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ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *ASTRAGALUS ERINACEUS*

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Tahir SAVRAN tarafından Yüksek Lisans tezi olarak sunulan "Isolation and Characterization of Saponins from *Astragalus erinaceus* (*Astragsalus Erinaceus'dan saponinlerin izolasyonu 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 02.07.2010 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *ASTRAGALUS ERINACEUS*

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In this study, nine glycosides were isolated from *Astragalus erinaceus* species and structures of the two of the isolated compounds determined as cyclocanthoside E and brachycoside B by using spectroscopic tecniques.

Key words: *Astragalus erinaceus*, cycloartane, saponin, cyclocanthoside E, brachycoside B

ÖZET

ASTRAGALUS ERINACEUS'DAN SAPONİNLERİN İZOLASYONU VE KAREKTERİZASYONU

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Bu çalışmada *Astragalus erinaceus* türünden dokuz tane glikozid izole edilerek, izole edilen bileşiklerden iki tanesinin yapısı spektral teknikler kullanılarak siklokanthosid E ve brakhiosid B olarak belirlenmiştir.

Anahtar Kelimeler: *Astragalus erinaceus*, sikloartan, saponin, siklokanthosid E, brakhioside B.

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ABBREVIATIONS

UV	Ultraviolet
IR	Infra-red
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
1D- NMR	One-Dimensional Nuclear Magnetic Resonance
2D- NMR	Two-Dimensional Nuclear Magnetic Resonance
HMQC	Heteronuclear Multiple Quantum Coherence
HMBC	Heteronuclear Multiple Bond Coherence
CC	Column Chromatography
TLC	Thin Layer 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	Multiplet

1.INTRODUCTION

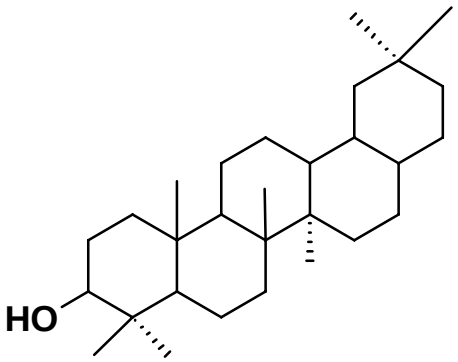
1.1.General Information about Saponins

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 1.1.)** (Kaufman, 1999). 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).

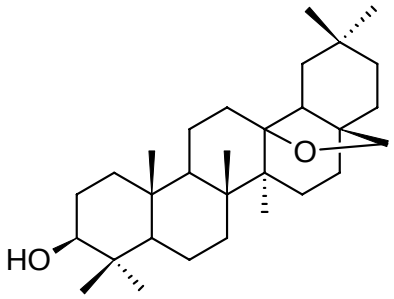
Saponins, generally known as non-volatile (Oleszek, 2002; Lanzotti, 2006; Vincken et al., 2007), are a major family of secondary metabolites that occur in a wide range of plant species (Hostettmann and Marston, 1995). Various members of this important family of plant secondary metabolites are exploited commercially for a variety of purposes including as drugs and medicines, precursors for hormone synthesis, adjuvants, foaming agents, sweeteners, taste modifiers and cosmetics (Osbourn, 2003).

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).

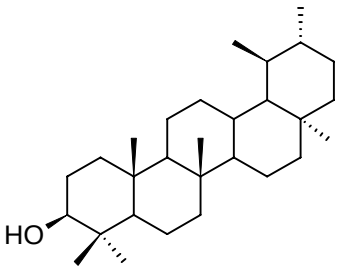
Most saponins isolated to date are triterpenoid (C_{30} aglycone-based) saponins (750 saponins with 360 different aglycones). At least basic triterpene aglycones **(Figure 1.1.)** exist. These structures have been well characterized and are published in the literature making structure elucidation of atypical aglycone relatively straightforward (Berger et al, 2001).



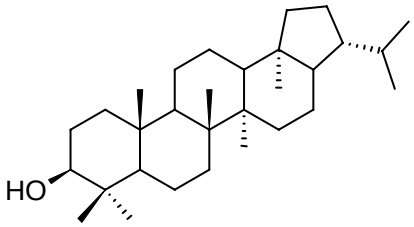
Triterpene



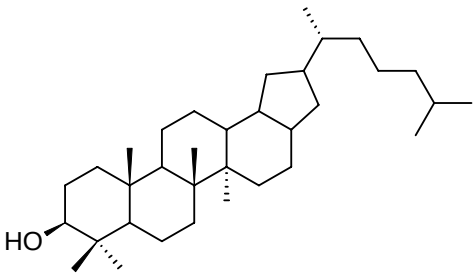
Oleanane



Ursane



Hopane



Dammarane

Figure 1.1. A Triterpene Skeleton and Four Common Triterpene Skeletons

Some saponins-containing plants have been employed for hundreds of years as soaps and this fact reflected in their common names; soapwort (*saponaria officinalis*), soaproot (*Chlorogalum pomeridianum*), sopapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), soapnut (*Sapindus makurossi*) **(**Hostettmann and Marston, 1995). Really, The name **'saponin'** is derived from the Latin word *sapo*, which means 'soap', because molecules form soap-like foams (oleszek, 2002; Vincken et al., 2007) in aqueous solution (Osbourn, 2003).

That is, 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 1.2.)** (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).

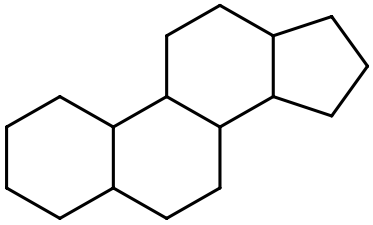
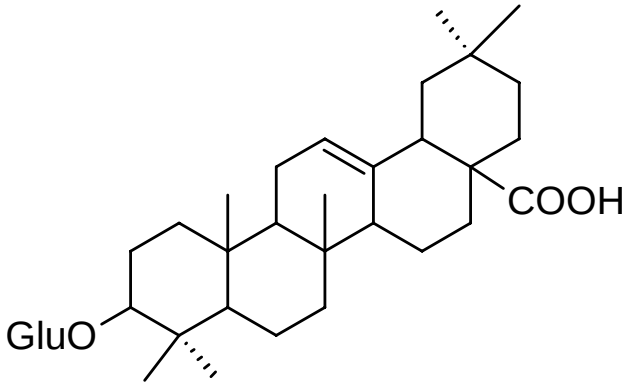


Figure 1.2. Basic Skeleton of steroid

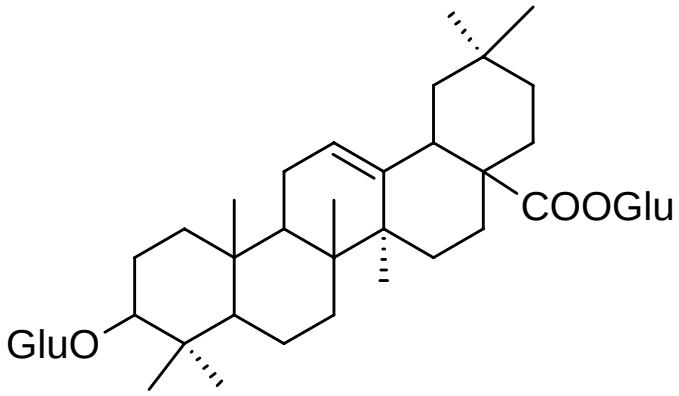
The carbohydrate part consist of one or more sugar moieties containing glucose, galactose, xylose, arabinose, rhamnose, or glucuronic acid glycosidically linked to a sapogenin (aglycone) **(Table 1.1.)**. Saponins that have one sugar molecule attached at the C-3 position are called ***monodesmosidic* 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 1.3.)** (Wina et al., 2005). ***Tridesmosidic* saponins** have three sugar chains and are seldom found. *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).

D-Glucose	Glc	L-Rhamnose	Rha
D-Galactose	Gal	D-Arabinose	Ara
D-Glucuronic acid	GlcA	D-Xylose	Xyl
D-Galacturonic acid	GalA	D-Fucose	Fuc

Table 1.1. Common Saponin Monosaccharide Groups



Monodesmosidic Saponins



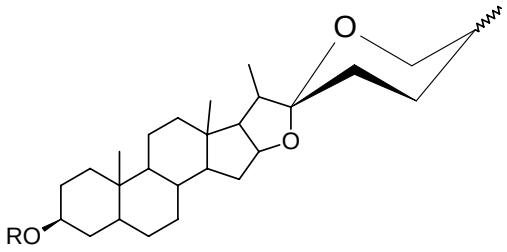
Bidesmosidic Saponins

Figure 1.3. *Monodesmosidic* and *Bidesmosidic* Saponins

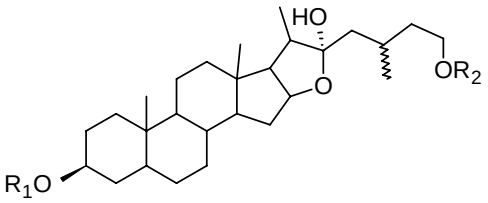
1.2. Classification and Occurrence

Saponins can be classified into two groups based on the nature of their aglycone skeleton. The first group consists of the **steroidal saponins (Figure 1.4., compound 1, 2, and 3)**, which are almost exclusively present in the monocotyledonous angiosperms. The second group consists of the **triterpenoid saponins (Figure 1.4., compound 4)**, which are the most common and occur

mainly in the dicotyledonous angiosperms. Some authors (Hostettmann and Marston, 1995) distinguish a third group called steroidal amines, which are classified by others as **steroidal alkaloids (Figure 1.4., compound 3)** (Sparg, 2004). All classes of aglycones may have a number of functional groups (-OH, -COOH, $-CH_3$) causing big natural diversity only because of aglycone structure (Oleszek, 2002).



1



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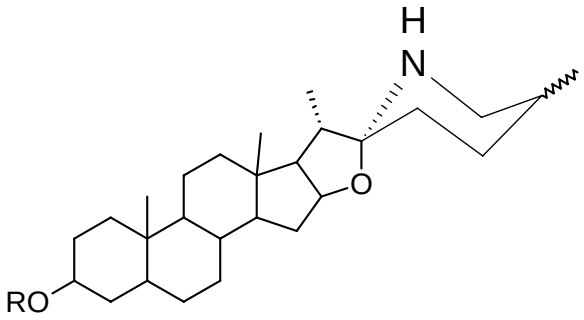
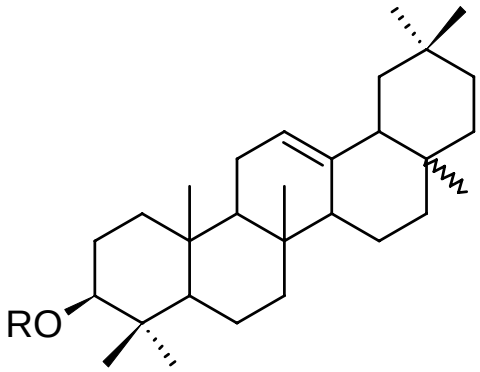


3

Steroidal Saponins

Figure 1.4. Classification of Saponins, R= sugar moiety.



4

Triterpenoid Saponins

Figure 1.4. Classification of Saponins, R= sugar moiety(Continued)

Steroidal saponins (C_{27} based aglycones) are structurally similar to triterpene saponins. In general, they possess similar physical and biological properties to the triterpene saponins. Fewer steroidal saponins have been found compared to the triterpene saponins steroidal saponins fall into two major and one minor classification: the spirostanol glycosides **(Figure 1.4., compound 1)**, the furostanol glycosides **(Figure 1.4., compound 2)**, and the steroidal alkaloids **(Figure 1.4., compound 3)**. The aglycones in steroidal saponins are also known as sapogenins (Berger et al, 2001).

Triterpenes can be mono-, bi-, or tridesmosidic. The prefix indicates the number of glycosyl moieties that exist. Steroidal saponins are typically monodesmosidic. The most common sugars possessed by saponins are D-glucose, L-rhamnose, L-arabinose, and D-xylose, D-apiose, D-quinovose, and D-allose have also been reported but are less common. Typically, a glycosyl moiety is attached to the aglycone at the 3-O position. However if the aglycone possesses additional oxygenated functionalities then additional glycosidal moieties may be present **(Figure 1.5.)**. The glycosidal moieties can be can be linear, branched, nitrogenated, oxidized, reduced, acetylated, or any combination thereof (Berger et al, 2001).

