progress in industrial microbiology

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PREFACE

The prostaglandins are a fascinating group of compounds because of the wide spectrum of pharmacological activity that they possess. So far, the clinical application of these compounds has been very limited, despite their apparently great potential. One of the factors which has inhibited wider application of the prostaglandins is the problem of isolating large quantities from the naturally occurring sources. In view of the recent advances in genetic engineering it must be possible that in the near future prostaglandins can be produced microbiologically. This could give a new stimulus to the study of chemically modified prostaglandins with unique pharmacological activities. Dr. Marsheck gives us in his article a complete survey of the work that has so far been done using micro-organisms to modify prostaglandins.

Pollution of the environment is a problem in all industrialized countries. Heavy metals are in general persistant toxic pollutants. Dr. Jeffries describes in his contribution the role of micro-organisms in detoxifying and recycling one of the most widespread and persistant of the heavy metal pollutants - mercury.

Another universal pollution problem in the disposal of high B.O.D. effluent, particularly the effluents of the bio-industry in the widest sense of the word. One of the most attractive disposal routes for bio-waste is anaerobic digestion, which results in the production of energy rich bio-gas, of which the most important component is methane. The organisms which are responsible for the biological production of methane are the subject of the very comprehensive review contributed by Dr. Taylor.

The physico-chemical upgrading of low grade metal ores is an expensive and energy consuming process. Micro-organisms, particularly Thiobacilli, have the ability to leach metals out of low-grade ores under normal ambient conditions of temperature and pressure. The article from Dr. Torma and Dr. Bosecker reviews comprehensively the current state of the art of microbial leaching.

In the very first volume of Progress in Industrial Microbiology in 1959, Dr. Woodbine wrote his now classic review article on microbial fat production. Now, 22 years later, we see that despite advances in our theoretical knowledge of oleaginous micro-organisms there is still very little industrial application outside the U.S.S.R. Dr. Ratledge in his thoughtful review on this subject summarizes our current knowledge and indicates where and why he thinks the time is approaching when the commercial exploitation may make good economic sense. The current availability of cheap acetic acid (chemically synthesized from methyl alcohol and carbon monoxide) may well help to tip the balance.

In a short but fascinating article Dr. Fowler reviews the current possibilities for the large scale production of plant cells with the concurrent production of specific plant biochemicals.

It has not been possible to include the promised second part of the article

of Dr. Fogarty and Dr. Kelly on microbial α-glucosides in this volume of Progress in Industrial Microbiology (part I appeared in volume 15) due to the indisposition of Dr. Kelly.

This will be the last volume of Progress in Industrial Microbiology which I shall edit. I would therefore like to finish on a personal note by thanking once again the authors of the volumes 14—16 for their willingness to contribute and their good humoured acceptance of changes and sometimes of frustrating delays. The final dedication is to my wife and two children who, without complaining, have so often had to take second place to P.I.M.

M.J. BULL

CONTENTS

MICROBIOLOGICAL BIOCONVERSION OF PROSTAGLANDINS

William J. Marsheck Jr., Ph.D. and Masateru Miyano, Ph.D.
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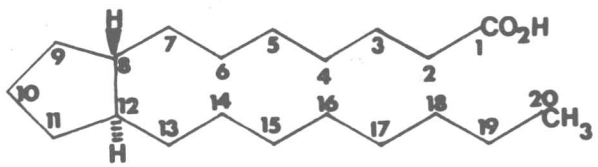
1. INTRODUCTION

The prostaglandins are a series of C_{20} oxygenated fatty acids having extensive and varied activities in mammalian systems. The name prostaglandin was originally given to a lipid factor in human seminal plasma having smooth muscle stimulating and vasodepressor properties. The occurrences in nature of the compounds are not limited to mammals, having been detected in insects, shellfish, and corals. All of the prostaglandins have a cyclopentane ring and are derivatives of the hypothetical substance prostanoic acid.



Prostanoic Acid

Fig. 1. Structure of the hypothetical substance which provides the basis of all prostaglandins.

The primary prostaglandins are divided into three types depending upon the functionalities on this ring

1) A-types (α,β-unsaturated ketones)
2) E-types (β-hydroxyketones)
3) F-types (1,3-diols).

Further division within each type can be made according to the number of carbon-carbon double bonds in the molecule, and this designation appears as a numerical subscript in the name. All of the naturally occurring prostaglandins except 15-epi-PGA_2 isolated from some coral represent a single stereoisomer from a relatively large set of possible diastereo-isomers.

