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(M. Sc. THESIS)

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *ASTRAGALUS GUMMİFER*

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Ömer YAŞARİKİZ tarafından Yüksek Lisans tezi olarak sunulan "Isolation and Characterization of Saponins from *Astragalus gummifer (Astragalus gummifer*'den Saponinlerin İzolasyonu ve Karakterizasyonu)" başlıklı bu çalışma, E.Ü. Lisansüstü Eğitim ve Öğretim Yönetmeliği ile E.Ü. Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 19.01.2012 tarihinde yapılan tez savunma sınavında aday oybirliği/~~oyçokluğu~~ ile başarılı bulunmuştur.

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ÖZET

ASTRAGALUS GUMMİFER'DEN SAPONİNLERİN İZOLASYONU VE KARAKTERİZASYONU

Ömer YAŞARİKİZ

Yüksek Lisans Tezi, Kimya Bölümü

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Bu çalışmada *Astragalus gummifer* türünün MeOH ekstresinden 6 tane sikloartan tip glikozit izole edilerek, izole edilen bileşiklerin yapısı spektral teknikler kullanılarak Cycloastragenol, Monoside, Cyclocanthoside E, Oleifolioside B, 3,6-di-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxy-cycloartane and Oleifolioside A olarak belirlenmiştir.

Anahtar Kelimeler: *Astragalus gummifer,* triterpenoid saponin, sikloartan

ABSTRACT

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *ASTRAGALUS GUMMIFER*

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M. Sc. Thesis, in Chemistry
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In this thesis, 6 cycloartane type glycosides were isolated from the MeOH extract of the *Astragalus gummifer* species and structures of the isolated compounds determined as Cycloastragenol, Monoside, Cyclocanthoside E, Oleifolioside B, 3,6-di-O-β-D-xylopyranosyl-3β, 6α, 16β,24(*S*), 25-pentahydroxycycloartane and Oleifolioside A by using spectroscopic techniques.

Key words: *Astragalus gummifer*, triterpenoid saponin, cycloartane

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CONTENTS

Page

CONTENTS (continued)

Page

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF SPECTRA

LIST OF TABLES

LIST OF SCHEMES

ABBREVIATIONS

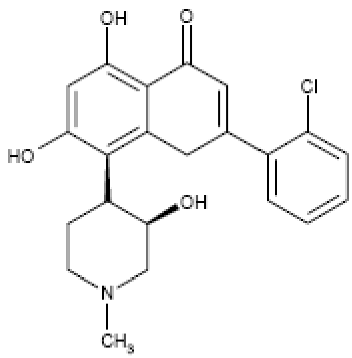
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
CC	Column Chromatography
CTLC	Centrifugal Thin Layer Chromatography
LPLC	Low-pressure Liquid Chromatography
MPLC	Medium-pressure Liquid Chromatography
HPLC	High Performance Liquid Chromatography
RP-VLC	Reverse Phase Vacuum Liquid Chromatography
SPE	Solid Phase Extraction
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	Multiple

1. INTRODUCTION

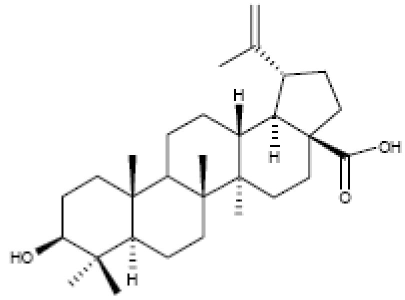
Natural products are an excellent source of complex chemicals possessing a wide variety of biological activities and, therefore, having great potential therapeutic value. They have been used for the treatment of various diseases for thousands of years. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Acadians. The "Ebers Papyrus", the best known Egyptian pharmaceutical record, which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC. The Chinese *Materia Medica*, which describes more than 600 medicinal plants, has been well documented with the first record dating from about 1100 BC. The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care (Shoeb, 2006).

Natural products often have an ecological role in regulating the interactions between plants, microorganisms, insects and animals. They can be defensive substances, antifeedants, attractants and pheromones. Chemotaxonomy provides another reason for examining the constituents of plants. Phytochemical surveys can reveal natural products that are "markers" for botanical and evolutionary relationships (Hanson, 2003).

Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation. Flavopiridol (**1**) is a synthetic flavone, derived from the plant alkaloid rohitukine, which was isolated from *Dysoxylum binectariferum* Hook. f. (Meliaceae). It is currently in phase I and phase tumors. Betulinic acid (**2**), a pentacyclic triterpene, is a common secondary metabolite of plants, primarily from *Betula* species (Betulaceae). Betulinic acid was isolated from *Zizyphus* species, e.g. *mauritiana*, *rugosa* and *oenoplia* and displayed selective cytotoxicity against human melanoma cell lines. The development of systemic and topical formulations of the agent for potential clinical trials by the NCI is ongoing. Two novel alkaloids, montamine (**3**) and schischkiniin (**4**) have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana.* Both of the alkaloids exhibited significant cytotoxicity against human cancer cell lines (Figure 1.1) (Shoeb, 2006).



(1)



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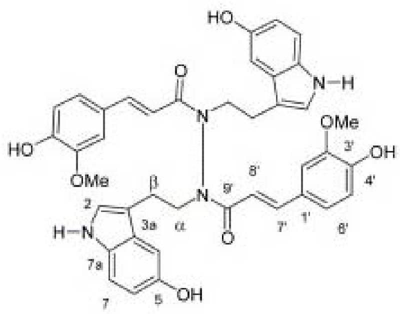

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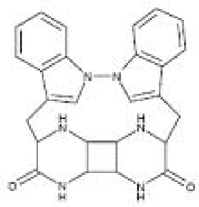


(4)

Figure 1.1. Plant-derived anticancer agents in clinical use

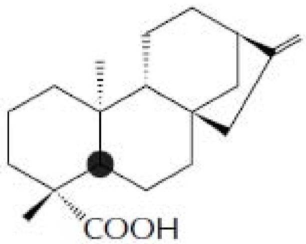
1.1 Primary and Secondary Metabolites

Natural product are organic compounds that area formed by living systems. The elucidation of their structures and their chemistry, synthesis and biosynthesis are major areas of organic chemistry (Hanson, 2003). There are two types of organic compounds that are synthesized in living organism, the primary metabolites and the secondary metabolites. The primary metabolites are essential for life functions and are universal in their distribution. They are fats, sugars and proteins. The secondary metabolites of plants are compounds with no apparent function in the primary metabolism of the organism, and these substances tend to be of restricted taxonomic distribution. The most common plant secondary metabolites occur in the following groups: alkaloids, anthraquinones, coumarins, essential oils (lower terpenoids and phenylpropanoids), flavonoids, steroids, and terpenoids (cardenolides, diterpenoids, iridoids, monoterpenoids, sesquiterpenoids [including sesquiterpene lactones], and triterpenoids) (Cannell, 1998).

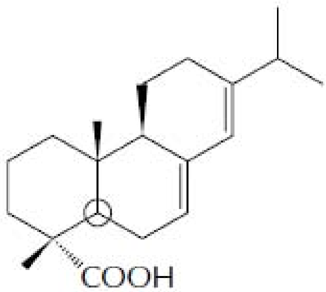
Primary and secondary metabolites cannot readily be distinguished on the basis of precursor molecules, chemical structures, or biosynthetic origins. For example, both primary and secondary metabolites are found among the diterpenes (C20) and triterpenes (C30). In the diterpene series, both kaurenoic acid and abietic acid are formed by a very similar sequence of related enzymatic reactions (Figure 1.2.); the former is an essential intermediate in the synthesis of gibberellins, i.e., growth hormones found in all plants, whereas the latter is a resin component largely restricted to members of the Fabaceae and Pinaceae. Similarly, the essential amino acid proline is classified as a primary metabolite, whereas the C6 analog pipecolic acid is considered an alkaloid and thus a natural product (Figure 1.2). Even lignin, the essential structural polymer of wood and second only to cellulose as the most abundant organic substance in plants, is considered a natural product rather than a primary metabolite. (Croteau et al., 2000)

In the absence of a valid distinction based on either structure or biochemistry, we return to a functional definition, with primary products participating in nutrition and essential metabolic processes inside the plant, and natural (secondary) products influencing ecological interactions between the plant and its environment. (Croteau et al., 2000).

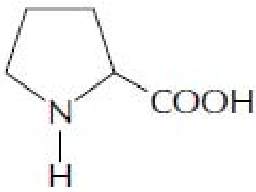
Primary metabolite **Secondary metabolite**



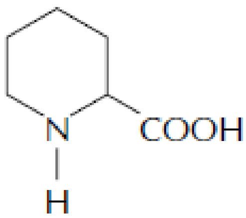
Kaurenoic acid



Abietic acid



Proline



Pipecolic acid

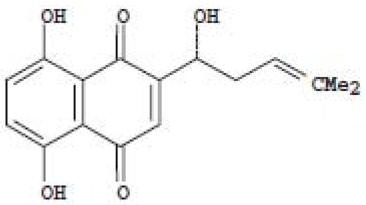
Figure 1.2. Kaurenoic acid and proline are primary metabolites, whereas the closely related compounds abietic acid and pipecolic acid are considered secondary metabolites.

Kaurenoic acid and proline are primary metabolites, whereas the closely related compounds abietic acid and pipecolic acid are considered secondary metabolites (Croteau et al., 2000).

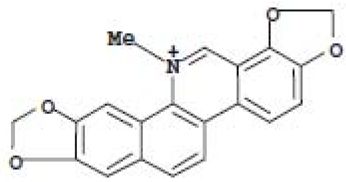
Plant secondary metabolites are unique resources for pharmaceuticals, food additives, and fine chemicals. They also provide original materials used in other areas. Besides direct extraction from plants, and chemical synthesis to provide those compounds or derivatives with similar uses, plant cell culture has been developed as a promising alternative for producing metabolites that are difficult to be obtained by chemical synthesis or plant extraction. However, in spite of four decades of efforts, production of plant secondary metabolites by plant cell culture technology is still facing many biological and biotechnological limitations. One of the major obstacles is the low yield of plant secondary metabolites in plant cell cultures. Since the major roles of plant secondary metabolites are to protect plants from attack by insect, herbivores and pathogens, or to survive other biotic and abiotic stresses, some strategies for culture production of the metabolites based on this principle have been developed to improve the yield of such plant secondary metabolites. These include treatment with various elicitors, signal compounds, and abiotic stresses (Yukimune et al., 1996; Zhao et al., 2000, 2001a,b,c; Zhang et al., 2004).

Many such treatments indeed effectively promote the production of a wide range of plant secondary metabolites, both in vivo and in vitro. However, the productivity is still rarely competitive for industrial application. Only the production of shikonin by Lithospermum *erythrorthizon* cell cultures and of taxol by Taxus cell cultures is successfully industrialized for commercial application. Considerable efforts are continuously being put in these studies for production of many other highly valuable compounds. (Zhao et al., 2005)

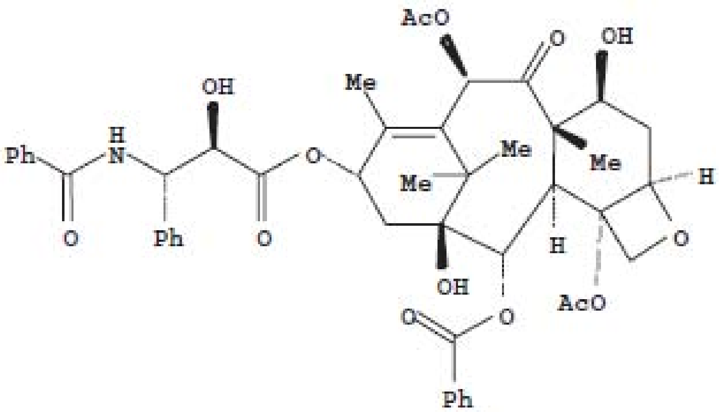
A recent work for improving production of plant secondary metabolites was mainly focused on the following aspects: I) manipulation of plant cell cultures to improve productivity of target compounds, through improving chemical processing and bioreactor performance or employing elicitors, abiotic stresses, and other approaches, regardless of their mechanisms (Zhong, 2001); II) studying signal transduction pathways underlying various effective strategies leading to biosynthesis of target secondary metabolites; III) studying transcription factors and their regulation mechanisms, including genetic manipulation of regulator genes to improve production of target secondary metabolites (Memelink et al., 2001); IV) cloning of secondary metabolite biosynthetic genes, and genetic modification of key genes to engineer the metabolic flux to target compounds (Verpoorte and Memelink, 2002); V) studying metabolic flux and profiling metabolic intermediates to understand whole pathways and overall regulation of target compound accumulation (Figure 1.3) (Sumner et al., 2003).



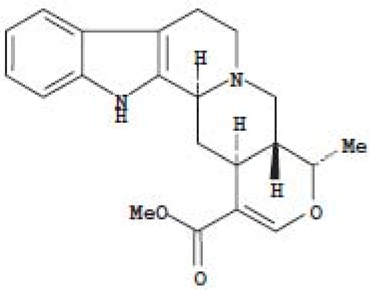
I. Shikonin, the only secondary metabolite that successfully produced by large-scale plant cell culture.

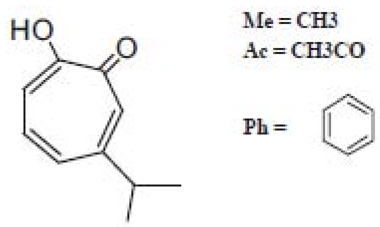


II. Sanguinarine, a benzylisoquinoline alkaloid produced by some poppy cell cultures.



III. Taxol, a diterpene compound from *Taxus spp*. has very strong anticancer activity. Studies on biosynthetic pathway and cell culture for its production are in process.





IV. Ajmalicine, an extensively studied terpenoid indole alkaloid from *Catharanthus roseus* cell culture.

V. β-Thujaplicin, an antifungi tropolone from *Cupressus lusitanica* cell culture, and the explanation for symbols in chemical structures.

Figure 1.3. Several typical plant secondary metabolites with special interests

Well known sources of secondary metabolites are plants, bacteria, fungi and marine organisms such as sponges, tunicates, corals and snails. Many secondary metabolites have proved invaluable as antibacterial or antifungal agents, anticancer drugs, cholesterol-lowering agents, immunosuppressants, antiparasitic agents, herbicides, diagnostics, and tools for research. Some of these have found to play a pivotal role in treatment or prevention of a multitude of biological disorders, many of which did not have any cure until these products were discovered.

In addition to their known activities and employment in combating disease, secondary metabolites reveal surprising additional activities which may be possible solutions to other diseases, some of which lack effective solutions. Many antibiotics, bacterial pigments, plant terpenoids, are also found to have anti-HIV, antitumor, anti-ageing, immunosuppressant, antiprotozoal and antihelminth activities, thus exhibiting multifarious applications in the sphere of medicine.

Unraveling the novel applications of known secondary metabolites and exploiting a myriad of sources as microbes, plants and higher animals for screening new secondary metabolites are paving the way to treat “untreatable diseases”, and help reduce mortality rates. Other activities of useful secondary metabolites in the battle against life-threatening diseases in the hope that this will catalyze further efforts to apply these useful compounds against other forms of human disease (Vaishnav and Demain, 2010).

1.1.1 The classes of secondary metabolites

The structures of secondary metabolites may seem to be bewilderingly diverse. However, the majority of these compounds belong to one of a number of families, each of which have particular structural characteristics arising from the way in which they are built up in nature, *i.e.* from their biosynthesis. The classes of secondary metabolites are:

- Polyketides and fatty acids
- Terpenoids and steroids
- Phenylpropanoids
- Alkaloids
- Specialized carbohydrates

Polyketides are formed by the linear combination of acetate units derived from the “building block” acetyl co-enzyme A. Terpenoids and steroids are assembled in nature from isoprenoid *C5* units derived from isopentenyl (3-methylbut-3-en-1-yl) pyrophosphate. These C5 units are linked together in a head-to-tail manner. They have characteristic specialized amino acids and peptides branched chain structure. A further group of natural products are those containing a phenylpropanoid. The amino acids are the building blocks for peptides and proteins. Although the amino acids are normally considered as primary metabolites, there are some unusual amino acids that are of restricted occurrence. Some antibiotics such as the penicillins are formed from small peptides. The alkaloids are a structurally diverse group of natural products containing nitrogen. The nitrogenous portions of the alkaloids are derived from amino acids such as ornithine, lysine, tyrosine or tryptophan (Hanson, 2003).

1.2 Terpenes

Terpenoids (terpene derivatives) constitute the largest class of natural products. About 30000 terpenes are known at present in the literature. For many, derivatives with more desirable biological properties have also been prepared. Many are being synthesized industrially. About 40 different monoterpene skeletons and over 200 sesquiterpene skeletons have been characterized. The many diverse functions that have been described to the terpenoids may be accounted for by the diversity in their structures: Many of the distinctive odors of plants are due to their volatile mono and sesquiterpene constituents. The growth regulating properties of terpenoids are well known; two major classes of growth regulators are the sesquiterpenoid abscisins and the diterpenoid gibberellins.

Terpenes are characterized by carbon skeletons constructed of isoprene units $(C5)_n$ (Figure 1.4), build up the carbon skeleton of terpenes; This is the isoprene rule 1 found by “RUZICKA” and “WALLACH”.

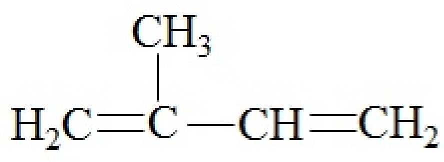


Figure 1.4. Isoprene

Terpenes are classified according to the number of isoprene units in the molecule (Figure 1.5).

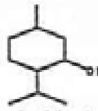
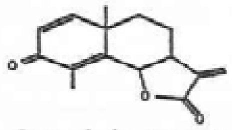
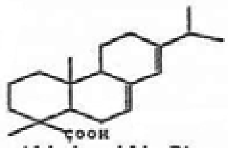
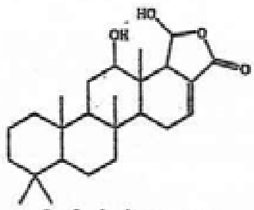
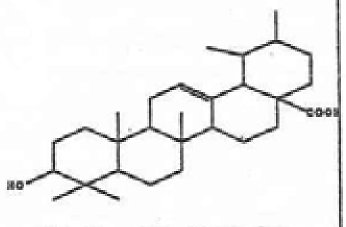
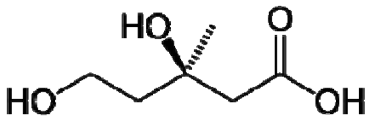
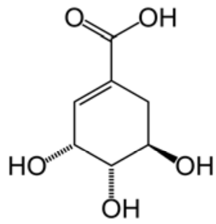
NO OF ISOPRENE UNITS	CARBON NO	CLASS	EXAMPLES
1	C_5	Isoprene	 in *Hamamelis japonica*
2	C_{10}	Monoterpenoid	 Menthol in *Mentha*
3	C_{15}	Sesquiterpenoid	 Santonin in *Artemisia*
4	C_{20}	Diterpenoid	 Abietic acid in *Pinus*
5	C_{25}	Sesterpenoid	 Scalarin in sponge
6	C_{30}	Triterpenoid	 Ursolic acid in *Euphorbia*
8	C_{40}	Tetra terpenoid	 β-Carotene in carrots
n	C_{5n}	Poly isoprenoid	 Rubber in *Hevea brasiliensis*

Figure 1.5. Classification of Terpenes

Terpenoids are derived from the mevalonic acid (Figure 1.6.A) and are typically found in all parts of higher plants. Other important groups of compounds attractive for insects are phenylpropanoids and green leaf volatiles (Metcalf and Metcalf, 1992). Phenylpropanoids present a C_6-C_3 structure and are derived from shikimic acid (Figure 1.6.B) (Harborne, 1994). Green leaf volatiles are six carbon alcohols, aldehydes, and related esters that are product of the degradation of the C_{18} linolenic and linoleic fatty acids (Hatanaka, 1993).



(A) mevalonic acid



(B) shikimic acid

Figure 1.6. Structure of mevalonic acid and shikimic acid

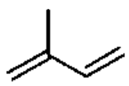
Conifer wood, balm trees, citrus fruits, coriander, eucalyptus, lavender, lemon, grass, lilies, carnation, caraway, peppermint species, roses, rosemary, sage, thyme, violet and many other plants or parts of those (roots, rhizomes, stems, leaves, blossoms, fruits, seed) are well known to smell pleasantly, to taste spicy, or to exhibit specific pharmacological activities. Terpenes predominantly shape these properties (Breitmaier, 2006).

In order to enrich terpenes, the plants are carved, e.g. for the production of incense or myrrh from balm trees; usually, however, terpenes are extracted or steam distilled, e.g. for the recovery of the precious oil of the blossoms of specific fragrant roses. These extracts and steam distillates, known as ethereal or essential oils are used to create fine perfumes, to refine the flavor and the aroma of food and drinks and to produce medicines of plant origin (phytopharmaca). The biological and ecochemical functions of terpenes have not yet been fully investigated (Breitmaier, 2006).

Many plants produce volatile terpenes in order to attract specific insects for pollination or otherwise to expel certain animals using these plants as food. Less volatile but strongly bitter-tasting or toxic terpenes also protect some plants from being eaten by animals (antifeedants). Last, but not least, terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants, as shown by preliminary investigations (Breitmaier, 2006).

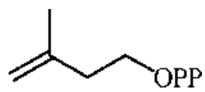
Terpenoids are usually found in the free form but some also exist as glycosides, organic acid esters, and also as bound to proteins. The free forms are lipid soluble and occur in the cytoplasm of the plant cell Essential oils may occur in glandular cells on the leaf surface. Carotenoids are located in chloroplasts in leaves and in chromoplasts in the petals. The terpenoid glycosides are partially water-soluble and can be transported into the cells or between the cells. The biological activity of terpenoid glycosides is well established. Many have been found in plants used in traditional medicine (Breitmaier, 2006).

Terpenes, or terpenoids, are represented with more than 8000 (Breitmaier and Teubner, 1999) compounds in this group of secondary metabolites comprising more than 80000 (Richter and Verlag, 1998) structures. According to the data given in the Chapmann & Hall/CRC *Dictionary of Natural Products* this number has already reached approximately 170000 in 2003. They show extraordinarily diverse structures and exhibit a large variety of physical and physiological properties. Their number increases every year with the addition of new structures, most of which have different biological effects (Richte and Verlag, 1996). In plants, the production of terpenoids is much more common than in animals or microorganisms. All isoprenoids, consequently terpenoids, are synthesized from one precursor "*isopentenyl diphosphate*" (IDP), which is the biological equivalent of isoprene (2-methyl-1,3-butadiene) (Figure 1.7). It was formulated hypothetically as the "*isoprene rule*" first by *Otto Wallach* in 1887 that, terpenoids are the sequential combinations of isoprene (C_5) units (Wallach, 1887).



Isoprene

2-Methyl-1,3-butadiene



Isopentenyl diphosphate

(IDP)

Figure 1.7. An isoprene unit and its biological equivalent, isopentenyl diphosphate.

The classification of terpenoids depends on the number of isoprene units present in the skeleton of the product. Compounds, which are composed of one isoprene unit, are called *hemiterpenes* (C_5), in the case of dimerization of two isoprene units they are called *monoterpenes* (C_{10}). *Sesquiterpenes* (C_{15}), *diterpenes* (C_{20}), *sesterterpenes* (C_{25}), *triterpenes* (C_{30}) and *tetraterpenes* (C_{40}) follow this sequence, each having an isoprene unit more than the preceeding one.

However, the biosynthetic routes to these terpenes do show some differences. The polymerization of an indefinite number of isoprene units results in polyisoprenes or polyprenes as in the case of natural rubber (Figure 1.8) (Breitmaier and Teubner, 1999).

