# Chapter 6

# BIOCATALYTIC CONVERSION OF OTHER LIPIDS

*In Chapters 4 and 5, lipase-mediated conversion of acylglycerols were presented. The Chapter 6 deals with biocatalytic modification of phospholipids, sphingolipids, steroids and fatty acids.*

## 6-1 Phospholipids

### 6-1-1 Utilization of phospholipids

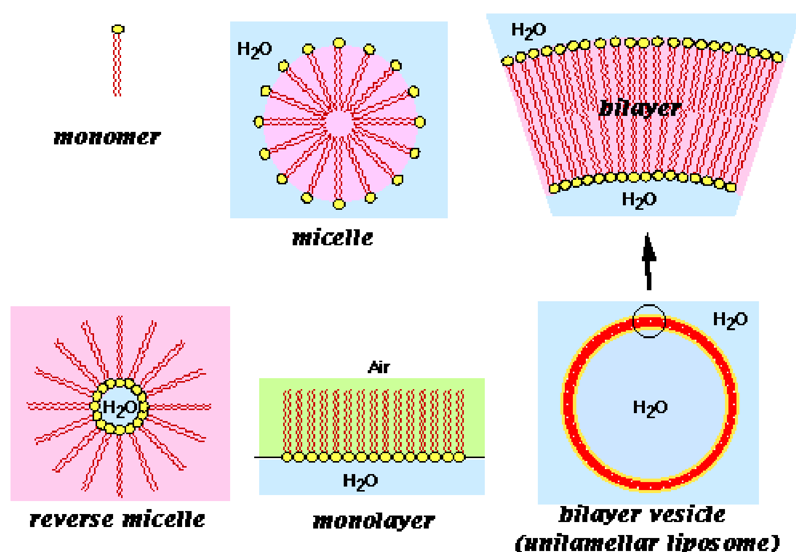


Figure 6-1-1: Structure of various physical forms of phospholipids.

Phospholipids are defined as lipids which contain phosphorus. Naturally occurring phospholipids are ubiquitous in all organisms. One of the most important characteristics of phospholipids is their amphiphilicity, which arises from the hydrophobic acyl or alkyl groups and hydrophilic polar head groups. Their amphiphilic nature makes it possible to form with water several aggregates such as micelles, reverse micelles and bilayer vesicles (Figure 6-1-1). Physiologically, phospholipids are major components of bio-membranes, which maintain the cell shape, support membrane proteins and provide the substrate for phospholipases involved in transmembrane signalling.

Physical properties, biocompatibility and nutritional functions of phospholipids make them useful in industrial fields such as food, cosmetics, and pharmaceuticals. In many cases, phospholipids seem to be employed for the benefits coming from the physical properties and biocompatibility rather than their nutritional benefits even in food industries. phospholipids can be used as emulsifiers, components of cosmetics, medical formulations, and for liposome preparations.

## 6-1-2 Phospholipases

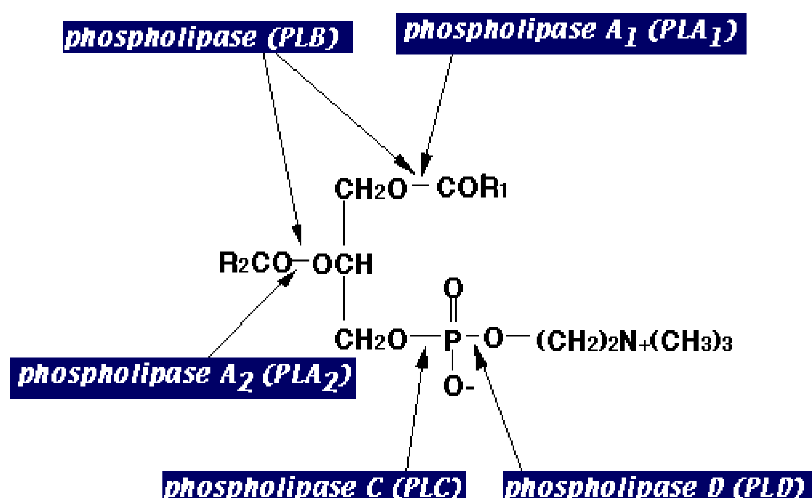


Figure 6-1-2: Mode of action of phospholipases on phosphatidylcholine.

