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Synthesis of KDKPPR analogues through alanine-scaning technique for affinity evaluation on NRP-1 receptor.

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TÍTULO: Synthesis of KDKPPR analogues through alanine-scaning technique for affinity evaluation on NRP-1 receptor.

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RESUMEN:

El trabajo fin de grado que se describe a continuación forma parte de un proyecto basado en el estudio de la terapia fotodinámica con el objetivo de mejorar el tratamiento anti cáncer que actualmente es considerado como uno de los principales métodos para el tratamiento de muchas enfermedades.

Por tanto, esta terapia fotodinámica puede ser utilizada para destruir células cancerígenas.El equipo de trabajo obtuvo el péptido **KDKPPR** que podría unirse con el receptor NRP-1 y por tanto con su co-receptor VEGFR-2 de gran importancia ya que ambos receptores son responsables de la regulación de la angiogénesis en el tratamiento del cáncer.

Una vez formado el péptido se fueron sustituyendo los distintos aminoácidos por alanina para poder estudiar cuál de ellos tenía mayor importancia, obteniéndose los siguientes péptidos: KDKPP**A**, KDKP**A**R, KDK**A**PR, KD**A**PPR, K**A**KPPR, **A**DKPPR para evaluarlos mediante el método ELISA TEST y así seguir trabajando con el que tuviera más afinidad con NRP-1.

PALABRAS CLAVE:

Terapia fotodinámica, NRP-1, síntesis de péptidos en fase sólida(spps), ELISA TEST, KDKPPR.

*"**SYNTHESIS** OF **KDKPPR** ANALOGUES THROUGH **ALANINE-SCANING** TECHNIQUE FOR AFFINITY EVALUATION ON NRP-1 RECEPTOR".*

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List of abbreviations:

Table 1 :*List of abbreviations.*

Symbol:	Meaning:
A	Alanine
Ala	Alanine
Arg	Arginine
Asp	Aspartatic acid
Boc	Tert.Butyloxycarbonyl or Tert.Butoxycarbonyl
D	Aspartatic acid
DMF	N,NDimethylformamide
ELISA	Enzyme-linked immunosorbent assay.
Fmoc	9-Fluorenylmethoxycarbonyl
HBr	hydrobromicacid
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
HF	Hydrofluoric acid
HPLC	High performance liquid chromatography
K	Lysine
LCPM	Laboratory macromolecular physical chemistry.
LRGP	Laboratory macromolecular physical chemistry.
LRGP	Reactions and laboratory engineering of processes.
Lys	Lysine
mbar	Milibars
mg	Milligrams
min	Minutes
ml	Milliliters
mm	Milimeters
mmol	Millimoles
Mol.Wt	Molecular weight
nm	Nanometers
NMM	4-Methylmorpholine 99% $C_5H_{11}NO$.
NMP	1-Methyl-2-pyrrolidinone.
NMR	Nuclear magnetic resonance spectroscopy.
NRP-1	Neuropilin-1.
P	Proline.
PDT	Photodynamic therapy.
ppm	Parts per million.
Pro	Proline.
PS	Photosensitizer.
R	Arginine.
ROS	Reactive oxygen species.
SPPS	Solid phase peptide synthesis.
TFA	Trifluoroacetic acid.

TFMSA	Trifluoromethanesulfonic acid.
TIPS	Triisopropylsilane.
VEGFR	Vascular endothelial growth factor.
Symbol:	**Meaning:**
^{1}H	Proton.
2D	Second dimension.
3D	Third dimension.
4D	Fourth dimension.

1. INTRODUCTION:

1.1. PHOTODINAMIC ANTICANCER THERAPY :

1.1.1. PHOTODINAMIC THERAPY (PDT):

Photodynamic therapy (PDT), sometimes called photochemotherapy, is considered to be one of promising methods in the treatment of many diseases, disorders and infections. In recent years, it has shown good capability in early cancertreatment and palliation of advanced cancer[1].

It is a form of phototherapy using nontoxic light-sensitive compounds that are exposed selectively to light (within specific wavelength), whereupon they become toxic to targeted malignant and other diseased cells*(Figure 1)*[2].

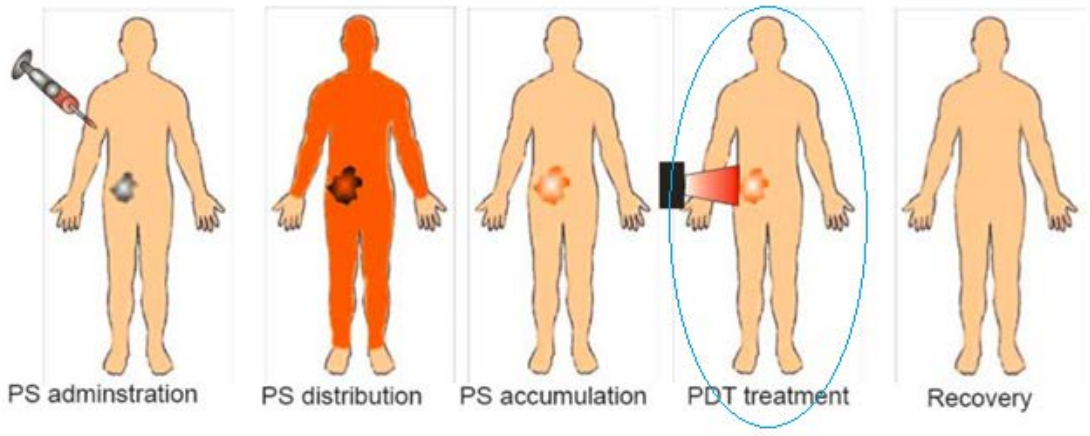


Figure 1- Schematic illustration of the mechanism of PDT

Thephotosensitizer (PS) is injected systemically and after sufficient time to allow itsaccumulation in the lesion, light is delivered to produce reactive oxygen species (ROS).

PDT involves three key components:

- **Photosensitizer (PS)**
- **Light source**
- **Tissue oxygen**

The combination of these three components leads to the destruction of nearby tissues following the exposure of the administered PS to light*in*[3]. It is important that the wavelength of the light is appropriate for excitation of the photosensitizer in order to produce the cytotoxic *reactive oxygen species*"**ROS**".

The Jablonski's diagram in *(Figure2)* described the reaction that happens following PS exposure to light. The absorption of photons by PS causes its transformation from ground state to a higher energy level and the energy will be released either through the formation of fluorescence or being transformed into a triplet state which still contain a high energy level. The excited triplet state interacts with ground state molecular oxygen to form reactive oxygen species (ROS). This process may occur by (1) electron transfer to form superoxide anion or by (2) energy transfer to produce singlet oxygen molecules[4],which will in turn cause destruction of nearby cells. All these reactions happen in a very short time; in nanoseconds, and hence the PS needs to be localized in the targeted area of cancer. For this reason, targeting of PS becomes an interesting topic to be explored to improve the delivery of PS and this will nevertheless increase the efficacy of PDT[2].

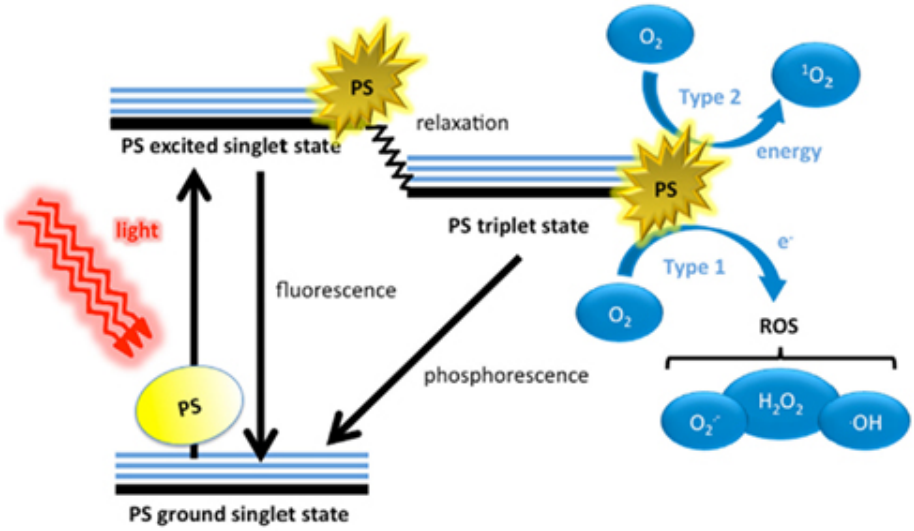


Figure 2- Jablonski's diagram.

Reactive oxygen species (ROS) are generated during PDT through *two types of reactions*:

❖ *Reaction (TYPE 1):*

The triplet (PS) can gain or donate an electron or hydrogen atom from a neighboring organic molecule and produce a super oxide anion radical (O_2^{-})*(Figure 3)*[1, 2].

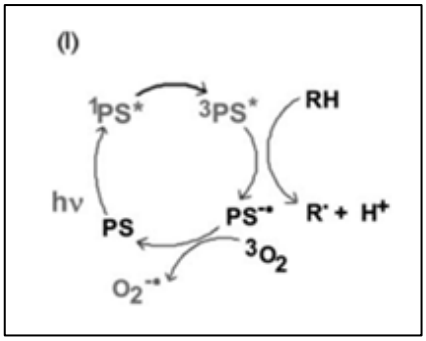


Figure 3 - Representation of the Type 1 photochemical mechanisms thought to operate in PDT.

❖ *Reaction (TYPE2):*

In this case, PS in its triplet states can transfer its energy ($^3O_2$) directly to molecular oxygen ($^1O_2$)*(Figure 4)*[1, 2].

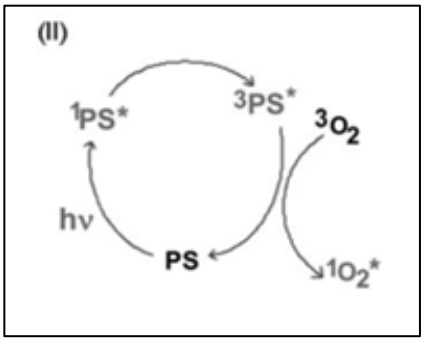


Figure 4 - Representation of the Type 2 photochemical mechanisms thought to operate in PDT.

1.1.2. MECHANISM OF PDT ADMINISTRATION AND ACTION:

The method of PDT consists of 4 steps *(Figure 5):*

- *Step 1:* Photosensitizer (PS) is injected into the bloodstream.

- *Step 2:*PS is absorbed by cells all over the body but will concentrate more in cancer cells.
- *Step 3:* After specific time length, PS will be accumulated in cancer cells and light at effective wavelength and light dose will be directed at them.

- *Step 4:*PS will cause the production ofreactive oxygen species (ROS) that will eventually destroy the nearby cells.

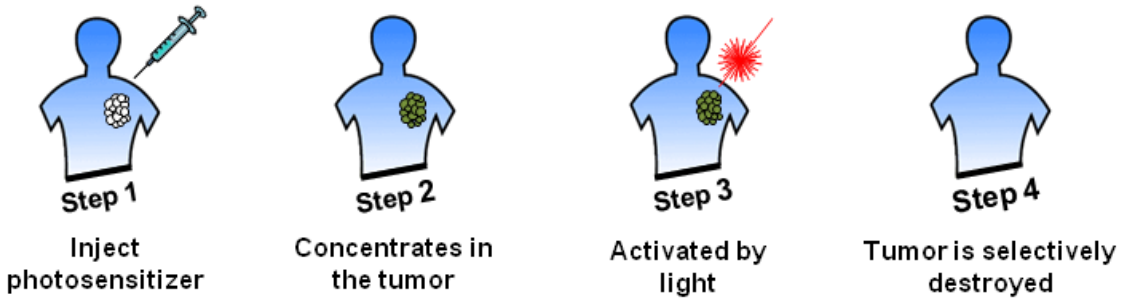


Figure 5- Steps to PDT.

1.1.3. ADVANTAGES AND DISADVANTAGESOF PDT:

Many studies have shown that PDT could produce good treatment outcome in the treatments of different cancers or pre-cancerous solid massand the following advantages are usually associated with PDT over surgery or radiotherapy:

Advantages:
- It is less invasive than surgery.
- It can be targeted very precisely.
- It has no long-term side effects when used properly and the therapeutic effects may become evidence in 48 to 72 hours.
- Usually it costs less than other cancer treatment options.
- It can be repeated many times at the same site if needed.

Disadvantages:
- It does not have the ability to treat metastasized cancers.
- Currently approved PS molecules can only act on the surface of the skin or a few (mm) within it, so it could notbe used to treat a large cancer or cancers that have grown deep into the skin or other organs.

1.2. PHOTOSENSITIZER:

1.2.1. *PHOTOSENSITIZER DISTRIBUTION IN TISSUES:*

Aphotosensitizeris a molecule thatcan be excited byabsorption of photon andis capable of transferringits energy to anacceptor molecule, and in the case of PDT, the energy is being transferred to oxygen molecules. Hence, in order to have a useful PS, the PS needs to have capability to absorb light in the visible region, with a bigger interest on molecules that have high visible absorbance in the red region *(Figure 6).*This is due to the fact that light could penetrate deeper into human tissues in this region and hence giving the possibility of using PDT for deep seated tumors[5].

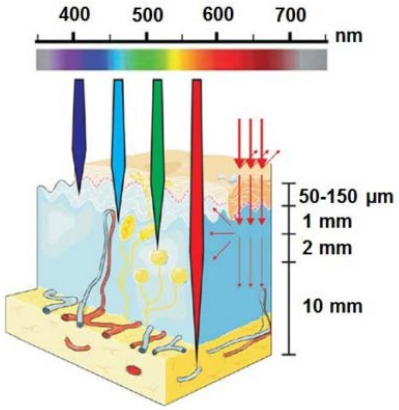


Figure 6 - Light penetrationinto the tissueas a function ofwavelength.

1.2.2. TYPES OF PHOTOSENSITIZER:

Historically, thecombination of a"drug"andlightin therapeuticapplications was started to be of interest since the beginning of 20th century, but it wasnot untilthe end of the century that « Food and Drug Administration, FDA » approved the first PS molecule for PDT application. Photofrin® which contained a mixture of haematoporphyrin derivatives was approved to be used for conditions includingpalliative treatment of esophageal cancer and non-small cell lung cancer. This product was also approved to be use in France, Germany, The Netherlands and Japan for the treatment of early and advanced stage of lung cancer, genitourinary and digestive tract cancer[6, 7].

- **Porphyrins photosensitizer:**

Aspreviously explained,red absorption is the desired penetration wavelength due to better tissue penetration. To date, the most useful basic compounds as PS are porphyrins, chlorins, and pthalocyanines*(Figure7)*. They have extensive aromatic systems and these molecules absorb light in the visible spectrum. There are also other classes of porphyrinoids use as PS for PDT such as: pheophorbides and texaphyrins[8].

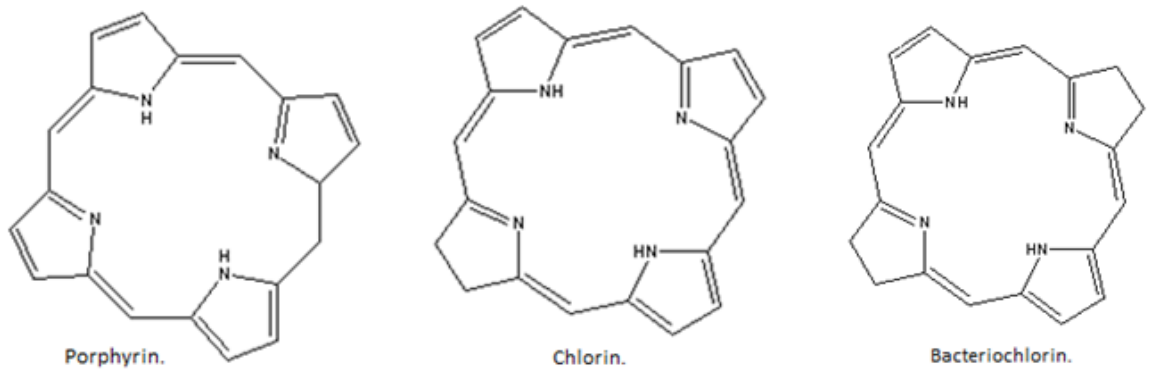


Figure 7 - Basic chemical structure of porphyrin, chlorin, bacteriochlorin.

1.3. ANGIOGENESIS IN CANCER:

Angiogenesis is a term used to describe the formation of new blood vessels. This process can be controlled by chemical signals in the body and can operate in two ways:

- repairing damaged blood vessels.
- forming new blood vessels.

Angiogenesis plays a fundamental role in the treatment of cancer because it is responsible of growth or expansion of all cells including cancer cells.

Metastasis is the process whereby cancer cells are disseminated throughout the body and can occur as a result of the presence of blood vessels following angiogenesis. Tumor uses the blood vessels to obtain oxygen and nutrients, and eventually transfer parts of the cells into blood circulation and the cells will continue growing in another part of the body.

Currently, there are many different treatment options for cancer available in clinical settings such as surgery, chemotherapy, radiotherapy, hormonal therapy and immunotherapy, to name a few. Photodynamic therapy is now one of the treatment options available for certain types of cancer.

PDT could be used to destroy the cancer cells itself through direct effect, or to destroy the blood vessels around the cancer cells through an indirect effect. In addition, the discovery of a peptide, KDKPPR by our research team in Nancy has opened up a possibility to directly target the Neropilin-1 (NRP-1) receptor. The NRP-1 is a co-receptor of VEGFR-2 and both are responsible in the regulation of angiogenesis process in cancer.

Hence in this aspect, our team is currently working in improving PDT through targeting of the NRP-1 receptor described above. By doing so, it is believed that the delivery of PS that will be conjugated with the targeting peptide could be better and ensuring improved PDT outcome[9].

1.3.1. DIFFERENTS RECEPTORS FOR ANGIOGENESIS:

VEGFR or vascular endothelial growth factor receptors are a group of receptors responsible in the angiogenesis process occurring in human body. Its ligand, the VEGF (vascular endothelial growth factor) is the one responsible in 'switching on' angiogenesis process as needed.

There are three variants of the VEGFR receptors:
- ***VEGFR-1***
- ***VEGFR-2***
- ***VEGFR-3***

Eversince the discovery of VEGFs, its receptors VEGFR-1,-2, -3 and their co-receptor NRP-1, multiple studies were being conducted in order to understand more in term of their function in angiogenesis. The information available in the literature now has enables the formulation of active compounds to target this receptor and/or halt the progression of angiogenesis[10]. By doing so, these receptors could be used as a target point besides being able to stop the growth of cancer by blocking them.

VEGFR-1:
This receptor works in soluble form in the transmembrane of cells, being very effective for the treatment arthritis or rheumatism.

VEGFR-2:
It hasa key role inthe treatment ofphysiologicalandpathologicalangiogenesisby controlling theproliferation and migration ofendothelial cells.

VEGFR-3:
It is important at the beginning of the angiogenesis and embryogenesis.It regulates vascular and lymphatic endothelial cell function.

Neuropilin-1:
NRP-1 is a co-receptor of VEGFR-2 and they control the formation of new blood vessels through the process of angiogenesis[10].

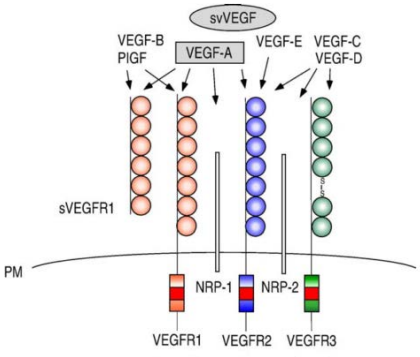


Figure 8 - VEGF and VEGF receptors.

1.4. VEGF & NRP-1 TARGETING PEPTIDES:

VEGFfactors could be classified into different isoforms; VEGF - **A**, VEGF - **B**, VEGF - **C**, VEGF - **D**, VEGF- **E**.Of all the possiblevariantsthe most importantforthe development ofangiogenesisisVEGF-A and it is also needed during pregnancyandinmaintainingblood pressure. VEGF-A itself has different isoforms, depending on the amino acids contained in the protein sequence in which they can be between 121-206 amino acids.There are five VEGF-A isoforms that are commonly found: VEGF-A_{121}, VEGF-A_{145},VEGF-A_{165}, VEGF-$A_{189}$and VEGF-A_{206}. Between all these isoforms, **VEGF-A_{165}** *(Figure 9)*isthe most important for pathological angiogenesis[11].

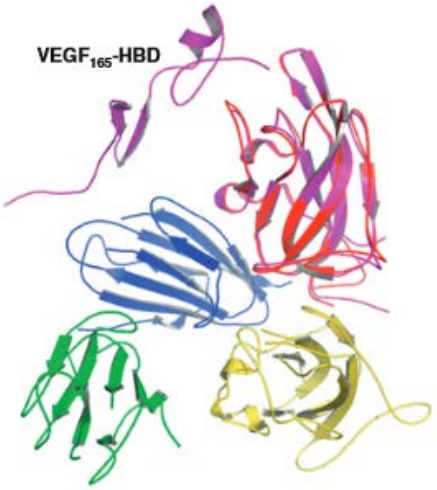


Figure 9 - $VEGF_{165}$

In 2006, our team reported the discovery of a heptapeptide, ATWLPPR that had showed good ability to target the NRP-1 receptor (Tirand 2006). However, due to *in vivo* instability problem that aroused, the quest to find new NRP-1 targeting peptides with improved stability was started and five peptides were found to have good targeting capability. These peptides were discovered through different approaches involving docking simulation studies and ELISA tests[12, 13].
The Peptides are:

*Four penta-peptides:

DKPPR
DKPRR
TKPPR
TKPRR

*One hexa-peptide:

CDKPRR

2. OBJECTIVES:

The discovery of the five peptides described above has given the possibility of improving PDT through peptide-based targeting. From all these peptides, DKPPR and TKPRR are two unpublished and novel findings for the research group. Based on ELISA test, DKPPR-PS-conjugate and TKPRR-PS-conjugate showed improved receptor inhibitory capability as compared to ATWLPPR-PS conjugate (Kamarulzaman 2016).

Kamarulzaman[13]has described that the presence of Arginine (Arg, R) amino acid at C-terminal position showed strong interaction with NRP-1. In an unpublished data, the addition of Lysine (Lys, K) in front of DKPPR, producing KDKPPR had shown further improvement of IC_{50} value against NRP-1 receptor. Hence, it was evidence that KDKPPR may have better targeting capability and it was indeed very interesting to further explore the characteristic of this peptide sequence.

This project is designed to explore the **importance** of **each amino acid in** the **KDKPPR** sequence through a technique called **Alanine-scanning**. Through this technique, each amino acid was changed systematically with Alanine (Ala, A) and tested through ELISA test to investigate the importance of the substituted amino acid.

In addition, there were also several other techniques that were learned through-out the process:

- Learning how tosynthesizepeptideby using the technique***« Solid phase peptide synthesis (SPPS) »***, the purification by RP-HPLC and subsequently storage of peptide.

- Learning how to **interpret**the**results** of « ***mass spectra*** » « ***NMR*** (Proton, TOCSY, COSY) » and « ***ELISA TEST*** ».

- **Evaluating**the binding ability of the different peptides formed towards NRP-1 receptor through ELISA test and determining the best results.

- Learning to **work** according to good laboratory practice**in the laboratory** and learning to work in team spirit.

3. ALANINE SCANNING:

3.1. METHOD:

In this study, a technique called Alanine-scanning was implemented in which each amino acid in the KDKPPR sequence was systematically substituted with Ala to investigate the importance of the substituted amino acid. Ala was used because it is the smallest chiral amino acid. In addition the substitution with Ala also eliminates side chain interactions without altering the conformation of main amino acid backbone[14].

3.2. PEPTIDES TO SYNTHESIZE:

Each amino acid in the KDKPPR sequence was replaced with Ala as presented in Figure below:

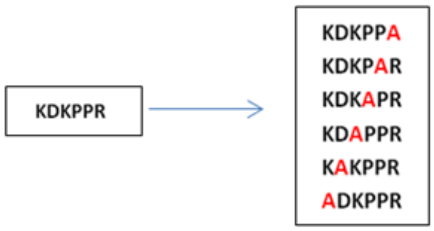


Figure 10 - Peptides with alanine.

In addition to the Ala-substituted peptides, three other peptides were also synthesized; (1) a peptide with Arg substituting Lys position - KDRPPR, (2) a retro peptide and (3) a retro-inverso peptide *(Figure-11).*The retro-inverso peptide which is formed by D-amino acids is believed to have better stability as compared to the native KDKPPR.

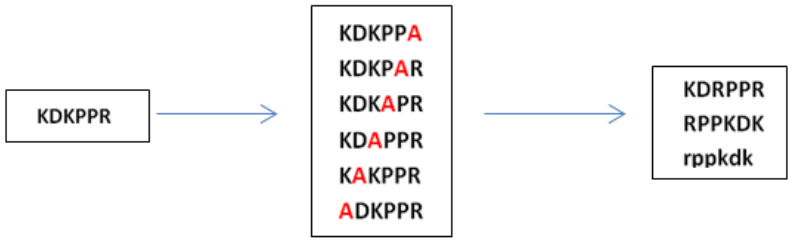
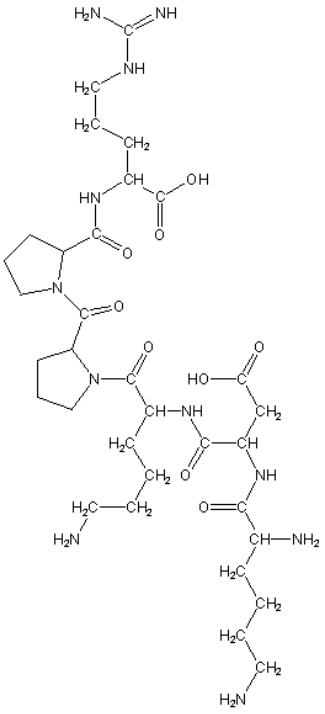
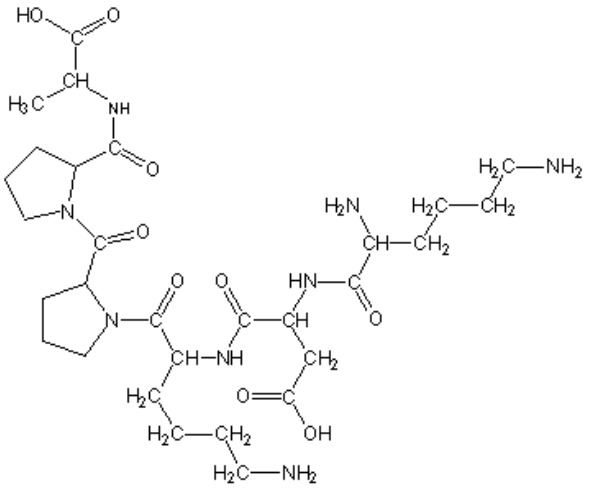
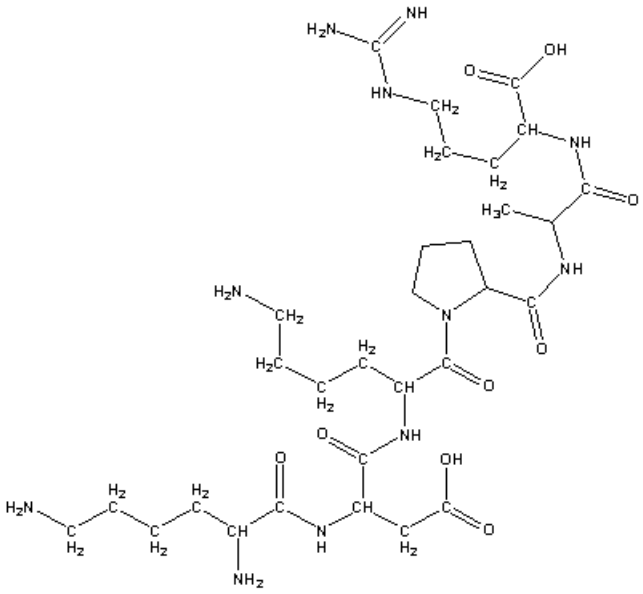
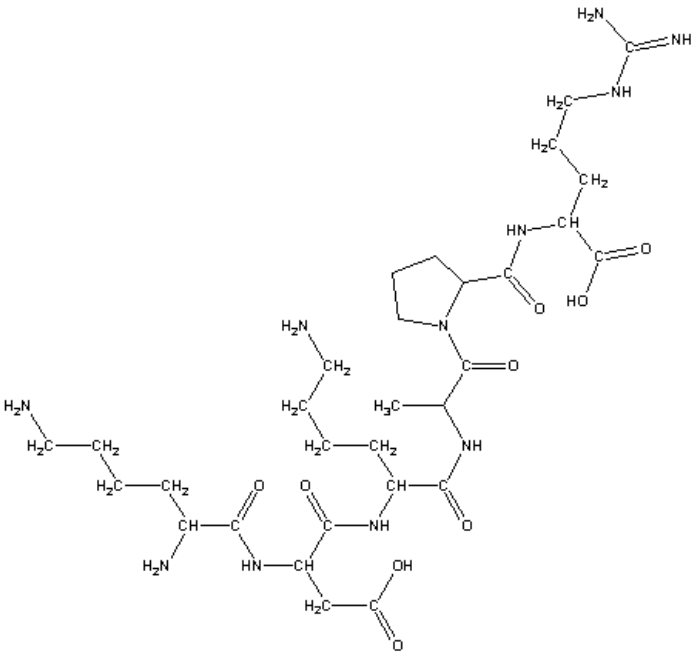


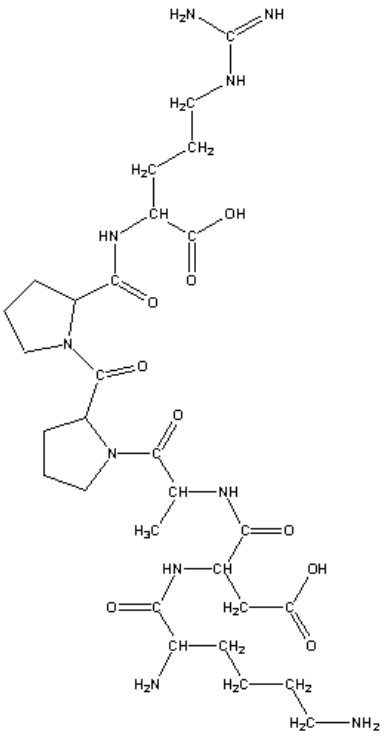
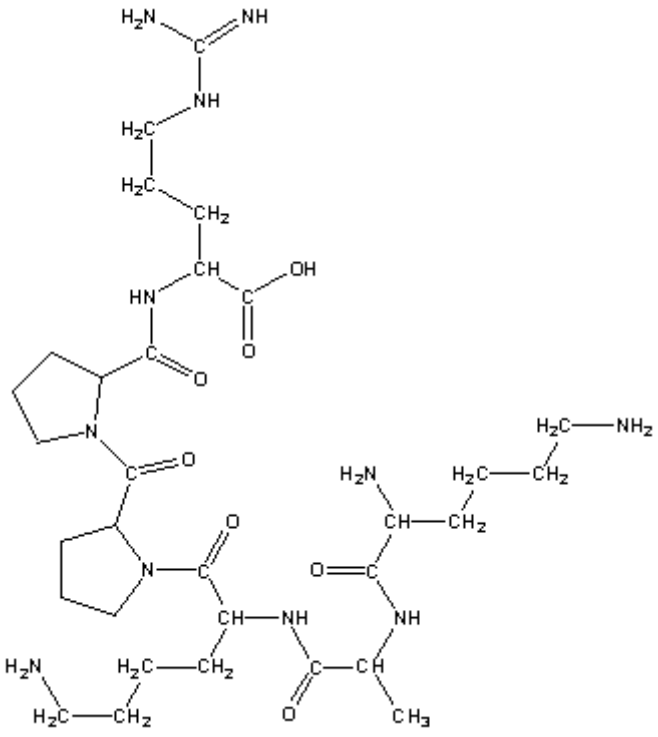
Figure 11 - Peptides to be formed.

