TURUN AMMATTIKORKEAKOULU
TURKU UNIVERSITY OF APPLIED SCIENCES

Bachelor's Thesis

DEVELOPMENT OF SMALL MOLECULE INHIBITORS FOR PROLYL OLIGOPEPTIDASE

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Laboratory Technology

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TURKU UNIVERSITY OF APPLIED SCIENCES ABSTRACT

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Title: Development of small molecule inhibitors for prolyl oligopeptidase

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The subject of this thesis work was prolyl oligopeptidase inhibitors. The aim was to prepare a series of syntheses that would result in a number of different final products with basic function. The thesis work was commissioned by the University of Antwerp, the Department of Medicinal Chemistry.

Prolyl oligopeptidase is a serine protease. It is specialised in hydrolysing peptide bonds with proteins that have a proline ring. This function is important because it means prolyl oligopeptidase can participate in the degradation of neuropeptides. Many neuropeptides contain a proline ring because it protects them from most enzymes.

This means that prolyl oligopeptidase might have an important role in neurological diseases. The studies have shown that there is a connection between prolyl oligopeptidase and learning and memory disorders such as Alzheimer's disease.

Therefore, the inhibitors of prolyl oligopeptidase could be a base for new drugs in treating these diseases. Some studies imply that the enzyme activity is increased correlating with the disease. The inhibition of prolyl oligopeptidase could be a part of the cure for the disease. There has been an ongoing project at the University of Antwerp for producing new prolyl oligopeptidase inhibitors for a few years. In this thesis work the focus is on inhibitors with basic decorating substituents.

The process of producing a new inhibitor molecule takes all in all a nine-step synthesis. The first eight steps are for building up a scaffold and in the ninth step the decorating group is added. The final products are analysed by mass spectrometry, NMR and HPLC.

All the desired products could be produced and purified. Unfortunately there was not enough time to perform testing of inhibitor activity. In that sense the work still needs to be continued.

Keywords: prolyl oligopeptidase, neurological diseases, synthesis

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TURUN AMMATTIKORKEAKOULU TIIVISTELMÄ

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Tekijä: Vaula Metso	
Työn nimi: Pienikokoisten prolyylioligopeptidaasi-inhibiittorien valmistus	
Ohjaaja: Pieter Van Der Veken, Ph.D. (Antwerpenin yliopisto) Jouko Vihanto, FT (Turun ammattikorkeakoulu)	
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Työn tarkoituksena oli valmistaa inhibiittoreita prolyylioligopeptidaasille. Prolyylioligopeptidaasi on seriiniproteaasi, joka on erikoistunut pilkkomaan pienikokoisia peptidejä, joissa on proliinirengas. Ihmisellä prolyylioligopeptidaasia esiintyy ympäri kehoa, mutta erityisesti sen sijainti aivoissa on terveydelle tärkeä. Prolyylioligopeptidaasi on yksi niistä entsyymeistä, joka voi osallistua neuropeptidien hajoittamiseen.

Tämä ominaisuus tekee siitä tärkeän aivojen toimintaan liittyvien sairauksien hoidossa. Prolyylioligopeptidaasilla on havaittu yhteys muun muassa Alzheimerin tautiin sekä kaksisuuntaiseen mielialahäiriöön. Prolyylioligopeptidaasin toimintamekanismi näissä sekä muissa sairauksissa ei ole vielä täysin selvitetty, mutta tutkimuksia tehdään koko ajan.

Vaikka toimintamekanismia ei ole vielä selvitetty, on kuitenkin kannattavaa ryhtyä etsimään tehokkaita inhibiittoreita prolyylioligopeptidaasille. Entsyymin aktiivisuuden lisääntymisellä ja esimerkiksi Alzheimerin taudilla on havaittu korrelaatio. Tehokas inhibiittori voisi olla osana taudin hoidossa. Tätä työtä on tehty Antwerpenin yliopiston lääkekemian laitoksella jo useamman vuoden ajan. Tässä opinnäytetyössä on keskitytty erityisesti emäksisillä ominaisuuksilla varustettujen inhibiittorien valmistukseen.

Inhibiittoreita valmistettiin yhteensä viisi kappaletta. Koko prosessi oli yhteensä yhdeksänvaiheinen synteesi, jossa kahdeksan vaihetta tarvittiin rungon valmistukseen ja viimeisessä vaiheessa lisättiin molekyylit toisistaan erottava funktionaalinen ryhmä.

Lopputuotteet analysoitiin massaspektrometrillä, NMR:llä sekä HPLC:llä. Kaikkien suunniteltujen lopputuotteiden valmistuksessa onnistuttiin. Lopputuotteiden inhibitiotehoa ei ehditty kuitenkaan analysoimaan, joten siinä mielessä työtä on vielä jatkettava.

Hakusanat: prolyylioligopeptidaasi, neurologiset sairaudet, synteesi

Säilytyspaikka: Turun ammattikorkeakoulun kirjasto, Lemminkäisenkatu

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FIGURES

1 INTRODUCTION

This thesis work was done at the Department of Medicinal Chemistry at the University of Antwerp, Belgium. The aim of the study was to prepare inhibitors for prolyl oligopeptidase. The subject was based on the previous work that had been done in the prolyl oligopeptidase project. In this thesis work the focus was on preparing five inhibitors that would have the same scaffold but would differ in the decorating substituents.

Prolyl oligopeptidase is a serine protease that is found in many places in human body but the localization in brain is the most interesting for the researchers. There are studies that indicate that prolyl oligopeptidase is somehow related to neurological diseases. The studies are mainly focused on memory and learning disorders such as Alzheimer's disease. The exact function of prolyl oligopeptidase is yet to be clarified. The studies that show a correlation with a high prolyl oligopeptidase activity and a memory disorder was the basis of this thesis work and reason why just inhibitors were made. If an efficient inhibitor could be prepared it could come a drug against these diseases. In addition, it could be used to further test what would happen if the prolyl oligopeptidase would be inhibited in physiology.

The synthesis was planned based on the previous projects that had been going on among the research group. All in all a nine-step synthesis was planned to carry out. The steps were somewhat similar to what had been made previously. In the first eight steps the scaffold would built up and then in the last step a different basic decorating substituents would be added.

The additional aim of the thesis work was to test the inhibitory activity of the compounds. Due to time restrictions this was not done. So the exact potential for further drug development still remains to be examined.

2 THEORETICAL BACKGROUND

2.1 Prolyl oligopeptidase

Prolyl oligopeptidase (EC 3.4.21.26) is a serine protease. Walter *et al.* first discovered it in human uterus in 1971 and it was then named prolyl endopeptidase (1). When more information of functions of prolyl oligopeptidase (PO) was gathered it was discovered that it hydrolyses oligopeptidases instead of proteins like other endopeptidases and the name was proposed to be changed to prolyl oligopeptidase (2). The IUBM (International Union of Biochemistry and Molecular Biology) recommends now the name prolyl oligopeptidase because it describes the enzymes functions more accurately (3).

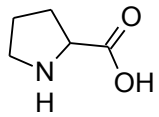


Figure 1: Proline.

Prolyl oligopeptidase is found in mammals, bacteria and even archaea, only exception being fungi (4). It is small sized, only 80 kDa, intracellular enzyme. Due to its localization in brain, it is considered to have a role in maturation and degradation of neuropeptides and peptide hormones (5). Most peptidases are not able to take part on hydrolysing the peptide bond at proline residues because proline (Figure 1) is an imino rather than an amino acid. Proline is an amino acid that has secondary amine group and that is why it is called imino acid. Many neuropeptides and peptide hormones contain one or more proline residues to protect then from enzymatic degradation. Proline-specific enzymes are therefore often required for processing such peptides. (6)

Serine proteases are proteases that share a common serine residue at their active site. The prolyl oligopeptidase family is a one family among the serine proteases. In addition to PO this family includes for example dipeptidyl peptidase IV (DPP IV), acylaminoacyl peptidase and oligopeptidase B. These proteases are specified in hydrolysing oligopeptides that are less than 30 amino acids. (4) Prolyl oligopeptidase hydrolyses oligopeptides after proline residues (7).

2.2 Prolyl oligopeptidase and diseases

According to recent studies, prolyl oligopeptidase might have a role in various neurological conditions. Two mainly studied groups are memory disorders, such as Alzheimer's disease and mental disorders like major depression and schizophrenia. The function of prolyl oligopeptidase in these various diseases is not yet fully understood. Moreover, the function varies a lot between the different types of diseases.

Many neuropeptides contain proline and therefore it has been proposed that PO might have a role in their metabolism. Arginine vasopressin (AVP), substance P (SP) and thyrotropin-releasing hormone (TRH) are all neuropeptides that are connected with learning and memory and they contain proline. The effect of the PO activity is however a complicated matter. There are studies reporting that PO activity is enhanced in the brains of patients with Alzheimer's disease (AD). On the other hand, there are also studies contradicting that result reporting that PO activity is reduced in AD patients. One explanation could be that PO has different role in different stages of AD. (8)

Recent studies have suggested that PO could participate in mood disorders. There is a hypothesis that PO is a regulator of inositol phosphate (InsP). Inositol depleting drugs (e. g. lithium) are used in the treatment of bipolar disorder (BD) and this is

how PO could be a part of BD. (9) There are also reports implying that there is a decrease of plasma PO activity in BD. The inhibition of PO has been shown to reverse the effect on lithium based mood stabilizer drugs. The matter is still under research investigating the exact mechanism and effect on BD. (10)

What can be gathered from the above mentioned is that a substantial research effort focusing on the role of PO and its functions in body is required. These diseases are just examples of the variety of theories of PO's role in diseases. For example there are also theories that PO could have a role in eating disorders and Parkinson's disease (11). At the same time the development of the inhibitors of prolyl oligopeptidase can be considered a valuable work: such compounds can play important roles in studying PO's role in physiology and in the future selected compounds can be developed further to produce efficient drugs against PO related diseases.

3 SYNTHETIC PROCESS

Most of the inhibitors that have been made this far are proline mimetics. In this thesis work, the lead compound was chosen to be the prolylproline motif. Proline has a cyclic structure that separates it from other amino acids. The reason for favouring proline is that prolyl oligopeptidase is a proline specified enzyme.

The aim of the syntheses was to prepare a number of end compounds that would share a common prolylproline-derivated scaffold, decorated with basic functional groups. There have been previous projects with the same scaffold but in this work, the goal was to add different groups with basic functions. The reason for this is that basic compounds are usually protonated at the physiological pH and therefore dissolve better in water. At the same time, the choice of substituents that have pK_a values of maximally 10, can be expected not to interfere with the compounds' ability to enter the Central Nerve System by passive diffusion. The last step was done using Cu^{I}-catalysed alkyne-azide cycloaddition, which meant that all the molecules added in the final reaction needed to have an alkyne terminus. Some of the reagents used in the last step were commercial and some of them had to be prepared in the laboratory.

All in all the synthetic process consisted of eight steps to prepare the scaffold and then one last step to add the functional group. Finally, five end products were prepared. The more detailed explanations of reaction mechanisms are described in the following chapters.

3.1 Esterification

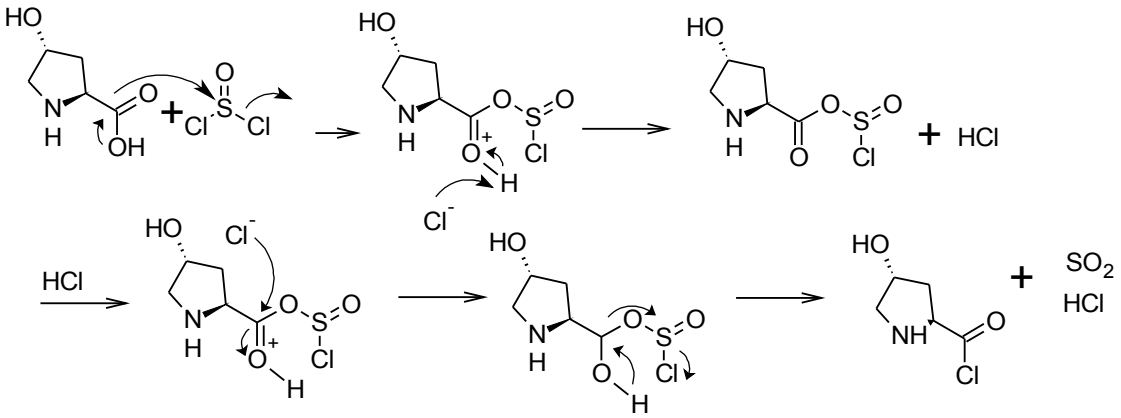


Figure 2. Formation of acyl chloride. (12 p. 295, modified)

2-hydroxyproline is first converted into an acyl halide using thionyl chloride. The carboxylic acid has a bad leaving group OH^-. In this reaction, it is turned into a good leaving group using a thionyl chloride, $SOCl_2$. Carboxylic acid attacks thionyl chloride forming HCl and an unstable intermediate that is highly electrophilic. The intermediate is protonated with HCl giving an electrophile that reacts with Cl^- forming again an unstable intermediate. This intermediate finally collapses into acyl chloride, SO_2 and HCl. The reaction is irreversible because SO_2 and HCl are gases and therefore lost from reaction mixture. (12 pp. 294-295) The reaction pathway for forming acyl chloride can be seen from Figure 2.

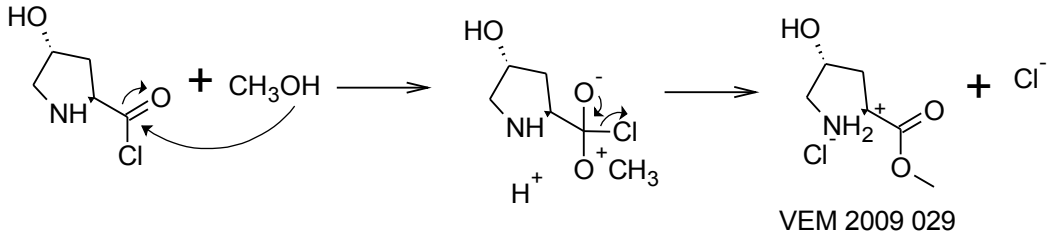


Figure 3. Esterification. (13 p. 300, modified)

Reaction is not ready yet since the acyl chloride still reacts. The methanol that is present in the reaction flask attacks the carbon atom at carbonyl group. This leads

to an unstable intermediate and finally to elimination of the chloride ion. (13 p. 300) The amide function in the proline reacts with the free chloride ions forming a chlorine salt. The esterification reaction and the formed molecule VEM-2009-029 are showed in Figure 3.

3.2 Protecting groups

When dealing with multifunctional chemical compounds it is sometimes necessary to protect one or more of the functional groups in the molecule to prevent them to react causing unwanted products. There is a variety of different protecting groups designed for different purposes. When choosing a protecting group there are many things that need to be taken into consideration. (14 p.1)

The protecting group has to be specific to that functional group which it is going to protect. The reactions that are performed to introduce protecting group and to cleave it should have a high yield and preferably be done without toxic chemicals. The protecting group should preferentially form a solid derivate, which is easy to purify. Protecting group should stay stable and not form new functional groups or affect the stereochemistry of the original compound. (14 p.1)

The BOC group

tert-Butyl carbamate, BOC group, is used to protect amine function mainly in peptide and heterocyclic syntheses. The BOC group is inert to many nucleophilic and electrophilic reagents and it does not hydrolyse easily in basic conditions. It can be cleaved using only strong acid. These characteristics make it a popular protecting group for amines. The structure of *tert*-Butyl carbamate is shown in Figure 4. (14, p.725)

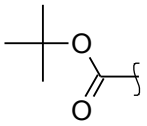


Figure 4. The BOC group. (12 p.655)

Usually protecting groups are put on using a carbamoyl chloride. This does not work with BOC group because *tert*-BuOCOCl is too unstable. In this thesis work, the BOC group is put on using the anhydride BOC_2O as it usually is the case. The structure of BOC_2O can be seen in Figure 12 above the reaction arrow. (12 p. 655)

3.3 Mesylation

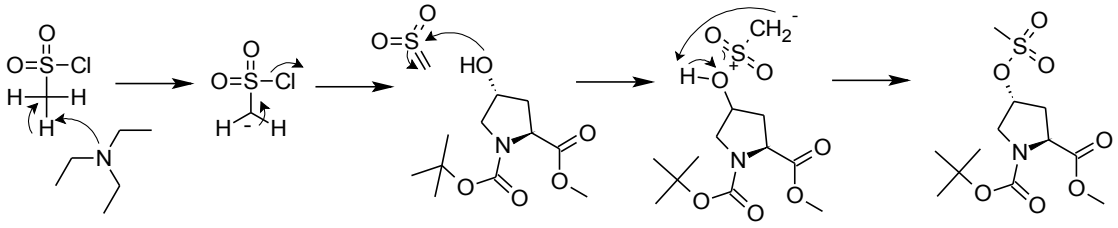


Figure 5. Mesylation. (12 p. 486, modified)

The BOC protected compound has still one hydroxyl group. It is converted to mesyl group to make a better leaving group for the next step. Methanesulphonyl chloride reacts with alcohols giving methanesulphonate esters if there is a base present (triethylamine in this reaction). First, the base reacts with the methanesulphonyl chloride so that an HCl molecule is eliminated. Then the formed sulphene reacts with the hydroxyl group forming the mesylate. The reaction mechanism can be seen in Figure 5. (12 pp. 485-486)

3.4 Replacing a mesyl group with an azide group

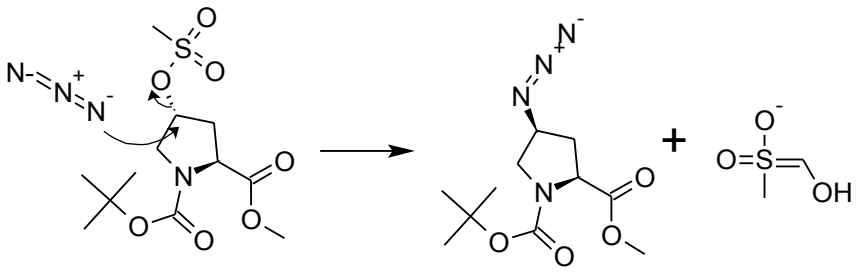


Figure 6. Replacing a mesyl group with an azide group. (12 p. 437, modified)

The azide molecule is a rod-shaped, linear molecule that is nucleophilic at both ends. That makes it able to insert itself into almost any electrophilic site. The nucleophilic end attacks the mesylated carbon forming an alkyl azide and eliminating the mesyl group. The reaction mechanism can be seen in Figure 6.

3.5 Hydrolysis of a methyl ester

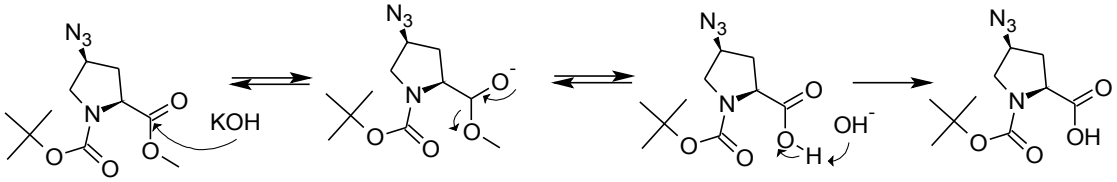


Figure 7. Hydrolysis of a methyl ester. (12 p. 291, modified)

The esterificated carboxylic acid needs to be converted back to acid because in the next step it is coupled with a pyrrolidine ring. Hydrolysis is done using a base, potassium hydroxide. The hydroxyl ion attacks the carbon atom in the carboxyl group and forms a carboxylic acid. For reaction mechanism see Figure 7.

3.6 Coupling with O-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate

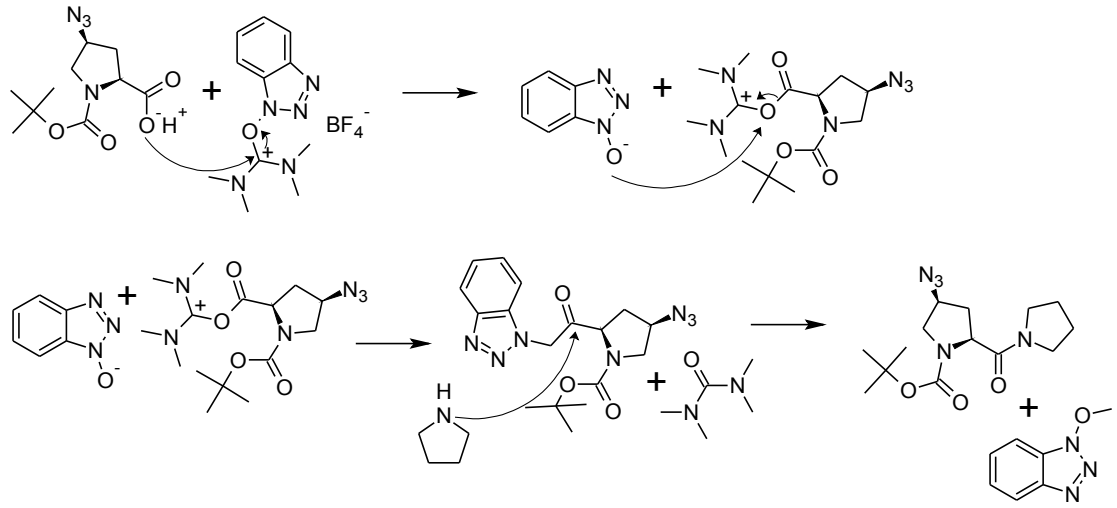


Figure 8. Coupling of a pyrrolidine ring using TBTU.

The hydroxyl group of the carboxylic acid reacts with the electron positive carbon of O-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) molecule forming a bond. The bond between carbon and oxygen is broken giving an extra electron to oxygen. This electronegative oxygen then attaches to carbonyl group. Being a good leaving group, this group makes the carbonyl group more accessible to the pyrrolidine ring. For reaction mechanism see Figure 8.