2. PROSTAGLANDIN SOURCES

The availability of different prostaglandins for microbial bioconversion studies has been limited. Chemical synthesis of natural prostaglandins usually provides sufficient quantities of dl-epimers. The biosynthesis of natural prostaglandins from poly-unsaturated fatty acids provides another source of compounds for microbial transformation studies. The biosynthesis by sheep seminal vesicular tissue has been described by Van Dorp et al (ref. 1) and Bergstrom et al (ref.2). This synthesis is catalyzed by the enzyme complex fatty acid cyclo-oxygenase which produces the necessary oxidative cyclization. The presence of double bonds at the

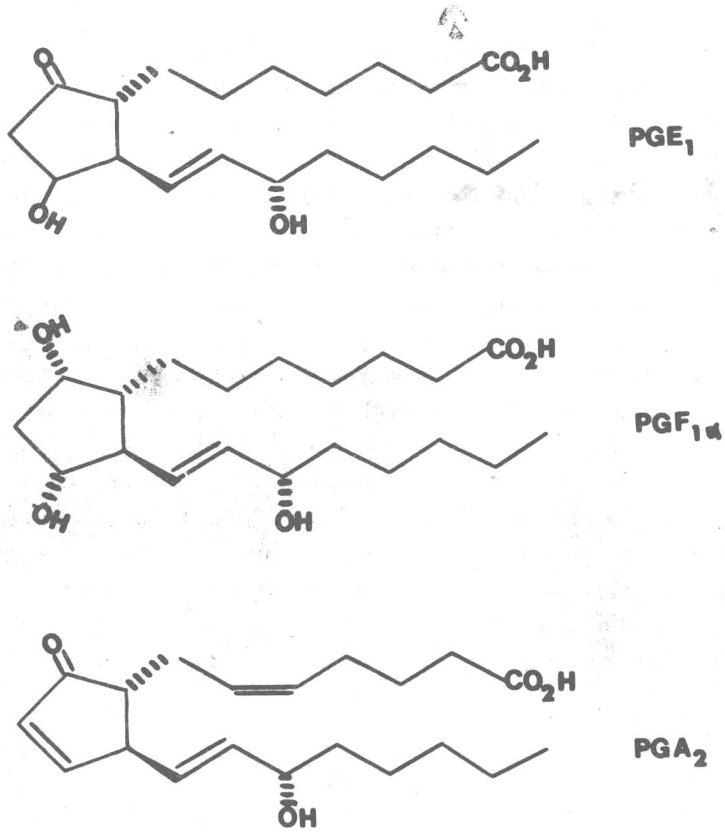


Fig. 2. Several naturally occurring prostaglandins.

6, 9, and 12 positions of the fatty acid substrate is essential for prostaglandin formation (ref. 3). Although this acitivity has been demonstrated in a variety of organ tissues, the ovine and bovine seminal vesicle glands contain highest activities (ref. 4). Using arachidonic acid as substrate, an acetone-pentane powder preparation of sheep seminal vesicular glands can convert arachidonic acid to prostaglandin E_2 (ref. 5). Cyclic prostaglandin endoperoxides (prostaglandin G_2 and H_2) are intermediates formed during the biosynthesis (ref. 6). These endoperoxides can be converted chemically or enzymatically to PGE_2, PGD_2, and PGF_2. The endoperoxides are also enzymatically transformed into physiologically very potent prostacycline (PGI_2) and thromboxane A_2 (TXA_2).

Conversion is limited by the fact that one or more of the products of the reaction inhibit the reaction. Gibson and co-workers have shown that this enzyme can be inhibited by certain oxaprostaglandin derivatives, i.e., 5-oxaprosta-13-

trans-enoate (ref. 7). The absence of 9- and 11- substituents and especially the 15-hydroxy group were required for the inhibition.

Bild and co-workers have claimed synthesis of a prostaglandin product, 9, 11, 15-trihydroxy-prosta-5, 13-dienoic acid, from arachidonic acid using the enzyme lipoxygenase-2 from soybeans (ref. 8). After reduction of the product, the compound reacted positively in a radioimmunoassay against rabbit anti-PGF_2 antibody.

