FORMATION OF A LYSINE-DERIVED PROTEIN CONJUGATE FROM REACTION OF (*E*,*E*)-MUCONALDEHYDE, A POTENTIALLY HEMATOTOXIC METABOLITE OF BENZENE, WITH SERUM ALBUMIN

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Vorgelegt von

Dipl.-LMChem. Matthias Stefan Emanuel

Vorsitzender der Prüfungskommission: Prof. Dr. rer. nat. Werner Thiel
1. Berichterstatter: Prof. Dr. med. Dr. rer. nat. Dieter Schrenk
2. Berichterstatter: Prof. Dr. rer. nat. Elke Richling

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DECLARATION

I hereby certify, to the best of my knowledge, that the present work described in this thesis is original in context and does not incorporate any ideas or material previously written, published, performed or created by other authors, unless noted otherwise.

The supply of this thesis is made on the understanding that its use in creating other written or published material is accompanied with appropriate acknowledgement.

This thesis or parts thereof have not been filed at other departments. I have not applied for doctoral studies previously. Cognizance of the graduate regulations act dated October 7th, 1999 is confirmed.

Homburg/Saar, May 15th, 2018

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To my family

ABSTRACT

Benzene is a natural constituent of crude oil and a product of incomplete combustion of petrol and has been classified as "carcinogenic to humans" by IARC in 1982 (IARC 1982). (*E,E*)-Muconaldehyde has been postulated to be a microsomal metabolite of benzene *in vitro* (Latriano *et al.* 1986). (*E,E*)-Muconaldehyde is hematotoxic *in vivo* and its role in the hematotoxicity of benzene is unclear (Witz *et al.* 1985).

We intended to ascertain the presence of (*E,E*)-muconaldehyde *in vivo* by detection of a protein conjugate deriving from (*E,E*)-muconaldehyde.

Therefore we improved the current synthetic access to (*E,E*)-muconaldehyde. (*E,E*)-muconaldehyde was synthesized in three steps starting from with (*E,E*)-muconic acid in an overall yield of 60 %.

Reaction of (*E,E*)-muconaldehyde with bovine serum albumin resulted in formation of a conjugate which was converted upon addition of $NaBH_4$ to a new species whose HPLC retention time, UV spectra, Q1 mass and MS^2 spectra matched those of the crude reaction product from one pot conversion of Ac-Lys-OMe with (*E,E*)-muconaldehyde in the presence of $NaBH_4$ and subsequent cleavage of protection groups.

Synthetic access to the presumed structure (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO)) was provided in eleven steps starting from (*E,E*)-muconic acid and Lys(Z)-OtBu*HCl in 2 % overall yield. Additionally synthetic access to (*S*)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-OH)) and (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS) was provided.

With synthetic reference material at hand, the presumed structure Lys(MUC-OH) could be identified from incubations of (*E,E*)-muconaldehyde with bovine serum albumin via HPLC-ESI^+-MS/MS.

Cytotoxicity analysis of (*E,E*)-muconaldehyde and Lys(MUC-CHO) in human promyelocytic NB4 cells resulted in $EC_{50} \approx 1$ µM for (*E,E*)-muconaldehyde. Lys(MUC-CHO) did not show any additional cytotoxicity up to 10 µM.

B6C3F1 mice were exposed to 0, 400 and 800 mg/kg b.w. benzene to examine the formation of Lys(MUC-OH) *in vivo*. After 24 h mice were sacrificed and serum albumin was isolated. Analysis for Lys(MUC-OH) has not been performed in this work.

ZUSAMMENFASSUNG

Benzol ist ein natürlicher Bestandteil von Rohöl und entsteht bei unvollständiger Verbrennung von fossilen Treibstoffen. Benzol wurde von der IARC 1982 als kanzerogen für den Menschen eingestuft (IARC 1982). (*E,E*)-Muconaldehyd ist als mikrosomaler Metabolit von Benzol *in vitro* postuliert worden (Latriano *et al.* 1986). (*E,E*)-Muconaldehyd ist hämatotoxisch *in vivo* and seine Rolle für die Hämatotoxizität von Benzol ist nicht geklärt (Witz *et al.* 1985).

Wir beabsichtigten die Präsenz von (*E,E*)-Muconaldehyd *in vivo* durch Detektion eines (*E,E*)-Muconaldehyd abgeleitetes Proteinkonjugates zu verifizieren.

Im Zuge dieser Arbeit wurde der synthetische Zugang zu (*E,E*)-Muconaldehyd verbessert. (*E,E*)-Muconaldehyd wurde in drei Schritten ausgehend von (*E,E*)-Muconsäure in einer Gesamtausbeute von 60 % synthetisiert.

Reaktion von (*E,E*)-Muconaldehyd mit Rinderserumalbumin resultierte in der Bildung eines Konjugates, welches nach Umsetzung mit Natriumborhydrid hinsichtlich HPLC-Retentionszeit, UV-Spektrum, Q1 Masse und MS^2 Spektrum mit dem übereinstimmte, welches eine Reaktionsmischung aus Ac-Lys-OMe mit (*E,E*)-Muconaldehyd in Gegenwart von Natriumborhydrid und anschließender Abspaltung der Schutzgruppen ergab.

Synthetischer Zugang zu der vermuteten Struktur (*S*)-2-Ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoat (Lys(MUC-CHO)) gelang in elf Schritten ausgehend von (*E,E*)-Muconsäure und Lys(Z)-OtBu*HCl in 2 % Gesamtausbeute. Zusätzlich gelang die Synthese von (*S*)-2-Ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoat (Lys(MUC-OH)) sowie (*S*)-2-Ammonio-6-((6-hydroxyhexyl)amino)hexanoat (IS).

Mit Zugang zu Referenzmaterial wurde die vermutete Struktur Lys(MUC-OH) in Inkubationen von (*E,E*)-Muconaldehyde mit Rinderserumalbumin nachgewiesen.

Die Cytotoxizität von (*E,E*)-Muconaldehyd und Lys(MUC-CHO) in humanen promyelozytischen NB4 Zellen resultierte in $EC_{50} \approx 1$ µM für (*E,E*)-Muconaldehyd. Lys(MUC-CHO) zeigte keine relevante Cytotoxizität im Konzentrationsbereich bis 10 µM.

B6C3F1 Mäusen wurde 0, 400 und 800 mg/kg KG Benzol peroral verabreicht um die Bildung von Lys(MUC-OH) *in vivo* zu untersuchen. Nach 24 h wurden die Tiere getötet und Serumalbumin isoliert. Lys(MUC-OH) wurde im Rahmen dieser Arbeit nicht mehr analysiert.

CONTENT

PROLOGUE

"WISHING, with the poor widow, to give something to the Lord's treasury out of our penury and poverty, we have dared to scale the difficult heights and to undertake a work beyond our strength. We have grounded our confidence of completion and the reward for our labor in the Good Samaritan, who, after giving two silver pieces for the care of the man left half-dead, promised to repay all the expenses of the caregiver, who might have to spend more. The truthfulness of the one making that promise delights us, but the immensity of the work terrifies us; the desire to make progress spurs us on, but the weakness of failure discourages us, and only the zeal for the house of God overcomes it."

Peter Lombard, *The Sentences*, Book 1: The Mystery of the Trinity, Prologue

Lk 22, 44

1. INTRODUCTION

BENZENE (Figure 1) is a natural constituent of crude oil and is formed as side product during incomplete combustion of organic matter. Due to its exceeding solvent properties, it is still used today in parts of rubber and paint industries as well as chemical manufacturing. "Since 1989 the concentration of benzene in [these] preparations is limited to 0.1 % (w/w) within the EU" except for gasoline which limits benzene content to a maximum of 1 % (v/v) since January of 2000 (EU 2008). Cause for this regulation roots in the toxicity of benzene.

In humans, cells of the blood forming organs are most damaged by chronic exposure to low levels of atmospheric benzene. A set of different parameters in blood count is affected adversely ultimately leading to an increased risk for malignancies in these tissues, specifically acute myelogenous leukemia and non-Hodgkin lymphomas. There is strong evidence that metabolites of benzene, acting alone or in concert, produce several genotoxic effects at the level of the pluripotent hematopoietic stem cell which results in chromosomal changes consistent with those seen in hematopoietic cancer (IARC 2012).

Despite extensive research cellular mechanisms leading to benzene induced myelotoxicity have not been fully understood.

Our research is focused on elucidating the role of (*E,E*)-muconaldehyde, an oxidative metabolite of benzene in benzene induced myelotoxicity (Figure 1). To this day, the *in vivo* presence of (*E,E*)-muconaldehyde has not been proven following exposure to benzene.

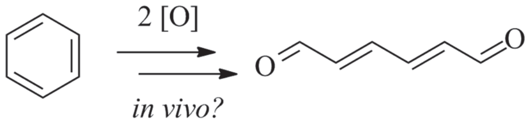

Figure 1 Oxidative formation of (*E,E*)-muconaldehyde from benzene *in vivo*

2. SCIENTIFIC BACKGROUND

2.1. USE OF BENZENE

Historically, benzene has been used as a component of inks in the printing industry, as a solvent for organic materials, as starting material and intermediate in chemical and drug industries (e.g. to manufacture rubbers, lubricants, dyes, detergents, pesticides), and as an additive to unleaded gasoline (NTP 2004; ATSDR 2007; Williams *et al.* 2008).

The primary use of benzene today is in the manufacture of organic chemicals. In Europe, benzene is mainly used for production of styrene, phenol, cyclohexane, aniline, maleic anhydride, alkylbenzenes and chlorobenzenes. It is an intermediate in synthesis of anthraquinone, hydroquinone, benzene hexachloride, benzene sulfonic acid and other products used in drugs, dyes, insecticides and plastics (Burridge 2007).

In the United States of America (USA), benzene is used primarily in the production of ethylbenzene, accounting for 52% of the total benzene demand in 2008. Ethylbenzene is mostly consumed by the manufacture of styrene, which is used in turn in synthesis of polystyrene and various styrene copolymers, latexes and resins. 22 % of the benzene demand in the USA goes to the manufacture of cumene (isopropylbenzene), nearly all of which is consumed in the production of phenol (IARC 2012).

Benzene is also used to manufacture chemical intermediates: cyclohexane, used in production of nylon monomers (15%); nitrobenzene, an intermediate for aniline and other products (7%); alkylbenzene, used in detergents (2%); chlorobenzenes, used in engineering polymers (1%); And miscellaneous other uses (1%) (Kirschner 2009). Benzene occurs naturally in petroleum products (e.g. crude oil and gasoline) and is also added to unleaded gasoline for its octane-enhancing and anti-knock properties (IARC 2012).

2.2. LEGAL PRODUCT CONCENTRATION LIMITS OF BENZENE

Since 1989, the concentration of benzene in preparations has been restricted to 0.1 % (w/w) within the European Union except for gasoline, which contains up to 1 % (v/v) benzene. Until 1999, the upper limit of benzene content in gasoline was 5 % (v/v) (EU 2008).

2.3. TOXICITY OF BENZENE

2.3.1. HEMATOTOXICITY

The most characteristic systemic effect resulting from intermediate and chronic benzene exposure is the arrest in development of blood cells. A common early clinical finding in benzene hematotoxicity is cytopenia, which is a decrease in cellular blood elements manifesting as anemia, leukopenia, or thrombocytopenia. Benzene associated cytopenias vary from a reduction in one (unicellular cytopenia) to all three (pancytopenia) cellular elements of the blood.

Benzene also causes a potentially lethal disorder called aplastic anemia. This disorder is characterized by reduction of all cellular components in the peripheral blood and in bone marrow, leading to fibrosis, an irreversible replacement of bone marrow tissue. Hematotoxicity of benzene is believed to be the prerequisite for development of different kinds of benzene related cancer (ATSDR 2007).

2.3.2. CARCINOGENICITY

Benzene is known to be carcinogenic in humans (group 1) causing a variety of different types of cancer of the blood forming system. With very few exceptions, overwhelming statistical evidence is provided for a correlation between exposure to benzene and increased risk for acute myeloid leukemia as well as acute non-lymphocytic leukemia. Besides, evidence for other types of cancer such as acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma and non-Hodgkin lymphoma is also well documented (IARC 1982).

Carcinogenicity of benzene can be reproduced in experimental animals, however there are differences in susceptibility (Huff *et al.* 1988). Mice show an overall greater capacity to metabolize benzene than do rats and cynomolgus monkeys (Sabourin *et al.* 1992; Sabourin *et al.* 1987) and develop more and more varied tumors than rats (Henderson 1996) . Therefor mice are considered today to be the animal model closest to mirroring benzene’s toxicity in humans.

As to the mechanism of benzene induced cancers, there is good experimental evidence that benzene metabolites produce multiple genotoxic effects at the level of the pluripotent hematopoietic stem cell resulting in adverse chromosomal changes. A variety of genotoxic changes, including chromosomal abnormalities, could be detected in workers exposed to benzene (IARC 2012).

2.3.3. CARCINOGENIC MODES OF ACTION

Despite a tremendous amount of mechanistic studies, the mode of action in benzene induced carcinogenicity still poses several unresolved questions. Several mechanisms acting alone or in consort are considered to be relevant in tumor formation.

1. Genotoxicity. It is believed that metabolites of benzene are responsible for genotoxic lesions. Several metabolites are reasonable candidates for genotoxicity and may be responsible for primary genotoxicity such as DNA mutations or damage and secondary genotoxicity such as clastogenity[1]. However, genotoxic potency of individual metabolites might be underestimated by synergistic effects of different metabolites (IARC 2012). Furthermore, metabolites of benzene might cause indirect DNA damage e.g. by formation of reactive oxygen species and raising the level of oxidative stress in sensitive target tissue[2].
2. Hematotoxicity. Proliferation stimuli of the damaged bone marrow cells as compensating response to cytotoxicity of benzene are reported (Aksoy 1989). As a result, enhanced proliferation of genetically modified cells might occur, thus favoring tumor formation. However, cases of leukemia without previous damage to the blood forming system have been reported. (Albertini *et al.* 2003; ATSDR 2007; Khan 2007; Rothman *et al.* 1996).
3. Topoisomerase-II inhibition. Topoisomerase-II is an enzyme essential for cell cycle. It cleaves both strands of DNA and recombines them with higher degree of coiling. Inhibition of Topoisomerase-II can lead to an increase of DNA double strand breaks thus marking an indirect genotoxic mechanism. 1,4-Benzoquinone and hydroquinone have been shown to be potent inhibitors of Topoisomerase-II *in vitro* (Bird *et al.* 2005; Eastmond *et al.* 2005; Lindsey Jr. *et al.* 2005; Whysner *et al.* 2004).
4. Mismatched DNA repair. Genetic alterations by benzene are thought to include DNA double strand breaks (DSBs) (see above). DSBs are repaired by either homologous recombination (HR) or nonhomologous end-joining (NHEJ). NHEJ is usually considered to be error prone "by causing small deletions or small insertions" (Hartwig 2010).
5. Poor DNA repair in bone marrow. Furthermore, a reduced capacity to repair DNA damage in bone marrow progenitor cells has to be considered. When comparing primary

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[1] The possible modes of action of individual metabolites are discussed in detail in section 2.6 (Benzene metabolism)

[2] Discussed in detail in section 2.6.2 (Phenol & hydroquinone)

human hematopoietic cells, CD34 progenitor cells show consistently lower repair capacities as compared to more differentiated CD34 cells (Ren *et al.* 2008; Hartwig 2010; BAuA 2012).

2.4. BENZENE EXPOSURE

The data presented here referring to benzene exposure is limited as much as possible to data referring to Germany. For more detailed information on exposure assessment of benzene in the European Union and Germany in particular, one might refer to the European Union Risk Assessment Report on benzene (EU 2008).

2.4.1. OUTDOOR EXPOSURE

Benzene is released from a number of anthropogenic sources. The main sources of environmental benzene are automobile exhaust emissions, losses via evaporation and emissions by refueling. Benzene in automotive exhaust stems from incompletely burned benzene and benzene produced in the combustion cycle through dealkylation of toluene and xylenes. From industrial sources benzene enters the environment primarily as fugitive emission from industrial intermediate production and processing operations as well as through air emissions from waste water treatment plants. Additionally, benzene is released from natural sources such as volcanos or forest fires.

Benzene is produced and emitted in industrial quantities. Because of its high volatility, benzene is emitted mainly to the air. Emissions to soil and water partly add to atmospheric emission. As a result, most of benzene is found in the air compartment (EU 2008).

Benzene emissions in Germany have decreased significantly from 66 214 t/a in 1990 to an estimated 2 887 t/a in 2010 (BUA 2006) (Table 1).

Table 1 Development of benzene emissions since 1990 in Germany (BUA 2006).

Emission	1990	1995	2000	2010
Benzene [t/a]	66 214	26 387	8 229	2 887 (est.)

Atmospheric benzene concentration limits are set by European and implemented by German law (Umweltbundesamt 2010). Legal bases are:

1. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe (EUP 2008).
2. *Neununddreißigste Verordnung zur Durchführung des Bundes-Immissionsschutzgesetzes Verordnung über Luftqualitätsstandards und Emissionshöchstmengen.* (39. BImSchV 2010).

In Germany, atmospheric benzene levels have decreased continually over the past years, underscoring the most recent enforced legal limit by the European Union of 5 µg/m³. (Umweltbundesamt 2010) (Figure 2). Current estimations of median atmospheric benzene concentrations range around 2 µg/m³ (Table 2).

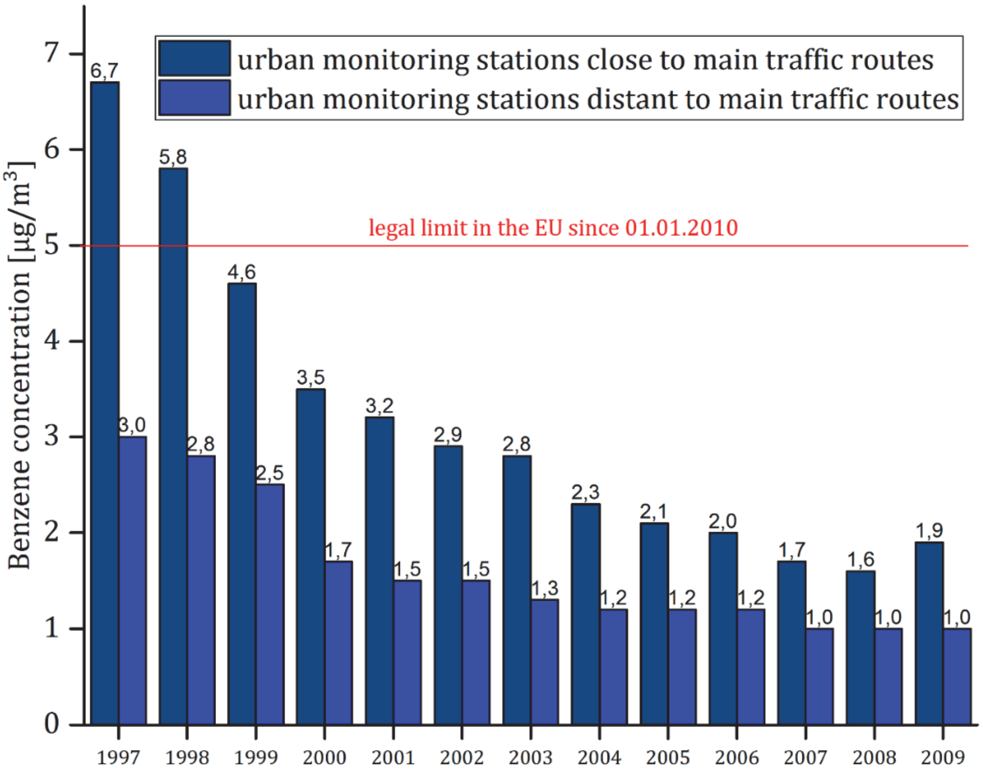

Figure 2 Latest data on median atmospheric benzene concentrations for selected monitoring stations in Germany, modified from (Umweltbundesamt, 2010)

Table 2 Median atmospheric benzene concentration (indoor + outdoor) estimation exposures in Germany, taken from (Umweltbundesamt, 2013).

Area	Benzene [µg/m^3]
Rural	1.70
Suburb	1.73
City	2.04

2.4.1.1. GASOLINE

As we have seen in section 2.2 the concentration of benzene in preparations is legally limited to 0.1 % (w/w) within the EU. However, an exception is gasoline, which is allowed to contain up to 1 % (v/v) benzene. Until end of 1999, the maximum content of benzene in gasoline was 5 % (v/v) (EU 2008).

Aside from the legal requirement, how much benzene can actually be detected in automobile fuels? One of the few reports in the literature listing benzene concentrations in gasoline separately, names 6140 mg L^{-1} or 0.96 % (v/v). According to the authors, this level reflects the current average for gasoline from US refineries, which is expected to fall from 1.05 % (v/v) in 2007 to 0.62 % (v/v) in 2015 (EPA 2010; Chin & Batterman 2012).

However, it must be noted that due to its volatility, benzene accounts for up to one fifth (m/v) of gasoline vapor (Chin & Batterman 2012). That is the reason why since 1998 gas stations in Germany must be equipped with systems that redirect excessive air in the tank back to the gas station upon refueling, so called *Gasrückführsysteme* (21. BImSchV 2014). This way gasoline emissions upon fueling are reduced by at least 85 % (LfU 2006).

2.4.1.2. DIESEL

Diesel fuel has a substantial higher boiling point than gasoline, which ranges between 160 – 360°C. Therefore, “the boiling range of diesel fuel […] largely excludes the presence of benzene […]” (IARC 1989). That is the reason why volatile organic compounds including benzene compositions of diesel and biodiesel blends have been reported “rarely”. (Chin & Batterman 2012). Nevertheless, diesel fuels may contain minor amounts of benzene (< 0.02 % (v/v)) (IARC 1989).

2.4.1.3. FUEL EXHAUST EMISSIONS

One might suspect, benzene emissions of diesel motors are lower than benzene emissions of gasoline motors since not only diesel reflects newer technology but also benzene content of diesel is by nature lower than benzene content of gasoline. To test this hypothesis, Louis *et al.* compared benzene emissions (among others) of 6 different engines with state of the art environmental protection technology (Louis *et al.* 2016) such as:

- Euro 4 gasoline
- Euro 5 gasoline with direct injection
- Euro 4 Diesel catalyzed Diesel particulate filter
- Euro 5 Diesel catalyzed Diesel particulate filter
- Euro 5 Diesel additive Diesel particulate filter

The following observations could be made:

1. There is no blanket advantage regarding benzene emissions when comparing diesel or gasoline motors even though diesel consumption is generally lower compared to gasoline motors.
2. Benzene emissions per driven km rank independently from gasoline or diesel motors between ~ 10 and 1000 µg/km.
3. Benzene emissions generally fallow the order urban area > rural roads > motorways.
4. Benzene emissions are substantially higher driving with cold machines compared to motors who reached optimal operating temperature.
5. In any scenario benzene emissions could be not be dropped below ~ 10 µg/km.

Since EURO 5 as well as the coming EURO 6 norm keep the amount of emission-related hydrocarbons of 0.1 g/km set by EURO 4, we expect no substantial change in benzene emissions for next generation cars (EUP 2007). Therefore, in the short term, fuel emissions will probably remain the single largest source of atmospheric benzene.

2.4.2. UPTAKE

Benzene can be absorbed by inhalative, dermal and oral exposure. The main route of exposure in the general population to benzene is via inhalation (BAuA 2012). However, atmospheric benzene degrades with a half-life of two to five days due to its reaction with hydroxyl radicals. Yet approximately half of inhaled benzene is taken up by the human body (Nomiyama & Nomiyama 1974). In contrast absorption of benzene via oral exposure is virtually complete however this route can be neglected in the general population due to the low concentration of benzene in foodstuff (see chapter 2.4.3.2 Intake by food). Dermal exposure is less than 1 % and therefor merely of relevance during exhaustive contact with benzene e.g. in the workplace (LfU 2006).

Total benzene uptake estimates for a person living in Germany have not been determined but are available for a person living in Canada and the United States and range around 200 µg/day for Canada (Table 3) (Hughes *et al.* 1994) and 320 µg/day for the United States (Wallace 1996). Since the current atmospheric exposure levels to benzene have decreased, these values seem to overestimate the current uptake levels and should not be overstressed.

Table 3 Daily uptake estimations for benzene in Canada by (Hughes *et al.*, 1994) taken from (WHO 2010).

Source	Intake
Ambient air	14 µg/day
Indoor air	140 µg/day
Food & drinking water	1.4 µg/day
Car related acitivies	49 µg/day
Total	**~ 200 µg/day**

2.4.3. INDOOR EXPOSURE

Domestic atmospheric concentration of benzene is dependent on two factors

1. Atmospheric benzene concentration outdoors
2. Additional sources for benzene formation indoors

2.4.3.1. INTAKE BY SMOKING

Combustion of tobacco products is the single largest domestic source of benzene. The estimated number of smokers in Germany as of 2015 counts 19 Mio people which is 26 % of the whole population (WHO 2015, p. 131).

“The average smoker (32 cigarettes per day) takes in about 1.8 milligrams (mg) of benzene per day. This amount is about 10 times the average daily intake of benzene by nonsmokers.” (ATSDR 2007)

Are non-smokers also endangered by other people’s smoking? Leaving aside multiple other toxic ingredients in cigarette smoke: Cigarette side stream smoke contains five to ten times as much benzene as mainstream smoke (NRC 1986, p. 30). Atmospheric benzene concentrations in households inhabiting smokers tend to exceed the median nonsmoking household level by 30 to 50 % in average (LfU 2006). Therefore, domestic passive smoking leads to an increase of 14–50 µg/day benzene in addition to the average daily intake of 200 µg/day (Nazaroff & Singer 2004). Since benzene is classified a genotoxic carcinogen, no safe level of exposure can be recommended[3]. We therefore strongly advice smokers to leave the apartment while smoking to prevent other inhabitants from additional exposure to carcinogenic agents including benzene and to even end their habit at all.

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[3] See section 2.5 (Risk assessment of benzene)

2.4.3.2. INTAKE BY FOOD

2.4.3.2.1. RELEVANCE

Recent studies measuring benzene concentrations in food support the conclusion that food and beverages are minor pathways for benzene exposure (Rose & Chin 1990; Wallace 1996; Duarte-Davidson *et al.* 2001; Bruinen de Bruin *et al.* 2005; WHO 2003; Smith *et al.* 2010). Therefore, in this work we restrain from a detailed dietary exposure assessment. However, since this work has been conducted at the department of food chemistry a relation of benzene to the original field of study seems appropriate. For this reason, elected sources of dietary exposure of benzene are be presented in the following section.

2.4.3.2.2. FOOD

Data regarding concentrations in and uptake of benzene from foods is limited, and the quality of the existing data is "highly questionable" (Bruinen de Bruin *et al.* 2005). Among others, benzene has been reported to occur in fruits, fish, vegetables, nuts, dairy products, beverages, and eggs (*ibd.*).

Eggs contained the highest concentrations of benzene up to 2100 ppb (uncooked) and 500–1900 ppb (hard-boiled)[4], followed by haddock (100 – 200 ppb), Jamaican rum (120 ppb), irradiated beef (19 ppb), heat-treated canned beef (2 ppb), and butter (0.5 ppb). Lamb, mutton, veal, and chicken contained < 10 ppb benzene if the meat was cooked.

A survey of more than 50 foods collected from 1991 to 1992 revealed that foods (including eggs) without added benzoates contained benzene at concentrations ≤ 2 ng/g. The concentration of benzene in foods containing benzoates and ascorbates ranged from < 1 to 38 ng/g (McNeal *et al.* 1993). In many foods, the presence of benzene is likely due to contamination from air (Smith *et al.* 2010). This conclusion is supported by the fact that the uptake of benzene decreases with a reduction in exposed surface of foods and contact time with air (Grob *et al.* 1990).

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[4] Concentration data in eggs from (IARC, 1982)

2.4.3.2.3. FORMATION THROUGH ADDITIVES

In addition to trace contamination of foods, benzene may be formed *in situ* in products containing certain food preservatives or additives. Benzoate salts, used as anti-microbial agents in certain carbonated beverages and naturally present in some fruits and their juices, may react with ascorbic acid to form benzene, especially in the presence of metal contaminants, sunlight and elevated temperatures. Ascorbic acid may be present either naturally from a fruit juice ingredient in the drink or added as an antioxidant food additive (Smith *et al.* 2010).

The mechanism proposed for formation of benzene in foods is depicted in Figure 3.

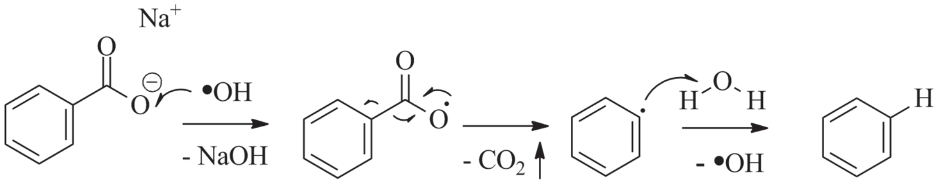

Figure 3 Proposed mechanism for benzene formation in foods from benzoic acid in the presence of transition metals such as Cu^{2+} and ascorbic acid. Taken from(Gardner & Lawrence, 1993)

2.5. RISK ASSESSMENT OF BENZENE

2.5.1. HOW BENZENE IS ASSESSED AS A CARCINOGEN AND WHY

There is no one-size-fits-all method used in risk assessment for genotoxic carcinogens. Rather there are different approaches which all have specific limitations. This circumstance makes it necessary to evaluate on a case by case basis which model is applied to evaluate (and communicate) the hazard that is conferred by the exposure to a genotoxic carcinogen.

2.5.2. THE MARGIN OF EXPOSURE (MOE) APPROACH

The MOE approach uses a reference point, also "point of departure" (POD), often taken from an animal study and corresponding to a dose that causes a low but measurable response in animals. This point of departure is then compared with total dietary intake estimates in humans. (EFSA 2005).

$$MOE = \frac{POD}{Exposure}$$

The EFSA Scientific Committee recommends the use of the $BMDL_{10}$ (benchmark dose lower confidence limit 10%) as the point of departure which is an estimate of the lowest dose which is 95% certain to cause no more than a 10% cancer incidence in rodents. The Scientific Committee also notes that the benchmark dose approach can be applied to human data when available (EFSA 2005).

The European Food Safety Authority argues that the margin of exposure approach should be applied "in cases where substances that are both genotoxic and carcinogenic have been found in food […]" (EFSA 2005). The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) seem to share this assessment, saying that "advice on compounds that are both genotoxic and carcinogenic should be based on estimated MOEs" (FAO/WHO 2005).

As shown above, benzene is commonly evaluated to be both genotoxic and carcinogenic and does occur in several foods. However, generally risk assessment of benzene is not performed based on application of MOE values, but rather by extrapolation from existing human data (Umweltbundesamt 2013). We will take a moment here to discuss why the MOE approach is generally not applied in the risk assessment in case of benzene.

The following reasons argue against the application of the MOE concept in risk assessment of benzene.

1. Poor quality of exposure data. The margin of exposure is calculated using various dietary intakes in humans (EFSA 2005). However, the reported data on benzene concentrations in foods are rife with controversy. Due to the potential formation of benzene e.g. in fruit juices, it is difficult to generalize which foods are major contributors to an individual's exposure to benzene. Further uncertainties abound as to the occurrence of benzene in food due to its volatility (Smith *et al.* 2010).
2. Dietary exposure is negligible. The intake of benzene by the general population through food products is estimated to be "less than 1.5 % of the inhalation exposure" (Bruinen de Bruin *et al.* 2005).
3. Relevance of animal data. Since the mode of action in benzene induced cancer formation has not been fully understood, interpretation of a MOE value derived from animal studies seems highly questionable (Cartus & Schrenk 2016).
4. Sufficient human data. Due to the ubiquitous sources found for benzene exposure at the workplace, especially in countries such as the United States or China, there are numerous studies dealing with the formation of malignancies in the blood forming system in humans after benzene exposure (Roller *et al.* 2006). Little if any extrapolation is necessary in these cases to calculate an effect dose which is correlated with an excess risk of 10 % for tumor formation (ED_{10})[5] (BAuA 2012).

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[5] Calculated for exposure at the workplace: 40 years for 8 h/day for 5 d/week

2.5.3. COMPARISON OF CARCINOGENIC POTENCY WITH OTHER ATMOSPHERIC CARCINOGENS

As we've seen benzene is evaluated to be carcinogenic. But how dangerous a carcinogen really is benzene? To assess the carcinogenic potency of benzene compared to other carcinogens, one must compare potencies for tumor formation upon exposure. Table 4 lists the estimated individual risk for additional formation of one malignant tumor of known or suspected atmospheric human carcinogens at an air concentration of 1 µg/m^3 after lifetime exposure (unit risk). One has to keep in mind, that risk estimates derive from extrapolation of studies with higher atmospheric compound concentrations. Therefor these data have to be regarded with certain caution. Nonetheless, these studies may serve to get a rough idea as to the magnitude of carcinogenic potency. As an example, within the margin of error for extrapolation, at a lifetime exposure of 1 µg/m^3 benzene[6] the additional formation of six malignancies of the blood forming system per 1 000 000 residents can statistically be accounted to exposure to benzene. This incidence puts benzene at the lower end of carcinogenic potency compared to other carcinogens occurring in the atmosphere.

Benzene (6×10^{-6}) is estimated to be more potent than vinyl chloride (1×10^{-6}) or trichloro-ethylene (4×10^{-7}) but less potent than e.g. arsenic (2×10^{-3}), chromium (VI) (4×10^{-2}) or benzo[a]pyrene (9×10^{-2}).

Table 4 Carcinogenic risk estimates of known or suspected human atmospheric carcinogens based on human studies[a], taken and modified from (WHO 2000)

Substance	IARC group	Unit risk[b]	Site of tumor
Acrylonitrile	2A	2×10^{-5}	Lung
Arsenic	1	2×10^{-3}	Lung
Benzene	1	6×10^{-6}	Blood forming system
Butadiene	2A	n.a.	Multisite
Chromium (VI)	1	4×10^{-2}	Lung
Nickel compounds	1	4×10^{-4}	Lung
Benzo[a]pyrene[c]	1	9×10^{-2}	Lung
Trichloroethylene	2A	4×10^{-7}	Lung, testis
Vinyl chloride	1	1×10^{-6}	Liver (mainly)

[a] Calculated with average relative risk model.

[b] Cancer risk estimates for lifetime exposure to a concentration of 1 µg/m^3.

[c] Based on a benzo[a]pyrene concentration of 1 µg/m^3 in air as a component of benzene-soluble coke-oven emissions.

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[6] Calculated for exposure at the workplace: 40 years for 8 h/day for 5 d/week

2.5.4. DOSE RISK ASSESSMENT

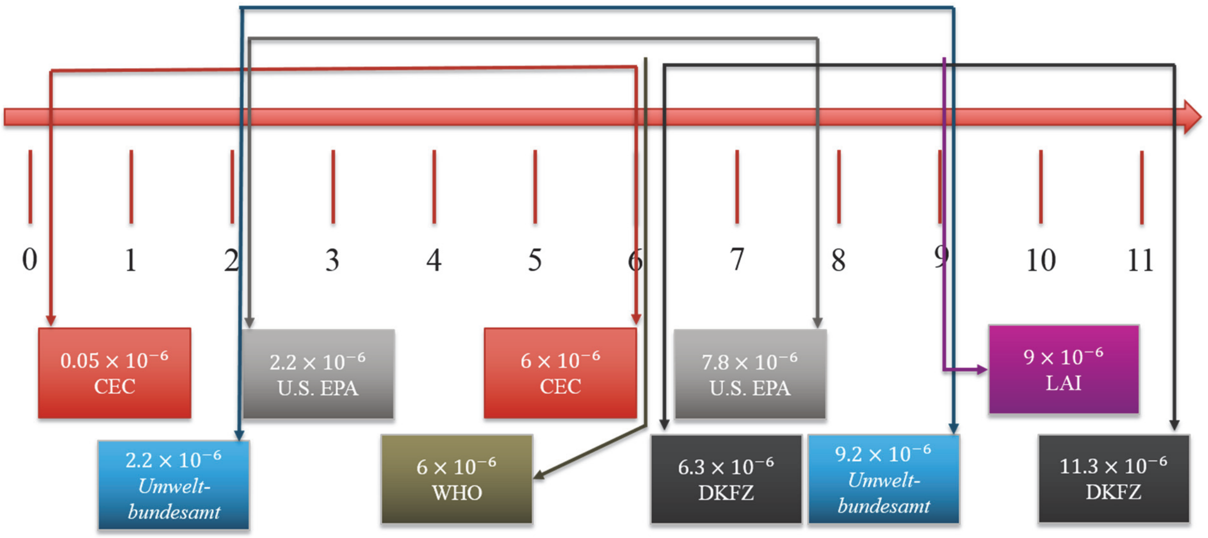


Figure 4 Graphic depiction of unit risk estimates (× 10^{-6}) for malignancies of the blood forming system for lifetime exposure to 1 µg/m^3 benzene. CEC (Commission of European Countries, 1998)), U.S. EPA (United States Environmental Protection Agency, 1998)), WHO (World Health Organization, 2000), DKFZ (Deutsches Krebsforschungszentrum, 1990), LAI (Länderausschuss für Immissionsschutz, 1992, 2004). Taken and modified from (Umweltbundesamt, 2013).

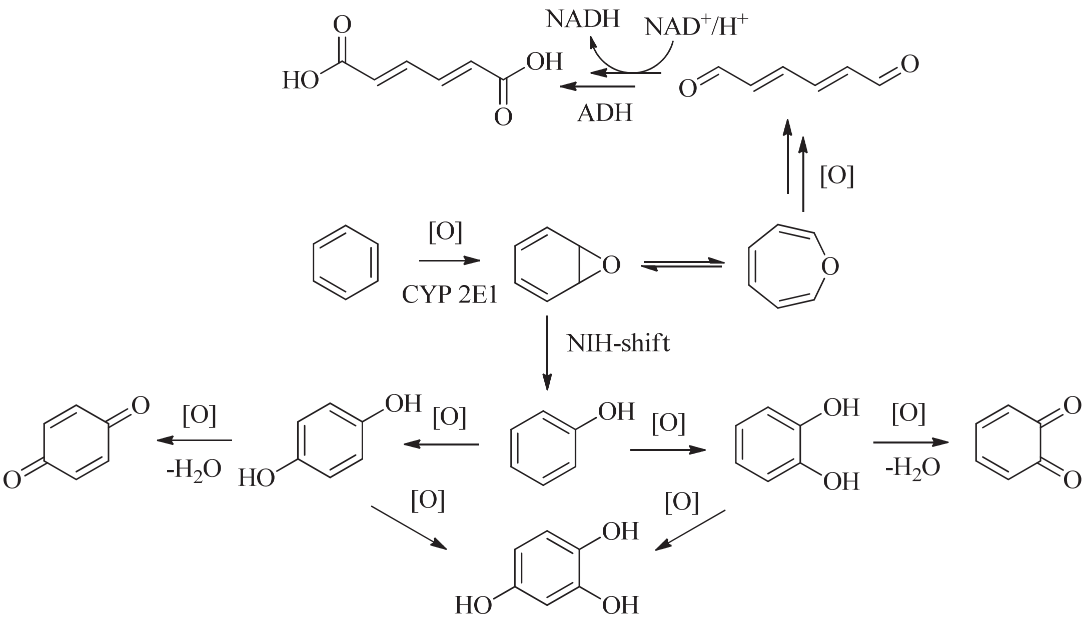
To show the variability of unit risk factors, Figure 4 depicts some unit risk estimates for malignancies of the blood forming system after lifetime exposure to benzene by different risk management institutions (Umweltbundesamt 2013). Unit risk estimates range from 0.05 – 11.3 × 10^{-6} and the median of 6 × 10^{-6} given above by the WHO is covered by every agency except for DKFZ and poses a reasonable middle ground for risk assessment.

Calculating from this number, at the current median rural background level for benzene in Germany of 1.7 µg/m^3 (Table 2), which is a conservative approach considering the many people living in cities, extrapolation results in an additional risk of 1 × 10^{-5} cases of leukemia or similar types of cancer of the blood forming system over lifetime exposure to benzene (WHO, World Health Organization 2000).

2.6. BENZENE METABOLISM

2.6.1. INTRODUCTION TO BENZENE METABOLISM

Benzene has to be metabolized to become carcinogenic (Ross 2000; Snyder 2004). CYP2E1 is considered to be the primary enzyme responsible for mammalian metabolism of benzene (Valentine *et al.* 1996; Nedelcheva *et al.* 1999; IARC 2012). Phase I metabolism of benzene is summarized in (Scheme 1).



Scheme 1 Phase I metabolism of benzene. Taken and modified from (Rappaport *et al.* 2010). ADH = Alcohol dehydrogenase, NIH = National Institute of Health

Metabolic activation of benzene involves cytochrome P450 (CYP)-dependent oxidation to benzene oxide, which exists in equilibrium with its tautomer oxepin. Most benzene oxide spontaneously rearranges to phenol, the primary Phase I metabolite of benzene (IARC 2012). This reaction has been named NIH-shift in remembrance to the location, where it has been reported first, the US National Institute of Health.

Oxidative metabolism of benzene oxide/oxepin is thought to open the aromatic ring, yielding reactive muconaldehydes (IARC 2012), initially the *Z,Z*-isomer which isomerizes to the *E,Z*- and subsequently to the *E,E*-isomer within minutes in presence of cellular nucleophiles such as glutathione (Henderson *et al.* 2005), the latter one being the only isomer claimed to be identified *in vitro* so far (Latriano *et al.* 1986).

Further hydroxylation of phenol leads to formation of catechol, hydroquinone and, to a lesser degree, trihydroxybenzene. Commonly accepted is the idea, that Phase I metabolism of benzene is toxicologically more significant than Phase II or III metabolism (conjugation and excretion). There is, however, dissidence within the scientific community, as to which extent the different metabolic routes of Phase I metabolism can be accounted for the hematotoxicity of benzene. There are currently three different approaches focusing on different Phase I metabolites (Ross 2000; EPA 2002):

1. Phenol & hydroquinone
2. Benzene oxide/oxepin
3. (E,E)-Muconaldehyde.

2.6.2. PHENOL & HYDROQUINONE

The first model focuses on phenol and hydroquinone as primary responsible agents in genotoxicity of benzene. This model has received growing support in the 1980s rooting from the observation that simultaneous high dosage of phenol and hydroquinone to mice resulted in a similar hematological profile found after benzene exposure (Eastmond *et al.* 1987). Formation of reactive benzoquinones such as p-benzoquinone is thereby considered to be responsible for cytotoxicity of benzene in the bone marrow. P-benzoquinone can be formed by oxidation from hydroquinone catalyzed by myeloperoxidase, an enzyme primarily found in the bone marrow (Bainton *et al.* 1971; Schlosser & Kalf 1989). The presence of phenol acts synergistically on the oxidation of hydroquinone (Smith *et al.* 1990).

Several objections in the literature argue against a key role of phenol and hydroquinone in benzene induced genotoxicity.

1. Peroxidases. The biological plausibility of this mechanism is mainly based on the peroxidase content of target tissues which would result in the production of reactive quinones. However, an absolute requirement for peroxidases for benzene induced hematotoxicity has not been demonstrated yet (Ross 2000).
2. Background levels. High background levels of 1,2- and 1,4-benzoquinone-albumin and -hemoglobin adducts have also been found in unexposed animals (McDonald *et al.* 1994).
3. Metabolism. Phenol itself does not reproduce the myelotoxicity of benzene. This issue is perhaps the major problem associated with the phenolic mechanism. Phenol is metabolized to hydroquinone, catechol and other polyphenolics, so its inability to reproduce the myelotoxicity of benzene is “puzzling” (Ross 2000).

These considerations argue against a key role of phenolic metabolites in benzene induced hematotoxicity.

2.6.3. BENZENE OXIDE / OXEPINE

The second model used for explanation of genotoxicity of benzene targets benzene oxide as primary responsible agent. Benzene oxide is the primary product of benzene oxidation (Tunek *et al.* 1978; Lovern *et al.* 1997; Lindstrom 1997), and therefore, a considerable amount of metabolism via this pathway could be expected. Benzene oxide released from the liver into systematic circulation has an estimated half-life of 7.9 minutes (Lindstrom 1997).

Although it was originally suggested that benzene oxide had little reactivity with proteins (Tunek *et al.* 1978) and primarily reacted with nucleic acids (Krewet *et al.* 1993), benzene oxide-protein adducts have been found in mice (McDonald *et al.* 1994) and workers exposed to benzene (Yeowell-O'Connell 1998).

Benzene oxide is therefore generated *in vivo* from benzene metabolism, released from liver into blood, and can reach the bone marrow[7]. Benzene oxide can react with both proteins and nucleic acids and must therefore be considered a viable candidate as a potential toxic metabolite of benzene (Ross 2000).

Several objections in the literature argue against a key role of benzene oxide in benzene induced genotoxicity.

1. Selectivity. It remains unclear why benzene oxide might exert toxicity selectively at the bone marrow (Ross 2000).
2. Reactivity. *In vitro* studies focusing the reactivity of benzene oxide revealed that the reactivity of benzene oxide with DNA is very low and can therefore hardly account for a significant genotoxic damage caused by benzene (Micova & Linhart 2012).
3. DNA-Adducts. 7-Phenylguanine, formed by reaction of benzene oxide with DNA, could not be detected in DNA isolated from mice treated with benzene as well as leukocyte DNA isolated from smokers (Zarth *et al.* 2014).

These findings argue against a key role of benzene oxide in benzene induced hematotoxicity

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[7] Blood circulation takes ca. 50 s in a 70 kg human (Lin 1995)

2.6.4. (*E,E*)-MUCONALDEHYDE

An additional metabolic pathway which cleaves the benzene ring also takes place in liver. Oxidation of benzene oxide leads to formation of (*E,E*)-muconaldehyde, a highly reactive $\alpha,\beta,\gamma,\delta$-unsaturated bis-aldehyde (Latriano *et al.* 1986). Metabolites of (*E,E*)-muconaldehyde including (*E,E*)-muconic acid are detected in the urine of benzene treated animals and benzene-exposed humans (Grotz *et al.* 1994; Zhang *et al.* 2012), giving evidence that the oxidative ring-opening pathway of benzene oxide is utilized *in vivo*. The administration of (*E,E*)-muconaldehyde or benzene to mice gives rise to similar toxic consequences and similarly leads to the excretion of (*E,E*)-muconic acid (Witz *et al.* 1985). (*E,E*)-Muconic acid has also been detected in the bone marrow of mice, indicating that (*E,E*)-muconaldehyde or one of its metabolites is capable of reaching this site (Zhang *et al.* 1997).

(*E,E*)-Muconaldehyde has been shown to be highly hematotoxic in mice (Witz *et al.* 1985). At the cellular level, (*E,E*)-Muconaldehyde inhibits the maturation of erythroid cells in the bone marrow (Snyder 2000) and interferes with gap–junction intercellular communication (Rivedal & Witz 2005). (*E,E*)-Muconaldehyde causes formation of micronuclei in bone marrow cells *in vivo* (Oshiro *et al.* 2001). As an unsaturated aldehyde, (*E,E*)-muconaldehyde reacts with primary amines of protein and DNA. Although not strongly mutagenic in cell-based assays, (*E,E*)-muconaldehyde has been shown to produce DNA damage in a variety of cell lines and the bone marrow *in vivo* (Witz *et al.* 1990; Chang *et al.* 1994). (*E,E*)-Muconaldehyde is cytotoxic by reaction with intercellular sulfhydryl groups which leads to damage in proteins and enzymes (Witz *et al.* 1985) and to the formation of DNA–protein crosslinks in cell lines (Schoenfeld & Witz 1999). The reaction of (*E,E*)-Muconaldehyde with glutathione proceeds rapidly, supposedly in a Michael addition type reaction among others (Witz 1989). In addition to its potent toxicity when administered to mice, (*E,E*)-muconaldehyde also exacerbates the toxic effects of hydroquinone (Witz *et al.* 1990; Short *et al.* 2006).

Several objections in the literature argue against a key role of (*E,E*)-muconaldehyde in benzene induced genotoxicity.

1. Selectivity. A reason for organ specific toxicity of (*E,E*)-Muconaldehyde towards the bone marrow instead of the liver, the location of its formation, is currently unavailable.
2. Diffusibility. Less than 0.05 % of (*E,E*)-Muconaldehyde administered to mice intraperitoneally or by intravenous injection reaches the bone marrow, and it is not clear in what form (*E,E*)-Muconaldehyde reaches the marrow (Zhang *et al.* 1997; Ross 2000).
3. Lack of evidence. Because of its profound reactivity, (*E,E*)-muconaldehyde itself has not been detectable *in vivo* following benzene administration, though it was reported to be detected following *in vitro* microsomal metabolism of benzene (Latriano *et al.* 1986; Grotz *et al.* 1994; Zhang *et al.* 1995; Short *et al.* 2006).

Especially the missing proof for the presence of (*E,E*)-muconaldehyde *in vivo* needs to be addressed assessing the role of (*E,E*)-muconaldehyde in benzene induced hematotoxicity.

2.7. PREVIOUSLY REPORTED CONJUGATES OF (*E,E*)-MUCONALDEHYDE

To assess the relevance of (*E,E*)-muconaldehyde lysine conjugates, we will consider previously reported conjugates deriving from (*E,E*)-muconaldehyde.

2.7.1. SYNTHETIC CONJUGATES

One purely synthetic conjugate of (*E,E*)-muconaldehyde has been reported in the literature (Bleasdale *et al.* 1993) (Figure 5). The missing yield and failure to crystallize the compound indicates, that polymerization issues upon reaction of (*E,E*)-muconaldehyde with propylamine have not been overcome.

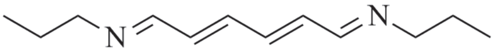

Figure 5 ((*E,E*)-hexa-2,4-diene-1,6-diylidene)bis(propanamine)

In our lab we were able to obtain analogue conjugates from reaction of (*E,E*)-muconaldehyde with simple amines such as benzylamine, aniline or L-valine methyl ester.

2.7.2. GLUTATHIONE (GSH)

Reaction between (*E,E*)-muconaldehyde and GSH occurs rapidly at physiological conditions. In the presence of 10 mM GSH half-life of (*E,E*)-muconaldehyde is calculated to be 6.2 s (Kline *et al.* 1993). Formation of two distinct GSH conjugates of (*E,E*)-muconaldehyde have been reported so far (Henderson *et al.* 2005) (Figure 6). However, identification was only achieved by means of mass spectrometric techniques. The authors were not able to isolate or synthesize these conjugates. One reason might be the inherent reactivity of both molecules. Both GSH conjugates still own reactive aldehyde moieties which e.g. are able to form inter- or intramolecular imines with primary amino groups.

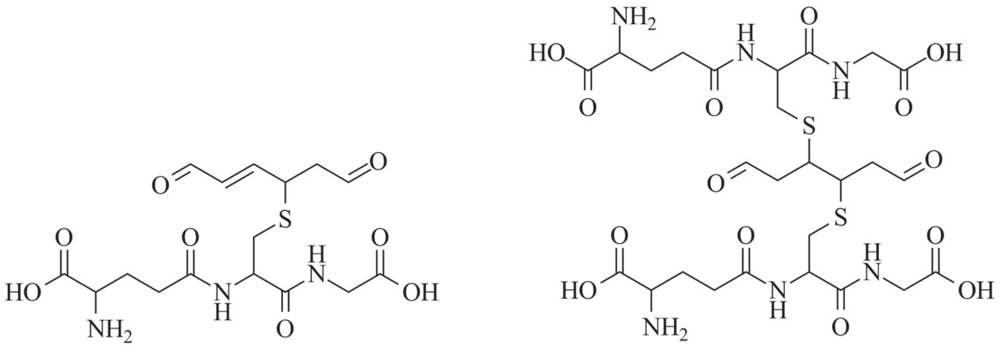

Figure 6 GSH conjugates detected after incubation with (*E,E*)-muconaldehyde (Henderson *et al.* 2005)

That is the reason, why the development of an analytical methodology failed. The question remains whether these GSH-conjugates of (*E,E*)-muconaldehyde are of biological relevance *in vivo*.

2.7.3. DNA

Harris *et al*. reported about DNA conjugates derived from (*E,E*)-muconaldehyde (Harris *et al.* 2011) (Figure 7). The reaction between (*E,E*)-muconaldehyde and desoxyguanosine is highly complex and involves at least 7 distinct intermediate steps, which are partly reversible. After two months (!) reaction time and subsequent reduction with sodium borohydride, four distinct stereoisomers could be isolated.

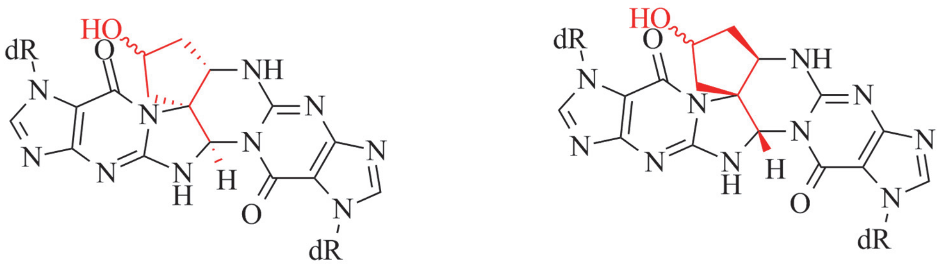

Figure 7 Final adducts formed after reaction of (*E,E*)-muconaldehyde with deoxyguanosine and reduction with sodium borohydride (Harris *et al.* 2011)

The authors did not report, whether these compounds could be detected after incubation of (*E,E*)-muconaldehyde with DNA, thereby giving rise to questions about the biological relevance of the compounds. Since in double stranded DNA guanine pairs with cytosine, it is doubtful whether a conjugate between two guanines could be formed. As in the case of GSH, biological relevance seems difficult to prove giving the complex reaction mechanism needed for formation.

2.7.4. PROTEIN

2.7.4.1. GENERAL REMARKS & SENSITIVITY

DNA adducts are merely available in trace quantities and are subject to enzymatic repair and cell division. For this reason, blood proteins may become more preferable monitors for the presence of reactive xenobiotic metabolites. The majority of chemical species that form DNA adducts also form protein conjugates. Few proteins have been considered worthwhile to investigate for their assignment as suitable biomarkers to benzene exposure. This is because some proteins quickly loose structural or functional integrity as a consequence of conjugate formation. Two prominent protein targets, that have been positively evaluated for biomarker monitoring purposes, are hemoglobin and serum albumin.

Hemoglobin as well as serum albumin are available in large quantities, neither one of them is target for enzymatic repair and both are easily available from blood samples, which favors their suitability as biomarkers (Barnes 2000) (Table 5).

Table 5 Biomacromolecules in blood used as monitor molecules, their availability and turnover rates (modified from (Törnqvist *et al.*, 2002))

Macromolecule	Type of sample	Amount available in blood	Turnover[8] [d]		
			Human	rat	mouse
Hemoglobin	Erythrocytes	~ 150 mg/ml	126	60	40
Serum albumin	Blood plasma	30–45 mg/ml	20	2.5	1.9
5’-Desoxynucleic acid (DNA)	Leukocytes	~ 6 mg/ml	Complex kinetics		

2.7.4.2. PROTEIN CONJUGATES OF (*E,E*)-MUCONALDEHYDE

There are currently no reports on distinct protein conjugates deriving from (*E,E*)-muconaldehyde. Taken together with the missing proof of existence for (*E,E*)-muconaldehyde *in vivo,* this missing link in understanding the role of (*E,E*)-muconaldehyde in myelotoxicity of benzene gave birth to the idea for this project.

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[8] Half-life for serum albumin

3. OBJECTIVES

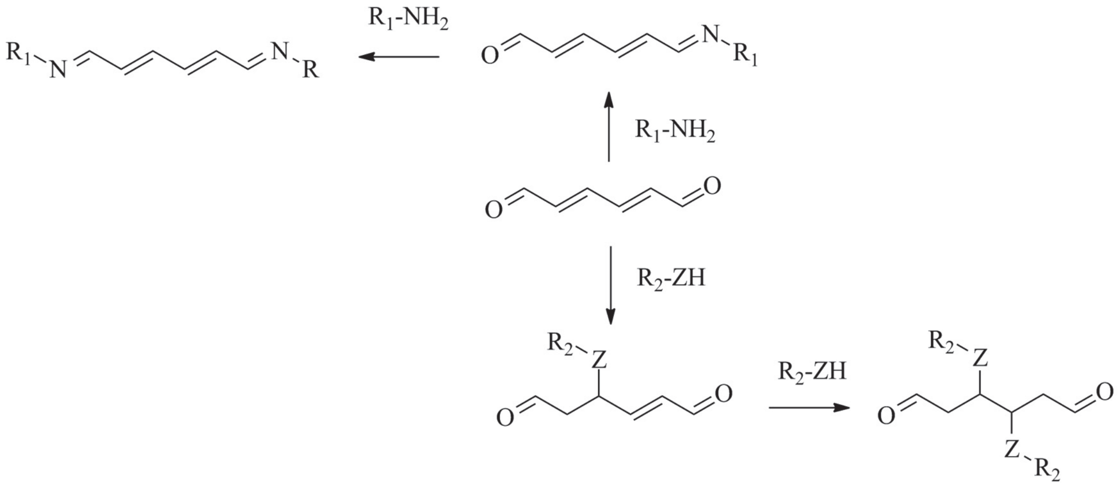
This project focused on formation of a protein conjugate derived from (*E,E*)-muconaldehyde which could be used to proof its existence *in vivo* indirectly. Therefore, the following steps needed to be accomplished.

1. Testing whether or not (*E,E*)-muconaldehyde forms a protein conjugate under physiological conditions.
2. If so, identification and characterization of this conjugate.
3. Establishment of an analytical procedure for this compound and testing whether this compound is formed *in vivo* following benzene administration.
4. Assessing *in vitro* toxicity of this compound to answer the question whether a transient conjugate of (*E,E*)-muconaldehyde might be responsible for genotoxicity of benzene.
5. Discussing the utility of this conjugate as a biomarker of benzene.

4. RESULTS

4.1. REACTION OF (*E,E*)-MUCONALDEHYDE WITH SINGLE AMINO ACIDS

In a first step (*E,E*)-muconaldehyde has been reacted with alpha amino protected amino acids in aqueous media simulating *in vivo* conditions (Figure 9 – Figure 11). Amino acids lysine (primary amines), histidine (secondary amines) and cysteine (thiol) were chosen based on several reports regarding their reactivity towards α,β-unsaturated carbonyls such as acrolein (Uchida *et al.* 1998; Esterbauer *et al.* 1991) and (*E,E*)-muconaldehyde itself (Bleasdale *et al.* 1996; Henderson *et al.* 2005) (Scheme 2).



Scheme 2 Simplified reaction outcome expectations for reaction of (*E,E*)-muconaldehyde with different nucleophiles, ZH = thiols or secondary amines. R_1,R_2 = alkyl

Fluorenylmethyloxycarbonyl (Fmoc) was chosen as an α-amino protecting group due to its characteristic UV-absorbance spectrum and to mimic amino acid reactivity within the protein (Figure 8). Every potential amino acid adduct deriving from reaction with (*E,E*)-muconaldehyde should therefore own the characteristic UV spectrum provided by Fmoc even if no other chromophores are present within the molecule.

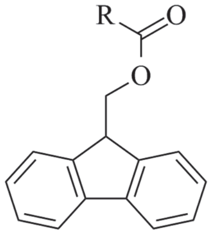


Figure 8 Fluorenylmethyloxycarbonyl (Fmoc)

Reactions were run for 48 hours with equimolar concentrations at three different pH values of 3, 7 and 10 before HPLC analysis. Chromatograms at pH = 3 and pH = 10 are not depicted here since they point to the same results as with pH = 7, they are however attached in the electronic attachments (cf. ME 34). The only major difference at basic pH was a reduction in UV absorbance intensity from (*E,E*)-muconaldehyde probably resulting from reduced stability at alkaline pH. Compounds were measured individually and chromatograms were compared with those of reaction mixtures. Reaction products were expected to elute in between the two educts due to their combined polarity.

Chromatograms of reaction mixtures of (*E,E*)-muconaldehyde with Fmoc-Lysine and Fmoc-Histidine however revealed no additional peaks aside from starting materials. Reaction of (*E,E*)-muconaldehyde with Fmoc-cysteine resulted in diffuse reaction products aside to complete consumption of (*E,E*)-muconaldehyde. One distinct peak between 5 to 6 minutes could be detected, however bearing no UV maximum at 266 nm characteristic for Fmoc. It was concluded therefore that no Fmoc group is still present within the molecule and thus this peak does not qualify as an amino acid adduct, rather a polymerization byproduct deriving from (*E,E*)-muconaldehyde generated by Fmoc-Cysteine.