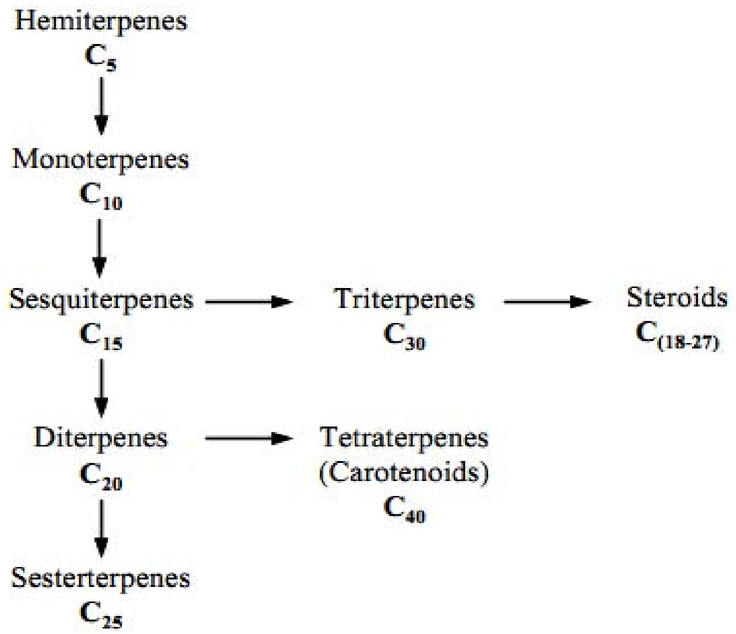


Figure 1.8. An overview over the classification of terpenes

Steroids (C27) belong to the group of isoprenoids as they originate from triterpenes. Labeling experiments showed that the formation of tetracyclic triterpenoids from 2,3-epoxysqualene is an introductory step in the formation of steroids (Breitmaier and Teubner, 1999). The loss of carbon atoms and rearrangement reactions during their biosynthesis from triterpenes makes the isoprene units of steroids unrecognizable. Therefore, they are classified separately from terpenes (Figure 1.9) (Thiele and Verlag, 1979).

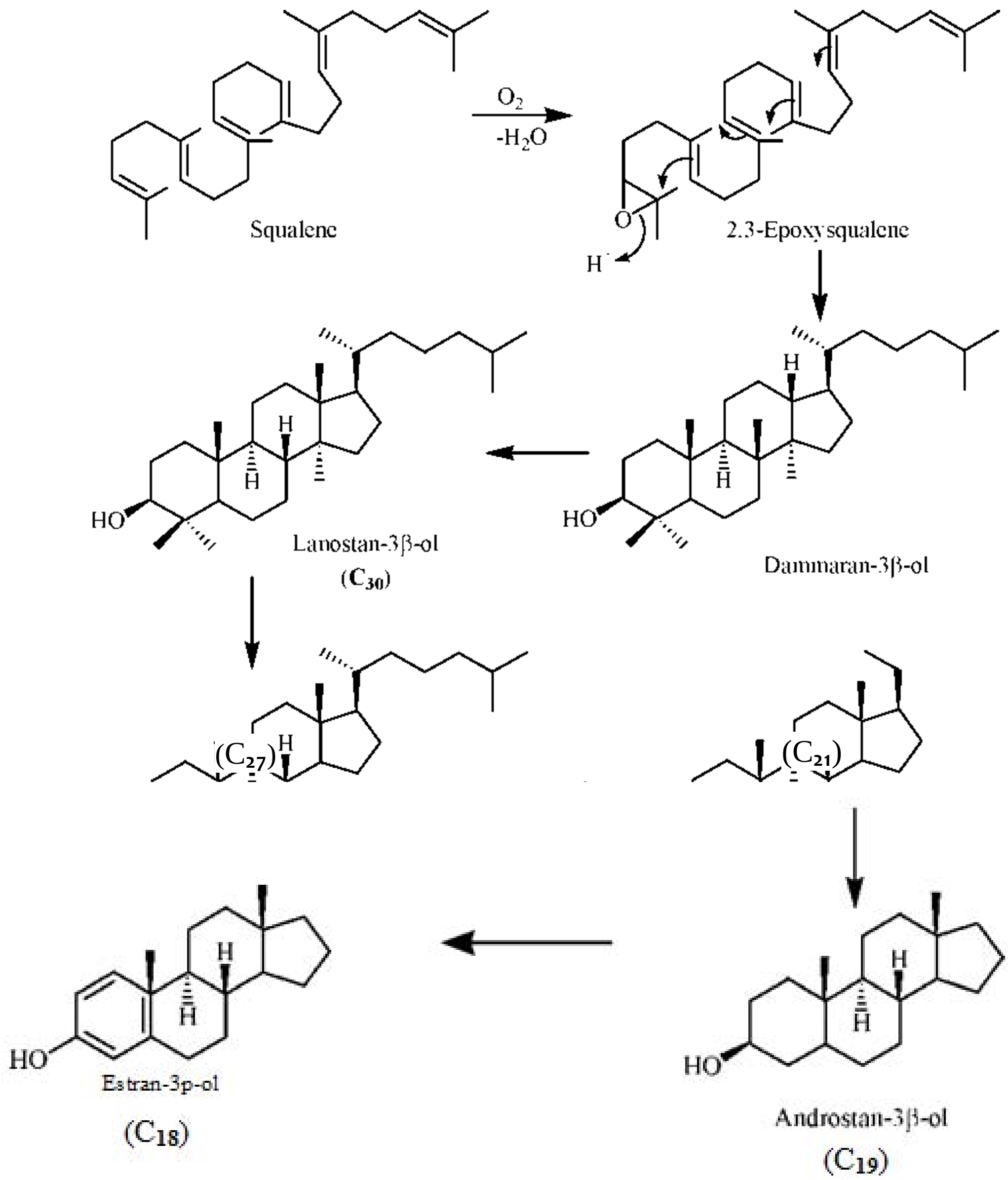


Fig. 1.9. The biosynthesis of some steroids from 2,3-epoxysqualene

Terpenes consist of 30 carbons or 6-isoprene units. $C_{30}H_{50}$ are called triterpene. About 5000 naturally abundant triterpenes are documented in the literature. Most of these are derived from squalane. They are composed of isoprene subunits and consist of approximately 30,000 identified compounds (Dzubak et al., 2006).

This subdivision forms by far the most extensive collection of saponins, comprising, to date, over 750 triterpene glycosides with 360 sapogenins. Most have either oleanane (*β*-amyrin) or dammarane skeletons, with ursanes, hopanes, lanostanes or lupanes assuming secondary importance with respect to distribution (Figure 1.10).

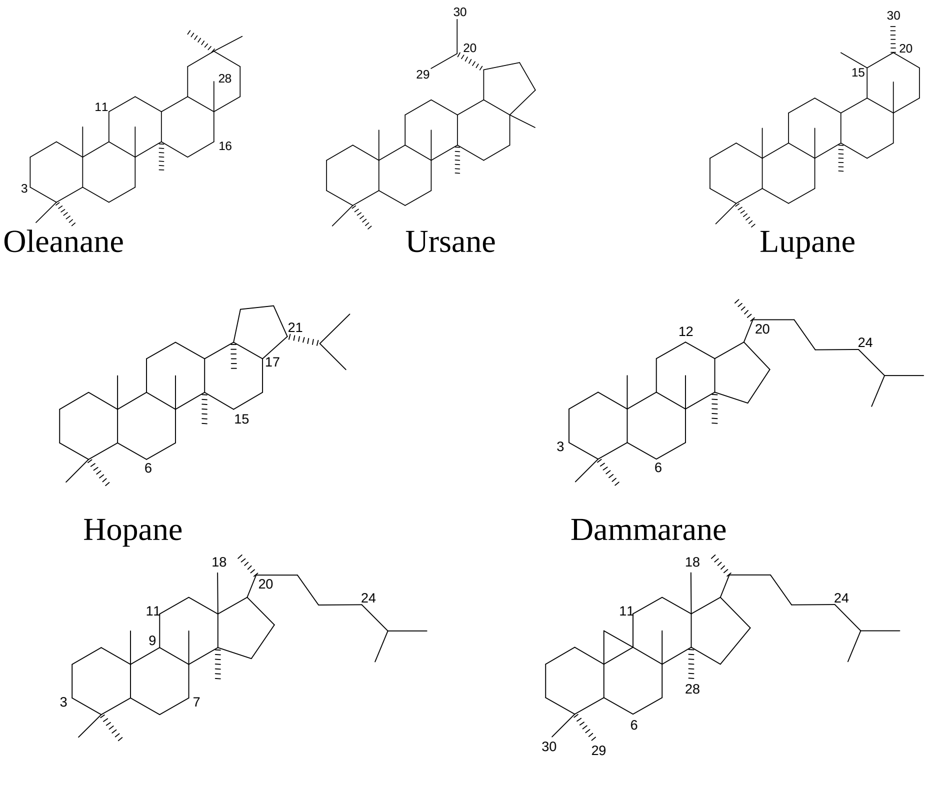


Figure 1.10. Triterpene aglycones (other than olean-12-en type)

There are numerous structural variants of the oleanane (or, more precisely, the olean-12-en) skeleton (Figure 1.11).

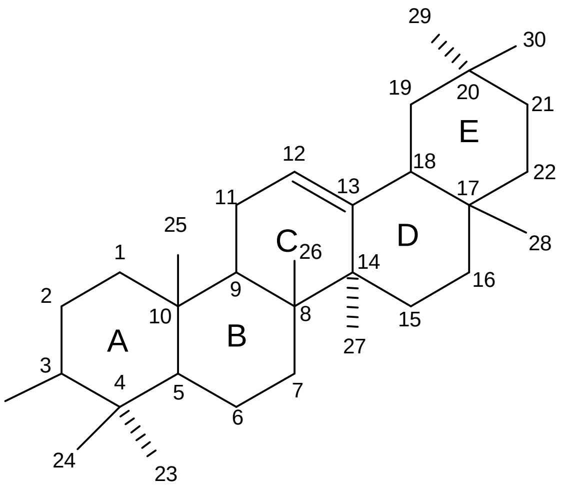


Figure 1.11. Olean-12-en skeleton

1.3 Saponins

The word Saponin is derived from Latin words "sapo" and "saponis" meaning soap. They are so called because saponins generally produce foam in aqueous solution. They are glycosides of triterpene aglycone part and one or more hexoses or pentoses sugar attached to it (Shibata, 1977).

Saponins are a special category of isoprenoid glycosides that form colloidal solutions with water and foam when shaken Removing the glycosides fraction yields aglycones known as sapogenins, which have either a terpenoid or steroid structure (Figure 1.12) (Barken, 2001). This foaming property, which is a well-known feature of most saponins, was used to search for plant saponin content. Froth formation after shaking in water solution is specific to most saponins, but some of them, especially those with two or three branched sugar chains do not form stable froth, and conversely some plant extracts not containing saponins may produce froth, providing misleading information (Oleszek, 2002).

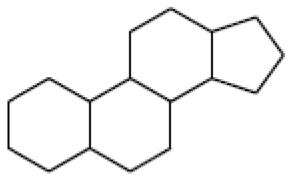


Figure 1.12 Basic Skeleton of steroid

Saponins are generally known as non-volatile (Oleszek, 2002; Vincken et al., 2007) and are a major family of secondary metabolites that occur in a wide range of plant species (Hostettmann and Marston, 1995). Various members of this important family of plant secondary metabolites are exploited commercially for a variety of purposes including as drugs and medicines, precursors for hormone synthesis, adjuvants, foaming agents, taste modifiers and cosmetics (Osbourn, 2003).

Mostly seeds, leaves, stem bark, roots and rhizomes of higher plants containing saponins and some marine animals e.g. seaslug and starfish also contain saponins. Many saponins showed characteristic antimicrobial and antifungal activities (Tschesche et al., 1965; Wagner and Horhamer, 1971).

Saponins are mostly found in plant kingdom because 76% of saponins are present in plant families. In 1970, Guvanor et al. investigated 1730 species from 104 families of plant growing in central Asia. He reported that 167 species contain triterpenoidal saponins and 127 species contain steroidal saponins (Guvanor et al., 1970, 1973). Steroidal saponins are important because there are the starting materials for the syntheses of steroidal hormones and related medicines. Some triterpenoid saponins have been shown to possess biological and pharmacological activities, which could be developed into important medicine to some medical uses (Tschesche and Wulff, 1973).

Saponins are high-molecular-weight triterpene glycosides containing a sugar group attached to either a sterol or other triterpene. They are widely distributed in the plant kingdom and composed of two parts: glycone and aglycone or genin (triterpene) (Figure 1.13). The aglycones or non-saccharide portion of the saponin molecule are called the genin or sapogenin. The classical definition of saponins is based on their surface activity; many saponins have detergent properties, stable foams in water, show haeomolytic activity, have a bitter taste and are toxic to fish (piscicidal) (Hostettmann and Marston, 1995).

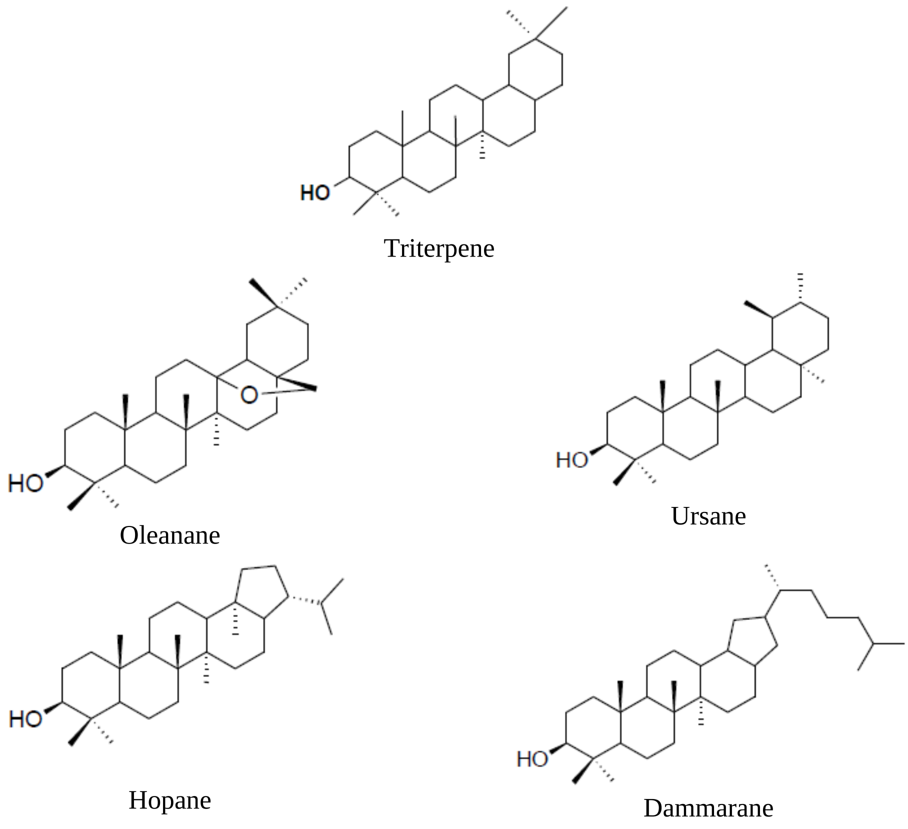
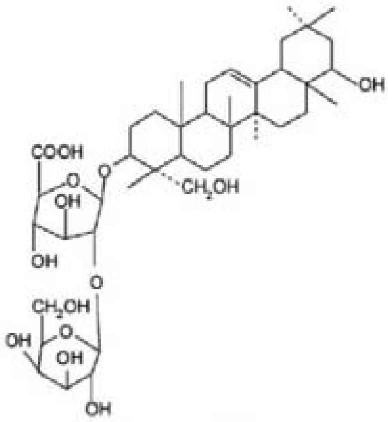


Figure 1.13. A Triterpene skeleton and four common triterpene skeletons

Saponins are steroid or triterpenoid glycosides, common in a large of plants and plant products that are important in human and animal nutrition (Francis et al, 2002). Yucca and *quillaja* saponins, for example, have both current and potential importance in animal and human nutrition (Singh et al., 2003).

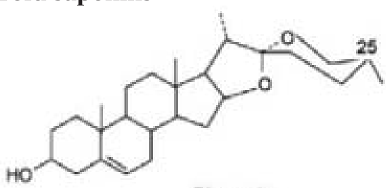
Saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin) linked to one or more oligosaccharide moieties (Figure 1.14).

1. Triterpene saponins



Soyasaponin III

2. Steroid saponins



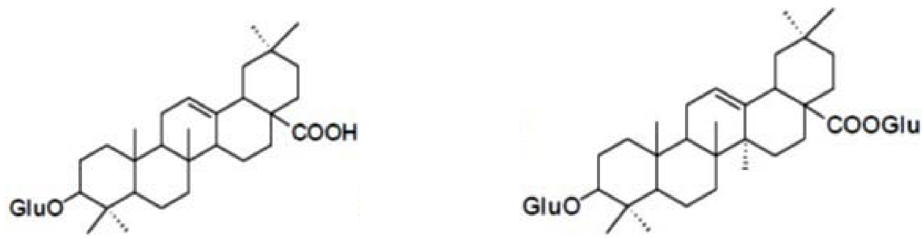
Diosgenin

Figure 1.14. Structure of triterpenoid and steroid saponins

The carbohydrate moiety consists of glucose, galactose, xylose, arabinose, rhamnose, pentoses, hexoses, or uronic acids. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provides saponins with strong surface-active properties that then are responsible for many of its adverse and beneficial effects. The primary biological effect of saponins is the interactions with cellular and membrane components. For example, saponins hemolysis red blood cells by nonspecific interactions with membrane proteins, phospholipids, and cholesterol of erythrocytes.

Saponins are characterized by their hemolytic activity and foaming properties and are responsible for imparting a bitter taste and astringency to plant materials containing high concentrations of saponins (Makkar et al., 2007).

All saponins have in common the attachment of one or more sugar chains to the aglycone. So saponins that have one sugar molecule attached at the C-3 position are called ***monodesmosidic saponins***, and those that have a minimum of two sugars, one attached to the C-3 through an ether linkage (Hostettmann and Marston, 1995) and one at C-22, or one attached through an ester linkage (acyl glycoside) at C-28 (triterpene saponins), as another view, or, an ether linkage at C-26 (furostanol saponins) (Hostettmann and Marston, 1995), are called ***bidesmosidic saponins*** (Figure 1.15) (Wina et al., 2005). ***Tridesmosidic saponins*** have three sugar chains and are seldom found (Figure 1.16) (Calis et al., 1999) Bidesmosidic saponins are easily transformed into monodesmosidic saponins by, for example, hydrolysis of the esterified sugar at C28 in triterpene saponins; they lack many of the characteristic properties and activities of monodesmosidic saponins (Hostettmann and Marston, 1995). [Greek desmos=chain) (Oleszek, 2002)].



Monodesmosidic Saponins **Bidesmosidic Saponins**

Figure 1.15. Monodesmosidic and bidesmosidic Saponins

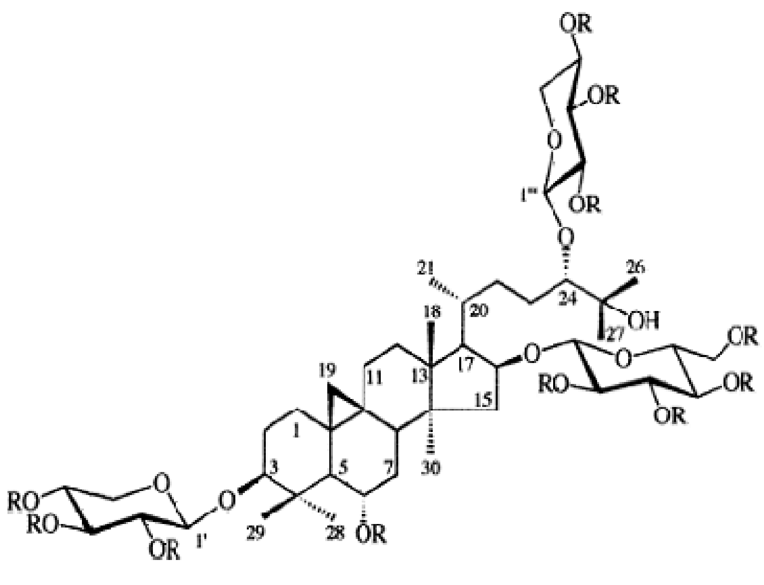


Figure 1.16. Cephalotoside A, a tridesmosidic cycloartane type glycoside

1.3.1 Classification and occurrence

Chemically, saponins as a group include compounds that are glycosylated steroids, triterpenoids, and steroid alkaloids (Wina et al., 2005). The aglycone or non-saccharide portion of the saponins are called the genin or sapogenin. Depending on the type of genin present, the saponins can be divided into three major classes (Hostettmann and Marston, 1995). The first group consists of the steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms and a steroidal aglycone, a C27 spirostane skeleton, generally comprising of a six-ring structure (Figure 1.17.A). In some cases, in fresh plant material, the hydroxyl group in the 26-position is engaged in a glycosidic linkage, and so the aglycone structure remains pentacyclic. This is referred to as a furostane skeleton (Figure 1.17.B). The second group consists of the triterpenoid saponins, which are the most common and occur mainly in the dicotyledonous angiosperms and a triterpenoid aglycone, which consists of a C30 skeleton, comprising of tetracyclic or pentacyclic structure (Figure 1.17.C) (Bruneton, 1995). And the third group called steroidal amines, which are classified by others as steroidal alkaloids (Bruneton, 1995). All classes of aglycones may have a number of functional groups (-OH, - COOH,-N,-CHO) causing big natural diversity only because of aglycone structure (Oleszek, 2002). The molecular weights of saponins range from 1000 to 1500 g/mol (Dorsaz and Hostettmann, 1986).

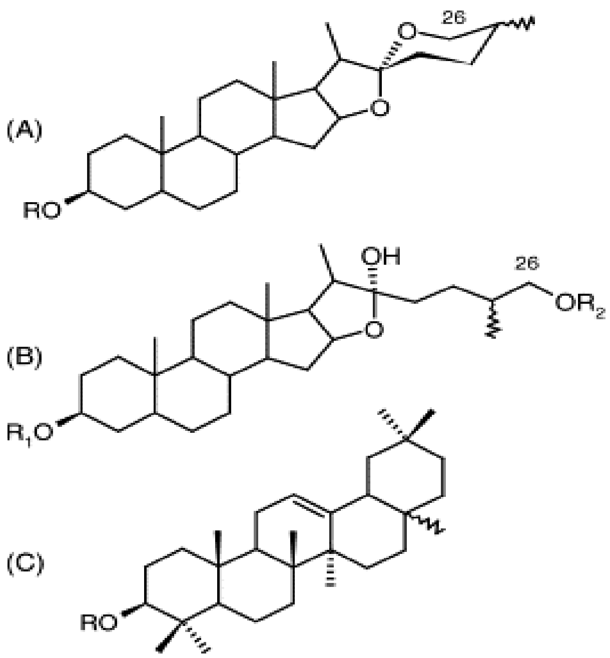


Figure 1.17. Aglycone skeleton of (A) stereoidal spirostane, (B) stereoidal furostone, (C) triterpenoid saponins. R = sugar moiety

Triterpene glycosides have either oleanane (β-amyrin) or dammarane skeletons, with ursanes, hopanes, lanostanes or lupanes assuming secondary importance with respect to distribution. The schematic correlation of main triterpene skeletones were given at Figure 1.18 (Nakanishi et al., 1974).

