Eva Fischereder

Enantioselective dealkylation of non-naturale *N*-ethyl benyzlisoquinoline substrates by the berberine bridge enzyme

Master Thesis

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Supervisor:

ao. Univ.-Prof. DI Dr. Wolfgang Kroutil

Department of Organic and Bioorganic Chemistry

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Dedicated to my parents

DANKSAGUNG

An dieser Stelle möchte ich all jenen Menschen Dank aussprechen, die durch ihre tatkräftige Unterstützung den Werdegang dieser Arbeit maßgeblich beeinflusst haben.

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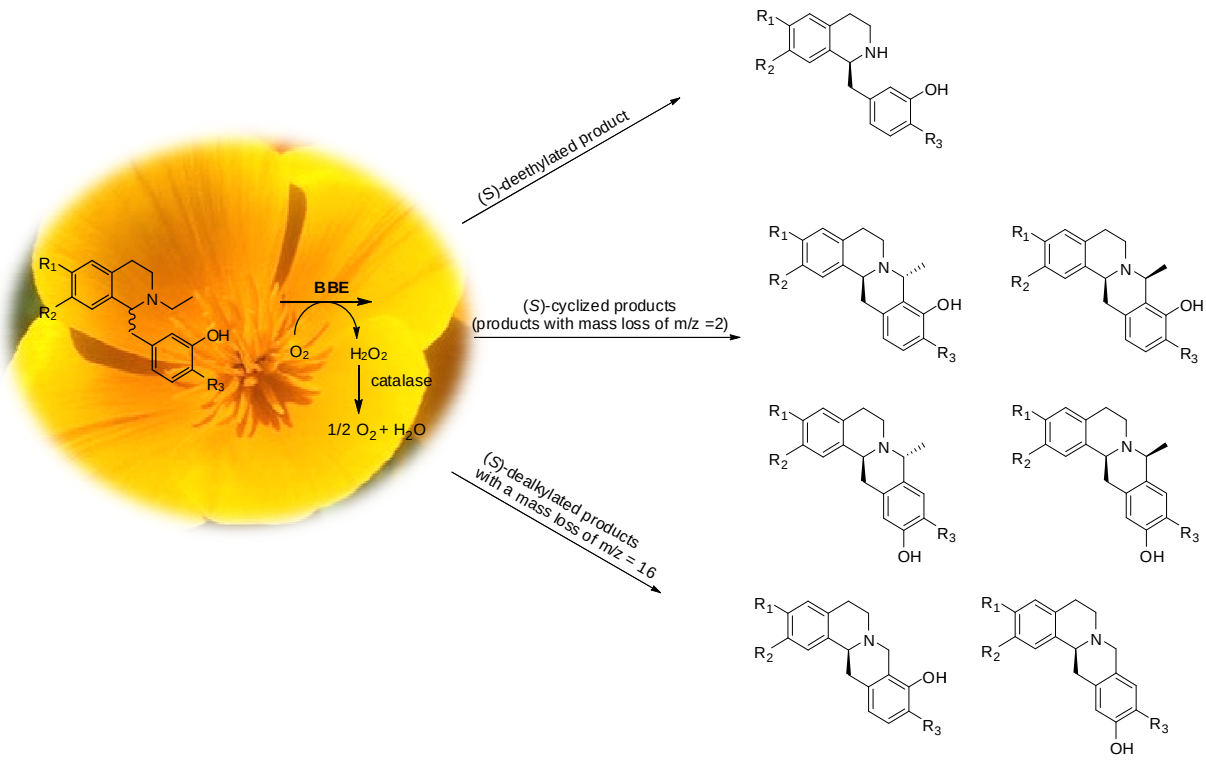
Wir konstruieren und konstruieren. Dabei ist Intuition noch immer eine gute Sache

(Paul Klee)

ABSTRACT

This master thesis deals with the synthesis of novel non-natural substrates for the biotransformation with the berberine bridge enzyme (BBE). California poppy (*Eschscholzia californica*) is the natural origin of the enzyme used. The plant's healing effects were already utilized by the American Indians to treat different diseases. The benzylisoquinoline alkaloids as well as the corresponding berbine alkaloids which were synthesized through the biotransformation by BBE are target molecules for many potent pharmaceuticals that are used e.g. as HIV-drugs, in the treatment of cancer or diseases of the nervous system like schizophrenia.

The substrates that were synthezised during this study harbor an additional prochiral center, achieved by the introduction of an ethyl group at the nitrogen of the tetrahydroisoquinoline scaffold. During the biotransformation of these substrates with BBE a completely new feature of the enzyme was discovered which demonstrates its promiscuity. The natural role of the enzyme is the enantioselective cyclization reaction of (*S*)-reticuline resulting in the formation of (*S*)-scoulerine. By applying the *N*-ethyl substrates to the enzyme it was observed that BBE is dealkylating the substrates. Within this biotransformation three different product classes were observed: (i) the cyclized products with a mass loss of m/z = 2; (ii) those that possess a mass loss of m/z = 16; (iii) and a deethylated product.



The structure of the deethylated product can be specified as a benzylisoquinoline lacking the ethyl group on the nitrogen, proven by comparison with a reference GC-MS spectrum.

For the cyclized products four different structures can be suggested. Those that harbor the hydroxy group at the C9 position of the berbine scaffold are the main products, whereas the corresponding C11-regioisomers are the minor ones.

Through the comparison with a GC-MS reference spectrum and retention time alignment on the HPLC, products with a mass loss of m/z = 16 can also be correlated to a cyclized structure lacking a methyl group at the newly formed bridging C8 atom.

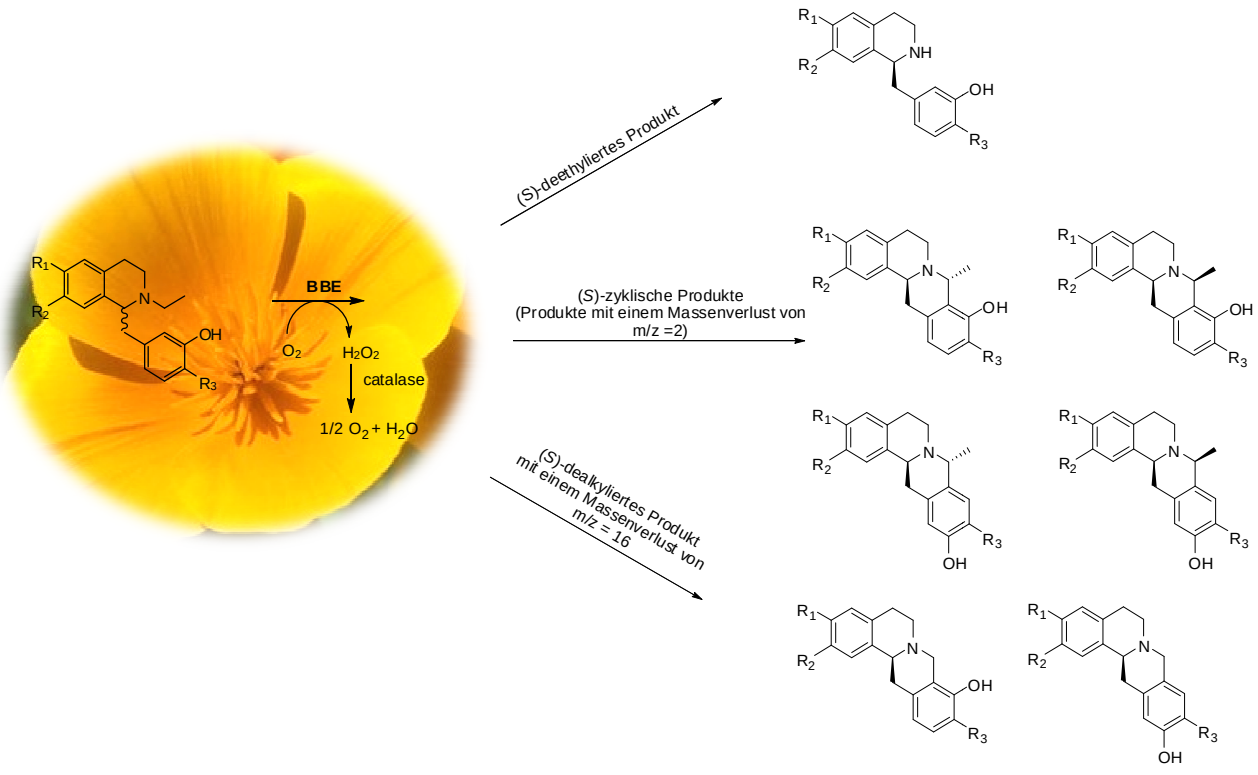
Interestingly, BBE is catalyzing an enantioselective dealkylation.

The newly discovered enzyme promiscuity of BBE leaves a lot of space for further investigations. The reaction mechanism leading to the novel formed products has to be proven and an adequate product characterization is necessary to complete the investigations. In the case of the cyclized products with a mass loss of m/z = 2 it has to be determined if the enzyme shows a stereopreference for the additionally introduced chiral center. Substrate and eventually enzyme engineering for this class of newly synthesized non-natural substrates will be the goals of further investigations based on this master thesis.

KURZFASSUNG

Diese Masterarbeit befasst sich mit der Synthese neuer Benzylisochinolinsubstrate für die biokatalytische Umsetzung mit dem Berberin-Brücken-Enzym (BBE), welches seinen natürlichen Ursprung im kalifornischen Goldmohn (*Eschschozia californica*) findet. Dieser Pflanze wurden bereits von den amerikanischen Ureinwohnern heilende Kräfte zugesprochen und von diesen als Arzneimittel eingesetzt. Die Benzylisochinolinalkaloide und deren entspechenden Berbinalkaloide, welche einerseits synthetisch hergestellt und andererseits durch die Biotransformation mit BBE erhalten wurden, stellen Ausgangsverbindungen vieler potentieller Pharmazeutiker dar, welche beispielsweise als HIV Medikamente, zur Behandlung von Krebs, oder Krankheiten des Nervensystemes wie Schizophrenie eingesetzt werden.

Die Verbindungen, welche im Laufe dieser Arbeit synthetisiert wurden beinhalten ein zusätzliches prochirales Zentrum, eingeführt durch die *N*-Ethylgruppe am Tetrahydroisochinolingrundgerüst. Bei der biokatalytischen Umsetzung dieser Subtrate mit BBE konnte eine neue Eigenschaft des Enzymes aufgezeigt werden und somit dessen Promiskuität belegt werden. Die natürliche Rolle des Berberin-Brücken-Enzymes ist die enantioselektive Ringbildung von (*S*)-Reticulin zu (*S*)-Scoulerin. Bei der Umsetzung der *N*-ethylierten Substrate mittels BBE stellte sich heraus, dass eine Dealkylierung der Benzylisochinolinverbindungen statt findet. Das vom Biokatalysator erzeugte Produktgemisch konnte in drei verschiedene Klassen unterteilt werden: (i) zyklische Produkte mit einem Massenverlust von m/z = 2; (ii) jene die einen Massenverlust von m/z = 16 aufweisen; (iii) und ein deethyliertes Produkt.



Durch den Vergleich des GC-MS Spektrums mit einer Referenzsubstanz kann die Struktur des deethyilierten Produktes als jene Benzylisochinolinstruktur identifiziert werden, welcher die Ethylgruppe am Stickstoff fehlt.

Für die zyklischen Produkte können vier Strukturen vorgeschlagen werden: die beiden Diastereomere, welche die Hydroxygruppe am C9 Atom des Berbingrundgerüsts tragen und die dazugehörigen Regioisomere, welche die funktionelle Gruppe am C11 Atom tragen und das Nebenprodukt darstellen.

Die Zuordnung der Produktgruppe mit einem Massenverlust von m/z=16, konnte durch Retentionszeiten-, sowie durch GC-MS Datenvergleich erfolgen. Diese Verbindungen können einer zyklischen Struktur zugeordnet werden, der eine Methylgruppe am C8 verbrückenden Atom im entstandenen Berbingerüst fehlt.

Bemerkenswerterweise transferiert BBE bei der Dealkylierung nur das (*S*)-Enantiomer zu den entsprechenden Produkten.

Die neu erweiterte Promiskuität von BBE lässt viel Spielraum für nachfolgende Fragestellungen, wie zum Beispiel der Aufklärung des Reaktionsmechanismus der neu geformten Produkte, als auch einer adequaten Produktcharakterisierung. Im Falle der zyklischen Produkte, die einen Massenverlusst von m/z = 2 aufweisen muss festgestellt werden, ob das Enzym eine gewisse Stereopräferenz für das neu eingeführte chirale Zentrum zeigt. Ebenso soll sowohl die strukturelle Modifikation der Substrate, als auch des Enzyms im Fokus weiterer Fragestellungen stehen.

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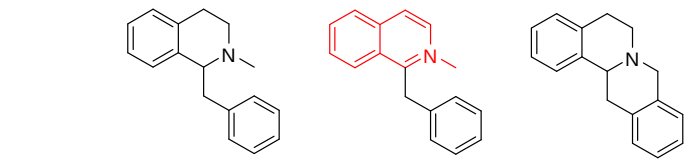
1 INTRODUCTION

1.1 Benzylisoquinoline alkaloids

1.1.1 Characteristics

The benzylisoquinolines (BIs) occupy a paramount position in alkaloid chemistry because they act as *in vivo* precursors for many of the naturally occurring isoquinolines like morphines, pavines, cularines, aporphines, protoberberines and others. They are either of the 1,2,3,4-tetrahydro type, or of the completely aromatic type including an isoquinoline system in their scaffold.[1,2]



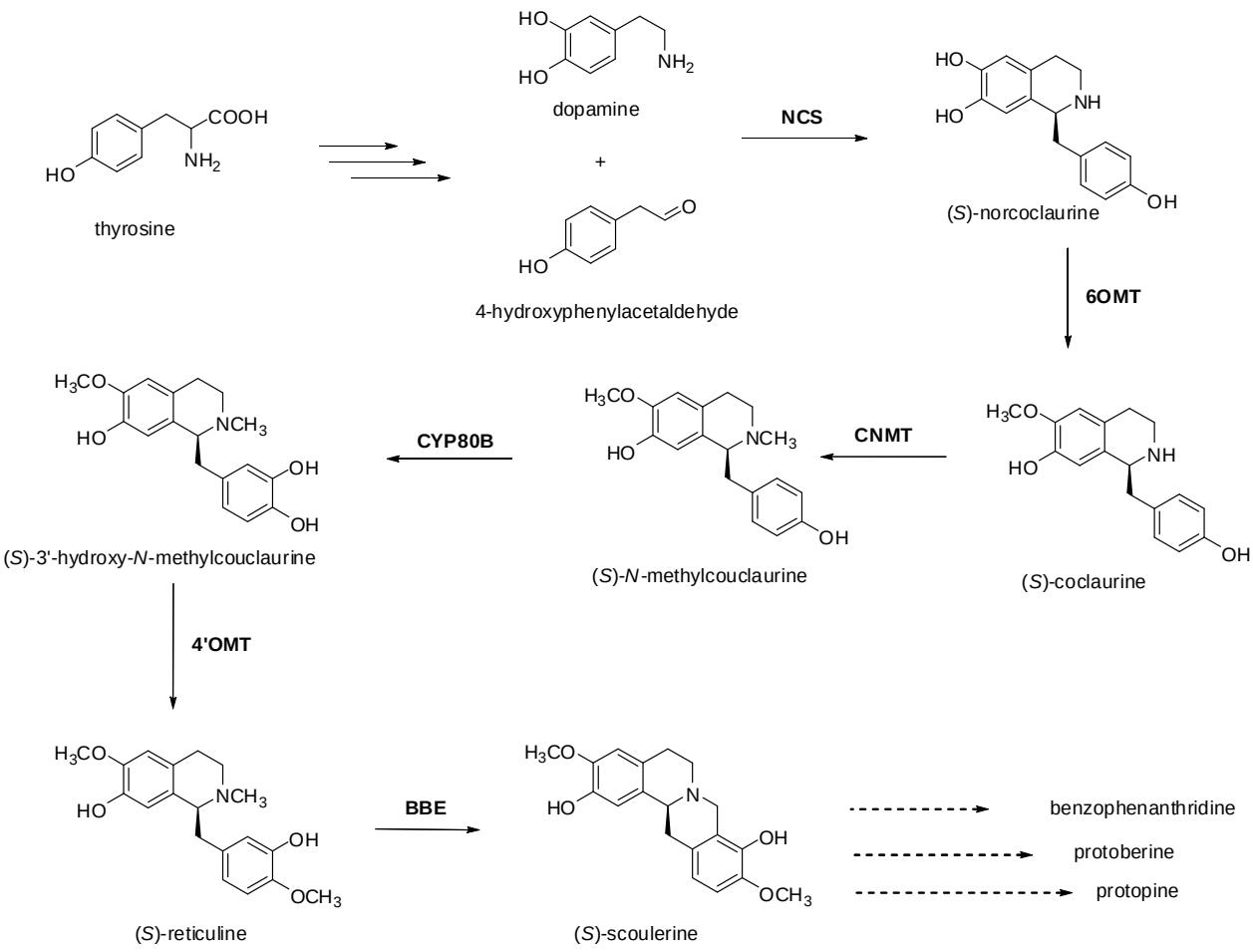
N-methyl-1,2,3,4-tetrahydro type BI *N*-methyl BI berbine

Scheme 1: Structural scaffold of two different types of benzylisoquinolines (isoquinoline marked in red) and the structure of berbine.

The compounds that are part of this master thesis belong to the 1-benzyl-1,2,3,4-tetrahydroisoquinolines and their biocatalytically obtained products to the berbine alkaloids.

1.1.2 Biosynthetic pathway in plants

These alkaloids are part of a large and diverse alkaloid group with more than 2500 defined structures. The pharmacological activity of benzylisoquinoline alkaloids (BIAs) is the reason why many of them are used as pharmaceuticals and is often a clue to their biological role in the plant. The biosynthesis begins with decarboxylation of tyrosine as well as by *ortho*-hydroxylation and deamination to dopamine and 4-hydroxyphenylacetaldehyd. Both originated substrates are condensed by norcoclaurine synthase (NCS) to form the trihydroxybenzylisoquinoline alkaloid (*S*)-norcoclaurine, which is the central precursor for all BIAs in plants (Scheme 2). [3]



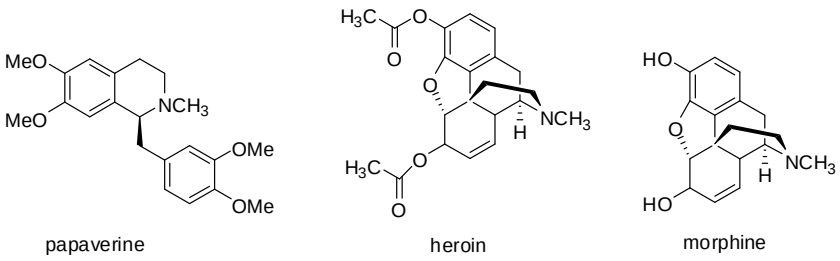
Scheme 2: Biosynthesis of benylisoquinoline alkaloids in plants. NCS…norcoclaurine-synthase, 6OMT…norcoclaurine-6-*O*-methyltransferase, CNMT…coclaurine *N*-methyltransferase, CYP80B…(*S*)-*N*-methylcoclaurine-3´-hyroxylase, 4'OMT…3'hydroxy-*N*-methylcoclaurine-4-*O*-methyltransferase, BBE…berberine bridge enzyme

(*S*)-Norcoclaurine is further subjected to a regioselective 6-*O*-methyltransfer as well as an *N*-methyltransfer *via* the enzymes norcoclaurin-6-*O*-methyltransferase (6OMT) and coclaurine *N*-methyltransferase (CNMT).[3] The following hydroxylation and methylation steps lead to (*S*)-reticuline which is a branch point intermediate in the biosynthesis of many BIA such as sanguinarine, macarpine,[4] berberine, palmatine[5] and morphinan alkaloids like morphine and codeine.[6] (*S*)-Reticuline can either be oxidized by the berberine bridge enzyme (BBE) to form (*S*)-scoulerine *via* the methylene bridge formation from the *N*-methyl group, or can be converted to the (*R*)-enantiomer which represents the first committed step in morphinan alkaloid biosynthesis.[3] Further biosynthetic steps convert (*S*)-scoulerine into the important benzophenanthridine, protoberbine and portopine alkaloids.

1.1.3 Biological significance

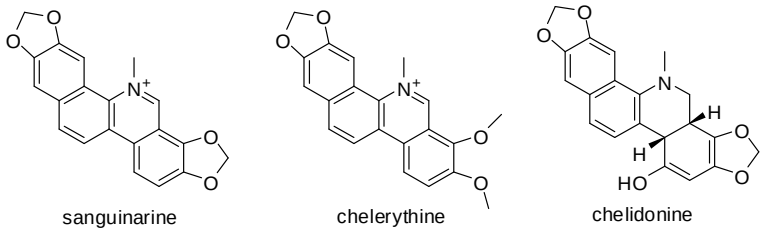
The potential biological activity of many benzylisoquinoline alkaloids led to their widespread application as pharmaceuticals, stimulants, narcotics and poisons.[3] The search for useful drugs of defined structure from plants began with the isolation of morphine from *Papaver somniferum* (opium poppy) in 1806 which is a strong sedative and analgesic agent.[7] Morphine

derivatives such as codeine and heroin, and papaverine are used as muscle relaxant, analgesic, as well as in the treatment of gastrointestinal spasms, heart disease and stroke (Scheme 3).[1,3]



Scheme 3: Structures of papaverine, heroin and morphine

The opium poppy is currently one of the most important renewable resources for pharmaceutical alkaloids, more than 100 different of them are derived from the amino acid L-tyrosine which have (*S*)-reticuline as a common intermediate.[7] *P. somniferum* also produces the benzophenanthridine alkaloid sanguinarine which has anitimicrobial properties and is known to be part of the chemical defense system in plants. Moreover sanguinarine as well as chelidonine and chelerythine (isolated from *Chelidonium majus*) are well known in cancer treatment (Scheme 4). They were reported to exert cell growth-inhibitory effect *via* the induction of apoptosis in numerous cancers cells. Furthermore, they are effective against certain tumors that are otherwise resistant to standard therapies.[8]



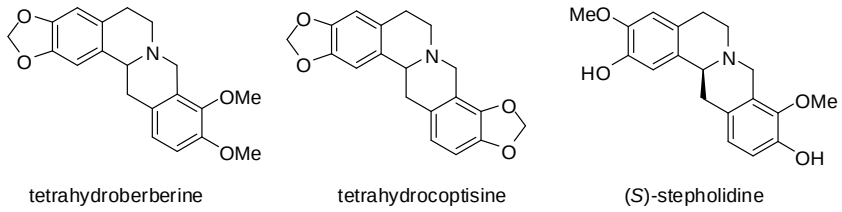
Scheme 4: Structures of sanguinarine, chelerythine and chelidonine

Also muscle-relaxing effects are attributed to various benzylisoquinolines, especially reticuline, norarmepavine, coclaurine and papaverine act antispasmodic, inhibiting the Ca^{2+} transport-system.[9] Furthermore reticuline has been shown to depress the central nervous system and to accelerate hair growth.[10,11]

Moreover the benzylisoquinoline alkaloids (*R*)-coclaurine and (*S*)-norcoclaurine isolated from *Nelumbo nucifera* (aquatic crop) together with quercetin 3-*O*-*β*-D-glucuronide are used as anti-HIV targets.[12]

Berbine alkaloids like tetrahydroberberine and tetrahydrocoptisine (Scheme 5) seem to have a depressant effect on the central nervous system and therefore are applied as potent sedatives.[13] Furthermore this class of alkaloids is used in the treatment of schizophrenia. (*S*)-Stepholidine

(Scheme 5) for example affects the dopaminergic system and reduces typical side effects.[14,15,16]

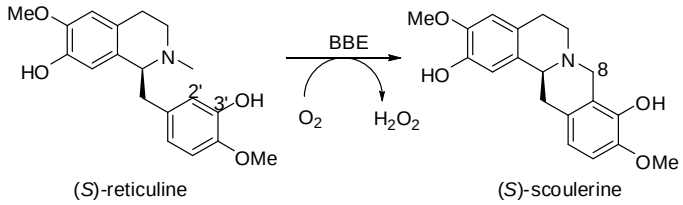


Scheme 5: Structures of tetrahydroberberine, tetrahydrocopisine, (*S*)-stepholidine

Berberine isolated from Chinese herb, has been recently identified as a cholesterol-lowering drug. Oral administration of berberine in 32 hypercholesterolemic patients for 3 months reduced serum cholesterol by 29%, triglycerides by 35% and LDL-cholesterol by 25%.[17]

1.2 Berberine bridge enzyme

The Berberine bridge enzyme (EC 1.21.3.3) catalyzes the enantioselective oxidative C-C-bond formation of its natural substrate (*S*)-reticuline to (*S*)-scoulerine under the consumption of molecular oxygen (Scheme 6). It belongs to the enzyme class oxidases and harbors a bicovalently linked FAD-cofactor.



Scheme 6: Bioconversion of (*S*)-reticuline to (*S*)-scoulerine at the expense of molecular oxygen, catalyzed by BBE

1.2.1 History

The berberine bridge enzyme (BBE) is a central enzyme of benzylisoquinoline alkaloid biosynthesis that channels the key intermediate (*S*)-reticuline towards the formation of diverse and important isoquinoline alkaloids like protoberberine, protopine and benzophenanthridine alkaloids.

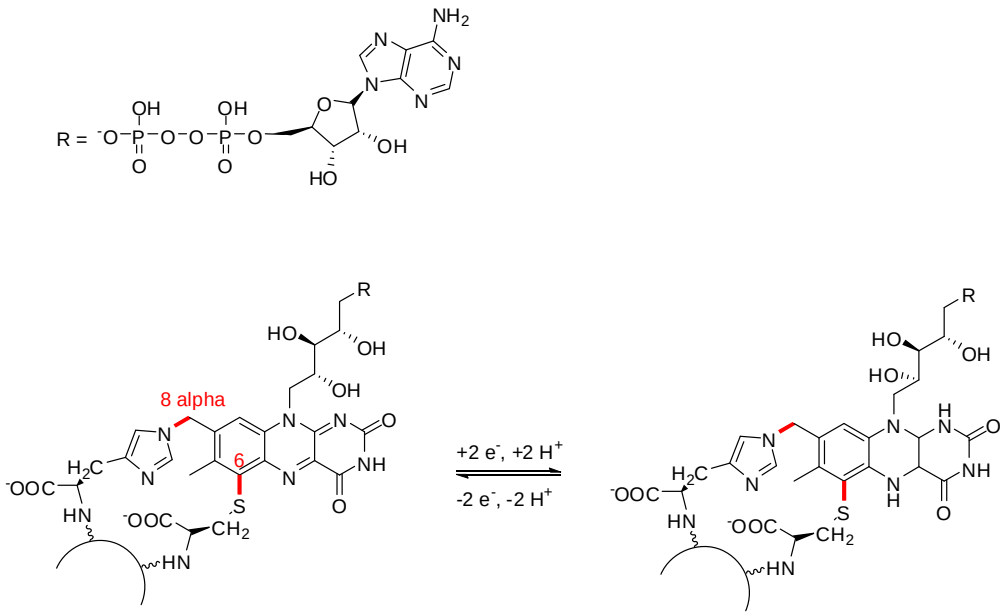
The enzyme activity is known in the families of *Papaveraceae*, *Berberidaceae*, *Fumariaceae*, *Menispermaceae* and *Ranunculaceae.*[18] For this thesis the enzyme is taken from the California poppy (*Eschscholzia californica*) which belongs to the family of the *Papaveraceae*. In earlier times this plant was used by American Indians as a traditional medicine.[19]

Figure 1: Picture of the California poppy (*Eschscholzia californica*)

In 1955 Sir Robert Robinson named the bridging C8 carbon between the isoquinoline nitrogen and the phenolic part in berbine alkaloids the "berberine bridging carbon" (Scheme 6).[20] Some years later in 1963 the two working groups of D. H. R. Barton and A. R. Battersby independently reported the first definitive evidence showing that this carbon atom was derived from the methyl group on the nitrogen found in benzylisoquinoline alkaloids.[21,22] Therefore they labeled the *N*-methyl group of *rac*-laudanosoline and *rac*-reticuline with ^{14}C-atoms and administered them to poppy plants. The isolation of the radioactive berbine alkaloids demonstrated that the bridging C8-atom was exclusively formed from the marked carbon atoms. The first isolation of the responsible enzyme from *Macleaya microcarpa* cell cultures (purification factor 7-fold) took place a decade later by Rink and Böhm.[23] They carried out the biotransfomations with ^{14}C-labeled *rac*-reticuline to (*S*)-scoulerine and named the biocatalyst "berberine bridge enzyme".[24] In 1984 Steffens *et al.* screened 21 different plants and 67 cell suspesion cultures for BBE activity.[18] The highest activity of the enzyme could be obtained using cell cultures of *Berberis beaniana* and therefore they purified it to homogeneity. The enzyme activity was conveniently monitored using *N*-CT_3-labeled *rac*-reticuline. (*S*)-Enantioselectivity of the enzyme led to (*S*)-scoulerine as the sole product. Dittrich and Kutchan reported in 1991 the first successful cloning experiment of berberine bridge enzyme cDNA isolated from elicited cell-suspensions of *Eschscholzia californica*.[25] This enabled the heterologous expression of BBE in *Saccharomyces cerevisiae*. Two years later the same working group heterologously expressed the enzyme in the fall army worm, *Spodoptera frugiperda*.[26] Therefore they were able to isolate 4 mg purified and active enzyme per liter cell culture. However, the level of protein expression achieved in this system was not enough to allow detailed investigations of the biochemical and structural properties of the enzyme. But recently the working group of P. Macheroux has been able to develop a high level expression of BBE in the methylotophic yeast *Pichia pastoris*.[27] *Via* a two-step chromatographic purification protocol, 120 mg of the enzyme could be obtained from 1 liter of fermentation culture.

1.2.2 Structure and location of the enzyme in the cell

The berberine bridge enzyme is a member of the recently discovered family of bicovalently flavinylated proteins. FAD is linked *via* its 8α-methyl group and the C6 atom of the isoalloxazine ring system to His104 and Cys166 of the enzyme (Scheme 7).[28]



Scheme 7: Bicovalent bond of the enzyme to the cofactor FAD

The first example of a covalent bond between FAD cofactor and a protein was discovered in 1956,[29] and since then a number of different types of linkages have been identified: 8α-histidylation, 8α-*O*-tyrosylation, 8α-*S*-cysteinylation and 6-*S*-cysteinylation. Half a century later bicovalent linkage of FAD was first revealed by elucidating the crystal structure of glucooligoscaccharide oxidase.[30] Based on this finding, a similar double covalent anchoring of FAD was recognized in other oxidases such as hexose oxidase (which belongs to the so called vanillyl-alcohol oxidase family), aclacinomycin oxidoreductase and BBE.[31]

The occurrence of this bicovalent FAD attachment raises the question of its role in enzyme catalysis.[32] Winkler *et al.* demonstrated the importance of this bond *via* mutagenetic analysis of the amino acid residues His104 and Cys166 of BBE (Figure 3). The Cys166 variant in which the substitution of cysteine against alanine took place, still had residual enzymatic activity, but reduced to ~6% of the turnover rate observed for the wild type berberine bridge enzyme.[32] The wild type protein exhibits a midpoint potential of +132 mV, which is the highest redox potential determined for any flavoenzyme so far. Removal of the cysteine linkage to FAD leads to a redox potential of +53 mV which is the expected range for flavoproteins with a single covalent attachment of FAD to a histidine residue. Furthermore the reductive half-reaction is highly influenced by the lack of the 6-*S*-cysteinyl linkage, resulting in a 370-fold decrease in the rate of flavin reduction.[32]

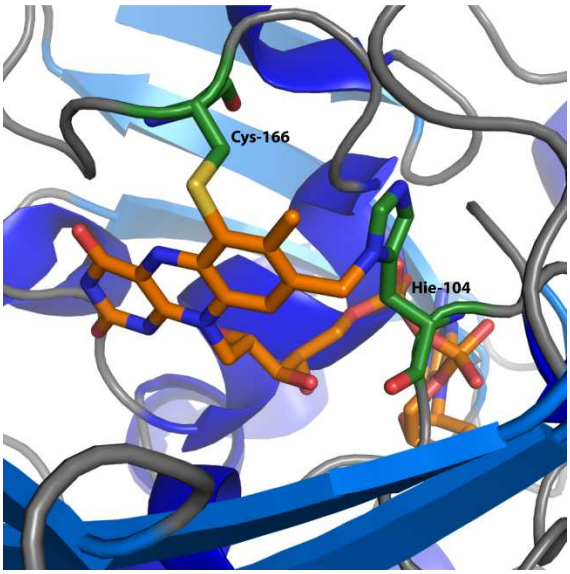


Figure 2: 3D model of the bicovalent linked FAD

The berberine bridge enzyme has a total length of 519 amino acids with a theoretical molecular mass of 58.599 Da including the covalently attached FAD cofactor.[33] It comprises two binding domains, a flavin adenine dinucleotide binding domain and an α/β domain with a seven-stranded, antiparallel β-sheet forming the substrate binding site, which bears mainly hydrophobic residues.[34] The substrate (*S*)-reticuline is bond in a deep cleft with the phenolic ring pointing towards the bottom of the binding site and is sandwiched between the FAD cofactor and the amino acid residues extending from the β-sheet of the central domain. The important active side amino acid Glu417 which is involved in the deprotonation of the C3'-hydroxy group of the phenolic moiety, is hydrogen bonded to this group.[34] Asn390 interacts with its carboxamide group with both, the OH group and the 4-methoxy group on the phenolic ring. The polar substituents of the isoquinoline scaffold are positioned close to Asp352 at the entrance to the active site. The *N*-methyl group of the substrate is placed between the C2' atom of the phenolic ring system and the N5 atom of the cofactor, with distances of 3.3 Å and 3.1 Å, respectively.[34]

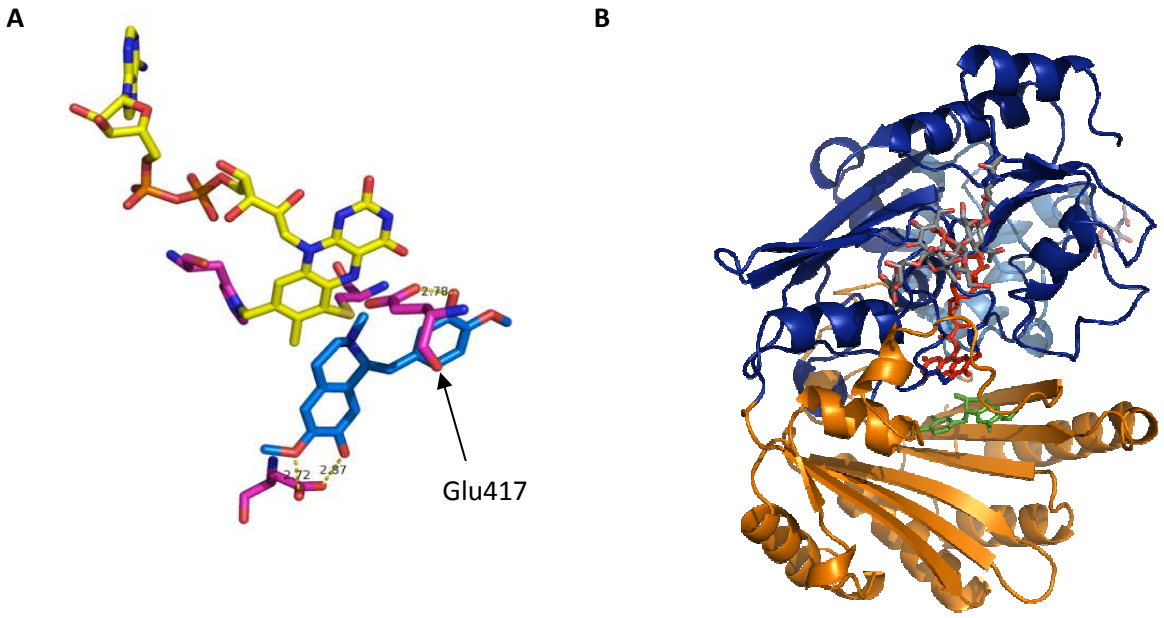
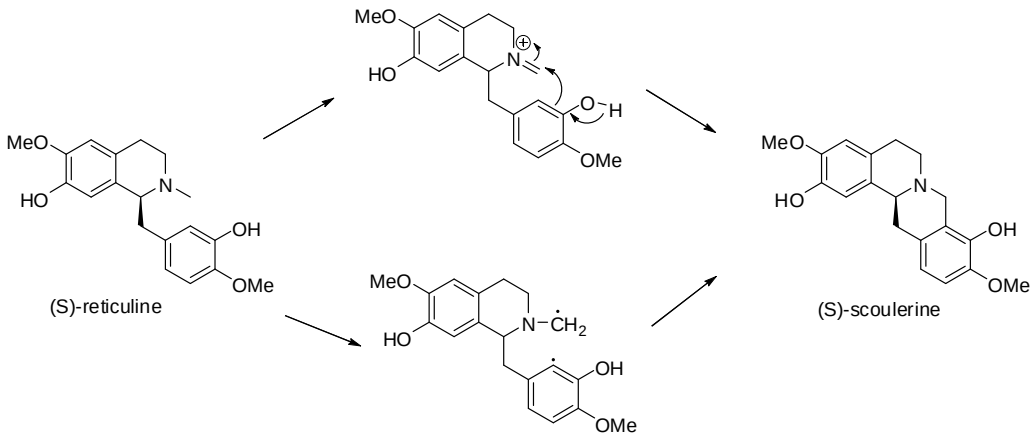


Figure 3: Crystal structure of *Eschscholzia californica* BBE in complex with reticuline. A: Arrangement of FAD and reticuline in the active site (reticuline in blue, FAD in yellow). B: Overview showing the FAD-binding (blue) and substrate-binding (orange) domains.

Concerning the location of the enzyme in the cells, enzymatic assays as well as immunoelectrophoresis showed that it is localized within a particle of the density ρ = 1.14 g/mL.[35-37] Furthermore this particle also contains the enzyme (*S*)-tetrahydropotoberberine oxidase (STOX) which together with the BBE is one of the eight enzymes involved in the biosynthesis of the isoquinoline alkaloid berberine.[37] Electron-microscopic examination of *B. wilsoniae var. subcaulialata* cells demonstrated that both enzymes originate from smooth vesicles (0.1-1 µm diameter) that occur frequently in clusters from small vacuoles.[37] The vesicles change their density according to the age of the cell culture and have to be permeable for H_2O_2 since BBE produces 1 mol of it per one mole of transformed reticuline. The small vacuoles formed by the agglomeration of the vesicles could move towards the central vacuole of the cell, merging with this compartment and thus releasing their osmiophilic material (protoberberine alkaloids) into the main vacuole.[37] Further investigations demonstrated that these small vesicles are derived from the endoplasmic reticulum (ER). BBE contains a unique targeting domain comprised of an *N*-terminal signal peptide containing 22 amino acids in *E. californica*.[38-42] This signal peptide directs the enzyme to the ER from which BBE is transported into the vacuole in order to deliver its biosynthetic products. Once the vesicles have fused to vacuoles the enzyme seems to lose its activity, due to the fact that the vacuolar pH was determined to be about 5 and the enzyme has its pH activity maximum at around 8.9.[38-42]

1.2.3 Enzymatic mechanism

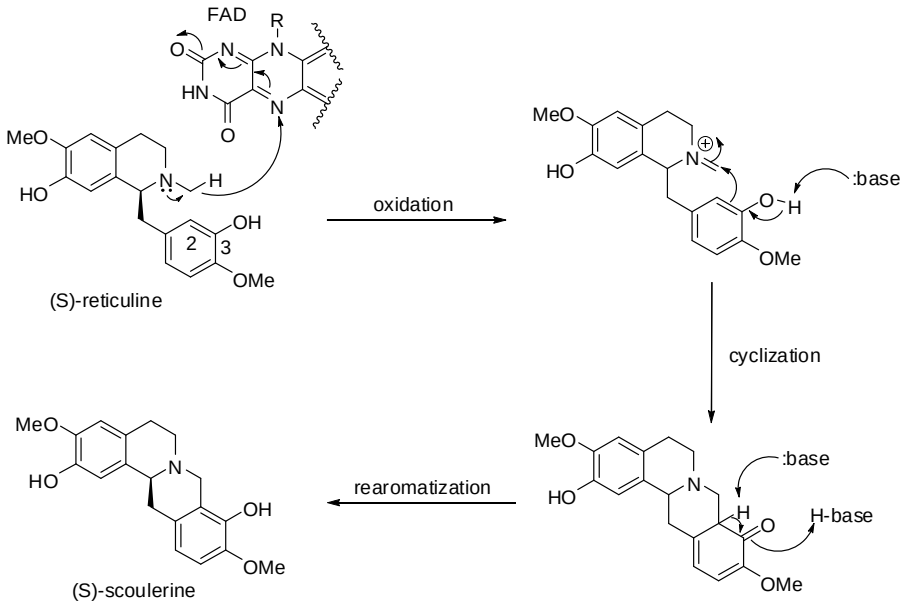
Based on the fact that the C-C-bond formation *via* the berberine bridge enzyme is unique in nature and cannot be mimicked by current chemical techniques, different approaches were established for the enzymatic mechanism which is not elucidated so far in detail. In the beginning of the investigations concerning the mechanism two different proposals were given. On the one hand the ionic mechanism *via* the formation of an iminium ion, and on the other hand the radical mechanism (Scheme 8).[25] In other words, the flavin can be reduced by either two one-electron transfers or one two-electron transfer from (*S*)-reticuline.



Scheme 8: Ionic versus radical mechanism by the enzymatic conversion of (*S*)-reticuline to (*S*)-scoulerine *via* BBE

The approach of the radical mechanism could be refuted by stopped-flow experiment, which gave no indication for a radical formation during the enzymatic reaction. Regarding the observed spectral characteristics of FAD, the direct transfer of the hydride from (*S*)-reticuline to the cofactor was clearly favored.[32]

Hence the enzymatic reaction was proposed to work out *via* an iminium ion formation realized by a two step process. The reaction starts with the oxidation of the *N*-methyl group to the corresponding iminium ion, followed by an ionic ring closure, initiated by the deprotonation of the C3'-OH group of the benzylisoquinoline moiety. Final rearomatization gives the reaction product (*S*)-scoulerine and completes the ionic mechanism.[32]

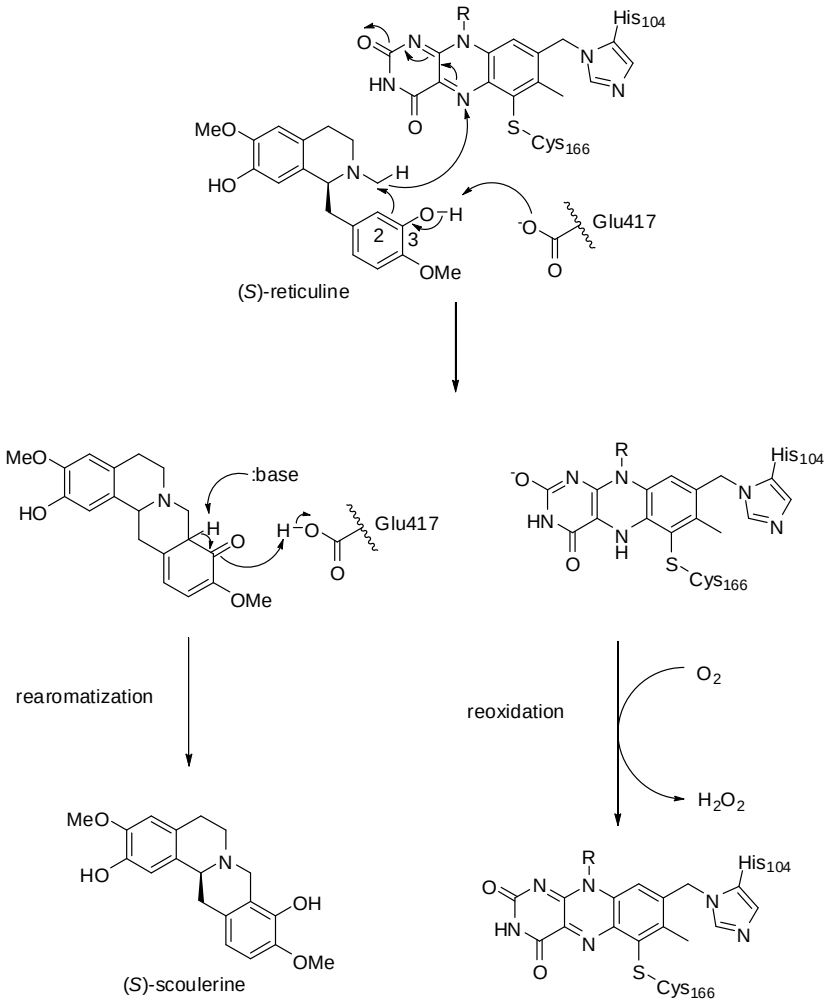


Scheme 9: Ionic BBE mechanism *via* iminium ion formation.

To improve the understanding of the BBE mechanism, the three-dimensional X-ray crystal structure of the enzyme from *Eschscholzia californica* was elucidated. It confirmed the previously indentified covalent attachment of the flavin cofactor to N1 of His104 and Cys166.[32-34] Based on the structure, mutagenesis experiments involving the three active-site amino acids (Tyr106, Glu417 and His459) were done. All three muteins could be expressed in nearly similar amounts as the wild-type protein and spectral characterization of the muteins indicated that the bicovalent flavin linkage was not affected by any of the three amino acid replacements.[34] The crystal structure of the complex with (*S*)-reticuline demonstrated the importance of the active-side amino acid Glu417 which is hydrogen bonded to the C3'-OH group of the substrate. The mutant E417Q in which glutamate was replaced through a glutamine showed 1,500-fold reduced reductive rate compared to the wild type.[34] Cofactor reduction became the rate-limiting step during turnover, but no effect on reoxidation of the cofactor was observed. Based on the kinetic parameters of the mutants it could be reasoned that the oxidation of the substrate's *N*-methyl group is not independent of the other reaction steps, like the two-step mechanism would suggest. The essential role of proton abstraction for substrate oxidation was further corroborated by the 30,000-fold slower reduction rate of the cofactor by replacing the natural substrate with laudanosine which bears a methoxy instead of a hydroxyl group at the C3´ position.[34]

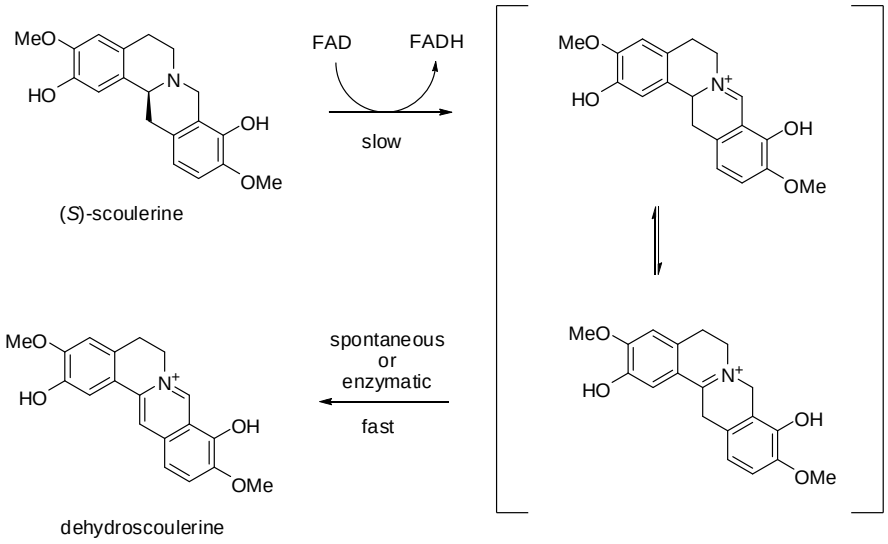
Recently Winkler *et al.* have proposed a concerted mechanism for the BBE reaction.[34] Thereby the important active-site amino acid Glu417 deprotonates the C3'-OH group of the substrate which strongly increases the nucleophilicity of the vicinal C2' carbon atom and facilitates the S_N2-type attack onto the *N*-methyl group of the isoquinoline backbone. This leads to carbon-carbon bond formation and concomitant transfer of a hydride to the flavin (Scheme 10). Another evidence for this mechanism lies in the position of the *N*-methyl group of the substrate and the FAD, which are in the appropriate constitution for performing hydride transfer. In contrast to previous findings, this mechanism incorporates both oxidation and ring closure into a single concerted step. Moreover, the mechanism demonstrated inversion of

configuration at the *N*-methyl group in agreement with earlier studies and implies that the stereochemical course of the reaction does derive from the stereospecifity of the S_N2-type reaction.[34]



Scheme 10: Concerted mechanism proposal for BBE

The same working group demonstrated that BBE is also able to further convert the biocatalytical reaction product (*S*)-scoulerine to dehydroscoulerine *via* a four electron oxidation (Scheme 11).[43]

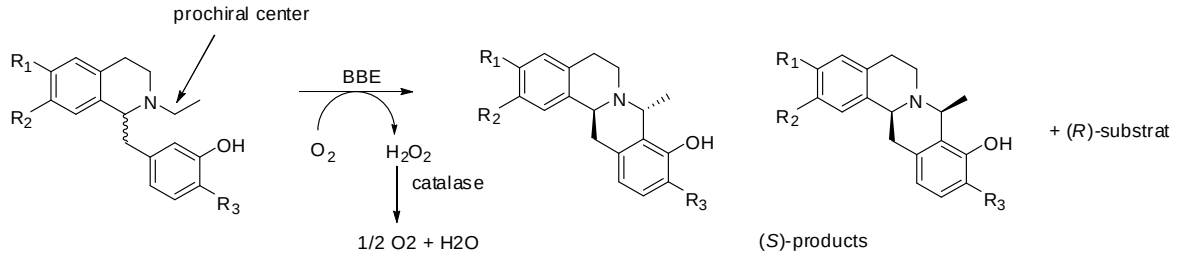


Scheme 11: Oxidation of (*S*)-scoulerine to dehydroscoulerine

Performing the reaction with an excess of the enzyme showed that scoulerine can be quantitatively converted into dehydroscoulerine. This finding can be rationalized either by a second enzymatic oxidation of the reaction intermediate or by spontaneous oxidation. Which scenario is correct has not been evidenced in detail till yet, but it is known that the further oxidation of the product to dehydroscoulerine is 3200 times slower than the conversion of (*S*)-reticuline to (*S*)-scoulerine.[43]

1.2.4 Diastereoselective enzyme variants

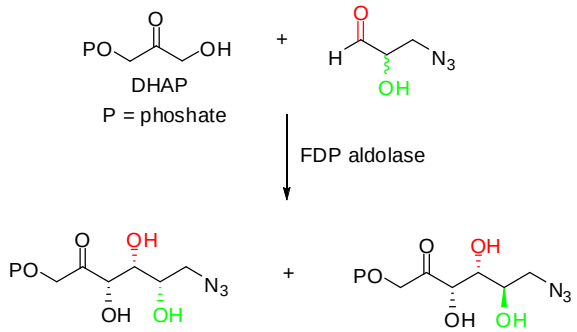
All benzylisoquinoline substrates dealed within this master thesis bear a racemic center at the C1 atom of the isoquinoline scaffold and a prochiral one introduced through an ethyl group located at the nitrogen. *Via* the ring closure by the berberine bridge enzyme a second chiral center would be formed at the ethyl moiety and through the enzyme's stereogenic preference only the substrate enantiomer with the (*S*)-C1 center undergoes cyclization (Scheme 12). The enzymatic transformation products are diastereoisomeres formed from substrates bearing a racemic and a prochiral center. This chapter presents other enzymes which are also able to form diastereoisomers, using substrates with the same characteristics.



Scheme 12: Diastereoselective cyclization of *N*-ethyl benzylisoquinoline substrates by BBE

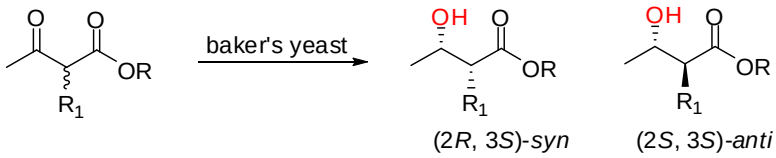
Aldolases are a class of enzymes which are able to form diastereoisomeres out of the same structural properties. They belong to the lyase family, which is capable to form carbon-carbon bonds in a highly stereoselective manner.[44] Aldol reactions catalyzed by aldolases are useful for the elongation of aldehydes by a two- or three-carbon unit. With few exceptions, the

stereochemical outcome of the aldol reaction is controlled by the enzyme and does not depend on the substrate structure. Therefore, the configuration of the carbon atoms adjacent to the newly formed C-C bond is highly predictable. By introducing a racemic center to the reacting aldehyde which undergoes the elongation reaction with the donor molecule diastereoisomers are obtained. For example fructose-1,6-diphosphate aldolase (FDP) isolated from rabbit muscle, catalyzes in its natural role the addition of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde-3-phosphate to form fructose-1,6-diphosphate. FDP accepts a wide range of aldehydes and therefore also those that bear a racemic center next to the carbonyl function (Scheme 13).[44]



Scheme 13: Stereospecific addition of DHAP and an racemic aldehyde *via* FDP aldolase

Another example for diastereoselective product formation is the important class of alcohol dehydrogenases which are able to reduce a broad range of ketones stereoselectively to furnish chiral secondary alcohols. During the course of reaction, the enzyme delivers the hydride preferentially either form the *re*- or the *si*-side of the ketone, to give (*R*)- or (*S*)-alcohols, respectively. The stereochemical course of the reaction mainly depends on the steric requirements of the substrate and can be predicted from the simple model which is generally referred to the "Prelog rule".[44] As a practical example the diastereoselective reduction of ketones *via* baker's yeast can be mentioned. Alpha-monosubstituted β-ketoester reduction using yeast leads to the formation of diastereomeric *syn*- and *anti*-β-hydroxyesters (Scheme 14). With small α-substituents, the formation of *syn*-diastereomers predominates, but the diastereoselectivity is reversed when increasing the substituent size.

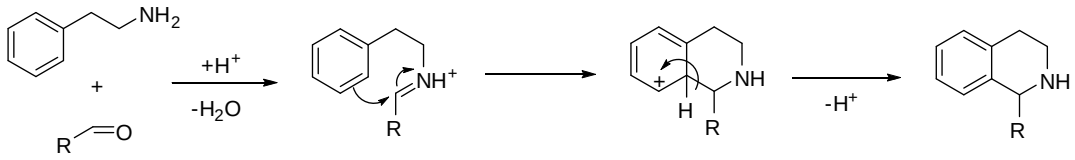


Scheme 14: Diastereoselective reduction of monosubstituted β-ketoesters *via* baker's yeast

1.3 Established synthetic pathways for benzylisoquinolines

1.3.1 The Pictet-Spengler condensation

The reaction was first discovered in 1911 by A. Pictet and T. Spengler when they condensed phenetylamine with methylal (dimethoxyethane) under the addition of hydrochloric acid resulting in the formation of tetrahydroisoquinoline.[45] Further investigations showed that the Pictet-Spengler reaction could be performed in non-acidic aprotic media as well as under the classical conditions of acidic catalysis.[46] Nucleophilic or rather electron-rich aromatic rings such as indole or pyrrole used in the structure of the amines give products in good yields under mild conditions (for example using TFA as an acid), while less nucleophilic aromatic rings such as phenyl give poor yields despite high temperatures and strong acids. If a hydroxyl group is represent in the *ortho* or *para* position of the aromatic ring in the amine's scaffold, acidic conditions are not required.[47,48] The Pictet-Spengler reaction starts with the condensation of the aldehyde and the amine under the loss of water and the *in situ* formation of the respective iminium ion. The electrophilicity of the imine double bond is the driving force of the following intramolecular aromatic substitution (S_EAr) which forms the tetrahydroisoquinoline product (Scheme 15). The Pictet-Spengler reaction can be considered as a special case of the Mannich reaction which originally consists of an amino-alkylation of an α-CH-acidic carbonyl compound with formaldehyde and ammonia, or any primary or secondary amine, forming the so called Mannich base as a final product.[49]

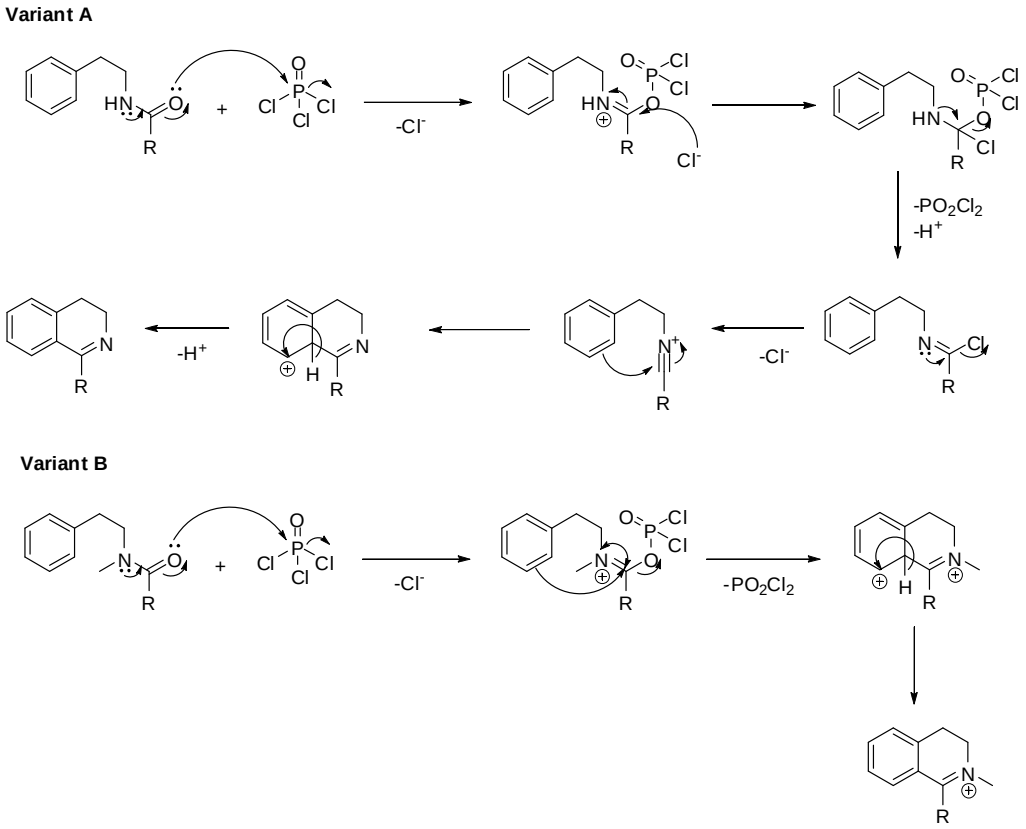


Scheme 15: Mechanism of the Pictet-Spengler condensation

1.3.2 The Bischler-Napieralski reaction

The most common method for the formation of benzylisoquinoline systems involves the use of the Bischler-Napieralki cyclization. The reaction was first established in 1893 by A. Bischler and B. Napieralski using phenetylacetamid as a substrate under the addition of phosphorus pentoxide in the same weight ratio and under heat.[50] The most widely used reagents in this reaction are phosphoryl chloride as well as phosphorus pentachloride, but thionyl chloride and carbonyl bromide have also been applied.[51,52] The first investigations of the reaction mechanism, using phosphorus pentachloride as a cyclization agent, propose the initial conversion of the amine to an imidoyl halide by a Lewis acid, followed by the formation of an nitrilium ion which further undergoes electrophilic ring closure (S_EAr). [53,54] When the used starting material is a tertiary amide the mechanism of the reaction is not the same as described above. Hence the cyclization takes place *via* simple phosphate elimination,

as the nitrilium ion cannot be formed.[55] Consequently two different reaction mechanisms can be introduced depending on the substrate and the reaction conditions (Scheme 16).



Scheme 16: The two different mechanisms for the Bischler-Napiralski reaction. Variant A: *Via* formation of the nitrilium ion for secondary amides, Variant B: For tertiary amides *via* elimination of the phosphate species to the cyclization product

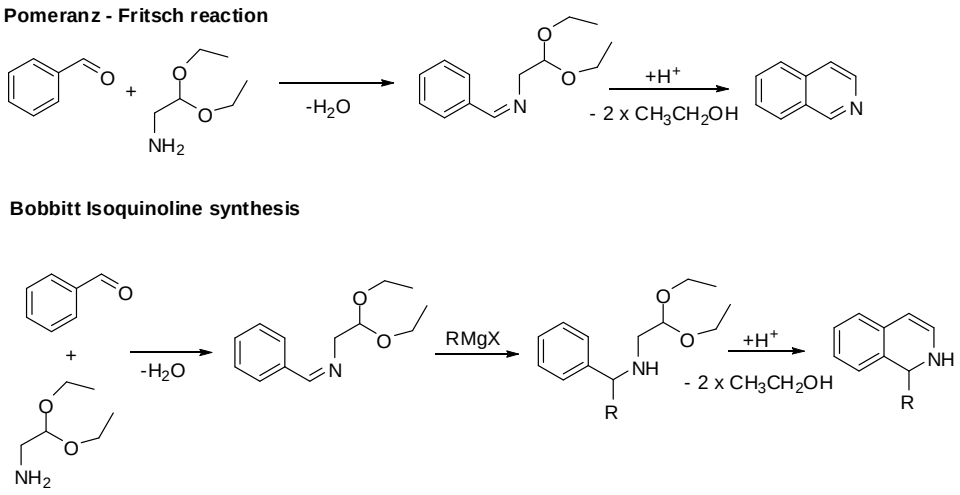
Further treatment with a reducing reagent like $NaBH_4$ subsequently gives the amine. The Bischler-Napieralski reaction strongly correlates with the Vilsmeier-Haack reaction in which aryl aldehydes or ketones are formed through reaction of an amide with an electron-rich arene under the use of phosphorus oxychloride. Both reaction mechanisms include an imidoyl chloride as intermediate.

