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**ISOLATION AND CHARACTERIZATION OF
SAPONINS FROM SOME *ASTRAGALUS* TAXA**

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ÖZET

**BAZI *ASTRAGALUS* TAKSONLARINDAN SAPONİNLERİN
İZOLASYONU VE KARAKTERİZASYONU**

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Astragalus L. (Leguminosae), yaklaşık 3000 türle çiçekli bitkiler aleminin en büyük cinsidir ve 224 tanesi endemik olmak üzere toplam 445 türle Türkiye florasında temsil edilmektedir (Davis, 1970; Aytaç, 2000).

Bazı *Astragalus* türlerinin kurutulmuş kökleri (Radix Astragali) geleneksel tıpta, hayvan ısırıkları ve zehirlenmelerinde, yaralanmalarda ve yanıklarda iyileştirici olarak, ayrıca lösemi ve rahim kanserinde sıklıkla kullanılmaktadır (Tang and Eisenbrand, 1992; Lev, 2002; Çakılcıoğlu and Türkoğlu, 2010).

Türkiye'deki *Astragalus* türleri üzerinde daha önce yapılan çalışmalarda bir seri oleanan ve sikloartan tipi triterpen saponin izole edilmiştir (Bedir et al., 1998b, 1999a,b, 2001b; Çalış et al., 1997, 2008b; Horo et al., 2010; Polat et al., 2009, 2010; Savran et al., 2012). Geçmiş çalışmalar göstermiştir ki *Astragalus* türlerinden izole edilen sikloartan ve oleanan tipi glikozitler immünostimülan (Çalış et al., 1997; Bedir et al., 2000a; Yeşilada et al., 2005), anti-protozoal (Özipek et al., 2005), antiviral (Gariboldi et al., 1995), sititoksik (Radwan et al., 2004), kardiyotonik (Khushbaktova et al., 1994), yara iyileştirici (Sevimli-Gür et al., 2011) ve adjuvan gibi (Nalbantsoy et al., 2011) ilginç biyolojik aktiviteler sergilemektedir. Özellikle, sikloartan glikozitlerin katı tümör (HepG2), kan kanseri (HL-60) ve ilaca dayanıklı tümörler (R-HepG2) gibi birçok kanser hücrelerine karşı antiproliferatif aktiviteleri kayda değerdir (Kikuchi et al., 2007).

Türkiye *Astragalus* türlerinden biyolojik olarak aktif bileşiklerin izolasyonuna ilişkin devam eden çalışmalarımız kapsamında *Astragalus aureus* Willd., *Astragalus tauricolus* Boiss. ve *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius* bitkileri çalışılmıştır.

A. aureus bitkisinden sekiz yeni sikloartan-tipi glikozitle (AAU1-AAU8) birlikte on adet bilinen sikloartan-tipi glikozit (AAU9-AAU18) izole edilmiştir. İzole edilen bu bileşiklerin yapıları 1D- ve 2D- NMR teknikleri ile ESIMS and HRMS analizleri kullanılarak belirlenmiştir. Tüm bu bileşiklerin kanser hücrelerine karşı sitotoksik aktiviteleri incelenmiş ve sadece AAU8 bileşiği 45µM konsantrasyonda insan meme kanser hücresine (MCF7) karşı aktivite göstermiştir.

A. tauricolus bitkinin butanol ekstresindeki triterpen glikozit profilinin belirlenmesine ilişkin HPLC-ESIMS'n'e dayalı denemeler yapılmıştır. HPLC-ESIMS'n incelemeleri sonrası, 10 tanesi daha önceden rapor edilmemiş olan 22 adet oleanan-tipi triterpen glikozit izole edilmiş ve yapıları 1D- ve 2D- NMR, ESIMS ve HRMS analizleri ile aydınlatılmıştır. İzole edilen ATA1-ATA12, ATA15, ATA17-ATA19 bileşiklerinin antiproliferatif aktiviteleri araştırılmış ve sadece ATA11'in 22 µM konsantrasyonda insan lösemi hücrelerine karşı aktivite gösterdiği belirlenmiştir. Test edilen diğer bileşikler, 1-50 µM konsantrasyon aralığında bu hücrelerde kayda değer bir toksisite yaratmamıştır.

A. angustifolius subsp. *angustifolius* var. *angustifolius* bitkisinden 6 yeni sikloartan-tipi (AAN1-AAN6), 4 adet yeni oleanan-tipi (AAN7-AAN10) triterpen glikozit ve 5 adet bilinen triterpen glikozit izole edilmiştir. Bu bileşiklerin yapıları 1D- ve 2D- NMR, ESIMS ve HRMS analizleri ile aydınlatılmıştır. Tüm bileşiklerin Hela (insan rahim kanser hücreleri), H-446 (insan akciğer kanser hücreleri), HT-29 (insan kolon kanser hücreleri) ve U937 (insan lösemi hücreleri) hücrelerine karşı antiproliferatif aktiviteleri incelenmiştir. Sadece AAN8 bileşiği sırasıyla Hela ve HT-29 hücrelerine karşı 36 ve 50 µM IC₅₀ değerleriyle zayıf etki göstermiştir

Anahtar Sözcükler: Leguminosae, *Astragalus aureus*, *Astragalus tauricolus*, *Astragalus angustifolius*, sikloartan, oleanan, saponin.

ABSTRACT

ISOLATION AND CHARACTERIZATION OF SAPONINS FROM
SOME *ASTRAGALUS* TAXA

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Astragalus L. (Leguminosae), one of the largest genera of flowering plants with about 3000 species, is represented in the flora of Turkey by 445 species, of which 224 are endemic (Davis, 1970; Aytaç, 2000).

The dried roots of some *Astragalus* species (Radix Astragali) are well known in traditional medicine as remedy for animal bites and poisons, wounds and burns, leukemia and uterine cancer. They are also famed for their antimicrobial, antiperspirant, anti-inflammatory, diuretic and tonic effects (Tang and Eisenbrand, 1992; Lev, 2002; Çakılcıoğlu and Türkoğlu, 2010).

Earlier investigations on Turkish *Astragalus* species resulted in the isolation of a series of oleanane- and cycloartane-type triterpene saponins (Bedir et al., 1998b, 1999a,b, 2001b; Çalış et al., 1997, 2008b; Horo et al., 2010; Polat et al., 2009, 2010; Savran et al., 2012). Previous studies have shown that cycloartane- and oleanane-type glycosides isolated from *Astragalus* species show interesting biological properties, including immunostimulating (Çalış et al., 1997; Bedir et al., 2000a; Yeşilada et al., 2005), anti-protozoal (Özipek et al., 2005), antiviral (Gariboldi et al., 1995), cytotoxic (Radwan et al., 2004), cardiogenic (Khushbaktova et al., 1994), wound healing (Sevimli-Gür et al., 2011), and adjuvant activities (Nalbantsoy et al., 2011). In particular, cycloartane glycosides have been reported for their antiproliferative activity against several cancer lines including solid tumor (HepG2), blood tumor (HL-60) and drug resistant tumor (R-HepG2) (Kikuchi et al., 2007).

As part of our ongoing research of new bioactive compounds from Turkish *Astragalus* species, we carried out a study on three *Astragalus* species, *Astragalus*

aureus Willd., *Astragalus tauricolus* Boiss and *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*.

Eight new cycloartane-type triterpene glycosides (**AAU1–AAU8**) were isolated from *A. aureus*, along with ten known cycloartane-type glycosides (**AAU9–AAU18**). Their structures were established by the extensive use of 1D and 2D-NMR experiments along with ESIMS and HRMS analyses. All of the compounds tested for their cytotoxic activities against a number of cancer cell lines. Among the compounds, only **AAU8** exhibited activity versus human breast cancer (MCF7) at 45 μ M concentration.

An approach based on HPLC–ESIMSⁿ experiments has been used to profile the triterpene glycosides occurring in the butanol extract of the *A. tauricolus*. On the basis of the results of the online screening by HPLC–ESIMSⁿ, 22 oleanane-type triterpene glycosides, including ten compounds never reported before, were isolated, and their structures were established by the extensive use of 1D and 2D-NMR experiments along with ESIMS and HRMS analysis. The antiproliferative activity of the isolated compounds **ATA1–ATA12**, **ATA15**, **ATA17–ATA19** was evaluated against a small panel of cancer cell lines. Only compound **ATA11** showed an IC₅₀ of 22 μ M against human leukemia cell line (U937). The other tested compounds, in a range of concentrations between 1 and 50 μ M, did not cause any significant reduction of the cell number.

Six new cycloartane-type (**AAN1–AAN6**) and four new oleanane-type (**AAN7–AAN10**) triterpene glycosides were isolated from *A. angustifolius* subsp. *angustifolius* var. *angustifolius*, together with five known triterpene glycosides. Their structures were established by the extensive use of 1D and 2D-NMR experiments along with ESIMS and HRMS analysis. All compounds were evaluated for their antiproliferative activity in Hela (human cervical cancer cells), H-446 (human lung cancer cells), HT-29 (human colon carcinoma cells), and U937 (human leukemia cells) cell lines. Only compound **AAN8** displayed a weak activity with IC₅₀ values of 36 and 50 μ M against Hela and HT-29 cell lines, respectively.

Keywords: Leguminosae, *Astragalus aureus*, *Astragalus tauricolus*, *Astragalus angustifolius*, cycloartane, oleanane, saponin.

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ABBREVIATIONS

BuOH	Butanol
CHCl ₃	Chloroform
CH ₂ Cl ₂	Dichloromethane
EtOAc	Ethylacetate
CH ₃ OH	Methanol
H ₂ O	Water
CD ₃ OD	Deuterated methanol
DMSO	Dimethylsulfoxide
UV	Ultraviolet
HPLC	High Performance Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
FAB-MS	Fast Atom Bombardment Mass Spectrometry
MALDITOF	Matrix-assisted Laser Desorption Ionization Time-of-flight
DQF-COSY	Double Quantum Filtered Correlation Spectroscopy
CC	Column Chromatography
GC	Gas Chromatography
FT-IR	Fourier Transform Infrared
TLC	Thin Layer Chromatography
RP-VLC	Reverse Phase Vacuum Liquid Chromatography

NMR	Nuclear Magnetic Resonance
1D-NMR	One-Dimensional Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Coherence
1D-TOCSY	One Dimensional Total Correlation Spectroscopy
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	Multiple

1. INTRODUCTION

1.1. General

The Fabaceae (Leguminosae) is a family of flowering plants comprising about 269 genera and 5100 species and is one of the largest plant families in the world and also in Turkey. It has 68 genera and more than 900 species in Flora of Turkey. This is a more important family of food plants, especially pulses (beans, gram, peas) and oil (soya, ground nut), but also tanbarks, timber, copal, gums, insecticides and cultivated ornamentals, as well as medicinal plants (Bağcı, 2006).

The flowering plant genus *Astragalus* (Fabaceae), common name "milkvetch" or "locoweed" ("loco" is Spanish for crazy), was already well known to western European botanists of the 17th century. A number of species of *Astragalus* from Western Asia (e.g., *A. gummifer* Lab., Iran and Turkey) are the source of "gum tragacanth" a dried gummy exudation tapped from roots or stems with hydrophilic and colloidal properties. It is used as an emulsifier, stabilizer and thickening agent in pharmaceuticals and foodstuffs. A few species are edible (as raw and cooked roots of *A. canadensis* L., legumes of *A. caryocarpus*, seed of *A. edulis* or leaves from *A. glycyphyllos* used as substitute for tea) (Motta, 1962) or have medicinal uses and some are used for livestock forage (*A. cicer* L.), to control erosion or as ornamental (Williams, 1981), but a large number of North American species are poisonous (e.g., *A. mollissimus* Torr.), especially for livestock and wild animals. In many cases, the toxins may be transferred to humans through meat or milk (Rios and Waterman, 1997).

Astragalus root is a very old and well known drug in traditional Chinese medicine and have been used to improve resistance to infections and to cure in immunological disorders and viral infections, and as hepatoprotective. It also stimulates the circulatory system and acts as a heart tonic. It is specifically used for symptoms of excessive sweating, deficient urination, prolapse of internal organs and rectum, and to speed the healing of burns and abscesses (Tang and Eisenbrand, 1992). "Astragali radix" (Huang qi in China; Japanese name, Ougi), is the dried root of *A. membranaceus* Bunge var. *mongholicus* Hsiao or *A. membranaceus* (Fisch.) Bunge. The Chinese name Huang qi translates as "yellow leader", referring to the yellow color of the root and its status as one of the most important tonic herbs. It is officially listed in the Chinese Pharmacopoeia and has been used for over 2000 years in traditional Chinese medicine (TCM)

prescriptions for the treatment of nephritis, diabetes, albuminuria, hypertension, cirrhosis, cancer, as well as a sedative and tonic (Li et al., 2001; Wang et al., 2004). Other traditional indications include wasting disorders, night sweats (Hong, 1986), chronic ulcerations and sores, numbness and paralysis of the limbs, and edema. (Bensky and Gamble, 1993). *Astragalus* is classically prescribed in combination with other Chinese medicinal herbs depending on the desired therapeutic effect and the exact diagnosis (Sinclair, 1998).

The herb has a sweet taste and the roots are sun-dried and then processed into a fine powder of encapsulation, tablets or capsules (2-3 per day). Textbooks of Chinese herbs recommend taking 9-15 g of the crude herb per day in decoction form. A decoction is made by boiling the root in water for a few minutes and then brewing the tea. Sometimes 3-5 ml of tincture three times per day are often recommended.

Astragali Complanati Semen, Shayuanzi, the dried ripe seed of *A. complanatus* R.Br. collected in late fall to early winter, is another *Astragalus* species listed in the Chinese Pharmacopoeia. It is used as tonic against polyuria and vertigo (Tang and Eisenbrand, 1992). *Astragalus* species are also used in traditional medicine in Bulgaria, Russia and other European and Asiatic countries. In modern Chinese medicine, it is used in Fu Zheng therapy to improve immune system parameters and it is the most frequently used promoter for other herbal therapies. Because its properties are somewhat similar to those of the more expensive herbs ginseng (*Panax ginseng*), it has been used as a substitute for that species (Ionkova et al., 1995). *Astragalus* is held in high esteem by the Chinese, who incorporated it into many others medicinal formulas. Herbal practitioners may suggest using this herb during treatment with chemotherapy as it stimulates the immune system. Current Western applications of *Astragalus* are primarily for restoring and strengthening the immune response, enhancing cardiovascular function, and increasing vitality (Monograph, 2003).

In Turkey, the roots and gum of *Astragalus* species are traditionally used in treatment of jaundice, sore throat, stomach ache, diabetes and cancer (Altundağ and Öztürk, 2011). Flowering parts of some species are also used against asthma, urethritis and as an expectorant (Tuzlacı and Şenkardeş, 2011). Additionally, some parts of the genus has been used against high blood pressure, stomach inflammation and as a pain killer (Güneş and Özhatay, 2011).

1.2. Botanical Description

The genus *Astragalus* belonging to the Leguminosae family is comprising the highest number of species among the Spermatophytes. While the exact number of species in this genus has not been established recently, it is estimated to be around 3.000 (Podlech et al., 1998). Although *Astragalus* sp. is widely distributed throughout the temperate regions of the world, located principally in Europe, Asia and North America, almost all of the species distributed throughout the Old World are found in Iran, Turkey and central Asian countries (Vural et al., 2008). In Turkey, it is represented by 445 species, of which 224 are endemic. They are classified in 62 sections (Özüdoğru et al., 2011; Aytaç et al., 2000; Davis, 1970). There are three different forms of the *Astragalus* species. They can be annual, perennial herbs and small shrubs (Davis, 1970).

1.2.1. *Astragalus aureus* Willd. (Sect. *Adiaspastus*)



Figure 1.1. *Astragalus aureus*

Astragalus aureus Willd. (Section: *Adiaspastus*) is a dwarf cushion-forming shrub, up to 25 cm. Leaf rachis spiny, 1-6 cm, straight, white-hairy or glabrescent, breaking off completely with age leaving bare woody stems. Leaflets 5-10 mm, linear-elliptic, often folded, mucronate, both surfaces glabrescent or sparsely white, spreading simple-hairy, usually glabrescent when mature, 4-6-paired. Stipules c. 10 mm, triangular-lanceolate, white-ciliate, united to more than

halfway, sometimes purple-tipped. Inflorescence globose, 10-20- flowered. Bracts 5-12 mm, linear-lanceolate to ovate, usually navicular, glabrous, sometimes purple-tipped. Bracteoles absent. Calyx tubular, 8-13 mm, whitepilose, tube 3-5 mm; teeth setaceous. Corolla bright yellow; standard 15-20 mm, platonychoid, usually retuse. Legume unknown. Fl. 7-8. Stony slopes, 2150-3300 m. It mainly grows at E. & N.E. Anatolia. Iran, Caucasia, Ir.-Tur. Element (Davis, 1970).

1.2.2. *Astragalus tauricolus* Boiss (Sect. *Malacothrix*)



Figure 1.2. *Astragalus tauricolus*

Astragalus tauricolus Boiss (Section: *Malacothrix*) is an endemic species which prostrate subcaulescent to caulescent perennial with a branched woody caudex; stems to c. 2 cm. Leaves 4-5 cm; leaflets 8-10 mm. elliptic, apex acute,

spreading, simple-subsericeous, 10-18-paired; stipules 7-10 mm, triangular-lanceolate, united for half their length. Peduncles 5-8 cm, slender. Inflorescence an oblong, 10-20-flowered spike which elongates in fruit. Bracts 5-7(-9) mm, lanceolate. Calyx 9-12 mm, tubular, inflating or splitting in fruit, shortly simple-villous with black and white hairs; teeth 3-4 mm, subulate. Corolla cream with purple centre, or yellowish purple; standard 20-25 mm, not ligulately attenuate. Legume 11-13 mm, ovoid, densely villous with long white hairs; beak curved, 2-3 mm. Fl. 5-8. Limestone slopes, screes, etc., 1370-3350 m. It mainly grows at S., C. & E. Anatolia. Ir.-Tur. Element (Davis, 1970).

1.2.3. *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius* (Sect. *Melanocercis*)



Figure 1.3. *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*

Astragalus angustifolius Lam., (Section: *Melanocercis*) is a dwarf, cushion-forming shrub. Leaves 2-6 cm, paripinnate; rachis spiny, leaflets 3-7 mm, elliptic to obovate, obtuse, sometimes mucronate. Both surfaces bifurcate-sericeous or glabrescent, (5-)7-12-paired; stipules 5-8 mm, lanceolate, white-hairy, joined to more than half-way. Peduncles shorter or longer than leaves. Flowers in 3-14-flowered racemes. Bracts 2-4 mm, lanceolate. Bracteoles- 1-2 mm, lanceolate. Calyx 6-10 mm, tubular, shortly $\pm$ adpressed black and white bifurcate-hairy; teeth 1-5 mm. Corolla pinkish-purple, yellow or whitish; standard 15-18 mm. Legume 8-10(-12) x 2-3 mm, oblong, densely $\pm$ adpressed whitehairy (sometimes

sparsely hairy with a few black hairs); beak c. 1 mm, straight. Peduncles of *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius* shorter than leaves, calyx teeth half as long as tube or less, corolla whitish or yellow, sometimes with a purple keel. Fl. 4-8. Steppe, 800-2900 m. It mainly grows N.W., W., S. & Inner Anatolia, and Greek Islands (Davis, 1970).

1.3. An Overview of the Chemistry of *Astragalus* Genus

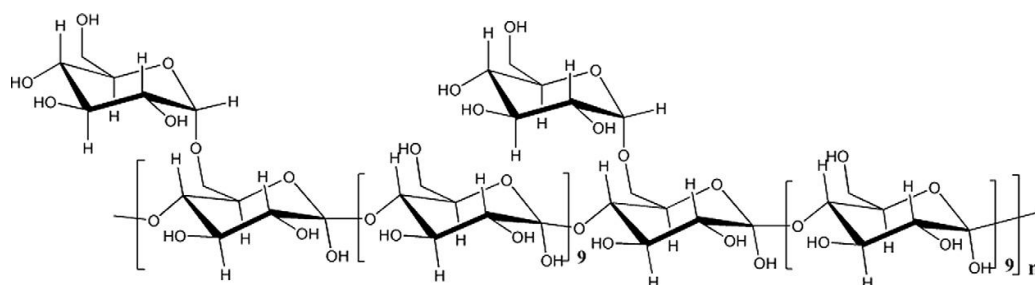
The genus *Astragalus* appears highly uniform from a chemical point of view, with two kinds of pharmacologically active principles and three different kinds of toxic compounds. In the former group the polysaccharides and the saponins stand out, and in the second, the indolizidine alkaloids (swainsonine 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 and isoflavones) in free and glycosidic forms; pterocarpan free and as glucosides; and organic acid derivatives (homopilosinic and phaseic acids) (Rios and Waterman, 1997).

Polysaccharides; The polysaccharides from *A. membranaceus* are the best known, and several research groups have isolated and purified them. *Astragalus* polysaccharides (APS) are considered as a group of possible bioactive components contributing to the beneficial effects of *Astragalus*. Several extractions of APS have been prepared and investigated for their monosaccharide compositions and molecular weights since the first one was purified from the roots of *A. membranaceus* in the 1980s (Niu et al., 2011).

Tang and Eisenbrand (1992) 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. Tomoda et al. (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-galacturonic acid 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).

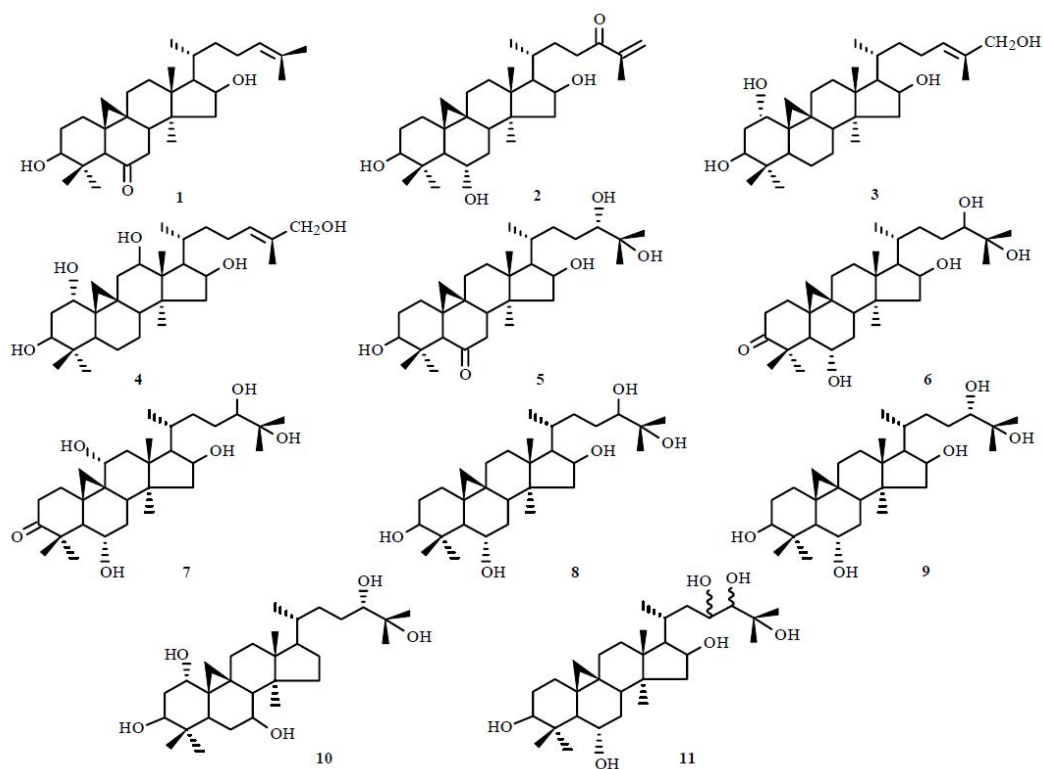
In 2011, Niu et.al., isolated a bioactive polysaccharide (APS) from the roots of *A. membranaceus* Bge. var. *mongolicus* (Bge.) Hsiao and characterized its chemical structure and potential health properties. The results showed that the APS was composed of α -D-glc residues with the estimated equivalent dextran molecular weight of 2.07×10^4 Da. Periodate oxidation analysis, 1D and 2D NMR spectroscopy demonstrated that APS has repeating (1 $\rightarrow$ 4)-linked backbone with a (1 $\rightarrow$ 6)-linked branch every 10 residues. The APS possessed scavenging activities against hydroxyl radicals and hydrogen peroxide, and showed chelating effect on ferrous ions. The APS was also able to bind cholic and chenodeoxycholic acids in vitro. In addition, APS was able to stimulate activity of purified mouse B cells without promoting T cell proliferation (Niu et al., 2011).



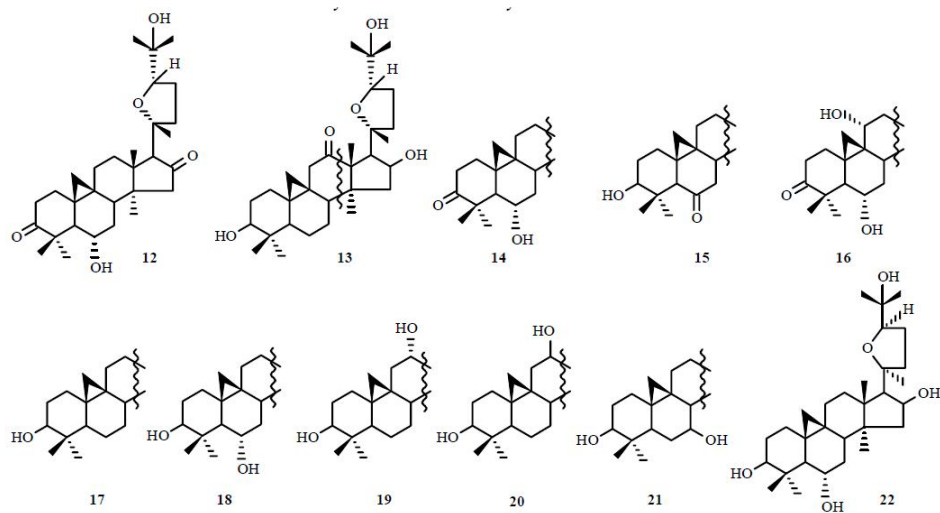
The repeating unit of APS.

Saponins: Cycloartane triterpenoids were first discovered in *Astragalus* plants. Plants of this genus drew attention due to their cycloartane production abilities. 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 $9\beta,19$ -cyclolanostane and are produced exclusively by photosynthetic eukaryotes. Cycloartanes dominate the known triterpenoids in plants of this genus (Mamedova and Isaev, 2004). During the past two decades, many researchers from different countries became to study the triterpenoid composition of many species from *Astragalus* genus. Mamedova and Isaev has been reviewed that a total of 30 structurally distinct genins have been found in the plants until 2004 (Mamedova and Isaev, 2004). The authors classified these substances into six structural types according to the side-chain structure: 1) cycloartanes with an acyclic side chain (1-11), 2) 20,24-epoxycycloartanes (12-22), 3) $16\beta,23;16\alpha,24$ -diepoxycycloartanes (23-26), 4) $16\beta,24;20,24$ -

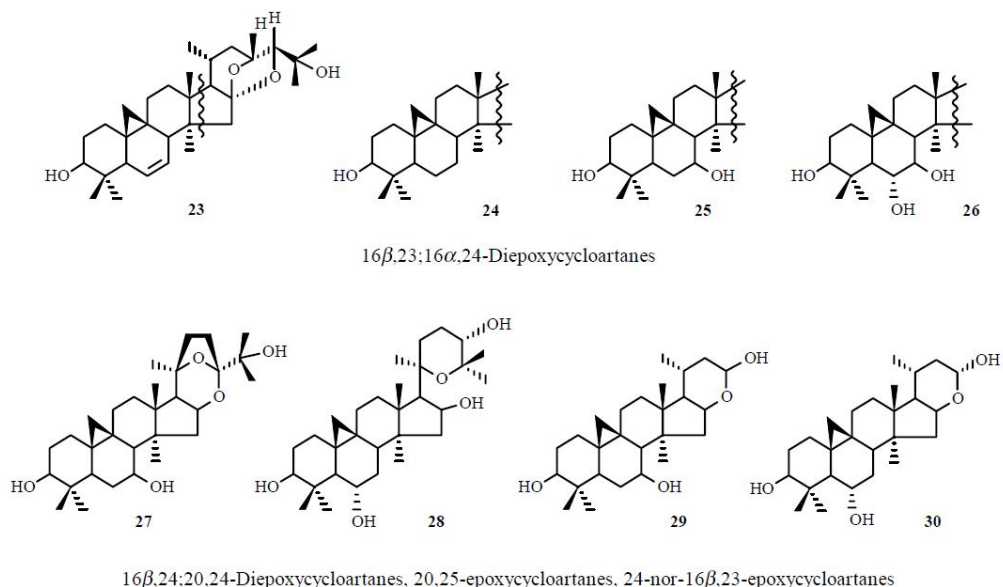
diepoxycycloartanes (27), 5) 20,25-epoxycycloartanes (28), 6) 24-nor-16 β ,23-epoxycycloartanes (29,30).



Cycloartanes with an acyclic side chain

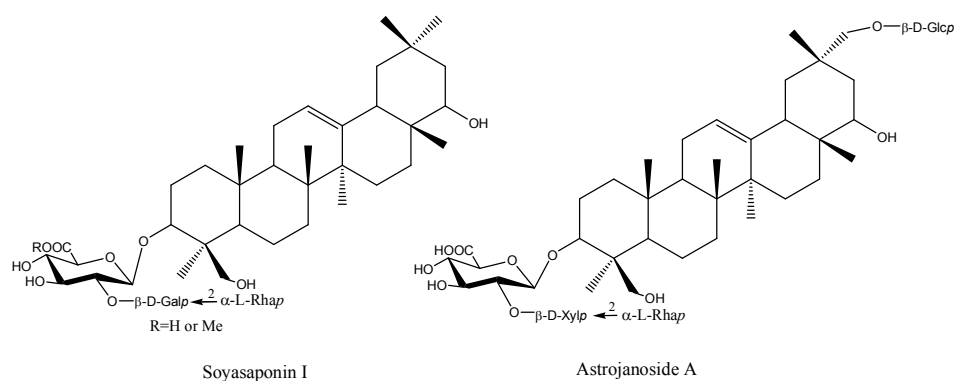


20,24-Epoxycycloartanes



The structures of **1-30** show that the side chains of the compounds differ substantially. 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 16 β -hydroxyl. A common structural element for cycloartanes with an acyclic side chain, 16 β ,23;16 α ,24-diepoxy cycloartanes, and 24-nor-16 β ,23-epoxy cycloartanes is a secondary CH₃-21 methyl. 20,24-Epoxy cycloartanes (12-22) represent the most numerous group. Of the four possible side-chain stereoisomers [two pairs of enantiomers: 20*R*,24*S* and 20*S*,24*R* (**1**) and 20*R*,24*R* and 20*S*,24*S* (**2**)], until 2004, only the first pair has been found in *Astragalus* plants. Side chains in 10 genins (12-21) have identical stereochemistry (20*R*,24*S*) whereas that of **22** is enantiomeric. Cyclosiversigenin (**18**) is the most widely distributed genin of *Astragalus* plant glycosides. Thus, it is not surprising that it was discovered first. Cyclosieversigenin is also known as cycloastragenol and astramembrangenin (Mamedova and Isaev, 2004).

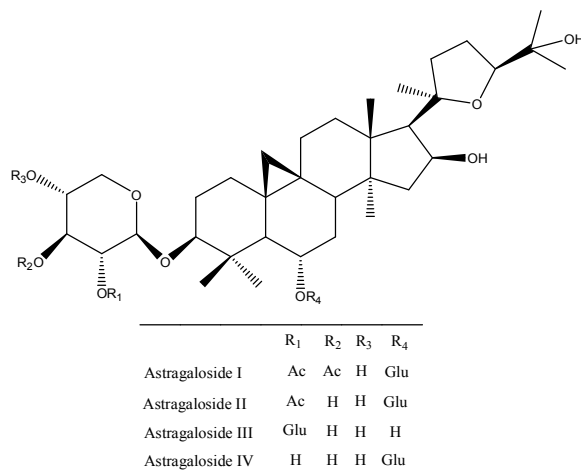
Oleanane- and lanostane-type triterpenoids are not common in *Astragalus* genus as cycloartanes. The most common oleanane- and lanostane-type saponins which were isolated from the genus given below.



Most of the isolated and identified compounds were based on the cycloartane skeleton, i.e. 9,19-cyclo-5 α ,9P-lanostane

Interest in the cycloartanes is due to the fact that cycloartenol, the initial compound of this series, is a key intermediate in the biosynthesis of phytosterols. However, the reason for the rising interest towards the cycloartane saponins isolated from the genus *Astragalus* is due to their wide range of biological activity, including immunostimulating (Çalış et al., 1997; Yeşilada et al., 2005; Bedir et al., 2000a), anti-protozoal (Özipek et al., 2005), antiviral (Gariboldi et al., 1995), cytotoxic (Radwan et al., 2004), cardiogenic (Khushbaktova et al., 1994), wound healing (Sevimli-Gür et al., 2011) and adjuvant (Nalbantsoy et al., 2011) activities (Rahman, 2002).

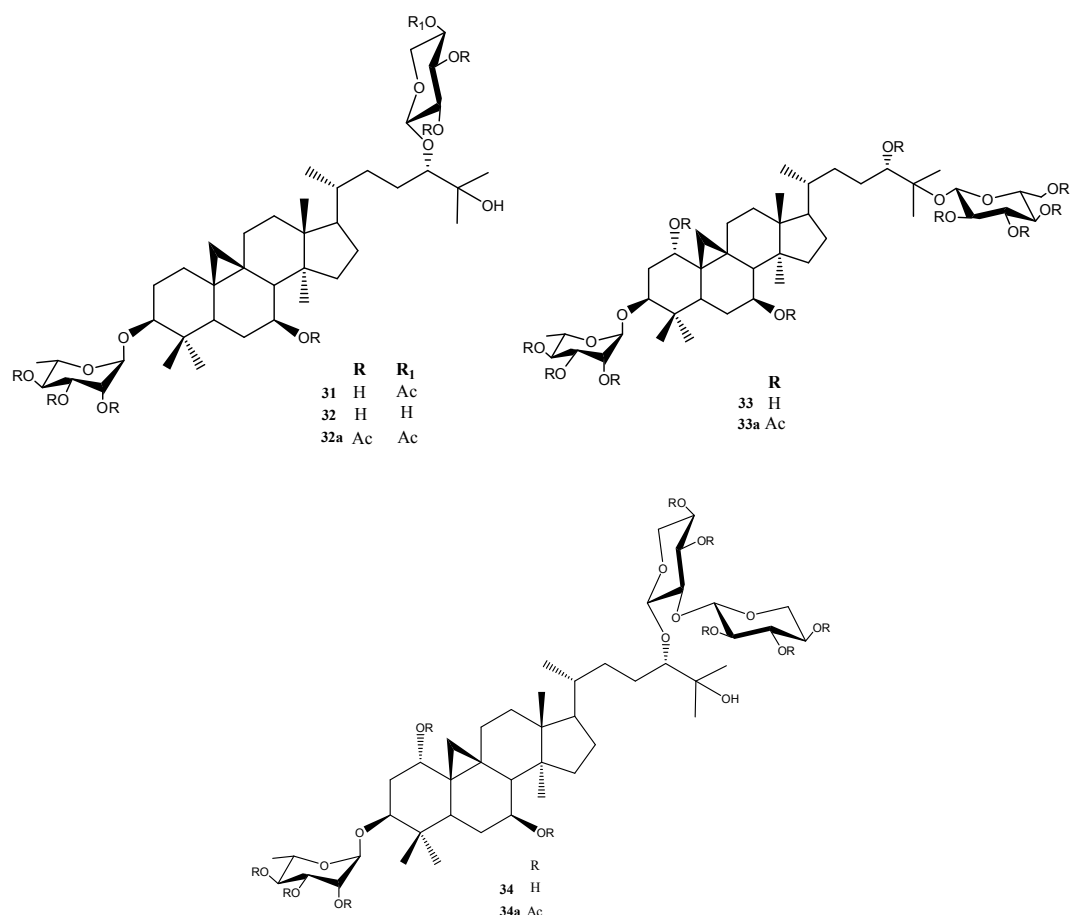
In general, triterpenoid saponins represent the major beneficial compounds responsible for the bioactivities and efficacies of *Radix Astragali* on human health. Especially, the four astragalosides, i.e., astragaloside I (AG I), astragaloside II (AG II), astragaloside III (AG III) and astragaloside IV (AG IV), belong to cycloartane-type triterpenoid saponins. They are considered as the major bioactive constituents with better pharmacological activities and possess antioxidant, antitumor, hepatoprotective, anti-diabetic, antimicrobial, antiviral and immunological activities. Thereinto, AG III and AG IV represent a pair of isomeric compounds (Yan et al., 2010).



1.3.1. Chemical and Biological Investigations on Turkish *Astragalus* sp.

Until now, 25 out of 445 Turkish *Astragalus* species, from 13 different sections, have been investigated for their secondary metabolite contents and structures of 64 new triterpene saponins and 5 new phenolic glycosides were identified besides known compounds.

In 1996, four novel cycloartane-type triterpene glycosides, macrophyllsaponins A-D (**31-34**) were isolated from the roots of *Astragalus oleifolius* (Sect. Macrophyllium). By means of chemical (acetylation, alkaline hydrolysis) and spectroscopic methods (IR, 1D- and 2D-NMR, FABMS), their structures were established as 3-*O*-α-L-rhamnopyranosyl-24-*O*-(4''-*O*-acetyl)-β-D-xylopyranosyl-1α,3β,7β,24(*S*),-25-pentahydroxycycloartane (**31**), 3-*O*-α-L-rhamnopyranosyl-24-*O*-β-D-xylopyranosyl-1α,3β,7β,24(*S*),-25-pentahydroxycycloartane (**32**), 3-*O*-α-L-rhamnopyranosyl-25-*O*-β-D-glucopyranosyl-1α,3β,7β,24(*S*),-25-pentahydroxycycloartane (**33**), and 3-*O*-α-L-rhamnopyranosyl-24-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-xylopyranosyl-1α,3β,7β,24(*S*),-25-pentahydroxycycloartane (**34**) (Çalış et al., 1996).

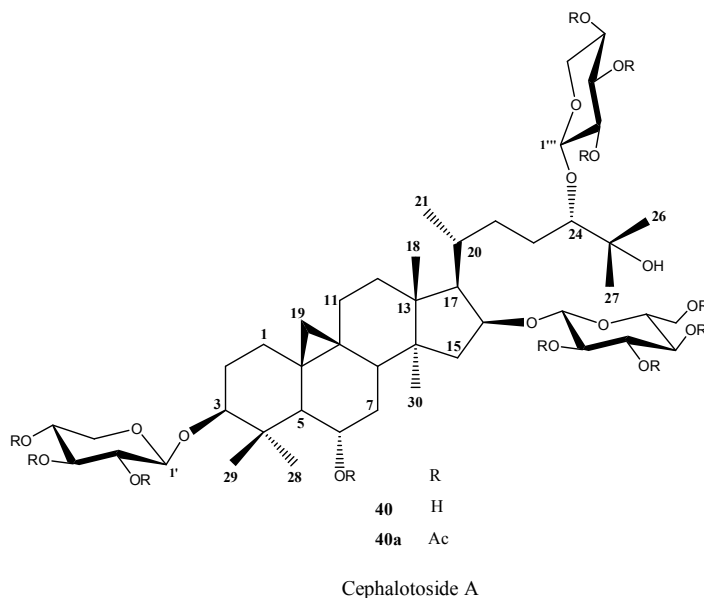


Çalış et al., were isolated eight known saponins; astrasieversianins II and X, astragalosides I, II, IV and VI, and cyclocanthosides E and G from the roots of *Astragalus melanophrurius* (Sect. Christiana). The isolates were evaluated in a broad range of bioassay systems and found to be inactive. Modest antibacterial activity was observed, however, as was immunomodulatory activity, as indicated by stimulation of isolated human lymphocytes (Çalış et al., 1997).

A novel cycloartane-type glycoside, cyclocephaloside I (**35**) was isolated from the roots of *Astragalus microcephalus* in addition to known glycosides cyclocanthoside E and astragaloside IV. The structure of **35** was determined by spectral (IR, ^1H and ^{13}C NMR, and FABMS) and chemical (acetylation) methods and established as 20,25-epoxy-3 β -(β -D-xylopyranosyl)oxy-6 α -(β -D-glucopyranosyl)oxy-cycloartane-16 β ,24 α -diol (Bedir et al., 1998a).

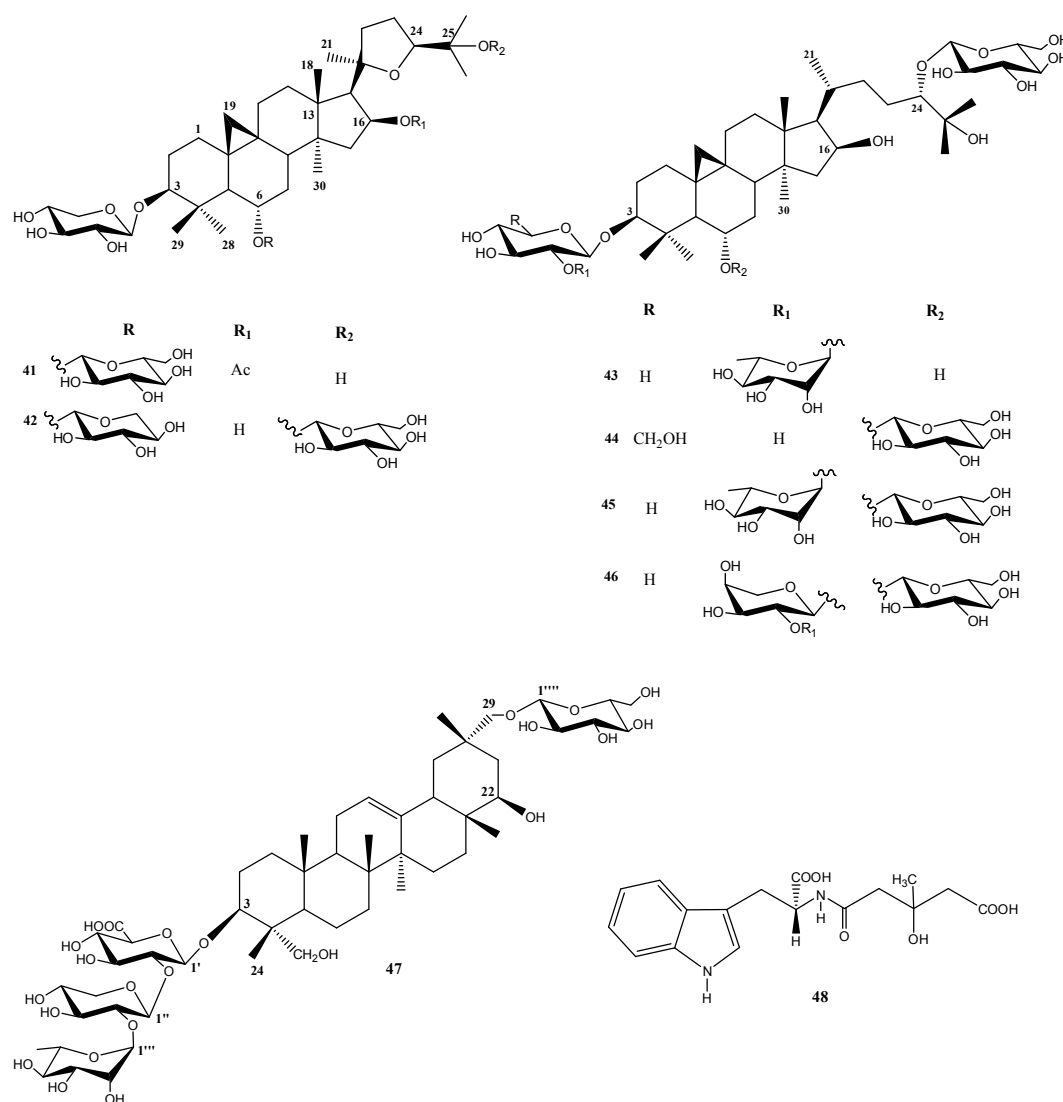
Chemical structures of compounds 36, 37, 38, and 39 are shown. Compound 36 is a complex steroid with multiple hydroxyl groups and a glycosidic linkage. Compound 37 is a steroid with a hydroxyl group and a glycosidic linkage. Compound 38 is a steroid with a hydroxyl group and a glycosidic linkage. Compound 39 is a steroid with an acetoxy group and a glycosidic linkage. The structures are shown in a 2D representation with stereochemistry indicated by wedges and dashes.

A new tridesmosidic cycloartane type glycoside cephalotoside A (**40-40a**) was isolated from the roots of *Astragalus cephalotes* var. *brevicalyx* (Sect. Rhacophorus) in addition to the known glycosides cyclocanthosides A-D and E. The structure of the new compound was determined by spectral and chemical (acetylation) methods and established as 3-(β -D-xylopyranosyl)oxy-16-(β -D-glucopyranosyl)oxy-24-(β -D-xylopyranosyl)oxy-cycloartane-6 β ,25-diol (Çalış et al., 1999).



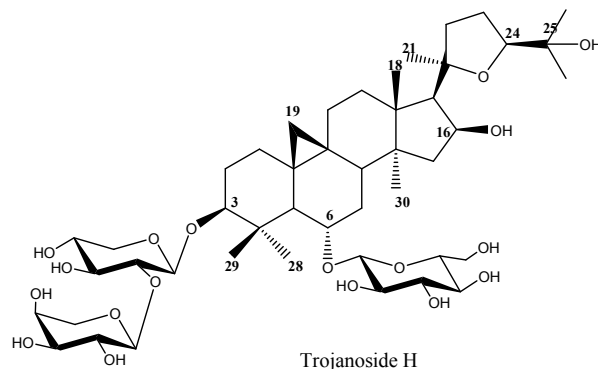
Six novel cycloartane-type glycosides were isolated from the roots of *Astragalus trojanus* (Sect. Pterophorus 1999). Two of these, compounds **41** and **42**, have (20*R*,24*S*)-epoxy-3 β ,6 α ,16 β ,25-tetrahydroxycycloartane as the aglycon, while compounds **43-46** possess 3 β ,6 α ,16 β ,25-(24*S*),25-pentahydroxycycloartane as the aglycon. The saccharide moieties linked to the C-3, C-6, and C-24 or C-25 positions of the aglycons in **41-46** contained either xylopyranose, glucopyranose, rhamnopyranose, or arabinopyranose units. Structure elucidation of compounds **41-46** was accomplished through the extensive use of 1D and 2D NMR techniques. In addition, a new oleanane glycoside (**47**) and a new tryptophan derivative (**48**) were also isolated and characterized. Compounds **41-47** were established as (trojanoside A): 3-*O*- β -D-xylopyranosyl-6-*O*- β -D-glucopyranosyl-16-*O*-acetoxy-(20*R*,24*S*)-epoxy-3 β ,6 α ,25-trihydroxycycloartane, (trojanoside B); 3-*O*- β -D-xylopyranosyl-6-*O*- β -D-xylopyranosyl-25-*O*- β -D-glucopyranosyl-(20*R*,24*S*)-epoxy-3 β ,6 α ,16 β ,25-tetrahydroxycycloartane, (trojanoside C): 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,25-(24*S*),25-pentahydroxycycloartane, (trojanoside D): 3-*O*- β -D-glucopyranosyl-6-*O*- β -D-glucopyranosyl-24-*O*- β -D-glucopyranosyl-

3 β ,6 α ,16 β , (24*S*),25-pentahydroxycycloartane, (trojanoside E): 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β , (24*S*),25-pentahydroxycycloartane, (trojanoside F): 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β , (24*S*),25-pentahydroxycycloartane, (astrojanoside A): 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24,29-tetrahydroxy-olean-12-en, respectively The structure of compound **48** was determined as N-[3-hydroxy-3-methyl-glutaroyl]-tryptophan, for which the trivial name achillamide is proposed. Astrasieversianin I, astrasieversianin II, astragaloside I, astragaloside IV, astragaloside VII, and brachyoside C were also isolated from the roots of *A. trojanus* (Bedir et al., 1999a).

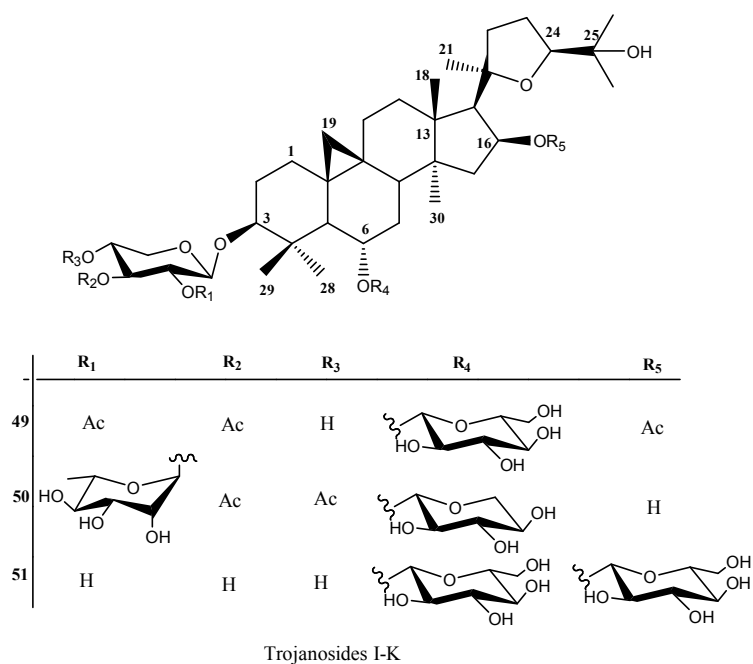


Isolation of trojanoside H were also reported from *A. trojanus* along with the known glycosides astragaloside II, astragaloside IV, astragaloside VII,

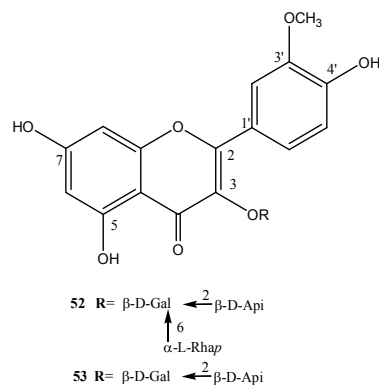
brachyoside B, brachyoside C and the pterocarpan derivative maackiain. The structure of trojanoside H was determined by spectral methods (1-D and 2-D NMR, and FABMS) and established as 3-*O*-β-[α-L-arabinopyranosyl(1→2)β-D-xylopyranosyl]-6-*O*-β-D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3β,6α,16β,25-tetrahydroxycycloartane (Bedir et al., 1999b).



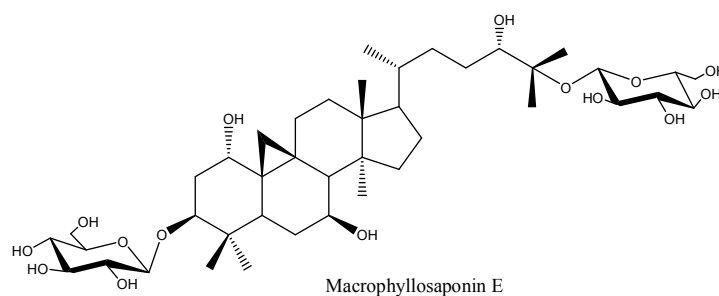
Another series of cycloartane saponins, Trojanosides I—K (**49**, **50** and **51**) were also isolated from the aerial parts of *Astragalus trojanus*. The structures of three new saponins were established as 3-*O*-β-(29,39-di-*O*-acetyl)-D-xylopyranosyl-6-*O*-β-D-glucopyranosyl-16-*O*-acetoxy-20(*R*),24(*S*)-epoxycycloartane-3β,6α,16β,25-tetrol (**49**), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-39,49-di-*O*-acetyl)-D-xylopyranosyl]-6-*O*-β-D-xylopyranosyl-20(*R*),24(*S*)-epoxycycloartane-3β,6α,16β,25-tetrol (**50**), 3-*O*-β-D-xylopyranosyl-6,16-di-*O*-β-D-glucopyranosyl-20(*R*),24(*S*)-epoxycycloartane-3β,6α,16β,25-tetrol (**51**). Astrasieversianin I, astrasieversianin II, astrasieversianin XV, astragaloside I, astragaloside II, astragaloside IV, astragaloside VII, and trojanoside H were also isolated from the aerial parts of *A. trojanus*, identified on the basis of their HR-ESI-MS, and NMR (¹H- and ¹³C-) data, in comparison with literature values. (Bedir et al., 2001a).



A new flavonol glycoside, isorhamnetin 3-*O*-β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside (**52**), in addition to the known glycoside, isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**53**) from the aerial parts of *Astragalus vulneraria* (Sect. *Vulneraria*) (Bedir et al., 2000b).

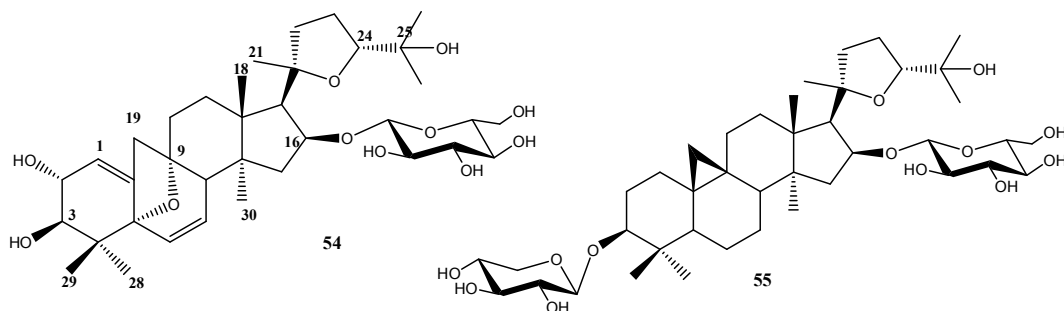


Same year, macrophyllsaponin E, a novel cycloartane-type triterpene, has been isolated from the roots of *Astragalus oleifolius* (Bedir et al., 2000c).

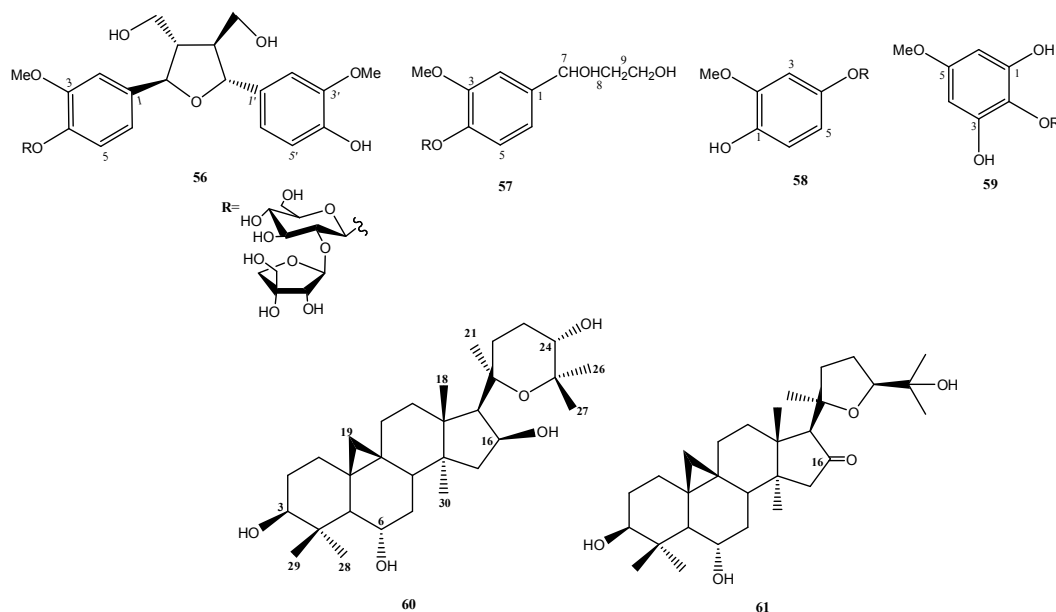


Bedir et al., were examined immunostimulatory effect of 19 cycloartane-type triterpene glycosides; macrophyllosaponins B-D, cyclocanthoside D and E, astrasieversianin II and X, astragaloside I, II, IV, VI, VII, Trojanoside A and H, cyclocephaloside I, brachyoside B, askendoside G, cephalatoside A, cycloastragenol which previously isolated from *A. oleifolius*, *A. prusianus*, *A. microcephalus*, *A. trojanus*, *A. cephalotes* and *A. melanophrurius* using a transcription-based bioassay for Nuclear Factor kappa B (NF-kappa B) activation in a human macrophage/monocyte cell line, THP-1. All compounds were inactive at 100 µg/ml except astragaloside I which increased NF-kappa B directed luciferase expression to levels about 65% as compared with maximal stimulation by *E. coli* lipopolysaccharide (LPS) at 10 µg/ml. None of the compounds were active at low dosage levels (0.1 µg/ml) in combination with 50 ng/ml LPS. Astragaloside I also Increased mRNA expression of the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) as measured using reverse transcriptase-polymerase chain reaction (RT)-PCR (Bedir et al., 2000a).

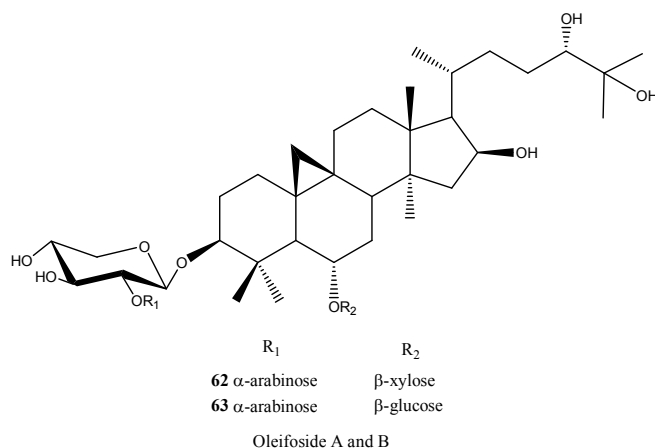
Two novel cycloartane-type glycosides (**54-55**), 16-*O*-β-D-glucopyranosyl-20(*S*),24(*R*)-5α-9-diepoxy-2α,3β,16β,25-tetrahydroxy-9,10-seco-cycloartan-1(10),6(7)-diene and 3-*O*-β-D-xylopyranosyl-16-*O*-beta-D-glucopyranosyl-20(*S*),24(*R*)-epoxy-3β,16β,25-trihydroxyhydroxycycloartane, were isolated from the roots of *Astragalus prusianus*. The 5-α-9-epoxy structural feature in **54** is encountered for the first time (Bedir et al., 2001b).



In 2001, Çalış et al., were isolated four new phenolic glycosides, β-apiofuranosyl-(1→2)-β-glucopyranosides (**56-59**), along with the cycloartane triterpenes 20(*R*),25-epoxy-3α,6β,16α,24β-tetrahydroxycycloartane (**60**) and 20(*R*),24(*S*)-epoxy-3β,6α,25-trihydroxycycloartan-16-one (**61**) from roots of *Astragalus zahlbruckneri* (Sect. Rhacophorus) (Çalış et al., 2001).



Another phytochemical investigation has been published in 2005 and two new cycloartane-type glycosides oleifoliosides A (**62**) and B (**63**) were isolated from the lower stem parts of *Astragalus oleifolius*. Their structures were identified as 3-*O*-[β -xylopyranosyl-(1 $\rightarrow$ 2)- α -arabinopyranosyl]-6-*O*- β -xylopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane and 3-*O*-[β -xylopyranosyl-(1 $\rightarrow$ 2)- α -arabinopyranosyl]-6-*O*- β -glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane, respectively, by means of spectroscopic methods (IR, 1D and 2D NMR, ESI-MS). Three known cycloartane glycosides cyclocanthoside E, astragaloside II and astragaloside IV were also isolated and characterized. All five compounds were evaluated for in vitro trypanocidal, leishmanicidal and antiplasmodial activities as well as their cytotoxic potential on primary mammalian (L6) cells. Except for astragaloside IV, all compounds showed notable growth inhibitory activity against *Leishmania donovani* with IC₅₀ values ranging from 13.2 to 21.3 μ g/ml. Only weak activity against *Trypanosoma brucei rhodesiense* was observed with the known compounds astragaloside II (4, IC₅₀ 66.6 μ g/ml) and cyclocanthoside E (3, IC₅₀ 85.2 μ g/ml), while all compounds were inactive against *Trypanosoma cruzi* and *Plasmodium falciparum*. None of the compounds were toxic to mammalian cells (IC₅₀'s > 90 μ g/ml) (Özipek et al., 2005).

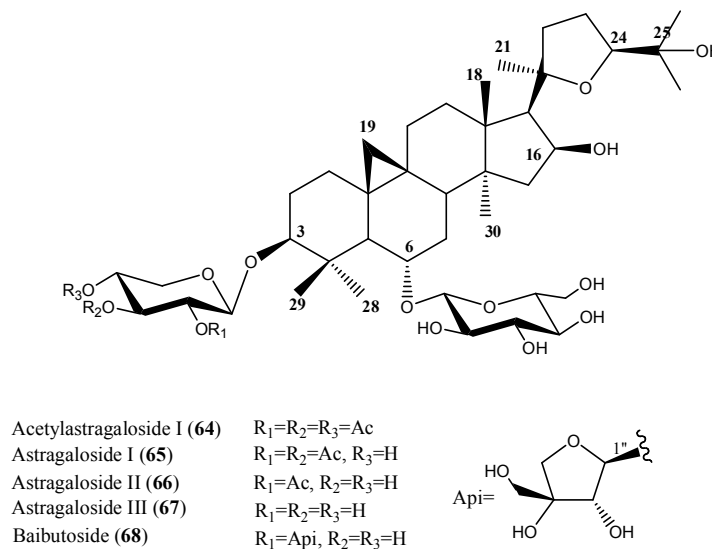


In 2005, a study was performed on another Turkish species; *Astragalus gilvus* (Sect. Christiana) and known cycloartane-type saponins, Astrasieversianins I, II, VI, VIII, X; Astragaloside IV were isolated (Tabanca et al., 2005)

In 2005, another study was performed for the investigation of immunostimulating effect of 13 cycloartane- and 1 oleanane-type triterpene saponins isolated from Turkish species (*Astragalus brachypterus*, *Astragalus cephalotes*, *Astragalus microcephalus*, and *Astragalus trojanus*), as well as methanol extracts from the roots of three *Astragalus* species (*Astragalus cephalotes*, *Astragalus oleifolius* and *Astragalus trojanus*). Cytokine concentrations of interleukins IL-1, IL-8 and TNF- α after bacterial lipopolysaccharide (LPS) and IL-2, IL-4 and INF- γ after phorbolacetate (PHA) stimulation were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits. All triterpene saponins tested in the present study (brachyoside A, brachyoside C, cyclocanthoside E, cycloastragenol, brachyosideB, cyclocephaloside II, astragaloside I, astragaloside II, astragaloside I, astragaloside VII, trojanoside A, trojanoside H, cyclocephaloside I, astrojanoside A: 3-*O*-(α -L-rhamnopyranosyl-(1-2)- β -D-xylopyranosyl-(1-2)- β -D-glucuronopyranosyl)-29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24,29-tetrahydroxy-olean-12-en showed a prominent IL-2 inducing activity between 35.9% and 139.6%. Among the extracts the highest score was obtained for *Astragalus oleifolius* (141.2%). Glycosides of 20,24-epoxy and 20,25-epoxy cycloartanes showed higher IL-2 inducing activity than those of acyclic-cycloartane derivatives as well as aglycone of 20,24-epoxy cycloartanes, cycloastrogenol. Especially the activity of astragaloside VII, a tridesmosidic glycoside of cycloastrogenol, was the most remarkable. The oleanane-type triterpene saponin also showed a prominent IL-2 inducing activity. IL-2 is a cytokine produced by activated T cells, which has

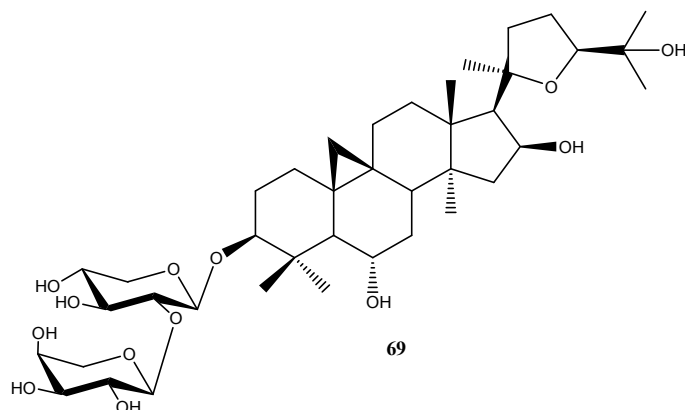
shown powerful immunostimulatory and antineoplastic properties (Yeşilada et al., 2005).

A new cycloartane-type triterpene glycoside, (20*R*,24*S*)-3-*O*-[β-D-apiofuranosyl-(1→2)-β-D-xylopyranosyl]-6-*O*-β-D-glucopyranosyl-3β,6α,16β,25-tetrahydroxy-20,24-epoxycycloartane, named baibutoside (**68**), has been isolated from the roots of *Astragalus baibutensis* (Sect. *Pterophorus*) along with four known glycosides, acetylastragaloside I, and astragalosides I, II, and IV. The antiprotozoal activities of the compounds **64–68** were assessed on a panel of parasites, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum*. All compounds were also tested on primary L6 mammalian cells (rat skeletal myoblasts), in order to determine their selective cytotoxicity. All compounds were devoid of activity against *L. donovani* and *P. falciparum*. Except for **68**, all pure metabolites displayed some growth inhibitory effect on *Trypanosoma brucei rhodesiense*, with acetylastragaloside I (**64**) being the most active one (IC₅₀ 9.5 μg/ml). Compound **64** also revealed promising activity against *T. cruzi*, with an IC₅₀ value of 5.0 μg/ml. On the other hand, acetylastragaloside I (**64**) possessed cytotoxic effect on mammalian cells, leading to narrow selectivity index values of 2.5 and 4.8 (Çalış et al., 2006).

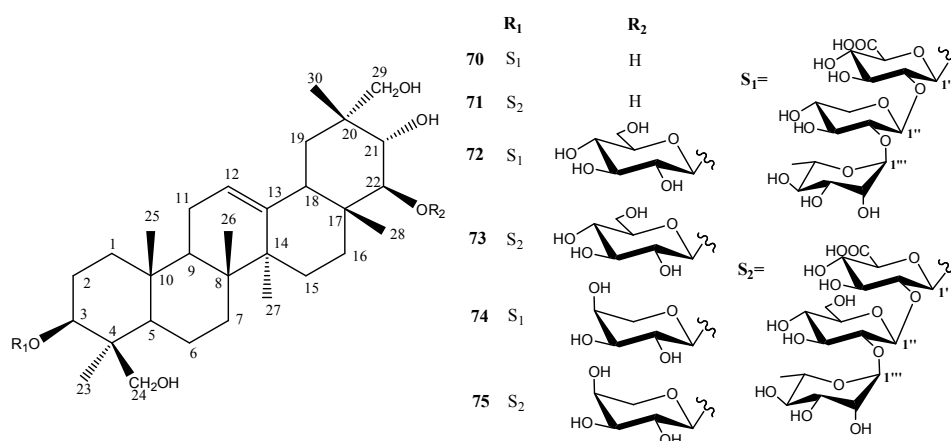


Together with two known cycloartane-type glycosides, askendosides D (3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-6-*O*-β-xylopyranosyl-cycloastragenol) and G (3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-16-*O*-β-glucopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane), also a new monodesmosidic cycloartane-type glycoside, elongatoside (**69**), was isolated from the roots of *Astragalus elongatus* (Sect. *Proselius*) and identified as 3-*O*-[α-

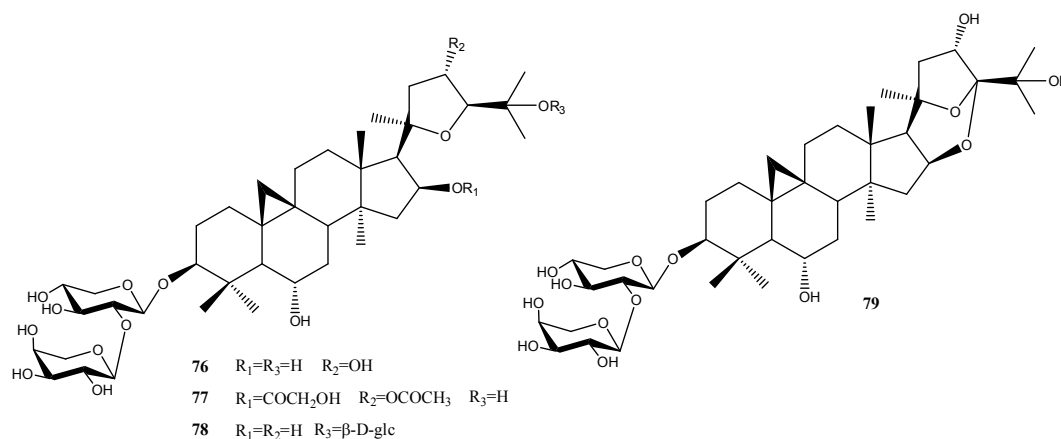
arabinopyranosyl-(1→2)-β-xylopyranosyl]-cycloastragenol. The isolated compounds were tested for the inhibition of proliferation and ICAM-1 expression in vitro using the human microvascular endothelial cell line (HMEC-1). Compound **69** showed weak activity in the ICAM-1 assay (Çalış et al., 2008).



In 2008, six new triterpene saponins, 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-21-epi-kudzusapogenol A (**70**), 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-21-epi-kudzusapogenol A (**71**), 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-22-*O*- β -D-glucopyranosyl-21-epi-kudzusapogenol A (**72**), 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-22-*O*- β -D-glucopyranosyl-21-epi-kudzusapogenol A (**73**), 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-22-*O*- α -L-arabinopyranosyl-21-epi-kudzusapogenol A (**74**), and 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl-22-*O*- α -L-arabinopyranosyl-21-epi-kudzusapogenol A (**75**), were isolated from the roots of *Astragalus flavescens* (Sect. Eustales), together with the known trojanoside B, azukisaponin V, and astragalosides IV, VII, and VIII (Avunduk et al., 2008a).

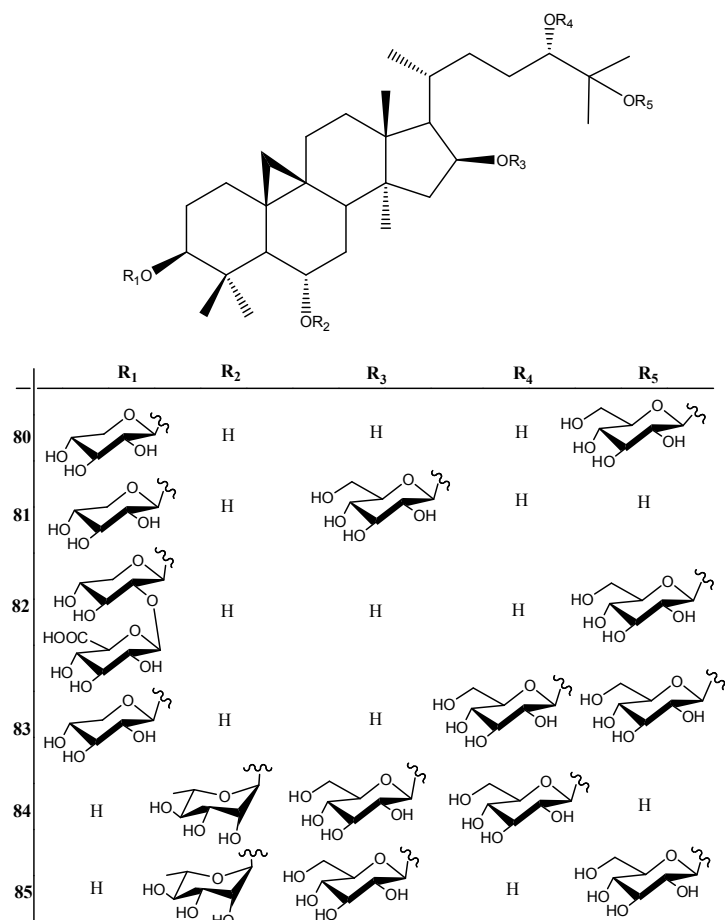


Another phytochemical work was performed in 2008 and four new cycloartane glycosides, 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-3 β ,6 α ,16 β ,23 α ,25-pentahydroxy-20(*R*),24(*S*)-epoxycycloartane (**76**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-16-*O*-hydroxyacetoxy-23-*O*-acetoxy-3 β ,6 α ,25-trihydroxy-20(*R*),24(*S*)-epoxycycloartane (**77**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-3 β ,6 α ,23 α ,25-tetrahydroxy-20(*R*),24(*R*)-16 β ,24;20,24-diepoxycycloartane (**78**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-25-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**79**), along with three known cycloartane glycosides; 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane, askendoside C and askendoside G were isolated from the MeOH extract of the roots of *Astragalus campylosema* ssp. *campylosema* (Sect. *Hololeuce*) (Çalış et al., 2008b).

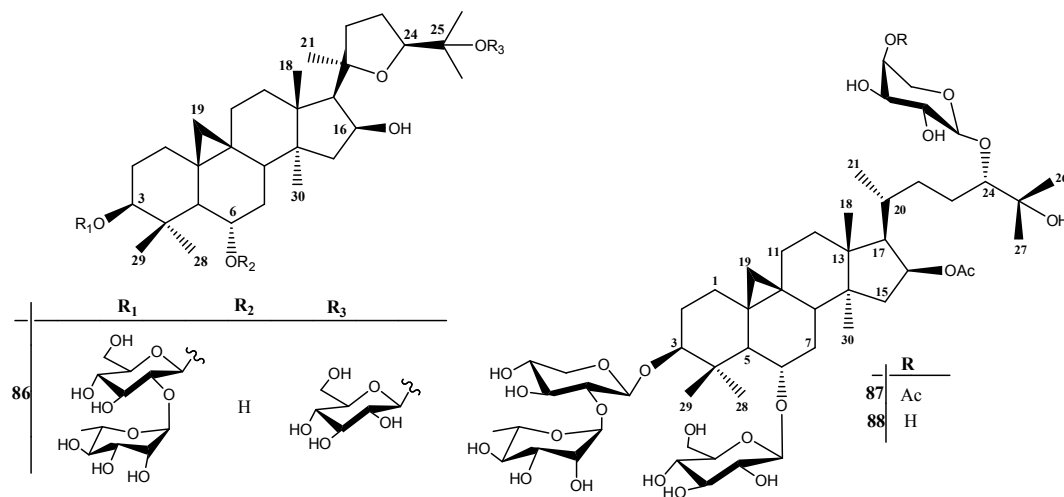


Five new cycloartane-type triterpene glycosides were isolated from the methanol extract of the roots of *Astragalus amblolepis* Fischer (Sect. *Rhacophorus*) along with one known saponin, 3-*O*- β -D-xylopyranosyl-16-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy-cycloartane (**85**). Structures of the new compounds were established as 3-*O*- β -D-xylopyranosyl-25-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**80**), 3-*O*-[β -D-glucuronopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-25-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy-cycloartane (**81**), 3-*O*- β -D-xylopyranosyl-24,25-di-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy-cycloartane (**82**), 6-*O*- α -L-rhamnopyranosyl-16,24-di-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy-cycloartane (**83**), 6-*O*- α -L-rhamnopyranosyl-16,25-di-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy-cycloartane (**84**) by using 1D and 2D-NMR techniques and mass spectrometry. In this study,

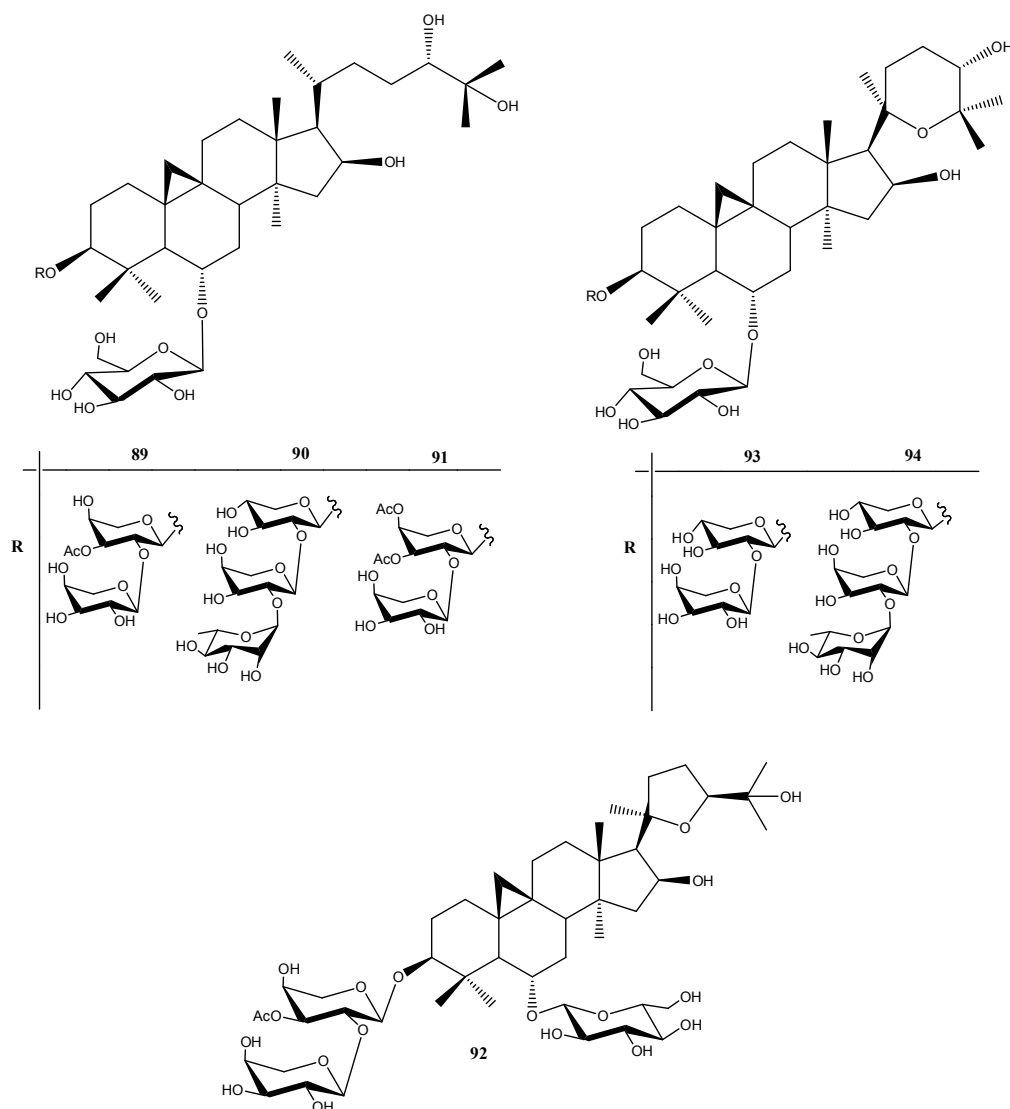
the glucuronic acid moiety in cycloartanes is reported for the first time (Polat et al., 2009).



In 2010, three cycloartane-type triterpene glycosides were isolated from *Astragalus wiedemannianus* (Sect. Pterophorus) together with eight known secondary metabolites namely cycloastragenol, cycloascauloside B, astragaloside IV, astragaloside VIII, brachyoside B, astragaloside II, astrachrysoside A, and astrasieversianin X. The structures of the new compounds were established mainly by a combination of 1D and 2D-NMR techniques as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-25-*O*- β -D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3 β ,6 α ,16 β ,25-tetrahydroxycycloartane (**86**), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-24-*O*- α -(4'-*O*-acetoxy)-L-arabinopyranosyl-16-*O*-acetoxy-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**87**), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-24-*O*- α -L-arabinopyranosyl-16-*O*-acetoxy-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**88**). The presence of an arabinose moiety on the acyclic side chain of cycloartanes is reported for the first time (Polat et al., 2010).



Six new cycloartane-type triterpene glycosides were isolated from *Astragalus icmadophilus* (Sect. Acanthophace) along with two known cycloartane-type glycosides, five known oleanane-type triterpene glycosides and one known flavonol glycoside. The structures of the new compounds were established as 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*-3-acetoxy- α -L-arabinopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**89**), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*- α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxy cycloartane (**90**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*-3,4-diacetoxy- α -L-arabinopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**91**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*-3-acetoxy- α -L-arabinopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**92**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(*R*),25-epoxycycloartane (**93**), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*- α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(*R*),25-epoxycycloartane (**94**) by the extensive use of 1D- and 2D-NMR experiments along with ESIMS and HRMS analysis. The first four compounds are cyclocanthogenin and cycloastragenol glycosides, whereas the last two are based on cyclocephalogenin as aglycone, more unusual in the plant kingdom, so far reported only from *Astragalus* spp. Additionally, two known cycloartane-type glycosides, oleifolioside B and astragaloside I, five known oleanane-type triterpene glycosides, azukisaponin V, azukisaponin V methyl ester, astragaloside VIII, astragaloside VIII methyl ester, 22-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -L-arabinopyranosyl]-3 β ,22 β ,24-trihydroxy-olean-12-ene and the flavonol glycoside narcissin were isolated. (Horo et al., 2010).



In another study; the hexane extracts of six *Astragalus* species (*A. atrocarpus*, *A. cristianus*, *A. galegiformis*, *A. macrocephalus*, *A. pinetorum* and *A. psoroloides*), which are grown in Eastern Anatolia, were isolated, and their mutagenic and antimutagenic properties were investigated by using *Salmonella typhimurium* TA1535, TA1537 and *Escherichia coli* WP2uvrA tester strains at 0.05, 0.5 and 5 $\mu\text{g}/\text{plate}$ concentrations. Known mutagens sodium azide (NaN_3), 9-Aminoacridine (9-AA) and N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) were used to determine antimutagenic properties of hexane extracts. The results showed that all hexane extracts, investigated in the present study, can be considered genotoxically safe because they do not have mutagenic activity at the tested concentrations. But, a great many of them have antimutagenic activity against 9-Aminoacridine known as a model intercalator agent. The inhibition rates obtained from the antimutagenicity assays ranged from 27.51% (*A. macrocephalus*–0.05 $\mu\text{g}/\text{plate}$) to 54.39% (*A. galegiformis*–5 $\mu\text{g}/\text{plate}$) (Güllüce et al., 2010).

In 2011, from the roots of *A. ptilodes* Boiss. var. *cariensis* Boiss. (Sect. *Pterophorus*), five known compounds; cyclosiversiosides E ($3\beta,6\alpha,16\beta,20R,24S$)-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostane-3,6-diyl bis[β -D-xylopyranoside]; 15.2 mg) and F ($3\beta,6\alpha,16\beta,20R,24S$)-20,24-epoxy-16,25-dihydroxy-3-(β -D-xylopyranosyloxy)-9,19-cyclolanostan-6-yl β -D-glucopyranoside; 10.1 mg), and astragaloside I ($3\beta,6\alpha,16\beta,20R,24S$)-3-[(2,3-di-*O*-acetyl- β -D-xylopyranosyl)oxy]-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostan-6-yl β -D-glucopyranoside; 11.8 mg) and cyclosiversioside A ($3\beta,6\alpha,16\beta,20R,24R$)-3-[(2,3-di-*O*-acetyl- β -D-xylopyranosyloxy)-20,24-epoxy-16,25-dihydroxy-9,19-cyclolanostan-6-yl β -D-xylopyranoside; 7.3 mg) were isolated (Linnek et al., 2011).

In 2011, another study was carried out for determination of in vitro growth stimulatory and in vivo wound healing properties of four cycloartane type saponins that are present in Turkish *Astragalus* species as major chemical entities (astragaloside IV, cycloastragenol, cyclocephaloside I and cyclocanthoside E). The obtained results indicated that cycloartane type saponins of *Astragalus* genus are capable of promoting wound healing based on proliferation and migration in scratch assay, proliferation in MTT assay, and in vivo wound model study. Although all the compounds increased both fibroblast proliferation and migration, the effects are most prominent for cycloastragenol (CA), astragaloside IV (AG) and cyclocanthoside E (CCE). The effect on migration was outstanding for CA at 1 ng/ml, whereas AG and CCE had their highest activities at 10 ng/ml. Parallel to above mentioned results, the highest proliferation rates at 10 ng/ml for AG and CCE, and 1 ng/ml for CA were observed in MTT assay. The results also showed that the topical treatments of *Astragalus* cycloartanes improve healing of subsequently induced abrasion skin wounds in rats compared to the control. At the end of 14 days treatment period, 5% CA preparation was found to be the most remarkable in the treated skin. Histological findings also showed that the CA treated group had a greater cell density, more regularly organized dermis (linear alignment) and more newly formed blood vessels compared to the other groups. Wound healing, cell proliferation and migration effects of the compounds were shown in **Fig.1.4**. (Sevimli-Gür et al., 2011).

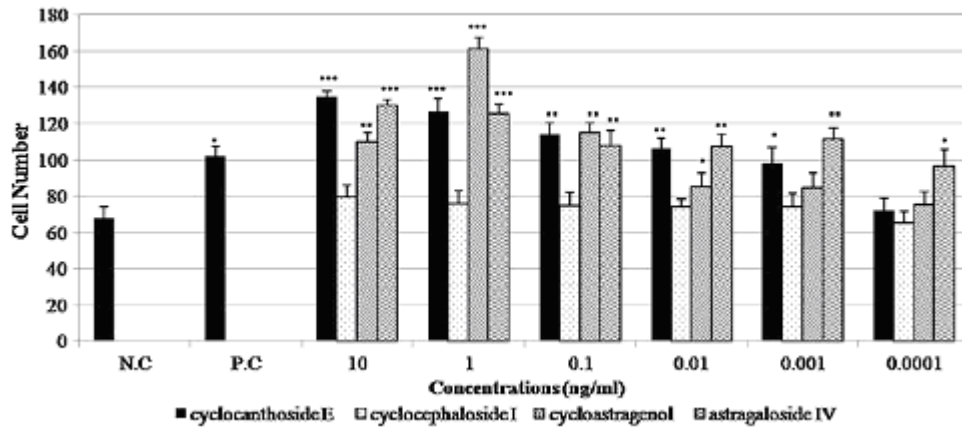
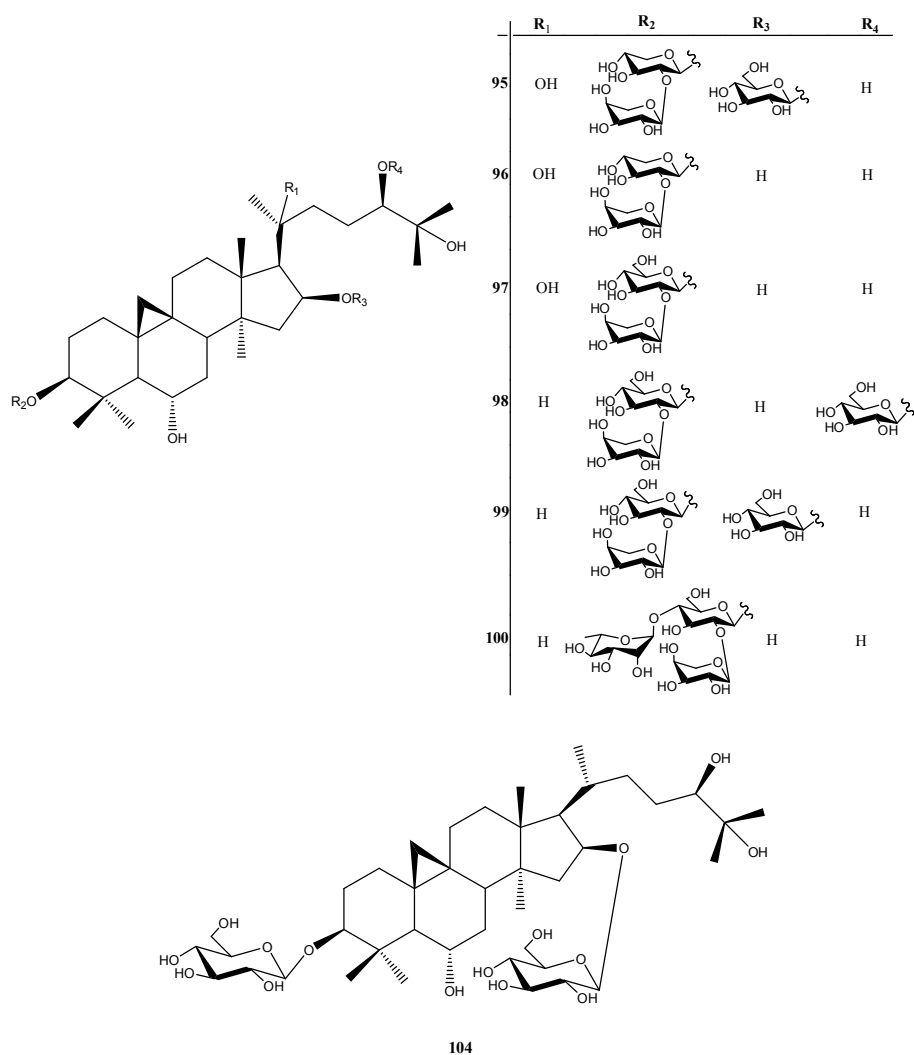


Figure 1.4. Wound healing, cell proliferation and migration effects of the compounds. As negative control group (NC) DMEM.HAM's/F- 12 medium with 0.1% DMSO, and as the positive control group (PC) 10 ng/ml of EGF were used. The data were obtained from three independent assays using three wells for each assay (mean-+SE). The resulting data were subjected to two-tailed paired t-test for statistical significance. * $p < 0.04$, ** $p < 0.001$, *** $p < 0.001$ versus the control (Sevimli-Gür et al., 2011).

The hemolytic activities of two immunomodulator *Astragalus* saponins Macrophyllosaponin B (MacB) from *Astragalus oleifolius* DC and astragaloside VII (Ast VII) from *Astragalus trojanus* Stev., and their adjuvant potentials on the cellular and humoral immune responses of Swiss albino mice against BSA were evaluated. The hemolytic activity of Mac B and Ast VII was determined using 0.5% rabbit red blood cell. For adjuvant activity, Swiss albino mice were immunized subcutaneously with BSA 100 μ g alone or with BSA 100 μ g dissolved in saline containing Ast VII (30, 60, 120 and 240 μ g), Mac B (30, 60, 90 and 120 μ g) or Freund's adjuvant on Days 1 and 15. Sera and splenocytes were collected 2 weeks after the last immunization for concanavalin A (Con A)-, lipopolysaccharide (LPS)- and BSA-stimulated splenocyte proliferation assay and measurement of BSA-specific antibodies in serum. Mac B and Ast VII showed a slight hemolytic effect, with 0.42% and 0.54% values, respectively, at the highest concentration of 500 μ g/ml. Mac B and Ast VII significantly enhanced the Con A-, LPS-, and BSA-induced splenocyte proliferation in the BSA-immunized mice especially at 120 and 240 μ g ($P < 0.001$), and 60, 90 and 120 μ g ($P < 0.05$, $P < 0.01$ or $P < 0.001$) doses, respectively. BSA-specific IgG, IgG1 and IgG2b antibody titers in serum were also significantly enhanced by Ast VII (120 μ g), Mac B (90 μ g) and Freund's as compared to the control group ($P < 0.01$ or $P < 0.001$). Moreover, the IFN- γ and IL-4 levels in the sera were detected using

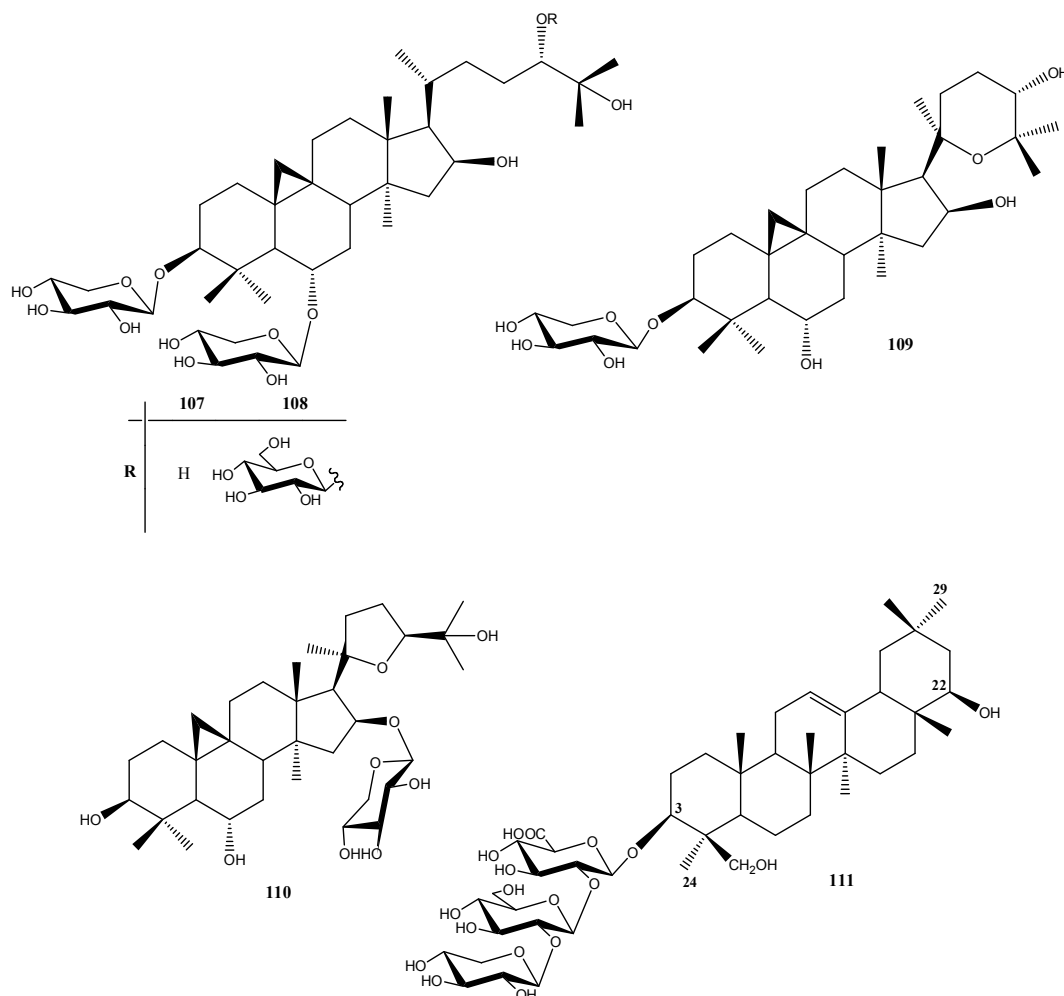
ELISA two weeks after the last immunization. Ast VII and Mac B were also found to stimulate IFN- γ production such as Freund's, two weeks after the last immunization at doses of 120 μ g and 90 μ g, respectively, as compared to the control. Results showed that Ast VII and MacB generate important specific antibody and cellular response against BSA in mice, proving their potentials as a new class saponin adjuvant (Nalbantsoy et al., 2011).

Studies on *Astragalus stereocalyx* Bornm (Sect. *Stereocalyx*) resulted isolation of six new cycloartane-type triterpene glycosides. The structures of new compounds determined as 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-16-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,20(*S*),24(*R*), 25-hexahydroxycycloartane (**95**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-3 β ,6 α ,16 β ,20(*S*),24(*R*),25-hexahydroxycycloartane (**96**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-3 β ,6 α ,16 β ,20(*S*),24(*R*),25-hexahydroxycycloartane (**97**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*R*),25-pentahydroxycycloartane (**98**), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-16-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*R*),25-pentahydroxycycloartane (**99**) and 3-*O*-{ α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)]- β -D-glucopyranosyl}-3 β ,6 α ,16 β ,24(*R*),25-pentahydroxycycloartane (**100**). Additionally, six known cycloartane-type glycosides, askendoside C (**101**), askendoside F (**102**), askendoside G (**103**), 3-*O*- β -D-glucopyranosyl-16-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*R*),25-pentahydroxycycloartane (**104**), elongatoside (**105**) and trojanoside H (**106**) were isolated. The antiproliferative activity of compounds **95–106** was tested in different cancer cell lines including HeLa (human cervical cancer), HT-29 (human colon cancer), U937 (human leukemia) and H446 (human lung cancer). Compounds **95–106** were tested in a range of concentrations between 1 and 50 μ M, but only few compounds showed a weak activity and only HeLa cell line was responsive to the treatment of some analyzed compounds. In particular, compound **104** exhibited an IC₅₀ value of 10 μ M against HeLa cells, while compounds **100**, **101** and **103** exhibited IC₅₀ values of 29.9, 31.5 and 24.4 μ M, respectively. For all the other compounds higher IC₅₀ values were observed (Yalçın et al., 2012).



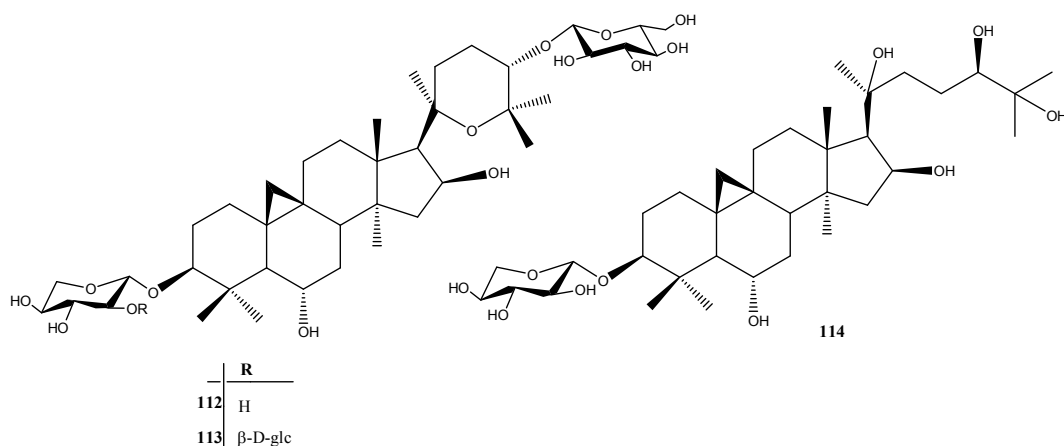
Another phytochemical study was performed on *Astragalus hareftae* (Sect. Acanthophae) which resulted in isolation of four cycloartane-(hareftosides A–D) and oleanane-type triterpenoids (hareftoside E) along with eleven known compounds. Structures of the compounds were established as 3,6-di(*O*- β -D-xylopyranosyl)-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**107**), 3,6,24-tri(*O*- β -D-xylopyranosyl)-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**108**), 3-*O*- β -D-xylopyranosyl-3 β ,6 α ,16 β ,25-tetra-hydroxy-20(*R*),25(*S*)-epoxycycloartane (**109**), 16-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**110**), 3-*O*-[β -D-xylopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-glucuronopyranosyl] soyasapogenol B (**111**) by the extensive use of 1D- and 2D-NMR experiments along with ESI-MS and HR-MS analyses. Known compounds were identified as cyclocanthoside E, macrophyllsaponin B, 3-*O*- β -D-xylopyranosyl-6,25-di-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane, oleifolioside B, cyclocephaloside I, astrasieversianin X, trojanoside B, cycloastragenol, astragaloside IV, brachyoside B, cyclodissectoside, and four known oleanane-type triterpene glycosides,

azukisaponin V, dehydroazukisaponin V, wistariasaponin D, astragaloside VIII (Horo et al., 2012).



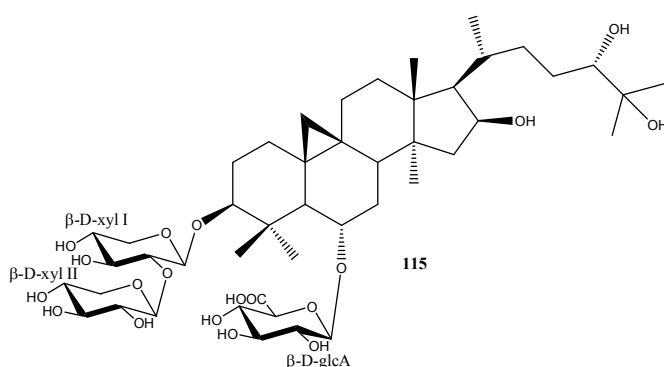
Phytochemical investigation on the CH_2Cl_2 extract of *Astragalus tmoleus* Boiss var. *tmoleus* (Sect. Pterophorus) led to the isolation and characterization of three known cycloartane-type saponins, astragaloside IV, cyclocephaloside II and astrasieversianin I by using various solid/liquid chromatographic techniques (Avunduk et al., 2012).

Three new cycloartane-type triterpene glycosides were isolated from the roots of *Astragalus schottianus* Boiss. (Sect. Rhacophorus). Their structures were established as 20(*R*),25-epoxy-3-*O*- β -D-xylopyranosyl-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxycycloartane (**112**), 20(*R*),25-epoxy-3-*O*-[β -D-glucopyranosyl(1 $\rightarrow$ 2)]- β -D-xylopyranosyl-24-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxycycloartane (**113**), 3-*O*- β -D-xylopyranosyl-3 β ,6 α ,16 β ,20(*S*),24(*S*),25-hexahydroxycycloartane (**114**) by the extensive use of 1D and 2D-NMR techniques and mass spectrometry (Karabey et al., 2012).



Four known cycloartane-type saponins; trojanoside H, astragaloside IV, astragaloside VIII and astrasieversianin X were isolated from the whole plant of *Astragalus pycnocephalus* var. *pycnocephalus* (Sect. Rhacophorus) and their inhibitory effects against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) were investigated (Koz et al., 2012).

Phytochemical investigation of *Astragalus erinaceus* (Sect. Rhacophorus) was resulted in isolation of a new cycloartane-type saponin, 3-*O*-[β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-glucuronopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**115**). Additionally, five known saponins cyclodissectoside, cycloastragenol, 6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane, oleifolioside B and 3,6-di-*O*- β -D-xylopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane were also identified (Savran et al., 2012).



1.4. Biological and Pharmacological Properties of *Astragalus* ssp.

1.4.1. Hepatoprotective and Antioxidative Effects of *Astragalus*

The hepatoprotective properties of *Astragalus membranaceus* extracts have been widely studied and, in some cases, the active principles have been described. Research has shown that its antioxidative properties can prevent liver damage. Studies on animals with toxic hepatic injury induced by CCl₄, indicates that *A. membranaceus* root extract prevents a decrease in hepatic glycogen content and raises the levels of total serum protein and albumin (Tang and Eisenbrand, 1992).

Han et al. (1989) studied the protective effect of *A. membranaceus* root extract against liver damage, using a model in which rats are exposed to a noise of 2 KHz/103 dB for one hour, and reported that an alteration in hepatic glycogen was a determining factor in liver damage. They observed a decrease in values of hepatic glycogen in the pretreated group in relation to the control one.

Zang et al. (1990) assayed the protective effect of the same drug after oral administration in a stilbenemide induced liver damage model. The ethanol extract of the roots reduced the alanine transaminase (SGPT) levels and the subacute toxicity of stilbenemide decreased. In 1992, Zang et al. reported the liver protective effects of the saponins isolated from *A. membranaceus* and *A. sieversianus* against chemical injury induced by CCl₄, D-galactosamine and acetaminophen in mice. In all cases, there were positive activities, and the saponins inhibited the rise in SGPT levels, decreased the malondialdehyde (MDA) content and increased the glutathione reduced (GSH) concentration in mouse liver.

Wang and Han (1992) studied a polysaccharide isolated from *A. mongholicus* as a possible hepatoprotector principle on *E. coli* endotoxin-induced liver damage in a mouse model. This polysaccharide increased the rate of survival in mice after endotoxin administration, and prevented the lowering of ATP levels, and adenylate energy change in mouse liver.

The water extract from *A. membranaceus* inhibited oxygen consumption and MDA production when it was assayed against the lipid peroxidation induced by ADP and FeSO₄ on rat heart mitochondria (Hong et al. 1994).

Sun et al., were carried out a study to investigate the effects of *Paeonia lactiflora* and *Astragalus membranaceus* extract on immunological liver injury in mice induced by *Bacillus Calmette-Guérin* and lipopolysaccharide (BCG/LPS) and to explore a possible mechanism. After administration of *P. lactiflora* and *A. membranaceus* (60, 120 and 240 mg/kg, intragastrically) daily for 10 days, the extract significantly reduced the degree of liver damage in BCG/LPS-induced liver injury, as well as the elevation of serum transaminase activities and level of nitric oxide in live injury mice. The extract also restored the decrease in superoxide dismutase and glutathione peroxidase activities and inhibited the formation of lipid peroxidative products. Moreover, *P. lactiflora* and *A. membranaceus* (60, 120 and 240 mg/kg, intragastrically) repressed high levels of tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) from peritoneal macrophages. In the primary cultured Kupffer cells, *P. lactiflora* and *A. membranaceus* also significantly decreased the production of TNF- α and IL-1 in cells stimulated with LPS (5 μ g/ml). These results suggested that *P. lactiflora* and *A. membranaceus* have a protective effect on BCG/LPS-induced liver injury mice, which might be associated with the antioxidant properties, ability to reduce nitric oxide production and suppression of Kupffer cell activity and pro-inflammatory mediator and cytokines production (Sun et al., 2008).

A synergistic hepatoprotective effect has been also revealed in combined use of *Astragalus* polysaccharides and *Schisandrae* lignans in an animal model (Yan et al., 2009a). Studies showed that Astragaloside IV, a purified saponin from *A. membranaceus*, has a protective effect on hepatic stellate cells (Liu et al., 2009) and antihepatitis B virus activities (Wang et al., 2009a).

The hepatoprotective and antioxidant effects of *Astragalus* polysaccharide (APS) on the carbon tetrachloride (CCl₄)- induced hepatocyte and liver injury in common carp in vitro and in vivo were investigated. In vitro, APS (200, 400 and 800 μ g/ml) was added to the carp primary hepatocytes before (pretreatment), after (post-treatment) and both before and after (pre- and post-treatment) the incubation of the hepatocytes with CCl₄ at 8 mM in the culture medium. APS at concentrations of 200, 400 and 800 μ g/ml significantly improved cell viability and inhibited the elevation of glutamate pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), lactate dehydrogenase (LDH) and malondialdehyde (MDA) and significantly increased the reduced level of superoxide dismutase (SOD). In vivo administration of APS at the doses of 1.5 and 3 g/kg in the diet for 60 days prior to CCl₄ intoxication significantly reduced the elevated activities of

GPT, GOT and LDH and increased the reduced levels of total protein and albumin in the serum; meanwhile, the reduced levels of SOD, glutathione and total antioxidant capacity (T-AOC) were markedly increased and the MDA formation was significantly inhibited in liver tissue. Overall results proved the hepatoprotective action of APS, which is likely related to its antioxidant activity. The results support the use of APS as a hepatoprotective and antioxidant agent in fish (Jia et al., 2012).

1.4.2. Effects of *Astragalus* subspecies on Immune System

A modification of the immune response could be responsible for the antiviral and anticancer activity of *A. membranaceus*. Studies have shown that astragalan I and II, the main constituents of the water extract from this species, potentiated the immunological response in mice after i.p. administration. They increased the weight and cell number of mouse spleen, elevated the response of mouse spleen against sheep red blood cells, and stimulated the phagocytic activity of peritoneal macrophages. When the compounds were given i.v. or p.o., the phagocytic fraction of peritoneal macrophages did not change (Tang and Eisenbrand, 1992).

The immunostimulant activity of *A. membranaceus* was studied by Sun et al. (1983a, b) using the local graft-versus host (GVH) reaction as a test assay for T-cell function, with samples from cancer patients and healthy donors. In a second test they used mononuclear cells of normal subjects, cancer and immunodepressed patients to study the effects on lymphocyte blastogenic responses. The results obtained made it possible to hypothesize that *A. membranaceus* aqueous extract must contain immunomodulatory principles. Five years later, Chu et al. (1988 a, b, c) fractionated and purified the immunostimulant extract using a Sephacryl S-200 column, and screened the activity of the different fractions by studying their effects on mononuclear cells derived from healthy normal donors, employing the local xenogeneic GVH reaction. Fraction 3 (F3), with an estimated molecular weight of 20.000 to 25.000, was the most active, with a potent immunorestorative activity in vitro. The activity was ratified in vivo, using cyclophosphamide-primed rats. After injection of 5.55 mg daily for 5 days, the cyclophosphamide-induced immunosuppression was completely reversed. The active fraction improved the response of lymphokine-activated killer (LAK) cell precursors, when it was applied together with the recombinant interleukin-2 (rIL-2). Finally the authors

propose the use of *A. membranaceus* plus rIL-2 to reduce the dose of this compound alone, with the consequent reduction in toxicity.

Wang et al. (1992) studied the synergism *A. membranaceus* extract on the cytotoxicity of rIL-2 generated cells against murine renal cell carcinoma and reported the same results as Chu et al. (1988c). In this research, the active fractions were purified by high-performance liquid chromatography. Similar results were obtained by Lau et al. (1994) when the capacity of *A. membranaceus* extract to inhibit the growth of murine cell carcinoma in vivo was studied. An investigation of the mechanisms involved in this action showed that splenocytes from mice transplanted with murine renal cell carcinoma responded less favourably to IL-2 in generating LAK cells, but this depression was restored with *A. membranaceus* administration. In conclusion, they suggest that this crude drug may exert the antitumor effect via augmentation of phagocyte and LAK activities.

The immunostimulant properties of the polysaccharide obtained from *A. membranaceus* has been studied by other researchers. D. C. Wang et al. (1989) used a local GVH reaction and the blastogenic response of lymphocytes in vitro as a test index for T-cell function. Samples were obtained from normal healthy donors and untreated patients with advanced cancer. Dose-dependent effects of polysaccharide were observed on the concavallin A and phytohaemagglutinin responses, and cell proliferation was inhibited at higher concentrations. They concluded that the mechanism of immunostimulation could be related to the suppressive T-cell activity. However, J. Wang et al. (1989) hypothesized that the immunostimulant activity of the *A. membranaceus* polysaccharide was related to the activity of the mouse complement C3. The effect in this case was investigated by immunofluorescence of the third component of the complement cleavage in mouse macrophages.

A. membranaceus extracts injected into normal, radiation and cyclophosphamide-immunodepressed treated or aging mice were able to enhance the antibody response to a T-dependent antigen. The activity was dependent on the carbohydrate content of the extract (Zhao et al., 1990).

A. membranaceus and *A. mongholicus* extracts enhanced the phagocytic function of the endothelial reticula and stimulated the immunological activities in normal and immunodepressed mice (Kang et al., 1989). Tomoda et al. (1992) isolated a glycan from the hot water extract of *A. membranaceus* roots and studied

its reticuloendothelial system potentiating activity in the carbon clearance system, where a net positive effect was demonstrated.

The polysaccharide from *A. mongholicus* administered i.p. to mice increased the immune response, and the amount of RNA in the spleen, but had no effect on thymus, heart or brain RNA, or on DNA metabolism (Tang and Eisenbrand, 1992), explained by immunological mechanisms on the basis of the results obtained in a study with urological neoplasm cells and bladder murine carcinomas. Rittenhouse et al. (1991) reported that *A. membranaceus* may exert its antitumor activity by abolishing tumor-associated macrophage suppression. The potentiation of the natural killer cytotoxicity of peripheral blood mononuclear cells in patients with systemic lupus erythematosus was demonstrated by Zhao (1992) using an enzyme-release assay. The activity was increased in the samples of healthy donors and patients with the pathology. The release of a natural killer cytotoxic factor by peripheral blood mononuclear cells was higher in the control group, and the levels of that factor correlated well with natural killer activity, and correlated negatively with the clinical effect.

In research on the secretion of tumor necrosis factors (TNF), astragalin increased the secretion of TNF- α and TNF- β from adherent and nonadherent human peripheral blood mononuclear cells in vitro (Zhao and Kong, 1993).

The results of studies on other species of *Astragalus* agree with those obtained for *A. membranaceus*. Li (1991) reported the immunomodulatory effect of saponins and polysaccharides isolated from *A. mongholicus*. Shimizu et al. (1991) isolated an acidic polysaccharide from the same species and demonstrated the reticuloendothelial system potentiating activity on a carbon clearance test. *A. propinquus* increased the production of IL-2 and IL-6 from spleen cells of autoimmune depressed mice and were able to reduce the level of TNF from their spleen cells (Chen et al., 1991).

Swainsonine obtained from *A. lentiginosus* increased lymphokine-activated killer-cell induction at suboptimal doses of IL-2. This alkaloid is an inhibitor of α -mannosidase II that causes accumulation of hybrid-type oligosaccharide-containing glycoproteins in mammalian cells, but the amount of swainsonine required to increase the LAK activity is 100–1000 times larger than the amount required to inhibit enzyme activity (Kang et al., 1993).

A. membranaceus extract (AME) had effect on enhancing human immuno-function and antitumor activity. It could be applied in clinical practice for immunomodulation and tumor treatment. AME could promote the proliferation of human peripheral blood mononuclear cells (PBMC), elevate the tumor cell killing activity of T-lymphocyte (CTL), strengthen the tumor cell phagocytosis and cytokines (TNF- α and IL-6) production of PBAM, and promote the IgG production of peripheral blood B cells (PBBC) (Wang et al., 2002, Liu et al., 2011).

Research shows *Astragalus* root stimulates the immune system in many ways. It increases the number of stem cells in bone marrow and lymph tissue and encourages their development into active immune cells. It appears to help trigger immune cells from a “resting” state into heightened activity. One study showed *Astragalus* root helps promote and maintain respiratory health. It also enhances the body’s production of immunoglobulin and stimulates macrophages. *Astragalus* can help activate T-cells and natural killer (NK) cells. In mice, oral doses of *Astragalus* enhanced several aspects of immunity, including superoxide anion production by peritoneal macrophages, potentiation of phagocytic function, increased thymus weight, and proliferation of splenocytes. *Astragalus* stimulated NK-cell activity of human peripheral blood lymphocytes and restored steroid-inhibited NK-cell activity (Monograph, 2003).

Kang et al. (2004) examined the effect of an Astragali Radix (AR), ethanol extract on both in vitro and in vivo murine CD4 T cells’ differentiation into Th1 and Th2 subsets. CD4 T cells from Balb/c mice were activated with anti-CD3/anti-CD28 mAb in the presence of AR for 2 days. AR treated cells showed an elevated level of IL-4 but a reduced level of IFN- γ secretion. In addition, in vitro Th1/Th2 polarization experiments revealed that AR enhanced the levels of IL-4 in Th2 cells but reduced the levels of IFN- γ in Th1 cells. To elucidate the effects of AR in Th1/Th2 lineage development during in vivo condition, AR was administrated orally to BALB/c mice. The results demonstrated that AR administration significantly increased IL-4 production in both the serum and supernatant of splenocyte culture, while IFN- γ secretion was diminished upon in vivo activation with anti-CD3 antibody. The obtained data clearly indicated that AR selectively alters Th1/Th2 cytokine secretion patterns and provides the pharmacological basis for AR’s clinical applications.

Li et al. (2008), reported that polysaccharopeptide (PSP) combined with APS as a new complex prescription (PSP + APS) could restore the immunological effects against adriamycin (AMD)-induced immunosuppression, such as the subset of leukomonocyte, the expression of IL-2/IL-2R in the spleen, and the thymus index. The results showed the immunomodulatory effects of this new formula (PSP + APS) were better than PSP alone and also could resist immunosuppression induced by AMD (Li et al, 2008).

Recent researches illustrated APS can significantly induce NO production and inducible NO synthase (iNOS) transcription through the activation of NF- κ B /Rel (Lee et al., 2005), increase the expression of IFN- γ , IL-4, IL-6, IL-10, and TLR4 mRNA in mice macrophage and enhance the immune response of bladder epithelial cell (Zhang et al., 2010; Yin et al., 2010a). A neutral homogeneous APS with molecular weight (MW) 3.6×10^4 Da can dose-dependent increase IgA, IgG, and IgM level in rats blood with stomach cancer and also play the renal protect effect on glomerulonephritis rats (Rui et al., 2009; Li et al., 2009) administration of APS containing β -D-(1 $\rightarrow$ 3)-galactans β -D-(1 $\rightarrow$ 6)-galactooligosaccharide side-chains to Peyer's patch cells can increase the production of bone marrow cells-proliferative cytokines (Kiyohara et al., 2010, Zhao et al., 2011).

A. membranaceus polysaccharides (APS) have been reported to have profound immunological functions such as suppressing tumor growth, improving humoral and cellular immunity, and regulating the expression of cytokines. In addition, APS have been shown to enhance the immune response in immunosuppressed mice. Furthermore, evidence has shown that APS are able to modulate mature of dendritic cells (Du et al., 2011).

Qin et al. (2012a) were aimed to determine the function of *A. membranaceus* extract (AME) on the AGE-induced inflammatory response in Ana-1 macrophages. The viability of cells treated with AME or AGEs was evaluated with the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] method. The secretion and mRNA levels of IL-1 β and TNF- α were measured by ELISA and RT-PCR, respectively. The activity of NF- κ B was assayed by EMSA. The phosphorylation of p38 MAPK was assessed by western blotting. The results showed that AME was not toxic to macrophages. The treatment of macrophages with AME effectively inhibited AGE-induced IL-1 β and TNF- α secretion and mRNA expression in macrophages. These effects may be mediated by p38 MAPK and the NF- κ B pathway. The results suggested

that AME can inhibit AGE-induced inflammatory cytokine production to down-regulate macrophage-mediated inflammation via p38 MAPK and NF- κ B signaling pathways and indicate that AME could be an immunoregulatory agent against AGE-induced inflammation in diabetes.

Qin et al. (2012b) were investigated the effects and mechanism of *Astragalus membranaceus* extract (AME) in the macrophage migration and immune response mediator release. The viability of Ana-1 macrophages treated with AME was evaluated by the MTT method. The secretion and mRNA levels of IL-1 β and TNF- α were measured by ELISA and RT-PCR, respectively. Macrophage migration was assayed by transwell assay. The activity of heparanase (HPA) was determined by a heparin-degrading enzyme assay. Results didn't show any toxicity of AME in macrophages. AME increased the activity of HPA, cell migration, mRNA levels and secretion of IL-1 β and TNF- α in macrophages. Pretreatment with anti-HPA antibody reduced cell migration, secretion of IL-1 β and TNF- α did not change the mRNA levels of IL-1 β and TNF- α significantly in AME-treated macrophages. This findings suggested that AME may increase the release of immune response mediator and cell migration via HPA to activate immune response in macrophages.