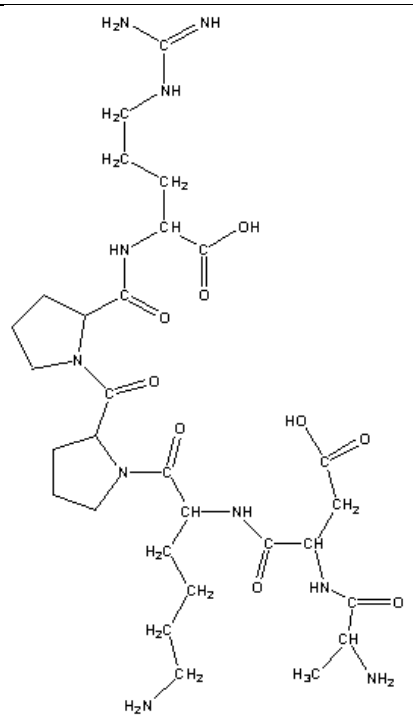
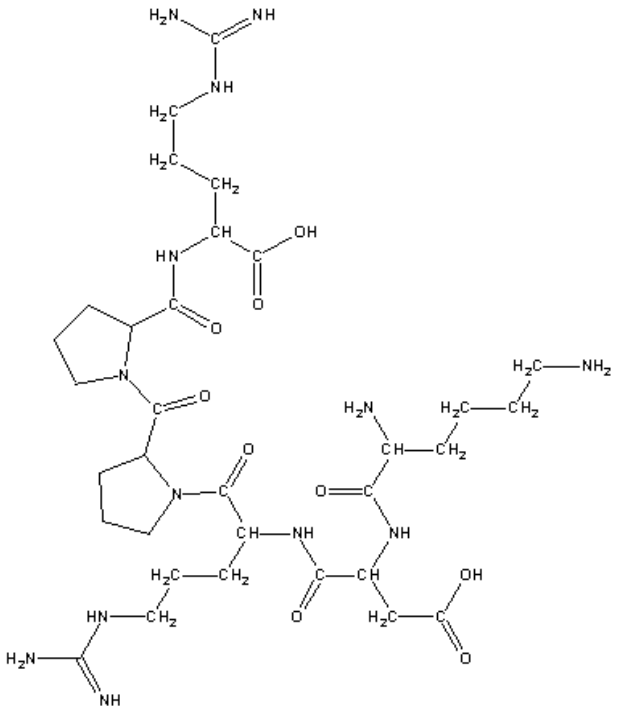
3.2.1. STRUCTURE AND CHARACTERISTICS OF THE SYNTHESIZED PEPTIDES:

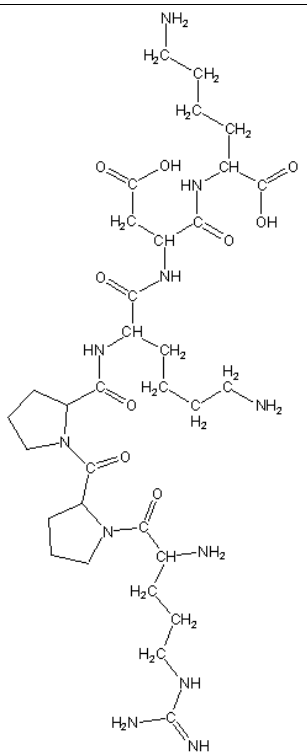
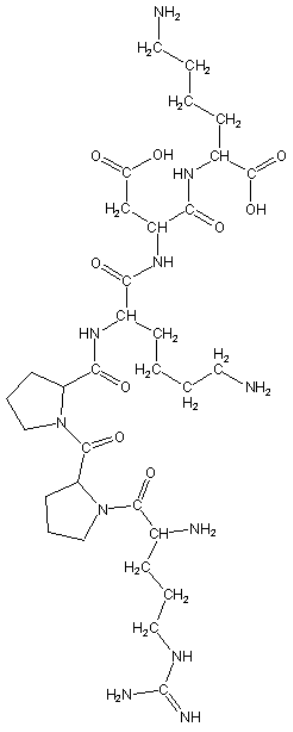
Table 2: *Structure and characteristics of the synthesized peptides.*

PEPTIDE	STRUCTURE
K-D-K-P-P-R $C_{32}H_{57}N_{11}O_9$ Exact Mass: 739,43 Mol. Wt.: 739,86	
K-D-K-P-P-A $C_{29}H_{50}N_8O_9$ Exact Mass: 654.37 Mol. Wt.: 654.76	

K-D-K-P-A-R $C_{30}H_{53}N_{11}O_9$ Exact Mass: 713,42 Mol. Wt.: 713,83	
K-D-K-A-P-R $C_{30}H_{53}N_{11}O_9$ Exact Mass: 713,42 Mol. Wt.: 713.83	

K-D-A-P-P-R $C_{29}H_{49}N_{10}O_9$ Exact Mass: 681,3 Mol. Wt.: 681,76	
K-A-K-P-P-R $C_{31}H_{57}N_{11}O_7$ Exact Mass: 695.44 Mol. Wt.: 695.85	

A-D-K-P-P-R $C_{29}H_{50}N_{10}O_9$ Exact Mass: 682,38 Mol. Wt.: 682,77	
K-D-R-P-P-R $C_{32}H_{57}N_{13}O_9$ Exact Mass: 767,44 Mol. Wt.: 767,88	

R-P-P-K-D-K $C_{32}H_{57}N_{11}O_9$ Exact Mass: 739,43 Mol. Wt.: 739,86	
r-p-p-k-d-k $C_{32}H_{57}N_{11}O_9$ Exact Mass: 739,43 Mol. Wt.: 739,86	

Note:*Software called ***"ChemDraw"was used to draw the structure of the peptides. By using this software, the*exact mass* and *molecular weight* of each molecule could also be calculated directly.

4. LABORATORY OPERATIONS:

4.1. THEORY:

When designing a peptide, it is necessary to consider several aspects such as the amino acid composition, length of peptide, solubility and the application in which the peptides are to be used.It is also very important to know the characteristic of the amino acids forming the peptide, because they have a strong influence on the purification of the peptide and therefore also on the peptides' solubility.
For example:
If a peptide contains many hydrophobic residues, its solubility in aqueous solution will be reduced and so their usability will also be reduced.In addition, a very hydrophobic peptide will also be more difficult to be obtained in large amounts with high purity. It is also recommended to have at least five amino acids when designing a peptide[15].

4.1.1. AMINO ACIDS AND THEIR PRINCIPAL CHARACTERISTICS:

An ***amino acid***is an organic molecule with an **amide group(-NH_2)**and **carboxyl group(-COOH).***(Figure12)*

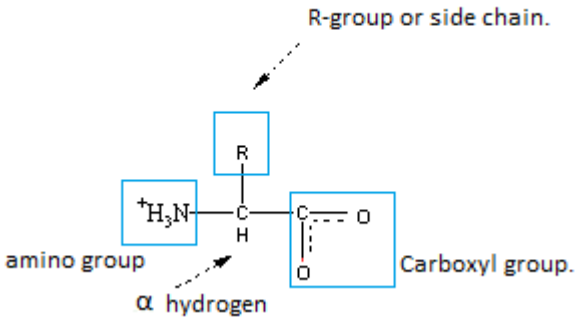


Figure 12 - Basic structure of amino acids.

The bond between two amino acids is called ***peptide bond*** and occurs by the condensation reaction between the amide group and the carboxyl group of another amino acid, releasing a molecule of water*(Figure 13).*Combination of multiple amino acids will form peptide. The synthesis of peptide is commonly occurring in cells, in an organel called ribosomes[16].

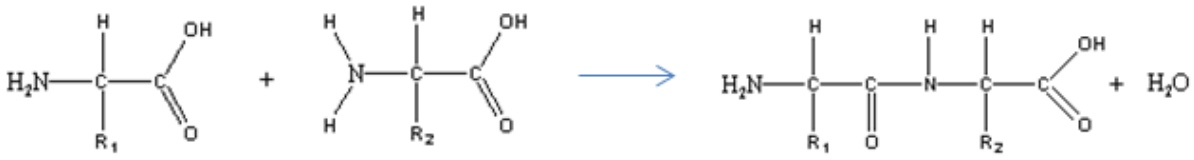


Figure 13 - Condensation reaction of a peptide bond.

- ***These are the amino acids that were used in this work:***

o ***Alanine:***

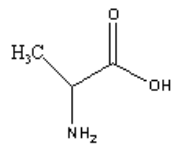


Figure 14 - Alanine structure.

*Abreviated:***Ala** o **A**.
Chemical formula:$C_3H_7NO_2$ or $CH_3CH(NH_2)COOH$.
Other names: 2-Aminopropanoic acid.

Characteristics:
Alanine is a non-polaramino acid.
The isomer -L- is one of the 20 amino acids encoded by the human genetic code, while the isomer -D- occurs in bacterial cell walls and in some antibiotic peptides. *(Figure 14)*[16].

o ***Arginine:***

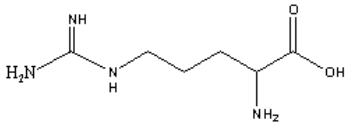


Figure 15 - Arginine structure.

*Abreviated:***Arg** o **R**
Chemical formula: $C_6H_{14}N_4O_2$
*Other names:*2-Amino-5-guanidinopentanoic acid.

Characteristics:
Arginine or L-arginine is a semi-essential amino acid.
It is important in many metabolic processes and treatment of diseases of the heart and blood pressure.
It is also used in diets for weight reduction and cholesterol.*(Figure 15)*[16].

- ***Aspartic - acid:***

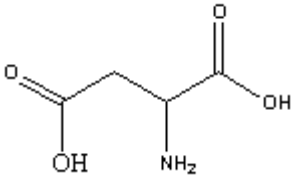


Figure 16 - Aspartic acid structure.

*Abreviated:***Asp** o **D**
Chemical formula:$C_4H_7NO_4$ or $HOOCCH(NH_2)CH_2COOH$
*Other names:*2-Aminobutanedioic acid.

Characteristics:
Aspartic acid is one essential amino acid not in humans.
This amino acid has an overall negative charge and plays an important role in the cycle of citric acid and urea.
From aspartic acid, it is possible to synthesize other amino acids as asparagine, arginine and lysine. *(Figure 16)*[16].

- ***Lysine:***

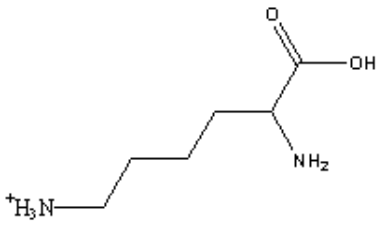


Figure 17 - Lysine structure.

*Abreviated:***Lys** o **K**
Chemical formula:$HO_2CCH(NH_2)(CH_2)_4NH_2$
Other names: 2,6-Diaminohexanoic acid
2,6-Diammoniohexanoic acid.

Characteristics:
Lysine is one of the amino acids synthesized by humans.
Chemically it acts as a base.
Animals could not synthesize lysine but they obtain this amino acid through ingestion of lysine-containing proteins. Plants on the other hand synthesize lysine from aspartic acid. *(Figure 17)*[16].

- ***Proline:***

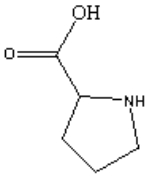


Figure 18 - Proline structure.

*Abreviated:***Pro** o **P**
Chemical formula:$C_5H_9NO_2$
*Other names:*Pyrrolidine-2-carboxylic acid

Characteristics:
Proline is an imino acid.
The primary amine on the α-carbon of semi-aldehyde glutamate forms a Schiff base with the aldehyde which is then reduced, yielding proline. *(Figure 18)*[16].

- ***Table 3 summarize the physicochemical characteristics of all these amino acids:***

Table 3 : *Characteristics of these amino acids.*

Amino Acid	Code	Hydropathy	Charge	pKa, NH_2	pKa,COOH	pK(R)	Solubility (mg/mlwater).
Alanine	A	Hydrophobic	N	9.87	2.35		50
Arginine	R	Hydrophilic	+	9.09	2.18	13.2	50
Aspartate	D	Hydrophilic	-	9.6	1.88	3.65	5
Lysine	K	Hydrophilic	+	10.28	8.9	2.2	100
Proline	P	Hydrophobic	N	10.6	1.99		50

4.2. EXPERIMENTAL METHOD:

4.2.1. SOLID PHASE PEPTIDE SYNTHESIS (SPPS)Step 1:

This methodwas pioneered by**Merrifield**and at this moment itis the most accepted method for obtaining peptides and proteinssynthetically[15, 17].

- ***Method used:***

The first thingto do inthis methodis to calculatethe amounts ofamino acidsandreagentsto beused. This is presented in ***"Annex-1".***

This design has three very important steps:**Protection**, **Deprotection** and **Coupling.**

a) Protection:

This stepis very important becauseamino acids havemultiplereactive groupswhichmust be protectedto avoidside reactionsandpreventramification.
Protecting groups are added to the amino acids and only leave one free carboxyl group and N-terminal is also protected by Fmocgroup. The protection is important to ensure that the reactionwill proceed in the right direction[18].
These protecting groups are temporary and they are easily removed. Commonly, all the amino acids obtained commercially are readily protected*(Figue-19).*

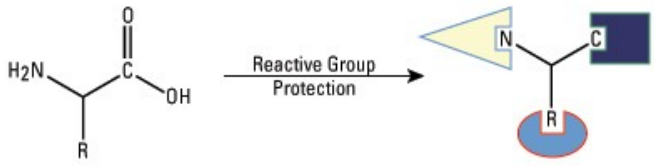


Figure 19 - Amino acid functional group protection.

There are two typesof protecting groups: *N-α-protecting groups and C-terminal protecting groups.*

- **N-α-protecting groups:**

FMOC *(9-Fluorenylmethoxycarbonyl):*
It is used as a protector for synthesis routine.*(Figure-20)*

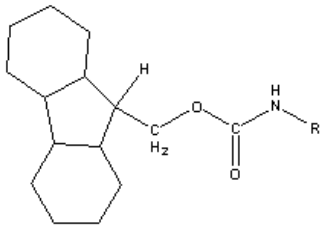


Figure 20 - Fmoc structure.

BOC (*Tert.Butyloxycarbonyl or Tert.Butoxycarbonyl):*
It is usedin complexsynthesis.

Characteristics:
- Stable to bases and nucleophiles.
- Unaffected by catalytic hydrogenation.(Figure-21)

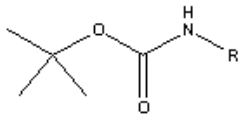


Figure 21 - Boc structure.

Note: Table 4 shows the necessary treatment for Fmoc and Boc according to the synthesis step.

Table 4: *Treatment for Fmoc and Boc in synthesis.*

Protecting Scheme	Deprotection	Coupling	Cleavage	Wash
Boc/Bzl	TFA	Coupling agent in DMF.	HF, HBr, TFMSA	DMF
Fmoc/tBut	Piperidine		TFA	

- **C-terminal protecting groups:**

Solid phase peptide synthesis does not use this kind of protection on resin because the resin works as a C-protecting group. There is also no protection on the C-terminal of amino acids because the synthesis necessitates the presence of free carboxylic group to form peptide bond.

b) *Deprotection:*

In this step, the Fmoc-protection of the N-terminal is removed by using piperidine. This is important to allow the formation of peptide bond with a new in-coming amino acid (which will have free carboxylic group).*(Figure-22)*

Figure 22 - Deprotection of Fmoc group from N-terminal.

FMOC: It is removed under basic conditions. Example: piperidine.

BOC:it is removed under acidic conditions. Example: TFA.

c) *Coupling:*

The coupling step between two amino acids starts with activation of the C-terminal carboxylic acid. Several agents could be used and in this study, HBTU was used. Following this activation, peptide bond could be formed with the previously deprotected N-terminal of the first amino acid. *(Figure-23)*

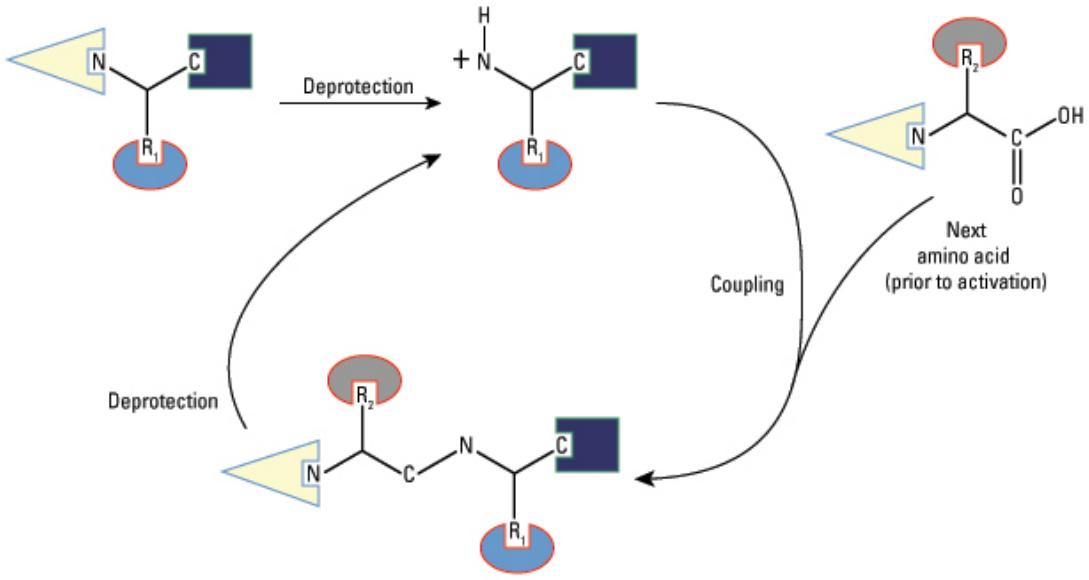


Figure 23 - Diagram of peptide synthesis.

Note:**"Annex-2"**showed the steps used by the SPPS in peptide synthesis. It is the example of KDKPPR, but the synthesis of the other peptides also follows the same steps.

- **Machine:**

Name :*« Intavis AG ResPep XL » Bioanalytical Instruments*

ResPep SL is a fully automated peptide synthesizer.It hasthe advantage thatmanypeptidescan be formedsimultaneouslyin parallel*(Figue-24)*[19].

Figure 24 - SPPS machine.

Parts of the machine:

- ***Plate and Mini-Column Module:***

  Each amino acid has a specific site where the needle goes automatically.

- ***Needle:***

  The needleis movingaboveall tubes, only taking thenecessary reactants in each synthesis step.

- ***Columnreactor:***

  Thisis the place where all the reactions take place and the peptide formed will be obtained. The resin is first added and then by the coupling of each amino acid will take place systematically.*(Figue-25)*

Figure 25 - Parts of spps machine.

- The amino acids, resins and other reactants used in this study, for the synthesis of peptide through SPPS.

  Amino acids:
  A:Fmoc-Ala-OH.
  D:Fmoc-Asp(OtBu)-OH.
  K:Fmoc-L-Lys(Boc)-OH.
  P:Fmoc-Pro-OH.
  r:Fmoc-D-Arg(Pbf)-OH.
  p:Fmoc-D-Proline.
  k:Fmoc-D-Lys-OH.
  d:Fmoc-D-Asp(OtBu)-OH.

Resins:
A-Ɵ: Fmoc-L-Ala-wang resin.
R-Ɵ: Fmoc-Arg(Pbf) – wang resin.
k-Ø : Fmoc-D-Lys(Boc)-wang resin.

Reagents:
Activator: HTBU (Benzotriazole-1-yl) tetramethyluroniumhexafluorophosphate.
Base: NMM (4-methylmorproline 99%)
Capmix: Acetic anhydride.
Piperidine.

Solvent:

DMF: N,N-Dimethylformamide**.**

Note:* The synthesisrequires around **8hours and**30minutes**obtaining a peptide with6aminoacids.

4.2.2. DRY + CLEAVAGE Step 2:

At the end of the peptide synthesis, it is necessary to remove residual DMF that could still be present with the peptide. Hence, a drying step is usually carried out by using a vacuum pump.

- ***Dry:***
- ***Machine and method used:***

The peptide is placedinto a dessicator that will be connected to a vacuum pump with nitrogen gas cooling system. *(Figue-26)*
Duration: **2 hours.**

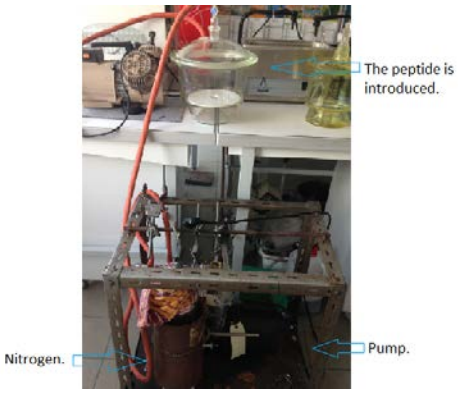


Figure 26 - Dessicator.

❖ *Cleavage:*

- ***Machine and method used:***

The next step after drying is cleavage and this step is important in order to remove all protection groups of the synthesized peptide. This step will also remove the peptide from the solid support*(Figure- 27)*. In our protocol, a combination of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water was used (95% TFA: 2.5% TIPS:2.5% water). This cleavage mixture was added into the crude peptide in the column reactor and left to agitate at room temperature for two hours.*(Figure 28)*

TFA = 5ml.
TIPS = 0.270ml.
Water = 0.135ml.

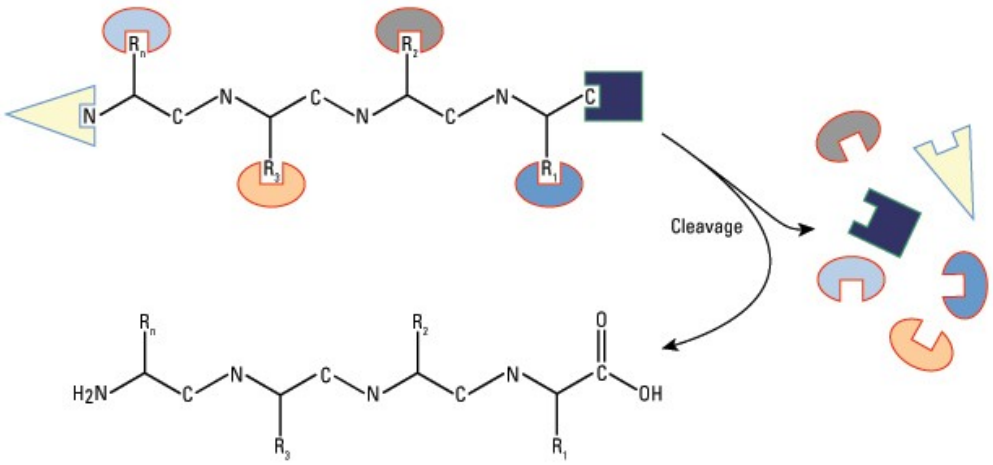


Figure 27 - Diagram of peptide cleavage after synthesis.

Figure 28 - Agitator.

At the end of the reaction time, the cleaved peptide was precipitated in cold diethyl ether and subjected tolyophilization to obtain dried crude sample ready for purification by reverse-phase high performance liquid chromatography (RP-HPLC).

4.2.3. LYOPHILISATION Step 3:

- ***Method and machine used:***

This step involvesfreeze-drying the cleaved peptideto remove tracesof TFA, TIPS and water that arestill presence with the peptide. The lyophilisation process is formed bythree steps: ***freezing***, ***primary drying*** and***secondary drying***.The first step to do is freezing the peptidetohave itin the form ofice (solid state). This step is done by adding an excess amount of water into the sample and subsequently immersing it into liquid nitrogen[20]. The frozen water will then be removed through sublimation in which it is directly changed from solid state into gaseous state. A very low pressure is needed in order to have an efficient sublimation process and usually a good pressure of 0.01 mbar is needed. During the removal of water, TFA and TIPS are being removed together, leaving the crude peptide sample in dried form.

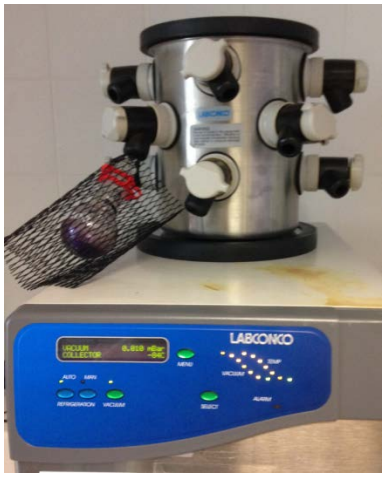


Figure 29 - Lyophilizater.

4.2.4. PURIFICATION - Reversed Phase High Performance Liquid Chromatography (RP-HPLC) Step 4:

The dried crude peptides following lyophilization needs to be purified to separate any incomplete peptide sequence or any unwanted amino acids that might be presence.Depending on the characteristics of the peptides, size, charge and hydrophobicity; there are differentmethodsofpurification such as[21]:

- size-exclusion chromatography
- ion exchange chromatography (IEC)
- partition chromatography
- High-performance liquid chromatography **(HPLC).**

- ***Method and machine used:***

The purification method chosen in this study was RP-HPLC. This method allows the recuperation of purified peptide with good purity by using a purification column and HPLC chain *(Figure-30).*It is always better to have the highest purity possible in order to have a good analysis result by NMR, mass spectroscopy and especially important for biological ELISA study.

Figure 30 - HPLC machine.

The lyophilized sample *(in step 3)* was solubilised in the solvent used for the HPLC analysis and filtered before injecting into the HPLC. The Waters® HPLC as presented in *(Figure 30)*has both analytical and preparative inlets and this allow the analysis of the sample in analytical amount before proceeding to the purification by preparative column***(Machery-Nagel VP 150/21 NUCLEOSIL 100-5 C18)***. The purification necessitates a gradient elution program to have a more efficient compound separation. The details of the purification process are presented in Table 5:

Table 5: Details of the purification.

Column	Machery-Nagel VP 150/21 NUCLEOSIL 100-5 C18		
Solvent	Water/Acetonitrile/0.1% Trifluoroacetic acid		
Gradient	Time (min)	Water (% conc.)	Acetonitrile (% conc.)
	0	95	5
	25	0	100
	30	0	100
	35	95	5
Flow rate	12 ml/min		
Detector	UV/Visible at 214 nm		
Pressure	1300 psi		

- ***Calculation ofretention time:***

Time it takes each substance to leave the chromatographic system. This retention is dependent on the molecular characteristics of each compound.

In this case to calculate the retention time must simply point the time it takes to appear our product in the chromatogram.

4.3. CHARACTERIZATION OF THE PEPTIDES:

4.3.1. MASS SPECTROMETRY (MS):

- **What does it consist of?**

Mass spectrometry (MS) isatechnique usedin laboratoriesto determine the presence of expected compound based on the mass/charge analysis of the sample. It is a simple and fast method in which the exact mass of the compound could be detected. The mass spectrometer available in the LCPM laboratory is: **LCMS-2020** (Liquid chromatograth mass spectrometer).

***Note:**The exact mass of the compoundis calculated directlyin the"ChemDraw" program.

4.3.2. NUCLEAR MAGNETIC RESONANCE (NMR):

- **What does it consist of?**

It is a group of scientific methods used to study and help to deduce the structure of organic, organometallic or biological molecules.This methodallows studying the presence of isotopeelement such asproton (^{1}H) and carbon(^{13}C). It is a non-destructive method because the sample keeps all its properties after being treated.It also hasthe advantage that it could produce structural informationin more than onedimension NMR(2D, 3D, 4D) and the results could be obtained in relatively quick and easy manner.

In this study, three different spectrums were produced by NMR method to help in the confirmation of peptide structures. The presence of all amino acid needs to be confirmed especially in this study whereby each amino acid is being changed systematically.

One-dimensional(1D) spectrum – Proton NMR (^{1}H): It gives information on all the hydrogen atoms present in the molecule.

Two-Dimensional(2D) spectrum (TOCSY, COSY):therelationship between different hydrogen atoms in the molecules was studied.

The NMR available in the LCPM laboratory is: BRUNKER (300MHz / 52mm).

The following images described in general the NMR shift (in ppm) of each proton from all amino acids used in this study. In TOCSY analysis, the correlation between two protons from unconnected carbons could be seen, whereas COSY only allows the correlation between protons from connected carbons. As an example, in the structure of alanine, there are three types of protons; the alpha-proton, beta-proton and proton for amide group. The correlation between beta-proton and amide-proton could be observed in TOCSY (red diamond spot) whereby in COSY, only correlation between beta-proton and alpha-proton could be seen (blue round spot) and subsequently another spot could be seen that correlate between alpha-proton and amide-proton. This analysis was done by using a program called DIA, which allows correlation to be done through formation of vertical and horizontal lines.

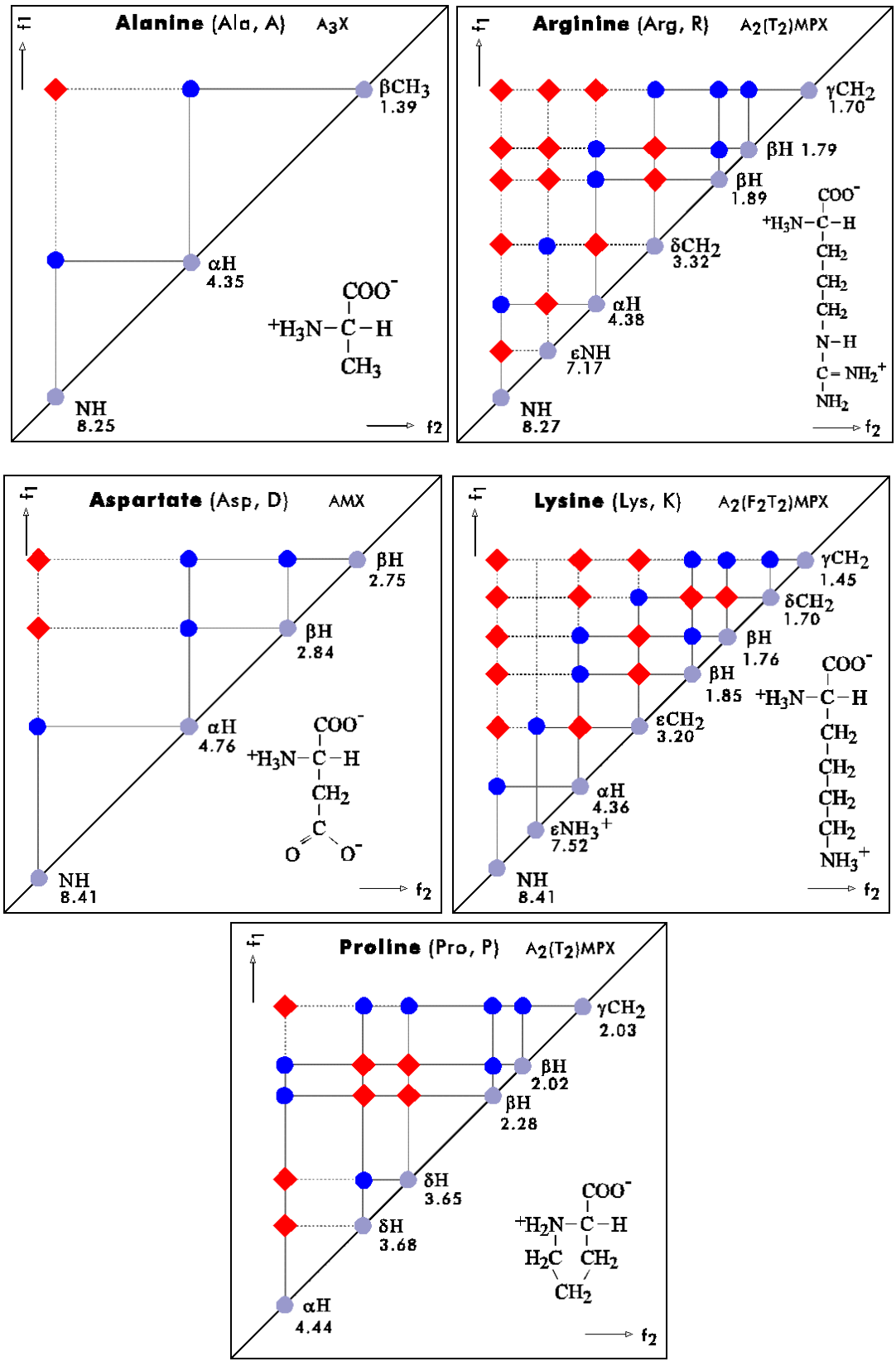


Figure 31 - Representation of amino acids in Tocsy and Cosy.

**** Note:***An example of the results obtained in the NMR can be seen in the next section of work.

4.3.3. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA TEST):

- **What does it consist of?**

It is a biological test that allows the detection of specific antigens that interact with specific antibody. It could be use in common antigen-antibody detection such as in HIV-detection or pregnancy tests or in other conditions such as in this study whereby the affinity of our synthesized peptide (antigen) will be tested on targeted NRP-1 receptor (antibody). An enzyme-linked antibody used in this study will catalyze a colour change that will indicate positive reaction.This technique was conducted by a team of biologists in our research group.

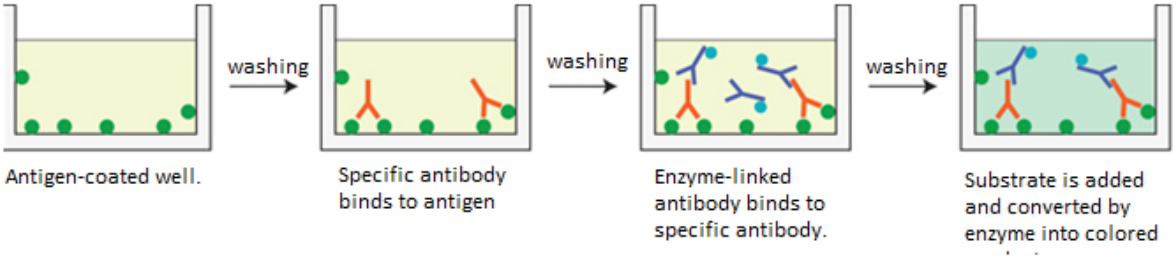


Figure 32 - Steps ELISA test.