1.3.3 Bobbitt isoquinoline synthesis

The synthesis is a modification of the Pomeranz-Fritsch reaction which is capable of forming isoquinoline out of benzaldehyde and aminoacetaldehyde diethyl acetal under acidic conditions (Scheme 17).[56]

Bobbitt *et al.* extended this reaction type for the synthesis of 1-alkyl-1,2,3,4-tetrahydroisoquinolines. The first reaction step leads to the formation of a Schiff base intermediate which is further alkylated by a nucleophilic attack from a Grignard reagent.[57] The obtained amine is cyclized under acidic conditions to afford the corresponding 1,2-

dihydroisoquinoline, which can be later reduced to result in the tetrahydroisoquinoline product.

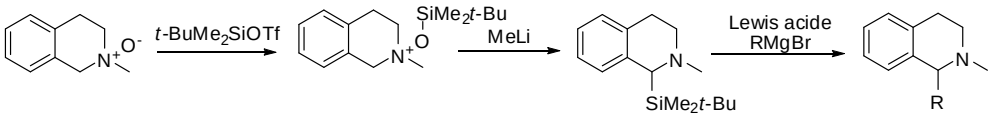


Scheme 17: Reaction sequence for the Pomeranz–Fritsch reaction and the Bobbitt isoquinoline synthesis

1.3.4 Alkylation of the vicinal C1-atom next to the nitrogen in the tetrahydroisoquinoline scaffold

1.3.4.1 Silicon Polonovski reaction

In 1927 Max and Michel Polonovski reported that the treatment of a tertiary amine *N*-oxide with acetic anhydride or acetyl chloride results in a rearrangement in which one of the alkyl groups attached to nitrogen is transferred to the vicinal carbon atom, and the *N*-acetyl derivative of the corresponding secondary amine and the aldehyde were obtained. The original work by the Polonovskis was mainly carried out on bicyclic tropane *N*-oxide derivatives and the reaction products were demethylated amides and formaldehyde.[58] The central feature of the Polonovski reaction is the formation of an α-acetoxyamine intermediate which reacts again with the anhydride to give the dealkylated product *via* an ammonium salt.[59,60] Some years later this reaction variant was modified by N. Tokitoh and R. Okazaki, instead of creating an α-acetoxyamine they obtained an α-siloxy amine. This was enabled by the *in situ* base-promoted rearrangement of a siloxyammonium salt obtained by treatment of a tertiary amine *N*-oxide with trialkylsilyltrifluoromethanesulfonate.[59] The C1-activation of the siloxy-group allowed a nucleophilic substitution by a Grignard reagent which led to the alkylation of the ring carbon atom next to the nitrogen (Scheme 18).[61]

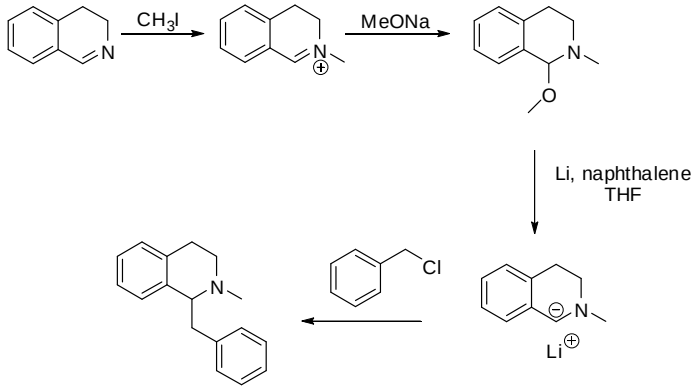


Scheme 18: Reaction scheme for the silicon Polonovski reaction.

The best results were obtained under the use of MeLi as a base and TMSOTf as a Lewis acid, but nevertheless the yields were quite moderate.

1.3.5 Alkali metal-mediated 1- and 4-substituted tetrahydroisoquinolines

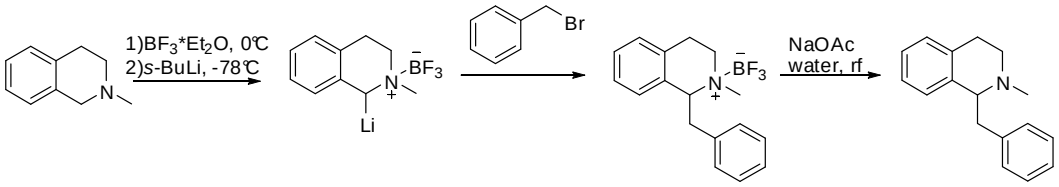
The reductive cleavage of 1-alkoxy-tetrahydroisoquinolines by a single electron transfer of a metal is highly regioselective, resulting in the cleavage of the C1-oxygen bond. Further treatment with different electrophiles led to the alkylation in different positions depending on the metal which was used. If lithium was used as an electron donor, C1-substitution was achieved, potassium reduction in contrast, led to the formation of a C4-substitution product.[62] For the successful accomplishment of this reaction type, catalytic amounts of naphthalene were added and all used tetrahydroisoquinoline educts had to be activated in the C1 position since otherwise a single electron transfer was not possible, because of the high electron density in this position, resulting from the proximity of the nitrogen atom (Scheme 19).



Scheme 19: Activation of the C1 position *via* alkali metal reduction

1.3.6 Activation of the C1 position *via* Lewis acids

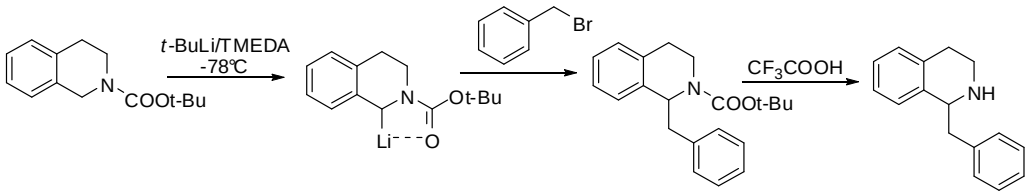
Due to its role as a Lewis acid, boron trifluoride is a good electron acceptor and therefore able to coordinate to the nitrogen lone pair and hence reduces the electron density at this position. Out of this fact deprotonation and further metalation at the C1 atom is greatly facilitated. Butyl lithium is a wide used metalation reagent in combination with the boron complex, whose metal ion can be easily replaced by various electrophiles (Scheme 20).[63]



Scheme 20: Pre-complexation with the Lewis acid (BF_3*Et_2O), followed by alkylation.

1.3.7 Activation of the C1 position *via* a carbamate formation

The activation of the C1 atom in the isoquinoline ring system can also be achieved through the reaction of the nitrogen atom with different electron withdrawing groups. The function of these groups lies in the stabilization of the incipient carbanion until the reaction with an electrophile is completed.[64] The versatility of the *t*-butoxycarbonyl (BOC) moiety as a protecting group for amines has been well documented and the group can be attached/removed easily. The *N*-protected tetrahydroisoquinoline further undergoes lithiation with organometallic reagents like BuLi. The intermediate is lithiated in the C1 position due to electronic stabilization through the carbamate (Scheme 21). Following addition of benzyl halides leads to the formation of the desired benzylisoquinoline.[64]



Scheme 21: Activation of the C1 position *via* protection of the nitrogen atom through BOC

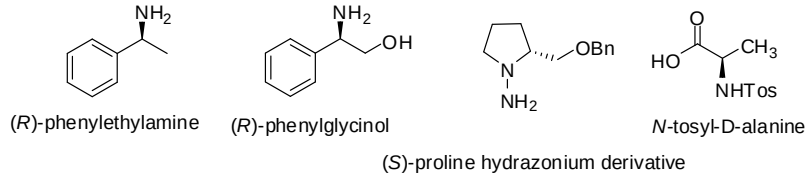
1.3.8 Asymmetric Synthesis

Asymmetric synthesis of isoquinoline alkaloids is generally based on two synthetic strategies: (i) stereochemical modification of the traditional, classical methods (Bischler-Napieralski reaction, Pictet-Spengler condensation and Pomeranz-Fritsch cyclization) and (ii) introduction of nucleophilic or electrophilic carbon units onto the C1 atom of the isoquinoline derivatives.[65]

1.3.8.1 Bischler – Napieralski cyclization

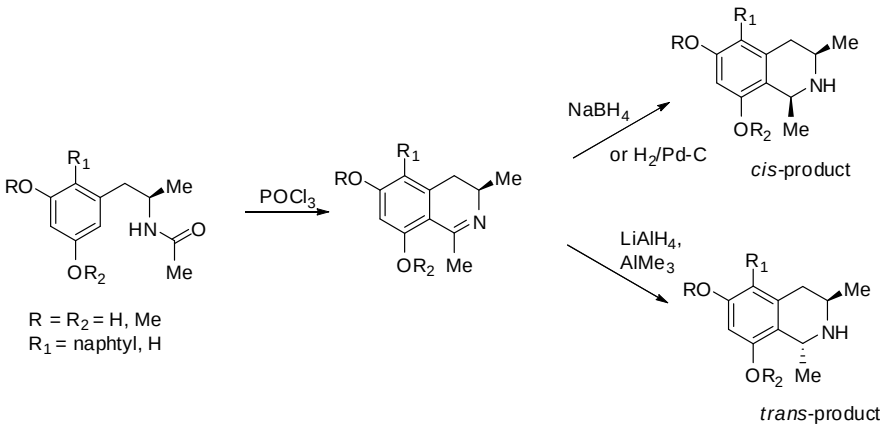
In this synthesis, β-arylethylamide is cyclized to get 1-substituted 3,4-dihydroisoquinoline, which is then reduced in the next step to the 1,2,3,4-tetrahydroisoquinoline derivative. The reduction step is crucial for the stereochemical outcome of the synthesis because it creates a stereogenic center, which can either be achieved in a diastereoselective or enantioselective way. One way to come along with this is the introduction of chiral auxiliaries which are

bound to the nitrogen in the isoquinoline scaffold and therefore directly affect the reduction side.[65] For this application chiral *N*-acyliminium salts that are equipped with *N*-protected amino acids placed on the nitrogen atom were generated *in situ* from dihydroisoquinolines. Among them *N*-tosyl-D-alanine and *N*-tosyl-L-proline chlorides used in combination with tetrabutylammonium borohydride as reducing agents show the highest degree of asymmetric induction.[66] Furthermore a chiral proline derivative with a hydrazonium functionality as well as (*R*)-1-phenylethyl-amine and (*R*)-phenylglycinol can be used as chirality inducing auxiliaries, combined with the reduction by sodium borohydride or palladium-hydrogen reduction (Scheme 22).[67-69]



Scheme 22: Different chiral auxiliaries for the asymmetric reduction of 3,4-dihydroisoquinolines

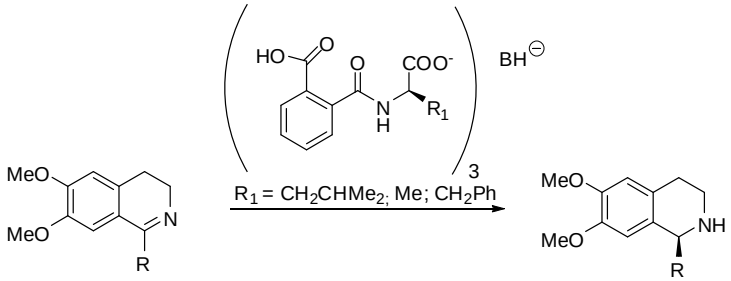
But there is not always the need for chiral auxiliaries to induce diastereoselectivity, if the dihydroisoquinoline that is formed *via* reduction owns a substituent in the C3 position, stereoselectivity can be introduced *via* the reduction conditions. For example acetamides prepared from chiral α-methyl-β-phenylethylamines can be either converted into *trans*-tetrahydroisoquinolines, using $LiAlH_4/Me_3Al$ as reduction reagents or yield in the *cis*-product with ($NaBH_4$ or H_2/Pd-C) (Scheme 23).[70]



Scheme 23: Diastereoselective reduction either *via* $NaBH_4$ or H_2/Pd-C leading to the *cis*-product or through $LiAlH_4/Me_3Al$ resulting in the *trans*-product. Requirement for this differentiation is the substitution in C3 position

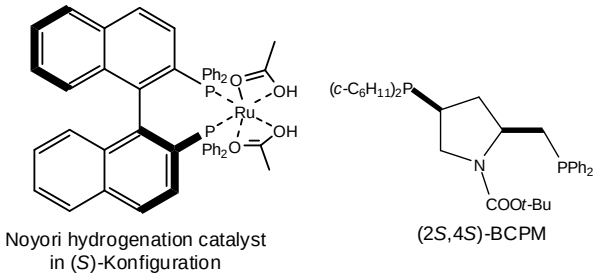
Enantioselective synthesis of isoquinoline alkaloids can be achieved either *via* chiral hydride reducing agents or through hydrogenation in the presence of chiral catalysts.[65] Sodium triacyloxy borohydrides prepared form $NaBH_4$ and *N*,*N*-phthaloyl-protected amino acids were used by Hajipour and Hantehzadeh in the synthesis of (*S*)-salsolidine or (*S*)-norcryptostyline

(Scheme 24).[71] In a similar synthetic strategy sodium borohydride was modified differently through the introduction of *N*-Cbz-proline.[72]



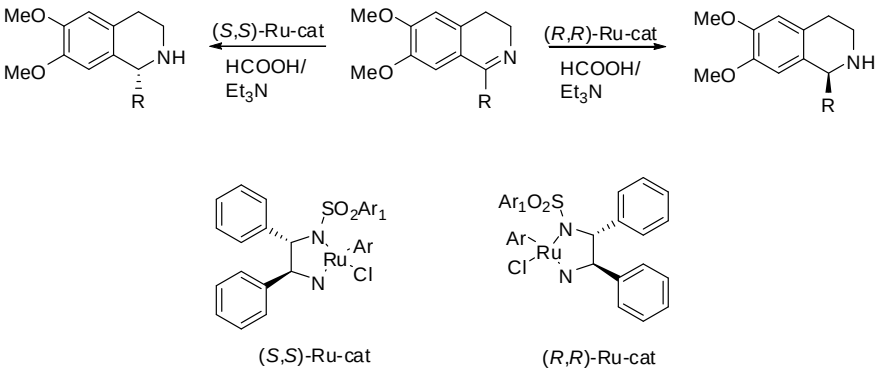
Scheme 24: Enantioselective reduction *via* triacyloxy borohydride

The probably most interesting achievements in the area of asymmetric hydrogenation were done by Noyori *et al.* in 1986. They applied a diphoshine-Ru(II) catalyst bearing the axially chiral ligand BINAP to *Z*-enamides yielding in excellent *ee*-values between 90 to 100% (Scheme 25). Notably the *E*-enamide substrates were inert to the present Ru-catalyzed hydrogenation conditions.[73,74] Other studies by C. A. Willoughby *et al.* used titanocene catalysts to control the stereochemistry of hydrogenation of cyclic imines, consequently the synthesis of isoquinolines with remarkable enantiomeric excess (*ee* = 98%) was achieved.[75] Morimoto *et al.* introduced Ir(I) complexes with (*S*)-BINAP and (2*S*,4*S*)-BCPM ligands for the reductive hydrogenation of dihydroisoquinolines to form alkaloids like (*S*)-norlaudanosine or (*S*)-salsolidine (Scheme 25).[76]



Scheme 25: Structure of the Noyori hydrogenation catalyst and (2*S*,4*S*)-BCPM

The method of choice in the stereoselective reduction of cyclic imines which was modified by many other working groups was introduced by Noyori *et al.* in 1996.[77] It represented an asymmetric hydrogen transfer of imines with formic acid and triethylamine catalyzed by a chiral *N*-sulfonated diamine-Ru(II)-η^6arene complex attainable in both enantiomeric forms (Scheme 26).

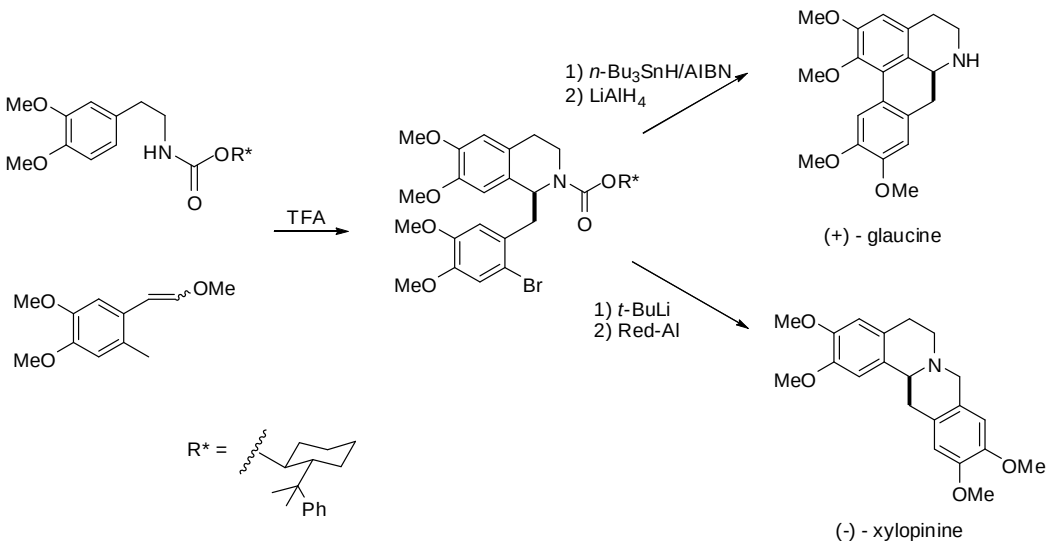


Scheme 26: Stereoselective reduction of cyclic imines by the chiral *N*-sulfonated diamine-Ru(II)-η^6arene complex

1.3.8.2 Pictet-Spengler condensation

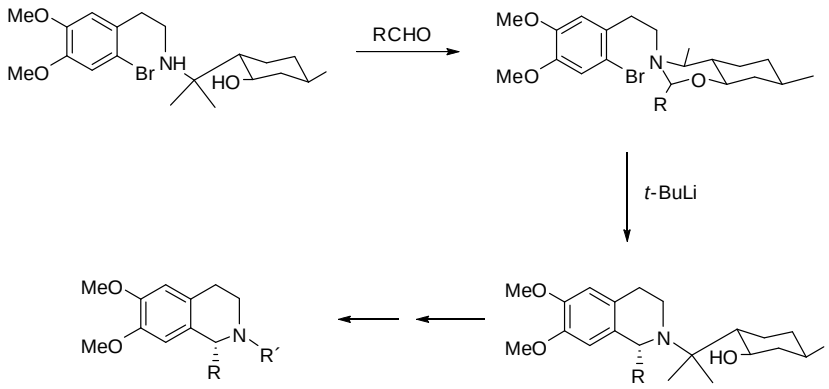
The Pictet-Spengler reaction involves the condensation of a β-arylethylamine with an aldehyde, ring closure and the formation of the stereogenic C1 center in a one pot process. This synthetic method represents a convenient method for the synthesis of tetrahydroisoquinoline derivatives.[65] In the condensation reaction that have been carried out in an asymmetric manner, the chirality transfer occurred from the chiral auxiliary introduced to either the amine or the aldehyde component, thus involving a diastereoselective synthesis.[65]

Comins *et al.* investigated the influence of a chiral auxiliary named (*R*)-*trans*-2-(α-cumyl)cyclohexyl, appended to the amine nitrogen. This amine structure in combination with a C2 bromine substituted aldehyde equivalent had a main contribution on the stereoselectivity in the cyclization step. It turned out that the bromine not only caused an increase in the diastereoselectivity in the ring formation (*de* = 77%) but also was helpful for separation of the diastereomeric products. By treatment with *n*-Bu_3SnH/AIBN and $LiAlH_4$, (+)-glaucine was obtained, where otherwise through the addition of *t*-BuLi and Red-Al (-)-xylopinine was achieved (Scheme 27).[78]



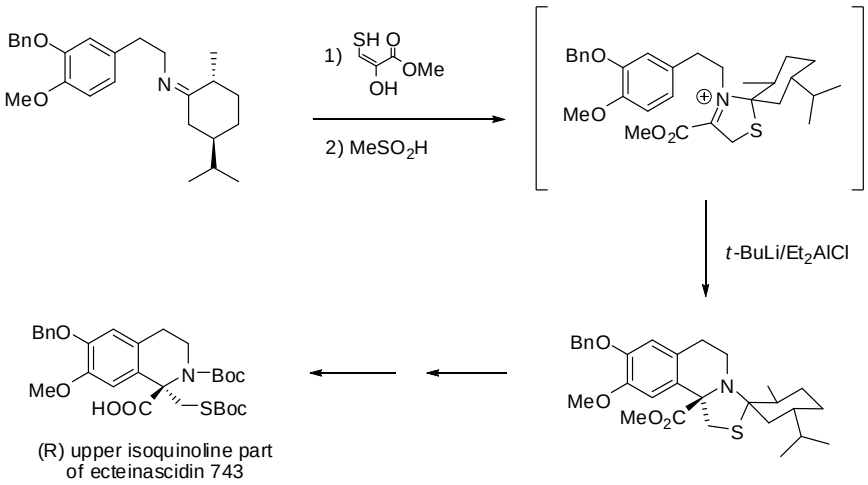
Scheme 27: Asymmetric Pictet-Spengler reaction through the chiral auxiliary (*R*)-*trans*-2-(α-cumyl)cyclohexyl, appended to the amine nitrogen

Another cyclohexyl-based chiral auxiliary placed on the amine nitrogen was derived from (*S*)-8-aminomenthol. The crucial step in this study was the incorporation of the aldehyde component into the *N,O*-acetal of perhydrobenzoxazine structure before the cyclization step took place. In this arrangement the intramolecular nucleophilic attack of the aromatic anion (generated by *t*-BuLi/Et_2AlCl) on the heterocycle occurred from the less hindered *si*-face to create the product with (1*R*) configuration.[79]



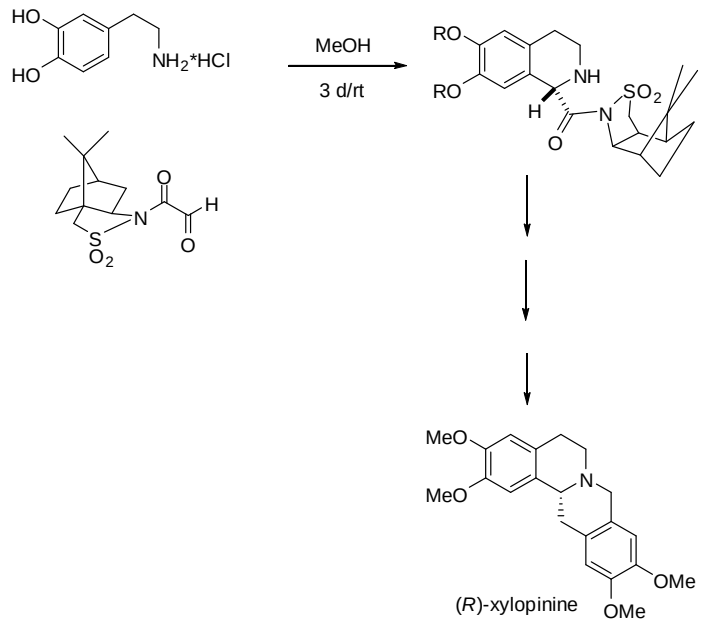
Scheme 28: Asymmetric Pictet-Spengler reaction using a chiral auxiliary derived from (*S*)-8-aminomethanol, appended to the amine nitrogen

Corey and Gin introduced a chiral imine obtained from β-arylethylamine and (+)-tetrahydocarvone to synthesize the upper isoquiniline part of the potent antitumor marine alkaloid, ecteinascidin 743. In the reaction with methyl mercaptopyruvate and methylsulfonic acid the educt was cyclized to form an iminium ion intermediate. Following Pictet-Spenger type condensation results in the formation of a tetrahydroisoquinoline, which further undergoes ester hydrolysis of the diastereomeric educt. The hydrolysis step allows the separation of the diastereoisomers, hence the (1*R*)-isomer hydrolyzed faster and could be transformed into the (1*R*)-final product after separation, acetal hydrolysis and BOC protection (Scheme 29).[80]



Scheme 29: Asymmetric Pictet-Spengler reaction using a chiral imine obtained from β-arylethylamine and (+)-tetrahydocarvone

Through the application of a chiral aldehyde in the Pictet-Spengler reaction Cazarnocki *et al.* synthesized (*R*)-xylopinine. Therefore they used an Oppolzer sultam substituted at the nitrogen with a glyoxyloyl group as an aldehyde equivalent and condensed this with dopamine hydrochloride. The product was of excellent diastereomeric excess (*de* = 86%) and another 6 steps led to the final product (*R*)-xylopinine (Scheme 30).[81]

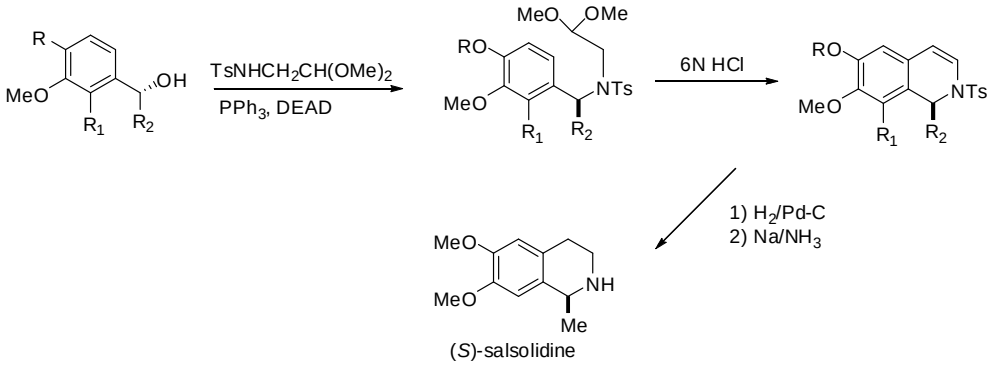


Scheme 30: Synthesis of (*R*)-xylopinine *via* asymmetric Pictet-Spengler reaction

1.3.9 Pomeranz-Fritsch synthesis

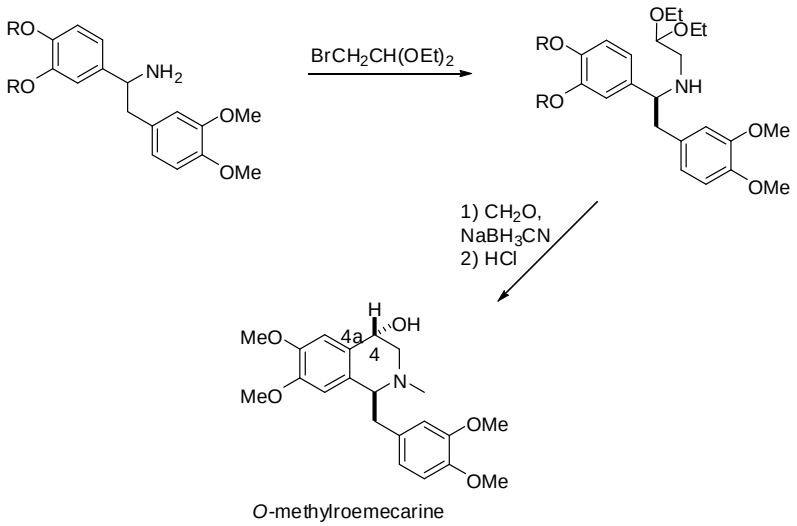
The original Pomeranz-Fritsch method represents an acid-catalyzed cyclization of benzaldehyde and aminoacetaldehyde diethylacetal to give fully aromatic isoquinolines. A useful modification for this synthetic approach was developed by Bobbitt to form tetrahydroisoquinolines.[57] Asymmetric Pomeranz-Fritsch-Bobbitt synthesis can be either achieved *via* aminoacetals from chiral benzyl alcohols on treatment with an aminoacetaldehyde acetal, or *N*-alkylation of chiral benzylamines with bromoacetaldehyde acetal. In another approach, addition of organometallic reagents to prochiral imines was carried out in the presence of external controllers of stereochemistry.[65]

Kaufman *et al.* introduced chiral benzyl alcohols in their synthesis of (*S*)-salsolidine. The treatment of the alcohol with *N*-tosylaminoacetaldehyde acetal under the Mitsunobu reaction condition produced the tosylamides in a satisfactory yield with enantioselectivity up to 95%. Further acidification followed by hydrogenation (H_2/Pd-C) and detosylation (Na/NH_3) yielded (*S*)-salsolidine (Scheme 31).[82]



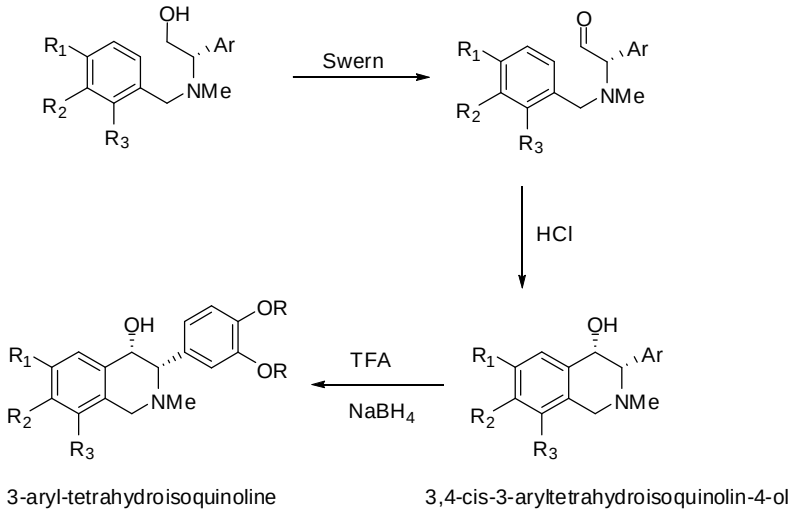
Scheme 31: Synthesis of (*S*)-salsolidine

The second alternative for the synthesis of asymmetric tetrahydroisoquinolines *via* the Pomeranz-Fritsch-Bobbitt reaction is the *N*-alkylation of optically active benzylamines with bromoacetaldehyde acetal followed by *N*-methylation and C4-C4a cyclization to result in *O*-methylroemecarine (Scheme 32) for example.[83]



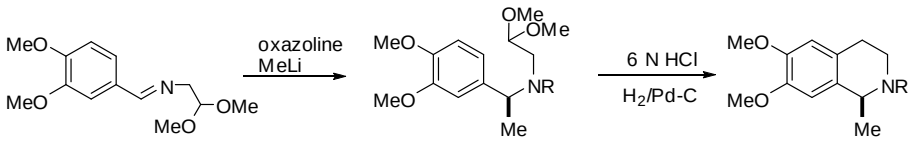
Scheme 32: Synthesis of *O*-methylroemecarine

In another series of experiments, chiral amines were oxidized *via* Swern oxidation to the corresponding aldehydes, which were then further cyclized under acidic conditions to give diastereomerically pure 3,4-*cis*-3-aryltetrahydroisoquinoline-4-ols. These structures can be further deoxygenated to give 3-aryl-substituted terahydroisoquinolines which are intermediates on the route to benzophenanthridines (Scheme 33).[84,85]



Scheme 33: Synthesis of 3-aryl-tetrahydroisoquinolines

As already mentioned organometallic reagents can be applied to the Pomeranz-Fritsch-Bobbitt reaction. Therefore methyllithium was used to alkylate the *E*-imine double bond stereoselectively. Best yields (92%) and enantioselectivity (79% *ee*) could be obtained in the presence of oxazolines as catalysts. The synthesis was completed by one pot cyclization/hydrogenolysis of the aminoacetal in 6 N HCl of the aminoacetal (Scheme 34).[86]

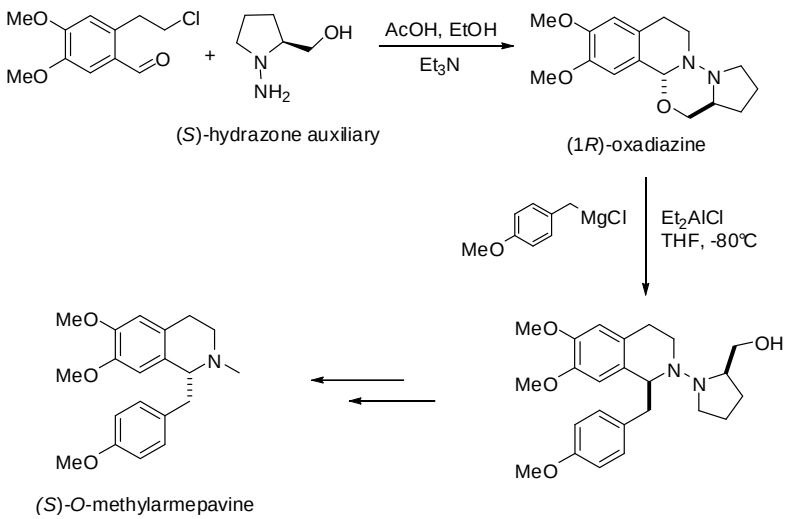


Scheme 34: Asymmetric Pomeranz-Fritsch-Bobbitt reaction *via* the alkylation of *E*-imines

1.3.9.1 C1-Cα connection

In this strategy the C-C bond between the isoquinoline ring C1 atom and the Cα substituent can be realized by two general synthetic ways: (i) addition of carbon nucleophiles to isoquinolines, or the corresponding isoquinolinium ions; (ii) *via* C1-alkylation of tetrahydroisoquinoline derivatives with electrophilic carbon reagents. The stereogenic center is created at this stage of the synthesis.[65]

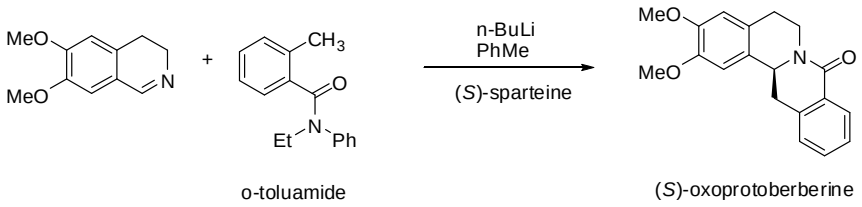
In the addition of carbon nucleophiles to isoquinoline derivatives, asymmetry is induced by a chiral auxiliary in most examples. Therefore a chiral hydrazonium ion introduced from Kibayashi can be applied. This method was already used for the reductive hydrogenation of C1 substituted Bischler-Napieralski dihydroisoquinolinium ions (see above 3.5.1). If the auxiliary bears a free hydroxyl group, (*R*)-oxadiazines are formed as intermediates, which strictly undergo inversion by the attack of a Grignard nucleophile. With this synthetic approach variant compounds like (*S*)-*O*-methylarmepavine could be formed (Scheme 35).[67]



Scheme 35: Synthesis of (*S*)-*O*-methylarmepavine

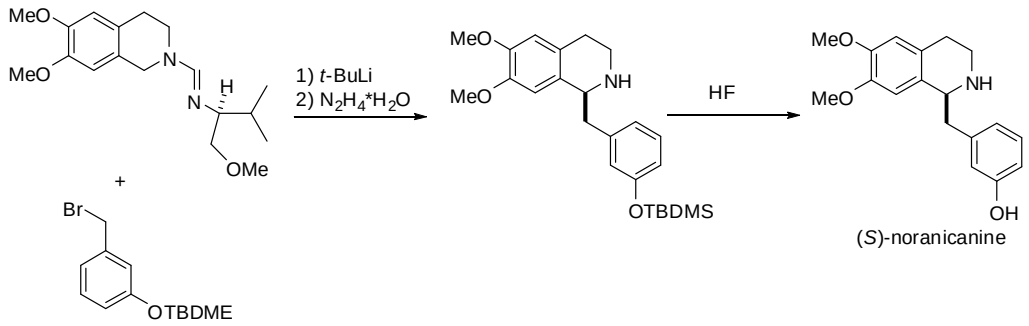
But there is not always the need for chiral auxiliaries. For example an efficient enantioselective synthesis of a protoberberine is based on (*S*)-sparteine addition of nonchiral *o*-toluamides which were lateral metalated. (*S*)-sparteine induced stereochemical preference caused by the highly sterically demanding nature of the assembly. The enantiomeric purity of the addition/cyclization product depended strongly on the nature of the residues of the amide. The best results (77% *ee* and 45% yield) were achieved when ethyl and phenyl groups were

used as N-substituents. The structure of (*S*)-sparteine restricted the conformational change of the amide group, thus positioned the chiral substituent in favored orientation which dominated the enantio-facial addition of the o-toluamide anion to 3,4-dihydroisoquinoline (Scheme 36).[87]



Scheme 36: Synthesis of an (*S*)-oxoprotoberberine

The synthesis of tetrahydroisoquinoline derivatives *via* alkylation of the C1-carbanion was strongly pushed forward by the formamidine carbanion chemistry of Meyers and co-workers.[88] During the past decade it turned out that it was quite problematic to generate a carbanion adjacent to nitrogen. The solution to this problem led to activation of the nitrogen with a suitable electron-withdrawing group increasing the kinetic acidity of the C1-proton and stabilize the carbanion by chelation. Meyers working group introduced chiral formamidines by simply affixing the appropriate group to the nitrogen which resulted in obtaining a highly stereoselective reaction. A very well known and high-performance fromamidine is derived from valinol methyl ether (VME). Using these chiral substrates the following carbanion formation *via* organo metal reagents is strongly facilitated, because of the double lone pair coordination from oxygen and nitrogen to the metal ion. Subsequent alkylation with *O*-silylated 3-hydroxybenzyl bromide, followed by cleavage of the silyl ether, results in the formation of (*S*)-noranicanine with an overall yield of 54% and high enantioselectivity (Scheme 37).[89,90]

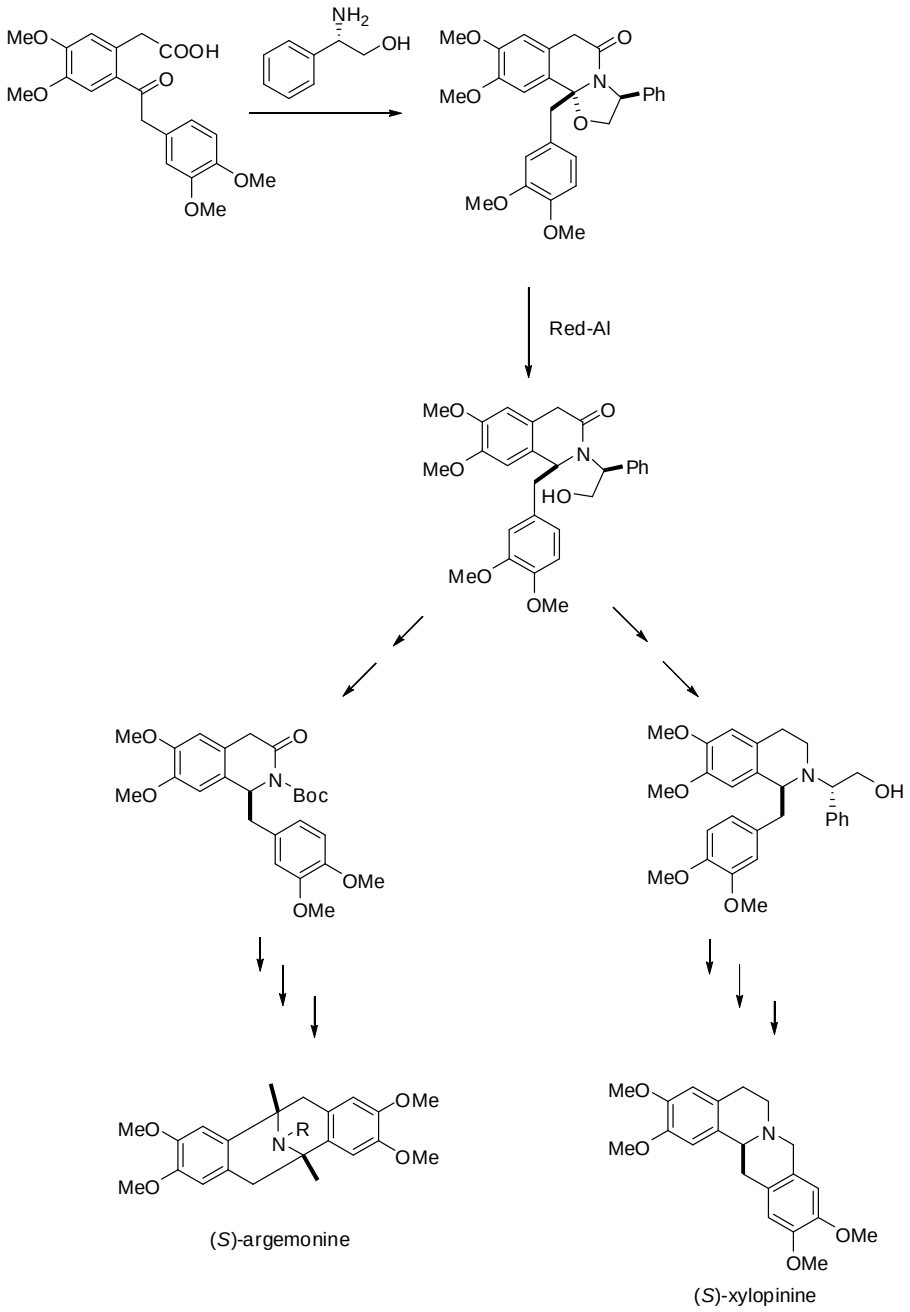


Scheme 37: Synthesis of (*S*)-noranicanine

1.3.10 Other synthetic options

Besides traditional methods for the synthesis of isoquinoline alkaloids, described in the previous section, there are many potent alternatives in which the ring closure occurs on other positions as discussed so far.[65]

For example Meyers and Munchhof synthesized several alkaloids from ketoacids that contain the alkaloids carbon framework.[91] They started with the condensation of the ketoacid with (*S*)-phenylglycinol which led to the formation of a chiral bicyclic lactam as a single isomer. Selective cleavage of the aminal C-O bond without affecting the carbonyl group (Red-Al, below -30°C) yielded 90% of the product lactam. At this point of the synthesis the resulted lactam can either be reduced with $LiAlH_4$ to the corresponding amine which than would be *N*-deprotected and further cyclized *via* Pictet-Spengler condensation to give the (*S*)-xylopinine as the product. The other possibility would be to cleave the C-N bond of the lactam first and then BOC-protect the nitrogen. Following reduction of the BOC-protected lactam led to the formation of a carbinolamine which further underwent ring closure to yield enantiopure (*S*)-argemonine (Scheme 37).[91]



Scheme 38: Synthesis of (*S*)-argemonine and (*S*)-xylopinine

1.4 References

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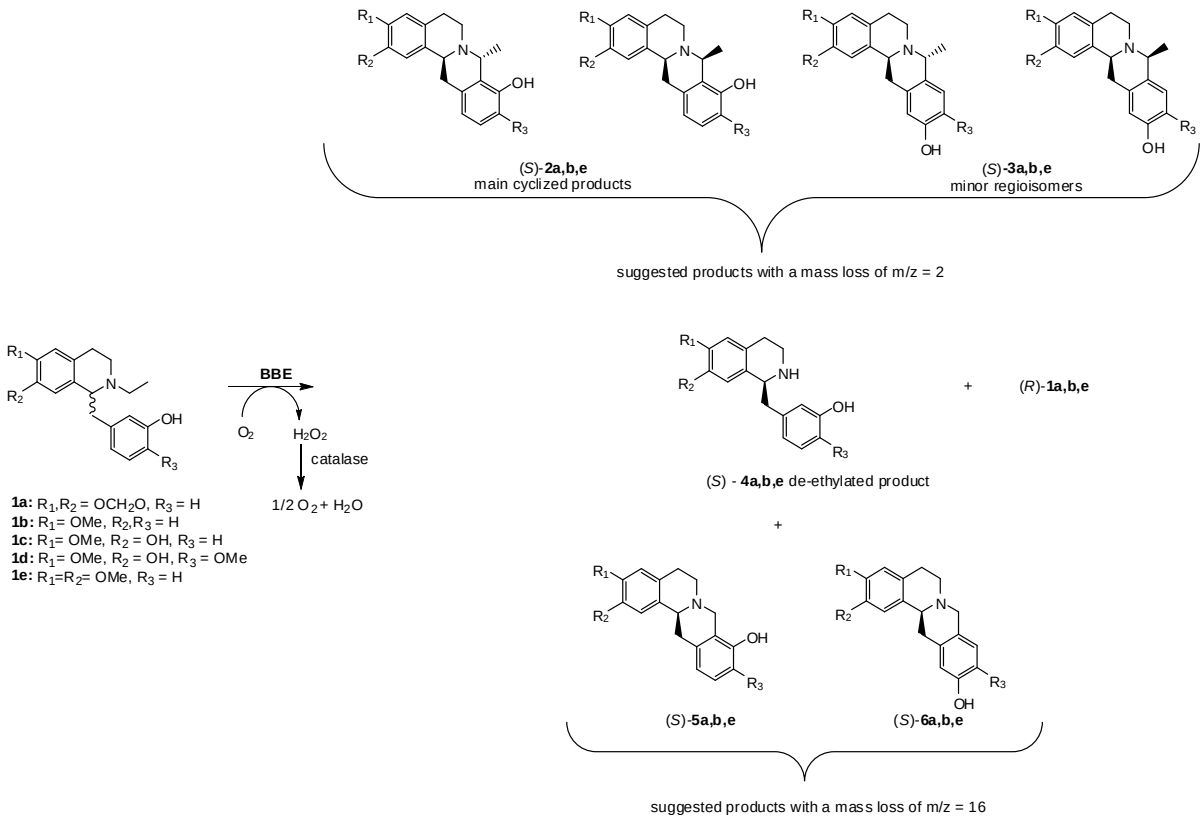
2 RESULTS AND DISCUSSION

2.1 Definition of the project

The aim of the project was to synthesize four different benzylisoquinolines (**1a-1d**) which should be further used in the biotransformation by the berberine bridging enzyme (BBE). This enzyme represents an outstanding and unique biocatalyst which enables the oxidative C-C bond formation of its natural substrat (*S*)-reticuline to (*S*)-scoulerine in plants. Recent investigations have shown that the enzyme is also able to cyclize a broad range of non-natural substrates, whereby only the (*S*)-enantiomer of the racemic benzylisoquinolines is transformed into optically pure (*S*)-berbines.[1] In contrast to various non-natural substrates that were tested for the conversion with BBE so far, the components investigated in this thesis bear an additional prochiral center achieved by an ethyl group attached to the isoquinoline nitrogen. As a result of this project it turned out that not only cyclization occurs, but also dealkylation (Scheme 39). The further task was to analyze if the enzyme transform the dealkylation reactions enantiospecifically as well as figuring out if the enzyme has a stereopreference for the newly introduced chiral center formed *via* the cyclization reaction.

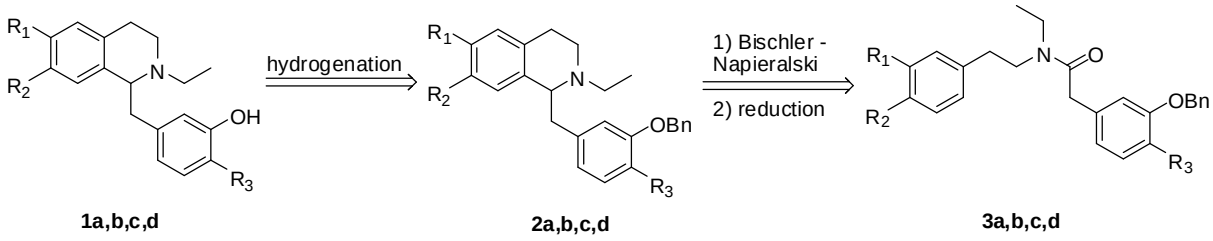


Scheme 39: Biotransformation of the *N*-ethyl benzylisoquinoline substrates **1a-e** by BBE

2.2 Organic synthesis of the four *N*-ethyl benzylisoquinoline substrates

Various strategies for the synthesis of benzylisoquinolines are known in the literature. The current methods for the synthesis of C1 substituted tetrahydroisoquinolines usually rely on three different strategies which are already discussed in the synthetic introduction part 1.4: (i) formation of the isoquinoline scaffold *via* a cyclization reaction either by the Pictet-Spengler or Bischler-Napieralski reaction (ii) alkylation of the C1 atom of the isoquinoline core by a nucleophile or an electrophile (iii) ring closure and formation of the scaffold *via* C4-C4a construction towards the Pomeranz-Fritsch reaction.

For the synthesis of the four different *N*-ethyl benzylisoquinoline substrates the Bischler-Napieralski reaction was chosen to build up the tetrahydroisoquinoline scaffold. The synthetic focus was laid on this reaction since it offered a broad scope of mild reaction conditions and resulted in moderate to high yields.[2]



Scheme 40: Retrosynthesis for the *N*-ethyl substrates **1a-d** using the Bischler-Napieralski reaction

The cyclization reaction of the amide was carried out using the Lewis acid $POCl_3$ which led to the formation of the corresponding 3,4-dehydroisquinoline derivatives. *Via* the following reduction with $NaBH_4$ the saturated 1,2,3,4-tetrahydroisoquinoline products were formed. Table 1 lists the yields of the cyclization reaction which resulted in high values for all of the four substrates.

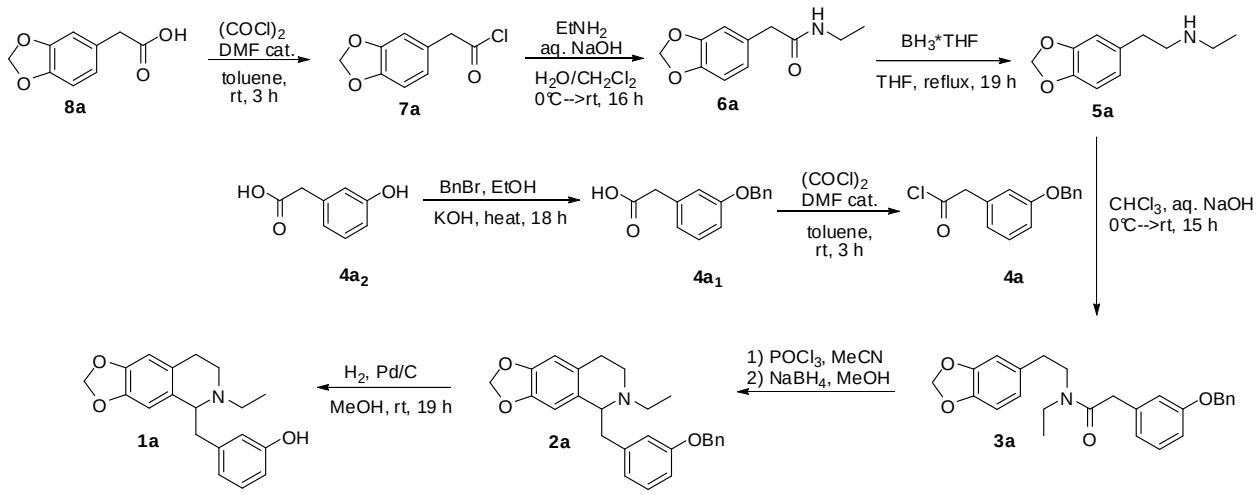
Table 1: Yields for the Bischler-Napieralski reaction in the reaction sequences of each substrate

	2a	**2b**	**2c**	**2d**
yield [%]	95	97	86	94

The phenolic moiety was protected with a benzyl group to avoid interactions with the Lewis acid. To obtain the free hydroxyl group which is absolutely necessary for the following biotransformation with BBE, the protective group was cleaved *via* hydrogenation using palladium on active charcoal under hydrogen atmosphere (Scheme 40).

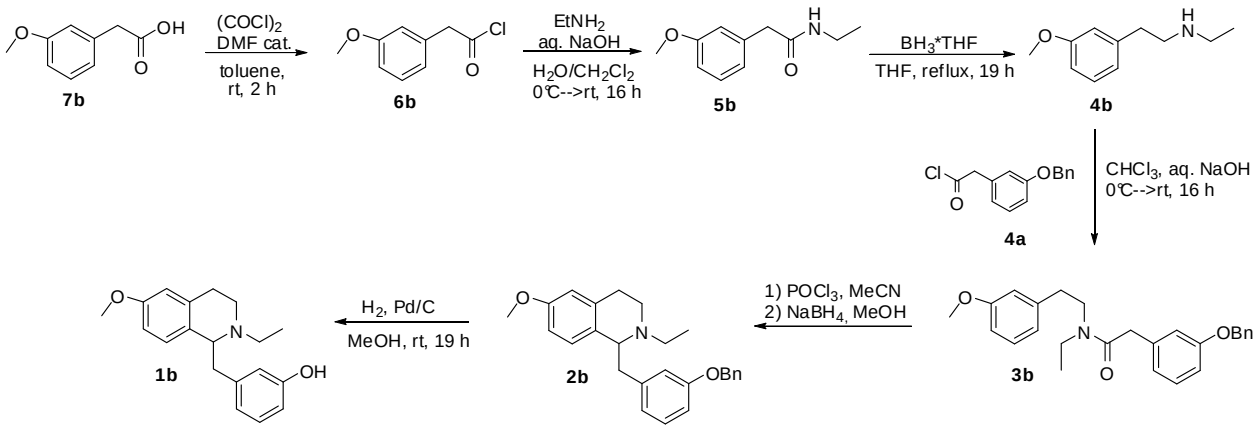
These three reactions described above are the synthetical main point in every substrate synthesis.

In the synthesis of 2-ethyl-1-(3-hydroxybenzyl)-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline (**1a**), the first step was the chlorination of (3,4-methylenedioxy)phenyl-acetic acid **8a** with oxalyl chloride (Scheme 41). The corresponding acyl chloride was converted into the *N*-ethyl amide **6a** *via* nucleophilic attack of ethylamine. Further mild reduction of the carbonyl group through the Lewis acid BH_3 resulted in the appropriate amine **5a**. The formation of the amide substrate **3a** for the Bischler-Napieralski reaction was achieved *via* the coupling of the previously formed amine **5a** with 3-benzyloxyphenylacetyl chloride **4a**. The chloride **4a** was synthesized from the corresponding acid that was first protected with benzylbromide and then converted into the acyl chloride by oxalyl chloride.



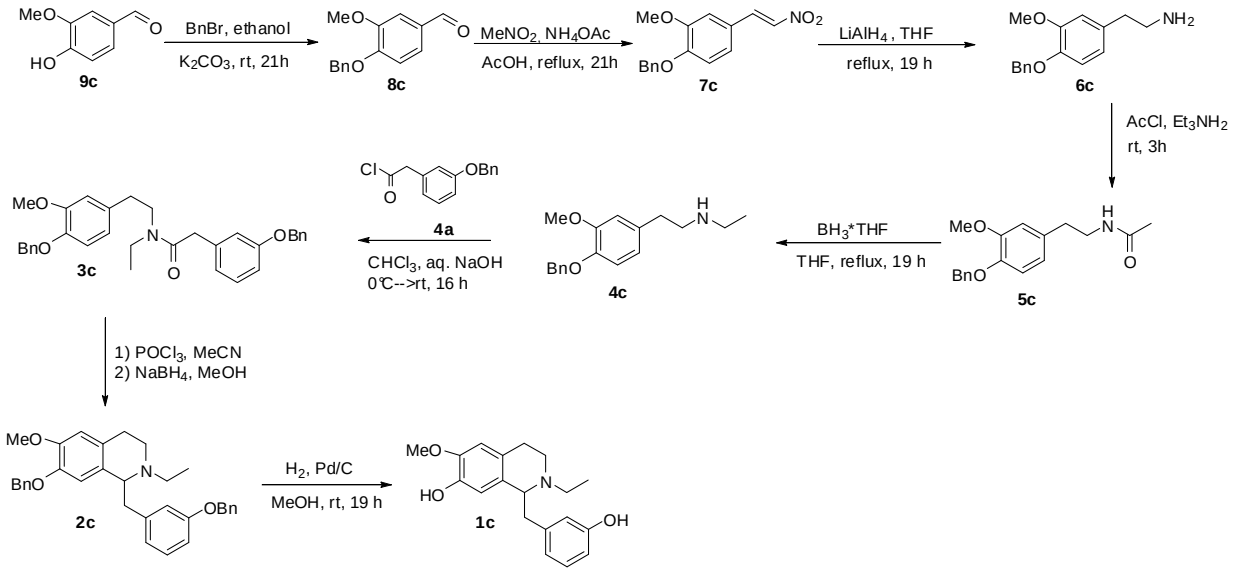
Scheme 41: Synthesis of substrate **1a**

The reaction sequence for the synthesis of 2-ethyl-1-(3-hydroxy)-6-methoxy-1,2,3,4-tetrahydroisoquinoline (**1b**) strongly resembled the one of substrate **1a**, exclusively the starting material was different. In this case 3-methoxyphenyl-acetic acid **7b** was used as precursor for the initial chlorination step. Again the same acyl chloride **4a** was applied for the formation of the amide **3b** which later underwent the cyclization reaction (Scheme 42).



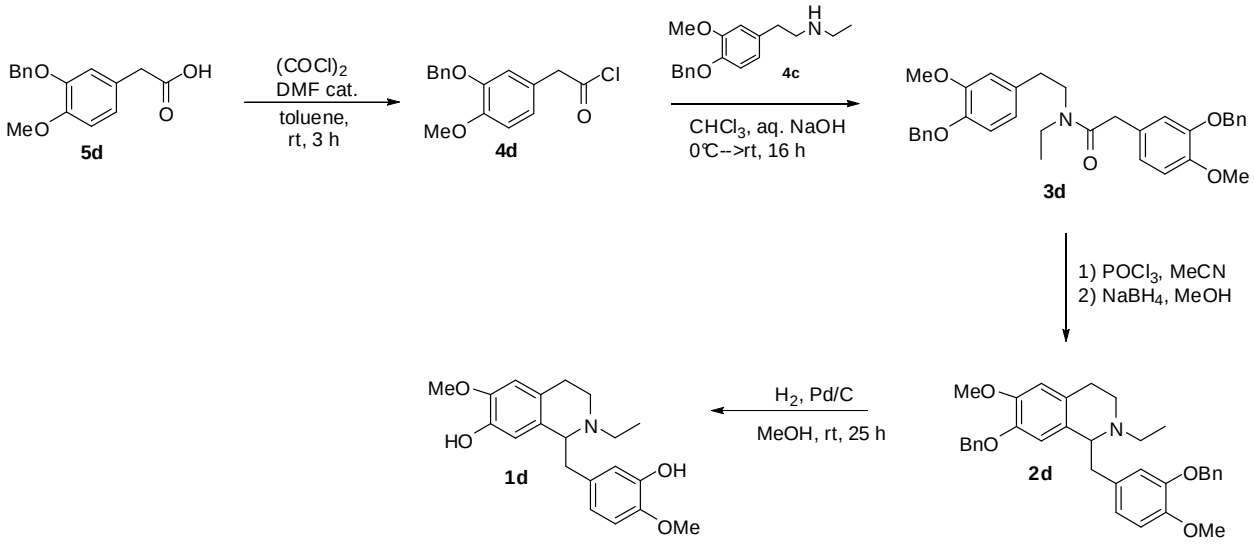
Scheme 42: Synthesis of substrate **1b**

For the synthesis of the third substrate 2-ethyl-6-methoxy-7-hydroxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (**1c**), vanillin **9c** was chosen as educt. First of all the free hydroxyl group was protected *via* benzylation (Scheme 43), subsequently the aldehyde underwent a Henry reaction with nitromethane to form the corresponding nitrostyrene **7c**. The Henry reaction strongly resembles the aldol reaction and therefore is also called "nitro-aldol reaction". In this case the deprotonated nitroalkane plays the role of the enolate ion formed in the reaction from which it derives. Addition of $LiAlH_4$ reduced the double bond as well as the nitro-group to yield the corresponding amine **6c**, which was further acetylated with acetyl chloride. Afterwards the carbonyl group of **5c** was reduced using BH_3 and the resulting *N*-ethyl amine **4c** reacted with 3-benzyloxyphenylacetyl chloride **4a** to form the precursor **3c** for the Bischler-Napieralski reaction.



Scheme 43: Synthesis of substrate **1c**

At least four reaction steps were required in order to form 2-ethyl-6-methoxy-7-hydroxy-1(-3-hydroxy-methoxybenzyl-1,2,3,4-tetrahydroisoquinoline (**1d**) (Scheme 44). To this end 2-(3-(benzyloxy)-4-methoxyphenyl)acetic acid **5d** was chlorinated to result in the desired acyl chloride which further underwent a S_N2 reaction with the *N*-ethyl amine **4c** which was synthesized in the reaction sequence of substrate **1c** to form amide **3d**. Then, according to previous synthetic strategies the Bischler-Napieralski reaction and a reduction with $NaBH_4$ followed by a palladium catalyzed cleavage of the protective group.



Scheme 44: Synthesis of substrate **1d**

The reaction sequences for the synthesized substrates **1a-d** are based on recently described *N*-methyl analogues investigated and synthesized by Schrittwieser *et al.*[2] For the introduction of the new prochiral center *via* the ethyl group located at the isoquinoline nitrogen, new reagents had to be used to elongate the alkyl part attached to the nitrogen. Due to the fact that the reaction sequence for each substrate was quite complex and at least consisted of four steps the linear overall yields are given in table 2.

Table 2: Linear overall yields and the according synthetic steps for each substrate synthesis

	substrate **1a**	substrate **1b**	substrate **1c**	substrate **1d**
synthetic steps	6	6	8	4
linear overall yield [%]	57	50	11	61

The tendency of table 2 is easy to interpret. The increasing reaction steps lead to a decrease in the overall yield, therefore the synthesis of substrate **1d** shows the highest yield. Consequently synthesis development always has to consider the influence of dropping yield over a long reaction sequence.