An important source of prostaglandin came from the discovery by Weinheimer and Spraggins of the presence of two prostaglandin derivatives, 15-epi PGA_2 and its acetate methyl ester in the Gorgonian Coral, Plexaura homomalla (ref. 9). Present in the air dried cortex at 0.2 percent and 1.3 percent respectively, these compounds are epimeric with the natural compound, PGA_2, at the allylic hydroxyl center, 15(R) vs. 15(S) natural configuration. Fifteen (R) prostaglandin E_2 and its methyl ester have also been found in gorgonian. Utilizing this abundant source of prostaglandin, Spraggins converted the 15(R) prostaglandin A_2 diester to the natural 15(S) form via acid-catalyzed epimerization (ref. 10). Bundy et al at The Upjohn Company have synthesized PGF_2 and PGE_2 from the 15(R) diester (ref. 11). Later, 15(S) prostaglandin A_2, E_2 (identical with mammalian prostaglandins) and their esters were isolated from Gorgonian Coral.

3. METHODOLOGY

3.1 Stability considerations. The handling of prostaglandins in conducting microbial bioconversions possesses special problems not generally encountered with most other compounds. The compounds generally existing as an oil are sensitive to mild acidic or basic conditions, and to oxidizing agents including oxygen in the air. The F-type prostaglandins are usually the most stable, being very stable to moderately basic aqueous medium conditions and only somewhat labile toward acidic conditions (ref. 12). The E-type prostaglandins are very labile toward both bases and acidic conditions giving rise to dehydration to A-type compounds. Furthermore, the A-type prostaglandins isomerize under basic conditions to B-type structures (ref. 13). It is apparent that careful pH monitoring of the medium when prostaglandin is present with the microbial cells is necessary. Depending upon the compound and reaction desired, a nitrogen atmosphere may be introduced into the bioconversion vessel after microbial growth at the time of prostaglandin addition. The prostaglandin may be added as a solvent solution to the microbial cells in the growth medium or the cells can be removed, washed, and resuspended in buffer before prostaglandin addition. Organic compounds in the growth medium can significantly influence the type and extent of the bioconversion.

Frequently, desired microbial oxygenations may be the first step in a degradative sequence. In a similar situation undesirable degradation of the steroid ring system during microbial transformation of steroids may be inhibited by the use of divalent metal ions, chelating agents, or the introduction of genetic

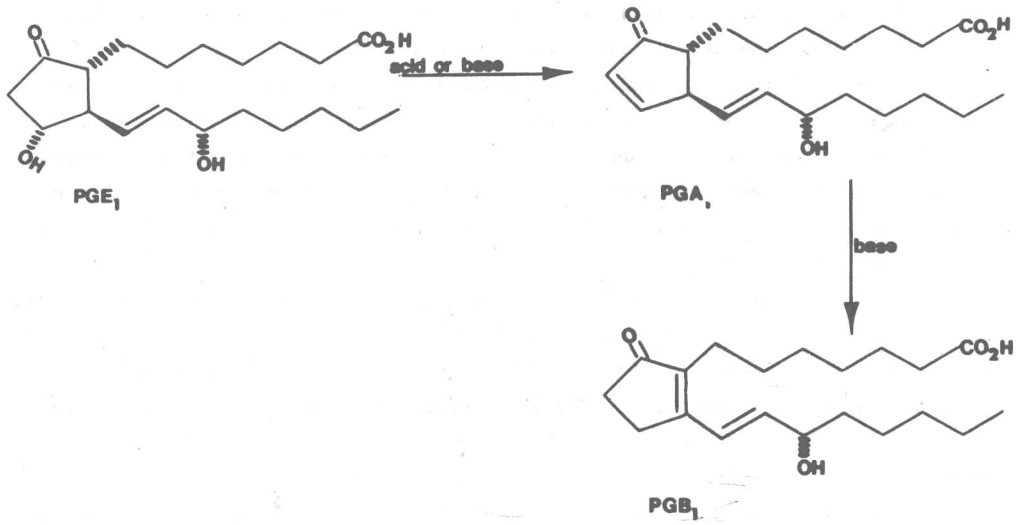


Fig. 3. Acid/base interconversion of natural prostaglandins (ref. 13)

mutations aimed at the enzyme responsible for initial attack or following reactions on the molecule. This degradation of substrate is especially important when costly prostaglandins are studied and becomes particularly critical when processes are scaled-up beyond the shake-flask stage to pilot-sized fermentors. Attack via beta or omega oxidation by the microorganism at the two prostaglandin side-chains is quite common. Certain advantages may be gained by the use of exospores of fungi or streptomyces rather than the vegetative growth stage. Conidiospores of Penicillium roqueforti have been used to transform fatty acids to ketones (ref. 14). Addition of chemical inhibitors might prevent degradation. Thysse reported on the use of acrylic acid to produce fatty acid accumulation when a Pseudomonas was grown on alkanes (ref. 15). Apparently β-oxidation was inhibited by acrylic acid via the inactivation of two Coenzyme A-dependent enzymes, acyl-CoA synthetase and 3-ketoacyl-CoA thiolase.

