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M.Sc. Thesis

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM ASTRAGALUS ORNITHOPODIOIDES SPECIES

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Özgür TAĞ tarafından Yüksek Lisans tezi olarak sunulan "Isolation and Characterization of Saponins from *Astragalus ornithopodioides* species(*Astragalus ornithopodioides* Türünden 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 21.06.2011 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM *ASTRAGALUS ORNITHOPOIDIDES* SPECIES

TAĞ, Özgür

M. Sc. in Chemistry

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In this study, 31 glycosides were isolated from *Astragalus ornithopodioides* species and structures of the 3 of the isolated compounds determined by using spectroscopic techniques.

Key words: *Astragalus ornithopodioides*, oleanen, saponin, apigenin

ÖZET

ASTRAGALUS ORNITHOPODIOIDES TÜRÜNDEN SAPONİNLERİN İZOLASYONU VE KARAKTERİZASYONU

Özgür TAĞ

Yüksek Lisans Tezi, Kimya Bölümü

Tez Yöneticisi: Yrd. Doç. Dr. Tamer KARAYILDIRIM

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Bu çalışmada *Astragalus ornithopodioides* türünden otuzbir tane glikozid izole edilerek, izole edilen bileşiklerden üç tanesinin yapısı spektroskopik teknikler kullanılarak belirlenmiştir.

Anahtar Kelimeler: *Astragalus ornithopodioides*, oleanen, saponin, apigenin

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SPECTRA

Spectrum Page

LIST OF SCHEMES

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
MPLC	Medium Pressure Liquid Chromatography
SPE	Solid Phase Extraction
s	singlet
d	doublet
t	triplet
q	quartet
m	multiplet

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. For sheer chemical diversity, biologically derived compounds are unrivalled. Such compounds are also invaluable springboards to new chemistries. Furthermore, natural products can define new drug targets. For example, different toxins have revealed numerous classes of ion channels. Many natural products have been developed into drugs which have revolutionized medicine. A classic example of a plant-based medicine is the discovery of taxol from the bark of the Pacific yew tree, which has been referred to as the most important anticancer drug of the past two decades. Today's plant-based prescription medicines come from plants belonging to only 95 of the estimated 250.000 species worldwide. More than 1000 of these plants have already been described as having significant anticancer properties but there is still a huge amount of screening and characterization to be done. The ability to exploit fully the active components from natural resources for their therapeutic value has previously been hampered by technical limitations. New enabling technologies that are being utilized by the pharmaceutical industry, coupled to a growing trend of consumer preference for naturally derived products, is spurring renewed efforts toward developing therapeutic agents from natural sources. In the past, the ability to exploit the active components from natural resources has been hampered in several ways. One of the obstacles that previously impeded herbal drug development was the lack of bioassays for screening efficacies of pharmaceutical interest.

Natural products have and will continue to be a major resource for therapeutic products. They present researchers with complete libraries of compounds of such diverse chemical structure that are virtually impossible to replicate in a synthetic chemistry laboratory. In addition, the structural elucidation of natural products, with identified activity or otherwise, will continually provide new scaffolds for synthetic chemists and structural biochemists.

Until recent years, chance discoveries have been the prime driving force in the identification of new natural products. This is set to change, not only because of the increasing awareness of the immensity of the resources available in the natural world but also because of the application of high-throughput methods to

the generation of natural product libraries, the screening process and the structural elucidation of active compounds (New et al., 2003).

1.1. General Information about Saponins

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, andfound in particular abundance in various plant species. Saponins are high-molecular-weight triterpene glycosides, consisting of a sugar moiety linked to a triterpene or steroid aglycone. They are now more conveniently defined on the basis of their molecular structure, namely as triterpene or steroid glycosides.

Saponins 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 is 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 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, 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).

Most saponins isolated to date are triterpenoid (C30 aglycone-based) saponins (750 saponins with 360 different aglycones). These structures have been well characterized and are published in the literature making structure elucidation of atypical aglycone relatively straightforward (Berger et al, 2001).

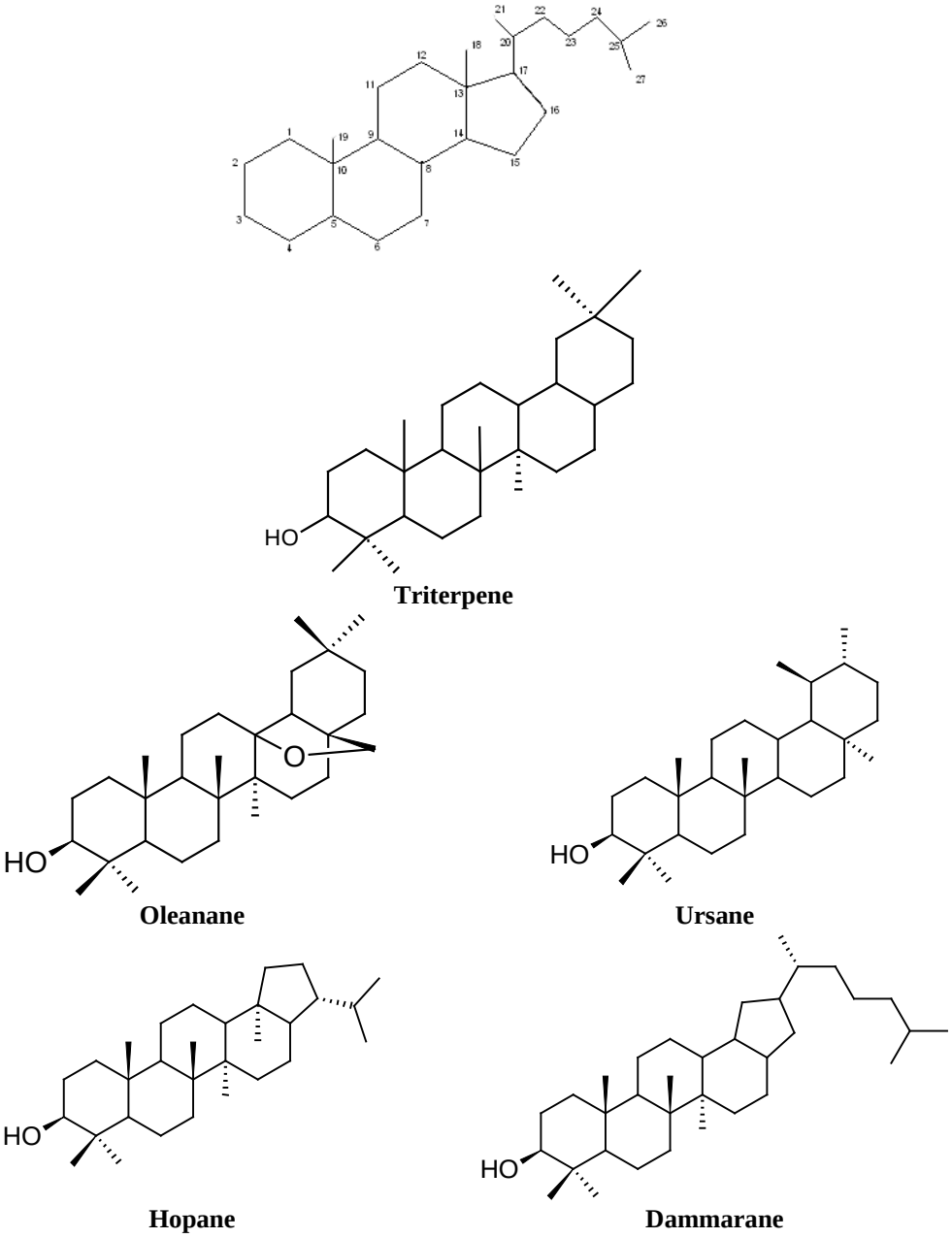


Figure 1.1. A Triterpene Skeleton and Four Common Triterpene Skeletons

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 saponari*a), soapberry (*Sapindussaponaria*), soapnut (*Sapindus makuros*si) (Hostettmann and Marston, 1995). Indeed, 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).

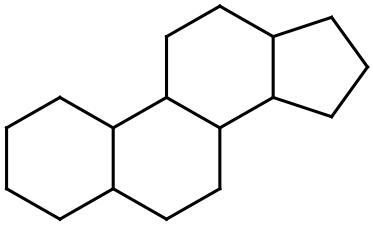
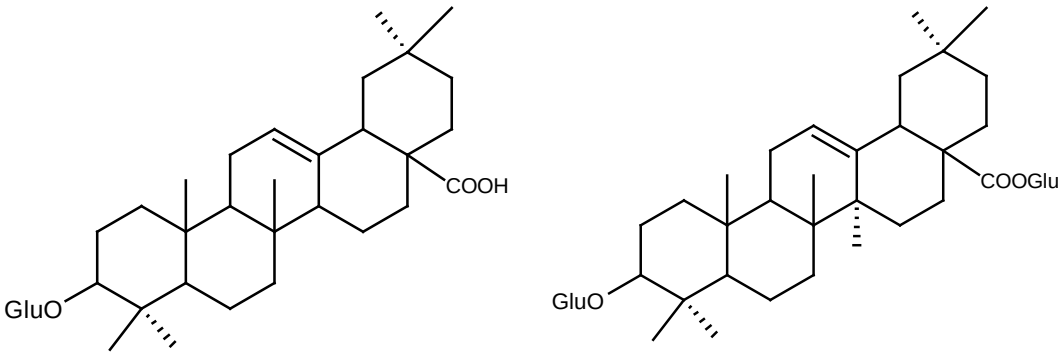


Figure 1.2. Basic Skeleton of steroid

All saponins have in common the attachment of one or more sugar chains to the aglycone. *Monodesmosidic* saponins have a single sugar chain, normally attached at C-3. *Bidesmosidic* saponins have two sugar chains, often with one attached through an ether linkage at C-3 and one attached through an ester linkage (acyl glycoside) at C-28 (triterpene saponins) (Figure 1.3) or an ether linkage at C-26 (furostanol saponins). *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 C-28 in triterpene saponins; they lack many of the characteristic properties and activities of monodesmosidic saponins (Wina et al., 2005).



Monodesmosidic Saponins *Bidesmosidic Saponins*

Figure 1.3. *Monodesmosidic* and *Bidesmosidic* Saponins

The carbohydrate part consist of one or more sugar moieties containing glucose, galactose, xylose, arabinose, rhamnose, or glucoronic acid glycosidically linked to a sapogenin (aglycone) (Table 1.1).

Table 1.1. Common Saponin Monosaccharide Groups

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

1.2. Biosynthesis

Plants have long been known to synthesize a plethora of small molecules, and plant genome sequencing is rein forcing 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 1.4). In addition tosterols (which are 6,6,6,5-tetracycles derived from lanosterolor 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 well defined 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 toone or more cyclic triterpene alcohols with up to six carbocyclic rings (Figure 1.4). 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, heterogonous expression, and biochemical characterization of the encoded protein shave provided substantial insight into the triterpenoid biosynthetic capabilities of plants. Cycloartenol synthase (CAS) converts oxidosqualene to cycloartenol through the protosteryl cation intermediate (Figure 1.4) and was the basal plant OSC from which others derived. The Arabidopsis CAS1 cDNA was cloned by screening extracts from a yeast *lanosterol synthase* 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 CAS activity through out seed plants.

The similarity of plant CAS genes to those in amoebae and bacteria suggests that the known *cycloartenol synthase* 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. Other enzymes that form the protosteryl cation have arisen from CAS gene duplication and diversification. For example, *Cucurbita pepo cucurbitadienol synthase* (CPQ) isclosely related (65-71 % identical) to cycloartenol and *lanosterol synthases* but produces cucurbitadienol (Figure 1.4), a precursor of cucurbitacins, which are bitter compounds that might act as anti-feedants. The sequence similarity of plant *cycloartenol, lanosterol*, and *cucurbitadienol synthases* is consistent with relatively recent divergence of *lanosterol* and *cucurbitadienol synthase* from an ancestral CAS via an evolutionary route that maintained the protosteryl cation intermediate (Figure 1.4). Several oxidosqualene cyclases that function in secondarymetabolism also have been reported to generate single product. *Lupeol synthases* cyclize oxidosqualene to the dammarenyl cation, promote ring expansion and annulationto the lupyl cation, and terminate by abstracting the C-29 proton to form lupeol (Figure 1.4.). *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.

