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Phytochemical analysis and acetylcholinesterase activity of lycopodium alkaloids from the club moss *Huperzia selago*

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Table of Contents

List of Tables

List of Figures

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AD	Alzheimer's Disease
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
CH_2Cl_2	Dichloromethane
HPLC	High pressure liquid chromatography
IC_{50}	The half maximum inhibitory concentration
MeOH	Methanol
$NH_4OH_{(33\%)}$	Ammonium hydroxide solution 30%-33%
NMR	Nuclear magnetic resonance
SPE	Solid phase extraction
TLC	Thin layer chromatography
VLC	Vacuum liquid chromatography

1 ABSTRACT

Substances with Acetylcholinesterase (AChE) inhibitory activity are an important therapeutic strategy for Alzheimer‘s disease (AD), a progressive neurodegenerative brain disease and the most common cause of dementia. The purpose of this research was to isolate Lycopodium alkaloids from *H. Selago* subsp. *appressa* and to elucidate their structure. Isolated and structural determinated alkaloids were afterwards tested on their AChE inhibitory activity.

Lycopodium alkaloids were isolated using SPE and HPLC chromatographic methods. Based on NMR experiments, structures of four alkaloids were elucidated including huperzine A, serratidine, lycopodine and 12-epilycodoline. The isolated alkaloids were tested for their in vitro inhibitory activity against AChE. Huperzine A is one of the lycopodium alkaloids that is already used in the treatment of AD in China. Beside the already expected huperzine A high efficacy as an inhibitor of AChE, serratidine also showed interesting inhibitory activity which makes it suitable for further investigation at higher concentrations. Results from lycopodine and 12-epilycodoline did not show significant AChE inhibitory activity. The AChE inhibitory activity of serratidine and 12-epilycodoline was estimated for the first time.

2 ZUSAMMENFASSUNG

Substanzen mit AChE inhibitorischer Aktivität sind besonders wichtige, wirksame therapeutische Mittel gegen AD, eine progressive neurodegenerative Erkrankung des Gehirns und die häufigste Ursache von Demenz. Das Ziel dieser Forschung war die Isoliereung und Identifizierung von Lycopodium Alkaloiden aus *H.selago* subsp. *apressa.* Isolierte und indentifizierte Alkaloiden wurden daraufhin auf ihre AChE Inhibition Aktivität getestet.

Im Rahmen dieser Diplomarbeit wurden die Lycopodium Alkaloide mithilfe von SPE und HPLC chromatographischen Methoden isoliert. Unter Anwendung von NMR Experimenten wurden die Strukturen der folgenden vier Alkaloiden aufgeklärt: Huperzin A, Serratidin, Lycopodin und 12-Epilycodolin. Die isolierten Alkaloide wurden auf ihre in vitro hemmende Aktivität gegen AChE getestet. Huperzin A gehört zu den Lycopodium-Alkaloiden und in China wird bereits in der Behandlung von Alzheimer Patienten verwendet. Neben der erfahrungsgemäß erwarteten Huperzin A hohen Wirksamkeit in der Hemmung der AChE, zeigte auch Serratidin eine bemerkenswerte inhibitorische Aktivität und ist damit interessant für weitere Untersuchungen in höheren Konzentrationen. Ergebnisse vom Lycopodin and 12-Epilycodolin zeigten keine erhebliche AChE Inhibition Tätigkeit. Die AChE Inhibition Aktivität von Serratidin und 12-Epilycodolin wurden zum ersten mal gemessen.

3 AIM OF THE STUDY

Huperzia selago is a club moss species growing in Iceland, which produces an array of lycopodium alkaloids. A few have been described already, including huperzine A which is a strong inhibitor of AChE.

The aim of this project is to isolate lycopodium alkaloids from *H. selago* subsp. *appressa* and to elucidate their structure. Further activity against AChE of the isolated alkaloids was tested. This study is a part of a larger study which will compare alkaloid content of two subspecies of *H. selago* subsp. *appressa* and subsp. *selago.*

Specific aims:

1. Isolation of lycopodium alkaloids using SPE and HPLC chromatographic methods
2. Elucidation of their structure using NMR spectroscopy
3. AChE inhibition test using the colorimetric method of Ellman

4 INTRODUCTION

4.1 Phytochemistry and medicinal plants

Humans have always relied upon Nature for their essential needs like food, clothes, flavours, aromas and medicines. The fundamental of the traditional medicine are plants which have been present for decades and still continue to be the source of new treatments (Gurib-Fakim, 2006). The research and discovery of new drugs based on plants involve different methods that combines botanical, phytochemical, biological and molecular facilities. The discovery of drugs from medicinal plant keeps on providing new and significant medication against different pharmacological targets e.g. cancer, AIDS, AD, malaria and pain (Balunas and Kinghorn, 2005). The reason why new discoveries in chemistry, biology and medicine are inspired by natural products is probably because of their tremendous structural and chemical diversity that cannot be outmatched by any databases of synthetic small molecules. Therefore natural products continue to be an important source of new medications and drug leads (Newman, 2012).

4.2 Club mosses

One of the first group of plants that appeared in the early Devonian, nearly 400 million years ago, were Lycopsids (club mosses and their relatives) (Mats E. Svensson, 2000). Club mosses include an early group of slow-growing, spore-forming plants that outlasted the late Silurian geological period about 400 million years. The botanical classification of club mosses is still a topic of different opinions and some taxonomists subscribe them to the family of Lycopodiaceae, divided into four genera: *Lycopodium, Lycopodiella, Huperzia* and *Diphasiastrum* while others have recommended seven or even more genera and pointed *Huperzia* in Huperziaceae, a separate family. There are worldwide more than 500 club moss species and the chemical content has been examined for only about 10% of the species (Elín Soffía Ólafsdóttir, 2013).


Figure 1: *H. selago*, GPS sample point: N 64°56‘93 and W 22°56‘64 (Fundneider Lorenz, 2016)


Figure 2: *H. selago*, GPS sample point: N 64°56‘93 and W 22°56‘64 (Fundneider Lorenz, 2016)

One among them is *H.selago* subsp. *appressa*, yellow-green moss with appressed leaves and bulbils that are always present in mature individuals. Shoots 5 till 15 cm, ca. 5 mm across. Its habitat above the tree-line is mostly in snow beds, scree slopes and damp heath (Jonsell, 2000). In Iceland it likes to grow buried in the gray moss hraungambri (*Racomitrium lanuginosum*) which covers the lava fields (see Figures 1 and 2).

4.3 Alkaloids

Alkaloids are a group of compounds widely distributed in the nature around the Earth. They can be structurally very different but they all are secondary metabolites that derive from the transamination process or from amino acids. Alkaloids are classified corresponding to the amino acids that give them their nitrogen atom, but steroid, terpenoid and purine alkaloids are important as well. The term “alkaloid” is not an easy task and it’s often a source of academic

arguments. It all began in the 1805 when Friedrich Sertürner, an apothecary's assistant from Westphalia, isolated one of the most important and most used alkaloids – morphine. That was a big step in pharmacology and chemistry. The first one who mentioned this term "alkaloid" was an apothecary from Halle, W. Meißner. He named these compounds alkaloids because they appeared "like alkali" But as a basic characteristic of alkaloids we can take the definition of Winterstein and Tier. According to them alkaloids have such characteristics as the basic character of a chemical structure, heterocyclic nitrogen as an element, a synthesis from amino acids or their derivatives, limited distribution in nature and greater or less toxicity (primarily on the central nervous system). For a long time, alkaloids have been a topic of research in pharmacology and organic chemistry (Aniszewski, 2015).

4.4 Lycopodium alkaloids

For this group of alkaloids, chemist and pharmacologist from all over the world showed their interest. The club mosses in the family Lycopodiaceae have been phytochemically investigated and a number of naturally existing Lycopodium alkaloids were preserved and characterized (Yu Tang, 2016). In the year of 1881, Bödeker isolated the first Lycopodium alkaloid, lycopodine, from the club moss *Lycopodium complanatum* (K., 1881). At present, more than 300 Lycopodium alkaloids have been characterized from about 54 species of Lycopodiaceae. They usually contain a skeleton of 16 carbon but still, sometimes they can have 32 carbons or even less than 16 carbons (Ma, 2004). Skeletal variation are mostly located in the D ring while the A, B and C ring are stable (Figure 3). There are different types of Lycopodium alkaloids. Their basic structure can be quinolizine, pyridine or α-pyridon. The first characterized and the most typical Lycopodium alkaloid is lycopodine. According to A.W.Ayer (W. A. Ayer, 1994) there are four structural classes: the lycodine class, the lycopodine class, the fawcettimine class and the miscellaneous group. Each of these groups has a characteristic compound and those are lycopodine, lycodine, fawcettimine and phlegmarine (Figure 3) (Ma, 2004).

It is very interesting to examine the lycopodium alkaloids as potential drug candidates because of their often rigid and stable molecular structure and their ability to interfere with diverse targets including the enzyme acetylcholinesterase (Elín Soffía Ólafsdóttir, 2013).

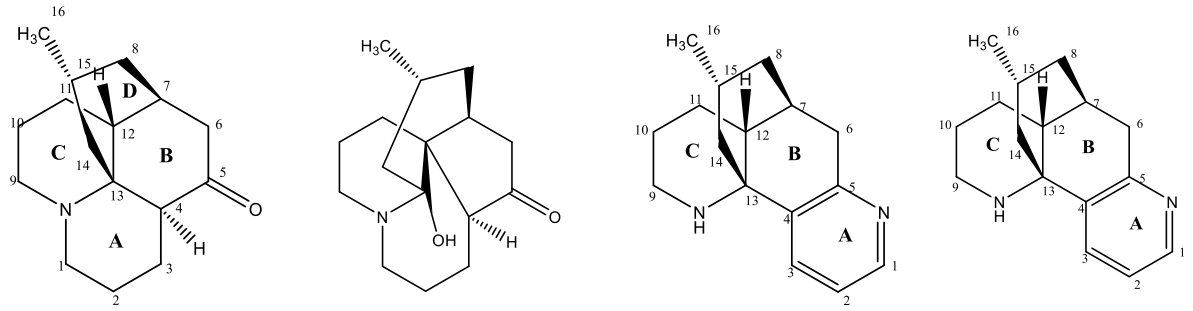


Figure 3: Representatives of the four subclasses of L. alkaloids: from left to right, lycopodine, lycodine, fawcettimine, phlegmarine

4.4.1 Lycopodine class

Lycopodine class alkaloids are the biggest group of known Lycopodium alkaloids and they also seem to be the most extensively distributed. But this may change in the future because only relatively few species of Lycopodium alkaloids have been examined in detail. Lycopodine (see Figure 3) was the first Lycopodium alkaloid that was characterized and it is the part of this group. Basic structure contains four connected six-membered rings. Ring A and C are quinolizidine ring system and the carbonyl group in ring B is usually at C-5 though it can be found at C-6. Lycopodine is not only the first identified but also the most typical compound in this class of Lycopodium alkaloids (Ma, 2004).

4.4.2 Lycodine class

Lycodine class is considered as a very important group of Lycopodium alkaloids because till now, most compounds with AChE inhibition activity belong to this class. Most prominent of them are huperzine A, huperizine B, N-methyl-huperizine B and huperzinine. Lycodine class also has four rings as a basic structure and the rings B, C and D are identical as in the lycopodine class and as for ring A, it is opened and altered to form a pyridone or a pyridine ring. The typical compound of this class is lycodine (see Figure 3). Huperzine A that has received some interest as a potential treatment for Alzheimer's disease (AD) is isolated from *H. serrata* and is the product of C ring splitting and elimination of C-9 which gives it a $C_{15}N_2$ skeleton (Ma, 2004).

4.4.3 Fawcettimine class

Fawcettimine class can be considered as the products of C4-C13 to C4-C12 bond migration from lycopodine class precursor. Fawcettimine group can be classified in two subgroups, alkaloids with carbinolamine form and alkaloids with keto-amine form. Compounds with carbinolamine form like fawcettimine (Figure 3), which has typical connection between N and C-13, are the most usual in the fawcettimine class. Alkaloids with keto-amine form have split the N-C13 bond of the carbinolamine in order to form a keto-amine (Ma, 2004).

4.4.4 Miscellaneous group

This group of Lycopodium alkaloids represents wholly variety of structural subjects. This is a miscellaneous group that contains all of the Lycopodium alkaloids that are not belonging in any of the first three classes. Typical and key compound included in this group is phlegmarine (Figure 3). The importance of the phlegmarine is reflected in its key role in the biosynthesis of the other Lycopodium alkaloids. It could be an intermediate in the biosynthesis of all Lycopodium alkaloids (Ma, 2004).

4.4.5 Lycopodium alkaloids isolated from *H. selago*

Lycopodium alkaloids from *H. selago* that already have been isolated and described are lycopodine (see Figure 3), 6α-hydroxylycopodine, lycodoline, 12-epilycodoline, acrifoline, α-obscurine, β-obscurine, selagoline, 6β-hydroxyhuperzine A (see Figure 4), huperzine A (see Figure 12) and serratidine (see Figure 13). Selagoline was isolated from H. selago collected in Iceland as a new natural product together with two known alkaloids, huperzine A and serratidine (Stærk, 2004).

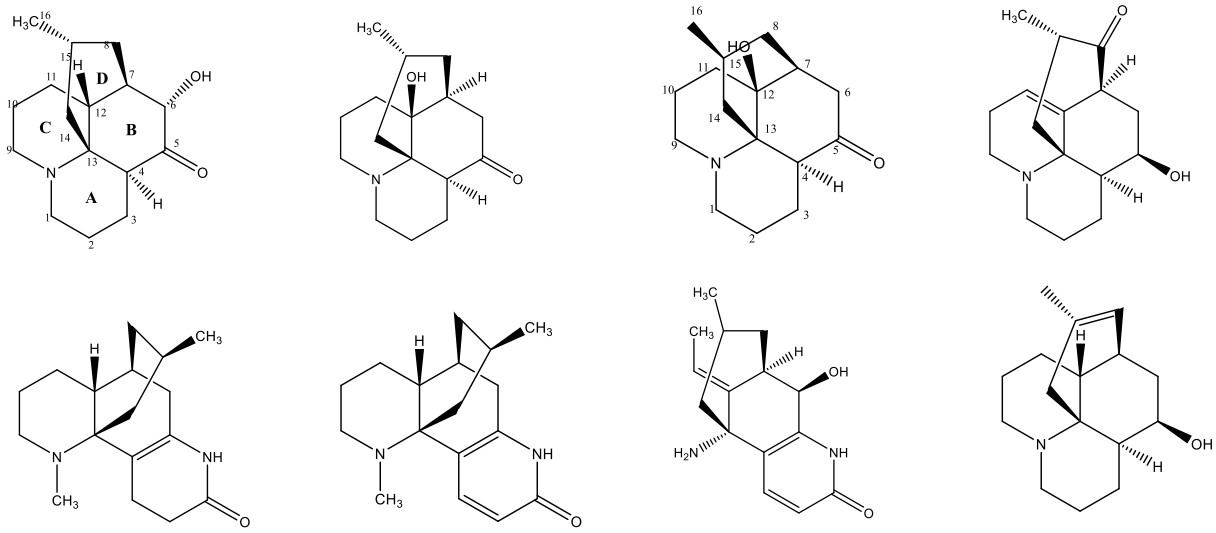


Figure 4: From left to right: 6α-hydroxylycopodine, lycodoline, 12-epilycodoline, acrifoline, α-obscurine, β-obscurine, 6β-hydroxyhuperzine A, selagoline

Table 1: Lycopodium alkaloids from *H. selago*

Alkaloid	Reference
acrifoline	(French, 1961, Halldórsdóttir, 2010)
12-epilycodoline	(Achmatowicz O, 1955, Achmatowicz O, 1956)
selagoline	(Stærk, 2004)
6β-hydroxyhuperzine A	(Stærk, 2004)
6α-hydroxylycopodine	(W. A. Ayer, 1990)
lycodoline	(Halldórsdóttir, 2010, Ma, 2004)
lycopodine	(Marion, 1944)
α-obscurine	(Ayer, 1962)
β-obscurine	(Ayer, 1962)

4.5 Alzheimer's disease

In the course of the twentieth century following the considerable rise in life expectancy, AD became most common disorder of late life. Loss of memory, perception, knowing, rationalizing and behavioural stability leads to global dementia and earlier death of the patient (Selkoe, 2015). Alois Alzheimer, German psychiatrist, was the first one that has described AD in 1907 (Alzheimer, 1907).

