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Extraction, Isolation and Structure Elucidation of Saponins from *Herniaria incana*

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Sammendrag

Det har blitt rapportert[1,2] at arter fra *Herniaria*-familien har flere medisinske anvendelser. To av disse, *Herniaria glabra* og *Herniaria hirsuta*, har begge blitt dokumentert til å inneholde saponiner.[3,4] Dette kan forklare hvorfor denne plantefamilien har helsefremmende effekter ettersom saponiner er kjent for sine mange biologiske egenskaper.[5i] På grunn av den lave mengden tilgjengelig informasjon om en av deres slektninger, *Herniaria incana*, er den en god kandidat for kjemisk analyse.

Målet med denne oppgaven var å bestemme det totale saponin-innholdet i *H. incana*, samt å ekstrahere, isolere og strukturbestemme saponinstoffer i denne arten.

Det totale saponin-innholdet ble bestemt ved hjelp av kolorimetri[6] med Ginsenosid Rb1 som standard. Målingene ble utført ved 550 nm, og resultatene ga et totalt saponin-innhold på 35.2 μg ginsenosid ekvivalent saponin-innhold per gram av planteekstraktet.

Saponiner fra *H. incana* ble isolert ved hjelp av TLC og kolonnekromatografi. Strukturen ble funnet ved bruk av en kombinasjon av 1D (^{1}H, ^{13}C) og 2D NMR teknikker (COSY-45, redigert HSQC, HMBC, H2BC, HSQC-TOCSY, NOESY og "^{1}H,^{1}H *J*-resolved" eksperiment) som *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D- glukopyranosyl-(1→6)-*O*-[β-D-6-*O*-acetylglukopyranosyl-(1→2)]-β-D-glukopyranosyl

Medicagen-28-at. Monosakkaridsekvensen ble også bekreftet av ESI-CID.

Abstract

Species of the *Herniaria* genus have been reported to have several medicinal uses.[1,2] Two of the species from this genus, *Herniaria glabra* and *Herniaria hirsuta*, have both been documented to contain saponins.[3,4] This can explain the health beneficial use of these plant species, since saponins are known for their many biological properties.[5i] However, the lack of information on one of their relatives, *Herniaria incana*, makes this species a target for further investigation.

The aim of this master thesis is to determine the total saponin content of *H. incana*, as well as to extract, isolate and closely investigate saponin compounds in this species.

The total saponin content was determined by colorimetry[6], using ginsenoside Rb1 as standard. Measurements were carried out at 550 nm, and the results gave a total saponin content of 35.2 μg ginsenoside equivalent saponin content per gram of the plant extract.

A saponin was isolated from *H. incana* by means of TLC and column chromatography. The structure was elucidated by the use of a combination of 1D (^{1}H, ^{13}C) and 2D NMR techniques (COSY-45, edited HSQC, HMBC, H2BC, HSQC-TOCSY, NOESY and ^{1}H,^{1}H *J*-resolved experiment) as *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D- glucopyranosyl-(1→6)-*O*-[β-D-6-*O*-acetylglucopyranosyl-(1→2)]-β-D-gluco-

pyranosyl Medicagen-28-ate. The monosaccharide sequence was also confirmed by ESI-CID.

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List of Symbols and Abbreviations

1D One-dimensional
2D Two-dimensional
ACN Acetonitrile
CC Column chromatography
CI Chemical ionization
CID Collision induced dissosiation
COSY (H, H)-correlated spectroscopy
DEPT Distortionless enhancement polarization transfer
DCM Dichloromethane
EI Electron impact
ESI Electron spray ionization
EtOAc Ethyl acetate
FAB Fast atom bombardment
H2BC Heteronuclear 2 bond correlation
HMBC Heteronuclear multiple bond correlation
HOHAHA Homonuclear Hartmann-Hahn
HPLC High performance liquid chromatography
HSQC Heteronuclear single quantum coherence
λ Wavelength
$\lambda_{\mathbf{max}}$ Wavelength of maximum absorption
MeOH Methanol
MS Mass spectrometry

NMR Nuclear magnetic resonance
NOE Nuclear overhauser efferct
NOESY Nuclear overhauser effect spectroscopy
ROESY Rotating-frame overhauser effect spectroscopy
RP Reverse phase
SPE Solid phase extraction
TLC Thin layer chromatography
TOCSY Total correlation spectroscopy
UV / VIS Ultraviolet-visible spectroscopy

Chapter 1

Introduction

It has been reported[1,2] that certain plants from the *Herniaria* genus have health beneficial properties. Both *Herniaria glabra* and *Herniaria hirsuta* have been used in traditional Moroccan medicine to treat kidney stones.[1,2] It has also been found that these plant species contain saponins.[3,4] This can explain the medicinal use of these plants, since saponins are compounds known for their versatile biological effects.[5i] However, there are no reported findings about saponin content and composition of one of their relatives, *Herniaria incana*. There is reason to believe that this species might contain saponins with similar properties, some of them potentially being new.

1.1 Strategy

The starting point of the investigation of *H. incana* was to determine its total saponin content. Since it was only necessary to quantify the saponin content, spectrophotometric determination was chosen as a fast and simple way of doing this.

Due to the fact that saponins usually occur in plants as a mixture of

structually related forms with very similar polarity, their separation remains a challenge. A suitable extraction method for the plant material needs to be applied. Usually the separation procedure consists of a number of different techniques, ranging from TLC to column chromatography and flash chromatography.[5c] All of these systems need to be adjusted and optimized to obtain pure compounds for structure elucidation.

Chapter 2

Theory

This chapter will give an overview of the general theoretical background of this work. It will give a review of the background information on the *Herniaria* genus and saponins as a class of compounds and a general introduction to their properties, importance and biological activity. Methods for structure elucidation of saponins are also reviewed. The last section concerns the separation techniques used, such as sephadex LH-20 as a stationary phase, reverse phase chromatography and VersaFlash as an improved version of column chromatography (CC).

2.1 The *Herniaria* genus

The *Herniaria* genus stems from the *Caryophyllaceae* (pink plant) family in the major group of *Angiosperms* (flowering plants) and is commonly refered to as rupture wort. The name Herniaria stems from the Latin words *hernia* (rupture) and *-aria* (alluding), which can be translated as a treatment of hernias. The plant is native to Europe, South America (Andes), central and west Asia and Africa.

Approximately 45 different species have been recorded.[7]

Herniaria is a biennial plant, and has a flowering period from May to Autumn. It has ripe fruit from June to October. The plant grows in dry, particularly sandy and sunny environments. It has small green leaves, its flowers are hypanthium cup-like (a cup-like or tubular enlargement of the secretion organ of a flower surrounding the female part of the flower or united with it) not abruptly expanded above. The flower has free sepals (the cup-like parts of the flower) of 0.6-1.2 mm, their shape being lanceolate to oblong and the leaves have a green to almost white colour and are hairy.[7,8] (Figure 2.1).

Figure 2.1 *Herniaria incana*.[9]

2.1.1 Medicinal use

It has been reported that *Herniaria glabra* has a variety of medicinal uses, such as treatment of catarrh of the bladder, dropsy, kidney stones, nerve inflammation, respiratory disorders, increase the flow of urine and removal of excess mucus from the stomach.[1] Rhiouani *et al.*[1] discuss the acute and sub-chronic toxicity of an aqueous extract

of *H. glabra* in rodents. Their results showed that *H. glabra* appears to be relatively non-toxic, but that higher doses can cause liver and kidney intoxication. They concluded that further clinical tests were needed to define a safe dose for humans.

Atmani *et al.*[2] have tested an aqueous suspension of *Herniaria hirsuta* on rats with kidney stones. Their preliminary results showed that the *H. hirsuta* extract increased the amount of crystals in the urine. This means that formation of crystals in the kidneys is reduced, and that the crystals can be swept away by the urine flow. Another study showed that there is reason to believe that the saponins extracted from *H. hirsuta* caused this effect.[10]

2.2 Saponins

Saponins are high-molecular-weight glycosides, consisting of a sugar unit(s) linked to a triterpene or a steroid aglycone. Many saponins have detergent properties. They lower the surface tension of aqueous solutions and therefore give stable foams when in contact with water. In fact, the name "saponin" stems from the latin word *sapo* (soap). Saponins are also known to cause haemolysis (lysis of erythrocytes with the release of hemoglobin), have a bitter taste, and be toxic to cold-blooded animals. Even though these attributes are not common to all known saponins, they are sometimes used to characterize this class of compounds. Many plant drugs and folk medicines, especially those that have origins in Asia, contain saponins. For this reason, there is a great interest in characterization and in the investigation of their pharmacological and biological properties.[5a, 11b]

The non-sugar or the aglycone unit of the saponin molecule is called the *sapogenin* or just the *genin*. The saponins can be divided into three major classes according to the structure of genin: *Triterpene* glycosides, *steroid* glycosides and *steroid alkaloid* glycosides. Figure

2.2 shows the different types of sapogenins found in the three classes of saponin.[5a, 11b]

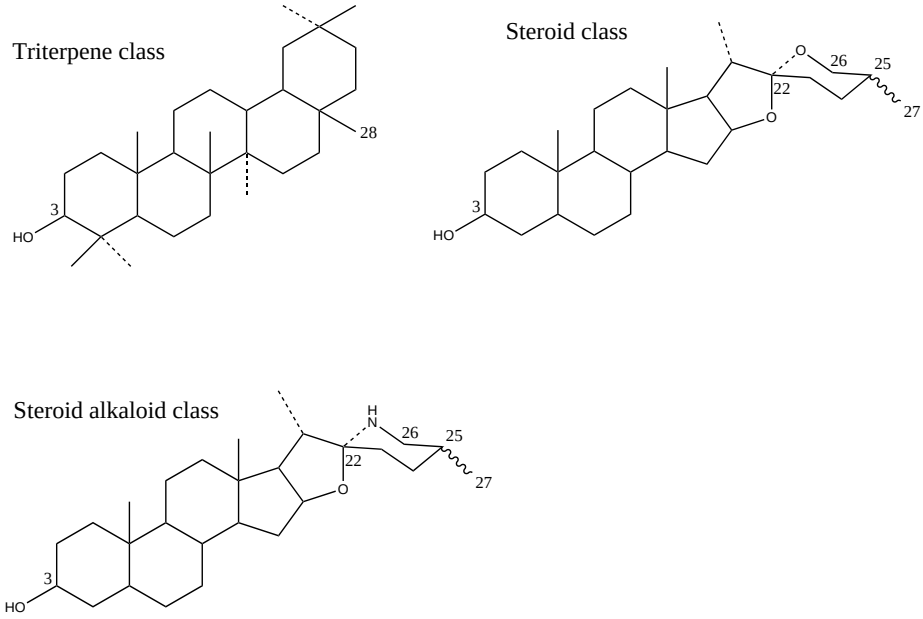


Figure 2.2 Skeletal types of sapogenin found in the three principal classes of saponins.

The common denominator for all saponins is the attachment of one or more sugar chains to the sapogenin. They can either be *monodesmosidic* (have a single sugar chain, usually attached at C-3) or *bidesmosidic* (two sugar chains attached to C-3 and C-28), see Figure 2.3.[5a]

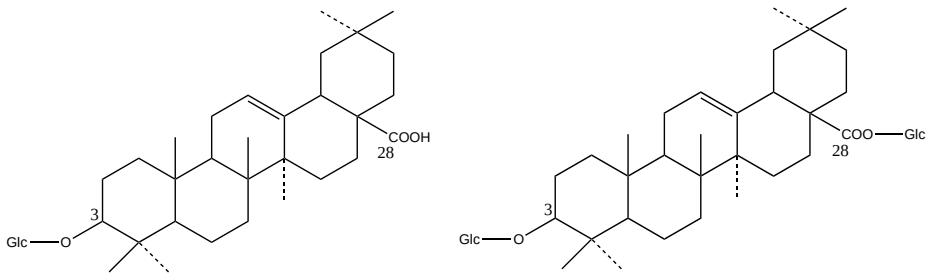


Figure 2.3 Monodesmosidic and bidesmosidic saponins.

2.2.1 Biosynthesis

Triterpenes and steroids are both built up from six isoprene units, both having in common that they are derived from *squalene*. The mechanism is probably via a ring-opening of squalene-2,3-epoxide, followed by a concerted cyclization (Figure 2.4).[5a]

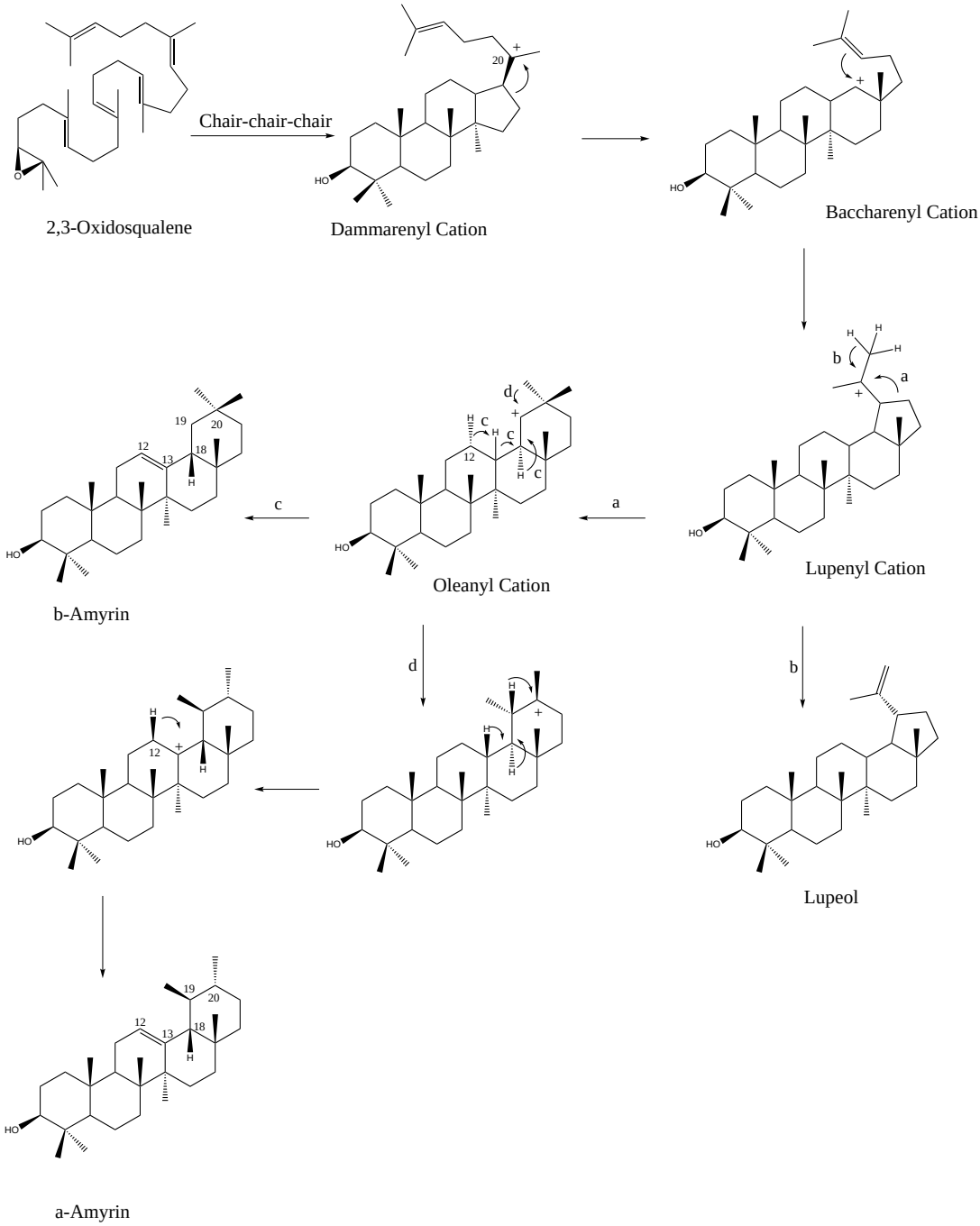


Figure 2.4 Biosynthesis of triterpenes and steroids.