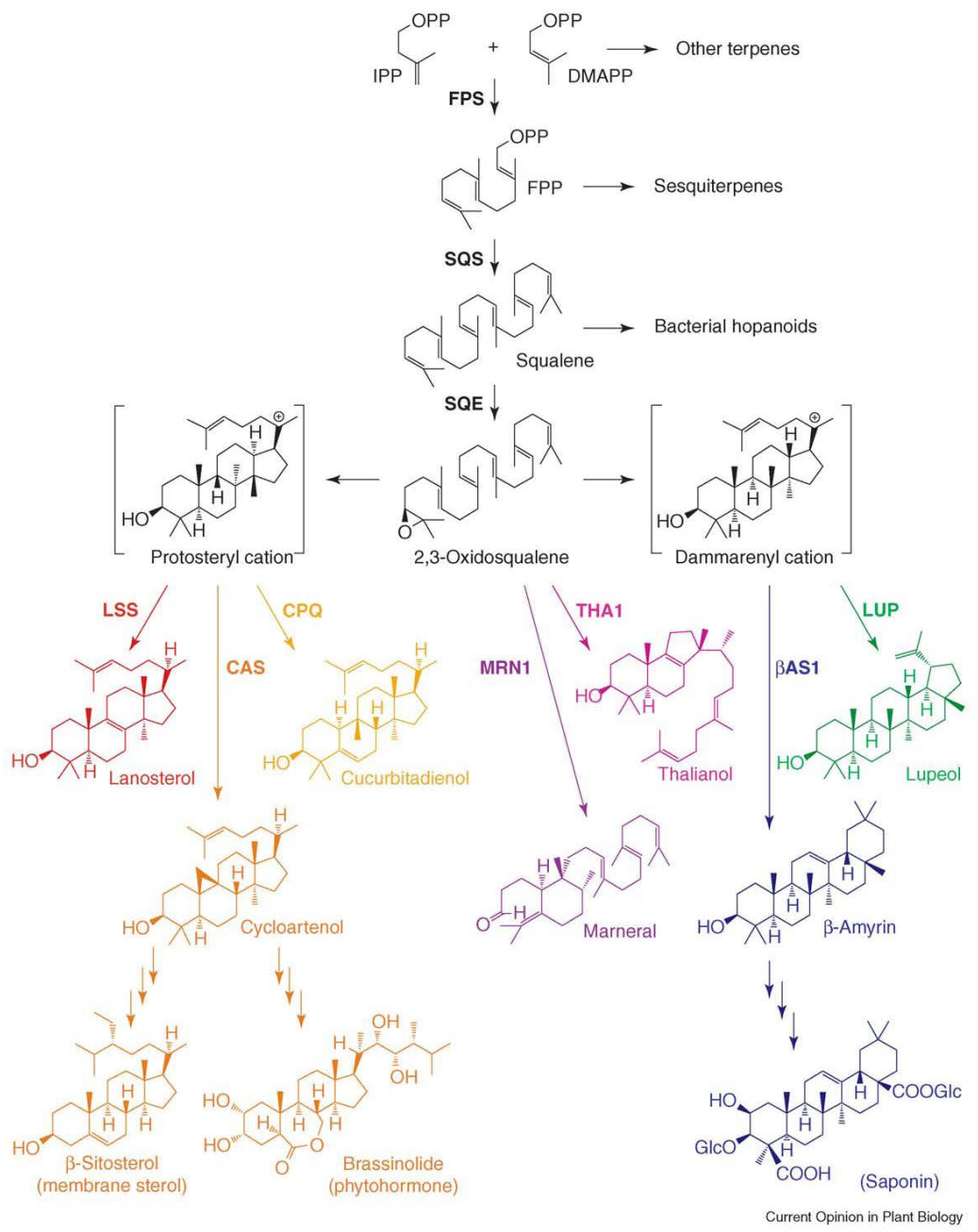


Figure 1.4. Simplified Scheme of Plant Triterpenoid Biosynthesis

β-Amyrin synthase 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 synthase* are considerably more distant from one another (48–50 % identical) than are the CAS enzymes (70–79 % identical), and independent origins of *β-amyrin synthase* 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 synthase*, less broadly distributed enzymes can also have high product selectivity. *Luffa cylindrica isomultiflorenol synthase* (LCIMS) cyclizes oxidosqualene through a dammarenyl cation to make isomultiflorenol, a bryonolic acid precursor. Temporal expression studies in L. cylindricacell 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. THA1converts oxidosqualene to the tricyclic alcohol thalianol (Figure1.4). Triterpenoids that have the methyl substitution and olefinic position of thalianol have not been found in nature. MRN1catalyzes an unusual cyclization reaction: oxidosqualene is converted to a bicyclic cation that undergoes rearrangement and A-ring cleavage to generate a monocyclic aldehyde (Figure1.4). 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 heterogonous 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 1.4).

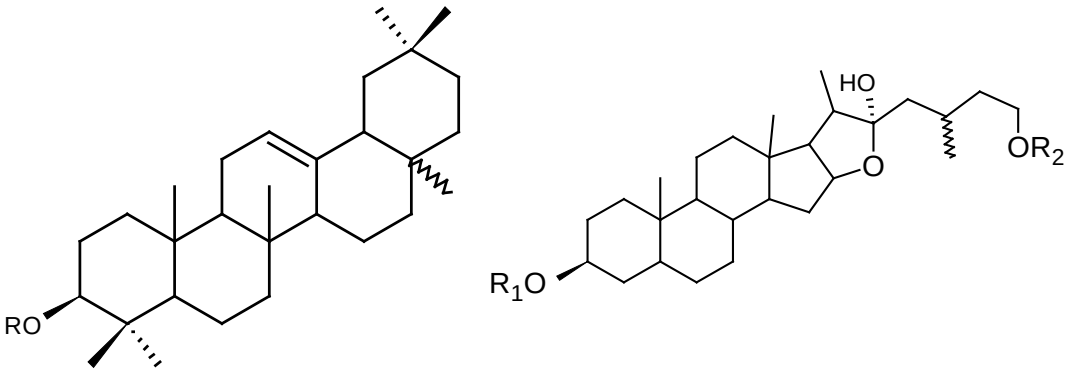
The first cloned SQE gene, from *Saccharomyces cerevisiae*, was identified by complementation of the yeast ergosterol biosynthetic mutant erg1. Two

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).

1.3. Classification and Occurrence

Saponins are often subdivided into two main classes, the triterpenoid and the steroid saponins (Figure 1.5) (Abe et al., 1993), which are both derived from the 30 carbon atoms containing precursor oxidosqualene (Haralampidis et al., 2002). The difference between the two classes lies in the fact that the steroid saponins have three methyl groups removed (i.e. they are molecules with 27 C atoms), whereas in the triterpenoid saponins all 30 C atoms are retained.



Triterpenoid Saponins **Steroidal Saponins**

Figure 1.5. Classification of Saponins, R= sugar moiety

Saponins are classified based on the biosynthesis of the carbon skeletons, it may be expected that similar types of saponins are found in similar plant orders. This expectation is based on the assumption that similar plant orders contain similar types of enzymes, and that these enzymes can probably catalyse similar biotransformations, ultimately leading to similar chemical structures (Vierhuiset al., 2001; Umezawa, 2003). Therefore, attempts were made to link the various carbon skeletons and their substitution patterns to plant orders.

Saponin biosynthesis proceeds via the isoprenoid pathwayin which 3 isoprene units (molecules containing 5 C atoms) are first linked in a head-to-tail manner to each other, resulting in the 15 C-atom molecule farnesyl pyrophosphate. Two farnesyl pyrophosphates are subsequently linked in a tail-to-tail manner to give a compound of 30 carbon atoms, called squalene (Holstein and Hohl, 2004). Squalene is oxidized to oxidosqualene, which is the common starting point for cyclization reactions in triterpenoid biosynthesis (Figure 1.6) (Abe et al., 1993; Haralampidis et al., 2002). Oxidosqualene is converted to cyclic derivatives via protonation and epoxide ring opening, which creates a carbocation that can undergo several types of cyclization reactions. After these cyclizations, subsequent rearrangements can proceed in different ways by a series of hydride shifts and/or methyl migrations, which lead to the formation of new carbocations. Finally, the carbocations are neutralized by proton elimination to give a double bond or a cyclopropanyl ring, or by reaction with water to give a hydroxyl group. The main cyclization and rearrangement reactions are shown that lead to the triterpenoid and steroid skeletons that have been found to occur in the saponins in literature search (Figure 1.6). The type of *cyclase* that is involved in the cyclization reaction primarily determines the skeleton that is formed (Figure 1.6). Many different kinds of cyclases (e.g. *cycloartenol synthase*, *lanosterol synthase*, *β-amyrin synthase*) have been described, and their mechanisms of action are well documented (Abe et al., 1993; Wendt et al., 2000; Wendt,2005; Haralampidis et al., 2002; Thoma et al., 2004). Cyclization of oxidosqualene to saponins can proceed in two ways, either via the ‘chair-chair-chair’ or via the ‘chairboat-chair’ conformation. An important difference between the two resulting skeletons lies in the stereochemistry, which is most clearly shown by the configurations of the C8 and the C14 atoms. After cyclization of the ‘chair-chair-chair’ conformation, the methyl group at the C8 atom is pointing upwards and the one at the C14 atom is pointing downwards, whereas the opposite is the case after cyclization of the ‘chair-boat-chair’ conformation (Figure 1.6).