2.3 Biotransformations

The transformations using the berberine bridge enzyme were performed with all five substrates outlined in Scheme 38 (2.1). Unfortunately only the substrates **1a**, **1b** and **1e** were converted by the enzyme in contrast to 2-ethyl-6-methoxy-7-hydroxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinolin (**1c**) and 2-ethyl-6-methoxy-7-hydroxy-1(-3-hydroxy-methoxybenzyl-1,2,3,4-tetrahydroisoquinoline (**1d**), where no product formation could be detected at all. Consequently only the results of the accepted substrates are shown and discussed.

2.3.1 Comparison of the wild type and the enzyme variant W165F for substrate 1a, 1b and 1e

To figure out if the wild type enzyme or its variant in which tryptophan is replaced by phenylalanine educes higher apparent conversions, **1a**, **1b** and **1e** were tested with both enzyme alternatives at reaction times of 2 and 24 hours. For substrates **1a** and **1b** the organic solvent formamide (10% v/v) was applied for the biotransformation whereupon for substrate **1e** 10% v/v formamide, DMSO and *N*-methylformamide were used. The wild type as well as the variant was applied at a concentration of 1.0 g/L. These values correspond to a molarity of 17 µM for both enzyme alternatives.

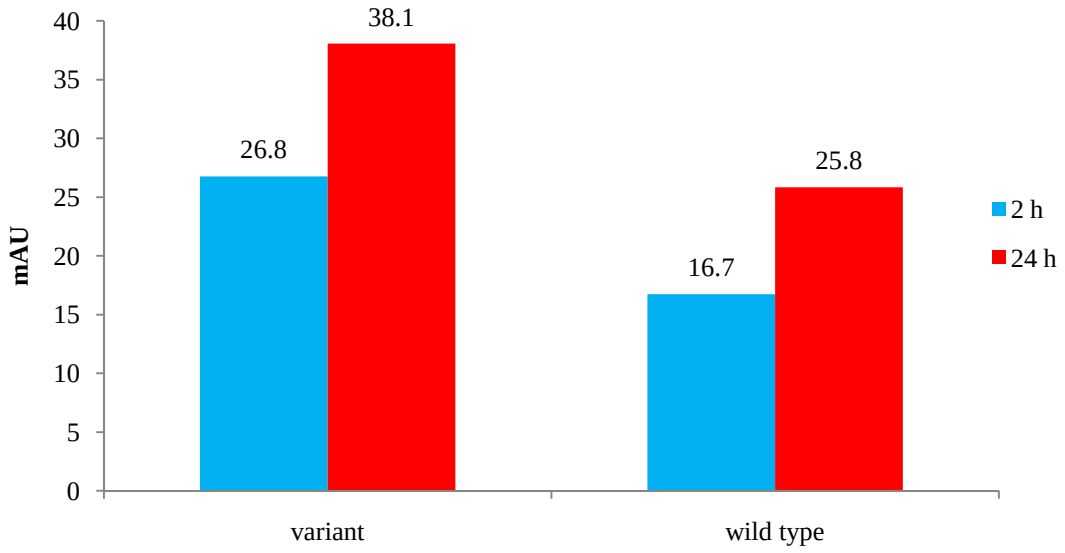


Figure 4: Apparent product formation for substrate **1a** either under useing the wild type enzyme or its variant W165F. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F or BBE wild type (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v formamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

In the case of substrate **1a** the enzyme variant showed higher apparent conversions at both reaction times, contrarily substrate **1b** gave almost the same values for the wild type as well as for the W165F variant. Notable at this point are the slightly higher apparent conversions for **1b** after two hours of reaction time compared to those of 24 hours.

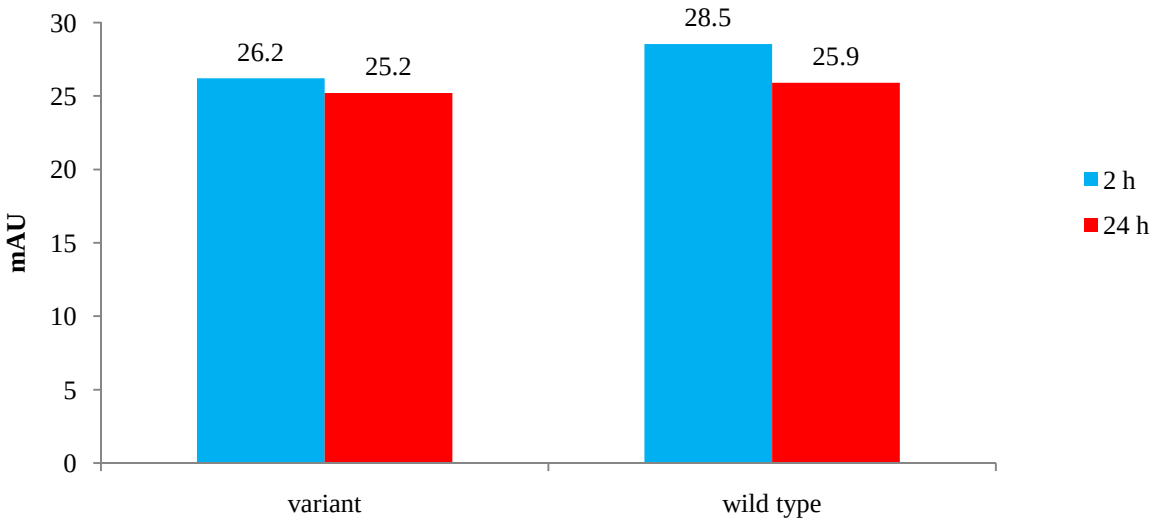


Figure 5: Apparent product formation for substrate **1b** either under using the wild type enzyme or its variant W165F. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F or BBE wild type (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v formamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

For substrate **1e** the apparent conversions of the variant were higher than those of the wild type when DMSO and *N*-methylformamide were used. In the case of formamide as organic solvent the results for both, the wild type and the variant, were similar.

The reason for the better or related results employing the enzyme variant W165F can be explained by the higher steric demand of the substrates. As already mentioned above when the variant was used, the aromatic amino acid tryptophan was substituted with phenylalanine. The substrates which bear an *N*-ethyl group in the tetrahydroisoquinoline scaffold required more space in the active site to be converted, compared to the natural substrate of BBE only exhibiting a less space-demanding methyl group.

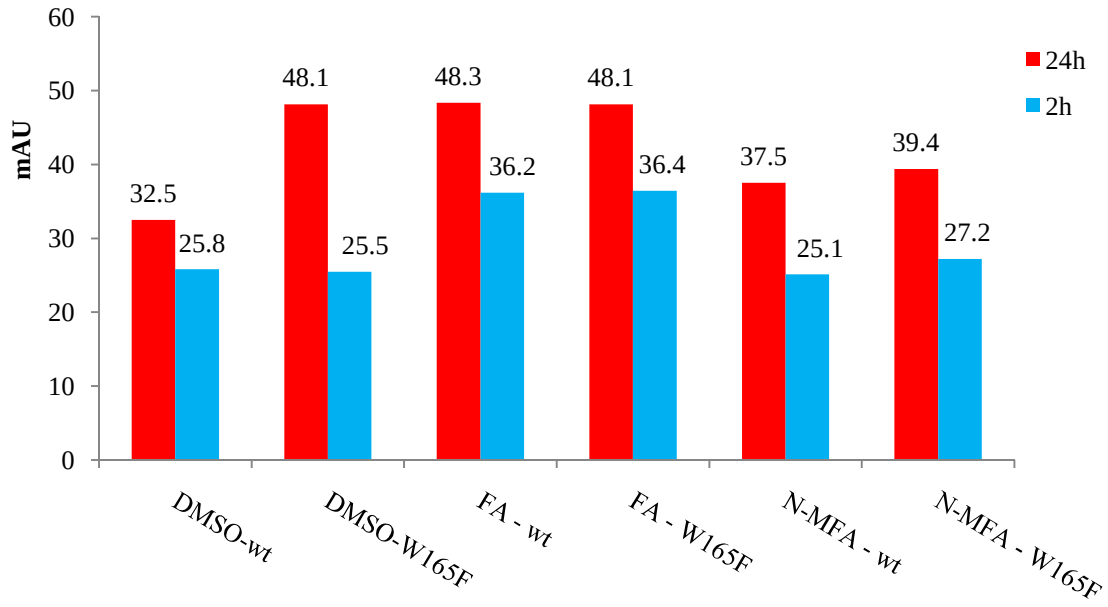


Figure 6: Apparent product formation for substrate **1e** either employing the wild type enzyme or the variant W165F. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F or BBE wild type (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v formamide, DMSO, *N*-methylformamide, 5 g/L catalase, 2 and 24 h, 40°C, 400rpm.

Comparison of the two enzyme alternatives suggested to employ variant W165F in all further biocatalytic experiments.

2.3.2 Solvent studies

To evaluate the tolerance of the enzyme towards organic solvents and to figure out in which of them the highest apparent conversion can be obtained, different organic solvents and one ionic liquid (EMimAc = 1-ethyl-3-methylimidazolium acetate) were tested. The screenings were performed using the BBE variant (W165F), 10% v/v organic solvent with 2 g/L of the corresponding substrate.

2.3.2.1 Substrate 1a

All tested organic solvents as well as the ionic liquid (EMimAc) were water-miscible. The highest apparent conversion for **1a** could be obtained with *N*-methylformamide (N-MFA) and formamide. Low conversions were detected when acetone, *t*-butanol or diphenyl ether were used. All other tested solvents showed moderate apparent conversions (Figure 7). The results for substrate **1a** as well as those for substrate **1b** should be seen with care because these compounds showed solubility problems in all solvents used. Their solubility could be improved by heating and ultrasonic bath treatment except for dissolving **1a** in EMimAc. After addition of the buffer to the pre-dissolved substrates, precipitation was observed. Hence the

results for the apparent conversions of **1a** and **1b** are not reliable, as they are not reproducible under these circumstances.

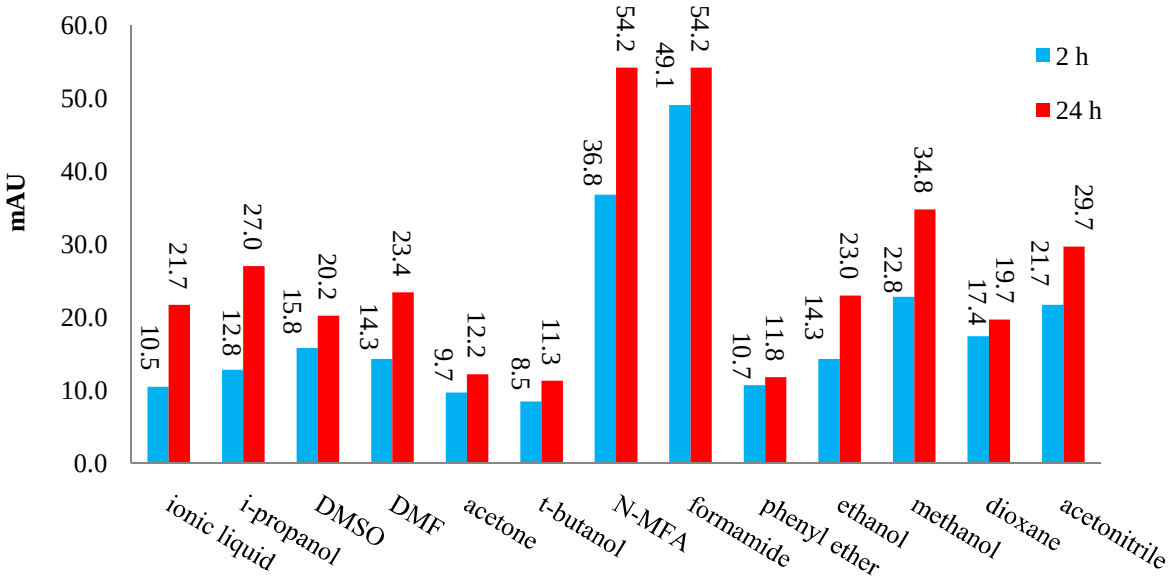


Figure 7: Apparent products formation for substrate **1a** in the presence of various solvents 10% v/v. Reaction conditions: 2 g/L substrate **1a** (6.4 mM). BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v organic solvent, 5 g/L catalase, 2 and 24 h, 40°C, 400rpm.

To identify the different products which contribute to the overall apparent conversions within the biotransformation by BBE, HPLC-MS measurements were applied. In case of substrate **1a** the 2 hour sample in formamide was taken to represent the composition of the reaction mixture after the given time period.

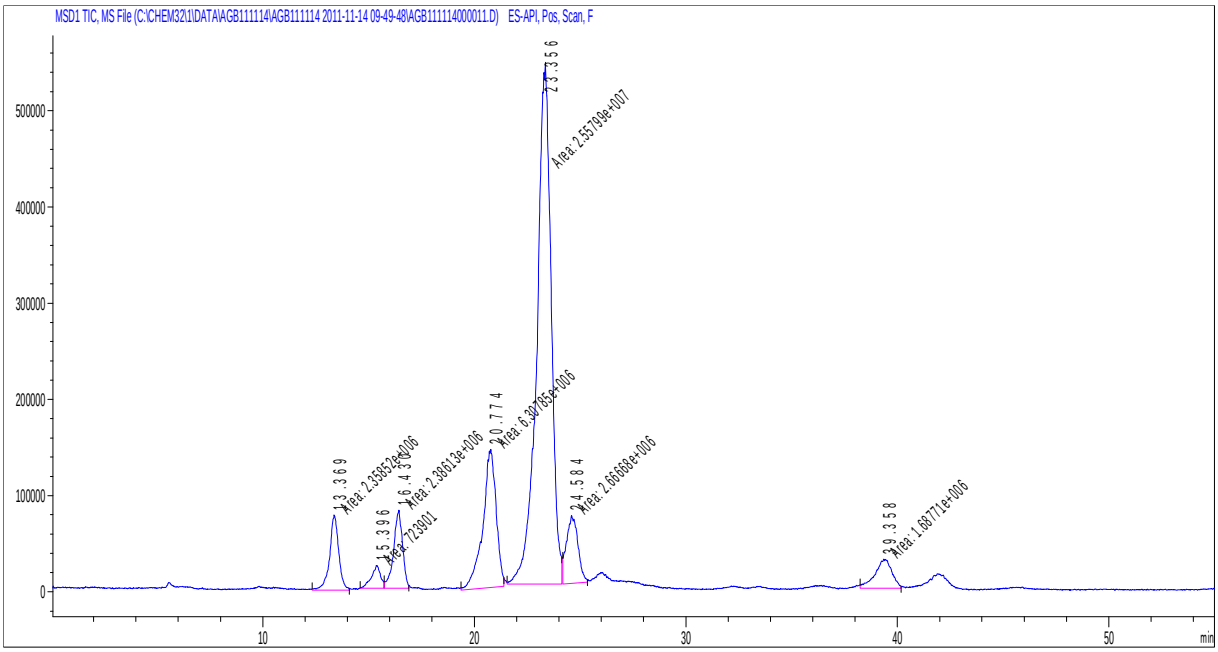


Figure 8: HPLC-MS measurements for substrate **1a**: MS detection (TIC = Total ion current) in the scan mode m/z = 250-500. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

Figure 8 shows the TIC of the HPLC-MS measurements from m/z = 250 to 500. Within this mass range all components of the reaction mixture are detectable. Figure 9 illustrates the substrate's HPLC-MS chromatogram in the SIM mode with an m/z ratio of 312 in accordance to its molecular mass.

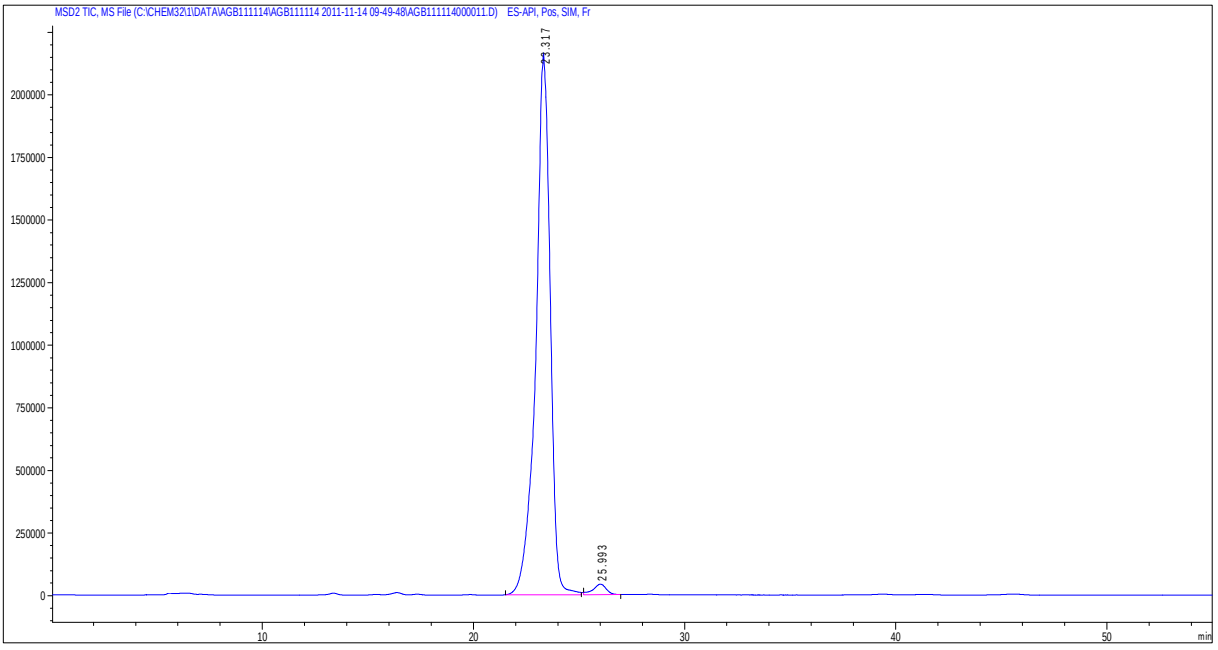
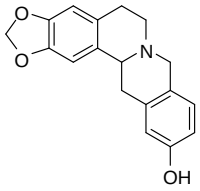
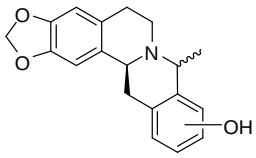
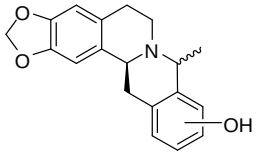
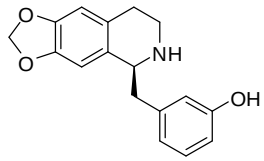
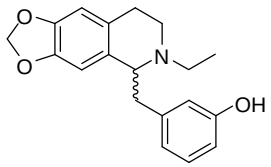
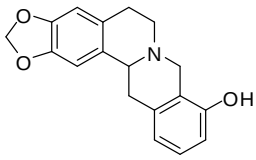
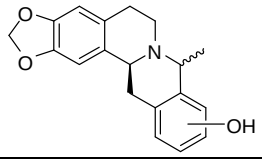


Figure 9: HPLC-MS measurements for substrate **1a**: MS detection in the SIM mode m/z = 312. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

Table 3 shows the masses, the species as well as the structure for a given retention time.

Table 3: Classification of the reaction mixture obtained by biotransformation of **1a** with BBE in formamide (10% v/v) after 2 h of reaction time

retention time [min]	species	formed mass [m/z]	suggested structure	observed reaction
13.36	$C_{17}H_{17}NO_3$ $[(M-H)^+]$	295.9		(*S*)-product with a mass loss of m/z =16
15.39	$C_{19}H_{19}NO_3$ $[(M-H)^+]$	310.0		(*S*)-product with a mass loss of m/z =2
16.43	$C_{19}H_{19}NO_3$ $[(M-H)^+]$	310.0		(*S*)-product with a mass loss of m/z =2
20.77	$C_{17}H_{17}NO_3$ $[(M-H)^+]$	284.0		(*S*)-deethylated product
23.35	$C_{19}H_{21}NO_3$ $[(M-H)^+]$	312.0		*rac*-substrate **1a**
24.58	$C_{17}H_{17}NO_3$ $[(M-H)^+]$	295.9		product with a mass loss of m/z =16
39.35	$C_{19}H_{19}NO_3$ $[(M-H)^+]$	310.0		(*S*)-product with a mass loss of m/z =2

Three different types of products were formed. The (*R*)-substrate was not converted by the biotransformation with BBE. According to the natural role of the investigated biocatalyst (*S*)-cyclized products were generated, according to the loss of m/z = 2. The absolute configuration of the novel chiral center that is introduced through the ring closure with the *N*-ethyl group cannot be determined. There is no information available about the compounds' fragemantion patterns since the MS selector is a single quadrupol and ionization of the compounds was done *via* ESI which is a very soft method and therefore fragmentation of the substances proceeds rarely. Two different cyclized products can be claimed. On the one hand the diastereoisomers **2a**, bearing the hydroxyl group at the C9 position of the berbine scaffold,

and on the other hand those which exhibit the functionality at the C11 carbon (**3a)** can be listed. Nevertheless no exact structural classification of the cyclized products to the three according retention times can be given. Figure 10 shows the HPLC-MS chromatogram of only the biotransformation products.

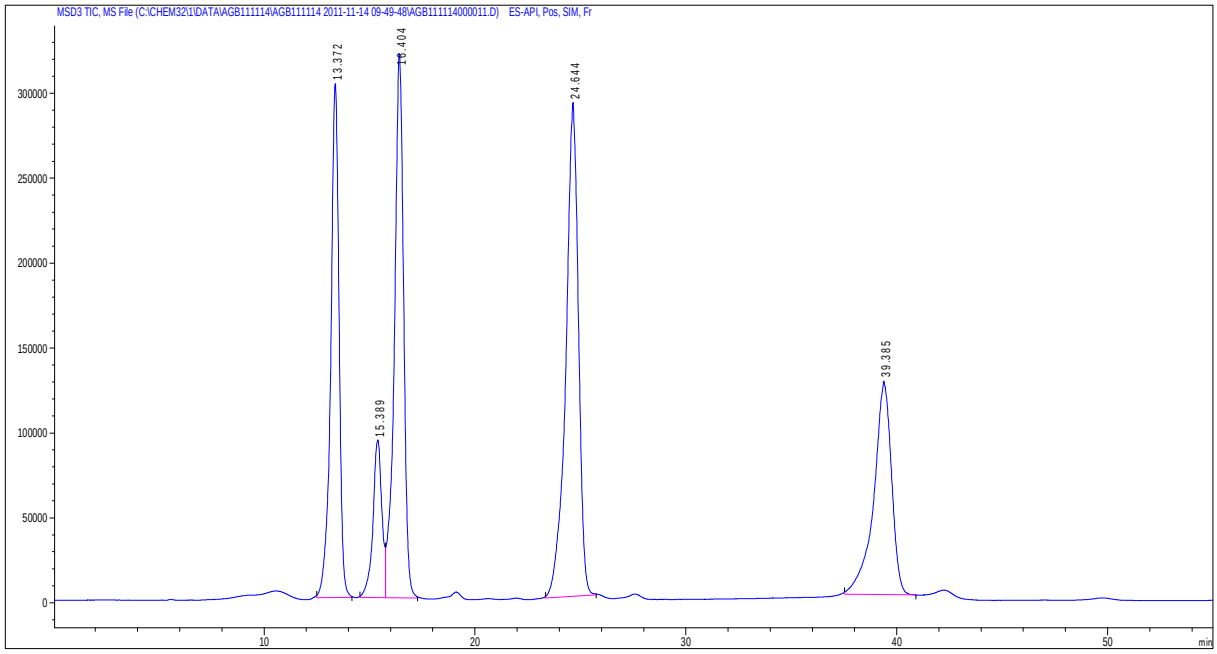


Figure 10: HPLC-MS measurements for substrate **1a**: MS detection in the SIM mode m/z = 310, 296, 284. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

2.3.2.2 Substrate 1b

Due to the similar solubility behavior of substrate **1a** and **1b** the organic solvents with the highest apparent conversions for **1a** were also tested for **1b**. As demonstrated in Figure 11 best results could be obtained with *N*-methylformamide followed by formamide and DMSO. The five other solvents also performed quite well, all apparent conversions after 24 hours of reaction time were higher than 30%. However, as already mentioned above, the reproducibility of the results is not ensured because of solubility problems.

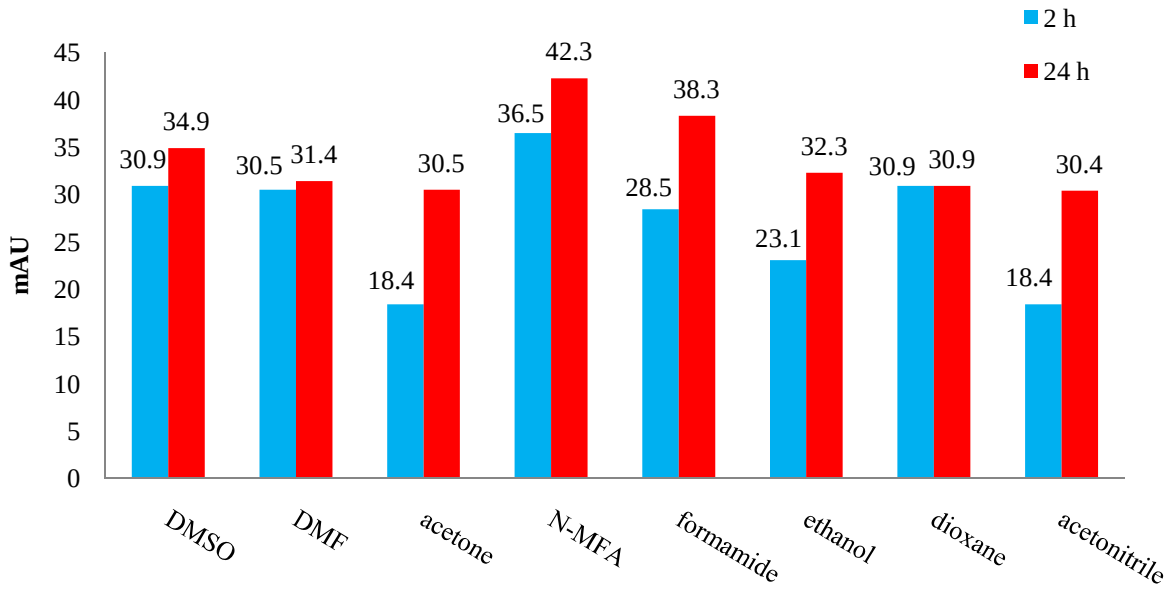


Figure 11: Apparent products formation for substrate **1b** in the presence of various solvents 10% v/v. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v organic solvent, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

Based on the results of the solvent study for substrates **1a** (see above) and **1b** formamide, *N*-methylformamide and DMSO were used for further experiments.

For the characterization of the biotransformation products, HPLC-MS chromatograms after two hours reaction time in *N*-methyl formamide are shown subsequently. These chromatograms are representative for the product conposition of each experiment carried out with substrate **1b**.

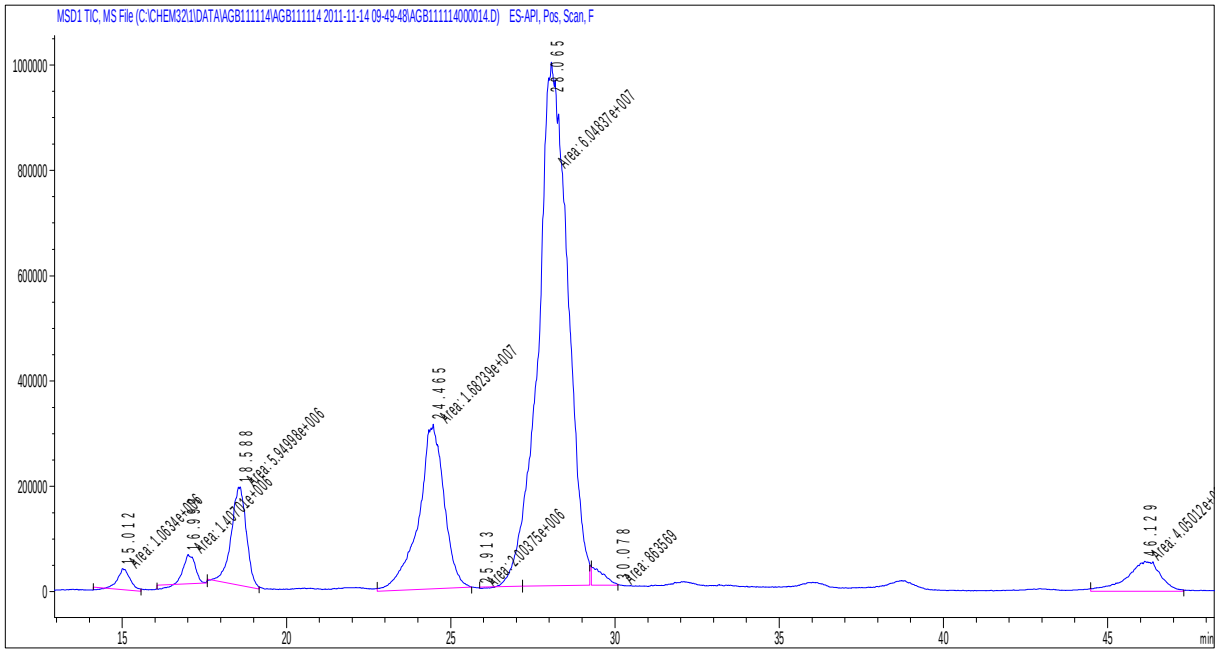


Figure 12: HPLC-MS measurements for substrate **1b**: MS detection in the scan mode m/z = 250-500. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% *N*-methyl formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

In the scan-mode of the HPLC measurements all products as well as the remaining (*R*)-substrate were detected (Figure 12). Figure 13 in contrast demonstrates the HPLC-MS chromatogram in the SIM mode correlating to the substate with an m/z ratio of 298

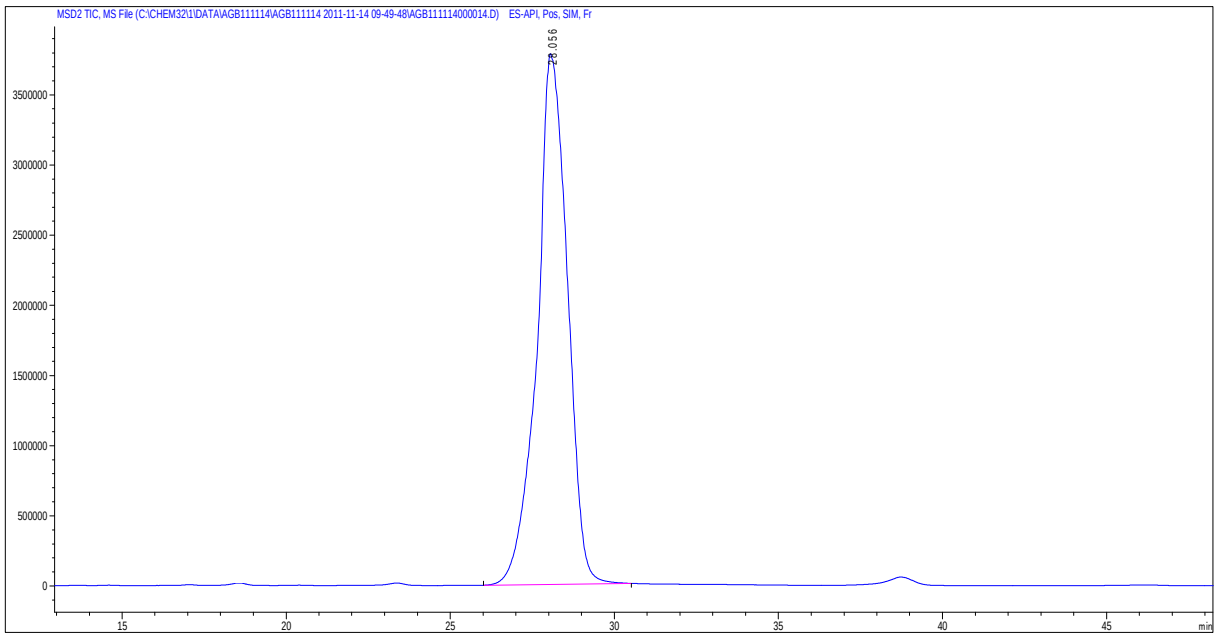
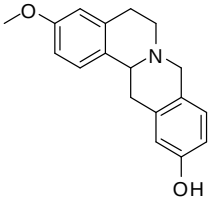
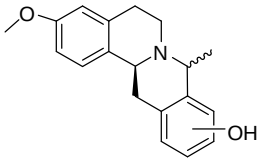
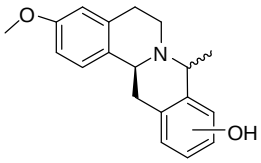
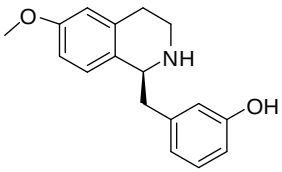
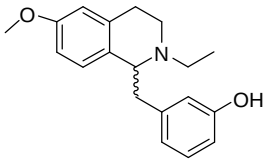
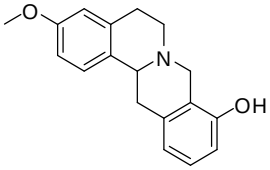
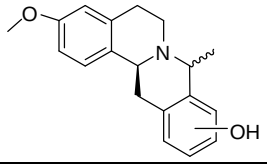


Figure 13: HPLC-MS measurements for substrate **1b**: MS detection in the SIM mode m/z = 298. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% *N*-methylformamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

Table 4 reflects the mass analysis correlating to each peak.

Table 4: Classification of the reaction mixture obtained by biotransformation of **1b** with BBE in formamide (10% v/v) after 2 h of reaction time

retention time [min]	species	formed mass [m/z]	suggested structure	observed reaction
15.01	$C_{18}H_{21}NO_2$ $[(M-H)^+]$	282.0		(*S*)-product with a mass loss of m/z =16
16.99	$C_{19}H_{21}NO_2$ $[(M-H)^+]$	296.0		(*S*)-product with a mass loss of m/z =2
18.58	$C_{19}H_{21}NO_2$ $[(M-H)^+]$	296.0		(*S*)-product with a mass loss of m/z =2
24.46	$C_{17}H_{19}NO_2$ $[(M-H)^+]$	270.0		(*S*)-deethylated product
28.06	$C_{19}H_{23}NO_2$ $[(M-H)^+]$	298		*rac*-substrate **1b**
30.07	$C_{19}H_{21}NO_2$ $[(M-H)^+]$	282.0		product with a mass loss of m/z =16
46.13	$C_{19}H_{21}NO_2$ $[(M-H)^+]$	296.0		(*S*)-product with a mass loss of m/z =2

The results of the HPLC-MS measurements strongly resemble those of substrate **1a**. The dealkylated as well as the expected cyclized products were formed during the biotransformation with BBE. Figure 14 reflects the peaks of the cyclized products measured on the HPLC-MS.

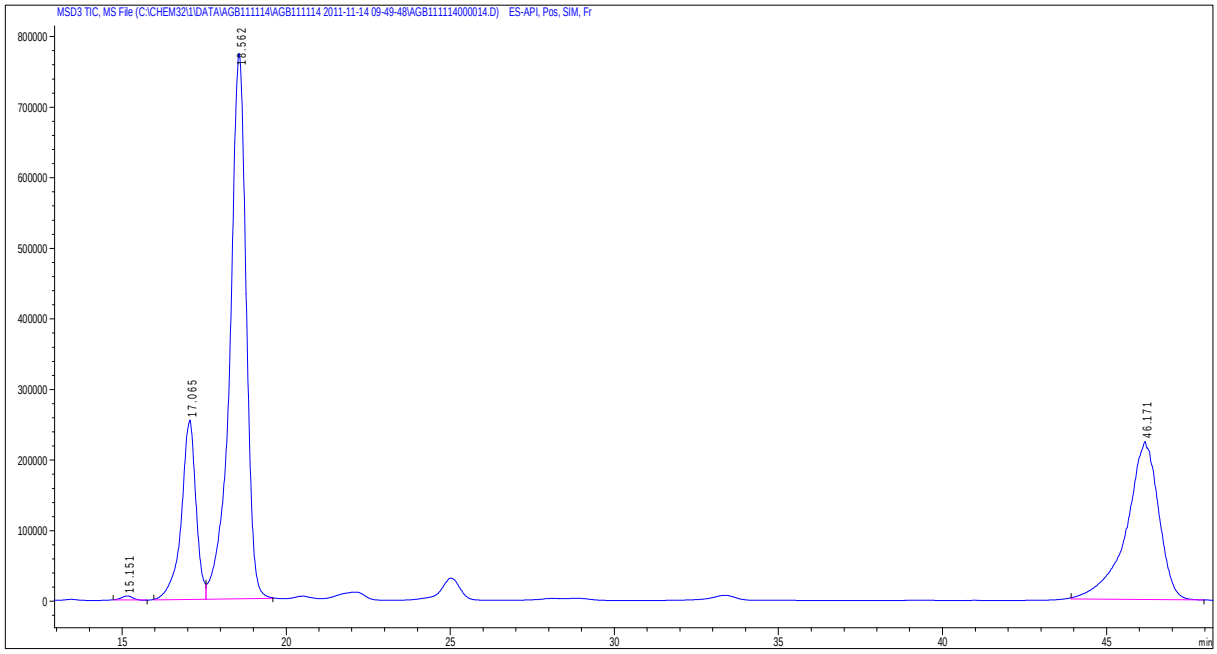


Figure 14: HPLC-MS measurements for substrate **1b**: MS detection in the SIM mode m/z = 296. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% *N*-methylformamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

2.3.2.3 Substrate 1e

2-Ethyl-6,7-dimethoxy-1-(3-hydroxybenzyl-1,2,3,4-tetrahydroisoquinoline (**1e**) showed a entirely different solubility behavior then **1a** and **1b**. It was easy to dissolve it, in each organic solvent. The highest apparent conversions were obtained using formamide and acetonitrile, lowest ones were achieved applying *t*-butanol. For all further experiments formamide was used as the solvent of choice.

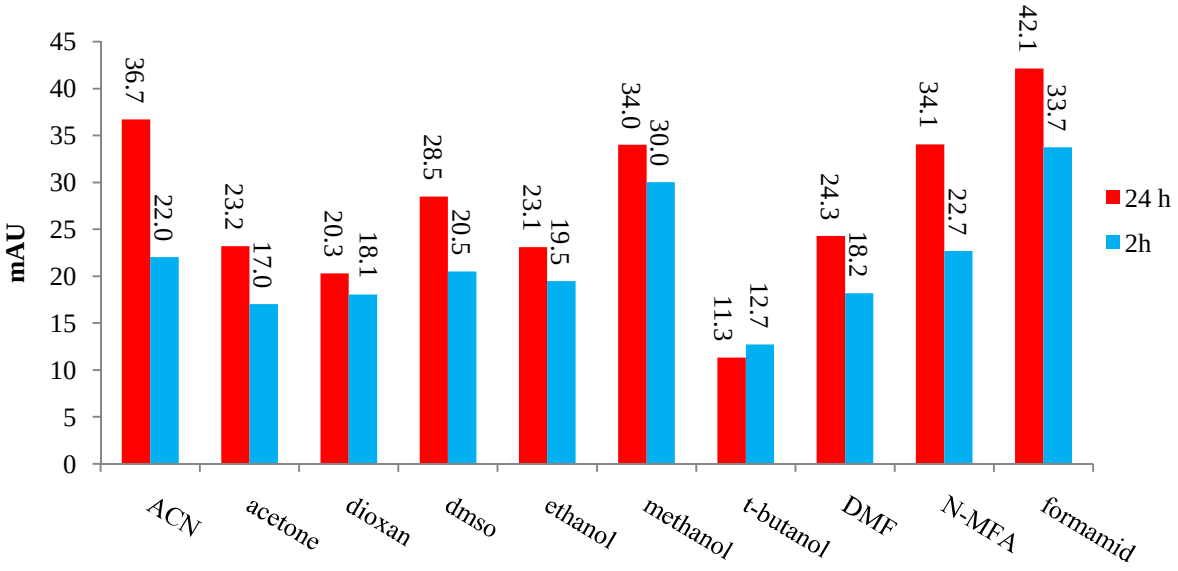


Figure 15: Apparent products formation for substrate **1e** in the presence of various solvents 10% v/v. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v organic solvent, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

For the characterization of the biotransformation products *via* HPLC-MS measurements, the sample with 10% v/v formamide after two hours of reaction time was taken to represent the composition of the reaction mixture for all given solvents. The results of the product characterization are listed below. Correlations between the formed products and the retention times are listed in Table 5.

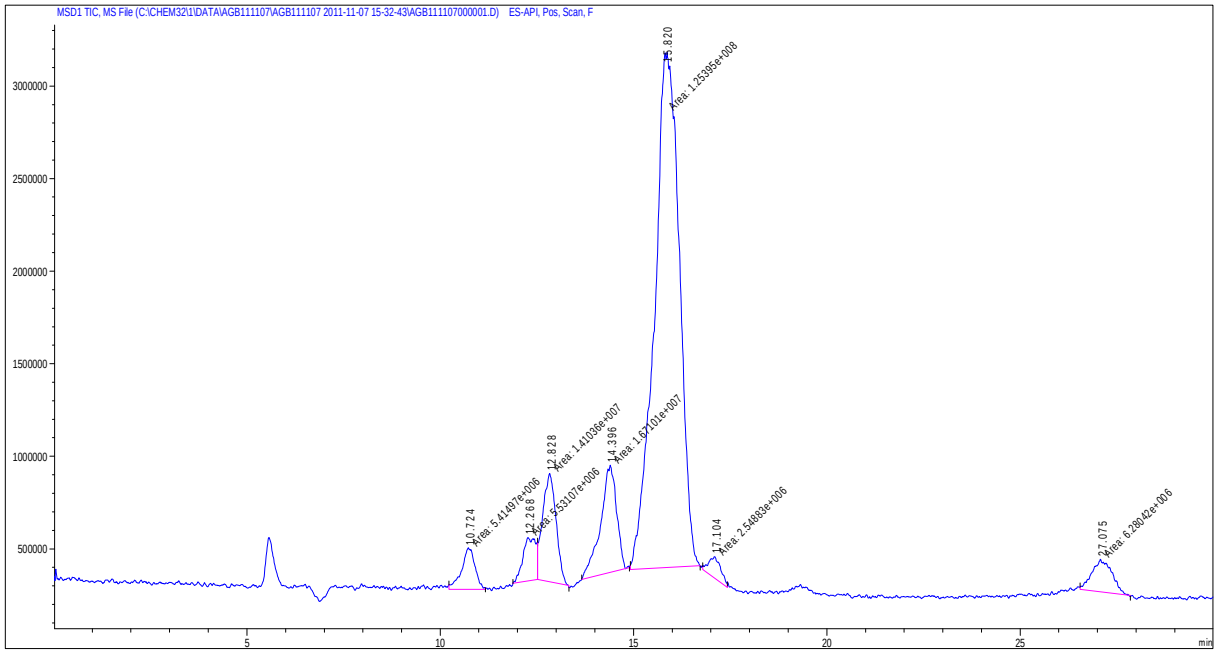


Figure 16: HPLC-MS measurements for substrate **1e**: MS detection in the scan mode m/z = 250-500. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

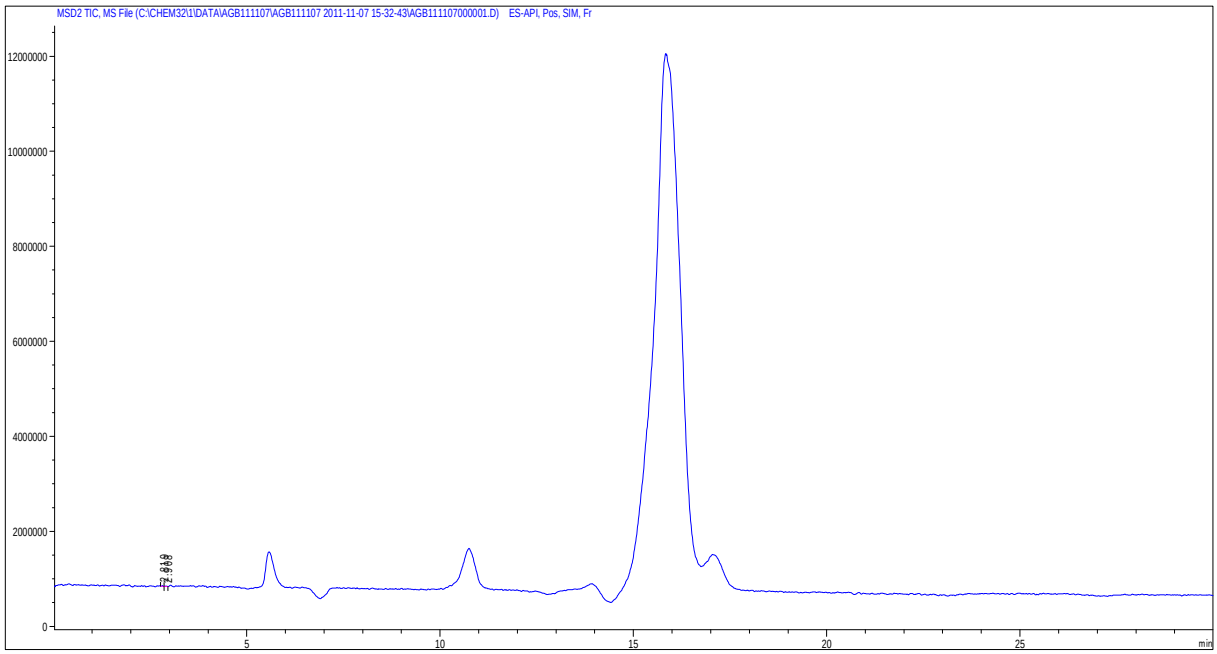
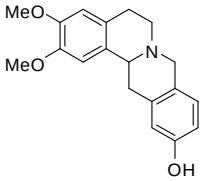
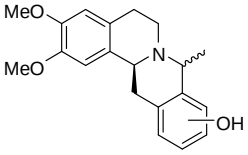
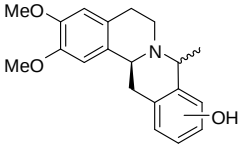
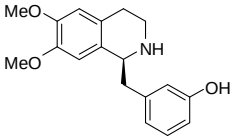
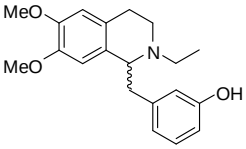
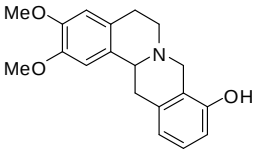
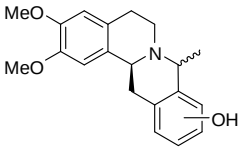


Figure 17: HPLC-MS measurements for substrate **1e**: MS detection in the SIM mode m/z = 328. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

Table 5: Classification of the reaction mixture obtained by biotransformation of **1e** with BBE in formamide (10% v/v) after 2 h of reaction time

retention time [min]	species	formed mass [m/z]	suggested structure	observed reaction
10.73	$C_{19}H_{23}NO_3$ $[(M-H)^+]$	311.9		(*S*)-product with a mass loss of m/z =16
12.29	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	326.0		(*S*)-product with a mass loss of m/z =2
12.85	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	325.9		(*S*)-product with a mass loss of m/z =2
14.39	$C_{18}H_{21}NO_3$ $[(M-H)^+]$	300.0		(*S*)-deethylated product
15.84	$C_{20}H_{25}NO_3$ $[(M-H)^+]$	328.0		*rac*-substrate **1e**
17.05	$C_{19}H_{23}NO_3$ $[(M-H)^+]$	311.9		product with a mass loss of m/z =16
27.11	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	325.9		(*S*)-product with a mass loss of m/z =2

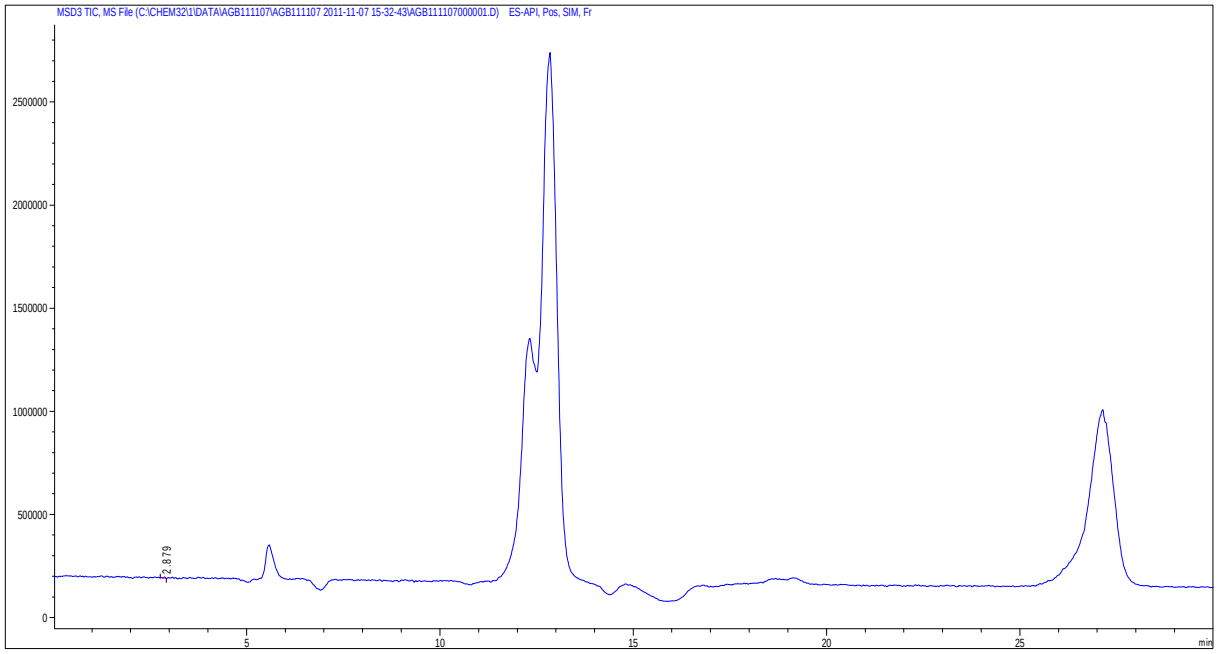


Figure 18: HPLC-MS measurements for substrate **1e**: MS detection in the SIM mode m/z = 326. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 2 h, 40°C, 400 rpm

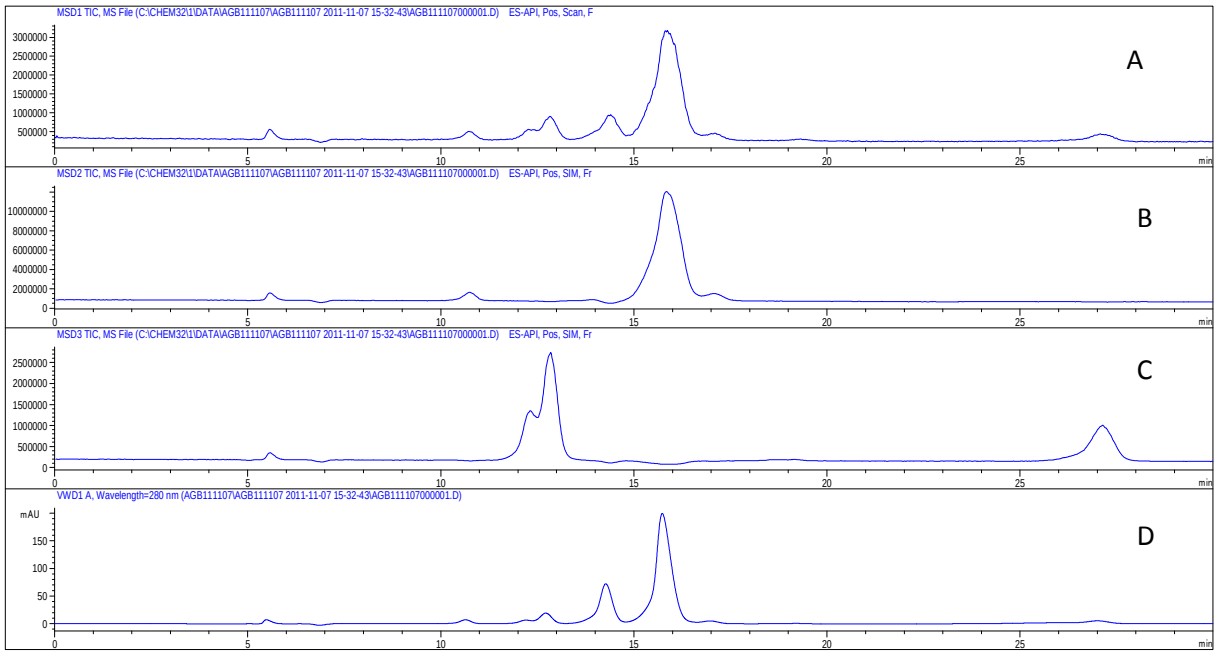


Figure 19: Overlay of the HPLC-MS measurements for substrate **1e**: A: scan-mode (m/z = 250-500). B: SIM-mode (m/z = 328). C: SIM-mode (m/z = 326). D: UV signal at 280 nm.

The evidence of the deethylated product structure which resembles the benzylisoquinoline scaffold dealkylated on the nitrogen atom, for each substrate (**4a**,**4b**,**4e**) respectively, was given through the comparison of **4e** with a reference spectrum of the synthesized dealkylated compound. The results of the GC-MS spectra matching are univocal, the accordance lies at 95%. Figure 20 demonstrates the high accordance of both spectra. Out of the fact that all tested and measured samples display equivalent fragmentation pattern and identical mass assignments it can be reasoned that in case of substrates **1a** and **1b** the deethylation products have to correlate to those of substrate **1e** despite unavailable reference chromatograms.

The same argumentation applies to the products with a mass loss of m/z = 16. Their structure also correlates to a cyclized species that lacks the methyl group at the bridging C8 atom. This component can bear the hydroxyl group either at the C9 atom (**5e**) of the berbine scaffold or at the C11 atom (**6e**). Comparison of the GC-MS spectrum of the biocatalytic product with a reference component demonstrates the high accordance (Figure 21). Furthermore the assignment of the products to the corresponding retention times can be done, since HPLC references were available too. This correlation can be transferred to substrates **1a** and **1b**.

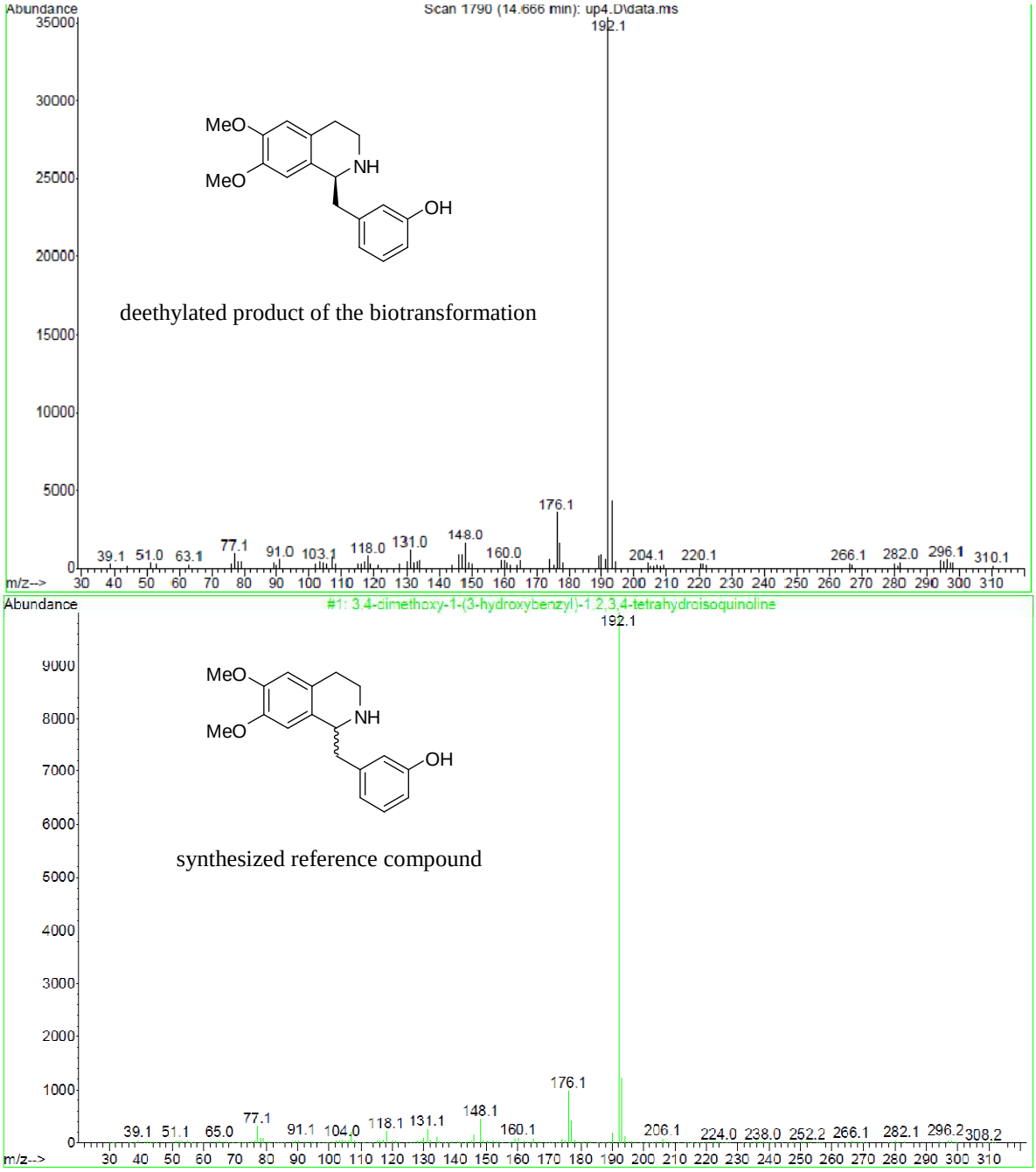


Figure 20: GC-MS chromatograms of the deethylated product **4e** and a synthesized reference compound. Upper spectrum: sample of the biotransformation with BBE (Reaction conditions: 2 g/L substrate 1e (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 m, pH 9), 10% formamide,5 g/L catalase, 2 h, 40°C, 400 rpm). Bottom spectrum: synthesized reference compound

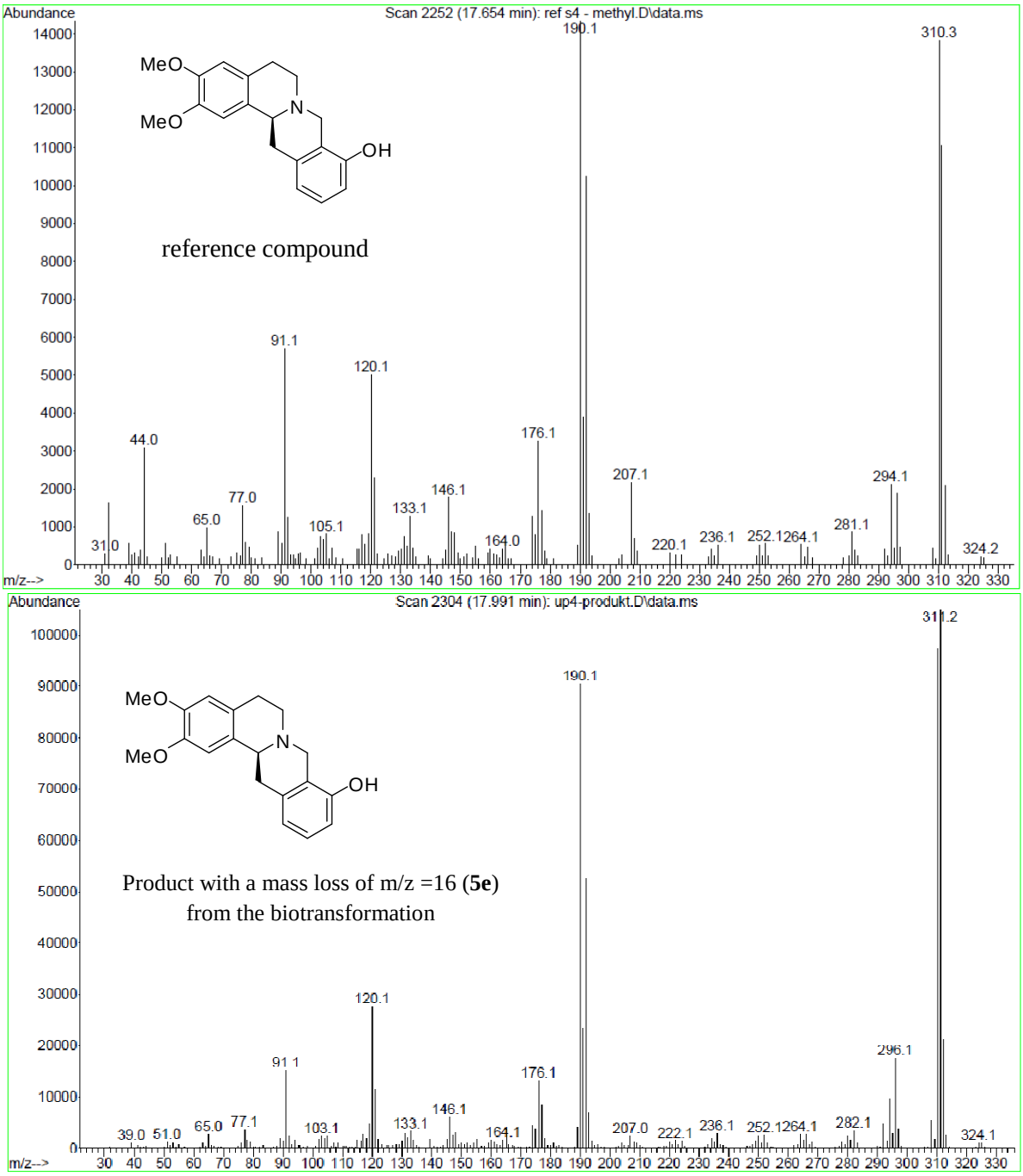


Figure 21: GC-MS chromatograms of the product with a mass loss of m/z = 16 **5e** and a reference component. Bottom spectrum: sample of the biotransformation with BBE (Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide,5 g/L catalase, 2 h, 40°C, 400rpm). Upper spectrum: reference compound

2.3.3 pH-studies for substrates 1a, 1b and 1c

After the identification of the organic solvents with the highest apparent conversions also the optimal pH of the reaction medium had to be determined. The working group of A. R. Battersby introduced the pH optimum for the aqueous buffer at a value of 9.[3]

The pH studies for substrate **1a** and **1b** were not only done for the optimized organic solvent respectively but three different solvents were tested because of already mentioned solubility problems. Hence formamide, *N*-methyl formamide and DMSO were used and the buffer's pH value was varied from 6 to 11. The whole pH-study was performed using the BBE variant W165F.

