclear receptor proteins. It is believed that the binding proteins may also function to channel the bound-retinoids into a particular metabolic pathway by interacting with specific enzymes. These beliefs have led to an interest in studying the structures and functions of these proteins. Some of the well characterized retinoid-binding proteins have been described in Chapter 1 and include SRBP, CRBP I, CRBP II, CRABP I, and CRABP II. In addition, photoaffinity labelling studies with tritiated all-trans-RA have discovered the existence of many yet to be characterized proteins that specifically bind RA.
The three-dimensional structures of these binding proteins, with and without the bound ligand, have been determined by X-ray crystallography. It has been found that the common feature of these molecules is a β-barrel, formed by two orthogonal β-sheets and an α-helix. This three-dimensional structure of these binding proteins is considered the prototype for a family of proteins which may all bind small hydrophobic molecules. The members of this family of proteins include β-lactoglobulin, purpurin, insect bilin-binding protein, placental protein 14, and androgen dependent secretory protein. Some of these proteins bind hydrophobic ligands and are thought to be involved in their transport. For example retinoids bind to RBP, β-lactoglobulin, and purpurin, while biliverdin pigments bind to bilin-binding protein, and cholesteryl esters bind to apolipoprotein D. The physiological functions of some of the proteins in this family are still not known.

Ligand binding studies by X-ray crystallography have revealed that SRBP and CRABPs bind their respective ligands in such a way that the ligand trimethylcyclohexenyl ring is bound innermost in the β-barrel and the terminal polar group extends toward the surface of the protein. On the contrary, even though CRBP I and CRBP II have been found to have similar β-barrel motifs, the CRBPs have been found to bind retinol with the terminal hydroxyl group innermost and the trimethylcyclohexenyl ring extending towards the outer surface of the binding pocket. Studies on localization and conformation of the protein-bound retinoids have therefore become increasingly interesting in the recent past. It has been found that retinal binds to opsin with an
extended side chain and a \textit{6-s-trans} conformation between the cyclohexene ring and the polyene side chain.\textsuperscript{215} It has also been suggested that RA binds to CRABP II in a twisted \textit{6-s-cis} conformation, while the conformation of RA when bound to CRABP I is still not clear.\textsuperscript{216} The preferred conformation for natural retinoids has earlier been predicted to be the one in which the planar extended side chain has a slightly twisted \textit{6-s-cis} relationship to the trimethylcyclohexenyl ring, based on solution NMR studies and the computational chemistry studies of uncomplexed retinoids.\textsuperscript{217,218}

\begin{center}
\includegraphics[width=\textwidth]{retinoids_conformers.png}
\end{center}

\textit{Figure 17. Different conformers of retinoids.}

The three-dimensional structure of \textit{\textbeta}-lactoglobulin B (BLG) has been determined by X-ray diffraction and is found to be remarkably similar to that of SRBP.\textsuperscript{142,144,145} Much like SRBP, BLG also has an eight-stranded anti-parallel \textit{\textbeta}-barrel flanked on one
side by an α-helix constituting a hydrophobic pocket.\textsuperscript{145} BLG is the major whey protein in bovine milk and therefore is available readily. BLG has been found to bind a number of hydrophobic molecules, in particular fatty acids and retinoids.\textsuperscript{219,220} Free fatty acids have been found to bind to BLG reversibly. Triglycerides also bind to β-lactoglobulin. Binding of retinol to BLG was first shown by Futterman and Heller in 1972. They showed that the fluorescence lifetime of retinol was enhanced by BLG from 3 to 10 ns and also BLG reduced the susceptibility of retinol to oxidation.\textsuperscript{219} Papiz \textit{et al.} suggested that retinol binds to BLG at the β-barrel, based on their model-building studies.\textsuperscript{145} Also, Cho \textit{et al.} suggested, based on their chemical modification studies, that the retinol binding site lies inside the β-barrel.\textsuperscript{221} On the other hand, Monaco \textit{et al.} suggested that retinol apparently binds in an external hydrophobic channel that is different from the β-barrel and therefore binding of retinol to BLG is different from binding of retinol to SRBP, in spite of their similar three dimensional structures.\textsuperscript{146} Therefore, it appears that BLG may have two different binding sites for retinol, a hydrophobic β-barrel and a hydrophobic pocket on the surface of the protein, although retinol binds to BLG only with a stoichiometry of 1:1.\textsuperscript{145,146,222} There is also a controversy as to whether BLG binds fatty acids and retinol at the same site or at different sites.\textsuperscript{223-225} It was also shown that BLG, while binding β-ionone and retinoids that have the β-ionylidene part in their structure, does not bind α-ionone which differs from β-ionone only in the placement of the cyclohexenyl double bond, which is not conjugated with the isoprenoid side chain.\textsuperscript{226}
This suggests that the structural constraints imposed on the retinoid due to the conjugation of the cyclohexenyl double bond with the isoprenoid side chain are essential for recognition and binding by BLG. Although BLG is often used as a model protein for retinoid-protein binding studies, the physiological function of this protein is still not known. It has been suggested that BLG may serve to bind and protect acid-labile ligands such as retinol and ultimately help in transporting those ligands from the maternal milk to the neonate, based on the protein's resistance to low pH and gastric proteolysis, although there is no direct evidence for the transport function.\textsuperscript{227,228} It has been suggested that specific receptors for BLG are present in the small intestine of the neonate calf and are probably involved in the uptake of vitamin A\textsuperscript{145}. BLG has also been proposed to be involved with the transfer of passive immunity, although there is no direct evidence for this function either.\textsuperscript{229} Therefore much remains to be learned about BLG in terms of its function as well as its primary ligand and the bound conformation of its ligands. Since BLG is readily available, similar to SRBP in structure, binds retinoids with reasonably
high affinity, and the conformations of BLG-bound retinoids is not known yet. we
decided to use it for studying the conformation of protein-bound retinoid.

3.2. NMR STUDIES OF PROTEIN-LIGAND INTERACTIONS

One of the methods that has been employed in studying the structures of proteins
in general and/or the conformation and localization of bound ligands is NMR
spectroscopy. There are many NMR methods that have been developed to study the three-
dimensional structural information of the protein-bound ligands depending on the nature
of the protein-ligand interactions. For example, transfer NOE measurements can be used
to study the bound conformation of weakly bound ligands but not tightly bound
ligands. Isotope-edited NMR and heteronuclear 3D NMR methods have been
developed to study the conformations of tightly bound ligands. These techniques
are used to selectively detect signals for protons attached to isotopically labelled ligand
when bound to the protein. Isotope-filtering techniques are used to detect the proton
signals of an unlabelled ligand when bound to an isotopically labelled protein.

The above mentioned isotope-edited NMR experiments have been less
extensively exploited for studying the protein bound conformations of the retinoids.
Using samples specifically $^{13}$C-labeled at different positions of retinal, Smith et al. found
that bacteriorhodopsin-bound retinal has a 6-\textit{s-trans} conformation based on the results of
solid-state magic angle spinning NMR (MAS NMR). Li and co-workers used
fluororetinol and performed $^{19}$F NMR studies. They found that when bound to CRBP
II, fluororetinol exchanges between magnetically distinct binding states, which may
correlate with the multiple conformations of bound retinol as observed in crystalline holo-
CRBP II.\textsuperscript{161,237} Li and co-workers have also used 6-fluorotryptophan substituted
CRBPs in their ligand binding studies and identified the presence of a tryptophan residue
in the retinol binding site.\textsuperscript{156} Further, Li and co-workers have also used specifically \textsuperscript{13}C-
labeled all-\textit{trans}-RA and have performed isotope-edited NMR methods to study the
CRBP-bound conformation of \textsuperscript{13}C-labeled RA.\textsuperscript{216} The different NMR techniques used
in their study include HMQC (heteronuclear multiple-quantum coherence), HMQC-
TOCSY (total correlation spectroscopy), and \textsuperscript{13}C-TOCSY-REVINEPT (reverse
insensitive nucleus enhancement by polarization transfer). By performing these NMR
experiments, they observed that CRABP II-bound RA has a 6-s-cis conformation with a
torsion angle of -60°.\textsuperscript{216} Using the same experiments they found that CRABP I-bound
RA had no single, static conformation and therefore its CRABP-I-bound conformation is
still uncertain.\textsuperscript{216}

3.3 NMR STUDIES OF \textit{β}-IONONE-\textit{β}-LACTOGLOBULIN B INTERACTIONS

As we have seen earlier, \textit{β}-ionone and the natural retinoids have similar structural
features, differing only by the length of the isoprenoid side chain. We have also seen
earlier that BLG binds \textit{β}-ionone and retinol and not \textit{α}-ionone, in which the cyclohexenyl
double bond is not conjugated with the isoprenoid side chain double bond. Apparent
dissociation constants of retinol-BLG and \textit{β}-ionone-BLG complexes have been found to
be 2 x 10^{-4} M and 6 x 10^{-7} M respectively.\textsuperscript{226,238} It therefore appears that the
conjugation of the double bonds and hence the conformation of the molecule about the C-6, C-7 single bond is more important than the length of the isoprenoid side chain in determining the binding specificity.

3.3.1 LIGANDS USED IN OUR STUDIES AND THEIR SYNTHESIS

The different ligands that have been used in our studies are unlabelled β-ionone, [8,10-\textsuperscript{13}C\textsubscript{2}]-β-ionone (18), [5-\textsuperscript{13}CH\textsubscript{3}]-β-ionone (19), and 4-oxo-β-ionone (20). Unlabelled β-ionone was purchased from Aldrich Chemical Co. Synthesis of ligands 18, 19, and 20 are described below. The labelled β-ionones were prepared and used in various NMR experiments to study the free and protein-bound conformation of β-ionone. By using the \textsuperscript{13}C-labelled β-ionones in HMQCNOE experiments, we hoped to observe the signals for the protons attached to the labelled carbons and the signals for those protons which show NOE to the protons attached to the labelled carbons. The observed NOE results from these experiments could then be used for predicting the conformation of protein-bound β-ionone.

3.3.1.2 SYNTHESIS OF [8,10-\textsuperscript{13}C\textsubscript{2}]-β-IONONE (18)

Doubly labelled β-ionone (18) was obtained in 58\% yield by Aldol condensation of β-cyclocitral and [1,3-\textsuperscript{13}C\textsubscript{2}]-acetone as shown in figure 19.\textsuperscript{239} The reaction mixture was periodically sampled by GC until all of the β-cyclocitral had been consumed, which
took about 9 days since the reaction mixture had limiting quantities of \( [1.3^{-13}\text{C}_2] \)-acetone. Compound 18, thus obtained from the aldol condensation, was identical to \( \beta \)-ionone in terms of its physical properties with \( ^1\text{H} \) NMR (CDCl\(_3\)) \( \delta \) 2.28 ppm (d, \( 3,-\text{CO}^{13}\text{CH}_3, J_{\text{CH}} = 124 \text{ Hz} \)), and 6.09 ppm (dd, 1, \( J_{\text{HH}} = 16.4 \text{ Hz}, J_{\text{CH}} = 123 \text{ Hz} \)). Also the mass spectrum obtained for 18 showed its molecular weight to be 194, which is consistent with its molecular formula \( \text{C}_{11}^{13}\text{C}_2\text{H}_{20}\text{O} \).

![Chemical structure](image)

Figure 19. Synthetic scheme for compound 18.