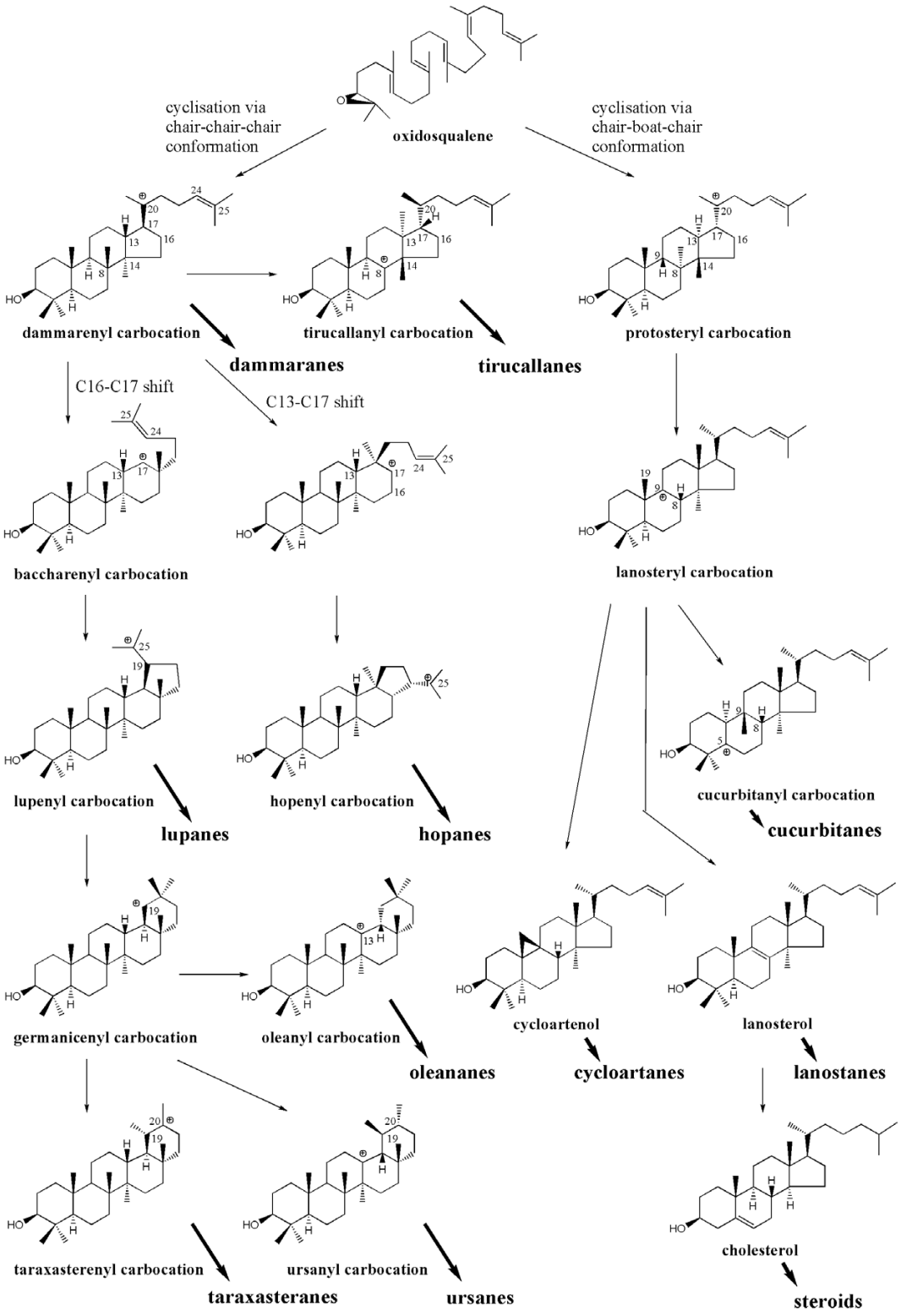


Figure 1.6. The cyclization of oxidosqualene to the various saponin skeletons

A proton-initiated cyclization of the ‘chair-chair-chair’ conformation results in the tetracyclic dammarenyl C20 carbocation, and all saponins derived from this carbocation are classified as *dammarane type saponins* (Ryu et al., 1997; Ma et

al., 1999; Chakravarty et al., 2001). A series of hydride and methyl shifts in the dammarenyl carbocation leads to the tirucallenyl C8 carbocation, and all saponins derived from this carbocation are classified as tiru*callane type saponins* (Teng et al., 2003). The 5-membered ring next to the C20 dammarenyl carbocation can expand either by a shift of the C16-C17 bond, or by a shift of the C13-C17 bond. A shift of the C16-C17 bond leads to the tetracyclic C17 baccharenyl carbocation and can be followed by a reaction with the C24-C25 double bond to produce the pentacyclic C25 lupenyl carbocation. All saponins derived from this carbocation are classified as *lupane type saponins* (Pambou Tchivounda et al., 1990; Elgamal et al., 1998; Xianget al., 2000; Yook et al., 2002). The lupenyl carbocation can be rearranged further, first to the C18 germanicenyl carbocation, and then via a series of hydride shifts to the C13 oleanyl carbocation. All saponins derived from this oleanylcarbocation are classified as *oleanane type saponins* (Sparget al., 2004). Oleanane type saponins have been isolated from a wide array of plants (Osbourn, 1996, 2003; Woldemichaeland Wink, 2002; Treyvaud et al., 2000; Voutquenneet al., 2003; Wandji et al., 2003), and this skeleton is also referred to as the *β*-amyrin skeleton (Haralampidis et al.,2002). A shift of the α methyl group in the germanicenyl carbocation produces the C20 taraxasterenyl carbocation, which can be deprotonated to yield *taraxasterane type saponins* (Yahara et al., 1997; Cheng et al., 2002). A methyl shift in the germanicenyl carbocation, followed by several hydride shifts, ultimately produces the C13 carbocation, which can be deprotonated to *ursane type saponins* (Babady-Bila et al., 1991; Amimoto et al., 1993; Zhao et al.,1997; Sanoko et al., 1999; Sahpaz et al., 2000). The ursane skeleton is also called the *α*-amyrin skeleton. The *α*-amyrin and *β*-amyrin skeletons are the cyclization products of distinct cyclases, *α-amyrin synthase* and *β-amyrin synthase*, respectively (Haralampidis et al., 2002).

A shift of the C13-C17 bond in the C20 dammarenyl carbocation leads to a C17 carbocation, which can be cyclized by a reaction with the double bond in the side chain to form the C25 pentacyclic hopenyl carbocation. All saponins derived from this carbocation are classified as *hopane type saponins* (Hamed et al., 1996; Meselhy and Aboutabl, 1997; Meselhy, 1998; Hamed and El-Emary, 1999; Sahuet al., 2001; Biswas et al., 2005). All hopane type saponins contain a hydroxyl group at the C3 atom.

From the proton-initiated cyclization of the ‘chair–boat–chair’ conformation of oxidosqualene, a tetracyclic protosteryl C20 carbocation is obtained, which undergoes a series of hydride and methyl shifts ultimately leading to the

intermediate C9 lanosteryl carbocation. This carbocationcan undergo further shifts of a methyl group and a hydride to the C5 cucurbitanyl carbocation. All saponins derived from this carbocation are classified as *cucurbitane type saponins* (Oobayashi et al., 1992). The lanosteryl carbocation may also undergo deprotonation of the C19 methyl group leading to formation of a cyclopropanering as is found in cycloartenol. All saponins derived from cycloartenol are classified as *cycloartane type saponins* (Choi et al., 1989; Xu et al., 1992; Xu and Xu, 1992; Kennelly et al., 1996; Sun and Chen, 1997; Zhao et al.,1997; Verotta et al., 1998, 2001; Bedir et al. 2000; Radwan et al., 2004).

Deprotonation of the lanosteryl carbocation gives lanosteroland all saponins derived from lanosterol are classified as *lanostane type saponins* (Pires et al., 2002; Mamedovaet al., 2003). Lanosterol can also undergo demethylation and isomerisation of the double bond, leading to cholesterol. The saponins derived from this skeleton are classified as *steroid type saponins* (Yahara et al., 1996a; Corea et al.,2005). The difference in deprotonation, to a cyclopropanering or a double bond, indicates that the cyclization is catalysed by different cyclases (*cycloartenol* and *lanosterol synthase*, respectively).

The 11 carbon skeletons shown in Figure 1.6, dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cucurbitanes, cycloartanes, lanostanes, and steroids, represent the end products of cyclization, rearrangement and degradation reactions, which cover the main saponin skeletons that have been found (Vincken et al., 2007).

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, *Abiesalba*, 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).

1.4. Distribution

Saponins are found in a wide variety of foods including asparagus, beans, blackberries, peas, potatoes, sugar beetand 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).

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

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

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).

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, it 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 of 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 oligosaccharite side chains will eventually be extract be into the butanolic phase. A further purification is then carried out, which involves liquid chromatography 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. Other classical methods are used to ascertain the presence of saponins in a crude plant extract, and to elucidate their composition through out purification steps. TLC and staining with dehydrating reagents containing aromatic aldehydes (such as anisaldehyde in sulphuric acid) are commonly used. The pure saponin may also be hydrolyzed 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 column 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 (C18, C8). 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).

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).

Saponins are generally extracted from plants through an alcoholic extraction of the defatted vegetable material. Due to the possible contemporary presence of acidic components (phenols and their acids, flavonoids, etc.) care should be taken about the pH of the alcoholic solution, which, if too low, can produce trans esterification. A subsequent useful step is the partition of the total dried alcoholic extract between *n*-butanol and water. This operation is important to eliminate mono- and di-saccharides which complicate further separations.

A typical isolation strategy is the preliminary purification of the *n*-butanol extract over dextran supports like Sephadex LH 20 or Fractogel TSK, followed by further fractionation of the crude saponin mixtures. A new generation of polymers has been exploited for the initial purification steps. They are highly porous polymers (Diaion HP-20, MCI gel CHP-20P, Amberlite XAD-2). Methanol-water or acetone-water solvent gradients are used.

The polar characteristics of saponins suggest to avoid unmodified silica gel stationary phases, which, if used, require water containing mobile phases to desorb the glycosides. Nevertheless, silica gel chromatography with chloroform-methanol-water as eluent is still the most popular and inexpensive method and is used in most separations (Verotta et al., 2001).

1.6.1. Chromatography

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.

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 mixtures are only partially separated. Other problems are the loss of material because of irreversible adsorption and the length of time required to perform the separations.

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, chemically derived silica packing have obtained increasing popularity due to their chemical stability and good separation efficiency. 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 et al., 2001).

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).

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.

Although most applications have involved silica gel sorbents, there is an increasing trend towards RP materials. RP flash chromatography enables the easy separation of saponins from other, more polar, components such as oligosaccharides (Hostettmann, and Marston 1995).

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 bar 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 good separation 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.

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).

High-Performance Liquid Chromatography (HPLC)

Chromatography by HPLC (High-performance liquid chromatography) is a powerful technique for obtaining multi-milligram 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 soya sapogenols A and B, separated on C18 column with MeCN:PrOH:H_2O:AcOH (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 et al., 2006).

Vacuum Liquid Chromatography (VLC)

The technique can be considered as preparative TLC run as a column, with a vacuum provided to speed up eluent flow rates. It differs from flash chromatography in that the column is allowed to run dry after each fraction is collected. This is similar to preparative TLC because plates can be dried after a run and then re-eluted. The chromatography column is packed with silica gel (generally 10-40 micrometer TLC grade) and the sample is eluted with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the elution strength. Application of a vacuum to the bottom of the column pulls the solvent through the sorbent.

In the last few years VLC has been increasingly used in the field of natural products because of its simplicity of operation. Separations of up to 30 g extract are possible (Hostettmann and Marston, 1995).

Over-Pressured Layer Chromatography (OPLC)

This is a modified planar chromatographic technique in which the vapor phase is eliminated by covering the sorbent layer with an elastic membrane under variable external pressure. The mobile phase is forced through the sorbent layer with a pump.

In over-pressured chromatography a horizontal thin-layer chromatography plate is covered by an elastic cushion. Pressure is applied to the cushion so that separations can performed on the TLC plate in the absence of vapor phase. Under forced-flow conditions of eluent, sample development is rapid. High efficiencies can be achieved due to the use of fine-particle sorbents and longer plates than those found in capillary-controlled systems. As separation times are short, diffusion effects are reduced. It is also possible to employ mobile phases with poor solvent wetting characteristics.

Applications to the separation of natural products include following: Anthraquinones, furocoumarins, secoiridoid glycosides, sesquiterpenes, diterpenes, cucurbitacins, steroid saponins and essential oils (Hostettmann and Marston, 1995).

1.6.2. Structure Determination

The complexity of many bioactive natural products containing sugar residues dictates the use of a combination of modern techniques dominated by those involving mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy for structure elucidation. In more and more cases the use of a single technique precludes a satisfactory unambiguous determination.

1.6.2.1. Mass Spectrometry

The choice of ionization method in MS depends on the polarity, lability and molecular weight of the compound to be analysed. It is principally the so-called 'soft' ionization techniques such as FAB and desorption/chemical ionization (D/CT) which are employed to obtain molecular weight and sugar sequence information for naturally occurring glycosides (Wolfender et al. 1992). These permit the analysis of glycosides without derivatization. In certain cases, fragmentations of aglycones are observed, but electron impact mass spectra (EI-MS) are more useful for this purpose.

