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From Soybean Phytosterols to Steroid Hormones

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1. Introduction

Phytosterols, which are structurally and physiologically similar to cholesterol, are a large group of steroidal triterpenes. They are essential to maintaining normal function in plant cell membranes. In recent years, some of their beneficial effects on human health have come to light. Phytosterols can lower intestinal cholesterol absorption, thereby reducing serum levels of low-density lipoproteins and the risk of atherosclerosis. The pharmaceutical industry has a long history of converting phytosterols to therapeutic steroid hormones by microbial transformation. One commercially available phytosterol product is a phytosterol mixture extracted from soybean oil deodorizer distillate. Soybean phytosterols usually include four sterols: β-sitosterol, stigmasterol, campesterol, and brassicasterol, all of which make good raw materials for the production of steroid hormones because of their typical A-ring molecular structure with a 3β-hydroxyl group and a 5,6-double bond (Fig. 1). Two kinds of steroid hormone intermediates can be produced from soybean phytosterols through microbial transformation. The first of these are the C19-steroids, which include androsta-4-ene-3,17-dione (AD), androsta-1,4-dien-3,17-dione (ADD), 9α-hydroxy-androstra-4-ene-3,17-dione, and testosterone; the second are the C22-steroids, such as 20-carboxy-pregna-l,4-dien-3-one and 20-hydroxymethylpregna-1,4-dien-3-one (Fig. 2). C19-steroids are the products of complete side chain cleavage. They can be used as precursors to almost all kinds of steroid hormones, including sex hormones, anabolic steroids, and even adrenocortical hormones. C22-steroids are the products of truncated side chain. These make good precursors to adrenocortical hormones. The chemical conversion of sapogenins to steroids is a well-established alternative to microbial transformation of phytosterols to steroids. This method has many shortcomings, however, such as higher costs, more steps, low yield, the waste of land resources, and the destruction of wild plant resources. In light of this, microbial conversion of soybean phytosterols to steroids shows great value in the synthesis of steroid hormones.

Here, we summarize our knowledge of the occurrence of phytosterols in soybeans and the technology that can be used to transform them from phytosterols to steroids through the regulation and modification of microbial catabolism. Based on analysis of the metabolic mechanisms of phytosterols and the bottlenecks inherent in the microbial transformation process, we will also discuss areas for development and improvement.

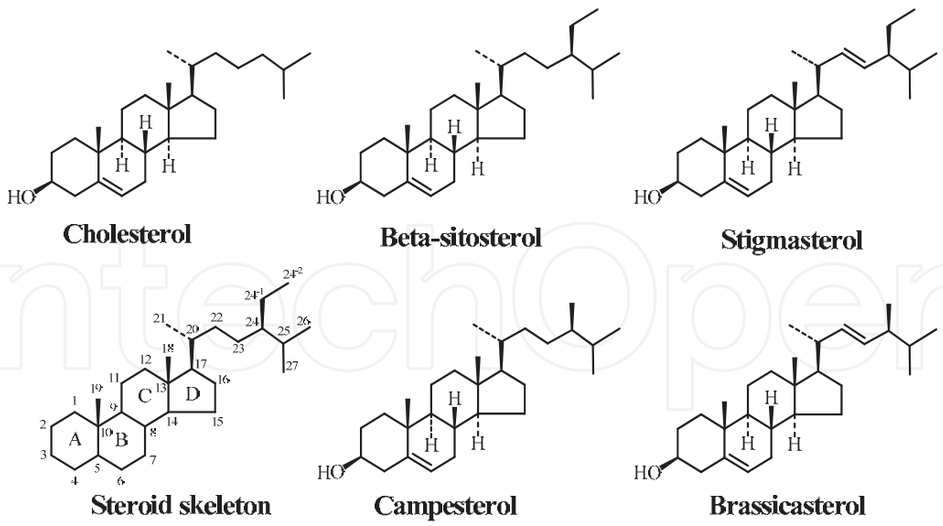

Fig. 1. Cholesterol and soybean phytosterols based on steroid skeleton

2. Soybean phytosterols

2.1 Soybean phytosterols and human health

Phytosterols are a group of steroid alcohols that occur naturally in plants. They are generally isolated during the process of producing vegetable oils, especially soybean oil. Soybean oil deodorizer distillate (SODD) is one main wastes of the soybean-processing process. It is rich in phytosterols and has become the main source of commercial phytosterols (Ramamurthi & McCurdy, 1993; Hirota, *et al.*, 2003, Benites, *et al.*, 2009). β-sitosterol, stigmasterol, campesterol, and brassicasterol are the four major types of soybean phytosterols soybean. They differ in the double bond at C22 and the substituents at C24 (Fig. 1). Just like cholesterol in animals, phytosterols regulate of the fluidity of plant cell membranes and feature in cellular differentiation and proliferation (Benveniste, 1986, Piironen, *et al.*, 2000).

Soybeans are a well-known health food, rich in quality protein, fatty acids, and other healthy components, including phytosterols. Because most people enjoy taking in high-fat, high-energy foods that raise the serum cholesterol level out of healthy range, dyslipidemia, together with its accompanied syndromes, hyperlipidemia, adiposis, and cardiovascular disease, are now threatening the health of citizens of the developed and developing world (Kes Niemi & Miettinen, 1987; Genest, *et al.*, 2003). In recent years, many studies have indicated that phytosterols may be beneficial as food additives because they can lower the absorption of cholesterol in intestines by 10% to 15% (Ostlund, *et al.*, 2003; St-Onge, *et al.*, 2003). The FDA has stated that daily intake of moderate amounts of phytosterols can reduce the risk of heart disease. The long-term intake of phytosterol-rich foods could efficiently diminish plasma cholesterol and atherosclerotic risk. For patients who cannot tolerate cholesterol-lowering drugs, it may be advisable to adopt a phytosterol-rich diet as a substitute (Ostlund, *et al.*, 2003).

2.2 Phytosterol biosynthesis

Like cholesterol, phytosterols are biosynthetically derived from squalene. However, the synthesis of phytosterols involves a relatively complicated mechanism. In plants, the

anabolism of phytosterols can be divided into two parts with squalene being the critical point (Fig. 3) (Piironen, *et al.*, 2000).