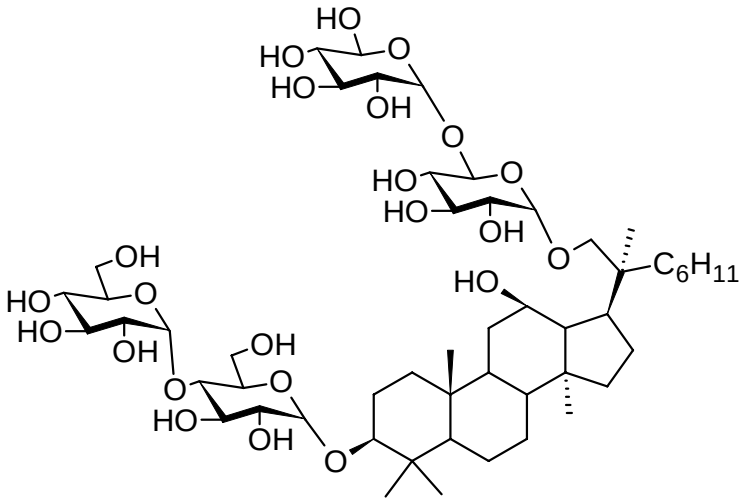


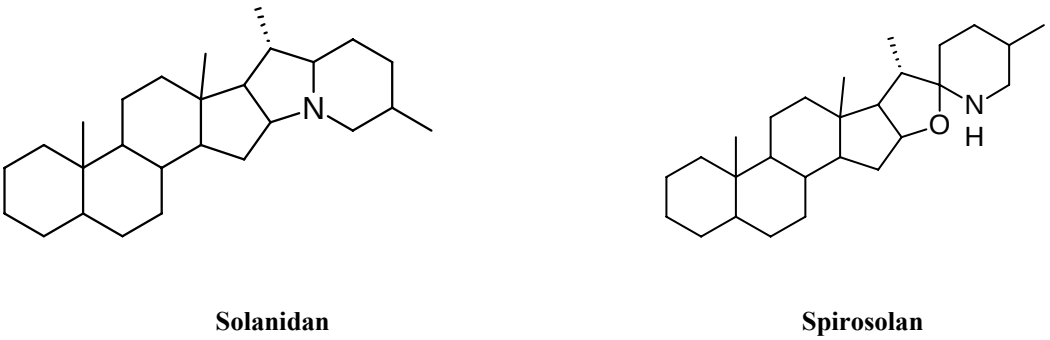
Figure 1.5. Gingengoside RB_2

The **steroids** are natural product derivatives from tetracyclic triterpenoids and are ubiquitous in the geosphere and the ambient environment (Ikan, 2008) and possess a cyclopentaperhydrophenanthrene (gonane) backbone **(Figure 1.2.)** (Breitmaier, 2006**)**. Moreover, the triterpenoids a class of terpenes, which are classified by the number of these C_5 isoprene (Hemiterpene) units that they contain. The classes are: Monoterpenoids (C_{10}), Sesquiterpenoids (C_{15}), Diterpenoids (C_{20}), Sesterterpenoids (C_{25}) (very rare) (Kaufman et al,, 1999), **Triterpenoids (C_{30})**, Carotenoids (C_{40}) (Tetraterpenes) (Hanson, 2000). In this thesis, we are concerned about saponins (triterpenoids). Therefore, the other terpenes will not been reviewed.

Practically all plant steroids are hydroxylated at C-3 and are in fact sterols (Kaufman et al., 1999). The availability, crystallinity and well-defined conformation of the steroids have meant that they have become suitable substrates with which to investigate the influence of steric factors on reaction rates and mechanisms (Hanson, 2000). Over 100 steroid sapogenins are known and most are from the furostan or spirostan skeleton **(Figure 1.4., compound 1 and 2)**. In all cases the C-18 and C-19 angular methyl groups are *β*-orientated and the C-21 methyl group has the *α*-configuration. There is sometimes a 5,6-double bond (Hostettmann and Marston, 1995).

There are two classes of steroid alkaloid sapogenin – the solanidans (such as solasodine), occur in plants of the Solanaceae, including the tomato and potato

(Hanson, 2000), and the spirosolans **(Figure 1.6.)** (Hostettmann and Marston, 1995).



Solanidan **Spirosolan**

Figure 1.6. The two Classes of steroid alkaloid sapogenin

On the other hand, triterpenoids are a major group of natural products in higher plants. The tetracyclic saponins, based on the lanostane, euphane, onocerane, and dammarane skeletons, are found mostly in vascular plants and have reported in sedimentary rocks. Many pentacyclic triterpenoids (e.g. the oleanane, ursane, teraxerane, lupine, fredelane, serratane, or bauerane skeleton) are characteristic natural product tracers for an origin from terrestrial higher plants. They occur as functionalized (e.g. alcohols, acids, ketones, esters) precursors but are not necessarily specific to individual classes of biota. The most frequently encountered compounds in sedimentary or contemporary environments are those derived from α- and β- amyrins (Ikan, 2008).

Triterpenes, the C_{30} terpenes, are based on six isoprene units. There are several important groups of triterpenes, including common triterpenes, steroids, saponins, sterolins, and cardiac glycosides. Among these is azadirachthin, a powerful insect antifeedant, first isolated in 1985 from Neem oil (Kaufman et al., 1999). Triterpenoids including squalene derivatives are protostanes, holostanes, cycloartanes, dammaranes, euphanes, tirucallanes, tetranortriterpenoids, quassinoids, lupanes, oleananes, fredelanes, ursanes, hopanes, isomalabaricanes and saponins (Connolly and Hill, 2008). These derivatives and others were previously reviewed by Breithmaier (2006), and Connolly & Hill (2008). Because of this, from these, cycloartanes and saponins will been only reviewed as largely in this thesis.

Saponins occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops the triterpenoid saponins are

common in plants used as herbs or for their health-promoting properties. Triterpenoids saponins have been detected in many legumes such as soyabeans, beans, peas, Lucerne, etc. and also in alliums (Lanzotti, 2006), tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenu-greek, yucca and ginseng. One example of an extensively studied group of triterpenoid saponins is produced from *Quillaja saponaria*, a tree native to the Andes region. The bark was peeled off and extracted with water by the indigenous peoples as a shampooing agent, and by the shamans as an overall curing agent. *Yucca schidigera* is the most common commercial source of steroids saponins (Francis et al., 2002).

Many sterols occur as glycosides typified by the steroidal saponins. These are responsible for the foaming produced by many plants (Hanson, 2000)

Saponins are found in a large number of plants and some animals (such as the sea cucumber). In plants, they occur in different parts such as root, tuber, bark, leaves, seed, and fruit. Triterpenoid saponins are found principally in dicotyledons while steroidal saponins occur in monocots. However, some plant species contain both triterpenoid and steroidal saponins. Avenacoside (steroidal), for example, occurs in oat leaves while avenacin (triterpenoid) is found in oat roots. Young leaves contain more saponins than mature leaves, but foliage saponins were found to be less haemolytic than root saponins (Wina et al., 2005). Besides, the simplest triterpene, squalene, was first isolated from fish liver oils. Subsequently, it has been found in plant oils and mammalian fats. The common tetracyclic triterpene lanosterol is a major constituent of wool fat and its esters are found in wood resin and the bark of many trees. A triterpene lactone, abietospiran, crystallizes on the surface of the bark of the silver fir, *Abies alba*, giving it a grey-white appearance. Glycyrrhetinic acid is a triterpene found in liquorice, and has healing properties in the treatment of peptic ulcers (Hanson, 2000).

1.3. Distribution

Saponins are found in a wide variety of foods including asparagus, beans, blackberries, peas, potatoes, sugar beet and tea. They occur in many different plant families, as evidenced by the isolation of saponins from phytochemical studies of many plant species over the years. **Table 1.2.**, only contains some species of Leguminosae family, provides a list of species from which saponins

have been isolated in the last 12 years (1998–2010). The Leguminosae have also been extensively investigated for saponins, in particular, species of *Acacia*, *Albizia* and *Astragalus*.

Many of these species have been chosen for phytochemical research based on ethnobotanical use. Of the roughly 200 species listed, 40% of the species were investigated based their traditional usage (Sparg et al., 2004).

Triterpenes are often high-melting colorless solids and are widely distributed among plant resin, cork, and cutin. Only a few of the common triterpenes are actually widely distributed among plants. These include the amyrins and ursolic and oleanic acid which are common on the waxy coatings on leaves and as a protective coating on some fruits. Other triterpenes include the *limonins* and the *cucurbitacins* (Kaufman et al., 1999).

Species	Saponin type	Species	Saponin type
Acacia concinna Wall.	Triterpenoid	*Astragalus microcephalus*	Triterpenoid
Acacia tenuifolia(L.) Willd.	Triterpenoid	*Astragalus amblolepis*	Triterpenoid
Acacia victoriae Benth.	Triterpenoid	*Astragalus sieversianus*	Triterpenoid
Albizia gummifera C.A. Sm.	Triterpenoid	*Astragalus campylosema Boiss. ssp.*	Triterpenoid
Albizia julibrissin Durazz.	Triterpenoid	*Astragalus Membranaceus*	Triterpenoid
Albizia lebbeck Willd.	Saponin mixture	*Astragalus unifoliolatus*	Triterpenoid
Albizia myriophylla Benth.	Triterpenoid	*Astragalus galegiformis L.*	Triterpenoid
Albizia procera Benth.	Triterpenoid	*Astragalus chivensis*	Triterpenoid
Albizia subdimidiata (Splitg.)	Triterpenoid	*Astragalus orbiculatus*	Triterpenoid
Astragalus Peregrinus	Triterpenoid	*Gleditsia sinensis Lam.*	Triterpenoid
A.unifoliolatus	Triterpenoid	*Astragalus caucasicus*	Triterpenoid
Astragalus trigonus DC.	Triterpenoid	*Lathyrus japonicus Willd.*	Triterpenoid
Astragalus australis (L.) R.Br.	Triterpenoid	*Lupinus oreophilus Phil.*	Triterpenoid
Astragalus baibutensis	Triterpenoid	*Medicago sativa L.*	Triterpenoid
Astragalus corniculatus Bieb.	Triterpenoid	*Spartium junceum L.*	Triterpenoid
Astragalus icmadophilus	Triterpenoid	*Swartzia schomburgkii Benth.*	Triterpenoid
Astragalus sieberi	Triterpenoid	*Trifolium spp.*	Triterpenoid
Astragalus gilvus	Triterpenoid	*Trifolium resupinatum L.*	Triterpenoid
Astragalus wiedemannianus	Triterpenoid	*Trigonella foenum-graecum (Willd.)*	Steroidal
Astragalus brachypterus	Triterpenoid	*Vigna angularis (Willd.)*	Triterpenoid

Table 1.2. Some species of Leguminosae family from which saponins have been isolated.

1.4. Biosynthesis

Plants have long been known to synthesize a plethora of small molecules, and plant genome sequencing is reinforcing the view that plants devote considerable efforts towards developing chemical solutions to biological problems. Terpenoids are metabolites of isopentenyl pyrophosphate (IPP) oligomers and comprise the largest group of plant natural products, with over 20 000 known members. Triterpenoids are synthesized from IPP via the 30- carbon intermediate squalene, and include sterols, steroids, and triterpenoid saponins **(Figure 1.7.)**. In addition to sterols (which are 6,6,6,5-tetracycles derived from lanosterol or cycloartenol), nearly 100 additional triterpenoid skeletons have been

described. Cycloartenol and lanosterol can serve as precursors to membrane sterols and steroid hormones. Other triterpenes have less welldefined roles, but many might act in plant defense. Triterpenoid carbon frameworks are cyclized by members of the oxidosqualene cyclase (OSC) family, which has expanded greatly in plants. In this review, we highlight recent progress in the study of the enzyme families that generate triterpene skeletal diversity.

Oxidosqualene cyclases (OSCs) convert oxidosqualene to one or more cyclic triterpene alcohols with up to six carbocyclic rings **(Figure 1.7.)**. Plants biosynthesize diverse triterpenoids and encode multiple OSC enzymes to form these skeletons (Cycloartenol, Lanosterol, Cucurbitadienol, *β*-Amyrin,..etc.). For example, the sequenced genomes of *Arabidopsis thaliana* and *Oryza sativa* (rice) encode 13 and nine apparent OSC enzymes, respectively. Genome mining, heterologous expression, and biochemical characterization of the encoded proteins have provided substantial insight into the triterpenoid biosynthetic capabilities of plants. Cycloartenol synthase (CAS) converts oxidosqualene to cycloartenol through the protosteryl cation intermediate **(Figure 1.7.)** and was the basal plant OSC from which others derived. The *Arabidopsis CAS1* cDNA was cloned by screening extracts from a yeast lanosterol synthase mutant (erg7) that was transformed with random *Arabidopsis* cDNAs for the ability to cyclize oxidosqualene. This isolation facilitated the subsequent homology-based identification of other OSCs. Plant CAS genes have now been cloned and characterized from numerous eudicots, several monocots, and a gymnosperm, consistent with biochemical evidence of CASactivity throughout seed plants. The similarity of plant CAS genes to those in amoebae and bacteria suggests 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 few plants (e.g. in the latex of several *Euphorbia* species. Intriguingly, the *Arabidopsis* protein most similar to CAS1 (65% identical) encodes a lanosterol synthase (LSS). The maintenance of CAS in all examined plant lineages (Abies, Avena, Euphorbia, etc.), despite an apparent ability of at least some plants to produce lanosterol, implies that plants require some cycloartenol metabolites that cannot be made from lanosterol. It is intriguing that Arabidopsis LSS contains residues that correspond to these mutations. This

precedent suggests that only a small evolutionary step could convert CAS to lanosterol synthase if natural selection favored a lanosterol route in plants.

