THESIS FOR THE DEGREE MASTER OF PHARMACY

Design and Synthesis of Novel Cyclopentapeptide Antagonist for the Chemokine Receptor CXCR4

By

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Øystein Eriksen

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Abstract

CXCR4 is a GPCR that by activation of its ligand CXCL12 is involved in the pathology of several diseases, examples being cancer and rheumatoid arthritis. It has also shown to play a crucial role for HIV-1 entry into T-cells and the development of AIDS. Several cyclopentapeptides (CPPs) based on the sequence D-Tyr-Arg-Arg-2-Nal-Gly of the lead compound FC131 have shown to have antagonistic activity. These CCPs are therefore targets for drug research. In this thesis a previously published 3-point pharmacophore for these CCPs is reproduced and a new 4-point pharmacophore is presented. Structural similarities of low-energy conformations of CPPs that fit these pharmacophores have been identified. A set of new conformational stabilized CPPs based on these findings have been designed and synthesized. A set of citrulline based analogs of FC131 have been synthesized as they will serve as probes to determine the nature of the Arg residues interaction with CXCR4

Introduction

1.1 Peptides as Drugs

Endogenous peptides are mediators in numerous biological processes, where they serve as e.g. neurotransmitters, hormones, growth factors, and immune response regulators. Thus, small molecules that regulate peptide signaling are of great interest as drugs. Unfortunately, the chemical structure of peptides severely limits their use as drugs due to unfavorable pharmacokinetic properties. Due to these limitations, *peptidomimetic* compounds, i.e. non-peptide compounds that mimic peptides and interact with peptide receptors, are preferred as drug candidates.[1]

1.2 CXCR4 as a Therapeutic Target

CXCR4 is a chemokine receptor of the G protein-coupled receptor (GPCR) family. CXCR4 is short for chemokine (C-X-C motif) receptor 4, the CXC motif meaning that the ligand protein has one amino acid between the first two cysteine residues.

GPRCs consist of seven transmembrane α-helices connected by intra-and extracellular loops. These receptors have its binding site extracellularly. Agonist will cause a cascade of signal transduction inside the cell. Normally GPCRs can bind different ligands, and similary one ligand can bind to different GPCRs. CXCR4 on the other hand has an almost monogamous relationship to its natural ligand; the chemokine stromal cell-derived-factor-1, SDF-1.[2, 3] SDF-1 has been assigned the name CXCL12 in the literature (Chemokine (C-X-C motif) ligand 12)

Interaction between the CXCR4 and its ligand is important in the migration of progenitor cells during embryologic development. It has been identified as crucial in the development of the cardiovascular, hematopoietic and nervous systems.[4] Its physiological role in adult life however is poorly disclosed, but CXCL12 has been identified as an attractant for leucocytes; and antagonist to the receptor causes mobilization of

hematopoietic stem cells into the blood stream.[5] Likewise prolonged activation of the receptor leads to retention of myeloid cells in the bone marrow. [6]

Though its natural function in adults is poorly understood it has been shown that CXCR4 plays a role in numerous diseases. CXCR4 is expressed on the surface of several types of cancer cells. It has also been shown that CXCL12 is abundant in tissue susceptible to metastasis. Migrating cancer cells will therefore be attracted to these organs. For a review see Tsutsumi *et al* 2006.[7]

Patients with rheumatoid arthritis (RA) have a high level of CXCL12 in their synovium and CXCR4 is highly expressed in memory T cells. RA is a complex disease, but a novel part of the pathology is the accumulation of memory T cells in the synovium. As with metastasis migrating memory T cells will find their natural ligand in the inflamed synovium. Binding of the ligand will also prevent apoptosis of memory T cells. The ligand also stimulates T cell migration. These momentums display a viscous circle that will cause the disease to progress. [8]

CXCR4 is also identified as a receptor in the entry of HIV into T-cells[9]. HIV is most commonly transmitted by the macrophage R5 strain. It is designated the name R5 because it uses the receptor CCR5 as a coreceptor for entry in macrophages. The R5 strain progenates to the X4 strain which uses CXCR4 as a coreceptor for entry in T-cells. This strain is most common in later stages of the disease and makes up for the majority of infections resulting in AIDS. Both strains utilize CD4 as the main receptor. For a review see Tsutsumi *et al* 2006.[7]

Thus, small-molecule CXCR4 antagonists have emerged as potential drugs for treatment of cancer, RA, and HIV/AIDS.

1.3 Cyclopentapeptide CXCR4 Antagonists

Several small-molecule CXCR4 antagonists have been described in the literature over the last decade, the most notable examples being mono-/bicyclams,[10] ALX40-4C (Ac-(D-Arg)$_9$-NH$_2$),[11] and KRH-1636.[12] In 2003, Fujii *et al.* discovered that certain cyclopentapeptides based on the sequence Arg-Arg-2-Nal-Gly-Tyr were highly potent

CXCR4 antagonists, and also displayed anti-HIV activity.[13] The structure-activity relationship (SAR) for this compound series has since been studied in more detail, mainly by substitution of individual amino acids, which has provided a useful picture of the structural requirements for binding to CXCR4. FC131 (cyclo(D-Tyr^1-Arg^2-Arg^3-Nal^4-Gly^5))(IC_{50} = 4 nM) has been most studied of these cyclopentapeptides and is considered a lead compound See Figure 1

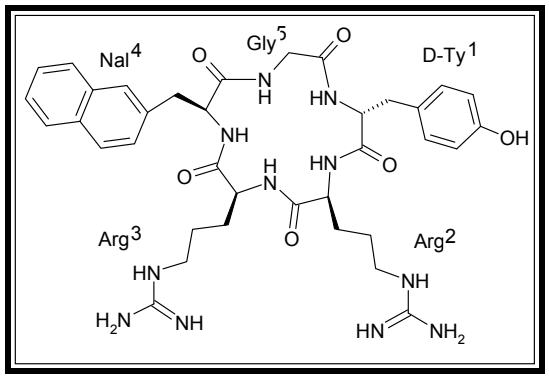

Figure 1: FC131.

1.4 Structure activity studies of FC131

It has been shown that replacing the Arg^2-residue of FC131 with alanine has minimal effect on antagonistic activity (Ala^{2}FC131 IC_{50} = 63 nM). In contrast alanine scans also showed that replacing Nal^4, D-Tyr^1 or Arg^3 completely diminished antagonistic activity[14]. Conserative substitution of D-Tyr^1,Arg^3 and Nal^4 with amino acids of similar characteristics also gives lower activity.[15] This implies that D-Tyr^1, Arg^3 and Nal^4 are essential for the activity of the parent compound, whereas Arg^2 is disposable. Based on this knowledge Våbenø *et al* developed a three point 3D pharmacophore for cyclopentapeptide CXCR4 antagonists. Ala^{2}FC131 (cyclo(D-Tyr^1-Ala^2-Arg^3-Nal^4)) was the reference compound for this pharmacophore.

D-Arg^{2}FC131(cyclo(D-Tyr^1-D-Arg^2-Arg^3-Nal^4.Gly^5)) (IC_{50} = 8 nM) has slightly lower activity than FC131.[13] The only difference between these two compounds is the stereochemistry of its second amino acid. Recently alanine scans of D-Arg^{2}FC131 showed that replacing D-Tyr^1 had only minimal effect on biological activity (D-Ala^1-D-Arg^{2}FC131, IC_{50} = 130 nM) .[16] In other words; provided D-Arg as the second amino acid D-Tyr^1 is not necessary for antagonism. The alanine scan also showed that D-Ala^{2}FC131 (cyclo(D-Tyr^1-D-Ala^2-Arg^3-Nal^4)) (IC_{50} = 230 nM) had lower activity than Ala^{2}FC131. [16]

Both these L/D-Ala^2 isomers have lower activity than their parent L/D-Arg^2 isomers. The activity of D-Ala^{2}FC131 is classified as medium and the three other is classified as high (Activity: FC131>D-Arg^{2}FC131>Ala^{2}FC131>D-Ala^{2}FC131).[16, 17]

These data indicate that there must be some conformational changes in the peptides when shifting from L- to D- amino acids in the Xaa^2 position. This could indicate that the backbone is involved in ligand binding, and not just serves as a scaffold for pointing side chains in the right direction. This has been indicated before as linear versions, as well as retro-inverso isomers of FC131 and D-Arg^{2}FC131 have low activities.[13, 18] NMR studies of the four compounds described have shown that the amide bond in D-Tyr^1-D-Xaa^2 is flipped 180˚ compared to the D-Tyr^1-L-Xaa^2 peptides. NMR studies also show that, for FC131 and Ala^{2}FC131, the amide bonds of Tyr^1-Xaa^2, Xaa^2-Arg^3 and Arg^4-Nal^5 is oriented in the same direction.[14]

Employing N-Me analogs of D-Arg and D-Ala in the Xaa^2 position causes an increase in activity that surpasses that of their parent compound (N-Me =N$^{\alpha}$-Me).[14, 16] NMR studies have shown that the D-NMe-Xaa^2 analogs have similar backbones as the L-Xaa^2 peptides. In other words N-methylation forces the amide bond of D-Tyr^1-D-Xaa^2 to flip 180˚, making the backbone similar to that of the L-Xaa^2 peptides. (See figure 2)

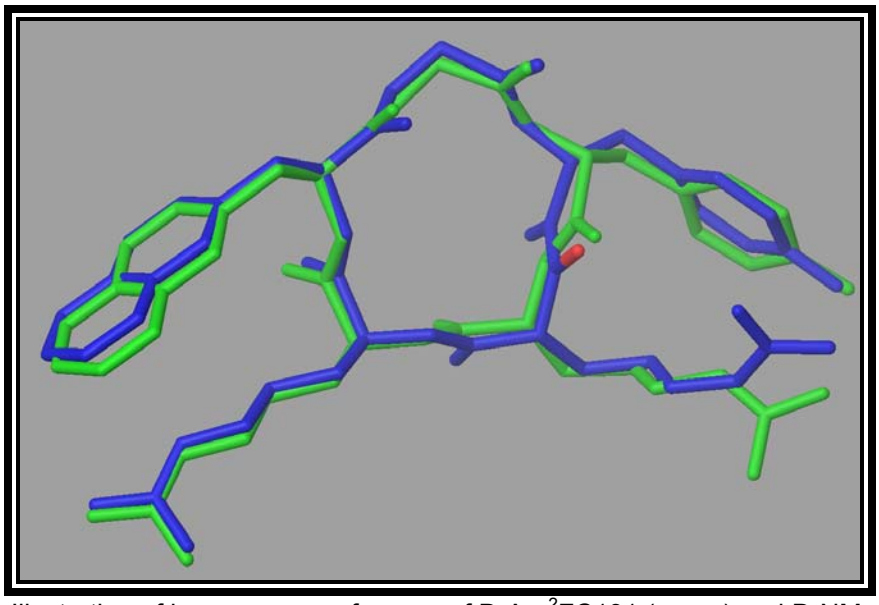

Figure 2: Illustration of low energy conformers of D-Arg^{2}FC131 (green) and D-NMe-Arg^{2}FC131 (blue). N-Me group is depicted in red. This illustration shows the different orientation of the D-Tyr^1-D-Xaa^2 amide bond of the two compounds. The illustration also serve to visualize the three amide bond orientated in the same plane as described above (blue figure: Carbonyls project downwards "into the paper").

Of pentapeptides known to date D-NMe-Arg^{2}FC131 (cyclo(D-Tyr^1-D-NMe-Arg^2-Arg^3-Nal^4-Nal^5))(IC_{50} = 3 nM) has the highest antagonistic activity for CXCR4. (Activity: D-NMe-Arg^{2}FC131>FC131>D-Arg^{2}FC131>D-NMe>Ala^{2}FC131>Ala^{2}FC131>D-Ala^{2}FC131)[16] This discloses a subtle pattern for binding of FC131 and its analogs to CXCR4. It seems like there are two 3-point pharmacophores that together form a 4-point pharmacophore. (See figure 3) Thus it is desired to investigate the role of Xaa^2 residues in the biological activity of FC131 and its analogs.

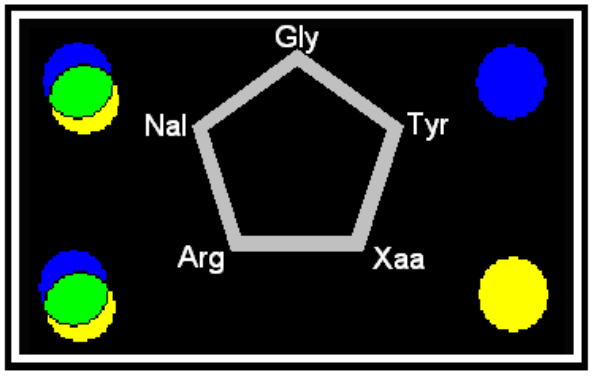

Figure 3: Blue and yellow each form a 3-point pharmacophore. Together blue and yellow make up the 4-point pharmacophore.

Importantly, this information should be transferable to non-peptides; thus, the Cyclopentapeptides represent a very attractive starting point for rational design of peptidomimetic CXCR4 antagonists with therapeutic potential. Elucidating the role of Xaa^2 will be valuable in the design of new lead compounds with potential of CXCR4 antagonism. Specifically it will provide the basis for the possibility of making of Arg^2-Arg^3-Nal^4 peptidomimetics. Such peptidomimetics are interesting since that specific fragment of FC131 share characteristics similar to other CXCR4 antagonist. One example is KRH-1636 which has a naphtyl group, Arg and an Arg-surrogate. See Figure 4.

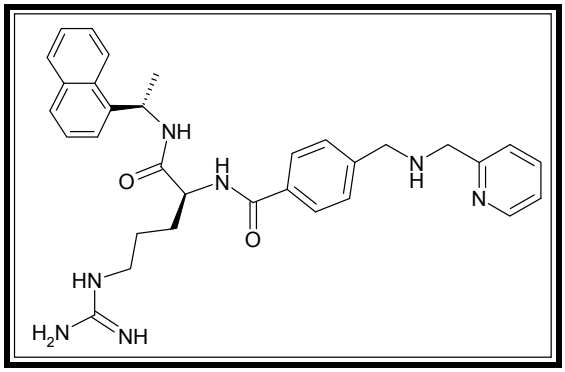

Figure 4:KRH-1636 displays similar characteristic to FC131 with its naphtyl group, Arg and an Arg-surrogate.

1.5 Objectives of the Thesis

The objectives of my thesis were: (1) to develop a 4-point pharmacophore for CPPs from the literature to investigate the role of Xaa^2 in binding to CXCR4; and (2) to design and synthesize a series of novel CPP CXCR4 antagonists to further explore the structure-activity relationship of this compound class.

2 Results and Discussion

2.1 Molecular Modeling Studies

Based on the background described above we wanted to develop a 4-point pharmacophore for CPP CXCR4 antagonists. In order to validate the results and the methods used for pharmacophore modeling in this thesis, we initially wanted to reproduce the 3-point pharmacophore of Våbenø *et al* [17].

2.1.1 Compound selection

From the 16 compounds in the publication by Våbenø *et al* 11 compounds were selected for deducing 3- and 4-point pharmacophores.[19] These compounds have activities classified as medium or high (Se article by Våbenø *et al* for details on classification).[19] The compounds are given in Table 1.

Table 1: Compound selection. Compounds **1b-1f** are analogs of FC131 (**1**), and they all have a guanidino group in their Xaa^2 residues giving them the potential to bind in both 3-point pharmacophores and the 4-point pharmacophore. Compounds **2b-2e** are analogs of Ala^2FC131 (**2**).

Compound	Name	Sequence	Affinity	Reference
1	FC131	cyclo(D-Tyr^1-Arg^2-Arg^3-Nal^4-Gly^5)	High	[13]
1b	D-Arg^2FC131	cyclo(D-Tyr^1-Arg^2-Arg^3-Nal^4-Gly^5)	High	[13]
1c	Trans-4-guanidino-Pro^2FC131	cyclo(D-Tyr^1-trans-4-guanidino-Pro^2-Arg^3-Nal^4-Gly^5)	High	[14]
1d	cis-4-guanidino-Pro^2FC131	cyclo(D-Tyr^1-cis-4-guanidino-Pro^2-Arg^3-Nal^4-Gly^5)	High	[14]
1e	D-Arg^2-D-Nal^4-FC131	cyclo(D-Tyr^1-D-Arg^2-Arg^3-D-Nal^4-Gly^5)	High	[13]
1f	RI L-Tyr^1-D-Nal^4FC131	cyclo (Tyr^1-Gly^5-Nal^4-D-Arg^3-Arg^2)	Medium	[18]
2	Ala^2FC131	cyclo(D-Tyr^1-Ala^2-Arg^3-Nal^4-Gly^5)	High	[14]
2b	D-NMe-Ala^2FC131	cyclo(D-Tyr^1-D-NMe-Ala^2-Arg^3-Nal^4-Gly^5)	High	[14]
2c	D-Ala^2FC131	cyclo(D-Tyr^1-D-Ala^2-Arg^3-Nal^4-Gly^5)	Medium	[14]
2d	Pro^2FC131	cyclo(D-Tyr^1-Pro^2-Arg^3-Nal^4-Gly^5)	Medium	[14]
2e	NMe-Ala^2FC131	cyclo(D-Tyr^1-NMe-Ala^2-Arg^3-Nal^4-Gly^5)	Medium	[14]

2.1.2 Starting conformations

The low energy conformations for compound **1-1f** and **2-2e** used by Våbenø *et al* were made available. These compounds had been energy minimized in vacuum with a dielectric constant (ε) of 80 using the OPLS-AA force field (OPLS: Optimized Potential for Liquid Simulations).[19, 20] The term force field refers to the set of mathematical equations, variables and parameters used to simulate the potential energy of a system of particles, in this case the particles are atoms; hence the term virtual solvent/vacuum.

The program Phase, developed by Scrödinger inc. was used for pharmacophore generation in this thesis..[21-24] Phase has different recommended settings than those used by Våbenø *et al*.[19] The recommended environment settings is either vacuum with a distant depended dielectric constant of 4(ε=4r), or a Generalized Born/Surface Area water model (GB/SA).[25] Moreover Phase uses the OPLS-2005 force field.[24] Reference on this force field has not been found, but the Maestro manual states that it is an enhanced version of the OPLS-AA force field which provides larger coverage of organic functionalities. Due to the uncertainties in mimicking the transmembrane protein environment of the CXCR4 receptor, one therefore wanted to investigate the use of the different two environment settings in Phase, using the OPLS-2005 force field, in addition to the settings used by Våbenø.

Access to the ECEPP/2 starting conformations of Våbenø *et al* were also provided and this became the basis for ε=4r and GB/SB minimizations of **1-2e** (ECEPP: Empirical Conformational Energy Program for Peptides).[26] ECEPP/2 is a force field that mainly energy minimizes the peptide torsion angles. The starting conformations from ECEPP/2 had been obtained by a systemic conformational search, which generates molecular conformations by systematically rotating bonds by discrete increments. This generates a search tree that results in reasonable molecular conformations. (See Figure 5)

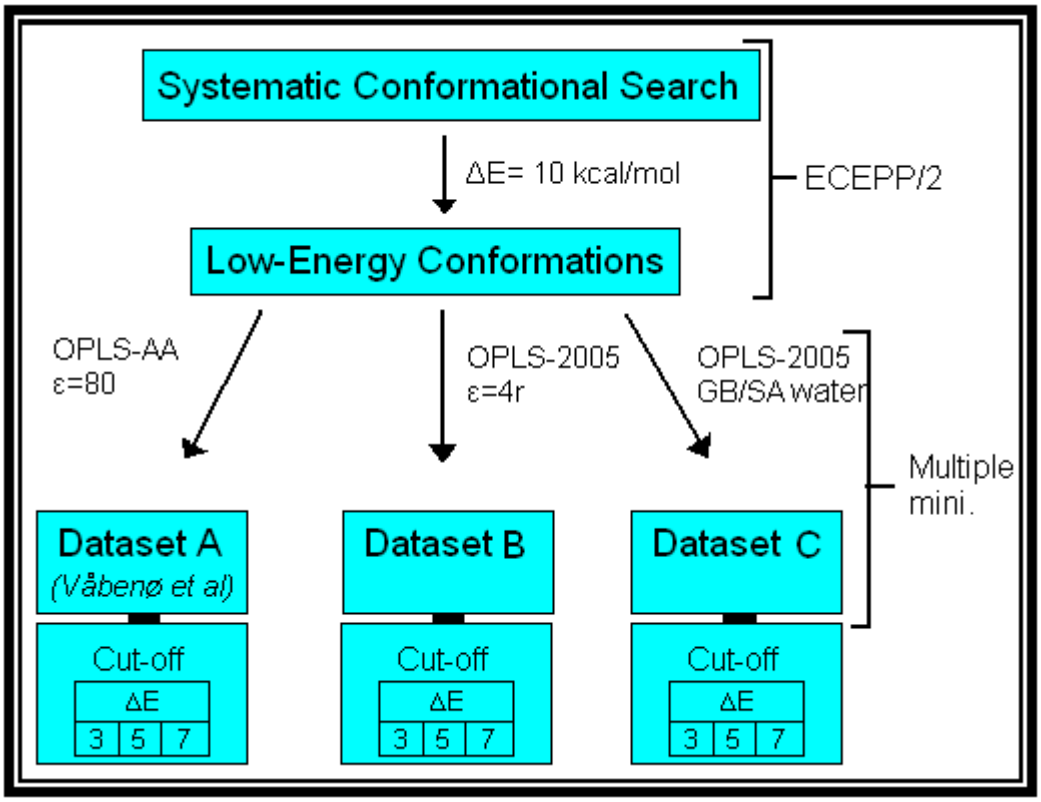

Figure 5: The figure show the systematically approach to develop low energy conformations. The figure also show the three datasets used in this thesis.

Dataset **B** and Dataset **C** were made by using the Multiple Minimization option in the MacroModel interface in Maestro.[24, 27]

The lowest energy conformations for all compounds were investigated using the Ramachandran plot.[28] The Ramachandran plot is a map over experimentally observed favorable and unlikely Phi and Psi values. Positive and Phi negative Psi values are forbidden in this plot, and this was seen for the L-Arg residue on the three lowest energy conformations for compound **1c** in Dataset **C**. For D-residues this plot is reversed, and negative Phi values and positive Psi values for the D-Tyr residue in compound **1b** were also identified in Dataset **C**. Since these conformations were considered computational artifacts they were removed from further investigation and the relative energy values were adjusted accordingly.

The final conformations for all three datasets were divided into groups with different cut-offs (ΔE: 3, 5 and 7 kcal/mol). The results are presented in Tables 2, 3 and 4.

Table 2: Number of conformations for Dataset **A**. (ΔE is in units of kcal/mol) The numbers of conformations generated for the energy cutoff of 3 kcal/mol shows minute differences from that of Våbenø *et al*.[19] This is believed to be due to a less accurate conversion from joules to calories by Våbenø

Compound	ΔE=3	ΔE=5	ΔE=7
1	217	1001	2213
1b	79	478	1332
1c	192	960	1951
1d	175	798	1485
1e	113	488	1128
1f	36	269	938
2	52	284	549
2b	146	682	1411
2c	84	515	1492
2d	81	508	1251
2e	146	561	1001

Table 3: Number of conformations for Dataset **B**
(ΔE is in units of kcal/mol)

Compound	ΔE=3	ΔE=5	ΔE=7
1	259	685	1342
1b	93	413	918
1c	109	546	1280
1d	256	662	1105
1e	138	133	924
1f	132	379	829
2	44	134	328
2b	145	526	1072
2c	115	473	1140
2d	142	539	1126
2e	131	359	708

Table 4: Number of conformations for dataset **C**
(ΔE is in units of kcal/mol)

Compound	ΔE=3	ΔE=5	ΔE=7
1	109	664	1828
1b	93	350	914
1c	83	415	867
1d	91	386	1048
1e	71	251	393
1f	39	266	1018
2	53	454	504
2b	99	407	989
2c	66	239	735
2d	95	335	906
2e	123	381	749

2.1.3 Pharmacophore Modeling

As mentioned above pharmacophore modeling was done with the Phase software.[23] Phase develops pharmacophore models from sets of conformers of different ligands. The program will also rank the hypotheses and display the conformers from each ligand with the closest fit for the pharmacophore

2.1.3.1 Reproducing 3-point model

Reproducing the 3-point pharmacophore of Våbenø et al. was done with Dataset **A** as it contains the conformers used in the original publication.[19]

The program was set to score hypotheses with four pharmacophoric sites , one positively charged group and three aromatic rings. Since, the two aromatic rings of Nal[4] can be counted as one feature this corresponds to a 3-point pharmacophore. If one knows the planar orientation of one of the aromatic rings there is only two potential orientations of the Nal[4] side chain. (See figure 6)

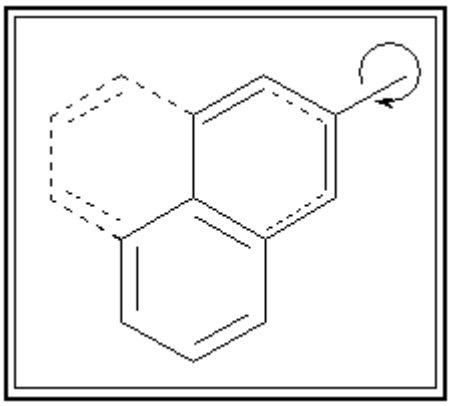

Figure 6. The only two possible orientations of Nal[2] if one knows the planar orientation of the C^{γ}-aromatic group. The C^{β}-C^{γ} bond displays free rotation and the two possibilities is obtained by rotating 180˚.

The results from the 3-point pharmacophore generation with 3 kcal/mol cut-off are shown in Figure 7 and Tables 5, 6 and 7.

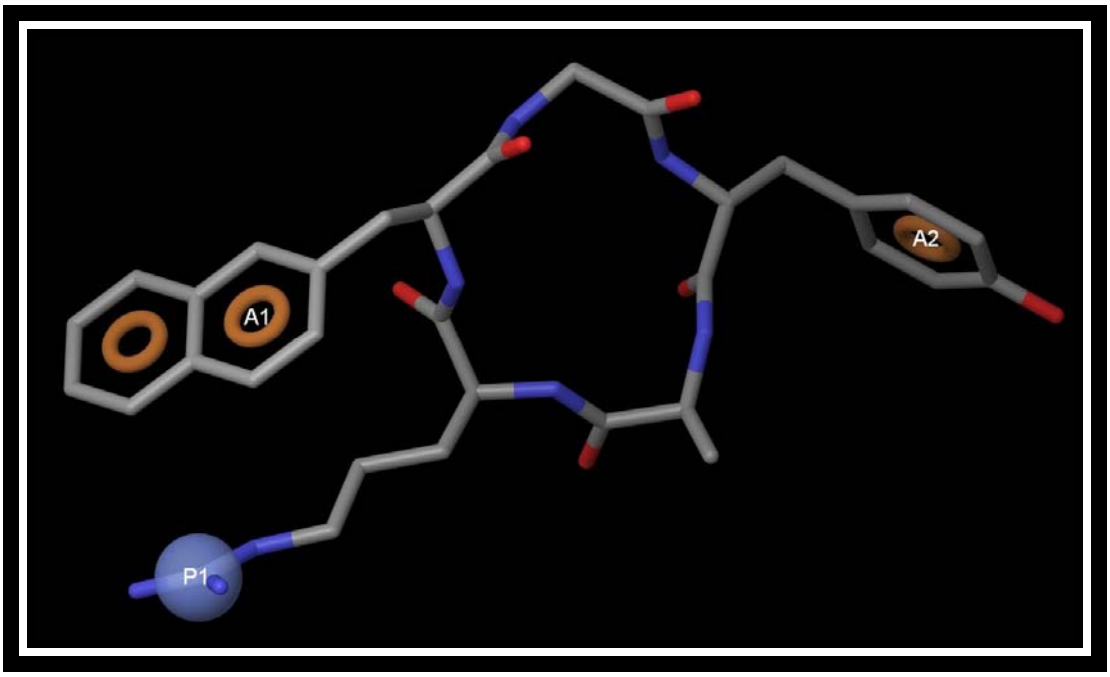

Figure 7: Pharmacophore model together with best fit for **2**.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Table 5: Torsion angles for **2** with best fit for pharmacophore model.

Residue	φ	ψ	ω	X_1	X_2	X_3	X_4
Gly	77,0	64,2	-176,2	-	-	-	-
D-Tyr	137,2	-103,6	-178,5	176,5	105,0	179,5	-
Ala	-68,3	-48,1	178,8	-	-	-	-
Arg	-136,3	-55,5	-177,8	-173,1	60,0	174,2	140,0
Nal	-115,2	83,5	-176,5	-62,0	116,9	-	-

Table 6: Measured distances between pharmacophore sites. Distances are given in Angstroms.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site		Distance
A1	A2	13,10
A1	P1	4,86
A2	P1	14,94

Table 7: Measured angles between pharmacophore sites.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site	Angle	
A1	$\angle A^2A^1P^1$	102,7
A2	$\angle A^1A^2P^1$	18,5
P1	$\angle A^1P^1A^2$	58,8

The result from 3-point pharmacophore generation with Dataset **A** supports the findings of Våbenø et al.[19] Torsion angles found are virtually identical, and by visual comparison of the two pharmacophores; they are undistinguishable. In this thesis the distances and angles of the actual pharmacophoric sites are also given. This validation shows that Phase is compatible with other methods, and that a comparison of the recommended settings of the program is feasible.

2.1.3.2 Generation of 4-point Pharmacophores

Only compounds with a guanidine-group in Xaa^2 were used to generate 4-point pharmacophores. These were the six compounds **1-1f.** The program was set to score hypotheses with two positive charges and three aromatic groups, a total of five pharmacophoric sites. As described above the two aromatic rings of Nal^4 will be counted as one, resulting in a 4-point pharmacophore.

It was initially required that 6 of 6 ligands should match the pharmacophore. This failed for all three datasets using energy cut-offs of 3- and 5kcal/mol respectively when adjusting to match 5 of 6 active ligands, pharmacophore generation succeeded using a cut-off of 5 kcal/mol. In theory this was not a problem since some compound could just as well bind to the receptor according to a 3-point pharmacophore, i.e. having a guanidino group at the Xaa^2 residue does not automatically prove that it displays a 4-point binding to CXCR4. However, for the three datasets there were four different compounds that did not fit the pharmacophore.

For dataset **A**; compound **1c** did not fit any pharmacophores generated**.**
For dataset **B**; compound **1f** did not 8/9 pharmacophores and compound **1b** did not fit 1/9 pharmacophores.
For dataset **C**; compound **1e** did not fit any pharmacophores generated.

Subsequently pharmacophore generation was attempted with a cut-off of 7kcal/mol and a fit for 6/6 compounds. This succeded with dataset **A** and **B**, but not for **C**.

Removal of Artificial Pharmacophores

It was expected that compound **1**, FC131, would have to fit pharmacophores with conformations that displayed the backbone characteristics deduced by NMR studies,[15] and this was set as a criterion. Other pharmacophores were not investigated. This means that the three amide bonds of Tyr^1-Arg^2, Arg^2-Arg^3 and Arg^3-Nal^4 are oriented in the same direction and that the amide bonds of Nal^4-Gly^1 and Gly^1-D-Tyr^2 are oriented in the opposite direction (see figure 2). Pharmacophores requiring other backbone conformations were not considered.

Pharmacophores for Dataset A; ε = 80

Pharmacophore $\mathbf{A_5}$

Pharmacophores will hereafter be denoted with a capital letter indicating which dataset it is derived from and a subscript indicating the energy cut-off used. For example $\mathbf{A_5}$ is derived from dataset **A** and a energy cut off of 5 kcal/mol for conformations are used in pharmacophore generation.

Results for $\mathbf{A_5}$ is given in Figure 8 and Tables 8-10

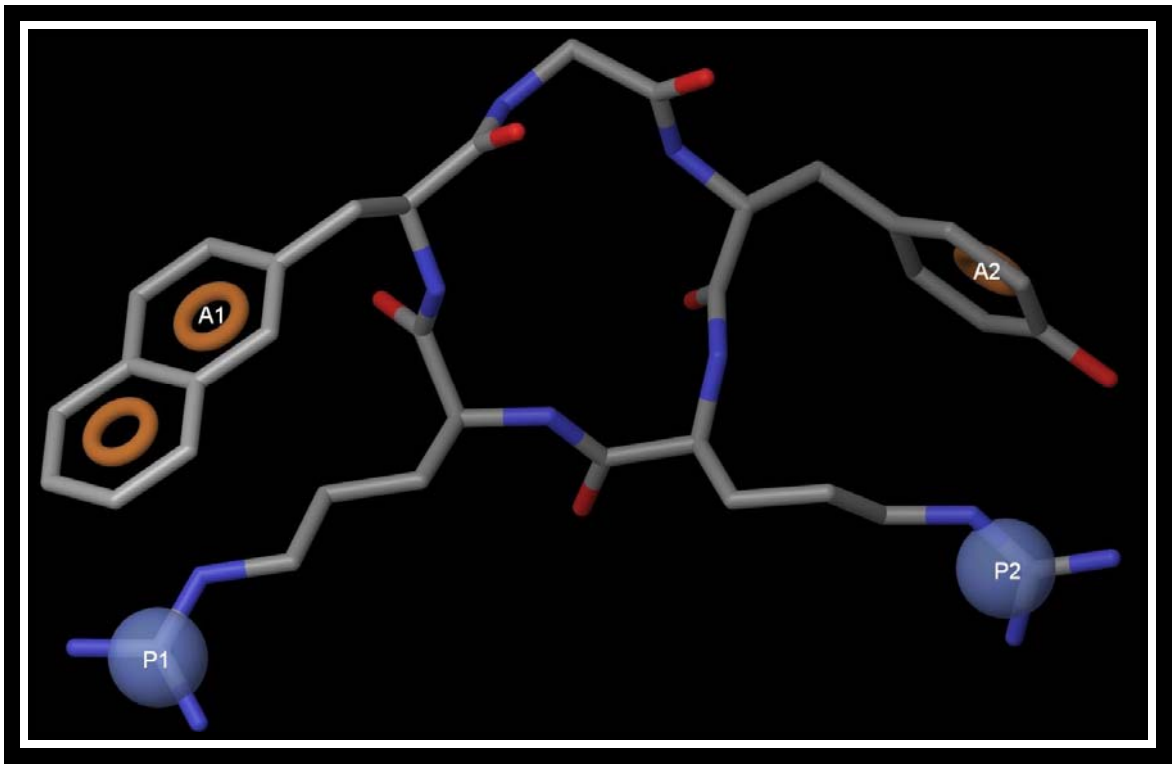

Figure 8: Pharmacophore model $\mathbf{A_5}$ together with best fit for FC131 (**1**).
A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1, P2= Positive site 2

Table 8: Torsion angles for FC131 (**1**) with best fit for pharmacophore model

Residue	φ	ψ	ω	X_1	X_2	X_3	X_4
Gly	71,4	64,4	-171,7	-	-	-	-
D-Tyr	139,9	-113,8	-175,5	179,6	105,3	-178,7	-
Arg	-71,4	-39,6	176,1	-57,2	-60,0	-174,2	138,5
Arg	-135,6	-60,6	-177,3	-173,4	174,8	-178,7	138,5
Nal	-112,4	87,9	-177,9	-62,3	120,4	-	-

Table 9: Measured distances between pharmacophore sites. Distances are given in Angstrom.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site		Distance
A1	A2	13,25
A1	P1	5,67
A1	P2	13,62
A2	P1	14,97
A2	P2	5,03
P1	P2	13,67

Table 10: Measured angles between pharmacophore sites.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site	Angle	
A1	$\angle A^2A^1P^1$	96,3
	$\angle A^2A^1P^2$	21,5
	$\angle P^1A^1P^2$	78,5
A2	$\angle A^1A^2P^1$	22,1
	$\angle A^1A^2P^2$	83,4
	$\angle P^1A^2P^2$	65,4
P1	$\angle A^1P^1A^2$	61,6
	$\angle A^1P^1P^2$	77,5
	$\angle A^2A^1P^2$	19,5
P2	$\angle A^1P^2P^1$	24,0
	$\angle A^1P^2A^2$	75,1
	$\angle P^1P^2A^2$	95,1

The pharmacophore and peptide-backbone for the 4-point-model is almost identical to the 3-point model. The only major difference is the addition of the extra positively charged pharmacophoric site in Xaa^2 and that the naphtyl moiety is flipped by 180^o.

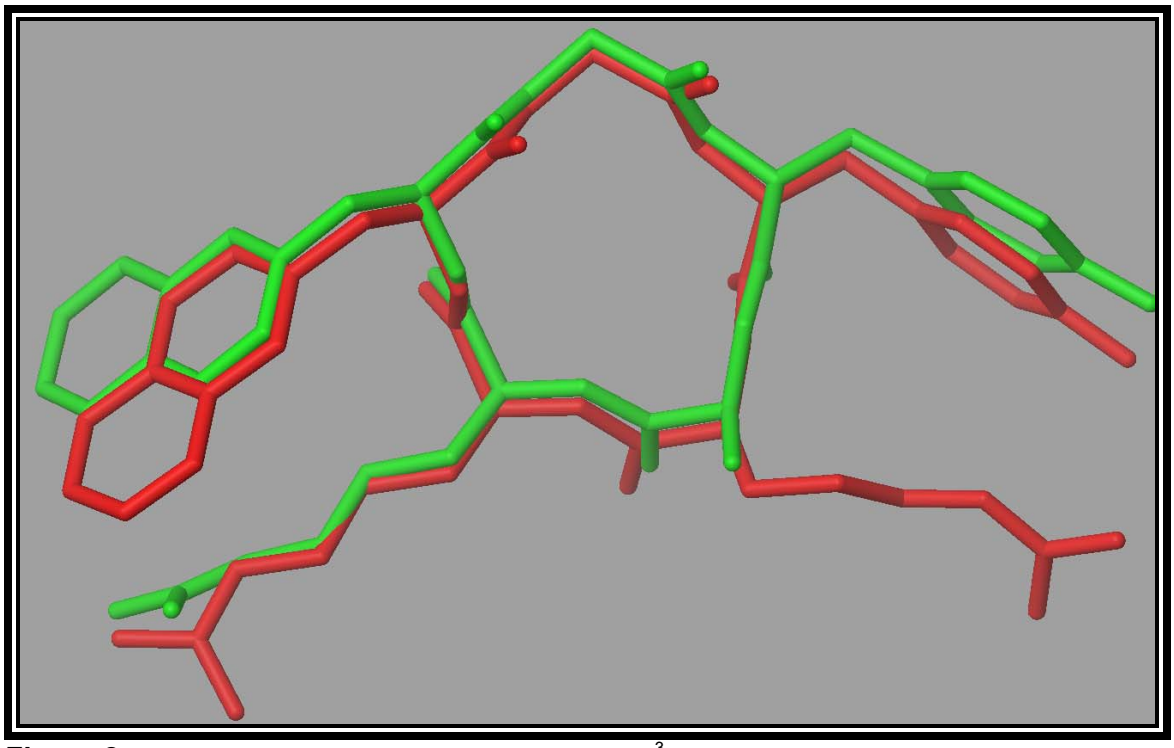

Figure 8: Superimposition of reference compound Ala^3FC131 **2** for the 3-point pharmacophore (green) and reference compound FC131, **1,** for $\mathbf{A_5}$ (red)

Pharmacophore **A_7**

The pharmacophore **A_7** is somewhat different from **A_5,** since The Arg^2 side chain points in a different direction. The backbone and the orientation of the other side chains are almost identical. See Figure 9

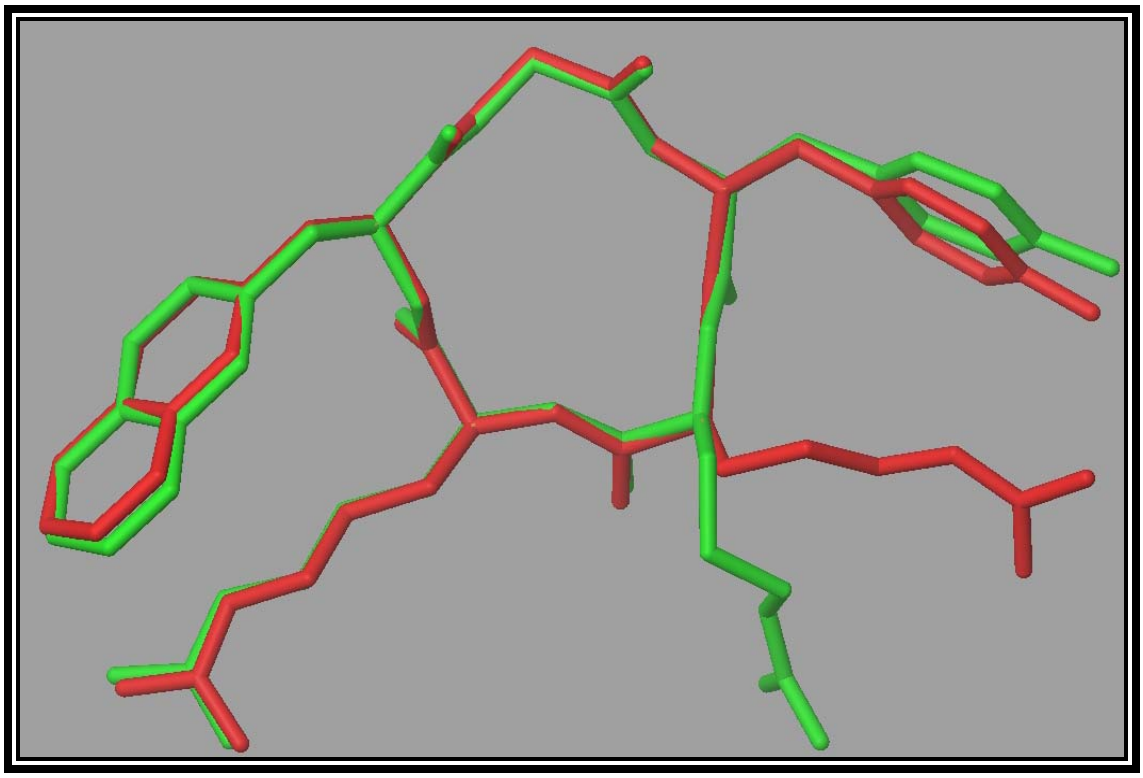

Figure 9: Superimposition of **1** with best fit for **A_5** (red) and **A_7** (green)

Pharmacophores for Dataset B; ε = 4r

Pharmacophore **B_5**

Results are given in Figure 10 and Table 11-13

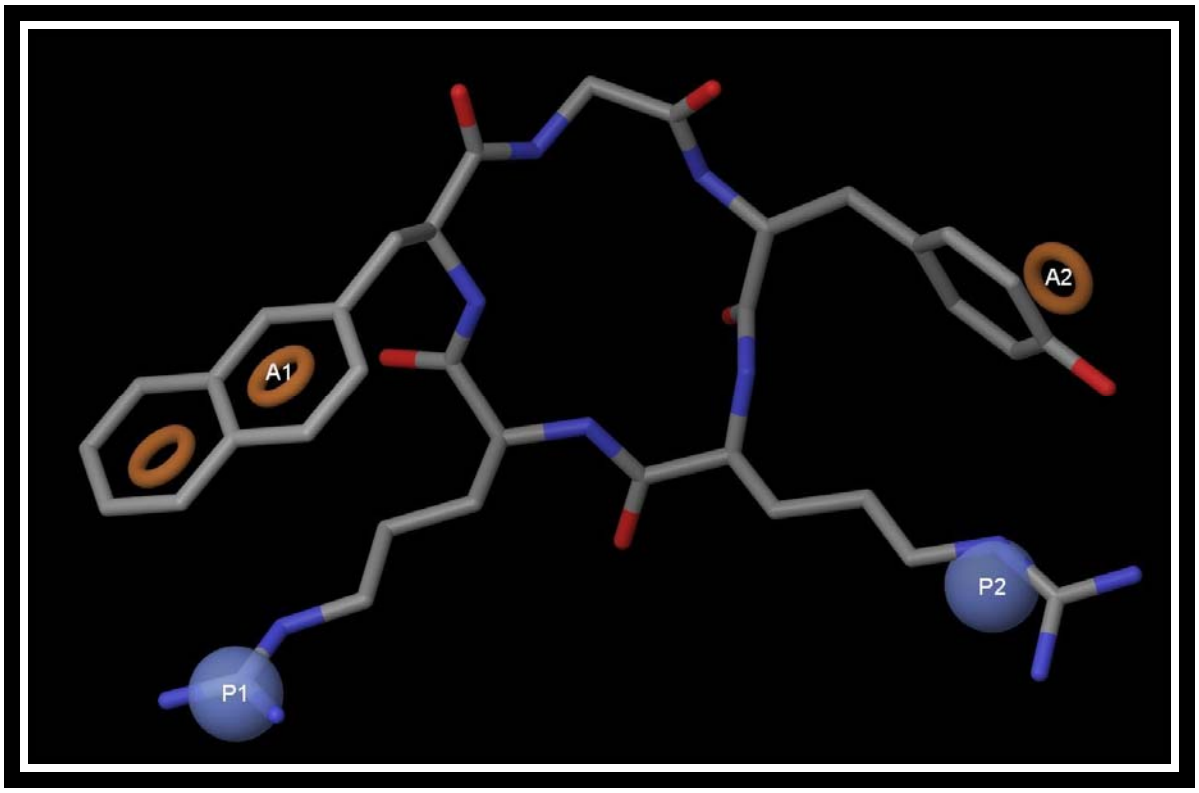

Figure 10: $\mathbf{B_5}$ together with best fit for **1**

Table 11: Torsion angles for **1** with best fit for $\mathbf{B_5}$.

Residue	φ	ψ	ω	X_1	X_2	X_3	X_4
Gly	117,0	61,0	-163,3	-	-	-	-
D-Tyr	148,2	-120,0	-178,1	172,5	95,3	-179,8	-
Arg	-83,4	-16,6	177,0	-62,2	174,2	-179,3	-158,8
Arg	-154,4	-34,7	-168,5	-171,7	173,1	-175,3	171,1
Nal	-129,2	25,8	-173	-60,4	119,1	-	-

Table 12: Measured distances between pharmacophore sites. Distances are given in Angstroms. (A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site		Distance
A1	A2	13,26
A1	P1	5,36
A1	P2	12,47
A2	P1	15,56
A2	P2	5,28
P1	P2	12,87

Table 13: Measured angles between pharmacophore sites.
(A1=Aromatic site 1, A2=Aromatic site 2, P1=Positive site 1)

Pharmacophore site	Angle	
A1	$\angle A^2A^1P^1$	105,3
	$\angle A^2A^1P^2$	23,4
	$\angle P^1A^1P^2$	82,0
A2	$\angle A^1A^2P^1$	19,4
	$\angle A^1A^2P^2$	69,9
	$\angle P^1A^2P^2$	50,6
P1	$\angle A^1P^1A^2$	55,3
	$\angle A^1P^1P^2$	73,7
	$\angle A^2A^1P^2$	18,5
P2	$\angle A^1P^2P^1$	24,3
	$\angle A^1P^2A^2$	86,7
	$\angle P^1P^2A^2$	111,0

A superimposition of **1** with best fit for $\mathbf{A_5}$ and $\mathbf{B_5}$ shows similarities of both the backbone and the four pharmacophore sites. There is only slight differences in the orientation of the Nal^4-Gly^5 amide bond and the spacial orientation of the Nal^4 side chain, however the naphtyl ring plane is the same. See Figure 11. A comparison of the measured distances and angles for the pharmacophores neither reveals any major differences.

