UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SCIENZE CHIMICHE

Corso di Laurea Magistrale in Chimica

TESI DI LAUREA MAGISTRALE

Synthesis and Reactivity of New Hydroxamates of Pterostilbene for Biomedical Applications

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... Ha viaggiato sospinto dal vento per otto anni:
ora in alto, ora in basso, sul mare e tra le nuvole,
sulle foreste, i deserti, le distese senza limite dei ghiacci;
poi una volta catturato, ha dato inizio
alla sua avventura nel mondo organico.
L'atomo del quale stiamo parlando è stato portato
dal vento lungo un filare di viti. Ha avuto il fortunato
destino di sfiorare una foglia, di penetrare al suo interno
e di essere qui fissato da un raggio di sole.

[Primo Levi, Il sistema periodico]

Riassunto

In questo lavoro di tesi si è voluto studiare la possibilità di utilizzare la funzione idrossamica per la protezione reversibile del gruppo ossidrilico in composti fenolici naturali: -OH → -O-N(R_1)COR_2.

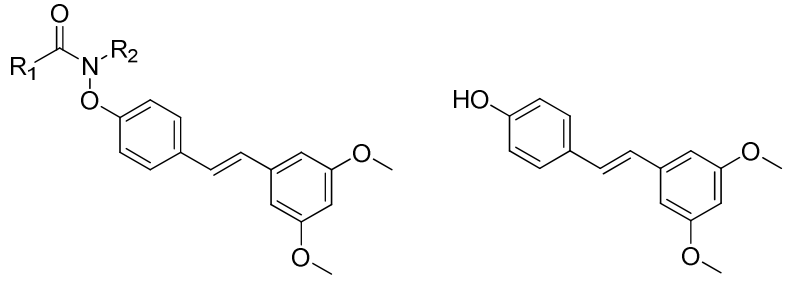
É noto che molti composti fenolici manifestano interessanti bioattività *in vitro* che possono trovare applicazione in campo farmaceutico. Tuttavia la biodisponibilità di questi composti è limitata dalle modificazioni della funzionalità fenolica durante il metabolismo di fase II in seguito a somministrazione orale.

È stata adottata la strategia del precursore farmaceutico per la protezione del sito soggetto a metabolizzazione al fine di incrementare l'assorbimento del farmaco.

La molecola modello del progetto di tesi è lo pterostilbene, un fenolo di origine naturale con grande potenziale per applicazioni in ambito farmaceutico.

Il lavoro di tesi è stato svolto in parte presso i laboratori della Dr.ssa Sabine Amslinger dell' Istituto di Chimica Organica dell' Università di Regensburg nell'ambito del progetto Erasmus.

Sono stati sintetizzati, purificati e caratterizzati, mediante spettrometria di massa e spettroscopia NMR ^{1}H e ^{13}C, undici nuovi derivati dello pterostilbene (Figura 1).



R_1 = *tert*-butoxy, OH, *tert*-butyl
R_2 = H, C(O)$CH_2OCH_2CH_2OCH_3$,
C(O)CH_3,C(O)$CH_2O(CH_2CH_2O)_2CH_3$,
Boc-(L)-Ala-CO,Boc-(L)-Ile-CO, CH_3

Pterostilbene

Fig. 1 *Pterostilbene and hydroxamate derivatives synthesized*

E' stato effettuato uno studio della reattività di questi derivati in soluzioni acquose a pH corrispondenti a quelli dei comparti biologici interessati alla somministrazione orale di farmaci (stomaco e primo tratto intestinale). La velocità di idrolisi ed i prodotti ottenuti dipendono molto dalla natura dei sostituenti R_1 ed R_2. Sono stati

individuati alcuni intermedi di reazione e proposti meccanismi di reazione anche riguardanti equilibri di isomeria E, Z. Alcuni dei composti sintetizzati presentano elevata stabilità sia in soluzioni fortemente acide (stomaco) che neutre (intestino) e si prospettano quindi come interessanti candidati per saggi più specifici. Sono attualmente in corso presso i laboratori del Dr. Mario Zoratti dell'Istituto di Neuroscienze del CNR prove di stabilità in sangue e studi *in vivo* mediante farmacocinetiche dopo somministrazione orale in ratti.

Summary

In this thesis work I explored the feasibility of using the hydroxamic functionality for the reversible protection of the hydroxy group of natural polyphenols: -OH → -O-N(R_1)COR_2.

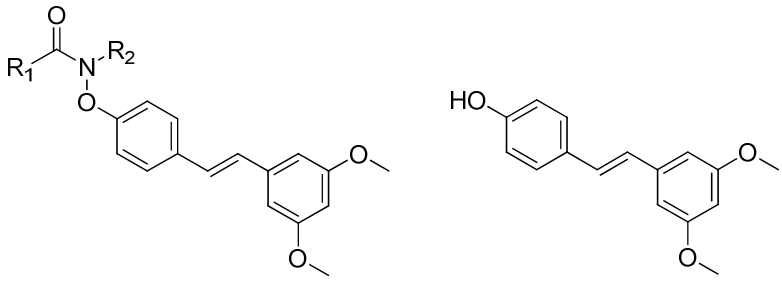
Many phenolic compounds exhibit, *in vitro*, interesting bioactivities with potential applications in human health care. However their bioavailability after ingestion is limited by the modifications of the phenolic functionality introduced by the enzymes of phase II metabolism.

The prodrug strategy has thus been adopted to protect the sensitive sites and to increase the absorption of the active molecule.

As model compound for the project I have used pterostilbene, a natural phenol with a vast potential for pharmaceutical applications.

The thesis work has been performed in part in the laboratory of Dr. Sabine Amslinger of the Institute of Organic Chemistry of the University of Regensburg within the Erasmus program.

Eleven new derivatives of pterostilbene (Figure 1) have been synthesised, purified and characterized by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy.



R_1 = *tert*-butoxy, OH, *tert*-butyl
R_2 = H, C(O)$CH_2OCH_2CH_2OCH_3$, C(O)CH_3,C(O)$CH_2O(CH_2CH_2O)_2CH_3$, Boc-(L)-Ala-CO,Boc-(L)-Ile-CO, CH_3

Pterostilbene

Fig. 1 *Pterostilbene and hydroxamate derivatives synthesized*

A study has been carried out of the reactivity of these derivatives in aqueous solution at pH values close to those of the body compartments involved in the absorption of orally administered drugs (stomach and first intestinal tract). The rate of hydrolysis and what products are obtained strongly depend on the nature of the R_1 and R_2 substituents. Some reaction intermediates have been identified and reaction

mechanisms proposed, regarding also E, Z isomerism equilibria. Some of the compounds obtain show considerable stability both in strongly acidic (stomach-like) and neutral (intestine-like) solutions, and are therefore interesting candidates for more elaborated assays. Stability tests in blood and pharmacokinetic studies *in vivo* (rat) are currently under way in the laboratory of Dr. M. Zoratti of the CNR Institute of Neuroscience.

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Introduction

The major aim of scientific research in pharmaceutical chemistry is the synthesis and study of drugs, or in other words substances applied in therapy.

A drug is an external substance, synthetically or naturally made, which issues a functional modification, positive or negative, through an interaction in a living being.

Polyphenols belong to a wide family of compounds, which are plant-derived and are well-known for their interesting pharmaceutical activities.

Pterostilbene is the model molecule chosen for this project and is a natural compound produced by some plants. It is a phenol and is known to afford favourable biomedical activities, including anti-inflammatory, antineoplastic, anti-oxidant and anti-diabetic effects.

Unfortunately, these potential bioactivities are not fully displayed *in vivo* because the bioavailability by oral administration is low. The inefficient adsorption and effective metabolizing of phenolic function does not allow to achieve a sufficient plasma level to execute the potential bioactivities.

The aim of this work is the synthesis and study of pterostilbene derivatives to be used as potential prodrugs.

Prodrug strategy is based on a drug derivatization to an inactive compound, which needs a chemical or enzymatic process to regenerate the active drug. In our case the major goal is to avoid modification by phase II metabolism by protecting the phenolic function. The promoiety must allow absorption and be bioreversible.

The project is focused on a new class of pterostilbene derivatives, where the phenolic group is derivatized by the hydroxamate function: -OH → -O-N(R_1)COR_2.

Substituents groups R_1 and R_2 are able to tune the chemical features and modulate the chemical stability of the N-O bond.

At this preliminary stage of the studies on protective function, different R substituent groups were developed.

The derivatives were synthesized, purified and characterized by ESI mass spectrometry and by ^{1}H NMR and ^{13}C NMR to the laboratory of Dr. Sabine Amslinger of Organic Department of the University of Regensburg.

Tests of stability in vitro and in solutions mimicking the gastrointestinal environment were developed at the laboratory of Dr. Mario Zoratti of the CNR Institute of Neuroscience.

Stability assays in whole rat blood, and *in vivo* pharmacokinetics of pterostilbene hydroxamates are still in progress.

Chapter 1 provides an overview on pterostilbene and the problem of low bioavailability, followed by a description of prodrug strategy as a potential solution of the problem. The synthesis of derivatives is fully described in Chapter 2. The results on stability assays and the discussion about the reactivity are reported in Chapter 3.

Finally, the experimental procedures and characterizations are described in Chapter 4.

CHAPTER 1

Pterostilbene and Prodrug design

This chapter contains a description of pterostilbene, an interesting natural compound with various potential pharmaceutical applications. A brief introduction on the general properties of this molecule is presented, followed by the explanation of the problem of its low bioavailability and the applied strategy to overcome it by prodrug design.

1.1 Pterostilbene

4-[(*E*)-2-(3,5- dimethoxyphenyl)ethenyl]phenol, by IUPAC convention, is commonly known as pterostilbene (Fig. 2).

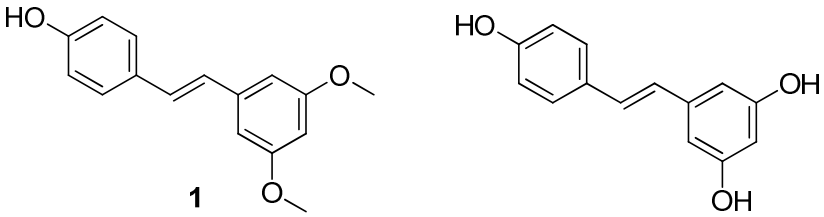


Fig. 2 *Pterostilbene (3,5-dimethoxy-4'-hydroxy-trans-stilbene) (**1**) and resveratrol (3,5,4'-trihydroxy-trans-stilbene)*

Pterostilbene is a stilbenoid, chemically related to resveratrol and classified as a benzylidene compound, which biochemically belongs to the family of phenylpropanoids.[1] It is a phytoalexin, which are antimicrobial and antifungine compounds produced by plants in response to infection.[2] Several molecules of the stilbenoid family are produced by plants in response to environmental challenges such as viral, microbial and fungal infections or excessive ultraviolet exposure.

Together with resveratrol, it has been isolated from several natural plant sources, notably blueberries; it has been identified also in *Vitis Vinifera* leaves, in infected as well as healthy and immature grape berries (Chardonnay, Gamay, Pinot Noir).[3] Pterostilbene has not been detected in wine[4], but it was found to be one of the active constituents in extracts of the heartwood of *Pterocarpus marsupium*.[5]

Chemically, pterostilbene is similar to resveratrol and, as this well-investigated counterpart, shows a range of powerful bioactivities, limited by a still unsatisfactory bioavailability; nonetheless, methylation of phenolic functions on 3,5 positions of resveratrol improves the transport into cells and increases the metabolic stability of the stilbenoid.

Bioavailability, pharmacokinetics, and metabolism of these phenols via oral dosing in rats have been compared, and the results suggests that the *in vivo* biological activity of equimolar doses of pterostilbene may be greater than that of resveratrol.[6]

The pharmacological activities of pterostilbene are summarized as anti-inflammatory, antineoplastic, and antioxidant actions via modulations of gene expression and enzyme activity, as discussed in some more detail below.[7] Furthermore, its pharmacological actions may alleviate diseases associated with oxidative damage, inflammation, aging, dyslipidemia and viral infection.

Antimicrobial effect

Pterostilbene exhibits potent antifungal properties that are 5-10 times stronger than those of resveratrol. It also exhibits antiviral effects. This protecting action is an important function of stilbenes in plants, and it may extend to humans and animals as well.[7]

Antineoplastic effect

The anticancer effect is exhibited through various molecular mechanisms.[8,9,10]

Studies show the actions of pterostilbene include modulation of signal transduction pathways, cell cycle regulatory genes, cell differentiation genes, oncogenes and tumor suppressor genes.[7]

An example of anticancer effect by inhibition of catalytic activity is shown on human recombinant cytochromes P450 CYP1A1. CYP1A1 and CYP1B1 are the inducible forms of cytochrome P450 expressed in extrahepatic tissues, which are responsible for the biotransformation of polycyclic aromatic hydrocarbons, heterocyclic amines and estradiol to carcinogenic intermediates.[11]

Antioxidant effect

Pterostilbene is able to reduce extracellular ROS.[12]

The localization of antioxidative effect allows the use of pterostilbene to target extracellular reactive oxygen species that are, among other things, responsible for tissue damage during chronic inflammation.

Anti-infiammatory effect

Pterostilbene exhibits moderate inhibition (IC_{50} = 19.8 μM) of cyclooxygenase (COX)-1 and is weakly active (IC_{50}= 83.9 μM) against COX-2, enzymes involved in the synthesis of mediators of inflammation.[7] It furthermore decreases the levels of tumor necrosis factor-α (TNF-α; a cytokine involved in systemic inflammation), in comparison with controls.[7]

Miscellaneous effects

Studies show that pterostilbene has hypolipidemic[13] and antidiabetic properties[14], and may be efficient in reversing the deleterious effects of aging such as cognitive function and working memory.[7,15]

1.2 Bioavailability of pterostilbene

Polyphenols are present in food as glycosylated derivatives. In blood, however, glycosylated derivatives are not found even in trace amounts, which indicate that hydrolysis of glycosides takes place during absorption.

Pterostilbene has a relatively poor bioavailability due to a built-in propensity to phase II metabolism. It is subjected to “detoxification”, consisting in the covalent modification of the hydroxyl groups by the sulfo-transferases (SULTs) and glucuronosyl-transferases (UGTs) of enterocytes and hepatocytes. Enzyme-catalyzed reactions are reported in Figure 3 and Figure 4. Sulfate and glucuronide metabolites are more soluble than their parent compounds and their elimination occurs via renal and biliary routes.[6]

Studies of *in vitro* metabolism in rat liver microsomes, and the detection of pterostilbene glucuronidated and sulfated metabolites in both serum and urine confirm a phase II metabolism of pterostilbene.[17]

When administered orally, pterostilbene shows a higher bioavailability than resveratrol, with higher total plasma levels of both the parent compound and metabolites. These differences in pharmacokinetics suggest that the *in vivo* biological activity of equimolar doses of pterostilbene may be greater than that of resveratrol.[6]

The dimethylether analogue to resveratrol may overcome some limitations to pharmacological efficacy.

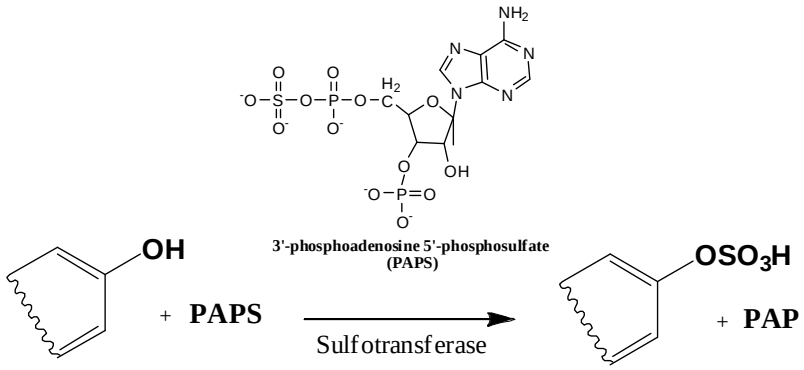


Fig. 3 *Enzyme-catalyzed sulfation of phenolic function with co-substrate 3'-phosphoadenosine-5'-phosphosulfate (PAPS)*

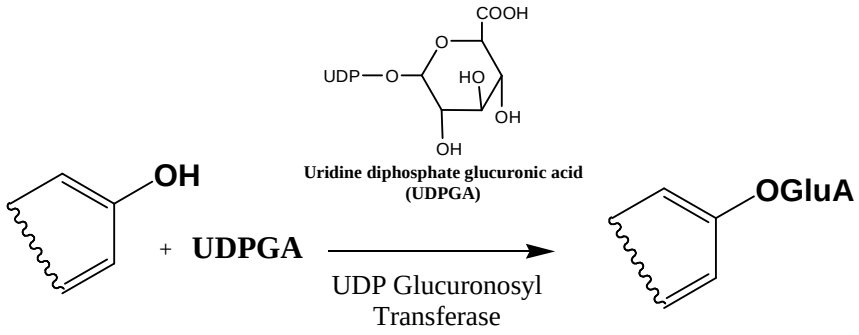


Fig. 4 *Enzyme-catalyzed glucuronidation of phenolic function with co-substrate Uridine diphosphate glucuronic acid (UDPGA)*

1.3 Prodrug strategy and prodrug design

As mentioned in paragraph 1.1, sulfate and glucuronide metabolites of pterostilbene are excreted more rapidly than their parent compounds via renal and biliary routes; they are furthermore likely to have lower bioactivity and a different set of pharmacological targets. Thus, it is desirable to limit the enzymatic conjugation of the phenolic function during phase II metabolism.

A possibility is to use the prodrug approach, widely used to enhance therapeutic efficacy and/or reduce adverse effects of drugs.

By Rautio's definition[18], prodrugs are bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation *in vivo* to release the active parent drug, which can then exert the desired pharmacological activity (Figure 5).[19]

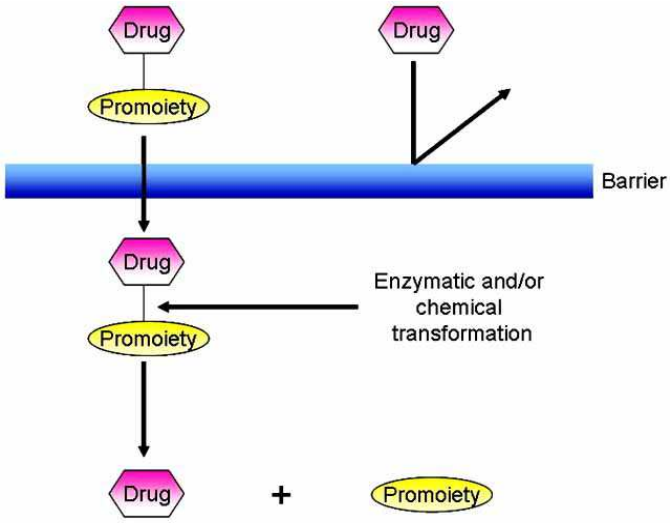


Fig. 5 *Regeneration of parent drug by enzymatic and/or chemical transformation*[19]

Prodrugs are chemicals with little (1000-times less potent than parent drug) or no pharmacological activity. In 2009, 5% to 7% of drugs approved worldwide were classified as prodrugs.

A chemical criterion helps to classify the prodrugs into four classes:[20]

- **Carrier-linked prodrugs**: Simple prodrugs which contain a covalent link between the drug and a strategically selected chemical/transport moiety or promoiety. Activation occurs by hydrolysis, oxidation or reduction.
- **Bioprecursors**: these do not contain a promoiety yet are activated by oxidation, reduction or hydrolysis.
- **Macromolecular prodrugs**: where the carrier is a macromolecule.
- **Drug–antibody conjugates**: where the carrier is an antibody.

A rational way of improving the efficiency of a drug is to manipulate the physicochemical properties by creating a prodrug with the potential to increase the rate of diffusion through biomembranes or increasing the solubility.

Another important strategy is to increase the targeting to a specific site or prolong the half-life.[19]

Increased aqueous solubility is able to overcome important factors limiting oral, transdermal, and parenteral bioavailability. Generally, charged promojeties can be used for this aim. It should be noted that the enhanced water solubility, and thus, the better oral bioavailability, can also be achieved by decreasing the crystal packing or by altering the melting point of the parent drug.

On the other hand, an increased lipophilicity is helpful for transcellular absorption, so it is necessary to balance these factors with sufficient aqueous solubility, otherwise oral bioavailability will become dissolution-limited.

Another validated concept in prodrug design is to protect the drug against rapid metabolic breakdown after activation; because the drug has to be transported via the circulation system to the target site where it carries out its mode of action.[21]

Finally, drug targeting to specific enzymes or membrane transporters is a widely used prodrugs approach and it is very efficient to achieve a high local concentration of the drug and to decrease unwanted side effects.

The prodrug strategy to drug design comprises versatile and powerful techniques which can be applied to a wide range of drug administration routes such as oral, transdermal, ocular, and parenteral applications.

Oral administration is the most common route of applying drugs, after which they are absorbed by the gastrointestinal system, attaining a measurable concentration in the circulatory stream.[19]

The fraction absorbed is largely controlled by the physico-chemical parameters of the drug or prodrug. The fraction absorbed can be increased by optimizing dissolution rate, solubility and/or membrane permeability.

1.4 State of the art and aims of the thesis

This thesis work is part of a research program addressing the problem of the low bioavailability of polyphenols (see paragraph 1.2) through the development of prodrugs. The project envisions the screening of various potential protective groups of phenol function to identify the functionality with optimal stability/reactivity features (Figure 6).

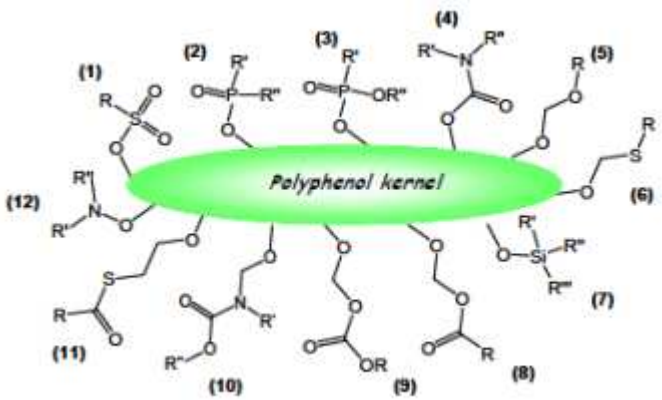


Fig. 6 *Types of potential protecting group screened in the search for an optimized linker for the protecting/functionalizing group in polyphenol prodrugs.*

Prodrugs meant for oral administration need to be stable under gastrointestinal conditions but must regenerate the natural compound in blood or other organs with appropriate kinetics (half-life in the order of several to many minutes) ("bioreversibility tuning"). Furthermore, the physico-chemical properties of the prodrug can be tailored through an appropriate choice of the substituent group(s) in order to optimize characteristics such as water solubility, membrane permeability, intestinal uptake. Some of the functionalities shown in Figure 6 have already been tested by Prof. Paradisi's research group[22,23]. The most promising one so far have is the N-monosubstituted carbamoyl linkage (number 4 in Fig. 6, with R'' = H). Its applications are being studied in other projects.

The aim of my project was to obtain model prodrug(s) containing the unusual -O-NR_1R_2 moiety (group 12 in Figure 6) and to test its suitability for prodrug construction, in particular its stability in aqueous environments. As model core compound for the study we chose pterostilbene, closely related to resveratrol and reportedly endowed with even more favourable biomedical properties.

In a preliminary phase of prodrug design information about the stability of conceivable derivatives was obtained from the literature. The simplest structures, with hydrogen and/or alkyl groups bonded to the N atom, did not offer sufficient perspectives of stability: phenoxy amine[24] and N-alkyl O-phenyl hydroxylamine[25] are classes of compounds too unstable and of too hard handling for the purposes of the project.

A common strategy to improve the stability of weak N-O bond is the introduction of a carboxyl group directly bonded to the nitrogen. N-Benzoylation is a typical reaction used to improve the stability of substituted hydroxylamines, taking advantage of the formation of a hydroxamate function. [25]

Various studies reported in the literature concern hydroxamic acids and their N-substituted esters, as well as their potential application in the pharmaceutical field, but the use of the hydroxamate function as protecting group is a recent innovation in prodrug design[26,27].

The strategy adopted for the syntheses was suggested by a recent study, which takes advantage of the irreversible activation of phenol by cleavage of a weak N-O bond by reducing nucleophiles.[27]

CHAPTER 2

Results and discussion

This chapter contains the description of the synthetic strategy and an overview on synthesized derivatives. Before that, the synthetic pathways and related problems are presented.

2.1 Synthesis

During my Thesis internship I have synthesized eleven new hydroxamate derivatives of pterostilbene (Fig. 7) with different R^1 and R^2 substituent groups as detailed in Tab. 1

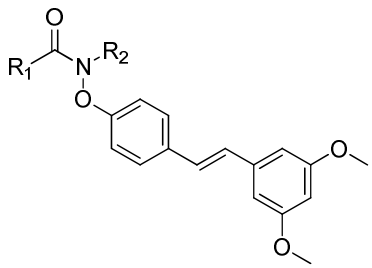


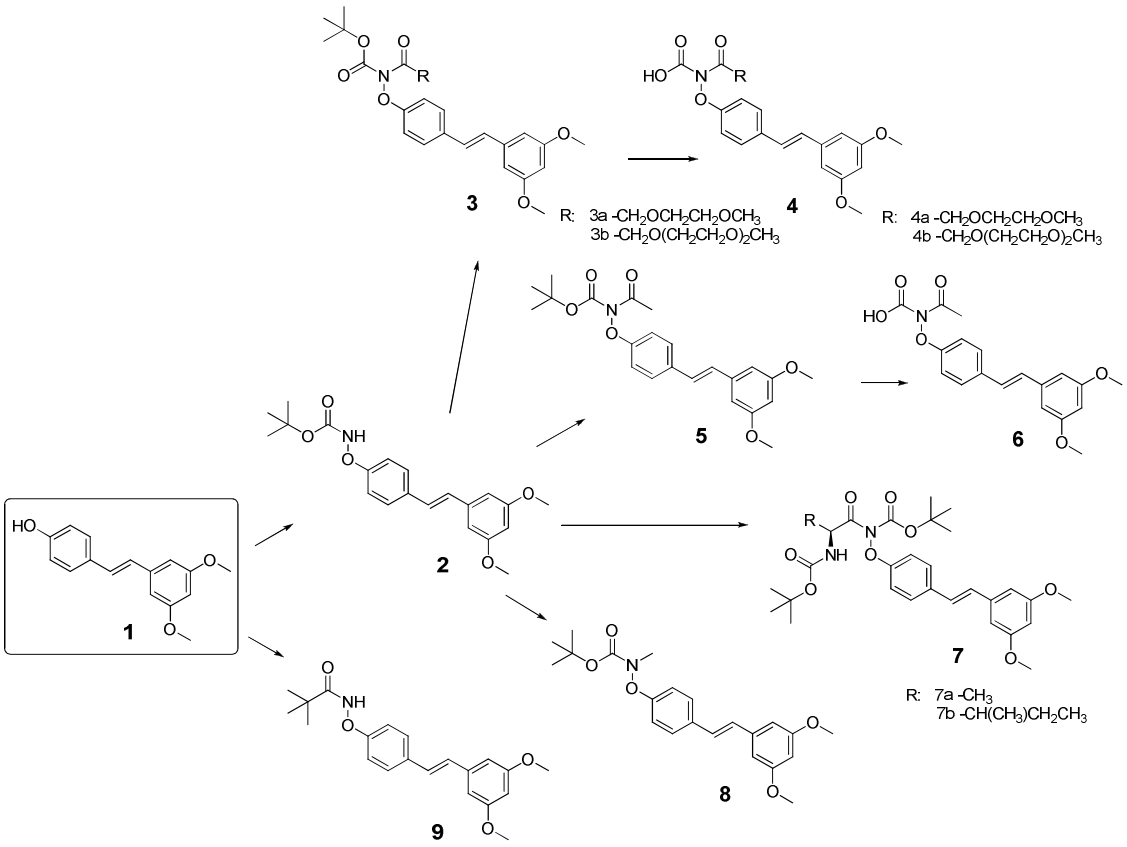
Fig 7 *Pterostilbene hydroxamate derivatives synthesized*

n.	R^1	R^2
2	*tert*-butoxy	H
3a	*tert*-butoxy	$C(O)CH_2OCH_2CH_2OCH_3$
3b	*tert*-butoxy	$C(O)CH_2O(CH_2CH_2O)_2CH_3$
4a	OH	$C(O)CH_2OCH_2CH_2OCH_3$
4b	OH	$C(O)CH_2O(CH_2CH_2O)_2CH_3$
5	*tert*-butoxy	$C(O)CH_3$
6	OH	$C(O)CH_3$
7a	*tert*-butoxy	Boc-(L)-Ala-CO
7b	*tert*-butoxy	Boc-(L)-Ile-CO
8	*tert*-butoxy	CH_3
9	*tert*-butyl	H

Tab. 1 *R_1 and R_2 substituent groups in the hydroxamate derivatives synthesized*

They will be tested as prodrugs to find out whether and under which conditions pterostilbene could be released via hydrolysis of the protecting group and regeneration of the free OH group.

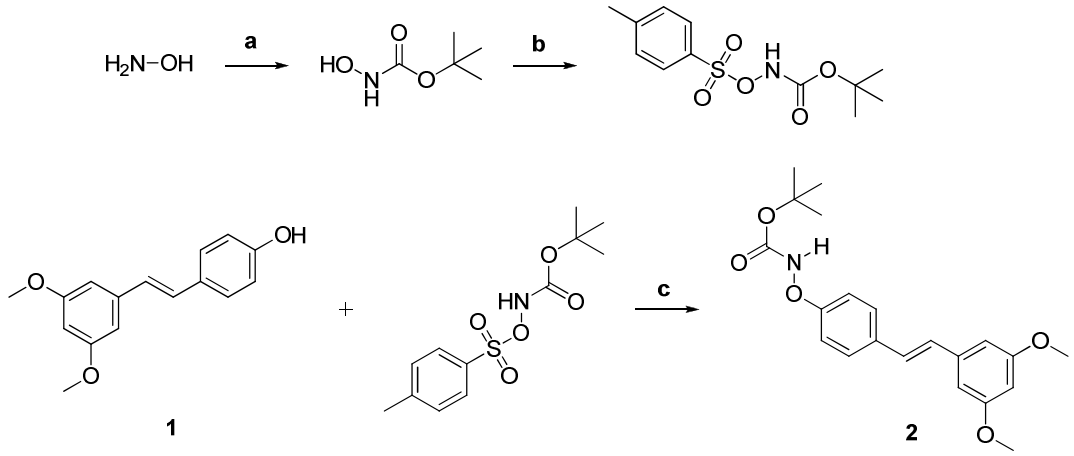
An overview of the pathways used to synthesize all of these hydroxamate derivatives starting from pterostilbene (**1**) is reported in Scheme 1. Two of these derivatives, **2** and **9**, were obtained via electrophilic amidation of pterostilbene. From **2**, then, four sets of derivatives were synthesized in which the hydrogen on the nitrogen atom is substituted by the following groups: methoxy oligoethylen glycol acetyl (**3a** and **3b**; **4a** and **4b**), acetyl (**5** and **6**), amino (**7a** and **7b**) and methyl (**8**).



Scheme 1. *General synthetic scheme to obtain the new hydroxamate derivatives*

2.1.1 Synthesis of *tert*-butyl N-pterostilbene carbamate (2)

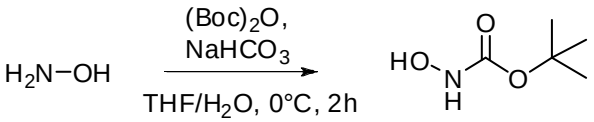
The *tert*-butyloxy hydroxamate of pterostilbene **2** was synthesized via three step synthesis as described in Scheme 2.



Scheme 2. *Synthesis of tert-Butyloxy hydroxamate of pterostilbene.*

The synthetic strategy was adapted from the literature, which reports the synthesis of N-acyl O-amino phenol derivatives of CBI-TMI (1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one), as prototype of the natural antitumor agent duocarmycin[27]. In this work it was found that the prodrug reacted to release the free drug with a half-life of 3 h.