1.4.3. Antitumor effects of *Astragalus*

The extract of *Astragalus membranaceus* roots (AR) prevented the cytotoxic activity of lymphocytes against YAC-1 cells from the depression by a carcinogen, N-butyl-N'-butanolnitrosoamine (BBN). It also protected the production of interleukin-2 and γ -interferon of lymphocytes from the depression by BBN. The results showed that the extract of AR exerted an anticarcinogenic effect in carcinogen-treated mice through cytotoxic activity and the production of cytokines (Kurashige et al., 1999; Liu et al., 2011).

The inhibition rates of AR on the different cancer cell lines were detected by trypan blue exclusion, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method, and tritium thymidine incorporation assay. Apoptosis was detected by DNA ladder method. The results indicated AR specifically inhibited gastric cancer cells growth in vitro and the mechanism was mainly cytostatic but not cytotoxic or inducing apoptosis (Lin et al, 2003; Liu et al., 2011).

The water extract of AR inhibited the proliferation of human hepatocarcinoma cells, SMMC-7721, and their mitochondria metabolic activity in vitro. Studies also showed this water extract decreased the weight of S180 tumor in tumor-bearing mice, and increased the T/B-lymphocyte ratio and the activity of peritoneal macrophages (Xiao et al, 2004; Liu et al., 2011).

The in vitro and in vivo anti-tumor effects of *A. membranaceus* were investigated. Five bioactive fractions were isolated from the root of *A. membranaceus*, the fraction designated as AI was found to be the most potent among the five fractions with respect to its mitogenicity on murine splenocytes. Besides investigating the cytostatic effect of AI, its activities on macrophage function, tumor necrosis factor production, induction of lymphokine-activated killer cell and tumor cell differentiation were also examined. The macrophage-like tumors and the myeloid tumors were found to be more sensitive to the cytostatic activity of AI, whereas the fibroblast-like tumors and the mouse Ehrlich ascites tumor appeared to be relatively resistant. Moreover, AI could effectively suppress the in vivo growth of syngeneic tumor in mice. Results showed that murine macrophage pretreated with AI had increased in vitro and in vivo cytostatic activities towards MBL-2 tumor. AI could also act as a priming agent for tumor necrosis factor production in tumor-bearing mice. Preincubation of mouse splenocytes with AI could induce in vitro lymphokine-activated killer-like activity towards WEHI-164 cell. Furthermore, AI was able to induce monocytic differentiation of both human and murine cells in vitro. AI administered in vivo could even partially restore the depressed mitogenic response in tumor-bearing mice. Collectively, the results showed that *A. membranaceus* could exhibit both in vitro and in vivo anti-tumor effects, which might be achieved through activating the anti-tumor immune mechanism of the host (Cho and Leung, 2007).

Upon the treatment with astragaloside IV (ASI), human hepatocellular carcinoma HepG2 cells were evaluated for the colonogenic survival and anchorage independent growth. The cellular proteins of treated and untreated cells were resolved by 2D polyacrylamide gel electrophoresis. The protein spots mostly altered by drug treatment were identified by mass spectrometry and subsequently verified by Western blotting using specific antibodies and RT-PCR technique using specific DNA primers. The results showed astragaloside IV attenuated the colonogenic survival and anchorage-independent growth of cancer cells (Qi et al, 2010; Liu et al., 2011).

Auyeung et al. (2012) had reported that *Astragalus* saponins (AST) exert promising anti-tumorigenic effects by suppressing the growth of HT-29 human colon cancer cells and tumor xenograft. Viability of AGS cells was measured by using the MTT reduction method. Western blotting was performed to examine the effect of AST on apoptotic- and cell growth-related protein expression. Effect of AST on cell cycle progression was also evaluated using PI staining. A Matrigel invasion assay was then employed to demonstrate the effect of AST on the invasiveness of gastric cancer cells. The expression of invasion-associated proteins (VEGF and MMPs) was also investigated. The results showed that AST could induce apoptosis in AGS cells by activating caspase 3 with subsequent cleavage of poly(ADP-ribose) polymerase. Besides, cell cycle arrest at the G2/M phase had been observed in AST-treated cells, leading to substantial growth inhibition. The anti-proliferative effect of AST was associated with the regulation of cyclin B1, p21 and c-myc. Results indicate that the number of AGS cells invaded through the Matrigel membrane was significantly reduced upon AST treatment, with concomitant down-regulation of the pro-angiogenic protein vascular endothelial growth factor (VEGF) as well as the metastatic proteins metalloproteinase (MMP)-2 and MMP-9. It was concluded that AST derived from the medicinal plant *Astragalus membranaceus* could modulate the invasiveness and angiogenesis of AGS cells besides its pro-apoptotic and anti-proliferative activities. These findings also suggest that AST has the potential to be further developed into an effective chemotherapeutic agent in treating advanced and metastatic gastric cancers.

1.4.4. Antiviral Properties of *Astragalus*

Many researchers have tried to demonstrate the antiviral activity of *Astragalus*. The best-studied species is again *A. membranaceus*, particularly against Coxsackie viruses. In 1987, Yang et al. reported the effect of *A. membranaceus* on rat beating heart cell cultures infected with the Coxsackie B-2 virus. In similar research, Yuan et al. (1989, 1990) reported the effect of the same species on the electrical activities of neonatal rat myocardial cell cultures infected with the same virus. Evaluation of the cells in the post-infected period showed changes in the beating percentage and cytopathic effect. Alterations in the electrical activities were measured by standard microelectrode techniques. In the infected group the beating percentage had decreased by 24 h, cytopathic effects appeared quickly, and the action potential amplitude, duration and rate of uptake showed a significant decrease between 24 and 96 h. Premature beats, tachycardia

and fibrillation were observed. In the treated group, the beating and the electrical activities were nearly normal, and a decrease in the cytopathic effect was observed in the myocardial cells treated with *A. membranaceus* extract. In conclusion, the authors confirm the protective effect of the drug in vitro against infection by Cocksackie B-2 virus and propose its possible use in prophylaxis and treatment of acute myocarditis caused by this virus.

In an in vivo experimental model, Yang et al. (1990a) evaluated the effectiveness of *A. membranaceus* in inhibiting Cocksackie B-3 virus propagation and in protecting the myocardium in mouse myocarditis. Four-week-old male BALB/c mice were used, and the parameters measured were gross, histopathologic and ultrastructural examinations of infected mice and those treated with *A. membranaceus* groups. It was observed that the severity of the myocardial lesions and the area involved diminished, and virus titre was also smaller in the *A. membranaceus* administered mice. The antiviral effect of this species could be mediated by an immunological response according to Wu et al. (1992). These authors evaluated the effect of *A. membranaceus* on mice with Cocksackie B-3 myocarditis using an antigen specific cytotoxic assay. On the basis of their results, they propose that the antiviral effect of *A. membranaceus* in acute viral myocarditis was mediated by immune reaction through the regulation of T lymphocytes.

Yang et al. (1990b) treated patients suffering from Cocksackie B viral myocarditis with *A. membranaceus* intramuscularly and compared with conventional therapy. They obtained a significant improvement in patients with *A. membranaceus* therapy and titres of IFN- α and IFN- γ increased in comparison with those before therapy. In an in vitro study, Wei et al. (1992) reported the effect of *A. membranaceus* against Cocksackie B-3 and Echo-10 viruses, and the influence of the crude drug on the susceptibility of human heart cells to viral infection. The *A. membranaceus* extract had no direct activity against the virus, but the cells pretreated with the extract before inoculating and treated after viral infection both showed lower susceptibility to the viral infection. They hypothesized that the activity could be mediated by the capacity of *A. membranaceus* extract to induce an enhancement of IFN production in the human myocardial cells. These results seem to be in agreement with the clinical double blind trials done by Qian and Li (1987), who studied the synergism of *A. membranaceus* and IFN as antiviral agents. In further work, the same authors (Qian et al., 1990) reported the synergic effect of *A. membranaceus* and

recombinant IFN- α 1 in the treatment of chronic cervicitis produced by papillomavirus type 16, *Herpes simplex* virus type 2 and cytomegalovirus infections. Wei et al. (1992) reported the effect of *A. membranaceus* against Echo-19 virus in human heart cells, and they suggested that the activity could be mediated by IFN production.

Several other reports about the activity of *A. membranaceus* against different kinds of viral infections have been published. Tang and Xiong (1990) studied the effect against epidemic haemorrhagic fever virus in mice, and they observed that the morbidity and infection rate of the treated mice were lower than in the control group. The effect seemed likely to be mediated through the enhancement of the immunological mechanism of the host because the drug had no effect on the replication of EHF virus in Hero E6 cells.

Abdallah et al. (1993) studied the in vitro anticancer and antiviral activity of triterpene glycosides isolated from *A. spinosus*. One of them, astragaloside II (Fig. 2), exhibited a cytoprotective effect on T-lymphocytes against HIV infection. However, Yao et al. (1992) screened several plant extracts against HIV virus, and obtained negative results in the case of *A. membranaceus*, the species from which Hirotani et al. (1994) isolated a series of astragalosides.

The protective effects of AR by i.g. administration and by ip injection against Japanese encephalitis virus (JEV) infection in mice were investigated. The studies indicated that the protective effect of AR by i.g. administration was based on a non-specific mechanism during the early stage of infection, before shifting to antibody production, and that macrophages played an important role in this resistance to JEV infection, e.g., by inducing the production of active oxygen (Kajimura et al, 1996b). On the other hand, the protective effect of AR was dependent on a non-specific mechanism during the early stage of infection, before it shifted to antibody production, and that peritoneal exudate cell (PEC) played an important role (Kajimura et al, 1996a). Astragalosides and APS inhibited hepatitis B virus (HBV) and the proliferation of human hepatocarcinoma HepG 2.2.15 cells. The expressions of hepatitis B surface antigen and hepatitis B e-antigen in HBVDNA-transfected HepG 2.2.15 cells were also inhibited (Zou et al, 2003). Another study indicated that ASI had the inhibitory effect on HBV replication in vitro with IC₅₀ of 13.2 mg/L. And ASI produced dose-dependent inhibition of HBsAg and HBeAg excreted by HepG 2.2.15 cells (Zhang et al, 2007). In the human HBV-transfected liver cell line HepG2 2.2.15, ASI effectively suppressed

secretion of HBV antigens. The inhibitory activity of ASI on secretion of HBV antigens was more potent than that of Lamivudine (3TC) without significant cytotoxicity. In duck hepatitis B virus (DHBV)-infected ducklings, ASI caused 64.0% inhibition at 120 mg/kg, 49.6% inhibition at 40 mg/kg, and 41.7% inhibition at 10 mg/kg to serum DHBVs and also reduced serum DHBV DNA levels. The results demonstrated that ASI possessed potent anti-HBV activity (Wang et al, 2009a; Liu et al., 2011).

The protective effects of *Astragalus* on the function of sarcoplasmic reticulum calcium ATPase (SERCA2) activity and endothelin system at acute and chronic periods of myocarditis mice induced by CVB3 infection were investigated. *Astragalus* feeding (2.2 mg/kg/day) could significantly increase the survival rate, alleviate pathological alterations and serum cardiac troponin I (cTnI), as well as restore impaired SERCA activity at the acute stage. Low affinity and capacity of ETR were reversed with *Astragalus* after the first CVB3 inoculation up to 7 days and after the second virus inoculation up to 150 days. In the meantime, the contents of cardiac ET-1 and ANP were reduced. Comparison the myocarditis mice treated with perindopril (0.44 mg/kg/day), an ACE inhibitor, showed that *Astragalus* achieved a similar effect on survival rate, SERCA2 and ET system. These results indicated that the beneficial effects of *Astragalus* and perindopril for treating viral myocarditis might be partly mediated by preserving the functions of SERCA 2 activity and ET system (Chen et al., 2006).

The effectiveness of two months treatment with Astragali compound (AC), containing AR and adjuvant components, was studied for the treatment of chronic viral hepatitis in 116 patients; 92 patients were given other drugs in regular clinical use for viral hepatitis (controls). The clinical efficacy of AC was significantly better in AC-treated patients than in controls. Negative seroconversions of HBV antigen e and HBV DNA were also significantly higher in AC-treated patients than in controls. Of eight duck viral hepatitis B models infected with DHBV and treated with AC, three showed negative seroconversion of DHBV DNA and serum DHBV DNA levels significantly decreased after AC administration compared with the controls; DHBV DNA was negative in biopsied liver tissue by in situ hybridization and immunohistochemistry in two ducks treated with AC. Pathological changes were milder in AC-treated ducks than in controls (Tang et al, 2009; Liu et al., 2011).

1.4.5. Effects of *Astragalus* Cardiovascular and Circulatory Disorders

Radix Astragali, a Chinese medicinal herb, has been widely used for treatment of cardiovascular diseases such as heart failure, angina pectoris, myocardial infarction and stroke in Asian countries (Zhao et al., 2008).

Liao et al., conducted a study to evaluate the efficacy of *A. membranaceus* in the treatment of coronary heart disease (CHD). In combination with *Codonopsis pilosula* (0.5 mg of each per ml, called CP-A), *A. membranaceus* was administered intravenously to 37 patients with CHD and 14 cases of acute myocardial infarction (AMI). Patients were divided into three groups: *Codonopsis pilosula*-*Astragalus* (CP-A) i.v., a dextrose group (20 mL 10% dextrose i.v.) and a Cedelanid-D (0.6 mg i.v. group). The drugs were administered at a single dose. Before treatment and 1 h following, the systolic time intervals (STI) were measured. STI parameters were evaluated as the mean values of five cardiac cycles. The results indicated that following administration of CP-A or Cedelanid-D, there was a significant increase in left ventricular performance and a decrease in PEP and PEP/LVET ratio. This suggests that CP-A exerts a positive inotropic action in patients with CHD, which may be responsible for improving dyspnea and fatigue (Liao et al., 1988).

In a large placebo-controlled clinical study in Beijing, 30 g of *A. membranaceus* was administered with a mixture of other herbs (15 g *C. pilosula*, 15 g *Poligonatum sibiricum*, 30 g *Salvia miltiorrhiza*, 15 g *Paeonia abovata* and 15 g *Curcuma aromatica*) to patients who had suffered AMI. In the 208 cases treated over 29 months, 181 of the patients improved, and 27 died. The mortality rate in the treated group was 13.0% compared to 19.0% for those in the placebo group. Before complementary, traditional, and Western medical treatments were combined in Chinese medical practice, the rate of mortality from AMI in China ranged as high as 33-56.5%. Once traditional techniques were combined, the mortality rate fell rapidly to 14.6% (Guo et al., 1983; McKenna et al., 2002).

The hypotensive effect of *A. membranaceus* was described and its active principle isolated by Hikino et al. (1976). It was identified as gamma-aminobutyric acid (GABA), and these authors observed a good correlation between the GABA content of pharmaceutical preparations and hypotensive activity in rats.

Other researchers have studied the effects of different *Astragalus* compounds against hypertension. Astramembrainnin I (Fig. 3), a saponin isolated from *A. membranaceus* roots, produced a hypotensive effect after i.v. administration to anaesthetized cats and rats (Tang and Eisenbrand, 1992). Castillo et al. (1993a, b) described the antihypertensive properties of 3-NPA isolated from some toxic species. This compound elicited a dose-dependent relaxation of precontracted rabbit aortic rings, which was not affected by the presence of antagonist drugs such as atropine, propranolol or brompheniramine, but was inhibited by methylene blue. Aortic rings precontracted with KCl were less sensitive to relaxation. These results suggest that at least part of the mechanism of vasodilatation is not related to an inhibition of calcium influx through the voltage-dependent Ca^{2+} -channel, nor to blocking a specific set of receptors. In vivo experiments demonstrated the hypotensive effect of 3-NPA in normotensive rats (i.v. acute administration) and renal hypertensive dogs (chronic oral administration). In both cases 3-NPA decreased the blood pressure and provoked bradycardia. The hypotensive effect was independent of the animal species and the administration route employed. On the other hand, 3-NPA had a negative effect on the inotropic and chronotropic properties of isolated guinea-pig auricles and inhibited the increase in contractile force and heart rate elicited by isoproterenol. The hypotensive effect of 3-NPA is probably a consequence of both the vasodilator and cardiodepressor activity. The mechanism of the former could be based on a guanylate cyclase stimulation (methylene blue inhibition), and the cardiac effects may be related to an inhibition of adrenergic mediated responses (isoproterenol antagonism).

From *A. membranaceus*, Wang (1992) obtained a saponin- enriched extract and studied its effect on isolated heart of rats. At doses of more than 50 mg/mL the extract showed a positive inotropic effect, but this turned negative at 30 mg/mL. The mechanism could be related to the cardiotonic glycosides, because the extract's behaviour was similar to that of strophanthidin K. Kudrin et al. (1987) studied the effects of *A. dasyanthus* extract on myocardial infarction in experiments with rats after coronary arterial ligation, and observed a moderate decrease in the damaged area in permanent and transient ischaemia.

Zhang WJ et al. (1997) investigated whether the saponin astragaloside IV (AS-IV), a 3-*O*- β -D-xylopyranosyl-6-*O*- β -D-glucopyranosylcycloastragenol, purified from the Chinese herb drug *Astragalus membranaceus*, which is used in traditional Chinese medicine to treat cardiovascular diseases, might affect the

fibrinolytic potential of cultured human umbilical vein endothelial cells (HUVECs). When HUVECs were conditioned with AS-IV, a dose (0.01-100 μ g AS-IV/ml)- and time-dependent decrease in plasminogen activator inhibitor type 1 (PAI-1) and an increase in tissue-type plasminogen activator (t-PA) synthesis were observed, which were significant from 1 μ g AS-IV/ml and from 12 h of incubation with 100 μ g AS-IV/ml. PAI-1 antigen decreased from 641 $\pm$ 86 to 318 $\pm$ 18 ng/10(5) cells/24 h, whereas t-PA antigen increased from 4.1 $\pm$ 0.3 to 9.7 $\pm$ 0.4 ng/10(5) cells/24 h after addition of 100 μ g AS-IV/ml. PAI-1 activity decreased to 30% of control level, whereas t-PA activity and t-PA-PAI-1 complexes reached a maximum stimulation of 3- and 5-fold over control levels, respectively, in the conditioned media of HUVECs treated with 100 μ g AS-IV/ml for 24 h. PAI-1-specific mRNA expression decreased to 55% (2.2 kb) and 72% (3.2 kb), 66% (2.2 kb) and 88% (3.2 kb), and 19% (2.2 kb) and 41% (3.2 kb) of control values after incubation for 6, 12 and 18 h, respectively, whereas t-PA-specific mRNA increased 2-, 2.5- and 1.4-fold in HUVECs treated with 100 μ g/ml AS-IV for 6, 12, and 18 h, respectively. The obtained data implies that in fact AS-IV can increase the fibrinolytic potential of cultured HUVECs not only by upregulating the expression of t-PA as NG-R1 does, but also by downregulating the expression of PAI-1 (Zhang et al., 1997).

In another study, Astragaloside IV was isolated and injected into 19 patients with congestive heart failure daily for two weeks. After two weeks symptoms of chest distress and dyspnea were alleviated in 15 of the 19 patients. Heart rate slowed from 88.21 $\pm$ 17.19 to 64.55 $\pm$ 13.06 beats/min ($P < 0.05$) (15) (Sinclair et al., 2003).

The saponins contained in *Astragalus* were found to have a positive effect on the function of the heart through the inhibition of the formation of lipid peroxides in the myocardium as well as by decreasing blood coagulation (Sinclair et al., 2003; Sinclair et al., 1998).

Ohkawara et al (2005) were investigated the inhibitory effects of astrapterocarpan, formononetin, and calycosin isolated from *Astragalus membranaceus* on platelet-derived growth factor (PDGF)-BB-induced proliferative response in rat vascular smooth muscle cells (A10 cells). Astrapterocarpan significantly inhibited PDGF-BB-induced cell proliferation and DNA synthesis in a concentration-dependent manner. This inhibition was not attributed to toxicity. In contrast, formononetin and calycosin had no effect. The

researchers next examined the effect of astrapterocarpan on PDGF-BB signal transduction. Astrapterocarpan inhibited PDGF-BB-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein (MAP) kinase. However, this compound had no effect on phosphorylation of PDGF- β -receptor, Akt kinase and p38 MAP kinase. These results indicated that astrapterocarpan inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and that this effect may be mediated, at least in part, by inhibition of the ERK1/2 MAP kinase cascade.

Zhang WD et al., were examined the effects of astragaloside IV on myocardial infarction and coronary flow in vivo and in vitro. The possible roles of its antioxidative and nitric oxide-inducing properties were also explored. The results showed that Astragaloside IV significantly reduced infarct size in dogs subjected to coronary ligation in vivo. Astragaloside IV also improved post-ischemic heart function and ameliorated reperfusion arrhythmias in rat hearts in vitro. The cardioprotection of astragaloside IV was accompanied by a significant increase in coronary flow both in vivo and in vitro. The nitric oxide synthase inhibitor, *N*-omega-nitro-L-arginine methyl ester partially abrogated astragaloside IV's protective effect on heart function. Myocardial antioxidative enzyme superoxide dismutase activity increased with astragaloside IV administration. These data suggested the potential roles of antioxidative and nitric oxide-inducing properties of astragaloside IV in its protection from myocardial ischemia (Zhang et al., 2006).

Zhao et al. (2008) were designed to evaluate the effect of Radix Astragali on myocardial dysfunction, cardiac remodeling and morphological alteration in an experimental model of autoimmune myocarditis, a clinical condition often resulting in dilated cardiomyopathy. Experimental autoimmune myocarditis was established with a subcutaneous injection of porcine cardiac myosin into rear footpad in Lewis rats. Radix Astragali treatment was delivered via an intravenous injection (0.2 ml/100 g body weight, daily) for 3 weeks. Results from transthoracic echocardiography indicated that experimental autoimmune myocarditis led to impaired myocardial contractile function which was reconciled by Radix Astragali. The experimental autoimmune myocarditis triggered profound inflammation and fibrosis in myocardium as assessed by hematoxylin and eosin (H and E) and Masson's trichrome staining. Interestingly, Radix Astragali significantly attenuated autoimmune myocarditis-induced myocardial inflammation and fibrosis. Similarly, Radix Astragali treatment alleviated

autoimmune myocarditis-triggered overt lymphocyte proliferation. Furthermore, Radix Astragali significantly attenuated elevated levels of the Th1 cytokines (IFN- γ and IL-2), and increased the Th2 cytokines (IL-4 and IL-10) in autoimmune myocarditis. Collectively, this data revealed that Radix Astragali effectively protected against cardiac functional and morphological aberrations in experimental autoimmune myocarditis.

The effect of *Astragalus membranaceus* on hemodynamic changes in adriamycin (ADR)-injured rat hearts were evaluated. Sprague-Dawley rats were divided into four groups: control, ADR only, ADR + low dose of *Astragalus* and ADR + high dose of *Astragalus*. Rats were injected intraperitoneally with 6 equal doses of ADR (cumulative dose, 12 mg/kg) over a period of 2 weeks. Treatment of *Astragalus* began 1 day before the onset of ADR injection and was given orally once a day for 50 days (3.3 or 10 g/kg/day). Five weeks after the final injection of ADR, rats treated with ADR only showed a significant inhibition of cardiac diastolic function accompanied by the presence of ascites, a remarkable reduction in body weight and heart weight as well as survival rate compared to the controls. Moreover, SERCA2a mRNA and protein expressions in hearts were obviously downregulated by ADR. However, this impaired cardiac function was significantly improved in both doses of *Astragalus* feeding groups. The amount of ascites was also reduced in a similar extent in these 2 groups. Only the high dose treatment of *Astragalus* significantly attenuated the changes of SERCA2a expression in injured hearts and improved survival. These results indicated that *Astragalus* could improve cardiac function of ADR-injured rat hearts, which was partly mediated by upregulation of SERCA2a expression (Su et al., 2009).

Anthracyclines are antitumor antibiotics with significant activity against solid and hematologic malignancies. One problem preventing more widespread use has been the development of cardiotoxicity. To determine whether antioxidant agents can reduce the cardiotoxicity of anthracyclines, a herb *Astragalus membranaceus* was introduced, which has been widely used for the treatment of cardiovascular diseases in China and was confirmed to be an effective antioxidant agent recently. Pre-treatment with *Astragalus membranaceus* significantly attenuated the daunorubicin-induced increases of reactive oxygen species ($p < 0.001$), apoptosis ($p < 0.05$) and the secretions of LDH ($p < 0.01$) in cultured neonatal cardiomyocytes. *Astragalus membranaceus* also raised the EC₅₀ of daunorubicin 1.24-fold. Compared with *Astragalus membranaceus*, N-acetyl-L-cysteine had similar effects on daunorubicin-induced cell injury, however,

superoxide dismutase reduced reactive oxygen species without attenuating apoptosis. The subcellular distribution of DNR was similar to the distribution of MitoTracker Red 580 in mitochondria, which was mainly in the cytoplasm around the nuclear membrane in cultured neonatal cardiomyocytes. In conclusion, the results suggested that *A. membranaceus* is potentially protective against daunorubicin cardiotoxicity by decreasing free radical release and apoptosis in cultured neonatal cardiomyocytes. The main subcellular distribution of daunorubicin may be in the mitochondria (Luo et al., 2009).

Zhao J. et al. (2012) were investigated the therapeutic mechanisms of Astragaloside IV (AGS-IV) at a network level by computer-assisted target identification with the in silico inverse docking program (INVDOCK). Targets included in the analysis covered all signaling pathways thought to be implicated in the therapeutic actions of all CVD drugs approved by US FDA. A total of 39 putative targets were identified. Three of these targets, calcineurin (CN), angiotensin-converting enzyme (ACE), and c-Jun N-terminal kinase (JNK), were experimentally validated at a molecular level. Protective effects of AGS-IV were also compared with the CN inhibitor cyclosporin A (CsA) in cultured cardiomyocytes exposed to adriamycin. Network analysis of protein-protein interactions (PPI) was carried out with reference to the therapeutic profiles of approved CVD drugs. The results suggested that the therapeutic effects of AGS-IV are based upon a combination of blocking calcium influx, vasodilation, anti-thrombosis, anti-oxidation, anti-inflammation and immune regulation.

1.4.6. Effect of *Astragalus* sp. on diabetes and diabetes related diseases

In a systematic screening of wild Egyptian plants, Shabana et al. (1990) studied the hypoglycaemic effects on alloxanized diabetic rats of some extracts of *Astragalus* species. Saponin astramembrainnin I, decreased carrageenan induced oedema in rats (p.o.), and inhibited the increase in vascular permeability induced by serotonin and histamine after i.v. or oral administration (Tang and Eisenbrand, 1992).

APS was proved to have the preventive effects on type 1 diabetes in non obese diabetic (NOD) mice. The APS group has lower incidence of diabetes, higher serum C-P levels, decreased degree of the lymphocytic inflammation of pancreatic islets, stronger proliferation of CD8 T subsets, and lower ratio of

CD4/CD8 subgroup in spleocytes than those of the normal solution group (Chen et al, 2001; Liu et al., 2011).

Astragalus polysaccharides have an effect to two-dimensionally regulate the level of blood glucose, which can increase the blood glucose of hypoglycemic animals or humans to normal level, and significantly lower the level of blood glucose, triglyceride and myocardial calcium, improve the abnormalities of myocardial ultrastructure and the metabolism of diabetic rats and mice, and inhibit the onset of type 1 diabetes in non-obese diabetic mice. *A. membranaceus* was reported to have an effect for diabetic complications, such as protecting the myocardium in diabetic nephropathy by inhibiting lipid peroxidation, prolonging the incubation period of late diabetic neuropathy by decreasing the motion nerve conduction velocity as an aldose reductase inhibitor, and exerting a beneficial effect on experimental diabetic nephropathy by suppressing the renal hypertrophy and micro albuminuria (Li et al., 2004).

It was reported that APS could reduce fasting plasma glucose (FPG) and blood lipids on insulin resistance of rats with type 2 diabetes. APS could significantly decrease the blood sugar and increase the content of the blood serum HDL of 2-DM IR rats (Liu et al, 2007).

A crude extract of AR inhibits the formation of Nε- (carboxymethyl) lysine (CML) and pentosidine during the incubation of bovine serum albumin with ribose. Astragalosides significantly inhibited the formation of both CML and pentosidine, and astragaloside IV had the strongest inhibitory effect among all the isolated compounds. That suggested that AR and astragalosides might be a potentially useful strategy for the prevention of clinical diabetic complications by inhibiting AGE inhibitors (Motomura et al, 2009).

Astragalus mongolicus (root) was able to reverse hyperglycemia and protect pancreatic islets in type 1 DM in NOD mice by changing the predominant form of lymphocytes/cytokines from Th1 to Th2, thus preventing lymphocyte infiltration of the pancreas. In addition, astragaloside IV (saponin extract of AM root) can ameliorate high glucose-induced podocyte adhesion by up regulating α -3- β -1 integrin and inhibiting the activation and overexpression of integrin-linked kinase. In diabetic rats treated with a preparations containing AM (root) and *Angelica sinensis*, attenuation of transforming growth factor (TGF)- β mRNA in the renal cortex was observed. The inhibitory effects on TGF- β were further substantiated

by the down-regulation of reactive oxygen species NF-kappa B signal pathway by a combination of AM (root) and *Arctium lappa* (fruit) in diabetic mice. Not only can AM (root) effectively treat diabetic nephropathy, it can also improve other renal diseases in animal models. AM (root) was observed to have significant anti fibrotic effects in rats with unilateral ureteral obstruction (UUO) by inhibiting myofibroblast activation and TGF- β 1 expression, and by inducing hepatocyte growth factor. AM (root) can also alleviate hyperlipidemia and proteinuria in rat models of nephritic syndrome by upregulating the expression of the hepatic low-density lipoprotein (LDL) receptor gene (Liet al., 2000) and reducing elevated levels of messenger RNA expression of arginine vasopressin (AVP) V2 receptor and AVP-dependent aquaporin-2 (Zhang et al., 2009).

Astragaloside II and isoastragaloside I isolated from AR selectively were reported to increase adiponectin secretion in primary adipocytes without any obvious effect on a panel of other adipokines. Adiponectin is an adipocyte-derived insulin-sensitizing hormone with antidiabetic, anti-inflammatory, and anti-atherosclerotic properties. Chronic administration of astragaloside II and isoastragaloside I in both dietary and genetic obese mice significantly elevated serum levels of total adiponectin and selectively increased the composition of its high molecular weight oligomeric complex. These results suggested that the two natural compounds might provide the lead as a novel class of therapeutics for obesityrelated diseases (Xu et al, 2009).

Liu M. et al. (2010) were examined whether treatment with *Astragalus* polysaccharide (APS) improves insulin sensitivity in insulin-resistant mice and whether this is associated with an improvement of dysregulated protein kinase B and glucose transporter 4 expressions in skeletal muscle. APS (700 mgkg⁻¹ day⁻¹) or vehicle was administered to 12-week-old diabetic KKAY and nondiabetic C57BL/6J mice for 8 weeks. Changes in body weight, blood glucose level, insulin resistance index, and oral glucose tolerance were routinely evaluated. The expressions of protein kinase B and glucose transporter 4 in skeletal muscle tissues were determined with Western blot. KKAY mice developed persistent hyperglycemia, impaired glucose tolerance and insulin resistance. Insulin-stimulated protein kinase B phosphorylation and glucose transporter 4 translocation were significantly decreased in KKAY compared to age-matched C57BL/6J mice. APS treatment ameliorated hyperglycemia and insulin resistance. Although the content of protein kinase B and glucose transporter 4 in KKAY skeletal muscle were not affected by APS, insulin-induced protein kinase B Ser-

473 phosphorylation and glucose transporter 4 translocation in skeletal muscle were partially restored by APS treatment. In contrast, APS did not have any effect on C57BL/6J mice. These results indicate that APS can regulate part of the insulin signaling in insulin-resistant skeletal muscle, and that APS could be a potential insulin sensitizer for the treatment of type 2 diabetes

Chen et al. (2012) were examined the effect of *Astragalus* polysaccharide (APS) on myocardial glucose and lipid metabolism in diabetes (DM) hamster and to explore its mechanism in intervention of DM cardiomyopathy. Low-dose-streptozotocin-induced hamsters (STZ, 40 mg/kg 93 days, i.p.) with blood glucose [13.9 mmol/L were considered as type 2 diabetic models. Blood glucose, serum lipid, insulin, C-peptide, myocardial enzyme levels, myocardial glycogen staining, myocardial ultrastructure, fluorescence quantitative RT-PCR detection of myocardial PPAR- α and the target genes (FATP, ACS) and GLUT4mRNA expression in normal control group, DM group and APS treatment group hamsters were measured. The results showed that there was significant glycolipid metabolic disorders in DM group compared with normal group. Glucose, glycosylated serum protein, myocardial enzymes and lipid levels in APS treatment group decreased significantly than DM group, but insulin and C-peptide levels was no difference. Myocardial glycogen staining and abnormal myocardial ultrastructure in APS treatment group were significantly improved than in DM group. Gene expression of myocardial PPAR- α and its target genes (FATP, ACS) in APS group were significantly lower than in DM group, while gene expression of GLUT4 in APS group was higher than DM group. It was concluded that APS can partially improve myocardial glucose and lipid metabolism disorders in diabetic hamsters and protect myocardium in some extent.

1.4.7. Other Pharmacological Effects of *Astragalus*

Wong et al. (1992) demonstrated the antimutagenic activity of *A. membranaceus* aqueous extract on aflatoxin B1-induced mutagenesis, using *Salmonella typhimurium* as a test strain and rat liver ultra-supernatant as the activating system. The results reinforce the use of this Chinese medicinal plant as a cancer chemopreventive agent, because the extract produced a concentration-dependent inhibition of histidine-independent reversant colonies induced by aflatoxin B1.

Eighteen Chinese medicinal plants were screened as stimulants of sperm motility (Hong et al., 1992), and *A. membranaceus* aqueous extract was active. However, other swainsonine-containing *Astragalus* can have adverse effects on male reproduction (Tulsiani et al., 1990).

Anti-inflammatory effect of *Astragalus* ssp. were also investigated. The effects of *Astragalus membranaceus* root (AR) extract on interleukin (IL-6) and tumor necrosis factor (TNF- α) production, prostaglandin E2 (PGE2) biosynthesis, and leukotriene C4 (LTC4) production from lipopolysaccharide (LPS)-stimulated human amnion cells were investigated. The data suggested that AR extract might play a role in inhibiting bacterial infection-associated preterm labor by suppressing the productions of IL-6, PGE2, and LTC4 by human amnion cells (Shon et al., 2002).

Studies investigated the effect of AR extract on IL-6 and TNF- α production, PGE2 and LTC4 released from IL-1 β -stimulated human amnion. The results indicated that AR had a broad anti-inflammatory effect in human amnion and might be considered a promising agent to protect preterm labor (Shon and Nam, 2003).

It was reported that AR displayed antiinflammation in zymosan air-pouch mice by reducing the expression of iNOS, COX-2, IL-6, IL-1 β , and TNF- α , and by decreasing the production of nitric oxide (NO). In a similar manner, AR reduced the expression of IL-6, iNOS, and COX-2 in LPS-treated Raw 264.7 cells. The data revealed that AR had an anti-inflammatory effect that was mediated by the MKP-1-dependent inactivation of p38 and Erk1/2 and inhibition of NF kappaB-mediated transcription (Ryu et al, 2008).

In conclusion, *Astragalus* species are a group of medicinal plants, whose active principles are principally saponins and polysaccharides. Some toxic principles have medicinal interest, for example swainsonine in cancer chemotherapy and 3-NPA as an antihypertensive principle. The use of *A. membranaceus* in Chinese traditional medicine seems to be justified as demonstrated by numerous studies. The potential use of polysaccharides from *Astragalus* species as immunostimulant agents is suggested and justified in several clinical studies. The effect on virus infections and cancer is related to its immunorestorative properties.

Two saponins, Astragaloside II and isoastragaloside I isolated from *A. membranaceus* selectively increased adiponectin secretion in primary adipocytes without any obvious effects on a panel of other adipokines. Furthermore, an additive effect on induction of adiponectin production was observed between these two compounds and rosiglitazone, a thiazolidinedione class of insulin-sensitizing drugs. Chronic administration of astragaloside II and isoastragaloside I in both dietary and genetic obese mice significantly elevated serum levels of total adiponectin and selectively increased the composition of its high molecular weight oligomeric complex. These changes were associated with an alleviation of hyperglycemia, glucose intolerance, and insulin resistance. By contrast, the beneficial effects of these two compounds on insulin sensitivity and glucose metabolism were diminished in adiponectin knockout mice. In conclusion, obtained results suggest that pharmacological elevation of circulating adiponectin alone is sufficient to ameliorate insulin resistance and diabetes and support the use of adiponectin as a biomarker for future drug discovery. The two natural compounds might provide the lead as a novel class of therapeutics for obesity-related diseases (Xu et al., 2009).

A lectin (AMML) from the roots of *Astragalus mongholicus* was extracted and purified by affinity chromatography technique. Human cervical carcinoma cell line (HeLa), human osteoblast-like cell line(MG63) and human leukemia cell line (K562) were used to check the effects of AMML on cell proliferation, apoptosis and cell cycle. Maximum growth inhibition (92%) was observed with HeLa cells, followed by K562 cells (84%) and MG63 (48%) cells. Morphological observation showed that AMML-treated HeLa cells displayed outstanding apoptosis characteristics, such as nuclear fragmentation and appearance of membrane-enclosed apoptotic bodies. The apoptosis of HeLa cells was confirmed by flow cytometry using Annexin V/FITC and propidiumiodide (PI) staining technique (Yan et al., 2009b).

AI (*Astragalus membranaceus* injection) combined with routine therapy has the therapeutic effect on acute attack of bronchial asthma. One hundred and eight patients with acute attack of bronchial asthma were randomly divided into two groups. The patients in routine treatment group were treated by conventional therapy as control group, while the patients in treatment group were treated with AI on the basis of routine treatment. After treatment, the levels of forced vital capacity (FVC), 1 s forced expiratory volume (FEV1), and peak expiratory flow

(PEF) in treatment group were significantly higher than those in control group (Zhang and Li, 2009; Liu et al., 2011).

The study was designed to investigate efficacy and safety of *Astragalus membranaceus* (AM) in the treatment of patients with seasonal allergic rhinitis (SAR). AM is an active component in the herbal and mineral complex (HMC) registered in Croatia as a food supplement Lectranal®. The study was designed as a 6-weeks, doubleblind, placebo-controlled clinical trial and conducted in 48 adult patients with a moderate to severe SAR. The treatment efficacy was evaluated by the mean change in the symptom score (TSS), quality of life (QoL), specific serum IgE and IgG, nasal eosinophils, and physicians' and patients' global evaluation. Compared to placebo, HMC significantly decreased the intensity of rhinorrhea while for other primary efficacy variables the treatment groups did not differ. In contrast, investigators and patients equally judged the treatment with HMC as more efficacious. In addition, the analysis of changes from baseline inside the groups for TSS, QoL, and 4 main symptoms of SAR were strikingly in favor of the active treatment. In patients with SAR due to weed pollen allergy, HMC significantly improved primary variables, reflective TSS and QoL. The study revealed a significant number of positive signals indicating the therapeutic effectiveness of the HMC in patients with SAR which should be further tested in larger, multicentre trials with more patients (Matkovic et al., 2010).

Yin et al. (2010b) were evaluated the protective effect of Astragaloside IV on Alzheimer's disease (AD) in rats induced by amyloid- β peptide ($A\beta_{1-42}$) and its potential therapeutic mechanism. 50 Male Sprague Dawley rats were divided into five groups (10 rats for each): control group, model group, treatment groups 1~3. 10 μ g $A\beta_{1-42}$ was injected bilaterally into the dorsal dentate gyrus of the hippocampus of rats in the model and treatment groups to prepare the AD models. 24h after modeling, Astragaloside IV administration, with different drug dosages of 20 mg/(kg•day), 40 mg/(kg•day) and 60 mg/(kg•day), was performed by gastric perfusion for rats in the treatment group 1~3. Later on, the cognitive ability of rats was examined by a series of behavioral tests, and the expression of Bcl-2 and Bcl-xl in the hippocampus of rats was detected by the fluorescein based Quantitative RT-PCR. The spontaneous alternation test in a Y maze and Morris water maze task have demonstrated that the repeated daily administration of Astragaloside IV at the doses of 20 mg/kg bw/day) ($p<0.05$), 40 mg/kg bw/day) ($p<0.01$), and 60mg/kg bw/day) ($p<0.01$) significantly ameliorated the impairment of performance caused by $A\beta_{1-42}$. Furthermore, Astragaloside IV also enhanced the

expression of Bcl-2 and Bcl-xl in hippocampal neurons of rats in a dosage-dependent manner. In conclusion; these findings suggested that Astragaloside IV could alleviate cognitive impairment and enhance the expression of Bcl-2 and Bcl-xl in hippocampus of rat models with AD.

Zhang et al. (2011) were investigated whether astragaloside IV is able to prevent the development of hypertension and endothelial dysfunction in fructose-fed rats. Rats were fed with 10% fructose in their drinking water for 8 weeks. From the beginning of week 5, two groups of fructose-fed rats were treated with 0.5 or 2 mg/kg, i.p., astragaloside IV. Another group of fructose-fed rats, injected with the same volume of vehicle (dimethylsulfoxide, DMSO) from week 5, served as the control group. At the end of the treatment period, blood pressure, blood glucose, glucose tolerance, blood insulin and lipids were determined. In addition, in vitro experiments were conducted at the end of the eight week treatment period to evaluate endothelium-dependent aortic vasorelaxation, as well as myocardial and aortic tissue levels of nitrate and nitrite (NOx) and cGMP. Fructose-fed rats developed clustering signs of metabolic syndrome, such as increased bodyweight, mild hypertension, hyperinsulinaemia, hypertriglyceridaemia, impaired glucose tolerance and impaired endothelium-dependent vasorelaxation. Administration of astragaloside IV reduced blood pressure and triglyceride levels in fructose-fed rats and high dose of astragaloside IV also improved glucose tolerance and endothelium-dependent vasorelaxation. The astragaloside IV-induced improvement in vasorelaxation was associated with increased levels of aortic NOx and cGMP and was abrogated by blockade of nitric oxide synthase with NG-nitro-l-arginine methyl ester (l-NAME). On the basis of its favourable effects on lipid metabolism, endothelium-dependent vasorelaxation and the nitric oxide–cGMP-related pathway, it was concluded that astragaloside IV may be useful in ameliorating food-induced metabolic syndrome.

Yan et al. (2012) evaluated the role of *Astragalus mongholicus* aqueous extract AMAE on spinal cord lipid peroxidation (LPO), antioxidant status, apoptosis index and inducible nitric oxide synthases (iNOS) positive expression rate in rats. Male albino rats of Wistar strain were divided into four groups: Group I, normal control rats; Group II, model rats suffering from spinal cord injury (SCI); Group III and V, AMAE rats supplemented with AMAE. Results showed that increased spinal cord LPO, apoptosis index, iNOS positive expression rate and decreased antioxidant enzymes activities were observed in model rats (Group II). Administration of AMAE significantly dose-dependently decreased spinal

cord LPO, apoptosis index, iNOS positive expression rate and increased antioxidant enzymes activities in AMAE rats. These findings demonstrated that AMAE can enhance the antioxidant status and decrease the incidence of free radical-induced LPO, and display strong neuroprotective activity in the experimental rats. (Neuroprotective action of *Astragalus mongholicus* aqueous extract in experimental rats suffering from spinal cord injury.

In another research, Xiong et al. designed a study to compare hearing gains from hearing impairment in 46 ears treated with Radix astragali (RA) with 46 ears treated with non-RA. RA was given intravenously daily for 10 days. There were no significant differences in clinical or audiological data between RA and non-RA groups. The results showed that the hearing gain at 250, 500, 1000, 2000, and 4000 Hz in RA group was much higher than that of non-RA group correspondingly ($P < 0.01$). Also, the hearing gain at PTA (pure-tone average of 250, 500, 1000, 2000, and 4000 Hz) in RA group was significantly higher than that of non-RA group ($P < 0.01$). It was concluded that the recovery of hearing was significantly better after treatment of RA than non-treatment of RA. RA can be valuable concurrent therapy for patients with SD (Xiong et al., 2012).

2. MATERIAL AND METHODS

2.1. General

Optical rotations were measured on a JASCO DIP 1000 polarimeter. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpinGmbH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD₃OD (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra. The NMR data were processed using UXNMR software. Exact masses were measured by a AB SCIEX Voyager DE mass spectrometer equipped with a 337 nm laser and delay extraction and operated in positive ion reflector mode. Samples were analyzed by MALDITOF mass spectrometry. A mixture of analyte solution and α -cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from adrenocorticotrophic hormone (ACTH) fragment 18-39 human at 2465.1989 Da and α -cyano-4-hydroxycinnamic acid at 190.0504 Da as internal standard. Butanol extract was analyzed by on-line HPLC–ESI-MSⁿ using a ThermoFinnigan Spectra System HPLC coupled with an LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose', CA, USA). HPLC separation was conducted on a C18 reversed-phase (RP) column (5 μ m, 2.1 mm $\times$ 250 mm; X-Terra MS C18; Waters, Milford, MA) at a flow rate of 0.2 ml/min. A gradient elution was performed by using H₂O (A) and CH₃CN (B), both added of 0.1 % acetic acid, as mobile phases. After a 3 min hold at 15 % B, elution was performed according to the following conditions: from 15 % B to 38 % B in 23 min; to 74 % B in 19 min; to 100 % B in 8 min, hold to 100 % B for 3 min. The column effluent was analyzed by ESI-MS in negative ion mode and the mass spectra were acquired and processed using the software provided by the manufacturer. The capillary voltage was set at -47 V, the spray voltage at 5 kV and the tube lens offset at 45 V. The capillary temperature was 280 °C. Data were acquired in MS¹ and MSⁿ scanning modes. By using a syringe pump (flow rate 5 μ l/min), each pure compound dissolved in H₂O/MeOH (1:1) was infused in the ESI source. Negative ESI-MSⁿ analyses were performed using the same conditions as those for HPLC–ESI-MSⁿ analysis. GC analysis was performed on a Thermo Finnigan Trace GC apparatus with a FID detector and using a I-Chirasil-Val column (0.32 mm x 25 m). Semi-preparative HPLC separations were carried out on a Waters 590 system equipped with a Waters R401 refractive index

detector, a Waters μ BondapackTM Prep C18 column (300 $\times$ 7.8 mm i.d.), and a Rheodyne injector. For thin-layer chromatography, silica gel F₂₅₄ (Merck 5554) precoated plates were used. Silica gel 60 (0.063-0.200 mm, Merck) was used for column chromatography. TLC analyses were carried out on silica gel 60 F254 (Merck 7734) eluting with CHCl₃- MeOH- H₂O (26:14:3). TLC plates were developed by spraying with 30% H₂SO₄, followed by heating at 120 °C.

During chromatographic studies the following solvent system were used;

I	CHCl ₃ :MeOH:H ₂ O	90:10:1
II	CHCl ₃ :MeOH:H ₂ O	80:20:2
III	CHCl ₃ :MeOH:H ₂ O	70:30:3
IV	CHCl ₃ :MeOH:H ₂ O	61:32:7
V	MeOH:H ₂ O	80:20
VI	MeOH:H ₂ O	60:40
VII	MeOH:H ₂ O	55:45
VIII	MeOH:H ₂ O	50:50
IX	MeOH:H ₂ O	40:60
X	MeOH:H ₂ O	32:68
XI	MeOH:H ₂ O	20:80

2.2. Plant Materials

2.2.1. *Astragalus aureus*

A. aureus Willd (Whole plant; Section: Adiaspastus) was collected from Karlıova, Bingöl, 40 km from Çat to Karlıova, from 2180 m altitude, East Anatolia, Turkey in July 2009, and identified by Fevzi Özgökçe (Department of Biology, Faculty of Science and Art, Yüzüncü Yıl University, Van, Turkey). Voucher specimen has been deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey (VANF 13646).

2.2.2. *Astragalus tauricolus*

Astragalus tauricolus (whole plant) was collected from Başet Mountain, from altitude of 3200 m, Hafik-Sivas, Turkey in July 2009. Samples of plant material were identified by Fevzi Özgökçe (Department of Biology, Faculty of Sciences and Art, Yüzüncü Yıl University, Van, Turkey). Voucher specimen has been deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey (VANF 13823).

2.2.3. *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*

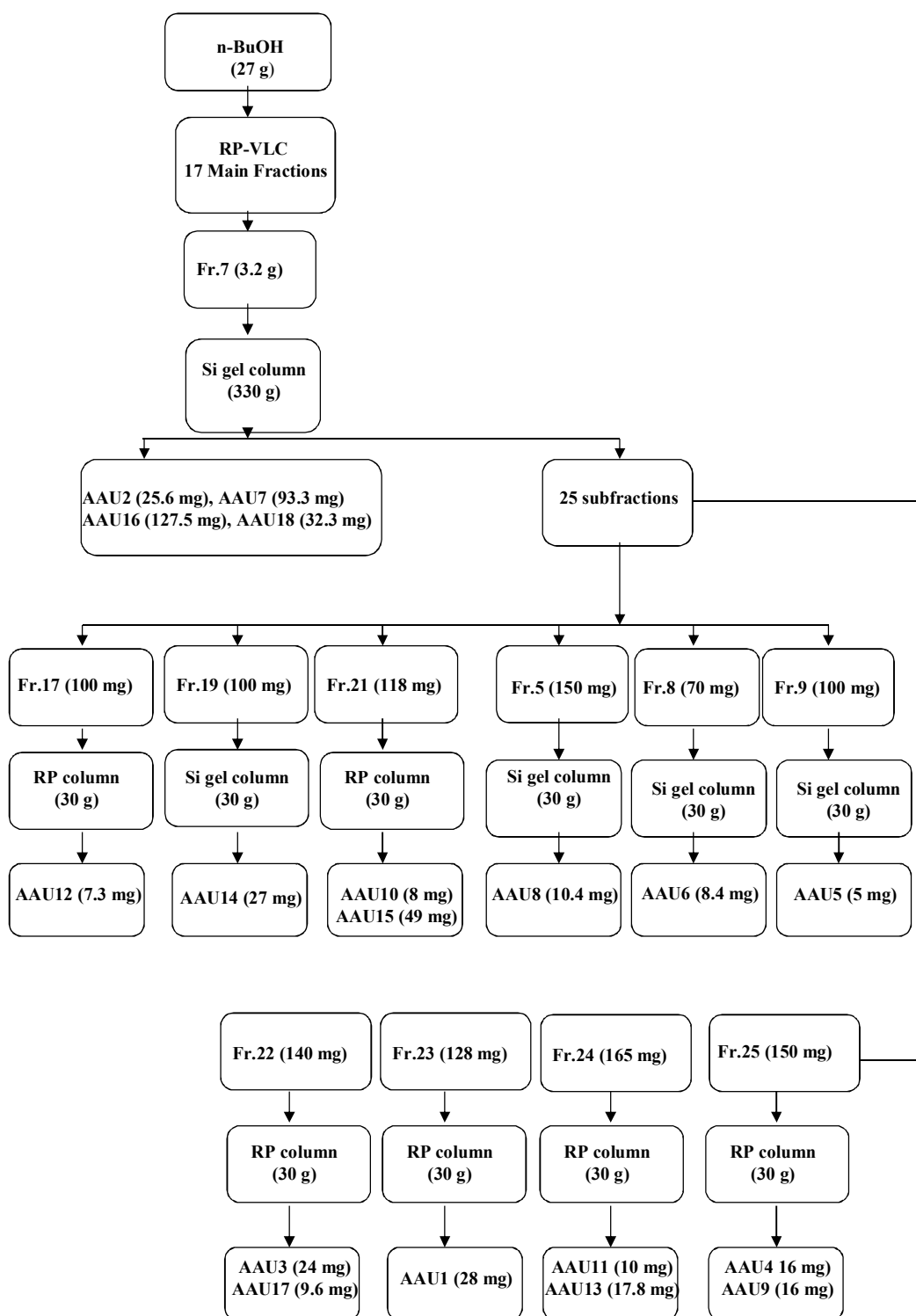
Astragalus angustifolius Lam. subsp. *angustifolius* var. *angustifolius* (whole plant) was collected from Çaltılı Village, Çingi hill, from altitude of 1200 m, Hafik-Sivas, Turkey in July 2009. Samples of plant material were identified by Serdar G. Senol (Department of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). Voucher specimen has been deposited in the Herbarium of Ege University, Izmir, Turkey (EGE 40790).

2.3. Isolation and Purification

2.3.1. *Astragalus aureus*

Air-dried and powdered plant material of *A. aureus* (Whole plant, 700 g) was extracted with MeOH (2 x 3.5 l) at 60 °C. After filtration, the solvent was removed by rotary evaporation yielding 72 g of extract. The MeOH extract was dissolved in H₂O (350 mL), and successively partitioned with n-hexane (2 x 200

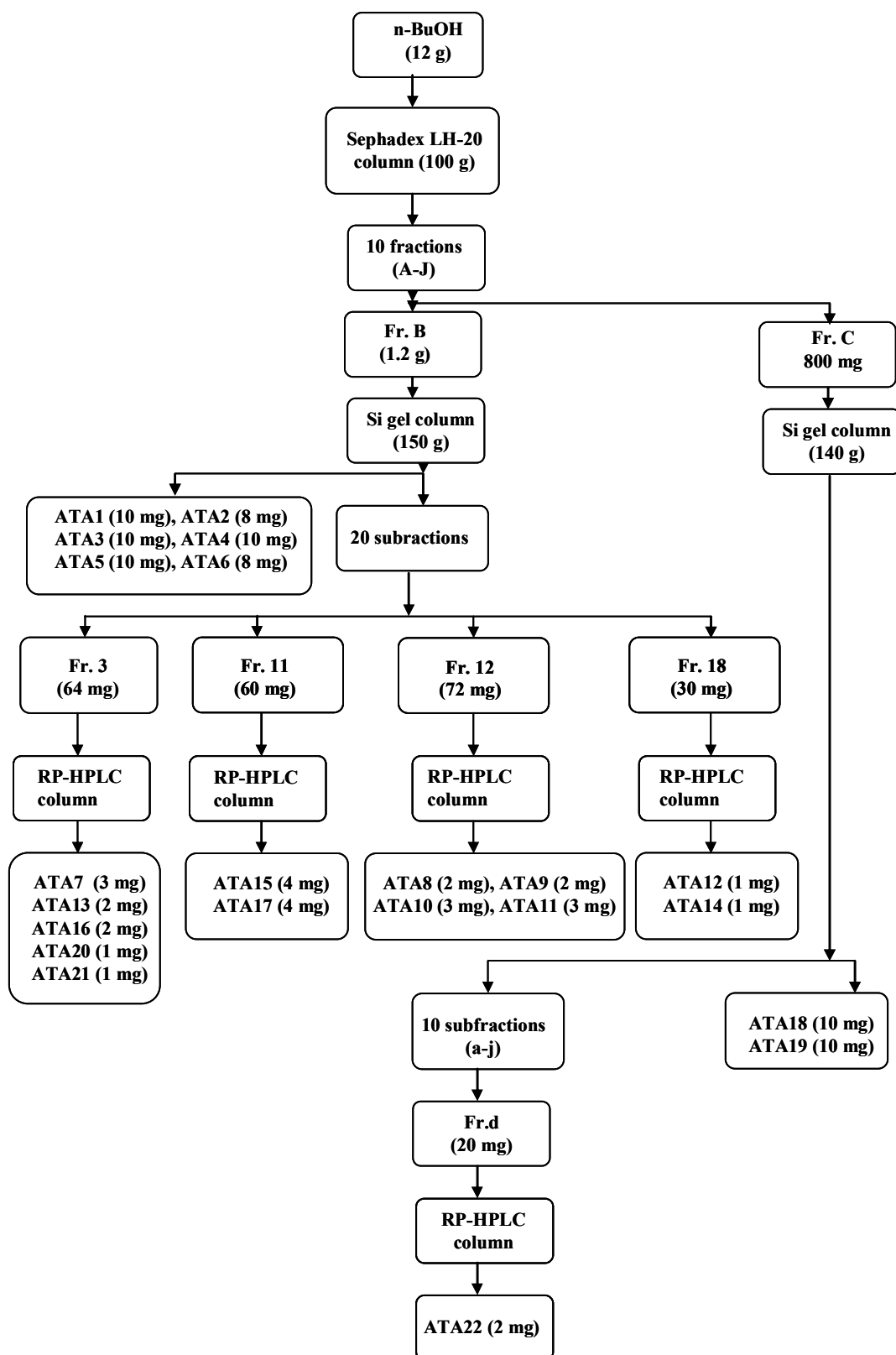
mL), EtOAc (2 x 200 mL) and *n*-BuOH saturated with H₂O (3 x 200 mL). The *n*-BuOH extract (27 g) was subjected to vacuum liquid chromatography (VLC) using reversed-phase material (Lichroprep RP-18, 25–40 µm, 150 g) employing H₂O (1000 mL), H₂O–MeOH (8:2, 1200 mL; 6:4, 2400 mL; 4:6, 3200 mL; 2:8, 1600 mL) and MeOH (800 mL) to give seventeen main fractions (1–17). Fraction 7 (3.2 g) was submitted to silica gel (330 g) column chromatography with the solvent system CHCl₃–MeOH–H₂O (90:10:1, 2000 mL; 80:20:2, 2500 mL; 70:30:3, 1500 mL) yielding **AAU7** (93.3 mg), **AAU18** (32.3 mg), **AAU2** (25.6 mg), **AAU16** (127.5 mg) and 25 subfractions. Subfraction 5 (150 mg) was subjected to silica gel (30 g) column chromatography. Elution was carried out with CHCl₃–MeOH–H₂O (90:10:1, 500 mL) to give **AAU8** (10.4 mg). Subfraction 8 (70 mg) was fractionated over an open column chromatography using silica gel (30 g). Elution was performed with CHCl₃–MeOH–H₂O mixtures (90:10:1, 500 mL) to afford **AAU6** (8.4 mg). Subfraction 9 (100 mg) was submitted to silica gel (30 g) column chromatography with the solvent system CHCl₃–MeOH–H₂O (90:10:1, 750 mL) to give **AAU5** (5 mg). Subfraction 19 (100 mg) was subjected to silica gel (30 g) column chromatography with the solvent system CHCl₃–MeOH–H₂O (90:10:1, 750 mL) to yield **AAU14** (27 mg). Subfraction 17 (100 mg) was applied to a reversed phase column (Lichroprep RP-18, 25–40 µm, 30 g) using MeOH–H₂O (6:4, 500 mL) to give **AAU12** (7.3 mg). Subfraction 21 (118 mg) was chromatographed on a reversed phase material (Lichroprep RP-18, 25–40 µm, 30 g), employing MeOH–H₂O (6:4, 500 mL) yielding **AAU15** (49 mg) and **AAU10** (8 mg). Subfraction 22 (140 mg) was purified on a reversed phase column (Lichroprep RP-18, 25–40 µm, 30 g) and eluted with MeOH–H₂O (6:4, 500 mL) to give **AAU17** (9.6 mg) and **AAU3** (24 mg). Subfraction 23 (128 mg) was subjected to a reversed phase column (Lichroprep RP-18, 25–40 µm, 30 g) employing MeOH–H₂O (6:4, 750 mL) to afford **AAU1** (28 mg). Subfraction 24 (165 mg) was applied to a reversed phase column (Lichroprep RP-18, 25–40 µm, 30 g) using MeOH–H₂O (6:4, 750 mL) to give **AAU13** (17.8 mg) and **AAU11** (10 mg). Subfraction 25 (116 mg) was chromatographed on a reversed phase material (Lichroprep RP-18, 25–40 µm, 30 g), employing MeOH–H₂O (6:4, 600 mL) to afford **AAU4** (16 mg) and **AAU9** (16 mg).



Scheme 2.1. Isolation of the new triterpenes from *Astragalus aureus*

2.3.2. *Astragalus tauricolus*

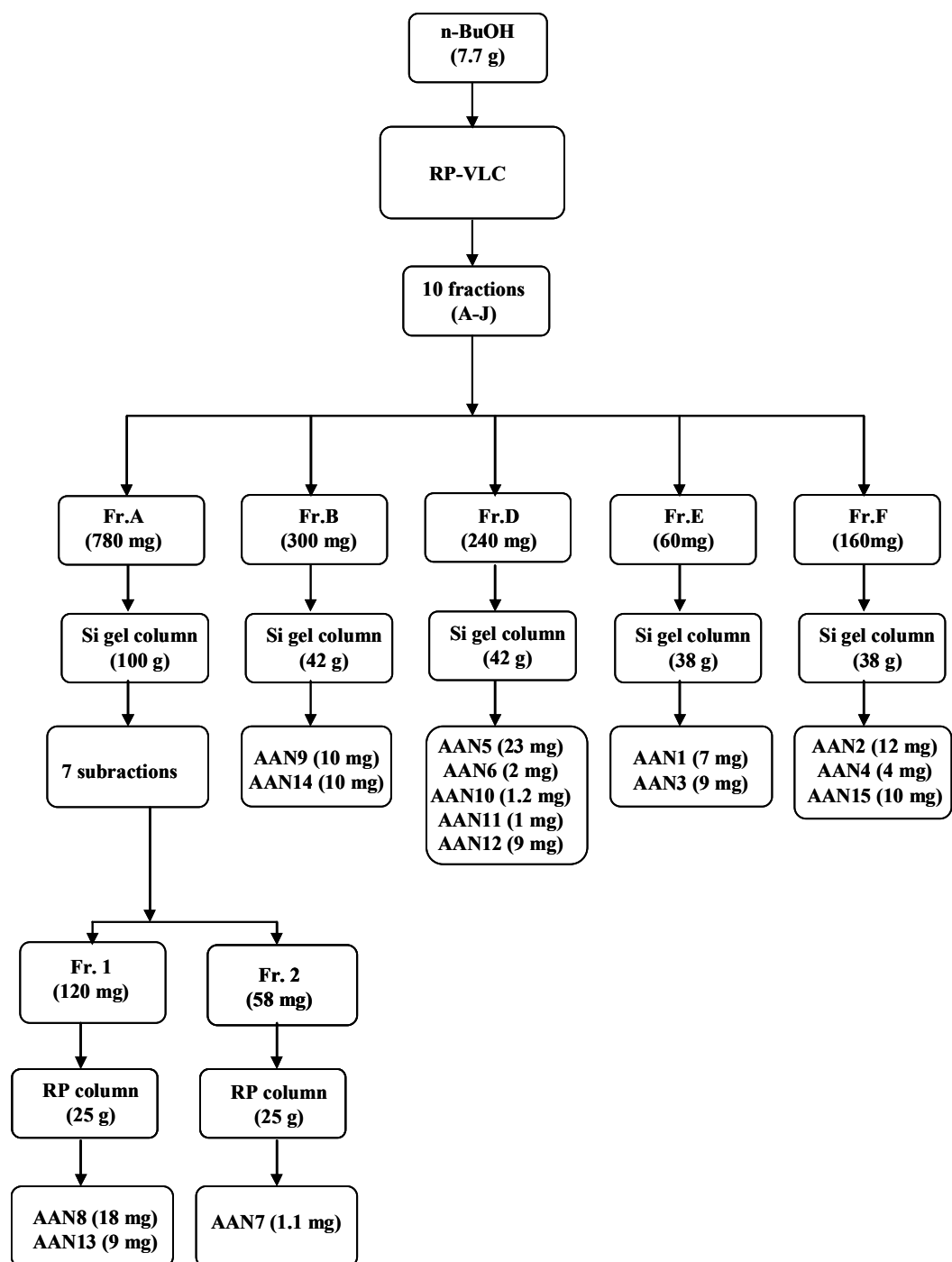
The air-dried and powdered plant material of *Astragalus tauricolus* was extracted with MeOH under reflux. After filtration, the solvent was removed by rotary evaporation to give crude extract. The residue was dissolved in water and then partitioned successively with *n*-Hexane, CHCl₃, and *n*-BuOH saturated with H₂O. The *n*-BuOH phase was chromatographed over Sephadex column chromatography to give ten main fractions (A-J). Fraction B (1.2 g) was subjected to a silica gel column (Merck.7734, 330 g) with the solvent system CHCl₃-MeOH-H₂O mixtures (80:20:2, 1000 mL; 70:30:3, 500 mL) to yield 20 subfractions and yielding **ATA1** (10 mg), **ATA2** (8 mg), **ATA3** (10 mg), **ATA4** (10 mg), **ATA5** (10 mg) and **ATA6** (8 mg). Subfraction 3 (64 mg) was subjected to RP-HPLC column chromatography with the solvent system MeOH-H₂O (55:44, 250 mL) to yield compounds **ATA7** (3 mg), **ATA13** (2 mg), **ATA16** (2 mg), **ATA20** (1 mg) and **ATA21** (1 mg). Subfraction 11 (60 mg) was submitted to RP-HPLC column chromatography with the solvent system MeOH:H₂O (32:68, 200 mL) yielding **ATA15** (4 mg) and **ATA17** (4 mg). Subfraction 12 (72 mg) was subjected to RP-HPLC column chromatography with the solvent system MeOH-H₂O (32:68, 250 mL) to yield compounds **ATA8** (2 mg), **ATA9** (2 mg), **ATA10** (3 mg), and **ATA11** (3 mg). Subfraction 18 (30 mg) was submitted to RP-HPLC column chromatography with the solvent system MeOH-H₂O (50:50, 200 mL) yielding **ATA12** (1 mg) and **ATA14** (1 mg). Fraction C (800 mg) was subjected to silica gel (140 g) column chromatography with the solvent system CHCl₃-MeOH-H₂O (70:30:3, 500 mL) to yield 10 subfractions (a-j) and yielding **ATA18** (10 mg) and **ATA19** (10 mg). Subfraction d (20 mg) was submitted to RP-HPLC column chromatography with the solvent system MeOH-H₂O (60:40, 250 mL) yielding **ATA22** (2 mg).



Scheme 2.2. Isolation of the new triterpenes from *Astragalus tauricolus*

2.3.3. *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*

Air-dried and powdered plant material of *Astragalus angustifolius* subsp. *angustifolius* (Whole plant, 367 g) was extracted with MeOH (3.5 l x 2) at 60 °C. The methanolic solution was then evaporated to dryness under reduced pressure and gave 28 g of a dark residue. This residue was suspended in H₂O (350 mL), and successively partitioned with *n*-hexane (200 mL x 2), EtOAc (200 mL x 2) and *n*-BuOH saturated with H₂O (200 mL x 3). The *n*-BuOH extract (7.7g) was subjected to vacuum liquid chromatography (VLC) using reversed- phase material (Lichroprep RP-18, 25-40 µm, 150 g) employing H₂O (1000 mL), H₂O–MeOH (8:2, 1200 mL; 6:4, 2400 mL; 4:6, 3000 mL; 2:8, 1600 mL) and MeOH (600 mL) to give ten main fractions (A-J). Fraction A (780 mg) was submitted to silica gel (100 g) column chromatography with the solvent system CHCl₃-MeOH-H₂O (80:20:2, 1500 mL; 70:30:3, 2000 mL) to give 7 subfractions. Subfraction 1 (120 mg) was chromatographed on a reversed phase material (Lichroprep RP-18, 25-40 µm, 25 g), employing MeOH-H₂O (8:2, 500 mL) yielding **AAN8** (15 mg) and **AAN13** (9 mg). Subfraction 2 (58 mg) was purified on a reversed phase column (Lichroprep RP-18, 25-40 µm, 25 g) and eluted with MeOH-H₂O (6:4, 500 mL) to give **7** (1.1 mg). Fraction B (300 mg) was subjected to silica gel (42 g) column chromatography with the solvent system EtOAc-MeOH-H₂O (100:17.5:13.5, 800 mL) to yield **AAN14** (10 mg) and **AAN9** (10 mg). Fraction D (240 mg) was submitted to silica gel (42 g) column chromatography, eluted with CHCl₃-MeOH-H₂O (80:20:2, 1500 mL; 70:30:3, 2000 mL) to give **AAN6** (2 mg), **AAN11** (1 mg), **AAN10** (1.2 mg), **AAN5** (23 mg) and **AAN12** (9 mg). Fraction E (60 mg) was subjected to silica gel (38 g) column chromatography with the solvent system CHCl₃-MeOH-H₂O (90:10:1, 600 mL; 80:20:2, 1500 mL) to yield **AAN3** (9 mg) and **AAN1** (7 mg). Fraction F (160 mg) was submitted to silica gel (38 g) column chromatography with the solvent system CHCl₃-MeOH-H₂O (90:10:1, 100 mL; 80:20:2, 1500 mL; 70:30:3, 2000 mL) to give **AAN4** (4 mg), **AAN2** (12 mg), **AAN15** (10 mg).



Scheme 2.3. Isolation of the new triterpenes from *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*

3. RESULTS AND DISCUSSION

3.1. *Astragalus aureus*

3.1.1. Structural Identification of Compound AAU1

The HRMALDITOF mass spectrum of **AAU1** (m/z 1057.5563 $[M+Na]^+$, calcd for $C_{51}H_{86}O_{21}Na$, 1057.5559) supported a molecular formula of $C_{51}H_{86}O_{21}$. The ESIMS spectrum showed a major ion peak at m/z 1057.65 which was assigned to $[M+Na]^+$. The MS/MS of this ion showed a peak at m/z 907.53 $[M+Na-150]^+$, corresponding to the loss of a pentose unit. In the MS^3 spectrum peaks at m/z 761.46 $[M+Na-150-146]^+$, corresponding to the loss of a deoxy-hexose unit, and 629.42 $[M+Na-150-146-132]^+$, due to the loss of a pentose unit, were observed. A detailed comparison of the NMR data (^1H , ^{13}C , HSQC, HMBC, COSY) of compounds **AAU1–AAU5** showed that the aglycon moiety was identical in all five compounds. In particular, the ^1H NMR spectrum of **AAU1** showed signals due to a cyclopropane methylene at δ 0.59 and 0.25 (each 1H, d, J = 4.2 Hz), six tertiary methyl groups at δ 1.28 (3H, s), 1.20 (3H, s), 1.18 (3H, s), 1.17 (3H, s), 1.03 (3H, s) and 1.01 (3H, s), a secondary methyl group at δ 0.98 (d, J = 6.5 Hz), and four methine proton signals at δ 4.48 (ddd, J = 8.0, 8.0, 5.2 Hz), 3.54 (ddd, J = 9.5, 9.5, 4.5 Hz), 3.41 (dd, J = 10.5, 2.4 Hz) and 3.23 (dd, J = 11.3, 4.0 Hz), which were indicative of secondary alcoholic functions. The NMR data of the aglycon moiety of **AAU1** were in good agreement with those reported for cyclocanthogenin with glycosidation shifts for C-3 (δ 89.6) and C-6 (δ 79.0) (Isaev et al., 1992). The ^{13}C -NMR chemical shift for C-24 can be regarded as a characteristic parameter in the determination of the configurations of C-24. In the case of the 24R configuration, the chemical shift for C-24 gives resonance at 79.9–80.6 ppm (Isaev et al., 1983, 1982; Agzamova and Isaev; 1998), while C-24 gives resonance at 77.0–78.2 ppm for the 24S configuration (Isaev et al., 1992; Bedir et al., 2000c; Hirotani et al., 1994a,b; Fadeev et al., 1988). The ^{13}C NMR data of compound **AAU1** are comparable to those reported for analogous compounds having a 24S configuration. The relative configurations of the oxygenated carbons were determined by the multiplicity of the vicinal proton–proton coupling constants to be β -OH for C-3 (δ 3.23, dd, J = 11.3, 4.0 Hz, H_{ax} -3), α -OH for C-6 (δ 3.54, ddd, J = 9.5, 9.5, 4.5 Hz, H_{ax} -6) and β -OH for C-16 (δ 4.48, ddd, J = 8.0, 8.0, 5.2 Hz, H_{ax} -16). Additionally, the relative configuration of C-20 was derived by the ROESY spectrum that showed key correlations between Me-

30 α (δ 1.01), H- 16 α (δ 4.48) and H-17 α (δ 1.74) signals, and between H-17 α and Me-21 α (δ 0.98) signals.

For compound **AAU1**, a secondary methyl group at δ 1.31 (d, J = 6.5 Hz) along with four anomeric protons at δ 5.06 (d, J = 1.2 Hz), 4.98 (d, J = 3.7 Hz), 4.49 (d, J = 7.5 Hz) and 4.33 (d, J = 7.5 Hz) were additionally observed. The chemical shifts of all the individual protons of the four sugar units were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analyses, and the ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (**Table 3.1**). These data showed the presence of one α -rhamnopyranosyl unit (δ 5.06), two β -xylopyranosyl units (δ 4.49 and 4.33) and one α -arabinopyranosyl unit (δ 4.98). The α configuration of the rhamnose unit was deduced from the H-1/C-1 J value = 169 Hz, measured from the residual direct correlation observed in the HMBC spectrum, in agreement with that reported for the α anomer of rhamnopyranose (Kasai et al., 1979).

For the L-arabinopyranosyl unit the value of $J_{\text{H1-H2}}$ coupling constant has been reported not to be diagnostic on its own, owing to the high conformational mobility of arabinopyranosides ($^4\text{C1} \rightarrow ^1\text{C4}$) (Breitmaier and Voelter, 1987). Evidence supporting an α -L-arabinopyranoside configuration in rapid conformational exchange was obtained from ROESY experiments. NOe's were observed from H_{ara}-1 to H_{ara}-2 and H_{ara}-1 to H_{ara}-3 as expected for $^1\text{C4}$ and $^4\text{C1}$ conformations respectively. The nOe H_{ara}-1-H_{ara}-3 would not be expected for both $^1\text{C4}$ - $^4\text{C1}$ - β -L-arabinopyranosides. An nOe was also observed between H_{ara}-1 and H_{ara}-5 as expected for an α -L-arabinopyranoside in a $^4\text{C1}$ conformation.

Glycosidation shifts were observed for C-2_{xyI} I (δ 79.9) and C- 2_{ara} (δ 75.9). An unambiguous determination of the sequence and linkage sites was obtained from the HMBC spectrum, which showed key correlation peaks between the proton signal at δ 4.49 (H-1_{xyI} I) and the carbon resonance at δ 89.6 (C-3), δ 4.98 (H-1_{ara}) and δ 79.9 (C-2_{xyI}), δ 5.06 (H-1_{rha}) and δ 75.9 (C-2_{ara}), and the proton signal at δ 4.33 (H-1_{xyI} II) and the carbon resonance at δ 79.0 (C-6). The D configuration of xylose unit and the L configuration of arabinose and rhamnose units were established after hydrolysis of **AAU1** with 1 N HCl, trimethylsilation and determination of the retention times by GC (De Marino et al., 2003).

On the basis of all these evidence, the structure of the new compound **AAU1** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-6-*O*- β -D-xylopyranosyl 3 β ,6 α ,16 β ,24(S),25pentahydroxycycloartane (**Fig. 3.1**).

Amorphous white solid; C₅₁H₈₆O₂₁; [α]_D²⁵ +37.2° (c 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3474 (>OH), 3035 (cyclopropane ring), 2953 (>CH), 1255 and 1068 (C–O–C); ESI–MS *m/z* 1057.65 [M + Na]⁺; MS/MS *m/z* 907.53 [M + Na-150]⁺, MS3 *m/z* 761.46 [M + Na-150–146]⁺, 629.40 [M + Na-150–146–132]⁺; HRMALDITOFMS [M + Na]⁺ *m/z* 1057.5563 (calcd for C₅₁H₈₆O₂₁Na, 1057.5559). ¹H (CD₃OD, 600 MHz) and ¹³C NMR (CD₃OD, 150 MHz) data: see **Table 3.1**.

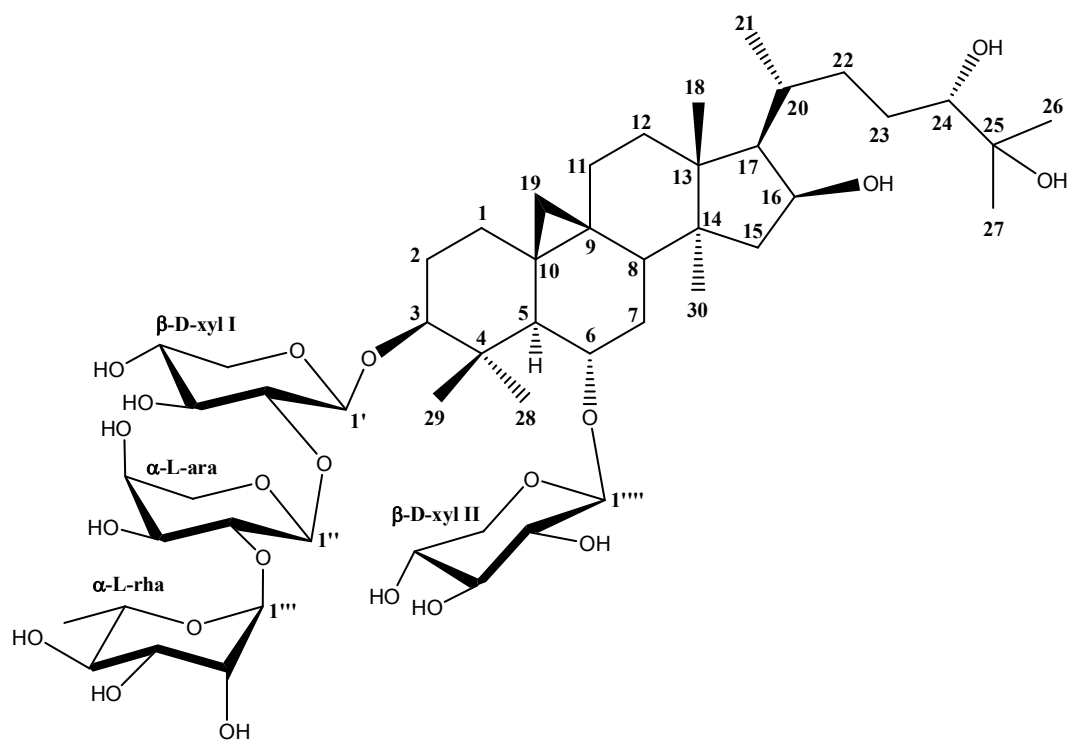
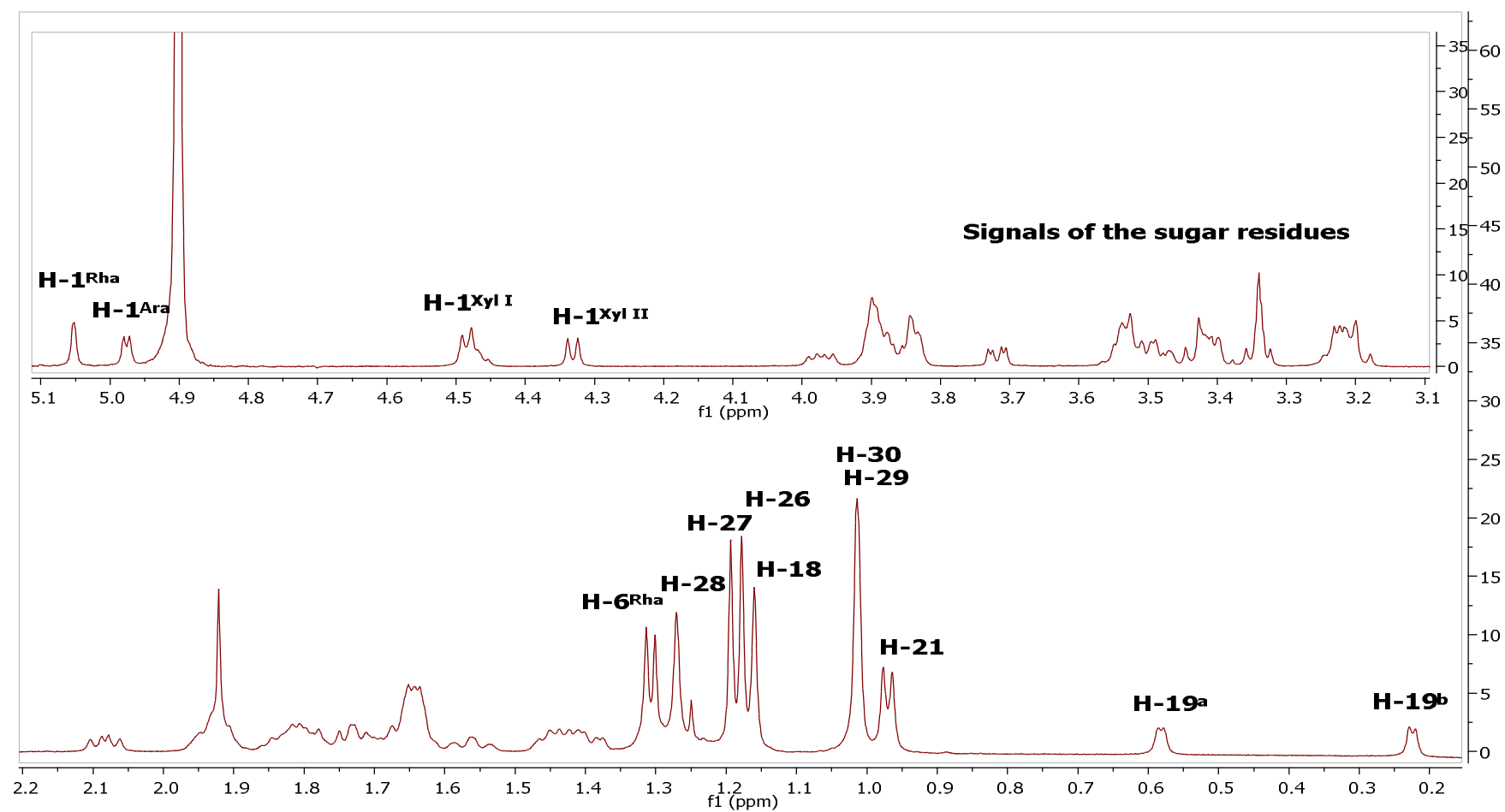


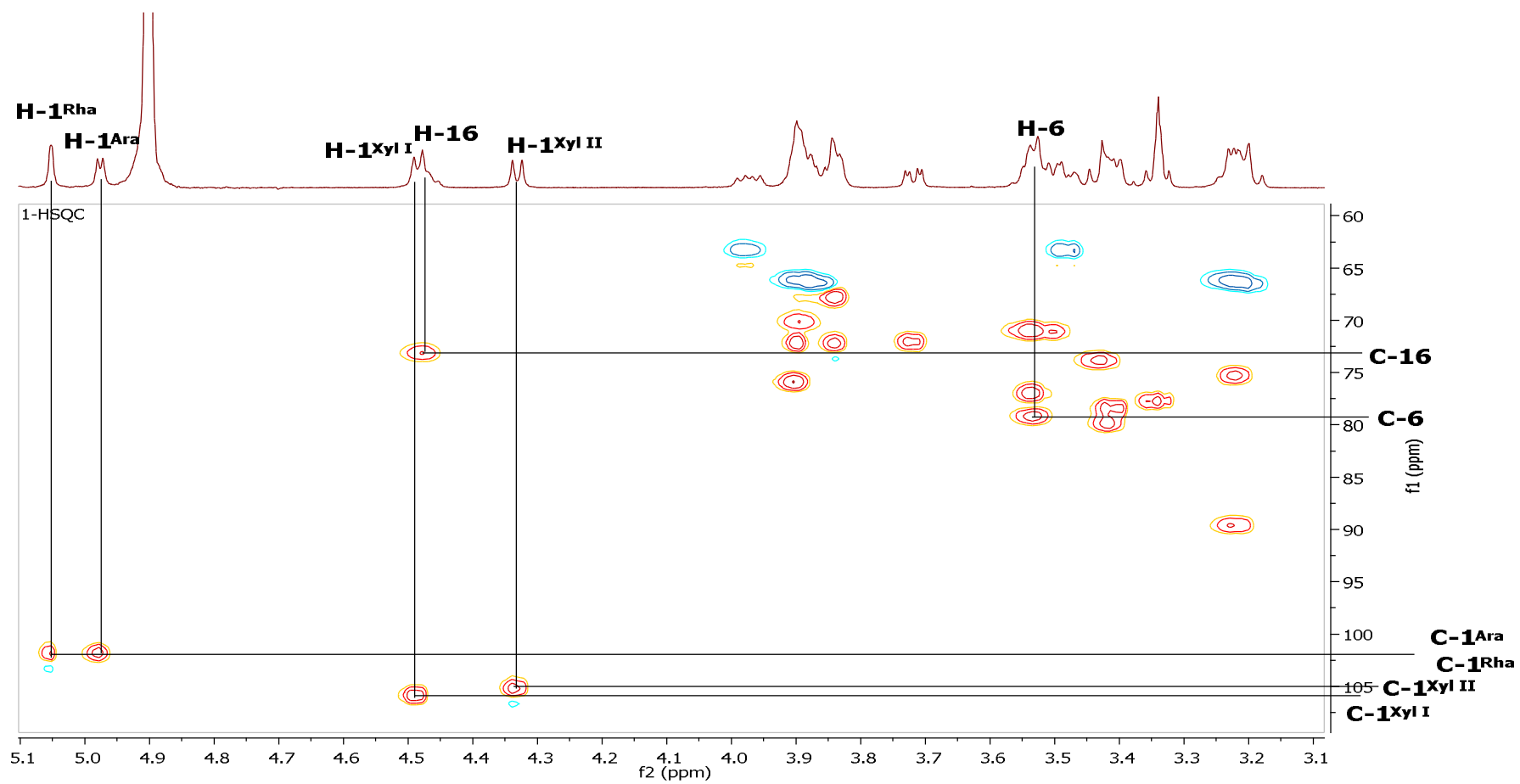
Figure 3.1. Structure of AAU1

Table 3.1. ^1H and ^{13}C Assignments of compound of AAU1 (150/600 MHz, δ ppm, in CD_3OD)

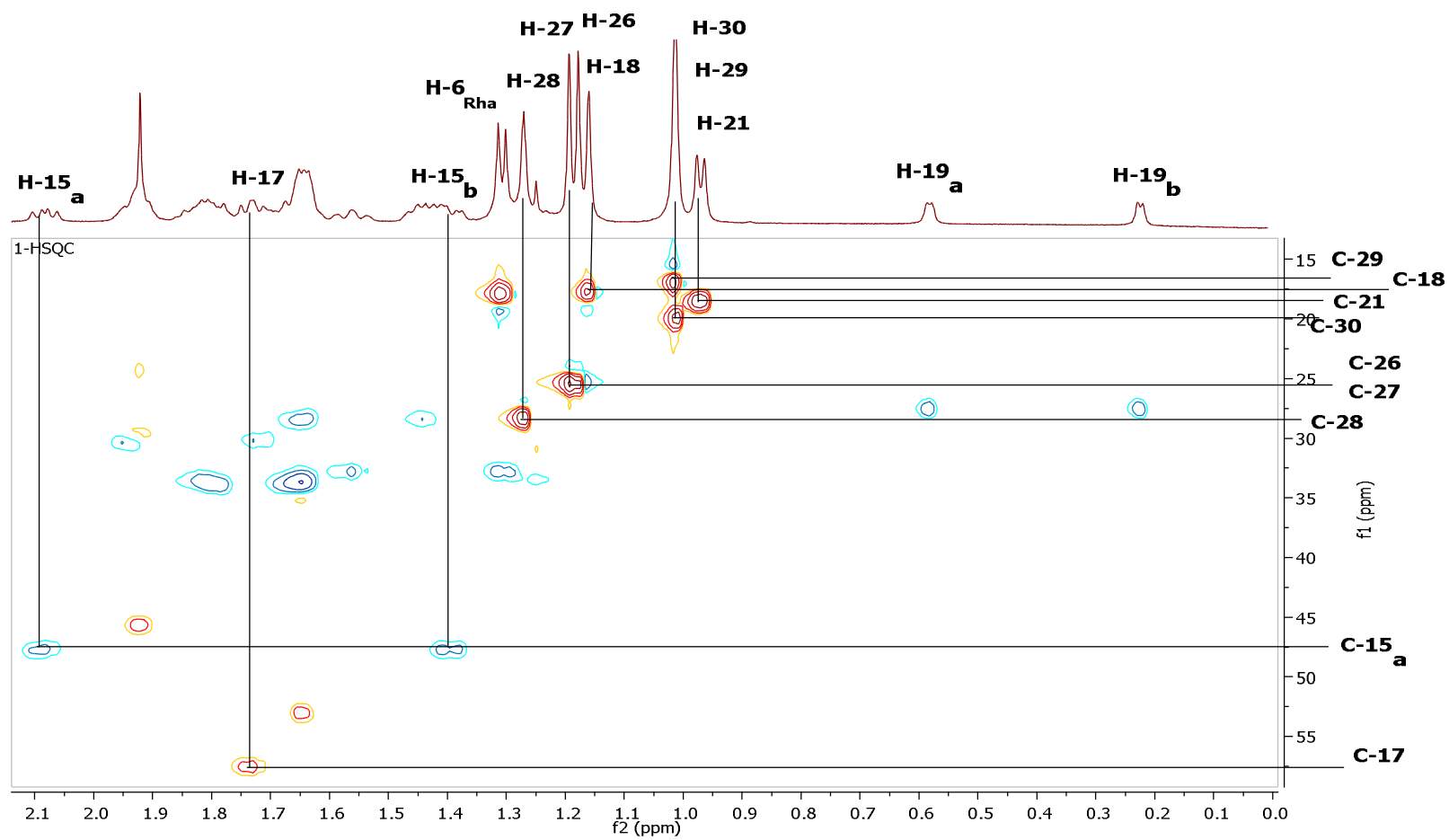
Position	δ_{C}	δ_{H} (J in Hz)
1	32.7	1.57, 1.31, m
2	30.2	1.97, 1.70, m
3	89.6	3.23, dd (11.3, 4.0)
4	42.5	-
5	52.9	1.66, d (9.5)
6	79.0	3.54, ddd (9.5, 9.5, 4.5)
7	34.3	1.80, 1.65, m
8	45.6	1.92, dd (11.9, 4.2)
9	21.9	-
10	29.1	-
11	26.8	1.85, 1.43, m
12	33.8	1.66, (2H) m
13	46.6	-
14	47.0	-
15	47.7	2.09, dd (12.7, 8.0) 1.40, dd (12.7, 5.2)
16	73.0	4.48, ddd (8.0, 8.0, 5.2)
17	57.6	1.74, dd (9.9, 8.0)
18	18.0	1.17, s
19	27.7	0.59, d (4.2), 0.25, d (4.2)
20	29.8	1.93, m
21	18.5	0.98, d (6.5)
22	34.1	1.81, 1.25, m
23	28.3	1.65, 1.44, m
24	78.1	3.41, dd (10.5, 2.4)
25	73.5	-
26	25.2	1.18, s
27	25.2	1.20, s
28	28.0	1.28, s
29	16.6	1.03, s
30	19.9	1.01, s
β -D-Xyl I (at C-3)-1'	105.9	4.49, d (7.5)
2'	79.9	3.42, dd (9.2, 7.5)
3'	77.0	3.54, t (9.2)
4'	71.0	3.54, m
5'	66.2	3.90, dd (11.7, 5.2); 3.23, t (11.7)
α -L-Ara (at C-2 _{xyl})-1''	101.8	4.98, d (3.7)
2''	75.9	3.90, dd (8.5, 3.7)
3''	72.1	3.84, dd (8.5, 3.0)
4''	67.9	3.84, m
5''	63.2	3.97, dd (11.9, 2.0); 3.48, dd (11.9, 3.0)
α -L-Rha (at C-2 _{ara})-1'''	101.8	5.06, d (1.2)
2'''	72.1	3.90, dd (1.2, 3.2)
3'''	72.1	3.72, dd (3.2, 9.3)
4'''	73.8	3.43, t (9.3)
5'''	70.1	3.89, m
6'''	17.9	1.31, d (6.5)
β -D-Xyl II (at C-6)-1''''	105.2	4.33, d (7.5)
2''''	75.4	3.22, dd (9.2, 7.5)
3''''	77.7	3.34, t (9.2)
4''''	71.0	3.50, m
5''''	66.3	3.86, dd (11.7, 5.2); 3.20, t (11.7)



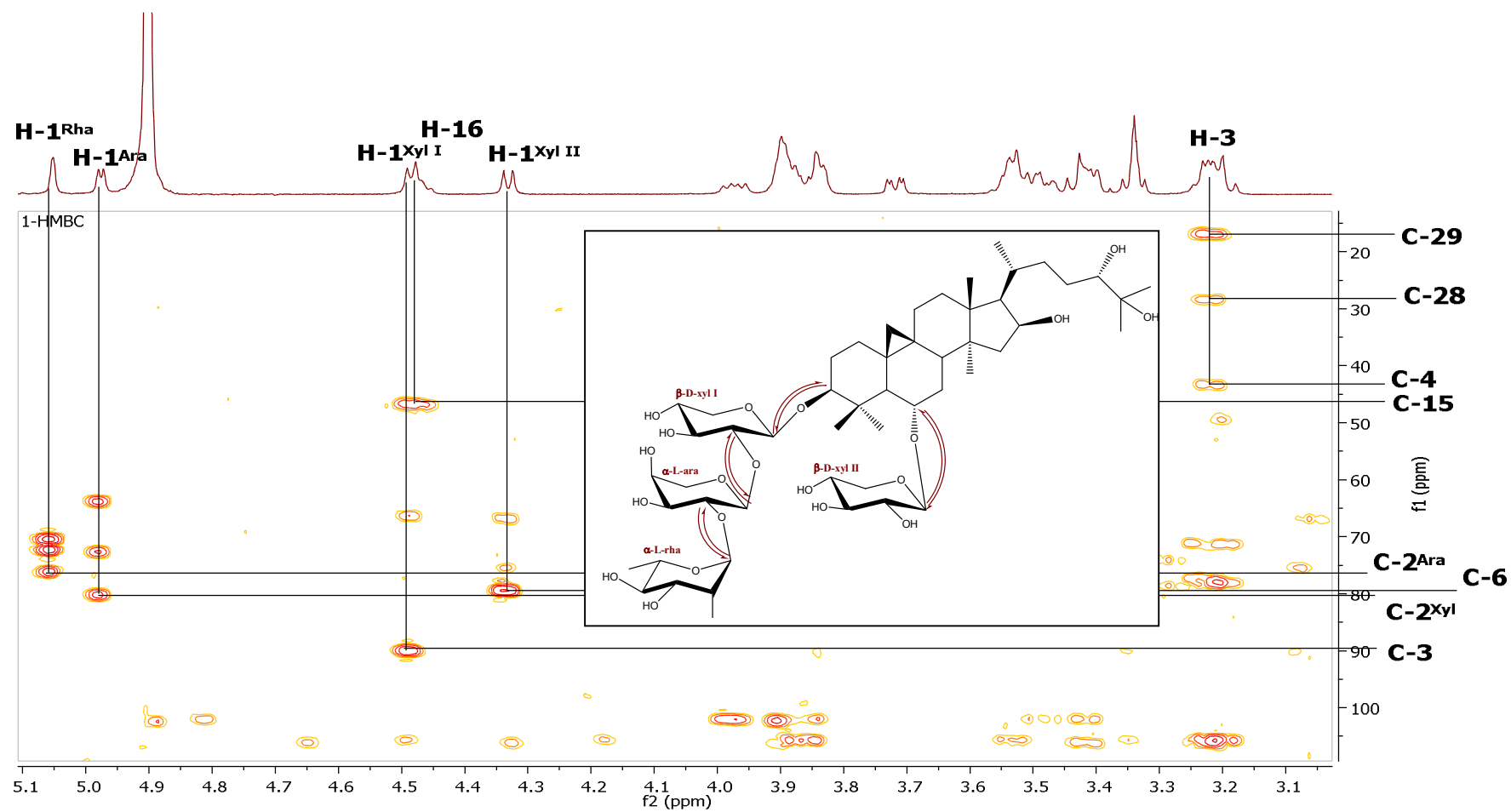
Spectrum 3.1. ^1H NMR Spectrum of AAU1 (600 MHz, CD_3OD).



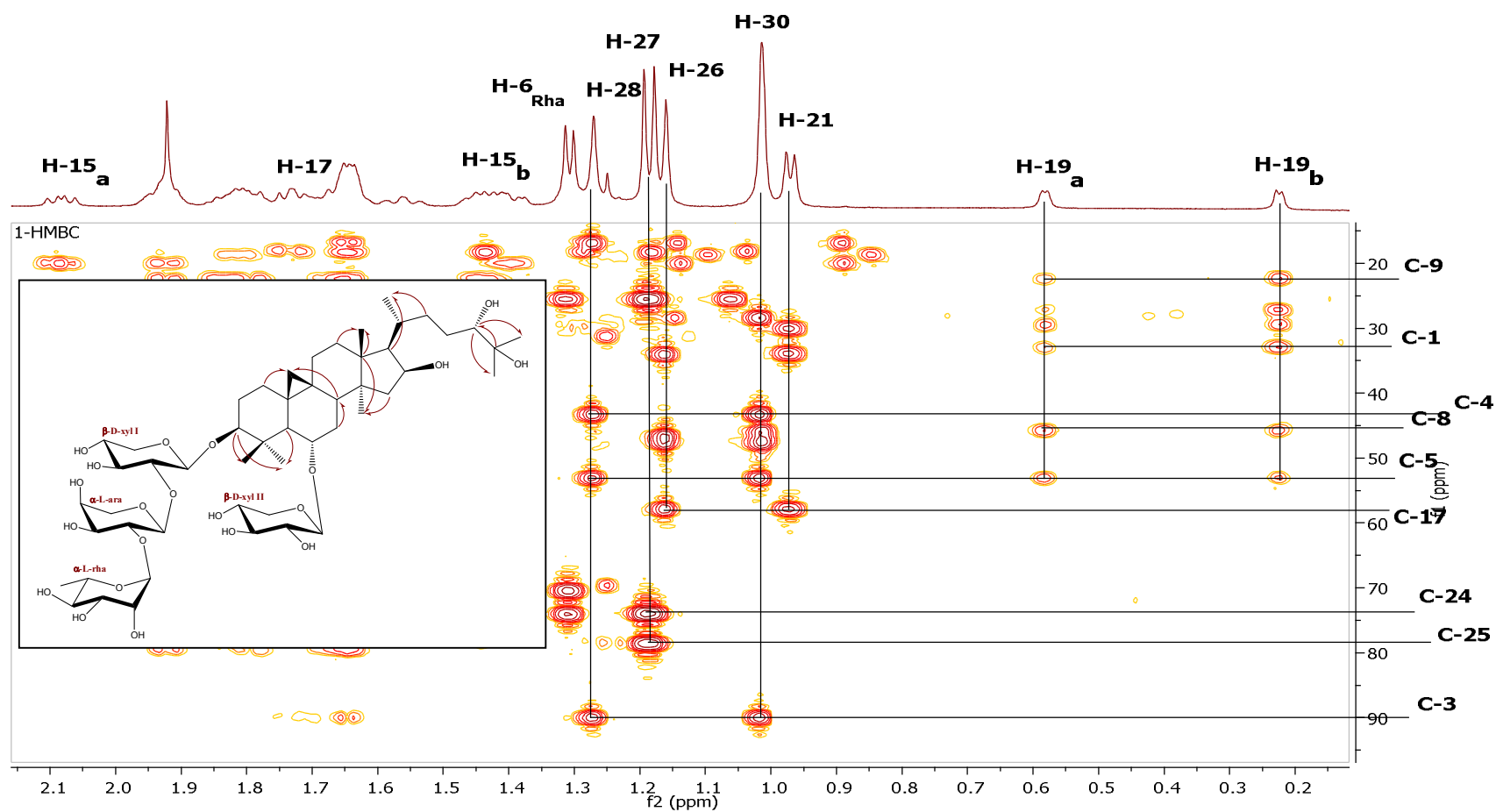
Spectrum 3.2. HSQC Spectrum of AAU1



Spectrum 3.3. HSQC Spectrum of AAU1



Spectrum 3.4. HMBC Spectrum of AAU1



Spectrum 3.5. HMBC Spectrum of AAU1

3.1.2. Structural Identification of Compound AAU2

The molecular formula of compound **AAU2** was established as $C_{40}H_{68}O_{13}$ by HRMALDITOFMS analysis (m/z 779.4561 $[M + Na]^+$, calcd for $C_{40}H_{68}O_{13}Na$, 779.4558). The ^1H NMR spectrum of **AAU2** showed signals for two anomeric protons at δ 4.31 (d, $J = 7.5$ Hz) and 4.32 (d, $J = 7.5$ Hz). The HSQC, HMBC, DQF-COSY and 1D-TOCSY data led to identify these sugar units as two β -xylopyranosyl units. The HMBC correlations between the proton signal at δ 4.31 ($H-1_{xyl I}$) and the carbon resonance at δ 89.6 (C-3) and the proton signal at δ 4.32 ($H-1_{xyl II}$) and the carbon resonance at δ 79.0 (C-6) allowed us to determine the linkage site of the sugar units. Thus, the new compound **AAU2** was elucidated as 3,6-di- O - β -D-xylopyranosyl-3 β ,6 α ,16 β ,24(S),25-pentahydroxycycloartane. (**Fig 3.2**)

Amorphous white solid; $C_{40}H_{68}O_{13}$; $[\alpha]_D^{25} +29.2^\circ$ (c 0.01 MeOH); IR $\nu_{max}^{KBr} cm^{-1}$: 3487 ($>OH$), 3043 (cyclopropane ring), 2950 ($>CH$), 1269 and 1059 (C–O–C); ESI–MS m/z 779.54 $[M + Na]^+$, MS/MS m/z 629.38 $[M + Na-150]^+$, 479.38 $[M + Na-150-150]^+$; HRMALDITOFMS $[M + Na]^+$ m/z 779.4561 (calcd for $C_{40}H_{68}O_{13}Na$, 779.4558). ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.2**.

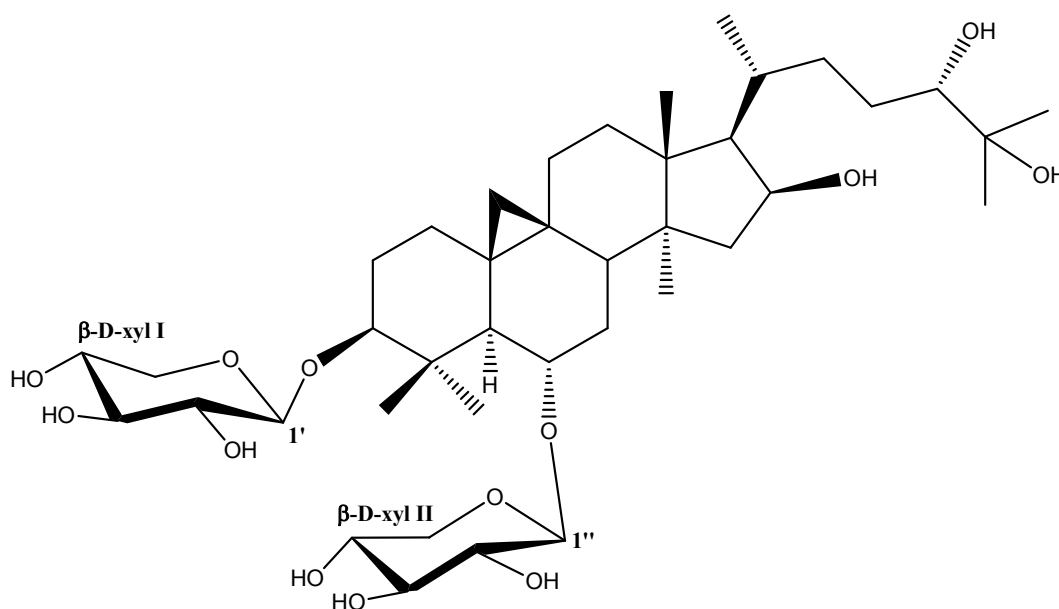
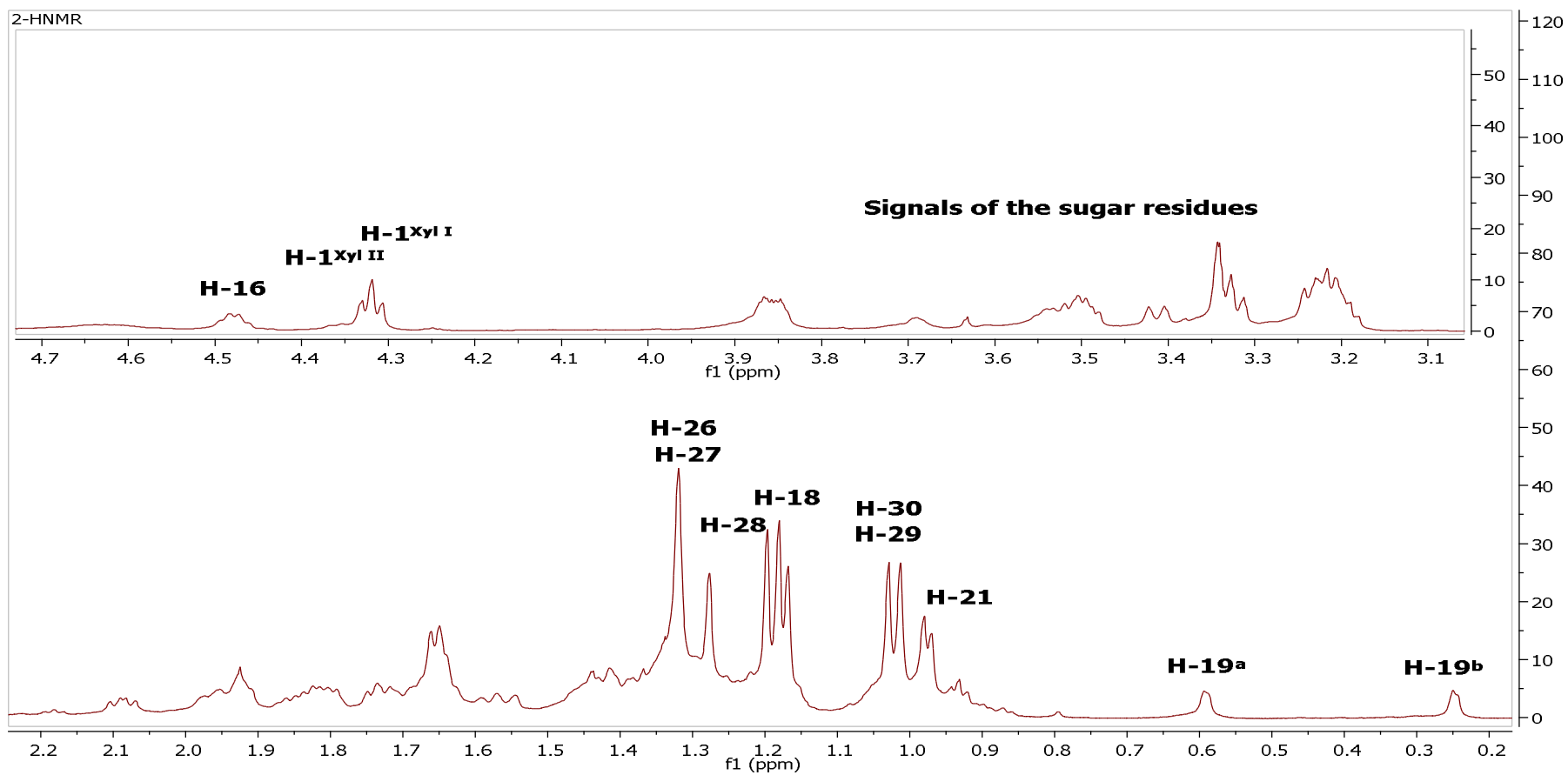


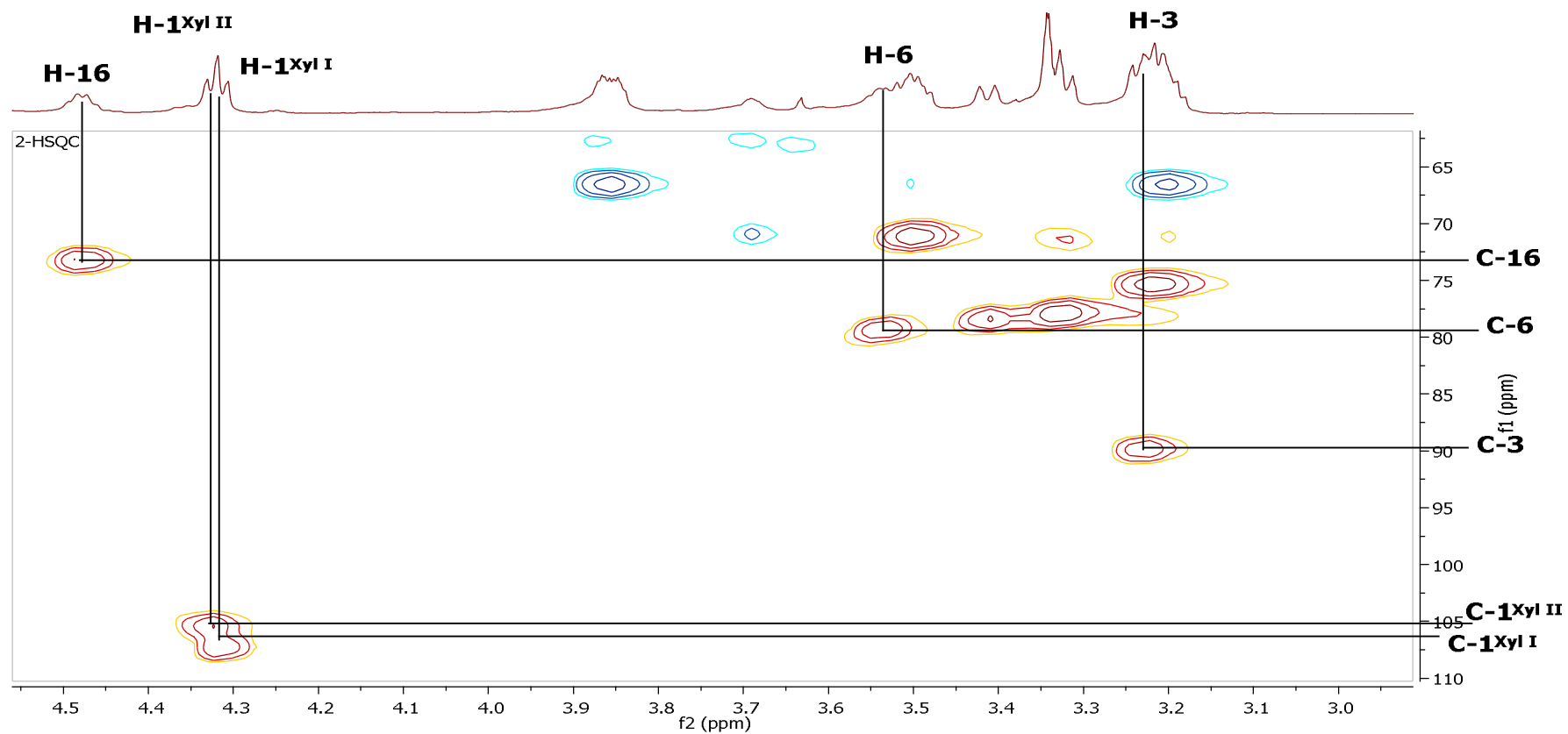
Figure 3.2. Structure of AAU2

Table 3.2. ^1H and ^{13}C Assignments of AAU2 (150/600 MHz, δ ppm, in CD_3OD)

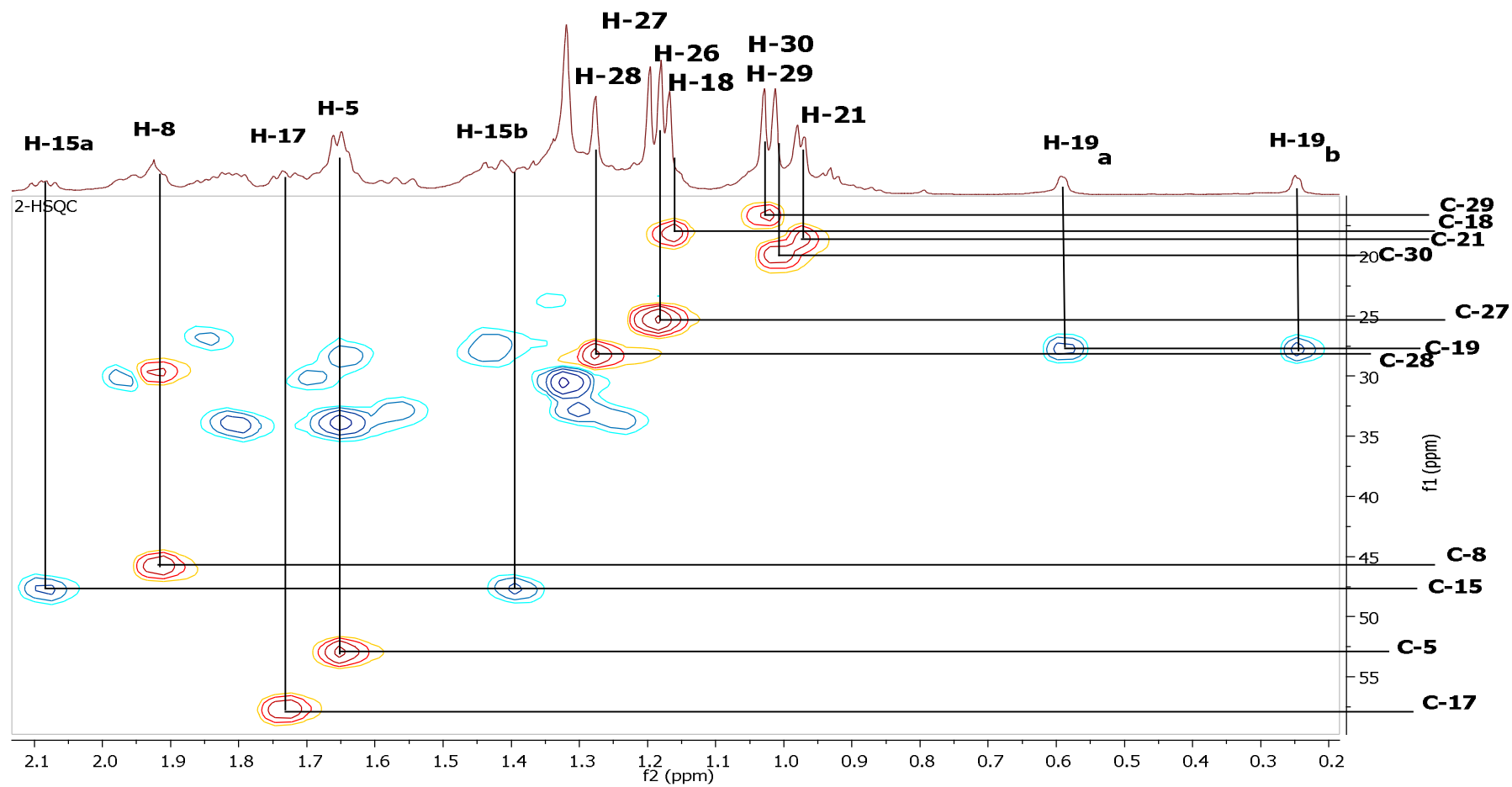
Position	δ_{C}	δ_{H} (J in Hz)
1	32.7	1.56, 1.30, m
2	30.1	1.96, 1.70, m
3	89.5	3.22, dd (11.3, 4.0)
4	42.5	-
5	52.9	1.66, d (9.5)
6	79.2	3.53, ddd (9.5, 9.5, 4.5)
7	34.3	1.80, 1.65, m
8	45.5	1.91, dd (11.9, 4.2)
9	21.9	-
10	29.1	-
11	26.7	1.85, 1.43, m
12	33.8	1.66, (2H) m
13	46.6	-
14	47.0	-
15	47.6	2.08, dd (12.7, 8.0) 1.40, dd (12.7, 5.2)
16	73.1	4.47, ddd (8.0, 8.0, 5.2)
17	57.5	1.74, dd (9.9, 8.0)
18	18.0	1.17, s
19	27.7	0.59, brs 0.25, brs
20	29.8	1.92, m
21	18.5	0.98, d (6.5)
22	34.1	1.80, 1.25, m
23	28.3	1.65, 1.44, m
24	78.2	3.41, dd (10.5, 2.4)
25	73.5	-
26	25.2	1.18, s
27	25.2	1.20, s
28	28.0	1.28, s
29	16.5	1.03, s
30	19.9	1.01, s
$\beta\text{-D-Xyl I (at C-3)-1'}$	106.9	4.31, d (7.5)
2'	75.1	3.22, dd (9.2, 7.5)
3'	77.7	3.33, t (9.2)
4'	71.0	3.50, m
5'	66.5	3.86, dd (11.7, 5.2), 3.20, t(11.7)
$\beta\text{-D-Xyl II (at C-6)-1''}$	105.1	4.32, d (7.5)
2''	75.1	3.22, dd (9.2, 7.5)
3''	77.7	3.33, t (9.2)
4''	71.0	3.50, m
5''	66.5	3.86, dd (11.7, 5.2) 3.20, t (11.7)



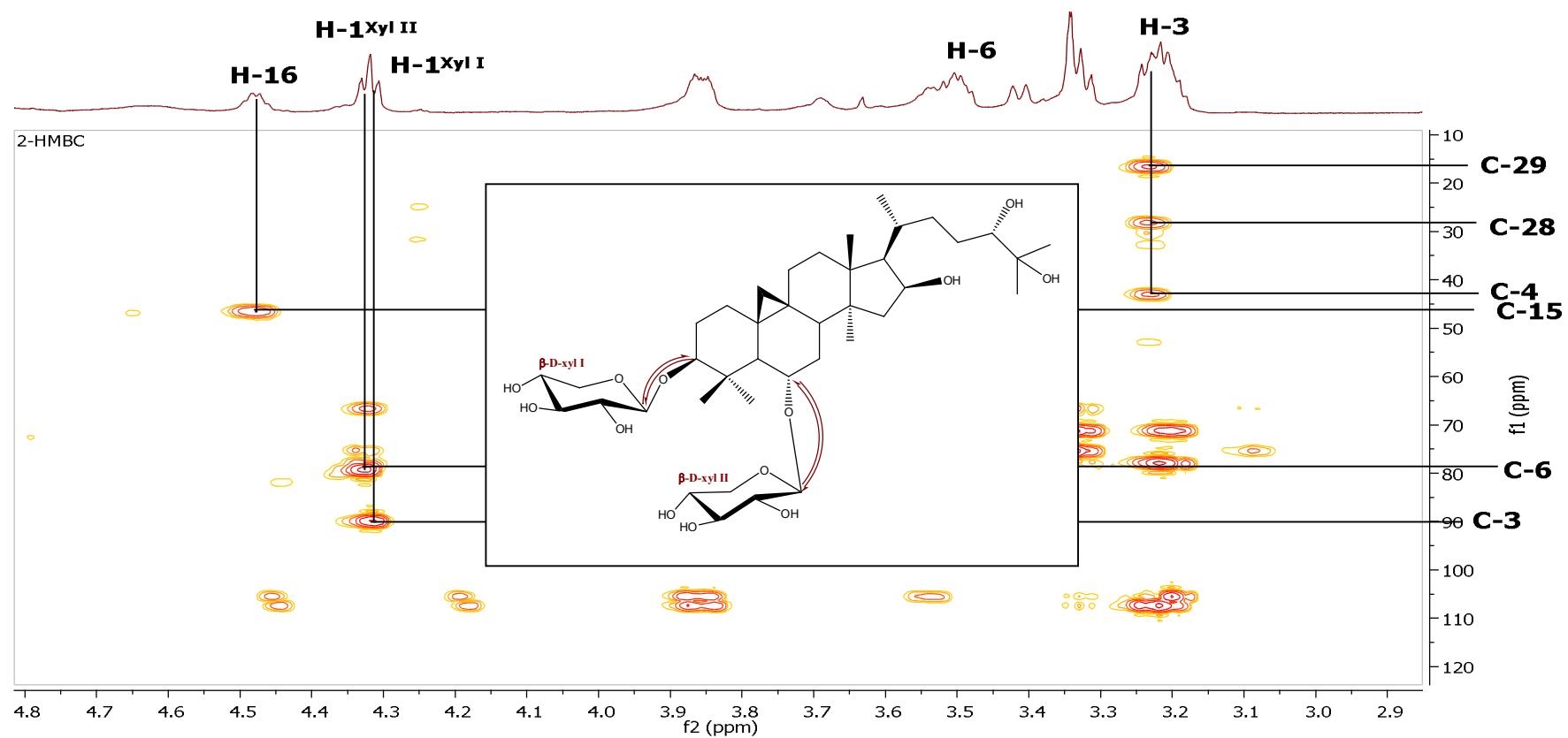
Spectrum 3.6. ^1H NMR Spectrum of AAU2 (600 MHz, CD_3OD)



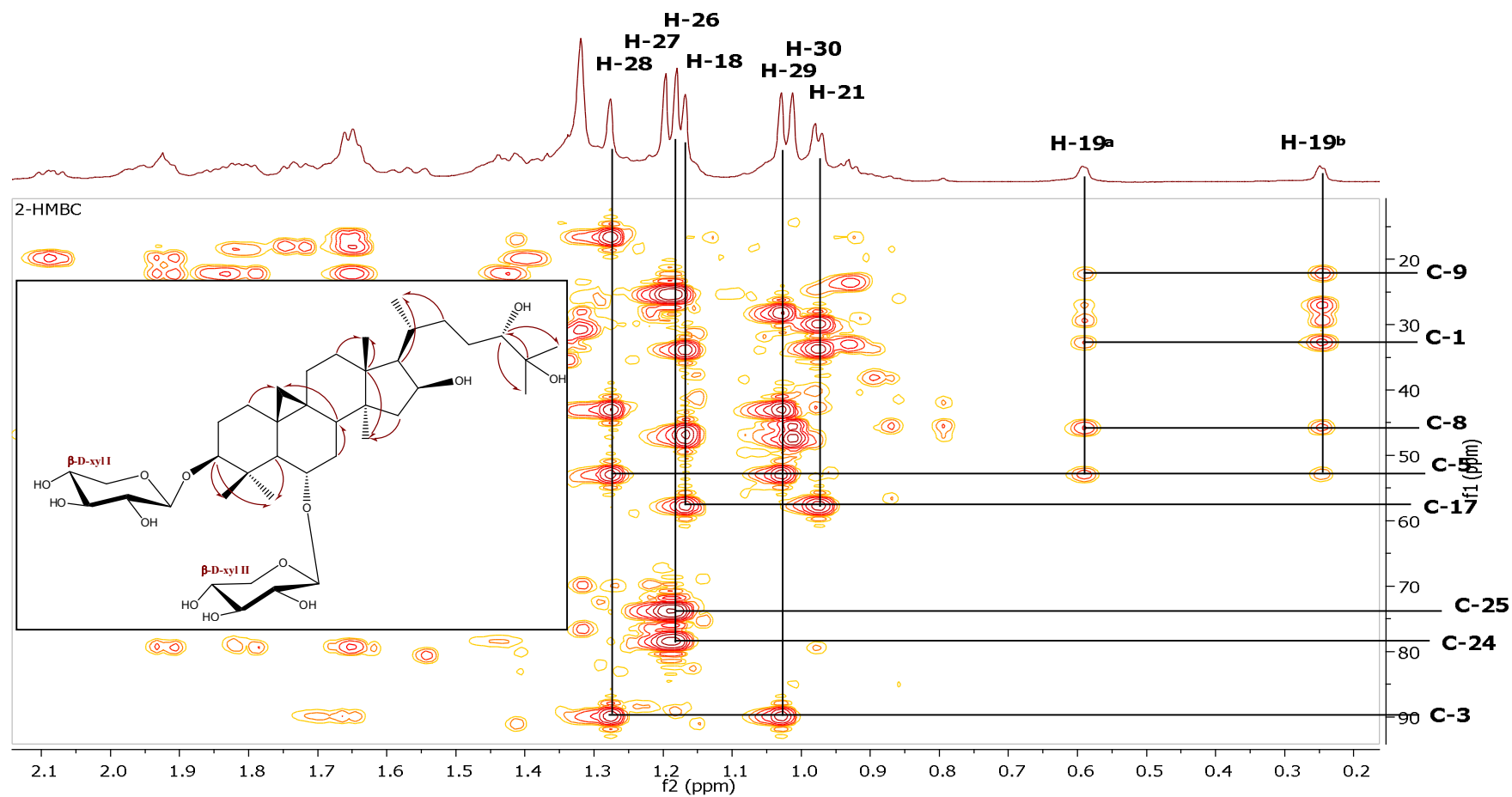
Spectrum 3.7. HSQC Spectrum of AAU2



Spectrum 3.8. HSQC Spectrum of AAU2



Spectrum 3.9. HMBC Spectrum of AAU2



Spectrum 3.10. HMBC Spectrum of AAU2

3.1.3. Structural Identification of Compound AAU3

The HRMALDITOF mass spectrum of **AAU3** (m/z 941.5090 $[M + Na]^+$, calcd for $C_{46}H_{78}O_{18}Na$, 941.5086) supported a molecular formula of $C_{46}H_{78}O_{18}$.