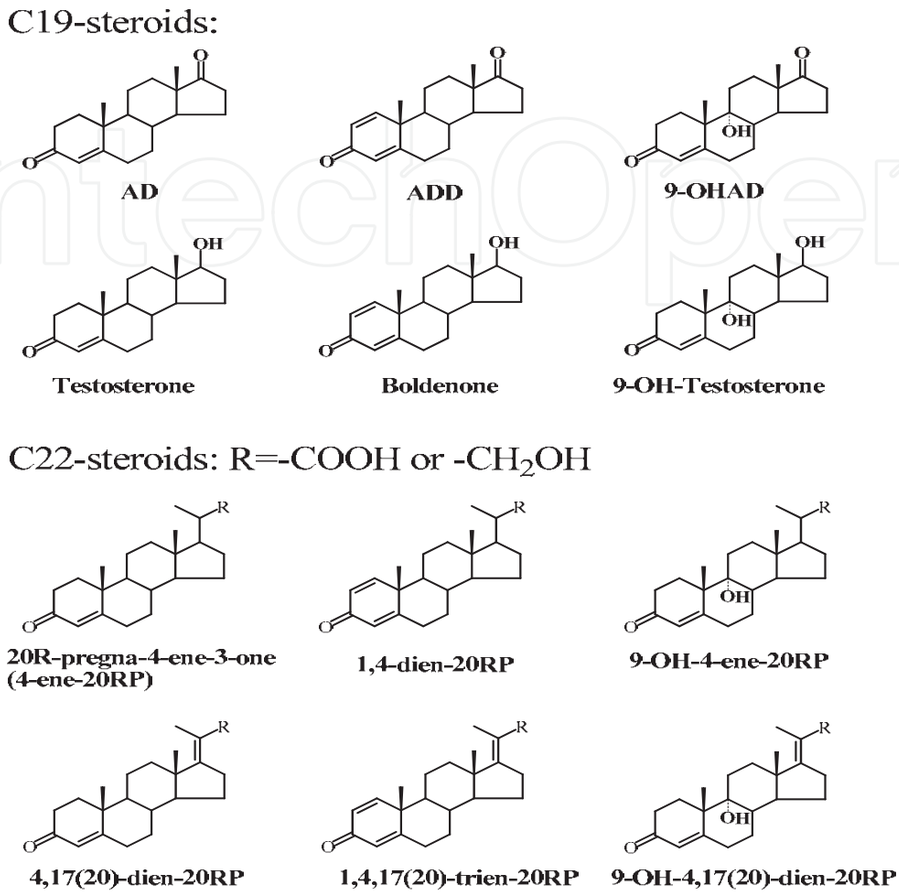

Fig. 2. Steroids derived from soybean phytosterols by microbial transformation

The pre-squalene pathway is a nucleus-accumulating process derived from isoprenoid, using isopentenyl pyrophosphate (IPP) as the fundamental building block. It is carried out by the mevalonic acid pathway (Guo, *et al.*, 1995). It has been determined that the series of enzymes involved in the pre-squalene pathway from acetyl coenzyme A to squalene, via the formation of 3-hydroxymethyl-3-glutaryl coenzyme A (HMG) are mevolonate, isopentenyl pyrophosphate (IPP), and farnesyl pyrophosphate (FPP). Within this process, 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMGR) is one predominant but controversial factor believed to determine the capability of phytosterol synthesis (Bach, 1986; Goldstein & Brown, 1990; Stermer, *et al.*, 1994). Squalene synthesis was reported to be involved in competition with sesquiterpene, which is regulated by sesquiterpene cyclase (SC) (Vogeli & Chappell, 1988). The activation of sesterpene synthesis would correspondingly result in the suppression of sterol accumulation. Consequently, the inhibition of SC should be regarded as an important control mechanism for enhancing sterol formation.

The post-squalene pathway mainly refers to the modification of branch chain on steroidal compounds. Conversion of cycloartenol to β-sitosterol and stigmasterol involves two

methylation steps, whereas conversion to campesterol and brassicasterol involves one. There are two different families of sterol methyl transferases (SMT 1 and SMT 2) known to be responsible for biosynthesis of the 24-methyl and 24-ethyl sterols, respectively (Nes & Venkatramesh, 1999; Bouvier-Navé, *et al.*, 1998). SMT has been found to be associated with the Δ5-sterol production. Most importantly, it has the highest degree of sterol specificity f any compound involved in the pathway. In other words, the ultimate concentration ratio of phytosterol components, campesterol/β-sitosterol, primarily depends on SMT activity.

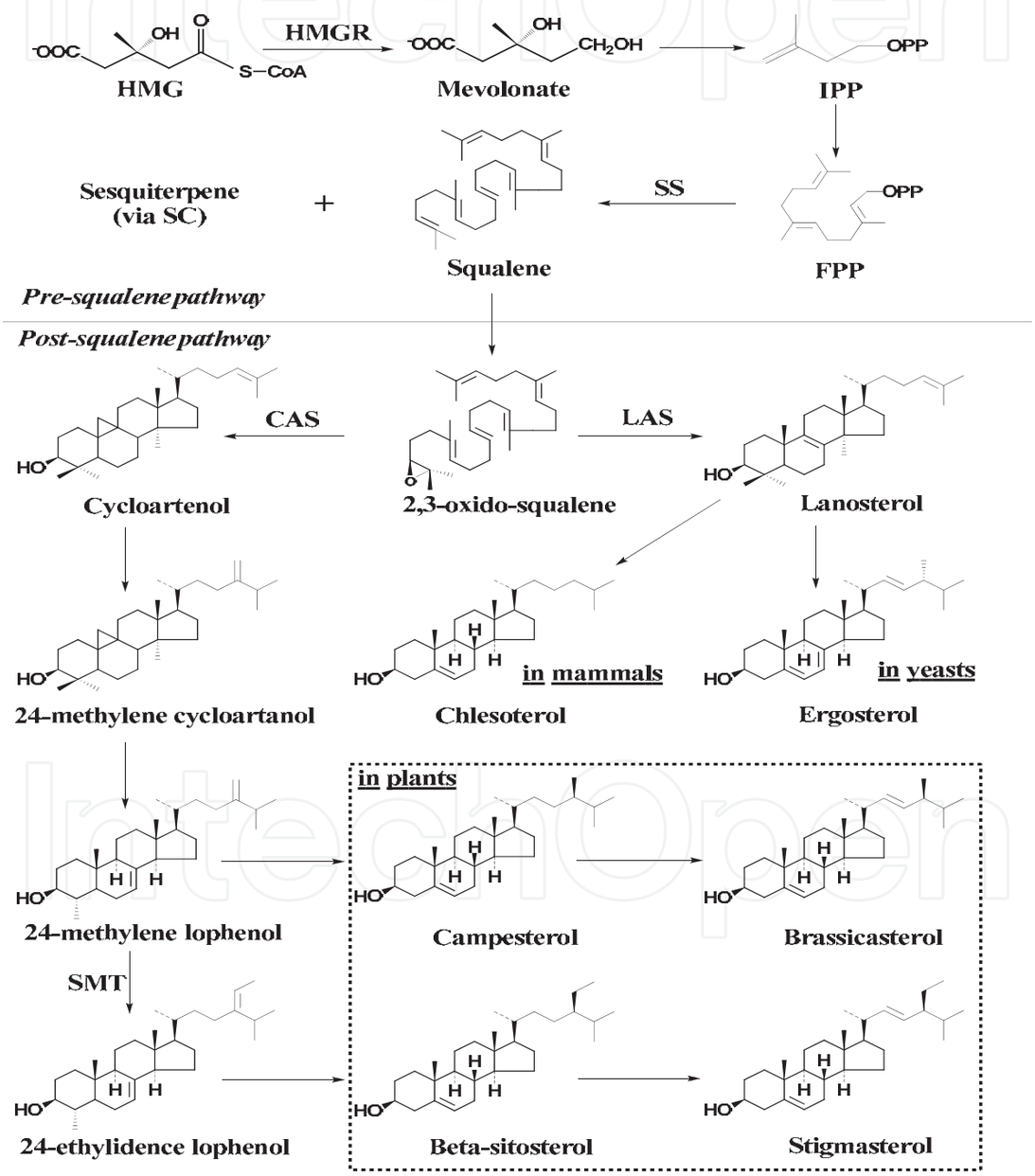

Fig. 3. Sterol synthesis in plants, yeasts and mammals.