Phospholipases are enzymes which hydrolyze phospholipids. Based on their mode of action, phospholipases are classified into several classes, $A_1$, $A_2$, B, C and D ($PLA_1$, $PLA_2$, PLB, PLC and PLD). Figure 6-1-2 shows the mode of action of each Phospholipases on phosphatidylcholine. $PLA_1$ and $PLA_2$ hydrolyze the acyl ester bond at *sn*-1- and 2-positions, respectively, while PLB hydrolyzes both. PLC and PLD act on the phosphodiester bond, but PLC cleaves the linkage between the glycerol and the phosphate, while PLD attacks the bond between the phosphate and the hydroxyl group of the polar head.

Enzymatic reactions catalyzed by phospholipases can be used for phospholipid processing or syntheses, as complements to chemical reactions. Among the five types of phospholipases (i.e. $A_1$, $A_2$, B, C and D types), $PLA_2$ and PLD are mostly studied with respect to their applications.

## 6-1-3 Production of lysolecithin

Lecithins are inferior to other food emulsifiers such as monoacylglycerol, sugar ester and polyglycerolester due to their strong hydrophobicity. In contrast, lysolecithin or lysophospholipid (usually 1-acyl-2-lyso form) has superior emulsifying properties because they are more soluble in water forming stable O/W-type emulsions. Industrially production of lysolecithin is shown in Figure 6-1-3. For this purpose, chemical methods are obviously complicated because of the difficulty in deacylation of the substrate specifically at the desired position.

Soybean lecithin and porcine pancreatic $PLA_2$ are used as starting material and catalyst, respectively. Soybean lecithin dispersed in aqueous buffer containing $Ca^{2+}$ ion as a cofactor is hydrolyzed by the enzyme. The hydrolyzate is dehydrated under reduced pressure,

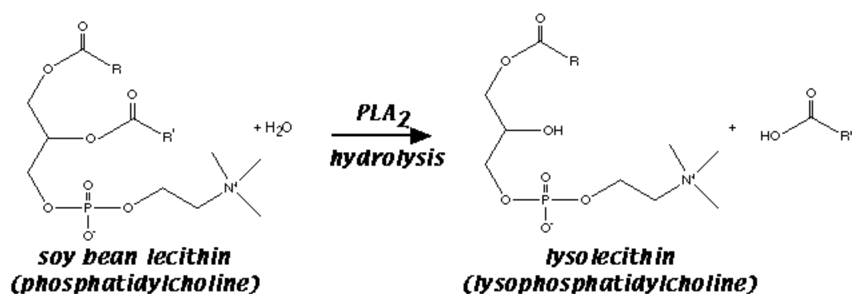


Figure 6-1-3: Production of lysolecithin by $PLA_2$ -mediated hydrolysis of soy bean lecithin.

followed by the removal of the fats, which are mainly liberated free fatty acids, with acetone. Then, the product is dried under reduced pressure to give the final product as a powder.

### 6-1-4 Modification of the head groups

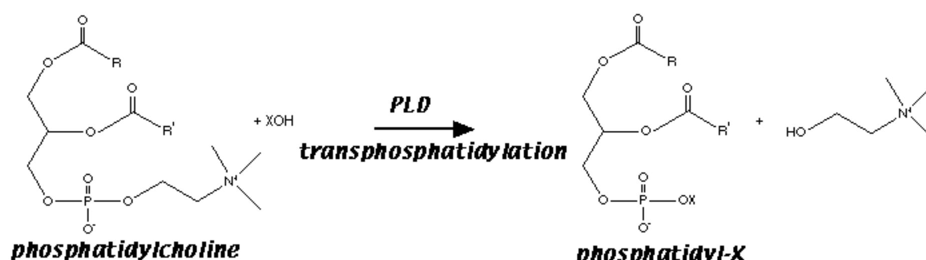


Figure 6-1-4: Transphosphatidylation catalyzed by PLD. Phosphatidylcholine is converted into "phosphatidyl-X" in the presence of hydroxyl compounds (XOH). When X is H, the reaction is hydrolysis.