The ^1H NMR spectrum of **AAU3** displayed, in addition to signals of the aglycon moiety, signals for three anomeric protons at δ 4.55 (d, $J = 7.5$ Hz), 4.33 (d, $J = 7.5$ Hz) and 4.31 (d, $J = 7.5$ Hz). On the basis of HSQC, HMBC, DQF-COSY and 1D-TOCSY correlations two β -xylopyranosyl units (δ 4.33 and 4.31) and one β -glucopyranosyl (δ 4.55) were identified. Glycosidation shifts for the aglycon moiety were observed for C-3 (δ 89.6), C-6 (δ 79.0) and C-25 (δ 81.2). Key correlation peaks in the HMBC spectrum were observed between the proton signal at δ 4.31 ($H-1_{xyI}$) and the carbon resonance at δ 89.6 (C-3), δ 4.33 ($H-1_{xyII}$) and δ 79.0 (C-6), and between the proton signal at δ 4.55 ($H-1_{glc}$) and the carbon resonance at δ 81.2 (C-25). The configuration of glucose and xylose was determined as D via hydrolysis followed by GC analysis.

Therefore, the structure of **AAU3** was established as 3,6-di-*O*- β -D-xylopyranosyl-25-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(S),25 pentahydroxycycloartane accommodating a very rare chemical feature in nature, a tridesmosidic arrangement. (**Fig. 3.3**)

Amorphous white solid; $C_{46}H_{78}O_{18}$; $[\alpha]^{25}_D +35.6^\circ$ (c 0.01 MeOH); IR $\nu^{KBr}_{max} cm^{-1}$: 3482 ($>OH$), 3040 (cyclopropane ring), 2945 ($>CH$), 1259 and 1051 (C-O-C); ESI-MS m/z 941.59 $[M+Na]^+$, MS/MS m/z 791.43 $[M+Na-150]^+$, MS^3 m/z 611.37 $[M+Na-150-180]^+$, MS^4 m/z 479.31 $[M+Na-150-180-132]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 941.5090 (calcd for $C_{46}H_{78}O_{18}Na$, 941.5086); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.3**.

Table 3.3. ^1H and ^{13}C Assignments of AAU3 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	32.6	1.57, 1.31, m
2	30.1	1.96, 1.70, m
3	89.6	3.23, dd (11.3, 4.0)
4	42.7	-
5	52.7	1.66, d (9.5)
6	79.0	3.54, ddd (9.5, 9.5, 4.5)
7	34.0	1.93, 1.65, m
8	45.7	1.92, dd (11.9, 4.2)
9	22.0	-
10	29.0	-
11	26.6	1.85, 1.44, m
12	33.7	1.66, (2H) m
13	46.3	-
14	47.2	-
15	47.4	2.08, dd (12.7, 8.0), 1.40, dd (12.7, 5.2)
16	72.9	4.48, ddd (8.0, 8.0, 5.2)
17	57.6	1.73, dd (9.9, 8.0)
18	17.9	1.17, s
19	27.4	0.59, brs
20	30.0	0.24, brs
21	18.3	1.90, m
22	18.3	0.97, d (6.5)
23	34.0	1.80, 1.25, m
24	28.4	1.59, 1.52, m
25	76.5	3.61, dd (10.5, 2.4)
26	81.2	-
27	22.4	1.25, s
28	23.0	1.29, s
29	28.0	1.27, s
30	16.3	1.03, s
31	19.6	1.01, s
$\beta\text{-D-Xyl I (at C-3)-1'}$	107.0	4.31, d (7.5)
2''	75.1	3.22, dd (9.2, 7.5)
3''	77.6	3.32, t (9.2)
4''	70.9	3.49, m
5''	66.3	3.86, dd (11.7, 5.2), 3.20, t (11.7)
$\beta\text{-D-Xyl II (at C-6)-1''}$	101.8	4.33, d (7.5)
2''	75.9	3.20, dd (9.2, 7.5)
3''	72.1	3.32, t (9.2)
4''	67.9	3.49, m
5''	63.2	3.86, dd (11.7, 5.2), 3.20, t (11.7)
$\beta\text{-D-Glc (at C-25)-1'''}$	97.6	4.55, d (7.5)
2'''	75.0	3.19, dd (7.5, 9.0)
3'''	77.9	3.40, t (9.0)
4'''	71.3	3.31, t (9.0)
5'''	77.5	3.31, ddd (3.5, 4.5, 9.0)
6'''	62.4	3.86, dd (3.5, 12.0), 3.68, dd (4.5, 12.0)

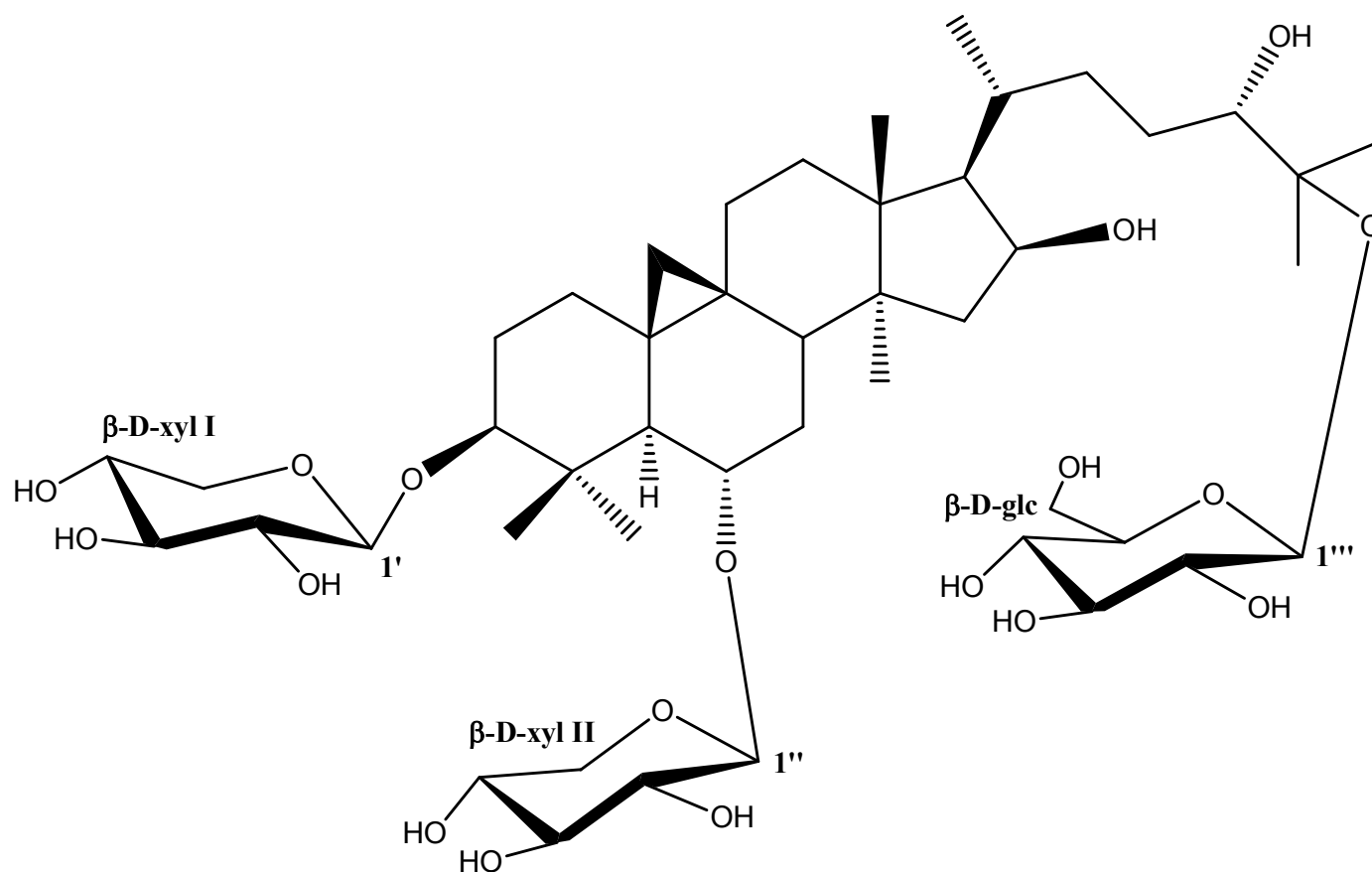
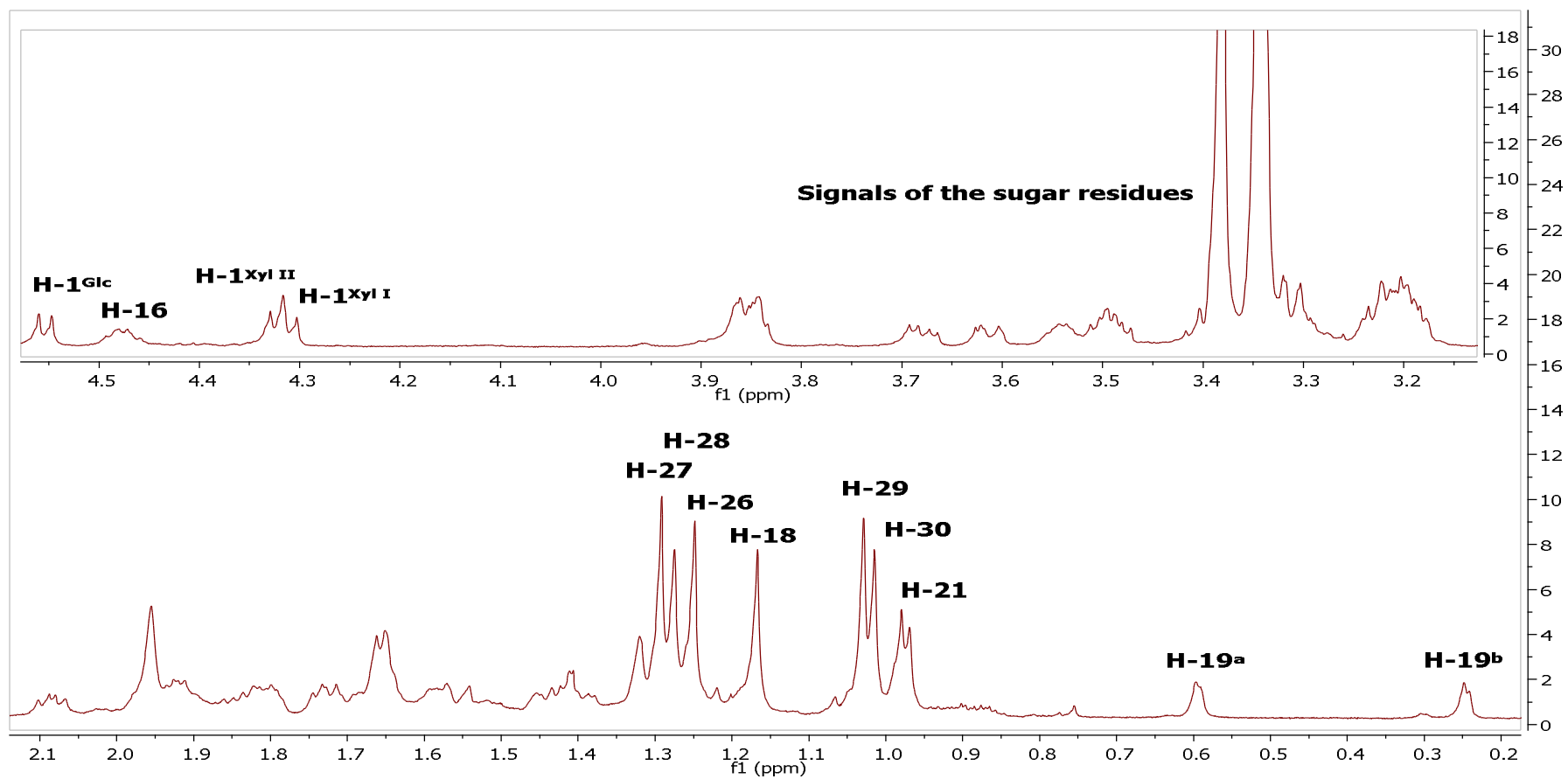
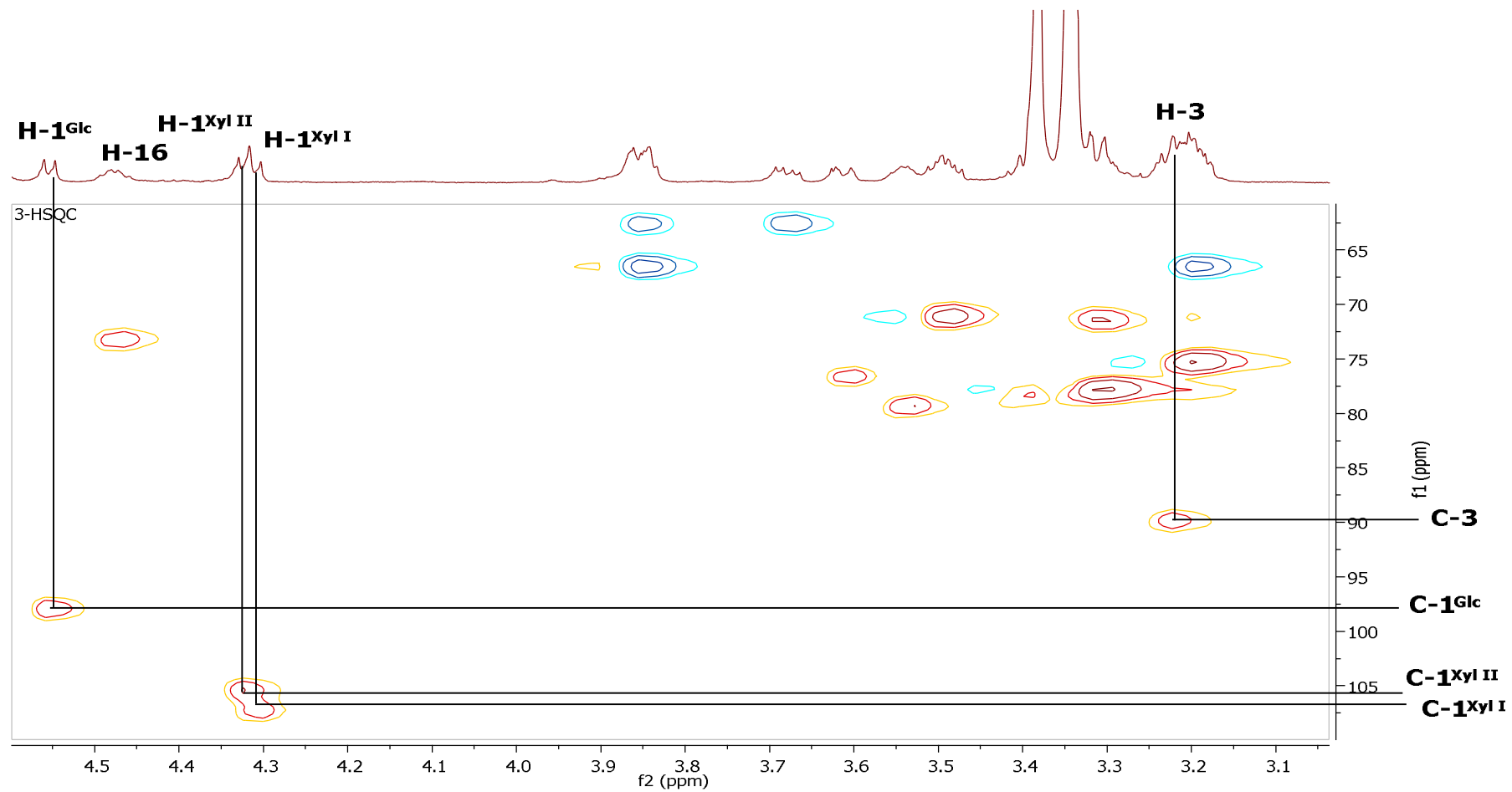


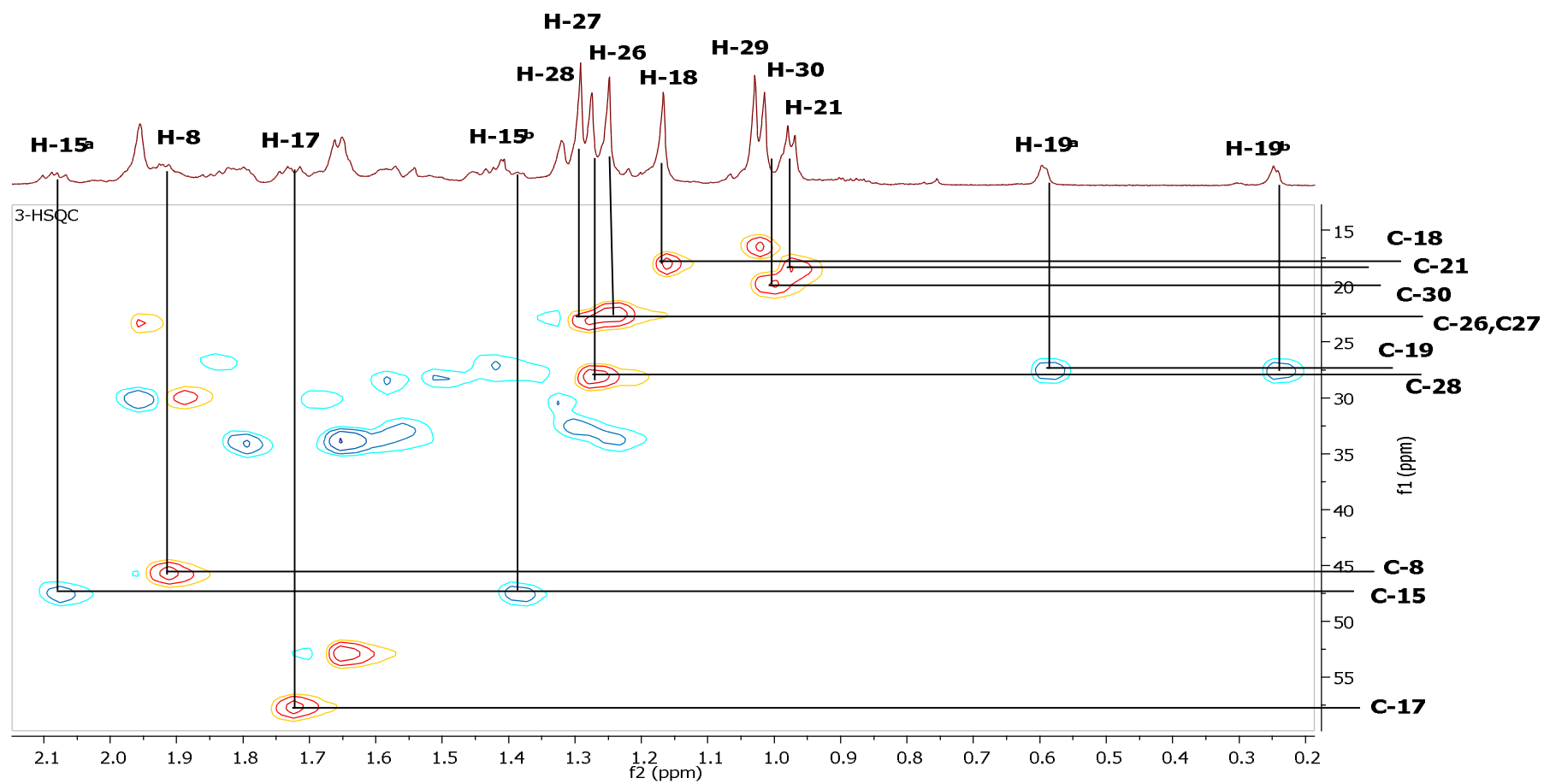
Figure 3.3. Structure of AAU3



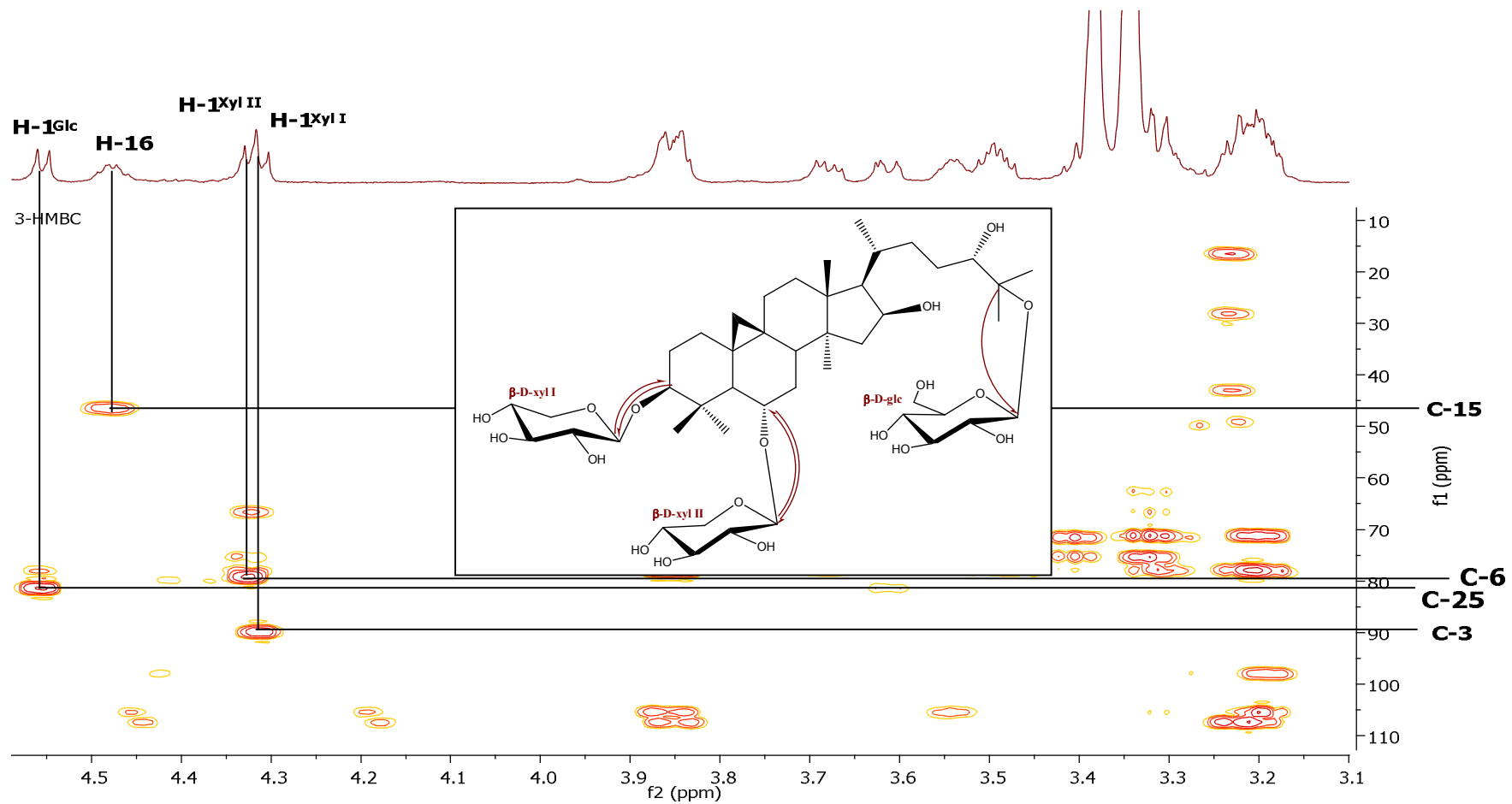
Spectrum 3.11. ^1H NMR Spectrum of AAU3 (600 MHz, CD_3OD)



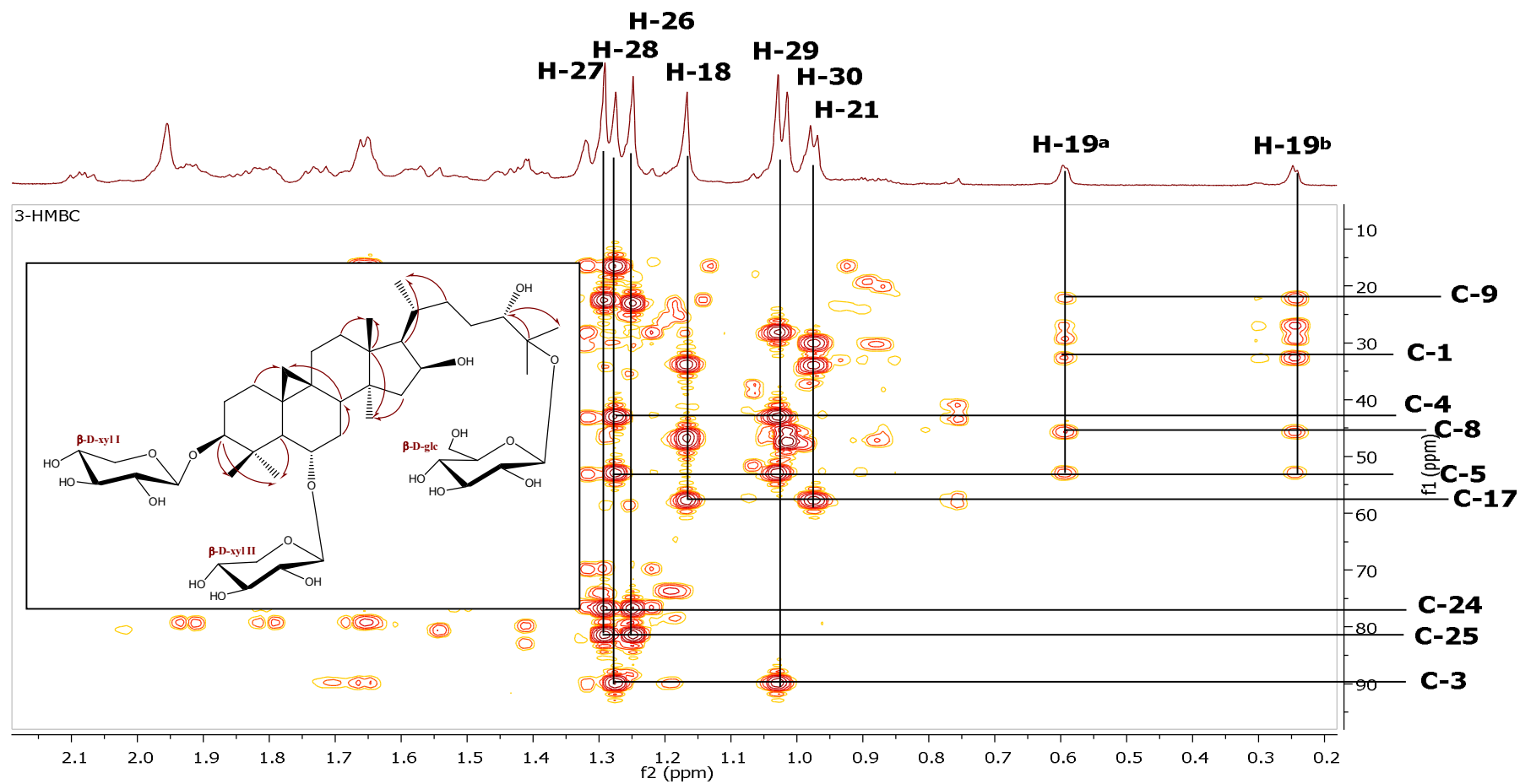
Spectrum 3.12. HSQC Spectrum of AAU3



Spectrum 3.13. HSQC Spectrum of AAU3



Spectrum 3.14. HMBC Spectrum of AAU3



Spectrum 3.15. HMBC Spectrum of AAU3

3.1.4. Structural Identification of Compound AAU4

The molecular formula of **AAU4** was established as $C_{47}H_{80}O_{19}$ by HRMALDITOFMS analysis (m/z 971.5197 $[M+Na]^+$, calcd for $C_{47}H_{80}O_{19}Na$, 971.5192). The comparison of the NMR data of compound **AAU4** with those of compound **AAU3** allowed us to determine that the two compounds differed only by the presence of a β -glucopyranosyl unit at C-6 in **AAU4** instead of the β -xylopyranosyl unit in **AAU3**.

Thus, compound **AAU4** was elucidated as 3-*O*- β -D-xylopyranosyl-6,25-di-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(S),25-pentahydroxycycloartane revealing another tridesmosidic glycoside. (**Fig. 3.4**)

Amorphous white solid; $C_{47}H_{80}O_{19}$; $[\alpha]^{25}_D +32.5^\circ$ (c 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}} \text{ cm}^{-1}$: 3477 ($>OH$), 3038 (cyclopropane ring), 2934 ($>CH$), 1263 and 1054 (C-O-C); ESI-MS m/z 971.56 $[M+Na]^+$, MS/MS m/z 791.46 $[M+Na-180]^+$, MS^3 m/z 611.40 $[M+Na-180-180]^+$, MS^4 m/z 479.32 $[M+Na-180-180-132]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 971.5197 (calcd for $C_{47}H_{80}O_{19}Na$, 971.5192); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.4**.

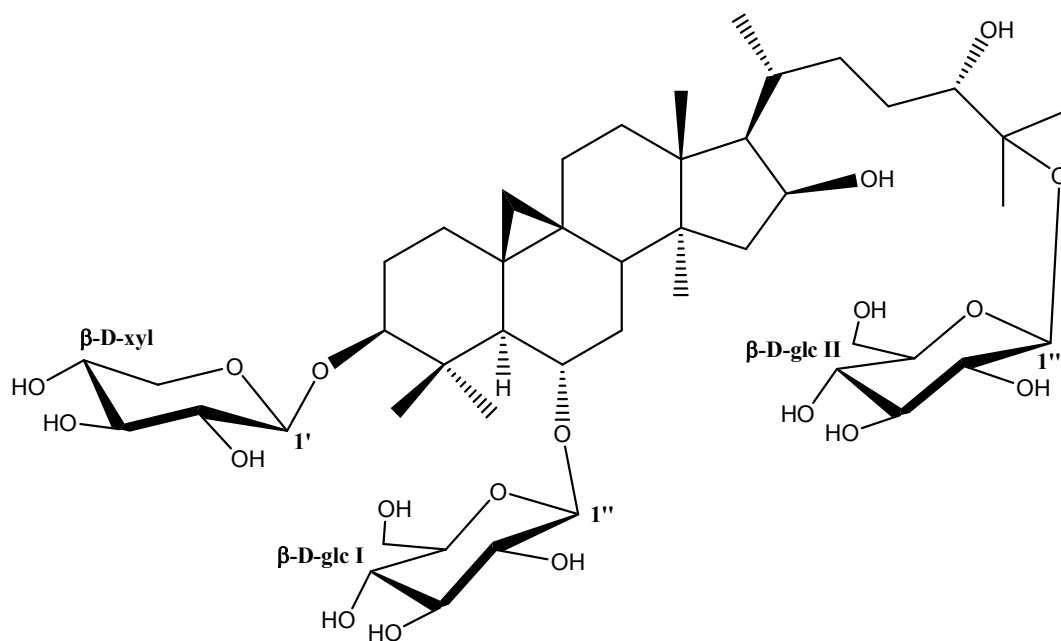
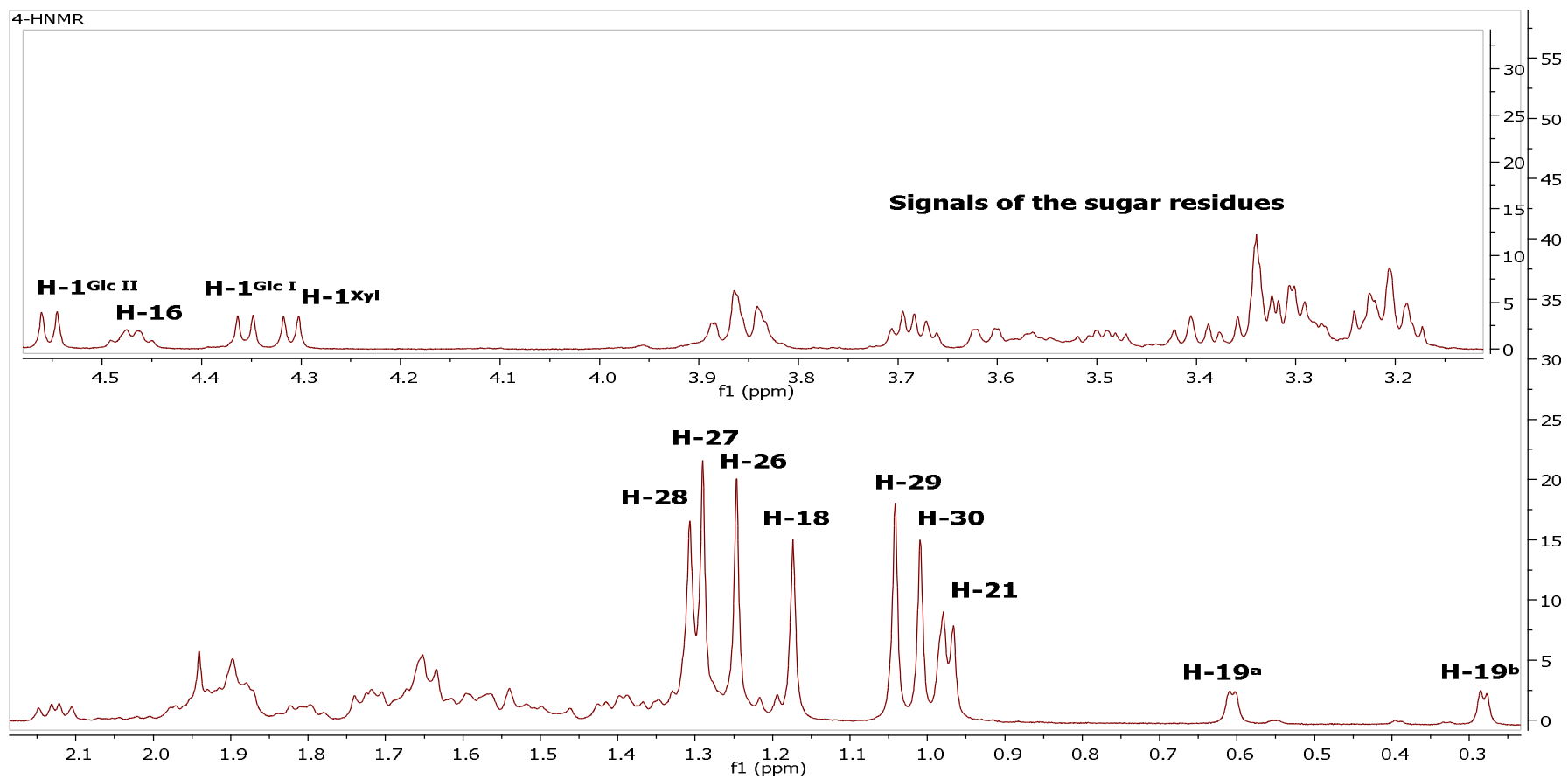


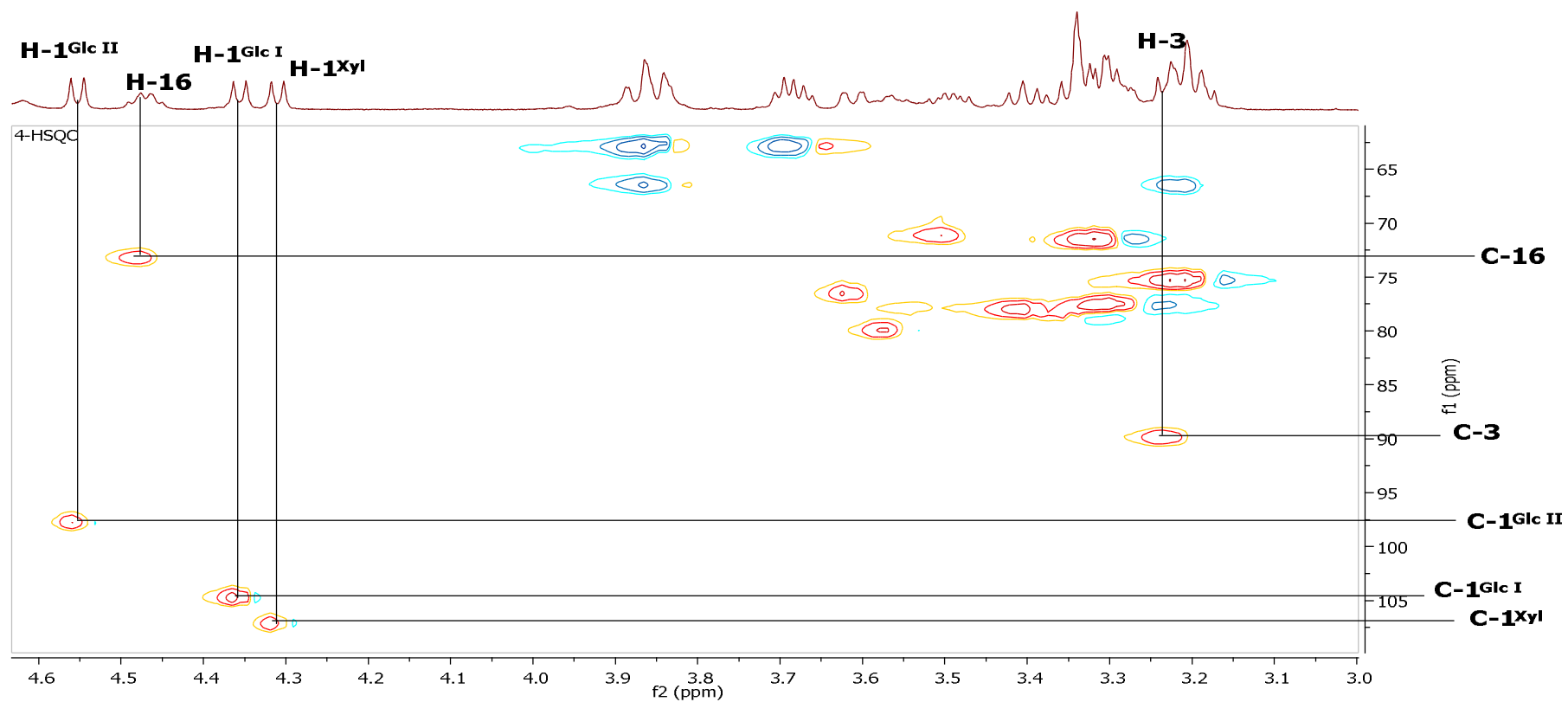
Figure 3.4. Structure of AAU4

Table 3.4. ^1H and ^{13}C Assignments of AAU4 (150/600 MHz, δ ppm, in CD_3OD)

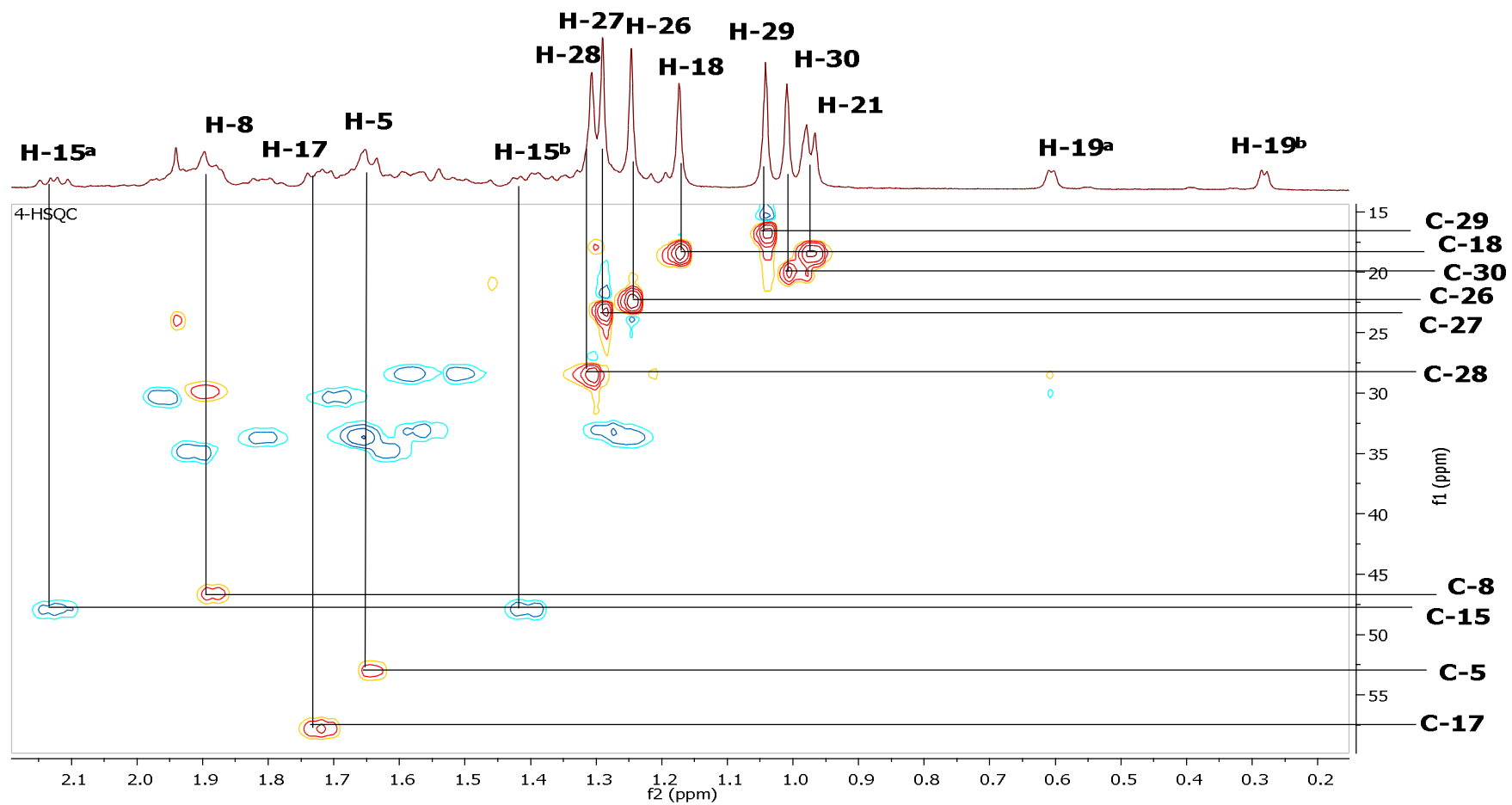
Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	32.5	1.57, 1.30, m
2	30.0	1.96, 1.70, m
3	89.6	3.22, dd (11.3, 4.0)
4	42.7	-
5	52.7	1.66, d (9.5)
6	79.2	3.53, ddd (9.5, 9.5, 4.5)
7	34.0	1.93, 1.65, m
8	45.6	1.92, dd (11.9, 4.2)
9	22.1	-
10	29.0	-
11	26.6	1.85, 1.44, m
12	33.7	1.66, (2H) m
13	46.3	-
14	47.2	-
15	47.4	2.07, dd (12.7, 8.0) 1.40, dd (12.7, 5.2)
16	72.9	4.48, ddd (8.0, 8.0, 5.2)
17	57.5	1.73, dd (9.9, 8.0)
18	17.9	1.17, s
19	27.4	0.59, d (4.2), 0.24, d (4.2)
20	30.0	1.90, m
21	18.3	0.97, d (6.5)
22	34.0	1.80, 1.25, m
23	28.4	1.59, 1.52, m
24	76.4	3.60, dd (10.5, 2.4)
25	81.0	-
26	22.4	1.25, s
27	23.0	1.29, s
28	28.0	1.27, s
29	16.2	1.03, s
30	19.6	1.01, s
$\beta\text{-D-Xyl (at C-3)-1'}$	106.8	4.31, d (7.5)
2'	75.0	3.22, dd (9.2, 7.5)
3'	78.0	3.34, t (9.2)
4'	71.0	3.50, m
5'	66.3	3.85, dd (11.7, 5.2) 3.21, t (11.7)
$\beta\text{-D-Glc I (at C-6)-1''}$	104.6	4.36, d (7.5)
2''	75.0	3.20, dd (7.5, 9.0)
3''	78.2	3.36, t (9.0)
4''	71.3	3.30, t (9.0)
5''	77.2	3.31, ddd (3.5, 4.5, 9.0)
6''	62.7	3.86, dd (3.5, 12.0) 3.68, dd (4.5, 12.0)
$\beta\text{-D-Glc II (at C-25)-1'''}$	97.5	4.55, d (7.5)
2'''	75.0	3.19, dd (7.5, 9.0)
3'''	77.9	3.41, t (9.0)
4'''	71.3	3.33, t (9.0)
5'''	77.4	3.28, ddd (3.5, 4.5, 9.0)
6'''	62.7	3.86, dd (3.5, 12.0), 3.68, dd (4.5, 12.0)



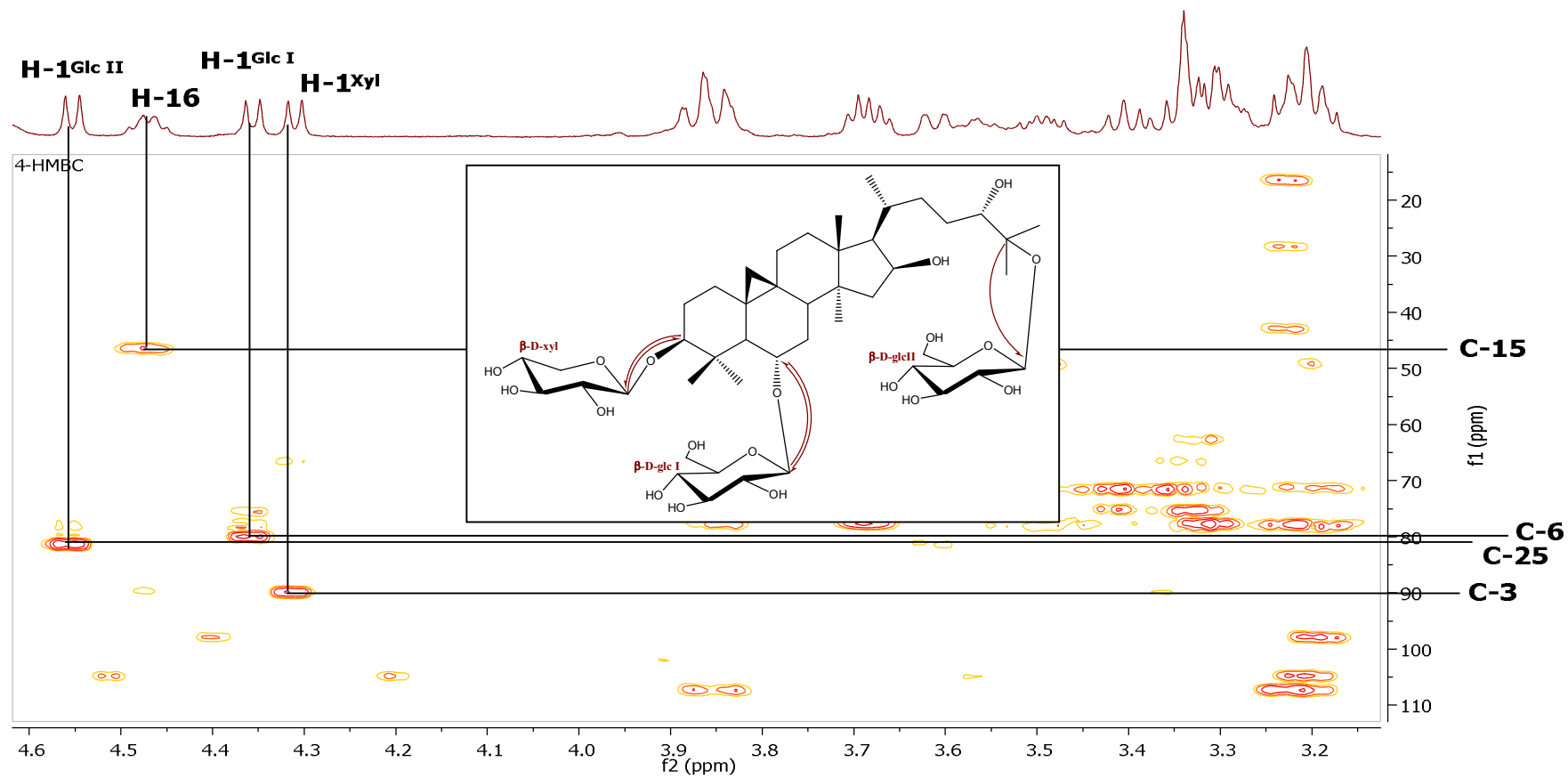
Spectrum 3.16. ¹H NMR Spectrum of AAU4 (600MHz, CD₃OD)



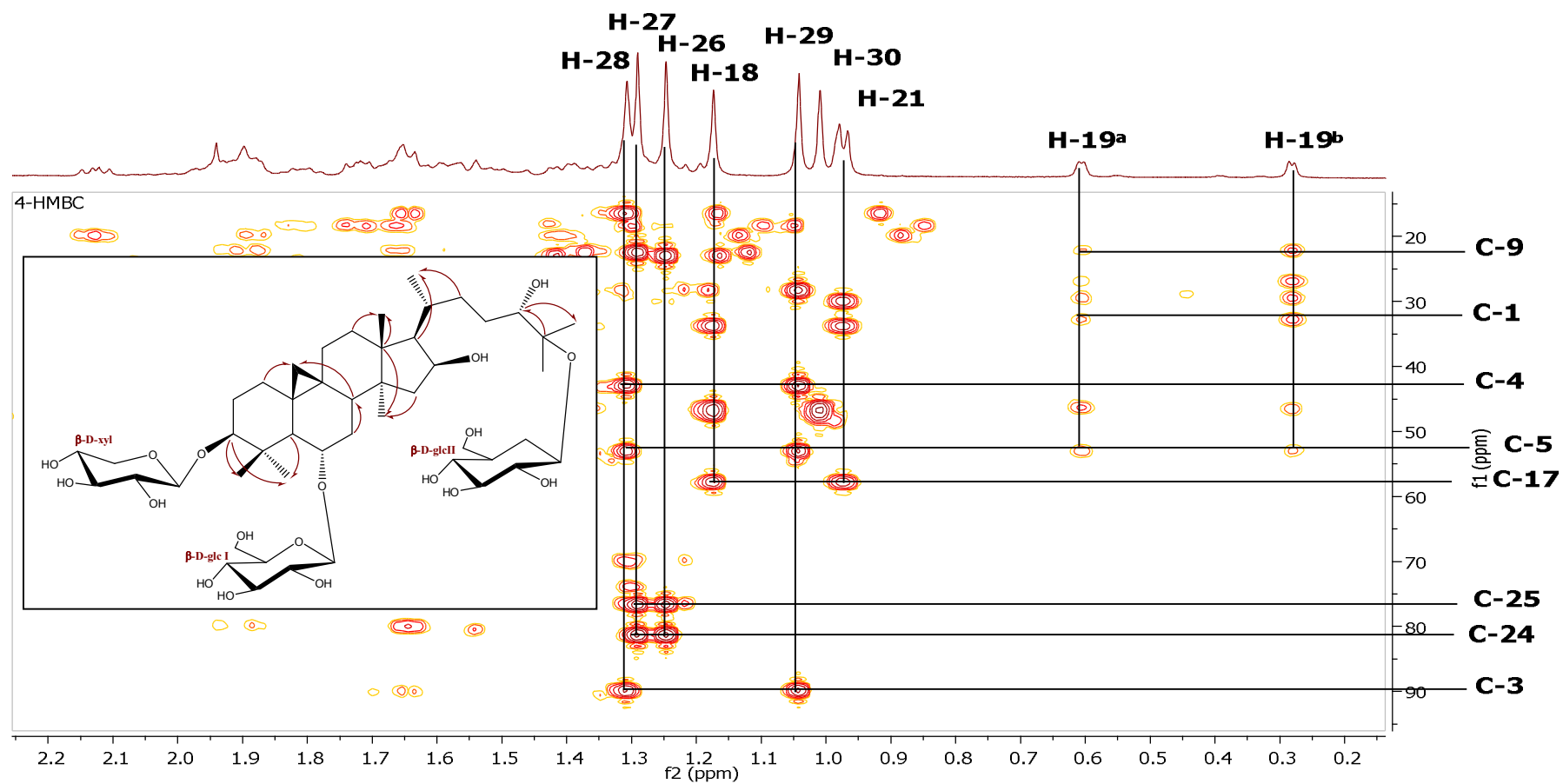
Spectrum 3.17 HSQC Spectrum of AAU4



Spectrum 3.18. HSQC Spectrum of AAU4



Spectrum 3.19. HMBC Spectrum of AAU4



Spectrum 3.20. HMBC Spectrum of AAU4

3.1.5. Structural Identification of Compound AAU5

The HRMALDITOF mass spectrum of **AAU5** (m/z 677.4246 $[M + Na]^+$, calcd for $C_{36}H_{62}O_{10}Na$, 677.4241) supported a molecular formula of $C_{36}H_{62}O_{10}$.

The ^1H NMR spectrum of **AAU5** displayed, in the sugar region, only one anomeric proton signal at δ 4.36 (d, $J = 7.5$ Hz). On the basis of 1D-TOCSY, HSQC and DQF-COSY data this sugar unit was identified as β -glucopyranosyl unit. The HMBC spectrum showed the correlation between the proton signal at δ 4.36 ($H-1_{glc}$) and the carbon resonance at δ 80.1 (C-6), allowing us to elucidate compound **AAU5** as 6- O - β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(S),25-pentahydroxycycloartane. (**Fig. 3.5**)

Amorphous white solid; $C_{36}H_{62}O_{10}$; $[\alpha]^{25}_D +27.8^\circ$ (c 0.01 MeOH); IR $\nu^{KBr}_{max} cm^{-1}$: 3485 ($>OH$), 3047 (cyclopropane ring), 2930 ($>CH$), 1254 and 1061 (C-O-C); ESI-MS m/z 677.56 $[M+Na]^+$, MS/MS m/z 497.37 $[M+Na-180]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 677.4246 (calcd for $C_{36}H_{62}O_{10}Na$, 677.4241); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.5**.

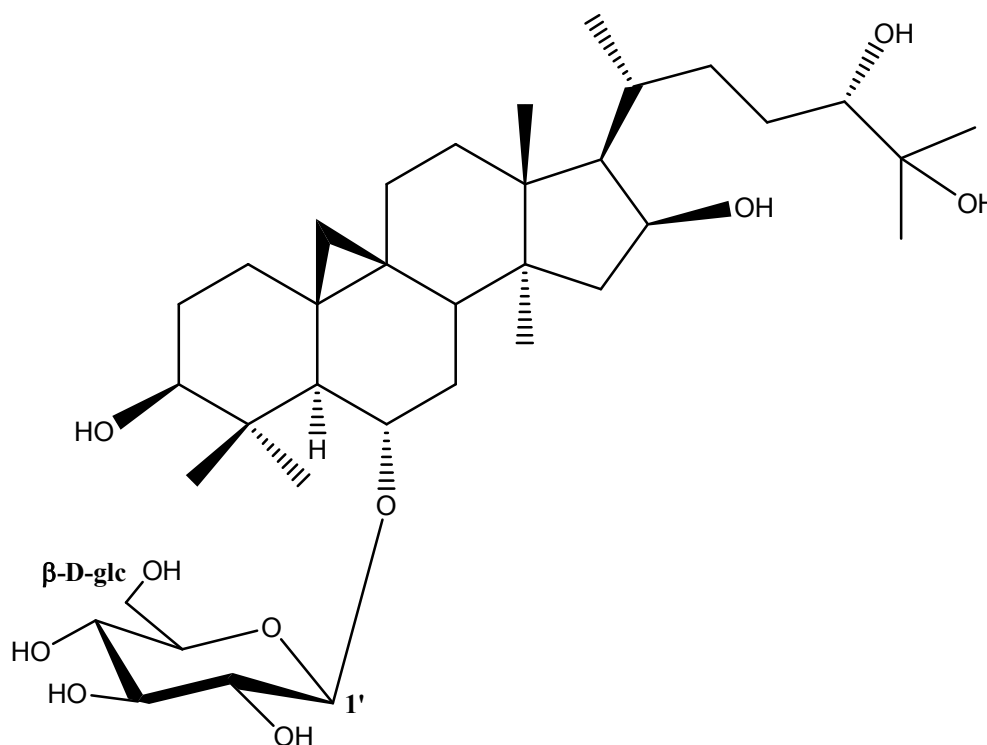
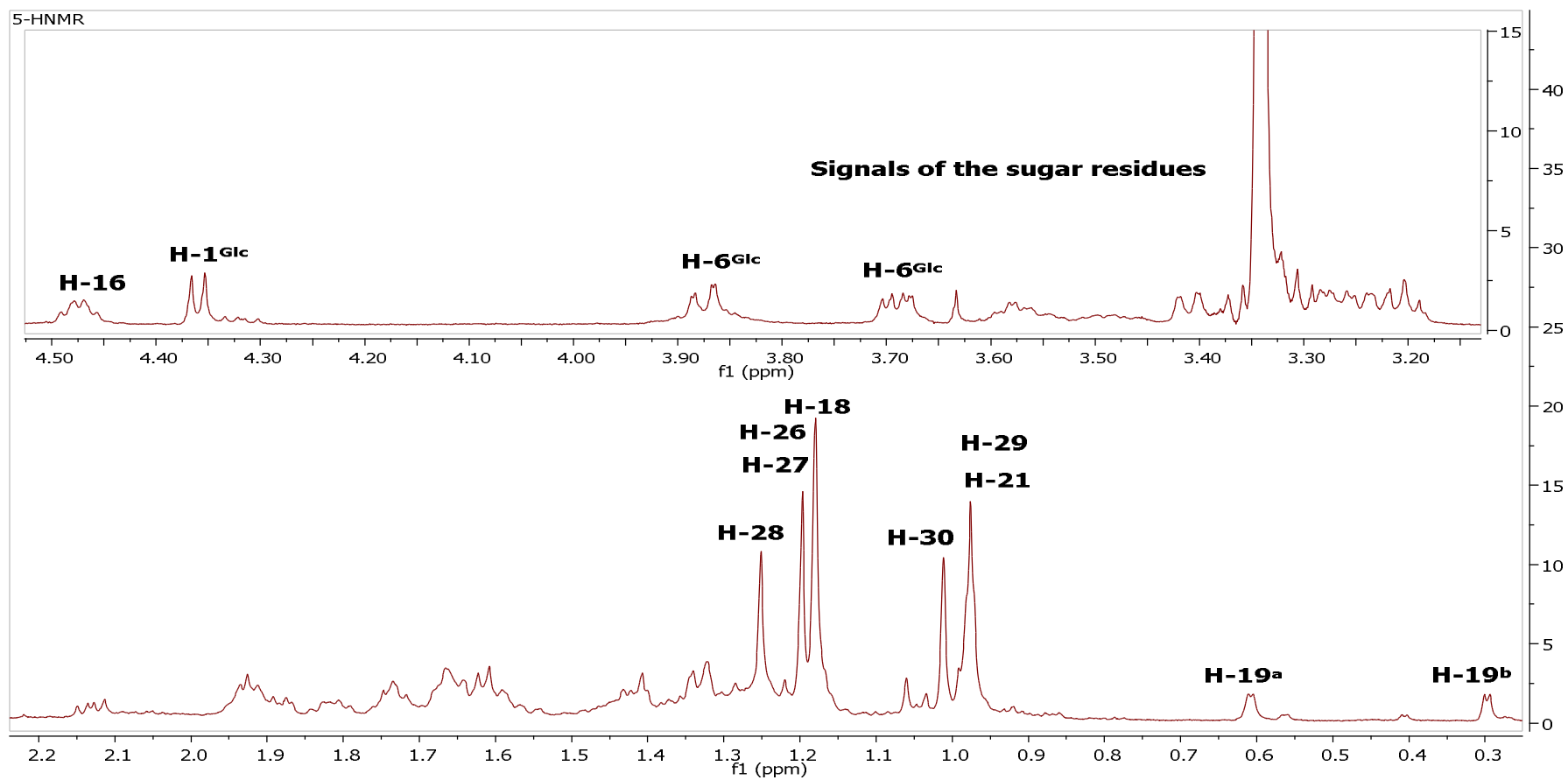


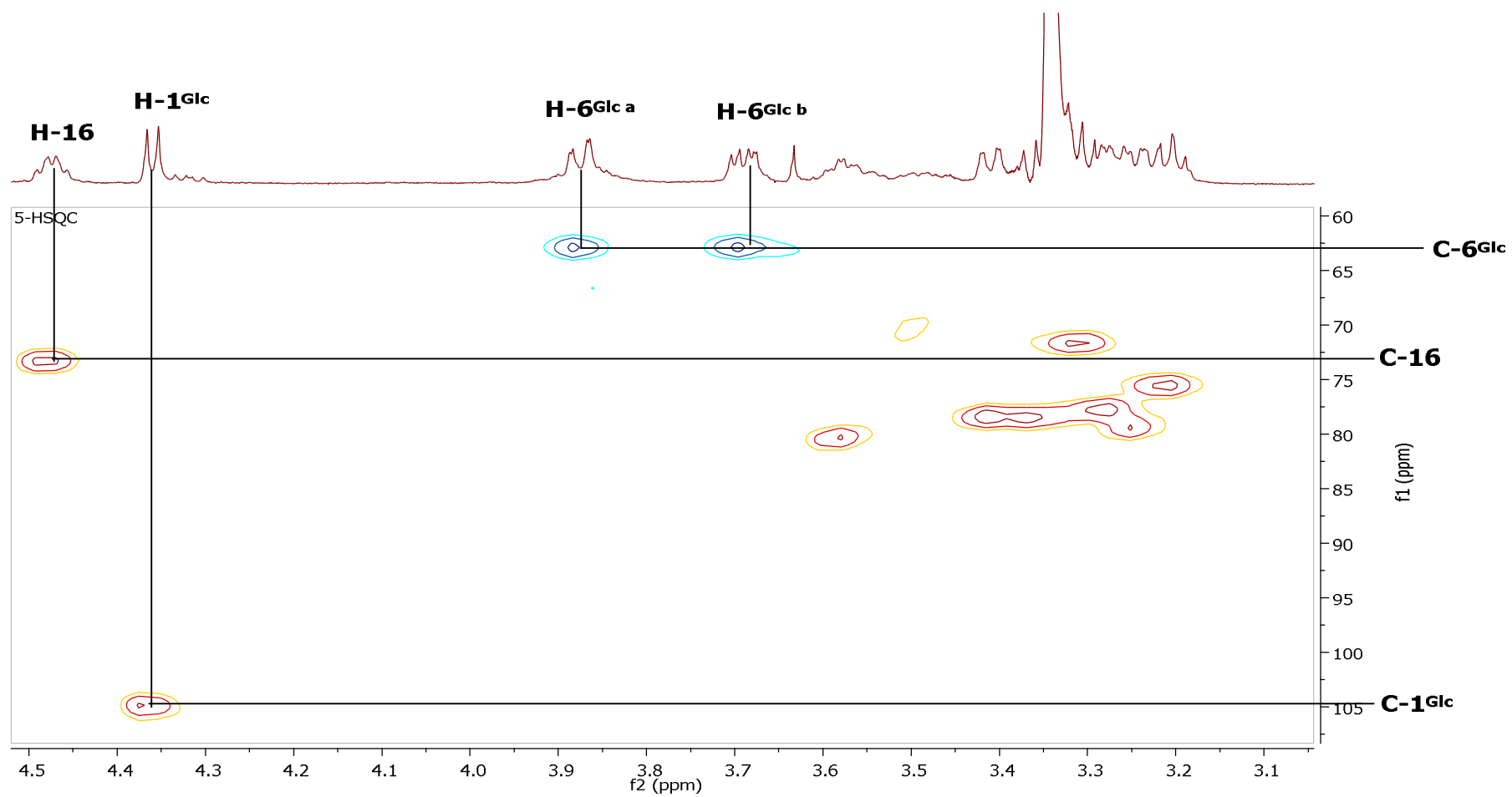
Figure 3.5. Structure of AAU5

Table 3.5. ^1H and ^{13}C Assignments of AAU5 (150/600 MHz, δ ppm, in CD_3OD)

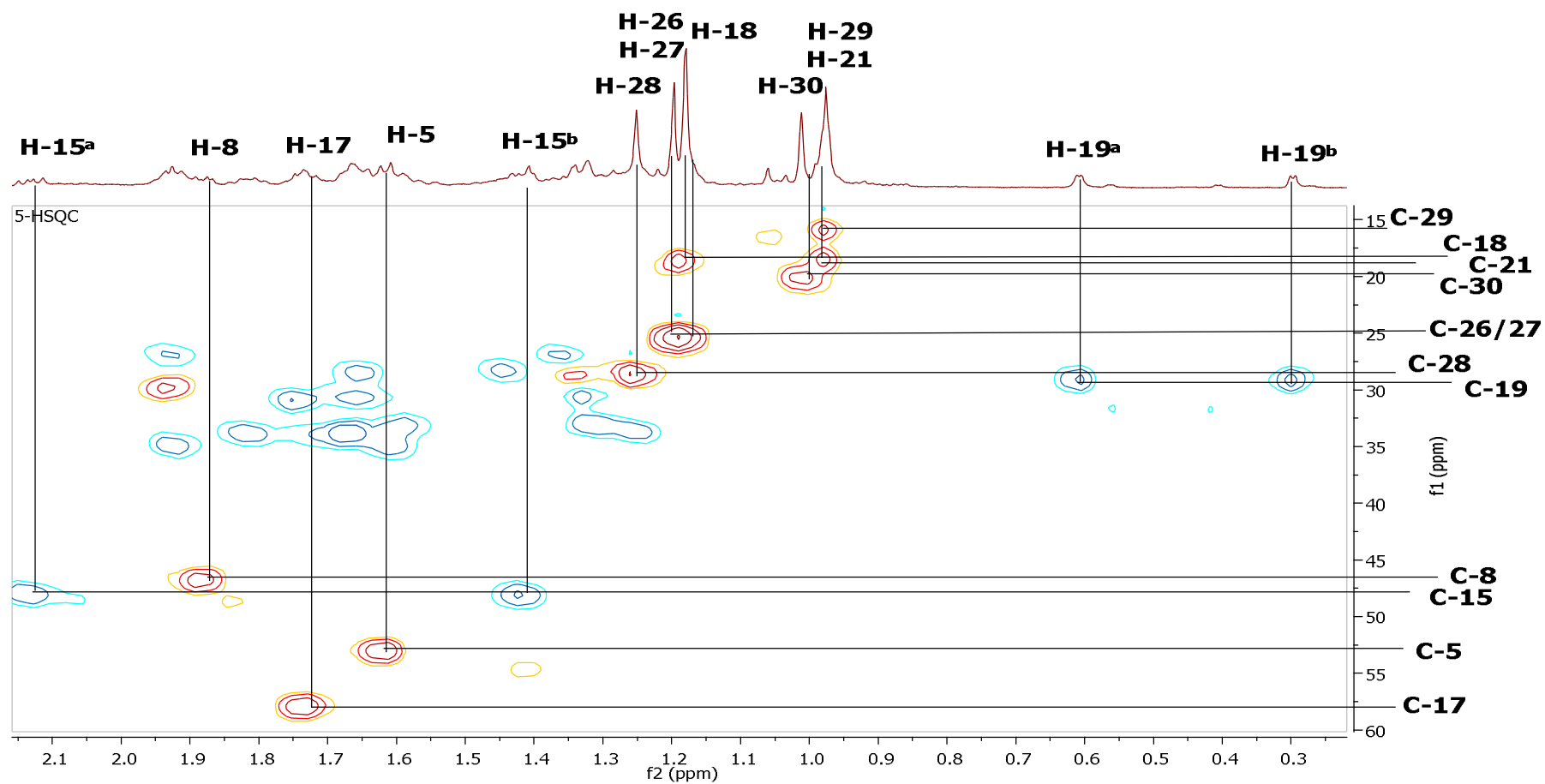
Position	δ_{C}	δ_{H} (J in Hz)
1	32.7	1.59, 1.32, m
2	30.7	1.75, 1.66, m
3	79.3	3.25, dd (11.3, 4.0)
4	42.6	-
5	52.9	1.62, d (9.5)
6	80.1	3.58, ddd (9.5, 9.5, 4.5)
7	34.8	1.93, 1.61, m
8	46.6	1.88, dd (11.9, 4.2)
9	22.3	-
10	29.7	-
11	26.6	1.93, 1.36, m
12	33.8	1.72, 1.67, m
13	46.6	-
14	47.9	-
15	47.9	2.13, dd (12.7, 8.0), 1.42, dd (12.7, 5.2)
16	73.0	4.47, ddd (8.0, 8.0, 5.2)
17	57.7	1.74, dd (9.9, 8.0)
18	18.5	1.18, s
19	29.0	0.61, d (4.2), 0.30, d (4.2)
20	29.6	1.93, m
21	18.3	0.98, d (6.5)
22	33.7	1.82, 1.26, m
23	28.2	1.65, 1.45, m
24	78.2	3.41, dd (10.5, 2.4)
25	73.8	-
26	25.2	1.19, s
27	25.5	1.19, s
28	28.4	1.25, s
29	15.7	0.98, s
30	19.9	1.01, s
$\beta\text{-D-Glc (at C-6)-1'}$	104.6	4.36, d (7.5)
2'	75.4	3.21, dd (7.5, 9.0)
3'	78.2	3.36, t (9.0)
4'	71.3	3.31, t (9.0)
5'	77.4	3.28, ddd (3.5, 4.5, 9.0)
6'	62.7	3.88, dd (3.5, 12.0), 3.69, dd (4.5, 12.0)



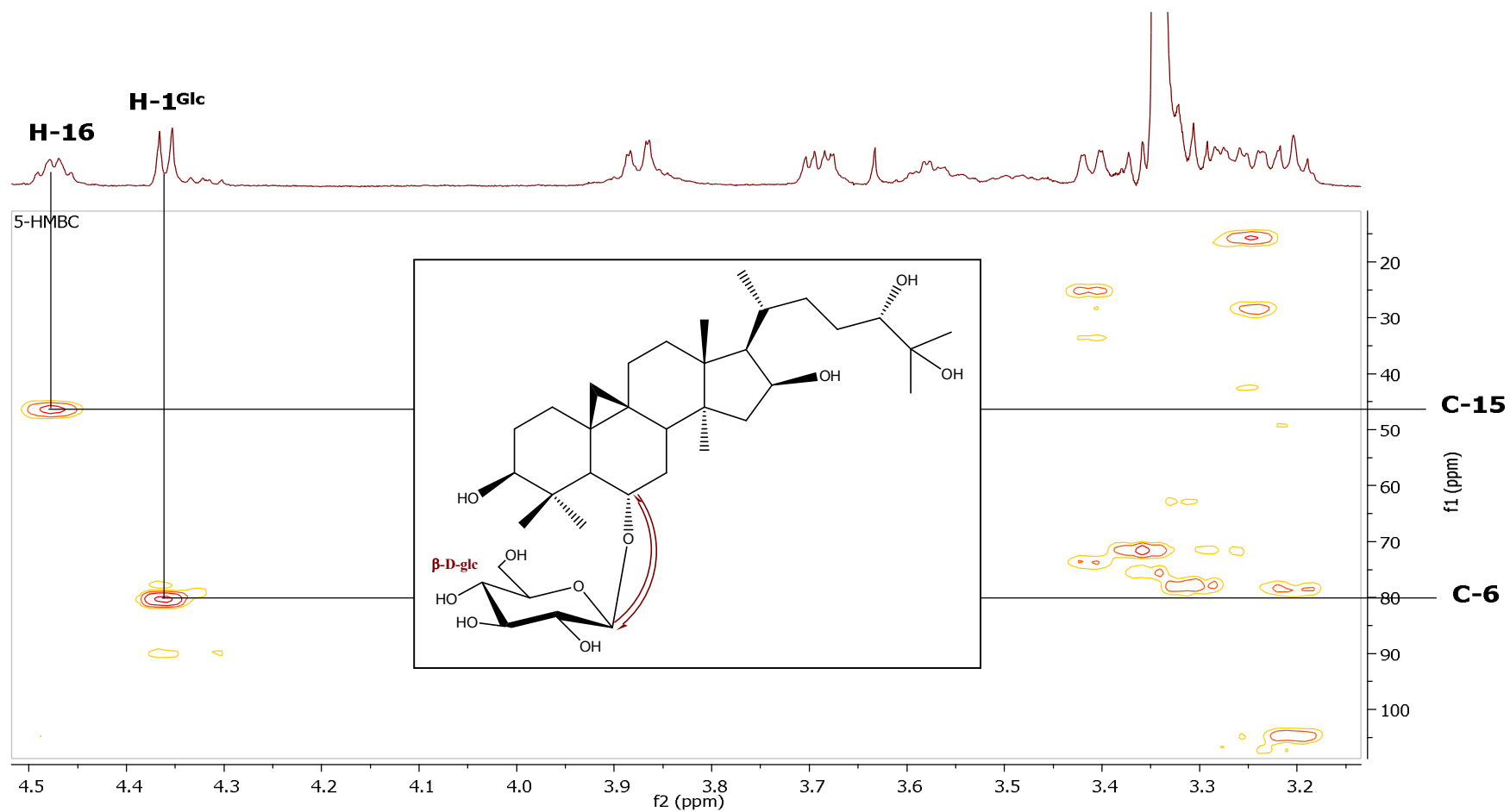
Spectrum 3.21. ^1H NMR Spectrum of AAU5 (600 MHz, CD_3OD)



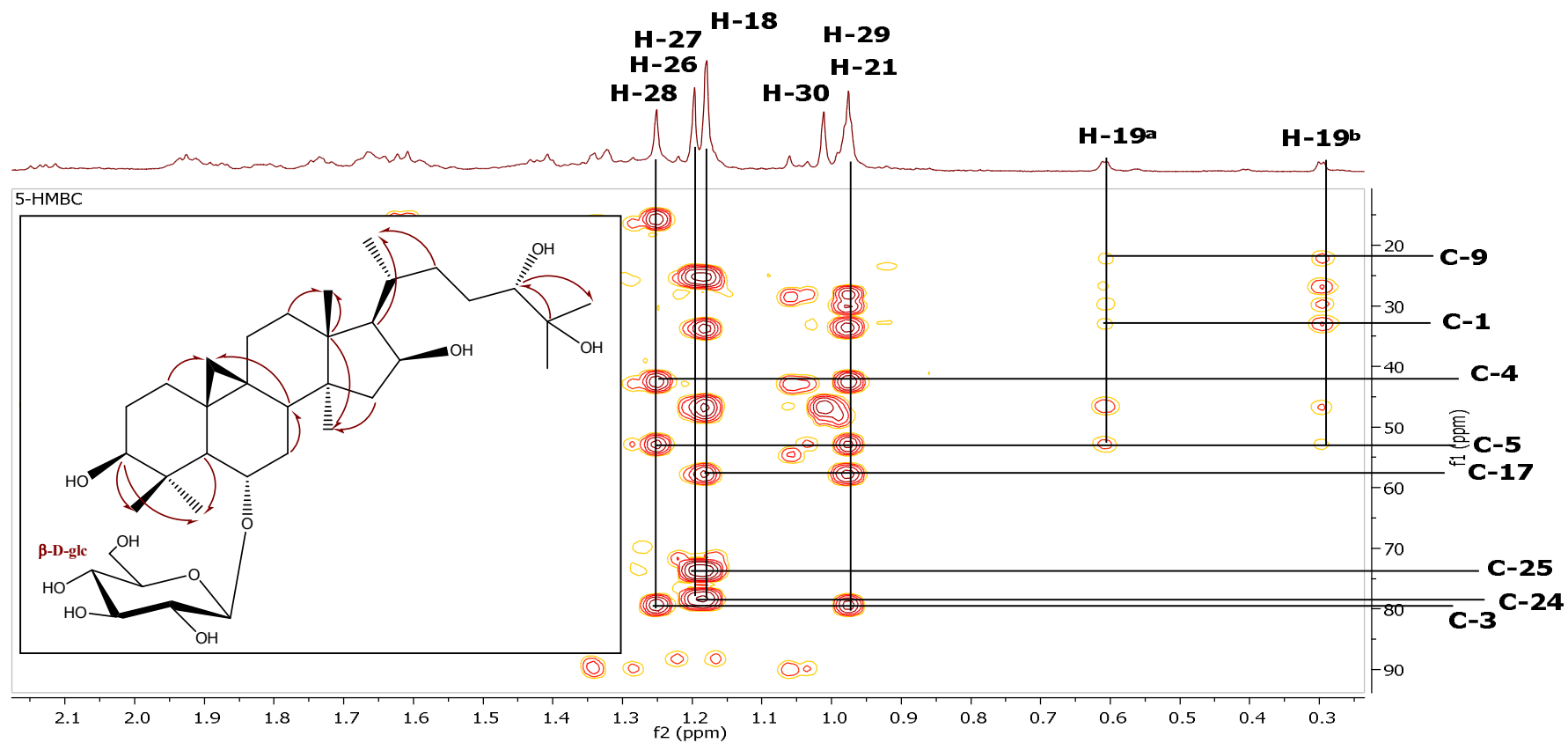
Spectrum 3.22. HSQC Spectrum of AAU5



Spectrum 3.23. HSQC Spectrum of AAU5



Spectrum 3.24. HMBC Spectrum of AAU5



Spectrum 3.25. HMBC Spectrum of AAU5

3.1.6. Structural Identification of Compound AAU6

HRMALDITOFMS analysis (m/z 777.4405 $[M + Na]^+$, calcd for $C_{40}H_{66}O_{13}Na$, 777.4401). The ESIMS mass spectrum showed the major ion peak at m/z 777.74 which was assigned to $[M + Na]^+$. In the MS/MS spectrum peaks at m/z 645.36 $[M + Na-132]^+$, corresponding to the loss of a pentose unit, 495.41 $[M + Na-150]^+$, corresponding to the loss of a further pentose unit, were observed. It was apparent from their ^1H and ^{13}C NMR data that **AAU6–AAU8** possessed the same aglycon moiety. In particular, the ^1H NMR spectrum of **AAU6** showed signals due to a cyclopropane methylene at δ 0.57 and 0.43 (each 1H, d, $J = 4.2$ Hz), seven tertiary methyl groups at δ 1.54 (3H, s), 1.49 (3H, s), 1.32 (6H, s), 1.22 (3H, s), 1.05 (3H, s) and 0.97 (3H, s) and four methine proton signals at δ 4.64 (ddd, $J = 8.0, 8.0, 5.2$ Hz), 3.47 (ddd, $J = 9.5, 9.5, 4.5$ Hz), 3.51 (br s) and 3.22 (dd, $J = 11.3, 4.0$ Hz). The NMR data of compound **AAU6** were in agreement with those reported for 3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(R),25-epoxycycloartane named cyclocephalogenin, previously reported from *Astragalus* spp. (Bedir et al., 1998a). The relative configurations of the oxygenated carbons C-3, C-6, C-16 and C-20 were determined as reported for compound **AAU1**. The α orientation of the –OH group at C-24 was determined by the ROE correlation between Me-27 (δ 1.32) and H-24 β (δ 3.51) signals.

For the sugar region in the ^1H NMR spectrum, two anomeric protons at δ 4.49 (d, $J = 3.7$ Hz) and 4.48 (d, $J = 7.5$ Hz) were observed. Complete assignments of the ^1H and ^{13}C NMR signals of the sugar portion were accomplished by 1D-TOCSY, HSQC and DQF-COSY experiments which led to the identification of one β -xylopyranosyl unit (δ 4.48) and one α -arabinopyranosyl unit (δ 4.49). The determination of the sequence and linkage sites was obtained from the HMBC correlations which showed key correlation peaks between the proton signals at δ 4.48 (H-1_{xy}) and the carbon resonance at δ 89.3 (C-3), and the proton signal at δ 4.49 (H-1_{ara}) and the carbon resonance at δ 82.9 (C-2_{xy}). Thus, compound **AAU6** was elucidated as 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(R),25-epoxycycloartane (**Fig. 3.6**).

Amorphous white solid; $C_{40}H_{66}O_{13}$; $[\alpha]_D^{25} +10.5^\circ$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3488 (>OH), 3053 (cyclopropane ring), 2939 (>CH), 1274 and 1050 (C–O C); ESI-MS m/z 777.74 $[M+Na]^+$, MS/MS m/z 645.36 $[M+Na-132]^+$, 495.41 $[M+Na-132-150]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 777.4405 (calcd for

$C_{40}H_{66}O_{13}Na$, 777.4401); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data see: **Table 3.6**.

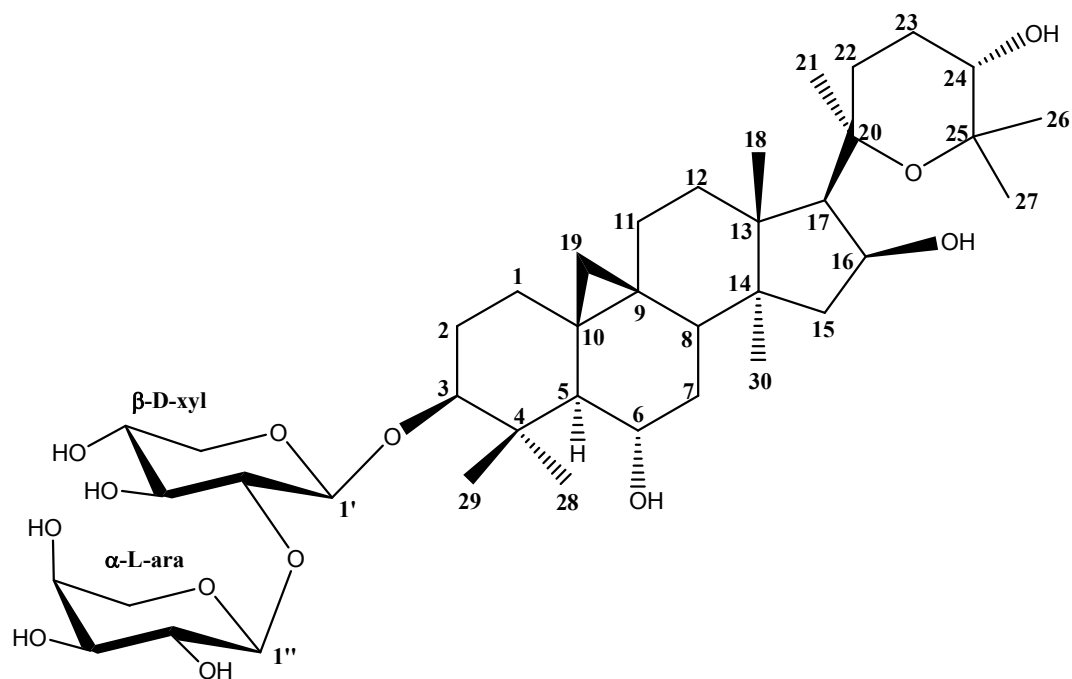
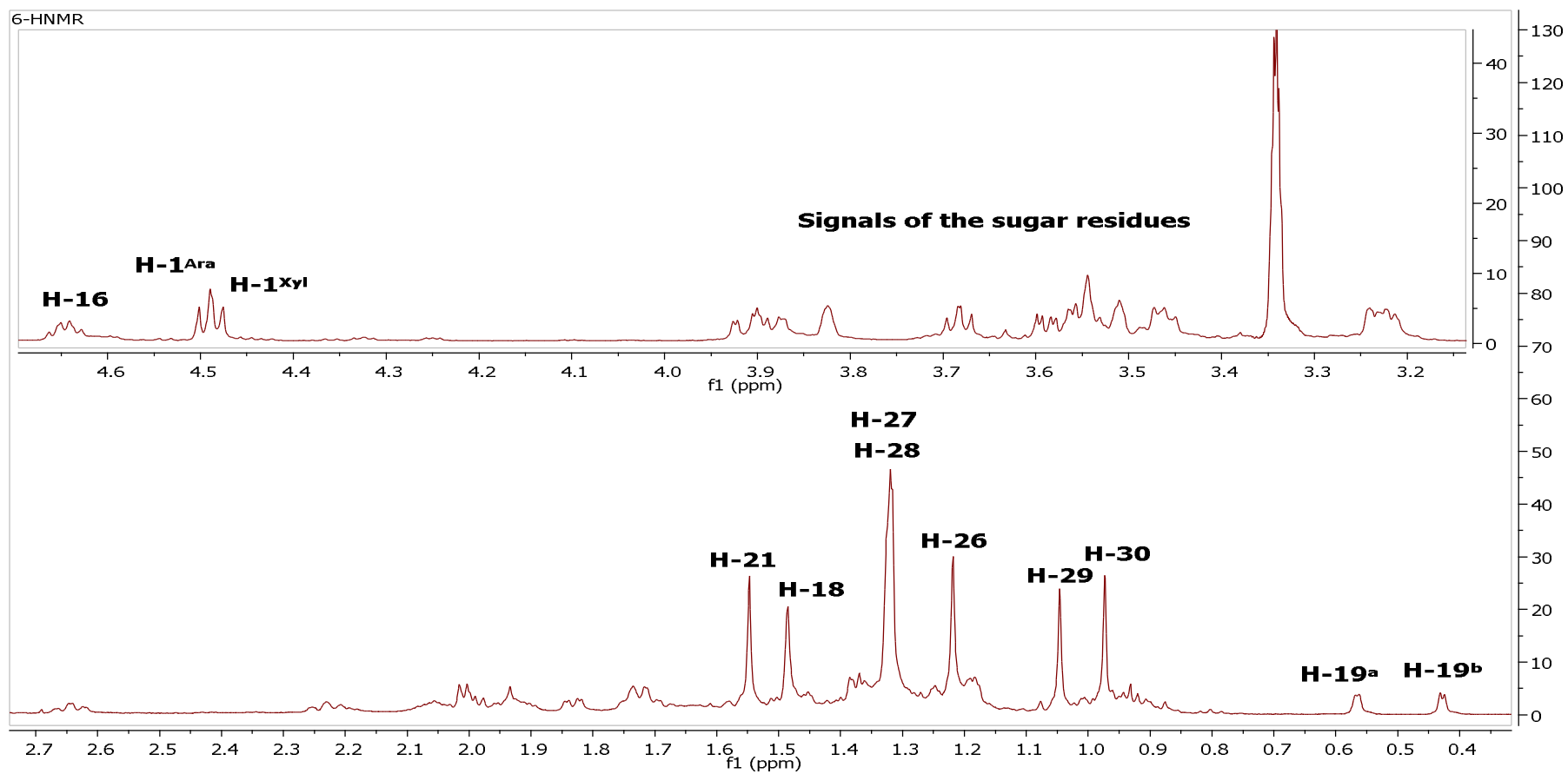


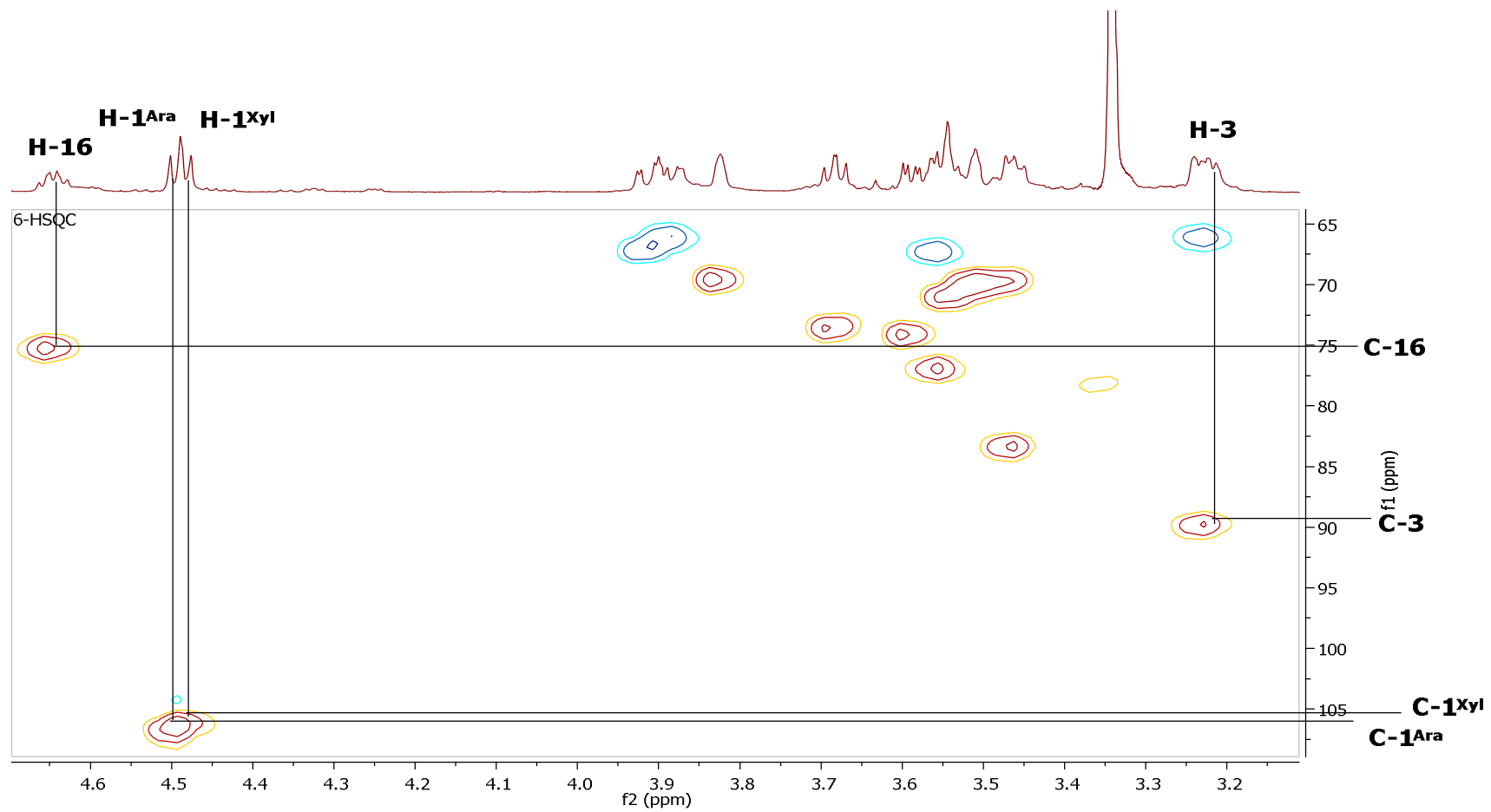
Figure 3.6. Structure of AAU6

Table 3.6. ^1H and ^{13}C Assignments of AAU6 (150/600 MHz, δ ppm, in CD_3OD)

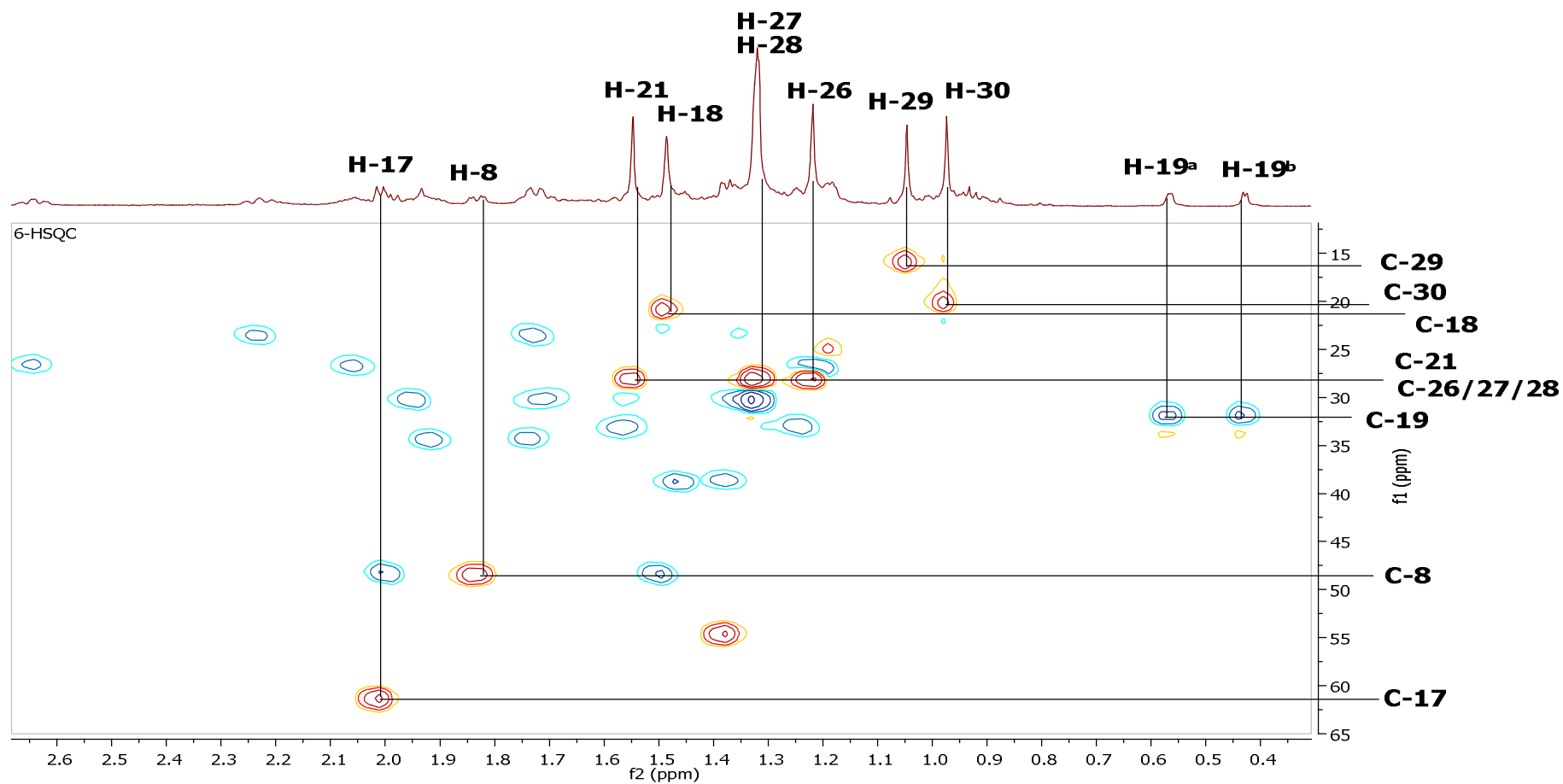
Position	δ_{C}	δ_{H} (J in Hz)
1	33.2	1.56, 1.24, m
2	30.2	1.94, 1.71, m
3	89.3	3.22, dd (11.3, 4.0)
4	43.1	-
5	54.3	1.38, d (9.5)
6	69.3	3.47, ddd (9.5, 9.5, 4.5)
7	38.7	1.46, 1.37, m
8	48.4	1.83, dd (11.9, 4.2)
9	21.7	-
10	30.4	-
11	26.8	2.06, 1.20, m
12	34.3	1.92, 1.73, m
13	46.4	-
14	47.5	-
15	48.2	2.00, dd (12.7, 8.0) 1.49, dd (12.7, 5.2)
16	74.8	4.64, ddd (8.0, 8.0, 5.2)
17	61.2	2.01, dd (9.9, 8.0)
18	21.0	1.49, s
19	31.8	0.57, brs, 0.43, d (4.2)
20	80.3	-
21	28.0	1.54, s
22	26.6	2.64, 1.23, m
23	23.7	2.23, 1.73, m
24	69.3	3.51, br s
25	76.5	-
26	28.2	1.22, s
27	28.2	1.32, s
28	28.2	1.32, s
29	16.2	1.05, s
30	20.4	0.97, s
$\beta\text{-D-Xyl (at C-3)-1'}$	105.2	4.48, d (7.5)
2'	82.9	3.46, dd (9.2, 7.5)
3'	76.3	3.55, t (9.2)
4'	70.5	3.54, m
5'	65.7	3.88, dd (11.7, 5.2), 3.22, t (11.7)
$\alpha\text{-L-Ara (at C-2}_{\text{Xyl}}\text{)-1''}$	105.9	4.49, d (3.7)
2''	73.0	3.68, dd (8.5, 3.7)
3''	73.7	3.59, dd (8.5, 3.0)
4''	69.1	3.89, m
5''	66.8	3.91, dd (11.9, 2.0) 3.55, dd (11.9, 3.0)



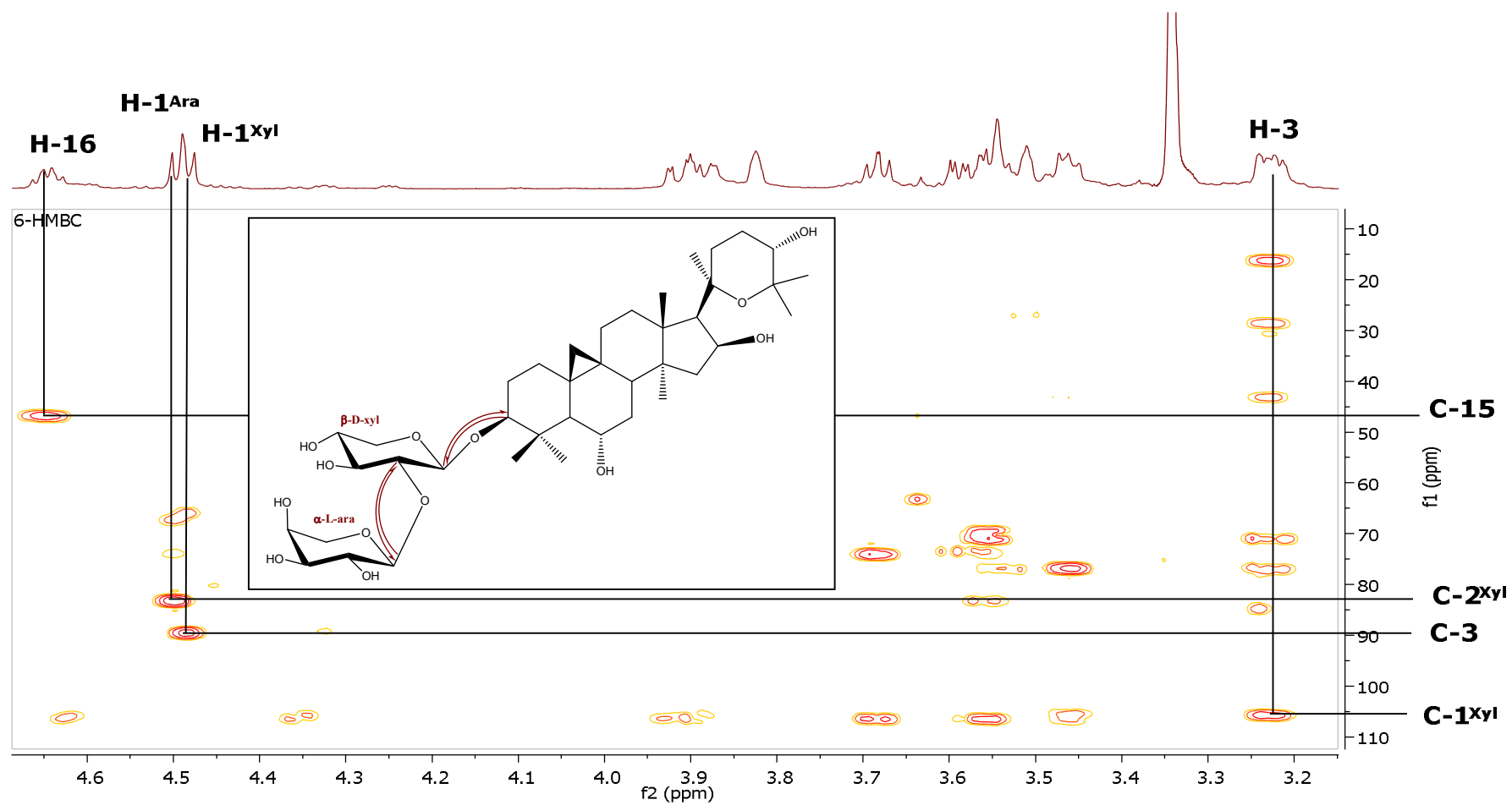
Spectrum 3.26. ^1H NMR Spectrum of AAU6 (600 MHz, CD_3OD)



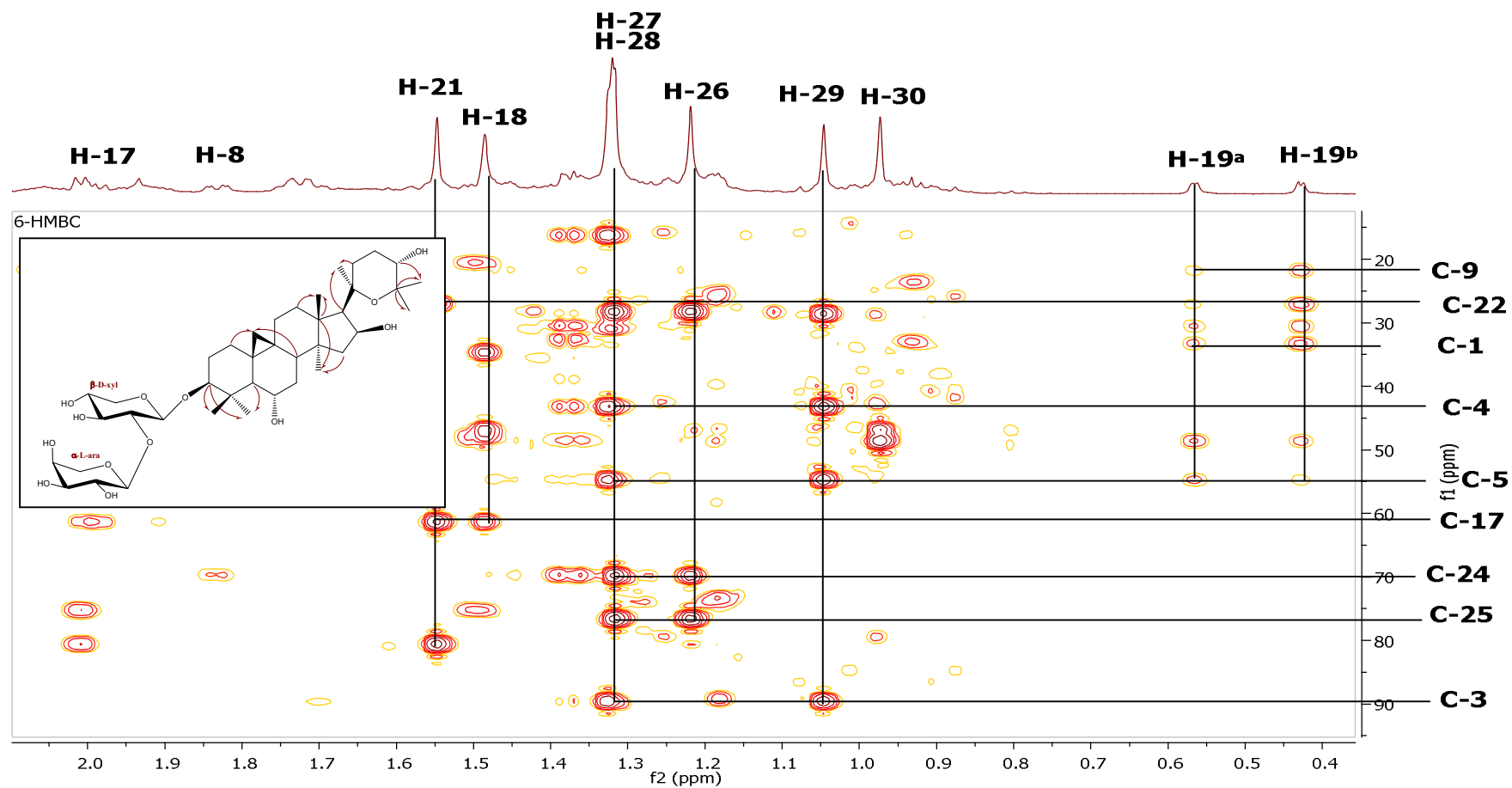
Spectrum 3.27. HSQC Spectrum of AAU6



Spectrum 3.28. HSQC Spectrum of AAU6



Spectrum 3.29. HMBC Spectrum of AAU6



Spectrum 3.30. HMBC Spectrum of AAU6

3.1.7. Structural Identification of Compound AAU7

The molecular formula of compound **AAU7** was established as $C_{36}H_{60}O_{10}$ by HRMALDITOFMS analysis (m/z 675.4089 $[M+Na]^+$).

The ^1H NMR spectrum of **AAU7** displayed, in the sugar region, only one anomeric proton signal at δ 4.36 (d, $J = 7.5$ Hz). On the basis of 1D-TOCSY, HSQC and DQF-COSY data this sugar unit was identified as β -glucopyranosyl unit. The HMBC spectrum showed the correlation between the proton signal at δ 4.36 (H-1_{glc}) and the carbon resonance at δ 80.2 (C-6), allowing us to elucidate compound **AAU7** as 6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(R),25-epoxycycloartane. (**Fig. 3.7**)

Amorphous white solid; $C_{36}H_{60}O_{10}$; $[\alpha]^{25}_D +15.2^\circ$ (c 0.01 MeOH); IR $\nu^{KBr}_{max} cm^{-1}$: 3479 ($>OH$), 3048 (cyclopropane ring), 2947 ($>CH$), 1257 and 1065 (C-O-C); ESI-MS m/z 675.47 $[M+Na]^+$, MS/MS m/z 495.29 $[M+Na-180]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 675.4089 (calcd for $C_{36}H_{60}O_{10}Na$, 675.4084); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data see: **Table 3.7**.