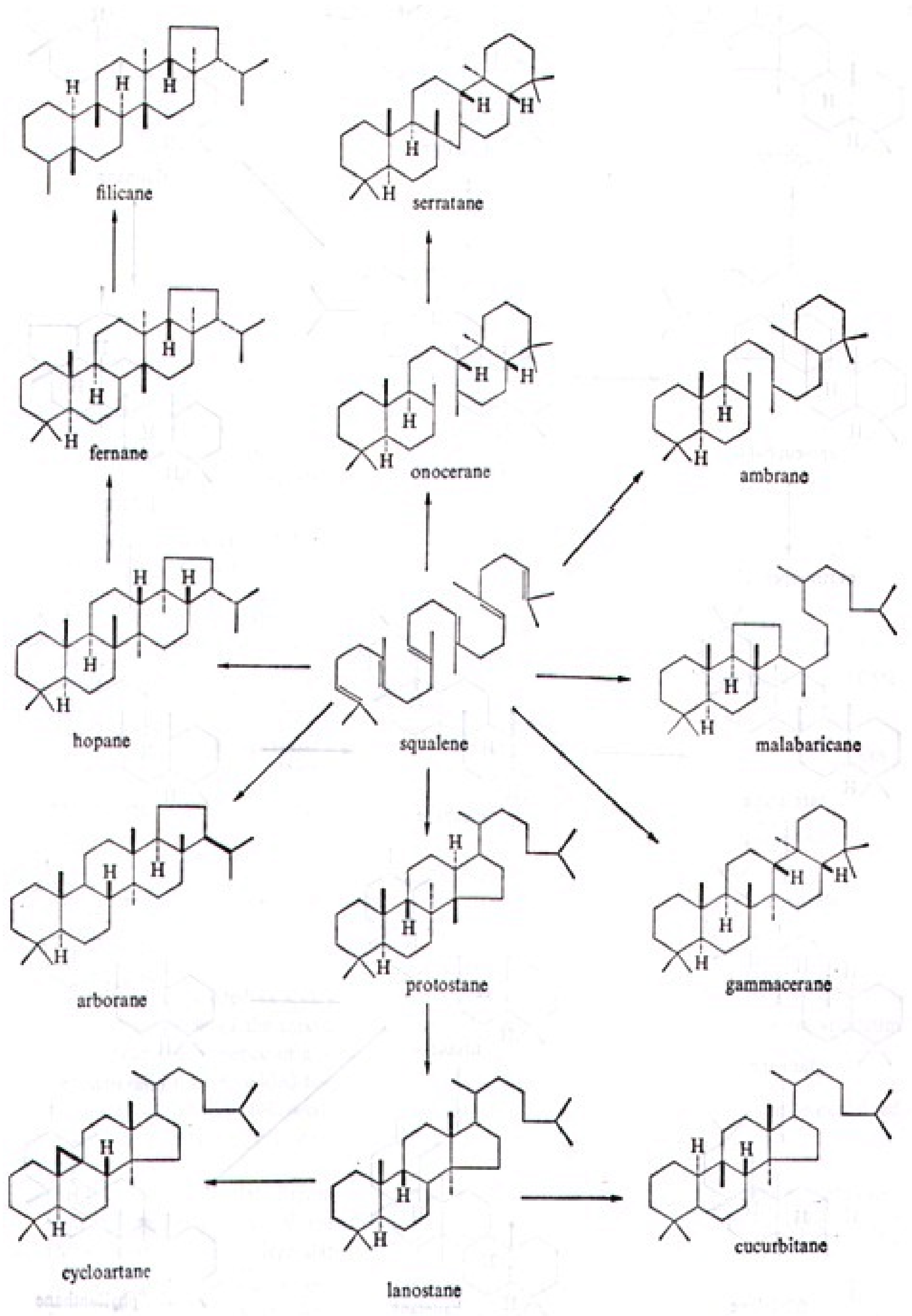


Figure.1.18. Schematic correlation of main triterpene skeletons

The pentacyclic triterpenoid skeletons exemplified by lupeol, α-amyrin, and β-amyrin (Figure 1.19) are frequently encountered in The form of triterpenoid saponins structures (Dewick and Paul, 2002).

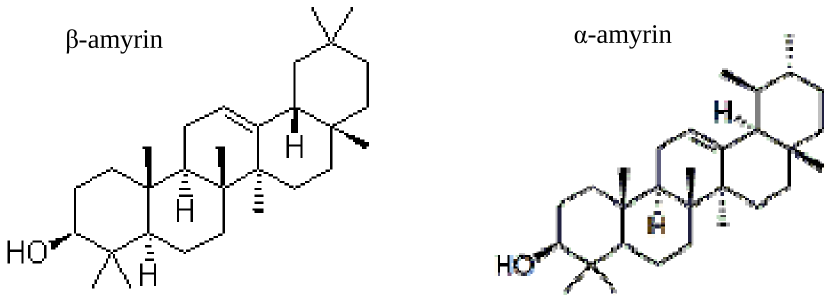


Figure 1.19. The pentacyclic triterpenoid skeletons, α-amyrin, and β-amyrin

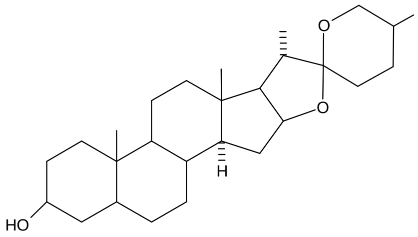
The triterpene saponins can be mono, bi, or even tridesmosidic. One sugar chain is often attached at C-3 and a second is frequently found to be esterified to the carboxyl group at C-17 of the aglycone (except certain dammarane glycosides and lanostane glycosides which have second or even a third glycosidically bound sugar chain). As monosaccharide units, D-glucose, L-rhamnose, L-arabinose and D-xylose occur widely (Hostettmann and Marston, 1995).

Steroid saponins are widely distributed in nature than the triterpenoid type. Monocotyledons are the main source of steroid saponins, with the Liliacea, Dioscoreaceae and Agavaceae providing many representatives.

The steroid saponins are divided into four groups depends on their aglycones (Figure 1.20) (Hostettmann and Marston, 1995).

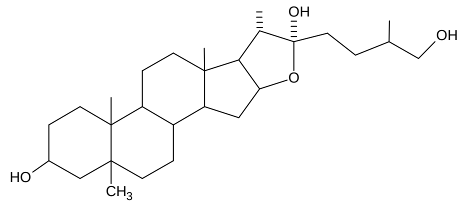
A. <u>SPIROSTANOL</u>

Sarsasapogenin



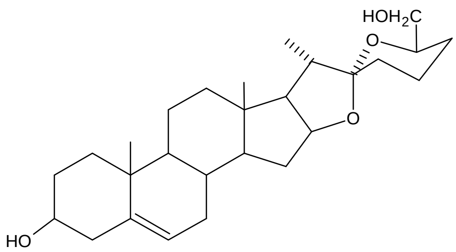
B. <u>FUROSTANOL</u>

(25 S) 5β-furostan-3 β, 22α, 26-triol



C. <u>NUATIGENIN</u>

Nuatigenin



D. <u>POLIPODO</u>

Polipodogenin

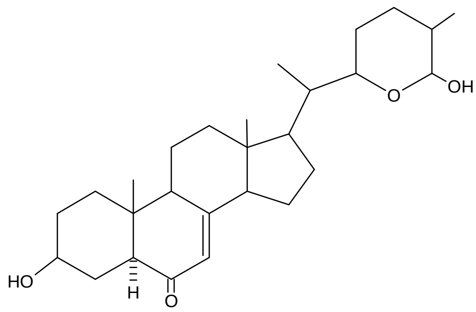
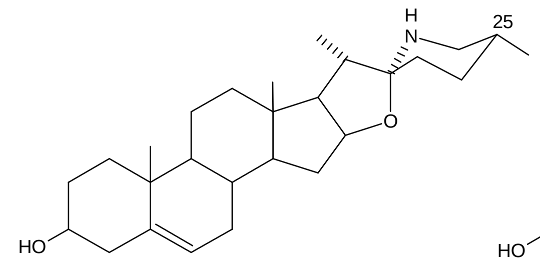


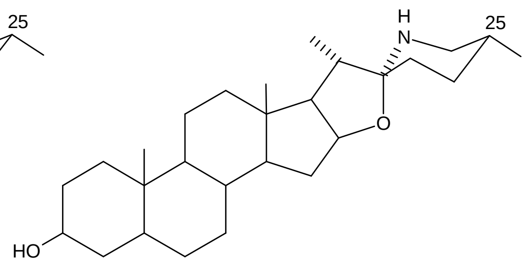
Figure 1.20 Classifications of Steroidal Saponins

The number and variety of naturally occurring steroid alkaloid glycosides can in no way be compared with the steroid glycosides. Even their distribution is limited and the Solanaceae family constitutes the largest source.

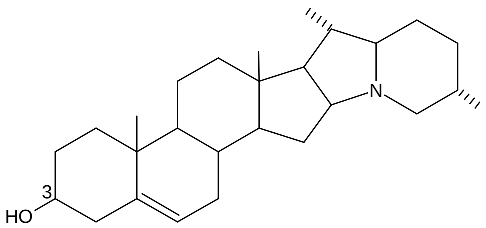
Glycoalkaloids are usually found in all organs of the plant, with the highest concentrations in regions of high metabolic activity, such as flowers, unripe berries, young leaves and sprouts.

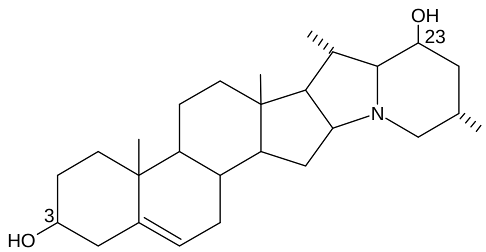
There are two basic skeletal types; the spirosolans and the solanidans. Examples of both types are given in Figure 1.21 (Hostettmann and Marston, 1995).





(A) Solasodine **(B)** Soladulcidine





(C) Solanidine **(D)** Leptinidine

Figure 1.21. Structures of spirosolan glycosides (A, B) and solanidan glycosides (C, D)

1.3.2 Pharmacological and biological activity of saponins

Triterpenoidal saponins have remarkable physiological and biological activities. There are number of drugs in traditional Chinese medicine which contain more saponins than alkaloids (Shibata et al., 1977)**.** It is not possible to describe the biological activities of saponins in detail here, but some salient features are mentioned. Saponins have also hemolytic properties and poisonous effect against fishes and shells. The pharmacological and biological activities of some important saponins containing plants are mentioned. The stem bark of <u>Akebiac quinata</u> (Lardizabalaceae) is used in Chinese medicine as an anti-inflammatory agent (Fujita et al., 1974). The roots of <u>Plletycodon grandi florum</u> DL. (Campanulaceae) also used in Chinese medicine as a combined expectorant and anti-tussive agent (Akiyama et al., 1972). The seeds of <u>*Aesculus*</u> <u>hippocastanum</u> (*Hippocastanaceae*) have been used in western folk medicine for the treatment of haemorrhoids and venous congestion and also show an anti-inflammatory action and it is administered orally for clinical use (Wulff and Tschesche, 1969). Saponins of <u>Bupleurum falcatum</u> L. are used as an important component of several prescriptions in Chinese medicine to resolve the tightness and resistance syndrome at the coastal margin that might be related to inflammation of the diaphragm and enlargement of liver caused by hepatitis. The main constituents of this drug are saponins named saikosaponins (Kubota and Hinoh, 1968). The saponins of the root of *polygala* tenuifolia (*polygalaceae*) are used in Chinese medicine as a sedative agent as well to strengthen nervous system. In western medicine the roots of polygala senega and senegae Radix are employed as an expectorant for bronchitis and asthema (Shibata et al., 1977).

Saponins isolated from *aralia mandshurica* and *callendule officinalis* represent a basis of new groups of medicines. *Aralia mandshurica* stimulate the granulocyte mother cells, phagocytosis of granulocytes and inhibits the tumor development in mice (Lutomski, 1983).

Saponins isolated from *zizyphus jujube* (*Rhamnaceae*) are used in Chinese medicine for strengthening the nervous system as a remedy for insomnia caused by both physical and mental strain. The constituents of this drug were isolated by Kawai et al., 1974.

Pharmacological studies of saponins shown that they possess sedative, antipyretic, anti-tussive and expectorant activities and also used to prevent stress ulcers, accelerates intestinal motility and shows anti-inflammatory activities

Licorice (liquorice), the root of *glycyrrhiza glabra* has been used since the ancient Greek time as expectorant, antitussive agent and an additive for sweetening, and as a drug for strengthening muscle and bone. It is also used as an antidote, the main function of licorice is a sweet tasting saponin (Shibata, 1977) , licorice extract that were effective clinically for treatment of gastric ulcer (Revers, 1946). Glycyrrhizin and its oleanane type aglycone revealed anti-ulcer (Khan and Suilivan, 1968) and anti-allergic action (Kawa et al., 1950; Kursu et al., 1954). The drug is also used as remedy of the Addison’s disease (Conn et al., 1968). In Chinese medicine licorice decoction is used to treat inflammation. In Europe licorice extracts were also used in a form of troche for similar purposes.

Panax ginseng (*Araliaceae*) is one of the most famous Chinese medicine used since ancient times and is equally popular even now. It is used as alternative, tonic, stimulant, carminative and demulcent. The root of *panax ginseng* is important in treatment of nervous disorder, anemia, heartache and nausea etc. Panaxin which increases muscular tone and gingenin supposed to show a beneficial effect on diabetes (Lily, 1980). Ginseng saponin is also used as remedy for bruise, hemorrhage and stimulated slight central nervous system. Antifatigue and tranquillizing activity were observed in ginsenoside (Khan and Suilivan, 1968; Takagi et al., 1974).

1.3.3 Biosynthesis

A brief biosynthetic summary of triterpenes and steroids is shown in Figure 1.22. They are built up of six isoprene units and have a common biosynthetic origin in that they are all derived from *squalene,* presumably via ring opening of squalene-2,3-epoxide (oxidosqualene), followed by a concerted cyclization. It is only recently that the corresponding *cyclases* have been characterized (Abe et *al.* 1993).

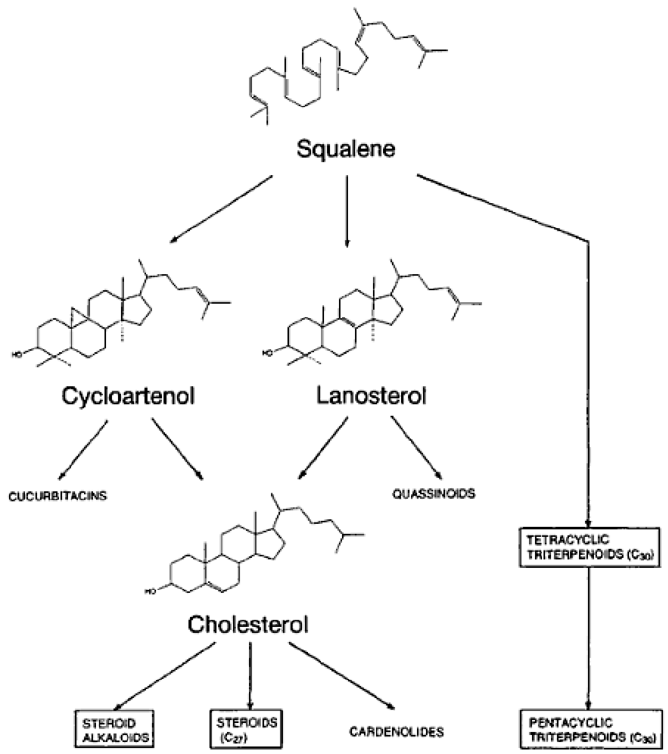


Figure 1.22. Biosynthesis of triterpenes and steroids

While the triterpenes have 30 carbon atoms, the steroids have only 27 carbons by virtue of the oxidative cleavage of three methyl groups from a C_{30} intermediate. Other details of the biosynthesis of steroids have been reviewed (Heftmann, 1968). Much work remains to be done to clarify the intermediates but at least the distinction between the cholesterol (C_{27}) and the triterpene (C_{30}) pathways is evident.

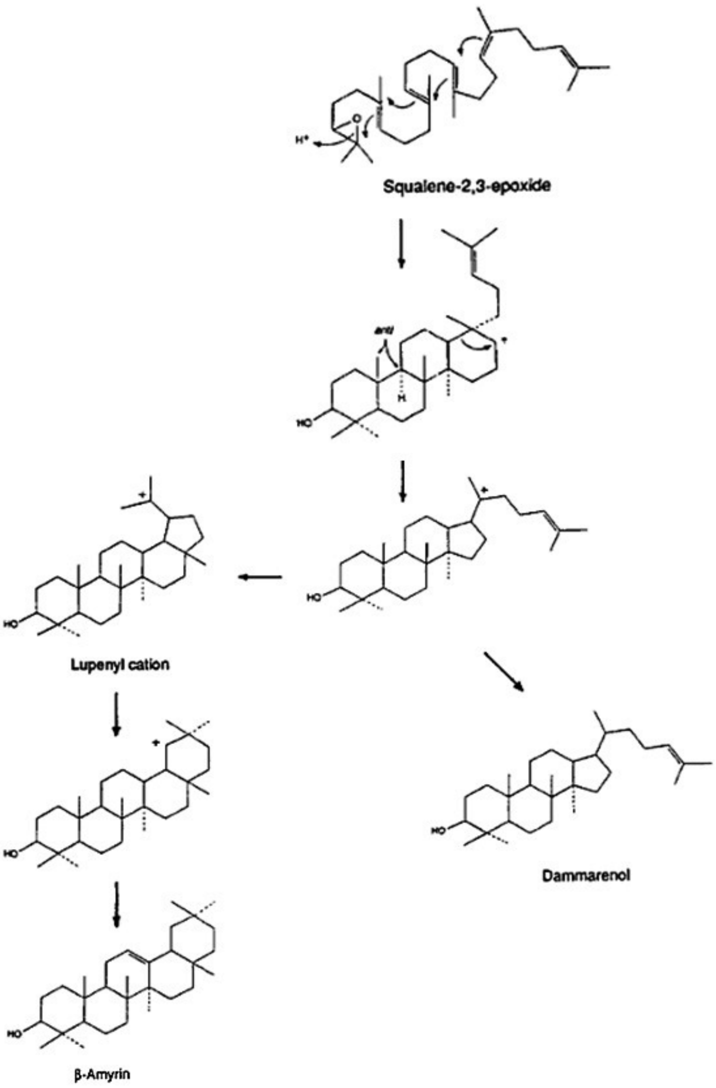


Figure 1.23. Cyclization of squalene-2,3-epoxide to C_{30} triterpenes

Figure 1.23 shows the cyclization of the chair-chair-chair-boat conformation of squalene-2,3-epoxide. Following the rearrangement of the tetracyclic carbonium ion, either a pentacyclic (e.g. β-amyrin) or tetracyclic (e.g. dammarenol) triterpene is formed (Luckner, 1990). More details are to be found in a review article by (Abe et al., 1993). In contrast, cyclization of the chair-boat-chair-boat conformation of squalene-2,3-epoxide, followed by several 1,2-rearrangements, leads to the formation of either cycloartenol or lanosterol (Figure 1.24).

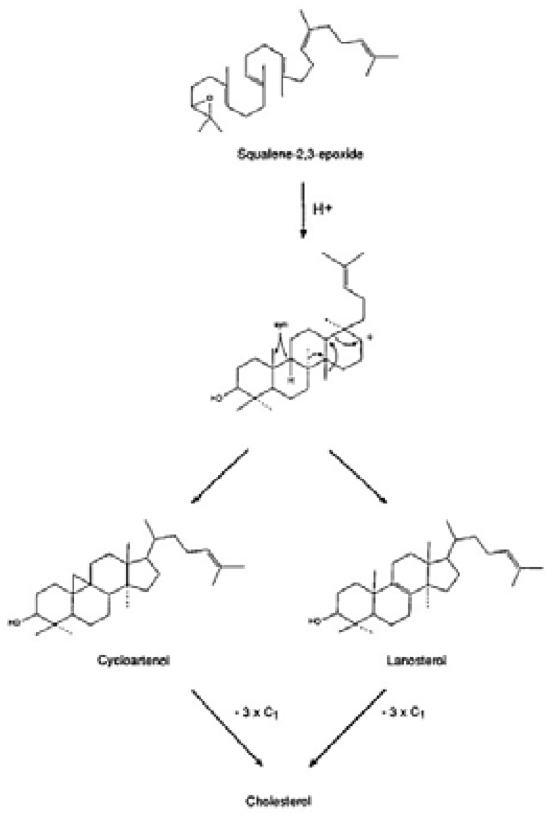


Figure 1.24. Cyclization of squalene -2,3-epoxide and formation of cholesterol

Oxidosqualene cyclases (OSCs) convert oxidosqualene to one or more cyclic triterpene alcohols with up to six carbocyclic rings (Figure 1.25). Plants biosynthesize diverse triterpenoids and encode multiple OSC enzymes to form these skeletons. *Cycloartenol synthase* (CAS) converts oxidosqualene to cycloartenol through the protosteryl cation intermediate (Figure 1.25) and was the basal plant OSC from which others derived. It can suggest that the known *cycloartenol synthases* are orthologs, and that cycloartenol synthase predates the emergence of plants. Lanosterol is the initial carbocyclic sterol precursor in animals, fungi, and trypanosomatids. Although substantial labeling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor, lanosterol biosynthesis has been demonstrated in a fewplants (e.g. in the latex of several Euphorbia species). β-Amyrin synthases forms the lupyl cation, but allow further ring expansion and some rearrangement before deprotonation to β-amyrin. Several OSCs from eudicots and monocots produce β-amyrin accurately. These β-amyrin synthases are considerably more distant from one another (48–50% identical) than are the CAS enzymes (70–79 % identical), and independent origins of β-*amyrin synthases* in eudicots and monocots have been proposed. Lupeol, β-amyrin, and their diverse metabolites are implicated in various plant processes. β-

Amyrin is a precursor of saponins, which are triterpene glycosides such as the antifungal saponin avenacin found in Avena roots (Phillips et al., 2006).

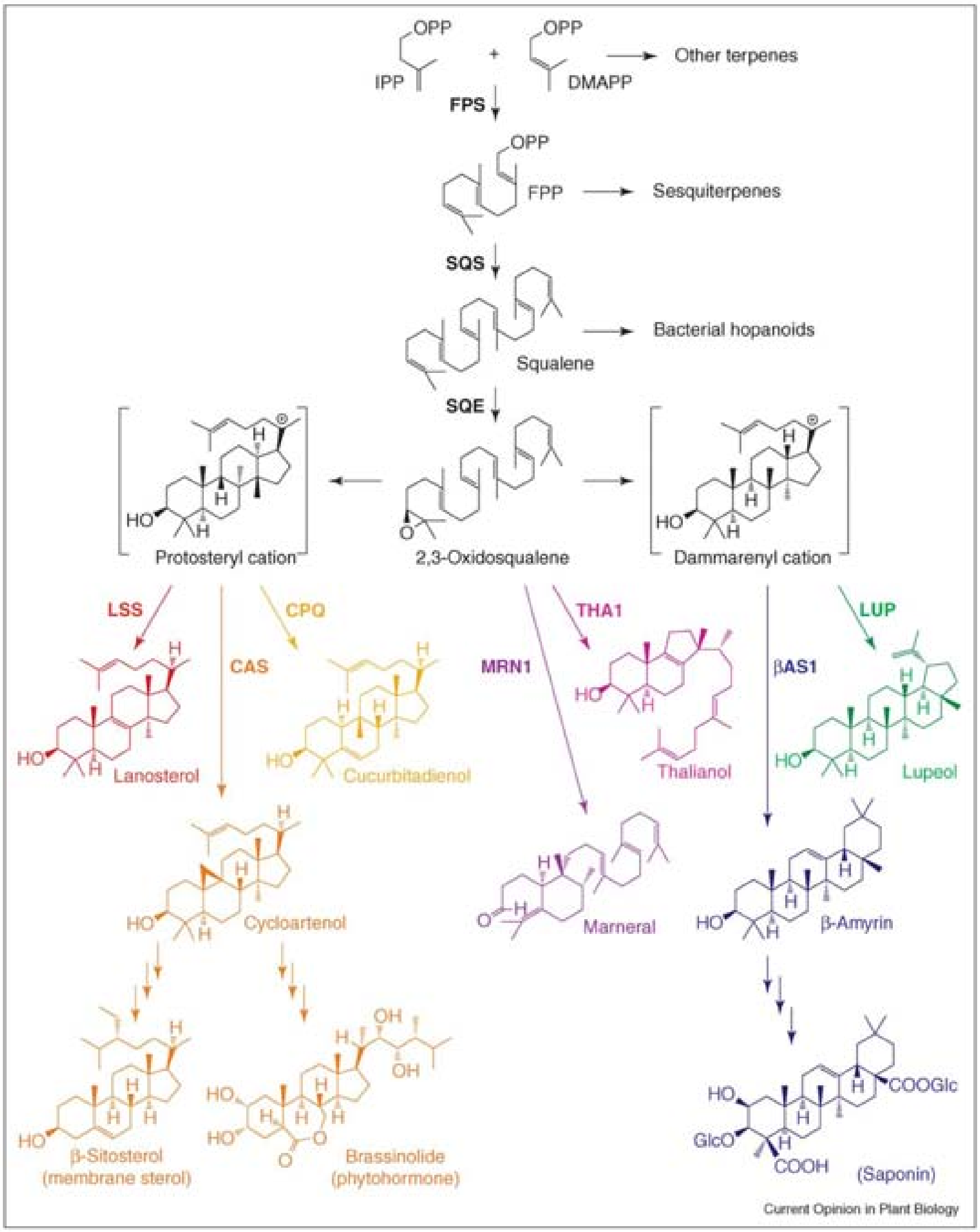


Figure 1.25. Simplified scheme of plant triterpenoid biosynthesis

Other important classes of secondary metabolites such as phytosterols, cardenolides, cucurbitacins, quassinoids and limonoids are also derived from squalene. There are similarities in the biosynthesis of cardenolides and steroid saponins. Both can start with cholesterol, both go through a 3-keto intermediate and both processes occur actively in *Digitalis* spp. (Heftmann, 1974). Like the saponins, many cardenolides give foams in aqueous solution but are classified separately because of their different biological activities (Figure 1.26).