3.2 Isolation. Removal of prostaglandins from aqueous broths is possible by suitable solvent extraction. The extract may then be chromatographed on silica gel or Sephadex LH-20 (Pharmacia) dextran gel. Solvent extraction requires prior acidification with an organic acid such as acetic, citric, or oxalic acid. Dehydration of PGE compounds to A-type prostaglandins is minimized when organic acids are used when compared to mineral acids. Thin-layer separation of compounds can be achieved on neutral and acidic silica gel or alumina (regular or preparative thin-layer plates) containing fluorescent indicator. The use of silica gel containing silver nitrate is required for separation of prostaglandins differing in

the degree of unsaturation (ref. 16). Increasing the amount of the silver nitrate will enhance the separation. Nondestructive visualization of the A- and B- type compounds or any other containing a conjugated double-bond is possible by their appearance as dark spots on the fluorescent background under short-wave ultra-violet light. Prostaglandin F and G compounds can be visualized by briefly exposing areas of the plate to a glowing-hot wire which causes these materials to quench fluorescence (ref. 13). Apparently, only the uppermost portion is exposed leaving the bottom layer unharmed.

Column chromatographic separation of the dried solvent extract can be carried out on Silic AR CC-4 (Mallinkrodt) using reversed-phase partition chromatography. Miyano et al have described a "magic column" procedure for the separation of epimeric mixtures of 15-keto PGE_1 (ref. 17). A mixture of benzene, methanol, and water (1.5:0.5:0.2) is shaken and allowed to stand in a separatory funnel. A slurry is prepared by combining the lower solvent phase with Silic AR CC-4 (100-200 mesh) and a column is prepared. The upper phase solvent is used to elute the prostaglandins from the column. Alternatively, solvent extracted material may be chromatographed on Sephadex LH-20 in organic solvents (ref. 16). This method may have advantages over silica gel chromatography in that it can be faster, and the loss of material during purification is low. For maximum separation the prostaglandin content in the crude extract should not be below ten percent. Partitioning between ether and buffer-ethanol may be employed for pre-purification of the crude mixture.

The solvent extraction of prostaglandins in very low concentrations from microbial growth media can be very difficult because of the formation of unmanageable emulsions. Amberlite XAD-2 (Rohm and Haas) has been shown to be effective in quantitatively absorbing prostaglandins from biological fluids, blood (ref. 18), and cerebrospinal and amniotic fluid (ref. 19). After removal by centrifugation and washing of the microbial cells, the aqueous solution is acidified. The solution is then slowly passed through the column under gravity flow. The absorbed prostaglandins are eluted with methanol.

3.3 Identification. Radioimmunoassay techniques have been successfully used for the identification of prostaglandins in mammalian metabolism studies as well as microbiological investigations (ref. 20, 21). Antibodies have been prepared for various A_2, B_1, E and F-type compounds including several metabolites. This method is extremely useful when nanogram per milliliter quantities of compounds are present and ordinary isolation and physico-chemical identification procedures are not sufficiently sensitive. When available in small amounts, prostaglandins may be biologically assayed by their action on smooth muscle tissue (ref. 22) or vasodepressor activity (ref. 23). Bygdeman et al, assaying the prostaglandin content of human seminal fluid, described a method for the quantitative determination of A, E, and F-type compounds (ref. 24). Group separation of PGE and PGF compounds was carried out by silicic acid chromatography followed by separation of E-type

compounds by thin-layer chromatography. Subsequent base treatment (0.5 M NaOH) produced dehydration of PGE_2 to the α,β-unsaturation system of PGA_2 compounds, which then rearranged to the doubly conjugated ketone system of PGB_2. This chromophore with λ_{max}^{MeOH} or EtOH at 278 mμ can be measured spectrophotometrically and quantitation achieved. The yield of the chromophore from PGE compounds was 90 percent; whereas, PGA and hydroxylated intermediates gave almost quantitative yield of B-type for spectrophotometric determination. Prostaglandin F compound yields must be determined by gas-liquid chromatography. The compounds are converted to their trimethylsilyl derivatives by treatment with Tri-Sil Z (Pierce Chemical Co.) and in some cases to their O-methyloxime derivatives by treatment with methoxylamine hydrochloride. After separation products may be structurally eludicated by infrared and mass spectroscopy.