Electron Impact MS (EI-MS)

The most common MS method, samples need to be volatilized and, of course, saponins cannot be analysed by this technique unless they are converted to permethyl or peracetyl derivatives; such derivatization is not applicable to saponins possessing more than four sugar residues (Komori et al. 1975). However, mention should be made here of the application of EI-MS to the structure elucidation of the aglycones obtained from saponins. It is possible to observe the molecular peak of the aglycone and also to arrive at conclusions about the structure of the terpenoid skeleton from the fragmentation pattern. One of the diagnostically important features of the EI-MS of aglycones possessing 12(13)-double bonds is the retro-Diels- Alder rearrangement which cleaves the molecule at ring C and enables information to be furnished about substitution in the ring systems (Figure 1.7) (Djerassi et al. 1962; Budzikiewicz et al. 1963; Karliner and

Djerassi, 1966). An especially good indication of the distribution of hydroxyl groups between rings A and B, and rings D and E can be obtained.

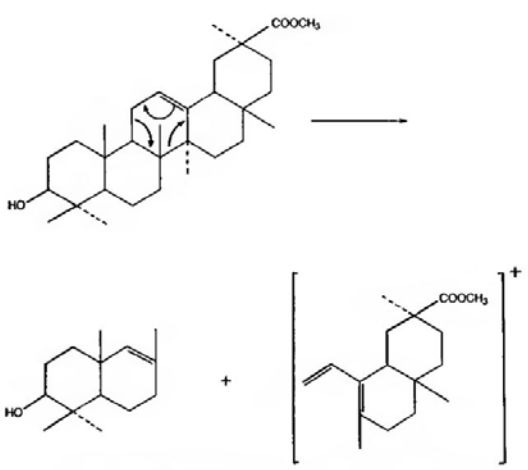


Figure 1.7. Retro-Diels-Alder Reaction of 12(13)-Unsaturated Oleananes

The EI-MS of peracetylated saponins gives useful information as to the construction of the sugar chain. For example, fragment ions corresponding to terminal glucose, (Glc)Ac4 (m/z 331), to terminal rhamnose, (Rha)Ac3 (m/z 273) and to (Rha-Glc)Ac6 (m/z 561) aided in the structure elucidation of a cyclamiretin A tetrasaccharide from *Ardisia crenata* (*Myrsinaceae*) (Wang et al. 1992).

1.6.2.2. Nuclear Magnetic Resonance (NMR)

Of all these modern methods for the structure elucidation of oligosaccharides and glycosides, NMR spectroscopy provides the most complete information, with or without prior structural knowledge (Agrawal, 1992). It is the only approach which can, in principle, give a complete structure without resort to any other method.

^{13}C-Nuclear Magnetic Resonance

Carbon-13 NMR spectroscopy, now widely used for the structure determination of saponins, is a fast and non-destructive method but requires quite large quantities of sample (mg amounts). Analysis of the spectra allows conclusions to be drawn about the following aspects:

- positions of attachment of the glycosidic chains to the aglycone,

- sequence, nature and number of monosaccharides,

- configuration and conformation of the inter glycosidic linkages,

- presence of acylglycosides in the chains (Ishii et al 1978,1984),

- nature of the aglycone (Doddrell et al. 1974; Tori et al. 1974; Blunt and Munro, 1980),

- structures of attached ester acids (Okada et al. 1980).

For assigning chemical shifts, it is very helpful to compare observed data with data reported for model and related compounds.

Triterpene saponins: As a guide to some of the typical chemical shifts in the ^{13}C-NMR spectrum of a triterpene saponin, the values obtained for the bayogenin glycoside are shown in Figure 1.8 (Domon and Hostettmann, 1984), It is of interest that sugar carbon resonances occur largely in a region distinct from that of the sapogenin moiety. Compilations of assignments of 13C-NMR signals for oleanane (Patra et al 1981; Agrawal and Jain, 1992), ursane, lupane (Wenkert et al. 1978; Sholichin et al. 1980), hopane (Wenkert et al. 1978; Wilkins et al. 1987) and lanostane (Parrilli et al. 1979) triterpenes have been made (Nakanishi et al. 1983).

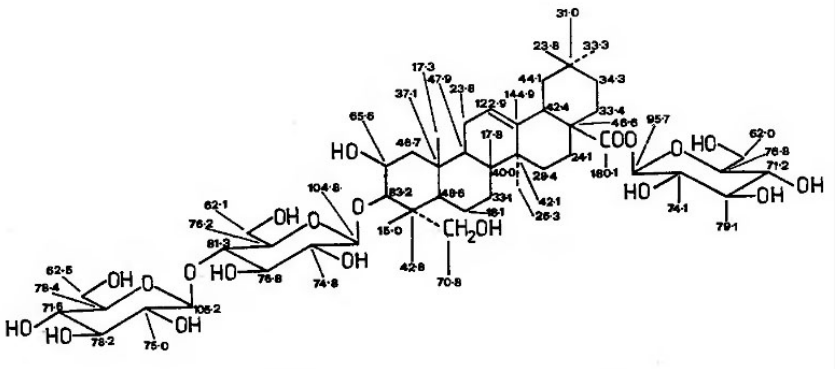


Figure 1.8. ^{13}C-NMR Data for a Typical Bidesmosidic Saponin

The number of anomeric carbons in a saponin is readily determined by ^{13}C-NMR, thus defining the number of individual sugar residues present. By comparing the chemical shifts with appropriate model sugars (Bock and Pedersen, 1974; Gorin and Mazurek, 1975; Seo et al. 1978; Agrawal et al. 1985), the ring size (furanose or pyranose form) and type of each monosaccharide can be established. Specific shifts for C-l, C-2 and C-3 (all appearing 4-14 ppm downfield in the furanose form) and C-5 (shifted 5-7 ppm upfield in the furanose form) are the markers which allow sugars of different ring sizes to be identified. The anomeric carbon atoms in pyranoses and their derivatives resonate at 90-110 ppm, while carbon atoms bearing secondary hydroxyl groups in pyranoses give signals at 65-85 ppm; carbon atoms carrying primary hydroxyl groups are found at 60-64 ppm (Bock and Thorgensen, 1982; Bock and Pedersen, 1983). A characteristic feature of saponins glycosylated at the carboxyl group is the appearance of the anomeric carbon of the carboxylic group-bound sugar residue at remarkably upheld positions (93-97 ppm) (Agrawal, 1992). The chemical shifts of anomeric carbons provide an easy by means of determining anomeric carbon configurations, i.e. either α- or *β*-glycosides (Seo et at. 1978; Kasai et al. 1979; Tanaka and Kasai, 1984). For example, smilagenin-3-*O*-α-D-glucopyranoside has an anomeric carbon signal at 98.7 ppm, which in smilagenin-3-*O*-*β*-D-glucopyranoside is at 103.1 ppm (Agrawal et al. 1985). The *α*-carbon of the aglycone is commonly displaced downfield by about 7 ppm on *β*-D-glucosylation, (5-L-rhamnosylation and *α*-L-arabinosylation, while the downfield shift for α-D-glucosylation, *α*-L-rhamnosylation and *β*-L-arabinosylation is somewhat smaller (c. 5-6 p.pлп.) (Kasai et al. 1979; Tanaka and Kasai, 1984).

^{1}H-Nuclear Magnetic Resonance

The ^{1}H-NMR spectra have characteristically proved complex and tedious to analyse. The vast majority of proton resonances of the carbohydrate moiety appear in a very small spectral width of 3.0-4.2 ppm, with subsequent problems of overlapping. These derive from the bulk of non-anomeric sugar methine and methylene protons which have very similar chemical shifts in different monosaccharide residues.

However, the methyl peaks of triterpenes are readily discernible and most proton resonance positions in oleanene, ursene and related skeletons have been assigned since the 1960s (Kojima and Ogura, 1989) by a variety of techniques.

Different 2-D NMR techniques

The use of HMQC and HMBC ^{13}C multiple- quantum coherence spectra is valuable not only for aglycone assignments but also for sugar sequence details. These experiments are analogous to ^{13}C–^{1}H heteronuclear correlated spectroscopy (HETCOR) but instead of observing ^{13}C, the more abundant ^{l}H is detected. It was possible to assign all the chemical shifts in the ^{1}H-NMR spectrum by considering ^{13}C-NMR data in conjunction with 2-D ^{l}H-detected HMQC and HMBC spectra. Cross peaks corresponding to two and three bond couplings were observed for nearly all possible correlations in the molecule. Similarly, long-range ^{1}H-^{13}C correlations in the HMQC and HMBC spectra enabled the determination of the sequence and positions of attachment of the sugar moieties.

1.7. *Astragalus* Genus

1.7.1. General on the Genus

The genus *Astragalus* L. is one of the largest and most widely distributed genera belonging to the family Leguminosae, comprising 455 species distributed mainly in the flora of Turkey (Davis, 1970; Calıs et al., 2008; Tabanca et al., 2005; Ozipek et al., 2005). Polysaccharides, saponins and flavonoids have been reported from *Astragalus* species (Tang and Eisenbrand, 1992; Bedir et al., 1998, 1999, 2000, 2001, 2005, 2009, 2010). 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).

1.7.2. Chemical Composition of *Astragalus* Species

The genus *Astragalus* appears highly uniform from a 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 indolizidinealkaloids (swainsonine and its N-oxide derivative, and lentiginosine), the nitro compounds endecaphyllins(nitropropionic acid-glucose derivatives) and 3-nitropropylglucosides, and the seleniferous derivatives (selenocysteine, -cystathionine, cystine, and methionine). There are other interesting compounds, such as flavonoids (flavonols, flavones, isoflavones, and flavylions) in free and

glycosidic forms; pterocarpans free and as glucosides; organic acid derivatives (homopilosinic and phaseic acids), (Rios and Waterman., 1997), and carotinoids such as *β*-carotene, lutein, zeaxanthine (Gigoshviiet al., 2003).

The polysaccharides from *A. membranaceus* are the best known, and several research groups have isolated and purified them. Tang and Eisenbrand, cited Astragalan I, II and III from *A. mongholicus* (*A. membranaceus* var.*mongholicus*). Astragalan I is a polysaccharide composed of D-glucose, D-galactose and D-arabinose in a molar ratio of 1.75:1.63:1, with a molecular weight of 36,300, while Astragalan II and III are composed of D-glucose only, with molecular weights of 12,300 and 34,000 respectively. Tomoda and coworkers (1992) purified a glycan from the hot water extract of the roots of *A. membranaceus*, constituted mainly of L-arabinoside, D-galactose, L-rhamnose and D-galacturonicacid in a molar ratio of 6:9:8:30, and Shimizu et al. (1991) isolated an acidic polysaccharide from *A. mongholicus*, composed of L-arabinoside, D-galactose, D-galacturonic acid, D-glucuronic acid in a molar ratio of 18:18:1:1, plus small amounts of acetyl groups and peptide moiety (Rios and Waterman., 1997).

1.7.2.1. 3-Nitropropylglucosides

3-nitro-1-propyl-*β*-D-gentiobioside (Majak and Benn, 1988), 3-nitropropyl-*β*-D-allolactoside (Majak, 1988), 3-nitro-1-propyl-*β*-D-laminaribioside (Benn and Majak, 1989) and 3-nitro-1-propyl-*β*-D-cellobioside (Long et al., 1992) have been isolated from the aerial parts of *Astragalus miser* var. *serotinus* and characterized by spectral studies.

In 1999, twenty milligrams samples of leaves of 460 specimens including 440 species of *Astragalus* from 48 sections, collected from the herbarium of the Research Institute of Forests and Rangelands, Tehran in Iran, were analysed for toxic aliphatic nitro compounds. Nitro compounds were found in 37 species from 20 of 48 taxonomic sections of *Astragalus* (Ebrahimzadeh et al., 1999).

Benn et al.(1997) were isolated a nitropropanyl isoxazolinone derivative from the *Astragalus canadensis* L. var. *mortonii* (Nutt) Wats. and *Astragalus collinus*.