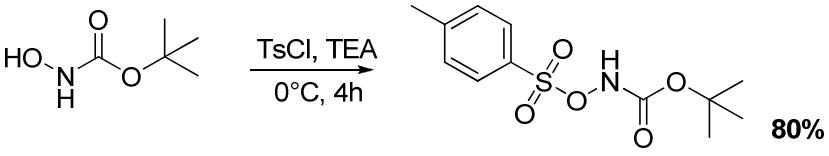
The first step of Scheme 2 (step **a**) is the Boc-protection reaction of hydroxylamine (eq. 1). The reaction was conducted under the typical conditions for the protection of amino groups using *tert*-butyl carbonate and a mild base.



(1)

The material was not exposed to heat, due to the relatively instability of the N-O bond, the hydroxylamine is a dangerous explosive on heating. The *tert*-butyloxy carbonyl group in the reaction product increases the stability of the weak N-O bond through electron withdrawing effects. The product is a hydroxamic acid, a good chelating agent. The chelating effect was observed by a colorimetric test, which showed a color change of an aqueous solution of ferric chloride from yellow to purple.

The second step (**b**) is a tosylation reaction of the hydroxyl group of the hydroxamate (eq. 2).



(2)

To avoid the decomposition of the starting material, this exothermic reaction was carried out at low temperature and with very slow addition of tosyl chloride. The introduction of a good leaving group, such as the tosylate, makes the nitrogen atom more electrophilic and thus useful as an amidating reagent in the next step of the synthesis. N-Tosyloxycarbamate can indeed be viewed as a synthetic equivalent of $^{+}$NHBoc (Figure 8).

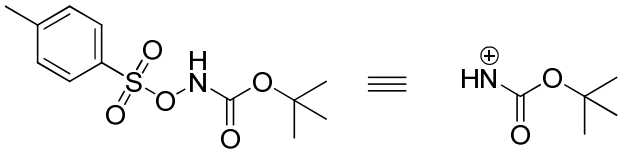
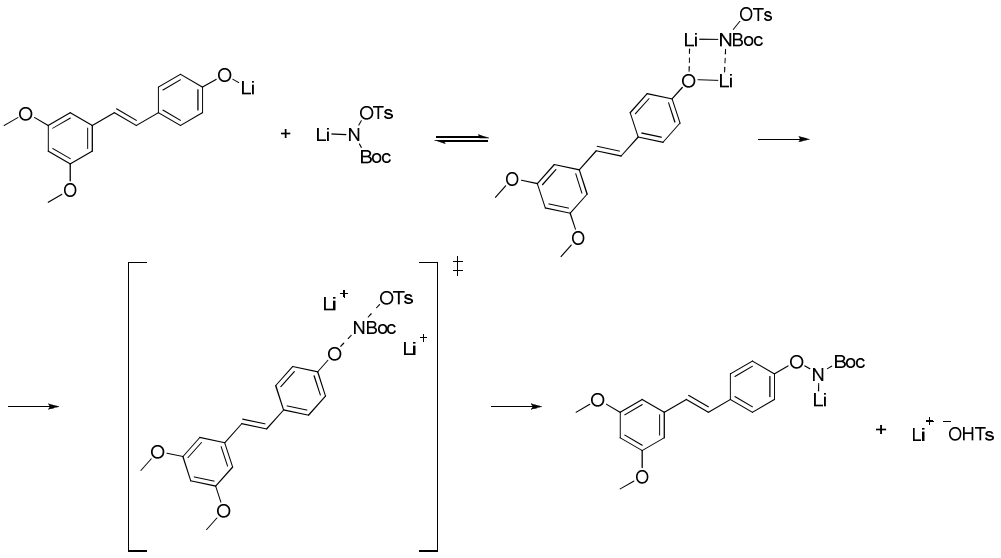


Figure 8 *Synthetic equivalent of $^{+}$NHBoc.*

The final step of the synthesis, **c** in Scheme 2, an electrophilic amidation reaction, was carried out in the presence of a base and afforded the desired product **2** in moderate yields (Table 2). The mechanism for this reaction which is proposed in literature[28] in the presence of the strong base LiHMDS is reported in Scheme 3, which shows the coordinating effect by the lithium cation.



Scheme 3. *Coordinating effect by the lithium cation on electrophilic amidation reaction in the presence of the base LiHMDS*

The influence of the metal coordinated to the nitrogen in metallated *tert*-butyl-N-tosyloxy carbamates on their reactivity in electrophilic aminations of carbanions is well known. [28]

Step **c** of Scheme **2** afforded at best a 37% yield of **2** when using LiHMSD as base. Attempts to improve the yield of this reaction using weaker amine bases, and increasing both the reaction time and temperature have been tried out. However, as reported in Table 2, the results were not improved and comparable yields were obtained also using longer reaction times and DMAP or TEA as base instead of LiHMDS.

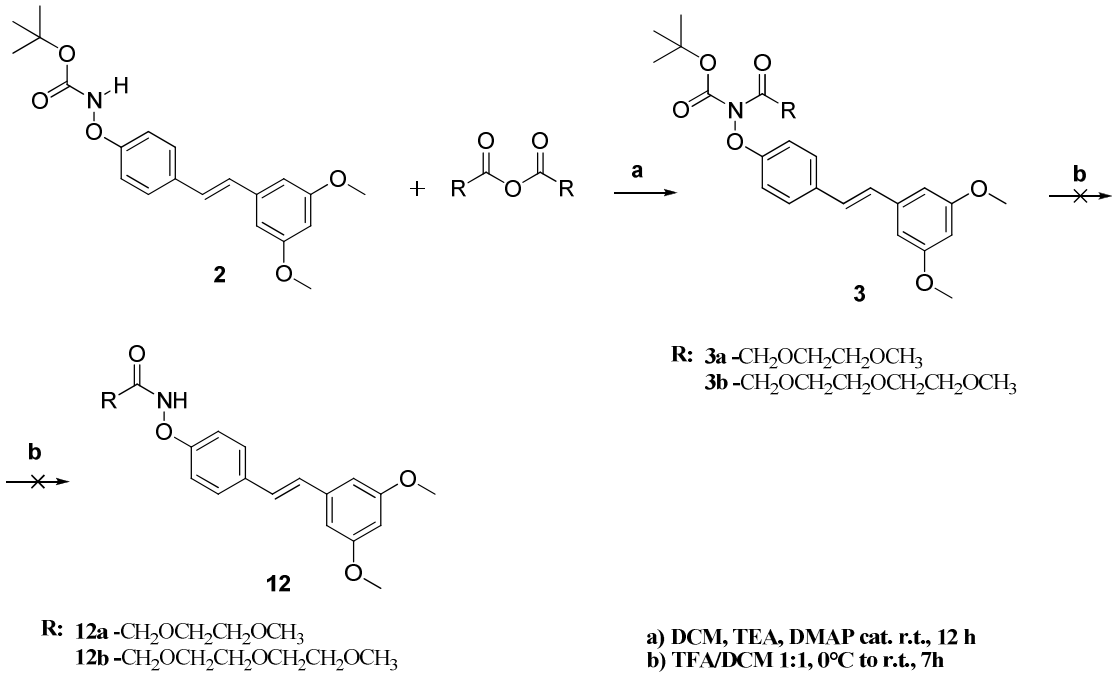
Solvent	Base	eq. Base	eq. Aminating agent	Time	Temperature	Yield of product **2** (%)
THF	LiHMDS	3	3	3 h	0°C → r.t.	**37**
THF	DMAP	1.5	1.5 x 4 times	5 gg	35°C	**38**
THF	TEA	1.5	1.5 x 4 times	5 gg	35°C	**27**

Table 2. Reaction conditions *of electrophilic amidation reaction*

Product **2** proved to have good stability and was purified without problems by flash chromatography, in accordance with the promoiety stability of prodrug reported in the literature[27].

2.1.2 Synthesis of *tert*-butyl N-(olygoethylen glycol acetyl)N-pterostilbene carbamates (3a, 3b) and N-(olygoethylen glycol acetyl)N-pterostilbene carbamic acid (4a, 4b)

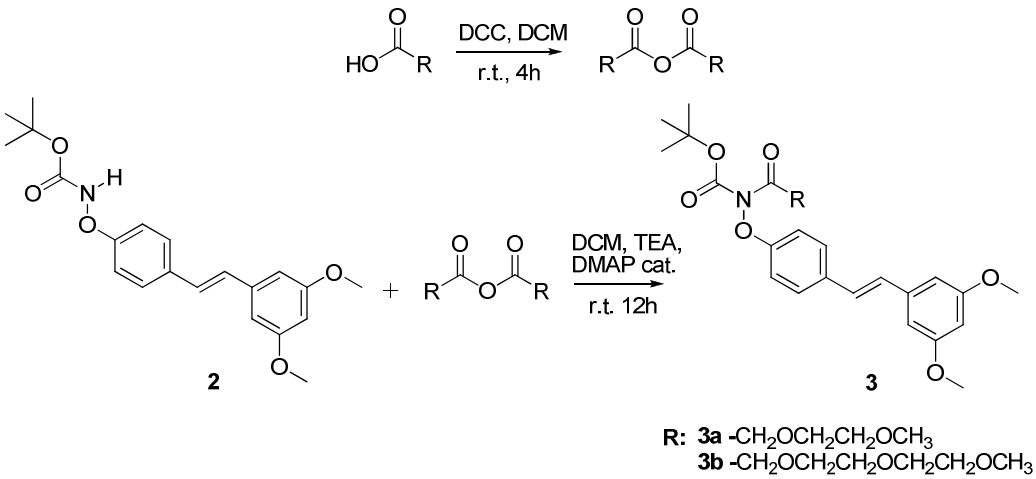
The next goal was to convert derivative **2** into prodrugs of greater hydrophilicity, notably the ethylen glycol substituted derivatives **3a-b** and the hydroxamate derivatives **12a-b** shown in Scheme 4.



Scheme 4. *Pathway attempted to synthesize olygoethylen glycol hydroxamate derivatives of pterostilbene*

.

The first step (**a**) of Scheme 4 is an acylation reaction on nitrogen to introduce the ethylen glycol acetyl chain. The anhydrides necessary for this reaction had to be synthesized from the corresponding acids, 2-(2-methoxyethoxy)acetic acid and 2-(2-(2-methoxyethoxy)ethoxy)acetic acid, via condensation induced by treatment with equimolar amounts of dicyclohexylcarbodiimide (DCC) in dichloromethane for 4 h at room temperature under nitrogen (eq. 3). The anhydrides were used directly, without further purification, in the subsequent step involving N-acylation of **2** to afford the desired product **3** (eq. 4).



(3)

(4)

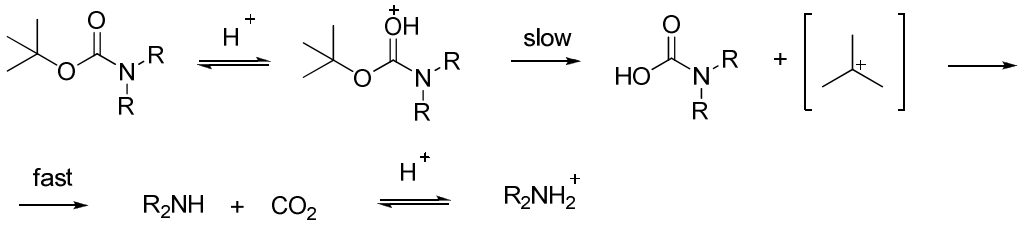
The acylation reactions were run in dichloromethane in the presence of an excess of TEA and of a catalytic amount of DMAP for 12 h at room temperature under nitrogen. The reactions afforded the products **3a** and **3b** in good yield (Tab.3)

Substituent group R =	% yield of product **3**
$-CH_2OCH_2CH_2OCH_3$	90
$-CH_2(OCH_2CH_2)_2OCH_3$	83

Tab.3 *Yield of products **3** obtained from reaction of **2** with the corresponding anhydride in the presence of TEA (4 eq), DMAP(in catalytic amount) in dichloromethane at room temperature for 12 h under nitrogen.*

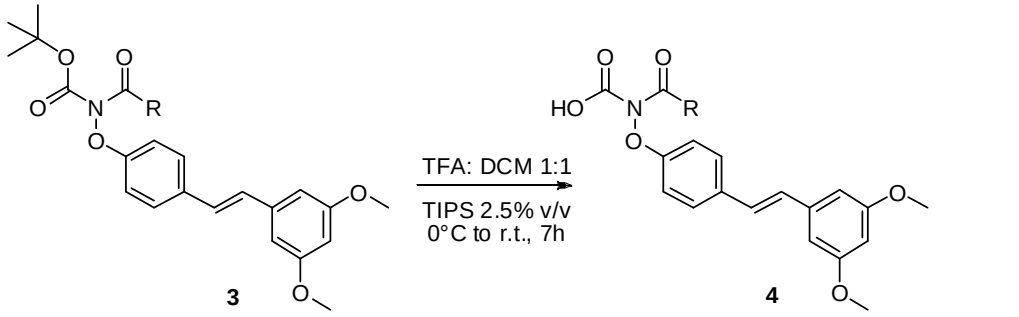
The reaction products showed a good stability.

The second step of the synthesis of Scheme 4 was intended to realize the deprotection reaction by removal of Boc to achieve the desired targets **10a** and **10b**. This reaction is usually afforded by treatment with a strong acid. The widely accepted mechanism for the acid-catalyzed deprotection of a Boc-protected amine involves a rapid equilibrium protonation of the Boc group, followed by a rate limiting fragmentation of the resultant protonated intermediate (Scheme 5). [29] In this mechanism it is assumed that the breakdown of the carbamic acid, initially produced by the reaction, is fast. [30]



Scheme 5 *Mechanism for the acid-catalyzed deprotection of a Boc protected amine*

However this reaction failed to produce from derivatives **3** the expected corresponding hydroxamate products **10**. The carbamic acids **4** were obtained instead (eq. 5).



(5)

R: **3a** $-CH_2OCH_2CH_2OCH_3$
3b $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

R: **4a** $-CH_2OCH_2CH_2OCH_3$
4b $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

For these reactions, the N-protected derivatives **3** were dissolved in a solution of dichloromethane and trifluoroacetic acid (1:1 v/v) in the presence of a small amount of triisopropylsilane (TIPS 2.5 % v/v). The reaction was run for 7 hours starting from an initial temperature of 0 °C and allowing the mixture to return to room temperature. Products **4** were obtained (Tab. 4).

Substituent group R =	% yield of product **4**
$-CH_2OCH_2CH_2OCH_3$	**44**
$-CH_2(OCH_2CH_2)_2OCH_3$	**44**

Tab.4 *Yield of products **4** obtained from reaction of **3** in TFA/DCM (1:1) from 0°C to room temperature for 7 h.*

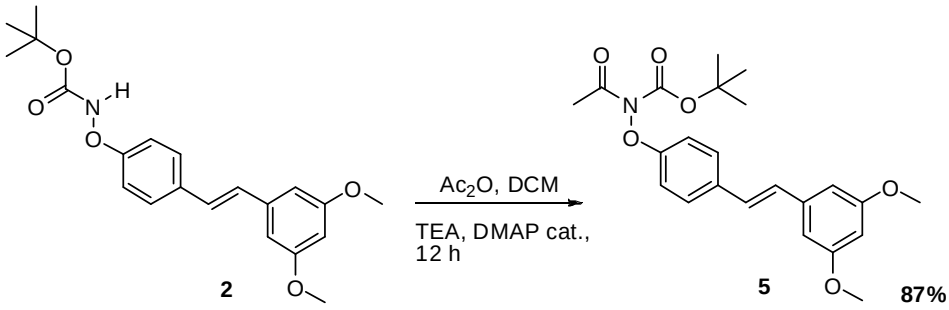
The addition of TIPS allows to trap the *tert*-butyl cation formed, thus avoiding a secondary reaction on the unsaturated pterostilbenic scaffold. Products **4** have limited chemical stability so that several attempts were necessary to find proper conditions for their isolation. Indeed products **4** proved to be unstable in silica gel, used as a

stationary phase for purification by chromatography (FSGC and PTLC), due to fast N-O bond cleavage with quantitative regeneration of pterostilbene. This cleavage was avoided by purifying the reaction products by means of HPLC, using a C18 reverse phase column. For details on the purification and characterization of the products see section 4.3.2.

To confirm and extend the unexpected results of incomplete Boc deprotection observed with derivatives **3a** and **3b**, the synthesis and study of an analogous derivative of simpler structure, namely the acetyl derivative **5**, was developed.

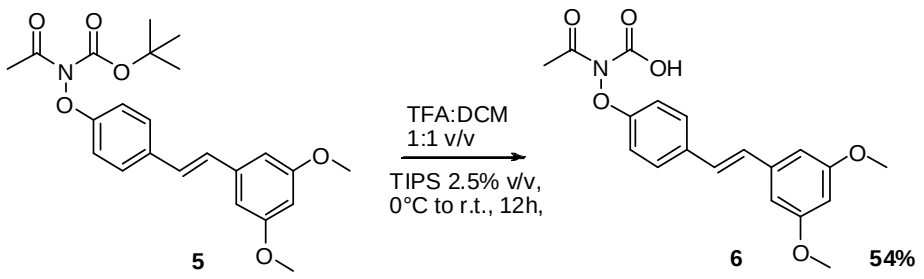
2.1.3 Synthesis of *tert*-butyl N-acetyl N-pterostilbene carbamate (5) and N-(acetyl) N-pterostilbene carbamic acid (6)

Product **5** was obtained in good yield by reaction of **2** with acetic anhydride in dichloromethane in the presence of an excess of TEA and of a catalytic amount of DMAP for 12 h at room temperature (eq. 6).The product is stable and was purified by flash chromatography on silica gel.



(6)

When subjected to the typical reaction conditions for Boc-deprotection, **5** behaved similarly to **3a** and **3b** producing the acid **6** (eq. 7) instead of the fully deprotected product, N-pterostilbene acetamide.



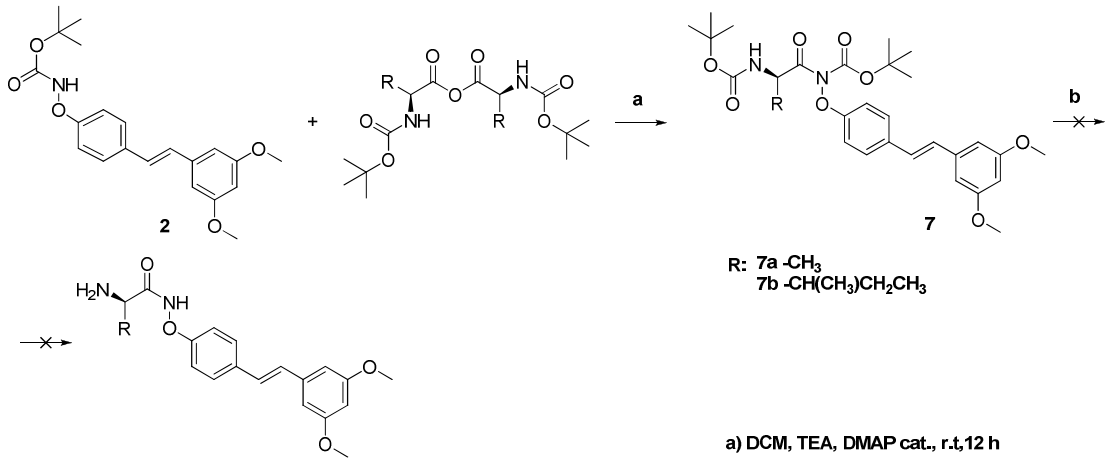
(7)

Similarly to the analogous derivatives **4a** and **4b**, **6** has limited stability which made its manipulation rather difficult. N-O bond cleavage, with consequent regeneration of

pterostilbene was observed during the purification by FSGC (Flash Silica Gel Chromatography) in acetone on silica gel. The product was therefore purified by HPLC, using a C18 reverse phase column. For details on the purification and characterization of the products see section 4.5.2.

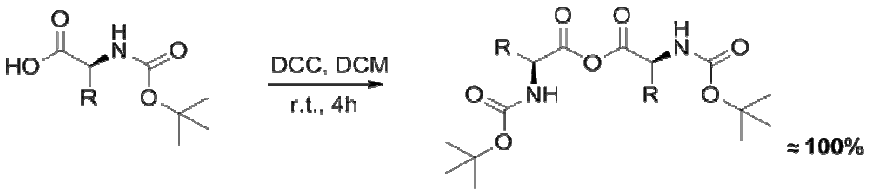
2.1.4 Synthesis of *tert*-butyl N-Boc protected amino acydyl N-pterostilbene carbamate (7a-b)

The amino acidyl hydroxamate derivatives **7a-b** were obtained from **2** following a similar pathway to that used for the ethylen glycol derivatives **3a-b** (step **a** in Scheme 4). From compounds **7a-b** I tried to obtain the derivatives **11a-b** which were of interest because of the possibility that the free amino group could promote an active transport of the prodrugs mediated by amino acid transporters.



Scheme 6 *Synthesis of C-amino hydroxamate derivatives of pterostilbene*

Step **a** of Scheme 6 is an N-acylation reaction of **2**. The required amino acidic anhydrides were synthesized in quantitative yield from the corresponding amino acids (eq. 8) and used directly, without further purification, in the following step (**a** in Scheme 6). This reaction was run in dichloromethane in the presence of an excess of TEA and a catalytic amount of DMAP at room temperature for 12 h. The products **7a** and **7b** were obtained in good yield (Tab.5)



(8)

Substituent group R =	% yield of product **7**
-CH_3 (**Ala** derivative **7a**)	**75**
-$CH(CH_3)_2CH_2CH_3$ (**Ile** derivative **7b**)	**73**

Tab.5 *Yield of 7 products obtained from reaction of **2** with the corresponding anhydride in presence of TEA (4 eq), DMAP cat. in dichloromethane at room temperature for 12 h.*

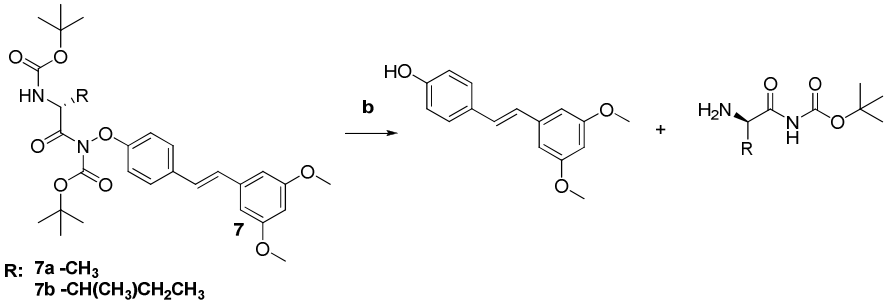
To develop more atom economic pathway to produce the acylating reagent for step **a** two attempts were experimented. The acylation of **2** was only successful when the anhydrides were used as acylating reagents. There was no reaction in the synthesis of derivative **7b** via activated ester using two different activating reagents, N-hydroxysuccinimide (NHS) and N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) under the reaction conditions reported in Tab. 6.

Amino acid	Solvent	Activating reagent	Base	Activating agent	Time	Yield
Boc-(L)-Ile-OH	DCM/DMF 9:1	NHS	DMAP	DCC	12	no reaction
Boc-(L)-Ile-OH	DMF	HBTU	TEA	-	12	no reaction

Tab.6 *Attempted alternative acylation reactions via activated ester*

The reason for this failure could be attributed to the less electrophilic character of activated esters with respect to anhydrides, or to steric hindrance around the reaction center.

All attempts to perform the next step, a Boc deprotection reaction of **7a** and **7b** (step **b** in Scheme 6) under strongly acidic conditions, failed to yield the desired products, **11a-b**, but gave pterostilbene instead (eq. 9).



(9)

A summary of all experimental conditions tested to perform the desired Boc deprotection reaction[31,32] is reported in Tab. 7a-b-c.

n.	Acid	Temperature (°C)	Solvent	Cleavage N-O bond
1	TFA	0	DCM	complete
2	TFA	-10	DCM	complete
3	TFA	-78	DCM	complete
4	TFA	-20	-	complete
5	TFA + 0.025% H_2O	-20	-	complete

Tab.7a *Attempted Boc deprotection using TFA*

n.	Acid	Temperature (°C)	Solvent	Cleavage N-O
1	HCl	r.t.	EtOAc	complete
2	HCl	r.t.	dioxane	complete

Tab.7b *Attempted Boc deprotection using HCl*

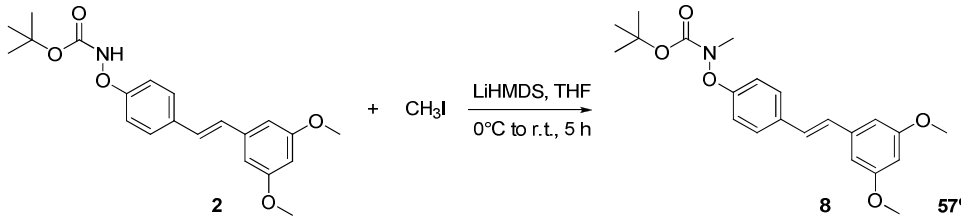
n.	Deprotecting agent	Temperature (°C)	Solvent	Cleavage N-O
1	TMS-Cl/ NaI	reflux	ACN	complete
2	TMS-Cl/ NaI	-20	$CHCl_3$	complete

Tab.7c *Attempted Boc deprotection using TMS-Cl/NaI*

The amino acidic promojety destabilizes the N-O bond of derivatives **7**, an effect which is confirmed by the results of stability assays reported at the Section 3.2.

2.1.5 Synthesis of *tert*-butyl N-(methyl) N-pterostilbene carbamate (8)

The derivative **8** was synthesized from **2** via direct N-methylation (eq. 10). The goal was to study a derivative of **2** with steric hindrance on the nitrogen.

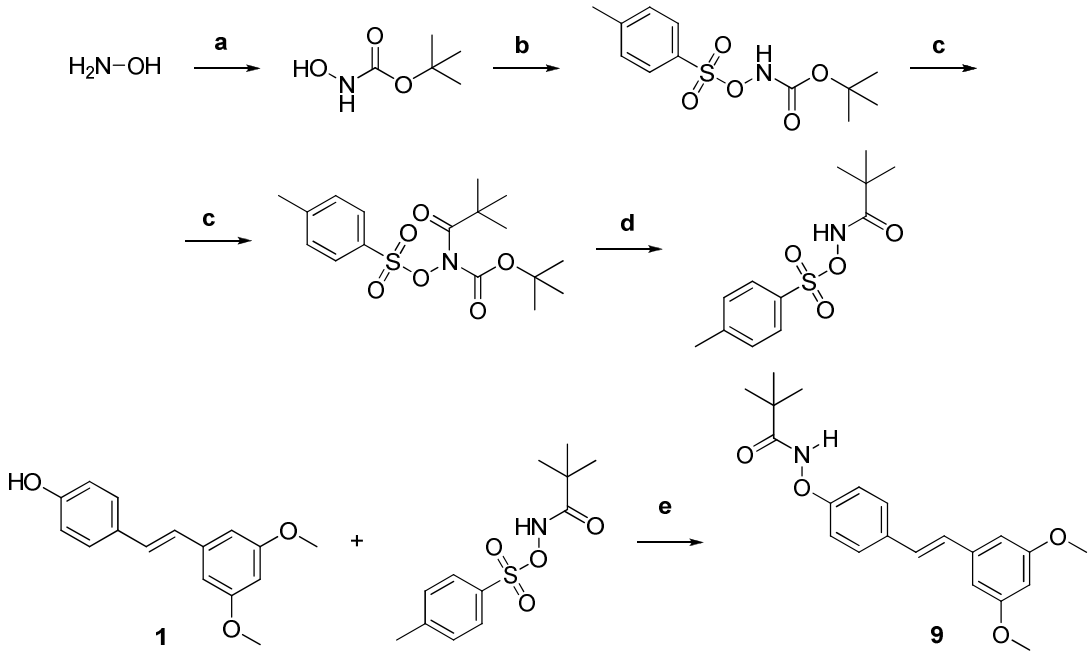


(10)

The product was easily synthesized using an excess of methyl iodide (2 eq.) and LiHMDS (1 eq.), as a strong base. Low reaction yield is explained by experimental observation of pterostilbene regeneration during addition of methylating agent. Excess of methylating agent, compared with the base equivalent, afforded a production of hydrogen iodide, which afforded the cleavage of the weak N-O bond.

2.1.6 Synthesis of N-pterostilbene pivaloylamide (9)

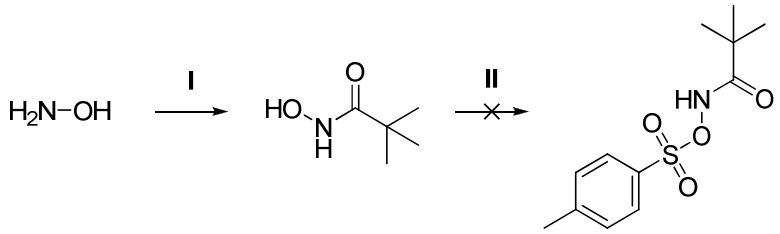
The synthesis of **9** was achieved via the 6-step procedure outlined in Scheme 7**.**



Scheme 7. *Synthesis of tert-butyl hydroxamate of pterostilbene*

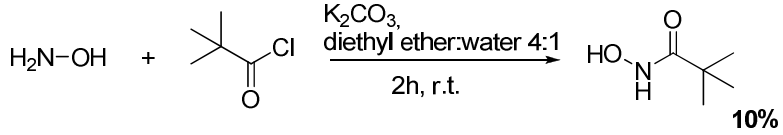
The first five steps (**a** - **d**) were necessary to synthesize the reagent N-(tosyloxy) pivaloylamide, which is then used as amidating agent in the last step **e** to achieve the desired product.

I tried a shorter route to synthesize N-(tosyloxy) pivaloylamide, as outlined in Scheme 8.



Scheme 8. *Attempted two step synthesis of N-(Tosyloxy) pivaloylamide*

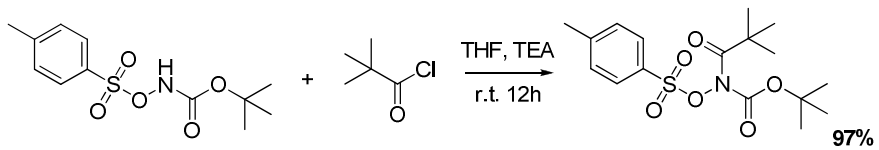
The first step of this pathway (**I**) afforded the desired product *tert*-butyl hydroxamic acid, although in low yield, via reaction of hydroxylamine with pivaloyl chloride (eq. 9).



(9)

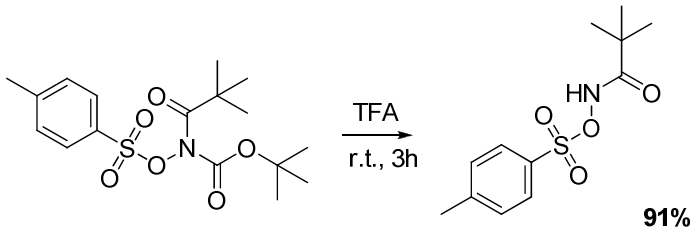
Unfortunately, all attempts to perform the second step (**II** in Scheme 8), failed to afford the expected product. The tosylation reaction was not successful using tosyl chloride in the presence of two different bases, DMAP and LiH. The failure to obtain the desired product is probably due to the low nucleophilicity of the reagent and the chemical instability of the product in the presence of a base.

Thus, the 5-step route of Scheme 7 was adopted. Steps **a** and **b** are the same as described in paragraph 1.1. It is interesting to compare reaction (9), which could not be performed, with reaction (2) which gave the tosylated product in 80% yield: it is probably due to the electron withdrawing effect of the oxygen in the alkoxy substituent. Step **c** was performed with pivaloyl chloride the in presence of base to give the acylated product (eq. 10).



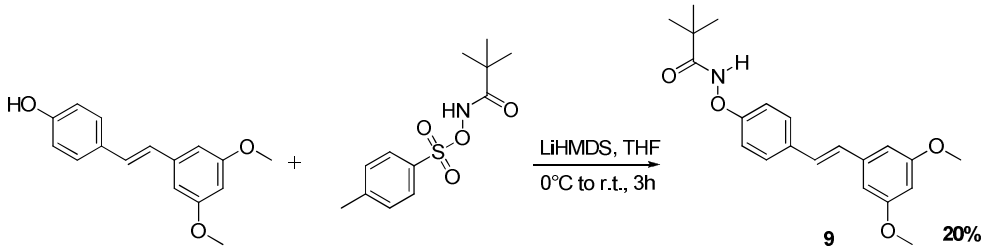
(10)

Step **d,** a Boc-deprotection reaction, afforded the desired product using TFA at room temperature (eq. 11).