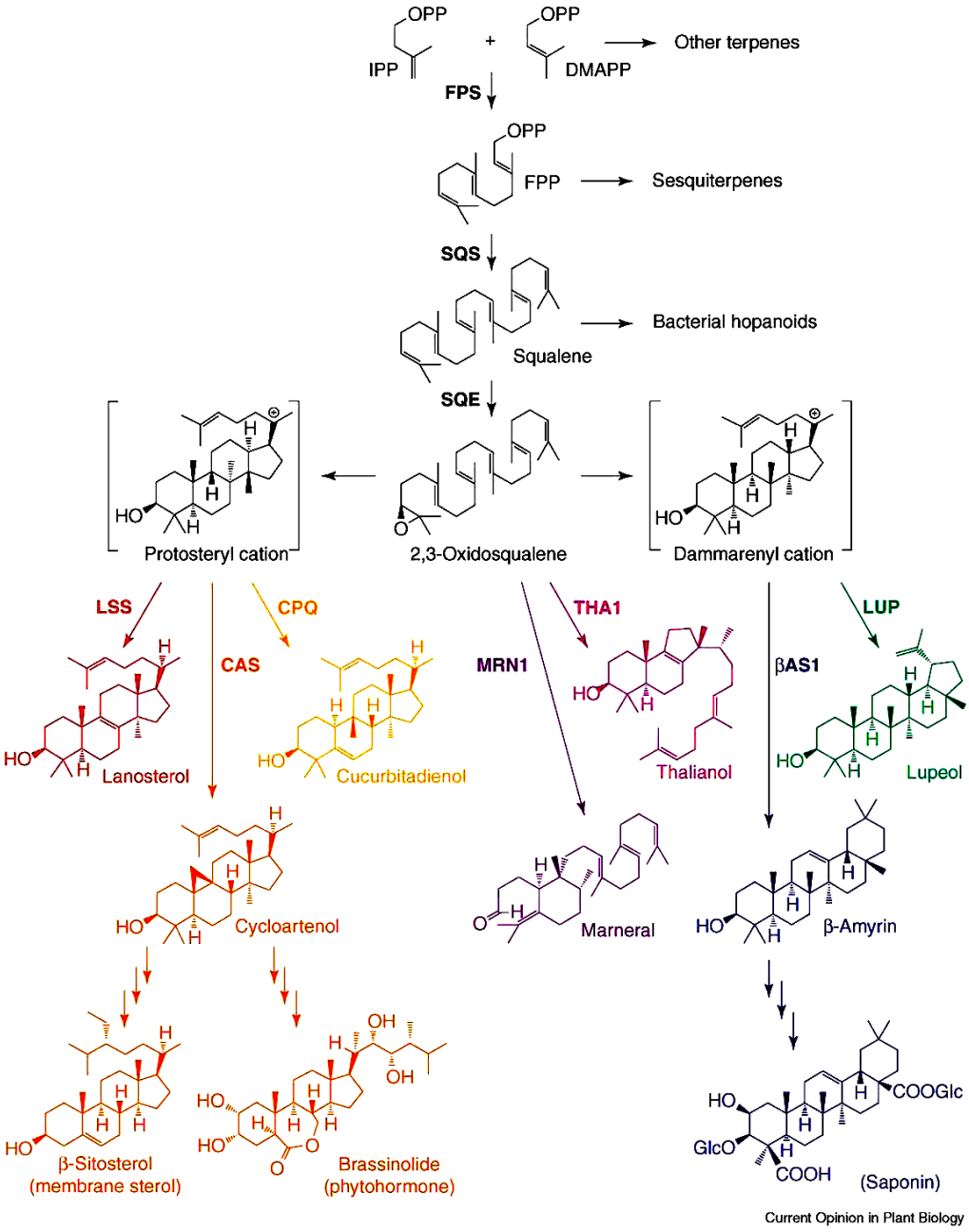


Figure 1.7. Simplified scheme of plant triterpenoid biosynthesis.

Farnesyl diphosphate synthase (FPS) isomerizes isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to farnesyl diphosphate (FPP), which squalene synthase (SQS) converts to squalene. Squalene epoxidase (SQE) oxidizes squalene to 2,3-oxidosqualene. OSC enzymes cyclize 2,3 oxidosqualene through cationic intermediates (e.g. the protosteryl cation, the dammarenyl cation

and others not shown) to triterpene alcohols or aldehydes. A few of the characterized OSC enzymes and products listed in **Table 1** are illustrated. OSC products can be further modified by multiple enzymes to form membrane sterols, brassinosteroids, saponins, and other compounds. bAS1, *β*-amyrin synthase; LUP, lupeol synthase; MRN1, marneral synthase; PP, diphosphate; THA1, thalianol synthase.

Other enzymes that form the protosteryl cation have arisen from CAS gene duplication and diversification. For example, Cucurbita pepo cucurbitadienol synthase (CPQ) is closely related (65–71% identical) to both cycloartenol and lanosterol synthases but produces cucurbitadienol (**Figure 1.7.)**, a precursor of cucurbitacins, which are bitter compounds that might act as antifeedants. The sequence similarity of plant cycloartenol, lanosterol, and cucurbitadienol synthases is consistent with relatively recent divergence of lanosterol and cucurbitadienol synthases from an ancestral CAS via an evolutionary route that maintained the protosteryl cation intermediate **(Figure 1.7.)**.

Several oxidosqualene cyclases that function in secondary metabolism also have been reported to generate single products. Lupeol synthases cyclize oxidosqualene to the dammarenyl cation, promote ring expansion and annulation to the lupyl cation, and terminate by abstracting the C-29 proton to form lupeol **(Figure 1.7.)**. Lupeol synthases are found in *Glycyrrhiza glabra*, *Betula platyphylla*, *T. officinale*, and *Olea europea*; these enzymes are 74–81% identical to one another and form a clade that is distinct from other characterized OSCs. The presence of eurosid and asterid genes in this clade suggests that an accurate lupeol synthase evolved before the divergence of asterids and eurosids.

β-Amyrin synthases also form 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. *β*-Amyrin and its metabolites tend to accumulate in specific tissues for the

localization of 31 *Medicago* saponins). Some of this localization could be transcriptional, as indicated by the tissue-specific expression of *β*-amyrin synthase genes in *Medicago*, *Lotus*, *Pisum, Centella*, *Glycyrrhiza*, and *Avena*.

In addition to lupeol and *β*-amyrin synthases, less broadly distributed enzymes can also have high product selectivity. *Luffa cylindrica* isomultiflorenol synthase (LcIMS1) is cyclizes oxidosqualene through a dammarenyl cation to make isomultiflorenol, a bryonolic acid precursor. Temporal expression studies in *L. cylindrica* cell cultures indicate that LcIMS mRNA levels correlate with bryonolic acid accumulation.

Genome mining in *Arabidopsis* has uncovered two enzymes that generate incompletely cyclized structures that have not been characterized by classical natural product isolation. THA1 converts oxidosqualene to the tricyclic alcohol thalianol **(Figure 1.7.)**. Triterpenoids that have the methyl substitution and olefinic position of thalianol have not been found in nature. MRN1 catalyzes an unusual cyclization reaction: oxidosqualene is converted to a bicyclic cation that undergoes rearrangement and A-ring cleavage to generate a monocyclic aldehyde **(Figure 1.7.)**. Marneral also has not been isolated from any natural source but is suggested as a biosynthetic precursor of iridals found in sword lilies; these monocots are only distantly related to the eudicot *Arabidopsis*. These examples demonstrate that heterologous expression of novel OSC enzymes can provide a means to mine plant genomes for new biosynthetic pathways and low-abundance natural products.

OSC enzymes are a major radiation point in the triterpenoid pathway and facilitate the production of numerous and diverse triterpenoids. Interestingly, plants also have multiple genes that are predicted to encode squalene epoxidase (SQE) enzymes, also known as squalene monooxygenases. SQEs catalyze the first oxygenation step in triterpene biosynthesis, converting squalene into the OSC precursor 2,3-oxidosqualene **(Figure 1.7.)**. The first cloned SQE gene, from *Saccharomyces cerevisiae*, was identified by complementation of the yeast ergosterol biosynthetic mutant erg1. Two *Medicago* squalene epoxidases that were identified by sequence similarity are able to rescue the yeast erg1 mutant defect when heterologously expressed, indicating that the encoded enzymes function as squalene epoxidases.

In addition to the two biochemically characterized *Medicago* SQEs, the sequenced genomes of *Oryza*, *Populus*, and *Arabidopsis* encode multiple predicted SQEs. Each of these plants possesses genes that are closely related (63–82% identical) to the characterized Medicago SQEs, but Arabidopsis also has three SQE-like genes (Philips et al., 2006).

1.5. Role in Plants

The physiological role of saponins in plants is not yet fully understood. While there are a number of publications describing their identification in plants, and their multiple effects in animal cells and on fungi and bacteria, only a few have addressed their function in plant cells. Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. Saponins may be considered a part of plants' defence systems, and as such have been included in a large group of protective molecules found in plants named 'phytoanticipins' or 'phytoprotectants' (Morrisey & Osbourn, 1999). The first term describes those saponins, such as A and B avenacosides in oat, that are activated by the plant's enzymes in response to tissue damage or pathogen attack (Gus-Mayer et al. 1994). The second describes those saponins that have a general anti-microbial or anti-insect activity. A glycosylated triterpenoid saponins from peas (*Pisum sativum*) was purified and characterized as a specific inhibitor of diguanylate cyclase, a key regulatory enzyme in the cellulose (Ohana et al. 1998). It has also been suggested that saponins could be a source of monosaccharides (Barr et al. 1998; Francis et al., 2002).

Generally, saponins are found in tissues that are most vulnerable to fungal or bacterial attack or insect predation. Therefore, one of their roles is to act as a chemical barrier or shield in the plant defense system. Alfalfa saponins are induced by insect attack and act as a deterrent to subsequent attacks. When alfalfa saponins were administered in the diet of larval and pupal stages, retarded growth, increased mortality, and decreased fecundity and fertility.Saponins also control rhizosphere bacteria in the soil (Wina et al., 2005).

1.6. Isolation and Characterization

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 from even well-known saponin-containing plants 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. The modern methods available for the separation and analysis of saponins have been well reviewed by Marston et al. (2000), Muir et al. (2000) and Schopke (2000). These methods will be only outlined in the present review.

There are several strategies available for the isolation of saponins. As a general rule, they begin with the extraction of the plant material with aqueous methanol or ethanol. Further processing of the extract is carried out after evaporation under reduced pressure, dissolution in a small amount of water and phase separation into n-butanol. It is currently recognized that this step sometimes undesirable, since only those saponins with short oligosaccharide side chains will eventually be extract be into the the butanolic phase. A further purification is then carried out, which involves liquid chromatograpy over a silica gel column, or a gradient elution from a polymeric support or liquid-liquid partition chromatography, or, as most commonly employed, HPLC separation. In most cases, certain of the above steps have to be repeated with a change of support or eluent to achieve high purity.

Once the saponin has been purified, it may be subjected to analytical methods including MS, proton and carbon NMR, and infrared Spectroscopy(IR). Other classical methods are used to ascertain the presence of saponins in a crude plant extract, and to elucidate their composition throughout purification steps. TLC and staining with dehydrating reagents containing aromatic aldehydes (such as anisyl aldehyde in sulfuric acid) are commonly used. The pure saponin may also be hydrolysed to verify the nature of its glycosidic moieties (Francis et al., 2002).

Due to the fact that saponins usually occur in plants as a mixture of structurally related forms with very similar polarities, their separation still remains a challenge. It is a usual practice in isolation of these compounds that a number of different separation techniques (TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC) should be used to obtain pure compounds for the structure and biological activity determination. Early

work on saponins included hot extraction of plant material with alcohol–water solutions followed by evaporation of alcohol and extraction of saponins into butanol (liquid–liquid extraction). However, hot extraction may disintegrate some labile functions (acylated forms) and produce artifacts rather than genuine saponins. Besides, extraction with methanol in some cases, especially for steroidal saponins may result in formation of methyl derivatives, not found originally in plants. Thus, for obtaining real composition of saponins, cold extraction with ethanol–water solutions should be rather recommended. In liquid–liquid extraction, some highly polar saponins (bidesmosides, tridesmosides) can be lost or extraction may not be quantitative. The alternative for liquid–liquid extraction is selective solid phase extraction (SPE) on number of sorbents (C_{18}, C_8). In SPE method, saponin extract (aqueous 10–20% methanol) can be loaded on preconditioned sorbent and washed with methanol–water. Ratio of methanol–water has to be optimized individually in preliminary tests for different classes of saponins on ready to use 1–2 cm^3 cartridges. The procedure is very convenient for preparation of highly purified saponin mixtures for column separations of for biological activity test (Oleszek and Bialy, 2006).

1.7. Astragalus Genus

1.7.1. 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; Çalış et al., 2008; Tabanca et al., 2005; Özipek 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; Çalış et al., 1999; Yeşilada et al., 2005; Çalış 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).

1.7.2. 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).

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. (Çaliş 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; Yeşilada 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).

1.7.3. 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 1.10.)**, the nitro compounds and 3-nitropropyl glucosides **(Figure 1.8 and 1.9)**, and the selenium compounds (Polat et al., 2009a, 2009b).

