PHYTOSTEROLS FROM THE SEEDS OF PETAI (*PARKIA SPECIOSA*)

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ABSTRACT

Steroids are found in plants, animals and fungi. All steroids are made in cells either from the sterols lanosterol (animals and fungi) or from cycloartenol (plants). Both lanosterol and cycloartenol are derived from the cyclization of the triterpene squalene. Triterpenes are terpenes consisting of six isoprene units and have the molecular formula $C_{30}H_{48}$ (e.g. carotenoids). The steroids derived from animal are the cholesterol while the steroids derived from plant is the phytosterols. There are more than 100 types of phytosterols have been reported in plant species, but the more abundant are sitosterol, stigmasterol and campesterol. The importance of phytosterols is their contribution towards lowering body cholesterol. Cholesterol is transported in blood in the form of lipoprotein which includes the high density (HDL) and low density (LDL) lipoproteins where LDL is the bad cholesterol. Too much LDL cholesterol in the blood can cause cholesterol to build up in the artery walls, leading to the narrowing of the arteries, Atherosclerosis. Thus dietary cholesterol intake is reduced in patients with such disease. Another way to reduce blood cholesterol is to consume phytosterols which compete with cholesterol absorption. The objective of this project is to find the new sources of phytosterols from plant found in Malaysia. The plant use in this project is Petai (*Parkia speciosa*). Petai is an edible legume, the seeds of which are already consumed in Malaysia as a minor vegetable. Although many legumes have been studied as sources of phytosterols very little is reported on Petai. The practical study is carry out by using extraction to extract phytosterols from Petai and chromatographic and spectrophotometric method to separate and identify the extracted phytosterols. Petai is a legume. Legumes like soybean are known to be rich in phytosterols.

ABSTRAK

Steroid ditemui pada tumbuhan, haiwan dan kulat. Semua steroid dibuat dalam sel-sel baik dari lanosterol sterol (haiwan dan kulat) atau dari cycloartenol (tumbuhan). Baik lanosterol dan cycloartenol berasal dari siklisasi dari Squalene triterpen. Triterpen adalah terpene terdiri daripada enam unit isoprena dan mempunyai formula molekul $C_{30}H_{48}$ (karotenoid misalnya). Steroid daripada haiwan ialah kolesterol dan daripada tumbuhan ialah pitosterol. Terdapat lebih daripada 100 jenis pitosterol telah dilaporkan dalam spesies tumbuhan, terutamanya sitosterol, stigmasterol dan campesterol. Kepentingan pitosterol adalah sumbangannya dalam menurunkan kolesterol badan. Kolesterol diangkut dalam darah dalam bentuk lipoprotein yang merangkumi kepadatan tinggi (HDL) dan kepadatan rendah (LDL) mana lipoprotein LDL adalah kolesterol jahat. Terlalu banyak kolesterol LDL dalam darah boleh menyebabkan kolesterol terkumpul di dinding arteri, menyebabkan penyempitan saluran darah, Atherosclerosis. Jadi makanan yang mengandungi kolesterol perlu dikurangkan bagi pesakit dengan penyakit tersebut. Cara lain untuk mengurangkan kolesterol dalam darah adalah dengan mengkonsumsi pitosterol yang bersaing dengan penyerapan kolesterol. Tujuan daripada projek ini adalah untuk mencari sumber baru pitosterol dari tumbuhan yang boleh dijumpai di Malaysia. Tumbuhan digunakan dalam projek ini adalah Petai (*Parkia speciosa*). Petai adalah kacang-kacangan yang boleh dimakan, benih-benih yang sudah diambil di Malaysia sebagai sayuran minor. Walaupun banyak kacang-kacangan telah dipelajari sebagai sumber pitosterol tetapi pitosterol daripada Petai jarang dilaporkan. Penyelidikan praktisnya adalah melaksanakan dan menggunakan ekstraksi untuk mengekstrak pitosterol dari Petai dan kaedah kromatografi dan spektrofotometri untuk memisahkan dan mengenal pasti pitosterol yang telah diesktrak. Petai adalah kekacang. Kekacang seperti kacang soya dikenali kaya dengan pitosterol.

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LIST OF SYMBOLS

α	Alpha
β	Beta
^0C	Degree Celsius
R_f	Retention factor
%	Percentage

LIST OF ABBREVIATIONS

HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
AHA	American Heart Association
C	Carbon
TLC	Thin layer chromatography
FS	Free sterols
SE	Sterol esters
SG	Steryl glycosides
ASG	Acylated steryl glycosides
HSE	Hydroxycinnamic-acid esters
UV-Vis	UV-Visible spectrophotometry
GC-MS	Gas chromatography-mass spectroscopy
HCl	Hydrochloric acid
KOH	Potassium hydroxide
mL	Milliliter
μL	Microliter
mg	Miligram
μg	Microgram
g	Gram
nm	Nanometer

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

There are hundreds of distinct steroids found in plants, animals and fungi. All steroids are made in cells either from the sterols lanosterol (animals and fungi) or from cycloartenol (plants). Both lanosterol and cycloartenol are derived from the cyclization of the triterpene squalene. Triterpenes are terpenes consisting of six isoprene units and have the molecular formula $C_{30}H_{48}$. All triterpenes are synthesized via a pathway that starts with reduction of HMG-CoA (six carbons) to mevalonate (six carbons). The steroids derived from animal are the cholesterol while the steroids derived from plant is the phytosterols. Figure 1.1 shows the steroid skeleton, sterols have a double bond, typically between C-5 and C-6 of the sterol moiety.