3. Conversion of soybean phytosterols to steroid hormones

3.1 Steroid hormones

Steroids are a class of endocrine hormones secreted by the sexual organs and the adrenal cortex in animals. Sterols are closely related to the regulation of physiological effects and the development of reproductive structures, bone, and brain. Many synthetic steroid hormones have been developed and used as pharmaceuticals to cure diseases resulting from the secretion defects of endocrine steroid hormones. Steroid hormones can be grouped into three categories according to their physiological functions: corticosteroids, sex steroids, and anabolic steroids. Corticosteroids can be further divided into two kinds: glucocorticoids and mineralocorticoids. Glucocorticoids play a critical role in regulating many aspects of metabolism and immune function. They also exhibit favorable anti-inflammatory potencies. Mineralocorticoids help maintain blood volume and control renal excretion of electrolytes (Craigie, *et al.*, 2009). Common corticosteroids include C21-steroids, such as hydrocortisone and prednisone. Sex steroids are a type of sex hormone. They include androgens, estrogens, and progestagens, which affect the sex differences and support reproduction (Callewaert, *et al.*, 2010). Anabolic steroids are similar to androgens, interacting with androgen receptors to increase muscle and bone synthesis (Kicman, 2008). Both sex and anabolic steroids have C19 structures. Aside from their endocrine roles, some steroids also perform antiviral, anticancer, cholesterol management, cardiovascular, and neuroprotective functions (Wang, *et al.*, 2007; Garcia-Segura & Balthazart, 2009).

Usually, steroid hormones are synthesized by semi-synthesis using natural steroids as starting materials. Sapogenins and phytosterols are two basic kinds of starting materials commonly used for the production of steroids. These form two main technical routes in the pharmaceutical industry (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). One is the chemical transformation of sapogenins such as diosgenin, hecogenin and solasodine to C21 steroids such as 16-dexosy-prognenolone. The other is the microbial transformation of phytosterols to C19 steroids or C22 steroids (Fig. 2). Compared to the chemical transformation of sapogenins, the microbial transformation of phytosterols has the advantage of a simple and environmental friendly process, a stable supply, and low costs.

3.2 Microbial transformation of phytosterols to valuable steroids

Early in the 1980s, the putative metabolic pathway for synthesizing sterols in microorganisms had already been proposed according to the identification of metabolic intermediates (Fig. 4). So far, the cleavage process of sterol nucleus has been extensively studied, and many of the steps in its multi-step reactions have been identified. However side chain cleavage has not been well studied until now. In 2007, van der Geize disclosed the gene clusters involved in sterol catabolism from *Rhodococcus* sp. RHA1 and *Mycobacterium* species, signifying a new starting point for the biotransformation of phytosterols (van der Geize, *et al.*, 2007).

As shown in Figure 4, the biotransformation of phytosterols is initiated by the oxidation of 3-hydroxyl-5-ene moiety of sterols to 4-ene-3-one moiety. This is carried out by cholesterol oxidases (Cho) (Aparicio & Martin, 2008; Doukyu, 2009). Then the transformation process bifurcates into two independent routes: steroid nucleus decomposition and side chain cleavage (Szentirmai, 1990).

3-ketosteroid-9α-hydroxylase (Ksh) and 3-ketosteroid-1-dehydrogenase (KstD) are the enzymes responsible for the decomposition of the steroid nucleus, which acts independently

and successively on rings A and B to form 1,4-dien-9α-hydroxy-steroids, structurally unstable chemicals that spontaneously evoke the opening of ring B (van der Geize, *et al.*, 2001, 2002a). Maintaining an intact steroid nucleus is key to producing valuable steroids from the catabolism of phytosterols. Kshs and KstDs have attracted much attention for their special roles in initiating cleavage of the steroid nucleus. One enzyme must be inactivated to block the decomposition of the steroid nucleus for the development of steroid-producing strains (van der Geize, *et al.*, 2000, 2002a; Petrusma, *et al.*, 2009). As shown in Figures 2 and 4, the inactivation of Kshs, KstDs, or both can result in the production of C19 steroids such as AD, ADD, and 9α-OHAD. In theory, the production of AD can be achieved via the inactivation of both Kshs and KstDs in a microbial cell, and the accumulation of ADD or 9α-OHAD can be achieved by disrupting the Ksh or KstD genes. Traditional mutagenesis-screening techniques have long been employed as a practical means of producing C19-steroid-producing strains (Donova, *et al.*, 2005c; Huang, *et al.*, 2006; Gulla, *et al.*, 2010). Nevertheless, C19 steroids such as AD, ADD, and testosterone are known to coexist in products (Donova, *et al.*, 2005c; Huang, *et al.*, 2006). This is one of the more significant deficiencies between the strains selected from random mutations; the subtle differences in structure between C19 steroids make them difficult to separate in industrial applications. This seriously increases production costs and limits the application of these mutant strains. In addition, mutant strains have other deficiencies, including residual decomposition of the steroid nucleus and reduced transformation efficiency, such as we observed in our study. These deficiencies can be attributed to the complex metabolic processes of the sterols and multi-isoenzymes of KstD and Ksh within microorganisms. These make it difficult to target and block all of the KstD and Ksh genes without interfering with other important genes. Genetic engineering can be considered a viable technique toward facilitating the development of ideal steroid-producing strains. However, genetic engineering was limited by the genetically undefined mechanisms of sterol catabolism until 2000 and 2002, when van der Geize *et al*. first revealed the KstD and Ksh genes responsible for the catabolism of sterols in *Rhodococcus erythropolis* SQ1 (van der Geize, *et al.*, 2000, 2002a). This team went on to describe the isoezymes of KstD and Ksh (van der Geize, *et al.*, 2002b, 2008). Notably, in 2007, they revealed the existence of a gene cluster encoding sterol catabolism in *Rhodococcus* and *Mycobacterium* species (van der Geize, *et al.*, 2007). The research of van der Geize and co-workers facilitated the development of ideal sterol-transformation strains for the synthesis of valuable C19-steroids. Subsequently, targeted disruption or augmentation of KstDs and Kshs was successfully used in the development of C19 steroid-producing strains (van der Geize, *et al.*, 2000; Wei, *et al.*, 2010).

Cleavage of the sterol C17-side chain has been well depicted as similar to the fatty acid β-oxidation and the genes likely to be responsible for this process have been shown to be part of the gene cluster responsible for sterol catabolism (Szentirmai, 1990; Van der Geize, *et al.*, 2007). However, the genetic definitions of these processes have yet to be outlined. Using mutagenesis-screening techniques, the C17-side chain cleavage can be blocked to produce products with truncated side chains, such as C22-steroids, which are valuable candidate intermediates for the synthesis of steroid hormones (Fig. 2) (Szentirmai, 1990). C22-steroid-producing strains are rarer than C19 strains, partly because of the complex enzyme systems likely to be involved in side chain cleavage.

In the terms of practical applications, both C19-steroids and C22-steroids can be readily used to produce higher value-added steroid hormones. The C19-steroids can be grouped by pharmacological activity into anabolic and androgen steroids. These steroids are already

used as the main precursors for all kinds of sex and anabolic hormones, largely because their structures are already similar to those of the desired final products (Malaviya & Gomes, 2008). The C19-steroids can also be used as precursors for the synthesis of C21 corticosteroids (Andrews, *et al.*, 1996). C19-steroids are highly important precursors of steroid hormones and can be used to synthesize almost all of the steroid hormones used in clinical settings. C22-steroids show their advantage in the synthesis of C21 corticosteroids, which can be achieved by simple oxidative decarboxylation (Toröa & Ambrus, 1990).