4. DIRECT MICROBIOLOGICAL SYNTHESIS

Several reports have been made of the microbial synthesis of prostaglandins from fatty acids or "de novo" biosynthesis. Researchers at The Upjohn Company in 1966 patented a "method of cyclizing fatty acids having a 1, 4-diene to the corresponding cyclopentylalkanoic acid" (ref. 25). This procedure was very broad in its use of fungi, streptomyces, bacteria, and yeast to convert arachidonic and other fatty acids to "substances" having smooth muscle stimulating and mammalian hypotensive activities. Apparently, no further development of this method has been published from this group.

A second U. S. patent claims the growth of Pseudomonas aeruginosa in a culture medium free of fatty acids for prostaglandin formation (ref. 21). Prostaglandins of the A, E, and F types are claimed to be produced in a trypticase soy broth supplemented with heat-inactivated human serum. Quantities of 8.7 ng/ml for PGF, 3.1 ng/ml for PGE, and 16.2 ng/ml for PGA type compounds were determined by radioimmunoassay technique.

Citing the substances as possible factors responsible for inflammation in acne vulgaris, Abrahamsson et al isolated "prostaglandin-like" E-type compounds from Propionibacterium acnes (ref. 26). This finding is significant not only in regard to a better understanding of the causes of acne lesions but also as a possible new source for the microbial synthesis of prostaglandins from fatty acids.

5. BIOCONVERSION

5.1 Classification. The metabolism of prostaglandins in animals and humans proceeds via numerous metabolic pathways depending upon the compound type. For example, A-type prostaglandins are metabolized by the liver, kidney, and possibly intravascular pathways while E compounds are metabolized primarily by the lung (ref. 27). These reactions, many of which are not well understood and occur in various tissues throughout the body, may be categorized into the following reaction

types:

1) Dehydrogenation of the C-15 hydroxyl to the corresponding ketone,

2) Reduction of the 13, 14-double bond,

3) Beta-Oxidation at the carboxylic end of the molecule,

4) Omega hydroxylations on the eight-carbon alkane side-chain,

5) Reduction of 9-ketone of PGE compounds,

6) Dehydration of cyclopentane ring, and

7) Isomerization of the ring 10, 11-double bond (PGA) to the 8, 12-position (PGB).

Microbial bioconversion of prostaglandins produces many of the same types of reactions found with mammalian cells. The use of microbes to produce metabolites of biologically important compounds for further testing and evaluation in living systems has been reported with other compounds. Marsheck and Karim used the fungus Chaetomium cochloides QM 624 to produce 6-oxygenated metabolites of the anti-aldosterone agent, spironolactone (ref. 28). The microbial hydroxylation of certain thiatriazole derivatives, effective in reducing blood pressure in laboratory animals, led to the isolation of compounds similar to mammalian metabolites (ref. 29).

5.2 Oxidations and reductions. Oxidation of the C-15 hydroxyl by the enzyme 15-hydroxy-prostaglandin dehydrogenase seems to initiate mammalian metabolism of prostaglandins. This reaction is apparently controlled by thyroid hormones (ref. 50). This enzyme along with the Δ^{13}-reductase which catalyzes the reduction of the 13, 14-double bond are present in all animal species and humans. As a consequence of these initial reactions, the primary prostaglandins have a very short half-life in the circulating blood. A decreased rate of metabolism of one prostaglandin PGF_2 is attained if the double bond in the carboxylic acid side-chain is shifted from the Δ^5 to Δ^4 position (ref. 31). Oxidation at C-15 is inhibited and subsequent β-oxidation is slowed.

The microbial oxidation of the C-15 hydroxyl has not been extensively reported. Jiu et al at Searle isolated 15-keto derivatives of PGA_2 after two-days incubation with the fungus Dactylium dendroides NRRL 2575 (ref. 32). Further incubation to seven days results in reduction of the 10, 11-double bond. Reduction of the 10, 11-double bond during bioconversions with A-type prostaglandins is a very common reaction particularly utilizing gram-negative bacteria, yeast, and some fungi. In the reaction with D. dendroides the 10, 11-double bond was believed to be isomerized to the 8(12)-position presumably through C-type prostaglandin ($\Delta^{11,12}$) intermediates. Prostaglandin C compounds are very unstable and easily transform to the B series in acid or base conditions (ref. 33). Similar ring double-bond isomerase activity has been detected in humans and animals (ref. 34).

Greenspan and Leeming have reported in the patent literature on the reduction of this ring double bond using PGA_2 (15S) and 15-epi-PGA_2 (15R). A 66 percent

conversion of 15-epi-PGA_2 to 11-deoxy-15-epi-PGA_2 using Streptomyces griseus NRRL 3231 is claimed with a number of other bacteria belonging to the Streptomyces, Pseudomonas, and Corynebacterium genera said to carry out the same conversion.