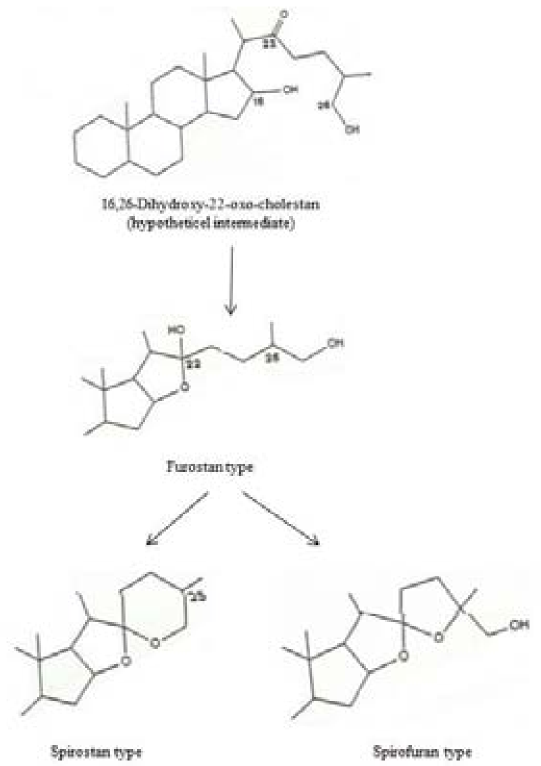


Figure 1.26. Biosynthesis of furostans and spirostans

1.4 Isolation and Analysis

The unique chemical nature of saponins demands tedious and sophisticated techniques for their isolation, structure elucidation and analysis. The task of isolating saponins from plant material is complicated also by the occurrence of many closely related substances in plant tissues, and by the fact that most of the saponins lack a chromophore. Thus, for many years, the complete characterization of saponins was not achieved. However, recently renewed interest in medicinal plants and foods alongside the dramatic evolution of analytical tools has resulted in a burst of publications presenting numerous novel saponins (Francis et al., 2002).

The isolation of pure saponins requires one or (as is almost always the case) more chromatographic separation steps in order to remove other polar constituents of alcoholic or aqueous plant extracts

A variety of modern separation techniques such as flash chromatography, DCCC, low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC) and HPLC are available, but a large number of the separations (especially the preliminary fractionation work) reported in the literature are still carried out by conventional open-column chromatography. The best results are usually achieved by strategies which employed a combination of methods.

1.4.1 Chromatography techniques

1.4.1.1 Open-column chromatography

Open column chromatography is often used as a first fractionation step for a crude saponin mixture but in certain cases may yield pure products .In general, though, the resolution is not high and complex mixture are only partially separated. Other problems are the loss of material because of irreversible adsorption and length of time required to perform the separation.

Silica gel chromatography with chloroform-methanol-water eluent is the most popular method and is still used in the majority of separations (Hostettmann and Marston, 1995).

As an addition to normal silica gel, coarse RP sorbents are now employed in the open-column chromatography of saponins. As long as the granulometry is not too fine and the columns not too long, gravity-fed columns are quite suitable. Rp-8 and rp-18 sorbents are the most frequent packing used. The commercially available particle sizes from 3 microns to 60 microns allow the use of vacuum chromatography (vlc) or medium to high pressure (mplc or hplc) chromatography depending on the desired load/resolution result. Thus, analytical and preparative separations can be performed; the analytical method can be easily transferred onto a preparative separation, if the chemistry of the sorbents is similar. The solvents of choice are mixtures of methanol-water or acetonitrile-water using gradient conditions (Verotta and El-Sebakhy, 2001).

RP chromatography is generally introduced after an initial silica gel separation step and enables a change in selectivity for the substances being separated. Another possibility is to introduce the reversed-phase separation after a DCCC step.

The use of dextran supports, as found in sephadex column packing, has been current practice for several years. Sephadex LH-20 finds the most frequent application but the ‘G’ series of polymers is not without interest. Typically, the polymeric supports are washed with water after loading the sample in order to elute monosaccharides, small charged molecules, such as amino acids, and other highly water soluble substances. Elution with a methanol-water gradient (or with methanol alone) is then commenced to obtain the saponin fractions. Other chromatographic techniques are employed for the isolation of pure saponins (Hostettmann and Marston, 1995).

1.4.1.2 Centrifugal thin-layer chromatography (CTLC)

The CTLC (Centrifugal Thin-Layer Chromatography) technique is a planar method related to preparative thin-layer chromatography (TLC) but without the need to scrape bands off the TLC plate. CTLC relies on the action of a centrifugal force to accelerate mobile phase flow across a circular TLC plate. The plate, coated with a suitable sorbent (1, 2 or 4 mm thickness), is rotated at 800 r.p.m. by an electric motor, sample introduction occurs at the center and eluent is pumped across the sorbent. Solvent elution produces concentric bands across the plate. These are spun off at the edges and collected for TLC analysis. Separations of 50-500 mg of a mixture on a 2 mm sorbent layer are possible. CTLC has been useful for the separation of a wide range of substrates from polyacetylenes to saponins and nucleotides. A limited number of applications in the separations saponins have been reported. For example a combination of ctlc with chloroform-methanol-water (100:30:3) and column chromatography was used for the isolation of ginsenosides. This method is relatively simple, fast, does not require scraping of bands the coated plate can be washed and regenerated step gradient elution is possible and the contact time between compound and stationary phase is reduced. In terms of loading capacity, a circular plate can tolerate the same amount of material as three ordinary tlc plates. The drawbacks are that coated plates are not commercially available, the maximum thickness of the layer is 4 mm and there is a restricted choice of stationary phases.

1.4.1.3 Flash chromatography

Flash chromatography is a preparative pressure liquid chromatography method which enables a considerable time saving when compared with conventional open-column chromatography. Ordinary glass columns are used but eluent is driven through a sorbent by compressed air or nitrogen, reaching a maximum pressure of about 2 bars at the top of the column. The granulometry of the sorbent is somewhat reduced because solvent is being delivered under pressure; resolution is consequently higher. Flash chromatography can be employed as a fast alternative to open-column chromatographic methods of preliminary fractionation; separations of 10 mg to 10 g of sample can be achieved in as little as 10 min. RP flash chromatography enables the easy separation of saponins from other, more polar, components such as oligosaccharides (Hostettmann and Marston 1995).

1.4.1.4 Low-pressure liquid chromatography (LPLC)

LPLC (Low-pressure liquid chromatography) is fast becoming one of the most popular methods for the isolation of pure saponins because of the speed of separation and ease of manipulation. LPLC employs columns containing sorbents with a particle size of 40-60 μm. High flow rates at pressures of up to 10 bars are possible and columns are mostly made of glass. Commercially available pre-packed columns in different sizes are ideal for the preparative chromatography of saponins in the 50-500 mg sample range. A high and uniform packing density guarantees a good separations efficiency. It is relatively easy to transpose analytical HPLC conditions onto an LPLC separation, given that the chemistry of the sorbents is similar. Most applications have been performed on RP sorbents, eluted with methanol-water mixtures. It is generally only pre-purified samples which are injected in this case.

1.4.1.5 Medium-Pressure liquid chromatography (MPLC)

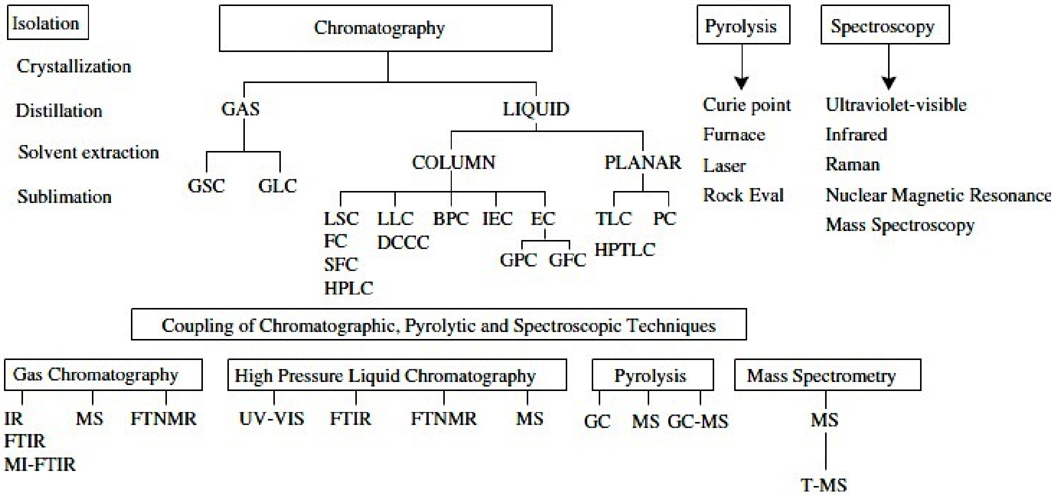
When relatively large amounts of pure saponins are required, mplc (medium-pressure liquid chromatography) is very useful. Unlike commercially available lplc equipment, gram quantities of sample can be loaded onto the column, while separations are run at pressures of up to 40 bars. The granulometry of the support normally lies in the 25-40 μm range and separations are, rapid, requiring considerably less time than open-column chromatography. A direct transposition of separation conditions from analytical hplc to mplc can be achieved on reversed-phase supports, thus facilitating the choice of solvent (Hostettmann and Marston, 1995).

1.4.1.6 High-Performance liquid chromatography (HPLC)

Chromatography by HPLC (High-performance liquid chromatography) is a powerful technique for obtaining multi-miligram quantities of saponins from mixtures of closely related compounds and, in this respect, is very frequently employed as a final purification step (Hostettmann and Marston, 1995). But the absence of a chromophore in saponins hampers their detection in ultraviolet light and allows non-specific detection in at 200-210 nm. Thus, most of published data are based on recording HPLC profiles at 200-210 nm. However, at this wavelength other than saponin components of the analyte may overlap with saponins making determination difficult. Only for 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone (DDMP) conjugated soyasaponins, which have an UV absorption maximum at 295 nm, glycyrrhetinic acid glycosides and cucurbitacins detection with UV-vis detectors could be successful. To overcome these problems and to be able to develop validated analytical methods for quality control of some products several trials were performed to apply evaporative light scattering detection (ELSD) for detection of saponins. This detector was successfully applied for measuring soyasapogenols A and B, separated on C18 column with MeCN-PrOH-H_2O-HOAc (80:6:13.9:0.1) in soybean. Validated HPLC method with ELSD was also developed for determination of major ginsenosides in samples of Chinese traditional medicine. Saponins were successfully separated on Spherisorb ODS2, C18 column in MeCN-H_2O gradient and quantified using calibration curves, with detection limits of 50 mg. (Oleszek and Bialy, 2006).

1.4.1.7 Counter current chromatograph (CCC)

Counter-current chromatography is a versatile but totally underused technique for the separation of plant metabolites. It provides efficient resolution of samples by a mechanism which relies solely on partition. In this respect, it is a method which is complementary to most other separation techniques. The technique is an all-liquid method, without solid phases, which relies on the partition of a sample between two immiscible solvents to achieve separation. The relative proportions of solute passing into each of the two phases are determined by the respective partition coefficients. Subsequent to the initial experiments, modern CCC has split into two basic directions. The first, which is called “CCC”, mainly involves apparatus with a variable gravity field produced by a double axis gyratory motion and a seal-free arrangement for the column (generally tubing wrapped around bobbins). The second has been termed “centrifugal partition chromatography", or CPC, and employs a constant gravity field produced by a single axis rotation, together with rotatory seals for supply of solvent. Separation takes place in cartridges or disks. CPC with cartridges or disks is a hydrostatic equilibrium system and can be likened to a static coil. If the coil is filled with stationary phase of a biphasic solvent system and then the other phase is pumped through the coil at a suitable speed, a point is reached at which no further displacement of the stationary phase occurs and the apparatus contains approximately 50% of each of the two phases. Steady pumping-in of mobile phase results in elution of mobile phase alone. This basic system uses only 50% of the efficient column space for actual mixing of the two phases. A more effective way of using the column space is to rotate the coil around its central axis while eluting the mobile phase. A hydrodynamic equilibrium is rapidly established between the two phases and almost 100 % of the column space can be used for their mixing. CCC with rotating coil instruments is an example of this latter mechanism. Counter-current chromatography benefits from a number of advantages when compared with the more traditional liquid-solid separation methods: (i) no irreversible adsorption; (ii) total recovery of injected sample; (iii) tailing minimized; (iv) low risk of sample denaturation; (v) low solvent consumption; and (vi) favourable economics (once the initial investment in an instrument has been made, no expensive columns are required and only common solvents are consumed). Although the efficiency (as represented by the number of theoretical plates) cannot match that of HPLC, the high selectivity and the high stationary to mobile phase ratio more than compensate. In HPLC, around 20% of the volume of the column is stationary (bonded) phase around the silica support, available for interaction with solute, while in CCC the figure for stationary phase content can be as high as 80%. Another advantage of CCC is the ability to reverse the flow direction and interchange the mobile and stationary phases ("reversed-phase" operation). Counter-current chromatography is an excellent alternative to circumvent the problems associated with solid-phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation. With these advantages, CCC is gaining popularity as a purification tool for natural products, and especially in the bioassay-guided fractionation of plant-derived compounds. The technique has evolved rapidly from the initial, time consuming applications with droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCCC), such that the new generations of instruments are referred to as high-speed CCC (or HSCCC) and high-performance CPC (or HPCPC) (Marston and Hostettmann, 2006). Classifications of different CCC instruments were given (Schema 1.1) (Hostettmann and Marston, 1995).



Schema 1.1 Chromatographic and spectroscopic techniques for detection and identification of organic compounds

1.5 Astragalus Genus

1.5.1 General on the Genus

The genus Astragalus belonging to the Leguminosae family is widely distributed throughout the temperate regions of the world, located principally in Europe, Asia and North America. About 2000 species have been described, 372 of them in North America and 133 in Europe (Polat, 2009a). *Astragalus* is a well represented species that numbers more than 2200 species growing worldwide (Mamedova and Isaev, 2004).

The genus *Astragalus L.* is one of the largest and most widely distributed genera belonging to the family Leguminosae, comprising 380 species distributed mainly in the flora of Turkey (Davis, 1970;Calıs et al., 2008; Tabanca et al., 2005; Ozipek et al., 2005). In the course of studies on Turkish *Astragalus* species several cycloartane- and oleanan-type triterpene glycosides were isolated and their structures were elucidated (Bedir et al., 1998a,b, 1999a,b, 2000; Calis et al., 1999; Yesilada et al., 2005; Calıs et al., 2006).

Astragalus L., the largest genus in the family Leguminosae, comprises 2000 species distributed mainly in the northern temperate regions and tropical African mountains and in particular it is represented by 32 species indigenous to Egypt (Perrone et al., 2008; Radwan et al., 2004).

Polysaccharides, saponins and flavonoids have been reported from *Astragalus* species (Tang and Eisenbrand, 1992; Bedir et al., 1998, 1999, 2000, 2001, 2005, 2009, 2010; Tabanca et al., 2005).

1.5.2 Medicinal and Biological Properties

Species of genus Astragalus are known to have numerous pharmacological activities and are used for medicinal purposes in many countries. The properties of these plants have been associated with its triterpene saponins and polysaccharides. The reason for the rising interest towards the triterpene saponins is due to their immunomodulatory, anti-cancer and antiviral activities. A number of reports have demonstrated that extracts from Astragalus species stimulated immune functions both in vivo and in vitro (Toshkova et al., 2007).

Some species of *Astragalus* genus, such as *Astragalus corniculatus, Astragalus glycyphyllos* (medical plants from the Bulgarian areas) and *Astragalus membranaceus*, are used as Medicinal plants. *A. membranaceus* could inhibit the development of tumor, decrease the toxic adverse effect of chemotherapy and elevate the immune function of organism. In the Bulgarian traditional medicine *A. glycyphyllos* and *A. corniculatus* is used as an antihypertensive, diuretic and anti-inflammatory remedy (Nikolov, 2006; Toshkova et al., 2007).

Astragalus, an herbaceous perennial native to northern China and Tibet has been widely used in China as a component of Fu-zheng therapy which is intended to augment the innate defenses of the individual against disease. A variety of in vitro and in vivo studies have identified astragalus' actions on the immune system including: increasing the proliferation of lymphocytes; increasing the cytotoxicity of natural killer cells; and increasing ing the secretion of tumour necrosis factor-alpha and beta. One controlled in vivo study of mice with renal cell carcinoma found that the mice receiving 500 mcg each of astragalus and Chinese privet (*Ligustrum lucidum* Ait.) intra-peritoneally daily for 10 days had a significantly higher cure rate than saline controls.

In addition, several studies have assessed the effects of astragalus (in combination with other herbs) as an adjunctive cancer therapy. One study of Fu-zheng therapy (which includes astragalus) as an adjunctive to conventional medical treatment in 572 cancer patients reported reduced bone marrow depression, fewer gastrointestinal adverse effects and protection of adrenal cortical function during chemotherapy and radiation treatments. Another study assessing the use of traditional Chinese medicine (including astragalus) as an adjunctive treatment to standard medical care in 54 consecutive patients with small cell lung cancer reported that the patients on average survived longer than patients treated with conventional medical treatment alone (based on previous statistics rather than a control group).

There are currently no known adverse effects or drug interactions associated with the ingestion of astragalus. It should also be noted that it is rarely given as a single herb, being used more often as one ingredient in traditional Chinese formulae. There is currently no clinical evidence that astragalus can be used as an alternative to conventional cancer treatment; however, further research into its role as an adjunctive treatment appears to be warranted (Smith et al., 1999).

Cycloartane saponins isolated from genus *Astragalus* exhibited a wide range of biological properties, including cardiotonic, analgesic, sedative, hepatoprotective, antiviral and immunostimulant activities (Verotta et al., 1998).

The roots of several *Astragalus* species are well-known in traditional medicine for the treatment of nephritis, diabetes, leukemia, uterine cancer and as an antiperspirant, diuretic and tonic (Calis et al., 2008). *Astragalus microcephalus* is used primarily in Turkey for the production of the economically important gum, tragacanth. *Astragalus* species are rich in cycloartane-type triterpene glycosides. Cycloartane- and oleanane-type glycosides from Astragalus species show interesting biological properties, including immunostimulating (Yesilada et al., 2005; Calis et al., 1997; Bedir et al., 2000), anti-protozoal (Ozipek et al., 2005), antiviral (Gariboldi et al., 1995) and cytotoxic activities (Radwan et al.,2004; Calıs et al., 2008). Some cycloartane glycosides have been shown to have antitumor and AIDS antiviral activity (Ozipek et al., 2005). Moreover, for example Astragaloside IV, a widely encountered 20,24-epoxy cycloartane glycoside found in Astragalus species, has been proven to be a neuroprotective agent and proposed as a potential agent in the treatment of Parkinson’s disease (Luo et al., 2004; Chan et al., 2009; Horo et al., 2010).

Chemical studies on Astragalus saponins have indicated the presence of cycloartane-type triterpenoid glycosides which were found to exert biological activities, e.g. antiinflammatory, analgesic, diuretic, hypotensive and sedative effects (Polat et al., 2009a, 2010).

In the district of Anatolia, located in South Eastern Turkey, an aqueous extract of the roots of Astragalus species is traditionally used against leukemia and for its wound healing properties. (Calis et al., 1997; Bedir et al., 2000**;** Polat et al., 2009a, 2010).

The former compounds of several *Astragalus* species are reported to possess anticancer and immunostimulating effects (Rios and Waterman, 1997; Bedir et al.2000; Yesilada et al., 2005). Astragalus polysaccharides are known to have anticancer and immune enhancing properties in both in vitro and in vivo experiments (Polat et al., 2009a).

1.5.3 Toxicity

The genus Astragalus appears highly uniform from chemical point of view, with two kinds of pharmacologically active principles and three different kinds of toxic compounds. In the former group, the polysaccharides and the saponins stand out, and in the second, the indolizidine alkaloids **(Figure 1.29)**, the nitro compounds and 3-nitropropyl glucosides **(Figure 1.27 and 1.28)**, and the selenium compounds (Polat et al., 2009a, 2009b).

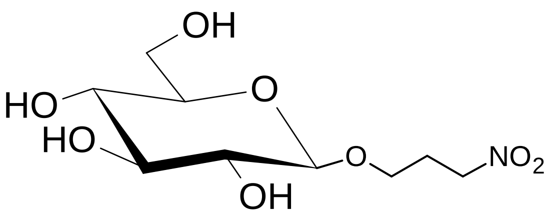


Figure 1.27. Miserotoxin (3-nitro-1-propyl-β- D-glucopyranoside)

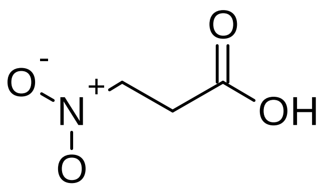
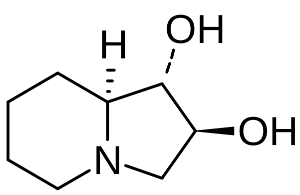
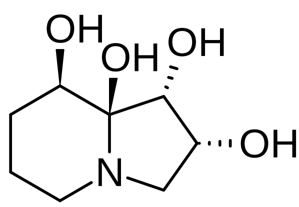


Figure 1.28. 3-nitropropionic acid(3- NPA)



Lentiginosine



Swainsonine

Figure 1.29. Indolizidine alkaloids glycosidase inhibitors occuring in *Astragalus* species

Some *Astragalus* species including toxic compounds are listed below (Polat et al., 2009b).

Species that include 3-nitropropyl compounds

A.miser, A.falcatus, A. glycyphyllos, A.hamosus, A.lusitanicus

Species that include Indolizidine alkaloid compounds

A. lentiginosus, A. pubentissimus, A. mollissimus, A.wootoni, A. thuseri, A. nothrosys

Species that include Selenium compounds

A. bisulcatus, A. Saurinus, A. praelongus, A.flavus, A.tenellus

1.6 Cycloartane Glycosides

(+)-Cycloartenol, also known as cyclobranol (**Figure 1.11.)**, from the fruits of *Strychnos nux vomica* (Loganiaceae), in the leaves of potato *Solanum tuberosum* (Solanaceae) and the seed of rice *Oryza sativa* (Poaceae), is one typical representative of more than 120 naturally abundant cycloartanes (Breitmaier, 2006).