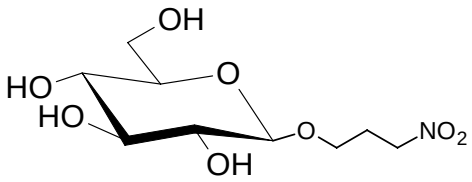


Figure 1.8. Miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside)

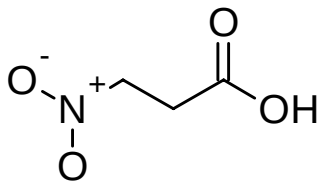
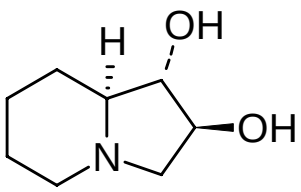
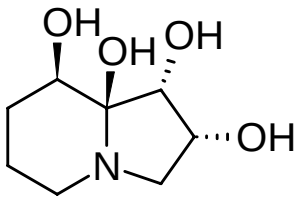


Figure 1.9. 3-nitropropionic acid(3- NPA)



Lentiginosine



Swainsonine

Figure 1.10. 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

1.8. 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).

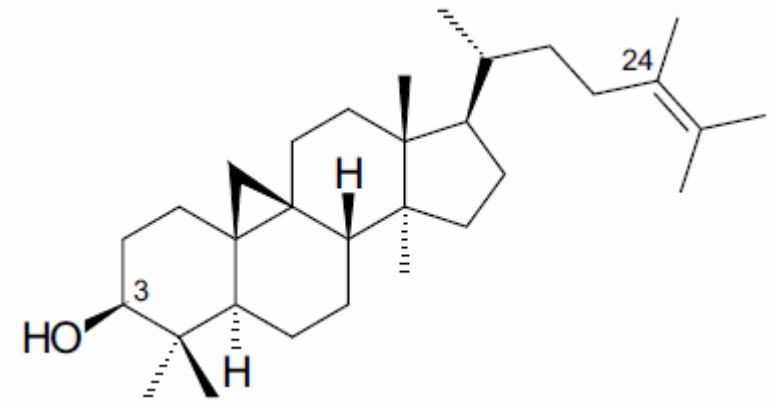


Figure 1.11. (+)-24-methylcycloarta-24-en-3*β*-ol (cyclobranol, cycloartenol)

Triterpenoids are often found among secondary plant metabolites. Triterpenoids of the cycloartane, lanostane, and oleanane **(Figure 1.1.)** 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 1.13.)** and lanostane **(Figure 1.12.)** triterpenoids. Cycloartanes are derivatives of 9*β*,19-cyclolanostane and are produced exclusively by photosyntheic eukaryotes.

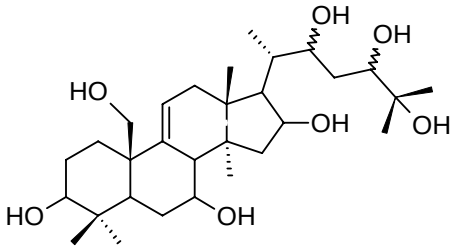


Figure 1.12. Lanostane triterpenoid (Orbigenin)

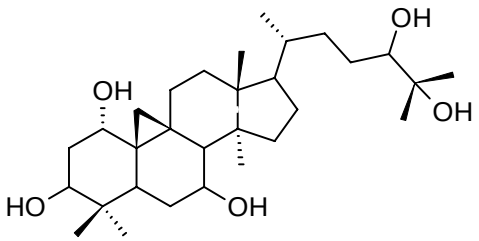


Figure 1.13. Cycloartane triterpenoid (Cyclomacrogenin B)

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 monosaccharide D-apiose is rather rare and found in two glycosides (Mamedova and Isaev, 2004).

1.8.1. 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., compound 1-6),**

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

3) 16*β*,23;16*α*,24-diepoxycycloartanes (**Figure 1.16., compound 13and14**),

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

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

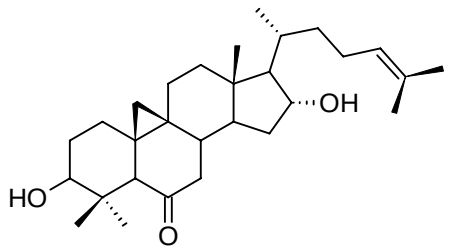
6) 24-nor-16*β*,23-epoxycycloartanes **(Figure 1.17., compound 17 and 18).**

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

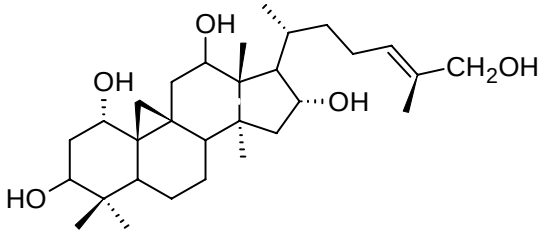
7) 16*β*,23; 22*β*,25-diepoxycycloartanes **(Figure 1.18., compound 19and20)**

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

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



1



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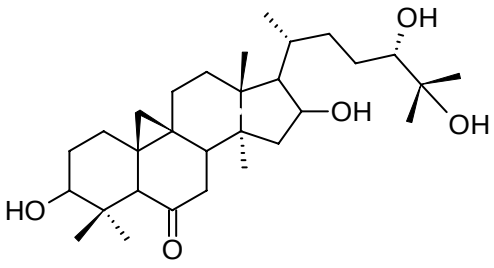

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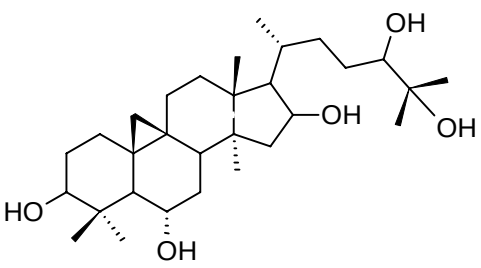

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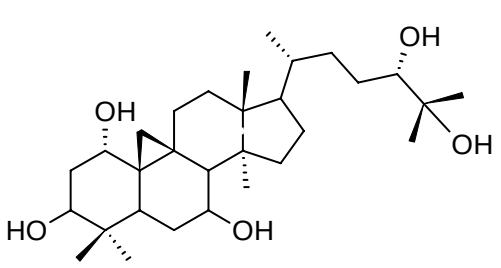

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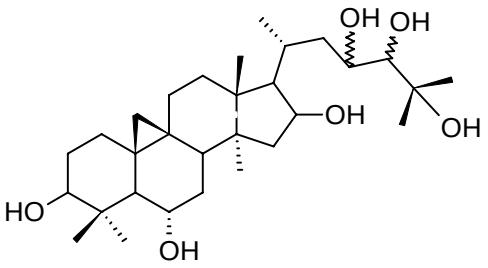

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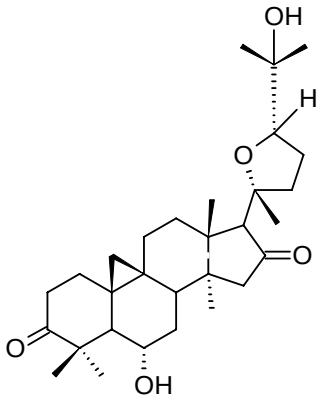
Figure 1.14. Some cycloartanes with an acyclic side chain

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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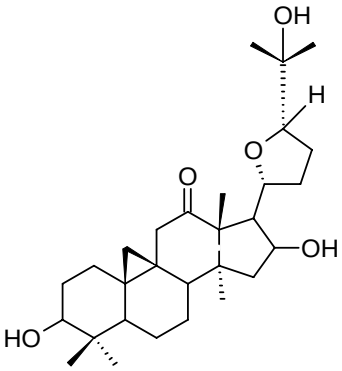

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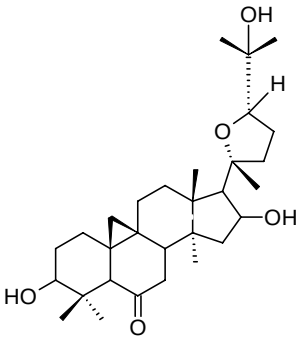

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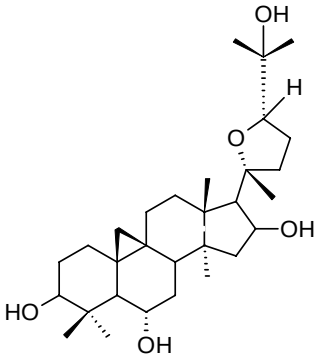

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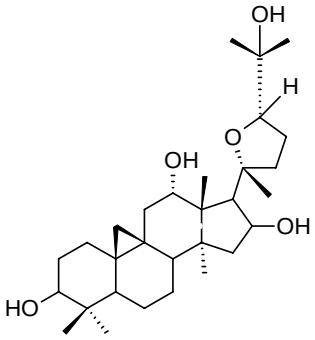

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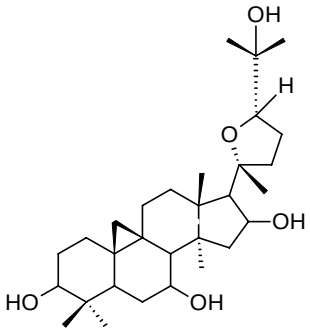

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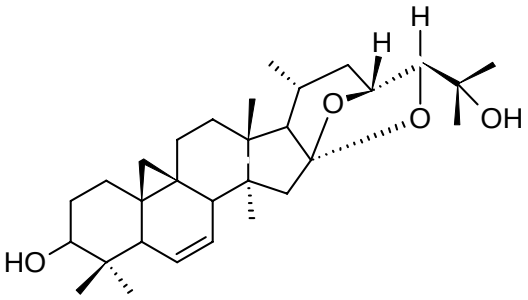
Figure 1.15. Some 20,24-epoxycycloartanes



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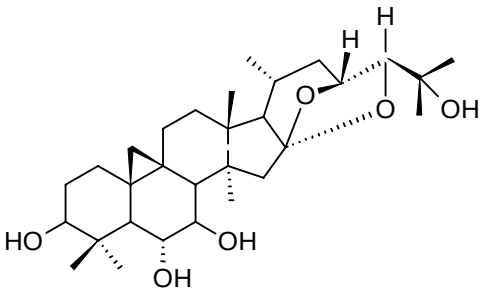

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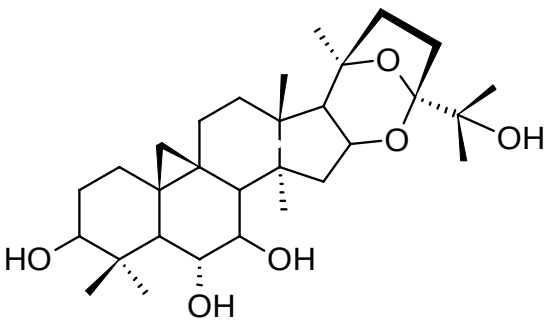
Figure 1.16. 16*β*,23;16*α*,24-diepoxycycloartanes



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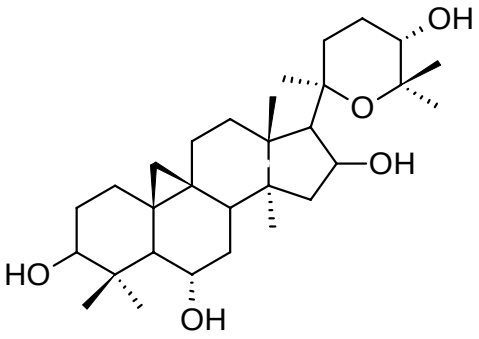

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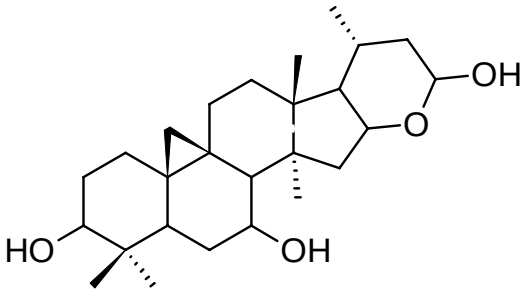

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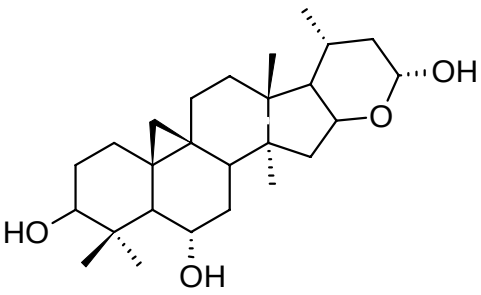

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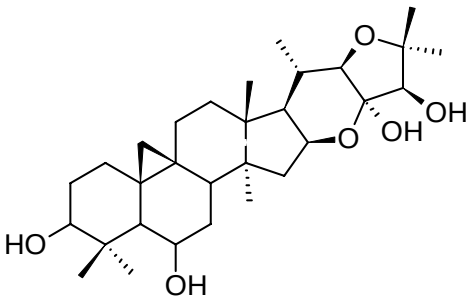
Figure 1.17. 16*β*,24;20,24-diepoxycycloartanes (15), 20,25-epoxycycloartanes (16), 24-nor-16*β*,23-epoxycycloartanes (17, 18)



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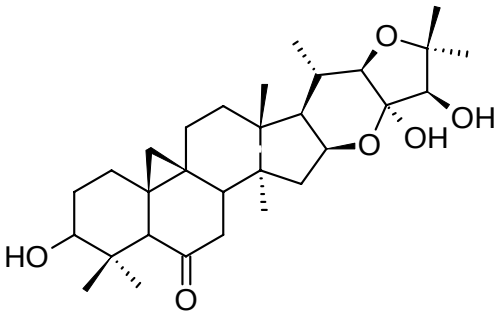

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Figure 1.18. 16*β*,23;22*β*,25-diepoxycycloartanes