AD is the most common type of dementia in the old-age, nearly two-thirds of all cases of dementia. It is a neurodegenerative disorder that affects dominant brain areas which contains the cortex and the limbic system. Gradual decline in memory followed with the damage of at least one other cognitive function it's the main characteristic of AD (Whitehouse PJ, 1981). There are diverse hypotheses about what are the causes of AD. A discovery in the late 1970s showed that the brains of patients with AD had a deficit in acetylcholine (Ach) (White, 1977), which mediates fast synaptic neurotransmission (see Figure 5). After this discovery a cholinergic hypothesis was born which expresses that the cognitive, functional and behavioural dysfunction correlated with AD are the result of incapacity to transfer neurologic impulses across cholinergic synapses (Heinrich, 2004). The amyloid hypothesis is the most recognized hypothesis and states that amyloid beta protein sediment, the main component of plaques found in post-obit brains of AD patients, is an important factor that contributes the AD. As claimed by the amyloid hypothesis, amyloid beta protein could be toxic to the brain, inducing brain anomalies and eventually programmed cell death. According to the third major hypothesis, tau hypothesis, clinical symptoms and the continuous neurodegeneration are caused by anomalies in the protein tau. This thesis specified that tau proteins pair with other threads of tau in order to form tangles inside neurons which cause the disrupting communication between neurons and eventually lead neurons to collapse and death. Beside those three main hypothesis, a lot of other hypothesis have been brought forward. Taking everything into consideration, ongoing research propose that the multiple factors provide the causes of AD and that the balance between these various causes could differ from patient to patient (Soldan et al., 2017).

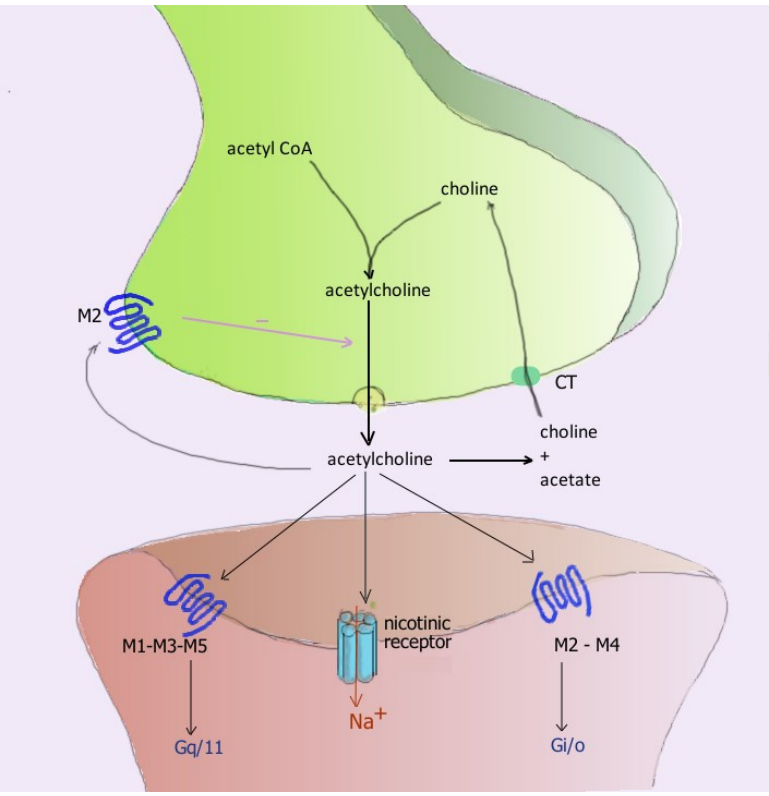


Figure 5: Acetylcholine processing in a synapse. After release acetylcholine is broken down by the enzyme AChE

4.6 Therapy

Most widely used drugs in treatment of AD are based on the cholinergic hypothesis (Soldan et al., 2017). Identification of neurochemical modification caused the development of the first generation of specific therapies for AD. Therefore, the first therapeutic approach to treating AD was to reduce the cholinergic neurotransmission through cholinesterase inhibitors (e.g. donepezil, galantamine, rivastigmine). However, research has also shown that AD is defined by modification in cortical and subcortical glutamatergic structures. As a result we have another therapeutic approach which is centred on NMDA receptor blockade (e.g. memantine) (Coman and Nemeş, 2017). It was found that the AD correlate with excessive release of glutamate in the brain, which can be noxious to neurons. In general, both types of drugs have shown some advancement in treatment of neuropsychiatric symptoms associated with AD, such as lethargy, delusion, aggression and hallucination (Soldan et al., 2017).

Despite the fact that both, cholinesterase inhibitors and NMDA receptor antagonist, have well-confirmed effectiveness, the clinical results of patients under such treatments are limited. They are only symptomatic treatment and do not cure the disease (Coman and Nemeş, 2017). A lot

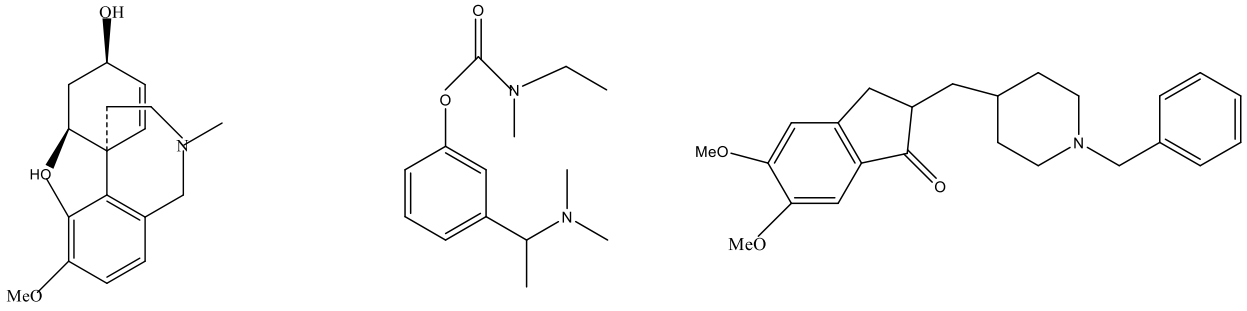
of other drug candidates are currently tested, for instance amyloid reducing agents, tau and tangle reducing drugs and stem cell therapies to replace damaged neurons (Soldan et al., 2017).

4.6.1 Acetylcholine and inhibition of its activity

Acetylcholinestrase (AChE) inhibitors delay the biochemical breakdown of acetylcholine and for this reason they are able to extend cholinergic neurotransmission. There are two types of cholinesterases existing in the human body, butyryl- and acetylcholinesterase. Levels of the butyrylcholinesterase increase as AD develops (while levels of AChE decrease) but the exact physiological role is still unknown. Both types of cholinesterases are detected in neuritic plaques. The most important element of the pathophysiology of AD is the beta-amyloid, and its sedimentation could be altered through the inhibition with cholinesterase inhibitors (Giacobini, 2000).

4.6.2 Currently accessible AChE inhibitors

Galantamine, rivastigmine and donepezil are currently available inhibitors of cholinesterase. Selective inhibitors of AChE, are galantamine and donepezil. Rivastigmine, at the same time, inhibits butyrylcholinesterase. Galantamine has an effect on neurotransmission by increasing the amount of preserving acetylcholine in the synapses (Giannopoulos, 2015). Structures of compounds that are used to treat AD can be seen in Figure 6.



Galantamine Rivastigmine Donepezil

Figure 6: Compounds that are used to treat Alzheimer's disease

And because two of the currently accessible drugs for AD are based on natural products (physostigmine-derivate rivastigmine and galantamine), there are many investigation on various plants that are seen as a potential source of new drugs (Russo P, 2013).

4.6.3 Huperzine A

Huperzine A, extracted from *Huperzia serrata,* is an alkaloid with AChE inhibitory activity. In China, Huperzine A has been clinically used to increase memory in AD. In US it is used as a memory increasing dietary supplement (Damar et al., 2017). Huperzine A compared to the AChE inhibitors that are at the moment on the market for treatment of AD (galantamine, rivastigmine, donepezil), has higher oral bioavailability, better penetration through the blood-brain barrier, longer continuation of AChE inhibitory activity and is also safer concerning side effects (Hügel, 2015).

Huperzine A is the most compelling lycopodium alkaloid so far tested for AChE inhibition activity. It has become a focus of research because it binds selectively and reversibly to the AChE and has a delayed biological half-life. Besides, preclinical studies point out that huperzine A has an overall neuroprotective effect which signify, although still not clinically proved, it's potential to modify the AD (Elín Soffía Ólafsdóttir, 2013). The primary goal in recent AChE inhibitory research has been to explain the relationship between structure and activity of huperzine A and to search for new analogues of its derivatives with even higher activity. Several groups have prepared derivatives of huperzine A and structurally cleared analogues, which aim to have longer continuation of activity, higher activity, less toxicity and may be prepared by more effective and much simpler methods in comparison with huperzine A itself (Ma, 2004).

5 RESULTS

5.1 Extraction

Extraction and fractionation of alkaloids from *H. selago* subsp. *appressa* were part of the bigger research project at the University of Iceland. Crude alkaloids fraction from *H. selago* subsp. *appressa* was fractionated on self-packed silica gel column. Six fractions, listed in the Table 16 were obtained. In this project, the focus was on fractions AVLC2, AVLC3 and AVLC4. A flowchart of the separated fractions is shown in Figure 7.

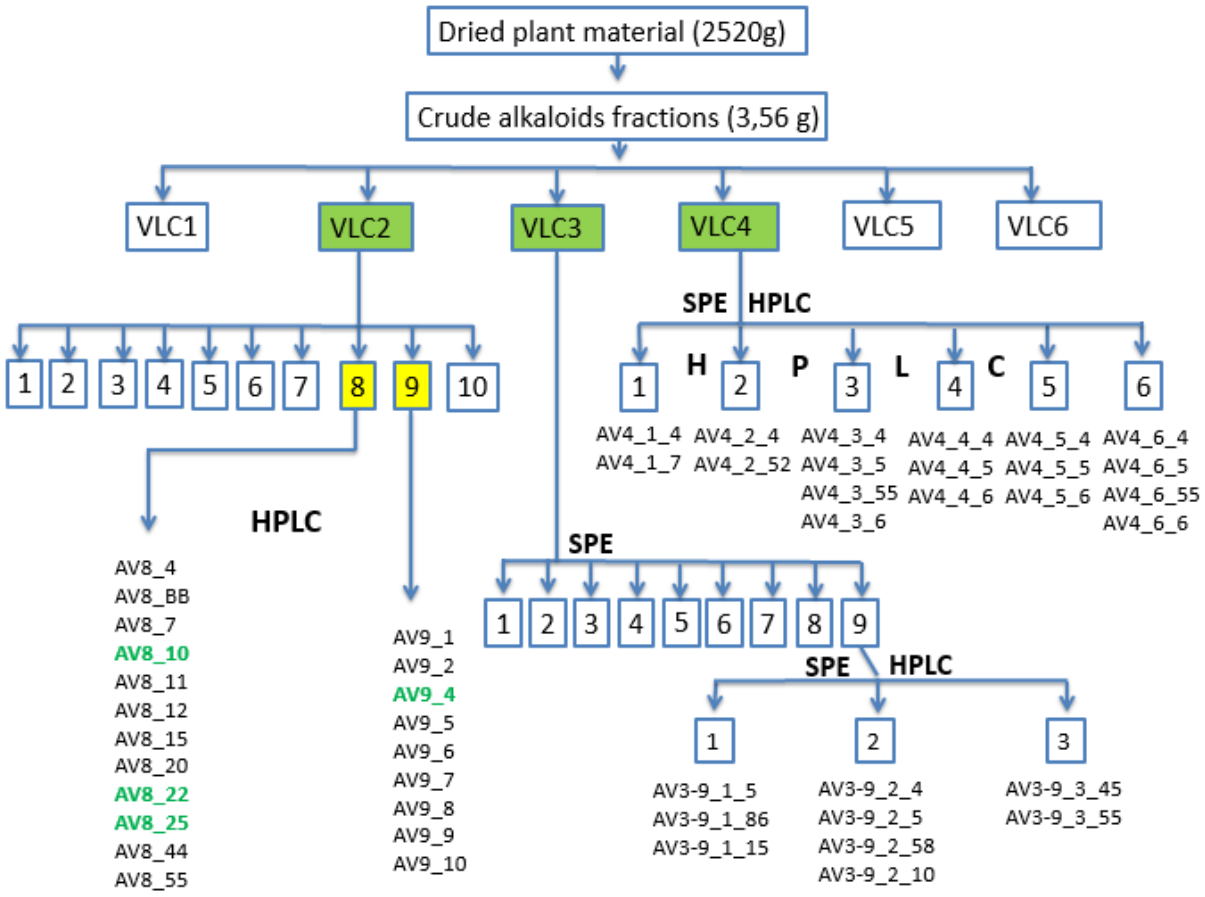


Figure 7: Flowchart of the SPE and HPLC process

5.2 SPE

Fractions AV3-9 and AV4 were fractionated on SPE. CH_2Cl_2 : MeOH : NH_4OH (85:14:1) was used for the mobile phase for those two fractions. At the end the column was washed with MeOH to get the more polar compounds out the column. Every 5th sample was spotted on TLC developed in appropriate mobile phase and visualized with Dragendorrf‘s reagent. Fractions were combined as described in the Table 2 and Table 3. The TLC plates of fractions AV3-9 and AV4 can bee seen in Figures 8 and 9. In the Table 2 and Table 3 are weights of combined SPE samples presented.