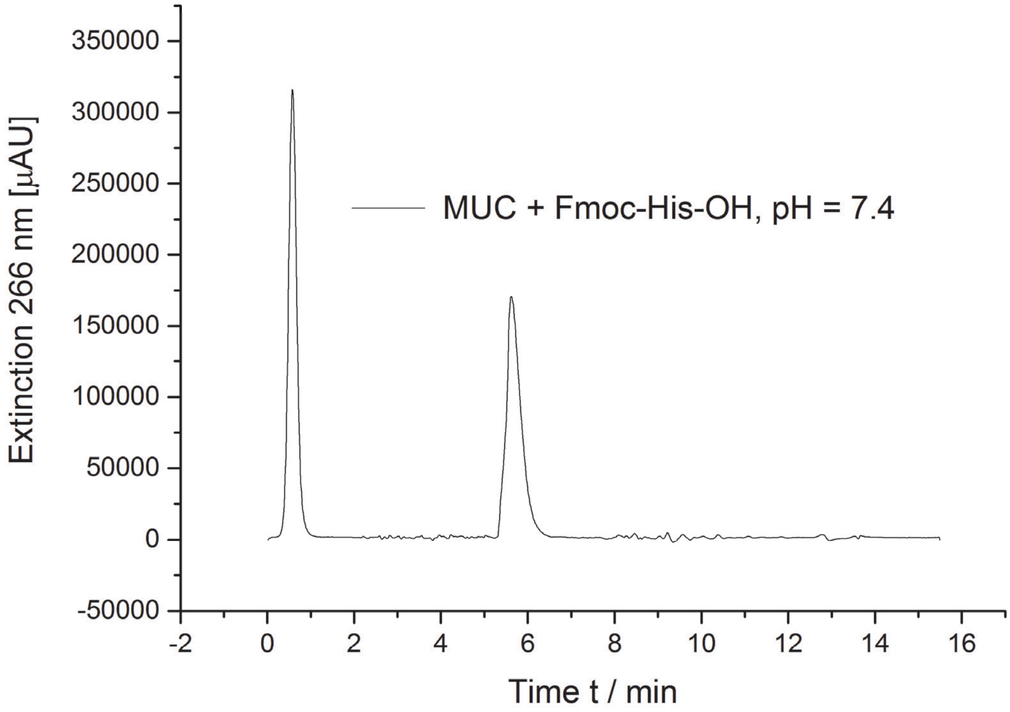


Figure 9 HPLC-UV chromatogram of a reaction mixture of (*E,E*)-muconaldehyde (0.5 mM) with Fmoc-His-OH (0.5 mM) in aqueous MOPS buffer (10 mM, pH 7.4) after 48 h.

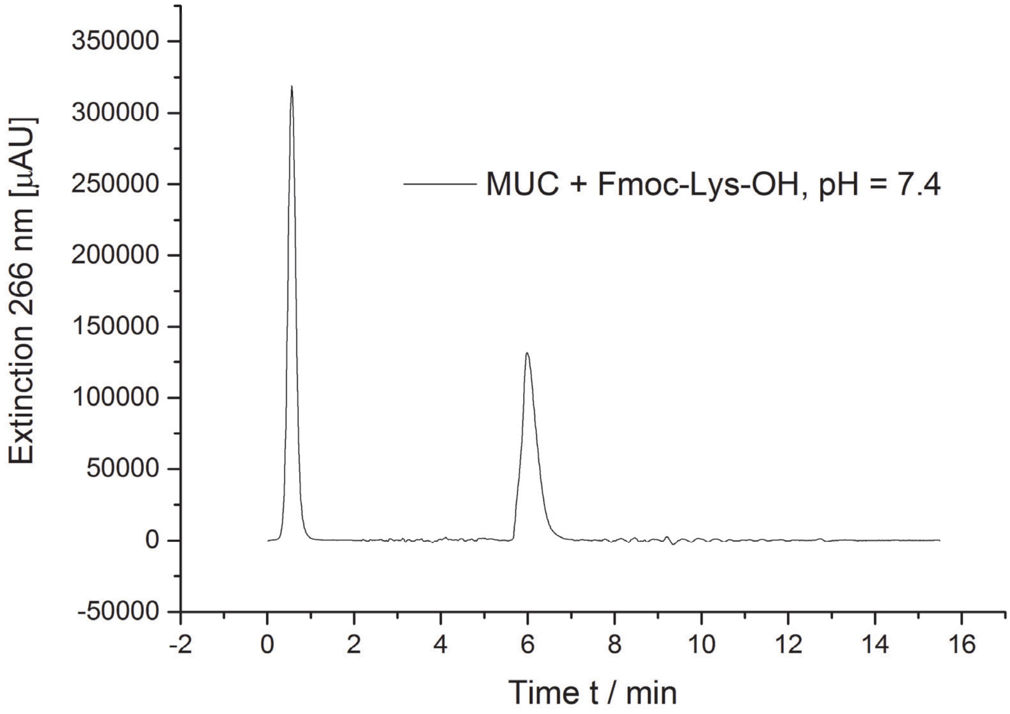


Figure 10 HPLC-UV chromatogram of a reaction mixture of (*E,E*)-muconaldehyde (0.5 mM) with Fmoc-Lys-OH (0.5 mM) in aqueous MOPS buffer (10 mM, pH 7.4) after 48 h.

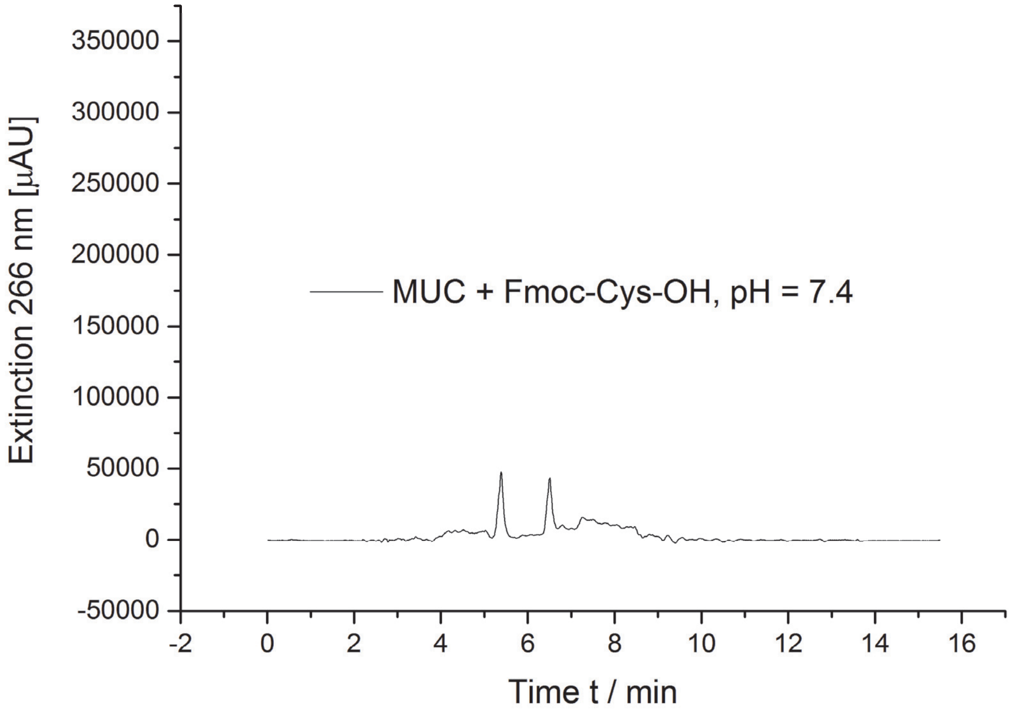


Figure 11 HPLC-UV chromatogram of a reaction mixture of (*E,E*)-muconaldehyde (0.5 mM) with Fmoc-Cys-OH (0.5 mM) in aqueous MOPS buffer (10 mM, pH 7.4) after 48 h.

4.2. REACTION OF (*E,E*)-MUCONALDEHYDE WITH BOVINE SERUM ALBUMIN

As a result of not being able to sort out a single amino acid forming a distinct individual amino acid conjugate, a "top down" approach was used next. (*E,E*)-muconaldehyde was tested in reaction with bovine serum albumin (BSA). BSA was chosen as a model protein because of its use in biomarker studies as well as its low UV absorbance above 300 nm which proved to be useful in monitoring the reaction. Additionally, in contrast to bovine hemoglobin, BSA owns a functional cysteine which is able to react with electrophiles and is used in exposure monitoring as biomarker, e.g. in the case of sulfur mustard (Noort *et al.* 1999).

Reaction of bovine serum albumin with (*E,E*)-muconaldehyde lead to a visible change in color from colorless to reddish (Figure 12). Spectroscopically, formation of two distinct absorbance maxima at 325 and 490 nm could be detected following the reaction (Figure 13). After 15 hours virtually no increase in absorbance could be seen for the absorbance maximum at 490 nm in contrast to the absorbance maximum at 325 nm. The absorbance maximum at 325 nm increased continually for more than three days indicating that reaction between (*E,E*)-muconaldehyde and bovine serum albumin still had not finished at that point (Figure 14).

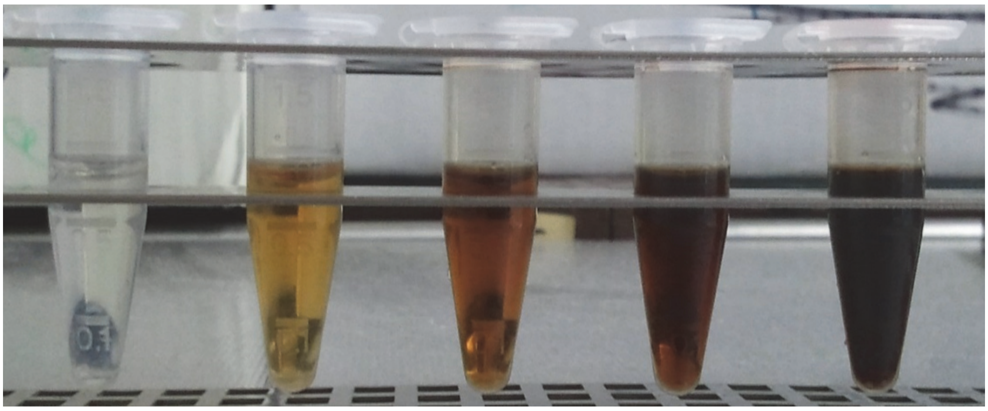


Figure 12 Reaction mixtures of bovine serum albumin (12 mg/ml) with varying concentrations of (*E,E*)-muconaldehyde (750 µM – 7.5 mM) after 24 h at pH = 7.4

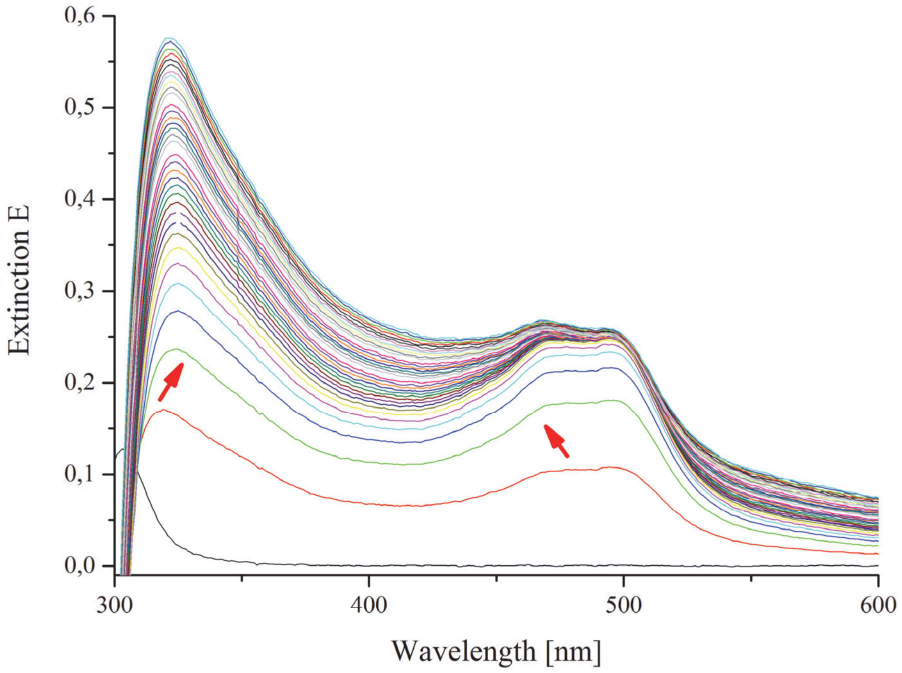


Figure 13 Overlay of UV/VIS-spectra (300 – 600 nm) taken every 2 hours over 72 h of a reaction mixture of bovine serum albumin (4 mg/ml) and (*E,E*)-muconaldehyde (100 µM). Red arrows indicate distinct shifts in absorption maxima. The black line indicates the UV/VIS-absorption immediately after mixture of reagents.

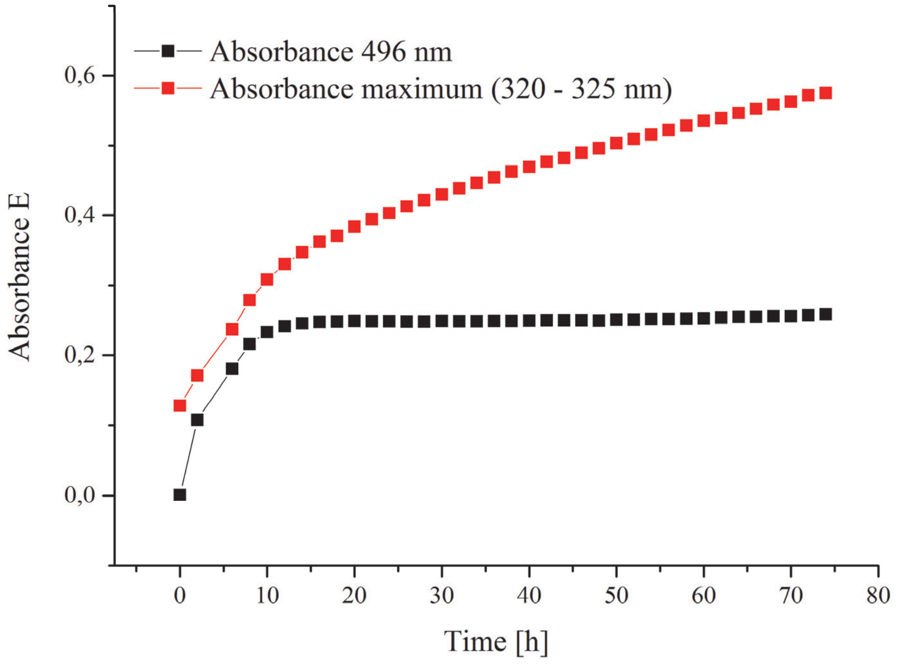


Figure 14 Progression of UV absorbance of two prominent absorbance maxima every 2 hours over 72 h of a reaction mixture of bovine serum albumin (4 mg/ml) and (*E,E*)-muconaldehyde (100 µM).

Due to the unusual reaction color the possibility of a non-covalent ligand reaction of (*E,E*)-muconaldehyde involving traces of transition metals present in the commercial protein sample such as iron or copper was considered. However, addition of EDTA (100 µl 0.1 M Na_2EDTA) to the reaction mixture consisting of (*E,E*)-muconaldehyde (1 ml, 0.1 mM) and bovine serum albumin (4 mg/ml) did not result in the extinction of the formed chromophore. Furthermore addition of Fe(III) (100 µl 60 – 6000 µM $Fe(III)(NO_3)_3$) to an aqueous solution of (*E,E*)-muconaldehyde (1 ml, 100 µM) did not result in the formation of the same chromophore.

Several methods for protein hydrolysis were employed to elucidate which amino acids were involved in the reaction. However, neither acidic (6 N HCl, 24 h, 110°C) nor alkaline (4.2 M NaOH, 24 h, 110°C) nor enzymatic hydrolysis (1% Pronase (w/w), 24 h, 37°C) resulted in preservation of the chromophore (Figure 15, cf. MSE 10). Furthermore addition of (*E,E*)-muconaldehyde to the reaction mixture (100 µl 0.1 mM) after enzymatic hydrolysis (1% w/w, 24 h, 37°C) of BSA (12 mg/ml) did not result in formation of the chromophore.

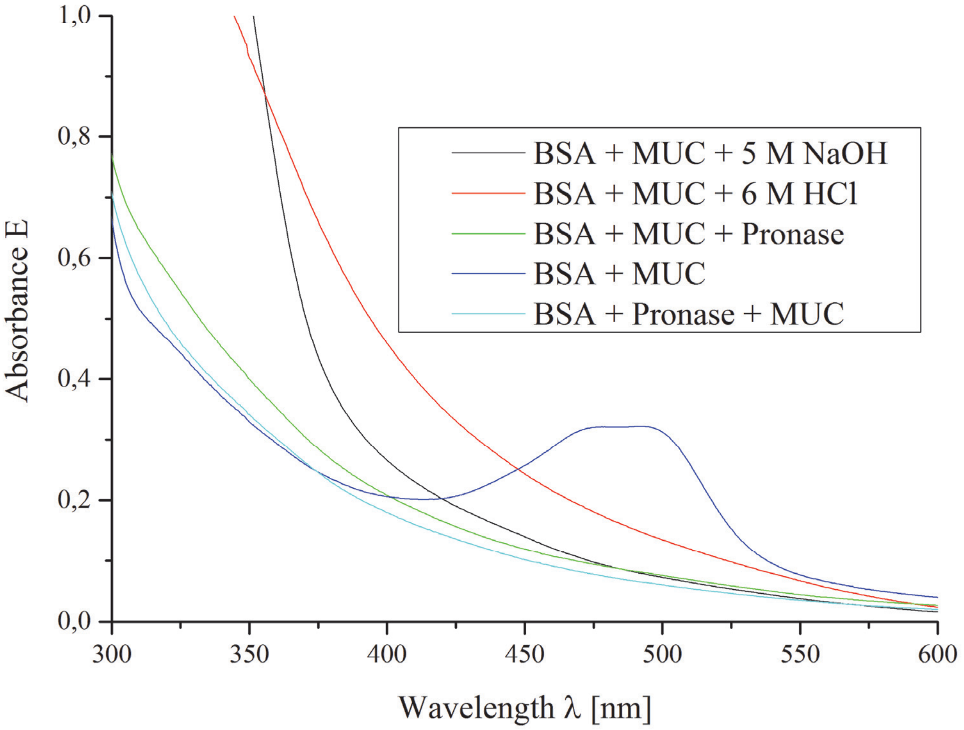


Figure 15 UV/VIS spectra from different reaction mixtures containing bovine serum albumin (BSA) (16 mg/ml) and (*E,E*)-muconaldehyde (0.1 mM) after hydrolysis.

The first hint in identification of the compound, which we were not able to interpret at the time, was the observation that addition of trichloroacetic acid led to a colorful intensification of the red chromophore followed by a slower decrease in intensity until no visible color remained.

4.2.1. DETERMINATION OF ENZYMATIC HYDROLYSIS EFFICIENCY

To assess enzymatic hydrolysis efficiency, a microplate-based detection of amino acids by derivatization with fluorescamine was employed (Bantan-Polak *et al.* 2001). Calibration was achieved using a polynomial fit with $R^2 = 1.00$ over 2.5 – 2000 µM using L-glycine as external calibration standard (Figure 16). Enzymatic hydrolysis using pronase, aminopeptidase M & prolidase was compared to acidic hydrolysis using 6 N HCl. Compared to acidic hydrolysis (relative fluorescence intensity (RFI) = 100 ± 3 %, n = 2) enzymatic hydrolysis resulted in RFI = 63 ± 5 %, n = 3 (Figure 17).

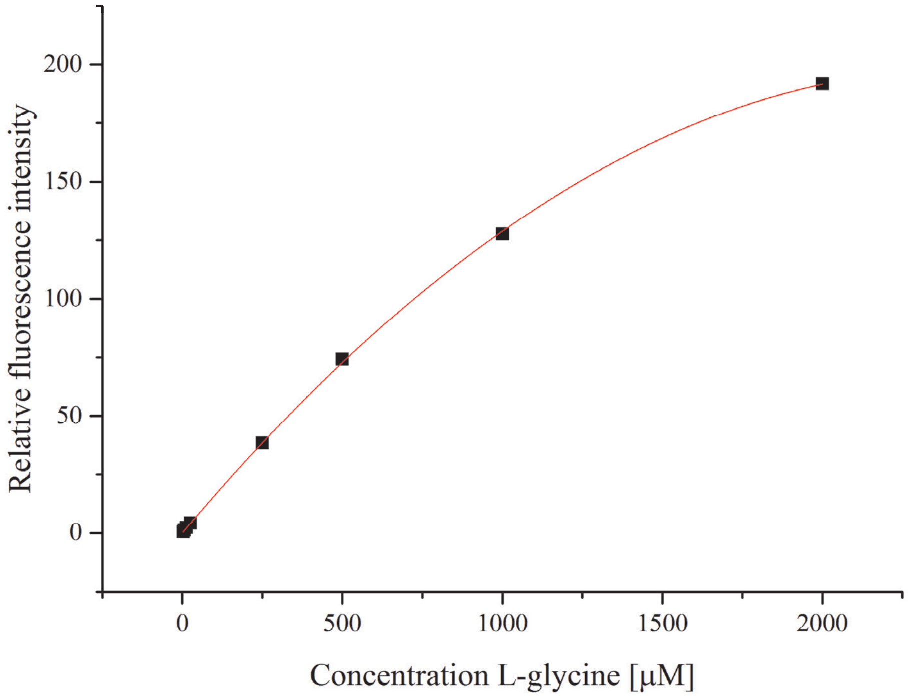


Figure 16 Calibration curve using microplate-based detection of amino acids by derivatization with fluorescamine using L-glycine (2.5 – 2000 µM) as external calibration standard (Bantan-Polak *et al.*, 2001)

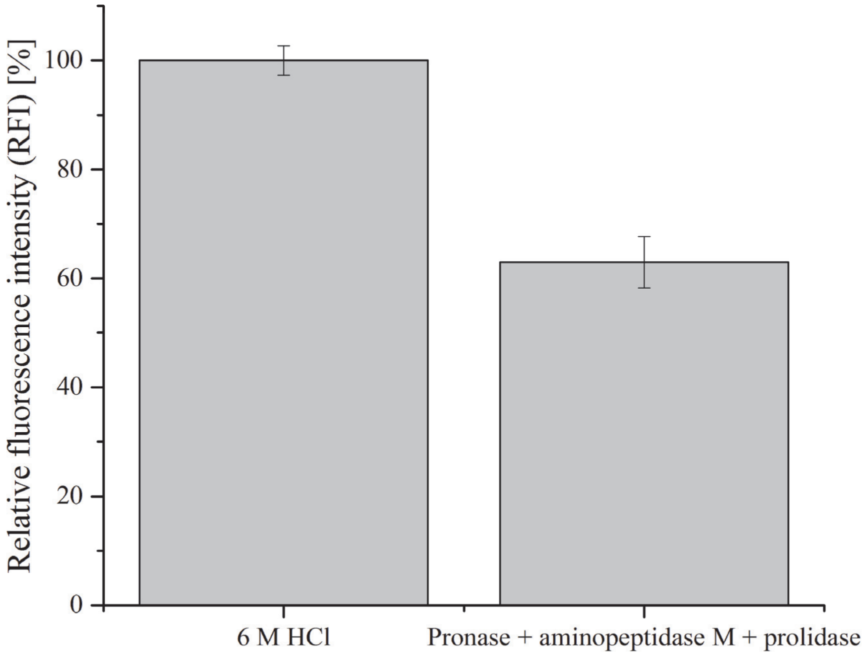


Figure 17 Relative fluorescence intensity (RFI) from hydrolysates of bovine serum albumin (3 mg/ml) using fluorescamine (Bantan-Polak *et al.*, 2001) after acidic hydrolysis using 6 N HCl (n = 2) or enzymatic hydrolysis using pronase, aminopeptidase M and prolidase (n = 3).

4.2.2. HPLC-UV ANALYSIS OF THE ENZYMATIC HYDROLYSATE

Following HPLC analysis of enzymatic hydrolysates of bovine serum albumin a distinct peak not present in control incubations without (*E,E*)-muconaldehyde could be detected. However, a distinct mass belonging to this signal could not be determined by mass spectrometric analysis (cf. ME 50).

A closer study of the literature revealed the second hint leading to a major breakthrough in the identification process of a compound related to the described peak. In Roger L. Lundblad's book *Chemical reagents for protein modification* the following note was found:

> *"Shapiro and coworkers investigated the reaction of PLP [sc. pyridoxal-phosphate] with rabbit muscle aldolase. The initial reaction produced a species with an absorbance maximum at 430-435 nm, reflecting the protonated Schiff base form of the PLP-protein complex. After reduction with sodium borohydride, the absorbance maximum was at 325 nm characteristic of the reduced Schiff base." (Lundblad 2015)*

Due to the similarity of the described chromophore with our unidentified compound we seized immediately on the idea of a reduction of the species with sodium borohydride.

Reduction of the crude reaction mixture with sodium borohydride diminished the red chromophore, leaving a faint yellow solution (Figure 18). However, no characteristic absorbance maximum above the common absorbance maximum of proteins of 280 nm could be detected. Upon HPLC-UV analysis a distinct signal not present in control incubations could be detected (Figure 19).

However, repetition of this reaction on a preparative scale and isolation of the peak failed to produce an evaluable NMR spectrum (Figure 20, cf. ME 40).

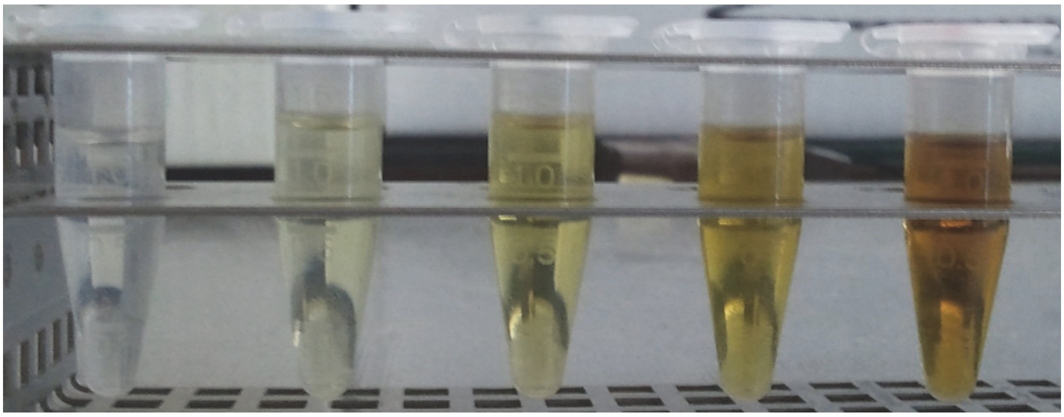


Figure 18 Reaction mixtures of bovine serum albumin (12 mg/ml) with varying concentrations of (*E,E*)-muconaldehyde (750 µM – 7.5 mM) after reduction with sodium borohydride.

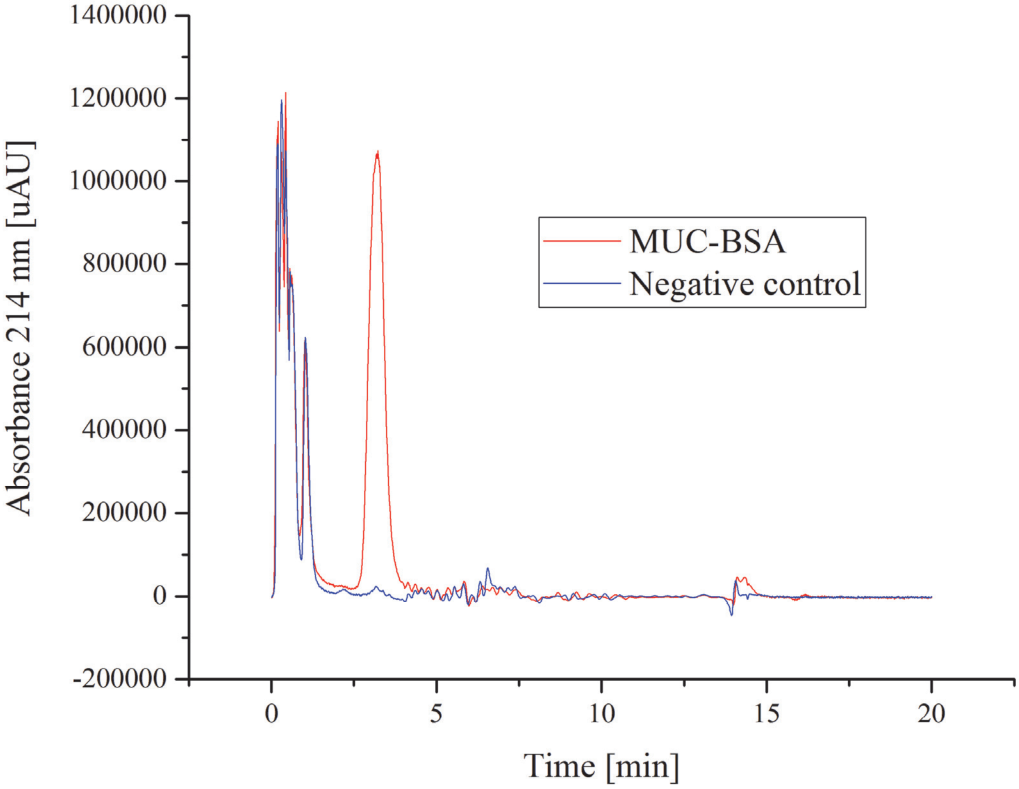
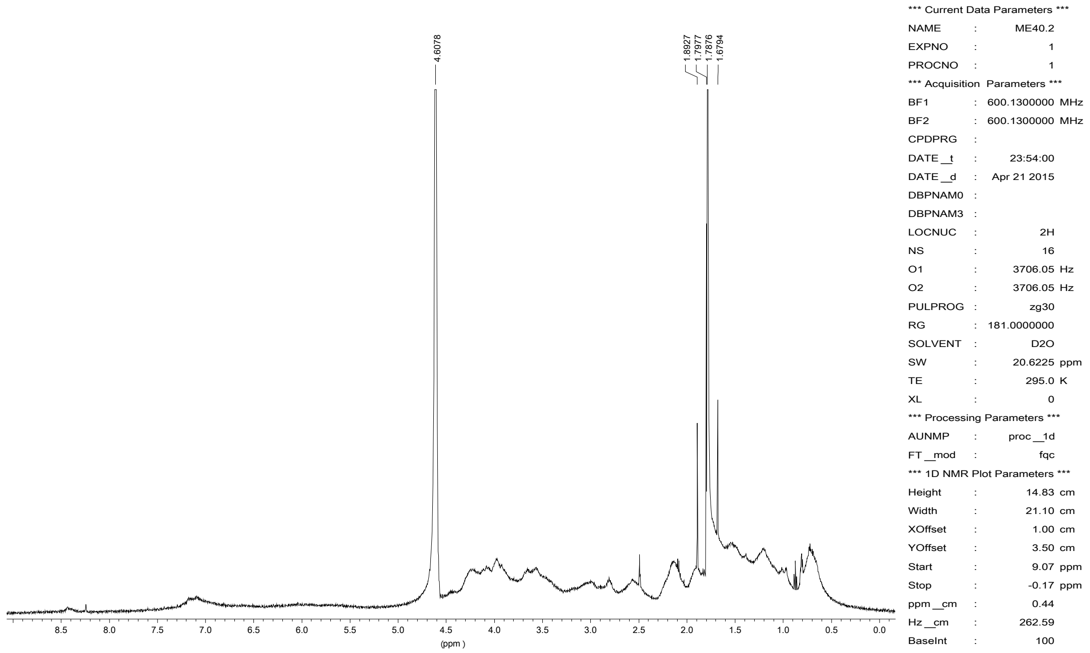


Figure 19 Comparison of HPLC-UV chromatograms (214 nm) of an enzymatic hydrolysate of bovine serum albumin reacted with (*E,E*)-muconaldehyde after reduction with sodium borohydride.