(11)

Finally, the last step in the overall sequence, **e** in Scheme 7, is analogous to the synthesis *tert*-butyloxy hydroxamate of pterostilbene **2** reported in Section 2.1.1 The same reaction conditions were used, i.e. 2.5 eq of the amidating agent and 2.5 eq. of base (LiHDMS) in THF starting at a temperature of 0°C and allowing the mixture to return to r.t. (eq. 12)



(12)

The importance to add the amidating agent at low temperature is proved by experimental results obtained in a reaction run under the same experimental conditions, except that the amidating agent was added in two portions: the first half (1.25 eq.) was added at 0°C and the second one (1.25 eq.) at room temperature. The yield in this case was drastically reduced from 20% to 4%, suggesting that at room temperature the amidating reagent is not stable in the presence of a strong base as LiHDMS.

Product **9** underwent partial reconversion to pterostilbene during purification with PTLC (acetone on silica gel). It was therefore purified by FSGC with a 1% TEA addition, to make less acid the stationary phase (Section 4.8.3).

CHAPTER 3

Stability of the synthesized derivatives at neutral and acidic pH

This chapter contains the results of stability studies on the synthesized new prodrugs in aqueous solutions at pH values which are characteristic of physiological compartments of interest for oral administration: strongly acidic, as in the stomach, and nearly neutral, as in the intestine. A brief introduction on the protocols used to perform the stability assays is presented, followed by a description of the results and their discussion.

3.1 Experimental procedures

Chemical stabilities were studied by monitoring concentration vs time profiles of the desired prodrug and products by means of HPLC-UV analysis as detailed in Chapter 4. Two aqueous reaction media were considered for these experiments, notably aqueous HCl (pH = 1) and PBS (pH = 6.8).

3.2 Results and discussion

The tested compounds (**2**, **3a**, **4a**, **4b**, **5**, **6**, **7a**, **8** and **9**) show a remarkable range of reactivity. With regard to the most important issue concerning the release of pterostilbene, the results, shown and discussed in the following paragraphs, indicate that this was either not observed or negligible, with the only exception of compound **7a**. Therefore, the hydroxamate function might be useful as protection of phenolic groups in the development of prodrugs for oral administration.

A few of the tested derivatives proved to be stable under both acidic and neutral pH as shown, for example in Figs. 9 – 10 for compound **2**. It is seen that the HPLC-UV traces of samples taken at time zero and 24 h after incubation at 37°C in either acidic or neutral solutions contain only the chromatographic peak due to **2**. Similar behavior was observed with compounds **8** (Figs. 11-12) and **9** (Figs 13-14).

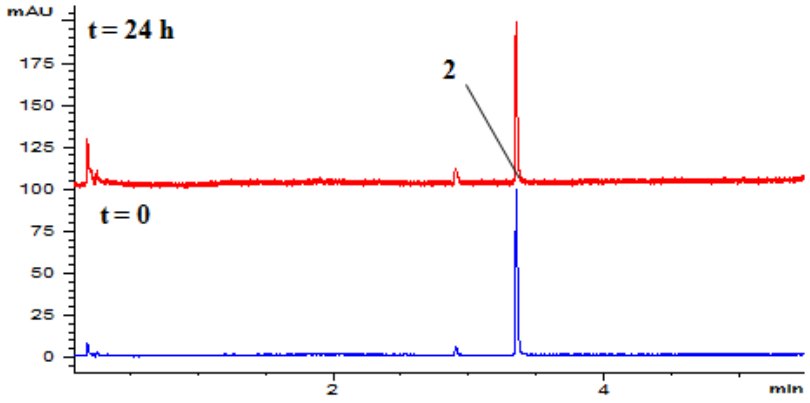


Fig. 9 *Product **2** in HCl 0.1N (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (5μM).*

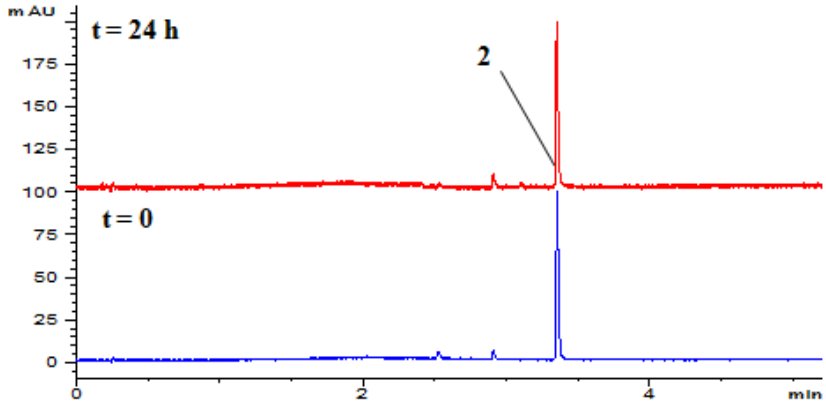


Fig. 10 *Product **2** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

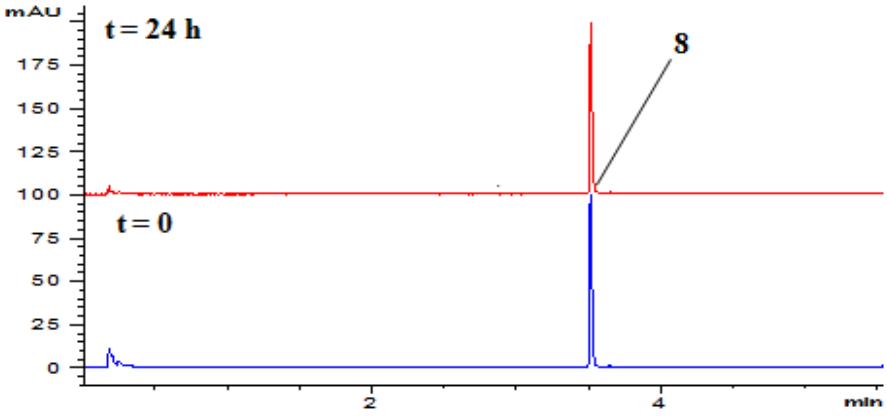


Fig. 11 *Product **8** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation at 37°C (12μM).*

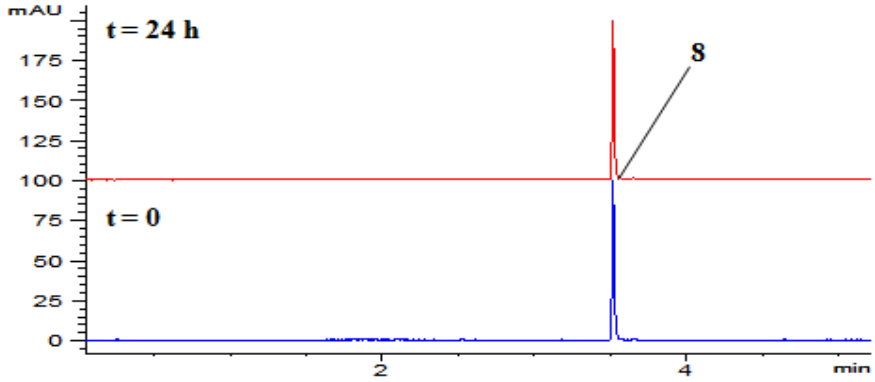


Fig. 12 *Product **8** in HCl 0.1N (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (12 μM).*

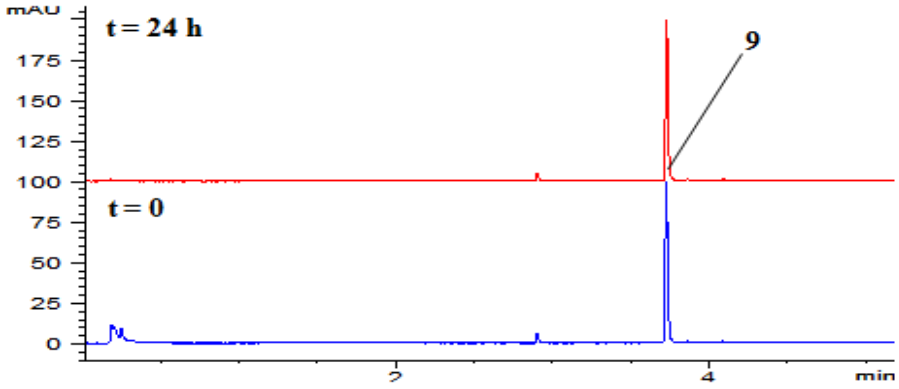


Fig. 13 *Product **9** in HCl 0.1N (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (15 μM).*

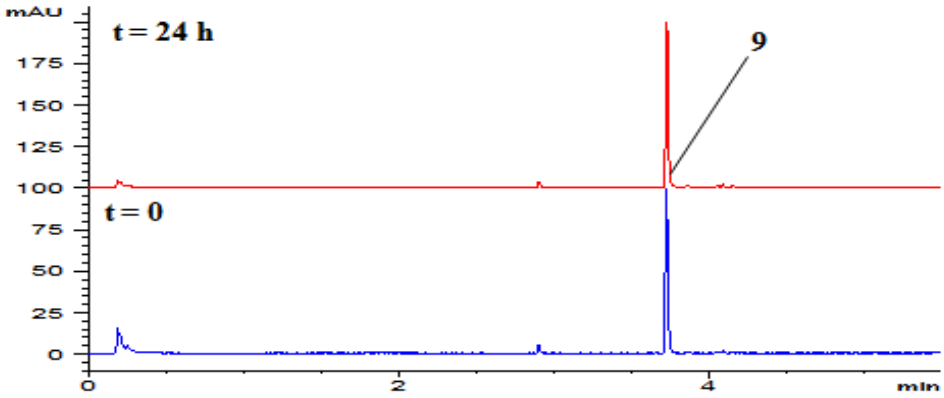


Fig. 14 *Product **9** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation at 37°C (15μM).*

All other derivatives tested undergo reaction to different extents and form different intermediates/products depending on the pH conditions and on the substituents present on the –N-O-PTS moiety. Notably all, except **5**, react faster at pH 6.8 than at pH 1.

In the case of compound **7a**, pterostilbene is the only product detected by HPLC analysis at any reaction time in either media (Fig. 15-17).

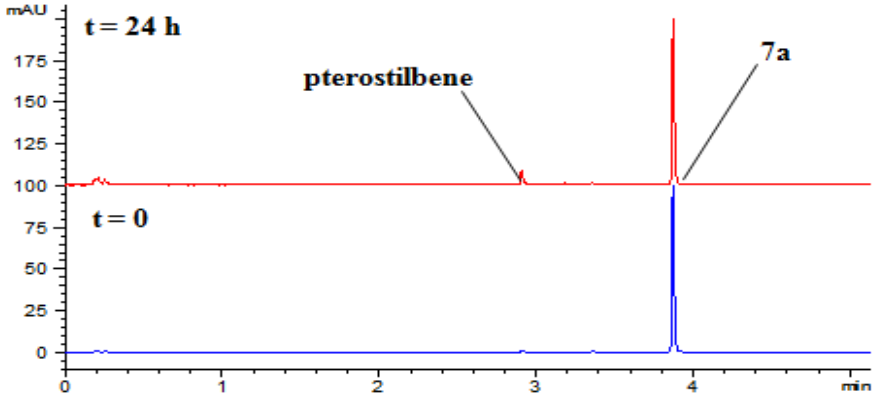


Fig. 15 *Product **7a** in HCl 0.1 M (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

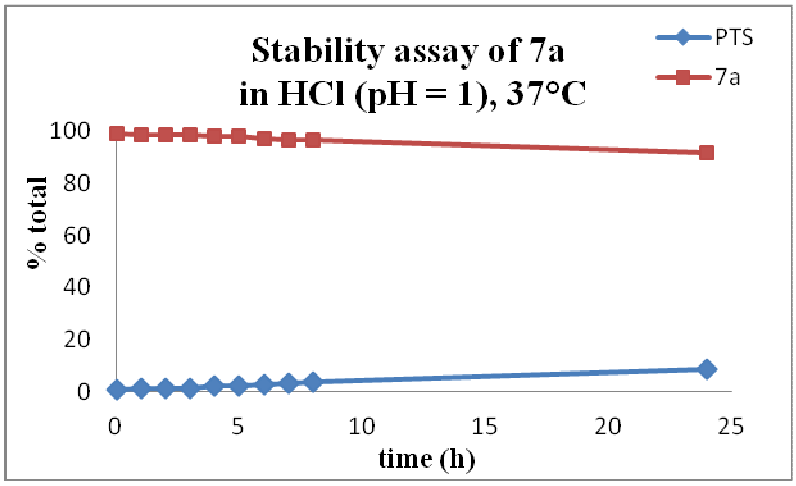


Fig. 16 *Stability assay of **7a** in HCl (pH = 1).*

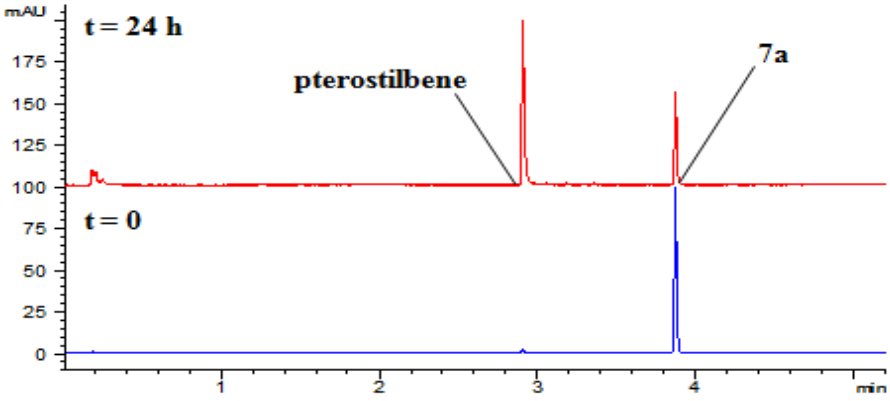


Fig. 17 *Product **7a** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

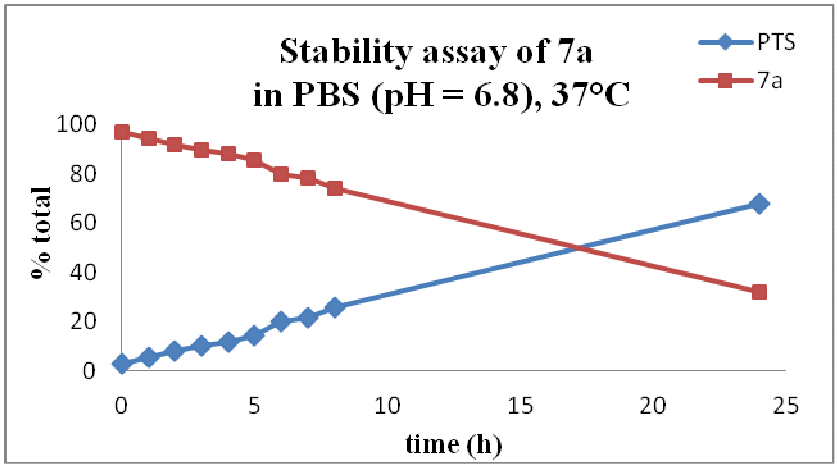


Fig. 18 *Stability assay of **7a** in PBS (pH = 6.8).*

Release of pterostilbene was also observed to a minor extent in the reaction of compounds **4a**, **4b**, and **6**, which display a more complex behavior, as described later.

The reactions of derivatives **3a** and **5** are similar and produce the same product. This was identified as **2** based on its retention time, matching that of an authentic sample, and by HPLC-ESI/MS analysis (Fig.19). As seen from Figs 20-22 and Figs. 24-26, no other peaks are detected. Moreover, the product is stable under the reaction conditions, consistent with the results reported above indicating that **2** does not react either in acidic or in neutral solution.

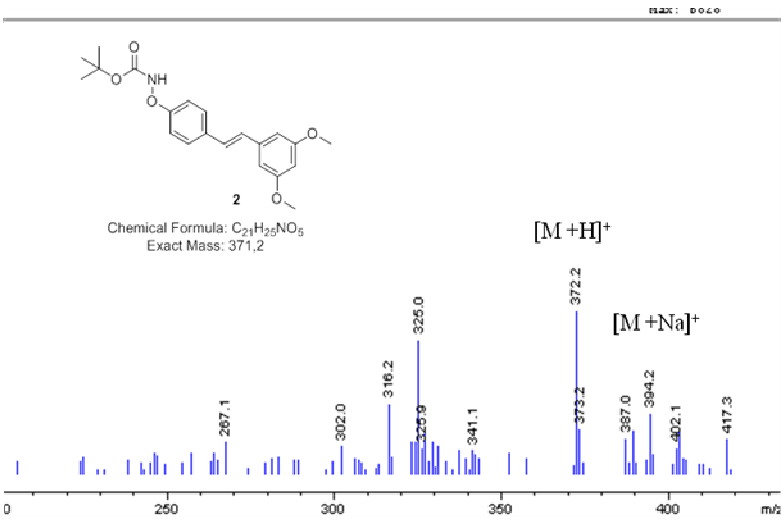


Fig. 19 *Mass spectrum of product **2** detected by HPLC-ESI/MS in the reaction of **5***

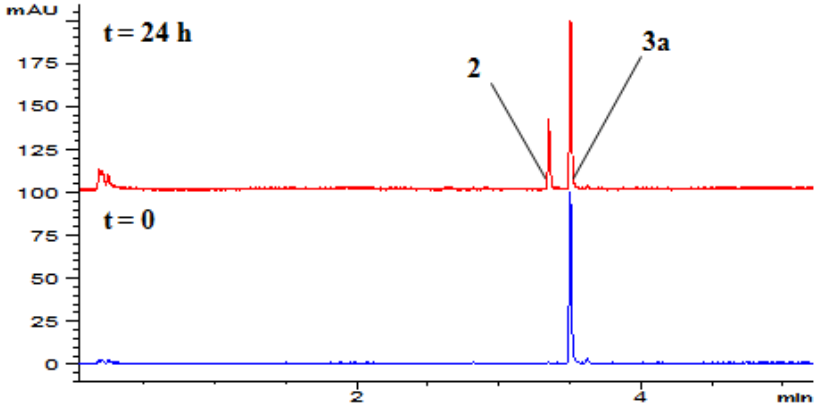


Fig. 20 *Product **3a** in HCl 0.1N (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

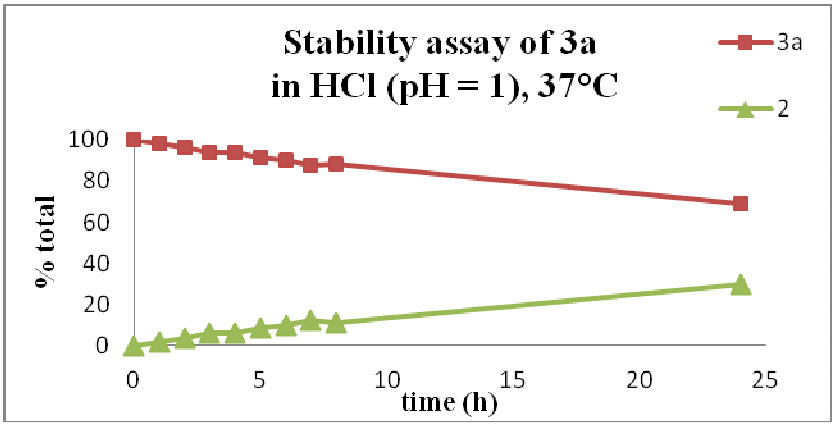


Fig. 21 *Stability assay of **3a** in HCl (pH = 1).*

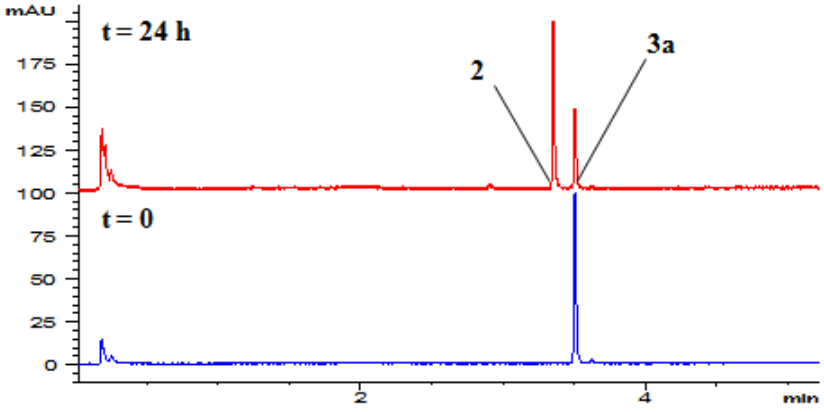


Fig. 22 *Product **3a** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

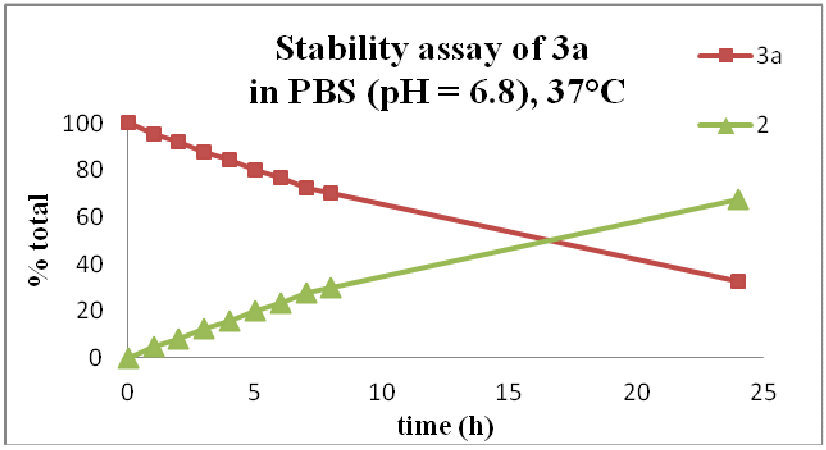


Fig. 23 *Stability assay of **3a** in PBS (pH = 6.8).*

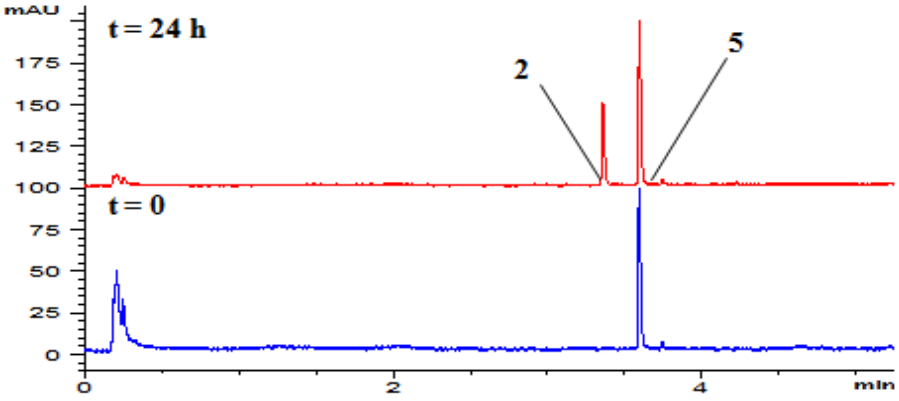


Fig. 24 *Product **5** in HCl 0.1N (pH = 1). Two chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 24 h of incubation at 37°C (5 μM).*

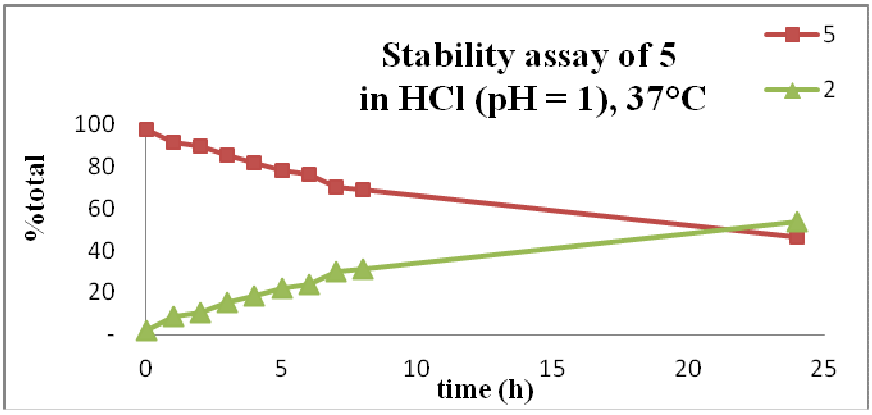


Fig. 27 *Stability assay of **5** in HCl (pH = 1).*

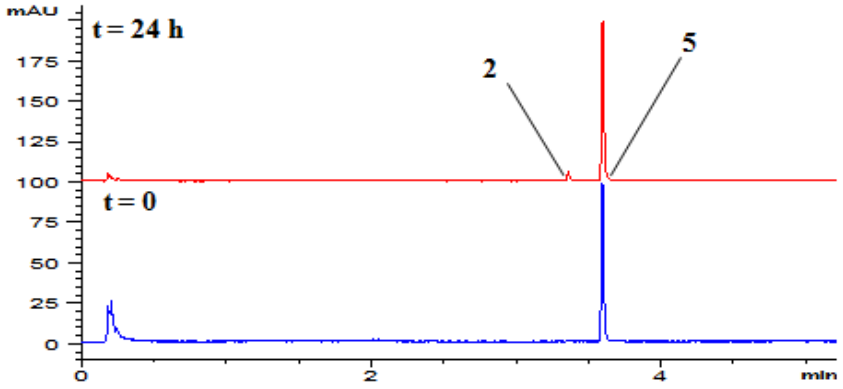


Fig. 26 *Product **5** in PBS (pH = 6.8). Two chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 24 h of incubation of incubation at 37°C (5 μM).*

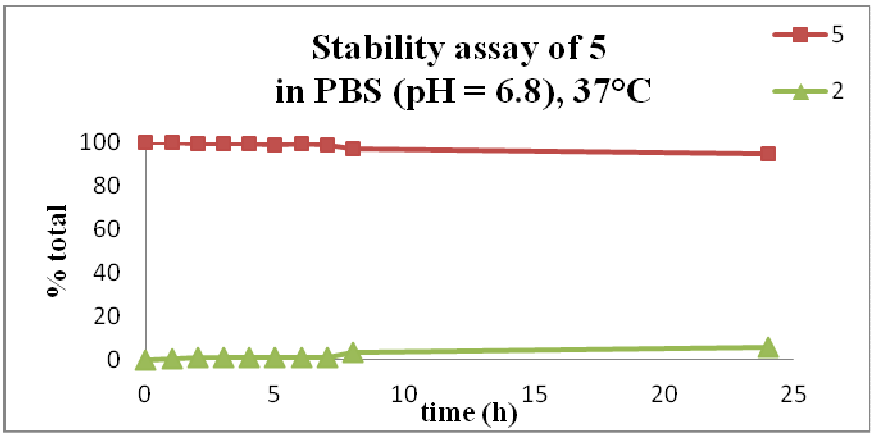
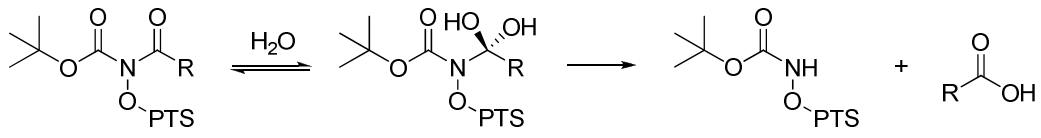


Fig. 25 *Stability assay of **5** in PBS (pH = 6.8).*

A possible mechanism for the hydrolysis of **3a** and **5** to product **2** is shown in Scheme 9.



Scheme 9 *Suggested pathway for hydrolysis of derivatives **3a** and **5** to product **2**.*

A deacylation mechanism of hydroxamic acid derivatives via tetrahedral intermediate is reported in the literature[33].

The behavior of compounds **4a**, **4b** and **6** is rather more complex. In addition to pterostilbene, mentioned above, other products were detected by HPLC analysis.