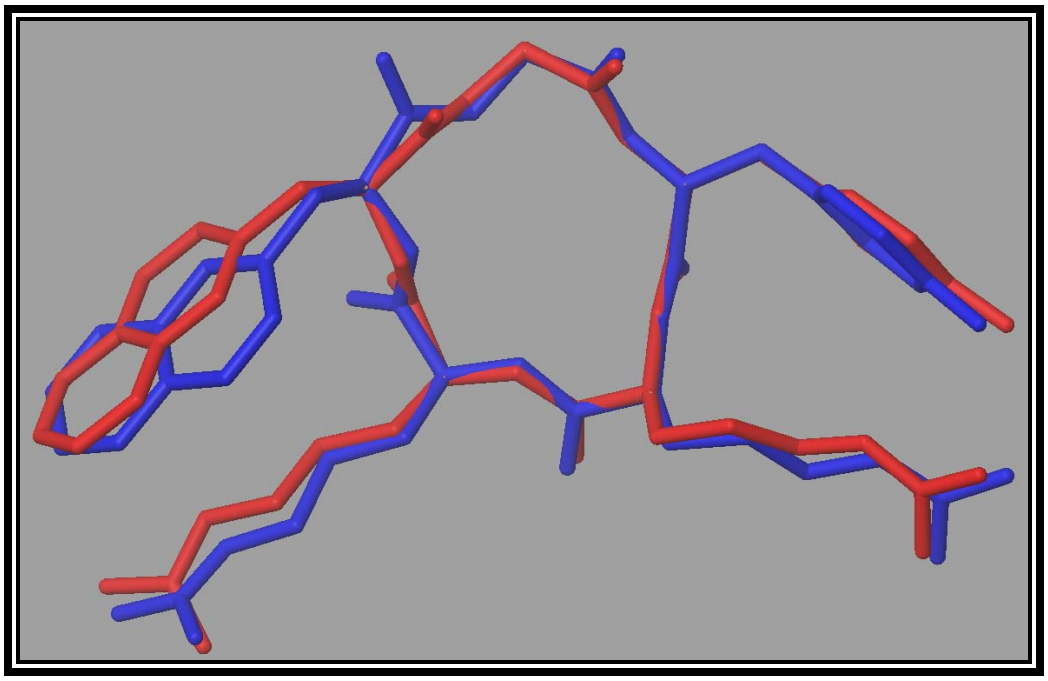

Figure 11: Superimposition of the reference compound **1** of pharmacophore $\mathbf{A_5}$ (red) and $\mathbf{B_5}$ (blue)

Pharmacophore $\mathbf{B_7}$

The pharmacophore $\mathbf{B_7}$ is somewhat different from $\mathbf{B_5}$, again as a result of a different orientation of the Arg^2 side chain of the reference compound **1**. The backbone and the orientation of the other side chains are almost identical. See Figure 12

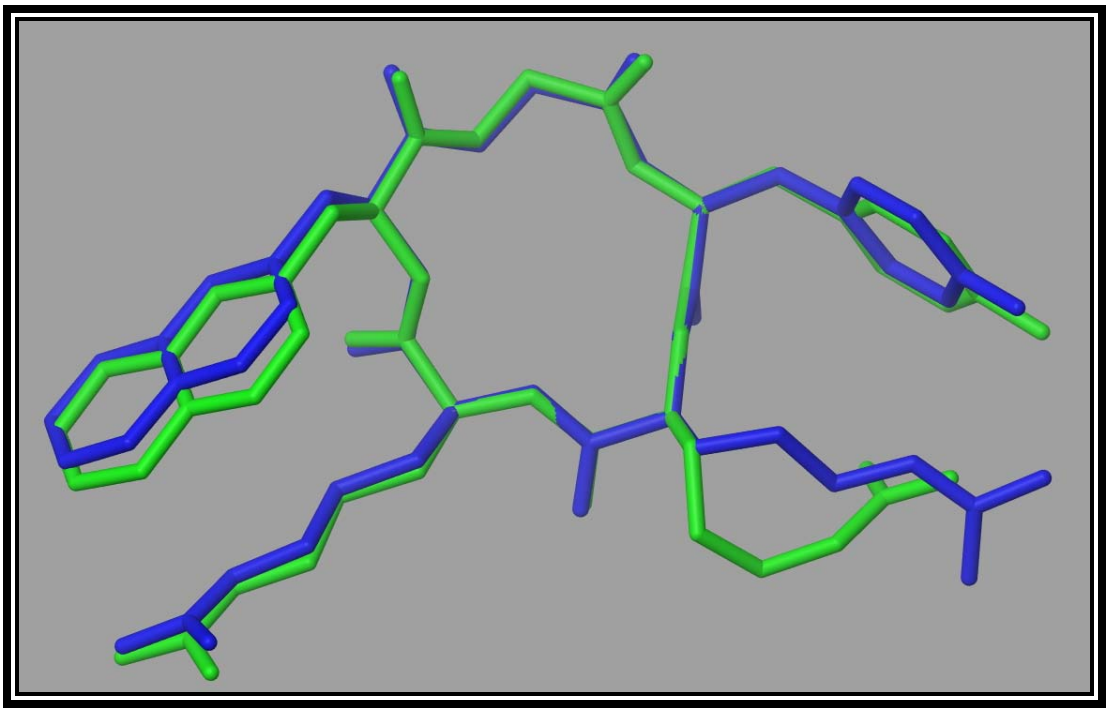

Figure 12: Superimposition of **1** with best fit for $\mathbf{B_5}$ (blue) and $\mathbf{B_7}$ (green). The positions of the guanidino group of Arg^2 is different.

Pharmacophores for Dataset C; GB/SA

The backbone criterion for reference compound **1** was not fulfilled for any pharmacophores generated, even with an energy cut-off of 10 kcal/mol and Dataset **C** was subsequentally abandoned from further reaserch.

Observations:

Superimposing **1** and **1b** with best fit for the pharmacophore $\mathbf{A_5}$ shows that the two Arg^2-residues together form a 6-membered ring in the chair conformation.

This observation could explain the fact that the two compounds have similar activities, despite having different stereochemistry in Xaa^2. See figure 13 and 14.

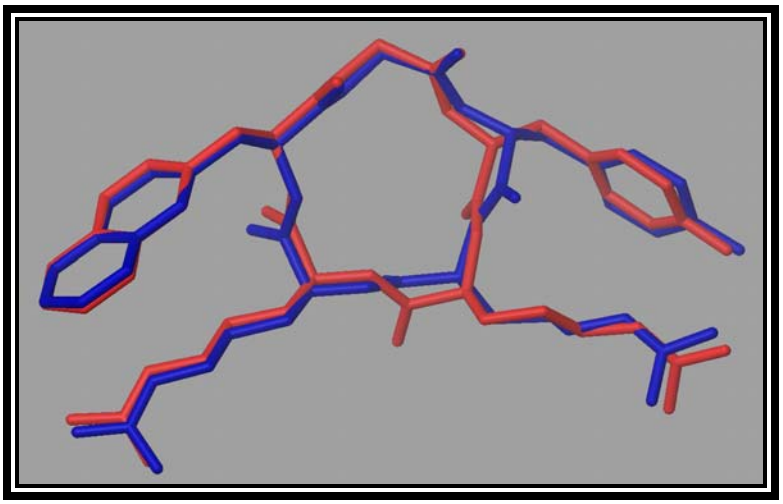

Figure 13: $\mathbf{A_5}$: **1** and **1b** superimposed. The Arg^2 residues form a 6-membered ring.

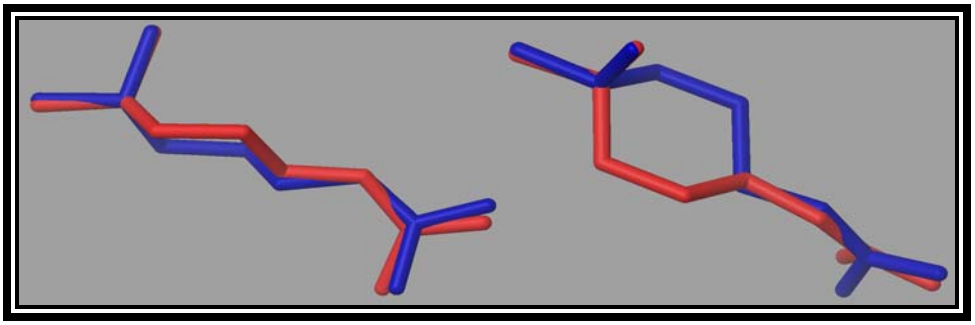

Figure 14: Superimposition of the Arg^2 residues from **1** and **1b** with best fit for $\mathbf{A_5}$

This could not be seen for the $\mathbf{B_7}$ and $\mathbf{B_5}$ conformations as no conformation for **1b** suitable for pairing such a ring was generated. However the conformations of the reference compound **1** for $\mathbf{B_7}$ and $\mathbf{B_5}$ showed pseudo-6-rings which was fully superimposable with cyclohexane as shown in Figure 15

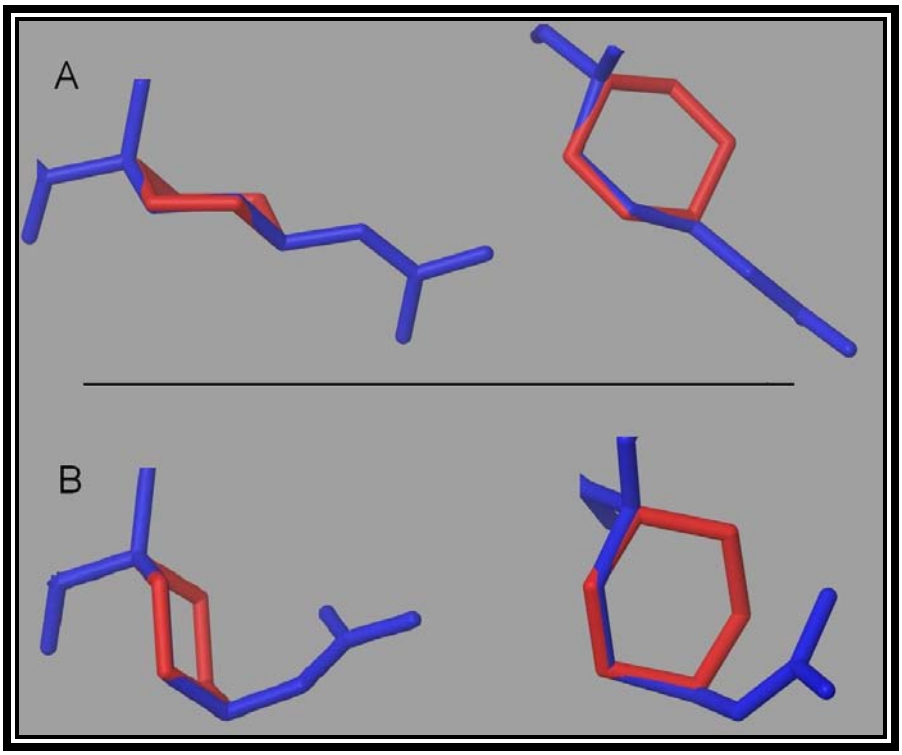

Figure 15: A: Arg-residue from the reference compound **1** (blue) for $\mathbf{B_5}$ superimposed on cyclohexane (red). B: Arg-residue from the reference compound **1** (blue) $\mathbf{B_7}$ superimposed on cyclohexane (red).

2.2 Design of target compounds

The top ranking pharmacophores generated φ, ψ angles of ~ -60˚,-60˚ in the Xaa^2 residue of the reference compounds. This was consistent for almost all compounds with fit for pharmacophores. Such angles are coherent with helix formation. This angle can be stabilised with disubstituted amino acids. One amino acid commonly used for this purpose is α-aminoisobutyric (Aib)(see figure 16) [29, 30]. Incorporating this amino acid instead of Ala^2 in **2** may increase the effect of the compound due to backbone stabilization.

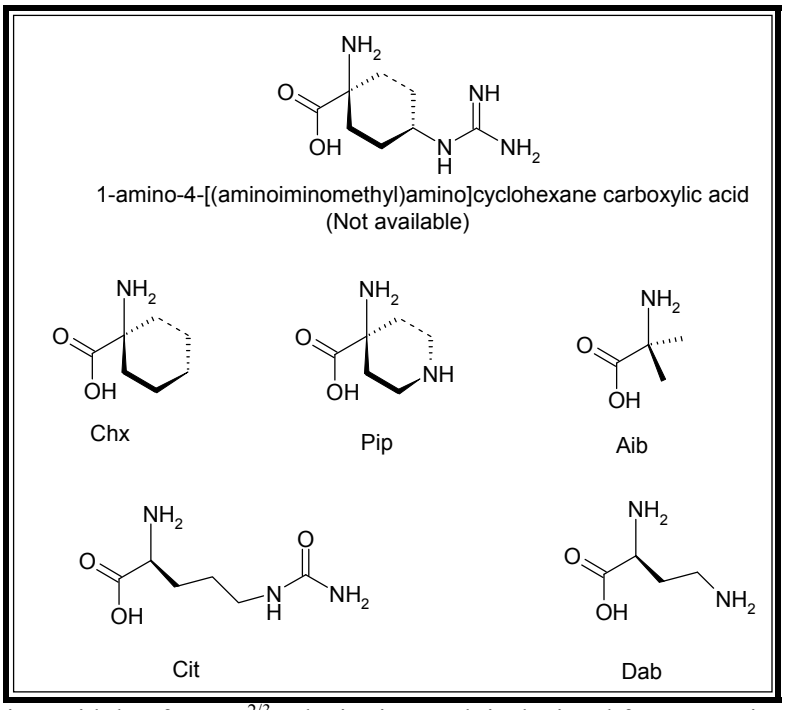

Figure 16: Amino acid that for $Xaa^{2/3}$ substitution. Dab is depicted for comparison with Pip as it is not a standard amino acid.

4-point pharmacophores showed that the D/L-Arg^2- residues of **1** and **1b** displays pseudo 6-membered rings protruding from the α-carbon. Incorporating such a ring into arginine results in 1-amino-4-[(aminoiminomethyl)amino]cyclohexane carboxylic acid (see figure 16). Replacing the Arg^2 residue of **1** with such a residue will give the guanidino group a more fixed position and may increase the activity of the compound. The constrained side chain will also stabilize the backbone like the Aib residue. In other words it is a very interesting subject for substitution with Arg^2 in **1**. However; such an amino acid was not available for purchase.

4-piperidine-carboxylic acid (Pip) was available (See figure 16). Replacing Ala^2 with alpha,gamma-diamonobutyric acid (Dab) in **2** has shown to increase the activity of the compound.[14] Pip can be compared with the activity of this compound. It will give the nitrogen moiety a more fixed position due to constraint in the piperidine ring. It will also stabilize the backbone. However, in the case of increased activity it is important to know if it is due to the more fixed position of the nitrogen or if it is a result of backbone stabilization. 1-aminocyclohexane carboxylic acid (Chx) was therefore also interesting for substitution in the Xaa^2 position. This amino acid will display the 6-membered ring

with no nitrogen moiety and thereby shed light on the effect of the constrained position of nitrogen in Pip. Chx will also be interesting in comparison to Aib.

It was also wanted to investigate if one could substitute any of the two arginines in **1** with citrulline without drastically reducing the biological activity. Arginine will be ionized at biological pH and that makes for poor ADME properties. Citrulline will not be ionized and citrulline containing analogs of **1** may serve as a better lead compounds for drug development if they maintain its parent compound activity.

The eight target compounds for synthesis in this thesis are given in table 14.

Table 14: Target compounds for synthesis.

Compound	Abreviation	Sequence
1	FC1231	Cyclo(D-Tyr^1-Arg^2-Arg^3-Nal^4-Gly^5)
2	Ala^{2}FC131	Cyclo(D-Tyr^1-Ala^2-Arg^3-Nal^4-Gly^5)
3	Aib^{2}FC131	Cyclo(D-Tyr^1-Aib^2-Arg^3-Nal^4-Gly^5)
4	Chx^{2}FC131	Cyclo(D-Tyr^1-Chx^2-Arg^3-Nal^4-Gly^5)
5	Pip^{2}FC131	Cyclo(D-Tyr^1-Pip^2-Arg^3-Nal^4-Gly^5)
6	Cit^{2}FC131	Cyclo(D-Tyr^1-Cit^2-Arg^3-Nal^4-Gly^5)
7	Cit^{3}FC131	Cyclo(D-Tyr^1-Arg^2-Cit^3-Nal^4-Gly^5)
8	Cit^{2,3}FC131	Cyclo(D-Tyr^1-Cit^2-Cit^3-Nal^4-Gly^5)

1 and **2** was made as reference compounds.

2.3 Synthesis of Target Compounds 1-8

2.3.1 Synthetic strategy

The overall synthetic strategy was to synthesize linear pentapeptides by solid-phase peptide synthesis (SPPS), followed by cyclization in solution, side chain deprotection, and purification.

It is important to choose the linear precursors carefully. This is because cyclization position plays an important role in ring closure. It has been shown that cyclization of pentapeptides with large or small residues on both termini results in low yields.[31] In other words; higher yields are obtained when cyclizing between one small and one large residue. Furthermore; disubstituted residues on either of the termini may give low yields due to steric hindrance surrounding the terminals,

Our target compounds, with the exception of compound **2**, all have four large residues, or three large and one disubstituted. It is therefore necessary to cyclize between Gly^5-Nal^4 or D-Tyr^1-Gly^5. Low yields when cyclizing with an N-terminal L-Tyr have been reported. Using D-Tyr the yield was higher if the C-terminal had a small L-residue. One last thing to consider is that cyclization is associated with risk of racemization. In this thesis it was chosen to cyclize between the D-Tyr^1 and Gly^5 residue. This is because glycine does not have a stereocenter, and therefore cannot epimerize.

Tyr, Arg and Pip have reactive side chains that need to be protected during synthesis of linear precursors and in cyclization. It was chosen to use Pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for Arg, *tert*-butyl (tBu) for Tyr, and *tert*-Butyl carbamate (Boc) for Pip. These have shown to give good protection of the reactive moieties.[32]

Table 15: Linear precursors for the synthesis of **1-8**

Compound	Sequence
9	H_2N-D-Tyr^1(tBu)-Arg^2(Pbf)-Arg^3(Pbf)-Nal^4-Gly^5-OH
10	H_2N-D-Tyr^1(tBu)–Ala^2-Arg^3(Pbf)-Nal^4-Gly^5-OH
11	H_2N-D-Tyr^1(tBu)–Aib^2-Arg^3(Pbf)-Nal^4-Gly^5-OH
12	H_2N-D-Tyr^1(tBu)–Chx^2-Arg^3(Pbf)-Nal^4-Gly^5-OH
13	H_2N-D-Tyr^1(tBu)–Pip^2(Boc)-Arg^3(Pbf)-Nal^4-Gly^5-OH
14	H_2N-D-Tyr^1(tBu)-Cit^2-Arg^3(Pbf)-Nal^4-Gly^5-OH
15	H_2N-D-Tyr^1(tBu)-Arg^2(Pbf)-Cit^3-Nal^4-Gly^5-OH
16	H_2N-D-Tyr^1(tBu)-Cit^2-Cit^3-Nal^4-Gly^5-OH

2.3.2 Pilot experiment

Before starting full scale synthesis of **1-8**, pilot experiments were performed. Specifically, it was of interest to optimize the cyclization step by exploring different solvents and coupling agents.

We had access to batches of crude linear **9** and **10** from the master course BIO-3311: Synthetic peptides, Design and applications in biotechnology and drug development. These linear precursors had been cleaved from the resin using a solution of acetic acid: trifluoroethanol: dichloromethane (DCM) (1:1:3) and were therefore present as acetate salts. Initial attempts to cyclize these compounds had failed, since the cyclization step resulted in N-acetylation instead of cyclization. To facilitate the desired head-to-tail cyclization, the acetate counter ion present in these batches had to be removed.

2.3.2.1 Extraction of acetate ion

Removal of the acetate ion was attempted by dissolving linear **9** in DCM and extracting the acetate ion with an equivalent amount of 10% aqueous $NaHCO_3$. See figure 17.

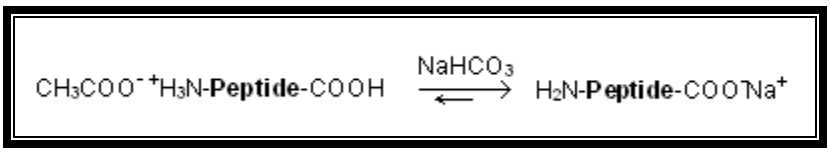

Figure 17: Ion extraction in 10% aqueus NaHCO

This mixture emulsified heavily. Separation of the aqueous and organic phase took 1,5 hours. The process was repeated two times, and the organic phase was dried for one hour with $MgSO_4$ and filtered. DCM was removed in vacuo. Two batches of linear **9** were extracted during pilot experiment. Both gave yields of ~50 %.

Table 16: Batch 1:

Initial mass	30,0 mg
Fields:	
Mass:	15,1 mg
Mol:	$1{,}13 \cdot 10^{-5}$
Percent	50,3%,

Table 17: Batch 2

Initial mass	100,0mg
Yields:	
Mass:	50,0 mg
Mol:	$3{,}75 \cdot 10^{-5}$
Percent	50,0%

MS analysis showed that the desired peptide MW = 1307 was obtained after extraction (see appendix, Figure A-1).

Table 18: M/Z for linear peptide **9**

$[M+H]^+$	1308,8 Da
$[M+Na]^+$	1330,8 Da
$[M+K]^+$	1346,7 Da
$[M+H,\text{-Pbf}]^+$	1056,7 Da

HPLC analysis revealed that the product was 98,12% pure with a retention time (R_t) of 28,2 minutes (see appendix, Figure A-2). Regarding HPLC; the peptide bond shows absorption maximum at 214nm.[33, 34] Therefore it is most suited to check for impurities in peptides. However certain solvents also display absorption at 214 nm. The HPLC chromatogram shows peaks relative to the one with most absorption. Giving a solvent with high absorption it will be able to mask impurities at 214 nm. In this case UV detection at 214 nm showed that the peptide was 100% pure. Detection at 254 nm gave the detection of impurities. (see appendix Figures A-2, A-3).

This was due to N,N-dimethylmethanamide (DMF) added to the HPLC sample to help solvation. i.e DMF is the the biggest peak in the chromatogram at 214 nm. For the rest of the thesis HPLC to check for purity was done at 214 nm without solvents with UV absorption at 214 nm. During cyclization UV detection was done at 254 nm.

Ethyl acetate was also tried as organic solvent in the extraction, but this emulsified even more. Extraction with DCM was, in lack of better alternatives, chosen as a method for removing the acetate counter ion. It gave low yields, but still provided sodium salts suitable for cyclization.

2.3.2.2 Cyclization Experiments.

For the synthesis ot the target compound we wanted to identify a solvent that promotes cyclization of the linear precursors. Since one would be working with high dilution it was desired that the solvent also could be easily removed.

Experiment 1: Solvent effect

DCM is a volatile solvent that is easily removed by evaporation and was used as a solvent for the first pilot experiment

15,1 mg of the sodium salt of the crude linear **9** was dissolved in DCM (4,0 ml DCM / mg peptide) and 2 mol equivalents 3-(Diethoxy-phosphoryloxy-3H-benzo[d][1,2,3]tiazin-4-one (DEPBT)[35] and 2 mol equivalents N,N-Diisopropylethylamine (DIPEA) was added. The solution was left for 24 hours at room temperature and analyzed by MS.

MS revealed that linear **9**, (MW=1307), was the major constituent and the protected cyclic product (MW=1289), seemed absent. The cyclic peptide should weigh 18 Da less than the linear peptide and no such masses were seen after 24 hours. (see Table 18 and appendix, Figure A-4)

Table 18: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+H]^+$	1308,9 Da
$[M+Na]^+$	1330,9 Da
$[M+K]^+$	1346,9 Da
$[M+H,-Pbf]^+$	1056,8 Da

2 equivalents DIPEA was added to the solution, which was left for another 24 hours and analyzed by MS. MS revealed no change.

0,5 ml DIPEA (126 equivalents) and 2 equivalents DEPBT was added, and the solution was analyzed by MS after another 24 hours. MS showed little change.

The peptide solution was then split into two batches. Batch 1 was added 3 equivalents DEPBT, and Batch 2 was added 5,0 ml DMF. Both batches were left overnight and analyzed by MS. MS analysis of Batch 1 revealed that there had been some cyclization, but linear peptides still remained as the major constituent.

(see Table 19, 20 and appendix, Figure A-5)

Table 19: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+H]^+$	1308,7 Da
$[M+Na]^+$	1330,8 Da
$[M+K]^+$	1346,8 Da
$[M+H,-Pbf]^+$	1056,7 Da

Table 20: M/Z for cyclic peptide

M/Z for cyclic peptides	
[M+H]+	1290,7 Da
[M+Na]+	1312,7 Da
[M+K]+	-
[M+H,-Pbf]+	-

MS analysis for Batch 2 revealed cyclized peptides as the major constituent. (See Table 21, 22 and appendix Figure A-6)

Table 21: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+H]^+$	-
$[M+Na]^+$	1330,8 Da
$[M+H,-Pbf]^+$	-

Table 22: M/Z for cyclic peptide

M/Z for cyclic peptide	
[M+H]+	1290,7 Da
[M+Na]+	1312,7 Da
[M+H,-Pbf]+	1038,7 Da

Reflections on experiment on solvent effect

With DCM coupling rate was minimal over 96 hours, even with high concentrations of both DIPEA (130 equivalents) and DEPBT (7 equivalents). DCM is moderately polar, but it may not be sufficient for the polar amide bond mechanism. A polar mechanism is a mechanism that involves the transfer of proton and/or has the potential for hydrogen bonding.

Adding DMF to the mixture initiated cyclization. DMF is a polar aprotic solvent well suitable for polar reaction mechanisms, but it has a low evaporation rate and is difficult to

remove from a solution. It is widely used in SPPS, but SPPS has the benefit that one can filter of DMF.

DMF was chosen for this thesis

Experiment 2: Coupling reagents

HBTU,[36] the coupling reagent for our linear peptides is not a satisfactory coupling reagent for cyclization due to formation of N-terminally guanidinated peptides.[37] It was therefore desired to evaluate the effect of other coupling reagents for cyclization.

DEPBT: DEPBT is an organophosphorus coupling reagent that has shown resistance against racemization and yielded high yields in cyclization of pentapeptides.[31, 35]
See Figure 18

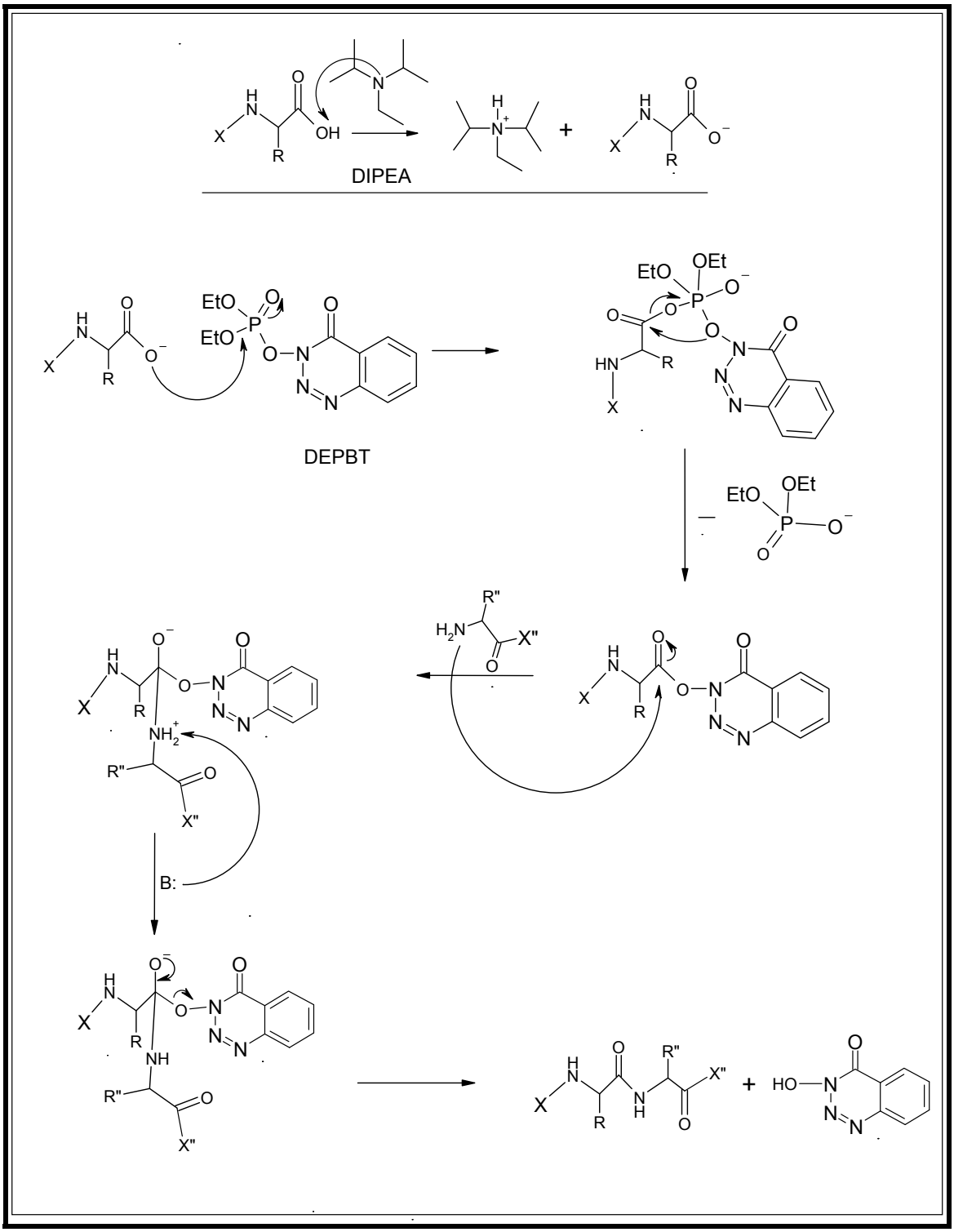

Figure 18: Proposed reaction mechanism for DEPBT. [35]

HPLC of DEPBT was done to distinguish it from peptide peaks during later analysis. See appendix figure A-7

25,0 mg ($1{,}88 \cdot 10^{-5}$ mol) linear peptide was dissolved in 100 ml DMF and 2 mol equivalents of DIPEA and 2 mol equivalents DEPBT were added.[35] Samples were analyzed with MS and HPLC after 8, 32 and 60 hours. After 8 hours no sign of reaction could be detected. After 32 hours MS showed major signs of linear peptide and minor signs of cyclic peptide (see Tables 23, 24 and appendix Figure A-8).

Table 23: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+Na]^+$	1330,8 Da
$[M+H,-Pbf]^+$	1056,7 Da

Table 24: M/Z for cyclic peptide

M/Z for cyclic peptide	
[M+Na]+	1312,7 Da
[M+H,-Pbf]+	1039,7 Da

After 60 hours HPLC showed that the cyclization was still not complete. HPLC shows a minor peak at R_t 28,22 minutes; as for the linear peptide. It also showed a peak with R_t 28,6 minutes. This is believed to be the peak for the cyclic peptide. The cyclic peptide is more hydrophobic than the linear and does will elute later (See appendix Figure A-9).

MS at 60 hours showed that there was both linear and cyclic peptide in the mixture, though cyclic peaks were more pronounced. (See Tables appendix Figure A-10).

Table 25: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+Na]^+$	1330,8 Da
$[M+H,-Pbf]^+$	1056,7 Da

Table 26: M/Z for cyclic peptide

M/Z for cyclic peptide	
[M+Na]+	1312,7 Da
[M+H,-Pbf]+	1039,7 Da

PyBOP: benzotriazol-1-yl-oxytripyrrolidinophosphonium (PyBOP);[38] a phosphonium based coupling agent, has proved to perform cyclizations,[39, 40] especially with a C-terminal Glycine,[40, 41] in high yield. As described earlier low yields have been reported when cyclizing peptides with an N-terminal tyrosine. DEPBT was the coupling agent when these problems were experienced.[31] DEPBT has been compared to a variety of coupling agents,[31, 35] but no comparisons concerning this specific problem has been found in the literature. Comparisons of PyBOP and DEPBT are scarce in the literature, but it is

reported that PyBOP is better than DEPBT in running reactions to depletion[41]. PyBOP was readily available in house and therefore a comparison of the two seemed called for. PyBOP mediates coupling through the formation of a benzotriazole-ester (OBt-ester) see Figure 19

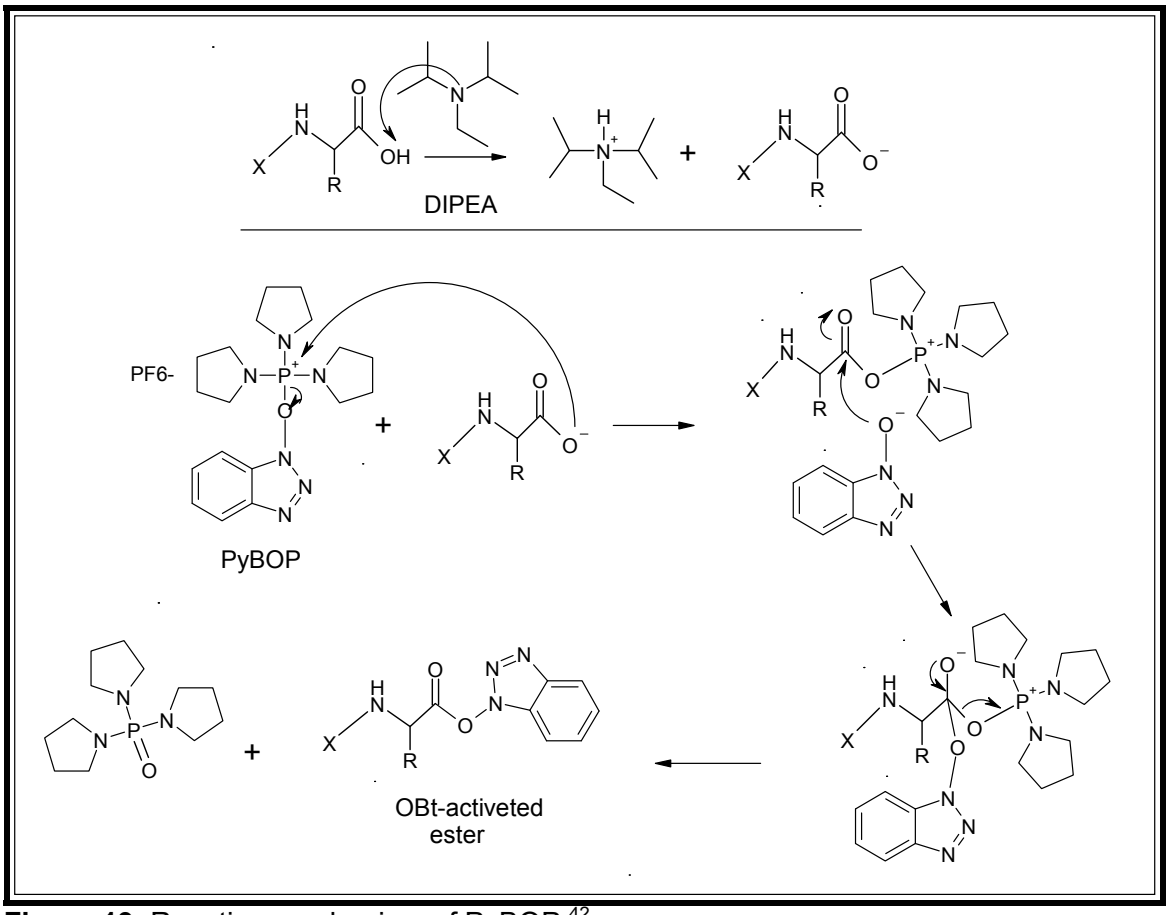

Figure 19: Reaction mechanism of PyBOP.[42]

Benzotriazole is polar and combined with oxygens in the carboxylic ester it results in diminished density of the electron cloud surrounding the carbonyl carbon, giving it a stronger partial positive charge. This mediates nucleophilic attack of lone pairs from the amines. OBt is also a good leaving group. See Figure 20

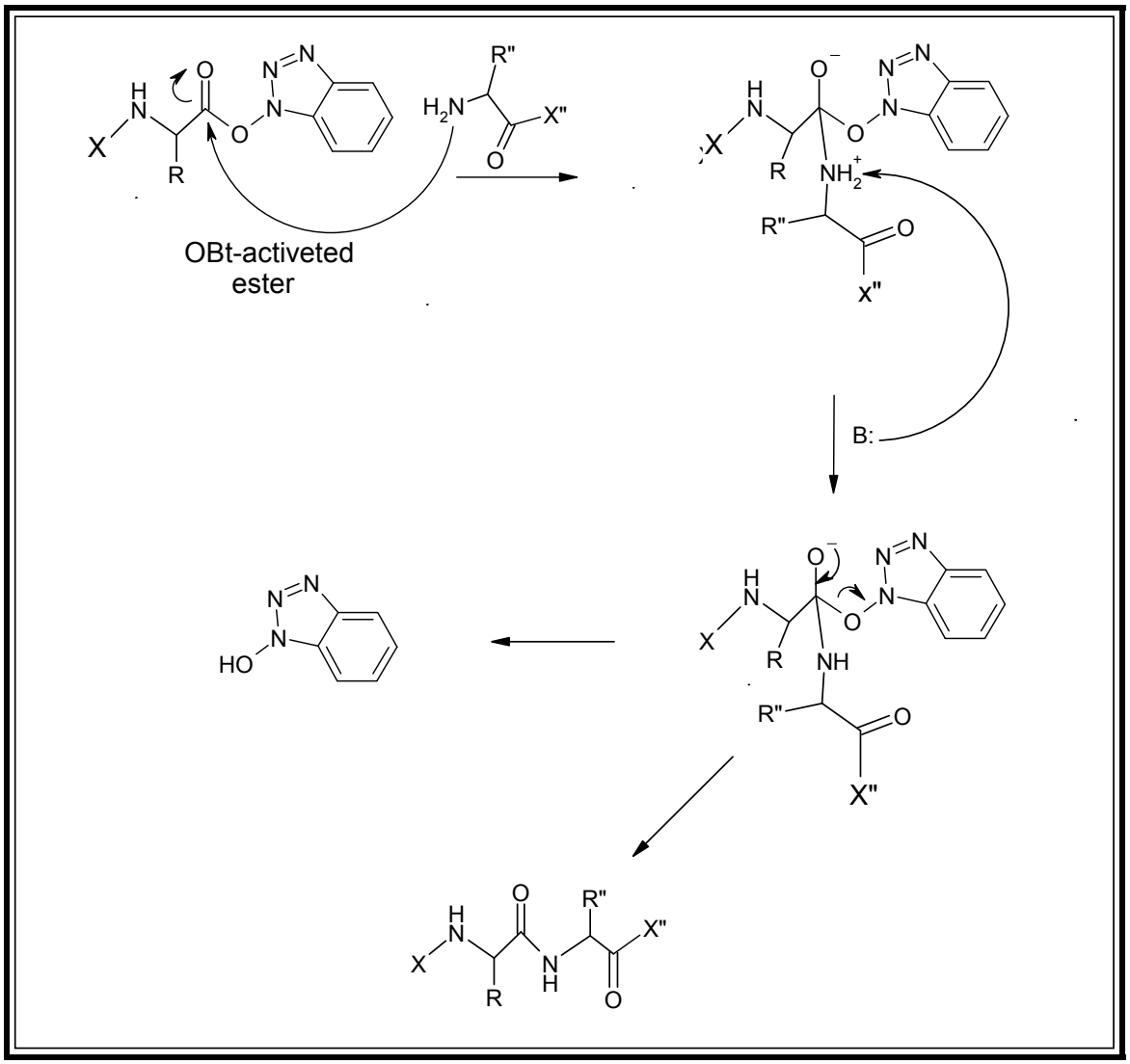

Figure 20: Reaction mechanism of amide bond formation from active OBt-ester

Samples were prepared in the same way as earlier except that PyBOP (2 equivalents) was used instead of DEPBT. HPLC of PyBOP was done to distinguish it from peptide peaks during later analysis (see appendix Figure A-11)

After 8 hours MS showed signs of both linear and cyclic peptide (see Table 27-28 and appendix Figure A-12).

Table 27: M/Z for linear peptide **9**

M/Z for linear peptide	
$[M+H]^+$	1308,7 Da
$[M+Na]^+$	1330,7 Da
$[M+H,-Pbf]^+$	1056,7 Da

Table 28: M/Z for cyclic peptide

M/Z for cyclic peptide	
[M+H]+	1290,7 Da
[M+Na]+	1312,7 Da
[M+H,-Pbf]+	-

After 32 hours HPLC showed that the reaction was complete. HPLC shows no peak at R_t 28,22 minutes; as for the linear peptide. It showes a peak with R_t 28.7 minutes. This is believed to be the peak for the cyclic peptide. (See appendix Figure A-13).

MS at 32 hours showed only cyclic peptides in the mixture (see Table 29 and appendix Figure A-14).

Table 29: M/Z for cyclic peptide **9**

M/Z for cyclic peptide	
$[M+H]^+$	1290,5 Da
$[M+Na]^+$	1312,5 Da
$[M+K]^+$	1328,5 Da
$[M+H,-Pbf]^+$	1038,5 Da

Reflections on experiment with coupling reagents

PyBOP was much faster than DEPBT. DEPBT did not run the reaction to completion in a reasonable time. In this thesis racemization was not a problem and DEPBT had no other advantages over PyBOP and was therefore abandoned from further research.

Removal of DMF

DMF was removed with extraction with DCM/H_2O. Peptides will remain in the organic phase wile DMF will distribute in both phases. Repeating the process will gradually deplete DMF from the mixture. DCM was removed in vacuo.

2.3.2.3 Cleavage of protecting groups

Protecting groups were cleaved using 95% TFA for 2 hours.[43] Cleaved protecting groups are highly reactive cations which can react with the unprotected peptide at the side chains, N- and C- terminals and even the peptide backbone. It is essential that these cations are scavenged.[32]

Water is used as a scavenger to remove tBu-cations, but it is only moderately effective and shows protection to a less degree from the Pbf- and Boc-cations.[43] Triisopropylsilan (TIS) is effective in scavenging all of these cations.[43, 44] Cleavage cocktail utilized in pilot experiment was TFA/TIS/H_2O (95:2,5:2,5).[43] The batch from experiments on PyBOP was cleaved with this cocktail. This showed to be effective and with no remnants of linear peptide according to MS. MS showed unprotected cyclic peptide to be the major component with [M^+] 730,4 Da. (See appendix Figure A-15)

2.3.2.4 Purification

The cyclic deprotected peptide was purified and lyophilized. Purification was done by HPLC with UV-detection at 214 nm. Detailed procedure is given in the Experimental section. HPLC showed the obtained product was 100% pure. (See appendix Figure A-16). MS showed that the product yielded had the right molecular weight. (See appendix Figure A-17).

Table 30: M/Z for cyclic peptide **9**

M/Z for cyclic peptide	
$[M+H]^+$	730,4 Da
$[M+Na]^+$	752,4 Da

Table 31: Yields

Yield	
Mass	6,8 mg
Mol	7,10·10-6
Percent	26,4 %

2.3.2.5 Conclusion from Pilot Experiment

PyBOP and DMF was the best choice for the work in this thesis.

After experiencing that it was very time consuming to remove DMF with extraction the procedure was revised. Cyclizations were to be performed in DCM/DMF (1:1), and solvents removed by evaporation in vacuo. Reducing the amount DMF with 50% would make it surmountable to remove DMF by evaporation. Removing DMF either with extraction or evaporation is a time consuming process, however evaporation has the advantage of very little manual effort.

Cyclizations of peptides have to be done in dilute solution to avoid dimerization. Pilot experiment were performed in 4 ml DMF per mg crude peptide. This would give a concentration of $1.9 \cdot 10^{-4}$ M – $3{,}1 \cdot 10^{-4}$ M for peptides to be cyclized. MS showed little dimerization, but to minimize dimerization 5 ml DMF pr mg crude peptide was used in the general procedure, giving a concentration of $1.5 \cdot 10^{-4}$ M – $2{,}5 \cdot 10^{-4}$ M.

During cyclization samples were not taken after 24 hours, but 24 hours reaction time was nevertheless used in the general procedure, with option to prolong reaction time if linear peptides remained.

A byproduct was identified during cyclizations. This compound has a molecular mass 45 Da bigger than cyclized peptides, or 27 Da bigger than the linear peptides. This byproduct was identified with the use of both DEPBT and PyBOP. However it was more pronounced with the use of DEPBT. Furthermore this compound was still seen after deprotection and could be isolated by preparative HPLC. This means that the byproduct is not caused by reactions on protective groups and is not some sort of adduct of desired peptides. Interestingly this byproduct was not seen when trying to cyclize in DCM. This led us to belive that the byproduct could be caused by a reaction with DMF, or rather hydrolyzed DMF. Hydrolyzed DMF forms formic acid and dimethylamine. Reaction of peptides with formic acid could result in N-formylation of the peptide corresponding to a mass increase of 27 Da. It was therefore decided to use synthetic grade DMF in the cyclizations. (JT Baker. Baker analyzed DMF)

Removal of acetic counter ion was successful, tough with low yields. This meant that one had to find another method for cleavage of the resin for synthesis of linear precursors **11-16**.

A protocol for synthesis of cyclic peptides from linear precursors was made, and is given in table 32.

Table 32: Protocol for synthesis of cyclic peptides from linear precursors.

1	Dissolve peptides in 5 ml DMF/DCM (1:1) per mg crude peptide. Analyze by MS and HPLC
2	Add 2 mol equivalents of PyBOP and DIPEA.
3	Analyze solution by MS and HPLC after 24 hours. If cyclization is complete proceed with next step.
4	Remove DMF/DCM in vacuo
5	Add 30 ml TFA/H_2O/TIS (95:2,5:2,5) and let stir for two hours.
6	Remove reaction mixture in vacuo.
7	Precipitate cyclic peptides in 100ml cold ether for 2 hours. Decant ether and repeat step. Analyze by MS and HPLC.
8	Purify peptides with preparative HPLC
9	Lyophilize peptides
10	Analyze peptides with MS, HPLC and NMR

2.3.3 Preparation of linear precursors

Compounds **9** and **10** were obtained as Na-salts by extraction of the corresponding acetate salts as described in Pilot Experiment while compounds **11**-**16** were prepared by SPPS.

2.3.3.1 Extraction of H_2N-D-Tyr^1(tBu)-Arg^2(Pbf)-Arg^3(Pbf)-Nal^4-Gly^5-OH (9)

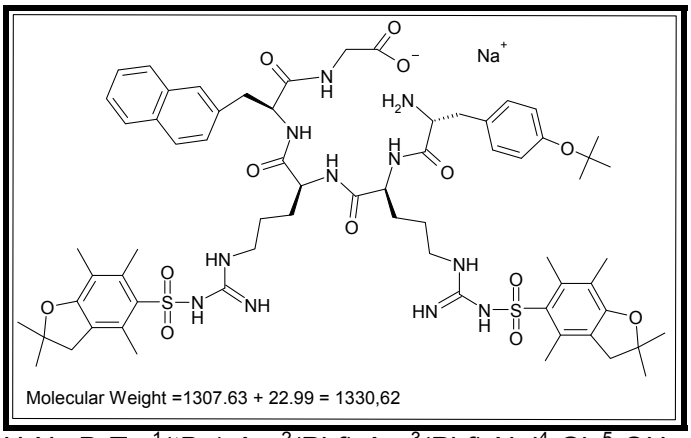

Figure 22: H_2N - D-Tyr^1(tBu)-Arg^2(Pbf)-Arg^3(Pbf)-Nal^4-Gly^5-OH

Same extraction as in Pilot Experiment: 199 mg of crude linear peptide yielded 117,3 mg, 58,9%, of the linear sodium salt.

HPLC showed that it had a purity of 97,45% with R_t 28,2 minutes. (See appendix Figure A-18). The HPLC trace was almost identical to HPLC done of compound **9** in pilot experiment. It was therefore postulated that the product had to be the same, and MS for identification was not done.

2.3.3.2 Extraction of H_2N-D-Tyr[1](tBu)-Ala[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH (10)

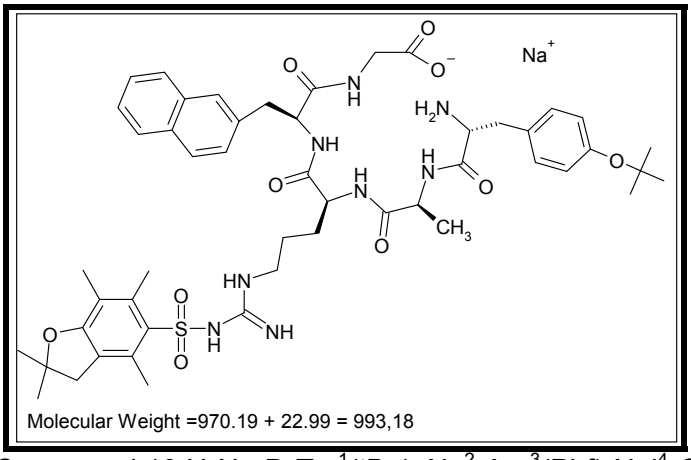

Figure 23: Compound 10 H_2N - D-Tyr[1](tBu)-Ala[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH

The crude linear precursors did not dissolve in neither DCM or acetyl acetate. It did dissolve in a mixture of DCM/DMF (7:1) A mixture of DCM/DMF 1:1 was used as the organic phase in extraction. DMF is miscible with both water and DCM. The concentration of DMF in the organic phase will therefore drop drastically for each extraction. An initial high concentration of DMF is therefore necessary to avoid precipitation during extraction.

148 mg of crude linear peptide yielded 103,3 mg, 69,8%, of the linear sodium salt.

HPLC showed two peaks. One bigger with a R_t 24,3 minutes, (86,93%), and a smaller with R_t 24,5 minutes (13,07%). (See appendix Figure A-19). MS showed that the correct MW = 971 was obtained. (See Table 33 and appendix Figure A-20)

The biggest peak of the HPLC chromatogram was assumed to be the linear peptide as no other major product could be seen in the mass spectrum.

Table 33: M/Z for linear peptide **10**

M/Z for linear peptide	
$[M+Na]^+$	993,5 Da
$[M+K]^+$	1009,5 Da
$[M+H,-Pbf]^+$	719,5 Da

2.3.3.3 Solid Phase Peptide Syntesis of 11-16

Synthesis of linear precursors **11-16** was performed by solid phase peptide synthesis (SPPS). SPPS refers to that peptides are bound to a solid phase, a resin that protects one terminal if the peptide, usually the C-terminus, of the peptide. This leaves only the N-terminal available for interaction. Amino acids used are protected on the N-terminal by a protecting group. Reactive side chains are also protected via protecting groups. This leaves only the C-terminal of amino acids and the N-terminal of the growing peptides available for interaction. After peptide bond formation the N-terminal protecting group is cleaved off leaving the peptide ready for coupling with the next amino acid.