1.7.2.2. Phenolic Compounds

A novel acylated flavonoid glycoside; complanatin from the seeds of *A. complanatus* (Cuiet al., 1991); Astragalin, kaempferol-3-*O*-*β*-D-robinobioside, kaempferol-3-rutinoside (nicotiflorine), and kaempferol-3-*O*-*β*-D-galactopyranosyl-(3′′,4′′)-di-*O*-*α*-L-rhamnopyranoside (ascaside) from *Astragalus tana* L., (Alaniya and Chkadua, 2000. A flavonol glycoside, isorhamnetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-[*α*-L-rhamno-pyranosyl-(1→6)]-*β*-D-galactopyranoside, and the known diglycoside, isorhamnetin-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside from the aerial parts of *Astragalus vulneraria* (Bedir et al., 2000). A new flavonol glycoside, kaempferol 3-*O*-*α*-L-rhamnopyranosy1-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranosyl-7-*O*-*α*-L-rhamnopyranoside, named astrasikokioside I, together with two flavonol glycosides, kaempferol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-7-*O*-*α*-L-rhamnopyrano-side and robinin from the aerial part of *Astragalus shikokianus* (Yaharaa et al., 2000); four phenolic glycosides, *β*-apiofuranosyl-(1→2)-*β*-glucopyranosides from the roots of *A. zahlbruckneri* (Calis et al., 2001), flavonol tetraglycosides; kaempferol-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-galactopyranoside, kaempferol-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-galactopyranoside, kaempferol-3-O-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-3-*trans*-p-coumaroylgalactopyranoside, kaempferol-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-3-*trans*-feruloylgalactopyranoside, kaempferol-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-4-*trans*-*p*-coumaroylgalactopyranoside, and kaempferol-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-[*α*-L-rhamnopyranosyl-(1→2)]}-*β*-D-4-*trans*-feruloylgalactopyranoside (Semmar et al., 2002a), besides rhamnocitrin-3-*O*-{[3-hydroxy-3-methylglutaroyl-(1→6)]-[*β*-D-apiofuranosyl-(1→2)]}-*β*-D-galactopyranoside, rhamnetin-3-*O*-{[3-hydroxy-3-methyl-glutaroyl-(1→6)]-[*β*-D-apiofuranosyl-(1→2)]}-*β*-D-galactopyranoside, kaempferol-3-*O*-[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranoside, and quercetin-3-*O*-{[*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→6)][*β*-D-apiofuranosyl-(1→2)]}-*β*-D-galactopyranoside from the leaves of *A. caprinus* (Semmar et al., 2002b). Two isoflavones, calycosin-7-*O*-*β*-D-glycoside and formononetin-7-*O*-*β*-D-glycoside, from *n*-butanol extract of the root of *Astragalus membranaceus* Bge. var. *mongholicus* (Ma et al., 2003); 7-hydroxy-3′,5′-

dimethoxyisoflavone besides diadzen, genisten, luteolin, apigenin, and apigenin-7-*O*-neohesperidoside from the aerial parts of *Astragalus peregrinus* (Abd El-Latif et al., 2003); and isoflavonoids, formononetin, 9, 10-dimethoxypterocarpan 3-*O*-α-D-glucoside, ononin, calycosin 7-*O*-Glc and calycosin from the roots of *Astragalus mongholicus* Bunge (Leguminosae) (Yu et al., 2005) were isolated.

1.7.2.3. Triterpenoids

Cycloartane triterpenoids

Cycloartane triterpenoids were first discovered in *Astragalus* plants. Plants of this genus drew attention to themselves after it was established that they produce cycloartane triterpenoids. These studies turned a new page in the study of Astragalus plants. Since then, their content of cycloartane methylsteroids and glycosides came under intense scrutiny in many scientific centers of the world. Cycloartanes are derivatives of 9β,19-cyclolanostane and are produced exclusively by photosynthetic eukaryotes. Cycloartanes dominate the known triterpenoids in plants of this genus (Mamedova and Isaev, 2004).Until 2004, 152 cycloartanes have been described from *Astragalus* plants.

Classification of these substances is based on structural features of the side chains. The known cycloartane methylsteroids of *Astragalus* plant scan be divided into six structural types according to the side-chain structure:

- Cycloartanes with an acyclic side chain,
- 20,24-Epoxycycloartanes,
- 16β,23;16α,24-Diepoxy-cycloartanes,
- 16β,24;20,24-Diepoxycycloartanes,
- 20,25-Epoxycycloartanes,
- 24-Nor-16β,23-epoxycycloartanes.

Cycloartane glycosides

During the first decade of research on the triterpenoid content of *Astragalus* plants, about 40 cycloartane glycosides were discovered (Isaev, et al., 1986) such as Cycloastragenol and astragenol (Kitagawa et al., 1983a), astragaloside III, astragaloside V, and astragaloside VI (Kitagawa et al., 1983b), astragaloside VII and VIII (Kitagawa et al., 1983c), astragaloside I, II and IV, acetylastragaloside I and isoastragalosides I and II (Kitagawa et al., 1983d). The number reached 122 in subsequent years. Some cycloartane glycosides isolated from *Astragalus* plants during the recent years are listen in Table 1.2 (Mamedova and Isaev, 2004).

Oleanane triterpenoids

Oleanane triterpenoids from Astragalus plants are not common. Soyasapogenol B and the new triterpenoid sapogenin II were obtained from the total triterpenoids of the aerial organs of *Astragalus glycyphyllos* L. (Table 1.2). Astragaloside VIII is a trioside of soyasapogenol B and was isolated from roots of *Astragalus membranaceus* Bunge. Its structure differs from that of soyasaponin I, which is found in certain representatives of the bean family, in that it has a β-D-xylopyranose instead of a β-D-galactopyranose (Table 1.2). In addition to methyl ethers of astragaloside VIII and soyasaponin I, four new unnamed glycosides were isolated from seeds of *Astragalus complanatus* R. Br (Table 1.2) (Mamedova, and. Isaev, 2004).

1.7.3. 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 triterpenesaponins 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. corniculatusis* 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 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 (Çalış 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 immune stimulating (Yesilada et al., 2005; Çalış 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; Çalış 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 neuro protective 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. anti inflammatory, analgesic, diuretic, hypotensive and sedative effects (Polat et al., 2009a, 2010).

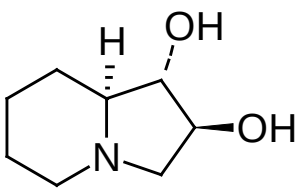
In the district of Anatolia, located in South Eastern Turkey, an aqueous extract of the roots of *Astragalus* species is traditionally used against leukemia and for its wound healing properties. (Calis et al., 1997; Bedir et al., 2000; Polat et al., 2009a, 2010).

The former compounds of several *Astragalus* species are reported to possess anticancer and immune stimulating 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).

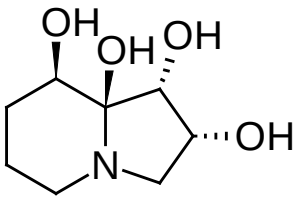
1.7.4. Toxicity

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 1.9), the nitro

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



Lentiginosine



Swainsonine

Figure 1.9. Indolizidine alkaloids glycosidase inhibitors occuring in *Astragalus* species

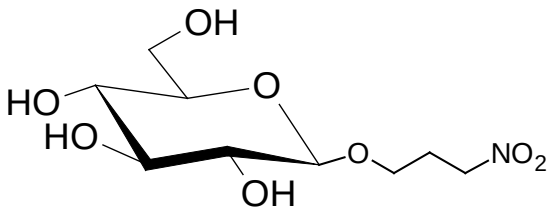


Figure 1.10. Miserotoxin (3-Nitro-1-propyl-*β*-D-glucopyranoside)

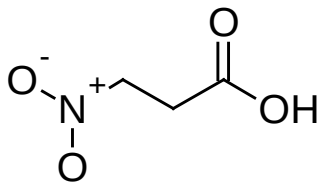


Figure 1.11. 3-Nitropropionic Acid (3-NPA)

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*

2. MATERIAL AND METHODS

2.1. General

Optic rotations were measured using a Perkin Elmer 341 Model Polarimeter. For spectroscopic identifications, the NMR spectra were recorded on a Varian instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C) in *d6*-DMSO 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 device. The spots were detected by 20 % H_2SO_4 water spraying reagent onto the TLC plates followed by heating the plates to 110 $^{\circ}C$ until the spots become visible.

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

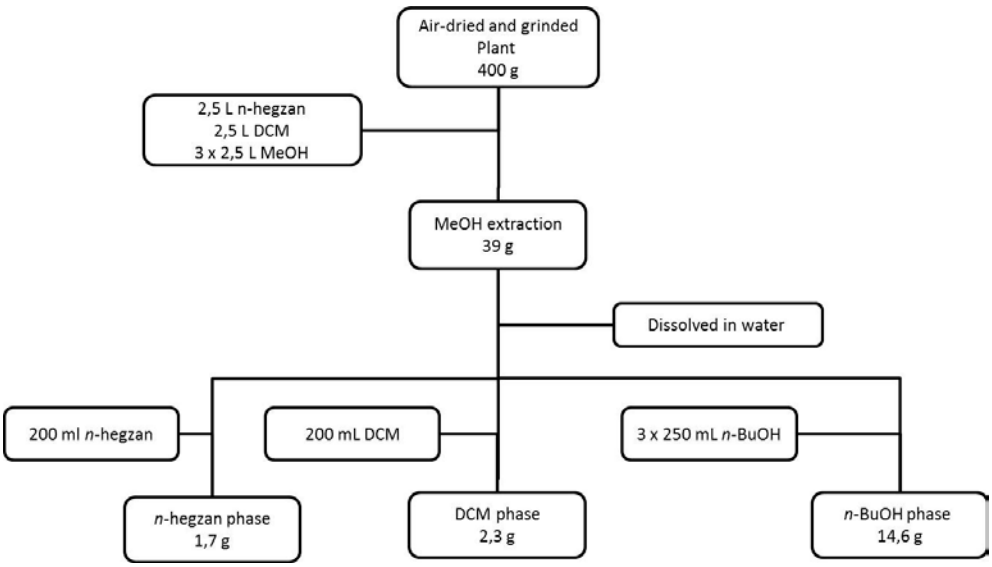
I	CH_2Cl_2:MeOH:H_2O	80:20:2
II	CH_2Cl_2:MeOH:H_2O	75:25:2.5
III	CH_2Cl_2:MeOH:H_2O	70:30:3
IV	CH_2Cl_2:MeOH:H_2O	61:32:7
V	CH_2Cl_2:MeOH:H_2O	64:50:10
VI	MeOH:H_2O	8:2
VII	MeOH:H_2O	7:3
VIII	MeOH:H_2O	6:4
IX	MeOH:H_2O	5:5
X	MeOH:H_2O	4:6
XI	MeOH:H_2O	3:7
XII	MeOH:H_2O	2:8

2.2. Plant Material

Astragalus ornithopodides was collected from Gürpınar in Van. Plant material was identified by Fevzi Özgökçe (Department of Biology, Faculty of Sciences, Yüzüncü Yıl University, Van, Turkey). A voucher specimen was deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey (VAN9F13822).

2.3. Isolation and Purification

The dried and grinded plant of *A. ornithopodides* (400 g) were extracted with *n*-hexane (2.5 L), dichloromethane (DCM, CH_2Cl_2) (2.5 L) and methanol (MeOH) (3x2.5 L) at room temperature. After filtration, the solvent was removed by rotary evaporation yielding 39 g of extract. The MeOH extract was dissolved in H_2O (100 mL), and successively partitioned with *n*-hexane (200 mL), CH_2Cl_2 (200 mL), and *n*-BuOH saturated with H_2O (3x250 mL) (Scheme 2.1).



Scheme 2.1. Extraction Procedure of *Astragalus ornithopodides*

The *n*-BuOH extract (14.6116 g) was subjected to column chromatography (CC) using sephadex material (100 g) employing H_2O:MeOH (8:2) to give 9 main fractions. (Scheme 2.2).