3.7 Removal of the di-*tert*-butyl dicarbonate protecting group

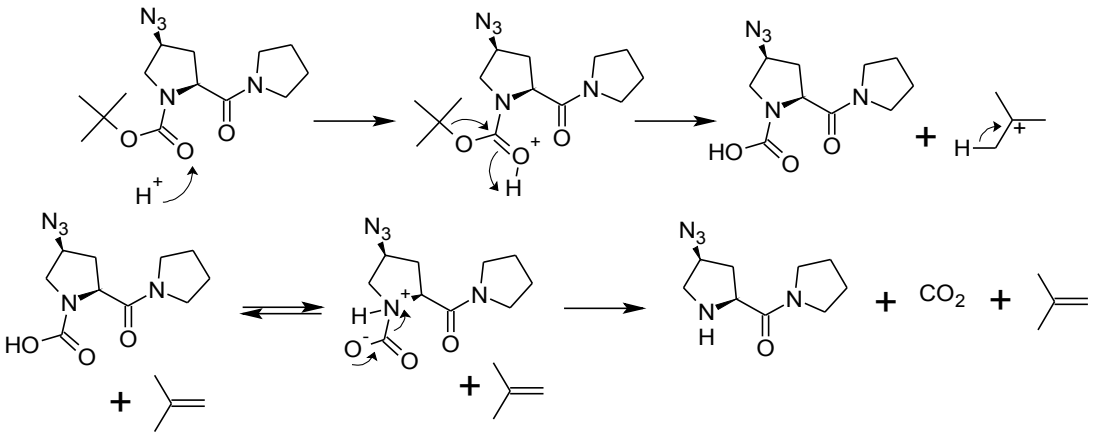


Figure 9. Removal of the BOC protecting group. (12 p. 655, modified)

BOC protection can be removed with acid, in this case trifluoroacetic acid, TFA, was used. TFA is used to protonate the oxygen in the carbonyl group. The protonated oxygen then attacks the hydrogen, which leads to the loss of *t*-butyl cation. The rest of the molecule then decarboxylates to an unprotected molecule. The reaction mechanism can be seen in Figure 9.

3.8 Addition of a phenylbutanoic group

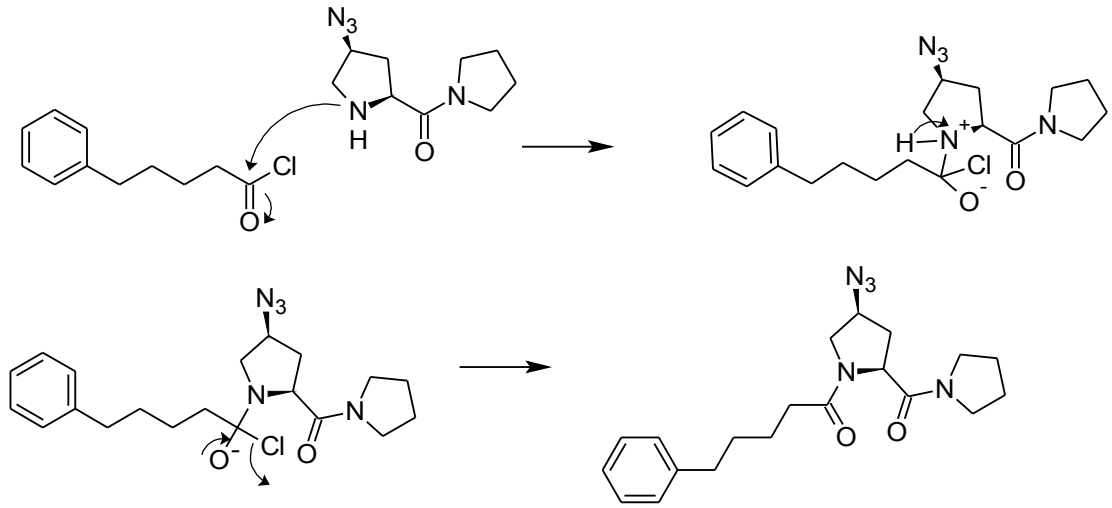


Figure 10. Addition of a phenylbutanoic group. (12 p. 284, modified)

The amine was deprotected in the previous step to make it accessible for the coupling reaction. The reaction is a simple reaction with the acyl chloride derived from phenylbutanoic acid to form an amide. The amine attacks the carbonyl carbon in the acyl chloride eliminating chloride ion. The reaction mechanism can be seen in Figure 10. In previous experiments with the PO inhibitors it was noticed that positioning a phenylbutanoic group in this part of the inhibitor affected less with the inhibition rate of the molecule. That means that it was better to replace BOC protecting group with phenylbutanoic group so that the inhibition would be most effective.

3.9 Copper-(I)-catalysed 1,3-dipolar addition

The Cu^{I}-catalysed 1,3-dipolar addition of an azide and an alkyne is used in this work to couple the scaffold and the functional group together in the last step of syntheses. It is classified as “click chemistry”, a relatively new group of synthetic methods. Click chemistry has gotten its name from the fact that the reactions are fast and they require only benign reaction conditions and simple work-up and purification procedures. The mechanism of the copper(I) catalysed version of this reaction has only partially been unravelled. (15)

4 METHODS AND INSTRUMENTATION OF LABORATORY EXPERIMENTS

4.1 Flash chromatography

Flash chromatography is a purification method. It is similar to traditional column chromatography but it is done under a pressure to speed up the elution time. The sample that is to be purified is placed on a top of a column usually filled with silica (normal phase). (16 p. 179) Sample can be either dissolved in a small amount of toluene or attached to silica or kieselguhr. The column is then attached to the pump and the elution is performed. The product and impurities are collected into tubes. From every collected tube, a small sample is applied to a TLC plate. The plate and MS are then used to detect the tubes with the pure compound.

With this method, the separation quality is not the best but it is faster than many other methods. One run could be something from 10 minutes to a couple of hours. In this work, the purifications that were made took about half an hour. This method works best for samples that have only few components. (16 p. 179)

In this work, some of the compounds were purified using column chromatography. A glass column is filled with silica and the silica-bound sample is added to the top. The elution flow can be forced to go faster by pumping air to the column.

4.2 Thin layer chromatography and preparative TLC

Thin layer chromatography (TLC) is a fast and practical way of following the flow of syntheses. It is usually done using plastic, aluminium or glass plates covered

with silica (SiO_2) or alumina (Al_2O_3) (16 p. 256). The materials for TLC are affordable and the work process is easy to learn. That is why it is so commonly used in organic chemistry to examine the reaction process. In these reactions a common UV active TLC-plate was used and UV light and ninhydrin colouring were used for detection.

Preparative TLC is an ordinary TLC scaled up. It is an analytical technique that can also be used to isolate compounds. The adsorbent layer, usually silica, is much thicker (0.5-2.0 mm) and the plate itself is larger (12x12 inch) (17 p. 276). With one plate a large amount, 10 - 100 mg, of product can be purified. The product is applied to the plate as a streak 1 cm from the bottom and 2 cm from the edges. The width of the streak depends on how much product there is to be purified. Same plate can be used for multiple products if there is enough space. After the plate is developed, the right band can be scraped off and then product is separated from the adsorbent with suitable solvent. (17 p. 80)

5 SYNTHESES

5.1 Esterification

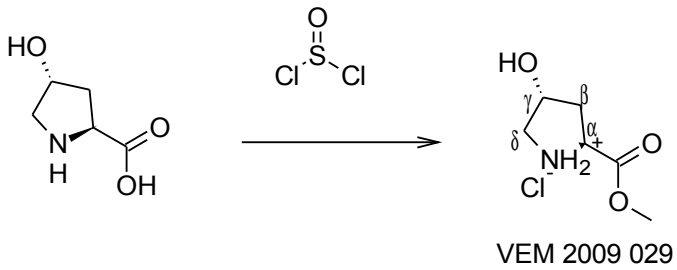


Figure 11. Synthesis of VEM-2009-029.

A 500 ml round bottomed flask was filled with argon through a rubber septum. The flask was then charged with dry methanol (150 ml) and cooled in an ice bath. Sulphurous dichloride (12.45 ml, 172 mmol) was added to methanol carefully. 2-hydroxyproline (15 g, 114 mmol) was added to the solution. The ice bath was removed and the solution was allowed to warm up to room temperature. Then the solution was heated to 70 °C with a reflux condenser and refluxed for 2 hours. Methanol was evaporated and the crude product was precipitated and washed with ether. The starting material and the final product are drawn in Figure 11. For NMR and MS spectra of the product see Appendix 1.

Yield: 92 %

ES-MS: m/z 146.1 = $[M^+H]^+$

^{1}H NMR (D_2O, 400 MHz) *δ*: 2.0 (1H, m, β1), 2.2 (1H, m, β2), 3.1 (1H, d, δ1), 3.2 (1H, m, δ2), 3.5 (3H, s, CH_3), 4.4 (H_2O and underneath it α and γ)

5.2 Di-*tert*-butyl dicarbonate protection

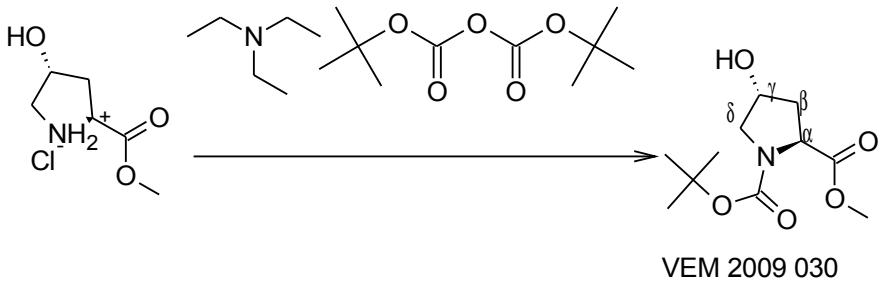


Figure 12. Synthesis of VEM-2009-030.

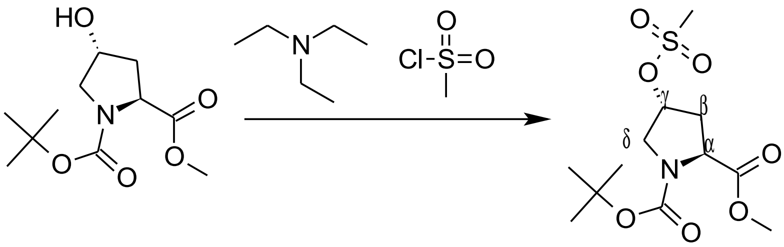
VEM-2009-029 (19 g, 105 mmol) was dissolved in dichloromethane (DCM, 200 ml) and cooled in an ice bath. Triethylamine (43.5 ml, 314 mmol) was added. Di-*tert*-butyl dicarbonate (22.83 g, 105 mmol) was dissolved in DCM (50 ml) and the mixture was added to the reaction solution dropwise. The ice bath was removed and the reaction was stirred for overnight. DCM was evaporated and the residue was dissolved in ethyl acetate (EtOAc, 150 ml). The solution was washed with 1 M HCl (2×75 ml), dried over sodium sulphate, filtered and evaporated under reduced pressure. The starting material and the final product are drawn in Figure 12. For NMR and MS spectra of the product see Appendix 2.

Yield: 80 %.

ES-MS: m/z 513.1 = $[2M^{+}Na]^{+}$, m/z 268.1 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.1 (9H, s, BOC), 1.8 (1H, m, β1), 2.0 (1H, m, β2), 3.3 (2H, m, δ), 3.4 (3H, s, CH_3), 4.1-4.2 (2H, m, γ+α)

5.3 Mesylation



VEM 2009 031

Figure 13. Synthesis of VEM-2009-031.

VEM-2009-030 (20 g, 82 mmol) was dissolved in DCM (100 ml) and triethylamine (16.73 ml, 122 mmol) was added. Solution was cooled in an ice bath. Methane sulphonyl chloride (6.94 ml, 90 mmol) was dissolved in DCM (50 ml) and the mixture was added to the reaction solution dropwise. The ice bath was removed and the solution was stirred for 2 hours. DCM was evaporated and the residue was dissolved in EtOAc (150 ml). The solution was washed with 1 M HCl (2×75 ml), dried over sodium sulphate, filtered and evaporated under reduced pressure. Product was dissolved in EtOAc and then precipitated with hexane, filtered and washed with hexane. The starting material and final product are drawn in the Figure 13. For NMR and MS spectra of the product see Appendix 3.

Yield: 92 %

ES-MS: m/z 669.1 = $[2M^{+}Na]^{+}$, m/z 346.2 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.1 (9H, m, BOC), 2.0 (1H, m, β1), 2.3 (1H, m, β2), 2.7 (3H, s, SCH_3), 3.2 (2H, s, δ), 3.5 (3H, s, OCH_3), 4.1 (1H, m, α), 5.0 (1H, s, γ)

5.4 Converting a mesyl group to an azide group

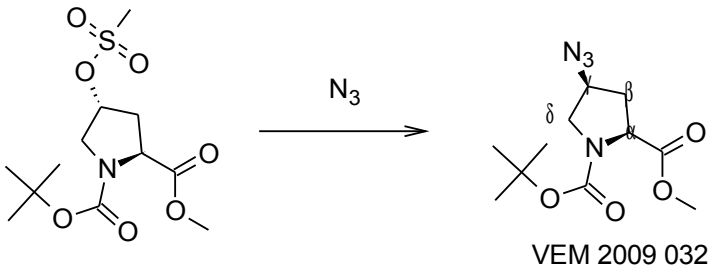


Figure 14. Synthesis of VEM-2009-032.

VEM-2009-031 (24 g, 74.2 mmol) was dissolved in dimethylformamide (70 ml). Sodium azide (9.65 g, 148 mmol) was added and the solution was heated to 85 °C. The solution was stirred for 3.5 hours at 85 °C. The reaction solution was dissolved in EtOAc (200 ml). The solution was washed with H_2O (2×150 ml), dried over sodium sulphate, filtrated and evaporated under reduced pressure. The starting material and final product are drawn in Figure 14. For NMR and MS spectra of the product see Appendix 4.

Yield: 95 %.

ES-MS: m/z 563.1 = [2M+Na]+, m/z 293.1 = $[M^+Na]^+$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.1 (9H, d, BOC), 1.9 (1H, m, β1), 2.2 (1H, m, β2), 3.2 (2H, s, δ), 3.4 (3H, s, CH_3), 3.8 (1H, m, α), 4.0-4-1 (1H, m, γ)

5.5 Hydrolysis of a methyl ester

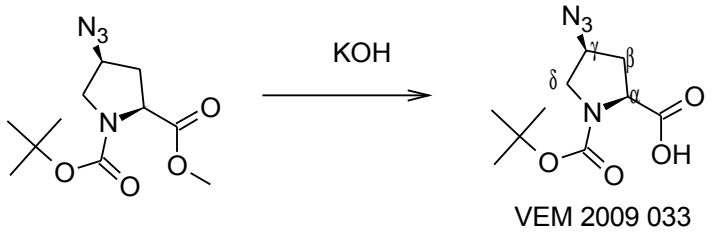


Figure 15. Synthesis of VEM-2009-033.

VEM-2009-032 (19 g, 70.3 mmol) was dissolved in methanol (20 ml). Potassium hydroxide (1 M solution in methanol, 70.3 ml, 70.3 mmol) was added to the solution which was then stirred for 28 h. Methanol was evaporated under reduced pressure. The residue was dissolved in water (75 ml) and washed with ether (50 ml). The water layer was acidified with 2 M HCl to pH 1 and then extracted with EtOAc (3×100 ml). The combined organic layers were dried over sodium sulphate, filtrated and evaporated under reduced pressure. The starting material and the final product are drawn in Figure 15. For NMR and MS spectra of the product see Appendix 5.

Yield: 79 %.

ES-MS: m/z 535.1 = $[2M^{+}Na]^{+}$, m/z 279.1 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$ + TMS, 400 MHz) δ: 1.5 (9H, d, BOC), 2.1-2.5 (2H, m, β), 3.5 (2H, s, δ), 4.1 (1H, m, α), 4.4 (1H, m, γ), 6.5 (1H, s, OH)

5.6 Coupling of a pyrrolidine ring using O-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate

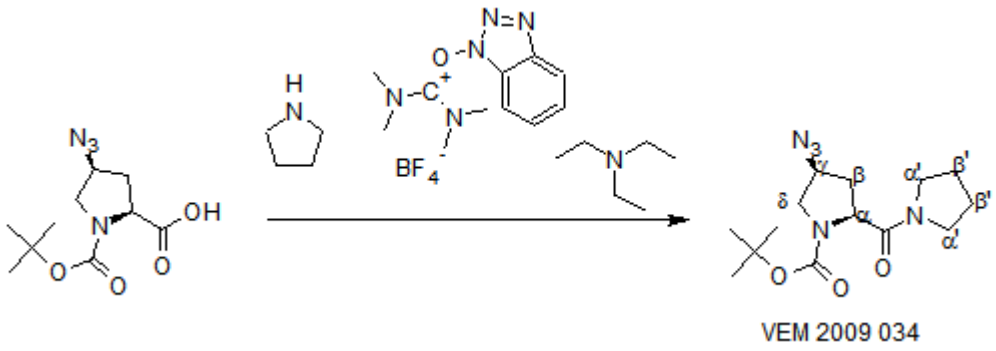


Figure 16. Synthesis of VEM-2009-034.

VEM-2009-033 (14 g, 54.6 mmol) was dissolved in DCM (100 ml) together with triethylamine (19.06 ml, 137 mmol). TBTU (14.63 g, 45.7 mmol) and pyrrolidine (3.75 ml, 45.7 mmol) were added and the reaction was stirred overnight. DCM was evaporated and the crude product was redissolved in EtOAc and washed with H_2O, saturated $NaHCO_3$ and saturated NaCl. The organic layer was dried over sodium

sulphate, filtered and evaporated under reduced pressure. The crude product was purified using column chromatography (gradient: hexane to EtOAc) yielding white crystalline mass. The starting material and the final product are drawn in Figure 16. For NMR and MS spectra of the product see Appendix 6.

Yield: 65 %.

ES-MS: m/z 641.2 = $[2M^{+}Na]^{+}$, m/z 332.2 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.1 (9H, d, BOC), 1.5-1.7 (3H, m, β'+β), 3.0-3.2 (4H, m, α'), 3.4-3-6 (2H, m, δ), 3.7 (1H, m, α), 4.1-4.2 (1H, m, γ)

5.7 Removal the di-*tert*-butyl dicarbonate protecting group

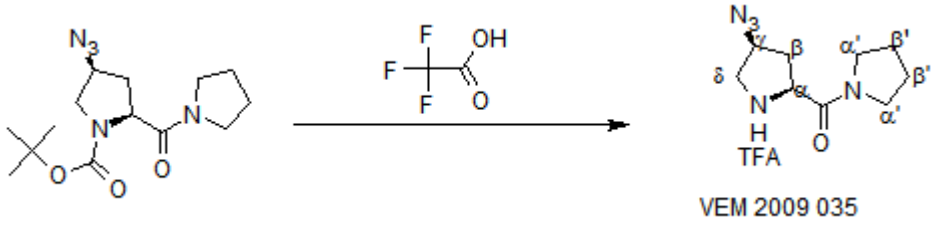


Figure 17. Synthesis of VEM-2009-035.

VEM-2009-034 (9.22 g, 29.8 mmol) was dissolved in DCM (90 ml) and trifluoroacetic acid (TFA, 90 ml, 1168 mmol) was added. The reaction was stirred for 30 minutes and then the volatiles were evaporated under reduced pressure. Most of the TFA was co-evaporated with hexane. The starting material and the final product are drawn in Figure 17. For NMR and MS spectra of the product see Appendix 7.

Yield: 195 % (TFA forms a salt with the product and it is hard to get rid of. Yield of 150 % to 200 % is usually considered acceptable.)

ES-MS: m/z 419.3 = $[2M^{+}H]^{+}$, m/z 210.2 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.6-1.8 (6H, m, β+β'), 2.5 (4H, m, α'), 3.2 (2H, m, δ), 3.4 (1H, m, α), 4.2-4.4 (1H, m, γ), 8.8 (1H, s, NH)

5.8 Addition of a phenylbutanoic group

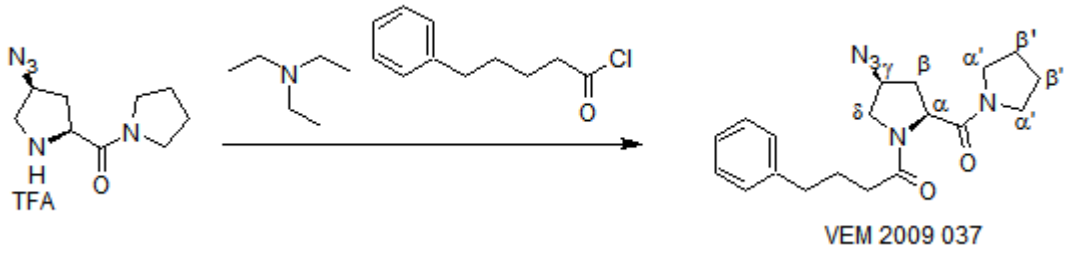


Figure 18. Synthesis of VEM-2009-037.

VEM-2009-035 (9.13 g, 29.8 mmol) was dissolved in DCM (75 ml) and triethylamine (12.39 ml, 89 mmol) was added. The solution was cooled in a water bath. 4-phenylbutanoyl chloride (5.44 g, 29.8 mmol) was dissolved in DCM (5 ml) and added to the solution dropwise. The solution was stirred for 1h 30 minutes. The solution was washed with 1 M HCl, dried over sodium sulphate, filtered and evaporated under reduced pressure. The crude product was purified using column chromatography (gradient: hexane to EtOAc) yielding white crystalline mass. The starting material and the final product are drawn in Figure 18. For NMR and MS spectra of the product see Appendix 8.

Yield: 43 %.

ES-MS: m/z 733.1 = $[2M^{+}Na]^{+}$, m/z 378 = $[M^{+}Na]^{+}$, m/z 356.2 = $[M^{+}H]^{+}$

^{1}H NMR (MEOD, 400 MHz) δ: 1.6 (4H, m, β') 1.7 (2H, m, CH2), 2.1 (2H, m, CH2), 2.4 (4H, m, α'), 3.0 (2H, m, CH2), 3.1 (2H, m, CH2), 3.2 (2H, m, β), 3.9 (1H, m, α), 4.3 (1H, t, γ), 6.8-7.0 (5H, m, Ar)

5.9 Introduction of an imidazole substituent

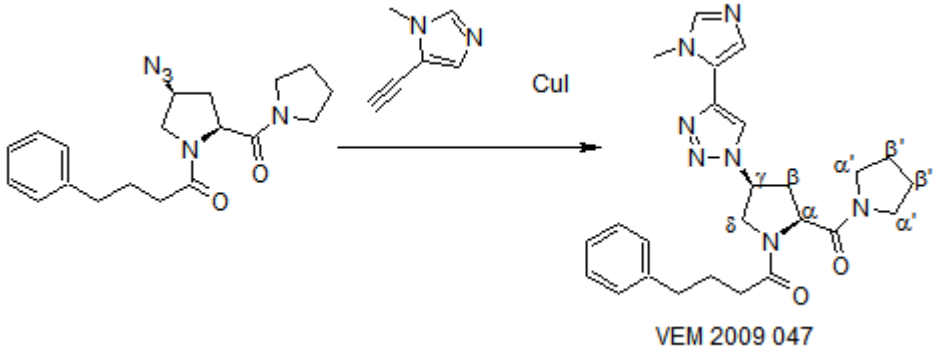


Figure 19. Synthesis of VEM-2009-047.