In addition to hydrolysis, PLD catalyzes a kind of transesterification, in which polar head groups of phospholipids are replaced with other hydroxyl compounds. This reaction (so called transphosphatidylation) can be used to prepare a phospholipid with a particular polar head group (Figure 6-1-4). Various phospholipids can be synthesized from naturally abundant ones such as phosphatidylcholine or lecithin and corresponding hydroxyl compounds.

The reaction is typically carried out in a bi-phasic system consisting of a water-immiscible organic solvent (e.g. diethylether and ethylacetate) containing lipids and an aqueous solution of enzyme and hydroxyl compounds (i.e. acceptor compounds such as ethanolamine, glycerol or serine). The transphosphatidylation undergoes even in such a water-rich system under the optimized conditions, although PLD is intrinsically a hydrolytic enzyme. Thus, there is no need to control water content in the reaction system or dehydrate the enzyme prior to the reaction, unlike the cases of lipase-catalyzed transesterification reactions. An advantage of the bi-phasic system is that the desired product is soluble in the organic phase and can be separated easily from the aqueous phase by a simple phase separation.

Figure 6-1-5: Phosphatidylserine

**Production of phosphatidylserine:** One industrial application of transphosphatidylation is production of phosphatidylserine (PS). PS is a type of natural phospholipid which is abundant especially in animal brain. Recently, PS has taken much attention as it shows therapeutic effects on several memory-related disorders (Figure 6-1-5). The possible natural sources for production of PS are animal organs such as bovine brain. However, these animal organs might not be suitable for human use as it may mediate infectious diseases such as brain spongiform encephalopathy. In addition, animal organs themselves are not likely appropriate sources of PS for industrial scale production because of low availability. From the reason, PS is industrially produced from soybean lecithin and L-serine by PLD-catalyzed transphosphatidylation (Figure 6-1-6).

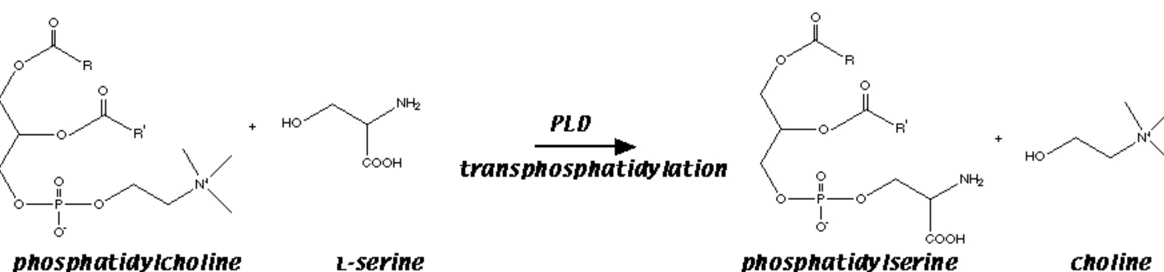


Figure 6-1-6: Synthesis of phosphatidylserine by transphosphatidylation.

## 6-1-5 Use of phospholipases to phospholipid analysis

Phospholipases can be used for chemical analysis of phospholpids. Some typical examples are shown briefly.

**$PLA_2$ :** $PLA_2$ is used for fatty acid distribution analysis of phospholipids (Figure 6-1-7). A phospholipid sample to be analyzed is chemically converted to fatty acid methylesters, and the composition of them (Total FA composition) is analyzed. Whereas, the phospholipid sample is hydrolyzed with $PLA_2$, to form free fatty acids (released from *sn*-2 position) and lysophospholipids (having fatty acids at *sn*-1 position). The free fatty acid fraction and lysophospholipid fraction, (which are separated by thin-layer chromatography), are