3.3.1.3 SYNTHESIS OF \( [5^{-13}\text{CH}_3] \)-\( \beta \)-ionone (19)

The synthetic scheme for \( ^{13}\text{C} \)-labelled \( \beta \)-ionone 19 is shown in figure 20. This synthetic scheme was patterned after the scheme employed for the synthesis of multiply labelled RA by McDermott et al. with slight modifications. Cyclic \( \beta \)-ketoester 23 was synthesized in two steps from 6-methylhept-5-en-2-one 21, as previously described. in
good yields. The anion of 21 prepared by reacting it with NaH in an inert atmosphere was reacted with dimethyl carbonate to give 22. Cyclization of 22 to form 23 in 66% yield was achieved by treating with stannic chloride. Grignard reaction, using 5 equivalents of $^{13}\text{CH}_3\text{MgI}$, with 23 gave the hydroxyester 24 in 40% yield, after stirring for 5 hrs at reflux and 5 days at room temperature in ether. The reaction conditions for the preparation of 24 from 23 were optimized by reacting 23 with unlabelled Grignard reagent (CH$_3$MgI) under various reaction conditions, including reaction temperature, number of equivalents of the Grignard reagent used in the reaction, and the total time of the reaction. The number of equivalents of Grignard reagent used in the reaction was varied from 1 to 10 equivalents and we found that 5 equivalents of unlabelled Grignard reagent gave the best yield of unlabelled 24. Also, we found that gently refluxing the reaction mixture for 5 hrs, followed by stirring for 5 days at room temperature formed unlabelled 24 in better yields. Therefore we used the same reaction conditions for our synthesis of labelled 24, using 5 equivalents of $^{13}\text{CH}_3\text{MgI}$. Dehydration of this hydroxyester 24 using POCI$_3$ in pyridine gave the unsaturated ester 25 in 67% yield after chromatography. In the next step, the unsaturated ester 25 was reduced to the alcohol 26 by reacting with LiAlH$_4$ in ether. Although oxidation of this allylic alchol 26 to the corresponding aldehyde, $\beta$-cyclocitril, by using MnO$_2$, was achieved in good yields by McDermott et al., in our hands the MnO$_2$ oxidation of 26 proceeded very poorly and was very inconsistent. Further attempts to improve the efficiency of the reaction by azeotroping the MnO$_2$ in benzene to remove the bound water was not successful. Our
Figure 20. Synthetic scheme for [5-\textsuperscript{13}CH\textsubscript{3}]\(\beta\)-ionone 19.
attempts with different solvents as the reaction medium, besides changing the reaction time from 1 day to 7 days, were also unsuccessful. Our attempts to achieve the oxidation by Swern conditions also failed. Pyridinium chlorochromate oxidation of the allylic alcohol 26, however, gave the pure aldehyde 27 (labelled β-cyclocitral) in 71% yield.242 Aldol condensation of 27 with an excess of acetone, as described above, gave 19 identical to β-ionone, in 56% yield, with $^{13}$C NMR (CDCl$_3$) $\delta$ 21.68 ppm, and $^1$H NMR (CDCl$_3$) $\delta$ 1.75 ppm (d, 3, $J_{CH} = 126$ Hz) for 5-$^1$CH$_3$ group. Also the mass spectrum showed the M$^+$ as 193, consistent with its molecular formula C$_{12}$$^{13}$C$_1$H$_{10}$O.

3.3.1.4 SYNTHESIS OF 4-OXO-β-IONONE (20)

The 4-oxo-β-ionone was synthesised as previously described by Curley and Carson.243 Allylic bromination of commercially available β-ionone by treating it with N-bromosuccinimide, followed by bromine displacement under basic conditions, lead to the formation of 4-hydroxy-β-ionone in 56% yield after chromatographic purification. The 4-hydroxy-β-ionone was then taken in methylene chloride and stirred with MnO$_2$ at room temperature for 12 hours to give the 4-oxo-β-ionone 20 in 61% yield. The reaction scheme is shown in figure 21.
3.3.2 STUDIES OF PROTEIN-BOUND CONFORMATION OF β-IONONES

Solution conformations of β-ionone and structurally related compounds have earlier been studied using NMR spectroscopy. Based on these studies, the preferred conformation for these uncomplexed molecules has been predicted to be the one in which the planar isoprenoid side chain has a slightly twisted 6-s-cis relationship with the trimethylcyclohexenyl ring. They also estimated the torsion angle of 30-40°, based on computational chemistry studies, although these calculations could not distinguish
between the predicted 6-s-cis conformation and a twisted 6-s-trans conformation with a ring-side chain torsion angle of 150-160°.\textsuperscript{218} The \textsuperscript{1}H NMR spectra of retinoids have been described and assigned.\textsuperscript{244} The \textsuperscript{1}H NMR spectrum of β-ionone in ethanol-d\textsubscript{6} is shown in figure 22. It can be seen from this spectrum that the geminal methyl groups at position 1 are chemical shift equivalent. We have found that these geminal methyl groups are chemical shift equivalent even at -130°C (ethanol-d\textsubscript{6}), as have others although they did not pursue measurement below -100°C.\textsuperscript{245} This has also been found to be true for the natural retinoids with a trimethylcyclohexenyl ring as a common structural feature.

Additionally, we measured the steady state NOEs to the methyl groups for β-ionone, all-trans-RA, and 4-oxo-β-ionone and the results of these NOE difference experiments are summarized in Table 2. We carried out the NOE difference experiments on these compounds in an argon degassed solution of non-polar solvent (CDCl\textsubscript{3}) as well as a polar, protic medium (CD\textsubscript{3}OD). We found that the type of solvent did not cause any significant change in the relative steady-state NOE. Earlier it has been demonstrated that NOE build-up is proportional to the inverse sixth power of the distance between the irradiated nucleus and the observed nucleus, and therefore in the case of rigid molecules the observed NOEs can be used to determine the conformations of these molecules in solution.\textsuperscript{73,246,247} The ring-side chain dihedral angle could not be conclusively estimated from the results of the NOE difference experiments for the retinoids due to the flexibility of the retinoid molecules and the lack of an exact reference distance in these
Figure 22. $^1$H NMR spectrum of β-ionone in ethanol-d$_6$. 
Table 2. Nuclear Overhauser enhancements for RA, β-ionone and 4-oxo-β-ionone.

<table>
<thead>
<tr>
<th>Group irradiated</th>
<th>Compound</th>
<th>Solvent</th>
<th>Observed enhancement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H-7</td>
</tr>
<tr>
<td>1,1'-(CH₃)₂RA</td>
<td>RA</td>
<td>CDCl₃</td>
<td>12.0*</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>CD₂OD</td>
<td>8.6</td>
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<tr>
<td></td>
<td>β-ionone</td>
<td>CDCl₃</td>
<td>14.0</td>
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<tr>
<td></td>
<td>β-ionone</td>
<td>CD₂OD</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>4-oxo-β-ionone</td>
<td>CD₂OD</td>
<td>5.3</td>
</tr>
<tr>
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<td>RA</td>
<td>CDCl₃</td>
<td>2.6</td>
</tr>
<tr>
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<td>CD₂OD</td>
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<td>β-ionone</td>
<td>CD₂OD</td>
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<tr>
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<td>CD₂OD</td>
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<td>RA</td>
<td>CDCl₃</td>
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<td>CD₂OD</td>
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<tr>
<td></td>
<td>4-oxo-β-ionone</td>
<td>CD₂OD</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* = No enhancement observed. NI = Not integrated since coupling effects prevented accurate integration. NA = Not applicable, since corresponding proton not present in structure. a -Values reported typically vary ±0.1 - 0.4.
compounds. However, as we can see from the table, there are strong interactions between the 1.1'-methyl protons and 7-vinyl proton as well as the 5-methyl protons and 8-vinyl proton, therefore suggesting a preference for a twisted 6-s-cis relationship between the ring and the side chain.

Irradiation of the 5-CH₃ group showed strong NOE to the 4-CH₂ group as well (an enhancement of 9.3%). The small H-8/H-7 % NOE ratios observed for RA and β-ionone when the 5-CH₃ group is irradiated may be due to the dipolar relaxation via the 4-CH₂ protons and therefore we thought that by using [4,4-²H₂]-β-ionone to measure the steady state NOE we could eliminate this possibility, since in this compound there will be no protons in the 4 position. Our first attempted method to prepare [4,4-²H₂]-β-ionone was to protect it as a 9-ethylenedioxy ketal first, and then oxidize the 4 position in β-ionone followed by treatment with LiAlD₄ to give the corresponding [4-²H₁]-4-hydroxy-β-ionone as its 9-ethylenedioxy ketal. We thought that the hydroxyl group on the 4 position in this compound could then be reacted with toluenesulphonyl chloride to give the corresponding 4-tosylated compound. The tosyl group could then be replaced with dueterium by reacting with LiAlD₄. The [4,4-²H₂]-(9-ethylenedioxy ketal)-β-ionone could then be deprotected to give the desired [4,4-²H₂]-β-ionone. Although we were able to prepare the 9-ethylenedioxy ketal of β-ionone, the ketal protecting group was not stable towards our allylic oxidation conditions using NBS. When we tried the allylic oxidation of the 9-ethylenedioxy ketal of β-ionone to form 4-hydroxy(9-ethylene dioxy ketal)-β-ionone, the protecting group was removed and hence we isolated 4-hydroxy-β-ionone. Therefore we decided to use a different scheme for the synthesis of [4,4-²H₂]-β-ionone.
Our next attempt to prepare \( [4,4^{-2}H_2]-\beta\)-ionone was designed on the basis of allylic bromination of \( \beta\)-ionone followed by displacement with deuterium by using NaBD\(_4\). The \([4^{-2}H_1]\)-\( \beta\)-ionol thus obtained could then be oxidized to give \([4^{-2}H_1]\)-\( \beta\)-ionone. Repeating the whole sequence of reactions would give a mixture of both 4,4-didueterated and 4-mono-dueterated \( \beta\)-ionones, the 4,4-didueterated \( \beta\)-ionone hopefully being the major product due to the deuterium isotope effect. We thought repeating the synthetic sequences again may lead to mostly 4,4-didueterated \( \beta\)-ionone because of the isotope effects. Unfortunately, when 4-bromo-\( \beta\)-ionone was reacted with NaBD\(_4\), we did not get any identifiable product.

We then decided to synthesize 4-oxo-\( \beta\)-ionone, which also does not have any protons in the 4 position and therefore would probably behave similarly to \([4,4^{-2}H_2]\)-\( \beta\)-ionone. when measuring steady state NOEs. Our modelling studies of 4-oxo-\( \beta\)-ionone predicted a preferred conformation similar to that of RA and \( \beta\)-ionone. The preparation of 4-oxo-\( \beta\)-ionone has been described in the previous section. The H-8/H-7 % NOE ratio upon irradiation of 5-CH\(_3\) group in 4-oxo-\( \beta\)-ionone was found to be greater than that for \( \beta\)-ionone and is also consistent with a slightly twisted \( 6\)-\( s\)-cis ring-side chain conformation.

In order to estimate the torsion angles, we performed molecular modelling studies on these compounds. For our molecular modelling studies, we initially constructed RA, \( \beta\)-ionone, and 4-oxo-\( \beta\)-ionone with a relatively planar side chain conformation and an \( s\)-\( cis\) relationship about the C-6, C-7 single bond. These structures were then allowed to
relax to their respective calculated minimum energy conformation using CHARMm. These starting structures were chosen since the NMR studies, as well as the X-ray crystal structure of the common triclinic form of RA, suggested a preference for a relatively planar, conjugated side chain. From these energy minimized structures of RA, β-ionone and 4-oxo-β-ionone the ring-side chain dihedral angle was predicted to be 43°, 38°, and 43.5° respectively. A similar preferred conformation for RA was also predicted using other computational methods (MMP2 and SYBYL). These predicted torsion angles are shown in Table 3.