5.2.1 SPE of AV3-9 fraction

Table 2: Weights of combined SPE samples from fraction AV3-9

Fraction	Sample No.	Weight (mg)
1	3-9	7,78
2	12-23	12,8
3	24-55	12,36

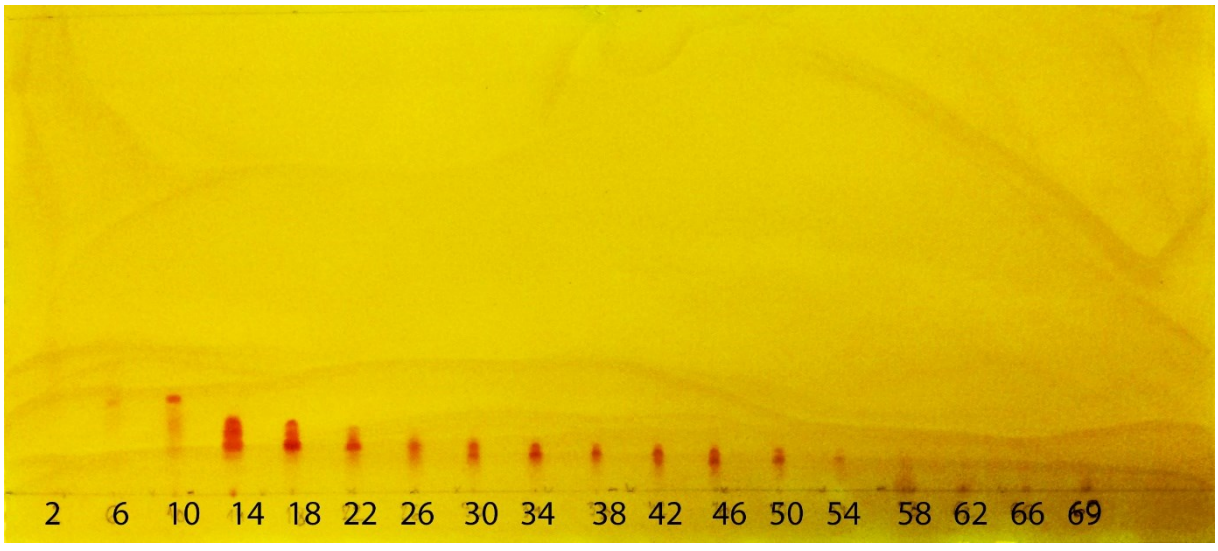


Figure 8: TLC plate from SPE of fraction AV3-9

5.2.2 SPE of AV4 fraction

Table 3: Weights of combined SPE samples from fraction AV4

Fraction	Sample No.	Weight (mg)
1	6-11	68,16
2	12-17	3,84
3	18-42	48,31
4	43-53	55,54
5	54-72	25,39
6	73-110	80,28

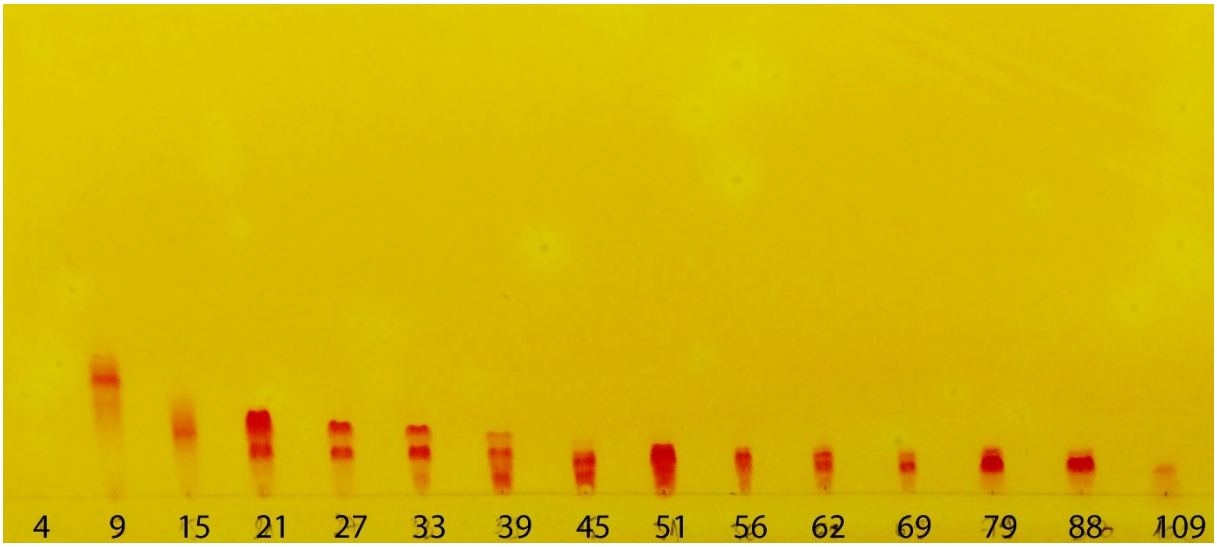


Figure 9: TLC plate from SPE of fraction AV4

5.3 HPLC

The Combined SPE fractions were separated on HPLC and all major peaks were collected. Collected HPLC fractions were evaporated, freeze dried and weighted. Those peaks that had enough amount (at least 1-2mg) were examined with proton NMR. Compounds AV8_10, AV8_22, AV8_25 und AV9_4, most interesting in this project, were investigated in details. Masses and retention times are presented in the table 4. In HPLC, a gradient elution was performed. Mobile phase consisted of acetonitrile and water basified to pH 10.5 with $NH_{3(aq)}$. During gradient elution the concentration of the mobile phase changed as shown in the table 5. The HPLC chromatograms of those compounds are presented in Figures 10 and 11.

Table 4: The weight and retention time of the compounds that were further examined with NMR

Peak	Weight (mg)	Retention time (min)
AV9_4	6.0	8.10
AV8_10	6.0	8.90
AV8_22	27	20.00
AV8_25	20	22.50

Table 5: Mobile phase concentrations

time (min)	Acetonitrile (%)	NH_3 + H_20 (%)
0	20	80
5	30	70
15	40	60
20	50	50
25	65	35
30	20	80
32	20	80

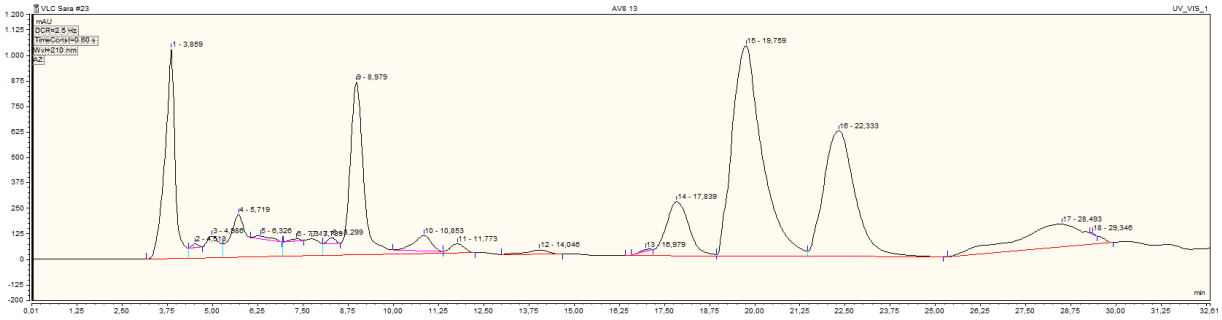


Figure 10: HPLC chromatogram of fraction AV8

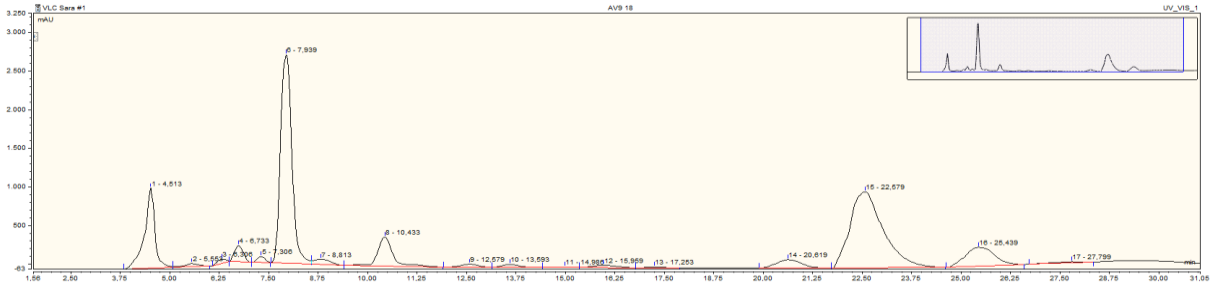


Figure 11: HPLC chromatogram of fraction AV9

5.4 NMR

After the analysis of the Peaks in HPLC chromatograms, twenty seven of them were sent to further NMR analysis. First, the proton NMR was examined and after that, according to their purity and to the amount of each peak, the carbon NMR was performed. For eleven peaks carbon spectra were recorded. The peaks that were examined in details are marked in green in the flowchart in Figure 7 and can be seen in Table 6.

Table 6: Alkaloids that were analysed

Peaks from HPLC	**Weight (mg)**
AV9_4	6.00
AV8_10	6.00
AV8_22	27.00
AV8_25	20.00

5.4.1 Analysis of compound AV9_4

According to the proton spectrum (Figure 12), the compound AV9_4 was really pure. The proton and carbon spectra were compared with spectra of known alkaloids form *H. selago* (see Appendix, Figure 22) and it came out that the compound AV9_4 is matching to huperzine A. This was confirmed by 2D NMR analysis (see Appendix, Figure 23) and after comparing the chemical shifts of proton spectra along with the known values which can be seen in Table 7.

Table 7: Chemical shifts from proton (^{1}H) spectra of compound AV9_4 and known values of Huperzine A. $CDCl_3$ was used as solvent for all spectra.

Position	Compound AV9_4 ^{1}H (ppm)/J in Hz	Huperzine A ^{1}H (ppm)/J in Hz (Dan Stærk, 2004)	Compound AV9_4 ^{13}C (ppm)	Huperzine A ^{13}C (ppm) (Liu J-S, 1986)
1			165.2	165.5, s
12			143.2	143.3, s
5				142.6, s
3	7.84, d J=9.4	7.90, d J=8.5	140.3	140.3, d
15			134.2	134.1, s
8	5.34, br d J=5.0	5.41, br d, J=5.3	124.3	124.3, d
4				122.9, s
2	6.34, d J=9.4	6.42, d J=9.5	117.1	117.0, d
11	5.43, q J=6.7	5.49, q J=6.7	111.4	111.2, d
13			54.4	54.4, s
14	2.08, 2H br s	2.11, br d J=16.8 2.15, d J=16.8	49.1	49.2, t
6	2.66, d J=16.8 2.82, dd J=16.9;5.1	2.72, dt J=16.8;1.7 2.89, dd J=16.8;5.3	35.4	35.2, t
7	1.61, d J=6.7	1.68, d J=6.7	32.9	32.9, d
16	1.48, br s	1.55, br s	22.7	22.6, q
10	1.61, d J=6.7	1.68, d J=6.7	12.4	12.3, q
NH	12.64	12.78		
NH2	1.9, s	1.70, br s		

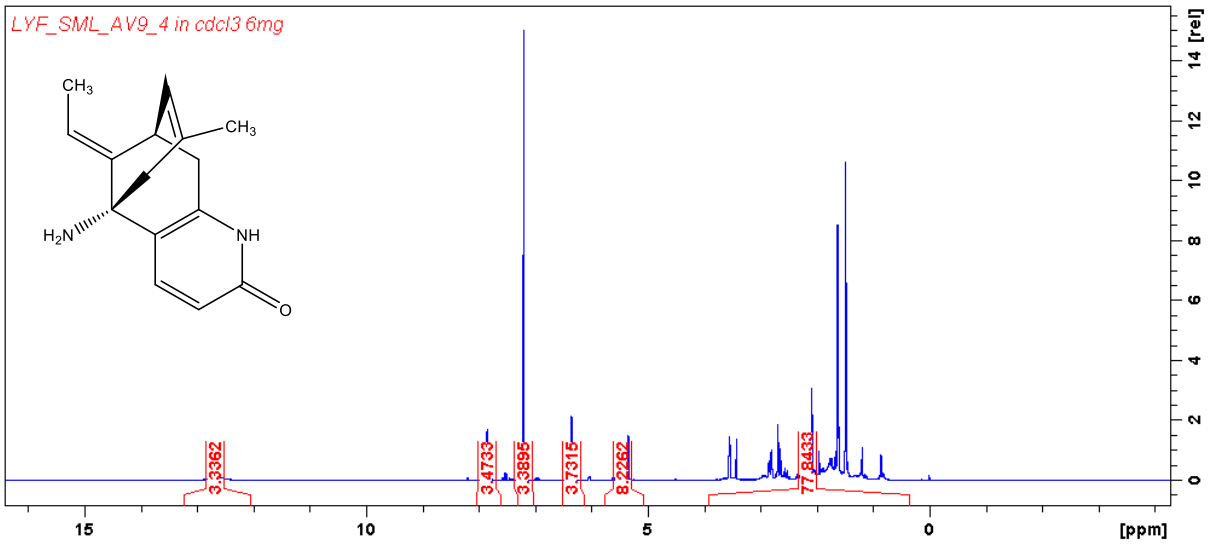


Figure 12: Proton spectra of compound AV9_4 and its chemical structure

5.4.2 Analysis of compound AV8_10

After comparison of all analysed peaks, compound AV8_10 looked very similar to the AV9_5 (see Appendix, Figure 24). From examination of the proton and carbon spectra it was proven that it is the same compound. But because of the purity and much more amount of the compound AV8_10, it was decided to take this one for the further analysis. After detailed carbon spectra analysis and comparing the compound AV8_10 with other known alkaloids from *H. selago*, it was concluded that it matches to serratidine. The proton spectra of compound AV8_10 and its molecular structure can be seen in Figure 13. In order to come to this conclusion, DEPT-135 spectra (see Appendix, Figure 25) was also necessary because on the carbon spectra the two of 16 carbon signals were overlapping which led to the wrong conclusion that there were only 15 carbon atoms in the structure of the compound AV8_10. After analysing the DEPT-135 spectra not only that two signals around 25 ppm were to discover (carbon spectra was showing only one) but it also showed the difference between the CH_3-, CH- group (positive signal) and CH_2-group (negative signal).

Table 8: Chemical shifts from carbon (^{13}C) spectra of compound AV8_10 and known values of serratidine. $CDCl_3$ was used as solvent for all spectra.

carbon atoms	compound AV8_10 ^{13}C (ppm)	serratidine ^{13}C (ppm) (Jesper Larsen, 2003)
5	208.5	208.6
12	144.0	144.8
11	114.6	114.6
7	73.2	73.3
13	60.0	59.4
6	55.4	55.3
4	53.5	53.8
8	51.6	51.7
1	48.4	48.3
9	45.2	45.2
14	36.3	36.2
10	25.4	26.0
15	25.3	25.3
2	22.6	23.0
16	22.1	22.1
3	19.6	19.8

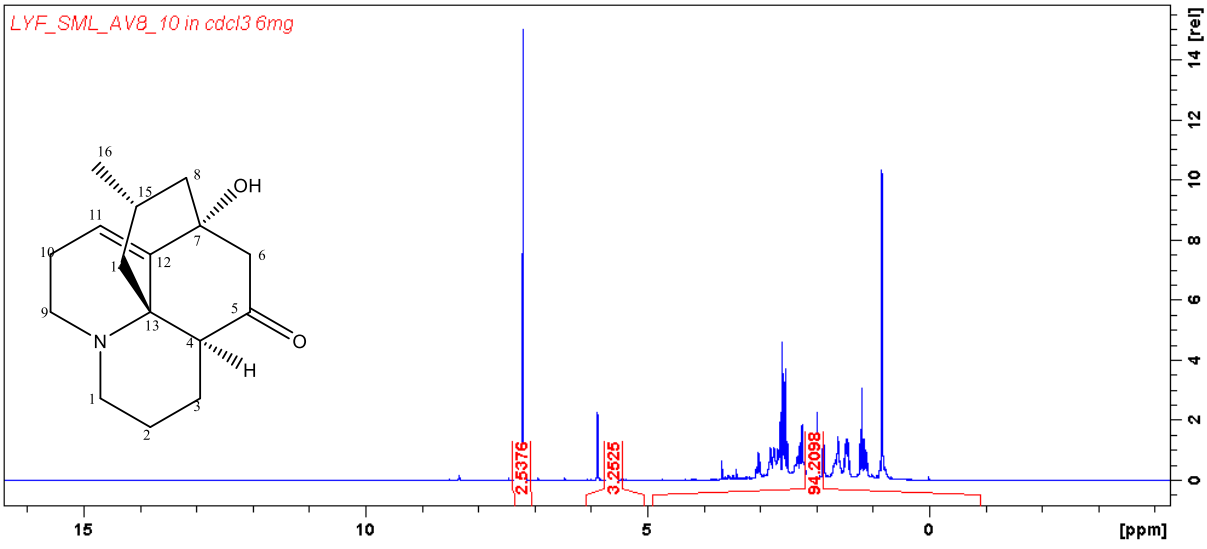


Figure 13: Proton spectra of compound AV8_10 and its chemical structure

5.4.3 Analysis of compound AV8_22

Compound AV8_22 showed a lot of similarities with the Peak AV9_9 and after some further comparison of proton (see Appendix, Figure 26) and carbon NMR as well as 2D NMR (see Appendix, Figure 27), it was confirmed that it's the same compound. Based on purity and amount of compound, compound AV8_22 (Figure 14) was chosen for more detailed analyses. Proton (see Appendix, Figure 28) and carbon (see Appendix, Figure 29) NMR spectra of compound AV8_22 was also compared to the known alkaloids from *H. selago*. Carbon NMR showed clearly that the compound AV8_22 is actually alkaloid lycopodine. Chemical shifts of carbon spectra along with the known values can be seen in the Table 9.