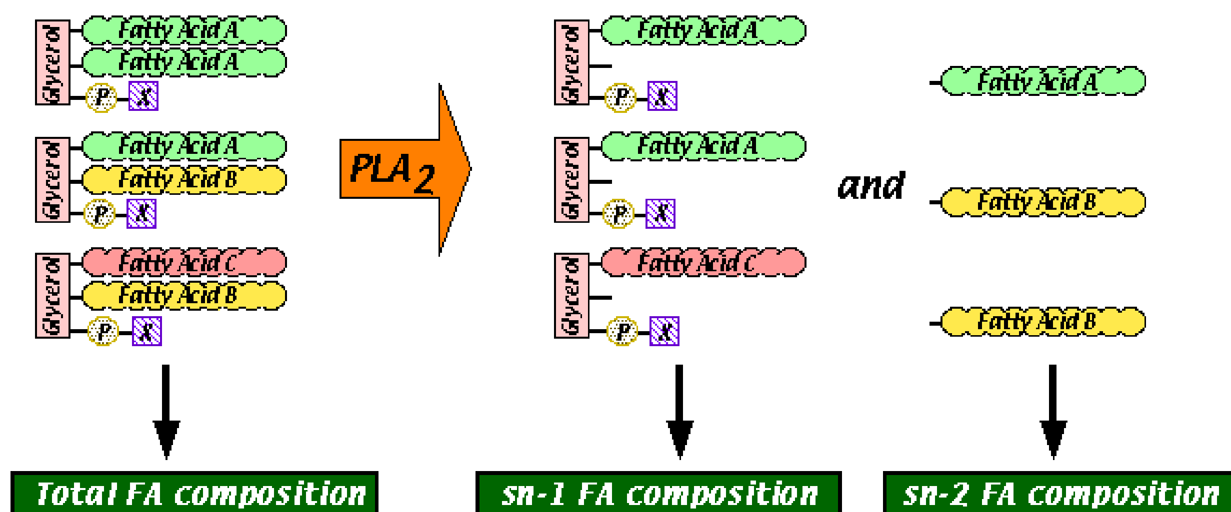


Figure 6-1-7: Analysis of fatty acid distribution with the help of $PLA_2$.

converted to fatty acid methylesters and the fatty acid composition is analyzed. From the data obtained, fatty acid distribution of the sample can be estimated.

**PLD:** Hydrolysis of phospholipids with PLD generates phosphatidic acid and the corresponding hydroxyl compounds such as choline, serine, glycerol and ethanolamine. Quantification of each of these water-soluble hydroxyl compounds enables quantification of each phospholipid class. For example, in quantification of phosphatidylcholine (Figure 6-1-8), a sample containing phospholipids is hydrolyzed with PLD. With the help of co-existing choline oxidase, the generated choline is converted to glycine betaine and $H_2O_2$ is formed. The $H_2O_2$ is reacted with phenol and 4-aminoantipyrine in the presence of peroxidase, resulting in the formation of red-colored quinone dye, which can be quantified spectrophotometrically. Using this principle, a kit for testing blood phospholipid is developed and used for medical diagnosis.

# 6-2 Sphingolipids

## 6-2-1 Use of sphingolipids

Sphingolipids are lipids containing sphingosine moiety. Since these sphingolipids are not as abundant in natural source as glycerophospholipids, their bulk usage (e.g. like as food emulsifiers) seems difficult. Recently, however, much attention has been paid to these lipids due to their specific biological functions involved in cellular recognition event and transmembrane signaling.

One remarkable application is in cosmetic industry to add ceramide (a kind of sphingolipid) in some cosmetics as moisture-keeping agent for skin. Extracts from plant and from microorganisms (*Sphingomonas* and Yeast) and chemically synthesized preparations are industrially used. Ones from plans and microorganisms are also used for food as supplements.