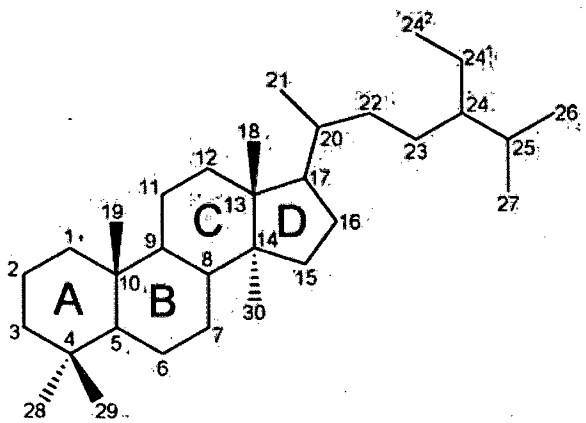


Figure 1.1: Steroid skeleton

Source: Cantrill (2008)

Cholesterol is a waxy steroid metabolite found in the cell membranes and transported in the blood plasma of all animals (Barter et al., 2007). It is an important component for the manufacture of bile acids, steroid hormones, and Vitamin D. Cholesterol in human body either comes from dietary or the steroid metabolism in human. The animal fats from dietary consist of complex mixtures of triglycerides, with lesser amounts of phospholipids and cholesterol. Cholesterol being carried around the body in blood by molecules called lipoproteins. There are two main lipoproteins which are low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The good cholesterol HDL carries cholesterol away from the cells and back to the liver, where it is either broken down or passed from the body as a waste product. However, the bad cholesterol LDL is the main cholesterol transporter and carries cholesterol from liver to the cells that need it. If there is too much cholesterol for the cells to use, this can cause a harmful build-up in blood. Too much LDL cholesterol in the blood can cause cholesterol to build up in the artery walls, leading to heart diseases such as narrowing of the arteries, Atherosclerosis (Lewington et al., 2007). Figure 1.2 shows the structure and numbering of cholesterol.

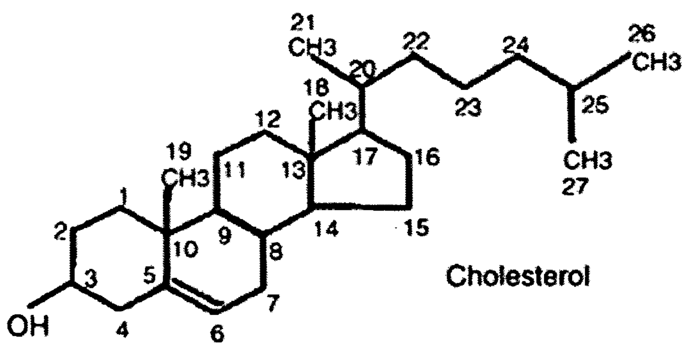


Figure 1.2: Structure and numbering of cholesterol

Source: Moreau et al. (2002)

According to American Heart Association (AHA), 2011, the drugs are most commonly used to treat high cholesterol elevated low density lipoprotein are the Statins (or HMG CoA reductase inhibitors), e.g., atorvastatin. Statins can lower LDL cholesterol. However, they have less effect than the fibrates or niacin in reducing

1.2 PROBLEM STATEMENT

Atherosclerosis is the heart disease due to the cholesterol deposition in the arteries. The drugs are most commonly used to treat high cholesterol which for elevated low density lipoprotein (LDL) cholesterol is the Statins (or HMG CoA reductase inhibitors), e.g., atorvastatin. Statins can lower LDL cholesterol. However, they have less effect than the fibrates or niacin in reducing triglycerides and raising HDL cholesterol. Recently, phytosterols have received much attention because of their cholesterol-lowering properties. Early phytosterol-enriched products contained free phytosterols and relatively large dosages were required to significantly lower serum cholesterol. Statin only help in cholesterol lowering by inhibit cholesterol synthesis in human body but it can't reduce the cholesterol absorption of dietary cholesterol. However, phytosterols can inhibit cholesterol absorption possibly by competitively inhibiting its incorporation into the mixed micelles in the small intestine although other mechanisms cannot be excluded. Phytosterols only differ from cholesterol by their side chain configuration, allowing them to compete with cholesterol for absorption from the gastro-intestinal tract (Heinemann et al., 1993; Jong et al., 2003 and Rozner and Garti, 2006). This characteristic of competition forms the basis for the cholesterol-lowering properties of phytosterols.