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DATE _t   :      23:54:00
DATE _d   :   Apr 21 2015
DBPNAM0   :
DBPNAM3   :
LOCNUC    :            2H
NS        :            16
O1        :       3706.05 Hz
O2        :       3706.05 Hz
PULPROG   :          zg30
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XL        :             0
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Stop      :         -0.17 ppm
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Hz _cm    :        262.59
BaseInt   :           100
```

Figure 20 ^{1}H NMR spectrum of preparative eluate of the isolated additional HPLC peak after reaction of bovine serum albumin with (*E,E*)-muconaldehyde and subsequent reduction with sodium borohydride and enzymatic hydrolysis. No specific NMR signals can be detected.

A possible explanation for failure of successful compound isolation lies in the inability of enzymatic hydrolysis to cleave all peptide bonds of the protein. Therefor many different short peptides attached to (*E,E*)-muconaldehyde might elute simultaneously so that there is no chance to single out a distinct amino acid conjugate. In retrospect, a lack of chemical stability furthermore compromised a successful compound isolation.

4.2.3. HPLC-MS ANALYSIS OF THE ENZYMATIC HYDROLYSATE

Mass spectrometric analysis revealed a distinct Q1 mass of m/z = 241.1 belonging to this new signal observed after reaction of (*E,E*)-muconaldehyde with bovine serum albumin following reduction with sodium borohydride and enzymatic hydrolysis (Figure 19 – Figure 23, cf. ME 43). A reasonable structure for m/z = 241.1 confirming the observation from Shapiro and coworkers (see above) would be the reduced Schiff base of a (*E,E*)-muconaldehyde-Lysine conjugate with a calculated m/z ($M+H^+$) = 241.1 (Figure 24).

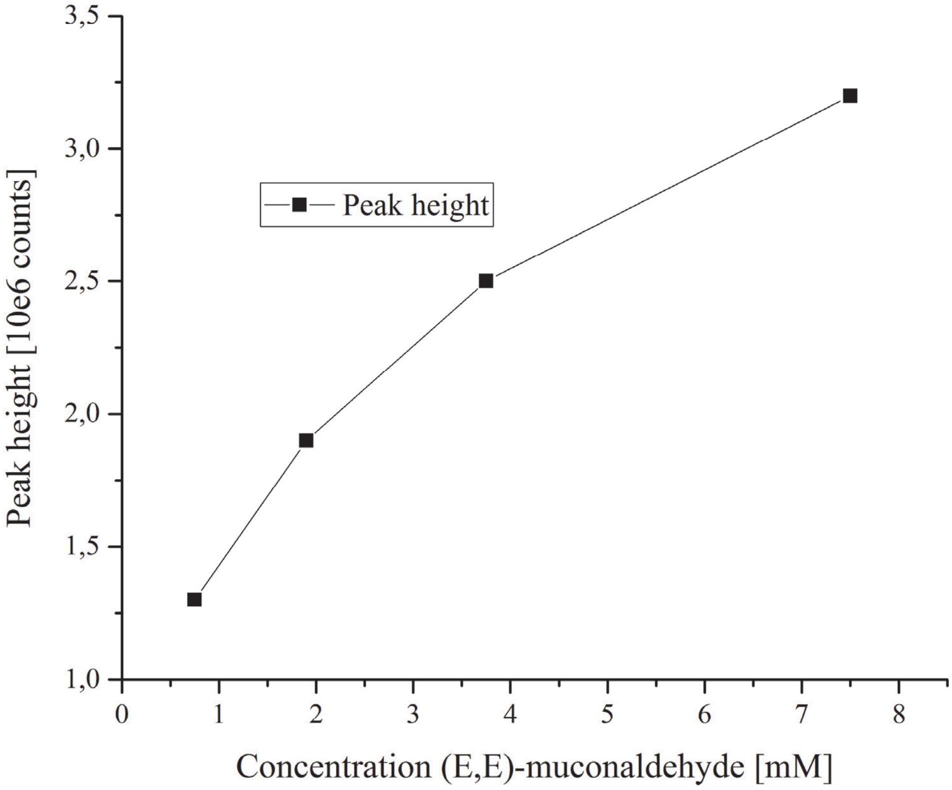

Figure 21 Progression of Peak height of the new HPLC-MS-Signal with m/z = 241.1 at RT = 5.8 min detected from an enzymatic digests of bovine serum albumin (12 mg/ml) after reaction with different concentrations of (*E,E*)-muconaldehyde (0.6 mM – 6 mM), reduction with sodium borohydride (2 mg) and enzymatic hydrolysis of the protein using pronase

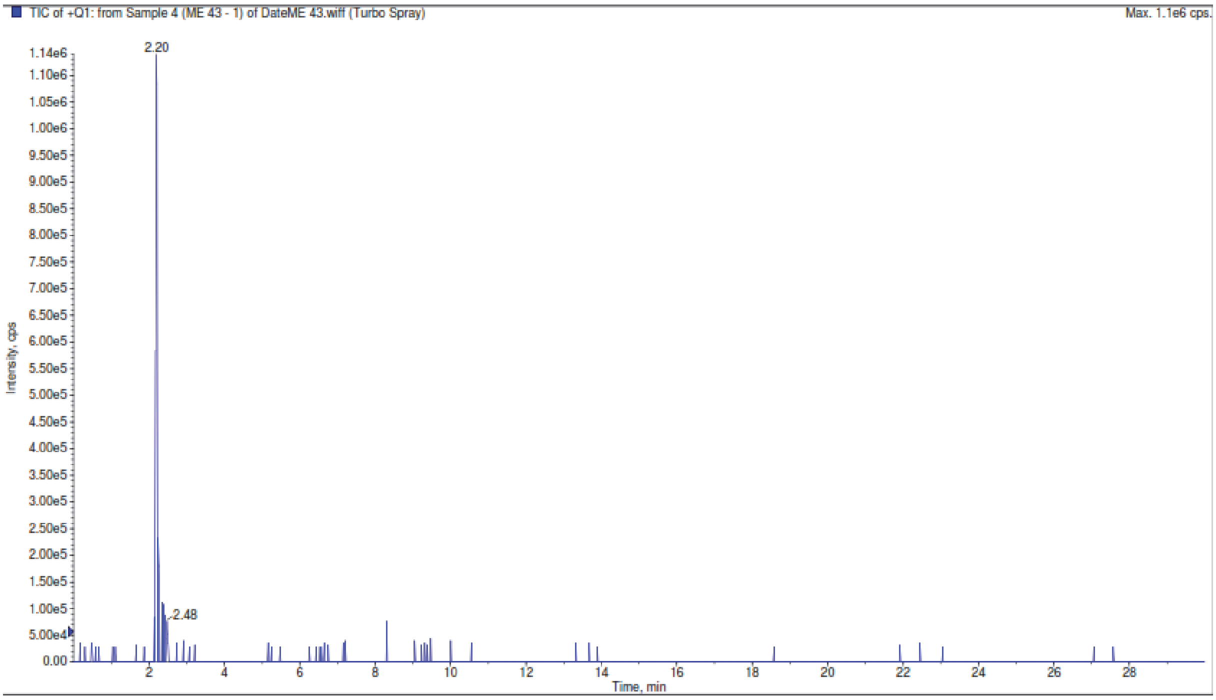

Figure 22 HPLC-MS chromatogram (XIC) for m/z = 240.5-241.5 from an enzymatic digests of bovine serum albumin (12 mg/ml) without reaction with (*E,E*)-muconaldehyde, reduction with sodium borohydride (2 mg) and enzymatic hydrolysis of the protein using pronase.

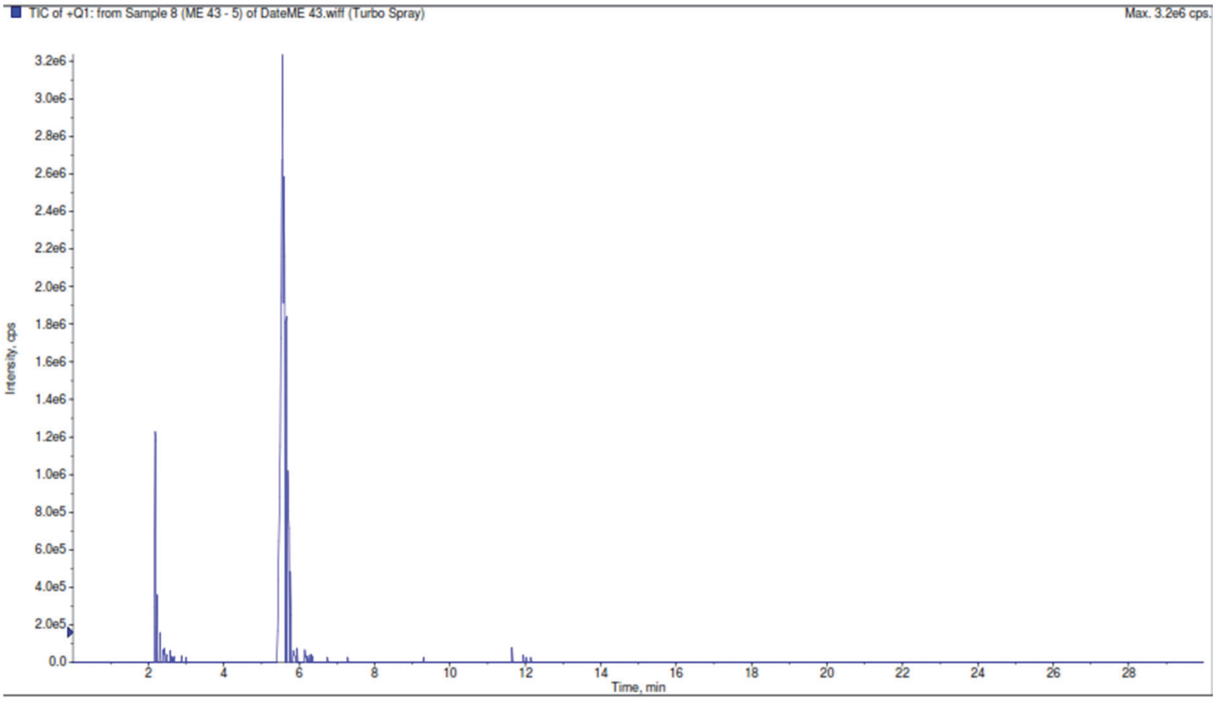

Figure 23 HPLC-MS chromatogram (XIC) for m/z = 240.5-241.5 from an enzymatic digests of bovine serum albumin (12 mg/ml) after reaction with (*E,E*)-muconaldehyde (6 mM), reduction with sodium borohydride (2 mg) and enzymatic hydrolysis of the protein using pronase.

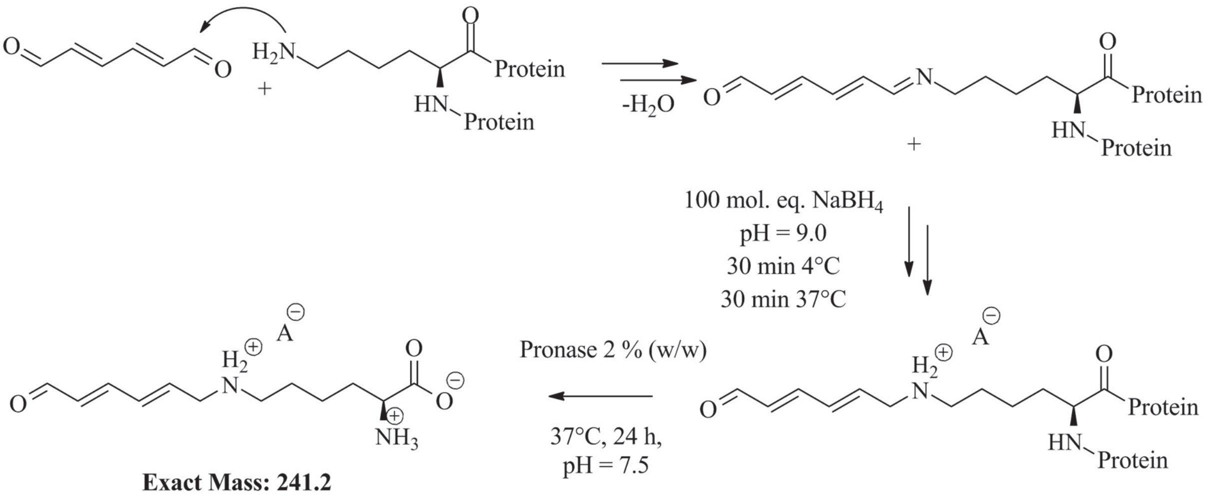

Figure 24 Structural proposal for the initial reaction product from reaction of bovine serum albumin with (*E,E*)-muconaldehyde upon reduction with sodium borohydride and enzymatic hydrolysis.

4.2.4. ONE POT CONVERSION OF N^{α}-ACETYL LYSINE METHYL ESTER WITH (*E,E*)-MUCONALDEHYDE

In an attempt to verify the assumption that mass signal 241.1 from incubation of (*E,E*)-muconaldehyde with bovine serum albumin belongs to a substituted lysine species, a one pot conversion was developed using N^{α}-acetyl lysine methyl ester (Ac-Lys-OMe).

Protection groups of the lysine component were changed from Fmoc for the following reasons:

1. The possibility could not be precluded that protection of the carboxyl group of the lysine compound (as in the protein) was necessary to form the conjugate[9].
2. If both carboxyl group and amino groups of the lysine compound are protected, adequate solubility for synthesis in aqueous media may be not given because of increased hydrophobicity.
3. The acetyl and methyl group are both small protecting groups, therefore Ac-Lys-OMe is sufficiently soluble in water[10].
4. Both acetyl and methyl ester protecting groups are enzymatically cleavable by the same conditions using one single enzyme, e.g. aminoacylase I.

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[9] No reaction was observed previously from reaction of (*E,E*)-muconaldehyde with Fmoc-Lys-OH, which possesses an unprotected carboxyl moiety (see section 4.1 Reaction of (*E,E*)-muconaldehyde with single amino acids)

[10] Aqueous solubility of Ac-Lys-OMe*HCl > 50 mg/ml at 25°C (Sigma-Aldrich 2018)

Aminoacylase I from *Aspergillus melleus* is a readily available and inexpensive enzyme mainly used in the industrial production of enantiopure L-amino acids from their N-acetyl derivatives, and also hydrolyzes the esters and amides of natural and non-natural amino acids with high enantioselectivity. (Youshko *et al.* 2004).

After reaction of Ac-Lys-OMe with (*E,E*)-muconaldehyde and subsequent reduction with sodium borohydride as well as enzymatic cleavage of the protecting groups using aminoacylase I, a signal was detected whose HPLC retention time (Figure 25), UV spectrum (Figure 26), Q1 mass (Figure 27) and MS^2 spectrum[11] (Figure 28 & Figure 29, cf. KS 111/114) matched those of the signal detected after incubation of (*E,E*)-muconaldehyde with bovine serum albumin (cf. ME 42/46). Therefore, we got evidence enough to verify the structure of the chromophore formed upon reaction of (*E,E*)-muconaldehyde with BSA.

Next, we strived to achieve synthetic access to the compound m/z = 241.1 for three main reasons.

1. Identification. Synthesis of a compound is necessary to conclude the verification process, that the substance obtained analytically belongs to the postulated structure.
2. Analysis. Optimization of analytical procedures and quantification requires the possession of the analyte as reference material.
3. Toxicological testing. To assess the relevance in benzene toxicity, employment of the substance in toxicological screening is necessary.

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[11] MS^2 spectra were performed by Katrin Schmidt.

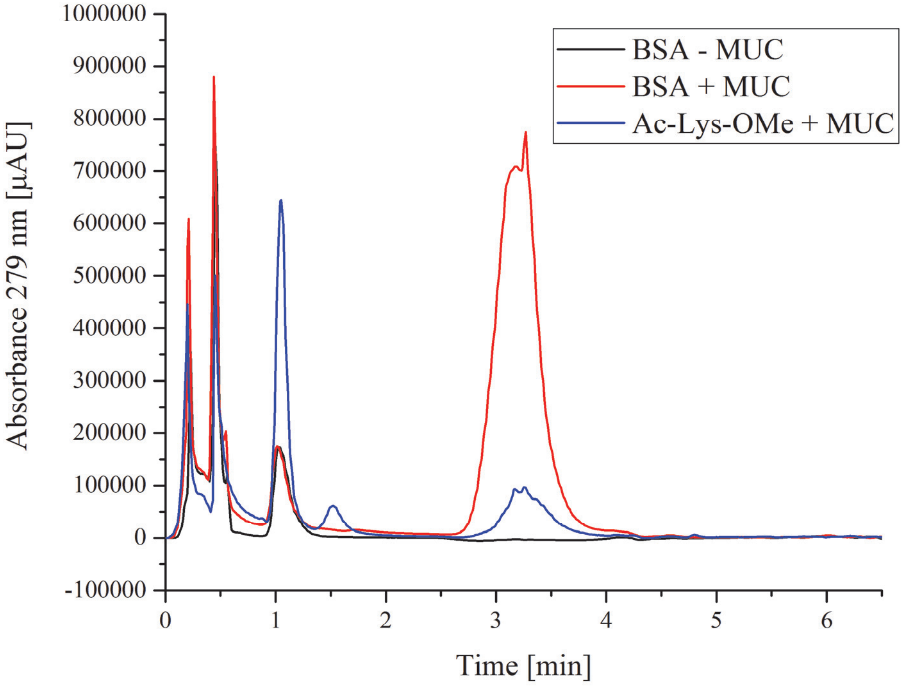


Figure 25 Overlay of HPLC-UV-chromatograms from analysis of reaction mixtures of bovine serum albumin with (red) or without (black) (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic hydrolysis or reaction of Ac-Lys-OMe with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic cleavage of protecting groups (blue).

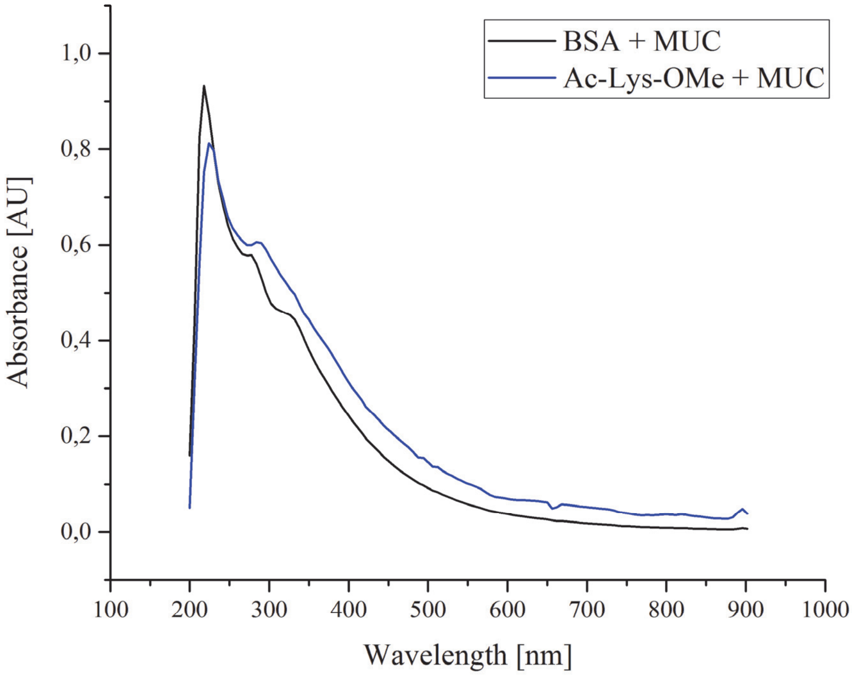


Figure 26 Comparison of UV-Spectra of the additional HPLC signal observed after reaction of bovine serum albumin with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic hydrolysis (black) or reaction of Ac-Lys-OMe with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic cleavage of protecting groups (blue).

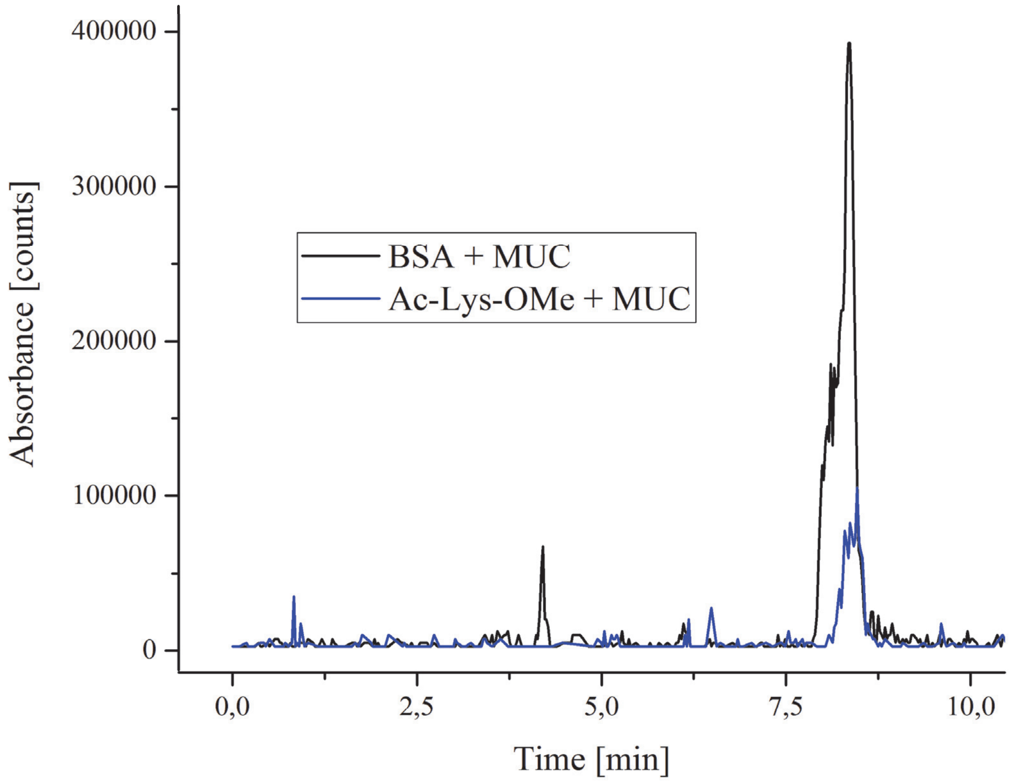


Figure 27 Comparison of HPLC -MS signals (XIC) for m/z = 240.5-241.5 after reaction of bovine serum albumin with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic hydrolysis (black) or reaction of Ac-Lys-OMe with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic cleavage of protecting groups (blue).

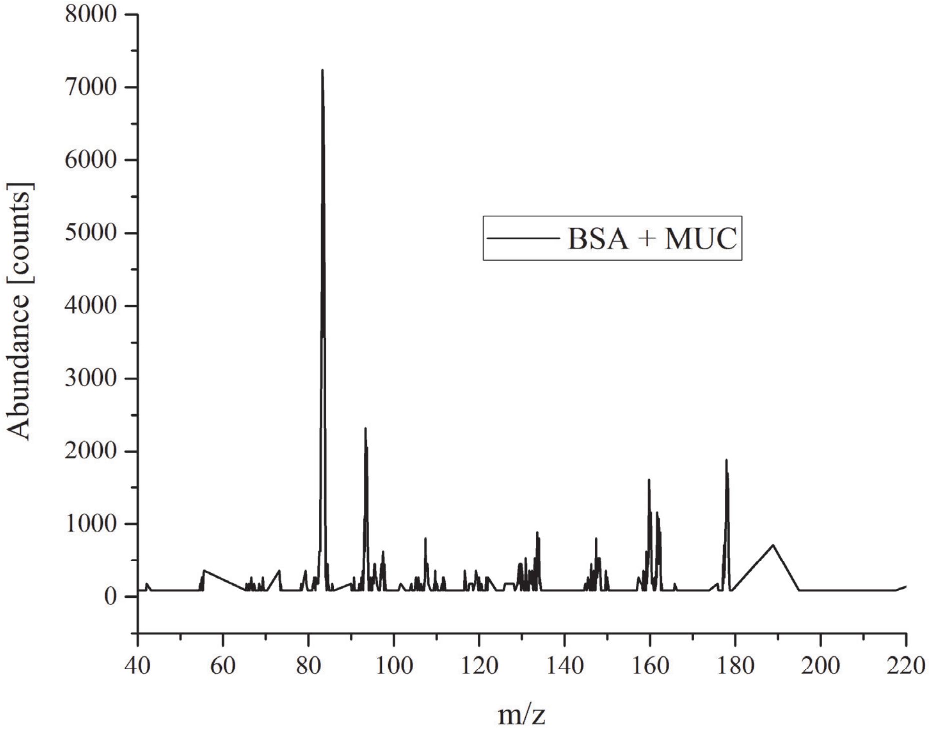


Figure 28 MS² spectrum of the additional HPLC MS signal (Q1) with m/z = 241.1 after reaction of bovine serum albumin with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic hydrolysis.

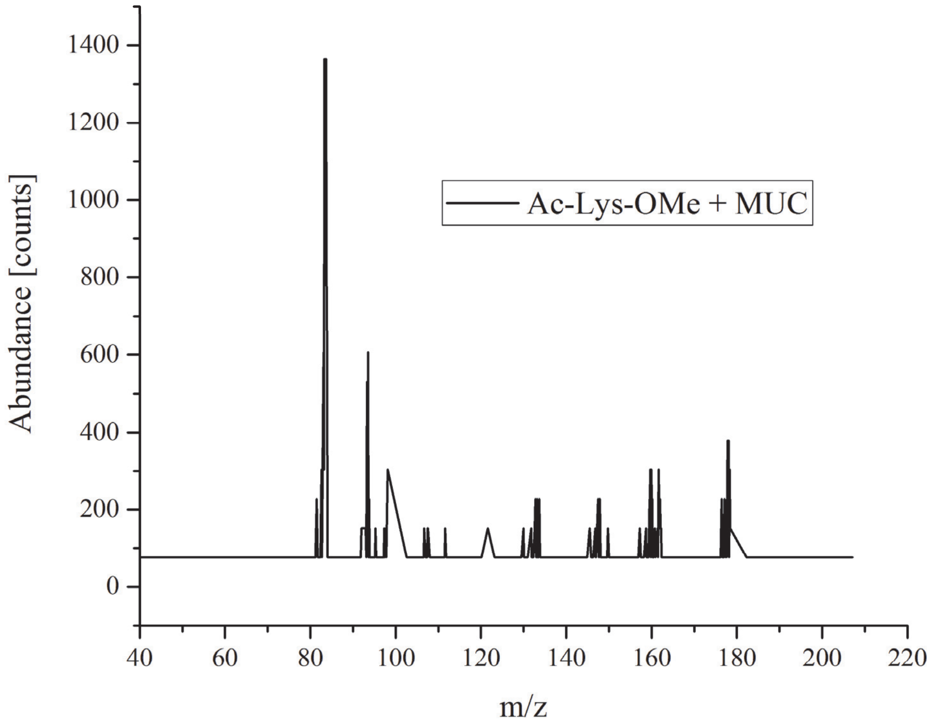
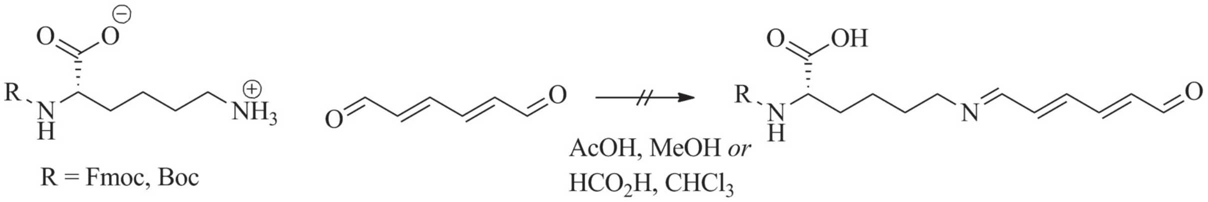


Figure 29 MS² spectrum of the additional HPLC MS signal (Q1) with m/z = 241.1 after reaction of Ac-Lys-OMe with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic cleavage of protecting groups.

4.3. SYNTHESIS OF (*E,E*)-MUCONALDEHYDE-LYSINE CONJUGATES

4.3.1. PREPARATORY STEPS

At first attempts for the synthesis of imine conjugates were undertaken in an effort to maintain simplicity of the synthetic route by directly coupling commercially available monoprotected lysine derivatives with (*E,E*)-muconaldehyde according to protocols reported in the literature for structurally similar terephtalaldehyde (Pettersson *et al.* 2008; Kumar *et al.* 2009) (Scheme 3).



Scheme 3 First attempt for imine conjugate formation starting from (*E,E*)-muconaldehyde

However, observations made from these reactions were that no monomeric product could be isolated. Supposedly due to (*E,E*)-muconaldehyde induced polymerization a rapid coloration of the reaction mixture from colorless to black was seen. At the end of the reaction a black residue could be recovered which was insoluble in most organic solvents. Additionally, no product spot could be detected by thin layer chromatography on either silica gel or aluminum oxide.

To preclude the possibility of the amine being the cause for this behavior, reactions were repeated with simple amines such as alanine methyl ester (Figure 30) with the same result.

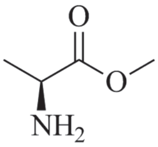
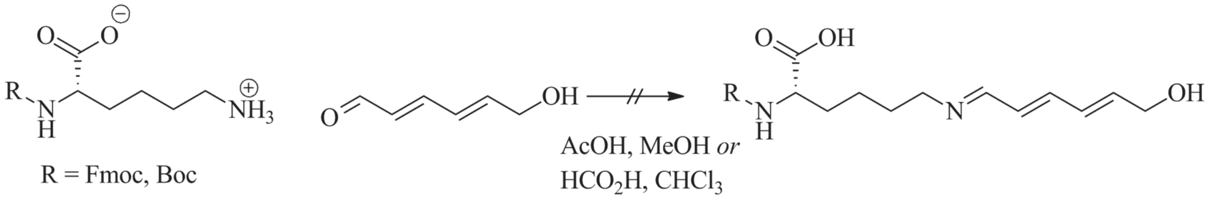


Figure 30 L-Alanine methyl ester

At the time, we attributed (*E,E*)-muconaldehyde induced polymerization to the bifunctionality of the molecule and concluded that removal of one aldehyde function would lead to imine formation. Therefore we performed reduction of one aldehyde moiety starting from (*E,E*)-muconaldehyde with sodium borohydride. However, repetition of imine formation according to scheme 2 with (*E,E*)-6-hydroxyhexa-2,4-dienal also lead to formation of unidentifiable black polymerization products (Scheme 4).



Scheme 4 Second attempt for imine conjugate formation starting from (*E,E*)-6-hydroxyhexa-2,4-dienal

To facilitate monitoring the reactions by TLC, the amino compound was switched to benzylamine (Figure 31) in following experiments due to its UV active chromophore.

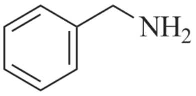


Figure 31 Benzylamine

We reasoned that despite reduction of one aldehyde moiety there might be linking possible between the remaining aldehyde moiety and the hydroxyl group within the molecule, therefore protection of the hydroxyl function should lead to inhibition of polymerization upon reaction with simple amines. Therefore we synthesized (*E,E*)-6-((*tert*-butyldimethylsilyl)oxy)hexa-2,4-dienal (Figure 32) starting from commercial available muconic acid (see section 4.3.2 below).

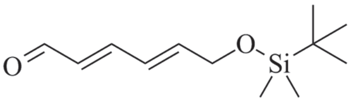


Figure 32 (*E,E*)-6-((*tert*-Butyldimethylsilyl)oxy)hexa-2,4-dienal

To our surprise, reaction of (*E,E*)-6-((*tert*-butyldimethylsilyl)oxy)hexa-2,4-dienal with benzylamine proceeded analogously as previous reactions leading to polymerization and formation of insoluble black organic matter (Figure 33).

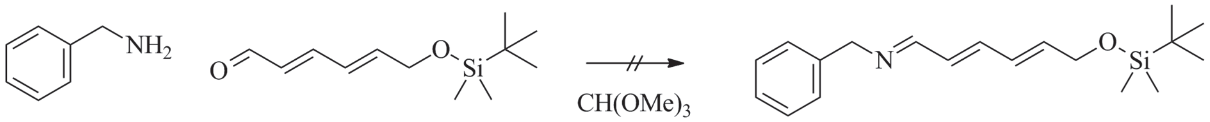


Figure 33 Third attempt for imine conjugate formation starting from (*E,E*)-6-((*tert*-butyldimethylsilyl)oxy)hexa-2,4-dienal

We concluded from this reaction that it is not the bifunctionality of (*E,E*)-muconaldehyde that is responsible for polymerization upon reaction with amines. This conclusion could be confirmed by successful reaction of benzylamine with commercially available (*E*)-hexa-2-enal (Figure 34). *N*-((*E*)-Hex-2-en-1-ylidene)-1-phenylmethanamine could be successfully isolated in quantitative yield. Reaction protocol hereby was changed to guarantee most safe reaction conditions by use of trimethyl orthoformate which is promising due to being an aprotic solvent as well as its ability to scavenge traces of water generating as side product from imine formation to yield methanol (Look *et al.* 1995).

Figure 34 **Reaction of benzylamine with (*E*)-hexa-2-enal to *N*-((*E*)-hex-2-en-1-ylidene)-1-phenylmethanamine**

A second important observation came with successful isolation of *N*-((*E*)-hex-2-en-1-ylidene)-1-phenylmethanamine. *N*-((*E*)-Hex-2-en-1-ylidene)-1-phenylmethanamine degraded upon contact to silica gel or aluminum oxide used for reaction monitoring in thin layer chromatography. Instability on silica gel makes purification by column chromatography virtually impossible. Therefore, we concluded in accordance with the literature that linear conjugates imines not adjacent to an aromatic system e.g. imines of benzaldehyde or terephtaldehyde, are not stable enough to be purified by chromatography on silica gel or aluminum oxide (Oberg & Rovis 2011).

Formation of a substituted bis-imine derived from (*E,E*)-muconaldehyde with isopropylamine is reported in the literature (Bleasdale *et al.* 1993). In our lab we were also able to isolate several substituted bisimines derived from (*E,E*)-muconaldehyde with different simple amines. Key to isolation is their ability to crystallize from the reaction mixture after filtration and simultaneous removal of polymerization byproducts. However, this approach comes with great loss of isolated product yield. Furthermore, this approach does not seem applicable with biological substrates being not suited for crystallization such as protected lysine compounds.

Final key to prevention of polymerization was achieved by kinetically controlling the reaction progress. Thereby both reactants are not mixed in high concentrations but rather a diluted solution of the amine is added dropwise to a diluted solution of the aldehyde compound (final concentration 150 mM). Using this protocol we were able for the first time to isolate (*E,E*)-muconaldehyde derived imines in 80 % yield after crystallization (Figure 35).

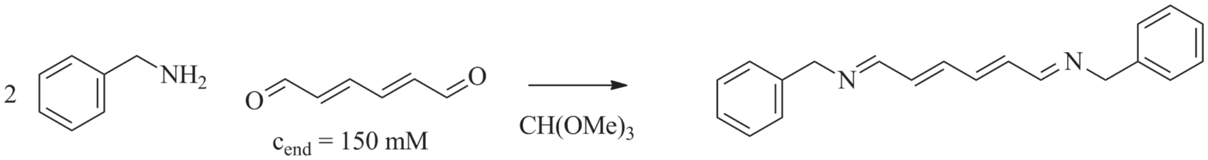


Figure 35 Successful reaction of benzylamine with (*E,E*)-muconaldehyde

We could find additional proof that polymerization upon reaction with amines could be attributed to the presence of an *α,β,γ,δ*-unsaturated aldehyde moiety rather than bifunctionality of (*E,E*)-muconaldehyde by reaction of benzylamine with commercial available (*E,E*)-hexa-2,4-dienal which upon reaction without kinetically controlled reaction conditions resulted in rapid polymerization (Figure 36).

Figure 36 Fourth attempt for imine conjugate formation starting from benzylamine with (E,E)-hexa-2,4-dienal

To circumvent isolation of the crude imine which could not be purified from starting material or side products we decided to look for a protocol to reduce the formed imine *in situ* yielding the secondary amine. Promising results delivered a protocol from Abdel-Magid which employed sodium triacetoxyborohydride in dichloroethane (Abdel-Magid *et al.* 1996). This approach takes advantage of the reduced potency of sodium triacetoxyborohydride compared to sodium borohydride as a reducing agent so that reduction of aldehydes and imines proceeds too slowly at room temperature to be of synthetic use whereas imines are reduced to secondary amines at sufficient rate. Using this protocol we were able to isolate (*E,E*)-*N*-benzylhexa-2,4-dien-1-amine (Figure 37).

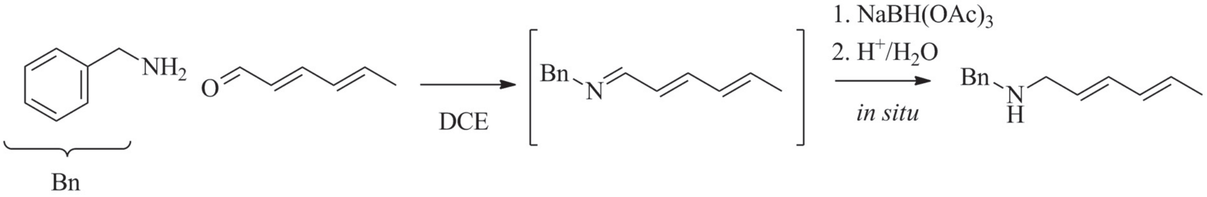


Figure 37 Successful reaction of benzylamine with (*E,E*)-hexa-2,4-dienal in dichloroethane (DCE) using a modified procedure given by (Abdel-Magid *et al.* 1996).

Since (*E,E*)-*N*-benzylhexa-2,4-dien-1-amine inhabits no imine group but a secondary amine we were able to detect this species using thin layer chromatography on silica gel using visualization with ninhydrin producing a yellow spot, typically seen for secondary amines (Bottom *et al.* 1978). At this point we resumed the synthesis of Lys(MUC-CHO).

4.3.2. FINAL SYNTHETIC PROCEDURE TO (*E,E*)-MUCONALDEHYDE LYSINE CONJUGATES

Synthetic access to the compound m/z = 241.1 referred to as (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO)), (*S*)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-OH)) and (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS) was finally accomplished by the following procedure (Scheme 5).

Lys(MUC-CHO) was synthesized from commercial available (*E,E*)-muconic acid, which was esterified with methanol in presence of catalytic amounts of acetyl chloride to give the corresponding methyl ester (**1**) in 95% yield. The ester was dissolved in chloroform and reduced to the alcohol (**2**) with diisobutyl aluminium hydride in quantitative yield. Regio preferred mono protection of the alcohol was accomplished in dimethyl formamide (DMF) with *tert*-butyldiphenylchlorosilane (TBDPS-Cl) with catalytic diisopropylethylamine. Flash chromatography yielded the mono protected alcohol (**3**) in 61 % yield aside from the bis protected alcohol (not shown). The alcohol was cleanly oxidized with manganese oxide in dichloromethane in 70 % yield to give the desired aldehyde (**4**).

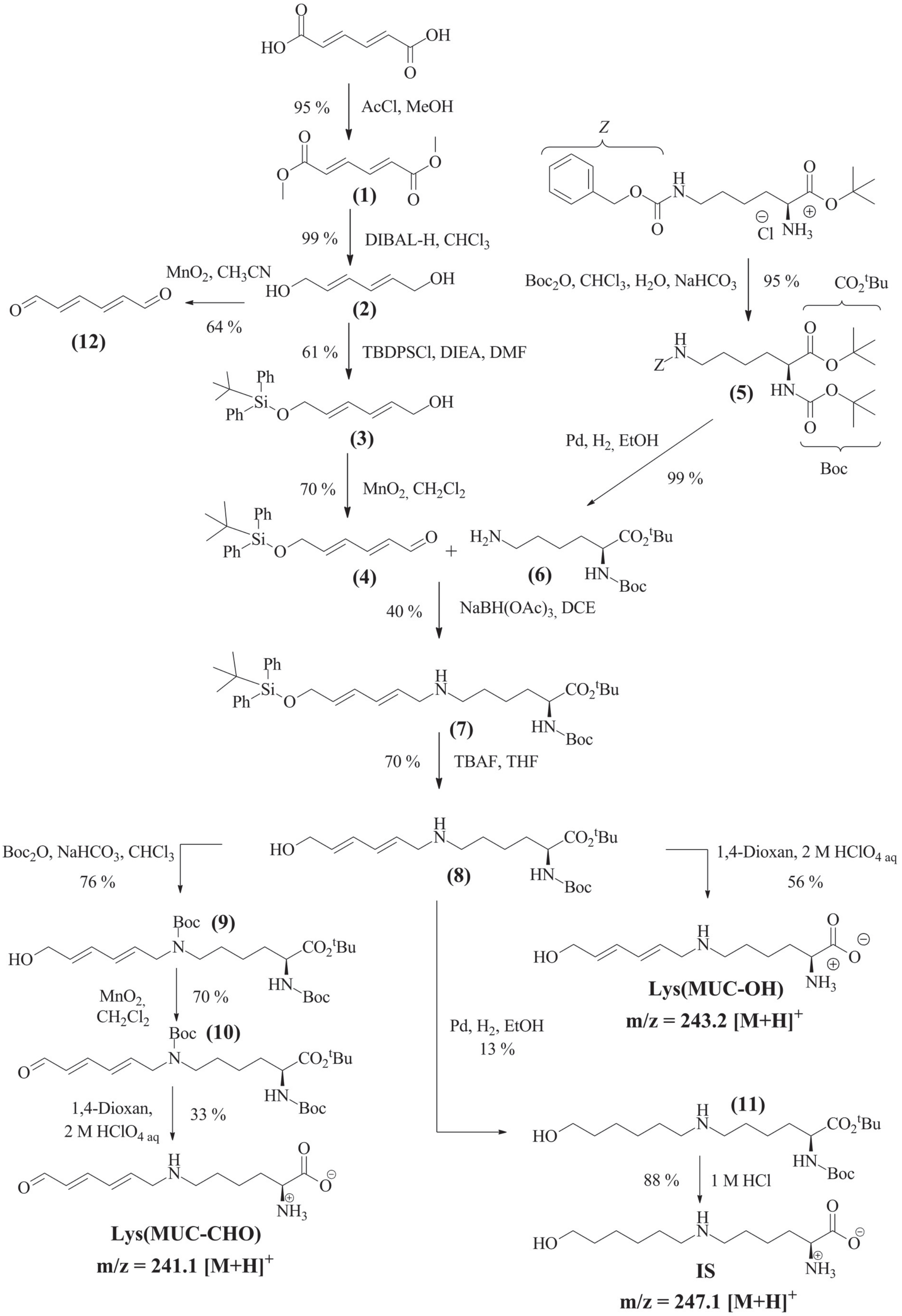
N$^{\alpha}$-Boc-Lys-O^{t}Bu synthesis commenced from commercial available H-Lys(Z)-O^{t}Bu*HCl which was deprotonated with aqueous sodium bicarbonate and reacted with di-*tert*-butyl dicarbonate in chloroform to yield *N*$^{\alpha}$-Boc-Lys(Z)-O^{t}Bu (**5**) in quantitative yield after flash chromatography. The protecting group of the epsilon amino function of the protected lysine was readily removed by hydrogenation in ethanol with elementary hydrogen over palladium on carbon as catalyst to yield *N*$^{\alpha}$-Boc-Lys-O^{t}Bu (**6**) quantitatively.

Coupling of the aldehyde (**4**) and *N*$^{\alpha}$-Boc-Lys-O^{t}Bu (**6**) was achieved by careful addition of the aldehyde to a diluted solution of the amine and reduction of the formed Schiff base *in situ* with sodium triacetoxyborohydride ($NaBH(OAc)_3$). The resulting secondary amine (**7**) was recovered in 40 % yield after purification by column chromatography. Removal of the silyl ether was readily accomplished by tetrabutylammonium fluoride (TBAF) in tetrahydrofuran in 70 % yield after purification using flash chromatography to give the alcohol (**8**).

For the synthesis of Lys(MUC-CHO), the secondary amine (**8**) was protected with Boc using di-*tert*-butyl dicarbonate in chloroform in 76 % yield after purification by flash column chromatography to give the amino protected alcohol (**9**). The amino protected alcohol was oxidized cleanly with manganese oxide in dichloromethane in 70 % yield to the corresponding amino protected aldehyde (**10**). Final removal of two Boc groups and one *tert*-butyl ester was performed in dioxane after the addition of aqueous perchloric acid to yield Lys(MUC-CHO).

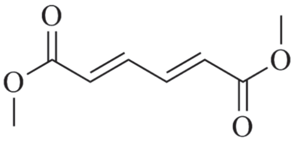
Lys(MUC-OH) was prepared analogously omitting alcohol oxidation with manganese oxide. Purification was achieved using semipreparative HPLC chromatography in 56 % yield.

IS was synthesized by two additional steps after removal of the silyl ether by hydrogenation in ethanol with elementary hydrogen over palladium on carbon as catalyst to yield the saturated alcohol (**11**), however yield was merely 13 %. Final removal of one Boc group and one *tert*-butyl ester was performed in 1 M aqueous hydrochloric acid in 88 % yield.



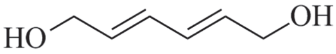
Scheme 5 Final synthetic procedure to (*E,E*)-muconaldehyde lysine conjugates Lys(MUC-OH), Lys(MUC-CHO) and IS

4.3.3. SYNTHESIS OF (*E*,*E*)-DIMETHYL HEXA-2,4-DIENEDIOATE (**1**)



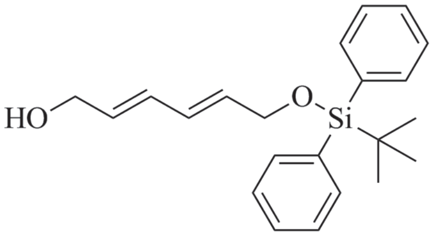
Synthesis of (*E*,*E*)-muconic acid dimethyl ester was performed in accordance to the procedure given by Gudipati by acidic esterification of (*E*,*E*)-muconic acid in methanol (Gudipati 2002). Addition of acetyl chloride to the reaction mixture leads to generation of hydrochloric acid *in situ* which proves efficient in catalysis of the reaction. The ester (**1**) was recovered in 95 % yield after crystallization at - 20°C.

4.3.4. SYNTHESIS OF (*E*,*E*)-HEXA-2,4-DIENE-1,6-DIOL (**2**)



Synthesis of (*E*,*E*)-hexa-2,4-diene-1,6-diol was performed in accordance to a procedure given by Gudipati by reduction of the ester (**1**) with diisopropyl aluminium hydride in chloroform (Gudipati 2002). The diol (**2**) was recovered in 99 % yield after crystallization at - 20°C. Due to the high polarity of the diol it was essential to stick closely to the protocol, otherwise substantially decreased yields were observed potentially since much of the product could not be extracted anymore once transferred into aqueous phase.

4.3.5. SYNTHESIS OF (*E*,*E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)-HEXA-2,4-DIEN-1-OL (**3**)



Synthesis of (*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)-hexa-2,4-dien-1-ol (**3**) was performed with a modified procedure for silylation of 1,n-primary diols given by Yu (Yu *et al.* 2000). Reaction of the diol with TBDPS-Cl in presence of excess diisopropylethylamine (Hünig's base) yielded the desired mono-TBDPS ether in 61 % alongside TBDPS-OH and the bis-$TBDPS_2$ ether. The pure mono ether (**3**) was isolated from side products by column chromatography on silica gel with 25 % ethyl acetate in n-hexane as eluent.

The protocol was chosen because of the reported regioselectivity for silylation of primary 1,n-diols which were to result in high concentrations of the mono silylated product, up to 90 % according to the authors. However, we could confirm only minor improved yields when comparing this protocol to conventional reaction conditions for silylation e.g. using trimethylchlorosilane (TMCS) and imidazol in DMF, which produced yields of 40 % (Corey & Venkateswarlu 1972). The advantage of using TMCS instead of TBDPS-Cl comes with the reduced molecular weight of the protecting group (151 vs. 275 g/mol). Since the crude reaction product needs to be purified by SC because of the presence of the bis-silylated diol and hydrolyzed silyl reagent, the factor to determines the scale size of the reaction is crude product weight.

Table 6 shows comparison of both methods using the maximum available SC for purification with d = 6 cm. Therefore, we conclude that under these conditions both methods are comparable and can be used compatibly. Corey's protocol should be preferred when larger reaction scale than ~ 3.6 g crude product weight is possible. Aside, from an ecological point of view Yu's protocol is disadvantageous for two reasons.

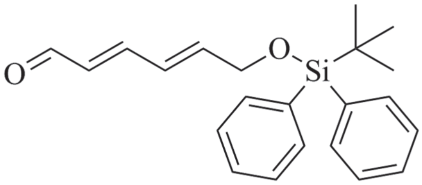
1. The excessive need for auxiliary base instead of the stochiometric amounts needed by Corey.
2. Higher price of the reagents and lesser atomic econometry producing more waste.

Table 6 Comparison of yields for silylation of (*E,E*)-hexa-2,4-diene-1,6-diol (2). Estimated maximum amount of starting material is calculated for crude product weight of 3.6 g for SC using a glass column with d = 6 cm

Protocol	Starting Material	Product Yield (3)	Est. max. starting Material	Est. max. product Yield (3)
Yu et al., 2000 (TBDPS-Cl)	8.2 mmol	4.9 mmol (61 %)	9.0 mmol	5.5 mmol
Corey et al, 1972 (TMCS)	5.3 mmol	2.6 mmol (40 %)	14 mmol	5.6 mmol

Conclusively for future experiments we recommend using the protocol given by Corey for silylation of (*E*,*E*)-hexa-2,4-diene-1,6-diol (**2**) instead of the protocol given by Yu *et al* (Corey & Venkateswarlu 1972; Yu *et al.* 2000).

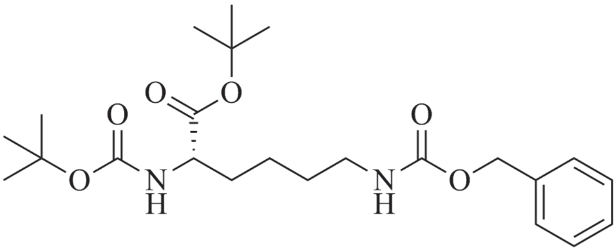
4.3.6. SYNTHESIS OF (*E,E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)HEXA-2,4-DIENAL (4)



Synthesis of (*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dienal (**4**) performed in accordance to the literature (Fernández & Tojo 2006).

(*E,E*)-6-((*tert*-Butyldiphenylsilyl)oxy)-hexa-2,4-dien-1-ol (**3**) was dissolved in dry dichloromethane and reacted with excess manganese dioxide at room temperature for 3 hours. The aldehyde (**4**) was recovered in 70 % yield in analytical purity after filtration and evaporation of the solvent.

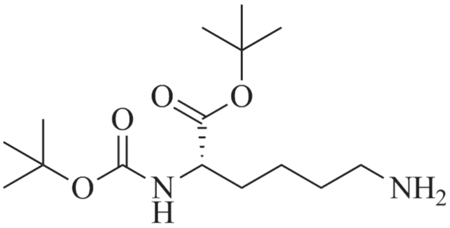
4.3.7. SYNTHESIS OF *N*α-BOC-LYS(Z)-O^T^BU (**5**)



Synthesis of *N*α-Boc-Lys(Z)-O^{t}Bu (**5**) was performed in accordance to a procedure given by Bergeron (Bergeron *et al.* 1997).

H-Lys(Z)-O^{t}Bu*HCl was thereby dissolved in chloroform in the presence of aqueous sodium bicarbonate solution and di-*tert*-butyl dicarbonate was added. The reaction mixture was refluxed for 1.5 h. The desired Boc protected amine (**5**) was recovered in 98 % yield after separation from unreacted starting material by flash column chromatography on silica gel with 25 % ethyl acetate in n-hexane as eluent.

4.3.8. SYNTHESIS OF *N*α-BOC-LYS-O^T^BU (**6**)

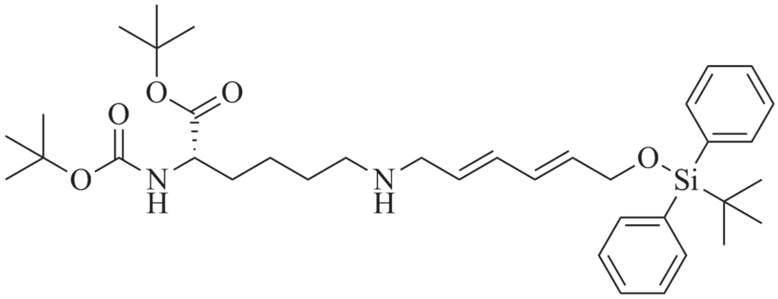


Synthesis of *N*α-Boc-Lys-O^{t}Bu (**6**) was performed with a modified procedure taken from Bergeron (Bergeron *et al.* 1997).

*N*α-Boc-Lys(Z)-O^{t}Bu (**5**) was dissolved in ethanol and reduced with palladium on carbon in an atmosphere of hydrogen at an overpressure of about 30 cm water. The product could be recovered in quantitative yield.

The original protocol operated with the addition of equimolar amount of hydrochloric acid to the reaction mixture to produce the corresponding ammonium hydrochloride. Using this protocol, we observed considerable loss of the acid sensitive Boc protecting group. Omission of addition of hydrochloric acid yielded the free amine while fully preserving Boc. The product (**6**) was recovered quantitatively in analytical purity after filtration and evaporation of the solvent.

4.3.9. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-(((*E,E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)HEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (7)



Synthesis of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate (**7**) was performed with a modified procedure taken from (Abdel-Magid *et al.* 1996). N^{α}-Boc-Lys-OtBu (**6**) was dissolved in dichloroethane and (*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dienal (**4**) was added dropwise as solution in dichloroethane. After addition was complete $NaBH(OAc)_3$ was added and the reaction mixture was stirred overnight. The secondary amine (**7**) was recovered in 40 % yield after separation from unreacted starting material and several side reaction products by column chromatography on silica gel with 15 % 2-propanol in toluene with 1 % triethylamine as eluent.