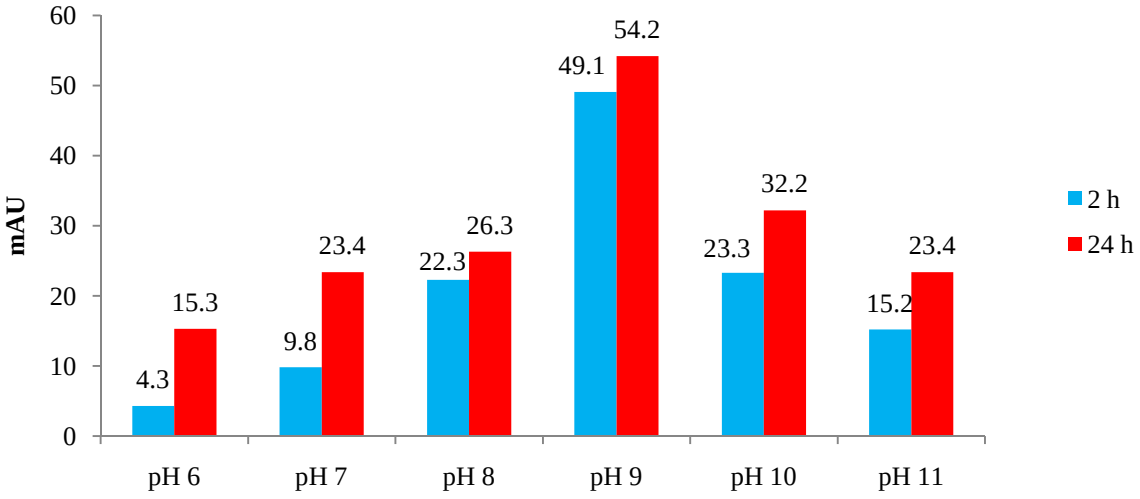


Figure 21: Apparent products formation for substrate **1a** at varied pH values ranging from 6 to 11 in the presence of formamide. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM. pH 6-11), 10% v/v formamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

Employing formamide as an organic solvent for substrate **1a** the highest apparent conversions were obtained at pH 9. Up to this pH the values increased constantly, but at higher pH values a slight decrease can be observed.

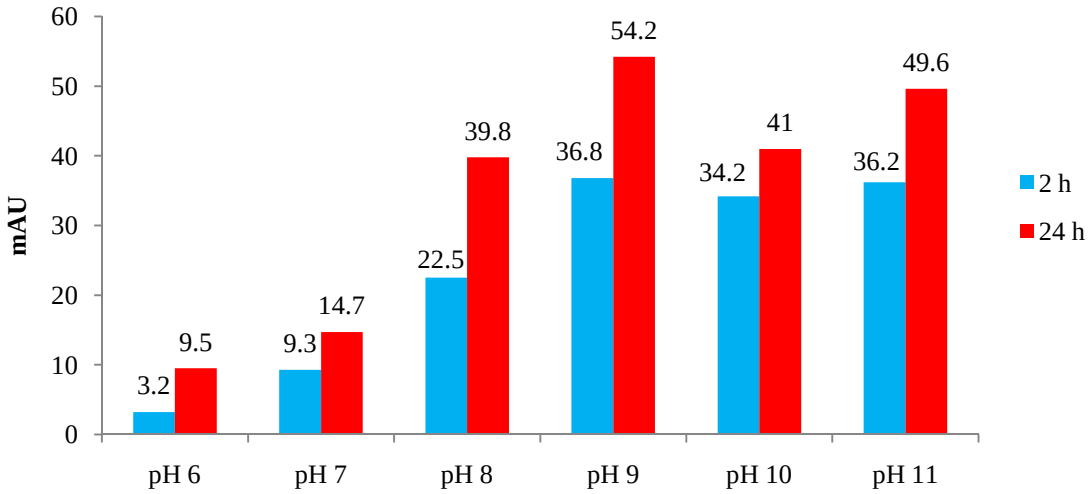


Figure 22: Apparent products formation for substrate **1a** at varied pH values ranging from 6 to 11 in the presence of *N*-methyl formamide. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v *N*-methylformamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

Using *N*-methylformamide the pH optimum was also obtained at pH 9, but the pH dependence is not similar to the one observed applying formamide. In this case the pH values do not decrease constantly afer reacing the optimum.

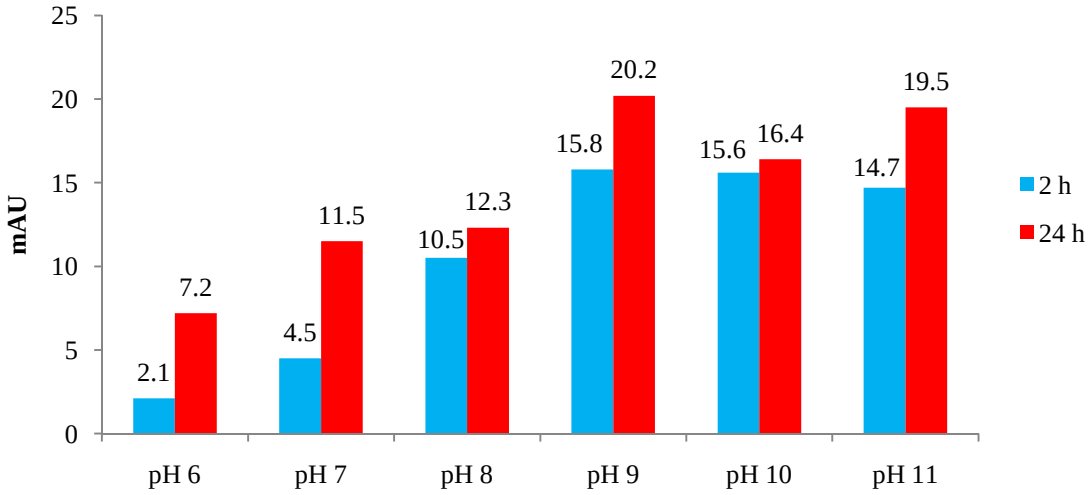


Figure 23: Apparent products formation for substrate **1a** at varied pH values ranging from 6 to 11 in the presence of . Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v DMSO, 5 g/L catalase, 2 and 24 h, 40°C. 400 rpm

When DMSO was used as organic solvent the dependence of the apparent conversion on the pH is similar to the *N*-methyl formamide system. Hence the pH optimum of all three organic solvents correlates with each other, a pH value of 9 is taken for all further experiments using substrate **1a**.

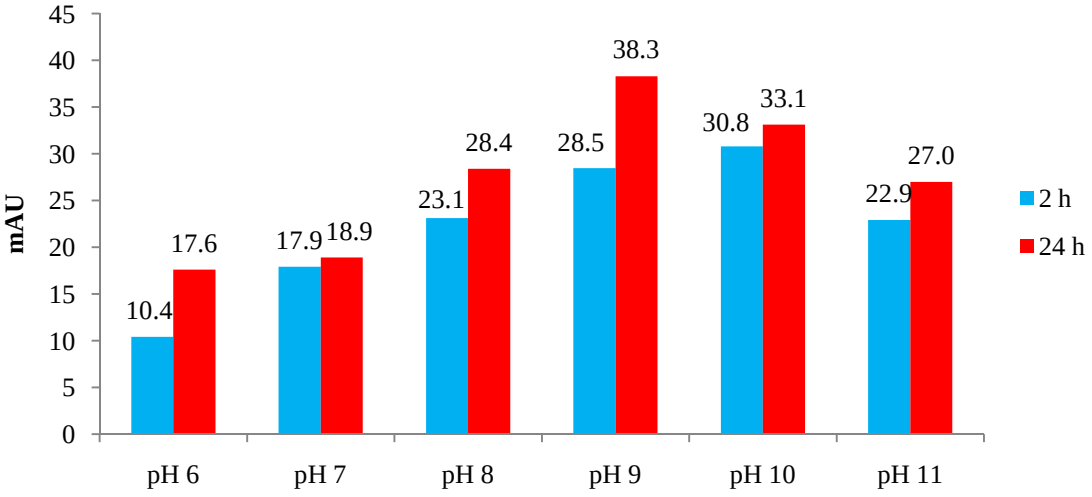


Figure 24: Apparent products formation for substrate **1b** at varied pH values ranging from 6 to 11 in the presence of formamide. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v formamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

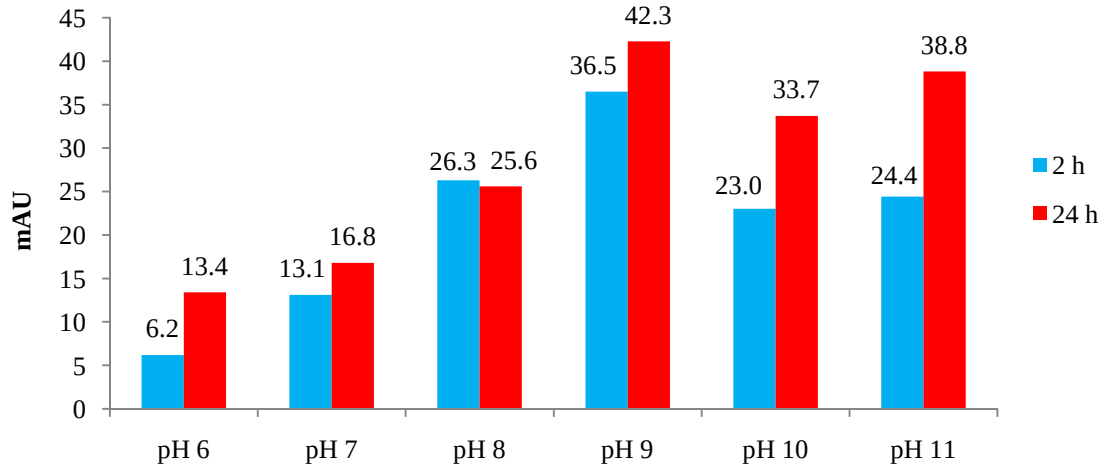


Figure 25: Apparent products formation for substrate **1b** at varied pH values ranging from 6 to 11 in the presence of *N*-methyl formamide. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v *N*-methylformamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

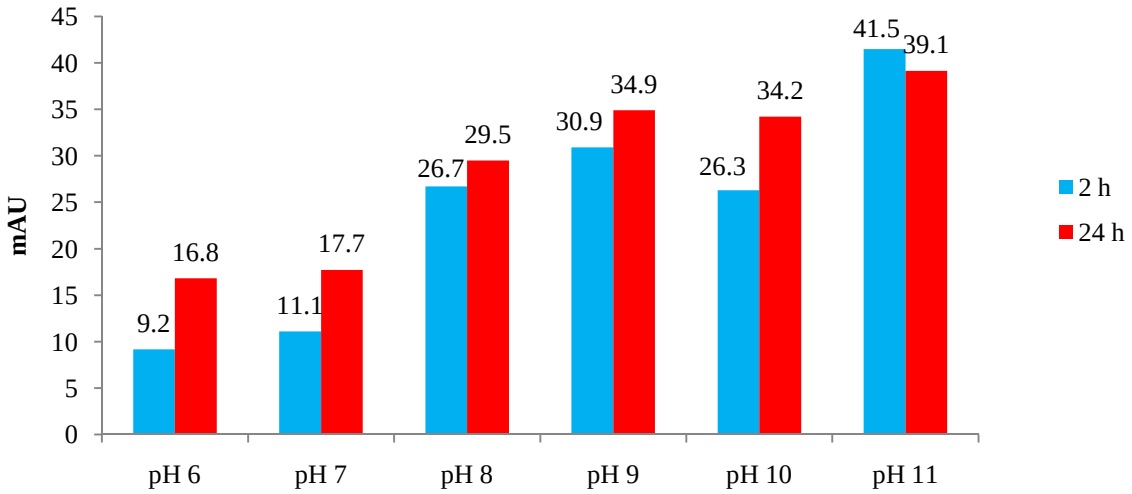


Figure 26: Apparent products formation for substrate **1b** at varied pH values ranging from 6 to 11 in the presence of DMSO. Reaction conditions: 2 g/L substrate **1b** (6.7 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v DMSO, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

In the case of substrate **1b** *N*-methyl formamide and formamide used as organic solvents, led to highest apparent conversions at pH 9. Under the addition of 10% v/v DMSO another characteristic was discovered. For this organic solvent the pH optimum of the reaction lies at 11 followed by the pH 9 reaction system. However, it cannot be ensured if a pH of 11 indeed represents the optimal value because of already mentioned solubility problems. Hence the buffer's optimal pH value can be declared as 9 in accordance to the formamide and *N*-methylformamide systems.

For the pH-study of substrate **1e** formamide, the organic solvent with the highest apparent conversions determined above was used to evaluate the influence of the pH. The pH optimum for this substrate was rather broad ranging from pH 9 to 11. All those values led to apparent conversions above 45 percent and therefore were usable to convert the racemic substrate to the (*S*)-enantiomeric products in high yield. For the following preparative transformations as well as for the time studies with substrate **1e**, pH 9 was used.

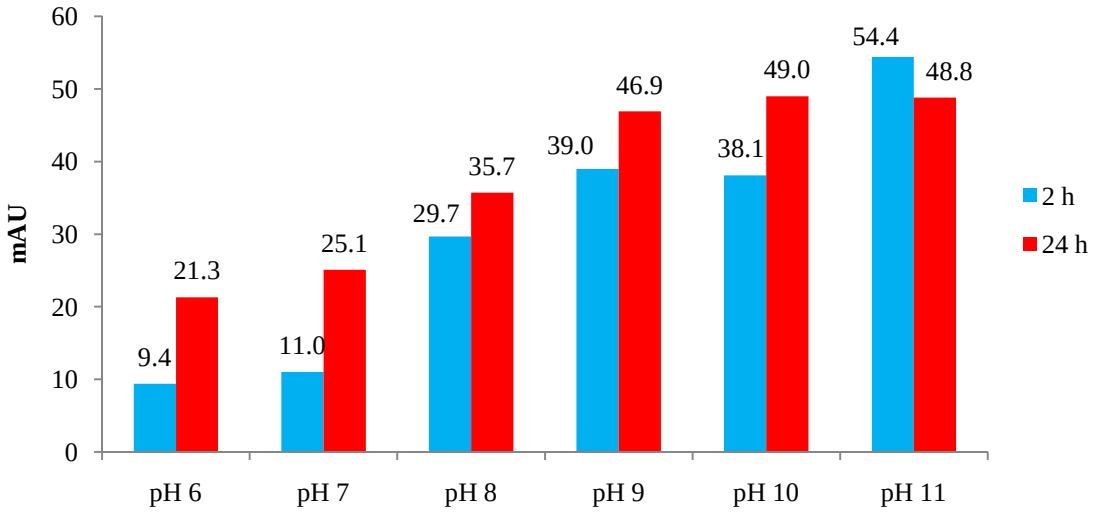


Figure 27: Apparent products formation for substrate **1e** at varied pH values ranging from 6 to 11 in the presence of formamide. Reaction conditions: 2 g/L substrate 1e (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 6-11), 10% v/v formamide, 5 g/L catalase, 2 and 24 h, 40°C, 400 rpm

2.3.4 Concentration studies for substrates 1a, 1b and 1e

To determine at which substrate concentration the highest product formation can be obtained three different concentrations for each substrate were tested: 1g/L, 2g/L and 4g/L. Furthermore an overview of the product ratios after different reaction times (2 and 24 hours) should be given. Dimethylsulfoxide (10% v/v) and toluene (70% v/v) served as organic solvents in these experiments. For the biotransformation the enzyme variant W165F was used to convert the substrates.

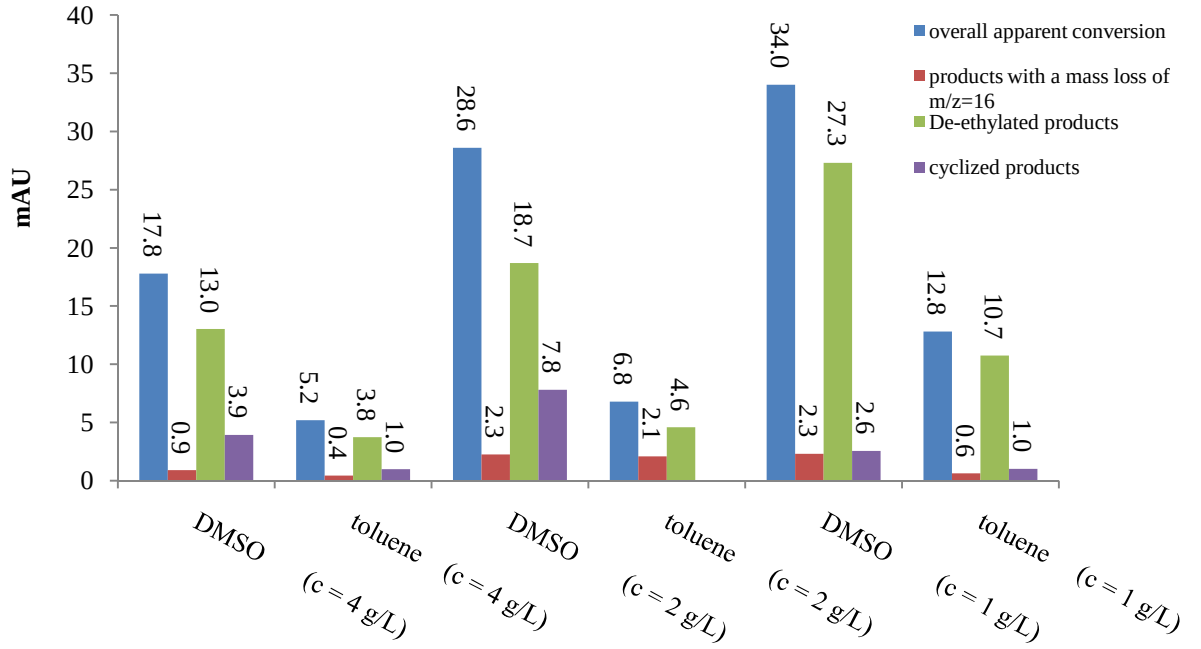


Figure 28: Apparent products formation for the concentration study with substrate **1e (**2 h**)**. Reaction conditions: 1,2,4 g/L substrate **1e** (3.1, 6.1, 12.2 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 2h, 40°C, 400 rpm

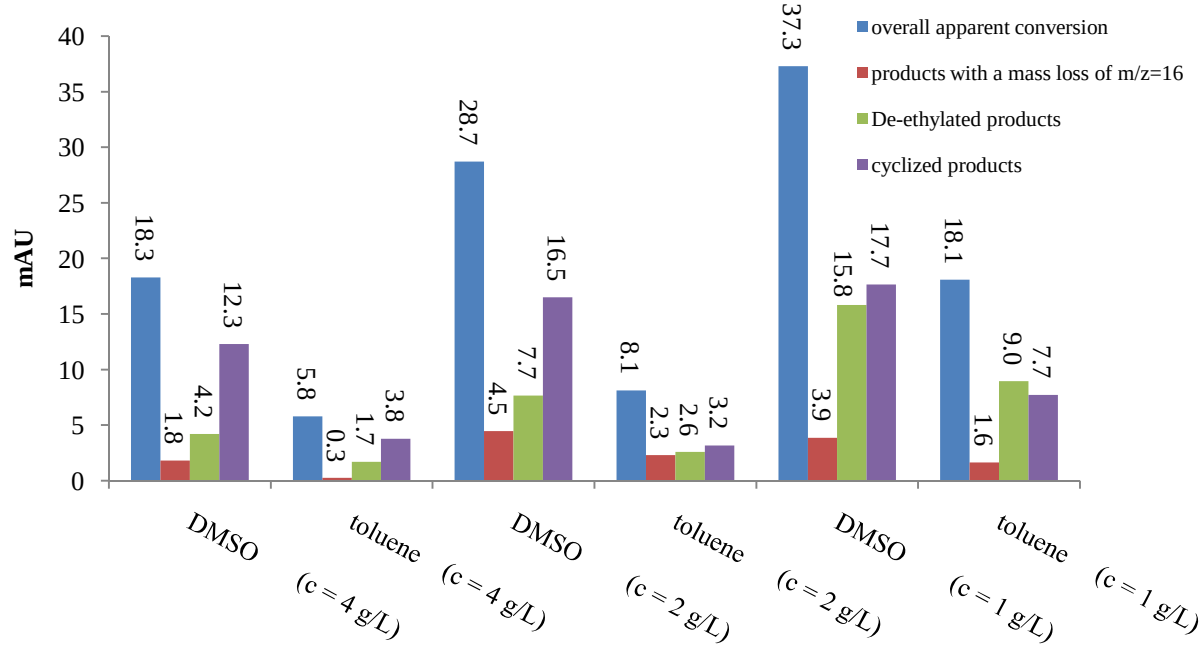


Figure 29: Apparent products formation for the concentration study with substrate **1e** (24 h). Reaction conditions: 1,2,4 g/L substrate **1e** (3.1, 6.1, 12.2 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 24h, 40°C, 400 rpm

The highest apparent conversions after 2 and 24 hours can be achieved using a concentration of 1 g/L, followed by 2 g/L and 4 g/L, but these results should not be mistakenly understood as the highest product concentrations formed through the biotransformation. In order to determine which substrate concentration led to the highest molarity for the three different biotransformation product classes, the corresponding concentrations of the products have to be calculated. Hence the exact epsilon values for the formed compounds were not determined or a standard calibration done the molarity of the products was not known at this point. Logically it would be concluded that the highest concentrations of the products will be found at the highest amounts of substrate introduced (4g/L), since the concentrations are not known, a possible substrate inhibition at increased concentrations cannot be verified or denied.

By taking a closer look at the samples with 24 hours reaction time the distribution of the products changed fundamentally. After two hours reaction time the deethylated products represented the main share of the reaction products but after a day their amount strongly decrease and those of the cyclized products increase notably.

For the overall apparent conversion of **1a** the same relationship can be observed as in the case of **1e**. The lowest concentration resulted in the highest apparent conversion with DMSO as an organic solvent, whereas for the concentration of 4 g/L the reverse case occurred. Furthermore the deethylated product showed the highest amount for the formed products in the case of 1 and 4 g/L substrate.

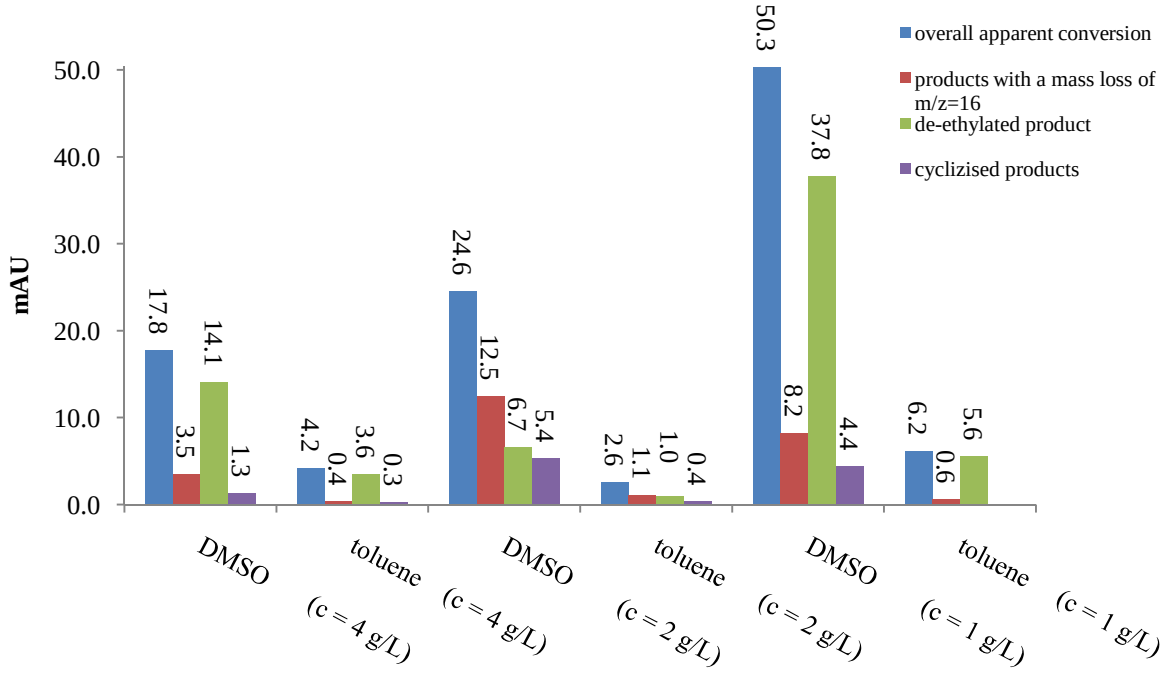


Figure 30: Apparent products formation for the concentration study with substrate **1a (**2 h). Reaction conditions: 1,2,4 g/L substrate **1a** (3.2, 6.4, 12.8 mM). BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 2h, 40°C, 400 rpm

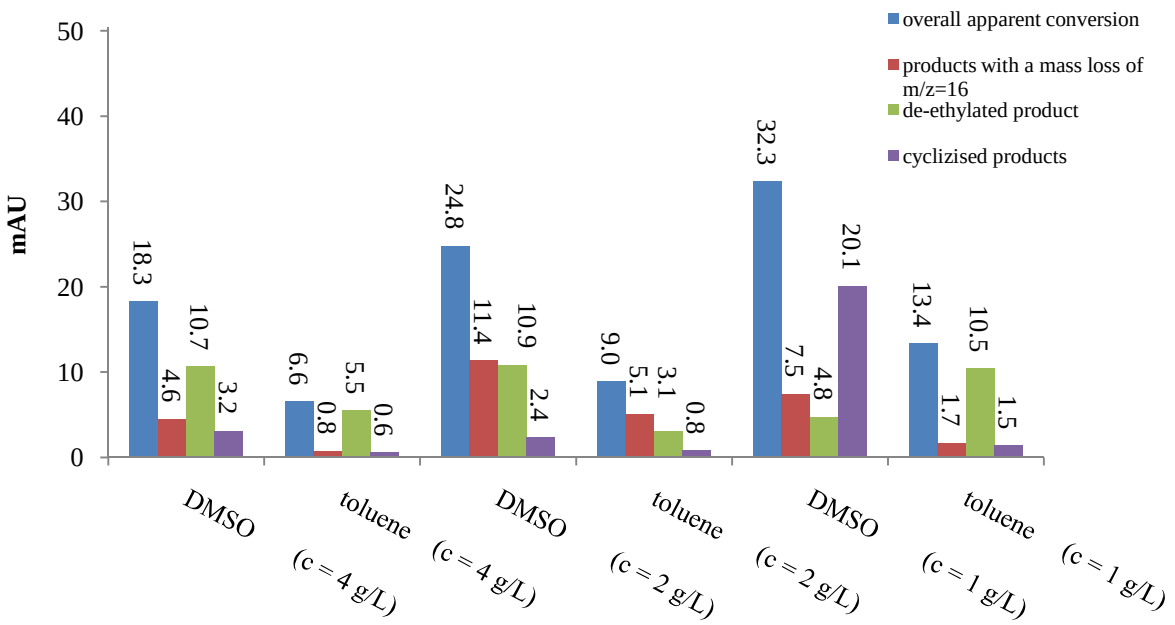


Figure 31: Apparent products formation for the concentration study with substrate **1a** (24 h). Reaction conditions: 1,2,4 g/L substrate **1a** (3.2, 6.4, 12.8 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 24h, 40°C, 400 rpm

After 24 hours reaction time the samples with the lowest concentration again showed the highest apparent conversion, but the remarkable fact was that the overall conversion values of 1 g/L **1b** in DMSO decreased from 50 to 32% which is quite unexpected and could lead to the assumption that the products vaporized. This is quite unlikely due to the fact that the corresponding substrate has a melting range of 139-142°C. The reason for these results could be found in the solubility problems concerning substrate **1a** and **1b**. Both are difficult to dissolve in the corresponding solvents and precipitated after addition of the buffer. Consequently the findings have to be treated with care.

For **1b** the same results were obtained.

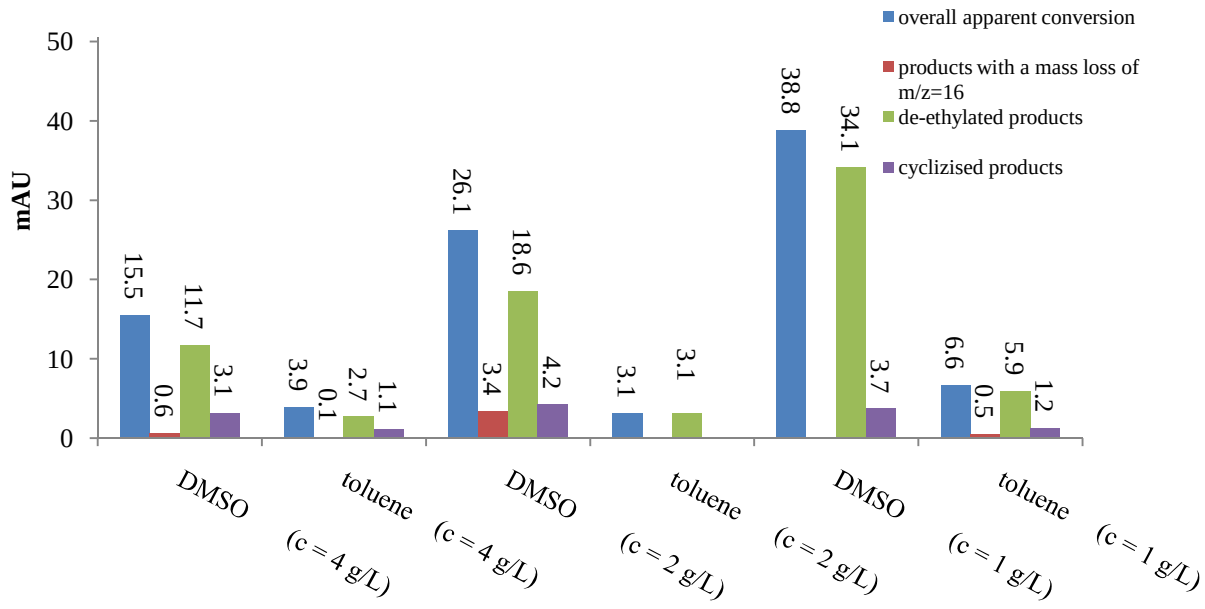


Figure 32: Apparent product formation for the concentration study with substrate **1b** (2 h). Reaction conditions: 1,2,4 g/L substrate **1b** (3.3, 6.7, 13.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 2h, 40°C, 400 rpm

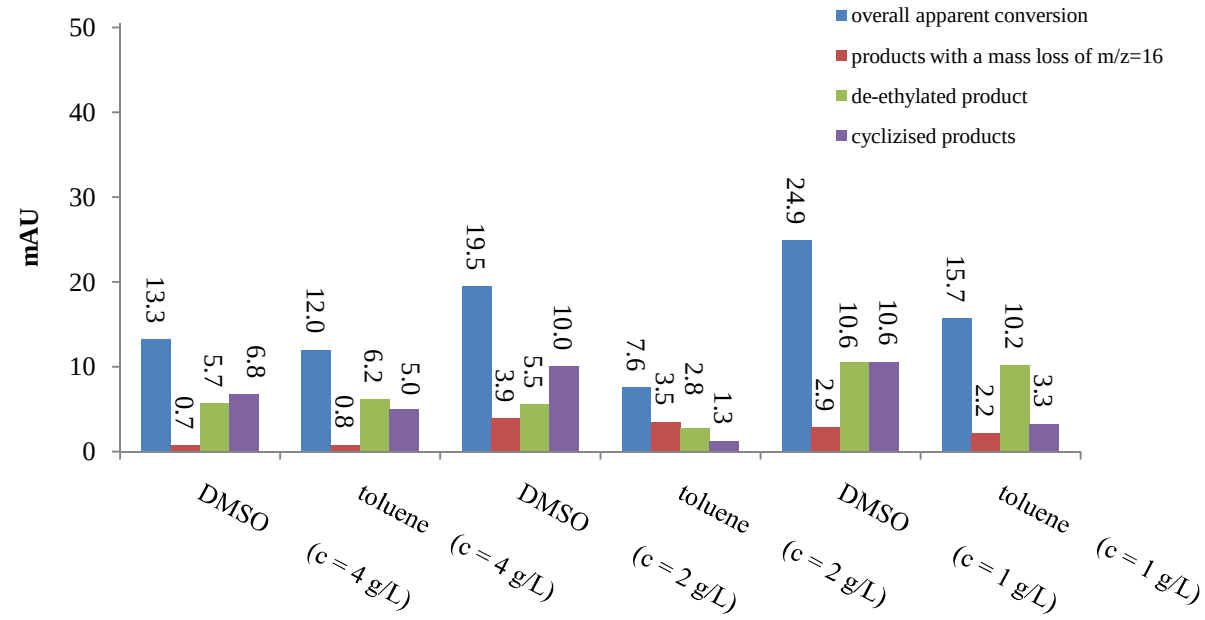


Figure 33: Apparent product formation for the concentration study with substrate **1b** (24 h). Reaction conditions: 1,2,4 g/L substrate **1b** (3.3, 6.7, 13.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM. pH 9), 10% v/v DMSO/toluene, 5 g/L catalase, 24h, 40°C,400 rpm

Comparison of both reaction times (2h, 24h) also showed a decrease of the apparent conversion in the case of 2 and 1 g/L in DMSO, as it could be observed in the case of substrate **1a**. Solubility problems as already mentioned above are claimed as the reason for this decrease.

Finally it can be concluded for nearly all samples of **1a**, **1b** and **1c**, that after 2 h reaction time the product mixture is strongly dominated by the deethylated products followed by the cyclized products and those with a mass loss of m/z = 16. A reaction time of 24 h resulted in quite different ratios. The percentage of the deethylated product was reduced to approximately a half compared to the values after two hours of reaction time. For the cyclized products the reverse trend is observed and also the amount of the products with a mass loss of m/z = 16 increased over the reaction time but nevertheless they only contribute to low percentages to the product composition.

2.3.5 Result of the co-solvent study with toluene for substrate 1a

Due to the fact that substrates **1a** and **1b** exhibited solubility problems and precipitated after pre-dissolving in the corresponding organic solvent by addition of the buffer, different amounts of toluene as a co-solvent were added to 10% v/v DMSO and the standard buffer. The addition of toluene started at 35% v/v and rose up to 55%. Toluene at an amount of 35% was taken as the initial point, since at this volumetric concentration the substrate did not precipitate any more after the addition of the buffer. 55% v/v were taken as the final concentration, because the enzyme should not decrease its activity. To conclude if the addition of toluene enhances the reproducibility the tests were done with substrate **1a**.

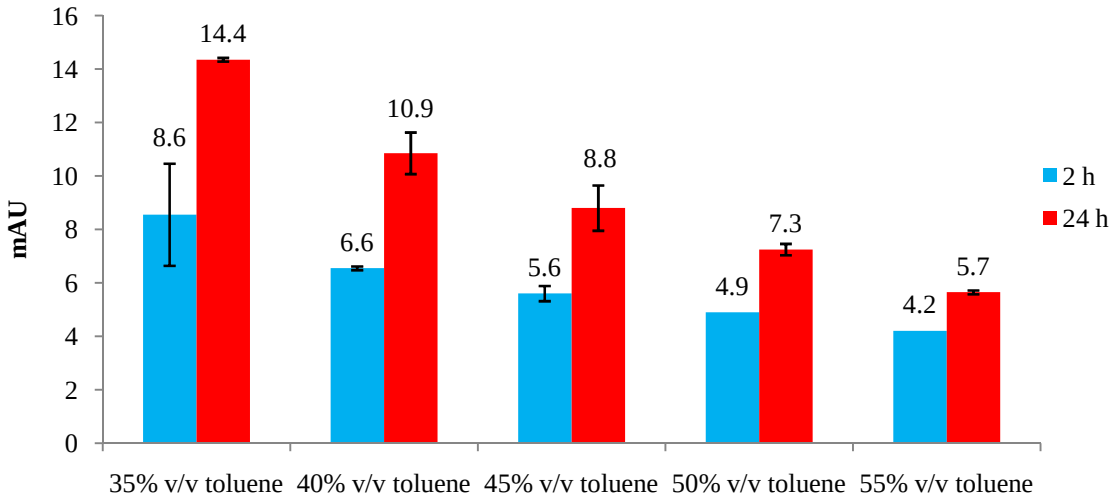


Figure 33: Apparent products formation for substrate **1a** at varied amounts of toluene as a co-solvent additional to DMSO. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO + 35-55% v/v toluene, 5 g/L catalase, 2 and 24 h. 40°C, 400 rpm. Error ranges represent standard deviations of triplicate experiments

Indeed the solubility problems can be eliminated through the addition of toluene as a co-solvent, but it can clearly be seen in figure 33 that this additive strongly decreases the apparent conversion with increasing amounts. Concerning the reproducibility of the samples triplicate determinations were prepared and the corresponding standard deviations illustrated in the diagram. The accurate values for the deviations are listed in table 6, which reveals that the values drop with rising amounts of toluene.

Table 6: Standard deviations for the co-solvent study with toluene for substrate **1e**

	standard deviation
35% v/v toluene 2 h	1.9
35% v/v toluene 24 h	0.1
40% v/v toluene 2 h	0.1
40% v/v toluene 24 h	0.8
45% v/v toluene 2 h	0.3
45% v/v toluene 24 h	0.8
50% v/v toluene 2 h	0.0
50% v/v toluene 24 h	0.2
55% v/v toluene 2 h	0.0
55% v/v toluene 2 h	0.1

Recapitulatory it can be mentioned that the addition of the co-solvent is helpful to keep the substrate in solution, and the reproducibility can be improved by applying amounts of 50-55% v/v toluene. Moreover the apparent conversion strongly decreases through the addition of toluene compared to those values obtained by simple addition of 10% v/v DMSO. Consequently the usage of toluene is not a real alternative for the biotransformations with substrate **1a** or **1b**.

2.3.6 Variation of the solvent concentration for substrate 1a

With the results of the co-solvent studies in hand the amount of the used water-miscible organic solvents was increased from 10 to 20% v/v. In this case the three solvents *N*-methyl formamide, formamide and DMSO were applied. Through the extended amount of organic solvent the substrate was dissolved easier. Furthermore different agitation angles (0° and 45°) and a higher agitation speed (changed from 400 to 500 rpm) were tested, to monitor the influence of the mixing on the apparent conversion and the reproducibility. All experiments with 20% v/v organic solvent were performed in triplicate and compared with 10% v/v of the solvent at same conditions.

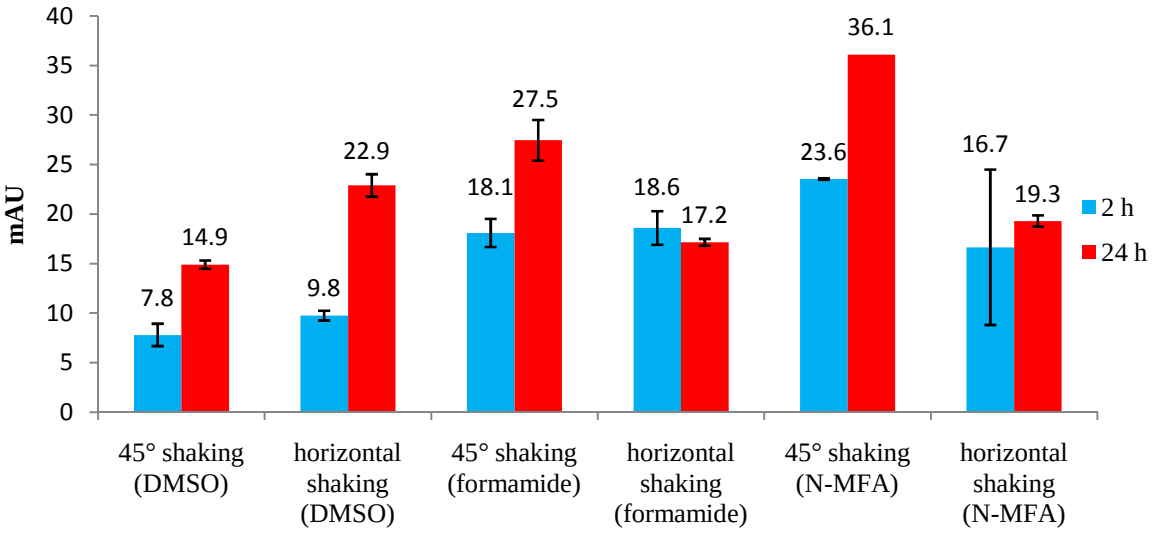


Figure 34: Apparent products formation for substrate **1a** for addition of 20% v/v organic solvent. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 20% v/v DMSO/formamide/*N*-methylformamide, 5 g/L catalase, 2 and 24 h, 40°C, 500 rpm, agitation angle (0°= horizontal/45°). Error ranges represent standard deviations of triplicate experiments

Table 7: Standard deviations for the addition of 20% v/v organic solvent and varying agitation angles

	standard deviation
45° shaking (DMSO) 2 h	1.1
45° shaking (DMSO) 24 h	0.4
horizontal shaking (DMSO) 2 h	0.5
horizontal shaking (DMSO) 24 h	1.1
45° shaking (formamide) 2 h	1.4
45° shaking (formamide) 24 h	2.1
horizontal shaking (formamide) 2 h	1.7
horizontal shaking (formamide) 24 h	0.4
45° shaking (*N*-MFA) 2 h	0.1
45° shaking (*N*-MFA) 24 h	0.0
horizontal shaking (*N*-MFA) 2 h	7.8
horizontal shaking (N-MFA) 24 h	0.6

Besides the positive effect of higher solvent concentrations on the solubility, this effect was not reflected for the reproducibility of the experiment. By comparing the standard deviations for the addition of 20% organic solvents to those from different amounts of toluene as a co-solvent, the values for this experiment are slightly higher. In the case of toluene as a co-solvent the largest standard deviation was 1.9%, through the addition of 20% v/v organic solvent the highest value was obtained by horizontal shaking of *N*-methyl formamide after two hours (7.8% standard deviation). For the experiments using 20% v/v of different organic

solvents, it concluded that the standard deviations are higher compared to those observed with toluene because of the lower dissolving effect resulting from the smaller volumetric amounts of organic solvent used.

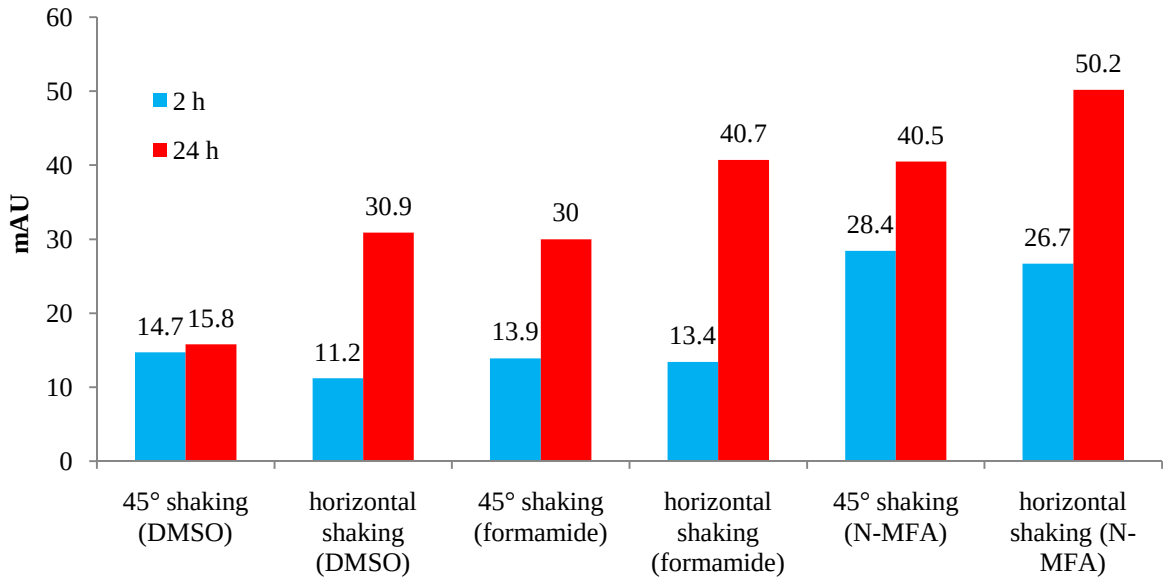


Figure 35: Apparent product formation for **1a** under the addition of 10% organic solvent. Reaction conditions: 2 g/L substrate **1a** (6.4 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v DMSO/formamide/*N*-methylformamide, 5 g/L catalase, 2 and 24 h, 40°C, 500 rpm, agitation angle (0°/45°)

Comparison of the apparent conversions after addition of 10% or 20% v/v organic solvent afforded the same results in case of all solvents. By increasing the amount of organic solvents the products' percentage decreases considerably. These results can be interpreted as followed: higher amounts of organic solvents cause a loss of enzyme activity. This supposition is strongly enhanced by frequently observed trends concerning biocatalysts. For enzymes which are dissolved in a monophasic aqueous-organic solution, as in the case of water miscible-solvents, a rule of thumb says that these solvents can be applied at concentrations up to 10% of the total volume. Only for some rare enzyme/solvent combinations even 50-70% of co-solvent may be used. If the proportion of the organic solvent exceeds a certain threshold, the essential structural water is stripped from the enzyme's surface leading to deactivation.[4]

2.3.7 Time study for substrate 1e

In order to describe the enzyme-substrate system for **1e** in detail, different enzyme concentrations were applied and the apparent conversion monitored over 24 h of reaction time.

The following four enzyme concentrations were tested 17.4, 8.6, 5.7, 3.4 µM of variant W165F. Formamide was used as solvent due to the previous given results of the solvent study. The substrate was applied at a concentration of 2 g/L (6.1 mM). Figure 36 reflects the correlation between the products to substrate ratios and the enzyme concentration over 24 h. The highest apparent conversion of 49% was reached with 17.4 µM of the variant but also the lower concentration of 8.6 µM performed well and pushed the reaction towards 43% after 24 h.

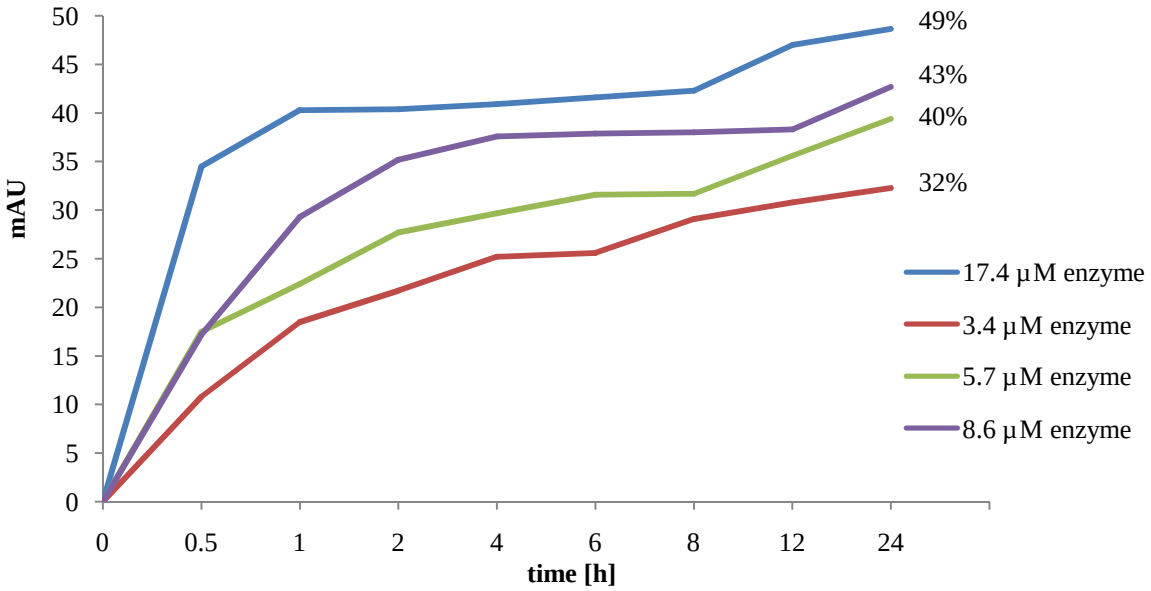


Figure 36: Time course of apparent conversion of substrate **1e** under the use of different enzyme concentrations. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (17.4 µM, 8.6 µM, 5.7 µM, 3.4 µM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% v/v formamide, 5 g/L catalase, time span (0.5-24h), 40°C, 400 rpm

To define the productivity of the biocatalyst the turn-over number (TON) was determined for each concentration.

Table 8: Correlation between the different enzyme concentrations,TON and space time yield for **1e**

Enzyme concentration [µM]	TON	Space time yield [g/Ld]
17.4	351	1
8.6	709	0.85
5.7	1070	0.79
3.4	1794	0.63

Since the turn-over number denotes the number of substrate molecules converted per number of catalyst molecules used within a given time span, the highest TON values are achieved applying an enzyme concentration of 3.4 µM. The corresponding space time yields are also

listed in table 8, whereupon highest apparent conversions at 17.4 µM enzyme concentration were associated to a space time yield of 1 g/Ld and a TON of 351.

2.3.8 Preparative scale-up experiments for substrate 1e

Performing a preparative transformation, 200 mg of substrate **1e** (61 mM) were shaken for 24 h employing the BBE variant W165F (1.01 mL, final concentration: 1g/mL: 0.017 mM) and formamide in 10% v/v. The HPLC measurements after the work-up revealed 46% apparent conversion. To isolate the remaining substrate the crude mixture was purified by flash chromatography and yielded 28.3 mg of (*R*)-**1e** (*ee* = 86%). NMR-spectra data and the bellow shown HPLC-MS measurements reflect the results of the described preparative transformation.

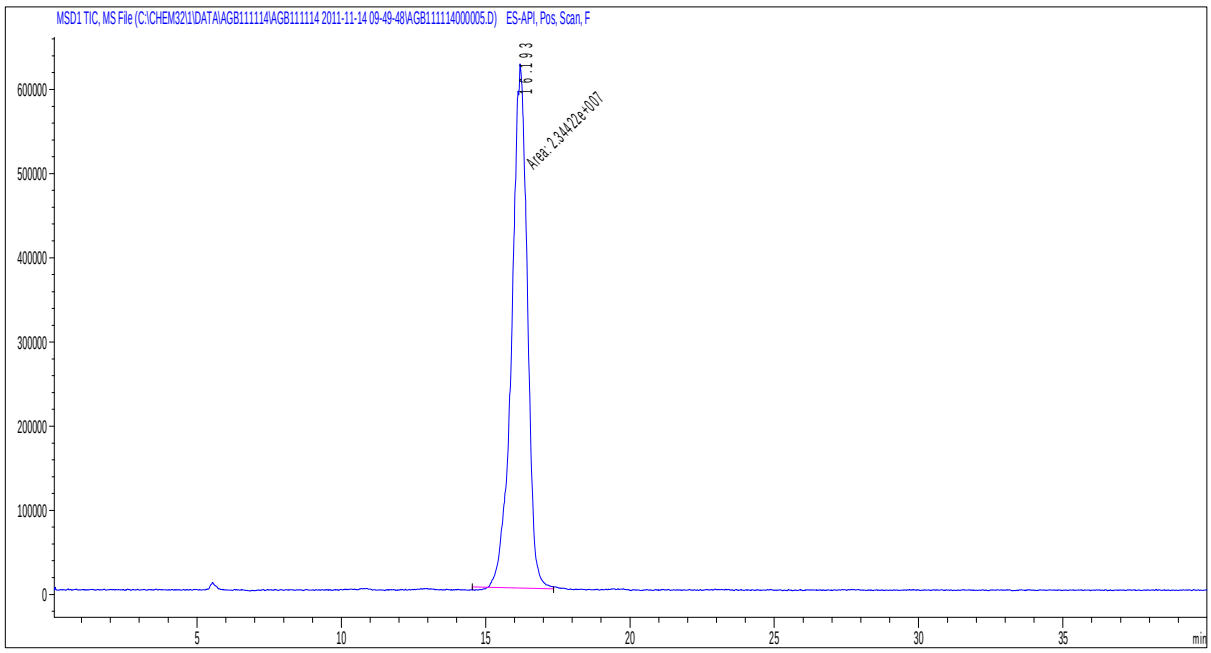


Figure 37: HPLC-MS measurements for substrate **1e** after purification of the preparative transformation: MS detection in the scan mode m/z = 280-500. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.17 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 24 h, 40°C, 400 rpm

The HPLC-MS measurements of the purified substrate show that it is totally free from any product.

Another preparative experiment was carried out under the same reaction conditions as described above, however, the reaction time was minimized to half an hour. After this time span an apparent conversion of 29% was determined. Figure 38 shows the HPLC-UV chromatogram of the reaction mixture after the work up.

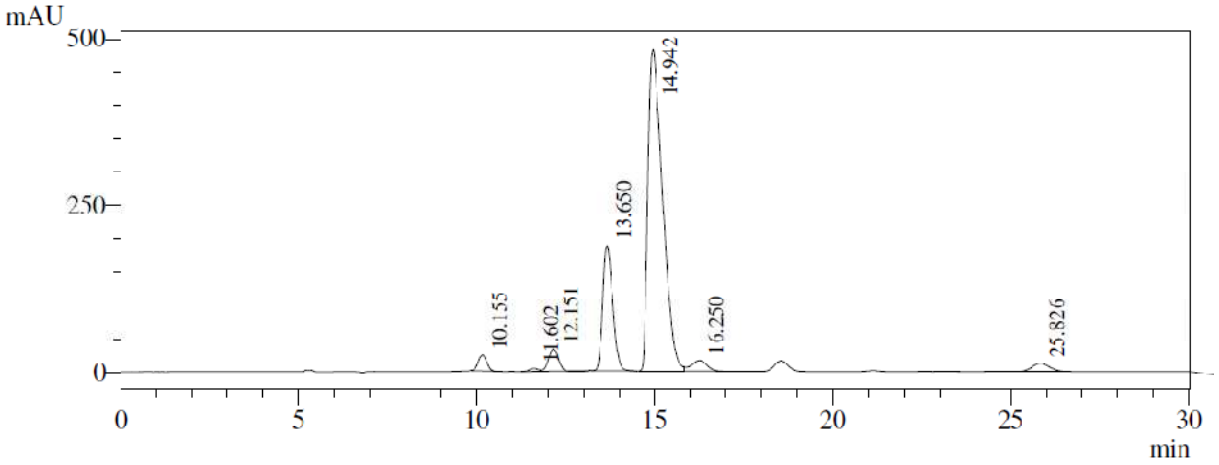


Figure 38: HPLC-UV chromatogram for the preparative transformation of **1e** after 0.5 hours reaction time. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.17 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 0.5 h, 40°C, 400 rpm

After purification of the reaction mixture *via* column chromatography, the products (15 mg) were separated from the substrate. The first purification step was followed by acidification, extraction and basification of the products which yielded in 8.4 mg final product. Figure 39 displays the HPLC-UV chromatogram after the two purification steps.

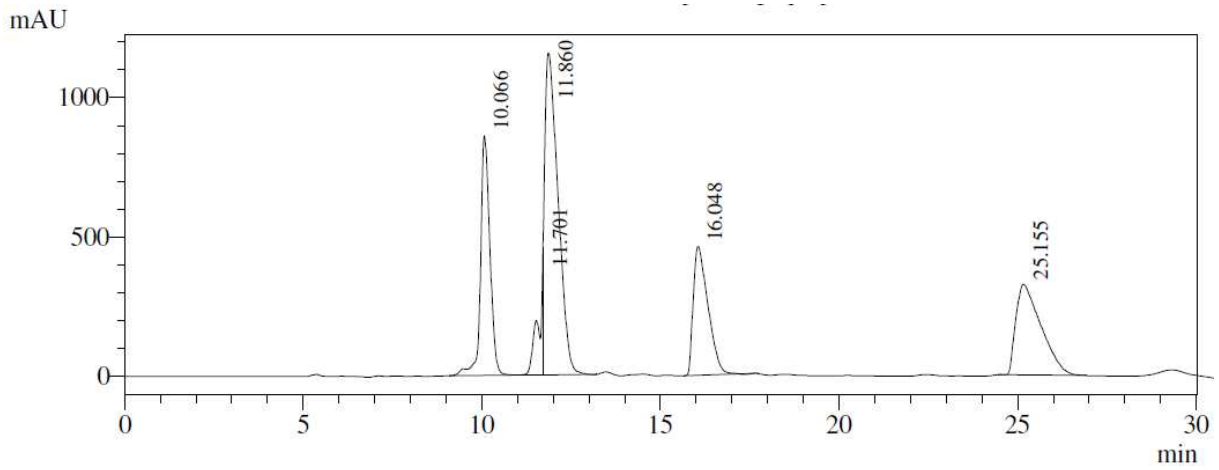


Figure 39: HPLC-UV chromatogram of substrate **1e** after purification of the preparative transformation. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 0.5 h, 40°C, 400 rpm

Comparison of both chromatograms (figure 38 and 39) shows that the peak at a retention time of 14.9, which represents the substrate, disappeared as well as the one at 13.6 minutes which correlates to the deethylated product (see 2.3.2.3.). The acidification and basification step which was in fact done to remove assumed impurities of the flash chromatography solvent, seemed to induce the loss of the deethylated product. The remaining components were measured on the HPLC-MS to again confirm the product composition of the biotransformation for substrate **1e** which was already demonstrated for a corresponding sample of the solvent study (see 2.3.2).

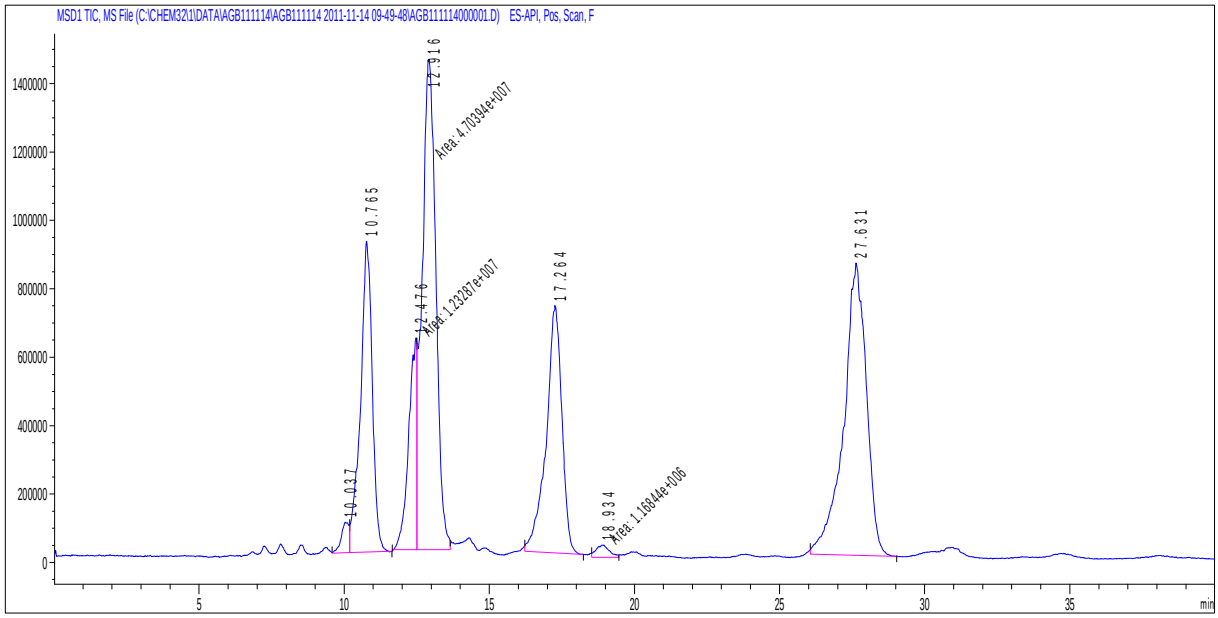
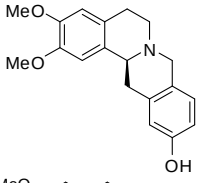
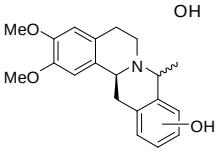
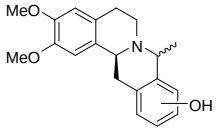
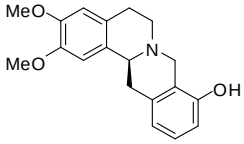
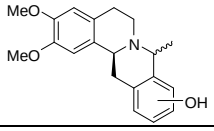


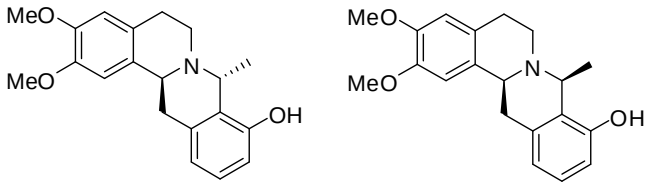
Figure 40: HPLC-MS chromatogram of substrate **1e**: MS detection in the scan mode m/z = 250-500. Reaction conditions: 2 g/L substrate **1e** (6.1 mM), BBE variant W165F (0.017 mM), buffer (Tris-HCl 50 mM + $MgCl_2$ 10 mM, pH 9), 10% formamide, 5 g/L catalase, 0.5 h, 40°C, 400 rpm

Figure 40 displays all formed products, the corresponding mass analysis is in accordance to the already identified products. Table 9 shows the identified products.