Table 9: Chemical shifts from carbon (^{13}C) spectra of compound AV8_22 and known values of lycopodine. $CDCl_3$ was used as solvent for all spectra.

Position	**Compound AV8_22 ^{13}C (ppm)**	**Lycopodin ^{13}C (ppm) (Halldorsdottir et al., 2013)**
5	213.6	213.9
13	59.8	60.0
9	47.2	47.5
1	46.6	46.8
12	44.9	45.2
14	43.2	43.4
4	42.9	43.2
6	42.8	43.1
8	42.5	42.7
7	36.7	37.0
10	26.1	26.3
15	25.3	25.5
11	25.1	25.4
16	22.4	23.1
3	19.5	19.7
2	18.8	19.0

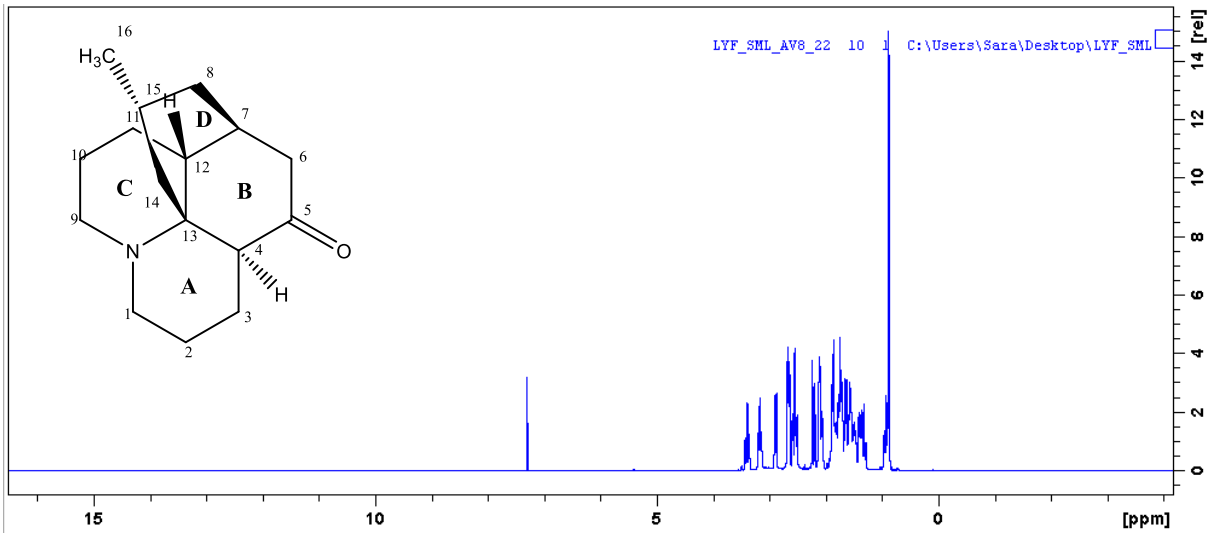


Figure 14: Proton spectra of compound AV8_22 and its chemical structure

5.4.4 Analysis of compound AV8_25

Comparing all of 16 NMR spectra that were the result of this project, it was concluded that the compound AV8_25 and AV9_10 are the same compound (see Appendix, Figure 30). Further analysis of compound AV8_25 and its comparison to all known alkaloids from *H. selago* led to conclusion that this alkaloid is matching to 12-epilycodoline. In order to come to this conclusion, the proton and carbon NMR (see Appendix, Figure 31) were compared. What was also required, is the HSQC spectrum (see Appendix, Figure 32) which allowed better understanding of correlation between the carbon atom and its attached protons. The proton spectra of compound AV8_25 and its chemical structure can be seen in Figure 15.

Table 10: Chemical shifts from carbon (^{13}C) spectra of compound AV8_25 and known values of 12-epilycodoline. $CDCl_3$ was used as solvent for all spectra.

Position	Compound AV8_25 ^{13}C (ppm)	12-epilycodolin ^{13}C (ppm)(W.A.Ayer, 1990)
5	211.9	210.5
12	70.1	70.1
13	62.3	62.5
4	52.1	52.3
1	48.6	48.7
9	47.8	48.0
6	43.8	44.0
7	40.7	40.9
8	37.3	37.5
11	30.2	30.3
14	25.5	25.6
2	25.1	25.2
15	24.5	24.6
16	22.6	22.6
10	20.9	21.0
3	18.5	18.7

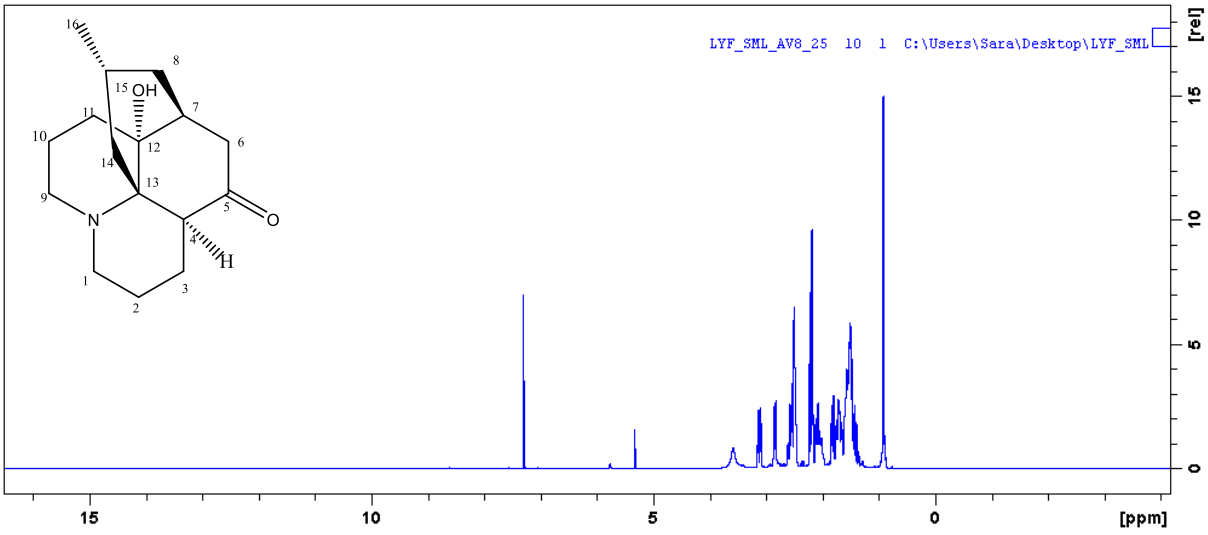


Figure 15: Proton spectra of compound AV8_25 and its chemical structure

5.5 AChE Inhibition Analysis

The AChE inhibition was measured for compound AV9_4 (huperzine A), compound AV8_10 (serratidine), compound AV8_22 (lycopodine) and compound AV8_25 (12-epilycodoline). As a reference, physostigmine was used.

5.5.1 Physostigmine as a reference

Table 11: Inhibition percentage of measured concentrations of physostigmine

Konc. µM	Inhib. %
0,16	44,8
0,32	58,3
0,64	70,7
1,28	88,2
2,56	94,2
5,00	96,2

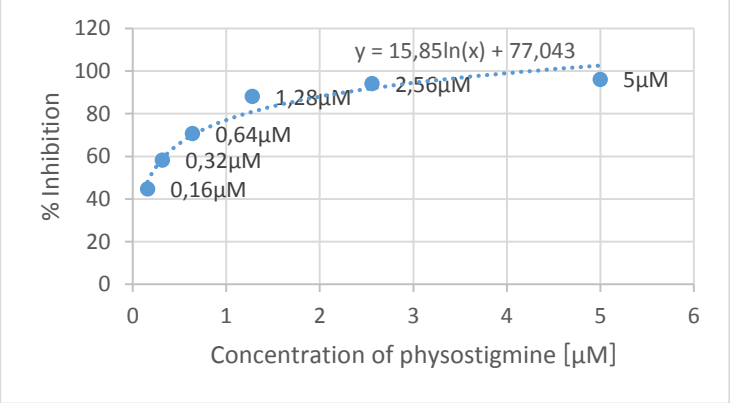


Figure 16: AChE inhibition of physostigmine in different concentration

Using the following equation:

$$y = 15{,}85\ln(x) + 77{,}043$$

From the logarithmic trendline (Figure 16), the IC_{50} value was estimated.

The IC_{50} of physostigmine was 0.18 µM which was the expected value (Halldórsdóttir, 2010).

5.5.2 Analysis of compound AV9_4

Table 12: Inhibition percentage of measured concentrations of AV9_4

Conc. µM	Inhib.
0,001	0,86
0,01	1,5
0,05	12,23
0,1	29,83
1,00	86,7
10,00	97,21

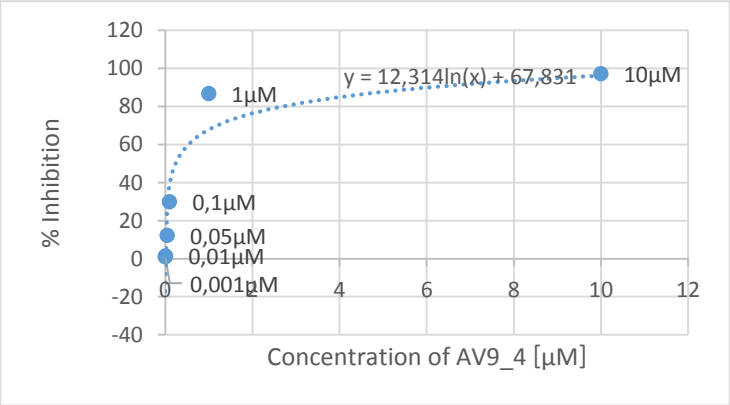


Figure 17: AChE inhibition of AV9_4 in different concentration

Using the following equation:

$$y = 12{,}314\ln(x) + 67{,}831$$

From the logarithmic trendline (Figure 17), the IC_{50} value was estimated.

The IC_{50} of huperzine A was 0.235 µM.

5.5.3 Analysis of compound AV8_10

Table 13: Inhibition percentage of measured concentrations of AV8_10

Conc. µM	Inhib.%
25	23,22
50	36,4
100	49,16

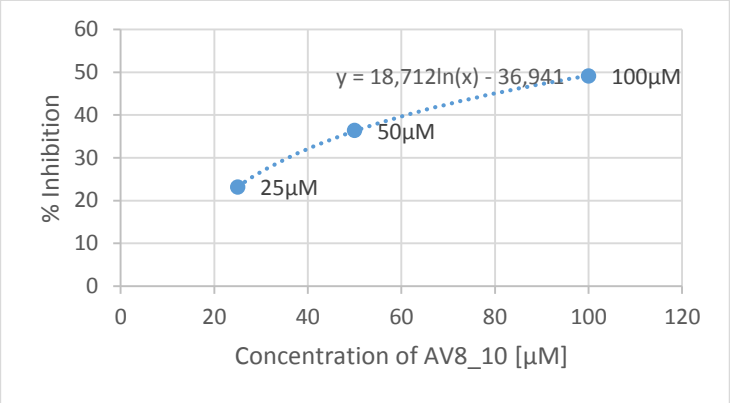


Figure 18: AChE inhibition of AV8_10 in different concentration

Using the following equation:

$$y = 18{,}712\ln(x) - 36{,}941$$

From the logarithmic trendline (Figure 18), the IC_{50} value was estimated.

The IC_{50} of serratidine was 104,17µM.

5.5.4 Analysis of compound AV8_22

Table 14: Inhibition percentage of measured concentrations of AV8_22

Conc. µM	Inhib.%
25	9,7
50	20,72
100	30,13

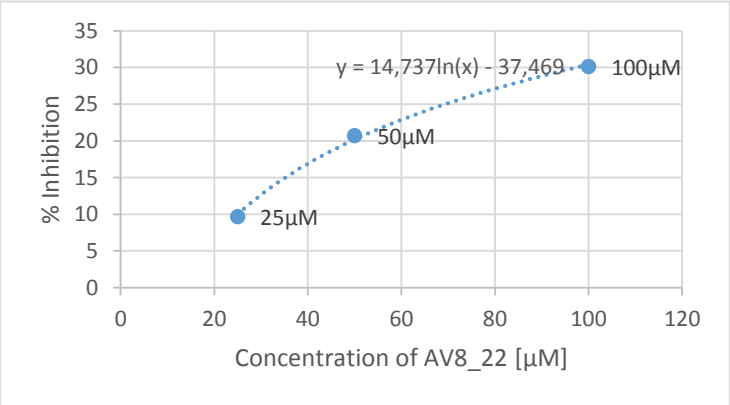


Figure 19: AChE inhibition of AV8_22 in different concentration

Using the following equation:

$$y = 14{,}737\ln(x) - 37{,}469$$

From the logarithmic trendline (Figure 19), the IC_{50} value was estimated.

The IC_{50} of lycopodine was 380 µM.

5.5.5 Analysis of compound AV8_25

Table 15: Inhibition percentage of measured concentrations of AV8_25

Konc. µM	Inhib.%
2,5	8,5
500	20,5

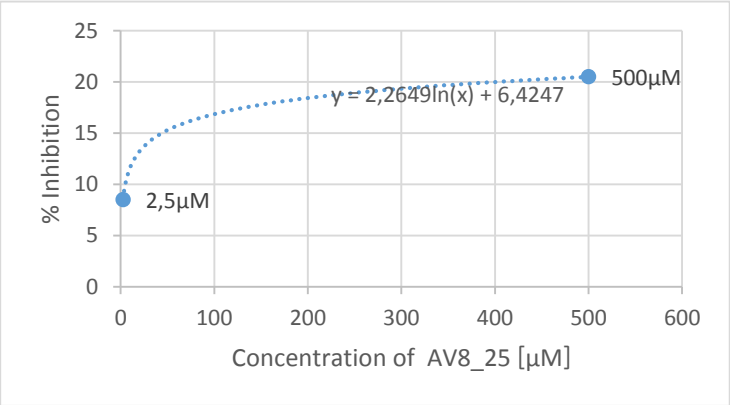


Figure 20: AChE inhibition of AV8_25 in different concentration

Using the following equation:

$$y = 2{,}2649\ln(x) + 6{,}4247$$

From the logarithmic trendline (Figure 20), the IC_{50} value was estimated.

The IC_{50} of 12-epilycodoline was more than 400 µM.

The data used for the calculations can be found in Appendix Table 17-21.