By using excess of the reagents followed by filtering and washing after each coupling, it is possible to optimize the coupling of every amino acid in the sequence. See Figure 24 for an overview of SPPS in this thesis

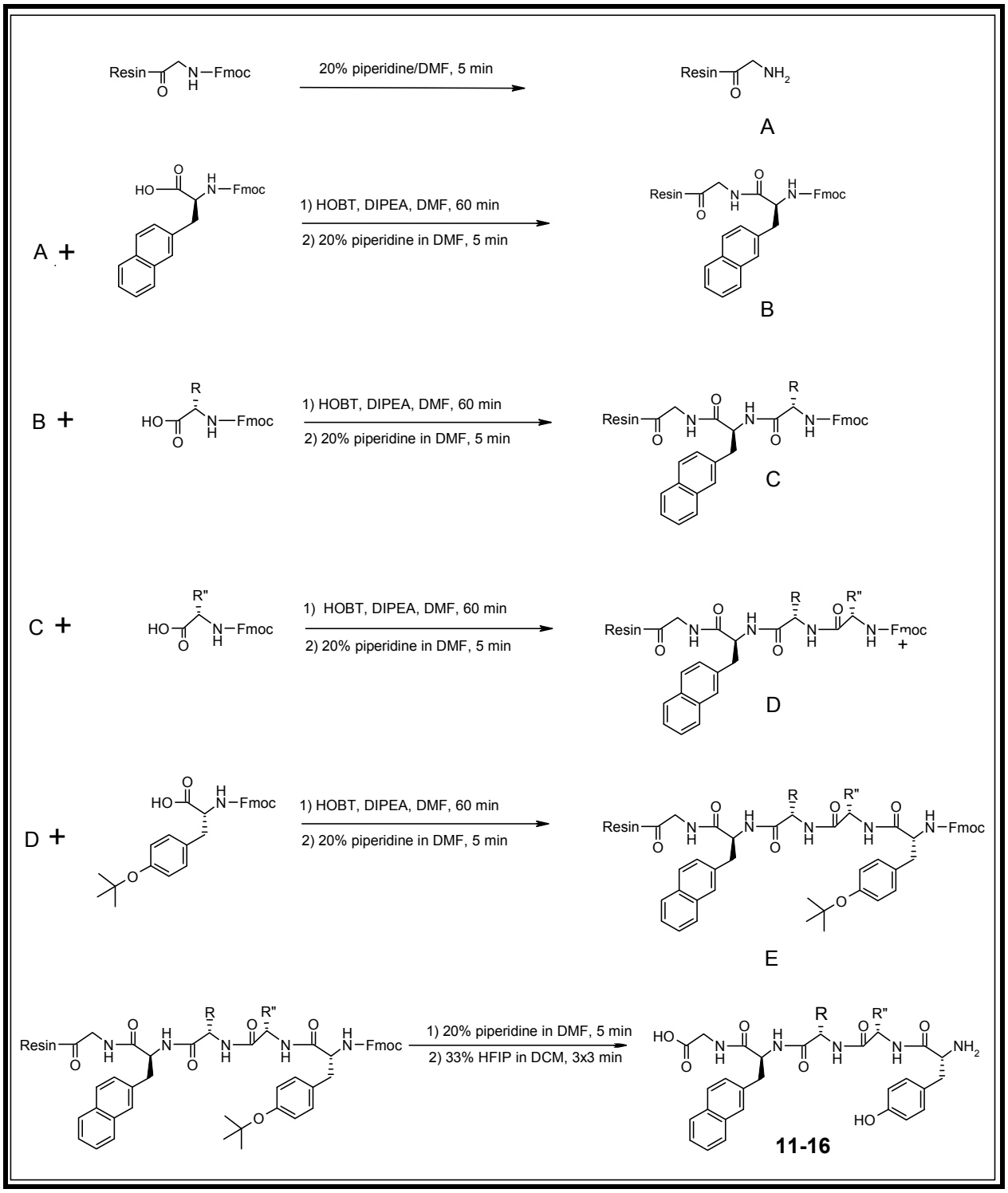

Figure 24: Reaction scheme of peptide synthesis.

Fmoc-chemistry: Fmoc (9*H*-fluoren-9-ylmethoxycarbonyl) chemistry is most commonly used in SPPS and was chosen in this thesis. Fmoc is an N-terminal protecting group that can be cleaved of with a base and leaves acid labile protecting groups of the peptide intact. Cleavage of Fmoc was done with 20% piperidine. See Figure 25

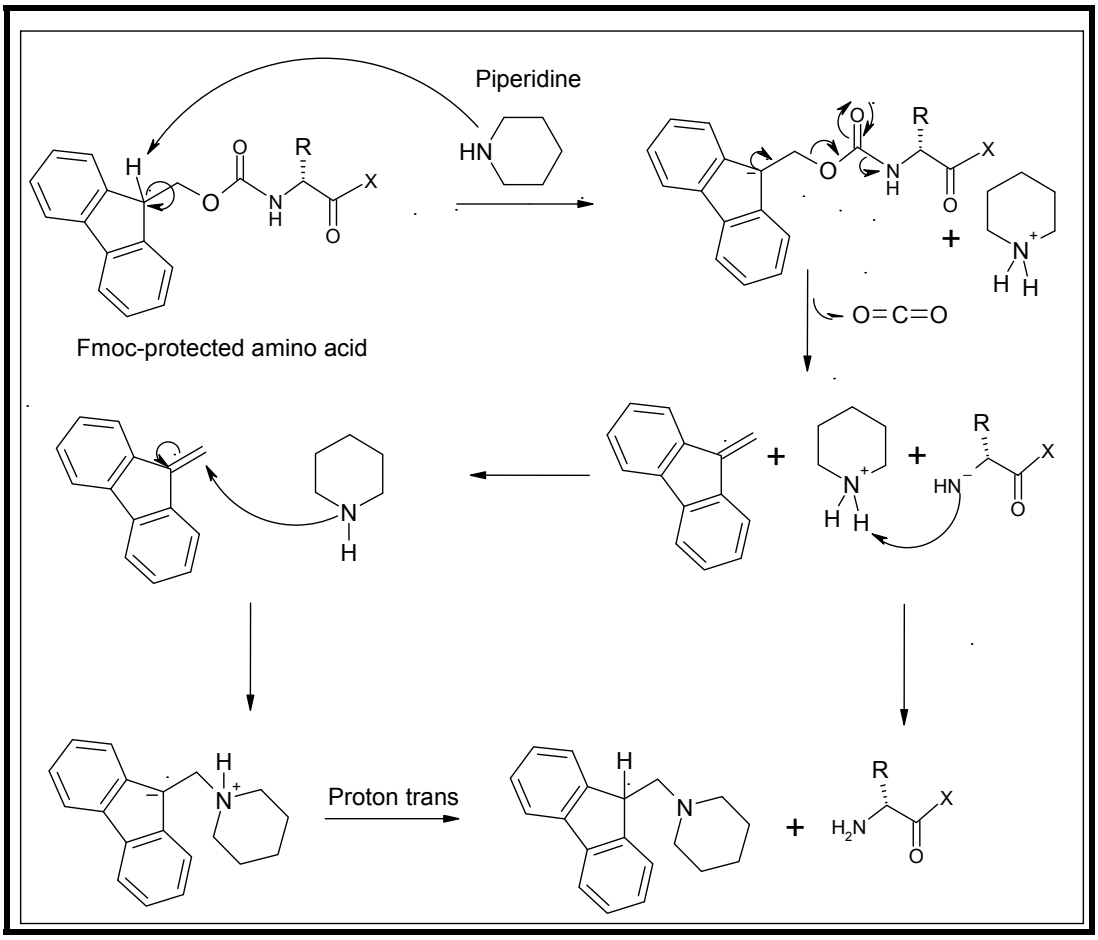

Figure 25: Cleavage of Fmoc from amino acids

Coupling reagent: HBTU (O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate) is a guanidium based coupling reagent that was used for all linear peptides. HBTU, as PyBOP mediates coupling via formation of HOBt, giving the activated OBt-ester. See Figure 26.

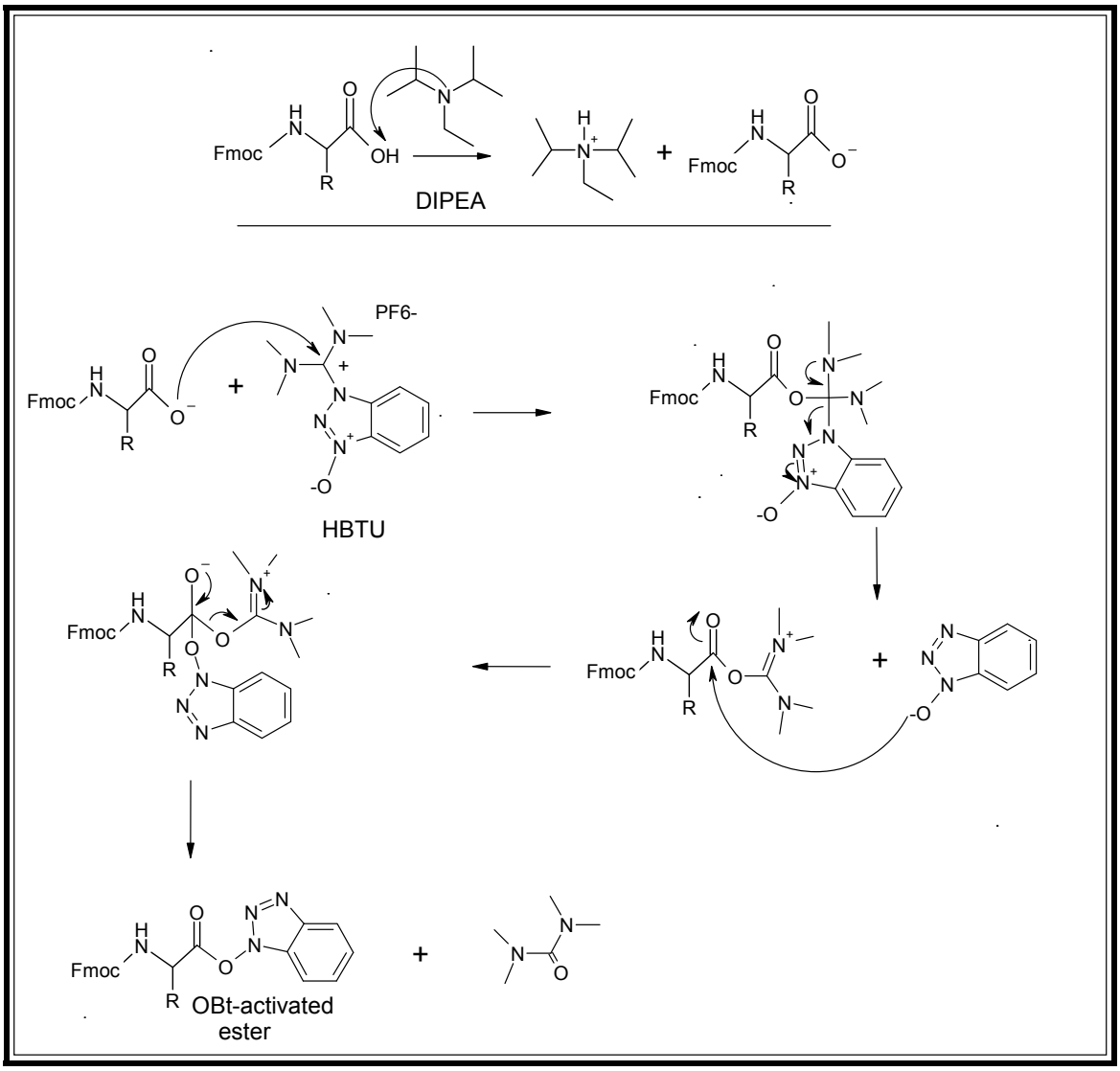

Figure 26: Reaction mechanism of HBTU resulting in the active OBt-ester.

Base is needed in the coupling to activate carboxyl groups of Fmoc protected amino acids for reaction. To avoid cleavage of Fmoc the base has to be sterically hindered. Sterically hindered bases also protects against epimerization due to abstraction of the α-carbon hydrogen. DIPEA is a sterically hindered base that was used for all couplings in this theses. The activated ester is analogous to the ester formation in the DEPBT mechanism.

Resin: The Fmoc-Gly-Novasyn TGT resin, which is a Fmoc protected Glycine preloaded to a Novasyn TG resin via a 4-carboxytrityl linker was used for all peptides. 4-carboxytrityl cleaves under weakly acidic conditions leaving the protecting groups intact

Cleavage from resin: Cleavage with AcOH:TFE:DCM yields peptides not readily suitable for cyclization. (See Pilot Experiment). Cleavage in this work was done with 33 % HFIP (1,1,1,3,3,3-Hexafluor-propanol),[45] which is an acidic alcohol, for 3 minutes followed by filtration. The process was repeated two times. Cleavage from the resin results in cations that could react with peptides. This cation is resin bound and filtering thereby reduces the possibility of such side reactions.

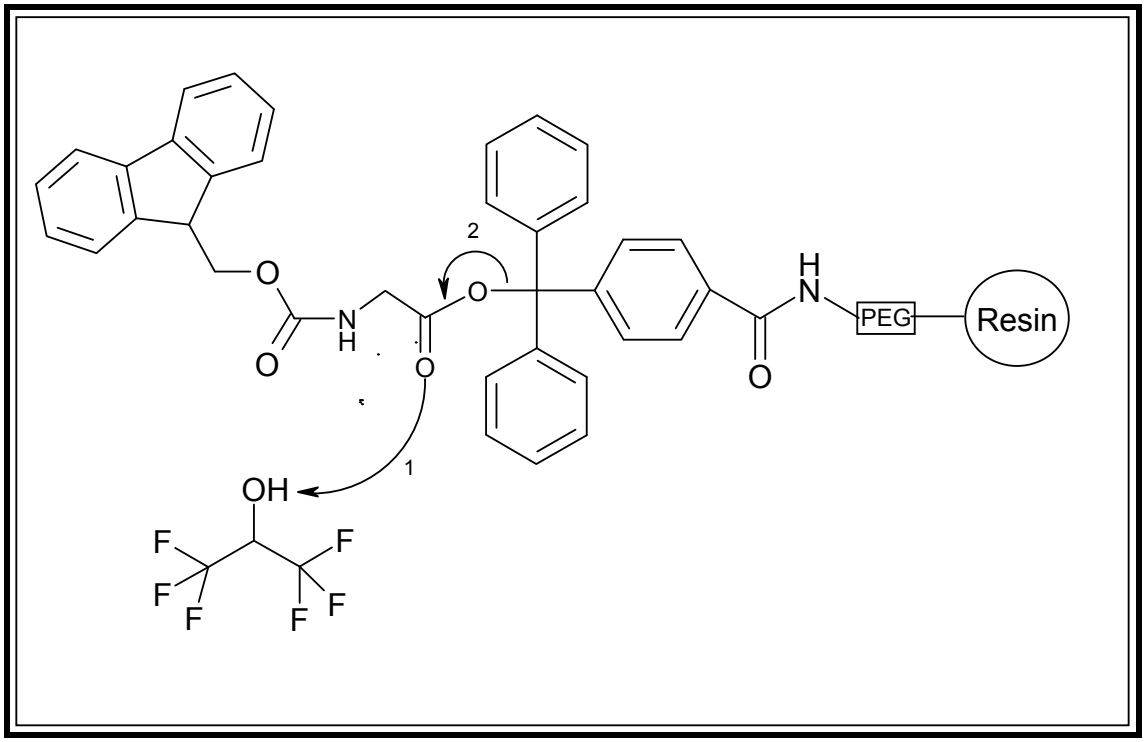

Figure 27: Fmoc-protected glycine bound to a 4-carboxytrityl linker. Also shown is the basic reaction mechanism for cleavage from the resin.

Synthesis of H_2N_2- D-Tyr[1](tBu)-Aib[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH (11)

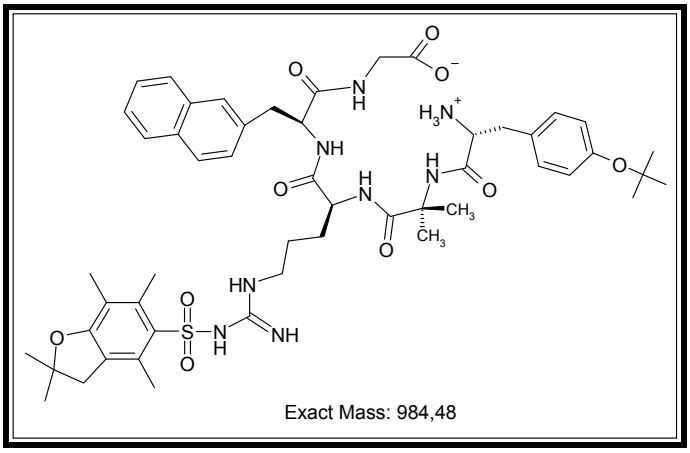

Figure 28: H_2N - D-Tyr[1](tBu)-Aib[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH (**11**)

HPLC showed that it had a purity of 100% with R_t 24,8 minutes. (See appendix Figure A-21). MS showed that the right product was obtained. (see Table 32 and appendix Figure A-22)

Table 34: M/Z for linear peptide **11**

M/Z for linear peptid	
$[M+Na]^+$	1007.5 Da
$[M+H,-Pbf]^+$	733,4 Da

Table 35: Yields:

Yields:	
Mass:	115 mg
Mol:	1,16·10-4
Percent:	77,9 %

Synthesis of H_2N - D-Tyr[1](tBu)-Chx[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH (12)

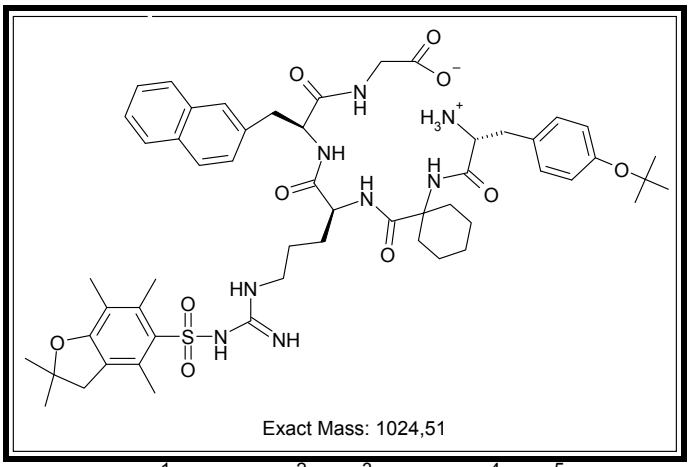

Figure 29: H_2N - D-Tyr[1](tBu)-Chx[2]-Arg[3](Pbf)-Nal[4]-Gly[5]-OH (**12**)

HPLC analysis revealed that the synthesis was not fully completed. It showed two peaks of similar intensity, R_t 21,9 minutes (43,43) % and R_t 26,3 minutes (56,57) %. (See appendix Figure A-23)

MS analysis revealed that the last amino acid did not couple successfully. It showed masses consistent with the loss of tyrosine, giving a tetrapeptide with MW=805. Nevertheless MS also showed that the desired peptide had been obtained; with greater peak intensity than the byproduct (see appendix Figure A-24). This could indicate that the last peak of the HPLC is the desired peptide.

Table 36: M/Z for linear **12**

M/Z for linear peptide	
[M+H]+	1025,7 Da
[M+Na]+	1047.7 Da
[M+K]+	1063,6 Da
[M+H,-Pbf]+	773,5 Da

Table 37: Yields

Yields:	
Mass:	63,4 mg
Mol:	6,19·10-5
Percent:	41,2 %

Yields are calculated from 56,57% of crude yield which was 112 mg.

M/Z for byproduct: $[M+H]^+$ 806,4 Da, $[M+Na]^+$ 828,4 Da, $[M+K]^+$ 844,4 Da.

Synthesis of H_2N -D-Tyr^1(tBu)-Pip^2(Boc)-Arg^3(Pbf)-Nal^4-Gly^5-OH (13)

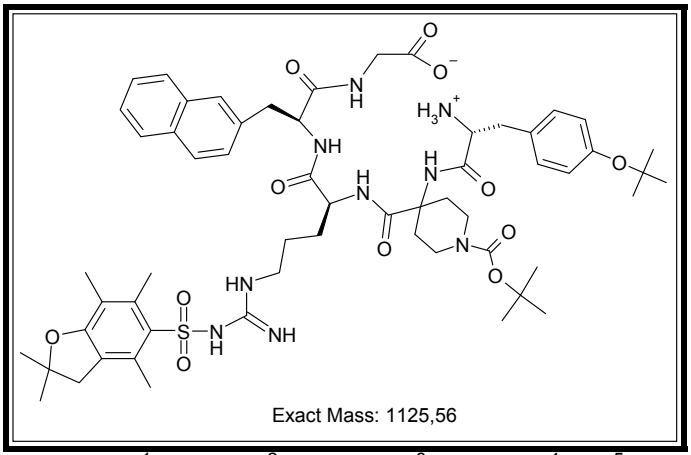

Figure 30: H_2N -D-Tyr^1(tBu)-Pip^2(BOC)-Arg^3(Pbf) -Nal^4-Gly^5-OH (**13**)

HPLC analysis revealed that the synthesis was not fully completed. It showed two peaks of different intensity, R_t 23,3 minutes (79,97 %) and R_t 26,9 minutes (20,09 %). (See appendix Figure A-25) As for **12** MS analysis revealed that the last amino acid did not couple successfully. It showed masses consistent with the loss of tyrosine, giving a tetrapeptide with MW=906. MS also showed that the desired peptide had been obtained, but with lower intensity in peaks than the byproduct (see appendix Figure A-26). This indicates that the last peak of the HPLC is the desired peptide.

Table 38: M/Z for cyclic peptide **13**

M/Z for linear peptid	
[M+H]+	1126,7 Da
[M+Na]+	1148.7 Da
[M+K]+	1164,5 Da

Table 39: Yields

Yields:	
Mass:	24,7 mg
Mol:	$2{,}19 \cdot 10^{-5}$
Percent:	14,6 %

Yields are calculated from 20,09% of crude yield which was 123 mg.

M/Z for byproduct: [M^+] 907,5 Da, [M+Na] 929,4 Da, [M+K] 945,4 Da.

Synthesis of H_2N - D-Tyr^1(tBu)-Cit^2-Arg^3(Pbf)-Nal^4-Gly^5-OH (14)

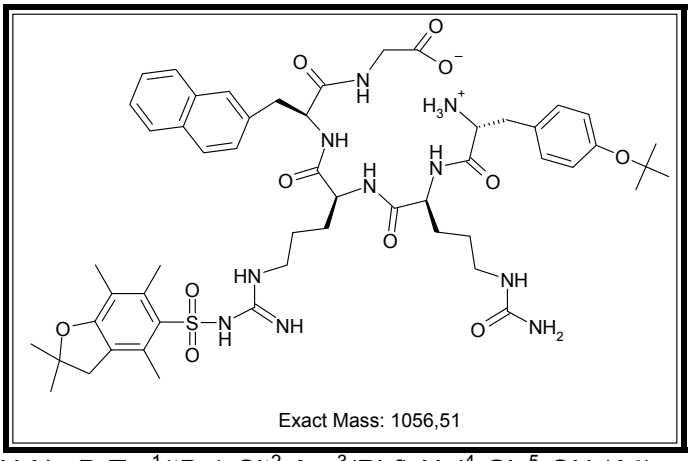

Figure 31: H_2N - D-Tyr^1(tBu)-Cit^2-Arg^3(Pbf)-Nal^4-Gly^5-OH (**14**)

HPLC showed that it had a purity of 100% with R_t 23,7 minutes. However, visually one could se peaks not recognized by the software. (see appendix Figure A-27 MS showed that the correct product was obtained. (see appendix Figure A-28

Table 40: M/Z for linear peptide **14**

M/Z for linear peptide	
[M+H]+	1057.5 Da
[M+Na]+	1079.5 Da
[M+K]+	1095.5 Da
[M+H,-Pbf]+	805,4 Da

Table 41: Yields

Yields:	
Mass:	123 mg
Mol:	$1{,}16 \cdot 10^{-4}$
Percent:	77,6 %

Synthesis of H_2N - D-Tyr^1(tBu)-Arg^2(Pbf)-Cit^3-Nal^4-Gly^5-OH (15)

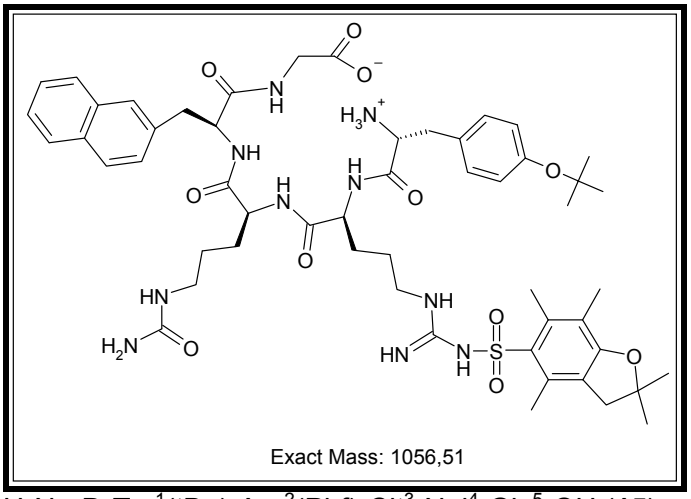

Figure 32: H_2N - D-Tyr^1(tBu)-Arg^2(Pbf)-Cit^3-Nal^4-Gly^5-OH (**15**)

HPLC showed that it had a purity of 96,2% with R_t 22,8 minutes. (See appendix Figure A-29). MS showed that the right product was obtained. (see appendix Figure A-30)

Table 42: M/Z for linear peptide **15**

M/Z for linear peptide	
[M+H]+	1057.6 Da
[M+Na]+	1079.6 Da
[M+K]+	1095.6 Da
[M+H,-Pbf]+	805,5 Da

Table 43: Yields

Yields:	
Mass:	122 mg
Mol:	$1{,}15 \cdot 10^{-4}$
Percent:	77,0 %

Synthesis of H_2N -D-Tyr^1(tBu)-Cit^2-Cit^3-Nal^4-Gly^5-OH (16)

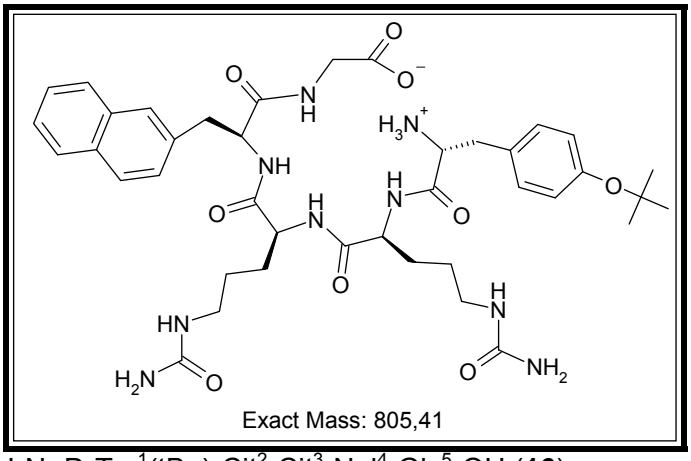

Figure 33: H_2N -D-Tyr^1(tBu)-Cit^2-Cit^3-Nal^4-Gly^5-OH (**16**)

HPLC showed that it had a purity of 94,2% with R_t 17,7 minutes. (See appendix Figure A-31). MS showed that the right product was obtained. (see appendix Figure A-32)

Table 44: M/Z for linerar peptide **16**

M/Z for linear peptide	
[M+H]+	806,5Da
[M+Na]+	828,5 Da
[M+K]+	844,4 Da

Table 45: Yields

Yields:	
Mass:	112 mg
Mol:	$1{,}39 \cdot 10^{-4}$
Percent:	94,5 %

2.3.4 Synthesis of 1-8 from 9-16

Cyclizations of all peptides were done after the protocol developed in Pilot Experiment unless otherwise stated.

2.3.4.1 Synthesis of FC131 (1)

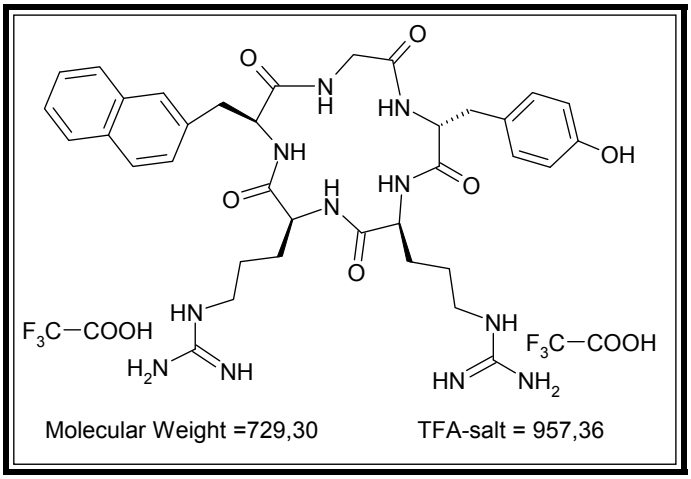

Figure 34: The figure shows cyclic unprotected **1.** Protected **1** has MW=1289

HPLC after 24 hours revealed that there were some of the starting peptide was left (which has a R_t of 28,2 minutes). HPLC showed two major products in the mixture and some other impurities. The two major products had retention times of 24,1 minutes and 28,6 minutes. (See appendix Figure A-33)

MS after 24 hours showed the cyclic peptide and the same types of impurities seen in the pilot experiment. (See appendix Figure A-34)

Table 46: M/Z for cyclic proteceted **1**

M/Z for cyclic protected peptide	
$[M+H]^+$	1290,8 Da
$[M+Na]^+$	1312,8 Da
$[M+H,-Pbf]^+$	1164,5 Da

The final product was a white hydroscopic powder. Final HPLC showed that the product was 98,9% pure. (See appendix Figure A-35). MS showed that the right product was

obtained; [M+H] 730,2. (See appendix Figure A-36) The ^{1}H-NMR spectrum was fully consistent with the desired target structure **1**. (see appendix Figure A-37)

Table 47: Yields of **1**

Yields:	
Mass:	16,5 mg
Mol:	$1{,}72 \cdot 10^{-5}$
Percent in cyclization	19,5%

Combined with the 6,8 mg produced under Pilot experiment a total of 23.3 mg ($2{,}43 \cdot 10^{-5}$ mol) were obtained.

2.3.4.2 Synthesis of Ala^{2}FC131 (2)

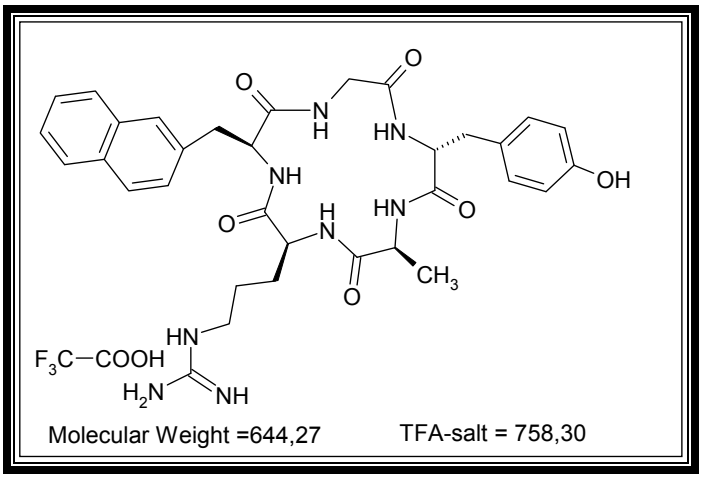

Figure 35: The figure shows cyclic unprotected **2.** Protected **2** has MW=952

HPLC after 24 hours indicated that there were nothing of the starting peptide left (wich would have a R_t of 24,3 minutes). HPLC showed four major products in the mixture and some other impurities. The four major products had retention times of 16,2 minutes, 24,1 minutes, 24,8 minutes (smallest) and 26,35 minutes (largest). It is possible that there had been a shift in retention times dough. PyBOP that usually elutes at 8,7 minutes eluted here at 9,2 minutes. The smallest peak in the chromatogram may therefore be the linear starting peptide, but this was not seen in MS analysis. (See appendix Figure A-38)

MS after 24 hours showed that the desired cyclic peptide was obtained with [M+Na] 975,5. Other major M/Z was 1020,5, 1047,5 and 1074,5. (See appendix Figure A-39) These share the same mass differences as impurities seen in the pilot experiment and in cyclization of **1**.

1020,5 Da is 27 Da more than the sodium adduct of the linear peptide. 1047,5 is again 27 Da more than 1020,5 and finaly 1074,5 is 27 Da more than 1047,5 Da. These numbers makes it a possibility that there is a repetitive reaction that is causing the impurities.

Rmoving of protective groups, purification and lyophilization gave a white hydroscopic powder. Final HPLC showed that the product was 96,5% pure. (See appendix Figure A-40). MS showed that the right product was gained; MW 644,27. (See appendix Figure A-41). NMR spectrum are given in appendix (Figure A-42)

Table 48: Yields for **2**

Yields:	
Mass:	16,2 mg
Mol:	$2{,}1 \cdot 10^{-5}$
Percent in cyclization	20,5%

2.3.4.3 Synthesis of Aib²FC131 (3)

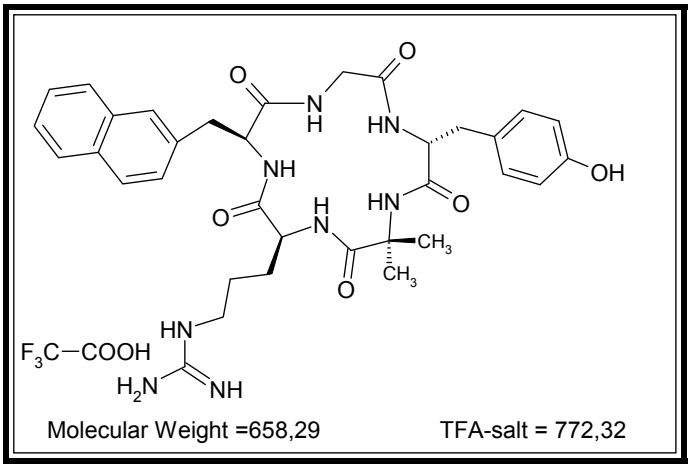

Figure 36: The figure shows cyclic unprotected **3.** Protected **3** has MW=967

HPLC after 24 hours showed only one peak other than that of PyBOP. It had a retention time of 26,1 minutes. As expected the cyclic compound eluted later than the linear peptide. (See appendix Figure A-43)

Mass spectroscopy confirmed that the reaction had resulted in cyclic peptide. See appendix Figure A-44)

Table 49: M/Z for cyclic protected **3**

M/Z for cyclic peptides	
$[M+H]^+$	967,5 Da
$[M+Na]^+$	989,5 Da
$[M+K]^+$	1005,5 Da
$[M+H,-Pbf]^+$	715,5 Da

Peptides were deproteced, purified and lyophilized. The final product was a white powder. MS showed that the product obtained had the desired mass MW 658,2. (See appendix Figure A-45). HPLC showed the peptide to be 100% pure (see appendix Figure A-46). NMR spectrum are given in appendix Figure A-47

Table 50: Yields for **3**

Yields:	
Mass:	41,2mg
Mol:	$5{,}3 \cdot 10^{-5}$
Percent in cyclization:	46,0 %,
Total percent	35,6 %

2.3.4.4 Synthesis of Chx²FC131 (4)

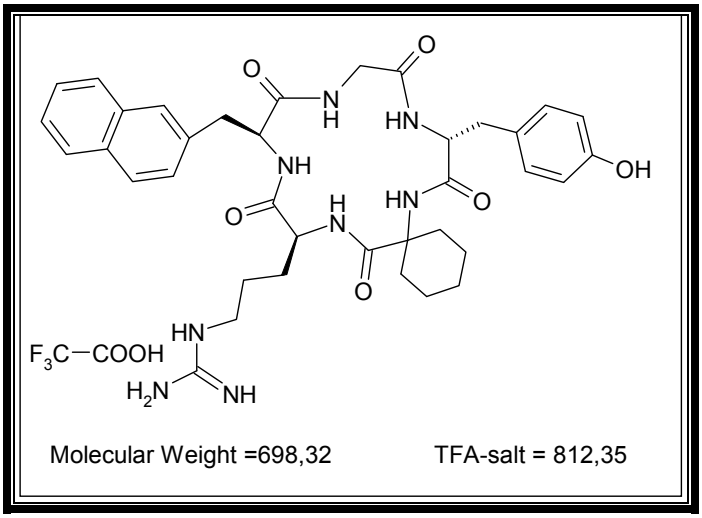

Figure 37: The figure shows cyclic unprotected **4.** Protected **4** has MW=1006

As tyrosine had failed to couple efficiently there were a significant amount of unwanted tetrapeptides in the crude batch of peptides. To avoid the formations of dimers of tetrapeptides and pentapeptides one increased the dilution during cyclization. Instead of 5 ml solvent per milligram, 8 ml solvent per milligram was used. Ideally one could have purified the linear pentapeptides before cyclization to avoid the problem entirely. However, at the time no means of purifications was available.

HPLC after 24 hours showed one major peak and many minor ones. This was expected since tetrapeptides will form dimers rather than cyclize due to restrains in the backbone. Both peaks from HPLC on the crude batch were not to be seen, indicating that reaction was completed. The major peak had a R_t of 27,6 minutes. (See appendix Figure A-48)

Mass spectroscopy confirmed that the reaction had resulted in cyclic peptide. See appendix Figure A-49

Table 51: M/Z for cyclic protected **4**

M/Z for cyclic peptides	
$[M+H]^+$	1007,5 Da
$[M+Na]^+$	1029,5 Da
$[M+K]^+$	1045,6 Da
$[M+H,-Pbf]^+$	755,4Da

Peptides were deproteced, purified and lyophilized. The final product was an off-white powder. MS showed that the product had the correct mass MW 698,3. (See appendix Figure A-50) HPLC showed the peptide to be 98,1 % pure (see appendix Figure A-51)

NMR spectrum are given in appendix Figure A-52

Table 52: Yields for **4**

Yields:	
Mass:	22,8 mg
Mol:	$2{,}8 \cdot 10^{-5}$
Percent in cyclization:	45,3 %
Total percent	18,7 %

2.3.4.5 Synthesis of Pip²FC131 (5)

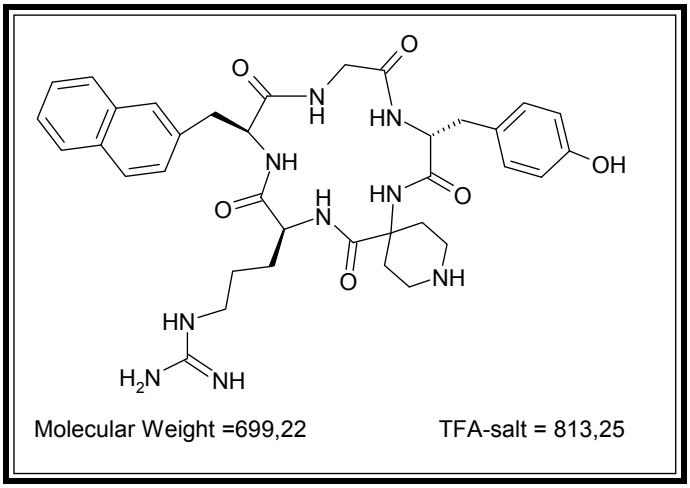

Figure 38: The figure shows cyclic unprotected **5.** Protected has a MW=1107

As in the synthesis of Chx^2FC131 8 ml solvent per milligram peptide was used to avoid dimerization.

HPLC after 24 hours showed one major peak and many minor ones. Both peaks from HPLC on the crude batch were not seen, indicating that the reaction was complete. Two compounds had R_t higher than the linear pentapeptides, as one expects from cyclized peptides. R_t 27,8 and R_t 28,2. (See appendix Figure A-53)

Mass spectroscopy confirmed that the reaction had resulted the desired cyclic peptide, but other M/Z showed greater intensity. (See appendix Figure A-54)

Table 53: M/Z for cyclic protected **5**

M/Z for cyclic peptides	
$[M+Na]^+$	1130,7 Da
$[M+K]^+$	1146,6 Da
$[M+H,\text{-Pbf}]^+$	856,5 Da

Peptides were deproteced, purified and lyophilized. The final product was an off-white powder. MS showed that the product had the correct mass MW 699,2. (See appendix Figure A-55), but HPLC showed the peptides only be 78,97 % pure (see appendix Figure A-56). This was much lower than the threshold of 95% purity for further investigation of peptides, and this compound will unfortunately not be tested for biological activity at this stage. The mass yield was 2,3 mg

2.3.4.6 Synthesis of Cit²FC131 (6)

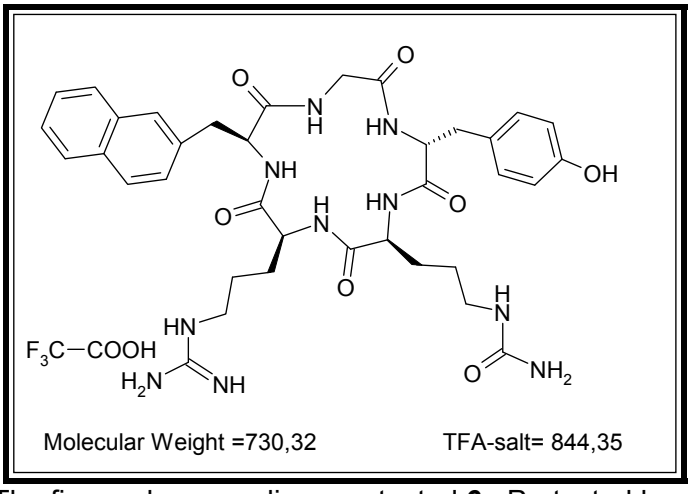

Figure 39: The figure shows cyclic unprotected **6.** Protected has a MW=1038

After 24 hours HPLC showed no remnants of the original peak from the linear peptide remained. The chromatogram showed that some byproducts had been generated. One compound had much grater intensity than other peaks and was assumed to be the cyclized peptide It had a retention time of 24,8 minutes. (See appendix Figure A-57)

Mass spectroscopy confirmed that the reaction had resulted in cyclic peptide. See appendix Figure A-58)

Table 54: M/Z for cyclic protected **6**

M/Z for cyclic peptides	
$[M+H]^+$	1039,6 Da
$[M+Na]^+$	1061,6 Da
$[M+K]^+$	1077,5 Da
$[M+H,-Pbf]^+$	787,5 Da

Peptides were deproteced, purified and lyophilized. The final product was a white powder sensitive to static electricity. HPLC showed the peptide to be 100% pure (see appendix Figure A-59) MS showed that the product obtained had the desired mass MW 730,3. (See appendix Figure A-60).

NMR spectrum are given in appendix Figure A-61

Table 55: Yields for **6**

Yields:	
Mass:	40,3 mg
Mol:	$4{,}7 \cdot 10^{-5}$
Percent in cyclization:	41,1 %,
Total percent	31,8 %

2.3.4.7 Synthesis of Cit[3]FC131 (7)

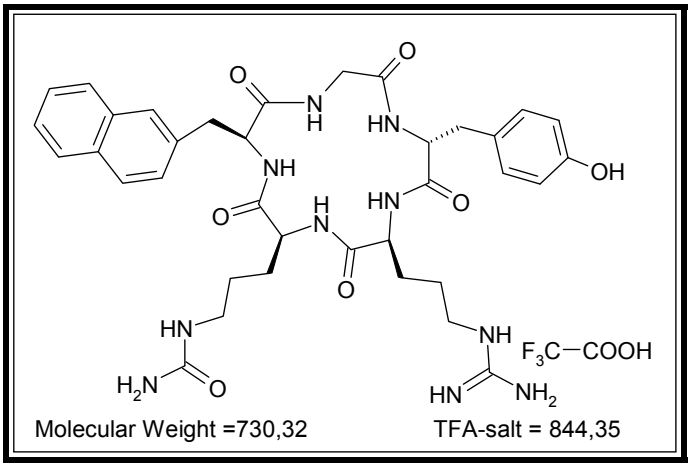

Figure 40: The figure shows cyclic unprotected **7.** Protected has a MW=1038

After 24 hours HPLC showed no remnants of the original peak from the linear peptide remained. The chromatogram showed that little byproducts. There where thre mayor peaks in the chromatogram and two of them being remnants of PyBOP. The compound assumed to be the cyclized peptide had a retention time of 24,7 minutes. (See appendix Figure A-62). Mass spectroscopy shoed masses coherent with cyclic peptides. See appendix Figure A-63)

Table 56: M/Z for cyclic protected **7**

M/Z for linear peptides	
$[M+H]^+$	1039,6 Da
$[M+Na]^+$	1061,6 Da
$[M+K]^+$	1077,5 Da
$[M+H,\text{-Pbf}]^+$	787,5 Da

Peptides were deproteced, purified and lyophilized. The final product was a white powder sensitive to static electricity. HPLC showed the peptide to be 96,6% pure (see appendix Figure A-64) MS showed that the product obtained had the correct mass MW 730,2. (See appendix Figure A-65). NMR spectrum are given in appendix Figure A-66

Table 57: Yields for **7**

Yields:	
Mass:	28,3mg
Mol:	$3{,}4 \cdot 10^{-5}$
Percent in cyclization:	29,1 %
Total percent	22 ,3%

2.3.4.8 Synthesis of Cit^{2,3}FC131 (8)

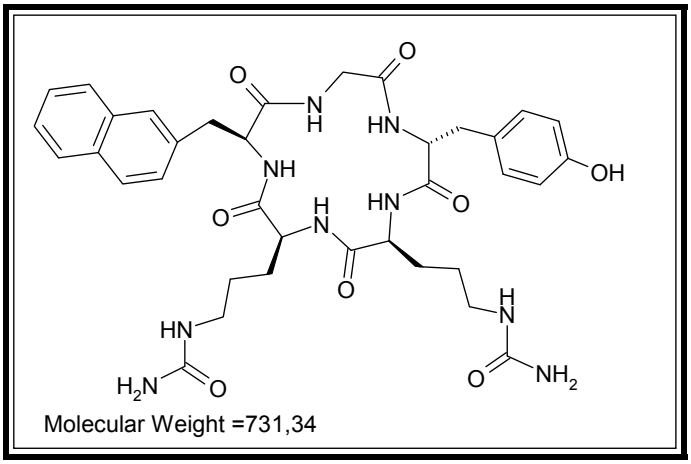

Figure 41: The figure shows cyclic unprotected **8.** Protected has a MW=787

HPLC after 24 hours showed that no remnants of the linear peptide remained. The chromatogram showed little byproducts. There where three mayor peaks in the chromatogram and two of them being remnants of PyBOP. The compound assumed to be the cyclic peptide had a retention time of 19,0 minutes. (See appendix Figure A-67)

Mass spectroscopy showed masses coherent with cyclic peptides. See appendix Figure A-68)

Table 58: M/Z for cyclic protected **8**

M/Z for cyclic peptides	
$[M+Na]^+$	810,5 Da
$[M+K]^+$	826,4 Da

Peptides were deproteced, purified and lyophilized. The final product was a white powder sensitive to static electricity. HPLC showed the peptide to be 96,6% pure (see appendix

Figure A-69) MS showed that the product obtained had the correct mass MW 731,3. (See appendix Figure A-70). NMR-spectrum are given in appendix figure A-71.

Table 59: Yields for **8**

Yields:	
Mass:	22,6 mg
Mol:	$3{,}6 \cdot 10^{-5}$
Percent in cyclization:	26,2 %,
Total percent	24,5 %

3 Conclusions

It has been determined that the recommended settings in Phase do not give similar results. The use of ε=4r gives more consistent results, and the results are similar to that of other methods of virtual environment mimicking.

In this thesis one has succeeded in generated 4-point pharmacophores for CCP antagonist for CXCR4. Indications of pseudo-6-membered rings have been identified in the Arg^2 side chain for the potent antagonist **1** and **1b** that fit these pharmacophores. Angles in the peptide backbone that can be stabilized with helix inducing residues have also been identified. New CCP have been designed and synthesized based on these findings.

8 compounds have been synthesized via SPPS. Using HOBT/DIPEA in DMF coupling of tyrosine to sterically hindered amino acids was not complete and caused low yields. This can be caused by many reasons, but further research is called for using other coupling reagents and/or different ways for N-terminal protection. Failure of Fmoc-deprotection is common for bulky amino acids and may be the reason for low yields.

There have been identified byproducts when cyclizing pentapeptides cleaved of resin with acetic acid. Removing the acetate ion diminished the problem with acetylating, but gave other byproducts. HFIP has been used with great success to avoid ions interfering with cyclizations.