Table 9: Classification of the reaction mixture obtained by biotransformation of **1e** with BBE in formamide (10% v/v) after 0.5 h of reaction time

retention time [min]	species	formed mass [m/z]	suggested structure	observed reaction
10.76	$C_{19}H_{23}NO_3$ $[(M-H)^+]$	311.9		(*S*)-product with a mass loss of m/z =16
12.47	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	326.0		(*S*)-product with a mass loss of m/z =2
12.91	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	325.9		(*S*)-product with a mass loss of m/z =2
17.67	$C_{19}H_{23}NO_3$ $[(M-H)^+]$	311.9		(*S*)-product with a mass loss of m/z =16
27.63	$C_{20}H_{23}NO_3$ $[(M-H)^+]$	325.9		(*S*)-product with a mass loss of m/z =2

The product mixture was also subjected to NMR-spectroscopy. It mainly consists out of the following two cyclized diasterioisomeres **5e**, demonstrate in Scheme 44.



Scheme 45: Reaction products from the preparative biotransformation of **1e** after 0.5 h reaction time, determined *via* NMR-spectroscopy after removal of the deethylated product

2.4 Conclusion

The conversion of the *N*-ethyl tetrahydroisoquinoline substrates **1a**, **1b** and **1e** by the berberine bridge enzyme revealed a completely new feature of the enzyme. BBE in its natural role catalyzes the oxidative C-C bond formation of (*S*)-reticuline to (*S*)-scoulerine under the consumption of molecular oxygen. Recent studies by Schrittwieser, Resch, *et al.* demonstrated that the enzyme is able to cyclize a broad range of non-natural substrates with excellent yields.[1] In the case of the *N*-ethyl substrates which were synthesized during this master thesis it was found that the enzyme is also able to *N*-dealkylate benzylisoquinolines stereoselectively, representing an example of enzyme promiscuity. The demethylation activity of the enzyme was already described before by Macheroux *et al*, as well as by Kutchan and Dittrich. [5,6]

Through biotransformation three different product classes (i: products with a mass loss of m/z =16 units; ii: products with a mass loss of m/z =2; iii: deethylated product) were formed for all applied substrates and detected by HPLC-UV/MS and NMR measurements (Figure 41).

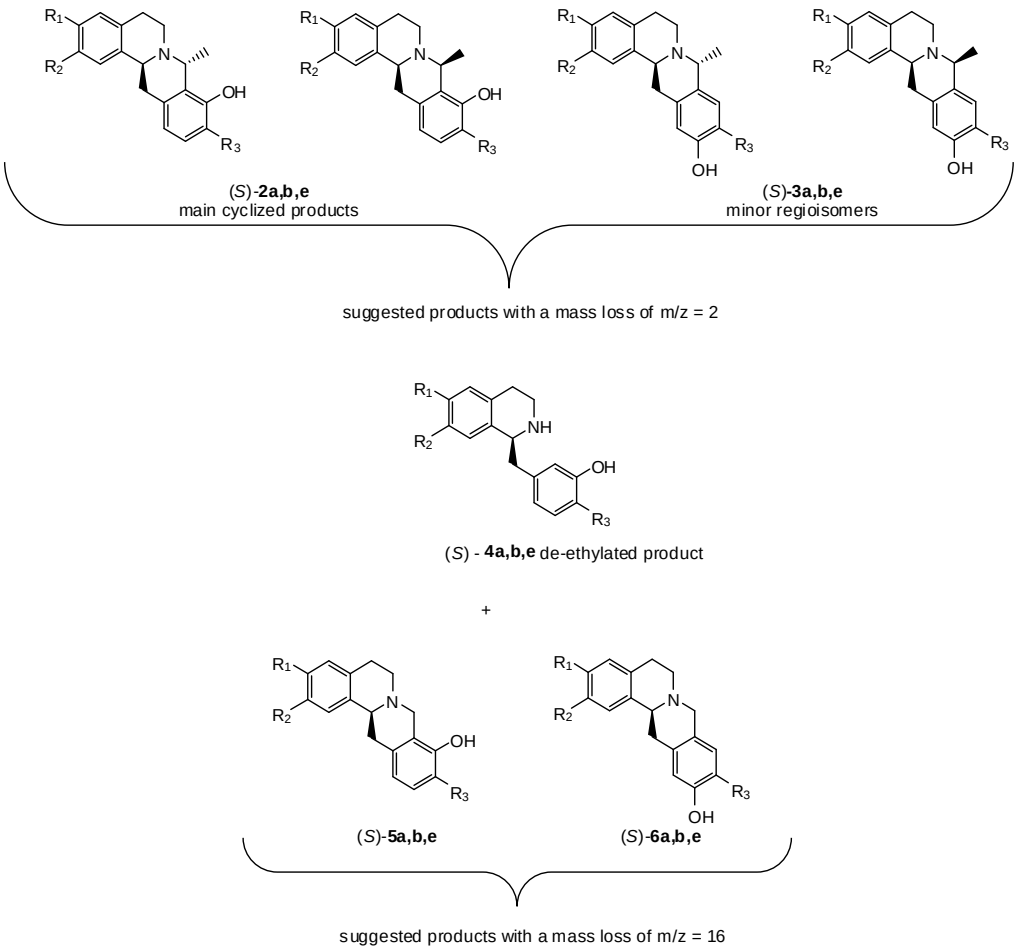


Figure 41: The three different product classes (i: products with a mass loss of m/z =16 units; ii: products with a mass loss of m/z =2; iii: deethylated product) formed within the biotransformation by BBE

For the products with a mass loss of m/z = 2 according to the HPLC-MS measurements, four different cyclized products are assumed. Both diastereoisomers (**2e**) could be detected in the proton NMR-spectrum of the preparative transformation with substrate **1e**. In recent studies with other non-natural substrates the regioisomers were always found as minor side products next to the cyclized ones.[2] Therefore also the two regioisomers (**3a**, **b**, **e**) are conceivable as products with the mass loss of m/z = 2. The structure of the deethylated products can be clearly identified by comparison of the GC-MS spectrum of **4e** with the one of the corresponding reference substance. Hence the product composition for each substrate is the same determined by HPLC-MS/UV measurements, it can be concluded that the deethylation site always remains the same for all substrates. Concerning the two product peaks with a substrate mass loss of m/z = 16 the cyclized structures lacking a methyl group on the bridging C8 carbon (**5/6-a,b,e**) can be suggested. These structural formations are evidenced through the comparison with a GC-MS spectrum of a reference substance in the case of **5/6e**, also a HPLC retention time alignment with references for the biotransformation products (**5/6-a,b,e**) were done.

The dealkylation activity of the enzyme can possibly be explained through the orientation of the substrate in the active site. In the case of the natural substrate reticuline the C2' atom of the phenolic moiety, the CH_3-group at the nitrogen in the tetrahydroisoquinoline scaffold and

the nitrogen N5 of the flavin cofactor are arranged in a defined angle of 126.8° to each other (Figure 42). When this arrangement is not given, the nucleophilic attack of the C2' atom belonging to the phenolic moiety onto the *N*-methyl group might be disfavored. When the *N*-ethyl-substrates are applied in the biotransformation with BBE, dealkylation proceeds as a main reaction pathway, possibly since the rotation of the ethyl group hinders the nucleophilic attack of the C2' carbon of the phenolic group. Consequently, hydride transfer to FAD leads to formation of an iminium ion formation. The latter further undergoes hydrolysis and eliminates acetaldehyde to form the corresponding deethylation product (Scheme 45).

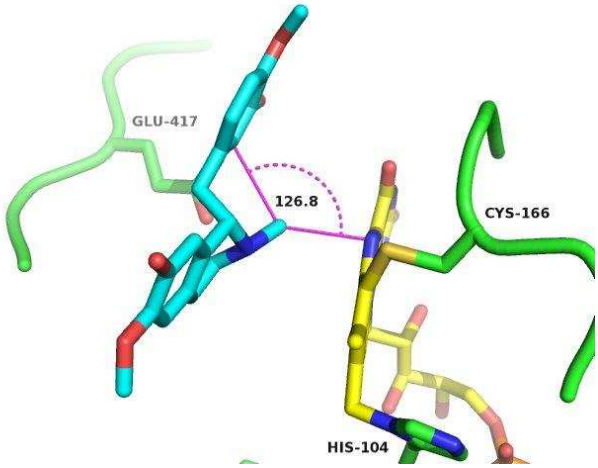
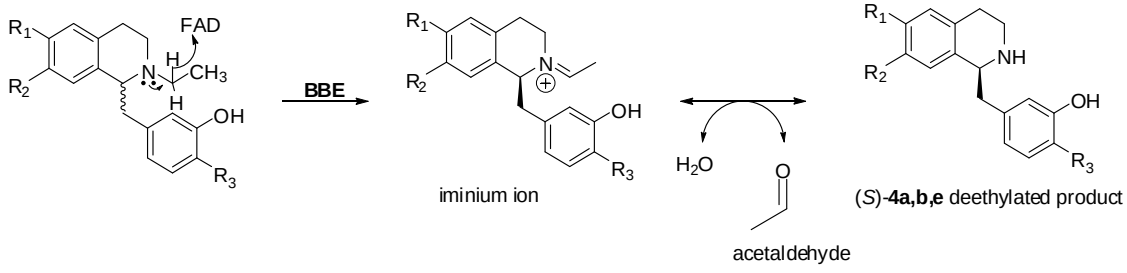


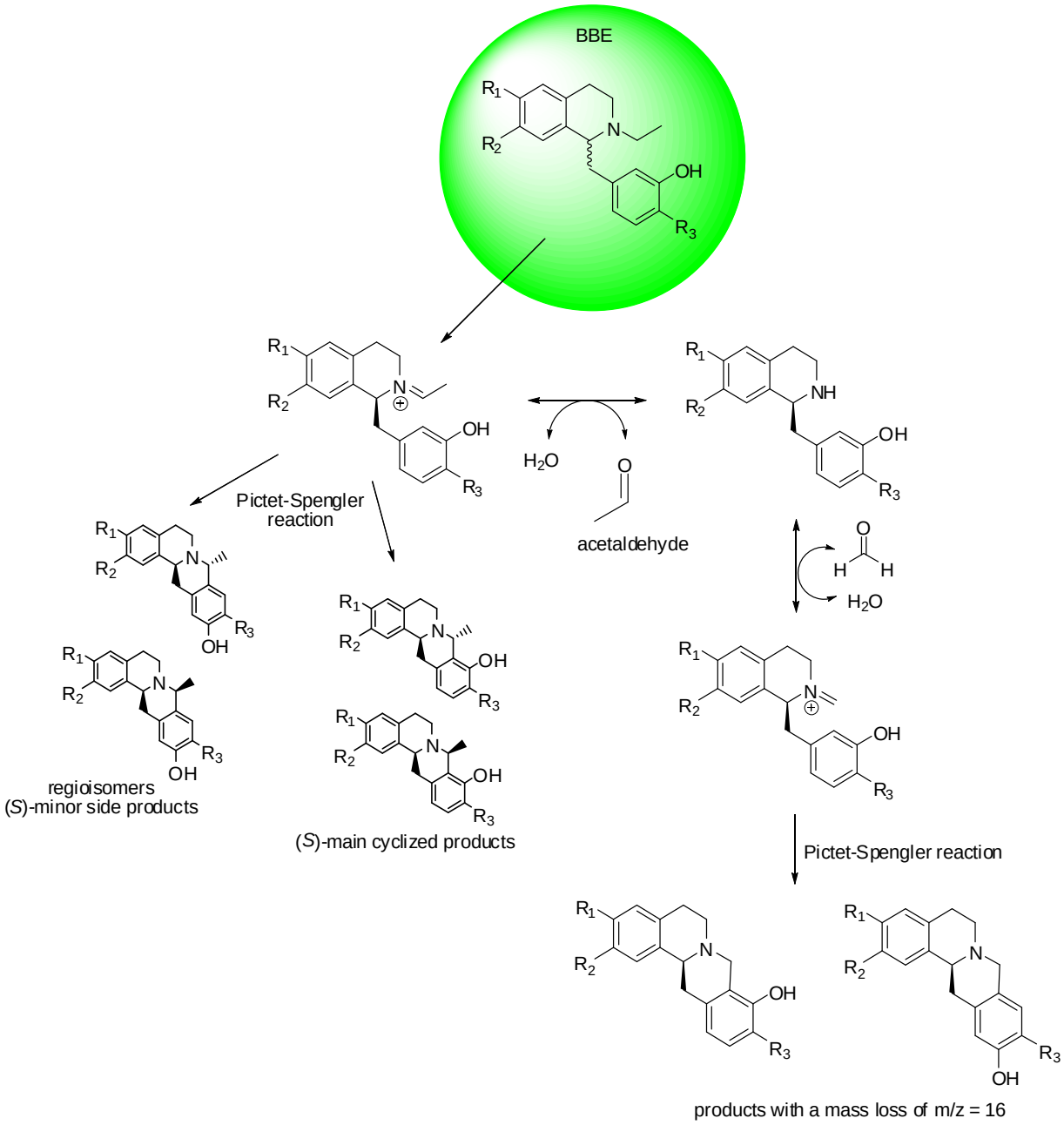
Figure 42: Orientation of reticuline (blue) to the flavin cofactor (yellow) in the active site of BBE. Picture was taken from the personal communication of Prof. Karl Gruber

The arguments given above might be the reason for the repressed cyclization reaction of the enzyme. Within all biotransformations a strong predominance of the deethylated products compared to the cyclized and ones with a mass loss of m/z =16 can be found for all substrates after two hours of reaction time (see 2.3.4).



Scheme 46: Suggested mechanism for the formation of the deethylated products

However, this ratio changes considerably after one day reaction time. The amounts of the cyclized products strongly increase and those of the deethylated products decrease compared to the results of the short-time experiments. It can be assumed that the deethylated products are transformed further into the cyclized ones and also into those with a mass loss of m/z = 16. It is conceivable that after longer reaction times the formed iminium ion at the isoquinoline nitrogen is able to diffuse out of the active site into the aqueous medium, were it probably could undergo a conventional Pictet-Spengler reaction to form the corresponding four cyclized products. Moreover it is very likely that the iminium ion there again hydrolyses to form the deethylated product, out of this one and in the presence of formaldehyde another imium ion is formed which is able to undergo the Pictet-Spengler reaction to end up in the cyclized products that lack the additional chiral centre through the loss of a methyl group. This assumption would offer a possible reaction mechanism for the products with a mass loss of m/z =16. Formaldehyde could be formed in this reaction through the oxidation of Tris which is a main component in the buffer, or traces of methanol (Scheme 46).



Scheme 47: Possible product formation in the aqueous medium in the presence of BBE

Nevertheless this assumptions need to be tested in further experiments to solve the remaining issues of this challenging and new feature of the berberine bridge enzyme.

The biotransformation with the four different synthesized *N*-ethyl substrates showed that only two of them are accessible for conversion with BBE. The one that harbors a bridged methylenedioxy substituent at the C6 and the C7 atoms of the aromatic part in the tetrahydroisoquinoline scaffold (**1a**) as well as **1b** which has a methoxy substituent at the C6 atom were transformed by BBE. The problem that occurred in connection with these substrates was the solubility issue, which were tried to overcome by addition of toluene as a

co-solvent (see 2.3.5) or *via* the increase of the amount of used organic solvents (see 2.3.6). Those experiments indeed helped to overcome the solubility problems but still led to significant deviations of the conversions. Consequently the values of all measurements for those two substrates have to be regarded with care.

Substrate **1e** which has two methoxy groups at the C6 and C7 positions was taken from recent synthetic experiments by Schrittwieser *et al.* and turned out to be the most suitable substrate. No solubility problems can be observed for **1e,** the defined optimized conditions for this substrate are: 10% v/v formamide, buffer at pH 9 and the use of the BBE variant W165F (0.017 mM).

The substrates **1c** and **1d** which possess the same substitution pattern as the natural substrate reticuline at the aromatic ring system of the tetrahydroisoquinoline scaffold (C6 = methoxy group; C7 = hydroxy group) were not converted by the enzyme at all. This is quite curious since **1d** strongly resembles reticuline, exclusively the *N*-methyl and the *N*-ethyl groups differ from each other. Nevertheless they were not adequate substrates for the biotransformation with BBE.

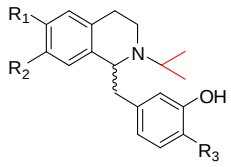
2.5 Outlook

The newly observed enzyme promiscuity created a novel area of investigations for the berberine bridge enzyme. A lot of questions arise through the found enantioselective dealkylation reaction concerning the mechanism of the enzyme and the formation of the different reaction products. Characterization and isolation of the latter should be the main goal of further experiments along with the synthesis of the corresponding reference compounds. Further questions, concerning the newly formed chiral center in the cyclized products introduced through the *N*-ethyl group, arise. Given that the cyclized products are formed in the active site of the enzyme it is possible that BBE has a stereopreference for this center, otherwise when they are formed in the aqueous medium of the enzyme through a normal Pictet-Spengler reaction (described above) the stereogenic center would probably be racemic since in this environment no asymmetric induction is given. To figure out if the cyclized product formation in the aqueous medium is possible the synthesized racemic deethylation product can be subjected to the reaction with acetaldehyde in the Tris-buffer. If the formation of the cyclized products can be observed it would be a strong evidence for the above given mechanistic proposal of the product formation outside of the enzyme. Moreover the reaction pathway suggested above for forming the products with a mass loss of m/z = 16 has to be clarified. This proposal could either be tested through the addition of formaldehyde to the biotransformation mixture to check if the amounts of the products increase, or through the reaction of the synthesized racemic deethylation product with formaldehyde in the buffer.

Furthermore the influence of the formed acetaldehyde in the deethylation reaction on the enzyme has to be determined. The presence of higher amounts of acetaldehyde particularly in preparative transformations can strongly inhibit the enyme in its role as a biocatalyst.

Besides, it would be very interesting to lower the enzyme concentration to a value where only the deethylated product is formed during the biotransformation, such that it can be isolated from the reaction mixture.

Substrate modification should also be a new goal for further investigations. By substituting another hydrogen atom against a second methyl group at the CH_2 group in the *N*-ethyl part of the substrate molecule, the steric demand of this group rises and therefore the adjustment of the right angle of 126° for the nucleophilic substitution and the concerted ring closure is hindered even more (Scheme 47). Another option is to selectively remove the acetaldehyde by enzymatic reduction.



Scheme 48: Possible substrate modification to force the formation of the deethylated product

Consequentely the formation of the cyclized products in the active site would be strongly decreased or even completely repressed, whereas the deethylation product could be found as the main or even sole product.

2.6 References

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[2] J. H. Schrittwieser, V. Resch, S. Wallner, W.-D. Lienhart, J. H. Sattler, J. Resch, P. Macheroux, W. Kroutil, *J. Org. Chem.* **2011**, *76*, 6703-6714.

[3] A. R. Battersby, R. J. Francis, M. Hirst, J. Staunton, *Proc. Chem. Soc.* **1963**, *198*, 268-268.

[4] K. Faber. *Biotransformations in Organic Chemistry*. Springer 6th edition. **2011.**

[5] A. Winkler, A. Lyskowski, S. Riedl, M. Puhl, T. M. Kutchan, P. Macheroux, K. Gruber, *Nat. Chem. Biol.* **2008**, *4*, 739-741.

[6] T. M. Kutchan, H. Dittrich, *J: Biol. Chem.* **1995**, *270*, 24475-24481.

3 EXPERIMENTAL SECTION

3.1 Materials

3.1.1.1 Chemicals for synthesis

Table 10: Provider and Lot. Number of the compounds used for synthesis

Compound	Provider	Lot. Number
Acetic acid	AnalaR NORMAPUR	10B260522
Acetyl chloride	Fluka	0001449293
Ammonium acetate	Fluka	349107/11195
Benzyl bromide	Sigma-Aldrich	12396MK
Borane tetrahydrofuran complex	Sigma-Aldrich	STBB7631
Dimethylformamide	Roth	29045510
Ethylamine (70% in water)	Riedel de Haen	020984
3-Hydroxyphenylacetic acid	Sigma-Aldrich	03514CH
Lithiumaluminiumhydride	Sigma-Aldrich	STBB5414
(3,4-Methylenedioxy)phenyl-acetic acid	Sigma-Aldrich	BCBB3195V
3-Methoxyphenylacetic acid	Sigma-Aldrich	S62586-289
Nitromethane	Sigma-Aldrich	12696LK
Oxalyl chloride	Sigma-Aldrich	03610DH
Palladium 10% on actived charcoal	Sigma-Aldrich	0001453211000900720
Phosphoryl chloride	Fluka	S5446822608B21
Potassium carbonate	Sigma-Aldrich	07718KU-060
Potassium hydroxide pellets	Fisher Scientific	0761782
Sodium borohydride powder	Sigma-Aldrich	STBB7441V
Sodium chloride	Merck	K3429684507
Sodium iodide	Merck	807B712023
Sodium sulfate	Acros Organics	A0296984
Triethylamine	Sigma-Aldrich	0001413140
Vanillin	Sigma-Aldrich	STBB1626

Organic solvents for synthesis were purchased from the following commercial sources: Tetrahydrofuran (VWR), toluene (VWR), acetonitrile (Roth), ethanol (Merck), methanol (Merck), dioxane (Fluka), dichloromethane (Roth), ethyl acetate (VWR), chloroform (VWR) . For drying acetonitrile and methanol 3Å molecular sieve (Fluka) was used and in the case of toluene 4Å were applied and the solvents were stored under argon atmosphere. In the case of THF, the solvent was distilled before use over Na/K. All other solvents and reagents were used without further purification.

3.1.2 Chemicals for biotransformations

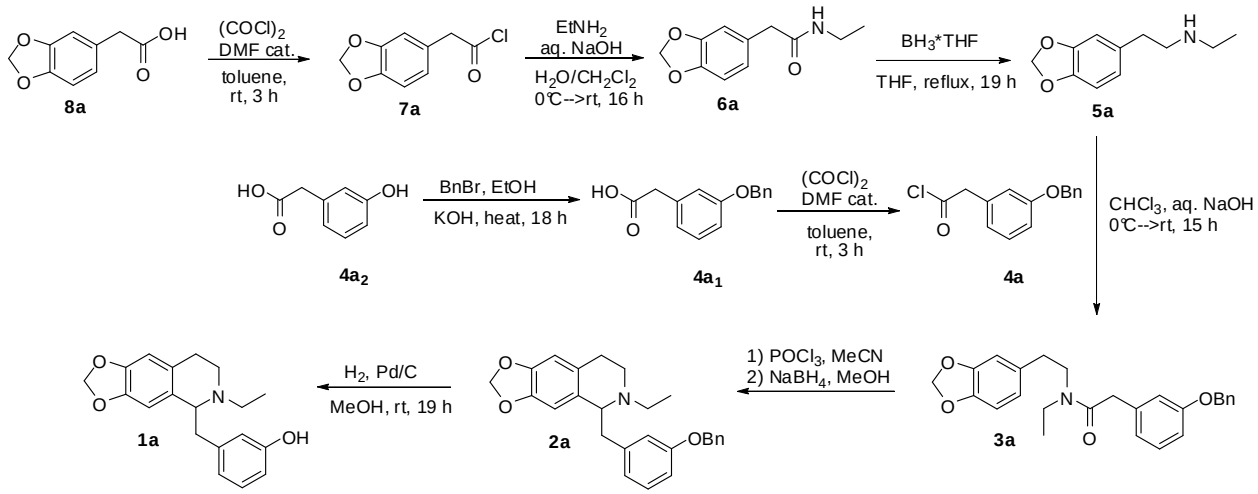
Table 11: Provider and Lot. Number of the compounds used for biotransformations

Compound	Provider	Lot. Number
Acetone	VWR	11E020958
Acetonitrile	Roth	0010/5CR
Ammonium formate	Rielel-de Haen	7200
Catalase from Bovine liver	Sigma-Aldrich	81H7146
t-Butanol	Riedel-de Haen	2008897
Dimethylformamide	Roth	29045510
Dioxan	Fluka	336924/1494
Ethanol	Merck	K42153128
1-Ethyl-3-methylimidazolium-acetate	Fluka	S3011055205B11
Formamide	Merck	K1566908
Magnesium chloride hexahydrate	Merck	TA519032945
Methanol	Roth	597851
N-Methylformamide	Sigma-Aldrich	89F3529
i-Propanol	Roth	675291
Sodium sulfate	Acros Organics	A0296984
Tris	Roth	280159067

Organic solvents for the biotransformations that are not named in the chemical table above were purchased from the following commercial sources: Toluene (Aldrich, 99.5%), dimethyl sulfoxide (Sigma Aldrich, 99.0%), ethanol (Sigma Aldrich, 99.5%) and diphenyl ether (Acros Organics, 99.0%). All solvents and reagents were used without further purification.

3.2 Synthesis

3.2.1 Synthesis of 2-ethyl-1-(3-hydroxybenzyl)-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline (1a)



Scheme 49: Synthesis of substrate **1a**

(3,4-Methylenedioxy)phenylacetyl chloride (7a): A solution of (3,4-methylenedioxy)phenyl-acetic acid **8a** (3.60 g, 20.0 mmol), oxalyl chloride (5.93 g, 46.7 mmol) and five drops of DMF in dry toluene was stirred at room temperature for three hours. The solvent was evaporated under reduced pressure to give 4.89 g (quant.) of a yellowish liquid. (3,4-Methylenedioxy)phenylacetyl chloride **2** was used in the following transformation without further purification. TLC (petrol ether/EtOAc = 1/1): R_f = 0.63. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.06 (2H, s, $ArCH_2$), 5.98 (2H, s, OCH_2O), 6.71-6.82 (3H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 52.7, 101.3, 108.8, 109.8, 123.1, 124.7, 147.6, 148.1, 172.1. The ^{1}H-NMR- and ^{13}C-NMR- data is in accordance with literature.[1]

(3,4-Methylenedioxy)phenyl-*N*-ethylacetamide (6a):[2] (3,4-Methylenedioxy)phenylacetyl chloride **7a** (4.89g, 24.6 mmol) was dissolved in CH_2Cl_2 (80 mL) and cooled to 0°C on an ice bath. A mixture of ethylamine (70% in H_2O; 4.19 g, 65.1 mmol) and 2 M NaOH (12 mL) was added dropwise to the solution during 1 h. Afterwards the ice bath was removed and the reaction mixture was stirred at room temperature overnight. Then the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 30 mL). The combined organic phases were washed with 2 N HCl solution (2 x 40 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 3.79 g (74%) of the amide **6a** as a slightly yellowish solid. Mp: 103-105°C. TLC (petrol ether/EtOAc = 1/1): R_f = 0.19. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.07 (3H, t, *J* = 7.2 Hz, NCH_2CH_3), 3.24-3.20 (2H, m, NCH_2CH_3), 3.47 (2H, s, $ArCH_2$), 5.53 (1H, bs, NH), 5.96 (2H, s, OCH_2O), 6.62-6.80 (3H, m, Ar). ^{13}C-NMR ($CDCl_3$,

75 MHz): δ = 14.7, 34.5, 42.4, 101.2, 108.6, 109.7, 122.6, 128.5, 146.9, 148.1, 170.9. MS (EI, 70 eV): m/z = 207 (M, 44), 135 (100), 106 (11), 77 (19), 51 (12), 44 (9).HRMS calculated for $C_{11}H_{13}NO_3$ [$(M)^+$]: 207.0898; found: 207.0892. No NMR reference data is available.

***N*-Ethyl-(3,4-methylenedioxy)phenethylamine (5a):**[2] To a solution of (3,4-methylenedioxy)phenyl-*N*-ethylacetamide **6a** (3.79 g, 18.0 mmol) in anhydrous THF (100 mL, BH_3*THF (1.0 M in THF; 100 mL, 100 mmol) was added. The mixture was refluxed for 19 h under argon atmosphere. After cooling to room temperature 6 N HCl solution (20 mL) was added cautiously and stirring was continued for half an hour. Then the mixture was concentrated under reduced pressure. The residue was basified by addition of 2 M NaOH solution (100 mL) and saturated with NaCl. Subsequently the mixture was extracted with EtOAc (5 x 40 mL) and the combined organic phases were washed with brine and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 3.60 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 90/9/1) afforded *N*-Ethyl-(3-methoxy)phenethylamine 2.96 g (84%) as a pale yellowish liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.40. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.07 (3H, t, *J* = 7.2 Hz, NCH_2CH_3), 1.13 (1H, bs, NH), 2.60 – 2.68 (2H, m, NCH_2CH_3), 2.70 (2H, t, *J* = 6.3 Hz, $ArCH_2$), 2.79 – 2.84 (2H, m, $ArCH_2CH_2$), 5.90 (2H, s, OCH_2O), 6.63 – 6.73 (3H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 15.4, 36.2, 44.1, 51.2, 100.9, 108.3, 109.1, 121.6, 134.0, 145.9, 147.7.MS (EI, 70 eV): m/z = 193 (M, 38), 122 (100), 107 (26),91 (23), 78 (11), 72 (14), 44 (14). HRMS calculated for $C_{11}H_{15}NO_2$ [$(M)^+$]: 193.1103; found: 193.1121. No NMR reference data is available.

3-Benzyloxyphenylacetic acid ($4a_1$):[3] A mixture of 3-hydroxyphenylacetic acid **$4a_2$** (9.01 g, 59.1 mmol), NaI (0.32 g, 2.07 mmol) and KOH (8.96 g, 15.9 mmol) in ethanol (300 mL) was heated to 90°C. Afterwards benzyl bromide (12.3 g, 70.9 mmol) was added dropwise while the solution was refluxed at 100°C for 18 h. Then the mixture was concentrated to approximately 50 mL and the residue was poured into water (300 mL) to give a slightly brownish solution. Upon acidification with concentrated HCl the product precipitated as yellowish needles. The solid was filtered and recrystallised from H_2O/HOAc (1/1, 150 mL) to yield 10.8 g (76%) 3-benzyloxyphenylacetic acid as a white solid. Mp: 122-124°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.65 (2H, s, CH_2-COOH), 5.09 (2H, s, Ph-CH_2-O), 6.91 - 6.97 (3H, m, Ar), 7.26 – 7.48 (6H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 41.1, 70.0, 113.7, 116.1, 122.2, 127.6, 128.0, 128.6, 129.7, 134.7, 136.88, 159.0, 177.9, MS (EI, 70 eV): m/z = 242 (M^+, 9), 91 (100), 65 (10). The ^{1}H-NMR data is in accordance with literature.[4]

3-Benzyloxyphenylacetyl chloride (4a): A solution of 3-benzyloxyphenylacetic acid (6.19 g, 25.6 mmol), oxalyl chloride (5.17 g, 40.7 mmol) and one five drops of DMF in dry toluene (75 mL) was stirred at room temperature for four hours. The solvent was evaporated under reduced pressure to give 8.16 g (quant.) of a yellowish liquid. 3-Benzyloxyphenylacetyl chloride was used in the following transformation without further purification. TLC (EtOAc): R_f = 0.78. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.13 (2H, s, CH_2-COCl), 5.09 (2H, s, $PhCH_2O$), 6.89-7.01 (3H, m, Ar), 7.27 – 7.48 (6H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 53.0, 70.0, 114.5, 116.2, 122.0, 125.3, 127.5, 128.1, 128.6, 129.1, 130.1, 132.6, 136.7, 159.2, 171.8. The ^{1}H-NMR- and ^{13}C-NMR-data is in accordance with literature.[1]

2-(3-Benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide (3a):[5] A solution of *N*-ethyl-(3,4-methylenedioxy)phenethylamine **5a** (3.07 g, 15.8 mmol), $CHCl_3$ (40 mL) and 3% NaOH solution (150 mL) was cooled to 0°C on an ice bath. Then a solution of 3-benzyloxyphenylacetyl chloride **4a** (8.16 g, 31.3 mmol) in $CHCl_3$ (20 mL) was added dropwise during one hour. Afterwards the ice bath was removed und stirring was continued for 15 h at room temperature. The phases were separated and the aqueous phase was extracted with $CHCl_3$ (3 x 60 mL). The combined organic phases were washed with 2 M HCl (100 mL), water (100 mL) and dried over Na_2SO_4. Then the solvent was evaporated under reduced pressure to give 10.5 g of a dark yellowish liquid. Flash chromatography (silica; petrol ether/EtOAc = 2/1) afforded 2-(3-benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide (6.6 g, 99%) of a pale yellowish liquid. TLC (petrol ether/EtOAc = 2/1): R_f = 0.47. NMR spectroscopy reveals that the product is a mixture of isomers (ratio *trans/cis* = 1.5/1). Based on the peak intensities as well as the DEPT, COSY and HSQC spectra, the NMR signals can be assigned to the isomers as follows:

trans-2-(3-Benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 0.96 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.68 (2H, t, *J* = 7.8 Hz, $ArCH_2$), 3.06 – 3.13 (2H, dd, J_1 = 6.9 Hz, J_2 = 7.2 Hz, $N\mathit{CH_2}CH_3$), 3.41 – 3.28 (2H, m, $ArCH_2\mathit{CH_2}N$), 3.58 (2H, s. $OCCH_2Ar$), 4.98(2H, s, OCH_2Ph), 5.82 (2H, s, OCH_2O), 6.44 – 6.84 (6H, m, Ar), 7.12 – 7.36 (7H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 14.1, 34.2, 40.9, 43.5, 47.8, 55.2, 69.9, 111.9, 113.3, 114.3, 115.3, 121.4, 121.3, 127.5, 128.6, 129.7, 136.9, 137.0, 140.9, 159.0, 159.7. No NMR reference data was available for this compound.

cis-2-(3-Benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.06 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.52 (2H, t, *J* = 7.8 Hz, $ArCH_2$), 3.41 – 3.28 (2H, m, $N\mathit{CH_2}CH_3$), 3.41 – 3.28 (2H, m, $ArCH_2\mathit{CH_2}N$), 3.43 (2H, s, $OCCH_2Ar$), 4.95 (2H, s, OCH_2Ph), 5.83 (2H, s, OCH_2O), 6.44 – 6.84 (6H, m, Ar), 7.12 – 7.36 (7H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 12.7, 35.8, 40.6, 41.1, 49.4, 55.2, 69.8, 111.9, 113.2, 114.6, 115.0,121.1, 121.3, 127.9, 129.4, 129.7, 136.9, 137.0, 139.8, 159.0, 159.8. No NMR reference data was available for this compound.

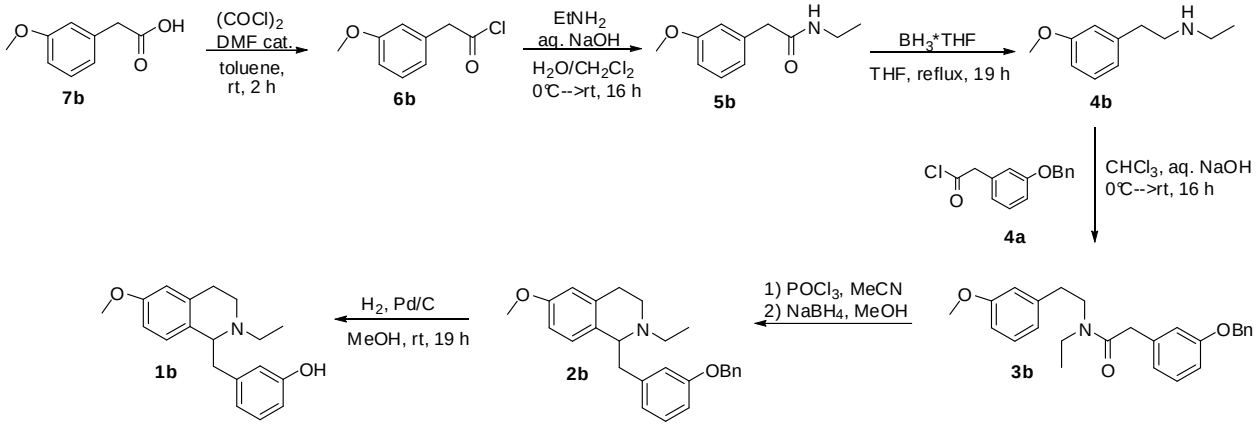
HRMS calculated for $C_{26}H_{27}NO_4$ [$(M)^+$]: 417.1940; found: 417.1955.

2-Ethyl-1-(3-benzyloxybenzyl)-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline (2a):[6] A solution of 2-(3-benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide **3a** (6.59 g, 15.9 mmol), dry acetonitrile (150 mL) and $POCl_3$ (7.84 g, 51.04 mmol) was refluxed for 4 h under argon atmosphere. The solvent and the excess of $POCl_3$ were evaporated under reduced pressure and the residue was taken up in methanol (100 mL). The resulting mixture was put under argon and cooled to -5°C on an ice/NaCl bath. Afterwards $NaBH_4$ (3.21 g, 84.95 mmol) was added in portions to the stirring mixture. The cooling bath was removed and stirring was continued for 14 h at room temperature under inert atmosphere. Then the solvent was evaporated under reduced pressure and the residue was taken up in half-saturated Na_2CO_3 solution (100 mL). The aqueous phase was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic phases were dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 7.35 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1) afforded 1-(3-benzyloxybenzyl)-6,7-methylenedioxy-2-

ethyl-1,2,3,4-tetrahydroisoquinoline (6.12 g, 95%) as a slightly orange liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.29. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.07 (3H, t, *J* = 6.9 Hz, $NCH_2\mathit{CH_3}$), 2.45 – 2.51 (1H, m, CH_2), 2.52 – 2.68 (2H, m, CH_2), 2.76 – 2.91 (3H, m, CH_2), 3.05 – 3.24 (2H, m, CH_2), 3.81 – 3.86 (1H, dd, J_1 = 6.6 Hz, J_2 = 9.9 Hz, CH), 5.06 (2H. s, OCH_2Ph), 5.88 (2H, s, OCH_2O), 6.19 (1H, s, Ar), 6.56 (1H, s, Ar), 6.76 – 6.86 (3H, m, Ar), 7.20 (1H, t, *J* = 7.8 Hz, Ar), 7.31 – 7.39 (5H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 13.2, 25.3, 41.7, 43.3, 47.5, 62.6, 69.9, 100.5, 108.2, 108.4, 112.3, 116.3, 122.4, 127.5, 127.5, 127.9, 128.6, 128.9, 131.0, 137.2, 141.8, 145.2, 145.8, 158.6. HRMS calculated for $C_{26}H_{27}NO_3$ [$(M-H)^+$]: 400.1913; found: 400.1937. No NMR reference data was available for this compound.

2-Ethyl-1-(3-hydroxybenzyl)-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline (1a):[7] A mixture of 2-ethyl-1-(3-benzyloxybenzyl)-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline **2a** (5.88 g, 14.6 mmol), acetic acid (2.35 g, 33.89 mmol), Pd 10% on active charcoal (0.6 g) and dry methanol (100 mL) was stirred for 19 h under H_2 atmosphere at room temperature. Then the solution was filtered through celite and washed with methanol (100 mL). The solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 (50 mL) and washed with half-saturated $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4 and the evaporation of the solvent under reduced pressure gave 4.42 g (96%) of 2-ethyl-1-(3-hydroxybenzyl)-6,7methylenedioxy-1,2,3,4-tetrahydroisoquinoline as a white solid. Mp = 186-189°C. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.33. ^{1}H-NMR (d-DMSO, 300 MHz): δ = 0.88 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.33 – 2.46 (2H, m, CH_2), 2.64 – 2.74 (3H, m, CH_2), 2.81 – 2.88 (1H, m, CH_2), 3.05 – 3.17 (1H, m, CH_2), 3.75 (1H, t, *J* = 6.3 Hz, CH), 5.88 (2H, d, *J* = 7.5 Hz, OCH_2O), 6.44 (1H, s, Ar), 6.54 – 6.59 (3H, m, Ar), 7.02 (1H, t, *J* = 7.8 Hz, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 13.5, 24.7, 41.8, 42.9, 47.1, 62.3, 100.7, 108.2, 108.6, 113.0, 116.9, 120.6, 127.8, 128.9, 128.9, 131.6, 137.2, 142.1, 145.3, 145.7, 157.3. HRMS calculated for $C_{19}H_{21}NO_3$ [$(M-H)^+$]: 310.1443; found: 310.1465. No NMR reference data was available for this compound.

3.2.2 Synthesis of 2-ethyl-1-(3-hydroxy)-6-methoxy-1,2,3,4-tetrahydroisoquinoline (1b)



Scheme 50: Synthesis of substrate **1b**

(3-Methoxy)phenylacetyl chloride (6b): A solution of (3-methoxy)phenyl-acetic acid **7b** (3.33 g, 20.0 mmol), oxalyl chloride (7.54 g, 59.4 mmol) and five drops of DMF in dry toluene was stirred at room temperature for two hours. The solvent was evaporated under reduced pressure to give 5.41 g (quant.) of a yellowish liquid. (3,4-methylenedioxy)phenylacetyl chloride was used in the following transformation without further purification. TLC (petrol ether/EtOAc = 1/1): R_f = 0.51. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.84 (3H, s, OCH_3), 4.14 (2H, s, $ArCH_2$), 6.84-6.93 (4H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 53.3, 55.3, 113.6, 115.2, 121.8, 125.3, 128.3, 129.1, 130.0, 132.5, 137.89. The NMR data is according to literature.[1]

(3-Methoxy)phenyl-*N*-ethylacetamide (5b):[2] (3-Methoxy)phenylacetyl chloride **6b** (5.41g, 29.4 mmol) was dissolved in CH_2Cl_2 (90 mL) and cooled to 0°C on an ice bath. To the solution a mixture of ethylamine (70% in H2O; 4.89 g, 76.0 mmol) and 2 M NaOH (13 mL) was added dropwise over 1h. Afterwards the ice bath was removed and the reaction mixture was stirred at room temperature overnight. Then the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 30 mL). The combined organic phases were washed with 2 N HCl solution (2 x 40 mL) and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 3.84 g (68%) of the amide **5b** as a slightly yellowish solid. Mp: 63-66°C. TLC (petrol ether/EtOAc = 1/1): R_f = 0.18. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.07 (3H, t, J_1 = 7.5 Hz, J_2 = 7.2 Hz, $NCH_2\mathit{CH_3}$), 3.20-3.29 (2H, m, $N\mathit{CH_2}CH_3$), 3.54 (2H, s, $ArCH_2$), 3.82 (3H, s, OCH_3), 5.49 (1H, bs, NH), 6.81 (H, d, J = 1.2 Hz, Ar), 6.83 (1H, s, Ar), 6.85 (1H, d, J = 1.8 Hz, Ar), 7.29 (1H, t, J_1 = 7.5 Hz, J_2 = 8.1 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 14.7, 34.5, 43.9, 55.2, 112.9, 115.1, 121.7, 130.0, 136.5, 160.0, 170.7. MS (EI, 70 eV): m/z = 193 (M, 38), 122 (100), 107 (26), 91 (23), 78 (11), 72 (14), 44 (14). HRMS calculated for $C_{11}H_{15}NO_2$ [$(M)^+$]: 193,1103; found: 193.1127. No NMR reference data is available.

***N*-Ethyl-(3-methoxy)phenethylamine (4b)**:[2] To a solution of (3-methoxy)phenyl-*N*-ethylacetamide **5b** (3.77 g, 19.5 mmol) in anhydrous THF (100 mL, BH_3*THF (1.0 M in THF; 100 mL, 100 mmol)) was added. The mixture was refluxed for 19 h under inert atmosphere. After cooling to room temperature 6 N HCl solution (20 mL) was added cautiously and stirring was continued for half an hour. Then the mixture was concentrated under reduced pressure. The residue was basified by addition of 2 M NaOH solution (100 mL) and saturated with NaCl. Subsequently the mixture was extracted with EtOAc (5 x 40 mL) and the combined organic phases were washed with brine and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 3.60 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 90/9/1) afforded *N*-ethyl-(3,4-methylenedioxy)phenethylamine 3.13 g (90%) as a pale yellowish liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.29. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.08 (3H, t, J = 7.2 Hz, $NCH_2\mathit{CH_3}$), 1.32 (1H, bs, NH), 2.62 – 2.69 (2H, dd, J_1 = 7.2 Hz, J_2 = 6.9 Hz, $N\mathit{CH_2}CH_3$), 2.78 (2H, t, J_1 = 6.6 Hz, $ArCH_2$), 2.85 – 2.90 (2H, m, $ArCH_2\mathit{CH_2}$), 3.78 (3H, s, OCH_3), 6.72 – 6.81 (3H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 15.3, 36.5, 44.1, 51.0, 55.2, 111.5, 114.5, 121.2, 129.5, 141.8, 159.8. HRMS calculated for $C_{11}H_{17}NO$ [$(M)^+$]: 179.1310; found: 179.1321. No NMR reference data is available.

2-(3-Benzyloxyphenyl)-*N*-(3-methoxyphenethyl)-*N*-ethylacetamide (3b):[5] A solution of *N*-Ethyl-(3-methoxy)phenethylamine **4b** (2.87 g, 16.1 mmol), $CHCl_3$ (30 mL) and 3% NaOH solution (150 mL) was cooled to 0°C on an ice bath. Then a solution of 3-benzyloxyphenylacetyl chloride **4b** (6.54 g, 25.09 mmol) in $CHCl_3$ (30 mL) was added dropwise over one hour. Afterwards the ice bath was removed und stirring was continued for 16 h at room temperature. The phases were separated and the aqueous phase was extracted with $CHCl_3$ (3 x 50 mL). The combined organic phases were washed with 2M HCl (100 mL), water (100 mL) and dried over Na_2SO_4. Then the solvent was evaporated under reduced pressure to give 10.7 g of a dark yellowish liquid. Flash chromatography (silica; petrol ether/EtOAc = 2/1) afforded 2-(3-benzyloxyphenyl)-*N*-(3-methoxyphenethyl-*N*-ethylacetamide (6.3 g, 97%) as a pale yellowish liquid. TLC (petrol ether/EtOAc = 2/1): R_f = 0.25. NMR spectroscopy reveals that the product is a mixture of isomers (ratio *trans/cis* = 1.5/1). Based on the peak intensities as well as the DEPT, COSY and HSQC spectra, the NMR signals can be assigned to the isomers as follows:

trans-2-(3-benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 0.96 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.75 (2H, t, *J* = 7.8 Hz, $ArCH_2$), 3.07 – 3.14 (2H, dd, J_1= 6.9 Hz, J_2= 7.2 Hz, $N\mathit{CH_2}CH_3$), 3.38 – 3.46 (2H, m, $ArCH_2\mathit{CH_2}N$), 3.46 (2H, s, $OCCH_2Ar$), 3.67 (3H, s, OMe), 4.97 (2H, s, OCH_2Ph), 6.54 – 6.85 (6H, m, Ar), 7.06 – 7.36 (7H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 14.1, 34.1, 40.9, 43.5, 47.7, 69.9, 100.8, 108.2, 109.2, 113.2, 115.3, 121.4, 121.7, 127.5, 128.6, 129.6, 131.9, 133.1, 136.8, 137.0, 145.9, 147.6, 159.0. No NMR reference data was available for this compound.

cis-2-(3-benzyloxyphenyl)-*N*-(3,4-methylenedioxy)phenethyl-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.06 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.58 (2H, t, *J* = 7.8 Hz, $ArCH_2$), 3.32 – 3.38 (2H, m, $N\mathit{CH_2}CH_3$), 3.32-3.38 (2H, m, $ArCH_2\mathit{CH_2}N$), 3.38 (2H, s, $OCCH_2Ar$), 3.69 (3H, s, OMe), 4.94 (2H, s, OCH_2Ph), 6.54 – 6.85 (6H, m, Ar), 7.06 – 7.36 (7H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ =12.8, 35.3, 40.6, 41.1, 49.4, 69.4, 100.9, 108.5, 109.1, 113.3, 115.0, 121.3, 121.6, 127.9, 128.6, 129.6, 131.9, 133.1, 136.8, 136.9, 146.3, 147.9, 159.1. No NMR reference data was available for this compound.

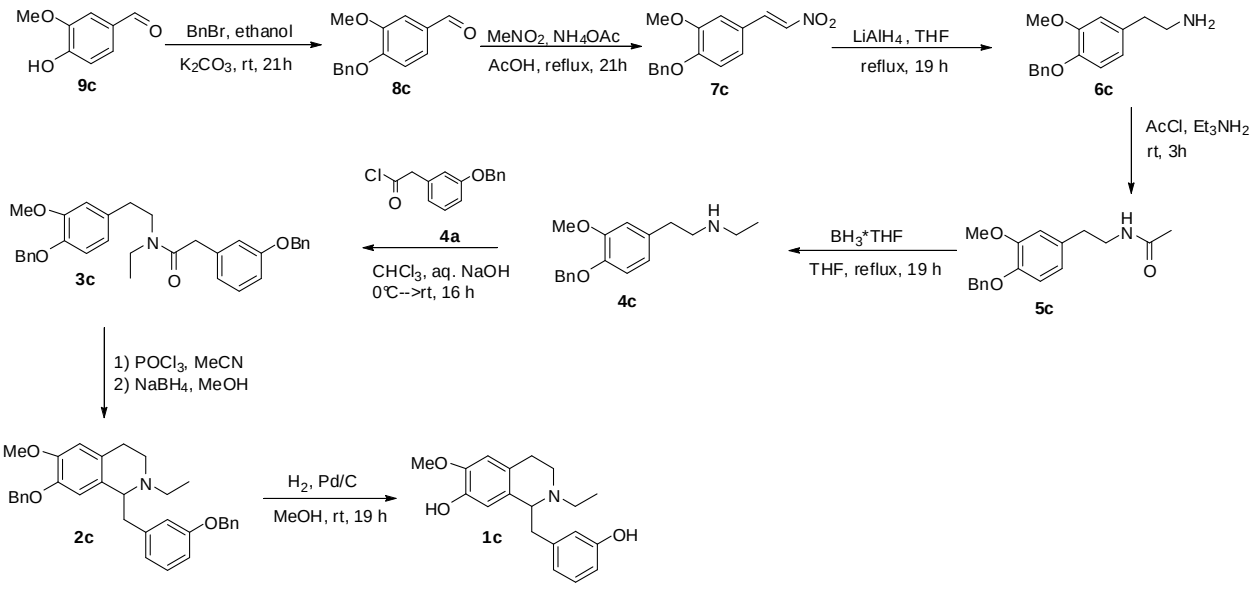
HRMS calculated for $C_{26}H_{29}NO_3$ [$(M)^+$]: 403.2148; found: 403.2154.

2-Ethyl-1-(3-benzyloxybenzyl)-6-methoxy-1,2,3,4-tetrahydroisoquinoline (2b):[6] A solution of 2-(3-benzyloxyphenyl)-*N*-(3-methoxyphenethyl)-*N*-ethylacetamide (6.45 g, 16.0 mmol), dry acetonitrile (150 mL) and $POCl_3$ (7.83 g, 51.2 mmol) was refluxed for 4 h under argon atmosphere. The solvent and the excess of $POCl_3$ were evaporated under reduced pressure and the residue was taken up in methanol (100 mL). The resulting mixture was put under argon and cooled to -5°C on an ice/NaCl bath. Afterwards $NaBH_4$ (3.35 g, 88.5 mmol) was added in portions to the stirred mixture. The cooling bath was removed and stirring was continued for 14 h at room temperature under argon atmosphere. Then the solvent was evaporated under reduced pressure and the residue was taken up in half-saturated Na_2CO_3 solution (100 mL). The aqueous phase was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic phases were dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 6.59 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1) afforded 2-ethyl-1-(3-benzyloxybenzyl)-6-methoxy1,2,3,4-

tetrahydroisoquinoline (5.84 g, 94%) of a slightly yellowish liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.29. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.10 (3H, t, *J* = , NCH_2CH_3), 2.56 – 2.72 (3H, m, CH_2), 2.76 – 2.98 (3H, m, CH_2), 3.08 – 3.26 (2H, m, CH_2), 3.78 (3H, s, OCH_3), 3.91 (1H, t, *J* = 6.6 Hz, CH), 5.04 (2H, s, OCH_2Ph), 6.59 – 6.63 (3H, m, Ar), 6.74 – 6.79 (2H, m, Ar), 6.83 – 6.87 (1H, dd, J_1 = 2.4 Hz, J_2 = 8.1 Hz, Ar), 7.19 (1H, t, *J* = 7.8 Hz, Ar), 7.34 – 7.47 (5H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 13.2, 25.8, 41.5, 43.3, 47.6, 55.1, 61.9, 69.9, 111.5, 112.3, 113.2, 116.3, 122.5, 127.5, 127.8, 128.5, 128.9, 129.3, 130.1, 135.7, 137.2, 141.8, 157.7, 158.6. HRMS calculated for $C_{26}H_{29}NO_2$ [(M-H)$^+$]: 386.2120; found: 386.2130. No NMR reference data was available for this compound.

2-Ethyl-1-(3-hydroxy)-6-methoxy-1,2,3,4-tetrahydroisoquinoline (1b):[7] A mixture of 2-ethyl-1-(3-benzyloxybenzyl)-6-methoxy-1,2,3,4-tetrahydroisoquinoline **2b** (5.76 g, 14.8 mmol), acetic acid (2.02 g, 33.6 mmol), Pd 10% on active charoal (0.6 g) and dry methanol (100 mL) was stirred for 19 h under H_2 atmosphere under room temperature. Then the solution was filtered through celite and washed with methanol (100 mL). The solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 (50 mL) and washed with half-saturated $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4 and the evaporation of the solvent under reduced pressure gave 4.04 g (91%) of 2-ethyl-1-(3-hydroxy)-6-methoxy-1,2,3,4-tetrahydroisoquinoline as a yellowish solid. Mp = 139-142°C. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.35. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.17 (3H, t, *J* = 7.2 Hz, NCH_2CH_3), 2.62 – 2.79 (4H, m, CH_2), 2.93 – 2.30 (2H, m, CH_2), 3.17 – 3.38 (2H, m, CH_2), 3.76 (3H, s, OMe), 4.02 (1H, dd, J_1 = 8.2 Hz, J_2 = 4.7 Hz, CH), 6.55 (1H, dd, J_1 = 7.0, J_2 = 4.3 Hz, Ar), 6.78 – 6.59 (1H, m, Ar), 7.10 (1H, t, *J* = 7.7 Hz, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 12.6, 24.5, 41.9, 42.8, 47.1, 55.2, 61.7, 111.2, 113.4, 113.7, 116.4, 121.7, 129.0, 129.4, 134.6, 141.1, 156.5, 157.9. HRMS calculated for $C_{19}H_{23}NO_2$ [(M-H)$^+$]: 296.1650; found: 296.1662.No NMR reference data was available for this compound.

3.2.3 Synthesis of 2-ethyl-6-methoxy-7-hydroxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinolin (1c)



Scheme 51: Synthesis of substrate **1c**

4-Benzyloxy-3-methoxybenzaldehyd (8c):[8] Vanillin (20.2 g, 131 mmol) was dissolved in ethanol (120 mL) and the solution was put under argon. K_2CO_3 (20.5 g, 146 mmol) as well as benzyl bromide (20.7 g, 10.1 mmol) was added to the mixture and stirring was continued for 21 h at room temperature under argon atmosphere. Then the solution was filtered and washed with CH_2Cl_2 (400 mL) and the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 (200 mL), washed with 5% NaOH solution (100 mL) and dried over K_2CO_3. Evaporation under reduced pressure yielded 33.4 g yellowish solid which was recrystalliesed from ethanol to give 27.5 g (87%) of 4-benzyloxy-3-methoxybenzaldehyd. Mp: 67-69°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 3.96 (3H, s, OCH_3), 5.27 (2H, s, $PhCH_2O$), 7.01 (1H, d, *J* = 8.1 Hz, Ar), 7.31 – 7.48 (7H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 56.1, 70.8, 109.3, 112.4, 126.6, 127.2, 128.2, 128.7, 130.3, 136.0, 150.0, 153.6, 190.9. The NMR data is according to literature.[8]

4-Benzyloxy-3-methoxy-β-nitrostyrene (7c):[8] A solution of 4-benzyloxy-3-methoxybenzaldehyd **8c** (27.3 g, 112.6 mmol), ammonium acetate (21.9 g, 28.2 mmol), nitromethane (22.2 g, 36 mmol) in acetic acid (250 mL) was refluxed for 21 h. The mixture was allowed to cool to approximately 60°C and was then poured onto ice water (500 mL). The product precipitated and CH_2Cl_2 (300 mL) was added until the whole precipitate was dissolved. Afterwards the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 80 mL). The combined organic phases were washed with water (200 ml), half-saturated Na_2CO_2 solution (200 mL), brine (200 mL) and dried over Na_2SO_4. The solvent was evaporated and the residue was recrystallized from ethanol (700 mL) to give 4-benzyloxy-3-methoxy-β-nitrostyrene as a yellowish solid (18.98 g, 59%). Mp = 120-123°C. ^{1}H-NMR

($CDCl_3$, 300 MHz): δ = 3.94 (3H, s, OMe), 5.23 (2H, s, OCH_2Ph), 6.90 – 9.95 (1H, m, Ar), 7.04 (1H, d, *J* = 2.1 Hz, Ar), 7.11 (1H, dd, J_1 = 2.1 Hz, J_2 =8.4 Hz, Ar), 7.32 – 7.46 (5H, m, Ar), 7.53 (1H, d, *J* = 13.8 Hz, *CH*=CH-NO_2), 7.96 (1H, s, *J* = 13.5 Hz, CH=*CH*-NO_2). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 56.1, 70.9, 113.5, 114.4, 123.1, 124.4, 126.3, 127.2, 128.2, 128.3, 128.7, 135.3, 136.1, 139.3, 150.0, 151.9. MS (EI, 70 eV): m/z = 285 (M, 7), 91 (100), 65 (7). The NMR data is according to literature.[8]

4-Benzyloxy-3-methoxy-phenethylamine (6c):[9] To a suspension of $LiAlH_4$ (6.36 g, 167.5 mmol) in anhydrous THF (120 mL) a solution of 4-benzyloxy-3-methoxy-β-nitrostyrene **7c** (18.3 g, 64.8 mmol) in dry THF (180 mL) was added dropwise over 1h under argon atmosphere. Then the mixture was refluxed for 19 h under argon atmosphere. Afterwards the suspension was allowed to cool to room temperature and water (13 mL), 15% NaOH solution (13 mL) and again water (39 mL) was added cautiously to deactivate the residues of $LiAlH_4$. After the addition, the mixture was stirred for 1 h at room temperature. The suspension was filtered through celite and the filter cake was washed with THF (3 x 100 mL). The solvent was evaporated under reduced pressure and the residue was taken up in 10% HCl solution (40 mL) and washed with ether (60 mL). The aqueous phase was basified with 2 M NaOH solution and extracted with ethyl acetate (3 x 50 mL). The combined organic phases were washed with water (100 mL), brine (100 mL) and dried over K_2CO_3. The solvent was evaporated and 4-benzyloxy-3-methoxy-phenethylamine (11.03 g, 65%) was obtained as a brown-yellowish solid. Mp = 70-73°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.55 (2H, bs, NH_2), 2.69 (2H, t, *J* = 6.6 Hz, CH_2-CH_2-NH_2), 2.94 (2H, t, *J* = 6.9 Hz, CH_2-CH_2-NH_2), 3.90 (3H, s, OMe), 5.23 (2H, s, OCH_2Ph), 6.68 (1H, dd, J_1 = 8.9 Hz, J_2 = 8.1 Hz, Ar), 6.76 (1H, d, *J* = 2.1 Hz, Ar), 6.83 (1H, d, *J* = 8.1 Hz, Ar), 7.28 – 7.47 (5H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 56.1, 70.9, 113.5, 114.4, 123.1, 124.4, 126.3, 127.2, 128.2, 128.3, 128.7, 135.3, 136.1, 139.3, 150.0, 151.9. MS (EI, 70 eV): m/z = 257 (M^+, 7), 228 (40), 137 (75), 91 (100), 65 (7). The NMR data is according to literature.[8]

***N*-(3-methoxy-4-phenoxyphenethyl)acetamide (5c):** A solution of 4-benzyloxy-3-methoxy-phenethylamine **6c** (12.7 g, 49.0 mmol), acetyl chloride (4.64 g, 59.0 mmol), triethylamine (6.02 g, 59.0 mmol) in CH_2Cl_2 was stirred at room temperature for 3 h. Then water (100 mL) was added, the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. *N*-(3-methoxy-4-phenoxyphenethyl)acetamide (14.8 g, 98%) was obtained as a yellowish solid. Mp = 89-92°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.95 (3H, s, CH_3-CO-N), 2.75 (2H, t, *J* =7.2 Hz, CH_2-CH_2-NH), 3.48 (2H, dd, J_1 = 6.9 Hz, J_2 = 12.9 Hz, CH_2-CH_2-NH), 3.89 (3H, s, OMe), 5.30 (2H, s, OCH_2Ph), 6.66 (1H, dd, J_1 = 8.9 Hz, J_2 = 8.1 Hz, Ar), 6.74 (1H, d, *J* = 1.8 Hz, Ar), 6.83 (1H, d, *J* = 8.1 Hz, Ar), 7.28 – 7.47 (5H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 23.3, 40.7, 45.8, 56.0, 71.7, 112.5, 114.4, 120.6, 127.3, 127.8, 128.5, 132.0, 137.3, 146.8, 149.8, 170.3. HRMS calculated for $C_{18}H_{21}NO_3$ [$(M)^+$]: 299.1521; found: 299.1543.