Table 3. Calculated minimum energy ring-side chain torsion angles.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Computational tool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHARMm</td>
</tr>
<tr>
<td>RA</td>
<td>43°</td>
</tr>
<tr>
<td>β-ionone</td>
<td>38°</td>
</tr>
<tr>
<td>4-oxo-β-ionone</td>
<td>43.5°</td>
</tr>
</tbody>
</table>

We also assessed the relative stability of the calculated minimum energy conformation with respect to the ring-side chain torsion angle by performing a grid conformational search about this torsion in β-ionone using the computational tool CHARMm. The energy minimized structure of β-ionone in CHARMm was taken, the torsion angle was incremented in 10° fixed steps, the remainder of the molecule allowed to relax after each increment, and the CHARMm energy recalculated during this search.
The calculated relative energy versus torsion angle was plotted as a graph and is shown in figure 23. In this figure, the positive angles represent motion of the H-7 proton toward the reader, while the negative angles represent the motion of the H-7 proton away from the reader. As we can see from this graph, a slightly twisted 6-s-cis conformation is preferred for β-ionone, while a twisted 6-s-trans conformation is also possible. Similar grid conformational searches on RA incremented in 5° fixed steps, using CHARMM or SYBYL, also had an identical profile. Although our prediction of a slightly twisted 6-s-cis conformation for these free retinoids is in agreement with the results of earlier works by Honig et al. and Rowan et al., their protein-bound conformations could be different from their uncomplexed conformations. Therefore we decided to study the BLG-bound conformation of β-ionone, by NMR techniques by using various 13C-labelled β-ionones.

It has previously been found that the secondary structure of BLG is altered from a predominantly β-strand to an α-helix, when exposed to buffers containing 20% ethanol by volume.224,249 Therefore for all our ligand-binding studies on BLG, we used buffers containing only 10% ethanol. It has been observed that bovine BLG exists as a monomer below pH 3.5 and also at physiological pH, although there is evidence that at pH 6.5 bovine BLG exists in an equilibrium mixture of monomer and dimer.250-252 Over a pH range of 8 to 9.5 BLG has been found to undergo slow, time-dependent changes, while above pH 9.5 it is denatured irreversibly. It has also been known that both the monomer and the dimer of BLG bind retinoids with equal affinity.238 The retinoids are very unstable at low pH values due to the conjugated double bonds in their structures.
Figure 23. Conformational analysis of β-ionone. The energy minimized structures of β-ionone for the indicated torsion angles (A, B, and C) are shown in next page.
Figure 23a. Structure of β-ionone with different torsion angles. A. Torsion angle of -120°. B. Torsion angle of -30°. C. Torsion angle of +39°.
Therefore we decided to study the retinoid-protein interactions at pH 7.0. Further, it has been known that at smaller concentrations (< 20 μM) BLG exists as a monomer while at high concentrations it exists as a dimer.\textsuperscript{222,252} We chose to use 1mM concentrations of BLG for our NMR experiments for sensitivity reasons.

We first prepared a saturated solution of β-ionone in 50 mM phosphate buffer, pH of 7.0, containing 10% ethanol, and we found that the concentration of β-ionone in this saturated solution was more than that required for our NMR studies (1mM), as estimated by UV spectrophotometry. When we prepared a 1mM β-ionone solution in deuterated ethanol/phosphate buffer, we observed a second set of broadened signals in the \textsuperscript{1}H NMR spectrum. Initially we thought the second set of signals might be due to the formation of the enolphosphate of β-ionone in the 10% ethanol/phosphate buffer. Examination of this ligand in buffer by \textsuperscript{3}P NMR showed only one signal for phosphorus in the buffer and hence the probable absence of an enol phosphate. This second set of signals observed in the \textsuperscript{1}H NMR spectra, however, disappeared upon further dilution with deuterated ethanol. We also extracted the ligand from the buffer with chloroform and found it to be β-ionone by \textsuperscript{1}H NMR in CDCl\textsubscript{3}, with no second set of signals. Therefore we suggest that the second set of signals present in the buffer solution was due to the presence of micelles of β-ionone in exchange with its free form in solution. Further, 1 mM of \textsuperscript{[8,10\textsuperscript{13}C\textsubscript{2}]}-β-ionone \textbf{18} in 10% deuterated ethanol/buffer was titrated with increasing concentrations of BLG (0, 0.1, 0.3, 1.0, and 2.0 mM) and \textsuperscript{1}H (shown in figure 24) and \textsuperscript{13}C NMR spectra were obtained for each step during the titration.
Figure 24. Titration of 1 mM of β-ionone in phosphate buffer with BLG. The vinyl region of the $^1$H NMR spectra after each addition of BLG are shown. Ratio of β-ionone to BLG is indicated.
We observed a progressive decrease in the second set of signals during the titration and the complete disappearance of the second set of signals for both the $^1$H and $^{13}$C NMR spectra was observed at 1-2 mM of BLG. Also, with increasing concentrations of BLG, we observed a downfield chemical shift and broadening of the $^{13}$C signals and an upfield shift and broadening of the vinyl proton signals for [8,10-$^{13}$C$_2$]-β-ionone 18. This effect was maximal at 1-2 mM of BLG. These observations are consistent with the loss of micelles of [8,10-$^{13}$C$_2$]-β-ionone 18 and its binding to BLG.

We then performed the HMQCNOE experiment with the BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18, with the $^{13}$C excitation frequency windowed around the labelled methyl carbon (around 26.5 ppm) and a mixing time of 150 ms. In the 2D HMQCNOE experiment the dipolar coupling to the protons attached to the $^{13}$C-labelled carbon atoms in the ligand were allowed to build up after the HMQC magnetization transfer from $^{13}$C to the attached $^1$H, by using the pulse sequence shown in figure 25 a. In this particular HMQCNOE experiment the dipolar coupling to the protons attached to the $^{13}$C-labelled methyl carbon in the ligand was allowed to build up after the HMQC magnetization transfer from the $^{13}$C-labelled methyl carbon to the attached protons. In this experiment we hoped to observe the signals for the protons attached to the labelled methyl carbon and also the signals for any other proton that would show NOE to those $^{13}$C-labelled methyl group protons. The results of this experiment would therefore enable us to identify the protons that are in close-proximity to the labelled methyl group. The result of this experiment is shown in figure 26. Significant NOEs from the $^{13}$C-methyl protons (2.19 ppm) to a prominent signal at 7.2 ppm as well as to a number of different signals from 0.5
Figure 25. Pulse sequences used in the 2D NMR experiments.

a. HMQCNOE

\[
\begin{array}{cccccccc}
\text{\textsuperscript{1}H} & 90 & \frac{1}{2}J_{\text{CH}} & \frac{1}{4}J_{\text{HH}} & \frac{1}{4}J_{\text{HH}} & 180 & \frac{1}{2}J_{\text{CH}} & 90 & t_m & 90 & t_2 \\
\text{\textsuperscript{13}C} & & 90 & 90 & & & & & & & \text{GARP} \\
\end{array}
\]

b. HMQCCOSY

\[
\begin{array}{cccccccc}
\text{\textsuperscript{1}H} & 90 & 180 & 90 & \frac{1}{4}J_{\text{HH}} & 180 & \frac{1}{4}J_{\text{HH}} & 90 & t_2 \\
\text{\textsuperscript{13}C} & & 90 & 90 & & & & & \text{GARP} \\
\end{array}
\]

c. HMQCTOCSY

\[
\begin{array}{cccccccc}
\text{\textsuperscript{1}H} & 90 & 180 & & & \text{DIPSI 2} & & t_2 \\
\text{\textsuperscript{13}C} & & 90 & 90 & & & & \text{GARP} \\
\end{array}
\]
Figure 26. HMQCNOE spectrum of BLG-bound 18. The $^{13}$C excitation frequency was windowed around labelled methyl group carbon.
to 2 ppm were observed. The resonance at 7.2 ppm is close in chemical shift to that of the H-7 vinyl proton of the protein-bound [8,10-^{13}C_2]-β-ionone. The upfield NOEs observed from 0.5 to 2.0 ppm most likely represent intermolecular NOEs to aliphatic amino acid side chain groups on BLG that are in close contact with the BLG-bound [8,10-^{13}C_2]-β-ionone 18.

We then performed a HMQCCOSY experiment with the BLG-bound [8,10-^{13}C_2]-β-ionone 18. In the case of the 2D HMQCCOSY experiment the scalar coupling to the protons attached to the ^{13}C-labelled carbon atoms were allowed to build up after the HMQC magnetization transfer from ^{13}C to ^{1}H, using the pulse sequence shown in figure 25 b. In this particular HMQCCOSY experiment the scalar coupling to the protons attached to the ^{13}C-labelled vinyl carbon was allowed to build up after the HMQC magnetization transfer from the vinyl ^{13}C to ^{1}H. By doing this experiment we hoped to observe the signal for the 8-vinyl proton, which is attached to the ^{13}C-labelled vinyl carbon, and the signal for the 7-vinyl proton, since it is is coupled to the 8-vinyl proton. Therefore, we hoped to establish the chemical shift of 7-vinyl proton of the protein-bound 18 as 7.2 ppm. The results of this experiment with the ^{13}C excitation frequency windowed around the labelled vinyl carbon atom (130.6 ppm) is shown in figure 27. The resonance at 7.2 ppm was in fact confirmed to be due to the H-7 vinyl proton of BLG-bound [8,10-^{13}C_2]-β-ionone 18. This result therefore suggests that the NOE signal observed at 7.2 ppm in the result of the HMQCNOE experiment shown in figure 26 is to the 7-vinyl proton of the protein-bound 18.
Figure 27. HMQCCOSY spectrum of 18. The $^{13}$C excitation frequency was windowed around labelled vinyl carbon.
We also performed another HMQCNOE experiment, this time setting the $^{13}$C excitation frequency windowed around the labelled vinyl carbon. The result of this experiment should show the signal for the 8-vinyl proton, which is attached to the labelled vinyl carbon, and the signals for protons that would show NOE to the 8-vinyl proton. Results of this experiment showed significant NOEs from the $^{13}$C-vinyl proton (6.2 ppm) to a resonance at 1.7 ppm as well as an upfield signal at 0.8 ppm, as can be seen in figure 28. The resonance at 1.7 ppm is most likely to be due to the protons of the methyl group on position 5 of the BLG-bound ligand, even though it is slightly upfield of the resonance for the same protons in the unbound β-ionone, while the NOE observed at 0.8 ppm is likely to be to one or both of the geminal methyl groups on C-1 of the BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18, even though these two methyl group protons have the same downfield chemical shift at 1.0 ppm in the case of free β-ionone.