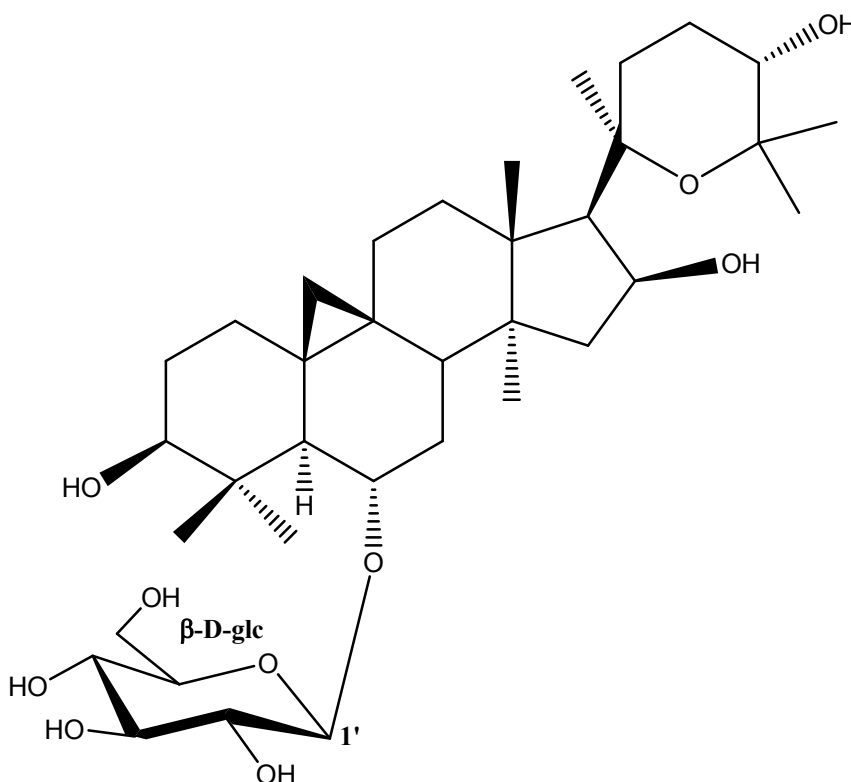
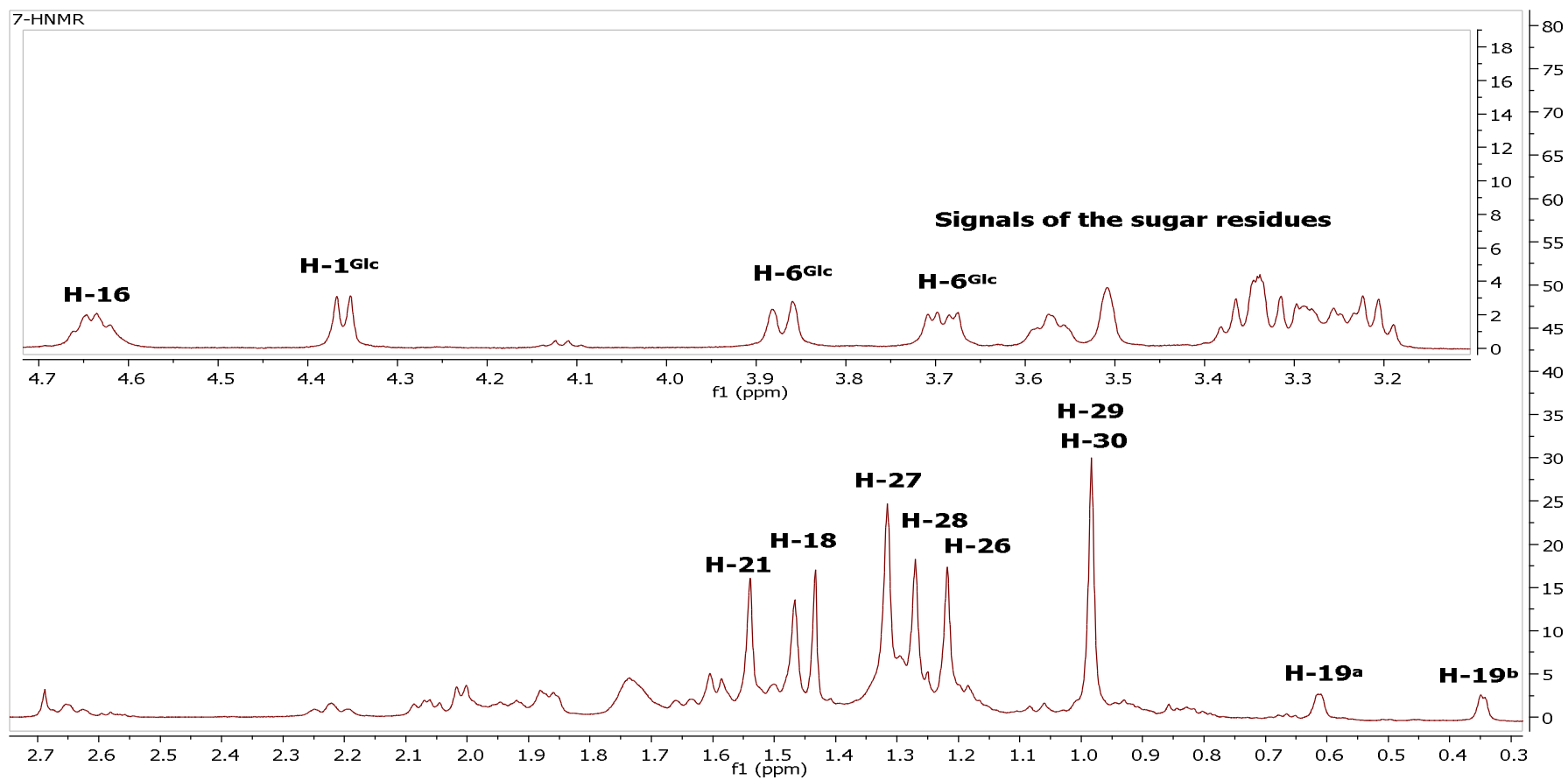


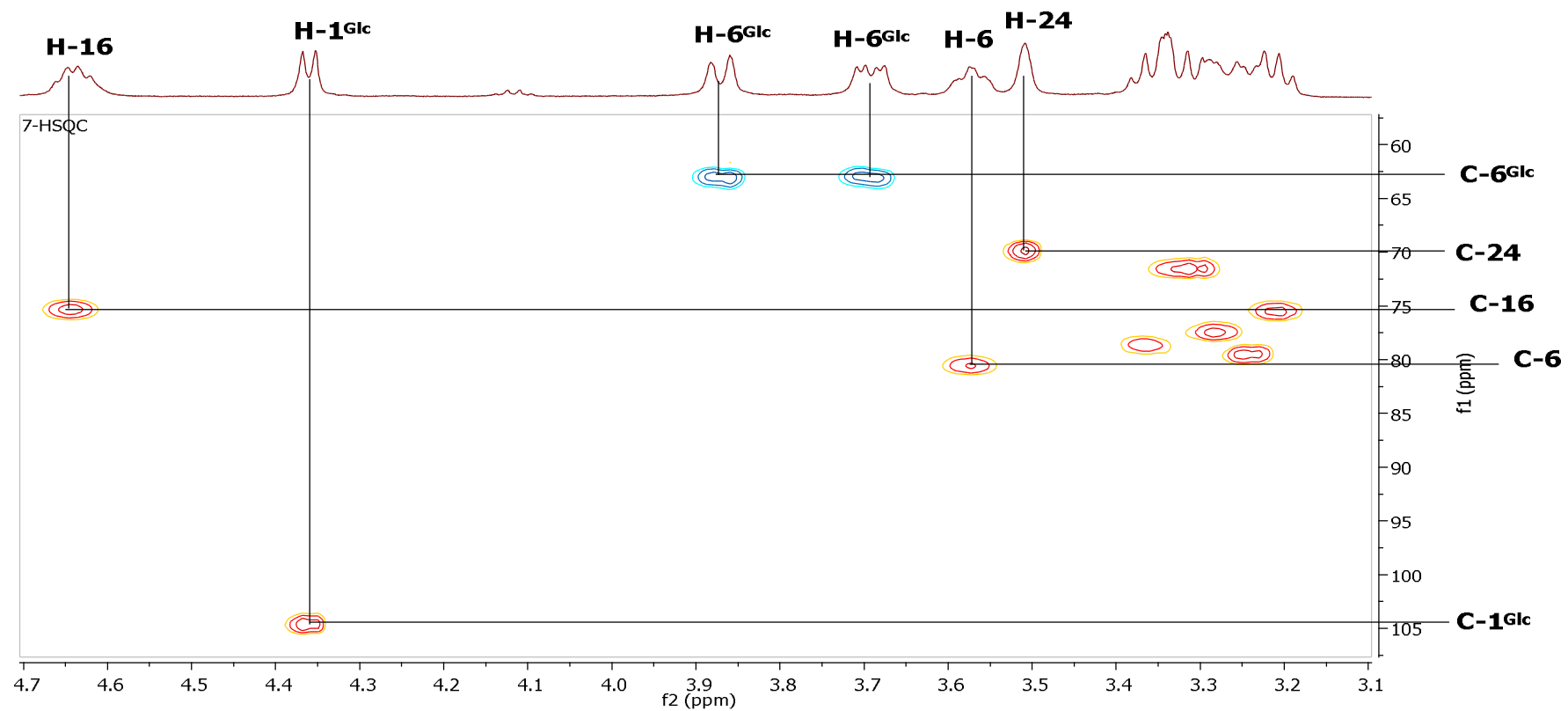
Figure 3.7. Structure of AAU7

Table 3. 7. ^1H and ^{13}C Assignments of AAU7 (150/600 MHz, δ ppm, in CD_3OD)

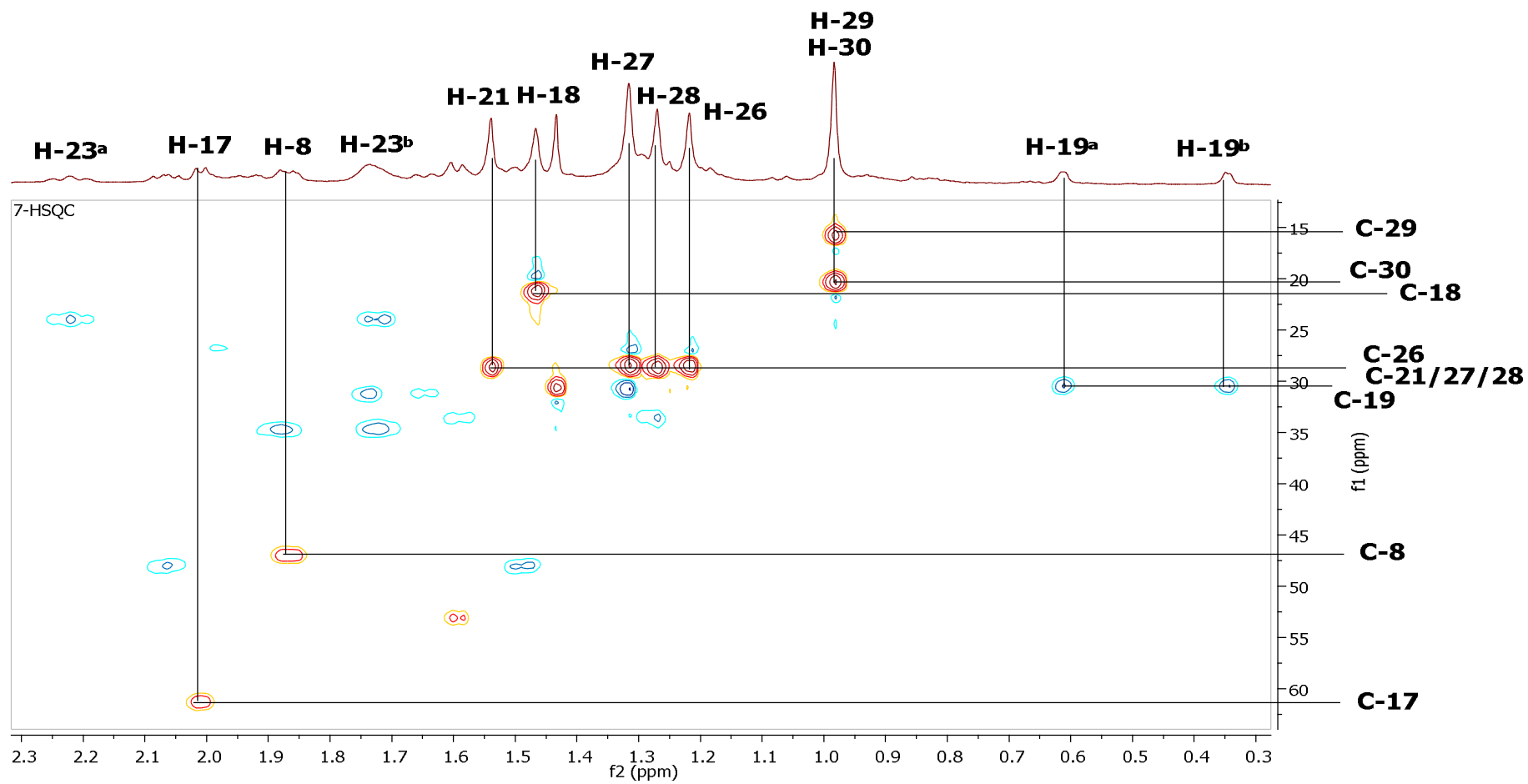
Position	δ_{C}	δ_{H} (J in Hz)
1	33.2	1.59, 1.29, m
2	31.0	1.74, 1.65, m
3	79.1	3.24, dd (11.3, 4.0)
4	42.2	-
5	52.7	1.60, d (9.5)
6	80.2	3.58, ddd (9.5, 9.5, 4.5)
7	35.1	1.92, 1.50, m
8	46.8	1.87, dd (11.9, 4.2)
9	21.8	-
10	29.9	-
11	26.3	1.99, 1.33, m
12	34.3	1.89, 1.73, m
13	46.5	-
14	47.7	-
15	47.7	2.07, dd (12.7, 8.0), 1.49, dd (12.7, 5.2)
16	74.9	4.64, ddd (8.0, 8.0, 5.2)
17	60.8	2.02, dd (9.9, 8.0)
18	21.0	1.47, s
19	30.1	0.62, brs 0.35, brs
20	80.2	-
21	28.3	1.54, s
22	26.5	2.64, 1.20, m
23	23.7	2.22, 1.73, m
24	69.4	3.51, br s
25	76.1	-
26	28.2	1.22, s
27	28.3	1.32, s
28	28.3	1.27, s
29	15.4	0.98, s
30	19.9	0.99, s
$\beta\text{-D-Glc (at C-6)-1'}$	104.6	4.36, d (7.5)
2'	75.4	3.21, dd (7.5, 9.0)
3'	78.2	3.36, t (9.0)
4'	71.3	3.31, t (9.0)
5'	77.4	3.28, ddd (3.5, 4.5, 9.0)
6'	62.7	3.88, dd (3.5, 12.0), 3.69, dd (4.5, 12.0)



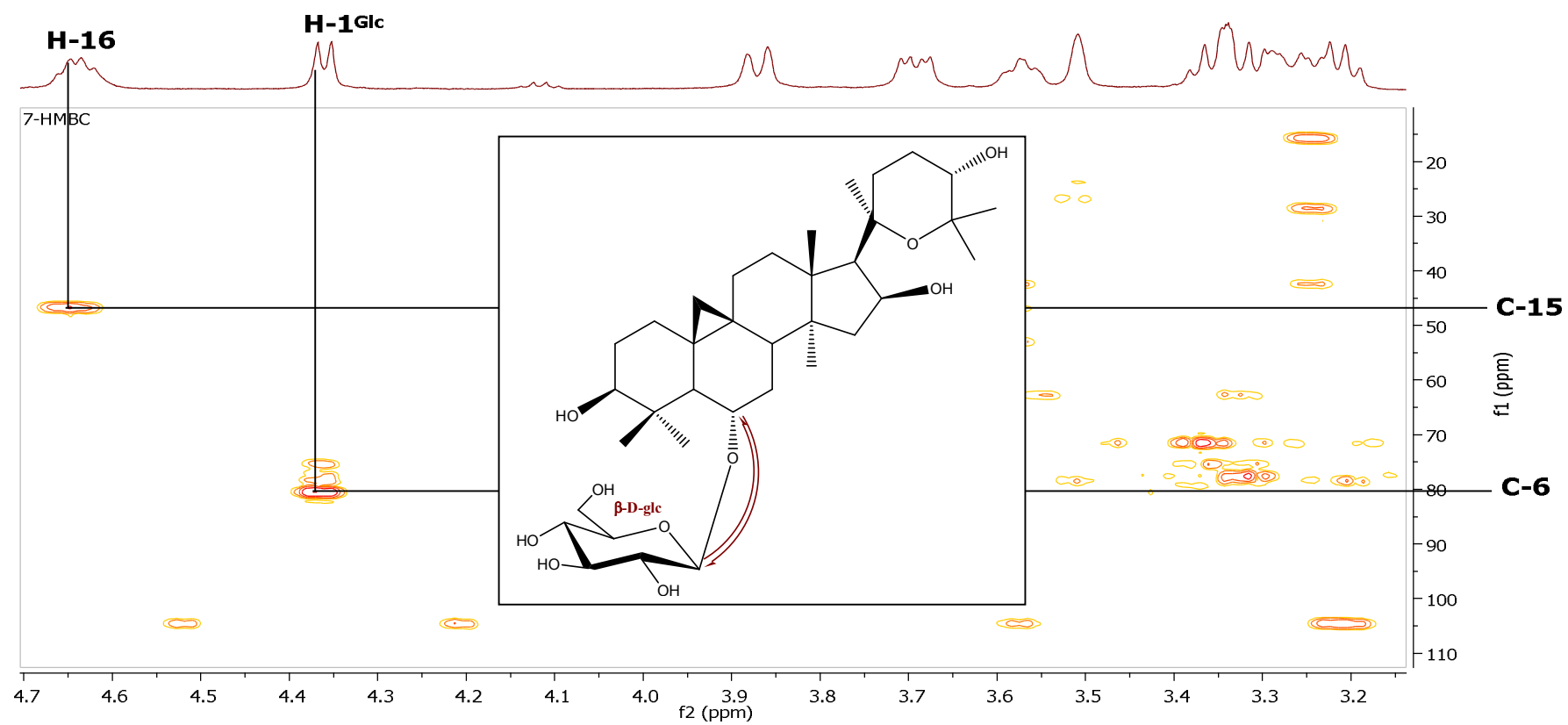
Spectrum 3.31. ^1H -Spectrum of AAU7 (600 MHz, CD_3OD)



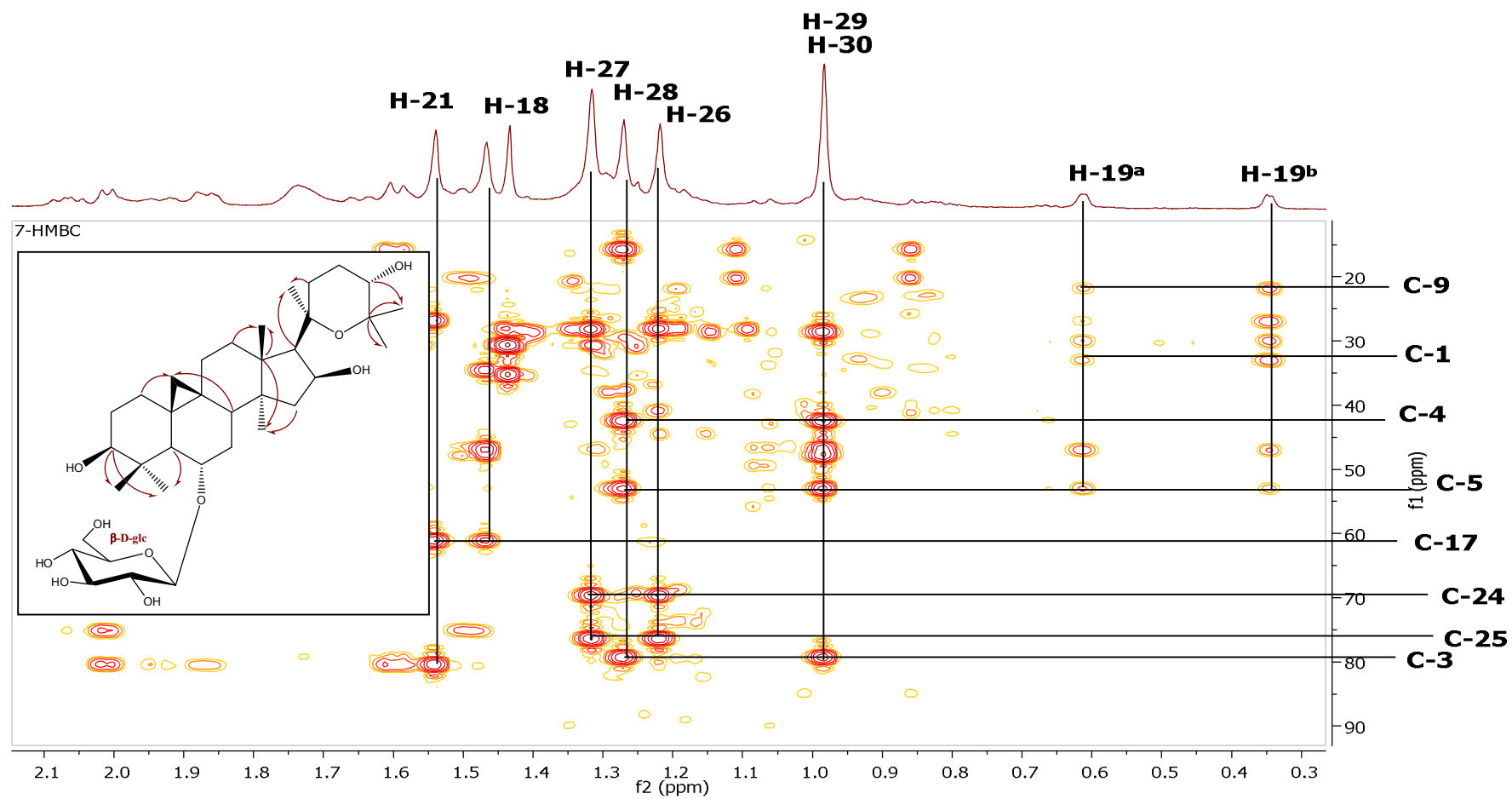
Spectrum 3.32. HSQC Spectrum of AAU7



Spectrum 3.33. HSQC Spectrum of AAU7



Spectrum 3.34. HMBC Spectrum of AAU7



Spectrum 3.35. HMBC Spectrum of AAU7

3.1.8. Structural Identification of Compound AAU8

The molecular formula of compounds **AAU8** was established as $C_{35}H_{58}O_9$ by HRMALDITOFMS analysis (m/z 645.3979 $[M+Na]^+$, calcd for $C_{35}H_{58}O_9Na$, 645.3983), respectively. The comparison of NMR data of compounds **AAU7** and **AAU8** showed that the two compounds differed only for the sugar unit at C-6. The combination of 1D-TOCSY, HSQC and DQF-COSY data led to determine this sugar unit as β -xylopyranose (δ 4.33, d, $J = 7.5$ Hz) for **AAU8**. Therefore, the structures of compounds **AAU8** was established as 6-*O*- β -D-xylopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(*R*),25-epoxycycloartane, respectively (**Fig. 3.8**).

Amorphous white solid; $C_{35}H_{58}O_9$; $[\alpha]_D^{25} +8.2^\circ$ (c 0.01 MeOH); IR $\nu_{\max}^{KBr}$ cm^{-1} : 3471 ($>OH$), 3050 (cyclopropane ring), 2943 ($>CH$), 1268 and 1057 (C-O-C); ESI-MS m/z 645.50 $[M+Na]^+$, MS/MS m/z 495.30 $[M+Na-150]^+$; HRMALDITOFMS $[M+Na]^+$ m/z 645.3983 (calcd for $C_{35}H_{58}O_9Na$, 645.3979); ^1H (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data see: **Table 3.8**.

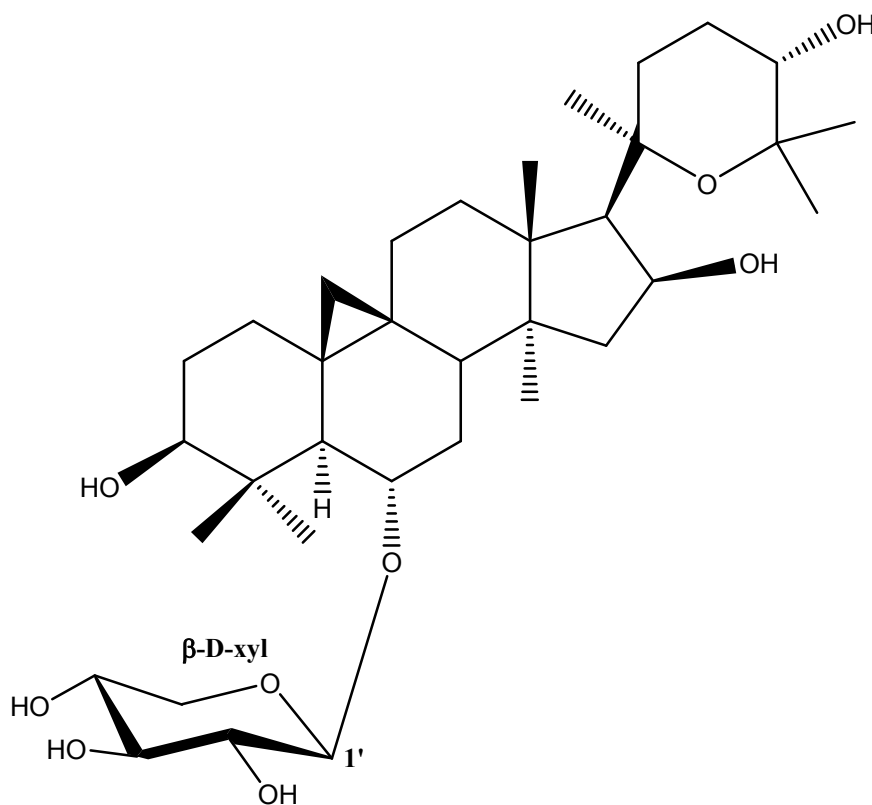
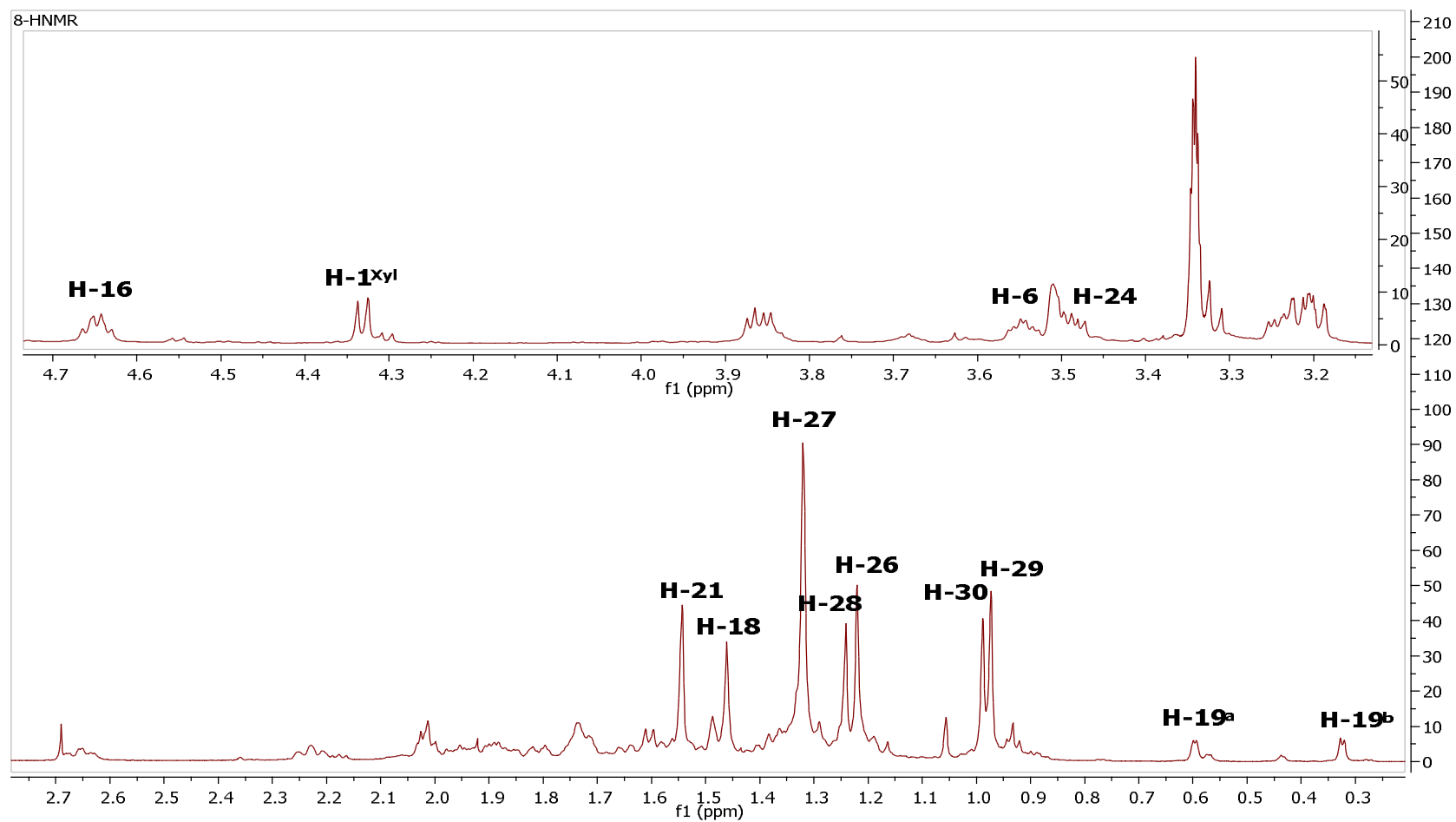


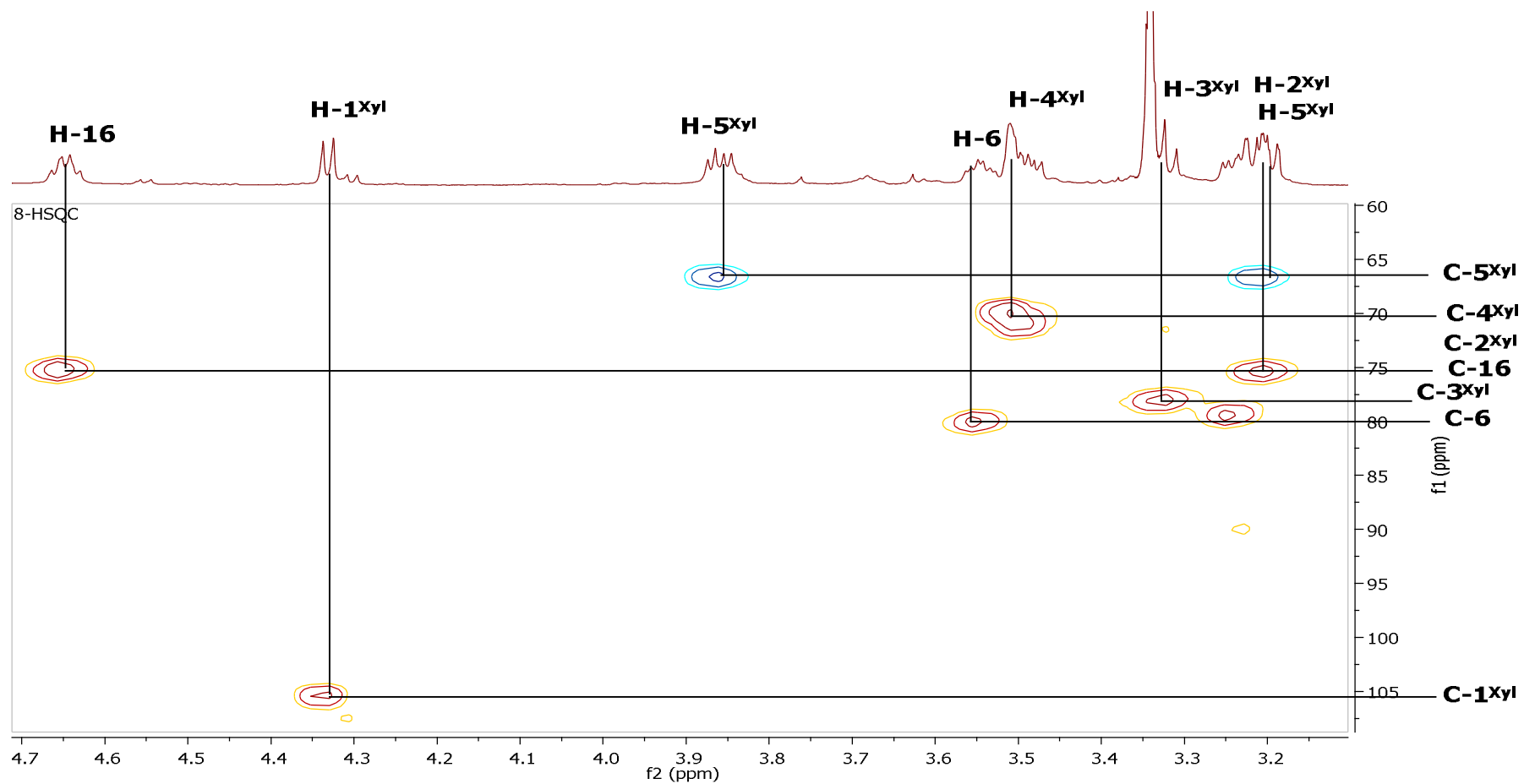
Figure 3.8. Structure of AAU8

Table 3.8. ^1H and ^{13}C Assignments of AAU8 (150/600 MHz, δ ppm, in CD_3OD)

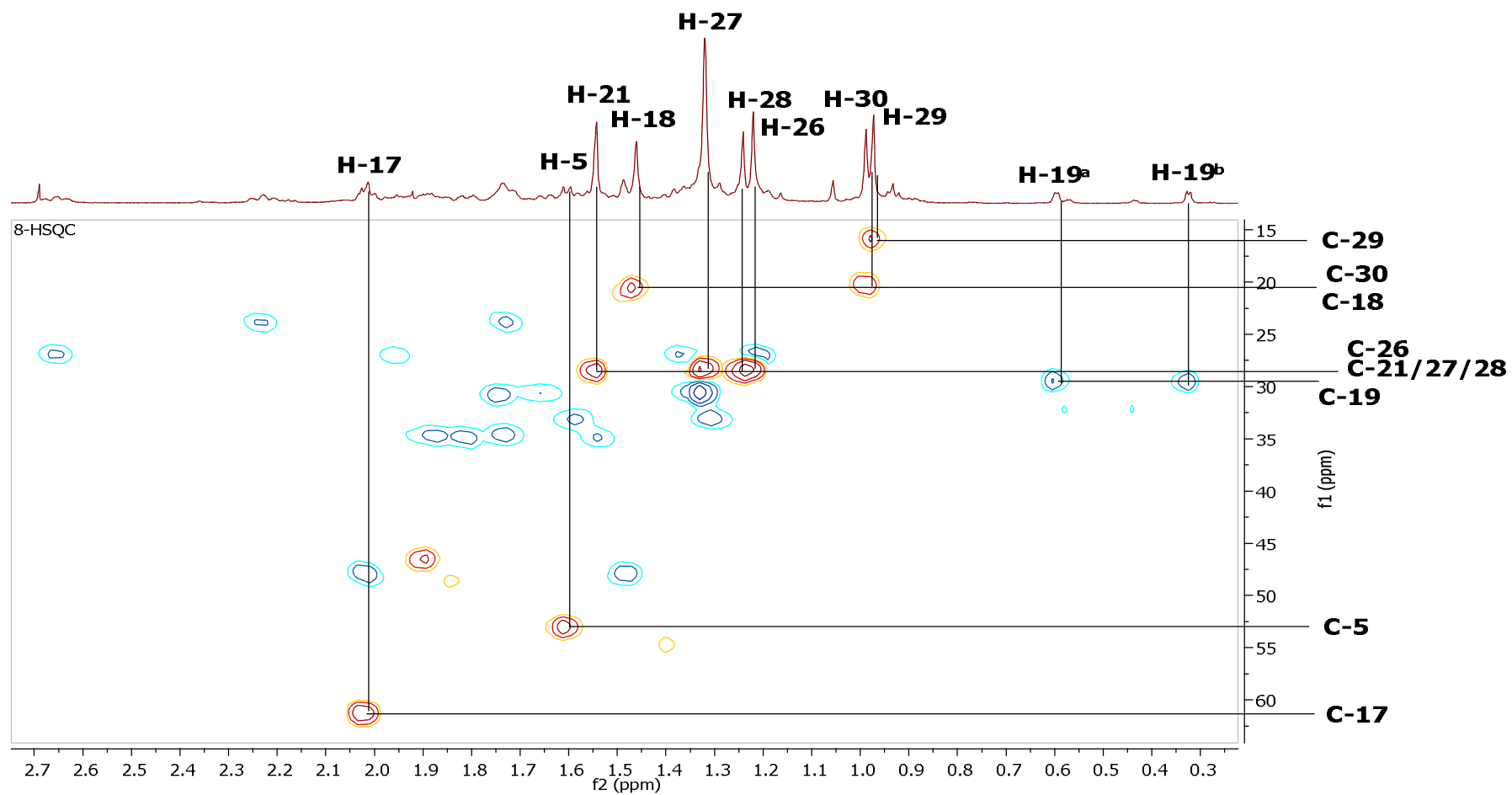
Position	δ_{C}	δ_{H} (J in Hz)
1	33.1	1.58, 1.27, m
2	31.1	1.74, 1.64, m
3	79.0	3.22, dd (11.3, 4.0)
4	42.2	-
5	52.7	1.60, d (9.5)
6	80.1	3.57, ddd (9.5, 9.5, 4.5)
7	35.1	1.92, 1.50, m
8	46.7	1.86, dd (11.9, 4.2)
9	21.8	-
10	29.9	-
11	26.3	1.99, 1.33, m
12	34.3	1.89, 1.73, m
13	46.4	-
14	47.5	-
15	47.5	2.06, dd (12.7, 8.0), 1.49, dd (12.7, 5.2)
16	74.7	4.63, ddd (8.0, 8.0, 5.2)
17	60.7	2.02, dd (9.9, 8.0)
18	21.0	1.47, s
19	30.1	0.62, d (4.2) 0.35, d (4.2)
20	80.1	-
21	28.3	1.54, s
22	26.5	2.63, 1.20, m
23	23.7	2.22, 1.73, m
24	69.3	3.50, br s
25	76.0	-
26	28.2	1.22, s
27	28.3	1.32, s
28	28.3	1.27, s
29	15.4	0.98, s
30	19.9	0.99, s
$\beta\text{-D-Xyl (at C-6)-1'}$	104.9	4.33, d (7.5)
2'	74.9	3.20, dd (9.2, 7.5)
3'	77.7	3.32, t (9.2)
4'	70.9	3.49, m
5'	66.2	3.86, dd (11.7, 5.2), 3.20, t (11.7)



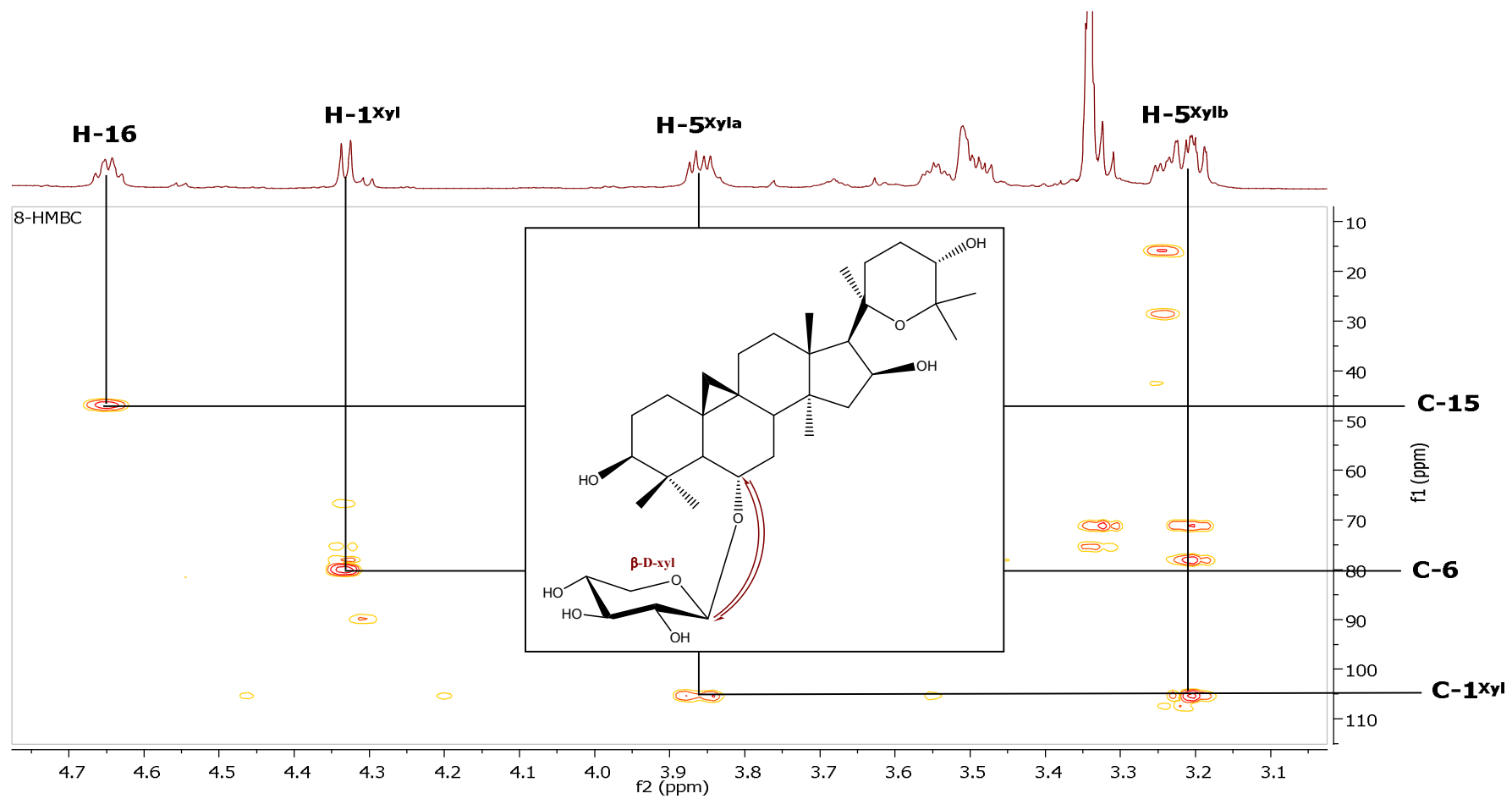
Spectrum 3.36. ^1H NMR Spectrum of AAU8 (600 MHz, CD_3OD)



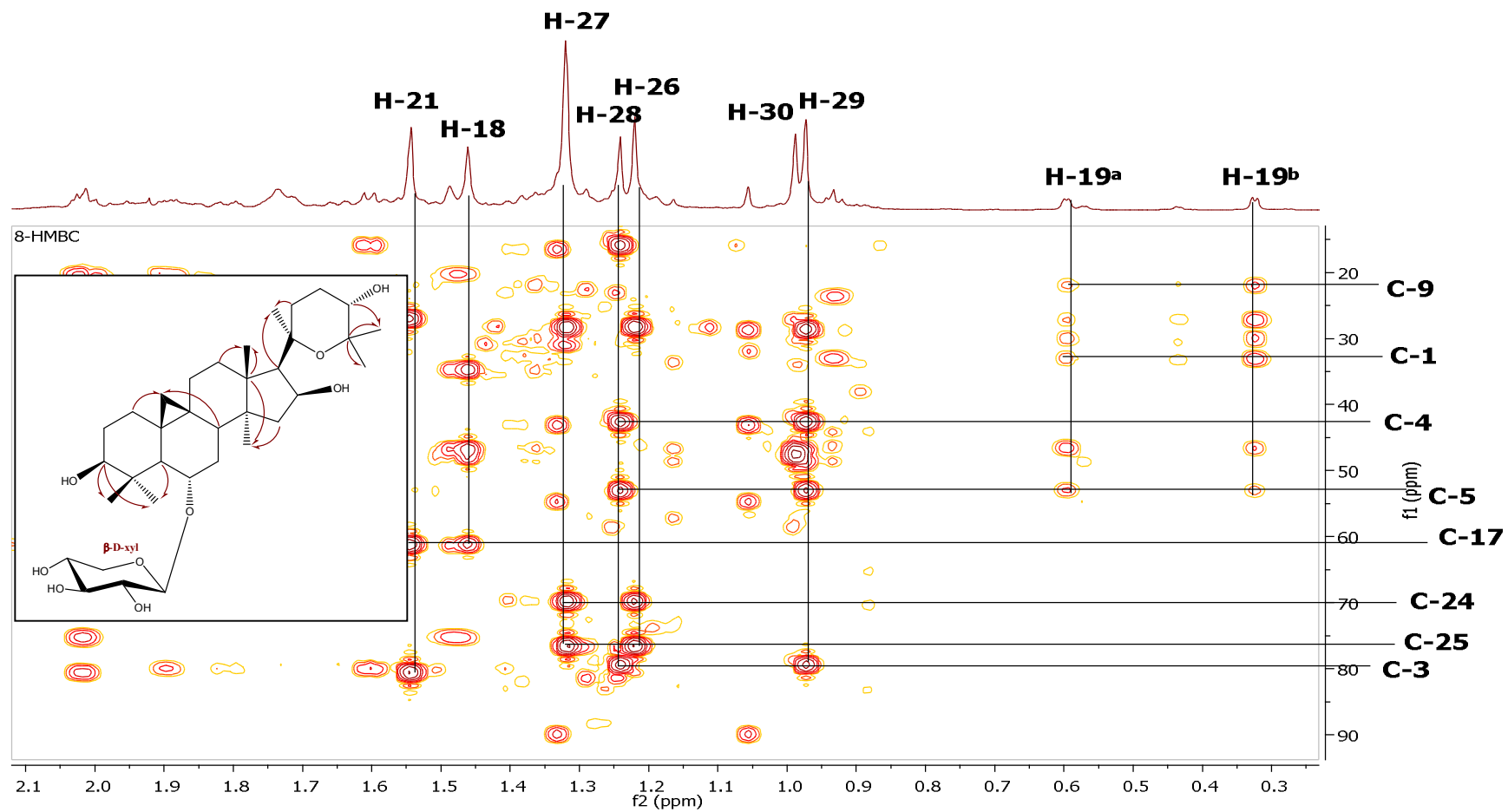
Spectrum 3.37. HSQC Spectrum of AAU8



Spectrum 3.38. HSQC Spectrum of AAU8



Spectrum 3.39. HMBC Spectrum of AAU8



Spectrum 3.40. HMBC Spectrum of AAU8

Additionally, ten known cycloartane-type glycosides, 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*- α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24(*S*),25-pentahydroxycycloartane (**AAU9**) (Horo et al., 2010), oleifolioside B (**AAU10**) (Özipek et al., 2005), cyclocanthoside G (**AAU11**) (Isaev et al., 1992), cyclocanthoside E (**AAU12**) (Isaev et al., 1992), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-*O*- α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(*R*),25-epoxycycloartane (**AAU13**) (Horo et al., 2010), 3-*O*-[α -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-xylopyranosyl]-6-*O*- β -D-glucopyranosyl-3 β ,6 α ,16 β ,24 α -tetrahydroxy-20(*R*),25-epoxycycloartane (**AAU14**) (Horo et al., 2010), cyclocanthoside F (**AAU15**) (Agzamova et al., 1999) cyclocephaloside I (**AAU16**) (Bedir et al., 1998a), cyclotrisectoside (**AAU17**) (Sukhina et al., 2007) and macrophyllsaponin B (**AAU18**) (Çalış et al., 1996) were isolated.

The antiproliferative activity of cycloartane glycosides has been reported against several cancer lines including solid tumor (HepG2), blood tumor (HL-60) and drug resistant tumor (R-HepG2) (Kikuchi et al., 2007). In particular 16 β ,23:23,26:24,25-triepoxy-, 16 β ,23:16 α ,24-diepoxy- and 16 β ,23;22 β ,25-diepoxycycloartane derivatives have been reported for their cytotoxic activity (Nian et al., 2010; Watanabe et al., 2002). Moreover cancer chemopreventive effects of natural and semisynthetic cycloartane-type and related triterpenoids have been reported (Tian et al., 2005). Leishmanicidal (Özipek et al., 2005) and immunomodulatory (Çalış, et al., 1997) activities have been reported for cyclocanthogenin and cycloastragenol derivatives.

On the basis of biological activities reported for cycloartane glycosides, the antiproliferative activity of compounds **AAU1-AAU18** was tested in several cell lines including HL-60 (human promyelocytic leukemia), MCF-7 (human breast cancer), HT-29 (human colon carcinoma), A549 (human lung adenocarcinoma), PC3 (human prostate cancer) and M14 (human melanoma). Compounds **AAU1-AAU18** were tested in a range of concentrations between 1-50 μ M, but only few compounds showed a weak activity. Compound **AAU17** exhibited an IC_{50} value of 32 μ M against MCF7 cells, while compound **AAU8** exhibited an IC_{50} value of 45 μ M against HL-60 cells. Compounds **AAU12** and **AAU14** showed an IC_{50} value of 50 μ M against A549 and PC3 cells. All the other compounds showed no activity (data not shown). Etoposide used as positive control showed IC_{50} values ranging from 0.5 μ M (HL-60 cells) to 16 μ M (A549 cells).

3.1.9. Acid Hydrolysis of compounds from *Astragalus aureus*

A solution (1 mg each) of compounds **AAU1** and **AAU3** in 1 N HCl (0.5 mL) was stirred at 80 °C for 4 h. After cooling, the solution was concentrated by blowing N₂ gas. The residue was dissolved in 1-(trimethylsilyl)-imidazole and pyridine (0.1 mL), and the solution was stirred at 60 °C for 5 min.

After drying the solution with a stream of N₂, the residue was partitioned between H₂O and CH₂Cl₂ (1 mL, 1:1 v/v). The CH₂Cl₂ layer was analysed by GC using an L-Chirasil-Val column (0.32 mm x 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5°C/min. The peaks of the hydrolysate of **AAU1** were detected at 8.94 and 9.81 min (α -L-arabinose and β -L-arabinose), 9.70 and 10.72 (α -L-rhamnose and β -L-rhamnose) and 10.99 and 12.02 (β -D-xylose and α -D-xylose). The peaks of D-xylose (10.98 and 12.01 min) and D-glucose (14.73 min) were detected in the hydrolysate of **AAU3**. Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine were detected at 8.92 and 9.80 (L-arabinose), 9.67 and 10.70 (L-rhamnose), 10.98 and 12.00 min (D-xylose), and 14.71 min (D-glucose).

3.2. *Astragalus tauricolus*

3.2.1. Structural Identification of Compound **ATA2**

HPLC-ESIMSⁿ analysis, in negative ionization mode on an ion trap analyzer, of the butanol extract of *Astragalus tauricolus* allowed us to obtain a preliminary plant metabolite profiling. In particular, the careful analysis of the multistage mass spectra of the compounds eluted from 22 to 42 min suggested the presence of 22 oleanane-type triterpene glycosides (**Table 3.19**), whose sugar composition could be inferred by checking neutral losses of 132, 146, 162 and 176 a.m.u., corresponding to different combinations of pentose, hexose, deoxyhexose and uronic acid units, respectively.

In order to unambiguously elucidate their structures by NMR experiments, the 22 compounds were isolated by size exclusion chromatography of the butanol extract, followed by further purification steps by silica gel chromatography and reversed-phase HPLC-RI.

The negative ESIMS spectrum of **ATA2** showed a major $[M-H]^-$ ion peak at m/z 1103 that was assigned to $[M-H]^-$. Its tandem mass spectrum highlighted the presence of a product ion at m/z 941, originated by neutral loss of a hexose sugar, along with two minor product ions at m/z 923 and 879, due to consecutive losses of water and carbon dioxide, respectively (**Table 3.9**). This latter neutral loss could be referred to the presence of a carboxyl group in the molecule.

The ^1H NMR spectrum of **ATA2** showed signals for six methyl groups as singlets at δ 1.39, 1.26, 1.17, 1.02, 0.92 and 0.88, one olefinic proton at δ 5.33 (H-12, t, $J = 3.5$ Hz), two oxygen-bearing methine protons at δ 3.35 (H-3, dd $J = 11.5, 4.2$ Hz), and 3.52 (H-22, brs), and signals for one primary alcoholic functions at δ 3.22 and 4.14 (H₂-24, d, $J = 11.5$) (**Table 3.9**) (Kapusta et al., 2005). A combination of 1D and 2D NMR techniques led to the assignment of the ^1H and ^{13}C NMR signals due to the A, B, C, D rings of a 3 β ,24-dihydroxyolean-12-ene sapogenol unit (Takeshita et al., 1989). Ring E was shown to contain an oxymethine at C-22 (δ_C 76.2, δ_H 3.52 brs) and an -COOH group, confirmed by the oxygenated carbon signal at δ 178.5 assignable to C-29 of the aglycon.

For the sugar portion, compound **ATA2** showed signals corresponding to four anomeric protons at δ 5.47 (d, $J = 7.5$ Hz), 5.23 (d, $J = 1.2$ Hz), 4.82 (d, $J = 7.5$ Hz), and 4.43 (d, $J = 7.5$ Hz) (**Table 3.9**). On the basis of 2D NMR data, one β -glucopyranosyl (δ 5.47), one α -rhamnopyranosyl (δ 5.23), one β -xylopyranosyl (δ 4.82) and one β -glucuronopyranosyl (δ 4.43) units were identified. The determination of the sequence and linkage sites was obtained from the HMBC correlations between the proton signals at δ 5.23 (H-1_{rha}) and the carbon resonance at δ 79.0 (C-2_{xy}), the signal at 4.82 (H-1_{xy}) with the carbon signal at 78.3 (C-2_{gluA}), the proton signal at δ 4.43 (H-1_{gluA}) and the carbon resonance at δ 91.6 (C-3) and the signal at 5.47 (H-1_{glc}) with the carbon signal at δ 178.5 (C-29). The D configuration of glucose, glucuronic acid and xylose units and the L configuration of rhamnose unit were established after hydrolysis of **ATA2** followed by GC analysis (De Marino et al., 2003). Thus, compound **ATA2** was identified as the new 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-29-*O*- α -L-glucopyranosyl-3 β ,22 β ,24-trihydroxyolean-12-en-29-oic acid. (**Fig. 3.9**)

Amorphous white solid; C₅₃H₈₄O₂₄; $[\alpha]_D^{25} +13.1$ (*c* 0.01 MeOH); IR ν_{max}^{KBr} cm⁻¹: 3428 (>OH), 2934 (>CH), 1680 (C=O), 1658 (C=C); HRMALDITOFMS

$[M+Na]^+$ m/z 1127.5262 (calcd for $C_{53}H_{84}O_{24}Na$, 1127.5250); ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data: see **Table 3.9**.

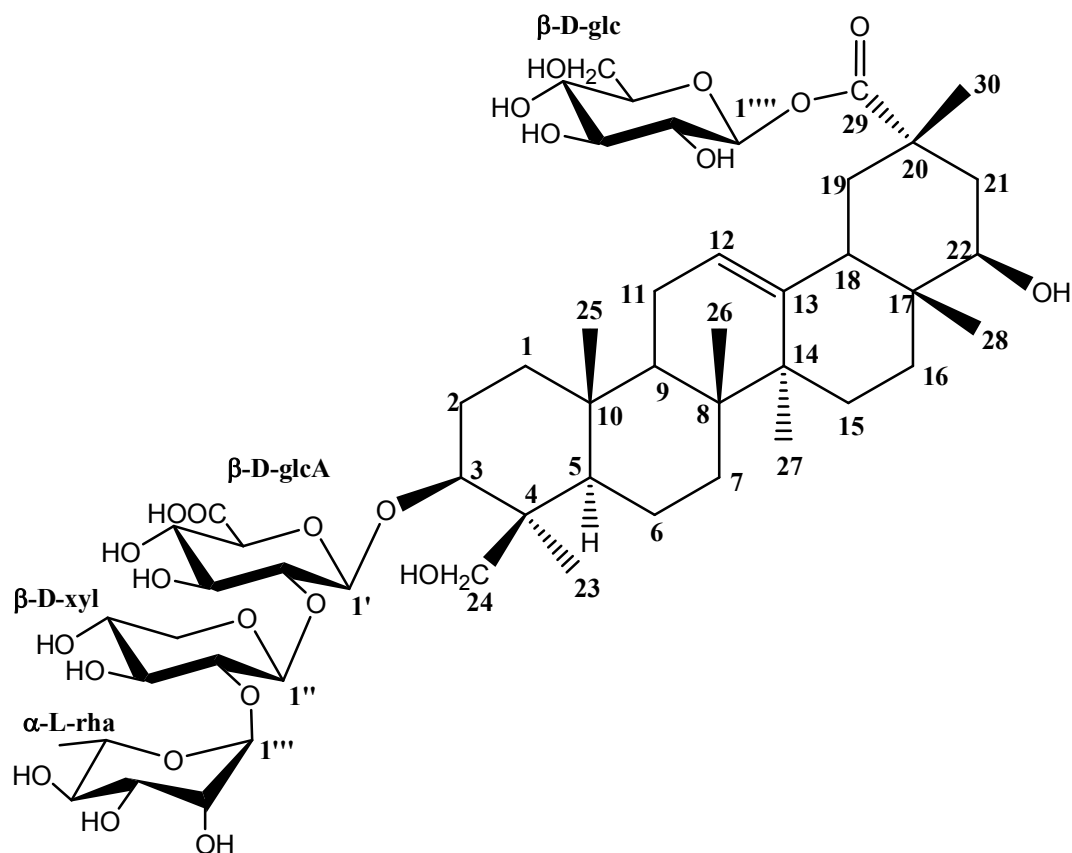
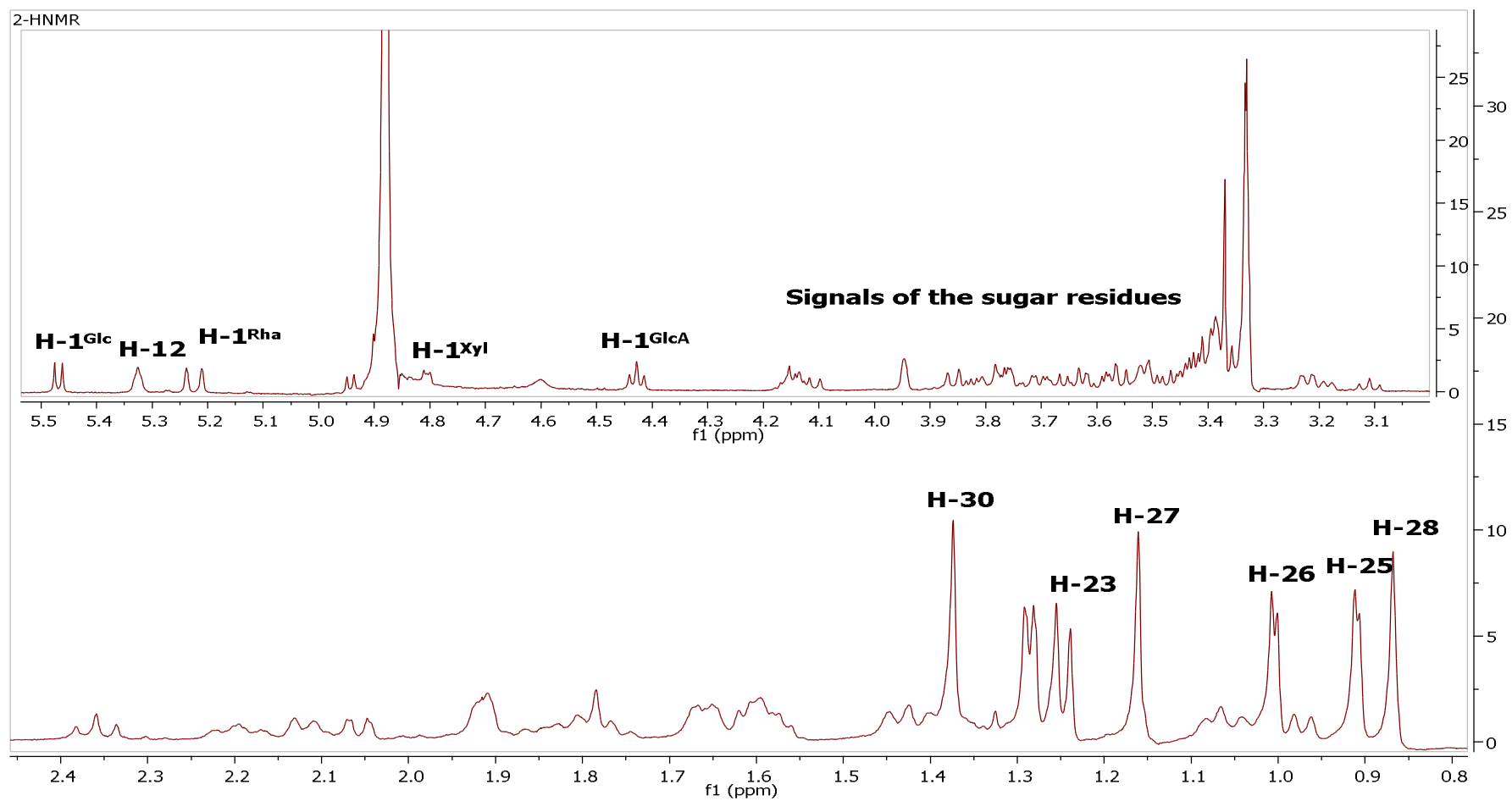


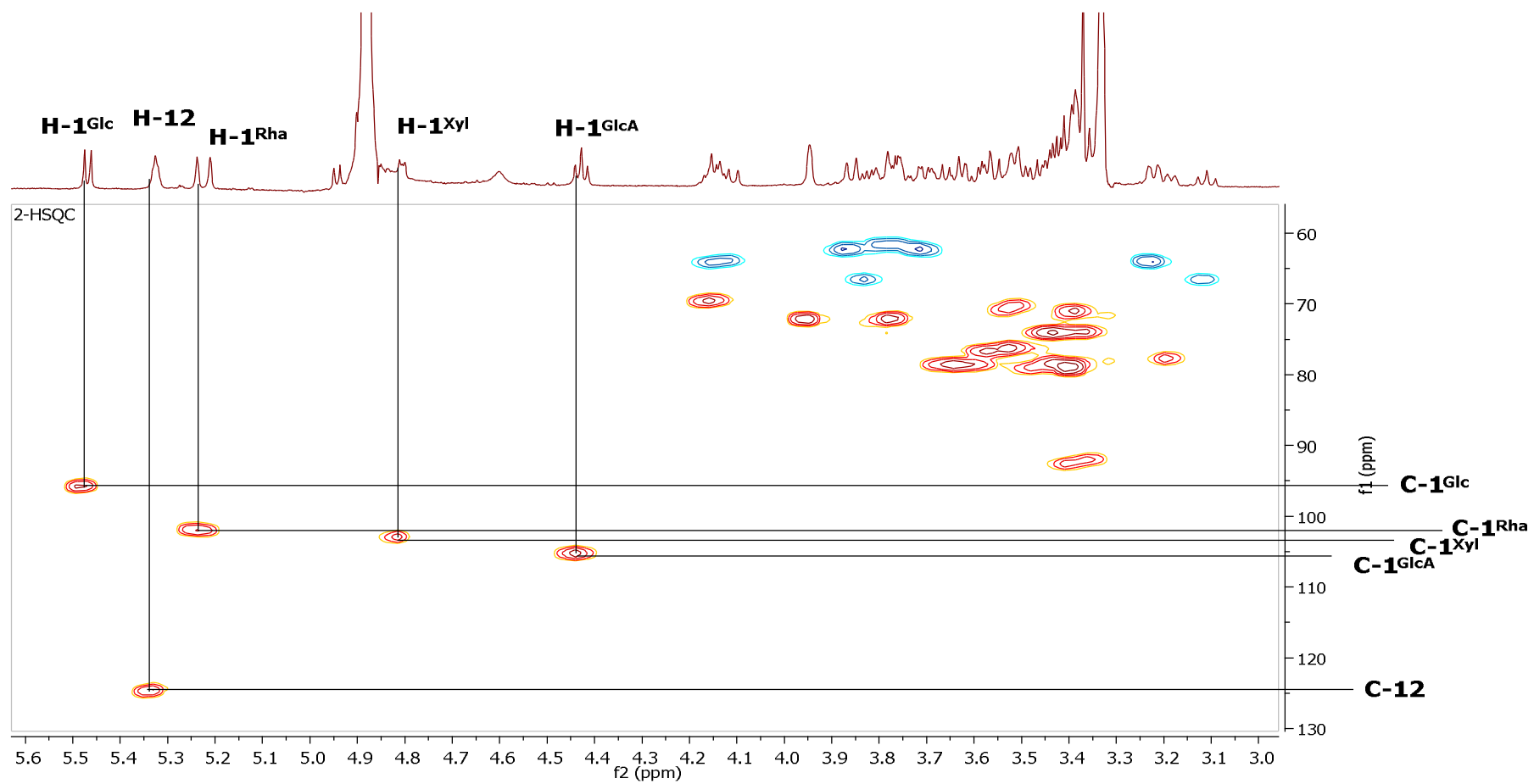
Figure 3.9. Structure of ATA2

Table 3.9. ^1H and ^{13}C Assignments of ATA2 (150/600 MHz, δ ppm, in CD_3OD)

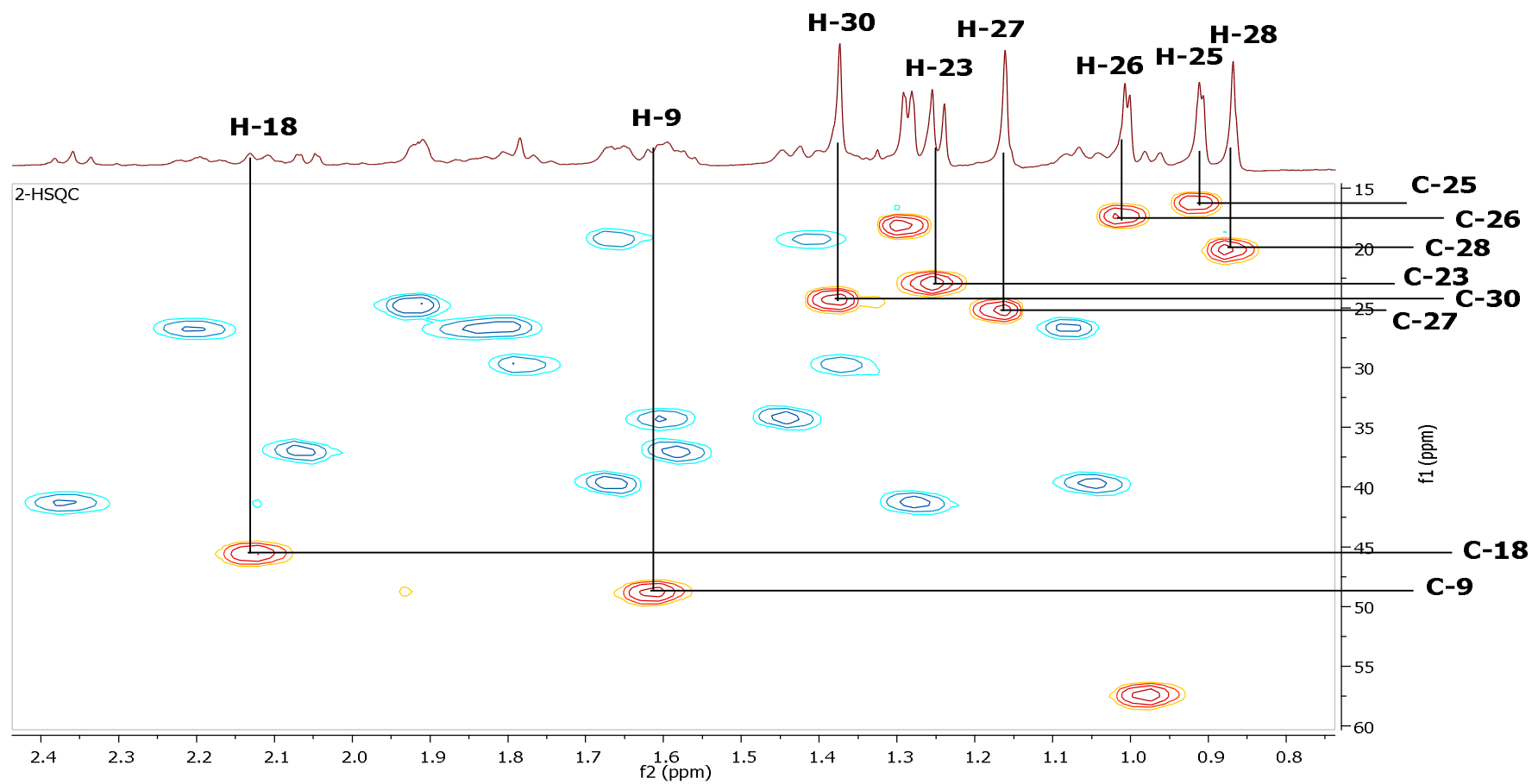
Position	δ_{C}	δ_{H} (J in Hz)
1	39.6	1.67, 1.07, m
2	26.7	2.20, 1.85, m
3	91.6	3.35, dd (11.5, 4.5)
4	44.5	-
5	57.2	0.99, m
6	19.3	1.66, 1.42, m
7	34.2	1.60, 1.44, m
8	40.6	-
9	48.6	1.62, m
10	37.0	-
11	24.6	1.92, (2H) m
12	124.4	5.33, t (3.5)
13	144.6	-
14	43.3	-
15	26.5	1.80, 1.09, m
16	29.6	1.78, 1.38, m
17	38.4	-
18	45.5	2.13, m
19	41.1	2.37, 1.28, m
20	42.8	-
21	36.6	2.07, 1.58, m
22	76.2	3.52, brs
23	22.8	1.26, s
24	63.9	4.14, d (11.5), 3.22, d (11.5)
25	16.1	0.92, s
26	17.1	1.02, s
27	25.1	1.17, s
28	19.9	0.88, s
29	178.5	-
30	24.1	1.39, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.0	4.43, d (7.5)
2'	78.3	3.59, dd (7.5, 9.0)
3'	78.4	3.63, dd (9.0, 9.0)
4'	73.8	3.43, dd (9.0, 9.0)
5'	76.4	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Xyl (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.82, d (7.5)
2''	79.0	3.39, dd (7.5, 9.2)
3''	76.4	3.58, t (9.2)
4''	70.2	3.52, m
5''	66.4	3.84, dd (5.2, 11.7), 3.12, t (11.7)
$\alpha\text{-L-Rha (at C-2}_{\text{xyl}}\text{)-1'''}$	101.6	5.23, d (1.2)
2'''	71.9	3.95, dd (1.2, 3.2)
3'''	71.9	3.78, dd (3.2, 9.7)
4'''	73.7	3.37, t (9.7)
5'''	69.3	4.16, m
6'''	17.8	1.27, d (6.5)
$\beta\text{-D-Glc (at C-29)-1''''}$	95.4	5.47, d (7.5)
2''''	73.8	3.38, dd (7.5, 9.0)
3''''	77.4	3.19, t (9.0)
4''''	70.9	3.39, t (9.0)
5''''	78.8	3.47, ddd (3.5, 4.5, 9.0)
6''''	62.1	3.87, dd(3.5, 12), 3.71, dd (4.5, 12)



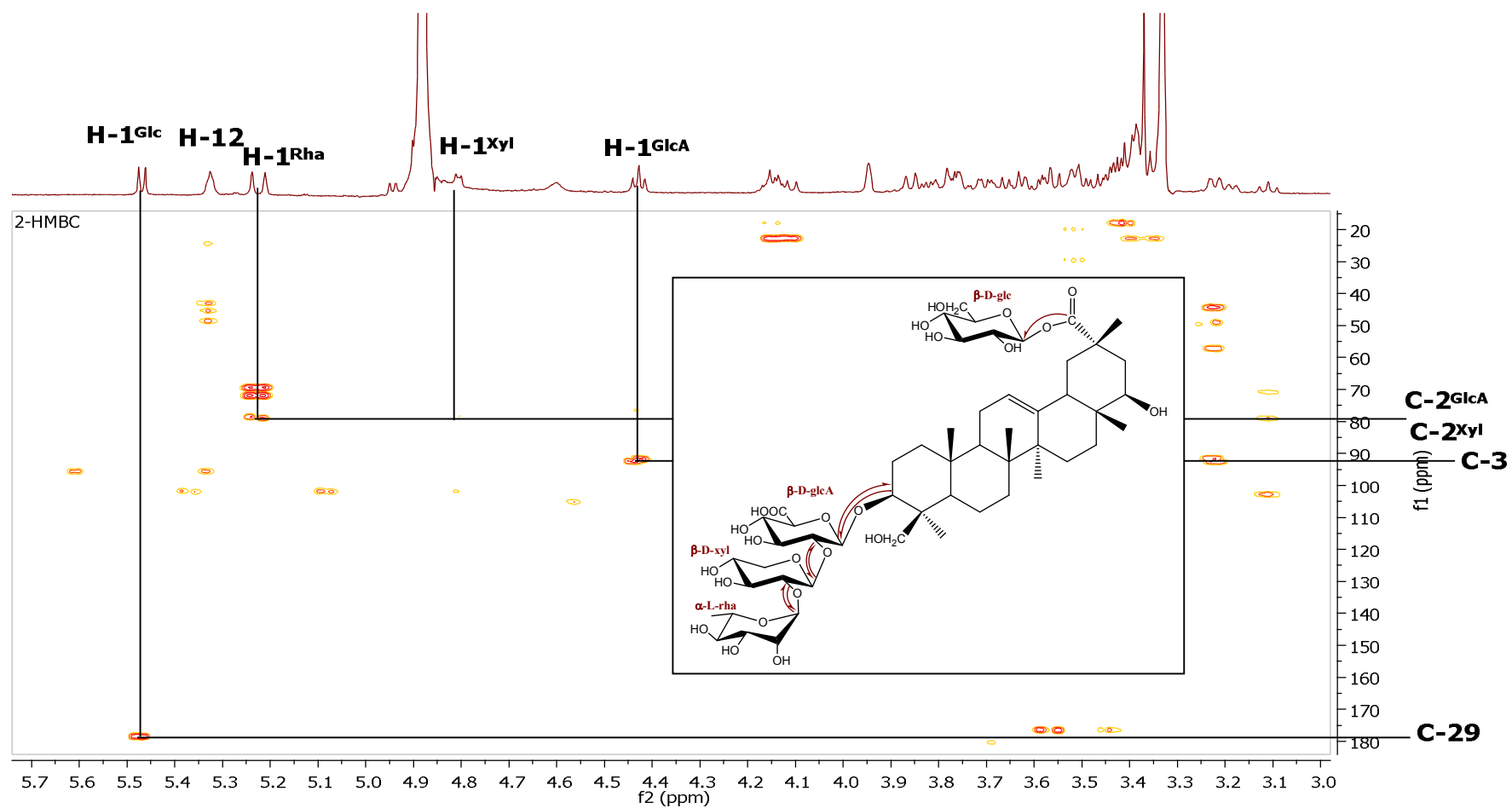
Spectrum 3.41. ^1H NMR Spectrum of ATA2 (600 MHz, CD_3OD)



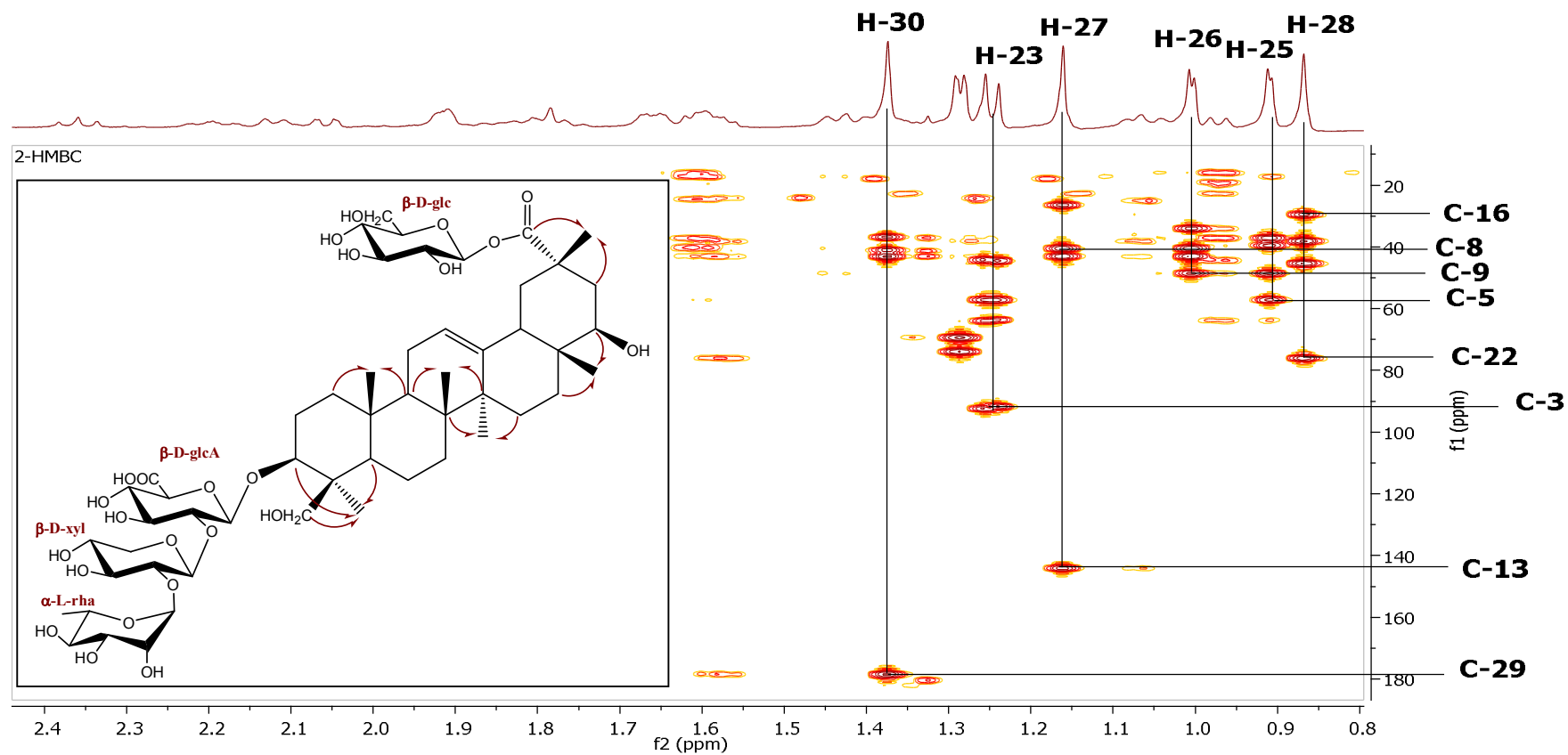
Spectrum 3.42. HSQC Spectrum of ATA2



Spectrum 3.43. HSQC Spectrum of ATA2



Spectrum 3.44. HMBC Spectrum of ATA2



Spectrum 3.45. HMBC Spectrum of ATA2

3.2.2. Structural Identification of Compound ATA3

The negative ESIMS spectrum of **ATA3** showed a major $[M-H]^-$ ion peak at m/z 1119. The ESIMSⁿ fragmentation pattern of the $[M-H]^-$ ion allowed to determine the sugar chain as made up of two hexoses, a deoxyhexose and an uronic acid units (**Table 3.10**). In particular, the ESIMS² spectrum showed a minor $[(M-18)-H]^-$ ion at m/z 1101 along with a more abundant $[(M-18-44)-H]^-$ ion at m/z 1057, originated by consecutive neutral losses of H₂O and CO₂, as already observed for **ATA2**. More informative was the ESIMS³ spectrum recorded for the ion at m/z 1101 showing the product ion at m/z 911, originated by neutral loss of a deoxyhexose unit from the $[(M-18-44)-H]^-$ ion, along with the product ions at m/z 749 and 587, resulting from consecutive losses of two hexose units from the ion at m/z 911. Finally, the check of the product ion at m/z 473 allowed confirming the presence of an uronic acid unit. This ion could be originated from the product ion at m/z 587 by neutral loss of 114 a.m.u., corresponding to a rearranged molecule derived from uronic acid after the losses of water and carbon dioxide. Thereby the product ion at m/z 473 could be identified as the aglycon ion.

The ¹H and ¹³C NMR signals of the aglycone of **ATA3** assigned from the 2D NMR spectra were almost superimposable on those of **ATA2**, except for the presence of a glycosylated hydroxymethyl group at C-29 (δ_C 80.0, δ_H 3.18 and 3.67, each d, $J = 9.7$ Hz), instead of a –COOH group. The location of the 29–CH₂OR group was derived from the resonance of Me-30 at δ 24.7 superimposable to that of abrisapogenol B (Takeshita et al., 1989). For the sugar portion, compound **ATA3** showed signals corresponding to four anomeric protons at δ 5.22 (d, $J = 1.2$ Hz), 4.81 (d, $J = 7.5$ Hz), 4.44 (d, $J = 7.5$ Hz) and 4.25 (d, $J = 7.5$ Hz) (**Table 3.10**). On the basis of 2D NMR data, the sugar units consisting of an α -rhamnopyranosyl (δ 5.22), two β -glucopyranosyl (δ 4.81 and 4.25) and a β -glucuronopyranosyl (δ 4.44) were identified. The determination of the sequence and linkage sites was obtained from the HMBC correlations between the proton signals at δ 5.22 (H-1_{rha}) and the carbon resonance at δ 79.1 (C-2_{glc}), the signal at δ 4.81 (H-1_{glc}) with the carbon signal at 78.4 (C-2_{gluA}), the proton signal at δ 4.44 (H-1_{gluA}) and the carbon resonance at δ 91.9 (C-3) and the signal at 4.25 (H-1_{glc}) with the carbon signal at 80.0 (C-29). Thus, compound **ATA3** was identified as the new 3-*O*-[α -L-rhamnopyranosyl-(1→2)- β -D-glucopyranosyl-(1→2)- β -D-glucuronopyranosyl]-29-*O*- α -L-glucopyranosyl-3 β ,22 β ,24,29-tetrahydroxyolean-12-ene. (**Fig. 3.10**)

Amorphous white solid; $C_{54}H_{88}O_{24}$; $[\alpha]_D^{25} +9.3$ (*c* 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3442 ($>\text{OH}$), 2930 ($>\text{CH}$), 1670 ($\text{C}=\text{O}$), 1655 ($\text{C}=\text{C}$); HRMALDITOFMS $[\text{M}+\text{Na}]^+$ m/z 1143.5573 (calcd for $C_{54}H_{88}O_{24}\text{Na}$, 1143.5563); ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data: see **Table 3.10**.

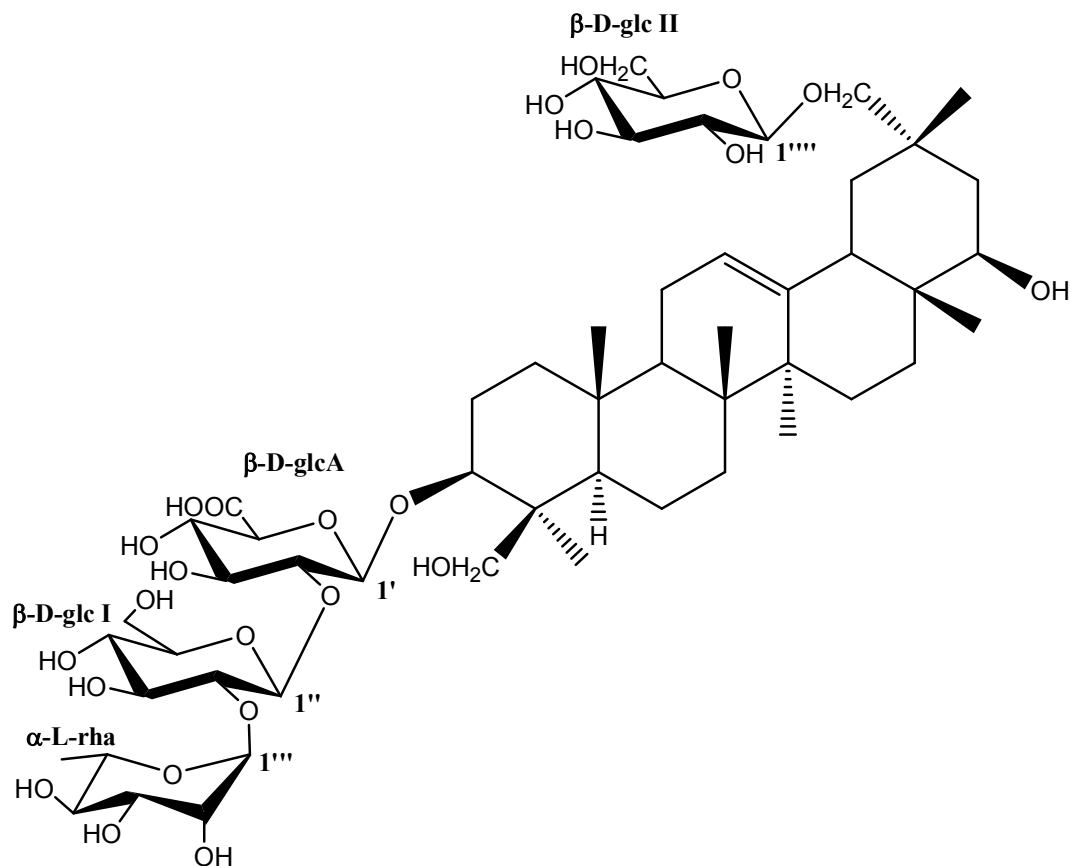
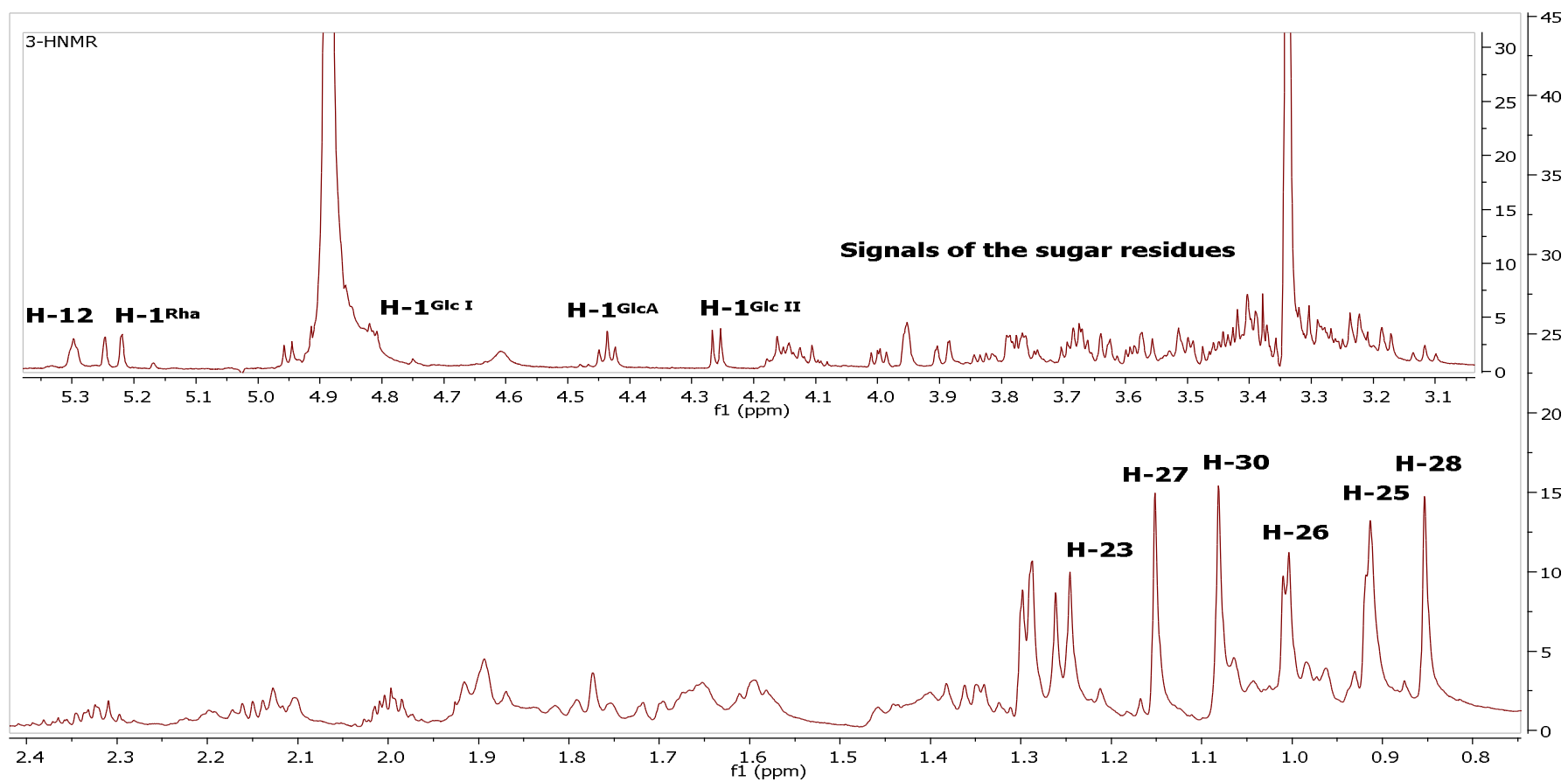


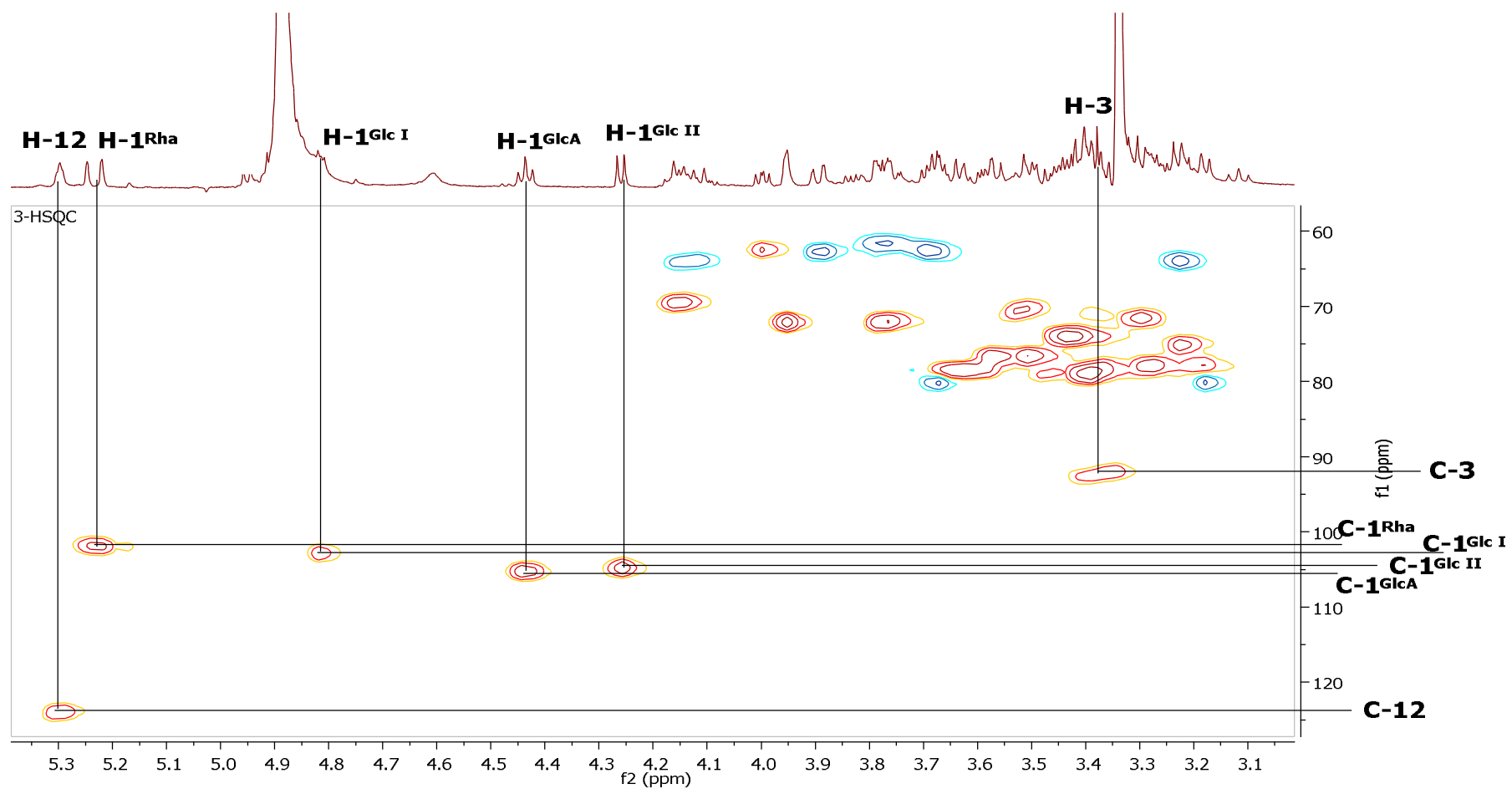
Figure 3.10. Structure of ATA3

Table 3.10. ^1H and ^{13}C Assignments of ATA3 (150/600 MHz, δ ppm, in CD_3OD)

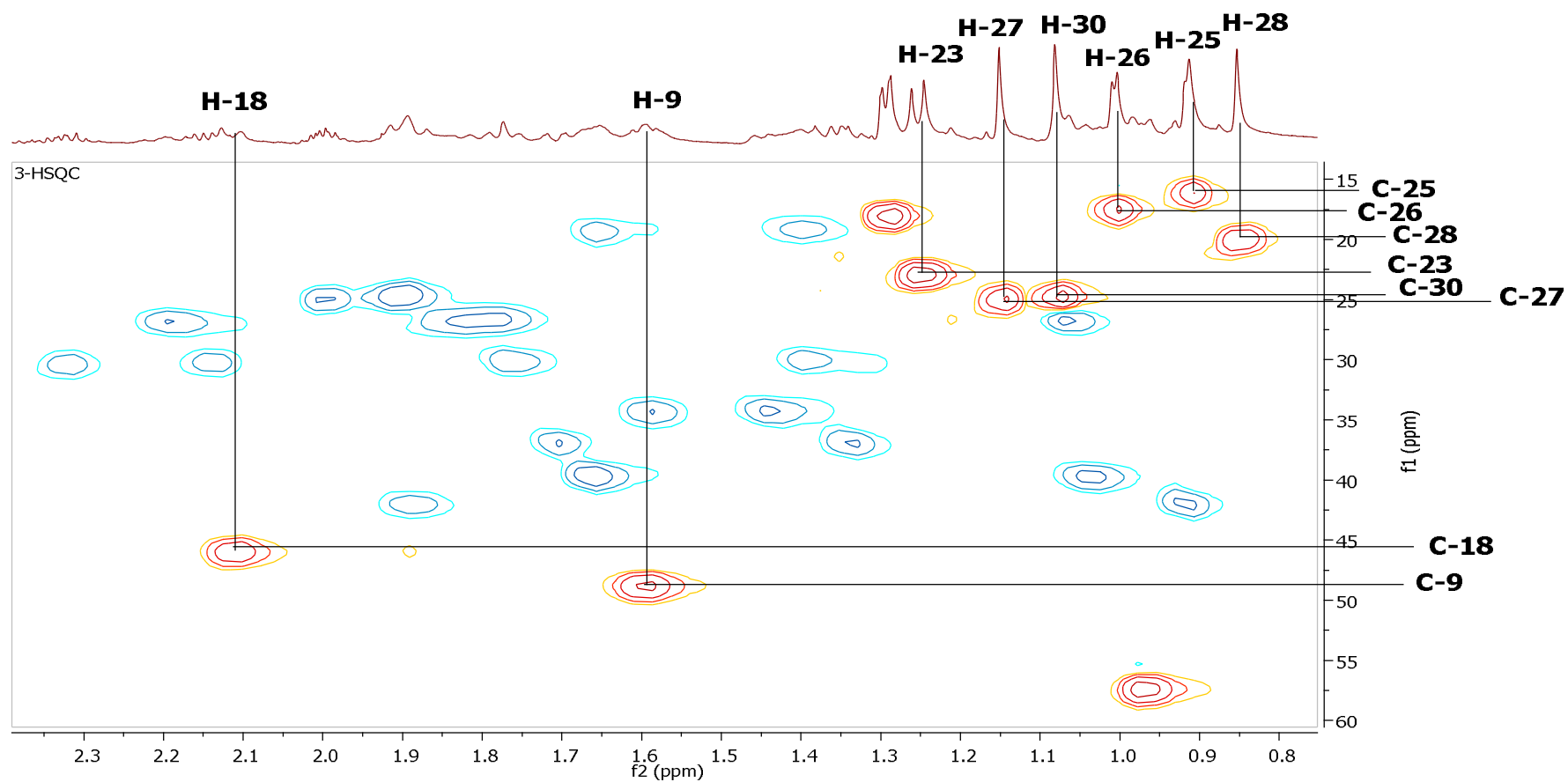
Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	39.9	1.66, 1.04, m
2	26.9	2.18, 1.80, m
3	91.9	3.36, dd (11.5, 4.2)
4	44.5	-
5	57.2	0.98, m
6	19.5	1.66, 1.39, m
7	34.3	1.60, 1.43, m
8	40.5	-
9	48.7	1.60, m
10	37.3	-
11	24.9	1.90, (2H) m
12	123.6	5.31, t (3.5)
13	144.7	-
14	43.3	-
15	26.6	1.80, 1.06, m
16	30.0	1.80, 1.31, m
17	38.4	-
18	46.0	2.02, m
19	41.9	2.38, 1.18, m
20	38.5	-
21	36.8	2.08, 1.45, m
22	77.0	3.52, brs
23	23.0	1.24, s
24	64.0	4.11, d (11.4), 3.23, d (11.4)
25	16.1	0.91, s
26	17.5	1.00, s
27	25.0	1.18, s
28	20.0	0.83, s
29	80.0	3.67, d (9.7), 3.18, d (9.7)
30	24.7	1.08, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.0	4.44, d (7.5)
2'	78.4	3.59, dd (7.5, 9.0)
3'	78.3	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5'	76.6	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Glc I (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	79.1	3.47, dd (7.5, 9.0)
3''	79.0	3.47, t (9.0)
4''	72.2	3.31, t (9.0)
5''	77.5	3.26, ddd (3.5, 4.5, 9.0)
6''	62.1	3.82, dd(3.5, 12), 3.71, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1'''}$	101.8	5.22, d (1.2)
2'''	71.9	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.8	3.43, t (9.7)
5'''	69.5	4.14, m
6'''	17.9	1.28, d (6.5)
$\beta\text{-D-Glc II (at C-29)-1''''}$	104.5	4.25, d (7.5)
2''''	75.0	3.22, dd (7.5, 9.0)
3''''	77.7	3.28, t (9.0)
4''''	71.5	3.28, t (9.0)
5''''	78.8	3.46, ddd (3.5, 4.5, 9.0)
6''''	62.5	3.86, dd(3.5, 12), 3.72, dd (4.5, 12)



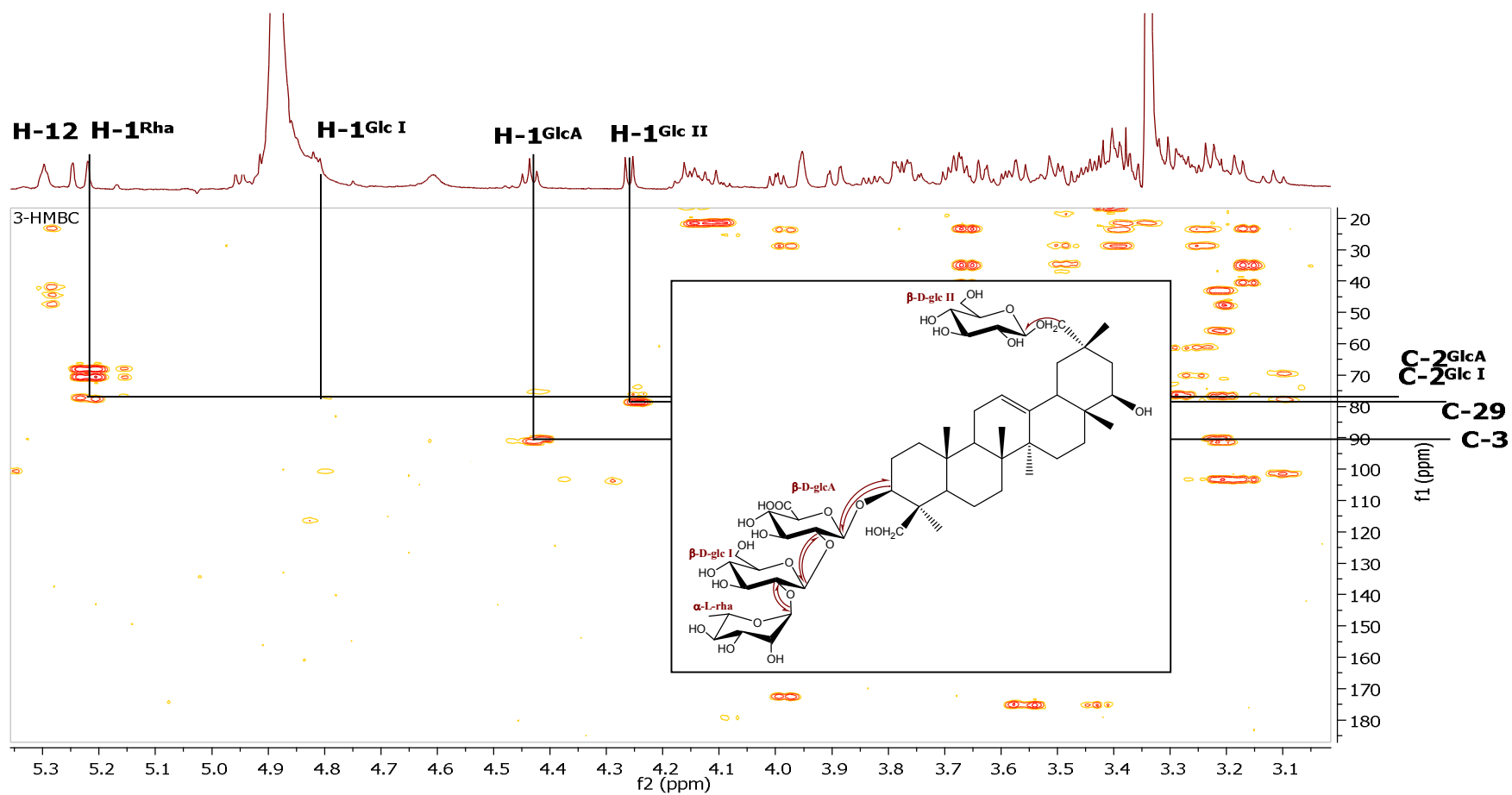
Spectrum 3.46. ^1H NMR Spectrum of ATA3 (600MHz, CD_3OD)



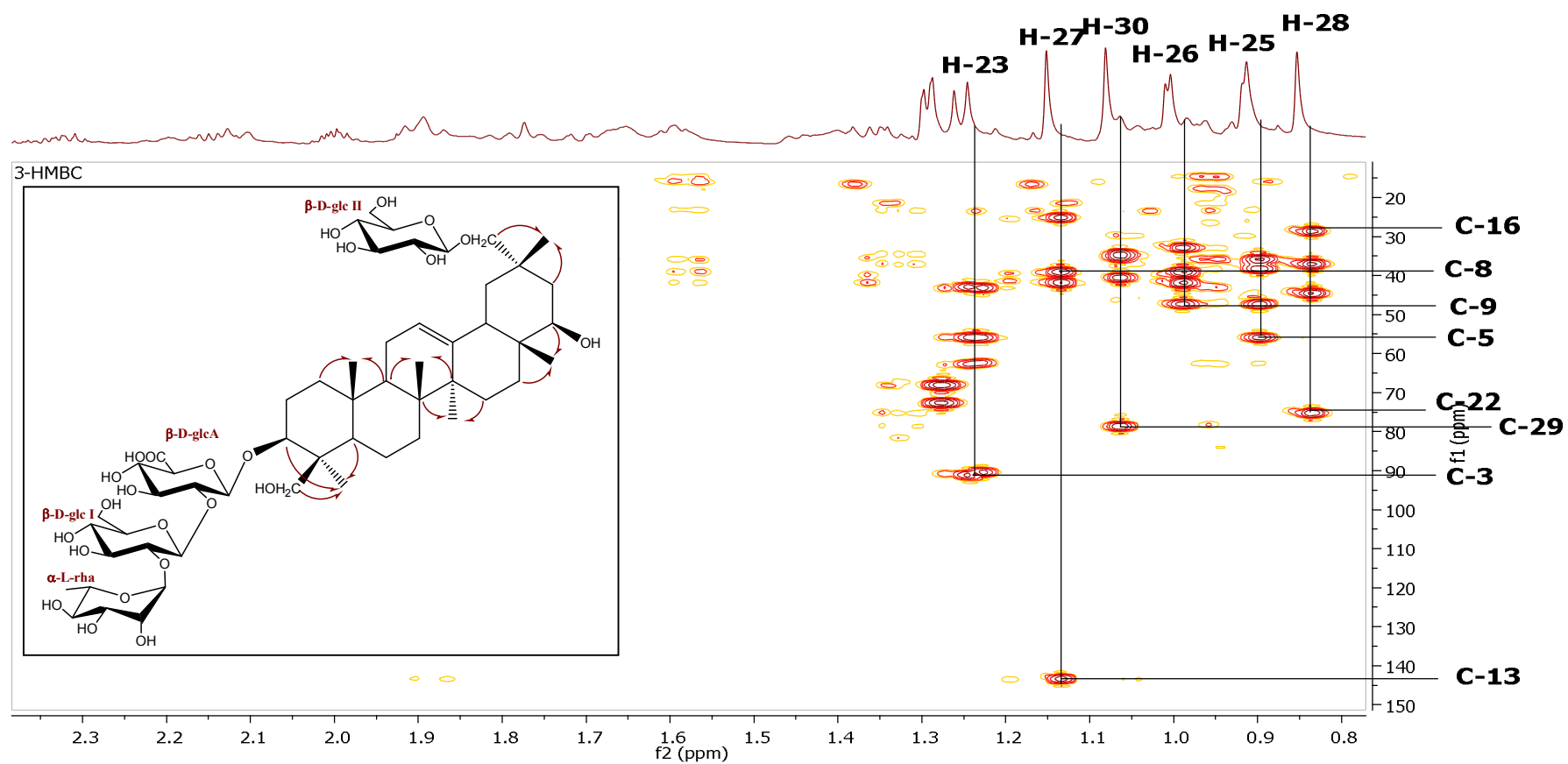
Spectrum 3.47. HSQC Spectrum of ATA3



Spectrum 3.48. HSQC Spectrum of ATA3



Spectrum 3.49. HMBC Spectrum of ATA3



Spectrum 3.50. HMBC Spectrum of ATA3

3.2.3. Structural Identification of Compound ATA4

The negative ESIMS spectrum of **ATA4** showed an abundant $[M-H]^-$ ion peak at m/z 1073. The tandem mass spectrum of **ATA4** showed two product ions originated by consecutive neutral losses of 18 and 44 a.m.u., that, according to the fragmentation behavior of **ATA2** and **ATA3**, were related to the product ion at m/z 619 obtained from the neutral loss of 114 a.m.u., revealing the presence of an uronic acid unit (**Table 3.11**). In confirmation of this, the ESIMS² spectrum showed, along with product ions originated by consecutive neutral losses of a deoxyhexose and a pentose from the $[(M-18-44)-H]^-$ ion, a product ion at m/z 619, obtained by loss of 114 a.m.u. Finally, the neutral loss of 146 a.m.u. from this latter ion produced the aglycon ion at m/z 473.

The ¹H NMR spectrum of **ATA4** showed signals for seven methyl groups as singlets at δ 1.25, 1.22, 1.09, 1.04, 0.98, 0.96, 0.91, one olefinic proton at δ 5.31 (H-12, t, $J = 3.5$ Hz), three oxygen-bearing methine protons at δ 3.36 (H-3, dd $J = 11.5, 4.2$ Hz), 3.54 (H-21, brs), 3.45 (H-22, d, $J = 3.5$) and signals for a primary alcoholic function at δ 3.25 and 4.11 (H₂-24, d, $J = 11.4$) (**Table 3.11**). The ¹³C NMR spectrum showed 53 signals, of which 30 were assigned to a triterpenoid moiety and 23 to a saccharide portion. In the HMBC spectrum, correlations between the proton signals at δ 0.96 (Me-29), and 1.09 (Me-30) and the carbon signal at δ 85.5 (C-21) revealed the location of a secondary alcoholic function at C-21; a further correlation between the proton signal at δ 0.98 (Me-28) and the carbon at δ 79.5 allowed us to assign the additional secondary alcoholic function at C-22. The β -orientation of the hydroxyl group at C-21 and the α -orientation of the hydroxyl group at C-22 were derived from the ROESY spectrum, which showed key correlation peaks between H-21 (δ 3.54) and Me-29 (δ 0.96) and H-19 α (δ 1.98), and between H-22 (δ 3.45) and Me-28 (δ 0.98). Detailed NMR studies allowed us to identify the aglycone of **ATA4** as C-22 epimer of soyasapogenol A (Kitawaga et al., 1982).

For the sugar portion, compound **ATA4** showed signals corresponding to four anomeric protons at δ 5.22 (d, $J = 1.2$ Hz), 4.81 (d, $J = 7.5$ Hz), 4.81 (d, $J = 1.2$ Hz), 4.44 (d, $J = 7.5$ Hz) (**Table 3.11**). On the basis of 2D NMR data, two α -rhamnopyranosyl (δ 5.22, 4.81), one β -xylopyranosyl (δ 4.81) and one β -glucuronopyranosyl (δ 4.44) moiety were identified. The determination of the sequence and linkage sites was obtained from the HMBC correlations which allowed us to identify the same chain sugar of compound **ATA2** located at C-3

and an α -rhamnopyranosyl linked at C-21 on the basis of correlation between the signal at 4.81 ppm with the carbon signal at δ 85.5. The D configuration of glucuronic acid and xylose units and the L configuration of rhamnose unit were established after hydrolysis of **ATA4** followed by GC analysis (De Marino et al., 2003). Thus, compound **ATA4** was identified as the new 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-21-*O*- α -L-rhamnopyranosyl-3 β ,21 β ,22 α ,24-tetrahydroxyolean-12-ene. (**Fig. 3.11**)

Amorphous white solid; C₅₃H₈₆O₂₂ [α]_D²⁵ +22.4 (*c* 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3450 (>OH), 2938 (>CH), 1680 (C=O), 1660 (C=C); HRMALDITOFMS [M+Na]⁺ *m/z* 1097.5515 (calcd for C₅₃H₈₆O₂₂Na, 1097.5508); ¹H and ¹³C NMR (CD₃OD, 600 MHz) data: see **Table 3.11**.

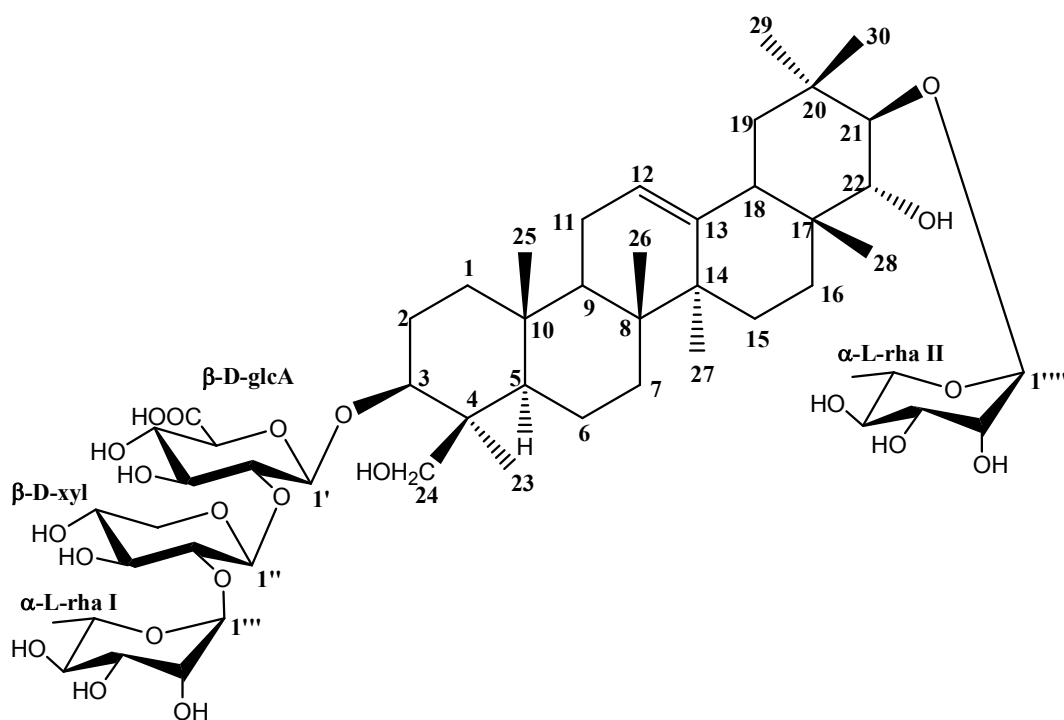
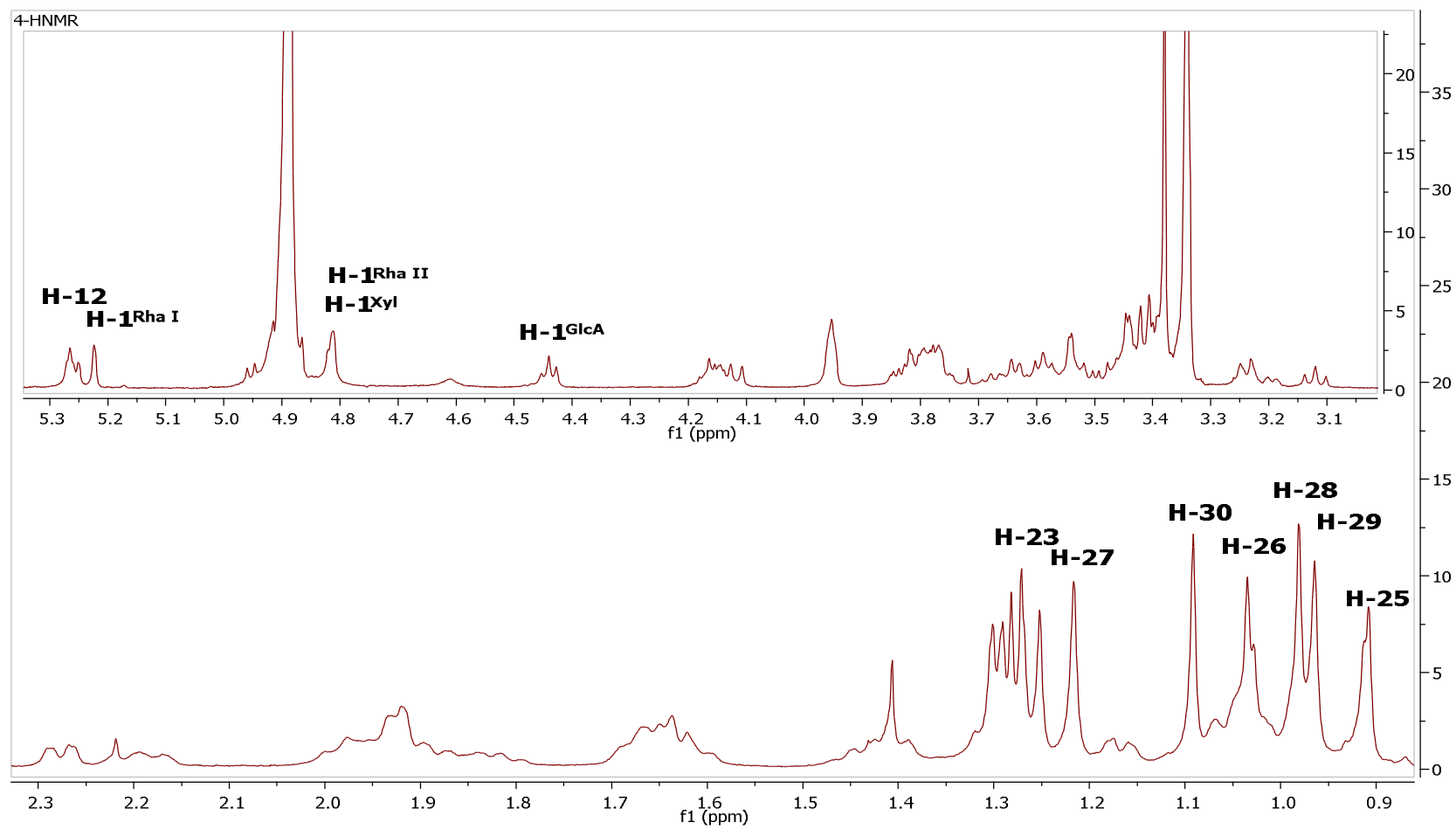


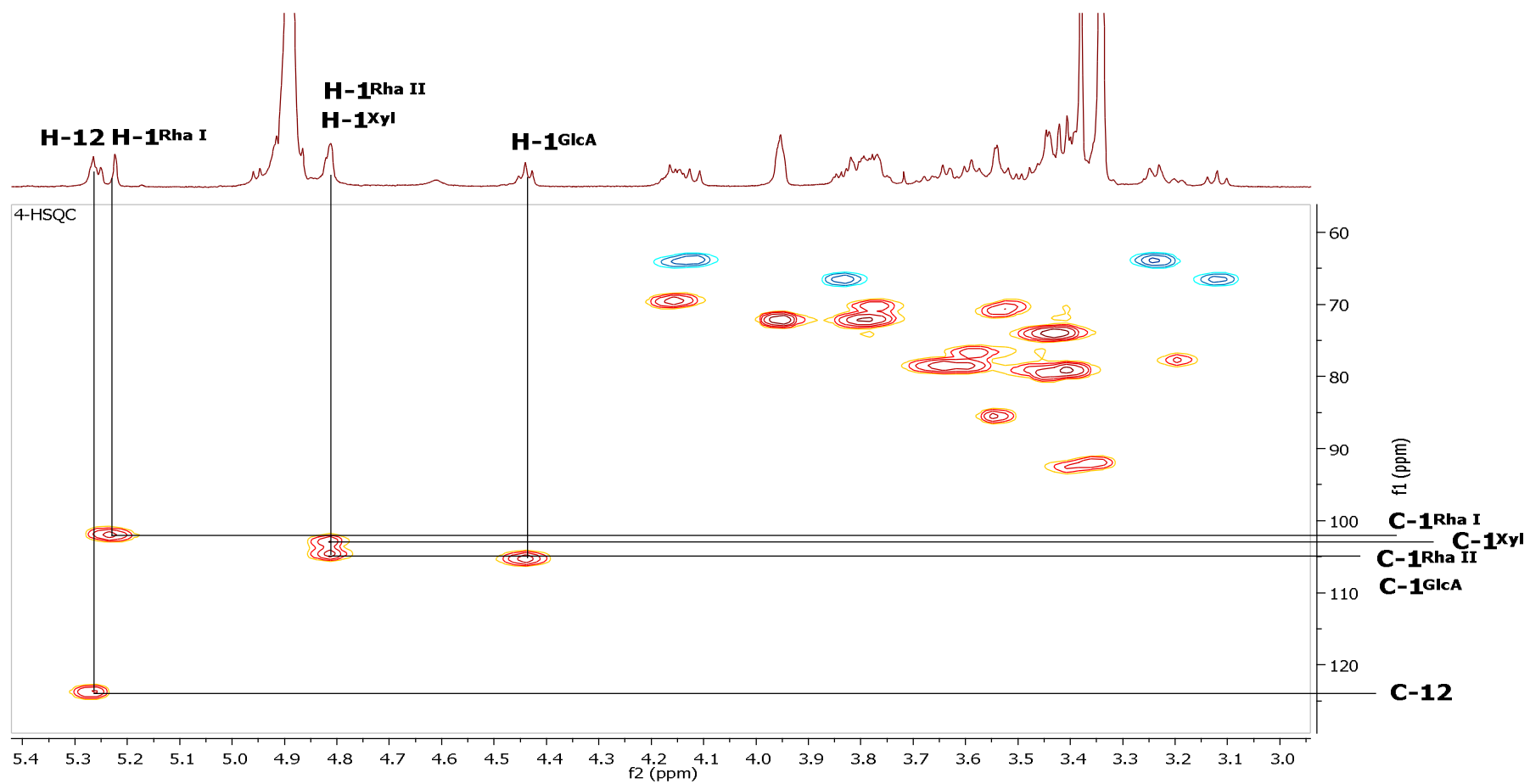
Figure 3.11. Structure of **ATA4**

Table 3.11. ^1H and ^{13}C Assignments of ATA4 (150/600 MHz, δ ppm, in CD_3OD)

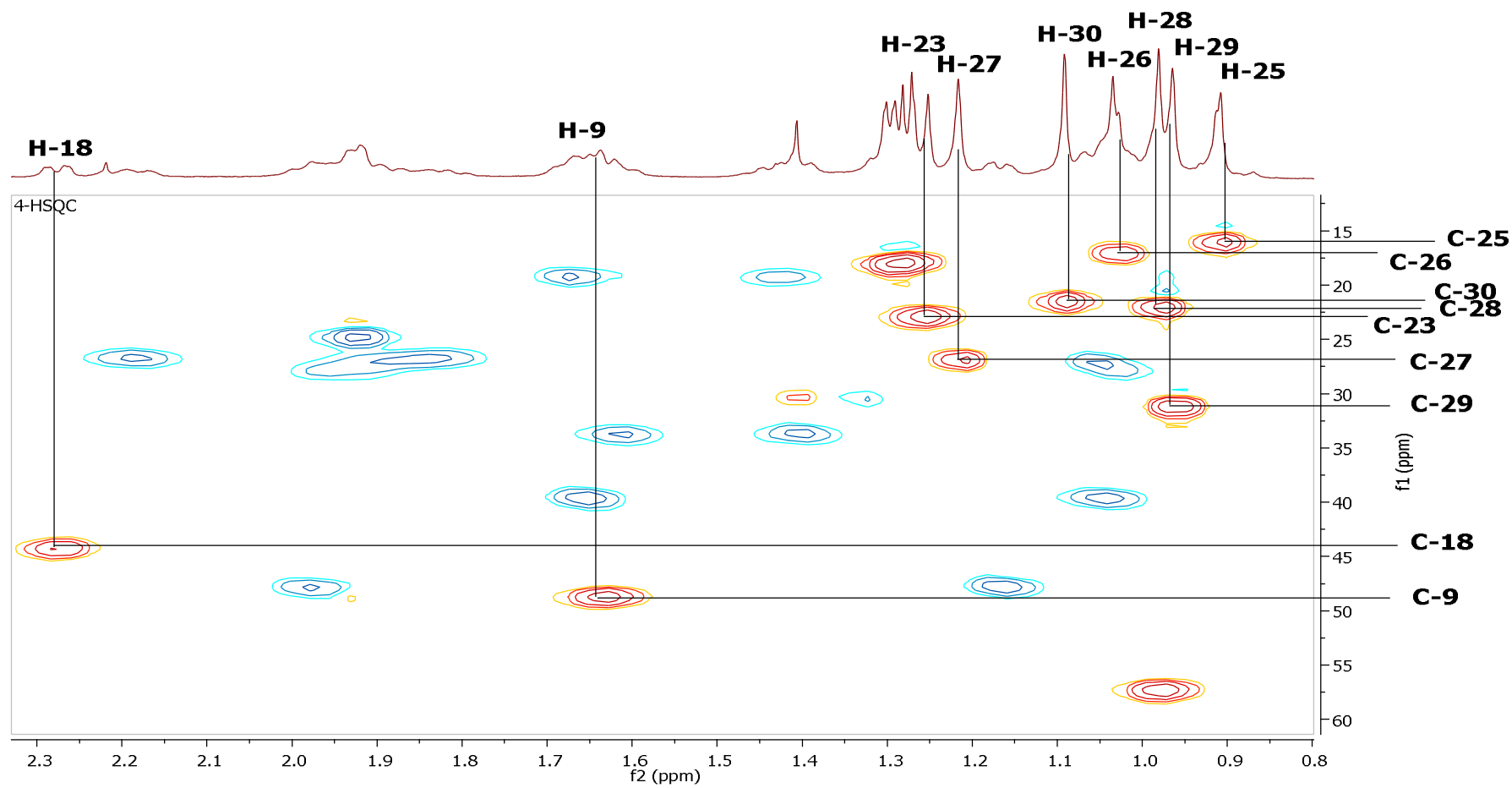
Position	δ_{C}	δ_{H} (J in Hz)
1	39.7	1.66, 1.05, m
2	26.7	2.19, 1.84, m
3	91.6	3.36, dd (11.5, 4.2)
4	44.7	-
5	57.2	0.98, m
6	19.2	1.67, 1.42, m
7	33.8	1.61, 1.42, m
8	40.9	-
9	48.8	1.64, m
10	37.3	-
11	24.7	1.93, (2H) m
12	123.4	5.31, t (3.5)
13	145.2	-
14	42.7	-
15	26.9	1.91, 1.06, m
16	28.0	1.95, 1.03, m
17	39.7	-
18	44.2	2.28, m
19	47.8	1.98, 1.17, m
20	37.0	-
21	85.5	3.54, brs
22	79.5	3.45, d (3.5)
23	22.8	1.25, s
24	63.8	4.11, d (11.4), 3.25, d (11.4)
25	15.9	0.91, s
26	17.0	1.04, s
27	26.7	1.22, s
28	22.0	0.98, s
29	31.2	0.96, s
30	21.2	1.09, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.1	4.44, d (7.5)
2'	78.3	3.59, dd (7.5, 9.0)
3'	78.4	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5'	76.6	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Xyl (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	79.0	3.40, dd (7.5, 9.2)
3''	76.6	3.58, t (9.2)
4''	70.6	3.53, m
5''	66.3	3.83, dd (5.2, 11.7), 3.12, t (11.7)
$\alpha\text{-L-Rha I (at C-2}_{\text{xyI}}\text{)-1'''}$	101.8	5.22, d (1.2)
2'''	71.9	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.9	3.43, t (9.7)
5'''	69.3	4.14, m
6'''	17.8	1.28, d (6.5)
$\alpha\text{-L-Rha II (at C-21)-1''''}$	104.4	4.81, d (1.2)
2''''	71.9	3.95, dd (1.2, 3.2)
3''''	71.9	3.78, dd (3.2, 9.7)
4''''	73.8	3.37, t (9.7)
5''''	70.1	3.77, m
6''''	18.0	1.29, d (6.5)



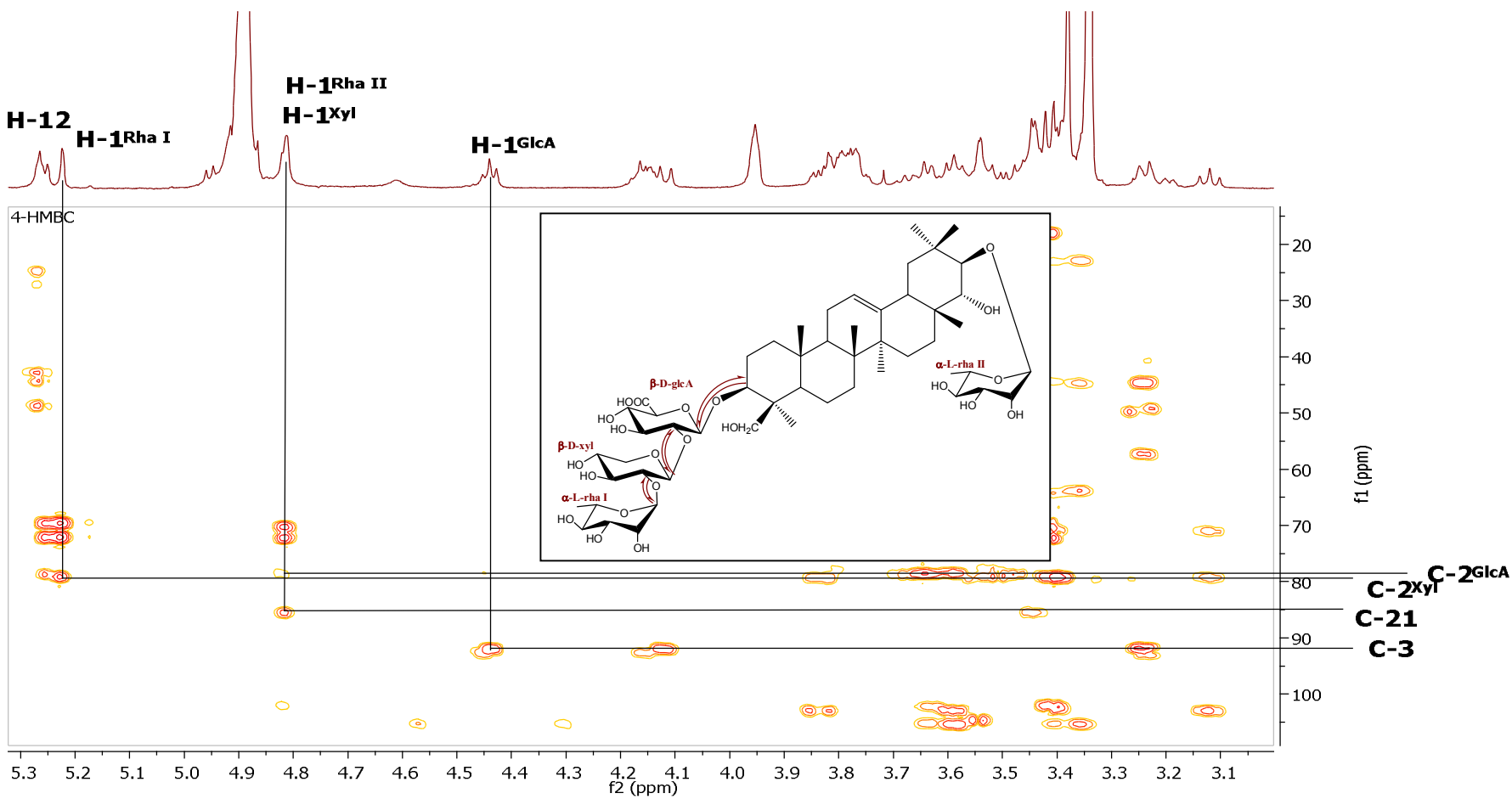
Spectrum 3.51. ^1H NMR spectrum of ATA4 (600 MHz, CD_3OD)



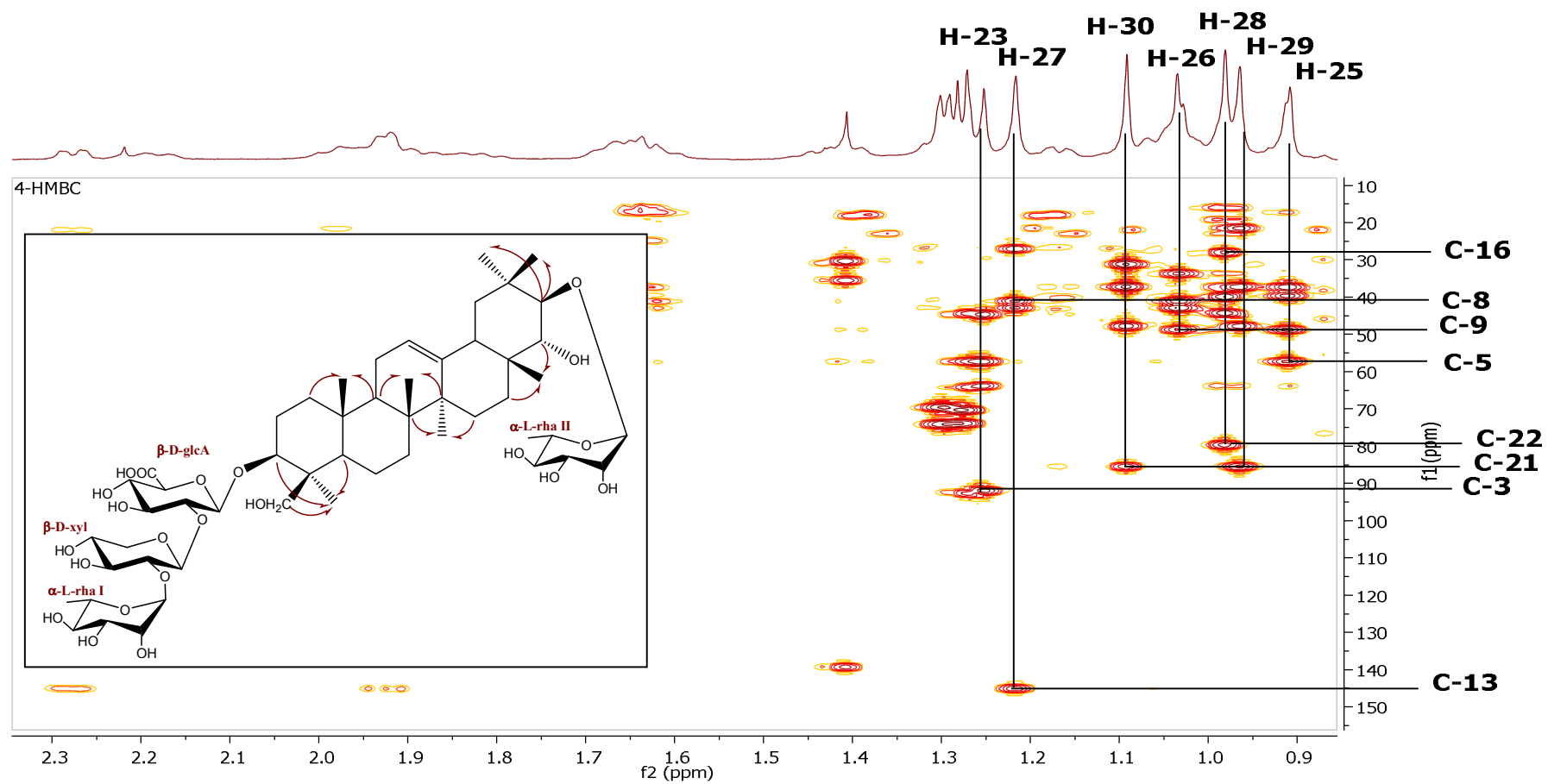
Spectrum 3.52. HSQC Spectrum of ATA4



Spectrum 3.53. HSQC Spectrum of ATA4



Spectrum 3.54. HMBC Spectrum of ATA4



Spectrum 3.55. HMBC Spectrum of ATA4

3.2.4. Structural Identification of Compound ATA5

The ESIMS spectrum of **ATA5** showed a major $[M-H]^-$ ion peak at m/z 1103, 30 a.m.u. greater than **ATA4**. The tandem mass spectrum showed a fragmentation pattern similar to that of compound **ATA4**, confirming four sugars but differing in the presence of a hexose unit instead of a pentose (**Table 3.12**).