Oxidative cleavage of three methyl groups from a C_{30} intermediate causes triterpenes to have 30 carbon atoms and the steroids to have 27.
A pentacyclic or a tetracyclic triterpene is formed after a cyclization of the chair-chair-chair-boat conformation of squalene-2,3-epoxide, followed by the rearrangement of the tetracyclic carbonium ion (Figure 2.5).[5b, 11b, 12]

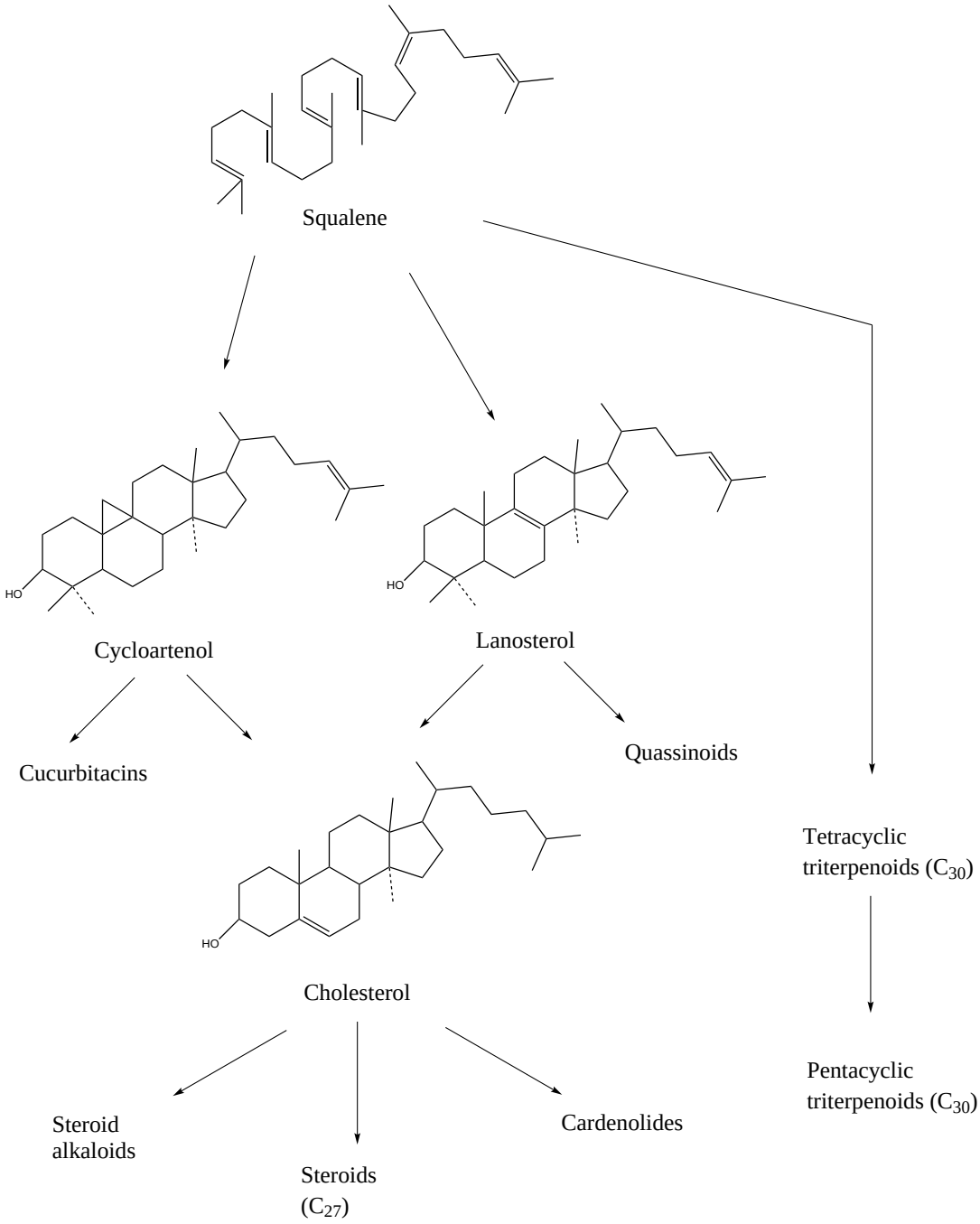


Figure 2.5 Biosynthesis of pentacyclic triterpenes from oxidosqualene.

2.2.2 Biological activity of triterpene saponins

There are many biological activities associated with saponins. Most of those arise from the chemical nature of saponins which have two constitutional moieties: The hydrophilic sugar and the usually lipophilic sapogenin. These properties are responsible for the interaction between saponins and cell membranes. Attributes such as their fungicidal and piscicidal (a chemical substance that is poisonous to fish) effects have been known for years, while new activities are continually being discovered.[5h]

Antifungal activity is related to the saponin content in many other species e.g. those of the *Phytolacca* (Phytolaccaceae) genus.[13,14]

As previously mentioned, some saponins have haemolytic activity. Sindambiwe *et al.*[15] tested a mixture of saponins derived from *Maesa lanceolata*, that showed a high haemolytic activity on human erythrocytes.

Li *et al.*[16] isolated two triterpenoid saponins from the stem bark of *Kalopanax pictus*. Both saponins showed significant anti-inflammatory activity. This activity was also documented by Kwak *et al.*[17] They investigated the triterpenoid saponin loniceroside C, isolated from *Lonicera japonica*, a medicinal plant known as an anti-inflammatory agent for centuries.

Saponins have also been reported to have antimicrobial,[18] cancer preventing[19] and antiviral activities.[20]

2.3 NMR and mass spectrometry of saponins

Saponins have a very complex structure, which is hard to elucidate. The benefit of using nuclear magnetic resonance (NMR) in combination with mass spectrometry (MS) is that this allows an examination of the *intact* saponin, instead of using cleavage reactions to cleave off sugar moieties from the sapogenin and analyze them separately.[5e]

2.3.1 NMR spectrometry

Proton and carbon-13 NMR spectrometry is widely used to determine the structure of saponins. By analysing the spectra, the following aspects can be ascertained: Where the glycosidic linkages to the aglycone are positioned; the number, sequence and nature of monosaccharide units; configuration of the interglycosidic linkages; presence of acyl glycosides in the chains; what kind of aglycone the saponin has, and the structures of eventually attached esters.
In the ^{1}H NMR spectra, difficulties arise. The proton resonances are prone to overlap, due to the majority of signals from the carbohydrate moieties that appear in the range from 3.0 to 4.2 ppm. The chemical shifts are similar, even though they derive from the same bulk of non-anomeric sugar methyne and methylene protons. Luckily, it is possible to assign proton shifts by combining different 1D and 2D NMR-techniques. By using 2D NMR the spectral crowding will be limited.[5g]

By use of various 2D NMR techniques, in a specially adapted procedure, Schröder *et al.*[3] determined the structure of a triterpene saponin from *Herniaria glabra* (Figure 2.6). They used methods such as H,H-COSY and TOCSY to establish the nature of the monosaccharide. TOCSY also confirmed the nature of the oligosaccharide. NOESY, ROESY and HMBC were used to find the sequence of

the oligosaccharide chain and the linkage site of glucuronic acid. The medicagenic acid aglycone was found by H,H-COSY, NOESY, ROESY and HMBC. The latter was also used to get information about the sequence of the oligosaccharide chain and binding sites to the aglycone. NOE or ROE was applied to get information on the conformation, while ^{13}C NMR resonances were assigned by DEPT-HMQC and HMQC.

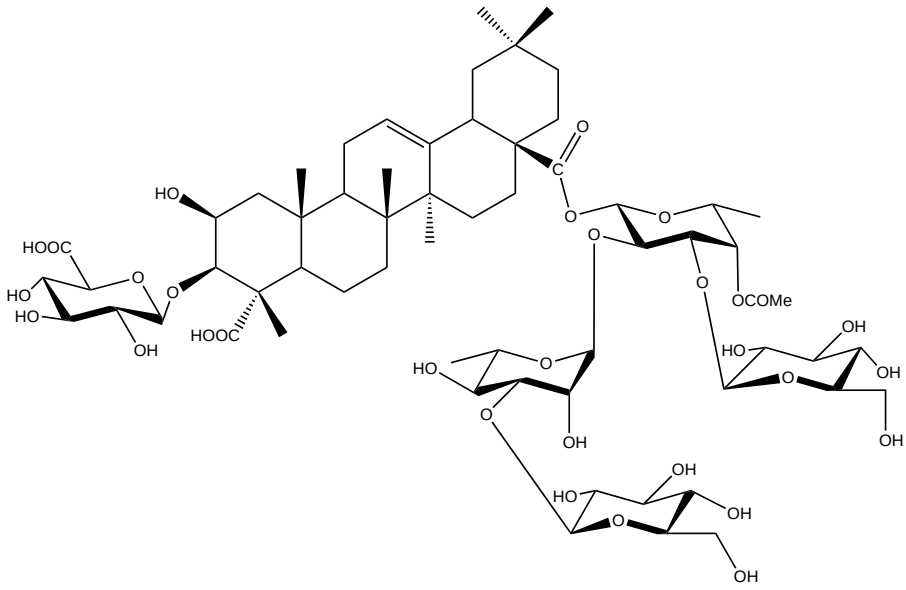


Figure 2.6 A triterpene saponin from *Herniaria glabra*.

2.3.2 Mass spectrometry

The ionization method in MS depends on the polarity, lability and molecular weight of the compound to be analysed. Previously, ionization techniques such as fast atom bombardment (FAB) and chemical ionization (CI) have been applied to find important structural information, like molecular weight and sugar sequence, for naturally oc-

curing glycosides. These techniques enabled analysis without derivatization of the glycosides.[5f] However, the matrix background generates a chemical noise, which reduces the sensitivity of the FAB method.[21]

For the most common MS method, electron impact (EI), samples need to be volatile and thermally stable. Saponins require conversion to permethyl or peracetyl derivatives in order to be analyzed by EI. This derivatization also has its limitations, since it is not applicable to saponins containing more than three sugar moieties.[22]

Electrospray ionization (ESI) has been reported[21,23,24] as a powerful tool in determing the molecular weight of saponins due to its high sensitivity, rapid analysis time and low levels of sample consumption. This ionization technique combined with collision induced dissociation (CID) can aid in identification of the backbone and glycosidic linkage sites of the saponins.[25] CID is a process where energy is transferred to an ion through collision with a neutral collision gas (He, N_2, Ar). This energy transfer is sufficient to result in bond cleavages and rearrangements of the selected ion. Fragmentation will be possible for gaseous ions that are otherwise perfectly stable.[26]

2.4 Sephadex LH-20

Sephadex LH-20 is a crosslinked dextran gel used in liquid chromatography. The separation is based on molecular size. In addition, it has the ability to separate according to selective absorption, as long as the compound has a molecular mass below 1000 g/mol.[27a] Sephadex LH-20 has been specifically developed for gel filtration of natural products, i.e. terpenoids, lipids, steroids and low molecular weight peptides, in organic solvents. It is also widely used for initial fractionation of crude extracts of highly polar compounds. A partial structure of Sephadex LH-20 is given in Figure 2.7.[28]

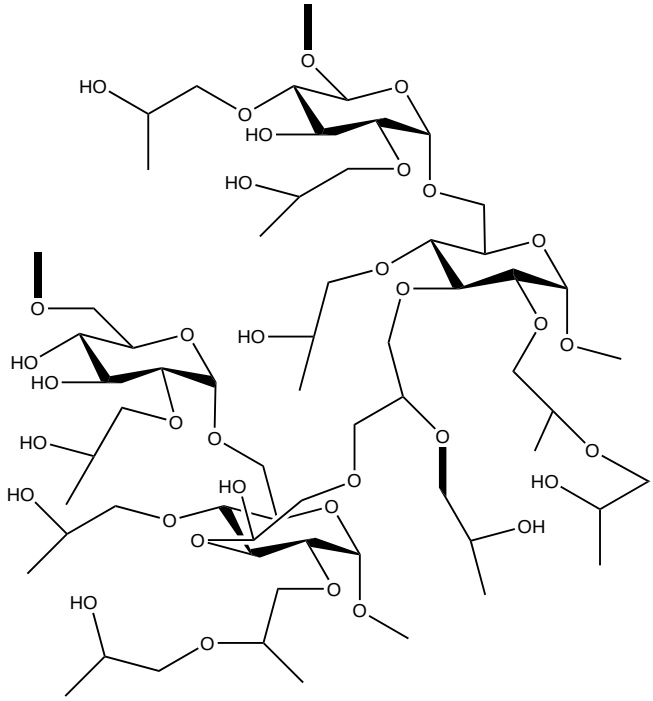


Figure 2.7 Partial structure of sephadex LH-20.