Considering the reaction conditions worked out in chapter 4.3.1 Preparatory steps, reaction was initially performed with a concentration of starting compounds of 150 mM. However these reaction conditions proved difficult to be performed large scale resulting often times in polymerization observed from reaction of (*E,E*)-muconaldehyde with primary amines. Following reaction was driven carefully more diluted with a final compound concentration of 40 mM which seemed to be uncritical for polymerization. However, product yield could not be increased above ~ 40 % despite an increased reaction time for up to 72 h. Refluxing of the reaction mixture was excluded since previous experiments under similar conditions using $CH(OMe)_3$ as solvent indicated increased tendency of the reaction components to polymerize upon heating.

Alternative reaction pathways for this step should be considered for future experiments since synthesis of the intact Schiff base can be precluded due to its instability and different methodologies for formation of secondary amines exist (Salvatore *et al.* 2001) (Figure 38).

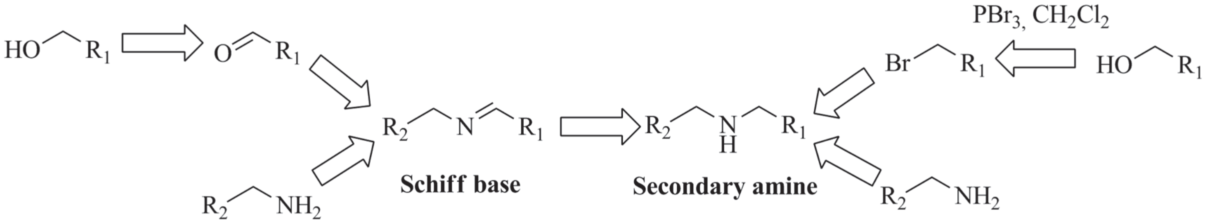


Figure 38 Alternative synthetic route for secondary amine formation

To replace the aldehyde component, formation of the alkyl bromide starting from the alcohol with PBr_3 might prove preferential because of

1. Prevention of polymerization. The coupling step consisting of the bromide and the protected lysine replaces the polymerization prone structural element (the $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde). Therefore, the reaction can be done with reactant concentrations > 40 mM resulting in higher yields than observed with the diluted amine/aldehyde setting.
2. Similarity to reports in the literature. Formation of (*E,E*)-hexa-2,4-dienyl bromide, which is a close analogue to our hypothetical starting compound, has been reported starting from the alcohol and reaction with PBr_3 in the literature (Ferri *et al.* 2015) (Figure 39). Therefore, there is good chance for positive reaction outcome.

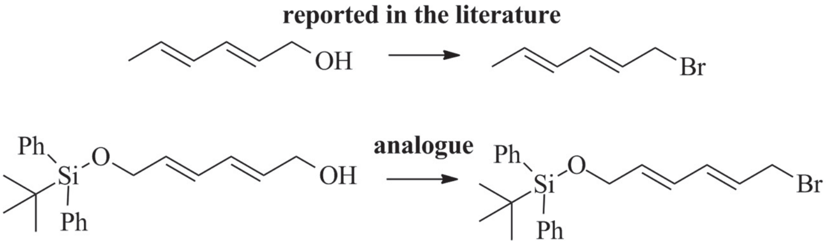
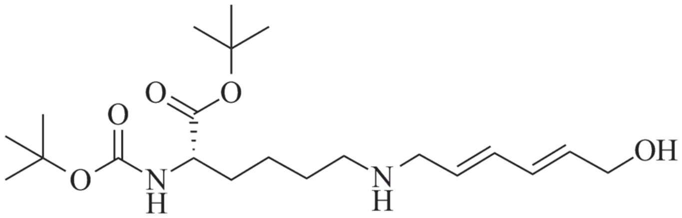


Figure 39 Comparison of synthetic routes for formation of *(E,E)*-muconaldehyde-lysine conjugates (Ferri *et al.*, 2015).

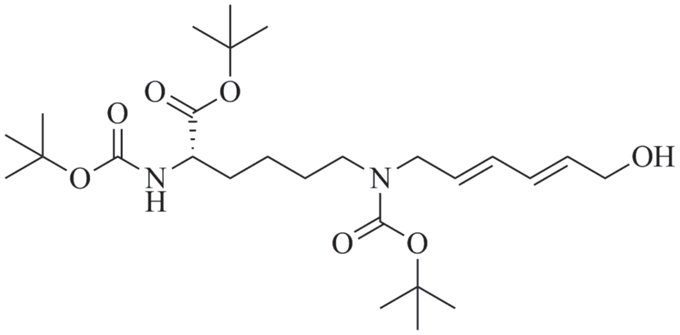
4.3.10. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-(((*E,E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (**8**)



Synthesis of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (**8**) was performed in accordance to a protocol given by Hanessian (Hanessian *et al.* 2011).

(*S*)-*tert*-Butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate (**7**) was dissolved in THF and equimolar amounts of a solution of tetrabutyl ammonium fluoride in THF was added. The reaction mixture was stirred overnight. The product (**8**) could be isolated in 70 % yield after separation from TBDPS-OH by flash column chromatography on silica gel with 40 % 2-propanol in toluene with 1 % triethylamine as eluent.

4.3.11. SYNTHESIS OF (*S*)-*TERT*-BUTYL 6-((*TERT*-BUTOXYCARBONYL)((*E,E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)-2-((*TERT*-BUTOXYCARBONYL)AMINO)HEXANOATE (**9**)

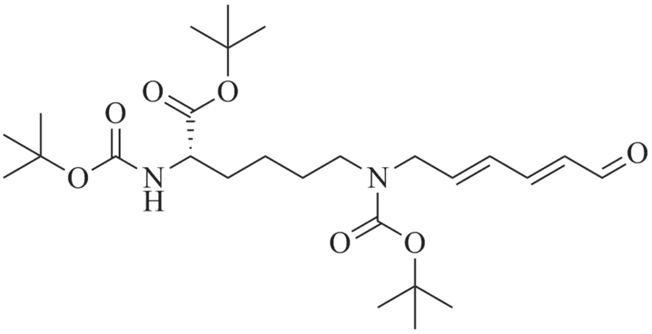


Synthesis of (*S*)-*tert*-butyl 6-((*tert*-butoxycarbonyl)((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (**9**) was performed in accordance to a procedure given by (Bergeron *et al.* 1997).

(*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (**8**) was thereby dissolved in chloroform in the presence of aqueous sodium bicaronate solution and di-*tert*-butyl dicarbonate was added. The reaction mixture was refluxed for 1.5 h. The desired amino protected alcohol (**9**) was recovered in 76 % yield after separation from unreacted starting material by flash column chromatography on silica gel using 40 % ethyl acetate in n-hexane as eluent.

The ^{1}H-NMR spectrum shows trace impurities in the area 5.0-6.5 ppm where proton signals adjacent to a double bond appear. Upon careful inspection, trace impurities surrounding the TLC spot could be detected as well. We therefore suppose that isomerization of the two conjugated double bonds occurred resulting in traces of (*E,Z*), (*Z,E*), and (*Z,Z*) isomers. Since no starting material was further available and the amount of impurities calculated from the ^{1}H-NMR spectrum was below 2 %, reaction cascade was continued.

4.3.12. SYNTHESIS OF (*S*)-*TERT*-BUTYL 6-((*TERT*-BUTOXYCARBONYL)((*E,E*)-6-OXOHEXA-2,4-DIEN-1-YL)AMINO)-2-((*TERT*-BUTOXYCARBONYL)AMINO)HEXANOATE (**10**)



Synthesis of (*S*)-*tert*-butyl 6-((*tert*-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (**10**) was performed by a modified procedure given by (Gudipati 2002).
(*S*)-*tert*-Butyl 6-((*tert*-butoxycarbonyl)((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (**9**) was dissolved in dichloromethane and excess manganese dioxide was added. The reaction mixture was stirred for 45 min, filtered and the solvent removed *in vacuo* to afford the product (**10**) in 70 % yield. Mass spectrometric analysis (HRGC-MS) revealed no distinct mass signal but rather massive fragmentation which could not be assigned with certainty to the loss of distinct structural elements.

Three different approaches were tested beforehand to perform oxidation to obtain the aldehyde moiety without protection of the free secondary amine, however unsuccessfully (Figure 40):

1. Aqueous oxidation. mIBX is a water-soluble analogue of 2-iodoxybenzoic acid (IBX) (Thottumkara & Vinod 2002). Oxidation of Lys(MUC-OH) with mIBX, did not result in traceable amount of the presumed Lys(MUC-CHO) via LC-MS (cf. ME 140+141) independent from chosen pH.
2. Aprotic oxidation. Oxidation of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (**8**) in the presence of manganese dioxide lead to polymerization as observed from reaction of (*E,E*)-muconaldehyde with primary amines. A possible explanation lies in the formation of a polymerization prone structural element in the course of the reaction, the $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde, in the presence of a nucleophile group, the secondary amine.

3. Diluted oxidation. Oxidation at ~ 40 mM product concentration using Dess Martin Periodane in dichloromethane prevented polymerization and two distinct signals could be detected on TLC and isolated preparatively: Despite presence of an aldehyde function according to the ^{1}H NMR, the lack of the signals for protecting groups Boc and *tert*-butyl ester indicated an unintended reaction outcome. Despite the spectroscopic purity of at least one of the compounds we were not able to conclude a reasonable structure (cf. ME 130).

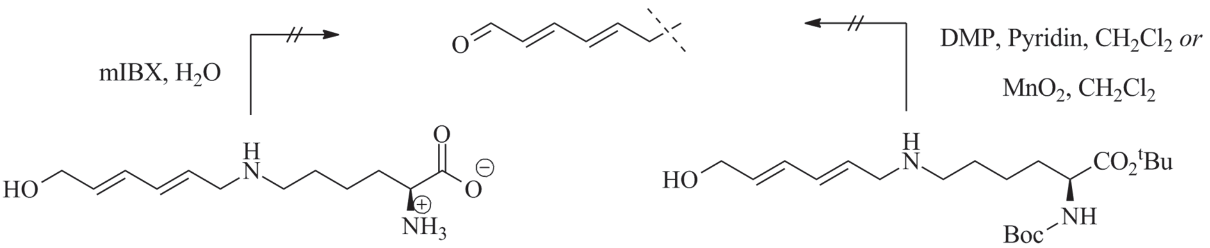


Figure 40 Failed synthetic approaches to formation of an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde moiety.

The problem that arises from the presence of an aldehyde function in the presence of a secondary amine is the combination of a nucleophilic center (the secondary amine) with a polymerization prone structural element (the $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde) (Figure 41). The same behavior could be observed in the reaction of (*E,E*)-hexa-2,4-dienal with benzylamine. In aprotic media such as dichloromethane protonation of the secondary amine cannot occur. That is the reason why, even though a conversion might have been successful, the intended product could not be isolated.

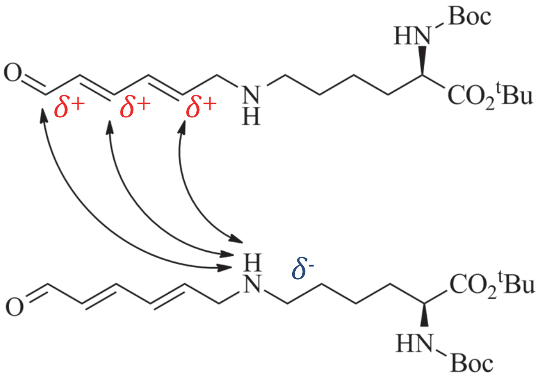
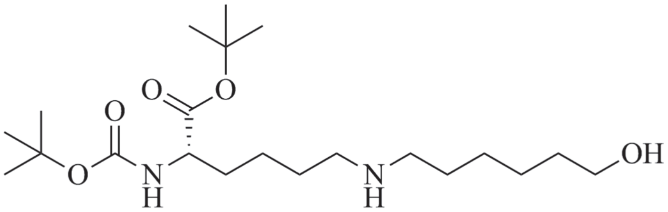


Figure 41 Reasonable locations for intermediate polar interactions leading to polymerization between nucleophilic and electrophilic sites in *(S)*-tert-butyl 6-((tert-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoate (10).

Therefore, we concluded protection of the secondary amine to be a requirement to simultaneously obtain the structural element of an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde in the presence of a secondary amine.

4.3.13. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-((6-HYDROXYHEXYL)AMINO)HEXANOATE (11)



Synthesis of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-((6-hydroxyhexyl)amino)hexanoate (**11**) was performed by hydrogenation with elementary hydrogen in ethanol. (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (**8**) was dissolved in ethanol and palladium on carbon was added. Apparatus was filled with hydrogen, and the reaction mixture was stirred for 7 hours at 30 cm H_2O. The reaction mixture was filtered, and the crude product was purified by column chromatography on silica gel using 80 % 2-propanol in toluene with 1 % triethylamine as eluent. The product could only be recovered in 13 % yield.

A side product could be recovered alongside with similar NMR characteristics suggesting side reactions might have taken place producing product dimers such as dimerization (cf. ME 118 V1) (Figure 42). Such behavior might have been caused by a too concentrated reaction mixture or an insufficient hydrogen atmosphere in the reaction chamber. Future experiments should ascertain sufficient solvent dilution and full and rapid flooding of the reaction chamber equipped with excess airspace to provide enough hydrogen to hydrogenate both double bonds simultaneously.

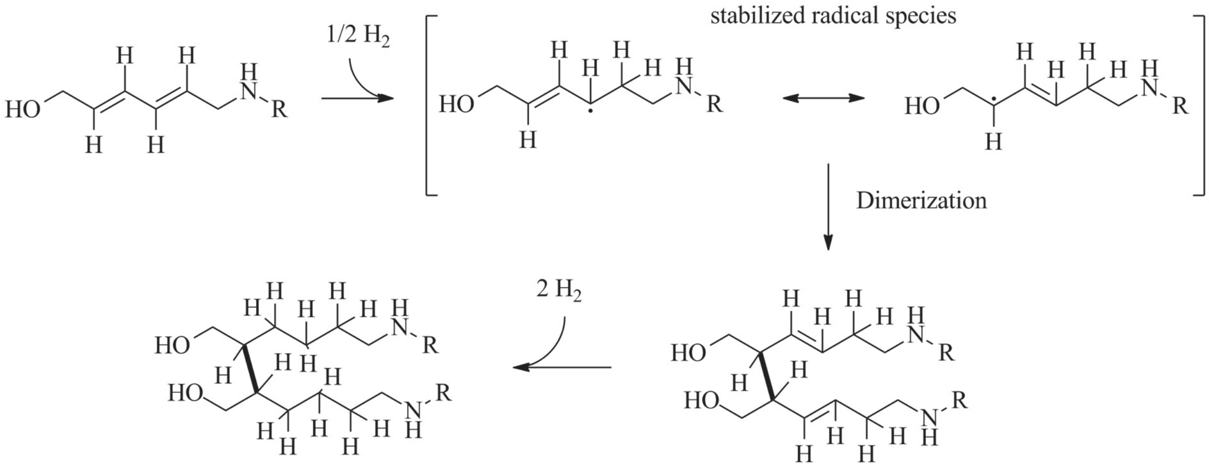
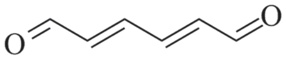


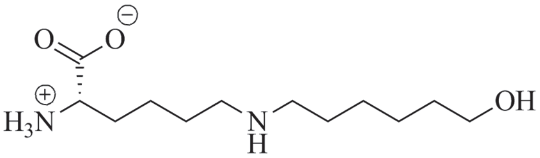
Figure 42 Hypothetical dimerization of two insufficiently hydrogenated radical species of (*S*)-tert-butyl 2-((tert-butoxycarbonyl)amino)-6-(((E,E)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (8)

4.3.14. SYNTHESIS OF (*E,E*)-MUCONALDEHYDE (**12**)



A novel synthetic route was developed for synthesis of (*E,E*)-muconaldehyde (**12**). (*E*,*E*)-hexa-2,4-diene-1,6-diol (**2**) was dissolved in acetonitrile and excess manganese dioxide was added. The reactions mixture was stirred for one hour, filtered and the solvens removed *in vacuo*, yielding (*E,E*)-muconaldehyde (**12**) as a bright yellow solid in 64 % yield.

4.3.15. SYNTHESIS OF (*S*)-2-AMMONIO-6-((6-HYDROXYHEXYL)AMINO)HEXANOATE (**IS**)



Synthesis of (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (**IS**) was performed by acidic removal of the protecting groups Boc and *tert*-butyl ester, which were chosen due to their similar conditions of removal. (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-((6-hydroxyhexyl)amino)hexanoate (**11**) was therefore taken up in 1 M deuterochloric acid in D_2O. After 31 h ^{1}H NMR indicated completion of the reaction and solvent was removed *in vacuo.* The product was obtained as yellow oil in 88 % yield. No product workup was performed since cleavage of both protecting groups results in gaseous byproducts isobutene and CO_2 aside from traces of *tert*-butanol (Figure 43).

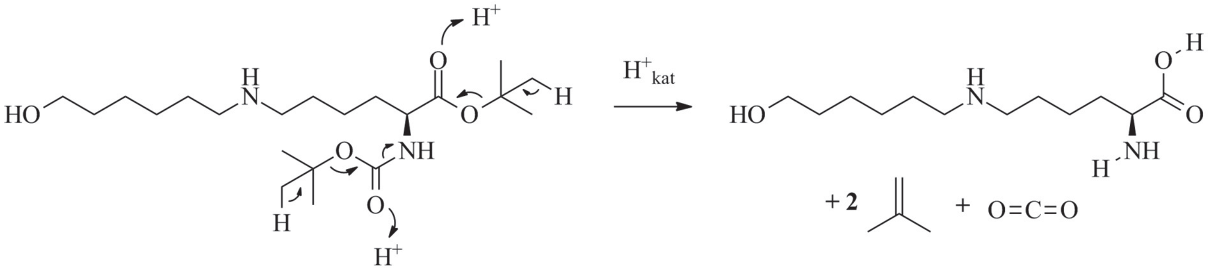


Figure 43 Mechanism of acid catalyzed Boc and tert-butyl ester cleavage shown for formation of (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS)

Mass spectrometric analysis for m/z = 247.2 $[M+H]^+$ revealed very little fragmentation which can be explained by the absence of ionizable structural elements (cf. ME 179, Figure 45). Detected fragments derive from the lysine residue which provides polar amino groups which are readily ionized via electrospray ionization (ESI) (Figure 44).

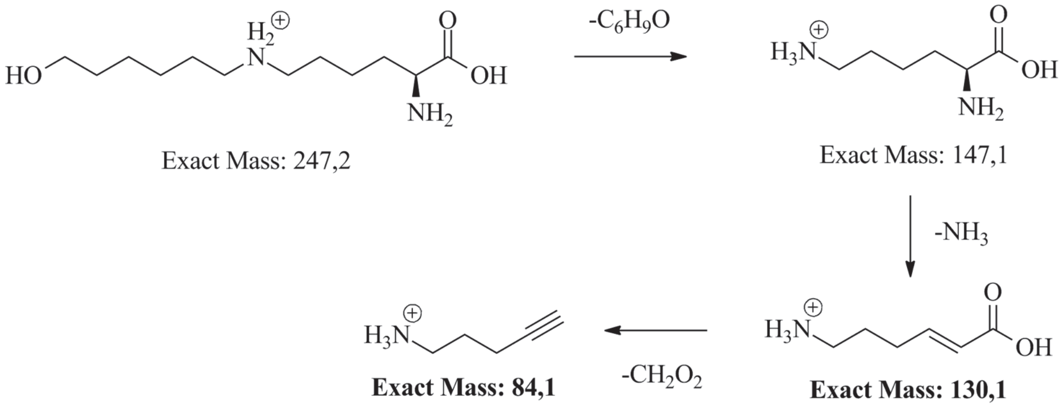


Figure 44 ESI^+-MS/MS Fragmentation proposal of (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS)

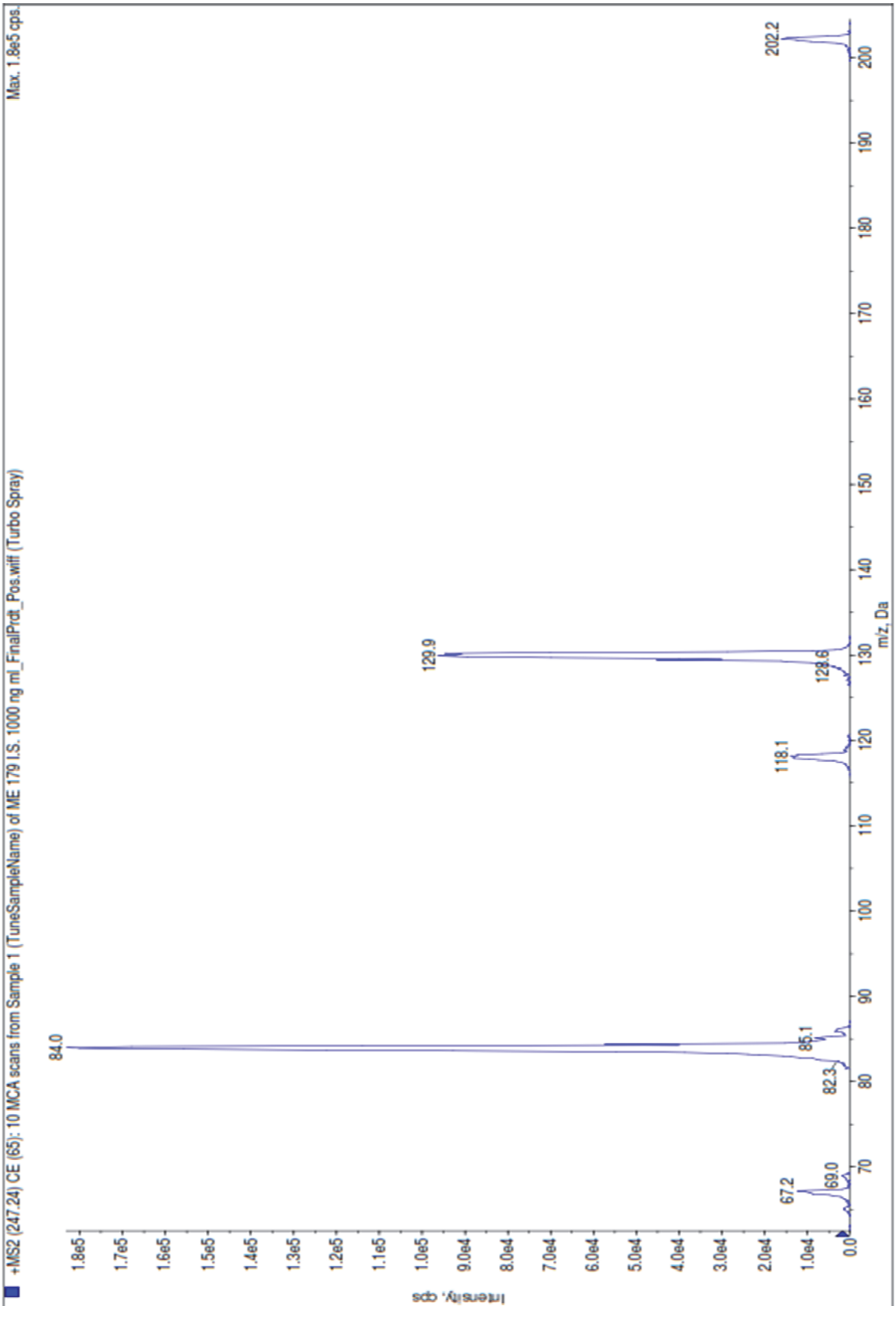
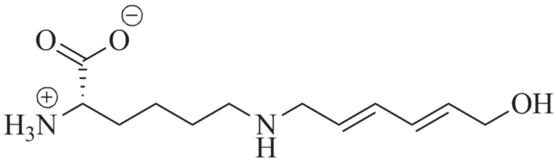


Figure 45 **ESI⁺-MS/MS spectrum of 1000 ng/ml (*S*)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS) for m/z (Q1) = 247.2 (M+H)⁺**

4.3.16. SYNTHESIS OF (*S*)-2-AMMONIO-6-(((*E,E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (LYS(MUC-OH))



Synthesis of (*S*)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (**Lys(MUC-OH)**) was performed by acidic removal of Boc and *tert*-butyl ester protecting groups from the reduced Schiff base (**8**) (Wuts 2014). Due to a low solubility of the starting material, a 1+1 mixture of 1,4-dioxane and aqueous 4 M perchloric acid was employed. One advantage from using perchloric acid comes from its easy removal by precipitation of potassium perchlorate upon neutralization with potassium hydroxide. Table 7 lists different reaction conditions tested for removal of Boc and *tert*-butyl ester.

Table 7 Reaction conditions tested for removal of Boc and tert-butyl ester

Reaction conditions
TFA in CH_2Cl_2 (1+1)
1 M HCl in Et_2O
Formic acid
1 M H_3PO_4
1 M HCl

Experiments were observed via HILIC-HPLC-UV (cf. electronic attachment ME 117 & 119). In contrast to the reactions performed in the absence of water, fewest side products were detected using aqueous acids. However, organic extraction workup of the product failed due to its high solubility in the aqueous phase. To separate the product from traces of UV-active impurities and residual potassium perchlorate the product was isolated using preparative HILIC-HPLC.

Future work up might attempt to employ ion-exchange resins and the products ability to change its state of ionization dependent of pH of the solution to remove potassium perchlorate from the reaction mixture.

Mass spectrometric analysis for m/z = 243.2 $[M+H]^+$ reveals ready fragmentation which can be explained by the presence of additional ionizable structural elements, i.e. two conjugated double bonds compared to IS (cf. ME 179, Figure 47). Aside from the known fragments of the lysine residue, additional fragments m/z = 79.1 and 96.9 can be accounted to the 1-hydroxy-hexa-2,4-dienyl side chain (Figure 46).

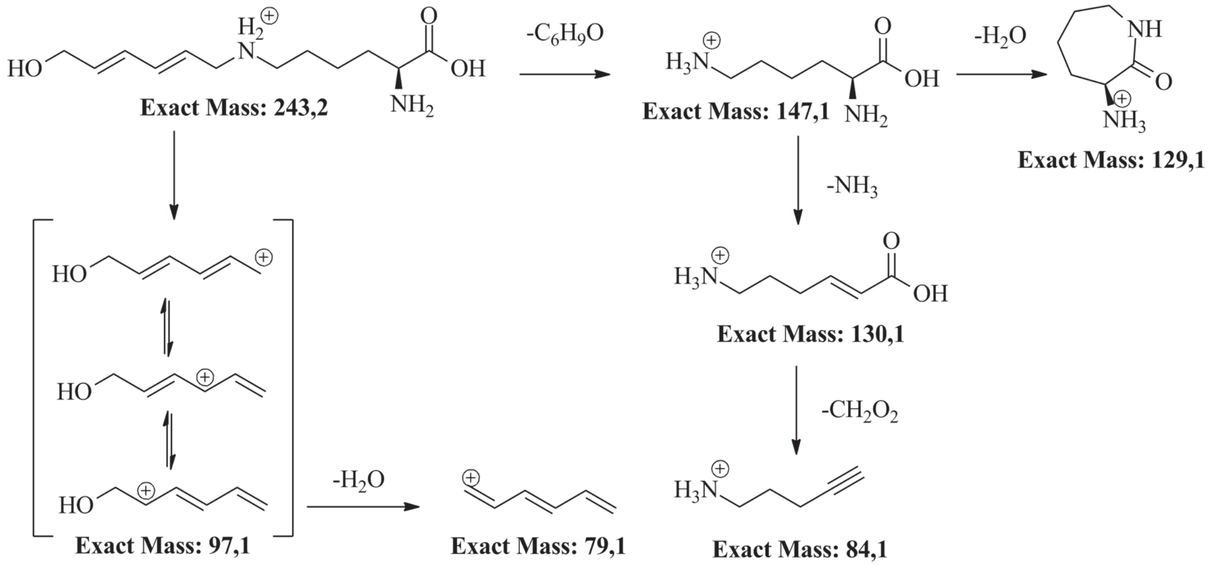


Figure 46 ESI⁺-MS/MS Fragmentation proposal of (*S*)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-OH))

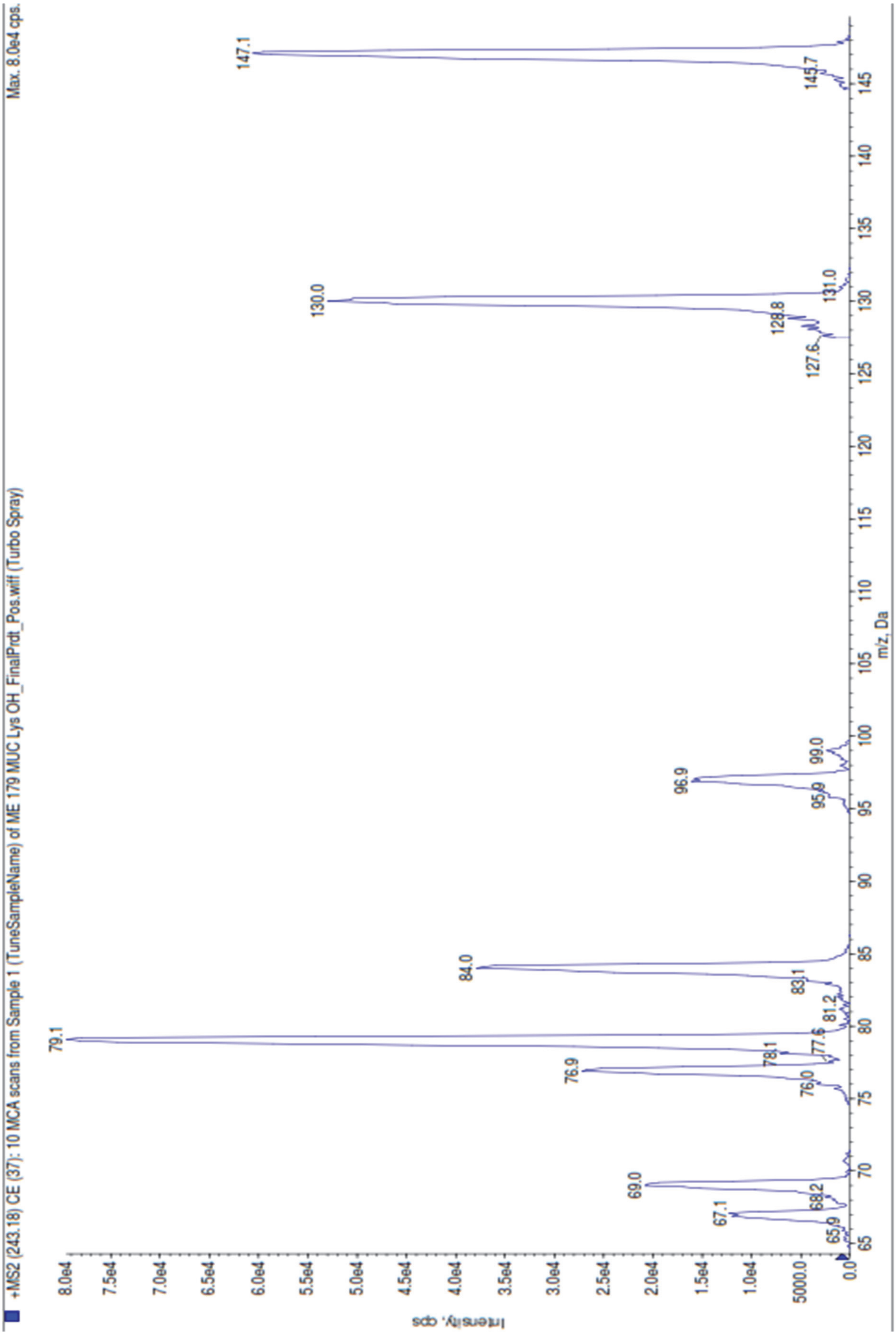
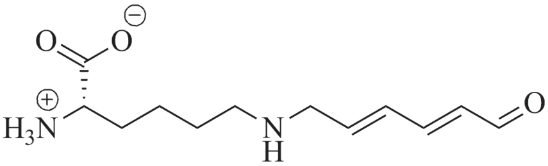


Figure 47 ESI$^+$-MS/MS spectrum of 250 ng/ml (*S*)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (IS) for m/z (Q1) = 243.2 [M+H]$^+$

4.3.17. SYNTHESIS OF (*S*)-2-AMMONIO-6-(((*E,E*)-6-OXOHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (**LYS(MUC-CHO)**)



Synthesis of (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (**Lys(MUC-CHO)**) was performed by acidic removal of Boc and *tert*-butyl ester similarly to Lys(MUC-OH). (*S*)-*tert*-butyl 6-((*tert*-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (**10**) was dissolved in a 1+1 mixture of 1,4-dioxan and 4 M $HClO_4$. After 16 h the product could be isolated via preparative HPLC in 33 % yield.

Resolution between the two signals seen in the reaction mixture was satisfactory so that attempts were made to isolate the product via solid phase extraction (Figure 48). However, degradation of the product was observed after elution (cf. ME 149).

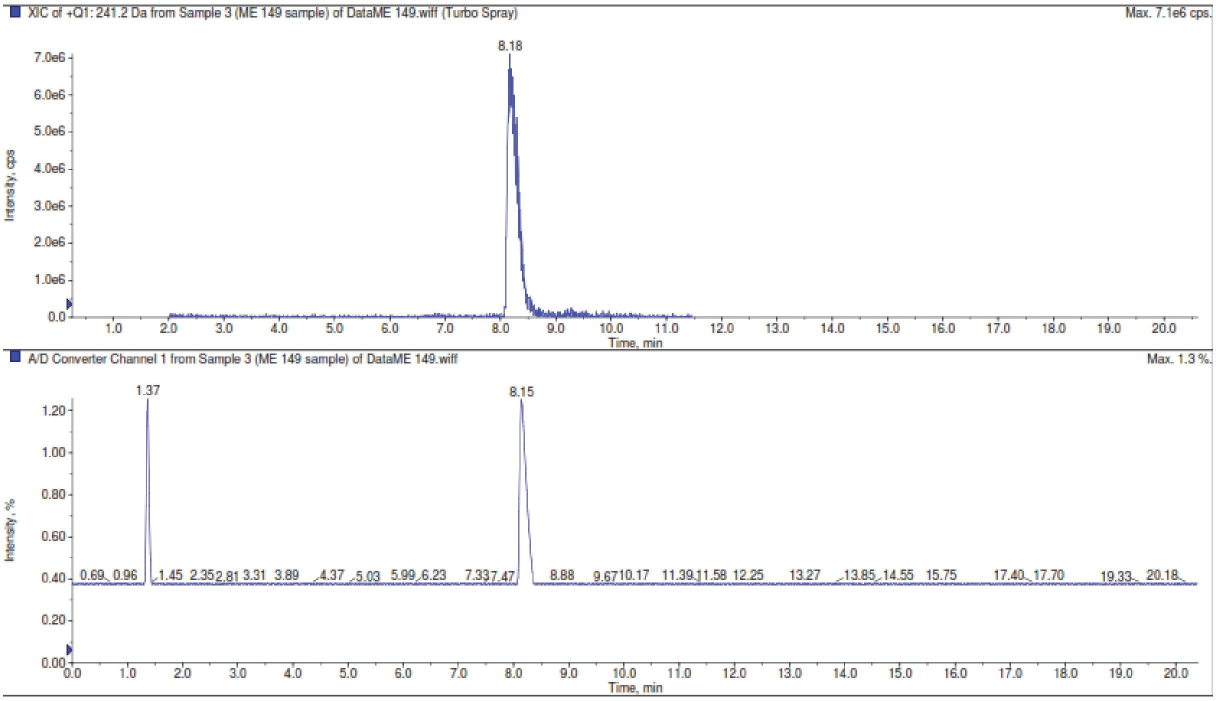


Figure 48 ESI$^+$-HPLC-MS (above) (XIC for m/z = 241.1) and HPLC-UV (below) (λ = 269 nm) spectra for the reaction mixture of (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO))

Mass spectrometric analysis for m/z = 241.1 $[M+H]^+$ revealed less fragmentation compared to Lys(MUC-OH) (cf. ME 164, Figure 50). Aside from the known fragments of the lysine residue, the only additional fragment m/z = 223.2 can be interpreted as a simultaneous loss of ammonia and the carboxyl group (Figure 49). This resilient behavior to fragmentation might indicate greater stability of the alkyl side chain to fragmentation from the parent molecule compared to Lys(MUC-OH).

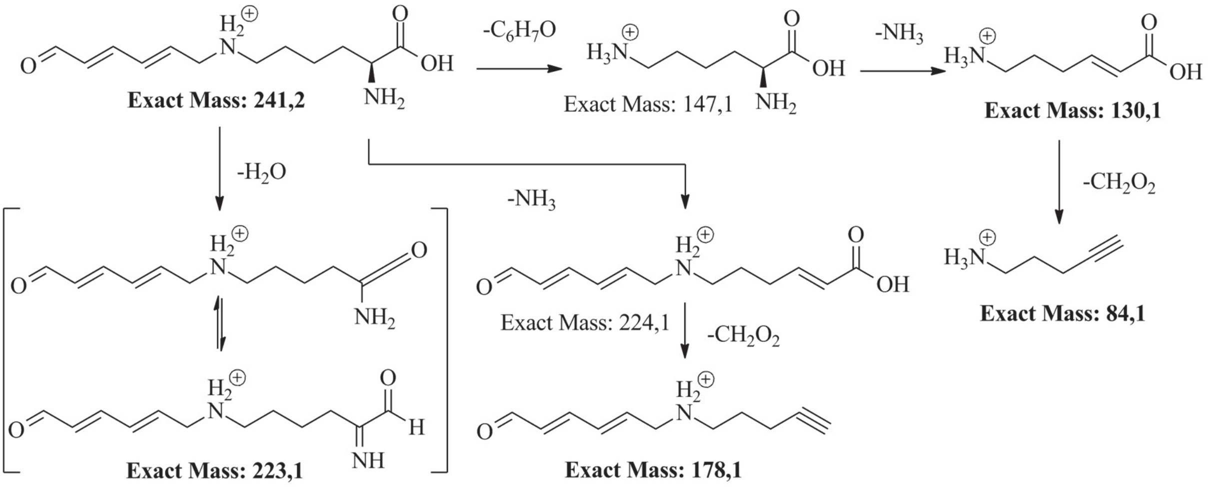


Figure 49 ESI⁺-MS/MS Fragmentation proposal of (*S*)-2-ammonio-6-(((E,E)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO))

Minor fragment signals can be seen with m/z = 99.0, 139.8, 158.8, 200.2 which were still present despite the absence of the substance and can likely be accounted to impurities within the solvent or the apparatus.

Isolation was attempted via concentration of the preparative HPLC eluate and subsequent lyophilization. However, two observations were made

1. Lyophilization resulted in reddish colorization of the product. Upon reconstitution, the product did not dissolve completely.
2. HPLC-UV analysis after reconstitution revealed degradation of the formerly uniform peak.

Therefore, isolation and determination of concentration for quantification was not possible due to lack of compound stability. For this reason, alternative ways of quantification were tested.

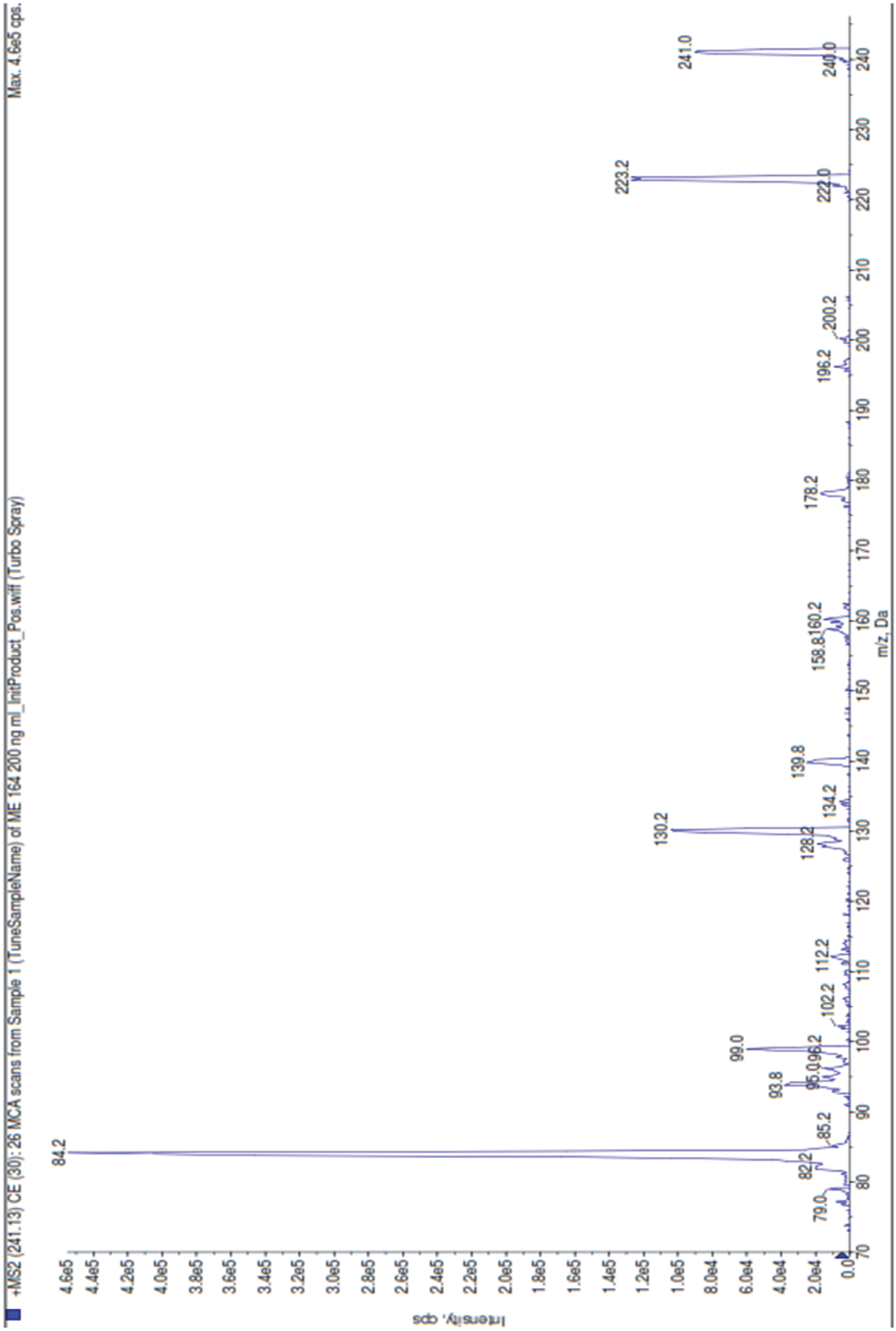


Figure 50 ESI⁺-MS/MS spectrum of 200 ng/ml (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO)) for m/z (Q1) = 247.2 [M+H]⁺

4.4. QUANTIFICATION OF LYS(MUC-CHO)

Quantification of Lys(MUC-CHO) was achieved using 2,4-dinitrophenylhydrazine (DNPH), a reagent commonly employed for total quantification of carbonyl compounds present in aqueous media (Yukawa *et al.* 1993) (Figure 51). The determination is performed by UV-spectroscopy. Thereby a calibration curve is prepared from a solution with known concentration of an arbitrary aldehyde and tested against the solution with unknown aldehyde concentration. The test is reported to be sufficiently accurate and does not require structural knowledge of the aldehyde which is to be determined. Nonetheless, we chose to employ (*E,E*)-hexa-2,4-dienal as a structural similar aldehyde to Lys(MUC-CHO).

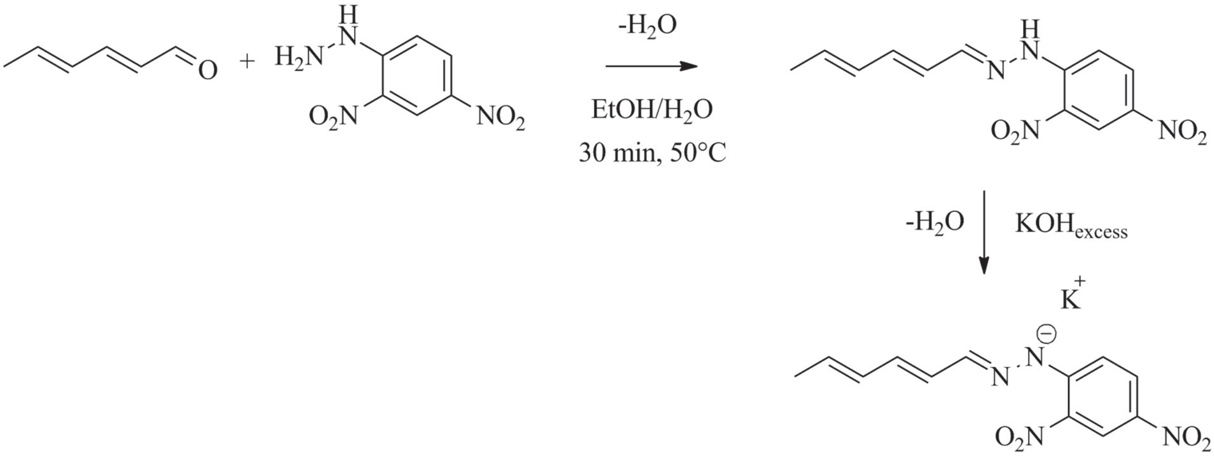


Figure 51 Mechanism of Derivatization with 2,4-dinitrophenylhydrazine (DNPH) shown for (*E,E*)-hexa-2,4-dienal and chromophore formation. Taken and modified from (Yukawa *et al.* 1993)

UV-spectra of DNPH conjugates of (*E,E*)-hexa-2,4-dienal and Lys(MUC-CHO) show similarity, facilitating quantification (Figure 52). Calibration using (*E,E*)-hexa-2,4-dienal was sufficiently linear in the calibration range of 17 – 130 µM, covering the analyte concentration of Lys(MUC-CHO) in the sample (Figure 53). Aldehyde concentration of the preparative HPLC eluate (about 125 ml) was determined to contain 28 mg/l Lys(MUC-CHO) (cf. ME 164/166).

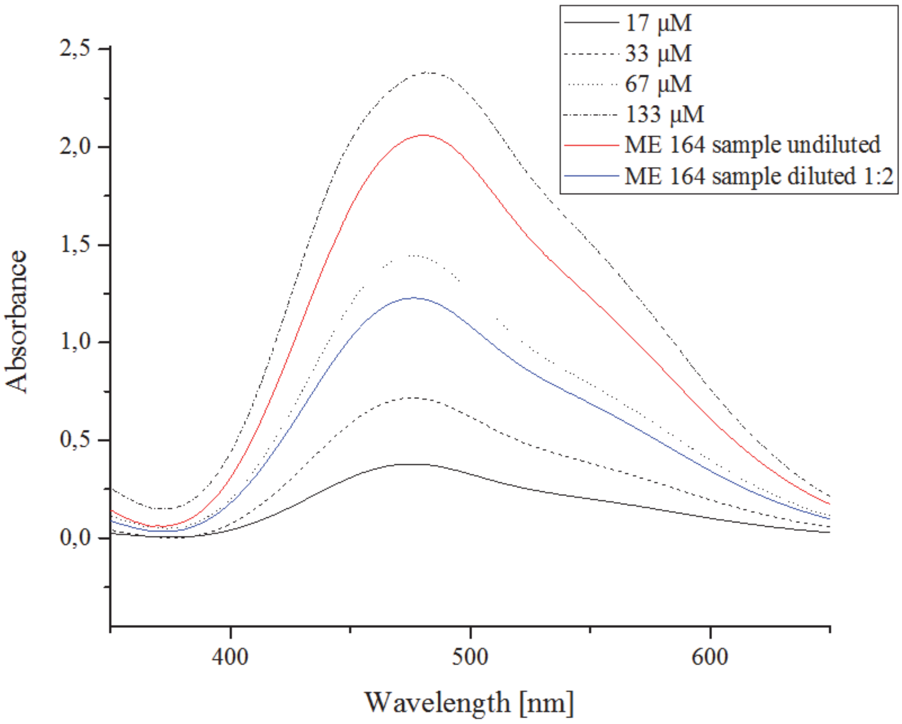
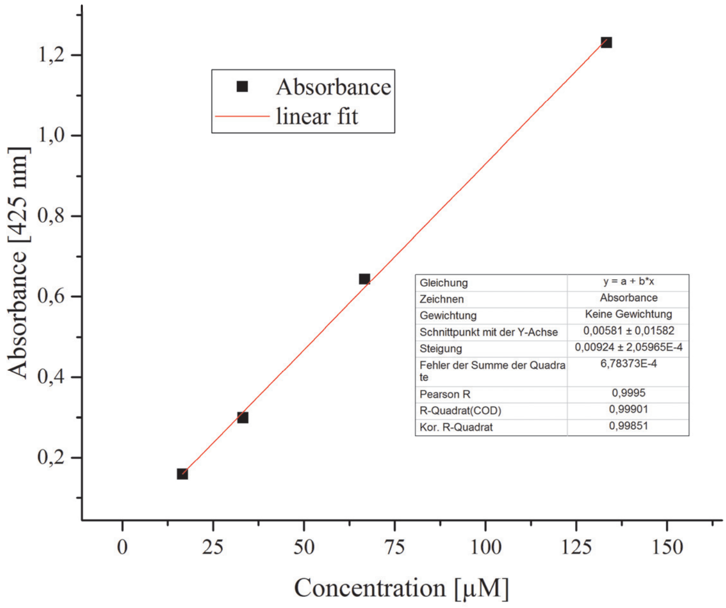


Figure 52 UV-spectra of (*E,E*)-hexa-2,4-dienal (black) and (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO)) (red and blue) after derivatization with 2,4-dinitrophenylhydrazine (DNPH)



Gleichung	y = a + b*x
Zeichnen	Absorbance
Gewichtung	Keine Gewichtung
Schnittpunkt mit der Y-Achse	0,00581 ± 0,01582
Steigung	0,00924 ± 2,05965E-4
Fehler der Summe der Quadrate	6,78373E-4
Pearson R	0,9995
R-Quadrat(COD)	0,99901
Kor. R-Quadrat	0,99851

Figure 53 Calibration curve of (*E,E*)-hexa-2,4-dienal at λ_{ex} = 425 nm after derivatization with 2,4-dinitrophenylhydrazine (DNPH)

4.5. HPLC-METHOD DEVELOPMENT FOR LYS(MUC-CHO)

A RP-HPLC-method for analysis of Lys(MUC-CHO) was developed using 0.1 % acetic acid and acetonitrile. Due to the ionic interactions peak shape deformation in width was observed unless a rash change in organic gradient of 20 % per minute was performed. Manual compound optimization resulted in three transitions suited for multiple reaction monitoring: 241.1 → 223.2, 130.2 and 84.2 (cf. ME 164, Figure 54). Calibration samples for Lys(MUC-CHO) were analyzed in the range of 0.1 – 1000 ng/ml. LOD for Lys(MUC-CHO) was estimated experimentally to be 0.5 ng/ml. However, we did not succeed in detection of Lys(MUC-CHO) in incubations of serum albumin with (*E,E*)-muconaldehyde. A possible explanation lies within a modified experimental protocol. Since animal experiments had started before finishing the analytical method development determining Lys(MUC-CHO), the modified workup procedure used in the animal experiments operated using higher amounts of sodium borohydride which supposedly lead to full reduction of the aldehyde moiety. Therefore, analytical focus was shifted to Lys(MUC-OH).

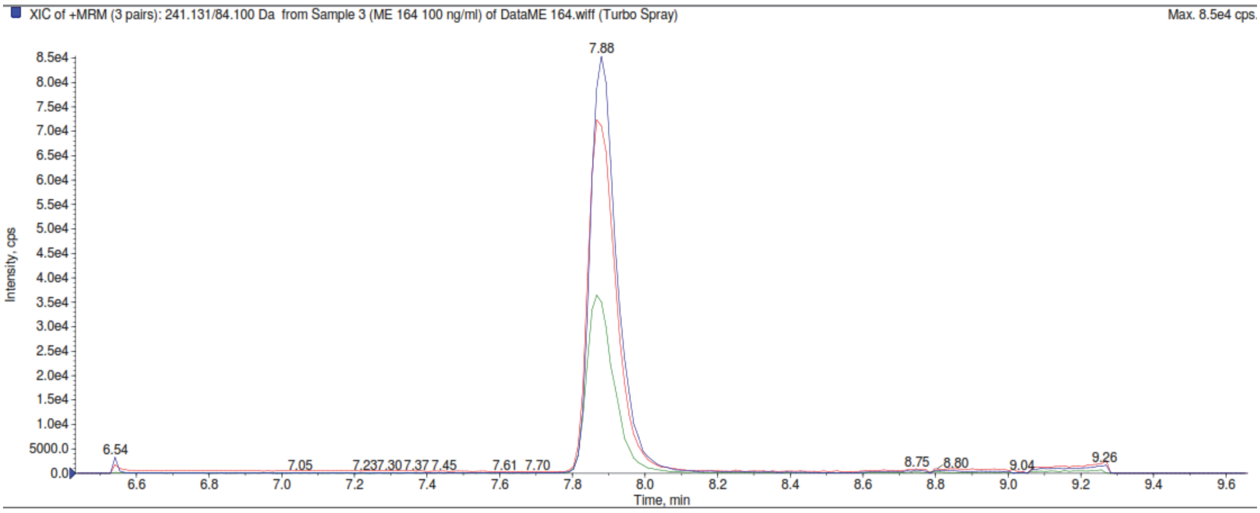


Figure 54 Excerpt from an HPLC-ESI-MS/MS chromatogram for Lys(MUC-CHO) (100 ng/ml) following MRM transitions 241.1 → 223.2 (red), 130.2 (green) and 84.2 (blue).

4.6. HPLC-METHOD DEVELOPMENT FOR LYS(MUC-OH)

A HILIC-HPLC-method for analysis of Lys(MUC-OH) was developed due to a lack of retention in polarly modified RP-HPLC using 10 mM ammonium acetate pH = 4.8. Manual compound optimization resulted in three transitions suited for multiple reaction monitoring: 243.2 → 147.1, 130.0 and 79.1 (Figure 55).

Following incubation of serum albumin with (*E,E*)-muconaldehyde, reduction with sodium borohydride and enzymatic hydrolysis using pronase, detection of Lys(MUC-OH) was achieved (Figure 56) (cf. ME 192).

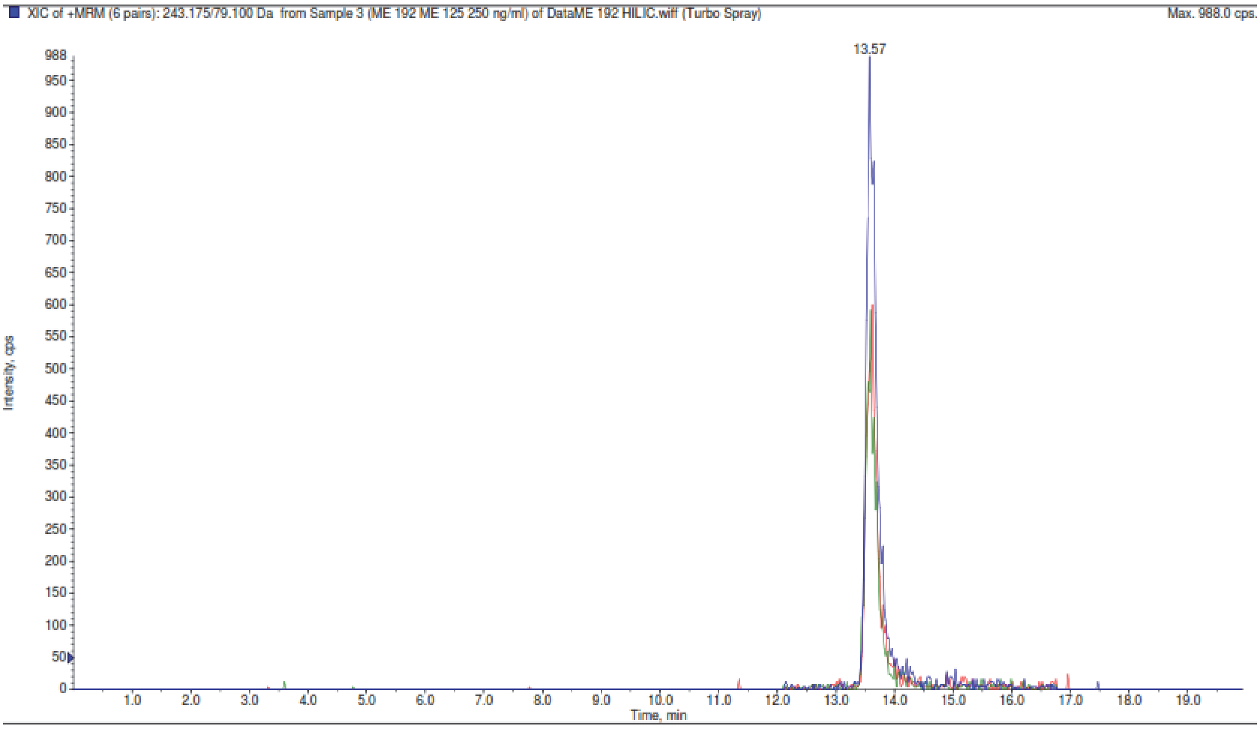


Figure 55 HILIC-HPLC-ESI$^+$-MS/MS chromatogram for Lys(MUC-OH) (250 ng/ml) following MRM transitions 243.2 → 147.1 (red), 130.0 (green) and 79.1 (blue)

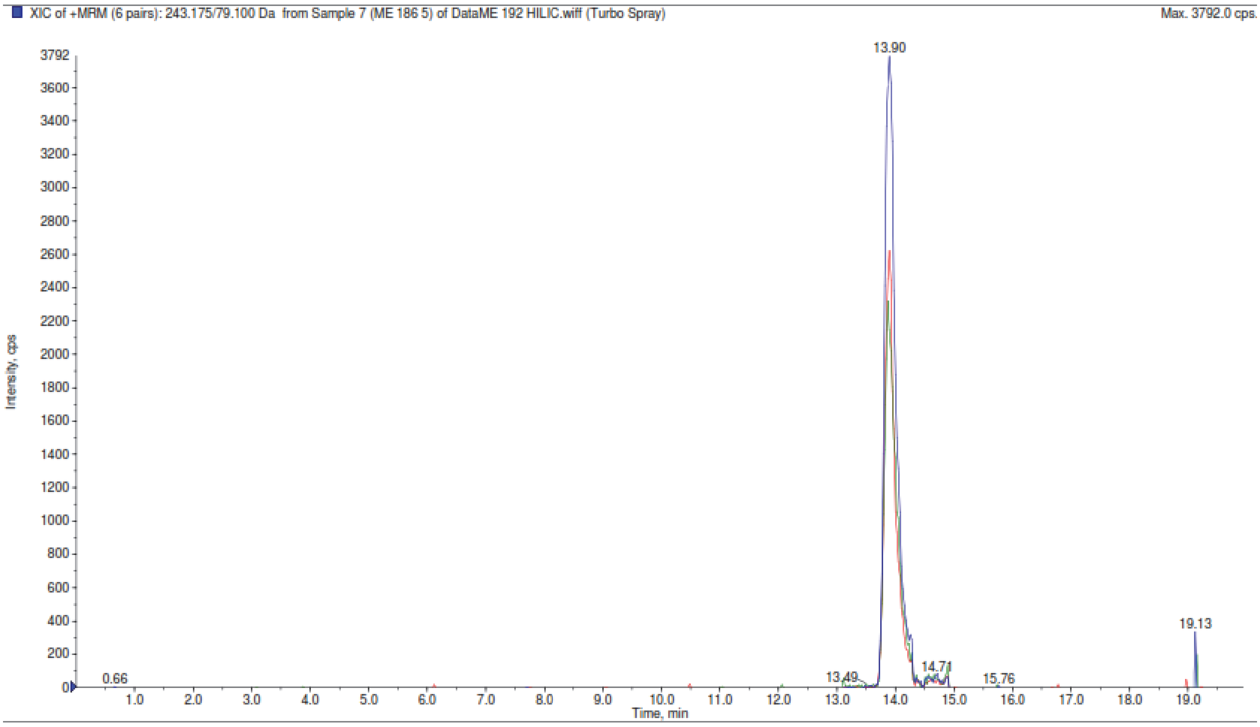


Figure 56 HILIC-HPLC-ESI$^+$-MS/MS chromatogram for incubation of serum albumin (16 mg/ml) with (*E,E*)-muconaldehyde (10 mM) following reduction with serum albumin (4 mg/ml) and subsequent hydrolysis using pronase (2 mg/ml) following MRM transitions 243.2 → 147.1 (red), 130.0 (green) and 79.1 (blue)

4.7. ANIMAL EXPERIMENT

4.7.1. SERUM ALBUMIN YIELD

Finally, we strived to ascertain the principal hypothesis of (*E,E*)-muconaldehyde formation *in vivo*. Therefore, B6C3F1 mice were exposed to 0, 400 and 800 mg/kg b.w. benzene. After 24 h mice were sacrificed, serum albumin was isolated. Protein content of serum albumin samples was determined after elution from blue sepharose affinity chromatography column using optical density measurement at 280 nm (Table 8). Linear calibration for serum albumin was achieved previously in the range of 0.5 – 5 mg/ml[12].

Table 8 Protein yields of serum albumin in animal experiment

Sample	Protein yield
ME 161 (Control)	12 mg
ME 163 (Control) sample 1	17 mg
ME 163 (Control) sample 2	24 mg
ME 165 (400 mg/kg bw) sample 1	18 mg
ME 165 (400 mg/kg bw) sample 2	27 mg
ME 167 (400 mg/kg bw) sample 1	19 mg
ME 167 (400 mg/kg bw) sample 2	30 mg
ME 171 (800 mg/kg bw) sample 1	20 mg
ME 171 (800 mg/kg bw) sample 2	25 mg
ME 172 (800 mg/kg bw) sample 1	27 mg
ME 172 (800 mg/kg bw) sample 2	15 mg

4.7.2. LYS(MUC-OH) DETERMINATION

Lys(MUC-OH) determination results in the animal experiment have not been included in this work due to technical failure of the mass spectrometer, which could not be reinstalled in due time. Since our earlier experiments indicated LOD above incubation concentrations of 100 µM (*E,E*)-muconaldehyde (cf. ME 192), detection of Lys(MUC-OH) might probably have been unsuccessful due to analytical limitations.

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[12] Calibration $y = 0.2489 \times x - 0.0005$ with $R^2 = 0.995$ performed by Christian Haase.

4.8. CYTOTOXICITY OF (*E,E*)-MUCONALDEHYDE AND LYS(MUC-CHO)[13]

In our lab, it is common to test cytotoxicity using hepatic cancer cell lines or primary hepatocytes. However, toxicity of benzene does not target the liver but rather the bone marrow. To shed some light onto the mechanism of benzene induced hematotoxicity it is therefore necessary to employ cell culture models with the toxic sensitivity of hematopoietic stem cells towards benzene. Today, there is no generally accepted cell culture model to mirror the hematotoxicity of benzene. In order to test the assumption that (*E,E*)-muconaldehyde or (*E,E*)-muconaldehyde-related conjugates such as Lys(MUC-CHO) might be responsible for benzene induced hematotoxicity, we strived to test these compounds in a suitable cell culture model.

The NB4 cell line was established in 1991 and is derived from the marrow of a 20 year old female patient who suffered from acute promyelocytic leukemia in relapse (Lanotte *et al.* 1991). Morphologically they are characterized as myeloblasts. Myeloblasts are cells in the early stage of differentiation from a common myeloid progenitor cell. Myeloblasts do still have a cell nucleus and are able to perform full cell cycle. After addition of e.g. all-trans retinoic acid these cells are able to restore cell maturation leading down the myeloid pathway to cells which are important for the human immune system such as basophils, neutrophils, eosinophils, macrophages or myeloid dendritic cells (*ibd.*).

Since acute myeloid leukemia is a disease pattern closely connected with exposure to benzene, we considered a promyelocytic cell line to be a suitable model to test hematotoxicity of (*E,E*)-muconaldehyde or (*E,E*)-muconaldehyde-related conjugates such as Lys(MUC-CHO). So far, hematotoxicity of (*E,E*)-muconaldehyde has been only been shown after application *in vivo*.

---

[13] Cytotoxicty analyses have been conducted in collaboration with the *Institute für Toxikologie* at *Universitätsmedizin Mainz*. FACS-analyses have been kindly provided by Viviane Ponath.

(*E,E*)-Muconaldehyde has been shown to be a potent cytotoxin in NB4 cells (Figure 57). Total cell death count after incubation with (*E,E*)-muconaldehyde for 24 h results in an EC_{50} = 0.98 µM. No margin of error could be calculated due to missing data points. Apoptosis and necrosis were determined above 2 µM however standard deviation increased dramatically, which was caused by interference in fluorescence by the (*E,E*)-muconaldehyde. Therefore, fluorescence-activated cell sorting (FACS) results above 2 µM could not be interpreted with sufficient accuracy. Increased apoptosis compared to control is statistically significant from 0.5 µM upwards. Increased necrosis compared to control is statistically significant from 2 µM.

Lys(MUC-CHO) does not show relevant cytotoxicity in NB4 cells compared to (*E,E*)-muconaldehyde (Figure 58). No EC_{50} could be calculated. Concentrations above 10 µM could not be tested since the substance was not available at a higher stock concentration in aqueous media.

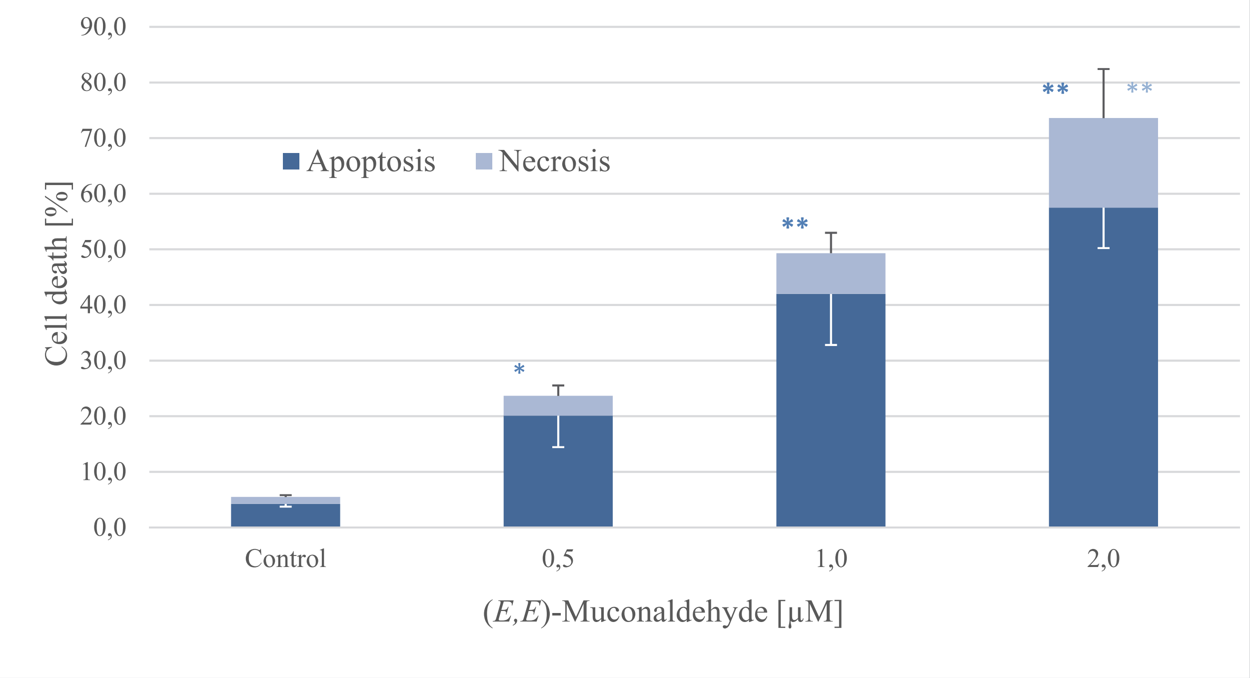


Figure 57 Cytotoxicity of NB4-cells after incubation with different concentrations of (*E,E*)-muconaldehyde for 24 h (n=4) with *p<0.05, **p<0.01.

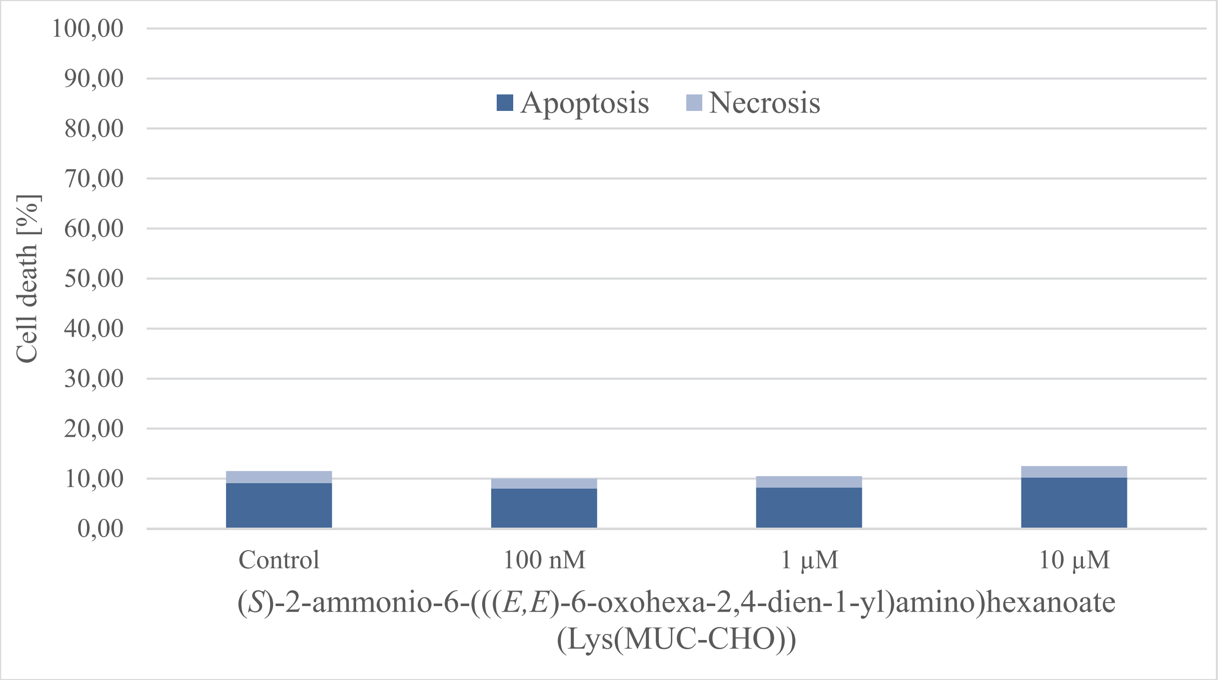
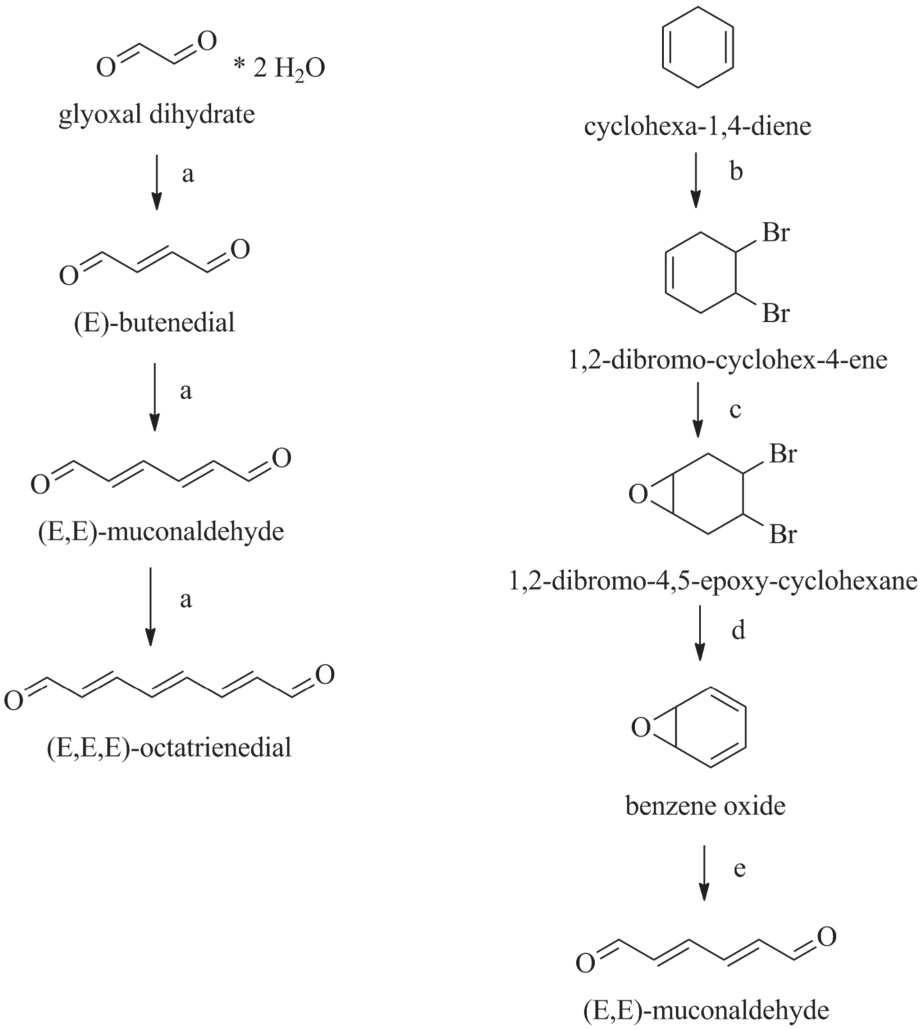


Figure 58 Cytotoxicity of NB4-cells after incubation with different concentrations of (*S*)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-CHO)) for 24 h (n=1).

5. DISCUSSION

5.1. SYNTHESIS OF (*E,E*)-MUCONALDEHYDE

(*E,E*)-Muconaldehyde has traditionally been prepared via either triphenylphosphoranylidene-acetaldehyde mediated homologous elongation of glyoxal or (*E*)-butenedial or via oxidation of benzene oxide (Scheme 6).