5. RESULTS:

5.1. PURIFICATION (HPLC):

Table 6 :*Retention time of the synthesized peptide in analytical and preparative HPLC.*

Peptide	Retention time-analytical *(min)*	Retention time-preparative *(min)*
KDKPPR	6.06	5.72
KDKPPA	6.71	6.81
KDKPAR	4.56	4.92
KDKAPR	4.84	5.56
KDAPPR	4.41	4.46
KAKPPR	4.52	4.56
ADKPPR	4.32	4.63
KDRPPR	5.75	6.38
RPPKDK	4.66	4.62
rppkdk	4.59	4.62

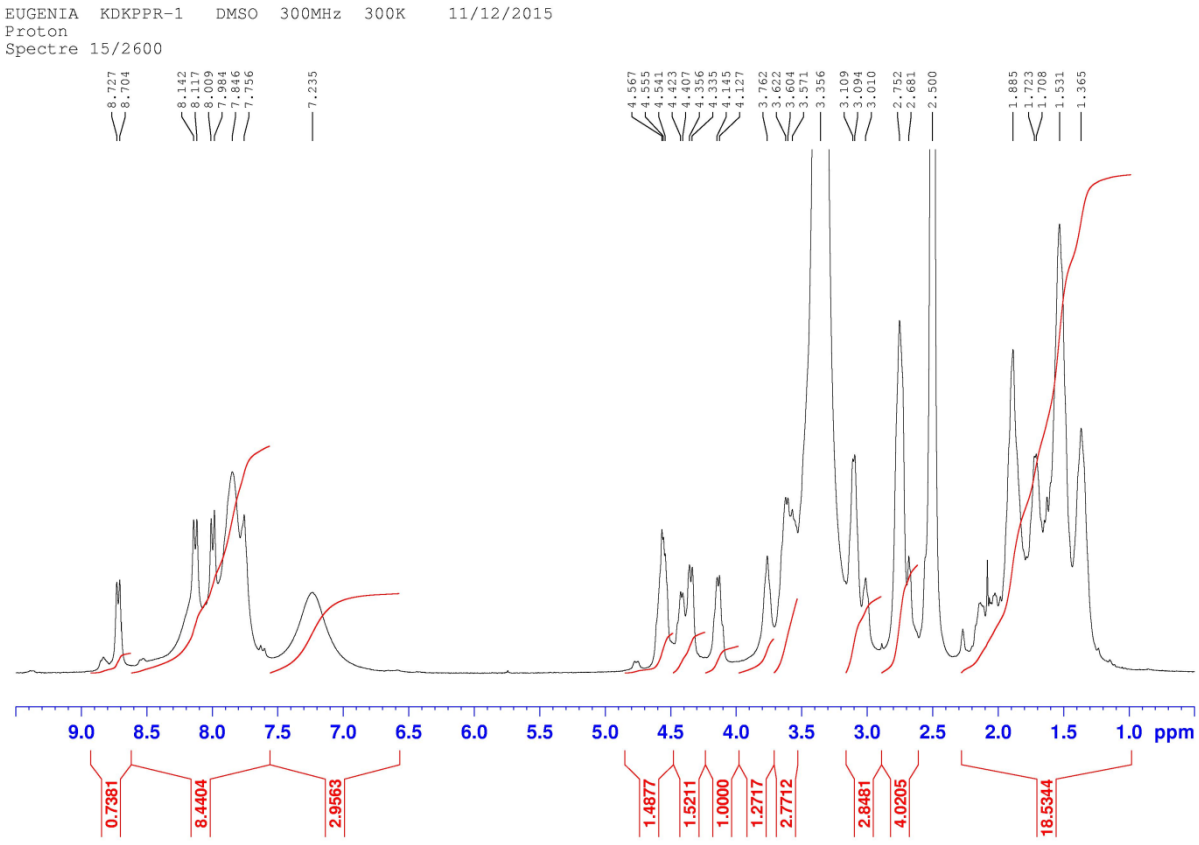
5.2. MASS SPECTRA:

Table 7 :*Results mass spectra.*

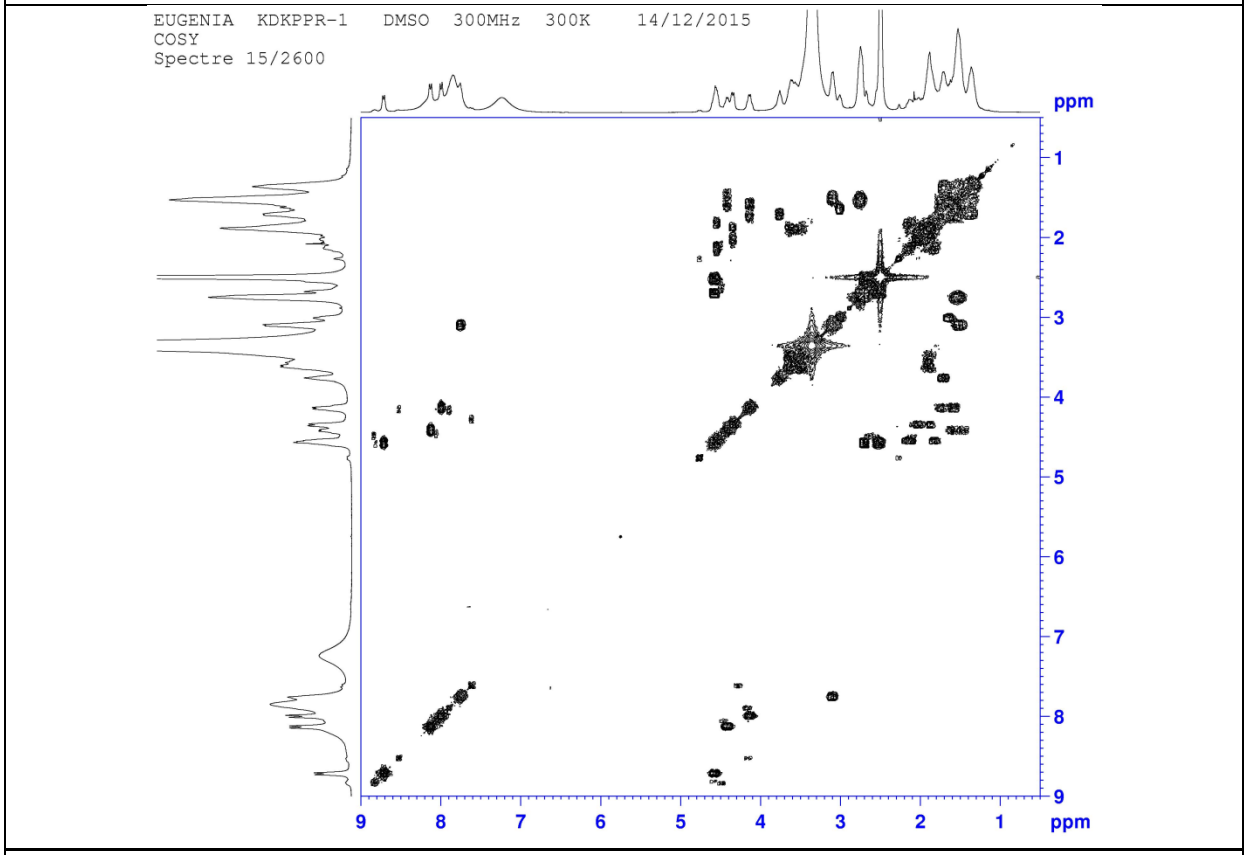
Peptide	Mass expected		Mass observed	
	M[+]	M[2+]		M[+]
KDKPPR	740.43	370.72	KDKPPR	740.43
KDKPPA	655.37	328.19	KDKPPA	655.37
KDKPAR	714.42	357.71	KDKPAR	714.42
KDKAPR	714.42	357.71	KDKAPR	714.42
KDAPPR	683.37	342.19	KDAPPR	683.37
KAKPPR	696.44	348.72	KAKPPR	696.44
ADKPPR	683.32	342.16	ADKPPR	683.32
KDRPPR	768.44	384.72	KDRPPR	768.44
RPPKDK	740.43	370.72	RPPKDK	740.43
rppkdk	740.43	370.42	rppkdk	740.43

5.3. NUCLEAR MAGNETIC RESONANCE (NMR):

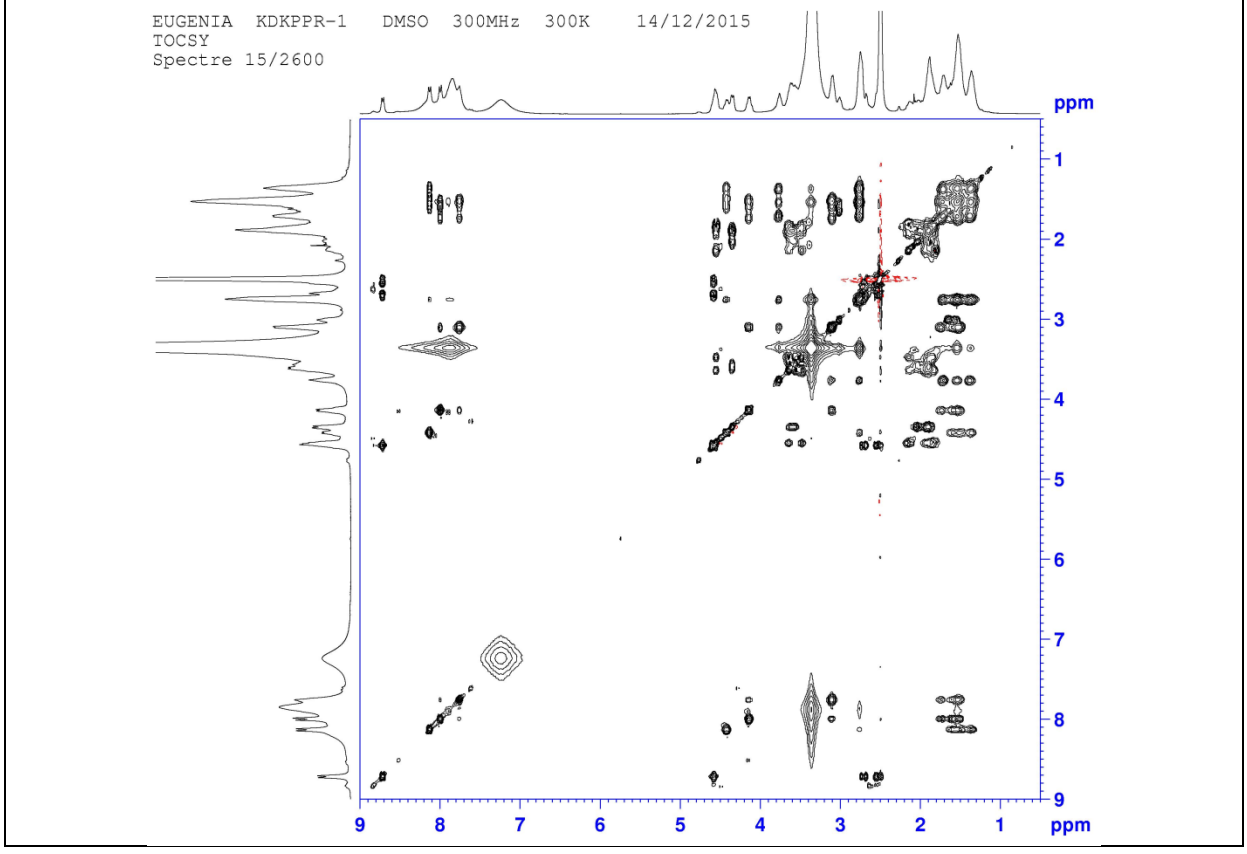
KDKPPR - Proton



KDKPPR - Cosy



KDKPPR - Tocsy



5.4. ELISA TEST:

I am waiting for the results.

5.5. CALCULATION OF THE YIELD OBTAINED:

$$\textbf{Expected yield (mg)} = \textbf{n (mmol)} \times \textbf{Mw}$$

Table 8 :*Yieldobtained.*

PEPTIDE	Amount obtained (mg)	Percentage (%)
KDKPPR	39.00	49.15
KDKPPA	51.00	77.93
KDKPAR	47.44	66.49
KDKAPR	35.37	49.58
KDAPPR	17.43	25.58
KAKPPR	22.00	31.63
ADKPPR	41.00	60.08
KDRPPR	39.00	50.82
RPPKDK	48.00	64.92
rppkdk	36.13	48.86

5.6. SUMMARY TABLE OF RESULTS:

Table 9 :*Summary of results.*

PEPTIDES								
NAME	OBTAINED(mg)	YIELD (%)	MOL. WEIGHT	MASS	PROTON	TOCSY	COSY	RETENTION(min)
KDKPPR	39,00	50,82	767,88	YES	YES	YES	YES	5,96
KDKPPA	51,00	77,93	654,76	YES	YES	YES	YES	6,81
KDKPAR	47,44	66,49	713,83	YES	YES	YES	YES	4,92
KDKAPR	35,37	49,60	713,83	YES	YES	YES	YES	5,16
KDAPPR	17,43	25,58	682,77	YES	YES	YES	YES	5,56
KAKPPR	22,00	31,63	695,85	YES	YES	YES	YES	4,46
ADKPPR	41,00	60,08	682,77	YES	YES	YES	YES	4,63
KDRPPR	39,00	50,82	767,88	YES	YES	YES	YES	5,96
RPPKDK	48,00	64,92	739,86	YES	YES	YES	YES	4,66
rppkdk	36,13	48,86	739,86	YES	YES	YES	YES	4,58

6. DISCUSSION and CONCLUSIONS:

Due to the inability to obtain ELISA result at the end of the attachment, it is not possible to draw any conclusion of the characteristics of the peptide synthesized, and of the importance of each amino acid in the interaction of KDKPPR on neuropilins-1 receptor, which is the main aim of alanine-scanning approach taken in this project. In due course, the discussion and conclusion will be made once the ELISA result is obtained.

7. BIBLIOGRAPHY:

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8. ANNEX:

8.1. ANNEX-1: Amounts of amino acids used in SPPS

Name	KDKPPR	
Derivatives	Amino acid (mg)	DMF (ml)
D	430	1,48
K	979	2,89
P	705	3,06
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R-Ø	175	

Name	KDKPPA	
Derivatives	Amino acid (mg)	DMF (ml)
D	430	1,48
P	705	3,06
K	979	2,89
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	7,2	36
Resin wang	(mg)	
A-Ø	175	

Name	KDKPAR	
Derivatives	Amino acid (mg)	DMF (ml)
A	325	1,54
D	430	1,48
P	353	1,53
K	979	2,89
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R**-Ø	164	

Name	KDKAPR	
Derivatives	Amino acid (mg)	DMF (ml)
A	325	1,54
D	430	1,48
P	353	1,53
K	979	2,89
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,1
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R**-Ø	164	

Name	KDAPPR	
Derivatives	Amino acid (mg)	DMF (ml)
A	325	1,54
D	430	1,48
P	705	3,06
K	490	1,45
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R-Ø	175	

Name	KAKPPR	
Derivatives	Amino acid (mg)	DMF (ml)
A	325	1,54
K	979	2,89
P	705	3,06
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R-Ø	175	

Name	ADKPPR	
Derivatives	Amino acid (mg)	DMF (ml)
A	325	1,54
D	430	1,48
K	490	1,45
P	705	3,06
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R-Ø	175	

Name	KDRPPR	
Derivatives	Amino acid (mg)	DMF (ml)
D	430	1,48
K	490	1,45
P	705	3,06
R	678	1,33
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
R-Ø	175	

Name	RPPKDK	
Derivatives	Amino acid (mg)	DMF (ml)
D	430	1,48
K	490	1,45
P	705	3,06
R	678	1,33
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
K-Ø	152	

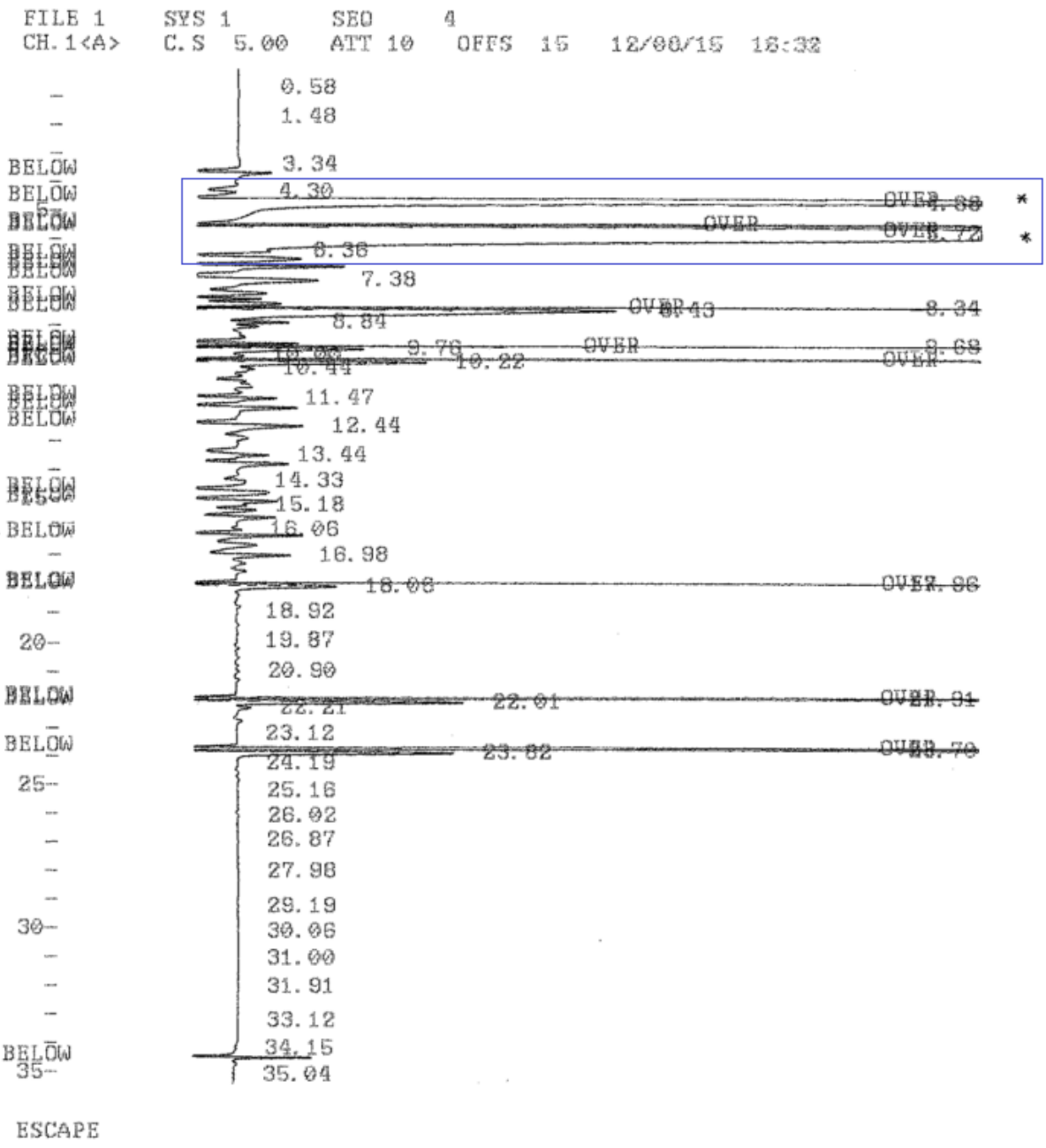
Name	rppkdk	
Derivatives	Amino acid (mg)	DMF (ml)
D	430	1,48
K	490	1,45
P	705	3,06
R	678	1,33
Reagents	Add (ml-g)	DMF (ml)
HBTU	2	8,6
NMM	1,2	2,6
Capmix	0,5	10,1
Piperidine	10,5	52,1
Resin wang	(mg)	
k-Ø	200	

8.2. ANNEX-2:Metod used in spps.

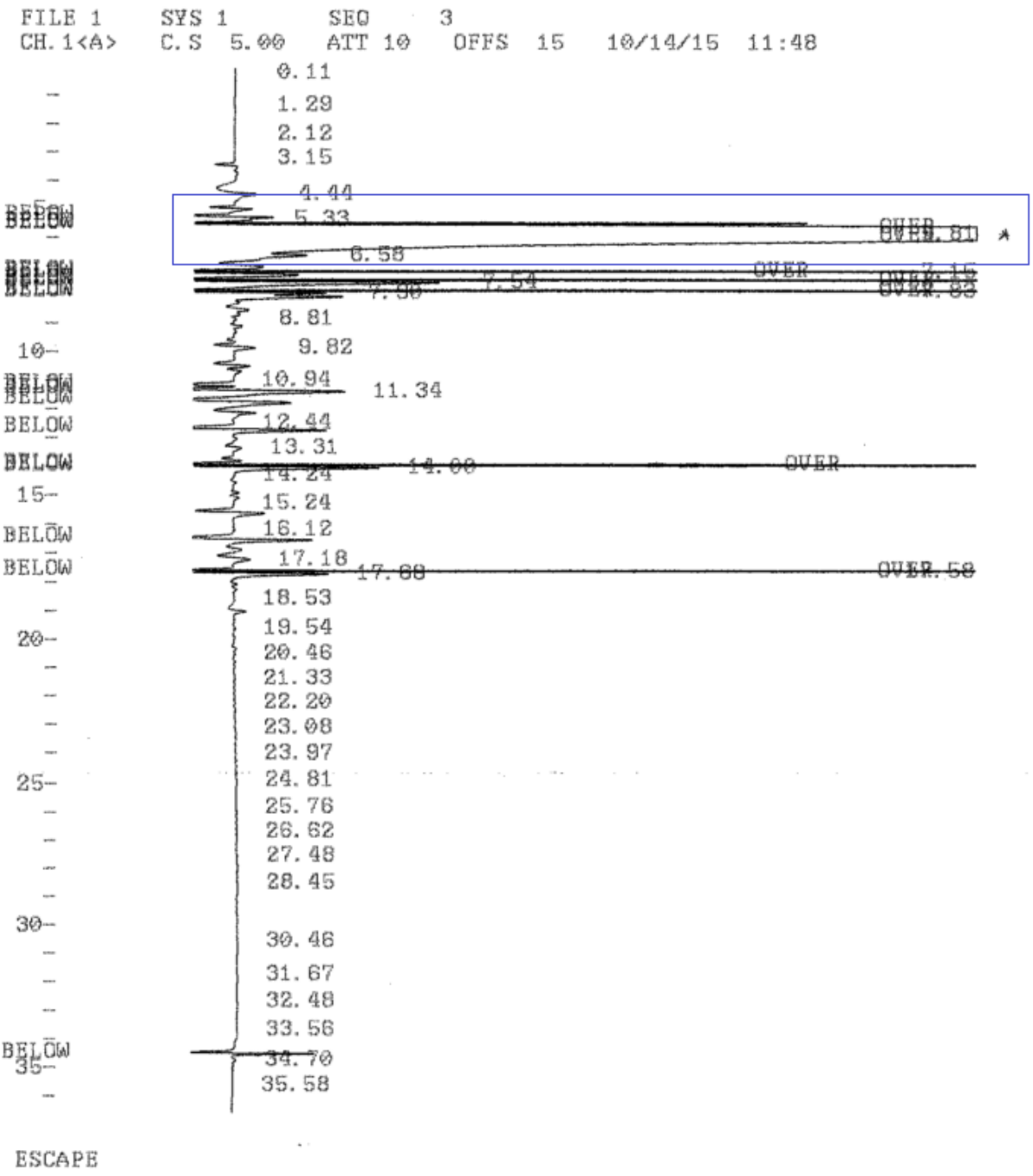
```
********** method-listing: KDKPPR x1 20-11-2015 100umole.MPM **********
********** path: C:\Documents and Settings\INTAVIS AG\Bureau\Eugenia\
** Software : MultiPep
** Version  : 22/04/2009

********** method ****************************

 Prepare

 1 Memo            Peptide synthesis using Fmoc deprotection
 2 RinseNeedle     1000 / 3900 ul
 3 WashColumns     2000 µl, Reservoir->Peptides
 4 WashColumns     2000 µl, DCM->Peptides
 5 WashColumns     2000 µl, Reservoir->Peptides
 6 WashColumns     2000 µl, Reservoir->Peptides
 7 WashColumns     2000 µl, Air->Peptides
 8 RinseNeedle     500 / 2000 ul
 ...

 Cycle :  1 -> 14  ( -> 5)

 9 Deprotection    2000 µl, Piperidine->Peptides
10 WashColumns     4500 µl, Air->Peptides
11 Deprotection    2000 µl, Piperidine->Peptides
12 Agitate         750/400 µl,  00:04 hh:mm
13 WashColumns     4500 µl, Air->Peptides
14 Deprotection    2000 µl, Piperidine->Peptides
15 Agitate         750/400 µl,  00:07 hh:mm
16 RinseNeedle     500 / 2500 ul
17 WashColumns     2000 µl, DCM->Peptides
18 WashColumns     2000 µl, DCM->Peptides
19 WashColumns     2000 µl, DCM->Peptides
20 WashColumns     2000 µl, EtOH->Peptides
21 WashColumns     2000 µl, EtOH->Peptides
22 WashColumns     2000 µl, EtOH->Peptides
23 WashColumns     2000 µl, Reservoir->Peptides
24 WashColumns     2000 µl, Reservoir->Peptides
25 WashColumns     2000 µl, Reservoir->Peptides
26 WashColumns     4500 µl, Air->Peptides
27 Coupling        830+250+20+840->Peptides /... /...
28 Agitate         750/400 µl,  00:18 hh:mm
29 WashColumns     4500 µl, Air->Peptides
30 WashColumns     2000 µl, DCM->Peptides,  3x
31 WashColumns     4500 µl, Air->Peptides
32 Coupling        830+250+20+840->Peptides /... /...
33 Agitate         750/400 µl,  00:18 hh:mm
34 WashColumns     2000 µl, DCM->Peptides,  3x
35 WashColumns     4500 µl, Air->Peptides
36 Capping         2000 µl, CapMixture->Peptides
37 WashColumns     2000 µl, DCM->Peptides
38 WashColumns     2000 µl, DCM->Peptides
39 WashColumns     2000 µl, DCM->Peptides
40 WashColumns     2000 µl, EtOH->Peptides
41 WashColumns     2000 µl, EtOH->Peptides
42 WashColumns     2000 µl, EtOH->Peptides
43 WashColumns     2000 µl, Reservoir->Peptides
44 WashColumns     2000 µl, Reservoir->Peptides
45 WashColumns     2000 µl, Reservoir->Peptides
46 WashColumns     4500 µl, Air->Peptides
 ...

 Final

47 Deprotection    2000 µl, Piperidine->Peptides
48 WashColumns     4500 µl, Air->Peptides
49 Deprotection    2000 µl, Piperidine->Peptides
50 Agitate         750/400 µl,  00:04 hh:mm
51 WashColumns     4500 µl, Air->Peptides
52 Deprotection    2000 µl, Piperidine->Peptides
53 Agitate         750/400 µl,  00:07 hh:mm
54 RinseNeedle     500 / 2500 ul
55 WashColumns     2000 µl, Reservoir->Peptides
56 WashColumns     2000 µl, Reservoir->Peptides
57 WashColumns     2000 µl, Reservoir->Peptides
58 WashColumns     2000 µl, EtOH->Peptides
59 WashColumns     2000 µl, EtOH->Peptides
60 WashColumns     2000 µl, EtOH->Peptides
61 WashColumns     2000 µl, DCM->Peptides
62 WashColumns     2000 µl, DCM->Peptides
63 WashColumns     2000 µl, DCM->Peptides
64 WashColumns     4500 µl, Air->Peptides,  2x
65 RinseNeedle     500 / 2500 ul
 ...

********** end of method *** ******************
```

8.3. ANEXX-3: Results of purification.

KDKPPR



KDKPPA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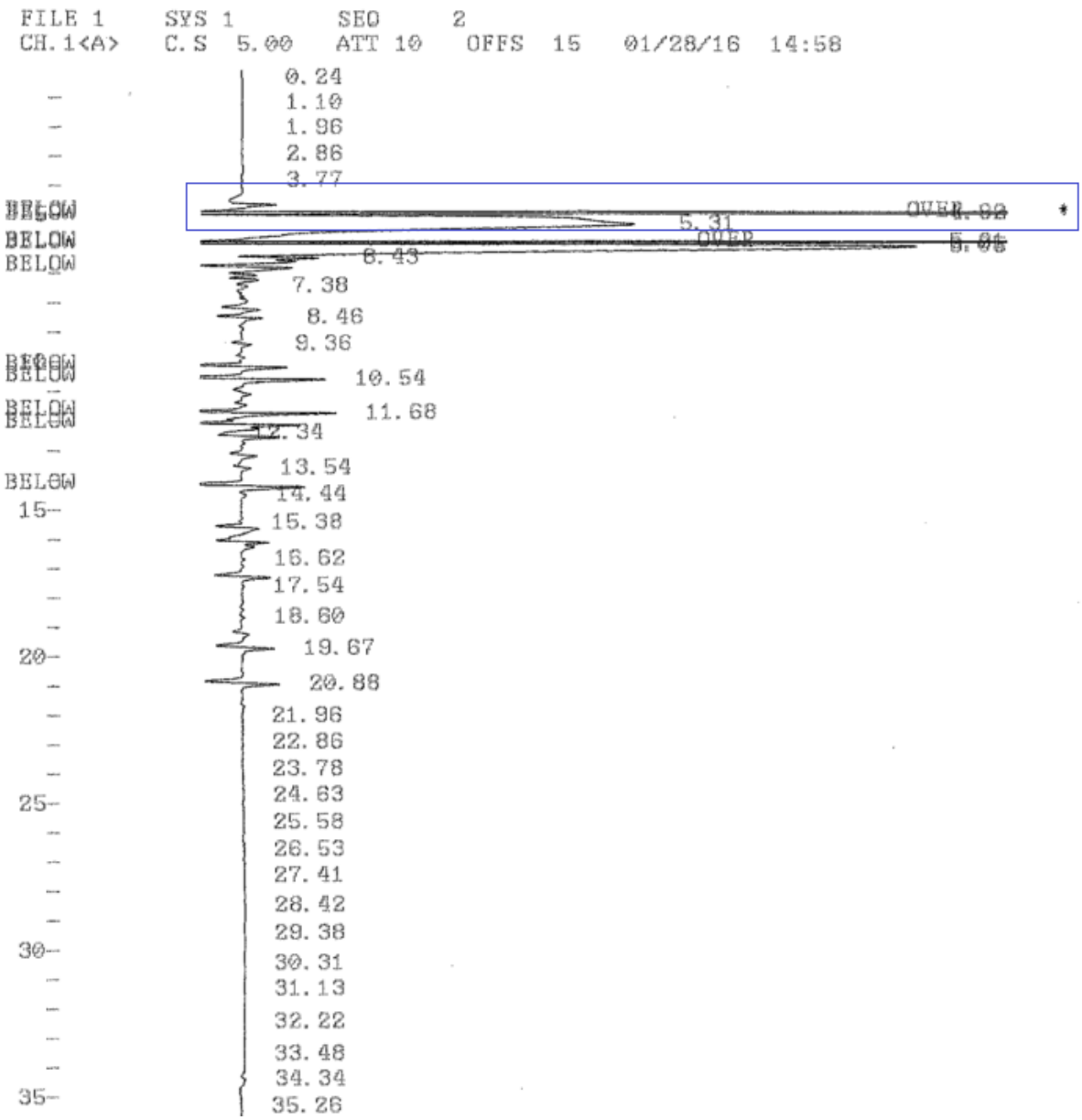
2.12
3.15
4.44
5.33
6.58
7.54
8.81
9.82
10.94
11.34
12.44
13.31
14.00
OVER
15.24
16.12
17.18
17.68
18.53
19.54
20.46
21.33
22.20
23.08
23.97
24.81
25.76
26.62
27.48
28.45
30.46
31.67
32.48
33.56
34.70
35.58
BELOW
ESCAPE

KDKPAR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0.24
1.10
1.96
2.86
3.77
BELOW
5.31
OVER
OVER
BELOW
6.43
BELOW
7.38
8.46
9.36
10.54
11.68
12.34
13.54
BELOW
14.44
15
15.38
16.62
17.54
18.60
19.67
20
20.88
21.96
22.86
23.78
24.63
25
25.58
26.53
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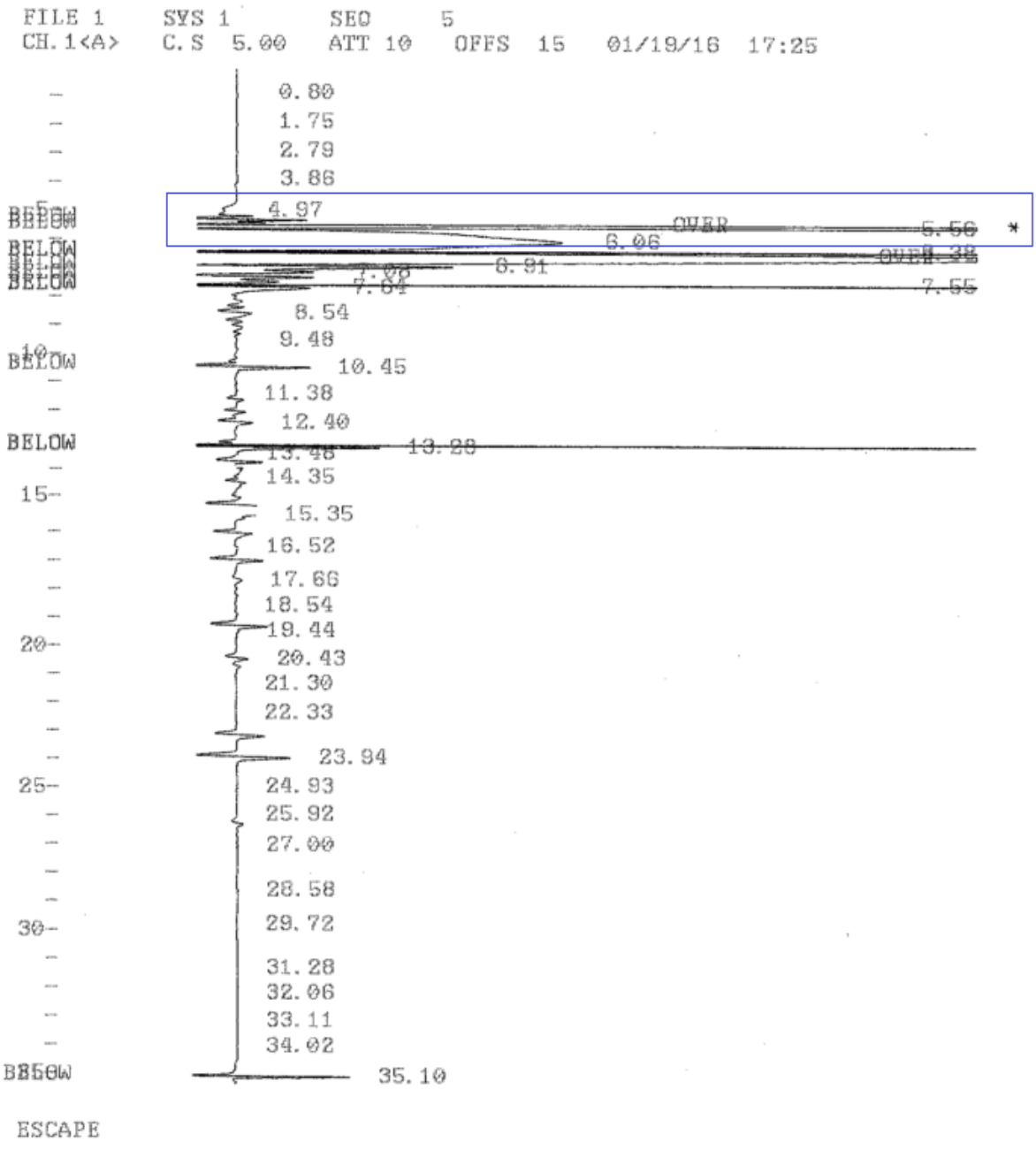
33.48
34.34
35
35.26