The ^1H and ^{13}C NMR signals of **ATA5** assigned from the 2D NMR spectra were almost superimposable on those of **ATA4** except for the characteristic signals of a glucopyranosyl moiety instead of a xylopyranosyl moiety on the sugar moiety linked at C-3 (**Table 3.12**). In the HMBC spectrum the (1 $\rightarrow$ 2) linkages between the rhamnose unit and glucose unit and between this latter and the glucuronic unit were verified by the cross-peaks between the proton signal at δ 4.81 (H-1_{glc}) and the carbon at δ 78.3 (C-2_{glcA}), and between δ 5.22 (H-1_{rha}) and δ 79.0 (C-2_{glc}). The structure of **5** was thus established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-21-*O*- α -L-rhamnopyranosyl-3 β ,21 β ,22 α ,24-tetrahydroxyolean-12-ene. (**Fig. 3.12**)

Amorphous white solid; $C_{54}H_{88}O_{23}$; $[\alpha]_D^{25} +21.1$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3430 ($>OH$), 2948 ($>CH$), 1670 (C=O), 1656 (C=C); HRMALDITOFMS $[M+Na]^+$ m/z 1127.5625 (calcd for $C_{54}H_{88}O_{23}Na$, 1127.5614). ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data see **Table 3.12**.

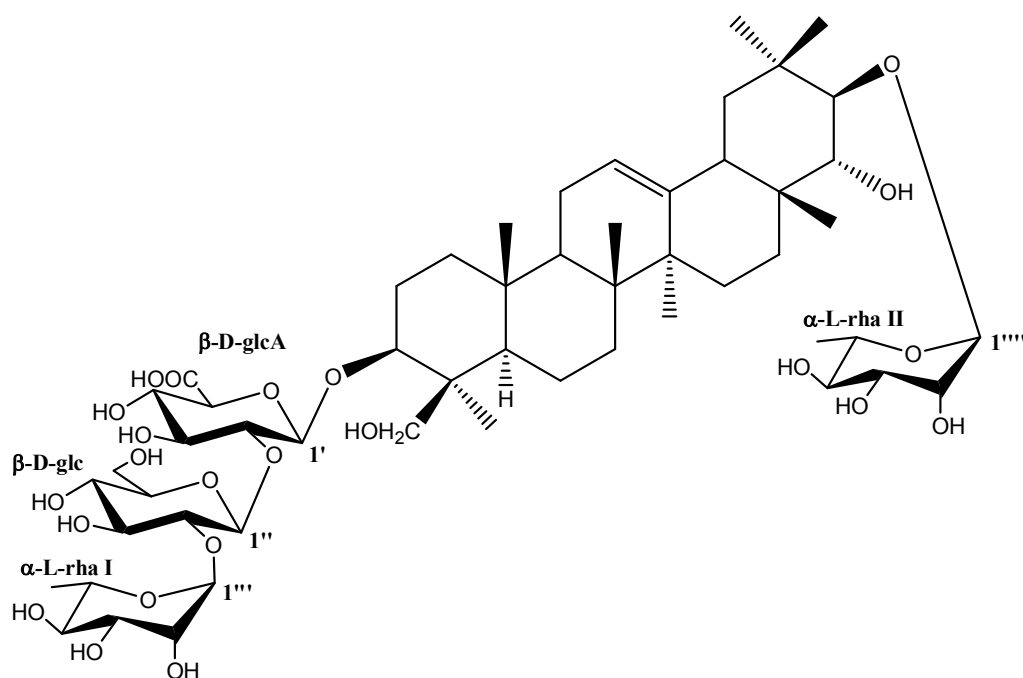
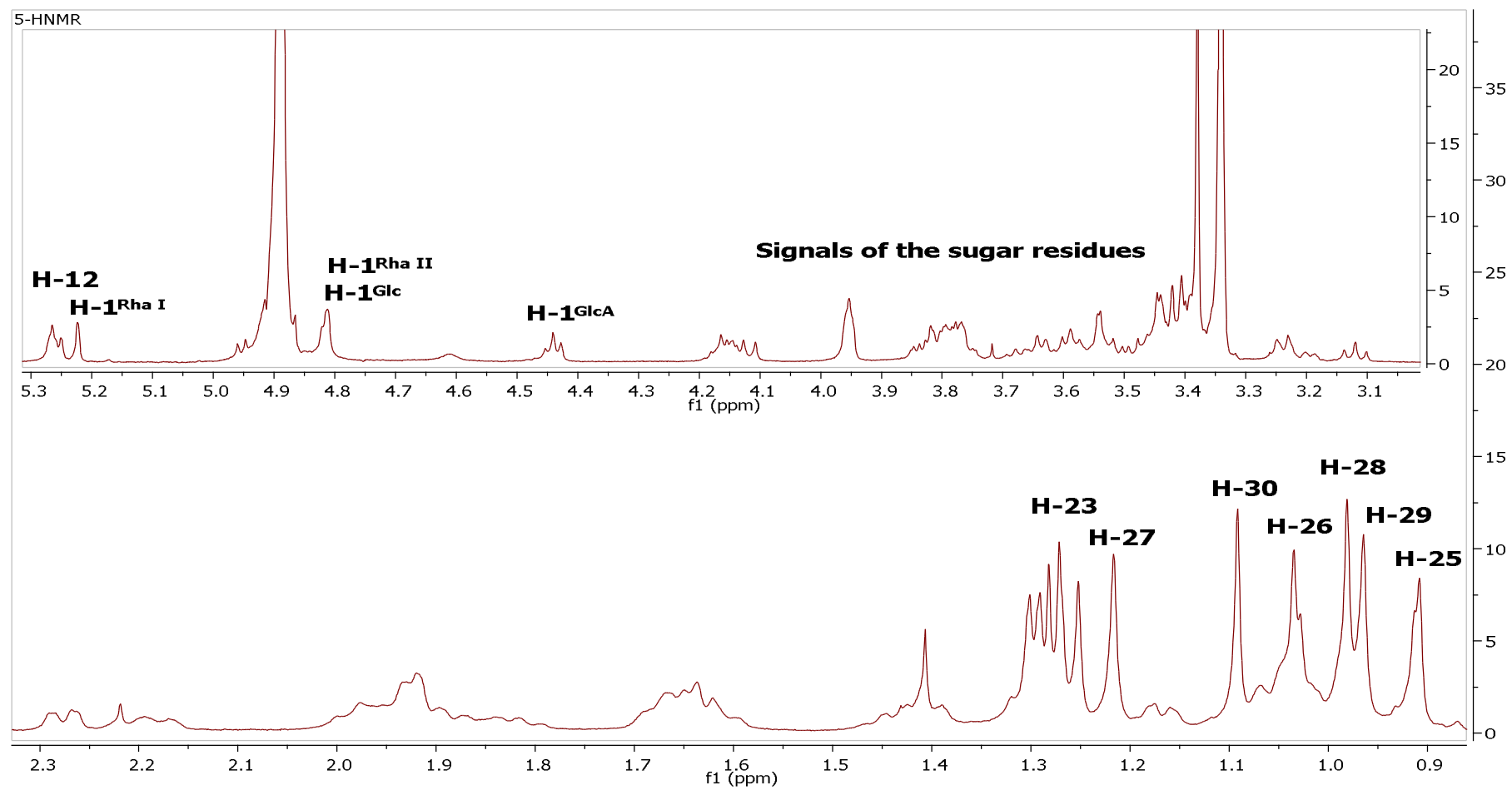


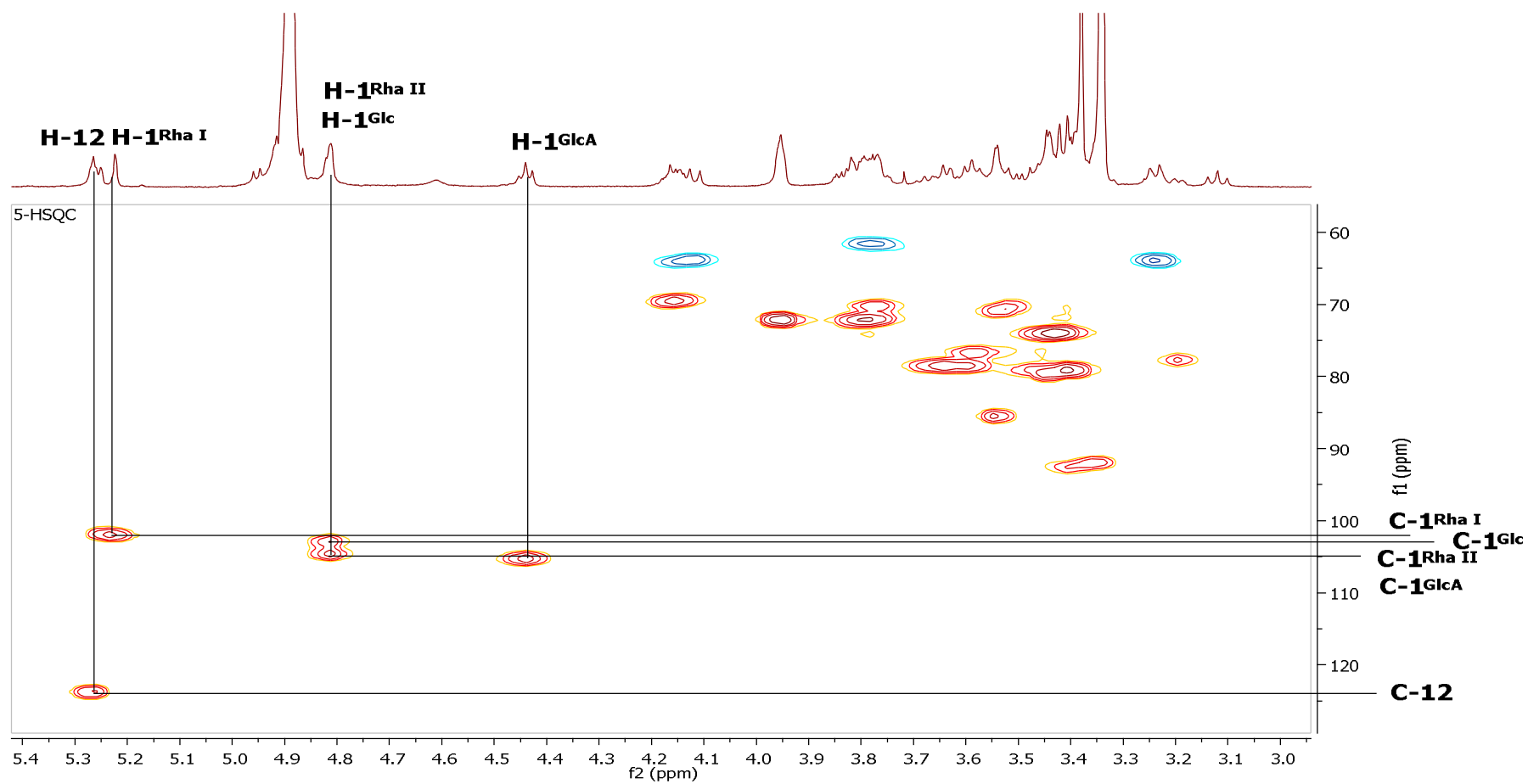
Figure 3.12. Structure of ATA5

Table 3.12. ^1H and ^{13}C Assignments of ATA5 (150/600 MHz, δ ppm, in CD_3OD)

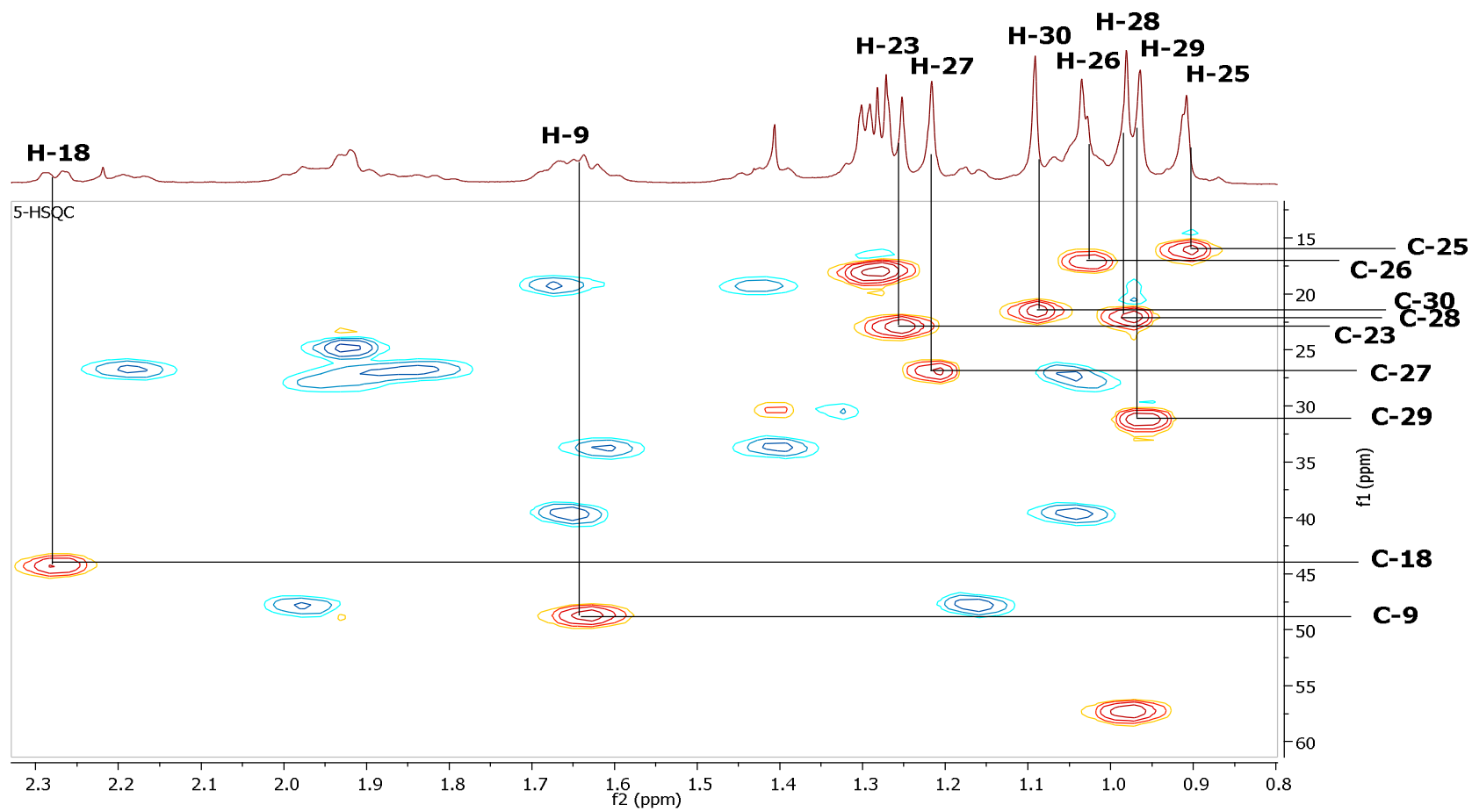
Position	δ_{C}	δ_{H} (J in Hz)
1	39.6	1.66, 1.04, m
2	26.7	2.18, 1.84, m
3	91.5	3.36, dd (11.5, 4.2)
4	44.7	-
5	57.1	0.97, m
6	19.2	1.67, 1.41, m
7	33.8	1.61, 1.42, m
8	40.8	-
9	48.8	1.63, m
10	37.3	-
11	24.5	1.92, (2H) m
12	123.3	5.30, t (3.5)
13	145.1	-
14	42.7	-
15	26.9	1.91, 1.06, m
16	28.0	1.94, 1.03, m
17	39.7	-
18	44.2	2.28, m
19	47.8	1.97, 1.17, m
20	37.0	-
21	85.5	3.54, brs
22	79.5	3.44, d (3.5)
23	22.8	1.25, s
24	63.7	4.10, d (11.4), 3.24, d (11.4)
25	15.9	0.90, s
26	17.1	1.04, s
27	26.5	1.22, s
28	22.0	0.97, s
29	31.2	0.95, s
30	21.2	1.09, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.1	4.44, d (7.5)
2'	78.3	3.59, dd (7.5, 9.0)
3'	78.4	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5'	76.6	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Glc (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	79.0	3.47, dd (7.5, 9.0)
3''	79.2	3.47, t (9.0)
4''	72.1	3.31, t (9.0)
5''	77.6	3.26, ddd (3.5, 4.5, 9.0)
6''	62.1	3.82, dd(3.5, 12), 3.71, dd (4.5, 12)
$\alpha\text{-L-Rha I(at C-2}_{\text{glc}}\text{)-1'''}$	101.8	5.22, d (1.2)
2'''	71.9	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.9	3.43, t (9.7)
5'''	69.3	4.14, m
6'''	17.8	1.28, d (6.5)
$\alpha\text{-L-Rha II (at C-21)-1''''}$	104.4	4.81, d (1.2)
2''''	71.9	3.95, dd (1.2, 3.2)
3''''	71.9	3.78, dd (3.2, 9.7)
4''''	73.8	3.37, t (9.7)
5''''	70.1	3.77, m
6''''	18.0	1.29, d (6.5)



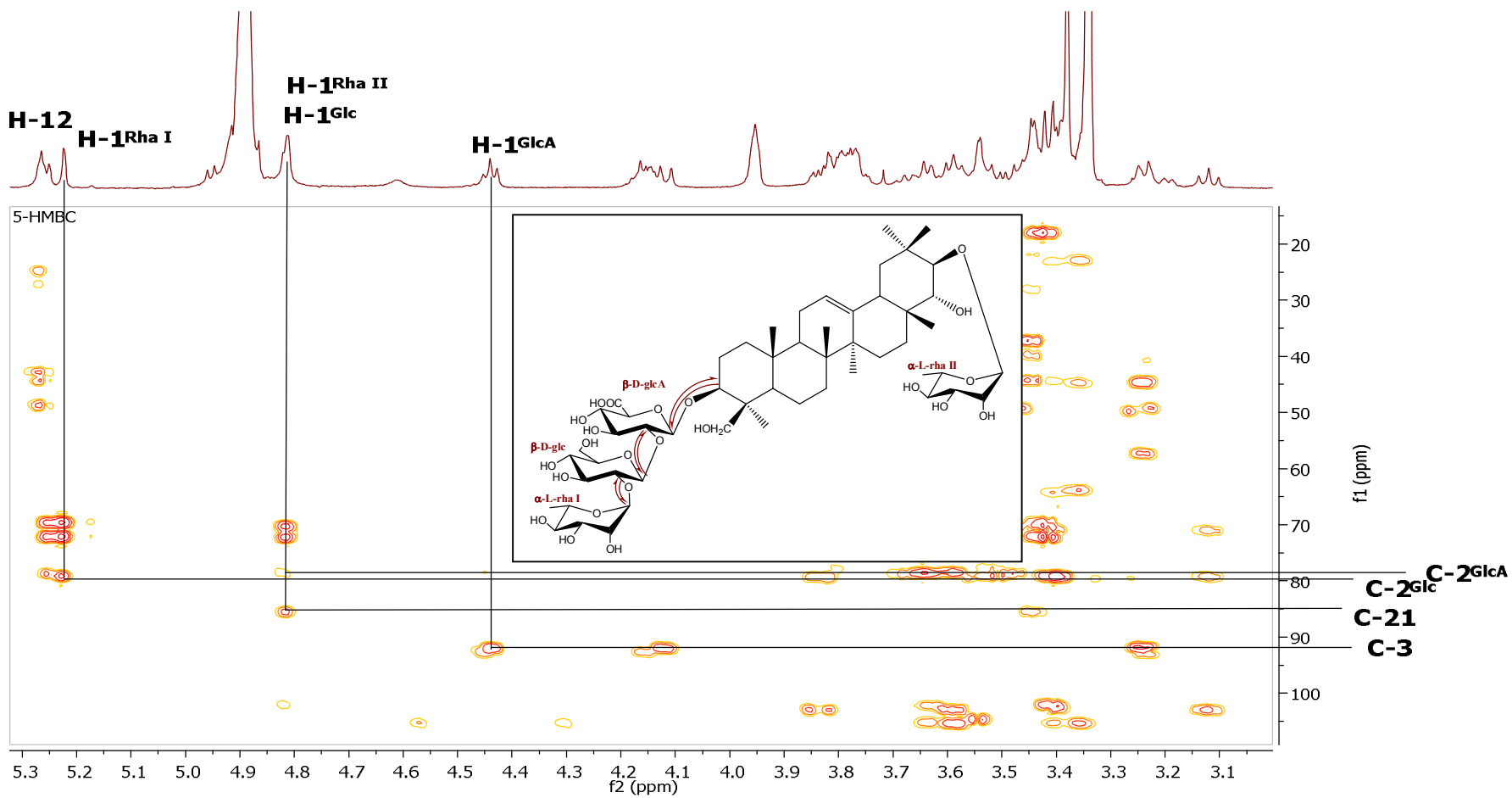
Spectrum 3.56. ^1H NMR spectrum of ATA5 (600 MHz, CD_3OD)



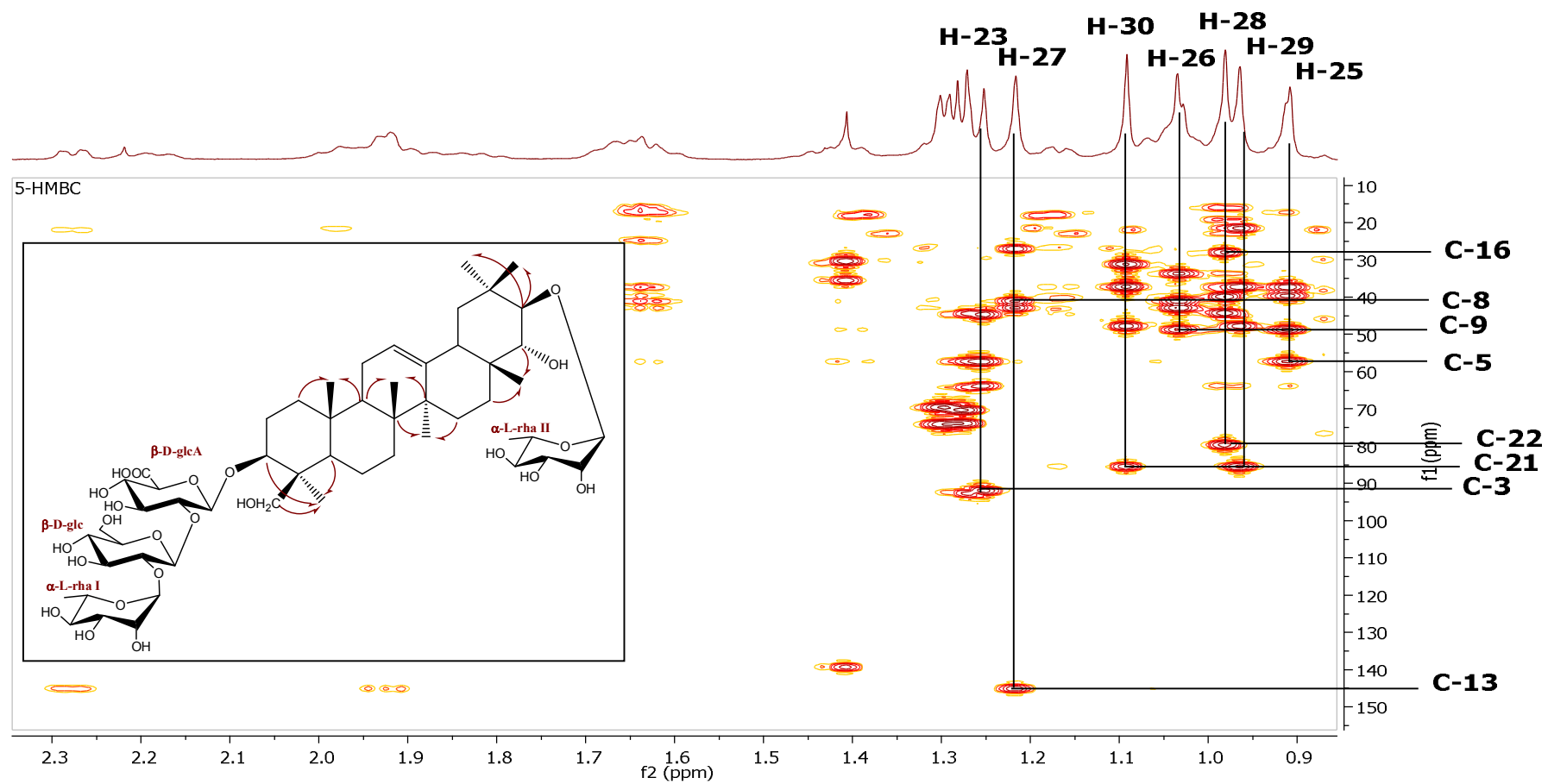
Spectrum 3.57. HSQC Spectrum of ATA5



Spectrum 3.58. HSQC Spectrum of ATA5



Spectrum 3.59. HMBC Spectrum of ATA5



Spectrum 3.60. HMBC Spectrum of ATA5

3.2.5. Structural Identification of Compound ATA6

A $[M-H]^-$ ion peak at m/z 1133 could be observed in the ESIMS spectrum of **ATA6**. Its ESIMS² spectrum promptly highlighted the presence of a hexose sugar by the product ion at m/z 971 (**Table 3.13**). Moreover, the fragmentation pattern of this latter ion gave useful indication about the sugar portion showing product ions formed by consecutive neutral losses of one deoxyhexose, one hexose, and one uronic acid unit, this latter following the same fragmentation behaviour shown by compounds **ATA2-ATA5**.

A detailed comparison of NMR spectroscopic data of compounds **ATA6** and **ATA2** demonstrated that both compounds possessed the same aglycon moiety. For the sugar portion, compound **ATA6** showed signals corresponding to four anomeric protons at δ 5.47 (d, $J = 7.5$ Hz), 5.23 (d, $J = 1.2$ Hz), 4.95 (d, $J = 7.5$ Hz) and 4.43 (d, $J = 7.5$ Hz) (**Table 3**). On the basis of 2D NMR data compound **ATA6** showed at C-3 the same sugar sequence as compound **ATA3** and a glucopyranosyl unit (δ 5.47) at C-29. Consequently, the structure of compound **ATA6** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24-trihydroxyolean-12-en-29-oic acid. (**Fig. 3.13**)

Amorphous white solid; $C_{54}H_{86}O_{25}$; $[\alpha]_D^{25} +15.6$ (c 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3435 ($>OH$), 2925 ($>CH$), 1660 ($C=C$); HRMALDITOFMS $[M+Na]^+$ m/z 1157.5363 (calc. for $C_{54}H_{86}O_{25}$, 1157.5356). ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data see **Table 3.13**.

Table 3.13. ^1H and ^{13}C Assignments of ATA6 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	39.5	1.67, 1.06, m
2	26.7	2.20, 1.85, m
3	91.5	3.34, dd (11.5, 4.5)
4	44.4	-
5	57.2	0.98, m
6	19.3	1.66, 1.42, m
7	34.2	1.60, 1.44, m
8	40.6	-
9	48.6	1.62, m
10	37.0	-
11	24.6	1.91, (2H) m
12	124.3	5.33, t (3.5)
13	144.5	-
14	43.3	-
15	26.5	1.80, 1.09, m
16	29.6	1.78, 1.38, m
17	38.4	-
18	45.5	2.13, m
19	41.1	2.37, 1.28, m
20	42.8	-
21	36.6	2.07, 1.58, m
22	76.2	3.51, brs
23	22.8	1.26, s
24	63.7	4.13, d (11.5), 3.22, d (11.5)
25	16.1	0.92, s
26	17.0	1.02, s
27	25.1	1.17, s
28	19.8	0.87, s
29	178.4	-
30	24.1	1.38, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.0	4.43, d (7.5)
2'	78.3	3.59, dd (7.5, 9.0)
3'	78.4	3.63, dd (9.0, 9.0)
4'	73.8	3.43, dd (9.0, 9.0)
5'	76.4	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Glc I(at C-2}_{\text{glc}}\text{)-1''}$	101.8	4.95, d (7.5)
2''	79.1	3.48, dd (7.5, 9.0)
3''	79.3	3.46, t (9.0)
4''	72.0	3.32, t (9.0)
5''	77.4	3.24, ddd (3.5, 4.5, 9.0)
6''	61.8	3.80, dd(3.5, 12), 3.70, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1'''}$	101.6	5.22, d (1.2)
2'''	71.9	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.7	3.43, t (9.7)
5'''	69.3	4.14, m
6'''	17.8	1.28, d (6.5)
$\beta\text{-D-Glc II(at C-29)-1''''}$	95.4	5.47, d (7.5)
2''''	73.8	3.38, dd (7.5, 9.0)
3''''	77.4	3.19, t (9.0)
4''''	70.9	3.39, t (9.0)
5''''	78.8	3.47, ddd (3.5, 4.5, 9.0)
6''''	62.1	3.87, dd(3.5, 12), 3.71, dd (4.5, 12)

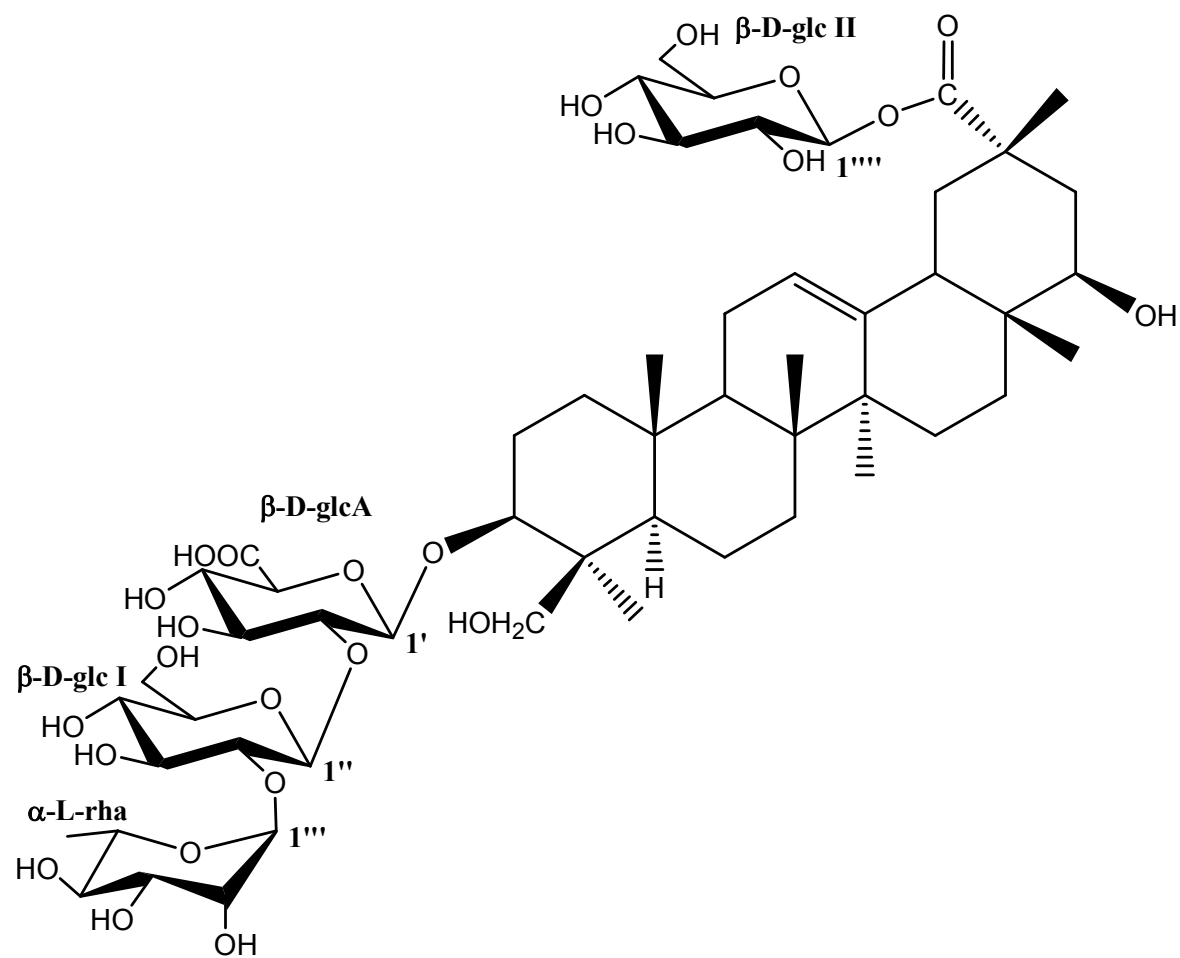
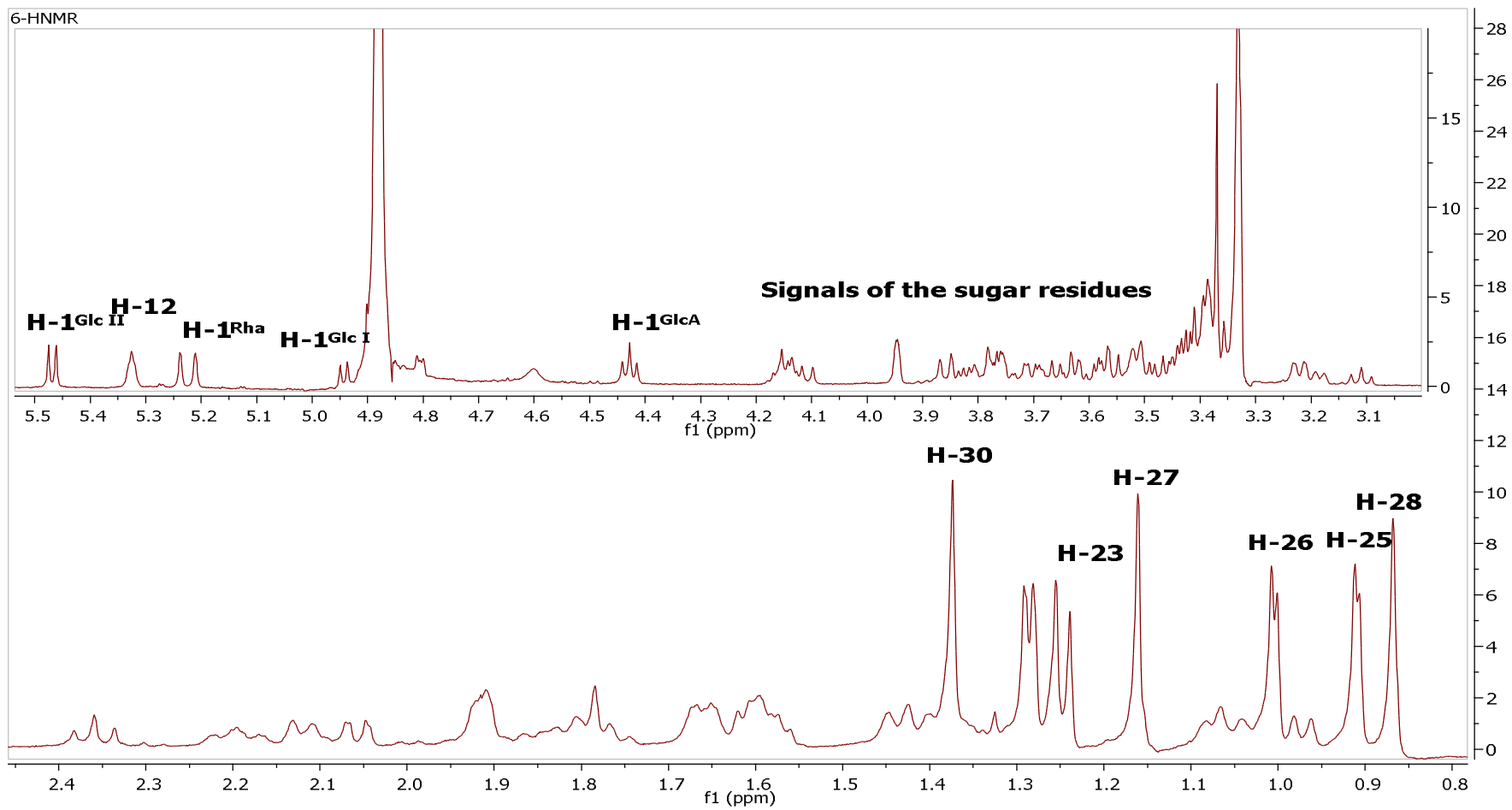
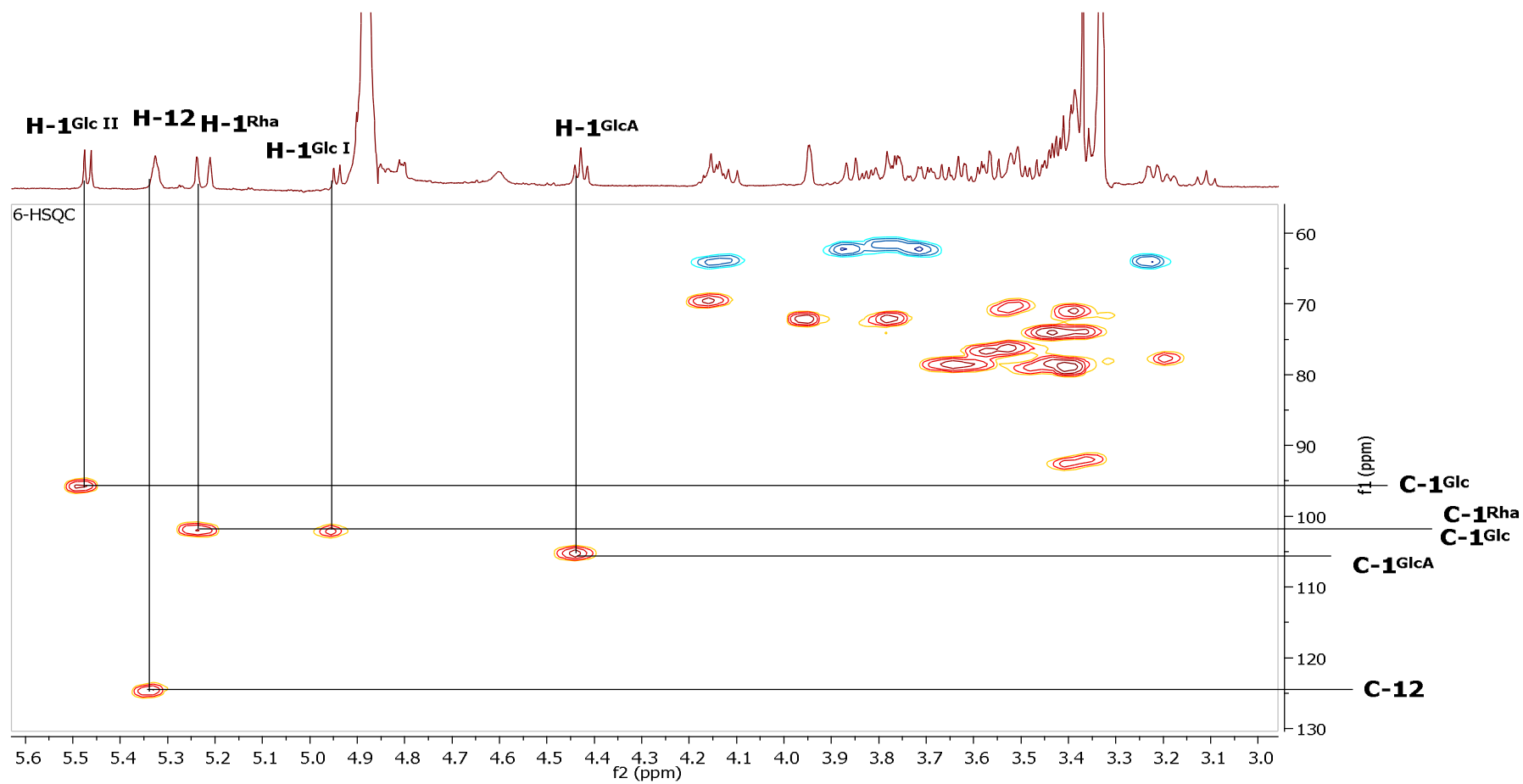


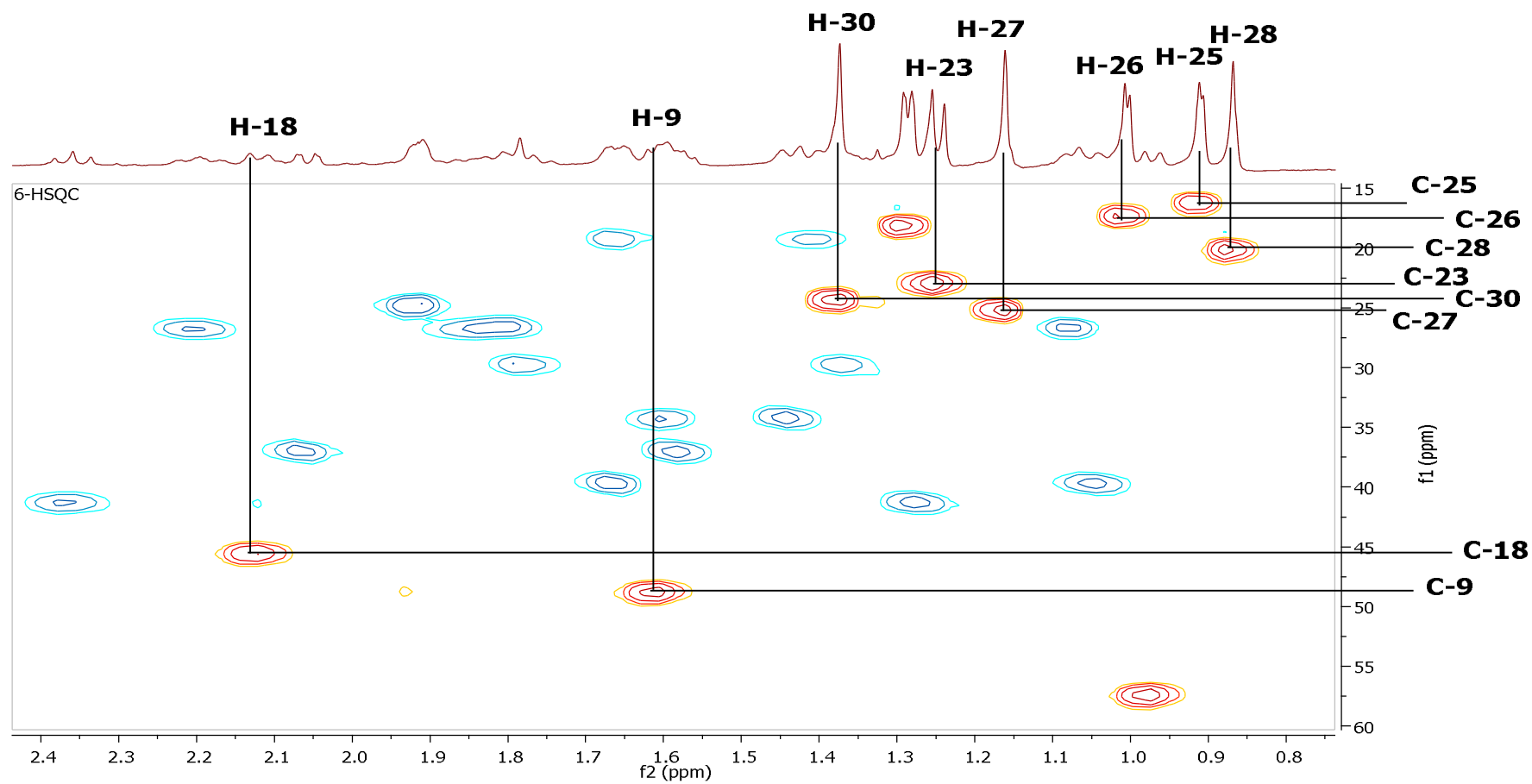
Figure 3.13. Structure of ATA6



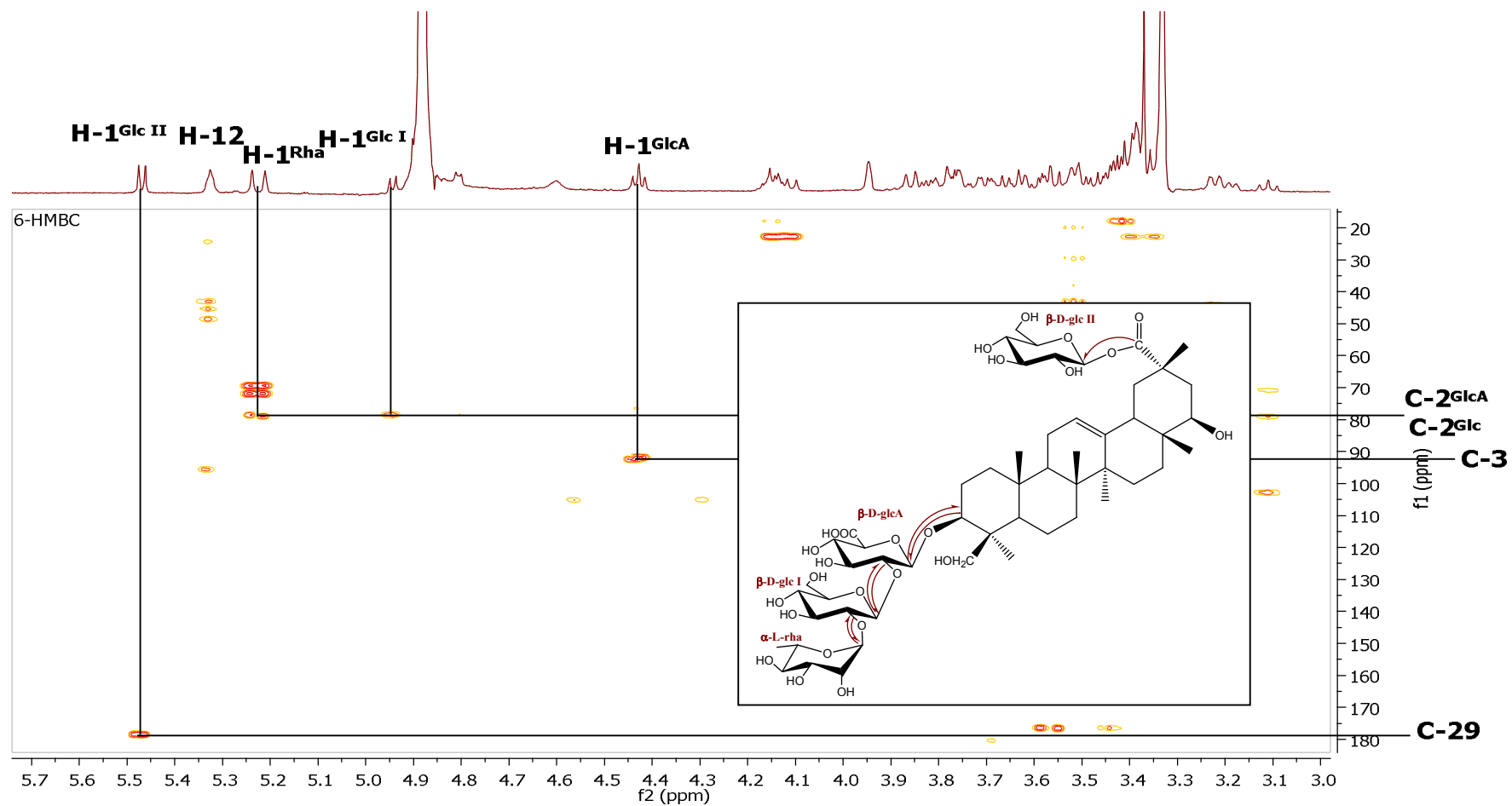
Spectrum 3.61. ^1H NMR spectrum of ATA6 (600 MHz, CD_3OD)



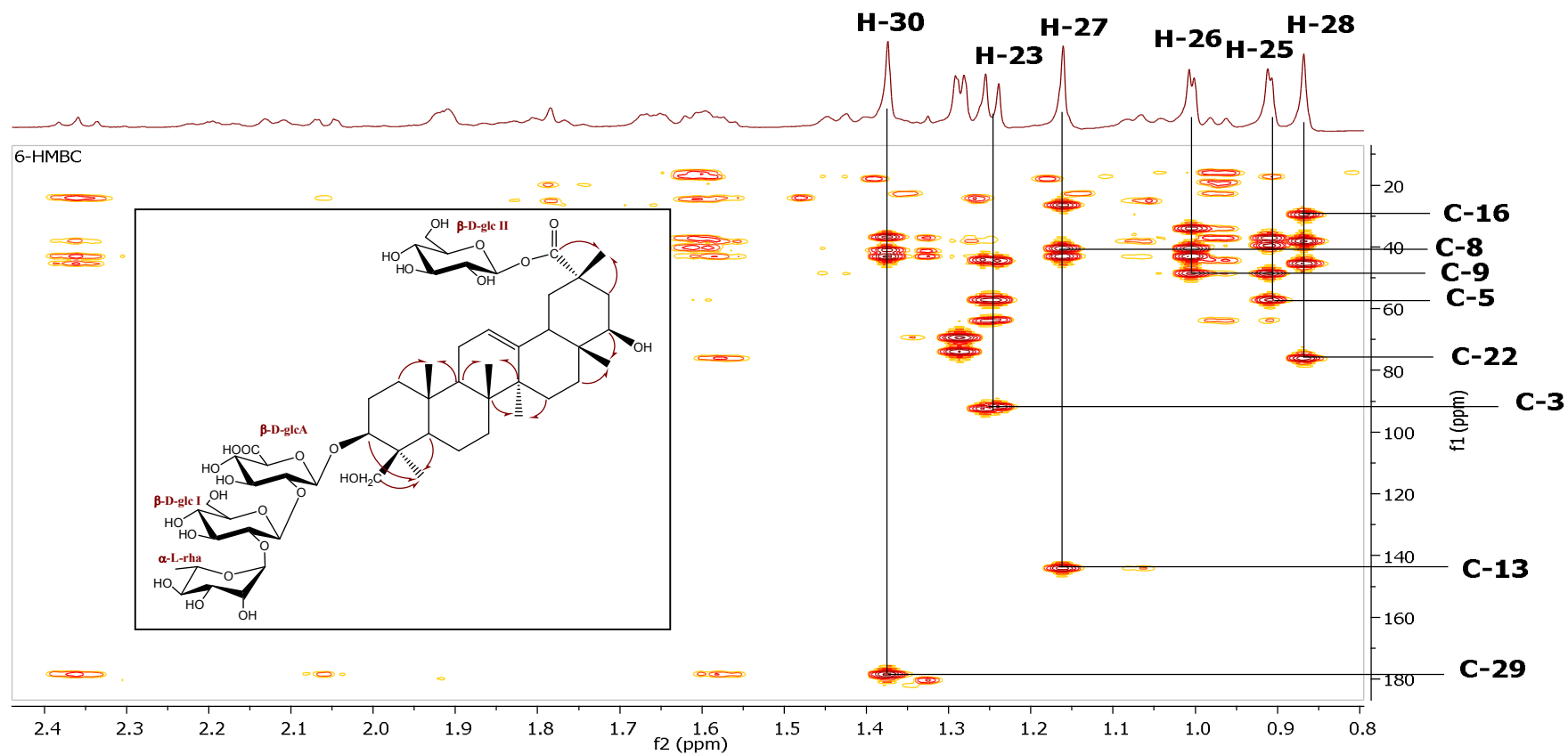
Spectrum 3.62. HSQC Spectrum of ATA6



Spectrum 3.63. HSQC Spectrum of ATA6



Spectrum 3.64. HMBC Spectrum of ATA6



Spectrum 3.65. HMBC Spectrum of ATA6

3.2.6. Structural Identification of Compound ATA7

The negative ESIMS spectrum of **ATA7** showed a major $[M-H]^-$ ion peak at m/z 1057, whose fragmentation yielded minor product ions at m/z 1039, 995, and 849, originated by consecutive neutral losses of a water, a carbon dioxide, and a deoxyhexose unit, respectively (**Table 3.14**). The presence in the ESIMS² of a major product ion at m/z 729 accounted for the overall neutral loss of two deoxyhexose units and two water molecules. Moreover, the product ion at m/z 579, formed by neutral loss of 150 a.m.u. from the major product ion, indicated the presence of one pentose.

The ¹H NMR spectrum of the aglycon portion of **ATA7** showed signals for seven tertiary methyl groups at δ 1.26, 1.17, 1.04, 1.00, 0.95, 0.92, 0.82, a typical signal of H-3ax at δ 3.40 (dd, $J = 11.5, 4.2$ Hz), a signal of H-22 at δ 3.30 (brs), and a characteristic olefinic proton at δ 5.31 (t, $J = 3.5$ Hz). The ¹³C NMR spectrum showed 53 signals, of which 30 were assigned to a triterpenoid moiety and 23 to the saccharide portion (**Table 3.14**). The NMR data of the aglycon moiety of **ATA7** were in good agreement with those of soyasapogenol B (Kitawaga et al., 1982), except for the downfield shift of C-3 at δ 91.9 and C-22 at δ 85.8, suggesting that **7** was a bidesmosidic saponin. The ¹H NMR spectrum of **7** showed signals for four anomeric protons at δ 5.23 (d, $J = 1.2$ Hz), 4.87 (d, $J = 7.5$ Hz), 4.82 (d, $J = 7.5$ Hz) and 4.45 (d, $J = 7.5$ Hz), which were identified by the combination of HSQC, HMBC and COSY data as two α -rhamnopyranose (δ 5.23 and 4.87), one β -xylopyranose (δ 4.82), one β -glucuronopyranose (δ 4.45). The sugar sequence and the linkage sites were determined by the HMBC which revealed the same sugar chain at C-3 observed in **ATA2**, and a rhamnopyranosyl unit (δ 4.87) linked at C-22 (δ 85.8). Thus, the new compound **ATA7** was identified as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-22-*O*- α -L-rhamnopyranosyl-3 β ,22 β ,24-trihydroxyolean-12-ene. (**Fig. 3.14**)

Amorphous white solid; C₅₃H₈₆O₂₁; $[\alpha]_D^{25} +19.6$ (*c* 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3443 (>OH), 2935 (>CH), 1668 (C=C); HRMALDITOFMS $[M+Na]^+$ m/z 1081.5567 (calc. for C₅₃H₈₆O₂₁Na, 1081.5559). ¹H and ¹³C NMR (CD₃OD, 600 MHz) data see **Table 3.14**.

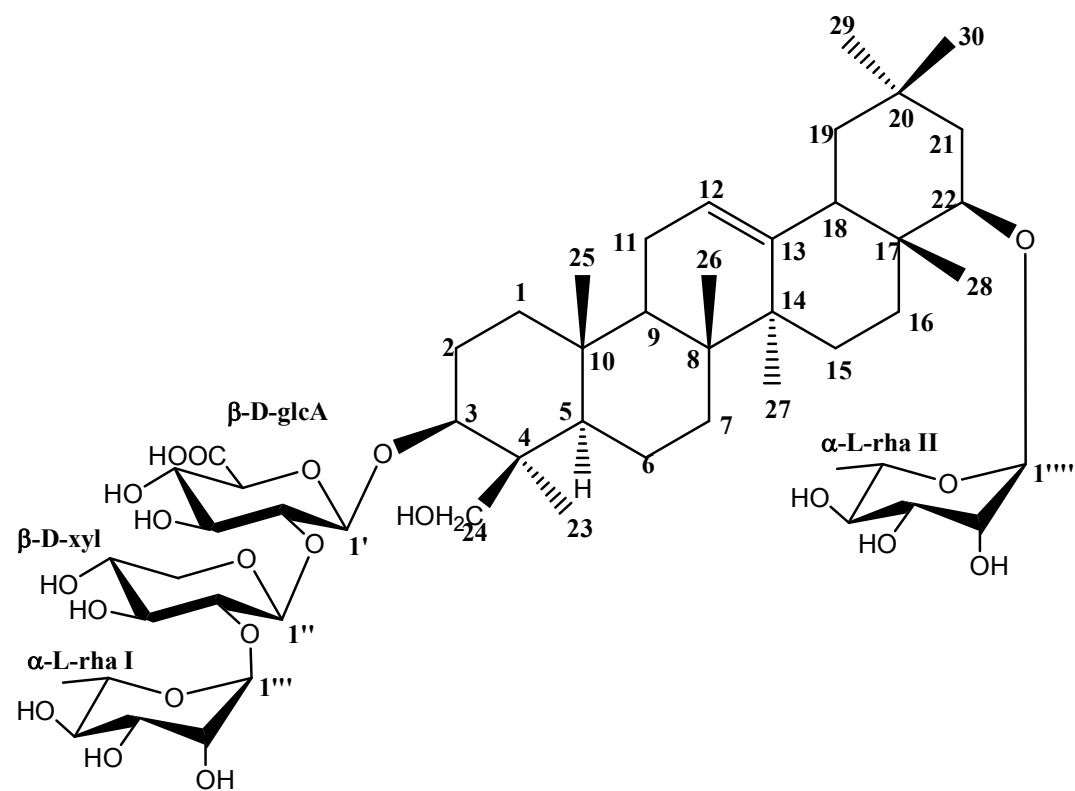
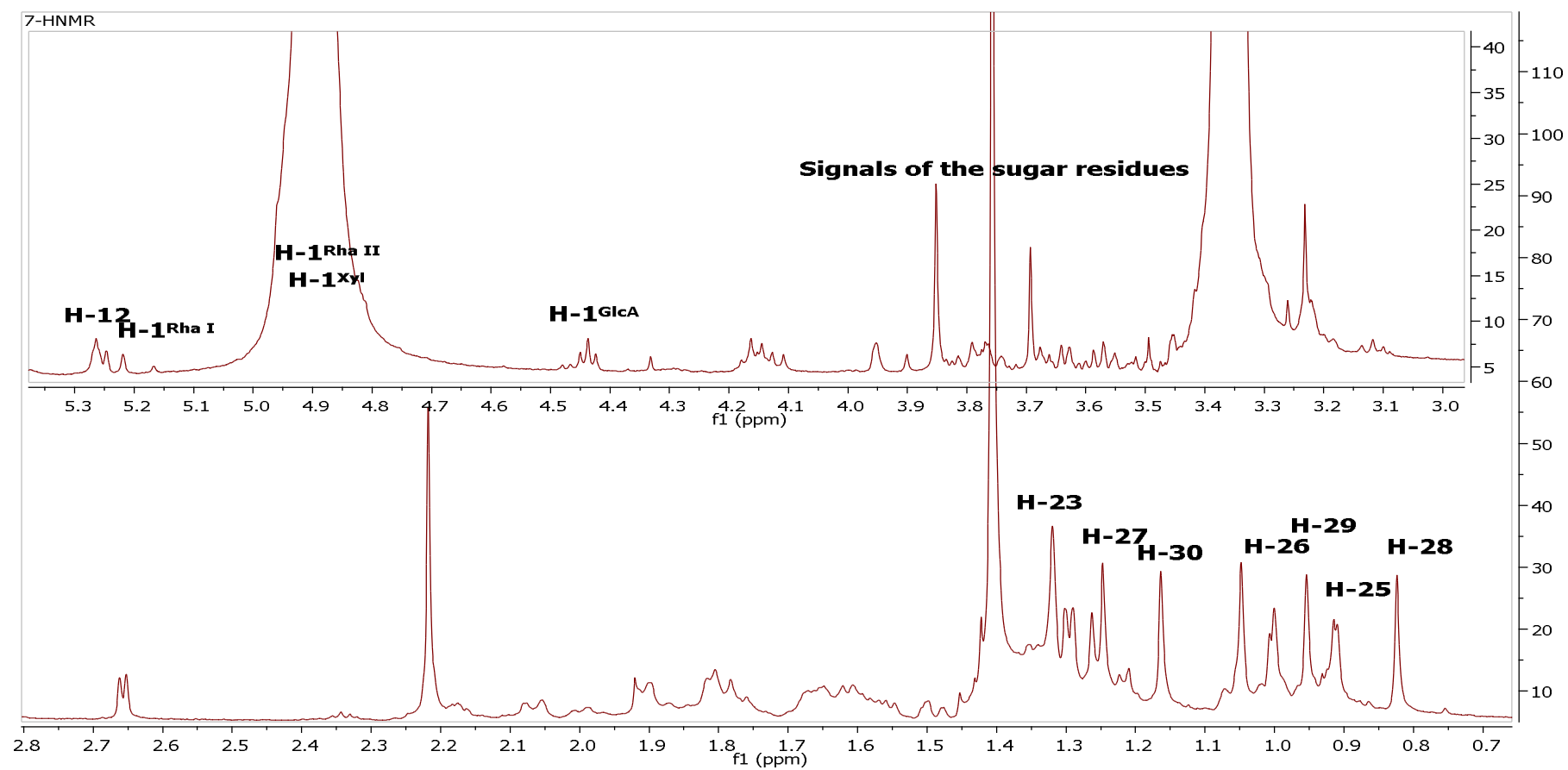


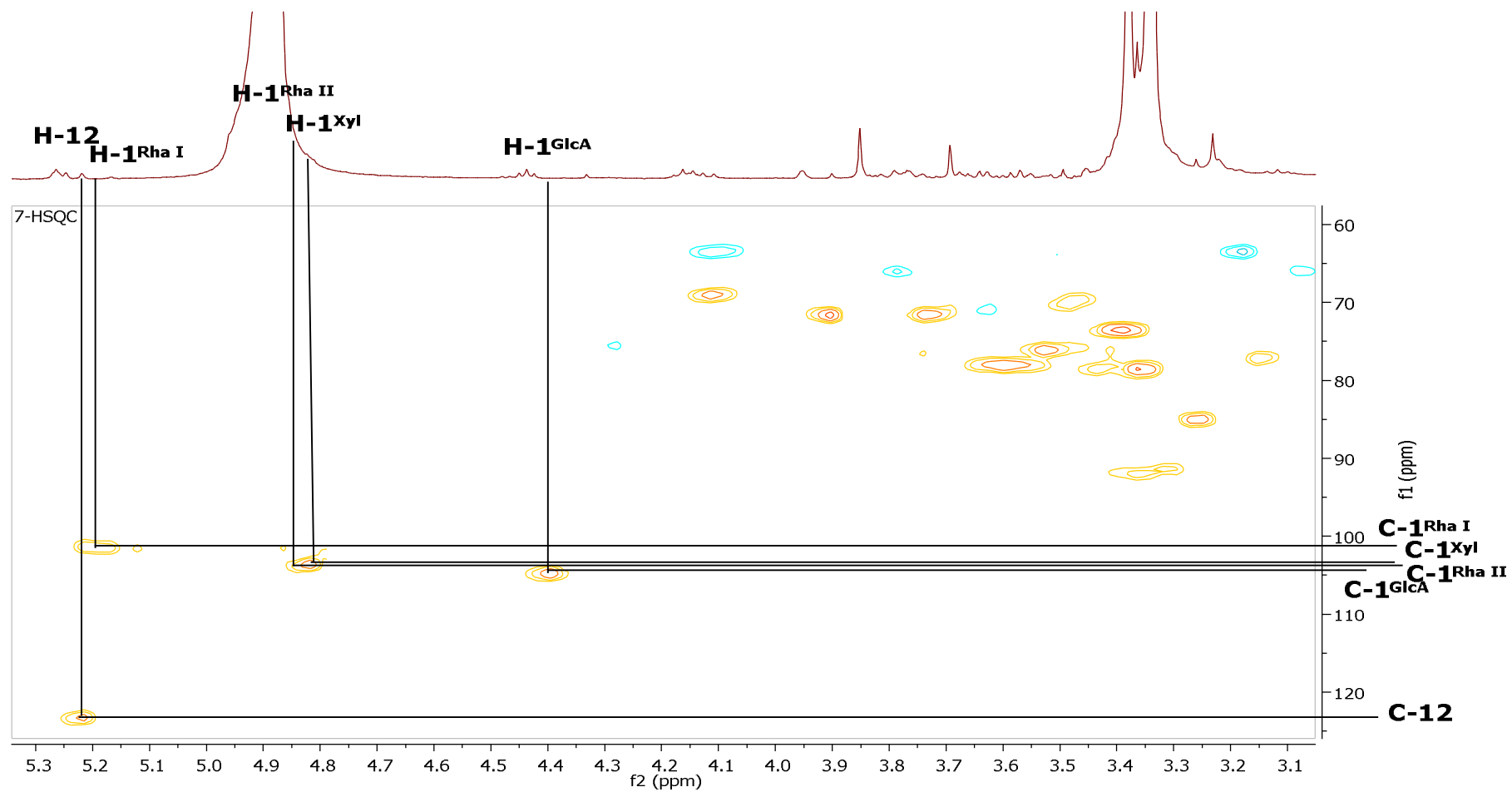
Figure 3.14. Structure of ATA7

Table 3.14. ^1H and ^{13}C Assignments of ATA7 (150/600 MHz, δ ppm, in CD_3OD)

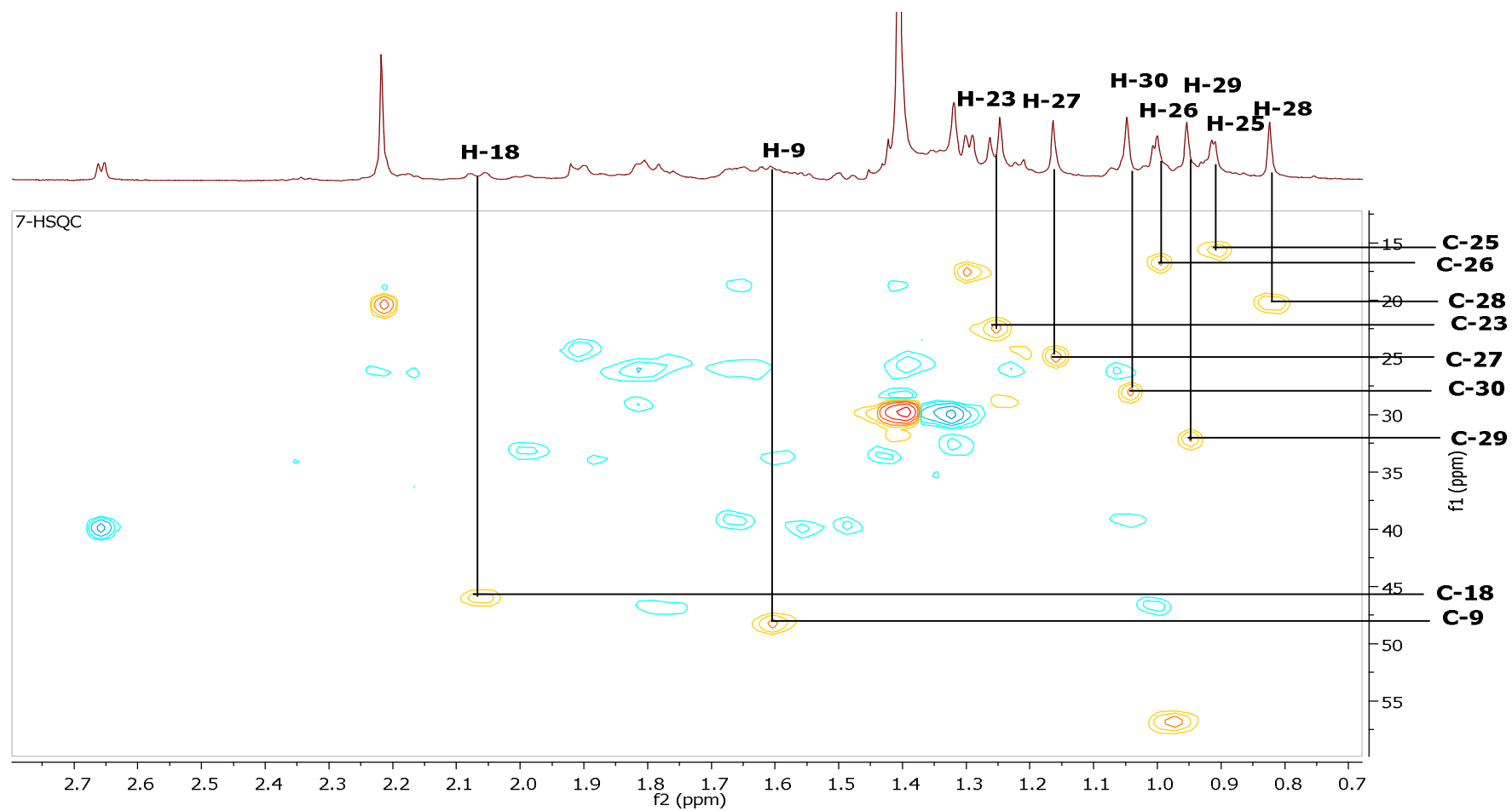
Position	δ_{C}	δ_{H} (J in Hz)
1	39.7	1.67, 1.05, m
2	27.0	2.17, 1.85, m
3	91.9	3.40, dd (11.5, 4.2)
4	44.5	-
5	57.3	0.98, m
6	19.7	1.66, 1.42, m
7	34.1	1.60, 1.43, m
8	40.6	-
9	48.6	1.60, m
10	37.3	-
11	24.7	1.91, (2H) m
12	123.4	5.31, t (3.5)
13	145.0	-
14	42.9	-
15	26.9	1.81, 1.07, m
16	29.6	1.81, 1.37, m
17	38.4	-
18	46.4	2.06, m
19	47.1	1.78, 0.99, m
20	31.4	-
21	40.2	2.65, 1.56, m
22	85.8	3.30, brs
23	22.8	1.26, s
24	64.0	4.12, d (11.4), 3.23, d (11.4)
25	16.2	0.92, s
26	17.4	1.00, s
27	25.3	1.17, s
28	20.7	0.82, s
29	32.6	0.95, s
30	28.5	1.04, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.2	4.45, d (7.5)
2'	78.4	3.64, dd (7.5, 9.0)
3'	78.3	3.59, dd (9.0, 9.0)
4'	73.9	3.44, dd (9.0, 9.0)
5'	76.4	3.57, d (9.0)
6'	176.0	-
$\beta\text{-D-Xyl (at C-2}_{\text{glcA}}\text{)-1''}$	102.6	4.82, d (7.5)
2''	78.8	3.40, dd (7.5, 9.2)
3''	76.4	3.57, t (9.2)
4''	70.3	3.52, m
5''	66.4	3.83, dd (5.2, 11.7), 3.11, t (11.7)
$\alpha\text{-L-Rha I (at C-2}_{\text{xyl}}\text{)-1'''}$	101.7	5.23, d (1.2)
2'''	72.0	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.9	3.37, t (9.7)
5'''	69.5	4.15, m
6'''	18.0	1.30, d (6.5)
$\alpha\text{-L-Rha II (at C-22)-1''''}$	104.4	4.87, d (1.2)
2''''	71.9	3.94, dd (1.2, 3.2)
3''''	71.9	3.77, dd (3.2, 9.7)
4''''	73.8	3.36, t (9.7)
5''''	70.1	3.78, m
6''''	18.0	1.28, d (6.5)



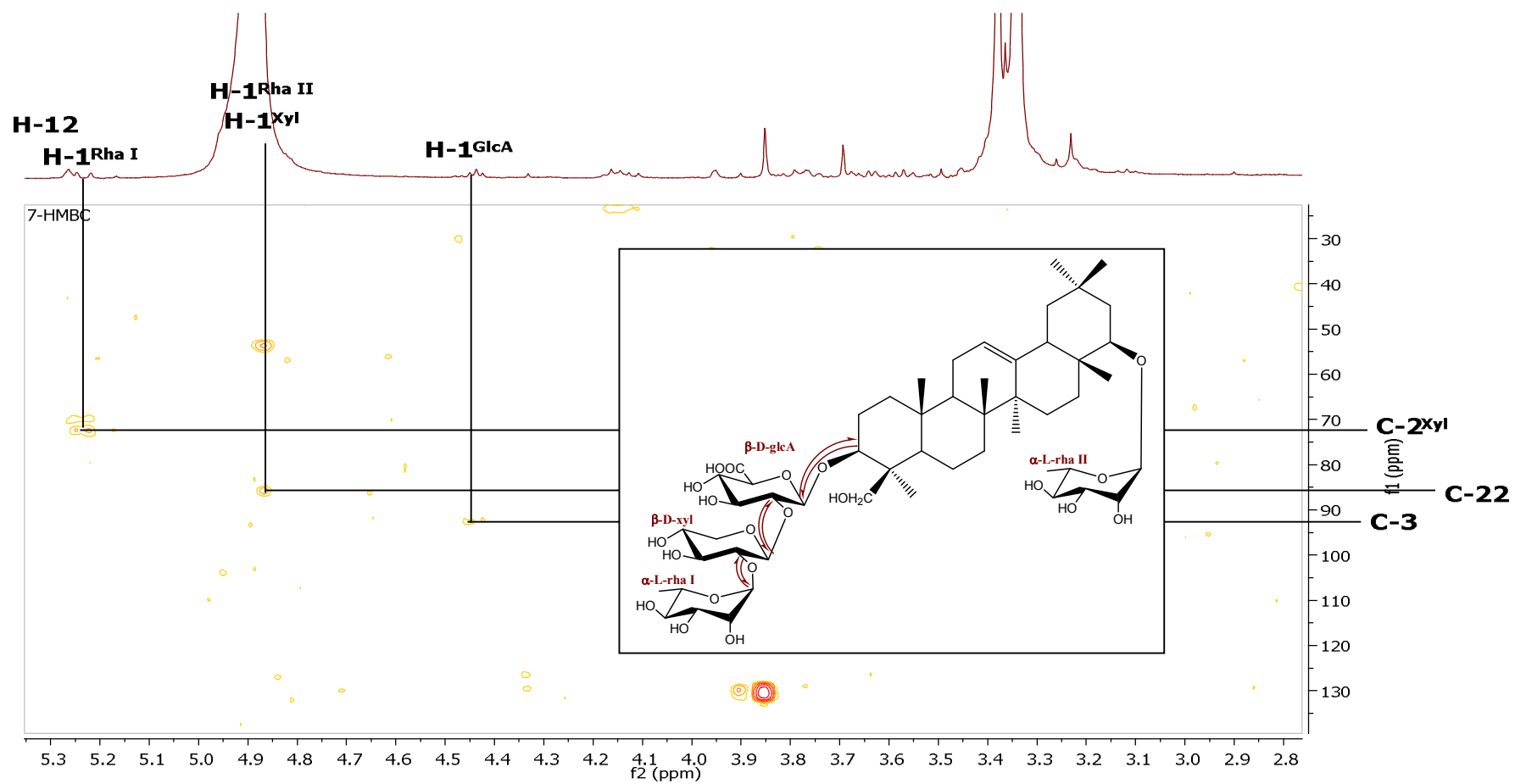
Spectrum 3.66. ^1H NMR spectrum of ATA7 (600 MHz, CD_3OD)



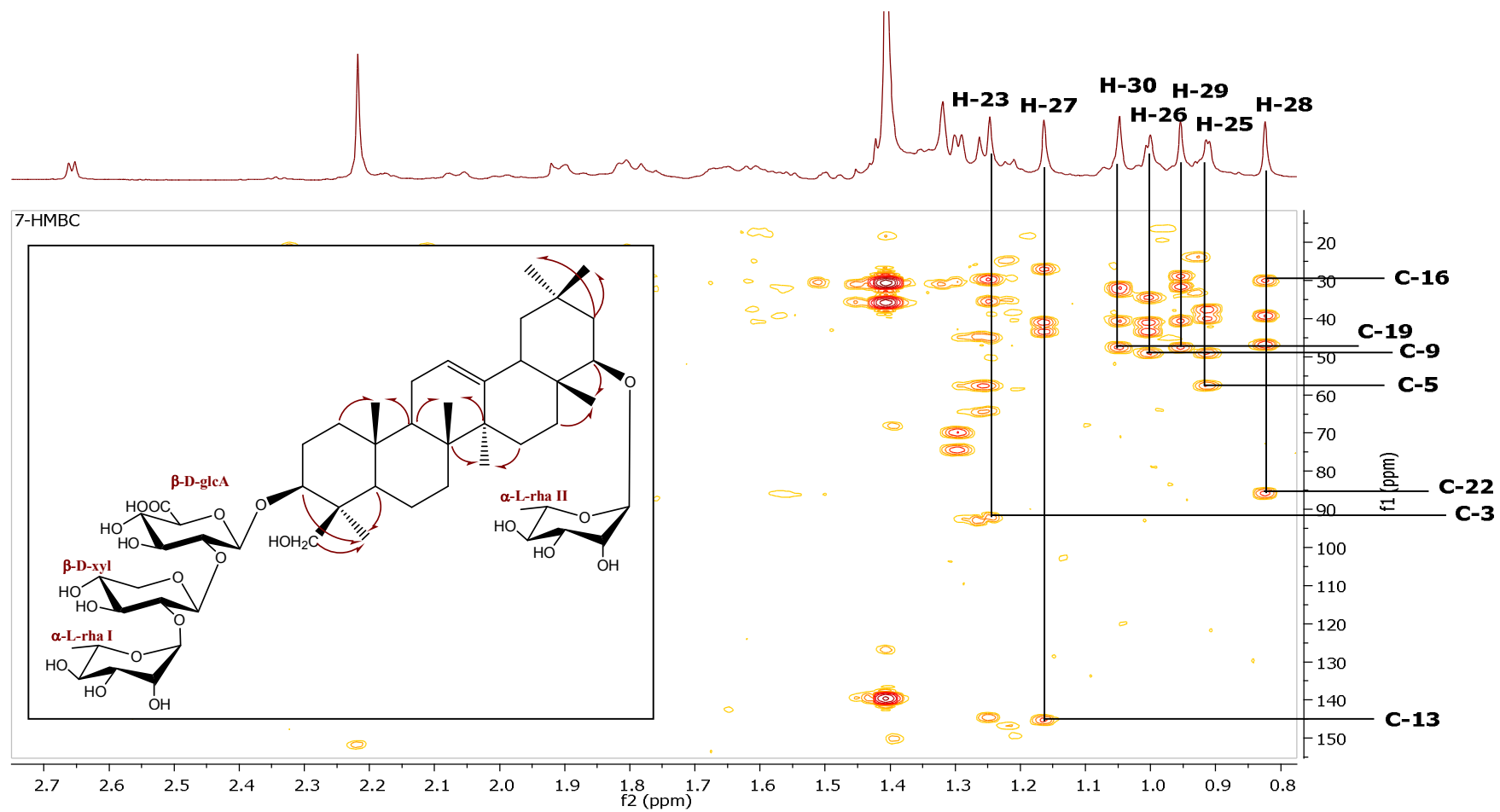
Spectrum 3.67. HSQC Spectrum of ATA7



Spectrum 3.68. HSQC Spectrum of ATA7



Spectrum 3.69. HMBC Spectrum of ATA7



Spectrum 3.70. HMBC Spectrum of ATA7

3.2.7. Structural Identification of Compound ATA9

The analysis of the ESIMS spectrum of **ATA9** highlighted a major $[M-H]^-$ ion peak at m/z 969. The ESIMS² spectrum showed a fragmentation pattern revealing the presence in the molecule of an uronic acid unit $\{[(M-18)-H]^-$, m/z 951; $[(M-18-44)-H]^-$, m/z 907; $[(M-18-44-146)-H]^-$, m/z 761; $[(M-18-44-146-162)-H]^-$, m/z 599; $[(M-18-44-146-162-114)-H]^-$, m/z 485}. In confirmation of this, the tandem mass spectrum showed one product ion at m/z 467 originated from the neutral loss of a whole uronic acid unit from the $[(M-146-180)-H]^-$ ion (**Table 3.15**).

The ¹³C NMR spectrum for the aglycon portion of **ATA9** in comparison to that of **ATA2** showed a distinct signal at δ_C 220.0. Moreover a detailed analysis of the NMR data suggested that the aglycon of **ATA9** differed from that of **ATA2** only for the presence of a keto group (**Table 3.15**). The HMBC correlation between the proton signal at δ 1.00 (Me-28) and the carbon resonance at δ 220.0 (C-22) confirmed the location of the keto group at C-22. For the sugar portion, compound **ATA9** showed proton and carbon resonances superimposable on those observed for compound **ATA2**. Thus, compound **ATA9** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-3 β ,24-dihydroxyolean-12-ene-22-oxo-29-oic acid. (**Fig. 3.15**)

Amorphous white solid; C₄₈H₇₄O₂₀; $[\alpha]_D^{25} +9.8$ (*c* 0.01 MeOH); IR $\nu_{\max}^{KBr}$ cm⁻¹: 3430 (>OH), 2938 (>CH), 1650 (C=C); HRMALDITOFMS $[M+Na]^+$ m/z 993.4682 (calc. for C₄₈H₇₄O₂₀Na, 993.4671). ¹H and ¹³C NMR (CD₃OD, 600 MHz) data see **Table 3.15**.

Table 3.15. ^1H and ^{13}C Assignments of ATA9 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	39.5	1.66, 1.07, m
2	28.8	2.21, 1.85, m
3	92.5	3.42, dd (11.5, 4.2)
4	44.4	-
5	57.1	1.00, m
6	19.3	1.69, 1.43, m
7	33.8	1.62, 1.43, m
8	40.6	-
9	48.6	1.69, m
10	37.2	-
11	24.7	1.97, (2H) m
12	123.4	5.32, t (3.5)
13	145.0	-
14	42.9	-
15	26.1	1.83, 1.16, m
16	28.4	2.27, 1.14, m
17	49.0	-
18	48.6	2.39, m
19	42.8	2.62, 1.65, m
20	47.1	-
21	47.8	2.17, 1.74, m
22	220.0	-
23	22.9	1.26, s
24	64.0	4.14, d (11.4), 3.23, d (11.4)
25	16.0	0.92, s
26	17.1	1.02, s
27	25.7	1.33, s
28	20.8	1.00, s
29	184.0	-
30	21.9	1.08, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.0	4.44, d (7.5)
2'	78.4	3.59, dd (7.5, 9.0)
3'	78.3	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5'	76.5	3.58, d (9.0)
6'	176.4	-
$\beta\text{-D-Glc (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	78.8	3.46, dd (7.5, 9.2)
3''	79.1	3.47, t (9.2)
4''	72.1	3.31, m
5''	77.4	3.27, ddd (3.5, 4.5, 9.0)
6''	62.1	3.81, dd(3.5, 12), 3.71, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1'''}$	101.6	5.24, d (1.2)
2'''	72.1	3.95, dd (1.2, 3.2)
3'''	72.0	3.78, dd (3.2, 9.7)
4'''	73.7	3.40, t (9.7)
5'''	69.5	4.16, m
6'''	18.1	1.30, d (6.5)

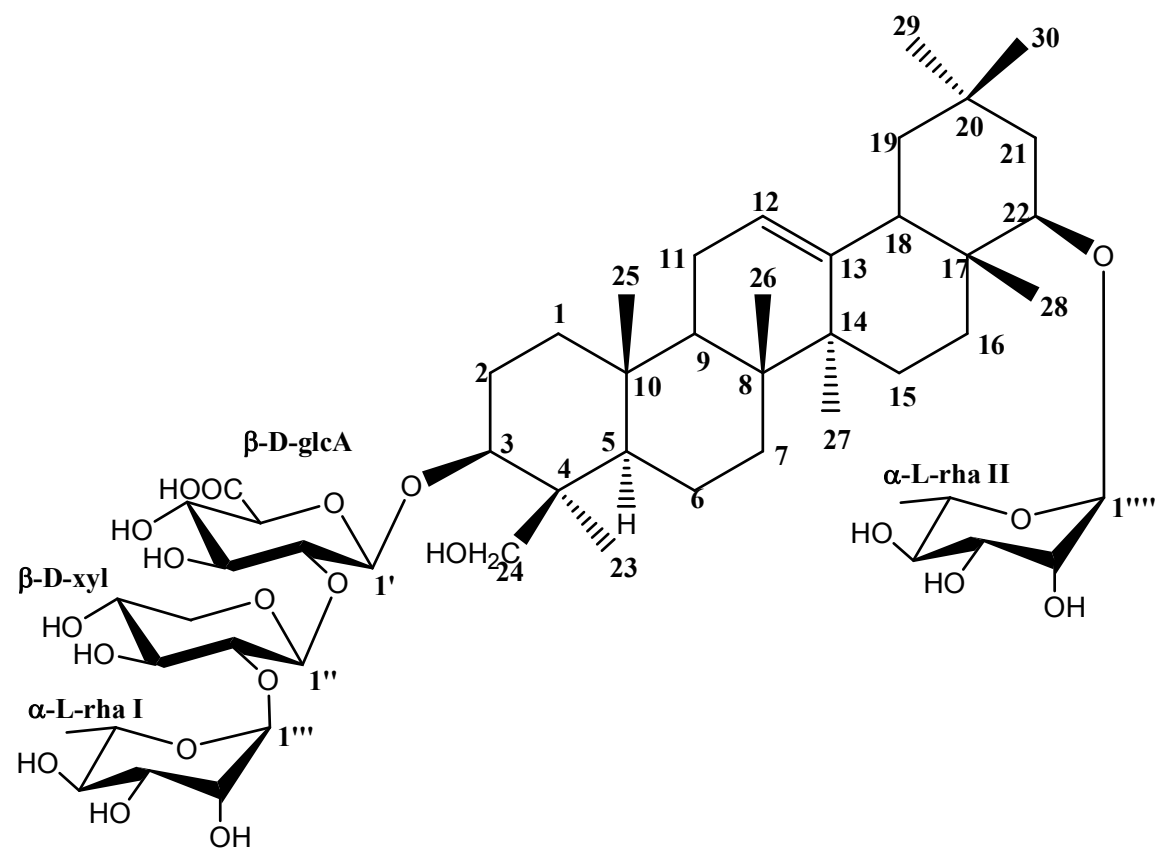
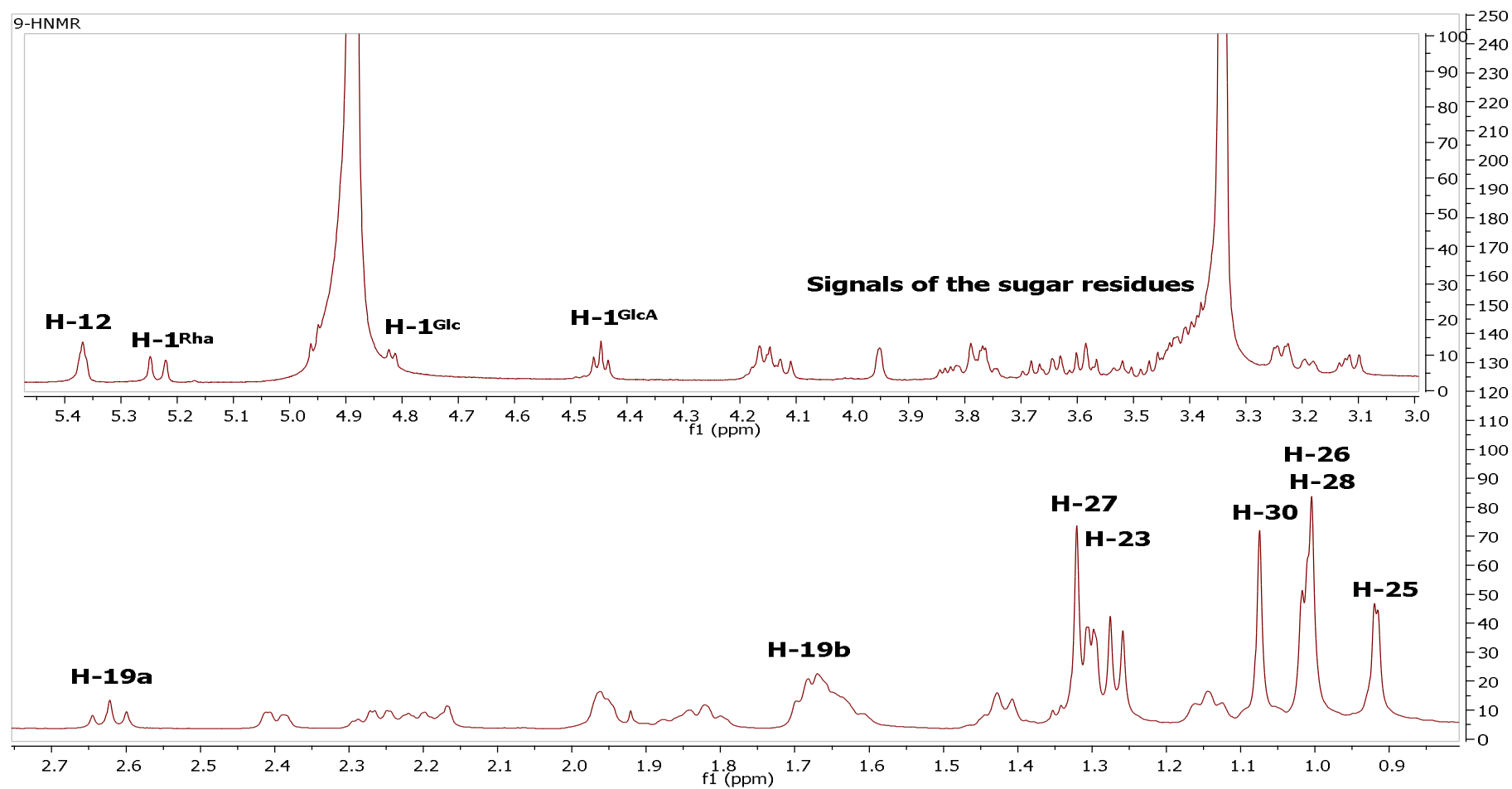
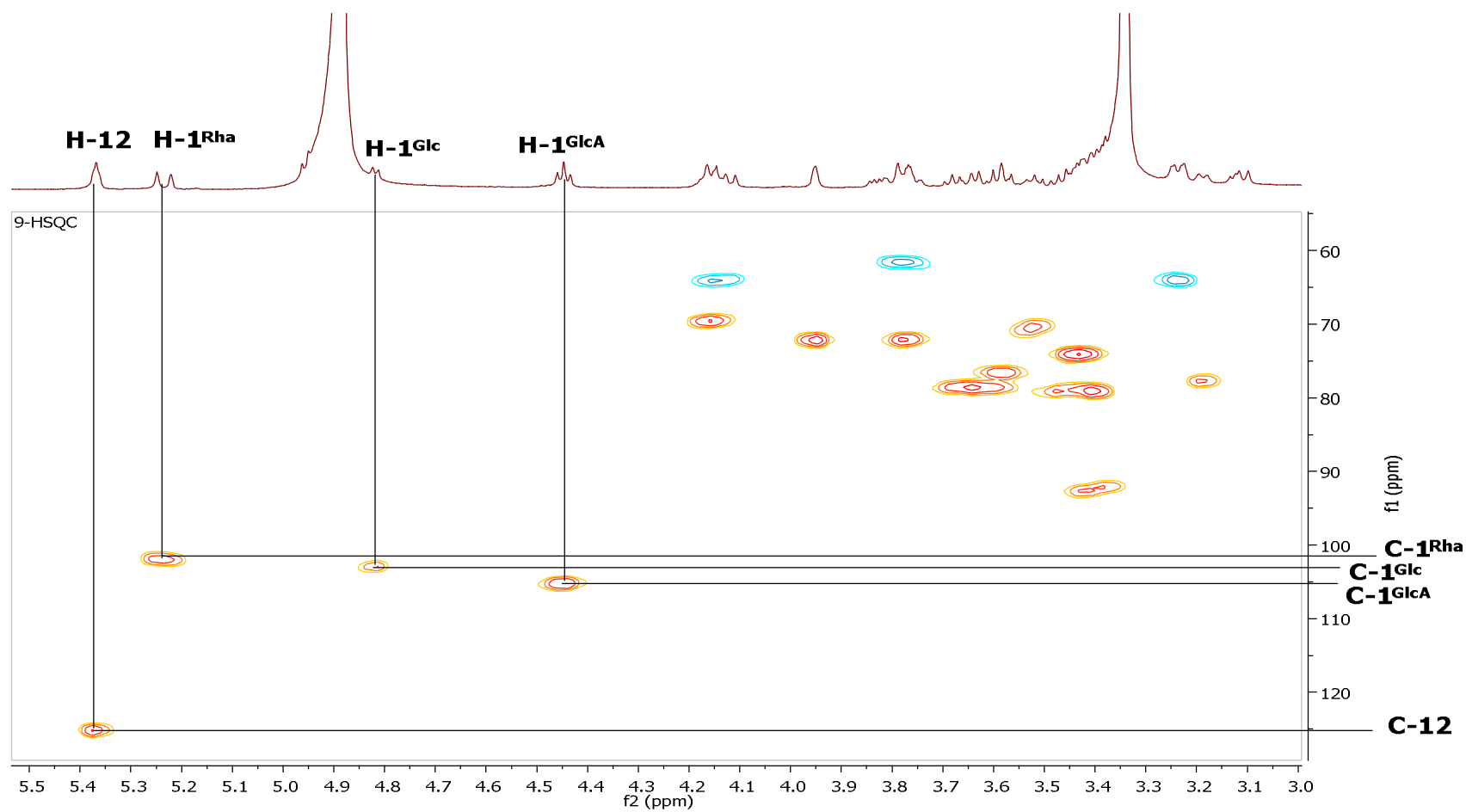


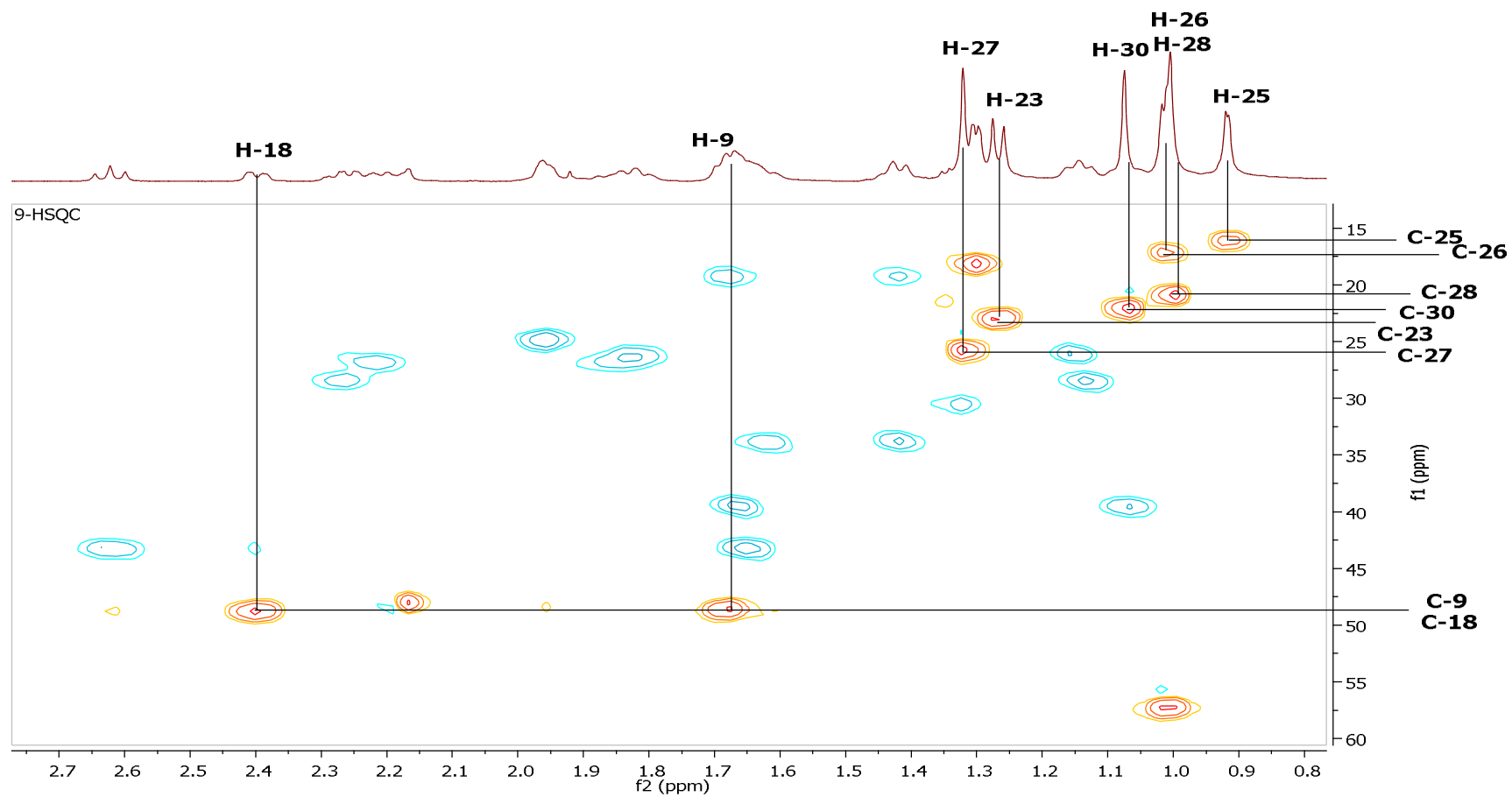
Figure 3.15. Structure of ATA9



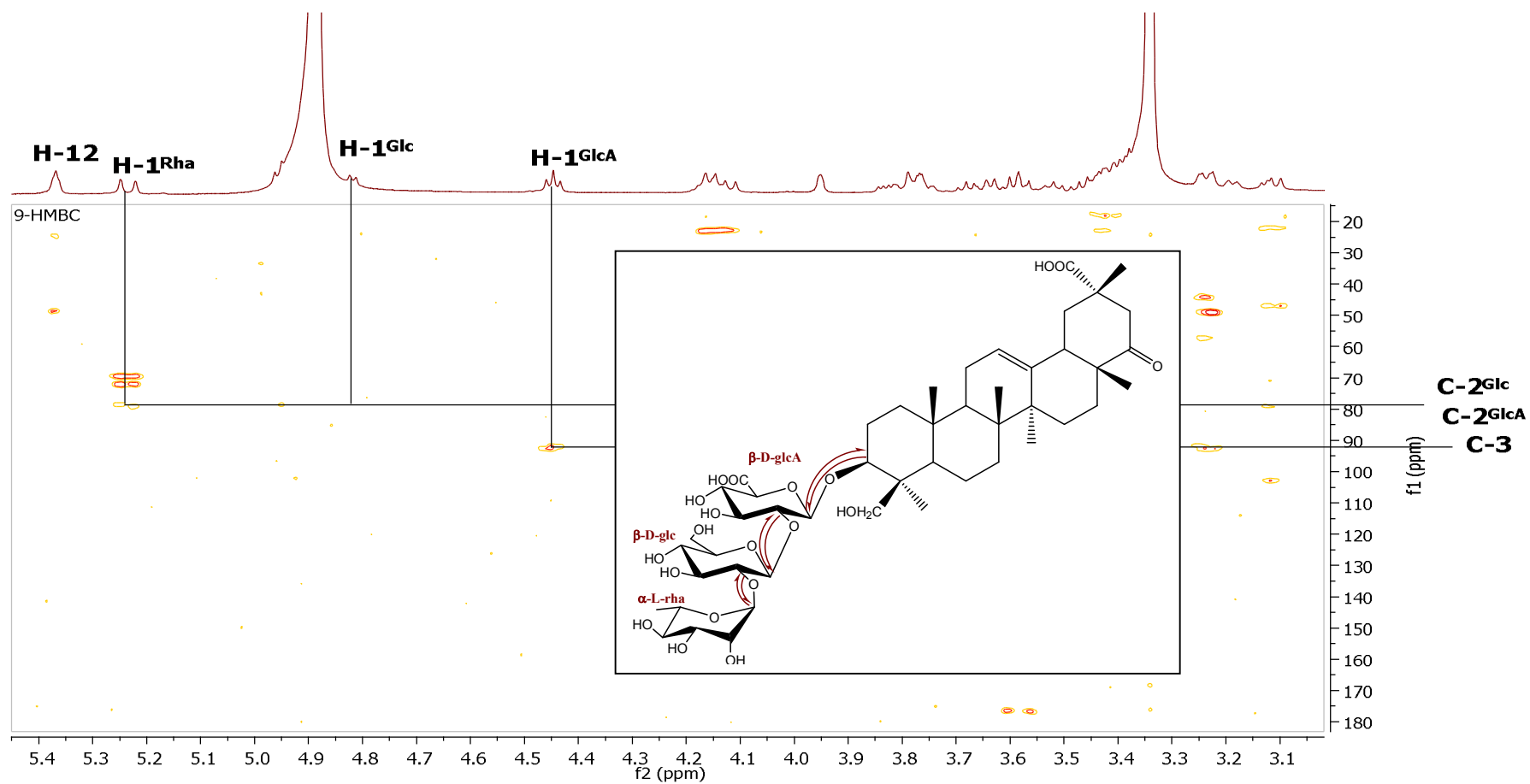
Spectrum 3.71. ^1H NMR spectrum of ATA9 (600 MHz, CD_3OD)



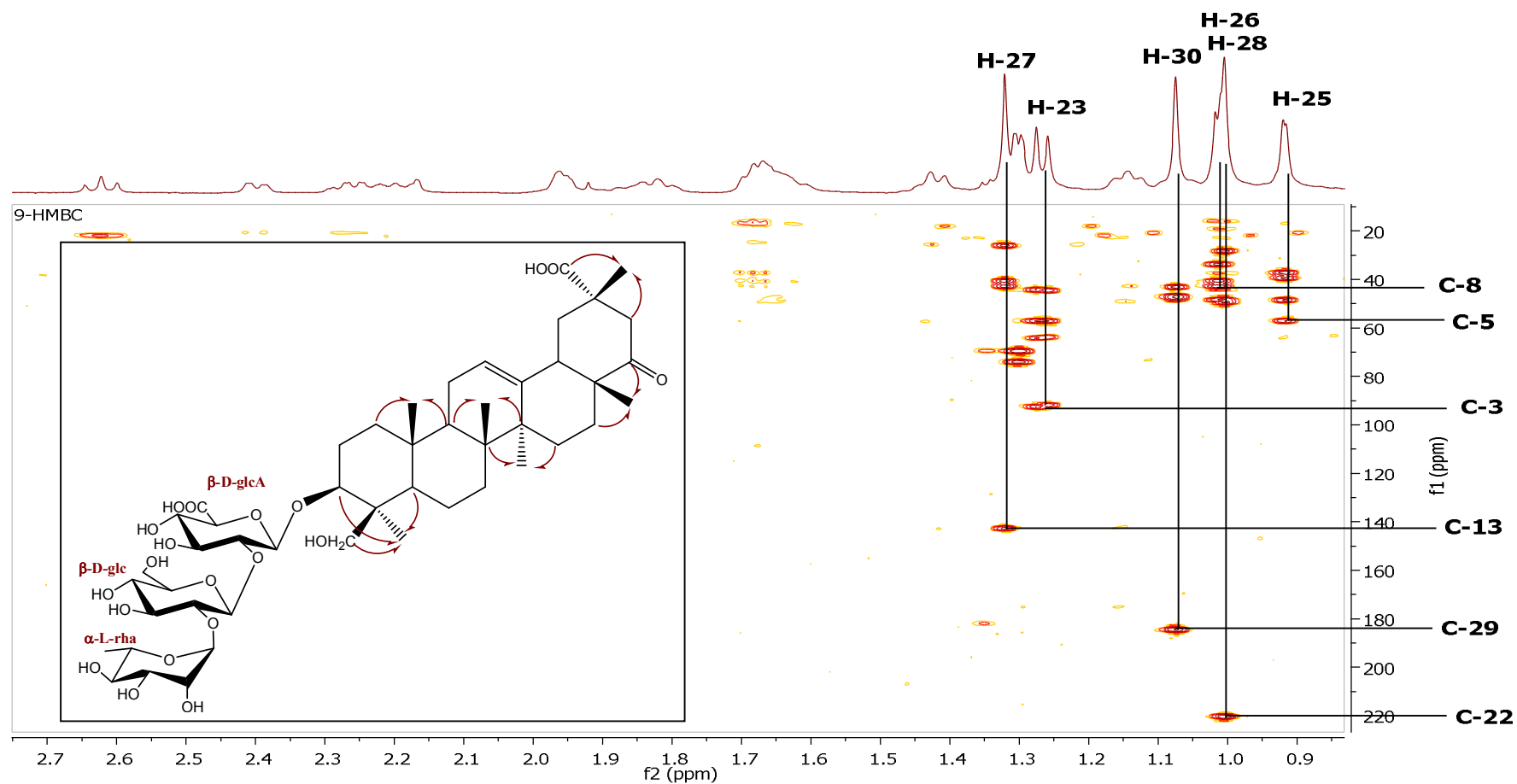
Spectrum 3.72. HSQC Spectrum of ATA9



Spectrum 3.73. HSQC Spectrum of ATA9



Spectrum 3.74. HMBC Spectrum of ATA9



Spectrum 3.75. HMBC Spectrum of ATA9

3.2.8. Structural Identification of Compound ATA11

The ESIMS spectrum of **ATA11** showed a major $[M-H]^-$ ion peak at m/z 973. Its fragmentation pattern allowed distinguishing the presence of three sugars, one deoxyhexose, one hexose, and one acid hexose, respectively (**Table 3.16**). A detailed analysis of the NMR data suggested that the aglycon of **ATA11** differed from that of **ATA5** only for the presence of a $-OH$ group at C-29 (δ 71.6). Moreover, the ^1H and ^{13}C NMR signals in the sugar region of **ATA11** assigned from the 2D NMR spectra were superimposable on those of **ATA5** except for the absence of a rhamnopyranosyl moiety linked at C-21 (**Table 3.16**). Thus, compound **ATA11** was identified as the new 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-3 β ,21 β ,22 α ,24,29-pentahydroxyolean-12-ene. (**Fig. 3.16**)

Amorphous white solid; $C_{48}H_{78}O_{20}$; $[\alpha]_D^{25} +16.8$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3438 ($>OH$), 2930 ($>CH$), 1655 ($C=C$); HRMALDITOFMS $[M+Na]^+$ m/z 997.4989 (calcd for $C_{48}H_{78}O_{20}Na$, 997.4982). ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data see **Table 3.16**.