A number of synthetic routes to natural prostaglandins have been reported. In many cases the introduction of secondary alcohols involves the reduction of the corresponding ketone. Generally, the reduction of this ketone by agents such as sodium borohydride results in a mixture of enantiomers in approximately a 50:50 mixture. This ratio may be shifted to favor one isomer if other polar functions are blocked by trimethylsilyl groups and bulky reducing agents are used (ref. 11). Chemical stereoselective reduction of ketones at C-9 and C-11 in the prostaglandin cyclopentane ring is possible using this approach; however, the stereoselective reduction of a C-15 ketone, removed some distance from the chiral influence of the ring, was more difficult. Miyano and Dorn have described a method for the preparation of dl-15-dehydroprostaglandin E_1 (ref. 35). An intermediate, dl-$\Delta^{8(12)}$-15-dehydroprostaglandin E_1, was found to be an excellent substrate for microbial 15-ketone reduction studies. The reduction of the 15-ketone and 13, 14-double bond of this compound was found to be a facile reaction carried out by many soil microorganisms. One such microbe, Flavobacterium sp. NRRL B-5641, produced a cis racemic mixture of $\Delta^{8(12)}$-PGE_1 (ref. 36) (Fig. 4). Several yeasts, particularly members of the genera Saccharomyces and Rhodotorula, and a number of fungi were capable of trans reductions giving racemic mixtures. Stereoselective trans reductions giving one optical isomer were achieved with several bacteria. Pseudomonas sp. NRRL B-3875 reduced dl-$\Delta^{8(12)}$-15-dehydroprostaglandin E_1 to produce optically pure 11-epi-$\Delta^{8(12)}PGE_1$ while Flavobacterium sp. NRRL B-3874 or Arthrobacter sp. NRRL B-3873 reduced to the 15-epi compound (Fig.5).

Schneider and Murray used yeast to reduce E-prostaglandins to F-type compounds (ref. 37). Noting that fermenting yeasts usually reduced ketones to secondary alcohols having an S configuration, the investigators were able to demonstrate the 9-ketone reduction of nat-PGE_1 and PGE_2 to PGF_1 and PGF_2 having the 9S or 9α-hydroxy configuration. Racemic PGE_1 and PGE_2 methyl esters were reduced to both isomeric C-9 alcohols (α-and β-alcohols). In an analogous reaction mammalian kidney preparations were also capable of converting F compounds to E-prostaglandins via 9-keto reduction (ref. 38).

Several laboratories investigating the total synthesis of natural prostaglandins have reported on the microbial reduction of key intermediates. Using Diplococcus uninucleatus Sih et al disclosed the asymmetrical reduction in 75 percent yield of a C-4 ketone to prepare 2-(6-carbomethoxyhexyl)-4(R)-hydroxycyclopentane-1,3-dione (ref. 39). The 4(S) epimer was produced by Mucor rammanianus in 46 percent yield (ref. 40) (Fig. 6). This reduction eventually results in the introduction of the hydroxy function at C-11 of nat PGE_1 or 11-epi PGE_1. Chirality at C-15 in this total synthesis can be introduced asymmetrically via microbial reduction of