***N*-ehyl-2-(3-methoxy-4-phenoxyphenyl)ethanamine (4c):**[2] To a solution of *N*-(3-methoxy-4-phenoxyphenethyl)acetamide **5c** (12.08 g, 40.0 mmol) in anhydrous THF (120 mL), BH_3*THF (1.0 M in THF; 200 mL, 200 mmol) was added. The mixture was refluxed for 19 h

under inert atmosphere. After cooling to room temperature 6 N HCl solution (40 mL) was added cautiously and stirring was continued for half an hour. Then the mixture was concentrated under reduced pressure. The residue was basified by addition of 2 M NaOH solution (200 mL) and saturated with NaCl. Subsequently the mixture was extracted with EtOAc (3 x 100 mL) and the combined organic phases were washed with brine and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 12.5 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1) afforded *N*-ethyl-2-(3-methoxy-4-phenoxyphenyl)ethanamine 7.14 g (63%) as a pale yellowish liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.25. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.09 (3H, t, *J* = 7.2 Hz, NH-CH_2*CH_3*), 2.66 (2H, dd, J_1 =7.2 Hz, J_2 = 14.1 Hz, NH-*CH_2*CH_2), 2.76 (2H, d, *J* = 6.3 Hz, Ph*CH_2*CH_2NH), 2.86 (2H, t, *J* = 6.0 Hz, PhCH_2*CH_2*NH), 3.88 (3H, s, OMe), 5.12 (2H, s, PhOCH_2), 6.68 (1H, dd, J_1 = 8.9 Hz, J_2 = 8.1 Hz, Ar), 6.76 (1H, d, *J* = 1.8 Hz, Ar), 6.82 (1H, d, *J* = 8.1 Hz, Ar), 7.28 – 7.38 (5H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 15.2, 35.9, 44.0, 51.1, 55.9, 71.2, 112.6, 114.3, 120.6, 127.3, 127.7, 128.5, 133.3, 137.4, 146.8, 149.8. HRMS calculated for $C_{18}H_{23}NO_2$ [$(M)^+$]: 285.1729; found: 285.1723.

***N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)phenyl)-*N*-ethylacetamide (3c):**[5]
A solution of *N*-ethyl-2-(3-methoxy-4-phenoxyphenyl)ethanamine (3.62 g, 12.7 mmol), $CHCl_3$ (40 mL) and 3% NaOH solution (150 mL) was cooled to 0°C on an ice bath. Then a solution of 3-benzyloxyphenylacetyl chloride **4a** (7.64 g, 29.3 mmol) in $CHCl_3$ (40 mL) was added dropwise over one hour. Afterwards the ice bath was removed und stirring was continued for 16 h at room temperature. The phases were separated and the aqueous phase was extracted with $CHCl_3$ (3 x 50 mL). The combined organic phases were washed with 2 M HCl (100 mL), water (100 mL) and dried over Na_2SO_4. Then the solvent was evaporated under reduced pressure to give 10.3 g of a dark yellowish liquid. Flash chromatography (silica; petrol ether/EtOAc = 2/1) afforded *N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)phenyl)-*N*-ethylacetamide (4.12 g, 64%) as a pale yellowish liquid. TLC (petrol ether/EtOAc = 1/1): R_f = 0.48. NMR spectroscopy reveals that the product is a mixture of isomers (ratio *trans/cis* = 1.5/1). Based on the peak intensities as well as the DEPT, COSY and HSQC spectra, the NMR signals can be assigned to the isomers as follows:

trans-*N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)phenyl)-*N*-ethylacetamide:
^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.06 (3H, t, *J* = 7.2 Hz, NCH_2*CH_3*), 2.81 (2H, t, *J* = 7.5 Hz, $ArCH_2$), 3.19 (2H, dd, J_1 = 7.2 Hz, J_2 = 14.4 Hz, N*CH_2*CH_3), 3.39 – 3.54 (2H, m, $ArCH_2$*CH_2*N), 3.54 (2H, s, $OCCH_2Ar$), 3.83 (3H, s, OMe), 5.08 (2H, s, OCH_2Ph), 5.13 (2H, s, OCH_2Ph), 6.59 (1H, dd, J_1 = 3.0 Hz, J_2 = 8.1, Ar), 6.65 (1H, dd, J_1 = 1.8 Hz, J_2 = 8.1 Hz, Ar), 6.67 – 6.95 (5H, m, Ar), 7.21 – 7.46 (10H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 14.1, 33.7, 41.1, 43.5, 47.9, 71.2, 112.6, 113.2, 114.3, 115.3, 127.5, 127.7, 127.9, 128.4, 128.5, 129.6, 131.5, 132.6, 136.9, 137.3, 147.6, 149.7, 159.1. No NMR reference data was available for this compound.

cis-*N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)phenyl)-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.17 (3H, t, *J* = 7.2 Hz, NCH_2*CH_3*), 2.65 (2H, t, *J* = 7.2 Hz, $ArCH_2$), 3.39 – 3.54 (2H, m, N*CH_2*CH_3), 3.39 – 3.54 (2H, m, $ArCH_2$*CH_2*N), 3.45 (2H, s, $OCCH_2Ar$), 3.87 (3H, s, OMe), 5.05 (2H, s, OCH_2Ph), 5.14 (2H, s, OCH_2Ph), 6.59 (1H, dd,

J_1 = 3 Hz, J_2 = 8.1, Ar), 6.65 (1H, dd, J_1 = 1.8 Hz, J_2 = 8.1 Hz, Ar), 6.67 – 6.95 (5H, m, Ar), 7.21 – 7.46 (10H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ =12.8, 34.9, 40.6, 40.9, 49.6, 69.4, 112.6, 113.2, 114.5, 115.1, 127.3, 127.8, 127.9, 128.5, 128.5, 129.6, 131.5, 132.6, 136.9, 137.2, 147.0, 149.8, 159.1. No NMR reference data was available for this compound.

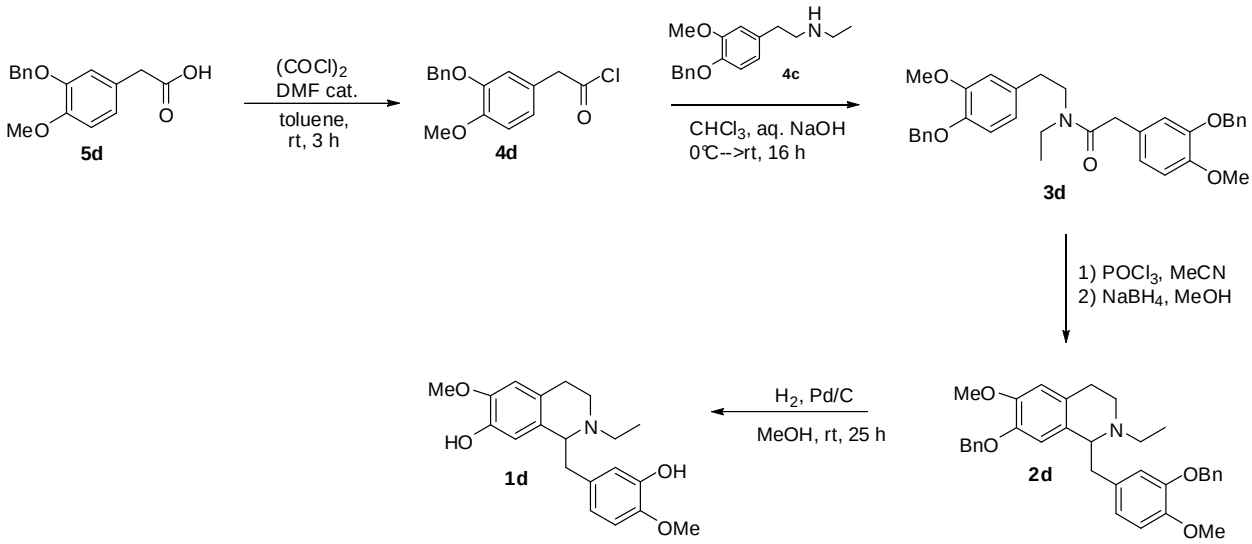
HRMS calculated for $C_{33}H_{35}NO_4$ [$(M)^+$]: 509.2566; found: 509.2597.

2-Ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)benzyl)-1,2,3,4-tetrahydroisoquinoline (2c):[6] A solution of *N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)phenyl)-*N*-ethylacetamide **3c** (4.12 g, 8.09 mmol), dry acetonitrile (100 mL) and $POCl_3$ (4.04 g, 25.09 mmol) was refluxed for 4 h under argon atmosphere. The solvent and the excess of $POCl_3$ were evaporated under reduced pressure and the residue was taken up in methanol (80 mL). The resulting mixture was put under argon and cooled to -5°C on an ice/NaCl bath. Afterwards $NaBH_4$ (1.70 g, 44.9 mmol) was added in portions to the stirring mixture. The cooling bath was removed and stirring was continued for 16 h at room temperature under iner argon atmosphere. Then the solvent was evaporated under reduced pressure and the residue was taken up in half-saturated Na_2CO_3 solution (100 mL). The aqueous phase was extracted with CH_2Cl_2 (4 x 100 mL) and the combined organic phases were dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 4.55 g of a red liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1) afforded 2-ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)benzyl)-1,2,3,4-tetrahydroisoquinoline (3.43 g, 86%) of a slightly orange liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.28. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.13 (3H, t, J = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.52 – 2.66 (1H, m, CH_2), 2.69 – 2.75 (3H, m, CH_2), 2.85 – 2.94 (2H, m, CH_2), 3.09 – 3.20 (2H, m, CH_2), 3.82 (3H, s, OMe), 4.78 (1H, dd, J_1 = 12 Hz, J_2 = 6.5 Hz, 5.02 (4H, s, 2 x OCH_2Ph), 6.05 (1H, s, Ar), 6.61 (1H, s, Ar), 6.71 (1H, d, J = 7.5 Hz, Ar), 6.77 (1H, t, J = 2.1 Hz, Ar), 6.86 (1H, dd, J_1 = 1.8 Hz, J_2= 8.1 Hz, Ar), 7.20 (1H, t, J = 7.8 Hz), 7.27 – 7.42 (10H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 13.2, 25.3, 40.9, 43.4, 47.6, 55.9, 55.9, 62.6, 69.3, 70.8, 111.8, 112.3, 114.1, 116.5, 122.7, 126.7, 127.3, 127.5, 127.6, 127.9, 128.4, 128.5,129.1, 129.5, 137.1, 137.3, 141.9, 145.5, 148.0, 158.7. HRMS calculated for $C_{33}H_{35}NO_3$ [$(M-H)^+$]: 492.2539; found: 492.2575. No NMR reference data was available for this compound.

2-Ethyl-6-methoxy-7-hydroxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (1c):[7] A mixture of 2-ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)benzyl)-1,2,3,4-tetrahydroisoquinoline **2c** (3.03 g, 6.13 mmol), acetic acid (1.22 g, 20.3 mmol). Pd 10% on activated charcoal (0.53 g) and dry methanol (80 mL) was stirred for 25 h under H_2 atmosphere at room temperature. Then the solution was filtered through celite and washed with methanol (200 mL). The solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 (60 mL) and washed with half-saturated $NaHCO_3$ solution (150 mL). The organic phase was dried over Na_2SO_4 and the evaporation of the solvent under reduced pressure gave 1.95 g (100%) of 2-ethyl-6-methoxy-7-hydroxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline as a yellowish solid. Mp = 76-80°C. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.33. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.01 (3H, t, J = 7.2 Hz. $NCH_2\mathit{CH_3}$), 2.42 – 2.90 (6H, m, CH_2), 3.01 (1H, dd, J_1 = 5.4 Hz, J_2 = 13.5 Hz, CH_2), 3.12 – 3.17 (1H, m, CH_2), 3.79 (3H, s, OMe), 4.04 (1H, dd, J_1 = 9.0. J_2 = 15.0 Hz, CH), 5.41 (2H, bs, 2 x OH), 6.11 (1H,

s, Ar), 6.45 (1H, s, Ar), 6.46 (1H, t, J = 7.8 Hz), 6.57 (1H, dd. J_1 = 5.7 Hz, J_2 = 13.8 Hz, Ar), 6.98 (1H, t, J = 7.8 Hz, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 12.5, 24.0, 41.5, 42.8, 47.1, 55.8, 61.9, 110.8, 113.6, 114.3, 116.5, 121.5, 124.7, 129.2, 129.7, 141.3, 143.3, 145.6, 156.4. HRMS calculated for $C_{19}H_{23}NO_3$ [$(M-H)^+$]: 321.1600; found: 312.1615. No NMR reference data was available for this compound.

3.2.4 Synthesis of 2-ethyl-6-methoxy-7-hydroxy-1(-3-hydroxy-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (1d)



Scheme 52: Synthesis of substrate **1d**

2-(3-(Benzyloxy)-4-methoxyphenyl)acetyl chloride (4d): A solution of 2-(3-(benzyloxy)-4-methoxyphenyl)acetic acid **5d** (4.39 g, 16.0 mmol), oxalyl chloride (4.65 g, 36.0 mmol) and five drops of DMF in dry toluene (120 mL) was stirred at room temperature for three hours. The solvent was evaporated under reduced pressure to give 5.34 g (quant.) of an orange liquid. 2-(3-(Benzyloxy)-4-methoxyphenyl)acetyl chloride was used in the following transformation without further purification. TLC (petrol ether/EtOAc = 1/1): R_f = 0.63.

***N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)-4-methoxyphenyl)-*N*-ethylacetamide (3d):** A solution of *N*-ethyl-2-(3-methoxy-4-phenoxyphenyl)ethanamine **4c** (3.52 g, 12.3 mmol), $CHCl_3$ (40 mL) and 3% NaOH solution (150 mL) was cooled to 0°C on an ice bath. Then a solution of 2-(3-(benzyloxy)-4-methoxyphenyl)acetyl chloride **4d** (5.34 g, 18.3 mmol) in $CHCl_3$ (100 mL) was added dropwise during one hour. Afterwards the ice bath was removed und stirring was continued for 16 h at room temperature. The phases were separated and the aqueous phase was extracted with $CHCl_3$ (3 x 50 mL). The combined organic phases were washed with 2 M HCl (100 mL), water (100 mL) and dried over Na_2SO_4. Then the solvent was evaporated under reduced pressure to give 8.93 g of an orange liquid. Flash chromatography (silica; petrol ether/EtOAc = 2/1) afforded *N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)-4-methoxyphenyl)-*N*-ethylacetamide (4.12 g, 64%) as a

pale yellowish liquid. TLC (petrol ether/EtOAc = 1/1): R_f = 0.44. NMR spectroscopy reveals that the product is a mixture of isomers (ratio *trans/cis* = 1.5/1). Based on the peak intensities as well as the DEPT, COSY and HSQC spectra, the NMR signals can be assigned to the isomers as follows:

trans-N-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)-4-methoxyphenyl)-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 0.90 (3H, t, *J* = 7.2 Hz, $NCH_2\mathit{CH_3}$), 2.66 (2H, t, *J* = 7.5 Hz, $ArCH_2$), 3.04 (2H, dd, J_1 = 7.2 Hz, J_2 = 14.4 Hz, $N\mathit{CH_2}CH_3$), 3.37 (2H, dd, J_1 = 6.0 Hz, J_2 = 7.8 Hz, $ArCH_2\mathit{CH_2}N$), 3.28 (2H, s, $OCCH_2Ar$), 3.73 (6H, s, 2 x OMe), 5.03 (4H, s, 2 x OCH_2Ph), 6.50 (1H, dd, J_1 = 1.8 Hz, J_2 = 7.1 Hz, Ar), 6.54 (1H, dd, J_1 = 1.8 Hz, J_2 = 8.1 Hz, Ar), 6.63 – 6.79 (5H, m, Ar), 7.18 – 7.37 (10H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 14.1, 33.7, 41.1, 43.3, 47.8, 56.1, 71.2, 70.9, 112.0, 114.3, 114.7, 114.3, 120.7, 127.4, 127.7, 128.5, 131.5, 132.6, 137.1, 146.6, 148.2, 148.6, 170.6. No NMR reference data was available for this compound.

cis-N-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)-4-methoxyphenyl)-*N*-ethylacetamide: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.03 (3H, t, *J* = 7.2, $NCH_2\mathit{CH_3}$), 2.51 (2H, t, *J* = 7.2, $ArCH_2$), 3.29 (2H, dd, J_1 = 7.5 Hz, J_2 = 13.5 Hz, $N\mathit{CH_2}CH_3$), 3.29 (2H, dd, J_1 = 7.5 Hz, J_2 = 13.5 Hz, $ArCH_2\mathit{CH_2}N$), 3.51 (2H, s, $OCCH_2Ar$), 3.77 (6H, s, 2 x OMe), 5.01 (2H, s, OCH_2Ph), 5.03 (2H, s, OCH_2Ph), 6.50 (1H, dd, J_1 = 1.8 Hz, J_2 = 7.1, Ar), 6.54 (1H, dd, J_1 = 1.8 Hz, J_2 = 8.1 Hz, Ar), 6.63 – 6.79 (5H, m, Ar), 7.18 – 7.37 (10H, m, Ar).^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 12.8, 34.9, 40.6, 40.9, 49.4, 56.1, 70.9, 70.8, 112.6, 114.4, 114.5, 120.7, 121.3, 127.3 113.2, 114.5, 115.1, 127.3, 127.7, 128.5, 131.5, 132.6, 137.3, 146.9, 148.3, 148.6, 149.8, 170.6. No NMR reference data was available for this compound.

HRMS calculated for $C_{34}H_{37}NO_5$ [$(M)^+$]: 539.2672; found: 539.2696.

2-Ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)-4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (2d):[6] A solution of *N*-(4-(benzyloxy)-3-methoxyphenethyl)-2-(3-(benzyloxy)-4-methoxyphenyl)-*N*-ethylacetamide **3d** (4.85 g, 8.90 mmol), dry acetonitrile (100 mL) and $POCl_3$ (4.46 g, 28.7 mmol) was refluxed for 4 h under argon atmosphere. The solvent and the excess of $POCl_3$ were evaporated under reduced pressure and the residue was taken up in methanol (80 mL). The resulting mixture was put under argon and cooled to -5°C on an ice/NaCl bath. Afterwards $NaBH_4$ (1.87 g, 49.4 mmol) was added in portions to the stirring mixture. The cooling bath was removed and stirring was continued for 16 h at room temperature under argon atmosphere. Then the solvent was evaporated under reduced pressure and the residue was taken up in half-saturated Na_2CO_3 solution (100 mL). The aqueous phase was extracted with CH_2Cl_2 (4 x 100 mL) and the combined organic phases were dried over Na_2SO_4. Evaporation of the solvent under reduced pressure yielded 5.76 g of a yellowish liquid. Flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1) afforded 2-ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)-4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (4.35 g, 94%) as a strong yellow liquid. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.17. ^{1}H-NMR ($CDCl_3$, 300MHz): δ = 1.09 (3H, t, *J* = 6.9 Hz, $NCH_2\mathit{CH_3}$), 2.46 – 2.52 (1H, m, CH_2), 2.62 – 2.68 (3H, m, CH_2), 2.69 – 2.89 (2H, m, CH_2), 3.01 (1H, dd, J_1 = 5.4 Hz, J_2 = 13.5 Hz, CH_2), 3.09 – 3.16 (1H, m, CH_2) 3.86 (6H, s, 2 x OMe), 4.79 (1H, dd, J_1 = 12 Hz, J_2 = 19.5 Hz, CH), 5.09 (4H, s, 2 x OCH_2Ph), 5.99 (1H, s, Ar), 6.55 (1H, d, *J*

= 2.1 Hz, Ar) 6.58 (1H, s, Ar), 6.67 (1H, d, *J* = 2.1 Hz, Ar), 6.78 (1H, d, *J* = 8.1 Hz, Ar), 7.26 – 7.36 (11H, m, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 13.2, 25.3, 40.4, 43.4, 47.6, 55.9, 55.9, 56.1, 62.3, 70.8, 71.0, 111.5, 11.7, 114.0, 114.1, 116.0, 122.7, 126.8, 127.2, 127.3, 127.6, 127.7, 128.4, 128.5, 129.4, 132.6, 137.3, 145.5, 147.7, 147.9, 148.1. HRMS calculated for $C_{34}H_{37}NO_4$ [$(M-H)^+$]: 522.2644; found: 522.2649. No NMR reference data was available for this compound.

2-Ethyl-6-methoxy-7-hydroxy-1(-3-hydroxy-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (1d):[7] A mixture of 2-ethyl-6-methoxy-7-(benzyloxy)-1-(3-(benzyloxy)-4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline **2d** (4.13 g, 7.89 mmol), acetic acid (1.38 g, 23.06 mmol), Pd 10% on activated charcoal (0.67 g) and dry methanol (80 mL) was stirred for 25 h under H_2 atmosphere under room temperature. Then the solution was filtered through celite and washed with methanol (200 mL). The solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2 (60 mL) and washed with half-saturated $NaHCO_3$ solution (150 mL). The organic phase was dried over Na_2SO_4 and the evaporation of the solvent under reduced pressure gave 2.72 g (100 %) of 2-ethyl-6-methoxy-7-hydroxy-1(-3-hydroxy-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline as a yellowish solid. Mp = 66-68°C. TLC (CH_2Cl_2/MeOH/NH_4OH = 90/9/1): R_f = 0.21. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 0.96 (3H, t, *J* = 6 Hz, NCH_2CH_3), 2.37– 2.76 (5H, m, CH_2), 2.78 – 2.95 (3H, m, CH_2), 3.07-3.17 (1H, m, CH_2), 3.75 (3H, s, OMe), 3.78 (3H, s, OMe), 6.25 (1H, s, Ar), 6.45 (1H, s, Ar), 6.46 (1H, t, *J* = 7.8 Hz), 6.49 (1H, d, *J* = 9 Hz, Ar), 6.65 (1H, d, *J* = 9 Hz, Ar), 6.72 (1H, s, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 12.9, 24.6, 40.4, 43.3, 47.3, 55.9, 55.9, 61.8, 110.4, 110.7, 114.1, 115.8, 120.9, 125.4, 130.7, 133.5, 143.2, 145.0, 145.2, 145.3, HRMS calculated for $C_{20}H_{25}NO_4$ [$(M-H)^+$]: 342.1705; found: 342.1698. No NMR reference data was available for this compound.

3.3 Biotransformations

3.3.1 Procedure for the concentration study for substrate 1a, 1b and 1e

For this experiment three different substrate stock concentrations were prepared, dissolved in aceton, and provided in 4 mL glass vials: 1 g/L, 2 g/L and 4 g/L. Aceton was removed under constant air flow. The substrate was redissolved in the organic solvent: toluene (350 µL; 70% v/v) and DMSO (50 µL; 10% v/v). Then an enzyme stock was prepared composed of the BBE –W165F (1.04 mg/mL), catalse (5 mg/mL) in a Tris-HCl buffer (50 mM, pH 9, 10 mM $MgCl_2$). After mixing the samples with enzyme buffer to a end volume of 500 µL the vials were closed with screw caps containing sealing gaskets and the samples were shaken in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) with 400 rpm at 40°C for two and 2 and 24 hours, under the protection of light. Then the reaction mixture was extracted with ethyl acetate (2 x 500 mL) and the organic phase was dried over Na_2SO_4. The residual ethyl acetate was removed under constant air flow and the crude product was redissolved in HPLC grade methanol (800 µL).

3.3.2 Procedure for solvent studies for substrate 1a, 1b and 1e

A stock solution of the substrates in acetone (2 mg/ mL) was prepared and aliquots containing 1 mg of substrate were transferred into glass vials (4 mL). Aceton was removed under constant air flow. The substrate was redissolved in the respective organic solvent (50 µL). Then an enzyme stock solution was prepared containing the BBE variant (W165F. 1.04 mg/mL), catalase (5 mg/mL) in a Tris-HCl buffer (50 mM, pH 9, 10 mM $MgCl_2$). After addition of the enzyme buffer (450 µL) to the pre-dissolved substrate, the vials were closed with screw caps containing sealing gaskets and the samples were shaken in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) with 400 rpm at 40°C for two and twenty four hours. Then the reaction mixture was extracted with ethyl acetate (2 x 500 mL) and the organic phase was dried over Na_2SO_4. The residual ethyl acetate was removed under constant air flow and the crude product was redissolved in HPLC grade methanol (800 µL).

3.3.3 Procedure for the pH studies for substrate 1a, 1b and 1e

The experiments were performed similar as described above except that the pH value of the Tris-HCl buffer (50 mM + 10 mM $MgCl_2$) varied from 6 to 11, adjusted by the addition of HCl or NaOH. *N*-methyl formamide, formamide and DMSO were used as organic solvents for substrates **1a** and **1b** and for substrate **1e** formamide was employed in 10% v/v. Reaction conditions remained the same as described above: 2 g/L substrate, 1.04 g/L BBE- W165F, 5g/L catalase and the mixture was shaken for 2 and 24 hours at 40°C and 400 rpm under light-protected conditions. Also the extraction procedure with ethyl acetate did not change.

3.3.4 Procedure for the activity comparison between the wild type and his variant W165F for the substrates 1a, 1b and 1e

For this procedure the optimized conditions of the time, pH and the solvent studies were chosen to carry out the comparison experiments concerning the products to substrate ratios between the wild type of BBE and its variant. Therefore the experiments were performed as described above: 2 g/L substrate, 1.04 g/L BBE- W165F or 0.63 g/L BBE wild type, 5 g/L catalase and the mixture was shaken for 2 and 24 hours at 40°C and 400 rpm under light-protected conditions. Also the extraction procedure with ethyl acetate did not change and the residual ethyl acetate was removed under constant air flow. The crude product was redissolved in HPLC grade methanol (800 µL).

3.3.5 Procedure for co-solvent studies with toluene for 1a

Since substrates **1a** and **1b** showed solubility problems, different percentages of toluene were added to the reaction mixture containing also DMSO and the enzyme buffer. Toluene amounts from 35, 40, 45, 50 and 55% v/v were added. Substrate **1a** (2 g/L) was dissolved in 50 µL of DMSO and the enzyme buffer amount [10 mM Tris-HCl, pH 9, 10 mM $MgCl_2$ + BBE-W165 F (1.04 g/L), catalase of a final contration of 5 g/L] was adjusted to the volume of toluene added to the reaction mixture (final reaction volume: 500 µL). Shaking was carried out in 4 mL glass vials closed with screw caps containing sealing gaskets at 40°C and 400 rpm in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) under light-protected conditions. Samples were taken after 2 and 24 hours. Furthermore different agitation angles were tested in the presence of 35% v/v toluene for substrate **1a**. The angels were choosen at 0° and 45°. The other reaction conditions remained the same except the agitation speed was increased to 500 rpm. All samples were extracted with ethyl acetate (2 x 500 mL) and the organic phase was dried over Na_2SO_4. The residual ethyl acetate was removed under constant air flow and the crude product was redissolved in HPLC grade methanol (800 µL).

3.3.6 Variation of the solvent amount for substrate 1a

As already mentioned in 3.3.5, due to solubility problems of substrate **1a** and **1b** the amount of the organic solvent (DMSO, *N*-methyl formamide, formamide) was increased to 20% v/v. For the procedure the substrate (2 g/L) was dissolved in 100 µL organic solvent and 400 µL enzyme buffer [10 mM Tris-HCl, pH 9, 10 mM $MgCl_2$ + BBE-W165F (1.04 g/L), catalase of a final contration of 5 g/L] was added. Shaking was carried out in 4 mL glass vials closed with screw caps containing sealing gaskets at 40°C and 400 rpm in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) under light-protected conditions, with 0° (horizontal) and 45° agitation angle. Samples were taken after 2 and 24 hours. The same procedure was done with 10% v/v of the corresponding organic solvents. The extraction procedure for the samples remain the same as described above.

3.3.7 Procedure for the time study experiments for substrate 1e

The time study for substrate **1e** was carried out using the optimized conditions for the biotransformation with four different enzyme concentrations. Therefore the substrate (2 g/L) was dissolved in 50 µL formamide and 450 µL enzyme containing buffer [10 mM Tris-HCl, pH 9,10 mM $MgCl_2$ + BBE-W165 F (1.04 g/L. 0.52 g/L. 0.34 g/L. 0.20 g/L), catalase of a final contration of 5g/L]. Shaking was carried out in 4 mL glass vials closed with screw caps containing sealing gaskets at 40°C and 400 rpm in an Incubator Mini Shaker (VWR, rotary, orbit 3 mm) under light-protected conditions. Samples were taken after 0.5, 1, 2, 4, 6, 8, 12 and 24 hours. For extraction the reaction mixture was extracted in ethyl acetate (2 x 500 mL) and the organic phase was dried over Na_2SO_4.

3.3.8 Preparative transformation – Upscaling 1

Substrate **1e** (200 mg. 0.61 mmol. final concentration: 2 g/L = 6.1 mM) was dissolved in 10% v/v formamide (10 mL) and buffer (89.9 mL, 10 mM Tris-HCl, pH 9, 10 mM $MgCl_2$) with BBE-W165F (1.01 mL enzyme solution, final concentration: 1 g/ mL = 0.17 mM) and catalase (500 mg crude preparation). The reaction mixture was shaken in a light-shielded Erlenmeyer flask (300 mL) in a thermo shaker at 40°C and 120 rpm for 24 hours. Then the phases were extracted with ethyl acetate (4 x 100 mL) and the phases were separated in the centrifuge (4 x 4 minutes at 4000 rpm). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1). The purification yielded in 28.3 mg of (*R*)-2-methyl-6,7-dimethoxy-1-(3-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline (**1e**). ^{1}H-NMR ($CDCl_3$. 300 MHz): δ = 1.15 (3H, t, *J* = 7.2 Hz. NCH_2CH_3), 2.51 (1H, dd, J_1 = 16.7 Hz, J_2 = 4.8 Hz, CH_2), 2.65 – 2.72 (3H, m, CH_2), 2.78-3.01 (2H, m, CH_2), 3.17-3.31 (2H, m, CH_2), 3.33 (3H, s, OMe), 3.74 (3H, s, OMe), 3.95 (1H, dd, J_1 = 9.8 Hz, J_2 = 3.1 Hz, 6.37 (1H, d, *J* = 7.5 Hz, Ar), 6.49 (1H, s, Ar), 6.62 (1H, d, *J* = 8.1 Hz, Ar), 6.78 (1H, s, Ar), 6.99 (1H, t, *J* = 7.8 Hz, Ar). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = 156.9, 147.5, 146.1, 141.4, 140.9, 129.6, 129.3, 127.9, 124.5, 121.8, 116.1, 114.0, 113.6, 111.3, 111.2, 61.8, 55.7, 55.7, 55.2, 53.4, 46.8, 42.9, 42.1, 29.7, 23.2, 12.5.

3.3.9 Preparative transformation – Upscaling 2

Substrate **1e** (105 mg, 0.32 mmol. final concentration: 2 g/L = 6.1 mM) was dissolved in 10% v/v formamide (5 mL) and buffer (44,45 mL. 10 mM Tris-HCl, pH 9, 10 mM $MgCl_2$) with BBE-W165F (525 µL enzyme solution, final concentration: 1 g/ mL = 0.017 mM) and catalase (263 mg crude preparation). The reaction mixture was shaken in a light-shielded Erlenmeyer flask (250 mL) in a thermo shaker at 40°C and 200 rpm for 0.5 hours. Then the phases were extracted with ethyl acetate (4 x 100 mL) and the phases were separated in the centrifuge (4 x 4 minutes at 4000 rpm). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (silica; CH_2Cl_2/MeOH/NH_4OH = 98/1/1). The purification step was done to separate the products which are formed after half an hour reaction time form the residual educt. After flash chromatographie 15 mg of the poduct mixture were obtained as a dark brownish liquid. The GC-MS measurements of the mixture displayed that there were still some impurities in the sample, therefore an acidic purification step was done. To the sample 10 mL of 6 N HCl was added and the aqueous phase was extracted with ethyl acetate (3 x 20 mL). Afterwards the aqueous phase was basified with 15% NaOH solution (30 mL) and extracted with CH_2CL_2 (3 x 20 mL). Then the combined dichloromethane phases were dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give 8.4 mg of a yellowish solid. A ^{1}H-NMR measurement was carried out on the product mixture: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 1.15 (3H, d, *J* = 3 Hz, NCHCH_3), 2.78-3.01 (4H, m, CH_2), 3.14-3.38 (2H, m, CH_2), 3.47-3.68 (2H, m, CH_2), 3.86 (3H, s, OMe), 3.89 (3H, s, OMe), 4.41-4.09 (2H, m, CH_2), 6.49-6.74 (4H, m, Ar), 6.90-7.06 (1H, m, Ar).

3.4 Analytics

3.4.1 Determination of the apparent conversion after the biotransformation

Conversions were measured by HPLC: Shimadzu, Communication Bus Module CBM-20 A, column oven CTO-20 AC, degasser DGU-20 A5, liquid chromatograph LC-20 AD, autosampler SIL-20 AC, diode array detector SPD-M20, C18 colunm (Phenomenex, LUNA C18, 0.46 cm x 25 cm, 5 µm). Eluent: ammonium formate (30 mM) and water/methanol/acetonitrile: 67:18:15 (isocratic), flow rate: 0.5 mL/min, detection wavelength: 280 nm.

Retention time:

1a: 24.7, **2a**+**3a**: 15.3, 16.4, 39.6, **4a**: 20.8, **5a**: 24.7, **6a**: 13.3

1b: 28.5, **2b**+**3b**: 16.9, 18.5, 46.8, **4b**: 24.6, **5b**: 29.5, **6b**: 14.9

1e: 16.1, **2e**+**3e**: 12.2, 12.8, 27.5, **4e**: 14.5, **5e**: 17.2, **6e**: 10.6

3.4.2 Determination of the optical purity (enatiomeric excess – *ee*)

The HPLC-system described above was equipped with a chiral column (Chiralcel OJ from Daicel Chemical Indrustries. 0.46 cm x 25 cm, Lot. No.: OJ00CE-NK006). Eluent: n-heptane (0.1 % formic acid)/2-propanol 70:30, flow rate: 0.5 mL/min (isocratic), detection wavelength: 280 nm.

Retention time:

1a: (*S*): 19.4 (*R*): 21.9

1b: (*S*): 14.7 (*R*): 16.7

1e: (*S*): 15.1 (*R*): 21.3

3.4.3 Identification of the substrates and products

NMR-Spektroskopie

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance III 300 MHz spectrometer with a 5 mm PABBO BB-probhead (^{1}H: 300 MHz. ^{13}C: 75 MHz). Chemical shifts are given in parts per million (ppm) relative to TMS (δ = 0 ppm) and coupling constants (*J*) are reported in Hertz (Hz). The spectra were processed and analyzed in MestReNova 5.2.5. Depending on the solubility of the substances $CDCl_3$ and *d*-DMSO were used as solvents.

MS-measurements

GC-MS

For the GC-MS measurements an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass-selecitive detector (electron impact, 70 eV) an Agilent HP-5ms (30 m x 0.25 µm) (5%-phenyl)-methylpolysiloxane phase column was used. Helium (column flow = 2 mL/min) was used as carrier gas.

High resolution MS analyses were performed using electron impact (EI) ionization at 70 eV and TOF mass selection.

HPLC-MS

For the HPLC-MS measurements an Agilent 1200 system was used: Agilent 1200 column thermostat SL 1 (temperature: 20°C). Agilent 1200 High Performance auto sampler SL 1 (injection volume: 8 µL), Agilent 1200 Binary Pump SL 1 (flow: 0.5 mL/min, run time: 50 min), Agilent 1200 variable Wavelenght detector SL 1(adjustment: 280 nm). As a column the Puropher Star (RP-18e, LiChroCART 250 mm x 4.6 mm, 5 µm) form Merck was chosen. For the MS analysis the ionization mode API-ES with positive polarity and a single quatrupol as a mass selector were used. Eluent: ammonium formate (30 mM) and water /methanol/acetonitrile: 67:18:15 (isocratic).

Melting point measurement

For these measurements an apparate of Gallenkamp MPD350 was used, values are uncorrected.

TLC and flash chromatography

Thin layer chromatography was carried out on silica gel 60 F_{254} plats. For flash chromatography silica gel from Merck was used. The products were visualized by UV (254 nm and 345 nm).

3.5 References

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4 APPENDIX

4.1 Abbreviations

4‘ OMT	(*S*)-3-hydroxy-*N*-methylcoclaurine 4‘-*O*-methyltransferase
6OMT	norcoclaurine 6-*O*-methyltransferase
AIBN	Azobisisobutyronitrile
AU	Arbitrary Units
ACN	acetonitrile
Asn	Asparagine
Asp	Aspartic acid
BINAP	2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl
BBE	Berberine bridge enzyme
BF_3	Boron trifluoride
BuLi	Butyllithium
BOC	Tert-butyloxycarbonyl
Cbz	Carbobenzyoxy
cDNA	Complementary deoxyribonucleic acid
CNMT	(*S*)-coclaurine *N*-methyl transferase
COSY	Correlation spectroscopy
CYP80B1	(*S*)-*N*-methylcoclaurine 3’-hydroxylase
Cys	Cysteine
Da	Dalton
de	Diastereomeric excess
DEPT	Distortionless enhancement by polarization transfer
DHAP	Dihydroxyacetone phosphate
DIBAL	Diisobutylaluminium hydride
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
ee	Enantiomeric excess
EI	Electron impact
EMimAc	1-ethyl-3-methylimidazolium acetate
Equiv.	Equivalents
ER	Endoplasmic reticulum
ES	Electron spray
Et_3N	Triethylamine
EtOH	Ethanol
FA	Formamide
FAD	Flavin adenine dinucleotide
FDP	Fructose-1,6-diphosphate aldolase
Glu	Glutamic acid
GC	Gas chromatograhy
GC-MS	Gas chromatography-mass spectroscopy
His	Histidine
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography

HRMS	High-resolution mass spectroscopy
HSQC	Heteronuclear single-quantum correlation spectroscopy
$LiAlH_4$	Lithium aluminium hydride
Me_3Al	Trimethylaluminium
MeOH	Methanol
Mp	Melting point
MS	Mass spectroscopy
NCS	Norcoclaurine synthase
MeLi	Methyllithium
MHz	Megahertz
$NaBH_4$	Sodium borohydride
NaOH	Sodium hydroxide
Na2SO4	Soldium sulfate
N-MFA	*N*-methylformamide
NMR	Nuclear magnetic resonance
P. pastoris	*Pichia pastoris*
rac	racemic
Red-Al	Sodium bis(2-methoxyethoxy)aluminumhydride
R_f	Retardation factor
rpm	Round per minute
S_EAr	Electrophilic aromatic substitution
S_N2	Bimolecular nucleophilic substitution
STOX	(*S*)-tetrahydroprotoberbine oxidase
TIC	Total ion current
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMEDA	Tetramethylethylenediamine
TMS	Tetramethylsilane
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TOF	Time of flight
TON	Turn-over number
Tris	Tris(hyroxymethyl)aminomethyne
Try	Trytophane
VME	Valinol methyl ether
v/v	Volume concentration (volume/volume)
UV	Ultraviolett
wt	Wild-type