1.3 THE OBJECTIVES OF THE RESEARCH

The objective of this project is to find the new sources of phytosterols from plant found in Malaysia. The plant use in this project is the Petai. Only limited research has been carried out on phytosterols of Petai. To our knowledge, in Malaysia, Petai has never been investigated. Therefore, Petai is the suitable plant to be selected and to be analyzing on phytosterols from its seeds.

1.4 SCOPE OF WORKS

i. To investigate the content of phytosterols in the pods (seed and fruit coat) of Petai (*Parkia speciosa*, family Leguminosae).

ii. To isolate and identify individual phytosterols compounds in Petai seeds and fruit coat, using TLC and spectroscopy.

iii. To compare phytosterol content and composition of Petai seeds with known rich sources of phytosterols such as soybean and peanut.

1.5 BENEFITS OF PROJECT

The benefits of applicability of this project is aim to raise public health awareness on cholesterol-lowering ability of Petai and to introduce phytosterols for industrial utilization as food additive. If Petai proves to be a good source of phytosterols, people will be made more aware of the fact so that they produce or consume more of this well-known vegetable.

CHAPTER 2

LITERATURE REVIEW

2.1 PHYTOSTEROLS

Phytosterols are the sterols derived from plants. Phytosterols are triterpenes that are important structural components of plant membranes, and free phytosterols serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell membranes (Fernandes and Cabral, 2007). All triterpenes are synthesized via a pathway that starts with reduction of HMG-CoA (six carbons) to mevalonate (six carbons). Six mevalonate units are then assembled into two farnesyl diphosphate molecules, which are combined to make squalene (30 carbons or ''three terpenes''). Enzymatic ring closure steps then form cycloartenol (also 30 carbons), and additional enzymatic reactions from common plant triterpenes such as phytosterols, triterpene alcohols, and brassinosteroids (Moreau et al., 2002) (refer to Figure 2.1).

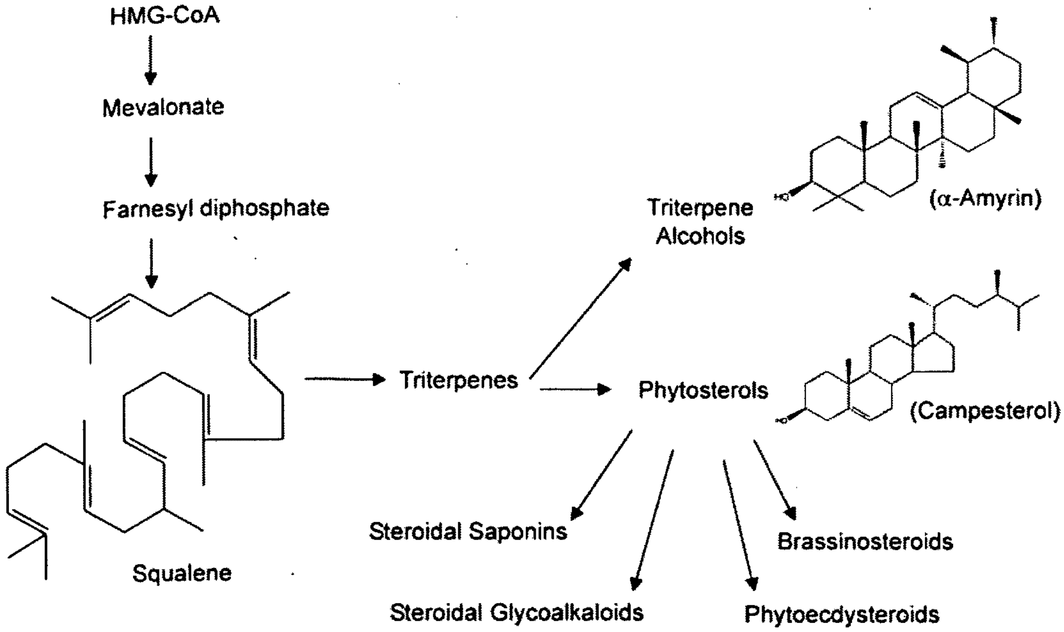
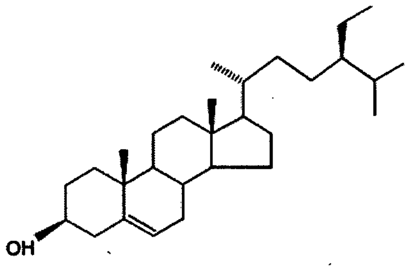