Table 3.16. ^1H and ^{13}C Assignments of ATA11 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	39.5	1.65, 1.05, m
2	26.7	2.20, 1.81, m
3	91.7	3.36, dd (11.5, 4.2)
4	44.6	-
5	57.2	0.98, m
6	19.0	1.68, 1.42, m
7	33.6	1.62, 1.39, m
8	40.8	-
9	48.7	1.64, m
10	37.0	-
11	24.7	1.92, (2H) m
12	123.3	5.32, t (3.5)
13	145.0	-
14	42.7	-
15	27.0	1.91, 1.03, m
16	27.5	1.90, 1.04, m
17	39.4	-
18	43.7	2.29, m
19	41.2	2.13, 0.99, m
20	41.0	-
21	70.7	3.84, brs
22	80.4	3.37, d (3.5)
23	22.8	1.25, s
24	63.8	4.12, d (11.4), 3.23, d (11.4)
25	16.0	0.91, s
26	17.1	1.03, s
27	26.3	1.23, s
28	22.0	0.98, s
29	71.6	3.45, d (9.7), 3.12, d (9.7)
30	16.7	1.00, s
$\beta\text{-D-GlcA (at C-3)-1'}$	104.9	4.43, d (7.5)
2'	78.4	3.59, dd (7.5, 9.0)
3'	78.4	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5'	76.5	3.57, d (9.0)
6'	177.1	-
$\beta\text{-D-Glc (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	78.9	3.46, dd (7.5, 9.2)
3''	79.1	3.47, t (9.2)
4''	72.2	3.31, m
5''	77.5	3.27, ddd (3.5, 4.5, 9.0)
6''	62.3	3.82, dd(3.5, 12), 3.71, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1'''}$	101.8	5.21, d (1.2)
2'''	71.8	3.95, dd (1.2, 3.2)
3'''	71.9	3.77, dd (3.2, 9.7)
4'''	73.9	3.43, t (9.7)
5'''	69.5	4.15, m
6'''	17.8	1.28, d (6.5)

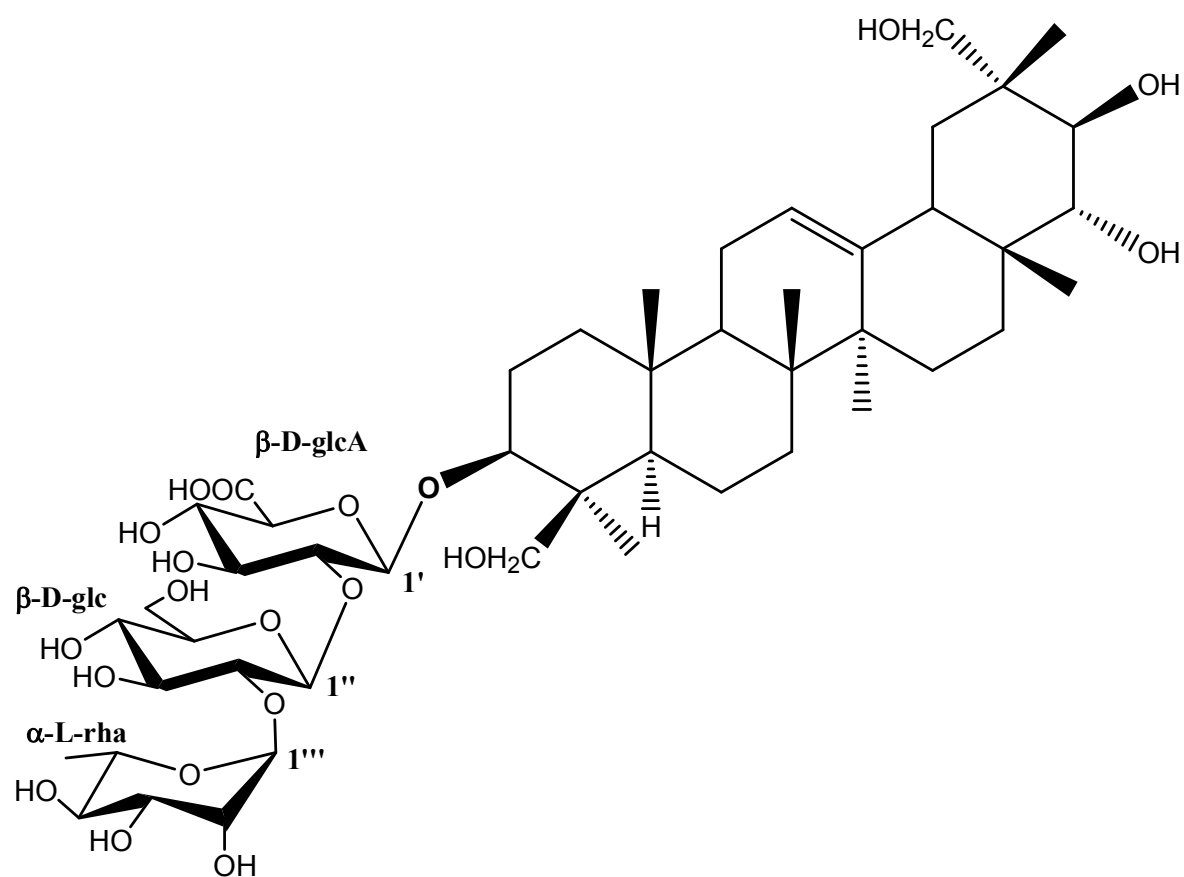
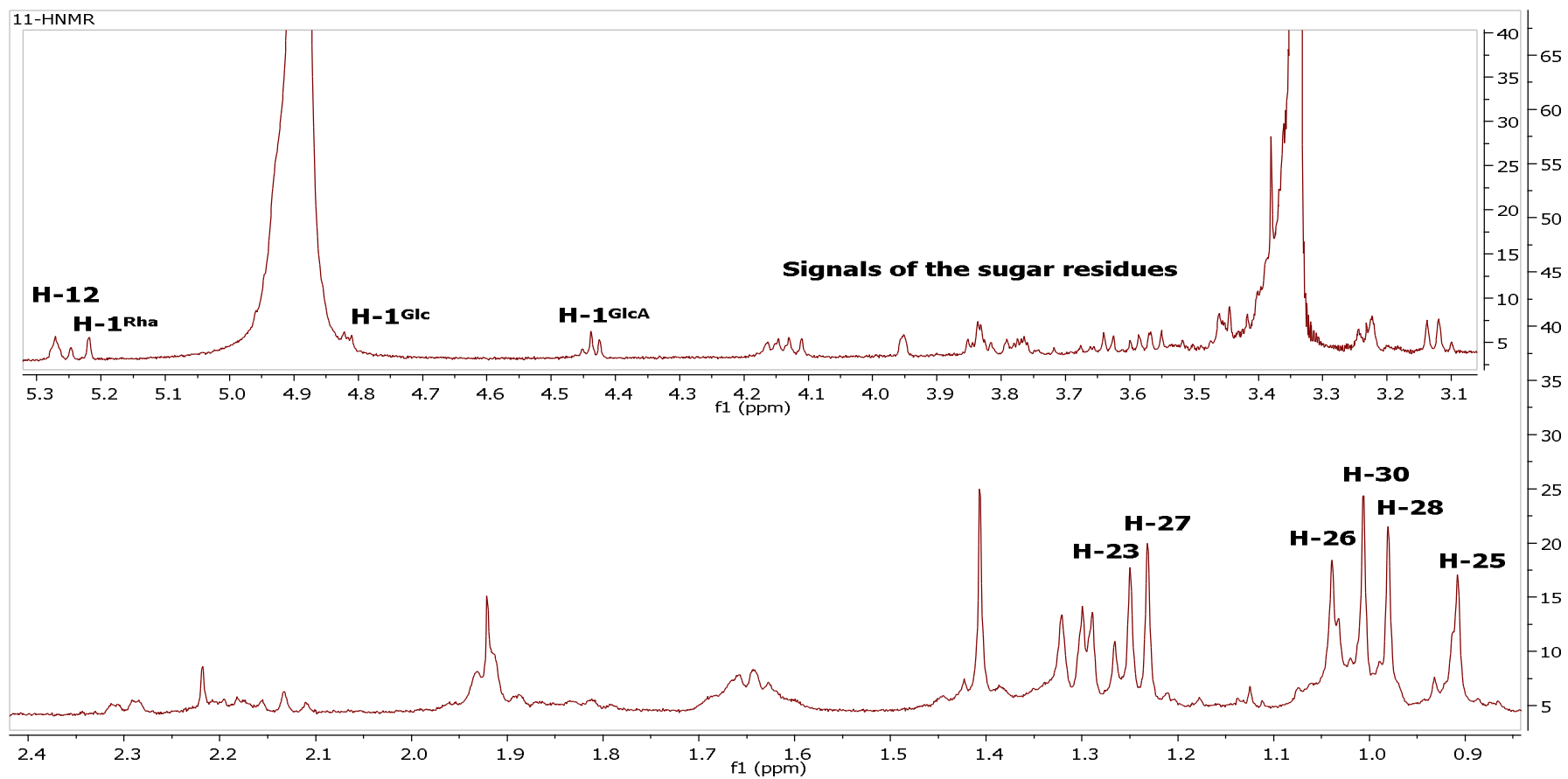
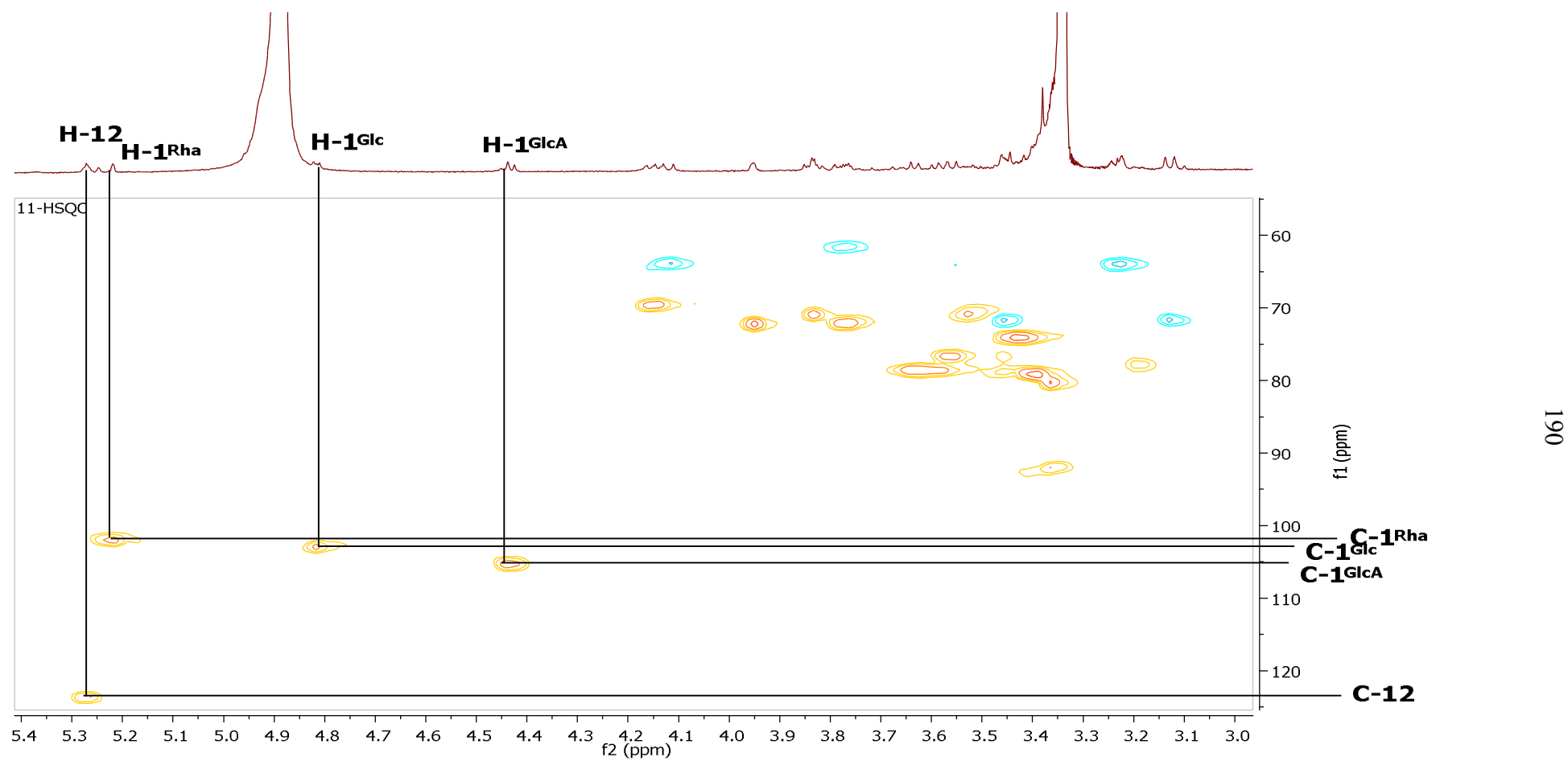


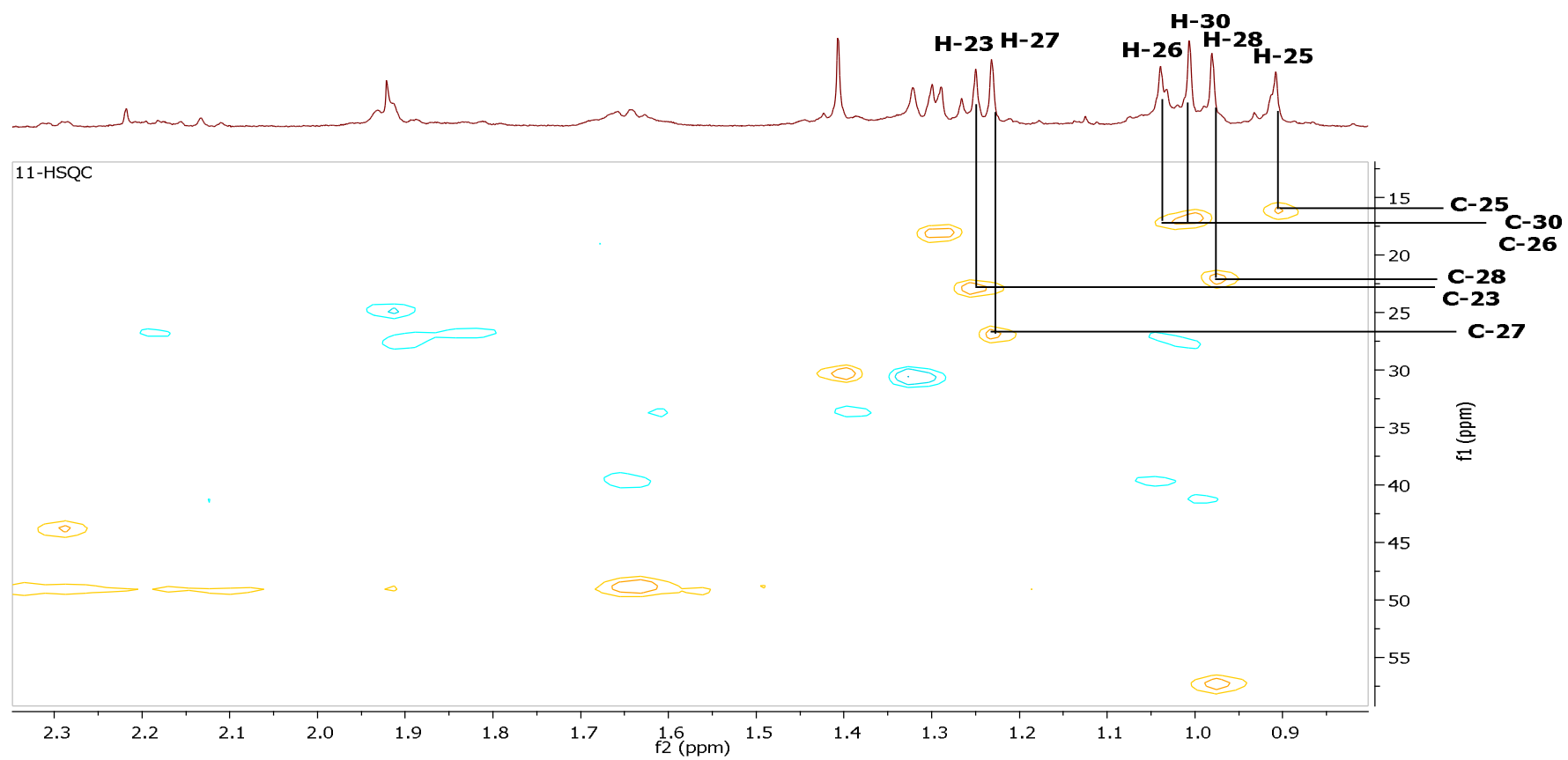
Figure 3.16. Structure of ATA11



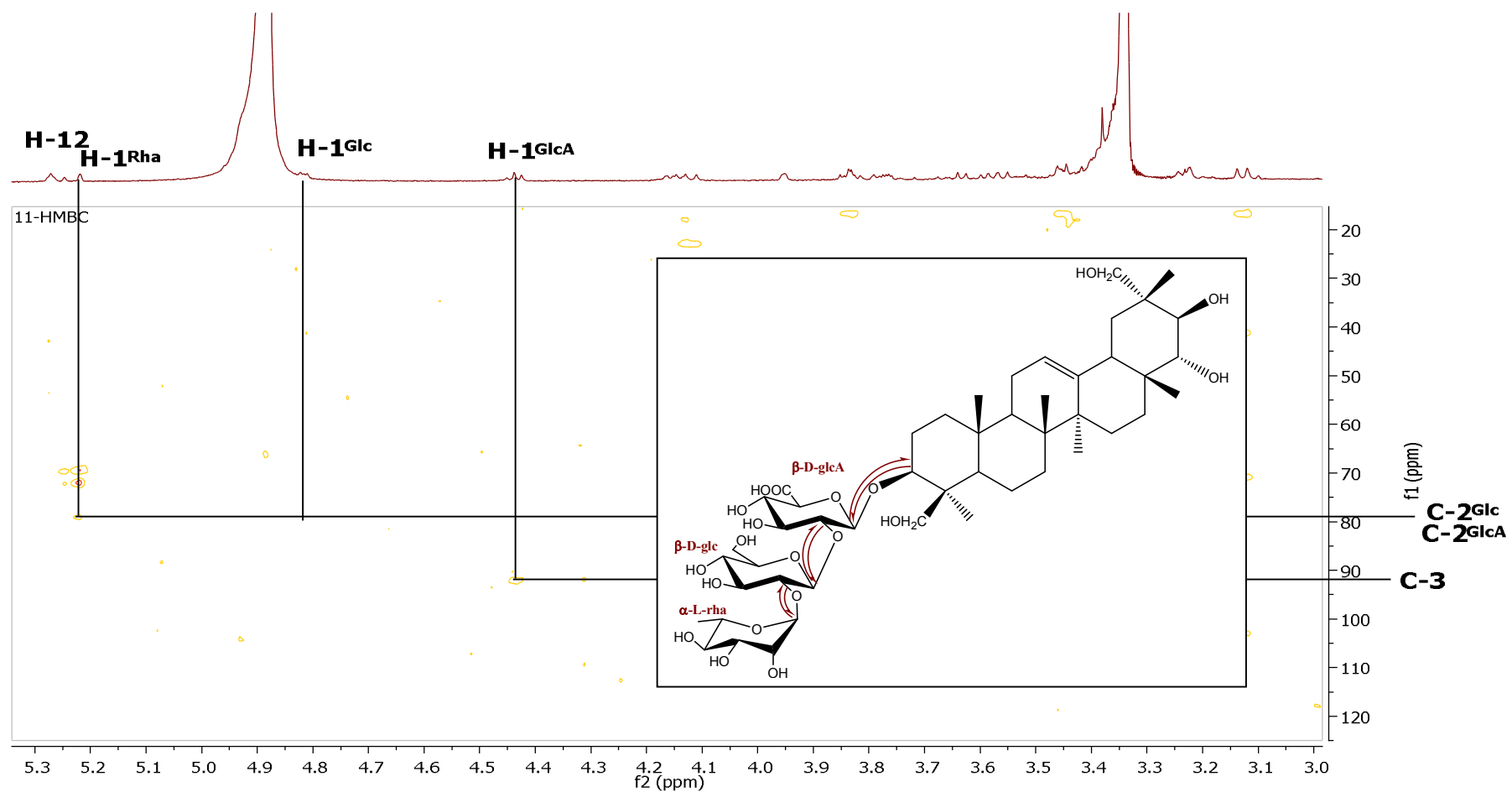
Spectrum 3.76. ^1H NMR spectrum of ATA11 (600 MHz, CD_3OD)



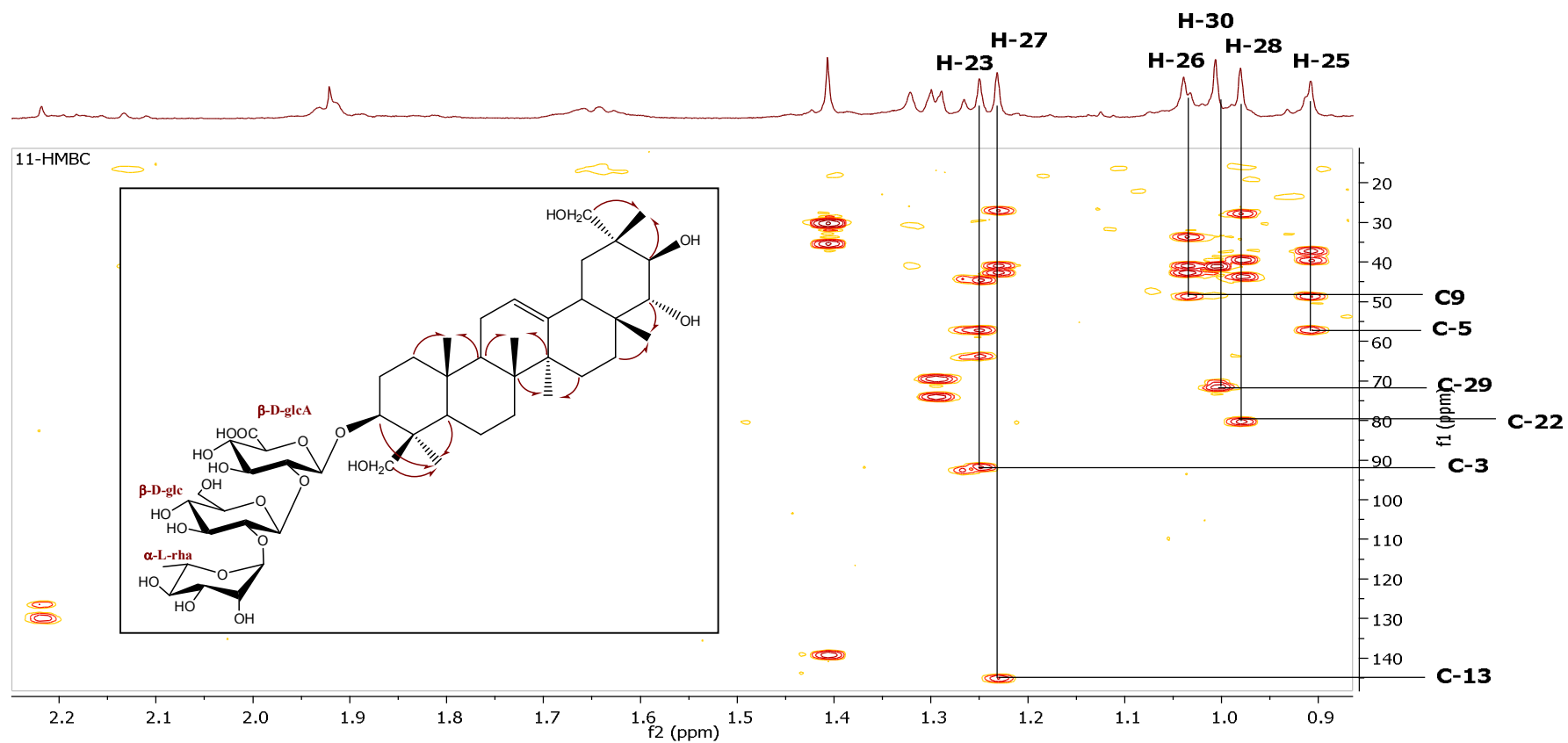
Spectrum 3.77. HSQC Spectrum of ATA11



Spectrum 3.78. HSQC Spectrum of ATA11



Spectrum 3.79. HMBC Spectrum of ATA11



Spectrum 3.80. HMBC Spectrum of ATA11

3.2.9. Structural Identification of Compound ATA12

The full negative ESIMS spectrum of **ATA12** exhibited a major $[M-H]^-$ ion peak at m/z 987, whose fragmentation in a mass tandem experiment was informative about the presence of two hexoses and one acid hexose (**Table 3.17**). The ^1H and ^{13}C NMR chemical shifts of **ATA12** were almost superimposable on those of **ATA6**, except for the absence of the terminal rhamnopyranosyl unit on the sugar chain linked at C-3. Thus, compound **ATA12** was identified as the new 3-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24,-trihydroxyolean-12-en-29-oic acid. (**Fig. 3.17**)

Amorphous white solid; $C_{48}H_{76}O_{21}$; $[\alpha]_D^{25} +16.8$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3445 ($>OH$), 2928 ($>CH$), 1655 ($C=C$); HRMALDITOFMS $[M+Na]^+$ m/z 1011.4785 (calc. for $C_{48}H_{76}O_{21}Na$, 1011.4777); ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data see **Table 3.17**.

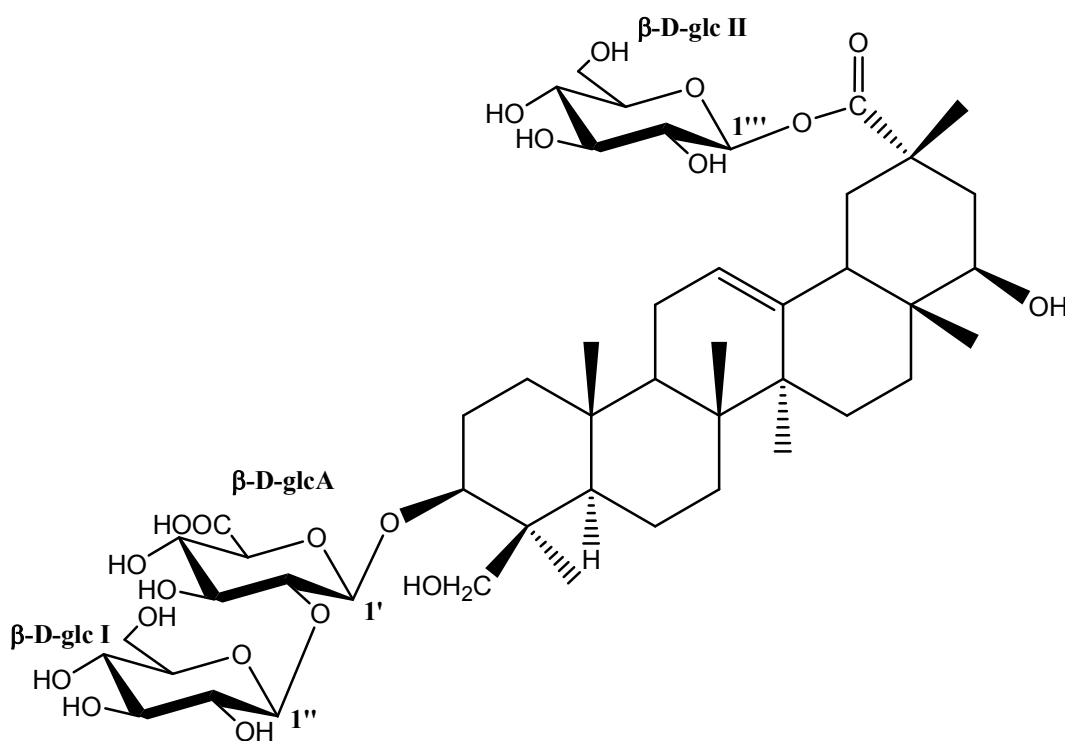
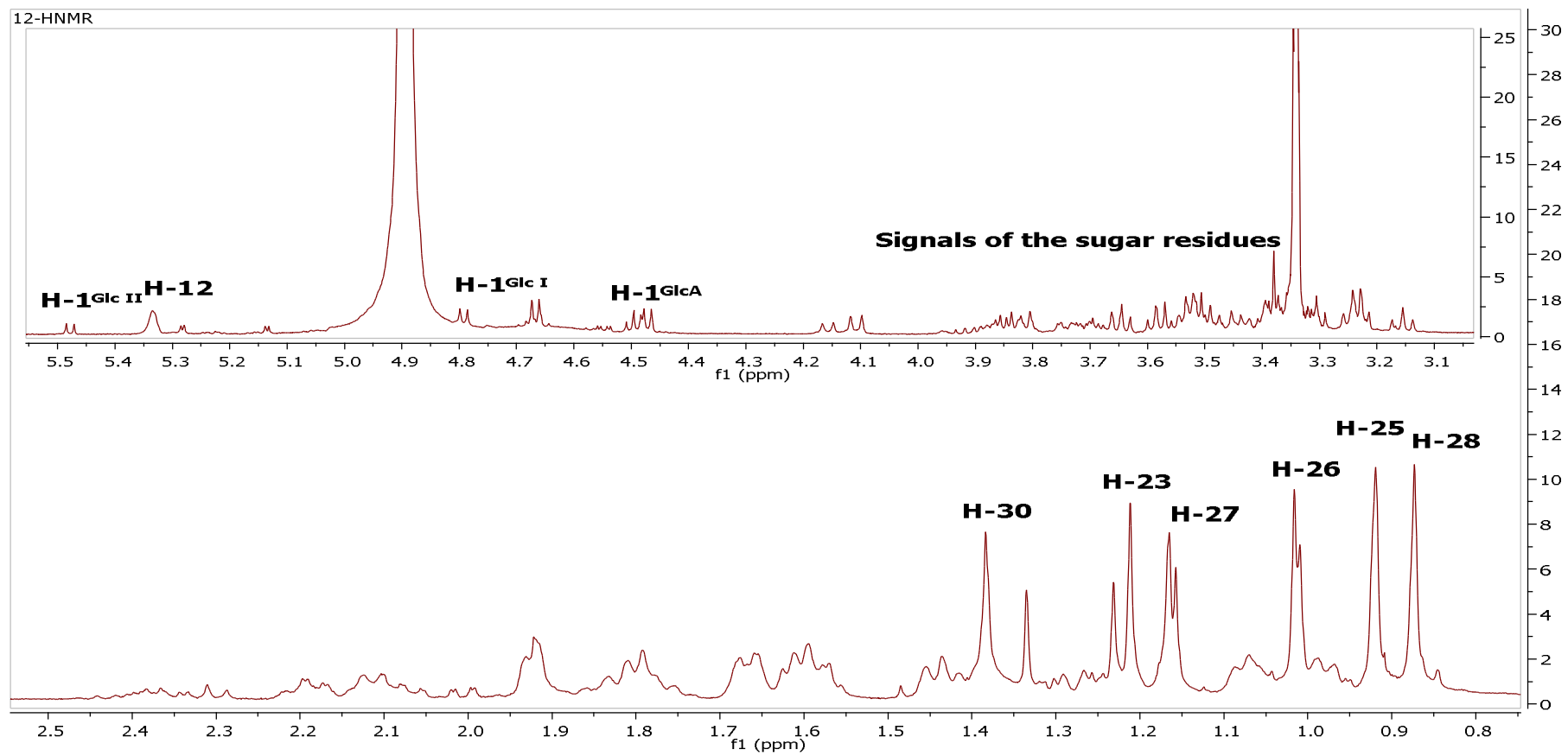


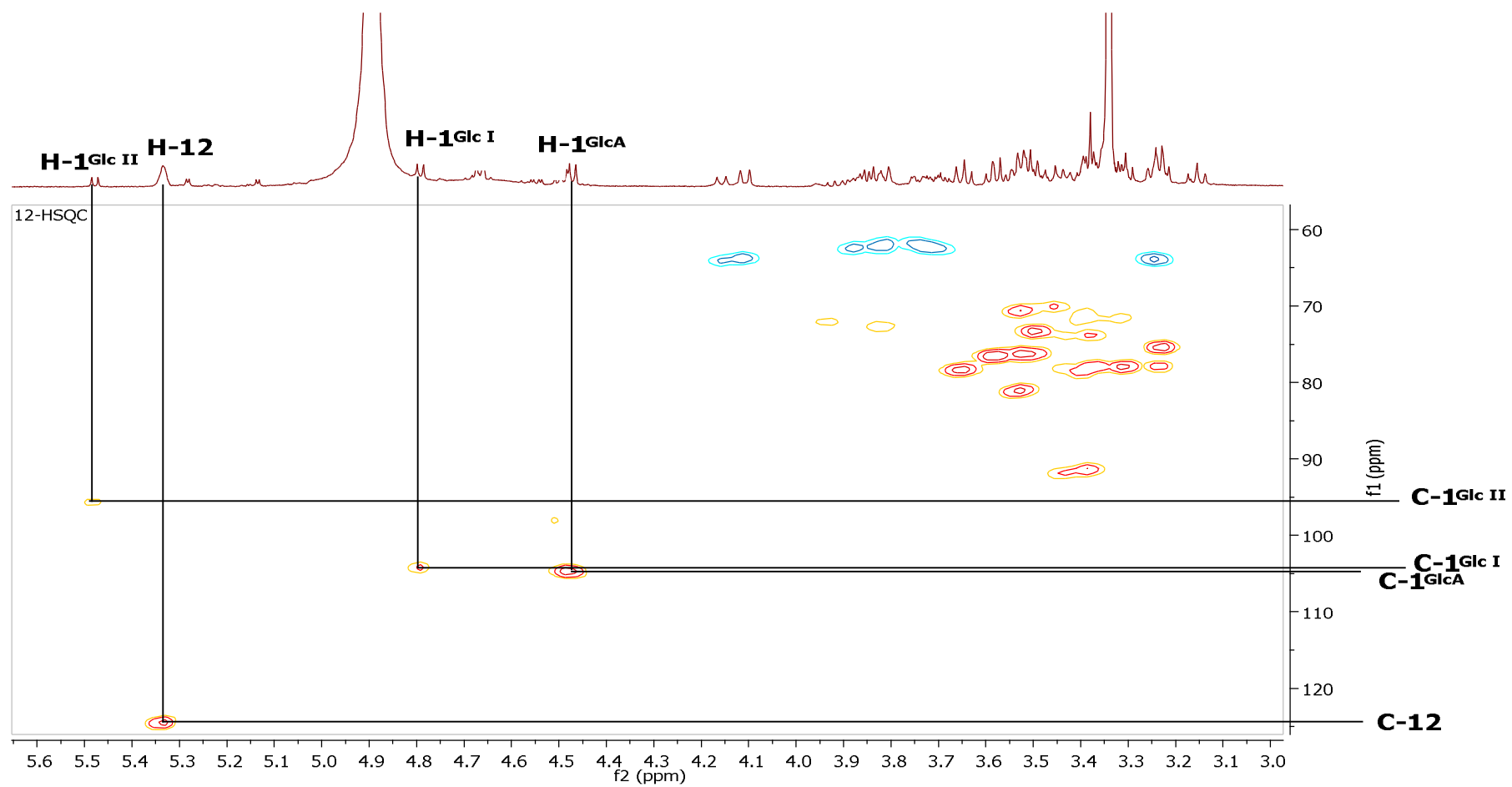
Figure 3.17. Structure of ATA12

Table 3.17. ^1H and ^{13}C Assignments of ATA12 (150/600 MHz, δ ppm, in CD_3OD)

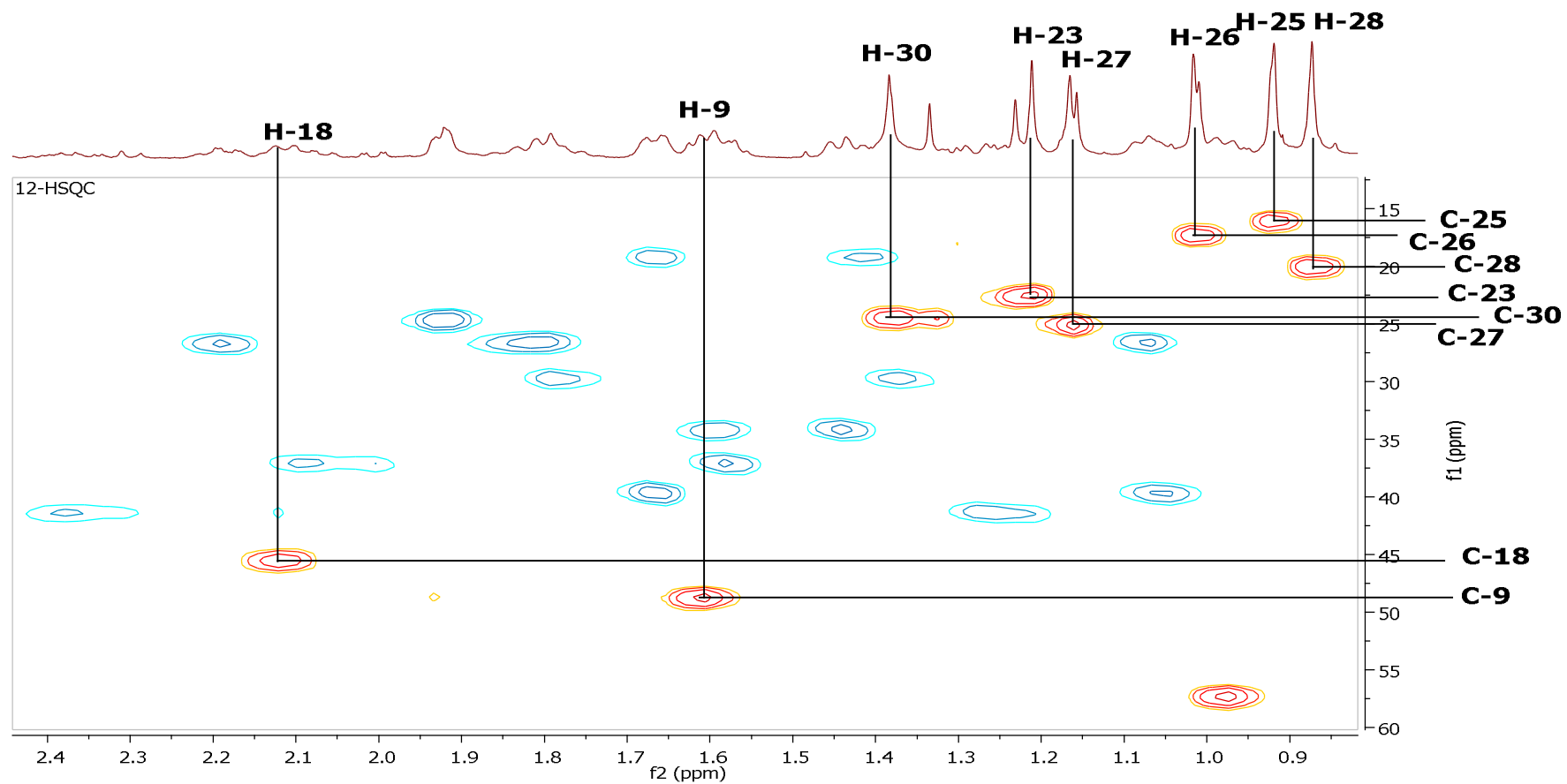
Position	δ_{C}	δ_{H} (J in Hz)
1	39.4	1.65, 1.05, m
2	26.7	2.21, 1.84, m
3	91.4	3.33, dd (11.5, 4.5)
4	44.4	-
5	57.1	0.97, m
6	19.3	1.65, 1.42, m
7	34.2	1.61, 1.44, m
8	40.6	-
9	48.7	1.61, m
10	37.0	-
11	24.6	1.90, (2H) m
12	124.2	5.32, t (3.5)
13	144.4	-
14	43.3	-
15	26.5	1.81, 1.09, m
16	29.6	1.78, 1.38, m
17	38.4	-
18	45.5	2.12, m
19	41.1	2.36, 1.28, m
20	42.7	-
21	36.6	2.06, 1.58, m
22	76.2	3.50, brs
23	22.8	1.25, s
24	63.5	4.12, d (11.5), 3.21, d (11.5)
25	16.0	0.91, s
26	17.0	1.00, s
27	25.0	1.16, s
28	19.7	0.86, s
29	178.3	-
30	24.0	1.37, s
$\beta\text{-D-GlcA (at C-3)-1'}$	104.5	4.44, d (7.5)
2'	80.8	3.59, dd (7.5, 9.0)
3'	78.2	3.64, dd (9.0, 9.0)
4'	73.8	3.43, dd (9.0, 9.0)
5'	76.3	3.58, d (9.0)
6'	177.1	-
$\beta\text{-D-Glc I (at C-2}_{\text{glcA}}\text{)-1''}$	102.7	4.81, d (7.5)
2''	79.0	3.45, dd (7.5, 9.2)
3''	79.6	3.50, t (9.2)
4''	72.0	3.32, (9.2)
5''	77.8	3.27, ddd (3.5, 4.5, 9.0)
6''	62.0	3.80, dd(3.5, 12), 3.71, dd (4.5, 12)
$\beta\text{-D-Glc II (at C-29)-1'''}$	95.6	5.47, d (7.5)
2'''	73.7	3.38, dd (7.5, 9.0)
3'''	77.7	3.38, t (9.0)
4'''	71.5	3.31, t (9.0)
5'''	77.9	3.23, ddd (3.5, 4.5, 9.0)
6'''	61.8	3.81, dd(3.5, 12), 3.71, dd (4.5, 12)



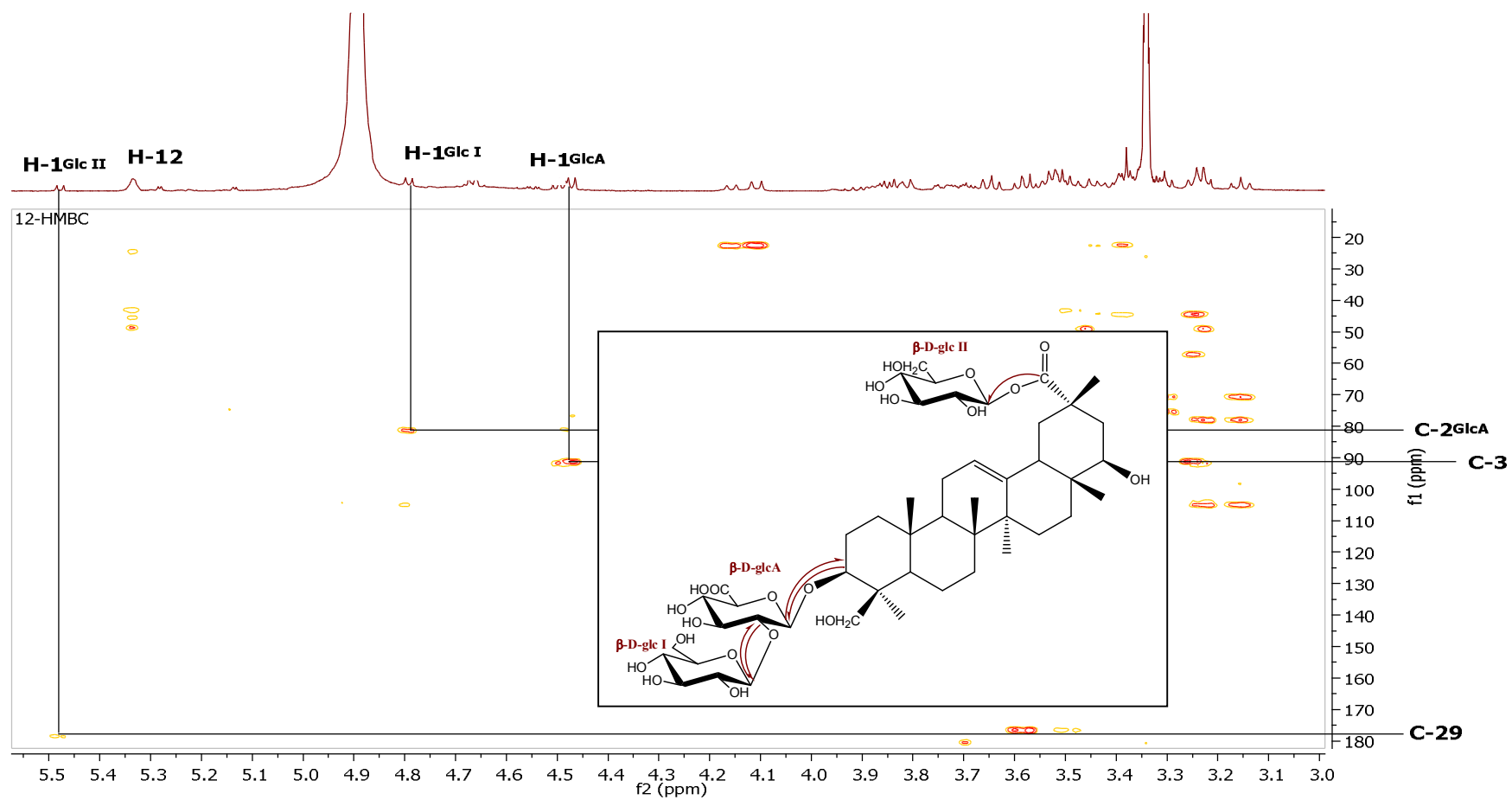
Spectrum 3.81. ^1H NMR spectrum of ATA12 (600 MHz, CD_3OD)



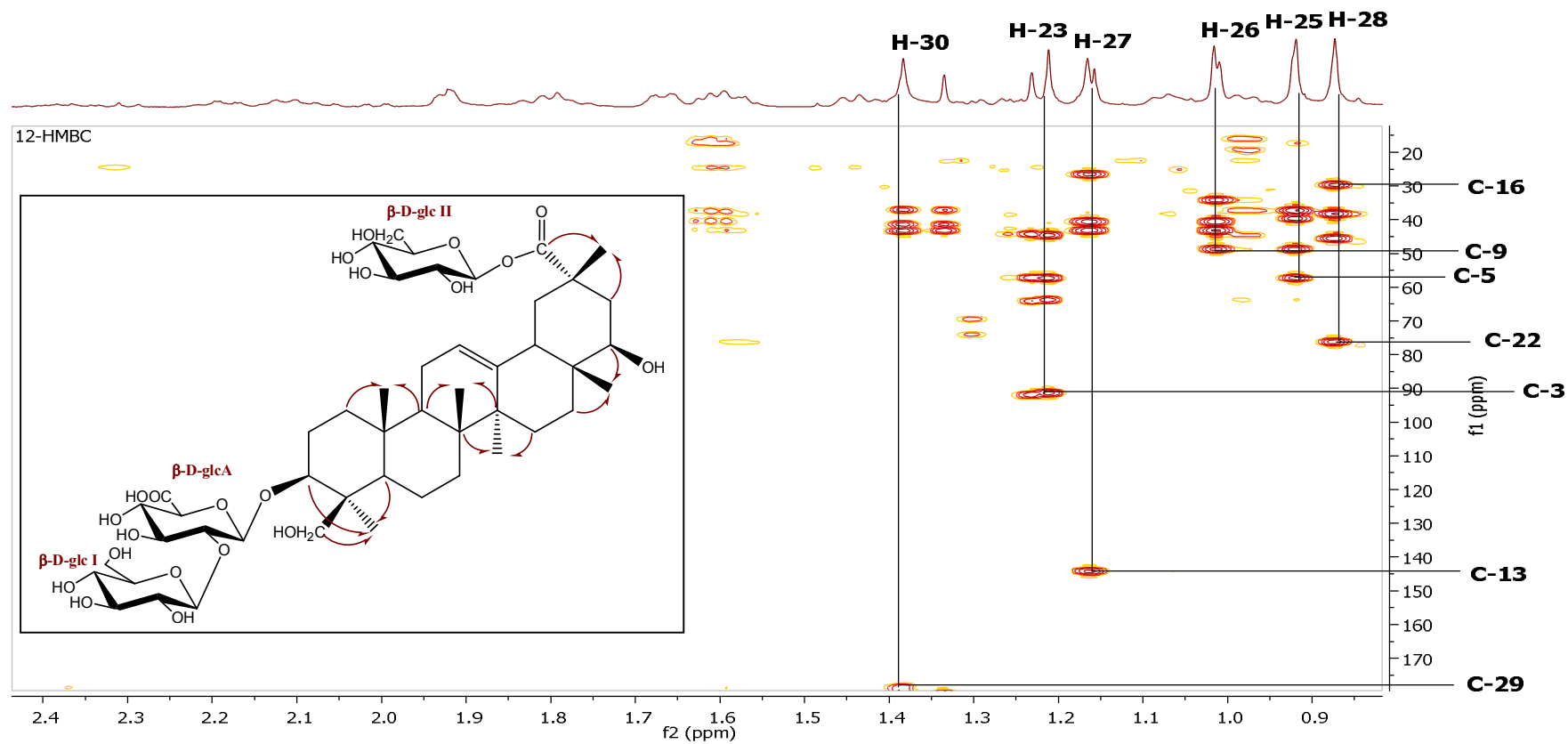
Spectrum 3.82. HSQC Spectrum of ATA12



Spectrum 3.83. HSQC Spectrum of ATA12



Spectrum 3.84. HMBC Spectrum of ATA12



Spectrum 3.85. HMBC Spectrum of ATA12

3.2.10. Structural Identification of Compound ATA14

The ESIMS spectrum of **ATA14** showed a major $[M-H]^-$ ion peak 30 a.m.u. greater than **ATA12** (**Table 3.18**). The analysis of the ESIMS² and ESIMS³ spectra allowed ascertaining that **ATA14** differed from **ATA12** for a pentose unit instead of a hexose one. The ¹H and ¹³C NMR signals of **14** assigned from the 2D NMR spectra were almost superimposable on those of **ATA12** except for the characteristic signals of a xylopyranosyl moiety instead of a glucopyranosyl moiety (**Table 3.18**). In the HMBC spectrum, the (1→2) linkage between the xylose and glucuronic acid units was substantiated by the cross-peak between δ_H 4.67 (H-1_{xy}) and δ 78.3 (C-2_{glcA}). Thus, compound **ATA14** was identified as the new 3-*O*-[β -D-xylopyranosyl-(1→2)- β -D-glucuronopyranosyl]-29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24, trihydroxyolean-12-en-29-oic acid. (**Fig. 3.18**)

Amorphous white solid; $C_{47}H_{74}O_{20}$; $[\alpha]_D^{25} +11.5$ (c 0.01 MeOH); IR $\nu_{\max}^{KBr}$ cm^{-1} : 3440 ($>OH$), 2934 ($>CH$), 1650 ($C=C$); HRMALTIDOFMS $[M+Na]^+$ m/z 981.4683 (calc. for $C_{47}H_{74}O_{20}Na$, 981.4671); ^1H and ^{13}C NMR (CD_3OD , 600 MHz) data: see **Table 3.18**.

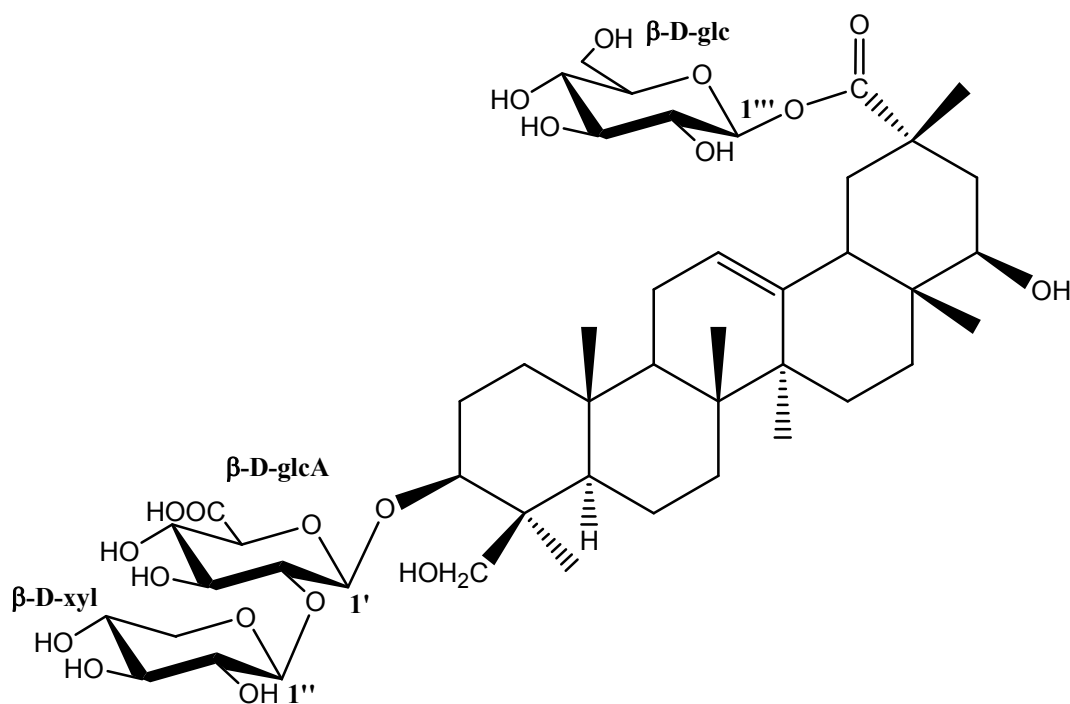
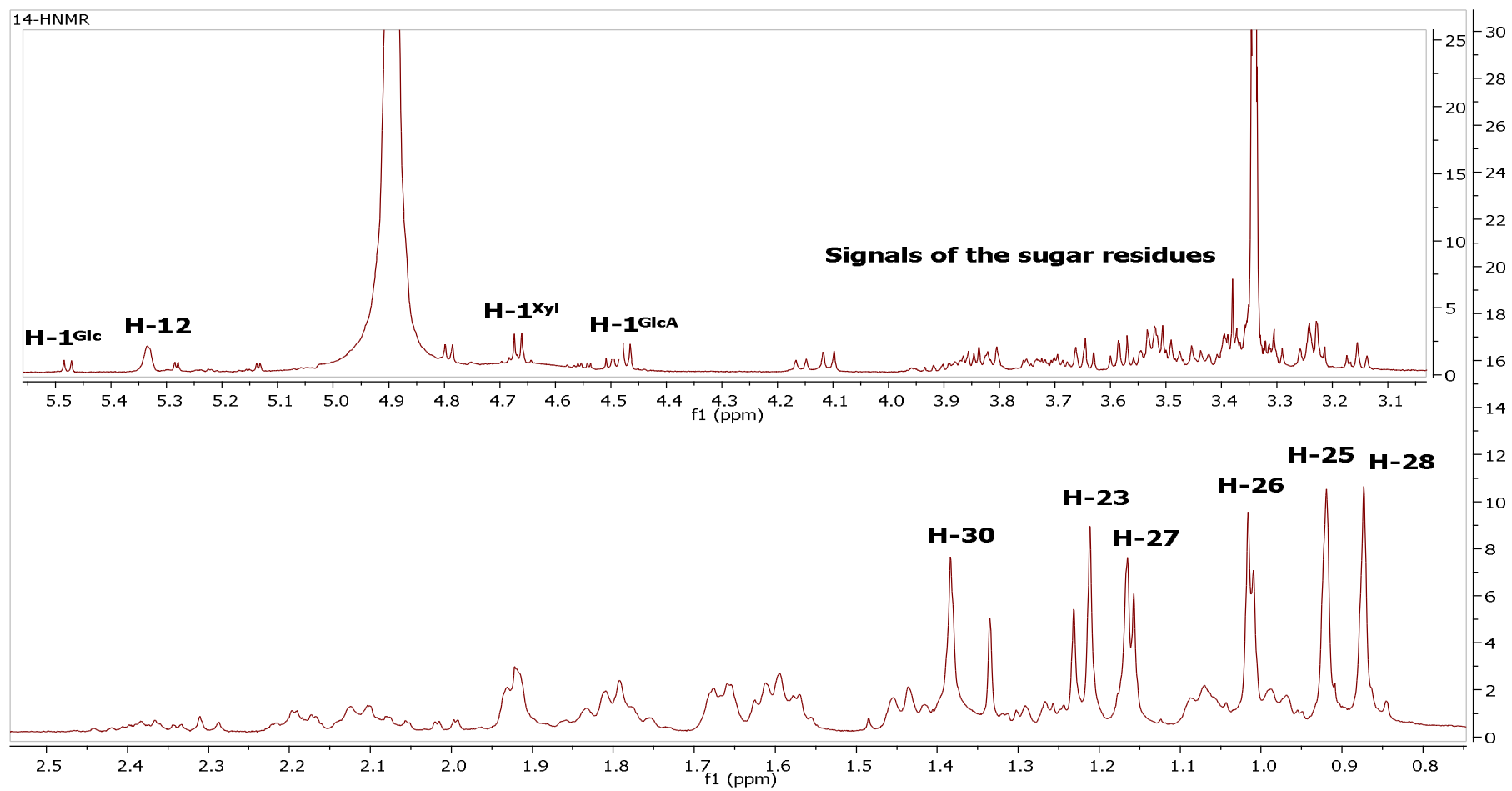


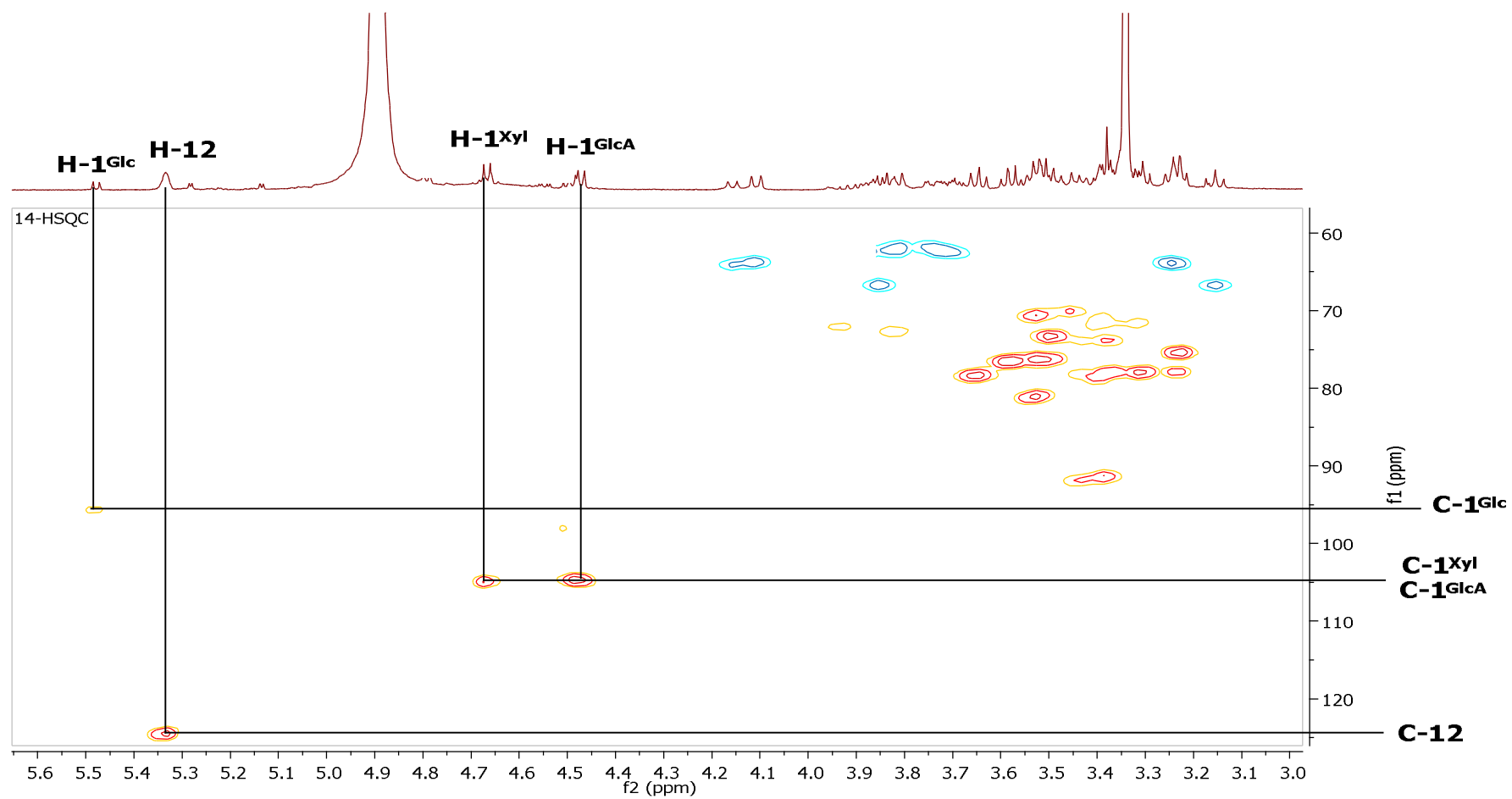
Figure 3.18. Structure of ATA14

Table 3.18. ^1H and ^{13}C Assignments of ATA14 (150/600 MHz, δ ppm, in CD_3OD)

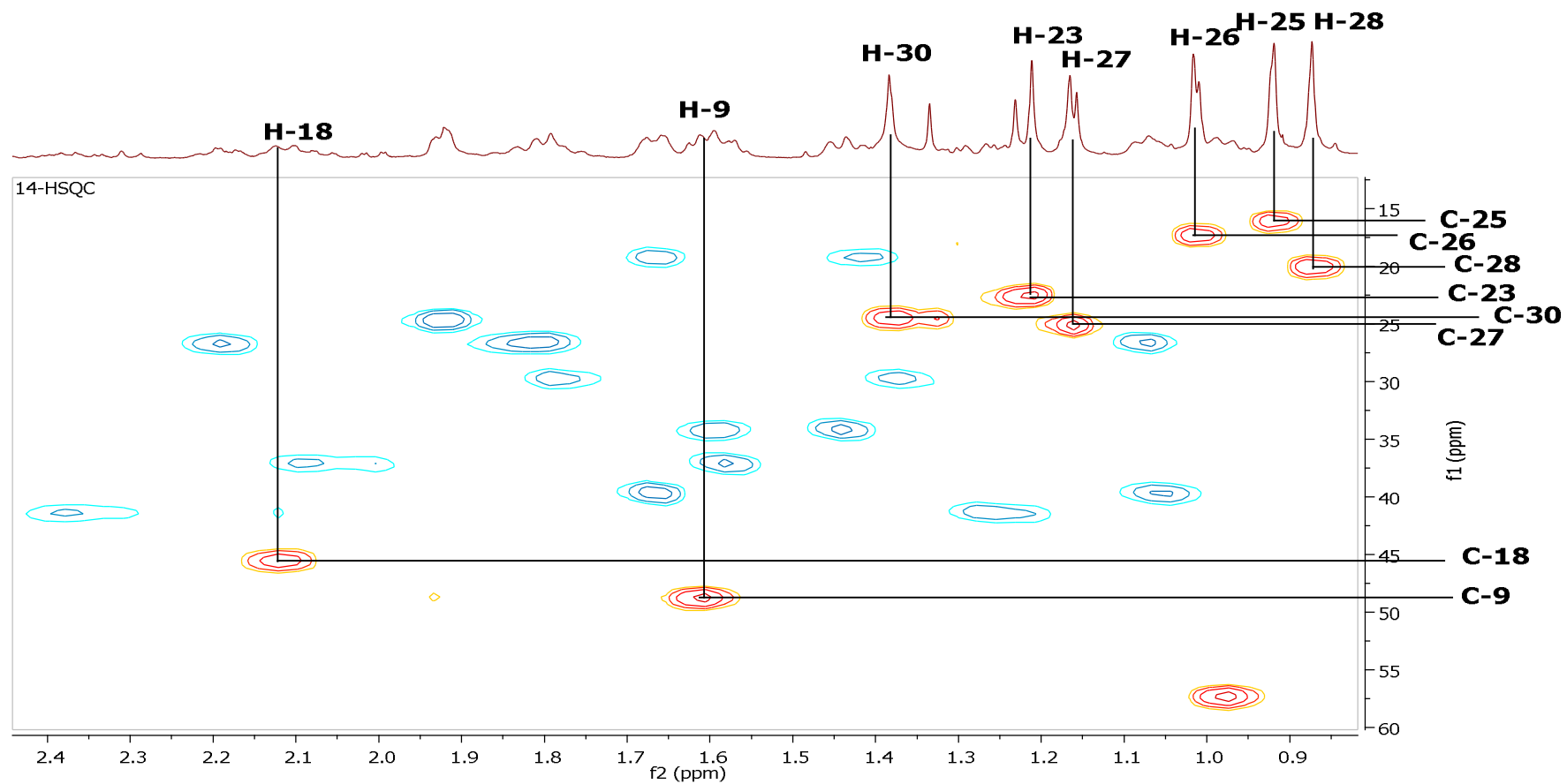
Position	δ_{C}	δ_{H} (J in Hz)
1	39.3	1.64, 1.05, m
2	26.6	2.21, 1.84, m
3	91.3	3.32, dd (11.5, 4.5)
4	44.4	-
5	57.0	0.957, m
6	19.3	1.64, 1.42, m
7	34.1	1.61, 1.44, m
8	40.5	-
9	48.6	1.60, m
10	37.0	-
11	24.6	1.90, (2H) m
12	124.1	5.31, t (3.5)
13	144.3	-
14	43.3	-
15	26.5	1.82, 1.09, m
16	29.6	1.78, 1.38, m
17	38.3	-
18	45.4	2.11, m
19	41.1	2.36, 1.28, m
20	42.6	-
21	36.6	2.06, 1.58, m
22	76.1	3.51, brs
23	22.8	1.25, s
24	63.4	4.11, d (11.5), 3.21, d (11.5)
25	16.1	0.90, s
26	17.0	1.00, s
27	25.1	1.16, s
28	19.6	0.85, s
29	178.2	-
30	24.0	1.36, s
$\beta\text{-D-GlcA (at C-3)-1'}$	105.1	4.44, d (7.5)
2'	78.3	3.59, dd (7.5, 9.0)
3''	78.4	3.64, dd (9.0, 9.0)
4'	73.9	3.43, dd (9.0, 9.0)
5''	76.6	3.58, d (9.0)
6''	177.1	-
$\beta\text{-D-Xyl (at C-2}_{\text{glcA}}\text{)-1''}$	104.8	4.67, d (7.5)
2''	75.3	3.23, dd (7.5, 9.2)
3''	76.0	3.52, t (9.2)
4''	70.5	3.52, m
5''	66.6	3.85, dd (5.2, 11.7), 3.16, t (11.7)
$\beta\text{-D-Glc (at C-29)-1'''}$	95.6	5.47, d (7.5)
2'''	73.7	3.38, dd (7.5, 9.0)
3'''	77.7	3.38, t (9.0)
4'''	71.5	3.31, t (9.0)
5'''	77.9	3.23, ddd (3.5, 4.5, 9.0)
6'''	61.8	3.81, dd(3.5, 12), 3.71, dd (4.5, 12)



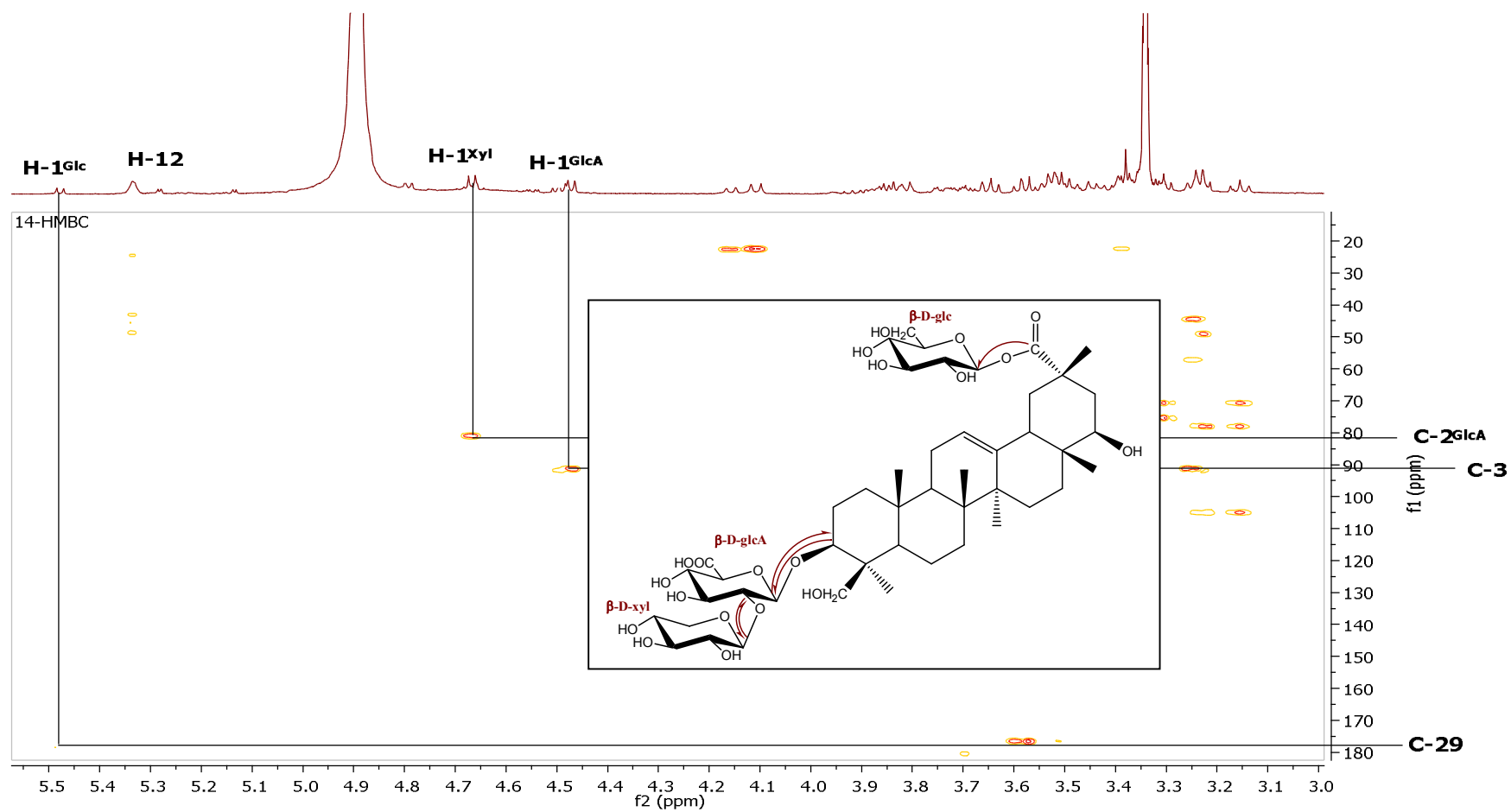
Spectrum 3.86. ^1H NMR spectrum of ATA14 (600 MHz, CD_3OD)



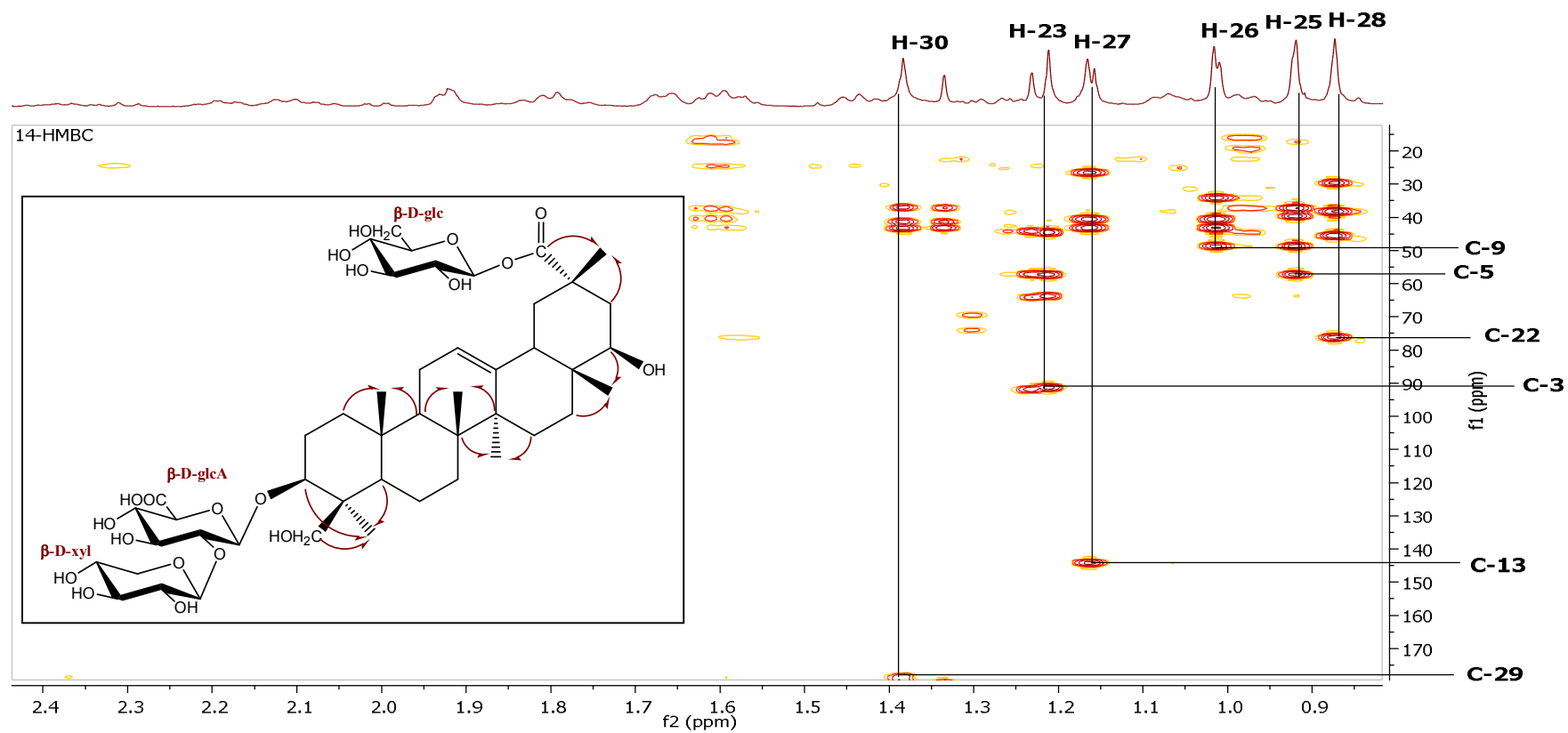
Spectrum 3.87. HSQC Spectrum of ATA14



Spectrum 3.88. HSQC Spectrum of ATA14



Spectrum 3.89. HMBC Spectrum of ATA14



Spectrum 3.90. HMBC Spectrum of ATA14

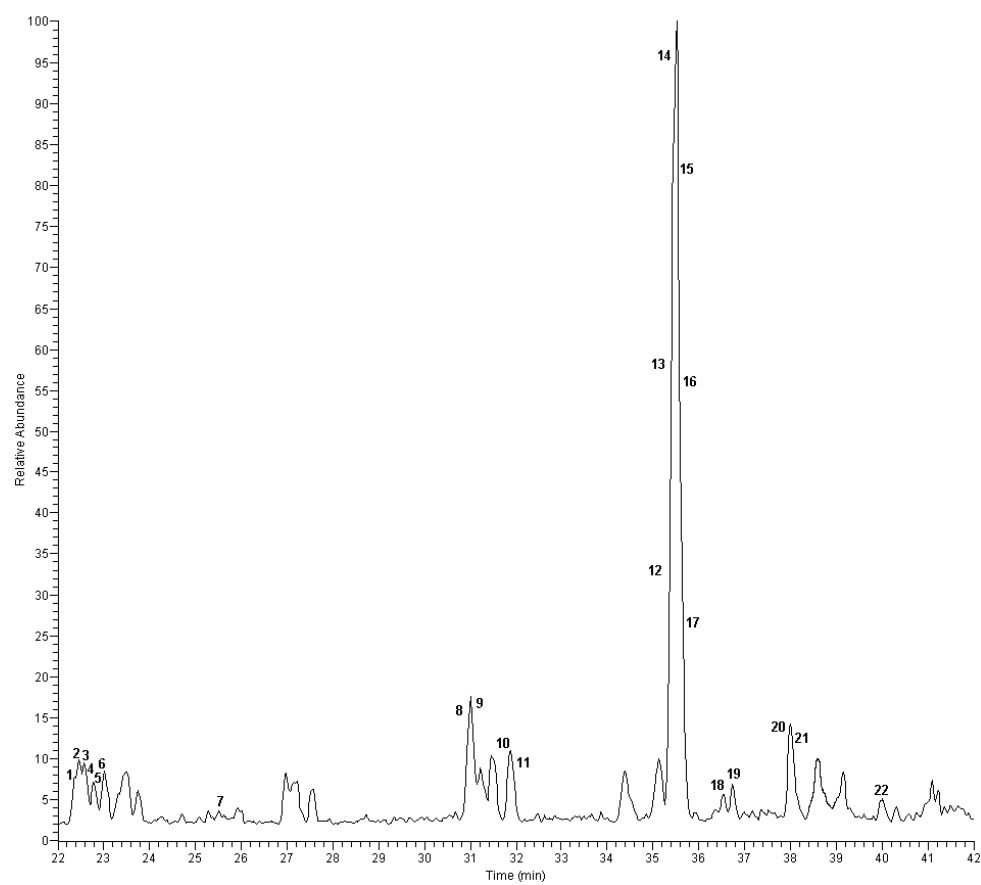


Figure. 3.19. HPLC-ESIMS profile of the butanol extract of *A. tauricolus* expanded in the time range from 22 to 42 min (oleanane-type saponin region)

Table 3.19. ESIMS and ESIMSⁿ Product Ions of Compounds ATA2-ATA7, ATA9, ATA11-ATA12, ATA14 Isolated from *A. tauricolus*.

Compound	MW	ESIMS	Product ions
ATA2	1104	1103 [M-H] ⁻	ESIMS ² 941 [(M-162)-H] ⁻
ATA3	1120	1119 [M-H] ⁻	ESIMS ³ of 941 923 [(M-162-18)-H] ⁻ , 879 [(M-162-18-44)-H] ⁻ ESIMS ² 1101 [(M-18)-H] ⁻ , 1057 [(M-18-44)-H] ⁻ , 973 [(M-146)-H] ⁻ , 911 [(M-18-44-146)-H] ⁻ , 793 [(M-146-180)-H] ⁻ , 749 [(M-18-44-146-162)-H] ⁻ , 635 [(M-18-44-146-162-114)-H] ⁻ ESIMS ³ of 1101 1057 [(M-18-44)-H] ⁻ , 955 [(M-18-146)-H] ⁻ , 911 [(M-18-44-146)-H] ⁻ , 775 [(M-18-146-180)-H] ⁻ , 749 [(M-18-44-146-162)-H] ⁻ , 587 [(M-18-44-146-162-162)-H] ⁻ , 473 [(M-18-44-146-162-162-114)-H] ⁻
ATA4	1074	1073 [M-H] ⁻	ESIMS ² 1055 [(M-18)-H] ⁻ , 1011 [(M-18-44)-H] ⁻ , 927 [(M-146)-H] ⁻ , 865 [(M-18-44-146)-H] ⁻ , 777 [(M-146-150)-H] ⁻ , 733 [(M-18-44-146-132)-H] ⁻ , 619 [(M-18-44-146-132-114)-H] ⁻ , 473 [(M-18-44-146-132-114-146)-H] ⁻
ATA5	1104	1103 [M-H] ⁻	ESIMS ² 1085 [(M-18)-H] ⁻ , 1041 [(M-18-44)-H] ⁻ , 957 [(M-146)-H] ⁻ , 895 [(M-18-44-146)-H] ⁻ , 733 [(M-18-44-146-162)-H] ⁻ , 619 [(M-18-44-146-162-114)-H] ⁻ , 473 [(M-18-44-146-162-114-146)-H] ⁻
ATA6	1134	1133 [M-H] ⁻	ESIMS ² 971 [(M-162)-H] ⁻ ESIMS ³ of 971 953 [(M-162-18)-H] ⁻ , 909 [(M-162-18-44)-H] ⁻ , 763 [(M-162-18-44-146)-H] ⁻ , 601 [(M-162-18-44-146-162)-H] ⁻ , 487 [(M-162-18-44-146-162-114)-H] ⁻
ATA7	1058	1057 [M-H] ⁻	ESIMS ² 1039 [(M-18)-H] ⁻ , 995 [(M-18-44)-H] ⁻ , 849 [(M-18-44-146)-H] ⁻ , 729 [(M-18-146-146-18)-H] ⁻ , 579 [(M-18-146-146-18-150)-H] ⁻
ATA9	970	969 [M-H] ⁻	ESIMS ² 951 [(M-18)-H] ⁻ , 907 [(M-18-44)-H] ⁻ , 823 [(M-146)-H] ⁻ , 761 [(M-18-44-146)-H] ⁻ , 643 [(M-146-180)-H] ⁻ , 599 [(M-18-44-146-162)-H] ⁻ , 485 [(M-18-44-146-162-114)-H] ⁻ , 467 [(M-146-180-176)-H] ⁻
ATA11	974	973 [M-H] ⁻	ESIMS ² 955 [(M-18)-H] ⁻ , 911 [(M-18-44)-H] ⁻ , 827 [(M-146)-H] ⁻ , 765 [(M-18-44-146)-H] ⁻ , 647 [(M-146-180)-H] ⁻ , 603 [(M-18-44-146-162)-H] ⁻ , 489 [(M-18-44-146-162-114)-H] ⁻
ATA12	988	987 [M-H] ⁻	ESIMS ² 969 [(M-18)-H] ⁻ , 923 [(M-18-44)-H] ⁻ , 825 [(M-162)-H] ⁻ , 763 [(M-18-44-162)-H] ⁻ , 649 [(M-18-44-162-114)-H] ⁻ , 469 [(M-18-44-162-114-180)-H] ⁻
ATA14	958	957 [M-H] ⁻	ESIMS ² 795 [(M-162)-H] ⁻ ESIMS ³ of 795 751 [(M-162-44)-H] ⁻ , 733 [(M-162-44-18)-H] ⁻ , 645 [(M-162-150)-H] ⁻ , 601 [(M-162-44-18-132)-H] ⁻ , 469 [(M-162-150-176)-H] ⁻

Additionally, twelve known oleanane-type glycosides, astrojanoside A (**ATA1**) (Bedir et al., 1999a), melilotus-saponin O2 (**ATA8**) (Hirakawa et al., 2000), 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-3 β ,21 β ,22 α ,24,29-pentahydroxyolean-12-ene (Gülcemal et al., 2012) (**ATA10**), azukisaponin V (**ATA13**) (Kitawaga et al., 1983a), wistariasaponin B2 (**ATA15**) (Konoshima et al., 1989), astragaloside VIII (**ATA16**) (Kitawaga et al., 1983b), wistariasaponin B1 (**ATA17**) (Konoshima et al., 1989), cloversaponin IV (**ATA18**) (Sakamoto et al., 1992), azukisaponin II (**ATA19**) (Kitawaga et al., 1983c), wistariasaponin D (**ATA20**) (Konoshima et al., 1991), dehydroazukisaponin V (**ATA21**) (Mohamed et al., 1995), and 3-*O*- β -D-glucuronopyranosyl-soyasapogenin B (**ATA22**) (Udayama et al., 1998) were isolated.

In conclusion, the proposed HPLC-ESIMSⁿ method resulted to be a prompt and effective tool to obtain a preliminary online profile of saponins in the butanol extract of *A. tauricolus* (**Fig. 3.19**). To the best of our knowledge, an HPLC-ESIMSⁿ approach has only been used to characterize astragalosides in *Radix Astragali* (Xu et al., 2007; Zu et al., 2009; Chu et al., 2010) but no HPLC-ESIMSⁿ study has been addressed to oleanane-type triterpene saponins in *Astragalus* spp.

Noteworthy, the phytochemical investigation of *A. tauricolus* shows only oleanane-type triterpene glycosides and no cycloartane-type glycosides, the main constituents of most *Astragalus* spp., sharing this peculiar feature with a limited group of *Astragalus* spp. (*A. hamosus*, *A. complanatus*, *A. sinicus*, and *A. corniculatus*) (Ionkova, 1991; Cui et al., 1992a, b; Krasteva et al., 2006, 2007).

The antiproliferative activity of compounds **ATA1–ATA12**, **ATA15**, **ATA17–ATA19** was tested in different cancer cell lines including human breast cancer (MCF-7), human lung adenocarcinoma (A549), human prostate cancer (PC-3) and human leukemia (U937) cell lines. The antiproliferative activity of the isolated compounds **ATA1–ATA12**, **ATA15**, **ATA17–ATA19** was evaluated against a small panel of cancer cell lines. Only compound **ATA11** showed an IC₅₀ of 22 μ M against human leukemia cell line (U937). The other tested compounds, in a range of concentrations between 1 and 50 μ M, did not cause any significant reduction of the cell number.

3.2.11. Acid Hydrolysis of compounds of *Astragalus tauricolus*

The configuration of sugar units was established after hydrolysis of **ATA2-ATA7**, **ATA9**, **ATA11**, **ATA12** and **ATA14** with 1 N HCl, trimethylsilation and determination of the retention times by GC operating in the experimental conditions previously reported by De Marino et al., 2003.

The peaks of D-glucuronic acid (15.83 min), D-glucose (14.74 min), D-xylose (10.98 and 12.01 min), and L-rhamnose (10.72 min) were detected in the hydrolysate of **ATA2**.

Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine were detected at 15.81 min (D-glucuronic acid), 14.71 min (D-glucose), 10.98 and 12.00 min (D-xylose) and 10.70 (L-rhamnose).

3.3. *Astragalus angustifolius* Lam. subsp. *angustifolius* var. *angustifolius*

3.3.1. Structural Identification of Compound AAN1

A detailed comparison of the NMR data of the sugar region (^1H , ^{13}C , 1D-TOCSY, HSQC, HMBC, DQF-COSY) and ESIMS data of compounds **AAN1-AAN4** showed that the disaccharide chain was identical in the four compounds. In particular, the ^1H NMR spectrum of compound **AAN1** showed for the sugar portion two anomeric proton doublets at δ 4.44 ($J = 7.5$ Hz), 5.42 ($J = 1.2$ Hz). In the HSQC spectrum, these protons correlated to carbons at δ 105.7 and 102.0, respectively (**Table 3.20**). Complete assignments of the ^1H and ^{13}C NMR signals of the sugar portion were accomplished by 1D-TOCSY, HSQC, HMBC and DQF-COSY experiments which allowed us to unambiguously assign all proton sugar signals and to identify one α -rhamnopyranosyl (δ 5.42) unit and one β -glucopyranosyl (δ 4.44) unit. The site of glycosidation on the aglycone of **AAN1** as well as the position of the interglycosidic linkage were determined by HMBC experiment, which showed long-range correlations between the anomeric proton signal at δ 4.44 (H-1_{glc}) and the carbon resonance at δ 89.9 (C-3), and between the anomeric proton signal at δ 5.42 (H-1_{rha}) and the carbon resonance at δ 79.7 (C-2_{glc}). The configuration of glucose and rhamnose units was established as D for glucose unit and L for rhamnose unit after hydrolysis of **AAN1** with 1 N HCl, trimethylsilation and determination of retention time by GC.[De Marino et al., 2003] Thus, the sugar sequence of compounds **AAN1-AAN4** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl].

The HRMALDITOF mass spectrum of **AAN1** (m/z 879.4722 [$\text{M}+\text{Na}$] $^+$, calcd for $\text{C}_{44}\text{H}_{72}\text{O}_{16}\text{Na}$, 879.4718) supported a molecular formula of $\text{C}_{44}\text{H}_{72}\text{O}_{16}$. The ESIMS mass spectrum showed the major ion peak at m/z 879 which was assigned to [$\text{M}+\text{Na}$] $^+$. The MS/MS of this ion showed peaks at m/z 803 [$\text{M}+\text{Na}-76$] $^+$, due to the loss of a hydroxyacetate molecule, at m/z 657 [$\text{M}+\text{Na}-76-146$] $^+$, corresponding to the loss of a deoxyhexose unit and at m/z 477 [$\text{M}+\text{Na}-76-146-180$] $^+$, ascribable to the loss of a hexose unit.

The ^1H NMR spectrum showed for the aglycone moiety signals due to a cyclopropane methylene at δ 0.58 (brs) and 0.39 (d, $J = 4.8$ Hz), seven tertiary methyl groups at δ 1.36, 1.33 (6H), 1.16, 1.14, 1.06, 1.05, four methine proton signals at δ 5.49 (ddd, $J = 8.0, 8.0, 5.6$ Hz), 3.77 (dd, $J = 8.2, 6.0$ Hz), 3.48 (ddd, J

= 9.7, 9.7, 4.5 Hz) and 3.27 (dd, $J = 11.3, 4.0$ Hz), indicative of secondary alcoholic functions, and signals at δ 4.14 and 4.07 (each, br s) corresponding to a primary alcoholic group (**Table 3.20**). On the basis of DQF-COSY, HSQC and HMBC spectra and by comparison of these data with those of cycloastragenol [Kitagawa et al., 1983a], it was observed that the aglycone of compound **AAN1** differed from cycloastragenol only by the presence of a $-\text{COCH}_2\text{OH}$ group. The HMBC correlation between the proton signal at δ 5.49 (H-16) and the carbon resonance at δ 175.7 ($-\text{COCH}_2\text{OH}$) confirmed the location of the $-\text{COCH}_2\text{OH}$ group at C-16. Thus full assignment of the ^1H and ^{13}C signals of the aglycone moiety of **AAN1**, based on DQF-COSY, HSQC and HMBC spectra, allowed us to identify the aglycone of **AAN1** as 16-*O*-hydroxyacetox-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane.

Consequently, the structure of compound **AAN1** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-16-*O*-hydroxyacetox-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**Fig. 3.20**).

Amorphous white solid; $\text{C}_{44}\text{H}_{72}\text{O}_{16}$; $[\alpha]_{\text{D}}^{25} +19.01$ (c 0.1 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3470 ($>\text{OH}$), 3040 (cyclopropane ring), 2950 ($>\text{CH}$), 1260 and 1058 (C-O-C); HRMALDITOFMS m/z $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{44}\text{H}_{72}\text{O}_{16}\text{Na}$, 879.4718, found 879.4722; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data see **Table 3.20**.

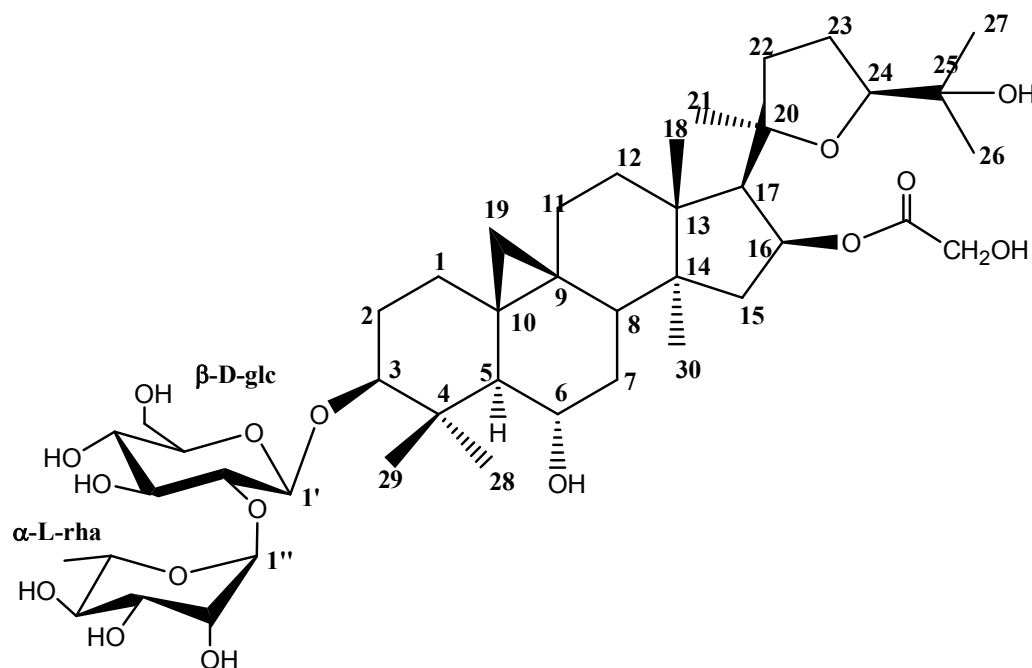
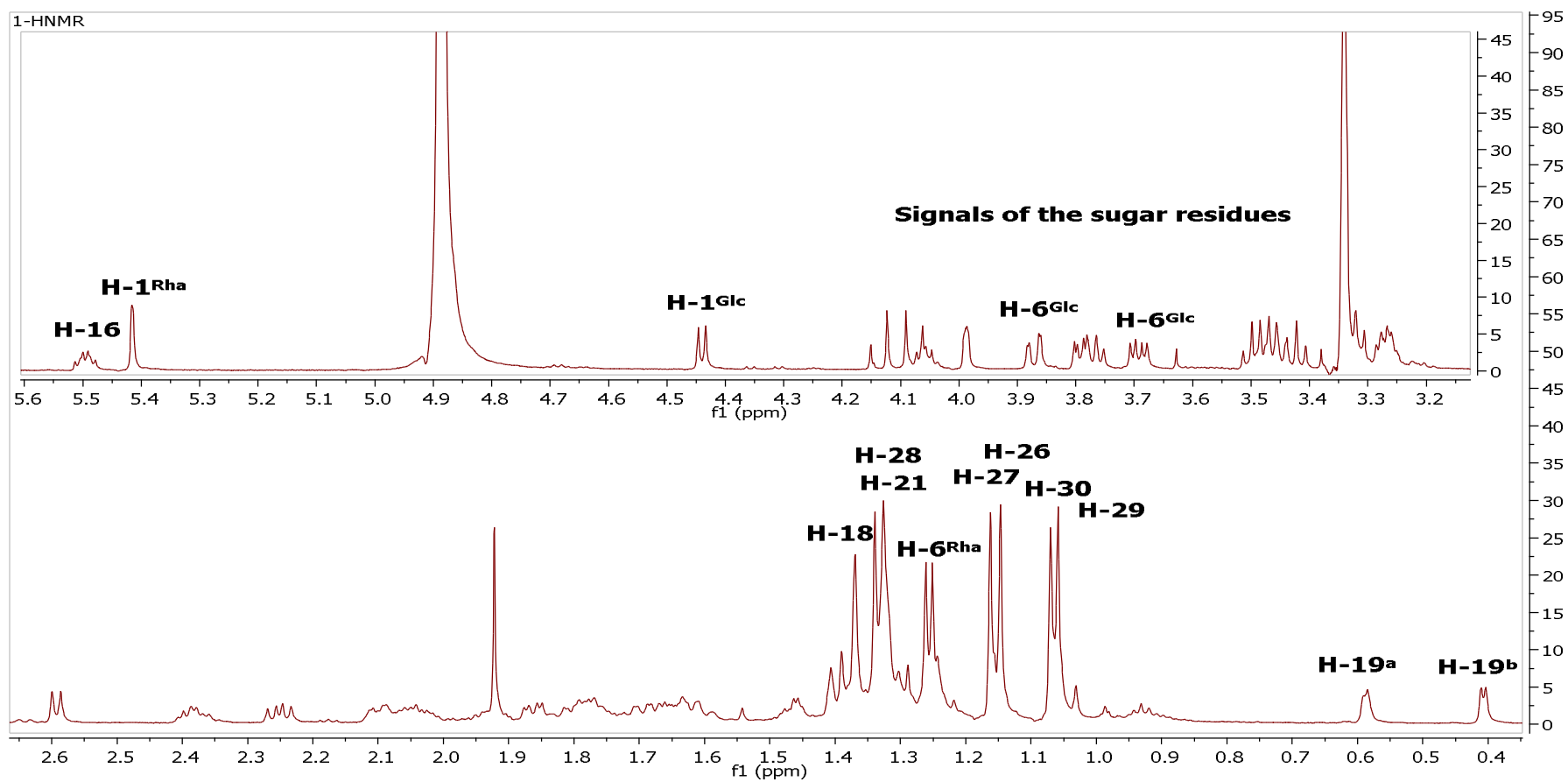


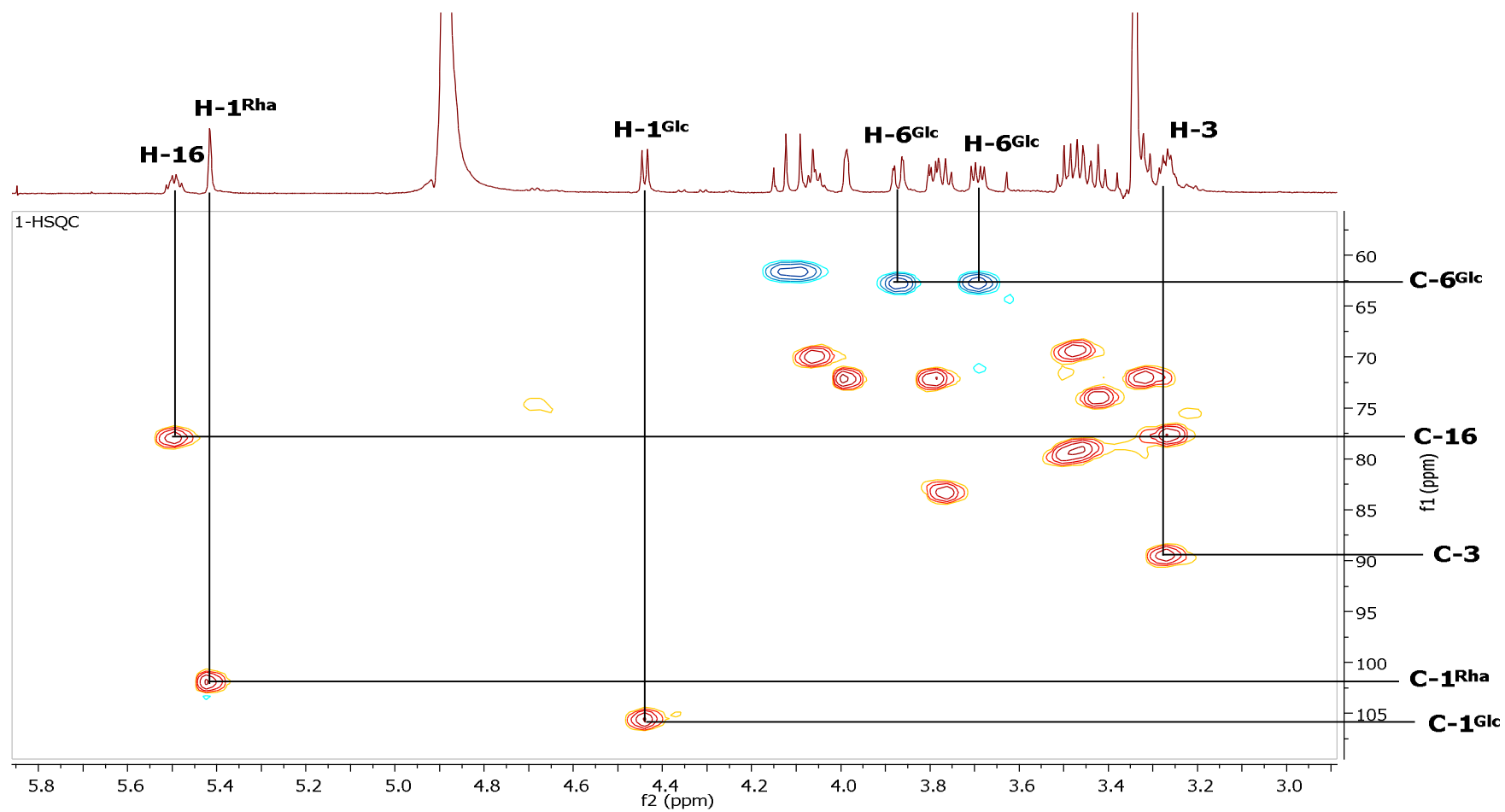
Figure 3.20. Structure of **AAN1**

Table 3.20. ^1H and ^{13}C Assignments of AAN1 (150/600 MHz, δ ppm, in CD_3OD)

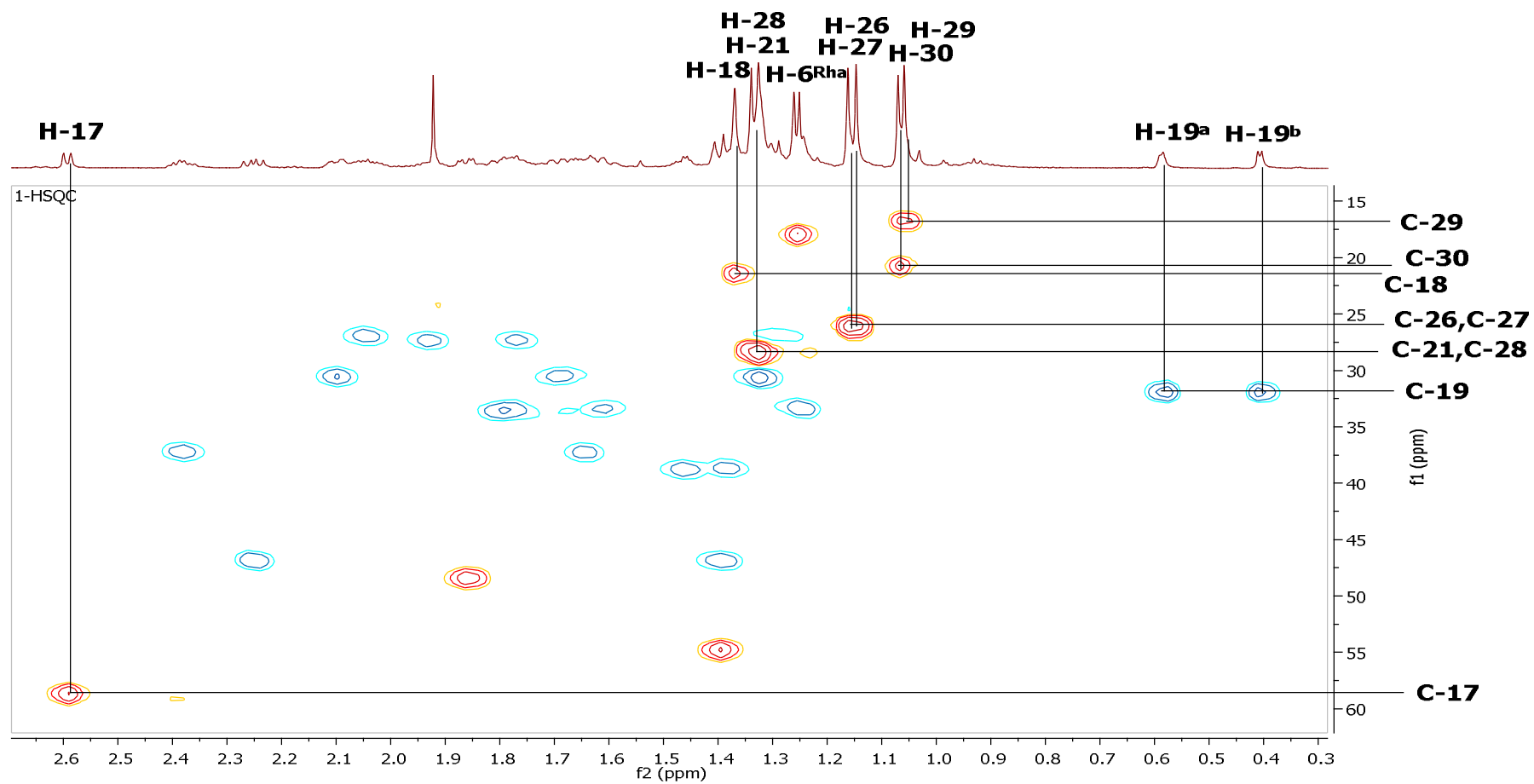
Position	δ_{C}	δ_{H} (J in Hz)
1	32.9	1.60, 1.24, m
2	30.5	2.10, 1.69, m
3	89.9	3.27, dd (11.3, 4.0)
4	42.9	-
5	54.6	1.39, d (9.7)
6	69.2	3.48, ddd (9.7, 9.7, 4.5)
7	38.6	1.47, 1.37, m
8	48.4	1.86, dd (11.3, 4.0)
9	21.2	-
10	29.9	-
11	26.8	2.05, 1.24, m
12	33.0	1.79 (2H) m
13	46.9	-
14	47.4	-
15	46.9	2.25, dd (13.7, 8), 1.38 dd (12.9, 5.6)
16	77.9	5.49, ddd (8.0, 8.0, 5.6)
17	58.4	2.59, d (8.0)
18	21.1	1.36, s
19	31.8	0.58, brs, 0.39, d (4.8)
20	86.8	-
21	28.4	1.33, s
22	37.0	2.38, 1.64, m
23	26.8	2.05, (2H) m
24	83.3	3.77 dd (8.2, 6.0)
25	72.3	-
26	25.9	1.14, s
27	25.9	1.16, s
28	28.6	1.33, s
29	16.4	1.05, s
30	20.7	1.06, s
COCH ₂ OH	175.7	-
COCH ₂ OH	61.7	4.14, 4.07, brs
β -D-Glc (at C-3)-1'	105.7	4.44, d (7.5)
2'	79.7	3.45, dd (7.5, 9.0)
3'	79.7	3.48, t (9.0)
4'	72.2	3.31, t (9.0)
5'	77.7	3.26, ddd (3.5, 4.5, 9.0)
6'	62.6	3.88, dd(3.5, 12), 3.69, dd (4.5, 12)
α -L-Rha (at C-2 _{glc})-1''	102.0	5.42, d (1.2)
2''	72.0	3.99, dd (1.2, 3.2)
3''	72.0	3.79, dd (3.2, 9.7)
4''	74.2	3.42, t (9.7)
5''	70.0	4.01, m
6''	17.6	1.23, d (6.5)



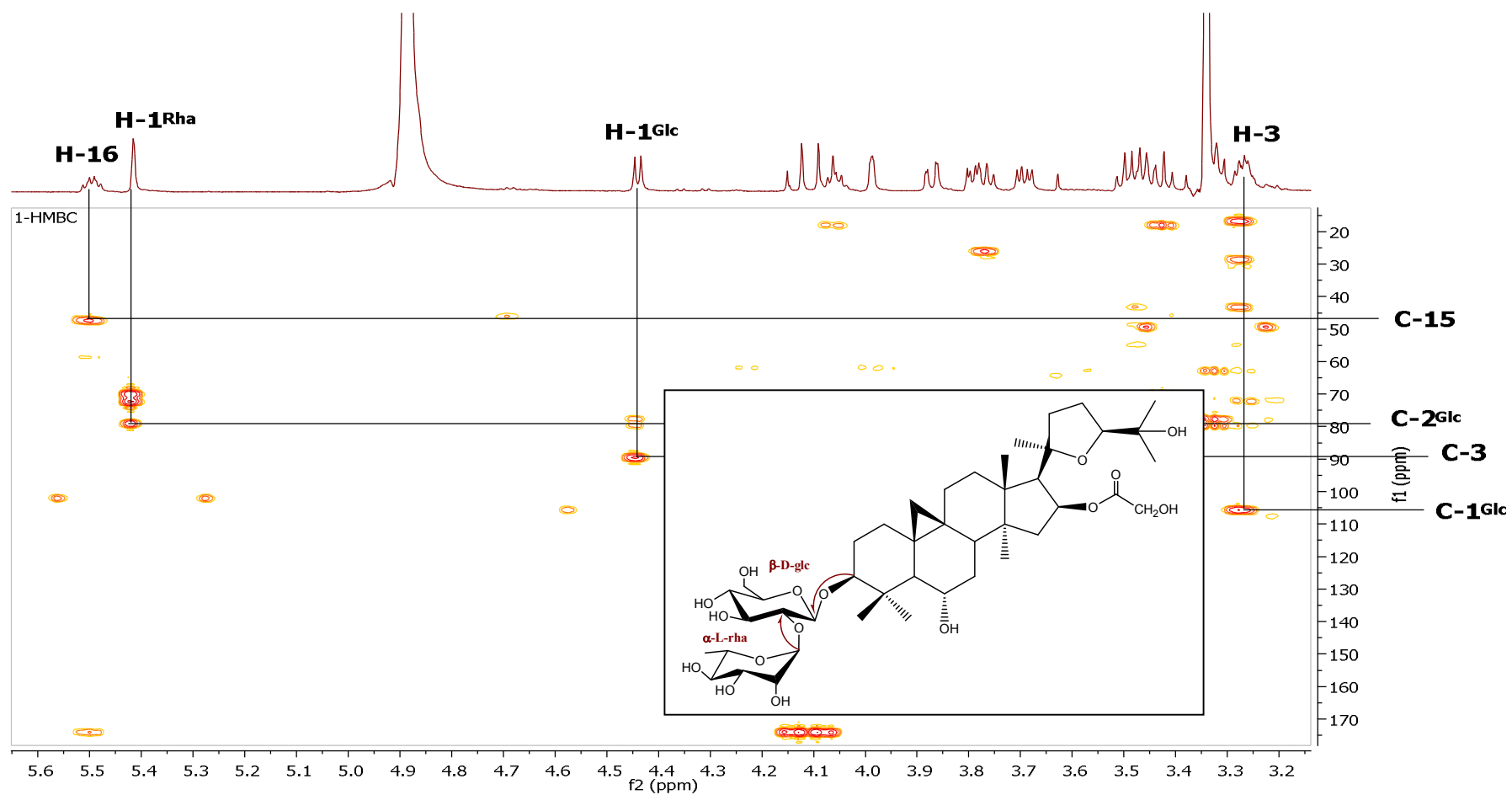
Spectrum 3.91. ^1H NMR spectrum of AAN1 (600 MHz, CD_3OD)



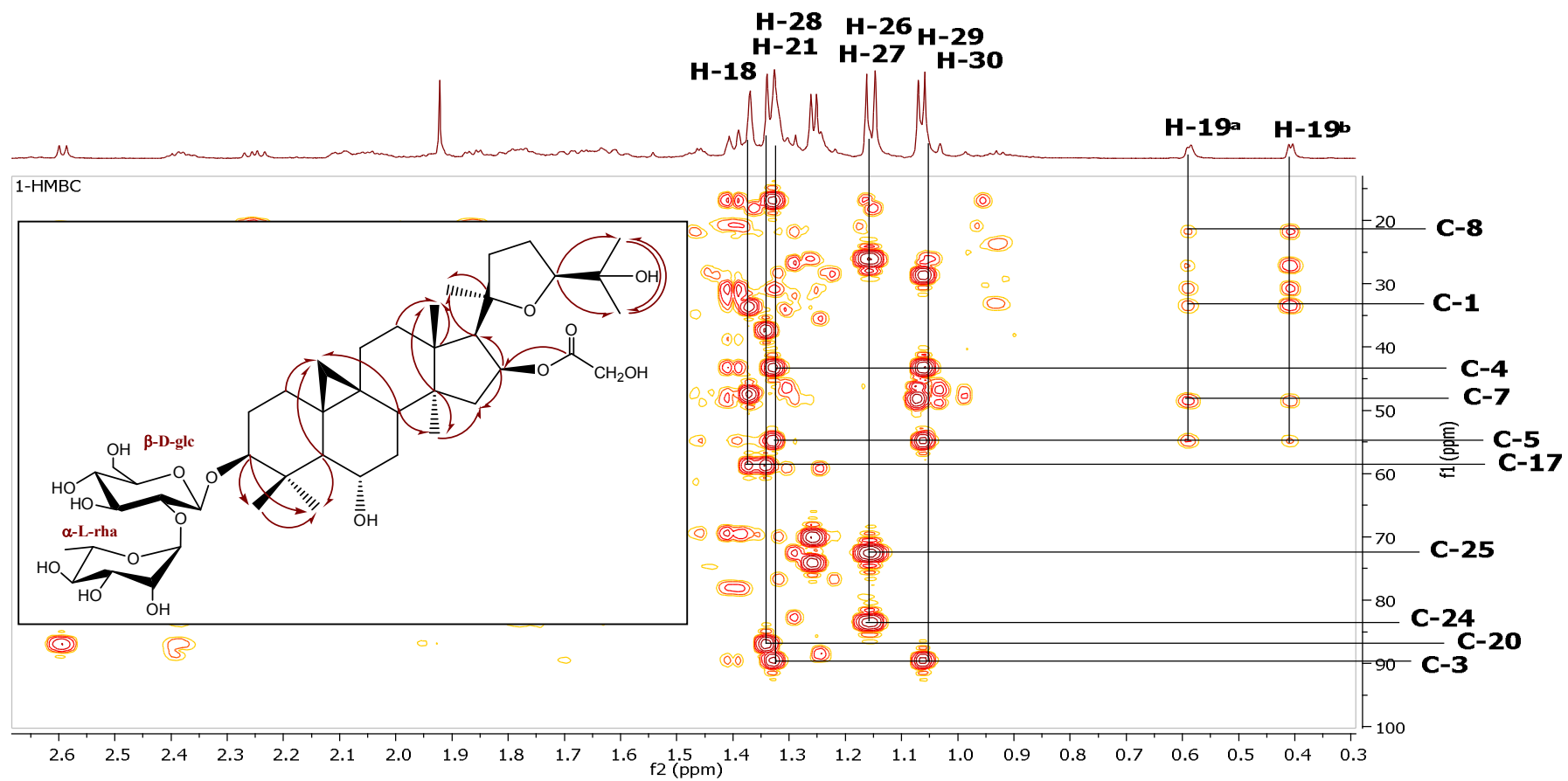
Spectrum 3.92. HSQC Spectrum of AAN1



Spectrum 3.93. HSQC Spectrum of AAN1



Spectrum 3.94. HMBC Spectrum of AAN1



Spectrum 3.95. HMBC Spectrum of AAN1

3.3.2. Structural Identification of Compound AAN2

The molecular formula of compound **AAN2**, isolated as an amorphous powder, was established as $C_{44}H_{72}O_{17}$ by HRMALDITOF mass spectrum (m/z 895.4669 $[M+Na]^+$, calcd for $C_{44}H_{72}O_{17}Na$, 895.4667). The positive ESIMS spectrum showed the sodiated ion peak at m/z 895 $[M+Na]^+$. Its MS/MS fragmentation showed peaks at m/z 819 $[M+Na-76]^+$, due to the loss of a hydroxyacetate molecule, at m/z 673 $[M+Na-76-146]^+$, corresponding to the loss of a deoxyhexose unit and at m/z 493 $[M+Na-76-146-180]^+$, due to the loss of a hexose unit.

The ^1H and ^{13}C NMR spectroscopic data of aglycone portion of **AAN2** were similar to those of **AAN1** except for the presence of signals due to an additional secondary alcoholic function (δ 4.47, ddd, $J = 8.8, 6.0, 3.5$ Hz; δ_C 74.5) (**Table 3.21**). In the HMBC spectrum cross-peaks between the proton signals at δ 4.47, 1.14 (H-26) and δ 1.16 (H-27) with the carbon at δ 72.6 (C-25) allowed us to locate this additional secondary alcoholic function at C-23. The α -orientation of hydroxyl group on C-23 was established by comparison with literature values (Çalış et al., 2008b). Therefore, the structure of compound **2** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-16-*O*-hydroxyacetoxo-3 β ,6 α ,16 β ,23 α ,25-pentahydroxy-20(*R*),24(*S*)-epoxycycloartane (**Fig. 3.21**).

Amorphous white solid; $C_{44}H_{72}O_{17}$; $[\alpha]_D^{25} +41.4$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3480 ($>OH$), 3035 (cyclopropane ring), 2945 ($>CH$), 1260 and 1055 (C-O-C); ESI-MS m/z 895 $[M+Na]^+$, MS/MS m/z 819 $[M+Na-76]^+$, m/z 673 $[M+Na-76-146]^+$, m/z 493 $[M+Na-76-146-180]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{44}H_{72}O_{17}Na$, 895.4667, found 895.4669; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.21**.