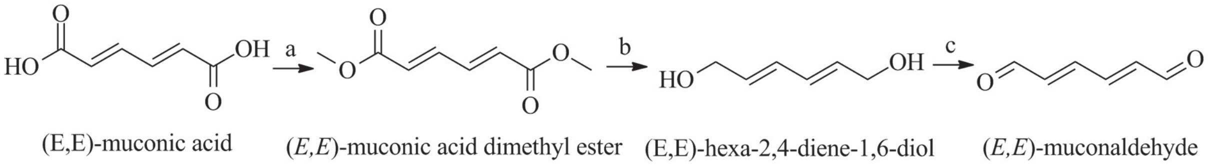


Scheme 6 Traditional syntheses of (E,E)-muconaldehyde (MUC). Reagents: (a) FMTP, DMF; (b) Br_2, CH_2Cl_2; (c) mCPBA, CH_2Cl_2; (d) DBU, Et_2O; (e) NBS, DMSO/H_2O

The first route has been conducted e.g. by Adger starting from furan which was oxidized *in situ* to (*E*)-butenedial (Adger *et al.* 1993). Twenty years ago Koßmehl accomplished synthesis of (*E,E*)-muconaldehyde similarly starting from glyoxal (Koßmehl & Bohn 1974). Both routes have come with difficulties. Adger's approach results in a 1:2 mixture of (*E,E*)- and (*E,Z*)-muconaldehyde. Koßmehl's approach has been revised in our laboratory. Unfortunately, it caused significant problems in specificity. Even though the (formylmethylen)-(tripheny1)phosphoran(FMTP)-reagent had been employed in exact stoichiometric amounts, substantial quantities of side products were formed due to the reactivity of the newly synthesized product. Whereas the reaction has to be conducted in the strict absence of water, glyoxal on the opposite is commercially provided as dihydrate. Therefore, even little stoichiometric differences in the reagent scale may lead to a different array of products.

Davies and Whitman established in 1977 the second route and synthesized (*E,E*)-muconaldehyde in a biomimetic fashion via oxidation of benzene oxide/oxepin. In our lab, the final conversion from benzene oxide to (*E,E*)-muconaldehyde using *N*-bromo-succinimide resulted in formation of a mixture of different stereoisomers and phenol. Purification by recrystallization twice from light petroleum – ether (1 + 1), did not provide sufficient purity. Separation of (*E,E*)-muconaldehyde could only be achieved using column chromatography. Unfortunately, distinct identical retention of stereoisomers of (*E,E*)-muconaldehyde did not allow for analytical purity under these conditions.

For synthesis of isomeric pure (*E,E*)-muconaldehyde we found the conversion of (*E,E*)-hexa-2,4-diene-1,6-diol in presence of manganese oxide to be advantageous compared to previously described methods (Scheme 7). The reaction occurs under neutral conditions in aprotic solvents with remarkable selectivity. No side products such as C2-homologues as in Adger's or Koßmehl's approach or traces of (*E,Z*)- or (*Z,Z*)-muconaldehyde using the route of Davies and Whitman could be detected. We account isomeric purity of (*E,E*)-muconaldehyde to the mechanism of the manganese oxide catalyzed oxidation preventing the formation of stereoisomers.



Scheme 7 Synthesis of (*E,E*)-Muconaldehyde (MUC). Reagents: (a) AcCl, MeOH; (b) DIBAL-H, $CHCl_3$; (c) MnO_2, CH_3CN

5.2. PREVIOUSLY REPORTED CONJUGATES

We report the formation of the distinct conjugate Lys(MUC-OH) upon reaction of (*E,E*)-muconaldehyde with serum albumin after reduction with sodium borohydride and enzymatic hydrolysis using pronase. To our knowledge, no distinct protein conjugate of (*E,E*)-muconaldehyde has been reported previously. In the following sections we will discuss:

1. Cytotoxicity of Lys(MUC-CHO) and consequences for benzene toxicity.
2. Potential *in vivo* metabolism of (*E,E*)-muconaldehyde-lysine conjugates.
3. Potential mechanisms of toxicity of (*E,E*)-muconaldehyde-lysine conjugates.
4. Relevance of (*E,E*)-muconaldehyde-lysine conjugates as potential biomarkers of benzene.
5. Future work regarding (*E,E*)-muconaldehyde-lysine conjugates in benzene toxicity.

5.3. CYTOTOXICITY OF (*E,E*)-MUCONALDEHYDE AND LYS(MUC-CHO)

(*E,E*)-Muconaldehyde has been tested previously for cytotoxicity in bacterial and mammalian cells (Table 9).

Table 9 Cytotoxicity estimates for (E,E)-muconaldehyde taken from the literature.

Cell model	$EC_{50\ est.}$	Reference
Chinese hamster ovary (CHO)	4 µM	(Witz *et al.* 1990)
Chinese hamster cell line (V79)	3 µM	(Chang *et al.* 1994)
Primary rat hepatocytes (PRH)	40 µM	(Witz *et al.* 1990)

Calculated $EC_{50} \approx 1$ µM in human NB4 cells seems in good concordance with values given in the literature for Chinese hamster ovary (CHO) and V79 cells given the fact that incubation procedures and assays used for cytotoxicity vary.

One might expect to see a more pronounced cytotoxic effect especially using a human & bone marrow derived cell line compared to bone marrow unrelated & mammalian cells from a different species. Such a positive result might have provided additional evidence to the hypothesis that (*E,E*)-muconaldehyde might be the key agent responsible for myelotoxicity of benzene in humans. However, our cytotoxicity results do not support this hypothesis. Does this result exclude the possibility of (*E,E*)-muconaldehyde being the responsible agent for myelotoxicity in humans?[14] There are different ways to interpret our results.

5.3.1. THE TEST SUBSTANCE

One could argue, (*E,E*)-muconaldehyde is missing additional cytotoxicity in NB4 cells compared to mammalian cells since (*E,E*)-muconaldehyde might not be the toxic agent responsible for myelotoxicity of benzene in humans or synergistic factors in the metabolism of benzene are the real cause for myelotoxicity and cannot be reproduced using one single metabolite. However, these reasons seem to be ruled out since (*E,E*)-muconaldehyde alone has been proven to be highly hematotoxic *in vivo* (Witz *et al.* 1985).

5.3.2. THE IN VITRO MODEL

One could further argue, NB4 cells are missing additional cytotoxicity compared to mammalian cells upon treatment with (*E,E*)-muconaldehyde due to the inapt transfer from an *in vivo* system to an *in vitro* model which mirrors the myelotoxicity of benzene.

Two main reasons argue in advance of this position.

1. Missing literature. To our knowledge, an *in vitro* model capable of mirroring myelotoxicity of benzene has not been reported yet, mirroring the difficulty in finding a system which incorporates both the myelotoxic sensitivity as well as the metabolic competence.[15]
2. Cell line limitations. NB4 cells might not be a suitable cell line for benzene derived myelotoxicity in humans since the metabolism, morphologic or physiologic characteristics of the cell line do not represent the *in vivo* situation and where cell damage might occur at an even earlier stage of cell maturation.

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[14] On the contrary, these results can also be interpreted as a strengthening of our favored working hypothesis which will be discussed at length in section: 5.5.3 Deliverance of (*E,E*)-muconaldehyde to the bone marrow via a proteinogenic carrier.
In short, it may not be organ specificity of a single metabolite, namely (*E,E*)-muconaldehyde, which causes myelotoxicity of benzene. Rather, based on our results, (*E,E*)-muconaldehyde seems to be ubiquitously toxic and organ selectivity might be explained by a carrier mechanism, which transports (E,E)-muconaldehyde to the bone marrow where it can exert its toxic effects.

[15] Meaning that substantially higher cytotoxicity in bone marrow derived cells can be detected upon treatment with benzene (or its metabolites) compared to cells not related to the bone marrow

5.3.3. SPECIFICITY

Due to the similarity of the cytotoxicity results in our work compared to the literature, it seems that cytotoxicity of (*E,E*)-muconaldehyde in NB4 cells is unrelated to physiological characteristics owned uniquely by bone marrow cells. Rather it seems that (*E,E*)-muconaldehyde shows unspecific potential for cytotoxicity in various kinds of cells. However, the missing organ specificity implies, that NB4 cells are no suitable model to mirror in specific regard to the myelotoxicity of (*E,E*)-muconaldehyde.

Since (*E,E*)-muconaldehyde has been proven to be highly hematotoxic *in vivo*, instead of selecting one cell line with specific limitations, isolation of primary bone marrow cells might be an alternative to study hematotoxicity of benzene. A problem with that solution is that bone marrow progenitor cells lean towards differentiation. Since research in this area is advancing slowly, cultivation of undifferentiated primary bone marrow cells poses a difficult undertaking. Alternatively, one could treat mice with benzene first and analyze afterwards the bone marrow cells more closely for the kind and magnitude of cellular damage.

Lys-(MUC-CHO) does not show any relevant cytotoxicity up to 10 µM in NB4 cells. Therefore, the unspecific mechanism of toxicity that provides cytotoxicity to (*E,E*)-muconaldehyde does not seem to apply in the case of Lys(MUC-CHO) despite the presence of a potential reactive structural element, an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde. Since NB4 cells do not seem to be a suitable cell model to analyze myelotoxicity of benzene (as discussed above), the question cannot be answered whether Lys(MUC-CHO) does in fact have cytotoxic properties pertaining to the myelotoxicity of benzene.

5.4. METABOLISM OF (*E,E*)-MUCONALDEHYDE-LYSINE CONJUGATES

5.4.1. FORMATION OF LYS(MUC-CHO) *IN VIVO*?

Lys(MUC-CHO) is generated *in vitro* after reduction of serum albumin modified with (*E,E*)-muconaldehyde using sodium borohydride. Can reduction of the Schiff base occur *in vivo*? Or are cytotoxicity data of Lys(MUC-CHO) biologically insignificant since Lys(MUC-CHO) resembles an artificial artifact of the original conjugate (Figure 59)?

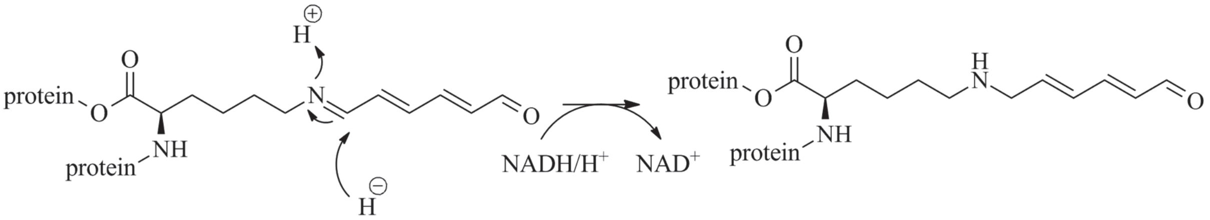

Figure 59 Possible formation of Lys(MUC-CHO) *in vivo* via reduction of the initially formed Schiff base

Studies examining this question have been performed in the past for reaction of proteins with acetaldehyde, the primary metabolite of ethanol, which covalently binds to proteinogenic lysine residues (Donohue *et al.* 1983; Moncada & Israel 1999; Thiele *et al.* 1994; Tuma *et al.* 1987; Tuma *et al.* 1991) (Figure 60).

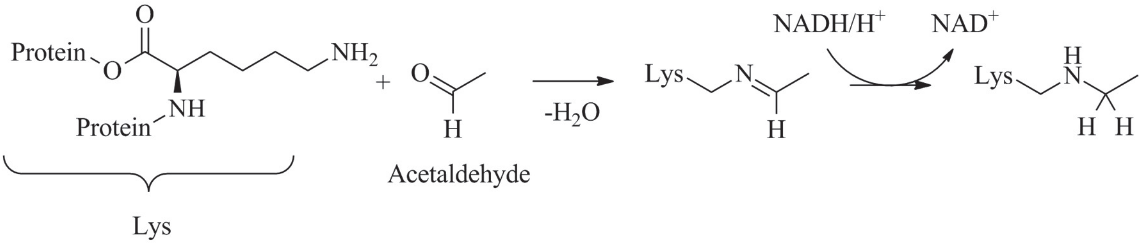

Figure 60 Proteinogenic lysine modification by acetaldehyde *in vivo.* Modified from (Nicholls *et al.* 1992)

In vitro studies to stabilize Schiff bases have relied on the use of non-physiological reducing agents such as sodium (cyano)borohydride. Tuma and coworkers showed that ascorbate but not glutathione was able to stabilize Schiff bases deriving from acetaldehyde (Tuma *et al.* 1984). However, other reducing agents may also be able to perform Schiff base reduction such as NADH. In the case of ethanol consumption, large amounts of NADH are formed in the liver by oxidation of ethanol. Thus, strongly reducing conditions prevailing in the liver may enhance reduction of Schiff base adducts (Nicholls *et al.* 1992).

Recent studies analyzing proteinogenic N^6-ethyl lysine after alcohol consumption refrained from additional reduction in the sample workup (Mabuchi *et al.* 2012). Additional reduction, e.g. via sodium cyanoborohydride, did not result in increases for analytically detected N^6-ethyl lysine (*ibd.*).

Taken these information into account, *in vivo* formation of Lys(MUC-CHO) by reduction of the initially formed Schiff base seems reasonable and might be relevant especially considering that (*E,E*)-muconaldehyde might be present in the liver at far lower concentrations compared to hepatic concentrations of acetaldehyde after alcohol consumption.

Therefore, we believe there is reasonable evidence that supports the formation of proteinogenic Lys(MUC-CHO) *in vivo*.

5.4.2. RESIDUAL REACTIVITY OF LYS(MUC-CHO)

Based on the presence of an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde moiety, Lys(MUC-CHO) bears residual reactivity towards cellular nucleophiles. Two specific reaction types can be postulated aside from unspecific polymerization in presence of nucleophiles as seen with (*E,E*)-hexa-2,4-dienal. The reaction with hard nucleophiles, e.g. amines, attacking the aldehyde carbon, followed potentially by intracellular NADH mediated reduction, or reaction with soft nucleophiles, e.g. thiols, attacking the β- and/or δ-carbon of the adjacent double bond acting as a Michael-type acceptor (Figure 61).

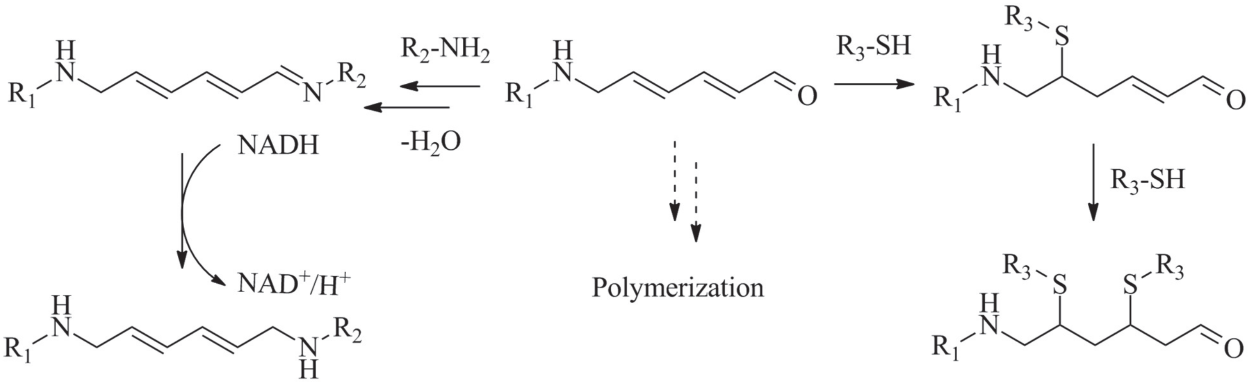

Figure 61 General reaction scheme for residual reactivity of Lys(MUC-CHO) (R_1-NH_2 = proteinogenic lysine) with amines (R_2-NH_2) and thiols (R_3-SH).

Therefore, reduced half-life of proteinogenic Lys(MUC-CHO) due to covalent interaction with physiological nucleophiles such as glutathione must be considered an important factor in determining its biological significance.

5.4.3. METABOLISM OF LYS(MUC-CHO)

Is Lys(MUC-CHO) expected to undergo further metabolism *in vivo* aside from further adduct formation with cellular nucleophiles?

Precedence is provided by the metabolism of acrolein, resulting in the formation of S-(3-oxopropyl)-mercapturic acid (OPMA) which bears structural resemblance to Lys(MUC-CHO) (Parent *et al.* 1998). Both reduction and oxidation of the carbonyl group can be observed following acrolein exposure in humans resulting in the urine excretion of S-(3-hydroxypropyl)-mercapturic acid (3-HPMA) (Carmella *et al.* 2007) and carboxypropyl-mercapturic acid (CEMA) (Ding *et al.* 2009) (Figure 62). Both compounds were detected recently at University of Technology in Kaiserslautern in the group of Prof. Dr. Elke Richling following digestion of commercially available potato crisps by five volunteers (Watzek *et al.* 2012).

On the assumption of metabolic competence at the location of protein hydrolysis, metabolites Lys(MUC-OH) and (*E,E*)-6-(((*R*)-5-ammonio-5-carboxylatopentyl)ammonio)hexa-2,4-dienoate (Lys(MUC-CO_2H)) are therefore reasonably expected to occur from *in vivo* metabolism of Lys(MUC-CHO) (Figure 63).

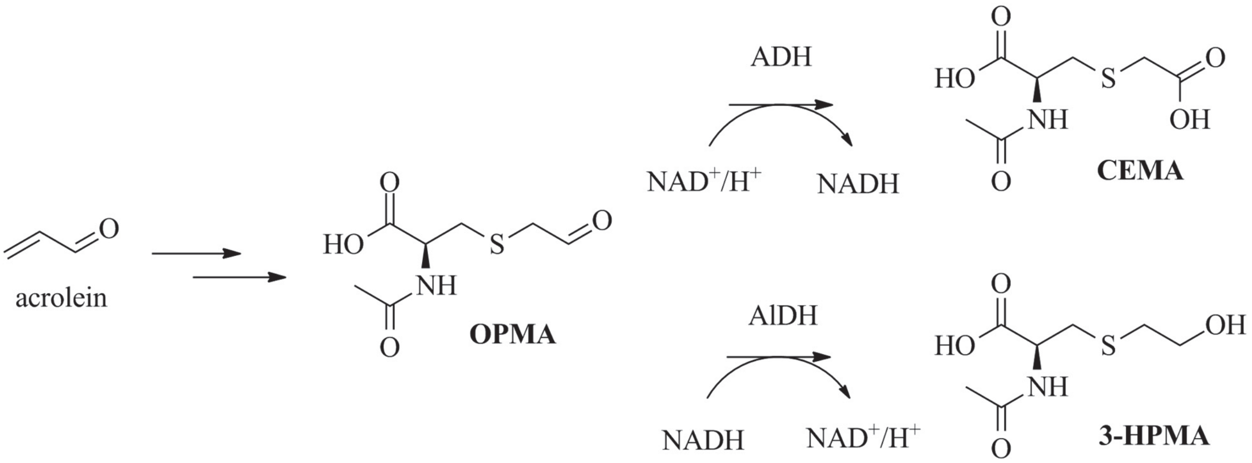

Figure 62 Metabolism of 3-Oxopropyl mercapturic acid (OPMA) to 3-Hydroxyproylmercapturic acid (3-HPMA) and Carboxyethyl mercapturic acid (CEMA) ADH = Aldehyde dehydrogenase, AlDH = Alcohol dehydrogenase. Taken and modified from (Parent *et al.* 1998)

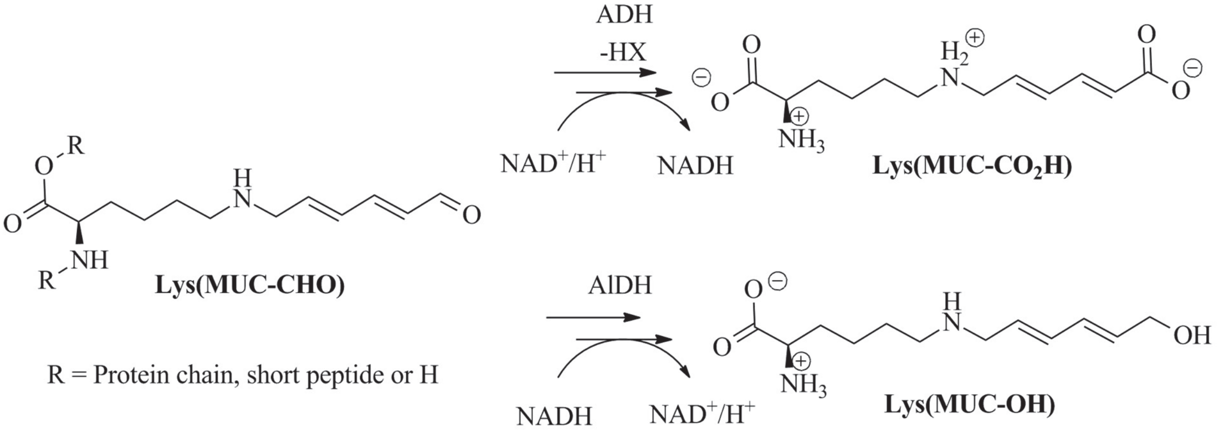

Figure 63 Proposal for further metabolism of (*R*)-2-ammonio-6-(((2,4)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate (Lys-MUC(CHO)) to (E,E)-6-(((*R*)-5-ammonio-5-carboxylatopentyl)ammonio)hexa-2,4-dienoate (Lys(MUC-CO_2H) and (*R*)-2-ammonio-6-(((E,E)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-OH)), ADH = Aldehyde dehydrogenase, AlDH = Alcohol dehydrogenase following protein hydrolysis.

Since either metabolic pathway leads to elimination of the electrophilic target (the aldehyde moiety), it may be regarded as detoxification of Lys(MUC-CHO).

5.5. MECHANISM OF TOXICITY

We will discuss the hypothesis that transient conjugates of (*E,E*)-muconaldehyde might be the reason for benzene mediated hematotoxicity. There might be three different modes of action.

1. Hapten formation
2. Inherent conjugate toxicity
3. Deliverance of (*E,E*)-muconaldehyde to the bone marrow via a proteinogenic carrier.

5.5.1. HAPTEN FORMATION

Haptens (gr. *haptein*) are low molecular weight compounds ($M_W < 1000$ g/mol) that must bind to a carrier molecule to become antigenic (Landsteiner & Jacobs 1936). “The carrier is usually an endogenous or exogenous protein to which the […] chemical is covalently bound. […] Prohaptens are chemicals that are not protein reactive unless they are metabolically activated to electrophilic species.” (Chipinda *et al.* 2011). Supposedly as a response to failure in recognition of an altered protein structure, the immune system is urged into antibody production, generating specific protein markers which can be detected in the blood following an immune response.

Benzene has been shown to elicit albeit weak immune responses in exposed workers (Dimitrova *et al.* 2005; Kirkeleit *et al.* 2006; Uzma *et al.* 2010). It is believed that HQ and 1,4-BQ are responsible agents for haptenic activity (Basketter & Gooodwin 1988; Basketter & Liden 1992; Rombach & Hanzlik 1997; Ewens *et al.* 1999).

There have been reports about haptens damaging the blood forming system, most notably methyldopa (Worlledge *et al.* 1966), high-dose intravenous penicillin (Ries 1975), and cefotetan, a cephalosporin (Garratty *et al.* 1992). However, pathologically these conditions can be described as *hemolytic* anemias, originating from antibodies targeting the late stage erythrocyte membrane. Hemolytic anemia is a condition not seen in chronic exposure to benzene.

As we’ve shown in section 2.3.1, hematotoxicity of benzene is thought to occur at a very early stage in hematopoiesis targeting bone marrow progenitor cells rather than fully developed erythrocytes. Since these two conditions differ by origin, we do not see experimental evidence to support the hypothesis, that (*E,E*)-muconaldehyde-lysine conjugates acting as haptens are be responsible for the myelotoxicity observed in chronic exposure to benzene.

5.5.2. INHERENT TOXICITY OF AMINO ACID CONJUGATES

5.5.2.1. FUNCTIONAL IMPAIRMENT OF TARGET PROTEINS

Another hypothetical mechanism of benzene toxicity operates by the impairment of functional proteins by covalent modification through (*E,E*)-muconaldehyde in the bone marrow.

There is only one publication addressing the reaction of (*E,E*)-muconaldehyde with proteins which addresses the decrease in free primary amino groups but leaves out functional impairment (Udupi *et al.* 1994). Therefore, we relate to structural analogues of (*E,E*)-muconaldehyde such as acrolein or 4-hydroxy-non-2-enal.

Incubation studies of purified proteins such as glyceraldehyde-3-phosphate dehydrogenase with acrolein or 4-hydroxy-non-2-enal reveal that proteinogenic lysine, histidine and cysteine residues are subject to adduct formation and enzyme function is impaired (Szweda *et al.* 1993; Uchida *et al.* 1994; Friguet *et al.* 1994; Ishii *et al.* 2003; Carbone *et al.* 2005; Tamamizu-Kato *et al.* 2007; LoPachin *et al.* 2008; Tran *et al.* 2014).

Due to structural analogy functional impairment of proteins by (*E,E*)-muconaldehyde seems plausible and might be in part responsible for myelotoxicity observed in chronic exposure to benzene. Organ specificity targeting the bone marrow seems plausible if the bone marrow can qualify as location of formation or location of uptake of (*E,E*)-muconaldehyde. However, cellular specificity towards early stage progenitor cells remains to be answered at this point.

To prove that amino acid conjugates generally can convey organ-specific toxic responses, we will illustrate with the example of GSH-HQ conjugates.

5.5.2.2. GLUTATHIONE-HYDROQUINONE CONJUGATES

In 1996, Bratton *et al*. discovered (glutathion-S-yl)-hydroquinone (GS-HQ) conjugates present in bone marrow following concomitant administration of phenol and hydroquinone (Bratton *et al.* 1997) (Figure 64).

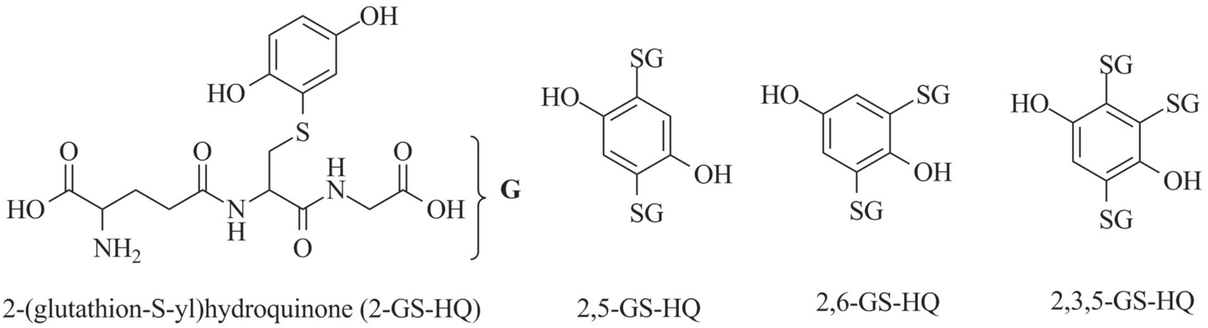

Figure 64 Glutathione-hydroquinone conjugates identified after concomitant administration of phenol & hydroquinone. Modified from (Bratton *et al.* 1997).

GS-HQ conjugates have been shown to cause erythrotoxicity in rats decreasing ^{59}Fe incorporation into reticulocytes (sc. bone marrow erythrocyte progenitor cells) and reduce the blood lymphocyte count (Lau *et al.* 2010). Redox cycling to glutathione benzoquinone conjugates is believed to be responsible for hematotoxic effects by formation of electrophilic species as well as ROS formation (Monks *et al.* 2010) (Figure 65).

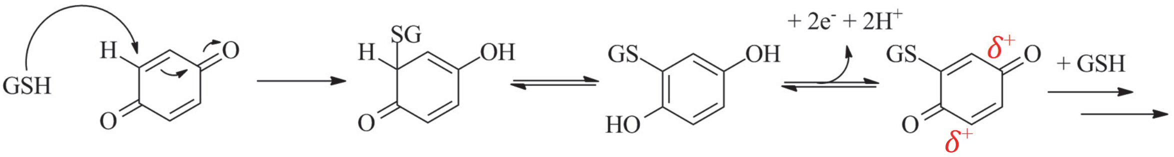

Figure 65 Redox cycling from GS-HQ to BQ-HQ possible. Taken and modified from (Monks *et al.* 2010).

Based on these findings, the authors conclude, that GS-HQ conjugates "may contribute to benzene-mediated hematotoxicity" (*ibd.*).

There are three reasons arguing against a key role of GS-HQ conjugates in benzene mediated hematotoxicity.

1. Nephrotoxicity. 2-GS-HQ & 2,3,5-GS-HQ have been shown to be potent nephrotoxins *ex vivo* (Hill *et al.* 1994), explaining the nephron carcinogenicity observed in rats following chronic exposure to hydroquinone (Shibata *et al.* 1991; Kari *et al.* 1992). However, nephrotoxicity is a condition observed neither in acute nor chronic poisoning with benzene.
2. Erythropoietin (EPO) ↓. EPO is a hormone in maturation process of erythrocytes. GS-HQ conjugates “are capable of damaging cells within the proximal tubules of the kidney (Lau *et al.* 1988), the major site of EPO production” (Lau *et al.* 2010). The question arising now whether hematotoxicity observed with GS-HQ conjugates is due to damage of the bone marrow by itself or indirectly due to depression of EPO. GS-HQ conjugates were “capable of indirectly inducing anemia by reducing serum EPO levels […] both 2,3,5-GS-HQ and 2,6-GS-HQ significantly reduced circulating EPO levels.” (*ibd.*)
3. GS-HQ levels after benzene administration. Even though GS-HQ conjugates could be detected after administration of PH & HQ, levels of GS-HQ conjugates after benzene administration are low, in fact so low, that reporting authors did not use coherent quantification units (e.g. nmol/2 femurs for phenol & hydroquinone administration vs. pmol/mg protein for benzene administration), thereby making it impossible to calculate a percentage of the fraction for GS-HQ formed in animals treated with phenol and hydroquinone versus animals treated with benzene. (*ibd.*)

Therefore, experimental evidence suggests GS-HQ conjugates not to be primarily responsible for benzene mediated hematotoxicity.

The example of GS-HQ conjugates shows that electrophilic amino acid conjugates of benzene metabolites are principally able to retain inherent organ specific toxic properties. Therefore, myelotoxicity of benzene occurring via formation of (*E,E*)-muconaldehyde-lysine conjugates does not appear to be an unreasonable hypothesis. The question arises: Does the literature provide evidence for the toxicity of related lysine conjugates?

5.5.2.3. LYSINE CONJUGATES OF BIS-ALDEHYDES

Literature about isolated lysine conjugates is restricted to biomarker analyses and toxicity of modified poly-L-lysine peptides, making it difficult to draw a comparison to cytotoxicity data of Lys(MUC-CHO).

Based on the missing positive cytotoxicity results for Lys(MUC-CHO) in NB4 cells, we cannot provide experimental evidence to add to the hypothesis that (*E,E*)-muconaldehyde-lysine conjugates might be responsible for the myelotoxicity observed in chronic exposure to benzene.

5.5.3. DELIVERANCE OF (*E,E*)-MUCONALDEHYDE TO THE BONE MARROW VIA A PROTEINOGENIC CARRIER

Another mechanism of benzene toxicity which we favor includes

1. Formation of (*E,E*)-muconaldehyde in the liver,
2. (Non-)covalent binding of (*E,E*)-muconaldehyde to a transient proteinogenic carrier
3. Excretion of the carrier into systemic circulation,
4. Selective uptake of the carrier into the bone marrow
5. Degradation of the proteinogenic vehicle followed by release of (*E,E*)-muconaldehyde itself or its conjugate

Release of (*E,E*)-muconaldehyde or (*E,E*)-muconaldehyde-lysine conjugates within hematopoietic stem cells might explain the organ specificity observed in benzene related hematotoxicity. We will address each point of this proposed mechanism of toxicity in the fallowing subsections.

5.5.3.1. BINDING OF (*E,E*)-MUCONALDEHYDE TO A TRANSIENT CARRIER

Based on our findings, two requirements seem to qualify a transient carrier for (*E,E*)-muconaldehyde.

1. Molecular size. In our experiments we did not observe covalent binding of (*E,E*)-muconaldehyde to N^{α}-Fmoc-lysine but rather to bovine serum albumin[16]. We therefore conclude that a transient carrier for (*E,E*)-muconaldehyde favors a proteinogenic carrier over a single amino acid or peptide-like carrier. Chemically, the reason for a favorable reaction outcome of (*E,E*)-muconaldehyde with serum albumin might be the partial hydrophobicity of (*E,E*)-muconaldehyde (log P = -0.38), which causes it to align to semi polar domains of the protein. Schiff base formation in areas with reduced activity of water is entropically favored since Schiff base formation occurs through loss of water.
2. Reversibility of covalent interaction. A second requirement for a transient (*E,E*)-muconaldehyde carrier to the bone marrow consists in the presence of amino acids capable of reversible conjugate formation. Kline *et al.* proposed reversible thiol conjugates e.g. with cysteine to act as transporters for (*E,E*)-muconaldehyde to reach the bone marrow (Kline *et al.* 1993) (Figure 66).

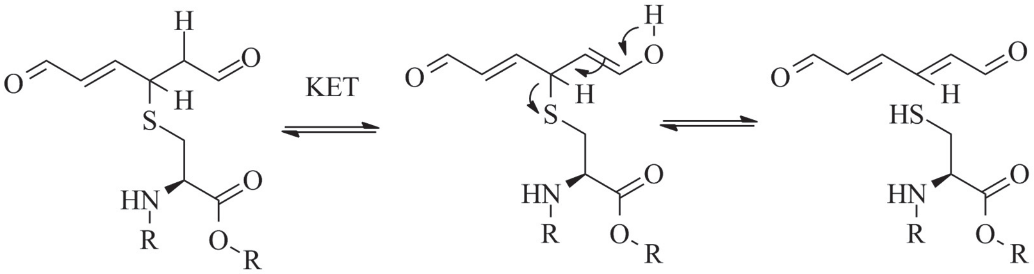

Figure 66 Reversibility of thioether formation of (E,E)-muconaldehyde with proteinogenic cysteine. R = protein chain.

Release of the aldehyde component from cysteine-conjugates has been shown for acrolein, a structural analogue to (*E,E*)-muconaldehyde (Ramu *et al.* 1995).

We add that not only thiol conjugates but also imine conjugate formation is reversible (Cordes & Jencks 1963). Schiff base conjugates of (*E,E*)-muconaldehyde with proteinogenic lysine might therefore serve as transient transport vehicles to reach the bone marrow (Figure 67).

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[16] In absence of sodium borohydride.

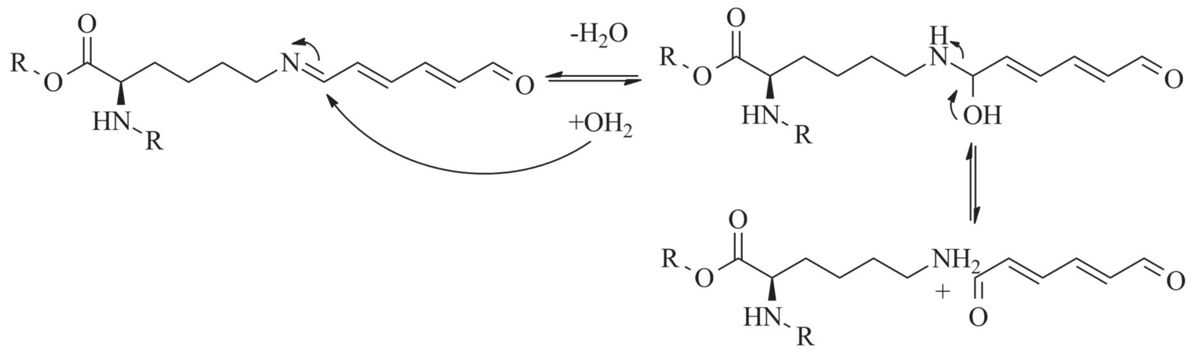

Figure 67 Reversibility of Schiff base formation of (*E,E*)-muconaldehyde with proteinogenic lysine. R = protein chain.

Absence of lysine and cysteine in the protein sequence does not necessarily exclude the suitability of a transient protein carrier as long as the protein contains semi polar domains suitable to temporarily accommodate (*E,E*)-muconaldehyde for transport to the bone marrow, thereby shielding it from reactive scavengers such as GSH.

Williams *et al.* specifically focused on identification of modified proteins following treatment with [^{14}C]benzene in mice (Williams *et al.* 2002). Modified liver and bone marrow proteins were reported including hemoglobin and several histones. However, the study does not seem suitable to answer the question regarding transient protein transporters of (*E,E*)-muconaldehyde for three reasons.

1. Choice of target proteins. No blood proteins were targeted for analysis.
2. Missing conjugate stabilization. No reducing agent was employed in order to stabilize (*E,E*)-muconaldehyde-lysine or GS-HQ conjugates.