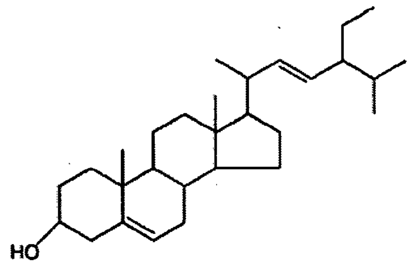
Figure 2.1: Biosynthesis of phytosterols and other triterpenes

Source: Moreau et al. (2002)

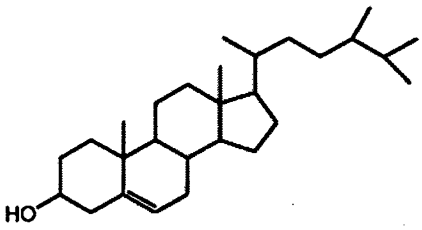
Most phytosterols contain 28 or 29 carbons and one or two carbon-carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain. More than 100 types of phytosterols have been reported in plant species, but the more abundant are sitosterol, stigmasterol and campesterol (Berger et al., 2004; Kritchevsky and Chen, 2005 and Moreau et al., 2002). Other relevant phytosterols that can be found in plants in minor amounts are brassicasterol, Δ^5-avenasterol, sitostanol and campestanol (Phillips et al., 2002). The biosynthesis and some representative of phytosterols are show in Figure 2.1 and Figure 2.2. Phytostanols are completely saturated forms of phytosterols and lack the carbon-carbon double bonds found in cholesterol and phytosterols (Miettinem and Gyllimg, 2004; Moreau et al., 2002; Nguyen, 1999; Piironen et al., 2000 and Pollak, 1953).



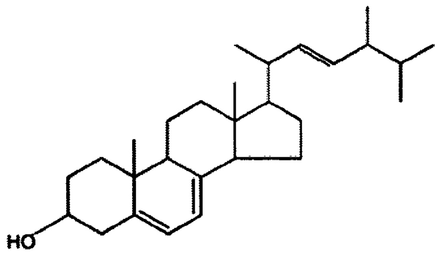
β-Sitosterol



Stigmasterol



Campesterol

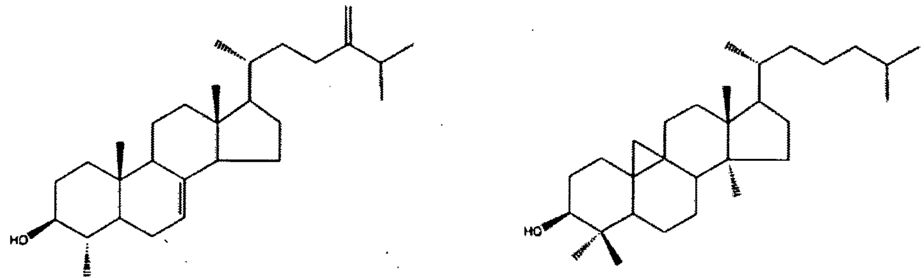


Ergosterol

Figure 2.2: Molecular structure of some representative phytosterols

Source: Fernandes and Cabral (2007)

Phytosterols is describe and catalog into three groups based on the number of methyl groups on carbon-4, two (4-dimethyl), one (4-monomethyl), or none (4-desmethyl). 4-Dimethylsterols and 4α-monomethylsterols are metabolic intermediates in the biosynthetic pathway leading to end-product, 4-desmethyl phytosterols, but they are usually present at low levels in most plant tissues. Cycloartenol and cycloartanol are examples of 4-dimethylsterols, and gramisterol is an example of a 4α-monomethylsterol (Moreau et al., 2002) (refer to Figure 2.3).



Gramisterol (24-methylenelophenol)

Cycloartanol

Figure 2.3: 4-Monomethyl and 4, 4-dimethylphytosterols

Cycloartanol, also called 9, 19-cyclo-5α, 9β-lanostan-3β-ol or cycloartan-3β-ol, $C_{30}H_{52}O$, MW 428.74, CAS # 4657-58-3. Found in rice bran oil and the rhizomes of Polypodium vulgare.

Gramisterol, also called 24-methylenelophenol, $C_{29}H_{49}O$, MW 412.69, CAS# 1176-52-9.

Source: Moreau et al. (2002)

4-Desmethylsterols include the 27-carbon sterol cholesterol (Figure 2.4) (ubiquitous and predominant in animals, but also generally present in plants at low levels) and all of the common 28-carbon (Figure 2.5) and 29-carbon (Figure 2.6) phytosterols, which are typically major membrane structural components in plant cells. Most 4-desmethyl phytosterols have a double bond between carbons 5 and 6 of the ring system and are thus called Δ^5 phytosterols. However, another group of common desmethylsterols that are abundant in plants of certain families have a double bond between carbons 7 and 8 instead of 5 and 6, and are hence referred to as Δ^7 phytosterols. Both Δ^5 and Δ^7 desmethylsterols can include a second double bond in the alkyl side chain, most frequently between carbons 22 and 23 or carbons 24 and 28 (carbons 24 and 24^1 in the 1989 IUPAC nomenclature) (Moreau et al., 2002).