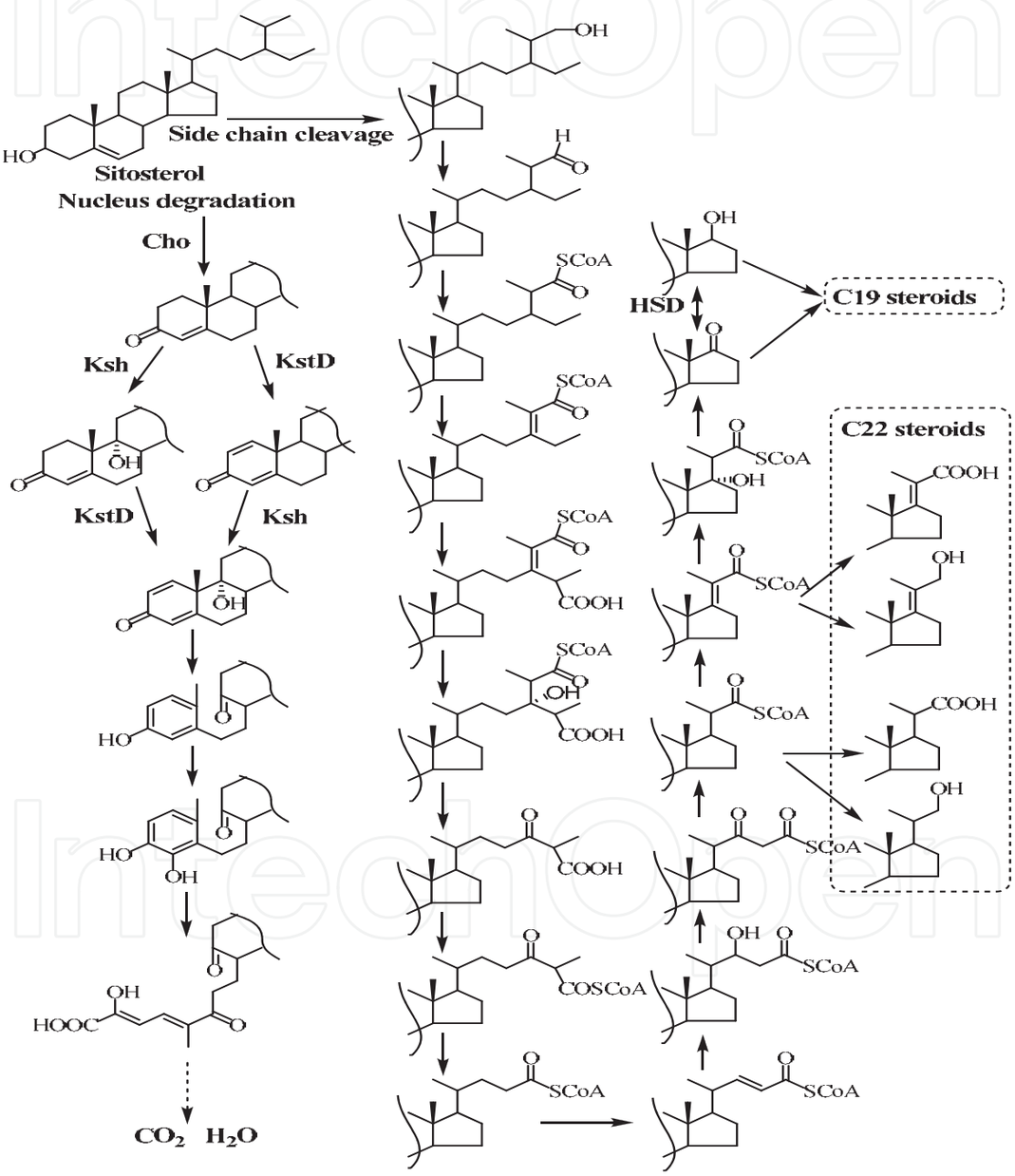

Fig. 4. Catabolic pathway of phytosterols (with β-sitosterol as substrate). (HSD represents the enzyme 17β-hydroxy steroid dehydrogenase.) (Kieslich, 1985; Szentirmai, 1990; van der Geize, *et al.*, 2007)

4. Inherent problems and solutions in strain improvement

The microbial metabolism of sterols has already been shown to be a promising means of preparing valuable steroids. During the second half of the twentieth century, many microbial strains were developed and tailored to synthesize many kinds of C19 and C22 steroids using phytosterols as substrates (Szentirmai, 1990). So far, however, the production of steroids from phytosterols by microbial transformation is still not widely used in the pharmaceutical industry. This may be because of two inherent problems in the phytosterol biotransformation process: (1) The low water solubility of phytosterols can lead to poor bioavailability. (2) The final products are toxic to microbial cells. Many technological strategies have been proposed to overcome these problems. These have been reviewed in detail by Malaviya and Fernandes (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). Here, we mainly focus on how to develop robust organisms that can withstand their own products.

4.1 Low water solubility and adaptive mechanisms

Most steroids have solubility below 0.1 mM. Phytosterols are more hydrophobic, their water solubility usually being no more than 1 μM. The poor solubility of phytosterols seriously retards their bioavailability because of inadequate mass transfer (Goetschel & Bar, 1992; Phase & Patil, 1994; Malaviya & Gomes, 2008). Many efforts have been carried out to improve the dispersity and dissolubility of phytosterols in reaction media. Among these, surfactant-facilitated emulsification, favored for its practicality and effectiveness, is a common means of enhancing the bioavailability of phytosterols (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). There are other strategies, but many of these seem inadequate in practice. For example, organic solvent facilitated dissolution significantly raises the complexity of the process and production costs and also results in environmental pressures. It is also not very productive.

The mechanism of sterol uptake by microbial cells has already been demonstrated: during the transformation process, microbial cells adhere to the surfaces of sterol particles, forming stable agglomerates. Sterol uptake takes place via the direct contact between the cells and the sterol particles (Atrat, *et al.*, 1991; Goetschel & Bar, 1992; Fernandes, *et al.*, 2003). This is an adaptive mechanism that allows microorganisms to use minimally water-soluble hydrocarbons as carbon sources. Like phytosterols, many polycyclic aromatic hydrocarbons (PAH), such as naphthalene and anthracene, also exhibit extremely low water solubility and are also poorly bioavailable (Johnsen & Karlson, 2004; Harms, *et al.*, 2010a, 2010b). Many microorganisms have been shown to use these hydrophobic hydrocarbons as carbon sources through similar evolutionary adaptations (Miyata, *et al.*, 2004; Heipieper, *et al.*, 2010; Parales & Ditty, 2010; Harms, *et al.*, 2010a). Generally, the microorganisms adapted to use hydrophobic hydrocarbons have the following physiological properties: (1) lipophilic cell walls and adaptive changes in the surface properties allowing direct adhesion to the hydrophobic substrates, (2) high affinity uptake systems such as active transporters and membrane-associated enzymes for initial degradation, (3) the ability to excrete biosurfactants or bioemulsifiers in order to increase the bioavailability of hydrophobic hydrocarbons (Wick, *et al.*, 2002; Heipieper, *et al.*, 2010; Miyata, *et al.*, 2004; Parales & Ditty, 2010; Johnsen & Karlson, 2004; Perfumo, *et al.*, 2010). Several studies regarding adaptive responses to PAH have been carried out with Gram-positive bacteria belonging to the mycolate-containing bacteria families, including *Mycobacterium*, *Rhodococcus*,

Corynebacterium, and *Nocardia*. Interestingly, mycolate-containing actinobacteria are also used in the transformation of phytosterols (Heipieper, *et al.*, 2010; Kim, *et al.*, 2010). Although these adaptive properties have not been well studied, some studies have shown that the similar adaptive responses play key roles in the transformation process of phytosterols (Atrat, *et al.*, 1991; Rajkhowa, *et al.*, 2000; Fernandes, *et al.*, 2003).