6 DISCUSSION

The aim of this project was to isolate lycopodium alkaloids from *H. selago* subsp. *appressa* and to elucidate their structure. AChE inhibition of the isolated alkaloids was also examined. Different analytical and chromatographic separation steps were performed. Fractions AV3-9 and AV4, which were part of the bigger research project at the University of Iceland, were fractionated on SPE. After fractionation on SPE columns, TLC was performed in order to get an overview in the content and distribution of alkaloids. Preparative HPLC was used for final separation of compounds from the combined SPE fractions. After the analysis of the collected peaks in HPLC chromatograms, twenty seven of them were sent to further NMR analysis. Four of them (huperzine A, serratidine, lycopodine and 12-epilycodoline) were selected to be investigated in details on the basis of proton signals of interest, amount and purity. After further NMR analysis and comparing the alkaloids with already known lycopodium alkaloids as described in chapter 4 above, it was confirmed that they are huperzine A (AV9_4), serratidine (AV8_10), lycopodine (AV8_22) and 12-epilycodoline (AV8_25). The structures of these four alkaloids were already known from other studies (Liu J-S, 1986, Jesper Larsen, 2003, Marion, 1944, Achmatowicz O, 1955) but not the activity of all of them. Serratidine and 12-epilycodoline were tested for their AChE inhibitory activity for the first time. Huperzine A showed, as expected, strong AChE inhibitory activity with it IC_{50} of 0.235 μM which is in agreement with previous studies (Tang and Han, 1999). Lycopodine did not inhibit the AChE enzyme with IC_{50}>400 μM which is in agreement with previous studies (Yang et al., 2016). The two remaining alkaloids investigated in this study, serratidine and 12-epilycodoline, were isolated from *H.selago* for the first time. The IC_{50} value of 12-epilycodoline was more than 400 μM and does not indicate AChE inhibitory activity of significance. On the other hand, serratidine showed a moderate activity with IC_{50} of about 100 μM. It would be interesting to take some further test in order to calculate its activity in some higher concentrations.

7 CONCLUSION

In conclusion; of the four known alkaloids isolated from the alkaloid fractions of *H.selago* subsp. *appressa*, two of them i.e. serratidine and 12-epilycodoline have not been isolated from this plant before. Further, the AChE inhibitory activity of serratidine and 12-epilycodoline were reported for the first time in this study. 12-epilycodoline did not show activity but serratidine showed a moderate inhibition of AChE. Serratidine could therefore be an interesting compound for further studies with respect to structure-activity relationship and possible synthesis of more potent analogues.

8 MATERIALS AND METHODS

8.1 Materials

Material	Manufacturer
Acetic acid (glacial 100% anhydrous)	Merck
Acetonitrile	Sigma-Aldrich
Acetylcholinesterase	Sigma-Aldrich
Acetylthiocholine iodide	Sigma
Albumin from bovine serum	Sigma-Aldrich
Ammonium hydroxide solution 30-33% ($NH_4OH_{(33\%)}$)	Sigma-Aldrich
Buffer A	Prepared at Hagi
Buffer C	Prepared at Hagi
Dichloromethane (CH_3CL_2)	Sigma-Aldrich/Fluka
Dragendorff's stock solution	Prepared at Hagi
DTNB (5,5'-Dithiobis(2-nitrobenzoic acid))	Sigma
Eserine (Physostigmine)	Sigma
Methanol (MeOH)	Sigma-Aldrich
Methanol-d4 (CD_3OD)	Aldrich

8.2 Instruments and Equipment

Instrument/Equipment	Manufacturer and model
Syringe filters	Phenomenex : NY 0.45 µm
Micropipettes 1-1000 µL	Thermo Scientific
Freeze dryer	Snijders scientific
HPLC	Dionex (Ultimate 3000)
HPLC column	Phenomenex : Gemini-NX 5u C18 (250 x 21.20 mm)
Magnetic stirrer	Heidolph MR3002
Microplate photometer	Thermo Scientific (Electron Corporation) : Multiskan Ascent
NMR (400 MHz)	Bruker Avance 400
Thermo (Orion 3 star)	pH meter
Rotavapors	Buchi RE111 Buchi RII
Analytical balance	Mettler Toledo MS105
SPE chamber	Phenomenex
SPE columns	Phenomenex (Strata SI-1 Silica (55um,70A) 10g/60mL Giga Tubes)
TLC plates	Merck Silica Gel 60 F254
UVLamp (245 and 365 nm)	Camag
Vacuum pump	Millipore
Vortex Genie 2	Scientific Industries

8.3 Methods

8.3.1 Extraction and fractionation of alkaloids from *Huperzia selago* subsp. *appressa*

VLC fractions which were examined in this project were part of the bigger research project at the University of Iceland. These fractions were prepared by a PhD student Natalia Kowal as described by Halldórsdóttir .

Briefly, dried and grained plant material was extracted with the mixture of methanol and dichlormethane (1:1). Extracts were dissolved in MeOH: H_2O (9:1) and defatted with petroleum ether. Methanol fraction was subjected to acid-base extraction (reagents used: 2% tartaric acid, ethyl acetate and saturated solution of sodium carbonate) yielding 3.56 g of crude alkaloids fraction.

8.3.2 Vacuum Liquid Chromatography

Crude alkaloids fraction from *Huperzia selago* subsp. *appressa* was fractionated on self-packed silica gel column. Six fractions, listed in the table 16 were obtained. Fractions AVLC2, AVLC3 and AVLC4 were used in this project.

Table 16: VLC fractions from *Huperzia selago* subsp. *apressa*

Fraction	Mobile phase		Amount
AVLC1	CH_2Cl_2:Me 98:2	700 ml	258 mg
AVLC2	**CH_2Cl_2:Me$_{(NH3)}$ 98:2**	**1000 ml**	**715.5 mg**
AVLC3	**CH_2Cl_2:Me$_{(NH3)}$ 90:10**	**1000 ml**	**1952 mg**
AVLC4	**CH_2Cl_2:Me$_{(NH3)}$ 80:20**	**600 ml**	**320.5 mg**
AVLC5	CH_2Cl_2:Me$_{(NH3)}$ 80:20	400 ml	41 mg
AVLC6	Me$_{(NH3)}$	900ml	77.8mg

8.3.3 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is a sample preparation method mostly used before other analytical methods (in this case HPLC) to concentrate and purify components in the sample. In this project, normal-phase SPE columns were used where a polar, silica based stationary phase and non-polar mobile phase are used. A disposable prepacked columns from Phenomenex were used. CH_2Cl_2 : MeOH : NH_4OH (85:14:1) was used for the mobile phase for VLC3-9 and VLC4. Fractions VLC8 and VLC9 were directly purified on HPLC. Before loading the sample, the column was conditioned with hexane, CH_2Cl_2 and with the mobile phase. The sample was dissolved in 2 mL of mobile phase and loaded on the column.. About 6 mL fractions were collected until all alkaloids were eluted. To ensure full elution, the column was washed with MeOH:$NH_{3(aq)}$ (99:1) in the last step.

8.3.4 Thin Layer Chromatography (TLC)

After fractionation on SPE columns, TLC was performed in order to get an overview in the content and distribution of alkaloids. Fractions containing similar compounds were combined together.

TLC separation was performed on TLC silica gel 60 F_{254} plates using a mobile phase system CH_2Cl_2 : MeOH : NH_4OH (90 : 9 : 1). The TLC plates were examined under UV light at 254 nm and 366 nm and subsequently developed with Dragendorff`s reagent, a solution for alkaloids detection, containing heavy metals salt (potassium bismuth (III) tetraiodide) which reacts with the nitrogen atom present in an alkaloid to form ion pair that forms an insoluble yellow to brick-red complexes. The mobile phase is non-polar and the stationary phase, silica gel, is polar. Therefore a non-polar compound will travel longer distance than a polar compound, because the non-polar compound binds less to the stationary phase traveling up the plate with the mobile phase. The mobile phase is basic because of the NH_4OH and this also influence the retention time of alkaloids.

8.3.5 High Performance Liquid Chromatography (HPLC)

HPLC is highly improved form of column chromatography where the solvent is pushed through the column under high pressure. In this study, preparative HPLC was used for final separation of compounds from the SPE fractions. Reversed phase chromatography was used. In order to have a good separation of alkaloids, which are basic compounds, a high pH of mobile phase was used. Mobile phase consisted of acetonitrile and water basified to pH 10.5 with $NH_{3(aq)}$. Samples were prepared in the mixture of mobile phase (1:1). Injections of 5-8 mg were conducted. Peak detection with UV lamp at 210 nm. The peaks were collected manually.

8.3.6 Nuclear magnetic resonance (NMR)

NMR is a technique for determining the structure of organic compounds. Although larger amounts of sample are needed, NMR is non-destructive spectroscopic method. It provide a way to determine an entire structure using one analytical method.

Samples were dissolved in 0.6 mL of deuterated MeOH (CD3OD) or $CHCl_3$ ($CDCl_3$). Analysis were performed at Raunvísindastofnun Háskóla Íslands by Sigríður Jónsdóttir. Analysis were performed on a 400 MHz spectrometer. First, the proton spectrum (^1H) was performed in order to select the fractions of highest interest. For the selected compounds, the carbon spectrum (^{13}C), DEPT-135 and 2D spectra (NOESY, COSY, HSQC and HMBC) were performed.

8.3.7 AChE inhibition assay

AChE inhibitory activity for selected compounds was evaluated using colorimetric method of Ellman.The reaction can be seen in figure 21. All compounds were tested at 6 concentrations.

AChE hydrolyses acetylthiocholine to thiocholine and acetate. Thiocholine in the presence of DTNB reacts to generate 5-thio-2-nitrobenzoate anion, which has a yellow color. The enzyme actvity is measured by following the increase of yellow color.

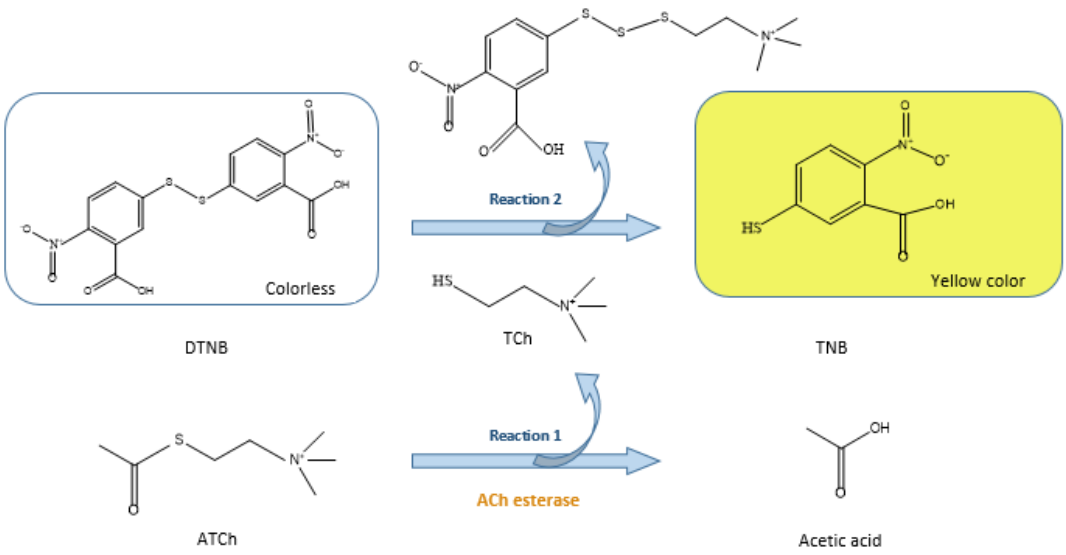


Figure 21: Ellman's reaction

In order to perform the assay, the following solutions were needed:

Buffer A: 50 mM Trizma, pH 8.0 with NaOH.

Buffer B: A 1 mg/mL bovine serum albumin (BSA) solution in buffer A.

Buffer C: 1.46g NaCl and 1.02g of $MgCl_2*6H_2O$ dissolved in buffer A.

DTNB solution: 1mM solution in buffer C

Substrate: 0.5 mM acetylthiocholine iodide (ATCI) solution in MilliQ-H_2O

Enzyme – AChE : 0.20 units/mL solution in buffer A

Reference – Physostigmine : 0.08-5.12 µM solutions of physostigmine in buffer A and Methanol (MeOH max 2% in the assay conditions).

Dragendorff‘s reagent is prepared by the following method:

Solution A: 0.85 g bismuth subnitrate

10 mL glacial acetic acid

40 mL distilled water

Solution B: 24 g potassium iodine

60 mL H_20

Dragendorff‘s stock solution : mixture of solution A and B (1:1)

Dragendorff‘s regent: 50 mL Dragendorff‘s stock solution

20 mL glacial acetic acid

100 mL destilled water

The TLC plates were dipped into the Dragendorff‘s reagent to visualize the spots. The alkaloids appeared as orange spots on a yellow background.