VEM-2009-037 (454 mg, 1.277 mmol) was dissolved in methanol (5 ml). 5-ethynyl-1-methyl-1H-imidazole (excess) and copper(I)iodide (catalytic amount) were added. The solution was stirred over weekend. Methanol was evaporated and the product was purified with column chromatography (gradient: hexane to EtOAc: 20% methanol) and preparative TLC plate (eluent: DCM: 5% methanol). The starting material and final product are drawn in Figure 19. For NMR and MS spectra together with HPLC chromatogram of the product see Appendix 9.

Yield: 5 %

Purity: 100 %

ES-MS: m/z 462.20 = $[M^+H]^+$, m/z 484.1 = $[M^+Na]^+$, m/z 500.3 = $[M^+K]^+$

^{1}H NMR ($CDCl_3$+TMS, 400 MHz) δ: 1.2 (2H, m, β'1), 1.9 (4H, m, CH_2×2), 2.3 (2H, m, β'2), 2.7 (2H, m, CH_2), 3.1 (2H, t, β), 3.5 (2H, m, CH_2N), 3.8 (1H, m, α'1), 4.0-4.3 (3H, m, α'2,3,4), 4.5-5.0 (2H, m, δ), 5.1 (1H, t, α), 5.5 (1H, s, γ), 6.3 (3H, s, CH_3), 7.0-7.3 (5H, m, Ar), 8.7 (3H, s, triazole, 2 × imidazole)

5.10 Introduction of a di-*tert*-butyl dicarbonate protected piperazine substituent

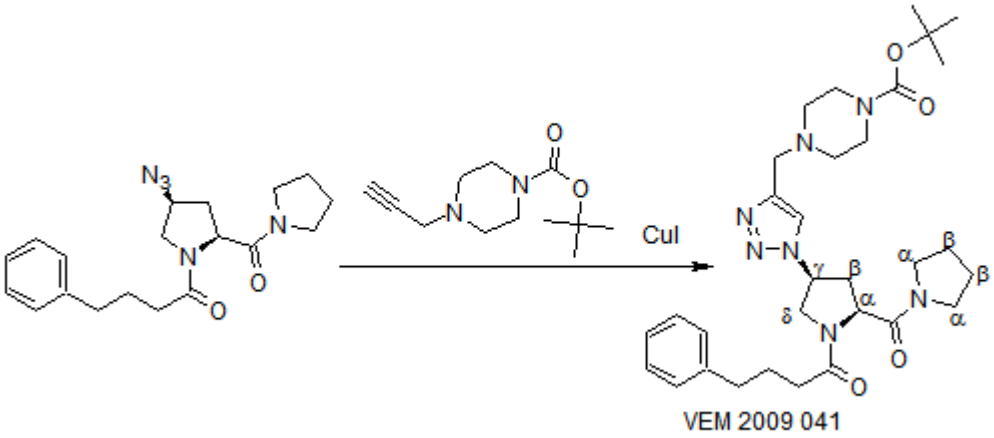


Figure 20. Synthesis of VEM-2009-041.

VEM-2009-037 (159 mg, 0.466 mmol) was dissolved in methanol (5 ml). *tert*-Butyl 4-(prop-2-ynyl)piperazine-1-carboxylate (excess) and copper(I)iodide (catalytic amount) were added. The solution was stirred over weekend. Methanol was evaporated and the product was purified with column chromatography (gradient: hexane to EtOAc: 20% methanol). The starting material and the final product are drawn in Figure 20. For NMR and MS spectra together with HPLC chromatogram of the product see Appendix 10.

Yield: 59 %

Purity: 99.65 %

ES-MS: m/z 602.4 = $[2M^{+}Na]^{+}$, m/z 580.4 = $[M^{+}Na]^{+}$

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.5 (9H, s, BOC), 1.9 (2H, m, β'1), 2.0 (2H, m, CH_2), 2.3 (2H, m, CH_2), 2.5 (2H, m, β'2), 2.7 (2H, m, CH_2), 2.9 (2H, s, β), 3.4 (4H, m, α'1 + α'2), 3.4 - 4.0 (8H, m, piperazine), 3.9 (1H, m, δ1), 4.2 (1H, m, δ2), 4.8 (1H, t, α), 5.4 (1H, s, γ), 7.1-7.3 (5H, m, Ar), 8.0 (1H, s, triazole)

5.11 Introduction of a morpholine substituent

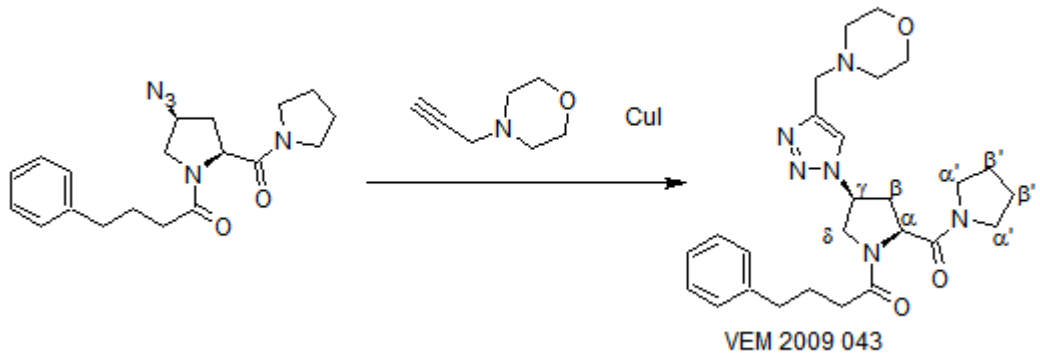


Figure 21. Synthesis of VEM-2009-043.

VEM-2009-037 (170 mg, 0.478 mmol) was dissolved in methanol (5 ml). 4-(Prop-2-ynyl)morpholine (excess) and copper(I)iodide (catalytic amount) were added. The solution was stirred over weekend. Methanol was evaporated and the product was purified with column chromatography (gradient: hexane to EtOAc: 20% methanol). The starting material and the final product are drawn in Figure 21. For NMR and MS spectra together with HPLC chromatogram of the product see Appendix 11.

Yield: 75 %

Purity: 100 %

ES-MS: m/z 503.2 = $[M^+Na]^+$, m/z 481.3 = $[M^+H]^+$

^{1}H NMR ($CDCl_3$+TMS, 400 MHz) δ: 1.9 (2H, m, β'1), 2.0 (2H, m, CH_2), 2.3 (2H, m, CH_2), 2.4 (2H, m, β'2), 2.6 (2H, m, CH_2), 2.9 (2H, s, β), 3.4 (2H, m, α'1), 3.5 (2H, m, α'2), 3.7-4.0 (9H, m, morpholine + δ1), 4.2 (1H, s, δ2), 4.8 (1H, t, α), 5.4 (1H, t, γ), 7.1-7.3 (5H, m, Ar), 8.0 (1H, s, triazole)

5.12 Introduction of a BOC protected amine substituent

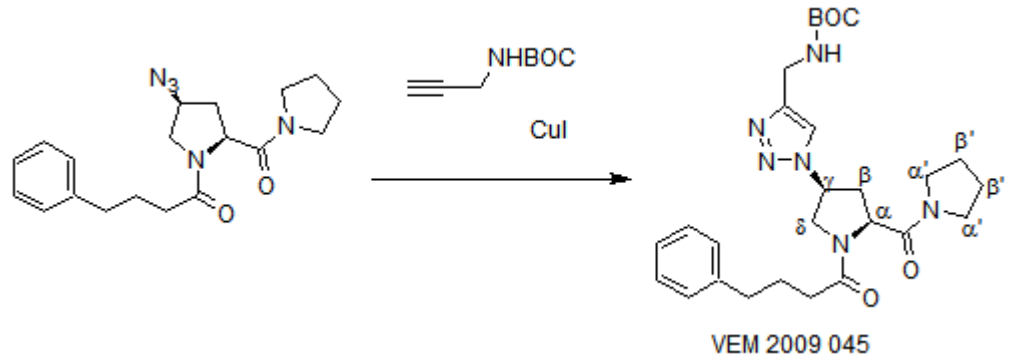


Figure 22. Synthesis of VEM-2009-045.

VEM-2009-037 (744 mg, 2.093 mmol) was dissolved in methanol (5 ml). *tert*-Butyl prop-2-ynylcarbamate (excess) and copper(I)iodide (catalytic amount) were added. The solution was stirred over weekend. Triethylamine was added to catalyse the reaction and the reaction was stirred overnight. Methanol was evaporated and the product was purified with column chromatography (gradient: hexane to EtOAc). The starting material and the final product are drawn in Figure 22. For NMR and MS spectra together with HPLC chromatogram of the product see Appendix 12.

Yield: 75 %

ES-MS: m/z 612.3 = $[M^+TEA]^+$, m/z 533.3 = $[M^+Na]^+$

^{1}H NMR ($CDCl_3$+TMS, 400 MHz) δ: 1.4 (9H, s, BOC), 1.8 (2H, m, β'1+β'2), 2.0 (2H, m, CH1), 2.3 (2H, m, CH2), 2.4 (1H, m, β'3), 2.6 (2H, m, CH2), 2.8 (1H, m, β'), 3.4 (4H, m, β1, NH, CH2), 3.5 (1H, m, β2), 3.9 (2H, m, α'1+2), 4.1 (1H, t, α'3), 4.4 (3H, d, α'4, δ1+2), 4.7 (1H, t, δ3), 5.2 (1H, t, α), 5.4 (1H, s, γ), 7.1-7.3 (5H, m, Ar), 7.8 (1H, s, triazole)

5.13 Removal of the BOC protecting group

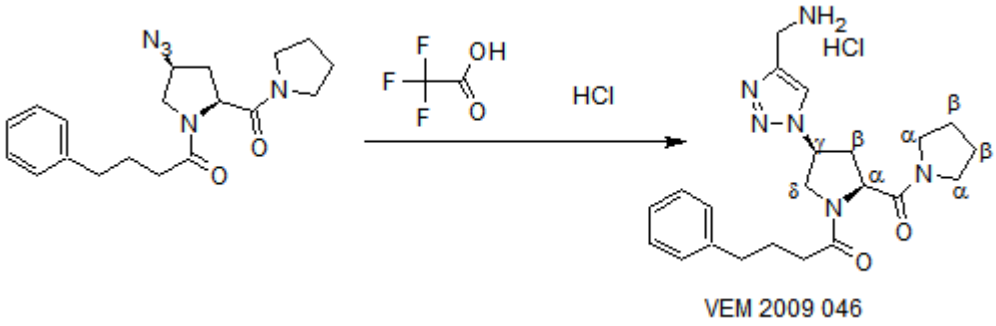


Figure 23. Synthesis of VEM-2009-046.

VEM-2009-045 (680 mg, 1.332 mmol) was dissolved in DCM (5 ml). 2,2,2-Trifluoroacetic acid (5 ml, 64.9 mmol) was added. The reaction was stirred for 1h 30 minutes. Then the volatiles were evaporated under reduced pressure. TFA is co-evaporated with hexane. The product was dissolved in methanol and 5-6 N HCl in 2-propanol was added. The volatiles were evaporated and the product was washed with ether. The starting material and the final product are drawn in Figure 23. For NMR and MS spectra together with HPLC chromatogram of the product see Appendix 13.

Yield: 97 %

Purity: 100 %

ES-MS: m/z 411.3 = $[M]^+$, m/z 434.3 = $[M^+Na]^+$, m/z 821.1 = $[2M]^+$

^{1}H NMR ($CDCl_3$+TMS, 400 MHz) δ: 1.2 (2H, m, β'1), 1.9 (4H, m, CH_2×2), 2.3 (2H, m, β'2), 2.5 (2H, m, CH_2), 3.1 (2H, t, β), 3.4 (2H, m, CH_2N), 3.8 (1H, m, α'1), 4.0-4.3 (3H, m, α'2,3,4), 4.5-5.0 (2H, m, δ), 5.1 (1H, t, α), 5.5 (1H, s, γ), 6.3 (2H, s, NH_2), 7.0-7.3 (5H, m, Ar), 8.7 (1H, s, triazole)

6 DISCUSSION

The purification of the products was challenging. In many of the products there was seen a small peak just next to the products signal. This means that the retention time of impurity is very close to the products and this makes purification difficult. This could be a result of different configurations that are formed in the last step of synthesis. Even though the exact mechanism of the copper-(I)-catalysed 1,2,3-triazole introduction is not fully understood there are some details that are more or less certain. That is, it can be predicted that the molecule that is added to the azide ring attaches itself to the carbon that is further away from the rest of the molecule. However, there still is a possibility that it is attached to the other carbon and this could be impurity that is formed. It could explain why it has so similar retention time.

This problem might be prevented by modification of reaction conditions. Also a better purification method would help with this problem. Flash chromatography is not accurate enough but for example a preparative HPLC could solve the problem.

Beginner's clumsiness can be seen from the NMR spectra. In some spectra (VEM-2009-033 for example), the TMS peak is too strong. In some samples there may have been too much solvent which could explain the widened peaks. It has to be taken consideration also that the produced products were not pure enough. Some of the products were later purified even more but it was done in a later phase when the testing of the compounds were started.

The synthesis has many steps which is not preferable. The more steps and phases the more impurities. It affects also the total yield. Maybe in this process the some steps could be somehow avoided or replaced. For example is it really necessary to first do the esterification and then hydrolyse again or could it be possible to just couple the pyrrolidine ring.

The yields of single steps could also be improved. Maybe with different reaction conditions some reactions could work better. For example the yields for click chemistry vary a lot. Of course the molecule and the amount of purification needed affects the result. Still, there are other ways for performing the click reactions than the above mentioned. Even small changes such as heating or adding a base could improve the yield. On the other hand it could also increase the amount of impurities or lead to unwanted reactions.

Should the development of these molecules into drugs become relevant all these aspects has to be taken into consideration. The inhibition activity of the prepared compounds is not yet known. The testing itself is a simple process that is done using a photospectrometric competition assay of prepared inhibitors with a PO substrate in the presence of the target enzyme. The results of the inhibitory activity was planned to be included in this thesis work but due to time limitations they had to be left out.

REFERENCES

1 Myöhänen, Timo T., García-Horsman, J. Arturo, Tenorio-Laranga, Jofre, Männistö, Pekka T. (2009) Issues About the Physiological Functions of Prolyl Oligopeptidase Based on Its Discordant Spatial Association With Substrates and Inconsistencies Among mRNA, Protein Levels and Enzymatic Activity. Journal of Histochemistry & Cytochemistry 57(9): 831-848.

2 Rawlings, Neil D., Polgar, László, Barret ,Alan J. (1991) A new family of serine-type peptidases related to prolyl oligopeptidase. Biochemical Journal 279:908.

3 http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/21/26.html

4 Venäläinen, Jarkko I., Juvonen, Risto O., Männistö, Pekka T. (2004) Evolutionary relationships of the prolyl oligopeptidase family enzymes. European Journal of Biochemistry 271:2705-2715.

5 Rosenblum, Jonathan S, Kozarich, John W. (2003) Prolyl peptidases: a serine protease subfamily with high potential for drug discovery. Current Opinion in Chemical Biology 7:469-504.

6 Polgár, L. (2002) The prolyl oligopeptidase family. Cellular and Molecular Life Sciences 59:349-362.

7 Jarho, Elina M., Venäläinen, Jarkko I., Juntunen, Juha, Yli-Kokko, Leena A., Vepsäläinen, Jouko, Christiaans, Johannes A.M., Forsberg, Markus M., Järvinen, Tomi, Männistö, Pekka T., Wallén, Erik A.A. (2006), An introduction of a pyridine group into the structure of prolyl oligopeptidase inhibitors. Bioorganic & Medicinal Chemistry Letters 16:5590-5593.

8 Toide, Katsuo, Iwamoto, Yohko, Fujiwara, Takako, Abe, Hiroyki. (1995) JTP-4819: A Novel Prolyl Endopeptidase Inhibitor with Potential as a Cognitive Enhancer. The Journal of Pharmacology and Experimental Therapeutics 247:1370-1378.

9 Robin, Williams S.B. 2004 Prolyl oligopeptidase and bipolar disorder. Clinical Neuroscience Research 4:233-243.

10 Breen G, Harwood AJ, Gregory K, Sinclair M, Collier D, St Clair D, Williams RSB. (2004) Two peptidase activities decrease in treated bipolar disorder not schizophrenic patients. Bipolar Disorders 6:156-161.

11 Brandt, Inger. Scharpé, Simon. Lamberi, Anne-Marie. (2007) Suggested functions for prolyl oligopeptidase: A puzzling paradox. Clinical Chimica Acta 377:50-61.

12 Clayden, Jonathan, Greeves, Nick, Warren, Stuart, Wothers, Peter. (2006) Organic Chemistry. Oxford University Press Inc. USA.

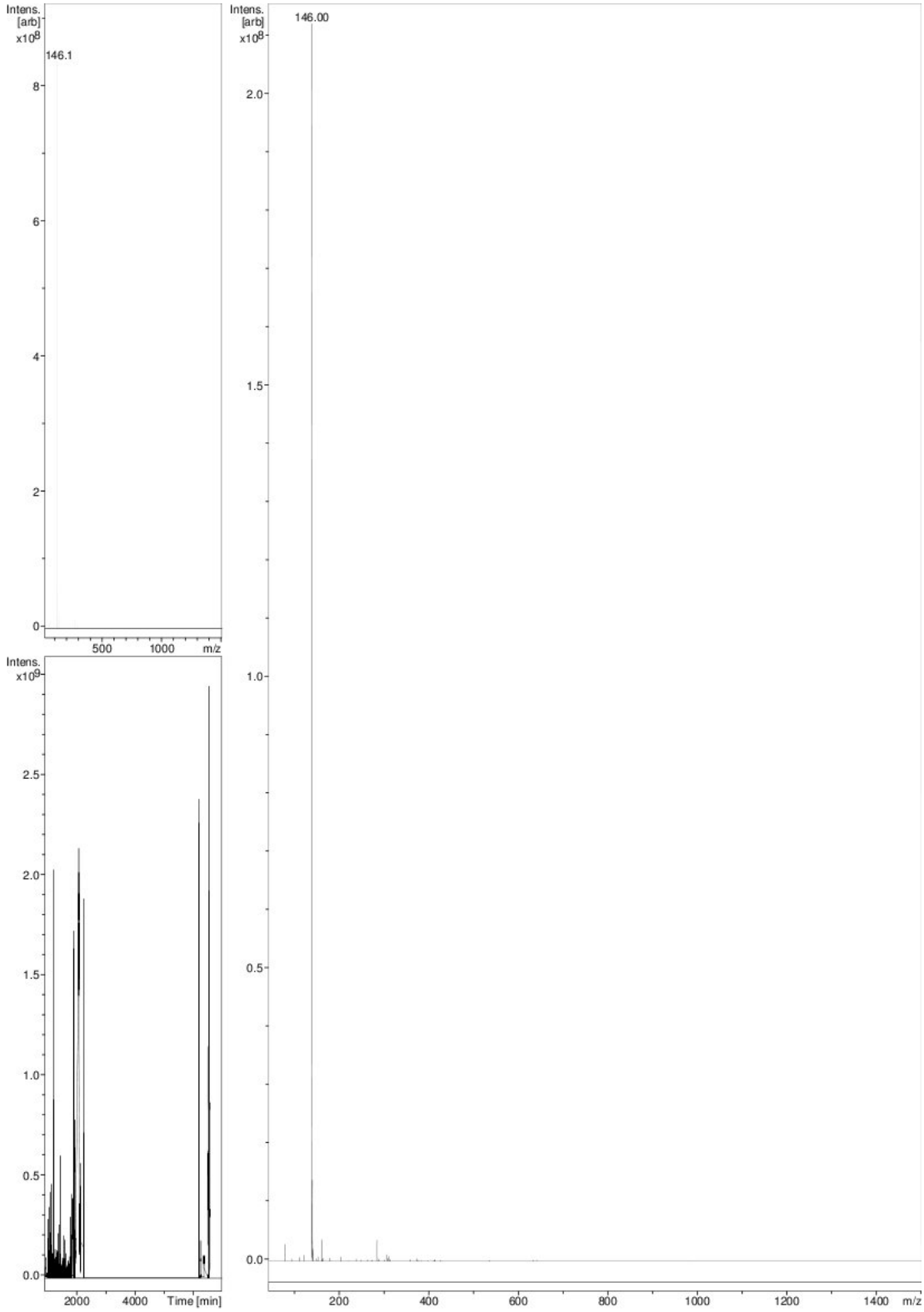
13 Bruice, Paula Y. (2006) Essential Organic Chemistry. Pearson Education Inc. USA.

14 Wuts, Peter G. M., Greene, Theodora W., (2007) Greene's Protective Groups in Organic Synthesis. 4th edition. John Wiley & Sons, Inc. USA.

15 Bock, Victoria D., Hiemstra, Henk., van Maarseveen Jan H. (2006) Cu^{I}-Catalyzed Alkyne-Azide "Click" Cycloadditions from a Mechanistic and Synthetic Perspective. Eur. J. Org. Chem. 51-68.