4.1.1 Cell envelope and adaptive changes affecting bioavailability

The mycolate-containing actinobacteria are well known as hydrophobic organisms. They have complex and extremely lipophilic cell envelopes containing large amounts of long-chained mycolic acids specific to this group of bacteria. Mycolic acids may reach up to 60% of the dry mass of the cell walls of some actinobacteria and typically range in size from 30–90 carbon atoms. These are often modified in structure by alkylation and hydroxylation and cross-linked to polysaccharide components, forming an exceptionally thick and rigid envelope. This envelope is of particular interest with respect to adaptations allowing the microorganism to degrade a broad range of hydrophobic solid compounds (Korycka-Machala, *et al.*, 2001; Heipieper, *et al.*, 2010; Kim, *et al.*, 2010). Many researches have also found that bacterial hydrophobic envelopes play a critical role in enhancing the uptake capacity of phytosterols (Atrat, *et al.*, 1991; Rajkhowa, *et al.*, 2000; Fernandes, *et al.*, 2003). Mycolate-containing bacteria are able to change their physiochemical surface properties by modifying the composition of their envelopes (Wick, *et al.*, 2002; Falkinham, 2009; Heipieper, *et al.*, 2010). In the case of mycobacteria, anthracene-grown cells respond to the low bioavailability of their hydrophobic substrate by modifying their cell envelopes via the synthesis of mycolic acids with longer alkyl chains. This significantly increases the hydrophobicity of the outer membrane, leading to up to 70-fold stronger adhesion to hydrophobic surfaces than glucose-grown cells (Wick, *et al.*, 2002). Although the adaptive changes of the cell envelope relevant to phytosterol-transforming activity are not well understood, cell wall lipid content has been proved to more than double in the presence of sterols. High lipid content and long-chain fatty acids in the envelope of sterol-grown cells is believed to contribute to the high adherence activity of these cells to sterols (Rajkhowa, *et al.*, 2000). It is conceivable that enhancing the hydrophobicity of the cell envelope by strain development based on natural adaptive mechanisms may be an effective means of overcoming the problem of low bioavailability.

It has been noted that the superficial envelope of mycolate-containing actinobacteria does not always seem to be advantageous with regard to phytosterol bioavailability. Some researchers have shown that the cell envelope might be a barrier to permeability, which is disadvantageous for phytosterol uptake and biotransformation. This can be improved by compounds such as vancomycin, glycine, lecithin, protamine, polymixin B nonapeptide, bacitracin, and ethambutol (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). Vancomycin, glycine, and bacitracin are effective reagents, affecting the peptidoglycan layer of the cell membrane. Ethambutol is can disrupt the biosynthesis of arabinogalactan in the cell envelope. Lecithin and protamine have been shown to alter the fatty acid profile of the cell envelope. Structurally speaking, in the envelope of mycolate-containing actinobacteria, mycolic acids attach to the 5'-hydroxyl groups of D-arabinose residues of arabinogalactan and form a mycolyl-arabinogalactan-peptidoglycan complex, becoming barriers to permeability for exogenous chemicals (Draper, 1998). The chemicals listed above have been found to affect the outer part (mycolic acid layer) of the cell envelope. This may motivate

research into enhancing the bioavailability and conversion productivity of phytosterols through strain improvement focusing on a structurally changed envelope.

4.1.2 Affinity uptake of sterols

In 1991, Atrat *et al*. described a model of flexible multi-component mesophase (FMCM) on the cellular uptake of sterol substrates via direct contact between cells and the substrate particles based on observation by freeze-fracture electron microscopy. In this model, they assumed that a FMCM exists between cells and sterol particles and that this FMCM mediates sterol uptake, forming a sharp concentration gradient to drive transport. At the same time, they considered that there should be channels composed of sterol-binding or steroid-transforming proteins connecting the cytoplasm with the cell surface to reinforce the transport of sterols across the thick cell walls (Atrat, *et al.*, 1991).

In 2007 and 2008, van der Geize, Mohn, and their colleagues disclosed and substantiated the idea that there is a steroid uptake system in the gene cluster encoding sterol catabolism (van der Geize, *et al.*, 2007; Mohn, *et al.*, 2008). The steroid uptake system is a kind of mce4 ("mammalian cell entry") locus encoded by an operon including 10 genes conserved in diverse mycolate-containing actinobacteria. This locus is composed of two transmembrane permease (supAB) genes and eight mce4 protein (mce4ABCDEFHI) genes (Mohn, *et al.*, 2008). The 10 genes have been shown to encode components of complex ATP-binding cassette ABC transporters. The deletion and inhibition of these genes has been shown to result in an absence of sterol uptake. Additionally, transcriptional analyses have indicated that the operon was up-regulated 4.0-fold during growth on cholesterol, relative to growth on pyruvate. This strongly supports the idea that these 10 genes are specifically involved in sterol uptake. Ultimately, Mohn speculated that the proteins encoded by the mce4 loci might form a complex of ABC uptake transporters in the membrane of mycolate-containing bacteria to mediate the movement of the sterol substrates across the thick, hydrophobic cell envelope (Mohn, *et al.*, 2008). This research on the biological function of the mce4 system provides us with new insight into the mechanism of active transport of phytosterols in the microbial transformation process.

Atrat also speculated that steroid-transforming proteins, especially extracellular enzymes, such as cholesterol oxidase (3β-hydoxysteroid dehydrogenase), might make up part of the FMCM structure and stimulate sterols transport (Atrat, *et al.*, 1991). Cholesterol oxidases are extracellular flavoenyzmes that catalyze the oxidation and isomerization of cholesterol to cholest-4-en-3-one (cholestenone or ketocholesterol), which is in charge of the first compulsory step in bacterial sterol catabolic pathways that transform sterols into sterones (Kreit & Sampson, 2009). These enzymes often occur in secreted and cell-surface-associated forms. This is closely related to their role in oxidizing exogenous sterols. Cholesterol oxidases are in a class of interfacial enzymes that catalyze reactions with membrane-soluble substrates. An enzyme is catalytically active at the contact membrane interface of cholesterol or other sterols (Sampson & Kwak, 2008). In a recent study, we identified two cholesterol oxidases, ChoM1 and ChoM2, from a gene cluster encoding sterol degradation in a *Mycobacterium* strain. ChoM1, a class I cholesterol oxidase, does not occur as a secreted form, while ChoM2, a class II cholesterol oxidase, can be secreted into the medium. The roles of these two ChoMs in the utilization of phytosterols have been demonstrated by genetic inactivation, which showed that both ChoM1 and ChoM2 are necessary for the *Mycobacterium* strain to use phytosterols as carbon sources. Notably, the deletion of ChoM2 led to the *Mycobacterium* strain failing to survive on a medium with phytosterols as the sole

carbon source. Both enzymes could enhance transformation efficiency by augmenting their activity in an ADD-producing strain. We also found *Mycobacterium* strains to make use of sterones to a greater degree than sterols. Cholesterol and phytosterols are indispensable to cellular membranes in animals and plants, respectively. They can exist in the plasma and mimic membranes. They function in altering the properties of lipids in membranes to maintain membrane integrity and fluidity. However, many studies have indicated that cholestenone dose not have any effect to change the properties of membrane lipids and is not stable in the membrane (Bacia, *et al.*, 2005; Beattie, *et al.*, 2005; Lintker, *et al.*, 2009). The differences in intra-membrane behavior between sterols and sterones and the physiological functions of cholesterol oxidases imply that cholesterol oxidases may be part of the sterol transport process. Although further investigation is still needed to confirm their specific roles on cellular sterol uptake, there is little doubt that the augmentation of cholesterol oxidases will improve phytosterol-transforming strains.