3. Harsh Sample workup procedures. Conditions during protein isolation, gel electrophoresis and staining reported are so harsh[17], that likely neither lysine conjugates from (*E,E*)-muconaldehyde nor GS-HQ conjugates[18] retained structural integrity.

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[17] Homogenization buffer contained 5 % mercaptoethanol, gels were equilibrated in 5 % dithiothreitol and staining was performed in 10 % acetic acid (Williams *et al.* 2002).

[18] GSH-HQ conjugates are reported to be unstable, especially in absence of a reducing agent (Kuhlman 2013).

5.5.3.2. Selective Uptake of a transient carrier of (*E,E*)-muconaldehyde into bone marrow

5.5.3.2.1. Liposomes

There are vast reports in the literature about artificially engineered colloidal particles, called liposomes, which can be manufactured to transport drugs specifically to the bone marrow. Liposomes are generally generated using four components: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, *N*-(3-carboxy-1-oxopropyl)-1,5-dihexadecyl glutamic acid (SA) and poly(ethylene glycol) (PEG) (Sou *et al.* 2011) (Figure 68).

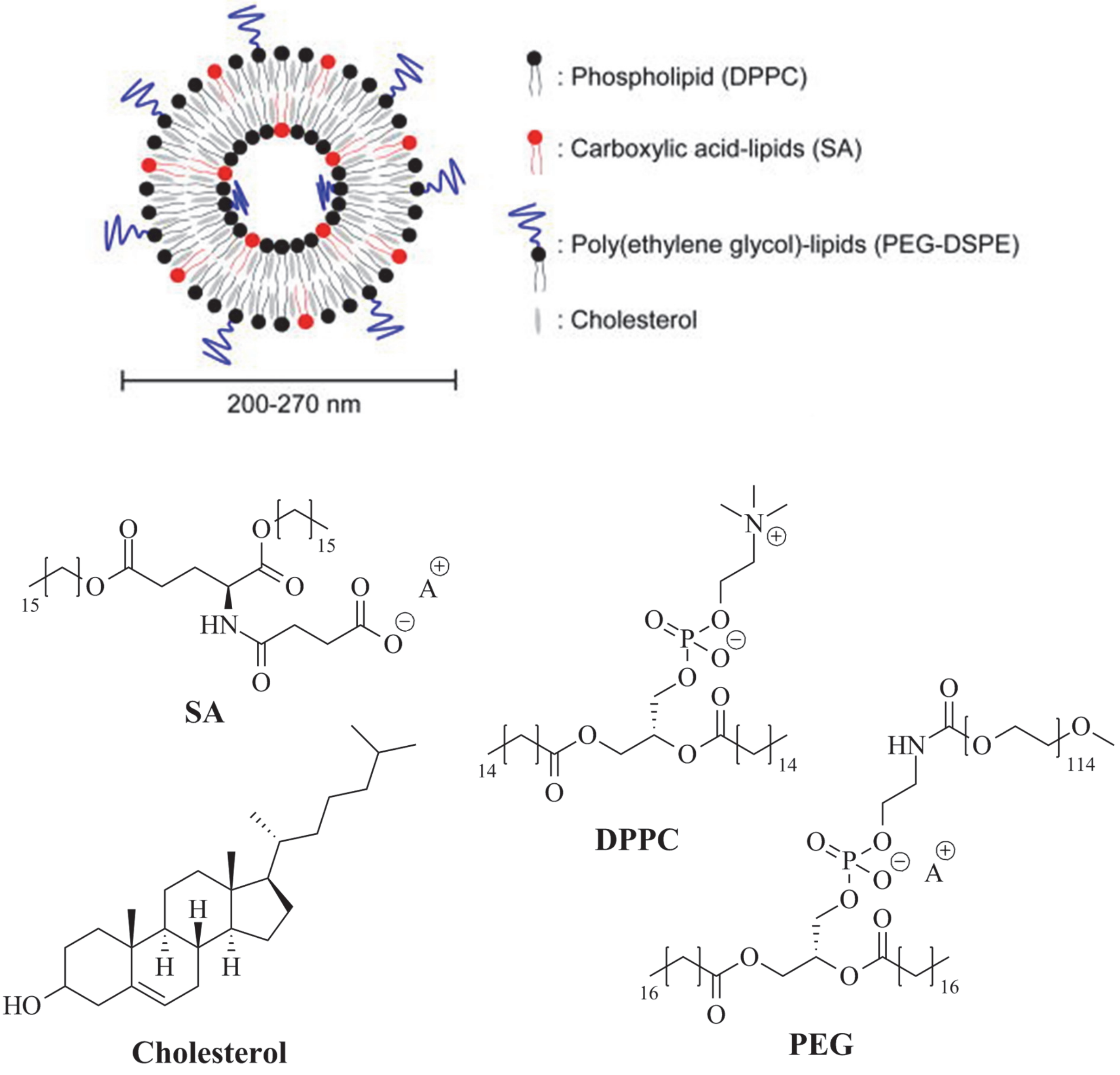

Figure 68 General lipid components of bone marrow-targeted liposomes. Taken and modified from (Sou *et al.* 2011).

While poly(ethylene glycol) lipids, cholesterol and phospholipids are interchangeable constituents, organ specificity for the bone marrow is achieved using carboxylic acid lipids.

Due to anionic carboxyl groups the liposome is attributed a negatively charged surface structure. This structural element causes endocytosis by bone marrow macrophages and selectivity is achieved (Sou *et al.* 2011).

Since anionic charged protein surface structures, e.g. by glutamic or aspartic acid, is no rare criteria of selection, many proteins and peptides should be to able enter the bone marrow via this route of uptake. Further selection of a suitable transient carrier of (*E,E*)-muconaldehyde is complicated by the fact, that bone marrow cells own more than one uptake mechanisms which can be used for transient carrier uptake.

However, serum albumin does not seem to be the appropriate transport vehicle for (*E,E*)-muconaldehyde to reach the bone marrow since it is neither the location of significant albumin distribution nor the location of albumin degradation (Rothschild *et al.* 1975; Peters 1995).

5.5.3.2.2. CELL-PENETRATING PEPTIDES

Cell-penetrating peptides are relatively short peptides, usually 5 – 40 amino acids, with the ability to access the cell interior using different mechanisms, including endocytosis. Cell-penetrating peptides are capable to promote the intracellular delivery of covalently or noncovalently conjugated bioactive cargoes (Langel 2007, Preface). (*E,E*)-Muconaldehyde could enter hematopoietic stem cells by using cell-penetrating peptides as a transient vehicle. Chemotherapeutics that have been shown to increase cellular uptake upon coupling with cell-penetrating peptides *in vivo* include doxorubicin (Rousselle *et al.* 2000), methotrexate (Kóczán *et al.* 2002), paclitaxel (Eldar-Boock *et al.* 2011) and organometal conjugates such as cymantrene (Hoyer *et al.* 2012).

Direct translocation mechanisms of cell-penetrating peptides as well as endocytosis mechanisms such as phagocytosis or pinocytosis exist to promote cellular uptake of cell-penetrating peptides. However, these mechanisms are ubiquitously present in all human cells and are nonspecific in the substance they transport. Therefore, mechanisms that might account for chronic toxicity of benzene have to consider substrate specificity. Receptor-mediated uptake mechanisms such as caveolin[19]- or clathrin[20]-mediated endocytosis might fill this gap since they are exhibit substrate specificity and might explain target organ selectivity (Kiss & Botos 2009; McMahon & Boucrot 2011)[21]. The ability of (*E,E*)-muconaldehyde to induce intermolecular cross links might thereby alter the cell-penetrating peptide structure so that substrate specificity is altered and ligand-receptor interaction increased[22]. Both clathrin and caveolin coated vesicles are able to follow the lysosomal degradation pathway (Kiss & Botos 2009; McMahon & Boucrot 2011).

Lysosomal lumen is acidic (pH ~ 4.6) and filled with over 60 different types of hydrolases including lipases, proteases, and glycosidases for catabolic degradation (Kolter & Sandhoff 2005). Degradation transporters are removed from lysosomes via specific exporters in the organelle membrane (Ruivo *et al.* 2009) or via vesicular membrane trafficking (Saftig & Klumperman 2009). While insoluble catabolites such as lipids are transported through vesicular trafficking, soluble catabolites e.g. carbohydrates and amino acids, are exported to the cytosol by specific transporters on the perimeter membrane (Ruivo *et al.* 2009; Sagné & Gasnier 2008). However, only a few lysosomal amino acid transporters have been identified. PQLC2/LAAT-1 is a lysosomal, H^+-dependent exporter of arginine/lysine (Jézégou *et al.* 2012). *Caenorhabditis elegans* lacking the LAAT-transporter exhibits accumulation of arginine and lysine in enlarged lysosomes (Liu *et al.* 2012; Xu & Ren 2015). (*E,E*)-Muconaldehyde-lysine conjugates could be potential substrates of PQLC2/LAAT-1 reaching the cytosol of hematopoietic stem cells engaging in activities related to myelotoxicity observed in chronic exposure to benzene.

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[19] Caveolin-mediated endocytosis is used e.g. for the uptake of serum albumin in endothelial cells (Lajoie & Nabi 2010).

[20] Clathrin-mediated endocytosis is employed e.g. in the uptake of transferrin (Motley *et al.* 2003).

[21] The first step in either mechanism constitutes interaction of the positively charged cell-penetrating peptide with negatively charged components of outer cell membrane such as heparan sulfate or the phospholipid bilayer. Clathrin resp. caveolin pits are thereby involved in the mechanism of uptake. Both clathrin and caveolin cover the intracellular side of the cellular membrane. They are required for invagination of the membrane and help to form the vesicles after binding of an extracellular ligand to the membrane receptor (Madani *et al.* 2011).

[22] Structure activity relationship studies have shown the importance of positive charges, especially arginine residues, in the uptake mechanism of cell-penetrating peptides (Elmquist *et al.* 2006; Madani *et al.* 2011; Wender *et al.* 2000).

Literature on cell-penetrating peptides specifically entering bone marrow derived cells is scarce. The synthetic peptide octa-arginine (R8) and the physiological protein HOXB4 have been reported to enter bone marrow derived cells (Nakamura *et al.* 2014; Langel 2007, pp. 259–271). However, the entry mechanism has not been fully elucidated, and it is likely that cells unrelated to the bone marrow can be entered similarly, which diminishes chances for organ selectivity.

Therefore, we have not been able to single out distinct carriers that fulfill the broad criteria outlined in section 5.5.3.1 for the proposed mechanism of toxicity, which is formation in the liver, excretion into systemic circulation, selective uptake followed by degradation in the bone marrow.

One reason might argue against the selection of a specific carrier.

1. Selectivity. Focusing on identification of a specific carrier must be regarded with caution, since selectivity might be implied in the choice of the proteinogenic carrier which (*E,E*)-muconaldehyde reacts with. But reactivity of (*E,E*)-muconaldehyde is not restricted to specific proteins or peptides but rather to certain functional groups such as amines or thiols. One could therefore argue: Why should (*E,E*)-muconaldehyde be myelotoxic when its reactivity reaches out to all sorts of proteinogenic carriers? The argument however can be strengthened if it can be proven, that carriers to the bone marrow have chemical superior reactivity with (*E,E*)-muconaldehyde compared to other carriers. Alternatively, increased susceptibility of hematopoietic stem cells towards toxicity of (*E,E*)-muconaldehyde compared to other cells should be considered, but such a finding would argue against the existence of a bone marrow specific carrier.

5.6. RELEVANCE OF (*E,E*)-MUCONALDEHYDE-LYSINE CONJUGATES AS BIOMARKERS TO BENZENE EXPOSURE

A biomarker of exposure is a marker in a biological system that indicates whether or not exposure to a certain chemical has taken place. A biomarker may be the compound itself or its metabolites measured in tissues, body fluids, or exhaled air. Biomarker may also be adducts formed in the DNA of white cells or conjugates with hemoglobin in red cells or with serum albumin (Medeiros *et al.* 1997).

To assess the utility of Lys(MUC-CHO) and Lys(MUC-OH) as potential biomarkers of exposure for benzene, we'll discuss briefly already established biomarkers for benzene. There are several established biomarkers of exposure available to for benzene.

5.6.1. UNMETABOLIZED BENZENE

Benzene has been used as one of the earliest biomarkers of exposure to benzene in the past. Measurement was performed by exhaled breath analysis to monitor short term workplace exposure (Srbova *et al.* 1950; Sherwood 1972a, 1972b; Berlin *et al.* 1980; Money & Gray 1989). Due to declined benzene concentrations at the workplace and complex analysis procedure, benzene analysis in breath nowadays is obsolete.

In contrast, urinary benzene has been considered as a biomarker of choice especially at atmospheric concentrations ranging below 1 ppm benzene because it is a specific, sensitive and non-invasive method (Ghittori *et al.* 1995; Ghittori *et al.* 1993; Ong *et al.* 1996; Mirzaei *et al.* 2016). Additionally, benzene analysis via solid phase micro extraction headspace gas chromatography (SPME-HS-GC) has simplified the analysis procedure greatly (Fustinoni *et al.* 1999; Waidyanatha *et al.* 2001; Mirzaei *et al.* 2016).

Other biomarkers of exposure proposed for benzene require metabolism to occur first to allow the desired analyte to be formed. Since the rations of these metabolite levels to unmetabolized benzene showed evidence of saturable CYP 450 metabolism, urinary benzene can also be regarded as a measure of the total internal dose of benzene (Mirzaei *et al.* 2016).

Before that, urinary phenol was used as a biomarker for benzene exposure but was abandoned due to lacking specificity for benzene (Ducos *et al.* 1990).

5.6.2. PHENOLIC METABOLITES

Since catechol and hydroquinone are formed by enzymatic hydroxylation of phenol, we will focus on phenol discussing its use as biomarker of exposure to benzene. Therefore, conclusions about the (un)suitability of phenol equally apply to catechol and hydroquinone.

Two reasons argue for phenolic metabolites as biomarkers for benzene.

1. Amount of biomarker formed. “In humans, phenol is the primary metabolite of benzene excreted in the urine accounting for 70–88% of the total urinary metabolites […] (Inoue et al., 1988; Kim et al., 2006b)” (Arnold *et al.* 2013).
2. Analytical procedures are easily available. Numerous “GC and HPLC methods have been described for the determination of phenol in the past 30–40 years (Angerer & Horsch, 1992; Weisel, 2010).” (Arnold *et al.* 2013)

However, one major drawback has caused researchers to avoid phenol as well as catechol and hydroquinone as biomarkers for benzene: The missing specificity for benzene. In humans formation phenol may be caused by sources other than benzene that confound the interpretation of air benzene exposure up to a concentration of approximately 5 ppm (Inoue *et al.* 1986) (Table 10). Humans ingest or produce endogenously through conversion of amino acids such as tyrosine or other simple phenols in the gut approximately 0.2 mg/kg-body weight of phenol per day (McDonald *et al.* 2001). Phenol is present in cigarette smoke (Hoffmann & Wynder 1986), and individual over-the-counter medicines have been shown to increase phenol excretion in the urine up to 40-fold (Fishbeck *et al.* 1978; McDonald *et al.* 2001; Arnold *et al.* 2013).

Table 10 Non-benzene sources of urinary phenol. Taken and modified from (Arnold *et al.* 2013)

Source	Amount	Reference
Diet and endogenous sources	0.2 mg/kg-bw/d	(McDonald *et al.* 2001)
Mainstream cigarette smoke	60–140 µg/cigarette	(Hoffmann & Wynder 1986)
Sidestream cigarette smoke	1.6–3.0 × mainstream smoke	(Hoffmann & Wynder 1986)
Over the counter medicines	Not quantified	(McDonald *et al.* 2001)

5.6.3. (*E,E*)-MUCONIC ACID

(*E,E*)-Muconic acid is the only ring opened metabolite used nowadays for benzene exposure assessment for atmospheric benzene concentrations greater than 0.5 ppm (Arnold *et al.* 2013). Interestingly, the excreted amount of (*E,E*)-muconic acid (2–25% of the total benzene uptake) in urine shows an inverse dose relationship: The higher the dose of benzene, the lower the relative excreted amount of (*E,E*)-muconic acid (Inoue *et al.* 1989; Boogaard & van Sittert 1996; Yu & Weisel 1996; Arnold *et al.* 2013).

However, there are background concentrations of (*E,E*)-muconic acid that are not explained by environmental benzene exposure may partly be due to dietary habits (Table 11). (*E,E*)-muconic acid is a metabolite of sorbic acid and sorbates can be present in various foodstuffs at concentrations up to 800 mg/kg (van Dokkum *et al.* 1982; Lück 1990; Serrano *et al.* 1991; Yu & Weisel 1996; Ruppert *et al.* 1997). For example, dietary supplementation with 500 mg sorbic acid significantly increases the mean urinary (*E,E*)-muconic acid excretion from 0.08 mg/24 h to 0.88 mg/24 h in eight non-smokers, although only 0.12% of the sorbic acid dose is excreted in urine as (*E,E*)-muconic acid (Ruppert *et al.* 1997). The authors conclude that a typical dietary intake of 6–30 mg/d sorbic acid accounts for 10–50% of the background (*E,E*)-muconic acid excretion in non-smokers, and for 5–25% in smokers (Ruppert *et al.* 1997; Arnold *et al.* 2013).

Table 11 Non-benzene sources of urinary (*E,E*)-muconic acid. Taken and modified from (Arnold *et al.* 2013)

Source	Amount	Reference
Diet (sorbitol) Europe	6–30 mg/d	(Ruppert *et al.* 1997)
Diet (sorbitol) USA	25 mg/d	(Yu & Weisel 1996)
Percentage of (*E,E*)-muconic acid in non-smokers attributed to dietary sorbic acid	10–50 %	(Ruppert *et al.* 1997)
Percentage of (*E,E*)-muconic acid in smokers attributed to dietary sorbic acid	5–25 %	(Ruppert *et al.* 1997)

Although there is no shortage in analytical procedures for (*E,E*)-muconic acid, its specificity is inadequate for the assessment of environmental benzene exposure when dietary sources are present simultaneously, “which is usually the case for western populations” (Arnold *et al.* 2013).

5.6.4. S-PHENYL MERCAPTURIC ACID (SPMA)

S-Phenyl mercapturic acid (SPMA) derives from the condensation of benzene oxide with glutathione (Figure 69). SPMA is considered a very specific short-term urinary biomarker of benzene (Arnold *et al.* 2013).

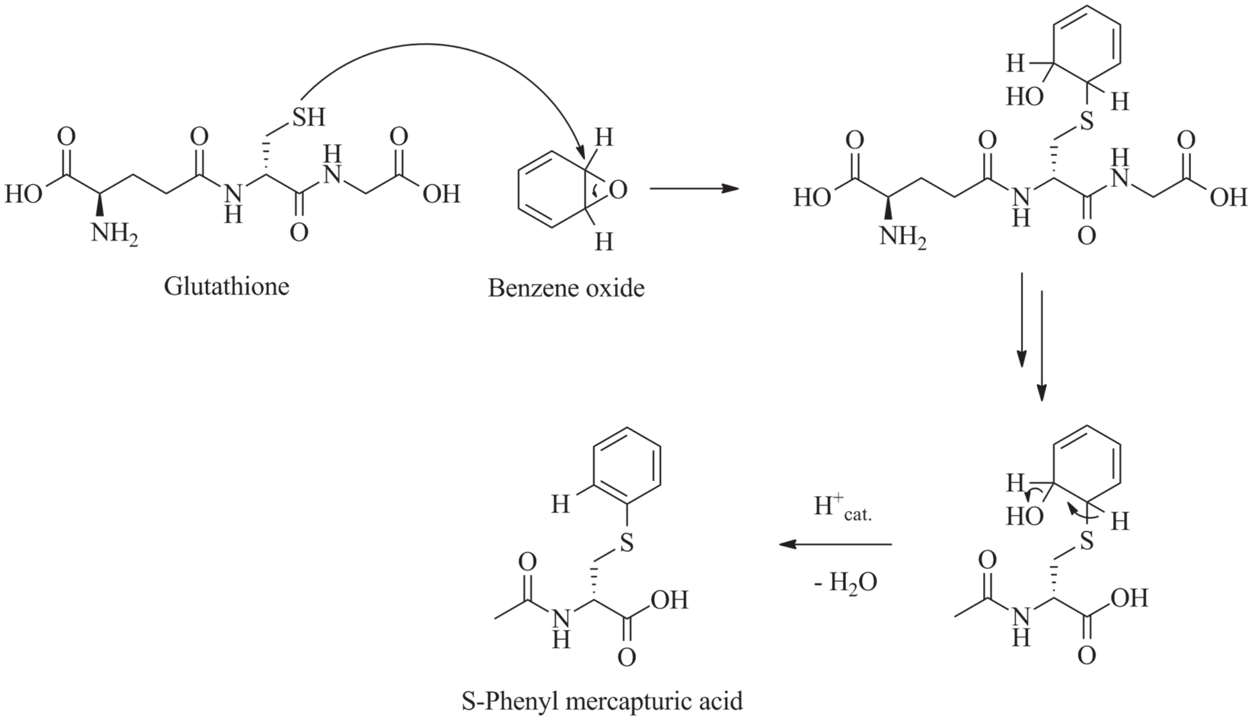


Figure 69 Formation of S-phenyl mercapturic acid (SPMA) *in vivo.* Modified from (ATSDR 2007).

Although SPMA is only a minor (0.01–0.9% of the dose) urinary metabolite of benzene in humans (Boogaard & van Sittert, 1995, 1996; Ghittori et al., 1999; Melikian et al., 2002; Stommel et al., 1989; van Sittert et al., 1993), the urinary concentration of SPMA is a reliable parameter to determine internal benzene exposures from recent sources (Arnold *et al.* 2013). There is currently no information indicating the formation of SPMA from sources other than benzene.

5.6.5. DNA ADDUCTS

Benzene has been shown to bind to DNA *in vivo* by radiochemical analysis (Lutz & Schlatter 1977; Synder *et al.* 1978; Snyder *et al.* 1978; Arfellini *et al.* 1985; Mazzullo *et al.* 1989; Turteltaub & Mani 2003). Metabolites of benzene that have been shown to covalently bind to DNA *in vitro* are benzene oxide (Norpoth *et al.* 1988; Norpoth *et al.* 1996), o- and p-benzoquinone (Levay *et al.* 1991; Pongracz & Bodell 1991; Gaskell *et al.* 2002), hydroquinone (Bodell *et al.* 1996; Gaskell *et al.* 2005a, 2005b) and (*E,E*)-muconaldehyde (Bleasdale *et al.* 1996; Harris *et al.* 2011).

However, literature about *in vivo* data is scarce. There are indications of DNA-adducts deriving from hydroquinone in mice following exposure to benzene (Bodell *et al.* 1993; Bodell *et al.* 1996). Also, N^7-Phenyl guanine, which is formed from reaction of benzene oxide with DNA, was shown to be in the urine of rats following exposure to benzene (Mueller *et al.* 1987) (Figure 70).

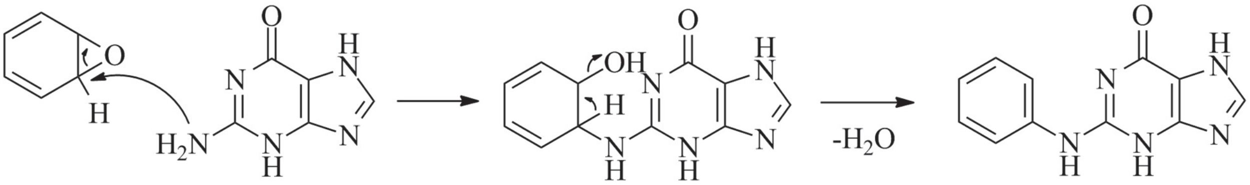


Figure 70 Formation of *N*⁷-phenyl guanine from reaction of benzene oxide with guanine (taken and modified from (Norpoth *et al.* 1996)).

However, there are currently no studies reporting on benzene derived DNA adducts in humans. There may be two reasons why.

1. <u>Missing funds</u>. Risk assessment has determined benzene to be genotoxic in humans (IARC 1982). It poses some difficulty to communicate the need for structural elucidation of DNA adducts, given the fact that the relevant information for risk assessment – benzene is genotoxic in humans at environmental concentrations – does not change even if the molecular structures of DNA adducts were to be identified.
2. <u>Low covalent binding index</u>. The ratio of benzene that finally forms DNA adducts at environmental benzene levels is very low. To quantify DNA adducts of benzene might therefore require expensive analytical equipment (Arnold *et al.* 2013).

Therefore, analysis of DNA adducts is not considered relevant assessing exposure to benzene in humans.

5.6.6. PROTEIN CONJUGATES

5.6.6.1. PROTEIN CONJUGATES DERIVED FROM PHENOLIC METABOLITES

Cysteine-S-yl hydroquinone is a conjugate postulated from reaction of 1,4-benzoquinone with cysteine sites of proteins (Figure 71). Reaction of 1,2-benzoquinone with cysteine yields the corresponding cysteine-S-yl catechol (Figure 72). Cysteine-S-yl hydroquinone and cysteine-S-yl catechol have been detected in hemoglobin and bone marrow proteins in rats and mice following benzene administration (Melikian *et al.* 1992; McDonald *et al.* 1994; Rappaport *et al.* 1996). Cysteine-S-yl hydroquinone has also been detected in human serum albumin following benzene exposure (Rappaport *et al.* 2002).

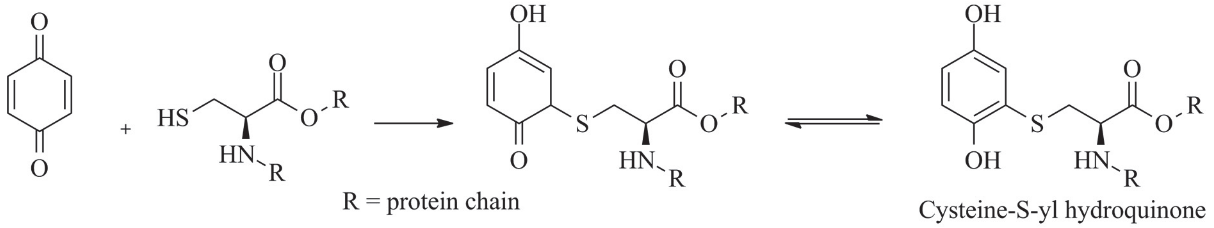


Figure 71 Formation of cysteine-S-yl hydroquinone (taken and modified from (Melikian *et al.* 1992)).

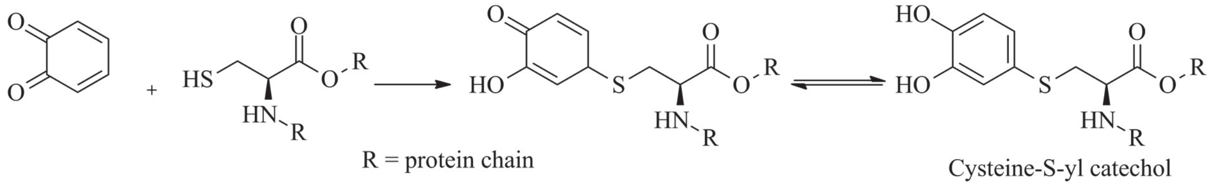


Figure 72 Formation of cysteine-S-yl catechol

There is one major drawback in employing protein conjugates of benzoquinones in exposure assessment of benzene: Their lack of specificity. As we've seen with phenolic metabolites used in exposure assessment, there are sources other than benzene which cause formation of phenol and, following metabolism, benzoquinones as well.

High levels of 1,4-benzoquinone conjugates in albumin are related to demographics, diet and lifestyle factors for persons occupationally not exposed to benzene (Lin *et al.* 2006). Therefore, 1,2- and 1,4-benzoquinone conjugates are not considered specific to benzene exposure (Arnold *et al.* 2013).

5.6.6.2. PROTEIN CONJUGATES DERIVING FROM BENZENE OXIDE / OXEPINE

S-phenyl cysteine (SPC) is a conjugate, which has been postulated from reaction of benzene oxide with cysteine sites of proteins, and has been found and measured in hemoglobin in rats and mice (Bechtold *et al.* 1992). Rat hemoglobin possesses an additional reactive cysteine at β-128, not present in humans, and consequently higher levels of SPC have been found in rats. Studies have also described the formation of SPC in the serum albumin of benzene-exposed workers (Yeowell-O'Connell *et al.* 1996; Barnes 2000; Rappaport *et al.* 2005).

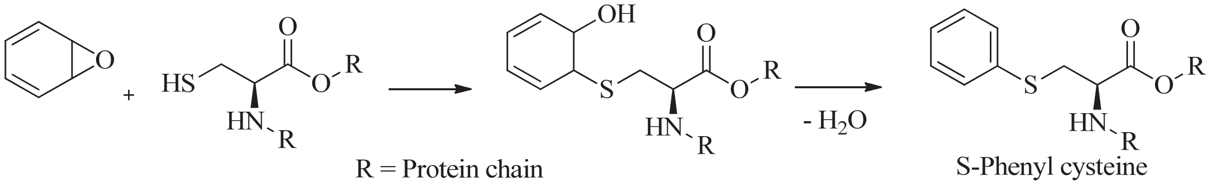


Figure 73 Formation of S-phenyl cysteine (SPC)

Hemoglobin and plasma protein adducts of benzene oxide are not considered sensitive enough to monitor environmental exposures (Arnold *et al.* 2013). Nonetheless, determination of S-phenyl cysteine levels in combination with short term biomarkers such as (*E,E*)-muconic acid or S-phenyl mercapturic acid might show promise to distinguish workers from occupational and non-occupational exposure to benzene (Bechtold & Henderson 1993).

5.6.6.3. PROTEIN CONJUGATES DERIVING FROM (*E,E*)-MUCONALDEHYDE

(*E,E*)-Muconaldehyde-lysine conjugates have not been proven to be formed *in vivo* upon exposure to benzene. Upon the premise of formation of (*E,E*)-muconaldehyde-lysine conjugates *in vivo*, different reasons argue for and against the employment as biomarkers of exposure to benzene.

Pro:

1. Specificity towards benzene. (*E,E*)-Muconaldehyde-lysine conjugates are formed by reaction of serum albumin with (*E,E*)-muconaldehyde. To our knowledge, there is neither relevant environmental exposure to (*E,E*)-muconaldehyde nor another environmentally relevant xenobiotic, which leads to formation of Lys(MUC-OH) *in vivo*. Therefore, we expect little background levels in the general population which marks (*E,E*)-muconaldehyde-lysine conjugates specific for exposure to benzene.
2. Mechanistic information. Analysis of (*E,E*)-muconaldehyde-lysine conjugates after benzene exposure could provide evidence for the formation of (*E,E*)-muconaldehyde *in vivo*. There is currently no other biomarker capable to answer the question if (*E,E*)-muconaldehyde is formed *in vivo*.

Contra:

1. Missing legal requirement for long term biomarker determination. Due to its toxicity, benzene exposure in the workplace has to be contained according to German law (BAuA 2014). However, samples for benzene biomonitoring have to be taken at the end of the work shift, and therefore, short term biomarkers are monitored (*ibd.*). In the case of benzene, urinary benzene, urinary (*E,E*)-mercapturic acid and urinary SPMA are accepted biomarkers of exposure to benzene in the workplace (*ibd.*). German law does not require to monitor human benzene exposures which date back more than one work shift. Therefore, analysis of long term biomarkers is not required.
2. Invasive sampling. Since protein conjugates between (*E,E*)-muconaldehyde and serum albumin are not excreted via urine, invasive sampling is necessary for biomarker analysis. This is not the case for urinary biomarkers.
3. Low levels of biomarkers. Even if oxidative ring opening of benzene might account for a major pathway of benzene metabolism at environmental concentrations (Rappaport *et al.* 2010), reactivity of (*E,E*)-muconaldehyde towards thiols such as

glutathione (Kline *et al.* 1993; Henderson *et al.* 2005) indicates that a considerable amount might be consumed before reaching target proteins. Therefore, biomarker levels are likely influenced from factors other than benzene concentration such as GSH-status.

4. Dose-response relationship. Ideally the relationship to exposure of a xenobiotic and the level of a corresponding biomarker is simply linear. However, (*E,E*)-muconaldehyde-lysine conjugates initially bear residual reactivity towards cellular nucleophiles due to the presence of an unsaturated aldehyde moiety. Additionally, Schiff base formation is complicated due to its dependency on reactant concentrations and the loss of water in aqueous media. However, (*E,E*)-muconaldehyde-lysine conjugate formation is facilitated at high concentrations of (*E,E*)-muconaldehyde and low (resp. high) concentrations of water (protein). Therefore, we assume no linear dose-response relationship between exposure to benzene and e.g. formation of Lys(MUC-OH).
5. Missing stability. Even though Lys(MUC-OH) is stable at physiological conditions, we assume degradation upon acidic or alkaline protein hydrolysis. In contrast, S-phenyl cysteine inherits a superior chemical stability making it suitable for acidic protein hydrolysis.
6. Well documented alternatives. There are many alternative long-term biomarkers which have been studied (see above). There is no evidence that analysis of (*E,E*)-muconaldehyde-lysine conjugates as biomarker of exposure to benzene bears advantage over established biomarkers such as urinary benzene or S-phenyl mercapturic acid.

Therefore (*E,E*)-muconaldehyde-lysine conjugates might not be suited as biomarkers of exposure to benzene. However, legitimacy for research purpose is provided to answer mechanistic questions concerning the formation of (*E,E*)-muconaldehyde following *in vivo* metabolism of benzene.

5.7. FUTURE WORK

5.7.1. PRESENCE OF (*E,E*)-MUCONALDEHYDE *IN VIVO*

In order to verify the presence of (*E,E*)-muconaldehyde *in vivo* following benzene exposure, analytical methodology of Lys(MUC-OH) has to be improved twofold.

1. Sample preparation. Residual amino acids and short peptides resulting from enzymatic protein hydrolysis need to be removed to obtain low mass spectrometric background noise. Since alkaline amino acids lysine, histidine and arginine can be readily separated from non-alkaline amino acids and short peptides by cation exchange chromatography (Thiele *et al.* 2008), application of solid phase extraction techniques on the rough enzymatic hydrolysate might be a reasonable strategy. After retention under slightly acidic conditions, which have Lys(MUC-OH) protonated bearing one net positive charge, alkaline amino acids may thereby be eluted comfortably by changing the solvent pH above the isoelectric point of lysine.
2. Sample analysis. Analytical HPLC-MS methodology presented in this work for Lys(MUC-OH) required the presence of ammonium acetate in the mobile phase. Future efforts targeting the need for buffer in the mobile phase are desirable. However, the use of buffer has been shown to be necessary for retention of Lys(MUC-OH) using zwitterionic stationary HILIC phases. So abdication thereof needs to be coupled with changing the stationary phase, which needs to fulfill one major priority: Satisfactory retention of highly polar, alkaline compounds in the presence of non-buffer additives tolerable for mass spectrometric analysis.