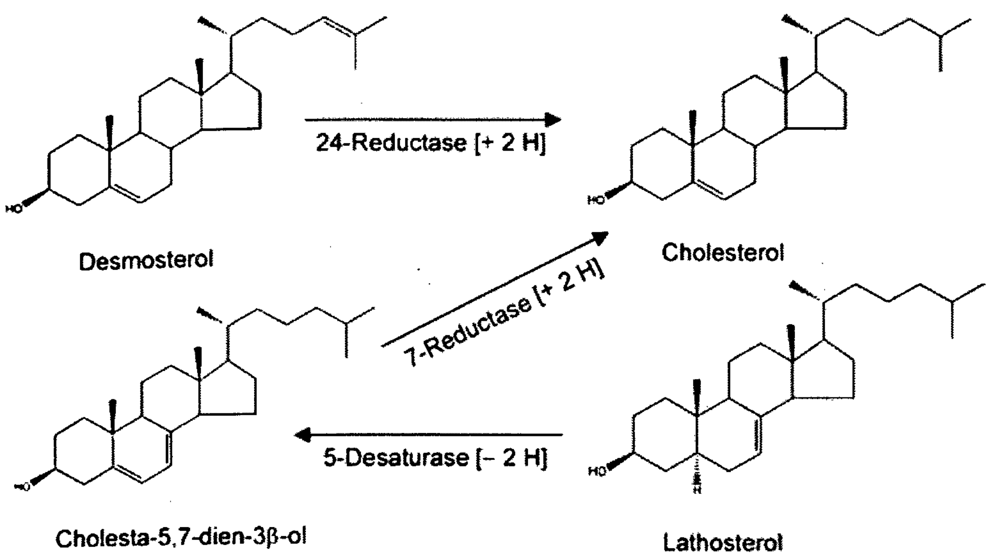


Figure 2.4: C27 4-desmethyl phytosterols

Cholesterol, cholest-5-en-3β-ol, $C_{27}H_{46}O$, mol. wt. 386.65, CAS# 57-88-5. The common sterol in animal tissues. Also occurs in the date palm, Phoenix dactylifera, and in many marine red algae (Rhodophyceae).

Desmosterol, also called cholesta-5, 24-dien-3β-ol, 24-dehydrocholesterol, $C_{27}H_{44}O$, MW 384.63, CAS# 313-04-02.

Lathosterol, also called 5α-cholest-7-en-3β-ol, $C_{27}H_{46}O$, mol. wt. 386.65. Lathosterol is the C27 precursor to phytoecdysteroids in spinach (Spinacia oleracea).

Cholesta-5,7-dien-3β-ol, also called 7-dehydrocholesterol, Δ^7-cholesterol, and Provitamin D_3, $C_{27}H_{44}O$, MW 384.63, CAS# 434-16-2.

Source: Moreau et al. (2002)

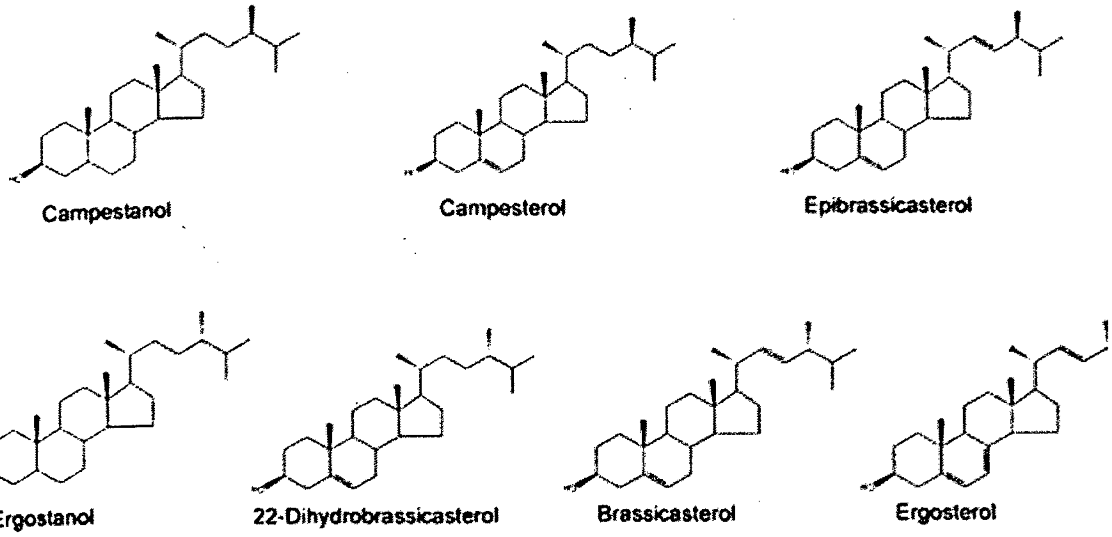


Figure 2.5: C28 4-desmethyl phytosterols. Note that some of these compounds are 24α (solid wedge) and some are 24β (dashed wedge). Catalytic hydrogenation (a method currently used in the production of commercial stanyl ester products) of the unsaturated C28 phytosterols can yield two 24-methyl epimers, campestanol (24α=24R) or ergostanol (24β=24S), depending on the phytosterol composition of the original material. Structures of common phytosterols