A protocol for cyclizing pentapeptides has been developed, and this protocol has been utilized with acceptable yield of cyclic peptides.

Introduction of helix inducers at the second amino acid seem to be beneficial for cyclizations. Peptides with Aib or Chx at this position gave high yields, but this will require more research to be determined.

4 Experimental

4.1 Molecular Modeling Studies

4.1.1 Starting conformations

To generate the two datasets Dataset **B** and Dataset **C** multiple minimization was performed using the program MacroModel[27] implemented in the Maestro[24] software. The OPLS-2005 force field with the TNCG algorithm was chosen with a maximum number of 5000 iterations and a convergence threshold of 0.0010. Further more minimization mode was minimization of conformers with comparison of heavy atoms and distinguishing of mirror image conformers. Energy window for saving structures was set to 41,8 kJ/mol.

Two parallels of this were performed; one used GB/SA water model with constant dielectric electrostatic treatment and a dielectric constant of 1, Dataset **C**. The other was performed in vacuum with distance dependent electrostatic treatment and a dielectric constant of 4($\varepsilon = 4r$), Dataset **B**

Problems relating to the iterations had on a previous occasion been identified. After consulting with the makers of Maestro one have started to alter the *.com file for each project using the multiple minimization. In each *.com file the MINI arg 2 is altered from a value of 1 to a value of 0.

4.1.2 Pharmacophore Generation

Pharmacophore generation were performed using Phase 2.5. Before one could generate any models one had to adjust the features of the conformers reqognised by the program. The positive charge of the guanidino group of the Arg-residue was reqonised by the program to be on just one of the three nitrogen atoms. In reality this is only one of the contributing structures to the resonance hybrid. The positive charge is delocalized by resonance stabilization throughout the whole guanidino group. For computational ease the positive charge was therefore centered on the carbon atom of the guanidino group.

Maximum tree depth was set to 5, initial box size was set to 32 Å and selectivity score turned of. All other adjustments were set to default.

4.2 List of chemicals

Chemical	Abbreviation	%	Supplier
Methanol	Me-OH	99,9	Merck KGaA
Methyl alcohol-d_4			CDN- isotopes Quebec, Canada
Dimethylformamide	DMF	99,8	Labscan Limited
Dimethylformamide	DMF	99,8	Malinchrodt Baker - B.V. Deventer Holland
Diethylether		99,7	Merck KGaA
Isopropanol		99,9	Arcus AS, Oslo Norway
Triflouroacetic acid	TFA	99,0	Sigma-Aldrich CH GmbH Steinheim, Germany
Triisopropylsilane	TIS	99,0	Sigma-Aldrich CH GmbH Steinheim, Germany
N-N-Diisopropylethyl amine	DIPEA	99,5	Sigma-Aldrich
Acetonitrile		99,8	Merck KGaA Darmstadt,Germany
Dichlormethane	DCM	99,8	VWR International AS Fontenary sous Bois, France
Fmoc-2-Nal-OH			Bachem AG Bubendorf, Switzerland
Fmoc-D-Tyr(tBu)-OH			Bachem AG Bubendorf, Switzerland
Fmoc-Arg(Pbf)-OH			Bachem AG Bubendorf ,Switzerland
N_α-Fmoc-L-citrulline		99,7	Sigma-Aldrich CH, Buchs, Switzerland
1-(Fmoc-amino)cyclohexane carboxylic acid		98,0	Sigma-Aldrich CH, Buchs, Switzerland
Fmoc-α-Me-Ala-Oh		97,0	Sigma-Aldrich CH, Buchs Switzerland
N-Boc-4-(Fmoc-amino)piperidine-4-carboxylic acid		97,0	Sigma-Aldrich CH, Buchs Switzerland
3-(Diethoxy-phosphoryloxy-3H-benzo[d][1,2,3]tiazin-4-one	DEBT		Bachem AG Bubendorf, Switzerland
Fmoc-Ala-OH		95,0	Sigma-Aldrich
(Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate	PyBOP	97,0	Sigma-Aldrich CH GmbH Buchs, Switzerland
Fmoc-Gly-Novasyn TGT			Novabiochem Germany
Ethylacetate			Merck KGaA, Darmstadt Germany
Dimethyl – d_6- sulfoxide			ISOTEC inc. Miamisburg OH, USA
1,1,1,3,3,3 – Hexafluor-2-propanol		99,8	Sigma-Aldrich Chemie GmbH Steinheim Germany

All evaporations of solvents were performed in vacuo at 37˚C. All solvents and reagents were analytical reagent grade.

4.3 MS

Mass spectra were recorded on a Waters Maldi micro MS using MassLynx V 4.0 SCN534 software. During purification of peptides mass spectra were recorded on a Micromass Quattro LC using Quattro Ultima MassLynx 3.4 software.

4.4 Analytical HPLC

Analytical RP-HPLC was performed on a Waters 2695 Separations Module with a Sunfire C18, 5µm, 4.6x250mm column using Empower Pro Empower 2 software.All HPLC analyses were performed using 94,9 % HPLC-grade H_2O, 5,0 % acetonitrile and 0,1 % TFA as Solution A. Solution B consisted of 94,9 % acetonitrile, 5,0 % HPLC grade H_2O and 0,1 % TFA. Samples for analytical HPLC were prepared of a sufficient quantity of analyte being added 1 ml of solution A and a sufficient quantity of solution B or for solvation. Samples from cyclization of peptides were prepared from 1,0 ml of the reaction mixture, DCM removed in vacuo and solution A ad 1 ml. During cyclization UV detection at 254 was set as a reference to avoid UV absoption of DMF. All other samples were done at 214 nm.

4.5 Preparative HPLC

All purifications were done by preparative HPLC. Preparative HPLC was performed with a Waters 717 Autosampler, Waters 600E System Controller, Waters 2487 Dual λ Absorbance Detector and a Waters Fraction Collector III using Empower Pro Empower 2 software. All samples were dissolved in 3,0 ml Solvent A unless stated. Injection volumes were 1,0ml and a 20 - 60 gradient of Solution B over 31 minutes were utilized as a standard. Fractions were analyzed by MS and HPLC. Fractions with the correct MW and purity over 95 % were pooled together. Fractions containing the correct MW, but with low purity were pooled, solvents removed in vacuo, and purified again.

4.6 Lyophilization

The final pooled fractions from preparative HPLC were lyophilized to give the final product. The final product was characterized by MS and HPLC.

4.7 NMR

^{1}H-NMR spectra were recorded on a 600 MHz Varian spectrometer and analyzed by VnmrJ. CD_3OD was used as solvent for compounds **1-7**, while DMSO-d_6 was used for **8** due to solubility problems in CD_3OD.

4.8 Pilot experiment

4.8.1 Solvent effect

NaHCO3, 10 g, was dissolved in 100 ml H_2O to give 100 ml 10 % $NaHCO_3$. Crude linear FC131 (30,4 mg) was dissolved in 30 ml DCM. The solution was washed with 30 ml 10 % $NaHCO_3$. This mixture emulsified. After separation (1,5 hours!) the aqueous phase was drained and the organic phase was washed two more times.

The organic phase was dried with $MgSO_4$ for one hour and filtrated. DCM was removed in vacuo. This yielded 15,1 mg ($1{,}13\cdot10^{-5}$ mol), of the sodium salt of the crude linear FC131. This was dissolved in 60,4 ml DCM and added 2 equivalents DEPBT (6,8 mg, $2{,}27\cdot10^{-5}$ mol), 2 equivalents DIPEA (3,4µl, $2{,}27\cdot10^{-5}$ mol). The solution was left for 24 hours at room temperature and analyzed by MS. MS revealed linear FC131 (MW 1308) as the mayor constituent and cyclic FC131 (MW 1290) as a minor one. The solution was added 2 equivalents DIPEA (3,4µl, $2{,}27\cdot10^{-5}$ mol), left for 24 hours and analyzed by MS. MS revealed no change. The solution was added 0,5 ml DIPEA (126 equivalents, $2{,}87\cdot10^{-3}$ mol), and 2 equivalents DEPBT (6,8 mg, $2{,}27\cdot10^{-5}$ mol), left for 24 hours and analyzed by MS. MS showed little change. The peptide solution was split in two batches. Batch 1 was added 3 equivalents DEPBT (5,1 mg, $1{,}70\cdot10^{-5}$ mol), and Batch 2 was added 5,0 ml DMF. Both batches was left overnight and analyzed by MS.

The extraction mixture emulsified hence extraction of acetate ion was also attempted with ethyl acetate as the organic solvent. Ethyl acetate coused more emulsification than DCM.

4.8.2 Coupling reagent effect

NaHCO3, 30 g, was dissolved in 300 ml H_2O to give 300 ml 10 % $NaHCO_3$. Crude linear FC131 (100 mg) was dissolved in 100 ml DCM. The solution was washed tree times with 100 ml 10 % $NaHCO_3$. The organic phase was dried with $MgSO_4$ for one hour and filtrated. DCM was removed in vacuo. This yielded 50,0 mg ($3{,}75 \cdot 10^{-5}$ mol) of the sodium salt of the crude linear FC131. This was dissolved in 200 ml DMF, analyzed by MS and RP-HPLC and split in two batches of 100 ml. Both batches should contain 25.0 mg ($1{,}88 \cdot 10^{-5}$ mol) FC131. Both batches was added 2 equivalent DIPEA (6,5µl, $3{,}76 \cdot 10^{-4}$ mol). One batch, batch A, was added 2 equivalents DEPBT (11,2 mg, $3{,}75 \cdot 10^{-5}$ mol) mg DEPT the other, batch B, was added 2 equivalents PyPOB (19,5 mg, $3{,}75 \cdot 10^{-5}$ mol). Both batches was left for 24 hours at room temperature. MS were performed after 8, 24, 32 and 60 hours. MS showed cyclization to be compleate in batch B after 24 hours. Cyclization was not complete in Batch A even after 60 hours. Batch A and DEPT were removed from further research.

4.8.3 Extraction of DMF

Batch B was added 100 ml DCM and washed three times with 100 ml H_2O to remove DMF from the solution. The organic phase was removed in vacuo. This yielded 25 mg product. MS and HPLC confirmed the product to be **9**.

4.8.4 Cleavage of protecting groups

10 ml of 95 % TFA / 2,5 % H_2O / 2,5 % TIS was added and left to stir for two hours. The solvent was removed in vacuo. The peptide was precipitated in cold diethyl ether, 50 ml, for one hour and the ether decanted. This was done three times. This yielded 18 mg and MS and HPLC showed this to be **9**. The crude cyclic peptide was purifed and lyophilized.

4.9 General Procedure for Solid Phase Peptide Synthesis of 11-16

The linear peptides **11-16** were prepared by continuous flow solid phase peptide synthesis on a Perseptive Biosystems Pioneer Peptide Synthesis System. Fmoc-Gly-Novasyn TGT resin (0,625 mg, 0,15 mmol) was used for all compounds. Fmoc-protected amino acids were added in 4 mol equivalents:

List of amino acids:

Fmoc-2-Nal-OH	(0,263 mg, 0,6 mmol)
Fmoc-L-Arg(Pbf)-OH	(0,389 mg, 0,6 mmol)
Fmoc-Me-Ala-OH	(0,195 mg, 0,6 mmol)
Fmoc-D-Tyr(tBu)-OH	(0,276 mg, 0,6 mmol
1-(Fmoc-amino)cyclohexanecarboxylic acid	(0,219 mg, 0,6 mmol)
N-Boc-4-(Fmoc-amino)piperidine-4-carboxylic acid	(0,280 mg, 0,6 mmol)
Nα-Fmoc-L-Citrulline	(0,238 mg, 0,6 mmol)

The "extended cycle", which is incorporated in the synthesizer's software package, was used for all compounds. This included the following: (1) pumping of 20% piperidine (flow rate, 5 mL/min for 5 min); (2) washing with DMF (flow rate, 30 ml/min, for 100 sec); (3) dissolution of Fmoc-amino acid (4-fold molar excess), HBTU (5-fold molar excess), and DIPEA (5-fold molar excess) in 3 ml of DMF; (4) recycling of the above (flow rate, 30 ml/min, for 60 min) and (5) washing with DMF (flow rate, 30 mL/min, for 30 s). The cycle was repeated for each amino acid. Final Fmoc was cleaved and resin shrunk with 53 ml isopropanol. The resins were washed with isopropanol and air dried over night. The resins were swelled in DCM and filtered. HFIP (5 ml, 33% in DCM) was added to the resins for 3 minutes and filtered off. This was repeated 2 times. DCM was removed from filtrate in vacuo. Peptides were precipitated in cold ether, the ether decanted and the precipitate air dried.

4.9.1 Synthesis of H_2N -D-Tyr^1(tBu)-Aib^2)-Arg^3(Pbf)-Nal^4-Gly^5-OH (11)

Compound **11** was synthesized according to the general procedure to give 115 mg (100 %) of the crude linear product. HPLC showed one major component (R_t = 24,8 min, 73,2 %), and MS confirmed that **11** (MW = 984,5) had been obtained.

4.9.2 Synthesis of H_2N -D-Tyr^1(tBu)-Chx^2-Arg^3(Pbf)-Nal^4-Gly^5-OH (12)

Compound **12** was synthesized according to the general procedure to give 63,4 mg (38,9 %) of the crude linear product. HPLC showed two major components (R_t = 21,9 min, 43,44%, R_t = 26,3 min, 56,57 %), and MS confirmed that **12** (MW = 1024,5) had been obtained. Yields were calculated assuming the last peak of the HPLC, the peak with greatest intensity, was the desired compound since MS showed grater intensity for the desired product. The crude mass was 112 mg.

4.9.3 Synthesis of H_2N -D-Tyr^1(tBu)-Pip^2(BOC)-Arg^3(Pbf)-Nal^4-Gly5-OH (13)

Compound **13** was synthesized according to the general procedure to give 24,7 mg (38,9 %) of the crude linear product. HPLC showed two major components (R_t = 23,3 min, 79,97%, R_t = 26,9 min, 20,09 %), and MS confirmed that **13** (MW = 1125,5) had been obtained. Yields were calculated assuming the last peak of the HPLC, the peak with minor intensity, was the desired compound since MS showed low intensity for the desired product. The crude mass was 123 mg.

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4.9.4 Synthesis of H_2N -D-Tyr^1(tBu)-Cit^2-Arg^3(Pbf)-Nal^4-Gly5-OH (14)

Compound **14** was synthesized according to the general procedure to give 123 mg (77,6%) of the crude linear product. HPLC showed one major components (R_t = 23,7 min, 96,2%), and MS confirmed that **14** (MW = 1056,5) had been obtained.

4.9.5 Synthesis of H_2N -D-Tyr^1(tBu)-Arg^2(Pbf)-Cit^3-Nal^4-Gly5-OH (15)

Compound **15** was synthesized according to the general procedure to give 122 mg (77,0%) of the crude linear product. HPLC showed one major components (R_t = 22,8 min, 96,2%), and MS confirmed that **15** (MW = 1056,5) had been obtained.

4.9.6 Synthesis of H_2N -D-Tyr^1(tBu)–Cit^2-Cit^3-Nal^4-Gly5-OH (16)

Compound **16** was synthesized according to the general procedure to give 112 mg (94,2%) of the crude linear product. HPLC showed one major components (R_t = 17,7 min, 94,2%), and MS confirmed that **16** (MW = 805,4) had been obtained.

4.10 General Procedure for Synthesis of 1-8 from 9-16

The linear precursor (**9-16**) was dissolved in 5 ml DMF/DCM (1:1) per mg crude peptide, and 2 mol equivalents of PyBOP and DIPEA were added. After completion of the cyclization (24 hours) the solvents were removed in vacuo. For side chain deprotection, 30 ml of the cleavage cocktail TFA/H_2O/TIS (95:2,5:2,5) was added, and the reaction was left for two hours before removing the reaction mixture in vacuo. The deprotected cyclic peptide was precipitated by adding 100 ml cold diethylether. After 2 hours the ether was decanted, and the step repeated. The crude product was purified by preparative HPLC, and finally lyophilized. The final products **1-8** were characterized by MS, HPLC and NMR.

4.10.1 Synthesis of FC131 (1)

Compound **1** was synthesized according to the general procedure, starting from a solution of precursor **9** (117,3 mg, $8{,}82\cdot10^{-5}$ mol) in 600 ml DCM/DMF 1:1, to which PyBOP (91,8 mg, $1{,}76\cdot10^{-4}$ mol) and DIPEA (31 µl, $1{,}76\cdot10^{-4}$ mol) were added. After lyophilization, 16,5 mg (19,5%, (in cyclization)) of **1** was obtained as a white powder. R_t = 21,2 min (98,88% purity); $[M+H]^+$ = 730,2. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **1**.

4.10.2 Synthesis of Ala^{2}FC131 (2)

Compound **2** was synthesized according to the general procedure, starting from a solution of precursor **10** (103,3 mg, $1{,}04 \cdot 10^{-4}$ mol) in 550 ml DCM/DMF 1:1, to which PyBOP (108,2 mg, $2{,}08 \cdot 10^{-4}$ mol) and DIPEA (36µl, $2{,}08 \cdot 10^{-4}$ mol) were added. After lyophilization, 16,2 mg (20,5%, (in cyclization)) of **2** was obtained as a white powder. R_t = 21,9 min (96,49% purity); $[M+H]^+$ = 645,2. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **2**.

4.10.3 Synthesis of Aib^{2}FC131 (3)

Compound **3** was synthesized according to the general procedure, starting from a solution of precursor **11** (115,0 mg, $1{,}01 \cdot 10^{-4}$ mol) in 775 ml DCM/DMF 1:1, to which PyBOP (121 mg, $2{,}08 \cdot 10^{-4}$ mol) and DIPEA (40µl, $2{,}23 \cdot 10^{-4}$ mol) were added. After lyophilization, 41,2 mg (35,6%) of **3** was obtained as a white powder. R_t = 22,6min (100% purity); $[M+H]^+$ = 659,2. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **3**.

4.10.4 Synthesis of Chx^{2}FC131 (4)

During cyclisation of **4** it was decided to increase the amount of solvent to 8 ml per mg peptide. Compound **4** was otherwise synthesized according to the general procedure. starting from a solution of precursor **12** ((111,9 mg, $1{,}09 \cdot 10^{-4}$ mol l) in 895 ml DCM/DMF 1:1, to which PyBOP (113,7 mg, $2{,}18 \cdot 10^{-4}$ mol) and DIPEA (38µl, $2{,}18 \cdot 10^{-4}$ mol) were added. After lyophilization, 22,8 mg (18,7%) of **4** was obtained as a white powder. R_t = 23,9min (98,12% purity); $[M+H]^+$ = 699,3. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **4**.

4.10.5 Synthesis of Pip^{2}FC131 (5)

During cyclisation of **5** it was decided to increase the amount of solvent to 8 ml per mg peptide. Compound **5** was otherwise synthesized according to the general procedure. starting from a solution of precursor **13**(123,4 mg, $1{,}10 \cdot 10^{-4}$ mol l) in 987 ml DCM/DMF 1:1, to which PyBOP (113,9 mg, $2{,}19 \cdot 10^{-4}$ mol) and DIPEA (38µl, $2{,}19 \cdot 10^{-4}$ mol) were added. After lyophilization, 2,3 mg of **5** was obtained as a white powder. R_t = 21,06min (78,97% purity); $[M+H]^+$ = 700,2. NMR was not done. Abandoned from further research due to impurities.

4.10.6 Synthesis of Cit^{2}FC131 (6)

Compound **6** was synthesized according to the general procedure, starting from a solution of precursor **14** (123,0 mg, $1{,}16 \cdot 10^{-4}$ mol) in 615 ml DCM/DMF 1:1, to which PyBOP (119,0 mg, $2{,}32 \cdot 10^{-4}$ mol) and DIPEA (39µl, $2{,}23 \cdot 10^{-4}$ mol) were added. After lyophilization, 40,3 mg (41,1%) of **6** was obtained as a white powder. R_t = 22,2min (100% purity); $[M+H]^+$ = 731,0. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **6.**

4.10.7 Synthesis of Cit^{3}FC131 (7)

Compound **7** was synthesized according to the general procedure, starting from a solution of precursor **15** (122 mg, $1{,}15 \cdot 10^{-4}$ mol) in 610 ml DCM/DMF 1:1, to which PyBOP (119,0 mg, $2{,}30 \cdot 10^{-4}$ mol) and DIPEA (39µl, $2{,}20 \cdot 10^{-4}$ mol) were added. After lyophilization, 28,3 mg (41,1%) of **7** was obtained as a white powder. R_t = 22,3 min (96,6% purity); $[M+H]^+$ = 731,2. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **7.**

4.10.8 Synthesis of Cit^{2,3}FC131 (8)

Compound **8** was synthesized according to the general procedure, starting from a solution of precursor **16** (112 mg, $1{,}39 \cdot 10^{-4}$ mol) in 610 ml DCM/DMF 1:1, to which PyBOP (144,6 mg, $2{,}80 \cdot 10^{-4}$ mol) and DIPEA (78,7µl, $2{,}80 \cdot 10^{-4}$ mol) were added. After

lyophilization, 22,6 mg (24,5%) of **8** was obtained as a white powder. R_t = 21,96 min (96,6% purity); $[M+H]^+$ = 699,2. The ^{1}H-NMR spectrum was fully consistent with the desired target structure **8.**

5 References

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APPENDIX

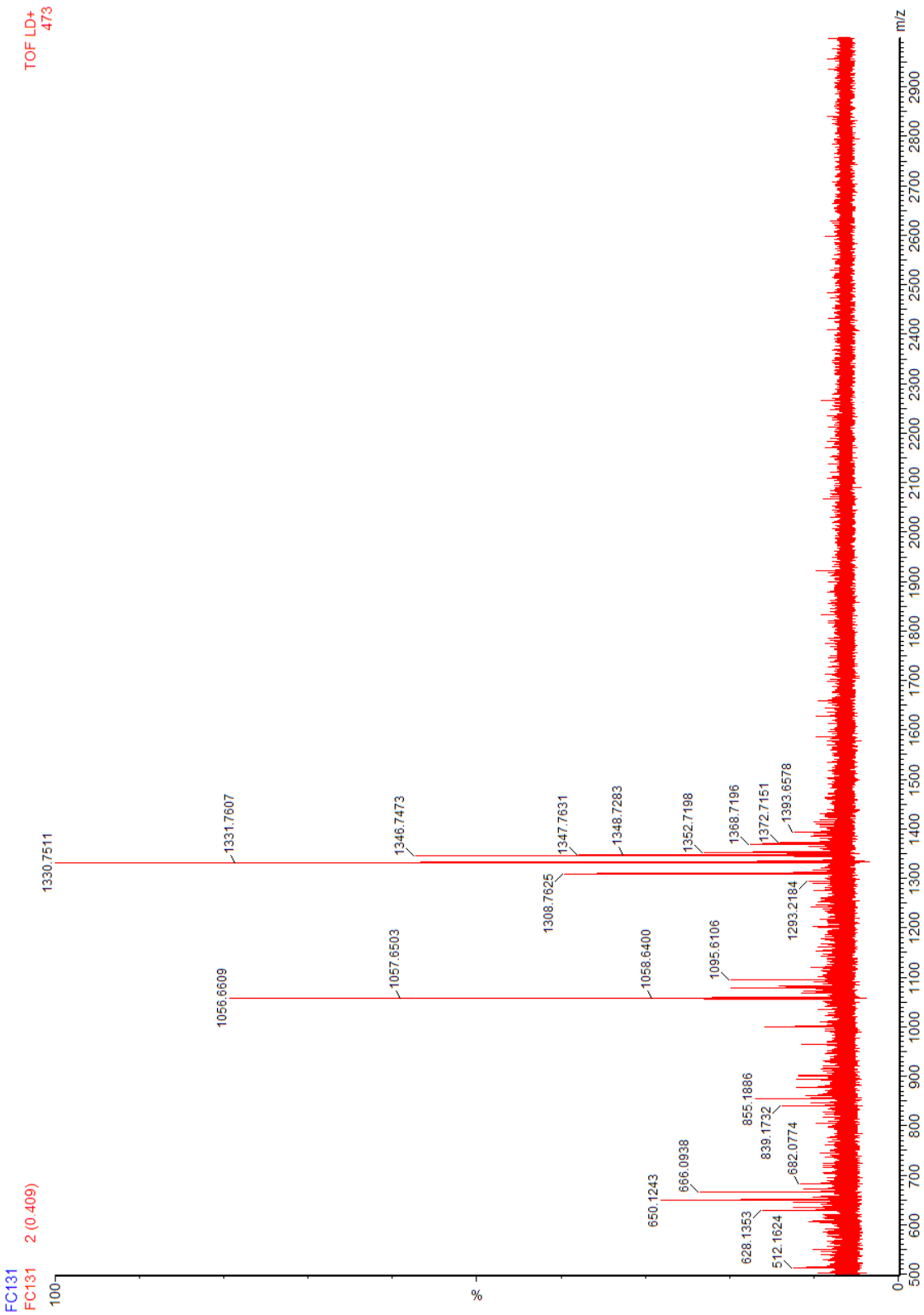
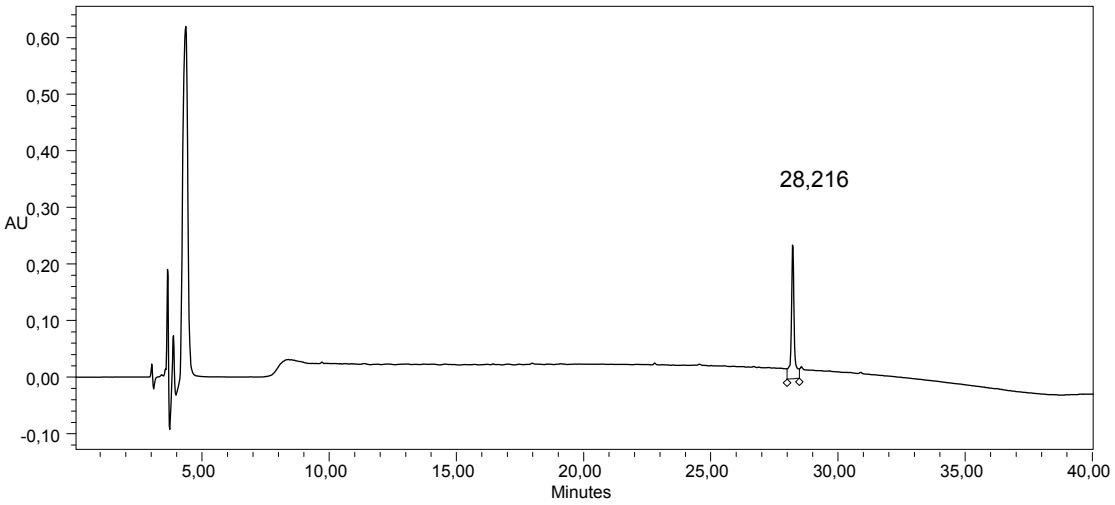


Figure A-1: MS analysis of compound **9** after extraction

SAMPLE INFORMATION

Sample Name:	FC131	Sample Set Name:	Oystein_FC131
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	67	Processing Method:	TH Default
Injection #:	1	Channel Name:	PDA single wavelength 214
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 214,0 nm
Run Time:	40,0 Minutes		



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W2690/5 Gradient Table

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1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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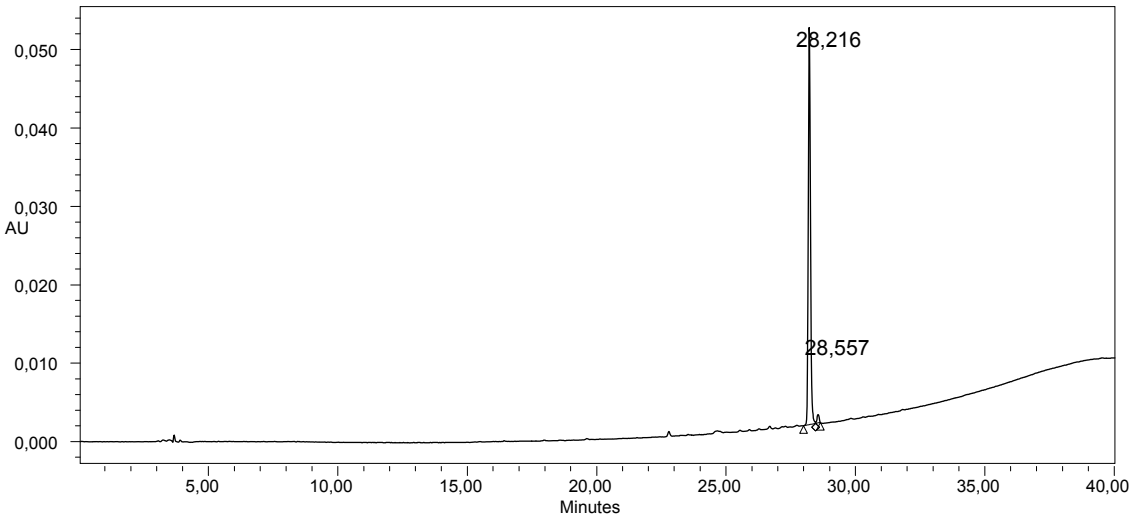
Figure A-2: HPLC analysis of compound **9**, wavelength 214 nm

20.05.2008 Øystein Eriksen

SAMPLE INFORMATION

Sample Name:	FC131	Sample Set Name:	Oystein_FC131
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	67	Processing Method:	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



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W2690/5 Gradient Table

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2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-3: HPLC analysis of compound **9**, wavelength 254 nm

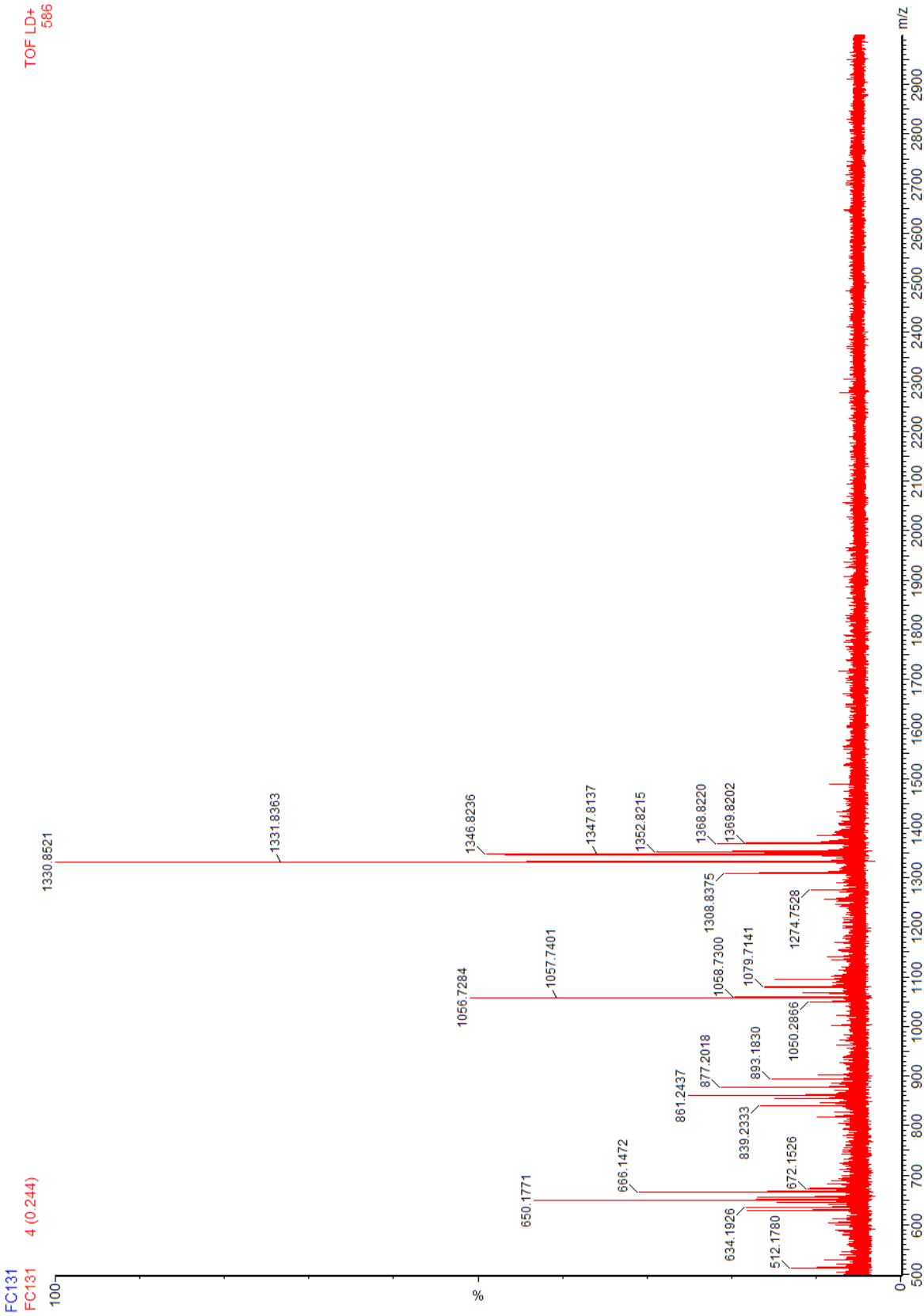


Figure A-4: Pilot Experiment: MS analysis of reaction mixture after 24 hours

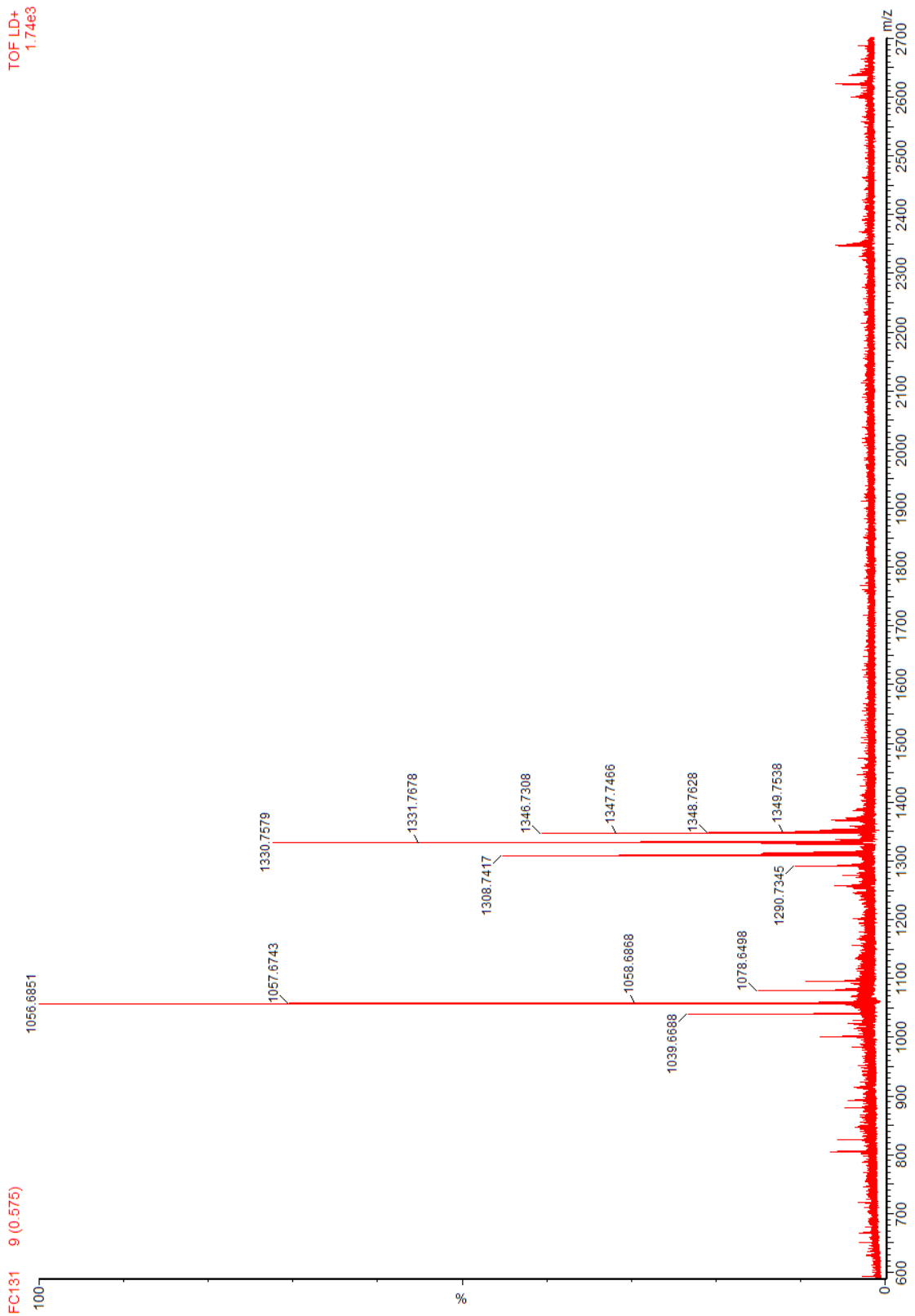


Figure A-5: Pilot Experiment: MS analysis of reaction mixture (Batch 1) after 96 hours

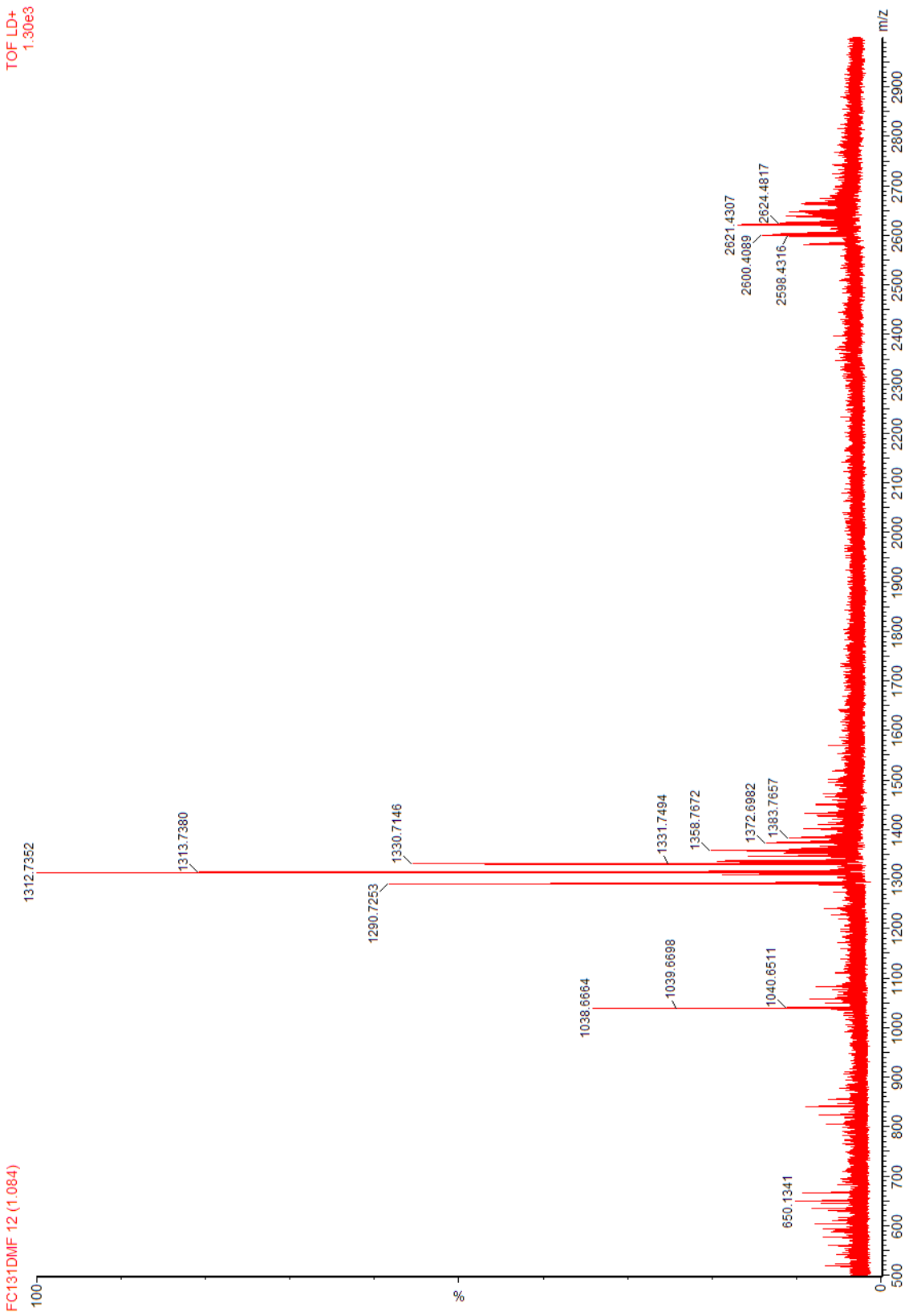
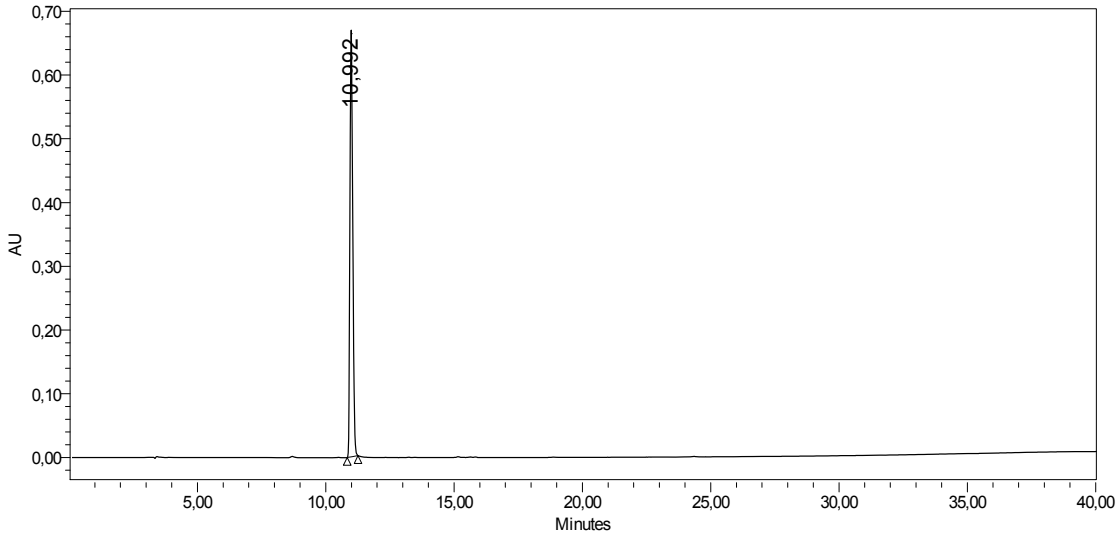


Figure A-6: Pilot Experiment: MS analysis of after 96 hours. Addition of DMF

SAMPLE INFORMATION

Sample Name:	DEPBT_blank	Sample Set Name:	Oystein
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	50	Processing Method	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



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W2690/5 Gradient Table

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2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-7: Pilot Experiment: HPLC analysis of DEPBT. Wavelength 254 nm

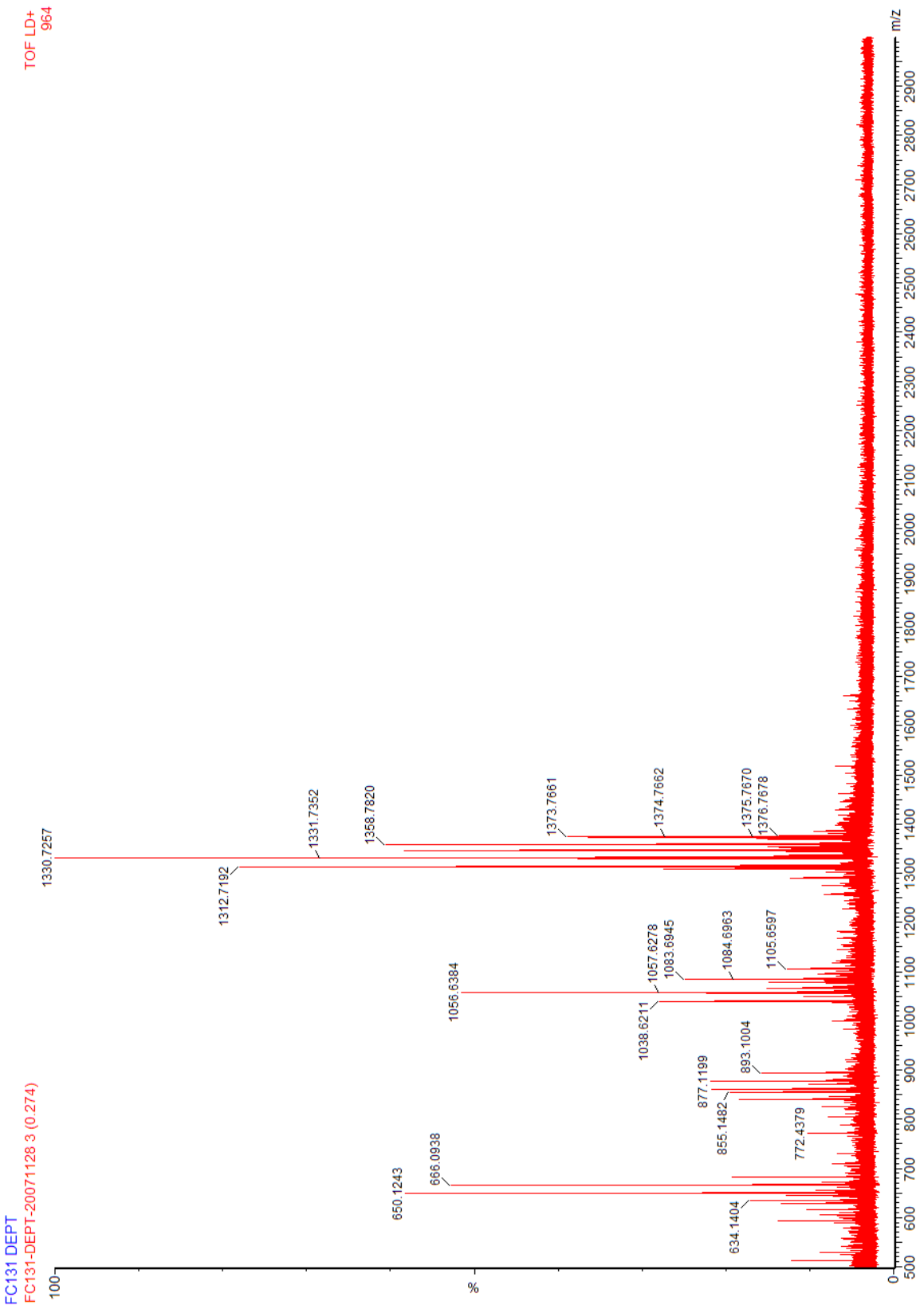
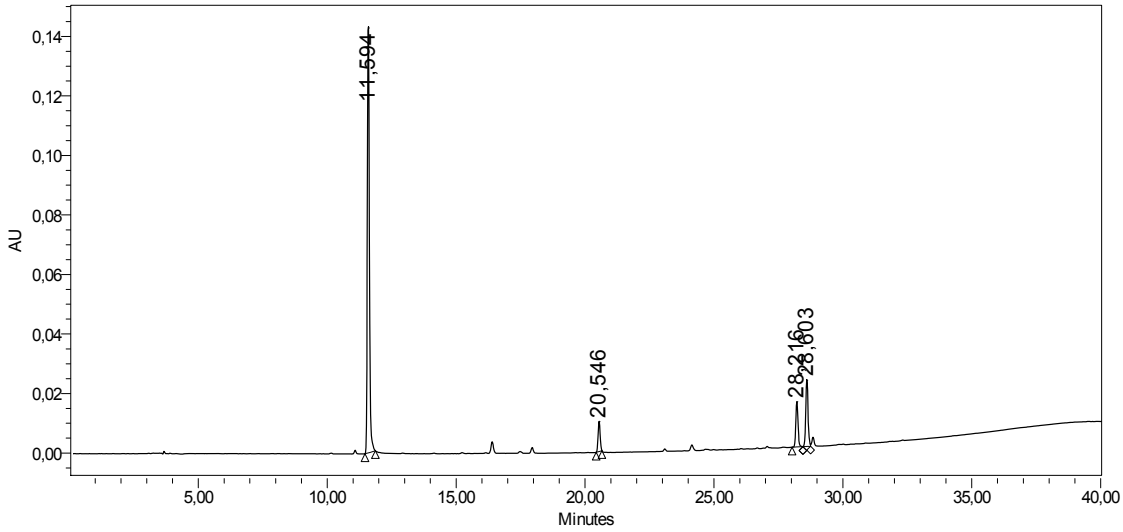


Figure A-8: Pilot Experiment: MS analysis of reaction mixture with DEPBT after 32 hours

SAMPLE INFORMATION

Sample Name:	FC131_DEPT		
Sample Type:	Unknown	Sample Set Name:	Oystein_FC131
Vial:	69	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

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2	20,546	56067	5,19	10111
3	28,216	89757	8,31	15037
4	28,603	137893	12,76	22377

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-9: Pilot experiment: HPLC analysis with DEPBT after 60 hours.