KDKAPR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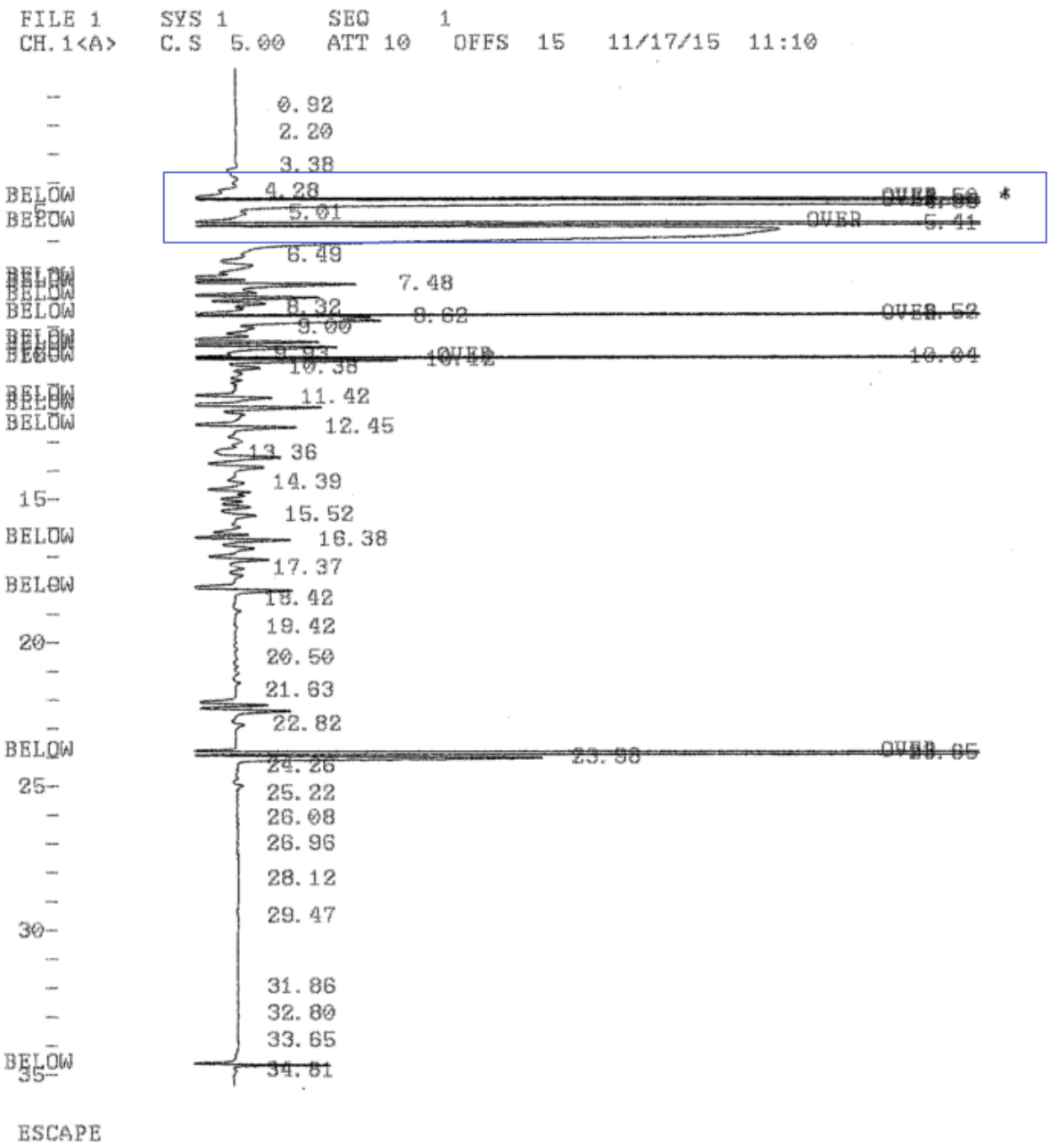
1.75
2.79
3.86
4.97
OVER
5.56
6.06
6.91
7.55
8.54
9.48
10.45
11.38
12.40
13.28
14.35
15.35
16.52
17.66
18.54
19.44
20.43
21.30
22.33
23.94
24.93
25.92
27.00
28.58
29.72
31.28
32.06
33.11
34.02
35.10
ESCAPE

KDAPPR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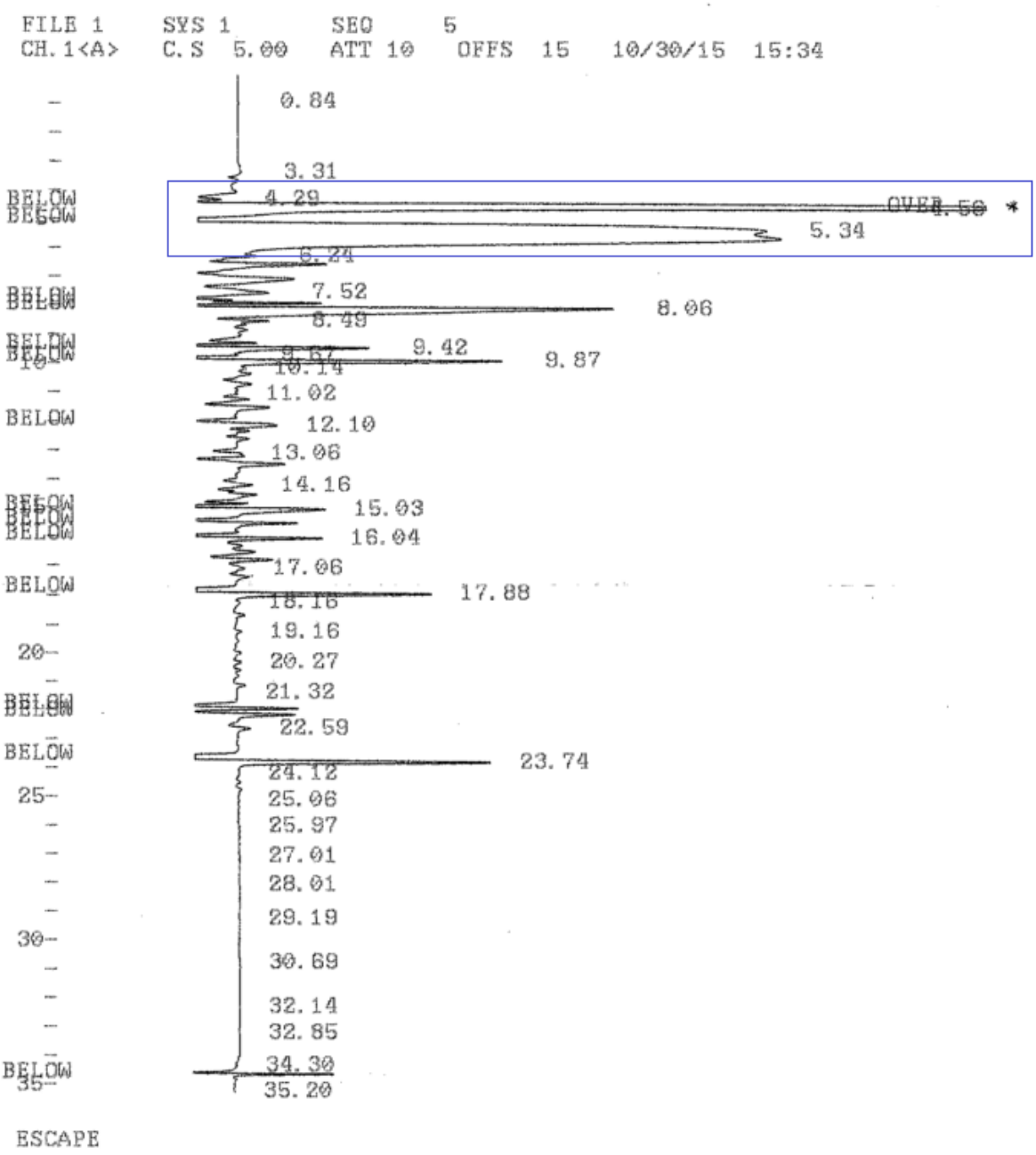
OVER 5.41
6.49
7.48
8.32
8.62
OVER 8.52
9.00
9.93
10.38
OVER 10.04
11.42
12.45
13.36
14.39
15.52
16.38
17.37
18.42
19.42
20.50
21.63
22.82
23.58
OVER 24.26
25.22
26.08
26.96
28.12
29.47
31.86
32.80
33.65
34.81
BELOW
15
20
25
30
35
ESCAPE

KAKPPR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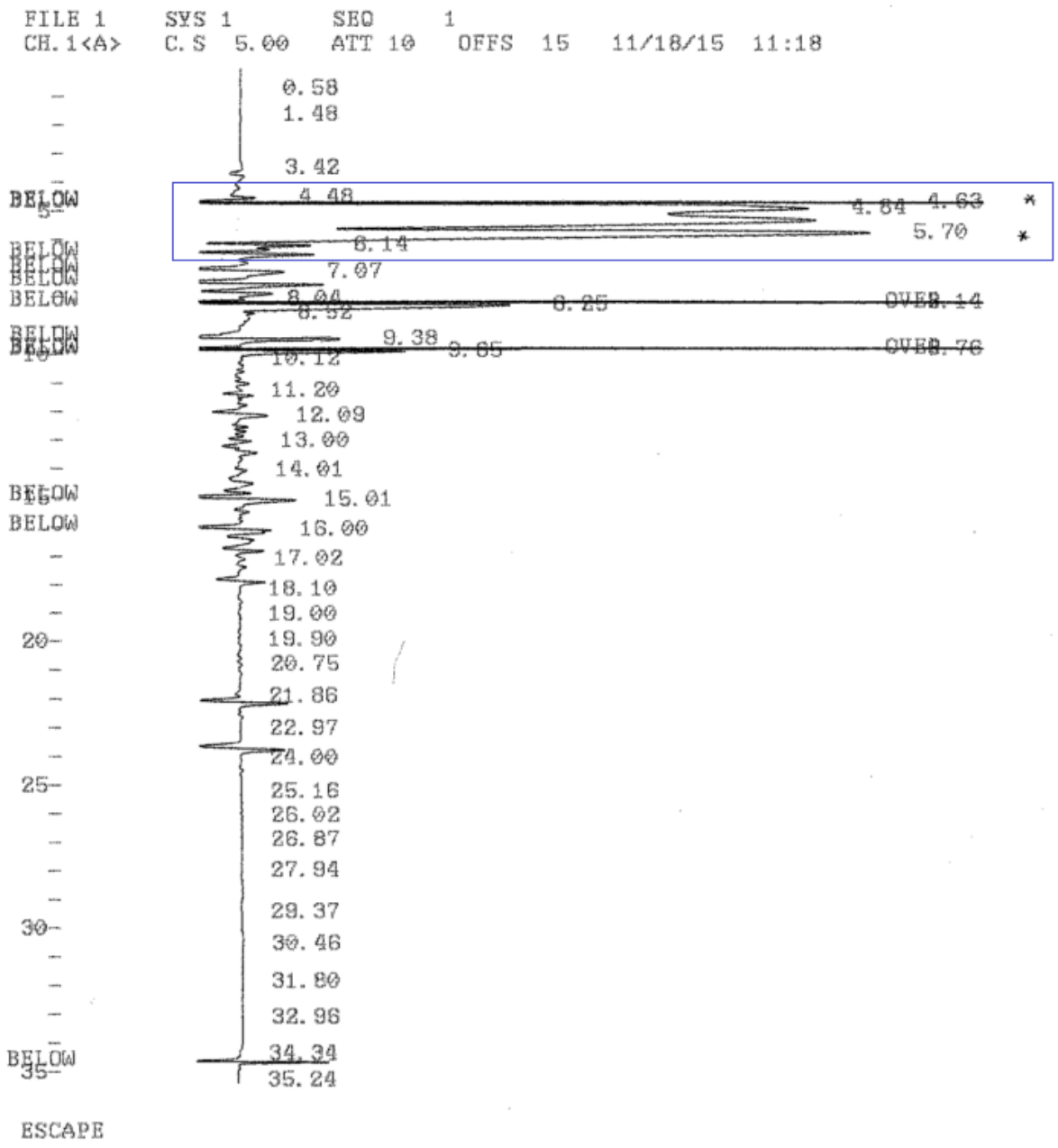
3.31
4.29
OVER 4.50 *
5.34
6.24
7.52
8.06
8.49
9.42
9.87
10.14
11.02
12.10
13.06
14.16
15.03
16.04
17.06
17.88
18.16
19.16
20.27
21.32
22.59
23.74
24.12
25.06
25.97
27.01
28.01
29.19
30.69
32.14
32.85
34.30
35.20
BELOW
ESCAPE

ADKPPR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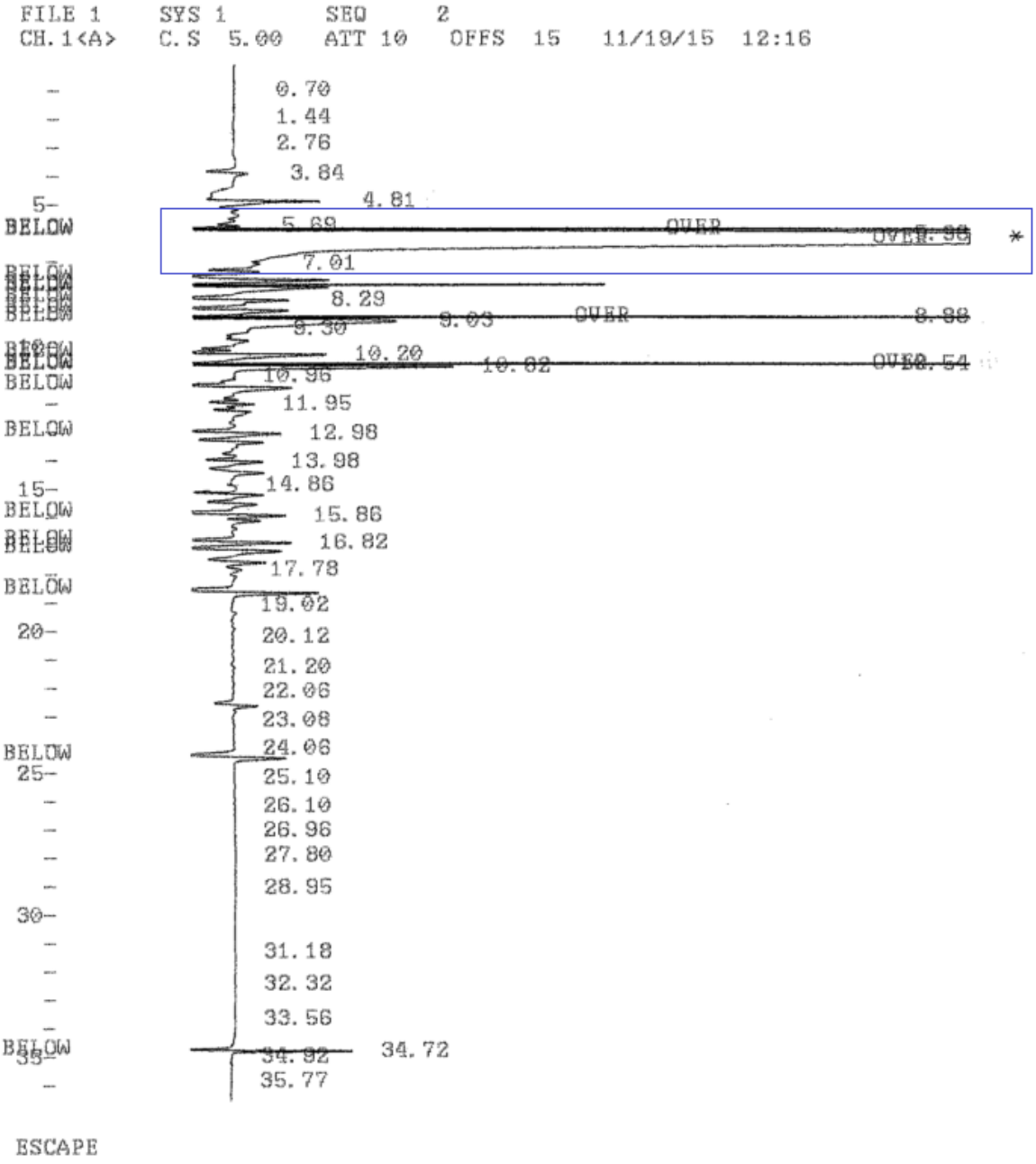
4.84
4.63
5.70
6.14
7.07
8.04
8.52
6.25
OVER
9.38
9.65
OVER
10.12
11.20
12.09
13.00
14.01
15.01
16.00
17.02
18.10
19.00
19.90
20.75
21.86
22.97
24.00
25.16
26.02
26.87
27.94
29.37
30.46
31.80
32.96
34.34
35.24
BELOW
ESCAPE

KDRPPR


FILE 1 SYS 1 SEQ 2
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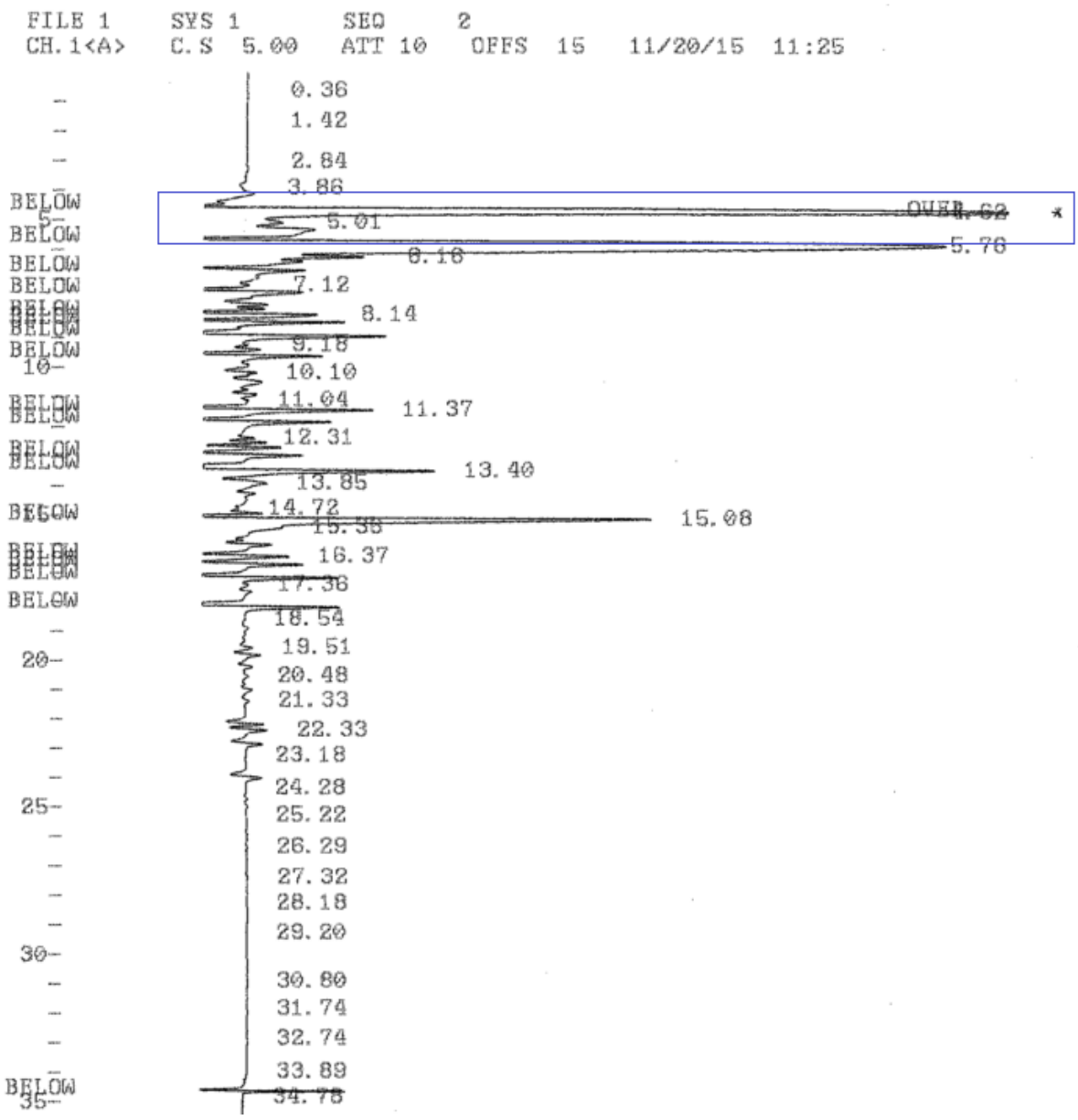
0.70
1.44
2.76
3.84
4.81
5.69
OVER
OVER
7.01
8.29
9.03
OVER
8.98
10.20
10.02
OVER
10.54
10.96
11.95
12.98
13.98
14.86
15.86
16.82
17.78
19.02
20.12
21.20
22.06
23.08
24.06
25.10
26.10
26.96
27.80
28.95
31.18
32.32
33.56
34.92
34.72
35.77
BELOW
ESCAPE

RPPKDK


FILE 1 SYS 1 SEQ 2
CH. 1<A> C.S 5.00 ATT 10 OFFS 15 11/20/15 11:25
0.36
1.42
2.84
3.86
5.01
5.76
8.16
7.12
8.14
9.18
10.10
11.04
11.37
12.31
13.40
13.85
14.72
15.08
16.37
17.36
18.54
19.51
20.48
21.33
22.33
23.18
24.28
25.22
26.29
27.32
28.18
29.20
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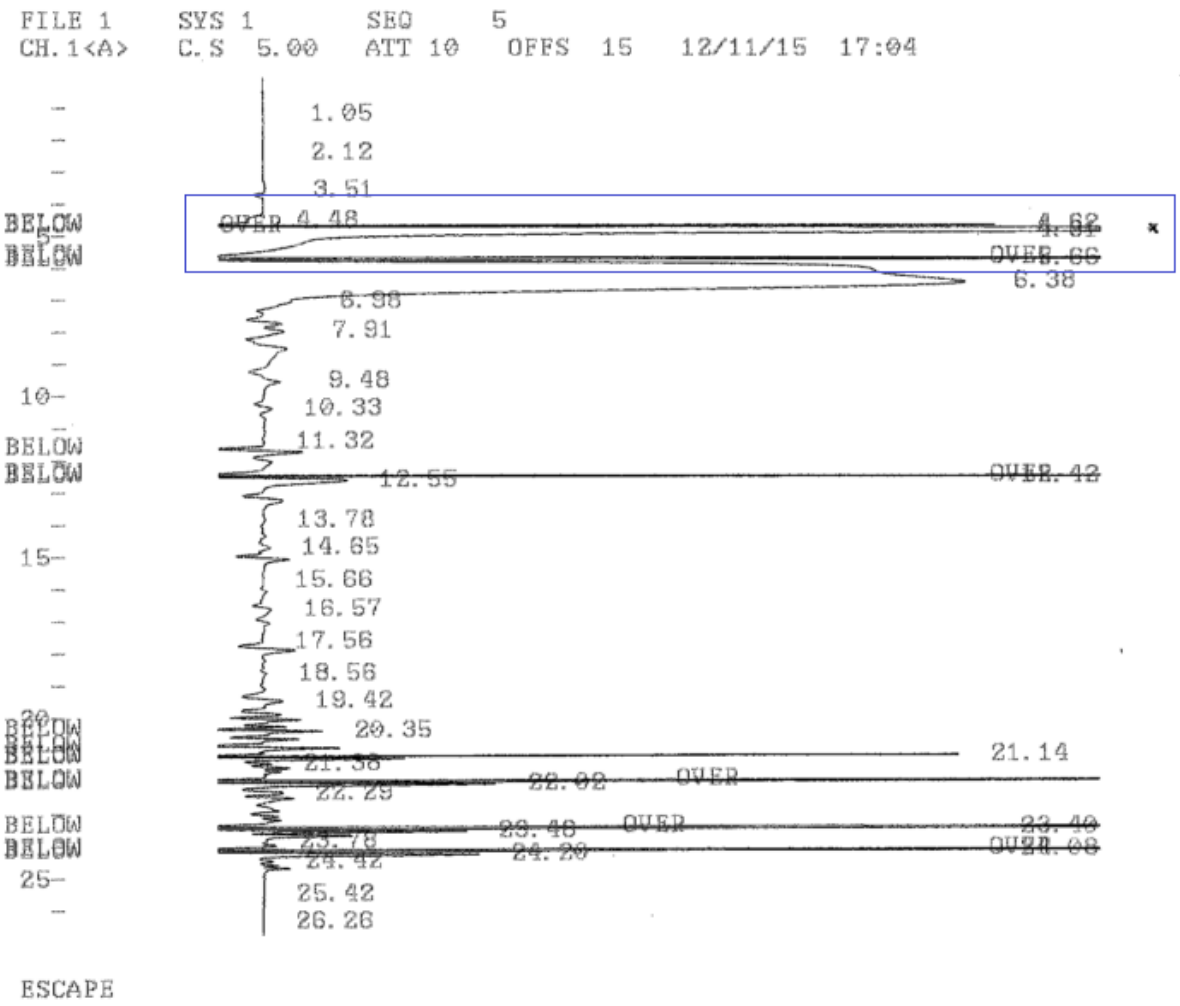
32.74
33.89
34.78
BELOW
5
10
20
25
30
35

rppkdk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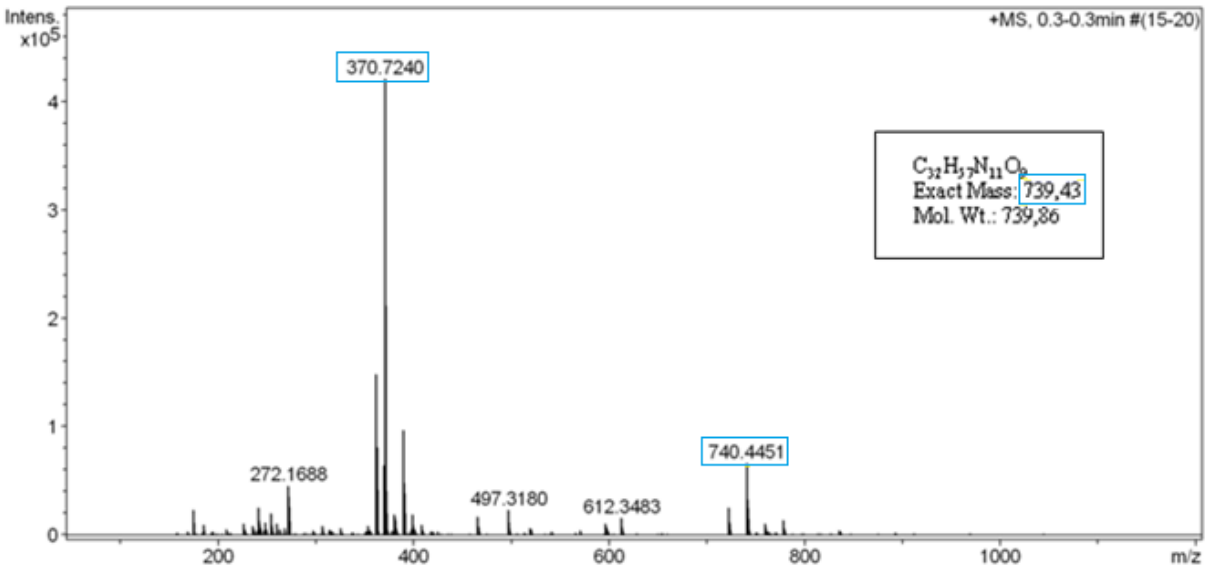
2.12
3.51
BELOW
OVER 4.48
4.62
BELOW
OVER.66
6.38
6.98
7.91
9.48
10
10.33
BELOW
11.32
BELOW
12.55
OVER.42
13.78
14.65
15
15.66
16.57
17.56
18.56
19.42
20
BELOW
20.35
BELOW
21.14
BELOW
22.02
OVER
22.29
BELOW
23.40
OVER
23.40
BELOW
24.20
OVER.08
25
25.42
26.26
ESCAPE

8.4. ANNEX-4: Mass spectrum graphics.

KDKPPR:



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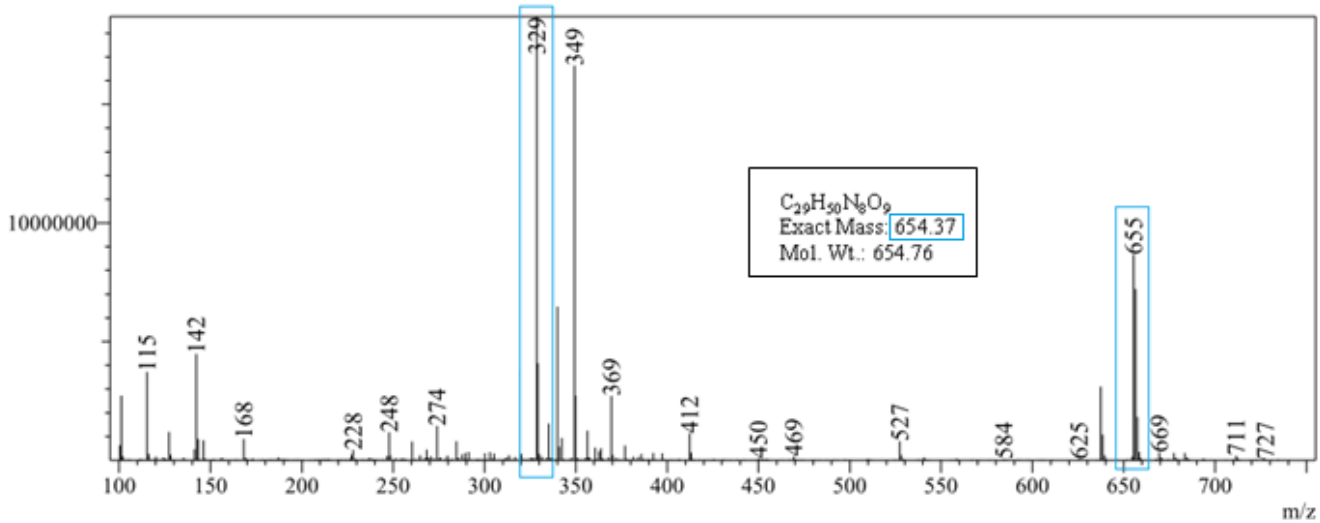

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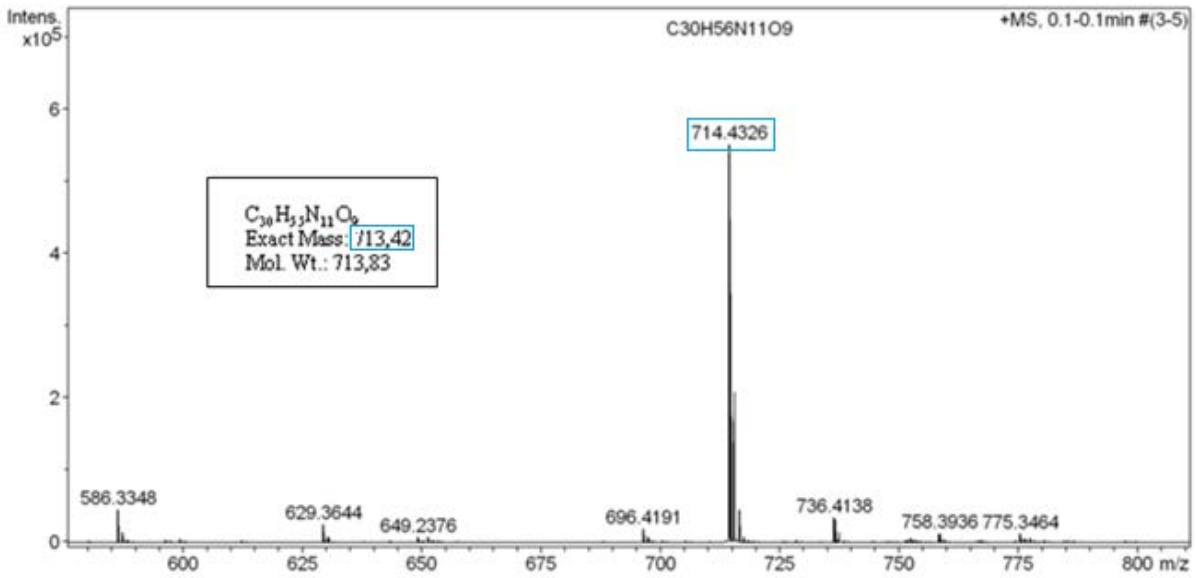