The hydroxypropylated dextran gel forms a straightforward, universal and powerful separating system with pure solvents like methanol, acetone, methylene chloride, chloroform, ethyl acetate and N-methyl-2-pyrrolidone as mobile phases.[27a] The name "sephadex" stems from separation, pharmacia dextran.[29]

Sephadex LH-20 has dual lipophilic (L) and hydrophilic (H) properties. Its lipophilic properties are derived from the isopropyl groups gained from the hydroxypropylation of sephadex G-25 (G= gel) (Figure 2.8).

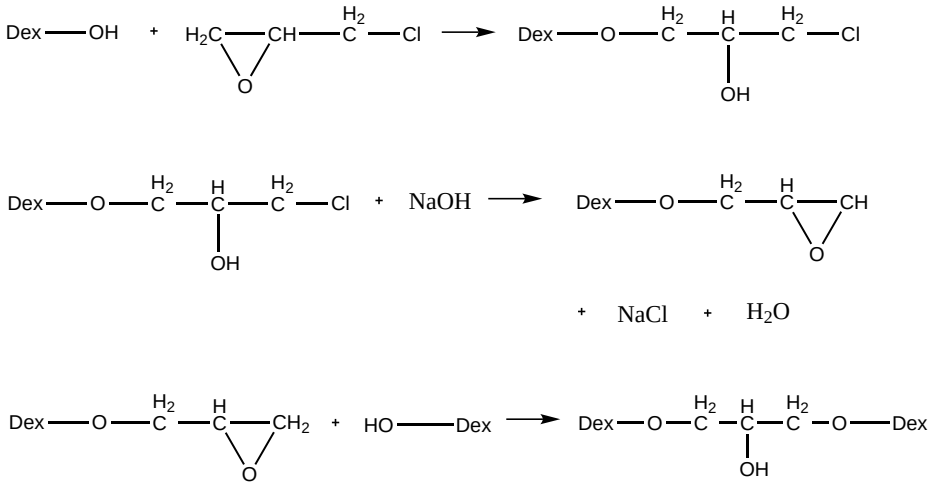


Figure 2.8 Synthesis of sephadex LH-20 from sephadex G-25.

Sephadex G-25 only contains hydrophilic properties, due to the numerous hydroxyl functions present. This dual nature of sephadex LH-20 gives it the ability to swell not only in solvents of weak and medium polarity, but also in those of higher polarity. The degree of swelling increases with increasing polarity of the solvent (mobile phase). The exclusion limit of sephadex LH-20 is 4000 g/mol, and will occur in water, methanol, dimethyl sulpoxide and N-methyl-2-pyrrolidone. In other words, all compounds with a molecular mass greater than 4000 g/mol can only pass through the interparticle spaces, and therefore elute without separation.[27b]

2.5 Reverse phase chromatography

When separating natural products, reverse phase (RP) flash chromatography is commonly used. This technique enables easy separation of saponins from other polar components, such as glycosides and oligosaccharides.[5c]

In reverse phase chromatography the stationary phase is non-polar (lipophilic) and the mobile phase polar (hydrophilic). The stationary phase is usually a modified silica with a surface bound to long-chained or substituted alkyl groups, or to other hydrocarbons. Solvents used as mobile phase are usually mixtures of water and water-miscible organic solvents such as MeOH, ACN or THF.[30]

The mechanism of retention varies depending on the properties of the RP material used. Retention is partially caused by direct interactions with the surface of the stationary phase or parts of the surface, and partially due to partition chromatography in a stationary phase containing solvent-solvated ligands. The most important interactions between the compound and the stationary phase are van der Waal forces. These relatively weak forces increase with molecular size. RP materials with long-chained hydrocarbon groups, such as C-18, give greater retention than hydrocarbons with shorter chains.[30]

In liquid chromatography, systems involving RP are commonly used. Polar molecules will have less affinity for the stationary phase and therefore elute faster. When increasing the polarity of the mobile phase, the elution becomes slower.[31]

It is also worth emphasizing the importance of the mobile phase being an aqueous solution. The general increase in retention with increasing solute size and reduced retention of polar solutes and ions, establish favorable intermolecular interactions with water. This is due to the high cohesive energy, hydrogen-bond acidity and dipolarity of water. This is the inverse of what is seen in normal phase chromatography. There are strong intermolecular interactions between water molecules that tend to promote self-association over interactions with different solvent or solute molecules.[32]

2.5.1 VersaFlash

VersaFlash is a high throughput flash purification (HTFP) technique. The main applications include flash chromatography for purification of synthetic products, isolation of target compounds from natural products and simplification of complex mixtures.[32]

VersaFlash is a system that utilizes spherical silica particles in pre-packed columns. The spherical particles optimize the uniformity of the cartridge bed, creating narrower bands, thus preventing bed shifting due to irregular particle abrasion. Spherical C-18 bonded silica will cause the target compound to elute in a narrower band by the use of less solvent, in comparison to irregular bonded silica in normal flash chromatography.[33]

Chapter 3

Results and discussion

3.1 UV-analysis

Ginsenoside Rb1 (Figure 3.1) was chosen to be the best suitable standard for this experiment since it shares a relatively similar basis structure with other saponins discovered for the *Herniaria* genus. Both the standard and the plant sample were measured at 550 nm, which is the $\lambda_{\max}$ measured for ginsenoside Rb1.

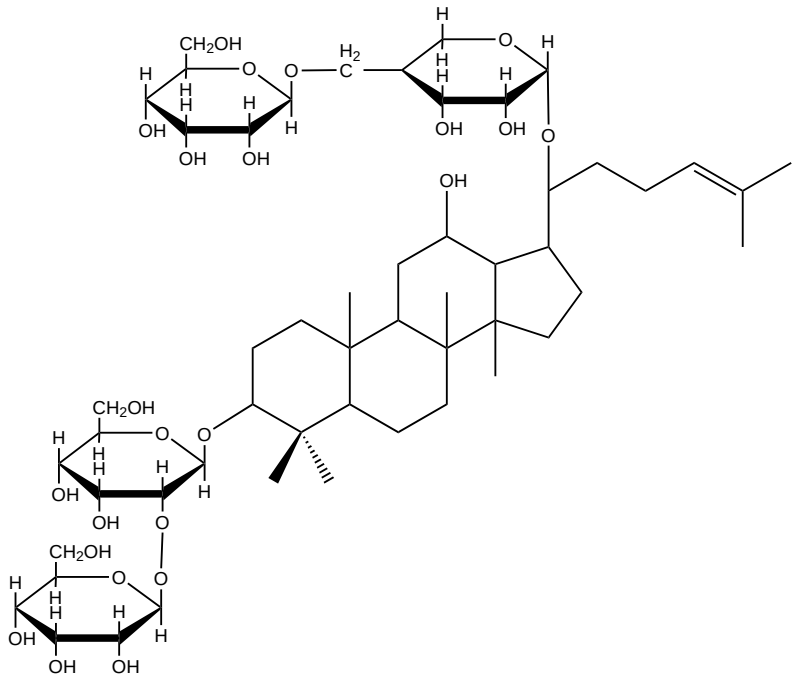


Figure 3.1 Structure of ginsenoside Rb1.

Since neither the standard nor the plant sample is UV active, it was necessary to add a chromogenic reagent. In this case a mixture of vanillin and acetic acid was used. Vanillin is easily oxidised and gave a deep purple colour when oxidized by perchloric acid. To ensure that the conditions were right, it was important that the vanillin-acetic acid solution was freshly made the same day it was used. The mechanism of this procedure with vanillin-acetic acid, perchloric acid and glacial acetic acid gives dehydration of the hydroxyl groups, and therefore increases the amount of double bonds, giving a conjugated system. Heating at 70 °C for 15 minutes was done to make sure that the reaction was complete. If the reaction mixture was heated excessively, the blank sample would get too deep a colour, increasing errors.

Concentrations of the standard and the plant sample, as well as the absorbance from the UV/VIS experiment are given in Table 3.1.

Table 3.1 UV/VIS absorbance measurements for the standard and the plant.

Concentration μg/mL	**Absorbance**
6.7	0.025
20.0	0.276
33.3	0.608
35.2*	0.635
46.7	0.929
60.0	1.226
73.3	1.421
86.7	1.604

Figure 3.2 shows the standard curve of ginsenoside Rb1. The equation from this curve,

$$f(x) = 0.0205x - 0.0862, \qquad R^2 = 0.991$$

was obtained by linear regression.

*Plant sample

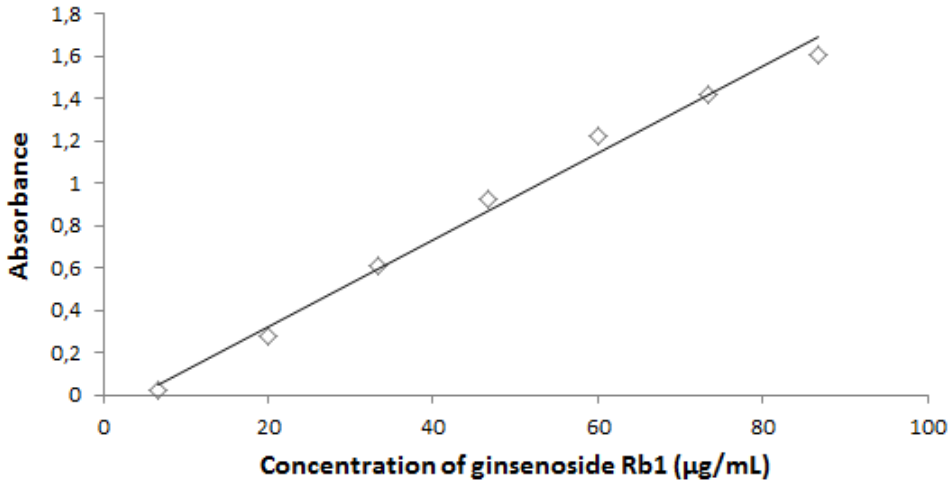


Figure 3.2 Standard curve of ginsenoside Rb1 measured at 550 nm.

From Figure 3.2, the total saponin content in *H. incana* was calculated to be 35.2 μg ginsenoside equivalent saponin content per gram of the plant extract.

3.2 Chromatography

As described in section 5.5.1, the extraction of the plant material was a tedious process. It was important that the plant was cut into small pieces to increase contact with the solvent. To assure a good separation, the methanolic plant extract was applied to a sephadex LH-20 (section 2.4) column. Four columns were run to avoid overloading the column. These separations gave eight combined fractions: A, B, C, D, E, F, G and H. The TLC analysis from these four columns is shown in section 3.2.1.

3.2.1 TLC analysis

The solvent systems used in TLC, methanol:chloroform 9:1 and 7:3, were able to differentiate and combine the fractions produced from the sephadex CC. However, these combinations needed further optimization on TLC to get the best possible R_f-value. Thirty-five different solvent systems were tested. The solvents used were toluene, diethyl ether, dichloromethane, chloroform, ethyl acetate, methanol, formic acid and water. The optimized systems for the fractions A-H, their R_f-values, colour of the spots after spraying with vanillin-sulphuric acid and heating, and their mass are shown in Table 3.2.

Table 3.2 Overview of normal phase TLC of the combined fractions from sephadex LH-20 CC.

Fraction	**Solvent system**	**R_f-value and colour**	**Mass**
A	MeOH:Et_2O 6:4	0.70 brown, 0.87 brown, 0.96 brown	1.19 g
B	MeOH:$CHCl_3$ 3:7	0.14 blue, 0.74 brown, 0.85 brown, 0.96 brown	3.56 g
C	$CHCl_3$:Et_2O 3:7	0.14 brown/blue, 0.33 brown/blue, 0.44 pale blue	1.52 g
D	MeOH:$CHCl_3$ 6:4	0.42 orange, 0.69 orange, 0.74 yellow, 0.79 blue, 0.83 brown, 0.88 brown, 0.94 brown	1.54 g
E	EtOAc:HCO_2H:H_2O 8:1:1	0.09 yellow, 0.23 ocher, 0.35 pale yellow, 0.48 pale yellow, 0.95 pale blue/violet	5.04 g
F	EtOAc:HCO_2H:H_2O 8:1:1	0.2 ocher, 0.26 pale yellow, 0.34 pale yellow, 0.45 pale yellow, 0.58 yellow, 0.95 pale blue/violet	0.38 g
G	MeOH:DMC 4:6	0.65 pale yellow, 0.90 yellow, 0.94 green	0.85 g
H	MeOH:DMC 4:6	0.89 yellow, 0.94 yellow	0.55 g

3.3 Fraction B

Of the fractions from Table 3.2, fraction B is a stand out because of its pure blue colour. After spraying with vanillin-sulphuric acid and heating the TLC plate, saponins often give a blue/violet colour.[34] Therefore, fraction B was selected for further separation (Figure 3.3). It consisted of five spots, three of which were most likely residue from fraction A, the blue target compound and a completely retained yellow impurity.

It has been reported that saponins often are soluble only in aqueous alcohol solutions, or pure alcohols.[5c] Since the TLC gave differences in polarity between the spots in fraction A and B (Table 3.2), where the latter was the most polar, an attempt was made to dissolve fraction A in a solvent with different polarity than methanol. If the A fraction was soluble in another solvent, then this solvent would extract the A parts in the B fraction, possibly giving a precipitation of B. The solvents used to try to extract A were diethyl ether, dichloromethane, ethyl acetate, chloroform and acetonitrile. Unfortunately, none of these solvents were able to dissolve A completely, ruling out simplification of the B fraction by this method.

Before fraction B could be subjected to VersaFlash C-18, an optimized system on RP TLC was needed. Thirty different solvent systems were tested. These were methanol:chloroform, methanol:water, methanol:dichloromethane, acetonitrile:water, methanol:water:formic acid, acetonitrile:water:formic acid, and ethyl acetate:water:formic acid in various ratios. Of the systems tested, methanol:water 6:4 gave the best separation and reduced tailing in comparison to the other systems. In this solvent system the B fraction showed spots with the following R_f-values: 0, 0.05, 0.16, 0.28 and 0.34. The latter belongs to the target compound, and the rest to the impurities.