4.1.3 Excretion of biosurfactants and bioemulsifiers and bioaccessibility and bioavailability of phytosterols

The bioaccessibility of hydrophobic hydrocarbons is considered an important part of their bioavailability. It depends on the contact surface area between microbial cells and substrates (Harms, *et al.*, 2010b). The smaller the particle size, the greater this area will be. Experimental observation has indicated that the most favorable arrangement to sterol absorption is substrate particles that are very similar in size to the intended microbial cells (Atrat, *et al.*, 1991). Although the size of the phytosterol particles can be affected by mechanical attrition, the hydrophobic phytosterols tend to self-aggregate in water to form agglomerates. This seriously limits their bioaccessibility and bioavailability. In most cases, surfactant-facilitated emulsification is used to inhibit the aggregation of phytosterols and keep the phytosterol particles homodisperse in the reaction medium. However, synthetic surfactants and emulsifiers are generally toxic to bacteria. They can solubilize cell membranes and induce enzymatic disorders, leading to necrosis and cell lysis (Li & Chen, 2009). In recent years, biosurfactants and bioemulsifiers have emerged as alternatives to synthetic surfactants and emulsifiers. They have attracted attention for their biocompatibility with cells. In natural environments, many bacteria secrete biosurfactants or bioemulsifiers to render minimally water-soluble carbon sources accessible (Li & Chen, 2009, Perfumo, *et al.*, 2010). Mycolate-containing actinobacteria such as *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocarida* have a catabolic capacity for phytosterols and have been shown to have the ability to produce biosurfactants (Perfumo, *et al.*, 2010). From this, it can be concluded that the selection and development of organisms with a robust ability to produce biosurfactants or bioemulsifiers may be an alternative to synthetic surfactants.

4.2 Toxic effects of steroid products on microorganisms

The cellular toxicity of steroids to microbial cells is one of the major factors limiting the productivity of conversion from phytosterols to steroid products. Some steroid products, such as AD and ADD, even at low concentrations, are believed to inhibit cell growth and respiration and suppress the enzyme activity in the sterol degradation pathway, causing low product yield (Zeillinger & Spona, 1986; Perez, *et al.*, 2003; Donova, 2007). Many attempts to overcome this problem are being investigated by segregating steroid products

from the reaction media through *in situ* product recovery and by developing mutants with improved tolerances to the steroid products (Perez*, et al.*, 2003; Malaviya & Gomes, 2008).

4.2.1 *In situ* product recovery

In situ product recovery is a common and effective means of reducing the negative effects of toxic products on microbial cells. Some special adsorbents, such as amberlite XAD-7 resin for AD and ADD, are often added to the reaction media to increase the yield of AD and ADD by absorbing steroid products (Huang*, et al.*, 2006; Malaviya & Gomes, 2008). Positive results have also been obtained by using polymers as reservoirs, reducing the concentration of steroid products in the reaction medium. For example, poly dimethyl siloxane and cyclodextrins have been used to recover ADD and AD from the reaction medium. This can improve transformation productivity. In addition, aqueous or organic-aqueous two-phase systems are also clean and effective tools for *in situ* product recovery. In two-phase systems, the water-immiscible organic phase acts as reservoir for toxic products. The significantly higher solubility of sterols in the organic phase allows the system to instantaneously recover toxic steroid products during biotransformation. For a more detailed discussion of *in situ* steroid recovery, we refer to the review by Malaviya and Gomes (2008).

4.2.2 Development of mutants with increased resistance to steroid products

The developing mutants with improved tolerance to toxic products may be a more effective means of solving the problem of toxic products than product sequestration. Perez *et al*. demonstrated that high concentrations of androstanes could be used to isolate tolerant mutants and increase androstane yield. In that case, colonies growing at least 1 mg/ml ADD in culture medium after nitrosoguanidine mutagenesis showed improved ADD yield, going from 18.81 mg/ml to 38.99 mg/ml (Perez*, et al.*, 2003). So far, the actual nature of the toxicity of steroids to microbial cells has not been elucidated. Similarly, the mechanisms by which microbial cells become resistant remain unknown. Even so, robust tolerance to toxic steroid products is one of the most important characteristics of improved phytosterol-transforming strains.

4.2.3 Eflux transporters for steroid products

In natural environments, tolerance to toxins is important for survival. In the case of phytosterol-transforming mycobacteria, there are two common mechanisms involved in resistance to toxicity: the rigid envelope acts as a barrier to extracellular toxins and the active multidrug efflux pumps remove intracellular toxins (Nikaido, 1994; De Rossi*, et al.*, 2006). In the phytosterol transformation process, most of the reactions occur intracellularly. Therefore, it is reasonable to conclude that multidrug efflux pumps play a role in resistance to toxic steroid products. Genes encoding drug efflux transporters have been identified in several mycobacterial species, including *M. fortuitum*, *M. tuberculosis*, and *M. smegmatis* (Ainsa, *et al*., 1998; Li, *et al*., 2004). These genes, which exist in the form of multi-copy plasmids or replicates in the genome, encode efflux proteins that transport tetracycline, fluoroquinolones, and other compounds outwards. Because steroid compounds resemble tetracycline and fluoroquinolones, it is reasonable that some non-specific efflux transporters may transport steroids outside the cell. These efflux transporters should be investigated for applicability to the development of phytosterol-transforming organisms resistant to toxic products.

4.3 Development of ideal organisms

Strain performance plays a central role in the commercial development of microbial conversion systems. In essence, the abovementioned problems can be attributed to deficient microbial strains. The fundamental solution is to develop robust, efficient, highly productive microbial strains. Random mutagenesis is the conventional breeding approach and has been the main means of developing custom phytosterol-transforming strains (Egorova, *et al.*, 2002; Donova, *et al.*, 2005a; Sripalakit, *et al.*, 2006). However, strains surviving multiple rounds of mutation are genetically undefined and vulnerable to further changes. Directed genetic modification may be a more feasible option for subsequent isolation of mutant strains with desired functions. Rational metabolic engineering provides a more effective and well-defined alternative to strain development than random mutagenesis. Strategies for developing the desired organisms by metabolic engineering can be summarized as follows: (1) inhibition of B-ring cleavage to maintain the steroidal nucleus by inactivating the key enzymes KstDs and Kshs; (2) overexpression of genes encoding rate-limiting reactions and blockage of downstream and branched reactions to increase the accumulation of desired product and reduce the production of unwanted by-products; (3) enhancement of cellular uptake of phytosterols by increasing the activity of active transporters and/or the permeability of the cell wall; (4) enhancement of cellular tolerance to toxic products and efflux capacity of toxic products.