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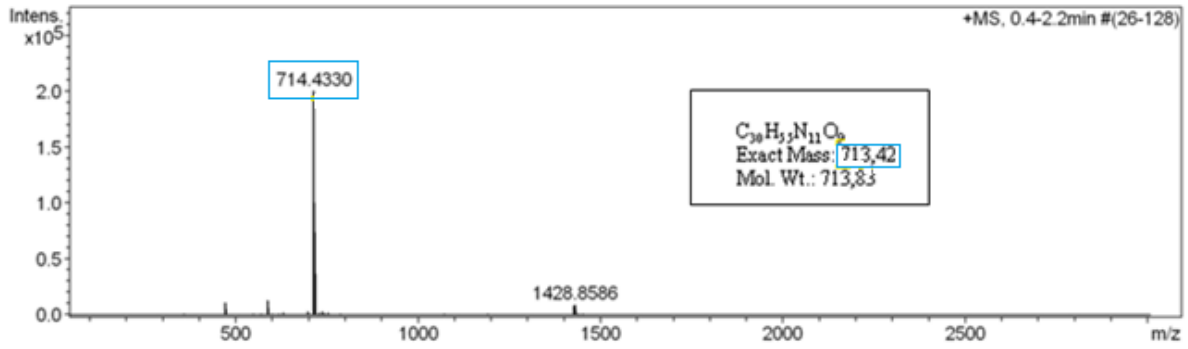

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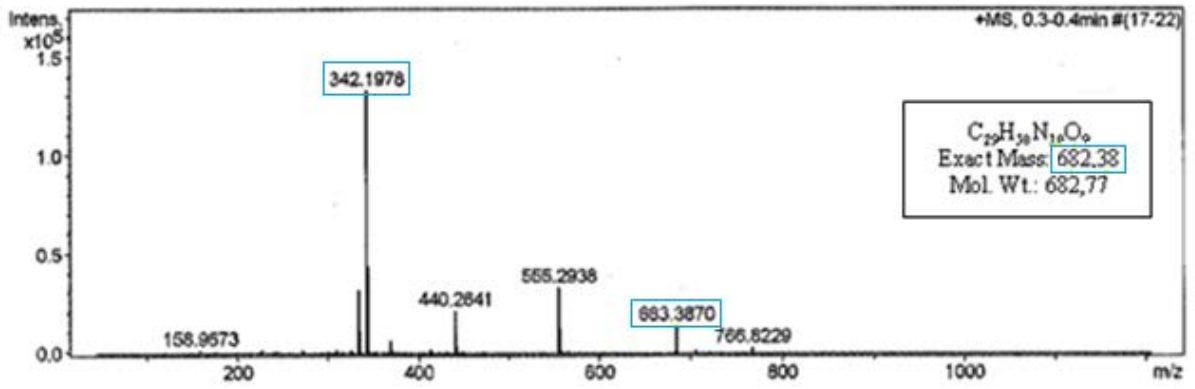

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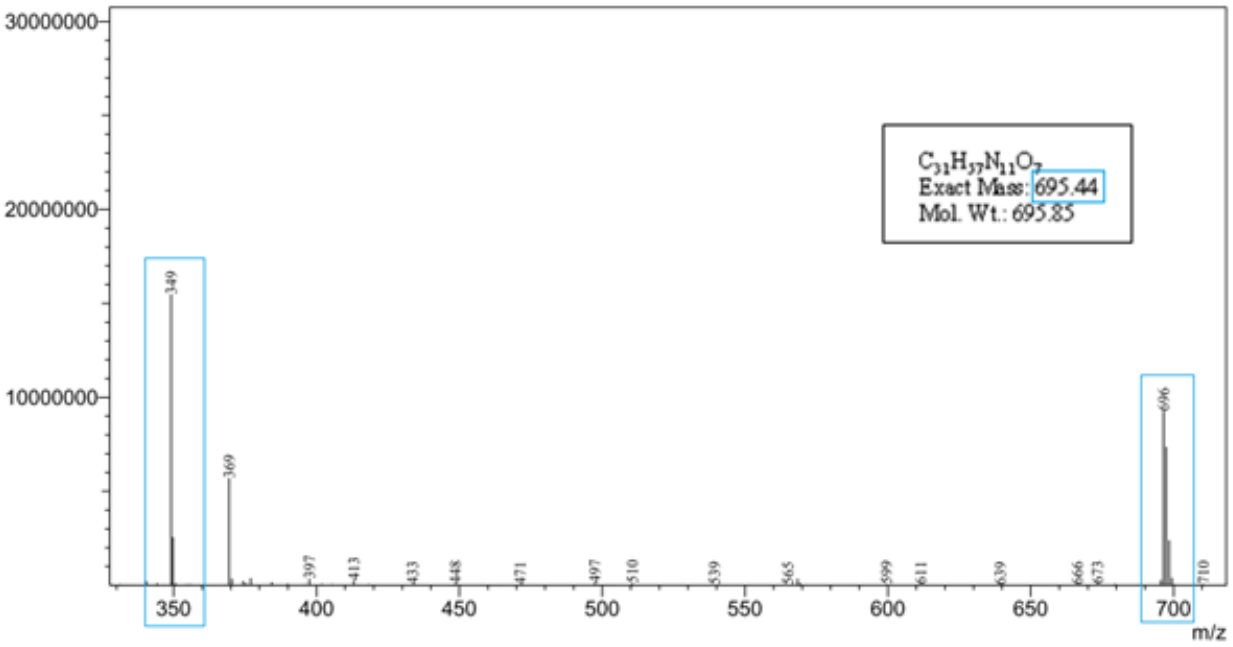

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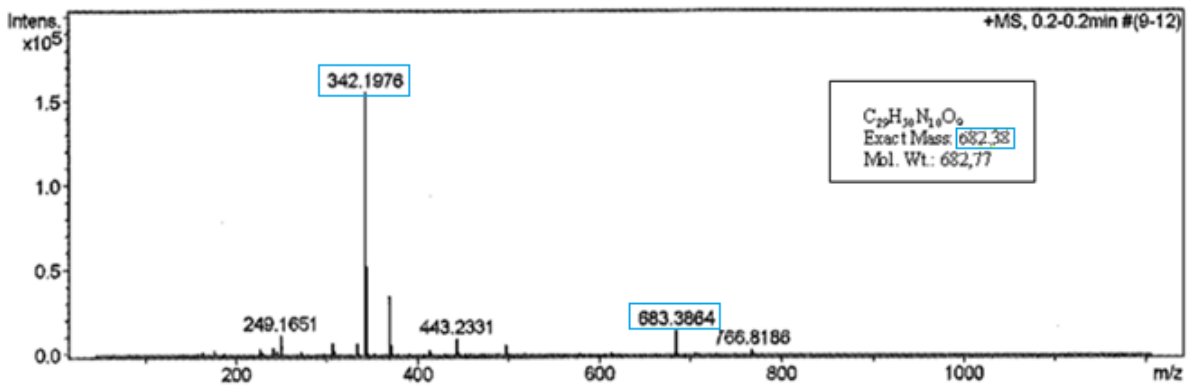

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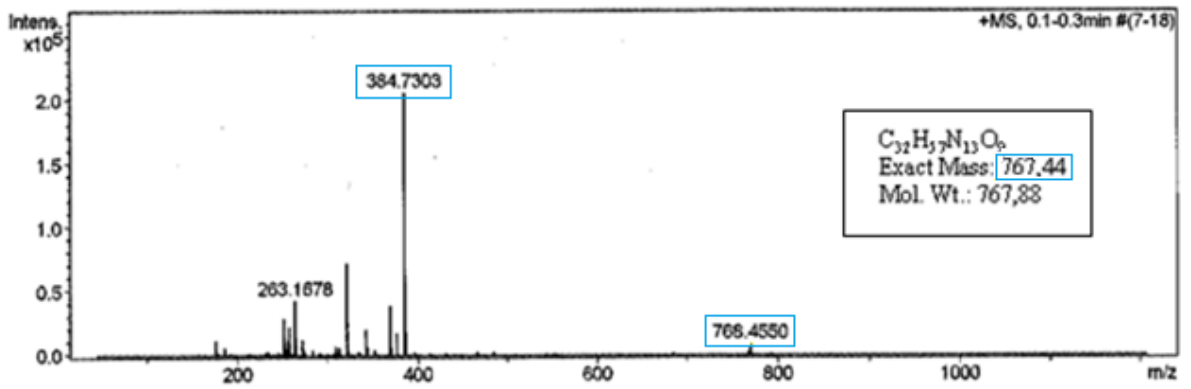
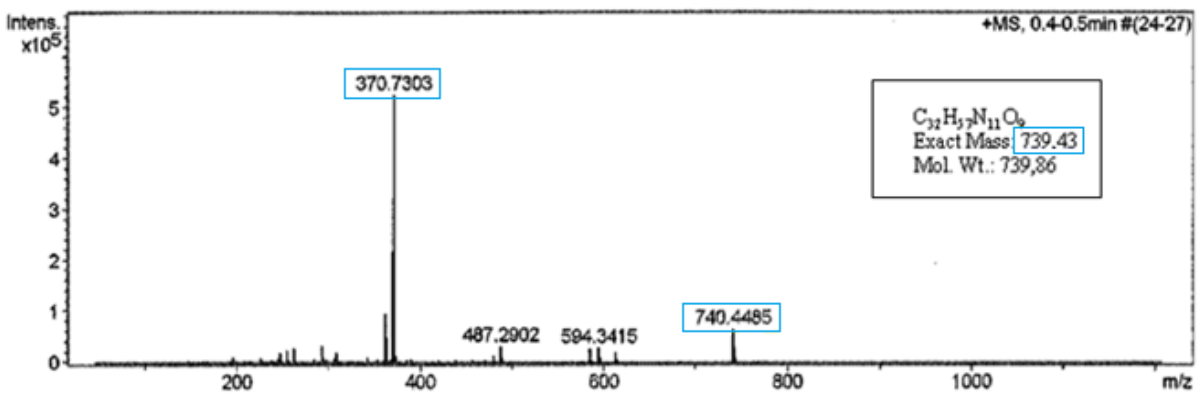
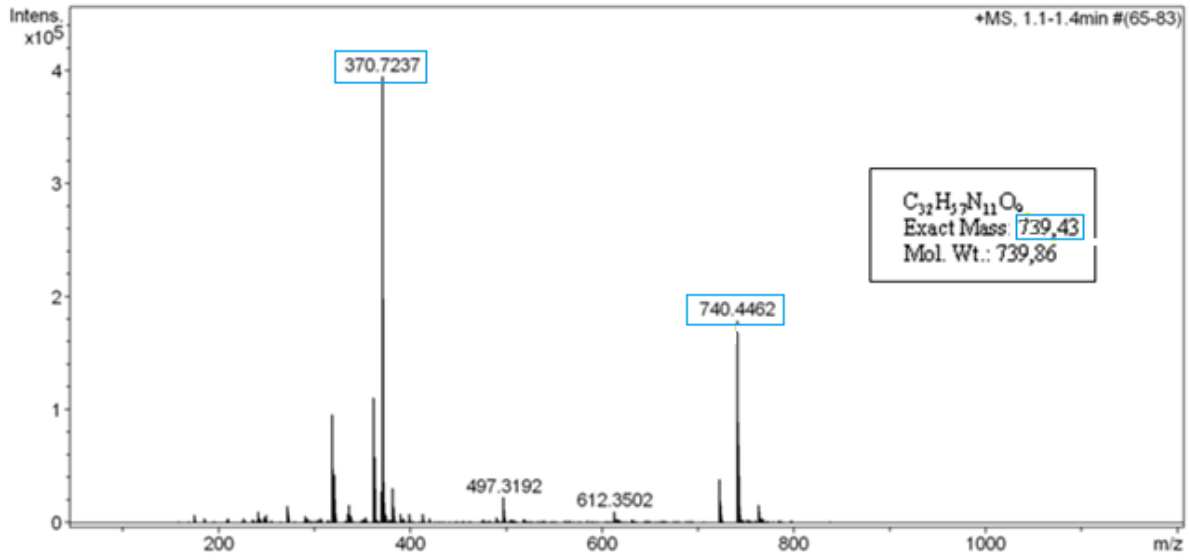


RPPKDK:

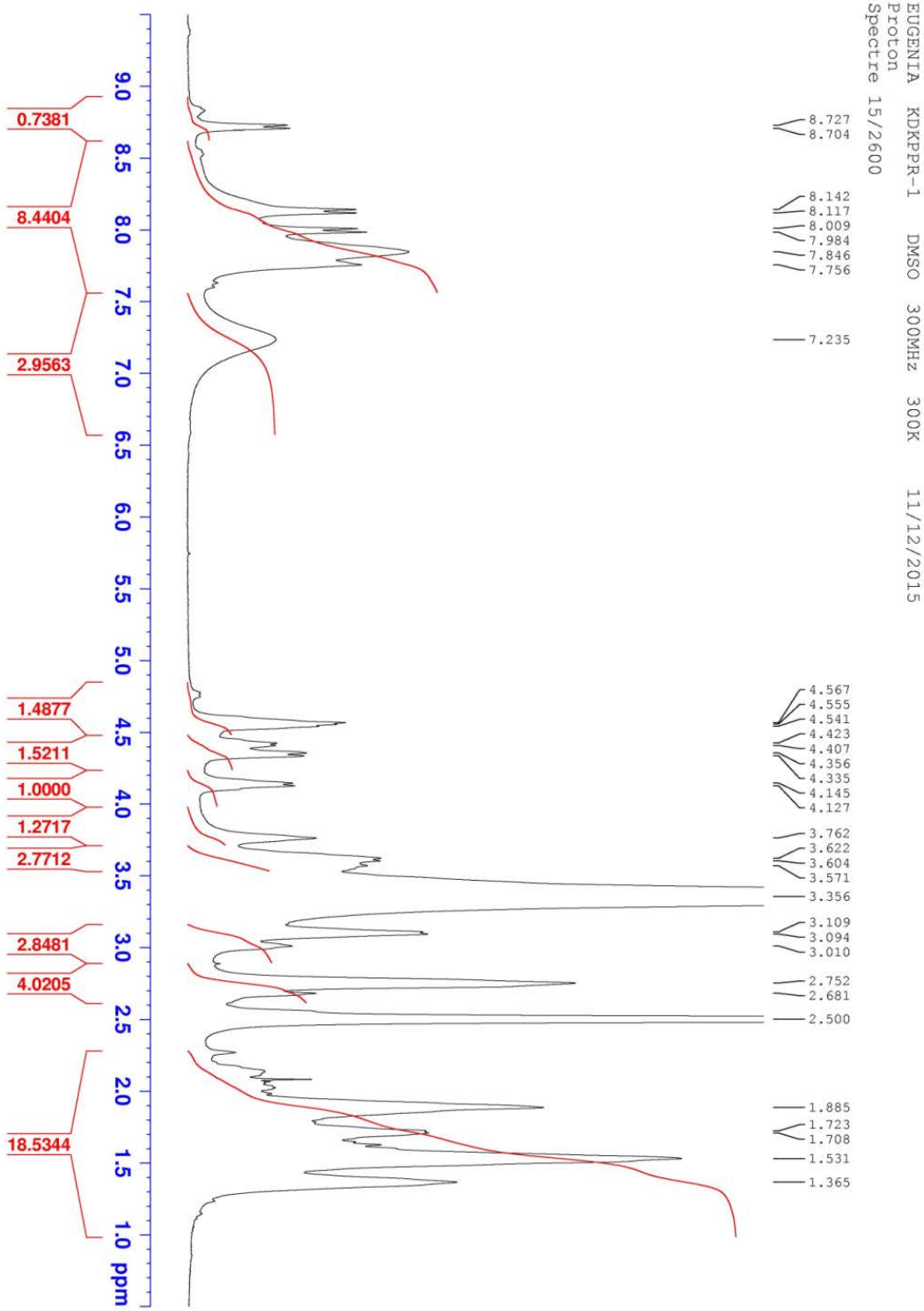


rppkdk:

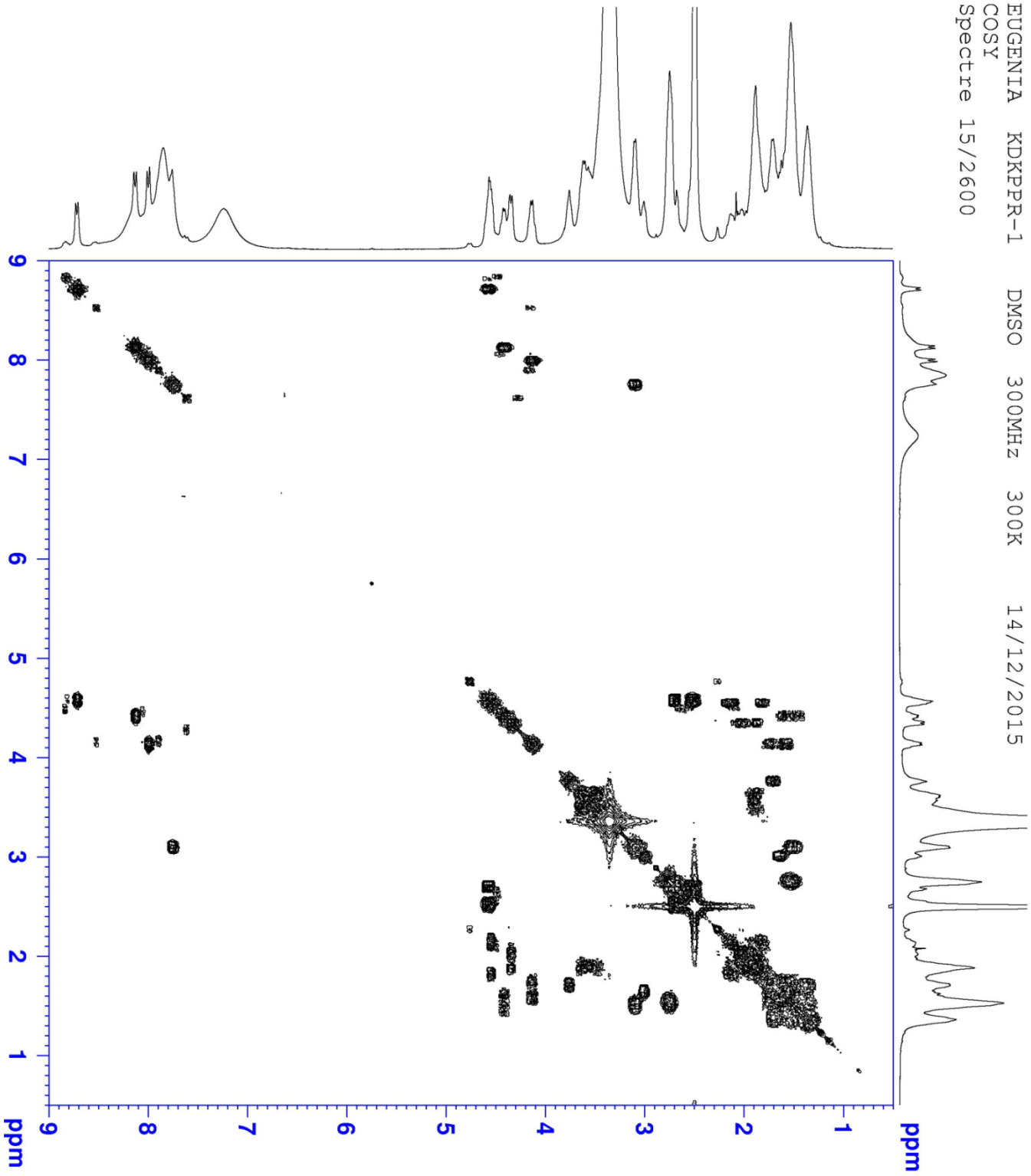

Intens.
x10^5
+MS, 1.1-1.4min #(65-83)
370.7237
740.4462
497.3192
612.3502
C32H57N11O6
Exact Mass: 739,43
Mol. Wt.: 739,86
m/z

8.5. ANNEX-5:*NMR graphics.*

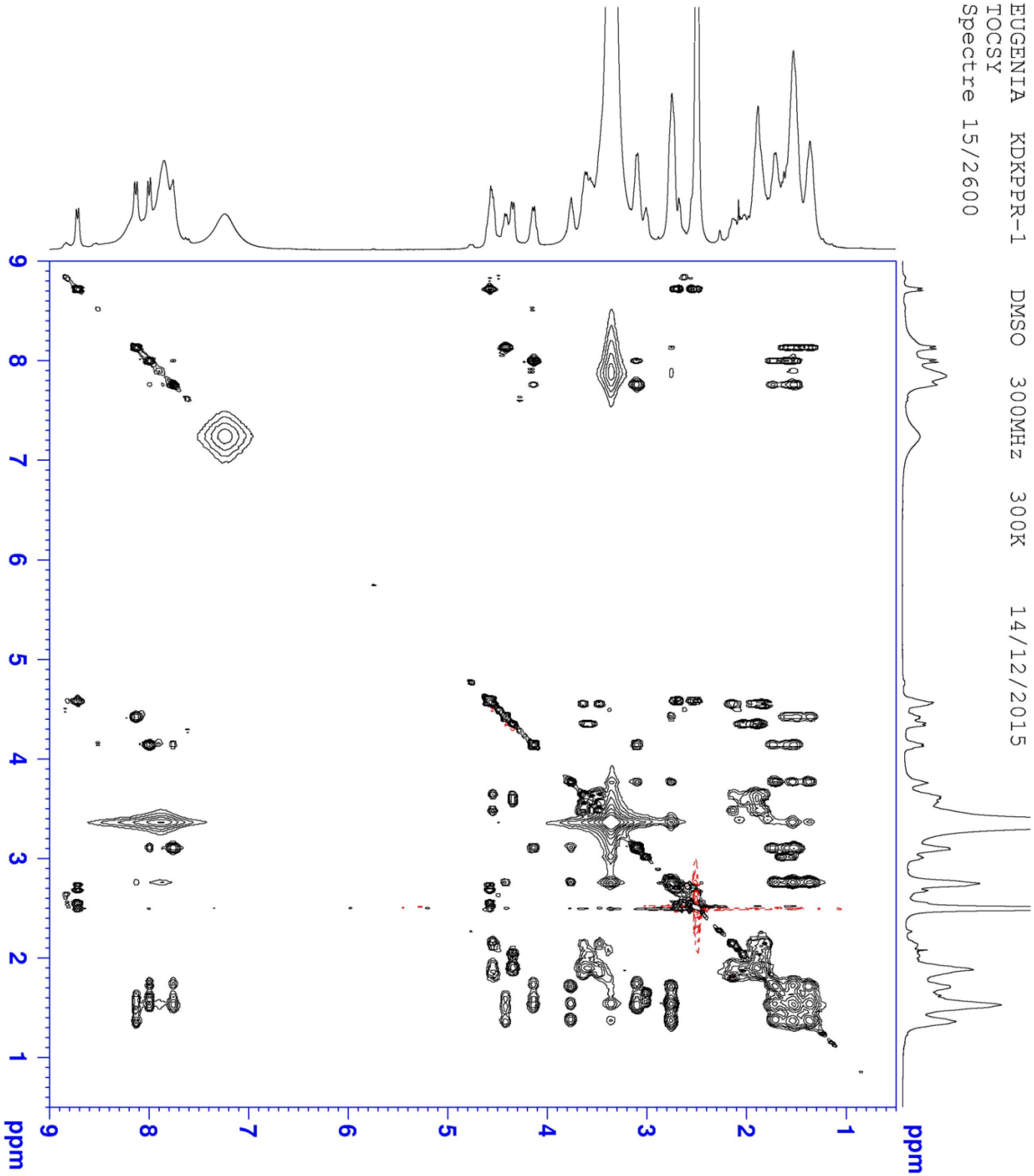
KDKPPR-Proton



KDKPPR-Cosy


EUGENIA KDKPPR-1 DMSO 300MHz 300K 14/12/2015
COSY
Spectre 15/2600
ppm
ppm

KDKPPR-Tocsy



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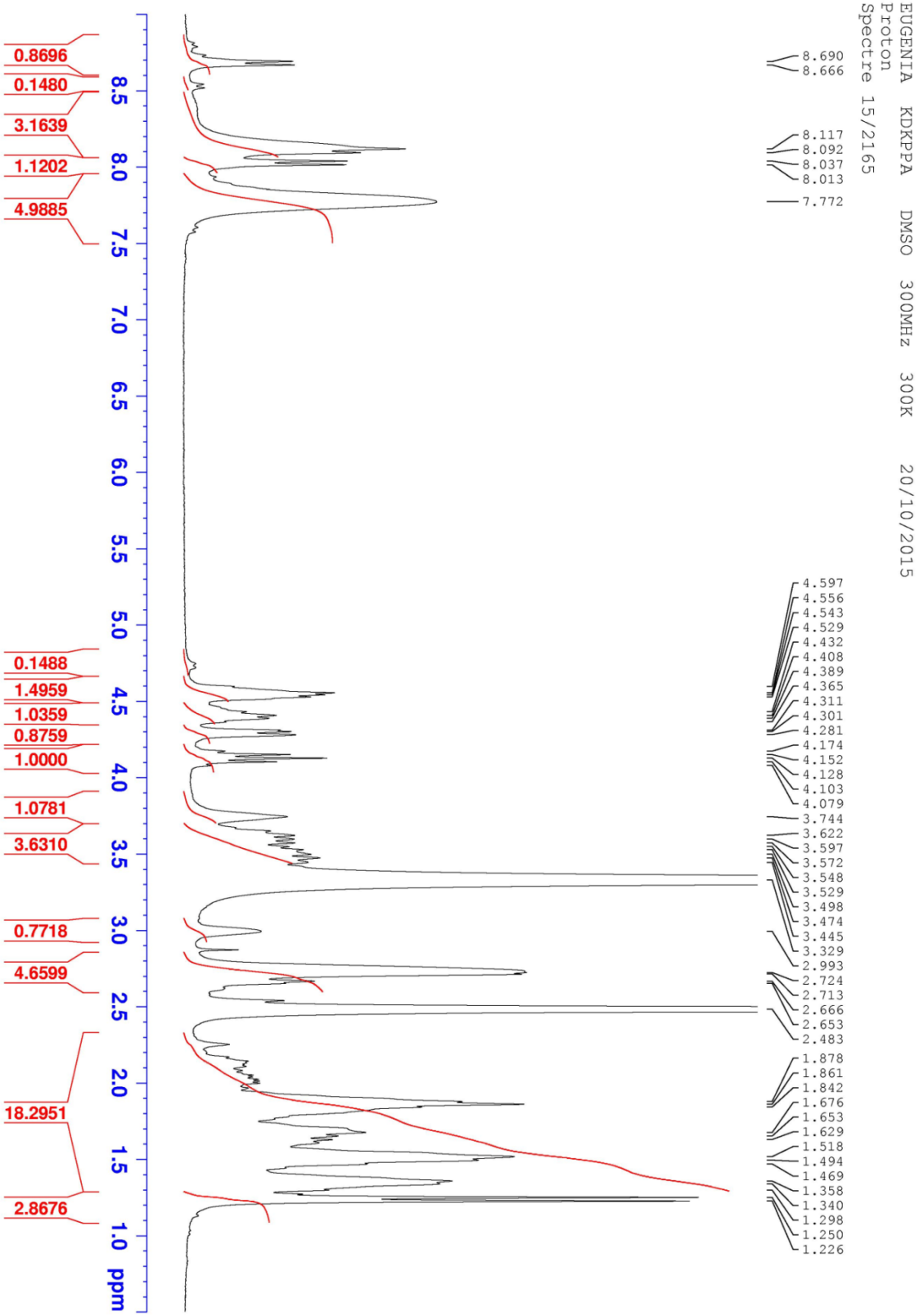