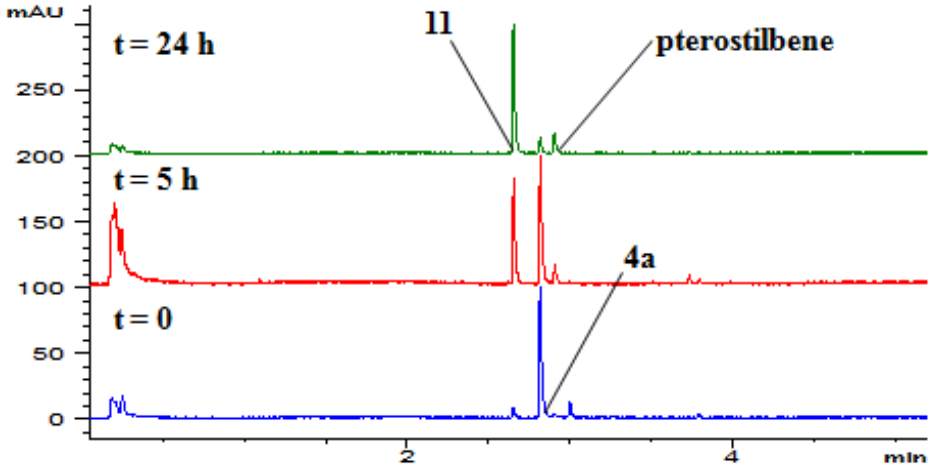


Fig. 28 *Product **4a** in HCl 0.1N (pH = 1). Three chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 5 h of incubation, and in green analysis after24 h of incubation at 37°C (15 μM).*

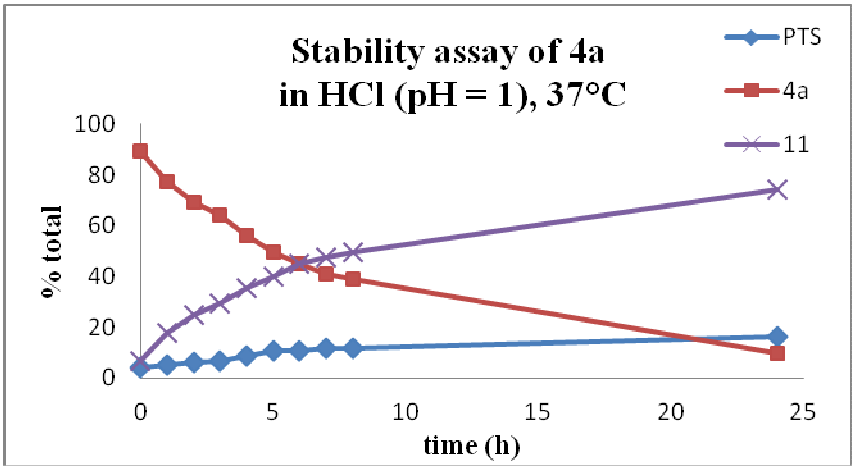


Fig. 29 *Stability assay of **4a** in HCl (pH = 1).*

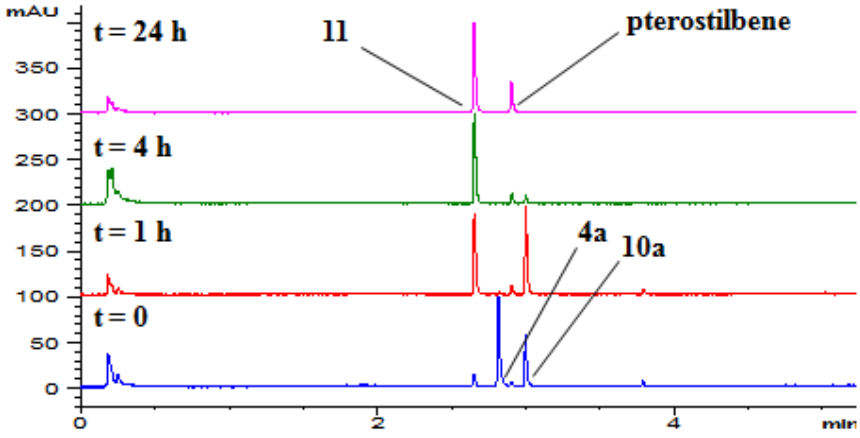


Fig. 30 *Product **4a** in PBS (pH = 6.8). Four chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 1 h of incubation, in green analysis after 4 h of incubation and in pink analysis after24 h of incubation at 37°C (15 μM).*

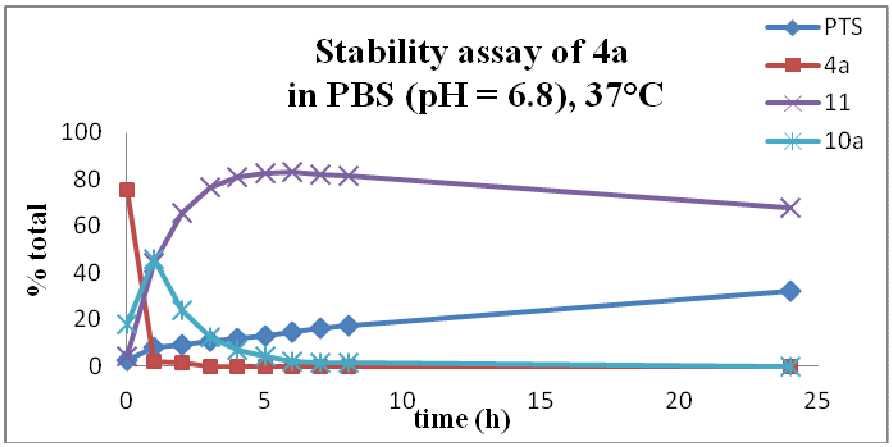


Fig. 31 *Stability assay of **4a** in PBS (pH = 6.8).*

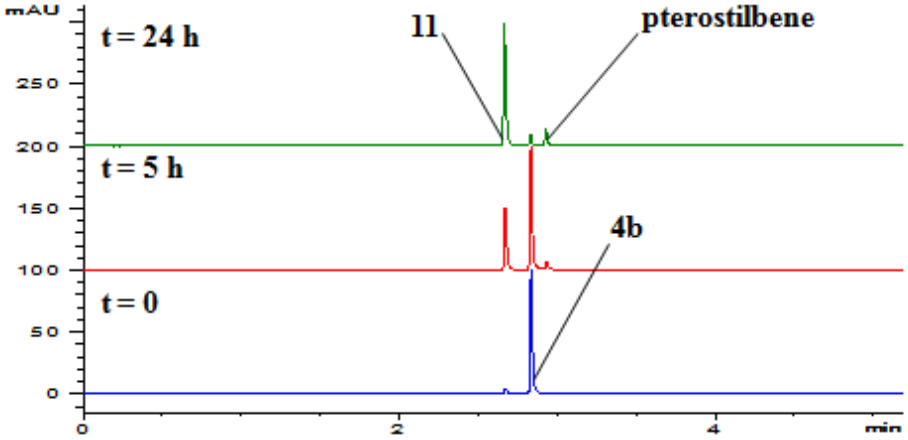


Fig. 32 *Product **4b** in HCl 0.1N (pH = 1). Three chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 5 h of incubation, and in green analysis after24 h of incubation at 37°C (15 μM).*

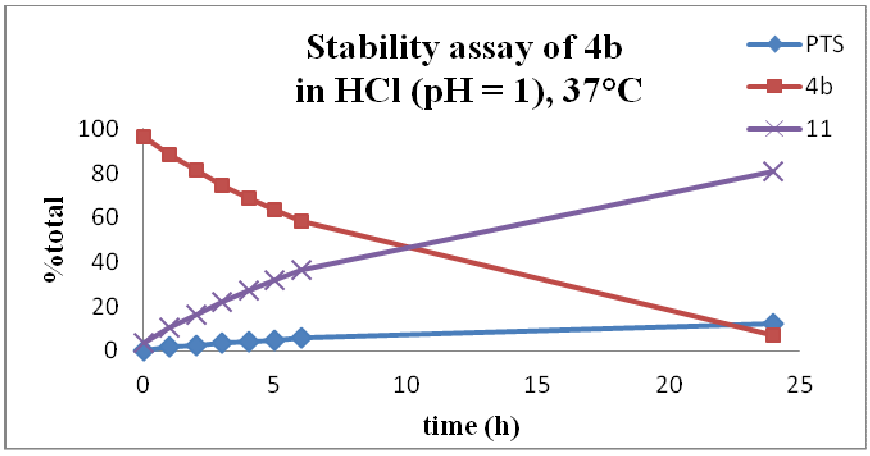


Fig. 33 *Stability assay of **4b** in HCl (pH = 1).*

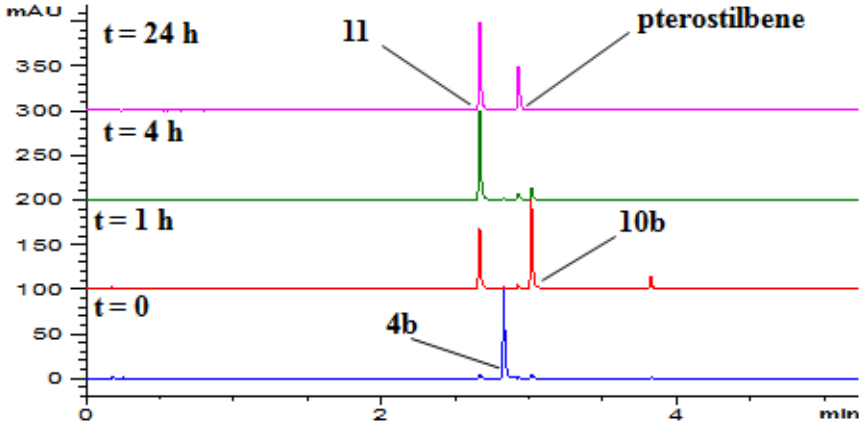


Fig. 34 *Product **4b** in PBS (pH = 6.8). Four chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 1 h of incubation, in green analysis after 4 h of incubation and in pink analysis after24 h of incubation at 37°C (15 μM).*

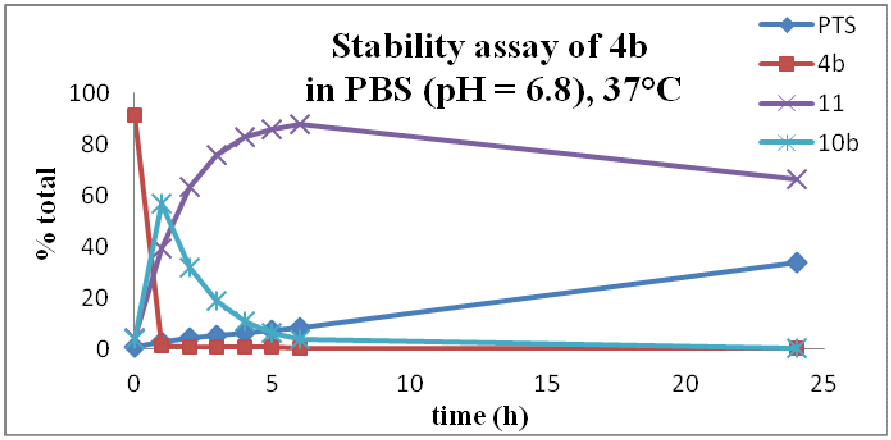


Fig. 35 *Stability assay of **4b** in PBS (pH = 6.8).*

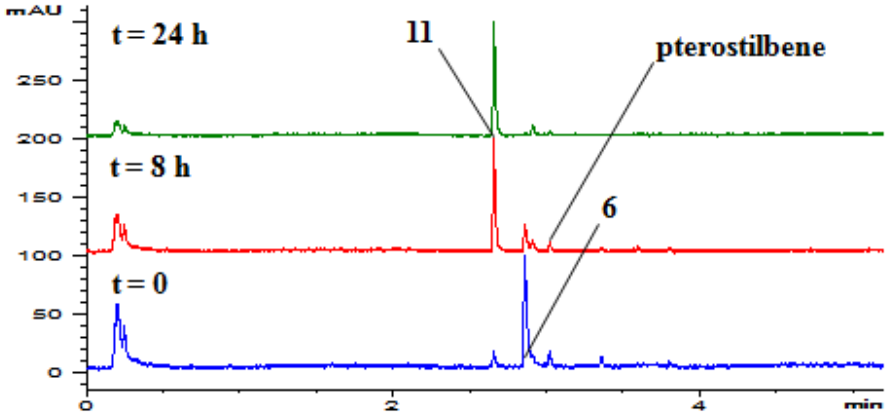


Fig. 36 *Product **6** in HCl 0.1N (pH = 1). Three chromatograms are shown: in blue analysis immediately after addition of the compound to HCl solution (t = 0), in red analysis after 8 h of incubation, in green analysis after 4 h of incubation and in pink analysis after24 h of incubation at 37°C (12 μM).*

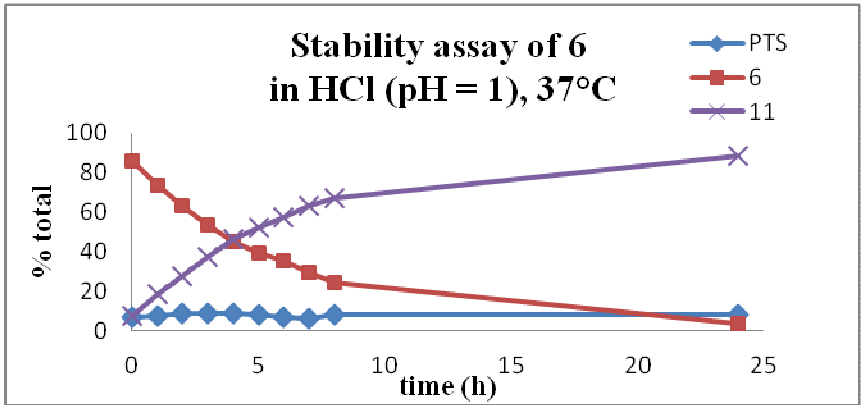


Fig. 38 *Stability assay of **6** in HCl (pH = 1).*

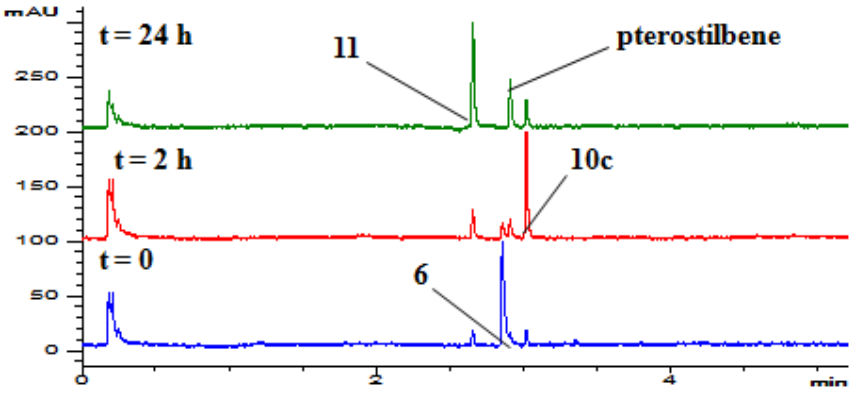


Fig. 39 *Product **6** in PBS (pH = 6.8). Three chromatograms are shown: in blue analysis immediately after addition of the compound to PBS solution (t = 0), in red analysis after 2 h of incubation, in green analysis after 24 h of incubation at 37°C (12 μM).*

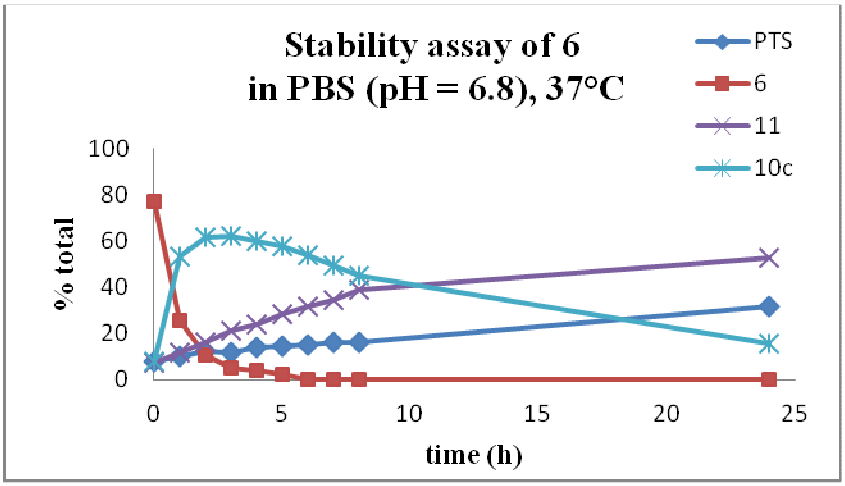


Fig. 40 *Stability assay of **6** in PBS (pH = 6.8).*

In particular, one common product is formed, both at pH 1 and 6.8, from all three prodrugs. Based on the results of HPLC-MS/ESI analysis, this product is assigned the structure of **11** (Fig. 41).

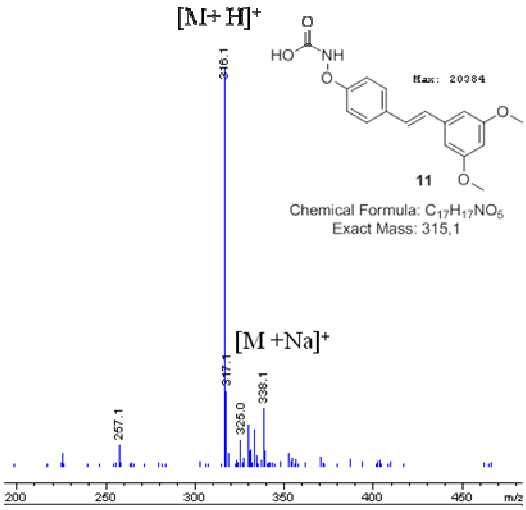
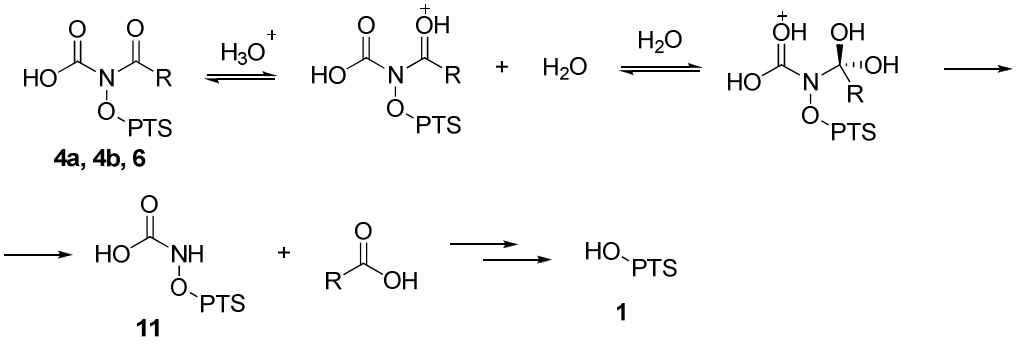


Fig. 41 *Mass spectrum of product **11** detected by HPLC-ESI/MS in the reaction of **6.***

Product **11** is possibly formed according to the pathway shown in Scheme 10.



Scheme 10 *Suggested hydrolytic pathway for derivatives **4a, 4b** and **6** in acid conditions (pH=1)*

Product **11** appears to be stable in acidic solution but somewhat less so at neutral pH.

In addition to **11** compounds **4a**, **4b** and **6** behave similarly also for the production, detected only in the experiments at pH 6.8, of a rather unstable species which builds up very rapidly and is then consumed in a slower reaction. These intermediate species are indicated as **10a**, **10b** and **10c** in Figs. 42

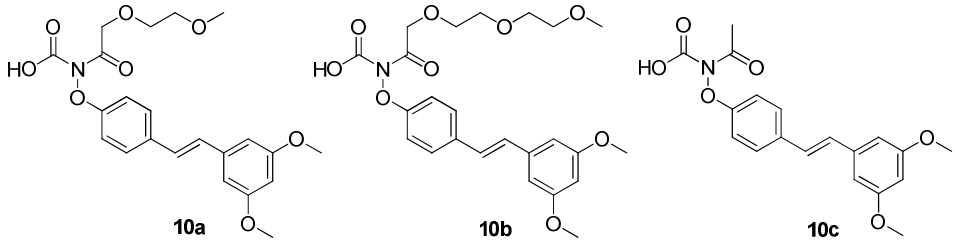
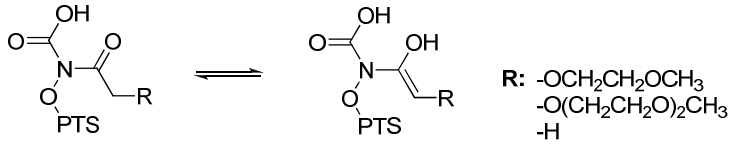


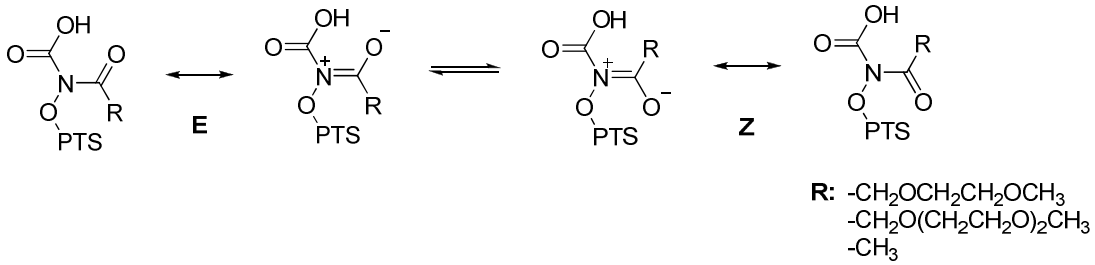
Fig.42 *Z-isomers **10a-b-c***

The identification of intermediates **10a, 10b** and **10c** was not so immediate. By means of HPLC-MS/ESI analyses it was found that each has the same mass as the

precursor prodrug, so they are isomers of **4a**, **4b** and **6**, respectively. In other words, derivatives **4a**, **4b** and **6** undergo some isomerization reaction in phosphate buffer solution. Two types of isomerism were considerd, namely a keto-enol tautomerism (Scheme 11) and a cis–trans isomerism about the C-N bond (Scheme 12).



Scheme 11 *Keto-enol tautomerism*



Scheme 12 E–*Z isomerism*

In the literature[34] a study of cis–trans isomerism about the C-N bond of N-substituted hydroxamic acids is reported. The same paper[34] also reports that NMR peaks of the enolic forms of N-substituted hydroxamic acids are only observable at low temperature (-15° C).

^{1}H and ^{13}C NMR spectra of derivatives **4a**, **4b** and **6** in $CDCl_3$ and DMSO-d_6 afforded the signals of a single isomer and enolic signals were not observed.

These results suggest the total or preponderant presence of one stable conformational isomer (Z or E) in solution. Furthermore, the ^{1}H NMR spectrum of **6** in DMSO-d6 shows a deshielded peak at 10.6 ppm, not revealed in $CDCl_3$, which is imputable to the proton of the carbamic function.

The low field shift of this proton is typical of a strongly intramolecularly hydrogen bonded proton, as is possible in the **E** isomer.

The **E** isomer could be stabilized by intramolecular hydrogen bond, but destabilized by steric interactions between the group R and the pterostilbene scaffold. Based on these considerations it can be assumed that **E** is the prevailing isomer of derivatives **4a**, **4b** and **6** in DMSO solution.

It is expected that the **E**/**Z** ratio is temperature and solvent dependent and indeed it was able to detect changes in the **E/Z** in experiments described below and followed by means of HPLC analysis.

A 1 µL aliquot of a solution of **4b** in DMSO (12 mM) was diluted in ACN (12 µM) at room temperature and analyzed by HPLC-ESI/MS. At t = 0 only one chromatographic peak was revealed and identified as **4b (**E isomer) (Figure 43). After one hour at room temperature two extra peaks were observed: **10b** (Z isomer) and **11** (Figure 41).

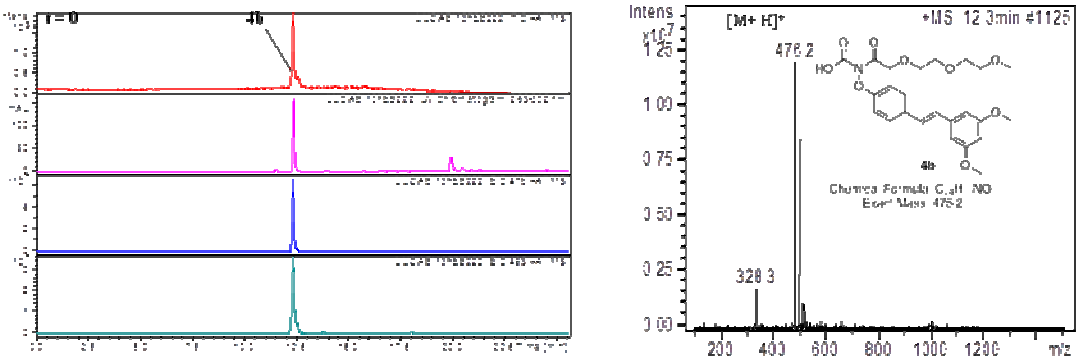


Fig. 43 *Chromatogram and mass spectrum of a fresh **4b** solution in ACN (t = 0) at r.t. (12 µM).*

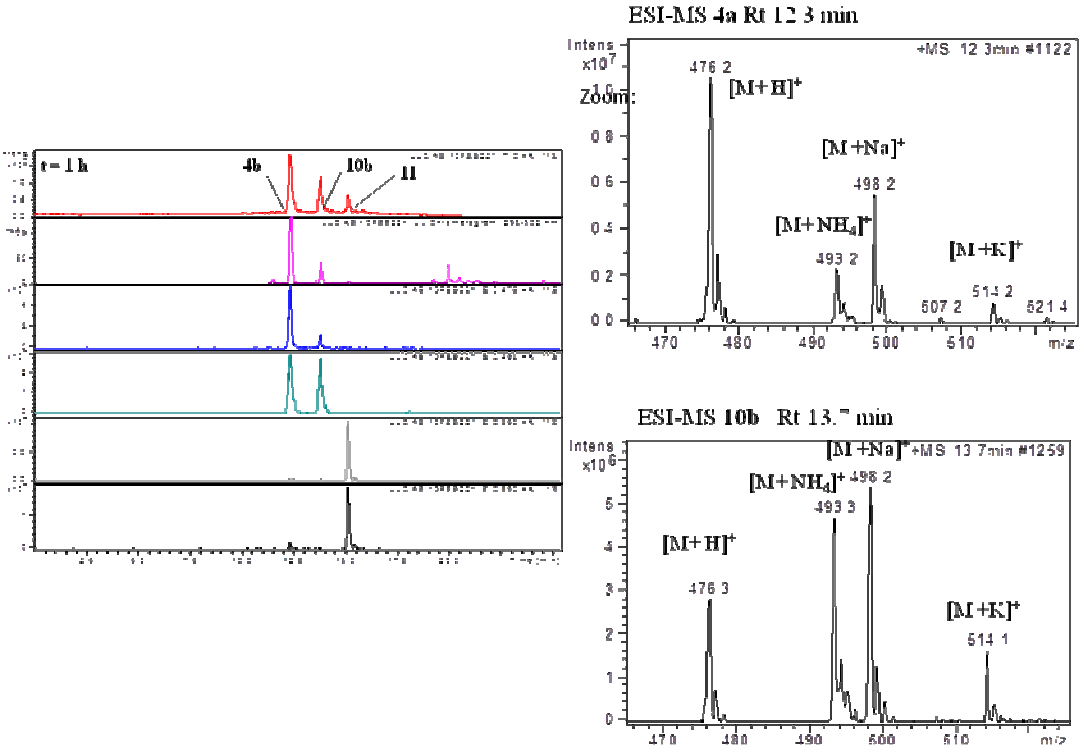


Fig. 44 *Chromatogram and mass spectra of a **4b** solution in ACN (t = 1h) at r.t. (12 µM).*

These results show that the **E**/**Z** ratio is solvent dependent and decreases in ACN.

The results showing the isomerization of **4b** to **10b** in ACN at room temperature are shown in Fig. 45.

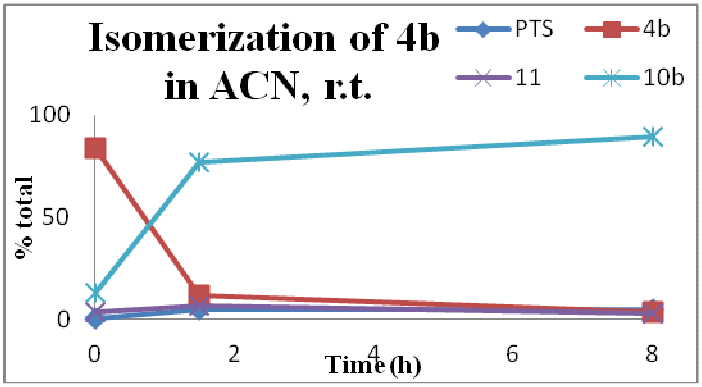


Fig. 45 *isomerization in ACN, r.t.*

The effect of solvent on this type of isomerizations was investigated with a similar experiment using derivative **6** in aqueous solution. A 1 µL aliquot of a solution of **6** in DMSO (15 mM) was diluted with water (15 µM) at 20°C and analyzed by HPLC. The overlaid chromatograms reported in Figure 47 show that the **E/Z** ratio is decreasing in time. The effect is more clearly visible in the bar plot shown in Fig. 47.

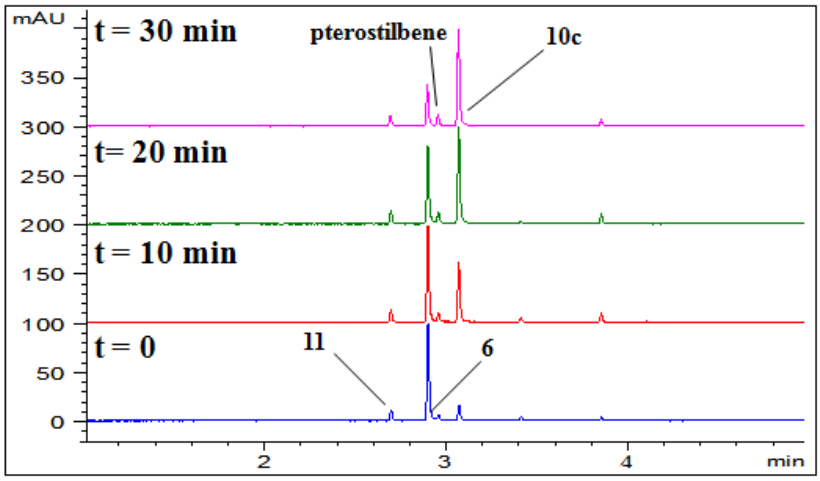


Fig. 46 *Overlais chromatogram of **4b** solution in DMSO diluted in water (t = 0, 10, 20, 30 min) at 20°C (15 µM).*

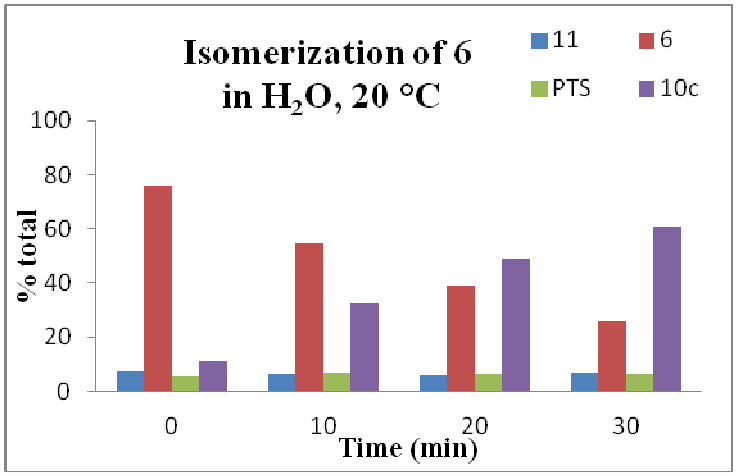


Fig. 47 6 *isomerization in H_2O, 20°C*

The effect of temperature was instead investigated by determining the **E/Z** ratio in two solutions of **6** in DMSO: H_2O = 4:1 incubated at different temperatures.

6 Solutions (15 mM) in DMSO: H_2O = 4:1 were diluted in DMSO: H_2O = 4:1 (15 μM) solutions at 20 °C and 37 °C. At the higher temperature a decrease of the E/Z ratio is observed as well as the hydrolysis of **11** to pterostilbene.

Overlaid chromatograms at 20 °C and 37 °C are reported in Figure 48.

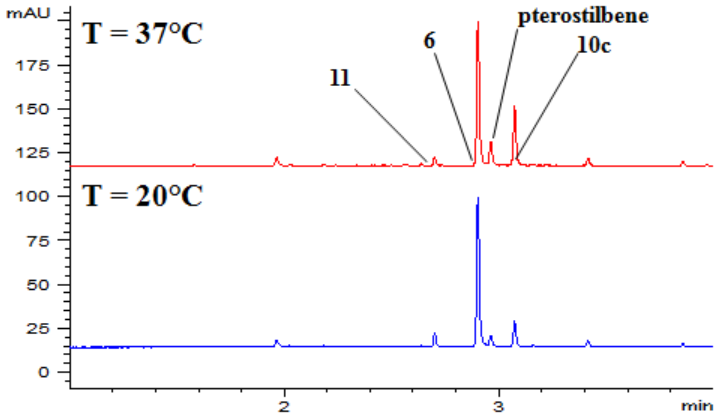


Fig. 48 *Product **6** in DMSO:H_2O 8:2. Two chromatograms are shown: in blue analysis immediately after addition of the compound at 20°C, in red analysis immediately after addition of the compound at 37°C (15 μM).*

Isomerization-temperature effect (**6**/**10c** ratio decrease with temperature raising) is reported in plot, Figure 49.

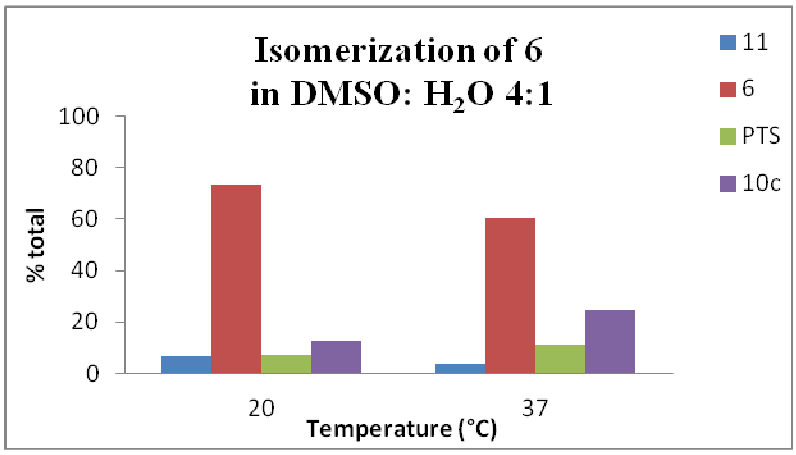


Fig. 49 I*somerization-temperature effect of **6** in DMSO:H_2O*

CHAPTER 4

Experimental section

This chapter contains the informations about materials, purification techniques, characterization methodsn and synthetic procedures.

4.1 Materials and procedures

4.1.1 Solvents and reagents

Pterostilbene was obtained from Wonda Science (North Waltham, MA, USA). Dimethylamino piridine (DMAP), Trifluoroacetic acid (TFA), Triisopropylsilane (TIPS), *p*-Toluenesulfonyl chloride (TsCl), Triethylamine (TEA), N-Boc hydroxylamine, *N*-hydroxysuccinimide, Boc-Ala-OH, Boc-Ile-OH, N,N-Dicyclohexylcarbodiimide (DCC), Triisopropyl silane (TIPS), Acetone, Tetrahydrofuran, Dichloromethane, Methanol, Petroleum Ether, Ethyl Ether, d-Chloroform, DMSO-d6 and HPLC grade Acetonitrile were obtained from Sigma Aldrich (Steinheim, Germany).

4.1.2 Purifications (FSGC, PTLC and preparative HPLC)

Column chromatography was performed on silica gel Geduran Si 60 (0.063-0.200 mm) by Merck. Preparative plates were prepared using silica gel 60 GF_{254} by Merck. Purifications by preparative HPLC were performed using a Shimadzu LC-8A with detector: absorption spectrophotometer UV-Vis at 300 nm and an inverse phase column (C_{18} functionalized silica) ACE 5 AQ with size of 150x21,2 mm.

4.1.3 Mass spectrometry

Mass spectra were obtained on Agilent Tech 6540 UHD, Finnigan MAT 95 or Agilent Technologies MSD SL Trap, equipped with electrospray source (ESI, ElectroSpray Ionization), ionic trap analyzer, connected with a binary pump (Agilent Technologies 1100 Series) and carried out in FIA (Flow Injection Analysis).