Since there is a long range coupling from the H-7 vinyl proton to the C-4 methylene protons and the methyl protons on C-5 of β-ionone, we then performed a HMQCTOCSY experiment with BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18. The 2D HMQCTOCSY experiment allows for a build up of strong couplings shown to the protons attached to $^{13}$C labelled carbon atoms, after the HMQC magnetization transfer from $^{13}$C to $^1$H, and the pulse sequence used for this experiment is shown in figure 25 c. In the case of HMQCTOCSY experiment with BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18, the $^{13}$C excitation frequency was set to that of the labelled vinyl carbon so as to see a TOCSY transfer from H-8 vinyl proton to the H-4 methylene proton and the methyl protons on C-5 through the H-7 vinyl proton. We hoped that this experiment would help us to establish
Figure 28. HMQCNOE spectrum of BLG-bound 18. The $^{13}$C excitation frequency was windowed around labelled vinyl carbon.
the chemical shift of the 5-CH$_3$ protons of protein-bound 18 as 1.7 ppm, and therefore that the NOE signal observed at 1.7 ppm in the result of the HMQCNOE experiment shown in figure 28 is to the 5-CH$_3$ group of the protein-bound 18. Surprisingly, the result of this experiment did not show any TOCSY transfer beyond the H-7 vinyl proton. We performed a selective TOCSY experiment on uncomplexed β-ionone in chloroform, so as to confirm that the TOCSY experiment could actually work. In the case of the selective TOCSY experiment, the H-8 vinyl proton was selectively excited in order to observe the TOCSY transfer from the H-8 vinyl proton to the 4-CH$_2$ and 5-CH$_3$ protons via H-7 vinyl proton. The results of this experiment showed the expected TOCSY transfer from H-8 vinyl proton to 4-CH$_2$ and 5-CH$_3$ protons via H-7 vinyl proton (data not shown). Therefore we believe that we performed the HMQCTOCSY experiment successfully, although we did not observe the expected TOCSY transfer. In the BLG-bound conformation of [8,10-$^{13}$C$_2$]-β-ionone 18, it is possible that the coupling constants between H-7 vinyl proton and 5-CH$_3$ protons, and H-7 vinyl proton and 4-CH$_2$ protons may have become vanishingly small, leading to the absence of TOCSY transfer beyond H-7 of the bound ligand. Alternatively, the small couplings may be unobservable because of the shortened relaxation times for the protein-bound ligand. In order to analyze the cause of the NOE at 0.8 ppm in the HMQCNOE experiment shown in figure 28, we then measured the NOE build-up with different mixing times. These NOE mixing time build-up curves, shown in figure 29, suggest that the NOE to the resonance at 0.8 ppm is influenced by spin diffusion. The volume of the ‘cross peak’ at 1.7 ppm divided by the
Figure 29. NOE mixing time build-up curves.
'diagonal peak' at 6.2 ppm increased linearly with increasing mixing time, as expected at early mixing times, never exceeded the theoretical maximum ratio of 1.0, and was not observed at long mixing times (600 ms). The ratio of the volume of the cross peak to that of the diagonal peak for the 0.8 ppm/6.2 ppm pair, on the other hand, showed a parabolic increase with increasing mixing times, and substantially exceeded a ratio of 1.0 at longer mixing times and therefore suggested a dominance of spin diffusion in the appearance of the NOE to the signal at 0.8 ppm. Spin diffusion is a phenomenon in which the rate of transfer of spin energy between nuclei becomes much larger than the rate of transfer of energy to the lattice and is frequently observed in NMR experiments involving macromolecules. It has been found that under such conditions the magnitude of the NOEs observed (one-dimensional as well as two-dimensional) does not have a simple relationship with the internuclear distance and may lead to relayed NOEs. Therefore the observed NOEs in these cases do not give much information about the conformation of molecules.

We then prepared the [5-13CH3]-β-ionone 19, and used it as a ligand in attempts to confirm that the NOE at 1.7 ppm observed in the HMQCNOE experiment with the labelled C-8 vinyl carbon is indeed to the 5-methyl group of BLG-bound [8.10-13C2]-β-ionone 18. The HMQCNOE experiment was performed with BLG-bound [5-13CH3]-β-ionone 19 with the 13C excitation frequency set to that of 13C-labelled 5-methyl group (22.6 ppm). Although significant NOE to aliphatic type resonances was observed from 0.8 ppm to 1.9 ppm, no measurable NOE to a resonance at 6.2 ppm was observed even at different mixing times (100 to 200 ms). The HMQC experiment with BLG-bound [5-
\(^{13}\text{CH}_3\)-β-ionone 19, however, showed a signal at 1.7 ppm (data not shown) suggesting that the resonance at 1.7 ppm is in fact due to the \(^{13}\text{C}\)-labelled C-5-methyl group protons of BLG-bound [5-\(^{13}\text{CH}_3\)]-β-ionone 19. This suggests that the NOE observed at 1.7 ppm in the HMQCNOE with BLG-bound [8,10-\(^{13}\text{C}_2\)]-β-ionone 18 as shown in figure 28 is to the 5-methyl proton signal.

The steady state NOE measurements obtained for RA, β-ionone, and 4-oxo-β-ionone predict a preferred solution conformation of these retinoids to be the one in which there is a 6-s-cis relationship between the isoprenoid side chain and the trimethylcyclohexenyl ring. The computational methods that we employed predicted a preferred torsion angle of 31-44°, consistent with the NMR experiments. The results of the HMQCNOE and HMQCCOSY experiments performed on BLG-bound \(^{13}\text{C}\)-labelled β-ionones strongly suggest that β-ionone also binds to BLG with a planar side chain with a 6-s-cis conformation similar to that shown below, in figure 30.

![β-ionone diagram](image)

**Figure 30.** Predicted conformation for BLG-bound β-ionone.
CHAPTER 4

CONCLUSIONS

Retinol and its analogs affect a number of physiological processes including growth and differentiation. Recently retinoids have generated interest as useful agents for the treatment of skin disorders and for cancer chemoprevention. Retinoids are believed to exert their biological activity by binding to the nuclear receptors, RARs and RXRs, even though their exact mechanism of action is not known yet. Metabolism of RA has been studied extensively. It has been found that some metabolites of RA, such as 13-cis-RA and retinoyl-β-glucuronide, are also biologically active while other metabolites, such as 5,6-epoxyretinoic acid and 4-hydroxyretinoic acid, are inactive. These studies have led to the development of synthetic retinoids such as etretinate, which are more resistant towards ring metabolism.

Etretinate has been indicated in the treatment of psoriasis. It is highly lipid soluble, has a long elimination half-life (about 100 days), and is also teratogenic. Acitretin, the free acid form of etretinate, has been found to be equipotent to etretinate, in terms of its antipsoriatic activity, and therefore etretinate is considered to be a prodrug of acitretin. Etretinate as well as acitretin have been less well studied than RA in terms of
their metabolism. Their mechanism of action is also not fully known yet. Owing to these reasons, there is an interest in studying the metabolism of etretinate, which could potentially lead to the identification of the mechanism of action of retinoids.

We performed microbial biotransformation studies, initially on the etretinate model compound 11, to develop methods for studying the biotransformation of etretinate and to identify some of the microorganisms that would appear to have the potential to biotransform etretinate. Of the twenty-one microorganisms that were used in our initial screening studies, *C. elegans* ATCC 9245 and *S. racemosum* ATCC 18192 looked promising in terms of biotransformation of compound 11. The biotransformation products obtained from these two organisms include phenol-\(O\)-demethylation products, ester hydrolysis product and the products formed by oxidation on benzylic positions. These biotransformations are similar to those found in the reported mammalian metabolites of etretinate.

Using these methods developed for the biotransformation of 11, we then studied the biotransformation of etretinate by *C. elegans* ATCC 9245 and *S. racemosum* ATCC 18192. In this study, we found that *S. racemosum* ATCC 18192 did not biotransform etretinate. On the other hand, *C. elegans* ATCC 9245 appeared to form at least two biotransformation products of etretinate. As mentioned earlier, etretinate is very lipid soluble, and in mammals it has been found to be present mostly stored in the subcutaneous fat compartment. The circulating etretinate has been found to be present predominantly in the protein-bound form. It is therefore possible that in our microbial biotransformation studies, the etretinate might have had problems getting transported
across the lipid membrane. It is also possible that etretinate itself and/or its biotransformation products might have been lost by adsorption to the mycelia. Also, in previous microbial biotransformation studies on β-ionone and RA, it has been found that the percent biotransformation decreased significantly in going from β-ionone, a simple model compound, to RA, for the same organism. Hartman et al. found that A. niger 16888 formed 4-hydroxy-β-ionone and 4-oxo-β-ionone in 61% and 8% yields respectively from β-ionone, but did not form any biotransformation product when RA was added to the culture. They also found that C. blakesleeana 8688a formed 4-oxo-β-ionol in 28% yield from β-ionone, but formed 4-oxo-RA and 2-hydroxy-RA from RA in only 1.3% and 2.2% yields respectively. Therefore, the solubility and stability of the substrate added may influence the biotransformation capabilities of the microorganisms. Since acitretin is more soluble than etretinate and is found to form etretinate in vivo in mammals, biotransformation studies on acitretin using the same microorganisms may provide more information on metabolism of acitretin and etretinate.

As mentioned earlier, retinoids are believed to exert their biological activity by binding to nuclear receptors. It has been observed that a number of proteins are involved in binding and transporting retinoids, and that some of these proteins have similar binding sites, although they have been suggested to bind different retinoids with different specificity. Some of these binding proteins are also believed to be involved in the metabolism of retinoids by interacting with the appropriate enzyme, after binding the retinoid. There has also been a controversy as to the protein-bound conformation of retinoids. The milk whey protein BLG-B has been found to bind retinoids with good
affinity and is often used as a model protein to study retinoid-protein interactions. Not much is known yet about the exact binding site for retinoids on the BLG. Therefore there is an increasing interest in studying the retinoid-protein interactions.

From the steady state NOE experiments performed using RA, β-ionone, and 4-oxo-β-ionone, we observed that the preferred solution conformation of these free retinoids is the one which has a slightly twisted 6-s-cis relationship between the trimethylcyclohexenyl ring and the isoprenoid side chain. Although we were not able to calculate the ring-side chain torsion angles from our NOE results for these free retinoids, due to their flexibility and a lack of known reference distance in these molecules, we found that the computational methods that we employed predicted a preferred torsion angle of 31-44°, which is consistent with the results of the NMR experiments. The results of the HMQCNOE and HMQCCOSY experiments performed on the BLG-bound 13C-labelled β-ionones strongly suggest that the retinoid model ligand binds to BLG with a planar side chain with a 6-s-cis conformation. The HMQCCOSY experiment further established that the only significant NOE observed from the 13C-labelled 9-CH3 group in BLG-bound [8,10-13C2]-β-ionone 18 is an intramolecular NOE to the H-7 vinyl proton consistent with the planar 6-s-cis side chain conformation. Examination of the NOEs from the H-8 vinyl proton of the BLG-bound [8,10-13C2]-β-ionone 18 in the HMQCNOE experiment showed only two prominent signals which we believe are again intramolecular and to the 5-CH3 group and to one or both of the geminal dimethyl groups of the BLG-bound [8,10-13C2]-β-ionone 18. However, the NOE mixing time build-up curves for these NOEs at 0.8 ppm and 1.7 ppm established that the NOE to the 0.8 ppm
resonance was dominated by spin diffusion and therefore the information from this NOE is unclear and not very useful. The result of the HMQCTOCSY experiment performed to establish if the observed NOE to the resonance at 1.7 ppm is indeed an intramolecular one to the 5-CH$_3$ group in BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18 did not show any TOCSY transfer from H-8 to the 4-CH$_2$ and 5-CH$_3$ groups via H-7 vinyl proton in BLG-bound [8,10-$^{13}$C$_2$]-β-ionone 18 that we hoped to observe due to the scalar coupling between these protons observed in free β-ionone in solution. In the BLG-bound conformation of [8,10-$^{13}$C$_2$]-β-ionone 18 these coupling constants may have become vanishingly small, leading to the absence of TOCSY transfer beyond H-7 of the bound ligand. Earlier, Karplus and co-workers developed a method to calculate the torsion angles for retinoids based on the homoallylic coupling constants observed for the H-7 vinyl proton and 4-CH$_2$ protons ($J_{74}$) and the H-7 vinyl proton and 5-CH$_3$ protons ($J_{75}$).$^{218}$ Calculations using these methods suggest that as the torsion angle in β-ionone approaches a value of either 0° or 180° these coupling constants become very small and eventually there will be no coupling observed at 0° or 180° torsion angle. Alternatively, the small couplings may be unobservable because of the shortened relaxation times for the protein-bound ligand. The HMQCNOE experiment performed with BLG-bound [5-$^{13}$CH$_3$]-β-ionone 19 showed no NOE to the H-8 vinyl proton. Instead, significant NOE was observed to aliphatic signals from 0.8 to 1.9 ppm. When cross-relaxation of the 5-CH$_3$ group in the bound ligand is measured, it is possible that the primary pathways for relaxation are to the 4-CH$_2$ group of the bound ligand and to the aliphatic amino acid side chains of BLG that are in close proximity with the bound ligand. The HMQC experiment performed with BLG-bound [5-
$^{13}$CH$_3$]-β-ionone 19 showed a resonance at 1.7 ppm, suggesting that the chemical shift for the protons of 5-CH$_3$ group in the bound ligand is 1.7 ppm. Therefore, the observed NOE from the H-8 vinyl proton to the resonance at 1.7 ppm is most likely to the 5-CH$_3$ group of the BLG-bound ligand. Thus, the results of the different NMR experiments with specifically labelled β-ionone bound to BLG suggest that β-ionone binds to BLG in such a conformation that the planar side chain has a twisted 6-s-cis relationship to the trimethylcyclohexenyl ring, although we were not able to calculate the exact ring-side chain torsion angle.