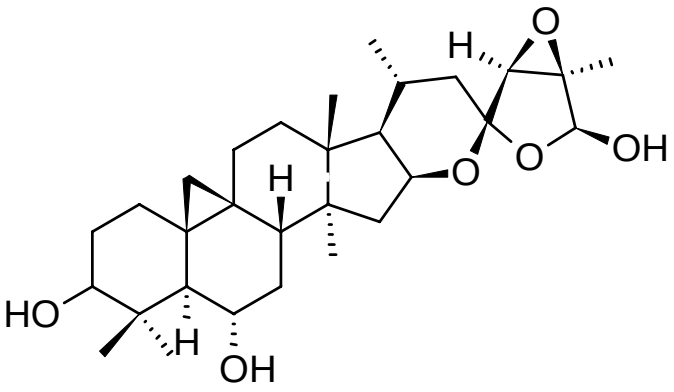

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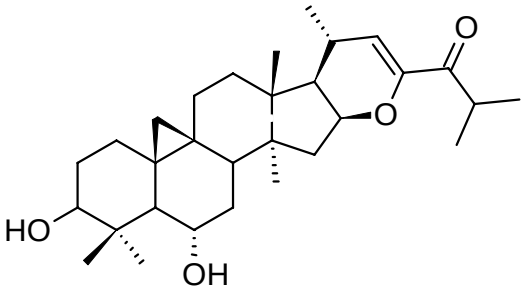
Figure 1.19. 16 *β*,23;23,26;24,25-triepoxycycloartanes



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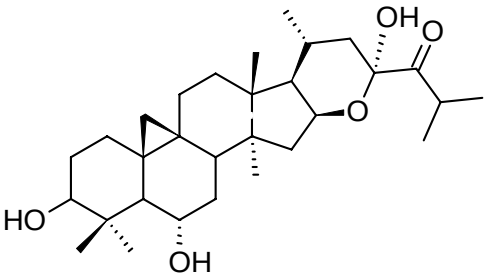

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Figure 1.20. 16*β*,23-epoxycycloartanes

1.8.2. Recent Reviews from *Astragalus* Species

Cycloartane triterpenoids were first discovered in *Astragalus* plants. Cycloartanes dominate the known triterpenoids in plants of this genus (Mamedova and Isaev, 2004).

Earlier investigations of *Astragalus* species resulted in the isolation of a series of cycloartane-type triterpene glycosides that these were reviewed by Isaev and Mamedova (2004) until 2004 and by Polat from 2005 to 2009 (Polat, E., 2009a, 2009b, 2010). Owing to this, we decided to research studies beginning from 2010 in this thesis.

Six cycloartane-type triterpene glycosides were isolated from *Astragalus icmadophilus* along with two known cycloartane-type glycosides, five known oleanane-type triterpene glycosides and one known flavonol glycoside. From these, the two compounds which are based on cyclocephalogenin as aglycone, more unusual in the plant kingdom, so far reported only from *Astragalus spp* (Horo et al., 2010).

For the first time three different natural compounds, isolated from hairy roots of *Astragalus membranaceus*, cultivated in airlift bioreactor were tested for their cytotoxic potential and apoptosis induction in a panel of human tumor cell lines. Root cultures, cultivated in bioreactor gave 18.5 gl^{-1} dry wt roots with the highest astragaloside production in vitro up to now — 1.64% (astragaloside I), 1.12% (astragaloside II) and 1.08% (astragaloside III). In this manner the production in airlift bioreactor can be used as means of reliable supply of

cycloartane saponins to extend the research to human clinical studies (Iankova, 2010).

Phytochemical efforts on the methanol extract of *Astragalus illyricus* have resulted in the isolation and identification of three cycloastragenol glycosides (astragalosides) namely, astraverrucin I or cycloaraloside A, astragaloside III and cyclounifolioside B (Barbic, 2010).

2. MATERIAL AND METHODS

2.1. General

The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C) in C_5D_5N using TMS as internal standart. Column chromatography was carried out a Silica gel 60 (Merck 7734) and Li Chroprep RP (C_{18}, Merck 9303). TLC was conducted on pre-coated silica gel 60 F_{254} aluminium sheets (Merck 5554) and RP-18 F_{254} (Merck) plates.

Compounds were detected at 254 and 366 nm UV lamp 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, VLC and TLC controls) the following solvent systems were used:

I	$CHCl_3$:MeOH	90:10
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
X	MeOH:H_2O	5:5

XI	$MeOH:H_2O$	4:6
XII	$MeOH:H_2O$	2:8

2.2. Plant Material

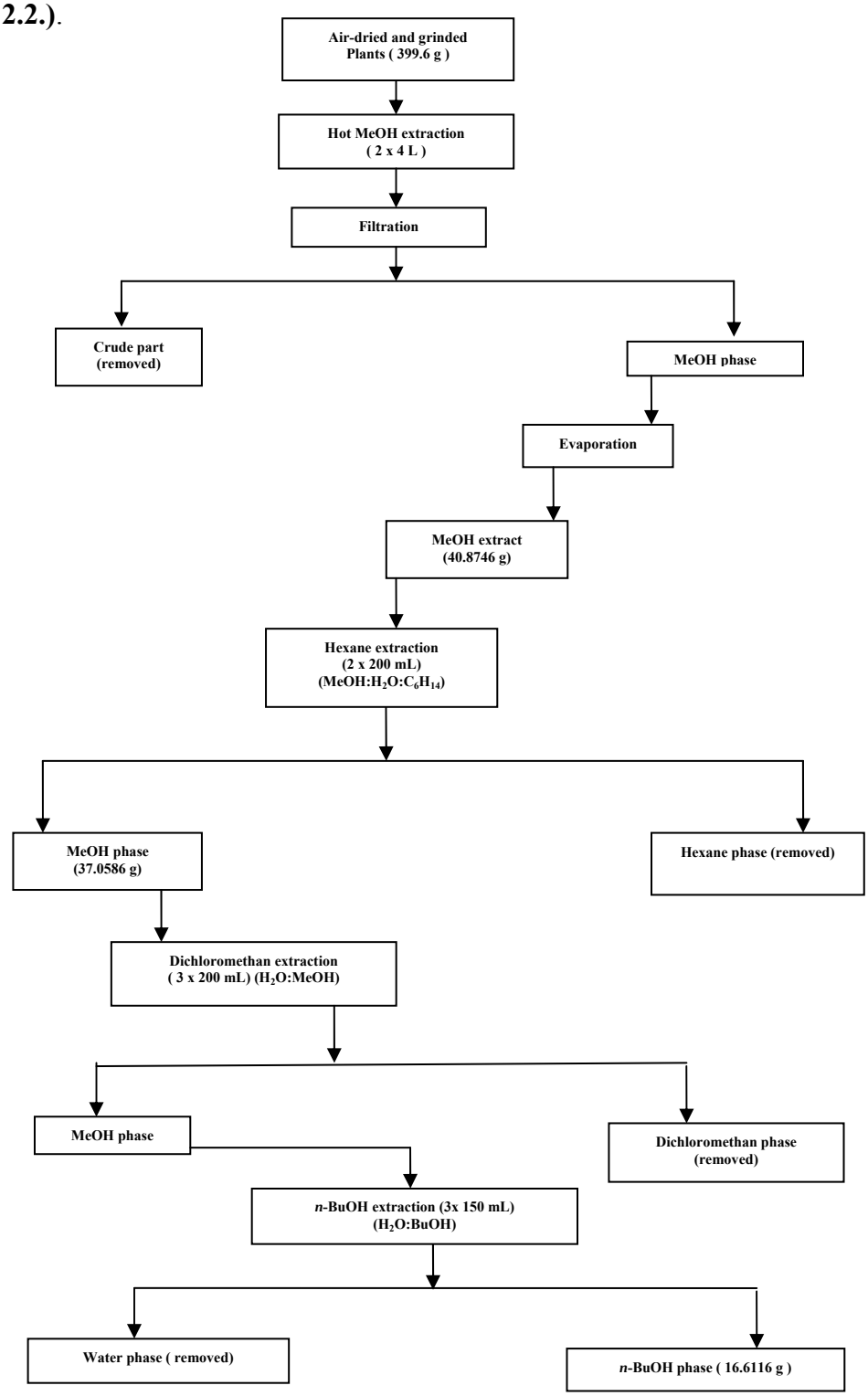
Astragalus erinaceus was collected from Van in Turkey 2009 Plant material was identified by Serdar G. Senol (Department of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). A voucher specimen was deposited in the Herbarium of Ege University, Izmir, Turkey (EGE).

2.3. Isolation and Purification

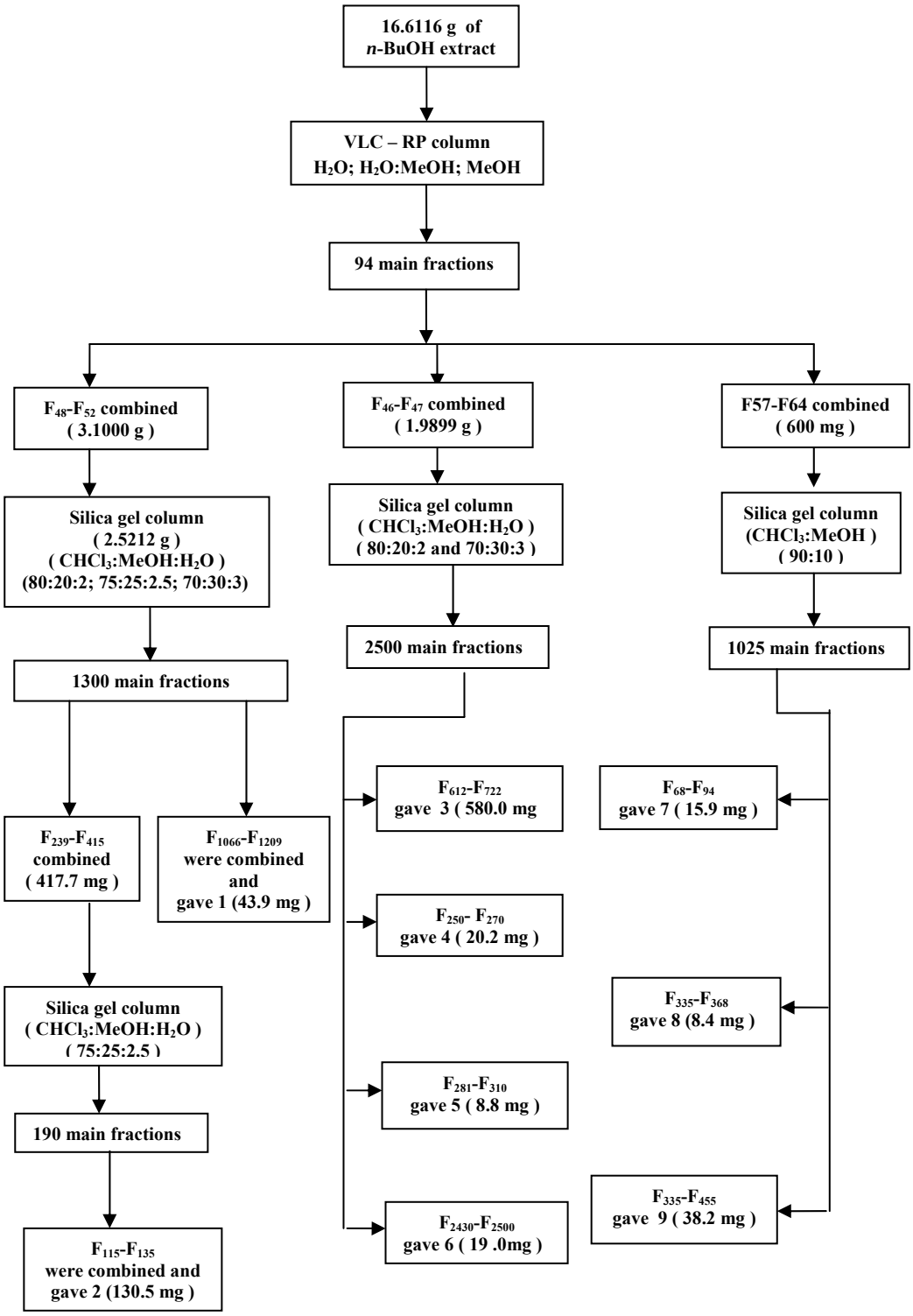
Air-dried and grinded plants (399.6 g) were extracted with MeOH (2x4 L) at 60 ^oC. After filtration, the solvent was removed by rotary evaporation yielding 40.8746 g of extract. The MeOH extract was dissolved in H_2O (200 mL), and successively partitioned with n-hexane (2x200 mL), CH_2Cl_2 (3x200 mL), and *n*-BuOH saturated with H_20 (3x150 mL)**(scheme 2.1)**.