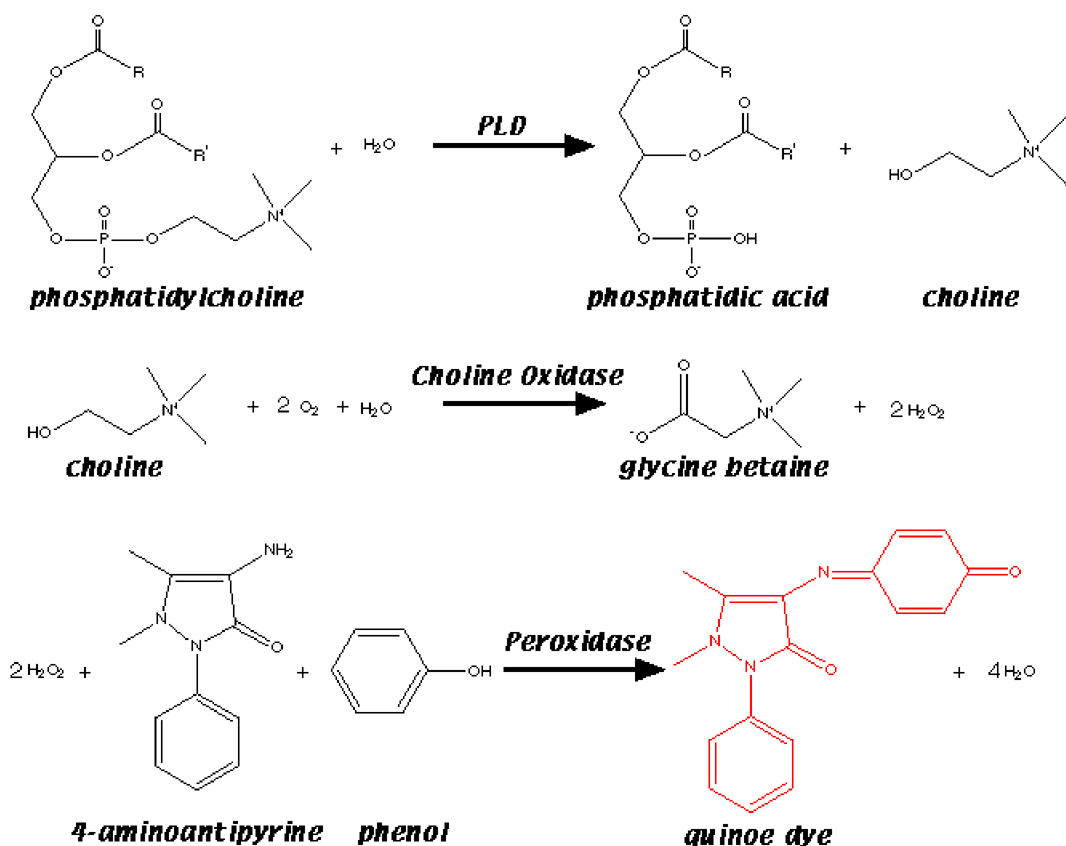


Figure 6-1-8: Quantification phosphatidylcholine.

## 6-2-2 Enzymatic modification of sphingolipids

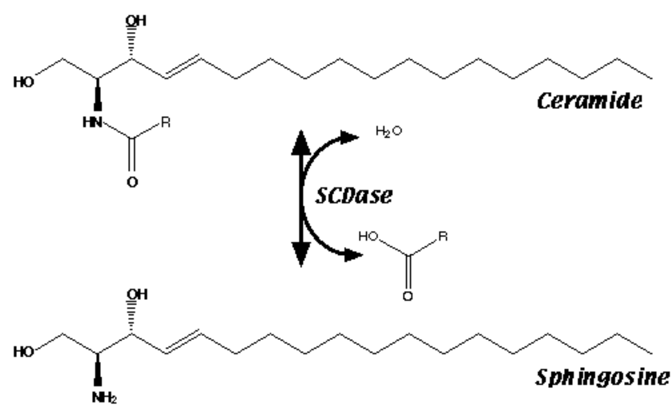


Figure 6-2-1: Modification of amide moiety with SCDase.

**Sphingosine-ceramide-N-acylase (SCDase)** is an enzyme which cleaves *N*-acyl linkage of sphingolipids. Hydrolysis of sphingolipids with SCDase gives the corresponding lysosphingolipids (Figure 6-2-1). This deacylation of the amide structure is possible by a non-enzymatic means such as acid hydrolysis. However, this chemical method often causes epimerization (racemization) of the configuration at C3 of the sphingosine moiety, resulting in formation of *(2S, 3S)*-form, which differs from the naturally occurring *(2S, 3R)*-form. In

contrast, the enzymatic method does not cause such an epimerization. In addition, SCDase catalyzes condensation between fatty acid and lysosphingolipids, enabling preparation of sphingolipids with various N-acyl groups by a simple enzymatic reaction.