Table 3.21. ^1H and ^{13}C Assignments of AAN2 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	33.0	1.58, 1.23, m
2	30.3	2.10, 1.69, m
3	90.7	3.58, dd (12.2, 3.5)
4	43.7	-
5	55.0	1.39, d (9.7)
6	69.5	3.47, ddd (9.7, 9.7, 4.5)
7	38.5	1.50, 1.37, m
8	49.0	1.83, dd (13.1, 3.5)
9	22.1	-
10	29.4	-
11	26.7	2.04, 1.24, m
12	33.6	1.80, (2H) m
13	47.3	-
14	47.7	-
15	46.8	1.98, dd (12.3, 8.0), 1.39, dd (12.3, 5.6)
16	78.2	5.49, ddd (8.0, 8.0, 5.3)
17	59.0	2.33, d (8.0)
18	21.2	1.35, s
19	32.0	0.59, brs, 0.41, d (4.8)
20	86.4	-
21	28.5	1.48, s
22	47.7	3.09, 1.67, m
23	74.5	4.47, ddd (8.8, 6.0, 3.5)
24	89.3	3.57, d, (6.0)
25	72.6	-
26	25.7	1.19, s
27	27.8	1.29, s
28	28.0	1.29, s
29	16.5	1.05, s
30	20.2	1.01, s
COCH ₂ OH	177.0	-
COCH ₂ OH	61.3	4.15, 4.08, brs
β -D-Glc (at C-3)-1'	105.5	4.45, d (7.5)
2''	79.6	3.45, dd (7.5, 9.0)
3''	79.6	3.48, t (9.0)
4''	72.2	3.31, t (9.0)
5''	77.6	3.26, ddd (3.5, 4.5, 9.0)
6''	62.7	3.88, dd (3.5, 12), 3.69, dd (4.5, 12)
α -L-Rha (at C-2 _{glc})-1''	102.0	5.43, d (1.2)
2''	72.3	3.99, dd (1.2, 3.2)
3''	72.3	3.79, dd (3.2, 9.7)
4''	74.1	3.42, t (9.7)
5''	70.0	4.07, m
6''	17.8	1.25, d (6.5)

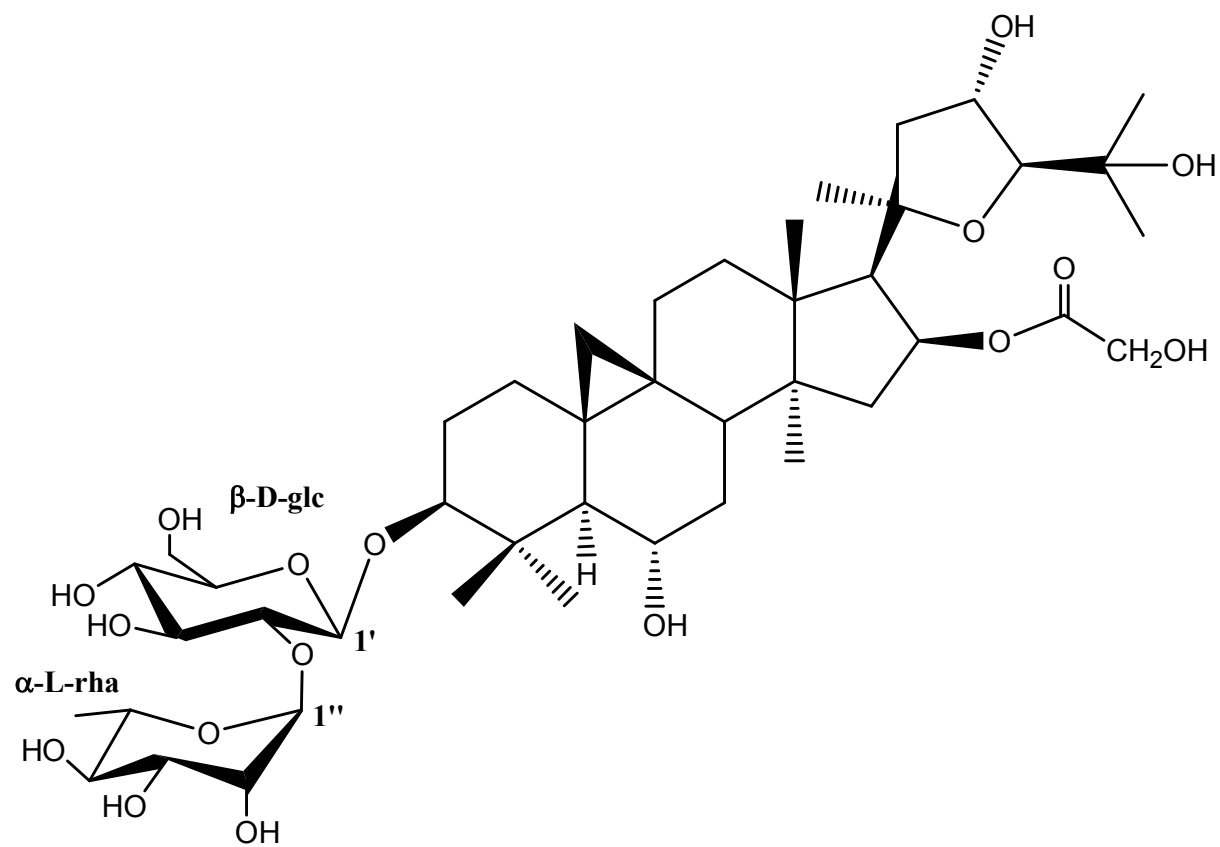
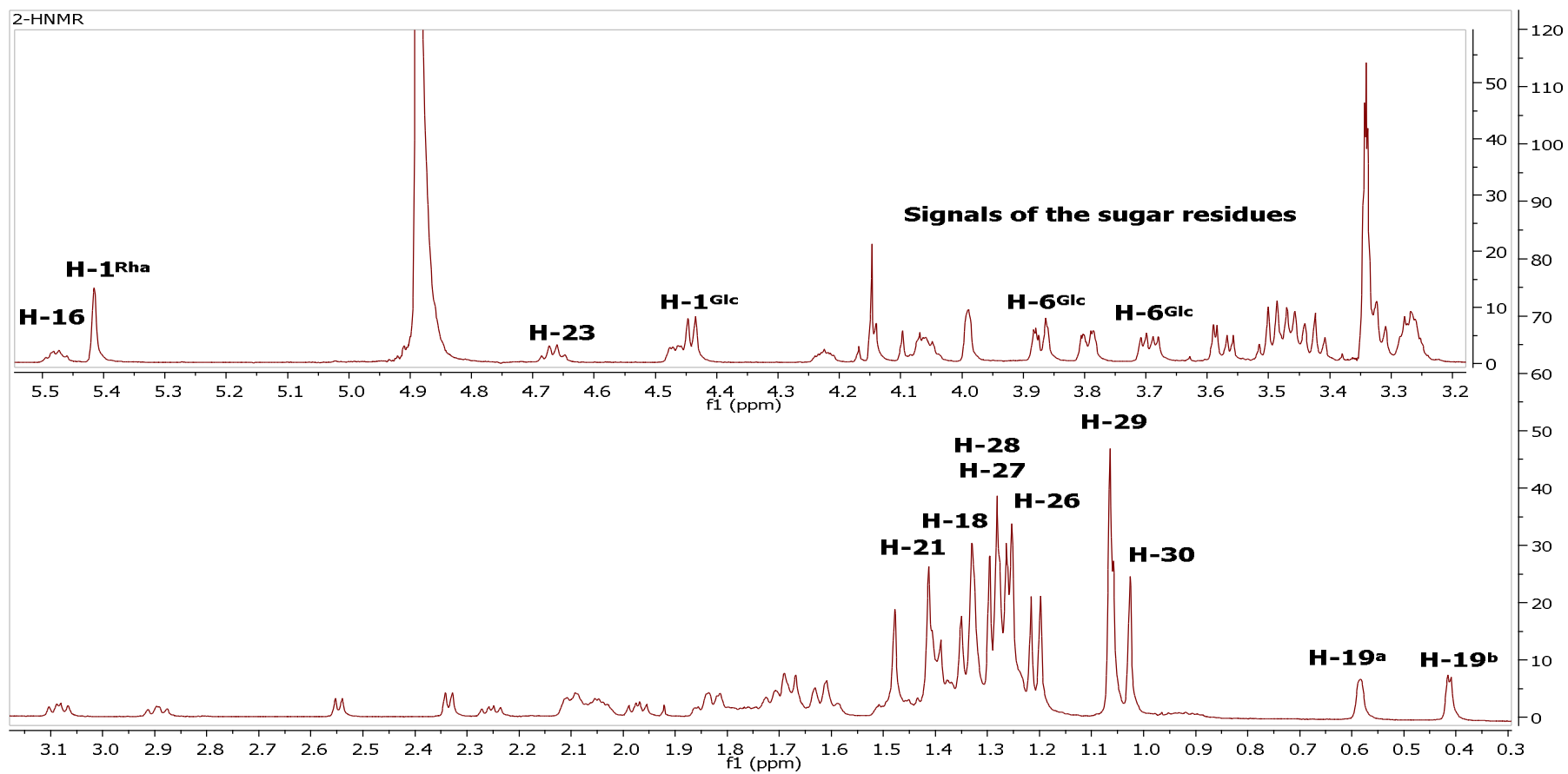
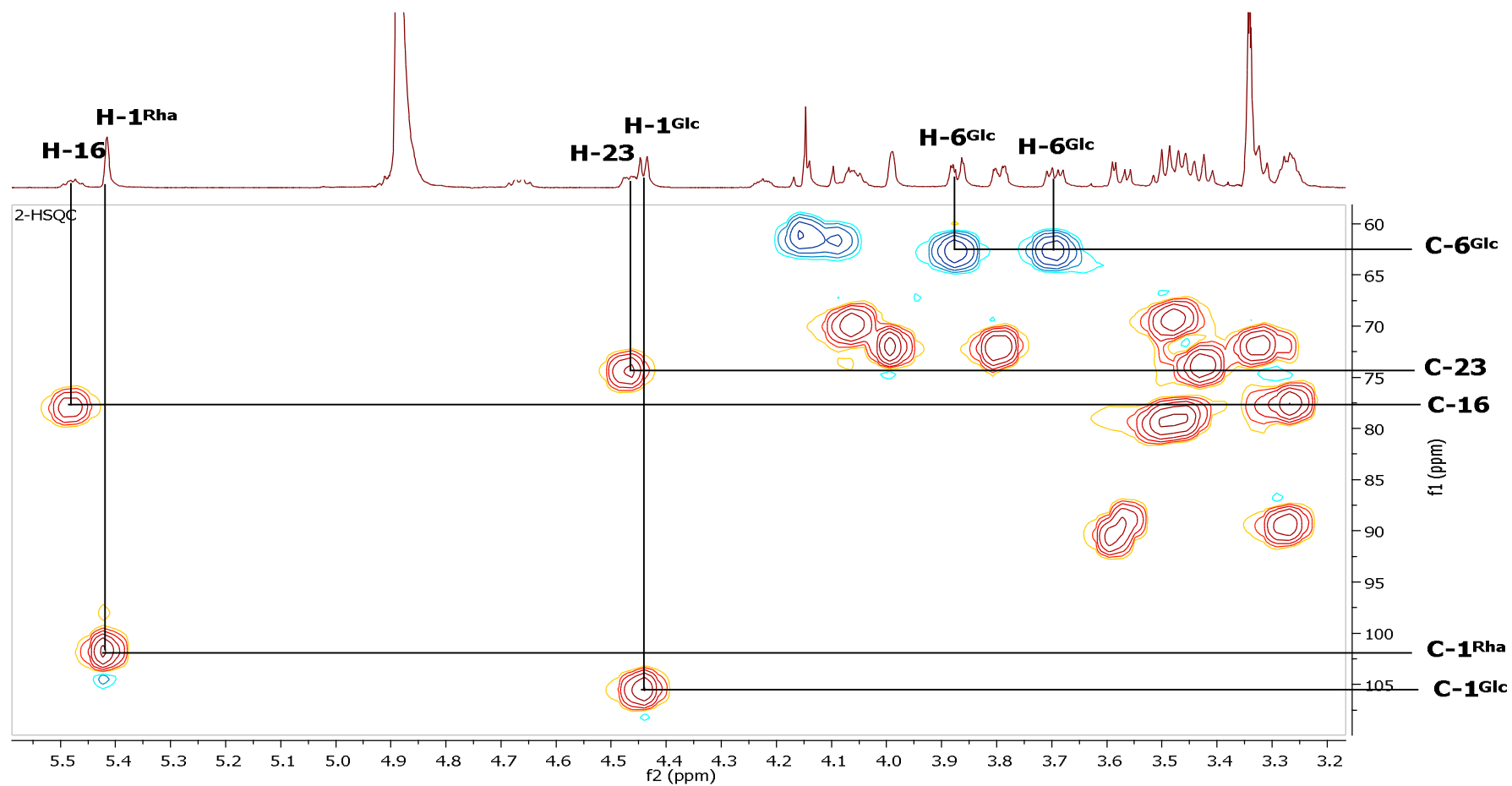


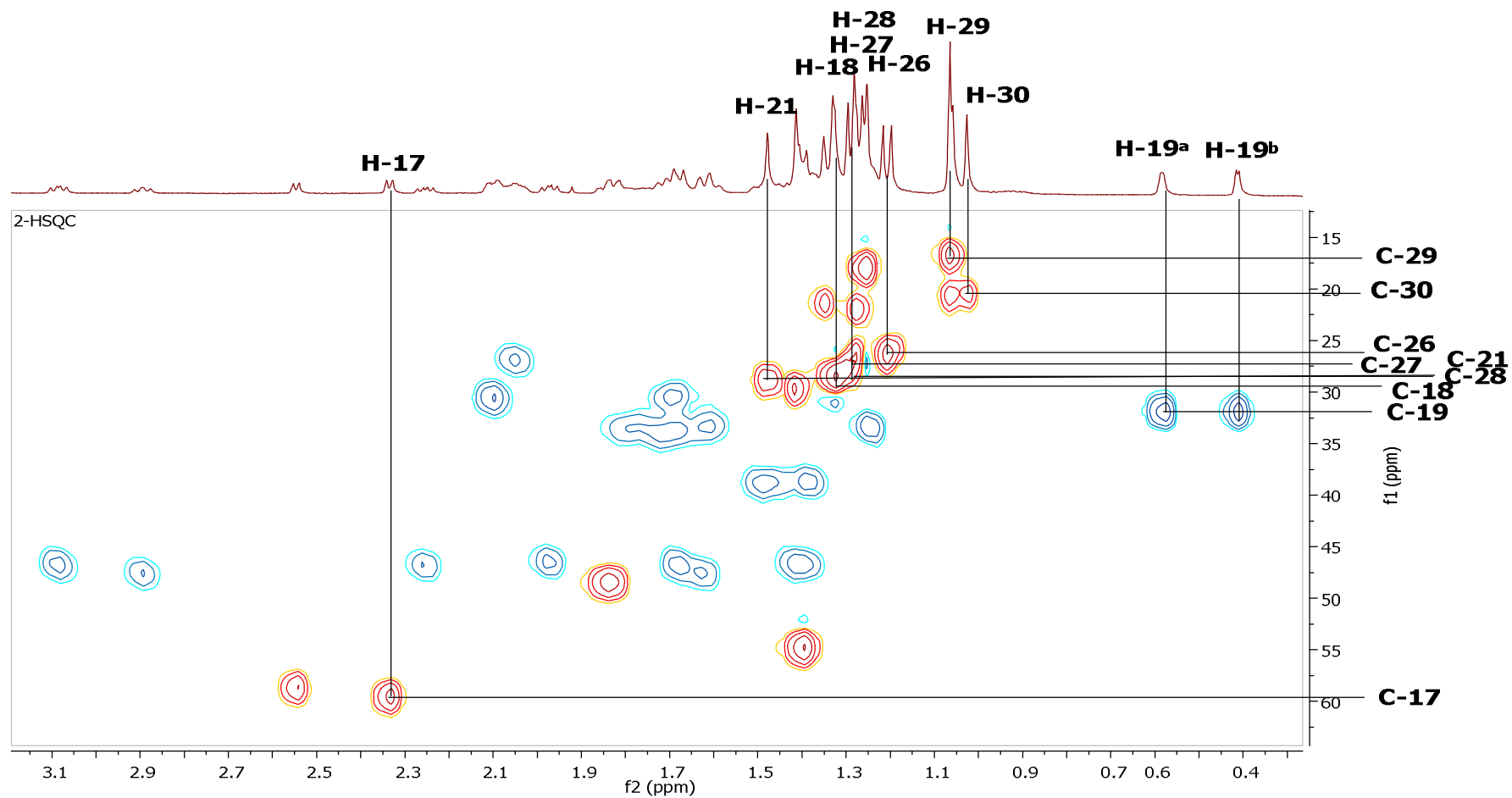
Figure 3.21. Structure of AAN2



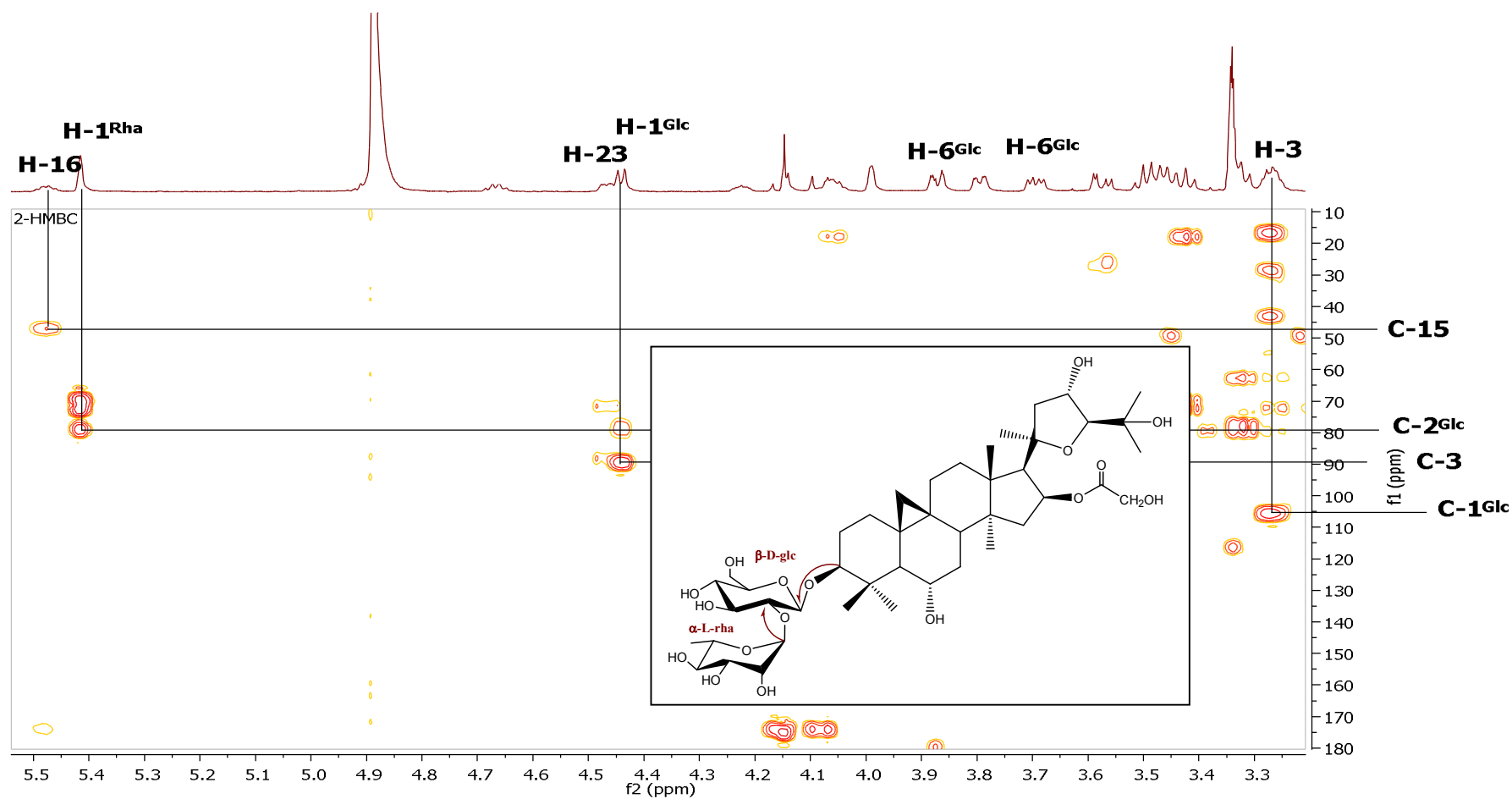
Spectrum 3.96. ^1H NMR spectrum of AAN2 (600 MHz, CD_3OD)



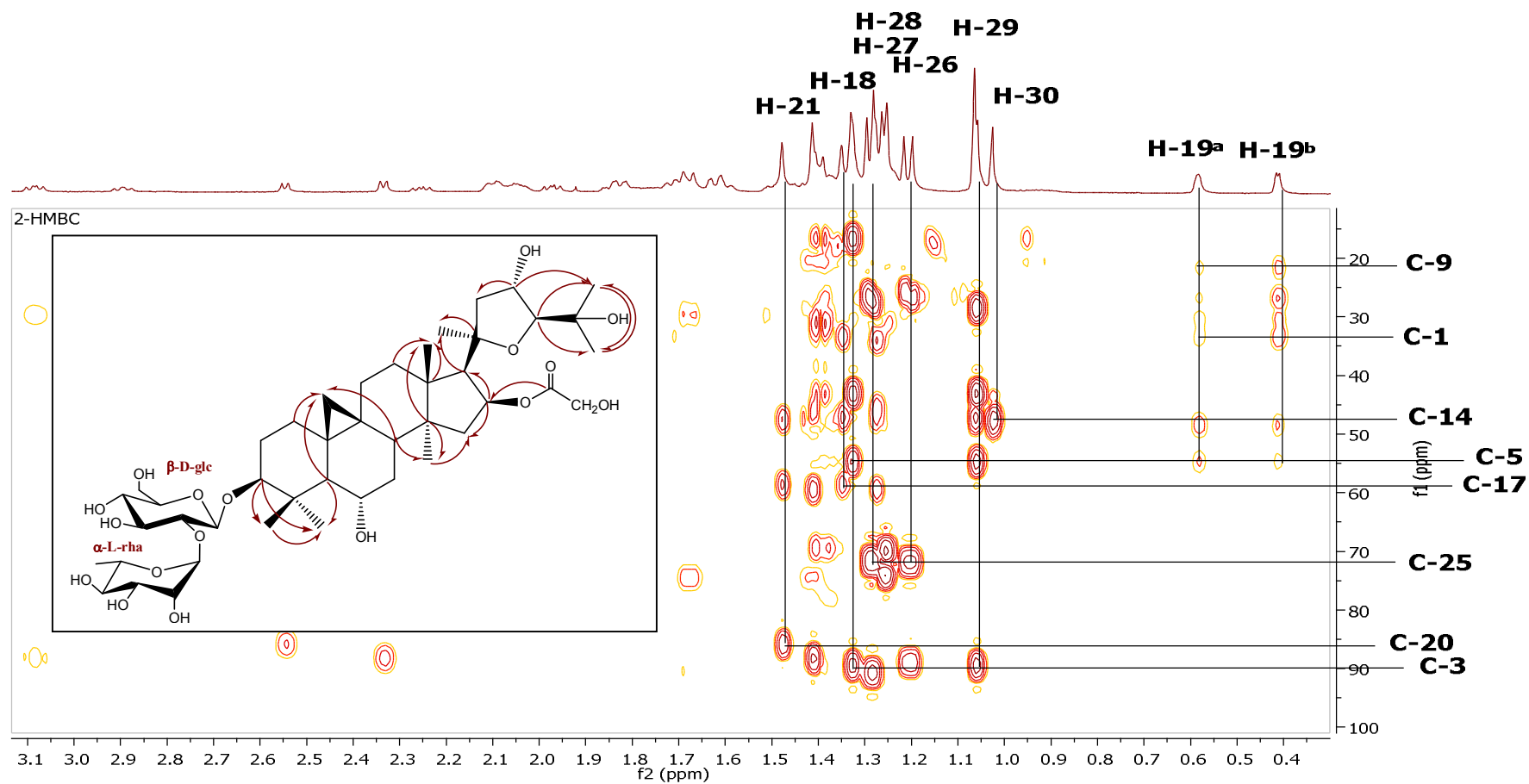
Spectrum 3.97. HSQC Spectrum of AAN2



Spectrum 3.98. HSQC Spectrum of AAN2



Spectrum 3.99. HMBC Spectrum of AAN2



Spectrum 3.100. HMBC Spectrum of AAN2

3.3.3. Structural Identification of Compound AAN3

Compound **AAN3** was isolated as an amorphous powder. The HRMALDITOF mass spectrum of **AAN3** showed a major ion peak at m/z 819.4512 $[M+Na]^+$ ascribable to the molecular formula $C_{42}H_{68}O_{14}$ (calc. for $C_{42}H_{68}O_{14}Na$, 819.4507). The positive ESIMS spectrum showed the sodiated ion peak at m/z 819 $[M+Na]^+$. Its MS/MS fragmentation showed peaks at m/z 673 $[M+Na-146]^+$, due to the loss of a deoxy-hexose unit, and at m/z 493 $[M+Na-146-180]^+$, corresponding to the loss of a hexose unit.

The NMR spectroscopic data of the aglycone portion of **AAN3** resembled that of cycloastragenol (Kitawaga et al., 1983d) except for the absence of a secondary alcoholic function. Instead, the typical resonance of a keto group (δ 222.2) was observed in the ^{13}C NMR spectrum (**Table 3.22**). The carbon resonance signals of D ring and Me-18 in **AAN3** suggested that the keto group was located at C-16. This hypothesis was confirmed by the HMBC experiment, which showed diagnostic long-range correlations between the proton signal at δ 1.28 (Me-18) and the carbon value at δ 66.9 (C-17), and between the proton signals at δ 2.98 (H-17) and 2.05 (H-15 α and H-15 β) with the carbon resonance at δ 222.2. From this evidence, in combination with the data of the sugar moiety above reported, the structure of **AAN3** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-3 β ,6 α ,25-trihydroxy-20(*R*),24(*S*)-epoxycycloartane-16-one (**Fig. 3.22**).

Amorphous white solid; $C_{42}H_{68}O_{14}$; $[\alpha]_D^{25}$ -23.1 (*c* 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3475 (>OH), 3030 (cyclopropane ring), 2948 (>CH), 1740 (C=O), 1258 and 1050 (C-O-C); ESI-MS m/z 819 $[M+Na]^+$, MS/MS m/z 673 $[M+Na-146]^+$, m/z 493 $[M+Na-146-180]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{42}H_{68}O_{14}Na$, 819.4507, found 819.4512; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.22**.

Table 3.22. ^1H and ^{13}C Assignments of AAN3 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	33.2	1.64, 1.27, m
2	30.3	2.12, 1.71, m
3	89.7	3.29, dd (11.3, 4.0)
4	42.9	-
5	54.7	1.44, d (9.7)
6	69.1	3.51, ddd (9.7, 9.7, 3.2)
7	38.7	1.50, 1.40, m
8	47.3	1.93, dd (12.0, 4.0)
9	21.0	-
10	30.0	-
11	26.5	2.05, 1.24, m
12	32.9	1.80, 1.70, m
13	46.6	-
14	43.0	-
15	51.8	2.05, brs
16	222.2	-
17	66.9	2.98, s
18	20.4	1.28, s
19	31.4	0.60, brs, 0.41, d (4.4)
20	86.3	-
21	25.0	1.25
22	35.9	2.40, 1.78, m
23	27.0	2.05, (2H) m
24	83.2	3.79, dd (10.5, 5.2)
25	72.5	-
26	25.5	1.13, s
27	27.7	1.24, s
28	28.1	1.33, s
29	16.6	1.06, s
30	19.8	1.21, s
$\beta\text{-D-Glc (at C-3)-1'}$	105.8	4.44, d (7.5)
2'	79.2	3.46, dd (7.5, 9.0)
3'	79.6	3.49, t (9.0)
4'	72.1	3.32, t (9.0)
5'	77.8	3.27, ddd (3.5, 4.5, 9.0)
6'	62.8	3.88, dd(3.5, 12), 3.69, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1''}$	102.2	5.42, d (1.2)
2''	72.1	4.00, dd (1.2, 3.2)
3''	72.1	3.79, dd (3.2, 9.7)
4''	74.0	3.42, t (9.7)
5''	70.0	4.06, m
6''	17.6	1.26, d (6.5)

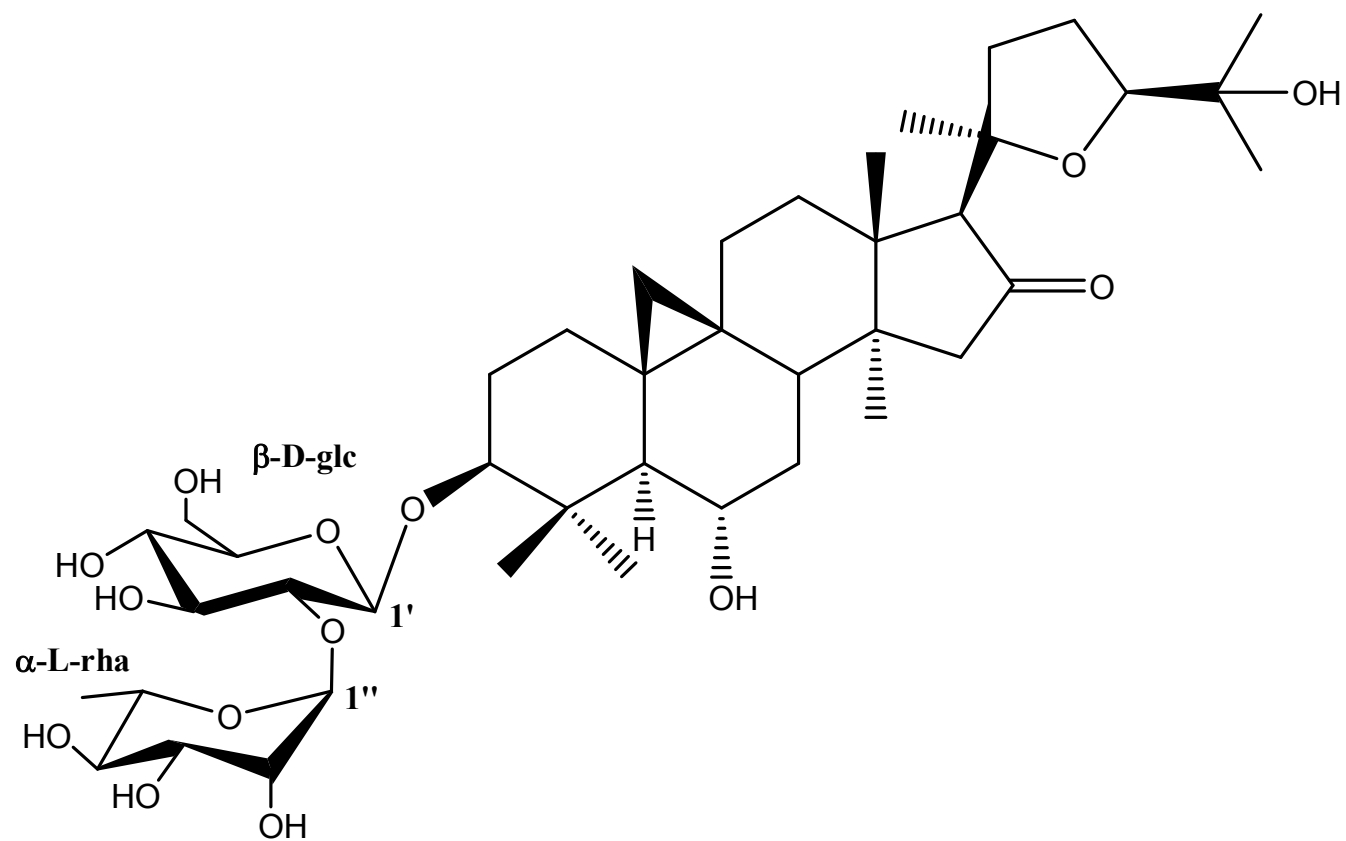
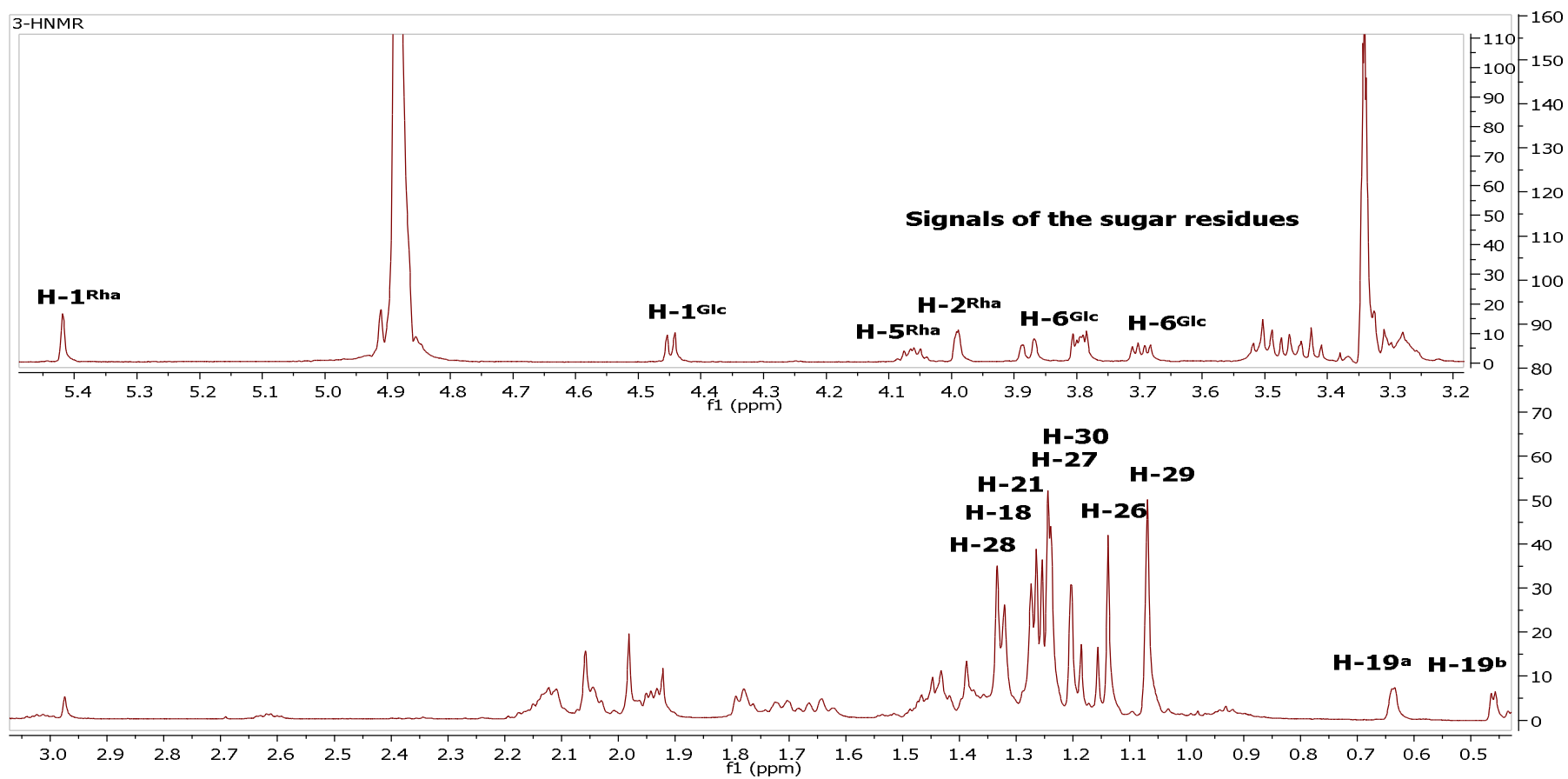
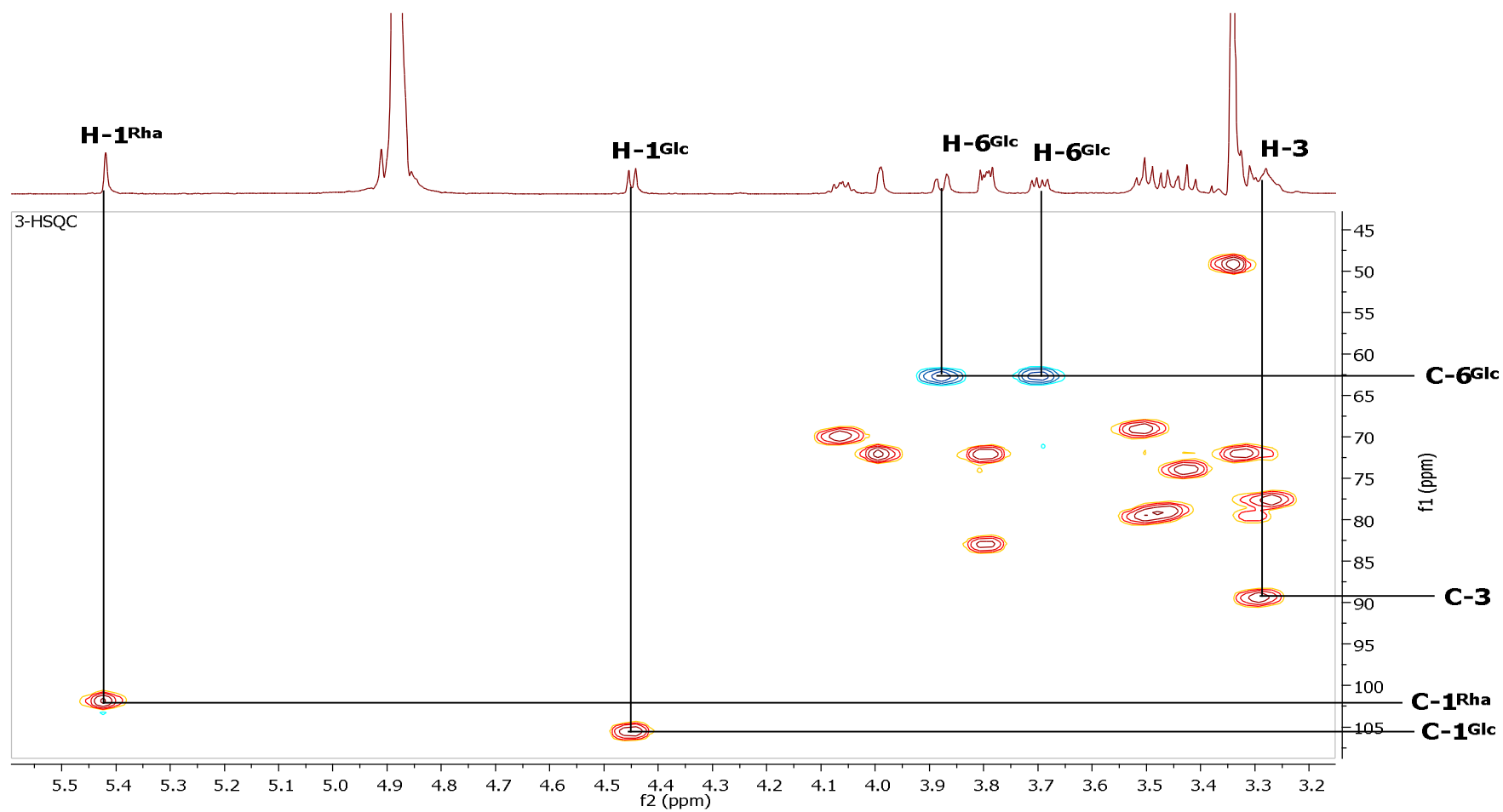


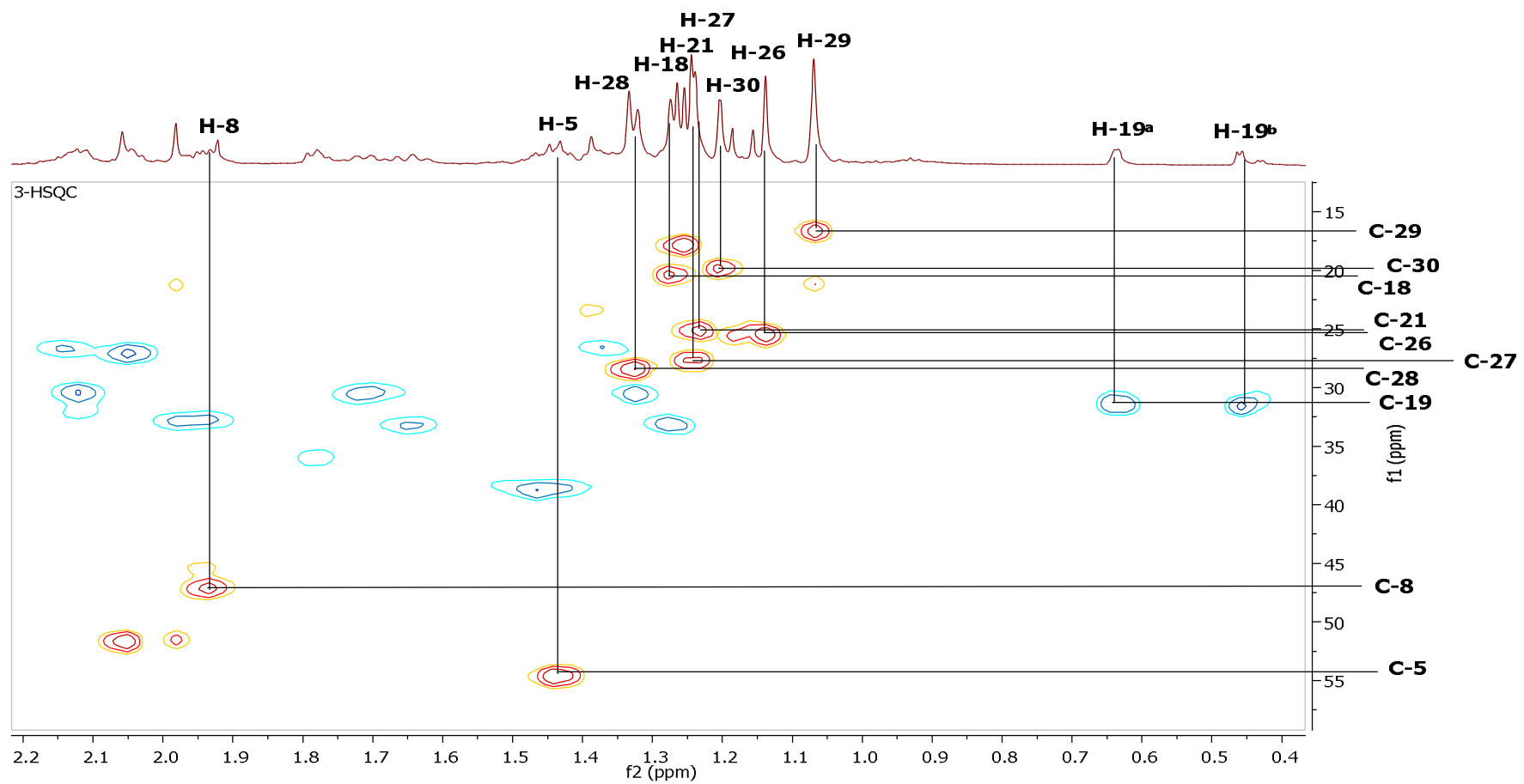
Figure 3.22. Structure of AAN3



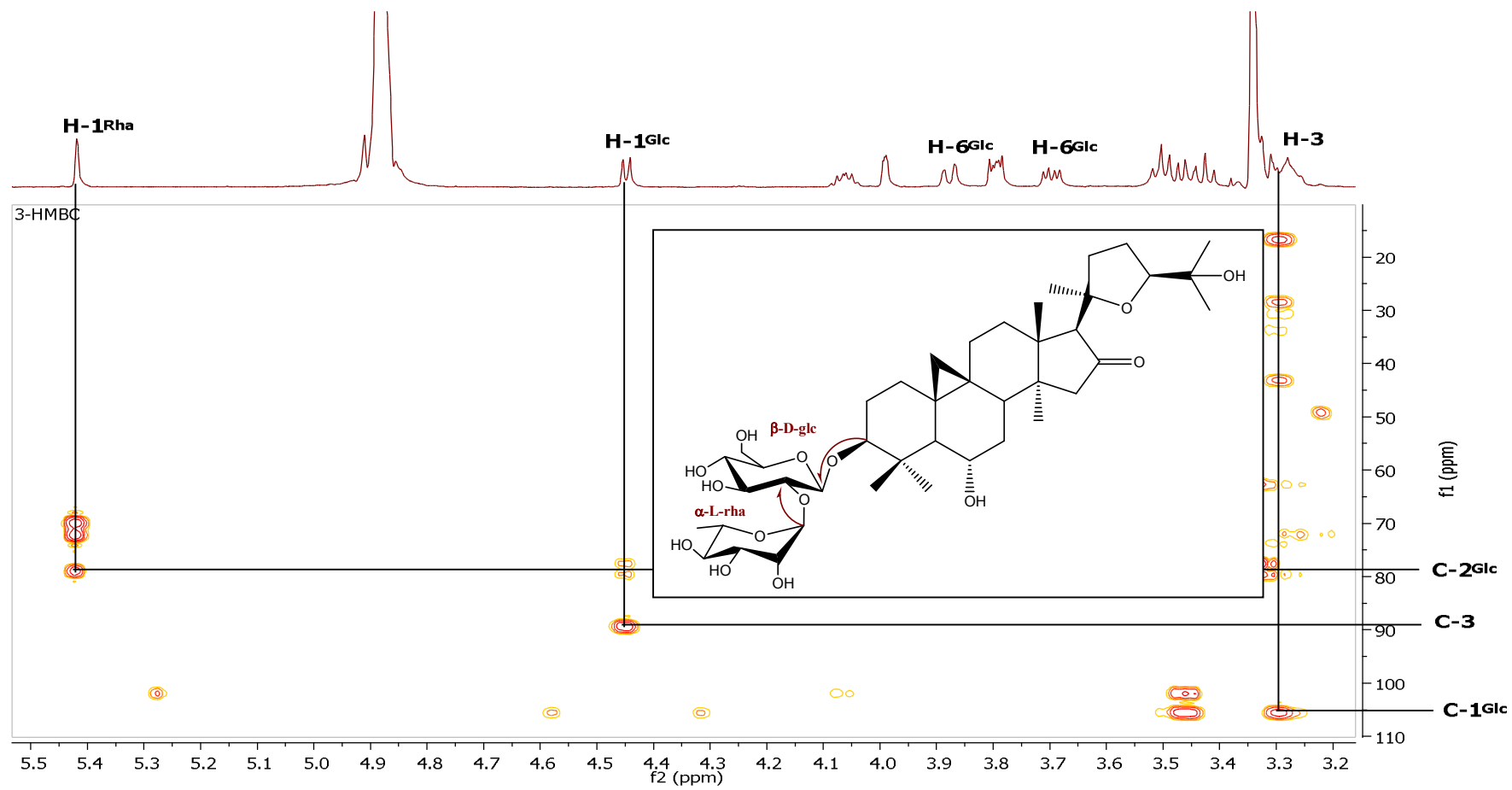
Spectrum 3.101. ^1H NMR spectrum of AAN3 (600 MHz, CD_3OD)



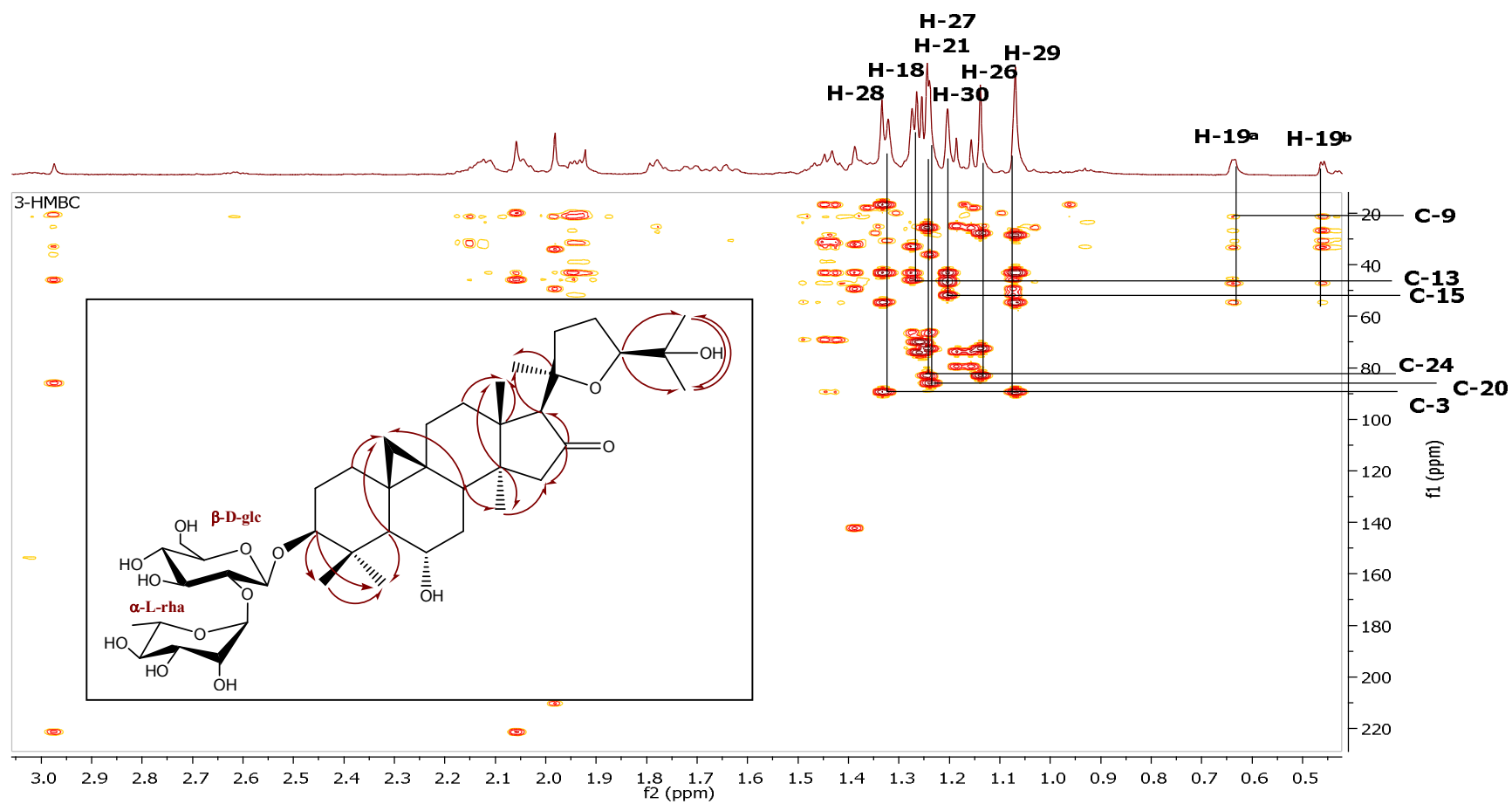
Spectrum 3.102. HSQC Spectrum of AAN3



Spectrum 3.103. HSQC Spectrum of AAN3



Spectrum 3.104. HMBC Spectrum of AAN3



Spectrum 3.105. HMBC Spectrum of AAN3

3.3.4. Structural Identification of Compound AAN4

Compound **AAN4** was obtained as a white amorphous powder. The HRMALDITOF mass spectrum of **AAN4** (m/z 821.4666 $[M+Na]^+$, calcd for $C_{42}H_{70}O_{14}Na$, 821.4663) supported a molecular formula of $C_{42}H_{70}O_{14}$. The ESIMS spectrum showed the major ion peak at m/z 821, which was assigned to $[M+Na]^+$. The MS/MS of this ion showed peaks at m/z 675 $[M+Na-146]^+$, due to the loss of a deoxyhexose unit, and at m/z 495 $[M+Na-146-180]^+$, corresponding to the loss of a hexose unit.

As concerning the aglycone moiety, the ^1H NMR spectrum showed highly shielded signals characteristic of methylene protons of a cyclopropane ring of an AX system (δ 0.59 and 0.41, each 1H, d, $J = 4.4$ Hz) and signals due to seven tertiary methyls (δ 1.44, 1.36, 1.33, 1.22, 1.17, 1.07 and 0.99) (**Table 3.23**). The full assignments of the proton and carbon signals of the aglycone moiety of **AAN4**, secured by DQF-COSY, HSQC and HMBC spectra, were in good agreement with those of cycloastragenol [31], except for the chemical shift of C-24 (δ 88.5), which could be attributed to a change of the absolute configuration at this center. The *R* configurations of C-20 and C-24 was derived by the ROESY spectrum, which showed key correlation peaks between Me-18 (δ 1.44) and H-23 β (δ 1.85), which in turn correlated with H-24 (δ 3.84) suggesting a β orientation for H-24. Further ROE correlations between H-17 (δ 2.27), Me-21 (δ 1.36), and Me-30 (δ 0.99) were observed. These data confirmed that the aglycone was the C-24 epimer of cycloastragenol. 20,24-Epoxy cycloartanes represent the largest group belonging to the class of cycloartane triterpenoids. Of the four possible side-chain stereoisomers [20*R*,24*S*, 20*S*,24*R*, 20*R*,24*R* and 20*S*,24*S*], to the authors' knowledge at present only the first two have been found in *Astragalus* plants (Mamedova et al., 2004). Therefore, this is the first report of a 20,24-epoxycycloartanes with 20(*R*),24(*R*) configuration.

On the basis of the obtained data, the structure of the new compound **AAN4** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*R*)-epoxycycloartane (**Fig. 3.23**).

Amorphous white solid; $C_{42}H_{70}O_{14}$; $[\alpha]_D^{25}$ -5.21 (*c* 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3460 ($>OH$), 3038 (cyclopropane ring), 2935 ($>CH$), 1250 and 1050 (C-O-C); ESI-MS m/z 821 $[M+Na]^+$, MS/MS m/z 675 $[M+Na-146]^+$, m/z 495 $[M+Na-146-180]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{42}H_{70}O_{14}Na$, 821.4663,

found 821.4666; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data : see **Table 3.23**.

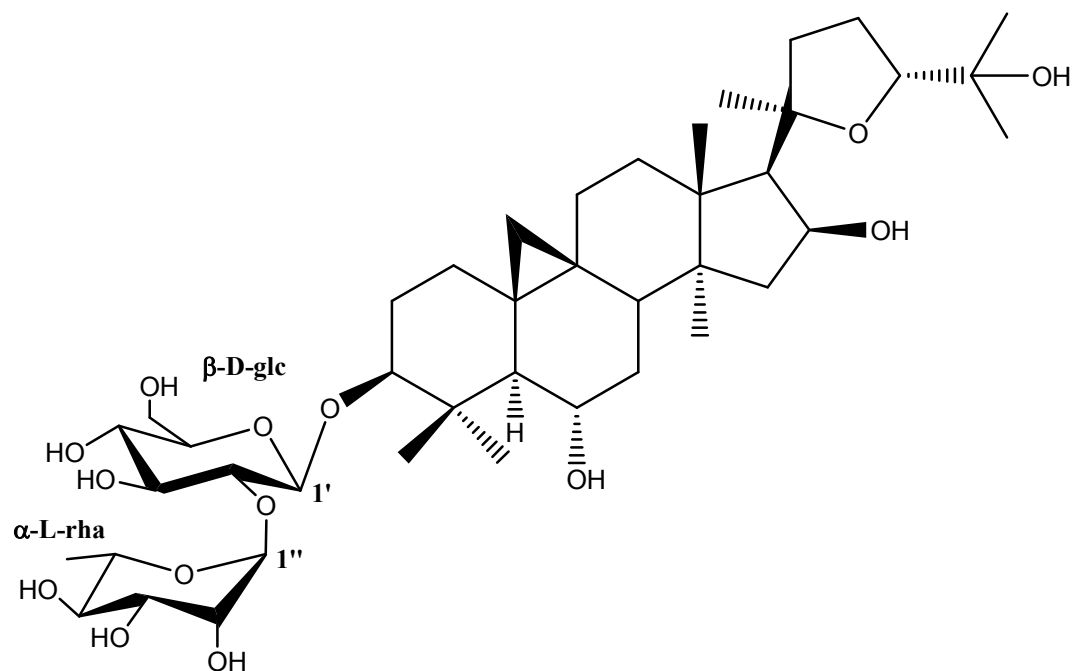
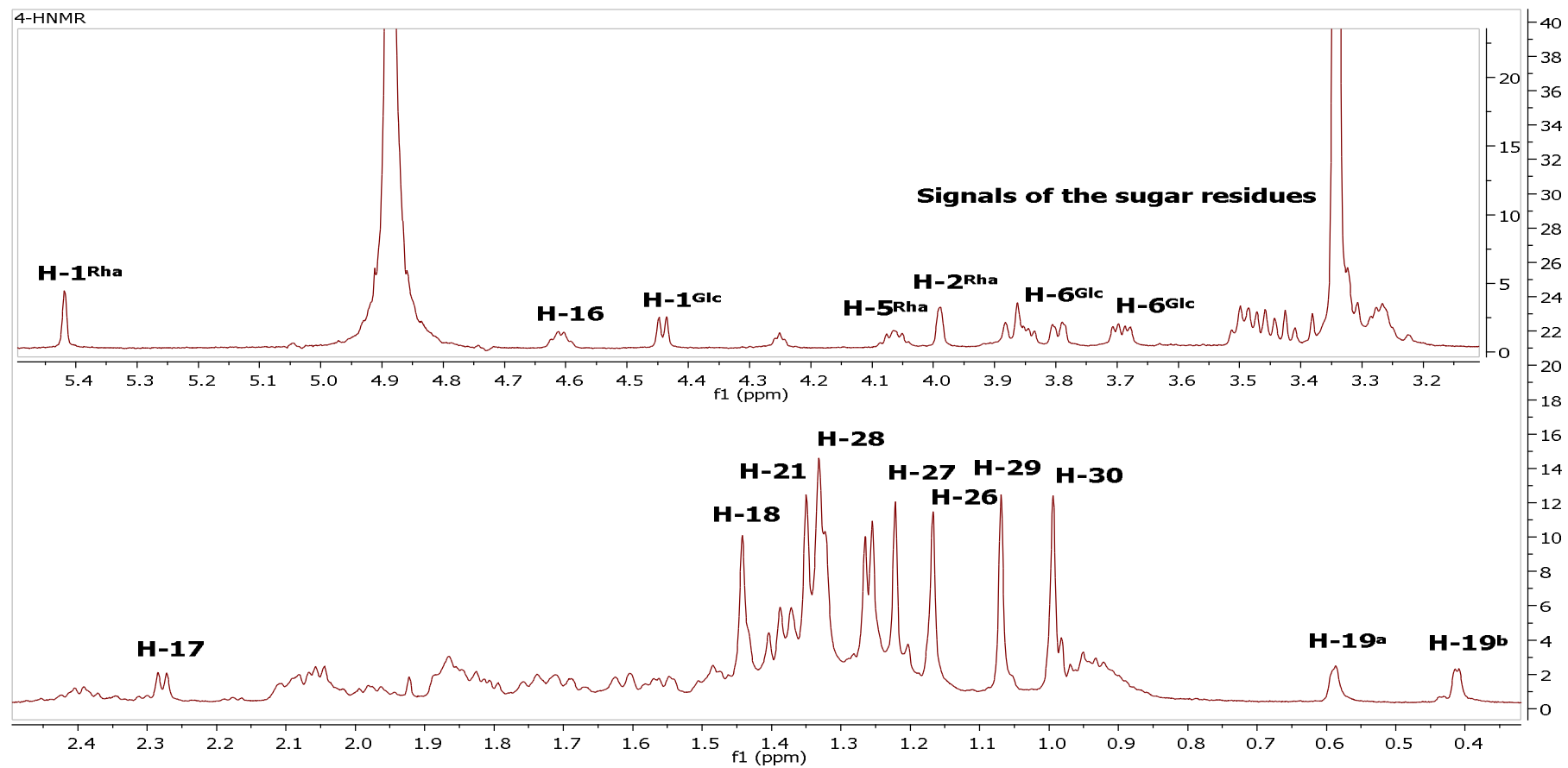


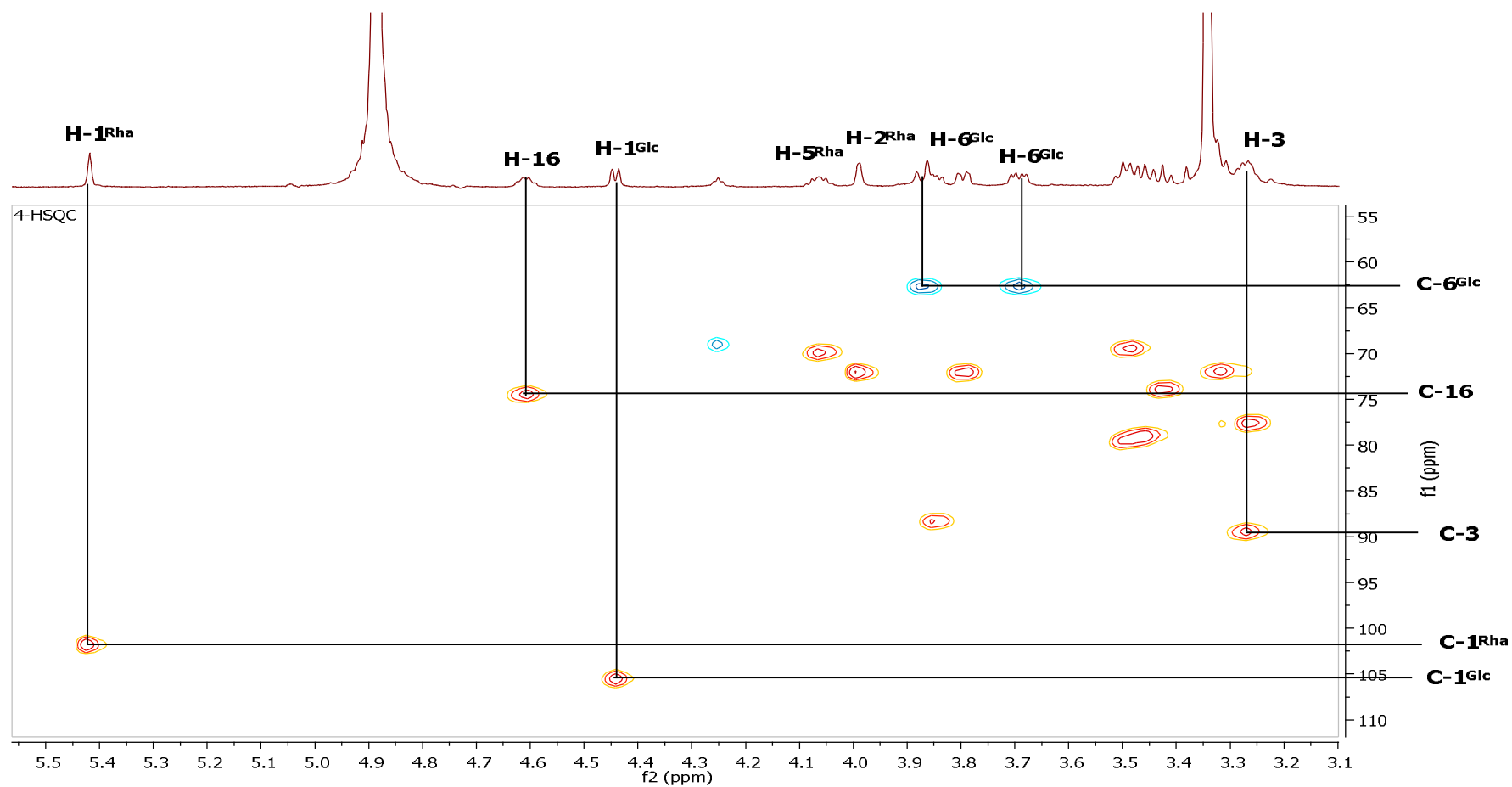
Figure 3.23. Structure of AAN4

Table 3.23. ^1H and ^{13}C Assignments of AAN4 (150/600 MHz, δ ppm, in CD_3OD)

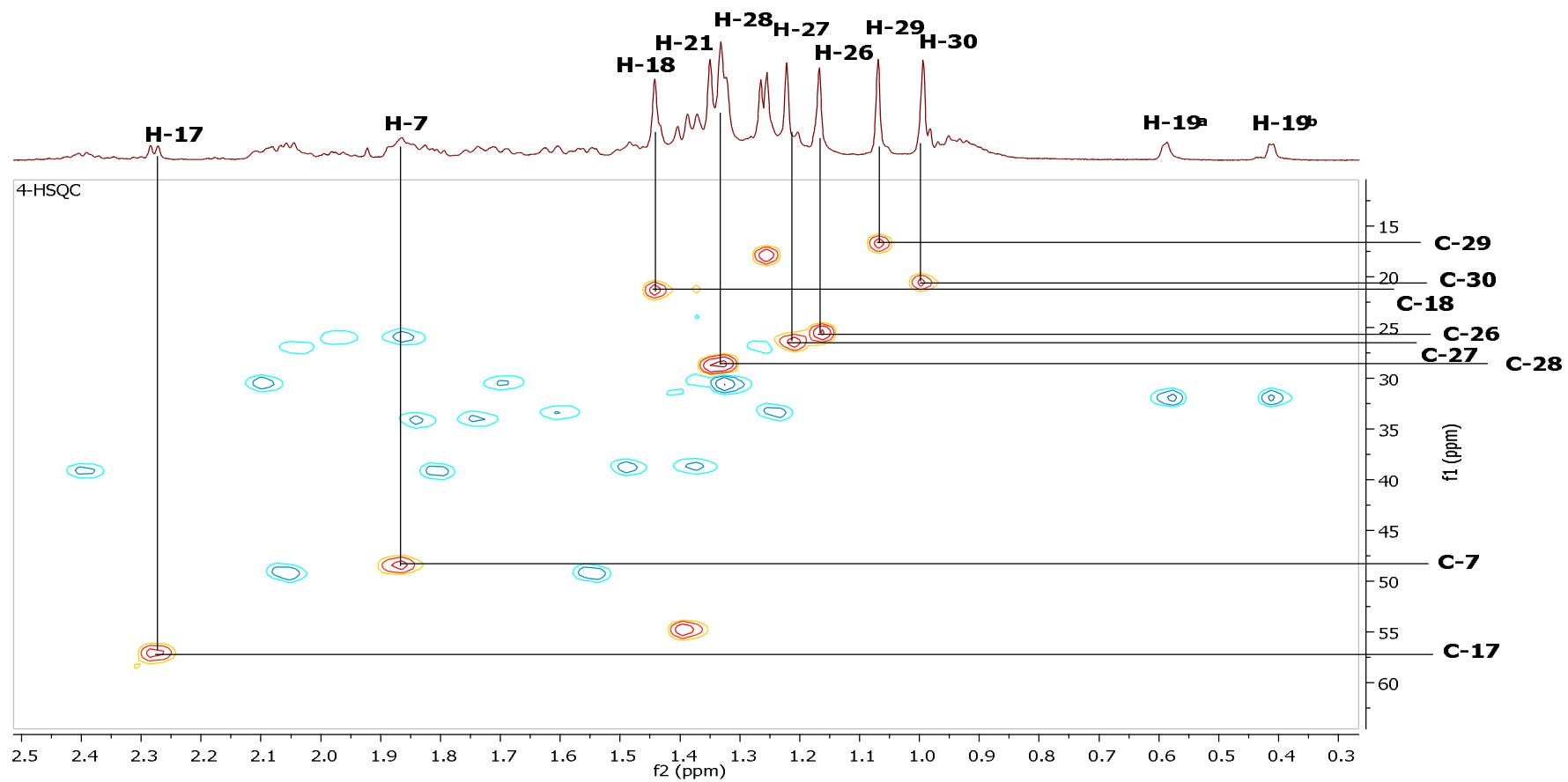
Position	δ_{C}	δ_{H} (J in Hz)
1	33.4	1.60, 1.23, m
2	30.5	2.10, 1.69, m
3	89.6	3.27, dd (11.3, 5.6)
4	43.3	-
5	54.8	1.39, d (9.7)
6	69.4	3.48, ddd (9.7, 9.7, 3.2)
7	38.8	1.49, 1.36, m
8	48.4	1.87, dd (12.0, 4.0)
9	21.6	-
10	30.1	-
11	27.2	2.05, 1.24, m
12	33.7	1.84, 1.72, m
13	47.3	-
14	47.8	-
15	49.2	2.06, dd (13.0, 8.0), 1.54, dd (13.0, 4.0)
16	74.1	4.62, ddd (7.3, 7.3, 5.6)
17	57.1	2.27, d (7.3)
18	21.1	1.44, s
19	31.9	0.59, brs, 0.41, d (4.4)
20	87.3	-
21	28.4	1.36, s
22	38.9	2.40, 1.80, m
23	25.9	1.98, 1.85, m
24	88.5	3.84 dd (10.5, 5.2)
25	72.0	-
26	25.1	1.17, s
27	26.1	1.22, s
28	28.7	1.33, s
29	16.6	1.07, s
30	20.3	0.99, s
$\beta\text{-D-Glc (at C-3)-1'}$	105.7	4.40, d (7.5)
2'	79.0	3.46, dd (7.5, 9.0)
3'	79.5	3.49, t (9.0)
4'	72.0	3.32, t (9.0)
5'	77.6	3.26, ddd (3.5, 4.5, 9.0)
6'	62.6	3.87, dd(3.5, 12), 3.69, dd (4.5, 12)
$\alpha\text{-L-Rha (at C-2}_{\text{glc}}\text{)-1''}$	102.1	5.42, d (1.2)
2''	72.1	3.99, dd (1.2, 3.2)
3''	72.2	3.79, dd (3.2, 9.7)
4''	73.9	3.42, t (9.7)
5''	69.8	4.06, m
6''	17.6	1.25, d (6.5)



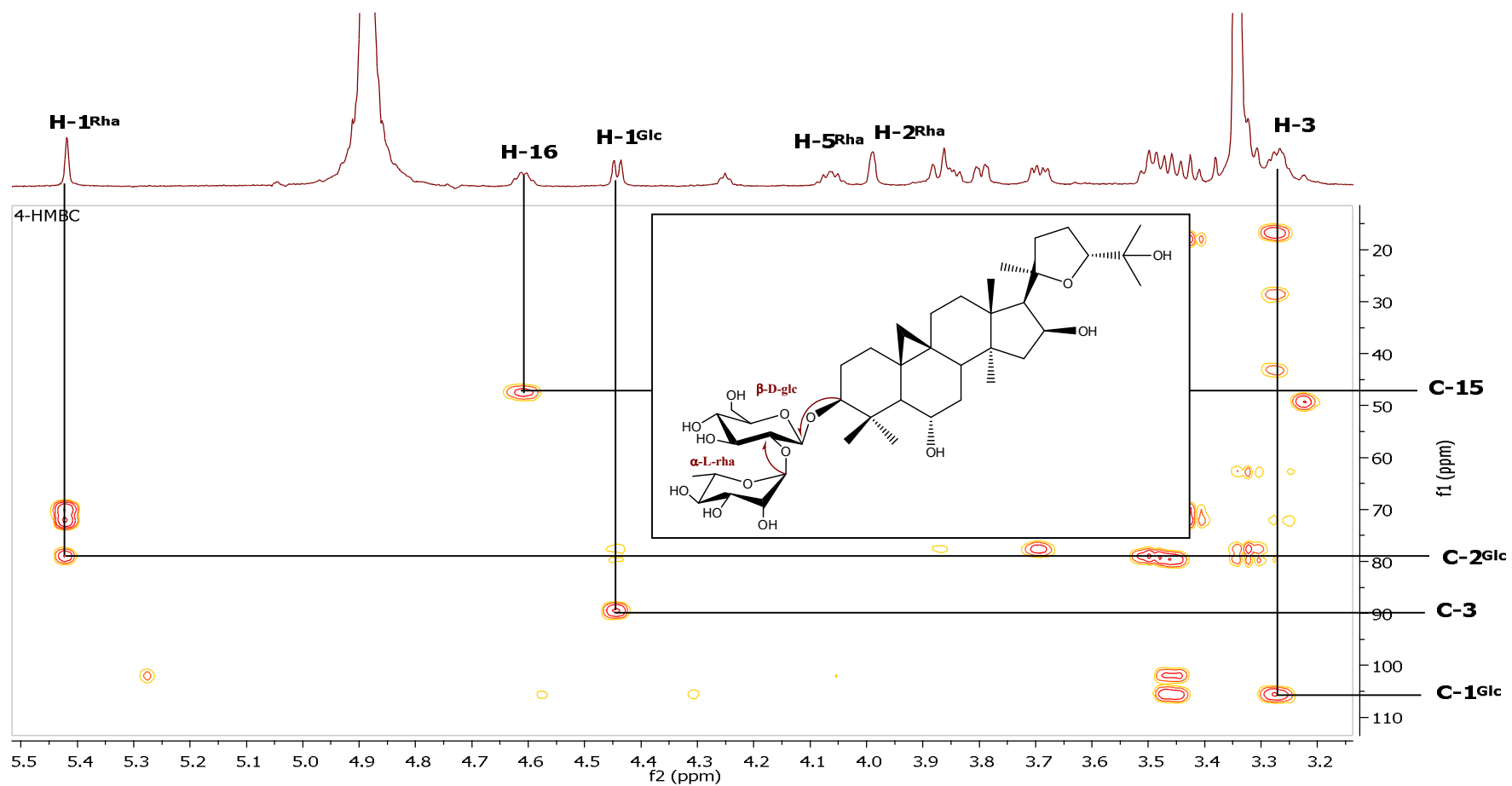
Spectrum 3.106. ¹H NMR spectrum of AAN4 (600 MHz, CD₃OD)



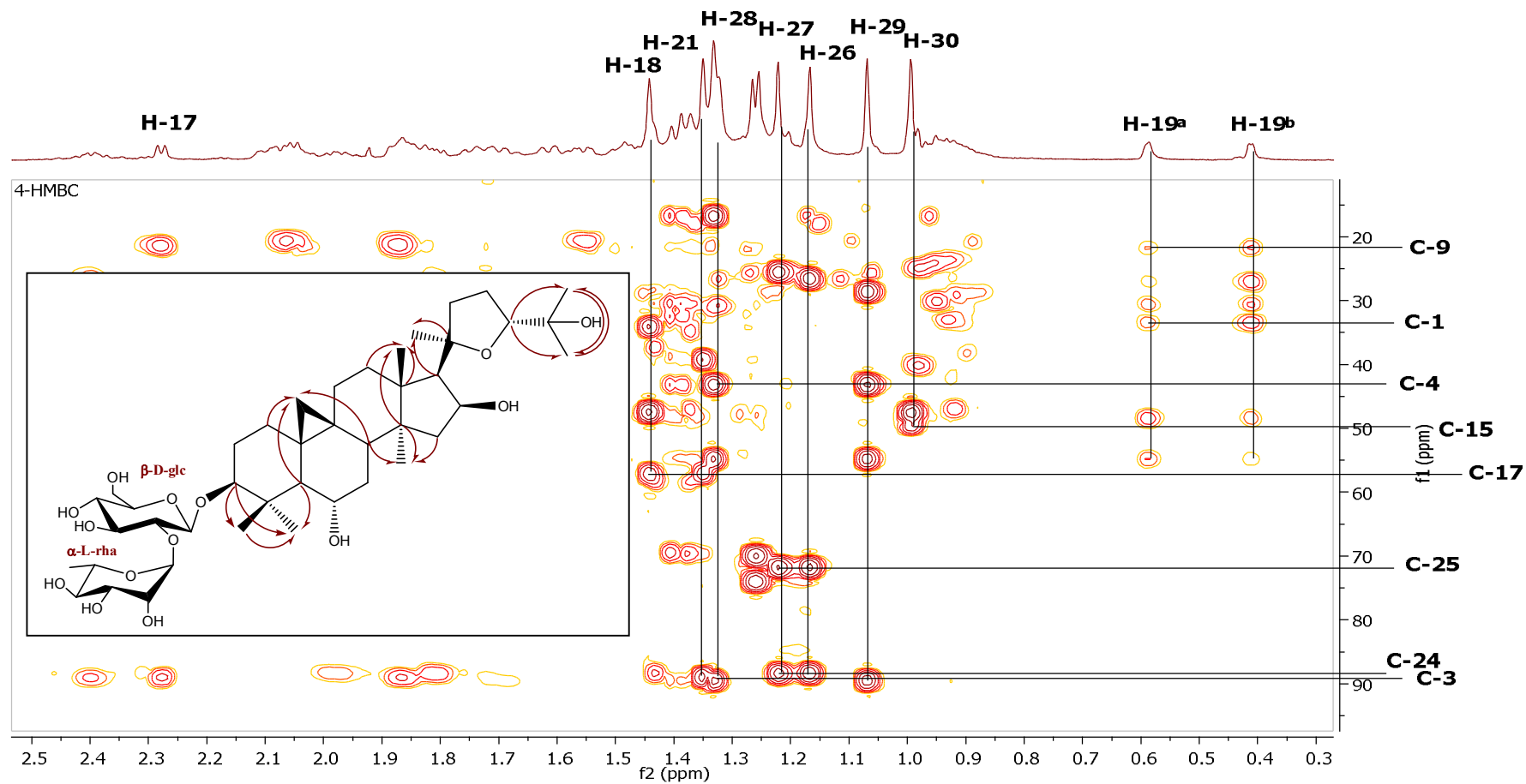
Spectrum 3.107. HSQC Spectrum of AAN4



Spectrum 3.108. HSQC Spectrum of AAN4



Spectrum 3.109. HMBC Spectrum of AAN4



Spectrum 3.110. HMBC Spectrum of AAN4

3.3.5. Structural Identification of Compound AAN5

Compound **AAN5** was obtained as an amorphous powder. The HRMALDITOF spectrum of **AAN5** (m/z 791.4561 $[M+Na]^+$, calcd for $C_{41}H_{68}O_{13}Na$, 791.4558) supported a molecular formula of $C_{41}H_{68}O_{13}$. The ESIMS spectrum showed the major ion peak at m/z 791 which was assigned to $[M+Na]^+$. The MS/MS of this ion showed peak at m/z 645 $[M+Na-146]^+$, due to the loss of a deoxyhexose unit, 495 $[M+Na-146-150]^+$, corresponding to the loss of a xylopyranosyl unit.

A detailed comparison of the NMR data (^1H , ^{13}C , HSQC, HMBC, COSY) of compound **AAN5** showed that the aglycone moiety was identical to that of **AAN4**. Moreover, the ^1H NMR spectrum of **AAN5** showed two anomeric proton doublets at δ 4.78 ($J = 1.2$ Hz) and 4.28 ($J = 7.5$ Hz) in the downfield region (**Table 3.24**), which correlated to carbon signals at δ 104.2 and 107.8, respectively, in the HSQC spectrum. The determination of the sequence and linkage sites was obtained from the HMBC correlations which showed key correlation peaks between the proton signals at δ 4.78 (H-1_{rha}) and the carbon resonance at δ 81.3 (C-6), and between the proton signal at δ 4.28 (H-1_{xy}) and the carbon at δ 89.7 (C-3). The D-configuration of xylose unit and the L-configuration of rhamnose unit were established after hydrolysis of **AAN5** followed by GC analysis (De Marino et al., 2003).

Thus, compound **AAN5** was identified as 3-*O*- β -L-xylopyranosyl-6-*O*- α -L-rhamnopyranosyl-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*R*)-epoxycycloartane (**Fig. 3.24**).

Amorphous white solid; $C_{41}H_{68}O_{13}$; $[\alpha]_D^{25} +15.1$ (*c* 0.01 MeOH); IR ν^{KBr}_{max} cm^{-1} : 3460 (>OH), 3035 (cyclopropane ring), 2940 (>CH), 1240 and 1050 (C-O-C); ESI-MS m/z 791 $[M+Na]^+$, MS/MS m/z 645 $[M+Na-146]^+$, m/z 495 $[M+Na-146-150]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{41}H_{68}O_{13}Na$, 791.4558, found 791.4561; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.24**.

Table 3.24. ^1H and ^{13}C Assignments of AAN5 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	33.0	1.58, 1.26, m
2	30.2	1.98, 1.70, m
3	89.7	3.23, dd (11.3, 4.0)
4	42.8	-
5	53.4	1.56, d (9.6)
6	81.3	3.45, ddd (9.7, 9.7, 3.2)
7	35.6	1.86, 1.41, m
8	47.5	1.86, dd (12.0, 4.0)
9	21.6	-
10	29.8	-
11	26.9	2.05, 1.24, m
12	33.9	1.83, 1.72, m
13	47.3	-
14	47.8	-
15	49.5	2.05, dd (13.0, 8.0), 1.56, dd (13.0, 5.6)
16	74.5	4.61, ddd (7.8, 7.8, 5.2)
17	57.1	2.28, d (7.8)
18	21.2	1.44, s
19	31.4	0.60, d (4.4), 0.42, d (4.4)
20	88.5	-
21	28.8	1.36, s
22	39.1	2.39, 1.80, m
23	25.9	1.94, 1.84, m
24	88.5	3.85, dd (10.5, 5.2)
25	71.9	-
26	25.5	1.17, s
27	26.2	1.22, s
28	28.7	1.21, s
29	16.9	1.05, s
30	20.4	1.00, s
β -D-Xyl (at C-3)-1'	107.8	4.28, d (7.5)
2'	75.5	3.21, dd (7.5, 9.2)
3'	77.7	3.31, t (9.2)
4'	71.4	3.49, m
5'	66.7	3.84, dd (5.2, 11.7), 3.19, t (11.7)
α -L-Rha (at C-6)-1''	104.2	4.78, d (1.2)
2''	72.5	3.86, dd (1.2, 3.2)
3''	70.0	3.71, dd (3.2, 9.7)
4''	73.7	3.41, t (9.7)
5''	72.3	3.61, m
6''	17.6	1.27, d (6.5)

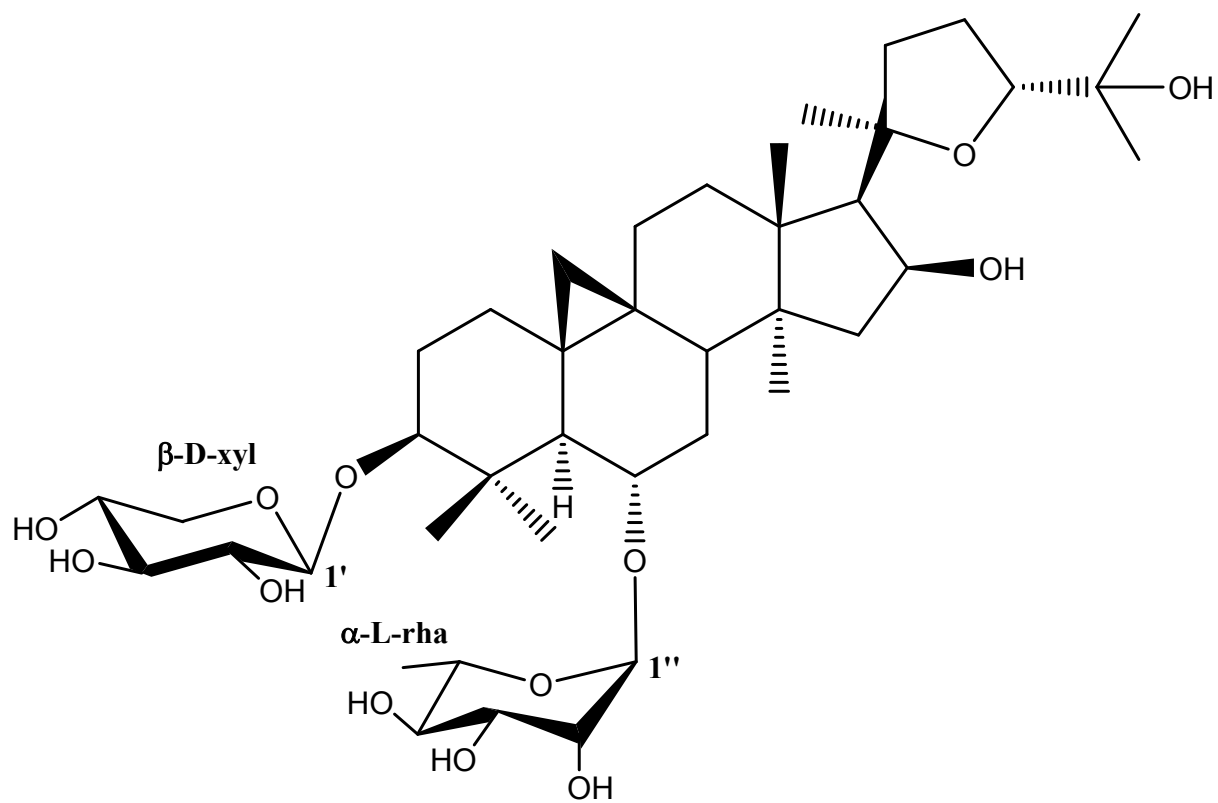
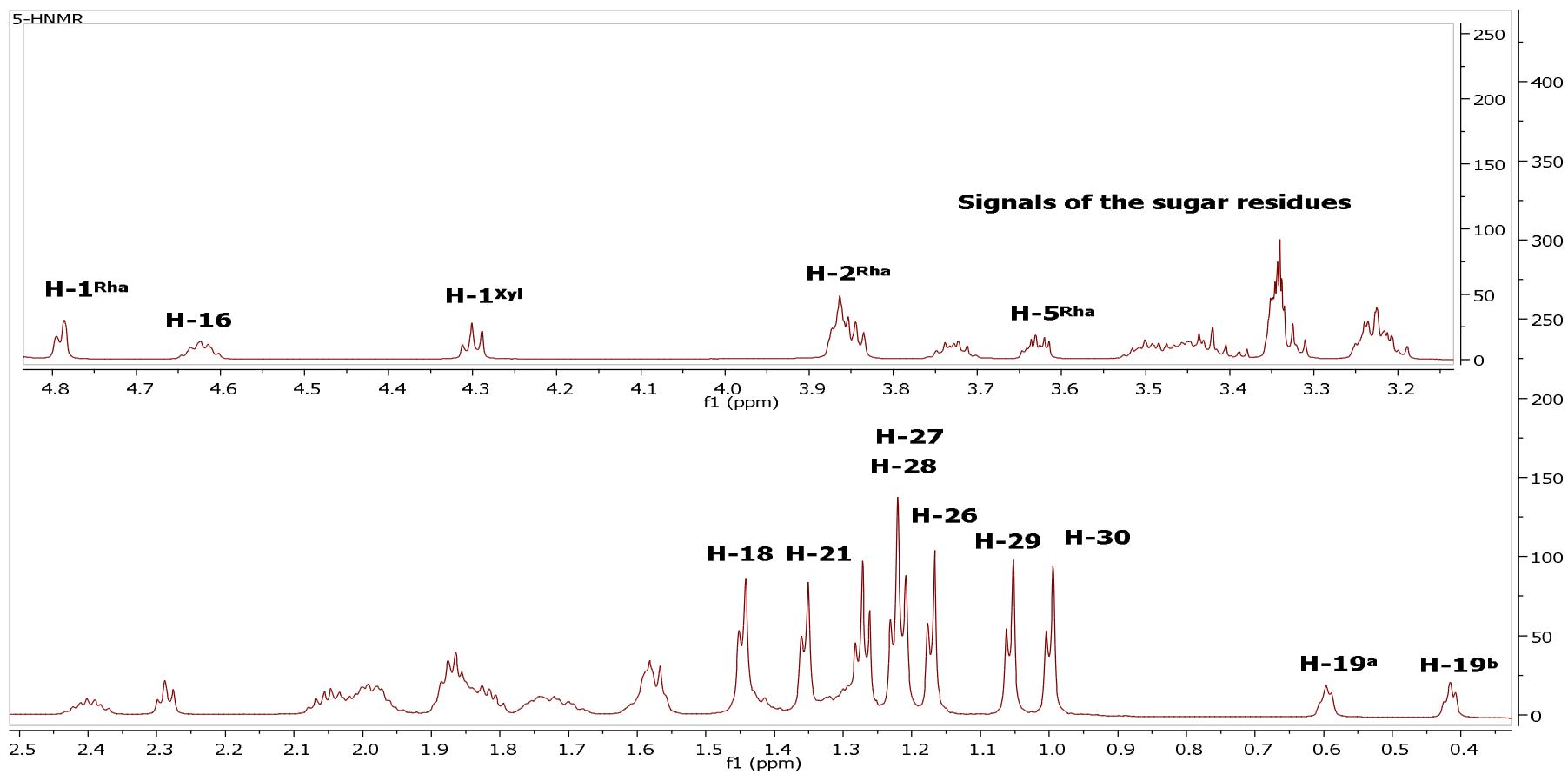
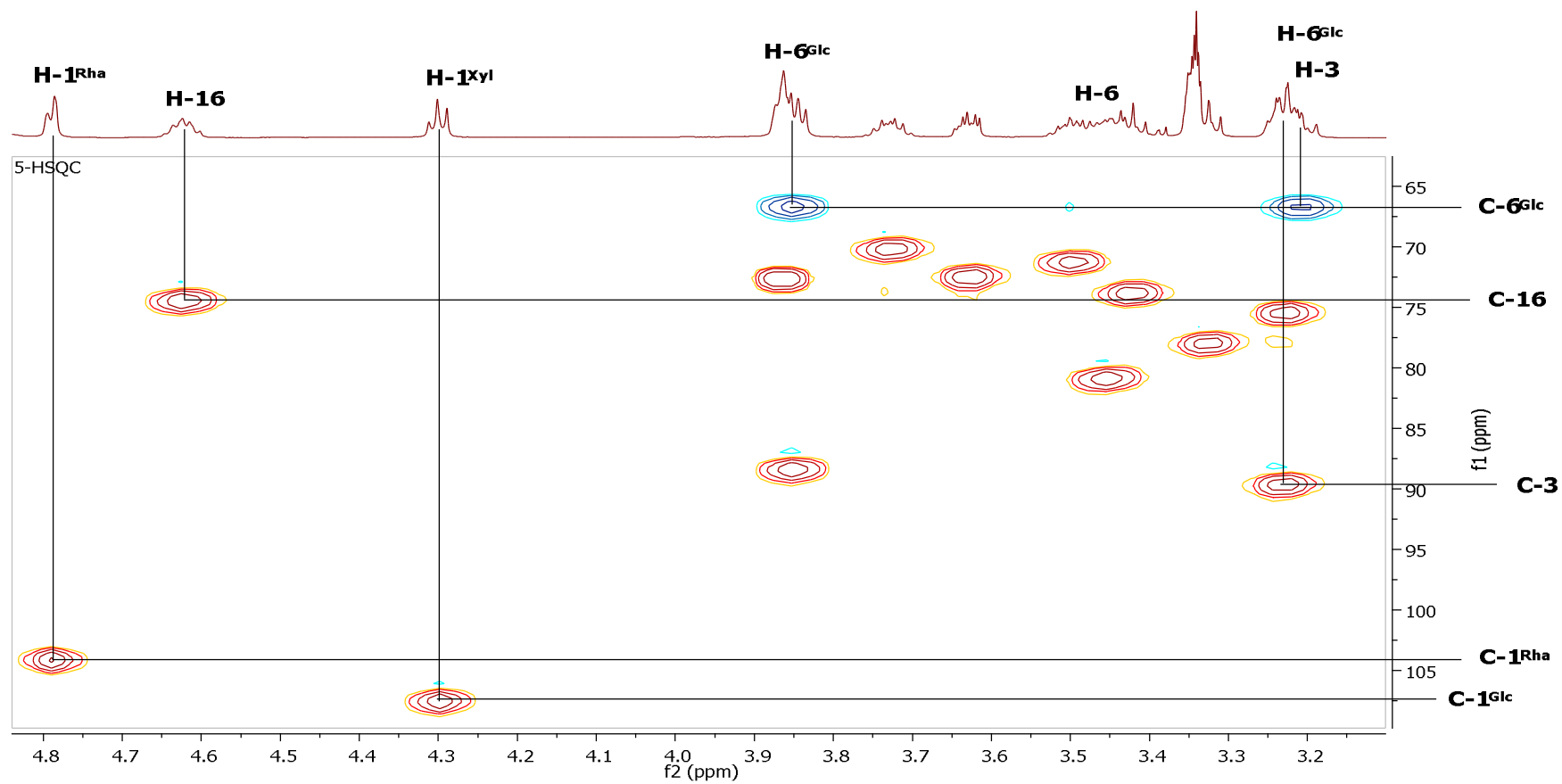


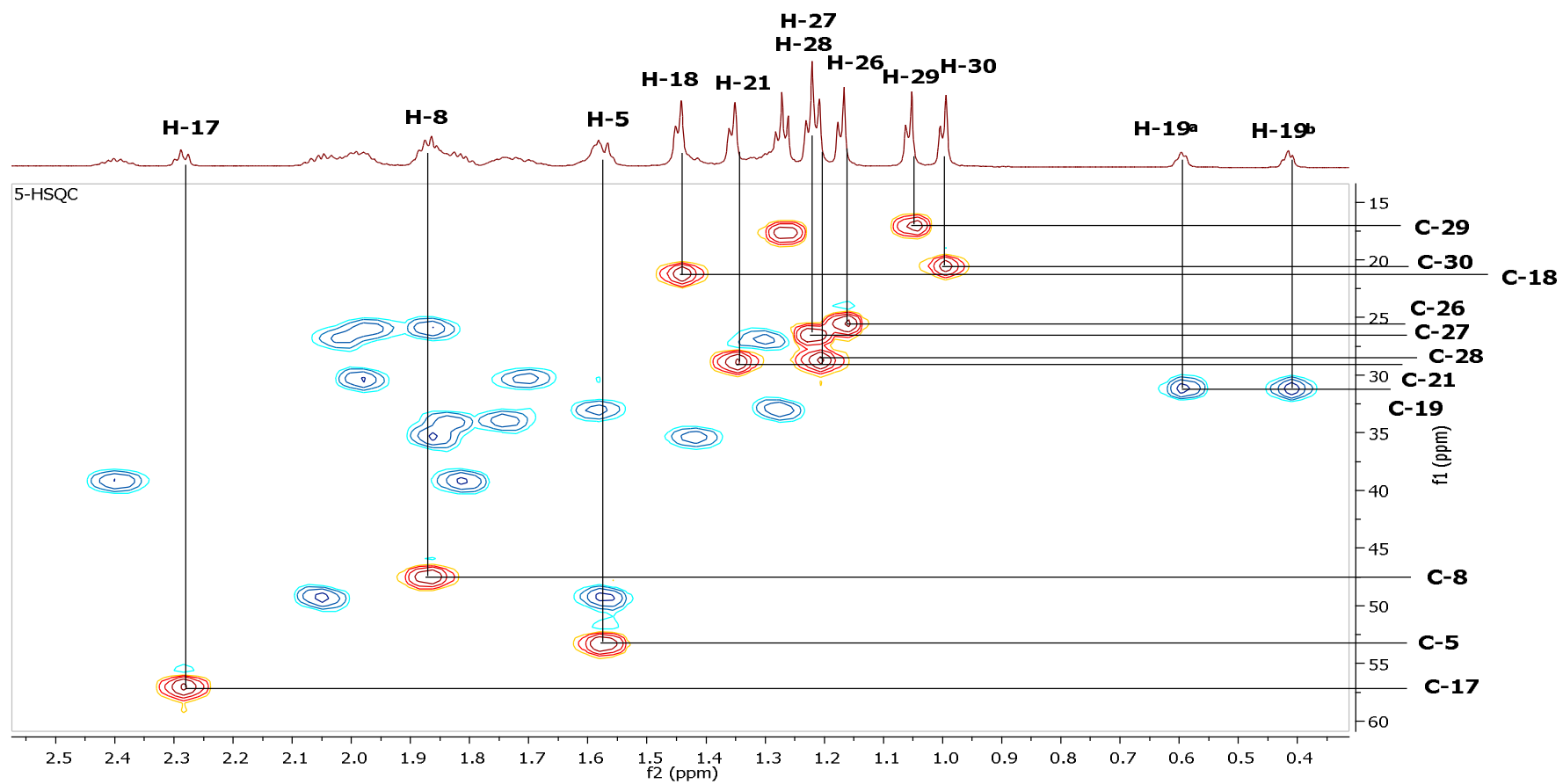
Figure 3.24. Structure of AAN5



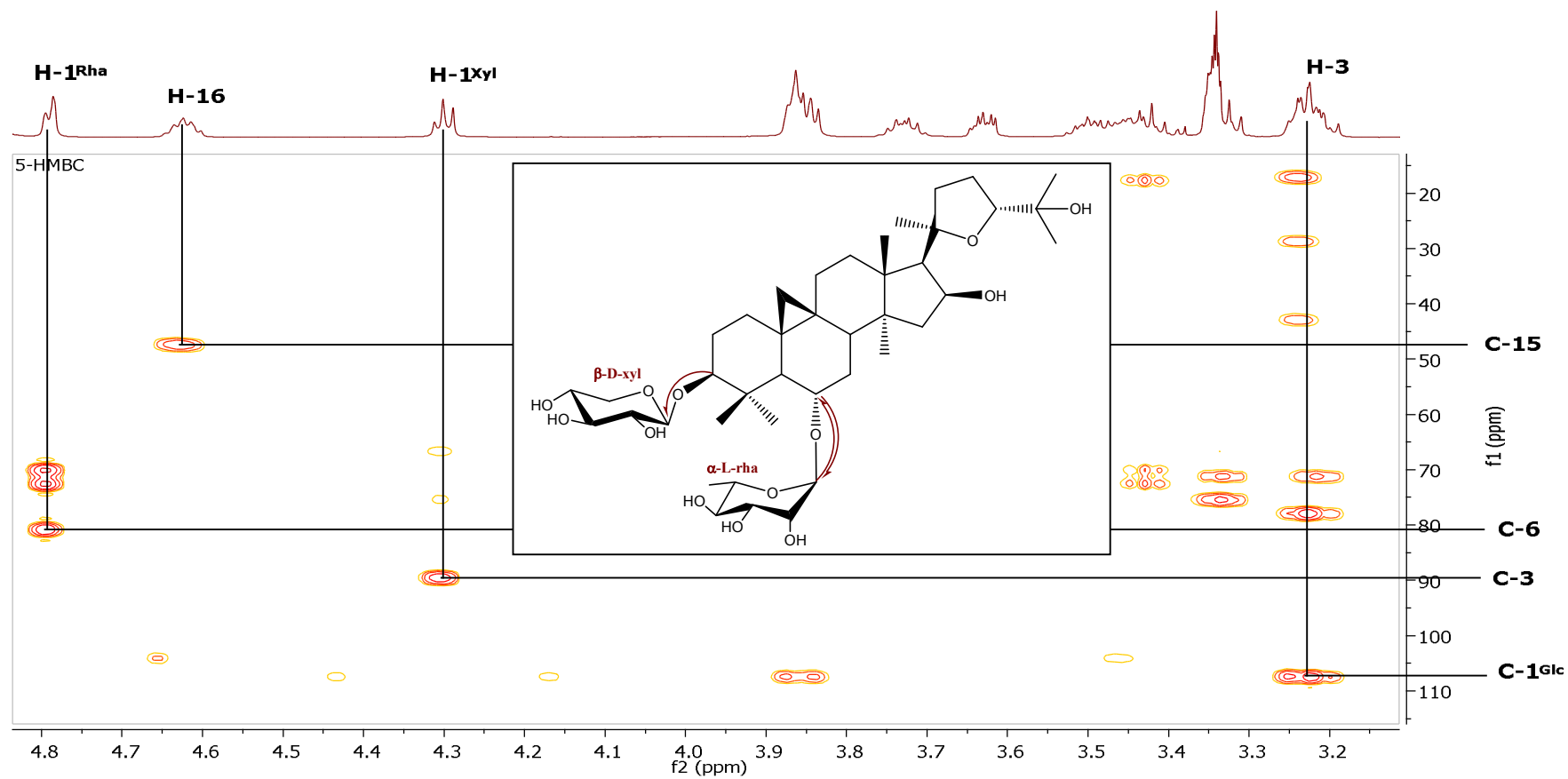
Spectrum 3.111. ^1H NMR spectrum of AAN5 (600 MHz, CD_3OD)



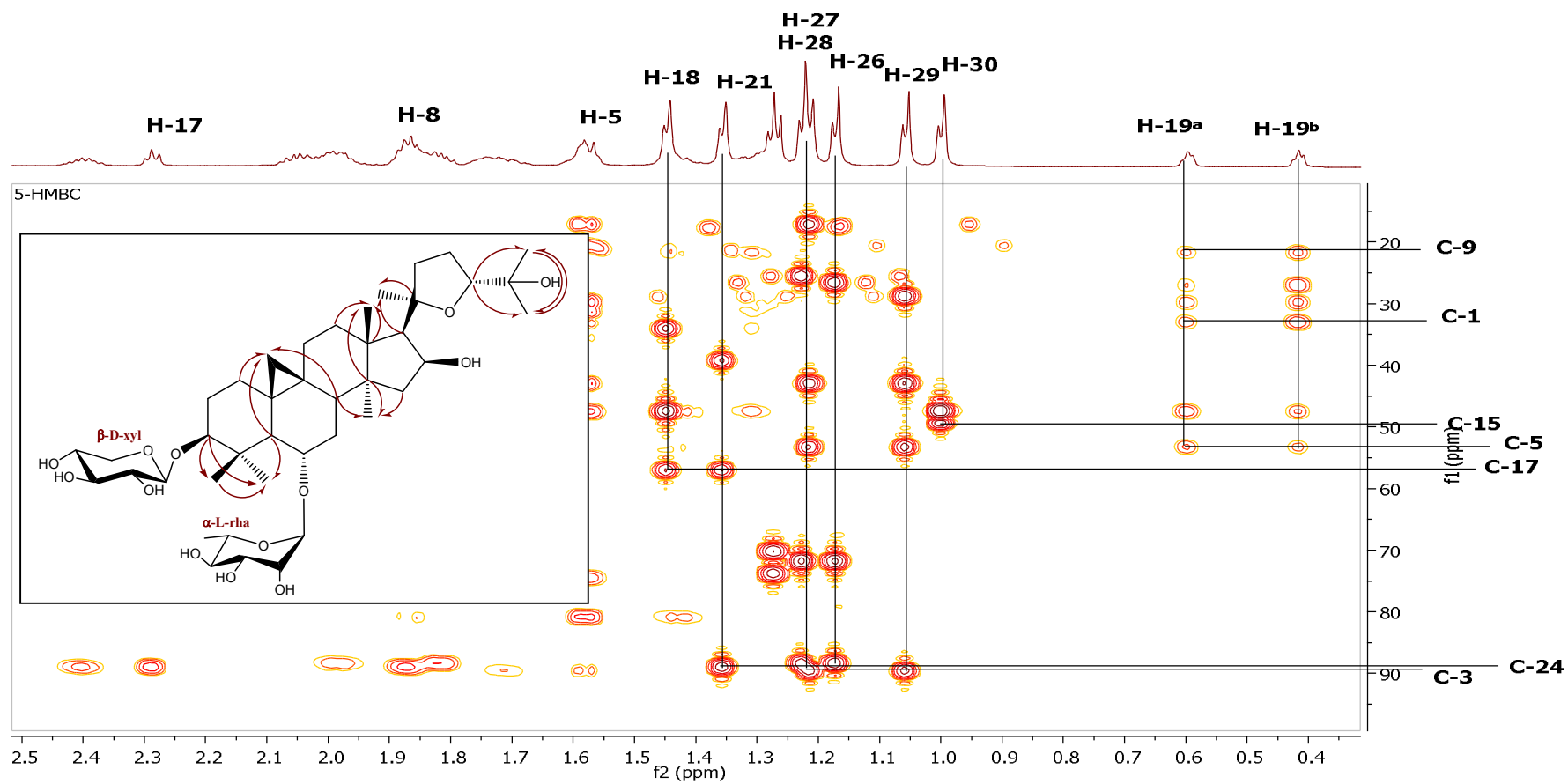
Spectrum 3.112. HSQC Spectrum of AAN5



Spectrum 3.113. HSQC Spectrum of AAN5



Spectrum 3.114. HMBC Spectrum of AAN5



Spectrum 3.115. HMBC Spectrum of AAN5

3.3.6. Structural Identification of Compound AAN6

The molecular formula of **AAN6** was established as $C_{36}H_{60}O_9$ by HRMALDITOF spectrum (m/z 659.4139 $[M+Na]^+$, calcd for $C_{36}H_{60}O_9Na$, 659.4135). The positive ESIMS spectrum showed the sodiated ion peak at m/z 659 $[M+Na]^+$. Its MS/MS fragmentation showed peak at m/z 513 $[M+Na-146]^+$ due to the loss of a deoxyhexose unit. A detailed analysis of the NMR data (^1H , ^{13}C , HSQC, HMBC, COSY) of compound **AAN6** revealed that the aglycone moiety was superimposable to that of **AAN4**.

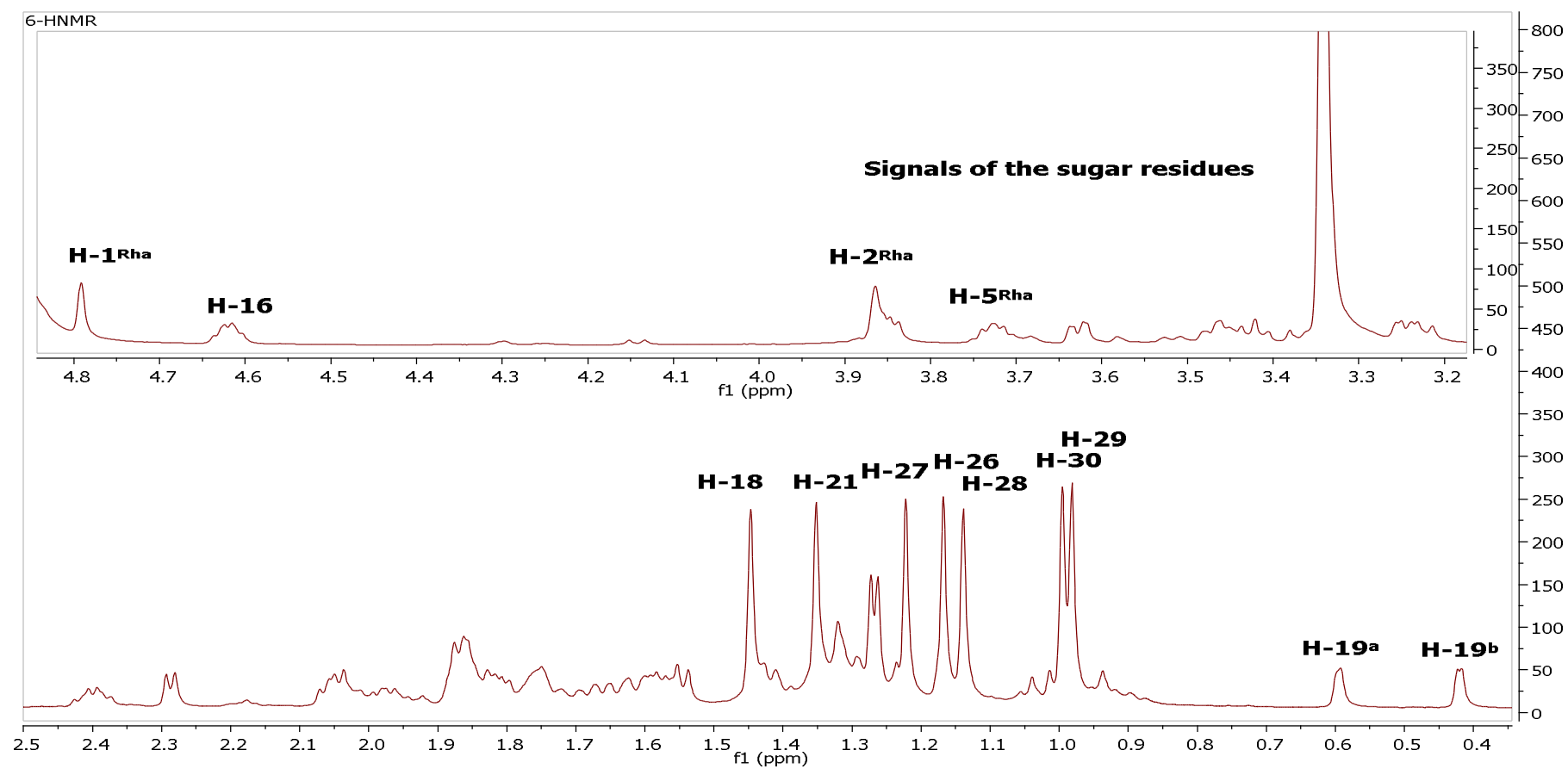
The ^1H NMR spectrum of **AAN6** displayed a signal for one anomeric proton at δ 4.80 (d, $J = 1.2$ Hz) (Table 1). On the basis of HSQC, HMBC, and COSY correlations one α -rhamnopyranose unit was identified. Key correlation peak in the HMBC spectrum was observed between the proton signal at δ 4.80 and the carbon resonance at δ 81.2 (C-6). The L-configuration of rhamnose unit was established after hydrolysis of 1 N HCl, trimethylsilation and determination of retention time on a chiral column by GC.

Therefore, the structure of **AAN6** was established as 6-*O*- α -L-rhamnopyranosyl-3 β ,6 α ,16 β ,25-tetrahydroxy-20(*R*),24(*R*)-epoxycycloartane (**Fig. 3.25**).

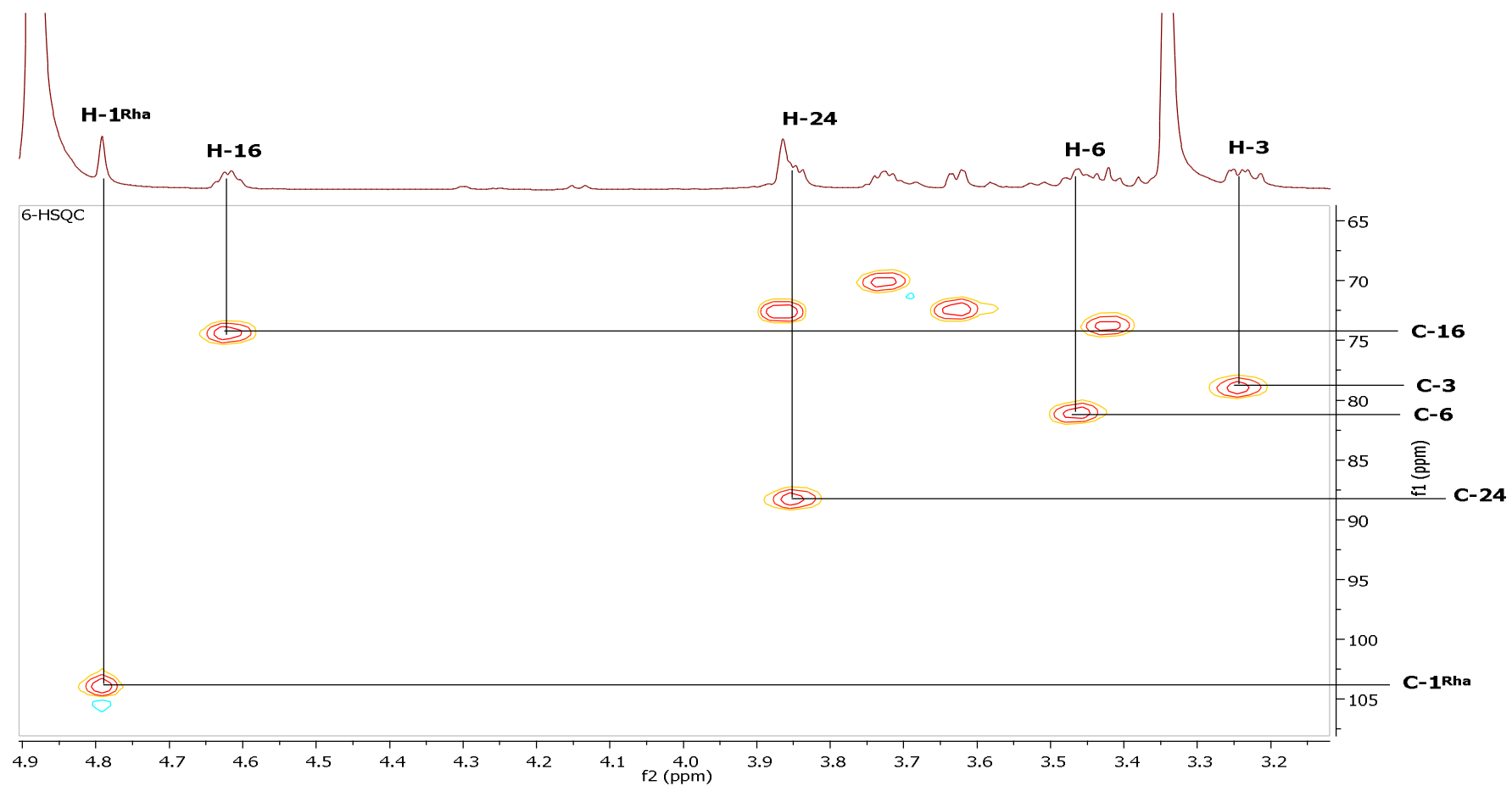
Amorphous white solid; $C_{36}H_{60}O_9$; $[\alpha]_D^{25}$ -8.09 (*c* 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3450 ($>OH$), 3042 (cyclopropane ring), 2938 ($>CH$), 1250 and 1052 (C-O-C); ESI-MS m/z 659 $[M+Na]^+$, MS/MS m/z 513 $[M+Na-146]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{36}H_{60}O_9Na$, 659.4135, found 659.4139; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.25**.

Table 3.25. ^1H and ^{13}C Assignments of AAN6 (150/600 MHz, δ ppm, in CD_3OD)

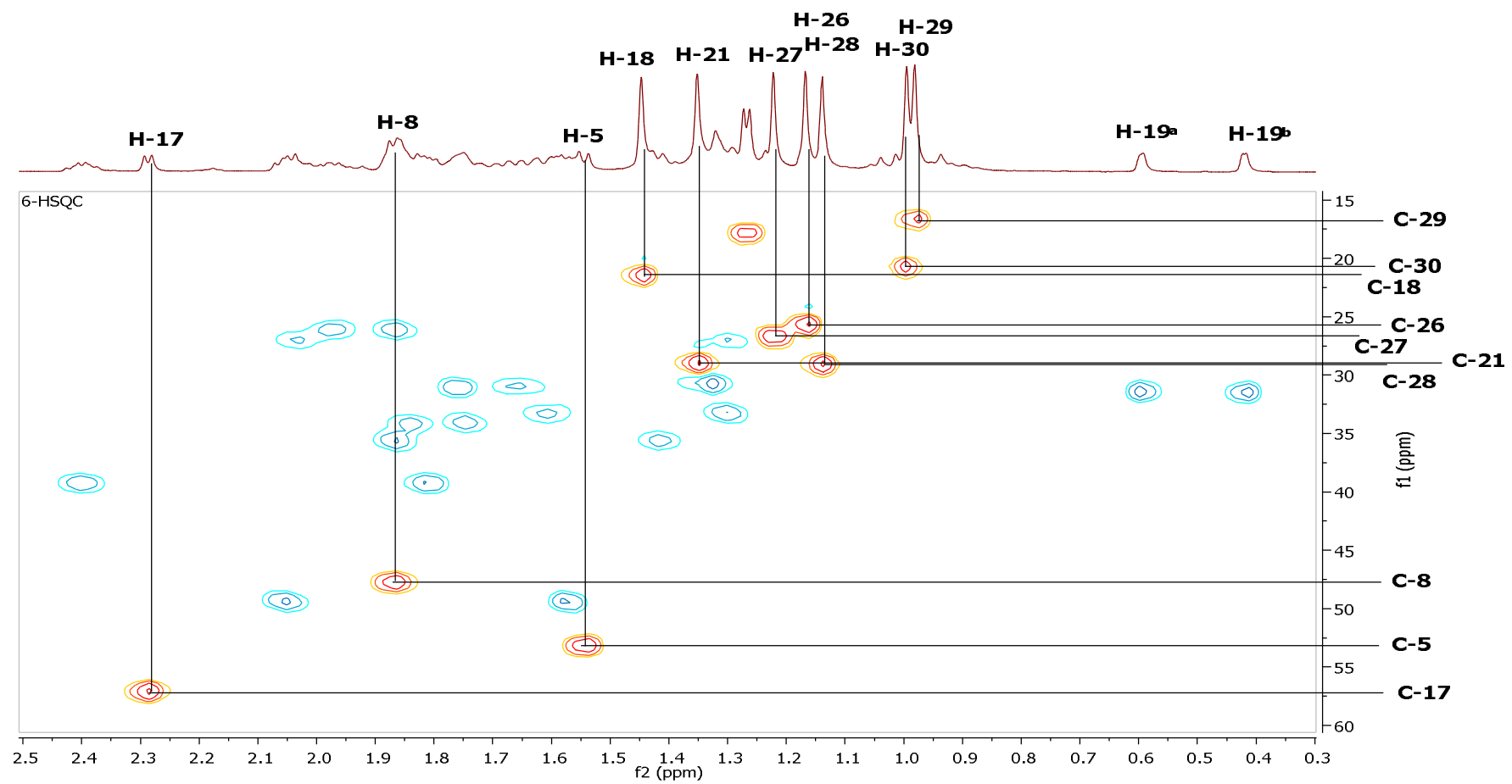
Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	32.9	1.61, 1.29, m
2	30.6	1.77, 1.64, m
3	79.3	3.24, dd (11.3, 4.0)
4	42.3	-
5	52.9	1.54, d (9.7)
6	81.2	3.46, ddd (9.7, 9.7, 3.2)
7	35.5	1.86, 1.40, m
8	47.6	1.86, dd (12.0, 4.0)
9	21.5	-
10	29.8	-
11	26.5	2.05, 1.24, m
12	33.9	1.85, 1.73, m
13	47.3	-
14	47.7	-
15	49.3	2.06, dd (12.9, 8.0), 1.57, dd (12.9, 5.6)
16	74.4	4.61, ddd (7.8, 7.8, 5.2)
17	56.9	2.29, d (7.2)
18	21.2	1.44, s
19	31.3	0.60, brs, 0.42, brs
20	88.8	-
21	29.0	1.35, s
22	39.0	2.40, 1.80, m
23	26.0	1.99, 1.86, m
24	88.4	3.84, dd (10.5, 5.2)
25	71.7	-
26	25.4	1.17, s
27	26.2	1.22, s
28	29.0	1.14, s
29	16.3	0.98, s
30	20.5	1.00, s
α -L-Rha (at C-6)-1'	104.2	4.80, d (1.2)
2'	72.6	3.86, dd (1.2, 3.2)
3'	70.1	3.71, dd (3.2, 9.7)
4'	73.8	3.42, t (9.7)
5'	72.3	3.61, m
6'	17.5	1.26, d (6.5)



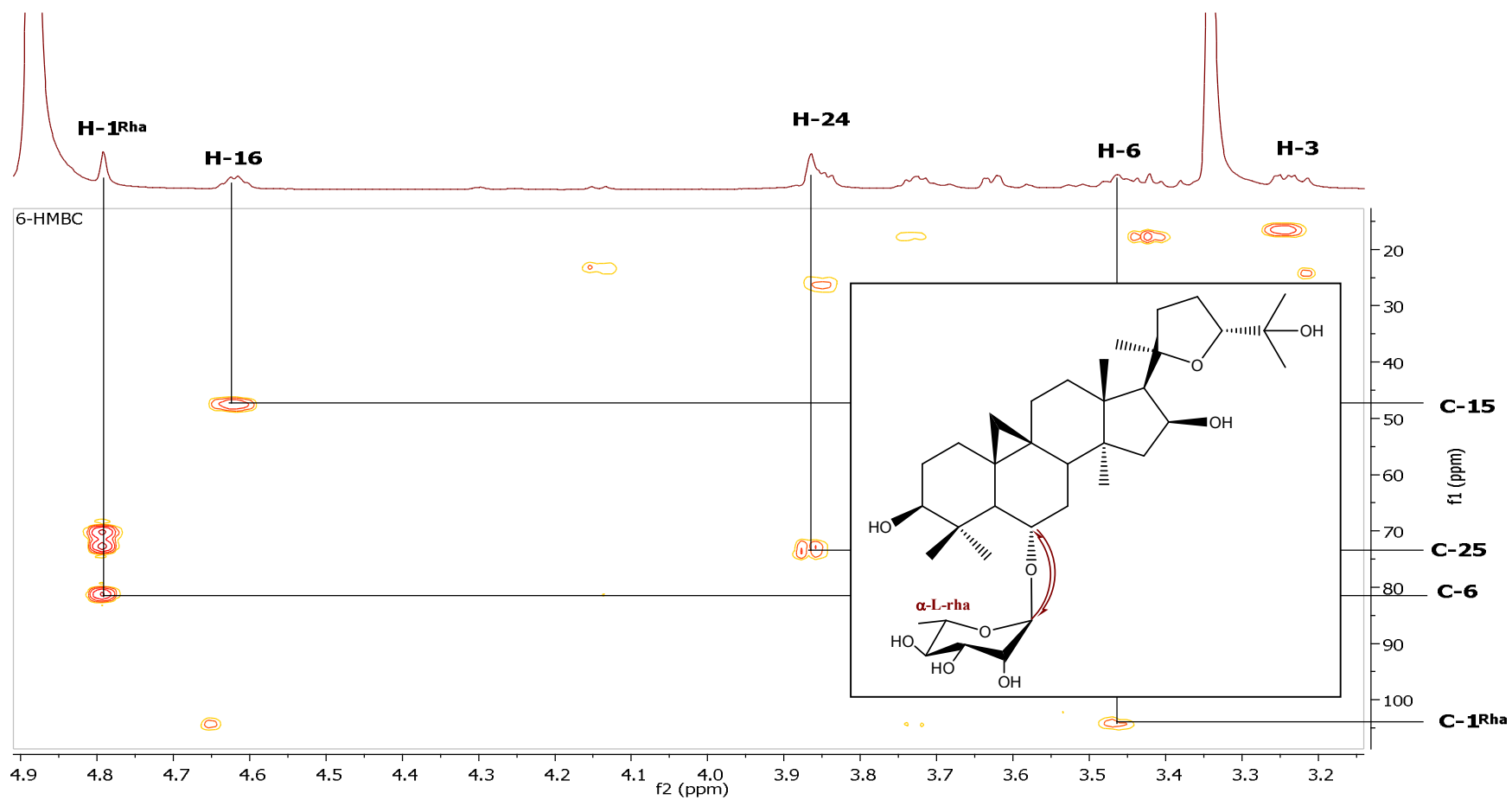
Spectrum 3.116. ^1H NMR spectrum of AAN6 (600 MHz, CD_3OD)



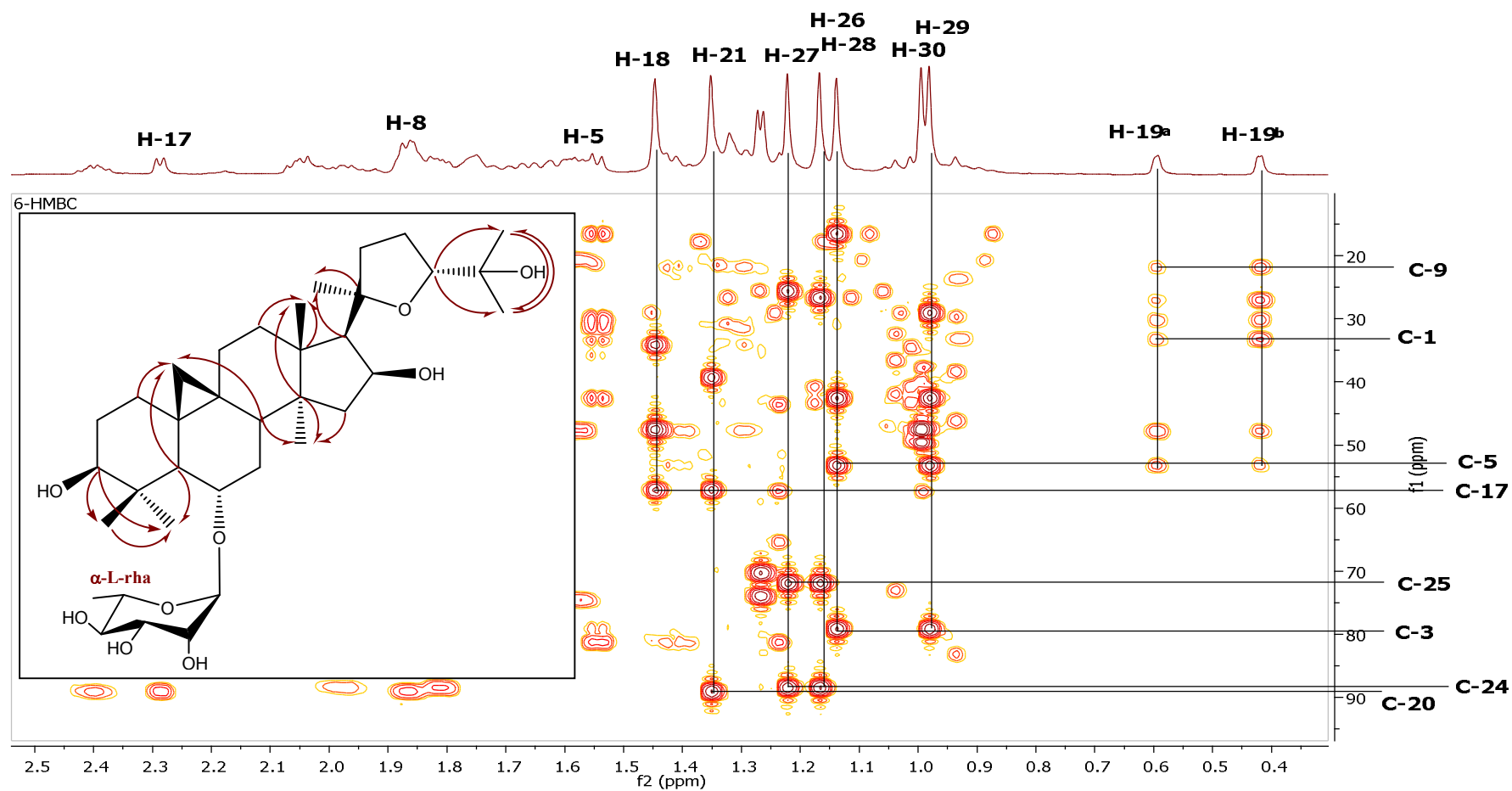
Spectrum 3.117. HSQC Spectrum of AAN6



Spectrum 3.118. HSQC Spectrum of AAN6



Spectrum 3.119. HMBC Spectrum of AAN6



Spectrum 3.120. HMBC Spectrum of AAN6

3.3.7. Structural Identification of Compound AAN7

A detailed comparison of sugar region NMR (^1H , ^{13}C , HSQC, HMBC, COSY) and ESIMS data of compounds **AAN7-AAN8** showed that the sugar chain was identical in the two compounds. In particular, for the sugar portion, compound **AAN7** showed signals corresponding to three anomeric protons at δ 5.23 (d, $J = 1.2$ Hz), 4.82 (d, $J = 7.5$ Hz), 4.44 (d, $J = 7.5$ Hz) (**Table 3.26**). On the basis of 2D NMR data one α -rhamnopyranosyl (δ 5.23), one β -xylopyranosyl (δ 4.82) and one β -glucuronic acid (δ 4.44) were identified. The determination of the sequence and linkage sites was obtained from the HMBC correlations between the proton signals at δ 5.23 (H-1_{rha}) and the carbon resonance at δ 79.3 (C-2_{xy}), the signal at 4.82 (H-1_{xy}) with the carbon signal at 78.8 (C-2_{gluA}), and the proton signal at δ 4.44 (H-1_{gluA}) and the carbon resonance at δ 92.4 (C-3). The D configuration of glucuronic acid and xylose units and the L configuration of rhamnose unit were established after hydrolysis of **AAN7** followed by GC analysis (De Marino et al., 2003). Thus, sequence of compounds **AAN7-AAN8** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranoside. Moreover, the aglycone of the compounds **AAN7-AAN10** was recognized to be an oleanane-type triterpene by ^1H NMR and ^{13}C NMR analysis (**Table 3.26**) (De Tommasi et al., 1998).