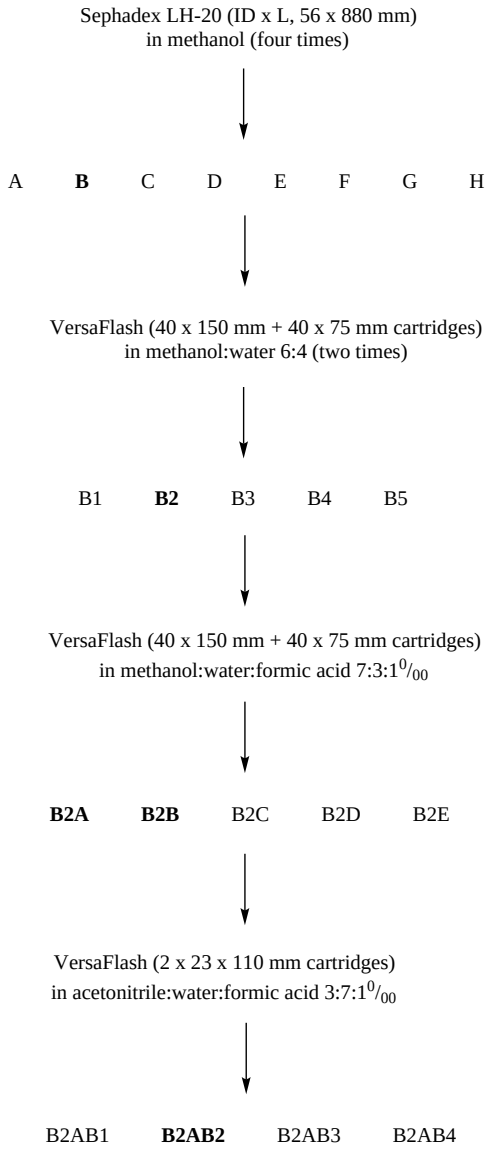


Figure 3.3 Separation scheme of the plant material.[†]

[†]The selected fractions are in bold.

Separation of the B fraction gave five new fractions: B1, B2, B3, B4 and B5 (Figure 3.3). These fractions were tested on RP TLC, using methanol:water 6:4 as a solvent system. Even though most of the impurities had been removed, the RP TLC still showed overlapping tailing with the target compound. To optimize a new RP TLC system, nine solvent systems were tested, such as methanol:water, methanol:dichloromethane, methanol:water:formic acid and acetonitrile:water in various ratios. Of these systems methanol:water:formic acid 7:3:1‰ gave the best separation, and showed that the B2 fraction contained most of the target compound, with R_f-values of 0.23 (impurity) and 0.51 (blue spot).

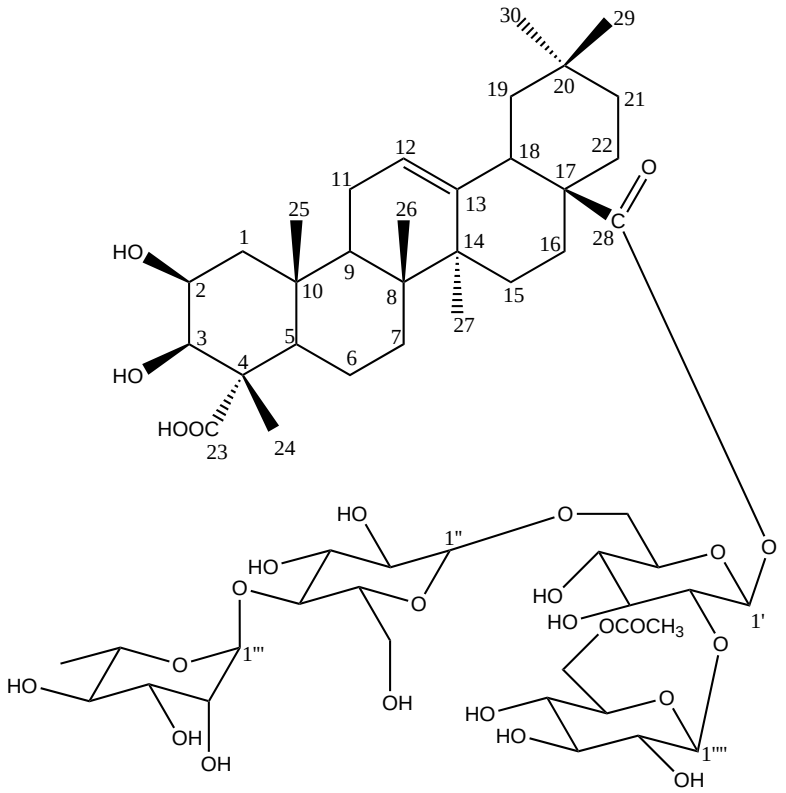
The B2 fraction was subjected to VersaFlash C-18, using methanol: water:formic acid 7:3:1 ‰ as solvent system. This gave five combined fractions: B2A, B2B, B2C, B2D and B2E (Figure 3.3). To optimize the solvent system for these fractions, two such systems were tested: Acetonitrile:water 3:7 and acetonitrile:water:formic acid 3:7:1‰, the latter having the best separation. Of the combined fractions, B2A and B2B contained three common spots with R_f-values of 0.22, 0.26 and 0.31, while B2B contained one more with a R_f-value of 0.18.

Both of these fractions were run separately on VersaFlash C-18, using acetonitrile:water:formic acid 3:7:1‰ as a solvent system. According to RP TLC, all the obtained sub-fractions were combined to four fractions: B2AB1, B2AB2, B2AB3 and B2AB4 (Figure 3.3). The B2AB2 fraction contained only one blue spot with a R_f-value of 0.22, in addition to a pale brown tail. To remove the tail, diethyl ether was added to B2AB2. The impurity was extracted, giving B2AB2 a yield of 45 mg.

3.4 Spectrometry

3.4.1 NMR spectrometry

Structure elucidation of the isolated compound was done by NMR. It revealed that the isolated compound was a triterpenoid saponin with four monosaccharide units (Figure 3.4).



1

Figure 3.4 Structure and numberings of atoms in **1**.

The assignment of ^{13}C NMR (δ C), ^{1}H NMR shifts (δ H), multiplicity (M) and coupling constants (J) for the triterpene and the monosaccharides is shown in Table 3.3 and 3.4, respectively. The numbering of the atoms are shown in Figure 3.4. ^{1}H and ^{13}C shifts are assigned in accordance to ^{1}H (Figure A.1), ^{13}C (Figure A.2), COSY-45 (Figure A.3), HSQC edited version (Figure A.4), HMBC (Figure A.5), H2BC (Figure A.6), HSQC-TOCSY (Figure A.7), NOESY (Figure A.8) and ^{1}H,^{1}H J-resolved experiment (Figure A.9 and A.10) NMR spectra (Appendix A).

Table 3.3 Assigned shifts for the triterpene in **1**.

Position	**Carbon**	δ **C**	δ **H**	**M**	***J*(Hz)**
1	CH_2	45.69	α 1.23, β 2.11	m, m	-
2	CHOH	72.17	α 4.07	m	-
3	CHOH	76.58	α 3.97	m	-
4	C	54.33 (52.20)‡	-	-	-
5	CH	52.95	α 1.59	m	-
6	CH_2	22.1	α 1.18, β 1.71	m, m	-
7	CH_2	33.9	α 1.32, β 1.53	m, m	-
8	C	41.2	-	-	-
9	CH	49.81	α 1.60	m	-
10	C	37.55	-	-	-
11	CH_2	24.8 (23.60)‡	α 1.94, β 2.01	m, m	-
12	CH	123.71	5.26	m	-
13	C	145.14	-	-	-
14	C	43.11	-	-	-
15	CH_2	29.79	α 1.00, β 1.72	m, m	-
16	CH_2	23.69 (24.70)‡	α 1.93, β 1.80	m, m	-

Continued on next page

‡Reported shift.[35]

Table 3.3 cont.

Position	**Carbon**	δ **C**	δ **H**	**M**	***J*(Hz)**
17	C	48.02	-	-	-
18	CH	42.58	α 2.83	dd	4.2/13.7
19	CH_2	47.19	α 1.16, β 1.70	m, m	-
20	C	31.72	-	-	-
21	CH_2	35.04	α 1.22, β 1.38	m, m	-
22	CH_2	33.30	α 1.66, β 1.58	m, m	-
23	COOH	182.10	-	-	-
24	CH_3	13.20	1.33	s	-
25	CH_3	17.38	1.28	s	-
26	CH_3	17.99	0.80	s	-
27	CH_3	26.60	1.16	s	-
28	COO-sugar	178.20	-	-	-
29	CH_3	33.64	0.91	s	-
30	CH_3	24.40 (23.60)‡	0.94	s	-

‡Reported shift.[35]

Table 3.4 Assigned shifts for the monosaccharides in **1**.

Position	δ **C**	δ **H**	**M**	***J*(Hz)**
β-D-Glucopyranose I				
1'	93.86	5.38	d	7.9
2'	79.10	3.67	m	-
3'	70.80 (75.20)[§]	3.45	m	-
4'	78.06	3.85	m	-
5'	78.19	3.54	m	-
6'	69.30	3.81/4.08	m/m	-
β-D-Glucopyranose II				
1"	104.29	4.41	d	7.6
2"	75.44	3.23	m	-
3"	76.80	3.47	m	-
4"	79.61	3.55	m	-
5"	76.95	3. 29	m	-
6"	61.94	3.66/3.81	m/m	-

Continued on next page

[§]Reported shift.[35]

Table 3.4 cont.

Position	δ **C**	δ **H**	**M**	***J*(Hz)**
α-L-Rhamnopyranose				
1”’	103.10	4.85	d	3.7
2”’	72.60	3.84	m	-
3”’	72.32	3.64	m	-
4”’	73.90	3.41	m	-
5”’	70.80	3.98	m	-
6”’	17.86	1.26	m	-
6-Acetyl-β-D-glucopyranose				
1””	104.37	4.79	d	7.8
2””	75.73	3.20	m	-
3””	78.13	3.37	m	-
4””	72.02	3.21	m	-
5””	75.30 (79.10)[§]	3.47	m	-
6””	65.55	4.18/4.34	m/m	-
Acetyl				
Me	21.11	2.09	m	-
CO_2	172.88	-	-	-

[§]Reported shift.[35]

The carbon sequences in compound **1** were confirmed by means of COSY-45, H2BC, HMBC and HSQC-TOCSY NMR experiments. Coupling constants were extracted from ^{1}H and ^{1}H, ^{1}H J-resolved NMR experiments, and are only given for non-overlapping protons. The conformation of the protons was assigned by coupling constants, as well as NOESY NMR experiment. The conformation of C-23 and C-24 in the triterpene in compound **1** was confirmed by the lack of NOESY signal, shown in Figure 3.5.

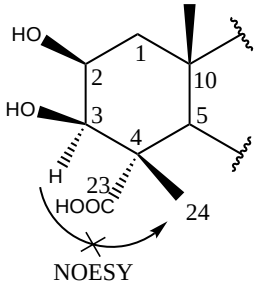


Figure 3.5 Conformation of C-23 and C-24 in **1**.

The point of attachment of the sugar groups, and the kind of NMR experiment used to confirm them, is shown in Figure 3.6.

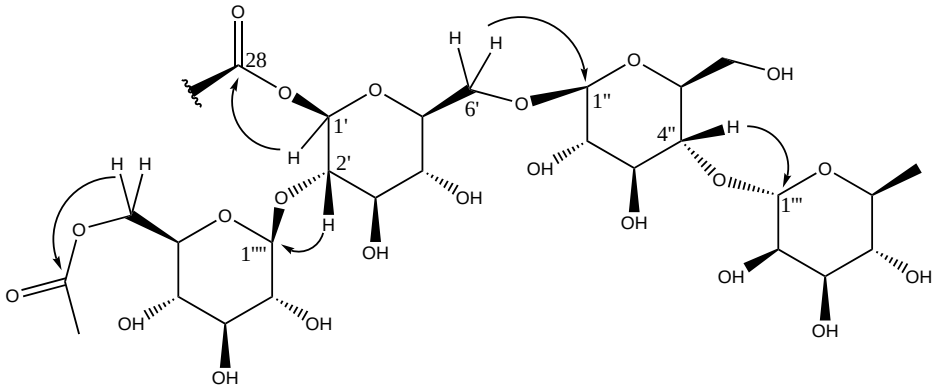


Figure 3.6 HMBC-confirmed connectivity of monosaccharide units.

Freiler *et al.*[35] have reported the same saponin as compound **1** (Figure 3.4), extracted from *Herniaria glabra*. All the primary data for **1** is similar to that of Freiler *et al.*[35], but there are some deviations in the assignment of carbon shifts (Table 3.3 and 3.4): Some of their carbon shifts in the triterpene, as well as two of the carbons in the sugars glucopyranose I and 6-acetyl-glucopyranose, seem to be interchanged, probably due to typing errors.

3.4.2 Mass spectrometry

The mass accuracy results from MS with an ESI source of compound **1** (Appendix B, Figure B.1) are summarized in Table 3.5. The sample was run in the positive mode. The protonated molecular ion was not detected; only ammonium and sodium adducts were identified. The obtained results are within the 2 ppm error range.

Table 3.5 Main MS adducts for **1**.

m/z	**Adduct**
1194.59	$[M + NH_4]^+$
1199.55	$[M + Na]^+$

Both of the adducts from Table 3.5 gave a molecular weight of 1176 g/mol. This corresponds to a molecular formula of $C_{56}H_{88}O_{26}$, which is the same as for the structure elucidated for compound **1** (Figure 3.4).