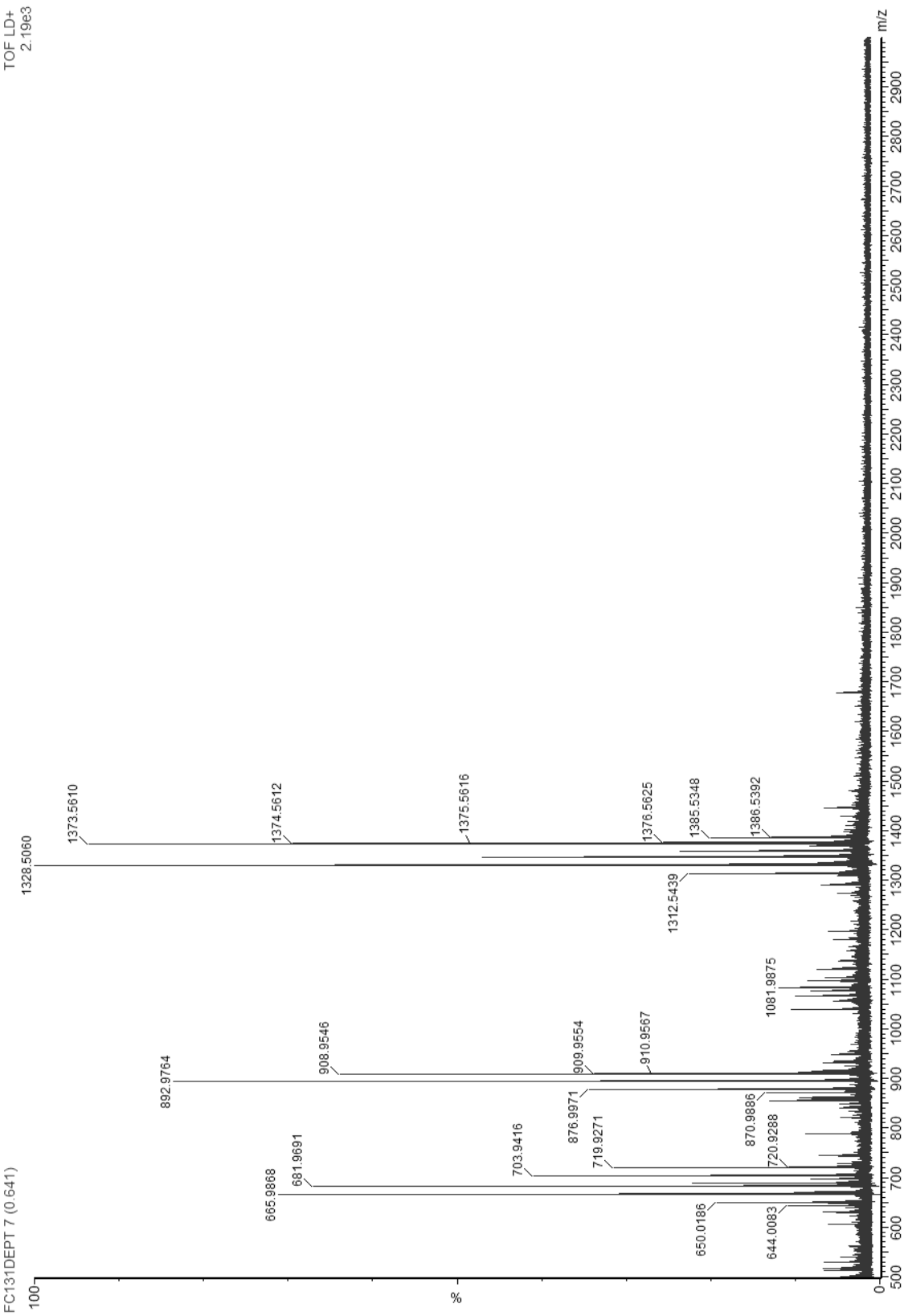
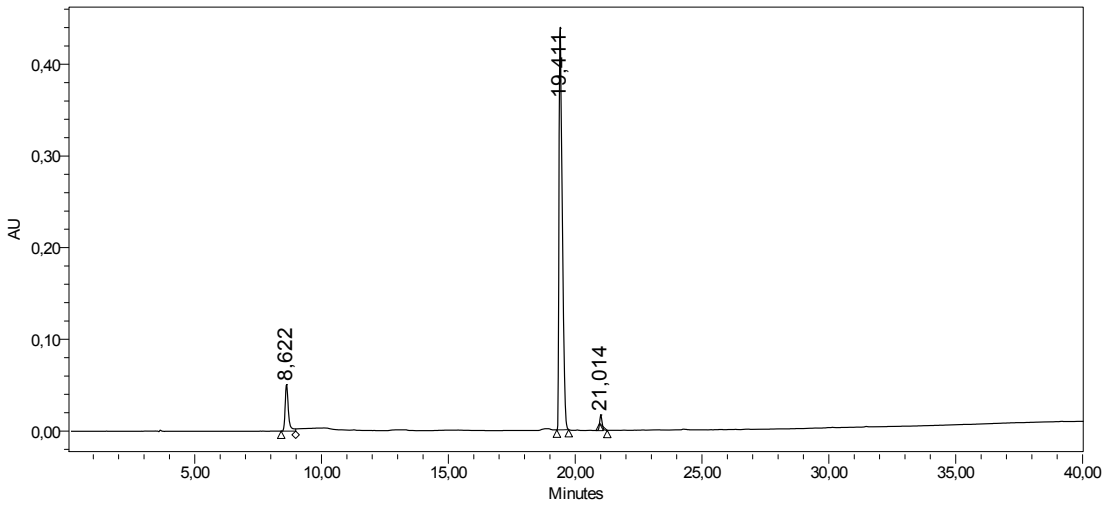


Figure A-10: Pilot Experiment: MS analysis of reaction with DEPBT after 60 hours.

SAMPLE INFORMATION

Sample Name:	PyBOP		
Sample Type:	Unknown	Sample Set Name:	Oystein_3
Vial:	65	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

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W2690/5 Gradient Table

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2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

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Figure A-11: Pilot experiment: HPLC analysis of PyBOP

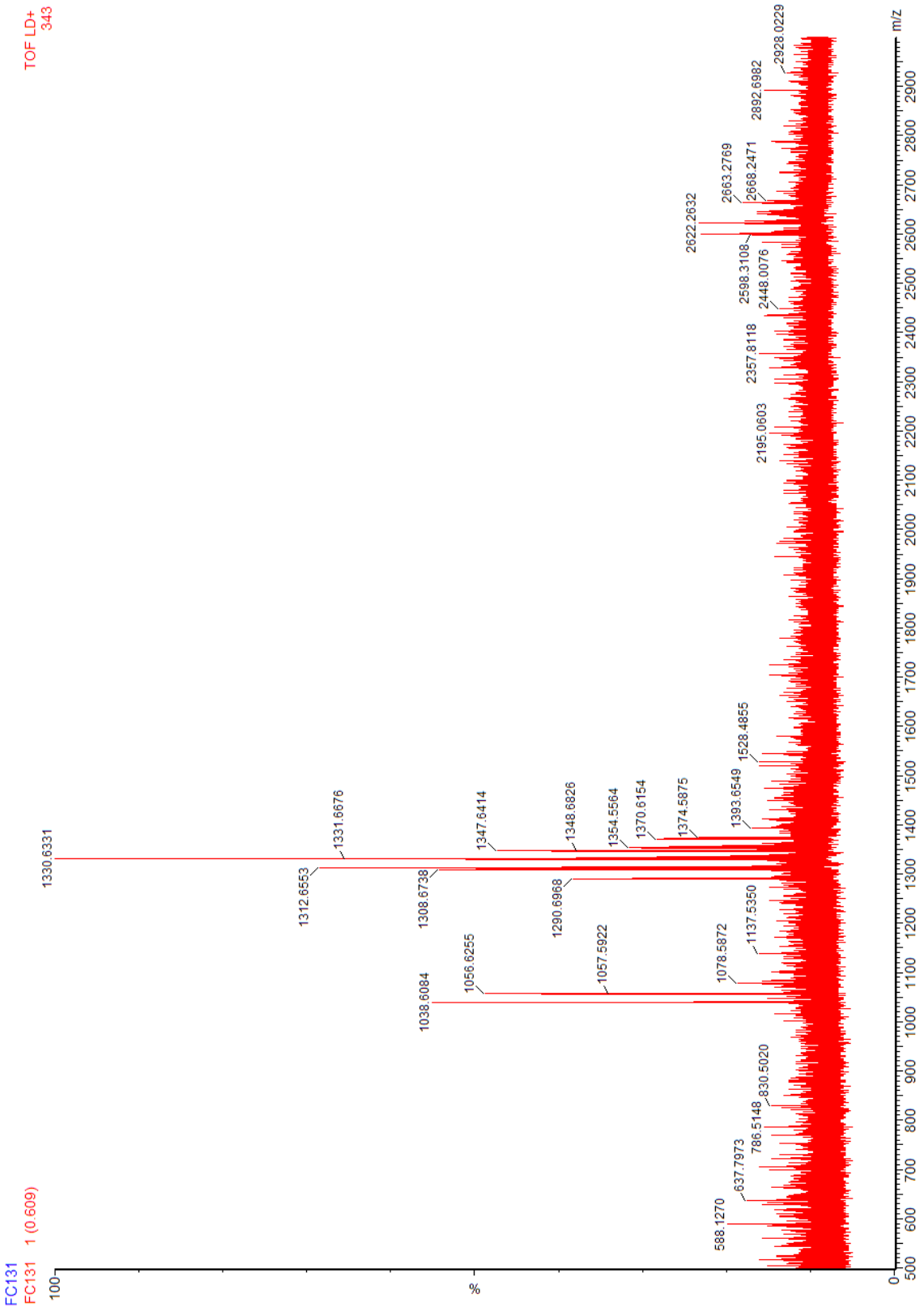
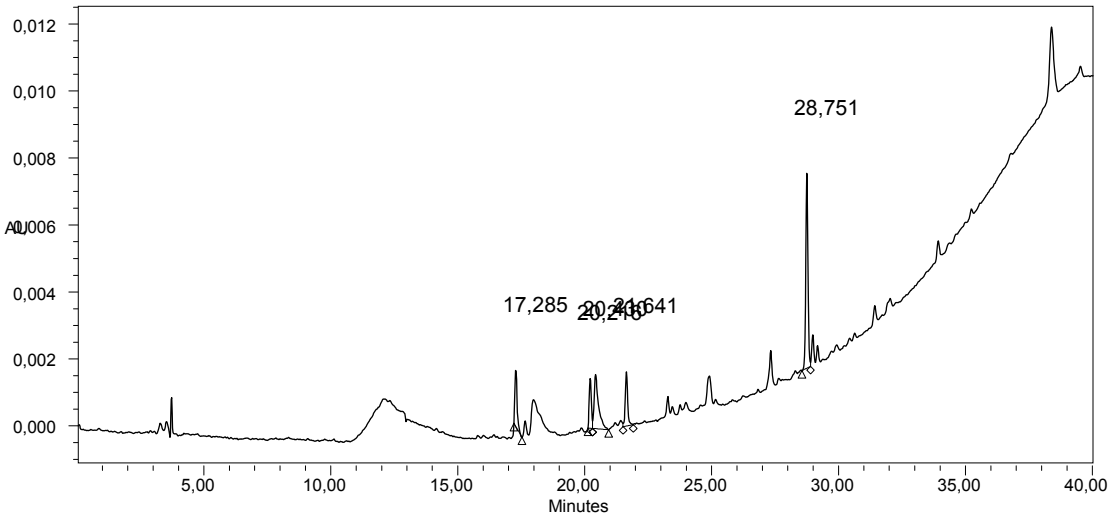


Figure A-12: Pilot Experiment: MS analysis of mixture with PyBOP after 8 hours.

SAMPLE INFORMATION

Sample Name:	FC131_PyBOP04122007		
Sample Type:	Unknown	Sample Set Name:	OysteinFC131_PyBOP04122007
Vial:	66	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method:	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	17,285	10356	12,23	1689
2	20,216	8611	10,17	1482
3	20,430	19533	23,06	1612
4	21,641	10632	12,55	1607
5	28,751	35567	41,99	5858

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-13: Pilot Experiment: HPLC analysis of mixture with PyBOP after 32 hours.

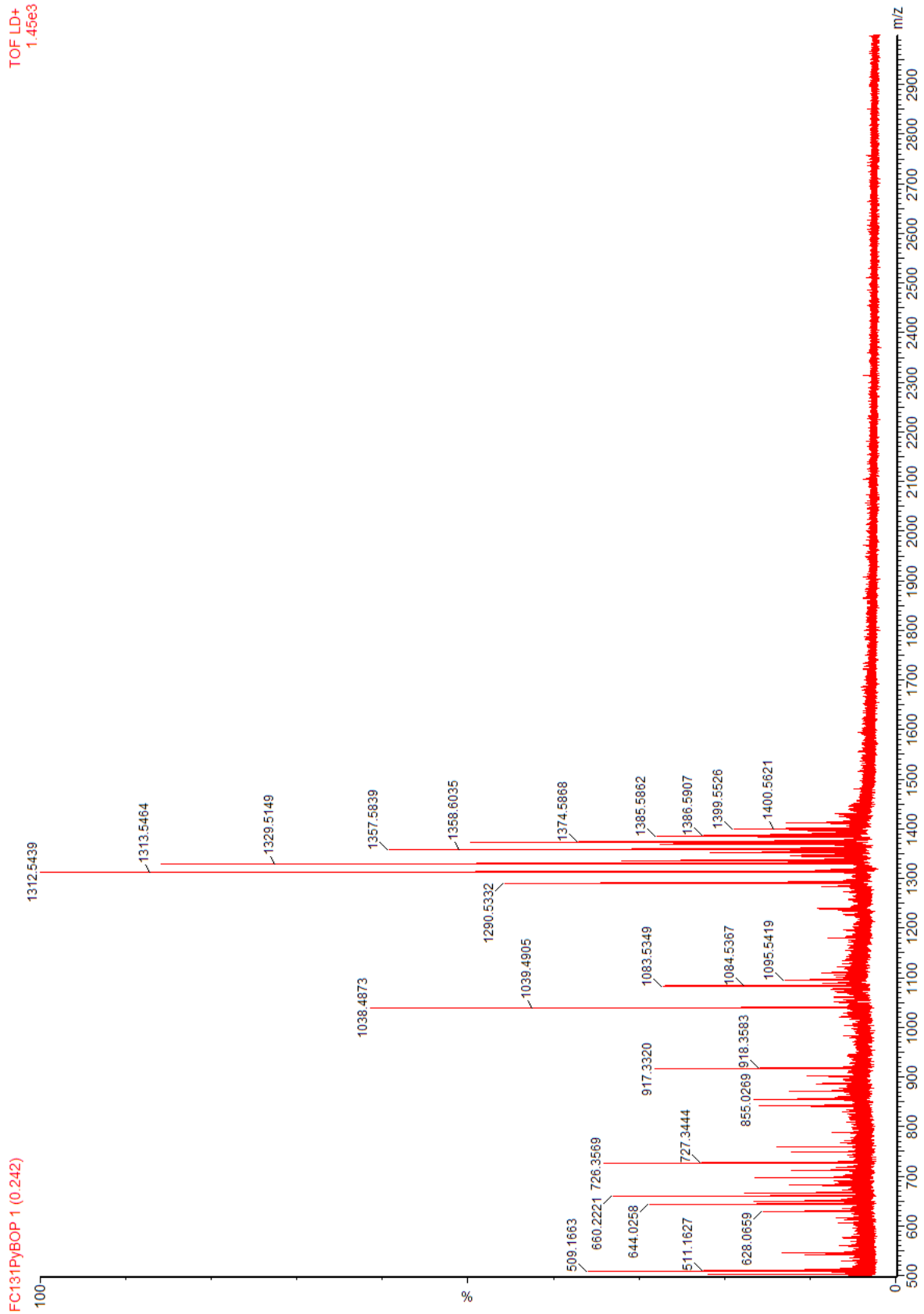


Figure A-14: Pilot Experiment: MS analysis of mixture with PyBOP after 32 hours.

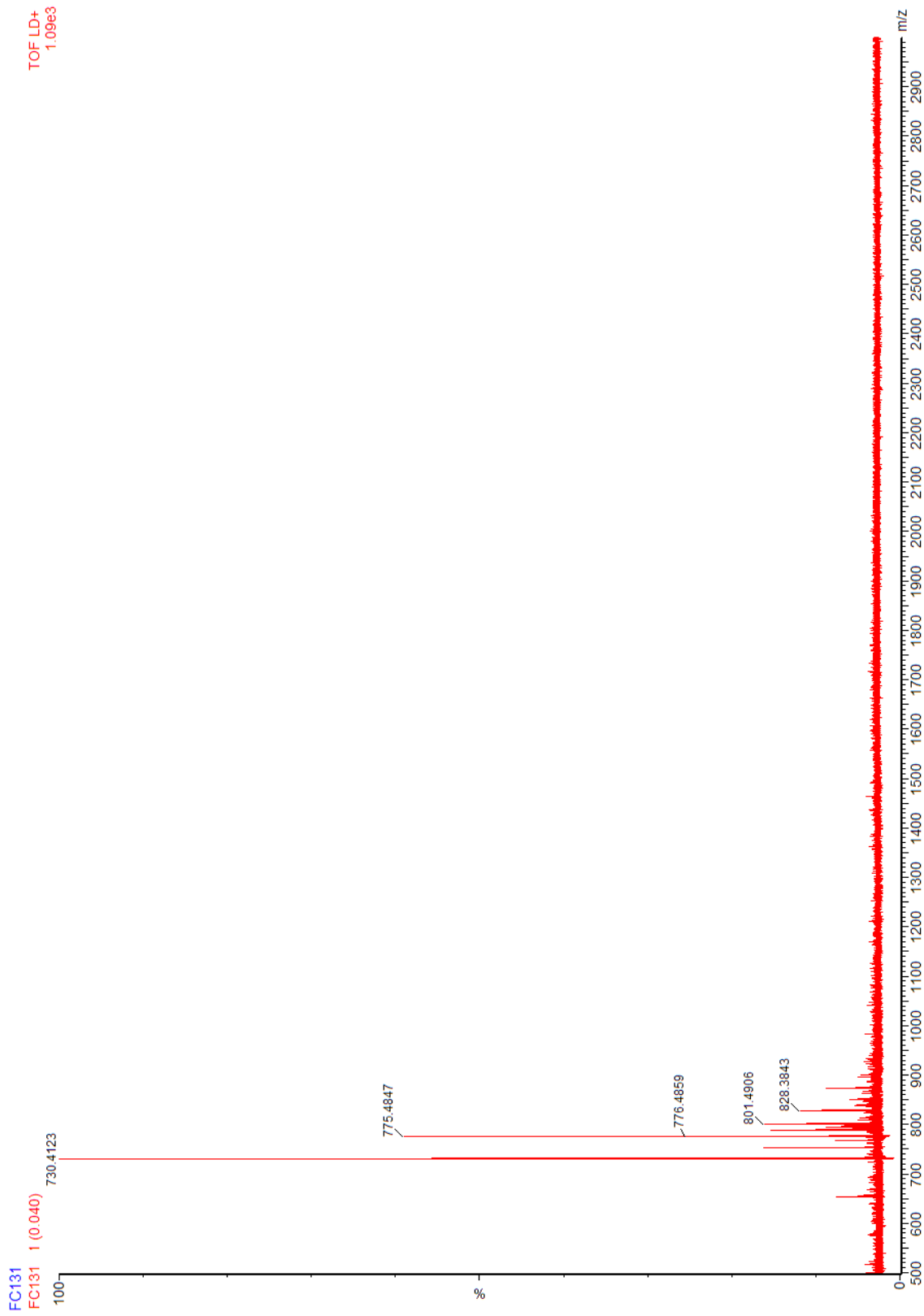
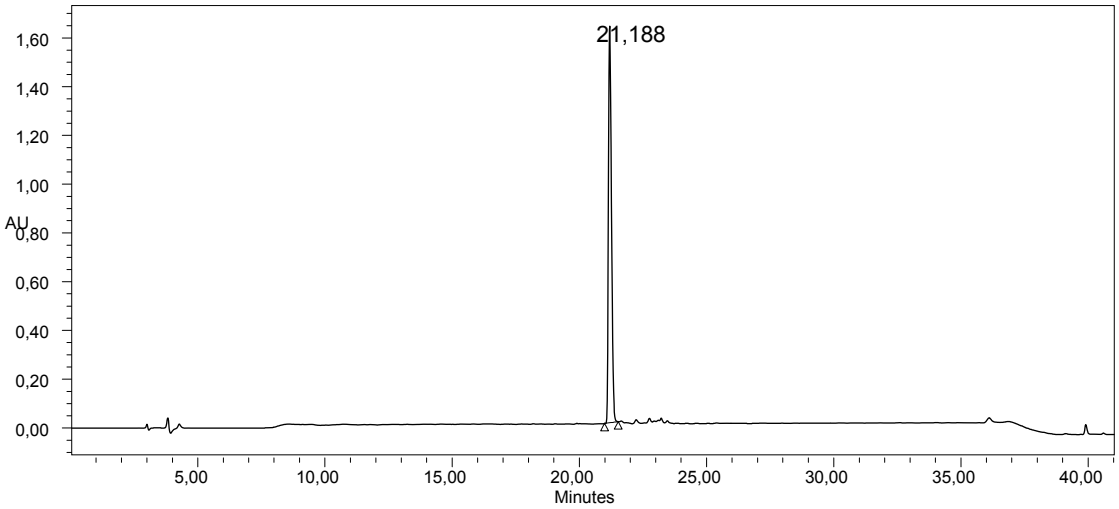


Figure A-15: Pilot Experiment: MS analysis of **1** after cleavage of protecting groups.

SAMPLE INFORMATION

Sample Name:	FC131_Final	Sample Set Name:	FC131_Final
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Vial:	69	Processing Method:	TH Default
Injection #:	1	Channel Name:	PDA single wavelength 214
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 214,0 nm
Run Time:	41,0 Minutes		



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,188	13719019	100,00	1630068

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Reported by: Øystein Eriksen
Report Method: Øystein Eriksen
Report Method ID 6746
Page: 1 of 1

Project Name: Farmasi - MBSs gruppe
Date Printed: 20.05.2008
06:39:35 Europe/Oslo

Figure A-16: Pilot Experiment: HPLC of purified FC131, compound **1**

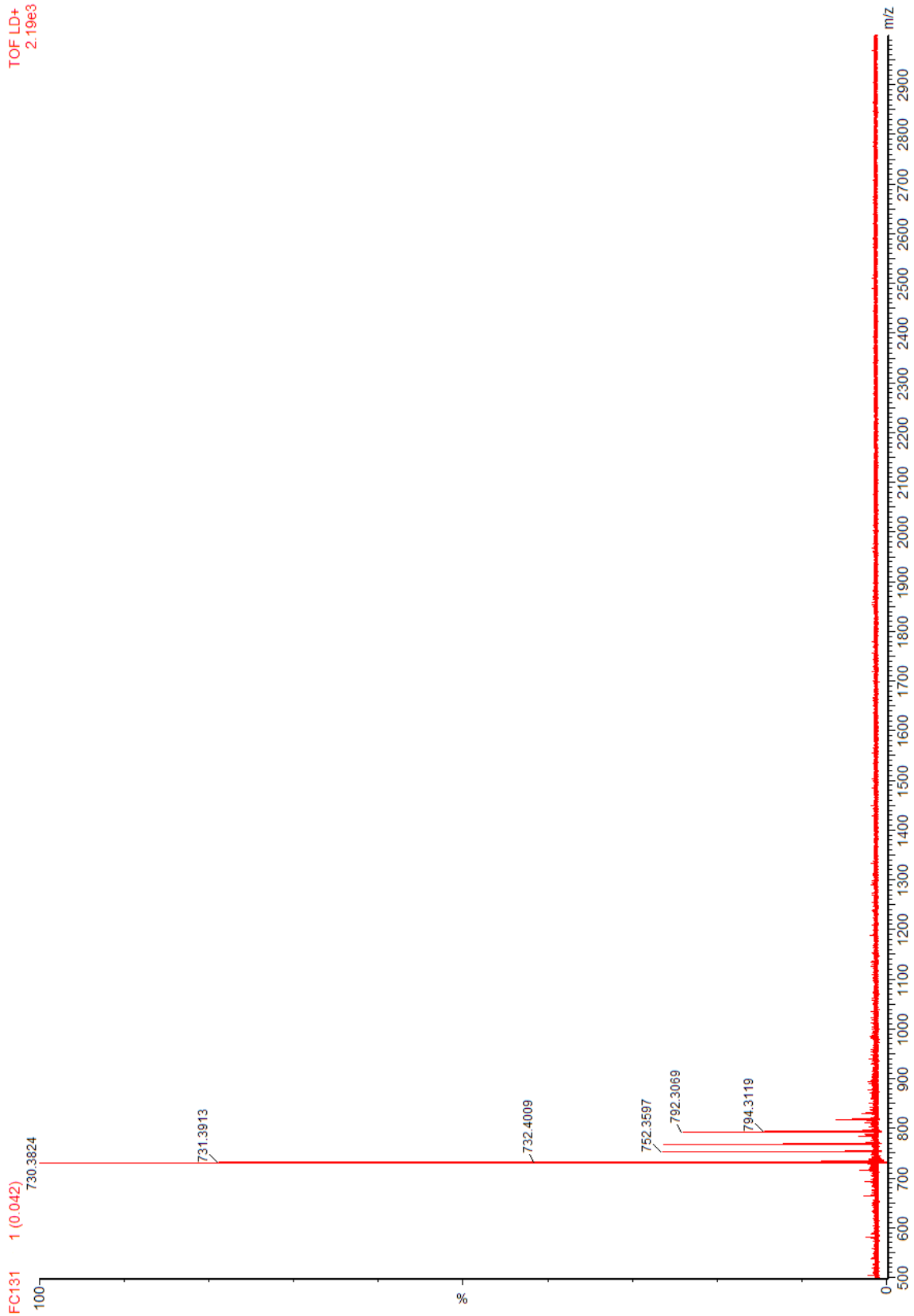
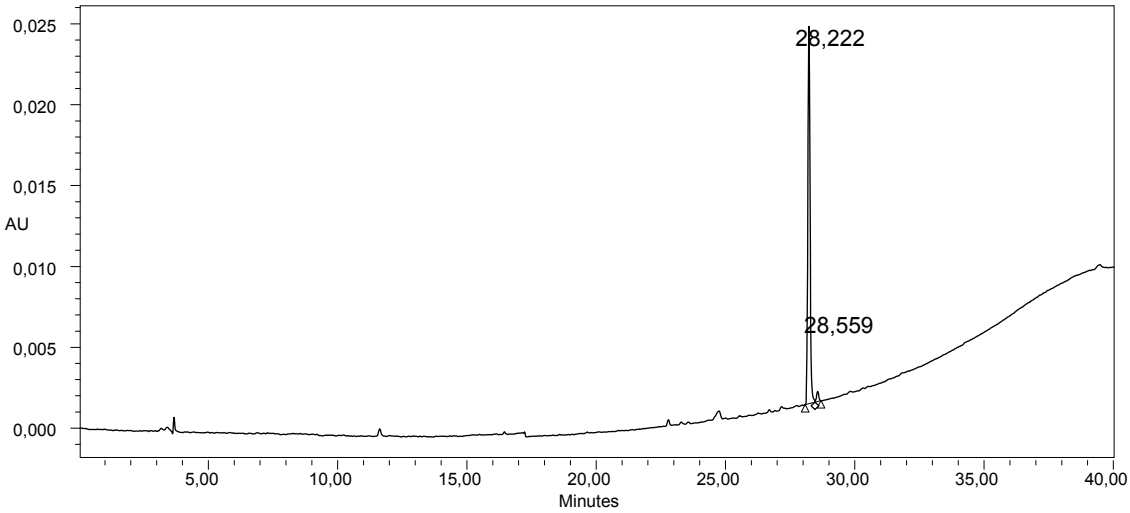


Figure A-17: Pilot Experiment: MS of purified FC131, compound **1**

SAMPLE INFORMATION

Sample Name:	FC131	Sample Set Name:	Oystein_FC131
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	66	Processing Method:	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	28,222	139167	97,45	23220
2	28,559	3642	2,55	611

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

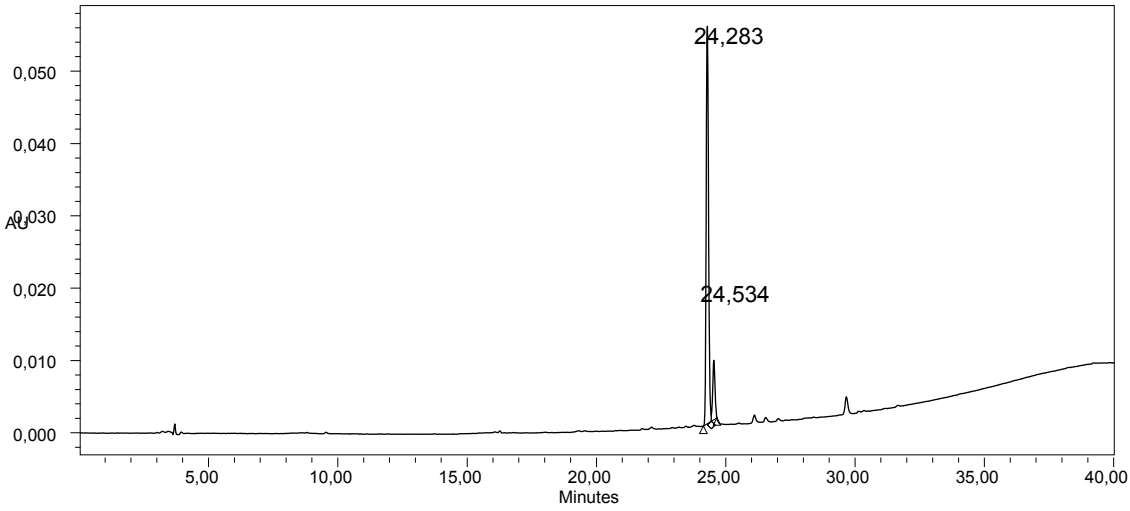
Revision History

This method contains 2 items in the revision history.

Figure A-18: HPLC analysis of compound **9** after extraction

SAMPLE INFORMATION

Sample Name:	FC131Ala_crude		
Sample Type:	Unknown	Sample Set Name:	Oystein_21012008
Vial:	65	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method:	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	24,283	340552	86,93	54758
2	24,534	51185	13,07	8208

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-19: HPLC analysis of compound **10** after extraction.

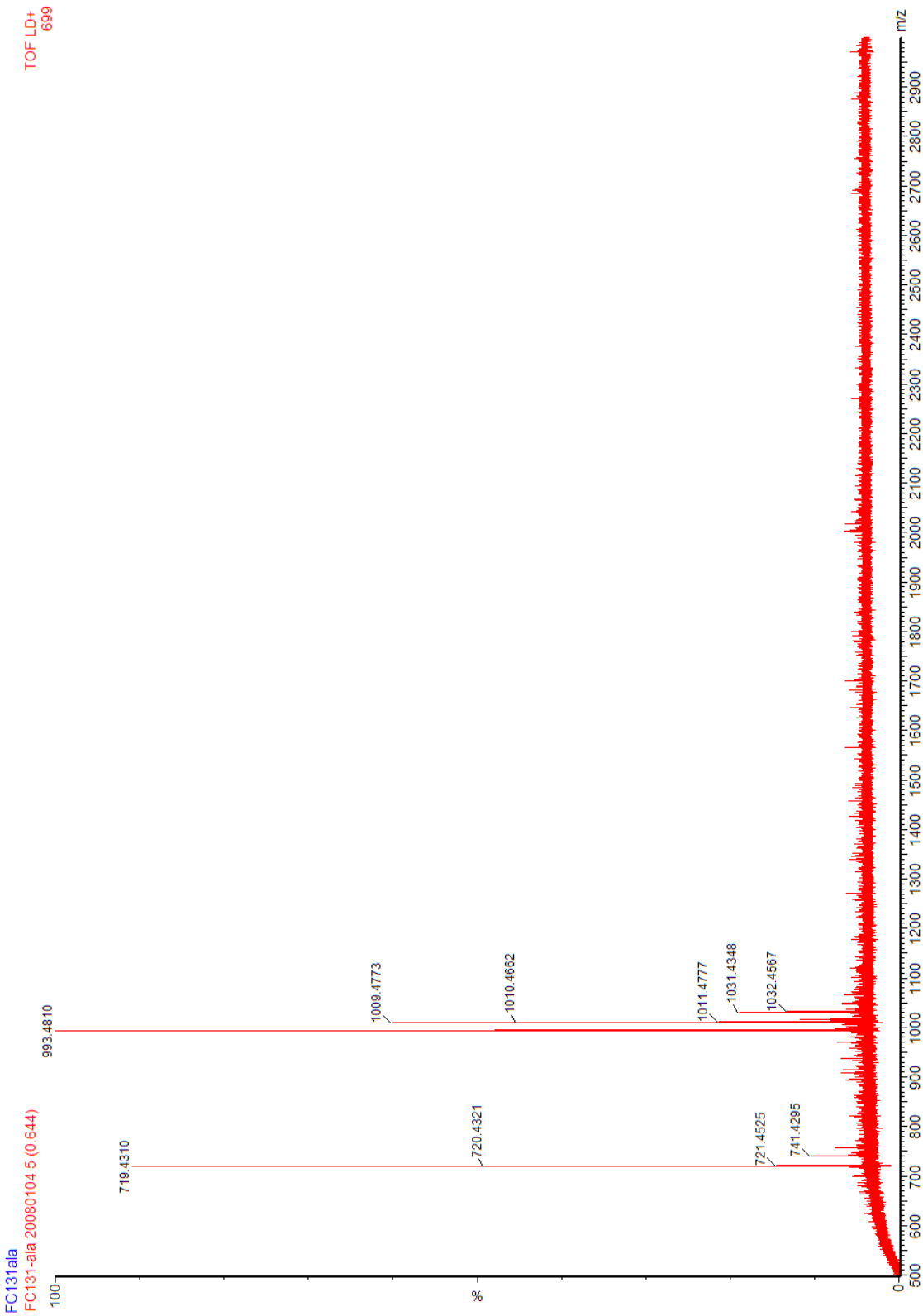
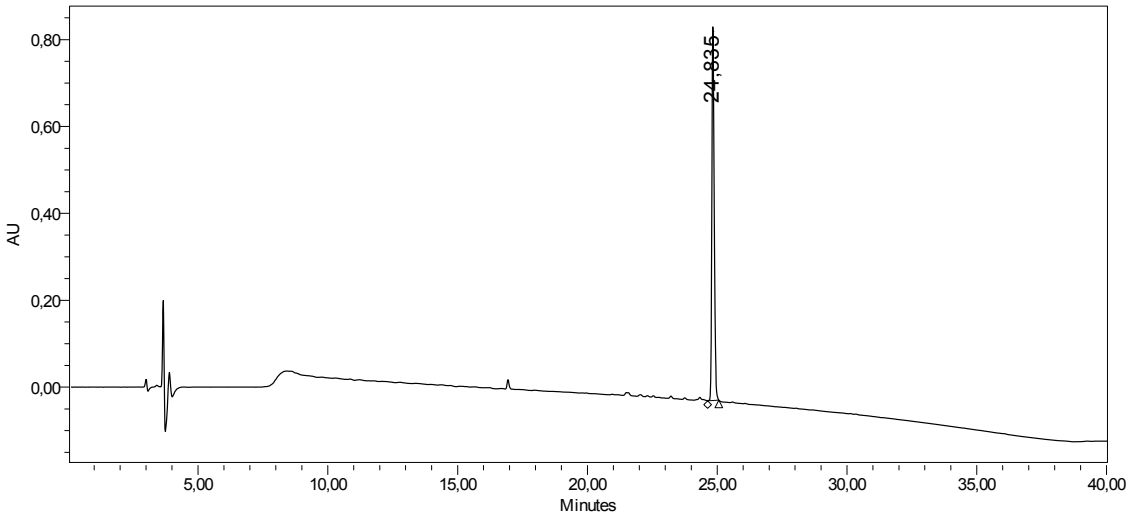


Figure A-20: MS analysis of compound **10** after extraction

SAMPLE INFORMATION

Sample Name:	FC131Aib		
Sample Type:	Unknown	Sample Set Name:	Oystein_21012008
Vial:	66	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	24,835	5484838	100,00	859965

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

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Page: 1 of 1

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20.05.2008
07:57:13 Europe/Oslo

Figure A-21: HPLC of compound **11**

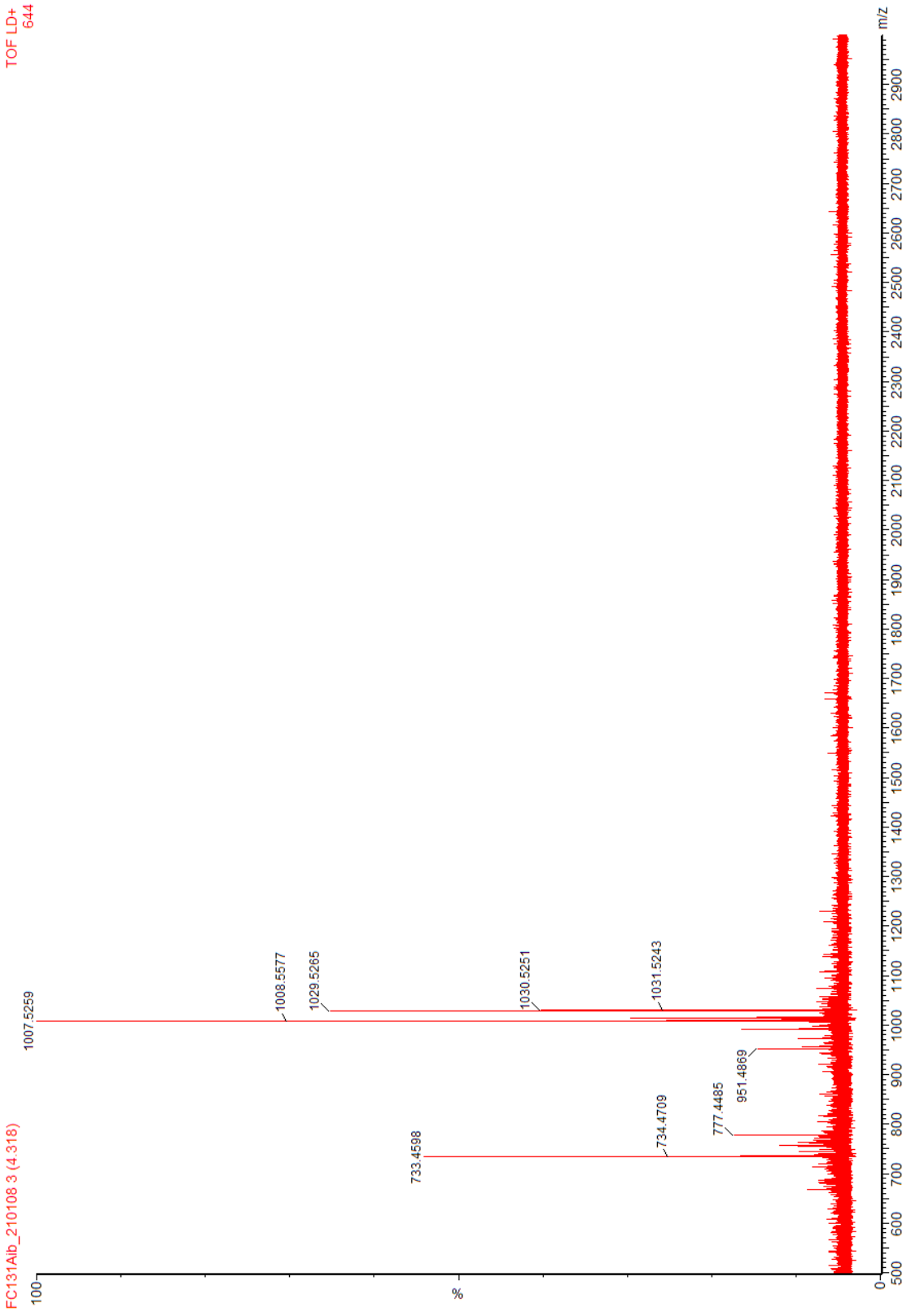
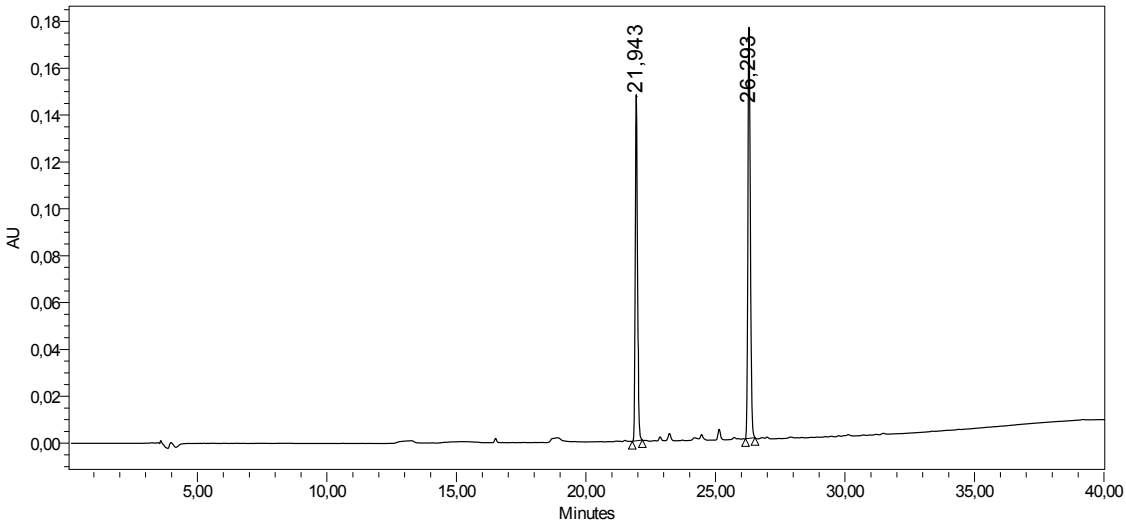


Figure A-22: MS analysis of compound **11**

SAMPLE INFORMATION

Sample Name:	FC131(Chx)		
Sample Type:	Unknown	Sample Set Name:	Oystein_3
Vial:	66	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	21,943	884462	43,43	146596
2	26,293	1152108	56,57	174534

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-23: HPLC analysis of compound **12**

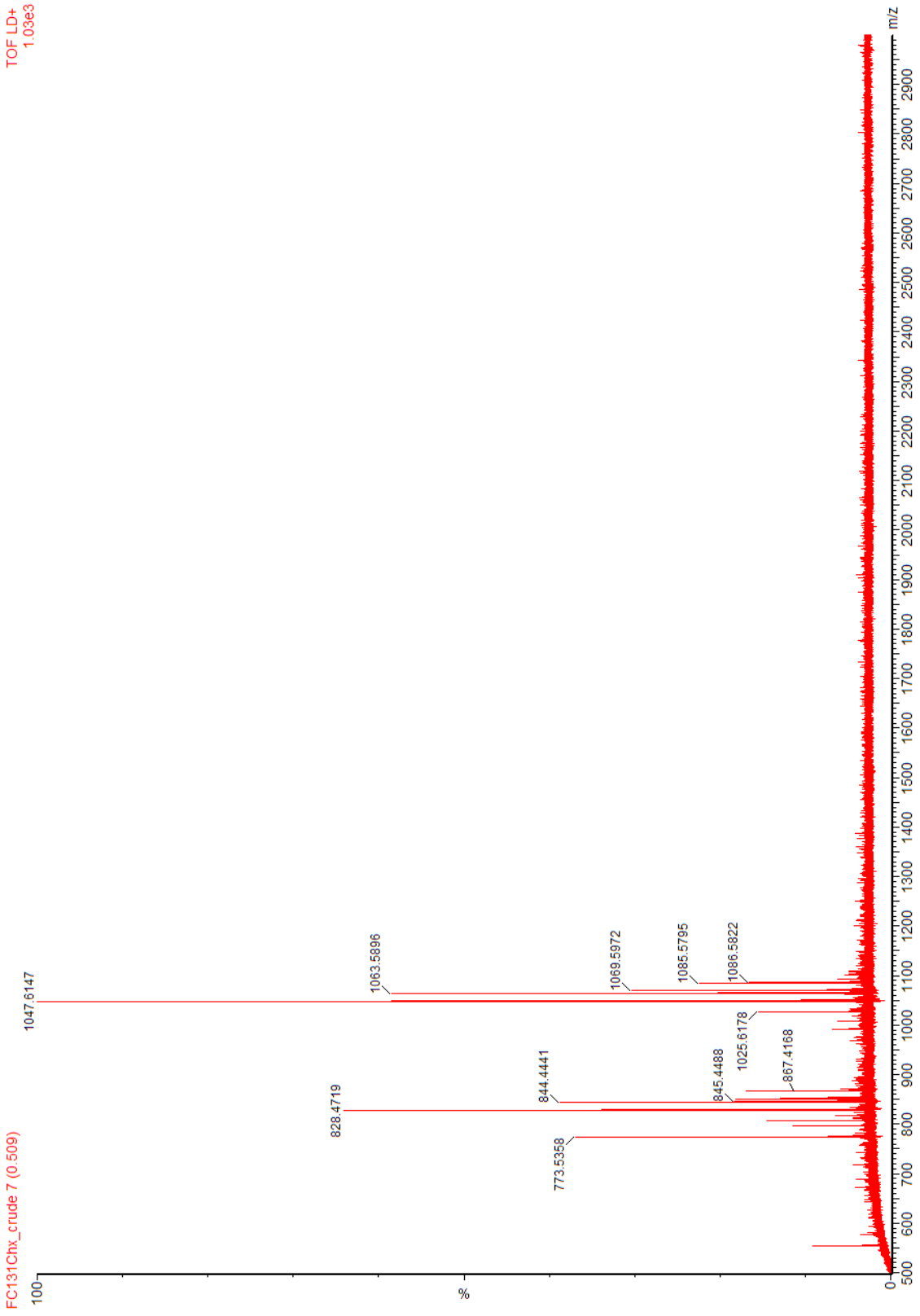
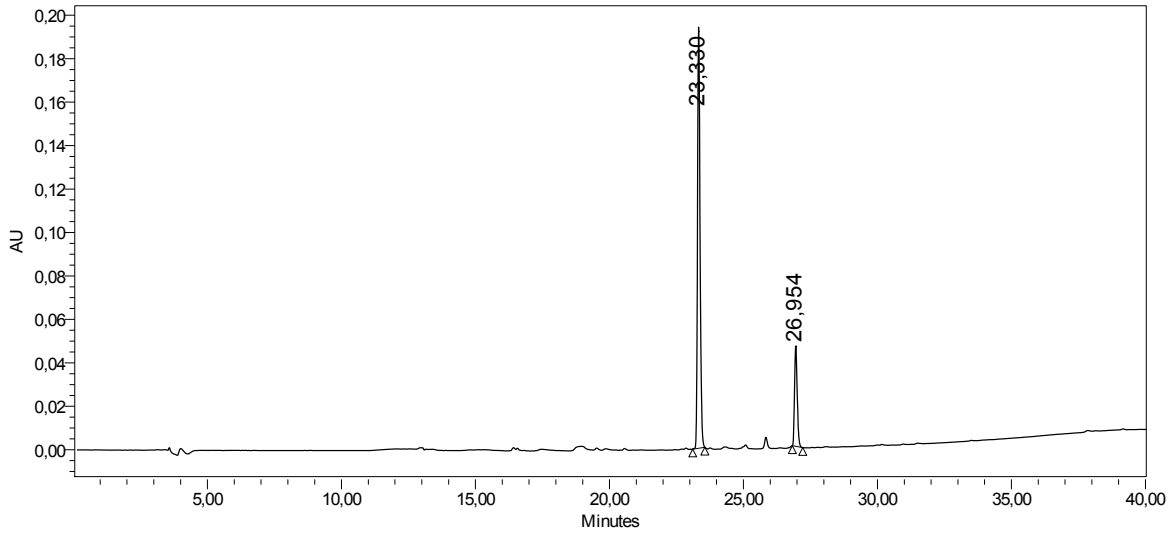


Figure A-24: MS analysis of compound **12**

SAMPLE INFORMATION

Sample Name:	FC131Pip_crude	Sample Set Name:	Oystein_6
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	65	Processing Method	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	23,330	1266030	79,97	192972
2	26,954	317076	20,03	46396

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Reported by: Øystein Eriksen
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Report Method ID 6746
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Project Name: Farmasi - MBSs gruppe
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20.05.2008
13:49:49 Europe/Oslo