Based on earlier studies of BLG-retinol complexes, it has been suggested that the Trp$^{19}$ residue of BLG is one of the important amino acids at the base of the β-barrel, and that it interacts with the β-ionylidene ring, while the Lys$^{70}$ of BLG lies near the outer surface of the β-barrel interacting with the polar end group.$^{145,219,221,226,238,256}$ On the other hand, based on X-ray crystallographic studies, Monaco et al. suggested that retinol binds to BLG at an external hydrophobic pocket rather than in the β-barrel, and that this hydrophobic pocket is lined mostly by aliphatic amino acids.$^{146}$ In our studies with the BLG-bound labelled β-ionones, we did not see any NOE that can be attributed to an intermolecular interaction between the bound ligand and any Trp residue of the BLG. Instead, we have observed some NOEs to the aliphatic regions from 0.8 ppm to 1.9 ppm. Some of these observed NOEs from the bound ligand are probably to the aliphatic amino acid side chains of BLG that are in close proximity with the bound ligand, which may support the suggestion that the external hydrophobic pocket may be the retinoid-binding
site in BLG. Further NMR studies with labelled ligands as well as labelled BLG should be of great value in identifying and characterizing the retinoid-binding site in BLG.
CHAPTER 5

EXPERIMENTAL

5.1 GENERAL AND ANALYTICAL METHODS

$^1$H and $^{13}$C NMR spectra were recorded using an IBM AC250 spectrometer operating at 250 MHz for $^1$H and 62 MHz for $^{13}$C measurements respectively. with CHCl$_3$ as an internal standard. The $^{31}$P NMR experiment was conducted using an IBM AC270 spectrometer operating at 109.3 MHz. All NOE difference measurements were done using an IBM AC270 spectrometer operating at 270 MHz for $^1$H measurements with CHCl$_3$ or CH$_3$OH as an internal standard. FTIR spectra were collected using an Analect RFX 40 instrument with a Diffuse Reflectance accessory attachment. Mass spectra were obtained in EI mode at The Ohio State University Campus Chemical Instrument Center on a Kratos MS-30 instrument. UV spectra were obtained from a Beckman DU-40 spectrophotometer. HPLC was conducted on a Beckman Instruments model 332 chromatograph with a model 164 UV detector using a Beckman-Ultrasphere ODS column (0.45 x 25 cm). GC was conducted on a Varian 3300 gas chromatograph equipped with a flame ionization detector, helium carrier gas (linear gas rate of 23.8 cm/sec.), and 30 m x 0.32 mm SPB-1 column (Supelco, Inc.).
Protein/Ligand NMR Studies: All 2D NMR experiments for the protein/ligand studies were conducted as variations of HMQC experiments using Bruker AM600 or AM500 spectrometers operating at 600.13 and 499.84 MHz for proton measurements and 150.92 and 125.68 MHz for carbon measurements respectively. All spectrometers were equipped with the Aspect 3000 data system and off-line data processing was performed using FELIX 2.1 or 2.3 (BIOSYM Technologies, San Diego, CA).

Energy Calculations: Ligand models were built and energy minimization calculations and conformation predictions were conducted using QUANTA/CHARMm, SYBYL, and MMP2. QUANTA 4.0 and CHARMm 22.2 (Molecular Simulations Inc., Burlington, MA) and SYBYL 5.22 (Tripos Associates, St. Louis, MO) were employed on Silicon Graphics IRIS 4D/70GT or Indigo Elan 4000 systems (Mountain View, CA). MMP2 (Quantum Chemistry Program Exchange, University of Indiana, Bloomington, IN) calculations were performed on a microcomputer.

Synthesis of 11: To 150 ml of methanol in a round bottom flask was added 100 mg of 2,4-cis, trans abscisic acid (Sigma chemical company, St. Louis, MO) followed by three drops of conc. H$_2$SO$_4$ and the reaction mixture was refluxed overnight. The solvent was then removed under vacuum, the residue was dissolved in methylene chloride, and washed with water followed by saturated sodium chloride solution. The organic layer was then dried using anhydrous MgSO$_4$ and concentrated to give the crude product which was then purified on a silica gel column using 30% ethyl acetate: hexanes as the mobile phase to give 365 mg of 11 (35% yield) as an amorphous solid in a 1:1 mixture of 2,4-cis, trans.
and 2,4-trans, trans isomers, as determined by HPLC and $^1$H NMR: UV(MeOH) $\lambda_{\text{max}}$ 323.5 nm, 251 nm; IR (KBr) 1710, 1620, 1500 cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 2.11 (s, 6. -CH$_3$), 2.12 (s, 3. -CH$_3$), 2.2 (s, 3. -CH$_3$), 2.25 (s, 3. -CH$_3$), 2.28 (s, 3. -CH$_3$), 2.31 (s, 3. -CH$_3$), 2.44 (s, 3. -CH$_3$), 3.69 (s, 3. -CO$_2$CH$_3$), 3.71 (s, 3. -CO$_2$CH$_3$), 3.8 (s, 6. -OCH$_3$), 5.70 (s, 1. 2-H), 5.79 (s, 1. 2-H), 6.20 (d, 1. 4-H, J = 16 Hz), 6.59 (s, 2. Ar-H), 6.99 (d, 2. 5-H, J = 16Hz), 7.7 (d, 1. 4-H, J = 16Hz); HRMS m/e (%): 274.1591(M$^+$, 77) (Calculated mass for C$_{17}$H$_{22}$O$_3$ = 274.15696).

MICROBIAL BIOTRANSFORMATION

Culture Methods for biotransformation of 11: All microorganisms except Aspergillus terreus OSU, were obtained from the American Type Culture Collection. All cultures were stored at 4°C in tightly capped mycophil (BBL) agar slants. A two-stage culture incubation procedure was used for all biotransformation studies.$^{204}$ Stage I cultures were initiated by pipetting 1 ml of an aqueous or normal saline spore suspension prepared from 7-day old mycophil slant cultures into 100 ml of sterilized PY (Pharmamedia-Yeast) medium in a 500 ml Erlenmeyer flask. The stage I cultures were incubated on a rotary shaker (Gyratory, New Brunswick Scientific Co.) at 250 rpm for 2 days at 25°C, and then 10 ml of stage I cultures were used to inoculate each stage II culture containing the same medium which were then incubated on a rotary shaker at 250 rpm and 25°C. All shake cultures were grown in PY medium which consists of the following (per liter of distilled water): Pharmamedia (Traders Oil Mill Co., Memphis, TN), 10 g; yeast extract, 5 g; D-glucose, 20 g; NaCl, 5 g; and K$_2$HPO$_4$, 5g.

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Screening methods for compound 11: Stage II cultures of the twenty-one microorganisms shown below were prepared as described above, in the culture methods. One day after starting the stage II cultures, 10 mg of 11 in 0.2 ml of ethanol was added to each of the stage II cultures. Another set of stage II cultures of all twenty-one organisms (shown in table below) which did not receive compound 11 served as controls.

The cultures were harvested after 7 days by exhaustively extracting with 1:1 ethyl acetate/hexanes. The mycelia were removed by vacuum filtration. The organic extracts were separated from the aqueous extracts, washed with saturated NaCl, dried using

Microorganisms used in our screening studies

<table>
<thead>
<tr>
<th>Aspergillus aculeatus ATCC 1034</th>
<th>A. ochraceus ATCC 1008</th>
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<tbody>
<tr>
<td>A. alliaceus ATCC 10060</td>
<td>A. ochraceus ATCC 18500</td>
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<tr>
<td>A. candidus ATCC 20022</td>
<td>A. parasiticus ATCC 15517</td>
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<tr>
<td>A. carbonarius ATCC 6277</td>
<td>A. terreus OSU</td>
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<tr>
<td>A. flavipes ATCC 1030</td>
<td>Cunninghamella elegans ATCC 9245</td>
</tr>
<tr>
<td>A. flavipes ATCC 11013</td>
<td>Streptomyces species ATCC 15077</td>
</tr>
<tr>
<td>A. flavipes ATCC 16795</td>
<td>Streptomyces griseus ATCC 10137</td>
</tr>
<tr>
<td>A. flavus ATCC 24741</td>
<td>Streptomyces griseus ATCC 13968</td>
</tr>
<tr>
<td>A. niger ATCC 10581</td>
<td>Mucor rouxii ATCC 24905</td>
</tr>
<tr>
<td>A. niger ATCC 11394</td>
<td>Syncphalastrum racemosum ATCC 18192</td>
</tr>
<tr>
<td>A. niger ATCC 16888</td>
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</tbody>
</table>
MgSO₄, the drying agent removed by vacuum filtration and concentrated to give crude organic extracts. The thin layer chromatograms (30% ethyl acetate/hexanes) of the organic extracts of the cultures fed with 11 were compared to those of the control cultures, visualized by fluorescence quenching under 254 nm UV light. The comparisons were made by semiquantitative inspection of the TLC plates as observed by the partial disappearance of the starting material as well as the appearance of more polar, potential metabolites. In this comparison, certain components present only in the chromatograms from cultures fed with 11 were believed to be biotransformation products, and those microorganisms that appeared to contain those products in appreciable quantities i.e., Cunninghamella elegans 9245 and Syncephalastrum racemosum 18192, were selected for further studies.