The fraction **2** (1.7 g) eluted with MeOH:H_2O (2:8, 400 mL; 4:6, 600 mL; 6:4, 600 mL; 8:2, 800 mL) and MeOH was submitted open column chromatography on

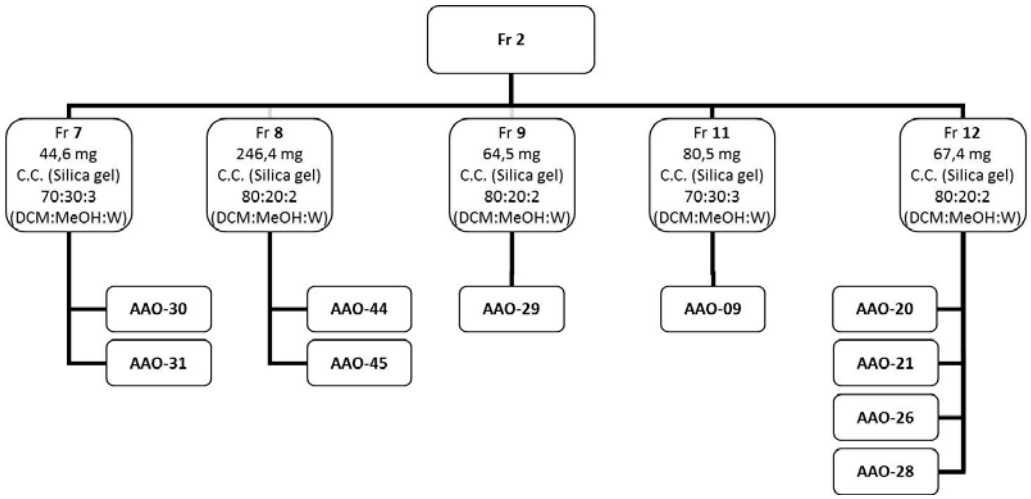
reverse phase (120 g) to give 13 main fractions (Scheme 2.3). A five of these main fractions were separated with different methods as shown on Scheme 2.3.

The fraction **4** (300 mg) was applied on normal phase silica gel (200 g) using CH_2Cl_2:MeOH:H_2O solvent system (70:30:3) to afford a pure compounds [**AAO-3**: 14.6 mg)] (Scheme 2.2).

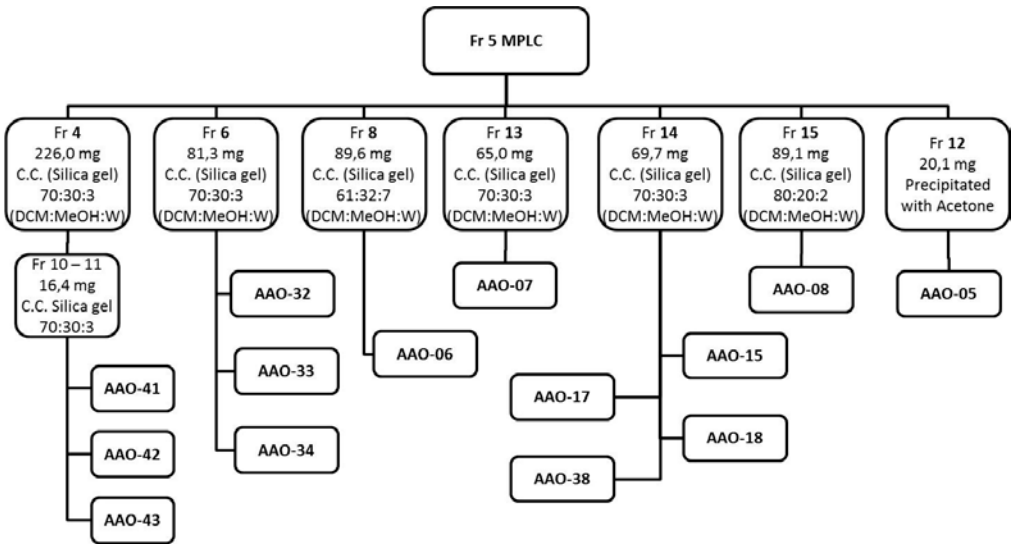
The fraction **5** (4.8 g) was fractionated by MPLC on RP using MeOH:H_2O (2:8, 1000 mL; 4:6, 1000 mL; 5:5, 1000 mL; 6:4, 800 mL; 8:2, 1200 mL) and MeOH to give 17 fractions (Scheme 2.2). The seven of these main fractions were separated with different methods as shown on Scheme 2.4. **AAO-05** (20.0 mg) was isolated from the sub-fraction **8** by acetone precipitation. The sub-fraction **12** gave the **AAO-06** (12.0 mg) over silica gel column by using CH_2Cl_2:MeOH:H_2O (61:32:7) solvent system.

The fraction **6** (5 g) eluted with MeOH:H_2O (2:8, 400 mL; 4:6, 600 mL; 6:4, 600 mL; 8:2, 800 mL) and MeOH was submitted open column chromatography on RP (120 g) to give 15 main fractions (Scheme 2.2). 3 of these main fractions were separated with different methods as shown on Scheme 2.5.

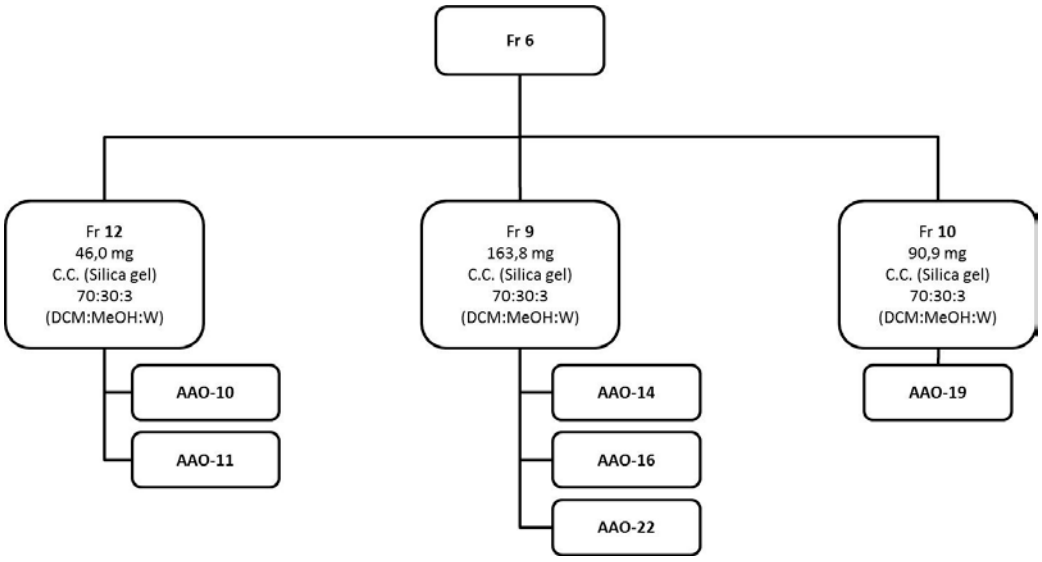
Scheme 2.2. Isolation Procedure of Compound **AAO-03**



Scheme 2.3. Isolation Procedure of Fraction **2**



Scheme 2.4. Isolation Procedure of Compounds **AAO-05** and **AAO-06**



Scheme 2.5. Isolation Procedure of Fraction **6**

3. RESULT AND DISCUSSIONS

3.1. Structural Identification of Compound AAO-03

A detailed comparison of the aglycon moiety NMR data (^{1}H-, ^{13}C-NMR, HMQC and HMBC) of compound **AAO-3**. The ^{1}H-NMR spectrum of **AAO-3** showed it's a phenolic compound. In the ^{1}H-NMR spectrum of **AAO-3**, the following signals for aglycone moiety H-6′ and H-2′ at 7.92 ppm (2H), H-3' and H-5′ at 6.92 ppm (2H), H-3 and H-8 at 6.83ppm (2H), H-6 at 6.44 ppm, anomeric proton H-1′′ at 5.06 ppm (d) were observed. For the sugar moiety of **AAO-03** in the ^{1}H-NMR spectrum, resonance for anomeric protons of the sugar moiety were observed at 4.74 ppm (d, H-1′′, *β*-D-glucose).

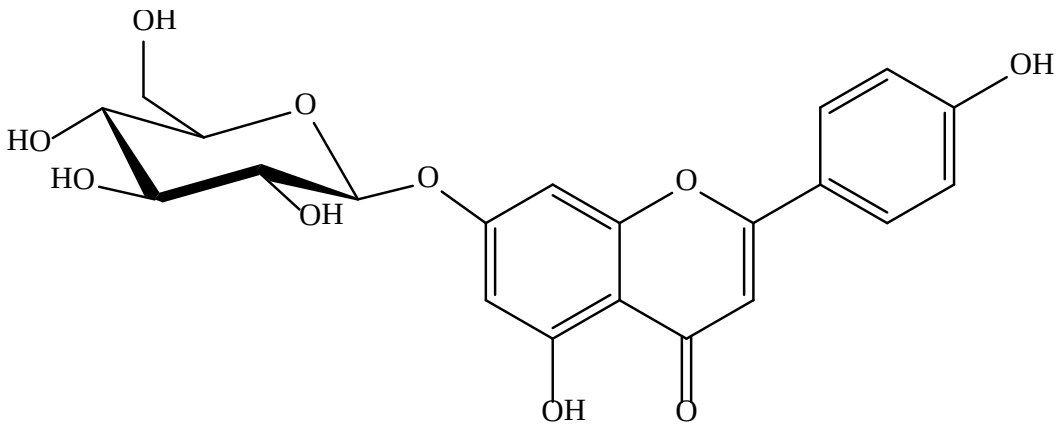
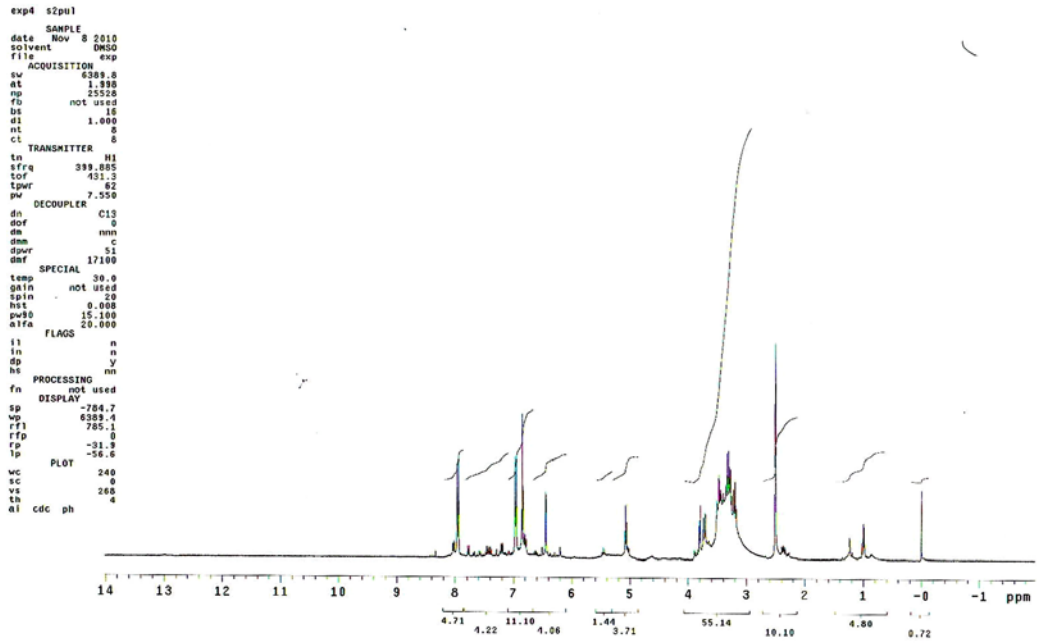
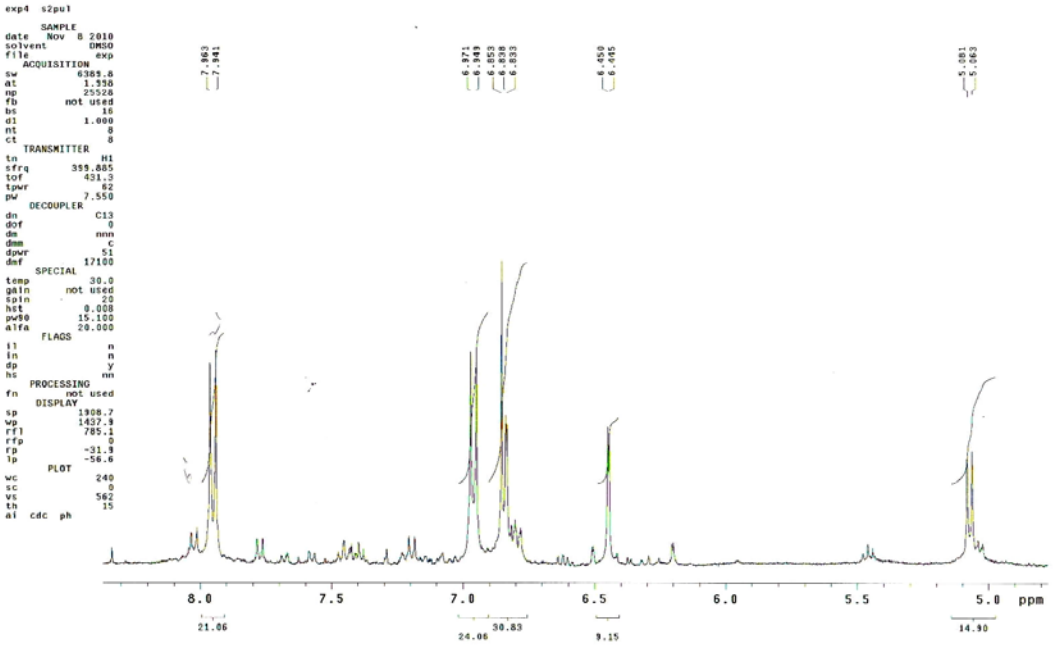