Ideal microorganisms should have the following characteristics:

(1) Lack of pathogenicity, ease of culture

Many organisms that can transform phytosterols into valuable steroids have been isolated. These include mycobacteria, which seem perform the best. This is why most of the microbial strains used in industrial and academic research are members of the genus *Mycobacterium* (Atrat, *et al.*, 1991; Fernandes, *et al.*, 2003; Perez, *et al.*, 2006). However, mycobacteria have two serious deficiencies. First, many *Mycobacterium* species are known to be stubborn pathogens. These include *M. tuberculosis* and *M. leprae*. Many other *Mycobacterium* species, although generally non-pathogenic, have been shown to be opportunistic pathogens (Malaviya & Gomes, 2008; Cassidy, *et al.*, 2009; van Ingen, *et al.*, 2009). For example *M. fortuitum* can cause local cutaneous disease, osteomyelitis, and joint infections (Wallace, *et al.*, 1983). The other problem is that mycobacteria are difficult to culture because of their complicated physiological states, rod-coccus growth cycle, slow growth rate, and special nutritional requirements (Falkinham, 2009; Kim, *et al.*, 2010). In addition, the process by which mycobacteria convert phytosterols to steroid hormones is complicated and can last more than a week. This reduces its economic benefit. For these reasons, it may be best to select safer and more rapidly growing organisms, such as those in the genera *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Brevibcterium*, *Rhodoccocus*, *Norcardia*, and others.

(2) Ease of modification

Unfortunately, genetic manipulation techniques suitable to most of the organisms with proven capacity for phytosterols conversion lags behind those that work on other bacterial species. This makes it difficult to improve these organisms by means of metabolic engineering. For example, it is difficult to perform genetic recombination on mycobacteria because of the high rate of illegitimate recombination and the lack of universal tools among *Mycobacterium* species (Kim, *et al.*, 2010). This has hindered the progress of strain improvement. In addition, phytosterol metabolism in microbes is a complex process. To

build an ideal strain, many metabolic reactions and physiological properties will need to be modified and regulated. An ideal phytosterol-transforming organism, therefore, must be easy to be modified, preferably by relatively simple genetic recombination techniques.

(3) Single-product production

Most phytosterol-transforming strains can transform phytosterols into many structurally similar products, such as the C19 steroids AD, ADD, and their derivatives, at the same time (Egorova, *et al.*, 2002; Donova, *et al.*, 2005a, 2005b, 2005c; Molchanova, *et al.*, 2007). Because the differences in the structures of these products are very subtle, it is difficult to separate and purify them. This significantly complicates the production process and increases production costs (Molchanova, *et al.*, 2007). An ideal microbial strain, therefore, must be able to transform phytosterols into only a single desired product.

(4) Powerful phytosterol uptake capacity

The extreme hydrophobicity of phytosterols seriously limits their bioavailability. As mentioned above, most of the strategies that enhance phytosterol uptake increase the complexity of the production process and add to its costs. However, many microorganisms already have numerous physiological adaptations that allow them to take up highly hydrophobic compounds, such as phytosterols. These adaptations can be further improved to enhance the organisms' phytosterol uptake capacity. The following strategies should be attempted: i) Increase the direct contact between microbial cells and phytosterol particles by changing the physiochemical properties of the cell envelope. ii) Enhance the active transport capacity for phytosterols by augmenting the activity of mce4 transporters. iii) Overexpress cholesterol oxidases. iv) Increase the bioaccessibility of phytosterols by reinforceing the excretion of biosurfactants and/or bioemulsifiers.

(5) Strong tolerance to toxic products

Although several methods of sequestering toxic steroids from the reaction media have been tested, the development of mutants with improved tolerance to steroid products may be a more effective way to bypass the toxic effects of steroid products on microbial cells (Molchanova, *et al.*, 2007; Perez, *et al.*, 2003; Malaviya & Gomes, 2008). An ideal organism should have physiological functions that reduce or eliminate the harmful effects of toxic products. Such organisms may be developed using strategies that allow the selection of evolutionary mutants by continuous, prolonged exposure to high concentrations of toxic products and augment efflux transporters.

5. The future of phytosterol production

Phytosterol catabolism in microbes is extremely intricate. It involves not only dozens of specific biocatalytic reactions but also cellular properties catering to phytosterol metabolism, such as phytosterol uptake systems and resistance to toxic products. Currently, only the genes directly encoding sterol catabolism were found as a cluster of 51 genes in *Rhodococci* and 80 to 82 genes in mycobacteria (van der Geize, *et al.*, 2007). Most of which have not been well characterized in function until now. The most urgent task is to clearly characterize and elucidate the mechanisms of phytosterol catabolism and the cellular properties related to phytosterol uptake, tolerance to toxic products, and global regulations.

Along with gradual in-depth research on the mechanisms by which phytosterols are transformed into steroid hormones, metabolic engineering may be useful to the

development ideal microbial "factories" for the production of desired steroid hormones from phytosterols. To meet the basic standards depicted in section 4.3, strain improvement by metabolic engineering should include three steps:

The first step is to rationally modify the catabolic phytosterol pathway toward formation of the target product, avoiding cleavage of the steroid nucleus and formation of any byproducts. The cleavage of the steroid nucleus can be prevented by disrupting the key enzymes in steroid B-ring cleavage, KstDs and Kshs (Fig. 2). The accumulation of byproducts can be eliminated by blocking side reactions and amplifying rate-limiting reactions. To produce ADD, for example, Kshs should be blocked and KstDs should be activated (Fig. 5). Several similar projects have already been successful in *Rhodococcus* species and *Mycobacterium* (van der Geize*, et al.*, 2007; Wei*, et al.*, 2010).

The second step should focus on economically feasible productivity. The strains developed in the first stage may be limited in efficiency. To increase their economic benefit, they should be reconstructed to avoid limitations in productivity. The phytosterol uptake capacity of the organisms should be enhanced by augmenting sterol transporters such as Mce proteins, and reducing the barrier effect of the cell envelope (Mohn*, et al.*, 2008; Korycka-Machala*, et al.*, 2005; Hoffmann*, et al.*, 2008). The tolerance of the microbial cells to toxic products should be improved. This may be achieved by enhancing the cells' ability to expel active products by augmenting or rationally designing specific efflux pumps or by reducing the sensibility of the strain to the toxic products. Negative regulators and feedback inhibitors limiting the phytosterol metabolism may also be of use and should be examined and deregulated. For example two TetR-type transcriptional regulators control sterol utilization in mycobacteria (Kendall*, et al.*, 2010).

The third step is to comprehensively reprogram phytosterol-transforming organisms based on systems biology. In brief, the metabolic flux from phytosterols to steroid products should be comprehensively investigated, modified, and optimized based on industrial requirements. Approaches to this include transcriptomics, proteomics, metabolomics, and computational modeling.