Figure A-25: HPLC analysis of compound **13**

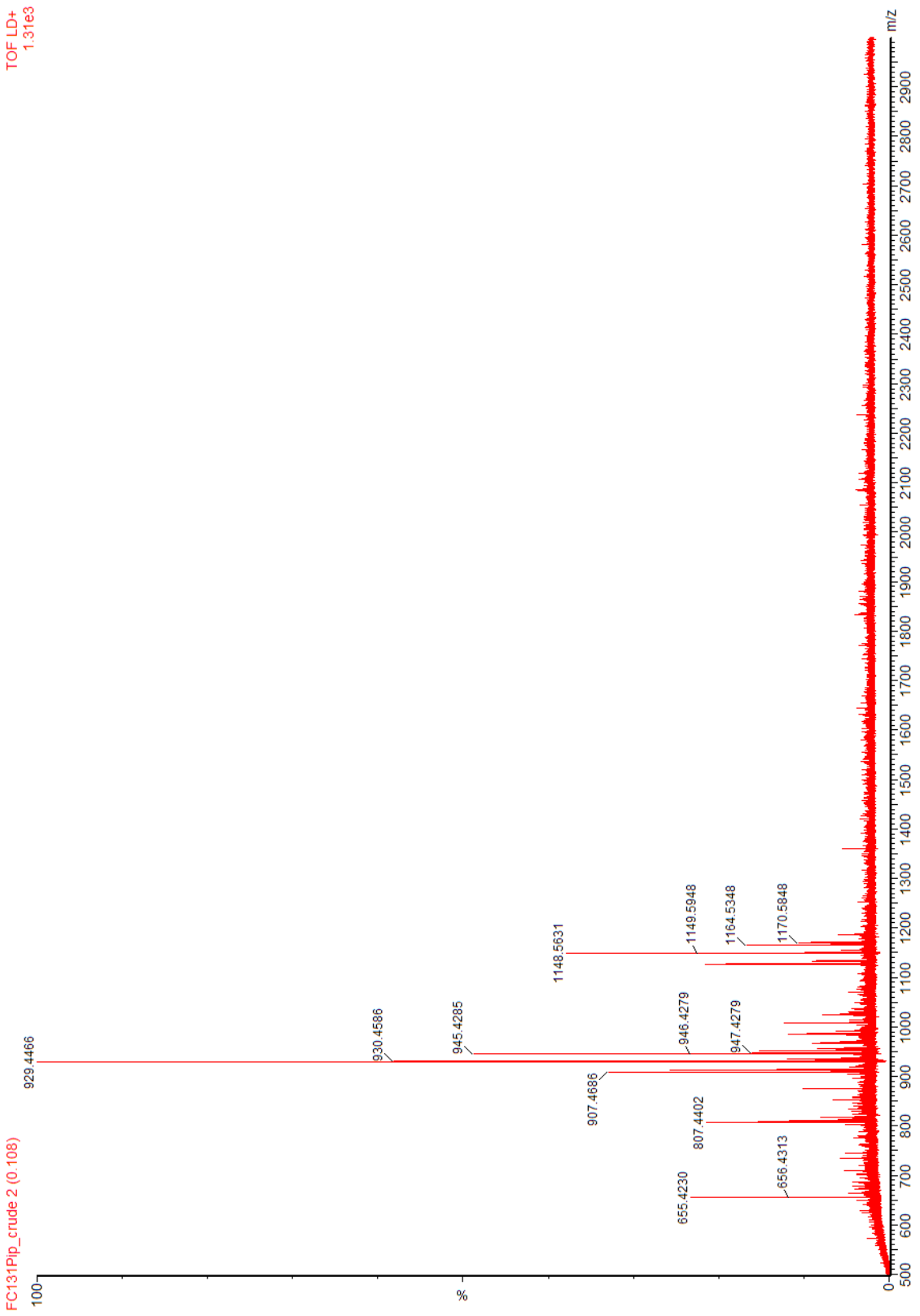
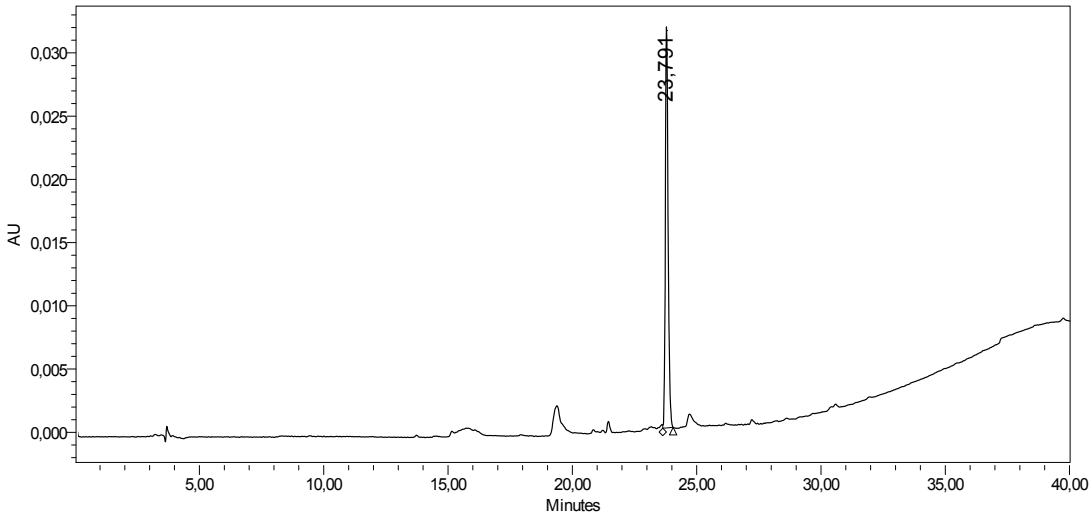


Figure A-26: MS analysis of compound **13**

SAMPLE INFORMATION

Sample Name:	FC131Cit2_crude		
Sample Type:	Unknown	Sample Set Name:	Oystein8
Vial:	63	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	23,791	243493	100,00	31709

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Reported by: Øystein Eriksen
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Report Method ID 6746
Page: 1 of 1

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20.05.2008
13:53:54 Europe/Oslo

Figure A-27: HPLC analysis of compound **14**

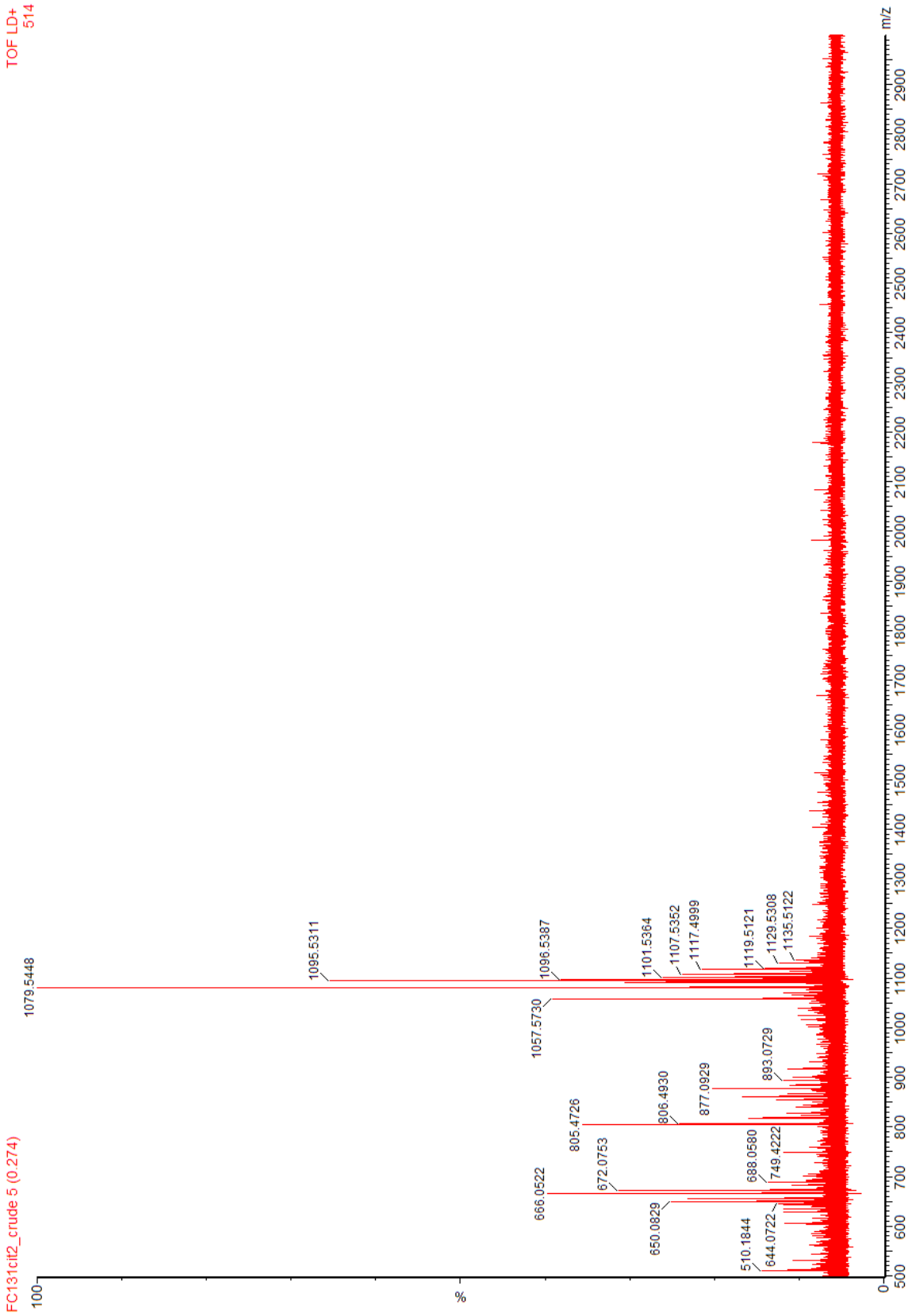
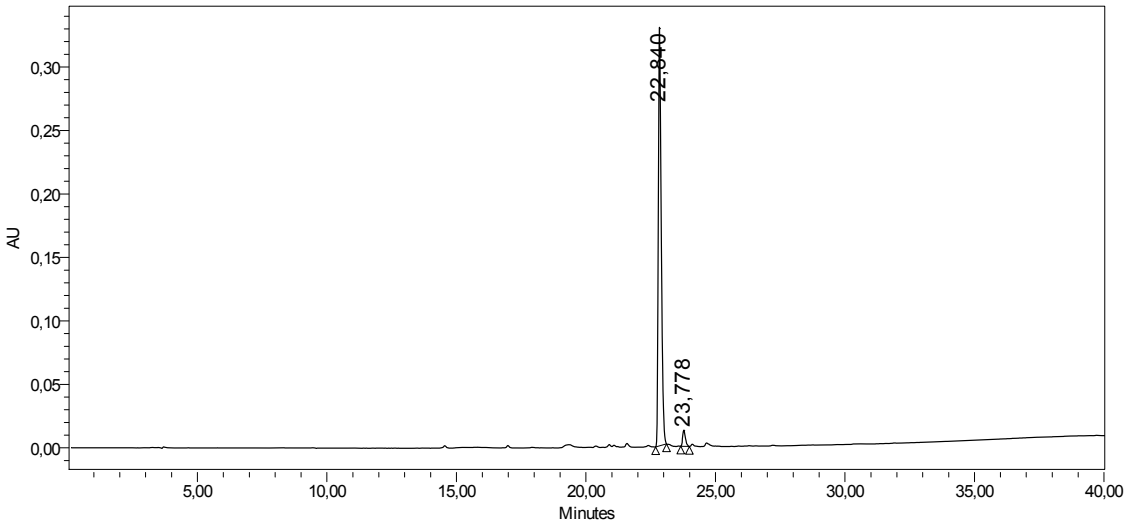


Figure A-28: MS analysis of compound **14**

SAMPLE INFORMATION

Sample Name:	FC131Cit3_crude		
Sample Type:	Unknown	Sample Set Name:	Oystein8
Vial:	61	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	22,840	2604516	96,53	328934
2	23,778	93630	3,47	12628

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-29: HPLC analysis of compound **15**

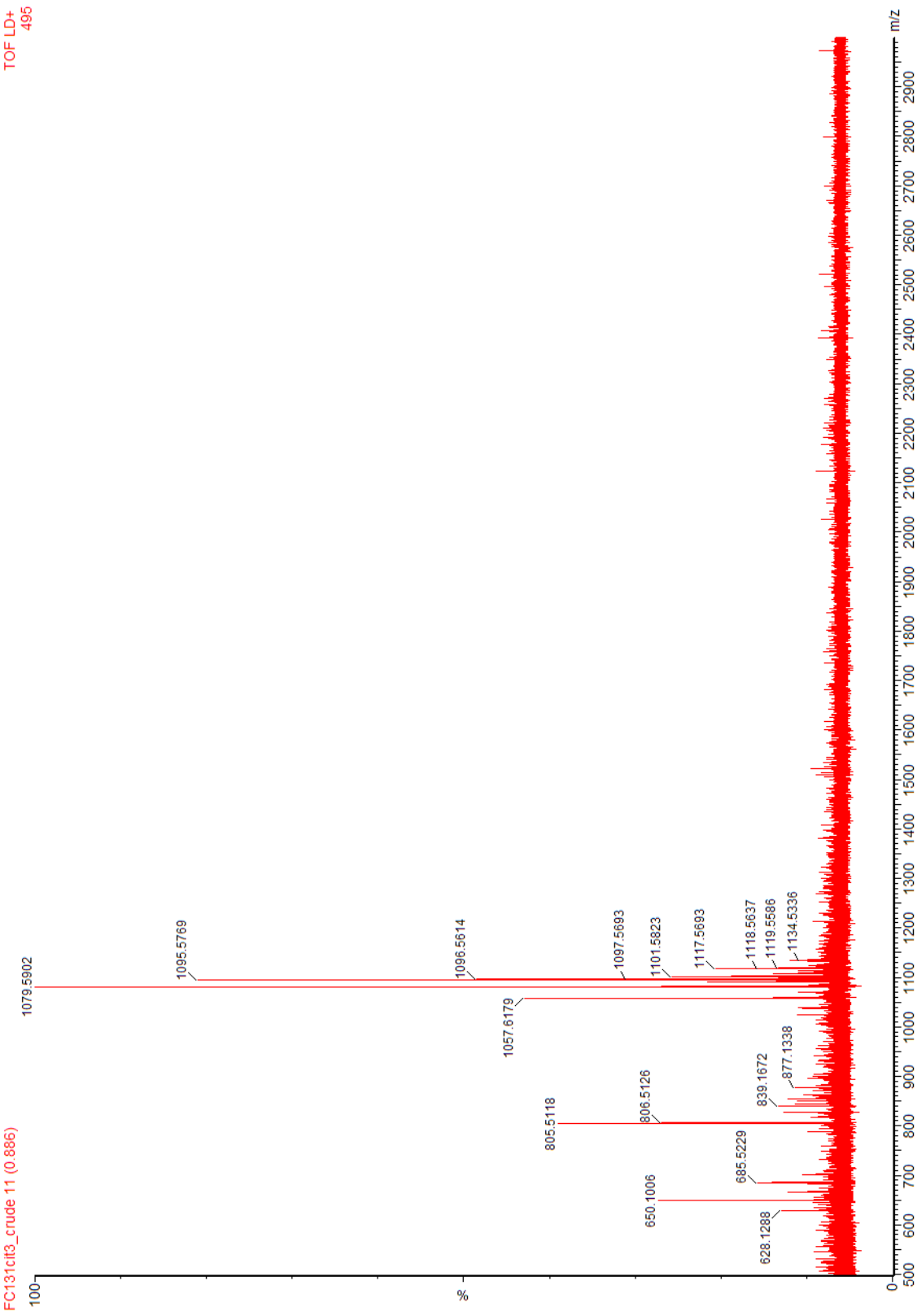
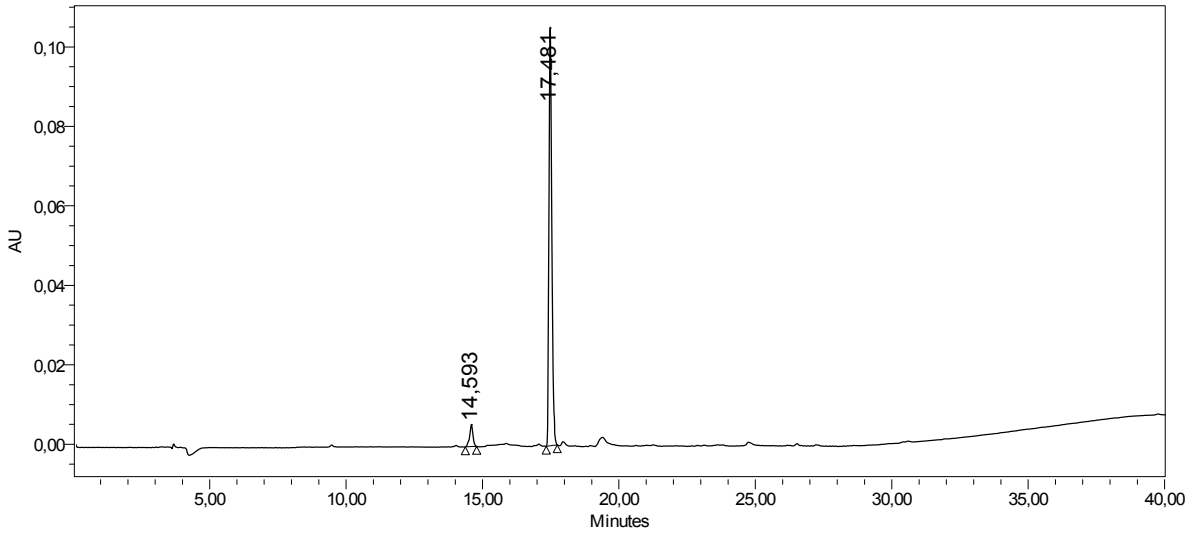


Figure A-30: MS analysis of compound **15**

SAMPLE INFORMATION

Sample Name:	FC131CitCit_crude	Sample Set Name:	Oystein8
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	68	Processing Method	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	14,593	47277	5,82	5559
2	17,481	764439	94,18	105699

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-31: HPLC analysis of compound **16**

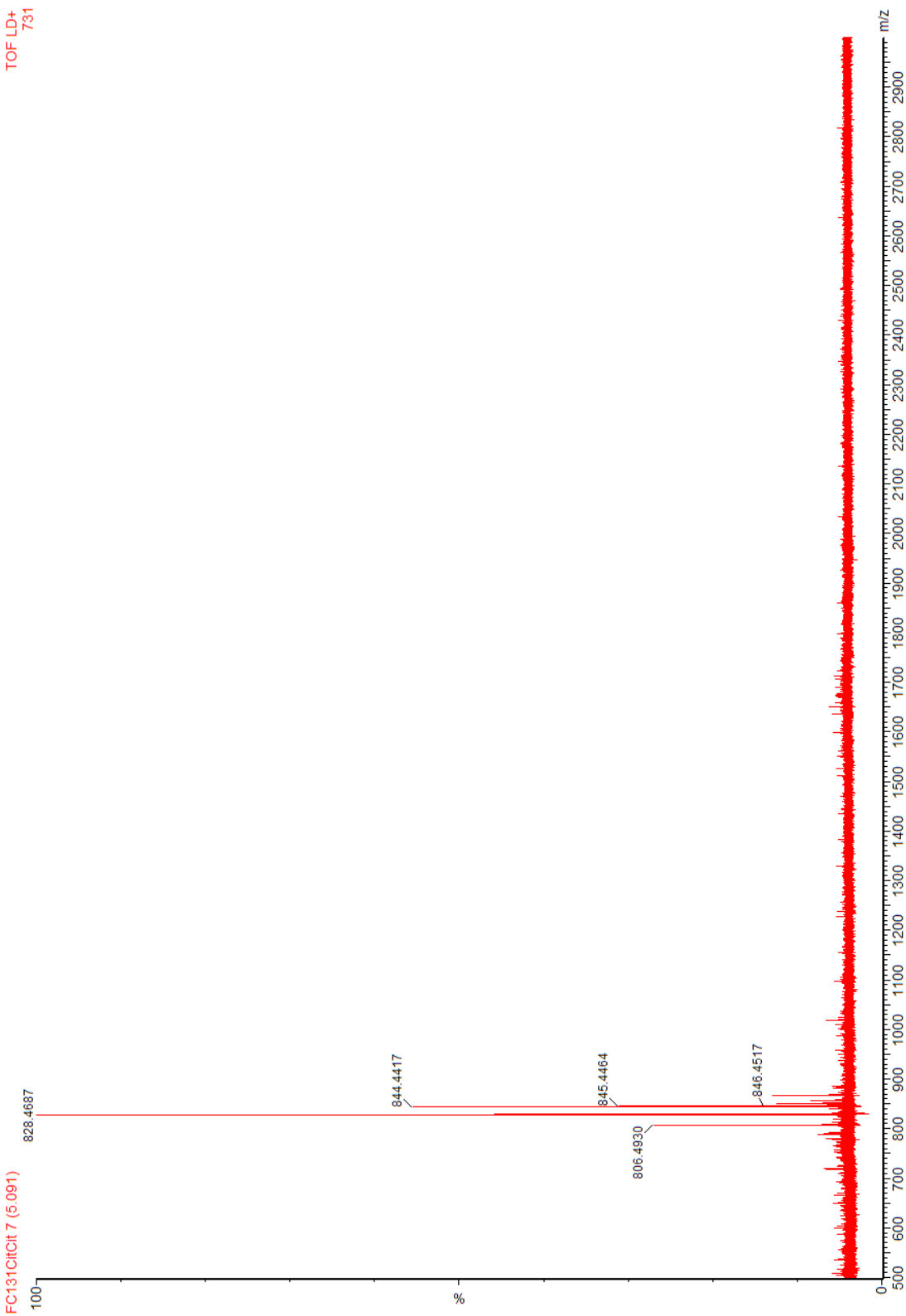
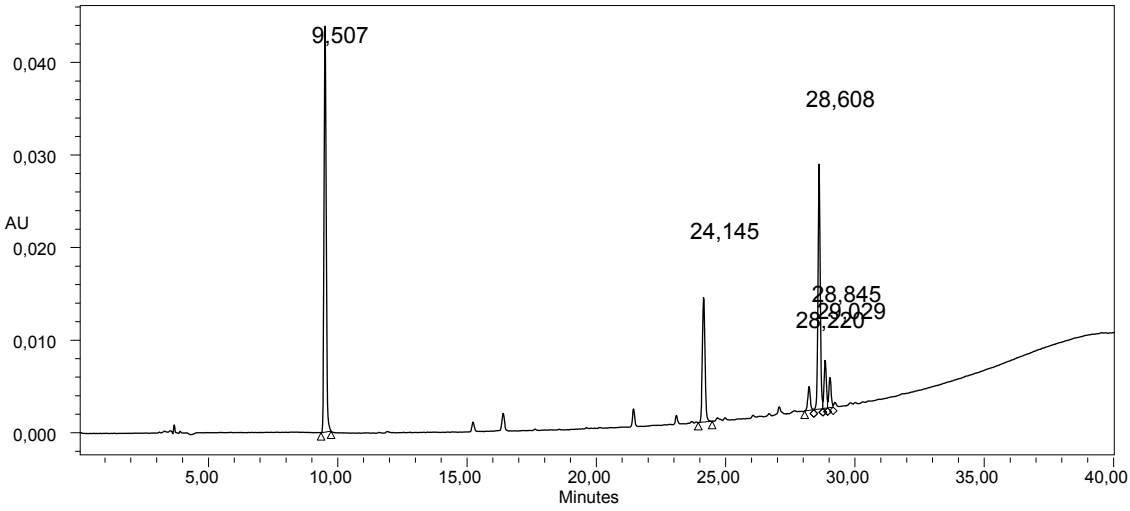


Figure A-32: MS analysis of compound **16**

SAMPLE INFORMATION

Sample Name:	FC131	Sample Set Name:	Oystein_FC131
Sample Type:	Unknown	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Vial:	68	Processing Method:	TH Default
Injection #:	1	Channel Name:	PDA Single wavelength 254
Injection Volume:	10,00 ul	Proc. Chnl. Descr.:	PDA 254,0 nm
Run Time:	40,0 Minutes		



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	9,507	256022	44,09	43484
2	24,145	95623	16,47	13445
3	28,220	16968	2,92	2585
4	28,608	161087	27,74	26279
5	28,845	30850	5,31	5172
6	29,029	20109	3,46	3258

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Page: 1 of 1

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13:42:35 Europe/Oslo

Figure A-33: HPLC analysis of protected **1**

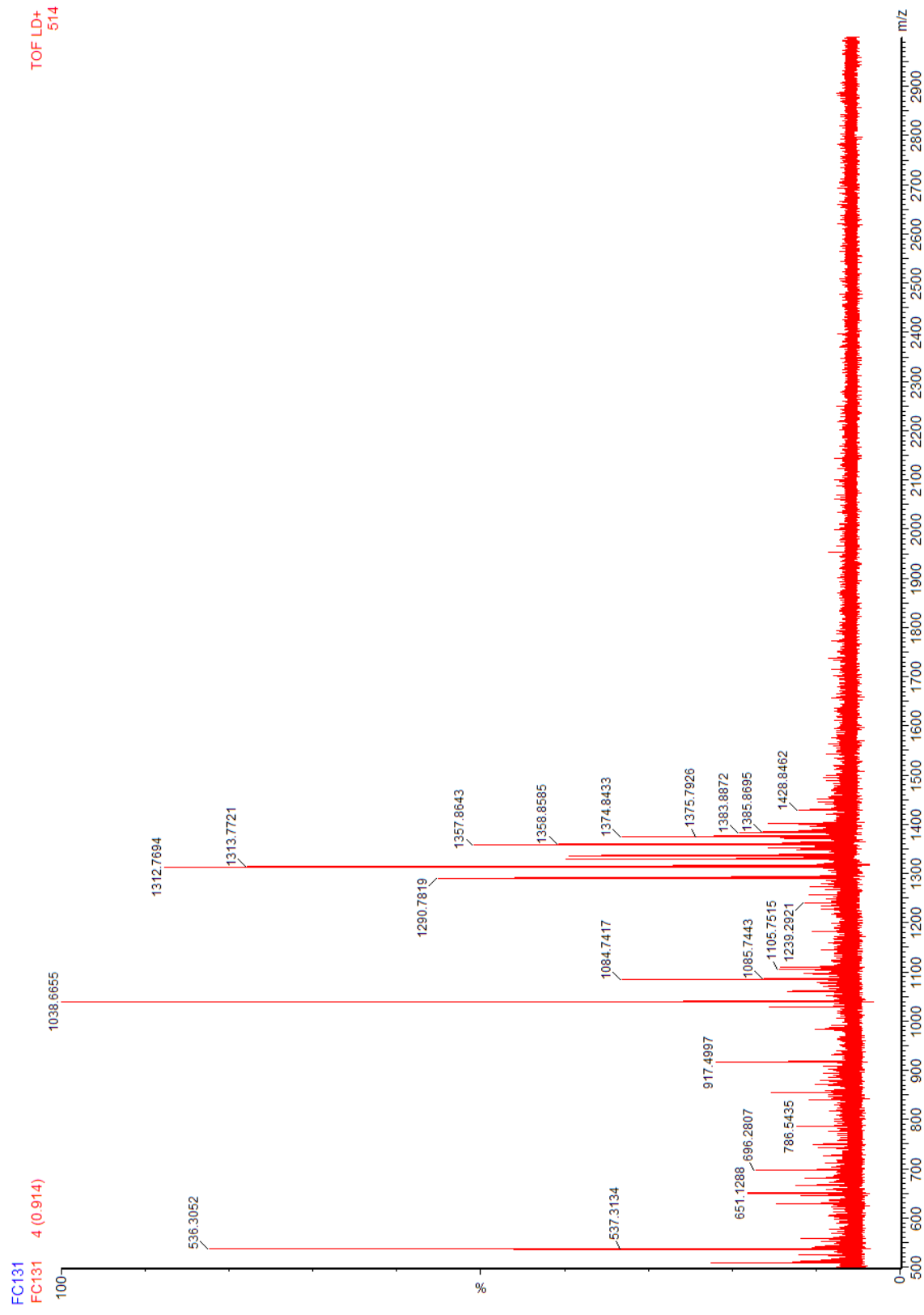
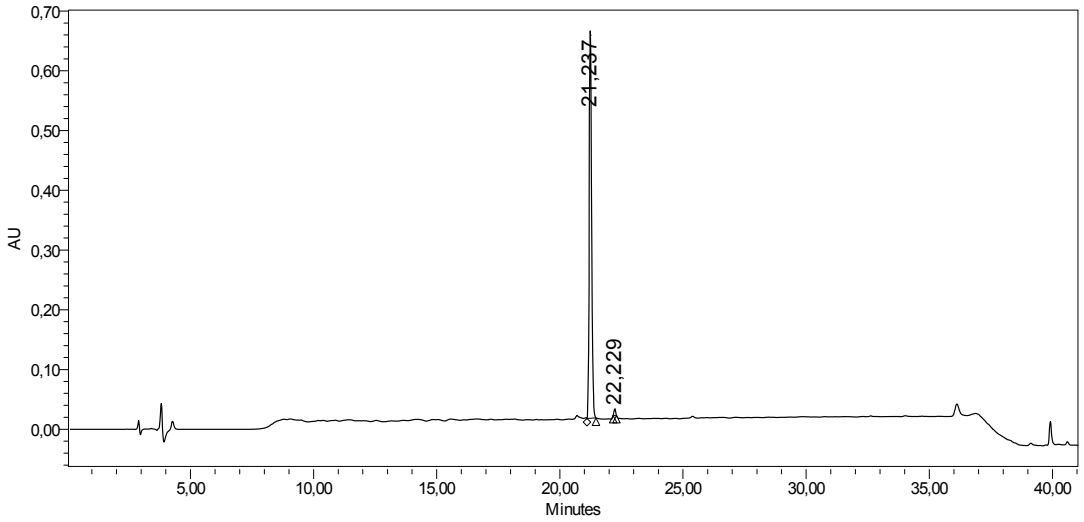


Figure A-34: MS analysis of protected **1**.

SAMPLE INFORMATION

Sample Name:	ØE036a_FC131_Final		
Sample Type:	Unknown	Sample Set Name:	ØE036a_FC131_Final
Vial:	68	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,237	4365775	98,88	647046

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Figure A-35: HPLC analysis of purified compound **1**

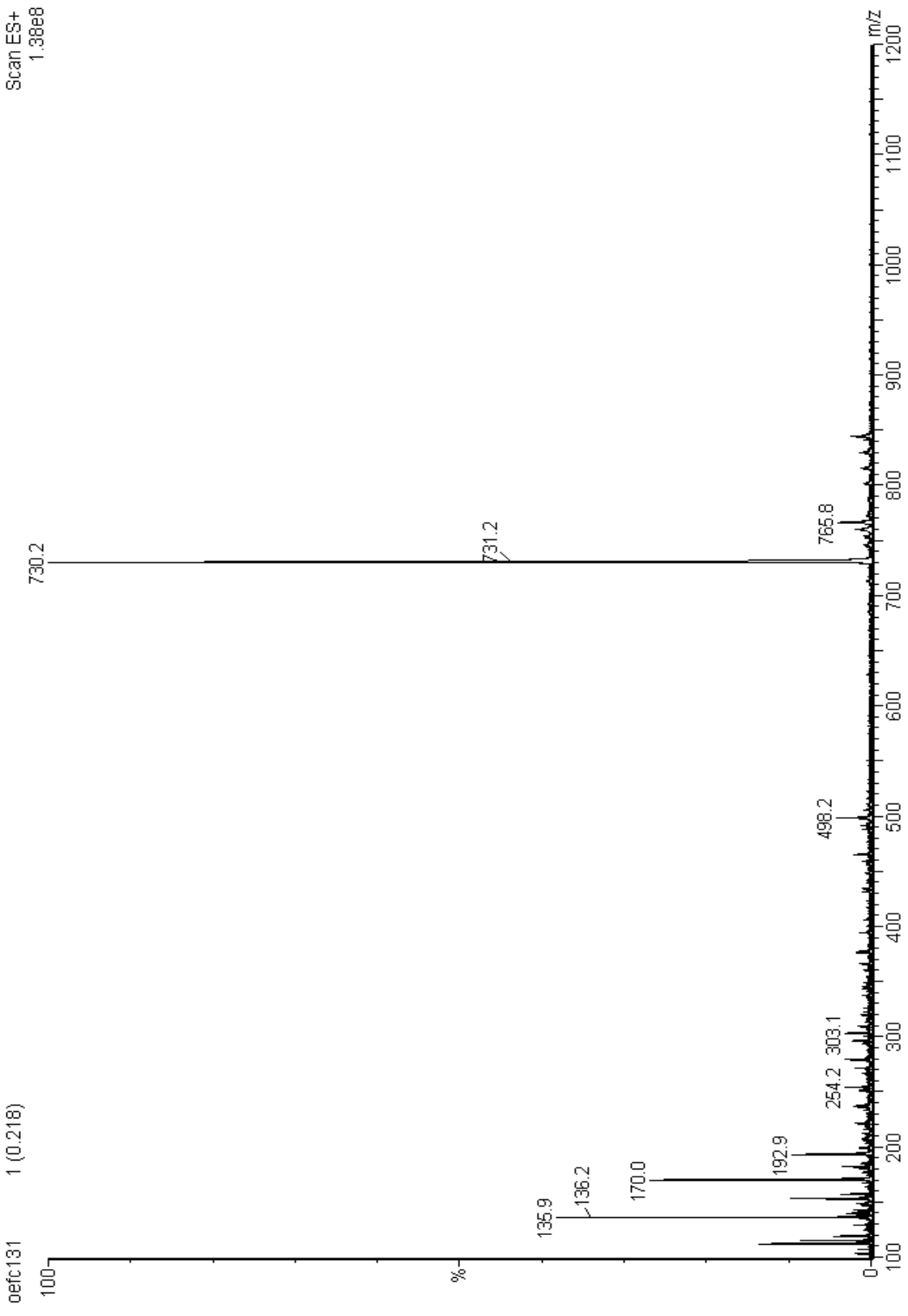


Figure A-36: MS analysis of purified compound **1**

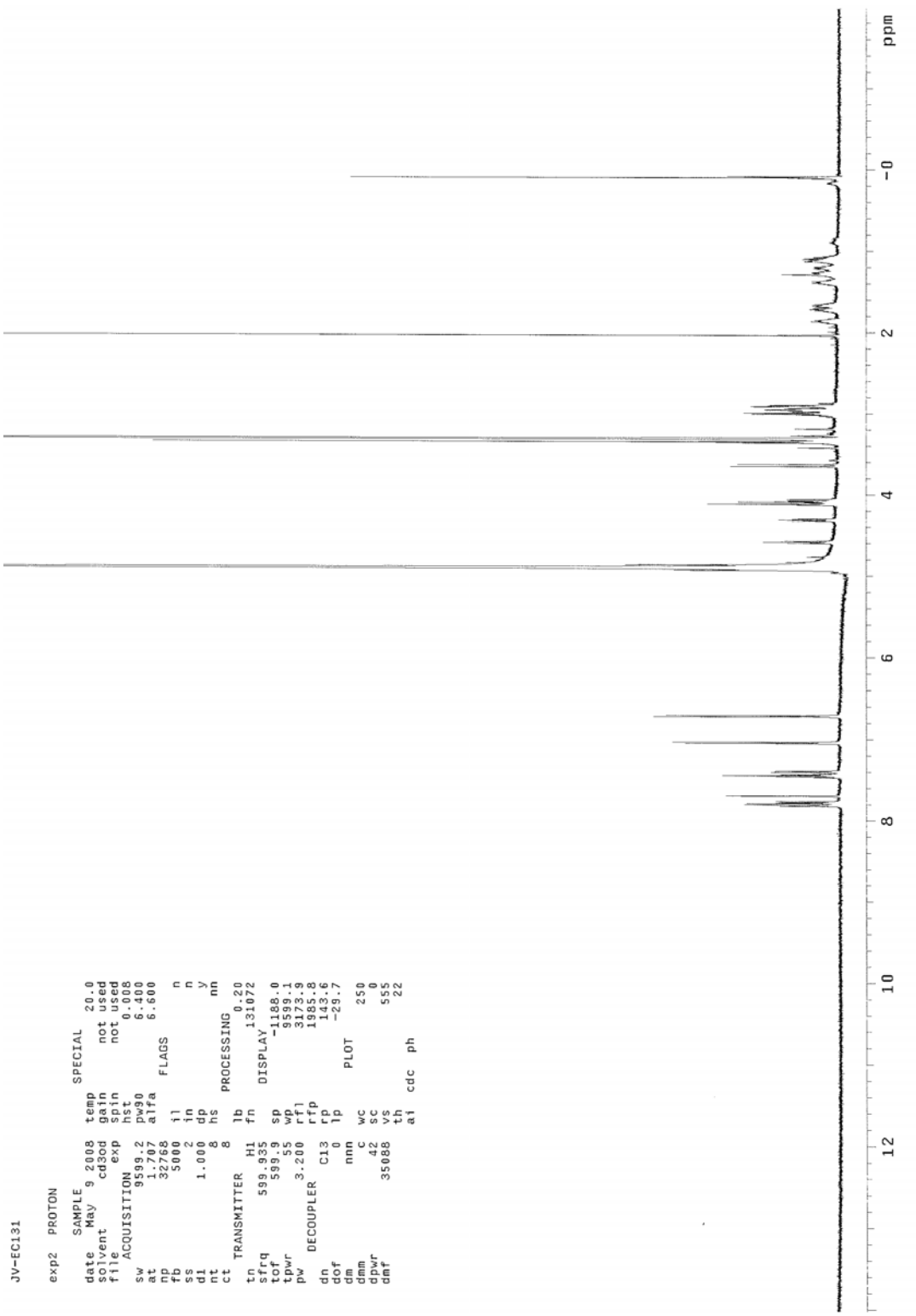
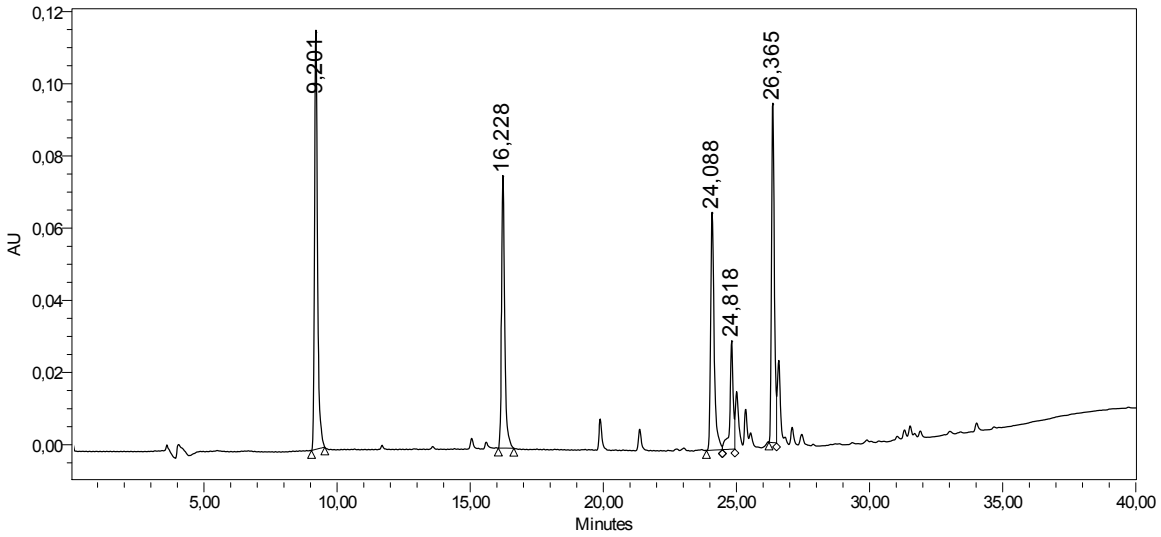


Figure A-37: NMR of compound **1**

SAMPLE INFORMATION

Sample Name:	FC131Ala_cyclic3		
Sample Type:	Unknown	Sample Set Name:	Oystein_12
Vial:	62	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	9,201	894839	28,99	115858
2	16,228	641109	20,77	76103
3	24,088	600216	19,44	65782
4	24,818	261886	8,48	29923
5	26,365	688982	22,32	94257

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-38: HPLC analysis of protected **2**

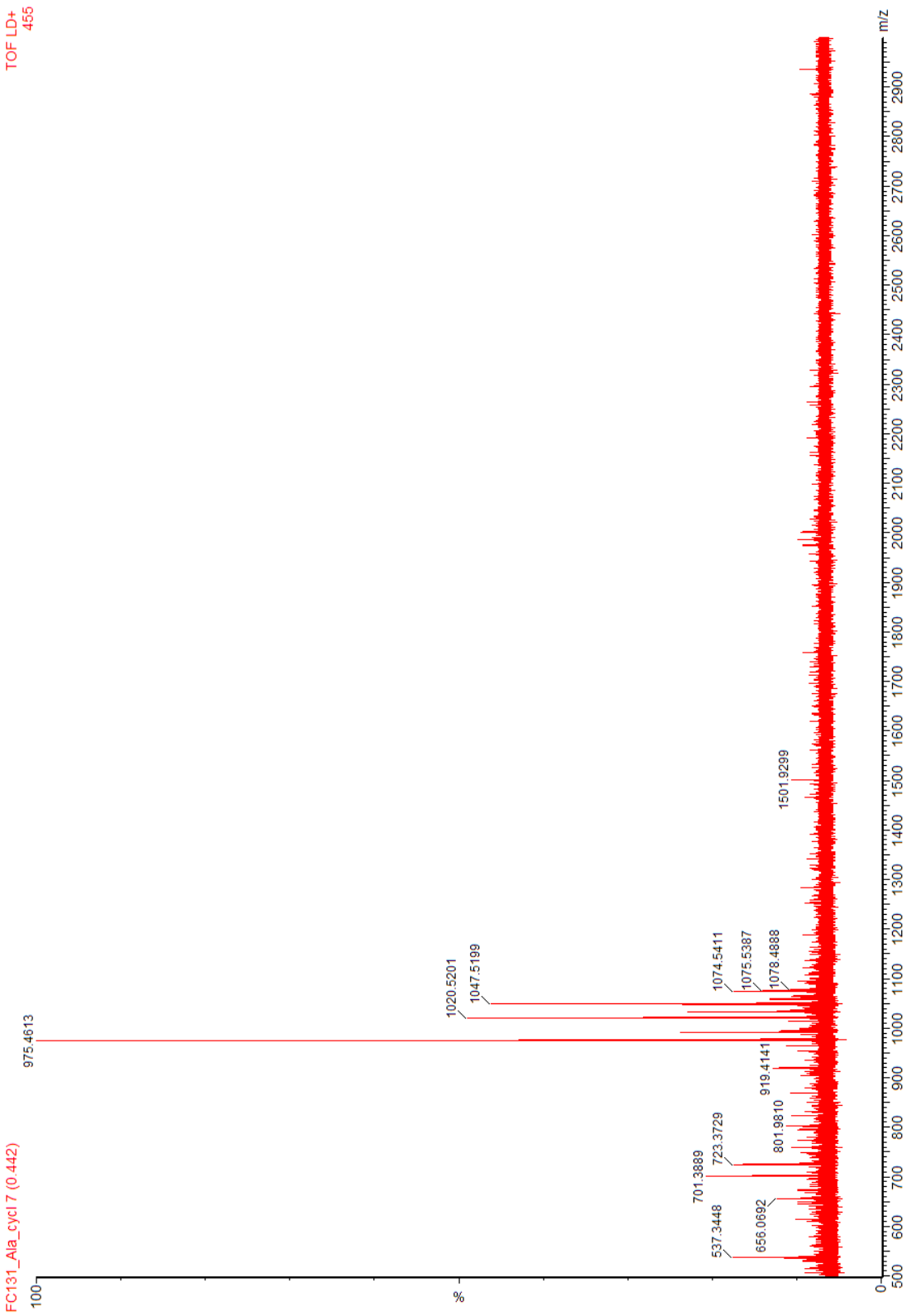
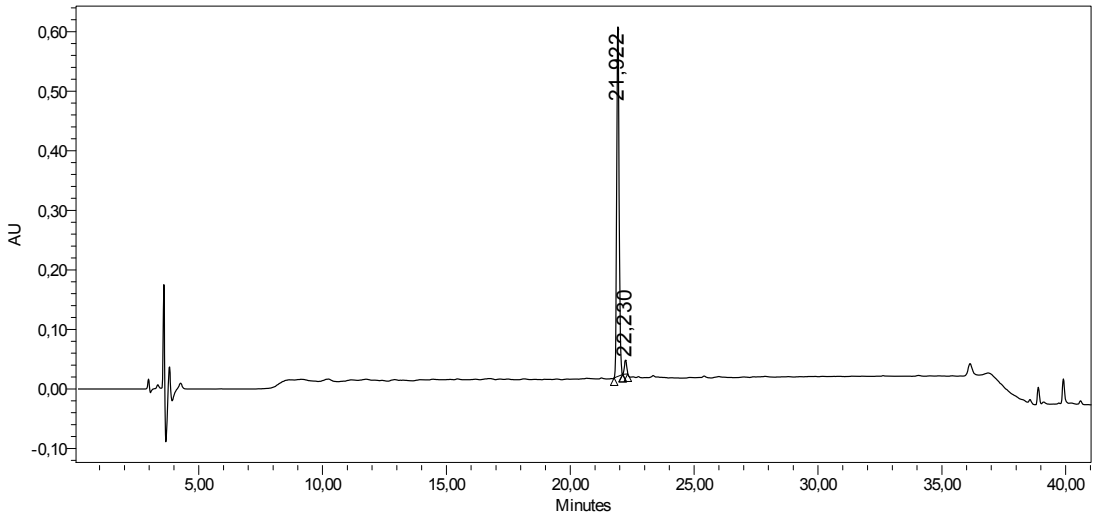


Figure A-39: MS analysis of protected **2**

SAMPLE INFORMATION

Sample Name:	ØE034b_FC131Ala3_Final		
Sample Type:	Unknown	Sample Set Name:	ØE034b_FC131Ala3_Final
Vial:	59	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,922	4004374	96,49	585529
2	22,230	145512	3,51	23521

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Reported by: Øystein Eriksen
Report Method: Øystein Eriksen
Report Method ID 6746
Page: 1 of 1

Project Name: Farmasi - MBSs gruppe
Date Printed:
20.05.2008
06:36:39 Europe/Oslo

Figure A-40: HPLC analysis of purified compound **2**

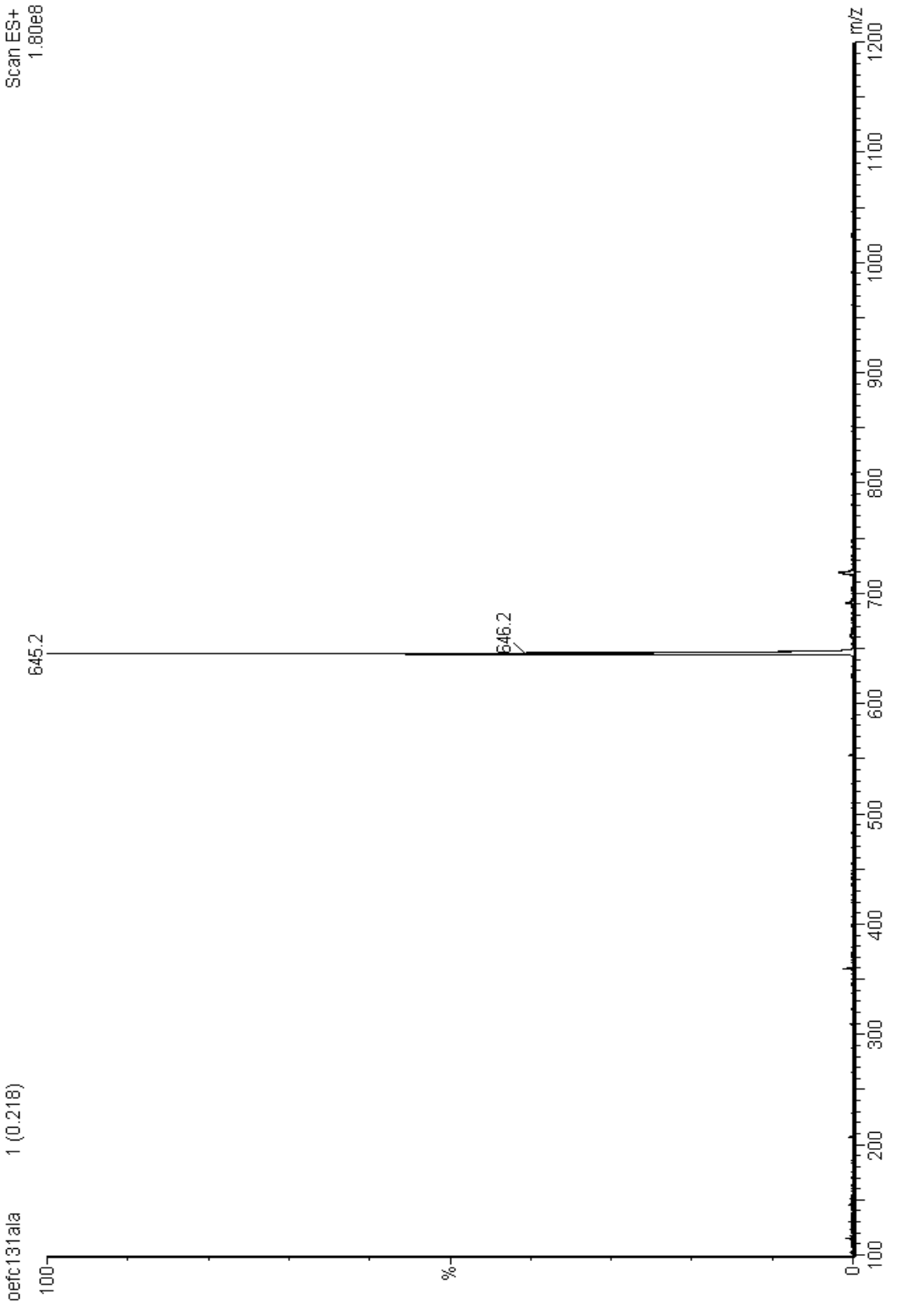


Figure A-41: MS analysis of purified compound **2**.