4.2 NMR and HR-MS spectra of all unpublished substrates

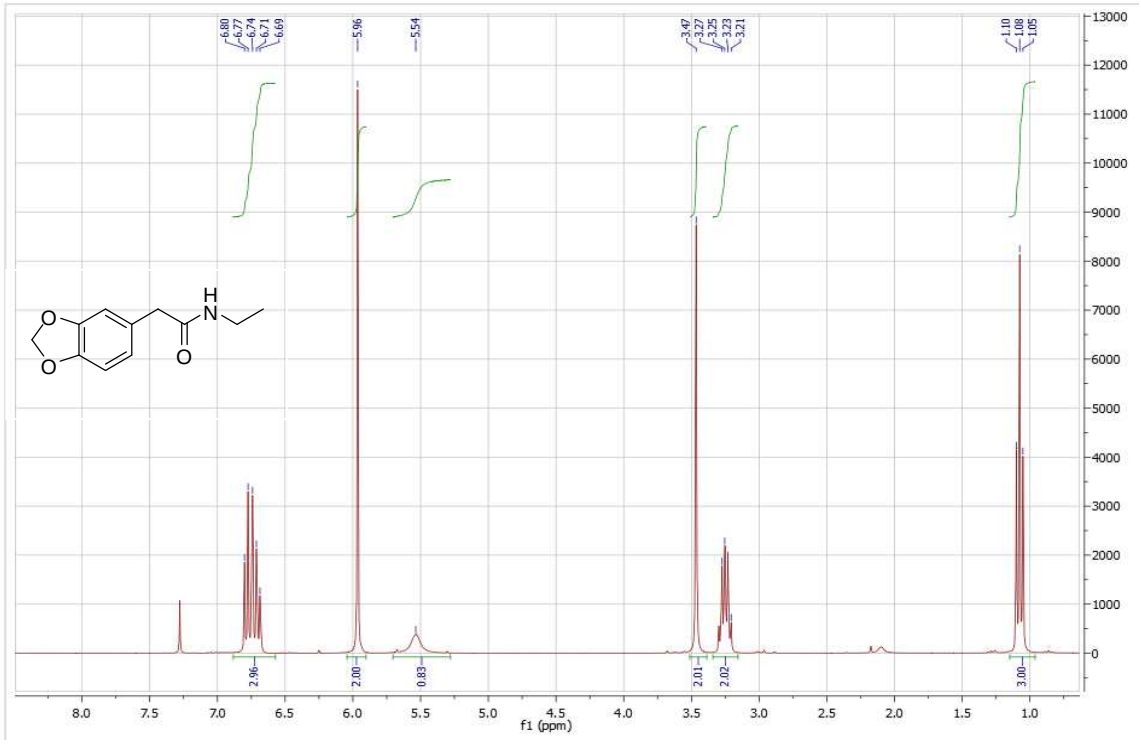

Figure 43: ^{1}H-NMR spectrum of **6a**

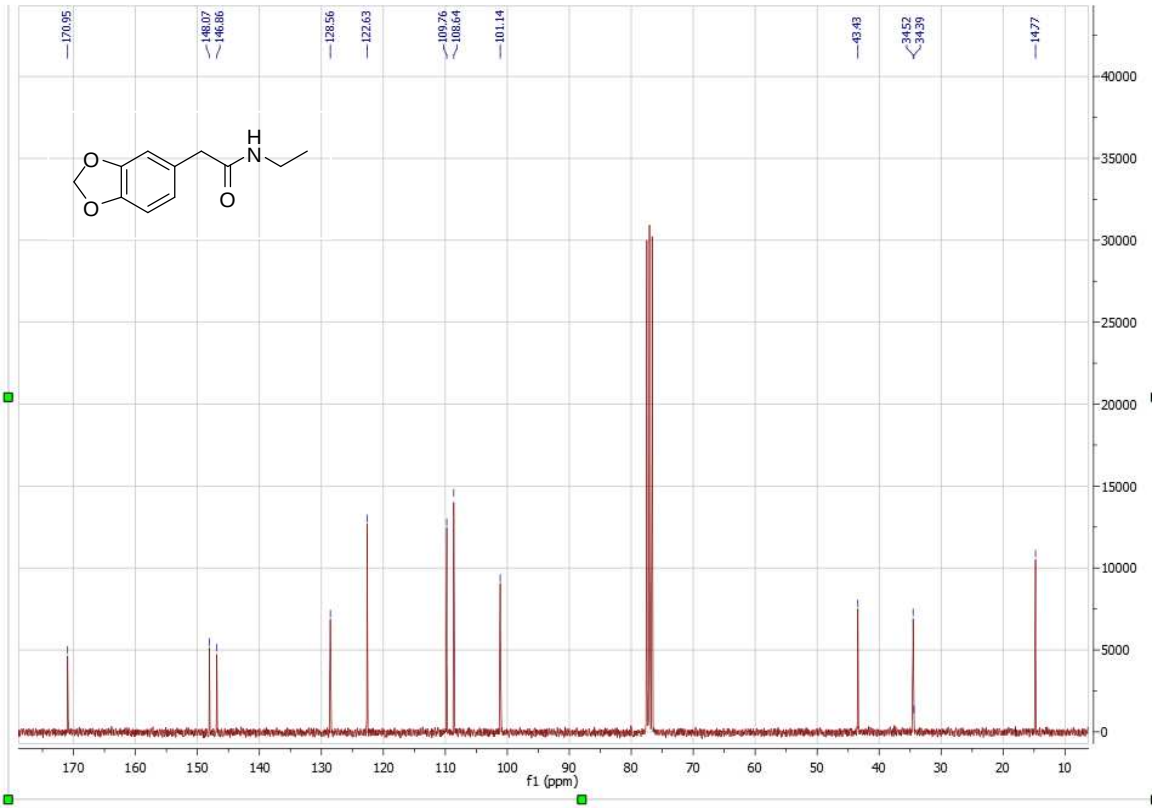

Figure 44: ^{13}C-NMR spectrum of **6a**

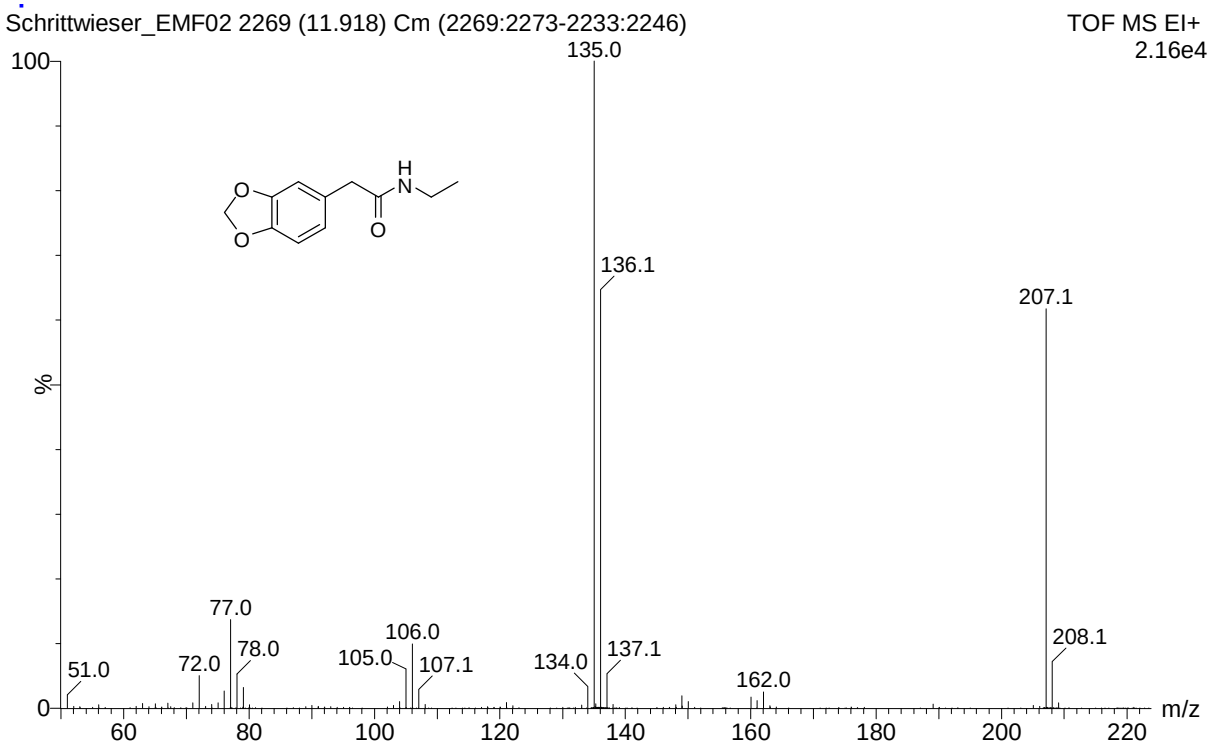

Figure 45: MS spectrum of **6b**

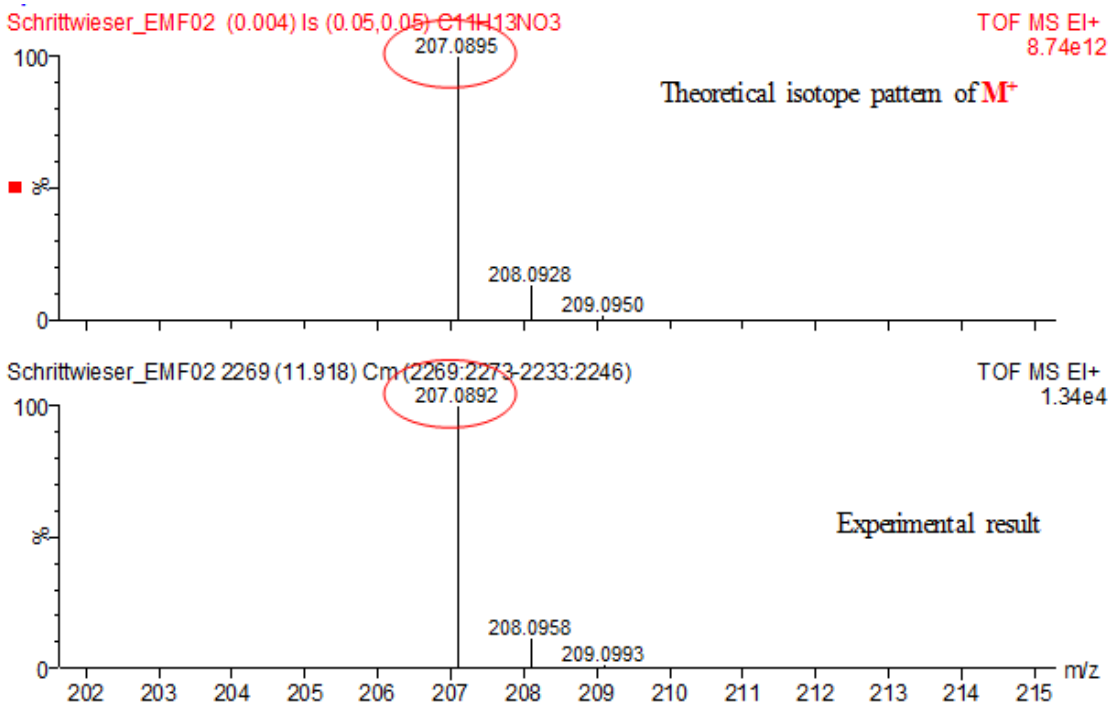

Figure 46: HR-MS spectrum of **6a**

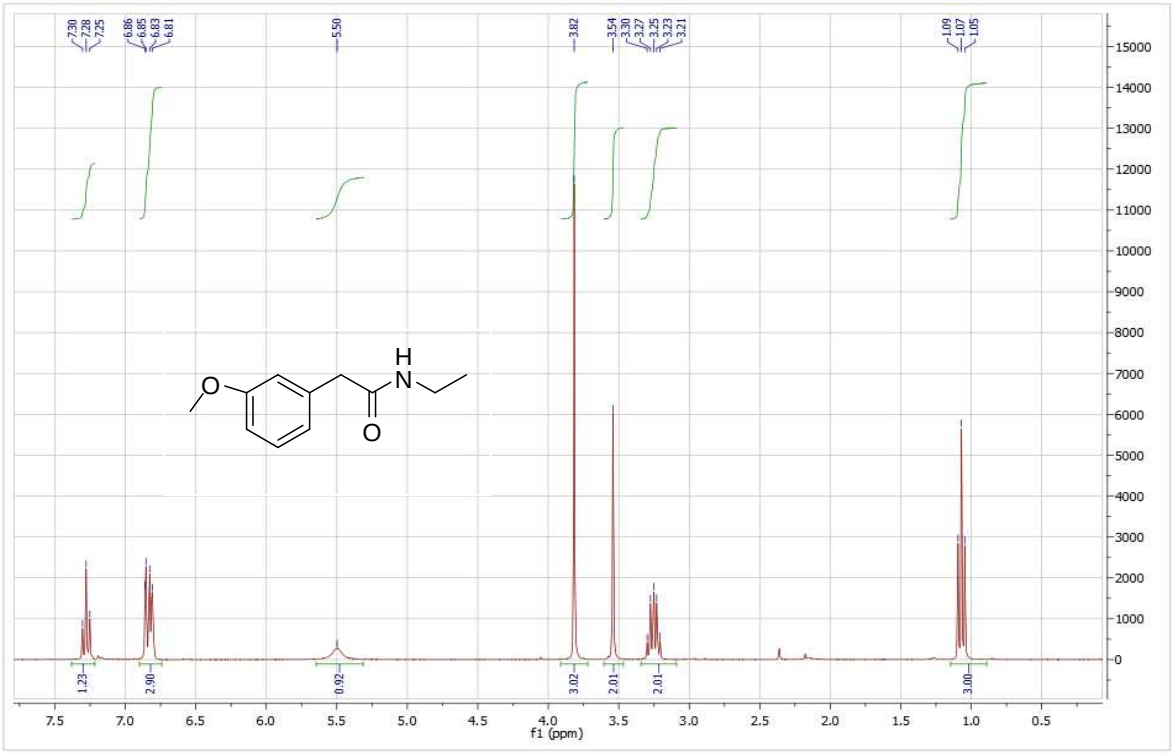

Figure 47: ^{1}H-NMR spectrum of **5b**

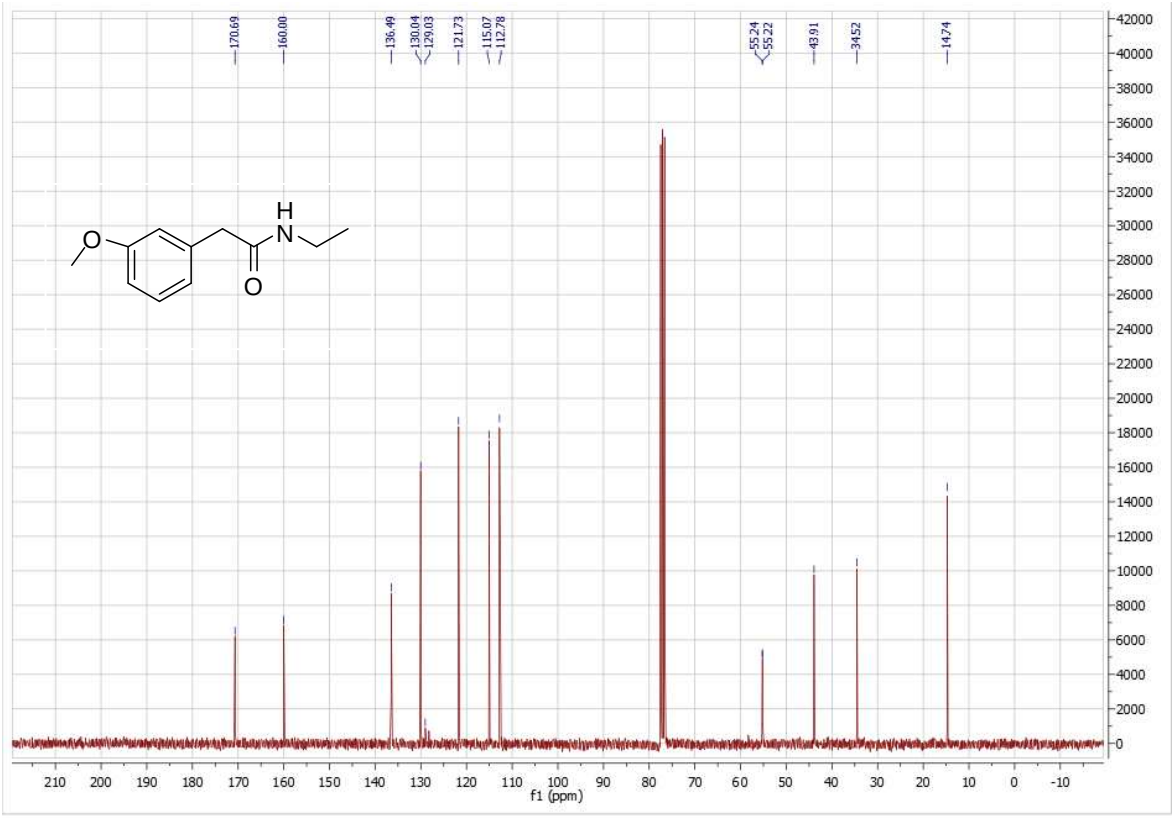

Figure 48: ^{13}C-NMR spectrum of **5b**

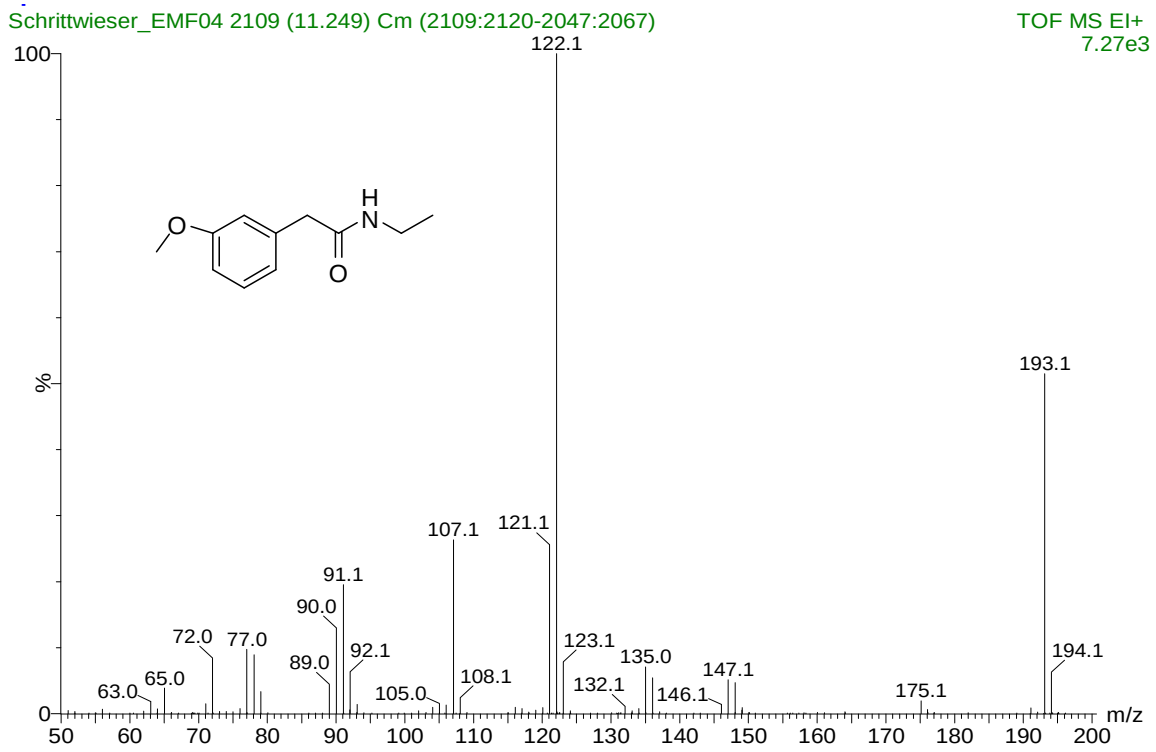

Figure 49: MS spectrum of **5b**

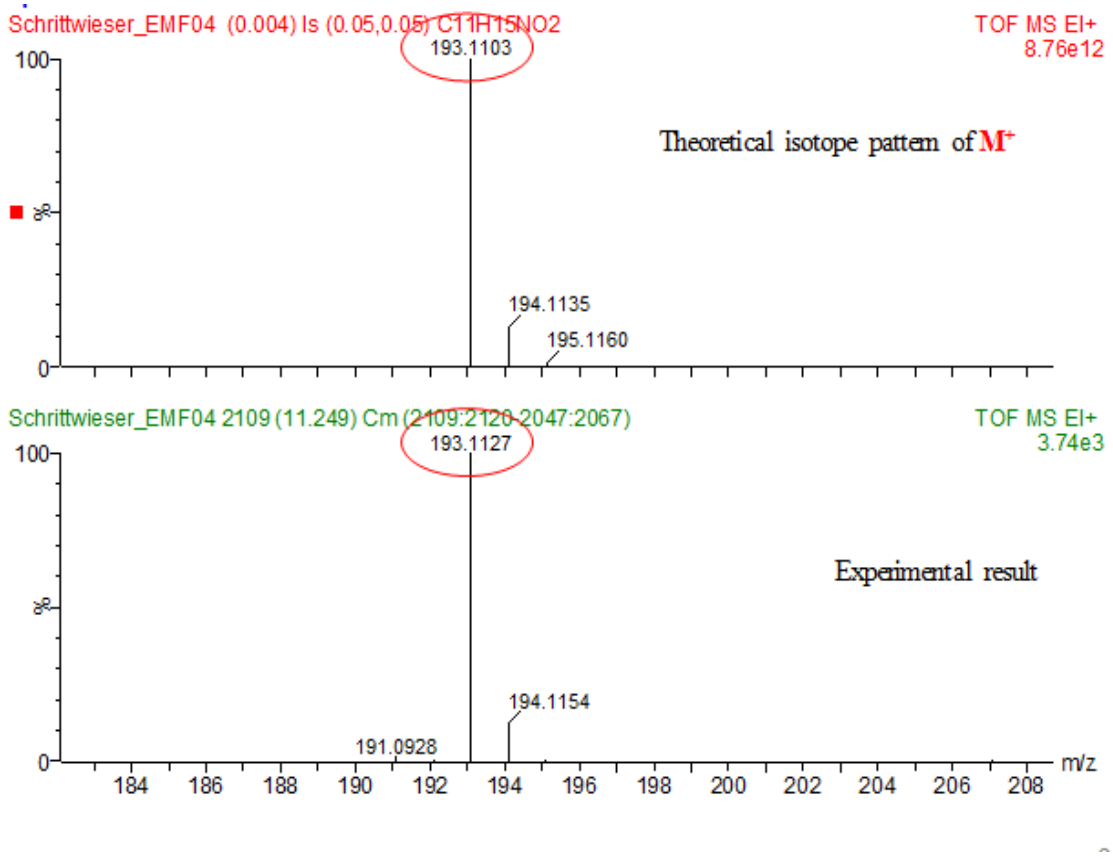

Figure 50: HR-MS spectrum of **5b**

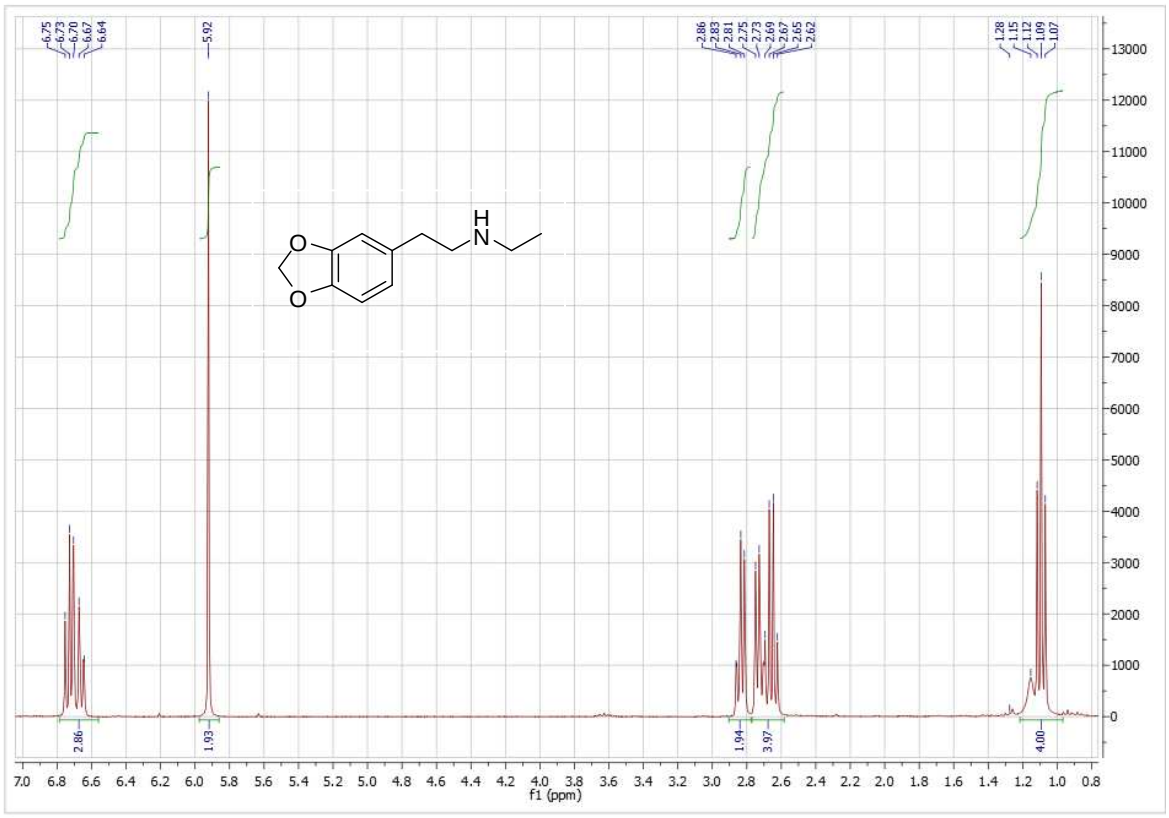

Figure 51: [1]H-NMR spectrum of **5a**

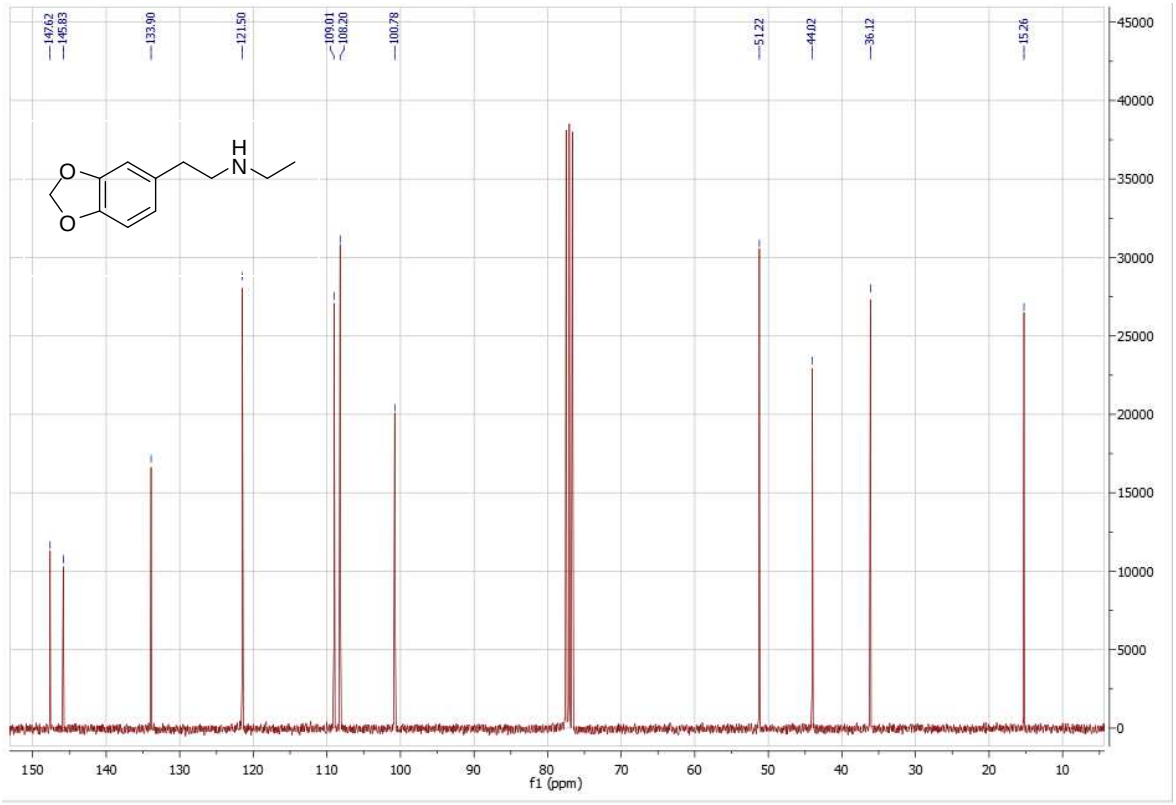

Figure 52: [13]C-NMR spectrum of **5a**

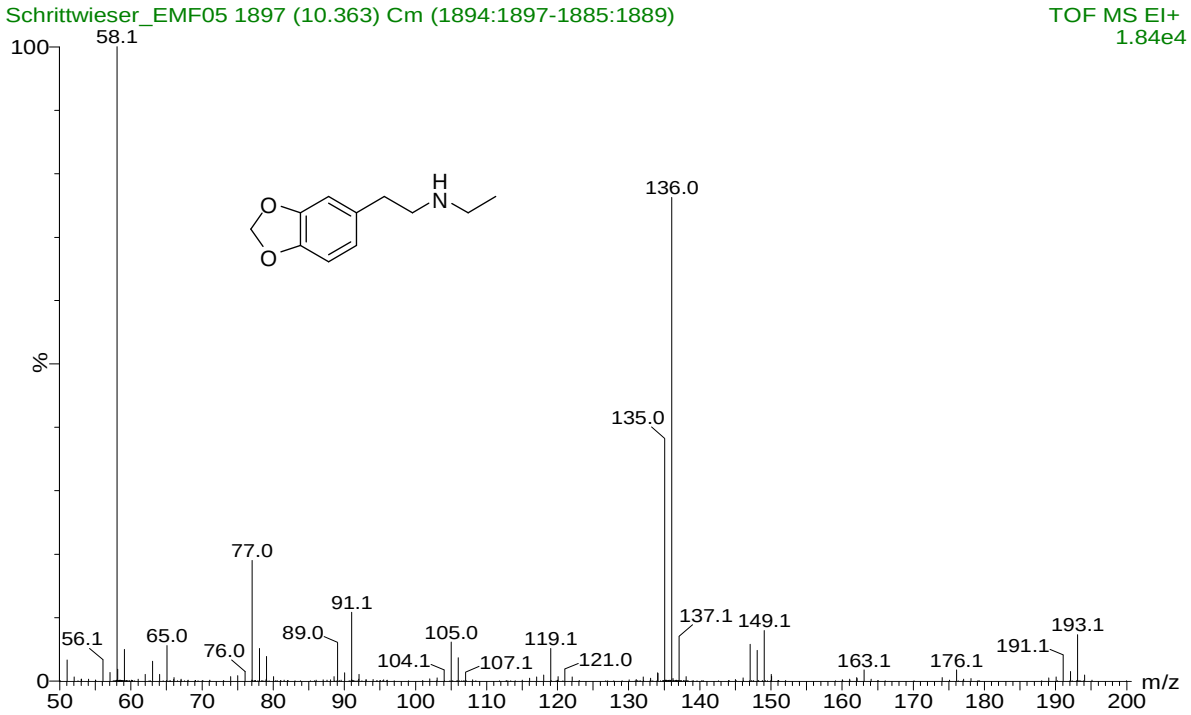

Figure 53: MS spectrum of **5a**

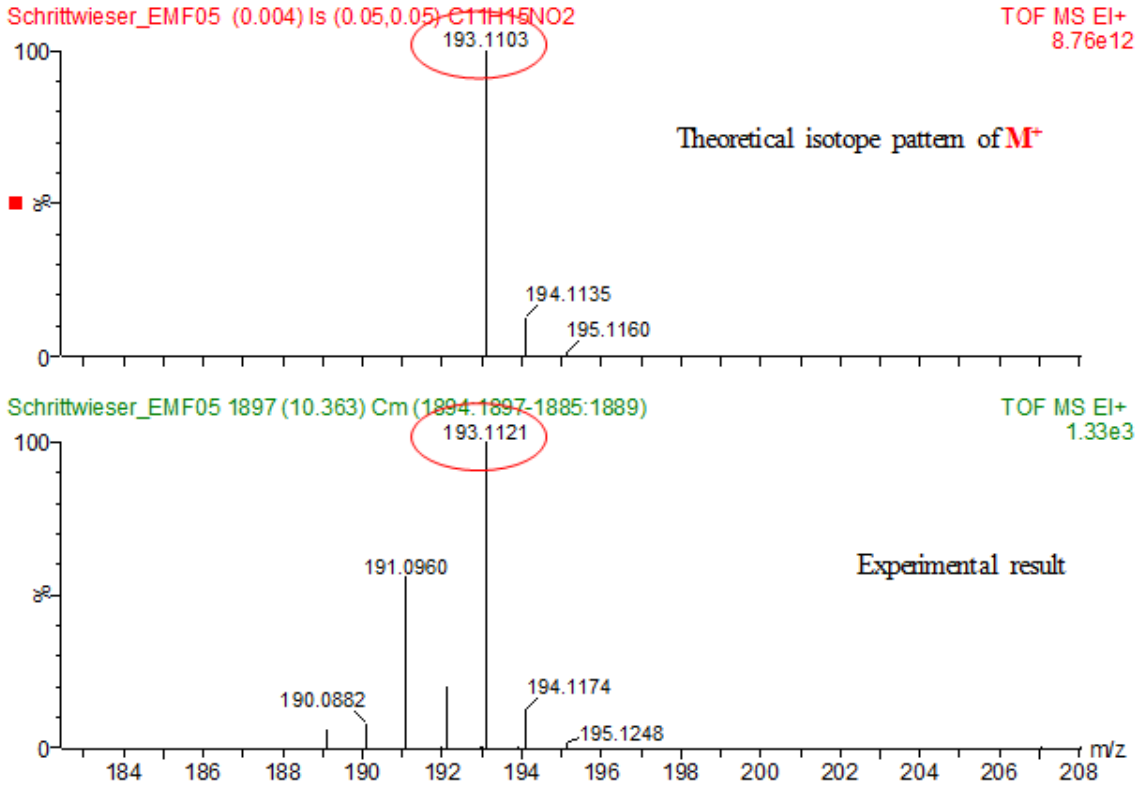

Figure 54: HR-MS spectrum of **5a**

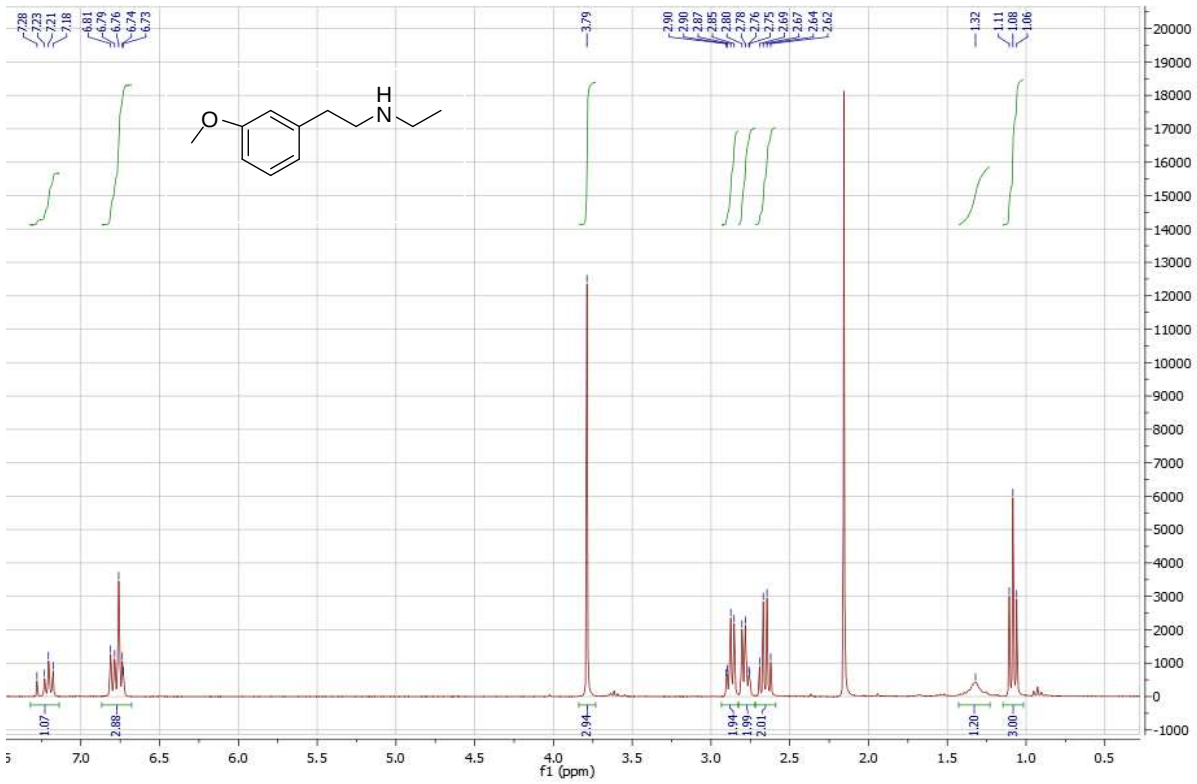

Figure 55: ^{1}H-NMR spectrum of **4b**

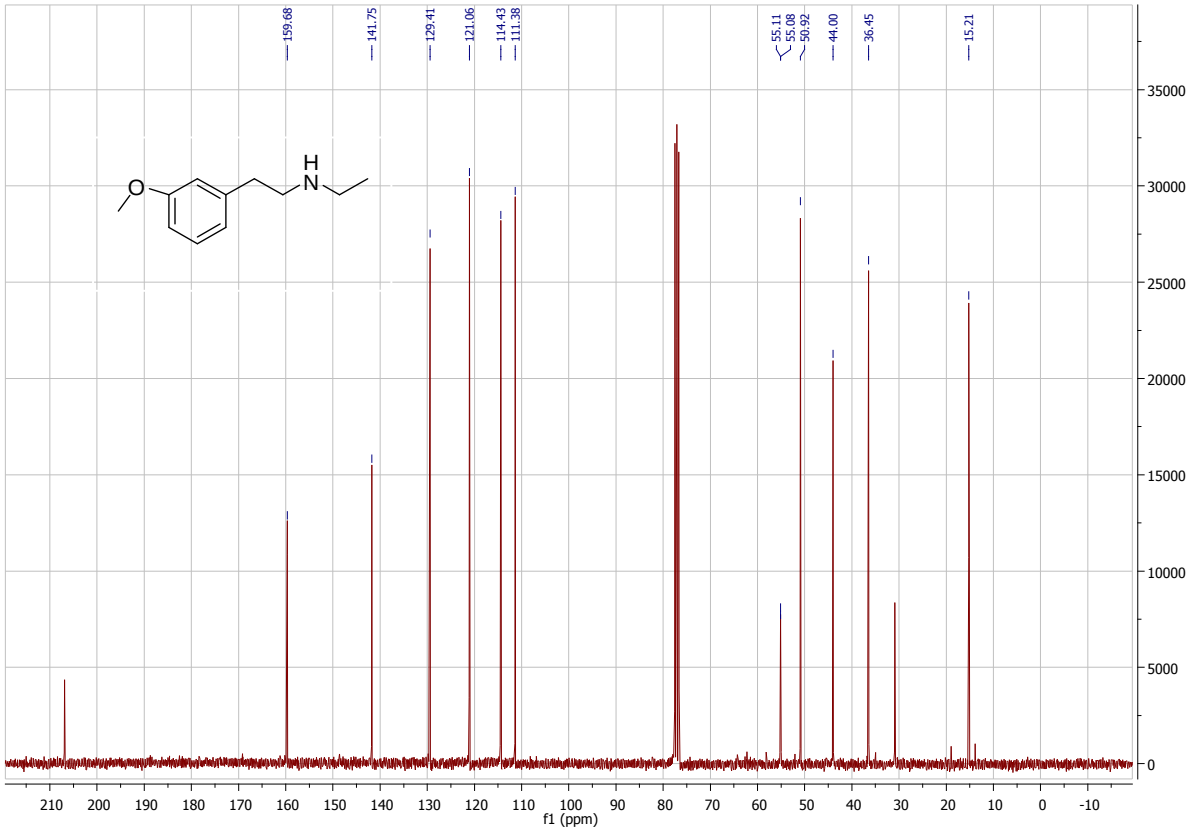

Figure 56: ^{13}C-NMR spectrum of **4b**

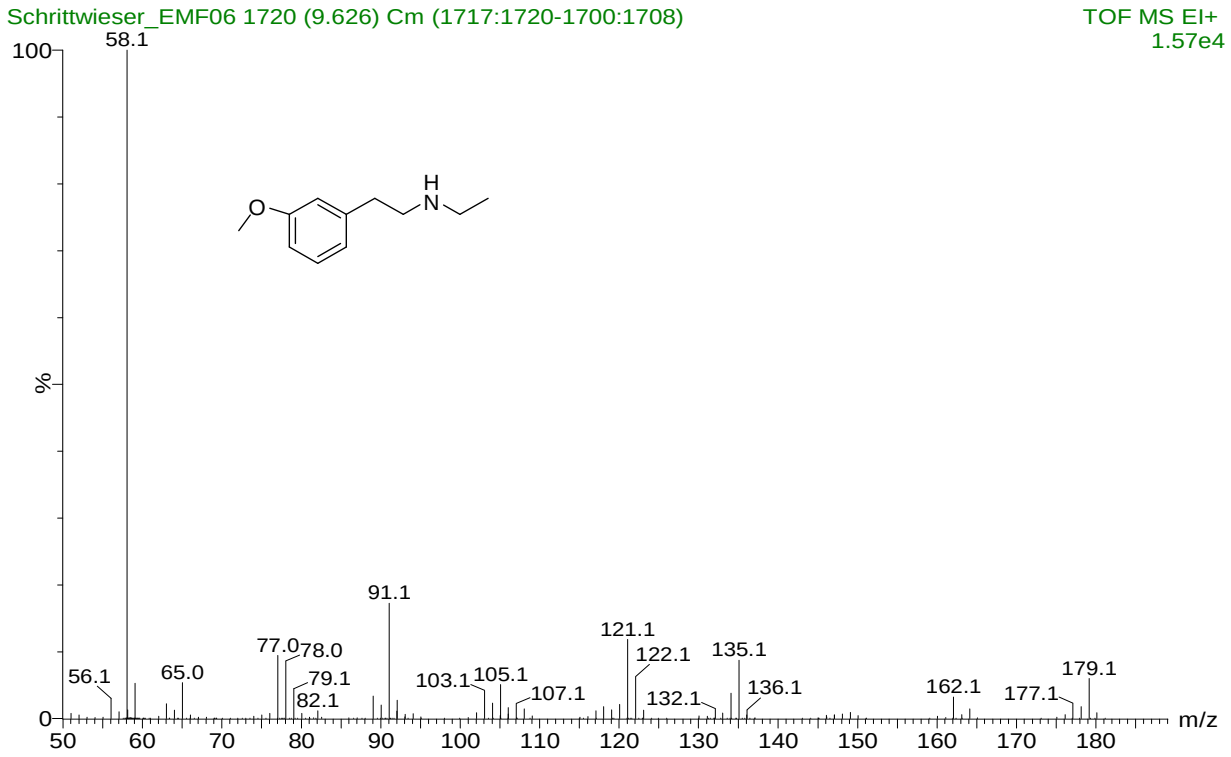

Figure 57: MS spectrum of **4b**

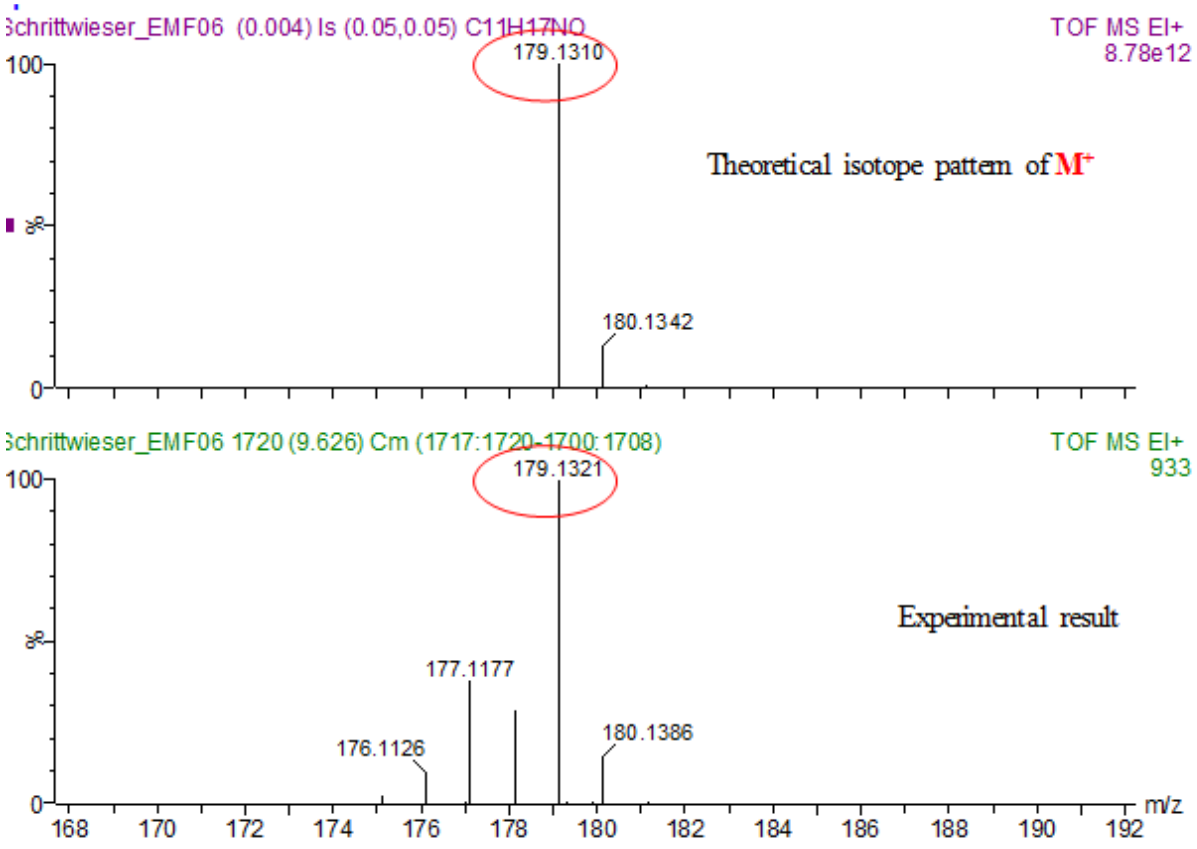

Figure 58: HR-MS spectrum of **4b**

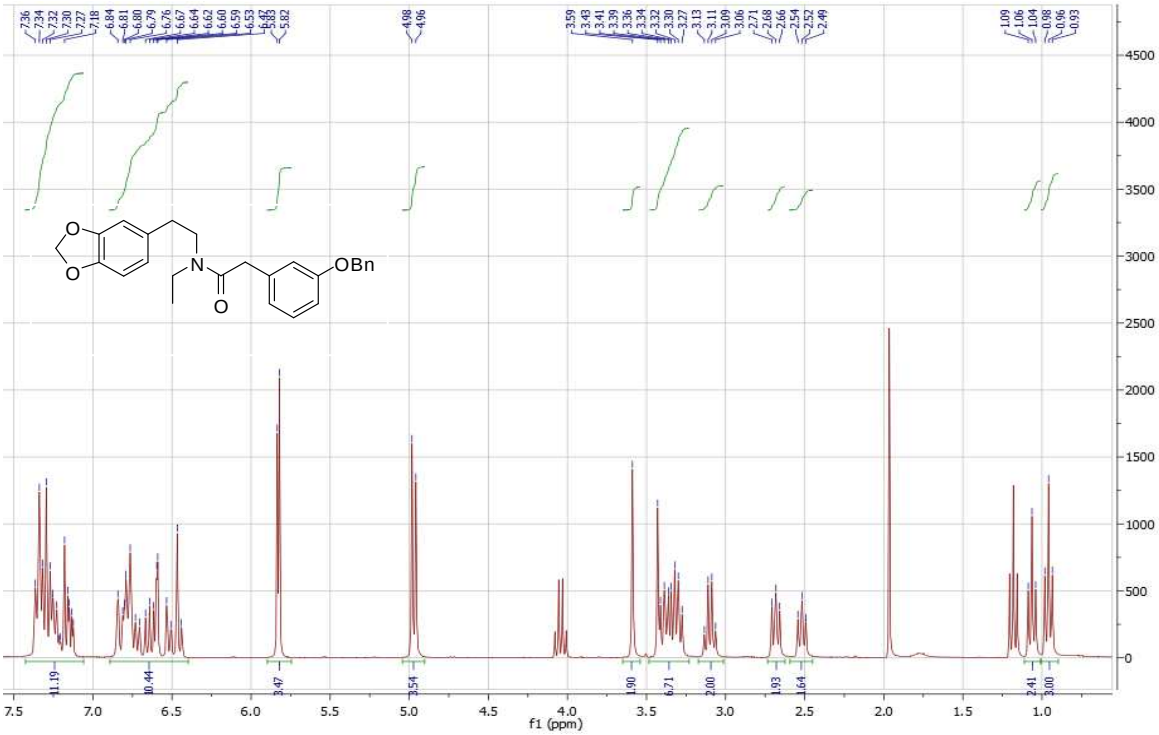

Figure 59: ^{1}H-NMR spectrum of **3a**

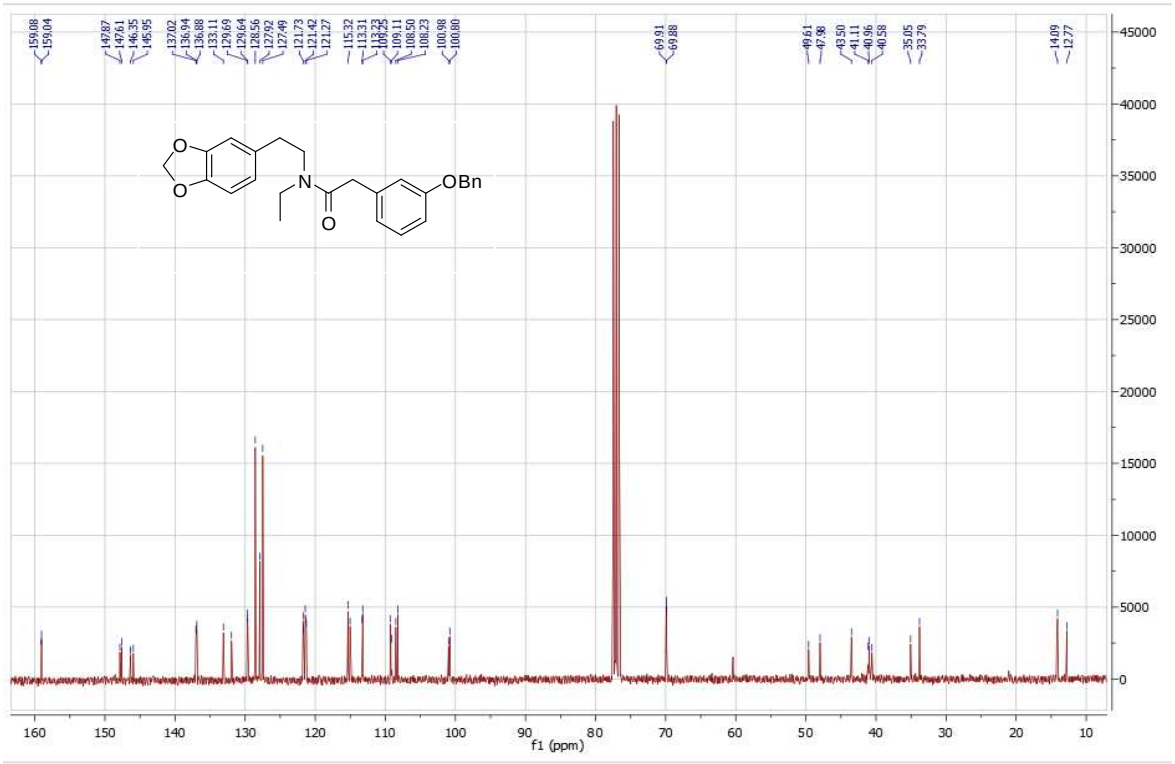

Figure 60: ^{13}C-NMR spectrum of **3a**

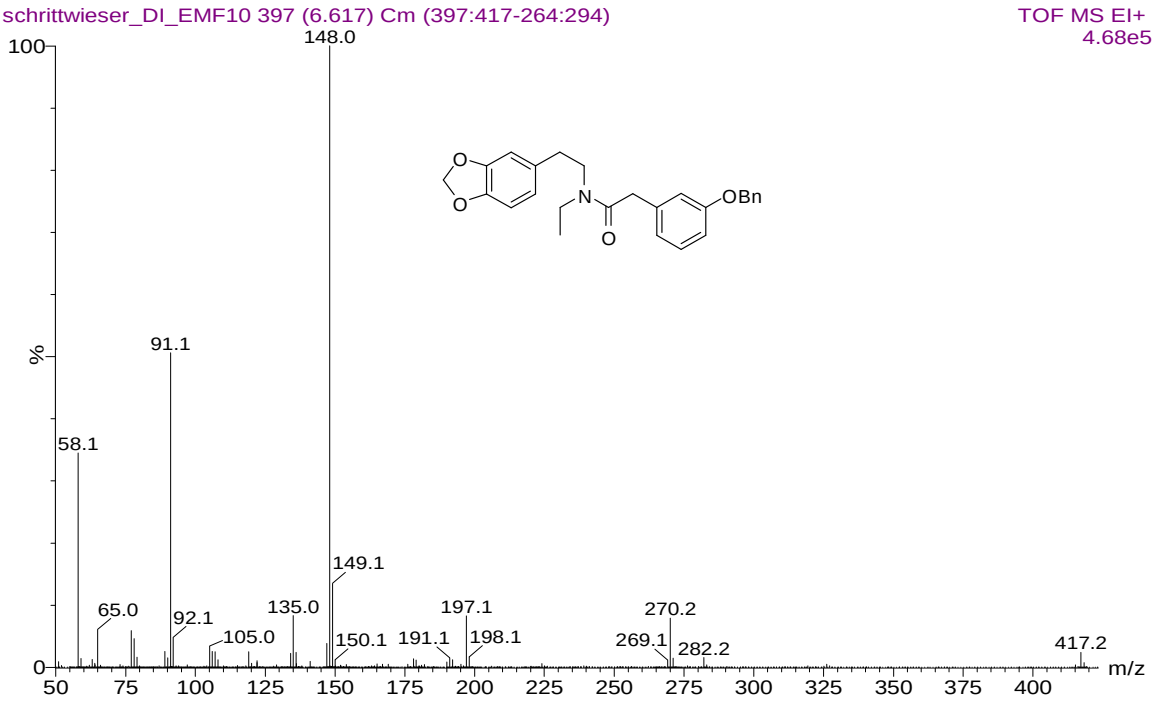

Figure 61: MS spectrum of **3a**

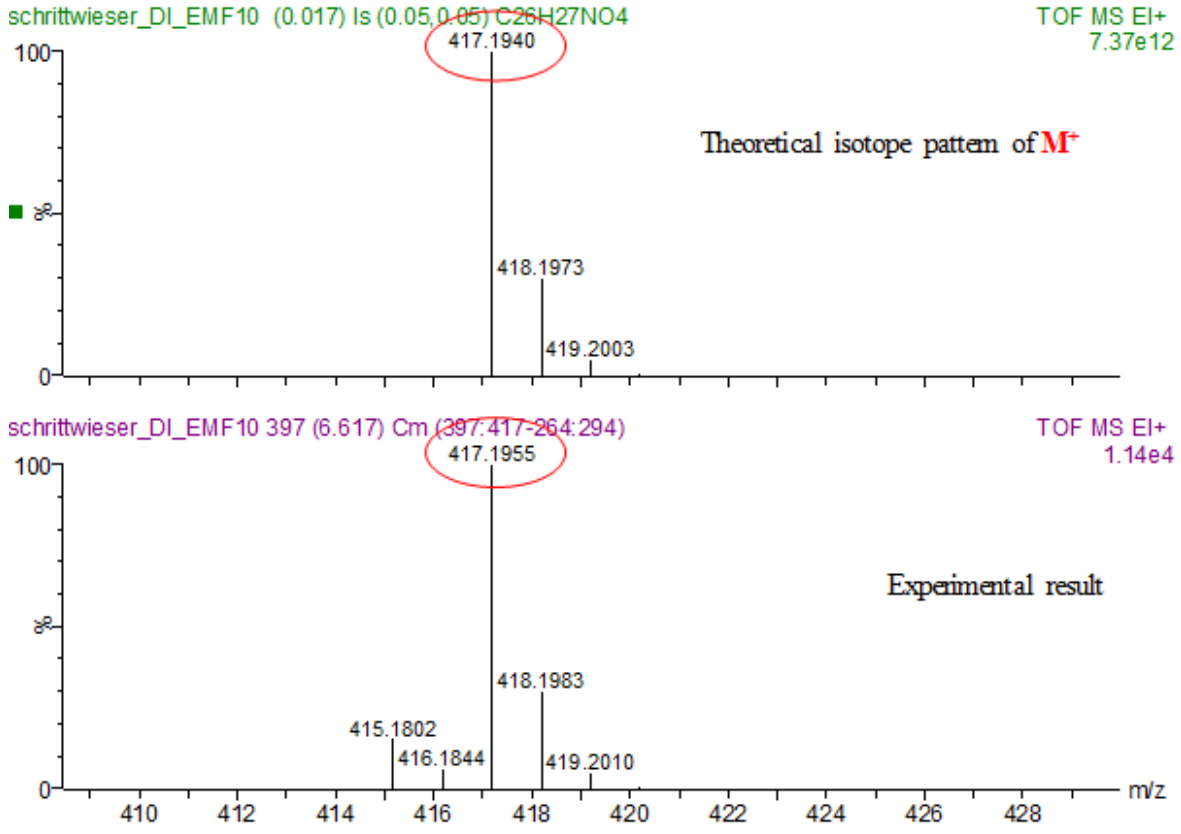

Figure 61: HR-MS spectrum of **3a**

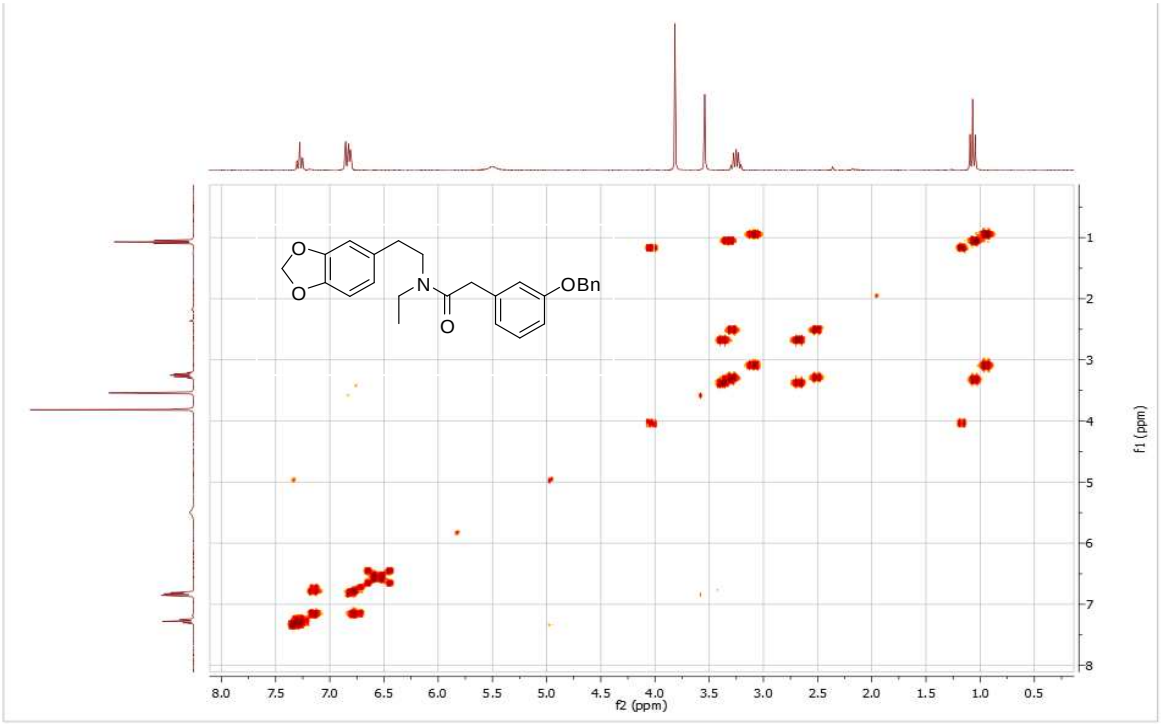

Figure 64: COSY-spectrum of **3a**

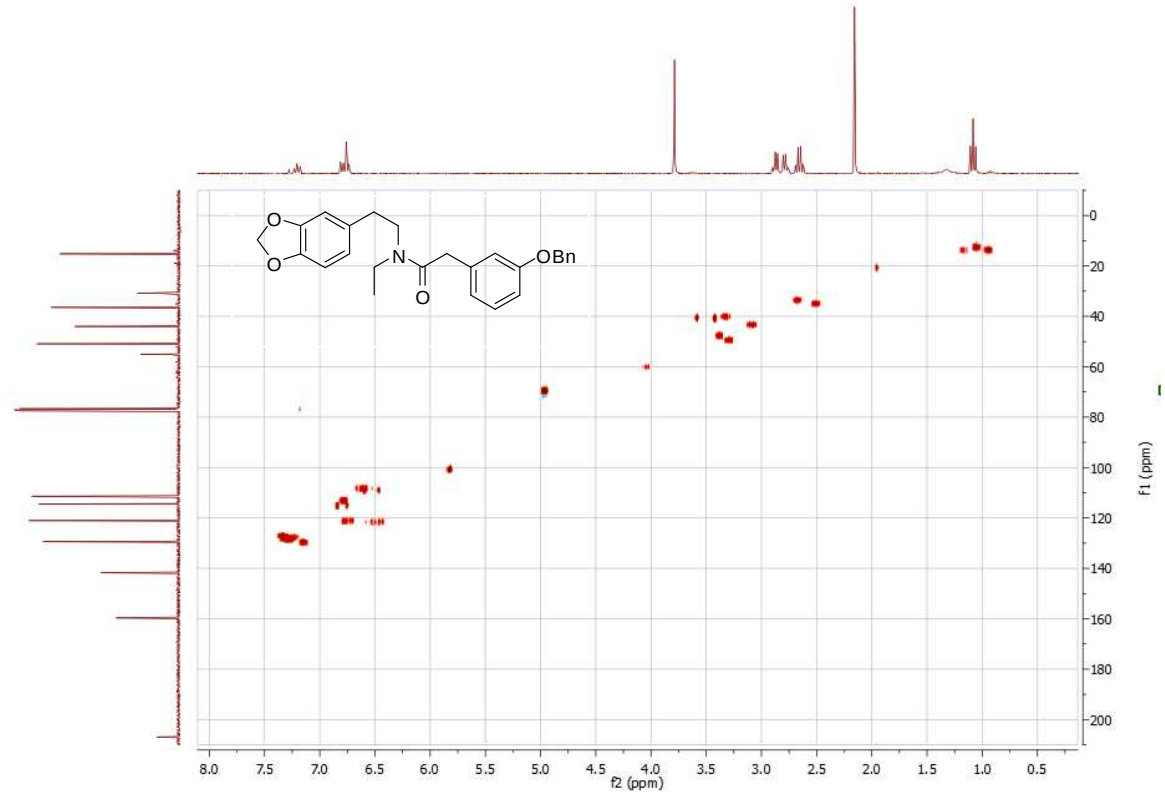

Figure 65: HSQC spectrum of **3a**

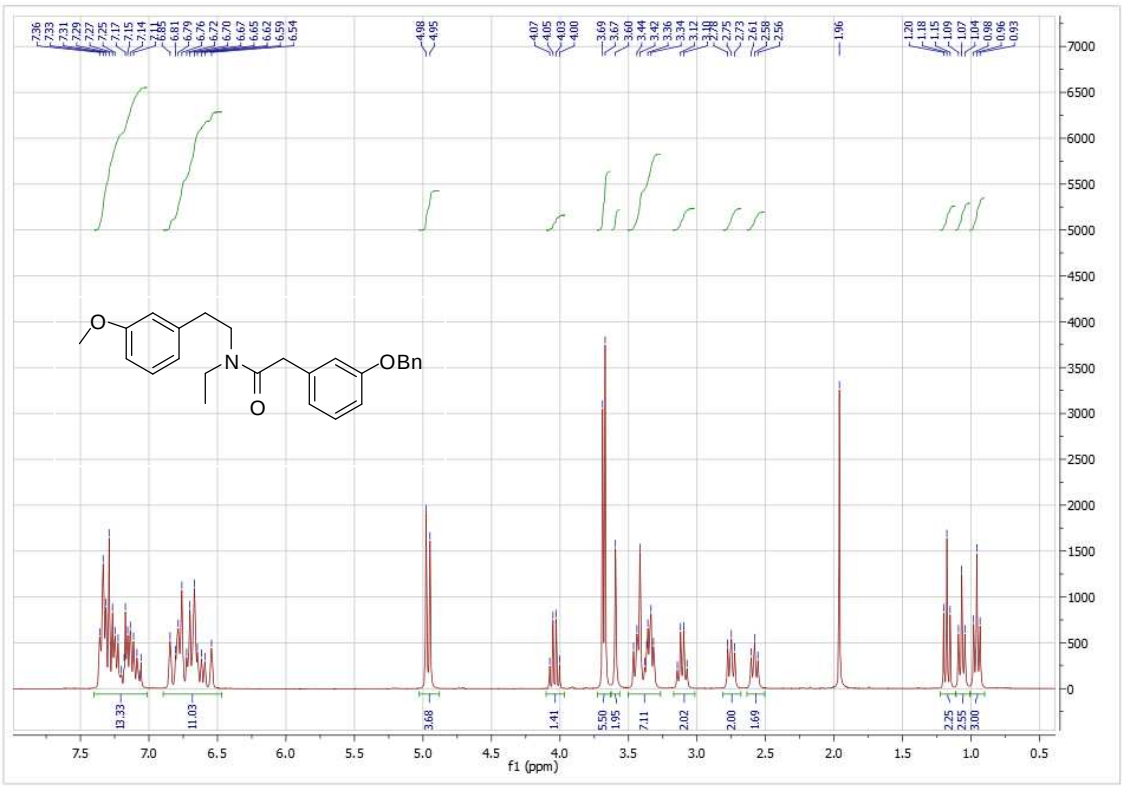

Figure 66: ^{1}H-NMR spectrum of **3b**

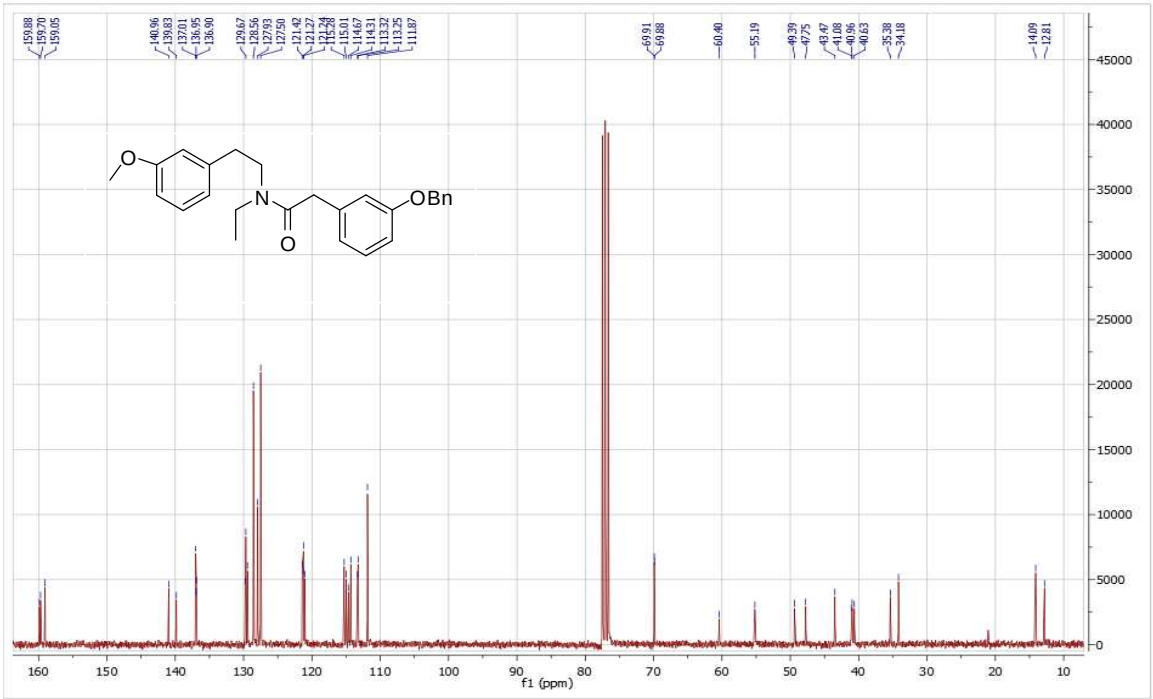

Figure 67: ^{13}C-NMR spectrum of **3b**