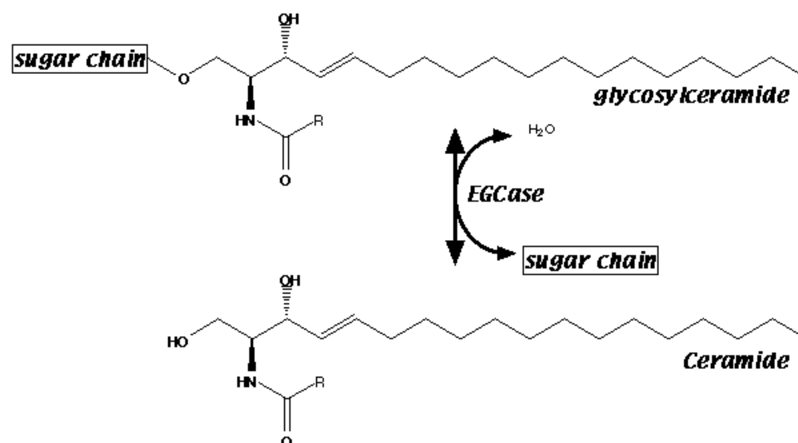


Figure 6-2-2: Modification of glycosyl linkage of glycosylceramides wth EGCase.

**Endoglycoceramidase (EGCase)** hydrolyzes glycoside bond of glycosylsphingolipids to release sugar chain and ceramide (Figure 6-2-2). This enzyme also catalyzes condensation of sugar chain and ceramide to give the corresponding glycosylsphingolipids. Using this reaction, various glycosylsphingolipids can be prepared.

These enzymatic reactions are not used for bulk production of sphingolipids, but very useful for preparation of sphingolipids as reagents for research purposes.

# 6-3 Steroids

## 6-3-1 Use of plant sterols for functional oil products

Phytosterols are sterols of plant origin including $\beta$-sitosterol, stigmasterol and campesterol (Figure 6-3-1). They are known to inhibit absorption of cholesterol in animal intestine, thereby reducing the blood cholesterol concentration. Interestingly, phytosterols themselves are not absorbed to animal body. Featuring this property, several food companies add phytosterols to their products (e.g. cooking oil, margarine) and launched as specialty food with cholesterol-reducing effect.

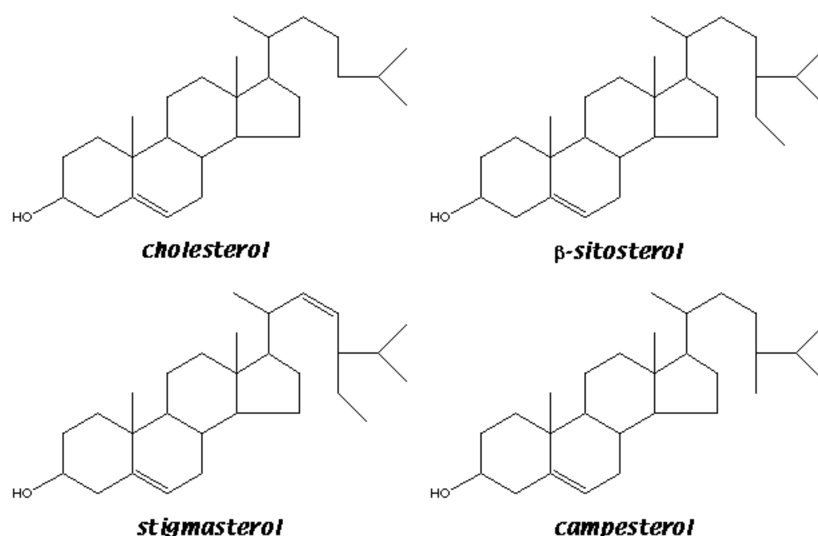


Figure 6-3-1: Structure of major sterols. Cholesterol is of animal origin, whereas phytosterols ($\beta$-sitosterol, stigmasterol and campesterol) are from plants.