The *n*-BuOH extract (16.6116 g) was subjected to vacuum liquid chromatograpy (VLC) using reversed-phase material (Lichroprep RP-18, 25-40 μm) employing H_2O (600 mL), H_2O-MeOH (8:2, 1650 mL; 6:4 , 2550 mL; 4:6, 1800 mL; 3:7, 3450 mL; 2:8, 2700 mL) and MeOH (1800 mL) to give 94 main fractions(1-94). First of all, Fractions 48-52 (2.5212 g) that rich in saponins were combined and applied to an open column chromatograpy using silica gel (370 g) as stationary phase. Elution performed with $CHCl_3$-MeOH-H_2O (80:20:2, 3700 mL), $CHCl_3$-MeOH-H_2O (75:25:2.5; 1900mL) and $CHCl_3$-MeOH-H_2O (70:30:3, 2000 mL) mixtures to yield 1300 main fractions. Fractions 1066-1209 were combined and afforded compound **1** (43.9 mg). From this column, fractions 239-415 (417.7 mg) were combined and fractionated over silica gel (114 g). Elution performed with $CHCl_3$-MeOH-H_2O (75:25:2.5, 1800 mL) and collected 190 main fractions. Fractions 115-135 gave compound **2** (130.5 mg). Secondly, Fractions 46 and 47 (1.9899 g) from VLC-RP were combined and subjected to silica gel (258 g) column employing with $CHCl_3$-MeOH-H_2O (80:20:2, 5650 mL: 70:30:3, 2100 mL) to give 2500 main fractions. Fractions 612-722, 250-270, 281-310 and 2430-2500 afforded compound **3** (580.0 mg), **4** (20.2 mg), **5** (8.8 mg) and **6** (19.0 mg), respectively.

Lastly, Fractions 57-64 eluted with H_2O:MeOH (3:7; 1050 mL) from VLC-RP also were rich in saponins. These were combined (600 mg) and applied to silica gel (100 g). Elution was started with $CHCl_3$-MeOH (9:1; 5250 mL) to yield 1025 main fractions. Fractions 68-94, 335-368 and 435-455 were combined and afforded compound **7** (15.9 mg), **8** (8.4 mg), **9** (38.9 mg), respectively **(Scheme 2.2.)**.



Scheme 2.1. Extraction of *Astragalus Erinaceus*



Scheme 2.2. Isolation of compounds from **1** to **9**

3. RESULT AND DISCUSSIONS

3.1. Structural Identification of Compound 3 (AER4)

A detailed comparison of the aglycon moiety NMR data (^{1}H, ^{13}C, HMQC and HMBC) of compound **3** (AER 4). The main features of a cyclopropane-type triterpene possesing an acyclic side chain were evident for compound 3(AER4): In particular, the ^{1}H-NMR spectrum of **3** (AER 4) characteristic signals due to a cyclopropane-methylene protons as an AX system (δ_H 0.22, 0.60, J_{AX}=3.22 Hz, H_2-19), six tertiary methyl groups at δ_H 1.36(3H, s), 1.42(3H, s), 1.01(3H, s), 2.01(3H, s), 1.47(3H, s), and 1.49(3H, s), respectively, H_3-29, H_3-18, H_3-30, H_3-28, H_3-27, H_3-26, a secondary methyl group at δ_H 1.10(d, *J*=5.6 Hz, H_3-21) (Bedir et al., 2001; Polat, 2009 b) **(Table 3.1.)**. Besides, four methine proton signals at δ_H 4.72(ddd, H-16), 3.70 (dt, H-6), 3.90(dd, H-24) and 3.57 (dd, H-3, *J*=11.6; 4.0 Hz), which were indicative of secondary alcoholic functions **(Table 3.1.)**. Addditonaly, the resonances for two anomeric protons were observed at δ_H 4.84(d, *J*=7.2 Hz) and 4.91(d, *J*=8.0 Hz), indicative of the presence of two α-linked sugar units **(Table 3.1)**. The HMBC spectrum showed two correlation peaks between the proton signal at δ 4.84 (d, *J*=7.2 Hz, H-1_{xyl}) and the carbon resonance at δ 88.9 (C-3), and proton signal at δ 4.91 (d, *J*=8.0 Hz, H-1_{Glu}) and the carbon resonance at δ 79.4 (C-6). Thus, compound **3** (AER4) **(Figure 3.1.)** was considered to be a cycloartane-type titerpene diglycoside and reported previously for **cyclocanthoside E** (Bedir, 1998b).

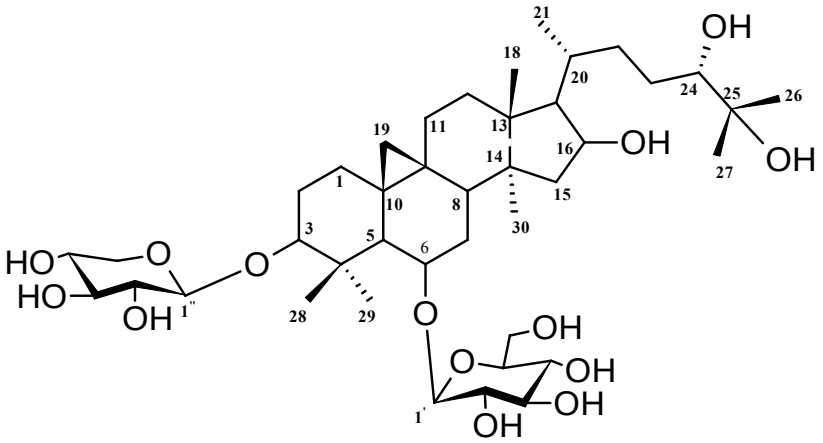
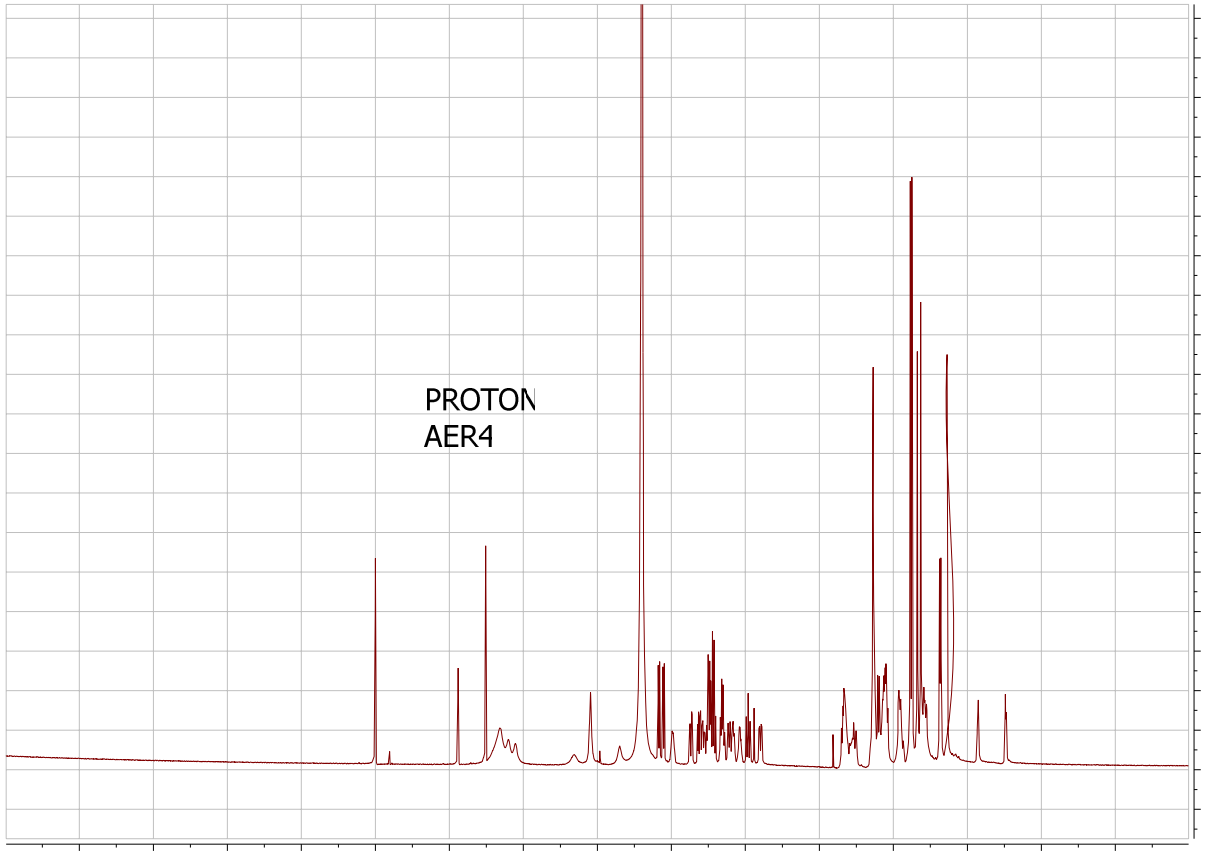


Figure 3.1. Structure of **3** (AER4)

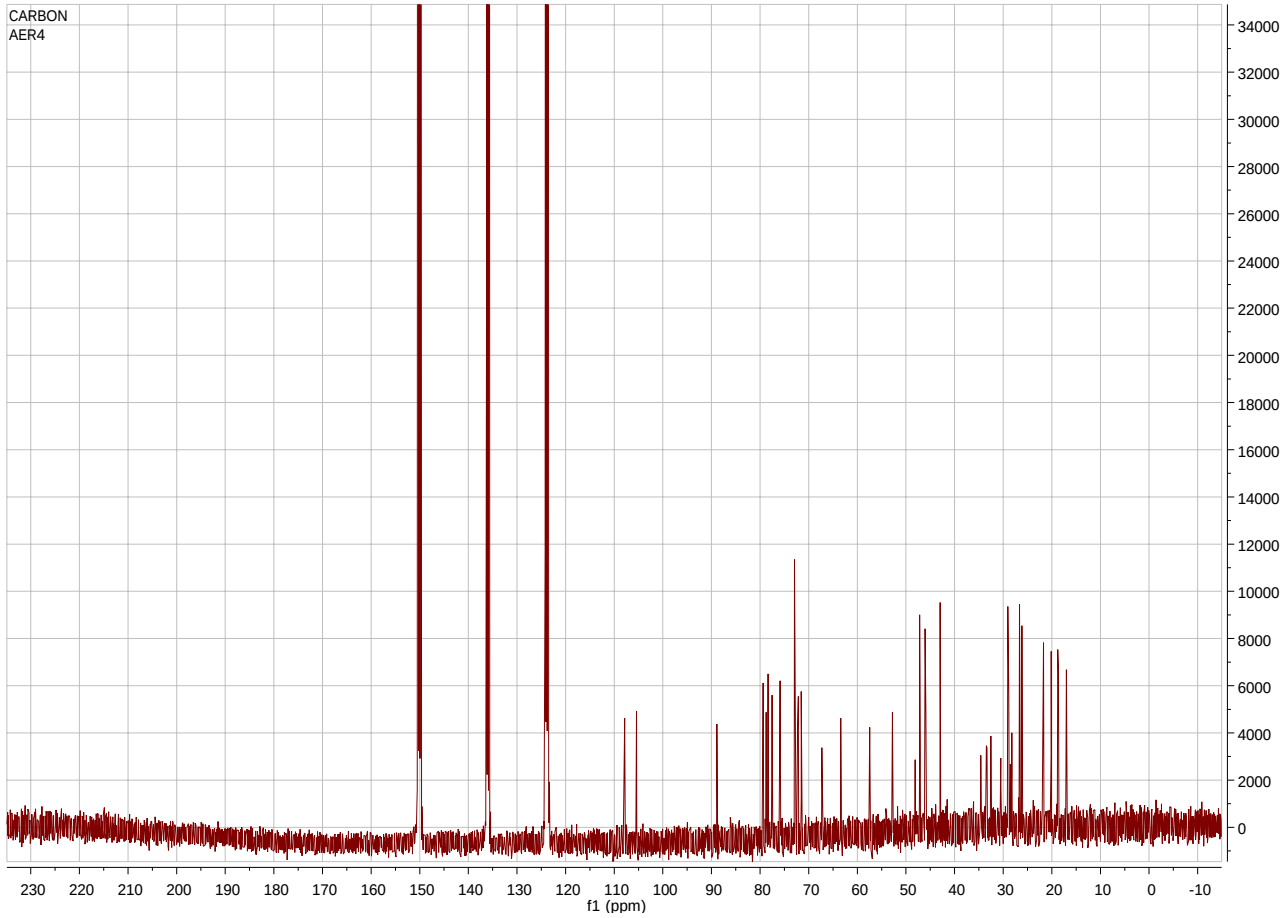
Table 3.1. ^{13}C and ^{1}H- NMR spectrum data of **3** (AER 4)

C / H	δ_C(ppm)	δ_H(ppm), *J*(Hz)		C / H	δ_C(ppm)	δ_H(ppm), *J*(Hz)
1	32.5	1.28m, 1.34 m		**24**	77.5	3.90 dd
2	30.5	2.38 m, 2.42 m		**25**	72.9	
3	88.9	3.57 dd(11.6,4.0)		**26**	26.1	1.49 s
4	43.0			**27**	26.8	1.47 s
5	52.8	1.95 d(8.8)		**28**	28.9	2.01 s
6	79.4	3.70 dt		**29**	17.0	1.36 s
7	34.6	2.24m, 2.28 m		**30**	20.13	1.01 s
8	45.9	2.0 m				
9	21.7			**Glu**		
10	29.0			**1'**	105.5	4.91 d(8.0)
11	26.6	1.58 m		**2'**	75.9	4.04
12	33.5	1.64 m, 1.66 m		**3'**	79.5	4.20
13	46.1			**4'**	72.2	4.16
14	47.2			**5'**	78.8	4.15
15	48.2	1.83 dd(12.0,6.0)		**6'**	63.5	4.32dd(12.0;6.0)
		2.40 dd(12.0,9.0)				4.55dd(12.0;5.2)
16	72.3	4.72 ddd				
17	57.5	1.81 m		**Xyl**		
18	18.78	1.42 s		**1''**	107.9	4.84 d(7.2)
19	28.5	0.22d; 0.60d(3.2)		**2''**	75.92	4.04
20	33.3	1.65 m		**3''**	78.4	3.89
21	18.7	1.10 d(5.6)		**4''**	71.6	4.22
22	33.3	1.65 m		**5''**	67.3	3.70 t(11.2)
23	28.2	1.85 m				4.36dd(11.2;5.2)