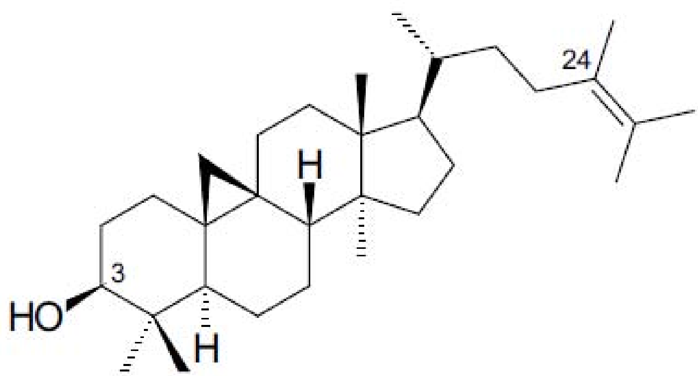


Figure 1.30. (+)-24-methylcycloarta-24-en-3β-ol (cyclobranol, cycloartenol)

Triterpenoids are often found among secondary plant metabolites. Triterpenoids of the cycloartane, lanostane, and oleanane **(Figure 1.30.)** types are observed. They all originate biogenetically from a single precursor, squalen- 2,3-oxide. Nevertheless, whereas cycloartanes and lanostanes are biogenetically related, oleananes have no biogenetic link to them. A peculiarity of phytosteroid biosynthesis is the generation of an additional 9,19-three-membered ring (cyclopropane) during cyclization of squalen-2,3-oxide with subsequent opening of this ring. This is responsible for the biogenetic relationship of cycloartane

(Figure 1.32.) and lanostane **(Figure 1.31.)** triterpenoids. Cycloartanes are derivatives of 9β,19-cyclolanostane and are produced exclusively by photosyntheic eukaryotes.

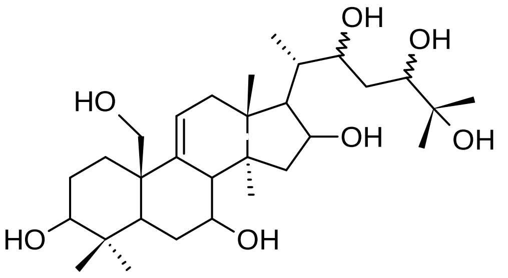


Figure 1.31. Lanostane triterpenoid (Orbigenin)

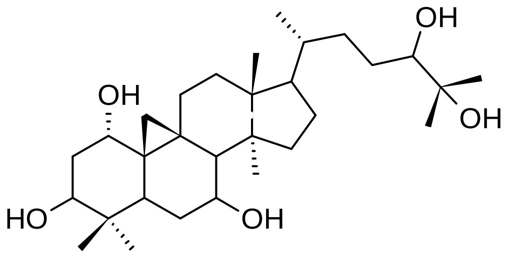


Figure 1.13. Cycloartane triterpenoid (Cyclomacrogenin B)

As a rule, the carbohydrate parts of the glycosides consist of D-glucose, D-xylose, L-arabinose, and L-rhamnose **(Table 1.1**). The branched monosccharide D-apiose is rather rare and found in two glycosides (Mamedova and Isaev, 2004).

1.6.1 Classification

Structures of cycloartane glycosides show that the side chains of the compounds differ substantially. Therefore, our Classification of these substances is based on structural features of the side chains. The known cycloartane methylsteroids of *Astragalus* plants can be divided into six structural types according to the side-chain structure (Mamedova and Isaev, 2004):

1) Cycloartanes with an acyclic side chain **(Figure 1.14., 1-6),**

2) 20,24-epoxycycloartanes **(Figure 1.15., 7-12),**

3) 16*β*,23;16*α*,24-diepoxycycloartanes (**Figure 1.16., 13 and 14**),

4) 16*β*,24;20,24-diepoxycycloartanes **(Figure 1.17., 15),**

5) 20,25-epoxycycloartanes (**Figure 1.17., 16**),

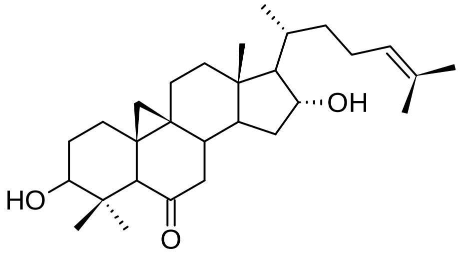
6) 24-nor-16*β*,23-epoxycycloartanes **(Figure 1.17., 17 and 18).**

Furthermore, There are also three classes which are reviewed in Ph. D thesis by Polat (2009b).

7) 16*β*,23; 22*β*,25-diepoxycycloartanes **(Figure 1.18., 19 and 20)**

8) 16*β*,23;23,26;24,25-triepoxycycloartanes **(Figure 1.19., 21)**

9) 16 *β*,23-epoxycycloartanes (**Figure 1.20., 22 and 23)**



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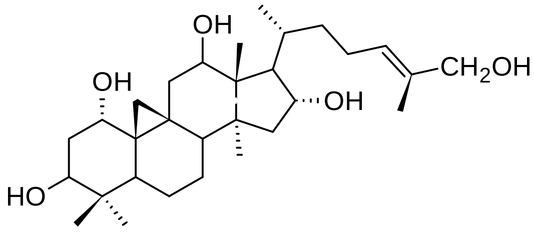

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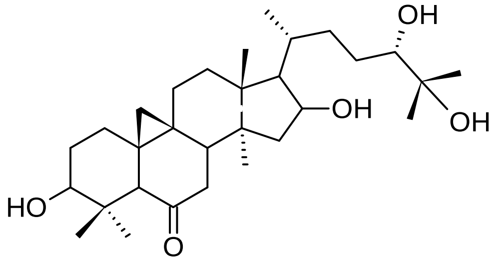

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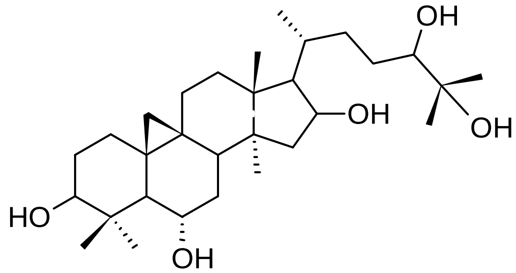

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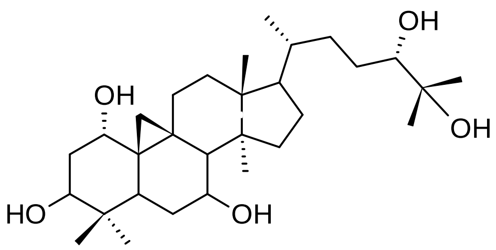

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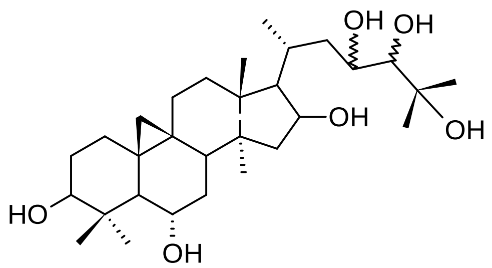

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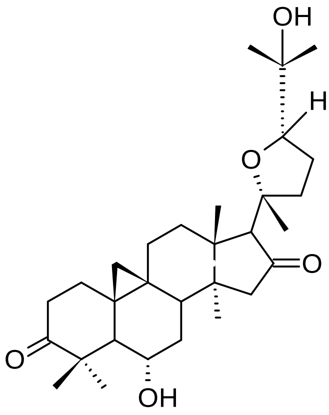
Figure 1.33. Some cycloartanes with an acyclic side chain

Triterpenoids of *Astragalus* plants are polyhydroxycompounds. Only genin **1** retains the intact side chain of cycloartenol. The side chain of the remaining compounds is oxidized to one degree or another. Most of the compounds contain in the acyclic side chain an α-diol on C-24–C-25 and a 16β-hydroxyl (Memedova and Isaev, 2004).



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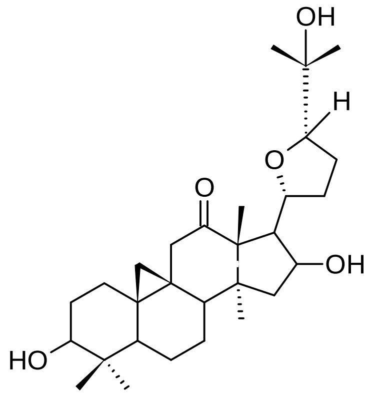

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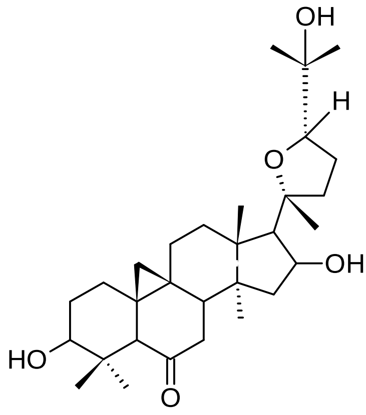

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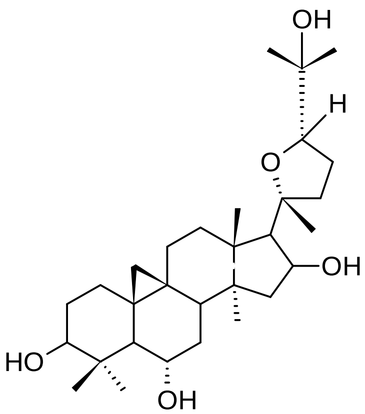

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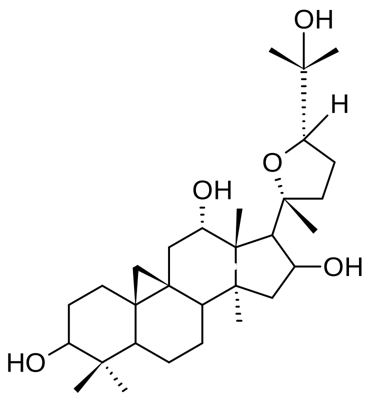

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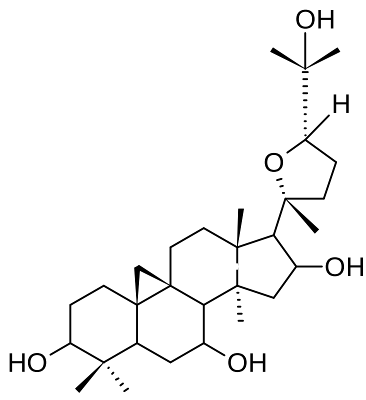

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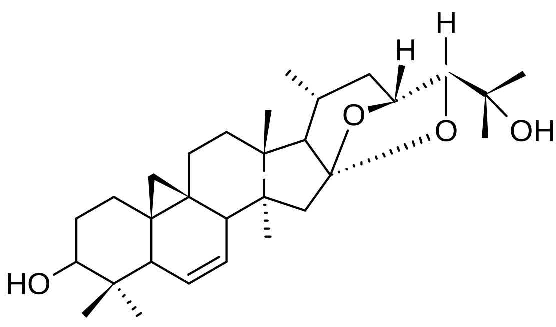
Figure 1.34. Some 20,24-epoxycycloartanes



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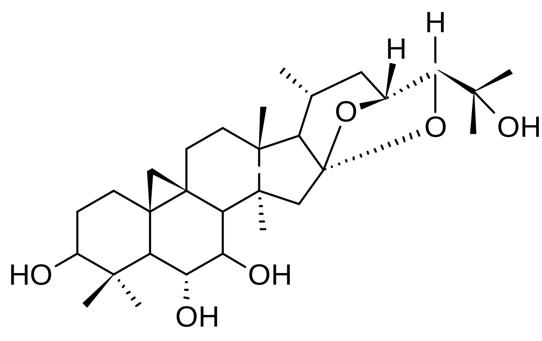

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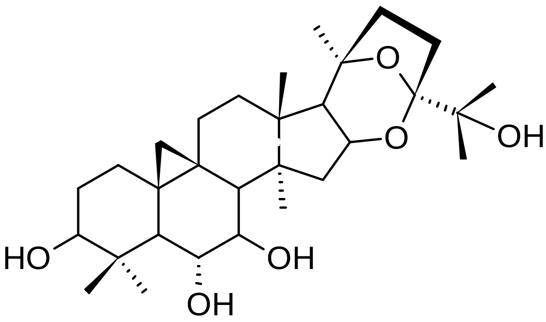
Figure 1.35. 16β,23;16α,24-diepoxycycloartanes



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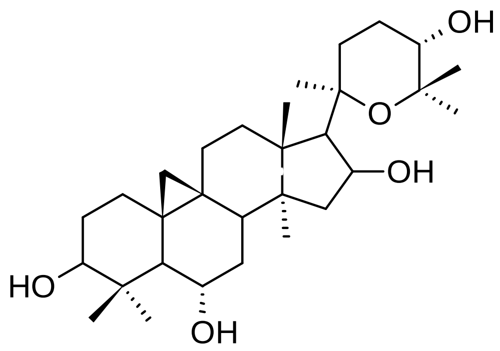

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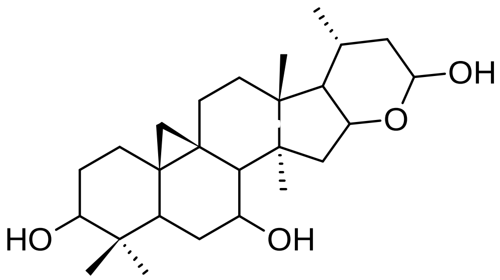

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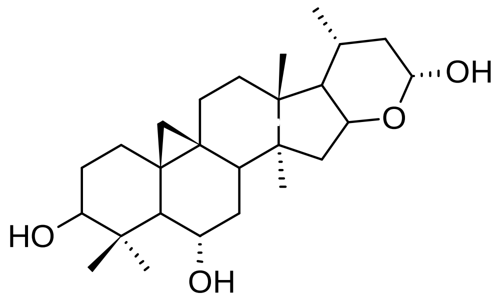

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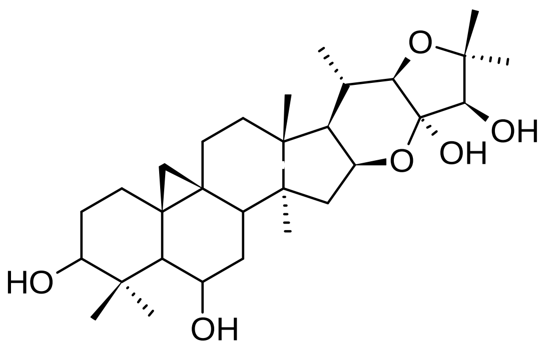
Figure 1.36. 16*β*,24;20,24-diepoxycycloartanes (15), 20,25-epoxycycloartanes (16), 24-nor-16*β*,23-epoxycycloartanes (17, 18)



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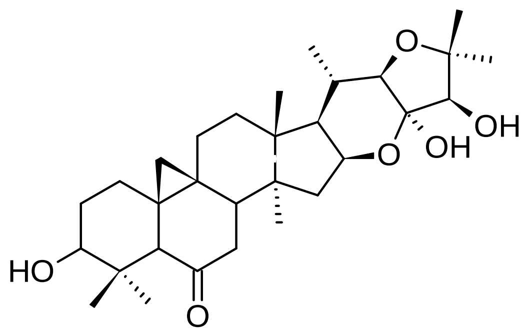

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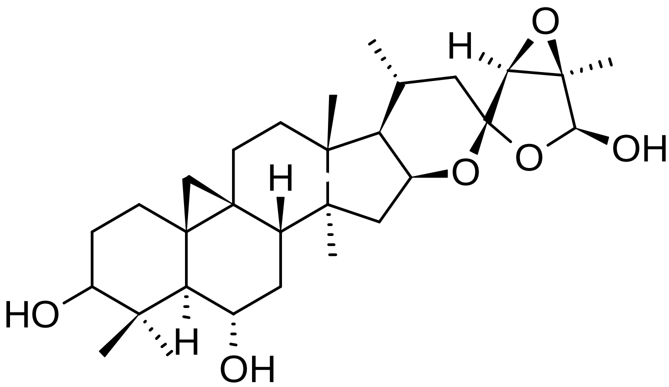
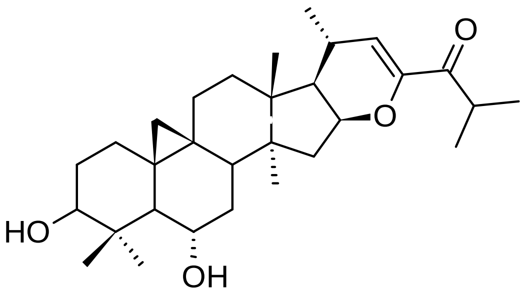
Figure 1.37. 16*β*,23;22*β*,25-diepoxycycloartanes



21

Figure 1.38. 16 *β*,23;23,26;24,25-triepoxycycloartanes



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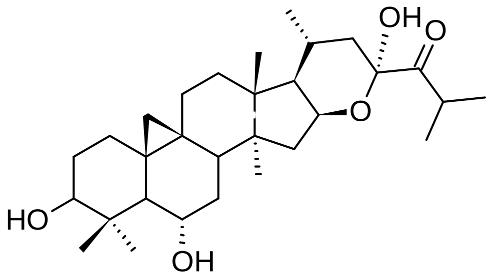


23

Figure 1.39. 16*β*,23-epoxycycloartanes

2. MATERIALS AND METHODS

2.1 General

In Spectroscopic identifications, optic rotations were measured using a Perkin Elmer 341 Model Polarimeter. The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 400 MHz (^1H) and 100 MHz (^{13}C) in *d6*-DMSO, using TMS as internal standard. 2-D NMR spectra (COSY, HMQC, HMBC) were run using a standard Bruker pulse programs. The delay for long-range coupling in the HMBC (1/2 J) was set to 63 msec. Column chromatography was carried out on silica gel 60 (Merck 7734) and Li Chroprep RP (C-18) (Merck 9303). Analytical TLC was conducted on pre-coated silica gel 60 F_{254} aluminium sheets (Merck 5554 and Merck 5569).

Compounds were detected at 254 and 366 nm UV lamb by using Desaga Uvis. The spots were detected by 20 % H_2SO_4 water spraying reagent onto the TLC plates followed by heating the plates to 110^o C until the spots become visible.

During chromatographic studies (CC and TLC controls) the following solvent systems were used:

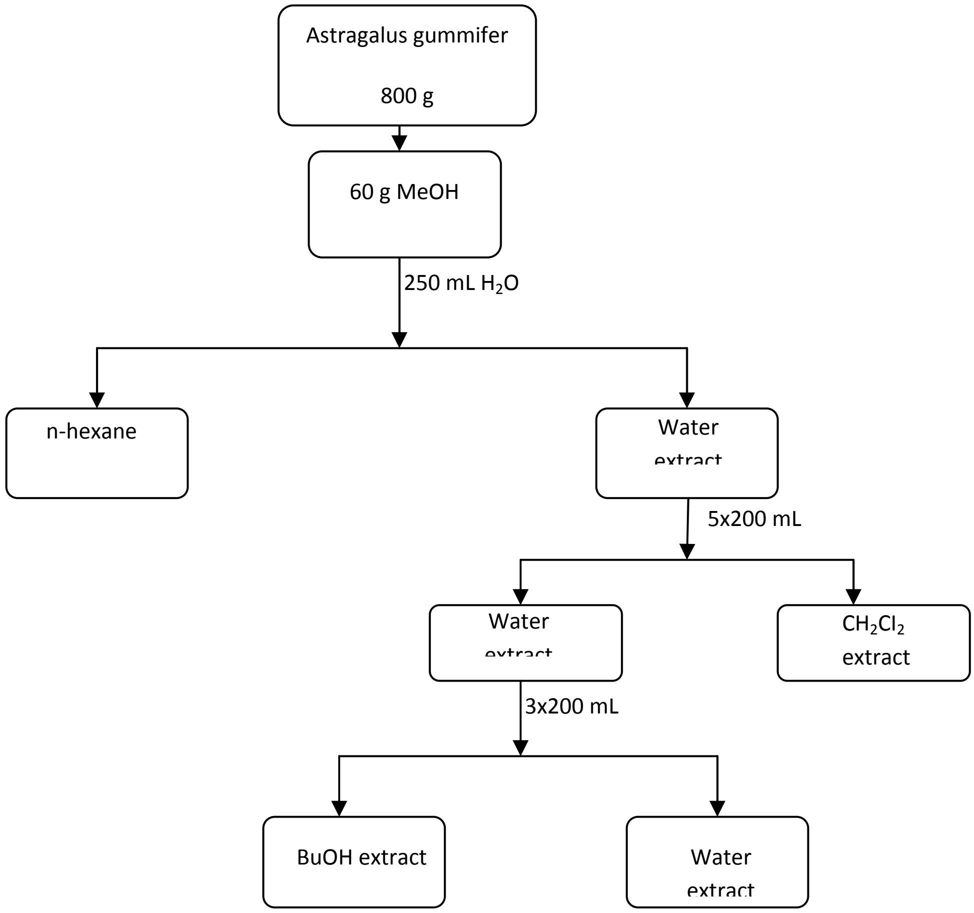
I	$CHCl_3$:MeOH	90:10:1
II	$CHCl_3$:MeOH:H_2O	80:20:2
III	$CHCl_3$:MeOH:H_2O	75:25:2.5
IV	$CHCl_3$:MeOH:H_2O	70:30:3
V	$CHCl_3$:MeOH:H_2O	61:32:7
VI	$CHCl_3$:MeOH:H_2O	64:50:10
VII	MeOH:H_2O	8:2
VIII	MeOH:H_2O	7:3
IX	MeOH:H_2O	6:4

2.2 Plant Material

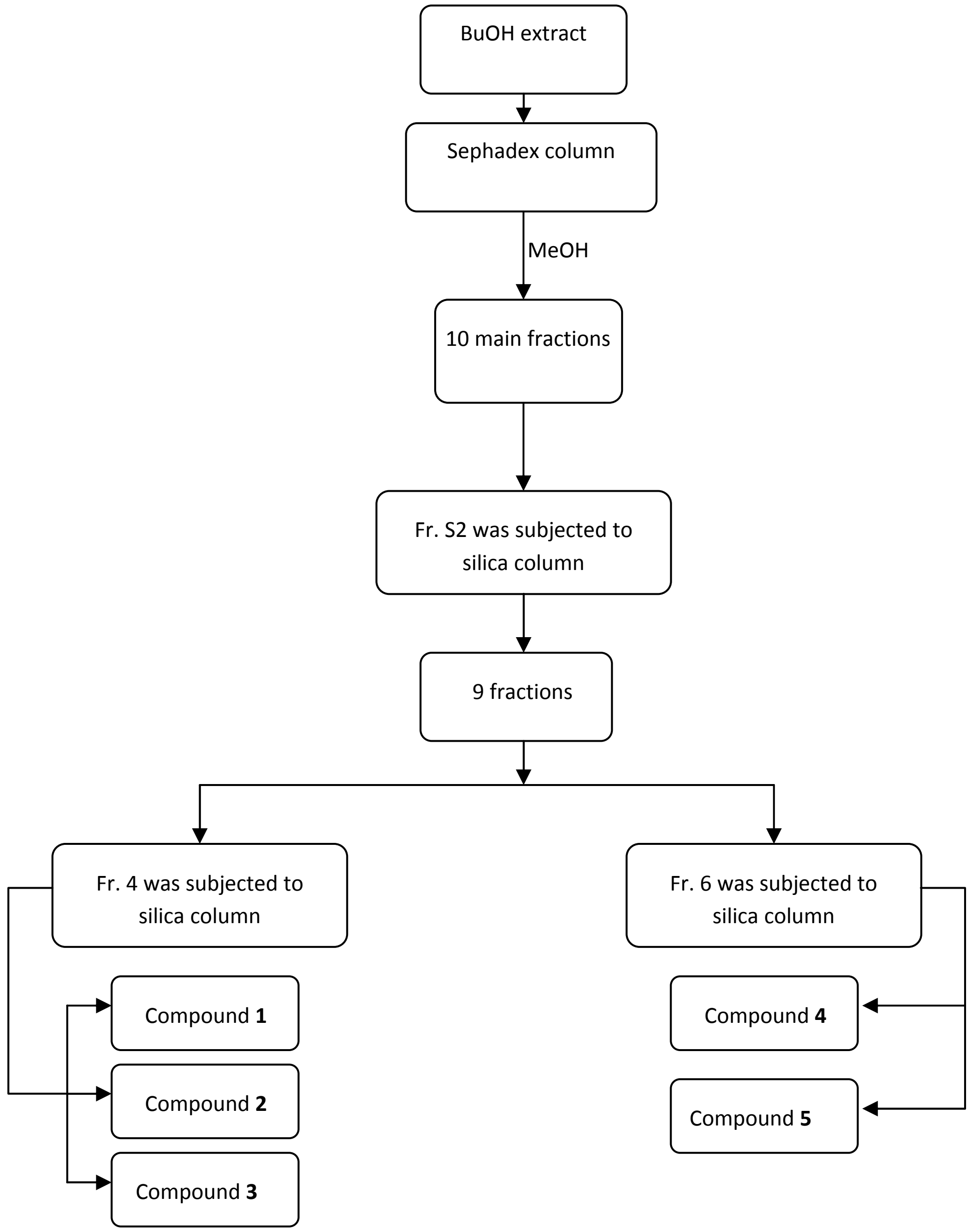
Astragalus gummifer was collected from Karadere Village, from altitude of 1750 m, Muş, East Anatolia, Turkey in 15.08.2010. Samples of plant material were identified by F. Ozgokce (Department of Biology, Faculty of Science and Art, Yuzuncu Yıl University, Van, Turkey). Voucher specimen has been deposited in the Herbarium of Yuzuncu Yıl University, Van, Turkey (VANF 13806).