4.1.4 NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded with a Bruker AV300 FT-NMR spectrometer, a Bruker AVII400 spectrometer, Bruker AVII500 spectrometer. The following abbreviations are used to explain the multiplicities: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet.

4.1.5 Protocol for stability assays

A 1 mL of a 5-15μM solution of the desired prodrug in the desired solvent was thermostatted at 37°C in the autosampler. Analysis of samples (2 μL) taken over time from solutions of the derivatives in HCl 0.1N and 0.1 M PBS (pH= 6.8) and analyzed using a reversed phase column (Zorbax RRHD Eclipse Plus C18, 1.8 μm, 50 × 2.1 mm i.d.). The eluate was monitored at wavelength 300 nm. The amount of compound in the samples was determined from the area of the corresponding chromatographic peaks and expressed as percent of the total (plotted vs. time of incubation at 37°C).

4.2 Synthesis of tert-butyloxy hydroxamate of pterostilbene

4.2.1 *tert*-butyl N-hydroxy carbamate

A solution of hydroxylamine hydrochloride (7.10 g, 102 mmol) and $(Boc)_2O$ (22.0 g, 101 mmol) in 1:1 THF:H_2O (220 mL) at 0° C was treated with $NaHCO_3$ (17.1 g, 203 mmol). The solution was stirred at 0° C for 2 h, after which it was diluted with ethyl acetate, washed with H_2O, and saturated aqueous NaCl, and dried ($MgSO_4$). The solvent was removed under reduced pressure to provide the product as a white solid (12.4 g, 92%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (s, 1H), 7.16 (s, 1H), 1.46 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 158.8, 82.1, 28.2.

CI-MS LR m/z 151 (M + NH_4^+, $C_5H_{15}N_2O_3^+$ requires 151).

4.2.2 *tert*-butyl N-Tosyloxy carbamate

Tert-Butyl N-hydroxycarbamate (5.01 g, 37.6 mmol) and TsCl (7.14 g, 37.5 mmol) in THF (60mL) at 0° C was treated with triethylamine (6.00 mL, 43.2 mmol). The resulting suspension was stirred at 0 °C for 4 h. The solution was filtered through course frit and the filtrate was concentrated under reduced pressure. The residue was dissolved in ethyl acetate, washed with 1N HCl, H_2O, and saturated aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated, and the resulting yellow solid

was purified by FSGC (8:2 PE: acetone) to provide the product as a pure white solid (8.66 g, 80%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 – 7.86 (m, 2H), 7.57 (s, 1H), 7.36 (d, *J* = 8.0 Hz, 2H), 2.46 (s, 3H), 1.30 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 145.9, 129.6, 129.6, 83.9, 27.2, 21.7.

ESI-TOF HRMS m/z 305.1169 ($M + NH_4^+$, $C_{12}H_{21}N_2O_5S^+$ requires 305.1166)

4.2.3 *tert*-butyl N-pterostilbene carbamate (2)

A solution of pterostilbene (604 mg, 2.35 mmol) in THF (4 mL) at 0° C was treated with LiHMDS (7.00 mL, 7.00 mmol, 1.0M in THF).The solution was stirred for 30 min, after which *tert*-Butyl tosyloxycarbamate (2.09 g, 7.28 mmol) was added and the solution was warmed to room temperature and stirred for 4 h. The reaction mixture was diluted with dichloromethane, washed with H_2O and saturated aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated under reduced pressure and purified by FSGC (30:70:5 PE:DCM:acetone) to afford 324 mg of product (37%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 – 7.45 (m, 2H), 7.17 – 7.11 (m, 2H), 7.00 (q, *J* = 16.3 Hz, 2H), 6.66 (d, *J* = 2.2 Hz, 2H), 6.40 (t, *J* = 2.2 Hz, 1H), 3.82 (s, 6H), 1.32 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 161.7, 157.0, 150.7, 139.9, 135.4, 129.5, 128.8, 128.1, 122.2, 105.3, 100.8, 82.7, 56.0, 26.9.

ESI-TOF HRMS m/z 372.1810 ($M+ H^+$, $C_{21}H_{26}NO_5^+$ requires 372.1805).

4.3 Synthesis of N-(2-(2-methoxyethoxy)acetyl)N-pterostilbene carbamic acid

4.3.1 *tert*-butyl N-(2-(2-methoxyethoxy)acetyl)N-pterostilbene carbamate (3a)

To a solution of 2-(2 methoxyethoxy) acetic acid (231 mg, 1.72 mmol) in DCM (4 mL) was added DCC (185 mg, 0.896 mmol). The reaction quickly became cloudy and was stirred at r.t. for 4 h. The reaction was filtered through a coarse frit and the filtrate concentrated under vacuum. Then it was diluted in diethyl ether (4 mL) and filtrated through microfilter (PTFE, 0.45 μm) and concentrated under vacuum. The product was dissolved in DCM (4 mL) and treated with a solution of **2** (74.2 mg, 0.199 mmol) in DCM (4 mL), TEA (110 μL, ρ= 0.73 g/mL 0.792 mmol) and a small amount of DMAP cat. The reaction mixture was stirred at r.t. for 12h and then it was concentrated under vacuum, diluted with ethyl acetate, washed with H_2O, saturated

aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated under reduced pressure and purified by FSGC (90:15 DCM:acetone) to afford 87.2 mg of product (90 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.48 (m, 2H), 7.17 – 7.09 (m, 2H), 7.08 – 6.93 (m, 2H), 6.64 (d, *J* = 2.3 Hz, 2H), 6.39 (t, *J* = 2.2 Hz, 1H), 4.63 (d, *J* = 1.1 Hz, 2H), 3.80 (s, 6H), 3.78 – 3.67 (m, 2H), 3.63 – 3.55 (m, 2H), 3.37 (s, 3H), 1.38 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.0, 161.0, 153.5, 149.3, 138.9, 135.8, 129.5, 127.7, 127.6, 121.37, 104.6, 100.1, 86.1, 71.9, 71.9, 70.8, 59.0, 55.3, 27.1.

ESI-TOF HRMS *m/z* 488.2281 (M+ H^+, $C_{26}H_{33}NO_8^+$ requires 488.2279)

4.3.2 N-(2-(2-methoxyethoxy)acetyl)N-pterostilbene carbamic acid (4a)

A solution of 3a (101 mg, 0.208 mmol) in DCM (2 mL) was treated with triisopropylsilane (50 μL, ρ= 0.77 g/mL 0.244 mmol) and trifluoroacetic acid (2 mL) at 0°C. The mixture reaction was allowed to achieve room temperature, was stirred for 7h, and then the solvent was taken off under a stream of nitrogen and washed with DCM and evaporated under vacuum. The reaction mixture was dissolved in DMSO (1mL) and purified by HPLC (program: 10→100%B (30 min); where B=ACN and A=H_2O) to afford 39.6 mg of product (44%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.5 Hz, 2H), 7.19 (d, *J* = 8.6 Hz, 2H), 7.01 (q, *J* = 16.3 Hz, 2H), 6.65 (d, *J* = 2.2 Hz, 2H), 6.40 (t, *J* = 2.2 Hz, 1H), 4.68 (s, 2H), 3.82 (s, 6H), 3.77 (dd, *J* = 5.4, 3.4 Hz, 2H), 3.62 (dd, *J* = 5.4, 3.4 Hz, 2H), 3.39 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.1, 161.1, 150.6, 149.3, 139.0, 135.9, 129.6, 127.9, 127.7, 121.6, 104.7, 100.2, 71.9, 71.3, 70.8, 59.0, 55.4.

ESI-TOF HRMS *m/z* 432.1661 (M+ H^+, $C_{22}H_{26}NO_8^+$ requires 432.1653)

4.4 Synthesis of N-(2-(2-(2-methoxyethoxy)ethoxy))acetyl)N-pterostilbene carbamic acid

4.4.1 *tert*-butyl N-(2-(2-(2-methoxyethoxy)ethoxy))acetyl)N-pterostilbene carbamate (3b)

To a solution of 2-(2-(2-methoxyethoxy)ethoxy)acetic acid (489 mg, 2.75 mmol) in DCM (5 mL) was added DCC (286 mg, 1.39 mmol). The reaction quickly became cloudy and was stirred at r.t. for 4 h. The reaction was filtered through a coarse frit and the filtrate concentrated under vacuum. Then, it was diluted in diethyl ether (4

mL) and filtrated through microfilter (PTFE, 0.45 μm), and concentrated under vacuum. The product was dissolved in DCM (5 mL) and treated with a solution of **2**(126 mg, 0.341 mmol) in DCM (1 mL), TEA (189 μL, ρ= 0.73 g/mL 1.362 mmol) and a small amount of DMAP cat. The reaction mixture was stirred at r.t. for 12h and then it was concentrated under vacuum, diluted with ethyl acetate, washed with H_2O, saturated aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated under reduced pressure and purified by FSGC (90:5 DCM:acetone) to afford 151 mg of product (83 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (t, *J* = 5.6 Hz, 2H), 7.15 – 7.08 (m, 2H), 6.98 (q, *J* = 16.4 Hz, 2H), 6.62 (d, *J* = 2.2 Hz, 2H), 6.36 (t, *J* = 2.2 Hz, 1H), 4.62 (s, 2H), 3.77 (s, 6H), 3.75 – 3.64 (m, 4H), 3.64 – 3.59 (m, 2H), 3.54 – 3.48 (m, 2H), 3.33 (s, 3H), 1.36 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 160.8, 153.4, 149.2, 138.8, 135.6, 129.3, 127.5, 121.2, 104.5, 100.0, 86.0, 71.8, 71.7, 70.7, 70.5, 70.3, 58.9, 55.2, 27.0.

4.4.2 N-(2-(2-(2-methoxyethoxy)ethoxy))acetyl)N-pterostilbene carbamic acid (4b)

A solution of **3b** (60.9 mg, 0.115 mmol) in DCM (2 mL) was treated with triisopropylsilane (200 μL, ρ= 0.77 g/mL 0.976 mmol) and trifluoroacetic acid (2 mL) at 0°C. The mixture reaction was allowed to reach room temperature, stirred for 12 h, and then the solvent was taken off under a stream of nitrogen and washed with DCM and evaporated under vacuum. The reaction mixture was dissolved in DMSO (1mL) and purified by preparative HPLC (program: 10→100%B (30 min); where B=ACN and A=H_2O) to afford 23.7 mg of SP047 (44%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.5 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 2H), 7.10 – 6.95 (m, 2H), 6.66 (d, *J* = 1.7 Hz, 2H), 6.41 (s, 1H), 4.68 (s, 2H), 3.88 – 3.76 (m, 8H), 3.74 – 3.66 (m, 2H), 3.67 – 3.60 (m, 2H), 3.58 (d, *J* = 5.0 Hz, 2H), 3.40 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 161.1, 150.7, 149.4, 139.1, 135.9, 129.6, 127.9, 127.7, 121.6, 104.8, 100.3, 72.2, 71.8, 71.3, 71.1, 70.2, 59.0, 55.5.

ESI- LRMS *m/z* 493.2183 (M+ NH_4^+, $C_{24}H_{33}N_2O_9^+$ requires 493.2181)

4.5 Synthesis of N-(acetyl) N-pterostilbene carbamic acid

4.5.1 *tert*-butyl N-acetyl N-pterostilbene carbamate (5)

A solution of **2** (95.5 mg, 0.257 mmol) in DCM (10 mL) was treated with acetic anhydride (128 μL, ρ= 1.08 g/mL 1.354 mmol), TEA (190 μL, ρ= 0.73 g/mL 1.370 mmol) and a small amount of DMAP cat. The mixture reaction was stirred at r.t. for 12h and then it was concentrated under vacuum, diluted with ethyl acetate, washed with H_2O, saturated aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated under reduced pressure and purified by FSGC (40:50:5 PE:DCM:acetone) to afford 92.9 mg of product (87%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.57 – 7.51 (m, 2H), 7.19 – 7.13 (m, 2H), 7.12 – 6.96 (m, 2H), 6.67 (d, *J* = 2.2 Hz, 2H), 6.41 (t, *J* = 2.2 Hz, 1H), 3.83 (s, 6H), 2.53 (s, 3H), 1.39 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.6, 161.0, 153.5, 149.6, 139.0, 135.6, 129.4, 127.8, 127.6, 121.4, 104.6, 100.1, 85.7, 55.4, 27.0, 25.3.

ESI-TOF HRMS *m/z* 414.1907 (M+ H^+, $C_{23}H_{28}NO_6^+$ requires 414.1911)

4.5.2 N-(acetyl) N-pterostilbene carbamic acid (6)

A solution of **5** (115 mg, 0.278 mmol) in DCM (2 mL) was treated with triisopropylsilane (200 μL, ρ= 0.77 g/mL 0.976 mmol) and trifluoroacetic acid (2 mL) at -10°C. The mixture reaction was allowed to reach room temperature and was stirred for 12h, then the solvent was taken off under a stream of nitrogen and washed with DCM and evaporated under vacuum. The mixture purified by FSGC (40:65:5 PE:DCM:acetone) to afford a mix of product and parent phenol. The product was purified by HPLC (program: 10→100%B (30 min); where B=MeOH and A=H_2O) to afford 46.2 mg of product (54%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (dd, *J* = 6.6, 4.7 Hz, 2H), 7.24 – 7.20 (m, 2H), 7.04 (q, *J* = 16.3 Hz, 2H), 6.67 (d, *J* = 2.2 Hz, 2H), 6.42 (t, *J* = 2.2 Hz, 1H), 3.84 (s, 6H), 2.64 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 168.8, 161.0, 149.4, 149.2, 138.9, 135.9, 129.5, 127.8, 127.6, 121.6, 104.6, 100.2, 55.4, 23.6.

ESI-TOF HRMS *m/z* 358.1286 (M+ H^+, $C_{19}H_{20}NO_6^+$ requires 358.1285)

4.6 Synthesis of N-Boc protected amino acydil tert-butyloxy hydroxamate of pterostilbene

4.6.1 *tert*-butyl N-(Boc-Ala) N-pterostilbene carbamate (7a)

To a solution of Boc-(L)-Ala (2.03 g, 10.7 mmol) in DCM (25 mL) was added DCC (1.31 g, 6.38 mmol). The reaction quickly became cloudy and was stirred at r.t. for 4 h. The reaction was filtered through a coarse frit and the filtrate concentrated under vacuum. Then it was diluted in diethyl ether (5 mL) and filtrated through microfilter (PTFA, 0.45 μm), and concentrated under vacuum. The anhydride was dissolved in DCM (20 mL) and treated with a solution of **2** (500 mg, 1.35 mmol) in DCM (20 mL), TEA (307 μL, ρ= 0.73 g/mL 2.217 mmol) and a small amount of DMAP cat. The mixture reaction was stirred at r.t. for 12h and then it was concentrated under vacuum, diluted with ethyl acetate, washed with H_2O, saturated aqueous NaCl, and dried ($MgSO_4$). The solvent was removed under reduced pressure and purified by FSGC (80:30:5 PE: EtOAc: acetone) to afford 548 mg of product (75 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 8.4 Hz, 2H), 7.20 – 7.15 (m, 2H), 7.03 (q, *J* = 16.3 Hz, 2H), 6.67 (d, *J* = 2.2 Hz, 2H), 6.41 (t, *J* = 2.2 Hz, 1H), 5.24 (s, 1H), 3.83 (s, 6H), 1.49 – 1.45 (m, 3H), 1.44 (s, 9H), 1.39 (s, 9H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 175.0, 161.0, 155.0, 153.1, 149.5, 139.0, 135.7, 129.4, 127.8, 127.6, 121.4, 104.6, 100.2, 86.1, 79.8, 55.3, 50.8, 28.3, 26.9, 19.3.

ESI-TOF HRMS *m/z* 565.2515 (M+ Na^+, $C_{29}H_{38}N_2NaO_8^+$ requires 565.2520)

4.6.2 *tert*-butyl N-(Boc-Ile) N-pterostilbene carbamate (7b)

To a solution of Boc-(L)-Ile (113 mg, 0.487 mmol) in DCM (1 mL) was added DCC (105 mg, 0.508 mmol). The reaction quickly became cloudy and was stirred at r.t. for 4 h. The reaction was filtered through a coarse frit and the filtrate concentrated under vacuum. Then it was diluted in diethyl ether (4 mL) and filtrated through microfilter (PTFE, 0.45 μm), and concentrated under vacuum. The product was dissolved in DCM (2 mL) and treated with a solution of **2**(19.4 mg, 0.0520 mmol) in DCM (2 mL), TEA (29 μL, ρ= 0.73 g/mL 0.209 mmol) and a small amount of DMAP cat. The mixture reaction was stirred at r.t. for 12h and then it was concentrated under vacuum, diluted with ethyl acetate, washed with H_2O, saturated aqueous NaCl, and dried ($MgSO_4$). The solvent was removed under reduced pressure and purified by FSGC (40:65:5 PE:DCM:acetone and 3:1 PE:acetone) to afford 22.3 mg of product (73 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.5 Hz, 2H), 7.18 (d, *J* = 8.2 Hz, 2H), 7.03 (q, *J* = 16.3 Hz, 2H), 6.67 (d, *J* = 2.3 Hz, 2H), 6.41 (t, *J* = 2.2 Hz, 1H), 5.16 (m, 1H), 3.83 (s, 6H), 1.98 (m,1H), 1.62 – 1.37 (m, 20H), 1.08 – 0.99 (m, 3H), 0.93 – 0.84 (m, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.3, 161.1, 155.9, 149.7, 139.1, 135.8, 129.5, 128.0, 127.7, 127.6, 121.5, 104.8, 100.3, 79.9, 77.3, 58.8, 55.5, 31.0, 27.1, 27.1, 23.3, 16.3, 11.8.

ESI-TOF HRMS *m/z* 607.2988 (M+ Na^+, $C_{32}H_{44}N_2NaO_8^+$ requires 607.2990)

4.7 Synthesis of *tert*-butyl N-(methyl) N-pterostilbene carbamate (8)

A solution of **2** (38.9 mg, 0.105 mmol) in THF (1 mL) at 0 °C was treated with LiHMDS (104 μL, 0.104 mmol, 1.0M in THF) and was stirred for 30 min. MeI (13 μL, 0.0209 mmol) was added, and then the solution was warmed to room temperature and stirred for 3 h. The solvent was removed under a stream of N_2, and the residue was purified by PTLC (50:50:5 PE:DCM:acetone) to afford the product (23 mg, 57%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 – 7.47 (m, 2H), 7.15 – 7.11 (m, 2H), 7.02 (q, *J* = 16.3 Hz, 2H), 6.66 (d, *J* = 2.2 Hz, 2H), 6.40 (t, *J* = 2.2 Hz, 1H), 3.83 (s, 6H), 3.32 (s, 3H), 1.36 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 161.0, 158.1, 150.6, 139.2, 134.7, 128.7, 128.2, 127.4, 121.7, 104.5, 100.1, 82.6, 55.4, 41.2, 27.1.

ESI-TOF HRMS *m/z* 386.1962 (M+ H^+, $C_{22}H_{27}NO_5^+$ requires 386.1962)

4.8 Synthesis of tert-butyl hydroxamate of pterostilbene

4.8.1 *tert*-butyl Pivaloyl(N-tosyloxy)carbamate

A solution of *tert-Butyl* N-Tosyloxycarbamate (1.03 g, 3.49 mmol) and trimethylacetyl chloride (2.14 mL, 17.4 mmol) in THF (15 mL) at 0 °C was treated with triethylamine. The solution was warmed to room temperature and stirred overnight (16 h). The reaction mixture was diluted with ethyl acetate, washed with H_2O and saturated aqueous NaCl, and dried ($MgSO_4$). The reaction mixture was concentrated and the residue purified by FSGC (1:1 PE: DCM) to provide the product as a white solid (1.11 g, 97%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (d, *J* = 8.4 Hz, 2H), 7.37 (d, *J* = 8.0 Hz, 2H), 2.46 (s, 3H), 1.33 (s, 9H), 1.29 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 181.2, 151.0, 146.0, 131.3, 129.7, 129.6, 85.5, 43.4, 27.5, 27.4, 21.7.

ESI-TOF HRMS m/z 394.1297 (28.25% M + Na^+, $C_{17}H_{25}NNaO_6S^+$ requires 394.1295) 272.0958 (100% M + H^+ - Boc).

4.8.2 N-(Tosyloxy) pivaloylamide

A vial containing *tert-Butyl* Pivaloyl(N-tosyloxy)carbamate (505 mg, 1.36 mmol) was treated with TFA (2 mL). The reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated under a stream of N_2 to provide the product (346 mg, 91%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.3 Hz, 2H), 7.31 (d, *J* = 8.2 Hz, 2H), 2.41 (s, 3H), 1.06 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 165.1, 140.7, 130.5, 128.9, 126.0, 52.8, 27.5, 21.3.

ESI-MS HR m/z 272.0947 (M +H^+, $C_{12}H_{18}NO_4S^+$ requires 272.0951)

4.8.3 N-pterostilbene pivaloylamide (9)

A solution of Pterostilbene (88.7 mg, 0.346 mmol) in THF (1 mL) at 0 °C was treated with LiHMDS (877 μL, 0.877 mmol, 1.0M in THF). The solution was stirred for 30 min, after which N-(Tosyloxy)pivalamide (239 mg, 0.880 mmol) was added and the solution was warmed to room temperature and stirred for 3 h. The reaction mixture was diluted with DCM, washed with H_2O and saturated aqueous NaCl, and dried ($MgSO_4$). The solution was concentrated and purified by FSGC (1:1 DCM:hexanes +1% TEA) to afford the product as a white solid (24.9 mg, 20%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.6 Hz, 2H), 7.12 (d, *J* = 8.6 Hz, 2H), 7.01 (dd, *J* = 40.1, 16.3 Hz, 2H), 6.66 (d, *J* = 2.2 Hz, 2H), 6.40 (t, *J* = 2.2 Hz, 1H), 3.83 (s, 6H), 1.40 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 160.9, 152.5, 150.5, 139.3, 134.1, 128.4, 128.4, 127.3, 121.9, 104.5, 100.0, 55.3, 50.8, 28.7.

ESI-TOF HRMS m/z 356.1857 (M +H^+, $C_{21}H_{26}NO_4^+$ requires 356.1856)

Conclusions

Eleven new hydroxamate derivatives of pterostilbene have been synthesized and characterized (Fig. 50) with the purpose of studying the properties of the hydroxamate functionality as a protecting group for phenolic hydroxyl groups.

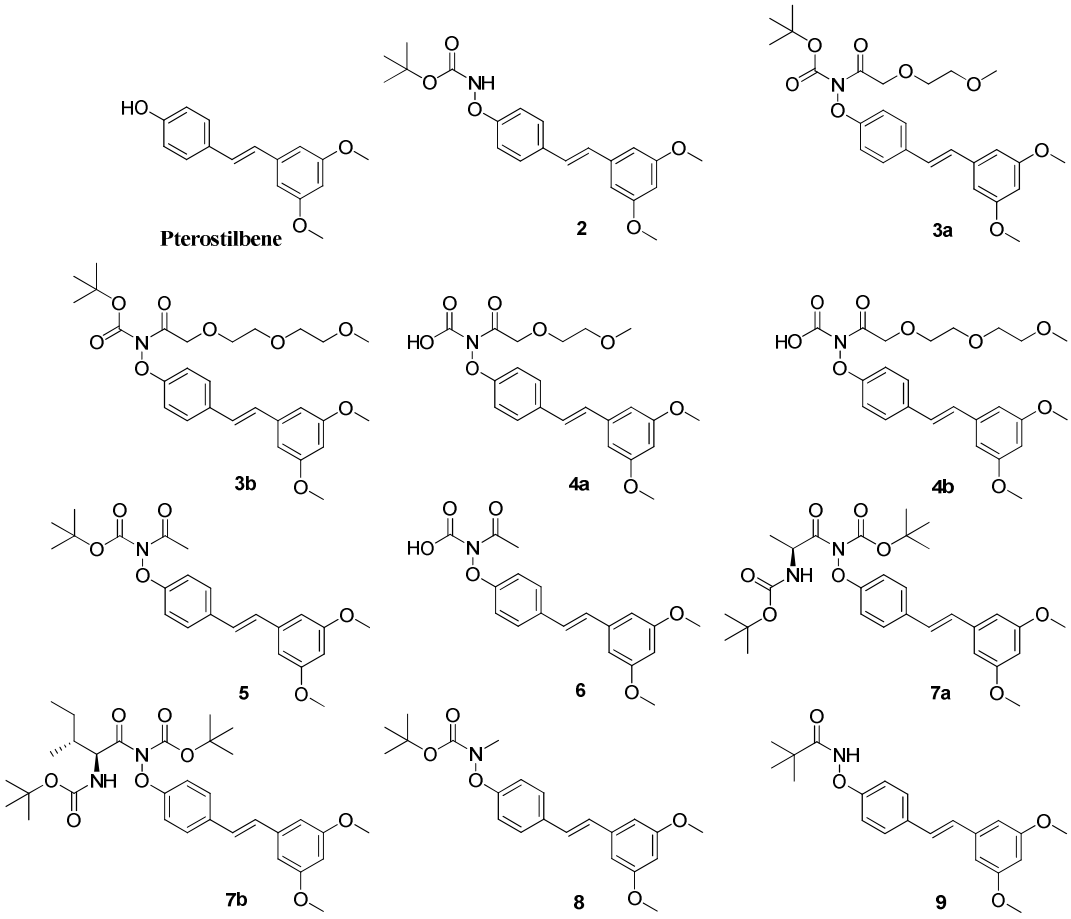


Fig. 50 *Pterostilbene and synthesized hydroxamate derivatives*

The interest in this research is related to the possibility of developing new prodrugs for oral administration. The reactivity of the new derivatives was studied in aqueous media at acidic and neutral pH which are characteristic of the stomach and first intestinal tract, respectively. I have found that the chemical stability of the N-O bond under these conditions changes significantly depending on the substituent groups on the hydroxamate function.

In all cases, with the only exception of derivative **7a**, a good stability for the protecting group was observed with respect to cleavage to regenerate pterostilbene. Other reactions were observed for some of the derivatives: kinetics, intermediates and products of these reactions were investigated by means of HPLC-ESI/MS

analysis. Notably, a cis–trans isomerization about the C-N bond has been identified and rationalized in the case of derivatives **4a**, **4b** and **6**,

Some of the derivatives I have synthesized, in particular derivatives **2**, **8** and **9**, show high stability in aqueous media mimicking gastric and intestinal pH values and are thus useful candidates for further bioassays which are underway. These include studies of stability in blood and pharmacokinetics in rats after oral administration and will be carried out by the group of Dr. Mario Zoratti of the CNR Institute of Neuroscience.

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APPENDIX

ABBREVIATIONS

ACN = Acetonitrile

Boc = tert-butyloxycarbonyl

Boc-Ala-OH = *N*-(*tert*-Butoxycarbonyl)-L-alanine

Boc-Ile-OH = *N*-(*tert*-Butoxycarbonyl)-L-isoleucine

$(Boc)_2O$ = Di-*tert*-butyl dicarbonate

CDCl3 = Deuterochloroform

DCC = Dicyclohexylcarbodiimide

DCM = Dichloromethane

DMAP = Dimethylaminipyridine

DMSO = Dimethylsulphoxide

DMSO-d6 = Hexadeuterodimethyl sulfoxide

ESI-MS = ElectroSpray Ionization Mass Spectrometry

EtOAc = Ethyl Acetate

FSGC = Flash Silica Gel Chromatography

HPLC = High Performance Liquid Chromatography

Ile = L-Isoleucine

LiHMDS = Lithium bis(trimethylsilyl)amide

Ala = L-Alanine

MeOH = Methanol

NMR = Nuclear Magnetic Resonance

PBS = Phosphate Buffer Solution

PTLC = Plate Thin Layer Chromatography

PTS = Pterostilbene

RT = Room Temperature

THF = Tetrahydrofurane

TsCl = *p*-Toluenesulfonyl chloride

UV = Ultraviolet

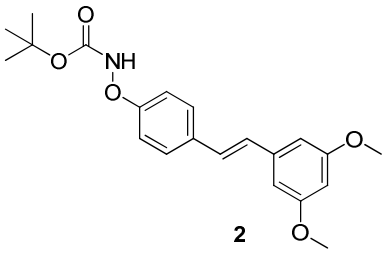
TEA = Triethylamine

TFA = Trifluoroacetic Acid

TIPS = Triisopropylsilane

TLC = Thin Layer Chromatography

ESI MASS AND NMR SPECTRA OF THE SYNTHESIZED COMPOUNDS



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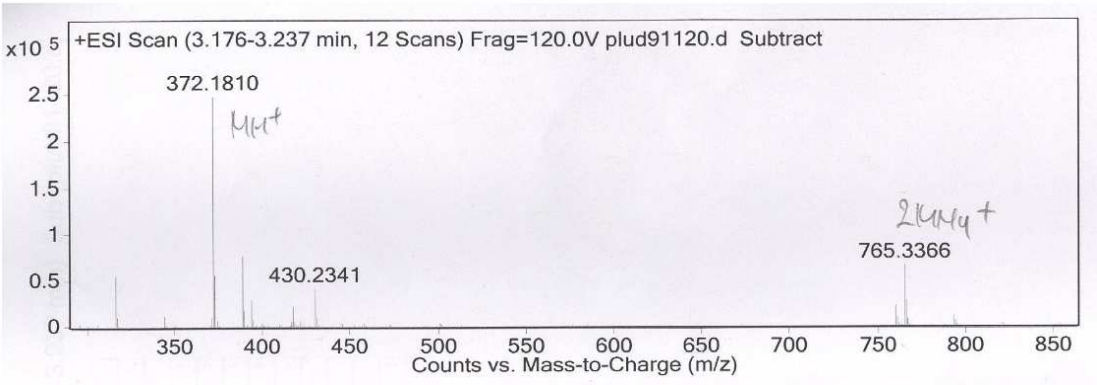

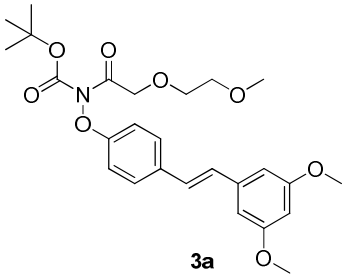

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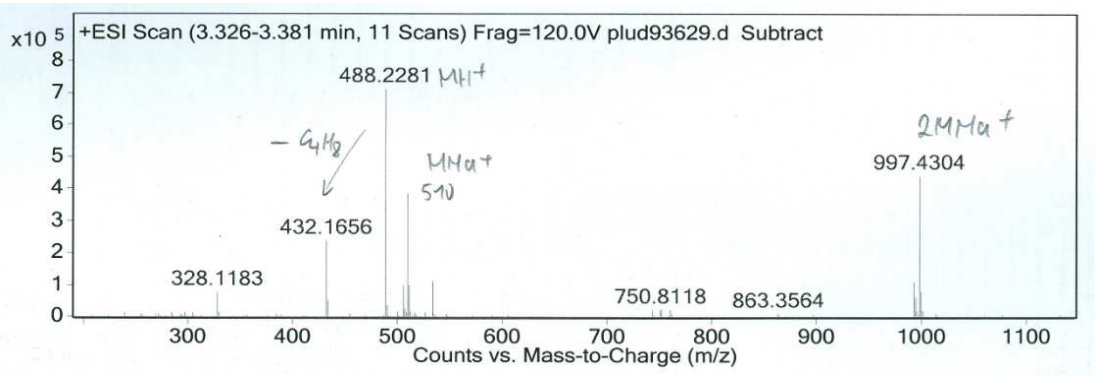