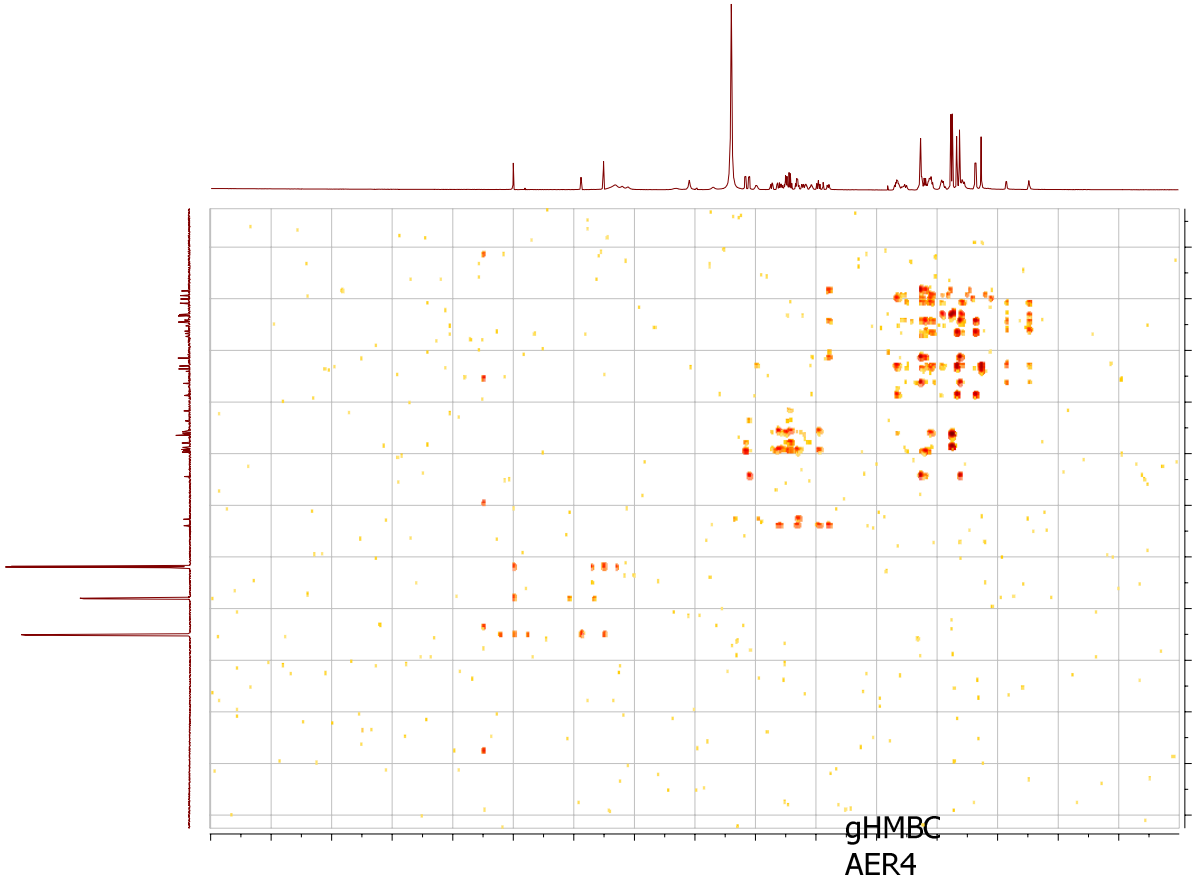
Spectrum 3.1 ^{1}H NMR spectrum of compound **3**



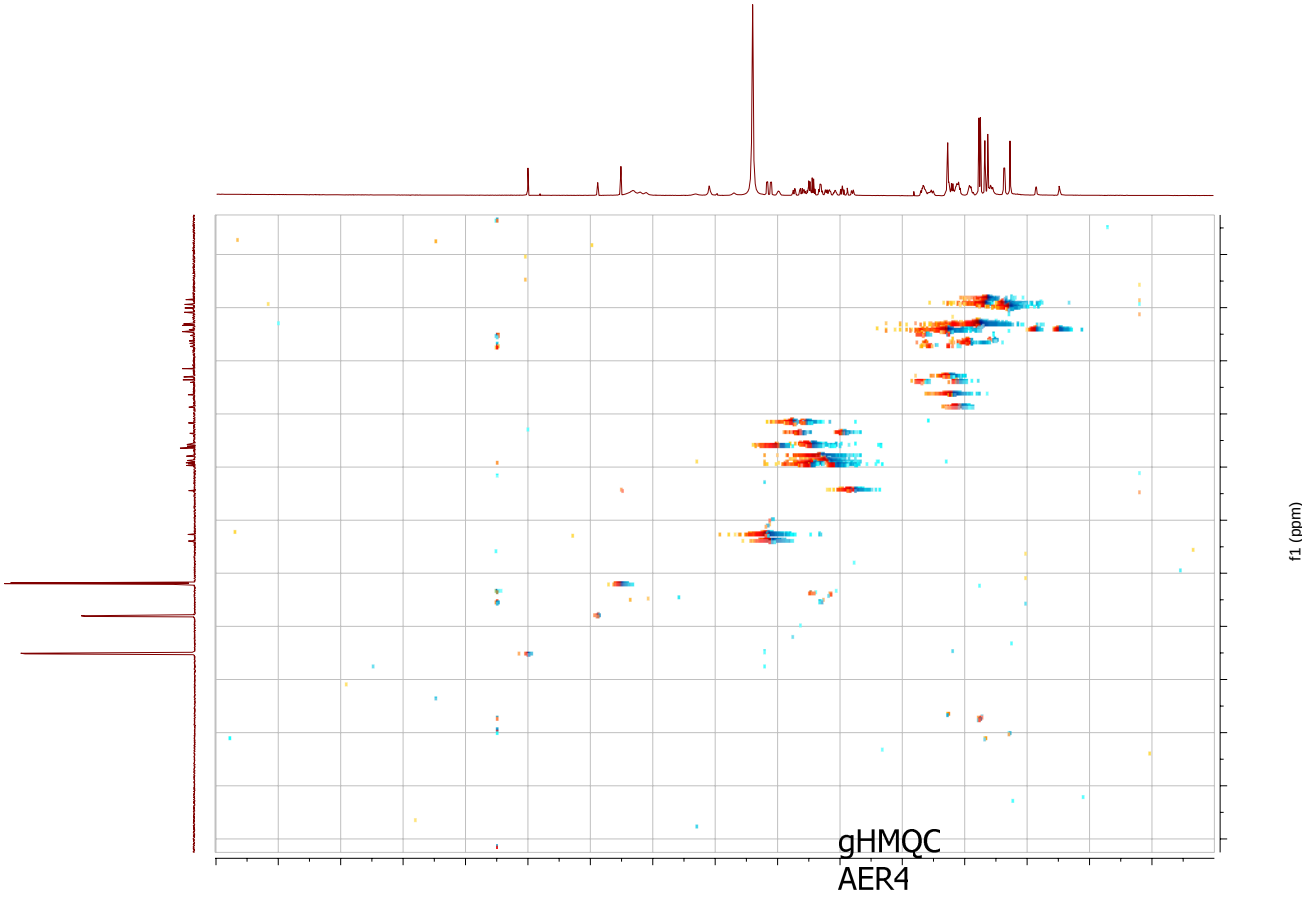
Spectrum 3.2 ^{13}C-NMR spectrum of compound **3**



Spectrum 3.3 HMBC spectrum of compound **3**



Spectrum 3.4 HMQC spectrum of compound **3**



3.2. Structural Identification of Compound 9(AER 10)

The NMR data (^{1}H, ^{13}C, HMQC and HMBC) showed, particularly ^{1}H NMR spectrum, characteristic signals cyclopropane-methylene protons as an AX system (δ_H 0.31 and 0.67, d, J_{AX}=3.6, 3.2 Hz, H_2-19) and seven tertiary methyl groups at δ_H 0.97(3H, s, H_3-30), 1.33(3H, s, H_3-21), 1.34(3H, s, H_3-26), 1.43(3H, s, H_3-29), 1.47(3H, s, H_3-18), 1.60(3H, s, H_3-27), 1.97(3H, s, H_3-28), and four methine proton signals at δ_H 4.96(ddd, H-6), 3.9(dd, H-24, J=8.8,5.6 Hz), 3.86(ddd, H-6), 3.15(dd, H-3, J=20,10 Hz) which were indicative of secondary alcoholic functions **(Table 3.2.)**. Furthermore, three oxygenated quarternary carbons (δ_C 87.6, 82.04, 71.6; C-20, C-24, and C-25, respectively). In addition, the ^{1}H NMR spectrum of **9** (AER10) clearly showed only one anomeric proton doublets at δ_H 4.95 (d, J=8.0 Hz), indicative of one *β*-linked sugar unit in the downfield region. This correlated to carbon at δ_C 105.5 in the HMQC spectrum. The ^{13}C NMR spectrum of **9** (AER10) displayed 36 carbon signals.

On basis of HMQC and HMBC spectra and by comparison with data of related compounds, all signals were assigned. Thus, the aglycon of **9** (AER10) was identified as acycloastragenol. From 1D- and 2D- NMR experiments, the attachment of the glucose moiety at C-6 (δ_C 80.17) of the aglycon was determined by means of the diagnostic glycosidation shift of this carbon atom and confirmed by the results of the HMBC spectrum. The resonances of C-3 (δ_C 78.6) and H_3-29 (δ_H 1.43) were indicative of an unsubstitued –OH group at C-3. Consequently, the structure of **9** (AER10) **(Figure 3.2.)** was elucidated as a cycloartane which have 20-24 epoxy side chain by comparing spectral data with those reported previously for **brachycoside B** (Bedir et al, 1998a,1998b, Polat, 2009b).

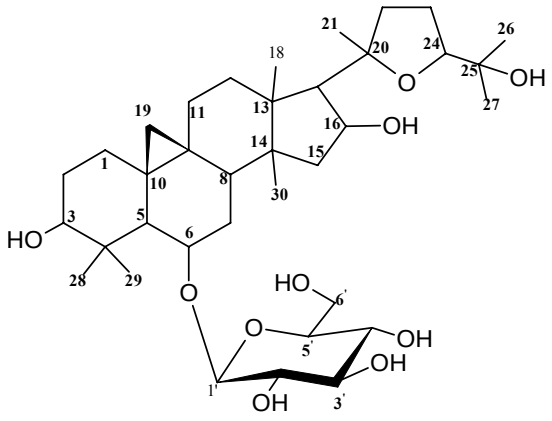
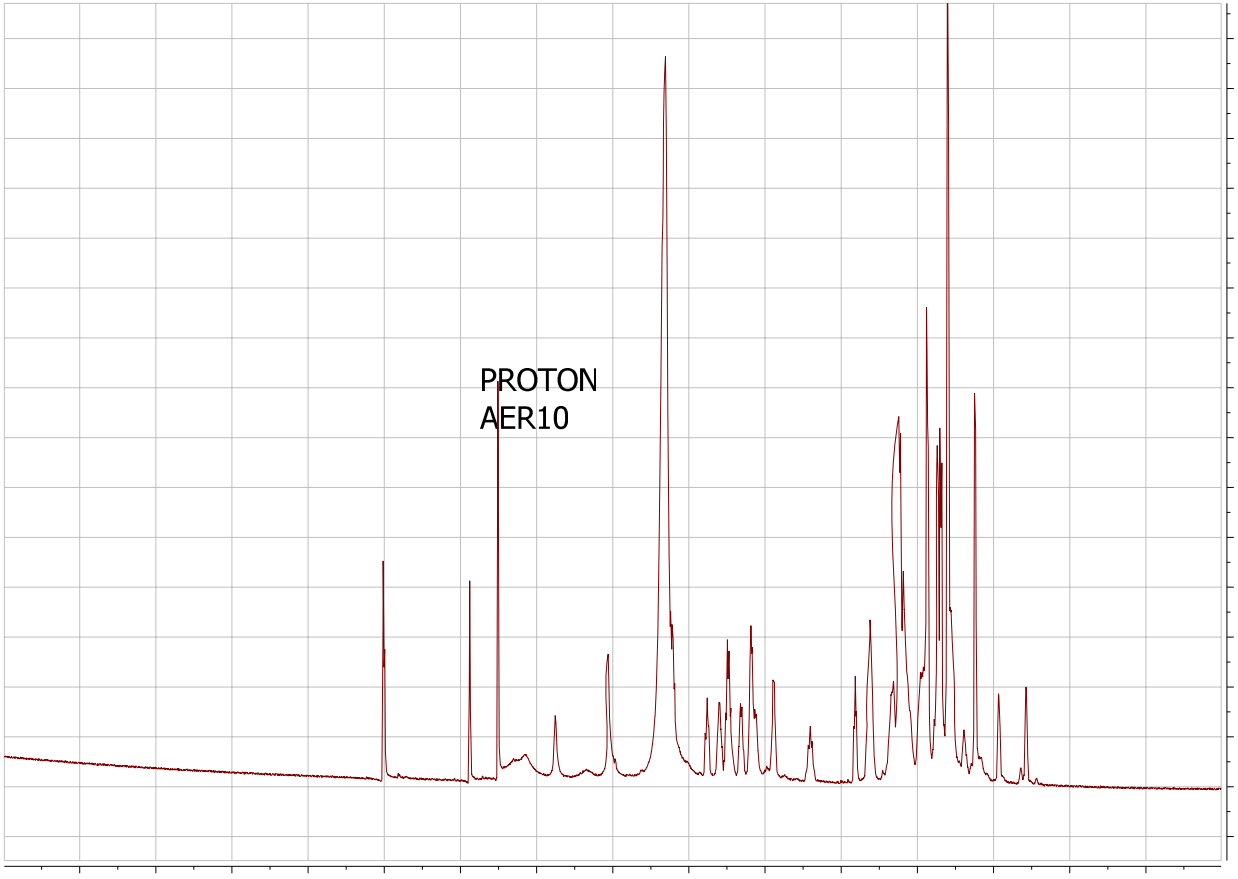