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10 APPENDIX

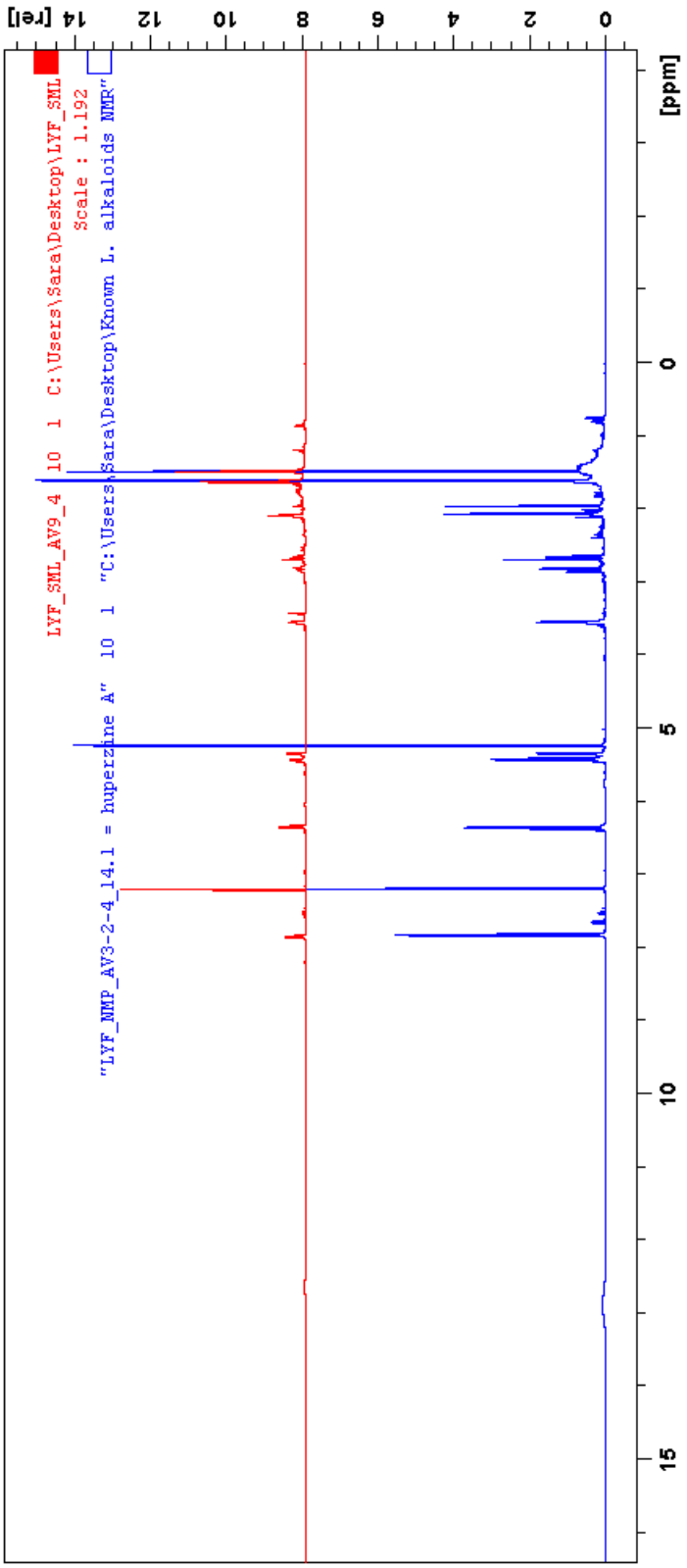

Figure 22*:* Proton spectra comparison between compound AV9_4 and huperzine A

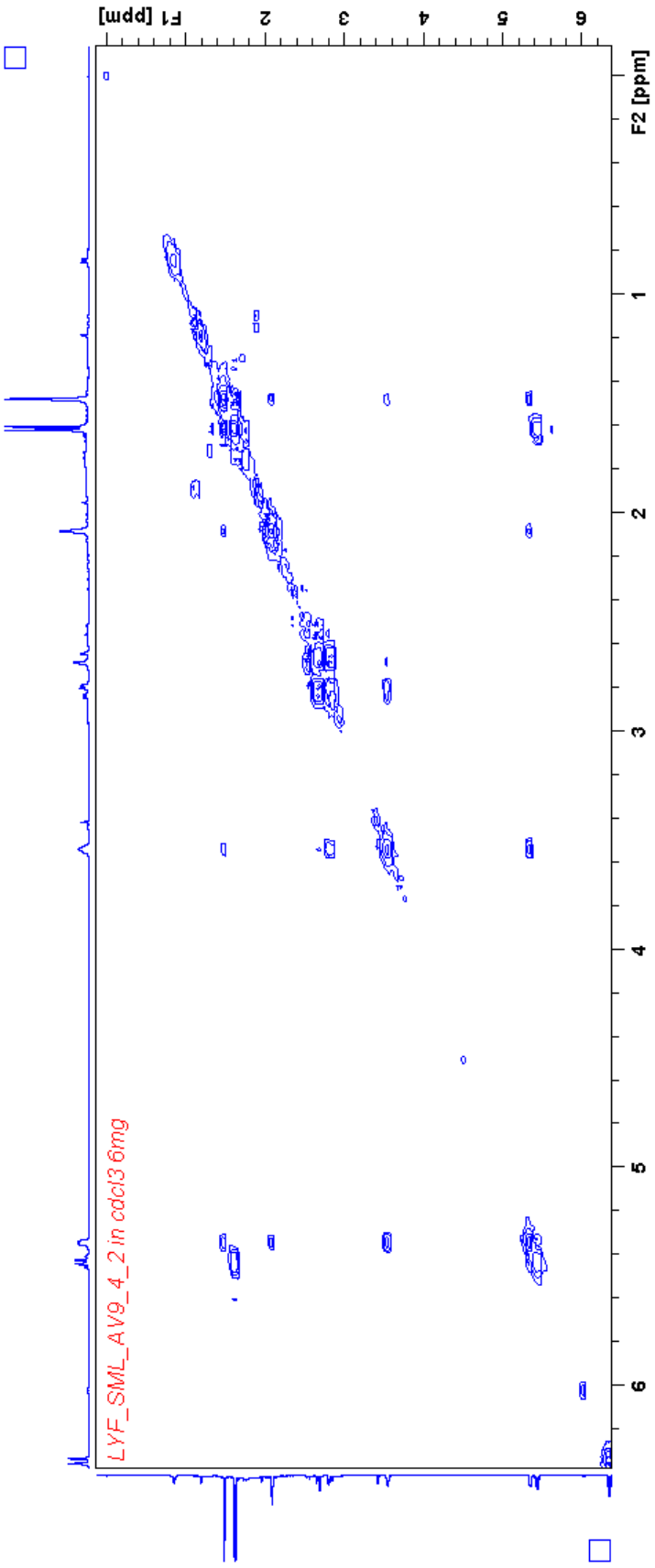

Figure 23: 2D NMR analysis of compound AV9_4

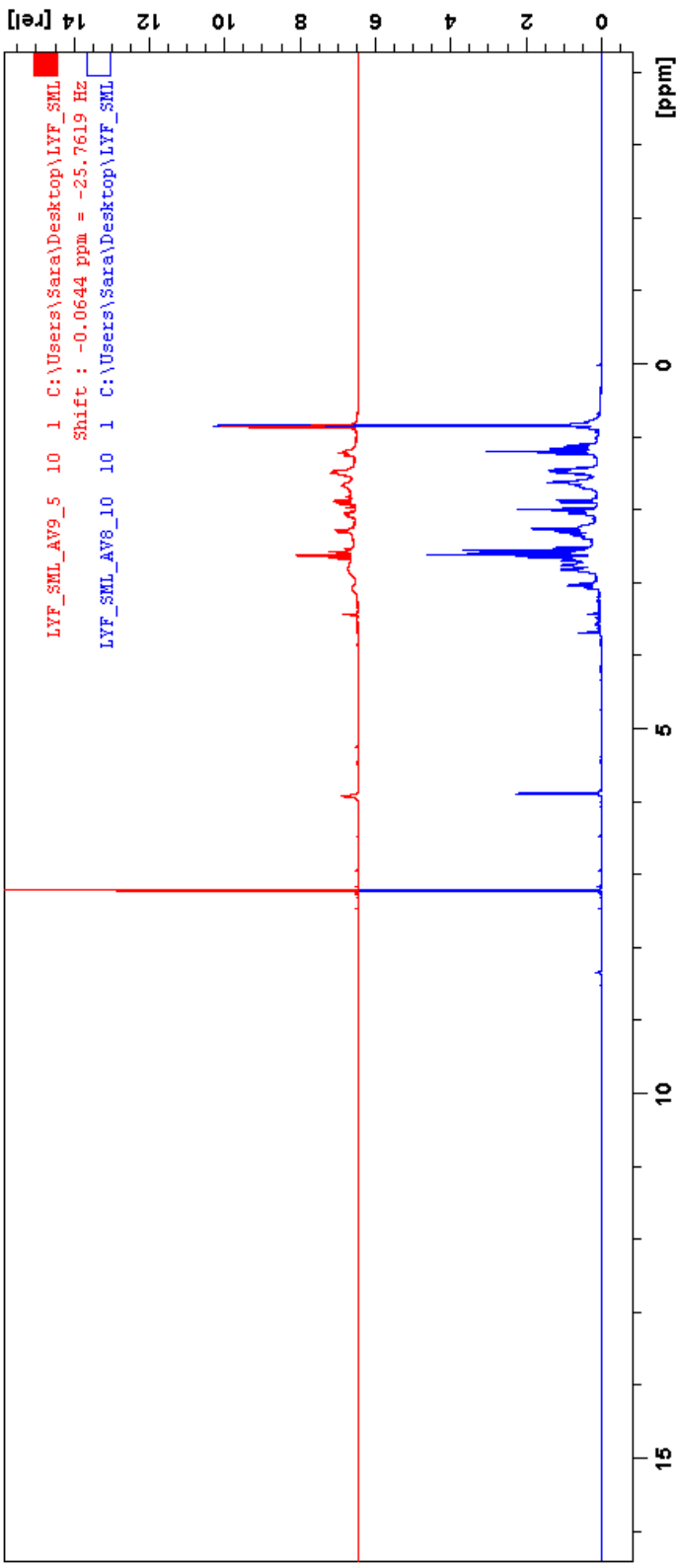

Figure 24: Proton spectra comparison between AV8_10 and AV9_5

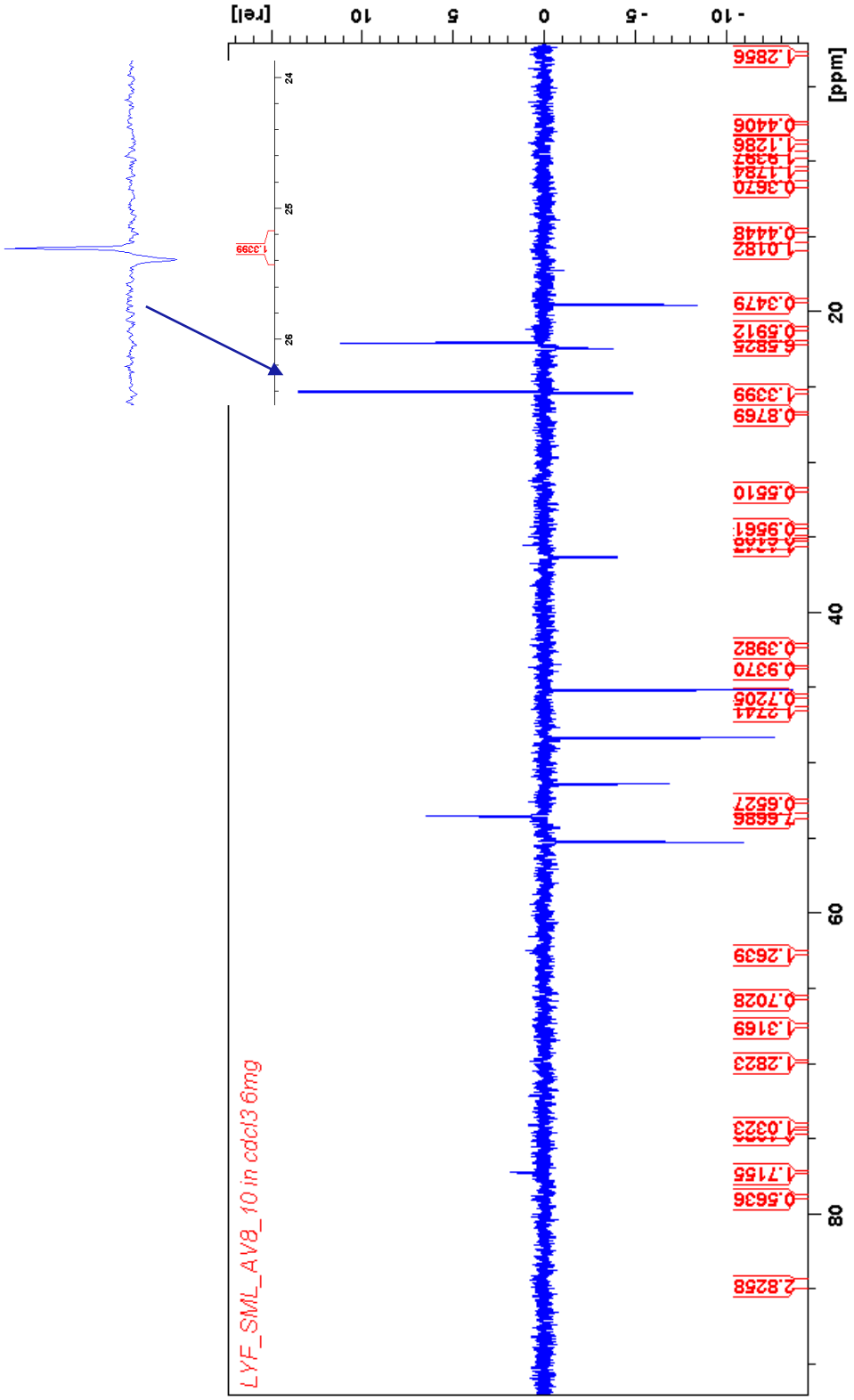

Figure 25: Compound AV8_10 DEPT-135 spectra

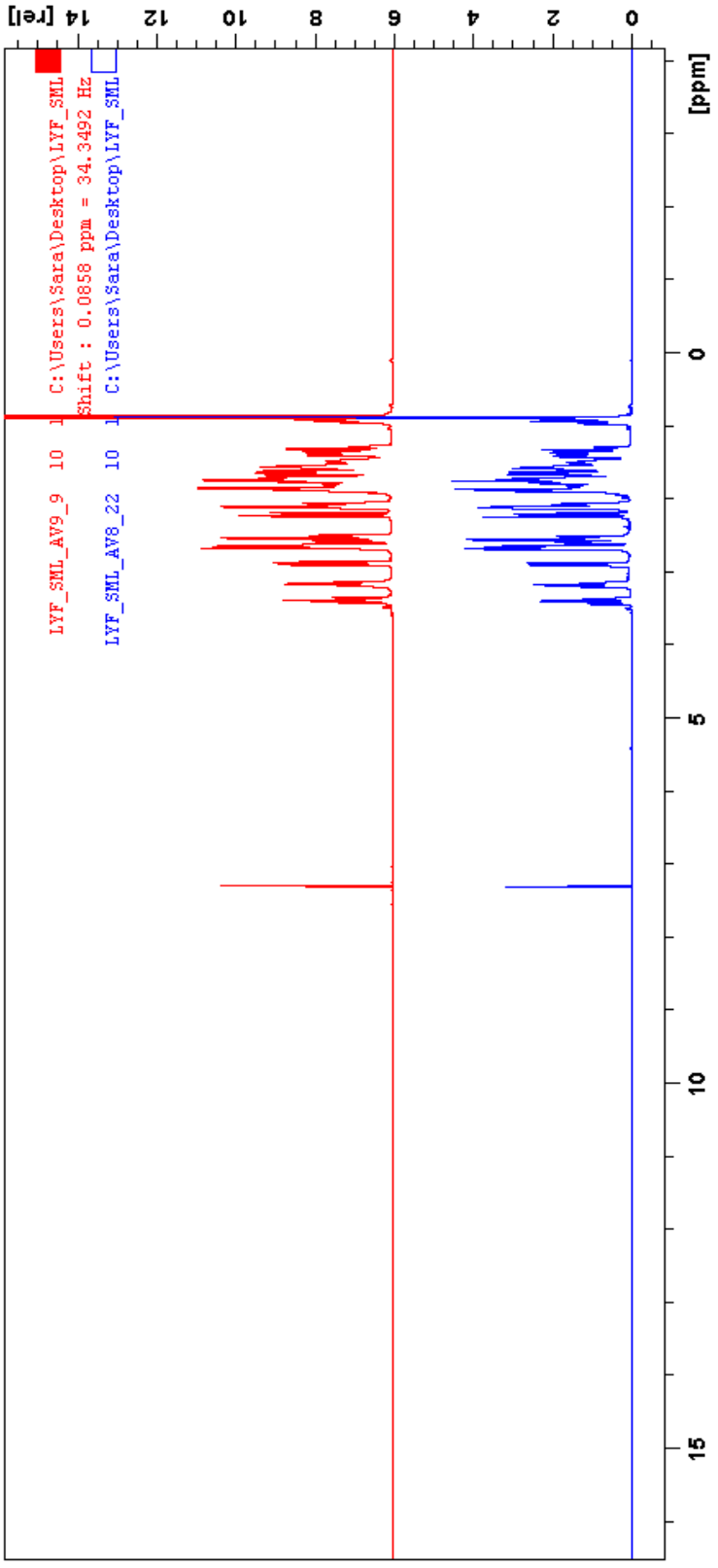

Figure 26: Proton spectra comparison between compound AV8_22 and AV9_9

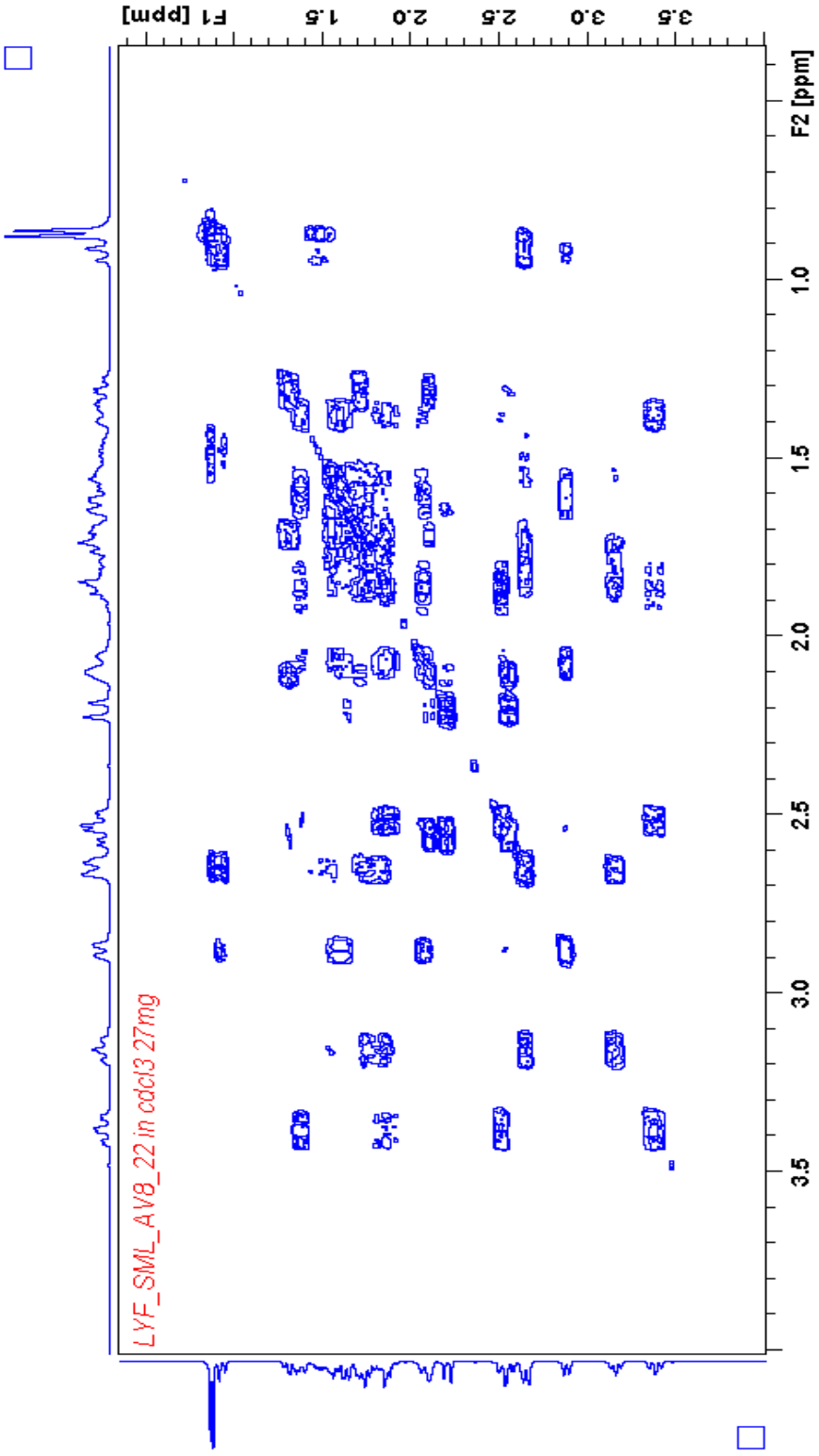

Figure 27: 2D NMR spectrum of compound AV8_22

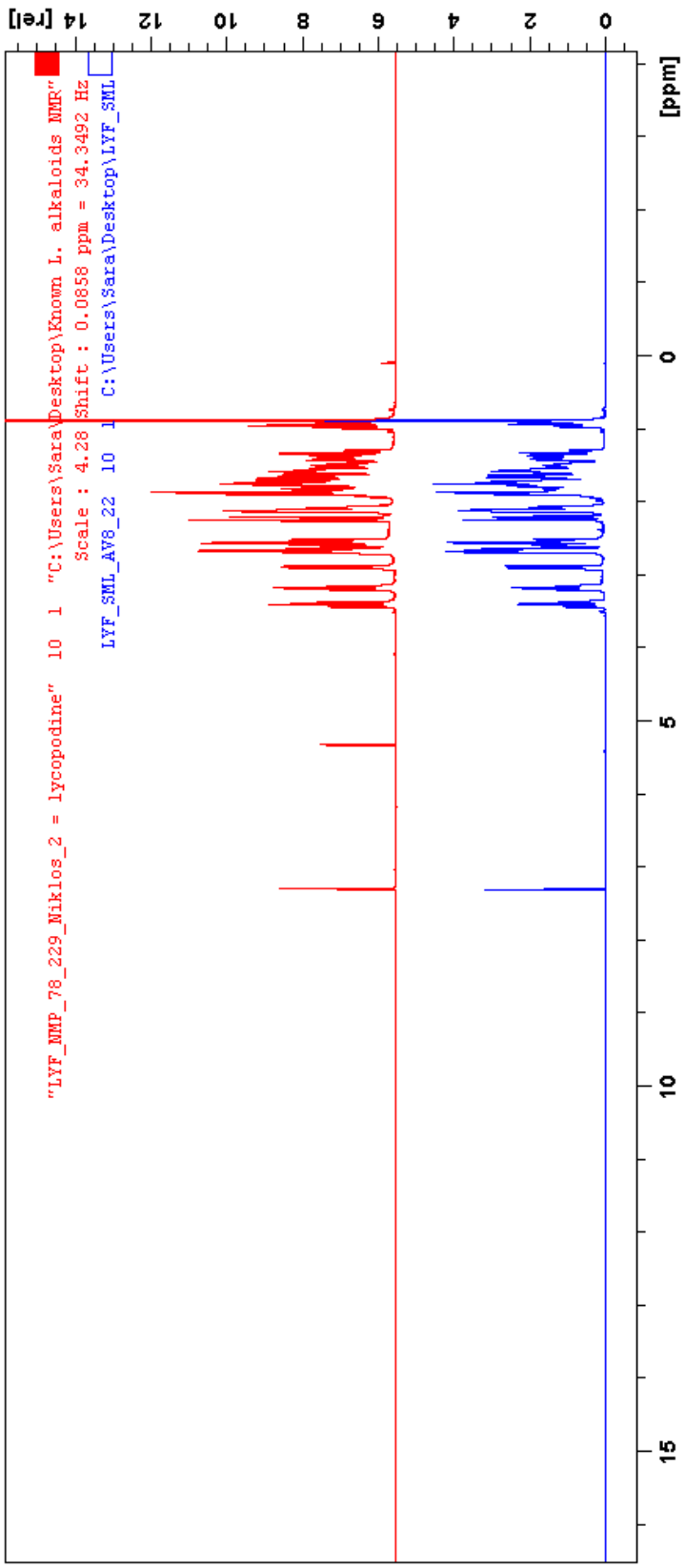

Figure 28: Proton spectra comparison between AV8_22 and lycopodine

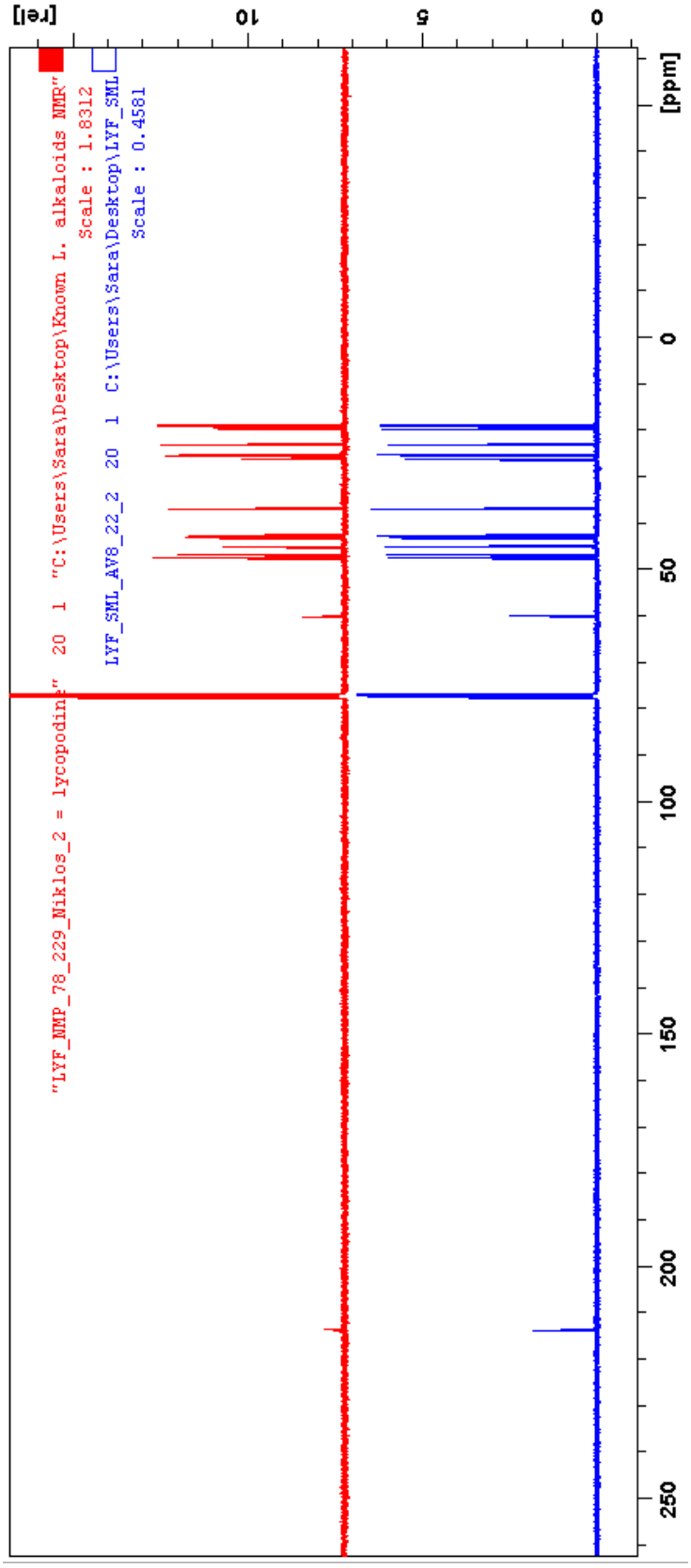

Figure 29: Carbon spectra comparison between AV8_22 and lycopodine

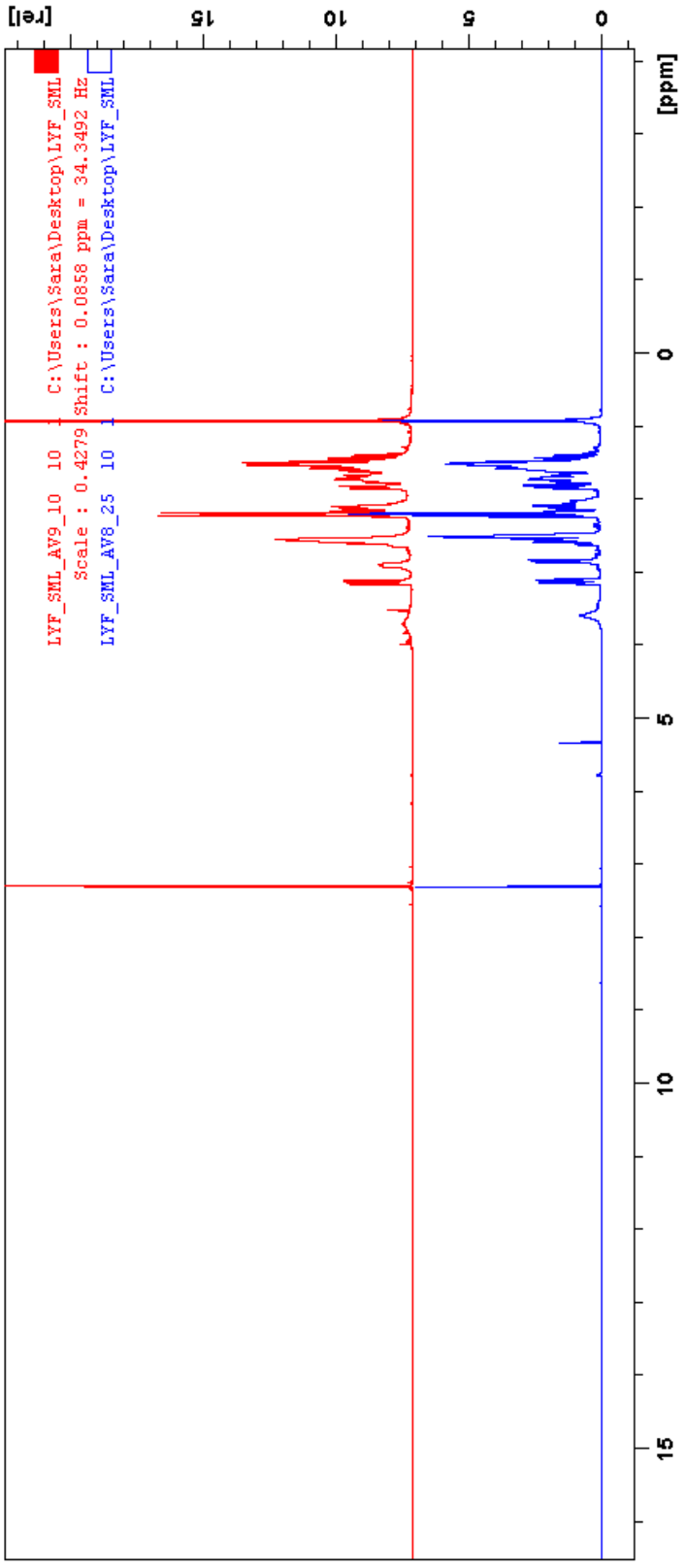

Figure 30: Proton spectra comparison between AV8_25 and AV9_10

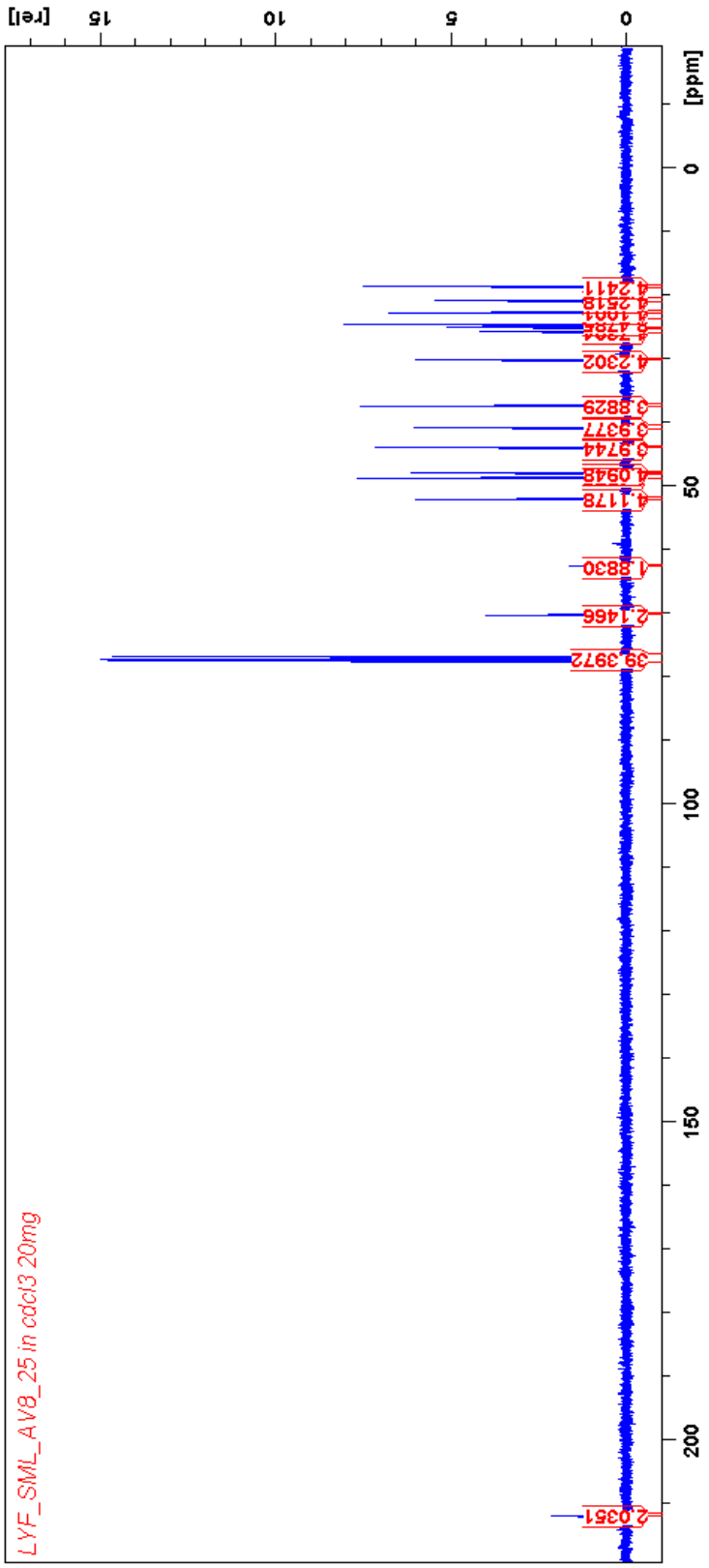

Figure 31: ^{13}C NMR spectrum of compound AV8_25

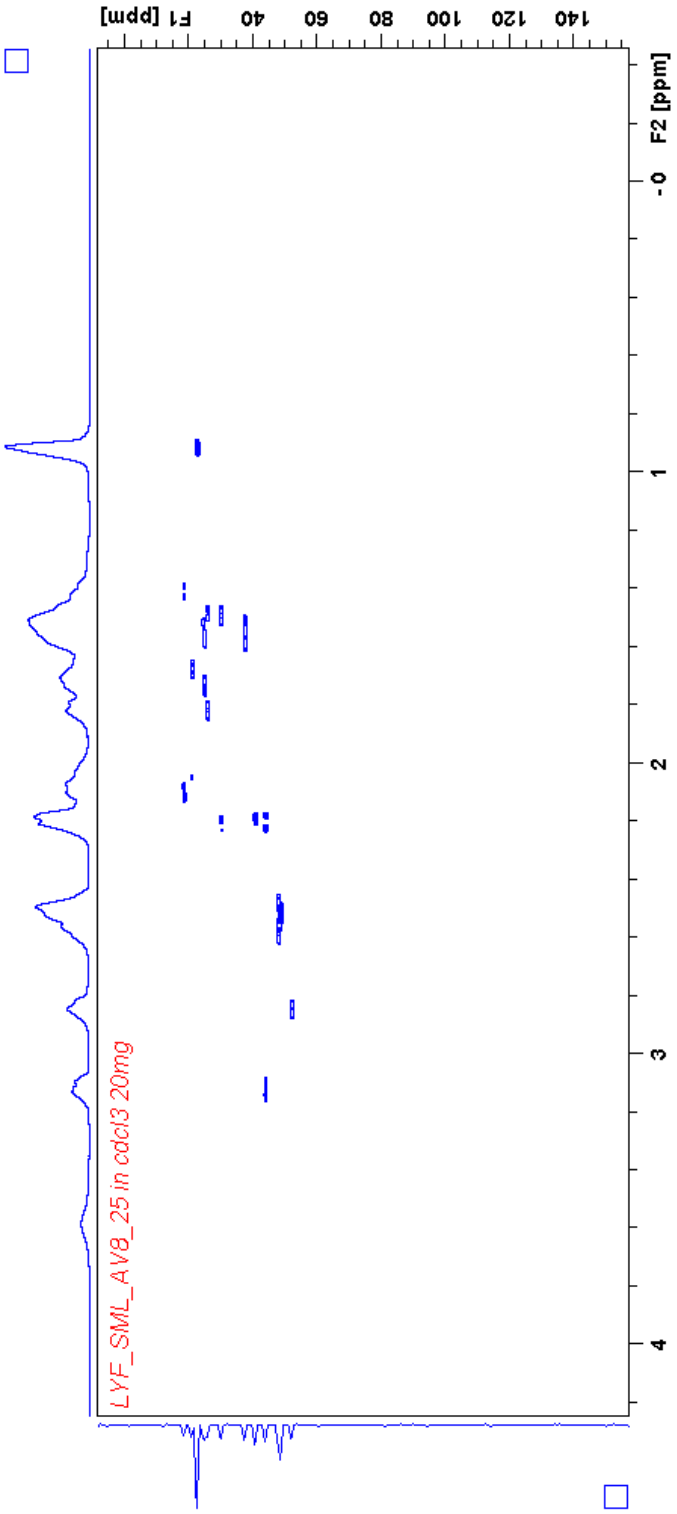

Figure 32: HSQC spectrum of compound AV8_25

Table 17: Measured concentrations of physostigmine

Vi1	Vi2	Vi3	Vi4	Vi5	Vi6	V0
0,246	0,243	0,251333	0,242333	0,241333	0,227	0,232
0,291667	0,29	0,300667	0,274	0,262667	0,236	0,287667
0,335333	0,329667	0,342	0,295667	0,275	0,239667	0,342667
0,378	0,366333	0,376	0,312	0,281667	0,242	0,399
0,418	0,4	0,405	0,324	0,286667	0,243667	0,453667
0,457667	0,432	0,429667	0,334667	0,289667	0,245667	0,507667
0,492667	0,46	0,451667	0,341	0,292	0,247333	0,561
0,528	0,486	0,470667	0,347333	0,294	0,249333	0,612667
0,559333	0,509	0,487	0,351333	0,296	0,251333	0,661667
0,591	0,530333	0,501	0,357333	0,298333	0,253	0,709
0,619	0,550333	0,513	0,361	0,3	0,255	0,754333
0,644667	0,568	0,524333	0,364	0,302667	0,256333	0,801667
0,670667	0,584333	0,534	0,368333	0,304667	0,258333	0,850667
0,693	0,599667	0,542	0,372	0,306333	0,260333	0,902333
0,713	0,614667	0,55	0,376	0,308667	0,262	0,952667
0,732333	0,630333	0,557333	0,379333	0,311	0,264	1,007
0,752333	0,645	0,565333	0,383	0,313	0,266	1,061333
0,771	0,659667	0,573	0,386	0,315667	0,268	1,117667
0,790667	0,674	0,579333	0,389667	0,318	0,269667	1,172333
0,811333	0,688667	0,587	0,393333	0,32	0,271667	1,233
0,829667	0,701	0,594667	0,396333	0,322333	0,274	1,283

Table 18: Measured concentrations of huperzine A

V1i	V2i	V3i	V4i	V5i	V6i	V0
0,285	0,285	0,272667	0,278667	0,269333	0,232	0,233667
0,285333	0,293	0,281	0,281	0,275667	0,232667	0,239
0,329667	0,338	0,327667	0,325	0,308	0,235667	0,284333
0,381667	0,390667	0,377333	0,372333	0,333667	0,236667	0,335
0,427667	0,438	0,421333	0,412667	0,351	0,237667	0,380333
0,479	0,492333	0,470667	0,457333	0,366333	0,238333	0,432