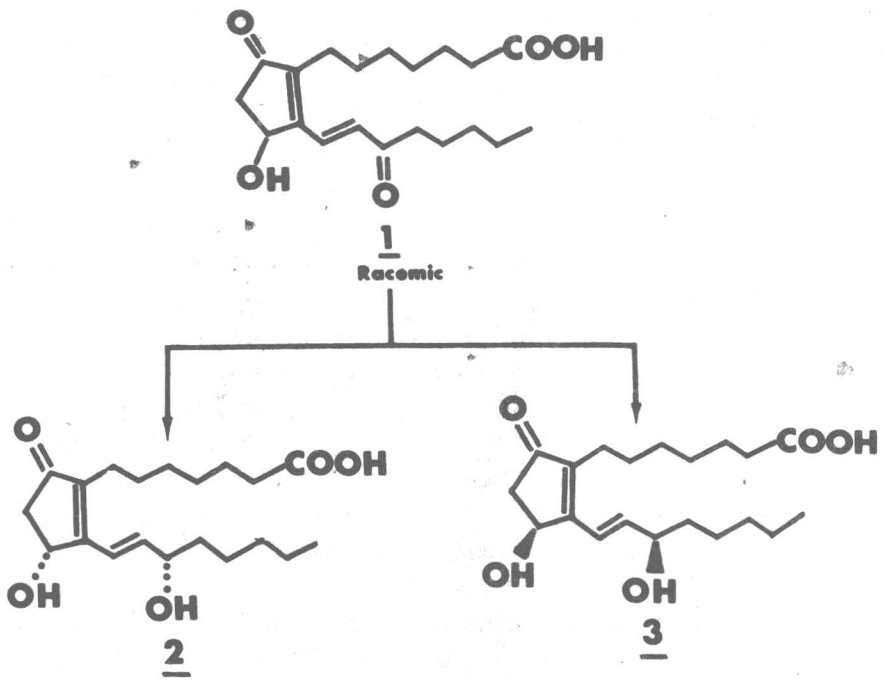


Fig. 4. Cis racemic reduction of a 15-keto prostaglandin by Flavobacterium sp. NRRL B-5641 (ref. 38)

1-iodo-1-octen-3-one (ref. 41) (Fig. 7). Ascomycetes, Phycomycetes or Fungi Imperfecti after growth are harvested, washed, and the mycelium suspended in buffer with the substrate. The resulting iodo alcohol after appropriate derivatization is reacted with the hydroxycyclopentanedione described above to give the prostaglandin structure. Other reports of the microbial reduction of ketone functions of prostaglandin intermediates have appeared in patent literature (ref. 42, 43).

5.3 Hydroxylations and degradation. The hydroxylation of prostaglandins by microorganisms has been demonstrated by several investigators. These types of reactions have been previously shown in human and mammalian prostaglandin metabolism studies. In all instances, the ability to hydroxylate the prostaglandin side-chains is one step in the oxidative degradation of fatty acids. Alpha oxidation occurs via hydroxylation at the C-2 position in the fatty acid chain. This reaction sequence ultimately leads to the decarboxylation of the hydroxy fatty acid giving an odd-numbered fatty acid. A second type, beta oxidation, results in the continual removal of two carbon acetate units in which the fatty acid is

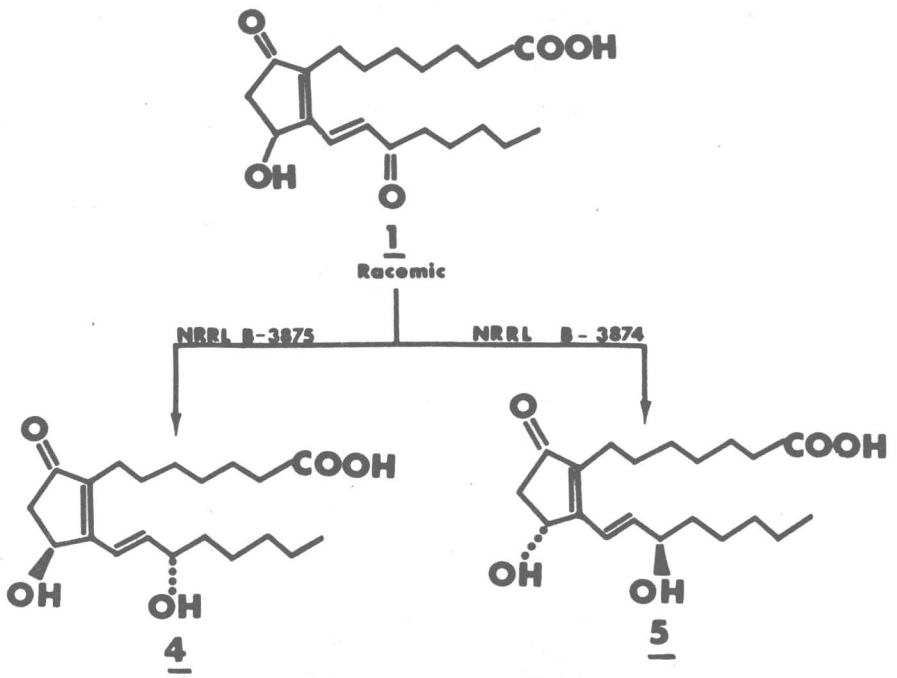


Fig. 5. Trans reduction of a 15-keto prostaglandin giving optically pure compounds (ref. 38)

converted to a α,β-unsaturated fatty acyl-CoA intermediate. Following stereospecific hydration and oxidation to a β-ketoacyl-CoA compound, the molecule is cleaved. Thirdly, of particular importance to a study of microbial bioconversion of prostaglandins is omega oxidation. This process results in the ω-hydroxylation of the fatty acid molecule which may then be oxidized further to the dicarboxylic acid. Following omega oxidation and the generation of a new carbonyl group, the beta oxidation enzyme system may shorten the molecule. The ω-hydroxylase of Pseudomonas oleovorans have been investigated and shown to be inducible and specific for the ω-oxidation of hydrocarbons as well as fatty acids (ref. 44).