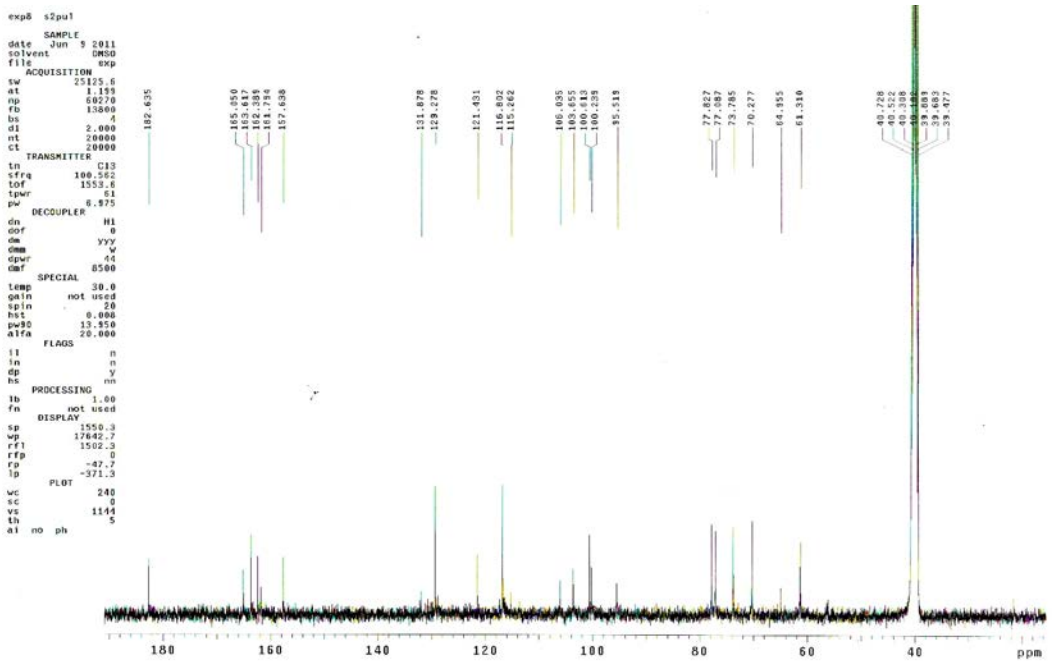
Figure 3.1. Structure of Compound **AAO-03**



Spectrum 3.1. ^{1}H-NMR Spectrum of Compound **AAO-03**



Spectrum 3.2. ^{1}H-NMR Spectrum of Compound **AAO-03** Sugar Moiety



Spectrum 3.3. ^{13}C-NMR Spectrum of Compound **AAO-03**

3.2. Structural Identification of Compound AAO-05

The ^{1}H-NMR spectrum of the aglycone moiety of **AAO-5** showed signals for seven tertiary methyl groups δ_H (0.73, 0.78, 0.83, 0.87, 0.95, 1.04, 1.08), four oxygen bearing methine groups [H-3 (3.87 ppm), H-2 (3.65 ppm), H-23 (3.09 ppm) and H-16 (3.21 ppm)], one olefinic proton at δ 5.15 . Additionally the resonances of three anomeric protons, indicative of the presence of three *β*-linked sugar units, were observed in the low-field region at δ 4.77 (d, *J*= 7.5 Hz), 4.65 (brs) and 5.08 (s). The ^{13}C-NMR spectrum of the aglycone moiety showed signal seven methyl groups (at δ 15.9, 18.5, 17.2, 20.9, 25.6, 28.9, 33.2), and olefinic carbon signals at 122.2 and 144.6 ppm (Figure 3.2).

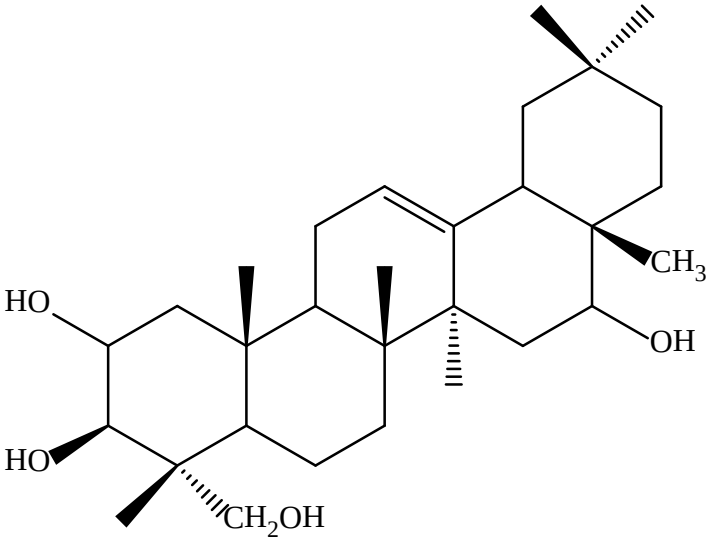
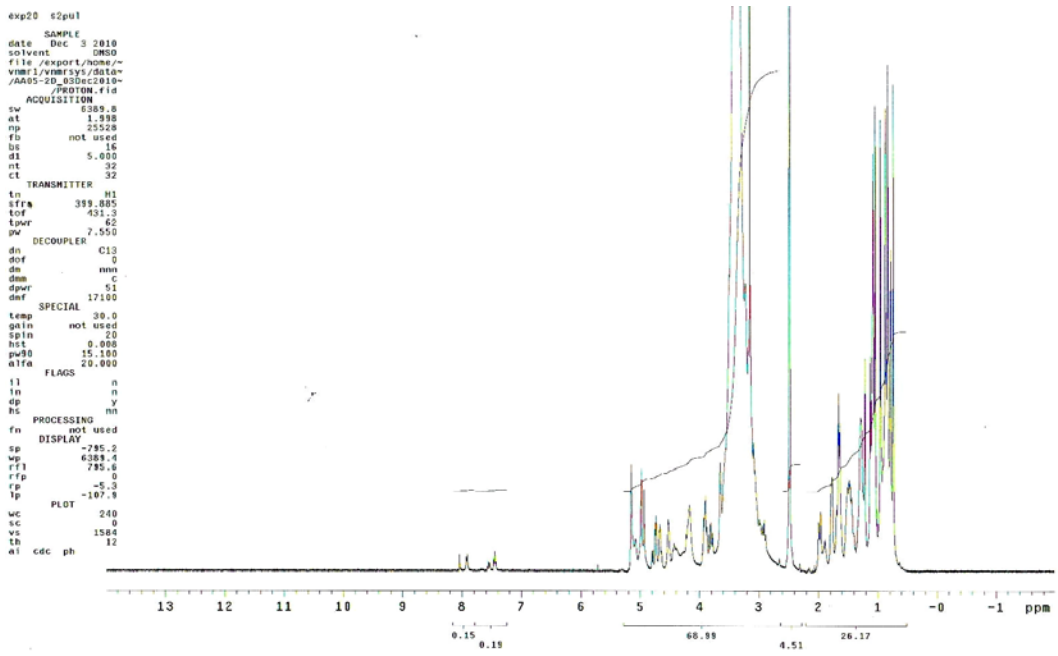
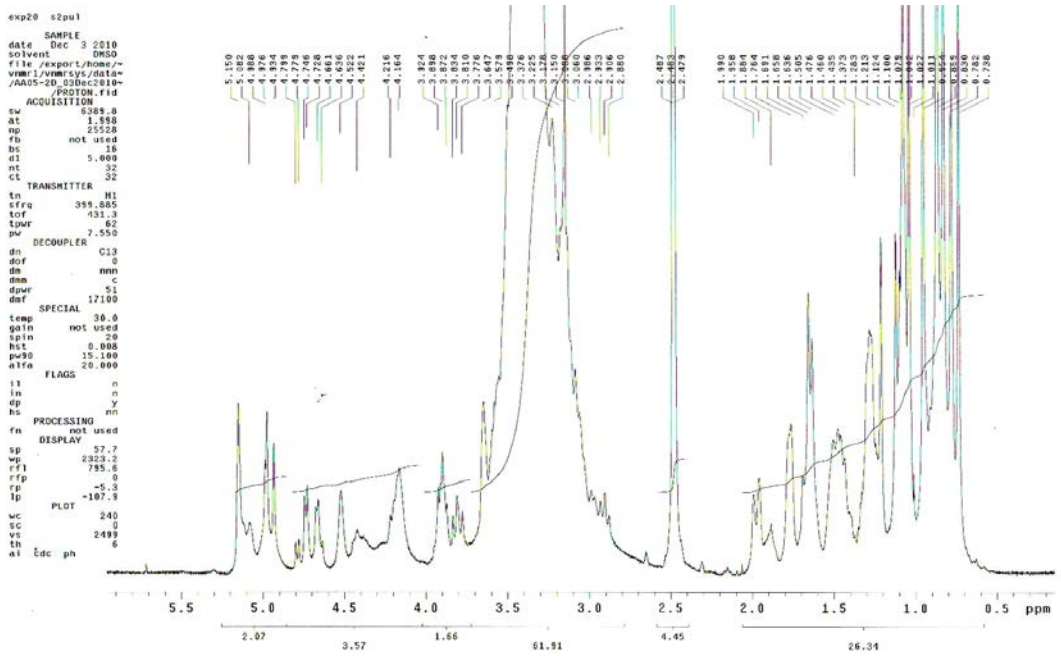