Campestanol (saturated) (24α=24R), also called (24R)-24-methylcholestan-3β-ol or (24R)-ergostan-3β-ol, $C_{28}H_{50}O$, MW 402.70, CAS # 474-60-2. Occurs naturally in corn fiber oil, almost exclusively as a ferulate or *p*-coumarate esters. Generated by catalytic hydrogenation of campesterol or epibrassicasterol.

Ergostanol (saturated) (24β=24S), also called (24S)-24-methylcholestan-3β-ol, $C_{28}H_{50}O$, MW 402.70. Not reported naturally but can be generated by catalytic hydrogenation of brassicasterol, 22-dihydrobrassicasterol, or ergosterol.

Campesterol (Δ^5) (24α=24R), also called (24R)-24-methylcholest-5-en-3β-ol, campest-5-en-3βol, or Δ^5-24α-methyl-cholesten-3β-ol, (24R)-ergost-5-en-3β-ol, $C_{28}H_{48}O$, mol. Wt. 400.68, CAS # 474-62-4. Widespread occurrence in plants.

22-Dihydrobrassicasterol (Δ^5) (24β=24S), also called ergost-5-en-3β-ol and 24-epicampesterol, $C_{28}H_{48}O$, mol. Wt. 400.68, CAS # 4651-51-8.

Brassicasterol ($\Delta^{5, 22E}$) (24β=24R), also called (22E)-ergosta-5, 22-dien-3β-ol, $C_{28}H_{46}O$, MW 398.66, CAS # 474-67-9. Found in rapeseed oil from Brassica napus.

Epibrassicasterol ($\Delta^{5,\,22E}$) (24α=24S), also called (22E)-(24S)-24-methylcholesta-5, 22-dien-3βol or (22E)-campesta-5, 22-dien-3β-ol, $C_{28}H_{46}O$, MW 398.66, CAS # 17472-78-5.

Ergosterol ($\Delta^{5,\,7,\,22E}$) (24β=24R), also called (22E)-ergosta-5, 7, 22-trien-3β-ol, $C_{28}H_{44}O$, MW 396.54, CAS # 57-87-4. Occurs in yeasts and many other fungi

Source: Moreau et al. (2002)

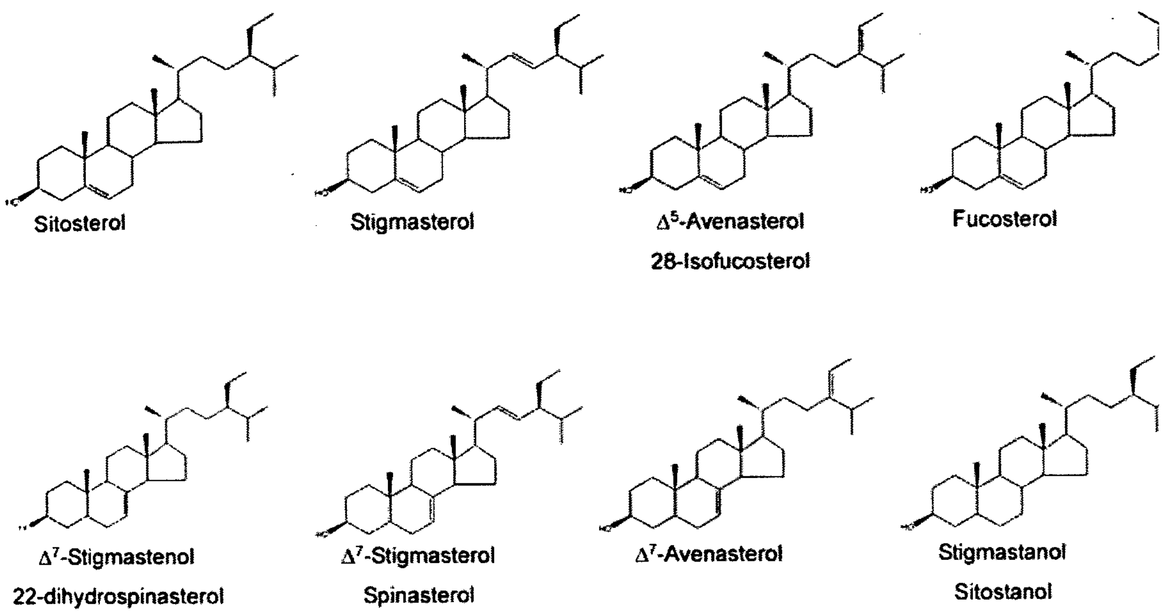


Figure 2.6: C29 4-desmethyl phytosterols. Note that catalytic hydrogenation (a method currently used in the production of commercial stanyl ester products) of unsaturated C29 phystosterols can yield stigmastanol and/or its 24 β epimer, depending on the phytosterol composition of the original material.