Following refinery in analytical methodology, we give great promise to the detection of Lys(MUC-OH) following benzene exposure *in vivo* and thereby finding evidence for the formation of (*E,E*)-muconaldehyde *in vivo*.

5.7.2. EXPLORATION OF MECHANISMS OF BENZENE TOXICITY

5.7.2.1. HAPTEN FORMATION

The possibility of hapten formation of (*E,E*)-muconaldehyde as promotor for benzene derived myelotoxicity can be excluded by repeated exposure of experimental animals towards (*E,E*)-muconaldehyde modified protein, such as serum albumin. After initial sensitization, immune response markers should be detected systematically in concert with early signs of myelotoxicity.

5.7.2.2. INHERENT TOXICITY OF LYS(MUC-CHO)

To answer the question whether the inherent toxicity of Lys(MUC-CHO) might be responsible for benzene induced myelotoxicity, animal experiments with Lys(MUC-CHO) as test substance need to be carried out. If adverse hematological and cytotoxic parameters can be detected as in the case of (*E,E*)-muconaldehyde, additional evidence would be provided that (*E,E*)-muconaldehyde or (*E,E*)-muconaldehyde-related biological conjugates might bear a responsibility in benzene induced myelotoxicity.

Alternatively, development of a suitable cell culture model for benzene induced myelotoxicity could be targeted. However, development of a suitable cell culture model for myelotoxicity of benzene is hindered for three main reasons:

1. Missing mechanistic knowledge. The missing knowledge of cellular and subcellular targets of benzene induced myelotoxicity hinders the choice of a suitable bone marrow cell type (Whysner *et al.* 2004; Snyder 2004; Smith 1996).
2. Requirement for different cell types. Toxicity of benzene requires metabolic activation predominantly by CYP2E1 (Gut *et al.* 1996; Valentine *et al.* 1996). CYP2E1 is present in bone marrow of mice, rats and rabbits, however CYP2E1 activity in these tissues is about 0.1 % of the corresponding activity in liver (Bernauer *et al.* 2000). Therefore, if one wants to refrain from working with synthetic metabolites, he has to bring together metabolic competence of liver cells as well as the target sensitivity of bone marrow cells in a single cell culture model.
3. Economic considerations. Myelotoxic effects in experimental animals following benzene administration have been well documented. To reach this level of biological resemblance using *in vitro* based methods probably requires costly investments for development, implementation and maintenance of a suitable cell culture model considering the price of experimental animals.

Therefore, *in vivo* studies providing information to the mechanism of toxicity in benzene exposed experimental animals seem to be preferable. However, these experiments are complicated by the missing stability of Lys(MUC-CHO). That is why studies aiming to determine half-life and degradation behavior of Lys(MUC-CHO) need to be taken into account.

5.7.2.3. SELECTIVE UPTAKE OF A TRANSIENT CARRIER OF (*E,E*)-MUCONALDEHYDE INTO BONE MARROW

Systemic application of caveolin- or clathrin mediated endocytosis inhibitors such as methyl-β-cyclodextrin or concanavalin A to keep bone marrow cells from taking up potential proteinogenic carriers of (*E,E*)-muconaldehyde would not qualify as legitimate options, since other receptor-mediated endocytosis mechanisms in the organism will be inhibited as well (Guo *et al.* 2015). Such a condition would not be sustainable for prolonged toxicological experiments. Selective genetic alteration of bone marrow cells resulting in specific PQLC2/LAAT-1, caveolin- or clathrin mediated endocytosis receptor knockouts would be preferable. Under the assumption that these animals are still viable, exposure to benzene could confirm whether these transport mechanisms were involved in benzene derived myelotoxicity.

Genetic alterations of bone marrow cells *in vivo* could be achieved either by using *in vivo* hematopoietic stem cell transduction (Richter *et al.* 2017) or by administration of genetically modified hematopoietic stem cells to an experimental animal that has undergone myeloablation by whole-body irradiation (Duran-Struuck & Dysko 2009).

6. EXPERIMENTAL SECTION

Chemical structures are drawn using *ChemDraw* 12.01 from *CamebridgeSoft*. This thesis has been written using Microsoft Word 2016 MSO 32-bit.

6.1. GENERAL

Proton nuclear magnetic resonance spectra and carbon nuclear magnetic resonance spectra were recorded on a *Bruker DPX 400* and *DPX 600* spectrometer. Signal shifts are given in parts per million (δ) relative to the residual proton signal. Signals for ^{1}H NMR coupling constants (J-values) are given in Hz.

Low-resolution mass spectra were obtained on an Agilent 6890 N equipped with an Agilent 7683 injector and an Agilent 5973 inert mass detector. High resolution gas chromatography mass spectrometry data (HRGC-MS) were obtained from the organic analysis facility of the organic chemistry department at the TU Kaiserslautern on a *GCT Premier Micromass* from *Waters* (EI, 70 eV*)*.

Thin layer chromatography was performed on commercially available alumina backed plates (25 x 25 cm), coated with silica gel 60 and fluorescence indicator (*ALUGRAM SIL G*) from *Macherey-Nagel*. Visualization was accomplished using ultraviolet light (254 nm) or heating the chromatogram after staining with a solution of 0.1 % (w/v) ninhydrin in 5 % acetic acid in butanol. Conventional chromatography was performed on silica gel 60, 63 – 200 µm, whilst flash column chromatography was performed on silica gel 60, 40 – 63 µm.

Dry solvents were stored in glassware that had been thoroughly dried under vacuum for at least ten minutes. Dried dichloromethane and chloroform were obtained by refluxing over phosphorous pentoxide for three hours. Tetrahydrofuran was dried over molecular sieves (40 nm).

All synthetic chemicals were purchased in analytical purity (p.a.) or alternatively highest grade available from *Sigma-Aldrich*, *Acros Organics*, *VWR, Alfa Aesar, Carl Roth* and *Carbolutions*. Exemptions are pronase E from *streptomyces griseus* min. 5.0 DMC-U/mg (Serva), aminopeptidase M from porcine kidney (Merck, 164598), prolidase from porcine kidney (Sigma Aldrich, P6675), acylase I from *Aspergillus melleus* (Sigma-Aldrich, 01818), (*E,E*)-hexa-2,4-dienal (Alfa Aesar, predominantly *trans, trans*, 95 %), benzene (Merck, ≥99.5 %, 1017822500) and corn oil (Mazola, *"100% reines Kaimöl"*). (*E,E*)-muconaldehyde, Lys(MUC-CHO), Lys(MUC-OH) and IS were synthesized as a part of this thesis.

6.2. UV/VIS-SPECTROSCOPY

Spectroscopic experiments were conducted on a *Cary 300* by *Varian* using 3 ml quartz glass cuvettes 10.00 mm in depth from *Hellma* for measurements below 350 nm and similar cuvettes (OS) or disposable 70 µl micro UV cuvettes from *BRAND* above 350 nm.

6.3. PREPARATIVE RP-HPLC-UV/VIS

Preparative reversed phase was conducted on an Agilent 1200 Series HPLC assisted with a binary high-pressure gradient system (G1361A), fraction collector (G1364B) and UV/VIS-multiwavelength detector (MWD G1315A). Two columns have been used: VDS Optilab C18-SE 250 × 20 mm and VDS Optilab HILIC-Z 250 × 5 mm.

6.4. ANALYTICAL HPLC-UV/VIS

Analytical HPLC-UV was performed on two different HPLC systems

- Jasco HPLC system – "Snoopy": Jasco 1500 equipped with a Jasco MD 2015 UV detector, Jasco PU-1580 HPLC pump, Jasco DG-1580-53 3-Line Degasser, Jasco LG-1580-02 Ternary Gradient Unit and a Jasco AS-1550 autosampler.
- Agilent HPLC system – "Bert": Agilent 1200 equipped with an Agilent G1315D UV detector, G1316A column oven, G1329A autosampler, G1311A HPLC pump and a G1322A degasser.

6.5. ANALYTICAL HPLC-MS/MS

Mass spectrometric analyses were performed on a *PE Sciex* (today *AB Sciex*) API 2000 equipped with an Agilent 1100er HPLC system using an Agilent G1314A VWD, G1316A column oven, G1313A autosampler, G1311A HPLC pump and G1322A degasser.

Compound optimization was performed by individual compound infusion at concentration of 200 – 1000 ng/ml at 10 µl/min.

6.5.1. LYS(MUC-CHO)

Lys(MUC-CHO) was analyzed using a Phenomenex Kinetex C18 150 × 4.0 mm, 5 µm, 100 Å. The gradient is listed in Table 12. Hardware specific parameters are listed in Table 13. Substance specific parameters are listed in Table 14.

Table 12 RP-HPLC gradient for Lys(MUC-CHO) with 0.1 % $HOAc_{aq}$ (A) and 0.1 % HOAc in CH_3CN (B)

Time	%A	% B	Flow [ml/min]
0	95	5	1
5	95	5	1
9.25	10	90	1
10.25	10	90	1
11	95	5	1
17.5	95	5	1

Table 13 Hardware related parameters for analysis of Lys(MUC-CHO) resulting from flow injection analysis (FIA). Potentials are given in eV.

Parameter	value
CUR	45
CAD	2
IS	5500
TEM	550
GS1	55
GS2	60

Table 14 Compound related parameters for analysis of Lys(MUC-CHO) resulting from substance specific tuning. Potentials are given in eV.

Q1	Q3	Time [ms]	DP	FP	EP	CEP	CE	CXP
241.131	84.100	400	11	370	6	10	25	12
241.131	223.100	400	11	370	6	10	13	12
241.131	130.100	400	11	370	6	10	17	18

6.5.2. LYS(MUC-OH)

Lys(MUC-OH) was analyzed using a VDS Optilab VDSpher PUR HILIC-Z 100 × 2.0 mm, 5 µm, 100 Å. The gradient is listed in Table 15. Hardware specific parameters are listed in Table 16. Substance specific parameters are listed in Table 17.

Table 15 HILIC-HPLC gradient for Lys(MUC-OH) with 10 mM NH_4OAc, 50 % acetonitrile , pH = 4.8 (A) and 10 mM NH_4OAc, 90 % acetonitrile , pH = 4.8 (B)

Time	%A	% B	Flow [ml/min]
0	0	100	0.2
2.5	0	100	0.2
10.5	100	0	0.2
14.5	100	0	0.2
15	0	100	0.4
22	0	100	0.4

Table 16 Hardware related parameters for analysis of Lys(MUC-OH) resulting from flow injection analysis (FIA). Potentials are given in eV.

Parameter	value
CUR	55
CAD	2
IS	4500
TEM	550
GS1	45
GS2	50

Table 17 Compound related parameters for analysis of Lys(MUC-OH) resulting from substance specific tuning. Potentials are given in eV.

Q1	Q3	Time [ms]	DP	FP	EP	CEP	CE	CXP
243.245	79.100	800	11	350	5	12	29	8
243.245	147.100	800	11	350	5	12	15	22
243.245	130.200	800	11	350	5	12	19	20

6.6. *IN VITRO* METHODS

6.6.1. REACTION OF (*E*,*E*)-MUCONALDEHYDE WITH SINGLE AMINO ACIDS

Table 18 Solutions and preparation thereof for incubation of (*E*,*E*)-muconaldehyde with single amino acids

Solution	Substance	Amount
0.1 M MOPS	MOPS + H_2O_{dd}	25 mg ad 1.2 ml
0.1 M NaH_2BO_3	$B(OH)_3$+ H_2O_{dd}	618 mg ad 100 ml
	8 M NaOH	Adjust to pH = 10
0.1 M $NaHCO_3$	$NaHCO_3$	68 mg
	H_2O_{dd}	10 ml
	Formic acid	Adjust to pH = 3
5 mM Fmoc-Lys-OH	Fmoc-Lys-OH	2.0 mg
	0.1 M HCl	Ad 1.2 ml
5 mM Fmoc-Cys-OH	Fmoc-Cys-OH	2.0 mg
	0.1 M HCl	Ad 1.1 ml
5 mM Fmoc-His-OH	Fmoc-His-OH	2.0 mg
	0.1 M HCl	Ad 1.1 ml
Sample diluent	1 % Formic acid$_{aq}$	1 ml
	Acetonitrile	1 ml
	H_2O_{dd}	6 ml

Table 19 Pipetting scheme for reaction of (E,E)-muconaldehyde with single amino acids

Solution	Volume/µl
Buffer	100
(*E*,*E*)-Muconaldehyde	100
Amino acid	100 each
H_2O_{dd}	Ad 1 ml

Reaction of (*E*,*E*)-muconaldehyde was performed with single amino acids as well as mixtures. Sample blanks were prepared by use of water instead of (*E*,*E*)-muconaldehyde. Samples were left for 48 h at 37°C at 120 rpm (TH 30, *Edmund Bühler*), diluted 1:5 with sample diluent and filtered over regenerated cellulose (0.2 µm). Samples were stored at 4°C until HPLC-UV analysis.

6.6.2. REACTION OF (*E*,*E*)-MUCONALDEHYDE WITH SERUM ALBUMIN

Table 20 Solutions and preparation thereof for incubation of (E,E)-muconaldehyde with serum albumin

Solution	Substance	Amount
20 mg/ml BSA solution	Bovine serum albumin (BSA)	200 mg
	H_2O_{dd}	Ad 10 ml
0.01 M MUC	(*E*,*E*)-Muconaldehyde	11 mg
	H_2O_{dd}	Ad 10 ml
0.5 M KH_2PO_4 buffer	KH_2PO_4	14 g
	H_2O_{dd}	Ad 0.2 l
	1 M KOH	Adjust to pH = 7.4
0.05 M NaH_2BO_3 buffer	$B(OH)_3$	1.6 g
	H_2O_{dd}	Ad 0.5 l
	8 M NaOH	Adjust to pH = 9.0
1.0 M $NaBH_4$ solution	$NaBH_4$	40 mg
	0.1 M NaOH (4°C)	Ad 1 ml
0.05 M NH_4HCO_3 buffer	NH_4HCO_3	2.0 g
	$CaCl_2$*2 H_2O	74 mg (1 mM)
	H_2O_{dd}	Ad 0.5 l
Pronase enzyme diluent	NaOAc	41 mg (10 mM)
	$CaCl_2$*2 H_2O	37 mg (5 mM)
	H_2O_{dd}	Ad 50 ml
	0.1 M HCl	Adjust to pH = 7.5
Pronase	Pronase E lyophil.	30 mg
	Pronase E enzyme diluent (4°C)	Ad 1 ml

To 800 µl of 20 mg/ml BSA solution is added 100 µl of 0.5 M KH_2PO_4 buffer and 100 µl of (*E,E*)-muconaldehyde solution. The resulting solution is incubated for 24 hours at 37°C.

The solution is applied to a 10 ml Zeba™ Spin Desalting Column (ThermoFisher, 7 kDa MWCO) equilibrated to 0.05 M sodium borate pH = 9 and centrifuged at 1500 × g for 2 minutes (Thermo Scietific 2016).

The resulting solution is transferred to a 1.5 ml reaction tube and put on ice, 100 µl of 1.0 M $NaBH_4$ solution is added and the reaction is left for 30 minutes with open lids.

The solution is applied to a 10 ml Zeba™ Spin Desalting Column (ThermoFisher, 7 kDa MWCO) equilibrated to 0.05 M NH_4HCO_3 buffer and centrifuged at 1500 × g for 2 minutes (Thermo Scietific 2016).

400 µl of the resulting solution is transferred to a 1.5 ml reaction tube, 6 µl of Pronase is added (1:50) (w/w) and the reaction mixture is incubated for 24 hours at 37°C.

Afterwards ethanol (-20°C) is added to 66 % (v/v) and the resulting mixture is stored at -20°C for at least 30 minutes. The mixture is centrifuged for 10 minutes at 12 000 × g at 4°C. The supernatant is stored at 4°C until subjection to HPLC analysis.

6.6.3. ONE POT CONVERSION OF N^{α}-ACETYL LYSINE METHYL ESTER WITH (*E,E*)-MUCONALDEHYDE

Table 21 Solutions and preparation thereof for one pot conversion of N^{α}-acetyl lysine methyl ester with (*E,E*)-muconaldehyde

Solution	Substance	Amount
0.04 M (*E,E*)-Muconaldehyde	(*E,E*)-Muconaldehyde H_2O_{dd}	44 mg Ad 10 ml
1 M EPPS buffer	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid H_2O_{dd} 1 M KOH	2.52 g Ad 10 ml Adjust to pH = 8.0
0.1 M N^{α}-Acetyl-Lysine-methyl ester	Acetyl-Lysine-methyl ester hydrochloride H_2O_{dd}	24 mg Ad 1 ml
0.1 M $NaBH_4$ solution	$NaBH_4$ 0.1 M NaOH (4°C)	40 mg Ad 10 ml
Acylase I	powder, brown, >0.5 U/mg	q.s.

To 50 µl of 40 mM (*E,E*)-muconaldehyde solution in water (740 µl) is added 100 µl of 1 M EPPS buffer and 10 µl of 0.1 M N^{α}-acetyl-Lysine-methyl ester solution. The reaction mixture is stirred overnight at 37°C at 120 rpm (TH 30, Edmund Bühler). The reaction tube is put on ice and 100 µl of 0.1 M $NaBH_4$ solution is added. The reaction tube is left on ice for 30 minutes and incubated at 37°C for another 30 minutes. Acylase I (2U) is added and the reaction mixture is stirred overnight at 37°C at 120 rpm. Ethanol (-20°C) is added to 66 % (v/v). The mixture is centrifuged for 10 minutes at 12 000 × g at 4°C. The supernatant is stored at 4°C until subjection to HPLC-UV analysis.

6.6.4. FLUORESCAMINE TEST

Table 22 Solutions and preparation thereof for fluorescamine test

Solution	Substance	Amount
20 mg/ml BSA solution	Bovine serum albumin (BSA)	200 mg
	H_2O_{dd}	Ad 10 ml
0.5 M HEPPS	3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS)	636 mg
	H_2O_{dd}	Ad 5 ml
	8 M NaOH	Adjust to pH = 8.0
Fluorescamine solution	Fluorescamine	6 mg
	Acetonitrile	Ad 6 ml
0.2 M Glycine	L-Glycine	15 mg
	H_2O_{dd}	1 ml
0.1 M NaH_2BO_3 buffer	$B(OH)_3$	62 mg
	H_2O_{dd}	Ad 10 ml
	8 M NaOH	Adjust to pH = 8.0
8 M NaOH	NaOH + H_2O_{dd}	3.2 g ad 10 ml
8 M HCl	HCl 25 %	q.s.
Pronase	Pronase E lyophil. + H_2O_{dd}	66 mg ad 1 ml
Aminopeptidase M	Aminopeptidase M, crystalline solution. Stored at -20°C.	
Prolidase	Prolidase lyo.	250 U
	0.1 M $Glutathion_{aq}$	20 µl
	0.1 M $MnCl_2$	20 µl
	H_2O_{dd} (4°C)	Ad 1 ml

The test is performed according to Table 22. Acidic hydrolysis samples were incubated for 24 h at 110°C. Enzymatic hydrolysis samples were first incubated with pronase for 24 h. Incubation was continued for 16 h after addition of aminopeptidase M. Prolidase was added last and samples were incubated for another 2 h.

Table 23 Pipetting scheme for fluorescamine test

Sample	Substance	Volume
Acidic hydrolysis Blank	H_2O_{dd}	150 µl
	8 M HCl	450 µl
Acidic hydrolysis	BSA 20 mg/ml	150 µl
	8 M HCl	450 µl
Enzymatic hydrolysis Blank	H_2O_{dd}	895 µl
	HEPPS pH = 8.0	50 µl
	0.1 M $CaCl_2$	10 µl
	0.1 M $MnCl_2$	10 µl
	Pronase	10 µl
	Aminopeptidase M	15 µl
	Prolidase	10 µl
Enzymatic hydrolysis	BSA 20 mg/ml	895 µl
	HEPPS pH = 8.0	50 µl
	0.1 M $CaCl_2$	10 µl
	0.1 M $MnCl_2$	10 µl
	Pronase	10 µl
	Aminopeptidase M	15 µl
	Prolidase	10 µl

1. Samples are diluted 1:225 (4 µl ad 900 µl).
2. 4 µl of each sample is mixed with 90 µl of fluorescamine solution, 15 µl of 0.1 M NaH_2BO_3 buffer, and 191 µl of H_2O_{dd} in wells of a flat-bottomed 96-well microplate.
3. Samples are allowed to react at room temperature for 5 min.
4. Relative fluorescence intensity (RFI) is measured with λ_{ex}=390 nm and λ_{em}=475 nm (Bantan-Polak *et al.* 2001).

6.6.5. 2,4-DINITROPHENYLHYDRAZINE (DNPH) TEST

Table 24 Solutions and preparation thereof for 2,4-Dinitrophenylhydrazine (DNPH) test

Solution	Substance	Amount
DNPH stock solution	2,4-Dinitrophenylhydrazine	38 mg
	Ethanol	Ad 100 ml
DNPH usage solution	DNPH stock solution	10 ml
	$HCl_{conc.}$	350 µl
KOH-solution	KOH	5.0 g
	$Ethanol_{aq}$ 80 % (v/v)	Ad 50 ml
0.5 M Hexa-2,4-dienal stock	Hexa-2,4-dienal	58 µl
	Ethanol	Ad 1 ml
0.1 mM Hexa-2,4-dienal usage	Hexa-2,4-dienal stock solution	20 µl
	Ethanol	Ad 1000 ml

Table 25 Pipetting scheme for 2,4-Dinitrophenylhydrazine (DNPH) test

Solution	Volume (µl)									
Sample tube	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
H_2O_{dd}	0	290	280	260	220	140	0	290	150	-
0.1 mM Hexa-2,4-dienal	0	10	20	40	80	160	300	-	-	-
Sample	-	-	-	-	-	-	-	10	150	300

1. 300 µl of DNPH usage solution is added to each sample tube, the resulting solution is vortexed briefly and incubated for 30 min at 50°C.
2. 240 µl of KOH-solution is added and the resulting mixture is briefly vortexed, afterwards centrifuged for 5 minutes at 13 000 × g.
3. 700 µl of supernatant is transferred to an empty 1 ml disposable cuvette and absorbance is measured at $\lambda = 479$ and 425 nm (Yukawa *et al.* 1993).

6.6.6. CELL CULTURE – CYTOTOXICITY VIA ANNEXIN V/PROPIDIUM IODIDE STAINING

Solution	Substance	Amount
Annexin Binding Buffer (10 × stock)	HEPES (0.1 M)	1.2 g
	NaCl (1.4 M)	4.1 g
	$CaCl_2*2\ H_2O$ (25 mM)	185 mg
	BSA (1%)	500 mg
	H_2O_{dd}	Ad 50 ml
	8 M NaOH	Adjust to pH = 7.4
PBS (1×)	NaCl	8.0 g
	KCl	200 mg
	NaH_2PO_4	920 mg
	KH_2PO_4	200 mg
	H_2O_{dd}	Ad 1 l
	1 M NaOH/1 M HCl	Ad pH = 7.4
AnnexinV-FITC	Ready to use solution MACS Miltenyi Biotec	
PI solution	Propidium Iodide	50 mg
	H_2O_{dd}	Ad 1 ml

Cell culture experiments were performed by Viviane Ponath from *Institut für Toxikologie* at *Universitätsmedizin Mainz*.

The promyelocytic cell line NB4 in cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal calf serum (FCS) and 0.5% gentamicin in a humidified incubator at 37°C and 5% CO_2.

Annexin V/propidium iodide staining allows for simultaneous detection of early apoptotic, late apoptotic, and necrotic cells. Fluorescein isothiocyanate (FITC)-labeled Annexin V (MACS Miltenyi Biotec) binds to phospholipid phosphatidylserine, which translocates from the inner leaflet of the plasma membrane to the outer leaflet in early and late apoptotic cells. Propidium iodide is the standard flow cytometric dye used to distinguish viable from nonviable cells. The membranes of dead and damaged, necrotic cells are permeable to propidium iodide, but usually negative for Annexin V (viable cells exclude propidium iodide). Late apoptotic cells are propidium iodide positive, but additionally stain positive for Annexin V (Noack *et al.* 2016) (Figure 74).

NB4 cells are seeded into 12-well plates at a density of 2 × 10^5 cells/ml; 24 h later, cells are treated with (*E,E*)-muconaldehyde or Lys(MUC-CHO) for 24 h. Subsequently, cells are collected in FACS tubes, put on ice and centrifuged for 5 min at 1200 × g at 4°C. The supernatant is aspirated, and the cells resuspended once with cold PBS (1 ml), and centrifuged again for 5 min at 1200 × g at 4°C. The supernatant is aspirated, and the cell pellet is resuspended in Annexin V Binding Buffer (1×, 50 µl) + AnnexinV-FITC (1.5 µl). Cells are incubated for 15 min on ice in the dark. The DNA of the cells is stained with Annexin V Binding Buffer (1×, 430 µl) + propidium iodide solution (10 µl). The suspension is vortexed briefly.

Samples are measured directly on a FACSCanto II flow cytometer (BD Biosciences).
Data is evaluated with the FACSDiva software 7.0 (BD Biosciences) (Noack *et al.* 2016).

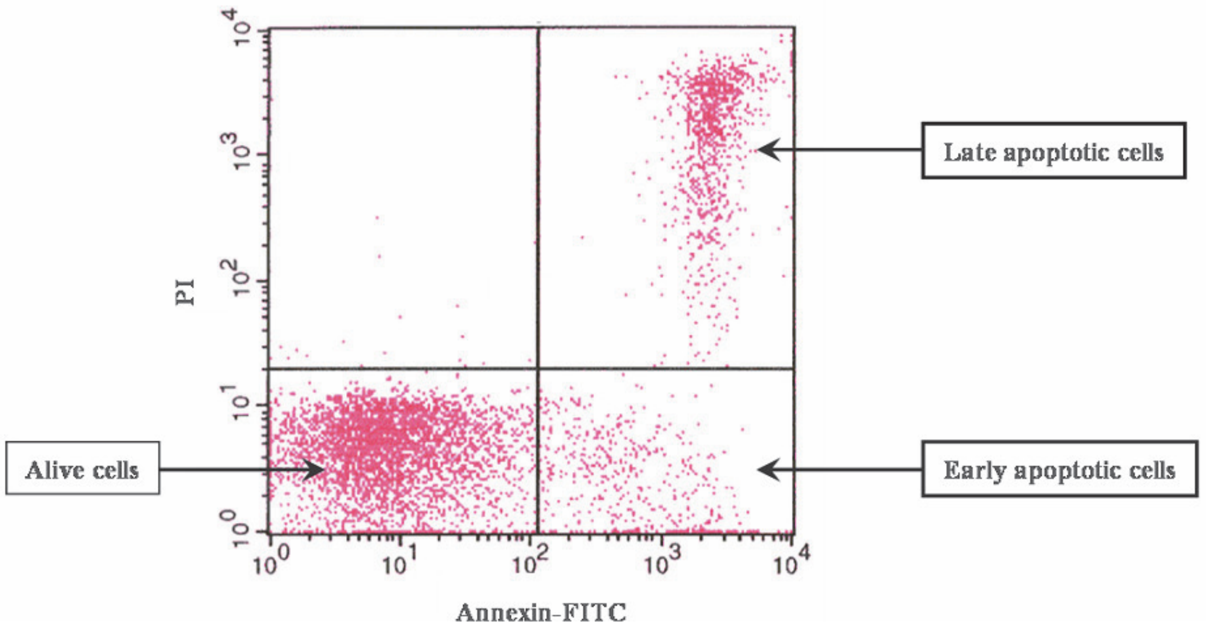


Figure 74 Exemplary Graph for FACS analysis of a cell population stained with Propidium iodide (PI) and AnnexinV-FITC. Early apoptotic cells are considered to die from apoptosis and late apoptotic cells are considered to die from necrosis. Picture by Viviane Ponath.

6.7. *IN VIVO* METHODS

6.7.1. ANIMAL EXPERIMENT

Table 26 Solutions and preparation thereof for animal experiment

Solution	Substance	Amount
Benzene 800 mg/kg b.w. solution	Benzene	V(benzene) [µl] = 1.06*b.w.(mouse [g]) - 4.4
	Corn oil	V(corn oil) [µl] = 250 µl – V(benzene)
Benzene 400 mg/kg b.w. solution	Benzene	V(benzene) [µl] = 0.74*b.w.(mouse)(g) - 8.5
	Corn oil	V(corn oil) [µl] = 250 µl – V(benzene)
Chloralhydrate solution (7 %)	Chloralhydrate	70 mg
	PBS (1×)	Ad 1 ml
PBS (1×)	NaCl	8.0 g
	KCl	200 mg
	NaH_2PO_4	920 mg
	KH_2PO_4	200 mg
	H_2O_{dd}	Ad 1 l
	1 M NaOH/1 M HCl	Ad pH = 7.4

The animal experiment was authorized by the *Landesuntersuchungsamt Rheinland-Pfalz* under the reference number 23 177-07/A 14-2-002 E 1.2.

Eleven $B_6C_3F_1$ male mice (~8 weeks of age; 25–30 g) were obtained from *Janvier Labs* (Genest saint isle, France) and left for a minimum of one week at animal facility of the TU Kaiserslautern before start of the experiment. Animals were housed one or two per cage in polycarbonate cages with hardwood chip bedding and paper tissues. They were given drinking water and food *ad libitum*, kept on a 12 h dark/light cycle, and maintained at about 22°C throughout the study.

Animals were divided into three groups. Three animals received no benzene, four animals each received 400 and 800 mg/kg bodyweight benzene dissolved in corn oil (250 µl) in a single dose by gavage. 24 h after treatment animals were anesthetized intraperitoneally with chloralhydrate using 100 µl per 10 g bodyweight. The Thorax is opened and 100 µl of Heparin (1000 U/ml, *Serva*, Heidelberg, Germany) is injected into the *vena cava inferior* after which a blood sample is taken with a hypodermic needle (21G) and a disposable syringe. The *vena porta* is punctuated and the liver perfused retrograde with PBS (42°C) until the red color of the liver has vanished. The liver is removed, immediately frozen in liquid nitrogen and stored at –80°C.

6.7.2. ISOLATION OF SERUM ALBUMIN

Table 27 Solutions and preparation thereof for isolation of serum albumin

Solution	Substance	Amount
0.9 % NaCl	NaCl	900 mg
	H_2O_{dd}	Ad 100 ml
0.2 M NaH_2BO_3 buffer	$B(OH)_3$	6.2 g
	H_2O_{dd}	Ad 0.5 l
	8 M NaOH	Adjust to pH = 9.0
1.0 M $NaBH_4$ solution	$NaBH_4$	40 mg
	0.1 M NaOH (4°C)	Ad 1 ml
0.5 M sodium citrate	Citric acid monohydrate	10.5 g
	H_2O_{dd}	Ad 100 ml
	8 M NaOH	Adjust to pH = 4.0
0.5 M KH_2PO_4 buffer	KH_2PO_4	14 g
	H_2O_{dd}	Ad 0.2 l
	1 M KOH	Adjust to pH = 7.4
2 M KCl	KCl	15 g
	H_2O_{dd}	Ad 100 ml
50 mM NH_4HCO_3 buffer	NH_4HCO_3	2.0 g
	$CaCl_2$*2 H_2O (1 mM)	74 mg
	H_2O_{dd}	Ad 0.5 l
Pronase	Pronase E lyophil.	20 mg
	H_2O_{dd} (4°C)	Ad 1 ml

About 0.6 ml of blood is transferred into a 1.5 ml Eppendorf tube and centrifuged for 10 minutes at 2500 × g and 4°C. The supernatant (blood plasma) is transferred to a new 2 ml Eppendorf tube and stored on ice. The resulting blood cell pellet is washed twice with 0.6 ml of 0.9 % sodium chloride solution and centrifuged again for 10 minutes at 2500 × g and 4°C.
The combined supernatants are centrifuged for 3 minutes at 9000 × g and 4°C.

The supernatant is applied to a 10 ml Zeba™ Spin Desalting Column (ThermoFisher, 7 kDa MWCO) equilibrated to 0.2 M sodium borate pH = 9 and centrifuged at 1500 × g for 2 minutes (Thermo Scietific 2016). 100 µl of 1.0 M $NaBH_4$ solution is added and the solution left on ice for 30 minutes.

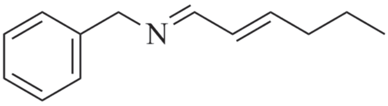
The resulting solution is diluted to a final volume of 10 to 15 ml with 50 mM sodium citrate pH = 4.0 and applied to a 2 ml Blue Sepharose CL-6B column equilibrated to 50 mM sodium citrate pH = 4.0 (GE Healthcare Life Sciences 2013). The column is washed with 10 ml of 50 mM sodium citrate pH = 4.0 and eluted with 10 ml of 1.6 M KCl, 50 mM KH_2PO_4, pH = 7.0. Protein content of fractions is checked by absorbance measurement at $\lambda = 280$ nm.

The resulting solution is applied to a 20 ml protein concentrator (ThermoFisher, 30 kDa MWCO, 88531) and concentrated according to the manufacturers protocol at 4700 × g to ~ 0.5 ml. The solution is diluted twice to 20 ml with 50 mM NH_4HCO_3 buffer and concentrated again to a final protein concentration of 25 mg/ml.

Pronase is added to a final concentration of 2 mg/ml. The solution is stirred for 72 h at 40°C at 120 rpm (TH 30, *Edmund Bühler*). Afterwards ethanol (-20°C) is added to 80 % (v/v) and the resulting mixture is stored at -20°C for at least 30 minutes. The mixture is centrifuged for 10 minutes at 12 000 × g at 4°C. The supernatant is concentrated to 100 µl by vacuum centrifugation. Ethanol (100 µl) is added, centrifuged for 5 minutes at 12 000 × g and the supernatant is stored at 4°C until subjection to HPLC-ESI$^+$-MS/MS analysis.

6.8. SYNTHESES

6.8.1. SYNTHESIS OF N-((E)-HEX-2-EN-1-YLIDENE)-1-PHENYLMETHANAMINE

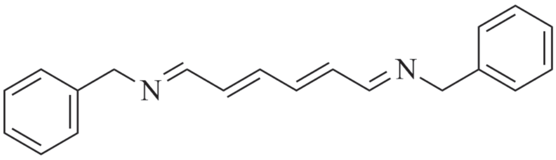


To a solution of benzyl amine (441 µl, 4.0 mmol) in trimethyl orthoformate (8 ml) is added (E)-hexa-2-enal (472 µl, 4.0 mmol) after five minutes. The reaction mixture is stirred for 8 hours, taken up in diethyl ether (25 ml) and extracted with water (3 x 50 ml). The organic extract is dried over sodium sulfate and the solvent is removed *in vacuo*.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 0.88 (t, *J*=7.40 Hz, 3 H) 1.42 (sxt, *J*=7.38 Hz, 2 H) 2.09 - 2.17 (m, 2 H) 4.56 (s, 2 H) 6.11 - 6.27 (m, 2 H) 7.07 - 7.26 (m, 5 H) 7.90 (d, *J*=8.03 Hz, 1 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 13.66, 21.69, 34.63, 65.00, 126.89, 128.00, 128.45, 130.68, 139.34, 146.02, 163.70