2.3 Isolation and Purification

Air-dried and powdered plant material of *Astragalus gummifer* (Whole plant, 800 g) was extracted with MeOH (3 x 4 l) at 60 ºC. The methanolic solution was then evaporated to dryness under reduced pressure and gave 60 g of a dark residue. This residue was suspended in H_2O (250 ml), and successively partitioned with *n*-hexane (2 x 200 ml), CH_2CI_2 (5 x 200 ml) and *n*-BuOH saturated with H_2O (3 x 200 ml). The *n*-BuOH phase was chromatographed over sephadex column chromatography to give ten main fractions (S1-S10). Fraction S2 (3 g) was submitted to silica gel column chromatography with the solvent system $CHCl_3$-MeOH-H_2O (80:20:2, 1500 ml; 70:30:3, 2000 ml) to give 9 fractions. Fraction 4 was subjected to silica gel column chromatography with the solvent system $CHCl_3$-MeOH-H_2O (80:20:2, 1000 ml; 70:30:3, 1000 ml) to yield **1** (6 mg), **2** (10 mg) and **3** (20 mg). Fraction 6 was submitted to silica gel column chromatography with the solvent system $CHCl_3$-MeOH-H_2O (80:20:2, 750 ml; 70:30:3, 1000 ml) to yield **4** (10 mg), **5** (10 mg).



Schema 2.1. Extraction of *Astragalus* gummifer



Schema 2.2. Isolation of compounds

3. RESULT AND DISCUSSIONS

3.1 Structural Identification of Compound 1

The ^{1}H NMR spectrum of **1** showed characteristic signals due to cyclopropane-methylene protons as an AX system (δ 0.41 and 0.58, AX system, J_{AX}= 4.2 Hz; H_2-19) and seven tertiary methyl groups (δ 0.98, 1.03, 1.16, 1.23, 1.26, 1.29, and 1.31). No anomeric proton was observed. This observation was supported by the HMQC and HMBC correlation. Thus, compound **1** was considered to be a cycloartane-type triterpen saponin in genin form. The remaining carbon and proton resonances consistent with $C_{30}H_{50}O_5$ for **1**. This implied six saturated ring systems because there were no olefinic protons. Additional functionalities on the genin included three geminal methine proton on oxygen-bearing carbon atoms (H-3, H-6, and H-16). The resonances for the oxygenated carbons also indicated the presence of three oxymethine carbons (δ 78.0, 68.8, and 73.4; C-3, C-6, and C-16, respectively) and three oxygenated quaternary carbons (δ 88.3, 81.4, and 72.5; C-20, C-24, and C-25, respectively). Consequently, the structure of **1** was established as cycloastragenol (Kitagawa et al., 1983).

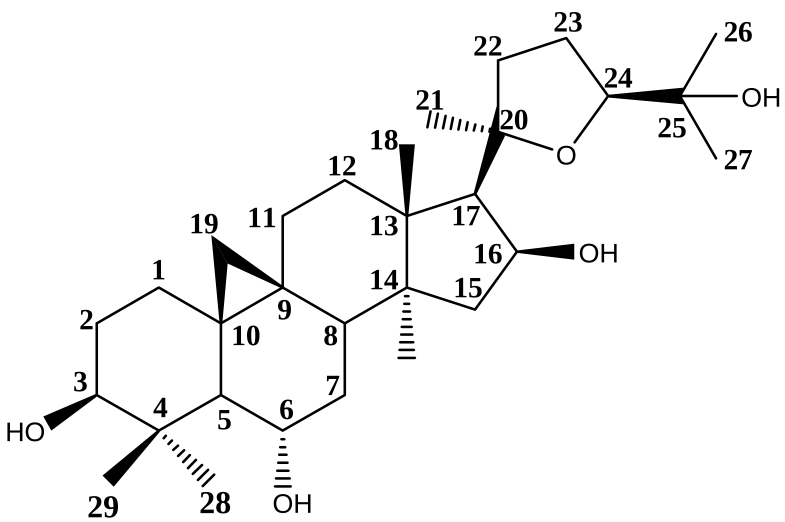
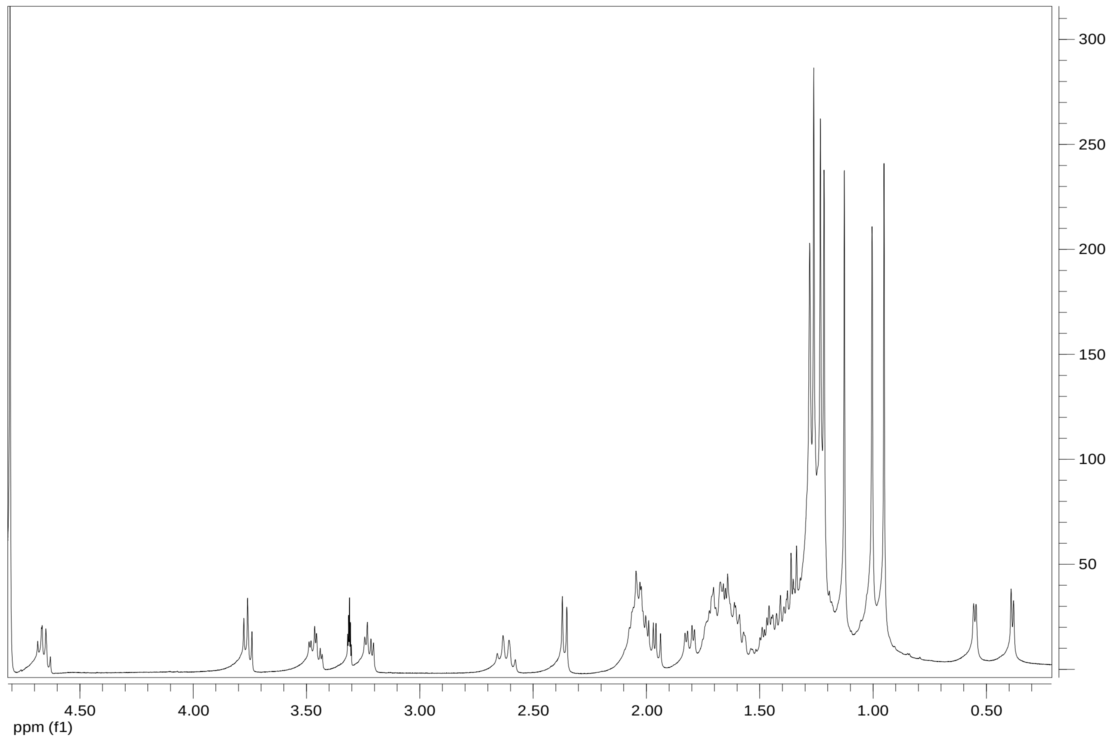


Figure 3.1. Structure of **1**

Table 3.1. ^{1}H- and ^{13}C-NMR Data of compound **1**

1		
	δ_C	δ_H (*J* in Hz)
1	32.2	1.61, 1.24, m
2	29.5	2.10, 1.71, m
3	78.0	3.23, dd (11.3, 4.0)
4	42.6	-
5	53.2	1.36, d (9.5)
6	68.8	3.47, ddd (9.5, 9.0, 4.5)
7	38.7	1.48,1.37, m
8	47.4	1.82 dd (11.8, 4.2)
9	21.5	-
10	30.9	-
11	26.8	2.06, 1.24, m
12	34.0	1.70, (2H) m
13	45.9	-
14	46.8	-
15	45.4	1.41, dd (12.6, 5.2)
		1.99, dd (12.6, 8.0)
16	73.4	4.70, ddd (8.0, 8.0, 5.2)
17	57.8	2.38, d (8.0)
18	29.0	1.31, s
19	30.8	0.41, d (4.2)
		0.58, d (4.2)
20	88.3	-
21	27.0	1.23, s
22	34.1	1.66, m
		2.63, m
23	26.2	1.98, m
		2.19, m
24	81.4.	3.78, dd (8.2, 6.0)
25	72.5	-
26	20.4	1.29, s
27	24.9	1.16, s
28	26.2	1.26, s
29	14.1	0.98, s
30	18.8	1.03, s



Spectrum 3.1. ^{1}H NMR spectrum of **1**

3.2 Structural Identification of Compound 2

^{1}H NMR spectrum of **2** showed signals due to a cyclopropane methylene at δ 0.54 and 0.40 (each 1H, d, J = 4.2 Hz), six tertiary methyl groups at δ 1.32 (3H, s), 1.21 (3H, s), 1.18 (6H, s), 1.05 (3H, s) and 0.98 (3H, s), a secondary methyl group at δ 0.98 (d, J = 6.5 Hz), and four methine proton signals at δ 4.27 (ddd, J = 8.0, 8.0, 5.2 Hz), 3.48 (ddd, J = 9.5, 9.5, 4.5 Hz), 3.41 (dd, J = 10.5, 2.5 Hz) and 3.23 (dd, J = 11.3, 4.0 Hz), which were indicative of secondary alcoholic functions. The NMR data of the aglycone moiety of **2** were in good agreement with those reported for cyclocanthogenin (Bedir et al., 1998a) with glycosidation shift for C-16 (δ 83.2). The chemical shifts of all the individual protons of the sugar unit were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analysis, and the ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum. An unambiguous determination of the sequence and linkage sites was obtained from the HMBC spectrum, which showed key correlation peaks between the proton signal at δ 4.35(H-1glc) and the carbon resonance at δ 83.2 (C-16). On the basis of all these evidence, the structure of the new compound **2** was established as 16-O-β-D-glucopyranosyl-3β, 6α, 16β, 24 (S), 25-pentahydroxycycloartane (Agzamova et al., 1998).

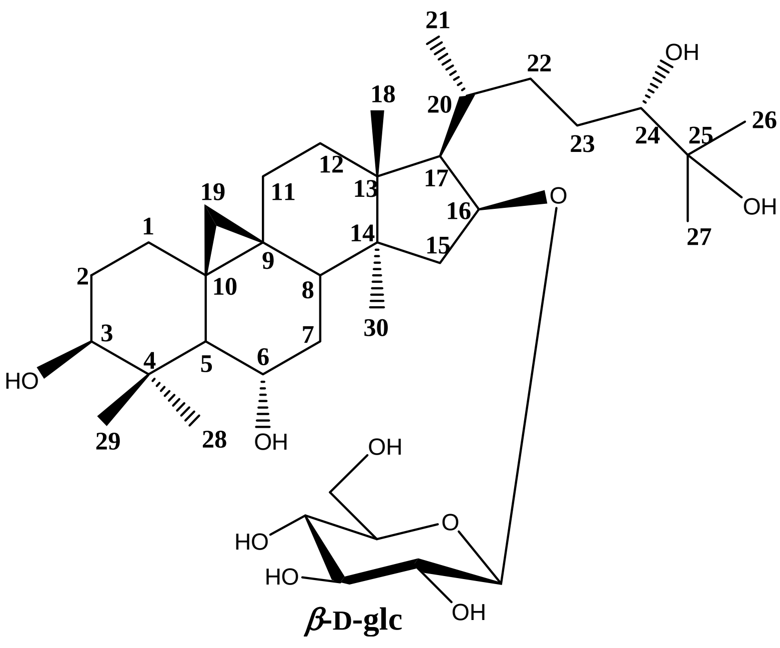


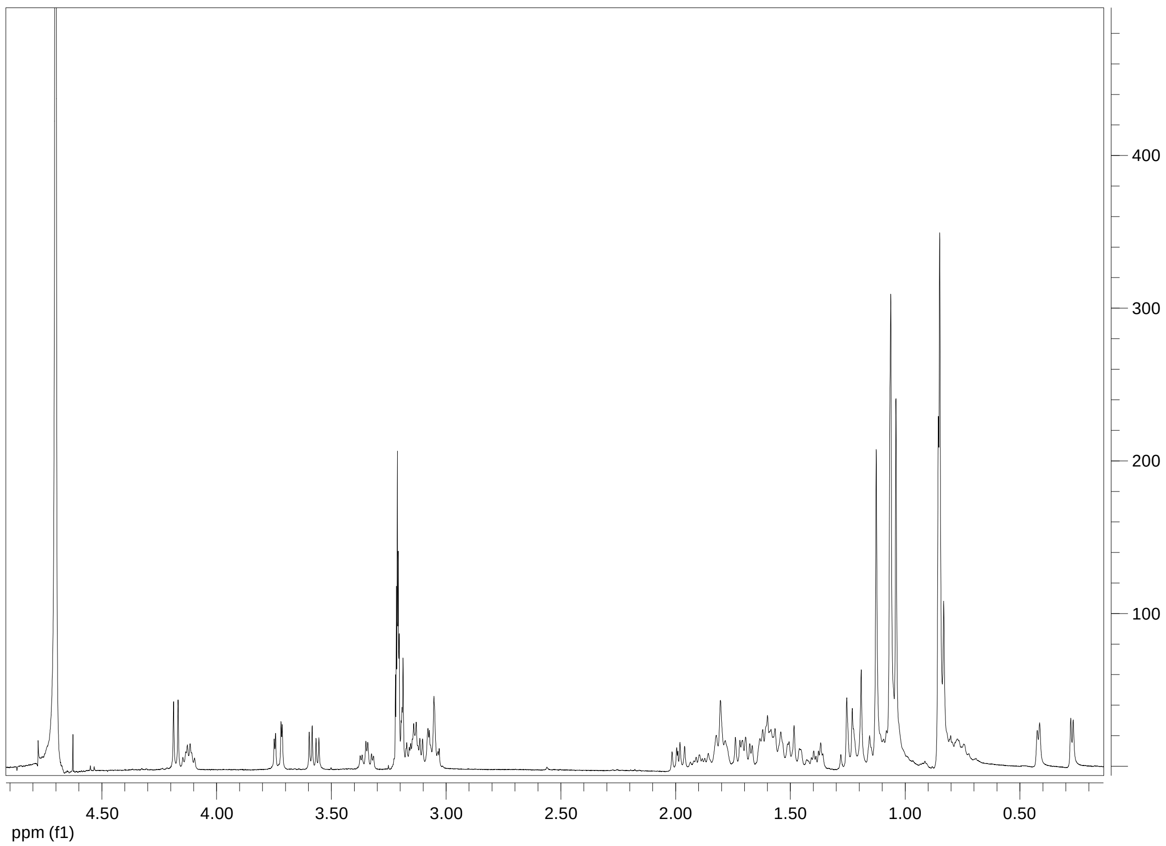
Figure 3.2. Structure of **2**

Table 3.2. ${}^{1}H$- and ${}^{13}C$-NMR Data of compound **2**

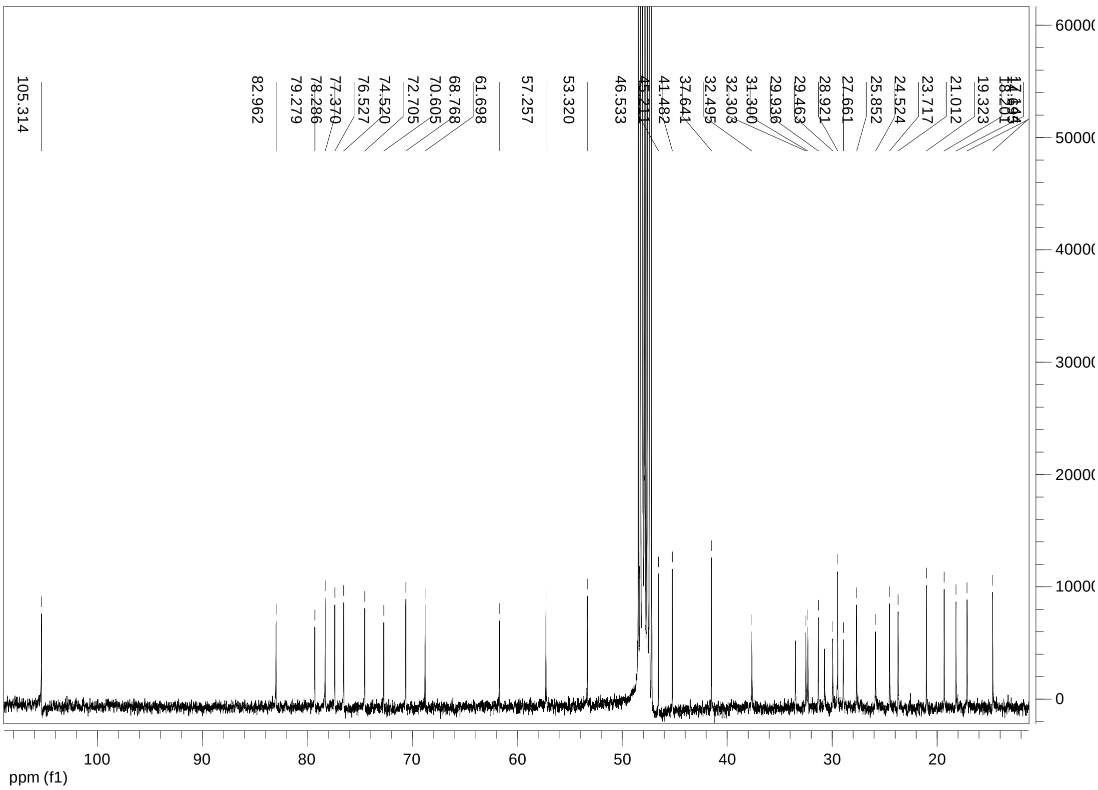
2					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	32.8	1.54, 1.25, m		β-D-glucose (at C-16)	
2	31.5	1.97, 1.68, m	1	104.6	4.35, d (7.5)
3	78.3	3.23, dd (11.3, 4.0)	2	75.5	3.22, dd (7.5, 9.0)
4	42.4	-	3	78.5	3.38, t (9.0)
5	53.9	1.39, d (9.5)	4	71.6	3.32, t (9.0)
6	68.2	3.48, ddd (9.5, 9.5, 4.5)	5	77.5	3.28, ddd (3.5, 4.5, 9.0)
7	38.5	1.50,1.36, m	6	62.9	3.88, dd(3.5, 12.0)
					3.71, dd(4.5, 12.0)
8	48.0	1.80, dd (11.8, 4.1)			
9	21.5	-			
10	29.7	-			
11	26.5	2.01, 1.21, m			
12	33.4	1.71, 1.67, m			
13	46.1	-			
14	47.5	-			
15	48.4	2.07, dd (12.6, 8.0)			
		2.06, dd (12.6, 5.1)			
16	83.2	4.27, ddd			
		(8.0, 8.0, 5.2)			
17	58.1	1.83, dd (9.8, 8.0)			
18	18.8	1.21, s			
19	31.4	0.54, d (4.2)			
		0.40, d (4.2)			
20	29.7	2.09, m			

Table 3.2. (continue)

2				
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
21	17.8	0.98, d (6.5)		
22	33.5	1.80, 1.24,m		
23	28.5	1.66, 1.39, m		
24	78.5	3.41, dd (2.5, 10.5)		
25	73.5	-		
26	24.8	1.18, s		
27	24.8	1.18, s		
28	28.1	1.32, s		
29	16.2	1.05, s		
30	20.0	0.98, s		



Spectrum 3.2.a. ^{1}H NMR spectrum of **2**



Spectrum 3.2.b. ^{13}C NMR spectrum of **2**

3.3 Structural Identification of Compound 3

A detailed comparison of the NMR data (^{1}H, ^{13}C, HSQC, HMBC, COSY) of compound **3** showed that the aglycone moiety was identical to that of **2**. Moreover, the ^{1}H NMR spectrum of **3** showed two anomeric proton doublets at δ 4.36 (J = 7.5 Hz) and 4.31 (J = 7.5 Hz) in the downfield region, which correlated to carbon signals at δ 104.6 and 106.8, respectively, in the HSQC spectrum. The determination of the sequence and linkage sites was obtained from the HMBC correlations which showed key correlation peaks between the proton signals at δ 4.36 (H-1glc) and the carbon resonance at δ 79.9 (C-6), and between the proton signal at δ 4.31 (H-1xyl) and the carbon at δ 89.6 (C-3). Thus, compound **3** was identified as 3-O-β-L-xylopyranosyl-6-O-β-D-glucopyranosyl-3β,6α,16β,25-tetrahydroxy-20(R),24(R)-epoxycycloartane (Isaev et al., 1992).