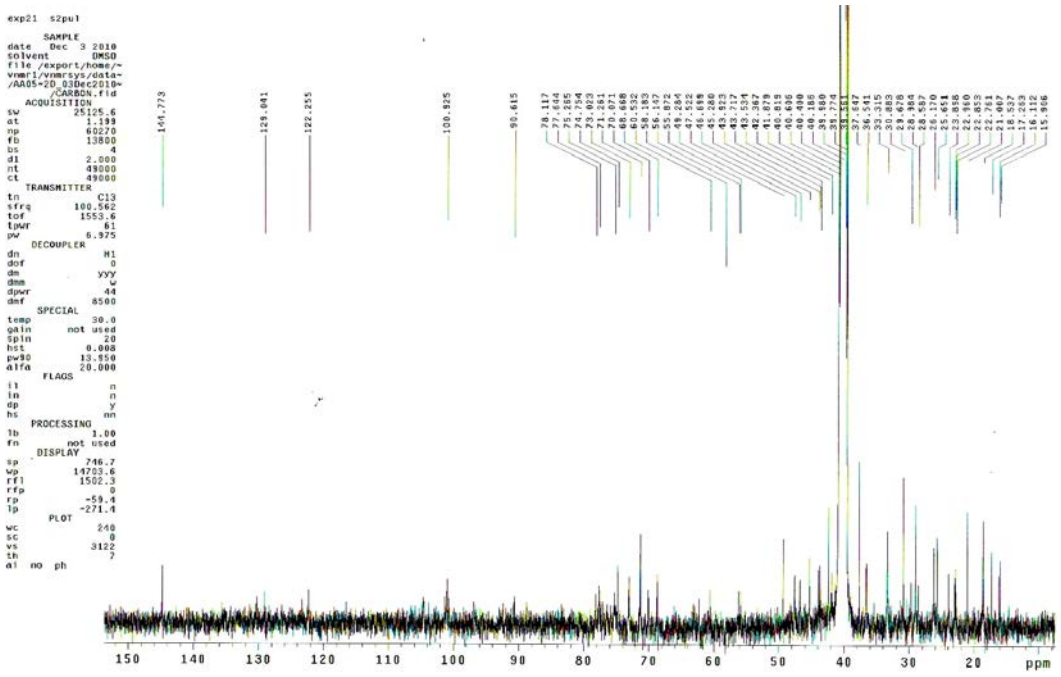
Figure 3.2. Aglycone Moiety of Compound **AAO-05**



Spectrum 3.4. ^{1}H-NMR Spectrum of Compound **AAO-05**



Spectrum 3.5. [1]H-NMR Spectrum of Compound **AAO-05** Sugar Moiety



Spectrum 3.6. [13]C-NMR Spectrum of Compound **AAO-05**

3.3. Structural Identification of Compound AAO-06

The [1]H-NMR spectrum of the aglycone moiety of **AAO-10** showed signals for seven tertiary methyl groups δ_H (at 0.68,0.72, 0.78, 0.82, 0.90, 1.00, 1.05

ppm). Further features were signals at δ_H 5.26 ppm (1H), respectively, to an olefinic function. The ^{13}C-NMR spectrum of the aglycone moiety showed signal seven methyl groups (at 15.9, 17.7, 19.4, 20.7, 22.8, 26.7, 33.2 ppm), olefinic carbon signals at 122.3, 144.6 and 173.7 ppm signal showed function a carboxylic acid (Figure 3.3).

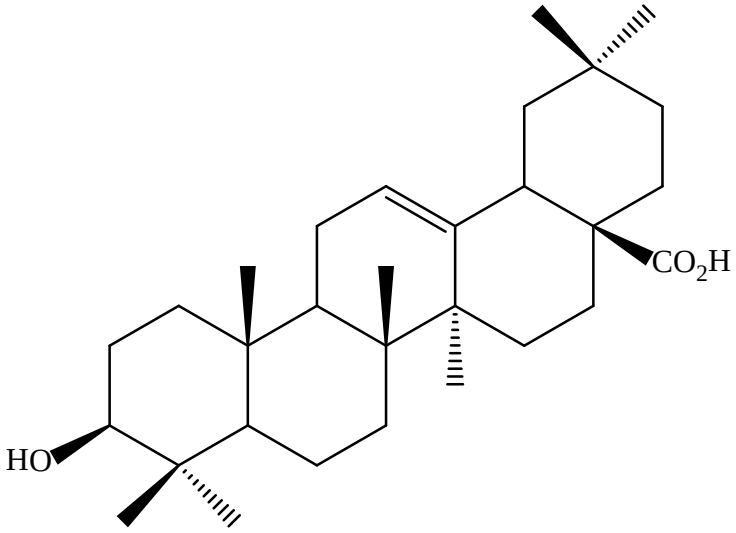
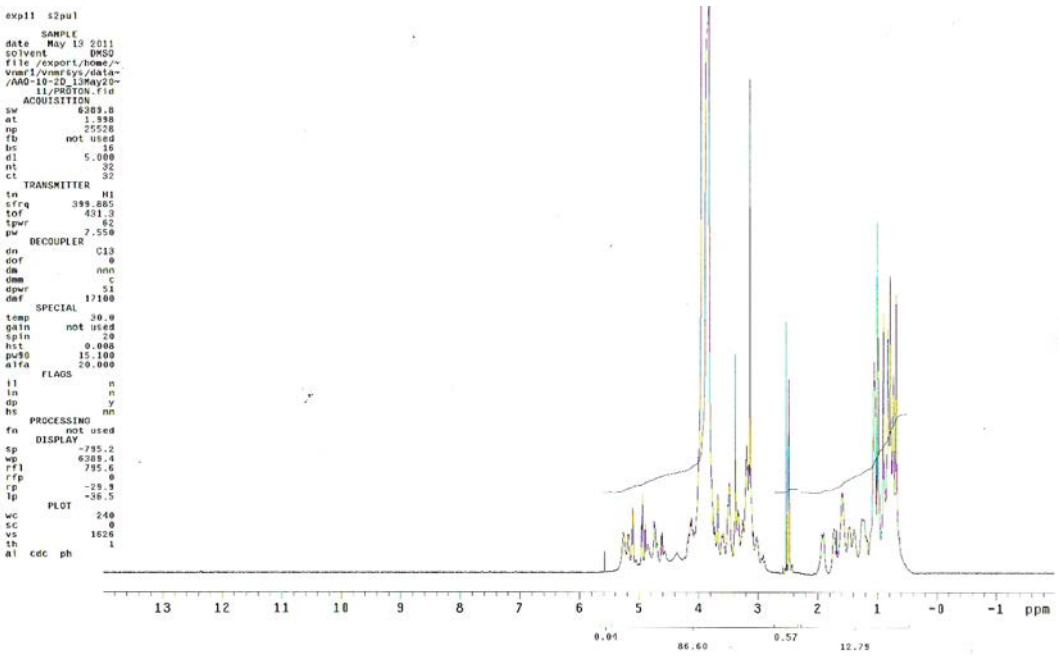
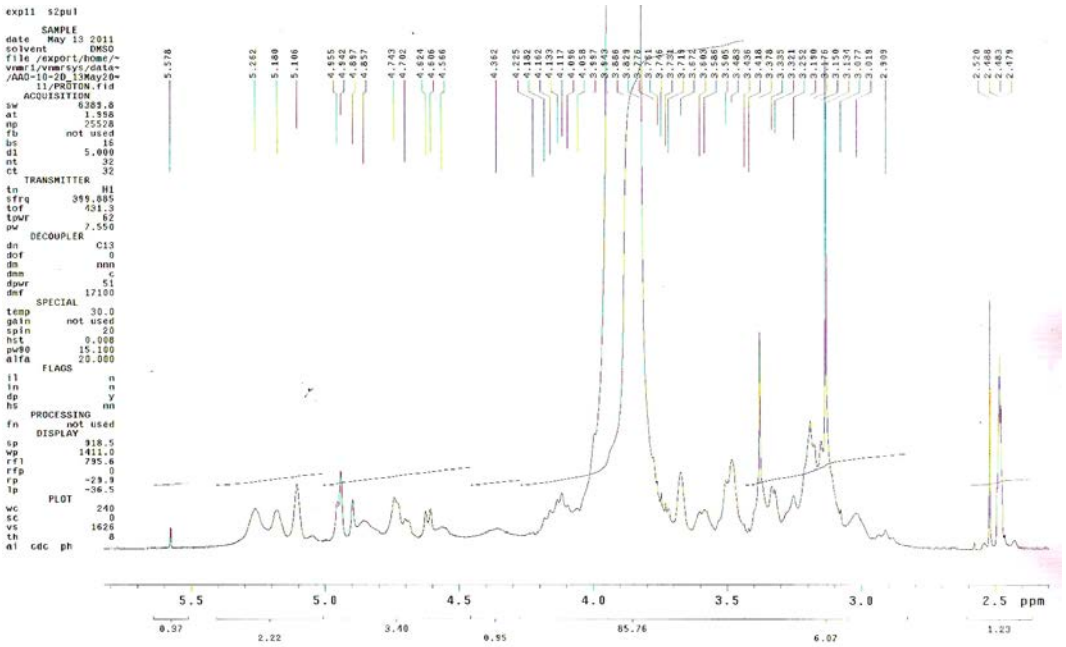


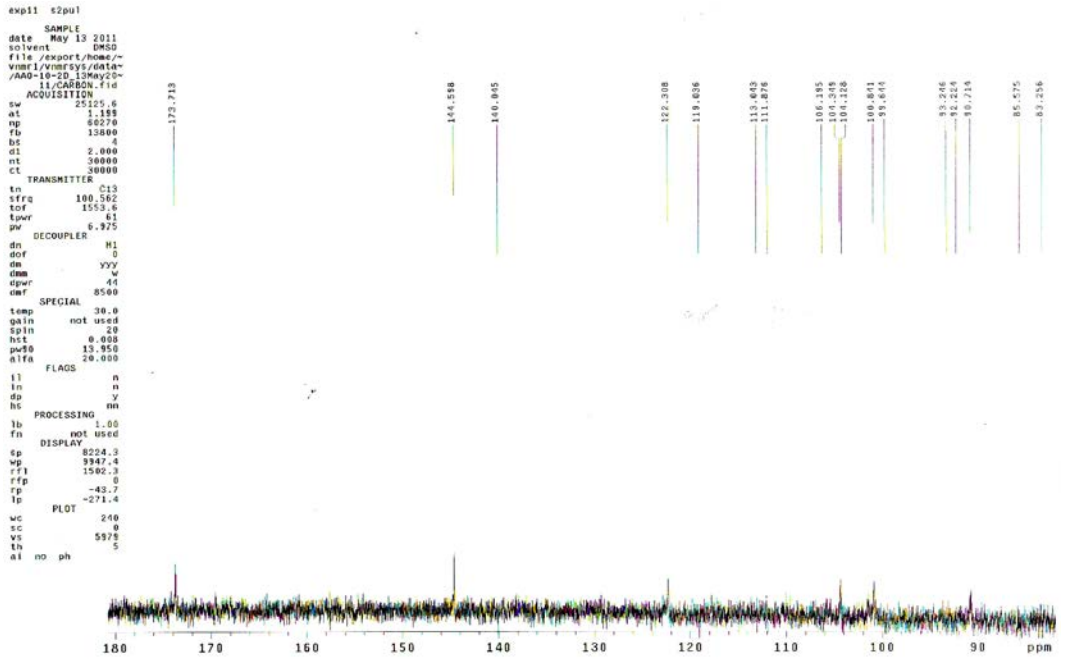
Figure 3.3. Aglycone Moiety of Compound **AAO-06**



Spectrum 3.7. ^{1}H-NMR Spectrum of Compound **AAO-06**



Spectrum 3.8. ^{1}H-NMR Spectrum of Compound **AAO-06** Sugar Moiety



Spectrum 3.9. ^{13}C-NMR Spectrum of Compound **AAO-06**

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