Microorganisms with good phytosterol conversion capacities may be not good candidates for modification by metabolic engineering. This is partly because of the complexity of physiological process of reprogramming the cell. Recently, metabolic engineering has become capable of creating novel, finely controlled metabolic and regulatory circuits that can produce desired products effectively. Reconstructing the transformation pathway from phytosterols to steroid homones in a heterologous host organism with suitable physiological trains, then, may an alternative to developing engineered organisms. In 2003, Szczebara and co-workers described a process that rerouted ergosterol biosynthesis in *Saccharomyces cerevisiae* to compatible brassicasterol and campesterol, two ingredients in the four soy phytosterols (Fig. 1) and then extended the pathway to produce hydrocortisone by mimicking hydrocortisone biosynthesis seen in the mammalian adrenocortex. In other words, an artificial, fully self-sufficient biosynthetic pathway was successfully built in a microbial host, suggesting that the production of steroid hormones may also take place in this simple, environmentally friendly, low-cost manner (Szczebara*, et al.*, 2003). This kind of revolutionary approach to the production of steroid hormones may be the future of the steroid pharmaceutical industry.

The integrated reconstruction of the phytosterol transformation system in microbes may become a reality in the near future, most likely in one of two ways.

The first way would be to rebuild the phytosterol transformation system in robust hosts that would be superior to existing phytosterol-transforming organisms in tolerance to high

concentrations of steroid hormones, phytosterol uptake capacity, simplicity of gene manipulation, and cell cultivation. This would need to be founded on a comprehensive understanding of the processes by which phytosterols are converted to products, including the degradation of C17-side chains. In order to facilitate the assembly and control of this system, the process of design and reconstruction should based on synthetic biology, standardizing the gene parts required, designing controllable and efficient gene circuits, developing functional modules, and integrating and optimizing the rebuilt system in the selected host. Therefore, a novel engineered organism with ideal phytosterol-transforming properties may be developed (Fig. 5A).

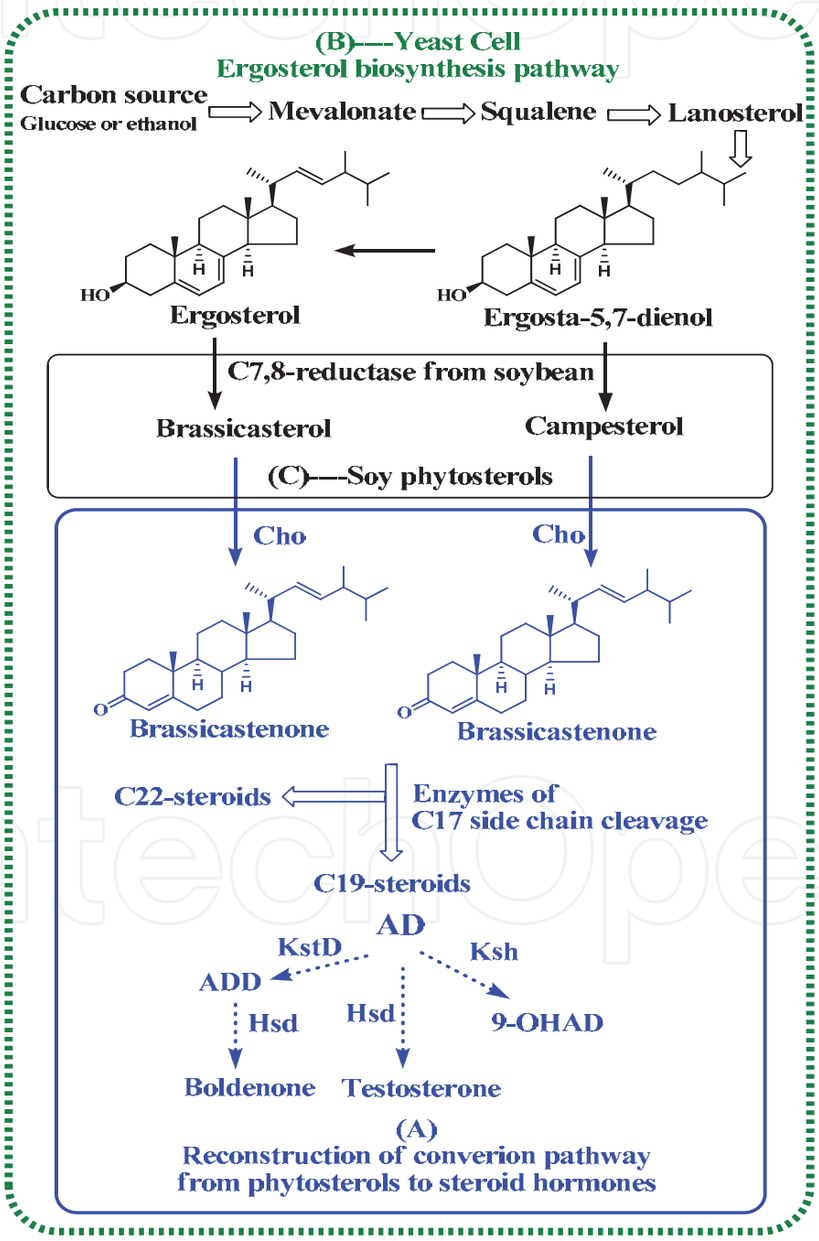

Fig. 5. Transformation pathway of phytosterols in *S. cerevisiae*.

The second way would be to learn from the works of Szczebara and Dumas and reconstitute the phytosterol conversion system in yeast (Szczebara, *et al*., 2003; Dumas, *et al*., 2006). Yeast is a proven producer of endogenous sterols, such as ergosterol and ergosta-5,7-dienol. Ergosterol and ergosta-5,7-dienol are similar to the precursors of phytosterols, and they can be converted to brassicasterol and campesterol by C7-reductases from plants (Hartmann, 2003; Szczebara, *et al*., 2003). Brassicasterol and campesterol are two components of the four main phytosterols found in soybeans (Fig. 1). Therefore, the endogenous sterol biosynthesis seen in yeast may be rerouted to produce soybean phytosterols by the introduction of C7-reductase from a plant, such as the soybean (Fig. 4C). In this way, the conversion pathway of soybean phytosterols to C19 steroids or C22 steroids can be reassembled (Fig. 4A) and then linked to the natural ergosterol biosynthetic pathway in yeast. This would result in steroid hormones in self-sufficient in yeast fed on a simple carbon source, such as glucose or ethanol (Fig. 4). The benefits of this method are that the many reactions involved in producing C19 or C22-steroids from phytosterols would be integrated in a single fermentation step, avoiding the need for the production or addition of phytosterols. Such a simple production process would revolutionize the synthesis of steroid hormones in industry and significantly lower production costs and environmental impact. Nevertheless, there are also some drawbacks to this technique. One important issue is the inherent difficulties of the yeast system to adapt to the exogenous biocoversion process, such as the toxicity of the steroids to the yeast organism. Much more additional research must be performed to improve the physiological properties of yeast before it can become an industrially advantageous means of producing steroid products.

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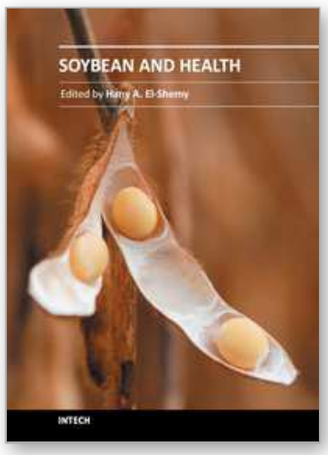

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