**Time course study:** Stage II cultures of Cunninghamella elegans 9245 and Syncephalastrum racemosum 18192 were prepared as described above. Compound 11 was added to each of the stage II cultures and the cultures were harvested 4, 6, 8, and 10 days after addition of the compound to the cultures, as described earlier. The extracts from different days were then compared by tlc as described previously and we found that 6 days of incubation was optimum for the biotransformation of 11 by both the organisms, based on the disappearance of the starting material as well as the appearance of more polar, potential metabolites in the thin layer chromatograms as visualized by fluorescence quenching under 254 nm UV light.
Production and Isolation of biotransformation products of 11: One day after initiating the stage II cultures as described above, 10 mg of 11 in 0.2 ml of ethanol was added to each of 20 flasks containing C. elegans 9245 and to another 20 flasks containing S. racemosum 18192 for biotransformation. For each organism, control cultures having no compound 11 added were incubated simultaneously. Six days after addition of 11, 100 ml of 1:1 ethyl acetate/hexanes was added to each of the culture flasks, and they were allowed to stand for 1 hr. The culture flasks were then filtered to remove the mycelia. The organic phase was then separated, washed with saturated sodium chloride solution, dried using anhydrous MgSO₄ and concentrated. The crude organic extracts were then analyzed by tlc on silica gel plates using 30 % ethyl acetate/hexanes as the mobile phase and compared to those of the controls. The products were then purified on silica gel columns and the isolated compounds were then used for further spectroscopic experiments.

XAD-2 resins (10 g), obtained from Supelco, Bellefonte, PA. were added to the aqueous phase and stirred for 30 minutes and then filtered. The XAD-2 resins were then washed with 100 ml of methanol, filtered and concentrated to give the crude water-soluble biotransformation products. These were then analyzed by tlc and purified by column chromatography.
Characterization of recovered 11 (C. elegans 9245 and S. racemosum 18192):
Twenty milligrams of compound 11 was recovered in fraction 1 from C. elegans 9245, while 25 mg of 11 was recovered in fraction 1 from S. racemosum 18192. The spectral data recorded on this isolated compound were identical to those of the starting material 11.
Characterization of 13 (C. elegans 9245): Purification of the organic phase from C. elegans 9245 as in isolation scheme shown above (page 117) gave 3 mg of compound 13 (1.7 % conversion) from fraction 3: UV (MeOH) $\lambda_{\text{max}}$ 315 nm, 250 nm; IR (KBr) 3600-3200 (broad), 3200-2400 (broad), 1690, 1600 cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 2.1 (s, 3. -CH$_3$), 2.2 (s, 3. -CH$_3$), 2.25 (s, 3. -CH$_3$), 2.4 (s, 3. -CH$_3$), 5.79 (s, 1. 2-H), 6.2 (d, 1. 4-H, $J = 16$Hz), 6.5 (s, 1. Ar-H), 6.99 (d, 1. 5-H, 16Hz), HRMS m/e (%) 246.12585 ($M^+$, 27) (calculated mass for C$_{15}$H$_{18}$O$_3$ = 246.12564).

Characterization of 14 (C. elegans 9245): After biotransformation, 3 mg of 14 was obtained from fraction 4 in the isolation scheme shown above (1.5 % conversion): UV (MeOH) $\lambda_{\text{max}}$ 323 nm, 247.5 nm; IR (KBr) 3600-3200 (broad), 3200-2400 (broad), 1680, 1590 cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 2.17 (s, 3. -CH$_3$), 2.28 (s, 3. -CH$_3$), 2.32 (s, 6. -CH$_3$), 2.37 (s, 3. -CH$_3$), 2.4 (s, 3. -CH$_3$), 3.8 (s,6. -OCH$_3$), 4.8 (s, 4. 3'-CH$_2$-OH), 5.7 (s, 1. 2-H), 5.8 (s, 1. 2-H), 6.2 (d, 1. 4-H, $J = 16$Hz), 6.6 (s, 2. Ar-H), 6.99 (d, 2. 5-H, $J = 16$Hz), 7.7 (d, 1. 4-H, $J = 16$Hz); HRMS m/e (%) 276.13668 ($M^+$, 42) (calculated mass for C$_{16}$H$_{20}$O$_4$ = 276.1362).

Methylation of 14: To a fraction of 14 (2 mg) dissolved in 5 ml of tetrahydrofuran in a 20 ml scintillation vial, was added 0.5 ml of methyl iodide and 10 mg of NaH (60% dispersion in mineral oil). The reaction mixture was stirred at room temperature for 10 hours and the solvent evaporated under reduced pressure. The residue was then partitioned between ether and water. The organic layer was washed with saturated sodium chloride...
solution, dried using MgSO₄, filtered to remove the drying agent, and the solvent evaporated to give the product. The product was obtained in too small an amount to conduct complete spectroscopic analysis. However, we were able to analyze it by mass spectrometry, which showed that the M⁺ for the product was 304 (calculated mass for C₁₈H₂₄O₄ = 304.1675).

**Characterization of 16 (S. racemosum 18192):** Compound 16 was obtained as a biotransformation product in 4 mg quantity from fraction 4 (2.12% conversion): UV (MeOH) λ_max 310 nm, 248.5 nm; IR (KBr) 3300-2400 (broad), 1700-1600, 1450 cm⁻¹; ¹H NMR (CDCl₃) δ 2.10 (s, 3, -CH₃), 2.19 (s, 3, -CH₃), 2.31 (s, 3, -CH₃), 2.39 (s, 3, -CH₃), 3.8 (s, 3, -OCH₃), 5.7 (s, 1, 2-H), 6.6 (s, 1, Ar-H), 7.0 (d, 1, 5-H, J = 16Hz), 8.05 (d, 1, 4-H, J = 16Hz); HRMS m/e (%) 260.14093 (M⁺, 25) (calculated mass for C₁₆H₂₀O₃ = 260.1413)

**Characterization of 17 (S. racemosum 18192):** Biotransformation product 17 was obtained in 6.5 mg (3.07 % conversion) from fraction 5: UV (MeOH) λ_max 310 nm, 250 nm; IR (KBr) 3600-2400 (broad), 1710-1600, 1450 cm⁻¹; ¹H NMR (CDCl₃) δ 2.15 (s, 3, -CH₃), 2.29 (s, 3, -CH₃), 2.33 (s, 3, -CH₃), 2.41 (s, 3, -CH₃), 3.7 (s, 3, -CO₂CH₃), 3.82 (s, 3, -OCH₃), 4.7 (s, 2, 3'-CH₂OH), 5.8 (s, 1, 2-H), 6.2 (d, 1, 4-H, J = 16Hz), 6.6 (s,1, Ar-H), 6.95 (d, 1, 5-H, J = 16Hz); HRMS m/e (%) 290.15069 (M⁺, 19) (calculated mass for C₁₇H₂₂O₄ = 290.15186).
**Methylation of 17**: To a fraction of 17 (3 mg) dissolved in 5 ml of tetrahydrofuran in a 20 ml scintillation vial, was added 0.5 ml of methyl iodide and 10 mg of NaH (60% dispersion in mineral oil). The reaction mixture was stirred at room temperature for 10 hours and the solvent evaporated under reduced pressure. The residue was then partitioned between ether and water. The organic layer was washed with saturated sodium chloride solution, dried using MgSO₄, filtered to remove the drying agent and the solvent evaporated to give the product. The product was obtained in too small an amount to conduct complete spectroscopic analysis. However, analysis by mass spectrometry showed an increase in molecular weight by 14 units to 304 (C₁₈H₂₄O₄).

**Extraction of etretinate from Tegison®**: Etretinate was extracted from Tegison® capsules (Roche Laboratories, Nutley, NJ), purchased from the pharmacy at The Ohio State University Hospitals (Columbus, OH). Tegison® was purchased in packs of 30 capsules, each capsule containing 25 mg of etretinate. The contents of the 30 capsules of Tegison® were emptied into a 250 ml round bottom flask equipped with a stir bar and 100 ml of ethyl acetate was added and the solution stirred at room temperature for 24 hours. The suspension was then filtered and the ethyl acetate evaporated under reduced pressure to give etretinate. The residue obtained from filtration, was then taken in a fresh 100 ml of ethyl acetate, stirred for 24 hours and treated in the same way as above. This extraction procedure was repeated for 7 days. Etretinate obtained after each extraction was pooled to provide 720 mg of etretinate. The ¹H NMR spectrum obtained on a sample of the extracted etretinate showed it to be apparently pure etretinate: UV (MeOH) λ_max 340.5
nm; IR (neat) 2900, 1707, 1584, and 1465 cm⁻¹; ¹H NMR (CDCl₃) δ 1.23 (t, 3, -O-C-CH₃), 2.09 (s, 3, 9-CH₃), 2.13 (s, 3, Ar-CH₃), 2.21 (s, 3, Ar-CH₃), 2.28 (s, 3, Ar-CH₃), 2.35 (s, 3, 13-CH₃), 3.8 (s, 3, O-CH₃), 4.16 (q, 2, CO₂-CH₂-) 5.78 (s, 1, 14-CH), 6.19-6.31 (m, 3, 8-CH, 10-CH, 12-CH), 6.6 (s, 1, Ar-H), 6.67 (d, 1, 7-CH), 7.0 (dd, 1, 11-CH); HRMS m/e (%) 354.2185 (M⁺, 87.46) (calculated mass for C₂₃H₃₀O₃ = 354.2196).

**Culture methods for biotransformation of etretinate:** C. elegans 9245 and S. racemosum 18192 were selected for studying the biotransformation of etretinate, based on the results of biotransformation studies on 11. One day after initiating the stage II cultures as described above, 10 mg of etretinate in 0.4 ml of ethanol was added to each of the four culture flasks containing C. elegans 9245 and to another 4 culture flasks containing S. racemosum 18192, for the initial biotransformation studies on etretinate. For each organism, a control culture, to which no etretinate was added, was also incubated simultaneously. Seven days after addition of etretinate, 100 ml of 1:1 ethyl acetate/hexanes was added to each of the culture flasks and were allowed to stand for 1 hr. The culture flasks were then filtered to remove the mycelia. The organic phase was then separated, washed with saturated sodium chloride solution, dried using anhydrous MgSO₄ and concentrated. The control cultures were also treated similarly. The crude organic extracts were then analyzed by tlc on silica gel plates using 20 % ethyl acetate/hexanes as the mobile phase and compared to those of the controls, for the possible presence of etretinate biotransformation products.
Production and isolation of biotransformation products of etretinate: Stage II cultures of *C. elegans* 9245 were started in 40 culture flasks as described above. One day after initiating the stage II cultures, 10 mg of etretinate in 0.8 ml of ethanol was added to each of the 40 flasks containing *C. elegans* 9245. After seven days the cultures were harvested as mentioned above by using 1:1 ethyl acetate/hexanes. The organic phase was then separated, pooled together, washed with saturated sodium chloride solution, dried using anhydrous MgSO₄ and concentrated. Column chromatography on silica gel using 10% methanol/chloroform gave one fraction containing retinoids. Further purification of this fraction on silica gel using 20% ethyl acetate/hexanes gave two fractions containing retinoids, which were then analyzed spectroscopically.