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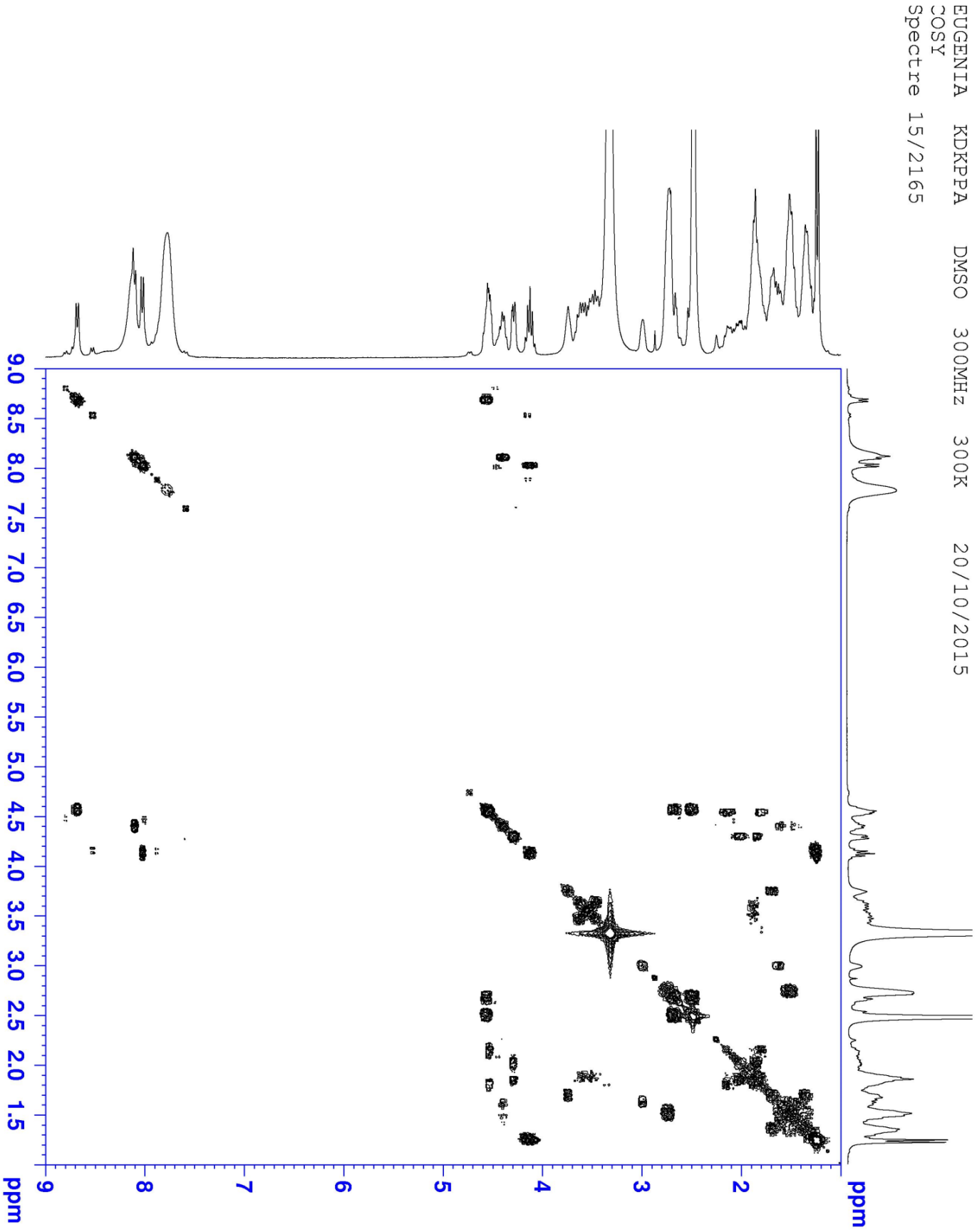
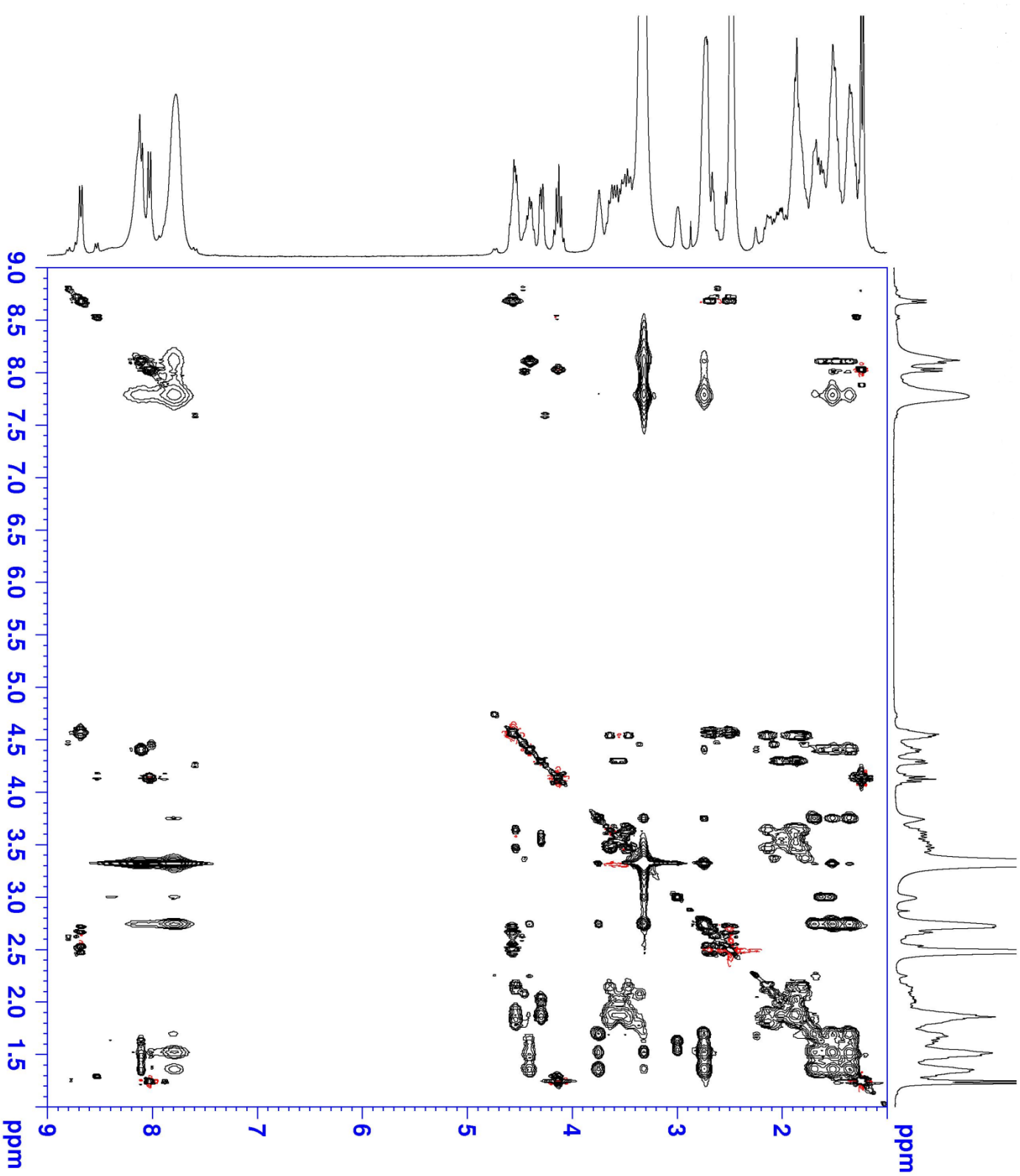
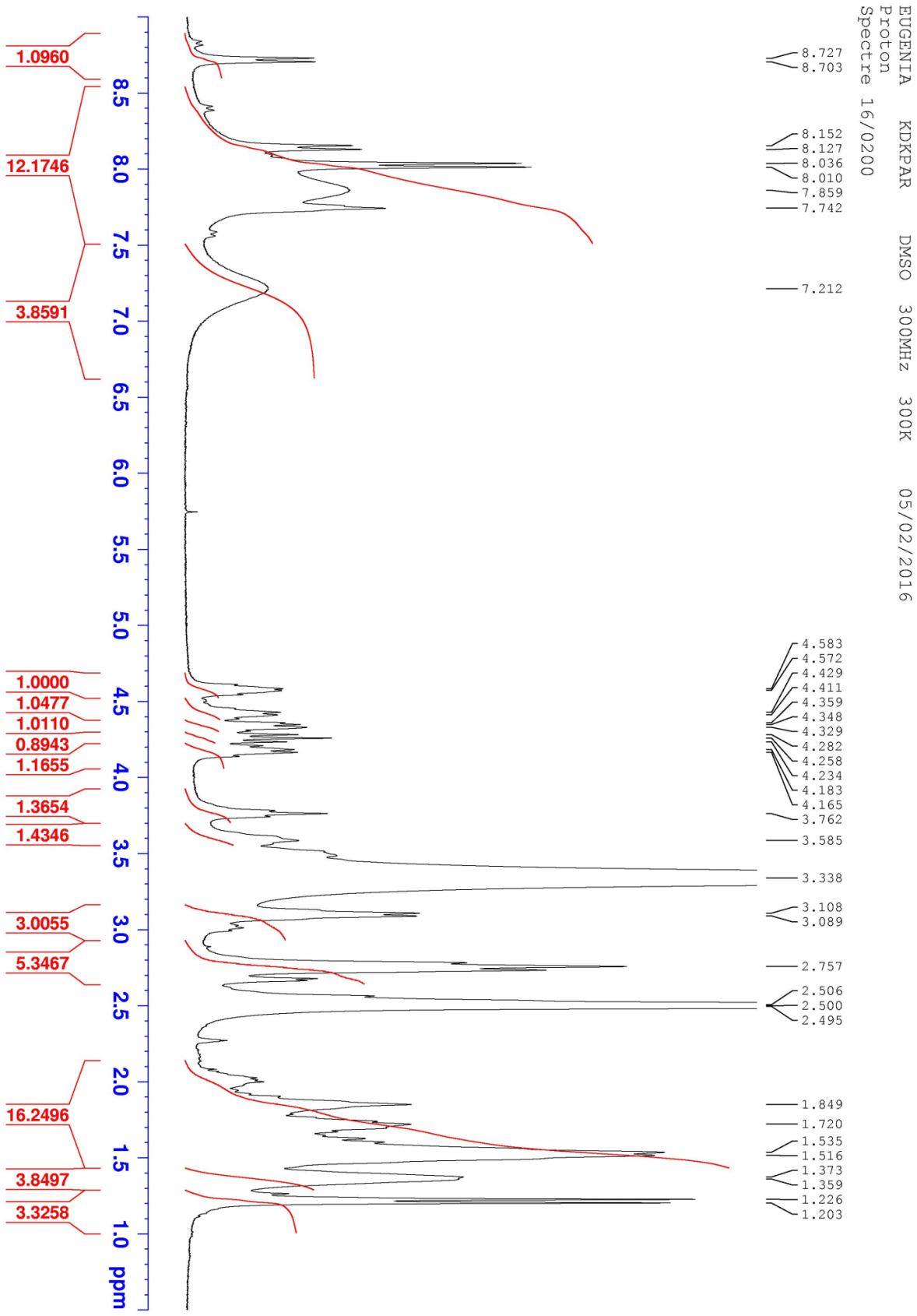

EUGENIA KDKPPA DMSO 300MHz 300K 20/10/2015
COSY
Spectre 15/2165
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5
ppm
9 8 7 6 5 4 3 2
ppm

KDKPPA-Tocsy



KDKPAR-Proton



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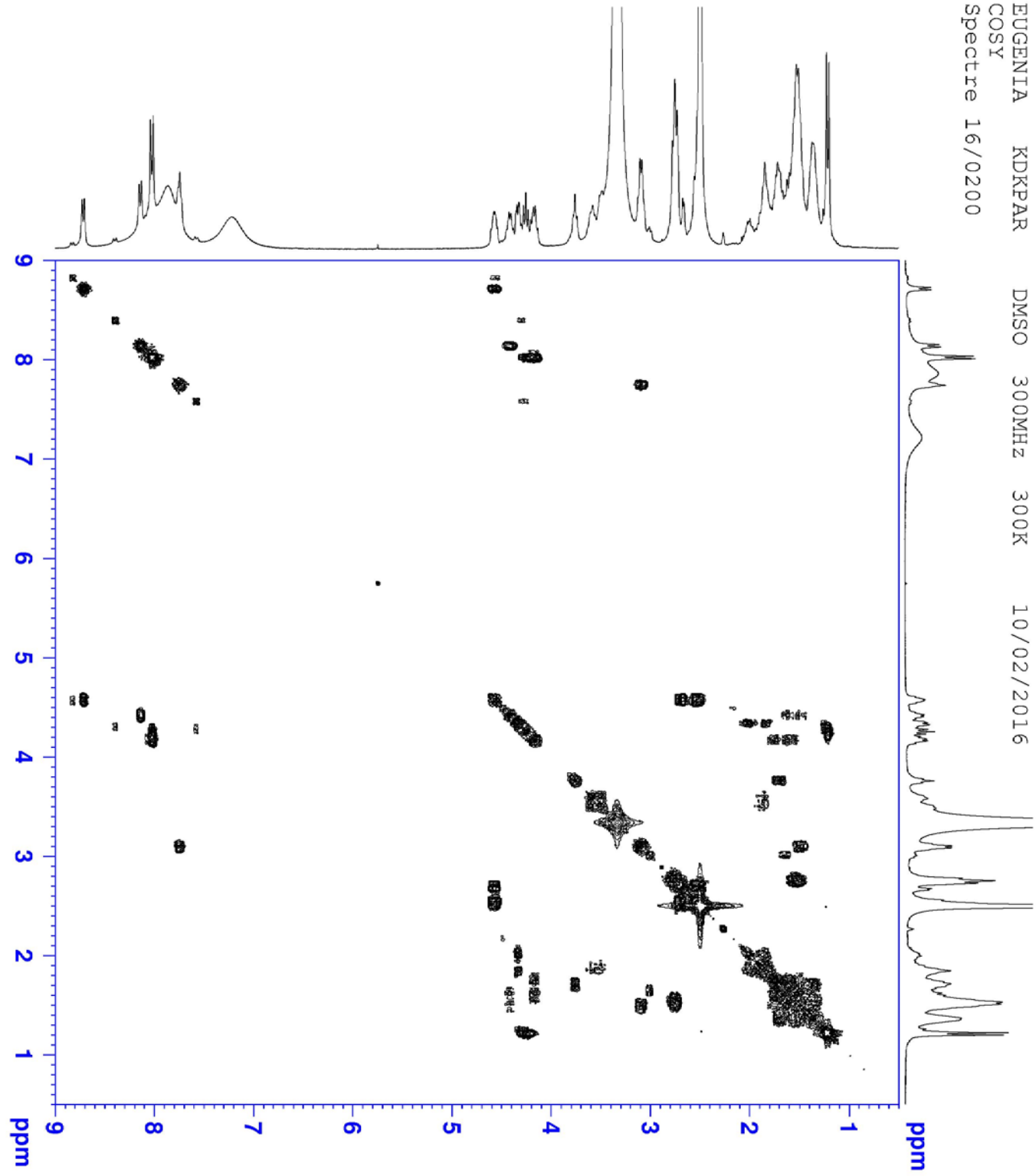
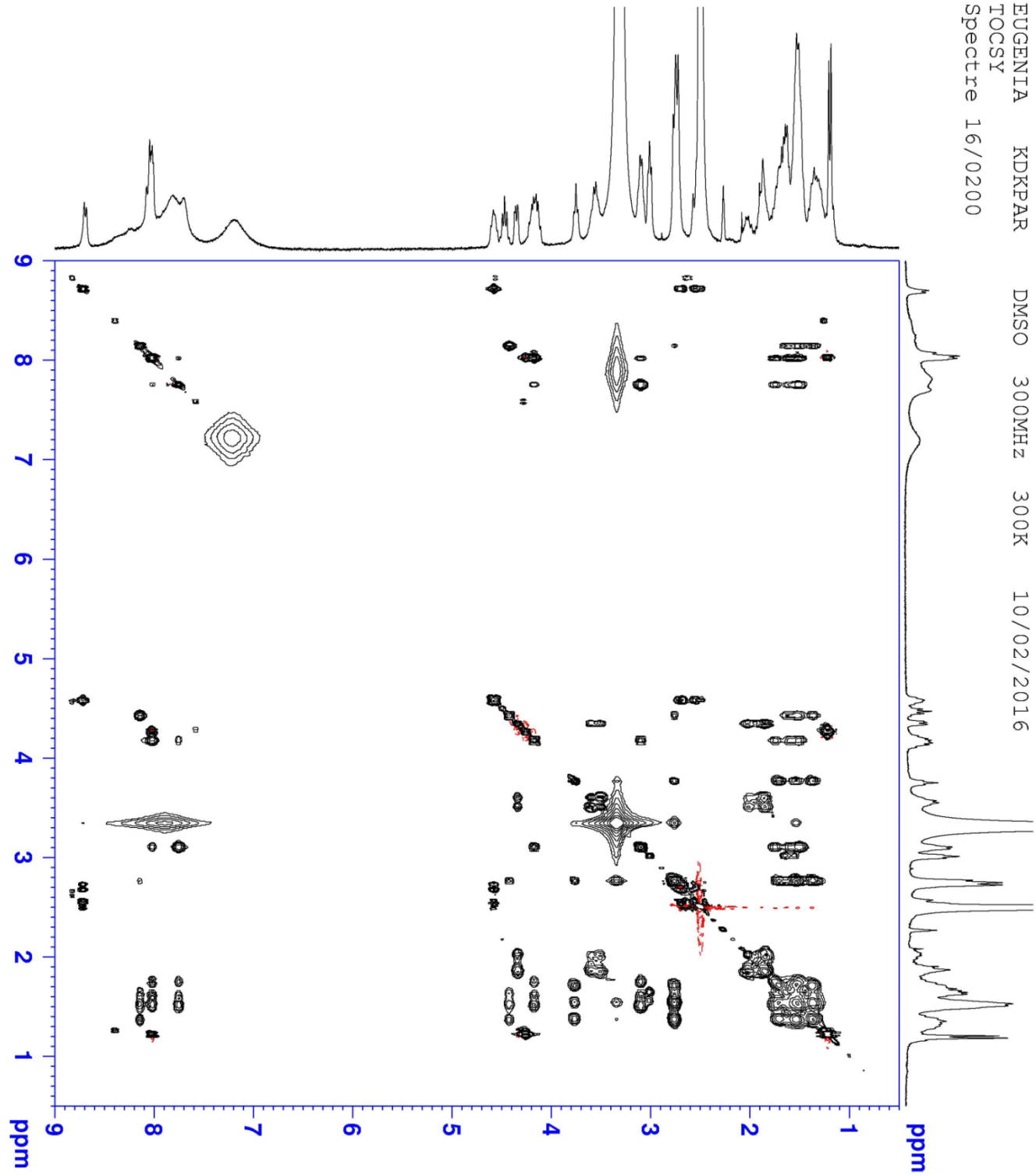

EUGENIA KDKPAR DMSO 300MHz 300K 10/02/2016
COSY
Spectre 16/0200
9
8
7
6
5
4
3
2
1
ppm

KDKPAR-Tocsy



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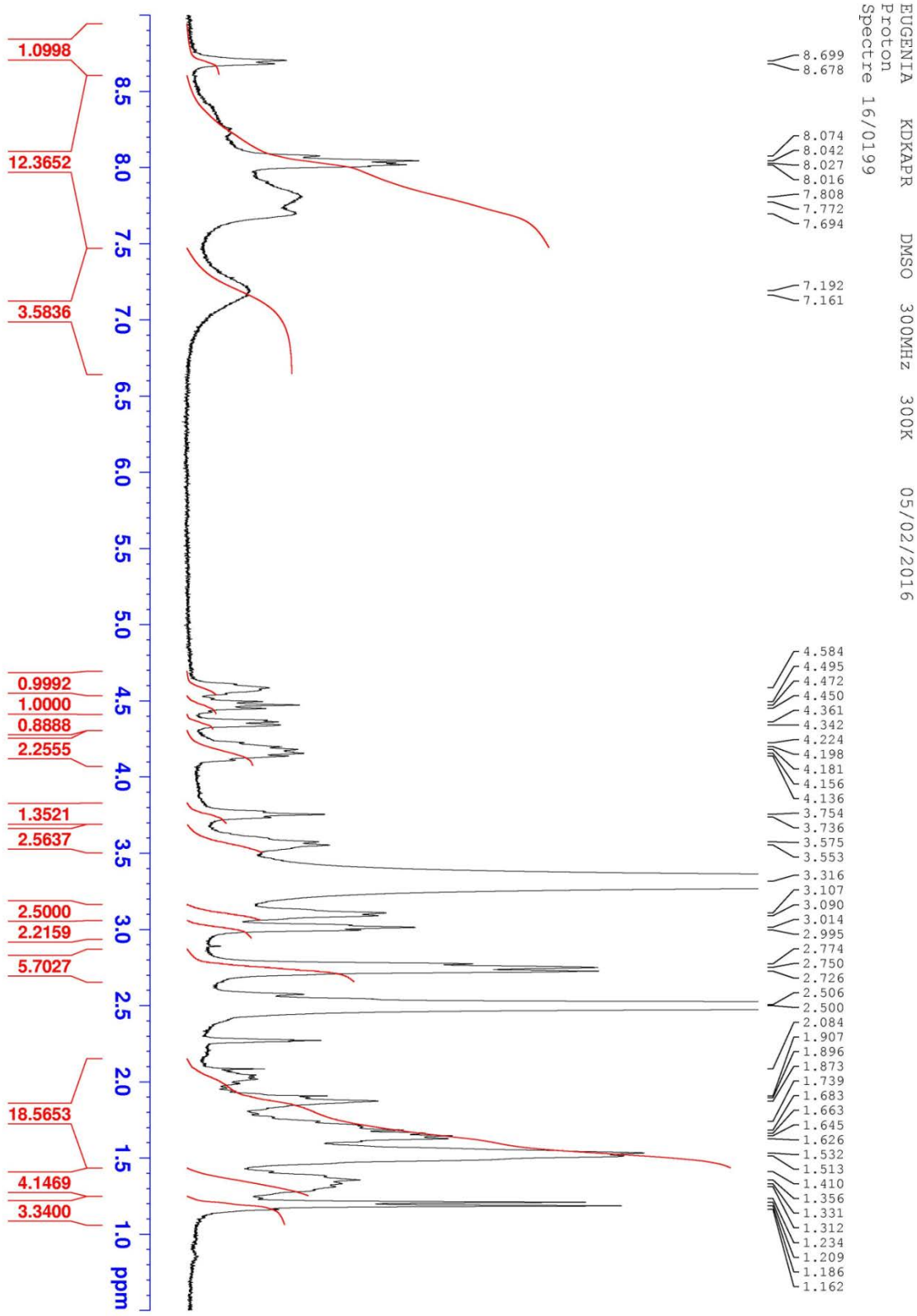

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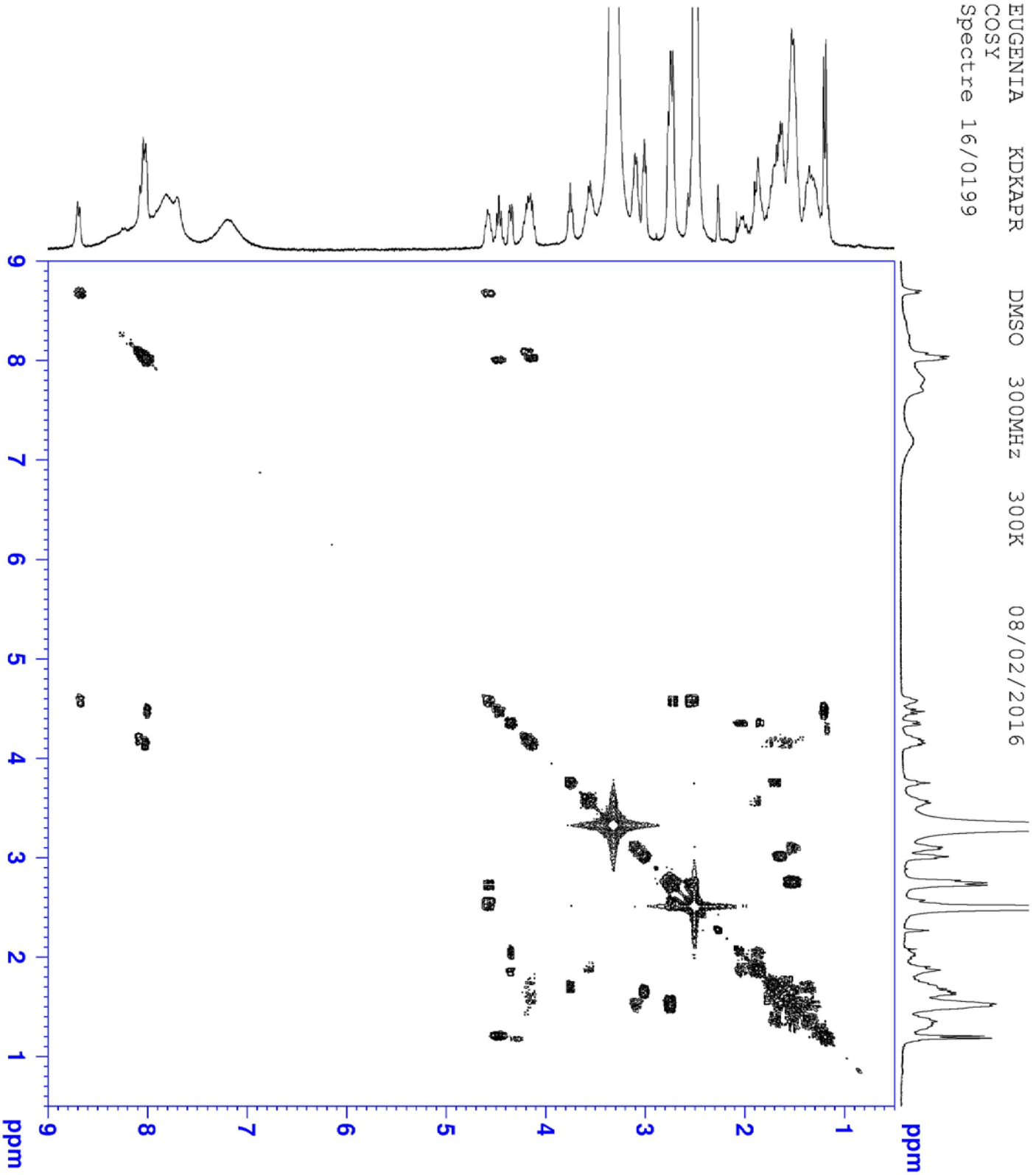
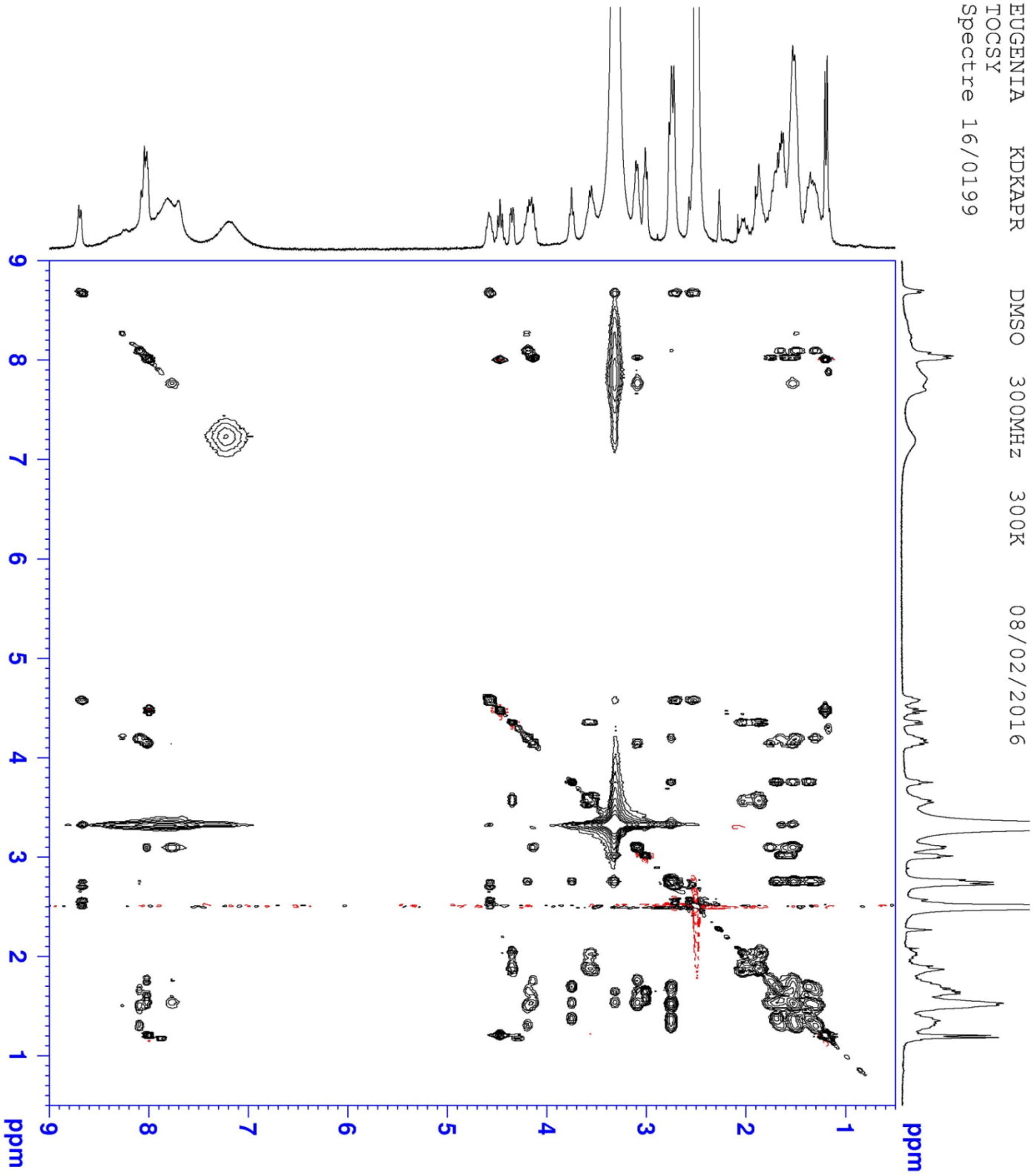

EUGENIA KDKAPR DMSO 300MHz 300K 08/02/2016
COSY
Spectre 16/0199
9
8
7
6
5
4
3
2
1
ppm

KDKAPR-Tocsy



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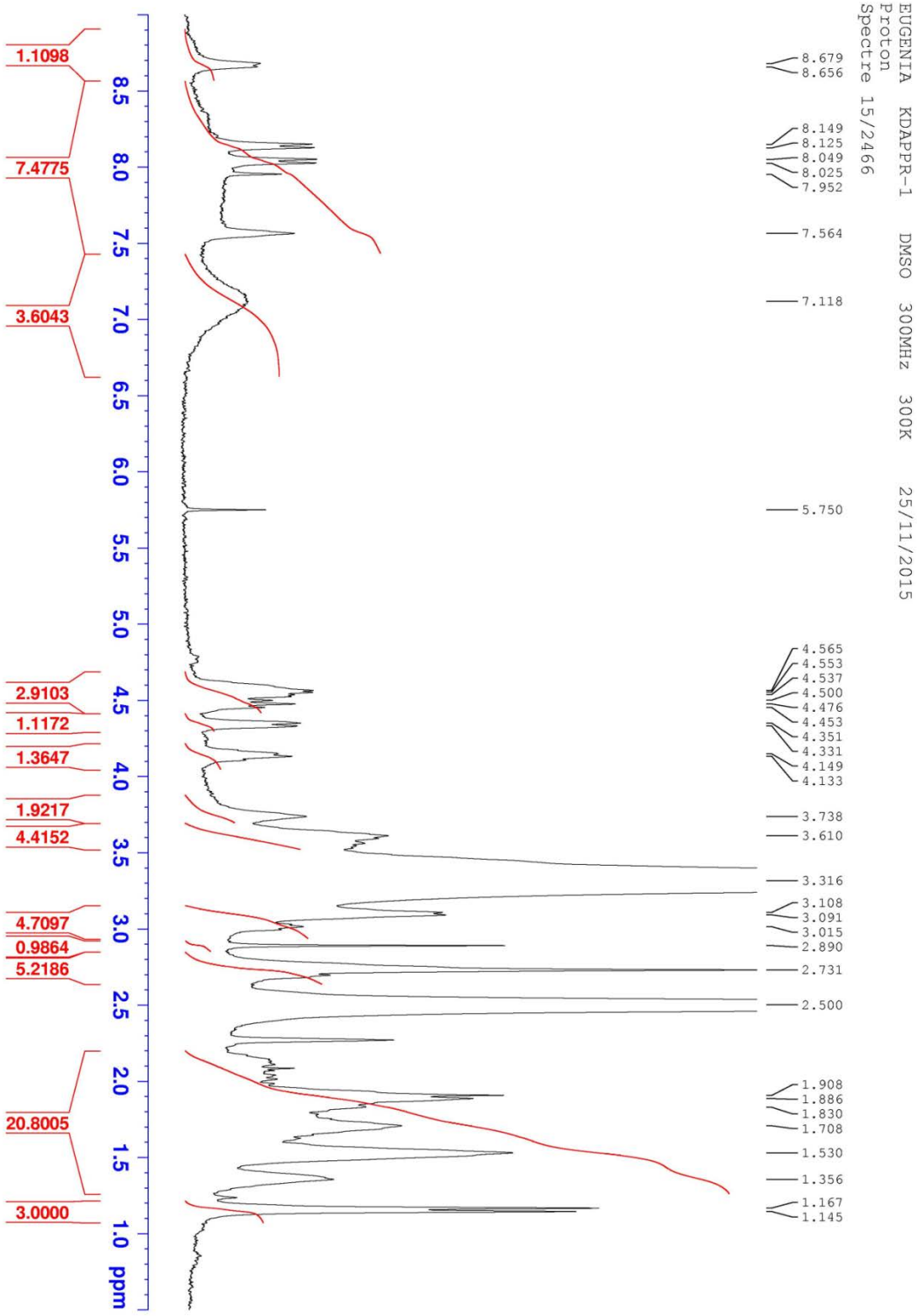

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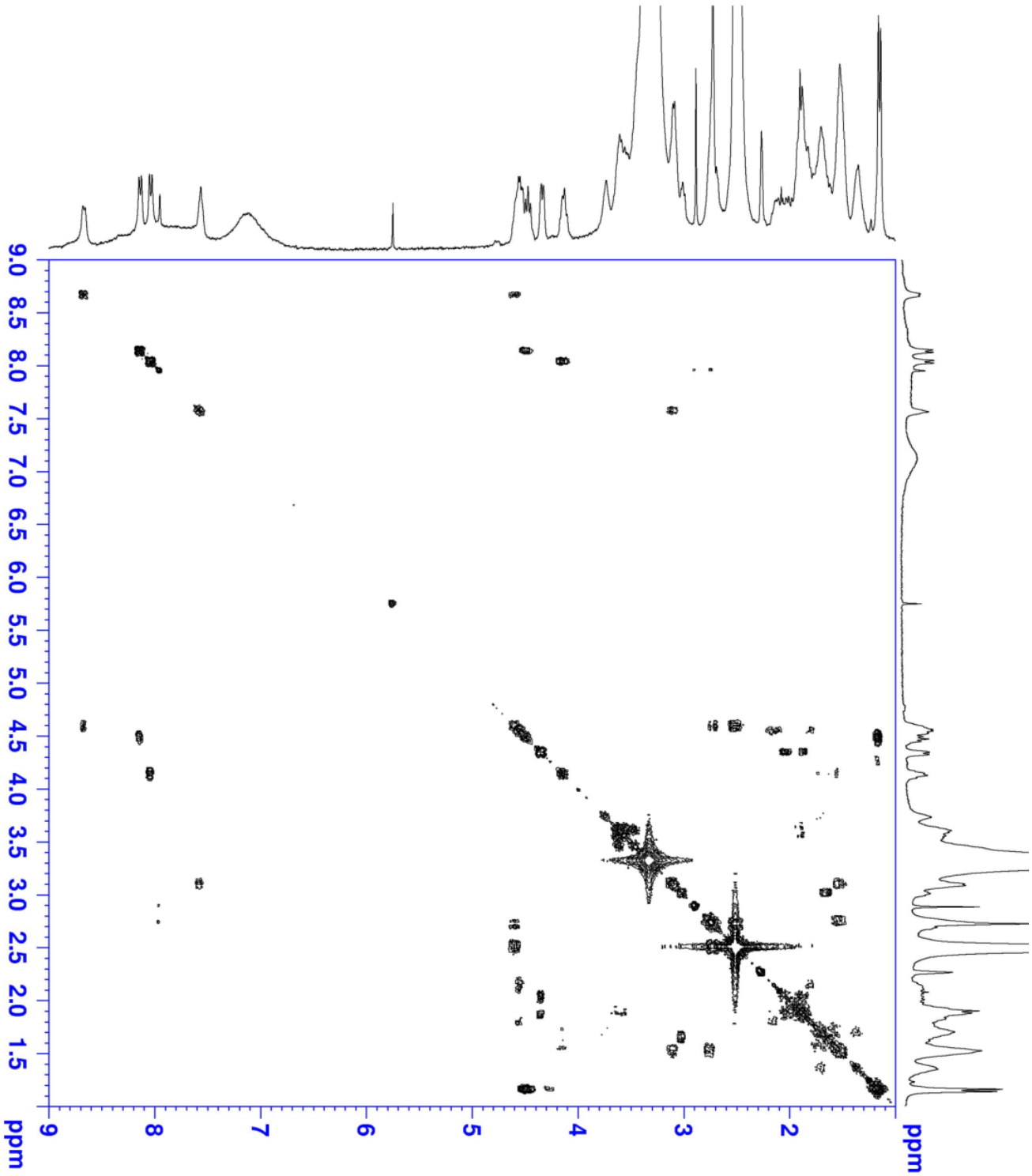