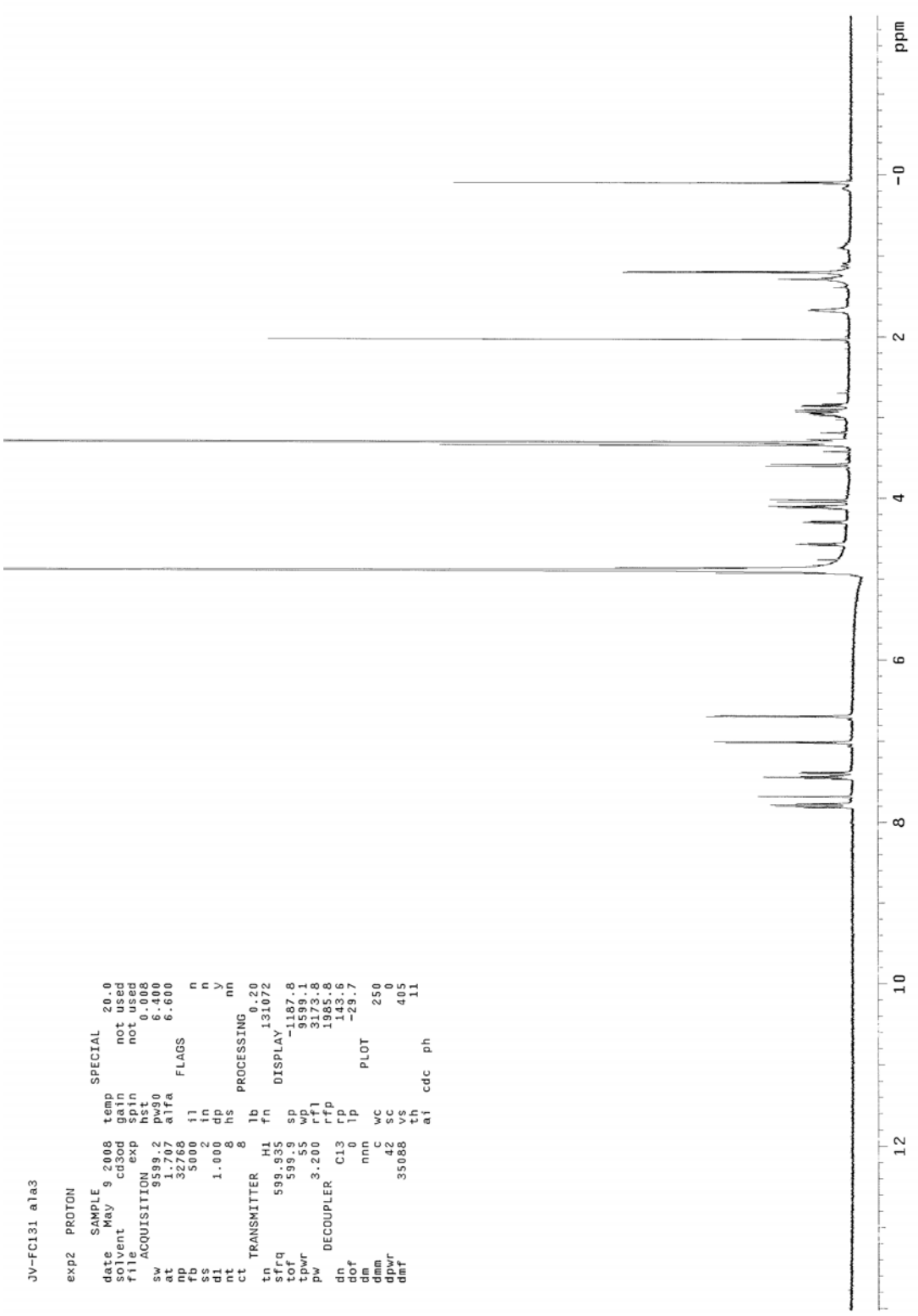
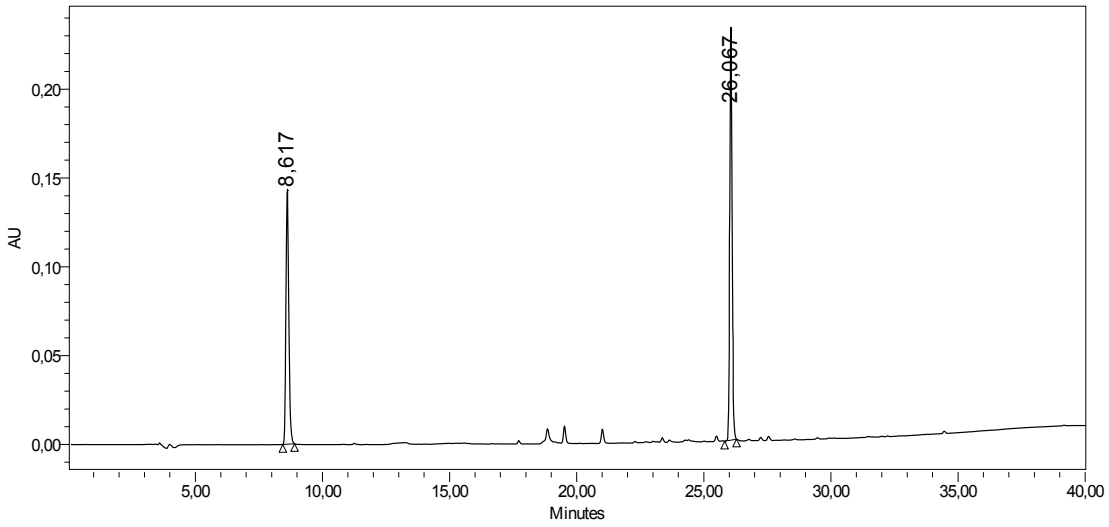


Figure A-42: NMR of compound **2**

SAMPLE INFORMATION

Sample Name:	FC131Aib005		
Sample Type:	Unknown	Sample Set Name:	Øystein_4
Vial:	63	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	8,617	1119828	42,56	143632
2	26,067	1511092	57,44	232384

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Reported by: Øystein Eriksen
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Page: 1 of 1

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Date Printed:
20.05.2008
13:47:52 Europe/Oslo

Figure A-43: HPLC of protected **3**

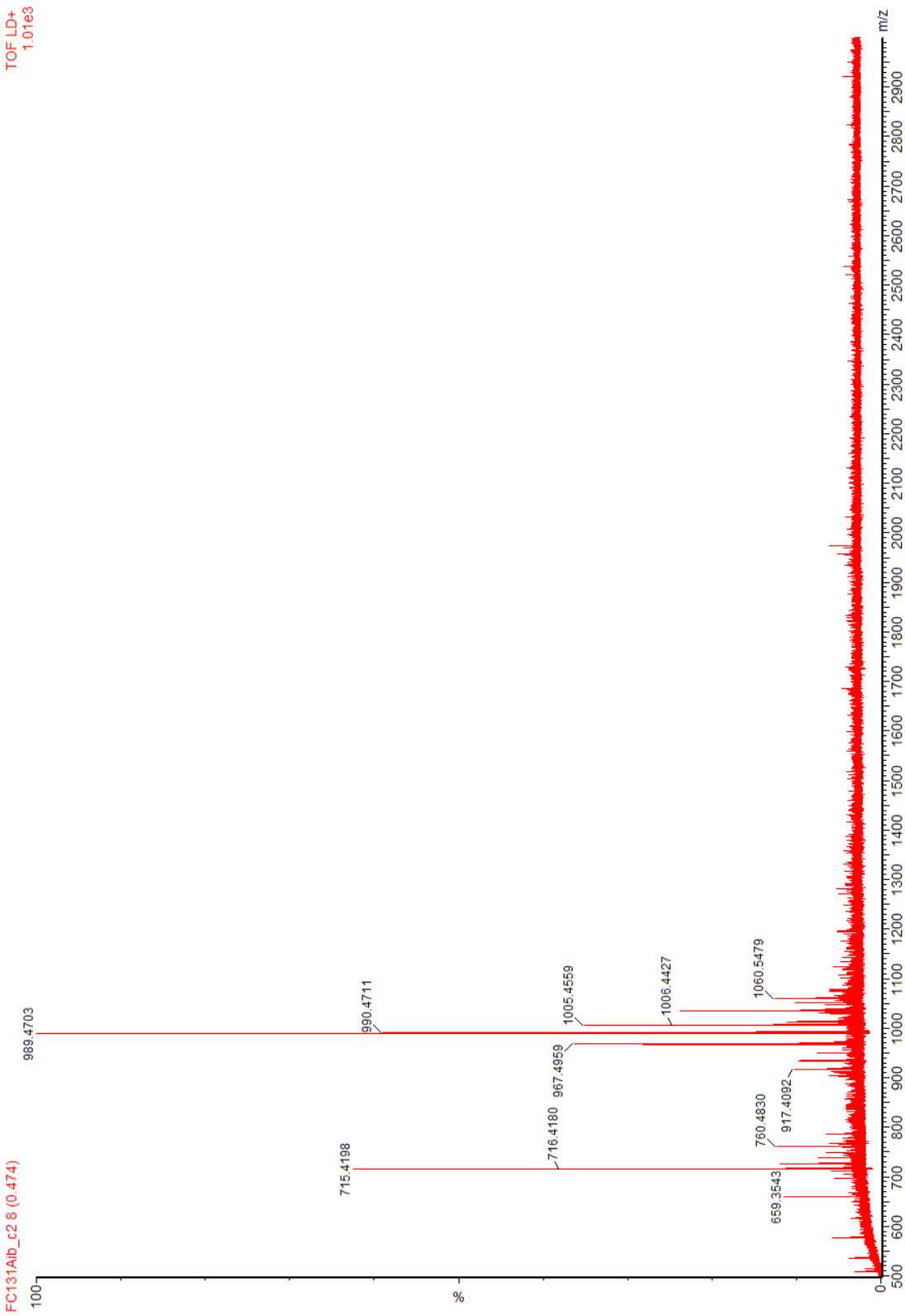
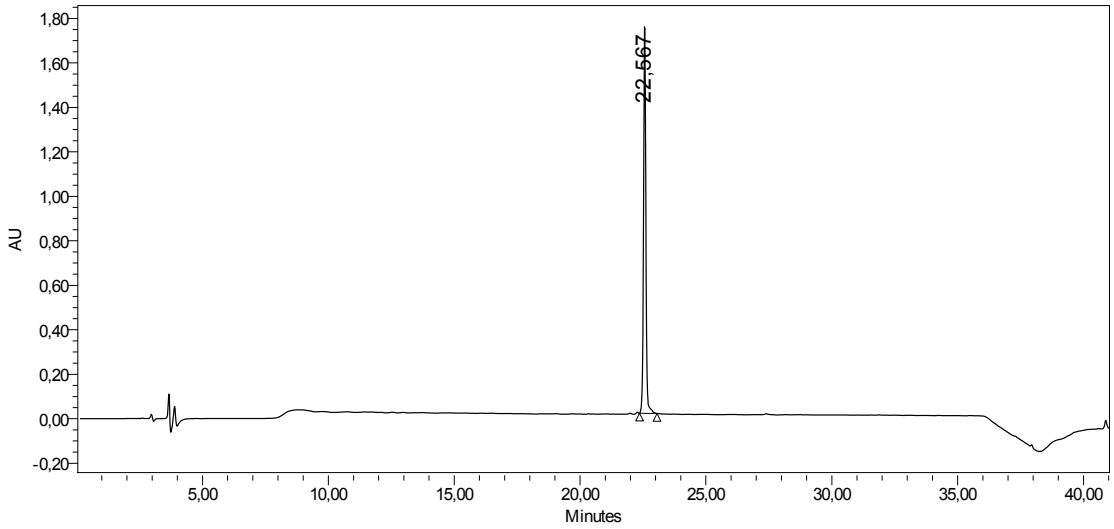


Figure A-44: MS analysis of protected **3**.

SAMPLE INFORMATION

Sample Name:	ØE035b_FC131Aib3_Final		
Sample Type:	Unknown	Sample Set Name:	ØE035b_FC131Aib3_Final
Vial:	67	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	22,567	11407097	100,00	1745534

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Figure A-45: HPLC of purified **3**

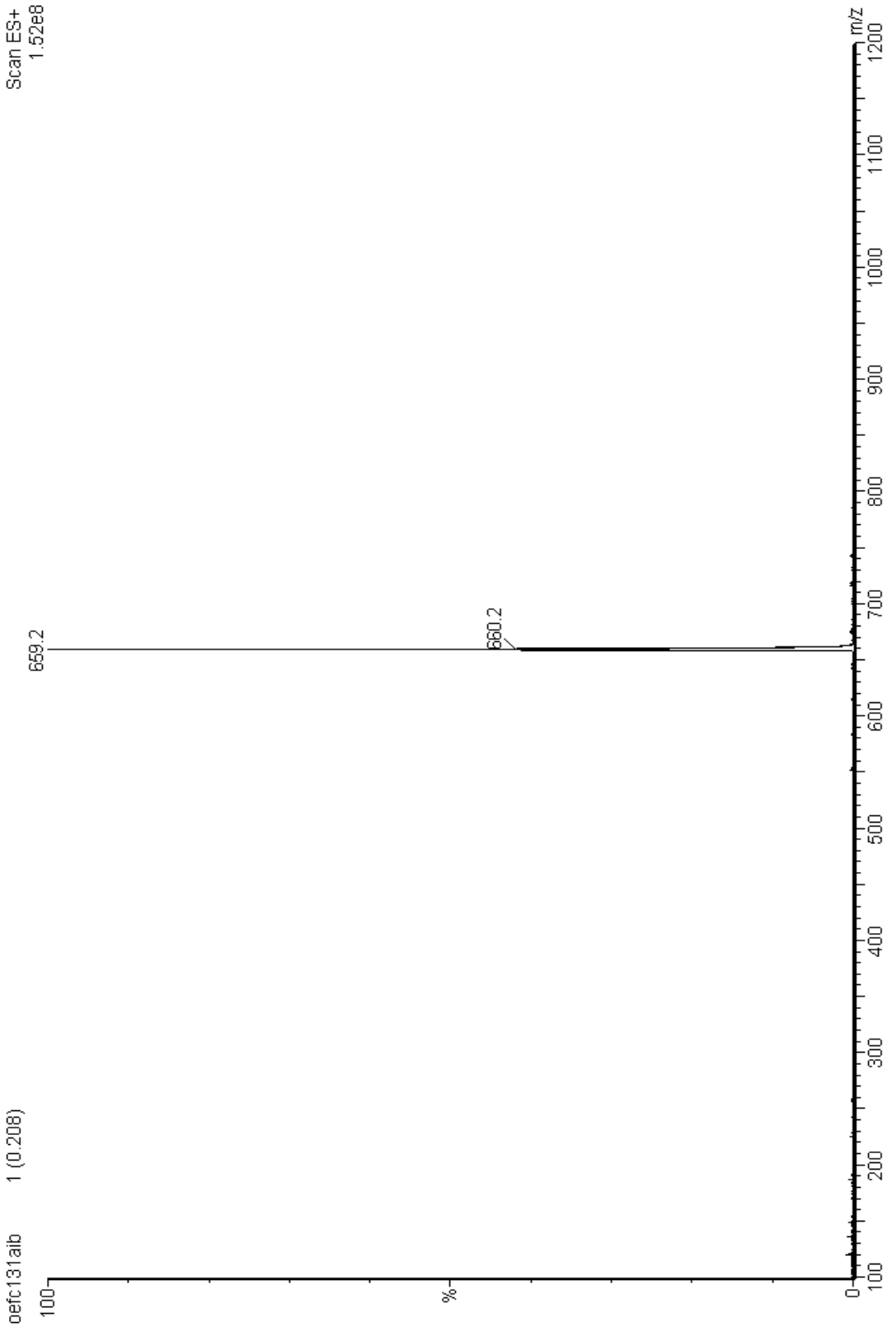


Figure A-46: MS analysis of purified **3**

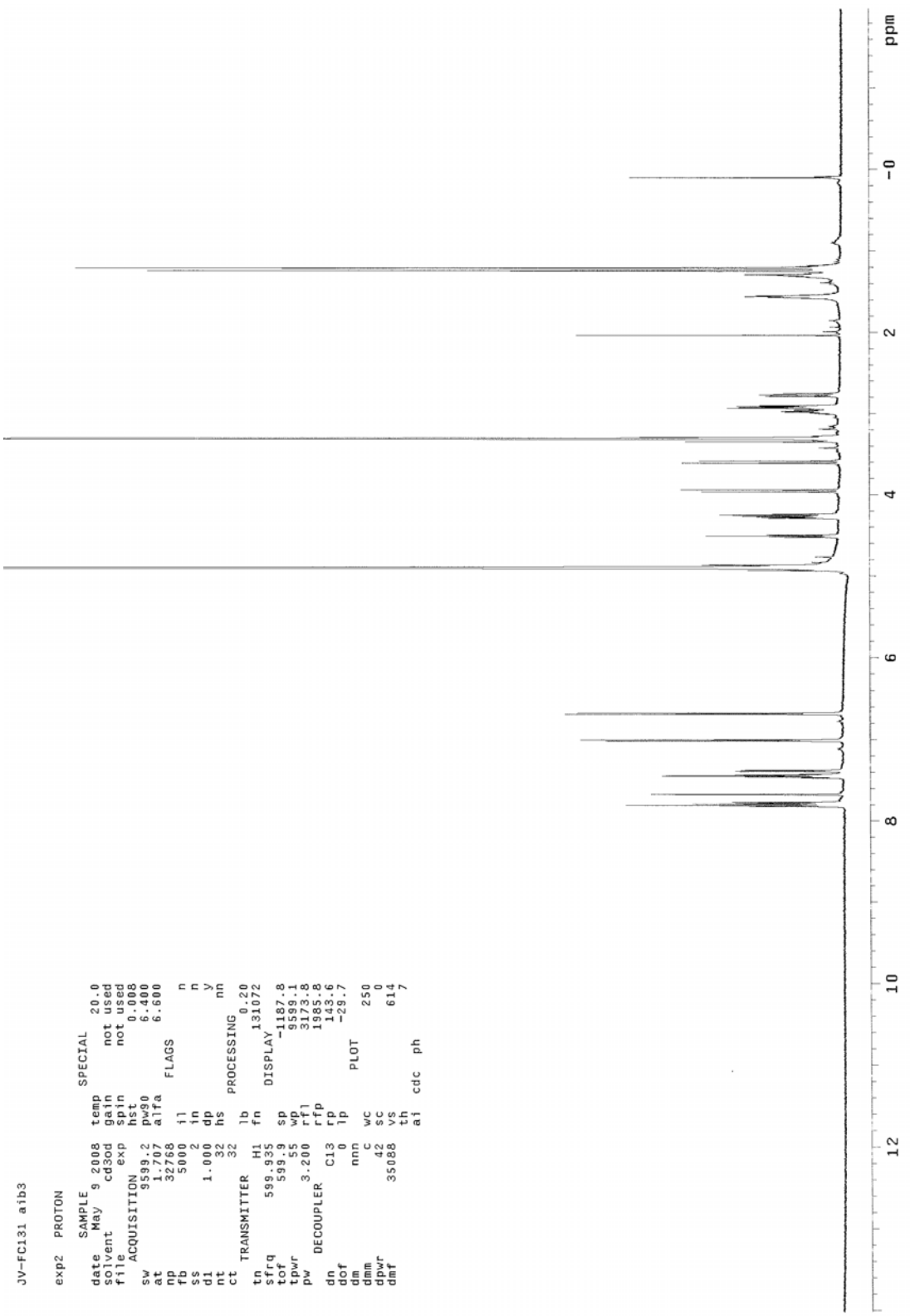
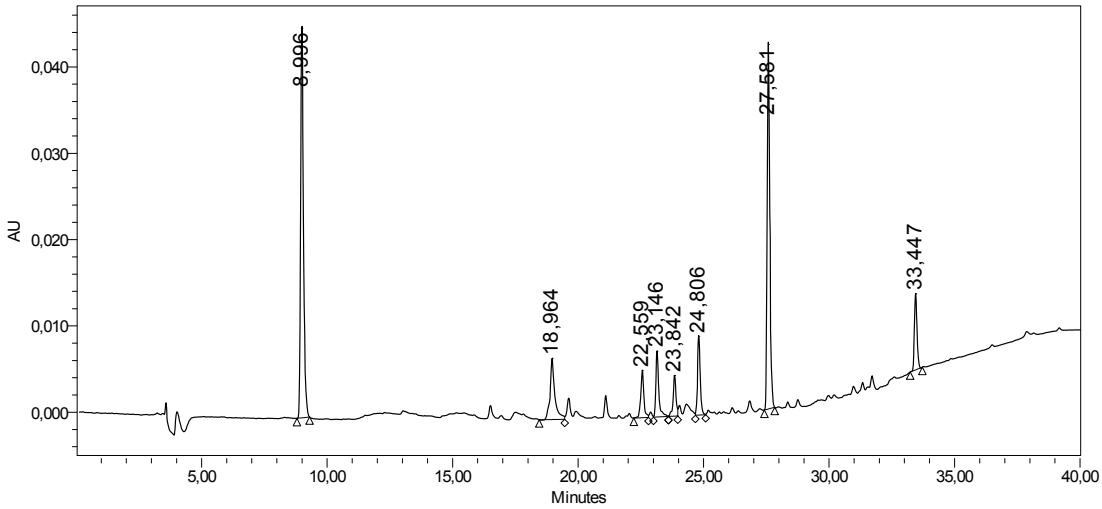


Figure A-47: NMR of compound **3**

SAMPLE INFORMATION

Sample Name:	FC131Chx_cyclic		
Sample Type:	Unknown	Sample Set Name:	Oystein_7
Vial:	64	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	8,996	359232	34,64	45419
2	18,964	92390	8,91	7112
3	22,559	46728	4,51	5521
4	23,146	60980	5,88	7656
5	23,842	37421	3,61	4760
6	24,806	67956	6,55	9204
7	27,581	308328	29,73	42443
8	33,447	64002	6,17	8858

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Reported by: Øystein Eriksen	Project Name: Farmasi - MBSs gruppe
Report Method: Øystein Eriksen	Date Printed:
Report Method ID 6746	20.05.2008
Page: 1 of 1	13:50:12 Europe/Oslo

Figure A-48 HPLC analysis of protected **4**

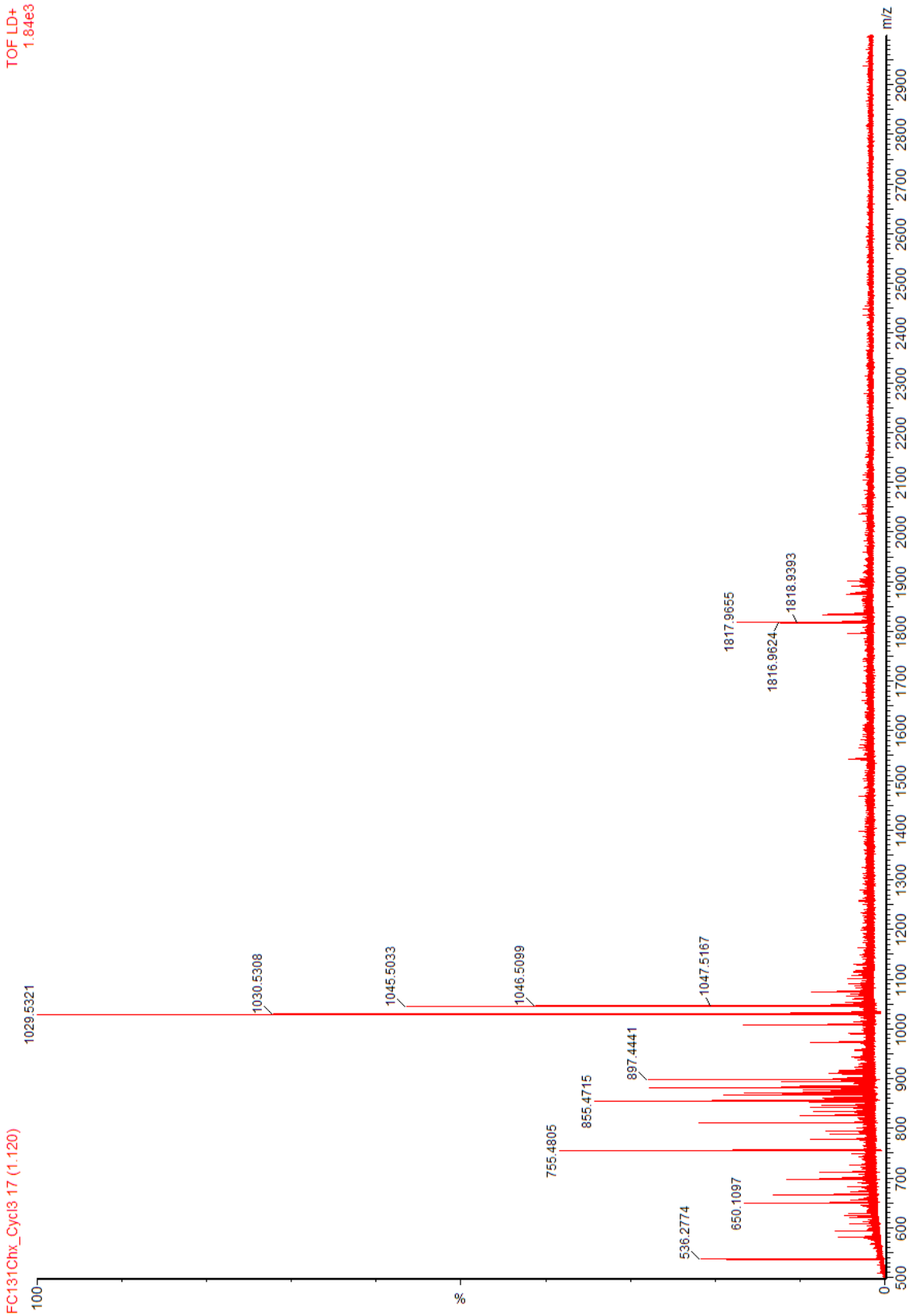


Figure A-49:MS analysis of protected **4**

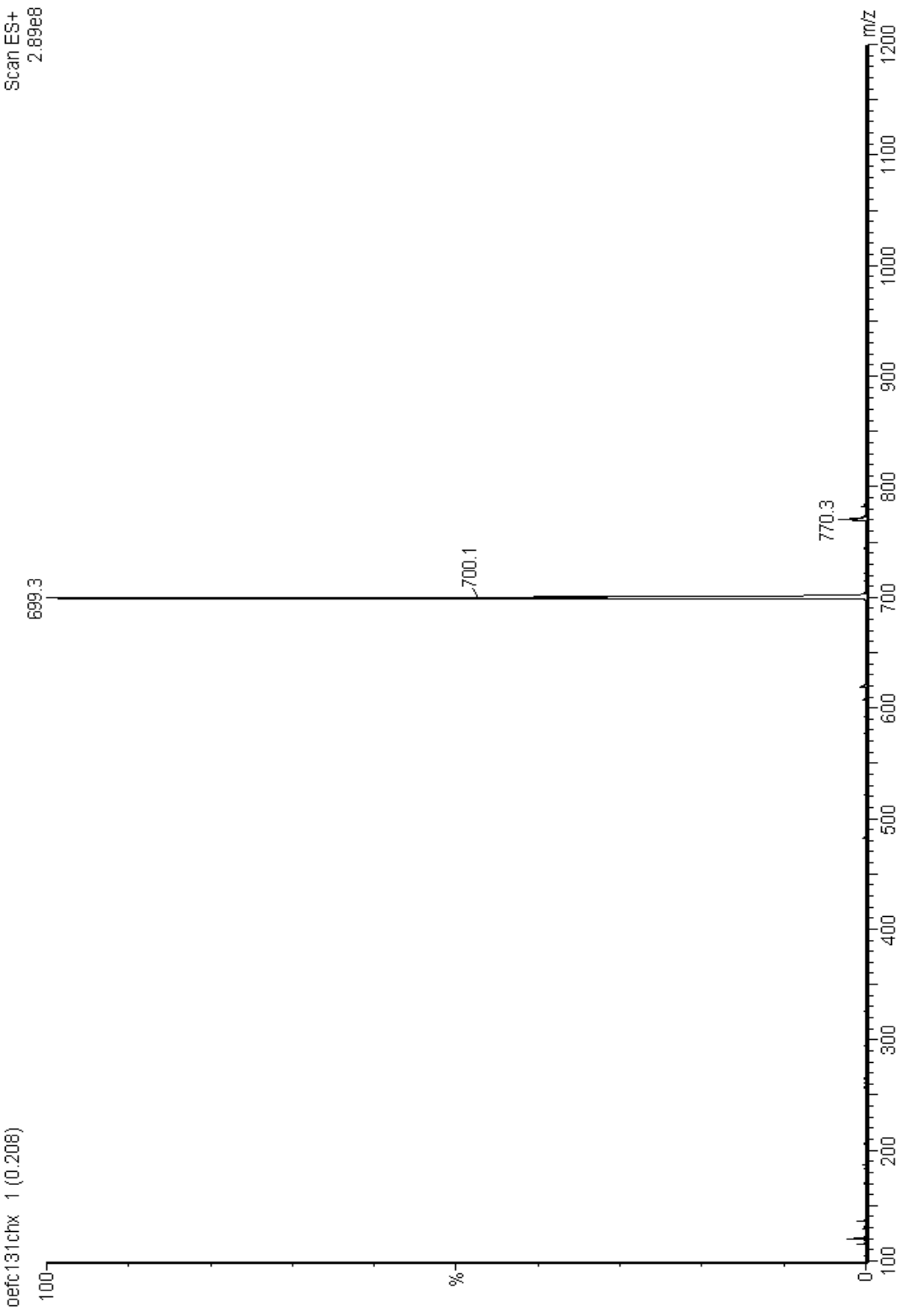
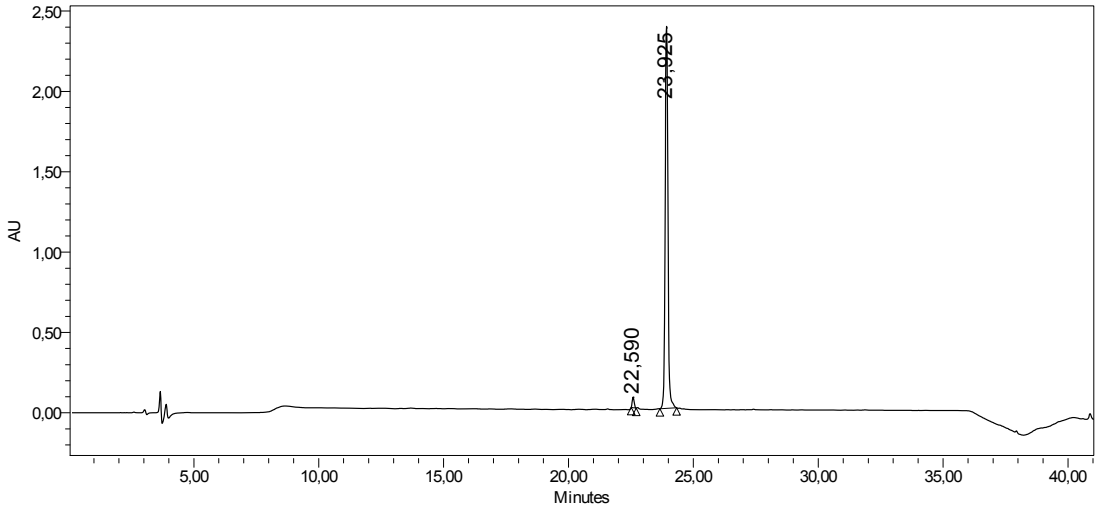


Figure A-50: MS analysis of purified **4**

SAMPLE INFORMATION

Sample Name:	ØE033_FC131Chx3_Final		
Sample Type:	Unknown	Sample Set Name:	ØE033_FC131Chx3_Final
Vial:	63	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	23,925	18536109	98,12	2384929

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Figure A-51 HPLC analysis of purified **4**

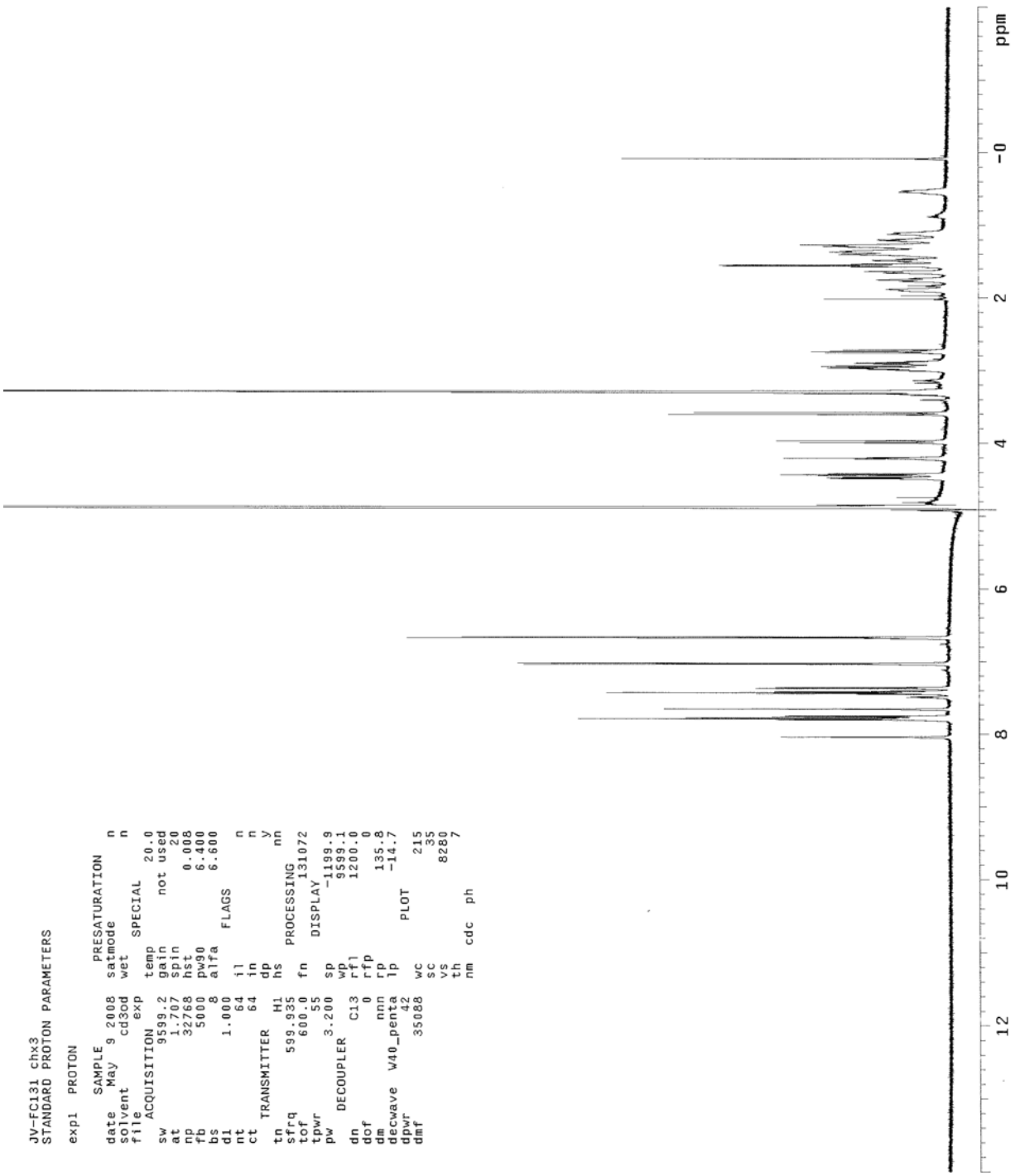
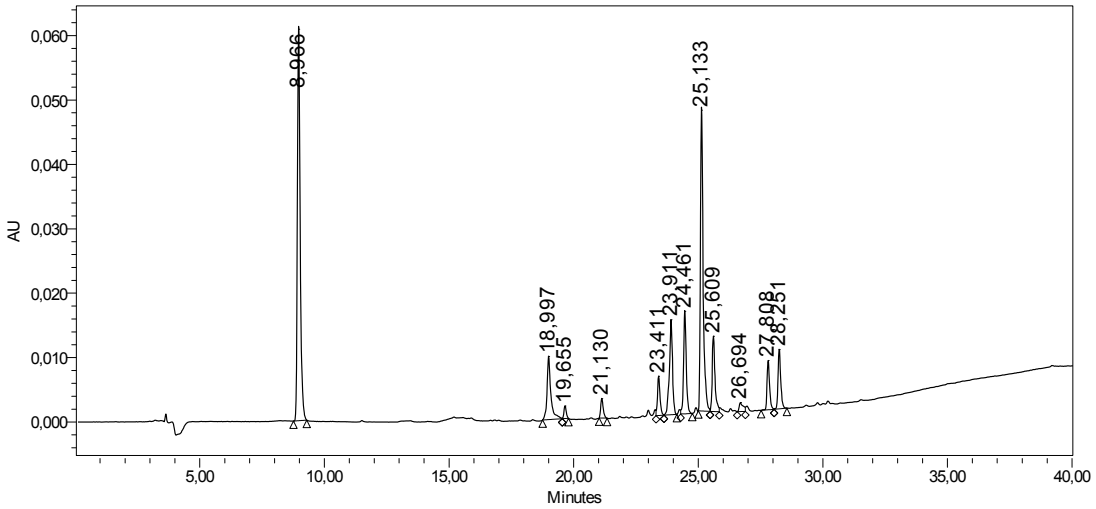


Figure A-52: NMR of compound **4**

SAMPLE INFORMATION

Sample Name:	FC131Pip_cyclic		
Sample Type:	Unknown	Sample Set Name:	Oystein_7
Vial:	66	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	8,966	479949	32,20	61419
2	18,997	103960	6,98	9835
3	23,411	42981	2,88	6076
4	23,911	126734	8,50	14680
5	24,461	118924	7,98	15929
6	25,133	354085	23,76	47297
7	25,609	89996	6,04	11643
8	27,808	54407	3,65	7657
9	28,251	69975	4,70	9319

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Reported by: Øystein Eriksen
Report Method: Øystein Eriksen
Report Method ID 6746
Page: 1 of 1

Project Name: Farmasi - MBSs gruppe
Date Printed:
20.05.2008
13:51:14 Europe/Oslo

Figure A-53: HPLC analysis of protected **5**

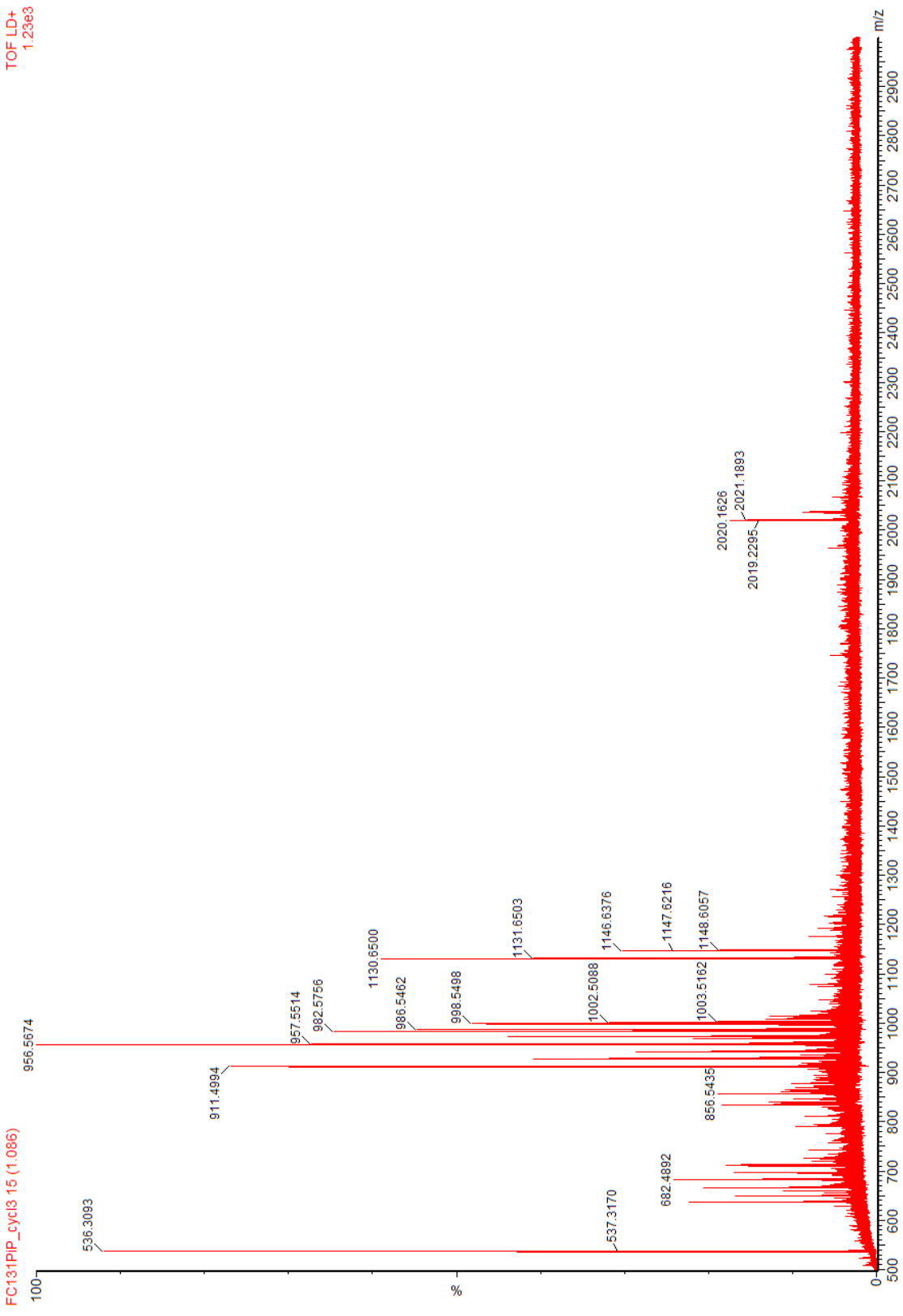


Figure A-54: MS analysis of protected **5**

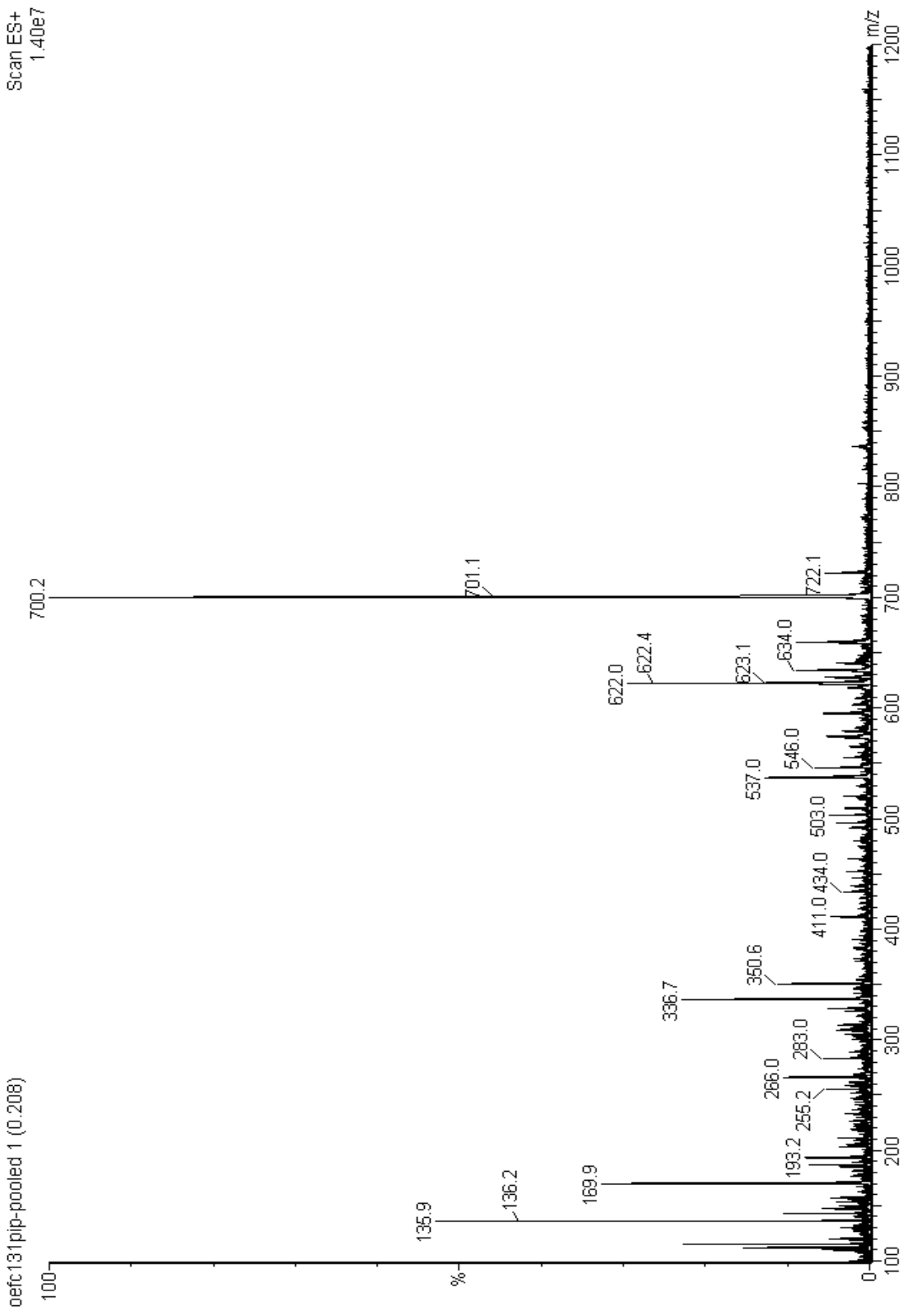
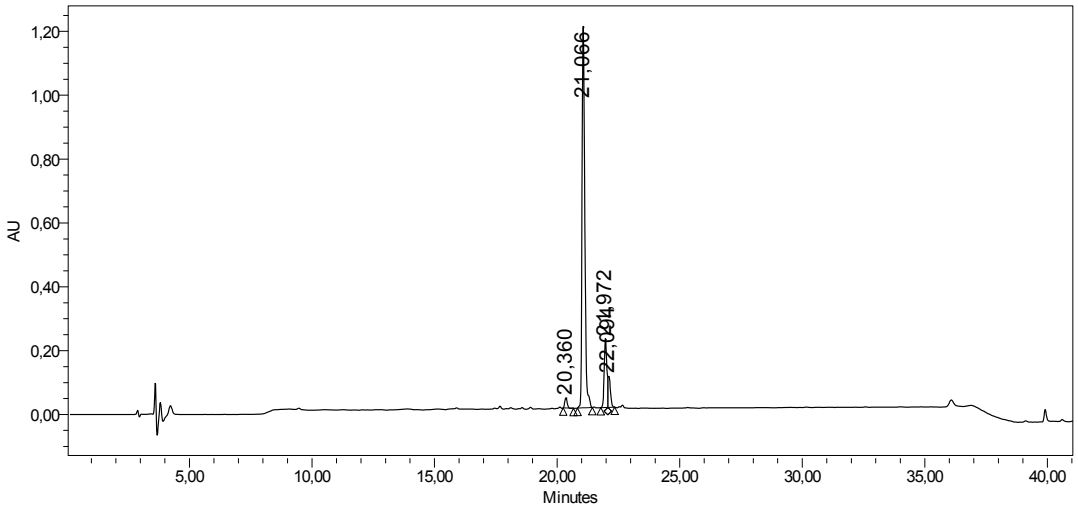


Figure A-55: MS analysis of purified **5**

SAMPLE INFORMATION

Sample Name:	ØE037_FC131Pip3_Final		
Sample Type:	Unknown	Sample Set Name:	ØE037_FC131Pip3_Final
Vial:	70	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,066	9382119	78,97	1192271
2	21,972	1585765	13,35	215573
3	22,094	692222	5,83	97064

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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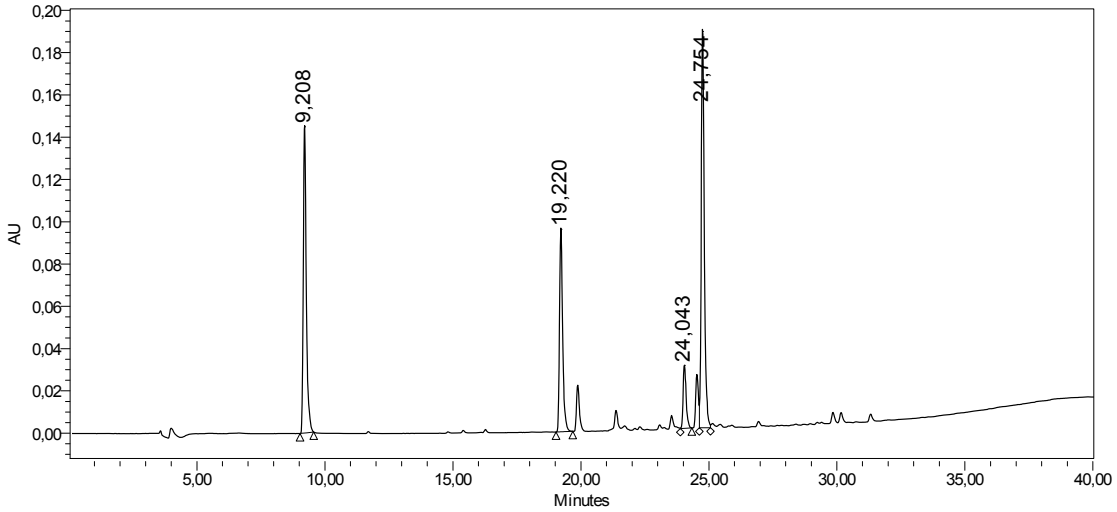
Report Method ID 6746
Page: 1 of 1

Project Name: Farmasi - MBSs gruppe
Date Printed:
23.05.2008
07:08:28 Europe/Oslo

Figure A-56: HPLC analysis of purified **5**

SAMPLE INFORMATION

Sample Name:	FC131Cit2_cyclic2		
Sample Type:	Unknown	Sample Set Name:	Oystein_9
Vial:	62	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	9,208	1188170	31,30	145683
2	19,220	820492	21,62	96760
3	24,043	244051	6,43	29891
4	24,754	1543041	40,65	189620

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 2 items in the revision history.