0,530333	0,547	0,518333	0,499333	0,378	0,239333	0,482667
0,58	0,601	0,565333	0,539667	0,386333	0,240667	0,533333
0,629	0,654333	0,610333	0,578	0,393	0,241333	0,582667
0,676667	0,705333	0,653333	0,613333	0,397667	0,242333	0,629667
0,723333	0,755333	0,695667	0,647667	0,401333	0,244	0,676
0,770333	0,802667	0,736667	0,680333	0,404333	0,245	0,722
0,816	0,848667	0,776667	0,711333	0,406333	0,246333	0,768667
0,860667	0,892333	0,814333	0,739667	0,408333	0,247667	0,812667
0,906333	0,935333	0,851333	0,768	0,410333	0,249	0,859333
0,95	0,976667	0,886667	0,795667	0,412	0,250333	0,904333
0,994667	1,017667	0,923333	0,82	0,413667	0,252333	0,950333
1,039333	1,057667	0,959333	0,843333	0,415333	0,253333	0,995
1,083667	1,086	0,996333	0,867	0,417333	0,254667	1,039333
1,126667	1,125333	1,032667	0,890667	0,419	0,256333	1,085333
1,171667	1,164333	1,069333	0,913	0,420333	0,257667	1,134
1,215667	1,203333	1,105667	0,938667	0,422667	0,259	1,183667

Table 19: Measured concentrations of serratidine

V1i	V2i	V3i	V4i	V5i	V6i	V0
0,267667	0,268667	0,274	0,258333	0,259333	0,251	0,226667
0,270667	0,274	0,277	0,265	0,264667	0,2565	0,234
0,315333	0,323333	0,329333	0,309667	0,303	0,286	0,286
0,366333	0,382	0,389	0,355	0,345	0,3165	0,344333
0,418333	0,439	0,447	0,397	0,383	0,347	0,401
0,471333	0,495667	0,504667	0,438	0,420333	0,3765	0,455667
0,523	0,551333	0,561333	0,477333	0,455	0,4055	0,507333
0,574	0,607333	0,616	0,516	0,489	0,432	0,556667
0,624667	0,662333	0,67	0,554333	0,523	0,458	0,603333
0,675	0,718	0,722667	0,592667	0,555667	0,4835	0,648
0,725667	0,773	0,773333	0,629	0,587667	0,508	0,692333
0,776333	0,828333	0,822	0,666333	0,618333	0,5315	0,737
0,828333	0,884	0,869333	0,702333	0,648333	0,556	0,781667
0,879667	0,94	0,916	0,738	0,677333	0,579	0,827667
0,931333	0,996667	0,961333	0,773667	0,704333	0,5995	0,874333
0,983	1,051333	1,008	0,808	0,732	0,62	0,923
1,034333	1,105333	1,053667	0,842333	0,757333	0,642	0,971
1,084333	1,158	1,101	0,877	0,781	0,6665	1,02
1,131667	1,208	1,150333	0,909667	0,804667	0,6835	1,069333
1,181667	1,258667	1,199333	0,942333	0,828667	0,706	1,117333
1,234333	1,309	1,249333	0,974333	0,850667	0,726	1,166667
1,282333	1,355	1,294333	1,002667	0,871	0,745	1,210667

Table 20: Measured concentrations of lycopodine

V1i	V2i	V3i	V4i	V5i	V6i	V0
0,329667	0,317	0,319	0,31	0,296333	0,290667	0,226667
0,328667	0,301	0,322667	0,311333	0,296667	0,295667	0,234
0,376	0,359667	0,371	0,355667	0,335333	0,336667	0,286
0,429333	0,412	0,427667	0,408667	0,378	0,380333	0,344333
0,484333	0,464333	0,482667	0,457667	0,420667	0,422667	0,401
0,538667	0,517	0,537	0,505667	0,463333	0,462667	0,455667
0,592	0,569333	0,59	0,553333	0,505667	0,501667	0,507333
0,644667	0,62	0,64	0,601	0,547333	0,539333	0,556667
0,697333	0,669333	0,689667	0,648333	0,587667	0,577667	0,603333