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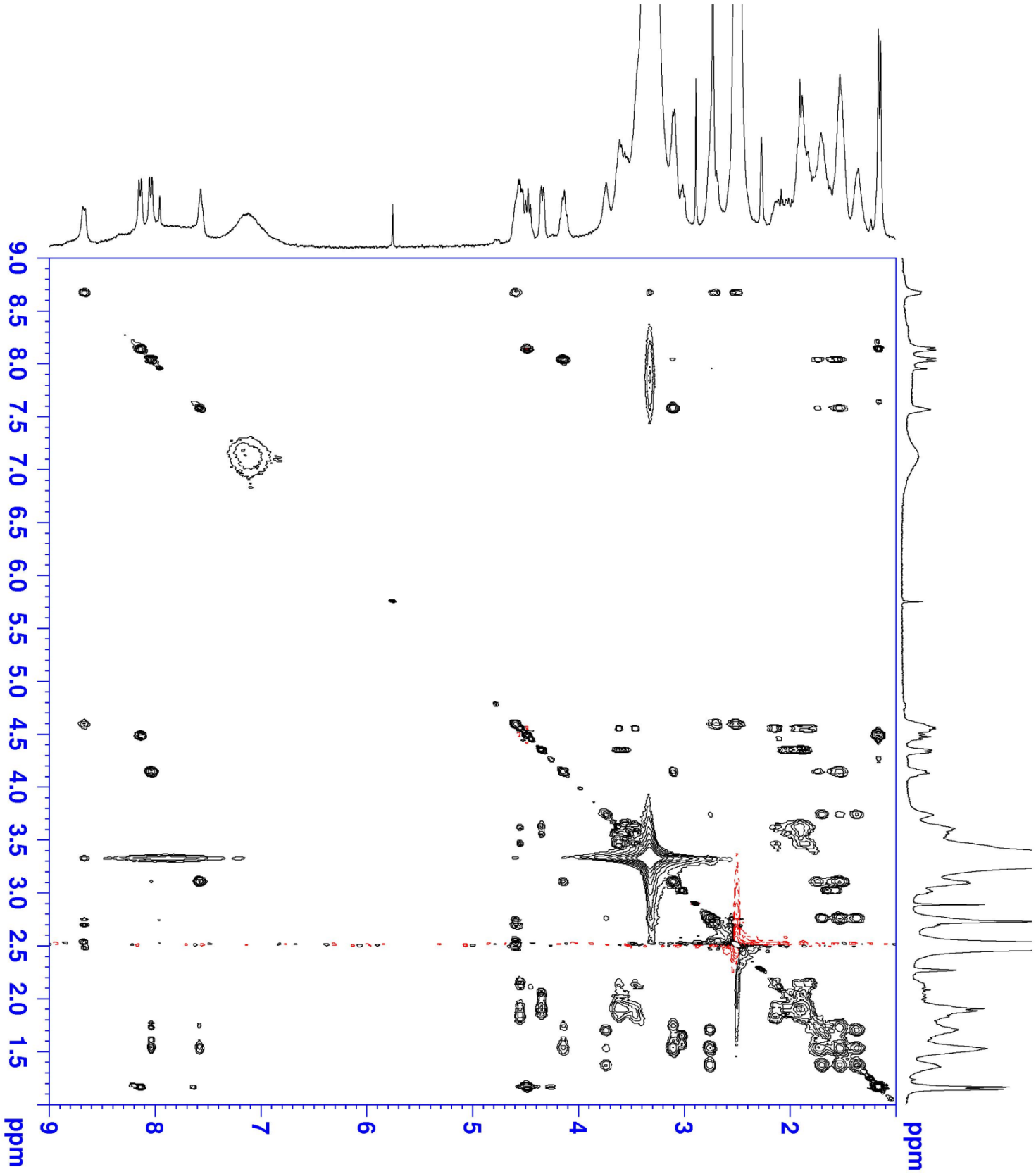

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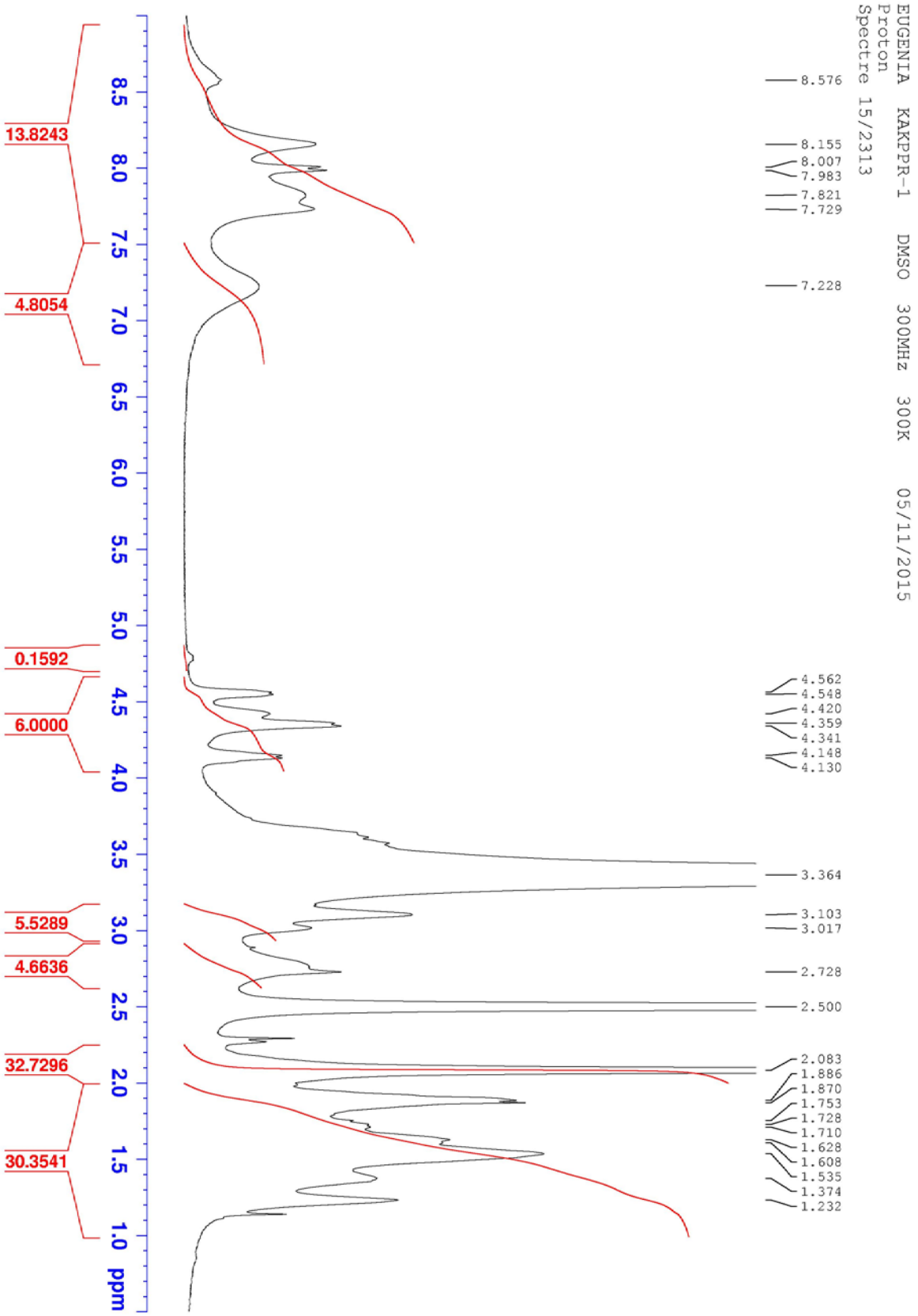

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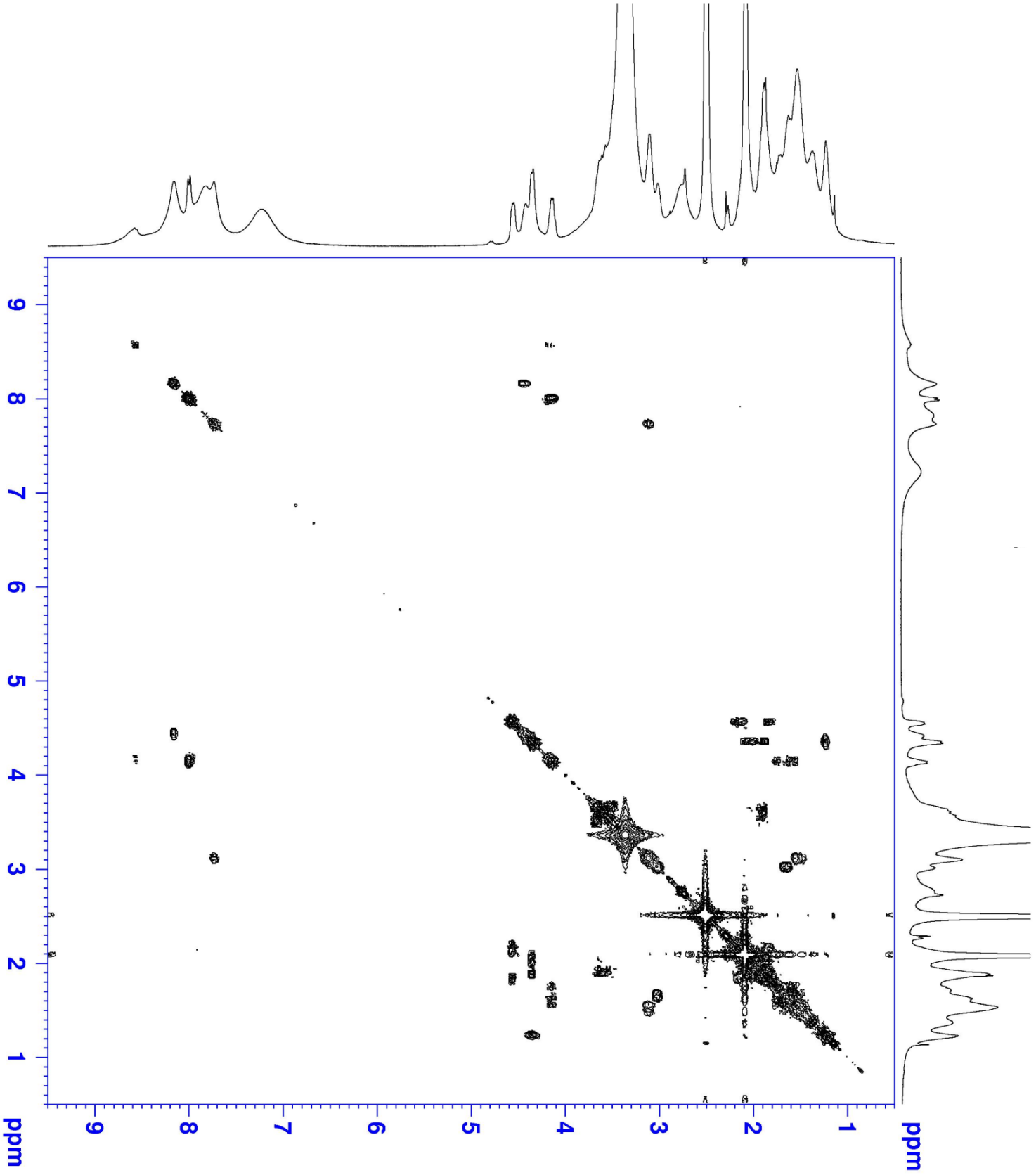

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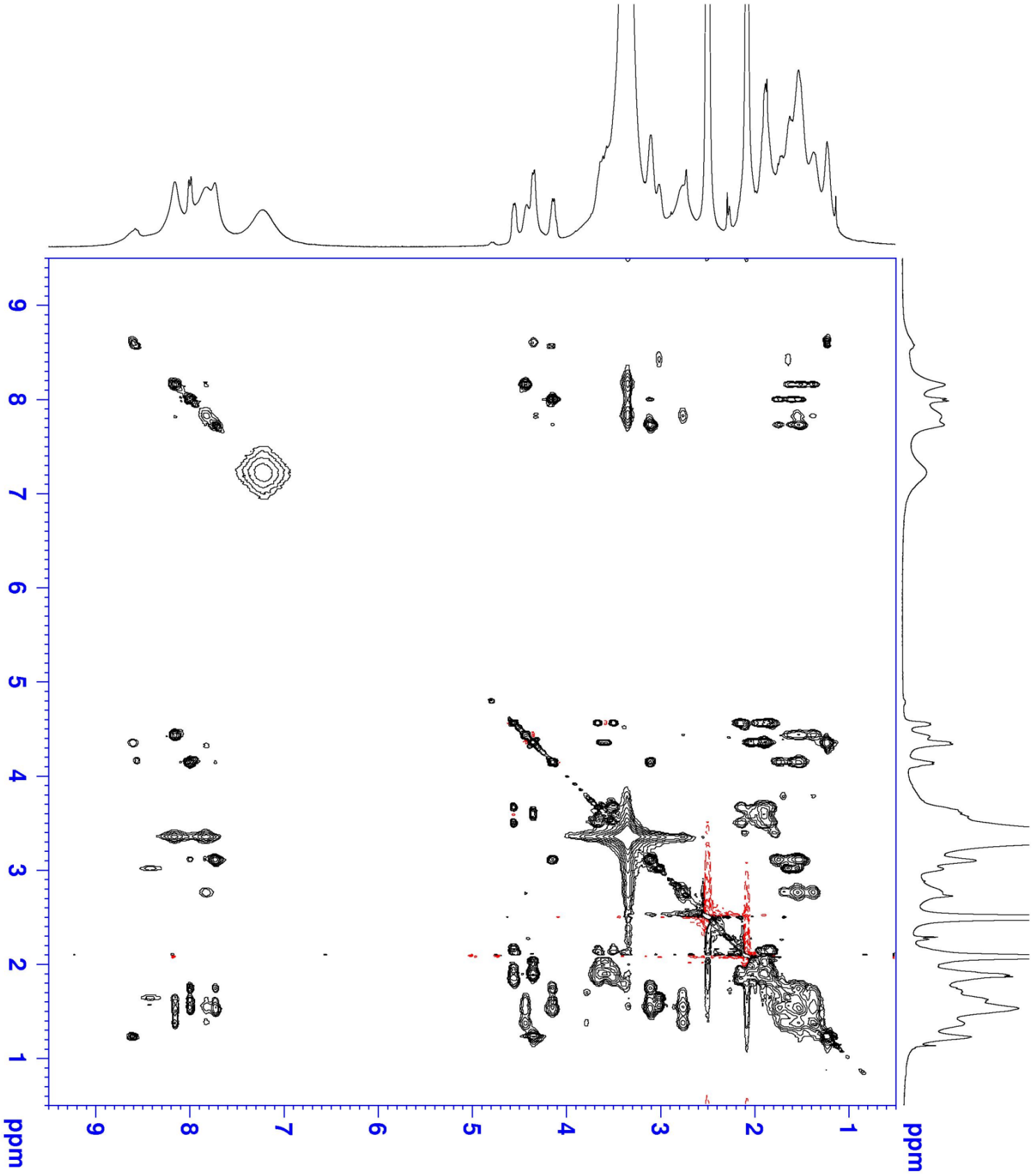

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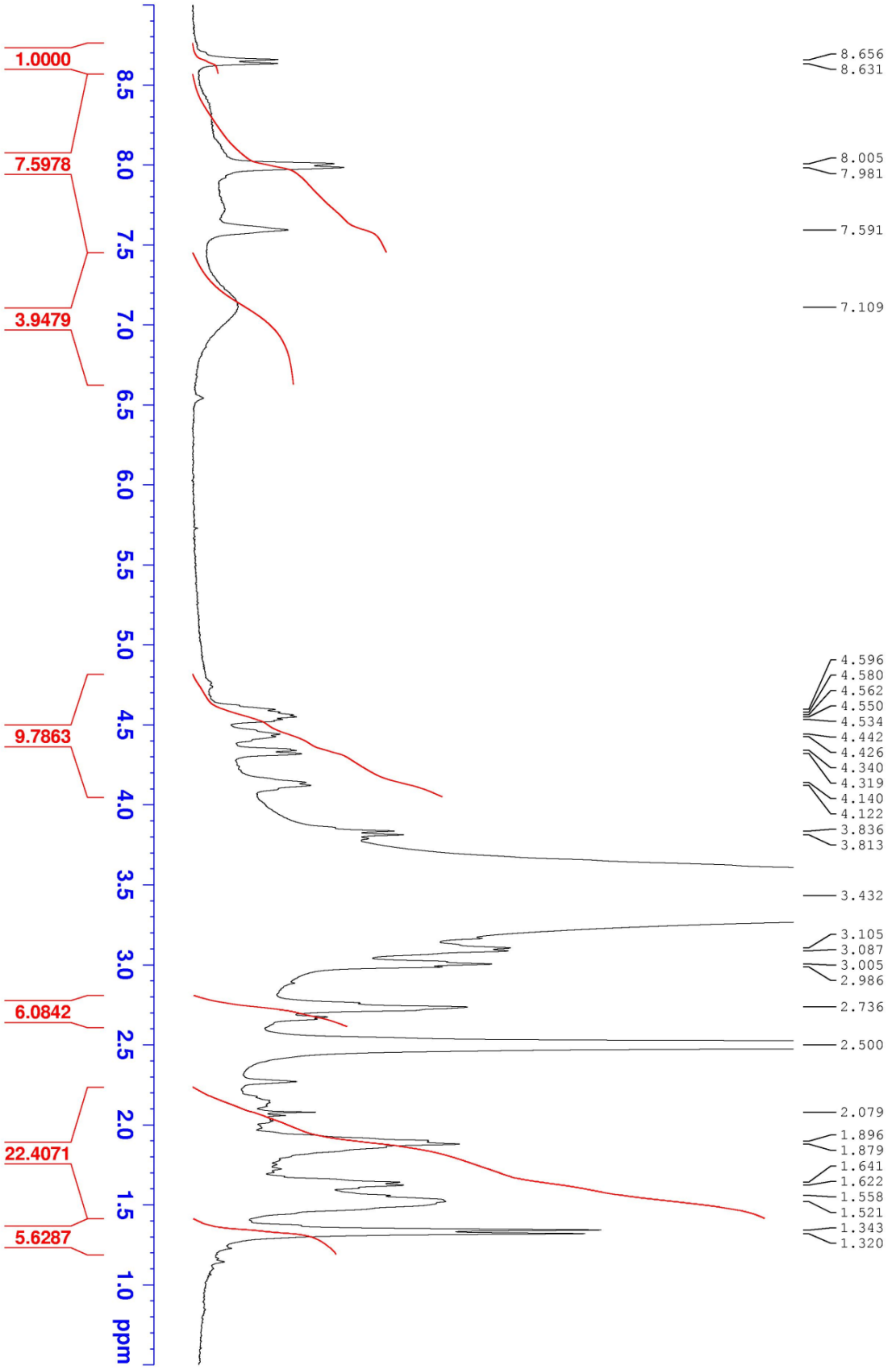

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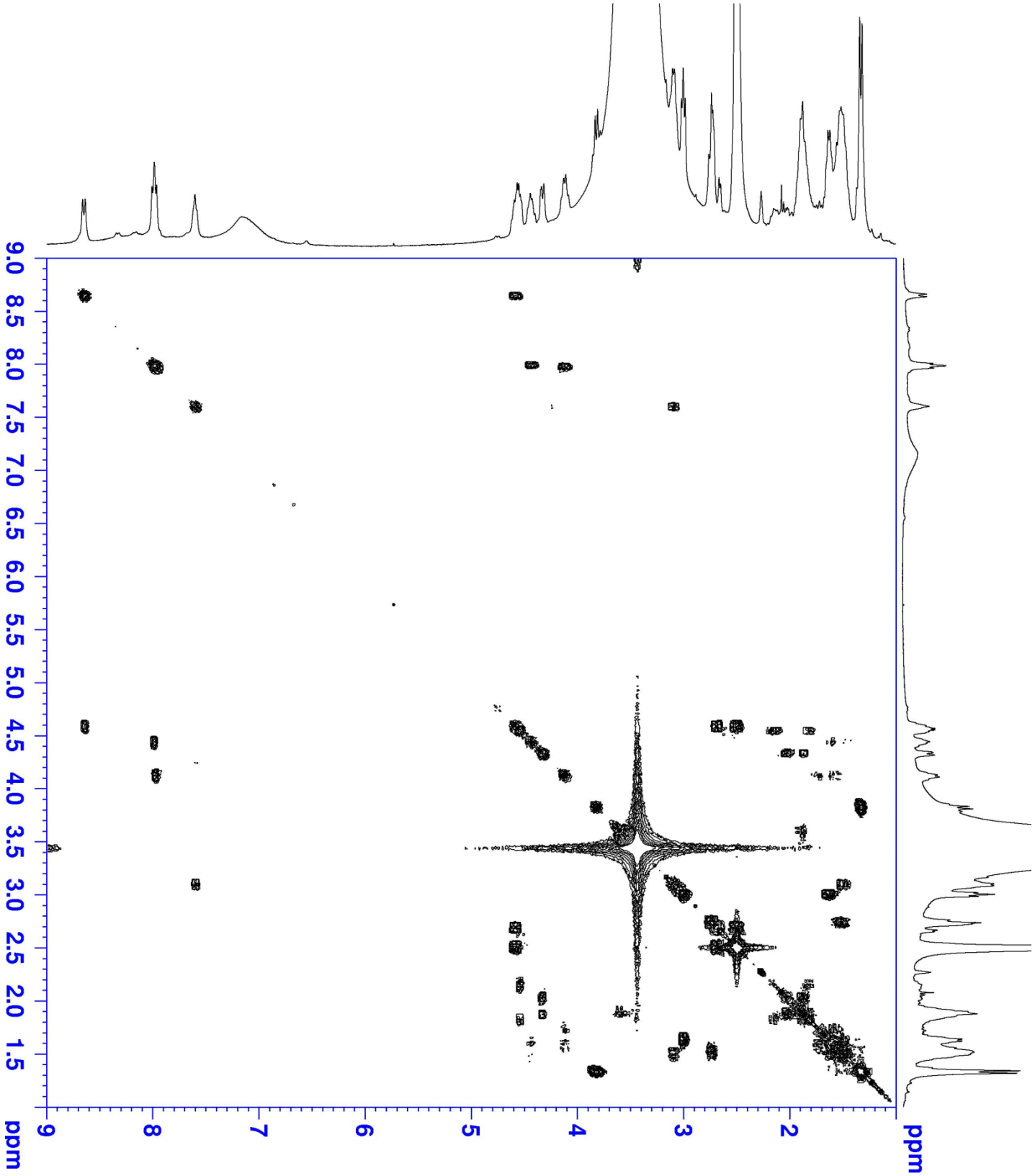

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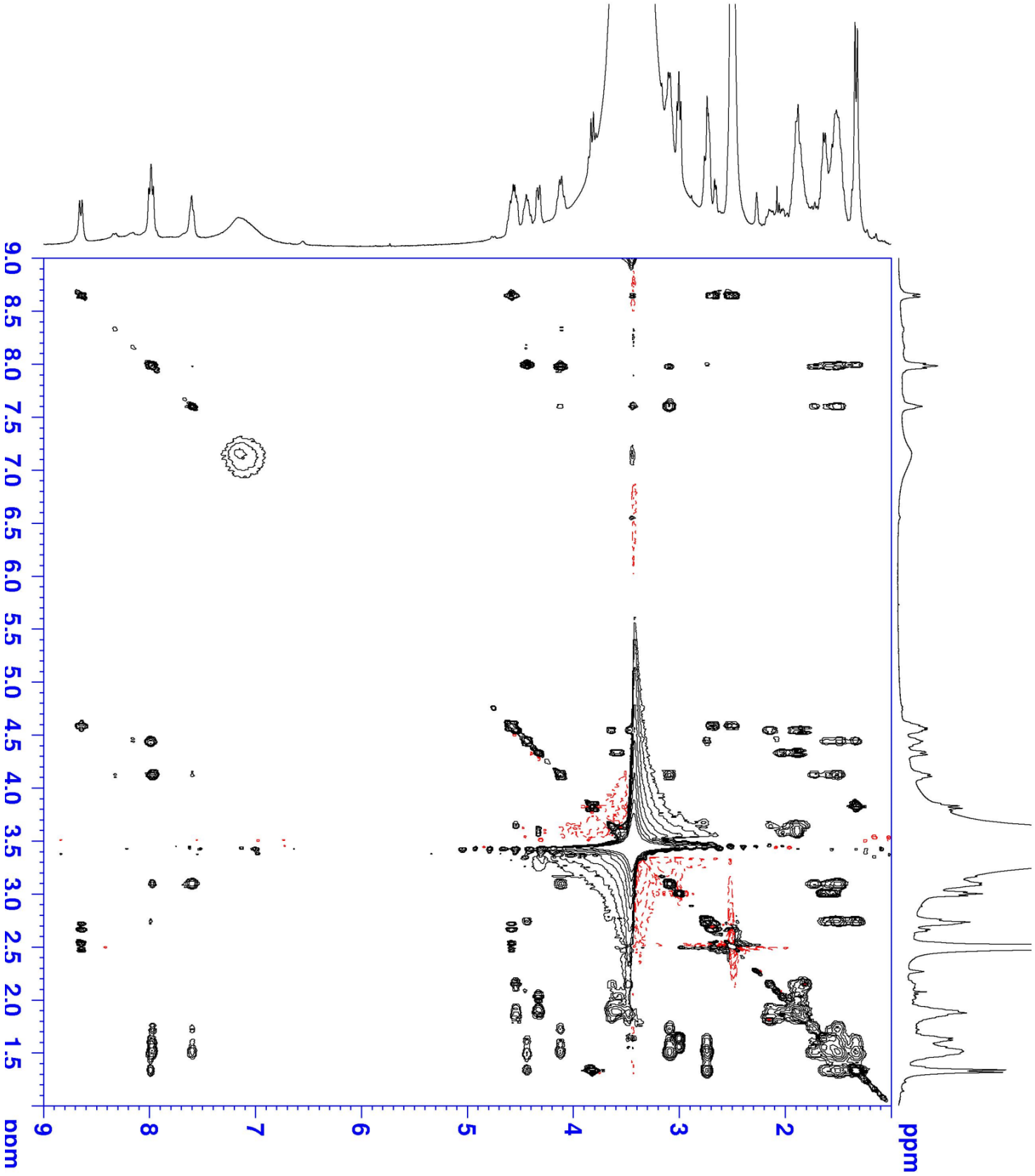

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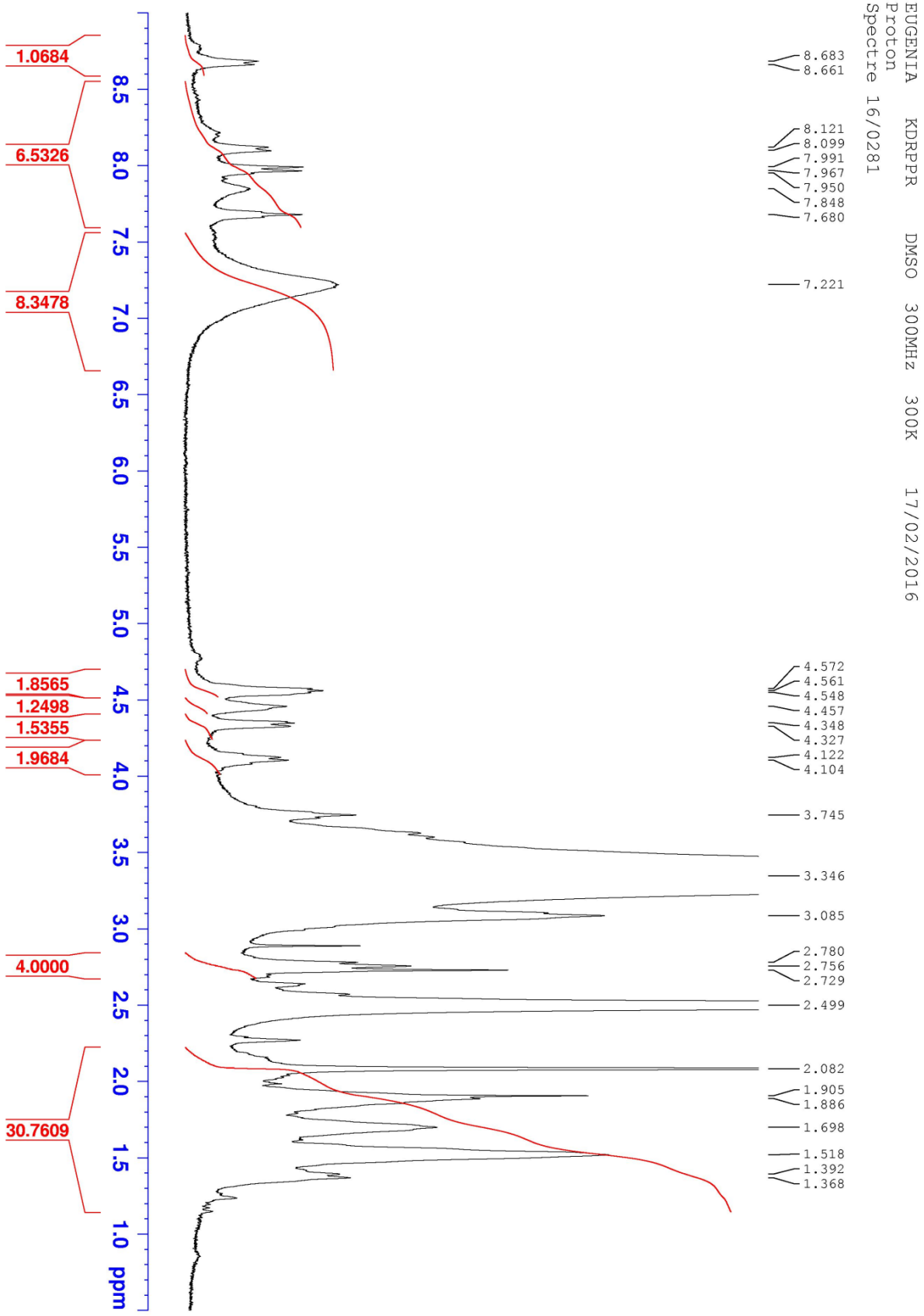

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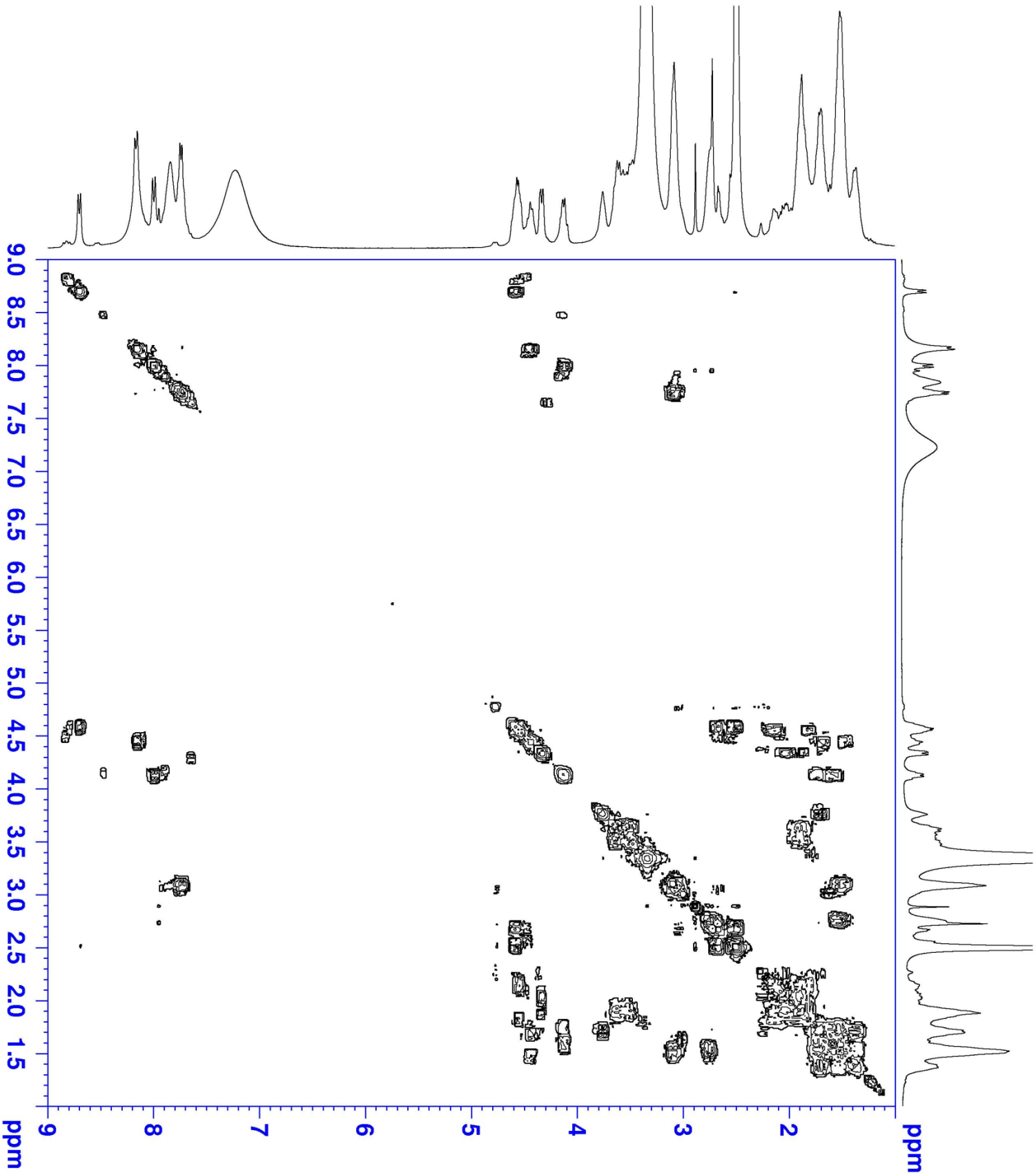