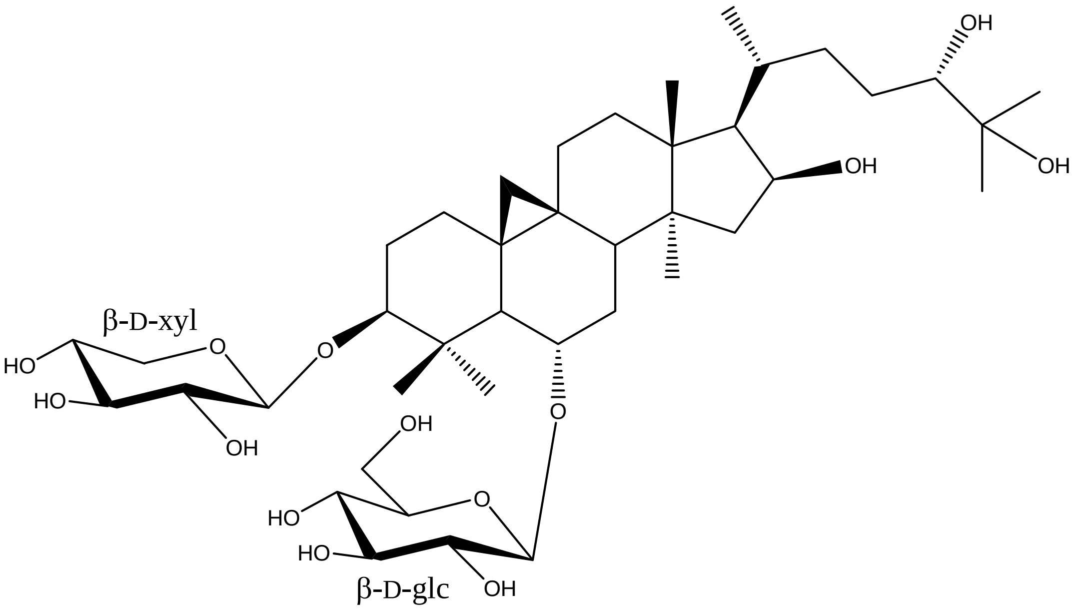


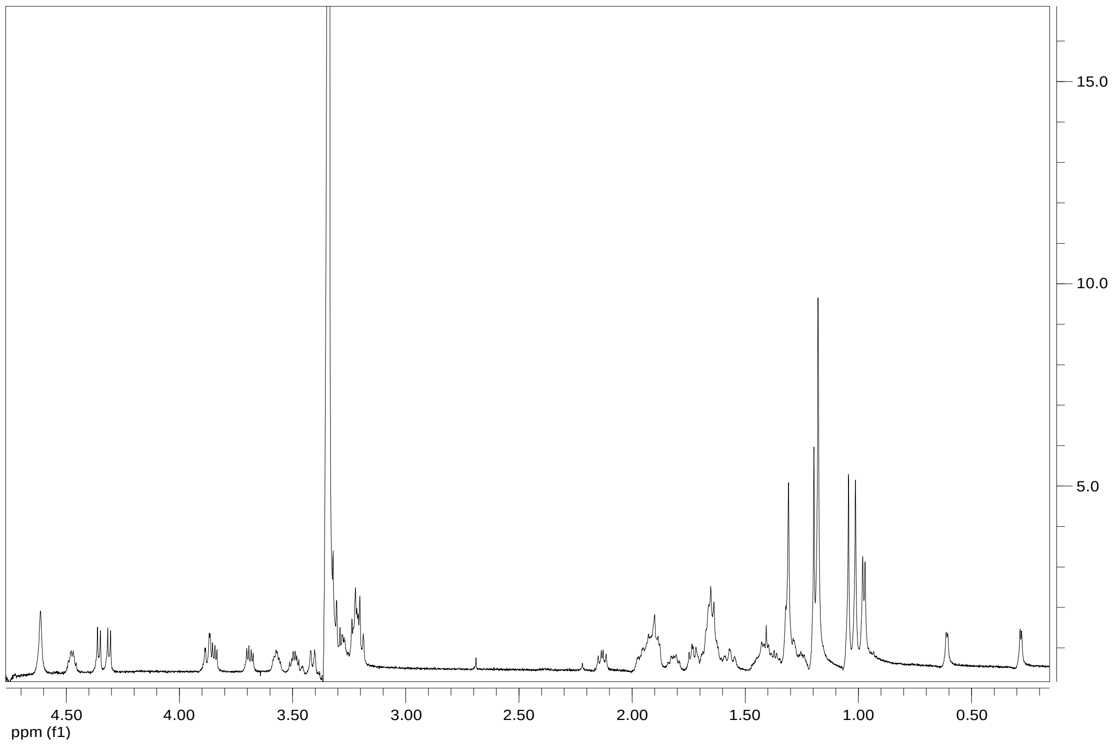
Figure 3.3. Structure of **3**

Table 3.3. ^{1}H- and ^{13}C-NMR Data of compound **3**

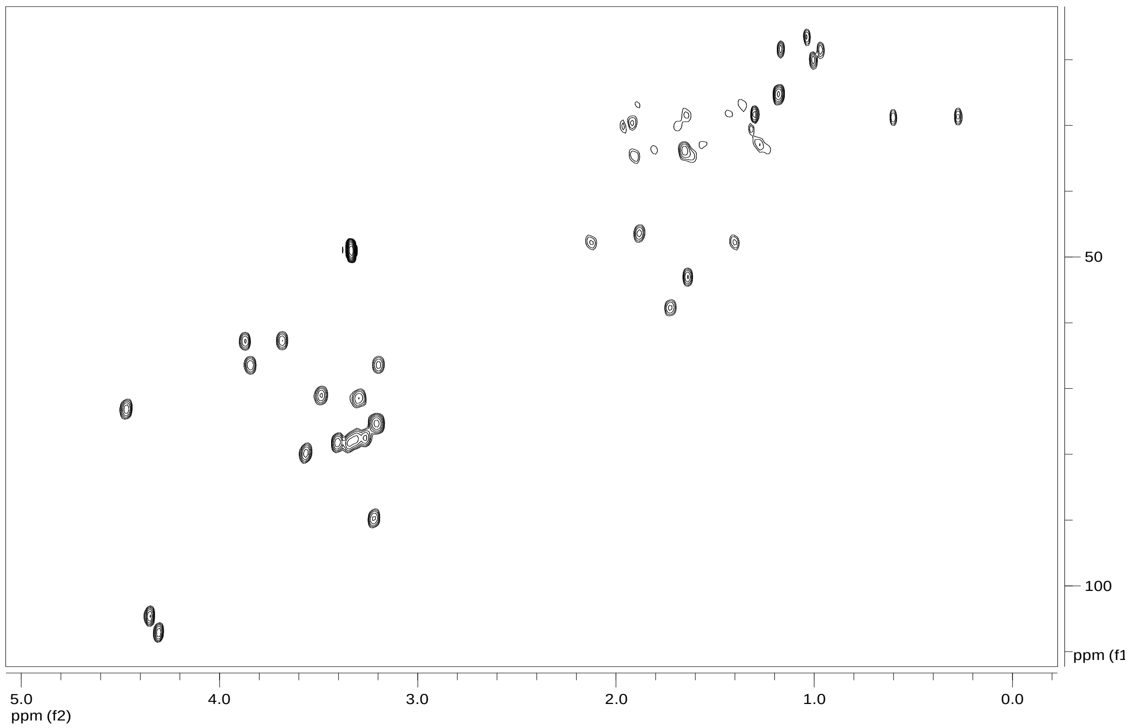
3					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	32.7	1.53, 1.24, m		β-D-xylose (at C-3)	
2	31.0	1.96, 1.67, m	1	106.8	4.31, d (7.5)
3	89.6	3.21, dd (11.3, 4.0)	2	75.5	3.22, dd (7.5, 9.0)
4	42.5	-	3	78.5	3.38, t (9.0)
5	53.7	1.39, d (9.5)	4	71.6	3.32, m
6	79.9	3.56, ddd (9.5, 9.5, 4.5)	5	66.2	3.90, dd (11.7, 5.2)
					3.23, t (11.7)
7	38.5	1.51,1.35, m		β-D-glucose (at C-6)	
8	48.0	1.78, dd (11.8, 4.1)	1	104.6	4.36, d (7.5)
9	21.4	-	2	75.5	3.22, dd (7.5, 9.0)
10	29.7	-	3	78.5	3.38, t (9.0)
11	26.5	2.01, 1.20, m	4	71.6	3.32, t (9.0)

Table 3.3. (continue)

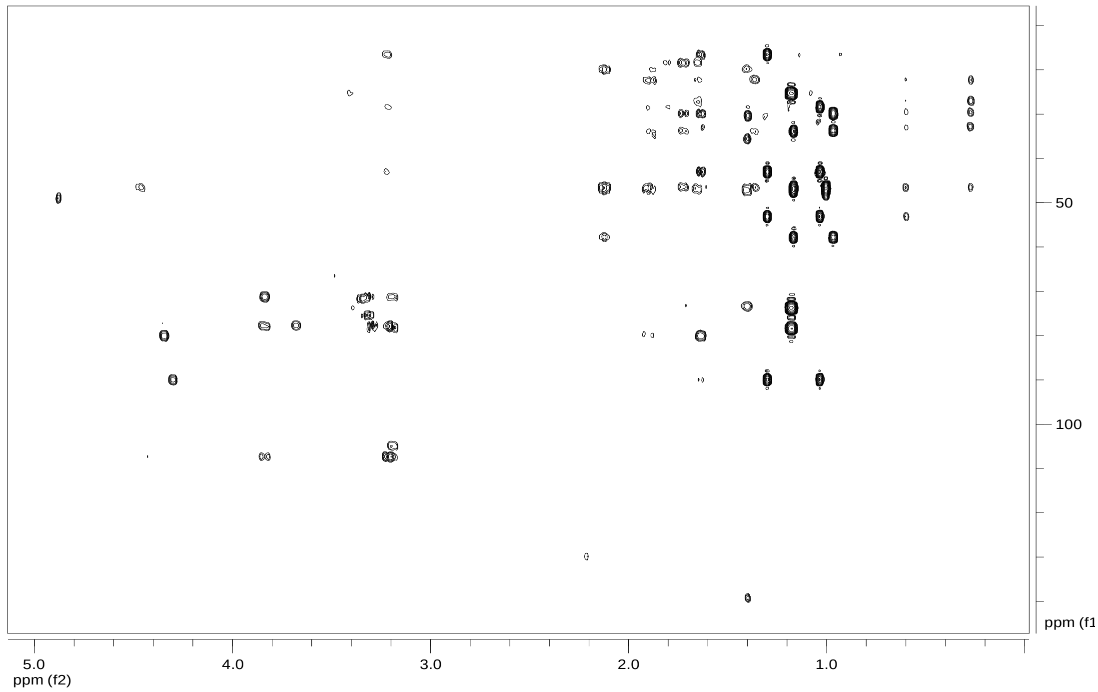
3					
	δ_C	δ_H (J in Hz)		δ_C	δ_H (J in Hz)
12	33.5	1.72, 1.66, m	5	77.5	3.28, ddd (3.5, 4.5, 9.0)
13	46.3	-	6	62.9	3.88, dd(3.5, 12.0)
					3.71, dd(4.5, 12.0)
14	47.5	-			
15	48.3	2.06, dd (12.6, 8.0)			
		2.05, dd (12.6, 5.1)			
16	73.0	4.48, ddd			
		(8.0, 8.0, 5.2)			
17	58.0	1.82, dd (9.8, 8.0)			
18	18.7	1.20, s			
19	31.3	0.53, d (4.2)			
		0.40, d (4.2)			
20	29.6	2.08, m			
21	17.8	0.97, d (6.5)			
22	33.5	1.80, 1.24,m			
23	28.6	1.65, 1.39, m			
24	78.4	3.40, dd (2.5, 10.5)			
25	73.4	-			
26	24.8	1.19, s			
27	24.8	1.19, s			
28	28.0	1.33, s			
29	16.1	1.05, s			
30	20.0	0.99, s			



Spectrum 3.3.a ^{1}H NMR spectrum of **3**



Spectrum 3.3.b. HSQC spectrum of **3**



Spectrum 3.3.c. HMBC spectrum of **3**

3.4 Structural Identification of Compound 4

A detailed comparison of the NMR data (^{1}H, ^{13}C, HSQC, HMBC, COSY) of compound **4** showed that the aglycone moiety was identical to that of **2**. The ^{1}H NMR spectrum of **4** displayed, in addition to signals of the aglycon moiety, signals for three anomeric protons at d 4.49 (d, J = 3.7Hz), 4.48 (d, J = 7.5 Hz) and 4.34 (d, J = 7.5 Hz). On the basis of HSQC, HMBC, DQF-COSY and 1D-TOCSY correlations one α-arabinopyranosyl (δ 4.49) one β-xylopyranosyl (δ 4.48) and one β-glucopyranosyl (d 4.34) units were identified. Glycosidation shifts for the aglycon moiety were observed for C-3 (δ 89.6) and C-6 (δ 79.8). Key correlation peaks in the HMBC spectrum were observed between the proton signal at δ 4.49 (H-1ara) and the carbon resonance at δ 79.8 (C-2xyl), δ 4.48 (H-1xyl) and δ 89.6 (C-3), and between the proton signal at d 4.34 (H-1glc) and the carbon resonance at δ 79.8 (C-6). Therefore, the structure of **4** was established as 3-O-[α-L-arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-6-O-β-D-glucopyranosyl-3β,6α,16β,24(S),25-pentahydroxycycloartane (Ozipek et al., 2005).

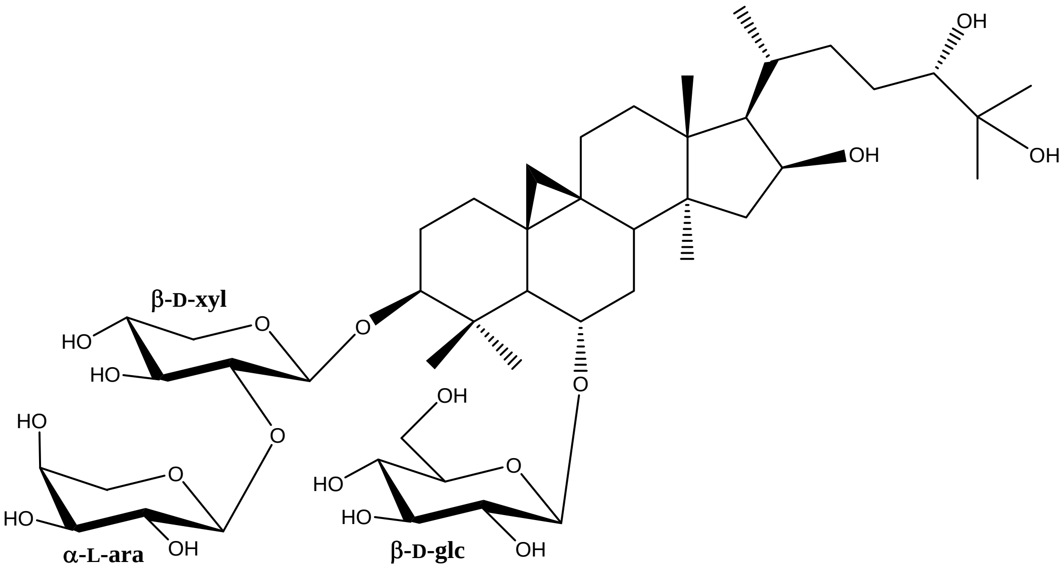


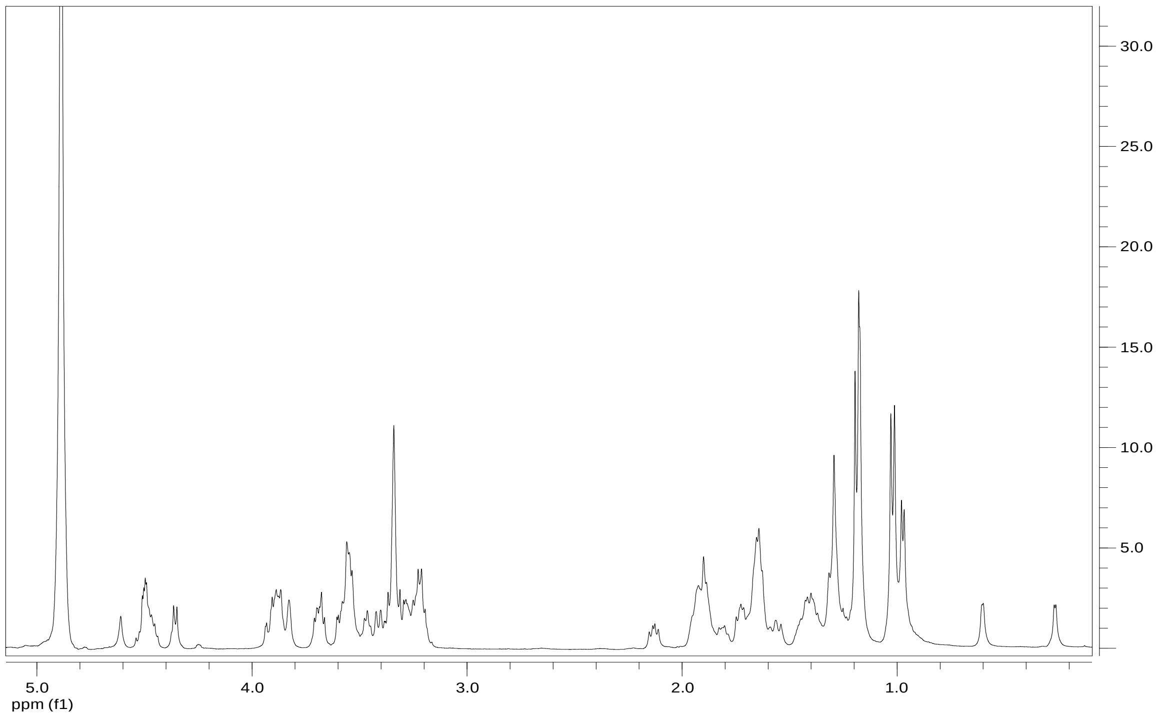
Figure 3.4. Structure of **4**

Table 3.4. ^{1}H- and ^{13}C-NMR Data of compound **4**

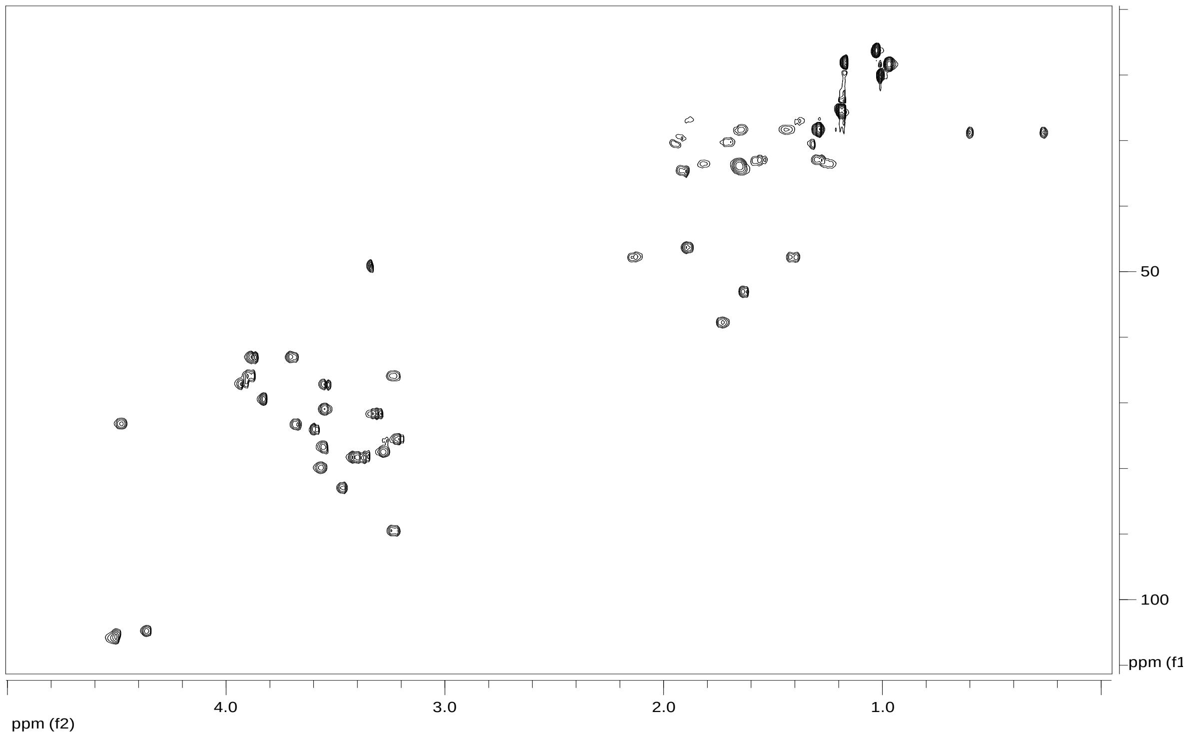
4					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	32.6	1.53, 1.24, m		β-D-xylose (at C-3)	
2	31.2	1.97, 1.67, m	1	105.2	4.48, d (7.5)
3	89.6	3.22, dd (11.3, 4.0)	2	79.8	3.41, dd (7.5, 9.0)
4	42.3	-	3	77.1	3.54, t (9.0)
5	53.8	1.38, d (9.5)	4	71.0	3.54, m
6	79.8	3.55, ddd (9.5, 9.5, 4.5)	5	66.2	3.90, dd (11.7, 5.2)
					3.23, t (11.7)
7	38.4	1.50,1.35, m		α-L-arabinose (at C-2xyl)	
8	47.9	1.79, dd (11.8, 4.1)	1	105.9	4.49,d (3.7)
9	21.4	-	2	73.0	3.67, dd (8.5, 3.7)
10	29.6	-	3	73.7	3.59, dd (8.5, 3.0)(
11	26.4	2.00, 1.20, m	4	69.0	3.89, m
12	33.3	1.70, 1.66, m	5	66.8	3.91, dd (11.9, 2.0)
					3.51, dd (11.9, 3.0)

Table 3.4. (continue)

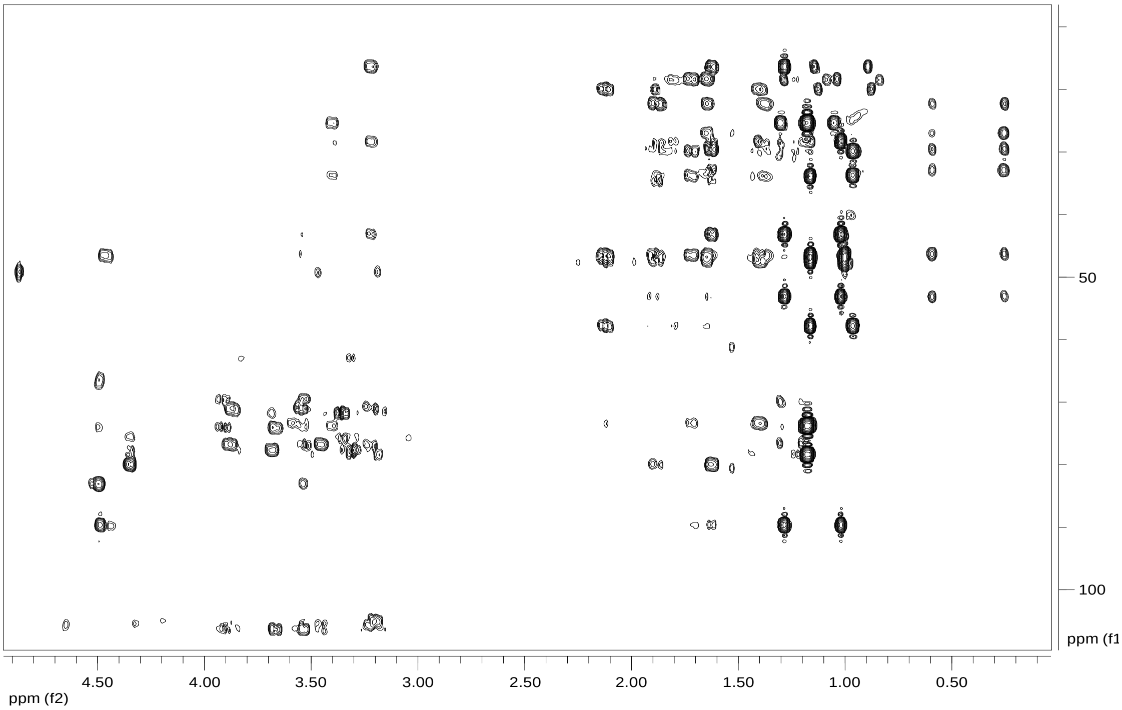
4					
	δ_C	δ_H (J in Hz)		δ_C	δ_H (J in Hz)
13	46.0	-		β-D-glucose (at C-6)	
14	47.4	-	1	104.5	4.34, d (7.5)
15	48.3	2.06, dd (12.6, 8.0)	2	75.4	3.23, dd (7.5, 9.0)
		2.05, dd (12.6, 5.1)			
16	73.0	4.40, ddd	3	78.4	3.36, t (9.0)
		(8.0, 8.0, 5.2)			
17	58.0	1.82, dd (9.8, 8.0)	4	71.4	3.31, t (9.0)
18	18.6	1.20, s	5	77.3	3.29, ddd (3.5, 4.5, 9.0)
19	31.2	0.53, d (4.2)	6	62.8	3.88, dd(3.5, 12.0)
		0.40, d (4.2)			
					3.71, dd(4.5, 12.0)
20	29.5	2.08, m			
21	17.7	0.97, d (6.5)			
22	33.4	1.80, 1.24,m			
23	28.4	1.65, 1.39, m			
24	78.3	3.40, dd (2.5, 10.5)			
25	73.2	-			
26	24.7	1.18, s			
27	24.7	1.18, s			
28	28.0	1.32, s			
29	16.1	1.04, s			
30	20.0	0.98, s			



Spectrum 3.4.a. ^{1}H NMR spectrum of **4**



Spectrum 3.4.b. HSQC spectrum of **4**



Spectrum 3.4.c. HMBC spectrum of **4**

3.5 Structural Identification of Compound 5

A detailed comparison of the NMR data (^{1}H, ^{13}C, HSQC, HMBC, COSY) of compound **5** showed that the aglycone moiety was identical to that of **2**. The ^{1}H NMR spectrum of **5** showed signals for two anomeric protons at δ 4.31 (d, J = 7.5 Hz) and 4.30 (d, J = 7.5 Hz). The HSQC, HMBC, DQF-COSY and 1D-TOCSY data led to identify these sugar units as two β-xylopyranosyl units. The HMBC correlations between the proton signal at δ 4.30 (H-1xyl) and the carbon resonance at δ 89.6 (C-3) and the proton signal at δ 4.31 (H-1xyl) and the carbon resonance at δ 79.9 (C-6) allowed us to determine the linkage site of the sugar units. Thus, the new compound **5** was elucidated as 3,6-di-O-β-D-xylopyranosyl-3β,6α,16β,24(S),25-pentahydroxycycloartane (Gulcemal, et al., 2011).