Sitostanol (saturated) (24α=24R), also called stigmastanol, stigmastan-3β-ol, 24α-ethylcholestan-3β-ol, $C_{29}H_{52}O$, MW 416.40, CAS # 19466-47-8. Occurs in corn fiber oil, almost exclusively as a ferulate ester or *p*-coumarate esters.

Sitosterol (Δ^5) (24α=24R), also called β-sitosterol, stigmast-5-en-3β-ol, 24α-ethylcholest-5-en-3β-ol, $C_{29}H_{50}O$, MW 414.71, CAS # 83-46-5. Widespread occurrence in plants.

Δ^7-Stigmastenol (7-Stigmastenol) (Δ^7) (24α=24S), also called, 22-dihydrospinasterol, stigmasta-7-en-3β-ol, $C_{29}H_{50}O$, MW 414.71, CAS # 521-03-9.

Stigmasterol ($\Delta^{5, 22E}$) (24α=24S), also called (22E)-stigmasta-5, 22-dien-3β-ol or 24α-ethylcholesta-5,22E-dien-3β-ol, $C_{29}H_{48}O$, MW412.69, CAS # 83-48-7. Widespread occurrence in plants.

Fucosterol ($\Delta^{5, 24E}$), also called [24(28) E]-stigmasta-5, 24(28)-dien-3β-ol, [24(240) E]-stigmasta-5, 24(240)-dien-3β-ol, or 24E-ethylidenecholesta-5, 24(28)-dien-3β-ol, $C_{29}H_{48}O$, MW 412.69, CAS # 17605-67-3. Found in coconut pollen, Cocos nucifera, and in many brown algae, e.g. Fucus vesiculosus.

Δ^5-Avenasterol (5-Avenasterol) ($\Delta^{5, 24Z}$), also called isofucosterol, 28-isofucosterol, 29-isofucosterol, 24Z-ethylidenecholesta-5,24(28)-dien-3β-ol, [24(28)Z]-stigmasta-5,24(28)-dien-3βol, or [24(240)Z]-stigmasta-5,24(280)-dien-3β-ol, $C_{29}H_{48}O$, MW 412.69, CAS # 18472-36-1. Found as a major phytosterol in oats and in significant levels in other plant materials.

Δ^7-Stigmasterol (7-Stigmasterol) ($\Delta^{7, 22E}$) (24α=24S), also called spinasterol, (22E)-stigmasta-7, 22-dien-3β-ol, $C_{29}H_{48}O$, MW 412.69, CAS # 481-18-4.

Δ^7-Avenasterol (7-Avenasterol) ($\Delta^{7,24Z}$), also called avenasterol, (24Z)-24-ethylidenecholesta-7, 24(28)-dien-3β-ol, $C_{29}H_{48}O$, MW 412.69, CAS # 23290-26-8.

Source: Moreau et al. (2002)

The common 29-carbon desmethylsterol stigmasterol (Figure 2.6), which includes both C5, 6 and (trans) C22, 23 double bonds, is, for example, designated as $\Delta^{5, 22E}$. For the C28 and C29 phytosterols the introduction of a methyl or ethyl group at C24 renders this position chiral and thus two epimers are possible. The nomenclature of the configuration of the C24 methyl, C24 ethyl, or C24 ethylidene groups on the C28 and C29 phytosterols requires some explanation. For the seven common C28 phytosterols listed in Figure 2.5, three (campesterol, epibrassicasterol, and campestanol) are 24α epimers (with the methyl group indicated as a "solid wedge"), and the other five phytosterols are 24β epimers (with the methyl group indicated as a "dashed wedge"). The 24-methyl epimers are also designated 24R and 24S, which are equivalent to 24α and 24β, respectively, unless there is a double bond at C22, 23, in which case the chirality is reversed (24R=24β and 24S=24α). With the C29 phytosterols, the good news is that almost all phytosterols are 24α-ethyl epimers. Unfortunately, three of the common C29 phytosterols have a double bond at C24, 28 (C24, 240) and the resulting ethylidene group can either be cis or trans. The C24 ethylidene in fucosterol is the trans isomer and is designated as a 24E, whereas the C24 ethylidene in Δ^5-avenasterol and Δ^5-avenasterol is the cis isomer and is designated as 24Z. Fortunately, the C22, 23 double bond in common phytosterols (brassicasterol, epibrassicasterol, stigmasterol, and 7-stigmasterol (spinasterol) only occur as 22E (Moreau et al., 2002).