## 6-3-2 Steroid fermentation

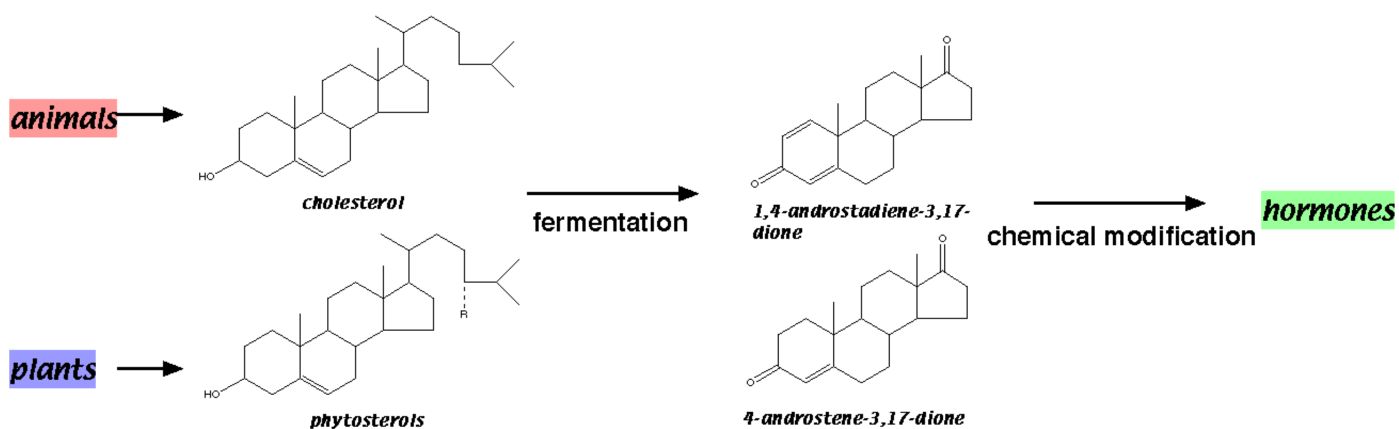


Figure 6-3-2: Sterol fermentation. Naturally occurring sterols (cholesterol and phytosterols) are converted to more complex steroids intermediates by the action of microorganisms. The obtained intermediates are further converted to various steroid hormone by chemical synthesis.

Steroid hormones have many biological functions. Various steroids hormones are used for medical purposes. One of the industrial production of steroids includes steroid fermentation, in which some important intermediates are produced from cholesterol or phytosterols using microbial fermentation (Figure 6-3-2). The intermediates are further modified by means of chemical syntheses into various hormones for medical uses.

## 6-3-3 Enzymatic modification steroid hormones

Many enzymatic methods for conversion of one steroid into another are industrially established. Examples are shown in Table 6-3-1. The reactions include oxidation, reduction and hydroxylation specifically at the desired positions of the substrates. Enzymes are very good at these specific modifications.

Generally, the enzymes required for the reactions are not isolated from the microbial cells. Instead the microbial cells (usually "resting cells") containing the enzyme are directly used as the catalyst. This "resting cell reaction" looks like fermentation, but they are different. In resting cell reactions, it does not matter whether the cells are dead or alive; the only important is that the enzyme is active. By contrast, in fermentation, the cells should be alive. Generally, resting cell reactions are used for one or a few-step conversion of the substrate as shown Table 6-3-1, whereas fermentation is for multi-step reactions (e.g. Figure 6-3-2.).

Table 6-3-1(a): Enzymatic conversion of steroids.

| Reaction | Substrate | Product | Scheme |
|---|---|---|---|
| $\Delta^1$-dehydrogenation | hydrocortisone | prednisolone | hydrocortisone → ($\Delta^1$-dehydrogenation) prednisolone |
| $\Delta^1$-dehydrogenation | 4-androstene-3,17-dione | 1,4-androstadiene-3,17-dione | 4-androstene-3,17-dione → ($\Delta^1$-dehydrogenation) 1,4-androstadiene-3,17-dione |
| $\Delta^1$-dehydrogenation | teststerone | $\Delta^1$-dehydroteststerone | teststerone → ($\Delta^1$-dehydrogenation) $\Delta^1$-dehydroteststerone |
| $\Delta^1$-dehydrogenation | cortexolone | 11$\beta$-deoxyprednisolone | cortexolone → ($\Delta^1$-dehydrogenation) 11β-deoxypredonisolone |

Table 6-3-1(b): Enzymatic conversion of steroids.