The HRMALDITOF spectrum of **AAN7** (m/z 967.4881 [$\text{M}+\text{Na}$] $^+$, calcd for $\text{C}_{47}\text{H}_{76}\text{O}_{19}\text{Na}$, 967.4879) supported a molecular formula of $\text{C}_{47}\text{H}_{76}\text{O}_{19}$. The ESIMS spectrum showed the major ion peak at m/z 967 which was assigned to [$\text{M}+\text{Na}$] $^+$. The MS/MS of this ion showed peak at m/z 821 [$\text{M}+\text{Na}-146$] $^+$, due to the loss of a deoxyhexose unit at m/z 689 [$\text{M}+\text{Na}-146-132$] $^+$, corresponding to the loss of a xylopyranosyl unit.

The ^1H NMR spectrum of **AAN7** showed signals for six methyl groups as singlets at δ 1.24, 1.23, 1.03, 1.00, 0.98, 0.90, one olefinic proton at δ 5.27 (H-12, d, $J = 3.5$ Hz), three oxygen-bearing methine protons at δ 3.35 (H-3, dd $J = 11.5$, 4.5 Hz), 3.83 (H-21, brs), 3.36 (H-22, dd $J = 3.5$) and signals for two primary alcoholic functions at δ 3.23 and 4.12 (H₂-24, d $J = 11.5$), and 3.12 and 3.45 (H₂-29, d, $J = 10.5$) (**Table 3.26**). In the HMBC spectrum, correlations between the proton signal at δ 3.83 and the carbon signals at δ 71.8 (C-29) and 16.8 (Me-30) revealed the location of a secondary alcoholic function at C-21; a further correlation between the proton signal at δ 0.98 (Me-28) and the carbon at δ 80.8 allowed us to assign the additional secondary alcoholic function at C-22. The β -

orientation of the hydroxyl group at C-21 and the α -orientation of the hydroxyl group at C-22 were derived from the ROESY spectrum, which showed key correlation peaks between H-21 (δ 3.83) with the proton signals H-29 (3.45) and H-19 α (2.12) and between H-22 (δ 3.36) and Me-28 (0.98). Detailed NMR studies allowed us to identify the aglycone of AAN7 as C-22 epimer of kudzusapogenol A (Kinjo et al., 1985).

Thus, compound AAN7 was identified as the new 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-3 β ,21 β ,22 α ,24,29-pentahydroxy-olean-12-ene (**Fig. 3.26**)

Amorphous white solid; C₄₇H₇₆O₁₉; [α]_D²⁵ -10.9 (*c* 0.01 MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3455 (>OH), 2945 (>CH), 1666 (C=C), 1252 and 1052 (C-O-C); ESI-MS *m/z* 967 [M+Na]⁺, MS/MS *m/z* 821 [M+Na-146]⁺, *m/z* 689 [M+Na-146-132]; HRMALDITOFMS *m/z* [M+Na]⁺ calcd for C₄₇H₇₆O₁₉Na, 967.4879, found 967.4881; ¹H NMR (CD₃OD, 600 MHz) and ¹³C NMR (CD₃OD, 150 MHz) data: see **Table 3.26**.

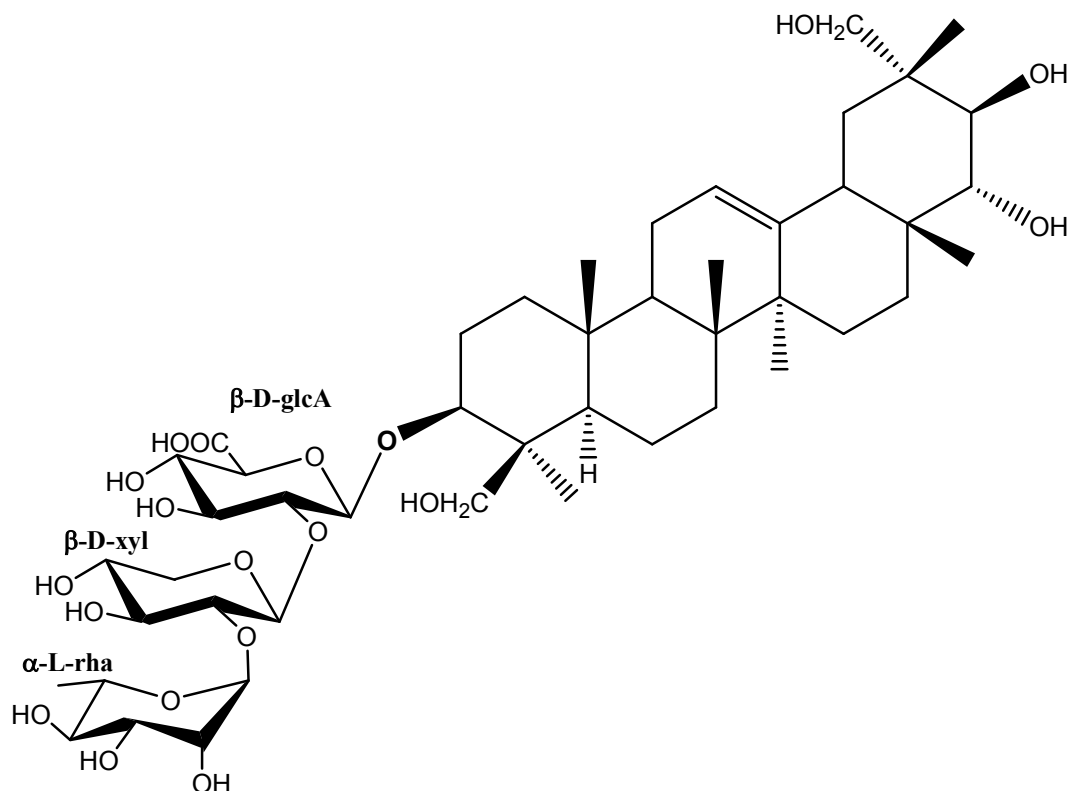
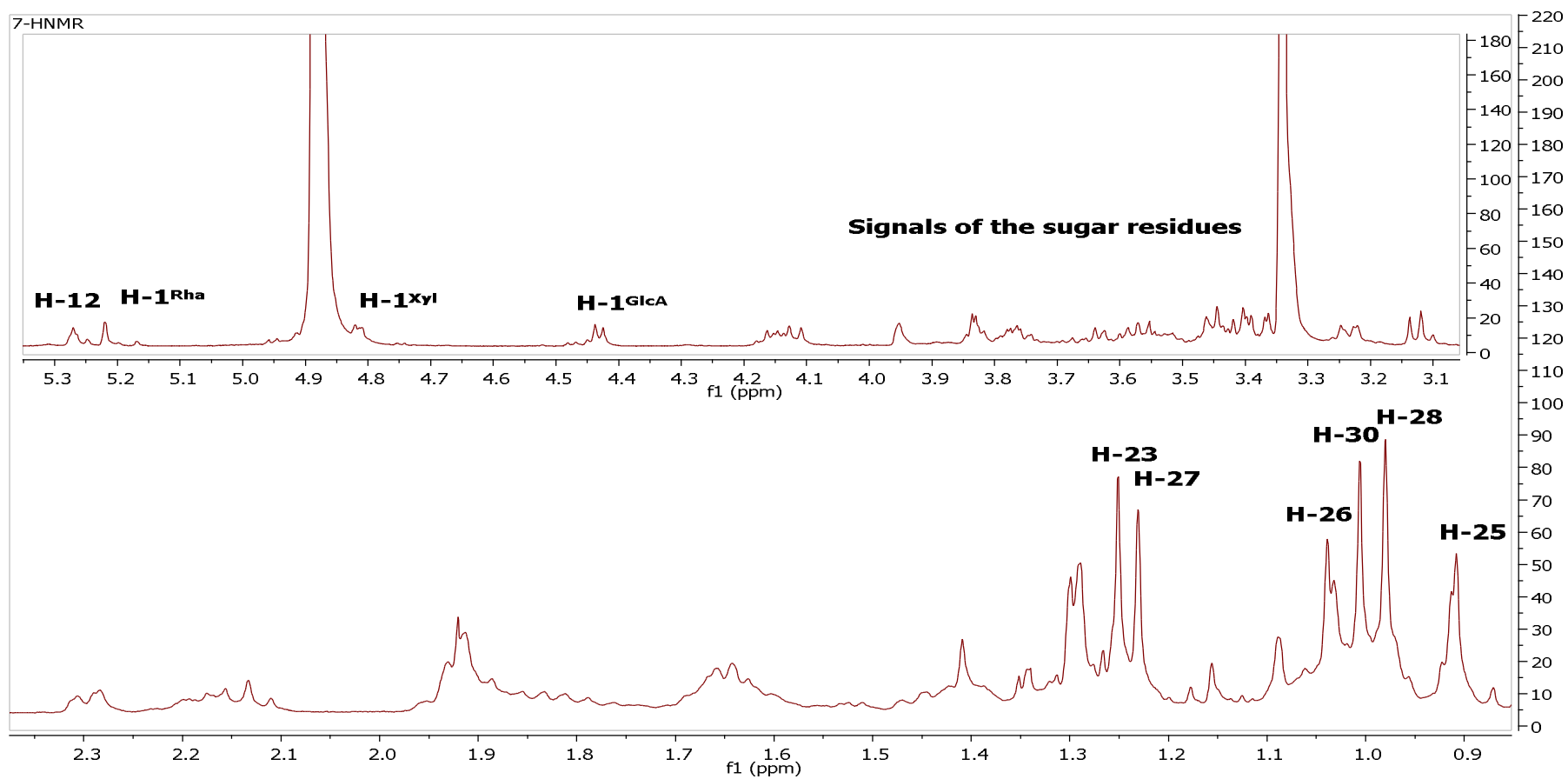


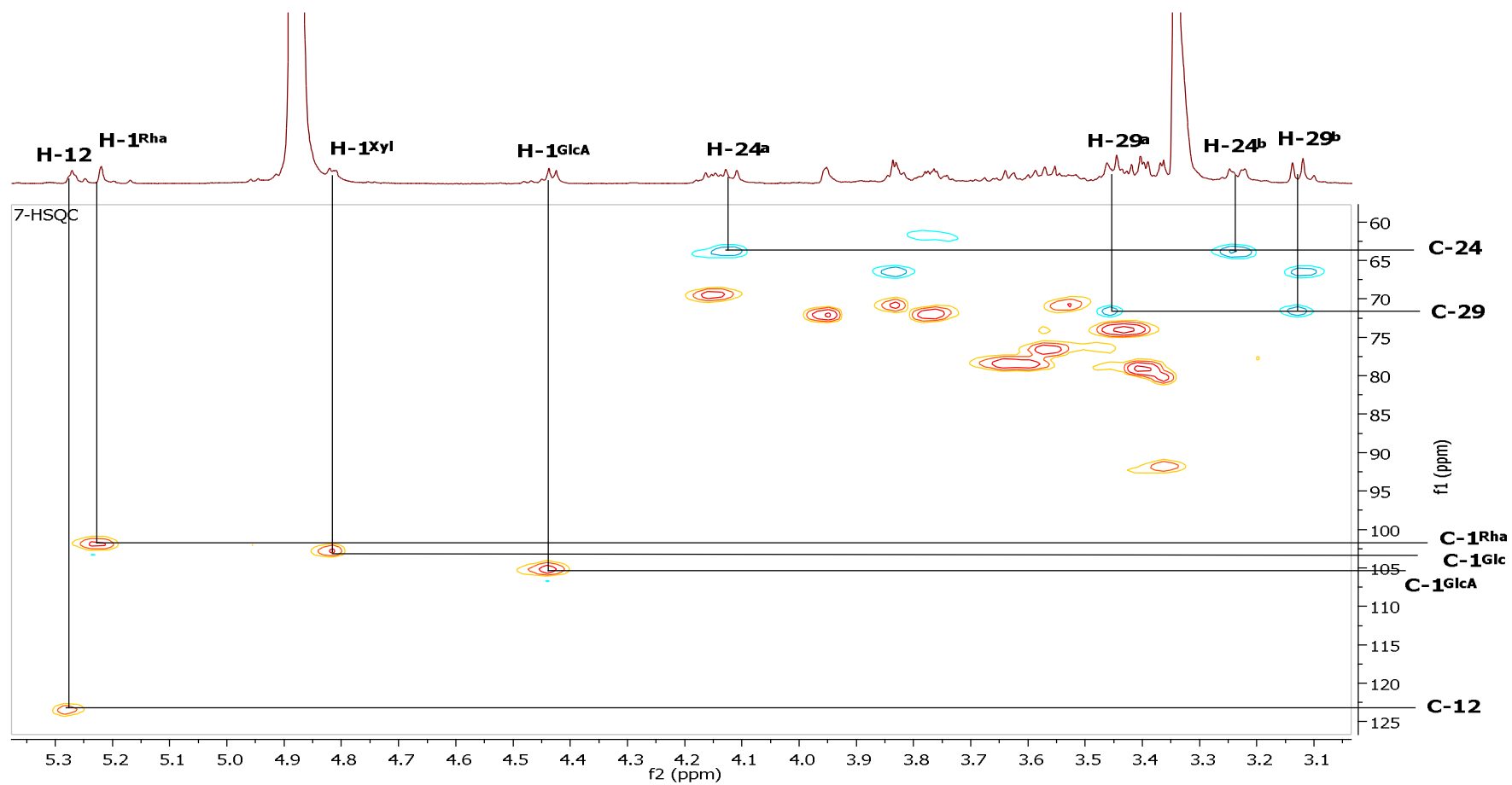
Figure 3.26. Structure of AAN7

Table 3.26. ^1H and ^{13}C Assignments of **AAN7** (150/600 MHz, δ ppm, in CD_3OD)

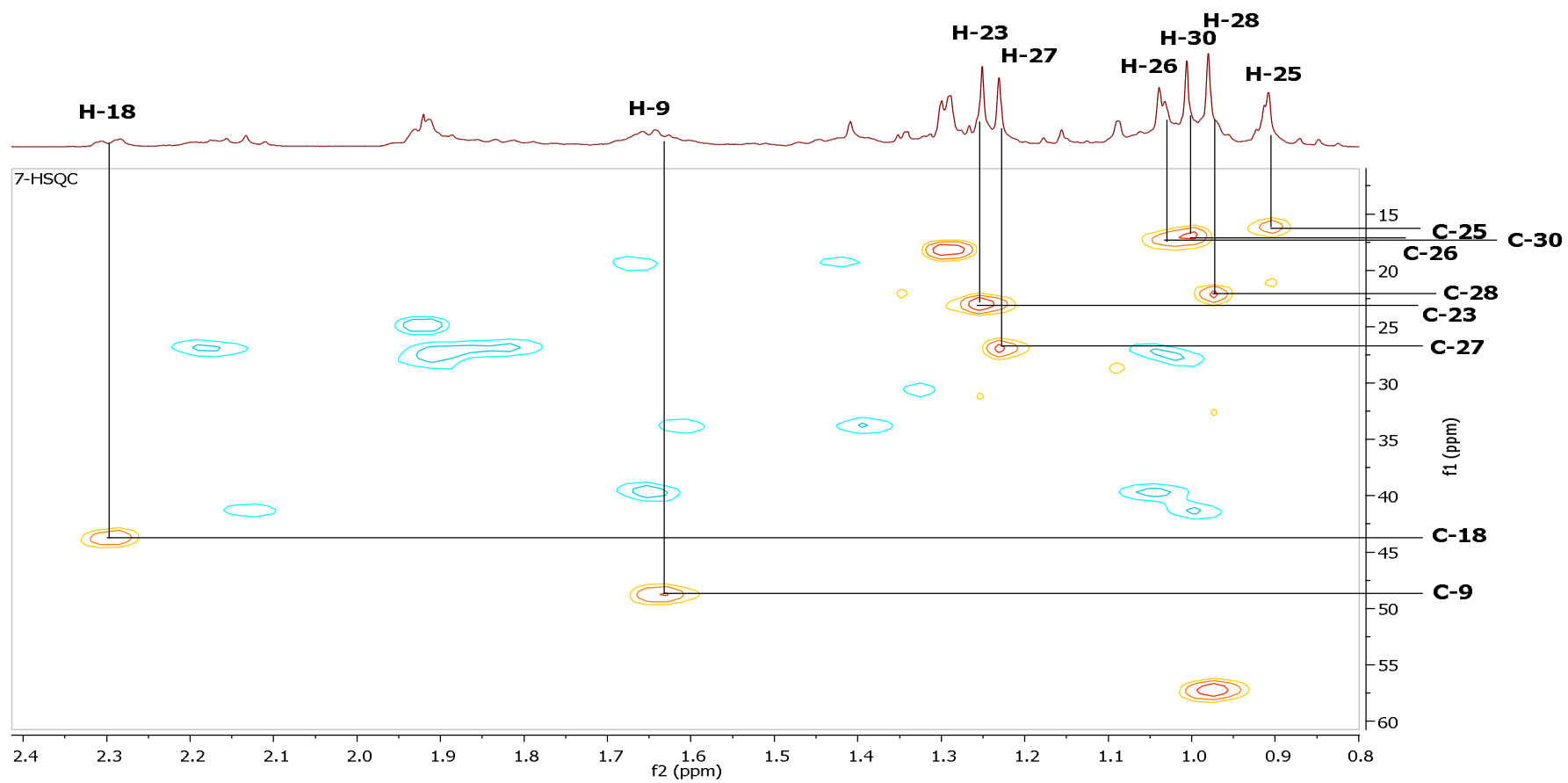
Position	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	39.7	1.64, 1.04, m
2	26.7	2.18, 1.80, m
3	92.4	3.35, dd (11.5, 4.5)
4	44.0	-
5	57.5	0.97, m
6	19.0	1.67, 1.41
7	33.8	1.61, 1.38, m
8	41.0	-
9	48.7	1.62, m
10	37.5	-
11	24.6	1.92, (2H) m
12	123.7	5.27, t (3.5)
13	146.0	-
14	43.1	-
15	27.3	1.90, 1.02, m
16	30.4	1.91, 1.00, m
17	39.7	-
18	43.9	2.29, m
19	41.3	2.12, 0.99, m
20	41.5	-
21	70.7	3.83, brs
22	80.8	3.36, d (3.5)
23	22.8	1.24, s
24	64.2	4.12, d (11.5), 3.23, d (11.5)
25	15.9	0.90, s
26	17.2	1.00, s
27	26.8	1.23, s
28	21.9	0.98, s
29	71.8	3.45, d (10.5), 3.12, d (10.5)
30	16.8	1.03
β -D-GlcA (at C-3)-1'	105.6	4.44, d (7.5)
2'	78.8	3.58, dd (7.5, 9.0)
3'	78.8	3.65, dd (9.0, 9.0)
4'	74.1	3.43, dd (9.0, 9.0)
5'	77.0	3.57, d (9.0)
6'	177.1	-
β -D-Xyl (at C-2 _{glcA})-1''	103.3	4.82, d (7.5)
2''	79.3	3.41, dd (7.5, 9.2)
3''	77.4	3.52, t (9.2)
4''	71.0	3.53, m
5''	66.6	3.83, dd (5.2, 11.7), 3.11, t (11.7)
α -L-Rha (at C-2 _{xyl})-1'''	102.3	5.23, d (1.2)
2'''	72.3	3.96, dd (1.2, 3.2)
3'''	72.2	3.76, dd (3.2, 9.7)
4'''	74.1	3.42, t (9.7)
5'''	69.7	4.15, m
6'''	17.6	1.26, d (6.5)



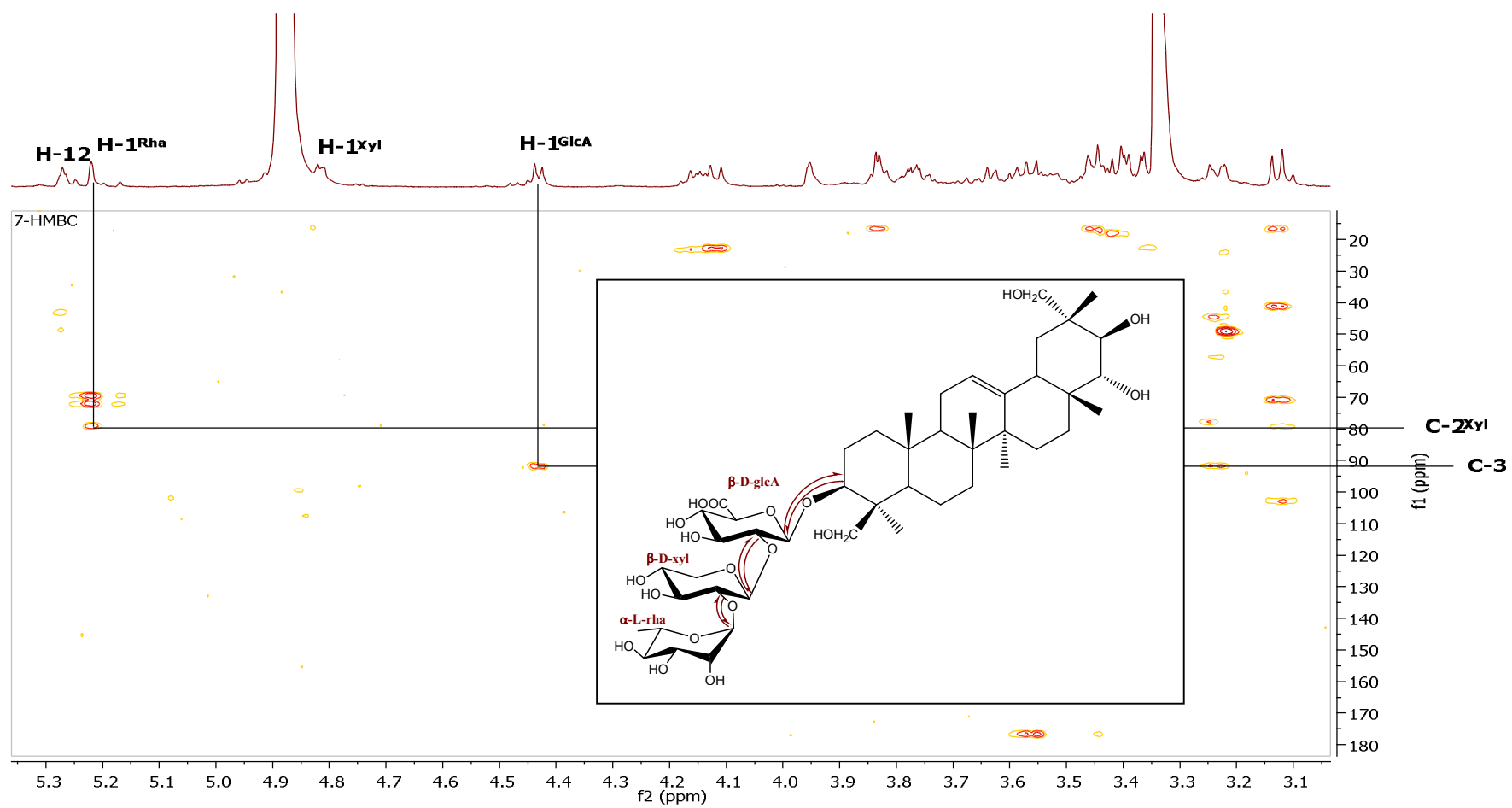
Spectrum 3.121. ^1H NMR spectrum of AAN7 (600 MHz, CD_3OD)



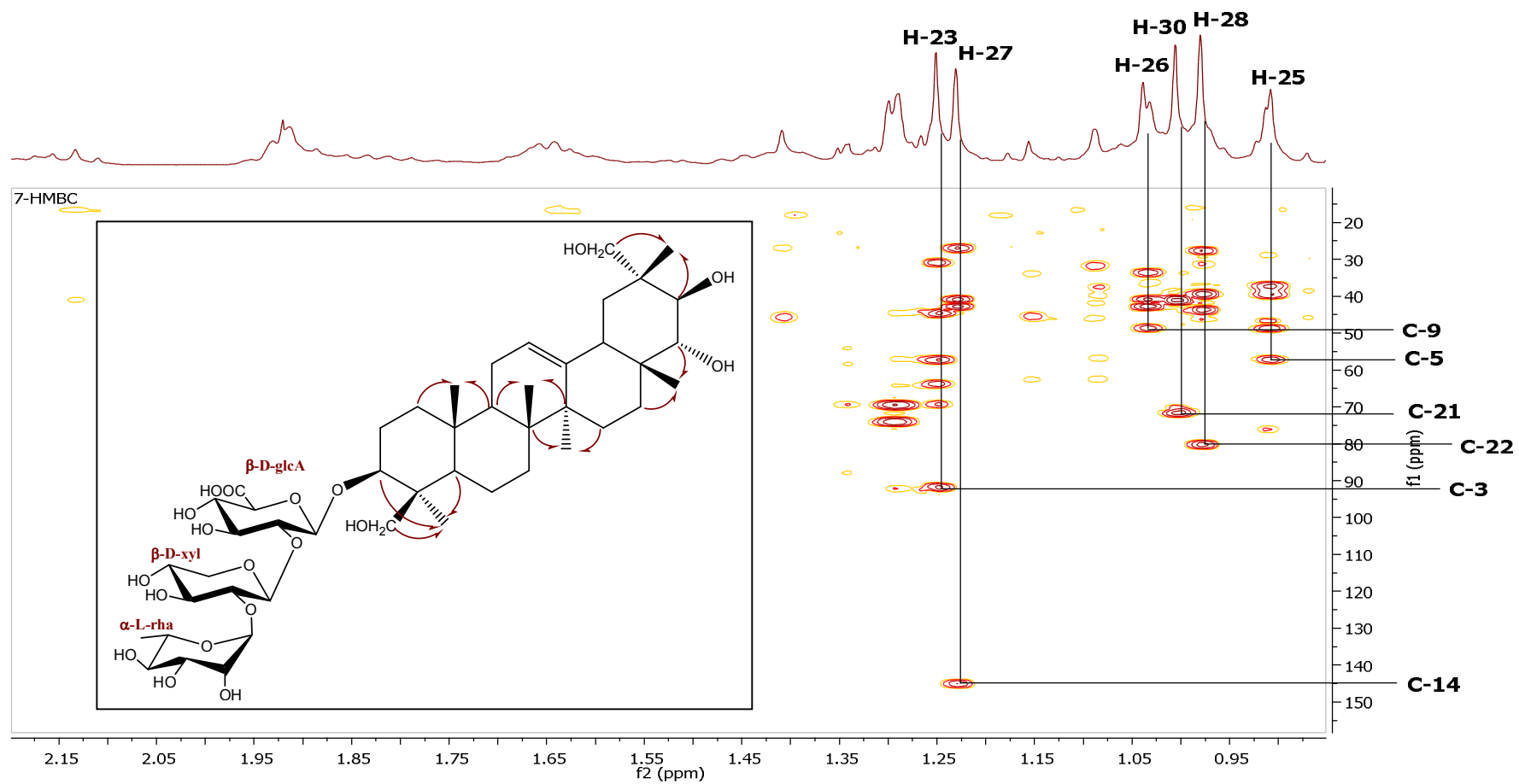
Spectrum 3.122. HSQC Spectrum of AAN7



Spectrum 3.123. HSQC Spectrum of AAN7



Spectrum 3.124. HMBC Spectrum of AAN7



Spectrum 3.125. HMBC Spectrum of AAN7

3.3.8. Structural Identification of Compound AAN8

The HRMALDITOF mass spectrum of **AAN8** (m/z 965.4726 $[M+Na]^+$, calcd for $C_{47}H_{74}O_{19}Na$, 965.4722) supported a molecular formula of $C_{47}H_{74}O_{19}$. The ESIMS spectrum showed the major ion peak at m/z 965 which was assigned to $[M+Na]^+$. In the MS/MS ion peaks at m/z 819 $[M+Na-146]^+$, due to the loss of a deoxyhexose unit, and at m/z 687 $[M+Na-146-132]^+$, corresponding to the loss of a xylopyranosyl unit, were observed.

The ^1H NMR spectrum of **AAN8** displayed signals for six tertiary methyl groups at δ 1.24 (6H), 1.18, 1.00, 0.91, 0.83, for an olefinic proton at δ 5.31 (t, $J = 3.5$ Hz), two oxygen-bearing methine protons at δ 3.36 (H-3, dd, $J = 11.5, 4.5$ Hz), 3.52 (H-22, brs) and one primary alcoholic function at δ 4.11 and 3.23 (H₂-24, d, $J = 11.5$ Hz) (**Table 3.27**). A combination of 1D and 2D NMR techniques led to the assignment of ^1H and ^{13}C NMR signals due to A, B, C and D rings of a 3 β ,24-dihydroxyolean-12-ene sapogenol unit (Kitawaga et al., 1983a), glycosylated at C-3. NMR data showed ring E containing an oxymethine at C-22 (δ_C 77.3, δ_H 3.52 brs) and a -COOH group at C-30 (δ_C 187.8).

Thus, the structure of compound **AAN8** was elucidated as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-3 β ,22 β ,24-trihydroxy-olean-12-en-29-oic acid (**Fig. 3.27**).

Amorphous white solid; $C_{47}H_{74}O_{19}$; $[\alpha]_D^{25} +7.12$ (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3451 ($>OH$), 2937 ($>CH$), 1735 (C=O), 1658 (C=C), 1248 and 1048 (C-O-C); ESI-MS m/z 965 $[M+Na]^+$, MS/MS m/z 819 $[M+Na-146]^+$, m/z 687 $[M+Na-146-132]$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{47}H_{74}O_{19}Na$, 965.4722, found 965.4726; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.27**.

Table 3.27. ^1H and ^{13}C Assignments of AAN8 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	39.7	1.66, 1.04, m
2	26.8	2.18, 1.80, m
3	92.6	3.36, dd (11.5, 4.2)
4	44.4	-
5	57.5	0.98, m
6	19.1	1.66, 1.39, m
7	34.7	1.60, 1.43, m
8	40.4	-
9	48.7	1.60, m
10	37.5	-
11	24.5	1.90, (2H) m
12	124.1	5.31, t (3.5)
13	145.5	-
14	43.5	-
15	26.3	1.80, 1.06, m
16	29.9	1.80, 1.31, m
17	38.2	-
18	46.4	2.02, m
19	42.8	2.38, 1.18, m
20	44.4	-
21	38.8	2.08, 1.45, m
22	77.3	3.52, brs
23	22.6	1.24, s
24	63.6	4.11, d (11.4), 3.23, d (11.4)
25	15.9	0.91, s
26	17.5	1.00, s
27	24.9	1.18, s
28	19.8	0.83, s
29	26.2	1.24, s
30	187.8	-
β -D-GlcA (at C-3)-1'	105.6	4.44, d (7.5)
2'	78.8	3.58, dd (7.5, 9.0)
3'	78.8	3.65, dd (9.0, 9.0)
4'	74.1	3.43, dd (9.0, 9.0)
5'	77.0	3.57, d (9.0)
6'	177.1	-
β -D-Xyl (at C-2 _{glcA})-1''	103.3	4.82, d (7.5)
2''	79.3	3.41, dd (7.5, 9.2)
3''	77.4	3.52, t (9.2)
4''	71.0	3.53, m
5''	66.6	3.83, dd (5.2, 11.7), 3.11, t (11.7)
α -L-Rha (at C-2 _{xyl})-1'''	102.3	5.23, d (1.2)
2'''	72.3	3.96, dd (1.2, 3.2)
3'''	72.2	3.76, dd (3.2, 9.7)
4'''	74.1	3.42, t (9.7)
5'''	69.7	4.15, m
6'''	17.6	1.23, d (6.5)

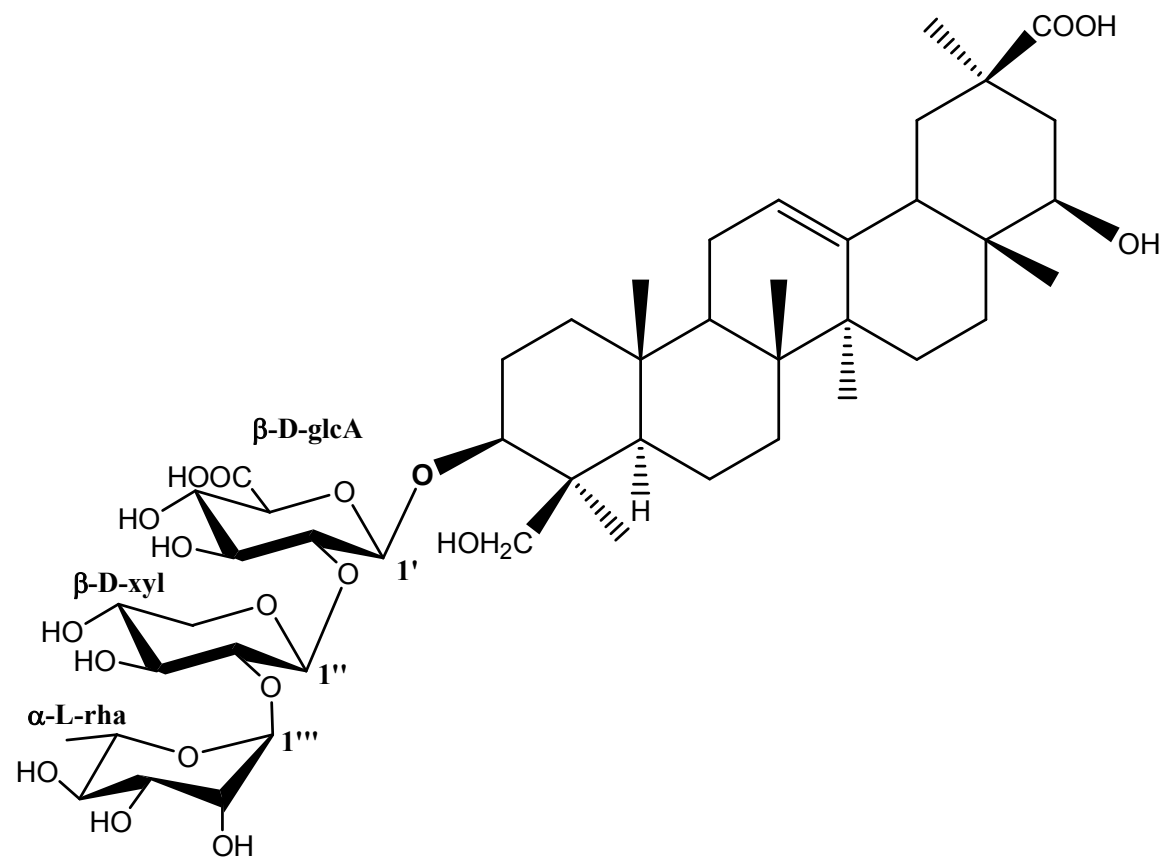
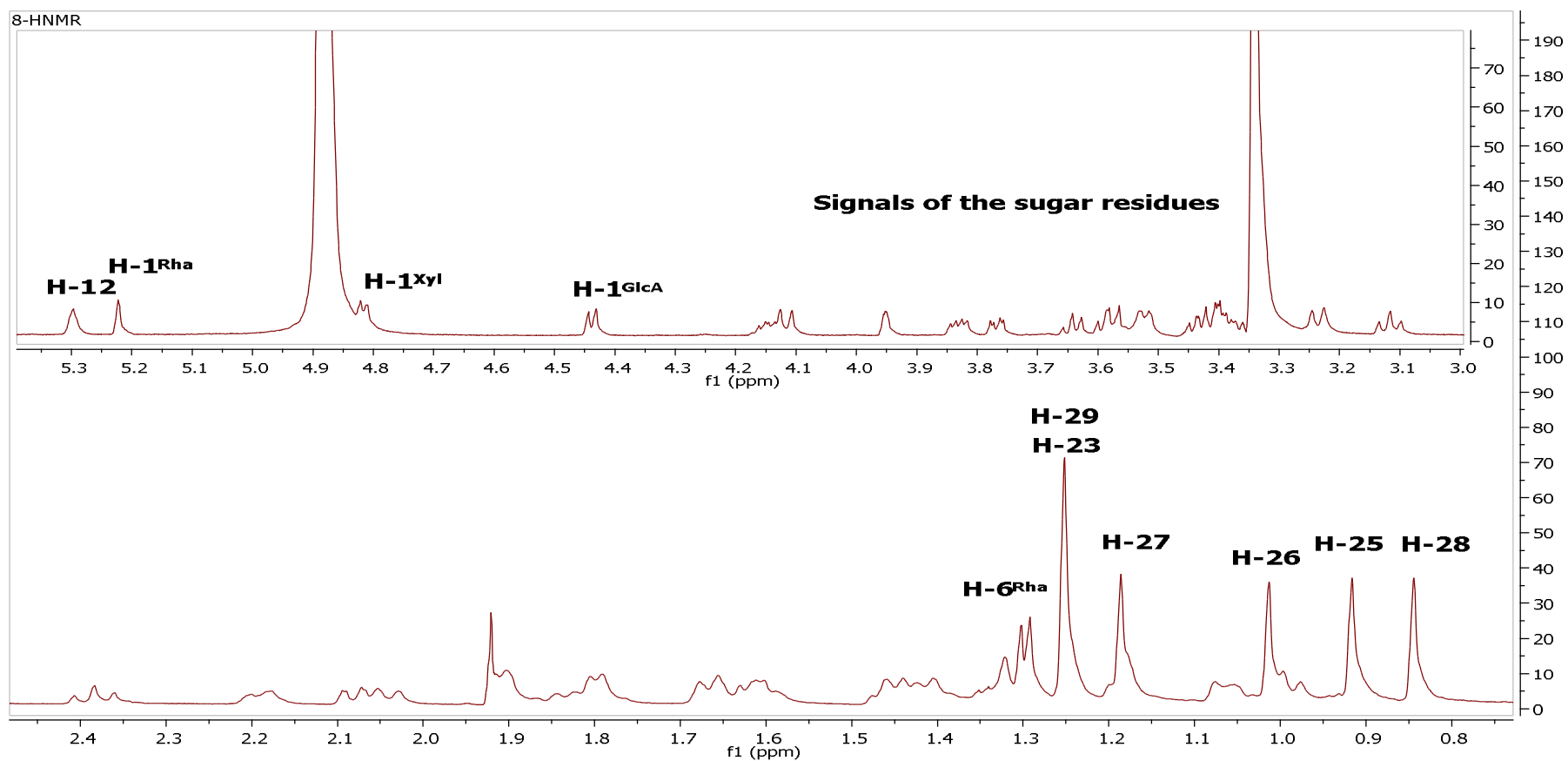
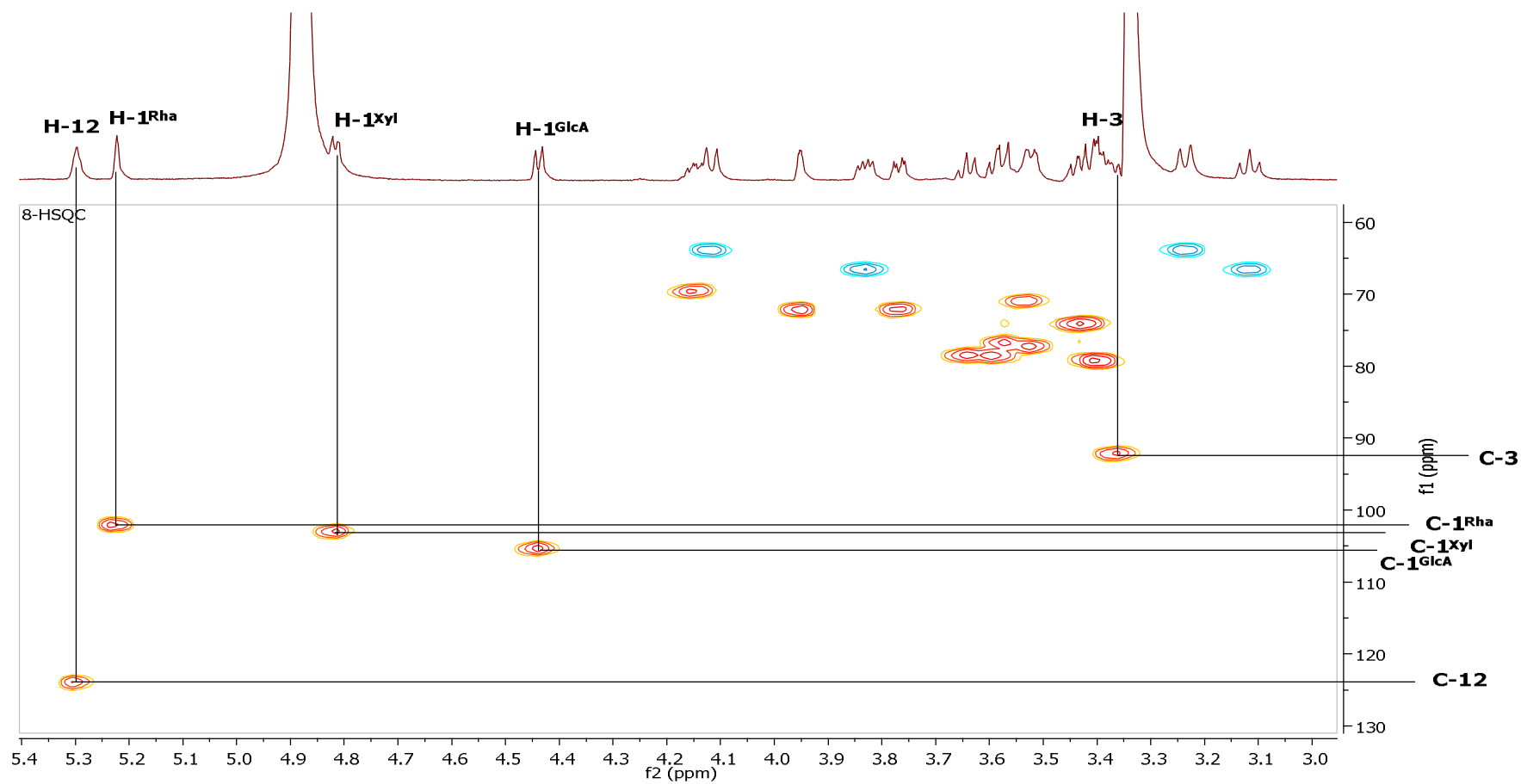


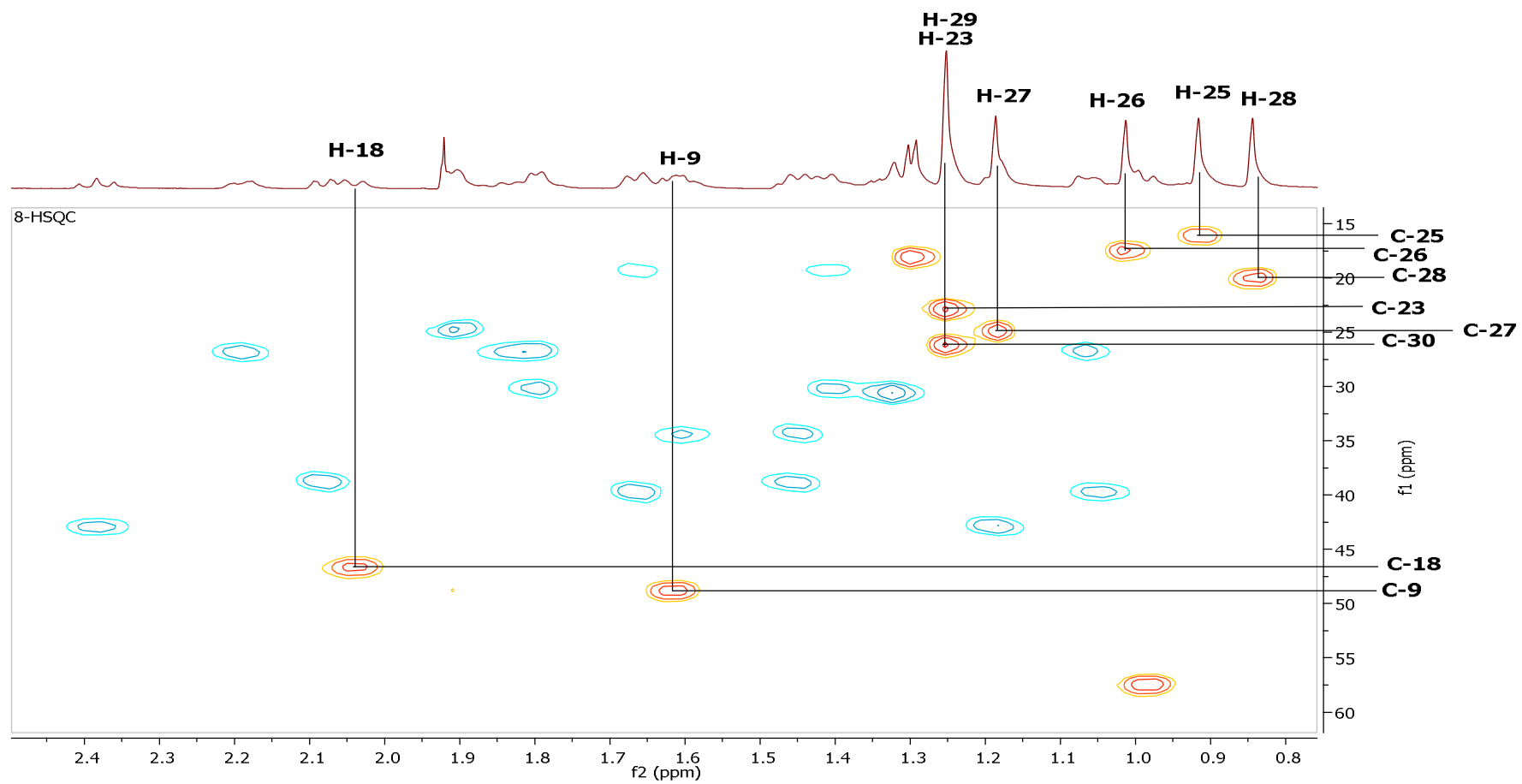
Figure 3.27. Structure of AAN8



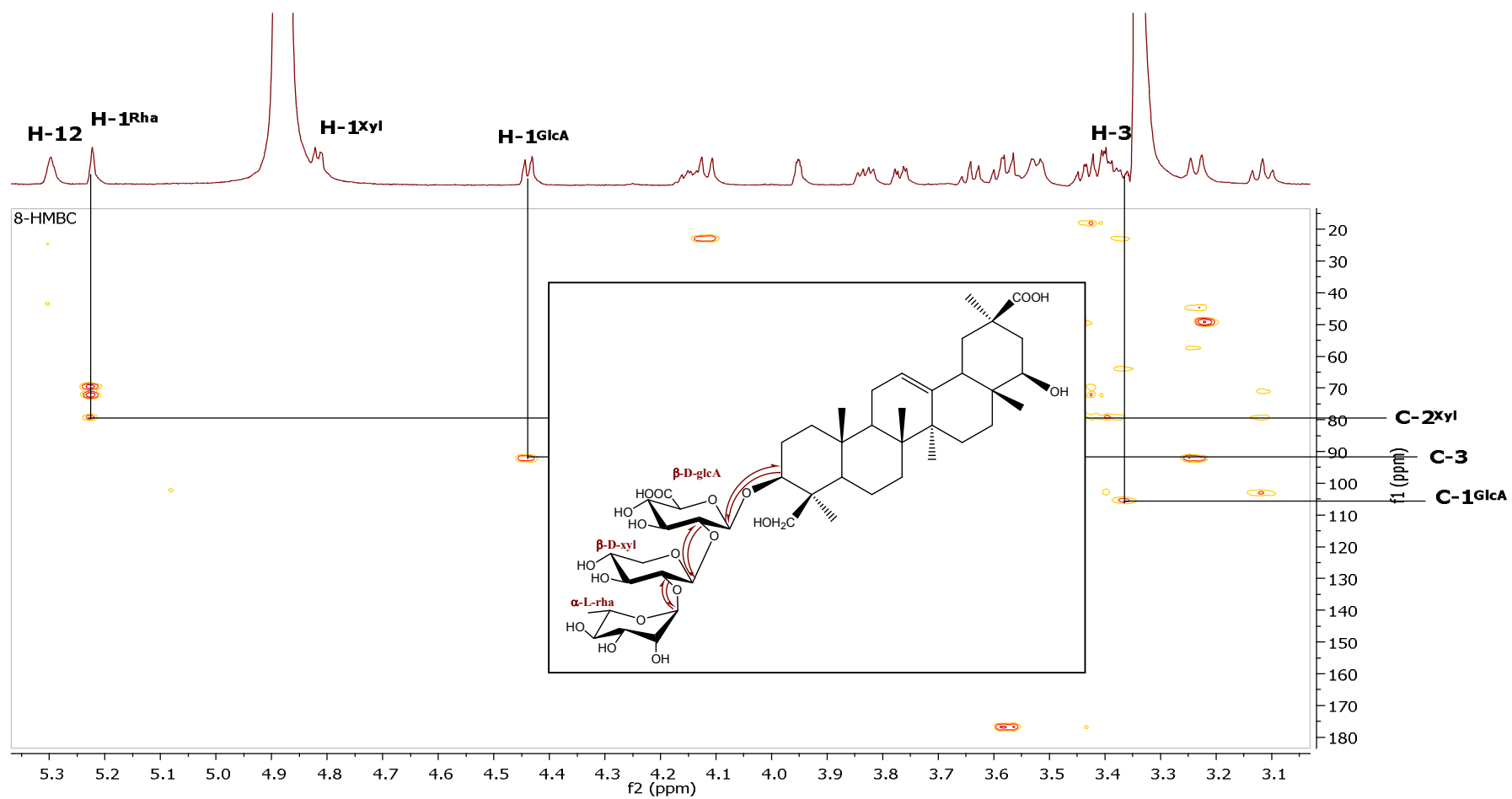
Spectrum 3.126. ^1H NMR spectrum of AAN8 (600 MHz, CD_3OD)



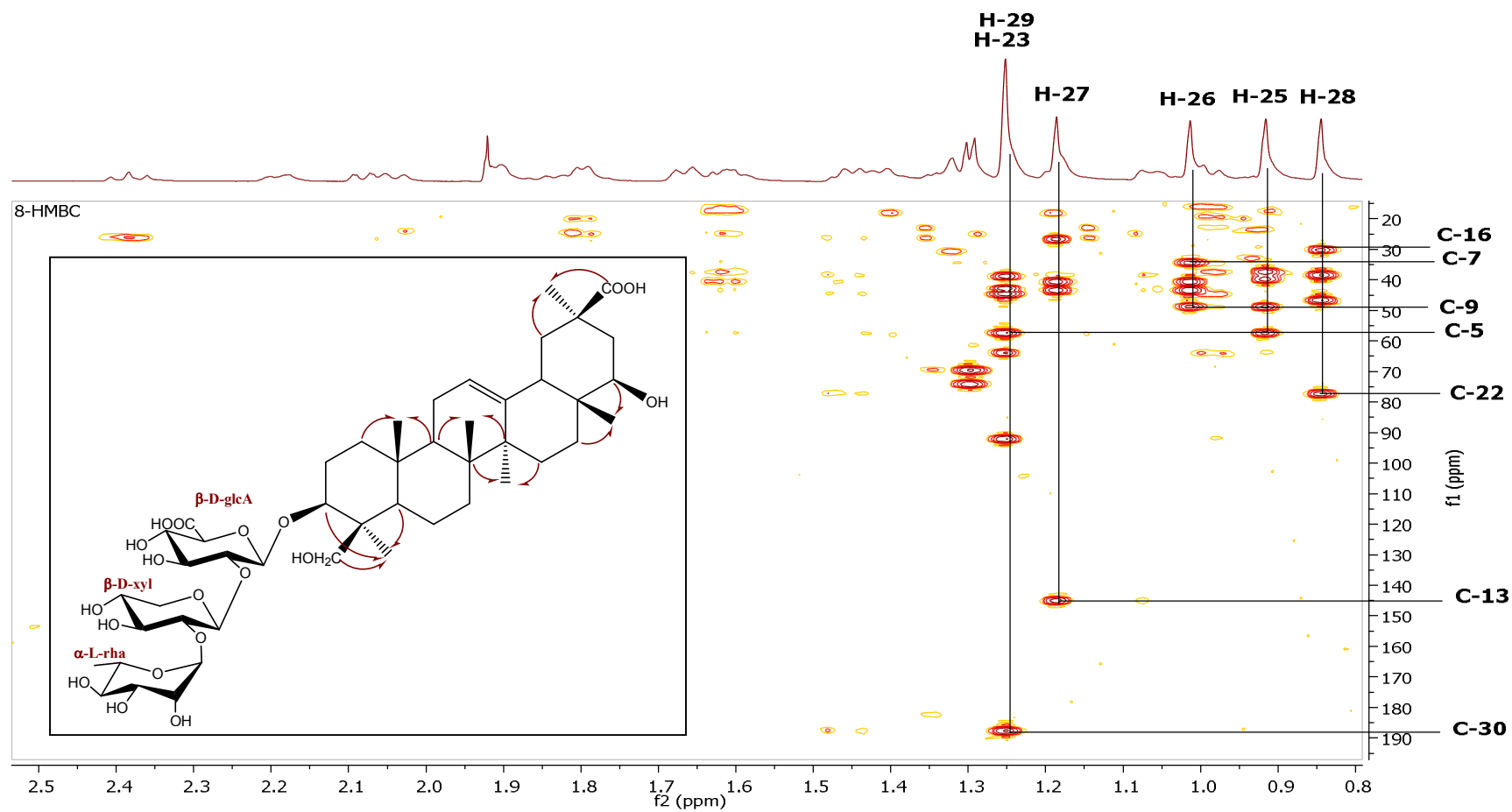
Spectrum 3.127. HSQC Spectrum of AAN8



Spectrum 3.128. HSQC Spectrum of AAN8



Spectrum 3.129. HMBC Spectrum of AAN8



Spectrum 3.130. HMBC Spectrum of AAN8

3.3.9. Structural Identification of Compound AAN9

The HRMALDITOF mass spectrum of **AAN9** (m/z 1067.5406 $[M+Na]^+$, calcd for $C_{52}H_{84}O_{21}Na$, 1067.5403) supported a molecular formula of $C_{52}H_{84}O_{21}$. The ESIMS spectrum showed the major ion peak at m/z 1067 which was assigned to $[M+Na]^+$. The MS/MS of this ion showed peaks at m/z 921 $[M+Na-146]^+$, due to the loss of a deoxyhexose unit, at m/z 789 $[M+Na-146-132]^+$, corresponding to the loss of a xylopyranosyl unit, and at m/z 613 $[M+Na-146-132-176]^+$, due to the loss of a glucuronopyranosyl unit.

The ^1H NMR spectrum displayed signals for seven tertiary methyl groups at δ 1.25, 1.16, 1.06, 1.00, 0.94, 0.93 and 0.90, and for an olefinic proton at δ 5.31 (1H, t, $J = 3.5$ Hz) (Table 4). These signals along with the carbon resonances in the ^{13}C spectrum for the methyl groups at δ 32.5, 28.8, 25.4, 22.8, 21.1, 17.5, 16.0 and the olefinic carbons at δ 145.1, 123.8, suggested that compound **AAN9** possessed an olean-12-ene skeleton as aglycone (Table 3.28). On the basis of COSY, HSQC and HMBC spectra and by comparison of these data with those of astragaloside VIII (Kitawaga et al., 1983b), compound **AAN9** differed from astragaloside VIII only by the presence of an additional α -arabinopyranosyl unit. In the HMBC spectrum, not only cross-peaks corresponding to the sugar chain at C-3 described above were observed but also an additional fourth anomeric proton signal at δ 4.27 assigned to the α -arabinopyranose. The correlation between the anomeric proton at δ 4.27 and the carbon resonance at δ 83.3 allowed us to locate this sugar at position C-22. The L-configuration of the arabinose unit was established after hydrolysis of **AAN9** followed by GC analysis on a chiral column (De Marino et al., 2003). Therefore, the structure of **AAN9** was established as 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-22-*O*- α -L-arabinopyranosyl-3 β ,22 β ,24-trihydroxy-olean-12-ene (Fig. 3.28).

Amorphous white solid; $C_{52}H_{84}O_{21}$; $[\alpha]_D^{25}$ -8.54 (c 0.01 MeOH); IR ν_{max}^{KBr} cm^{-1} : 3446 ($>OH$), 2930 ($>CH$), 1730 ($C=O$), 1648 ($C=C$), 1245 and 1052 ($C-O-C$); ESI-MS m/z 1067 $[M+Na]^+$, MS/MS m/z 921 $[M+Na-146]^+$, m/z 789 $[M+Na-146-132]^+$, m/z 613 $[M+Na-146-132-176]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{52}H_{84}O_{21}Na$, 1067.5403, found 1067.5406; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see Table 3.28.

Table 3.28. ^1H and ^{13}C Assignments of AAN9 (150/600 MHz, δ ppm, in CD_3OD)

Position	δ_{C}	δ_{H} (J in Hz)
1	39.5	1.67, 1.04, m
2	26.8	2.18, 1.82, m
3	91.9	3.35, dd (11.5, 4.2)
4	44.4	-
5	57.0	0.96, m
6	19.0	1.67, 1.41, m
7	33.7	1.60, 1.43, m
8	40.0	-
9	48.3	1.60, m
10	36.7	-
11	24.5	1.90, (2H) m
12	123.8	5.31, t (3.5)
13	145.1	-
14	42.5	-
15	26.1	1.79, 1.05, m
16	29.2	1.80, 1.38, m
17	36.8	-
18	46.9	2.12, m
19	47.0	1.78, 1.00, m
20	31.1	-
21	36.8	1.57, 1.37, m
22	83.3	3.44, brs
23	22.8	1.25, s
24	63.4	4.12, d (11.4), 3.23, d (11.4)
25	16.0	0.90, s
26	17.5	1.00, s
27	25.4	1.16, s
28	21.1	0.93, s
29	32.5	0.94, s
30	28.8	1.06, s
β -D-GlcA (at C-3)-1'	105.6	4.43, d (7.5)
2'	78.6	3.58, dd (7.5, 9.0)
3'	78.6	3.64, dd (9.0, 9.0)
4'	74.2	3.43, dd (9.0, 9.0)
5'	76.9	3.57, d (9.0)
6'	177.2	-
β -D-Xyl (at C-2 _{glcA})-1''	103.2	4.82, d (7.5)
2''	79.4	3.40, dd (7.5, 9.2)
3''	76.5	3.57, t (9.2)
4''	71.0	3.54, m
5''	66.6	3.84, dd (5.2, 11.7), 3.11, t (11.7)
α -L-Rha (at C-2 _{xyl})-1'''	102.3	5.23, d (1.2)
2'''	72.0	3.97, dd (1.2, 3.2)
3'''	72.1	3.78, dd (3.2, 9.7)
4'''	74.2	3.43, t (9.7)
5'''	69.7	4.16, m
6'''	17.5	1.25, d (6.5)
α -L-Ara (at C-22)-1''''	102.0	4.27, d (3.7)
2''''	74.2	3.60, dd (8.5, 3.7)
3''''	72.2	3.58, dd (8.5, 3.0)
4''''	69.0	3.86, m
5''''	65.7	3.89 dd (11.9, 2.0), 3.52, dd (11.9, 3.0)

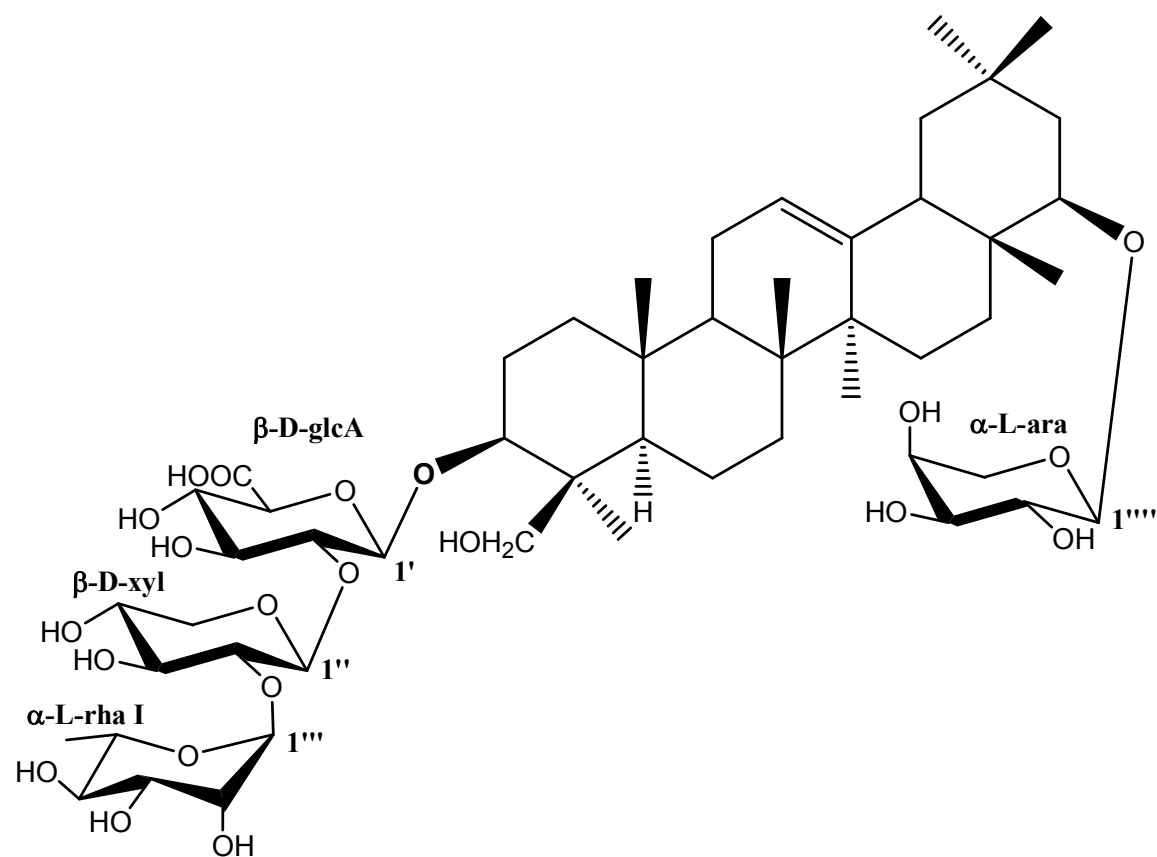
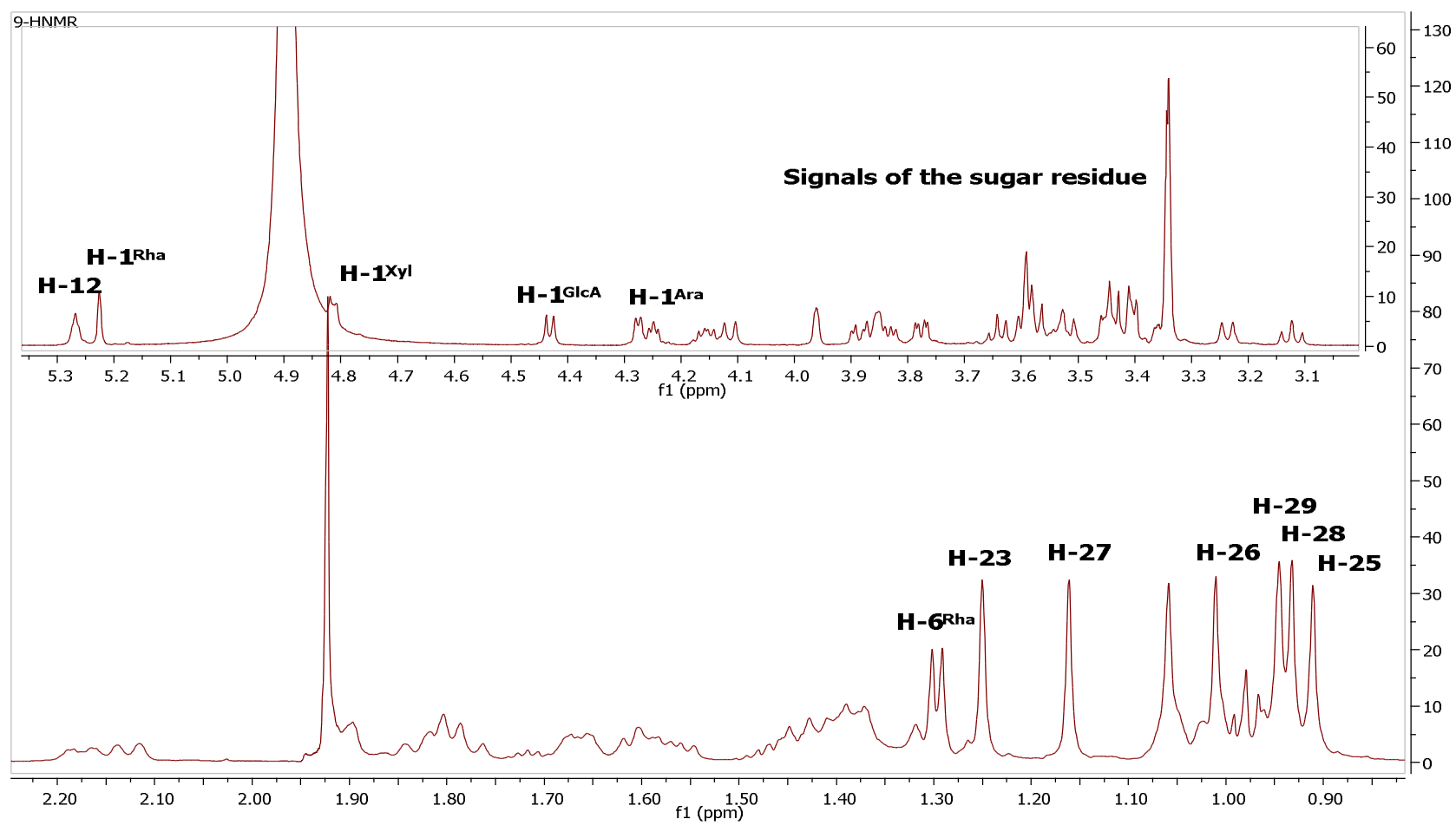
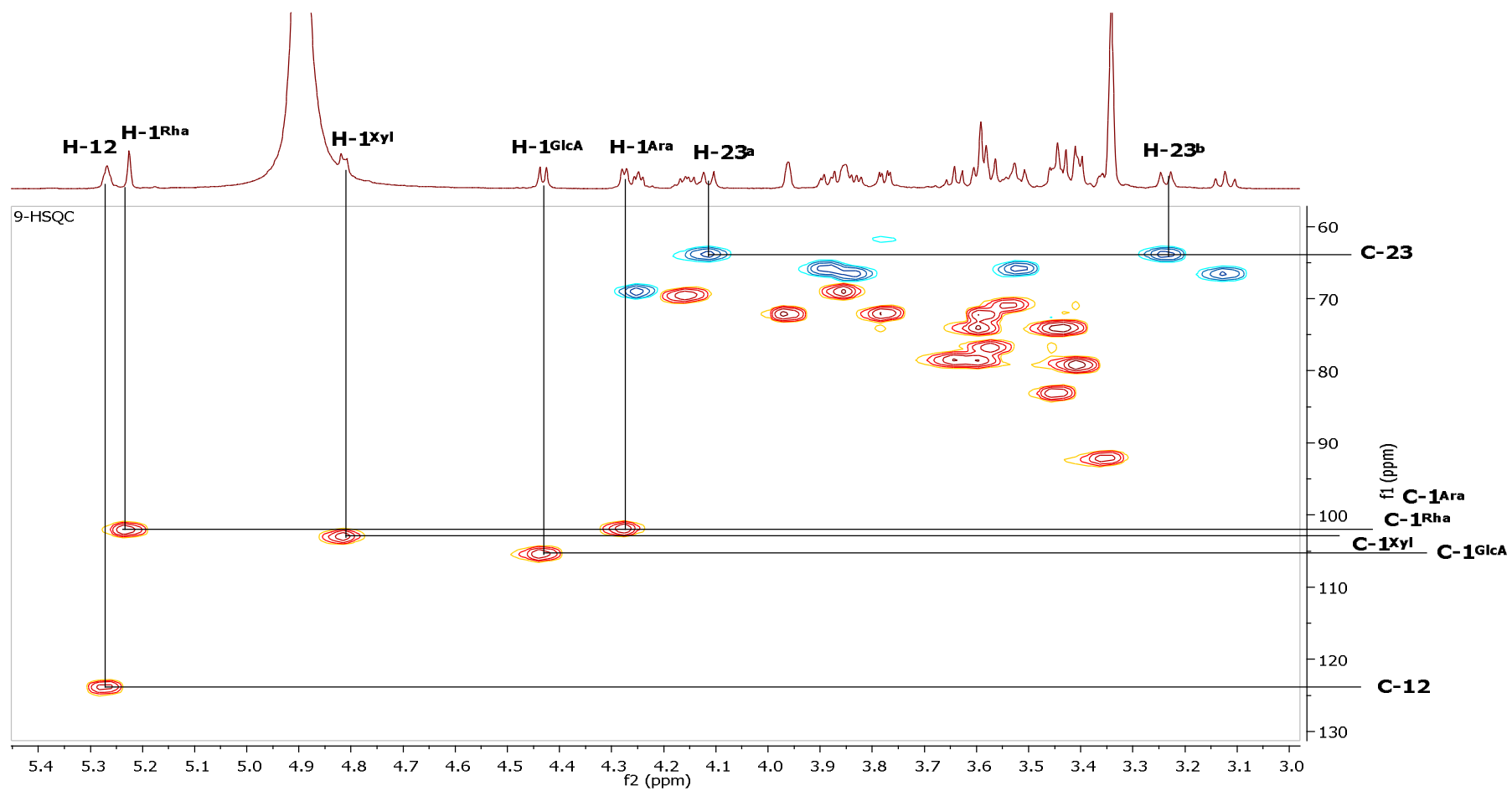


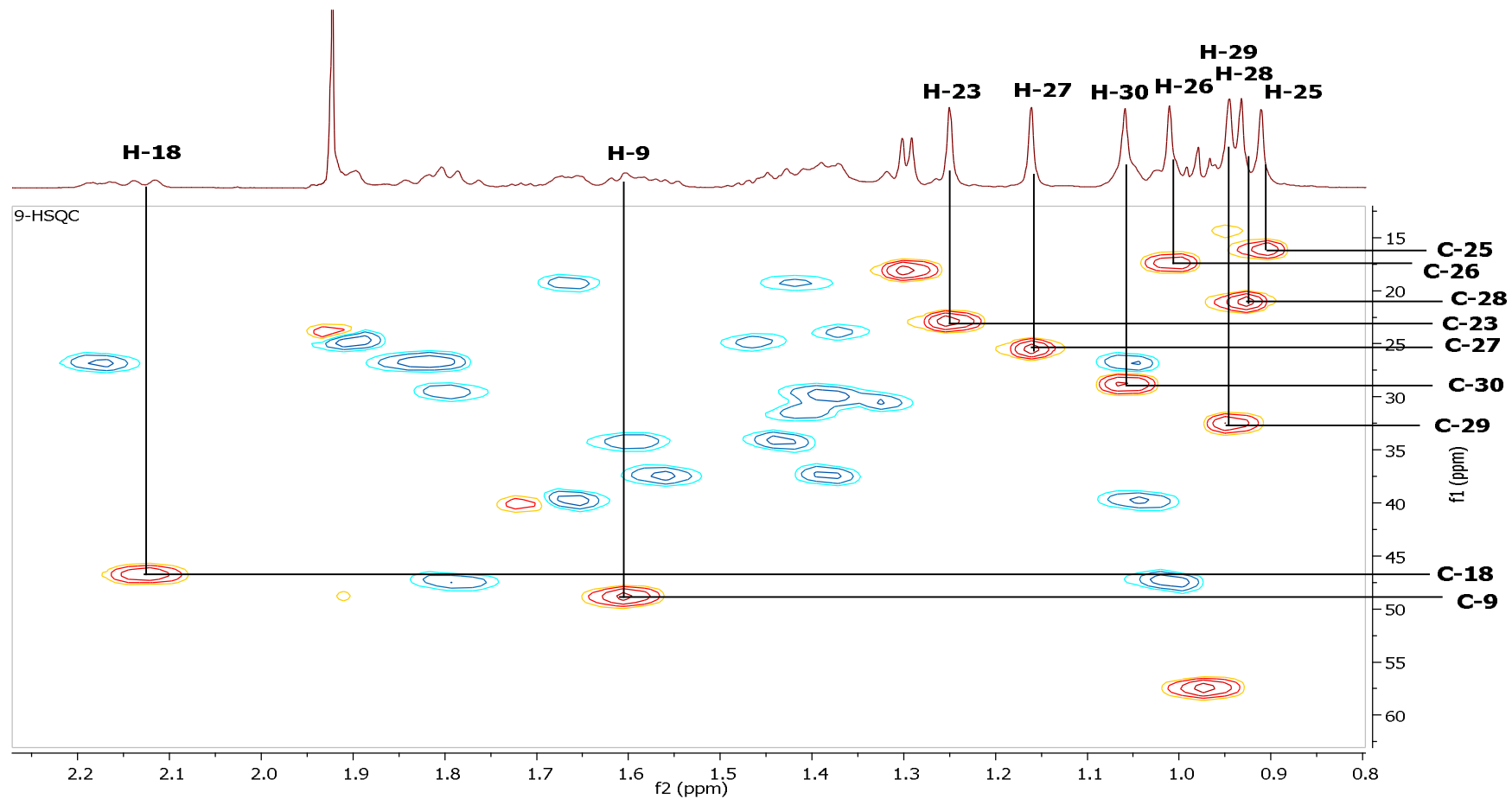
Figure 3.28. Structure of AAN9



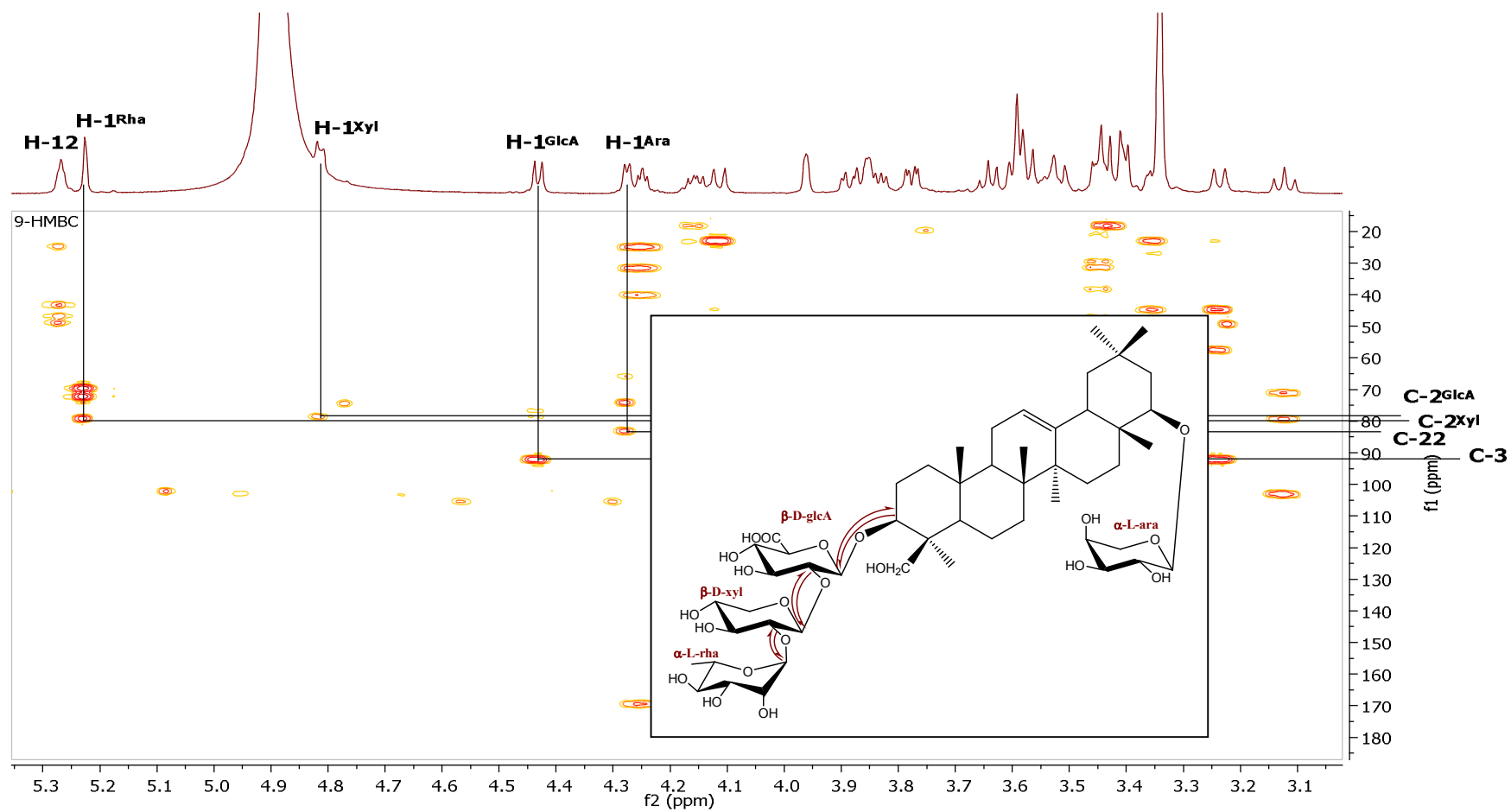
Spectrum 3.131. ^1H NMR spectrum of AAN9 (600 MHz, CD_3OD)



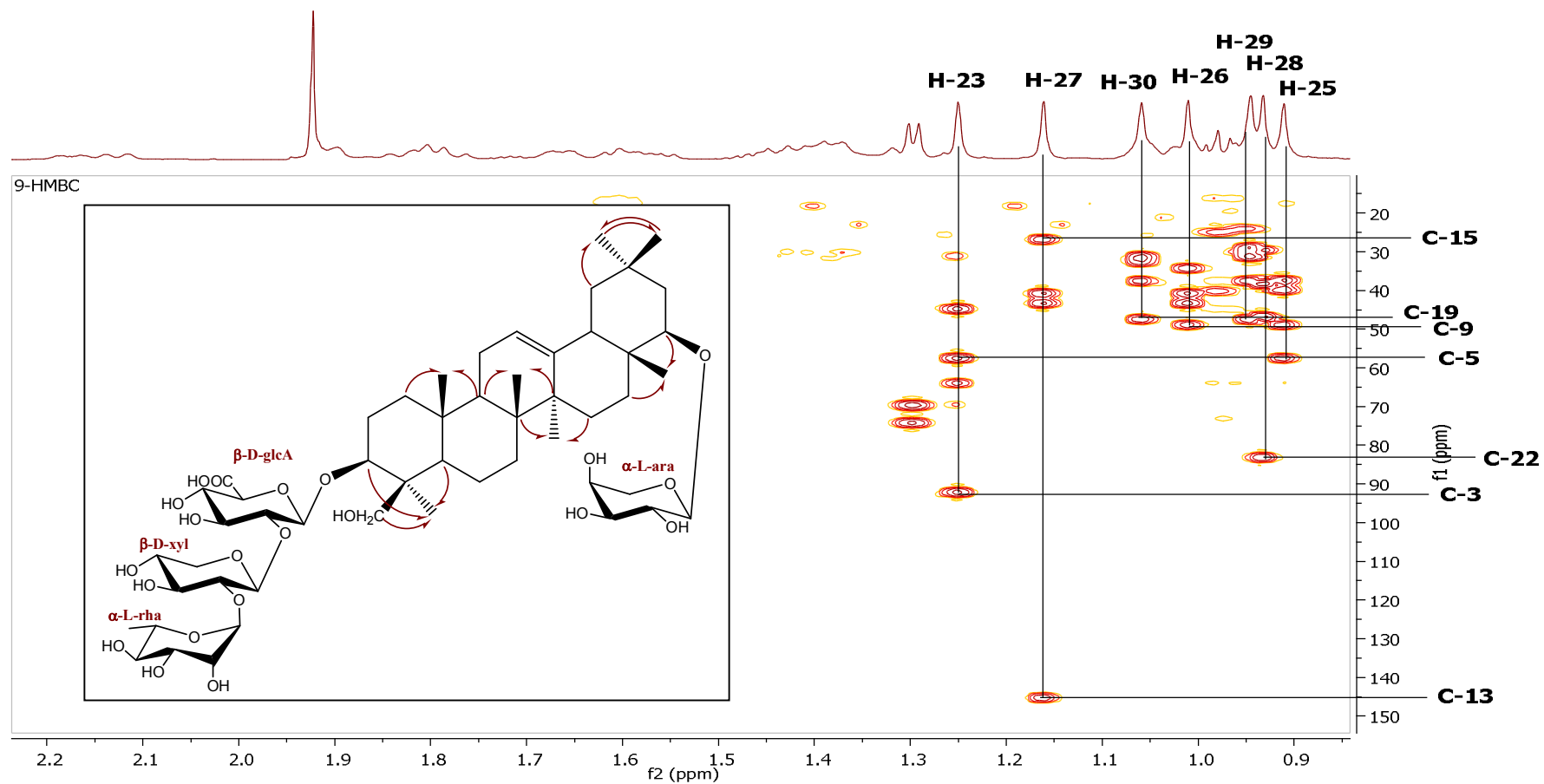
Spectrum 3.132. HSQC Spectrum of AAN9



Spectrum 3.133. HSQC Spectrum of AAN9



Spectrum 3.134. HMBC Spectrum of AAN9



Spectrum 3.135. HMBC Spectrum of AAN9

3.3.10. Structural Identification of Compound AAN10

The HRMALDITOF mass spectrum of **AAN10** (m/z 659.4139 $[M+Na]^+$, calcd for $C_{36}H_{60}O_9Na$, 659.4135) supported a molecular formula of $C_{36}H_{60}O_9$. The ESIMS spectrum showed the major ion peak at m/z 659, assigned to $[M+Na]^+$. The MS/MS of this ion showed the peak at m/z 439 $[M+Na-180]^+$, due to the loss of a hexose unit.

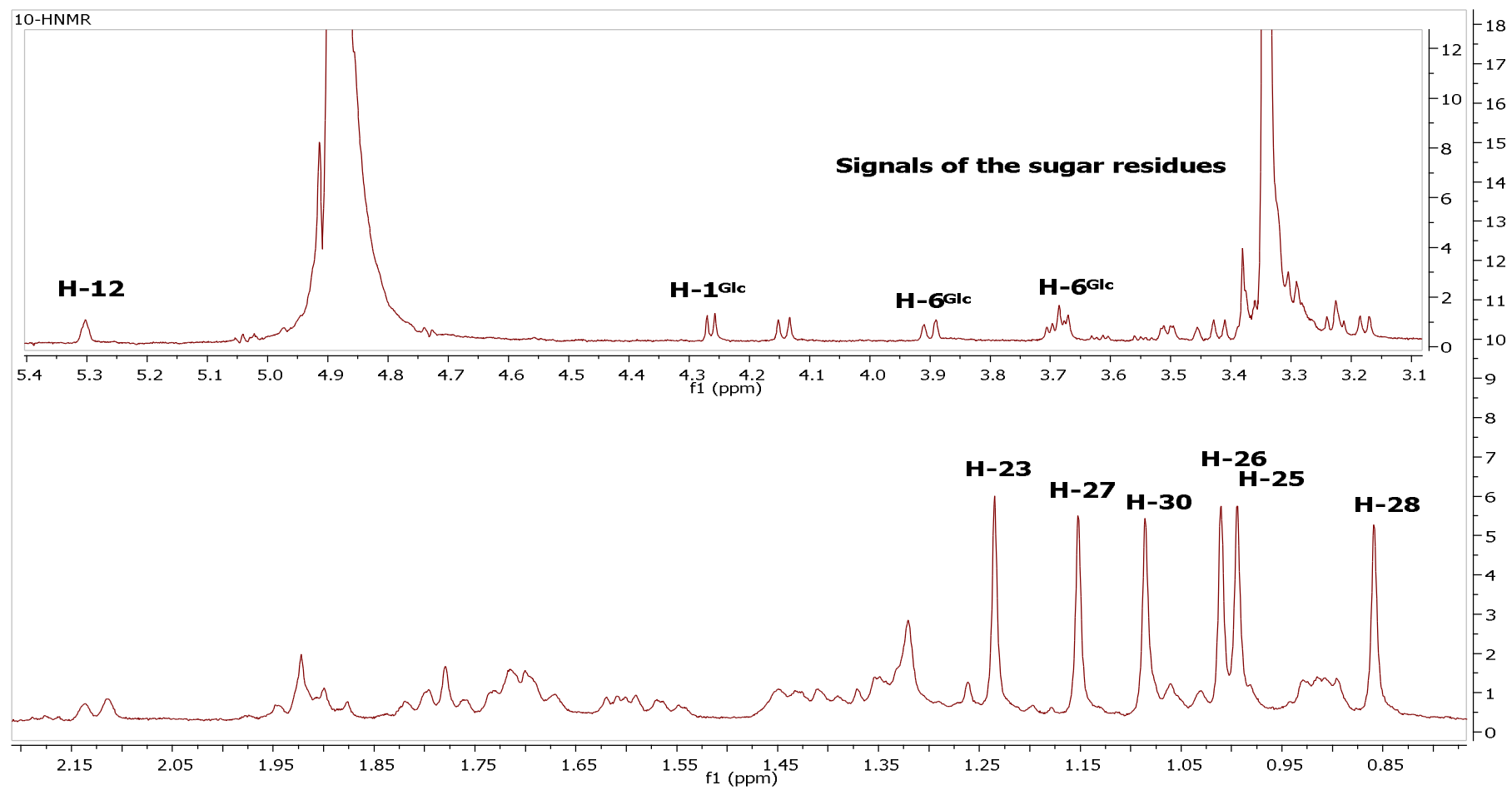
A combination of 1D and 2D NMR techniques led to the assignment of the ^1H and ^{13}C NMR signals due to the A, B, C, D rings of a 3 β ,24-dihydroxyolean-12-ene sapogenol unit (Kinjo et al., 1995; Miyao et al., 1996). Ring E was shown to contain an oxymethine at C-22 (δ_C 77.1, δ_H 3.50 brs) and a glycosylated hydroxymethyl group at C-29 (δ_C 80.2, δ_H 3.17 and 3.68, each d, $J = 9.7$ Hz). The location of the $-CH_2OR$ group at C-29 α was derived from the resonance of Me-30 at δ 24.4 superimposable to that of abrisapogenol B (Kinjo et al., 1995; Miyao et al., 1996). 1D and 2D NMR data also indicated the presence of a terminal β -D-glucopyranosyl moiety, which was located at C-29 on the basis of the HMBC correlation between the H-1 $_{glc}$ (δ 4.26) and C-29 (δ 80.2) signals. The D-configuration of glucose unit was established after hydrolysis of **AAN10** followed by GC analysis (De Marino et al., 2003).

Thus, **AAN10** was elucidated as 29-*O*- β -D-glucopyranosyl-3 β ,22 β ,24,29-tetrahydroxy-olean-12-ene (**Fig. 3.29**).

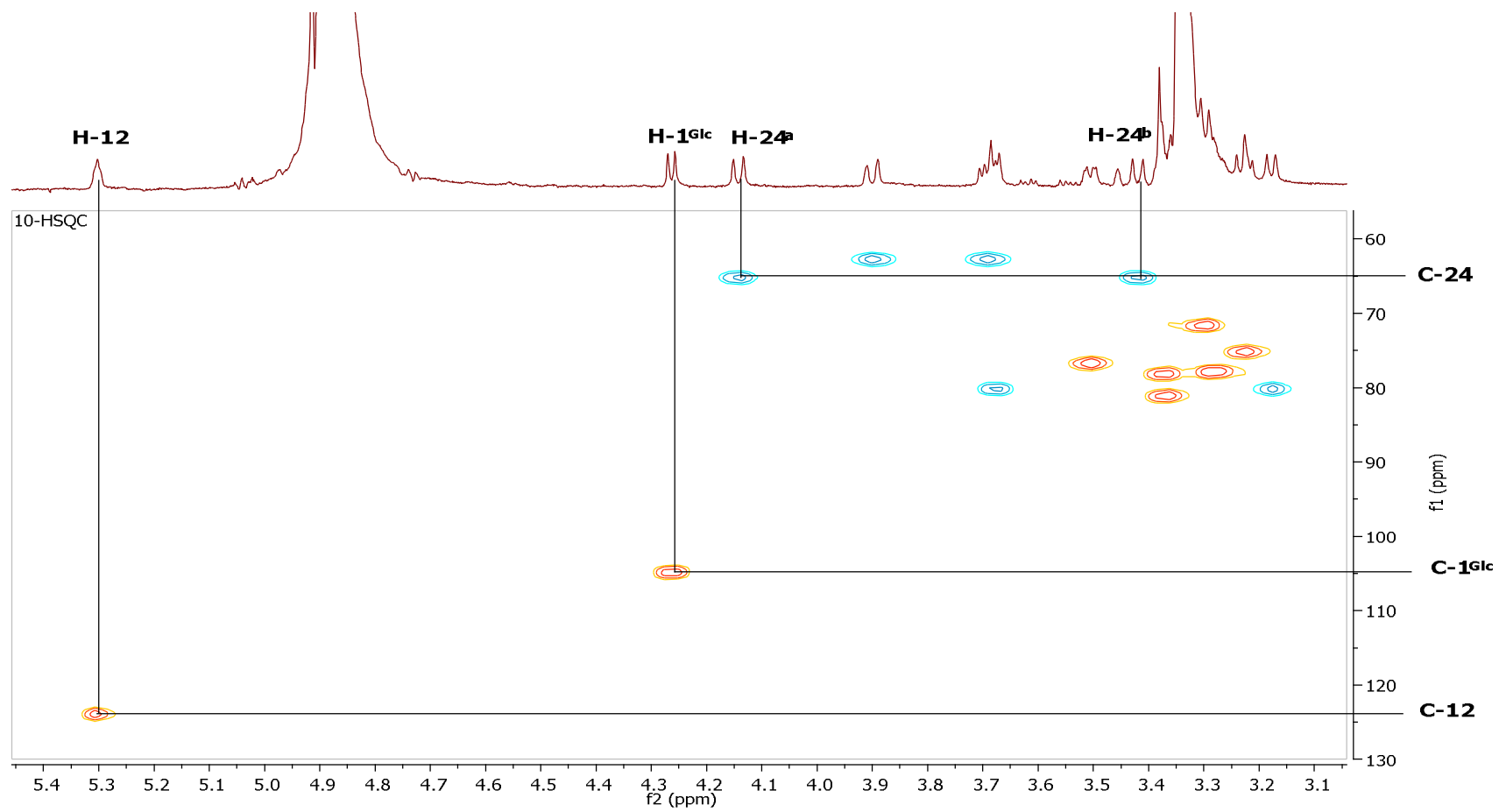
Amorphous white solid; $C_{36}H_{60}O_9$; $[\alpha]_D^{25} +27.7$ (c 0.01 MeOH); IR ν^{KBr}_{max} cm^{-1} : 3440 ($>OH$), 2933 ($>CH$), 1645 ($C=C$), 1240 and 1044 ($C-O-C$); ESI-MS m/z 659 $[M+Na]^+$, MS/MS m/z 439 $[M+Na-180]^+$; HRMALDITOFMS m/z $[M+Na]^+$ calcd for $C_{36}H_{60}O_9Na$, 659.4135, found 659.4139; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR (CD_3OD , 150 MHz) data: see **Table 3.29**.

Table 3.29. ^1H and ^{13}C Assignments of AAN10 (150/600 MHz, δ ppm, in CD_3OD)

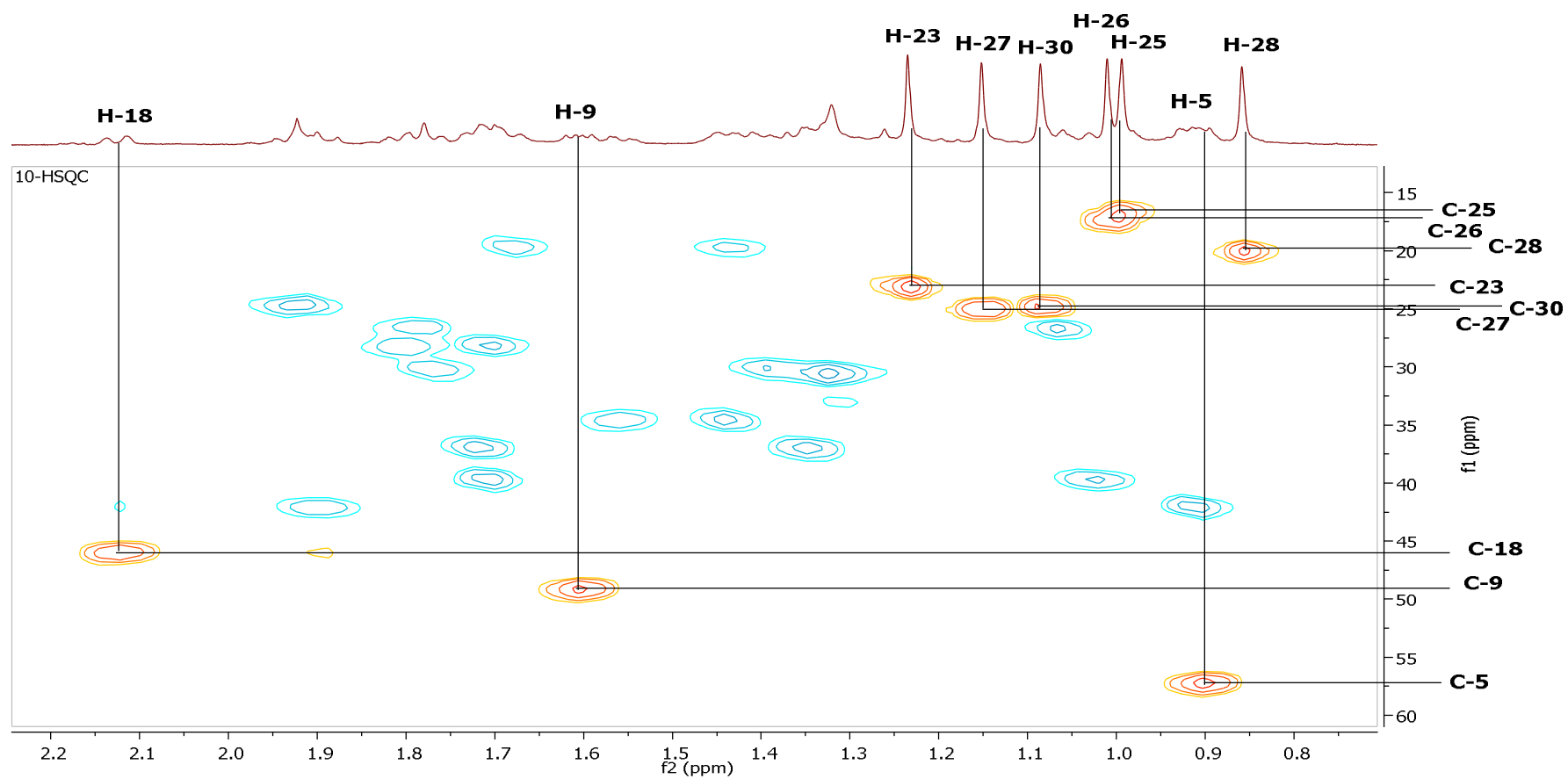
Position	δ_{C}	δ_{H} (J in Hz)
1	39.8	1.71, 1.01, m
2	28.4	2.18, 1.82, m
3	81.6	3.36, dd (11.5, 4.2)
4	43.8	-
5	57.3	0.89, m
6	19.4	1.68, 1.42, m
7	34.8	1.56, 1.43, m
8	40.5	-
9	49.0	1.59, m
10	37.6	-
11	24.5	1.91, (2H) m
12	124.1	5.31, t (3.5)
13	145.4	-
14	43.0	-
15	26.5	1.78, 1.06, m
16	29.9	1.78, 1.39, m
17	38.4	-
18	45.9	2.12, m
19	42.0	1.89, 0.90, m
20	36.2	-
21	36.8	1.72, 1.34, m
22	77.1	3.50, brs
23	23.0	1.24, s
24	65.3	4.14, d (11.4), 3.41, d (11.4)
25	16.6	0.99, m
26	17.1	1.01, m
27	25.0	1.15, m
28	19.9	0.85, m
29	80.2	3.68, d (9.7), 3.17, d (9.7)
30	24.4	1.08, s
$\beta\text{-D-Glc (at C-3)-1'}$	105.2	4.26, d (7.5)
2'	75.3	3.22, dd (7.5, 9.0)
3'	77.9	3.28, t (9.0)
4'	71.6	3.29, t (9.0)
5'	78.3	3.38, ddd (3.5, 4.5, 9.0)
6'	62.8	3.90, dd (3.5, 12), 3.69, dd (4.5, 12)



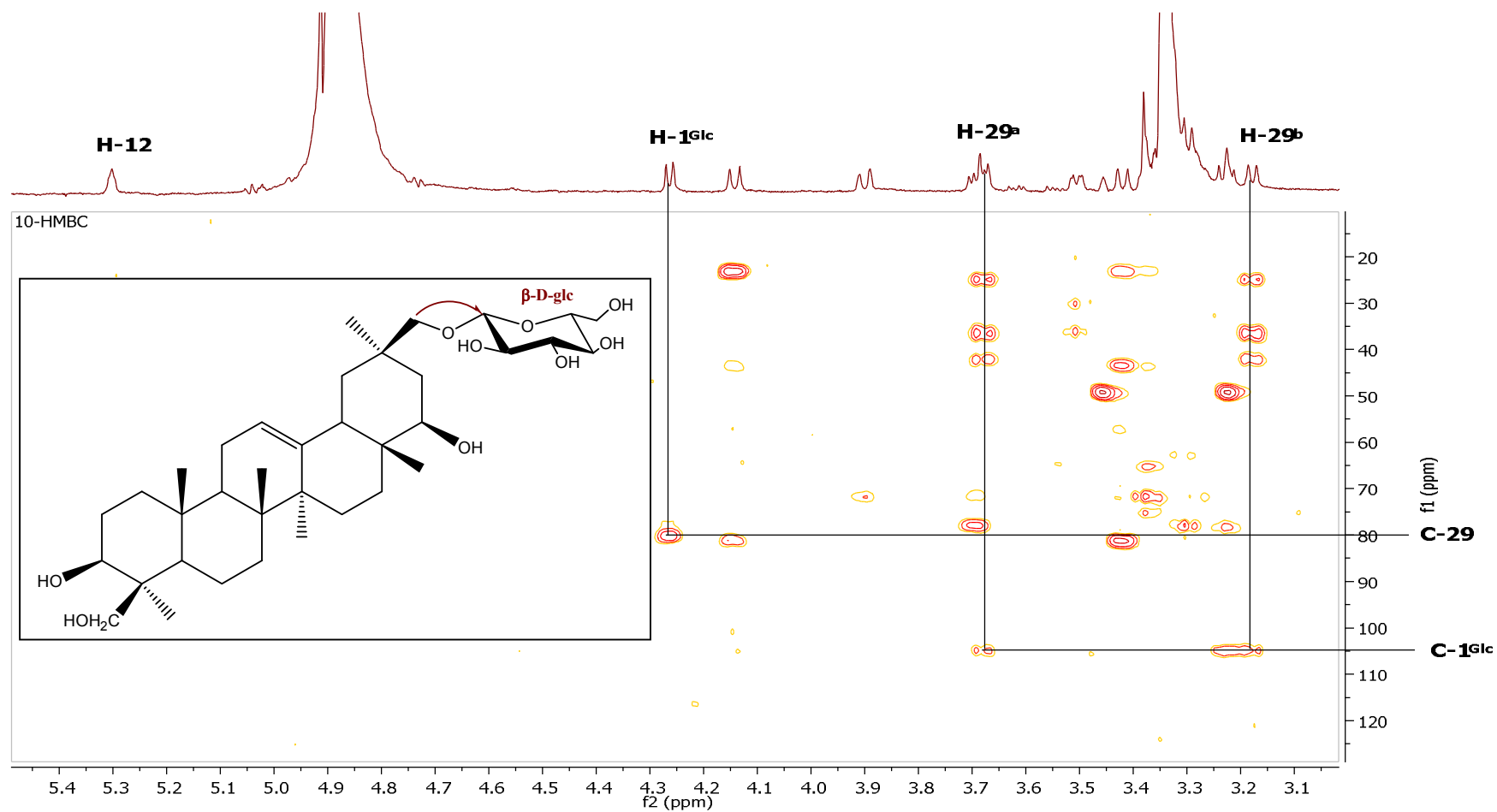
Spectrum 3.136. ^1H NMR spectrum of AAN10 (600 MHz, CD_3OD)



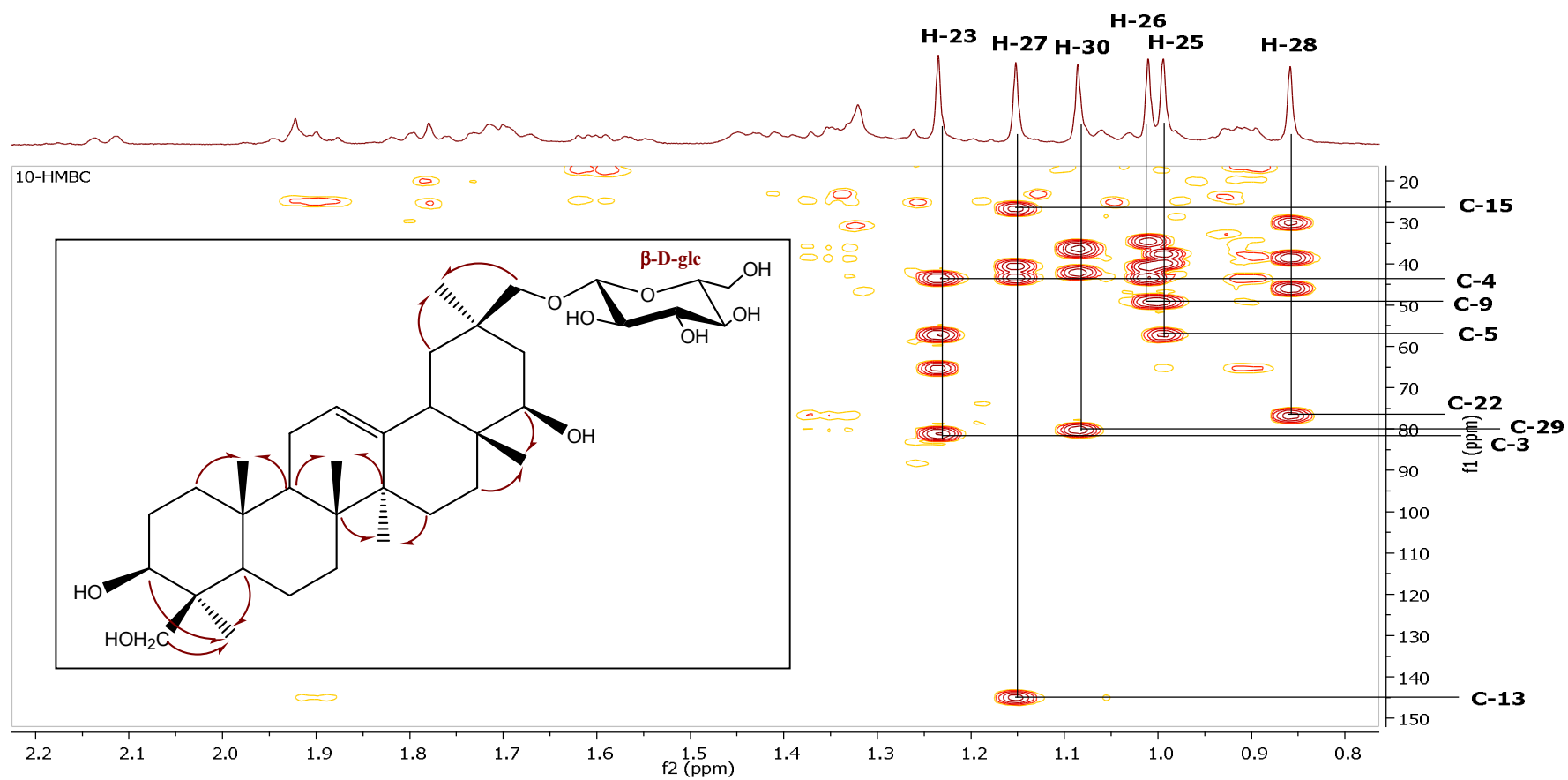
Spectrum 3.137. HSQC Spectrum of AAN10



Spectrum 3.138. HSQC Spectrum of AAN10



Spectrum 3.139. HMBC Spectrum of AAN10



Spectrum 3.140. HMBC Spectrum of AAN10

Additionally, three known cycloartane-type glycosides, 25-*O*-glucopyranosylcycloastragenol (**AAN11**) (Kitawaga et al., 1983b), cycloaraloside D (**AAN12**) (Isaev, 1991) and 3-*O*-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl]-25-*O*- β -D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3 β ,6 α ,16 β ,25-tetrahydroxycycloartane (**AAN13**) (Polat et al., 2010) and two known oleanane glycosides, astrajanoside A (**AAN14**) (Bedir et al., 1999a) and astragaloside VIII (**AAN15**) (Kitawaga et al., 1983b) were isolated.

On the basis of the cytotoxic activities exhibited by the cycloartane- and oleanane-type glycosides isolated from *Astragalus* species (Radwan et al., 2004; Kikuchi et al., 2007) and on the basis of cancer chemopreventive effects reported for the natural and semisynthetic cycloartane-type and related triterpenoids (Tian et al., 2005) the antiproliferative activity of compounds **AAN1-AAN15** was tested in different cancer cell lines including Hela (human cervical cancer cells), H-446 (human lung cancer cells), HT-29 (human colon carcinoma cells), U937 (human leukemia cells). In a range of concentrations between 1 and 50 μ M, none of the tested compounds, except compound **AAN8**, caused a significant reduction of the cell number when compared with controls. Compound **AAN8** displayed weak cytotoxicity against HeLa and HT-29 cell lines with IC₅₀ values of 36 and 50 μ M, respectively.

3.3.11. Acid Hydrolysis of compounds of *Astragalus angustifolius*

The peaks of the hydrolysate of **AAN1** were detected at 9.70 and 10.72 (α -L-rhamnose and β -L-rhamnose) and at 14.73 min (D-glucose). The peaks of the hydrolysate of **AAN5** were detected at 9.70 and 10.72 (α -L-rhamnose and β -L-rhamnose) and at 10.99 and 12.02 (β -D-xylose and α -D-xylose). The peaks of L-arabinose (8.94 and 9.81 min), L-rhamnose (9.70 and 10.72), D-xylose (10.98 and 12.01 min) and D-glucuronic acid (15.83 min) were detected in the hydrolysate of **AAN9**. For the hydrolysate of **AAN10** a peak at 14.73 min (D-glucose) was detected. Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine were detected at 8.92 and 9.80 (L-arabinose), 10.70 (L-rhamnose), 10.98 and 12.00 min (D-xylose), and 14.71 min (D-glucose), and 15.81 min (D-glucuronic acid).

As part of continuing studies on Turkish *Astragalus* species, we decided to carry out a full phytochemical study on three *Astragalus* species, *A. aureus*, *A. tauricolus* and *A. angustifolius*.

Eight new cycloartane-type triterpene glycosides (**AAU1–AAU8**) were isolated from *Astragalus aureus* Willd along with ten known cycloartane-type glycosides (**AAU9–AAU18**). Their structures were established by the extensive use of 1D and 2D-NMR experiments along with ESIMS and HRMS analyses. Compounds **AAU1–AAU5** are cyclocanthogenin glycosides, whereas compounds **AAU6–AAU8** are based on cyclocephalogenin as aglycon, more unusual in the plant kingdom, so far reported only from *Astragalus* spp. Moreover, for the first time monoglycosides of cyclocanthogenin (**AAU5**) and cyclocephalogenin (**AAU7**, **AAU8**) are reported. The antiproliferative activity of cycloartane glycosides has been reported against several cancer lines including solid tumor (HepG2), blood tumor (HL-60) and drug resistant tumor (R-HepG2) (Kikuchi et al., 2007). In particular 16b,23:23,26:24,25-triepoxy-, 16b,23:16a,24-diepoxy- and 16 β ,23;22 β ,25-diepoxycycloartane derivatives have been reported for their cytotoxic activities (Nian et al., 2010; Watanabe et al., 2002). Moreover, cancer chemopreventive effects of natural and semisynthetic cycloartane-type and related triterpenoids have been reported (Tian et al., 2005). Leishmanicidal (Özipek et al., 2005) and immunomodulatory (Çalis et al., 1997) activities have been reported for cyclocanthogenin and cycloastragenol derivatives. On the basis of the biological activities reported for cycloartane glycosides, the antiproliferative activity of compounds **AAU1–AAU18** was tested in several cancer cell lines including HL-60 (human promyelocytic leukemia), MCF-7 (human breast cancer), HT-29 (human colon carcinoma), A549 (human lung adenocarcinoma), PC3 (human prostate cancer). Compounds **AAU1–AAU18** were tested in a range of concentrations between 1 and 50 μ M, but only few compounds showed a weak activity. Compound **AAU17** exhibited an IC₅₀ value of 30 μ M against MCF7 cells, while compound **AAU8** exhibited an IC₅₀ value of 45 μ M against HL-60 cells. Compounds **AAU12** and **AAU14** showed an IC₅₀ value of 50 μ M against A549 and PC3 cells. All the other compounds showed no activity (data not shown). Etoposide used as positive control showed IC₅₀ values ranging from 0.5 μ M (HL-60 cells) to 16 μ M (A549 cells).

An approach based on HPLC-ESIMSⁿ experiments has been used to profile the triterpene glycosides occurring in the butanol extract of the whole plant of *Astragalus tauricolus*. On the basis of the results of the online screening by HPLC-ESIMSⁿ, twenty-two oleanane-type triterpene glycosides, including ten unreported compounds were isolated, and their structures were unambiguously elucidated by NMR spectroscopic data. Noteworthy, cycloartane-type triterpene glycoside, the main constituents of *Astragalus* spp., were not found. This peculiar

feature characterizes a very limited group of *Astragalus* spp. (*A. hamosus*, *A. complanatus*, *A. sinicus*, and *A. corniculatus*) (Ionkova, 1991; Cui et al., 1992a, b; Krasteva et al., 2006, 2007). The antiproliferative activity of compounds **ATA1–ATA12**, **ATA15**, **ATA17–ATA19** was tested in different cancer cell lines including human breast cancer (MCF-7), human lung adenocarcinoma (A549), human prostate cancer (PC-3) and human leukemia (U937) cell lines. The antiproliferative activity of the isolated compounds **ATA1–ATA12**, **ATA15**, **ATA17–ATA19** was evaluated against a small panel of cancer cell lines. Only compound **ATA11** showed an IC₅₀ of 22 µM against human leukemia cell line (U937). The other tested compounds, in a range of concentrations between 1 and 50 µM, did not cause any significant reduction of the cell number.

Fractionation of whole part of *Astragalus angustifolius* led to the isolation of six new cycloartane-type (**AAN1–AAN6**) and four new oleanane-type triterpene glycosides (**AAN7–AAN10**) along with five known triterpene glycosides. Their structures were established by the extensive use of 1D and 2D-NMR experiments along with ESIMS and HRMS analysis. Compounds **AAN1–AAN3** are glycosides of cycloastragenol, while compounds **AAN4–AAN6** show the C-24 epimer of cycloastragenol [20(*R*),24(*R*)] as aglycone, encountered for the first time in nature. The antiproliferative activity of compounds **AAN1–AAN15** was tested in different cancer cell lines including Hela (human cervical cancer cells), H-446 (human lung cancer cells), HT-29 (human colon carcinoma cells), and U-937 (human leukemia cells). In a range of concentrations between 1 and 50 µM, none of the tested compounds, except compound **AAN8**, caused a significant reduction of the cell number when compared with controls. Compound **AAN8** displayed weak cytotoxicity against HeLa and HT-29 cell lines with IC₅₀ values of 36 and 50 µM, respectively. Etoposide, used as a positive control, showed IC₅₀ values ranging from 2 µM (U-937 cells) to 12 µM (H-446 cells).

Our studies resulted in the isolation of 18 metabolites from *A. aureus*, 22 from *A. tauricolus* and 15 from *A. angustifolius*. By using mainly 1D and 2D NMR methods, structures of the compounds were determined to be cycloartane and oleanane type saponins.

From chemotaxonomy point of view, until now, 24 out of 445 Turkish *Astragalus* species from 10 different sections have been investigated for their secondary metabolite contents. Based on these studies, it was ascertained that

some sections; Sect. Pterophorus (*A. brachypterus*, *A. baibutensis*, *A. trojanus*, *A. ptilodes* var. *cariensis*, *A. wiedemannianus*, *A. tmoleus* var. *tmoleus*), Sect. Rhacophorus (*A. microcephalus*, *A. zahlbruckneri*, *A. cephalotes* var. *brevicalyx*, *A. amblolepis*, *A. pycnocephalus* var. *pycnocephalus*, *A. erinaceus*, *A. prussianus*, *A. schottianus*), Sect. Macrophyllium (*A. oleifolius*), Sect. Hololeuce (*A. campylosema* ssp. *campylosema*), Sect. Stereocalyx (*A. stereocalyx*), Sect. Christiana (*A. gilvus*, *A. melanophrurius*) and Sect. Proselius (*A. elongatus*), provided only cycloartane saponins, whereas both cycloartane and oleanane saponins were encountered in a few sections; Sect. Acanthophaea (*A. hareftae*, *A. icmadophilus*), and Sect. Eustales (*A. flavescens*).

Based on earlier phytochemical studies, one could readily claim that the cycloartane group is a chemical marker for the genus *Astragalus*. As parallel to this suggestion, phytochemical analysis of *A. aureus* (Sect. *Adiaspastus*) and *A. angustifolius* (Sect. *Melanocercis*) provided cycloartane type secondary metabolites. However phytochemical study on *A. tauricolus* from Sect. *Malacothrix* resulted in the isolation of twenty-two oleanane-type triterpene glycosides. It was remarkable to witness no trace of cycloartanes for the first time in an *Astragalus* species. This finding was also substantiated by ¹H NMR spectrum of the MeOH extract, in which characteristic signals of the cyclopropane ring in the upfield region (0.3-0.6 ppm, each d, AX signals) were not detected.

Consequently, although the occurrence of oleanane-based saponins was also reported in the genus *Astragalus*, cycloartanes extremely dominated the triterpenoid class. The chemical profile of *A. tauricolus* (Sect. *Malacothrix*) reveals that the presence of cycloartanes is not the case for all *Astragalus* members, presenting a unique position for the species. Further phytochemical studies are needed to support whether the absence of cycloartanes is a significant chemotaxonomic finding for the section *Malacothrix*.

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1. Prof. Dr. Ozgen ALANKUS CALISKAN, “Bazı Astragalus Türlerinden Doğal Bileşiklerin İzolasyonu, Kimyasal Yapılarının Belirlenmesi ve Stotoksik Aktivitelerinin İncelenmesi.”, TÜBİTAK, **2009-2013**.

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