Figure A-57: HPLC analysis of protected **6**

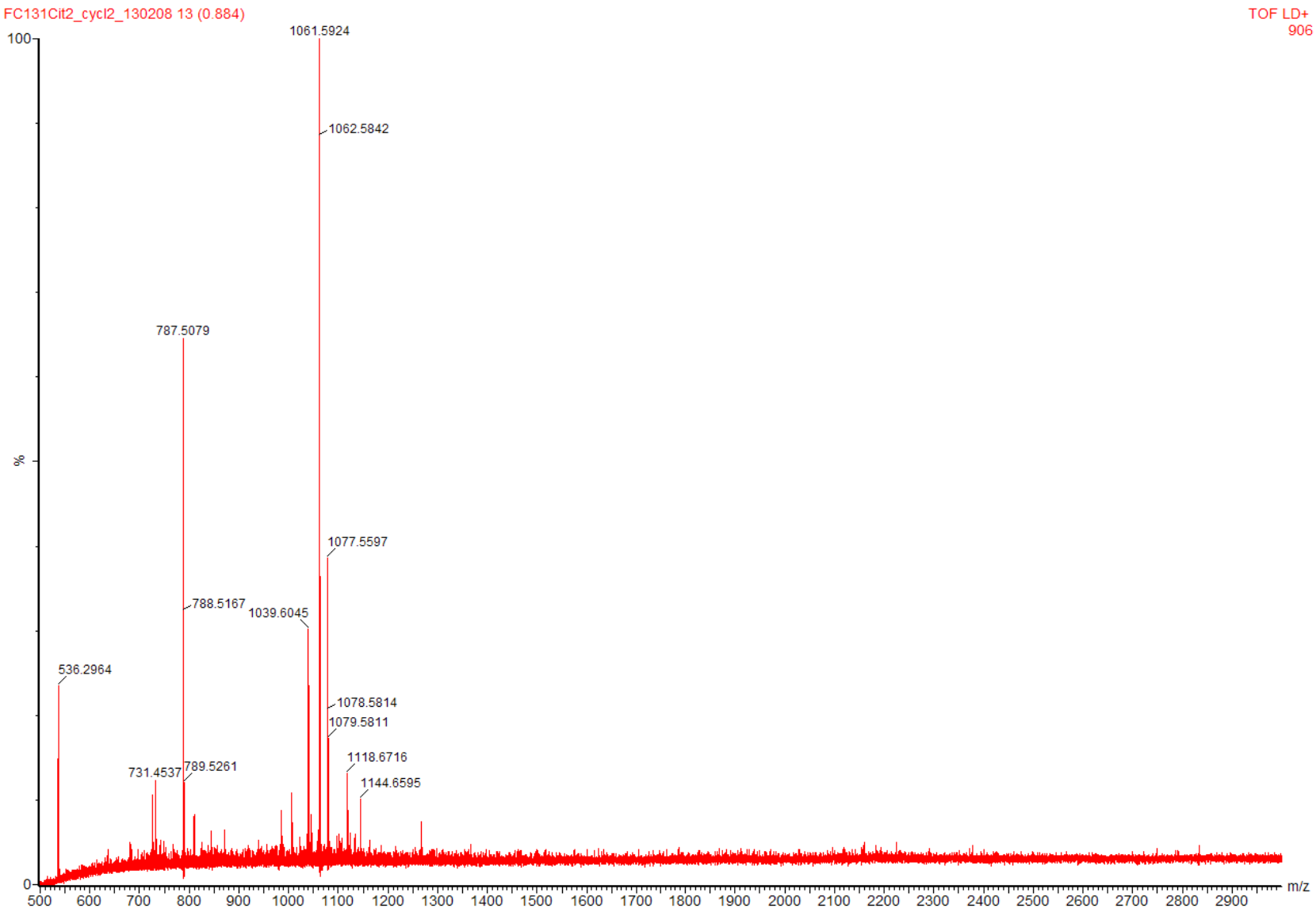

FC131Cit2_cycl2_130208 13 (0.884)
TOF LD+
906
100
%
0
1061.5924
1062.5842
787.5079
1077.5597
788.5167
1039.6045
536.2964
1078.5814
1079.5811
1118.6716
731.4537
789.5261
1144.6595
500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 2600 2700 2800 2900
m/z

Figure A-58: HPLC analysis of protected **6**

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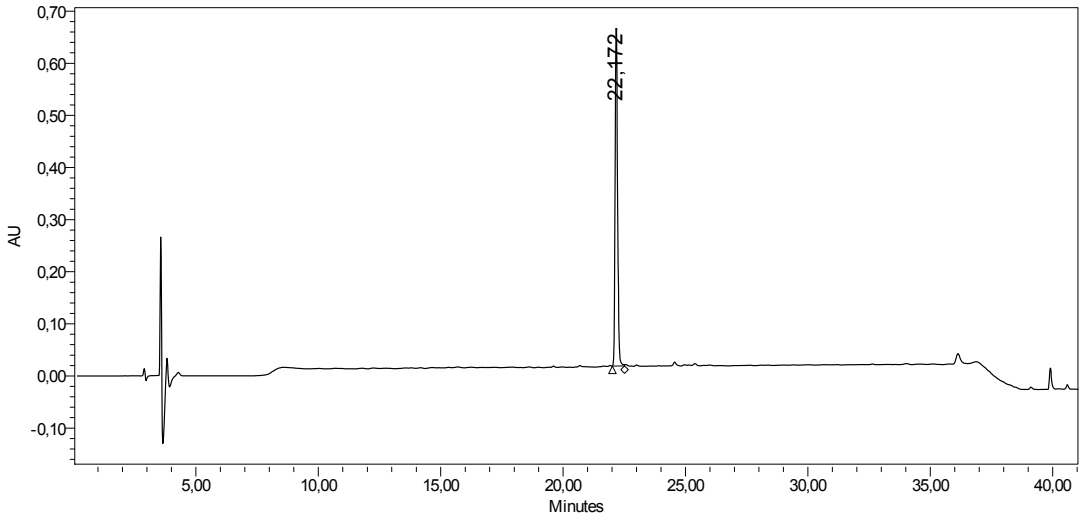
SAMPLE INFORMATION

Sample Name:	ØE031_FC131Cit3_Final		
Sample Type:	Unknown	Sample Set Name:	ØE031_FC131Cit3_Final
Vial:	61	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	22,172	4493907	100,00	654240

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

Reported by: Øystein Eriksen
Report Method: Øystein Eriksen
Report Method ID 6746
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06:41:12 Europe/Oslo

Figure A-59: HPLC analysis of purified **6**

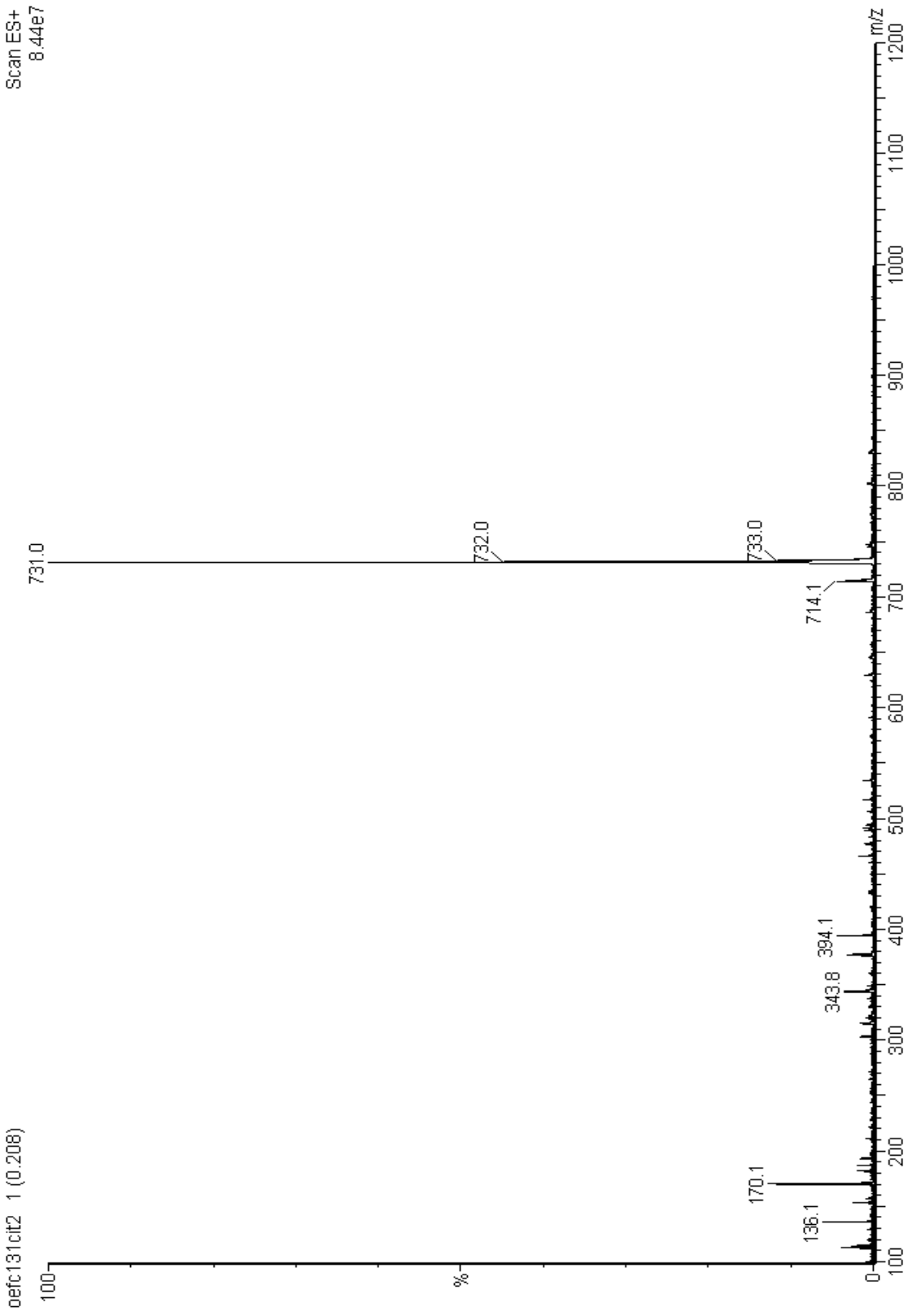

Figure A-60: MS analysis of purified **6**

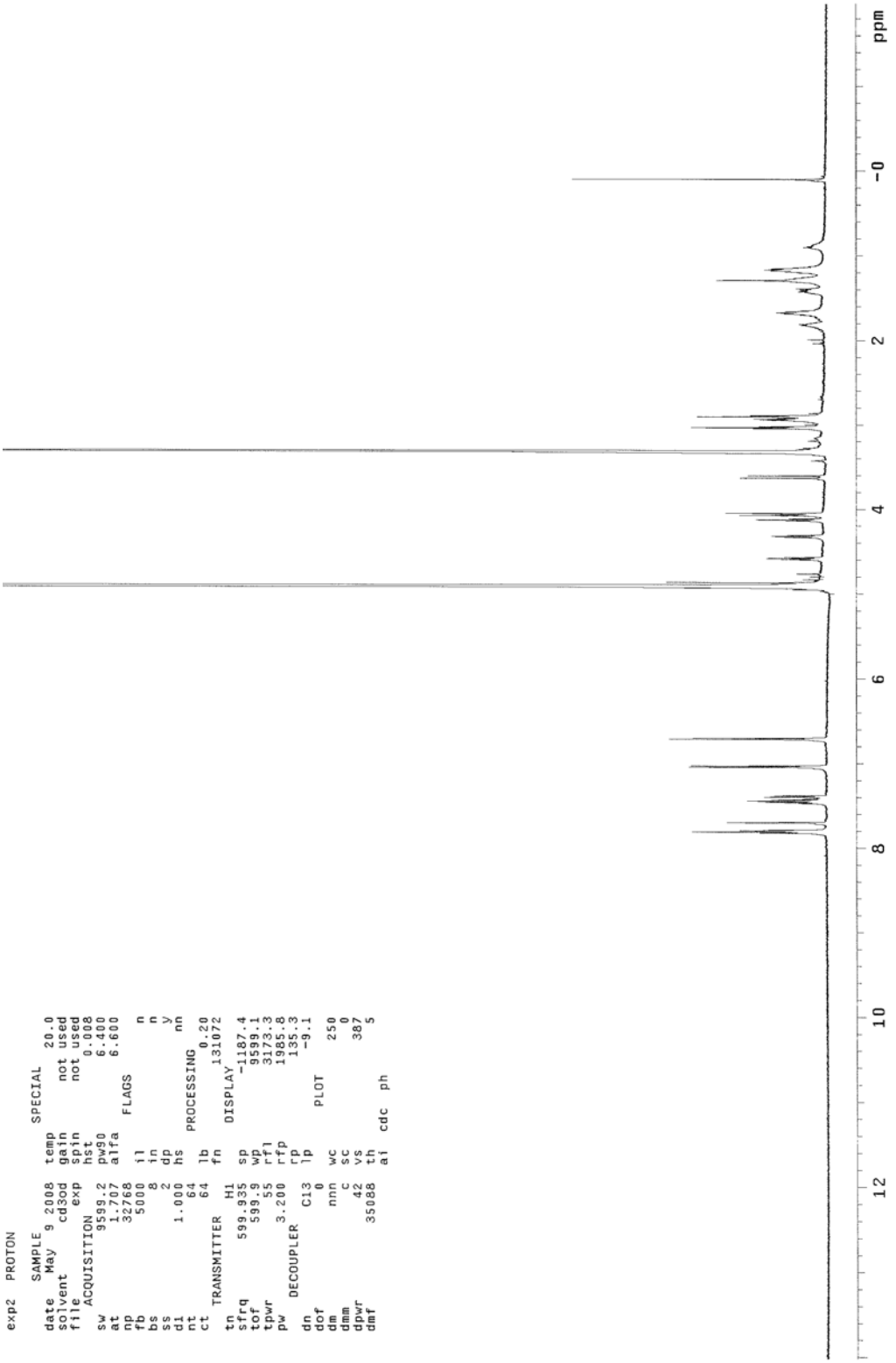

Figure A-61: NMR of **6**

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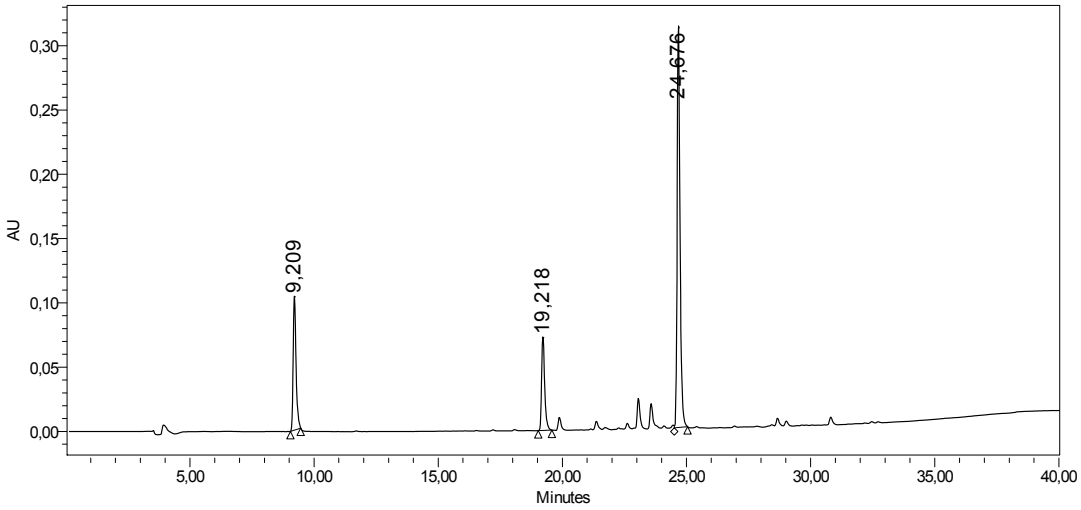
20.05.2008 Øystein Eriksen

SAMPLE INFORMATION

Sample Name:	FC131Cit3_cyclic2		
Sample Type:	Unknown	Sample Set Name:	Oystein_9
Vial:	61	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	9,209	839874	21,20	104053
2	19,218	623363	15,73	73124
3	24,676	2498774	63,07	311591

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-62: HPLC analysis of protected **7**

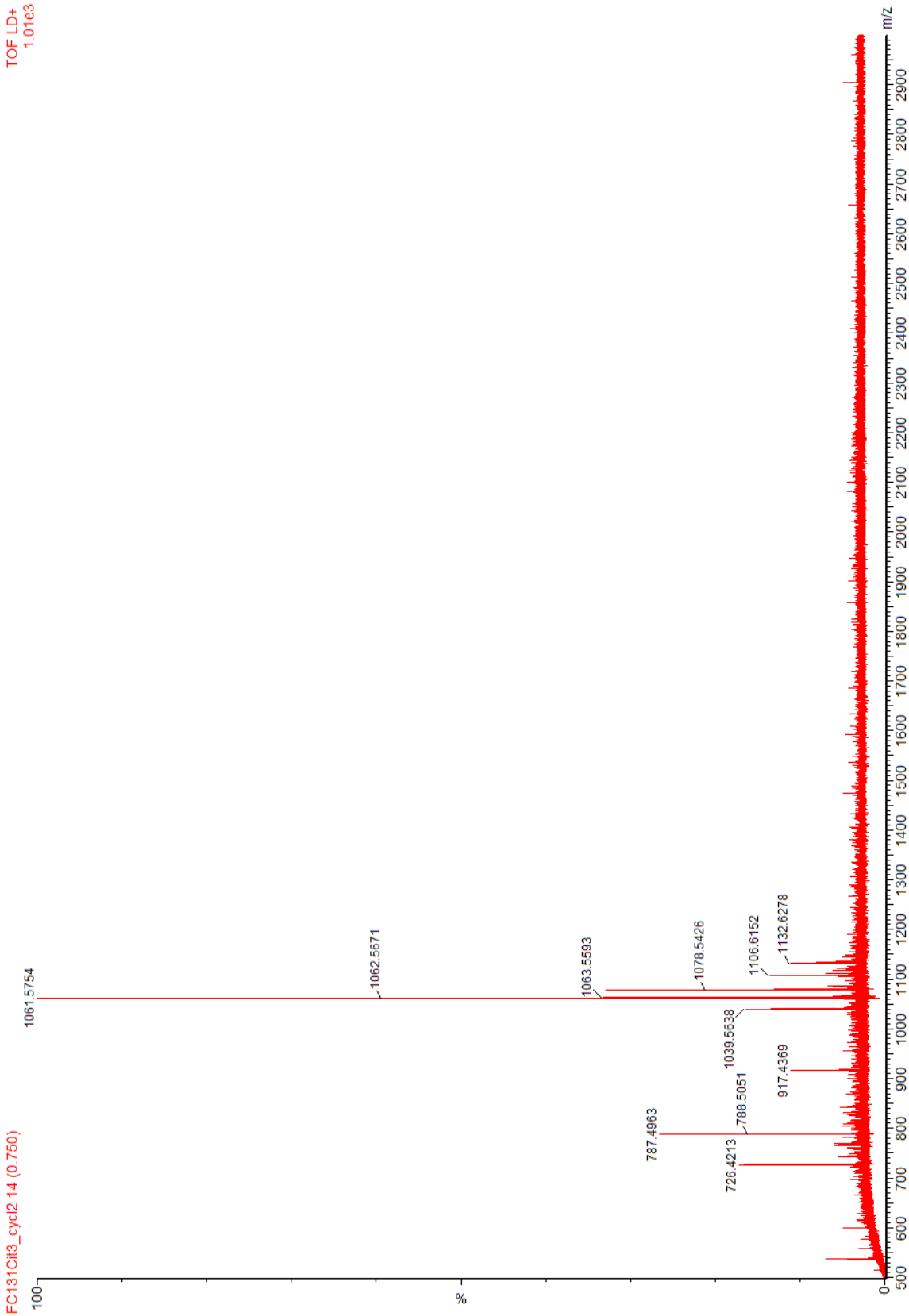
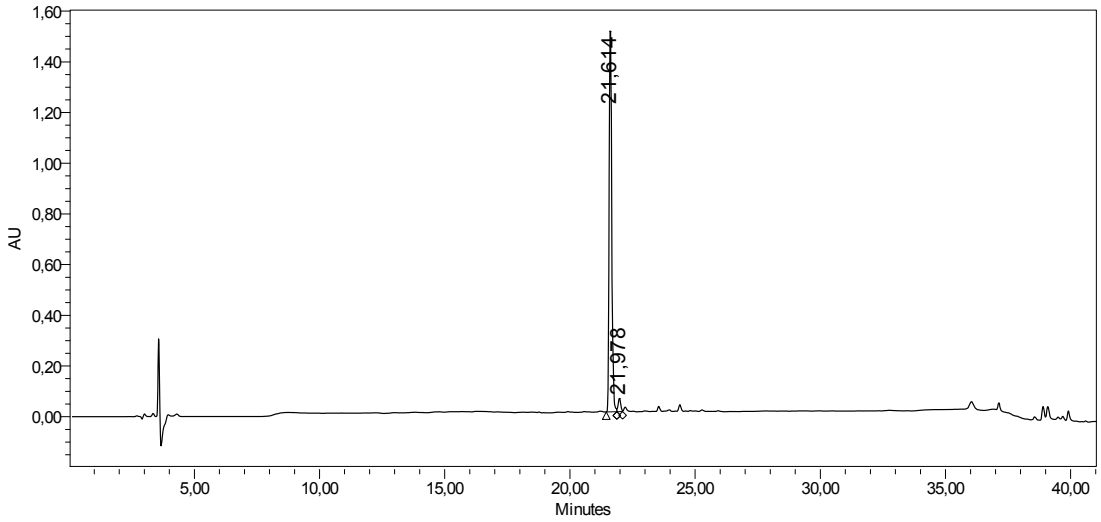


Figure A-63: HPLC analysis of protected **7**

SAMPLE INFORMATION

Sample Name:	ØE032_FC131Cit4_Final		
Sample Type:	Unknown	Sample Set Name:	Oystein_2
Vial:	57	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	20,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,614	10806108	96,60	1520980
2	21,978	380823	3,40	52081

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-64: HPLC analysis of purified **7**

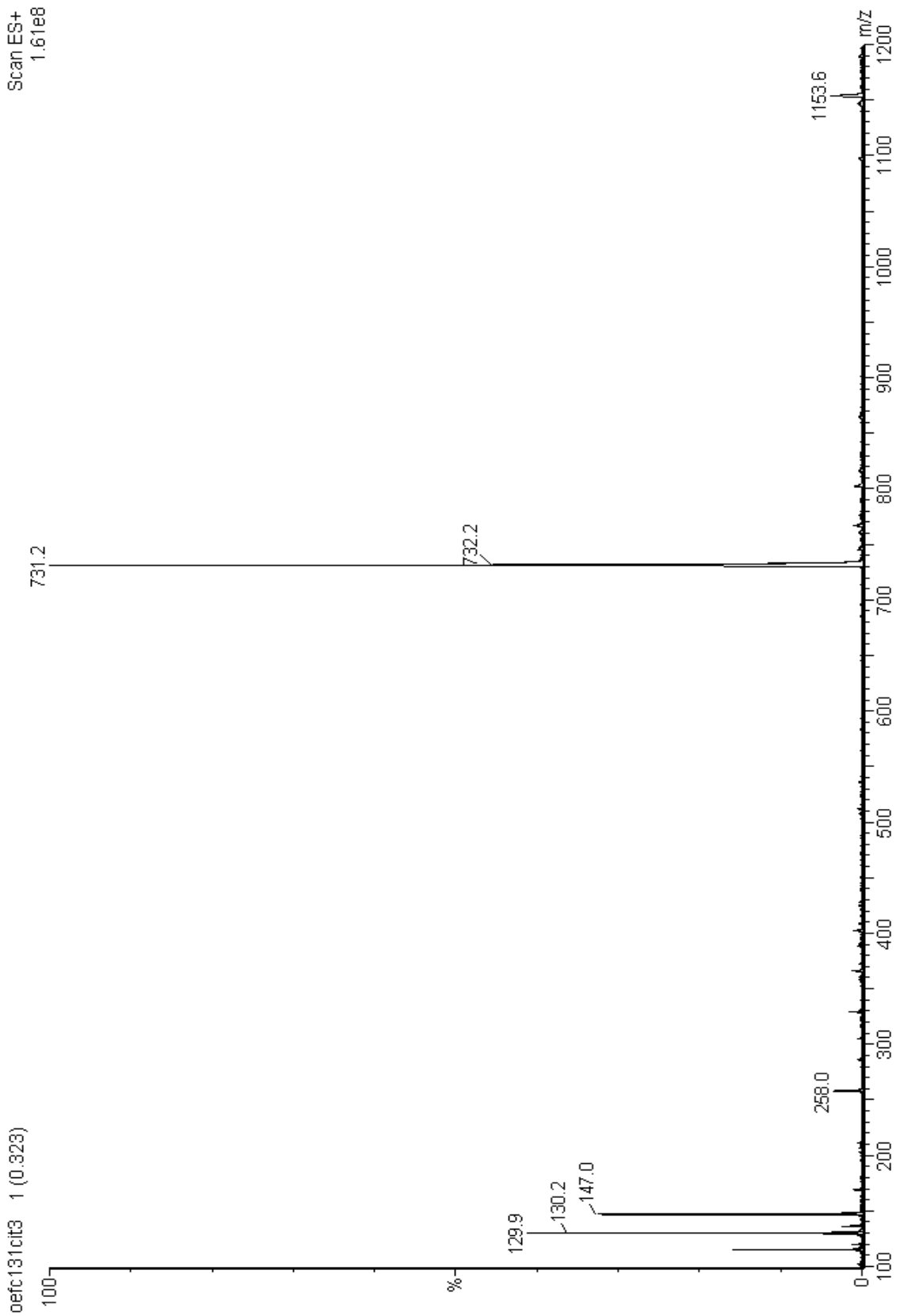


Figure A-65: MS analysis of purified **7**

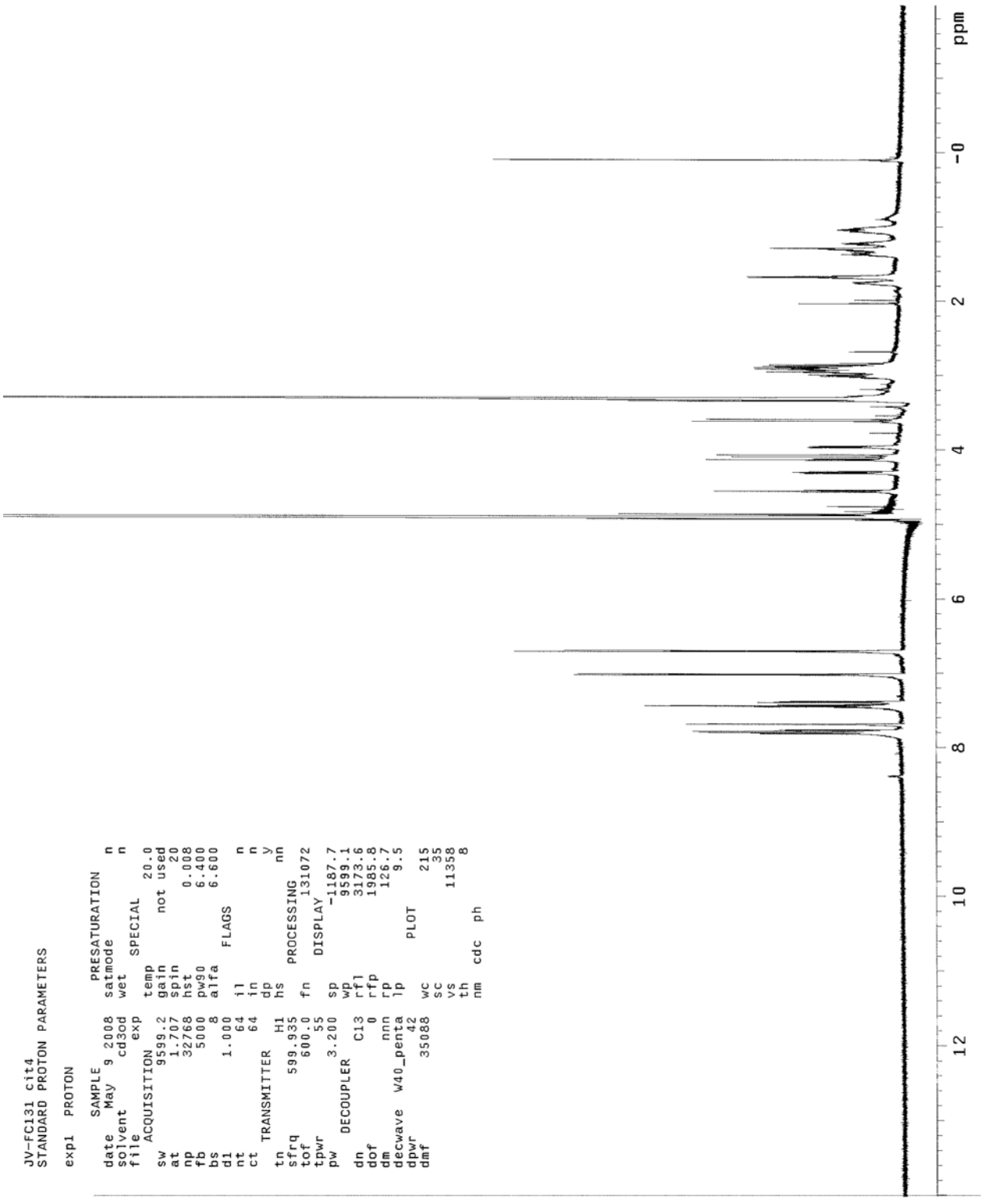
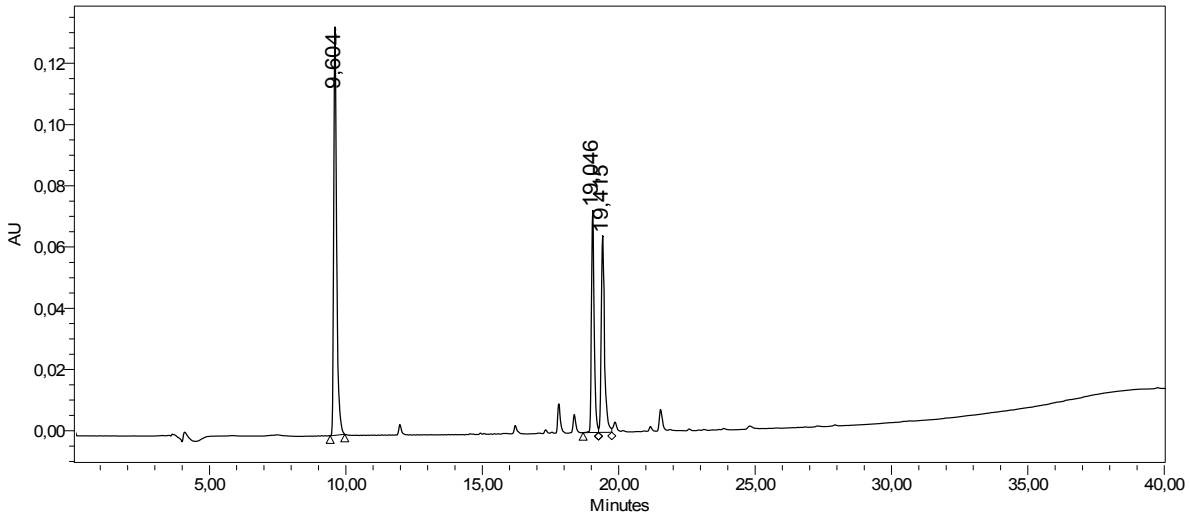


Figure A-66: NMR of **7**

SAMPLE INFORMATION

Sample Name:	FC131_CitCit_cyclic		
Sample Type:	Unknown	Sample Set Name:	Oystein_11
Vial:	62	Acq. Method Set:	Met Set Grad Krmsl C 0_95
Injection #:	1	Processing Method	TH Default
Injection Volume:	10,00 ul	Channel Name:	PDA Single wavelength 254
Run Time:	40,0 Minutes	Proc. Chnl. Descr.:	PDA 254,0 nm



Instrument Method: Instr Met Krmsl 0_95

Stored: 30.11.2007 10:26:44 CET

	RT	Area	% Area	Height
1	9,604	989948	48,98	134250
2	19,046	495708	24,53	72062
3	19,415	535514	26,50	64045

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	5,0	0,0	95,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

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Figure A-67: HPLC analysis of protected **8**

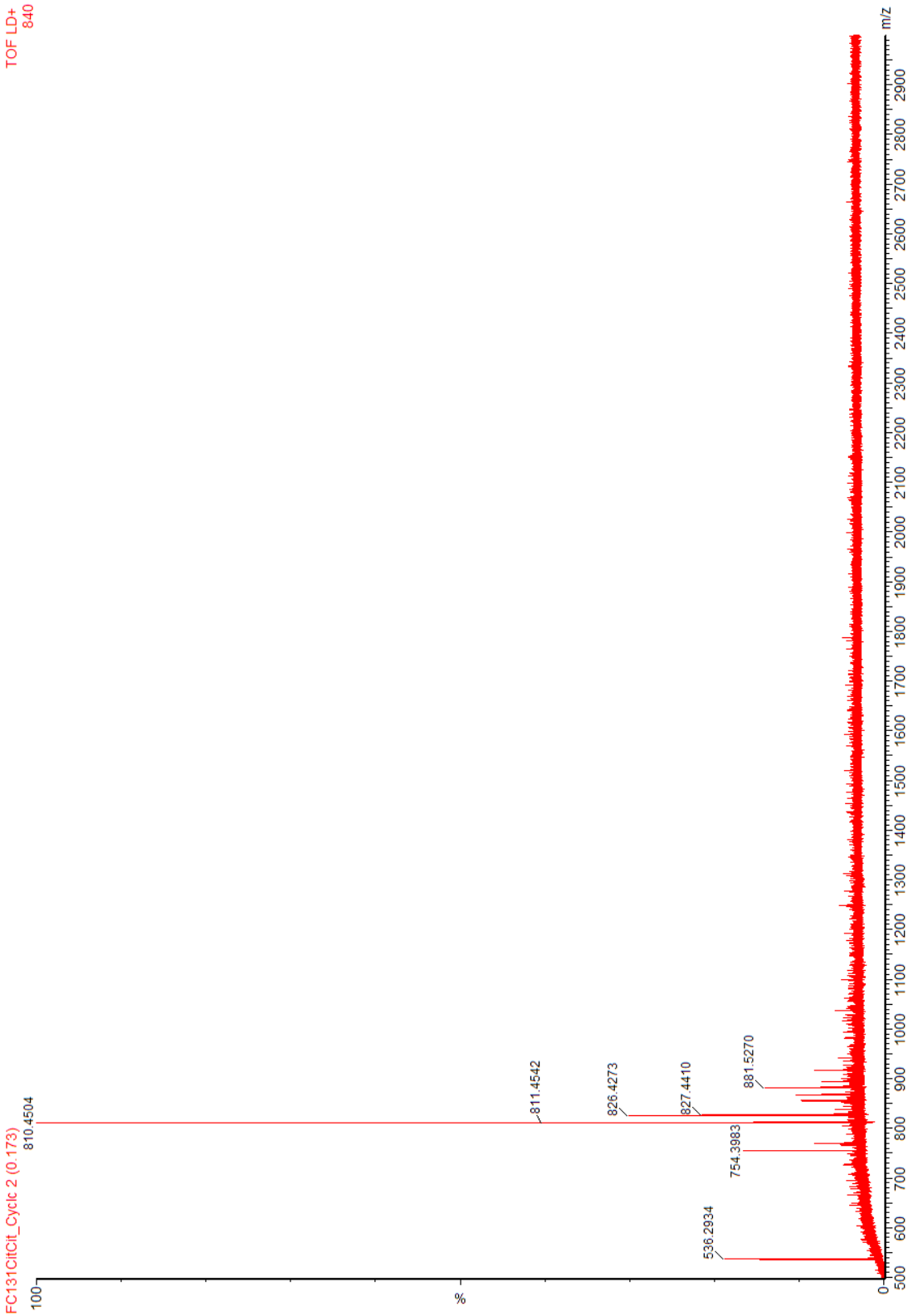
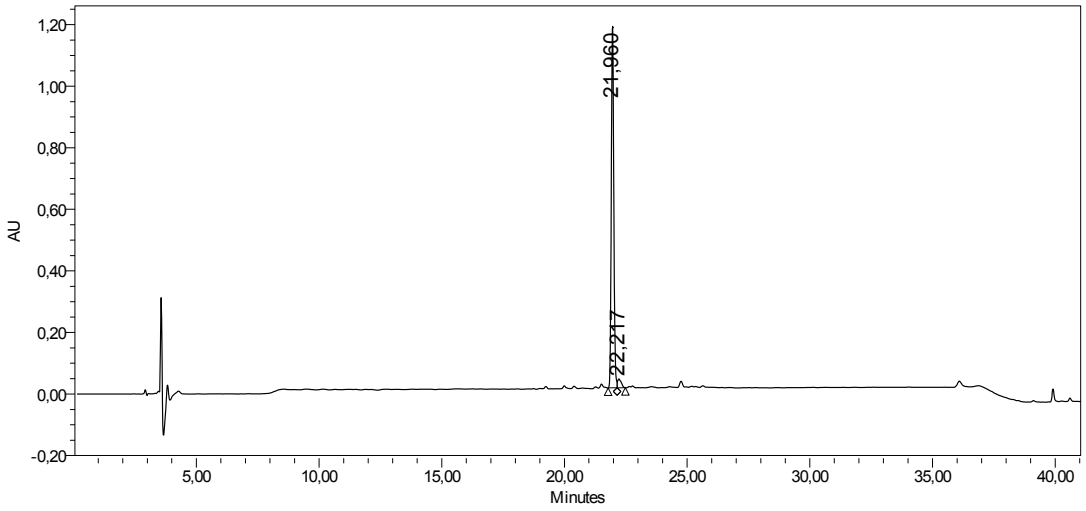


Figure A-68: HPLC analysis of protected **8**

SAMPLE INFORMATION

Sample Name:	ØE030_FC131Cit34_Final		
Sample Type:	Unknown	Sample Set Name:	ØE030_FC131Cit34_Final
Vial:	60	Acq. Method Set:	Met Set Grad Krmsl C 0_50
Injection #:	1	Processing Method	TH Default
Injection Volume:	20,00 ul	Channel Name:	PDA single wavelength 214
Run Time:	41,0 Minutes	Proc. Chnl. Descr.:	PDA 214,0 nm



Instrument Method: Instr Met Krmsl 0_50

Stored: 14.12.2007 14:26:10 CET

	RT	Area	% Area	Height
1	21,960	8279933	96,57	1179840
2	22,217	294268	3,43	28001

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1,00	100,0	0,0	0,0	0,0	
2	3,00	1,00	100,0	0,0	0,0	0,0	6
3	33,00	1,00	50,0	0,0	50,0	0,0	6
4	35,00	1,00	0,0	0,0	100,0	0,0	6
5	40,00	1,00	0,0	0,0	100,0	0,0	6
6	41,00	1,00	100,0	0,0	0,0	0,0	6

Revision History

This method contains 1 items in the revision history.

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Page: 1 of 1

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Figure A-69: HPLC analysis of purified **8**

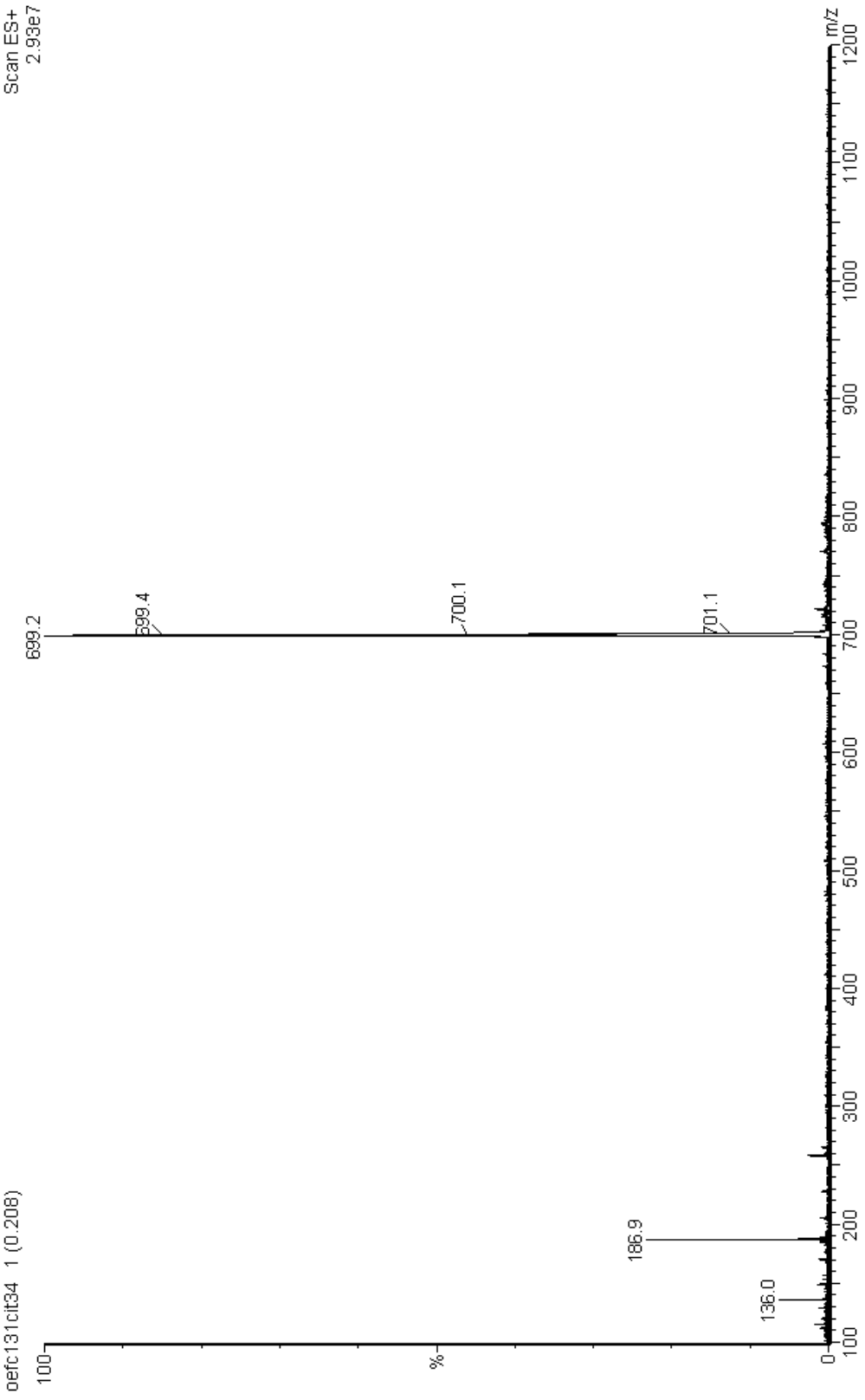

Figure A-70: MS of purified **8**

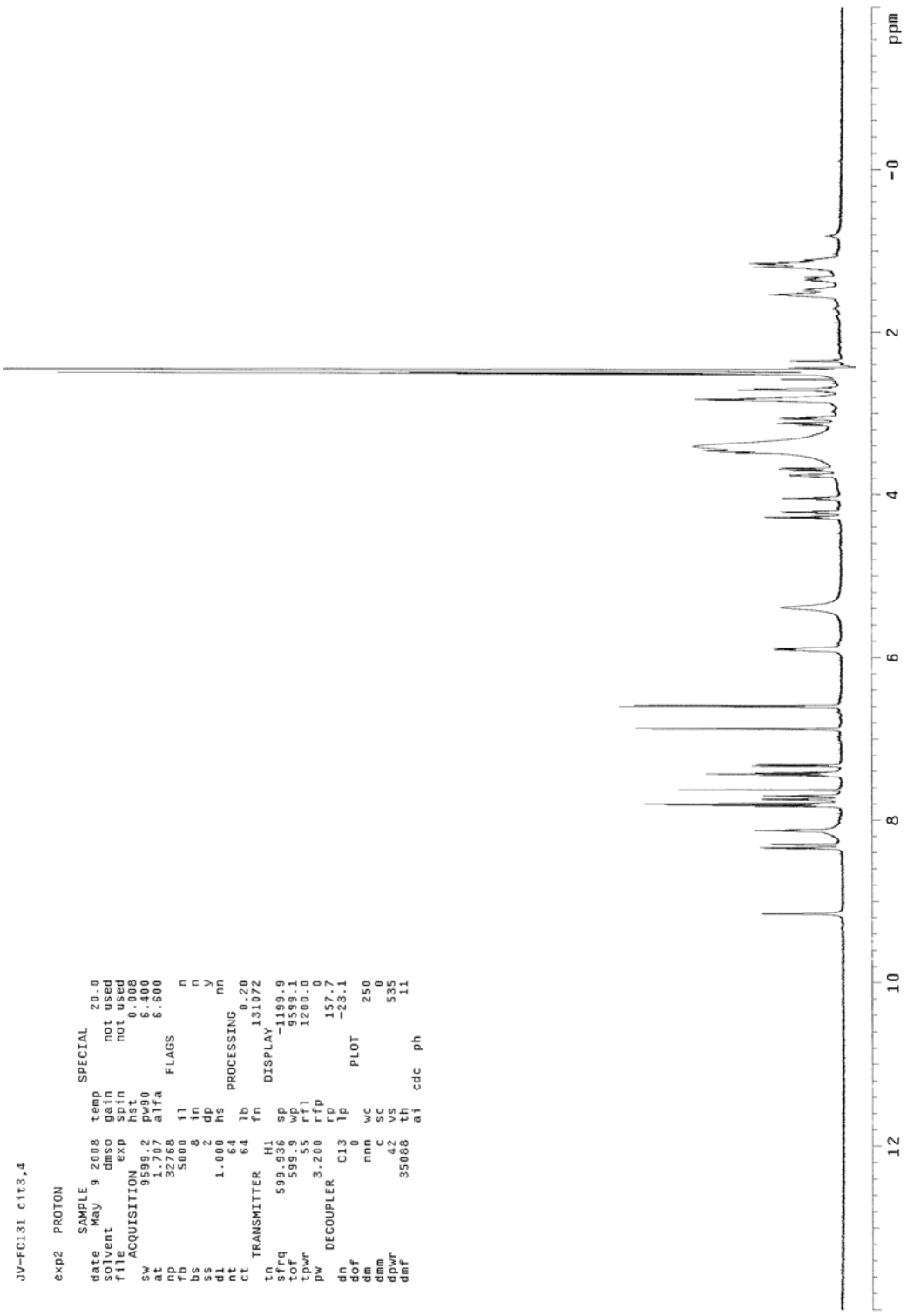

Figure A-71: NMR of **8**