16 Meloan, Clifton E. (1999) Chemical Separations Principles, Techniques, and Experiments. John Wiley & Sons, Inc. USA.

17 Zubrick, James W. (1997) The Organic Chem Lab Survival Manual. 4th edition. John Wiley & Sons, Inc. USA.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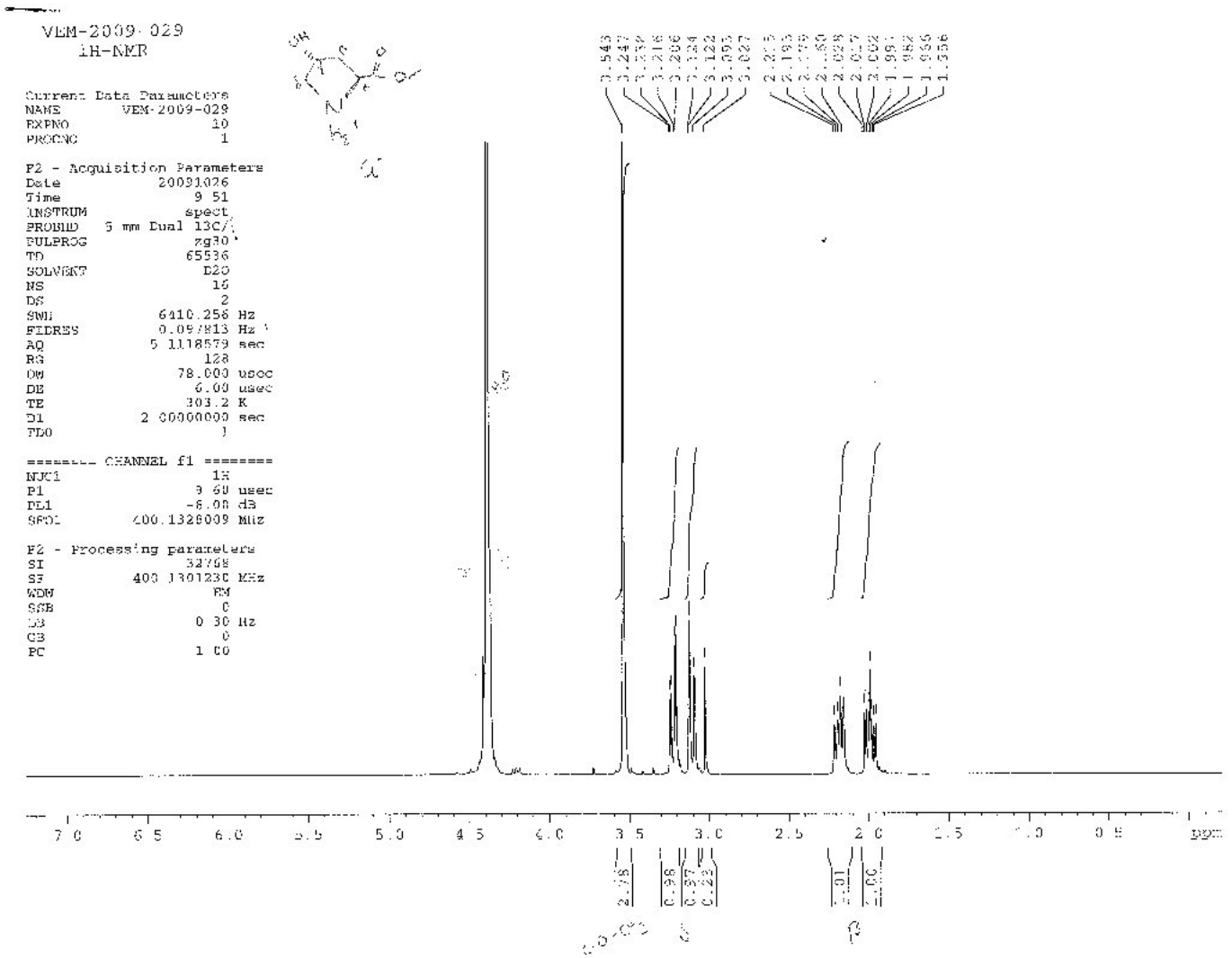
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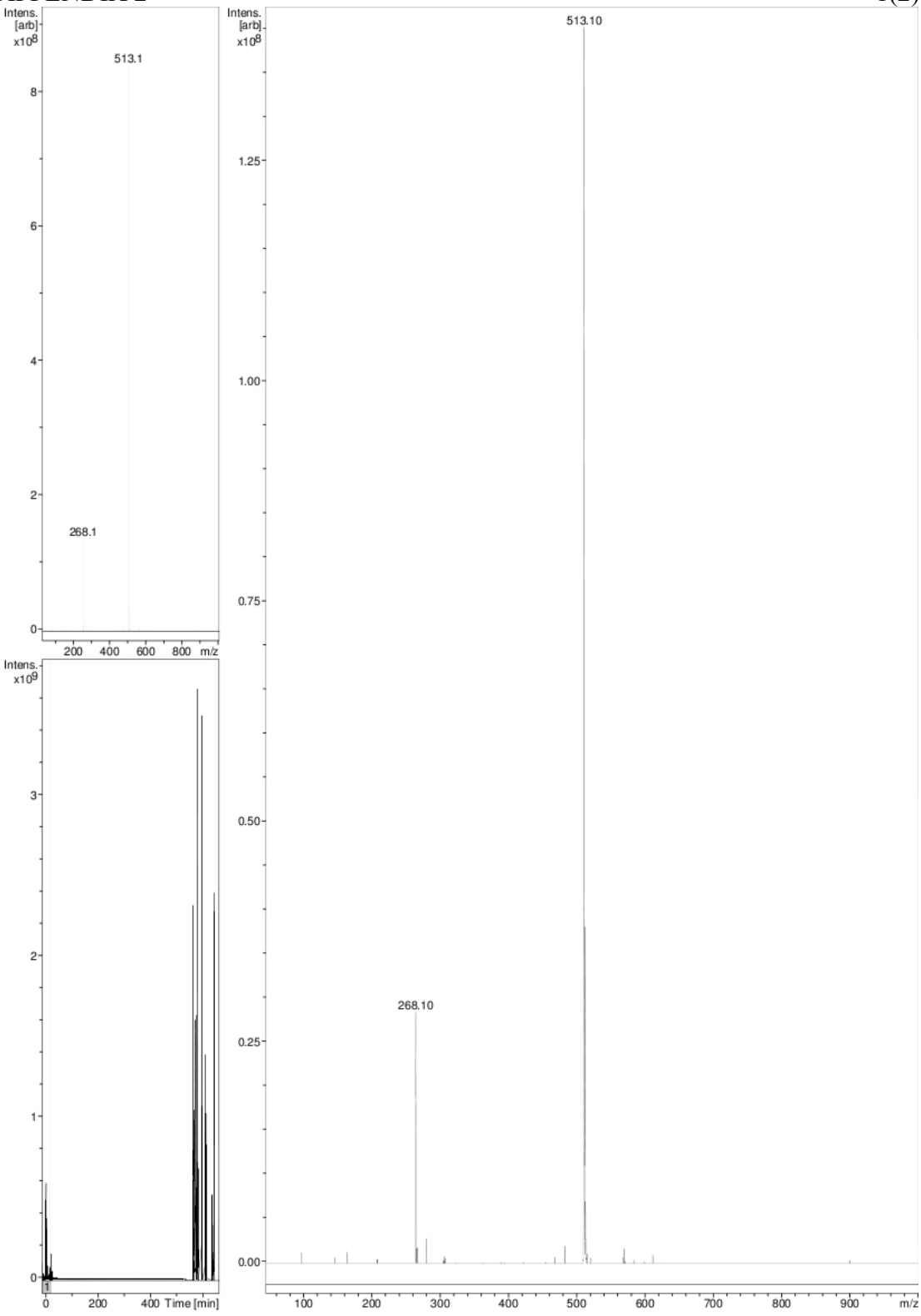
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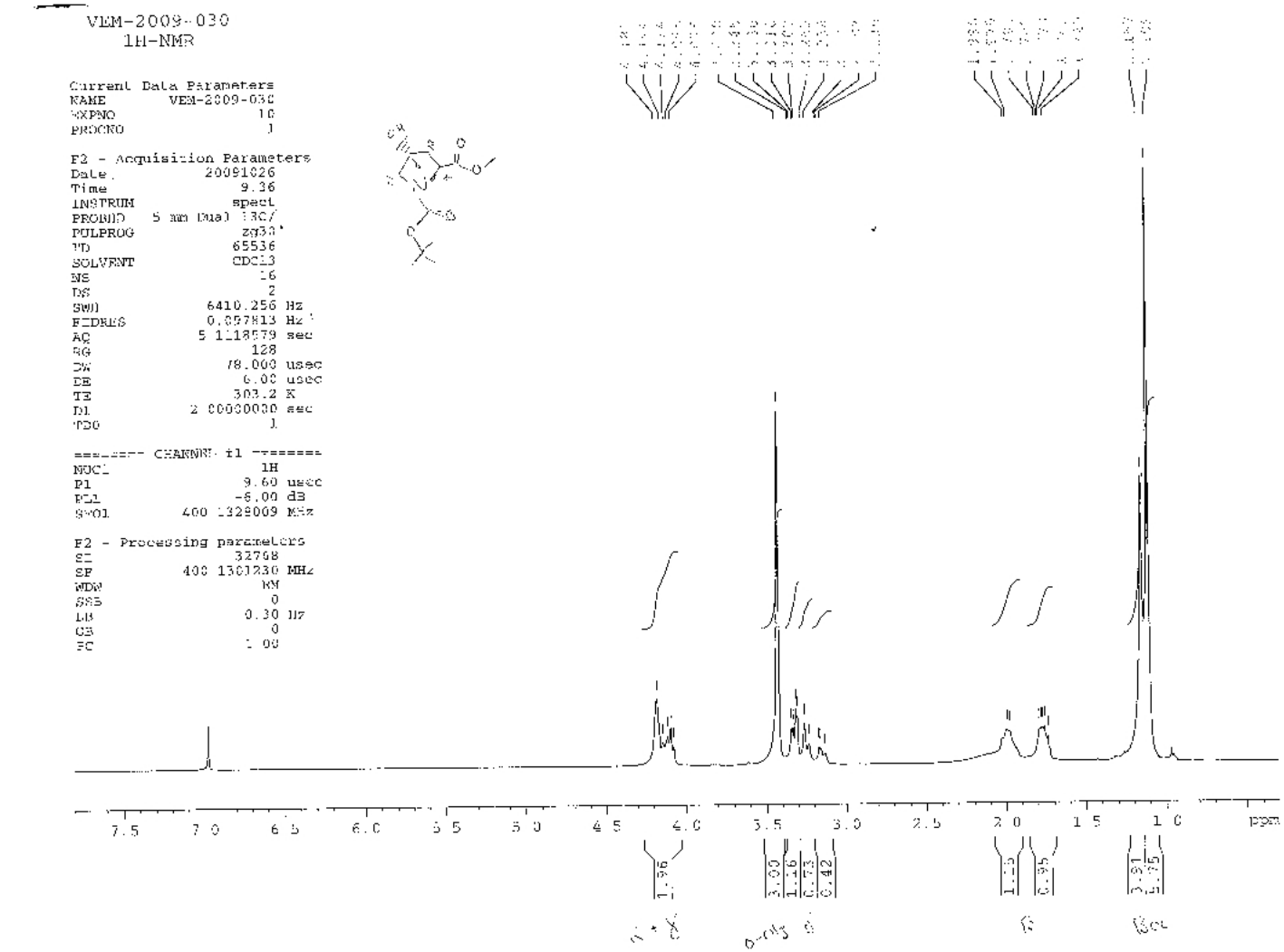
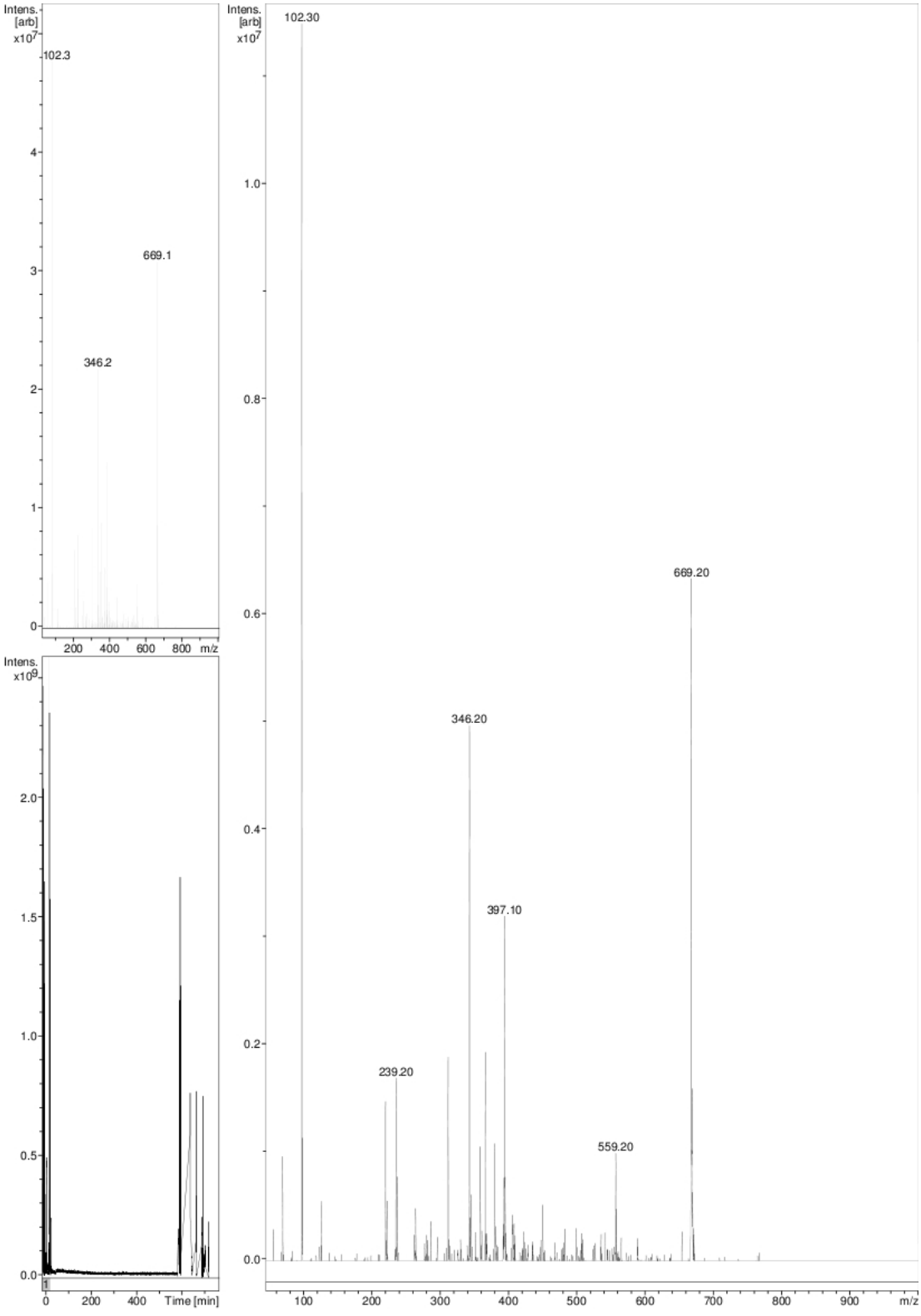
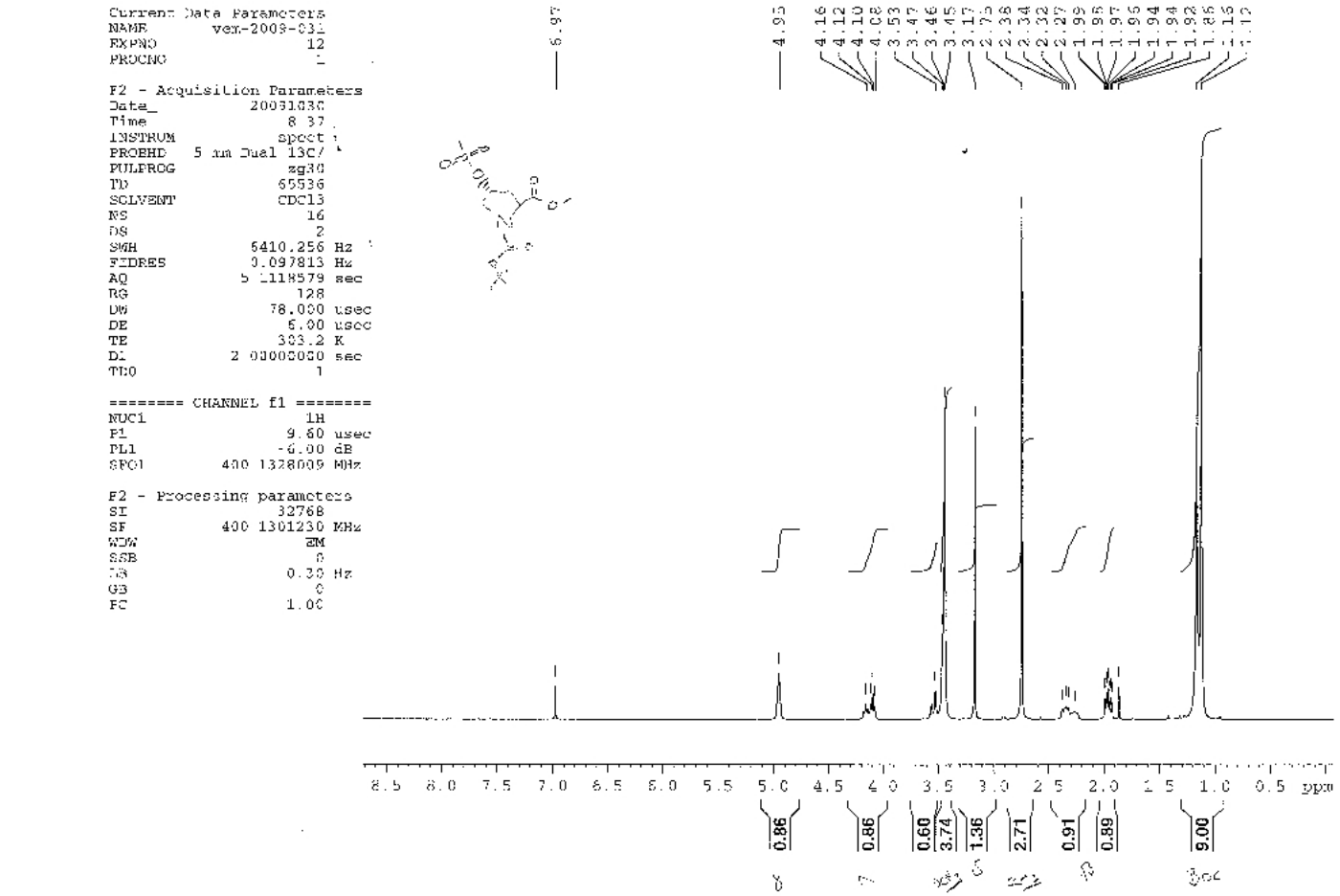
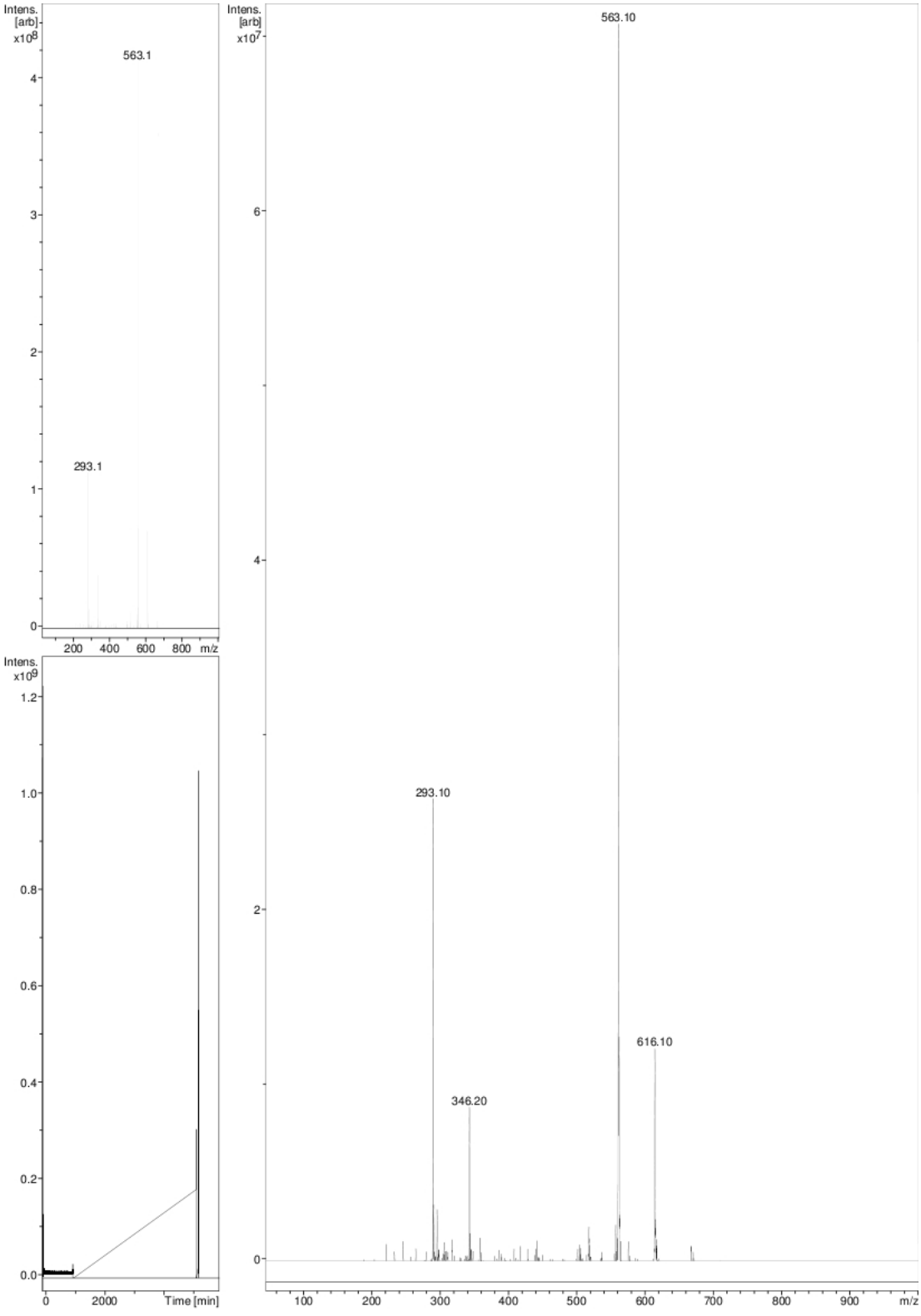

VEM-2009-030
1H-NMR
Current Data Parameters
NAME VEM-2009-030
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20091026
Time 9.36
INSTRUM spect
PROBHD 5 mm Dual 13C/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 6410.256 Hz
FIDRES 0.097813 Hz
AQ 5.1118079 sec
RG 128
DW 78.000 usec
DE 6.00 usec
TE 303.2 K
D1 2.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 9.60 usec
PL1 -6.00 dB
SFO1 400.1329009 MHz
F2 - Processing parameters
SI 32768
SF 400.1300230 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
1.96
3.00
1.16
0.43
0.42
1.00
0.65
3.91
1.95