In all plant tissues, phytosterols occur in five common forms (Figure 2.7): as the free alcohol (FS), as fatty-acid esters (SE), as steryl glycosides (SG), and as acylated steryl glycosides (ASG). The last three forms (SE, SG, and ASG) are generically called "phytosterol conjugates". In free phytosterols (FS), the 3β-OH group on the A-ring of the sterol nucleus is underivatized, whereas in the three conjugates the OH is covalently bound with another constituent. The OH group is ester-linked with a fatty acid in SE and linked by a 1-O-β-glycosidic bond with a hexose (most commonly glucose) in SG (first reported by Power and Salway, 1913). The third group of phytosterol conjugates, ASG, differ from SG by the addition of a fatty acid esterified to the 6-OH of the hexose moiety (first reported by Lepage, 1964). Seeds of corn and rice and other grains contain a fourth type of phytosterol conjugate, phytosteryl hydroxycinnamic-acid esters (HSE), in which the sterol 3β-OH group is esterified to ferulic or *p*-coumaric acid (Figure 2.7) (Moreau et al., 2002).

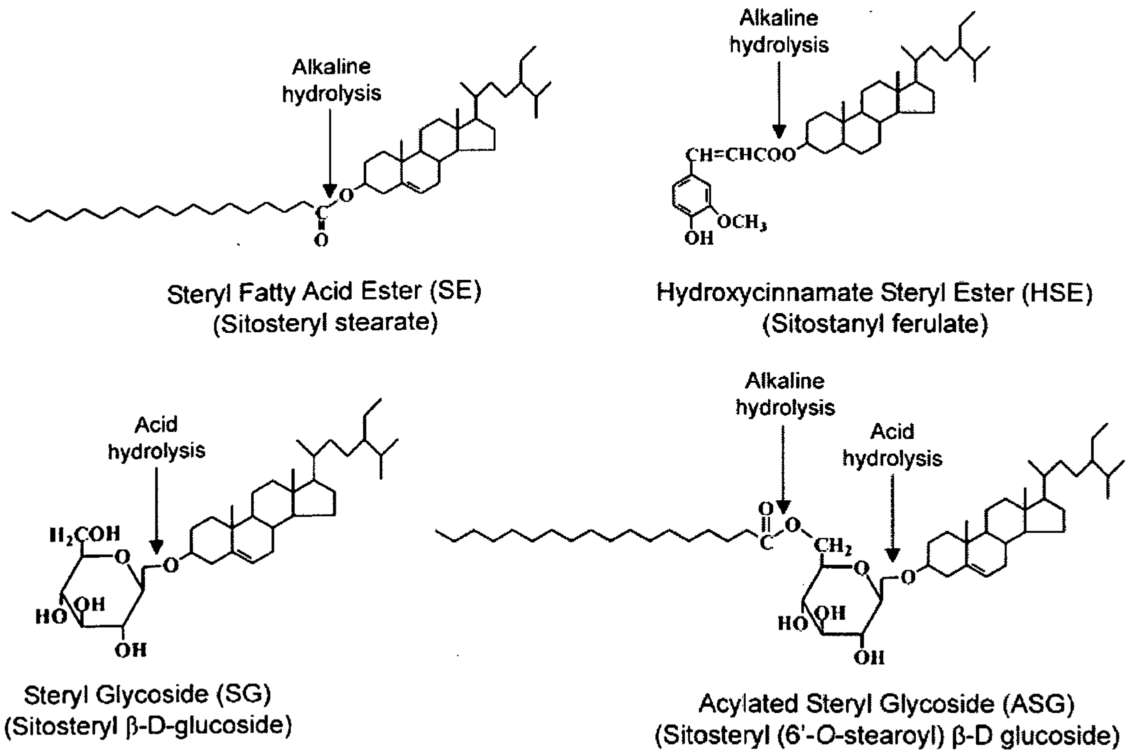


Figure 2.7: Structures of phytosterol conjugates. The sites of cleavage via alkaline hydrolysis (saponification) and acid hydrolysis are indicated with arrows.

Source: Moreau et al. (2002)

Phytosterols isolation in large scale is based in two major raw materials, vegetable oils and tall oil (Coss et al., 2000; Hayes et al., 2002 and Quilez et al., 2003). In edible oils phytosterols are mainly present in free and esterified forms (Phillips et al., 2002). One such phytosterol complex, isolated from vegetable oil, is cholestatin, composed of β-sitosterol, campesterol, stigmasterol and brassicasterol, and is marketed as a dietary supplement. Phytosterols play major roles in several areas, namely in pharmaceuticals (production of therapeutic steroids), nutrition (anti-cholesterol additives in functional foods, anti-cancer properties), and cosmetics (creams, lipstick). Table 2.1 shows the list of food sources which contain high phytosterol that help lower serum cholesterol.