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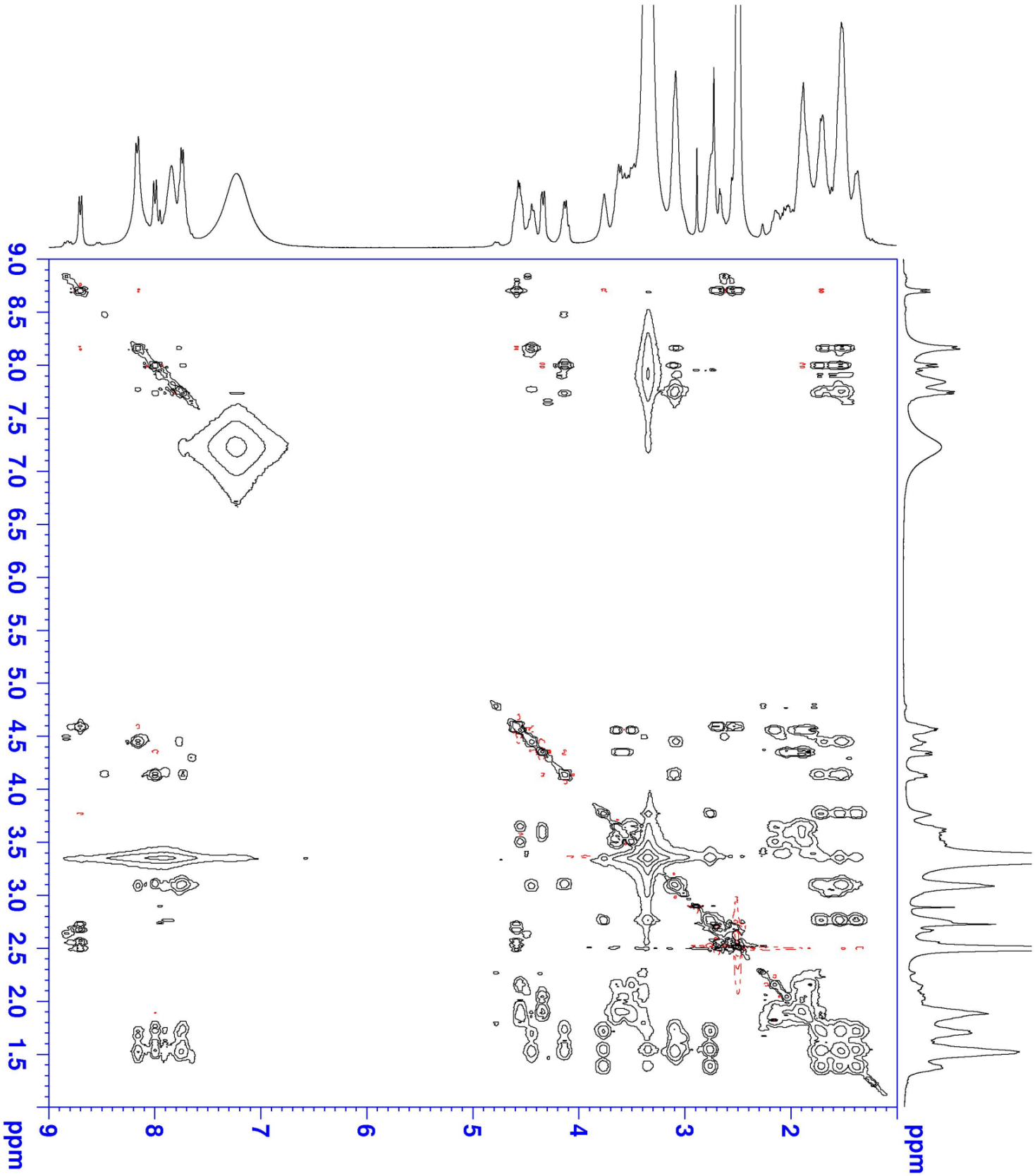

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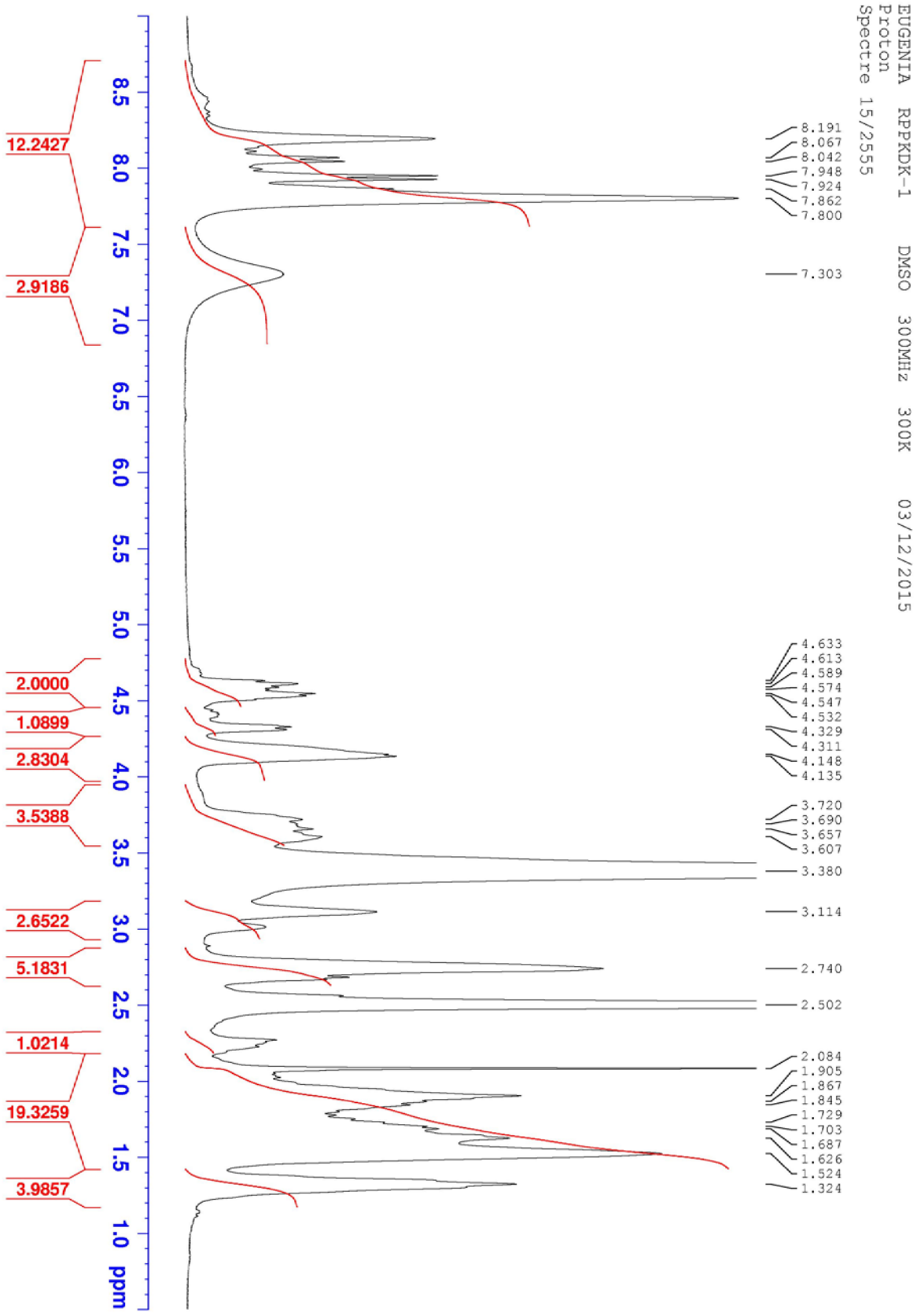

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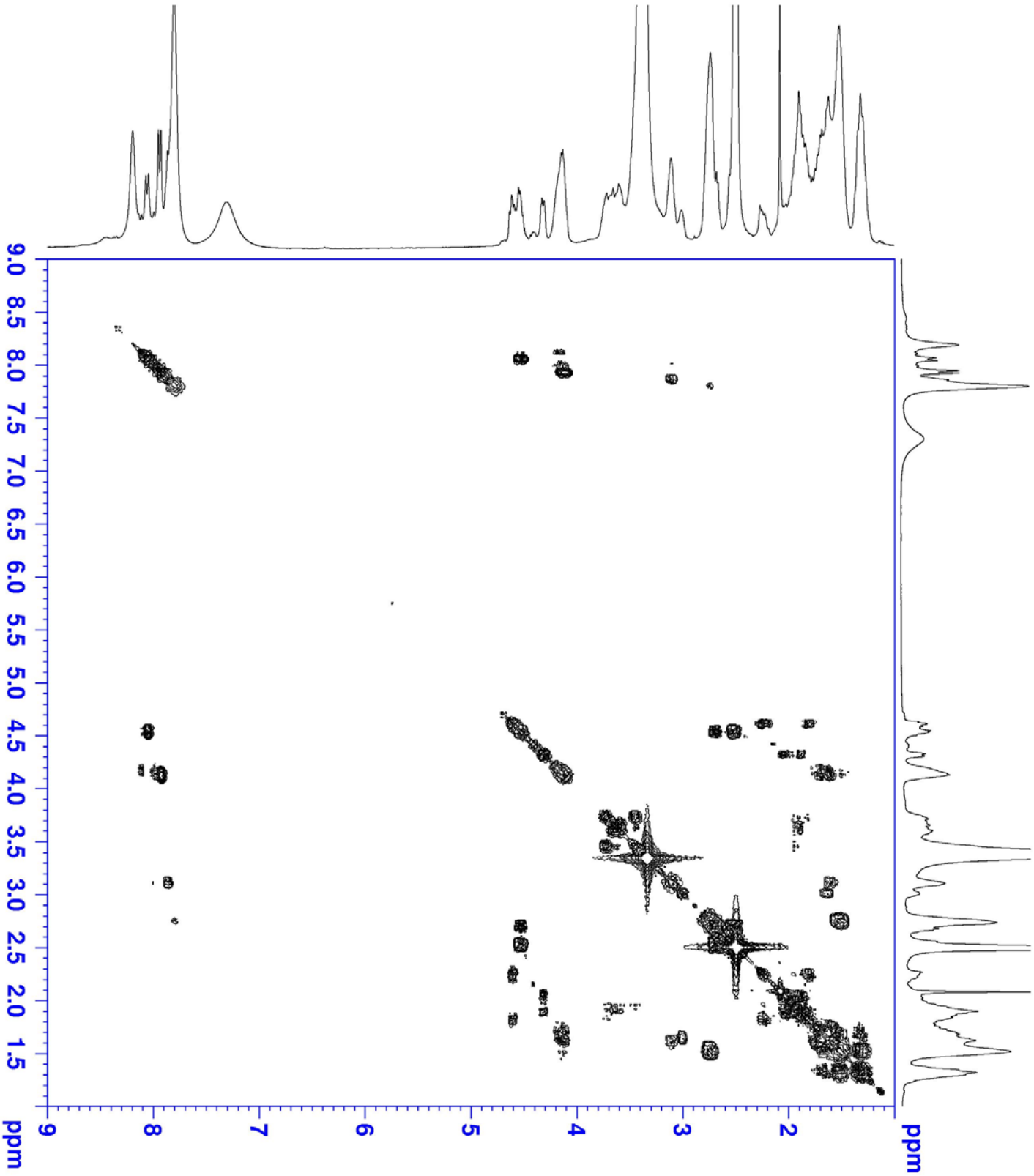

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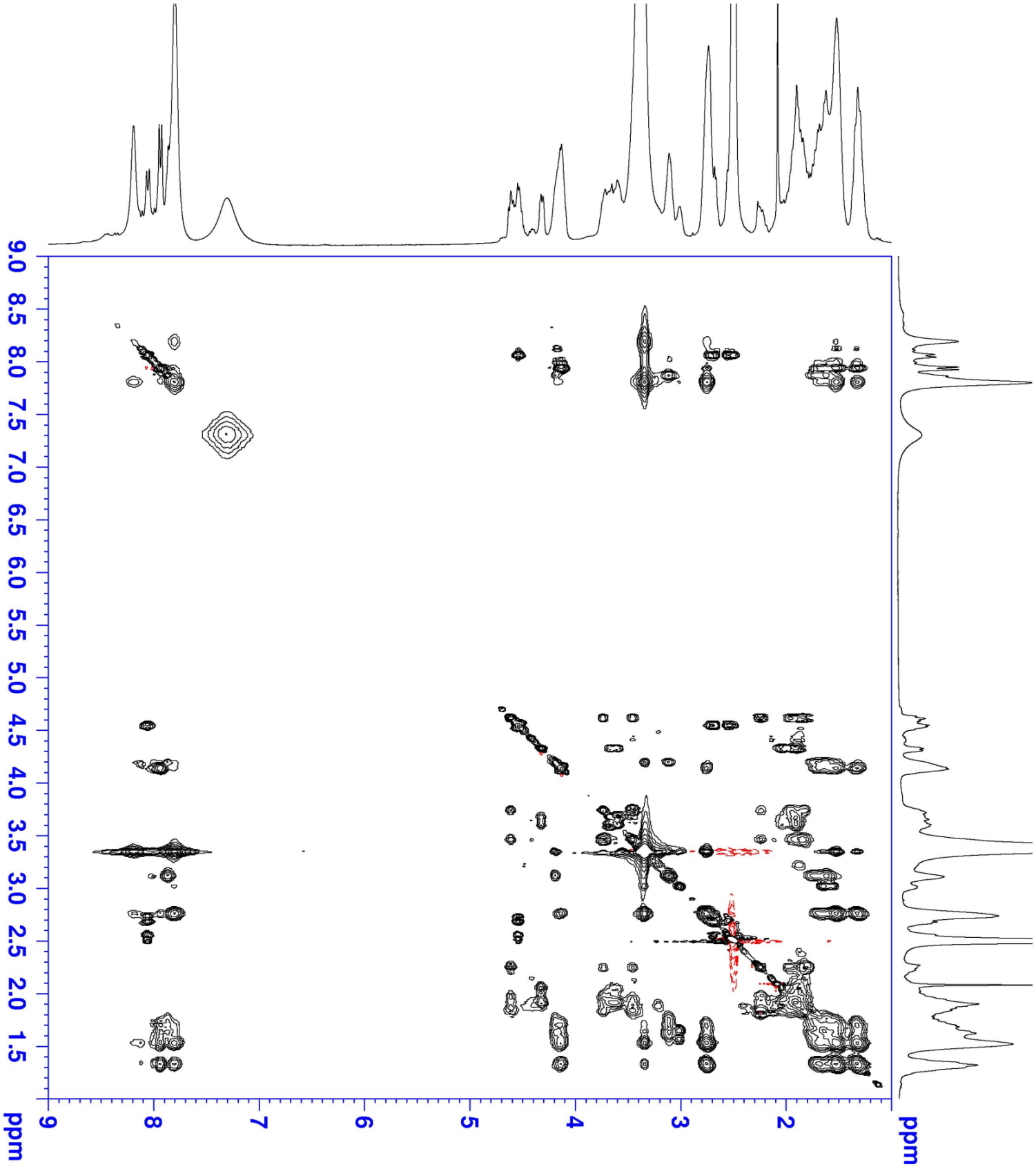

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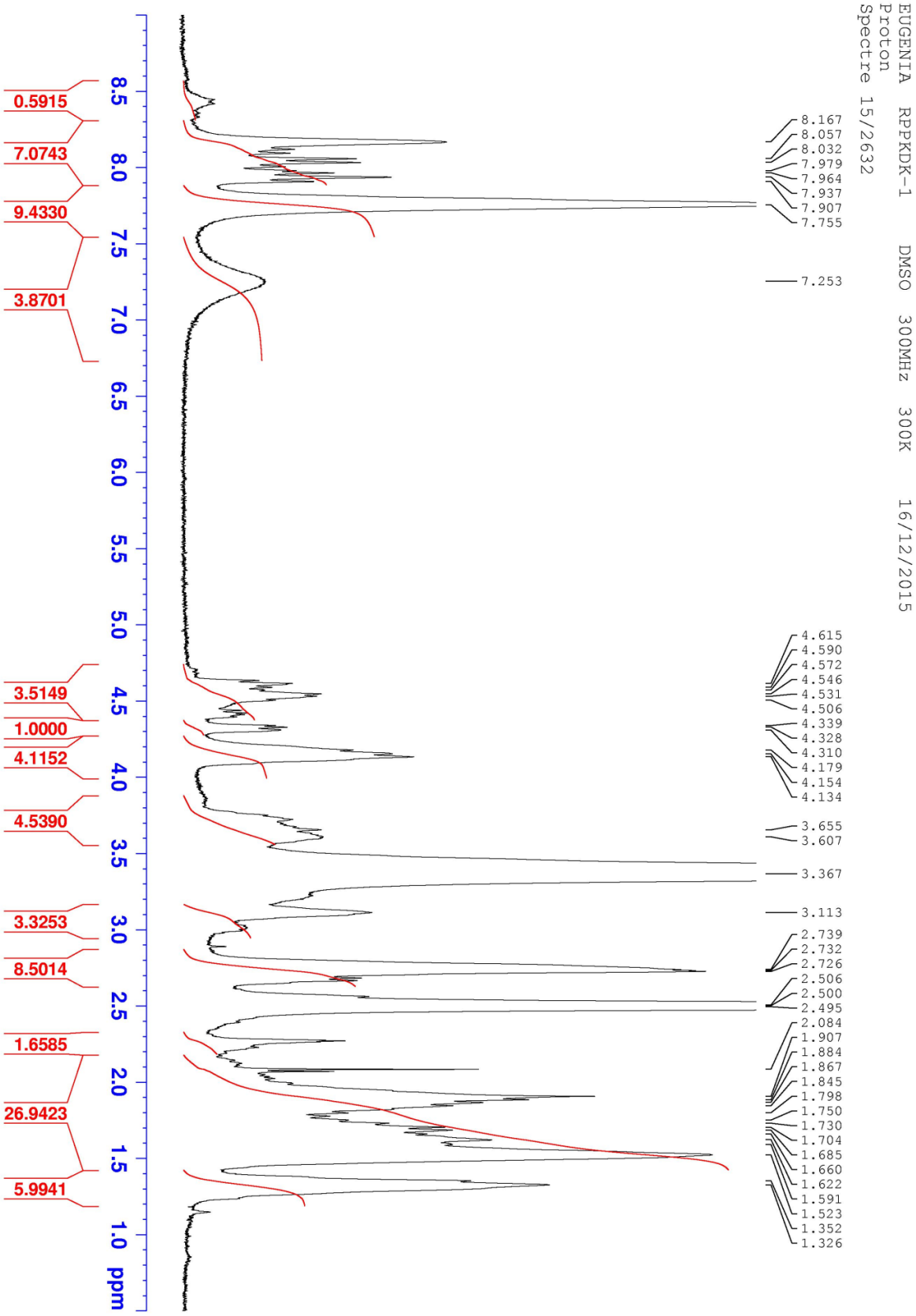

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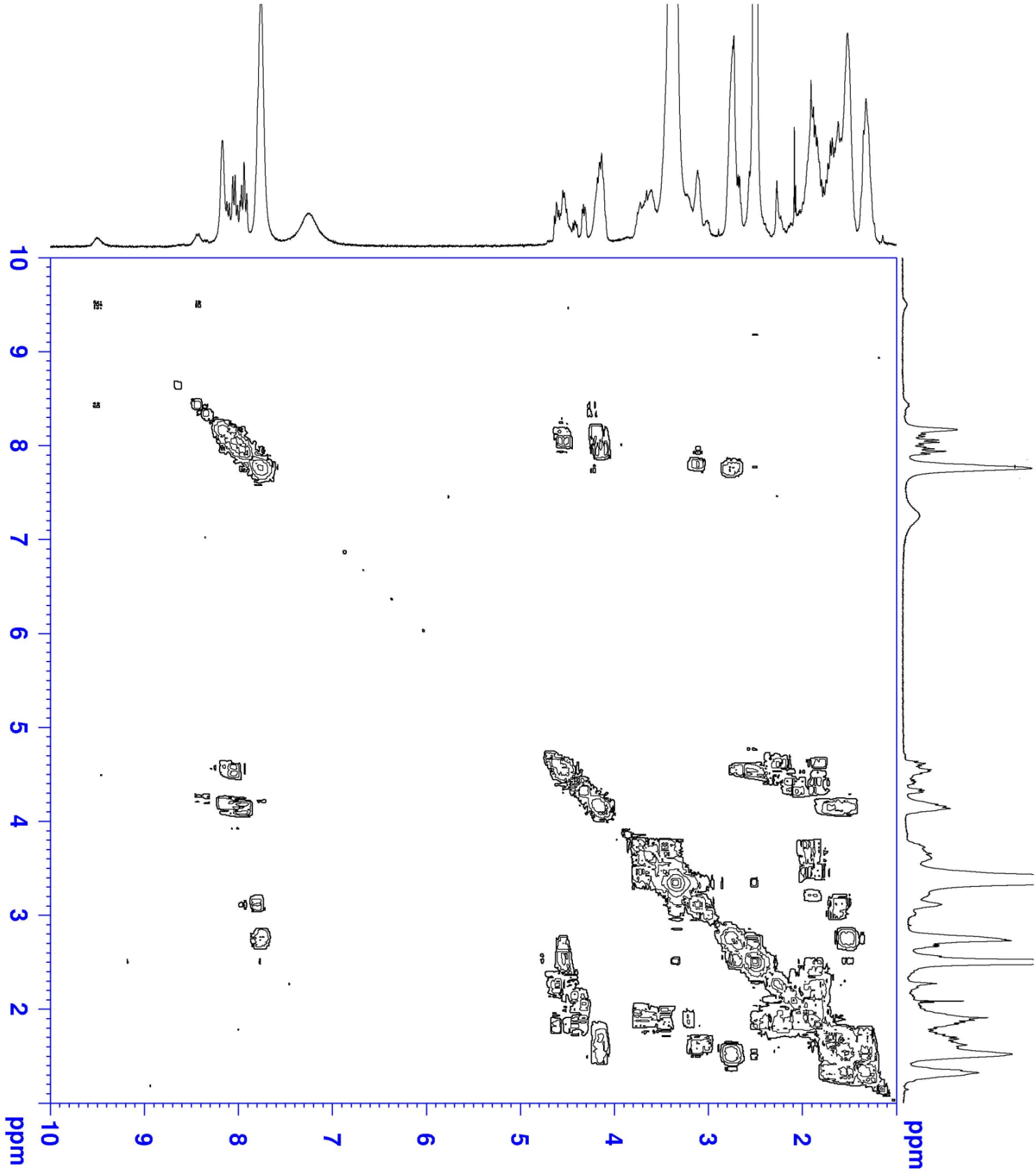

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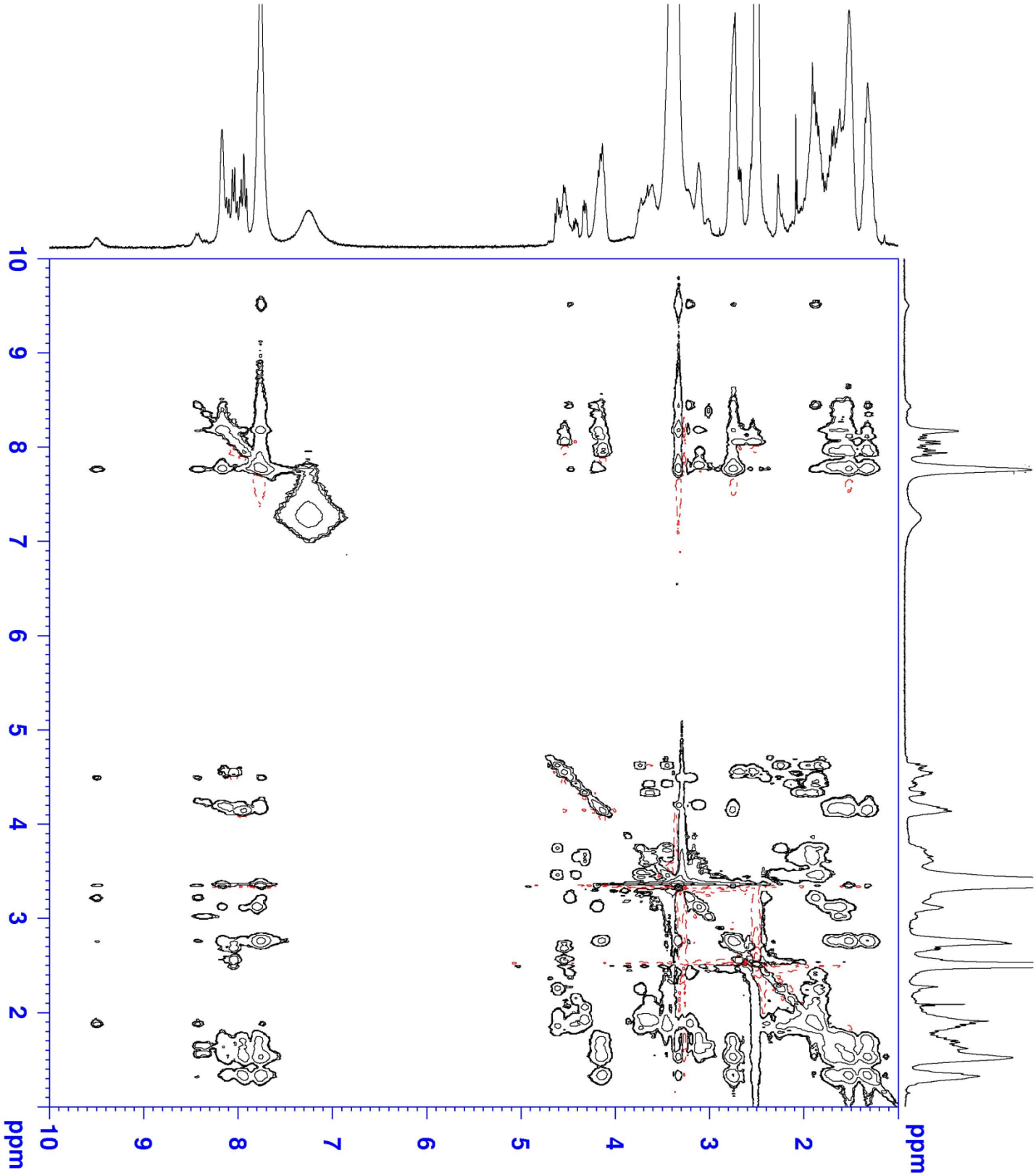


9. PERSPECTIVE:

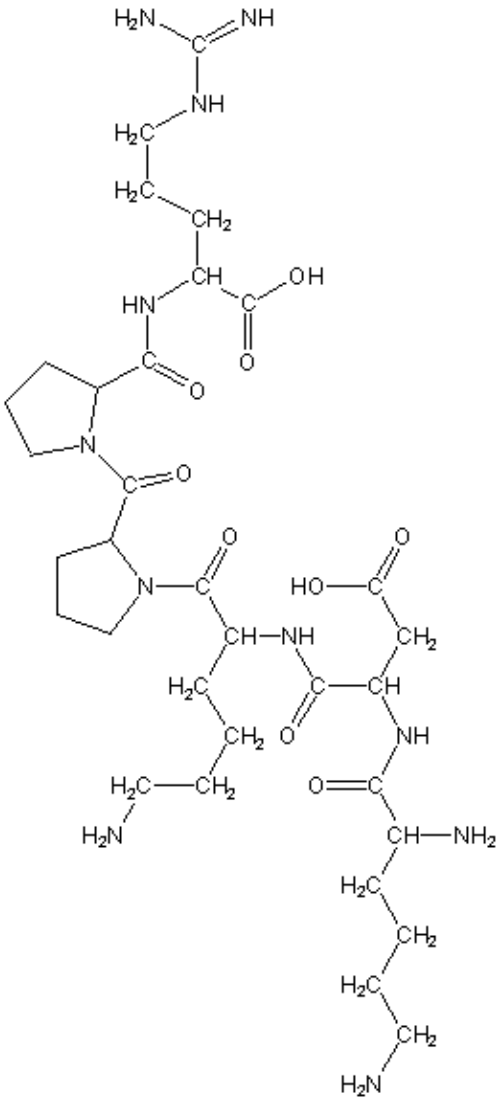
Once just built the 10 peptides described above it want to continue studying what the best stability presented and will continue to change the various amino acids which form the peptide.

That is why it was started designing with the same steps the following peptide:

K-d-K-P-P-R

- **Structure:**

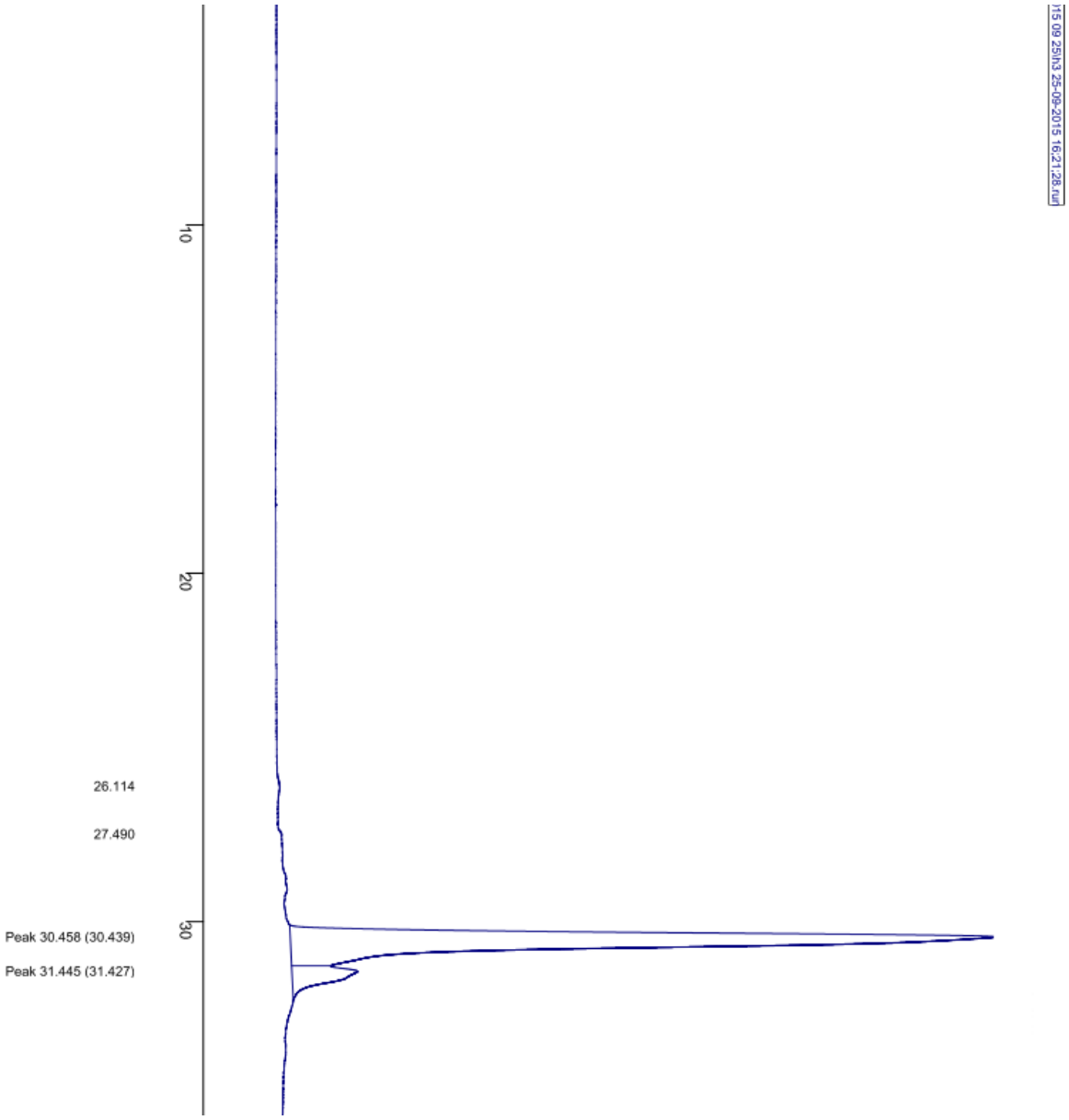
K-d-K-P-P-R



$C_{32}H_{57}N_{11}O_9$
Exact Mass: 739,43
Mol. Wt.: 739,86

With this peptide we were able to purify and send tests of mass spectra and NMR with the following results:

- **Purification (HPLC) :**



- Mass spectra :

**Note:* the result is still uncharacterized,We are awaiting the result of mass spectra to know which is our product

Because of time it has not been able to send ELISA test.

10. ACKNOWLEDGEMENT:

After these six months I have to thank all members of **PDTeam**, **LRGP**, **CRAN**, with whom I have had the opportunity to work.
Especially the PDTeam for taking the time in weekly meetings for my project and solving the problems that emerged in order to meet the objectives.

First to thank my tutor,***Règis VANDERESSE*** for accepting me as a student and giving to me the opportunity to workwith him to make the final project.

Second to thank the supervisors, ***Celine FROCHOT*** and ***Samir ACHERAR***, setting the objectives sought to be achieved.

Special thanks to**Amirah MOHD-GAZZALI** for having been with me at all times during these six months everything has taught me both professionally and personally.

Definitively everyone with whom I have shared this time both in the laboratory and in the office for having achieved that could come every day with a smile and it was very easy to work in the laboratory.

Finally thank both **ENSIC** as the University of Valladolid (**UVA**) for giving me the exchange in Nancy because It has been a great experience to evolve both professionally and personally.

Memory by: EUGENIA TERESA LONGARELA DEL HOYO.