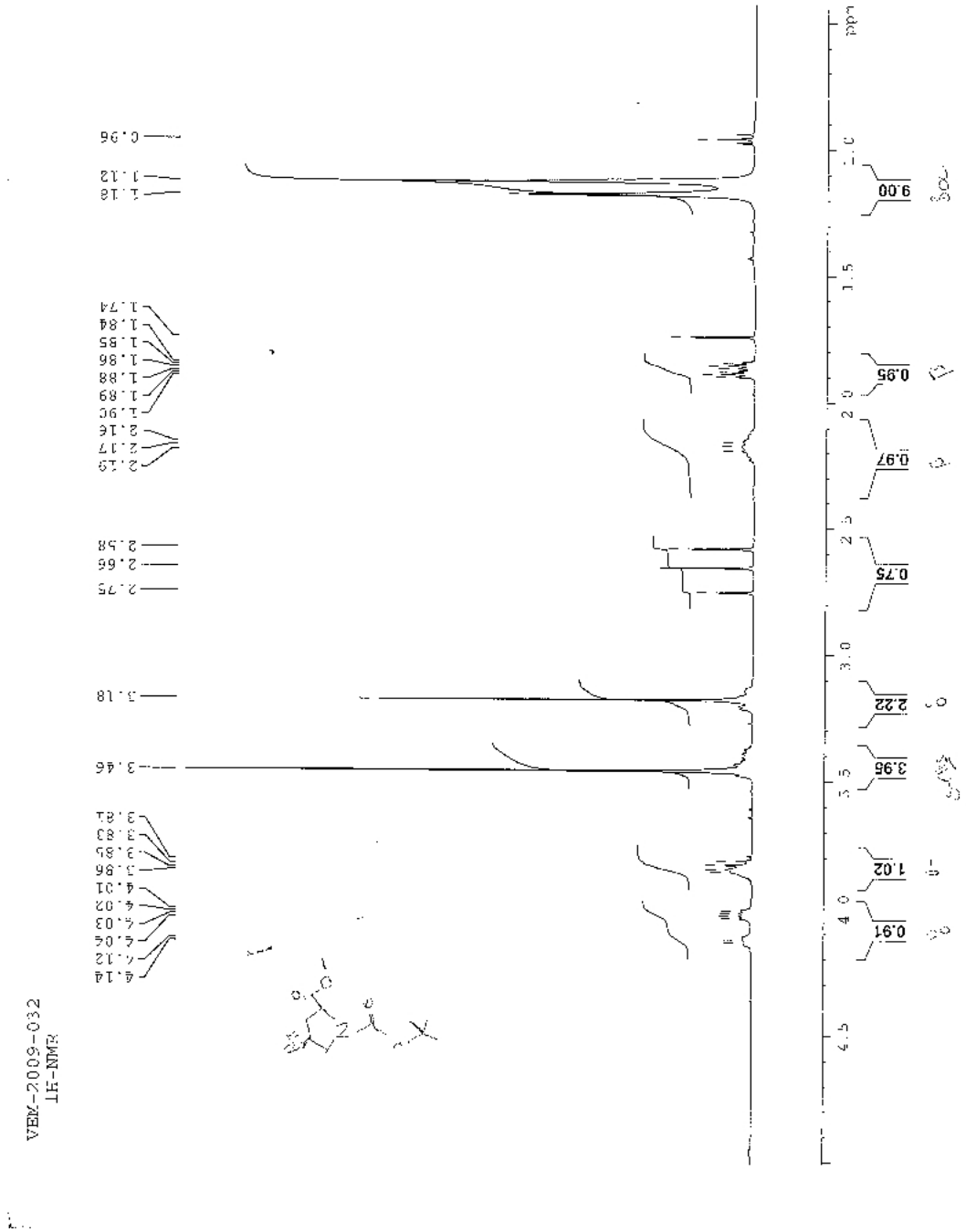

Intens.
[arb]
x10^7
102.3
669.1
346.2
200
400
600
800
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
102.30
669.20
346.20
397.10
239.20
559.20
100
200
300
400
500
600
700
800
900
m/z

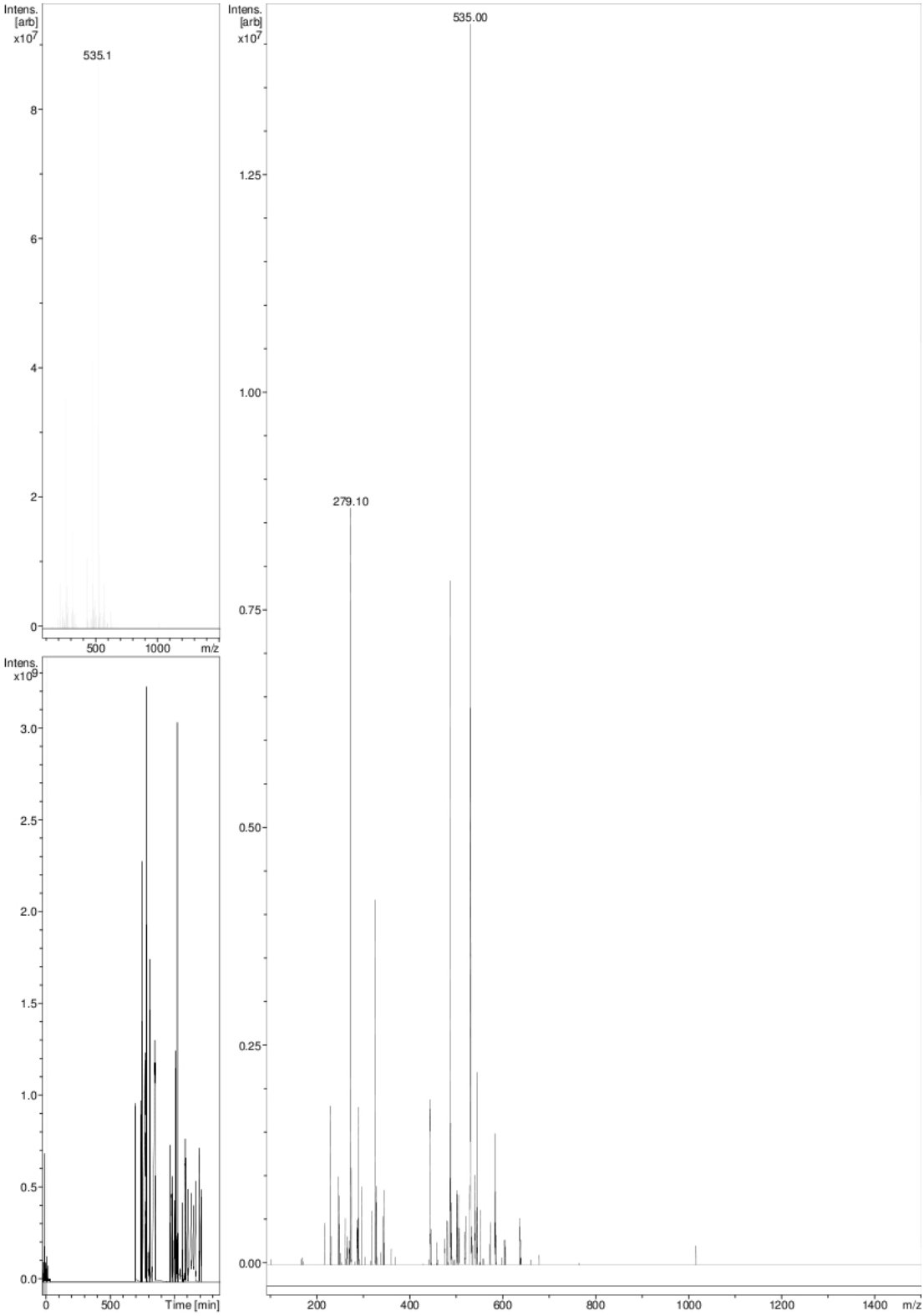
VEM-2009-31
1H-NMR

```
Current Data Parameters
NAME          vem-2009-31
EXPNO                  12
PROCNO                  1

F2 - Acquisition Parameters
Date_           20091030
Time                8.37
INSTRUM             spect
PROBHD   5 mm Dual 13C/
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             6410.256 Hz
FIDRES          0.097813 Hz
AQ              5.1118579 sec
RG                   128
DW                78.000 usec
DE                 6.00 usec
TE                303.2 K
D1           2.00000000 sec
TD0                    1

======== CHANNEL f1 ========
NUC1                  1H
P1                 9.60 usec
PL1               -6.00 dB
SFO1         400.1328009 MHz

F2 - Processing parameters
SI                 32768
SF          400.1301230 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00
```



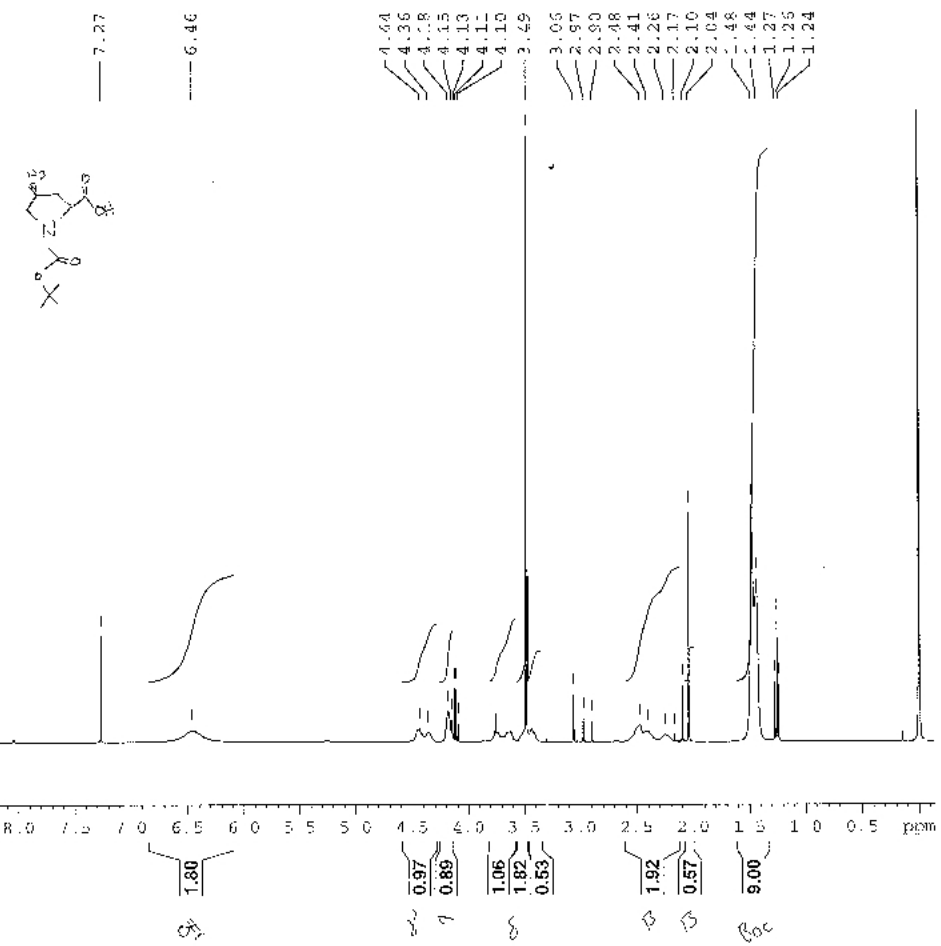

Intens.
[arb]
x10^8
563.1
293.1
200
400
600
800
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
563.10
293.10
616.10
346.20
100
200
300
400
500
600
700
800
900
m/z

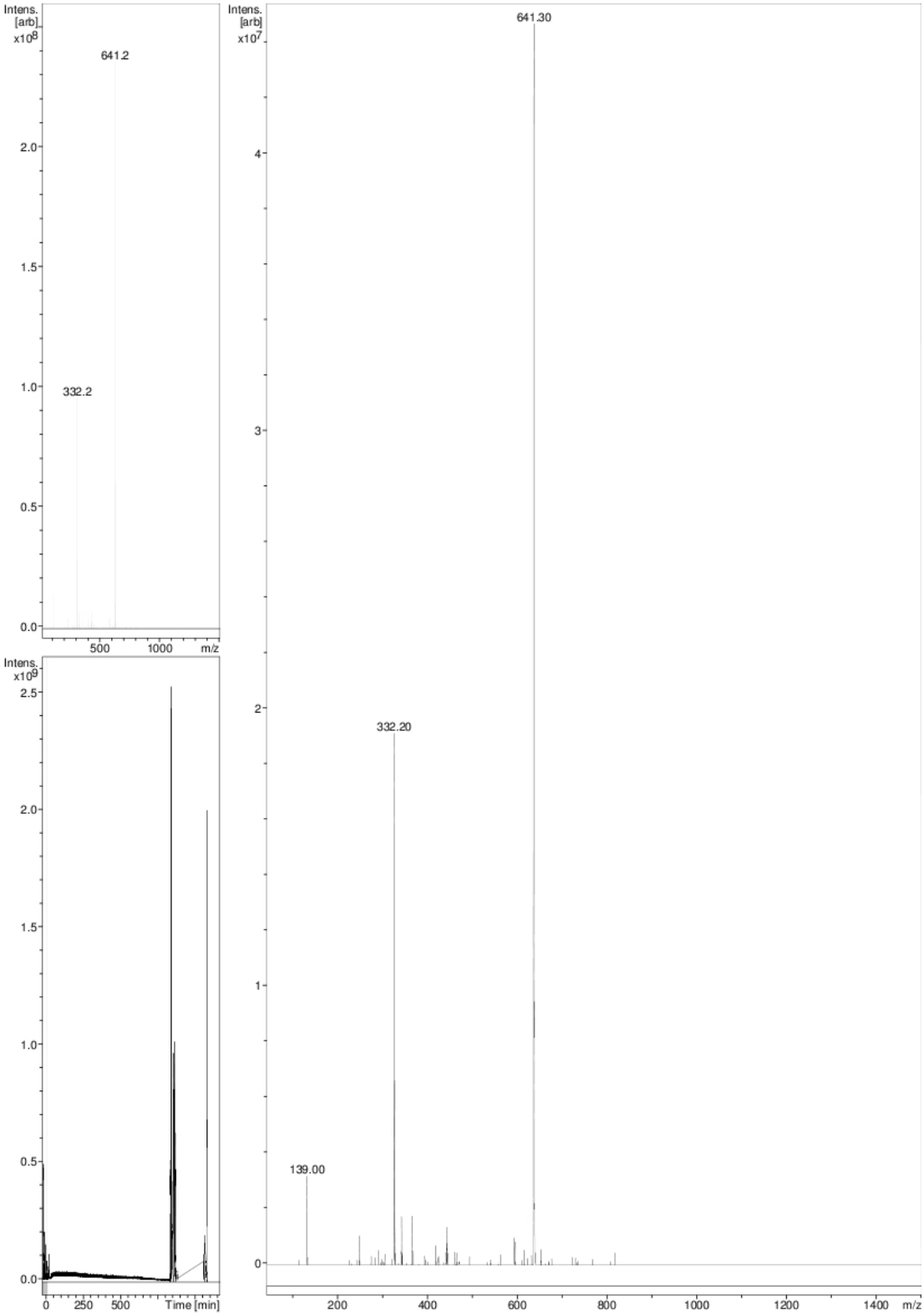

VBM-2009-032
1H-NMR
0.96
1.12
1.18
1.74
1.84
1.85
1.86
1.88
1.89
1.90
2.16
2.17
2.19
2.58
2.66
2.75
3.18
3.46
3.81
3.83
3.85
3.86
4.01
4.02
4.03
4.04
4.12
4.14
ppm
9.00
0.95
0.97
0.75
2.22
3.96
1.02
0.91


Intens.
[arb]
x10^7
535.1
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
535.00
279.10
m/z

VEM-2009-033
1H-NMR

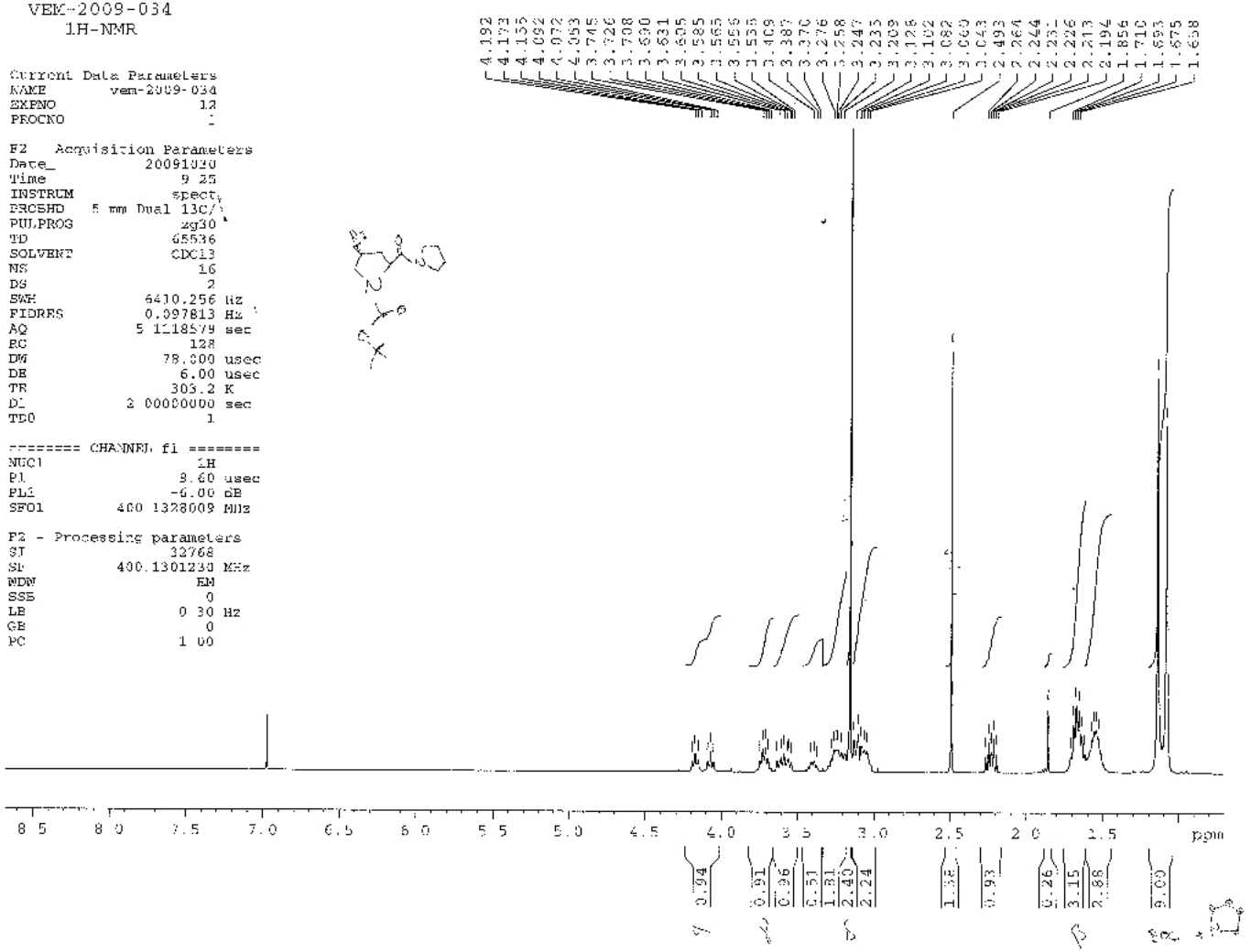
```
Current Data Parameters
NAME        vem-2009-033
EXPNO               12
PROCNO               1

F2 - Acquisition Parameters
Date_          20091030
Time              8.52
INSTRUM           spect
PROBHD   5 mm Dual 13C/
PULPROG           zg30
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   2
SWH           6410.256 Hz
FIDRES        0.097813 Hz
AQ            5.1118579 sec
RG                 128
DW               78.000 usec
DE                6.50 usec
TE              303.2 K
D1           2.00000000 sec
TD0                  1

======== CHANNEL f1 ========
NUC1                1H
P1                9.60 usec
PL1              -6.00 dB
SFO1        400.1328009 MHz

F2 - Processing parameters
SI                32768
SF          400.1300057 MHz
WDW                  EM
SSB                  0
LB                0.30 Hz
GB                   0
PC                1.00
```