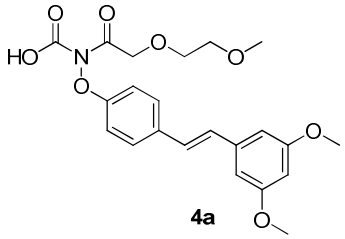

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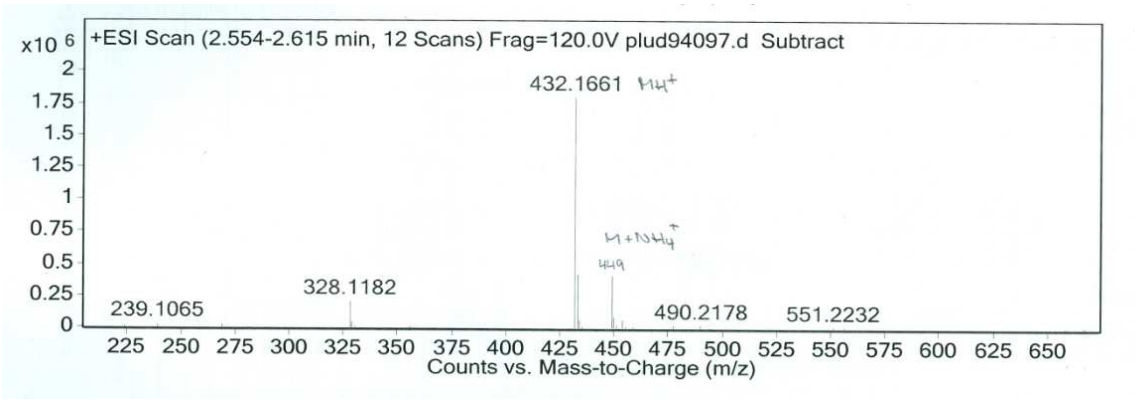
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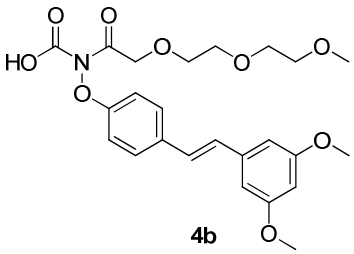
239.1065
490.2178
551.2232
Counts vs. Mass-to-Charge (m/z)



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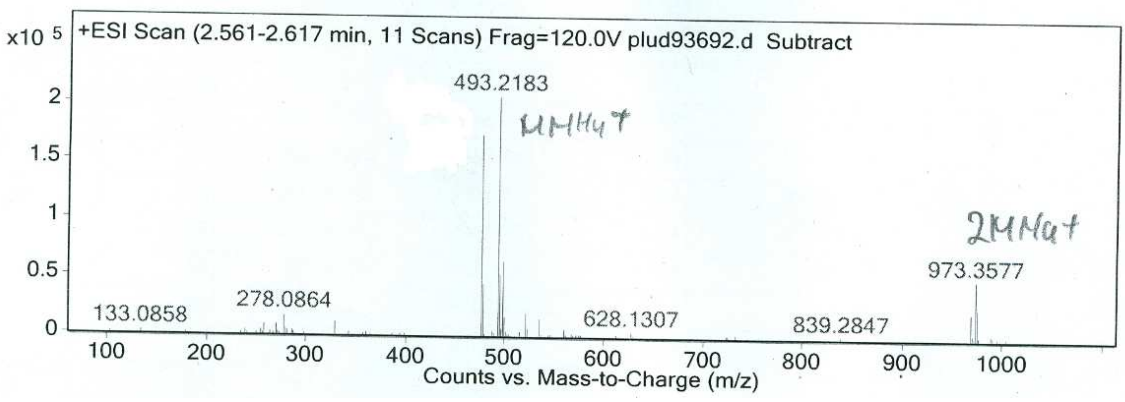
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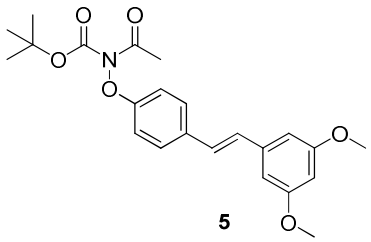
133.0858
628.1307
839.2847
Counts vs. Mass-to-Charge (m/z)



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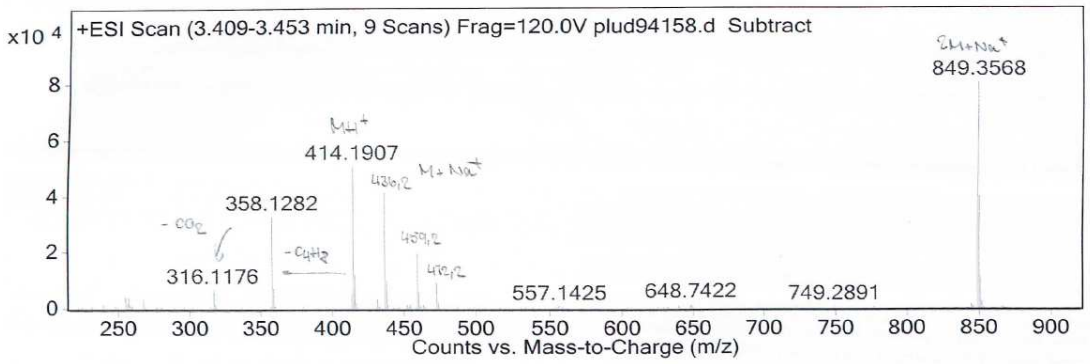
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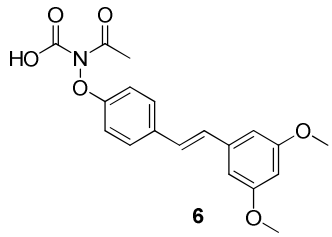
358.1282
316.1176
557.1425
648.7422
749.2891
Counts vs. Mass-to-Charge (m/z)



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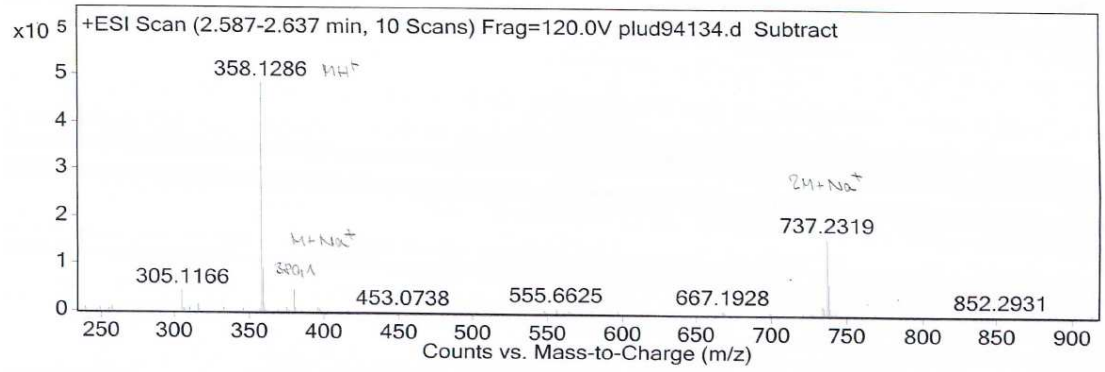
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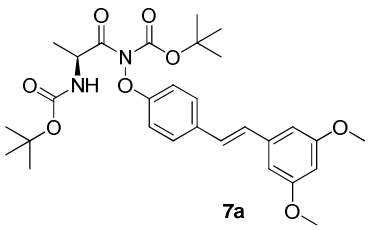
852.2931
Counts vs. Mass-to-Charge (m/z)



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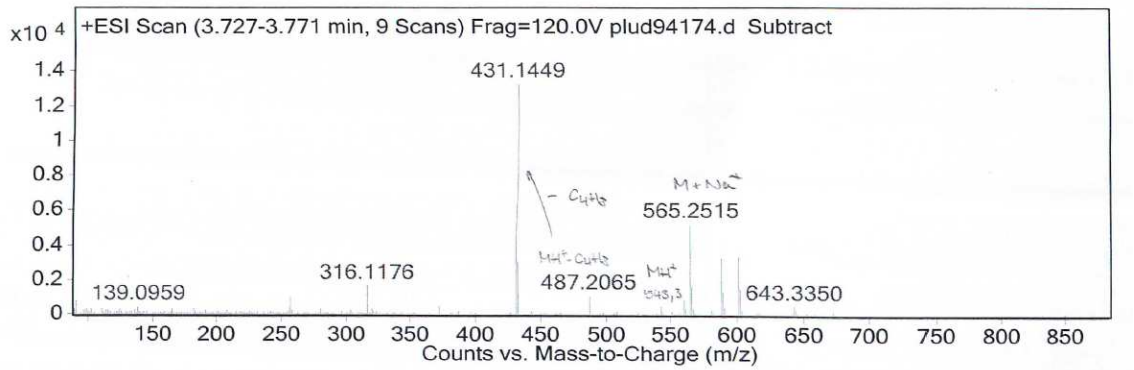
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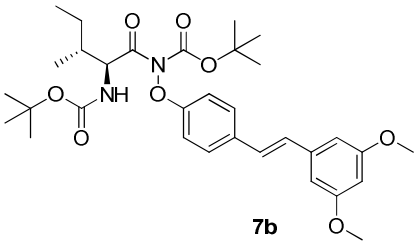
Counts vs. Mass-to-Charge (m/z)



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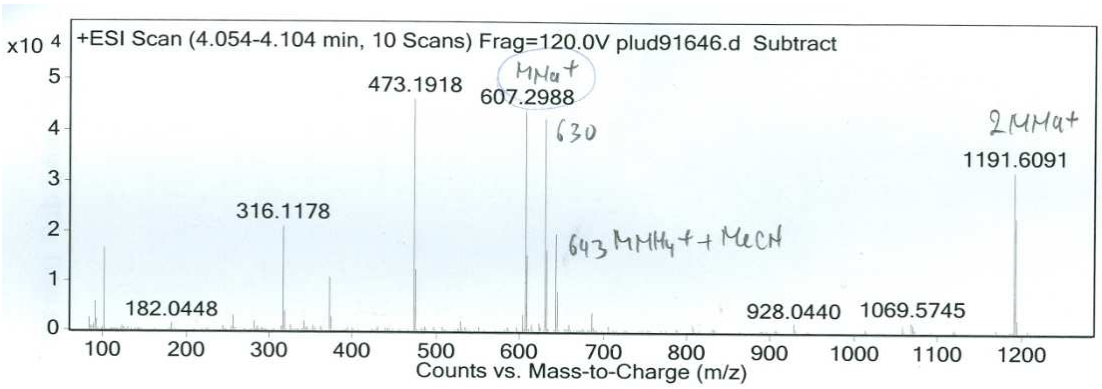
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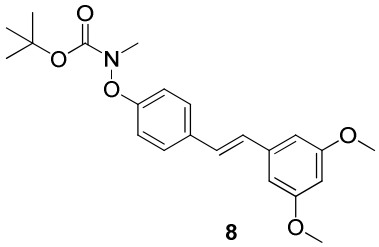
1069.5745
1191.6091
Counts vs. Mass-to-Charge (m/z)



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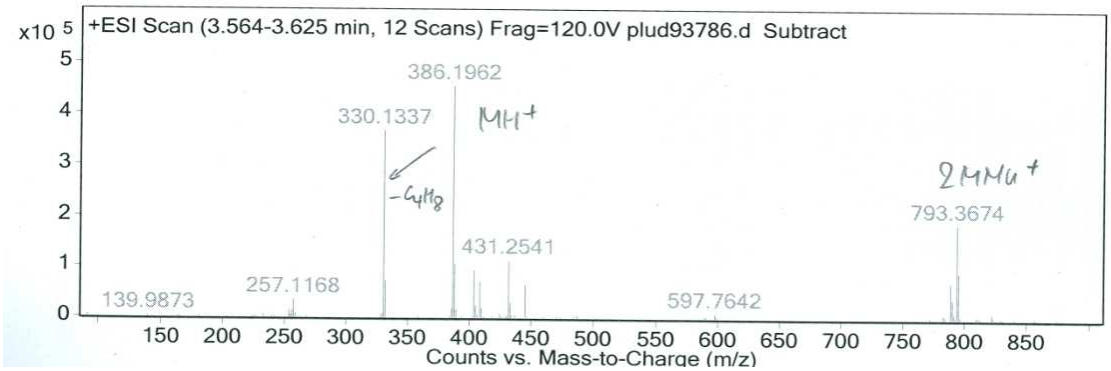
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386.1962
330.1337
MH+
-C4H8
2MH+
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139.9873
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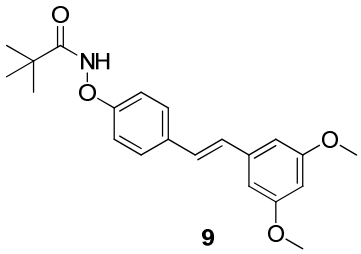
597.7642
Counts vs. Mass-to-Charge (m/z)



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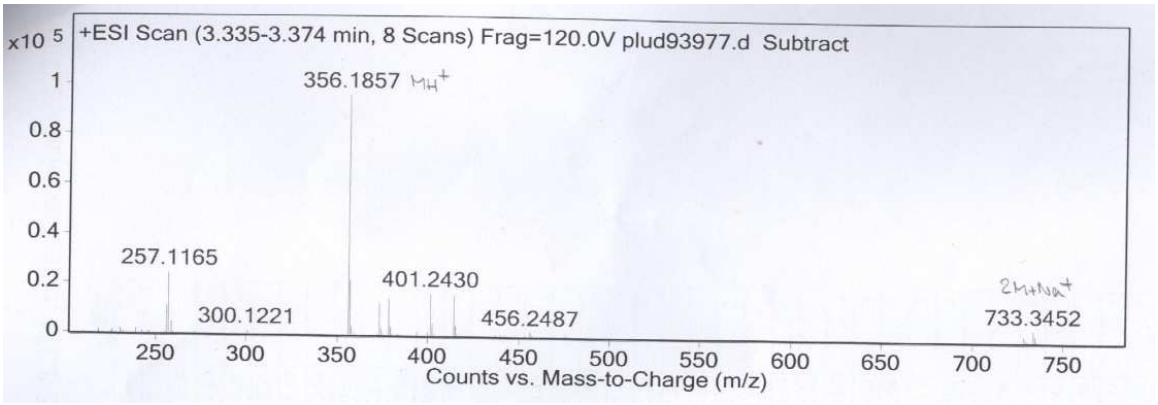
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257.1165
401.2430
300.1221
456.2487
2MH+Na+
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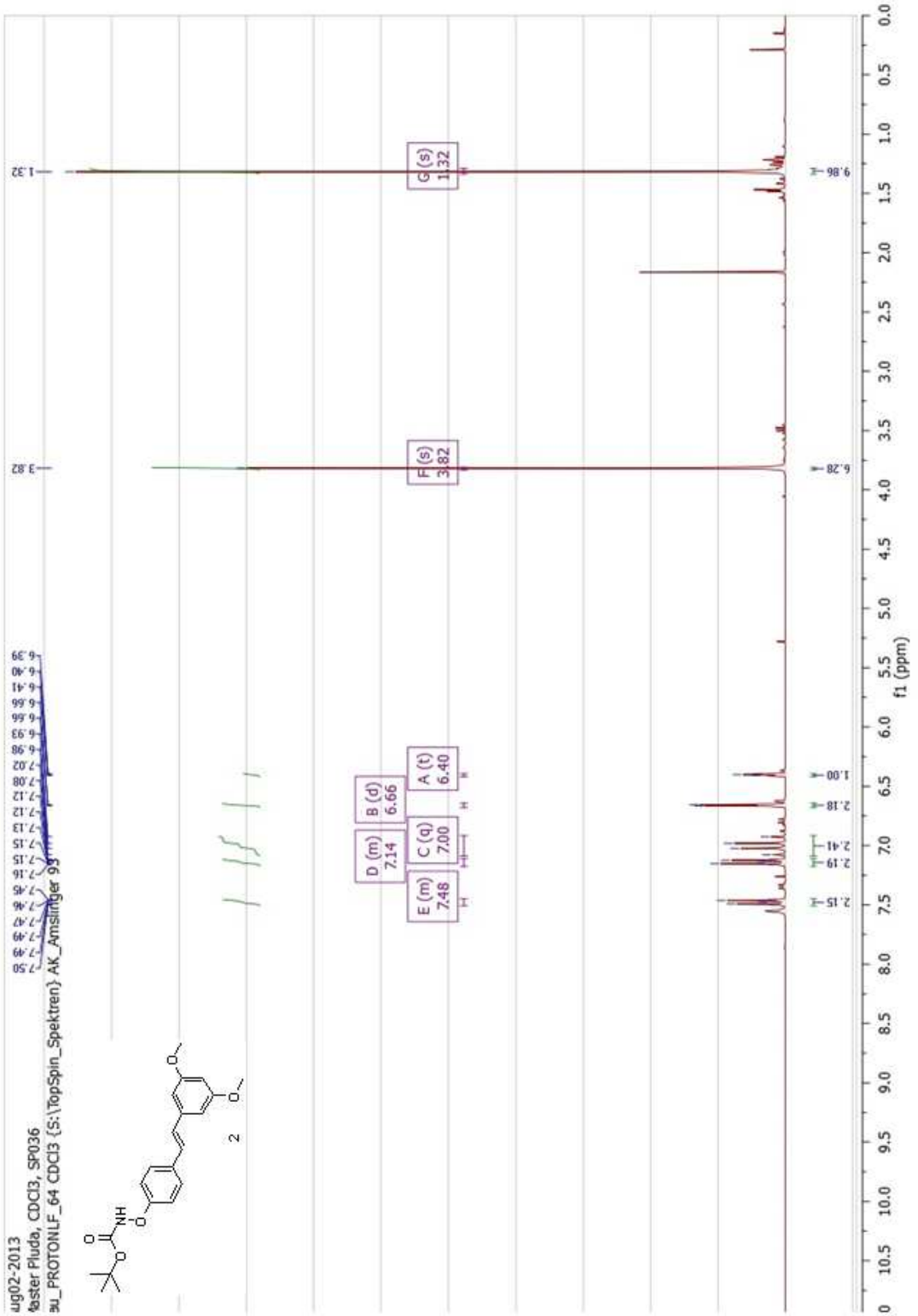
Counts vs. Mass-to-Charge (m/z)

Product 2 ^{1}H NMR (300 MHz, $CDCl_3$)



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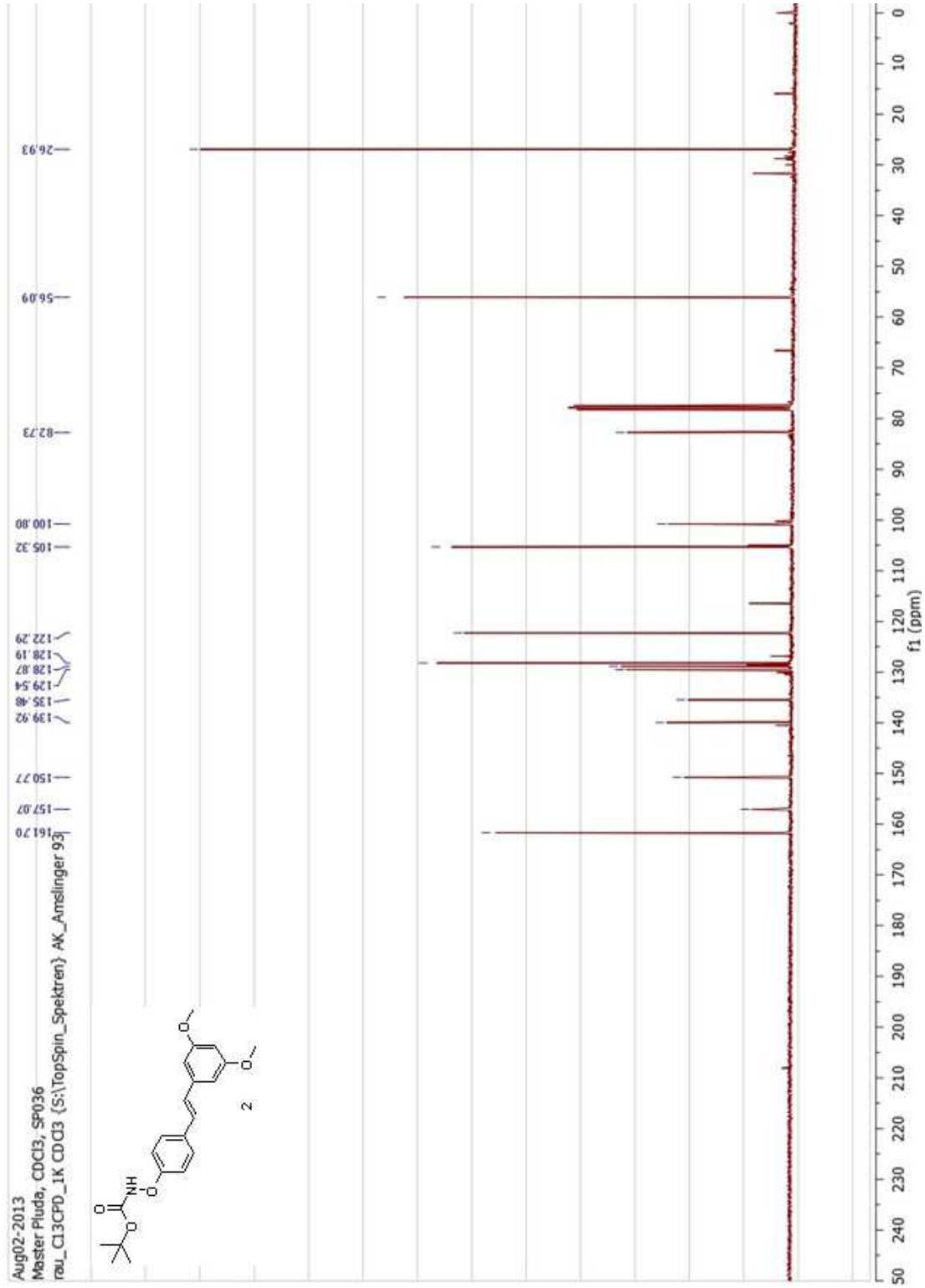

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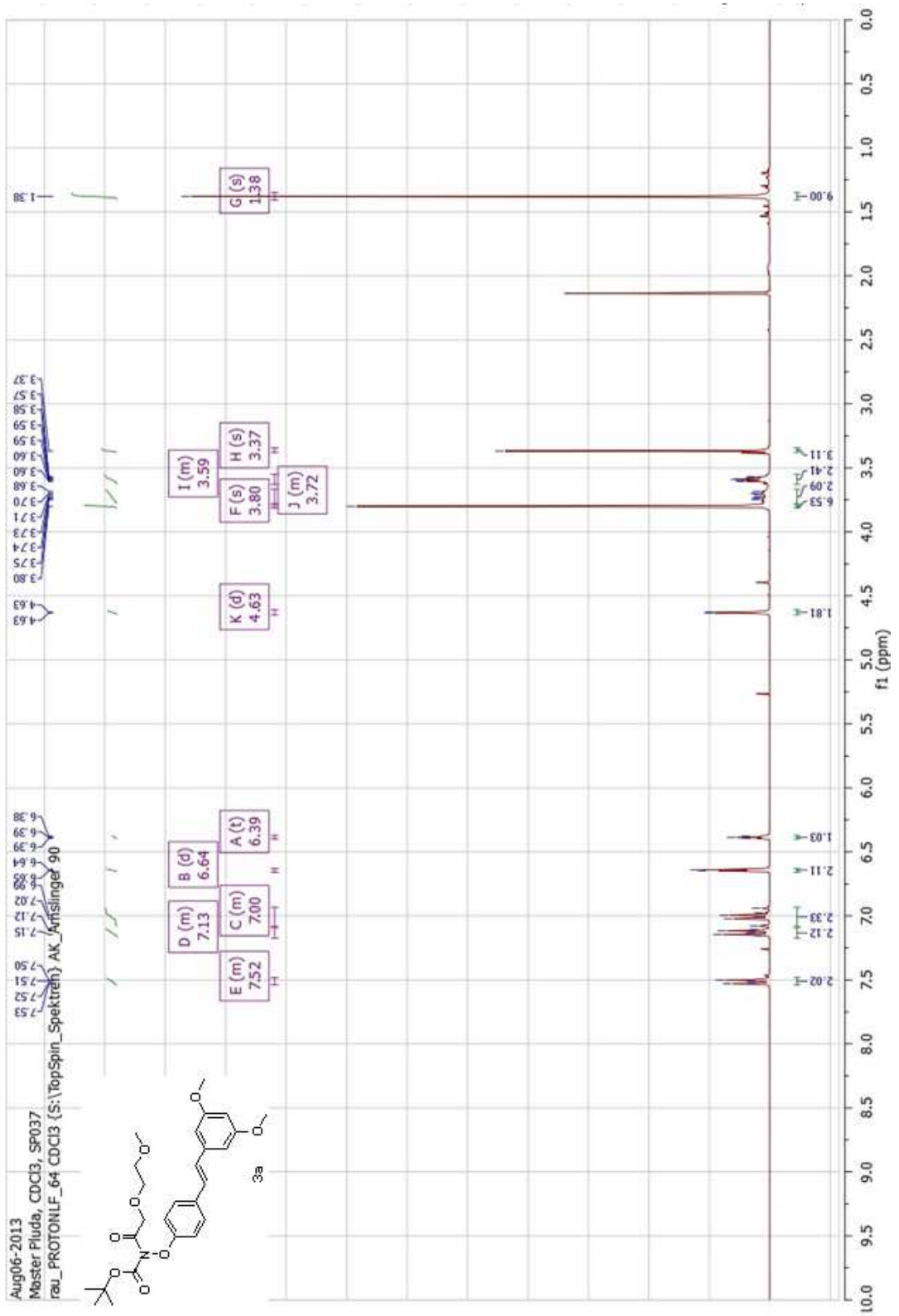

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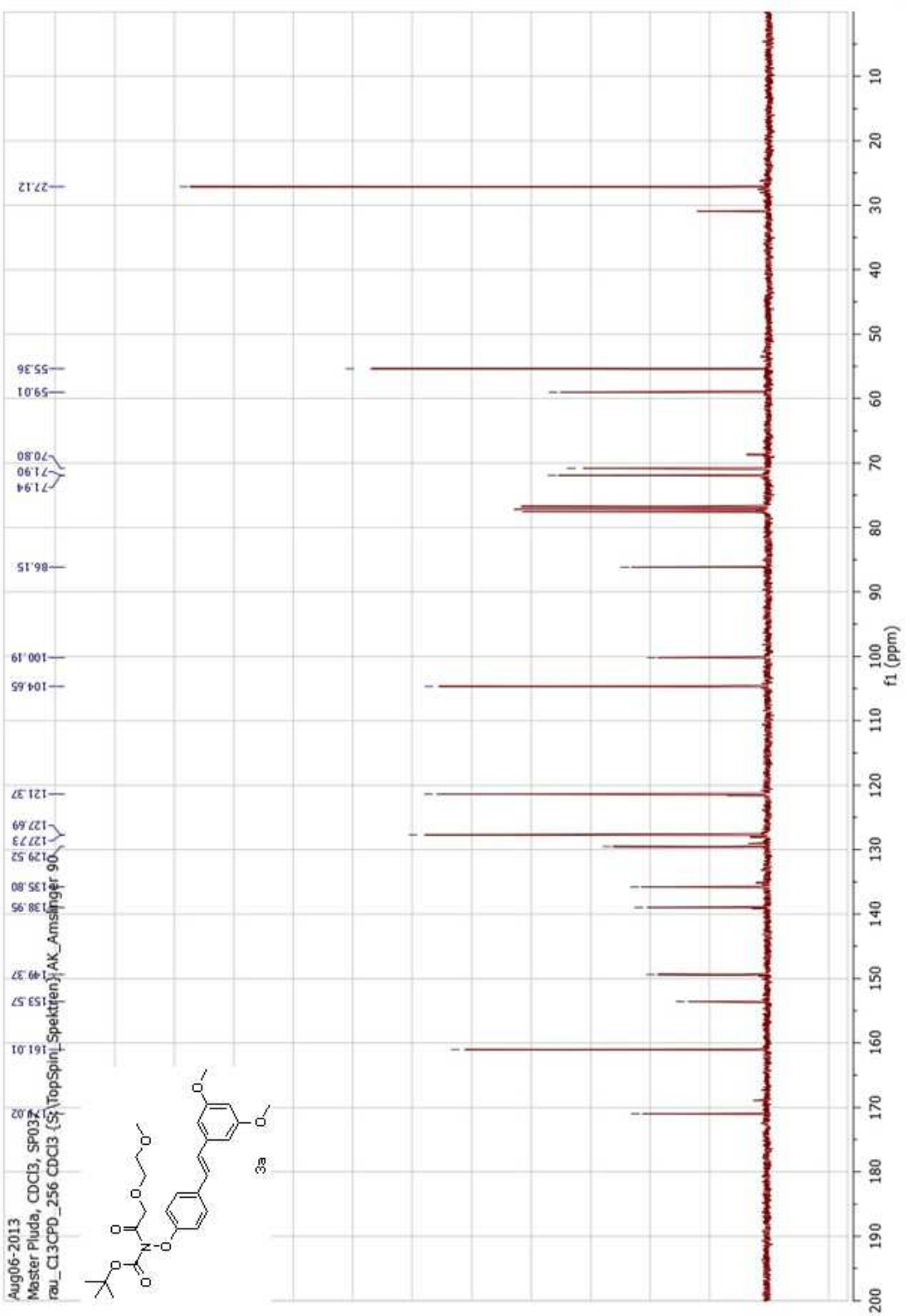

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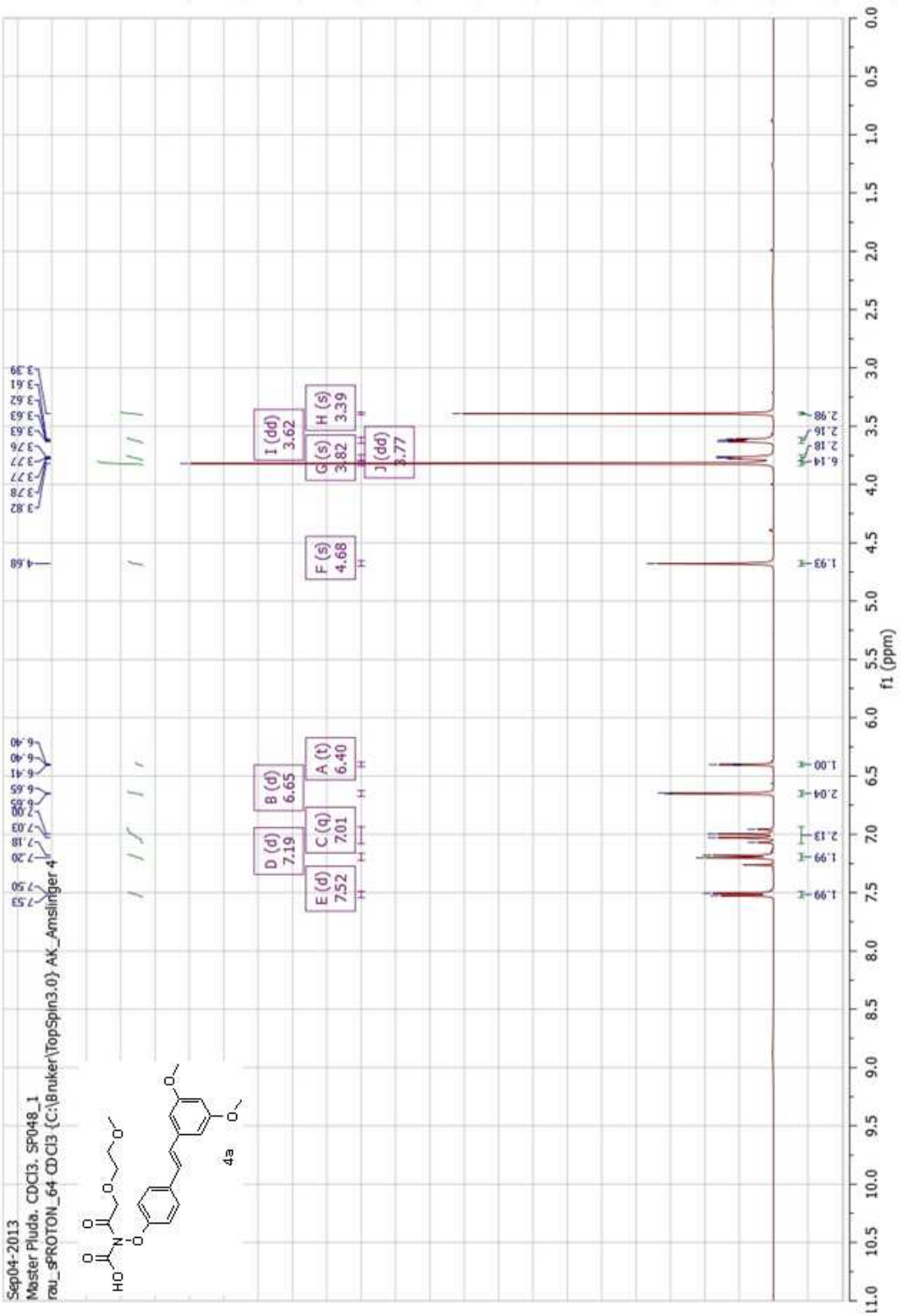