The sugar moieties in compound **1** were confirmed by running ESI-CID experiments in both positive and negative mode (Appendix B, Figure B.2 and B.3). For the positive mode, six main fragment ions, all of them considered to be sodium adducts, were observed at *m/z* 347, 389, 493, 551 and 697. The mass difference between the parent ion and the fragment ion *m/z* 697 was 501, corresponding to the loss of the triterpene unit. The loss of *m/z* 493 and 551 pointed to a loss of 6-Acetyl-β-D-glucose and α-L-Rhamnose, respectively. The fragment ions at *m/z* 347 and 389 indicated a loss of one glucose and one rhamnose sugar unit from the sugar moiety ion. The fragmentation pathway of the molecular ion $[M + Na]^+$ *m/z* 1199 is summarized in Figure 3.7.

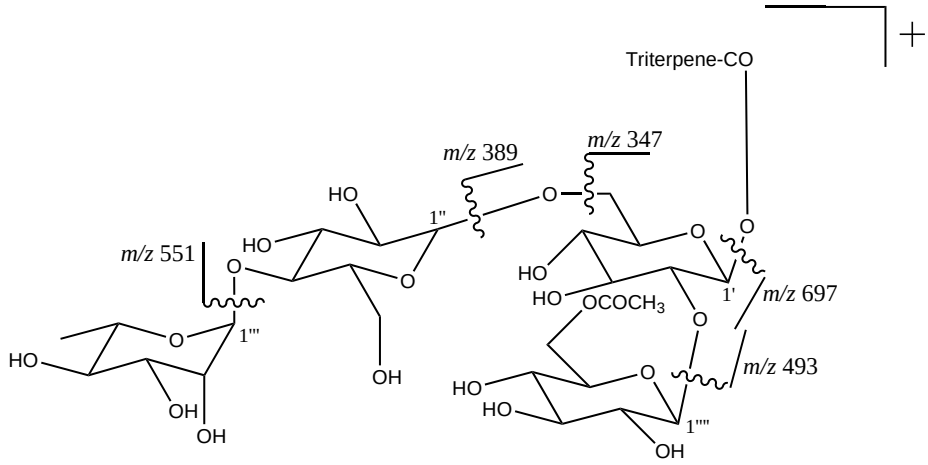


Figure 3.7 Proposed fragmentation pathway for $[M + Na]^+$ *m/z* 1199 of **1**.

In the negative mode ESI-CID experiment, the deprotonated molecular ion $[M - H]^-$ at *m/z* 1175 gave three daugther ions at *m/z* 483, 502 and 543. The two first corresponded to the triterpene unit after the loss of the four sugar moieties. The ion at *m/z* 543 pointed to a loss of α-L-Rhamnose. The fragmentation pathway for the deprotonated molecular ion $[M - H]^-$ *m/z* 1175 is shown in Figure 3.8.

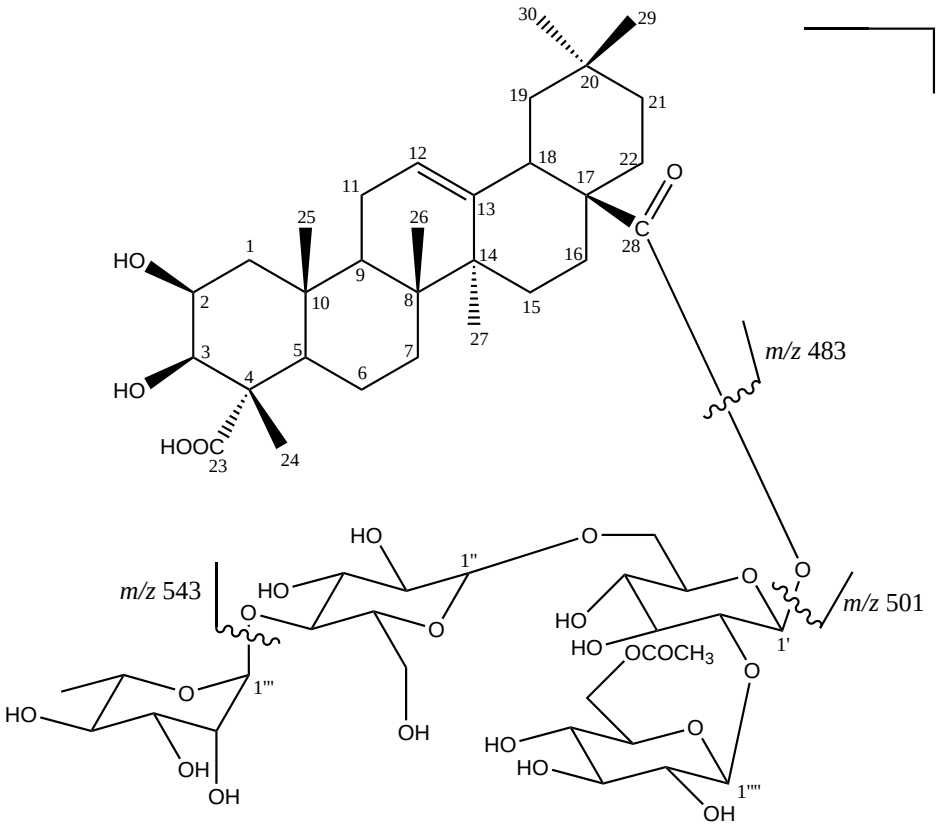


Figure 3.8 Proposed fragmentation pathway for [M - H]$^-$ *m/z* 1175 of **1**.

3.5 Future work

Based on TLC, several of the obtained fractions in this work displayed strong indications of containing saponins. As a logical continuation of this investigation, further work on their separation, isolation and structure elucidation is necessary. The identified saponin, as well as those that are yet to be isolated and identified, have to be tested for

various types of biological activity, which might eventually result in the discovery of new drug candidates.

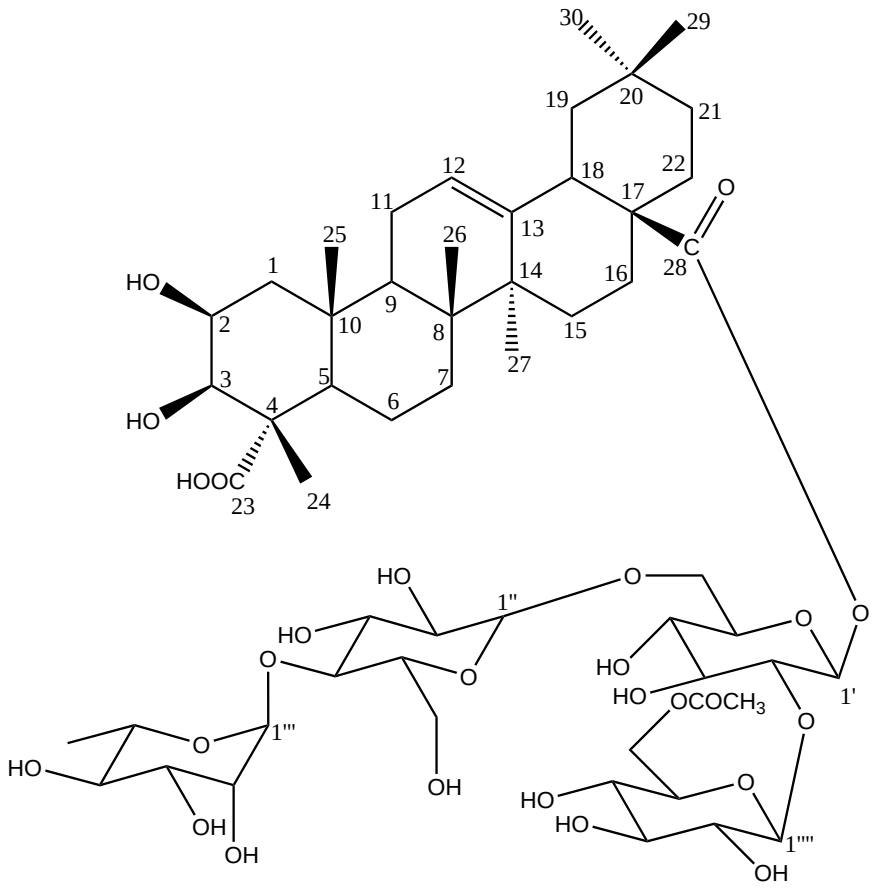
Chapter 4

Conclusion

The goals of this master project have been fully achieved by carrying out the proposed plan of research.

The total saponin content of *Herniaria incana* has been determined to be 35.2 μg ginsenoside equivalent saponin conent per gram of the plant extract, when using ginsenoside Rb1 as standard, measured at 550 nm.

A saponin has been extracted and isolated from *H. incana* by optimized conditions. The structure of compound **1** was elucidated by 1D and 2D NMR experiments as *O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D- glucopyranosyl-(1→6)-*O*-[β-D-6-*O*-acetylglucopyranosyl-(1→2)]-β-D-glucopyranosyl Medicagen-28-ate.



1

The sugar sequence was confirmed by ESI-CID experiments.

The identified saponin was also found in *H. glabra*[35], a close relative of *H. incana*. From a chemotaxonomic point of view, the findings of this work confirm the taxonomic positions of these plants and indicates that biosynthesis of saponins in these species is most likely

under genetic control.

For further insight into the composition of *H. incana*, the remaining fractions from the first separation step (Figure 3.3) should be investigated.

Chapter 5

Experimental

5.1 General

Solvents and chemicals were supplied by Sigma-Aldrich, Fluka Chemika, VWR and Merck.

5.2 Analysis

5.2.1 UV spectroscopy

UV-VIS spectra and absorption measurements were recorded on a Shimadzu UV mini 1240 single beam spectrophotometer. The samples were contained in quartz cells.

5.2.2 Chromatography

TLC analysis was done with Merck and Fluka Chemika 0.2 mm silica gel 60 on Al sheets plates, F_{254}, and Merck 2.0 mm silica gel 60 RP-18 glass plates, $F_{254}S$. The plates were developed by vanillin-sulphuric acid spray (0.25 g vanillin per 25 mL sulphuric acid) and heat. Column chromatography was performed on sephadex LH-20 gel, using a BIO-RAD BioLogic LP pump, and Supelco VersaFlash pre-packed VersaPak cartriges, using a Scilog pump. The eluent systems and length of column/cartridges are specified for each separation (Figure 3.3). The fractions were collected by ISCO fraction collector, model 2200.

5.2.3 NMR-spectrometry

^{1}H NMR and ^{13}C NMR data were recorded using a Bruker Avance 600 spectrometer operating at a proton frequency of 600.18 MHz, with a 5 mm triple-resonance cryo probe equipped with a z-gradient. The sample containing a solution of 25 mg of the substance in MeOD-d4 was measured at 298 K with the solvent signal as a reference. The data was analyzed with Bruker TopSpin 3.1. Pulse sequences from the Bruker library were used for following experiments:

1D NMR: ^{1}H, ^{13}C.

2D NMR: COSY-45, edited HSQC, HMBC, H2BC ,HSQC-TOCSY, NOESY and ^{1}H,^{1}H *J*-resolved experiment.

Shifts are assigned in Section 3.4.1. ^{1}H signal splitting patterns are abbreviated s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). All spectra are given in Appendix A.

5.2.4 Mass spectrometry

Accurate mass determination was performed on an Agilent 6520 QTOF MS instrument with an ESI source (SINTEF, Biotechnology).

ESI-CID experiments were performed using a LC-MS triple quadrupole (Waters Xevo TQS Acquity). All samples were dissolved in MeOH. A full scan of ions ranging from 50 to 1400 m/z was carried out in both positive and negative mode. All spectra are given in Appendix B.

5.3 Plant material

The plant material used in this work originates from Gabrovačko Brdo (Niš, Serbia). The plant was harvested in May/June 2009 by Bojan Zlatković (University of Niš, Department of Biology and Ecology, Niš, Serbia). A specimen of *H. incana* was deposited in the Herbarium collection under the acquisition number 6581 at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš.

The plant was dried for ten days at a shadowed place, and stored in a dark place protected from light. Soil and roots were removed, and the aerial parts were broken into smaller pieces and used for extraction. The total amount of cleaned plant was 426 g.

5.4 Determination of total saponin content

5.4.1 Preparation of sample solution

The dry aerial parts of *H. incana* (5.0 g) were defatted with petroleum ether (2 × 50 mL). The defatted phases were removed, and ethanol

(75%, 150 mL) was added. Reflux was done at 70 °C for four hours, the extract filtered and evaporated at 40 °C in rotavapour. The dry residue was extracted with *n*-butanol saturated with water (3 × 40 mL). The combined extracts were evaporated to dryness. The dry plant extract (yield 0.93 g, 18.6%) was dissolved in pure methanol to a volume of 25 mL.

To prepare the plant sample for UV-measurement, the following procedure was done: 50 μL of the plant sample was evaporated to dryness. A fresh solution of vanillin-acetic acid (5% w/v, 0.2 mL) and perchloric acid (0.8 mL) was added and kept at 70 °C for 15 minutes. The sample was cooled on ice for 20 seconds before adding glacial acetic acid (5 mL). The sample was scanned on UV/VIS at 550 nm.

5.4.2 Preparation of standard solution

The standard, ginsenoside Rb1 (2.0 mg), was dissolved in ethanol to a volume of 10 mL. The following dilutions were made; 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 mL. The dilutions were evaporated to dryness before adding vanillin-acetic acid (5% w/v, 0.2 mL) and perchloric acid (0.8 mL). They were then heated at 70 °C for 15 minutes. The samples were cooled on ice for 20 seconds and glacial acetic acid (5 mL) was added. The absorbance of the dilutions from the standard solution were measured at 550 nm.

5.5 Column chromatography

5.5.1 Extraction of plant material

The dry aerial parts of *Herniaria incana* were cleaned and cut into smaller pieces. The total amount of *H. incana* was 391.92 g. The dry

plant was exhaustively extracted three times with MeOH (80%), each time for 12 hours, and sonificated for 15 minutes every two hours. Filtered extracts were combined and the volume reduced to 500 mL on rotavapour at 40 °C. The methanol extract was defatted with hexane (4 × 250 mL), and then evaporated to dryness. The dry residue was suspended in water and extracted with *n*-butanol saturated with water (6 × 250 mL). The combined butanol extract was evaporated (rotavapour, 40 °C). Traces of solvent were removed by flushing with nitrogen for 5 hours, giving a yield of 17.89 g (4.6%).