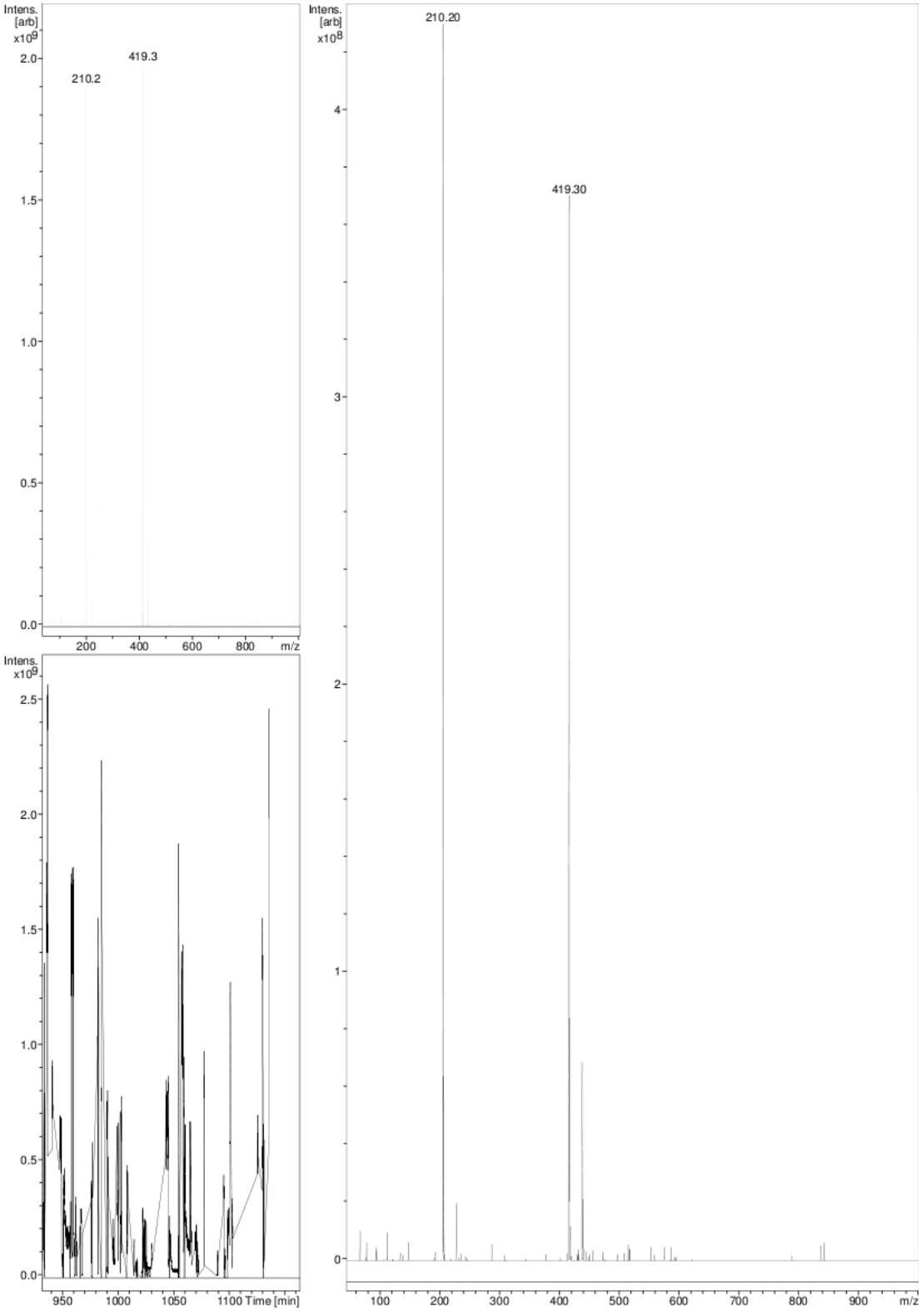


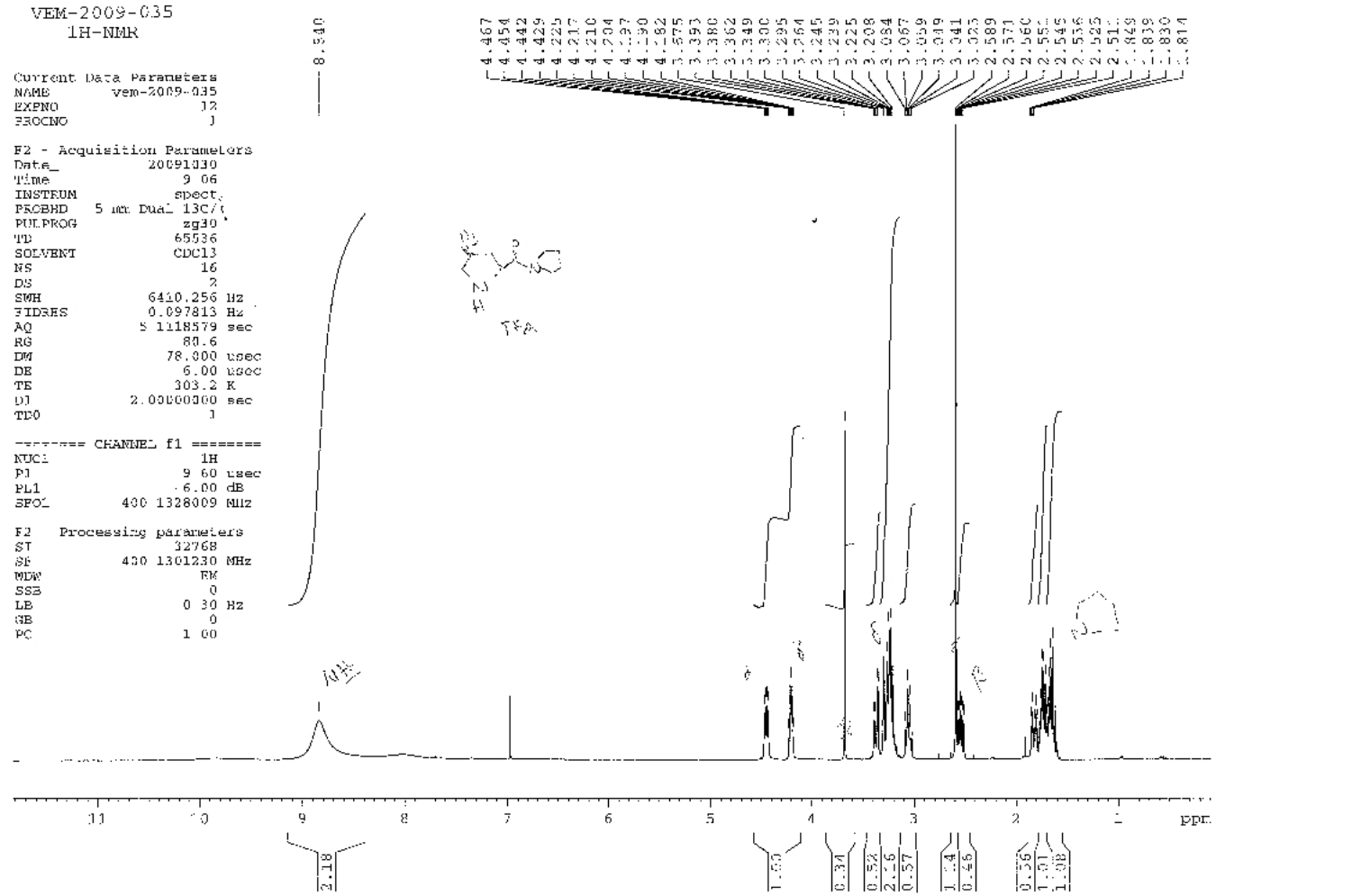

Intens.
[arb]
x10^8
641.2
332.2
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
641.30
332.20
139.00
m/z

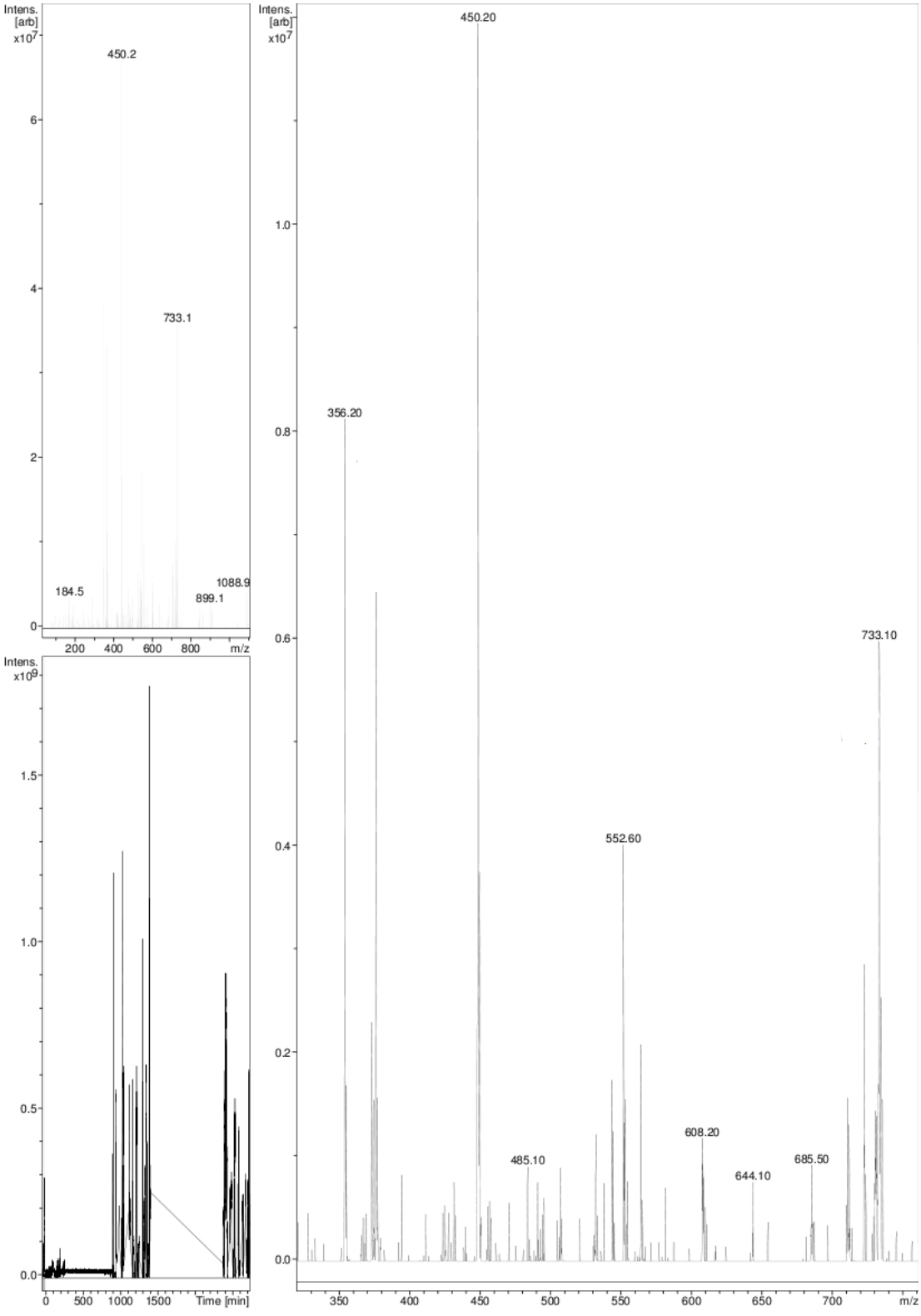
VEM-2009-034
1H-NMR

```
Current Data Parameters
NAME          vem-2009-034
EXPNO                   12
PROCNO                   1

F2 - Acquisition Parameters
Date_              20091020
Time                   9 25
INSTRUM                spect
PROBHD    5 mm Dual 13C/
PULPROG                zg30
TD                    65536
SOLVENT               CDCl3
NS                       16
DS                        2
SWH                6410.256 Hz
FIDRES             0.097813 Hz
AQ                 5.1118579 sec
RG                      128
DW                   78.000 usec
DE                    6.00 usec
TE                   303.2 K
D1                 2.00000000 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                     1H
P1                    9.60 usec
PL1                  -6.00 dB
SFO1          400.1328009 MHz

F2 - Processing parameters
SI                   32768
SF             400.1301230 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
```

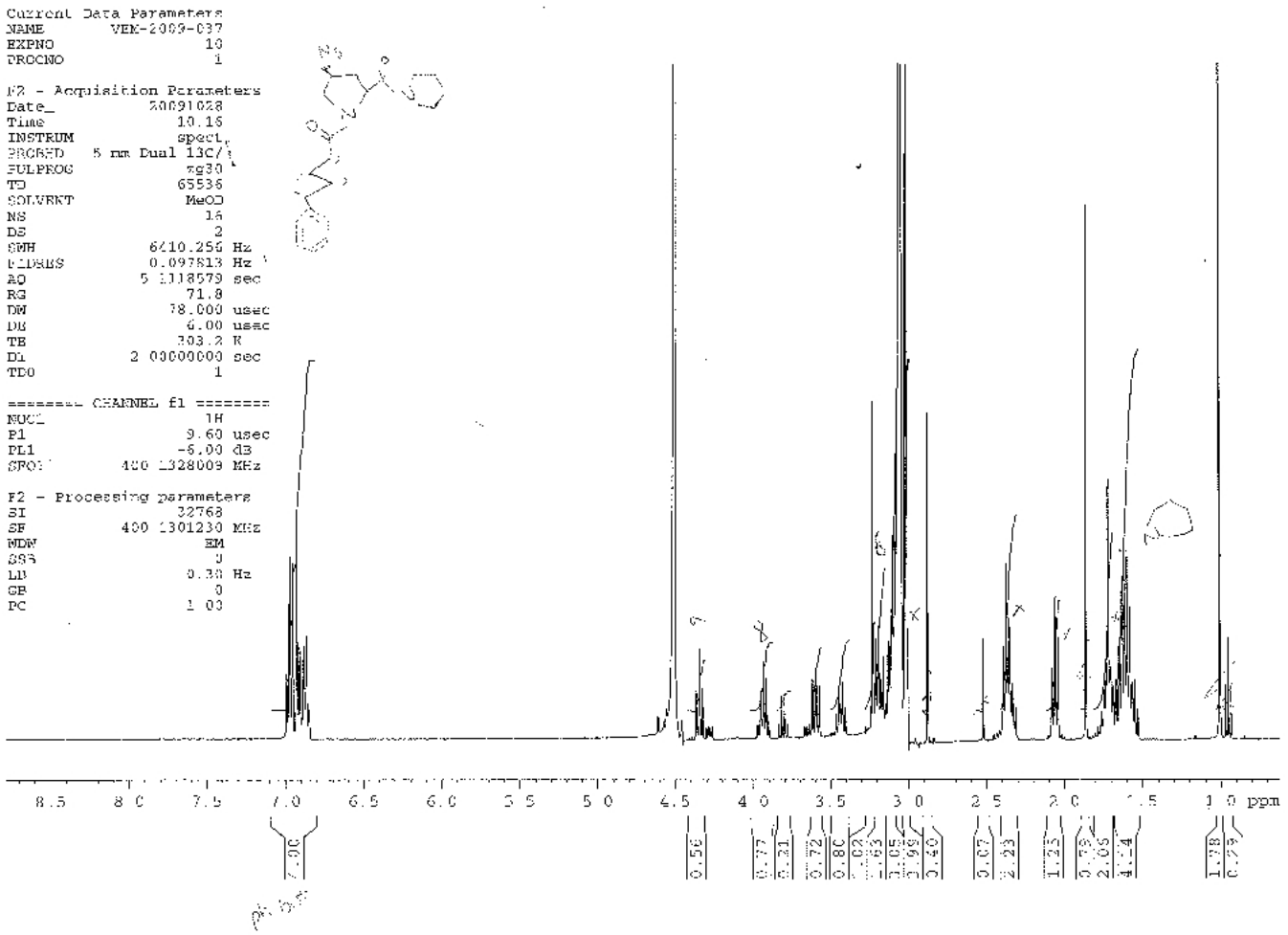


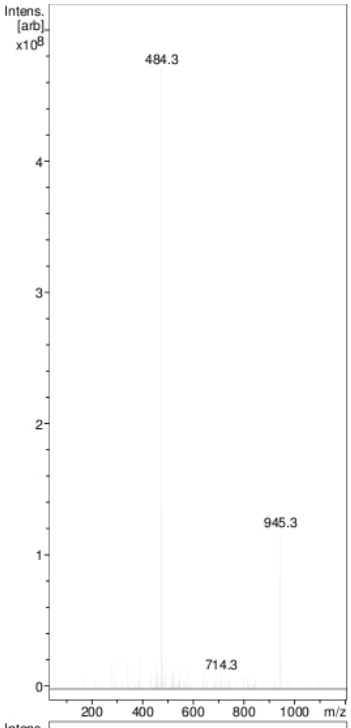

Intens.
[arb]
x10^9
2.0
1.5
1.0
0.5
0.0
419.3
210.2
200
400
600
800
m/z
Intens.
x10^9
2.5
2.0
1.5
1.0
0.5
0.0
950
1000
1050
1100 Time [min]
Intens.
[arb]
x10^8
4
3
2
1
0
210.20
419.30
100
200
300
400
500
600
700
800
900
m/z

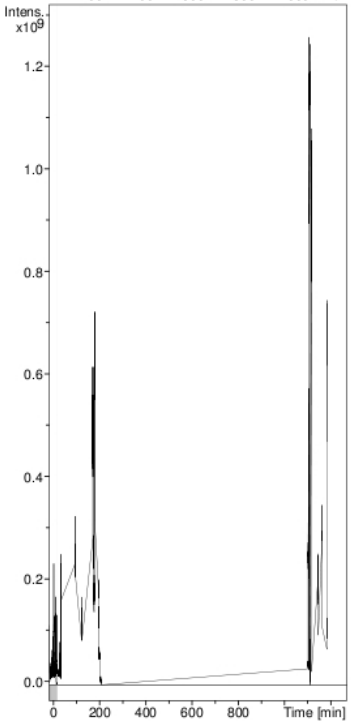

VEM-2009-035
1H-NMR
Current Data Parameters
NAME vem-2009-035
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20091030
Time 9 06
INSTRUM spect
PROBHD 5 mm Dual 13C/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 6410.256 Hz
FIDRES 0.097813 Hz
AQ 5.1118579 sec
RG 80.6
DW 78.000 usec
DE 6.00 usec
TE 303.2 K
D1 2.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 9.60 usec
PL1 -6.00 dB
SFO1 400.1328009 MHz
F2 - Processing parameters
SI 32768
SF 400.1301230 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
8.540
4.467
4.454
4.442
4.429
4.225
4.217
4.210
4.204
4.197
4.190
4.182
3.675
3.393
3.380
3.362
3.349
3.300
3.295
3.264
3.245
3.239
3.225
3.208
3.084
3.067
3.059
3.049
3.041
3.025
2.589
2.571
2.560
2.555
2.545
2.536
2.525
2.518
1.843
1.839
1.830
1.814
NH
ppm
2.18
1.00
6.34
0.52
2.16
0.57
1.74
0.46
0.36
1.01
1.08

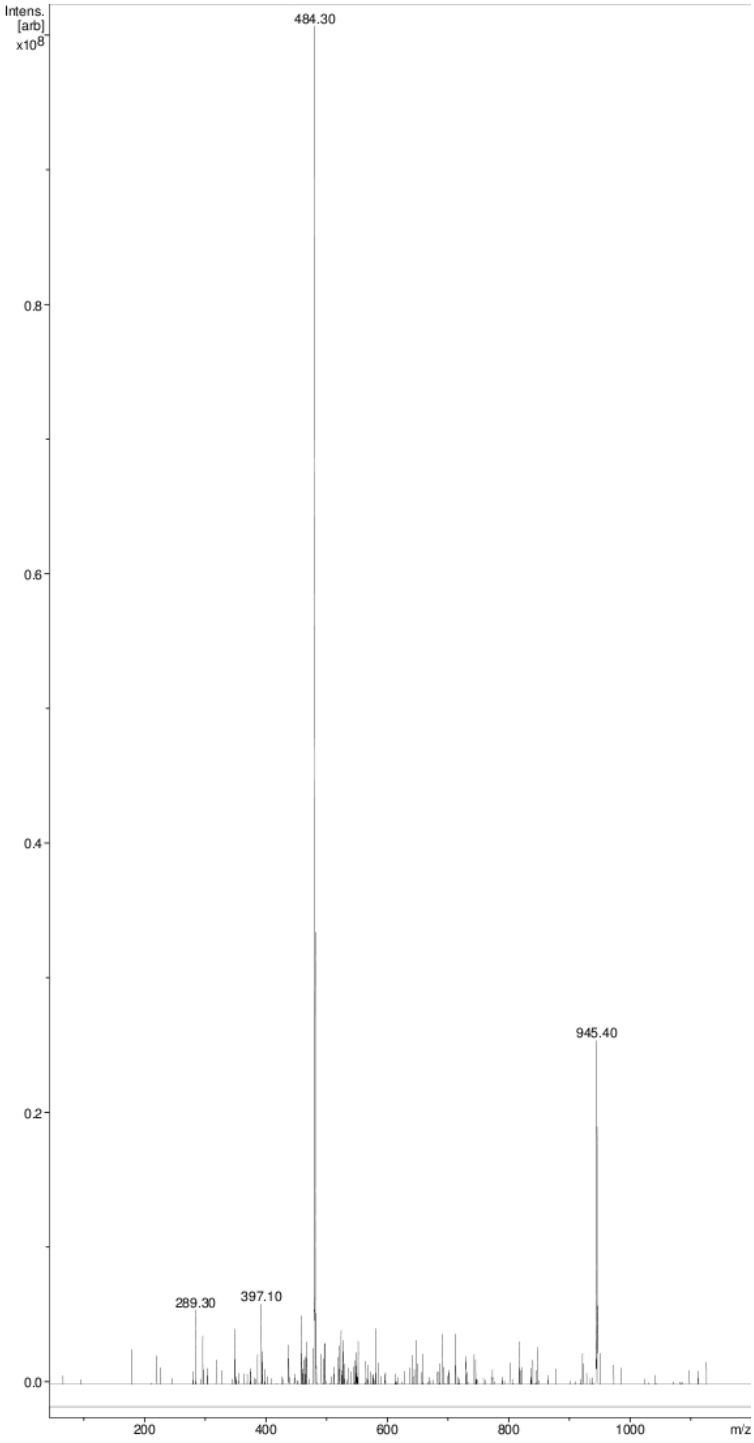

Intens.
[arb]
x10^7
450.2
733.1
184.5
899.1
1088.9
200
400
600
800
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
450.20
356.20
733.10
552.60
608.20
485.10
644.10
685.50
350
400
450
500
550
600
650
700
m/z

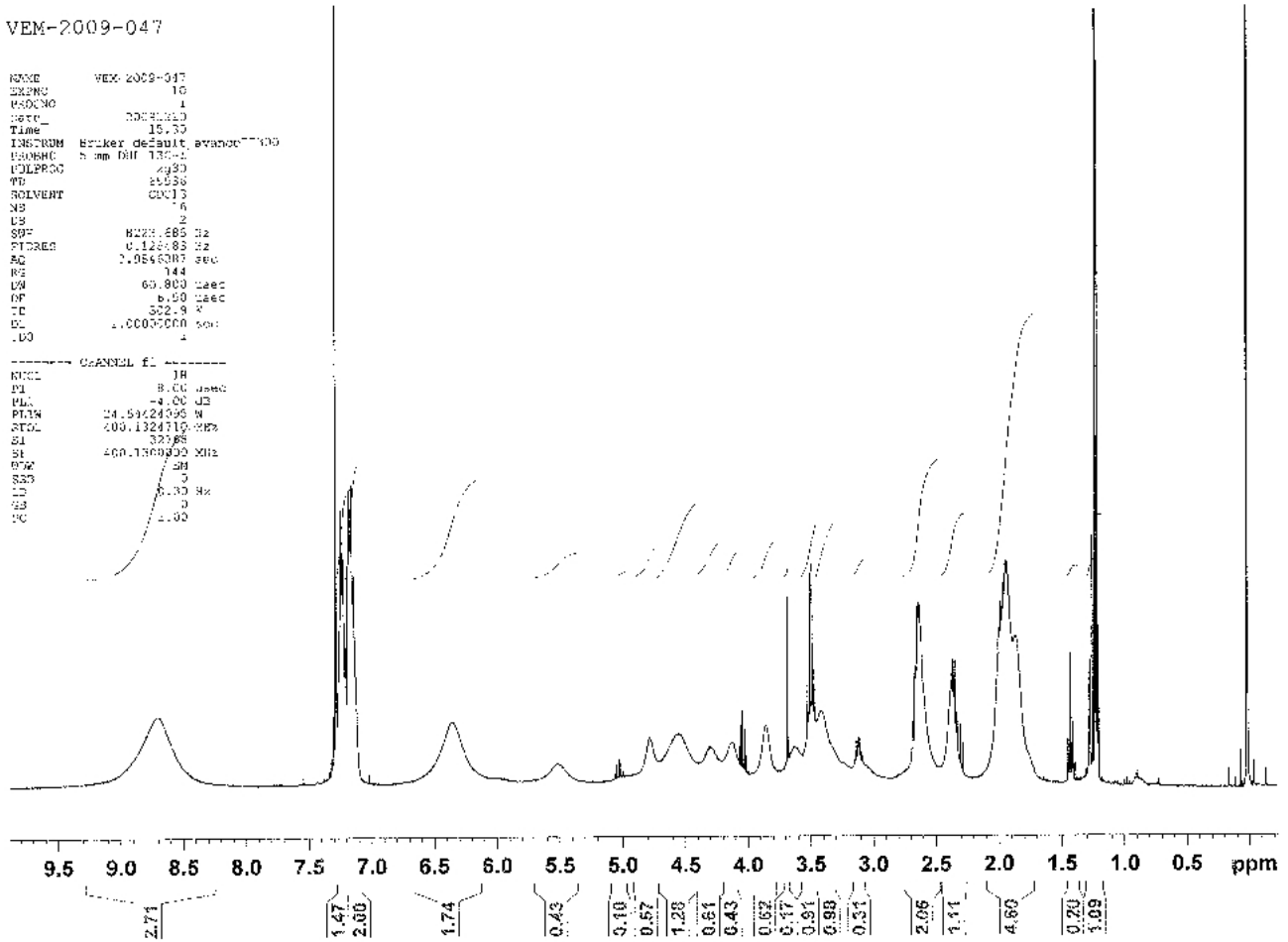
VEM-2009-037
1H NMR




Intens.
[arb]
x10^8
484.3
945.3
714.3
m/z


Intens.
x10^9
Time [min]