0,749	0,717	0,736333	0,695	0,626667	0,616333	0,648
0,801	0,766	0,783333	0,739333	0,666	0,652	0,692333
0,853	0,817333	0,83	0,782333	0,704333	0,684333	0,737
0,905667	0,870333	0,878	0,825	0,742667	0,717667	0,781667
0,958	0,926	0,926667	0,866667	0,780667	0,747667	0,827667
1,01	0,984	0,975667	0,907	0,818333	0,777667	0,874333
1,062	1,041333	1,026333	0,948	0,855	0,807	0,923
1,114	1,099667	1,078	0,988	0,890333	0,835333	0,971
1,16833	1,156667	1,130333	1,027333	0,925	0,861	1,02
1,22366	1,213667	1,183667	1,066	0,958667	0,888667	1,069333
1,281	1,27	1,237	1,104667	0,990667	0,914333	1,117333
1,33933	1,325667	1,289667	1,142	1,023333	0,938	1,166667
1,39033	1,374333	1,337	1,176	1,050333	0,958667	1,210667

Table 21: Measured concentrations of 12-epilycodoline

Vi1	Vi2	Vi3	Vi4	Vi5	Vi6	V0
0,297333	0,266	0,284	0,298	0,295667	0,299	0,232
0,354	0,301333	0,346	0,367667	0,362333	0,358667	0,287667
0,41	0,344667	0,400333	0,429333	0,424333	0,412333	0,342667
0,466667	0,388667	0,454667	0,488	0,485	0,464333	0,399
0,522667	0,433333	0,508	0,543333	0,544667	0,515667	0,453667
0,578	0,478	0,560667	0,598667	0,602333	0,567	0,507667
0,632	0,523333	0,613333	0,653333	0,657	0,618667	0,561
0,686333	0,568	0,666333	0,708333	0,707667	0,669	0,612667
0,739667	0,612333	0,718667	0,761667	0,757333	0,717333	0,661667
0,792333	0,656	0,77	0,813667	0,806667	0,765333	0,709
0,844	0,698667	0,820667	0,865333	0,855667	0,813667	0,754333
0,896	0,741	0,871	0,918	0,906667	0,861667	0,801667
0,946333	0,781	0,920667	0,971667	0,957667	0,907667	0,850667
0,996	0,821667	0,970333	1,026	1,008667	0,953	0,902333
1,043667	0,859667	1,018667	1,080333	1,060333	0,994667	0,952667
1,091333	0,897333	1,066	1,134667	1,113667	1,039667	1,007
1,139	0,933	1,114333	1,188	1,167	1,083667	1,061333
1,185333	0,969	1,160667	1,24	1,22	1,125333	1,117667
1,231333	1,003	1,206667	1,291	1,269333	1,167333	1,172333
1,275667	1,037333	1,255	1,340667	1,318667	1,206	1,233
1,314	1,068	1,296667	1,386	1,361333	1,241667	1,283

Table 22: VLC2 fractions 8 and 9

SAMPLE	Mass (mg)	NMR
AV8_4	4,0	✓
AV8_88	1,0	✓
AV8_7	4,0	✓
AV8_10	6,0	✓
AV8_11	2,0	✓
AV8_12	0,0	
AV8_15	1,0	✓
AV8_20	3,0	✓
AV8_22	27	✓
AV8_25	20	✓
AV8_44	1,0	✓
AV8_55	0,0	✓
AV9_1	1,4	✓
AV9_2	1,4	✓
AV9_3	1,3	✓
AV9_4	5,9	✓
AV9_5	2,2	✓
AV9_6	0,0	✓
AV9_7	0,0	✓
AV9_8	1,0	✓
AV9_9	26,8	✓
AV9_10	5,5	✓

Table 23: VLC3

SAMPLE	Mass (mg)	NMR
AV3-9_1_5	0,46	
AV3-9_1_86	0,49	
AV3-9_1_15	2,09	✓
AV3-9_2_4	1,60	
AV3-9_2_5	0,22	
AV3-9_2_58	0,69	
AV3-9_2_10	2,35	✓
AV3-9_3_45	3,44	✓
AV3-9_3_55	0,35	

Table 24: VLC4

SAMPLE	Mass (mg)	NMR
AV4_1_4	0,40	
AV4_1_7	0,31	
AV4_2_4	0,08	
AV4_2_52	0,71	
AV4_3_4	1,97	✓
AV4_3_5	2,99	✓
AV4_3_55	0,36	
AV4_3_6	0,04	
AV4_4_4	1,08	
AV4_4_5	0,00	
AV4_4_6	0,27	
AV4_5_4	1,84	✓
AV4_5_5	0,82	
AV4_5_6	0,26	
AV4_6_4	12,09	
AV4_6_5	2,03	✓
AV4_6_45	12,15	✓
AV4_6_6	1,50	✓