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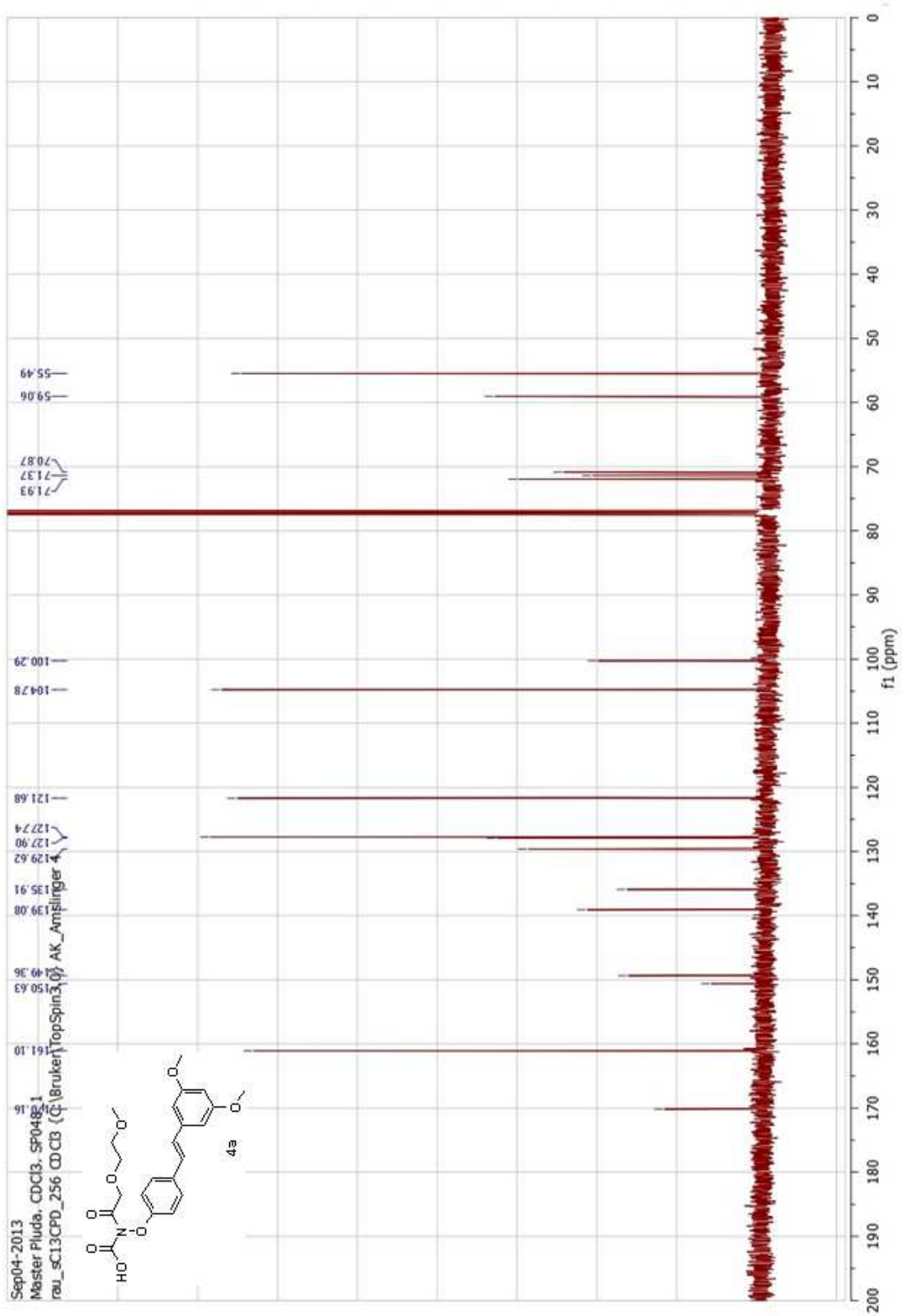

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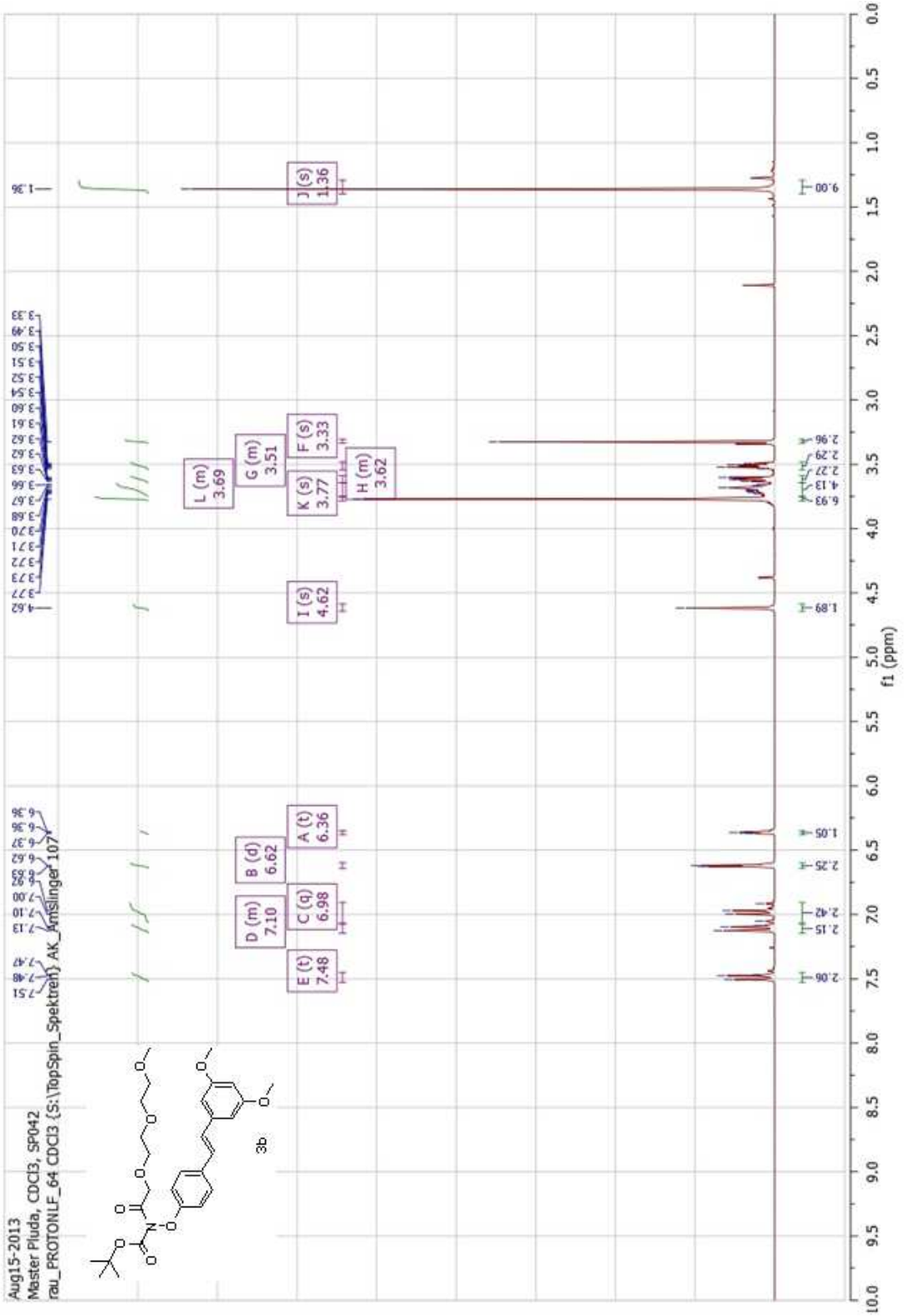
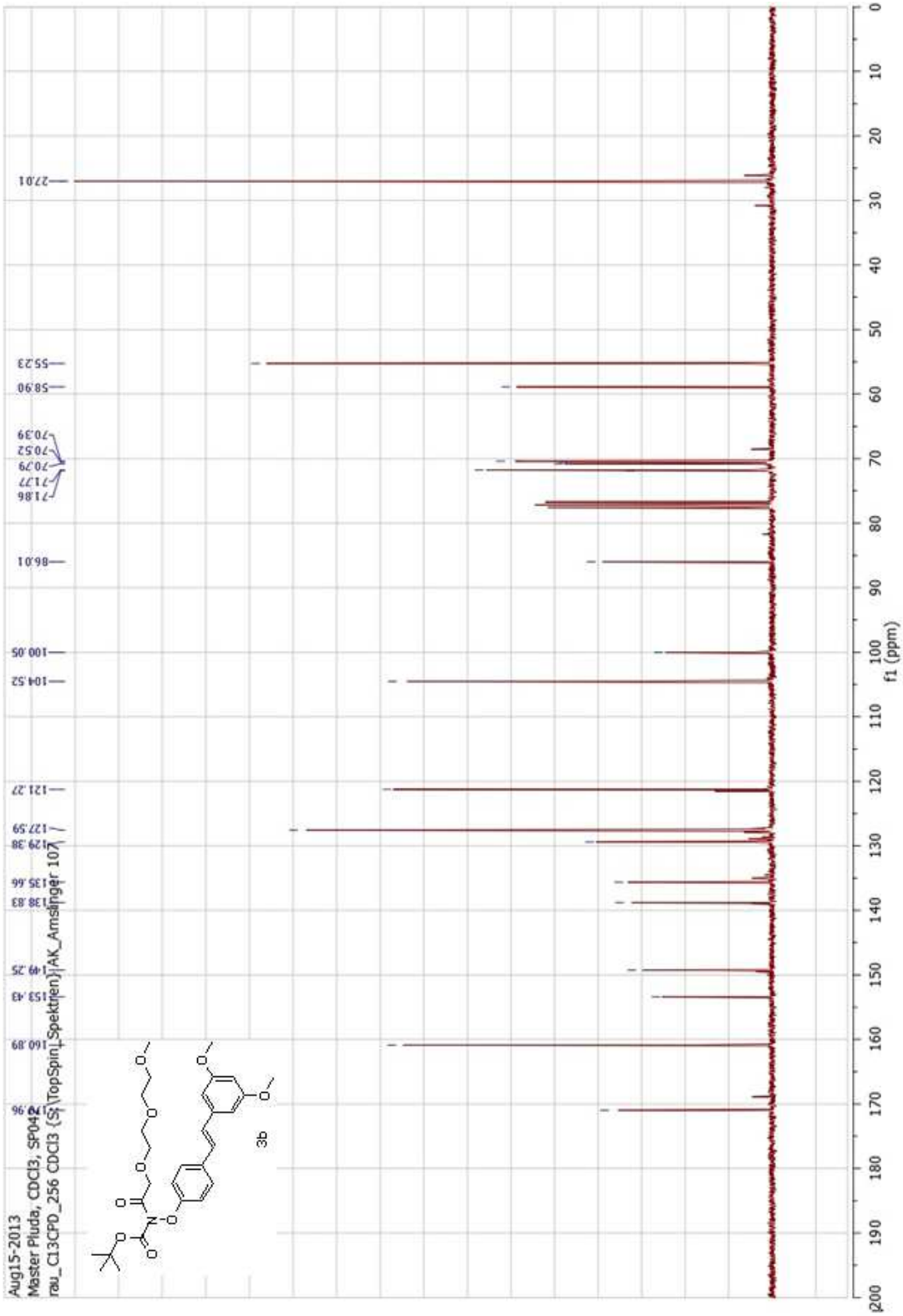


Product 3b ^{13}C NMR (75 MHz, $CDCl_3$)



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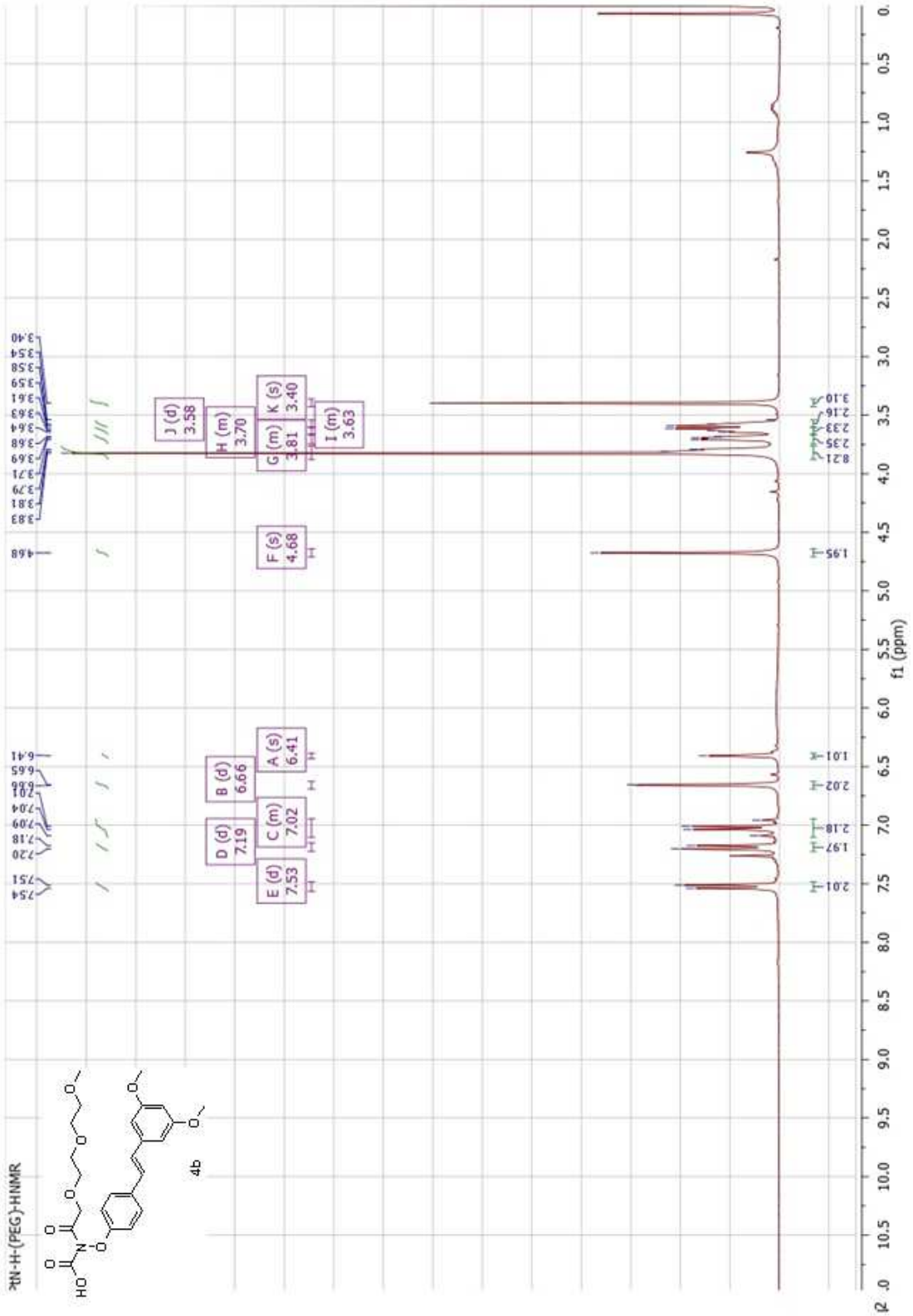
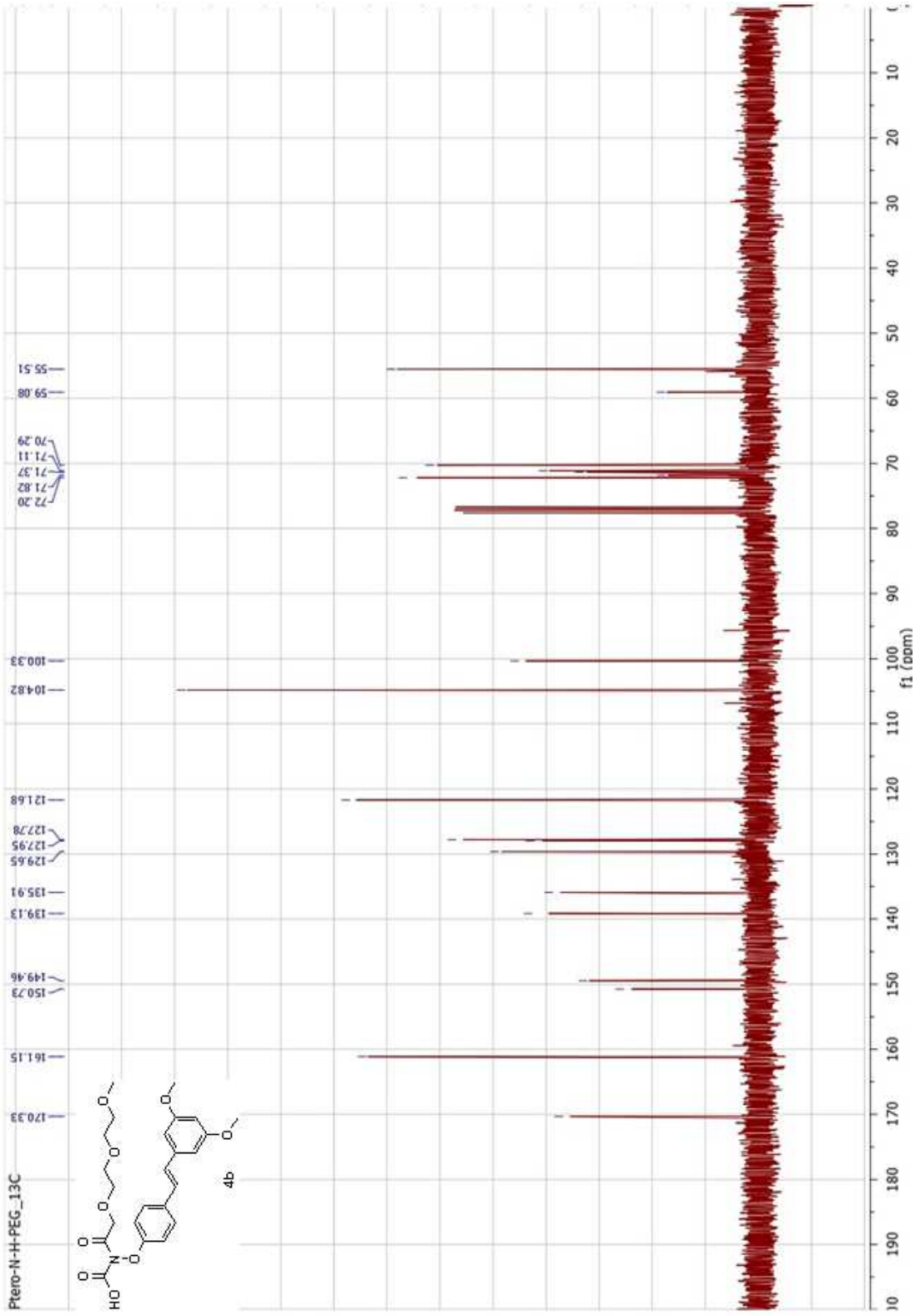
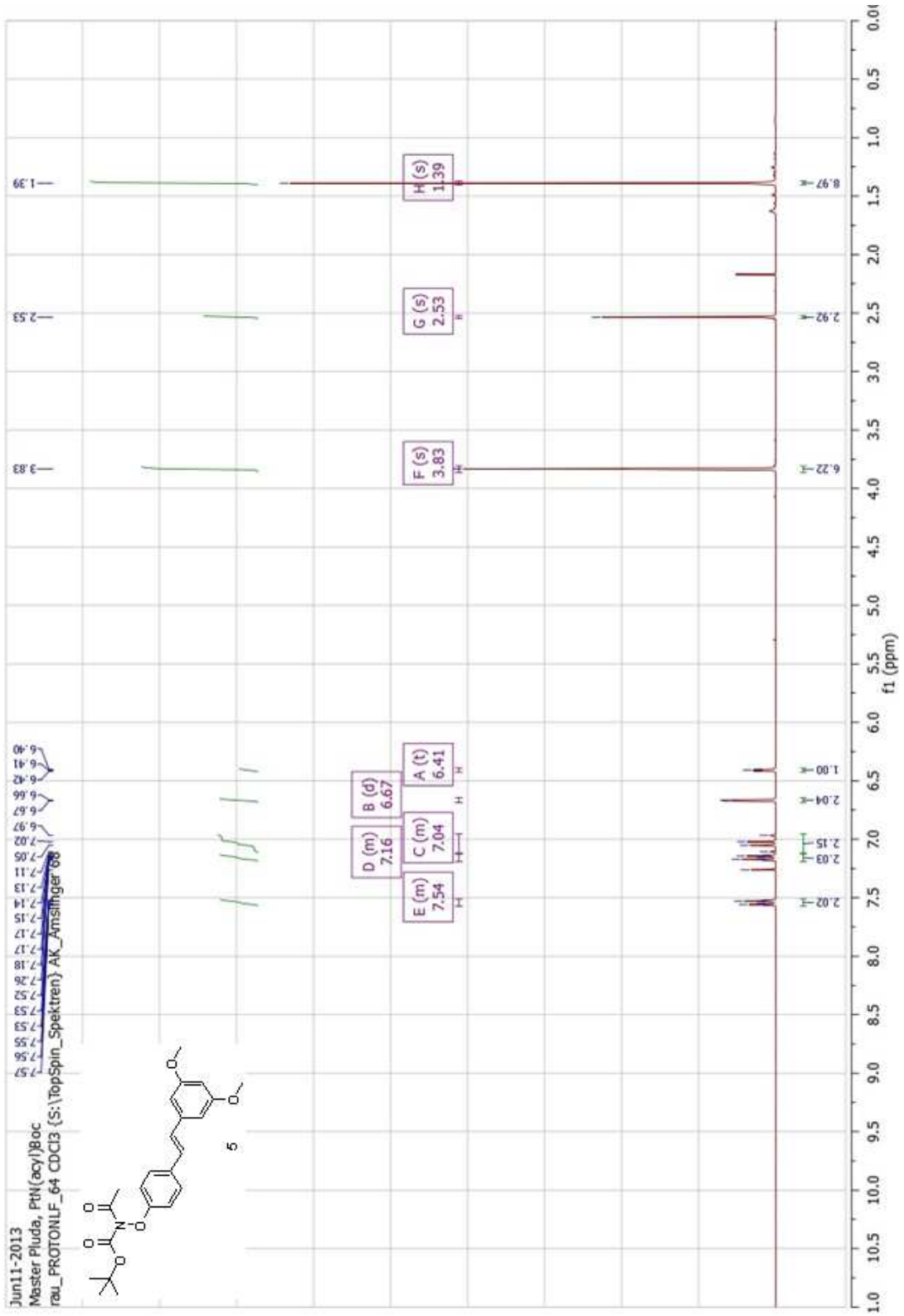


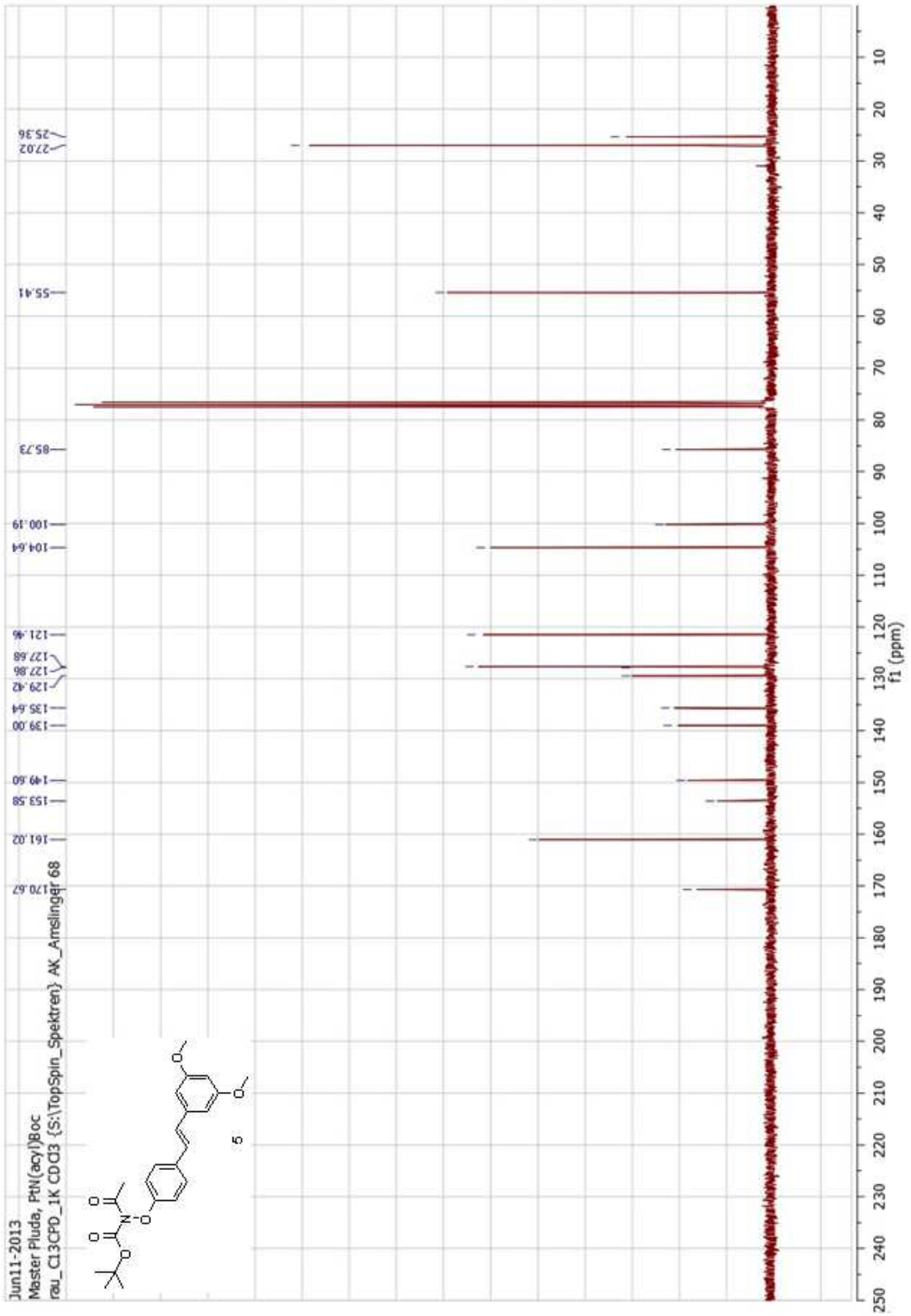
Product 4b ^{13}C NMR (75 MHz, $CDCl_3$)



Product 5 ^{1}H NMR (300 MHz, $CDCl_3$)



Product 5 ^{13}C NMR (75 MHz, $CDCl_3$)



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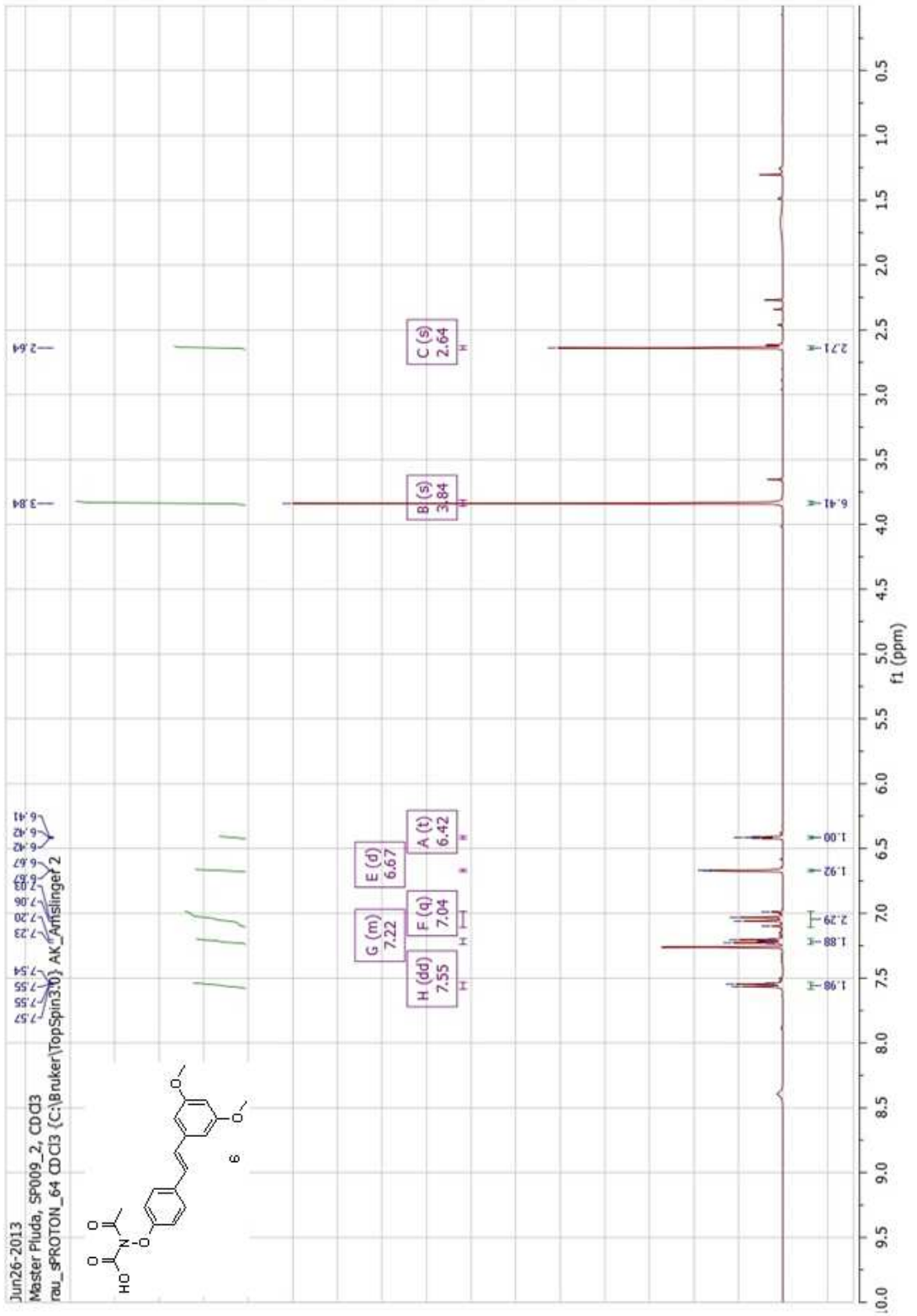