5.5.2 Separation

The dry plant extract was dissolved in methanol (150 mL), giving a concentration of 119 mg/mL. The methanolic plant extract was subjected to sephadex LH-20 CC (Inner diameter; 56 mm × length; 880 mm), using methanol as solvent. This separation was run four times, with 4 g of plant extract and a flow of 0.5–0.9 mL/min each time. Every second fraction was checked on TLC, using methanol:chloroform 9:1 and 7:3 as solvent systems. The optimized eluent systems for the combined fractions are specified in Table 3.2.

5.6 VersaFlash

Fraction B from the sephadex column was subjected to VersaFlash C-18 (Figure 3.3). Cartridges of 40 × 150 mm and 40 × 75 mm dimensions were connected into a longer column. The solvent system used was methanol:water 6:4. The separation was run twice with portions of 1.5 g (68 mg/mL) and a flow of 9–10 mL/min each time. The fractions were checked on RP TLC, using methanol:water:formic acid 7:3:1‰ as solvent system.

Fraction B2, 600 mg (65 mg/mL), was separated on VersaFlash C-18

(40 × 150 mm + 40 ×75 mm cartridges), using methanol:water:formic acid 7:3:1‰ as solvent system, and a flow of 9–10 mL/min. The fractions were checked on RP TLC, using acetonitrile:water:formic acid 3:7:1‰ as solvent system.

Fractions B2A and B2B were applied to VersaFlash C-18 (2 × 23 × 110 mm cartridges), using acetonitrile:water:formic acid 3:7:1‰ as solvent system. For RP TLC the same solvent system as for the column separation was used. B2A and B2B were combined into one fraction, B2AB2, ready for structure determination.

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Appendix A

NMR spectra

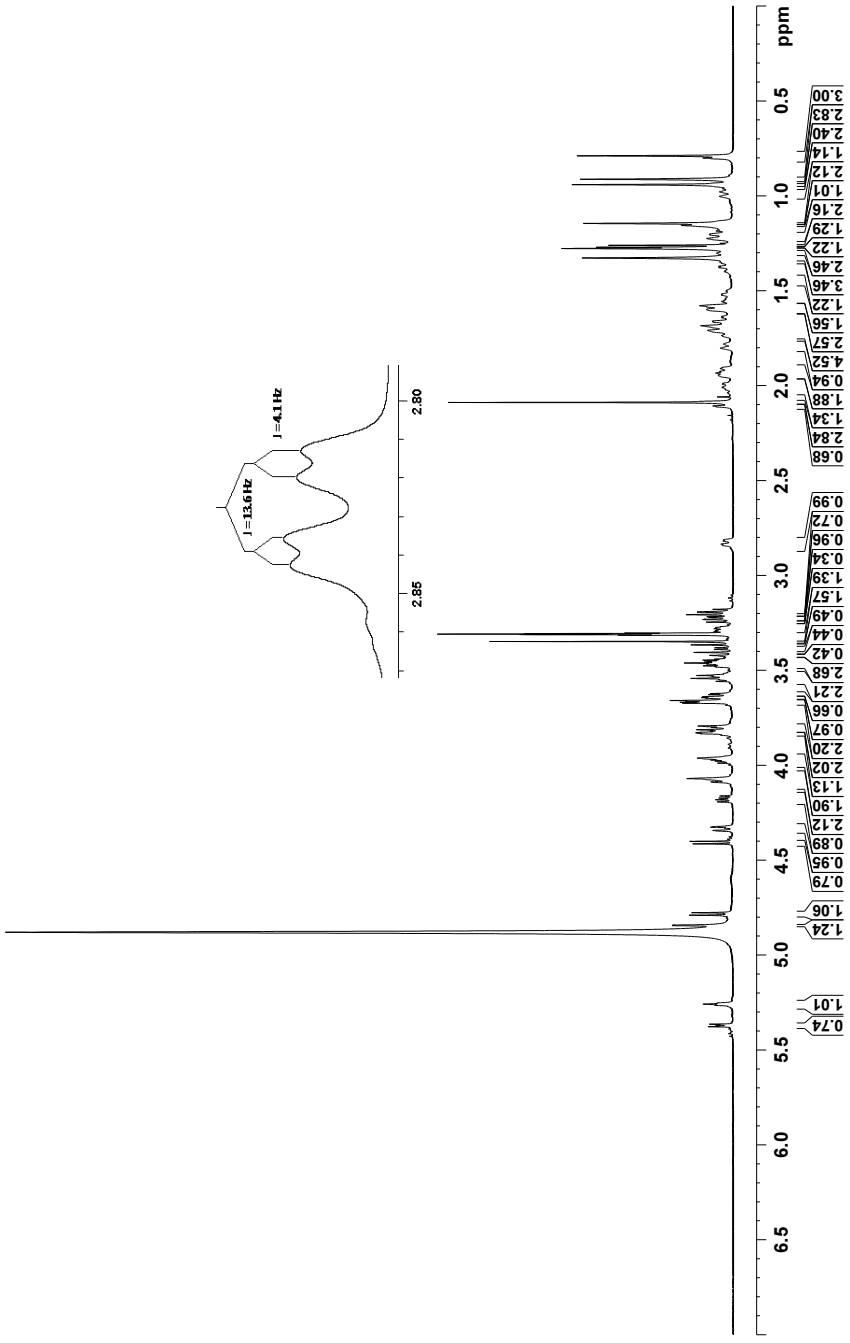

Figure A.1 ^{1}H NMR spectrum.

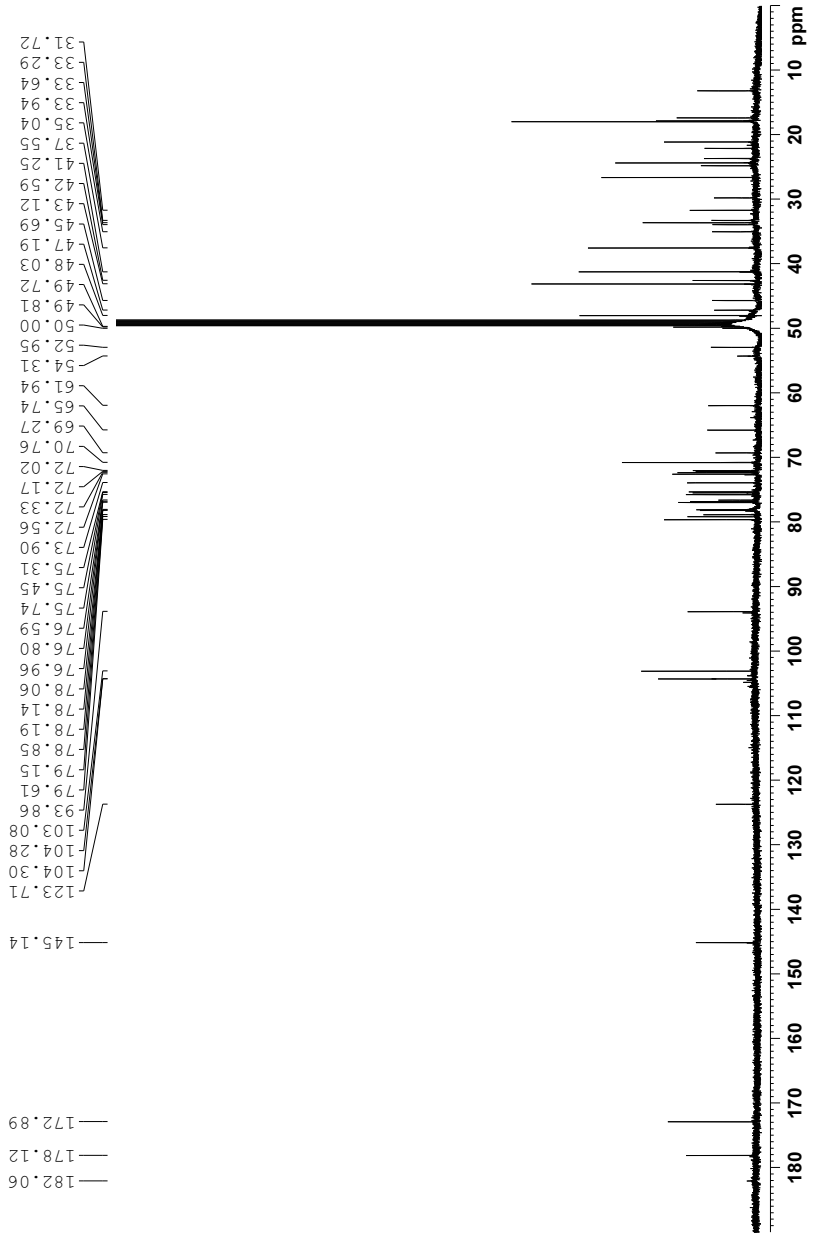

Figure A.2 ^{13}C NMR spectrum.

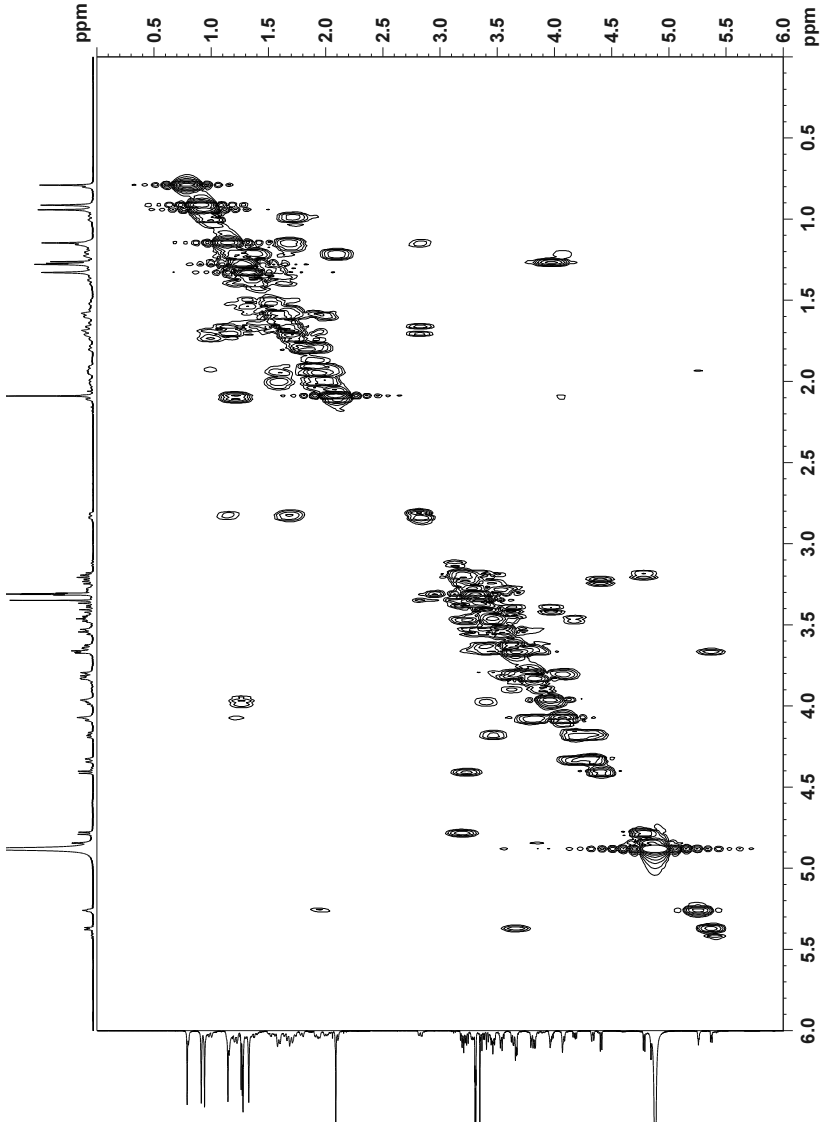

Figure A.3 COSY-45 NMR spectrum.

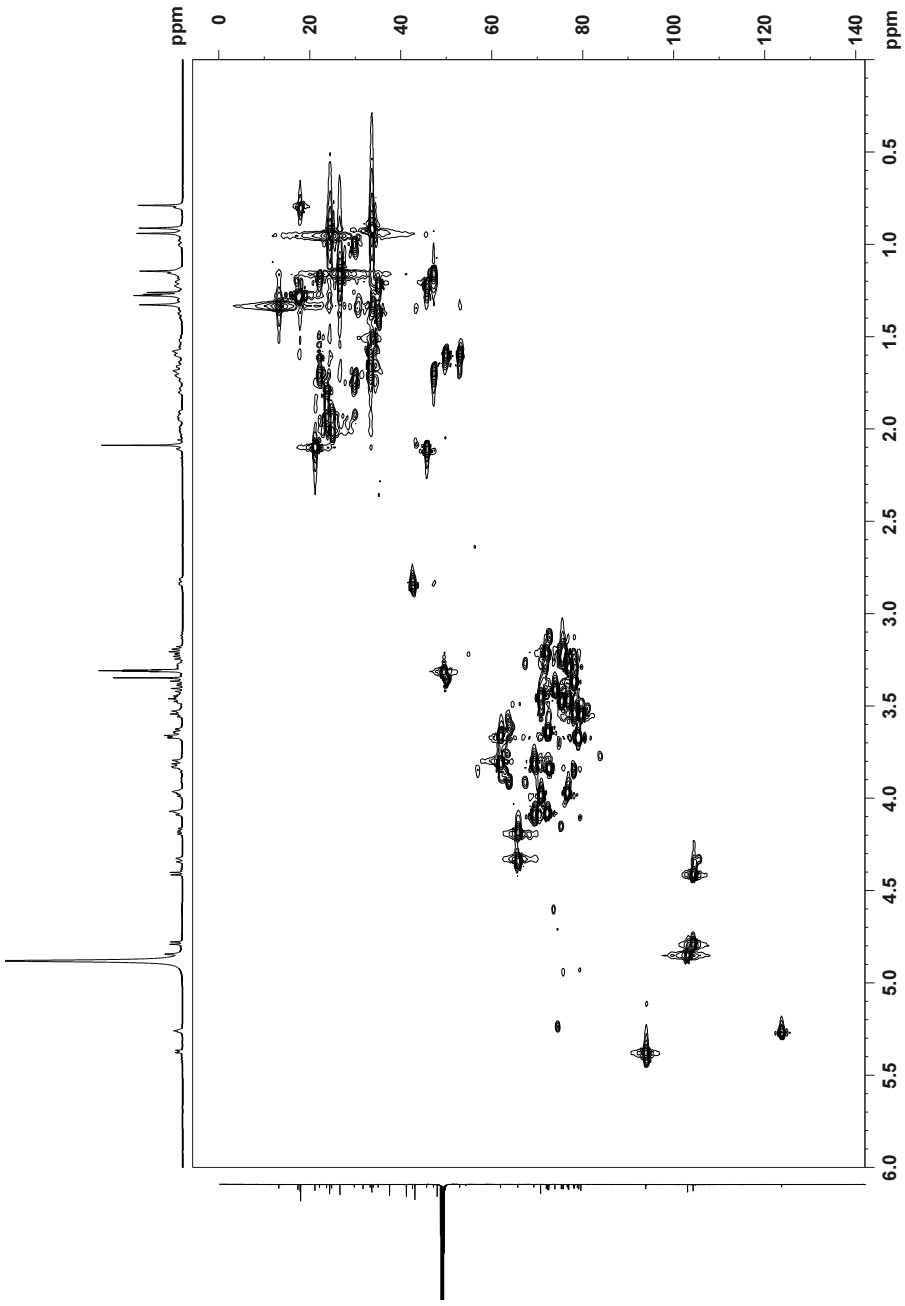

Figure A.4 Edited HSQC NMR spectrum.