XAD-2 resins were added to the aqueous phase and stirred for 10 minutes and then filtered, and washed with additional water. The resins were then stirred with 200 ml of methanol for 30 minutes, filtered to remove the resins and concentrated to give the crude aqueous extracts. Since the aqueous extract was sparingly soluble once methanol was evaporated, we were not able to purify various components from this extract. However, one of the components that was somewhat soluble in pyridine, was extracted by using 50 ml of pyridine. The solvent was then evaporated, to give a retinoid compound which was analyzed by ¹H NMR.
Characterization of recovered etretinate: Purification of the organic phase from *C. elegans* 9245, as in isolation scheme shown above, gave 24 mg of etretinate in fraction 1. The spectral properties obtained for this compound were identical to the spectral properties of standard etretinate.
**Characterization of isolated acitretin (C. elegans 9245):** Acitretin was obtained from fraction 2 in the isolation scheme shown above: \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 2.09 (s, 3, 9-CH\(_3\)), 2.13 (s, 3, Ar-CH\(_3\)), 2.21 (s, 3, Ar-CH\(_3\)), 2.28 (s, 3, Ar-CH\(_3\)), 2.35 (s, 3, 13-CH\(_3\)), 3.8 (s, 3, O-CH\(_3\)), 5.78 (s, 1, 14-CH), 6.19-6.31 (m, 3, 8-CH, 10-CH, 12-CH), 6.6 (s, 1, Ar-H), 6.67 (d, 1, 7-CH). 7.0 (dd, 1, 11-CH); HRMS \(m/e\) (%) 326.1849 (M\(^+\), 0.82), 281.1913 (M\(^+\)-CO\(_2\)H, 17.23) (calculated mass for C\(_{21}\)H\(_{26}\)O\(_3\) = 326.1882).

**Synthesis of methyl 3-oxo-7-methyloct-6-enoate (22):** A 60% mineral oil suspension of NaH (2.6 g, 65 mmol) was taken in a dry, three-necked round bottom flask equipped with a reflux condenser and an argon inlet. The sodium hydride was washed with 50 ml of hexanes to remove the oil and 100 ml of ether was added. A solution of dimethylcarbonate (6 g, 66.6 mmol) in 10 ml of ether was added and the suspension was stirred magnetically and heated to reflux. 6-Methyl-5-heptene-2-one (3.15 g, 25 mmol), obtained from Aldrich chemical company, was then added very slowly, over an hour. The reaction mixture was refluxed for 2 hrs after the addition was complete and allowed to remain at room temperature overnight. The solid mass that had formed overnight was then cooled in an ice-bath and a solution of methanol (5 ml) in ether (25 ml) was added slowly. The reaction mixture was broken up carefully with a glass rod and stirred for 2 hrs. The suspension formed was then poured into a mixture of ice and 10 ml of concentrated HCl. The aqueous layer was extracted twice with ether, and the combined ether layer washed with a saturated solution of sodium bicarbonate, dried using MgSO\(_4\), filtered to remove the drying agent, and the solvent evaporated to give 3 g (65% yield) of
22: $^1$H NMR (CDCl$_3$) δ 1.56 (s, 3. -CH$_3$), 1.63 (s, 3. -CH$_3$), 2.25 (m, 2. =C-CH$_2$-C-), 2.53 (t, 2. -CO-CH$_2$-C-), 3.4 (s 2. -CO-CH$_2$-CO-), 3.7 (s, 3. -CO$_2$CH$_3$), 5.0 (t, 1. -C=CH-).

Synthesis of 2-carbomethoxy-3,3-dimethylcyclohexanone (23)$^{241}$: To a stirring solution of 3 g (16.3 mmol) of 22 in 35 ml of methylene chloride in an ice-bath was added 15 ml of stannic chloride (1.0 M solution of SnCl$_4$ in dichloromethane) very slowly, in an inert atmosphere. The ice-bath was removed after the addition was complete, and the solution was stirred for 12 hours at room temperature. The reaction mixture was then diluted with twice its volume of ether and washed twice with 100 ml portions of 5% hydrochloric acid. The ether layer was then washed with water, dried, and the solvent evaporated to give the crude product 23. Two grams (66% yield) of 23 was obtained by column chromatography on silica gel using 5% ethyl acetate/hexanes as the mobile phase: $^1$H NMR (CDCl$_3$) δ 1.0 (s, 3. C - CH$_3$), 1.1 (s, 3, C-CH$_3$), 1.45 (m, 2, -C-CH$_2$-C-CO), 1.8 (m. 2. -CH$_2$-C-C-CO). 2.2 (m. 1. -CH$_3$(H$_6$)-CO), 2.55 (m. 1. -CH$_5$(H$_5$)-CO), 3.1 (s. 1, -CO-CH(C)-CO), 3.65 (s. 3. -CO$_2$CH$_3$).

Synthesis of [1-$^{13}$CH$_3$]-1,3,3-trimethyl-1-hydroxy-2-carbomethoxycyclohexane (24)$^{240}$: To a refluxing solution of 650 mg of Mg in 150 ml of ether in a three-necked round bottom flask equipped with an argon inlet, reflux condenser, and a dropping funnel, 3.8 g (27 mmol) of $^{13}$C-labelled methyl iodide (Cambridge Isotope Laboratories, 99.3 atom %) in 20 ml of ether was added. The reaction mixture was refluxed for an hour. One gram of 23 (5.4 mmol) in 30 ml of ether was added dropwise and the reaction mixture
was refluxed for 5 hrs. The reaction mixture was then stirred at room temperature for 5 days (until the solution became clear). The reaction mixture was then poured into 100 ml of water at which point the solution became cloudy again. To this solution, 10 ml of acetic acid was added slowly and stirred until the solution became clear again and the aqueous layer was extracted twice with 100 ml of ether. The combined ether layer was then washed with water, followed by saturated sodium bicarbonate, dried and the solvent removed under reduced pressure which gave crude 24. Purification by column chromatography on silica gel using 20% ethyl acetate in hexanes gave 436 mg of 24 (40% yield): $^1$H NMR (CDCl$_3$) $\delta$ 1.02. (d. $J_{CH} = 126$ Hz. 3. [5-$^{13}$CH$_3$]), 1.09 (s. 6. 1.1-(CH$_3$)$_2$). 1.4 (m. 2. 2-CH$_2$), 1.75 (m. 2. 3-CH$_2$). 1.9 (t. 2. 4-CH$_2$) 2.1 (s. 1. 6-CH), 3.72 (s. 3, CO$_2$CH$_3$); $^{13}$C NMR (CDCl$_3$) $\delta$ 31.0 (5-[13$^{13}$CH$_3$]) HRMS m/e (%) 201.14469 (M$^+$. 2.21) (calculated mass for $^{13}$C$_1$C$_{10}$H$_{20}$O$_3$ = 201.14466).

**Synthesis of [1-$^{13}$CH$_3$]-1,3,3-trimethyl-2-carbomethoxy cyclohexene (25)$^{240}$:**
To 1.0 g (5 mmol) of 24 in 20 ml of pyridine was added dropwise 2 ml of POCl$_3$ at 0°C. The reaction mixture was stirred at room temperature for 14 hrs. The reaction mixture was then diluted with 20 ml of petroleum ether. and slowly quenched with 10 ml of water at 0°C, at a rate of 1 drop of water per minute. The aqueous layer was then extracted with 100 ml of petroleum ether and washed with 20% hydrochloric acid (3 x 20 ml). The organic layer was then washed with saturated sodium chloride solution and the solvent removed under reduced pressure. which gave 606 mg (67% yield) of the dehydration product 25: $^1$H NMR (CDCl$_3$) $\delta$ 1.0 (s. 6. 1.1-(CH$_3$)$_2$), 1.45-1.68 (m, 4, 2-CH$_2$, 3-CH$_2$).
Synthesis of [1-$^{13}$CH$_3$]-1,3,3-trimethyl-2-hydroxymethyl cyclohexene (26): To 1.0 g (5.5 mmol) of 26 in dry ether in a round bottom flask, was added 420 mg (2 equivalents) of LiAlH$_4$, and the reaction mixture was stirred at room temperature for 4 hours. The reaction was then quenched by adding 100 ml of water. The reaction mixture was then taken in a separatory funnel, and the aqueous layer extracted with 100 ml of ether. The ether layer was washed with saturated sodium chloride solution, dried and the solvent was evaporated under reduced pressure. Compound 26 was obtained in 60% yield (507 mg) after column chromatography on silica gel using 20% ethyl acetate in hexanes: $^1$H NMR (CDCl$_3$) $\delta$ 1.1 (s, 6. -(CH$_3$)$_2$), 1.42 (m, 2. 2-CH$_2$), 1.59 (m, 2. 3-CH$_2$), 1.71 (d, $J_{CH} = 126$ Hz, 3. 5-['$^{13}$CH$_3$]), 1.98 (t, 2, 4-CH$_2$), 4.1 (s, 2, C-CH$_2$-O-); $^{13}$C NMR (CDCl$_3$) $\delta$ 19.2 (5-['$^{13}$CH$_3$]).

Synthesis of [5-$^{13}$CH$_3$]-β-cyclocitral (27): To 200 mg (1.3 mmol) of compound 26 dissolved in 49 ml of dichloromethane and 1 ml of pyridine in a round bottom flask was added 500 mg of pyridinium chlorochromate, prepared as previously described by Piancatelli et al.242 The reaction mixture was stirred for 2 hours. After 2 hours, 100 ml of dry diethyl ether was added and the supernatant liquid was decanted from a black, granular solid. The black solid was further washed with dry diethyl ether and the diethyl ether solutions were combined, and passed through a pad of florisil and the solvent was
removed under reduced pressure which gave 140 mg (71% yield) of 27. 

$^1$H NMR (CDCl$_3$) $\delta$ 1.09 (s, 6, 1,1-(CH$_3$)$_2$), 1.45 (m, 2, 2-CH$_2$), 1.61 (m, 2, 3-CH$_2$), 2.08 (d, $J_{CH} = 126$ Hz, 3. 5-[${}^{13}$CH$_3$]), 2.18 (t, 2, 4-CH$_2$), 10.1 (s, 1, 7-CHO); $^{13}$C NMR (CDCl$_3$) $\delta$ 21.8 (5-[${}^{13}$CH$_3$]); 

GC (100 to 175°C at 5°C/min) $t_R = 4.9$ min. (unlabelled $\beta$-cyclocitral, $t_R = 4.9$ min.).

**Synthesis of [S-']$^3$CH$_3$]-$\beta$-ionone (19):** 100 mg (0.65 mmol) of 27 was dissolved in 100 ml of acetone and taken in a three-necked round bottom flask equipped with a condenser, drying tube and an argon inlet. Three ml of 10% KOH was added and the reaction mixture was gently refluxed for 12 hrs. Acetone was removed under reduced pressure, and the residue was partitioned between 100 ml of ether and 100 ml of water. The ether layer was washed with saturated NaCl solution, dried and the solvent removed to give 70 mg (56% yield) of the compound 19 (greater than 95 atom % as determined by $^1$H NMR and mass spectrometry): UV(MeOH) $\lambda_{max}$ 293 nm ($\varepsilon$ 11,100); $^1$H NMR (CDCl$_3$) $\delta$ 1.05 (s, 6, 1,1-(CH$_3$)$_2$), 1.4 (m, 2, 2-CH$_2$), 1.62 (m, 2, 3-CH$_2$), 1.71 (d, $J_{CH} = 126$ Hz, 3. 5-[${}^{13}$CH$_3$]), 2.05 (t, 2, 4-CH$_2$), 2.28 (s, 3, 10-CH$_3$), 6.09 (d, $J_{HH} = 16.2$ Hz, 1, 8-CH), 7.22 (d, $J_{HH} = 16.2$ Hz, 1, 7-CH); $^{13}$C NMR (CDCl$_3$) $\delta$ 22.2 (5-[${}^{13}$CH$_3$]); HRMS $m/e$ (%) 193.1539 (M$^+$, 5.89) (calculated mass for $^{13}$C$_1$C$_{12}$H$_{20}$O = 193.15486); GC (100 to 175°C at 5°C/min) $t_R = 11.5$ min. (unlabelled $\beta$-ionone, $t_R = 11.5$ min.).