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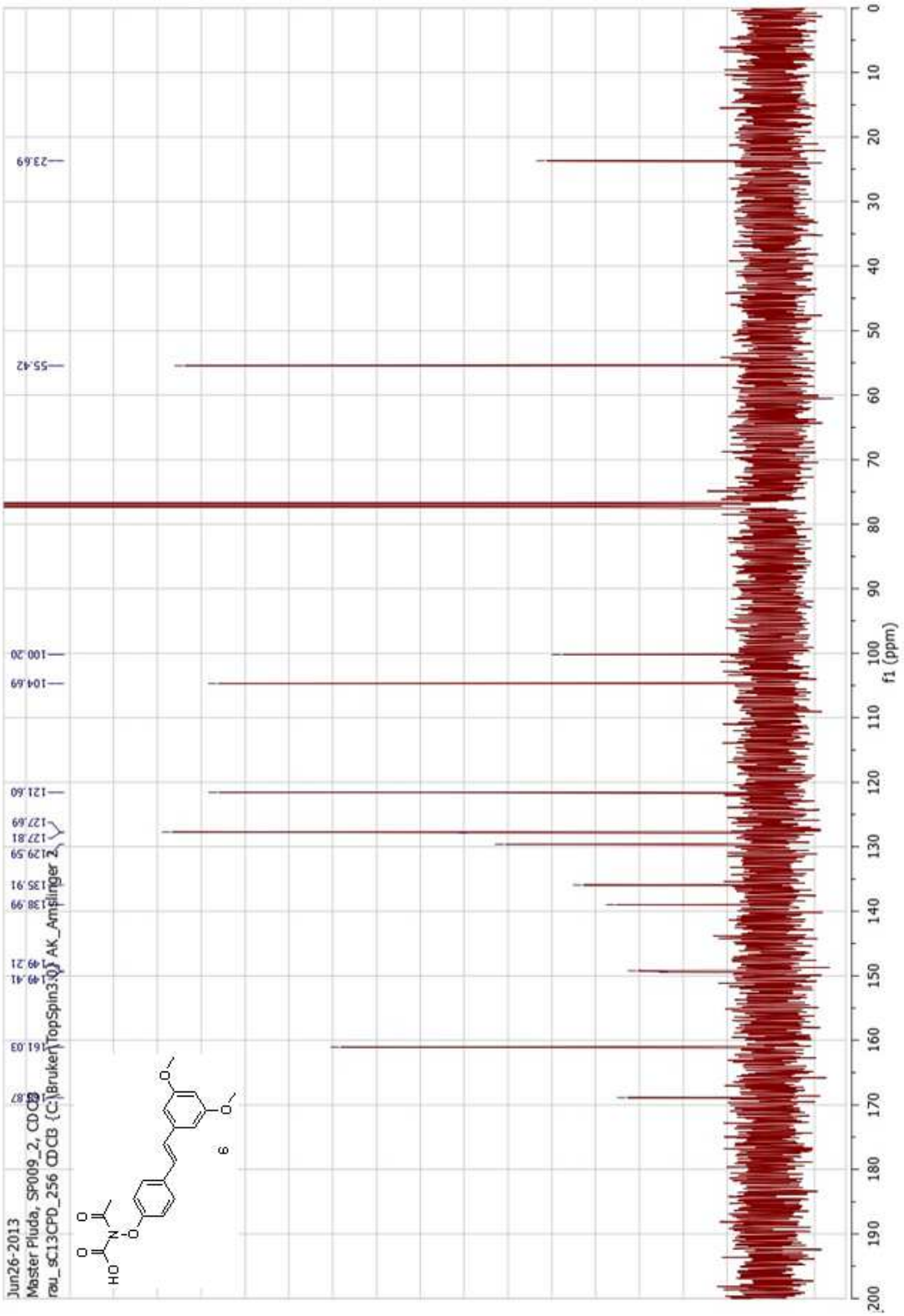

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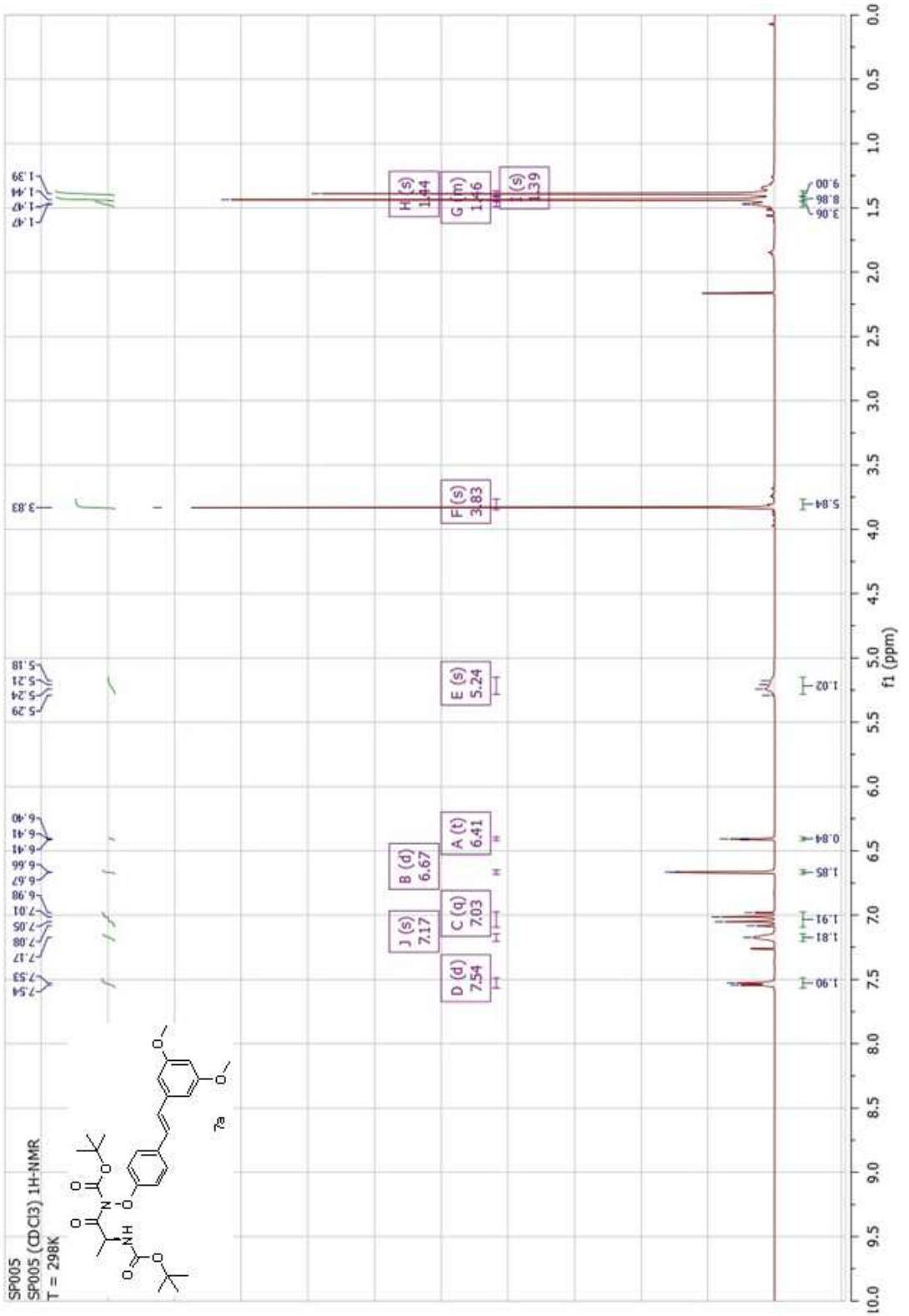

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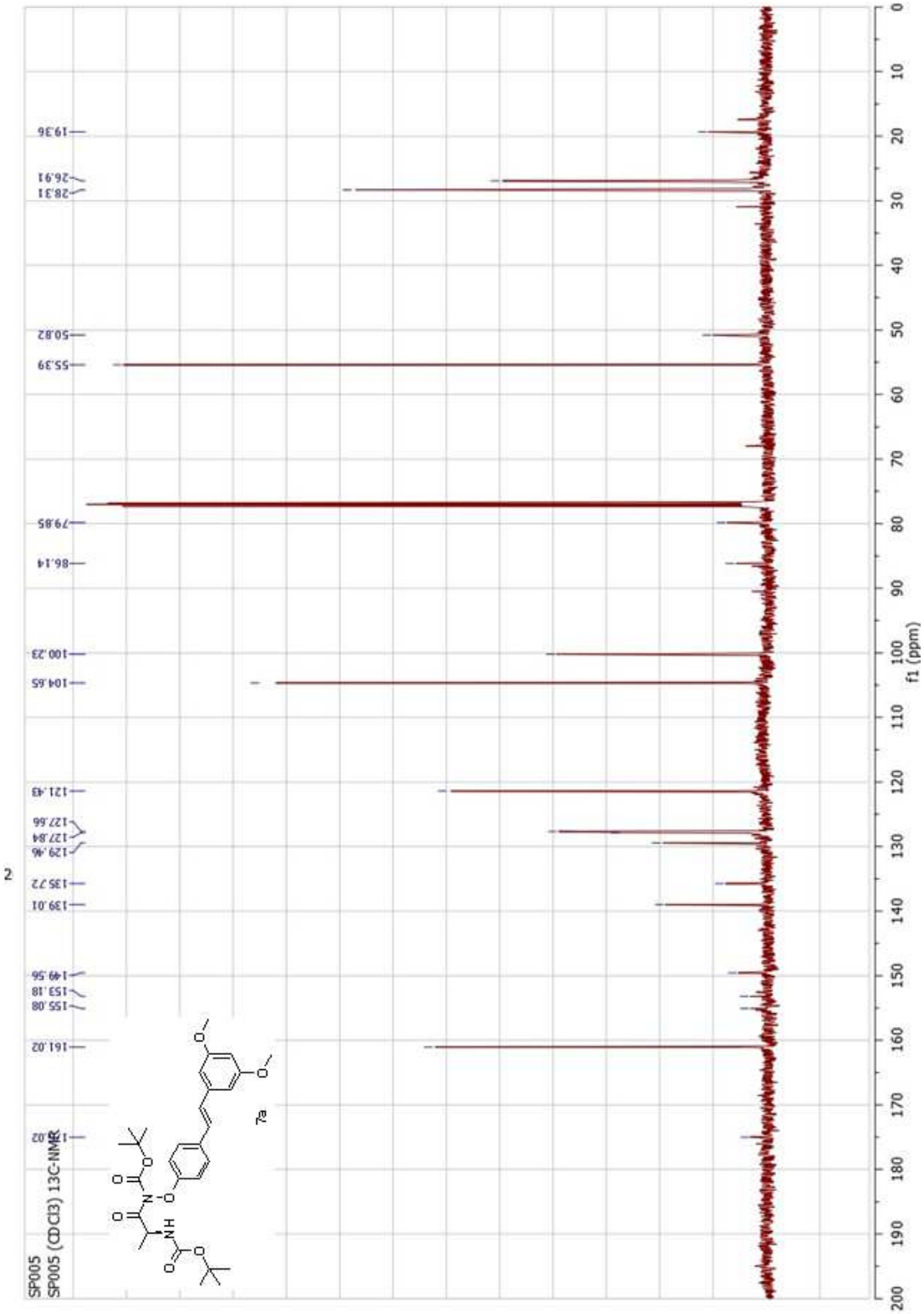
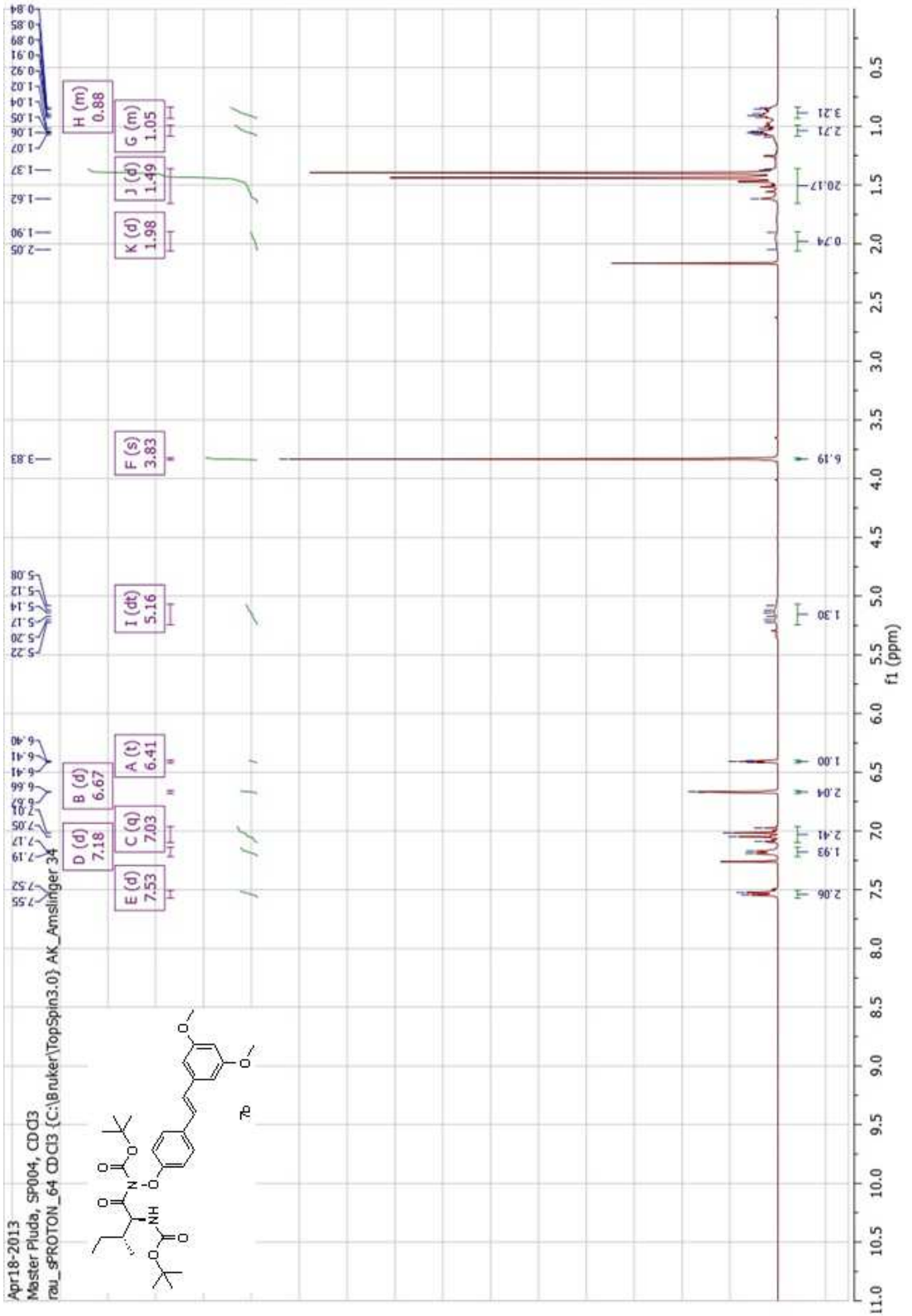
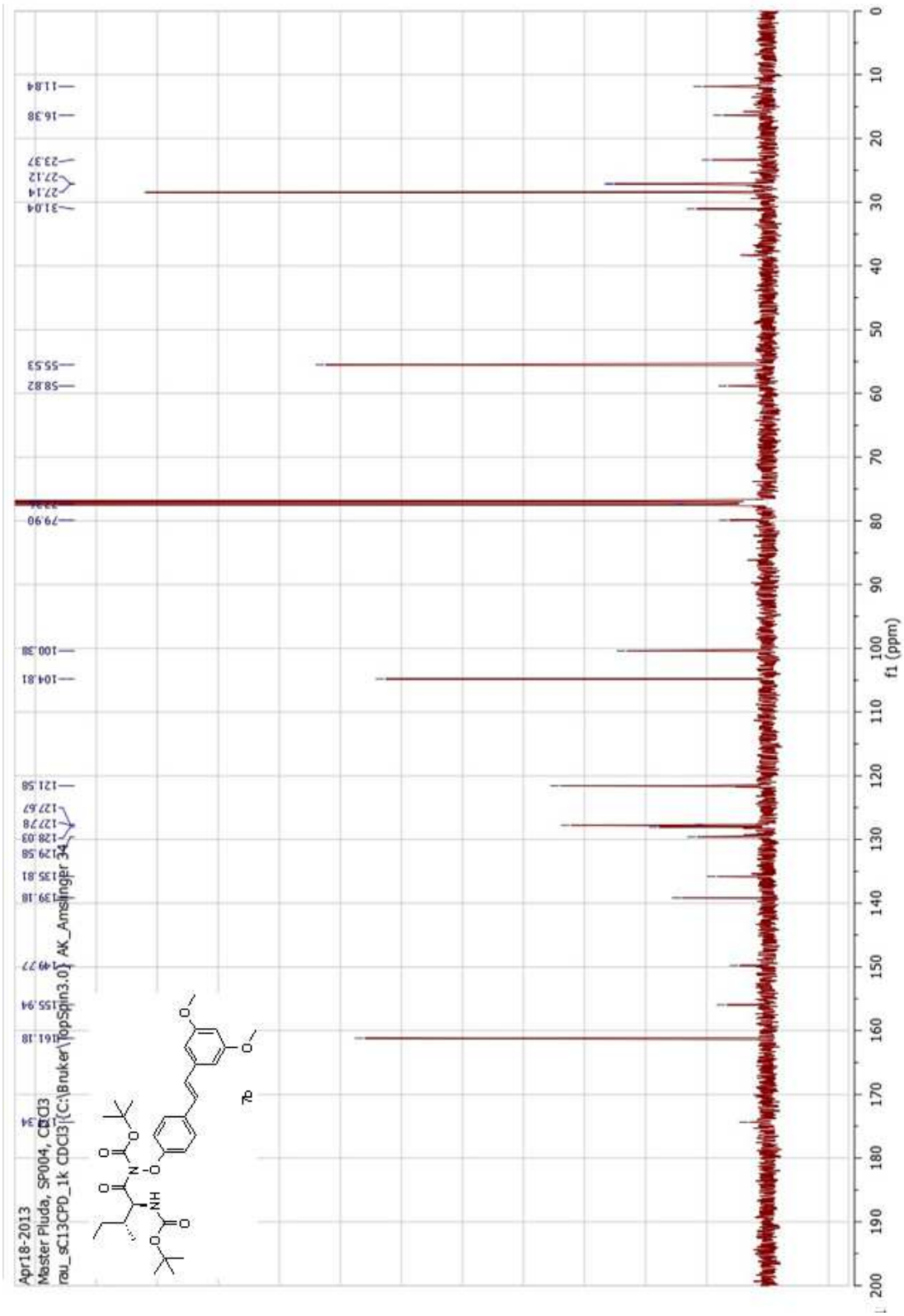


Product 7b ^{1}H NMR (400 MHz, $CDCl_3$)



Product 7b ^{13}C NMR (101 MHz, $CDCl_3$)



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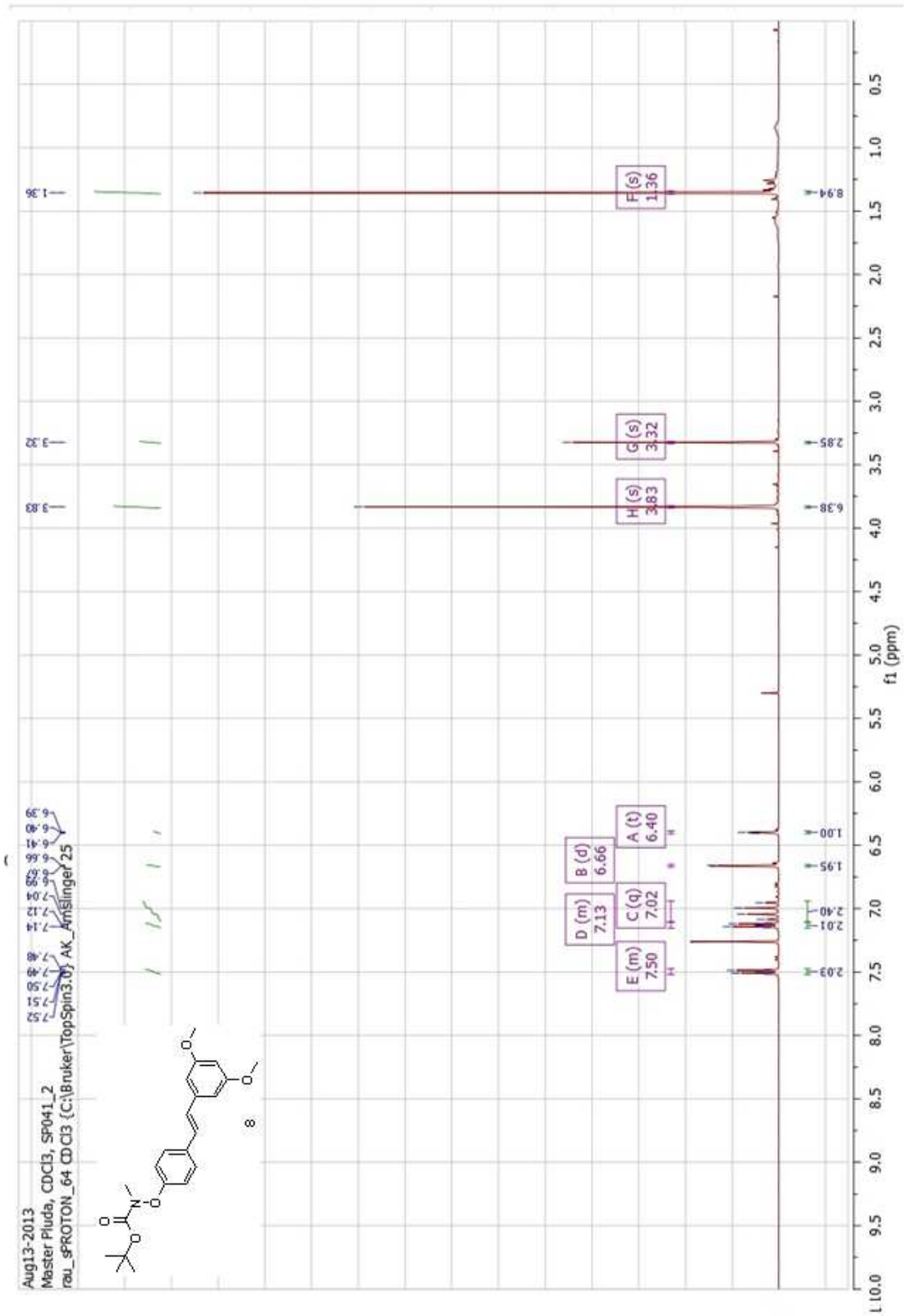
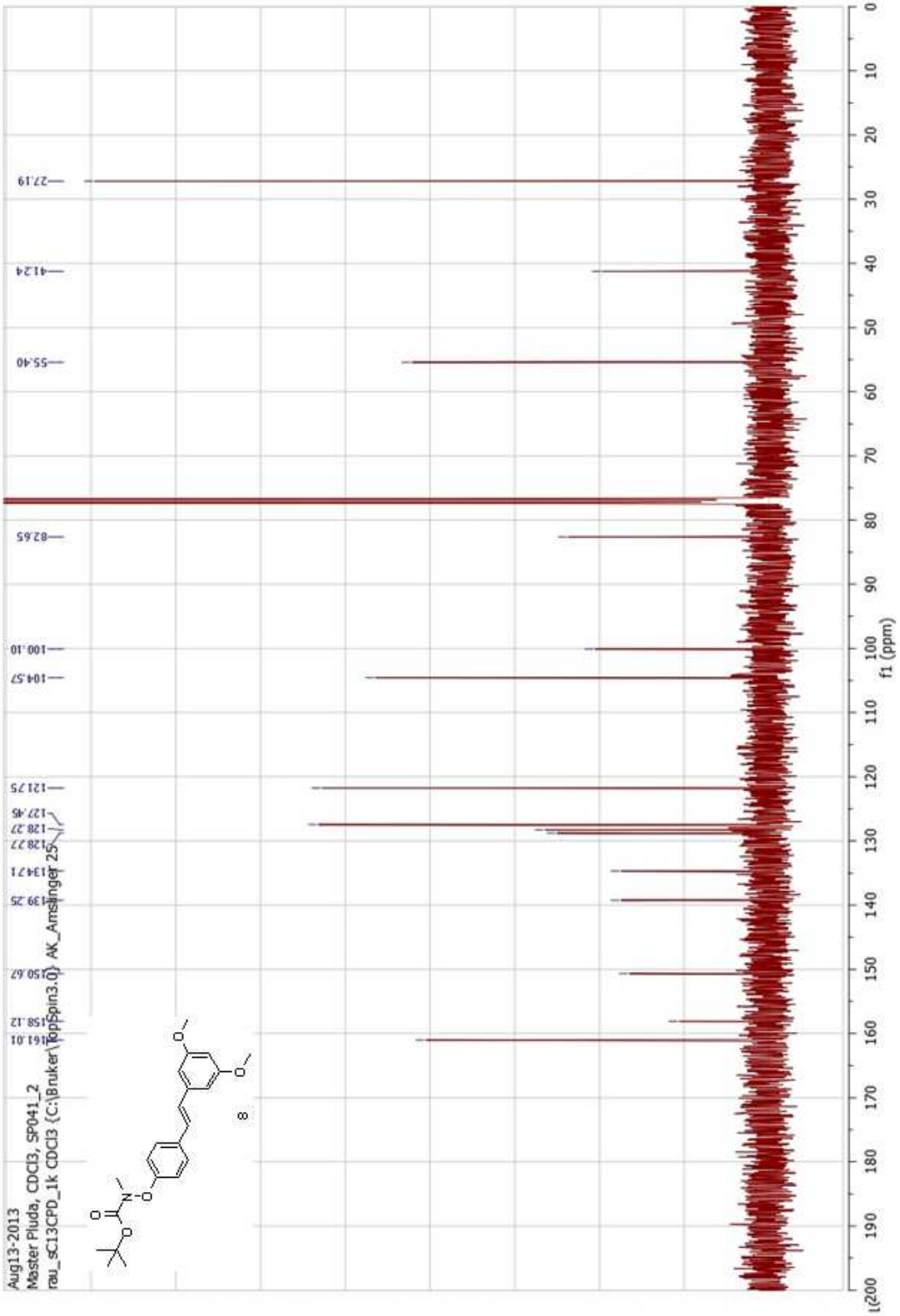
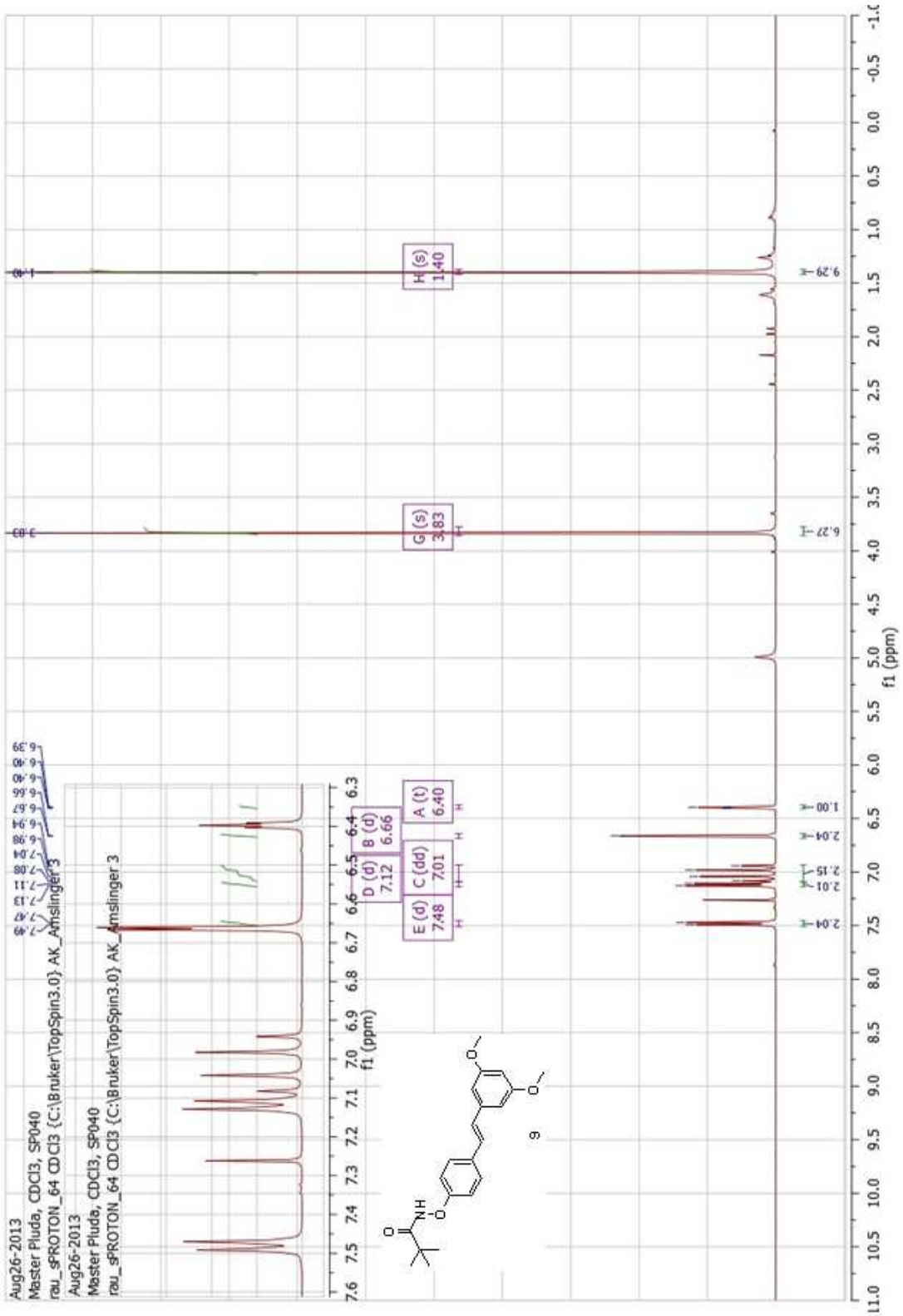


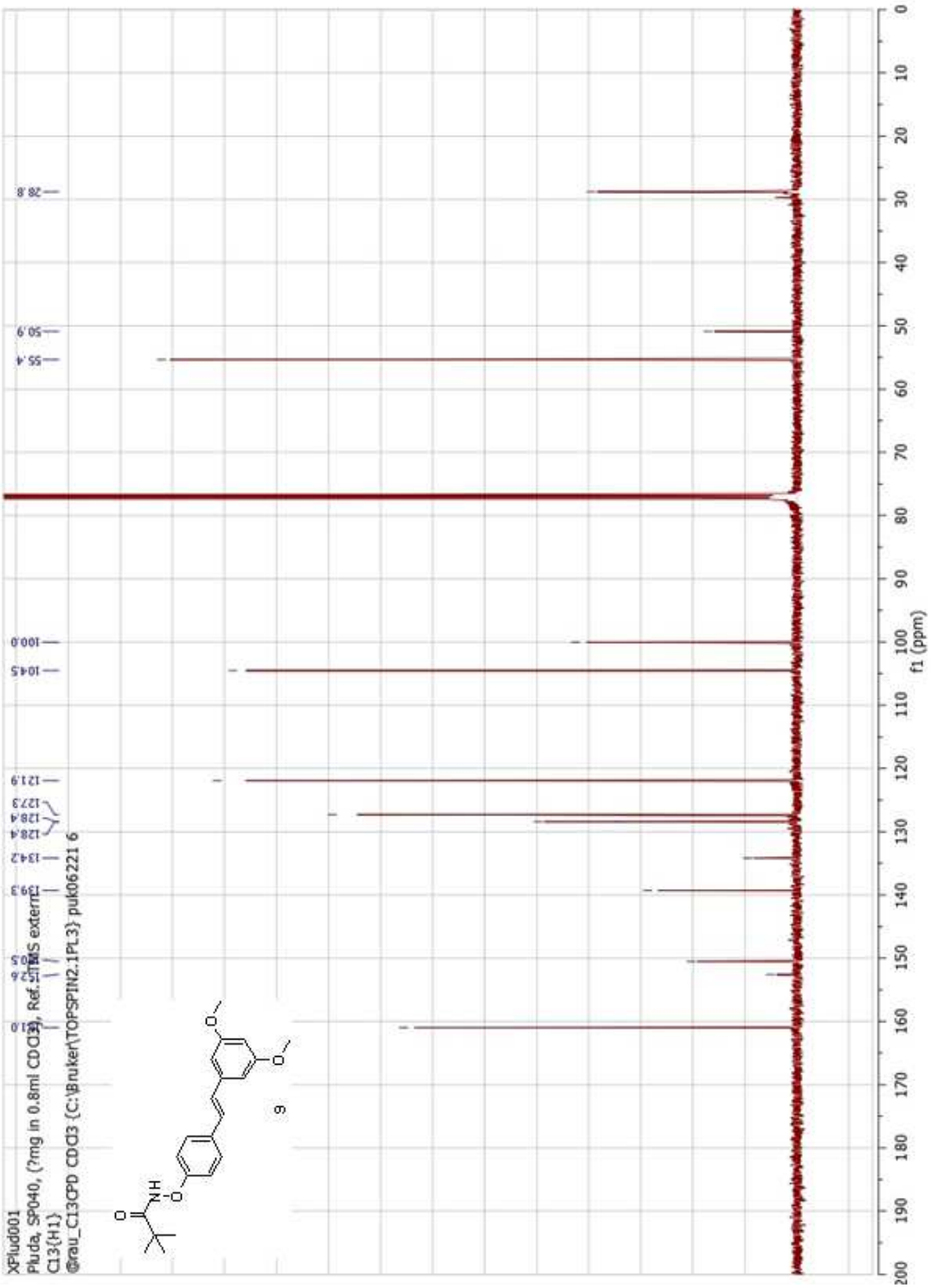
Product 8 ^{13}C NMR (101 MHz, $CDCl_3$)



Product 9 ^{1}H NMR (400 MHz, $CDCl_3$)



Product 9 ^{13}C NMR (101 MHz, $CDCl_3$)



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