Using the actinomycete Streptomyces ruber NRRL B-1268, the authors have reported the 17, 18, and 19-hydroxylation of 15-acetoxy-prostaglandin A_2 ethyl, methyl diester (ref. 45) (Fig. 8). After rapid reduction of the 10, 11-double bond and hydrolysis of the C-1 diester, the organism ω2-hydroxylated the prostaglandin yielding 19-hydroxy-15-acetoxy-10,11-dihydro PGA_2. Further hydrolysis

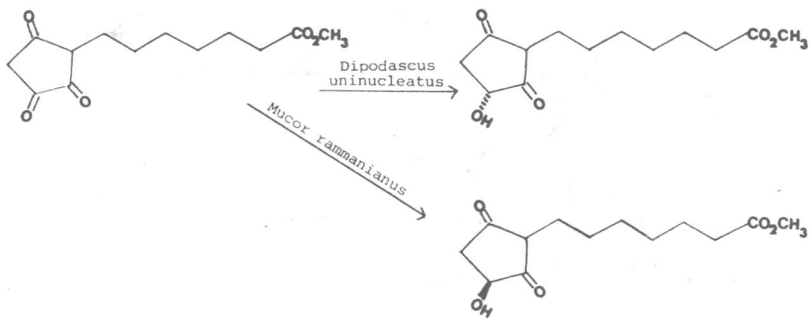


Fig. 6. Microbial C-4 reduction of a prostaglandin intermediate (ref. 42)

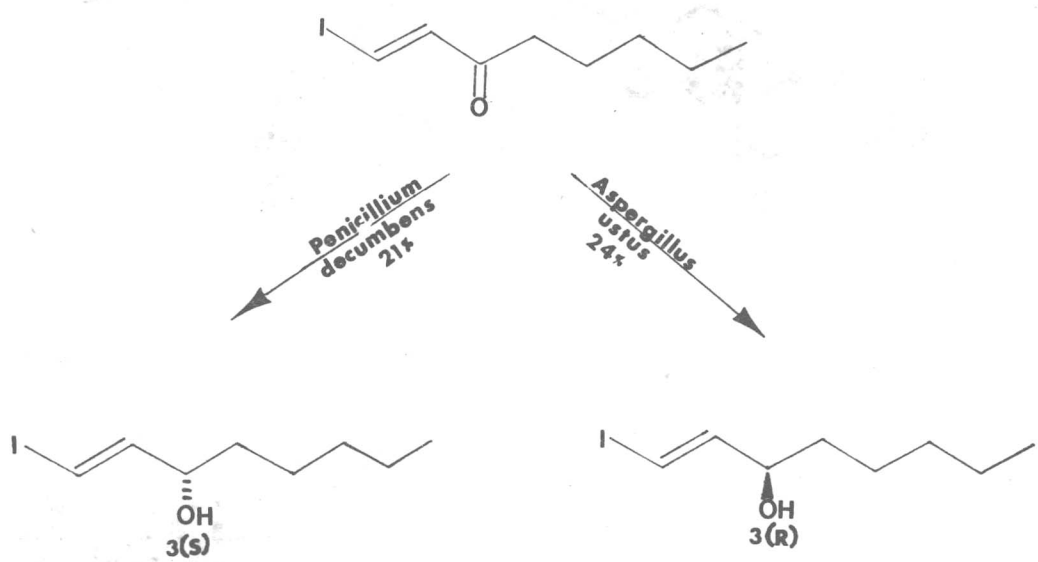


Fig. 7. Microbial reduction of 1-iodo-1-octen-3-one (ref. 43)

at C-15 produced 19-hydroxy-10,11-dihydro PGA_2. On the other hand, if hydrolysis of the 15-acetoxy group occurs early, hydroxylation is directed at C-18 and to a lesser degree C-17. Sebek et al screened streptomyces for hydroxylation of PGF_2 and PGE_2, and found that 10.7 percent of those tested gave hydroxylated products (ref. 46). One culture converted the prostaglandins into a mixture of 18- and 19-hydroxy derivatives in 20-24 percent total yield for PGF_2 and 40 percent for PGE_2 (Fig. 9). Investigators at Gist-Brocades in Holland have also reported on