| Reaction | Substrate | Product | Scheme |
|---|---|---|---|
| 9$\alpha$-hydroxylation | 4-androstene-3,17-dione | 9$\alpha$-hydroxy-4-androstene-3,17-dione | |
| 11$\beta$-hydroxylation | cortexolone | hydrocortisone | |
| 11$\alpha$-hydroxylation | progesterone | 11$\alpha$-hydroxy-progesterone | |
| 16$\alpha$-hydroxylation | estrone | 16$\alpha$-hydroxyestrone | |
| 16$\alpha$-hydroxylation | dehydro-epiandrostene | 16$\alpha$-hydroxydehydro-epiandrostene | |
| Dehydrogenation of 3$\beta$-hydroxyl | cholesterol | cholestenone | |
| Dehydrogenation of 3$\beta$-hydroxyl | $\beta$-sitosterol | $\beta$-sitosterone | |

Table 6-3-1(c): Enzymatic conversion of steroids.

| Reaction | Substrate | Product | Scheme |
|---|---|---|---|
| Dehydrogenation of 3$\beta$-hydroxyl | stigmasterol | stigmastenone | stigmasterol → dehydrogenation of 3β-hydroxyl → stigmastenone |
| Dehydrogenation of 3$\beta$-hydroxyl | dehydro-epiandrosterone | 4-androstene-3,17-dione | dehydroepiandrosterone → dehydrogenation of 3β-hydroxyl → 4-androstene-3,17-dione |
| Dehydrogenation at 3$\beta$-hydroxyl | pregnenorone | progesterone | pregnenorone → dehydrogenation of 3β-hydroxyl → progesterone |
| Dehydrogenation at 17$\beta$-hydroxyl | $\Delta^1$-dehydroteststerone | 1,4-androstadiene-3,17-dione | Δ1-dehydroteststerone → dehydrogeation of 17β-hydroxyl → 1,4-androstadiene-3,17-dione |
| Dehydrogenation at 17$\beta$-hydroxyl | $\beta$-estradiol | estrone | β-estradiol → dehydrogeation of 17β-hydroxyl → estrone |
| Dehydrogenation of 17$\beta$-hydroxyl | teststerone | 4-androstene-3,17-dione | teststerone → dehydrogeation of 17β-hydroxyl → 4-androstene-3,17-dione |

## 6-4 Conjugated fatty acids

### 6-4-1 Conjugated linoleic acid

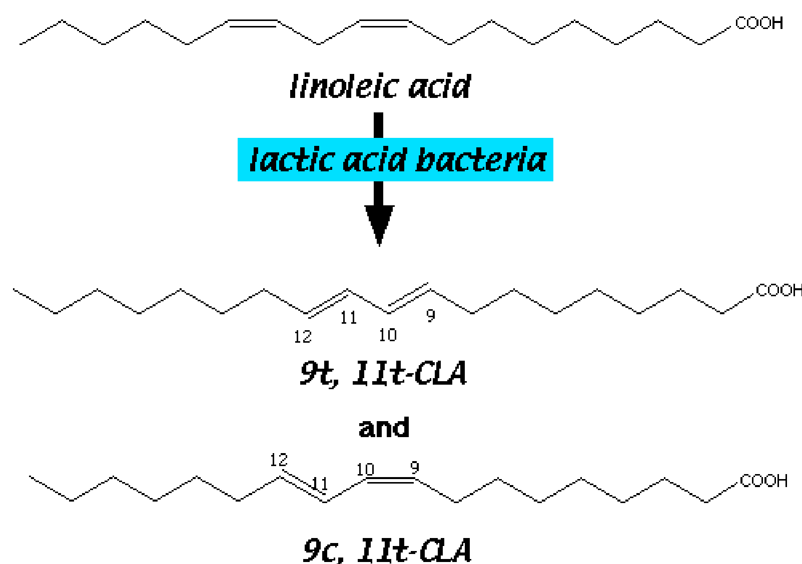


Figure 6-4-1: Conversion of linoleic acid to CLA using lactic acid bacteria.

Some conjugated fatty acids have biological effects such as body fat-reducing, anti-cancer etc. Currently, conjugated linoleic acid (CLA, a typical of conjugated fatty acid) are industrially produced by chemical isomerization of linoleic acid and sold as a supplement. Due to the random nature of the chemical reaction, however, the chemically produced CLA is usually mixture of various isomers. As an alternative way, bioconversion of linoleic acid to CLA using some lactic acid bacteria is being studied. Reaction of linoleic acid with the resting cells of a strain of lactic acid bacteria, *Lactobacillus plantarum* gives CLA. Although this reaction method is not industrialized yet, it is a promising option for production CLA.