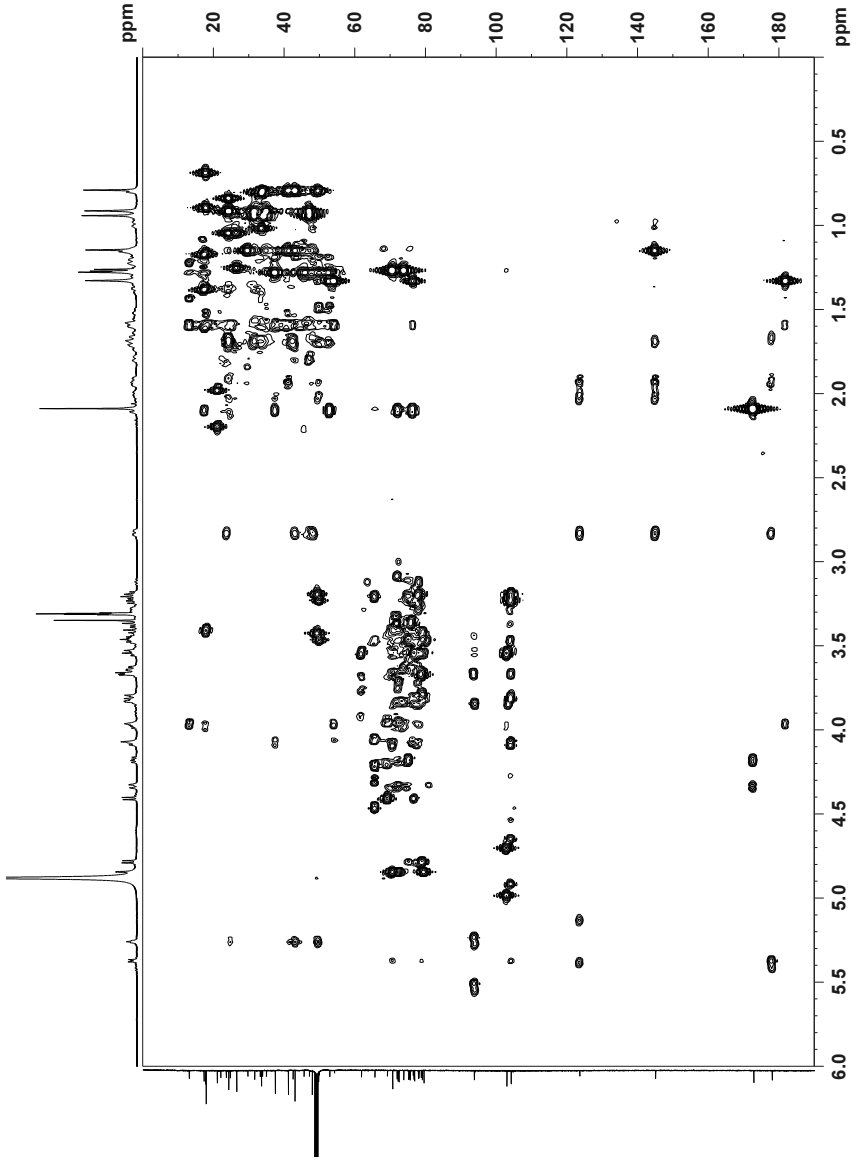

Figure A.5 HMBC NMR spectrum.

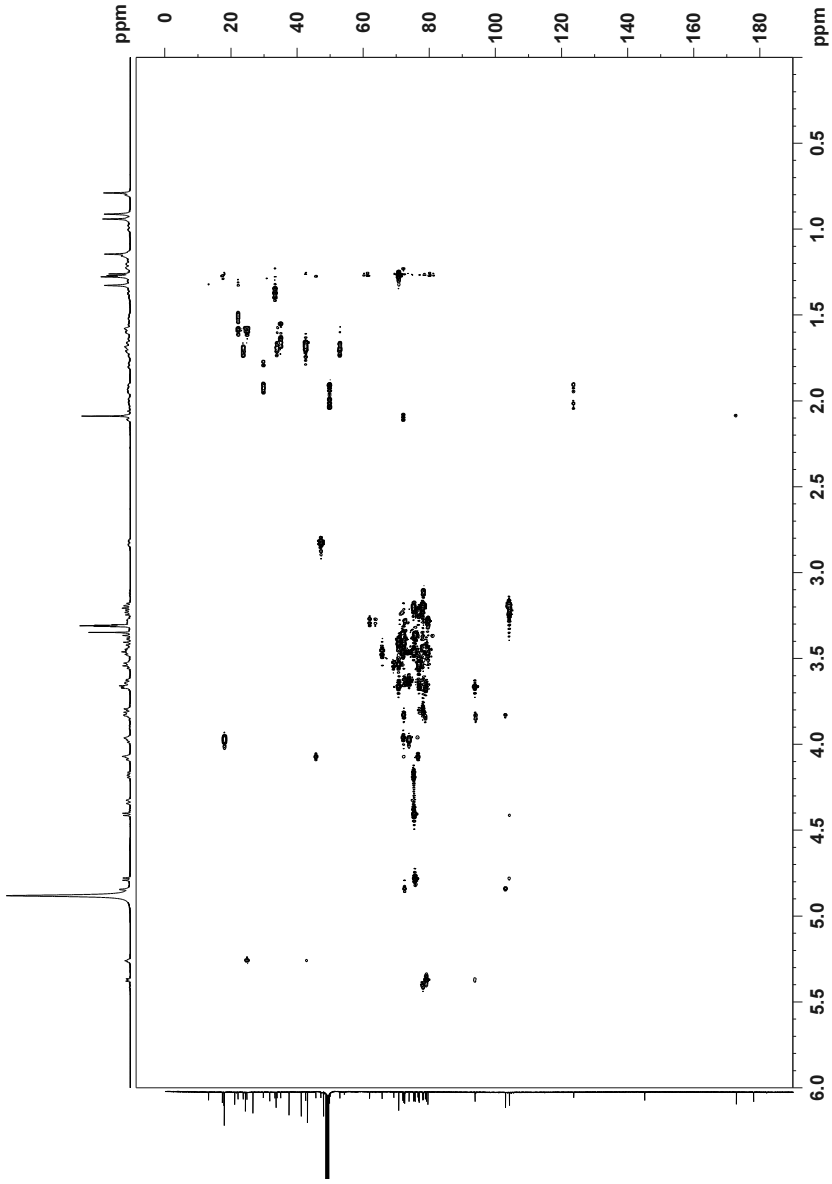

Figure A.6 H2BC NMR spectrum.

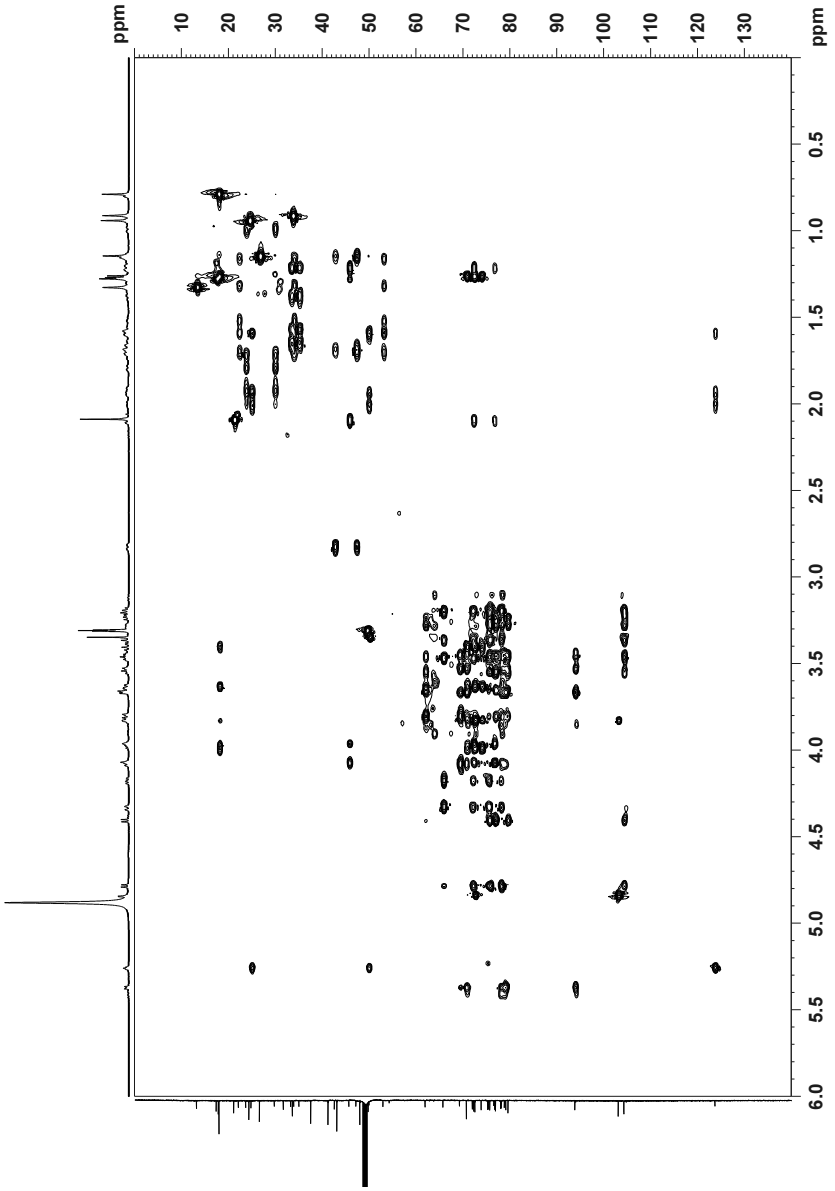

Figure A.7 HSQC-TOCSY NMR spectrum.

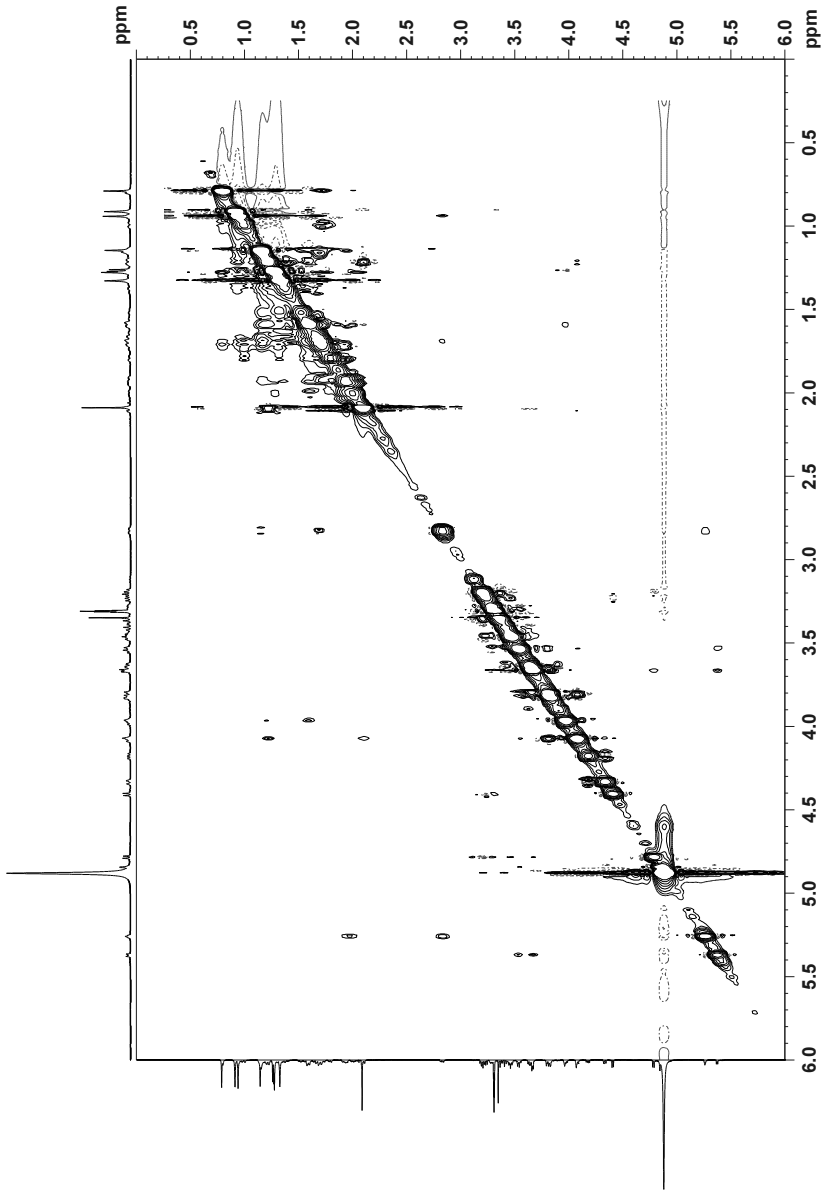

Figure A.8 NOESY NMR spectrum.