Intens.
[arb]
x10^8
484.30
945.40
289.30
397.10
m/z


VEM-2009-047
NAME VEM-2009-047
EXPNO 10
PROCNO 1
Time 15.33
INSTRUM Bruker default avance
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 8223.685 Hz
FIDRES 0.125483 Hz
AQ 3.9846080 sec
RG 144
DW 60.800 usec
DE 6.50 usec
TE 302.9 K
D1 1.00000000 sec
1.00 1
======== CHANNEL f1 ========
NUC1 1H
P1 8.00 usec
PL1 -4.00 dB
PL1W 24.59424896 W
SFO1 200.1324710 MHz
SI 32768
SF 200.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
2.71
1.47
2.06
1.74
0.43
0.19
0.57
1.28
0.61
6.43
0.62
0.17
0.91
0.98
0.31
2.06
1.11
4.60
0.20
1.09

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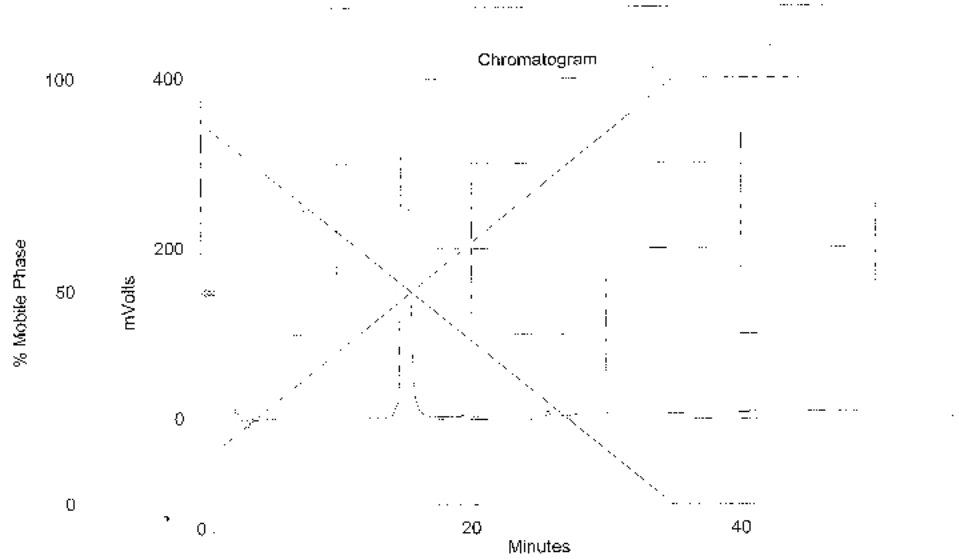
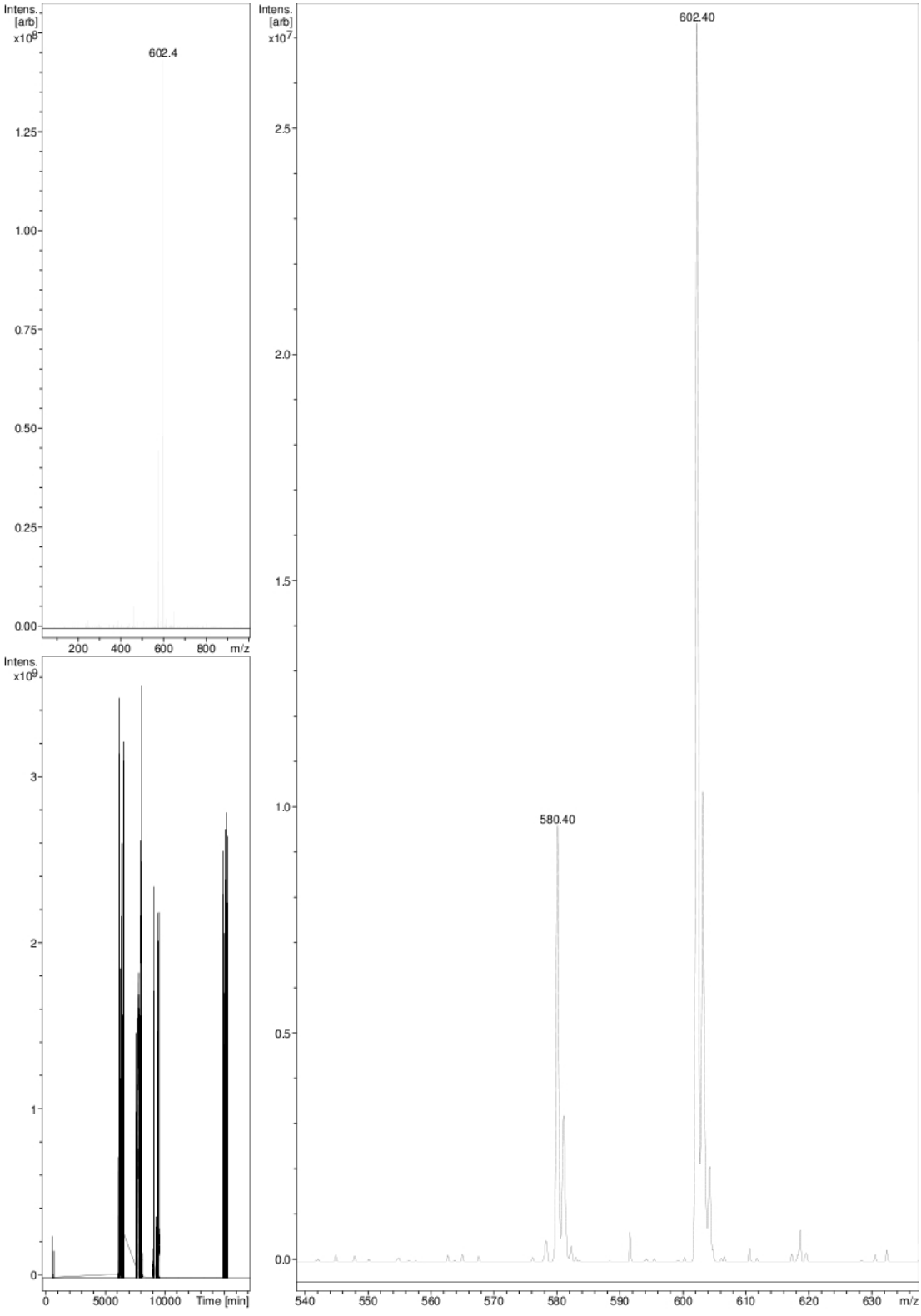


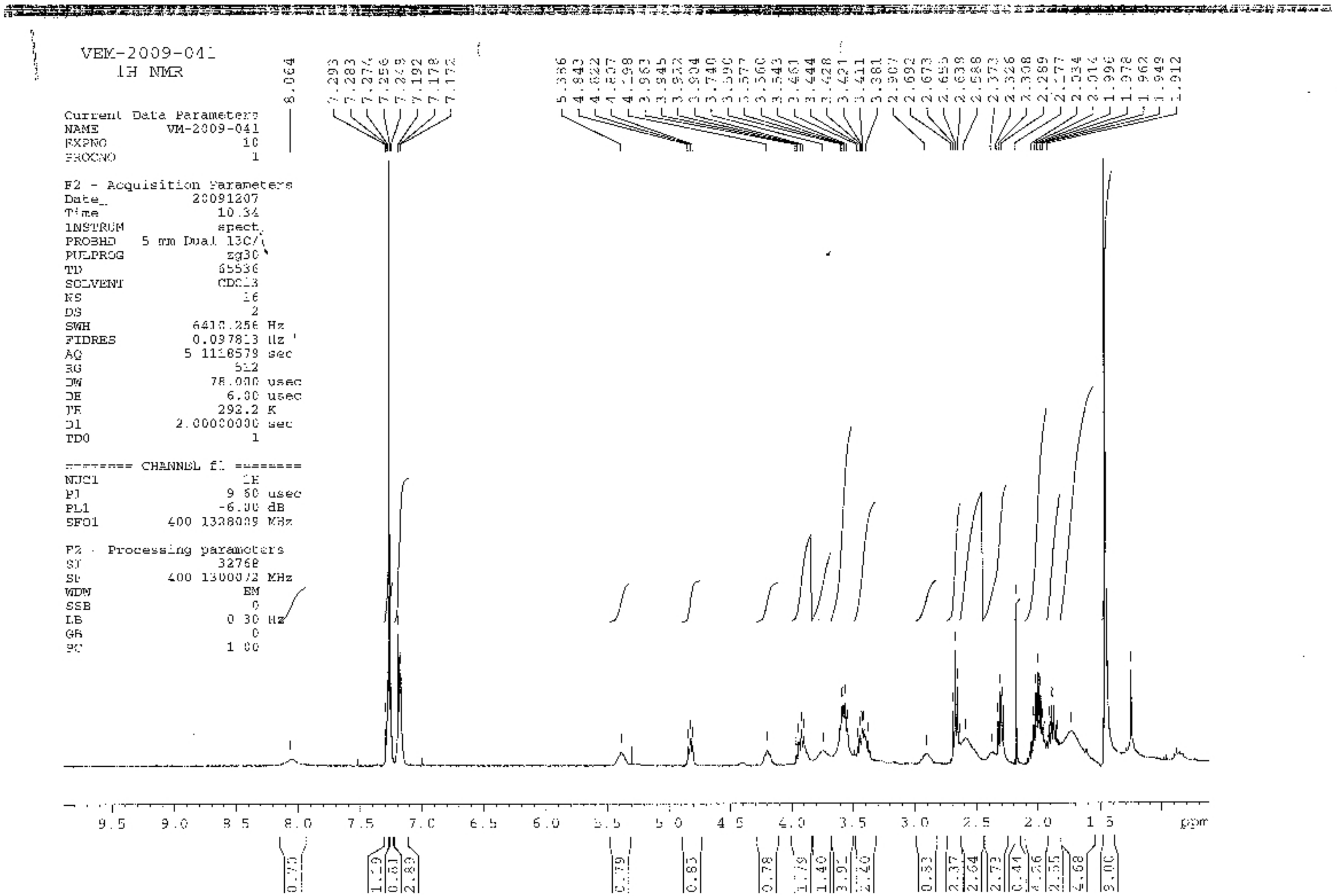
c:\gilson\unipoint\system1\gunther\gb101209.001\gb101209.gdt : 118, kanaal 1 : VEM-2009-047: Inj. Number: 7

ater

CN

Data File: c:\gilson\unipoint\system1\gunther\gb101209.001\gb101209.gdt
Date acquired: Thu Dec 10 2009 20:42:01
Control Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GCT
Sample name: VEM-2009-047


Intens.
[arb]
x10^8
602.4
1.25
1.00
0.75
0.50
0.25
0.00
200
400
600
800
m/z
Intens.
x10^9
3
2
1
0
0
5000
10000
Time [min]
Intens.
[arb]
x10^7
602.40
580.40
2.5
2.0
1.5
1.0
0.5
0.0
540
550
560
570
580
590
600
610
620
630
m/z


VEM-2009-041
1H NMR
Current Data Parameters
NAME VM-2009-041
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20091207
Time 10.34
INSTRUM spect
PROBHD 5 mm Dual 13C/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 6410.256 Hz
FIDRES 0.097813 Hz
AQ 5.1118579 sec
RG 512
DW 78.000 usec
DE 6.00 usec
TE 292.2 K
D1 2.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 9.60 usec
PL1 -6.00 dB
SFO1 400.1328009 MHz
F2 - Processing parameters
SI 32768
SF 400.1300072 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
8.064
7.293
7.283
7.274
7.256
7.249
7.192
7.178
7.172
5.386
4.843
4.822
4.807
4.198
3.963
3.945
3.932
3.904
3.740
3.590
3.577
3.560
3.543
3.461
3.444
3.428
3.421
3.411
3.381
2.907
2.692
2.673
2.655
2.639
2.588
2.373
2.326
2.308
2.289
2.177
2.034
2.014
1.996
1.978
1.962
1.949
1.912
ppm

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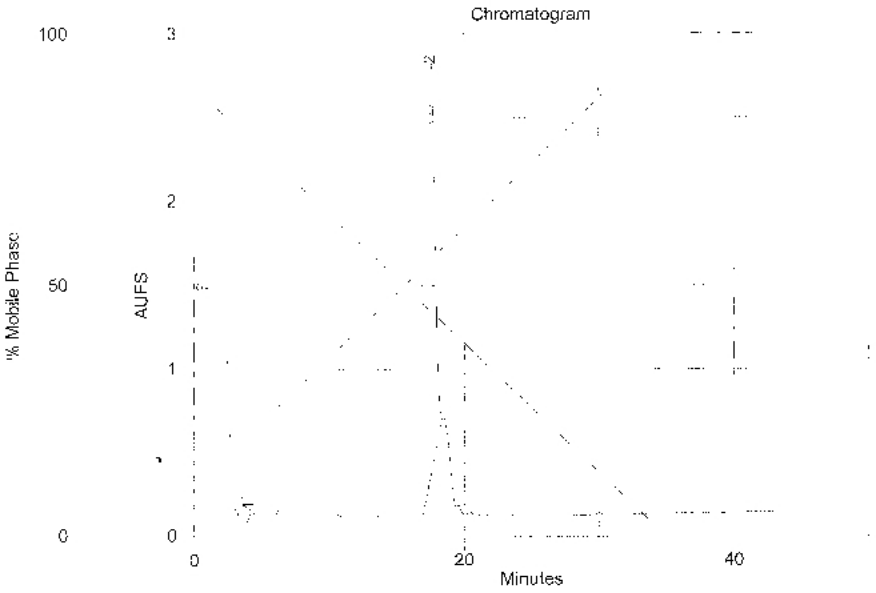


> c:\gilson\unipoint\system1\venkat\171109.001\171109.gdt : 118, kanaal 1 : VEM-2009-041: Inj. Number: 6

Water

ACN

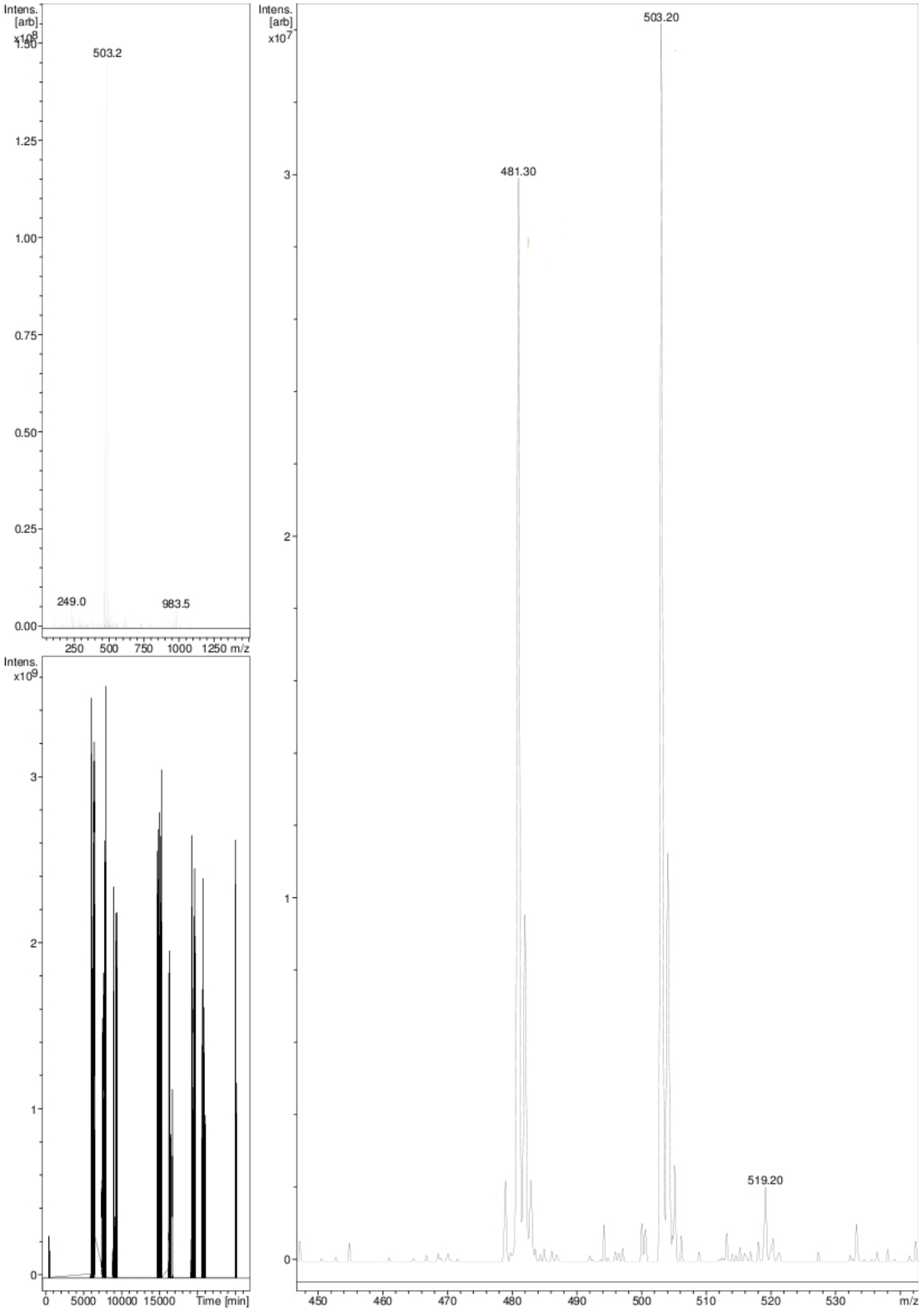
	Inj. Number	Peak Name	R. Time	Area	Area %	Sample Descrip.
1	6.00		4.11	39078.62	0.35	EM-2009-04
2	6.00	2	17.59	11103514.00	99.65	EM-2009-04

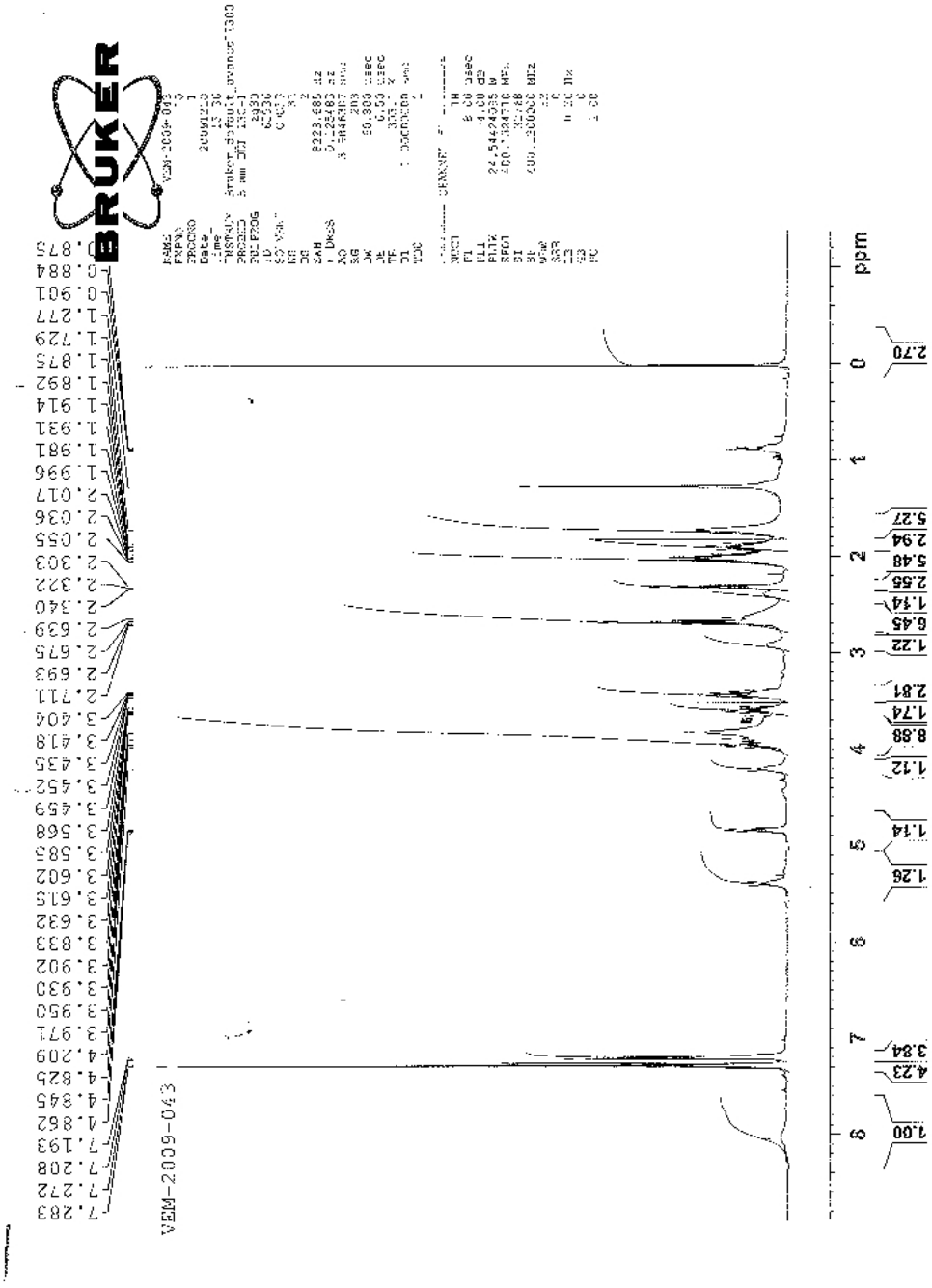
Data File: c:\gilson\unipoint\system1\venkat\171109.001\171109.gdt
Date acquired: Tue Nov 17 2009 16:55:36
Control Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GCT
Analysis Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GAN
Sample name: VEM-2009-041 Injection Number: 6 "Unknown"
Analyzed on: Wed Nov 18 2009 09:18:13