**Synthesis of [8,10-']$^{13}$C$_2$]-labelled $\beta$-ionone (18):** In a 5 ml conical reaction vial 100 mg (0.66 mmol) of $\beta$-cyclocitral (from Aldrich Chemical Company) was taken.
and 3 g (50 mmol) of [1,3-\textsuperscript{13}C\textsubscript{2}]-acetone (purchased from Isotec, Inc., 99 atom %) and 525 \mu l of 10% aqueous NaOH were added. The vial was sealed with a septum cap and immersed in a 63°C oil bath and stirred for 9 days and the reaction mixture was periodically checked for the complete consumption of \(\beta\)-cyclocitril by GC. After \(\beta\)-cyclocitril was completely consumed, the contents were transferred to a short-path still and the unreacted [1,3-\textsuperscript{13}C\textsubscript{2}]-acetone was recovered by distillation. The residue was then partitioned between ethyl acetate and water and the ethyl acetate layer was washed with saturated NaCl, dried, and concentrated to give the crude product as a light orange oil. Column chromatography (25% ethyl acetate/hexanes) gave 73 mg (58% yield) of compound 18\textsuperscript{239} (more than 98 atom % as determined by \textsuperscript{1}H NMR and mass spectrometry): UV(CH\textsubscript{2}Cl\textsubscript{2}) \(\lambda_{\text{max}}\) 293 nm (\(\varepsilon\) 11.100); \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 1.05 (s, 6. 1.1-(CH\textsubscript{3})\textsubscript{2}), 1.45 (m, 2. 2-CH\textsubscript{2}), 1.59 (m, 2. 3-CH\textsubscript{2}), 1.74 (s, 3. 5-CH\textsubscript{3}), 2.03 (t, 2. 4-CH\textsubscript{2}), 2.27 (d, \(J_{\text{CH}} = 124 \text{ Hz}\), 3. 10-[\textsuperscript{13}CH\textsubscript{3}]), 6.09 (dd, \(J_{\text{HH}} = 16.4 \text{ Hz}, J_{\text{CH}} = 126 \text{ Hz}\), 1. 8-[\textsuperscript{13}CH]), 7.23 (d, \(J_{\text{HH}} = 16.4 \text{ Hz}, 1. 7-\text{CH}\)); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\) 27.1 (10-[\textsuperscript{13}CH\textsubscript{3}]), 131.6 (8-[\textsuperscript{13}CH]); MS \(m/z\) (%) 194 (M\textsuperscript{+}, 16.9); GC (100 to 175°C at 5°C/min) \(t_R = 11.5 \text{ min}\). (unlabelled \(\beta\)-ionone. \(t_R = 11.5 \text{ min}\)).

**Synthesis of 4-hydroxy-\(\beta\)-ionone\textsuperscript{243}:** In an oven dried three-necked round bottom flask equipped with an argon inlet, reflux condenser, and a drying tube. 1.0 g (5.2 mmol) of \(\beta\)-ionone was taken and dissolved in 250 ml of chloroform and the solution was refluxed. Then 1.2 g (7.8 mmol) of NBS was added and the reaction mixture was stirred
at reflux for 15 minutes and then 400 mg (2.6 mmol) of additional NBS was added. The reaction mixture was refluxed for another 20 minutes and then cooled, concentrated under reduced pressure and the residue was suspended in hexanes. The hexanes suspension was filtered and the solvent removed under reduced pressure. The residue was dissolved in 100 ml of 9:1 acetone/water containing 600 mg of K$_2$CO$_3$ at 0° C and stirred at 0° C for 24 hours. The reaction mixture was then concentrated under reduced pressure, and extracted twice with ether. The ether layer was washed with saturated NaCl solution, dried, and the drying agent removed by vacuum filtration. The solvent was then removed under reduced pressure. Purification by column chromatography on silica gel using 30% ethyl acetate/hexanes afforded 602 mg (56% yield) of 4-hydroxy-β-ionone: UV(CH$_2$Cl$_2$) $\lambda_{\text{max}}$ 282 nm (ε 6700); IR (neat) 3420, 1660, 1610 cm$^{-1}$; $^1$H NMR (CDCl$_3$) δ 1.05 (s, 3, 1-CH$_3$), 1.07 (s, 3, 1-CH$_3$), 1.4 (m, 2, 2-CH$_2$), 1.7 (m, 2, 3-CH$_2$), 1.8 (s, 3, 5-CH$_3$), 2.3 (s, 3, 10-CH$_3$), 4.01 (t, 1, 4-CH), 6.1 (d, $J_{HH} = 15$ Hz, 1, 8-CH), 7.2 (d, $J_{HH} = 15$ Hz, 1, 7-CH).

**Synthesis of 4-oxo-β-ionone (20):** In a round bottom flask equipped with an argon inlet and a drying tube 500 mg (2.4 mmol) of 4-hydroxy-β-ionone was taken and dissolved in 100 ml of dichloromethane and the solution was cooled in an ice bath. To this reaction mixture at 0° C was added 1 g (11.5 mmol) of MnO$_2$ and the suspension stirred at room temperature for 12 hours. The reaction mixture was then filtered through a celite pad and the solvent removed under reduced pressure to give crude 20. Column chromatography on silica gel using 30% ethyl acetate/hexanes gave 300 mg (61% yield) of 20: IR (neat) 1670, 1617 cm$^{-1}$; UV(CH$_2$Cl$_2$) $\lambda_{\text{max}}$ 277 nm (ε 18,800); $^1$H NMR (CDCl$_3$)
\[ \delta 1.12 \text{ (s, 6, 1,1-(CH\textsubscript{3})\textsubscript{2})}, 1.73 \text{ (s, 3, 5-CH\textsubscript{3})}, 1.85 \text{ (t, 2, 2-CH\textsubscript{2})}, 2.3 \text{ (s, 3, 10-CH\textsubscript{3})}, 2.49 \text{ (t, 2, 3-CH\textsubscript{2})}, 6.12 \text{ (d, } J_{HH} = 16 \text{ Hz, 1, 8-CH}), 7.18 \text{ (d, } J_{HH} = 16 \text{ Hz, 1, 7-CH}). \]

MOLECULAR MODELLING STUDIES:

Ligand models were built and energy minimization calculations and conformation predictions were conducted using QUANTA/CHARMM, SYBYL, and MMP2. The ligands were built \textit{in vacuo}, initially with a relatively planar side chain conformation and a 6-s-cis ring-side chain relationship and were then allowed to relax to a calculated minimum energy conformation. These starting structures were chosen because the data derived from NMR studies, UV absorption of the retinoids, and the X-ray crystal structure suggests a preference for a relatively planar side chain for the retinoids. Energy minima calculations were done using the conjugate gradient method in CHARMM and Maximin2 in SYBYL. An energy cut-off value of 0.01 Kcal/mol was used for the minimization processes. For the conformational analysis, the energy minimized structure in CHARMM was taken and a grid conformational search about the ring-side chain torsion angle was performed. The energy minimized structure of \(\beta\)-ionone in CHARMM was taken, the torsion angle was incremented in 10\(^\circ\) fixed steps and the remainder of the molecule was allowed to relax after each increment. The CHARMM energy was then recalculated for each conformation of \(\beta\)-ionone during this search.
NMR EXPERIMENTS

NOE experiments for the microbial biotransformation products were obtained on their argon degassed solutions in deuterated chloroform with CHCl₃ as an internal standard, using an IBM AC 270 spectrometer operating at 270 MHz for ¹H measurements.

NOE difference measurements for free ligands β-ionone, RA, and 4-oxo-β-ionone were done at ambient temperatures, on their argon degassed solutions, using an IBM AC 270 spectrometer operating at 270 MHz for ¹H measurements with CHCl₃ or CH₃OH as an internal standard. In typical NOE experiments a sufficient number of transients, using a sweep width of 2000-2500 Hz over 16K data points, were acquired to ensure accurate integrations. Irradiations on resonances of interest, and one off-resonance control, were conducted cyclically in groups of eight scans preceeded by 2-4 dummy scans. An irradiation time of 2-5s, a pulse width of 2-4 μs (35-70°), and a low decoupling power (typically 60 dB attenuation of 200 mW channel) were used.

Sample preparation for ligand/protein studies

A stock solution of 50 mM phosphate buffer was prepared in D₂O, using D₃PO₄ and the pD was adjusted to 7.1 using 50 mM NaOD solution in D₂O.

The protein solutions for the NMR experiments were prepared by adding the appropriate quantity of BLG-B, from bovine milk, (Sigma chemical company, St. Louis, MO) to 500 μl of the 50 mM phosphate buffer prepared as mentioned above and once the protein has completely dissolved the pD of the solution was checked and adjusted to pD
7.1 using 50 mM NaOD solution in D$_2$O. The final volume of the protein solution was made up to 1.0 ml using D$_2$O. A fresh protein solution was made for each NMR experiment, since the protein appeared to be degrading after storage in the freezer for more than a week.

A stock solution of the ligand in ethanol was separately made using ethanol-d$_6$ and the appropriate quantity of the required ligand.

The protein-ligand solution was prepared by taking 450 µl of the protein solution prepared above and then adding 50 µl of the ligand solution in ethanol-d$_6$ to it, so that the final protein/ligand sample solution was 10% ethanol-d$_6$ in 50 mM phosphate buffer containing the required concentrations of BLG and the ligand.

2D NMR experiments

All 2D NMR experiments were conducted at 308°C, as variations of the HMQC experiment in which the dipolar coupling (NOE), scalar coupling (COSY) or strong coupling (TOCSY) to the protons attached to the $^{13}$C-labelled carbon atoms in the ligand were allowed to build up after the HMQC magnetization transfer from $^{13}$C back to $^1$H. The $^{13}$C chemical shifts were determined in separate experiments so that the t$_1$ dimension ($^{13}$C) of the HMQC experiment could be restricted to 12-24 ppm ranges around one of the $^{13}$C-labelled carbon atoms. The pulse sequences employed in these HMQCNOE, HMQCCOSY and HMQCTOCSY experiments are shown in figure 25 in Chapter 3.
Heteronuclear decoupling during data acquisition was accomplished by using a GARP sequence.

The HMQCNOE experiments (AM600) employed a $^1$H sweep width of 8929 Hz and spectra were recorded with mixing times of 50-600 ms with the $1/2J_{CH}$ delay tuned to the desired vinyl carbon-proton or the methyl carbon-proton coupling constants (160 and 127 Hz respectively) appropriate for the experiment. The HMQCCOSY experiment was performed with the same $^1$H sweep width but the $1/4J_{HH}$ delay was set to 5.5 ms instead of the optimal 13 ms to detect vinyl proton coupling because of signal decay. The HMQCTOCSY experiments (AM500) were performed with a 7463 Hz $^1$H sweep width, a DIPSI-2 spin-lock sequence and spin-lock mixing times of 17-105 ms.

For these 2D experiments in general, 96-320 scans of 4096 data points were collected in the $t_2$ dimension with a 1s relaxation delay before each scan. In the $t_1$ dimension, 64 experiments were usually used with 2-4 dummy scans between experiments. The $t_2$ data were generally zero-filled once and apodized with a $\pi/2$ phase-shifted sinebell square function while $t_1$ data were generally subjected to 2 levels of zero-fill and apodized with a $\pi/2$ phase-shifted sinebell function.
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