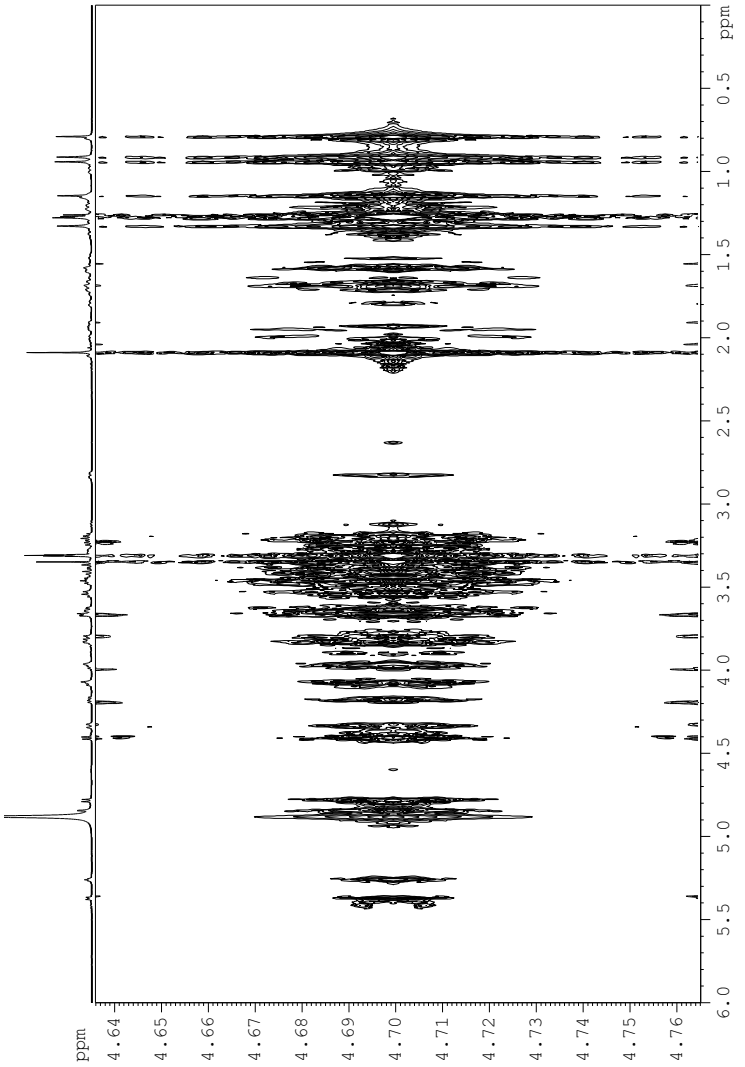

Figure A.9 ^{1}H,^{1}H *J*-resolved NMR spectrum.

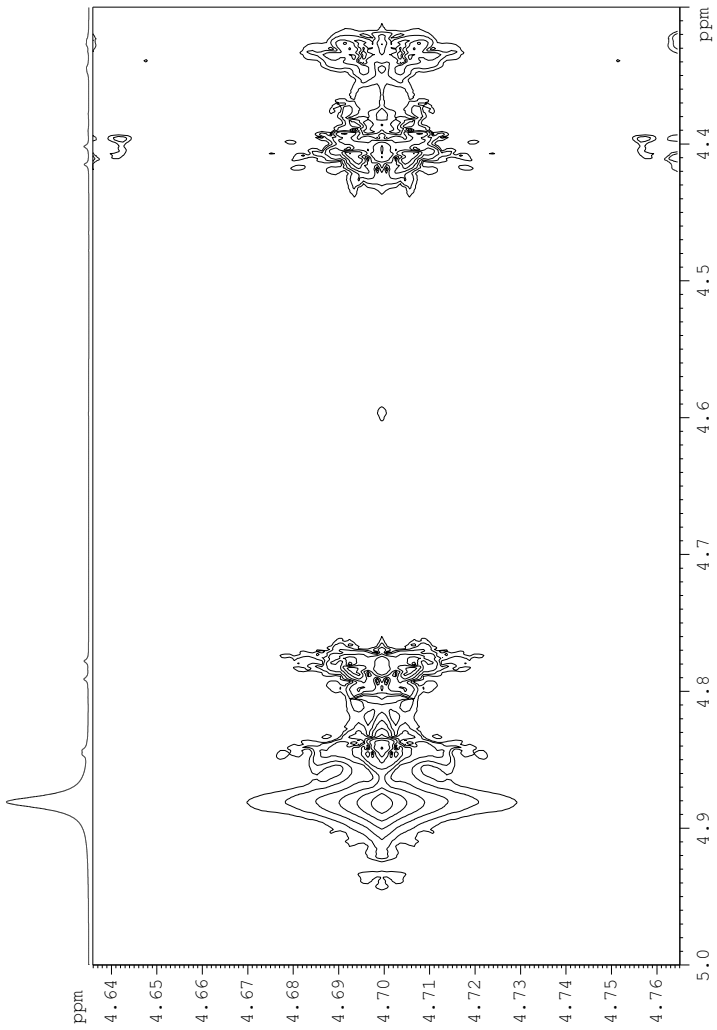

Figure A.10 Expanded ^{1}H,^{1}H J-resolved NMR spectrum.

Appendix B

MS spectra

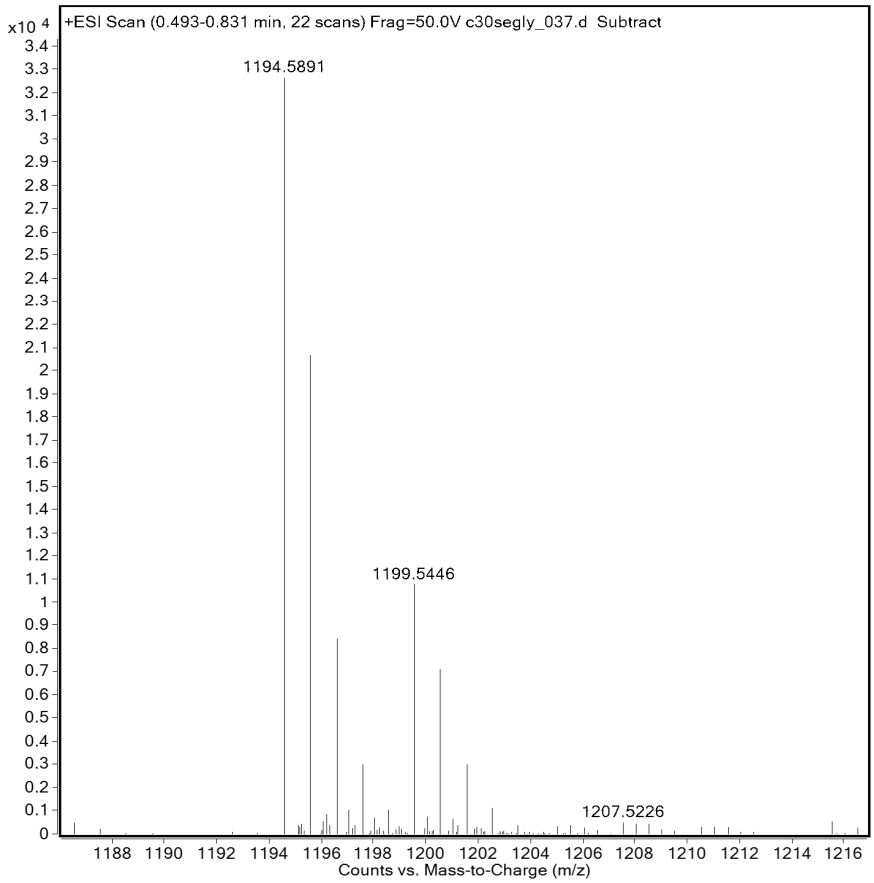

Figure B.1 ESI spectrum.

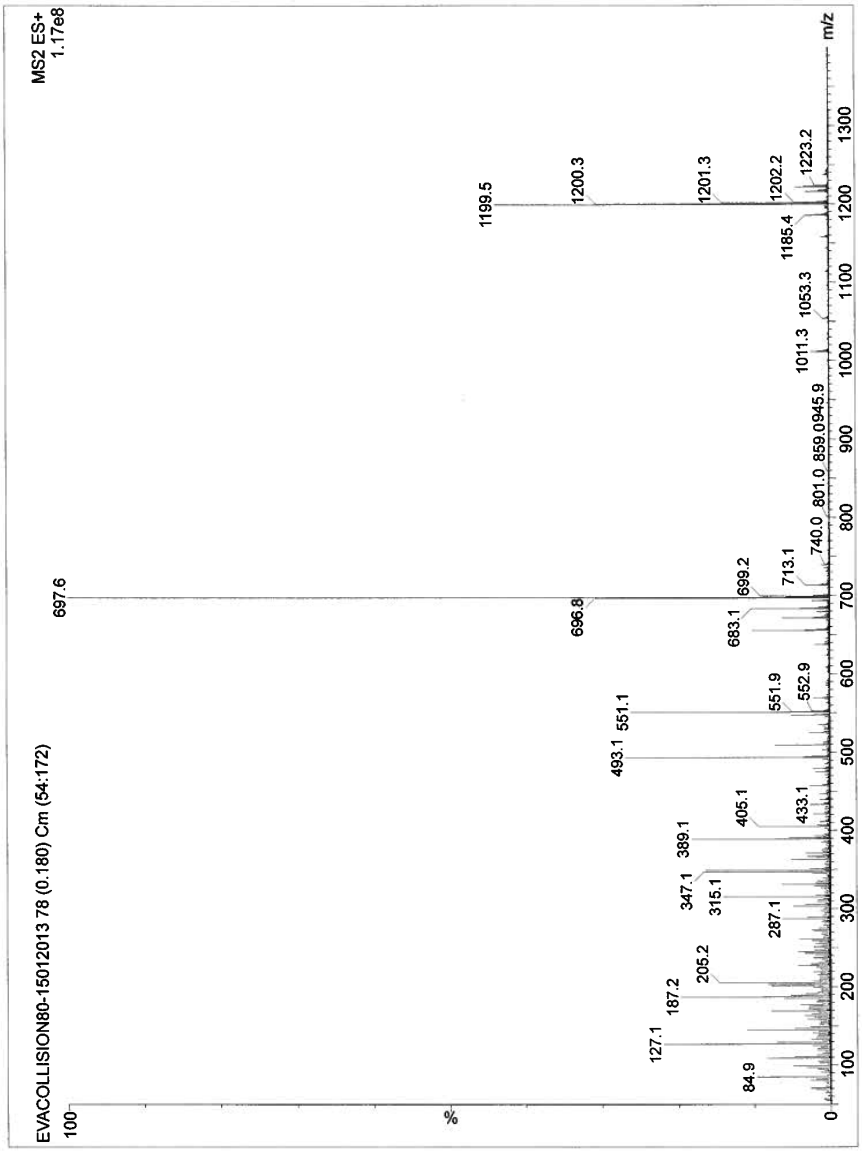

Figure B.2 ESI-CID in positive mode.

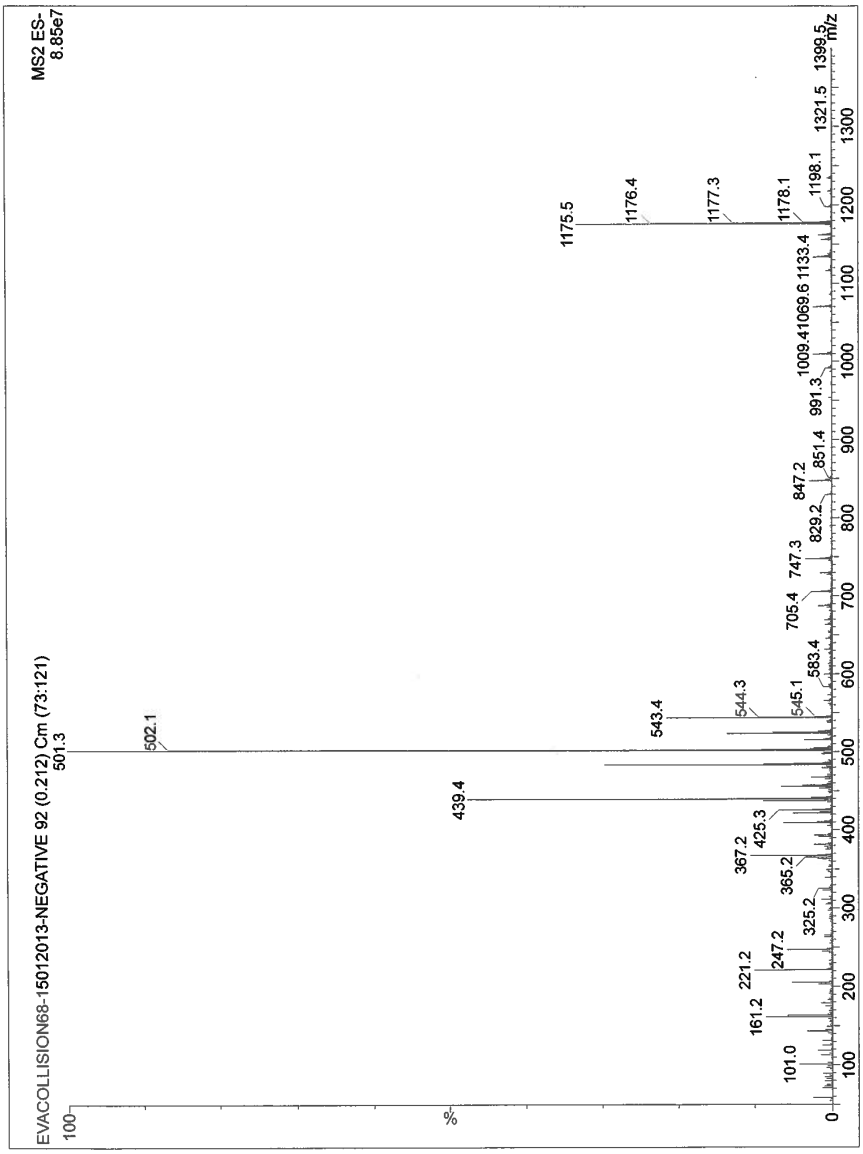

Figure B.3 ESI-CID in negative mode.