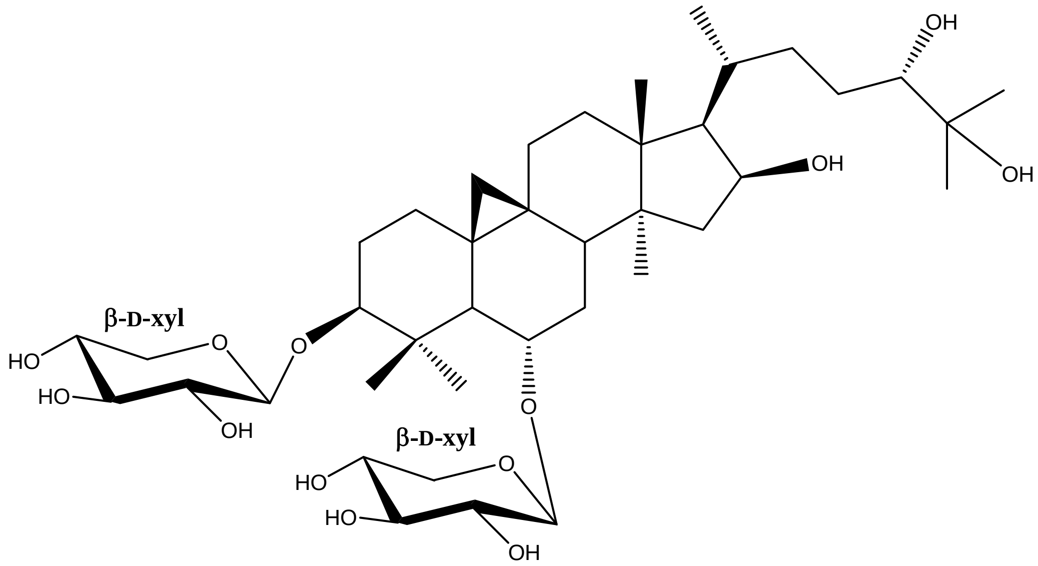


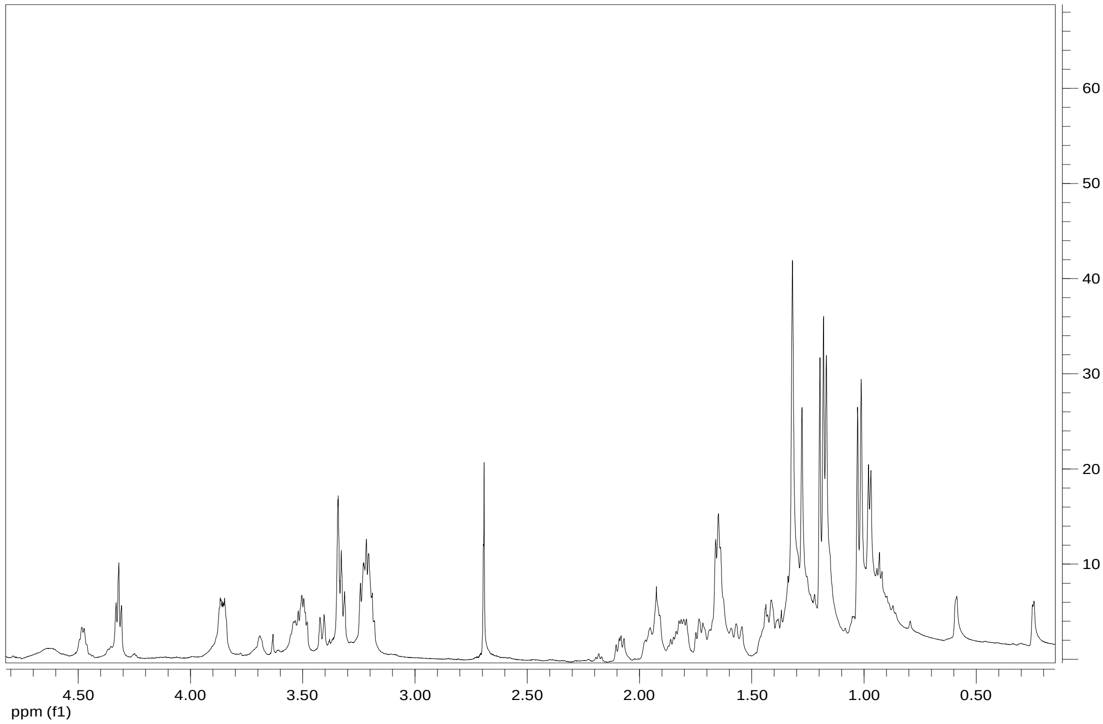
Figure 3.5. Structure of **5**

Table 3.5. ^{1}H- and ^{13}C-NMR Data of compound **5**

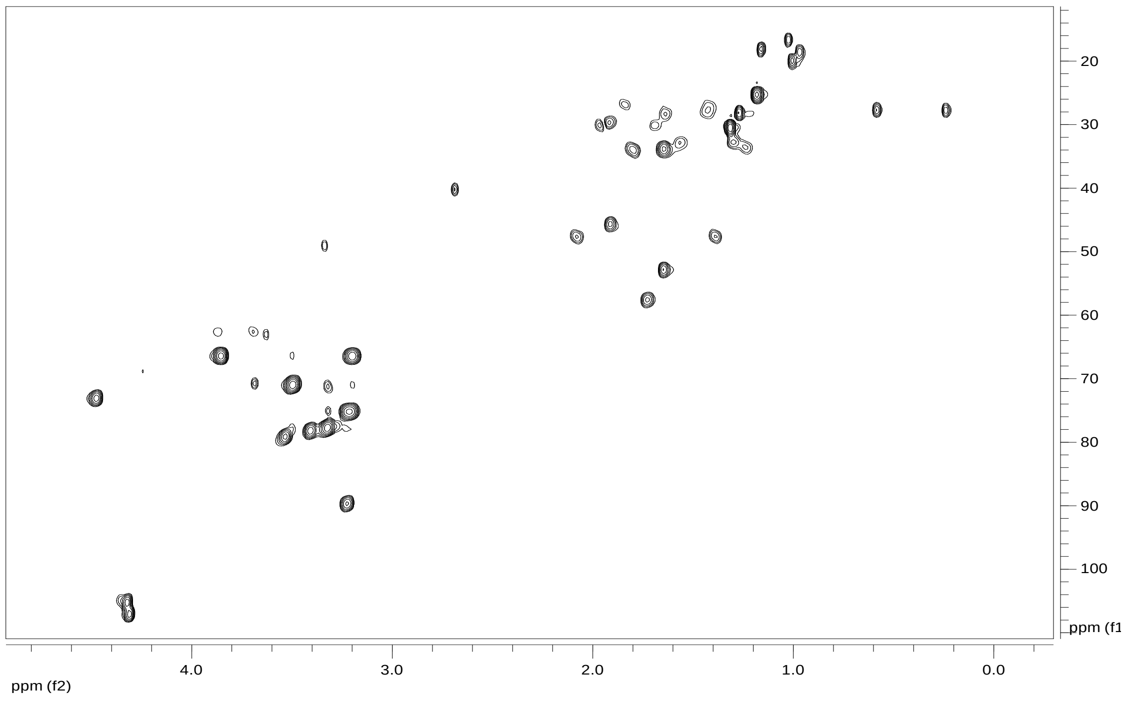
5					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	32.7	1.53, 1.24, m		β-D-xylose (at C-3)	
2	31.0	1.96, 1.67, m	1	106.8	4.30, d (7.5)
3	89.6	3.21, dd (11.3, 4.0)	2	75.0	3.21, dd (7.5, 9.0)
4	42.5	-	3	77.6	3.32, t (9.0)
5	53.7	1.39, d (9.5)	4	71.0	3.50, m
6	79.9	3.56, ddd (9.5, 9.5, 4.5)	5	66.5	3.86, dd (11.7, 5.2)
					3.23, t (11.7)
7	38.5	1.51,1.35, m		β-D-xylose (at C-6)	
8	48.0	1.78, dd (11.8, 4.1)	1	105.3	4.31, d (7.5)
9	21.4	-	2	75.5	3.22, dd (7.5, 9.0)
10	29.7	-	3	77.7	3.34, t (9.0)
11	26.5	2.01, 1.20, m	4	71.6	3.51, m

Table 3.5. (continue)

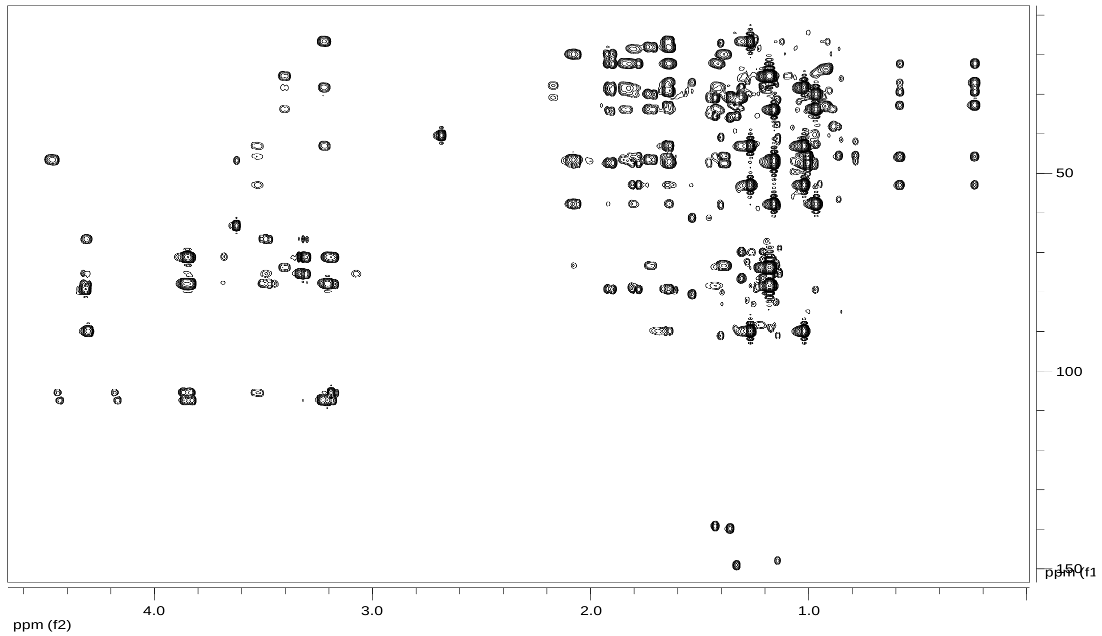
5					
	δ_C	δ_H (J in Hz)		δ_C	δ_H (J in Hz)
12	33.5	1.72, 1.66, m	5	66.5	3.86, dd(3.5, 12.0)
					3.23, dd(4.5, 12.0)
13	46.3	-			
14	47.5	-			
15	48.3	2.06, dd (12.6, 8.0)			
		2.05, dd (12.6, 5.1)			
16	73.0	4.48, ddd			
		(8.0, 8.0, 5.2)			
17	58.0	1.82, dd (9.8, 8.0)			
18	18.7	1.20, s			
19	31.3	0.53, d (4.2)			
		0.40, d (4.2)			
20	29.6	2.08, m			
21	17.8	0.97, d (6.5)			
22	33.5	1.80, 1.24,m			
23	28.6	1.65, 1.39, m			
24	78.4	3.40, dd (2.5, 10.5)			
25	73.4	-			
26	24.8	1.19, s			
27	24.8	1.19, s			
28	28.0	1.33, s			
29	16.1	1.05, s			
30	20.0	0.99, s			



Spectrum 3.5.a. ^{1}HNMR spectrum of **5**



Spectrum 3.5.b. HSQC spectrum of **5**



Spectrum 3.5.c. HMBC spectrum of **5**

3.6 Structural Identification of Compound 6

A detailed comparison of the NMR data (^{1}H, ^{13}C, HSQC, HMBC, COSY) of compound **6** showed that the aglycone moiety was identical to that of **2**. The ^{1}H NMR spectrum of **6** displayed, in addition to signals of the aglycon moiety, signals for three anomeric protons at δ 4.48 (d, J = 3.7 Hz), 4.47 (d, J = 7.5 Hz) and 4.31 (d, J = 7.5 Hz). On the basis of HSQC, HMBC, DQF-COSY and 1D-TOCSY correlations one α-arabinopyranosyl (δ 4.48) and two β-xylopyranosyl (δ 4.47 and 4.31) units were identified. Glycosidation shifts for the aglycon moiety were observed for C-3 (δ 89.8) and C-6 (δ 78.9). Key correlation peaks in the HMBC spectrum were observed between the proton signal at δ 4.48 (H-1ara) and the carbon resonance at δ 79.7 (C-2xyl), δ 4.47 (H-1xyl) and δ 89.8 (C-3), and between the proton signal at d 4.31 (H-1xyl) and the carbon resonance at δ 78.9 (C-6). Therefore, the structure of **6** was established as 3-O-[α-L-arabinopyranosyl-(1→2)-β-D-xylopyranosyl]-6-O-β-D-xylopyranosyl-3β,6α,16β,24(S),25-pentahydroxycycloartane (Özipek et al., 2005).

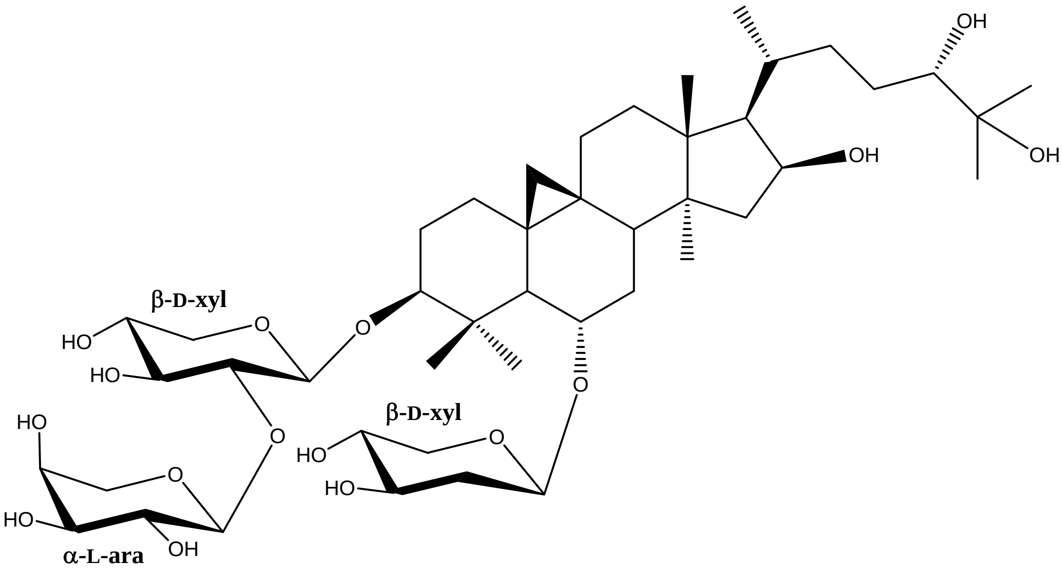


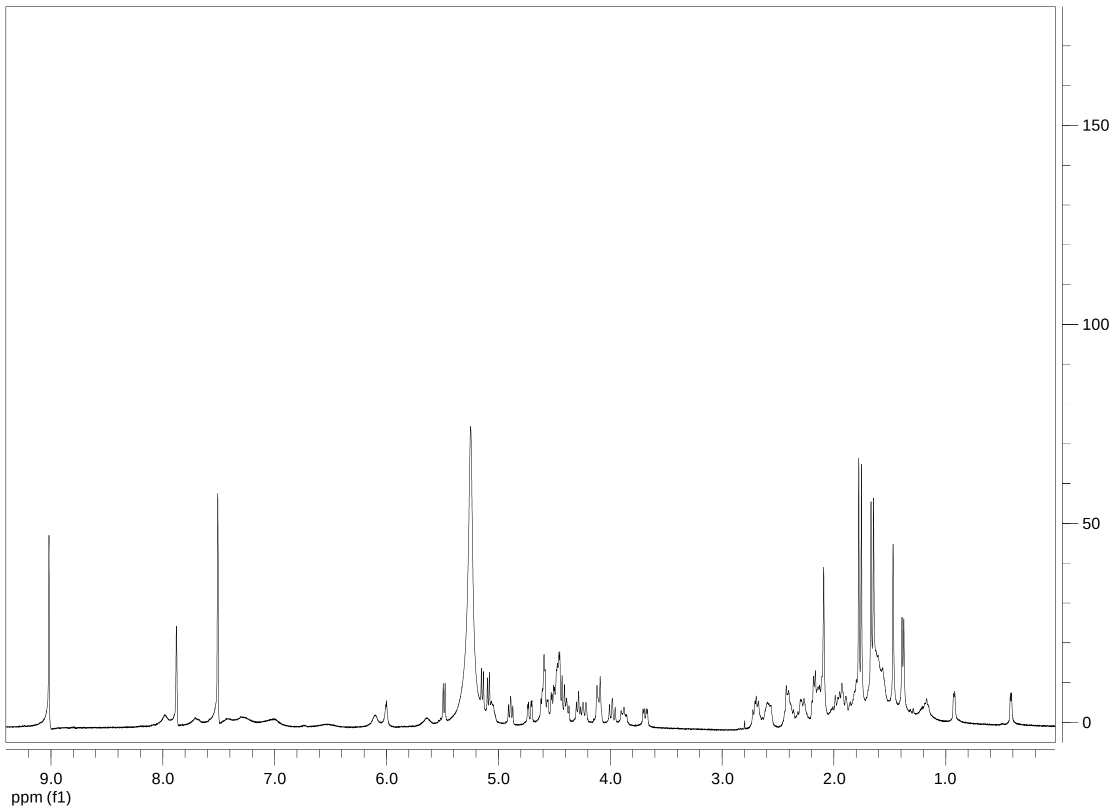
Figure 3.6. Structure of **6**

Table 3.6. ^{1}H- and ^{13}C-NMR Data of compound **6**

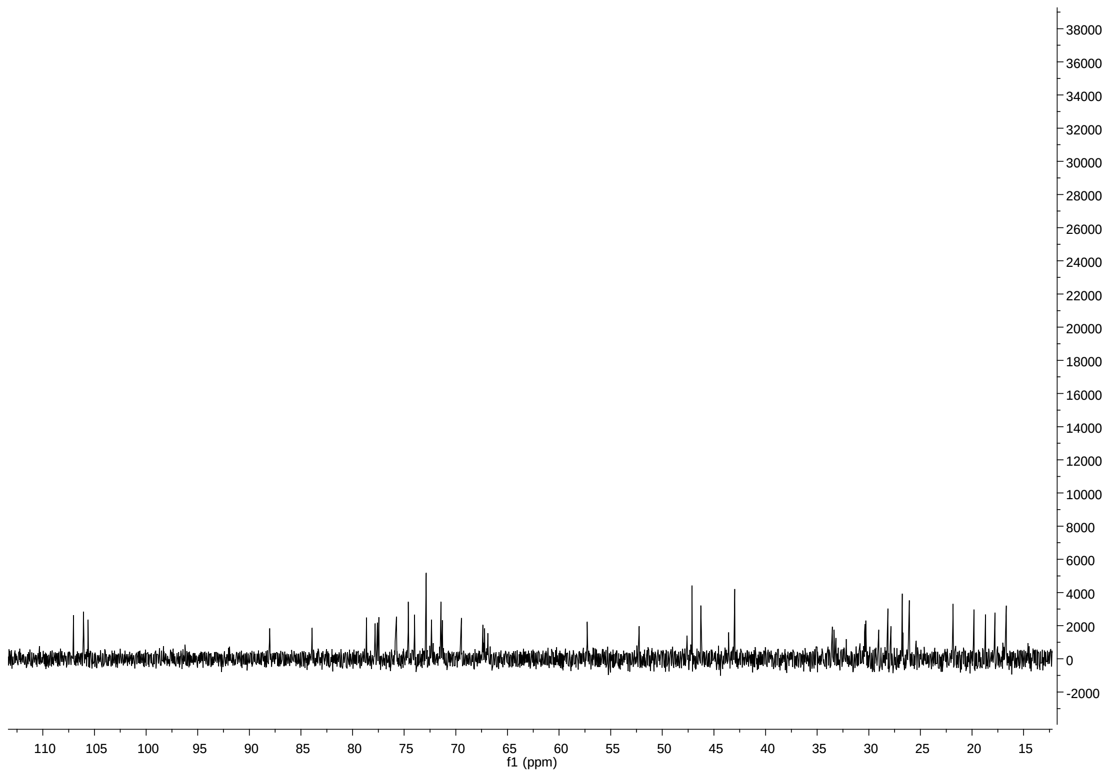
6					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	32.6	1.53, 1.24, m		β-D-xylose (at C-3)	
2	31.2	1.97, 1.67, m	1	105.1	4.47, d (7.5)
3	89.8	3.22, dd (11.3, 4.0)	2	79.7	3.40, dd (7.5, 9.0)
4	42.3	-	3	77.0	3.55, t (9.0)
5	53.8	1.38, d (9.5)	4	71.3	3.53, m
6	78.9	3.55, ddd (9.5, 9.5, 4.5)	5	66.2	3.90, dd (11.7, 5.2)
					3.23, t (11.7)
7	38.4	1.50,1.35, m		α-L-arabinose (at C-2xyl)	
8	47.9	1.79, dd (11.8, 4.1)	1	105.7	4.48,d (3.7)
9	21.4	-	2	73.1	3.67, dd (8.5, 3.7)
10	29.6	-	3	73.6	3.58, dd (8.5, 3.0)(
11	26.4	2.00, 1.20, m	4	69.2	3.88, m

Table 3.6. (continue)

6					
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
12	33.3	1.70, 1.66, m	5	66.8	3.91, dd (11.9, 2.0)
					3.51, dd (11.9, 3.0)
13	46.0	-		β-D-xylose (at C-6)	
14	47.4	-	1	105.3	4.31, d (7.5)
15	48.3	2.06, dd (12.6, 8.0)	2	75.5	3.22, dd (7.5, 9.0)
		2.05, dd (12.6, 5.1)			
16	73.0	4.40, ddd	3	77.7	3.34, t (9.0)
		(8.0, 8.0, 5.2)			
17	58.0	1.82, dd (9.8, 8.0)	4	71.6	3.51, m
18	18.6	1.20, s	5	66.5	3.86, dd(3.5, 12.0)
					3.23, dd(4.5, 12.0)
19	31.2	0.53, d (4.2)			
		0.40, d (4.2)			
20	29.5	2.08, m			
21	17.7	0.97, d (6.5)			
22	33.4	1.80, 1.24,m			
23	28.4	1.65, 1.39, m			
24	78.3	3.40, dd (2.5, 10.5)			
25	73.2	-			
26	24.7	1.18, s			
27	24.7	1.18, s			
28	28.0	1.32, s			
29	16.1	1.04, s			
30	20.0	0.98, s			



Spectrum 3.6.a. ^{1}H NMR spectrum of **6**



Spectrum 3.6.b. ^{13}C NMR spectrum of **6**

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