6.8.2. SYNTHESIS OF ((2,4)-HEXA-2,4-DIENE-1,6-DIYLIDENE)BIS(1-PHENYLMETHANAMINE)

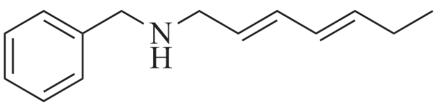


To a solution of benzylamine (104 µl, 0.94 mmol) in trimethyl orthoformate (2 ml) is added slowly a solution of (*E,E*)-muconaldehyde (52 mg, 0.47 mg) in trimethyl orthoformate (1 ml). The reaction mixture is stirred for 8 hours, the solvent is removed *in vacuo* and the crude product is taken up in diethyl ether (2 ml) and added to hexane (20 ml) at -20°C for crystallization.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 4.71 (s, 4 H) 6.51 - 6.66 (m, 2 H) 6.71 - 6.85 (m, 2 H) 7.22 - 7.41 (m, 10 H) 8.06 (d, *J*=8.91 Hz, 2 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 65.39, 127.10, 128.05, 128.55, 135.35, 138.77, 139.84, 162.59

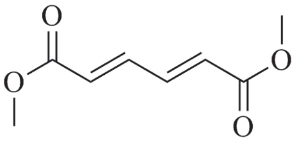
6.8.3. SYNTHESIS OF (*E,E*)-N-BENZYLHEPTA-2,4-DIEN-1-AMINE



To a solution of benzylamine (1.0 mmol, 111 µl) in dichloroethane (4 ml) is slowly added (*E,E*)-hexa-2,4-dienal (1.0 mmol, 116 µl) in dichloroethane (2 ml). Afterwards, sodium triacetoxyborohydride (1.4 mmol, 312 mg) is added and the reaction mixture is stirred overnight. The reaction mixture is quenched with saturated sodium bicarbonate solution (5 ml), and extracted with diethyl ether (2 × 10 ml). The combined organic extracts are dried over magnesium sulfate and the solvent is removed *in vacuo*.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.71 (d, *J*=6.78 Hz, 3 H) 3.24 (d, *J*=6.40 Hz, 2 H) 3.74 (s, 2 H) 5.57 – 5.66 (m, 2 H) 5.91 - 6.16 (m, 2 H) 7.19 - 7.29 (m, 5 H)
^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ ppm 18.03, 50.83, 53.20, 126.87, 128.13, 128.35, 128.73, 129.06, 131.07, 131.97, 140.24

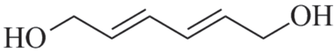
6.8.4. SYNTHESIS OF (*E,E*)-DIMETHYL HEXA-2,4-DIENEDIOATE (**1**)



Acetyl chloride (30 ml) is added slowly to an ice-cooled solution of (*E*,*E*)-muconic acid (10.0 g, 69 mmol) in methanol (200 ml), stirred at that temperature for 5 minutes and then refluxed for 2 h. Afterwards the reaction mixture is cooled to room temperature and the solvent removed under reduced pressure to yield the title compound (11.5 g, 98 %) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 3.79 (s, 6 H), 6.16 - 6.26 (m, 2 H), 7.28 - 7.38 (m, 2 H)
^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ 51.9, 128.0, 141.0, 166.3
HRMS for $C_8H_{10}O_4$: Calc. 170.0579; found 170.0587 (M^+)

6.8.5. SYNTHESIS OF (2,4)-HEXA-2,4-DIENE-1,6-DIOL (**2**)



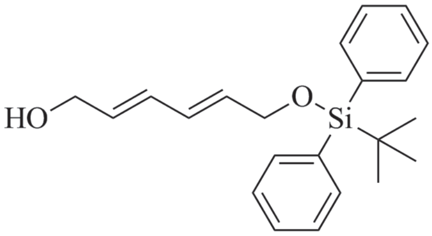
To an ice-cooled solution of (*E*,*E*)-dimethyl hexa-2,4-dienedioate (3.23 g, 19.0 mmol) in chloroform (190 ml) is added DIBAL-H (1.0 M in toluene, 95 ml) and the reaction mixture is stirred at 0°C for one hour. The reaction mixture is treated dropwise with methanol (19 ml) and stirred for additional 15 min at 0°C. Saturated aqueous sodium potassium tartrate (150 ml) is added to the reaction mixture and stirred at ambient temperature for one hour. The aqueous layer is extracted with ethyl acetate (3 x 100 ml). The combined organic layers are dried over $MgSO_4$ and concentrated. The title compound crystallizes at -20°C as colorless needles (2.05 g, 18.0 mmol, 95 %).

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 4.08 - 4.09 (d, *J*=5.27 Hz, 4 H) 5.74 - 5.84 (m, 2 H) 6.21 - 6.34 (m, 2 H)

^{13}C NMR (101 MHz, METHANOL-d_4) δ ppm 63.4, 131.5, 133.6

HRMS for $C_6H_{10}O_2$: Calc. 114.0681; found 114.0688 (M^+)

6.8.6. SYNTHESIS OF (*E,E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)HEXA-2,4-DIEN-1-OL (**3**)



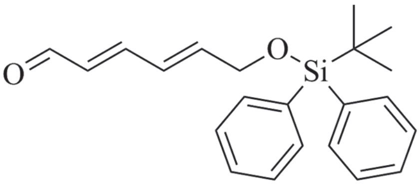
To a solution of (*E*,*E*)-hexa-2,4-diene-1,6-diol (913 mg, 8.2 mmol) in dry DMF (25 ml) at ambient temperature is added diisopropyl ethyl amine (82 mmol) and *tert*-butyldiphenylsilyl chloride (2.4 g, 8.6 mmol). The reaction mixture is stirred for 12 h followed by quenching with water (160 ml) and dilution with ethyl acetate (160 ml). The organic layer is separated and the aqueous layer is extracted with ethyl acetate (3 x 400 ml). The combined organic layers are extracted with 2 N aqueous hydrogen chloride solution (2 x 160 ml), saturated sodium bicarbonate solution (160 ml) and brine (160 ml), dried over magnesium sulfate, concentrated and the crude product is purified by flash column chromatography (25 % ethyl acetate in hexane) to yield the title compound (1.8 g, 45 %) as an oil.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.08 (s, 9 H) 4.17 - 4.30 (m, 4 H) 5.70 - 5.91 (m, 2 H) 6.20 - 6.39 (m, 2 H) 7.32 - 7.53 (m, 6 H) 7.61 - 7.77 (m, 4 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 19.2, 26.8, 63.4, 64.0, 127.7, 128.8, 129.4, 131.1, 131.3, 132.9, 133.6, 135.1

HRMS for $C_{22}H_{28}O_2Si$: Calc. 352.1859; found 334.1752 (M^+ - H_2O)

6.8.7. SYNTHESIS OF (*E,E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)HEXA-2,4-DIENAL (**4**)



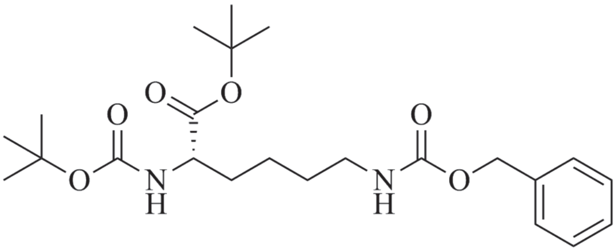
To a solution of (2,4)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-ol (1.7 g, 4.9 mmol) in dichloromethane (66 ml) at ambient temperature is added manganese oxide (4.8 g, 49 mmol). The reaction mixture is stirred for three hours, filtered and concentrated *in vacuo* to yield the title compound as a white solid (1.4 g, 82 %).

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.10 (s, 9 H) 4.37 (d, *J*=2.13 Hz, 2 H) 6.12 - 6.22 (m, 1 H) 6.27 - 6.36 (m, 1 H) 6.61 - 6.73 (m, 1 H) 7.09 - 7.20 (m, 1 H) 7.36 - 7.50 (m, 6 H) 7.68 (dd, *J*=7.91, 1.51 Hz, 4 H) 9.59 (d, *J*=8.03 Hz, 1 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 19.25, 26.75, 63.55, 126.83, 127.78, 129.86, 131.26, 133.03, 135.44, 143.84, 151.68, 193.87

HRMS for $C_{22}H_{26}O_2Si$: Calc. 350.1702; found 350.1672 (M^+)

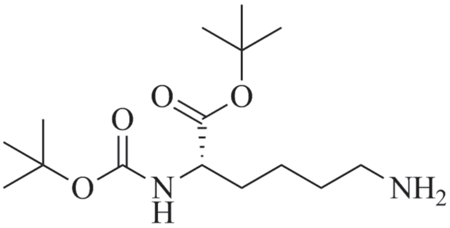
6.8.8. SYNTHESIS OF *N*$^{\alpha}$-BOC-LYS(Z)-O^{T}BU (**5**)



To a solution of H-Lys(Z)-OtBu*HCl (5.1 g, 13.4 mmol) in chloroform (40 ml) is added sodium bicarbonate (1.2 g, 14.2 mmol) in water (30 ml) and stirred vigorously. After 15 minutes di-*tert*-butyl dicarbonate (2.9 g, 13.4 mmol) in chloroform (20 ml) is added and the reaction mixture is refluxed for 1.5 hours. The reaction mixture is cooled to room temperature, the organic layer is separated and the aqueous layer is extracted with chloroform (2 x 40 ml). The combined organic extracts are dried over sodium sulfate, concentrated *in vacuo* and the crude product is purified by flash column chromatography (25 % ethyl acetate in hexane) to yield the title compound (5.5 g, 98 %) as an oil.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.30 - 1.38 (m, 2 H) 1.41 (s, 9 H) 1.43 (s, 9 H) 1.46 - 1.53 (m, 2 H) 1.53 - 1.65 (m, 1 H) 1.65 - 1.80 (m, 1 H) 3.14 (q, *J*=6.36 Hz, 2 H) 4.06 - 4.18 (m, 1 H) 5.06 (s, 2 H) 5.20 (d, *J*=6.40 Hz, 2 H) 7.20 - 7.38 (m, 5 H)
^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 22.11, 27.74, 28.09, 29.13, 32.23, 40.43, 53.51 , 66.24, 79.30, 81.48, 127.78, 127.84, 128.22, 136.45, 155.29, 156.29, 171.76
HRMS for $C_{23}H_{36}N_2O_6$: Calcd 436.2573; found 436.2594 (M^{+})

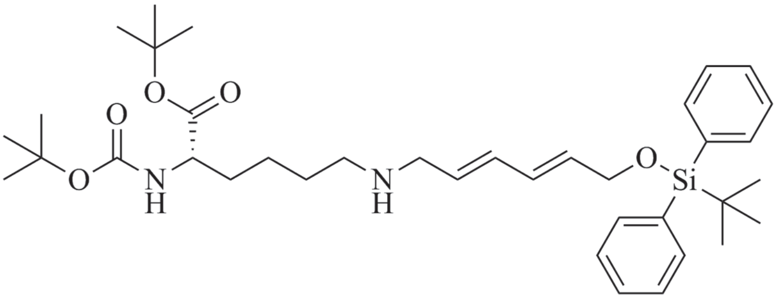
6.8.9. SYNTHESIS OF *N*$^{\alpha}$-BOC-LYS-O^{T}BU (**6**)



To a solution of *N*$^{\alpha}$-Boc-Lys(Z)-OtBu (5.8 g, 13.8 mmol) in ethanol (50 ml, technical grade) is added 10 % palladium on carbon (480 mg) and hydrogen gas is let in. After one hour, the solution is filtered, concentrated *in vacuo* to yield the title compound (3.9 g, 93 %) as an oil.

^{1}H NMR (400 MHz, METHANOL-*d*$_4$) δ ppm 1.39 - 1.48 (m, 2 H) 1.51 (s, 9 H) 1.53 (s, 9 H) 1.56 - 1.86 (m, 4 H) 2.69 (t, *J*=6.90 Hz, 2 H) 4.01 (dd, *J*=8.78, 5.02 Hz, 1 H)
^{13}C NMR (101 MHz, METHANOL-*d*$_4$) δ ppm 24.37, 28.44, 28.92, 32.66, 33.50, 42.41, 55.89, 80.42, 82.49, 158.20, 173.91
HRMS for $C_{15}H_{30}N_2O_4$: Calc. 302.2206; found 302.2183 (M$^+$)

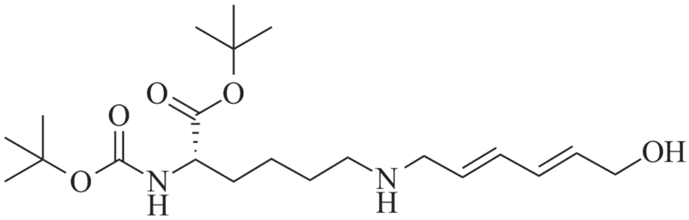
6.8.10. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-(((*E,E*)-6-((*TERT*-BUTYLDIPHENYLSILYL)OXY)HEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (7)



To a solution of *N*$^{\alpha}$-Boc-Lys-O^{t}Bu (500 mg, 1.7 mmol) in dichloroethane (20 ml) is slowly added a solution of (*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dienal (580 mg, 1.7 mmol) in dichloroethane (20 ml). After addition is completed, sodium triacetoxyborohydride (536 mg, 2.4 mmol) is added and the solution is stirred for 24 h. Saturated sodium bicarbonate solution (10 ml) is added and the mixture is stirred for 10 minutes. The layers are separated, and the aqueous layer is extracted with chloroform (2 x 20 ml). The combined organic extracts are dried over sodium sulfate and concentrated. The crude product is purified by column chromatography (20 % ethanol in ethyl acetate containing 1 % triethylamine) to yield the title compound (437 mg, 40 %) as an oil.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.04 - 1.09 (m, 9 H) 1.27 - 1.40 (m, 2 H) 1.45 (s, 9 H) 1.47 (s, 9 H) 1.49 - 1.70 (m, 4 H) 1.70 - 1.92 (m, 2 H) 2.62 (t, *J*=7.15 Hz, 2 H) 3.29 (d, *J*=6.27 Hz, 2 H) 4.17 (d, *J*=5.52 Hz, 1 H) 4.24 (d, *J*=4.52 Hz, 2 H) 5.07 (d, *J*=8.28 Hz, 1 H) 5.67 - 5.78 (m, 2 H) 6.12 - 6.36 (m, 2 H) 7.34 - 7.46 (m, 6 H) 7.63 - 7.78 (m, 4 H)
^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 19.21, 22.89, 26.77, 27.98, 28.31, 29.56, 32.77, 48.96, 51.39, 53.84, 64.02, 77.20, 79.54, 81.70, 127.62, 129.26, 129.60, 131.12, 131.33, 131.67, 133.59, 135.49, 155.37, 171.98
HRMS for $C_{28}H_{38}N_2O_2Si$: Calc. 636.3958; found 462.2703 ($M^+ - C_4H_9O - C_5H_9O_2$), 405.1998 ($M^+ - C_4H_9O - C_5H_9O_2 - C_4H_9$), 327.1529 ($M^+ - C_4H_9O - C_5H_9O_2 - C_4H_9 - C_6H_6$)

6.8.11. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-(((*E,E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (**8**)



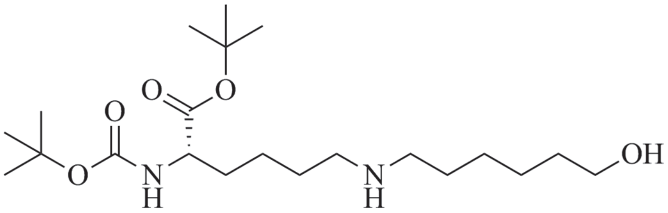
To a solution of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate (437 mg, 0.67 mmol) in THF (3 ml) is added tetra butyl ammonium fluoride (TBAF) (0.8 mmol, 800 µl of a 1.0 M solution in THF). The solution is stirred overnight. Saturated sodium bicarbonate solution is added and the aqueous phase is extracted three times with chloroform. The combined organic layers are dried over sodium sulfate and concentrated *in vacuo*. Purification is achieved by flash chromatography (40 % isopropanol in toluene containing 1 % triethylamine) to yield the title compound as a faint yellow oil (234 mg, 90 %).

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.25 - 1.40 (m, 2 H), 1.44 (s, 9 H) 1.46 (s, 9 H) 1.50 - 1.84 (m, 4 H) 2.62 (t, *J*=7.28 Hz, 2 H) 3.31 (d, *J*=6.53 Hz, 2 H) 4.09 - 4.25 (m, 3 H) 5.10 (d, *J*=8.16 Hz, 1 H) 5.70 - 5.87 (m, 2 H) 6.14 - 6.31 (m, 2 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 0.97, 22.84, 27.97, 28.30, 29.02, 32.68, 48.67, 51.04, 53.82, 62.94, 79.59, 81.75, 130.36, 130.69, 131.83, 132.25, 155.40, 171.95

HRMS for $C_{21}H_{38}N_2O_5$: Calc. 398.2781; found 398.2746 (M^+), 380.2657 ($M^+ - H_2O$)

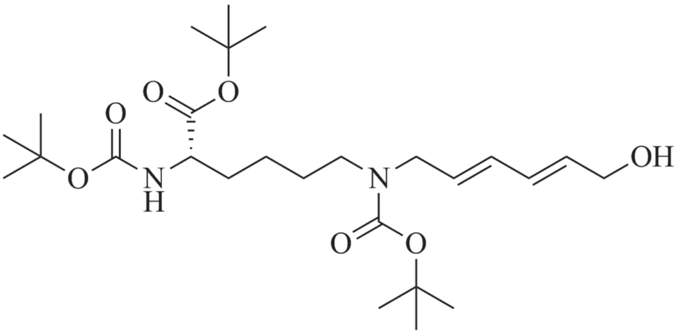
6.8.12. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-((6-HYDROXYHEXYL)AMINO)HEXANOATE (**9**)



To a solution of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (50 mg, 0.13 mmol) in ethanol (4 ml, technical grade) is added 10 % palladium on carbon (50 mg) and hydrogen gas is let in. After one hour, the solution is filtered, concentrated *in vacuo* and the crude product is purified by column chromatography (80 % isopropanol in toluene containing 1 % triethylamine) to yield the title compound (6.4 mg, 12 %) as an oil.

^{1}H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 1.32 - 1.82 (m, 14 H) 1.46 (s, 9 H) 1.48 (s, 9 H) 2.68 - 2.74 (m, 4 H) 3.66 (t, *J*=6.42 Hz, 2 H) 4.15 – 4.19 (m, 1 H) 5.15 (d, *J*=8.07 Hz, 1 H)
^{13}C NMR (151 MHz, CHLOROFORM-*d*) δ ppm 22.83, 25.42, 26.73, 28.00, 28.33, 28.62, 32.42, 32.69, 48.98, 49.10, 53.79, 62.57, 79.65, 81.84, 155.46, 171.91

6.8.13. SYNTHESIS OF (*S*)-*TERT*-BUTYL 2-((*TERT*-BUTOXYCARBONYL)AMINO)-6-(((*E,E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (10)

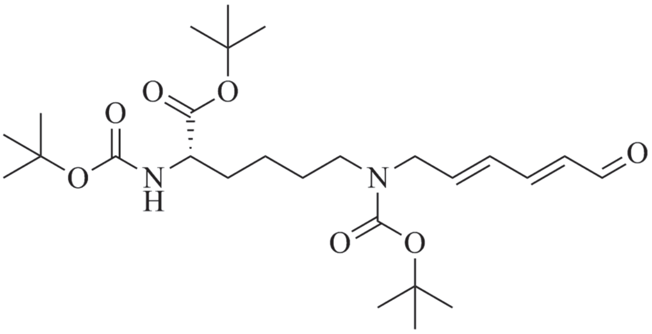


To a solution of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (300 mg, 0.75 mmol) in chloroform (10 ml) is added a solution of sodium bicarbonate (30 mg) in water (3 ml). Di-*tert*-butyl dicarbonate (167 mg, 0.75 mmol) in chloroform (2 ml) is added and the reaction mixture is refluxed for 1.5 hours. The reaction mixture is cooled to room temperature, the organic layer is separated and the aqueous layer is extracted with chloroform (2 x 5 ml). The combined organic extracts are dried over sodium sulfate, concentrated *in vacuo* and the crude product is purified by flash column chromatography (40 % ethyl acetate in hexane) to yield the title compound (285 mg, 76 %) as an oil.

^{1}H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 1.19 - 1.38 (m, 2 H) 1.43 (s, 9 H) 1.44 (s, 9 H) 1.45 (s, 9 H) 1.47 - 1.82 (m, 4 H) 3.11 – 3.16 (br. d, 2 H) 3.78 - 3.84 (br. d., 2 H) 4.04 - 4.15 (m, 1 H) 4.17 (d, *J*=5.45 Hz, 2 H) 5.01 – 5.09 (br. d., 1H) 5.62 (br. s., 1 H) 5.75 - 5.85 (m, 1 H) 6.09 (br. s., 1 H) 6.24 (dd, *J*=14.63, 10.80 Hz, 1 H)

^{13}C NMR (151 MHz, CHLOROFORM-*d*) δ ppm 22.35, 27.95, 28.28, 28.40, 29.63, 32.37, 46.20, 48.80, 53.83, 63.09, 79.45, 79.52, 81.63, 129.69, 130.39, 130.90, 131.98, 155.37, 155.44, 171.94

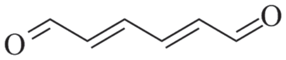
6.8.14. SYNTHESIS OF (*S*)-*TERT*-BUTYL 6-((*TERT*-BUTOXYCARBONYL)((*E,E*)-6-OXOHEXA-2,4-DIEN-1-YL)AMINO)-2-((*TERT*-BUTOXYCARBONYL)AMINO)HEXANOATE (**11**)



To a solution (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (250 mg, 0.50 mmol) in dichloromethane (25 ml) is added manganese dioxide (500 mg, 5 mmol) and the reaction mixture is stirred vigorously for three hours. The reaction mixture is filtered and concentrated to give the title compound (175 mg, 70 %).

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.21 - 1.39 (m, 2 H) 1.42 (s, 9 H) 1.44 - 1.48 (m, 18 H) 1.49 - 1.90 (m, 4 H) 3.17 (br. s., 2 H) 3.93 (d, *J*=15.43 Hz, 2 H) 4.13 (br. s., 1 H) 4.95 - 5.13 (m, 1 H) 6.12 (dd, *J*=15.37, 7.97 Hz, 1 H) 6.15 - 6.25 (m, 1 H) 6.27 - 6.42 (m, 1 H) 7.10 (dd, *J*=15.25, 10.73 Hz, 1 H) 9.54 – 9.56 (d, *J*=8.03 Hz, 1 H)
^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 22.30, 27.91, 28.23, 28.31, 29.58, 32.40, 46.79, 48.34, 48.77, 53.70, 79.48, 79.86, 81.66, 129.06, 131.44, 141.08, 151.08, 155.31, 171.80, 193.62

6.8.15. SYNTHESIS OF (*E,E*)-MUCONALDEHYDE (**12**)



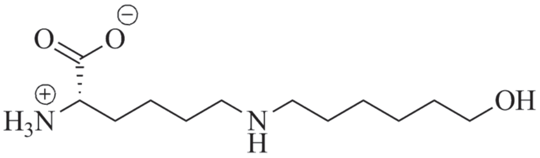
To a solution of (*E,E*)-hexa-2,4-diene-1,6-diol (243 mg, 2.1 mmol) in acetonitrile (25 ml) is added manganese(IV)-oxide (4 g, 42 mmol, 90 %) and the reaction mixture is stirred at room temperature for 45 min. Reaction progress is monitored via TLC (25 % ethyl acetate in hexane). After 45 min, the reaction mixture is filtered and concentrated to yield the title compound (151 mg, 1.40 mmol, 64 %) as a bright yellow solid.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) d ppm 6.39 - 6.65 (m, 2 H) 7.19 - 7.39 (m, 2 H) 9.72 (d, *J*=7.65 Hz, 2 H)

^{13}C NMR (101 MHz, CHLOROFORM-*d*) δ ppm 137.9, 146.4, 192.5

HRMS for $C_6H_6O_2$: Calc. 110.0368; found 110.0370 (M^+)

6.8.16. SYNTHESIS OF (*S*)-2-AMMONIO-6-((6-HYDROXYHEXYL)AMINO)HEXANOATE (**IS**)



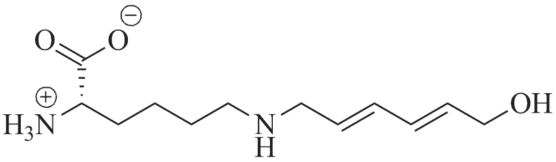
(*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-((6-hydroxyhexyl)amino)hexanoate (6.4 mg, 16 µmol) is taken up in DCl (0.5 ml, 1.0 M in D_2O) and left at ambient temperature for 31 h. The solvent is removed *in vacuo* to give the title compound as yellow oil (4.7 mg, 88 %).

^{1}H NMR (400 MHz, DEUTERIUM OXIDE) *δ* ppm 0.85 - 1.58 (m, 14 H) 2.48 - 2.61 (m, 4 H) 3.09 (t, *J*=6.65 Hz, 2 H) 3.53 + 3.63 (t, *J*=6.40 Hz, 1 H)

^{13}C NMR (101 MHz, ACETONITRILE-d_3) *δ* ppm 22.32, 25.28, 25.87, 26.22, 29.97, 30.40, 30.42, 31.69, 48.38, 53.25, 62.46, 172.44

HPLC-ESI$^+$-MS/MS: 247.2 → 202.2, 129.9, 84.0

6.8.17. SYNTHESIS OF (*S*)-2-AMMONIO-6-(((*E*,*E*)-6-HYDROXYHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (LYS(MUC-OH))



To a solution of (*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-6-(((*E,E*)-6-((*tert*-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate (10 mg, 16 µmol) in dioxane (0.5 ml) is added a aqueous solution of perchloric acid (0.5 ml, 4.0 M) and the solution is left at 4°C for 20 h. An ice cold aqueous solution of potassium hydroxide (1 ml, 2.0 M) is added and the solution is left at 4°C for 20 minutes following by centrifugation for 20 min at 4°C and 9 000 × g. The supernatant is purified by preparative hydrophilic interaction liquid chromatography using the following linear gradient using 0.1 % acetic acid (A) and acetonitrile (B) as mobile phase (Table 28).

Table 28 Preparative HPLC gradient for (S)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate with 0.1 % acetic acid (A) and acetonitrile (B)

Time	% B	flow
0	90	1
2	90	5
5	90	5
9	50	5
10	50	5

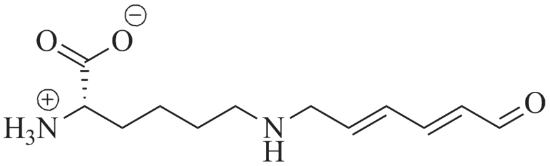
^{1}H NMR (400 MHz, D_2O+ ACETONITRILE-*d*$_3$) δ ppm 1.29 - 1.50 (m, 2 H) 1.59 - 1.71 (m, 2 H) 1.78 - 1.86 (m, 2 H) 2.93 - 3.03 (m, 2 H) 3.63 (d, *J*=7.28 Hz, 2 H) 3.67 (m, 1 H) 4.10 (d, *J*=5.52 Hz, 2 H) 5.67 (dt, *J*=15.00, 7.43 Hz, 1 H) 5.93 (dt, *J*=15.31, 5.65 Hz, 1 H) 6.21 - 6.32 (m, 1 H) 6.38 - 6.52 (m, 1 H)

^{13}C NMR (101 MHz, D_2O+ACETONITRILE-*d*$_3$) δ ppm 21.14, 24.84, 29.47, 45.75, 48.34, 54.07, 61.15, 120.78, 128.79, 134.40, 137.60, 174.09

λ_{max}(HPLC-UV/VIS) = 229 nm

HPLC-ESI^{+}-MS/MS: 243.2 → 147.1, 130.0, 96.9, 84.0, 79.1.

6.8.18. SYNTHESIS OF (*S*)-2-AMMONIO-6-(((*E,E*)-6-OXOHEXA-2,4-DIEN-1-YL)AMINO)HEXANOATE (LYS(MUC-CHO))



To a solution of (*S*)-*tert*-butyl 6-((*tert*-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (22.5 mg, 45 µmol) in dioxane (0.5 ml) is added a aqueous solution of perchloric acid (0.5 ml, 4.0 M) and the solution is left at 4°C for 20 h. An ice cold aqueous solution of potassium hydroxide (1 ml, 2.0 M) is added and the solution is centrifuged for 20 min at 4°C and 9 000 × g. The crude product is purified by preparative reversed phase high pressure liquid chromatography using the following linear gradient using 0.1 % acetic acid (A) and acetonitrile (B) as mobile phase (Table 29).

Table 29 Preparative HPLC gradient for (S)-2-ammonio-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate with 0.1 % acetic acid (A) and acetonitrile (B)

Time	% B	flow
0	5	1
3	5	15
7	5	15
11.5	95	15
13	95	15

^{1}H NMR (400 MHz, H_2O+D_2O+ACETIC ACID-d_4) δ ppm 1.35 - 2.0 (m, 6 H) 3.01 - 3.07 (m, 2 H) 3.80 (d, *J*=7.03 Hz, 2 H) 3.96 (t, *J*=6.53 Hz, 1 H) 6.23 - 6.36 (m, 2 H) 6.72 (dd, *J*=15.31, 10.79 Hz, 1 H) 7.37 (dd, *J*=15.31, 10.79 Hz, 1 H) 9.45 (d, *J*=8.03 Hz, 1 H)

^{13}C NMR (151 MHz, H_2O+D_2O+ACETIC ACID-d_4) δ ppm 20.91, 24.47, 28.81, 46.01, 47.75, 52.61, 132.03, 132.22, 134.45, 151.94, 168.31, 197.41

λ_{max}(HPLC-UV/VIS) = 265 nm

2,4-Dinitrophenylhydrazine-reactivity: positive

HPLC-ESI$^+$-MS/MS: 241.1 → 223.2, 178.2, 130.2, 84.2

7. DIRECTORIES

7.1. TABLE OF ABBREVIATIONS

Abbreviation	Translation
BSA	Bovine serum albumin
Boc	*tert*-Butyloxycarbonyl
CAT	Catechol (o-Dihydroxybenzen)
Cf.	(Lat. *conferatur*) eng. compare; reference to experiment number in the digital attachment
CYP	Cytochrom P-450
dd	Distilled twice
DMSO	Dimethylsulfoxide
EFSA	European Food Safety Authority
Est.	Estimated
FAO	Food and Agriculture Organization of the United Nations
GS-HQ	(Glutathion-S-yl)-hydroquinone
GSH	Glutathion (l-γ-Glutamyl-l-cysteinylglycin)
HILIC	Hydrophilic interaction liquid chromatography
HQ	Hydroquinone (p-Dihydroxybenzene)
i.e.	(Lat. *id est*) eng. that means
Ibd.	(Lat. *ibidem*) eng. in the same place; referring to the literature cited before
IS	(*S*)-2-Ammonio-6-((6-hydroxyhexyl)amino)hexanoate
ME/MSE	Refers to the corresponding reaction number in the electronic attachment
Mol. Eq.	Molar equivalent
MOPS	3-(N-morpholino)propanesulfonic acid
$NaBH(OAc)_3$	Sodium triacetoxyborohydride
Lys	L-Lysine
Lys(MUC-CHO)	(S)-2-Ammonio-6-(((*E*,*E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoate
Lys(MUC-OH)	(S)-2-Ammonio-6-(((*E*,*E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate
O^tBu	*tert*-Butyl ester
PH	Phenol

q.s.	(Lat. *quantum satis*) eng. as much as needed
RP	Reversed phase
ROS	Reactive oxygen species
sc.	(Lat. *scilicet*) eng. which means
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TBAF	Tetra butyl ammonium fluoride
TLC	Thin layer chromatography
USA	United States of America
WHO	World Health Organization
Z	Benzyloxycarbonyl

7.2. TABLE OF FIGURES

7.3. TABLE OF TABLES

8. ATTACHMENT

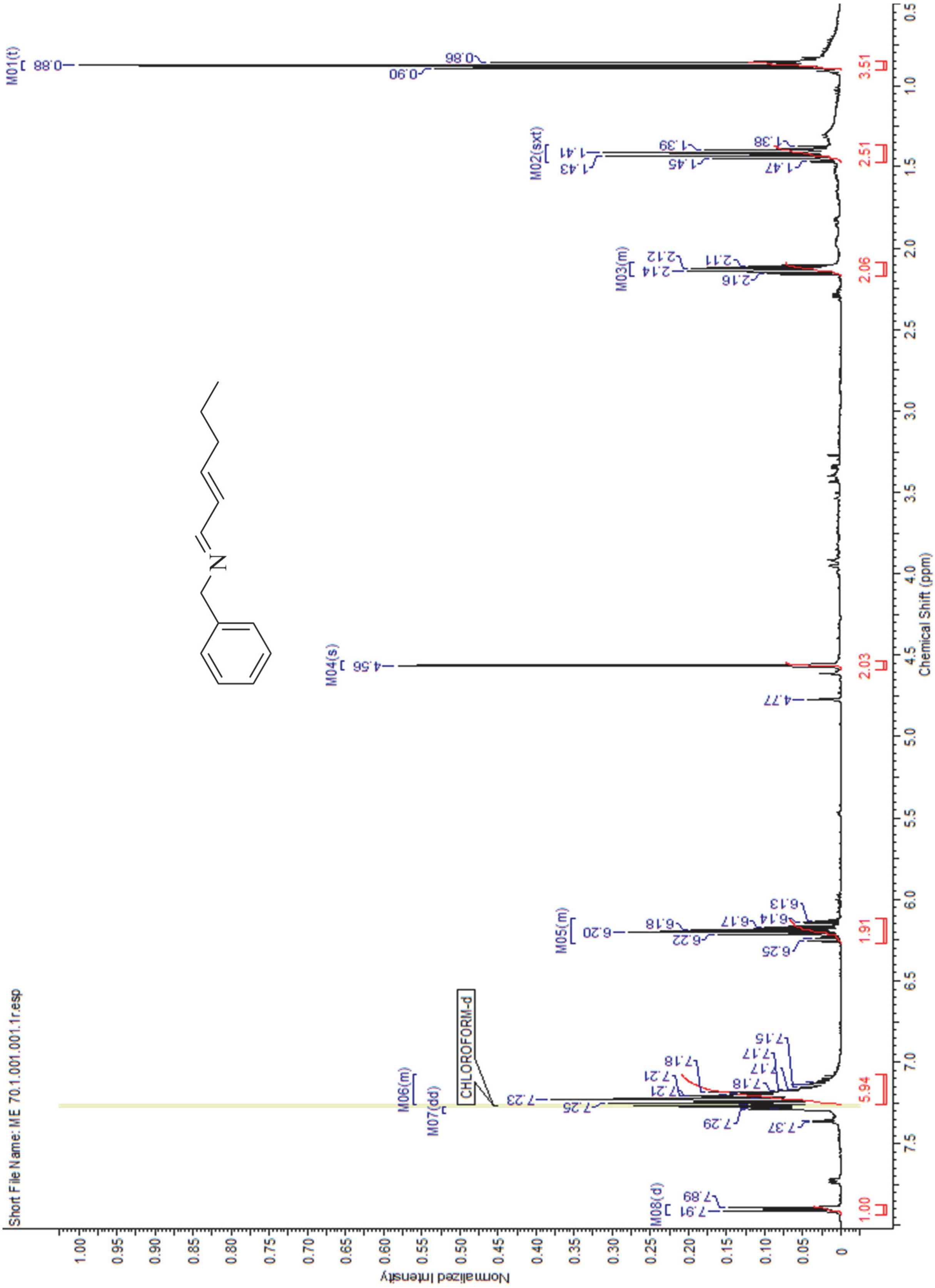


Figure 75 ¹H NMR spectrum of ((*E*)-hex-2-en-1-ylidene)-1-phenylmethanamine, 400 Mhz

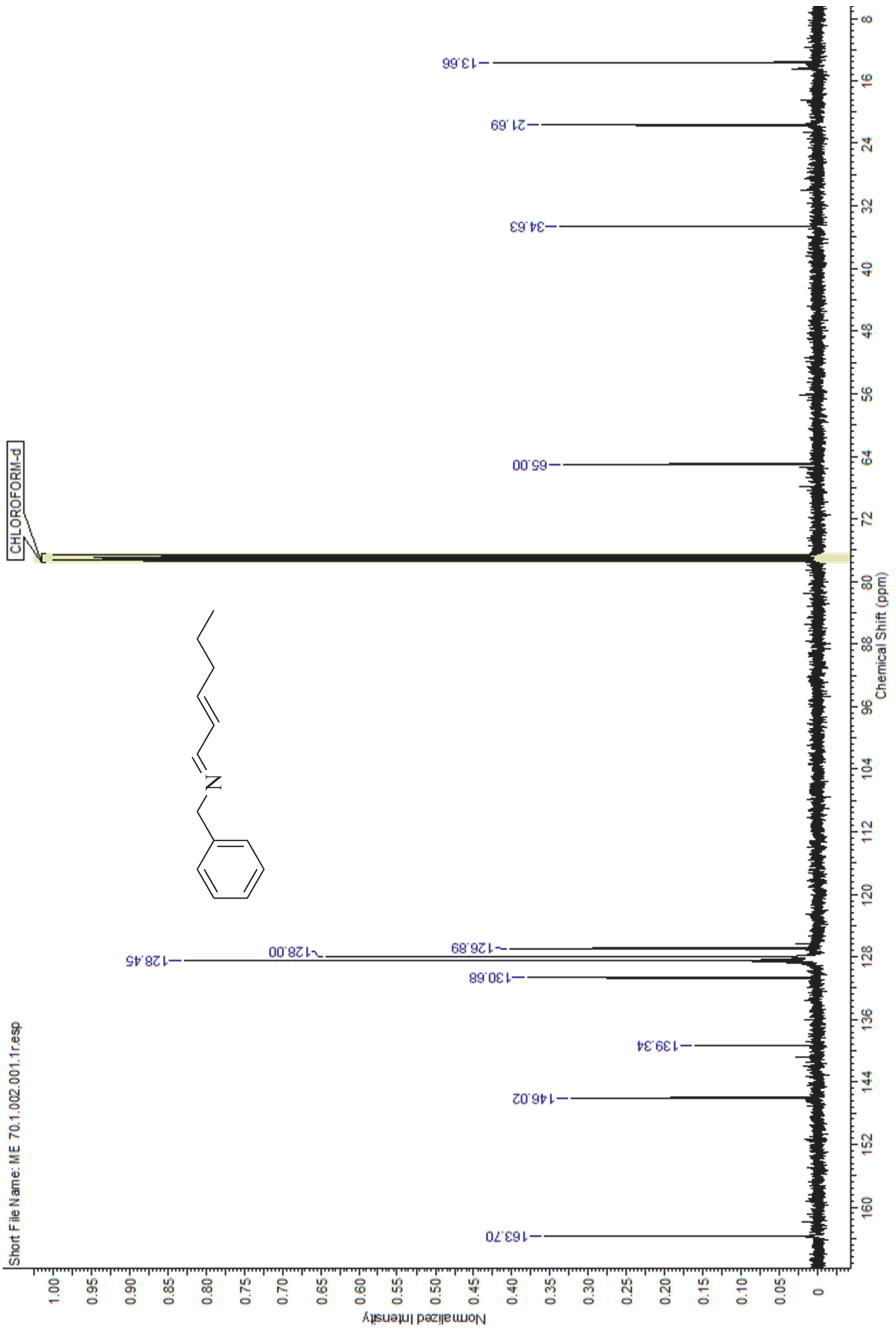


Figure 76 ^{13}C NMR spectrum of ((*E*)-hex-2-en-1-ylidene)-1-phenylmethanamine, 100 Mhz

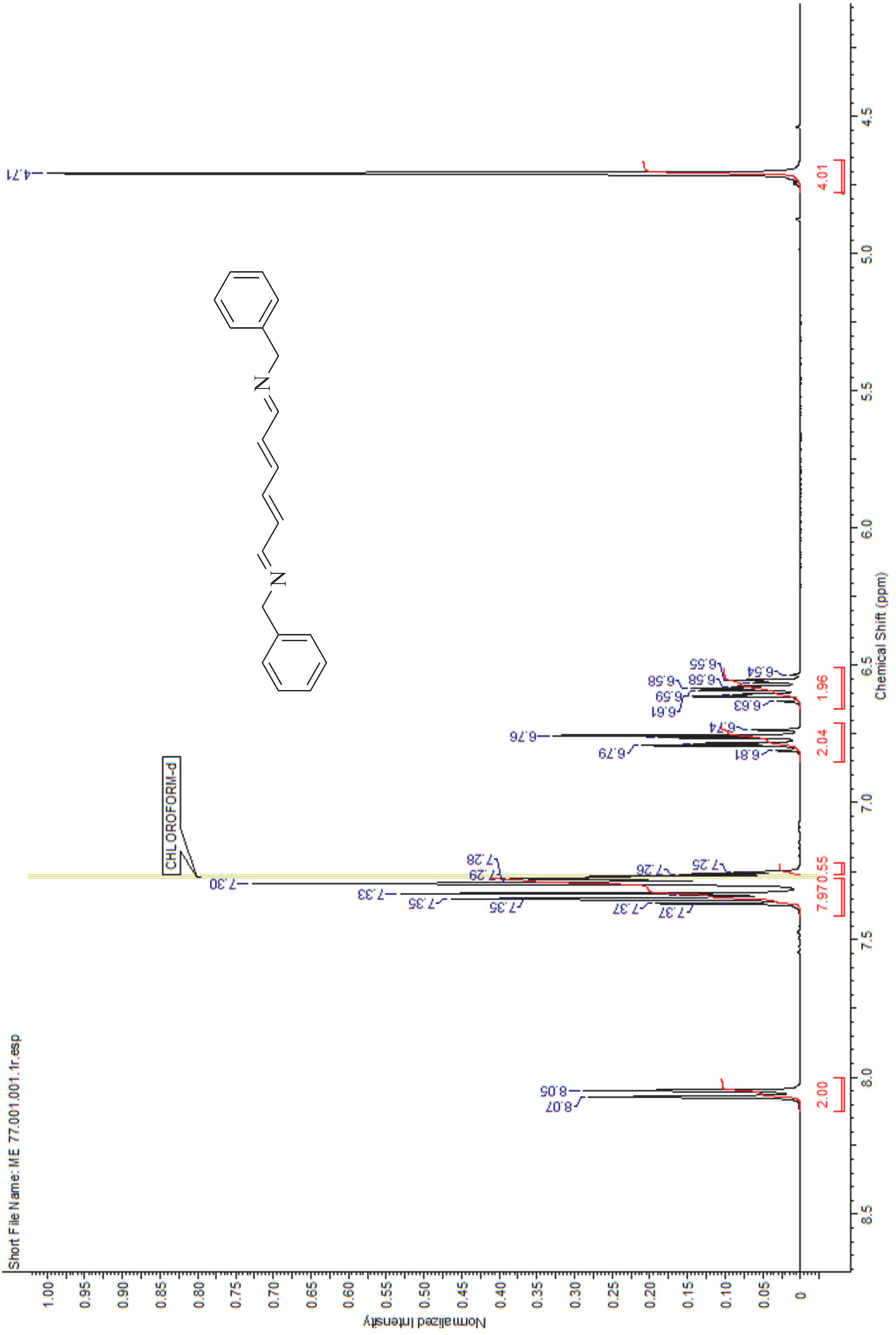


Figure 77 ^{1}H NMR spectrum of ((*E,E*)-hexa-2,4-diene-1,6-diylidene)bis(1-phenylmethanamine), 400 Mhz

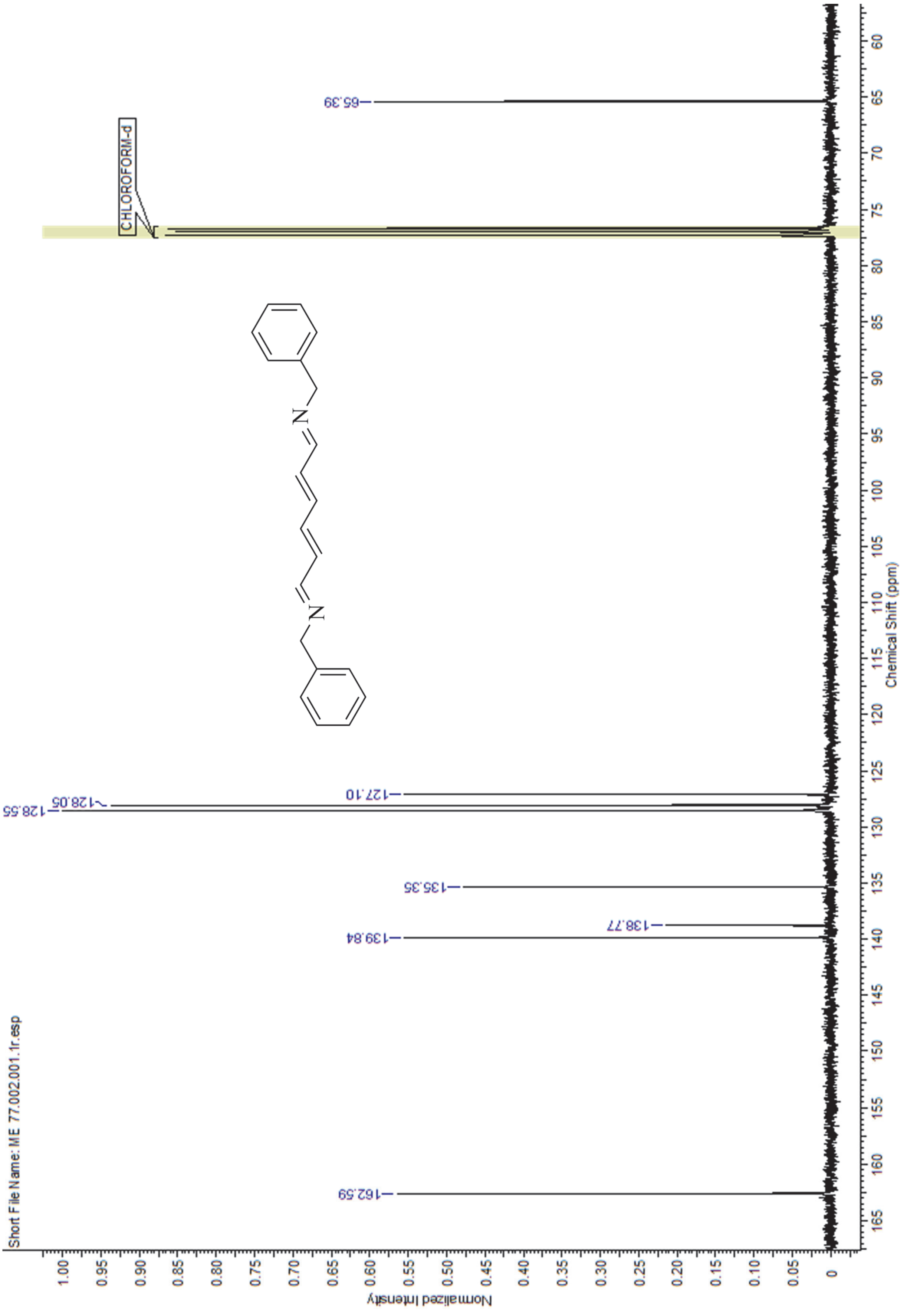


Figure 78 ^{13}C NMR spectrum of ((*E,E*)-hexa-2,4-diene-1,6-diylidene)bis(1-phenylmethanamine), 100 Mhz

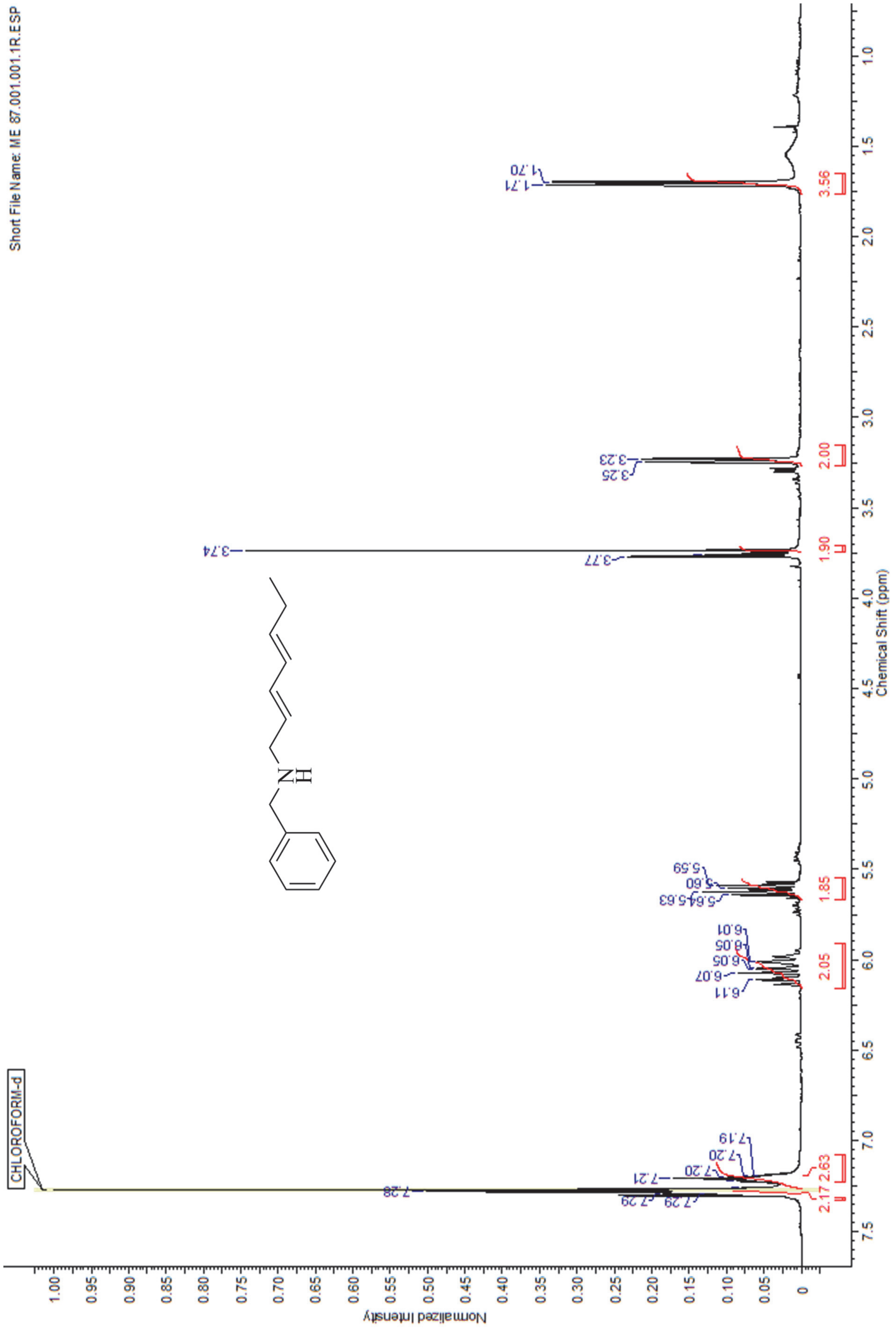


Figure 79 ^{1}H NMR spectrum of (E,E)-N-benzylhepta-2,4-dien-1-amine, 400 Mhz

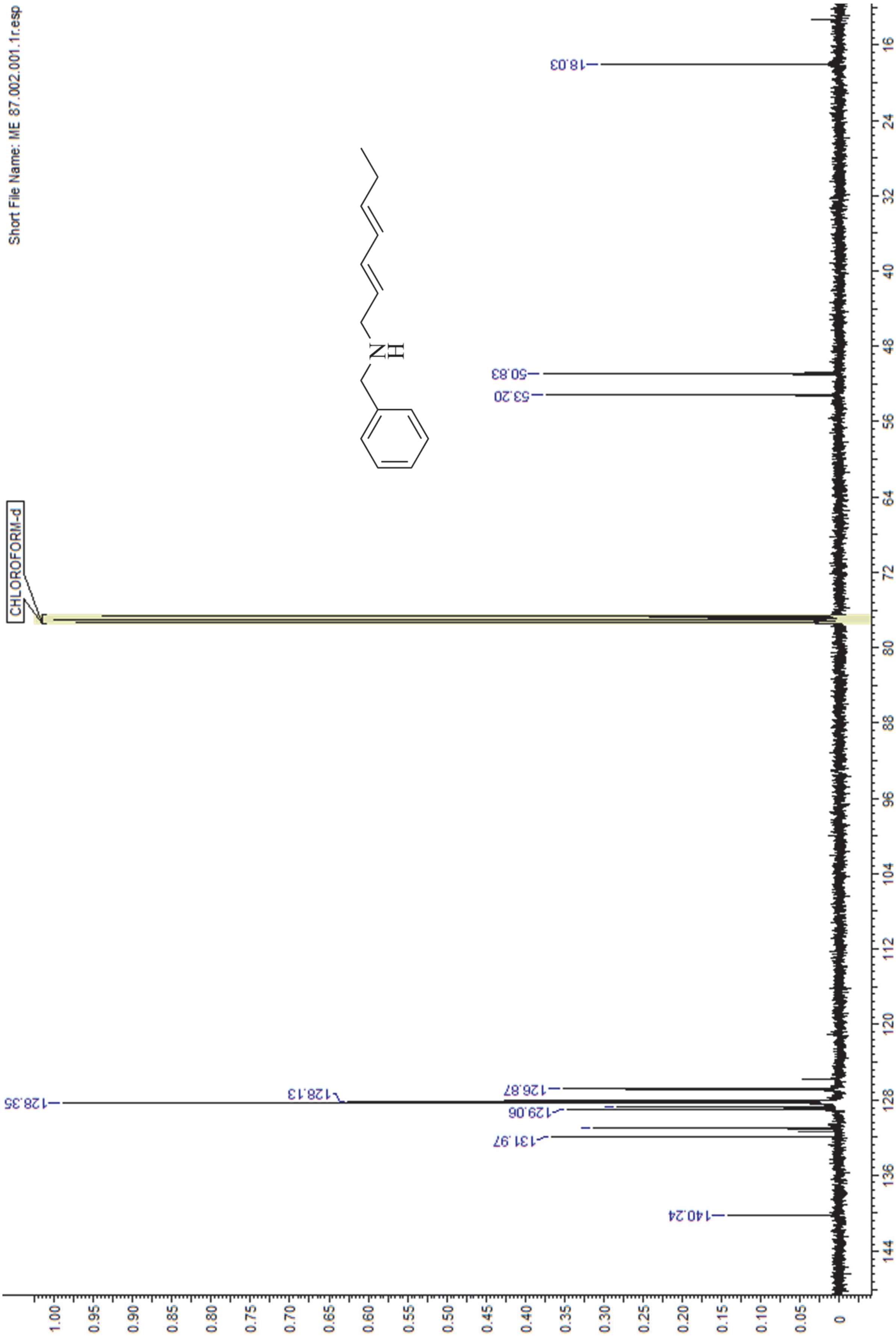


Figure 80 ^{13}C NMR spectrum of (E,E)-N-benzylhepta-2,4-dien-1-amine, 100 Mhz

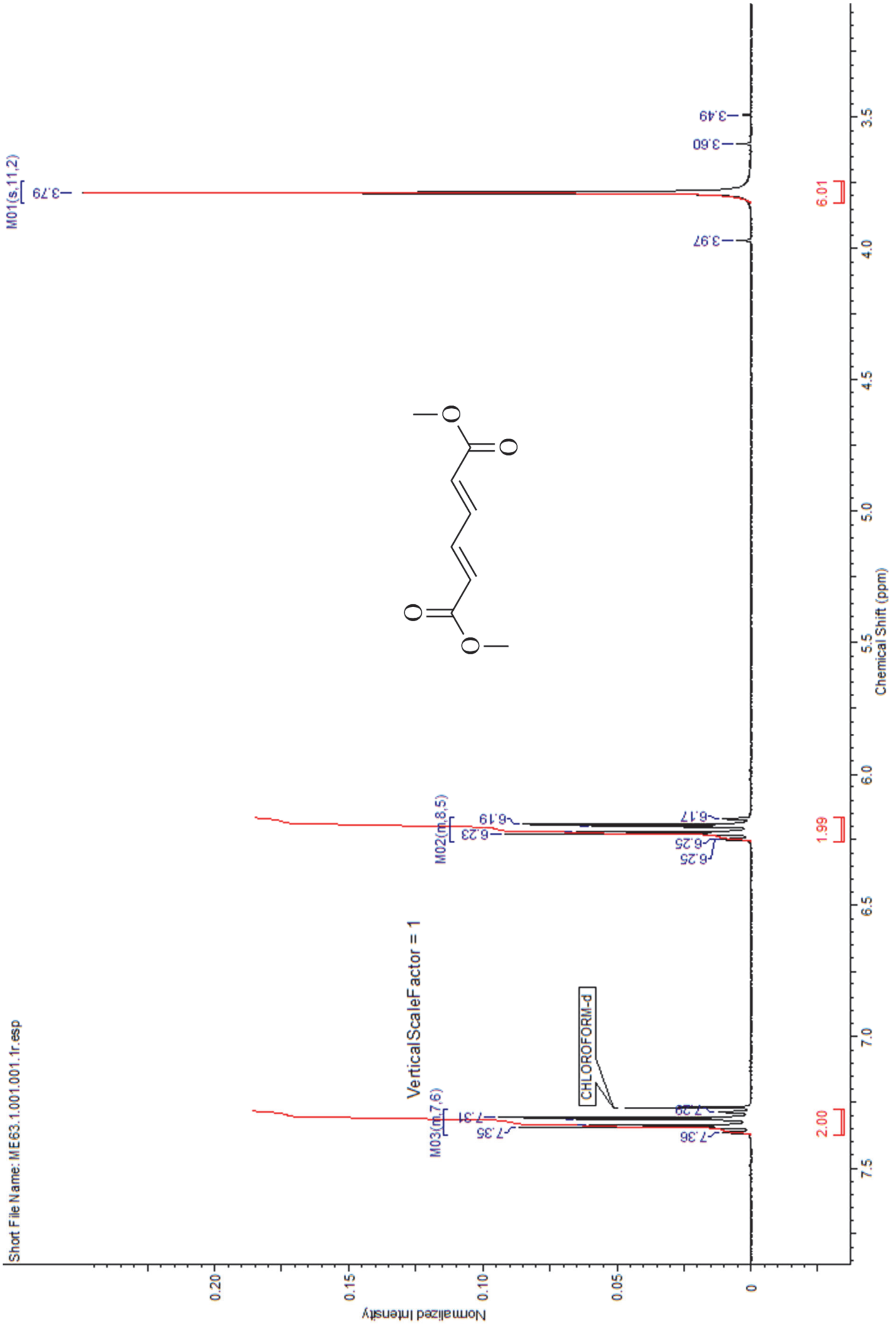


Figure 81 ^{1}H NMR spectrum of (*E,E*)-dimethyl hexa-2,4-dienedioate, 400 Mhz

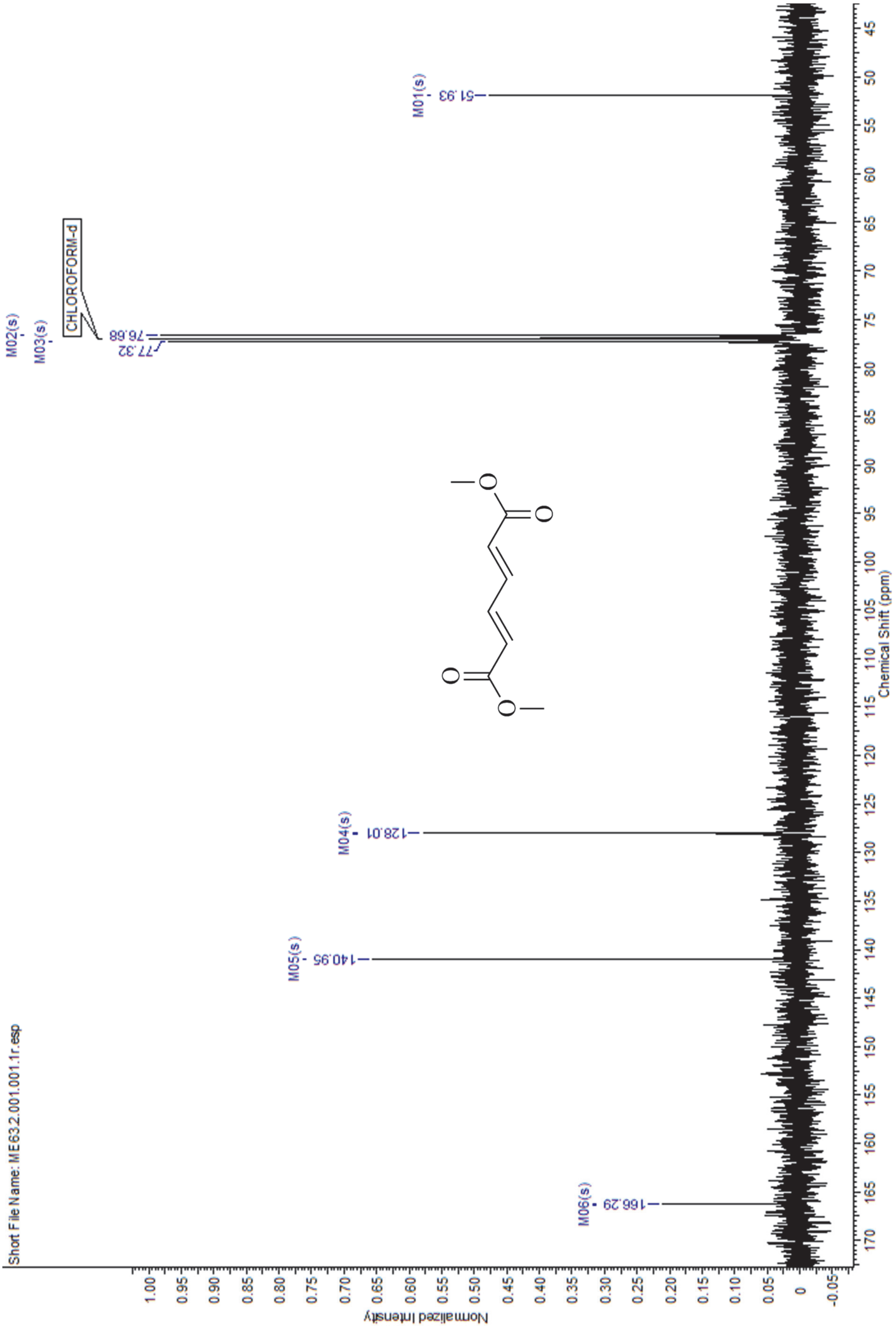


Figure 82 ^{13}C NMR spectrum of (*E,E*)-dimethyl hexa-2,4-dienedioate, 100 Mhz

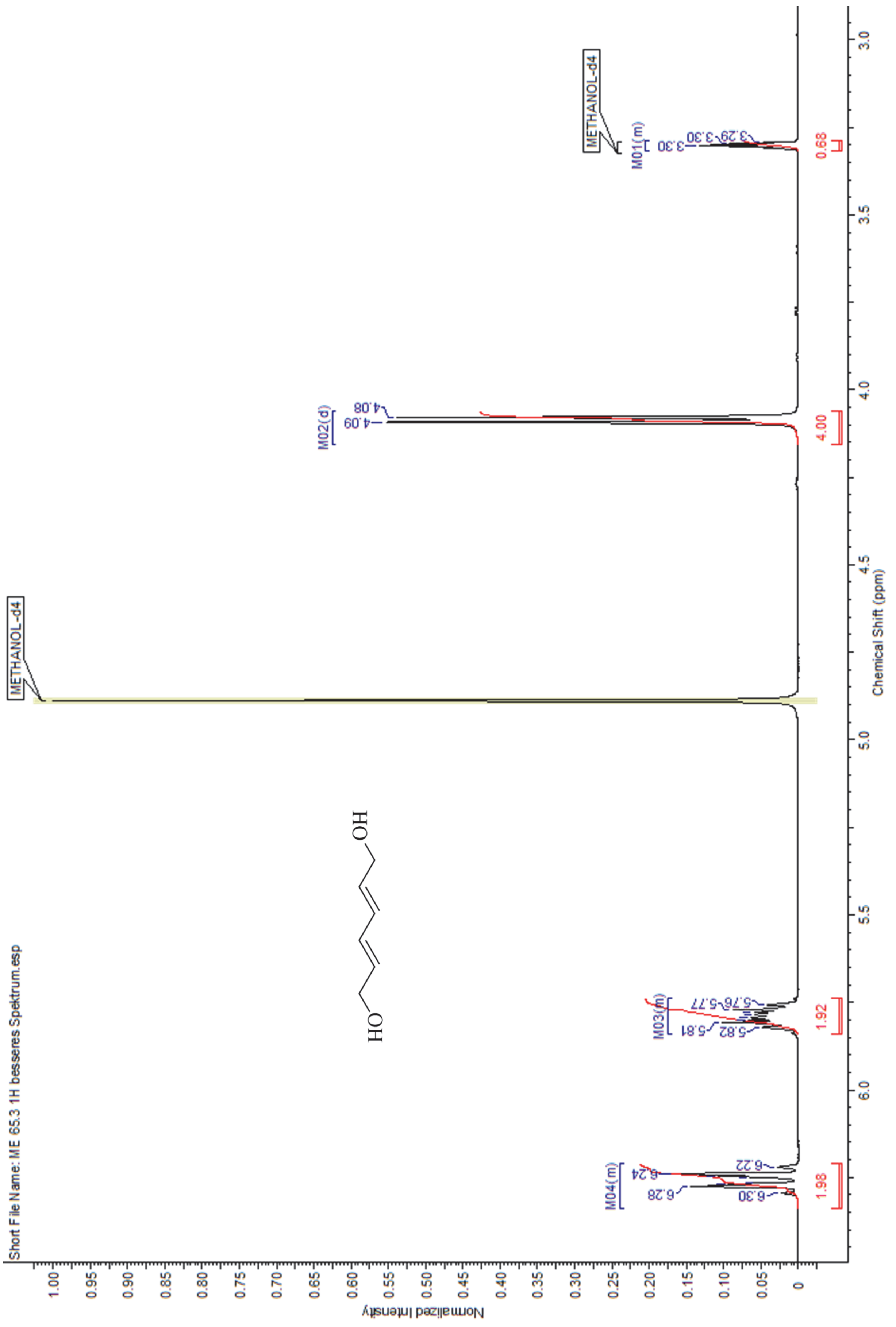


Figure 83 ^{1}H NMR spectrum of (*E*,*E*)-hexa-2,4-diene-1,6-diol, 400 Mhz

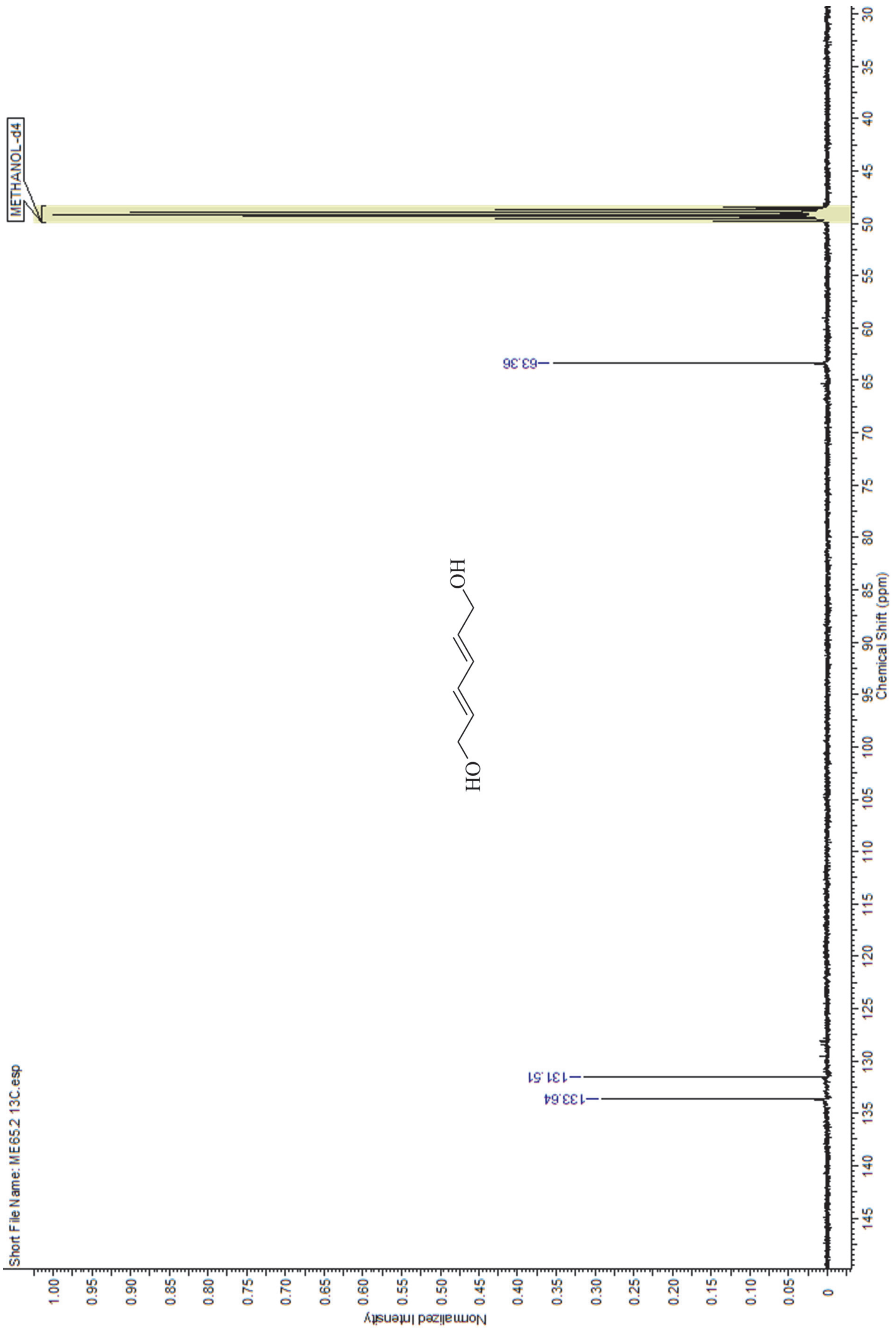


Figure 84 **^{13}C NMR spectrum of (*E*,*E*)-hexa-2,4-diene-1,6-diol, 100 Mhz**

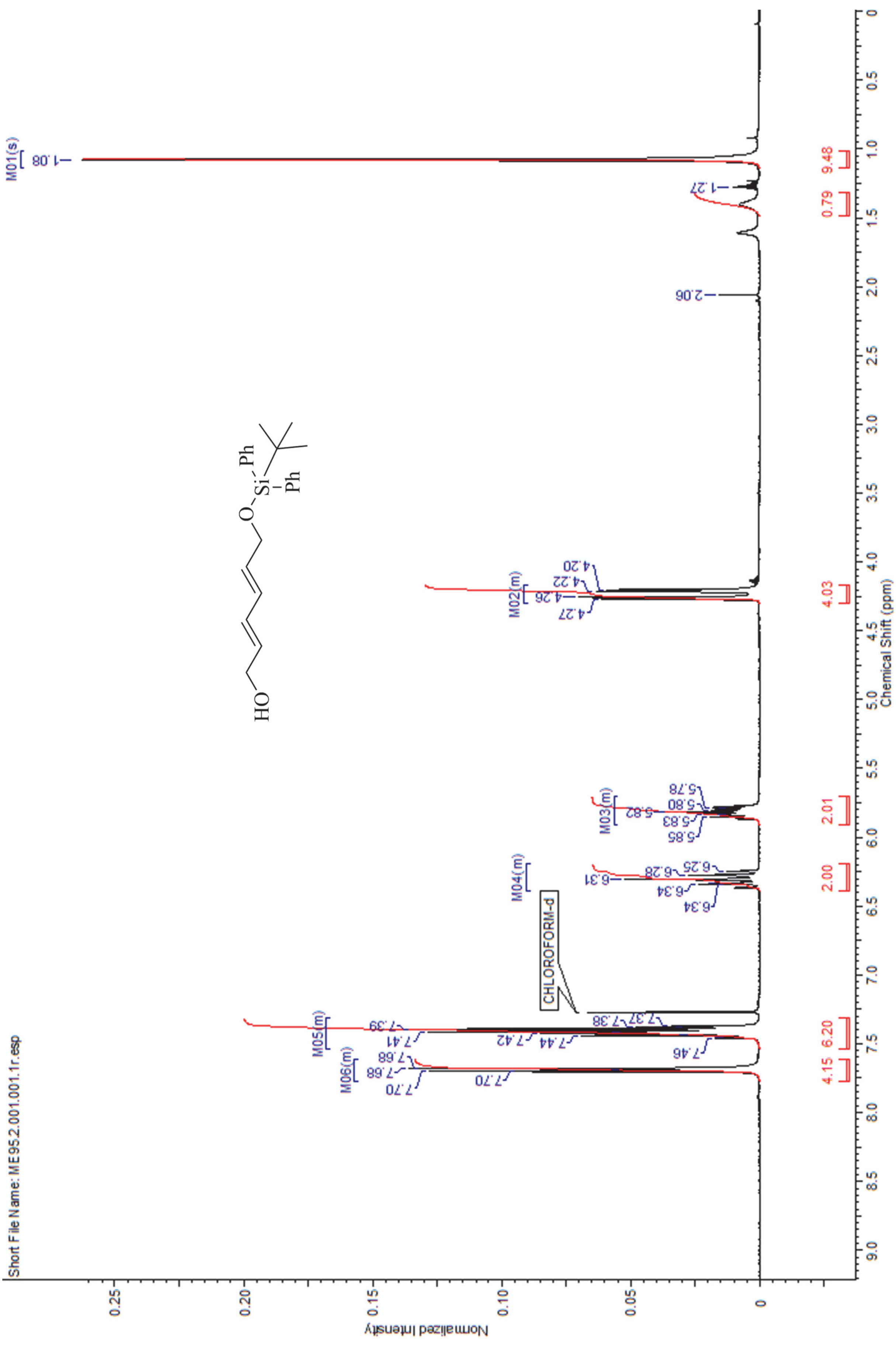


Figure 85 ^{1}H NMR spectrum of (*E,E*)-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-ol, 400 Mhz

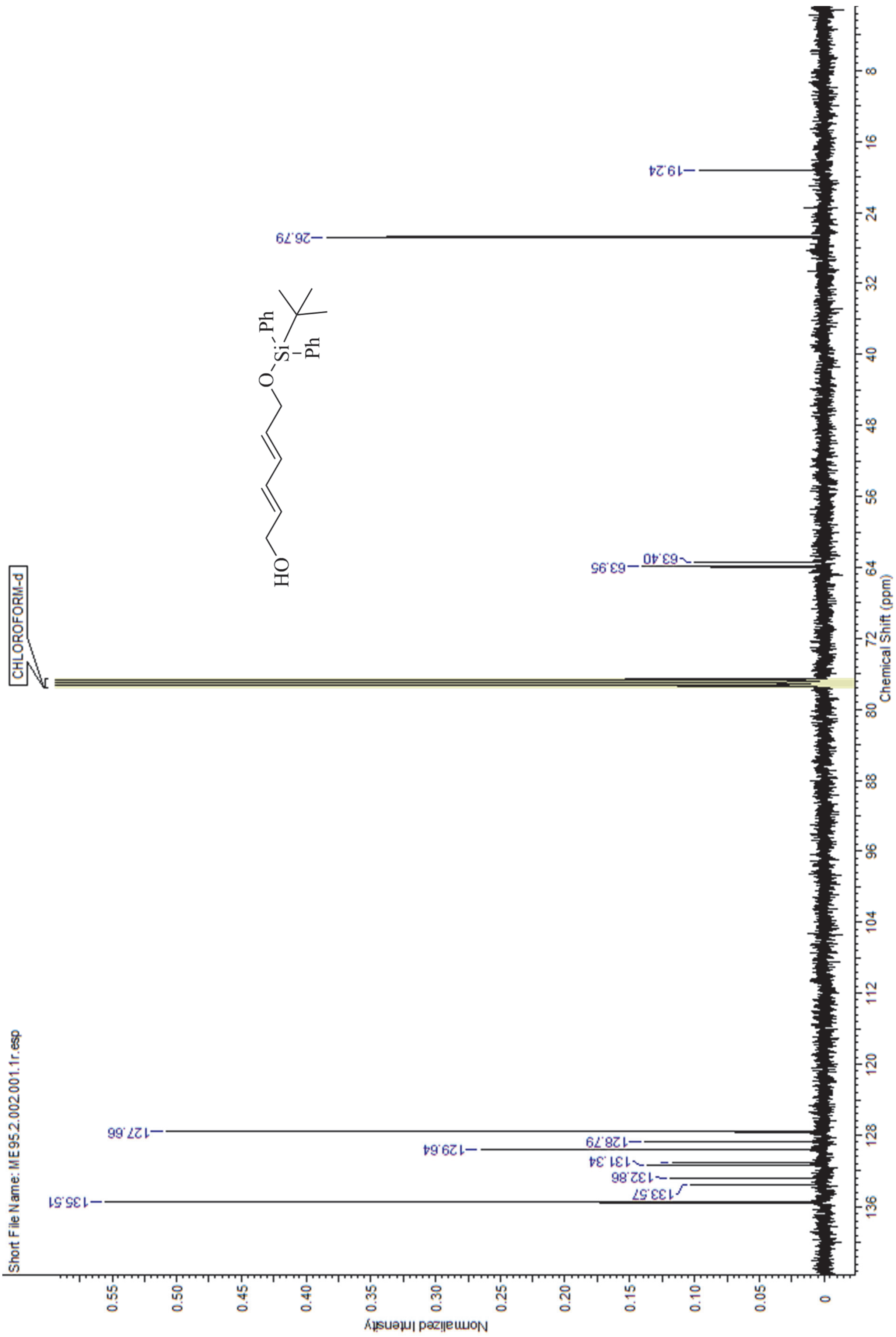


Figure 86 ^{13}C NMR spectrum of (*E*,*E*)-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-ol, 100 Mhz

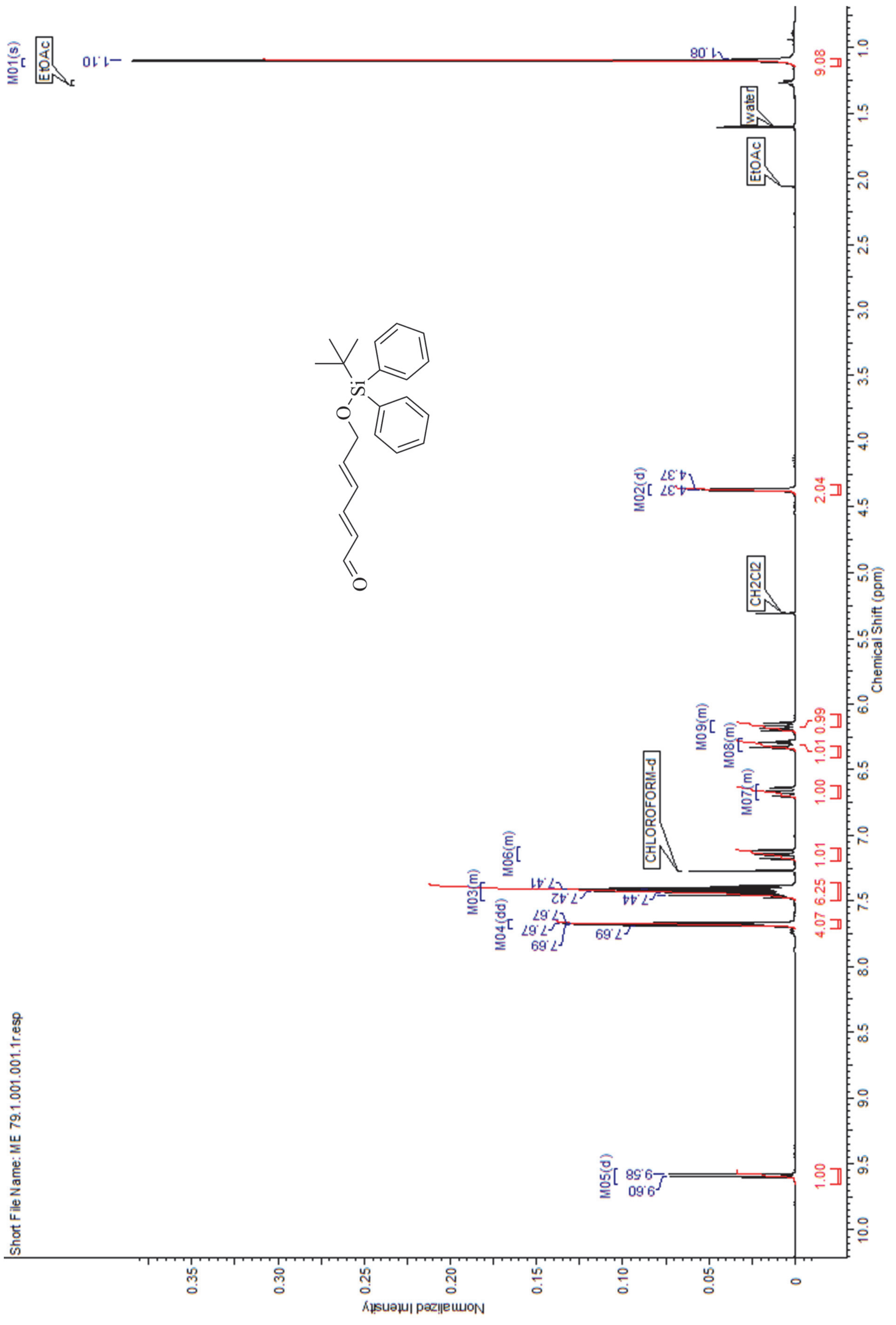


Figure 87 ^{1}H NMR spectrum of *(E,E)*-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dienal, 400 Mhz

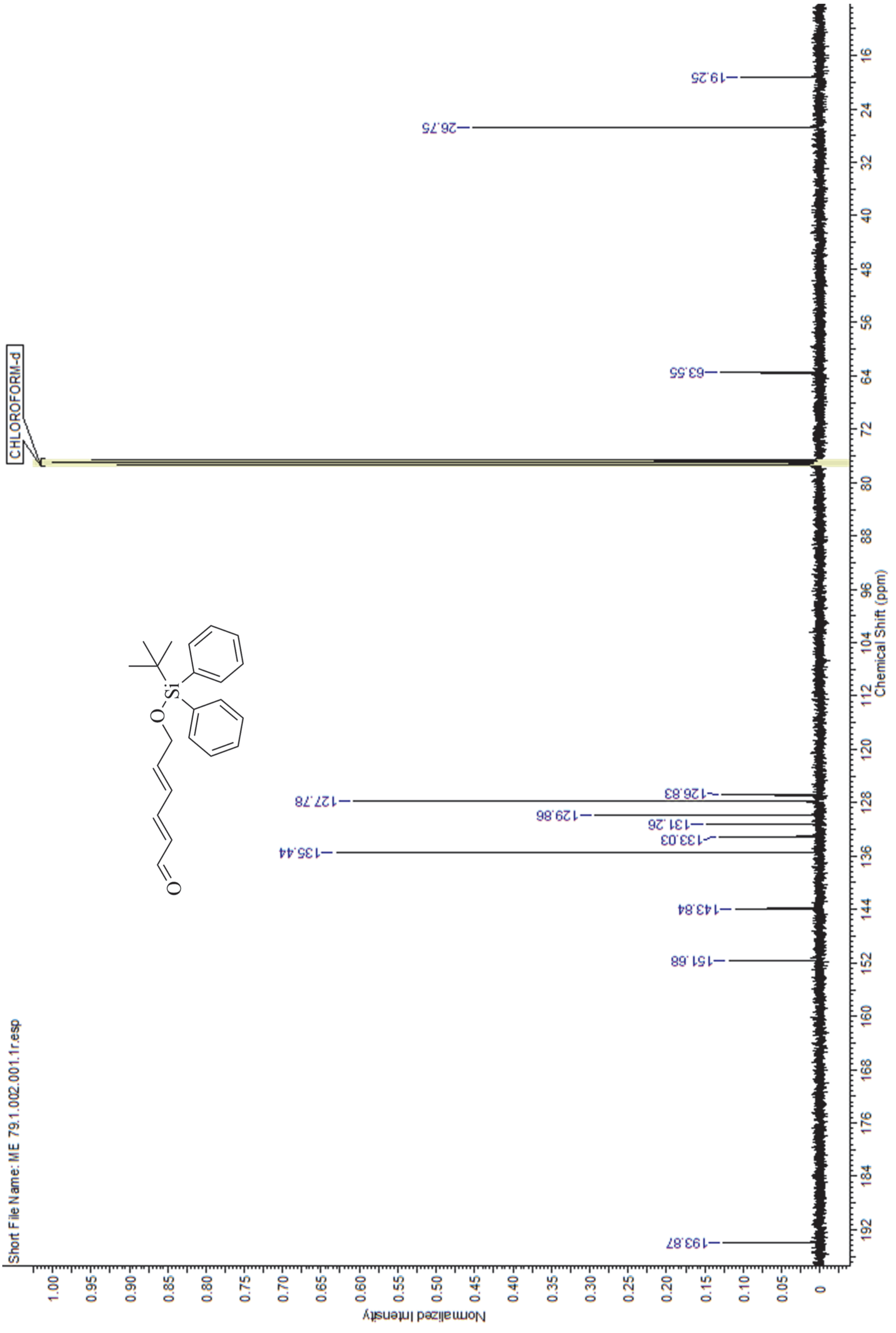


Figure 88 ^{13}C NMR spectrum of (*E,E*)-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dienal, 100 Mhz

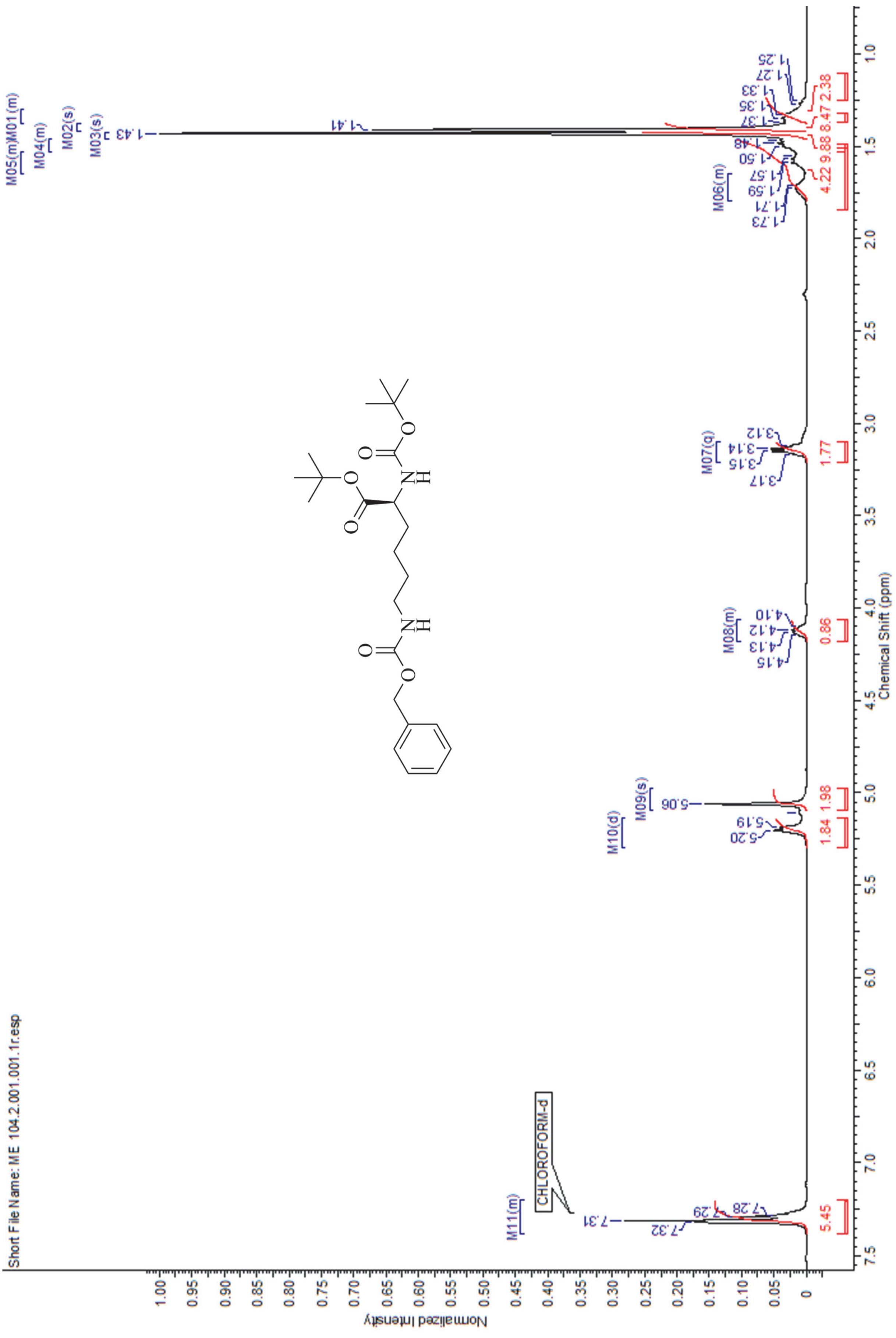


Figure 89 ^{1}H NMR spectrum of N^α-Boc-Lys(Z)-OtBu, 400 Mhz

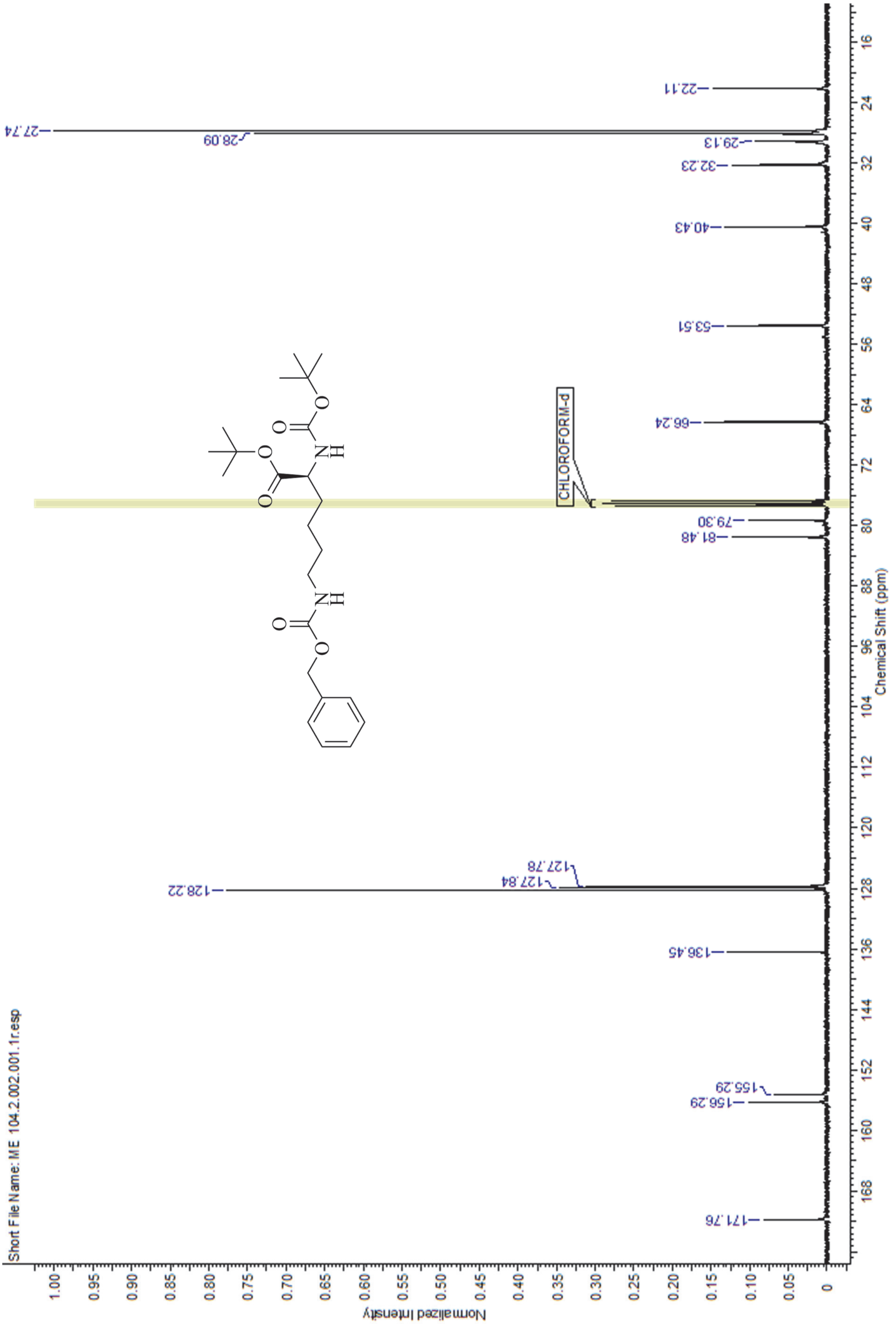


Figure 90 ^{13}C NMR spectrum of *N*$^{\alpha}$-Boc-Lys(Z)-OtBu, 100 Mhz

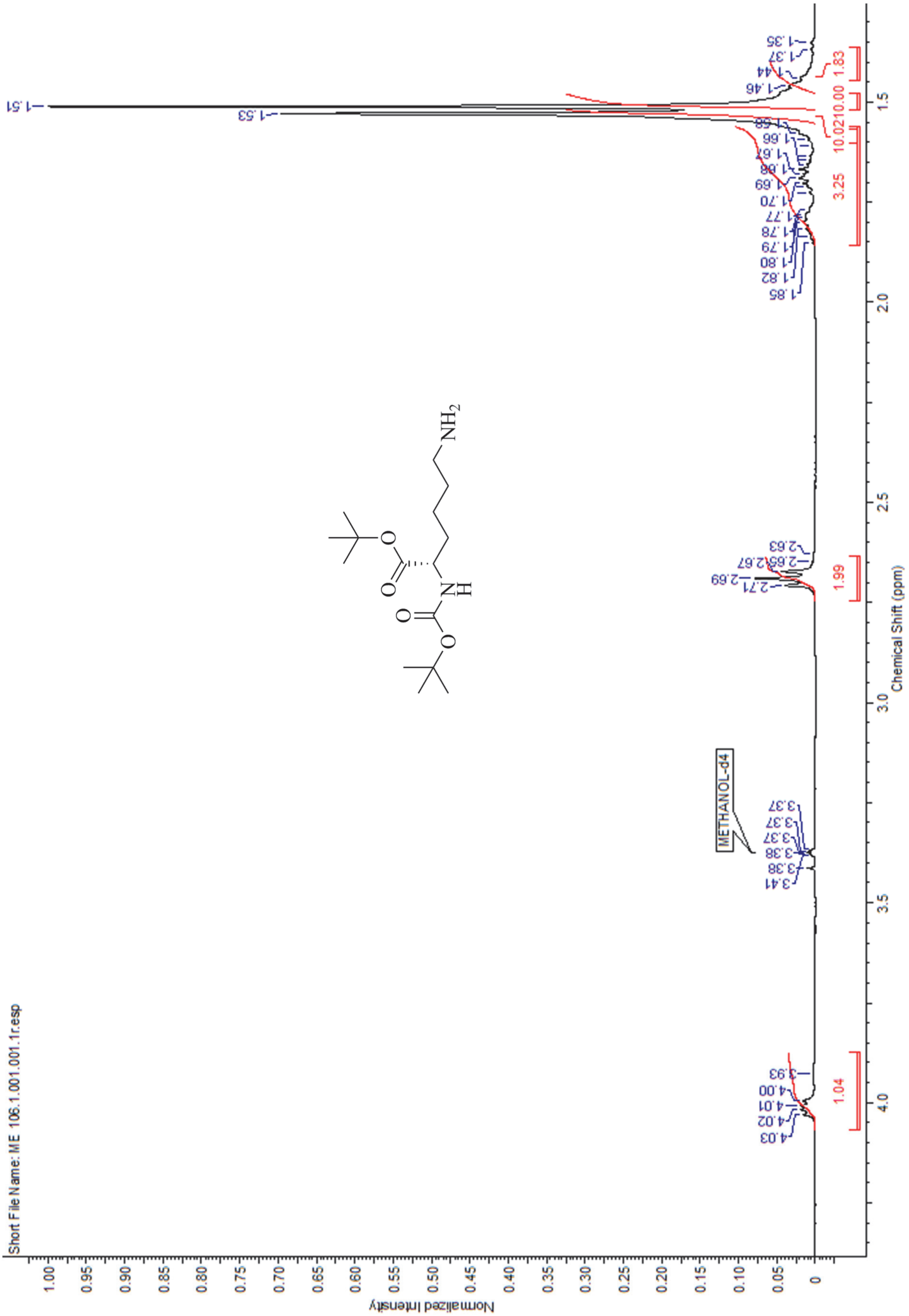


Figure 91 ¹H NMR spectrum of *N^α*-Boc-Lys-OtBu, 400 Mhz

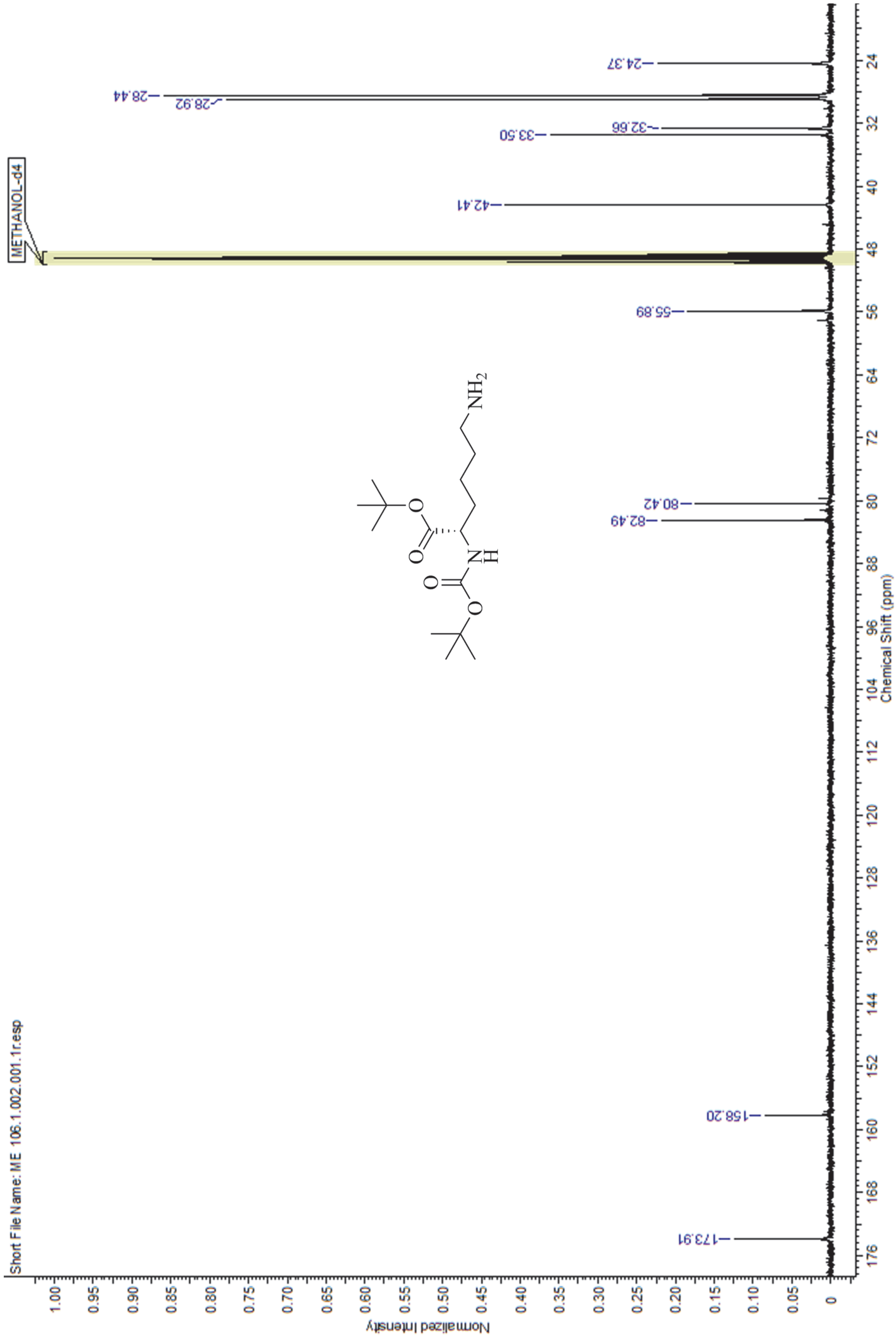


Figure 92 ^{13}C NMR spectrum of *N$^{\alpha}$*-Boc-Lys-OtBu, 100 Mhz

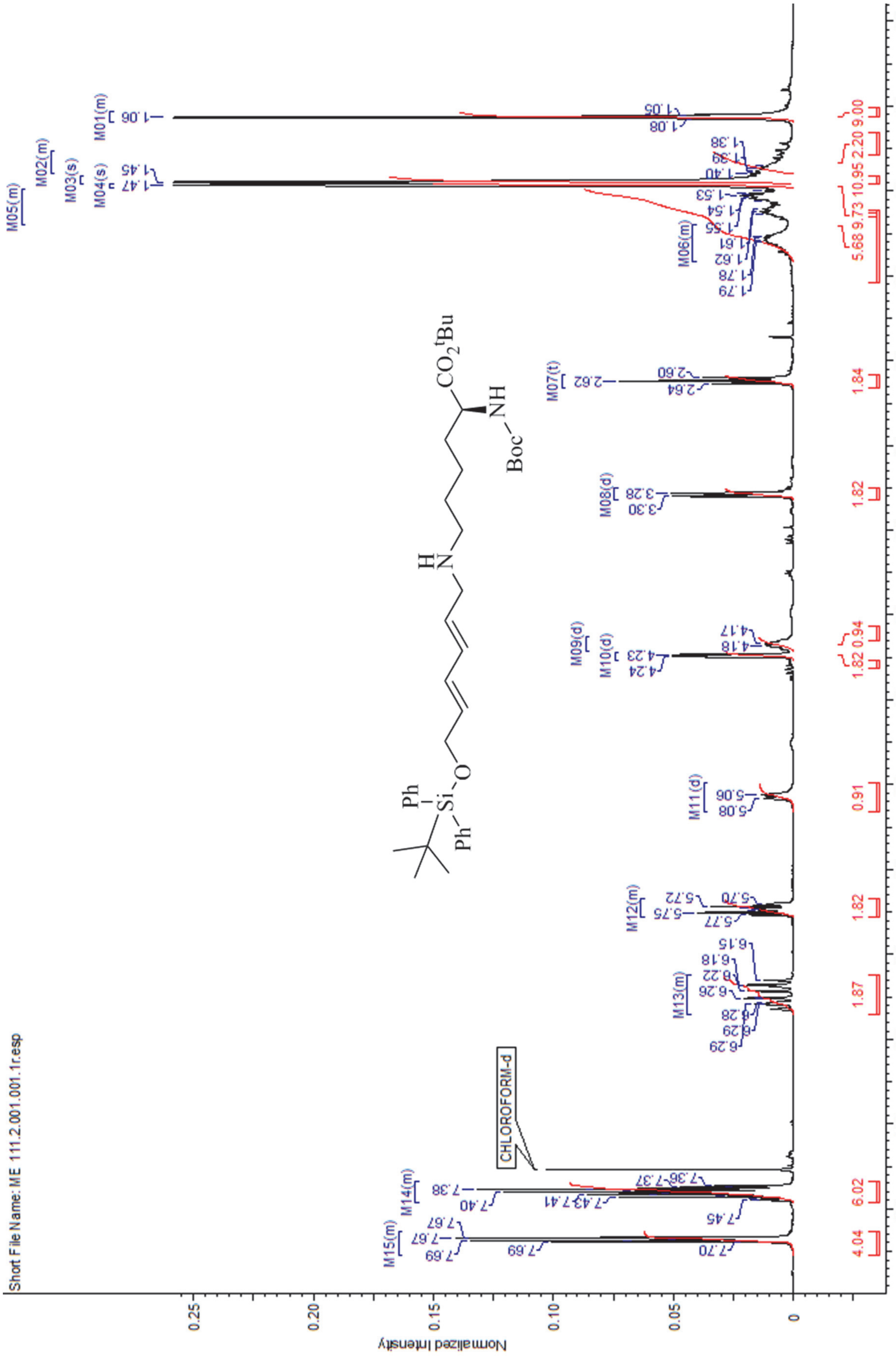


Figure 93 ^{1}H NMR spectrum of tert-butyl 2-((tert-butoxycarbonyl)amino)-6-((((*E*,*E*)-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate, 400 Mhz

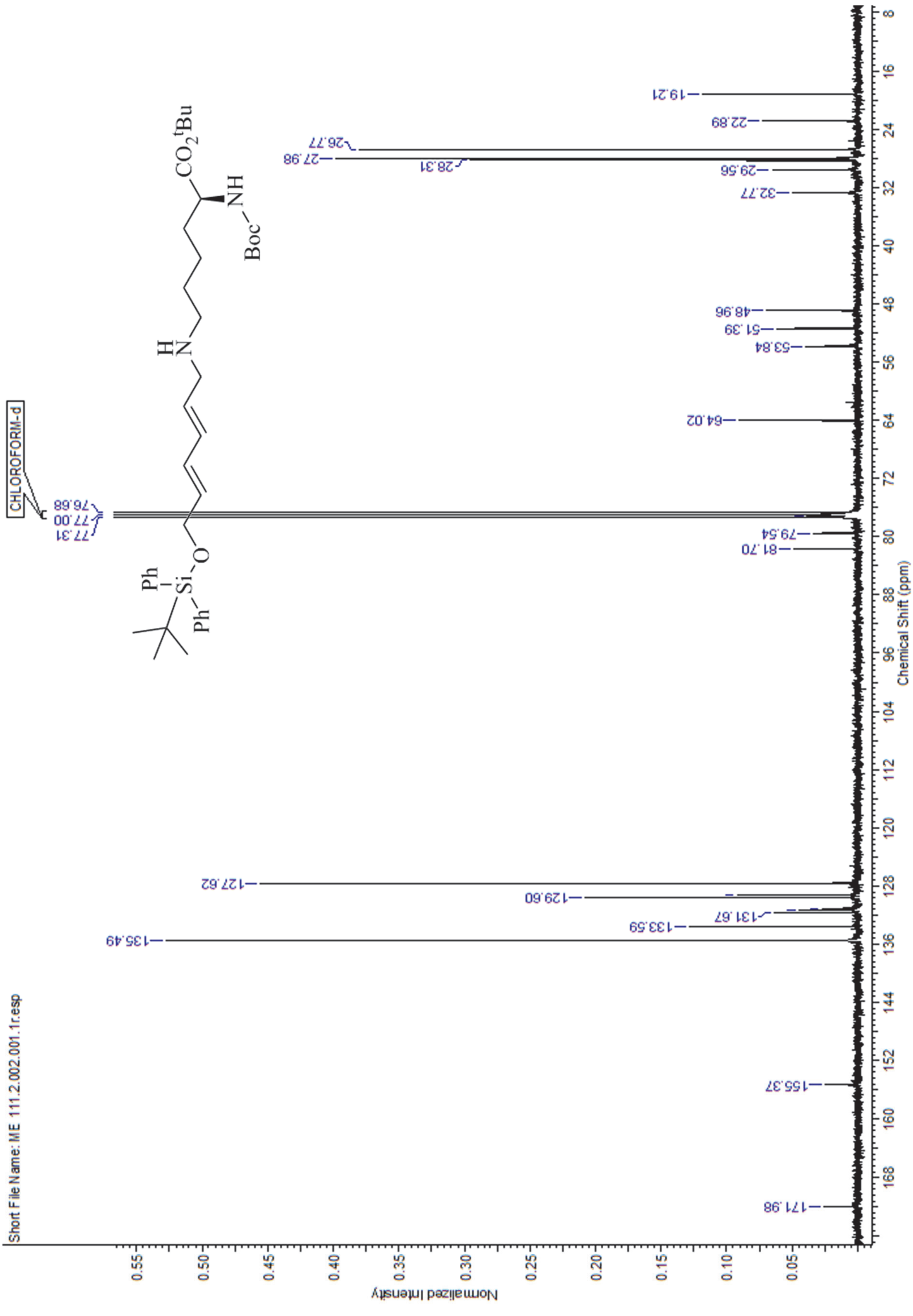


Figure 94 ^{13}C **NMR spectrum of tert-butyl 2-((tert-butoxycarbonyl)amino)-6-(((*E,E*)-6-((tert-butyldiphenylsilyl)oxy)hexa-2,4-dien-1-yl)amino)hexanoate, 100 Mhz**

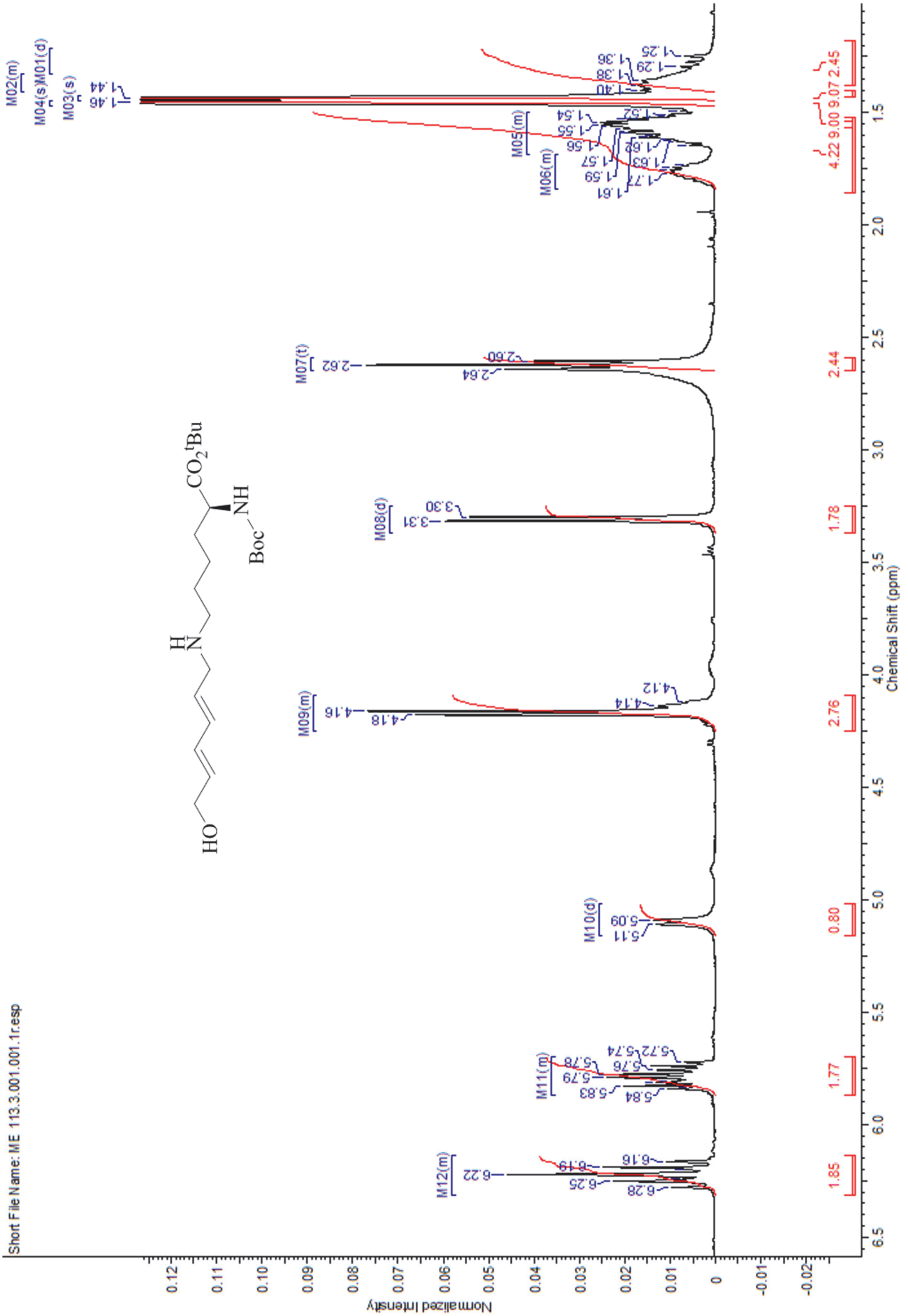


Figure 95 ^{1}H NMR spectrum of (*S*)-tert-butyl 2-((tert-butoxycarbonyl)amino)-6-(((*E*,*E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate, 400 Mhz

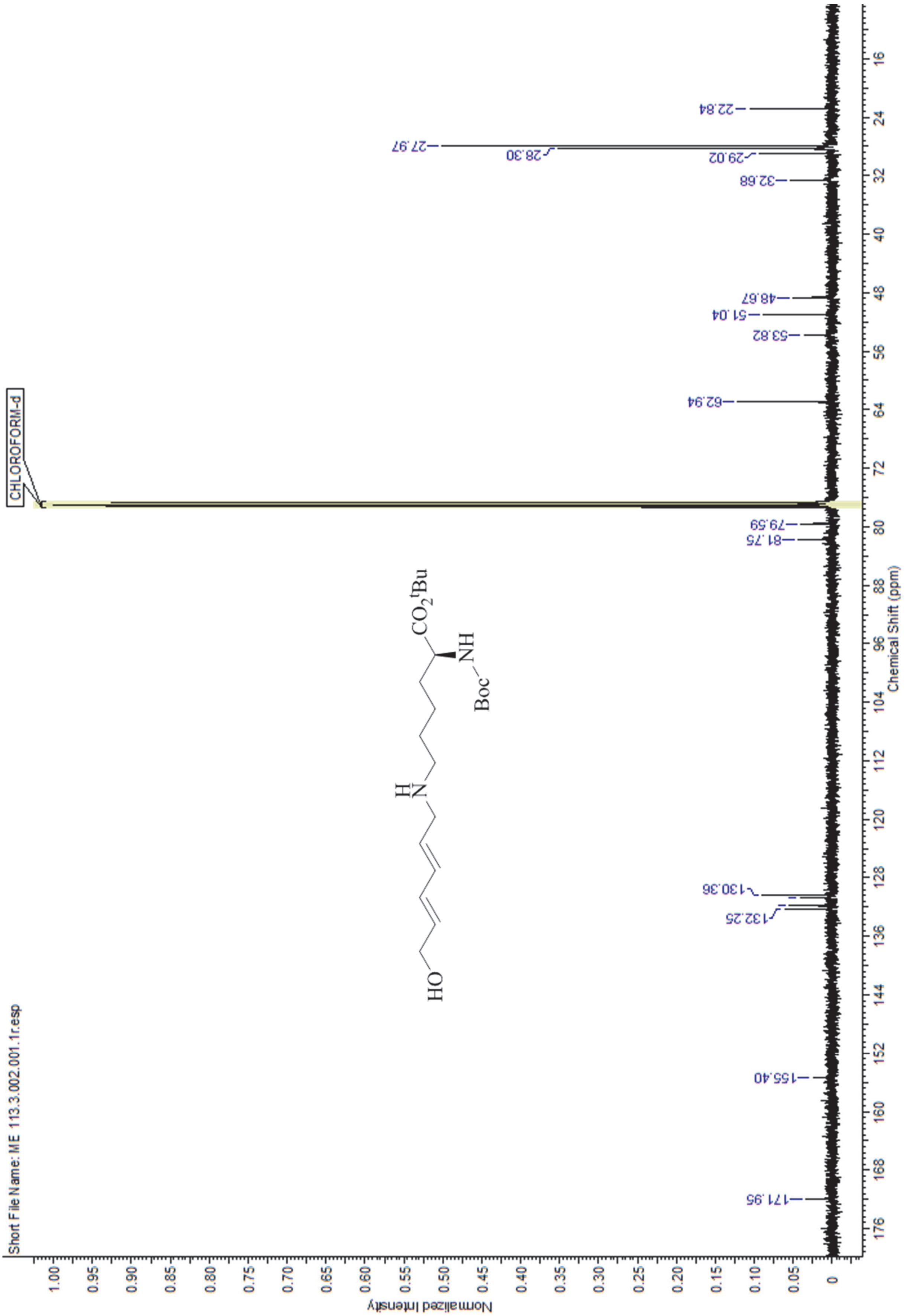


Figure 96 ^{13}C NMR spectrum of (*S*)-tert-butyl 2-((tert-butoxycarbonyl)amino)-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate, 400 Mhz

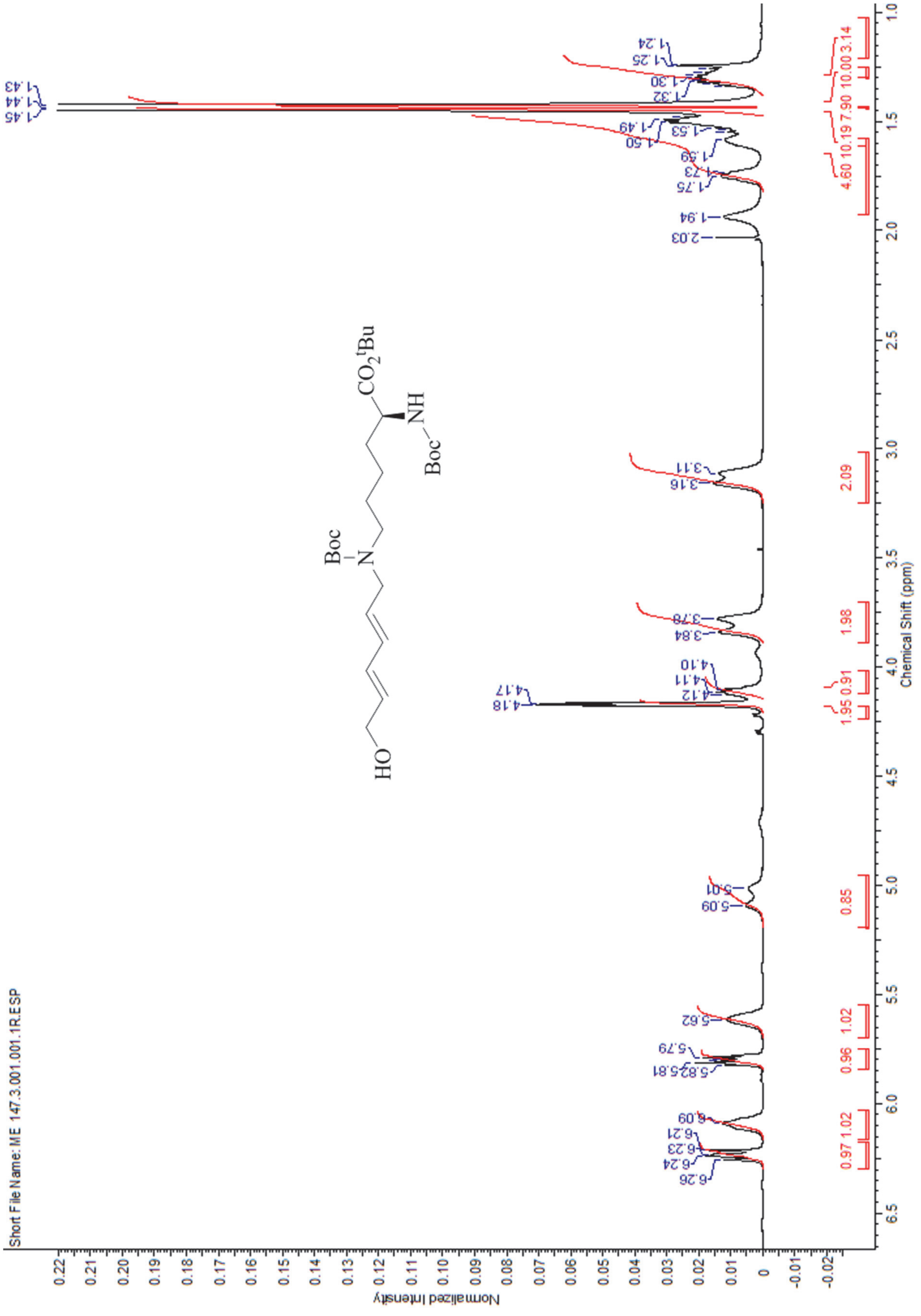


Figure 97 ^{1}H NMR spectrum of (*S*)-tert-butyl 6-((tert-butoxycarbonyl)((*E*,*E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoate, 600 Mhz

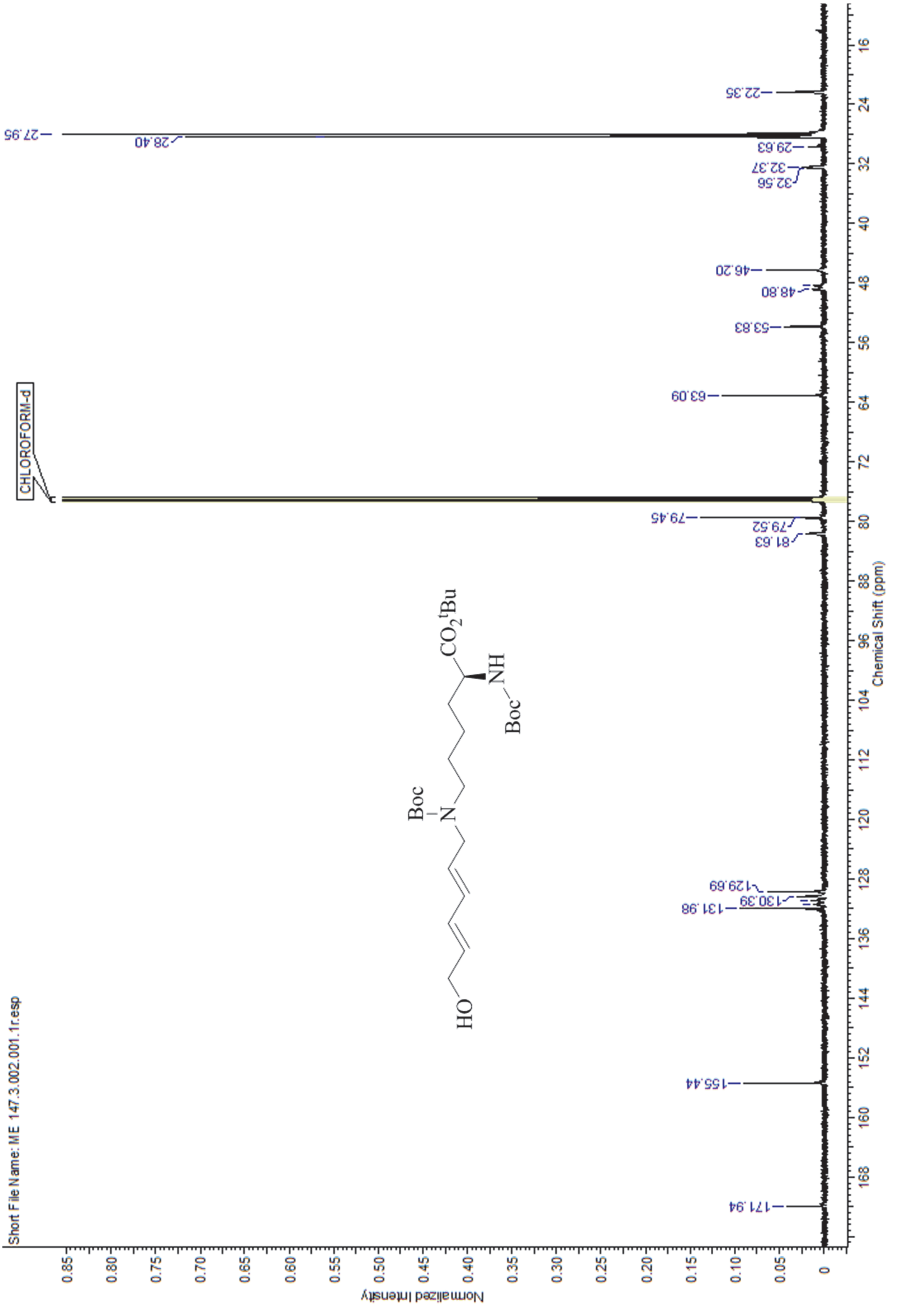


Figure 98 ^{13}C NMR spectrum of (*S*)-tert-butyl 6-((tert-butoxycarbonyl)((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoate, 150 Mhz

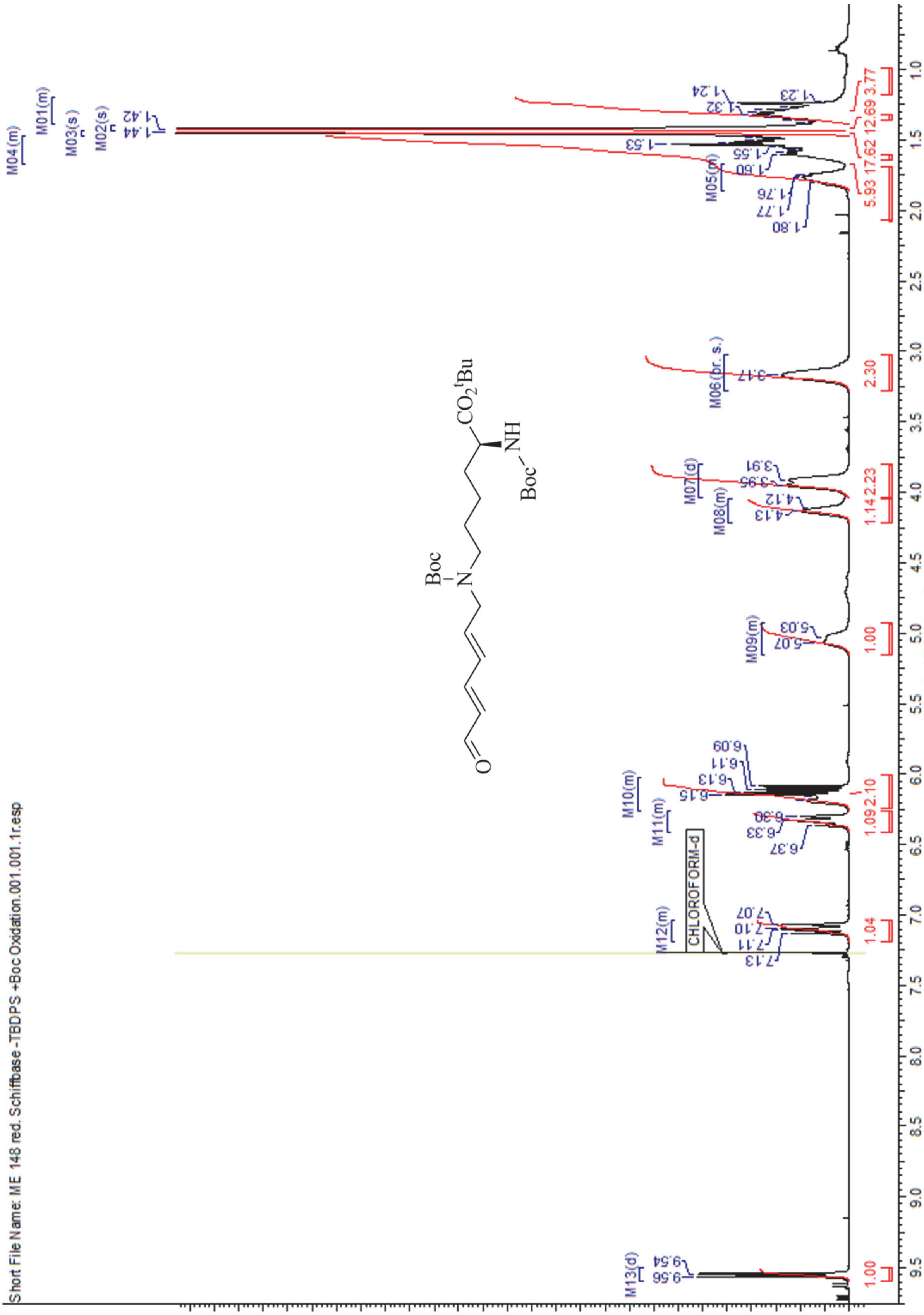


Figure 99 ^{1}H NMR spectrum of (S)-tert-butyl 6-((tert-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoate, 400 Mhz, residual solvent signal removed

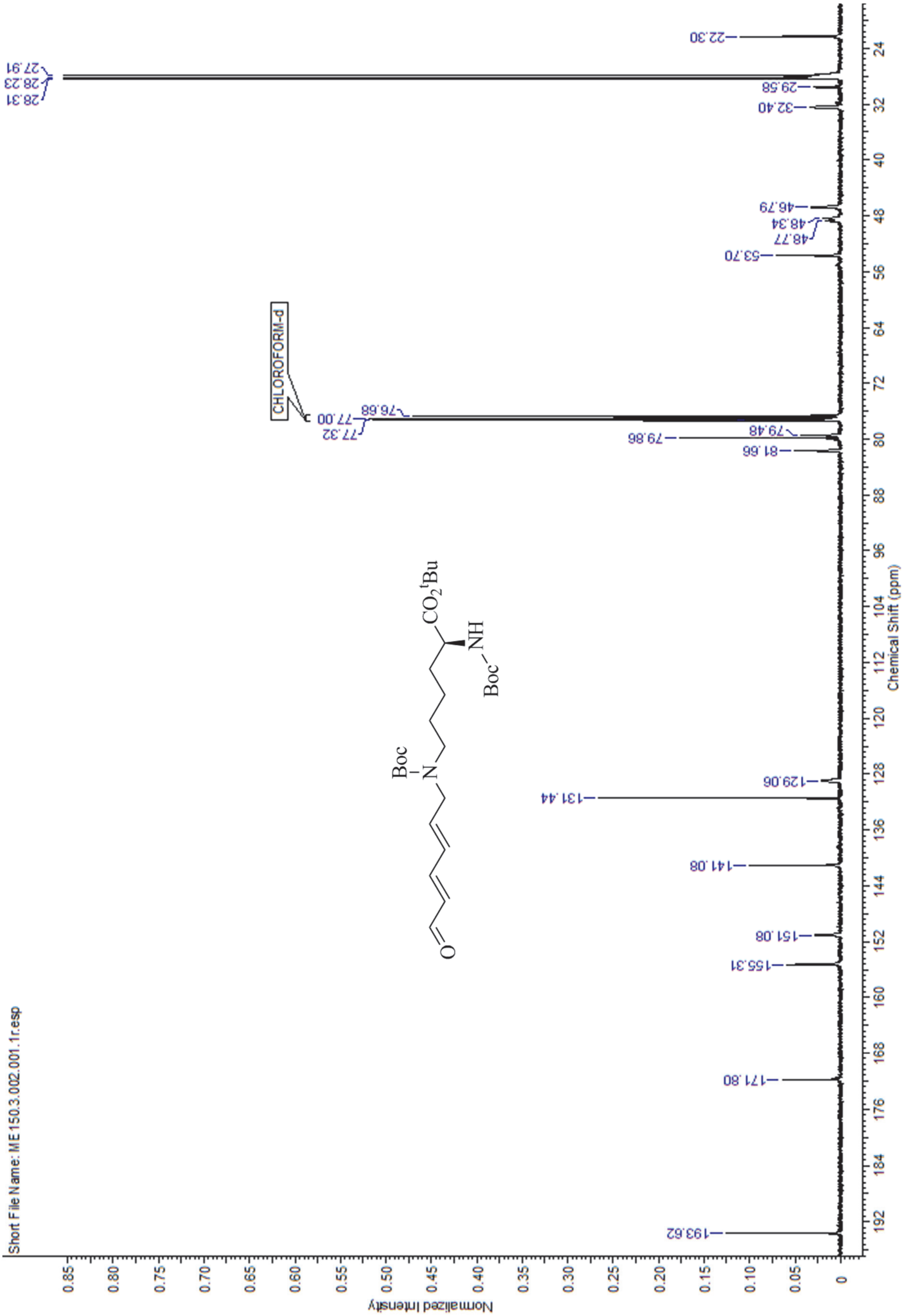


Figure 100 ^{13}C NMR spectrum of (S)-tert-butyl 6-((tert-butoxycarbonyl)((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)-2-((tert-butoxycarbonyl)amino)hexanoate, 100 Mhz, residual solvent signal removed

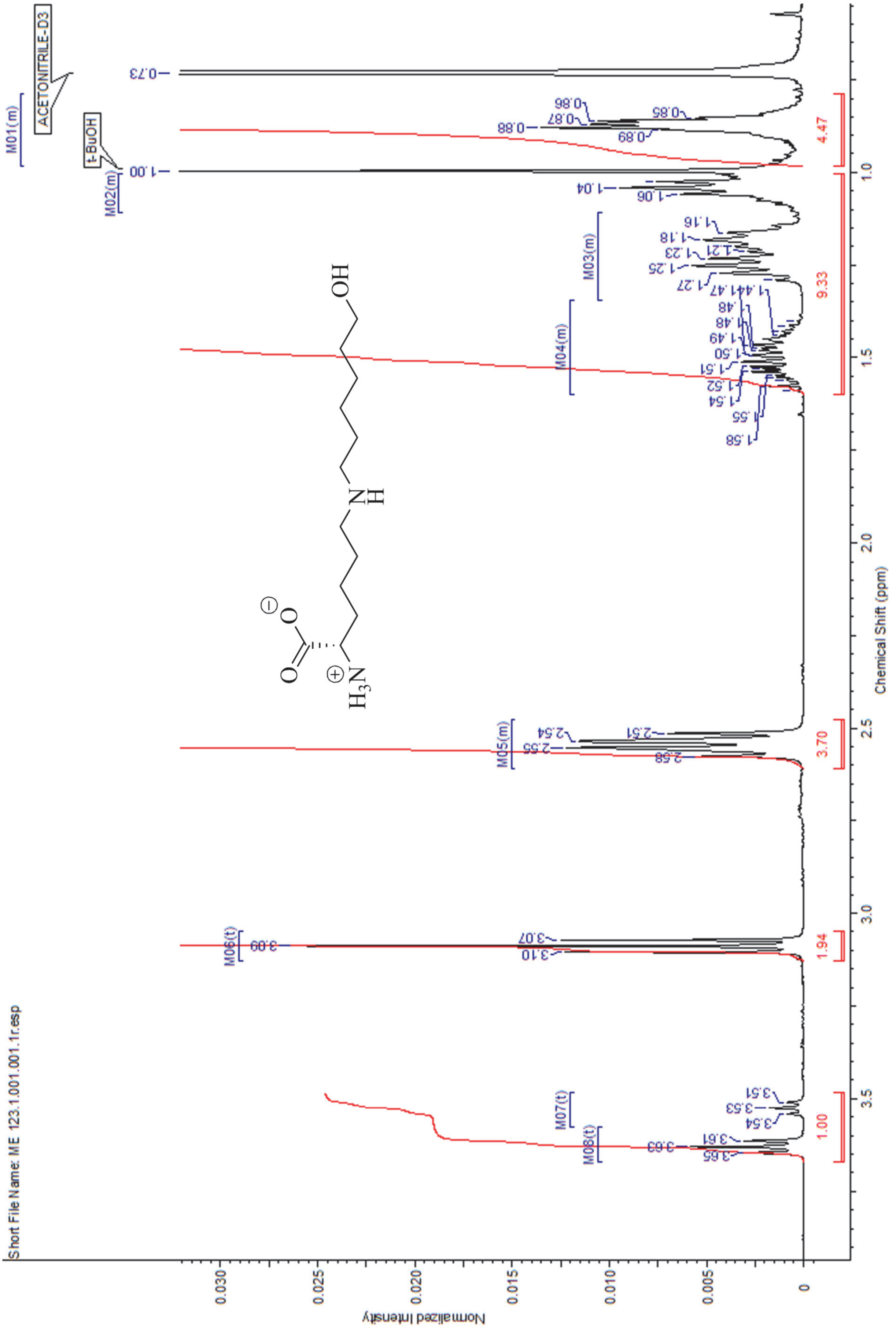


Figure 101 ^{1}H NMR spectrum of (S)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS), 400 Mhz

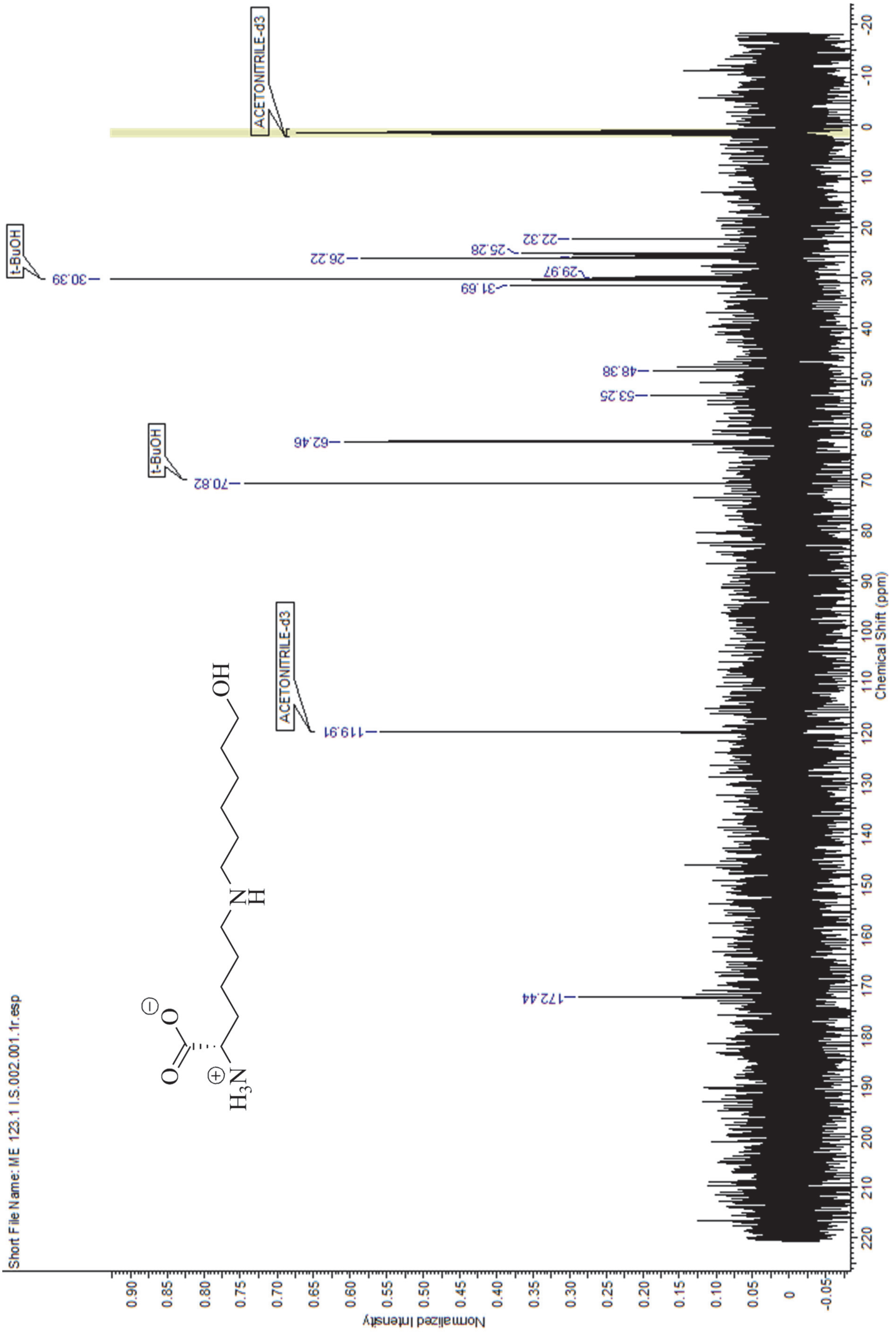


Figure 102 ^{13}C NMR spectrum of (S)-2-ammonio-6-((6-hydroxyhexyl)amino)hexanoate (IS), 100 Mhz

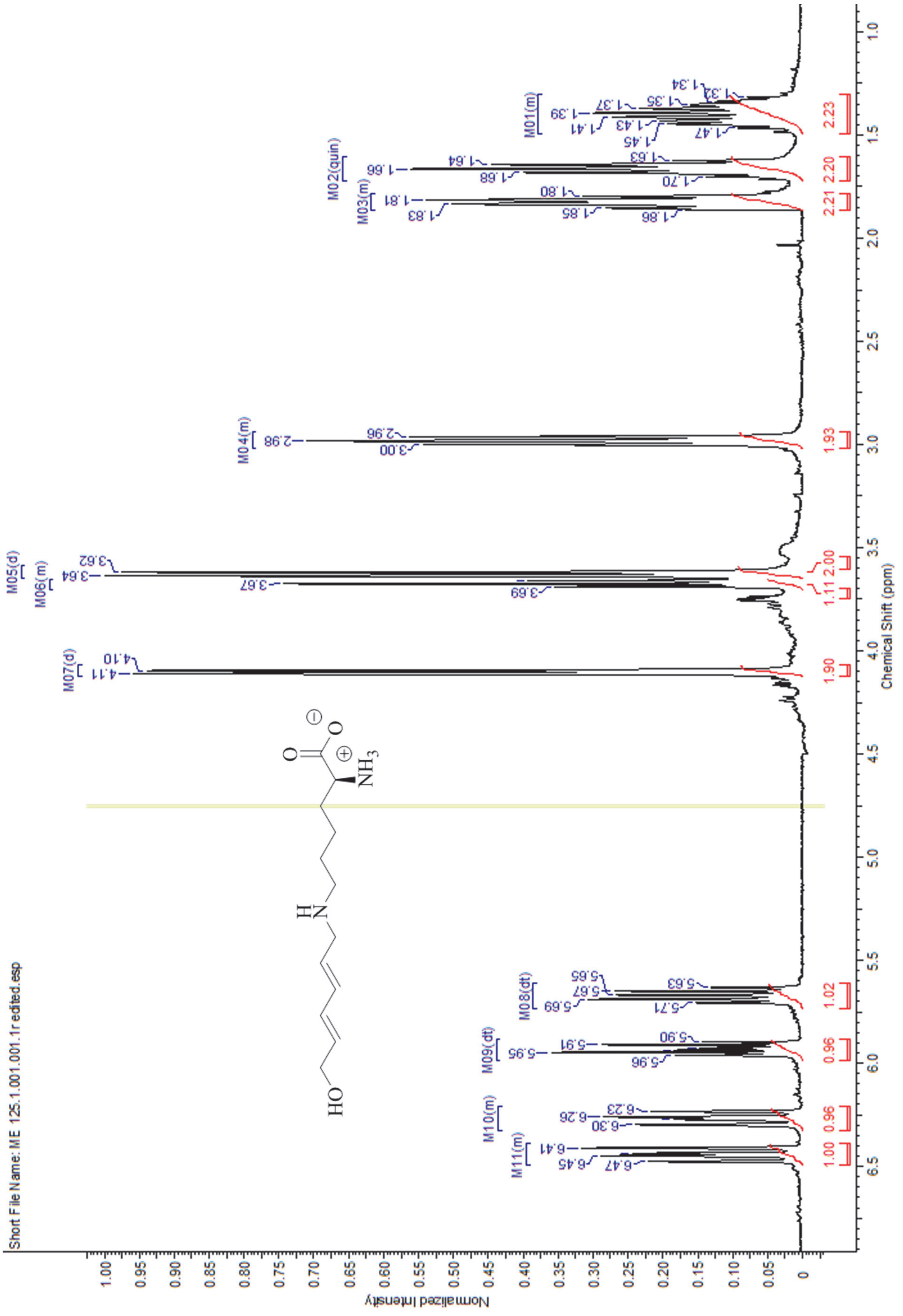


Figure 103 **^{1}H NMR spectrum of (S)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate (Lys(MUC-OH)), 400 Mhz, residual solvent signals removed**

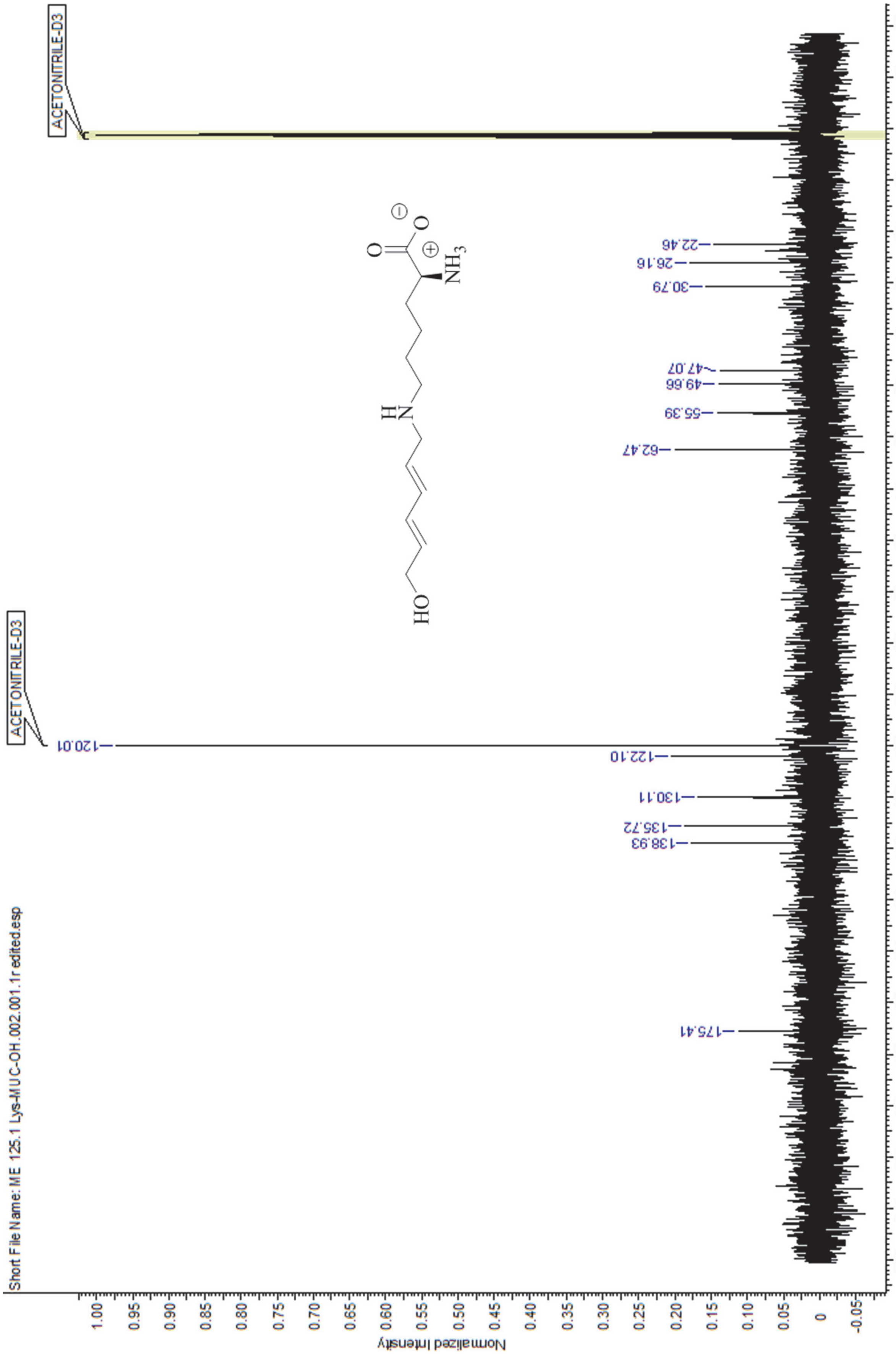


Figure 104 ^{13}C NMR spectrum of (S)-2-ammonio-6-(((*E,E*)-6-hydroxyhexa-2,4-dien-1-yl)amino)hexanoate, 100 Mhz, residual solvent signal removed

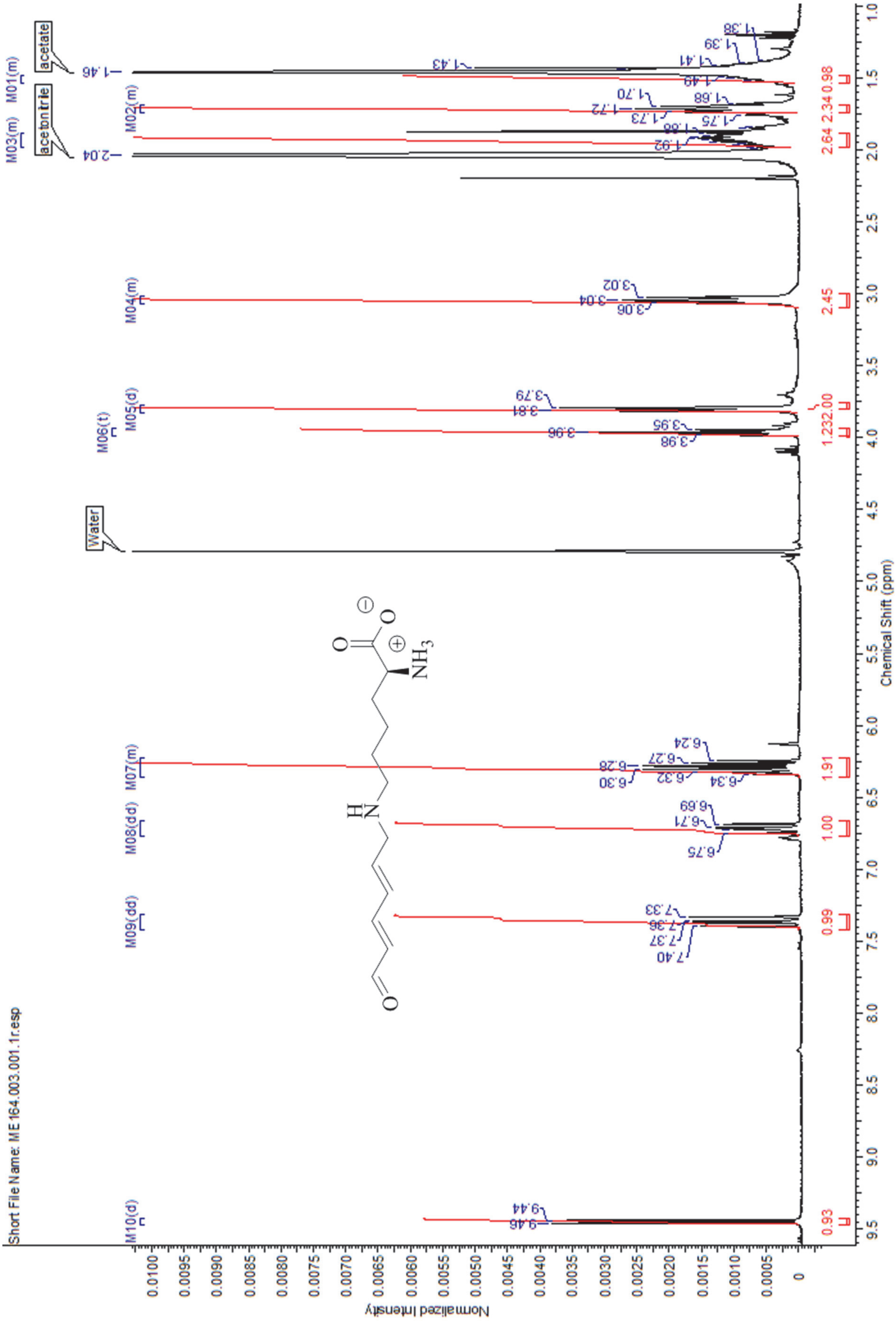


Figure 105 ^{1}H NMR spectrum of (S)-2-amino-6-(((*E,E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoic acid, 400 Mhz, residual solvent signal removed

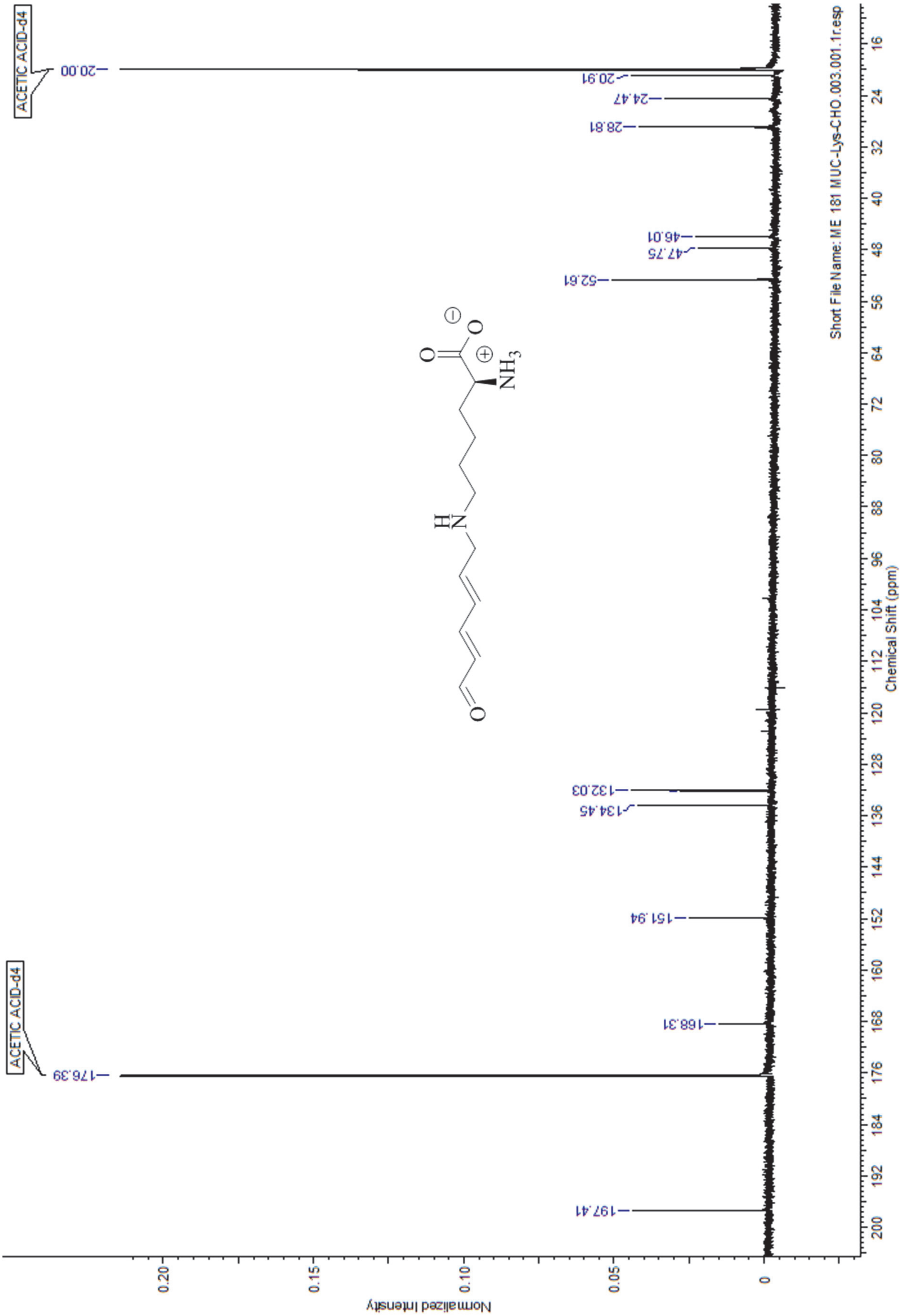


Figure 106 **^{13}C NMR spectrum of (*S*)-2-amino-6-(((*E*,*E*)-6-oxohexa-2,4-dien-1-yl)amino)hexanoic acid, 150 Mhz, residual starting compound signals removed**

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10. CURRICULUM VITAE

Surname	Emanuel	
First name	Matthias Stefan	
Studies	01.10.2009 – 31.03.2014	Study of food chemistry at University of Technology, Kaiserslautern (GER) (Final grade 1.3)
	14.01.2013 – 15.04.2013	Research internship at Gdánsk University of Technology (PL) as ERASMUS exchange student
Doctorate	01.04.2014 – 30.09.2017	Doctorate at the department of chemistry at University of Technology, Kaiserslautern (GER). Supervision by Prof. Dr. Dr. Dieter Schrenk (Final grade *sehr gut*)

Further skills: English, Latin, Old Greek, Basic French