Analysis Method Events

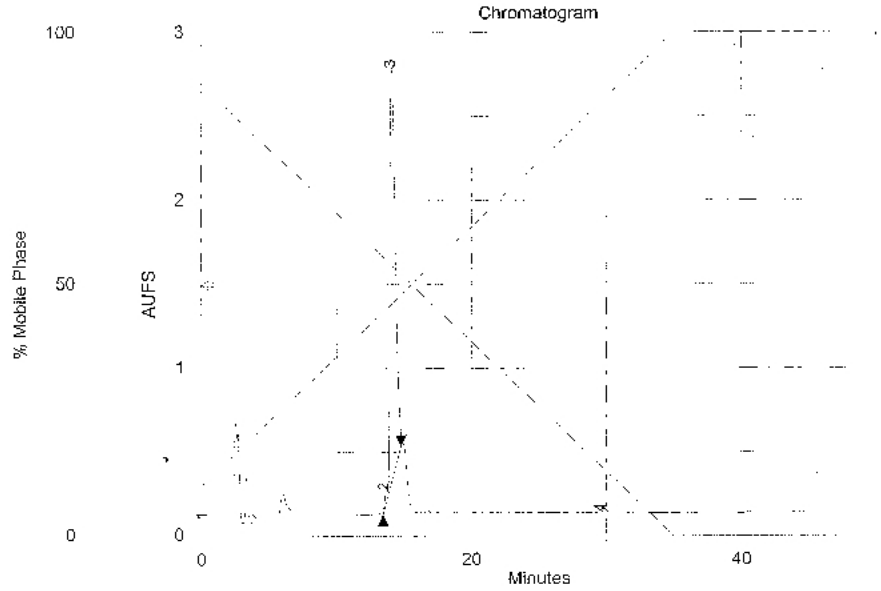
0	Inhibit Integration	
0	Disable Negative Peak Integration	initial default
0	Peak Width 0.1	initial default
0	Peak Sensitivity 20	initial default
0	Default Baseline	initial default
3.80	Enable Integration	
36.00	Inhibit Integration	

Channel Scales
<Auto range>
118, kanaal 1 0.00 to 100.00 mVolts (10 %offset), Data rate: 20.00 (points/second) <Analysis Channel>


Intens.
[arb]
x10^8
503.2
249.0
983.5
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
503.20
481.30
519.20
m/z


BRUKER
VEM-2009-043
ppm
0
1
2
3
4
5
6
7
8
2.70
5.27
2.94
5.48
2.55
1.14
6.45
1.22
2.81
1.74
8.88
1.12
1.14
1.26
3.84
4.23
1.00

Fri Nov 20 2009 09:08:24 Results Report Page 1



> c:\gilson\unipoint\system1\venkat\191109.001\191109.gdt : 118, kanaal 1 : VEM-2009-043: Inj. Number: 8

Water

ACN

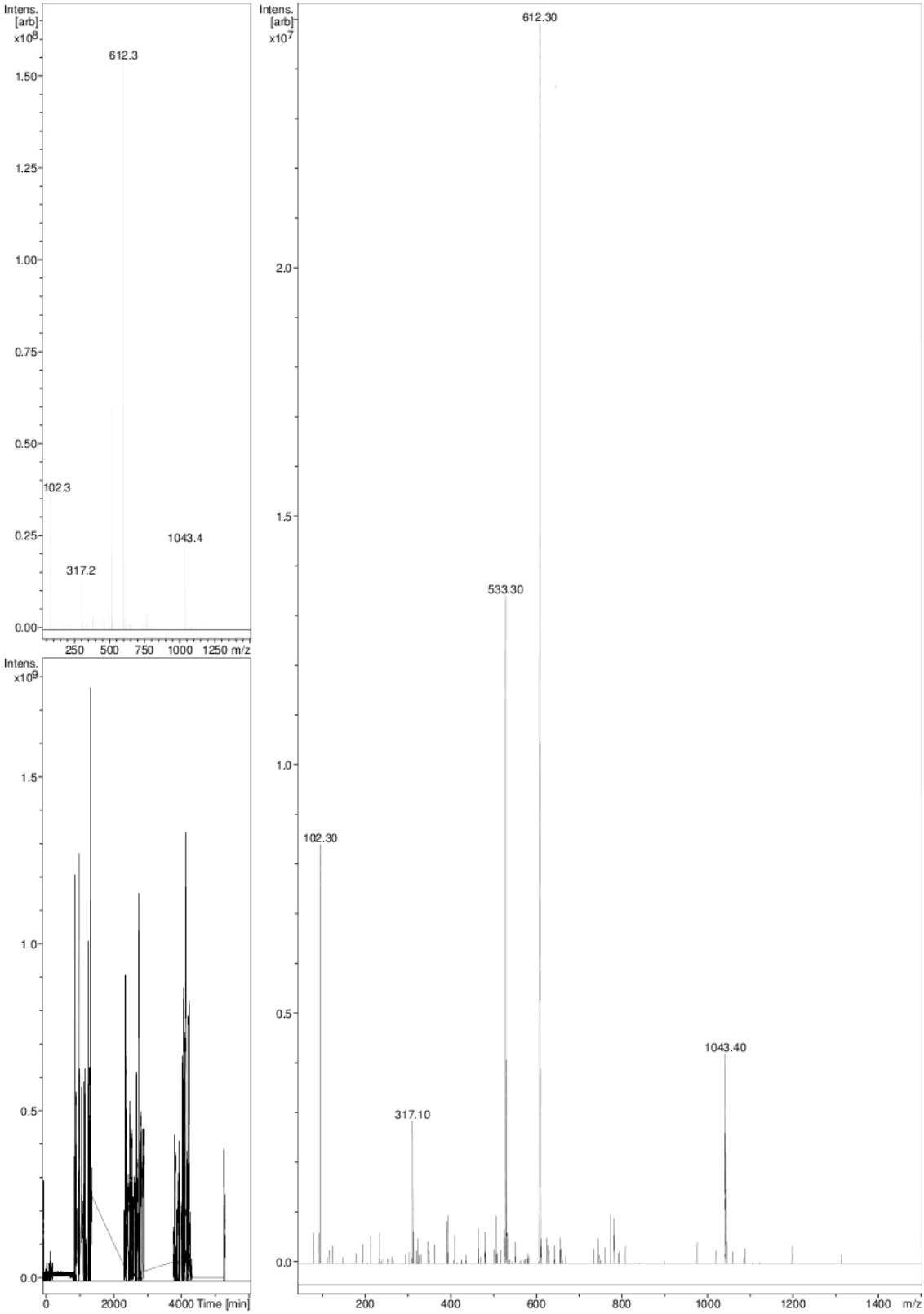
Inj. Number	Peak Name	R. Time	Area	Area %	Sample Descrip
1	8.00	3	14.08	11549494.00	100.00 EM-2009-04

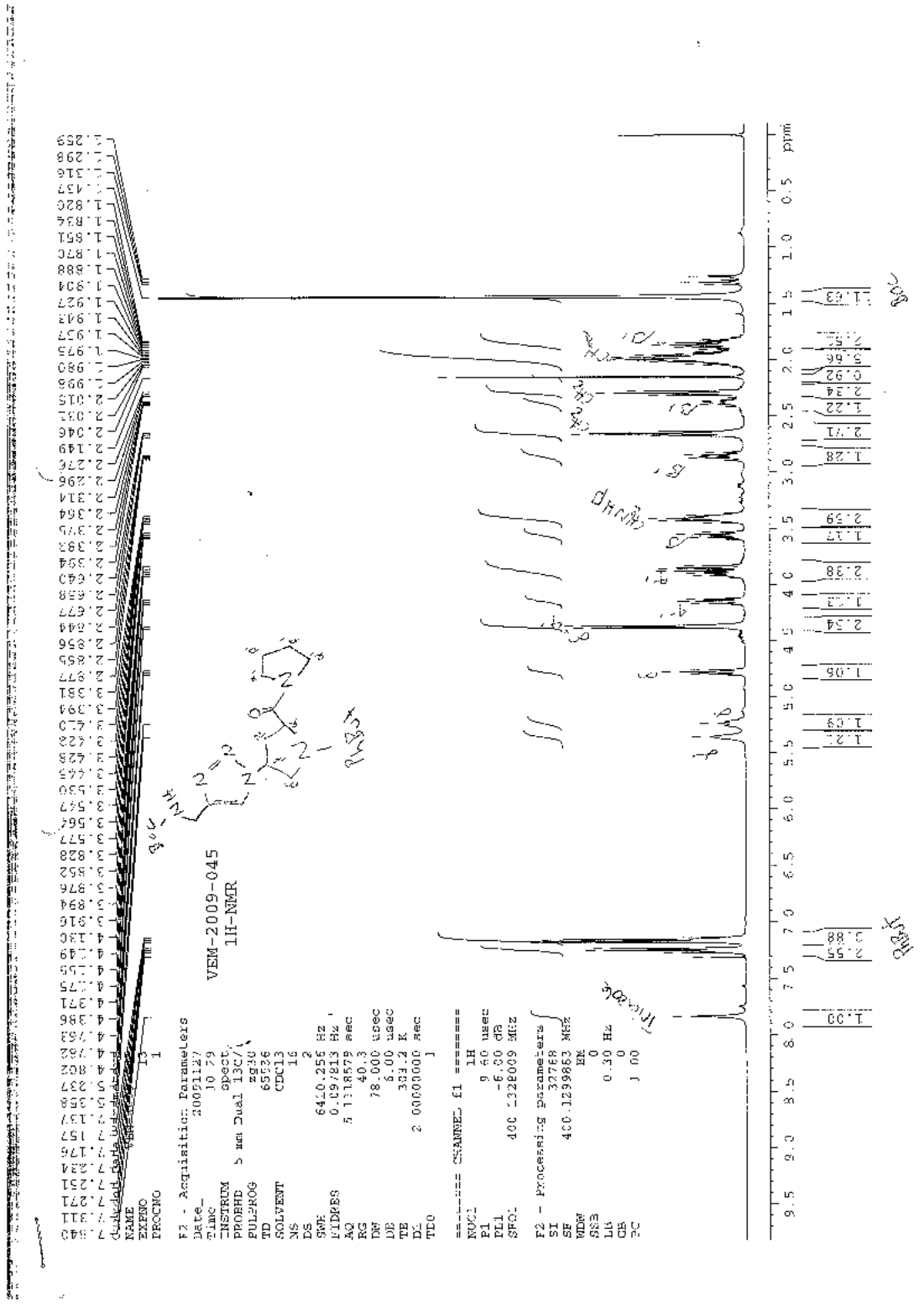
Data File: c:\gilson\unipoint\system1\venkat\191109.001\191109.gdt
Date acquired: Thu Nov 19 2009 21:36:35
Control Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GCT
Analysis Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GAN
Sample name: VEM-2009-043 Injection Number: 8 "Unknown"
Analyzed on: Fri Nov 20 2009 09:08:13
<Peak baselines manually adjusted>

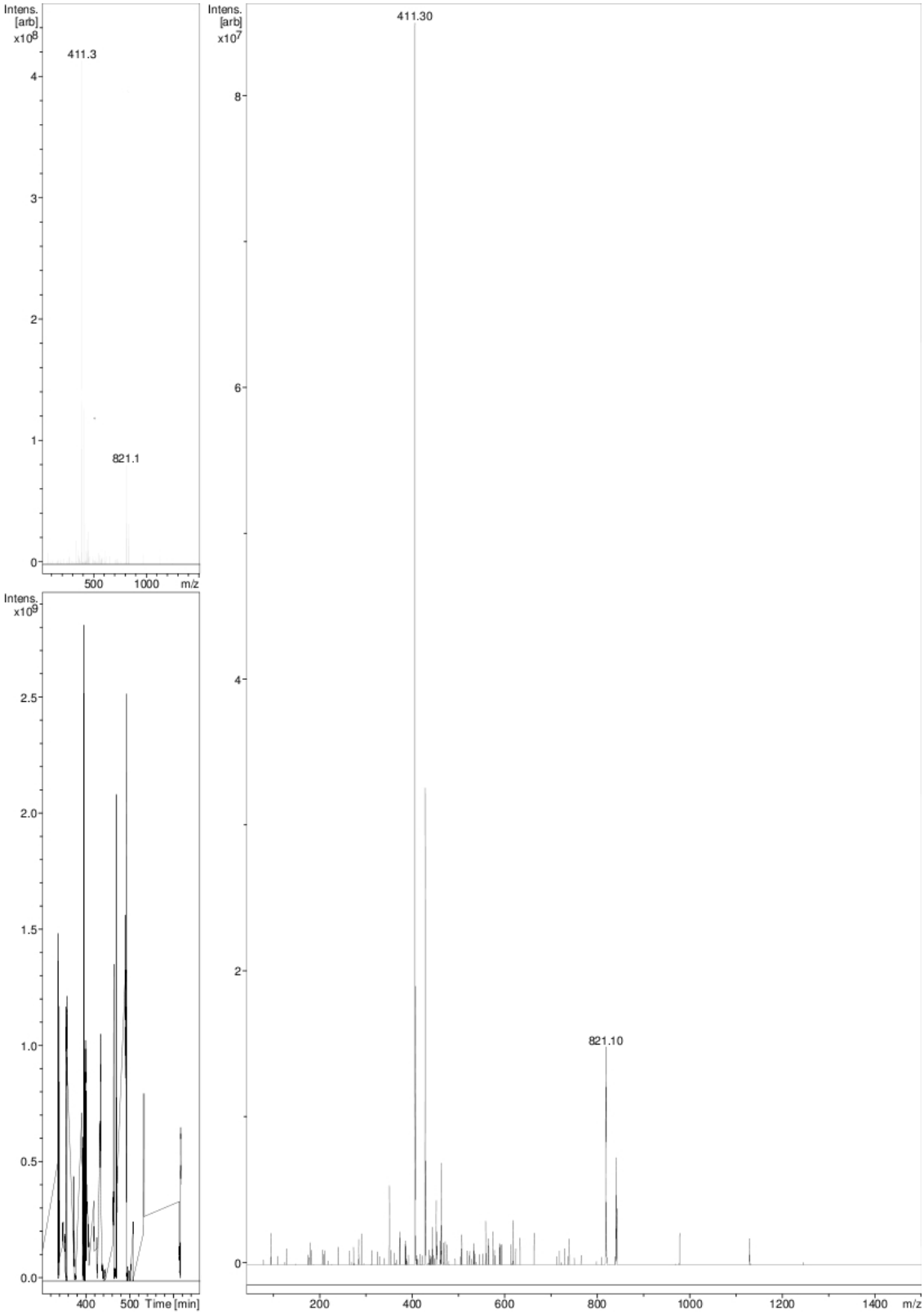
Analysis Method Events

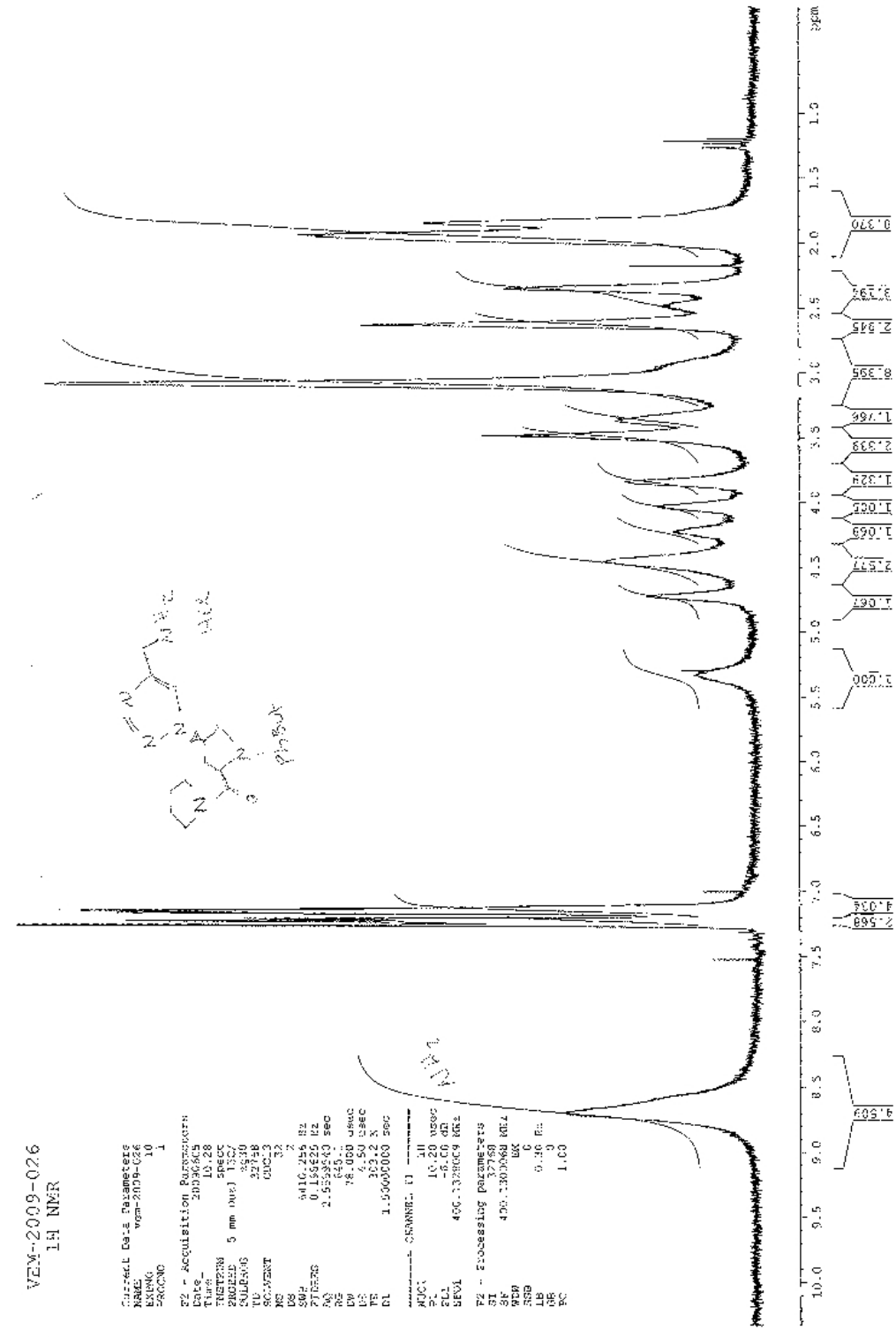
0	Inhibit Integration	
0	Disable Negative Peak Integration	initial default
0	Peak Width 0.2	initial default
0	Peak Sensitivity 20	initial default
0	Default Baseline	initial default
3.80	Enable Integration	
36.00	Inhibit Integration	

Channel Scales
<Auto range>
118, kanaal 1 0.00 to 100.00 mVolts (10 %offset), Data rate: 20.00 (points/second) <Analysis Channel>

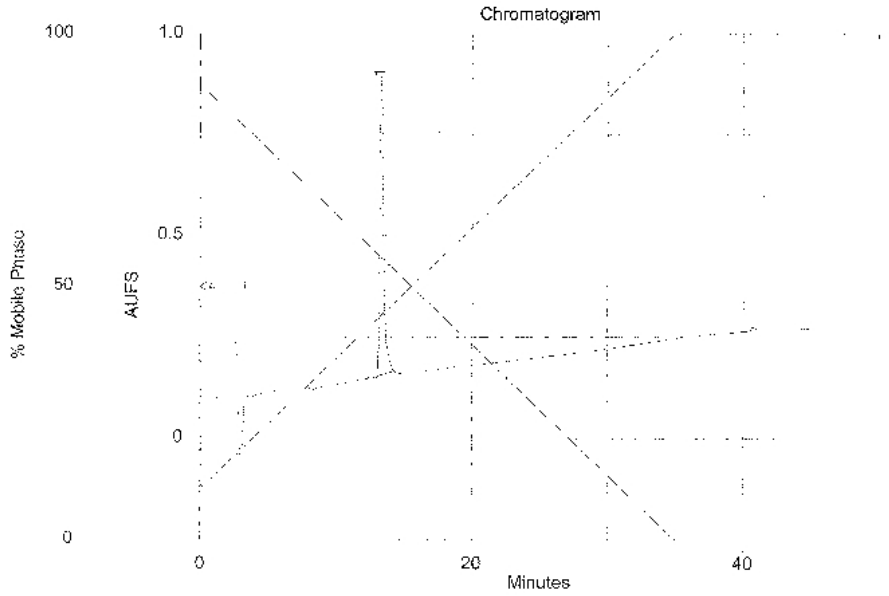

Intens.
[arb]
x10^8
612.3
1.50
1.25
1.00
0.75
0.50
102.3
0.25
1043.4
317.2
0.00
250 500 750 1000 1250 m/z
Intens.
x10^9
1.5
1.0
0.5
0.0
0 2000 4000 Time [min]
Intens.
[arb]
x10^7
612.30
2.0
1.5
533.30
1.0
102.30
0.5
1043.40
317.10
0.0
200 400 600 800 1000 1200 1400 m/z


VEM-2009-045
1H-NMR
F2 - Acquisition Parameters
Date_ 20091127
Time 10.29
INSTRUM spect
PROBHD 5 mm Dual 13C/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 6410.256 Hz
FIDRES 0.097813 Hz
AQ 5.1118579 sec
RG 40.3
DW 78.000 usec
DE 6.00 usec
TE 303.2 K
D1 2.00000000 sec
TD0 1
F2 - Processing parameters
SI 32768
SF 400.1299863 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
ppm


Intens.
[arb]
x10^8
411.3
821.1
m/z
Intens.
x10^9
Time [min]
Intens.
[arb]
x10^7
411.30
821.10
m/z


VEM-2009-026
1H NMR

Fri Jun 12 2009 13:36:18 Results Report Page 1



:\gilson\unipoint\system1\gunther\gb110609.001\gb110609.gdt : 118, kanaal 1 : VEM-2009-026: Inj. Number: 23
ter
N

Inj. Number	Peak Name	R. Time	Area	Area%	Sample Descrip...
23.00	1	13.29	2414138.00	100.00	EM-2009-02

Data File: c:\gilson\unipoint\system1\gunther\gb110609.001\gb110609.gdt
Date acquired: Fri Jun 12 2009 12:11:17
Control Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GCT
Analysis Method: C:\GILSON\UNIPOINT\SYSTEM1\PEPTANA.GAN
Sample name: VEM-2009-026 Injection Number: 23 *Unknown*
Analyzed on: Fri Jun 12 2009 13:35:53

Analysis Method Events

0	Inhibit Integration	
0	Disable Negative Peak Integration	initial default
0	Peak Width 0.1	initial default
0	Peak Sensitivity 20	initial default
0	Default Baseline	initial default
3.80	Enable Integration	
36.00	Inhibit Integration	

Channel Scales
<Auto range>
118, kanaal 1 0.00 to 100.00 mVolts (10 %offset), Data rate: 20.00 (points/second) <Analysis Channel>
10 mVolts == 0.1 AUFS