Figure 3.2. Structure of **9**(AER 10)

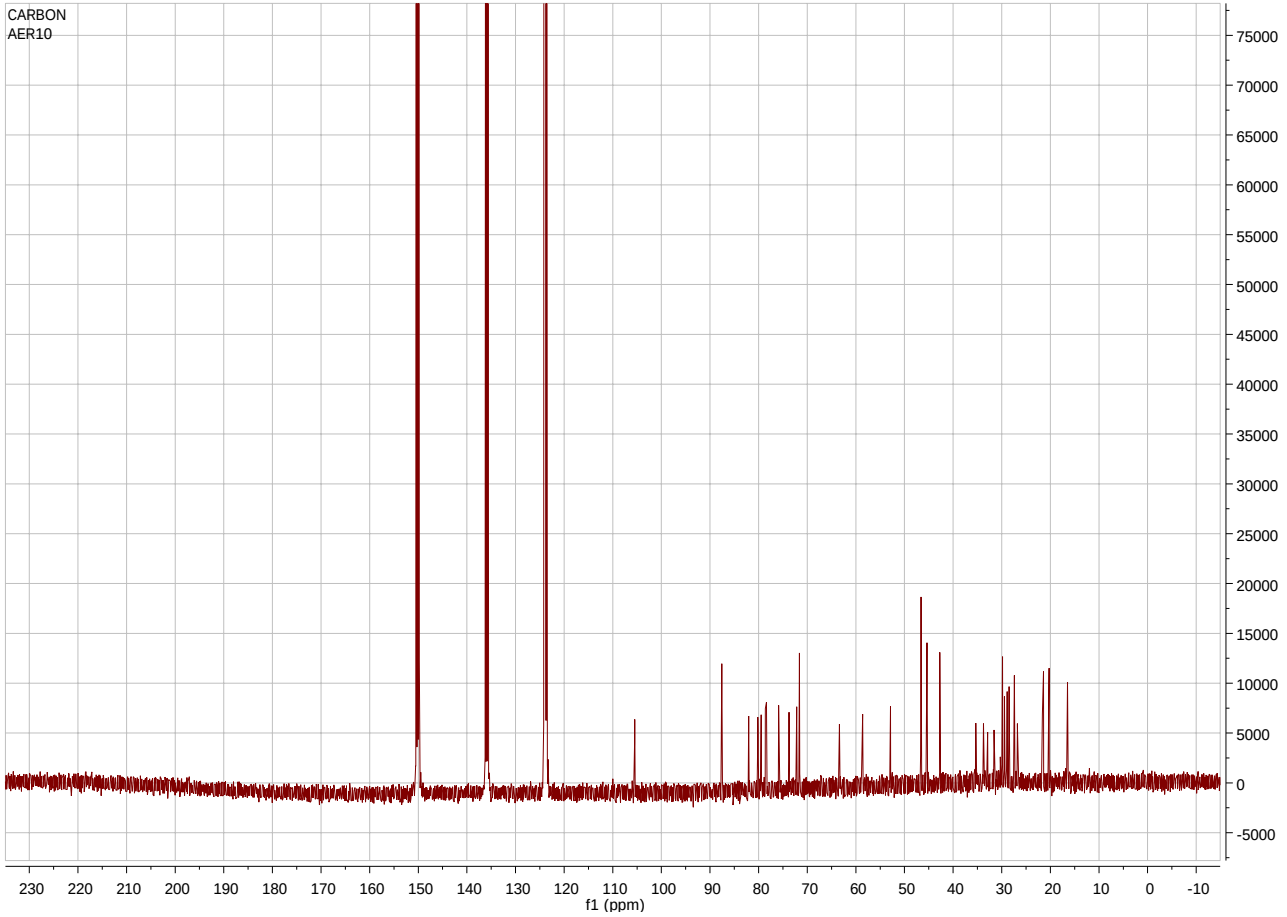
Table 3.2: ^{13}C and ^{1}H –NMR spectrum data of **9** (AER 10)

C / H	δ_C (ppm)	δ_H (ppm), *J* (Hz)
1	33.7 t	1.33 m, 1.73 m
2	31.6 t	2.05 m
3	78.6 d	3.62 dd(20; 10)
4	42.7 s	
5	52.9 d	1.96 m
6	80.17 d	3.86 ddd
7	35.3 t	1.68 m
8	46.6 d	2.02 m
9	21.7 s	
10	29.5 s	
11	26.7 t	1.33 m
12	35.3 t	2.37 m
13	45.4 s	
14	46.7 s	
15	46.2 t	2.4 m
16	73.8 d	4.96 ddd
17	58.6 d	2.55 d
18	21.41 q	1.47 s
19	29.8 t	0.31 d, 0.67 d(3.6,3.2)
20	87.6 s	
21	27.4 q	1.33 s
22	35.3 t	2.37 m
23	26.5 t	2.05 m
24	82.04 d	3.9 dd(8.8, 5.6)
25	71.6 s	
26	28.9 q	1.34 s
27	28.5 q	1.60 s
28	29.4 q	1.97 s
29	16.5 q	1.43 s
30	20.3 q	0.47 s
Glu		
1'	105.5 d	4.95 d (8.0)
2'	75.9 d	4.05 dd
3'	79.5 d	4.21 t
4'	72.2 d	4.20 m
5'	78.4 d	3.92 ddd
6'	63.4 t	4.43 dd(11.2) 4.5 dd(5.2)

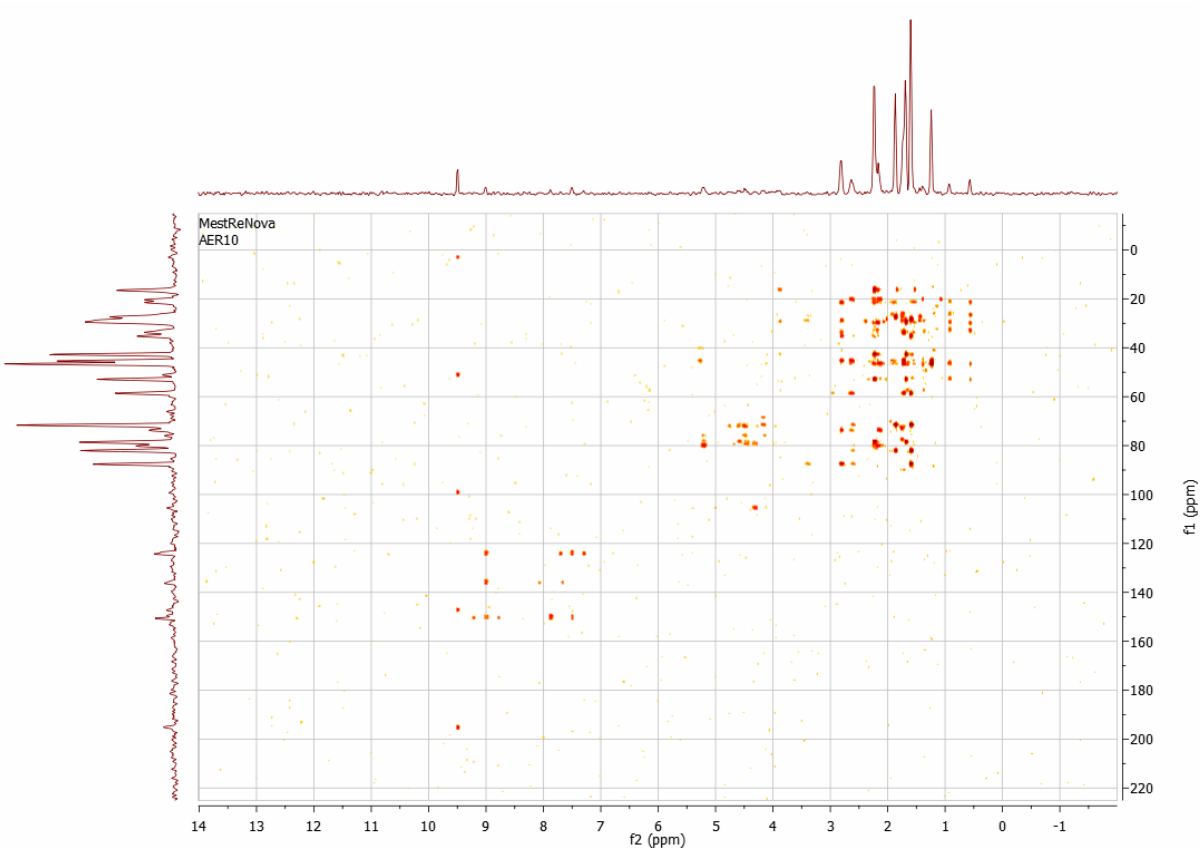
Spectrum 3.5 ^{1}H NMR spectrum of compund **9**



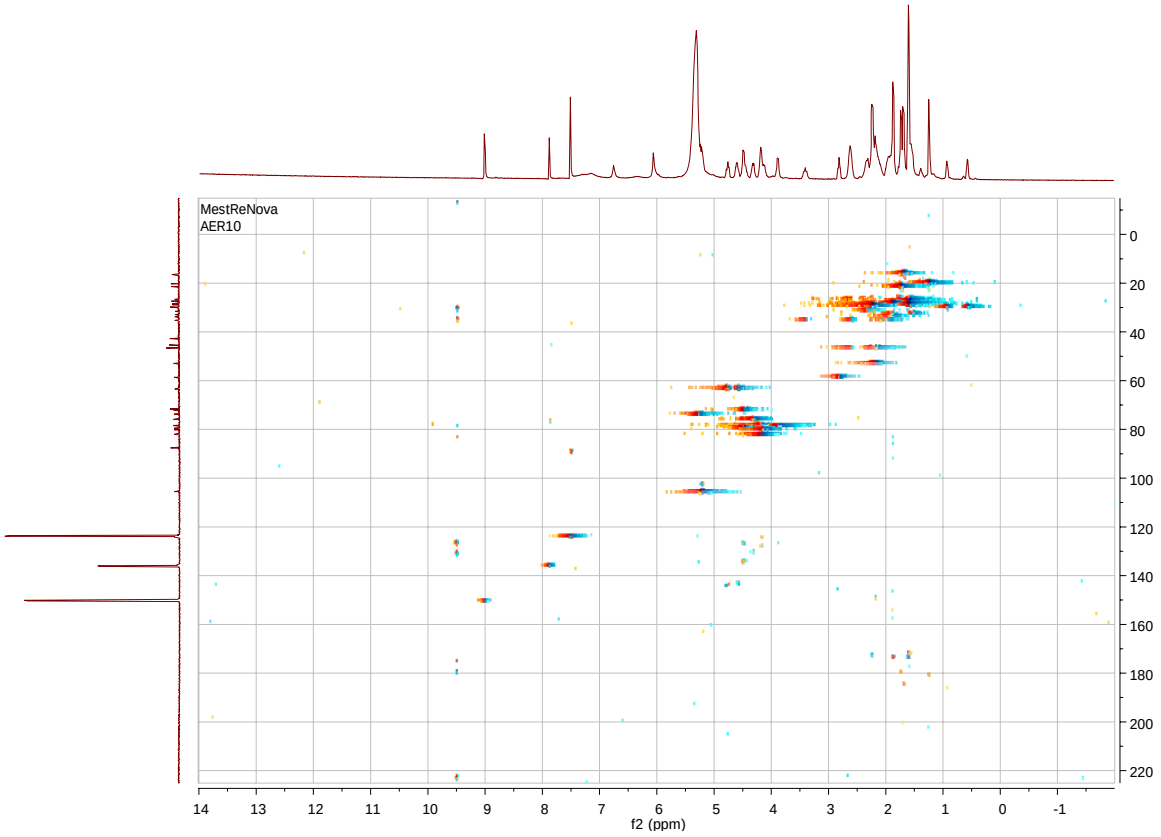
Spectrum 3.6 ^{13}C NMR spectrum of compound **9**



Spectrum 3.7 HMBC spectrum of compound **9**



Spectrum 3.8 HMQC spectrum of compound **9**



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