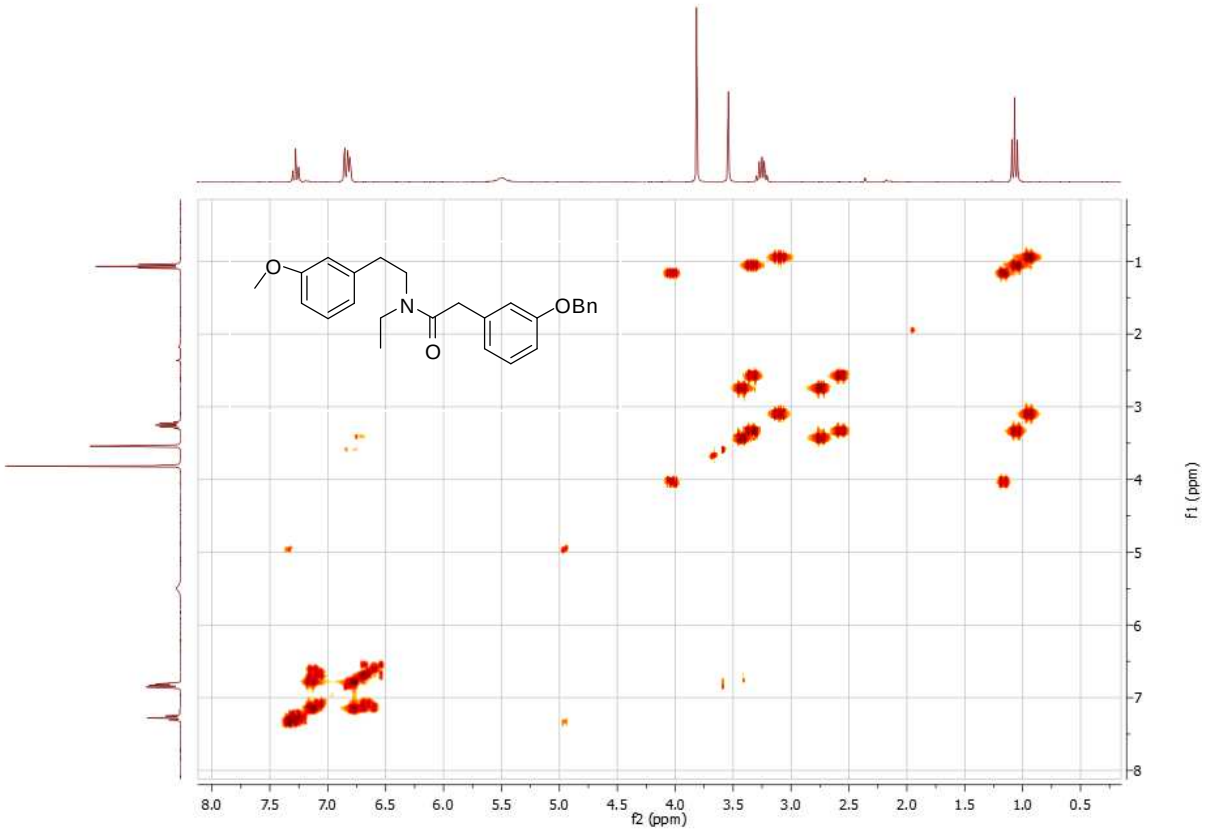

Figure 68: COSYspectrum of **3b**

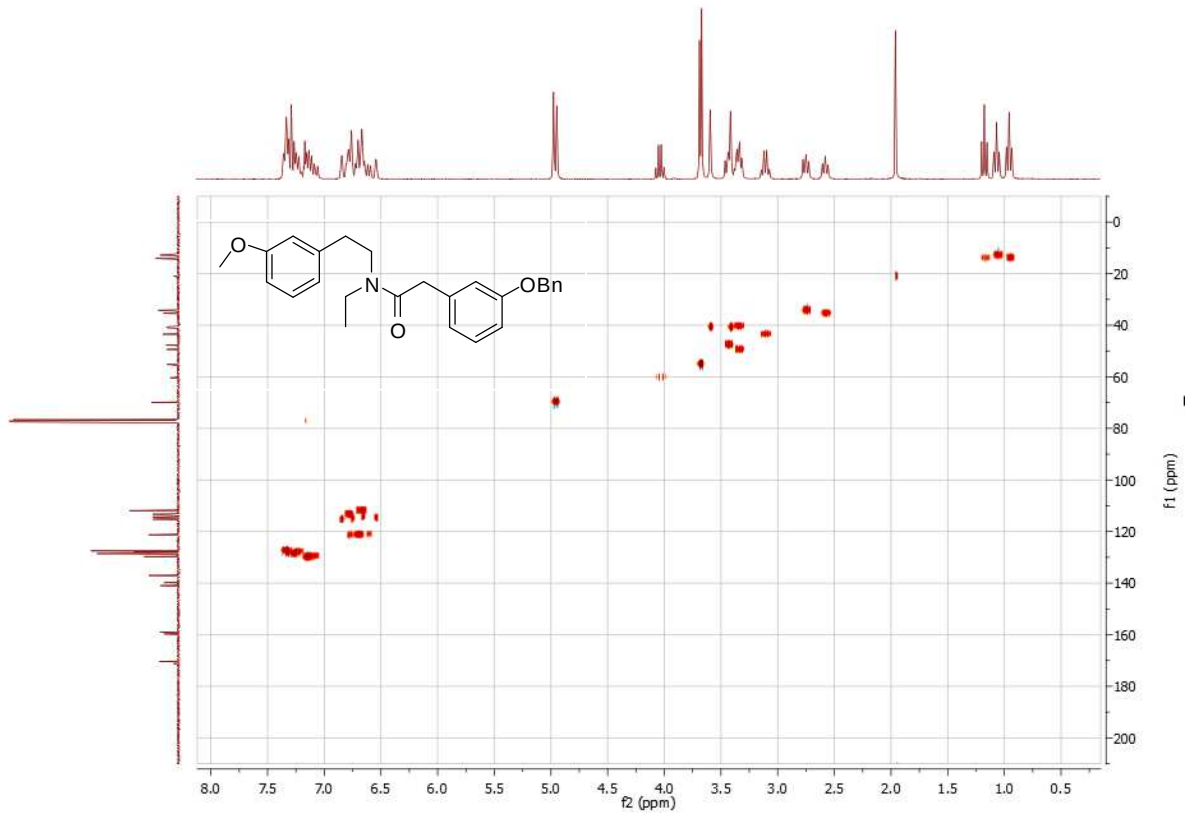

Figure 69: HSQC spectrum of **3b**

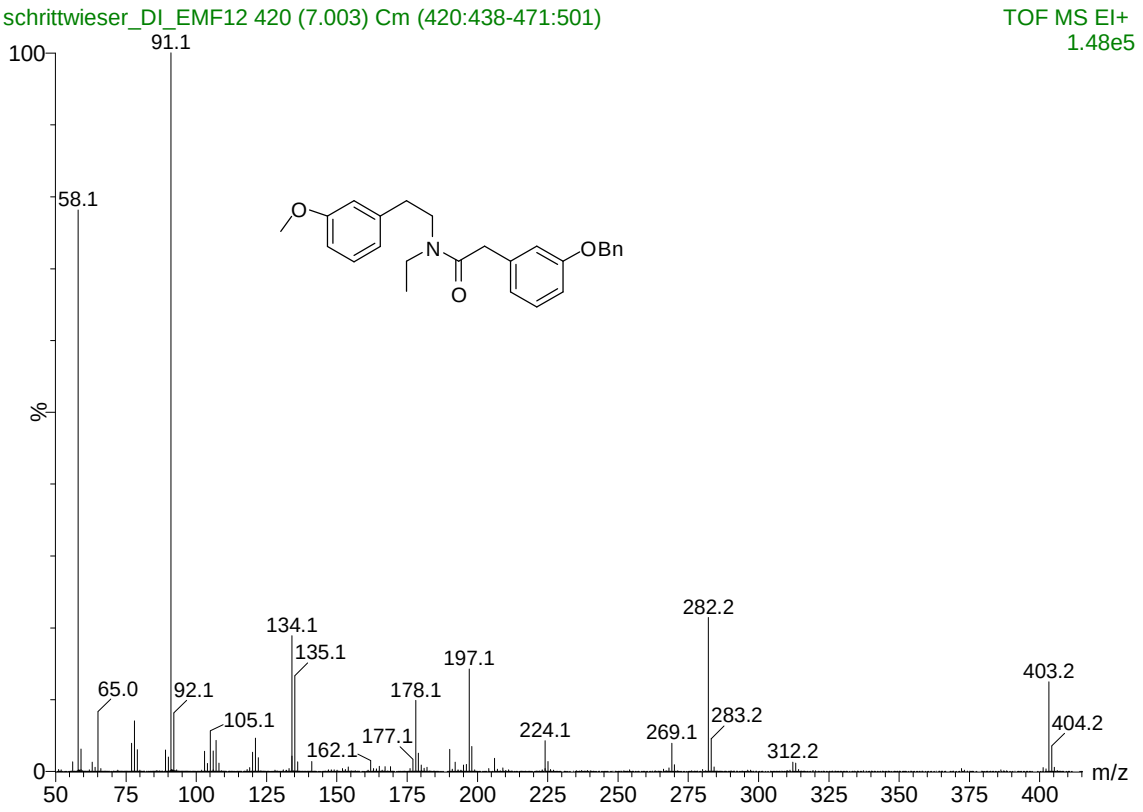

Figure 70: MS spectrum of **3b**

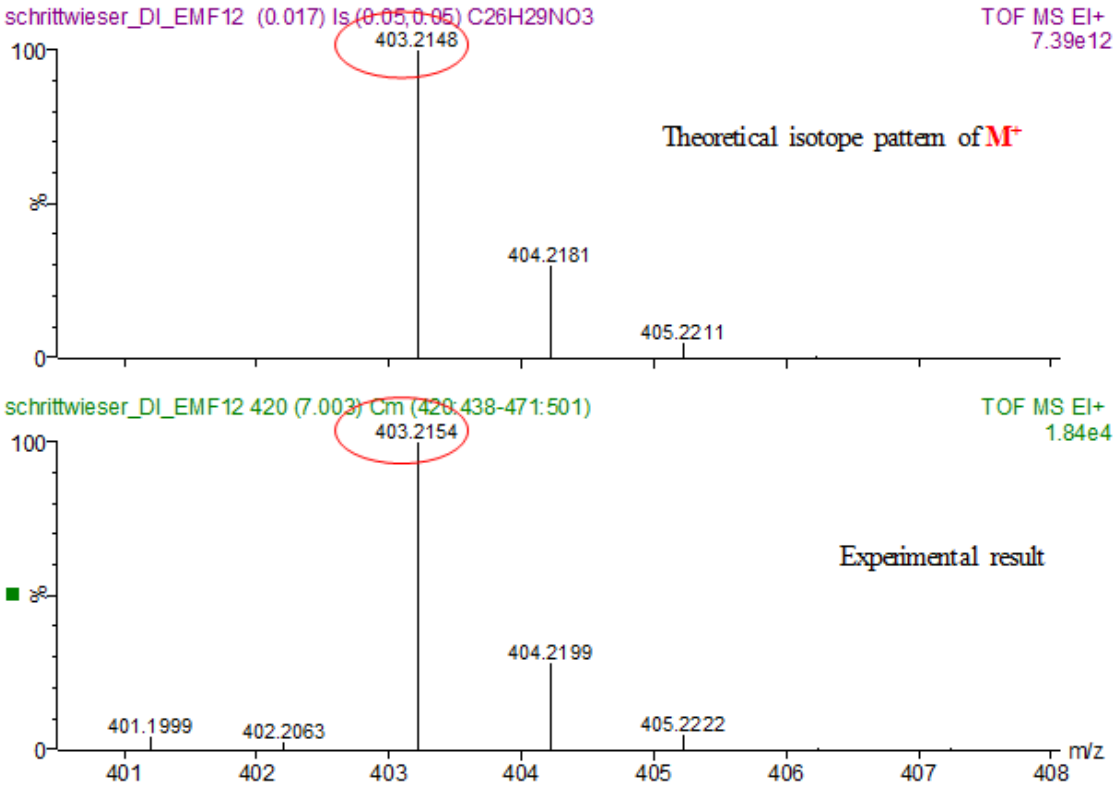

Figure 71: HR-MS spectrum of **3b**

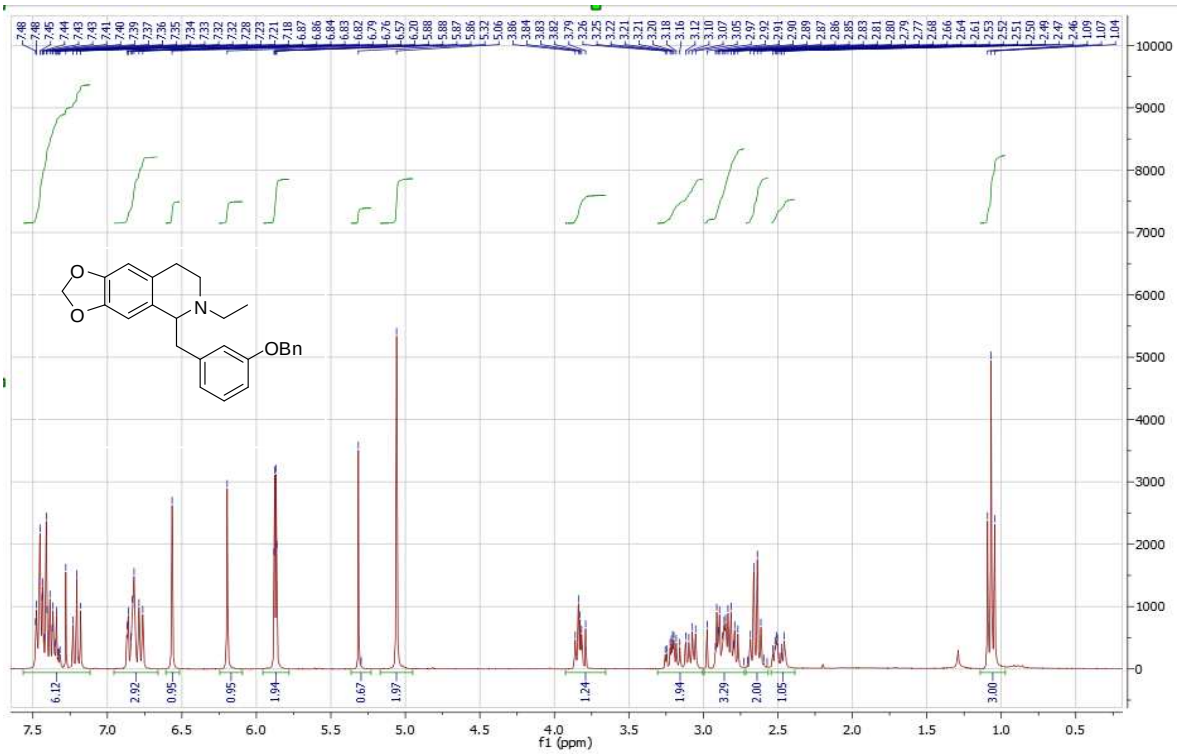

Figure 72: [1]H-NMR spectrum of **2a**

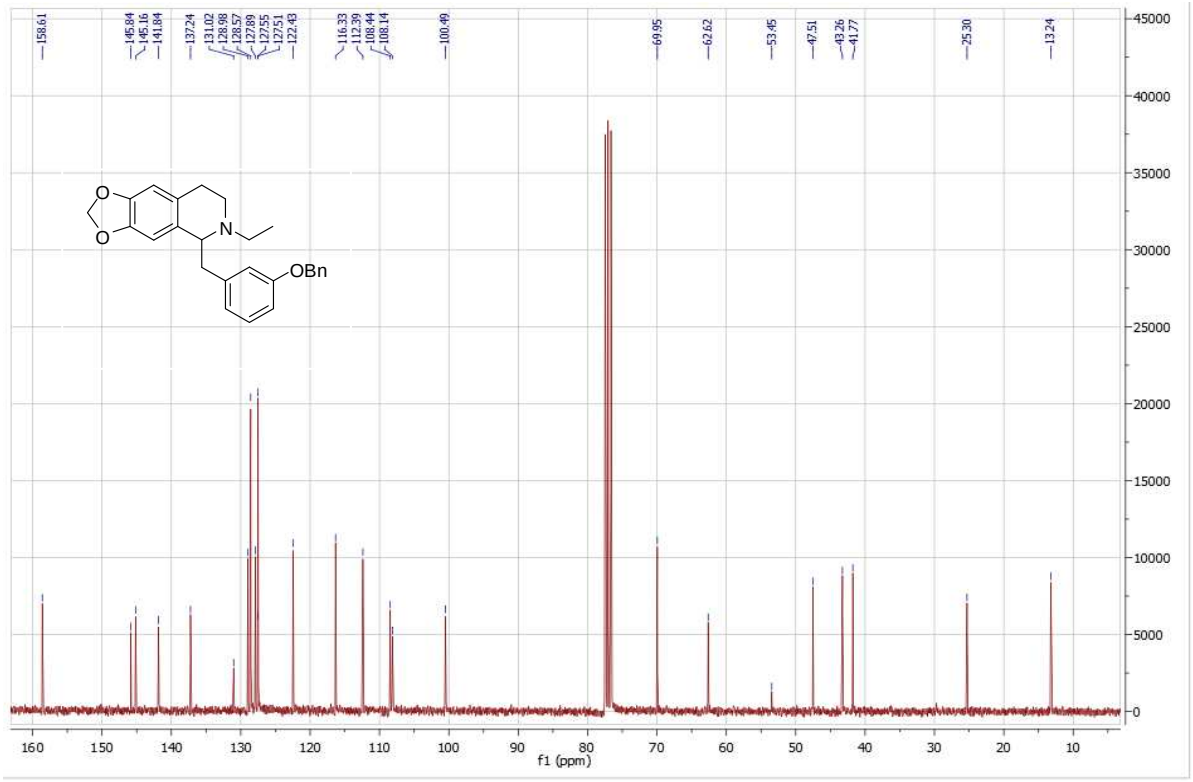

Figure 73: [13]C-NMR spectrum of **2a**

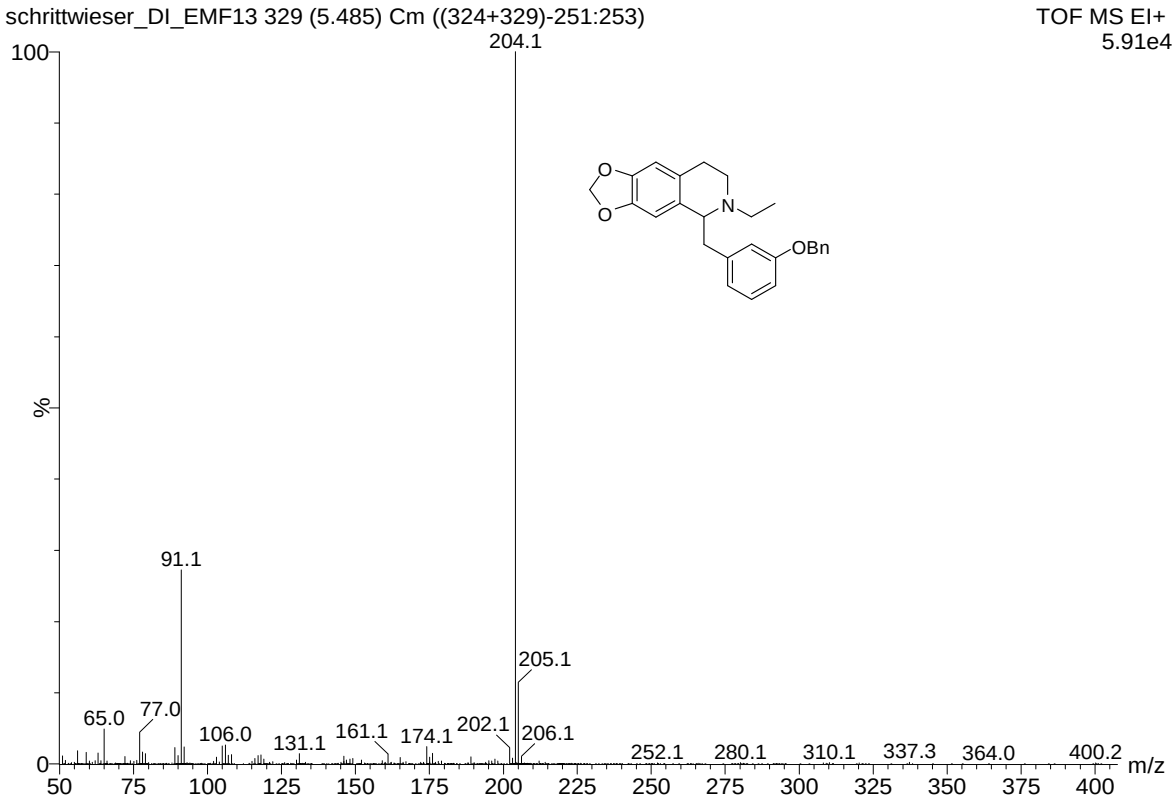

Figure 74:MS spectrum of **2a**

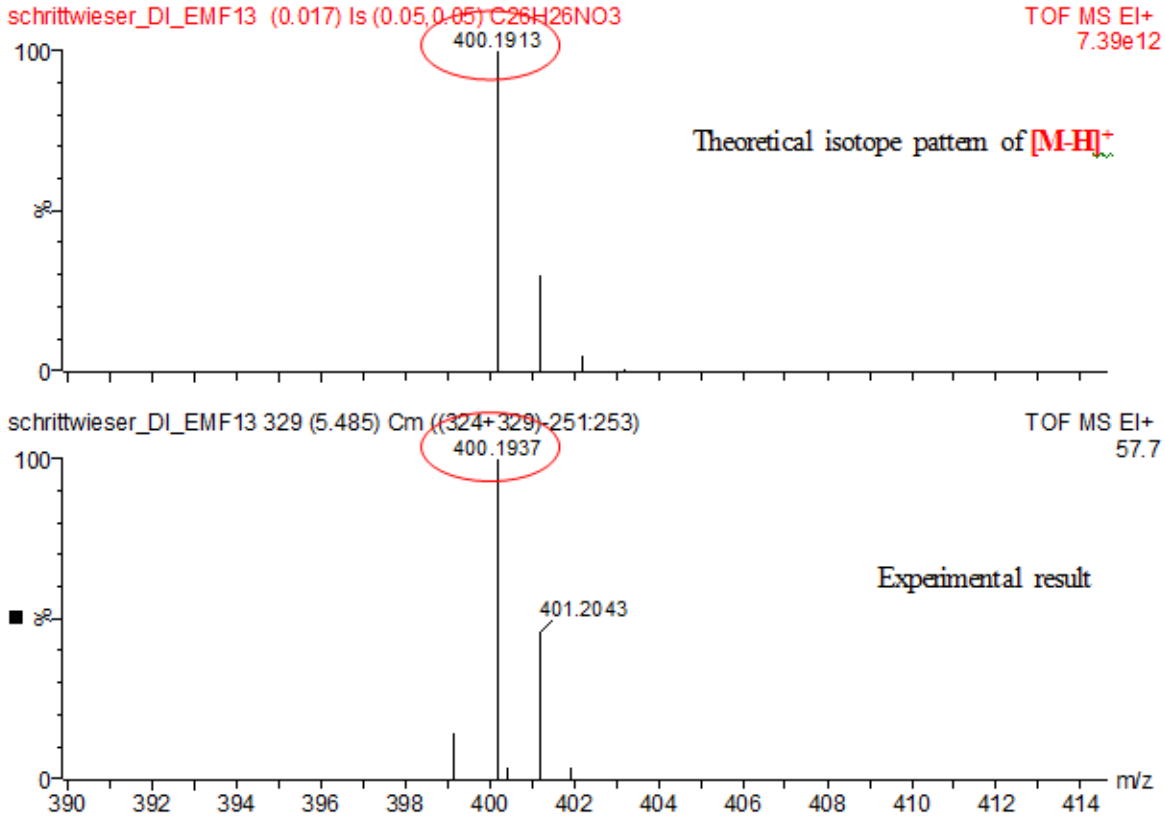

Figure 75: HR-MS spectrum of **2a**

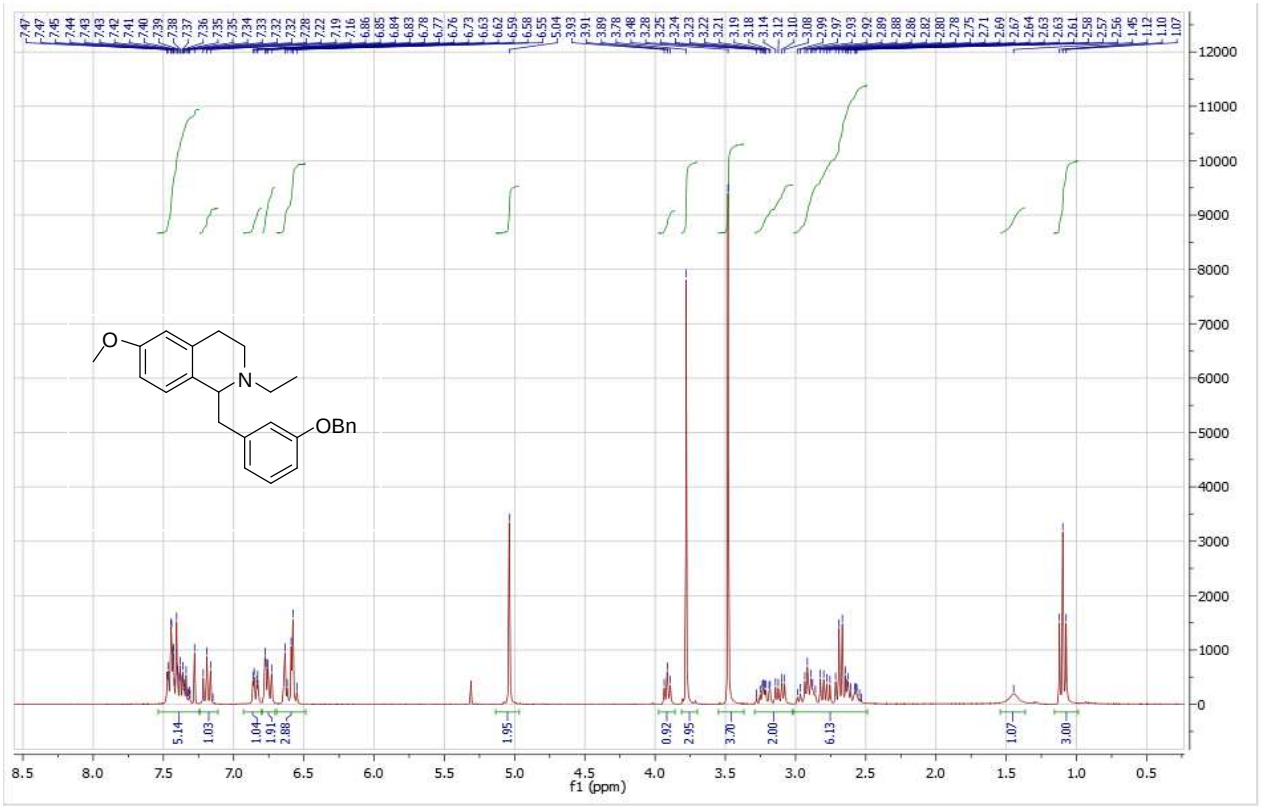

Figure 76: ^{1}H-NMR spectrum of **2b**

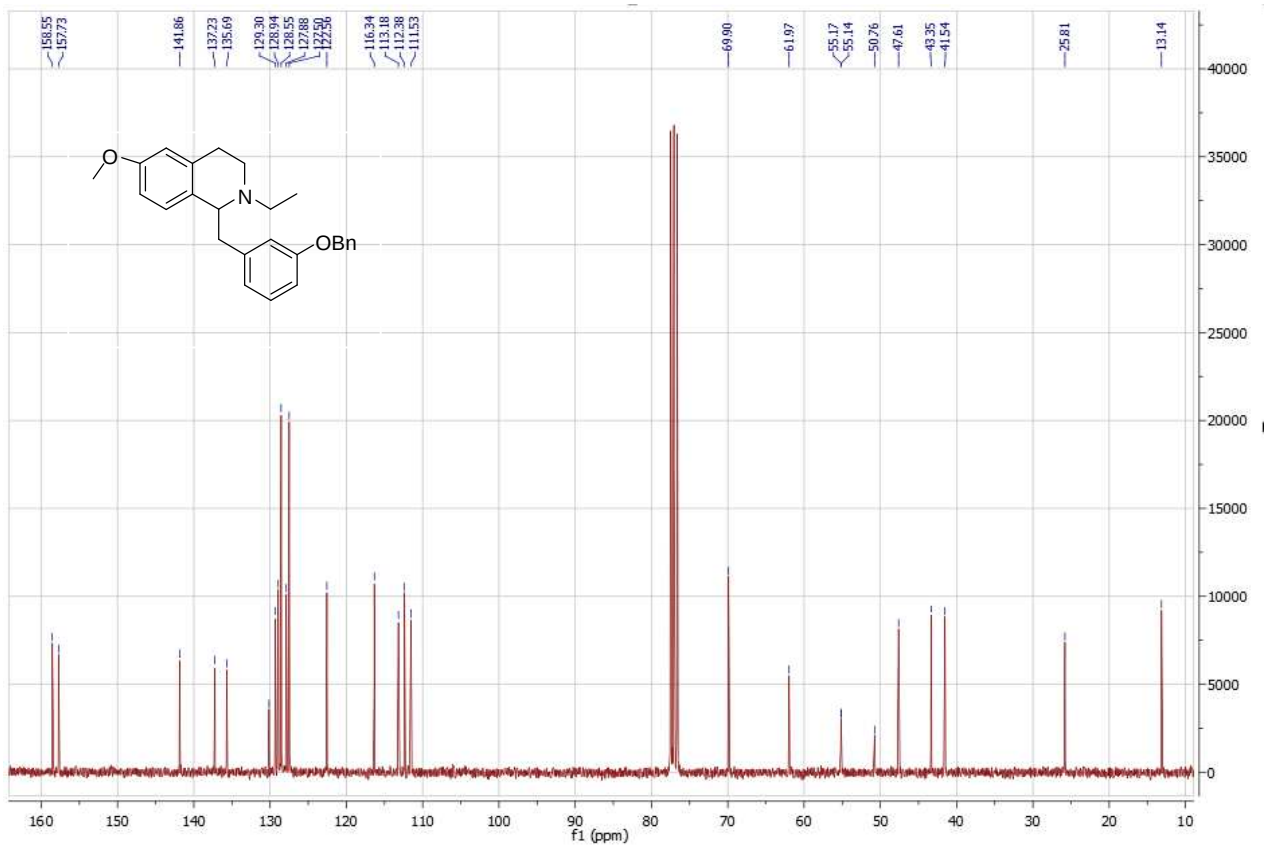

Figure 77: ^{13}C-NMR spectrum of **2b**

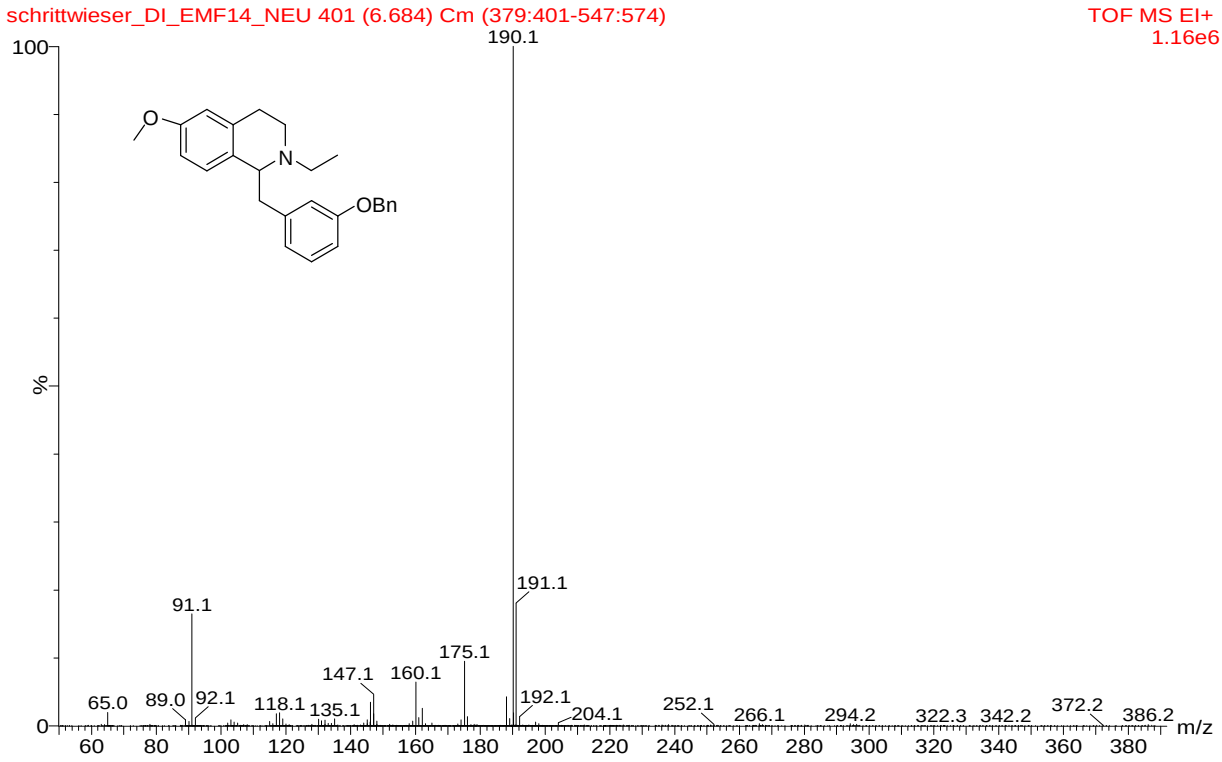

Figure 78: MS spectrum of **2b**

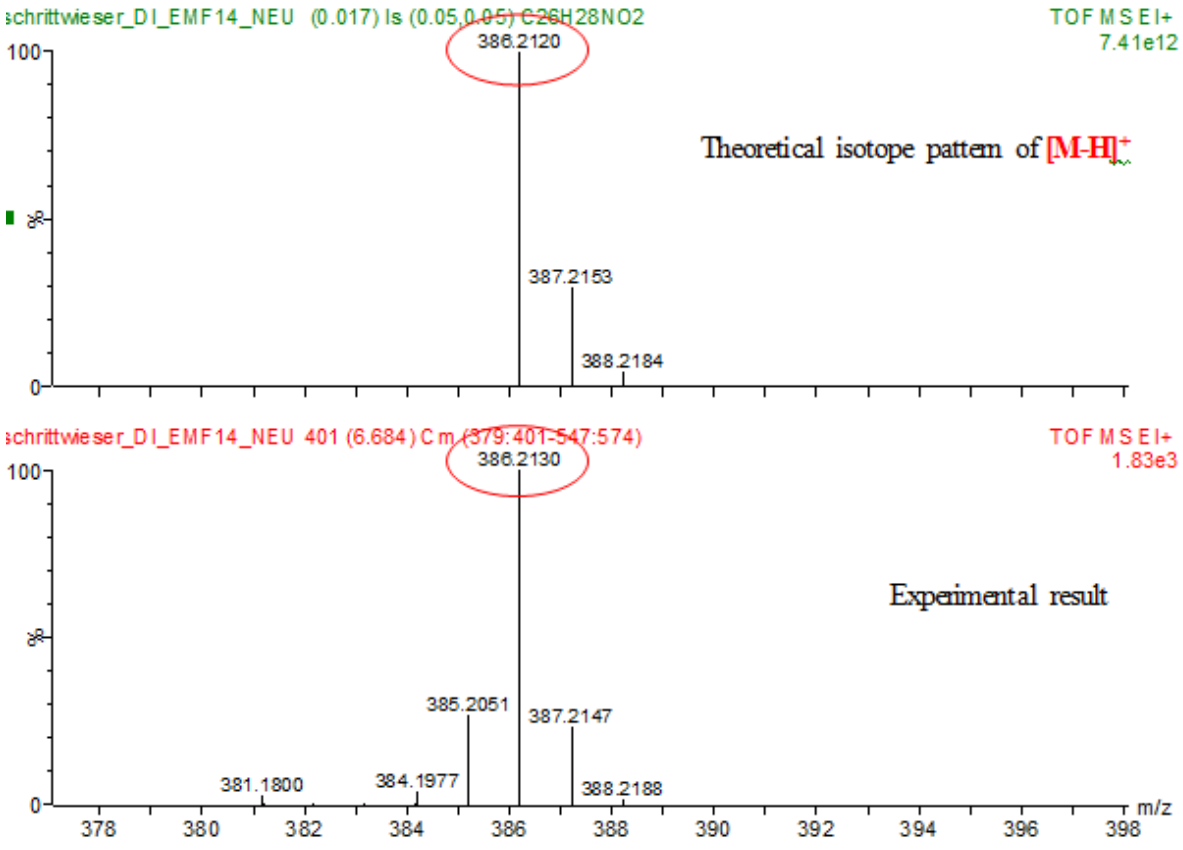

Figure 79: HR-MS spectrum of **2b**

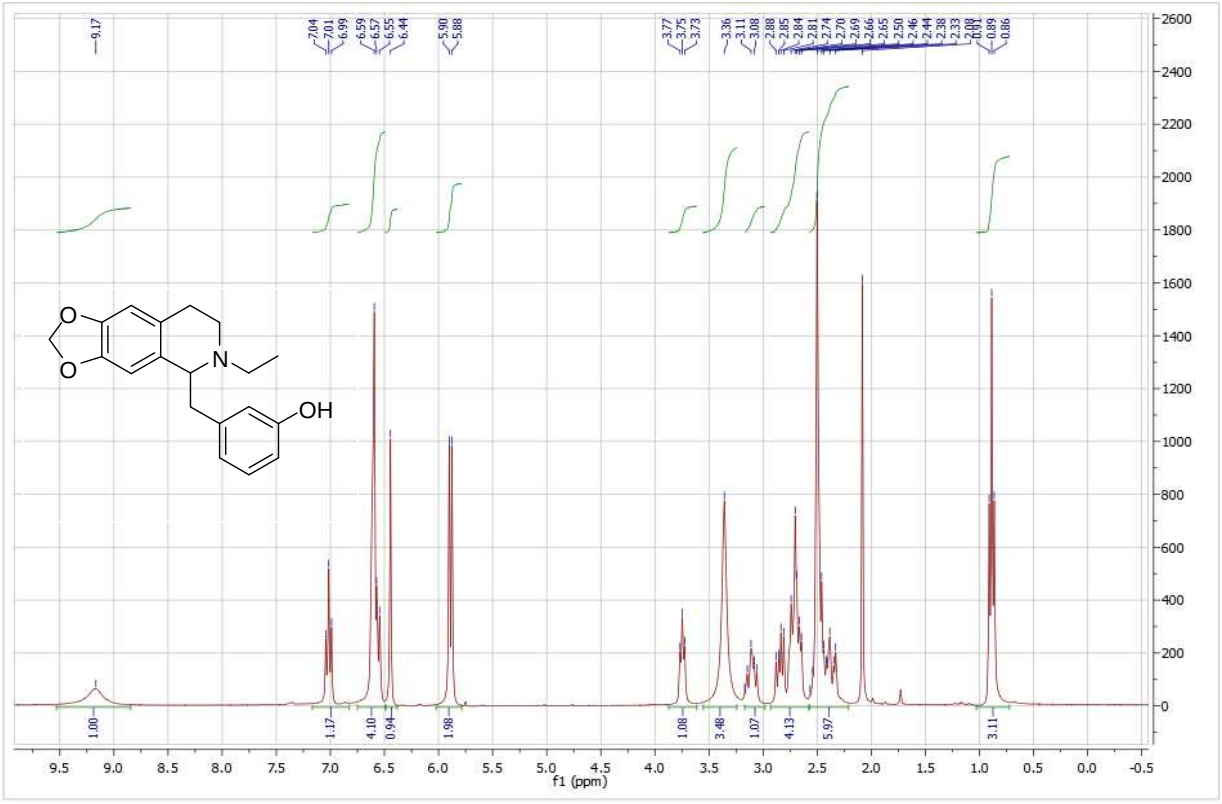

Figure 80: ^{1}H-NMR spectrum of **1a**

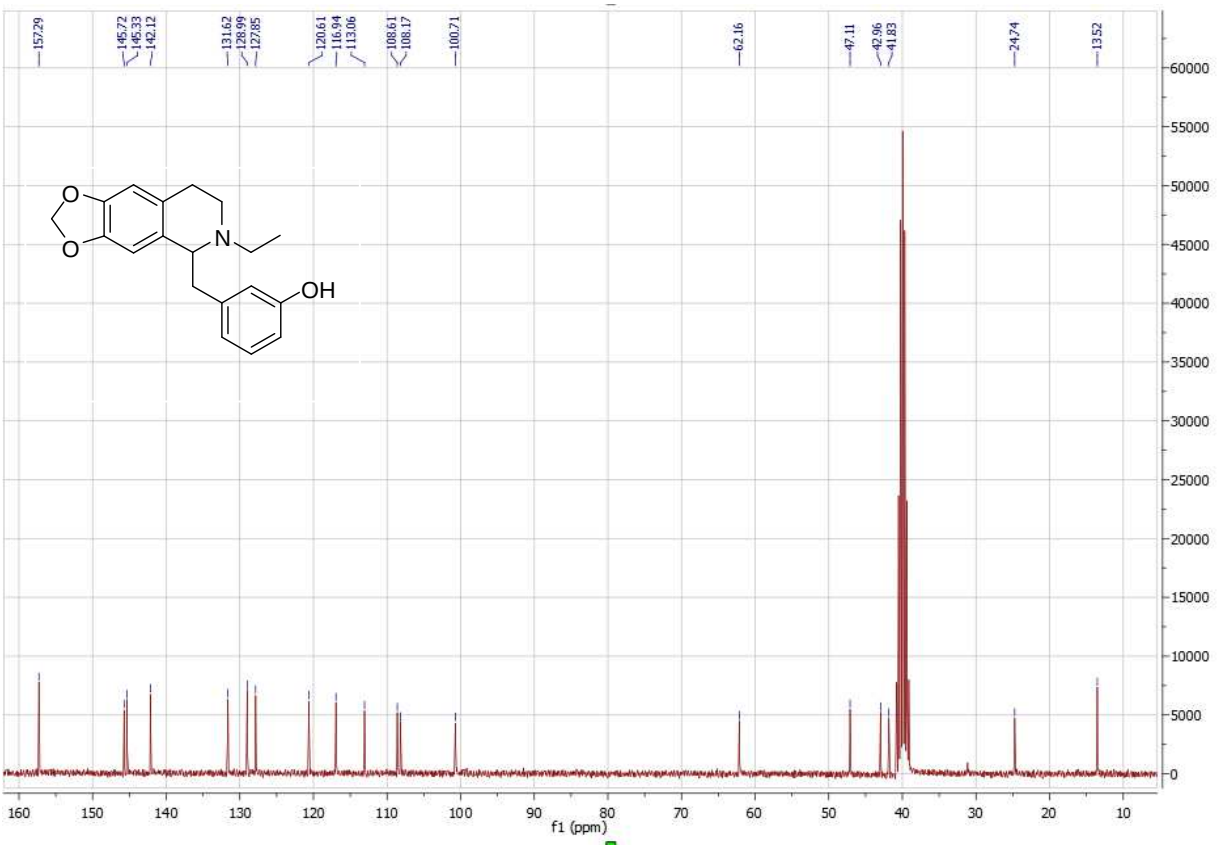

Figure 81: ^{13}C-NMR spectrum of **1a**

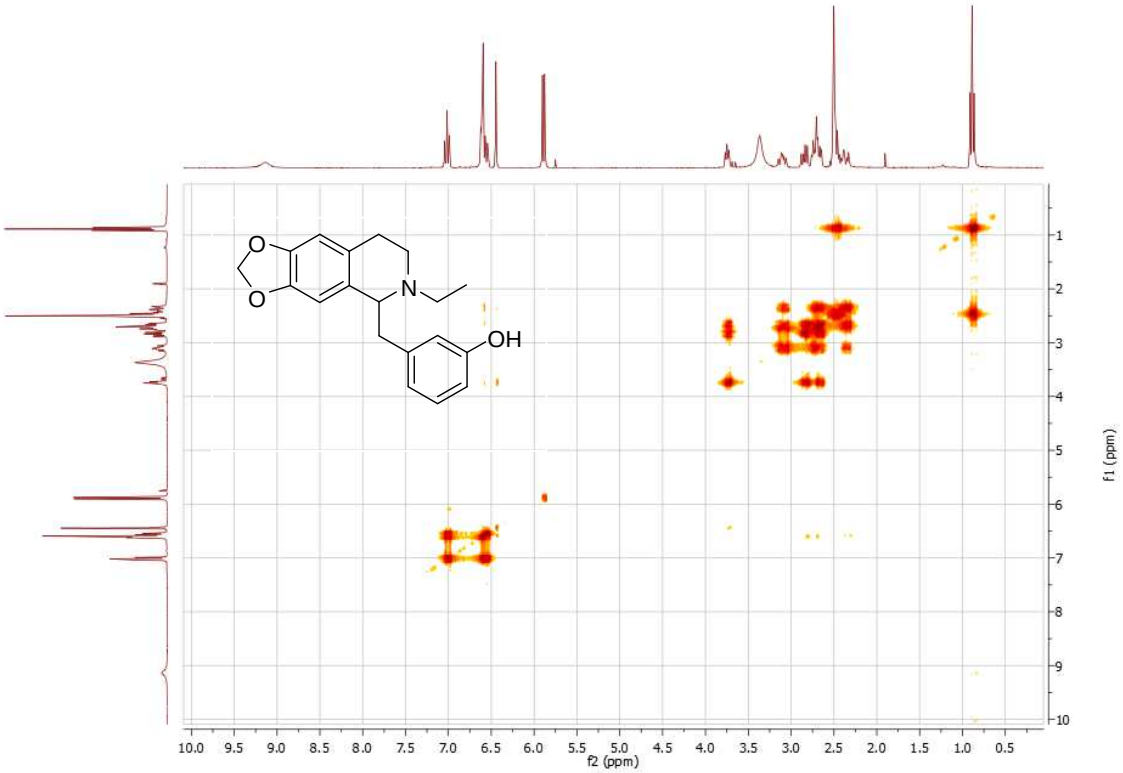

Figure 82: COSY spectrum of **1a**

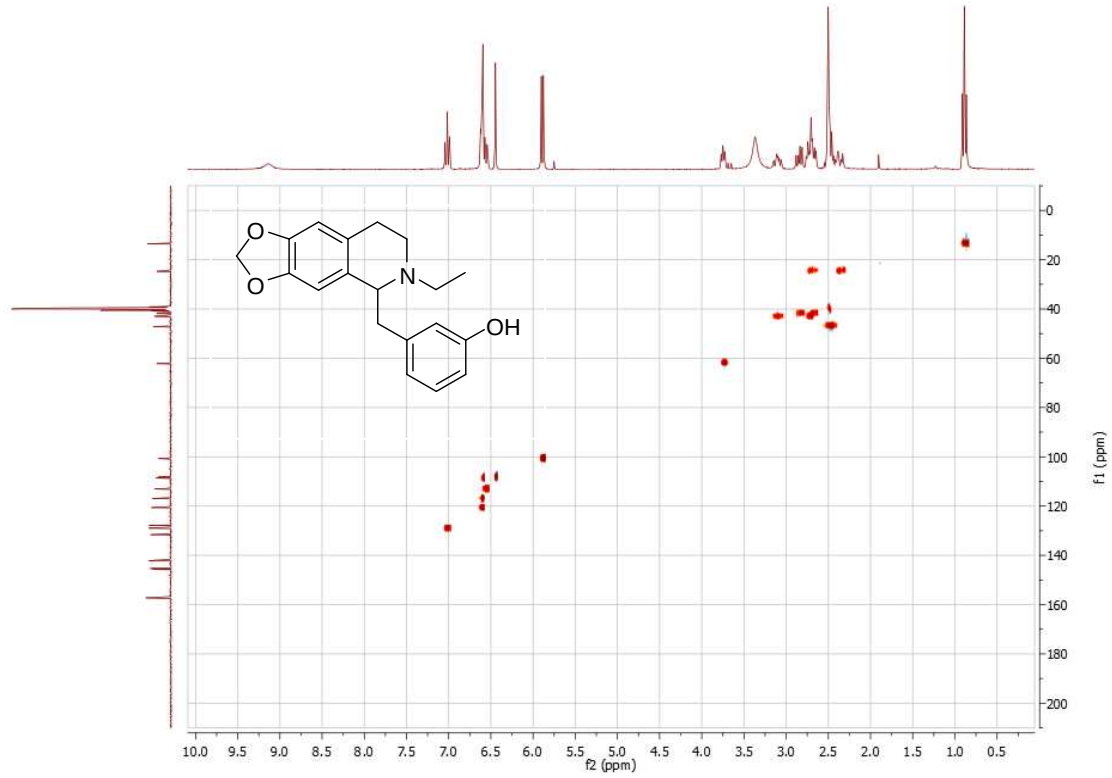

Figure 83: HSQC spectrum of **1a**

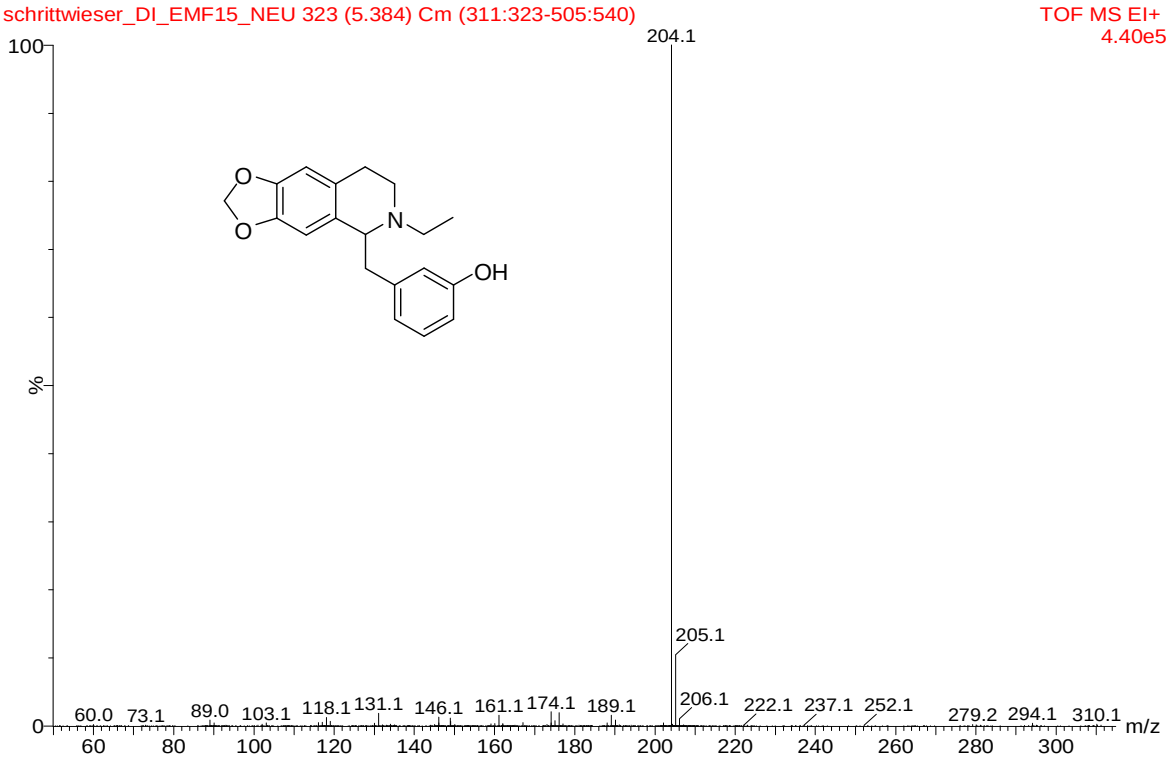

Figure 84: MS spectrum of **1a**

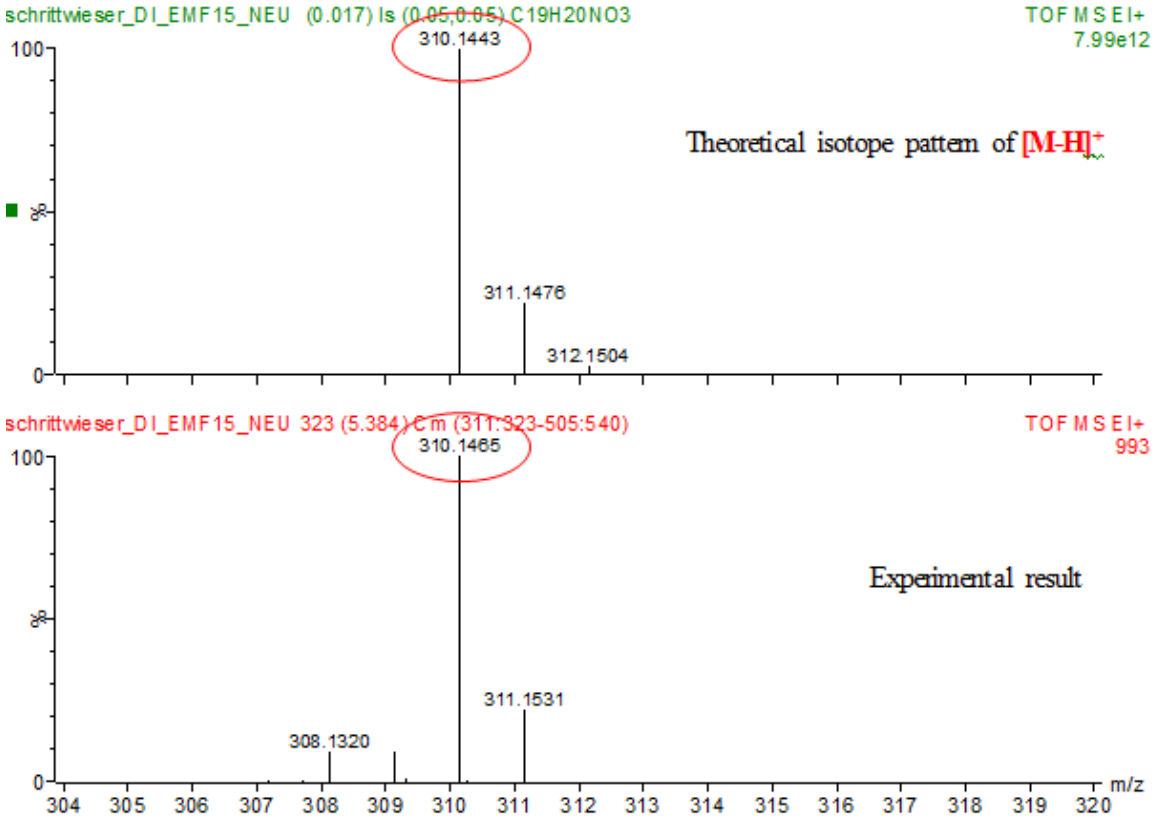

Figure 85: HR-MS spectrum of **1a**

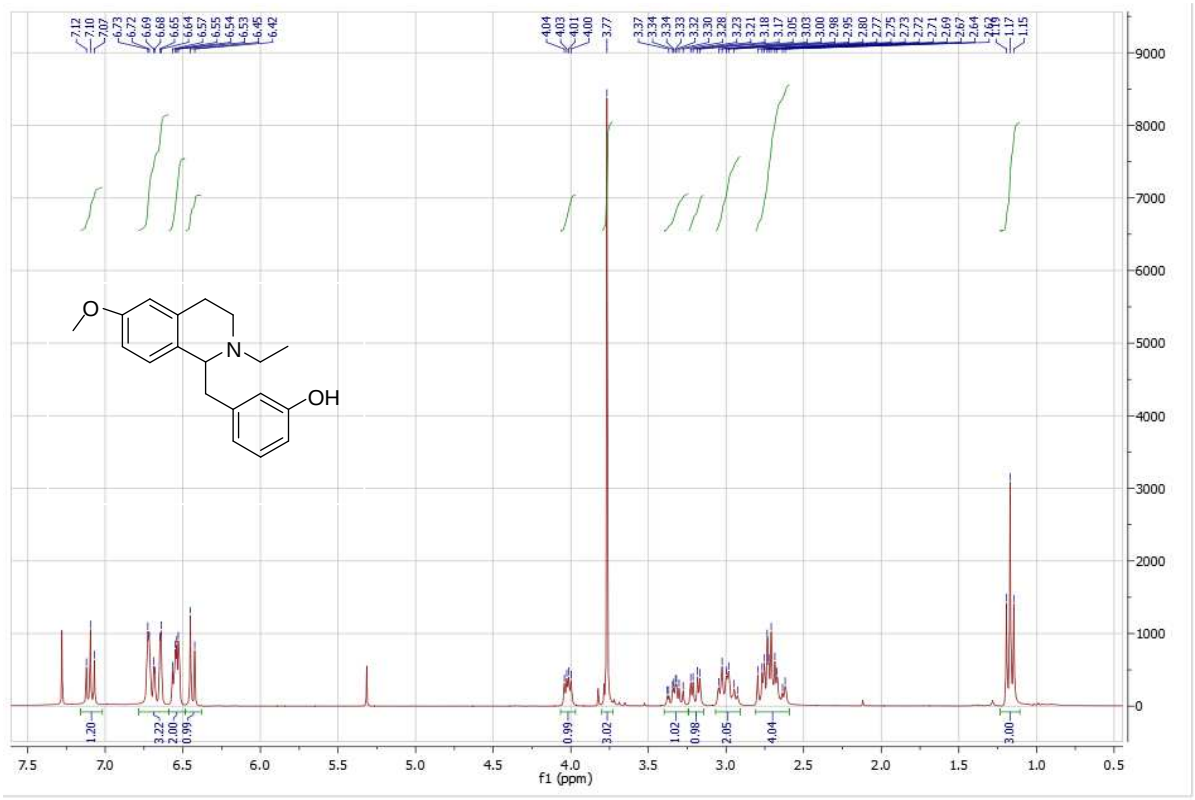

Figure 86: ^{1}H-NMR spectrum of **1b**

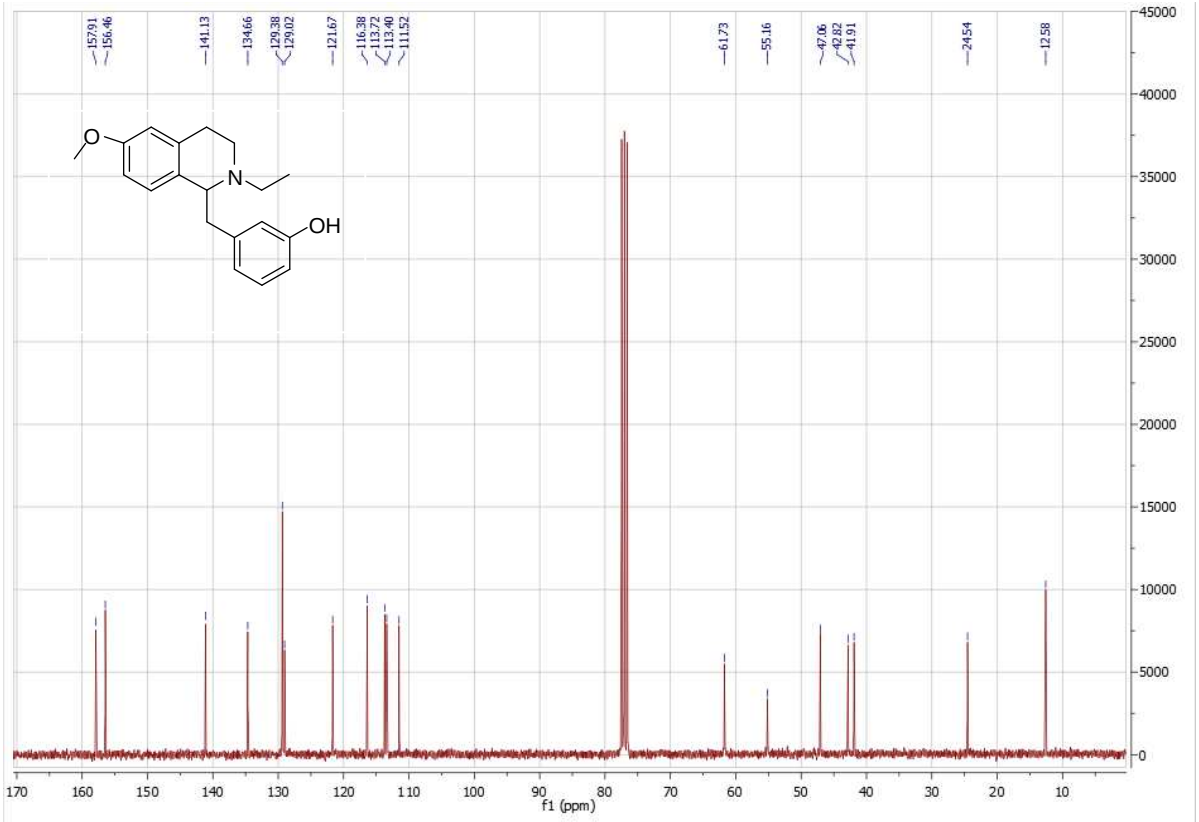

Figure 87: ^{13}C-NMR spectrum of **1b**

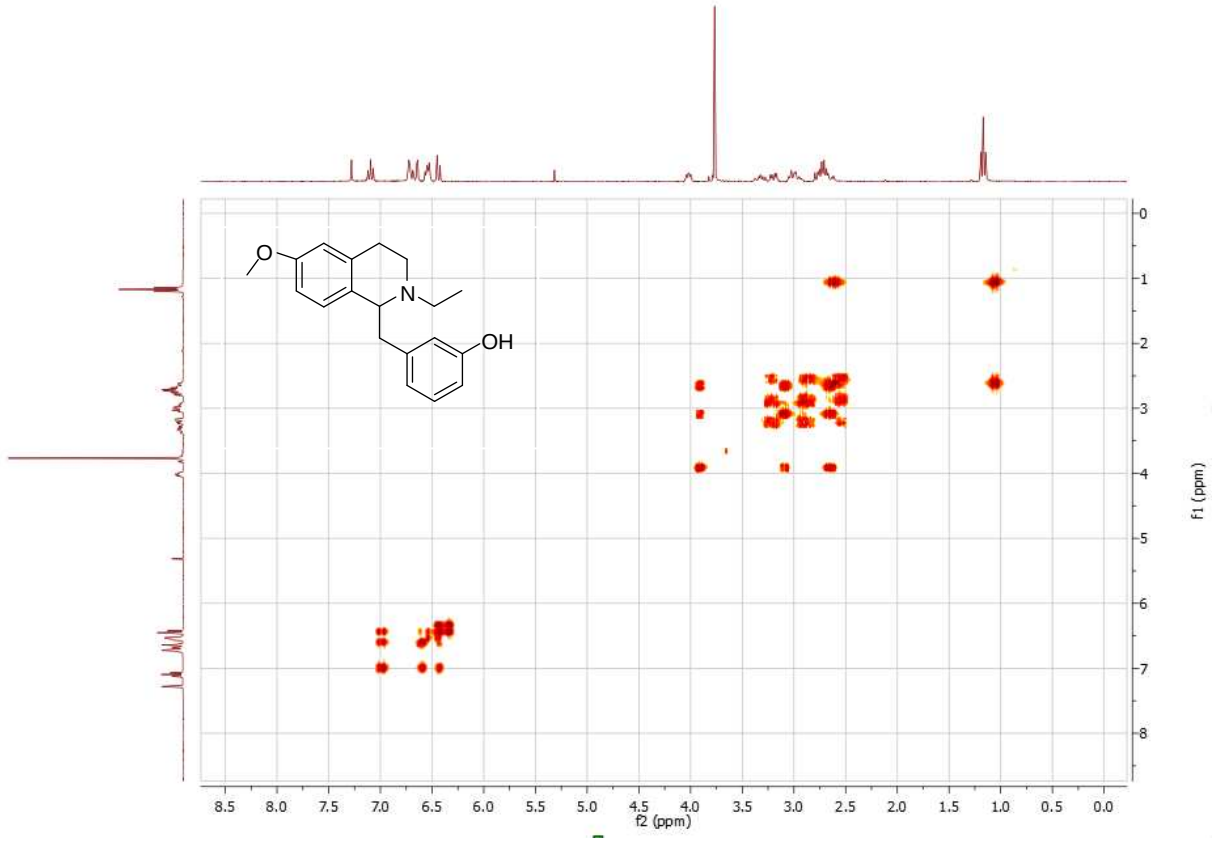

Figure 88: COSY-spectrum of **1b**

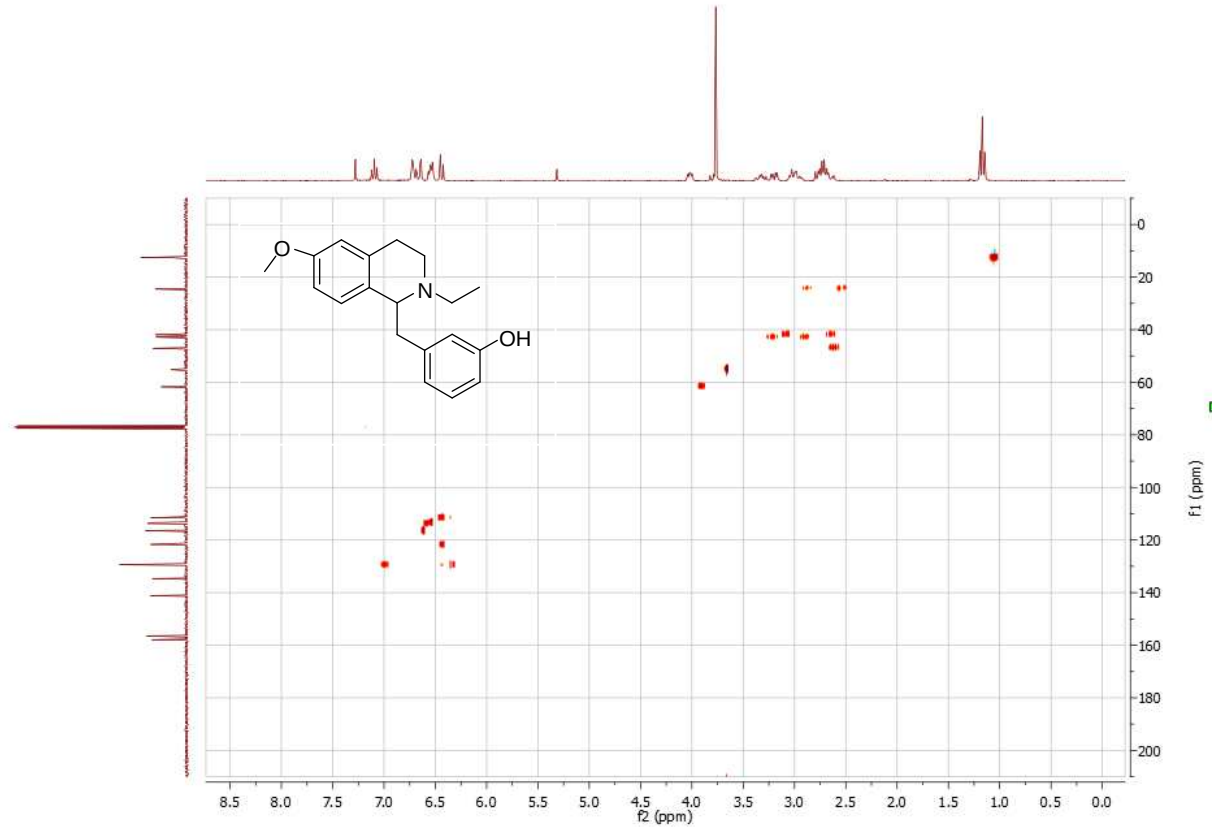

Figure 89: HSQC spectrum of **1b**

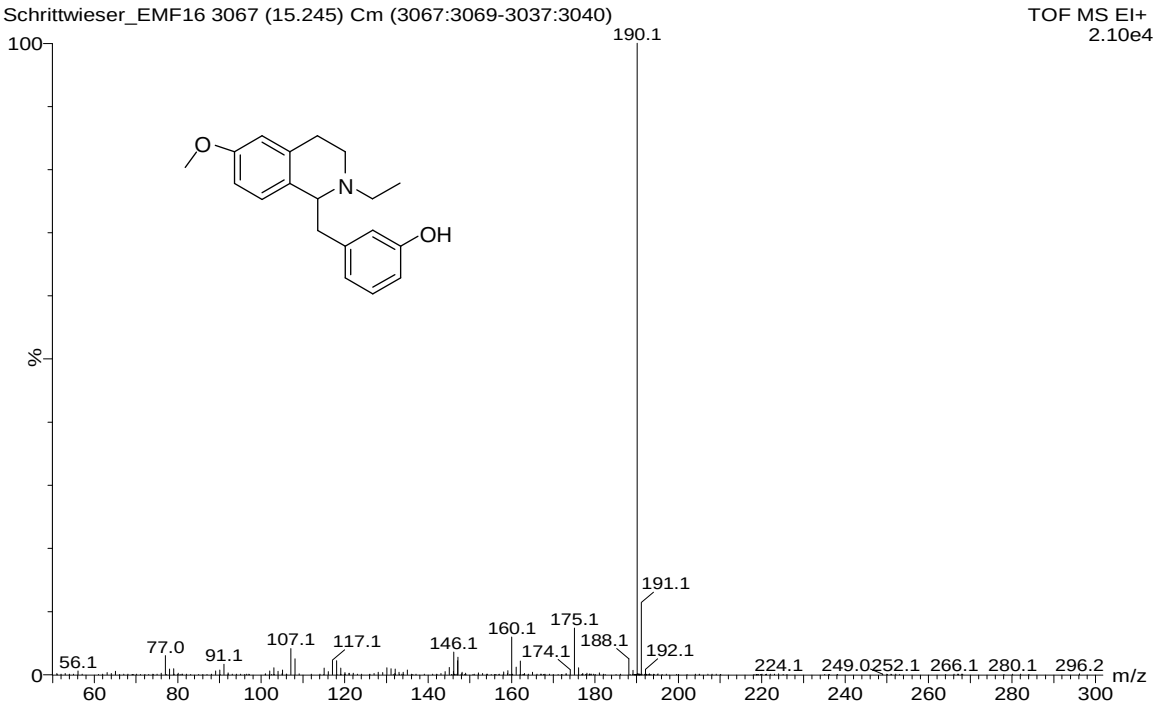

Figure 90: MS spectrum of **1b**

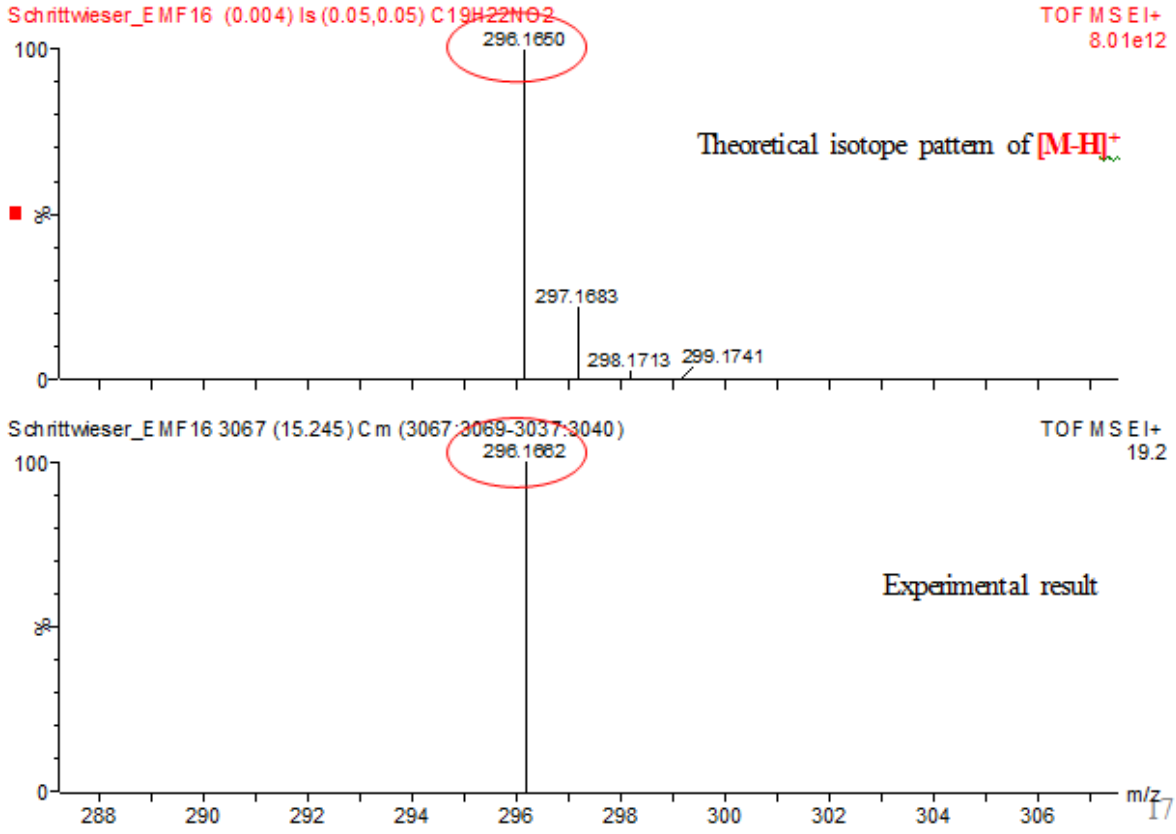

Figure 91: HR-MS spectrum of **1b**

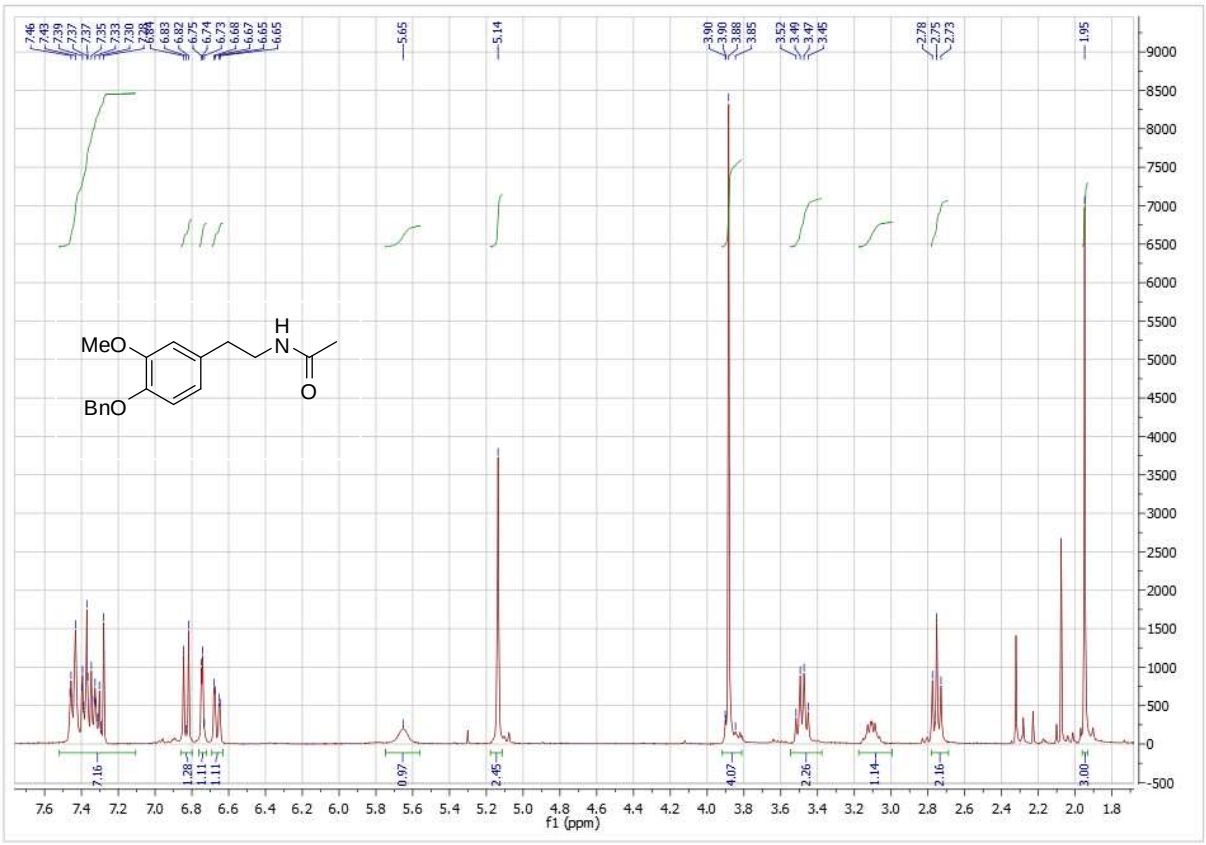

Figure 92: ^{1}H-NMR spectrum of **5c**

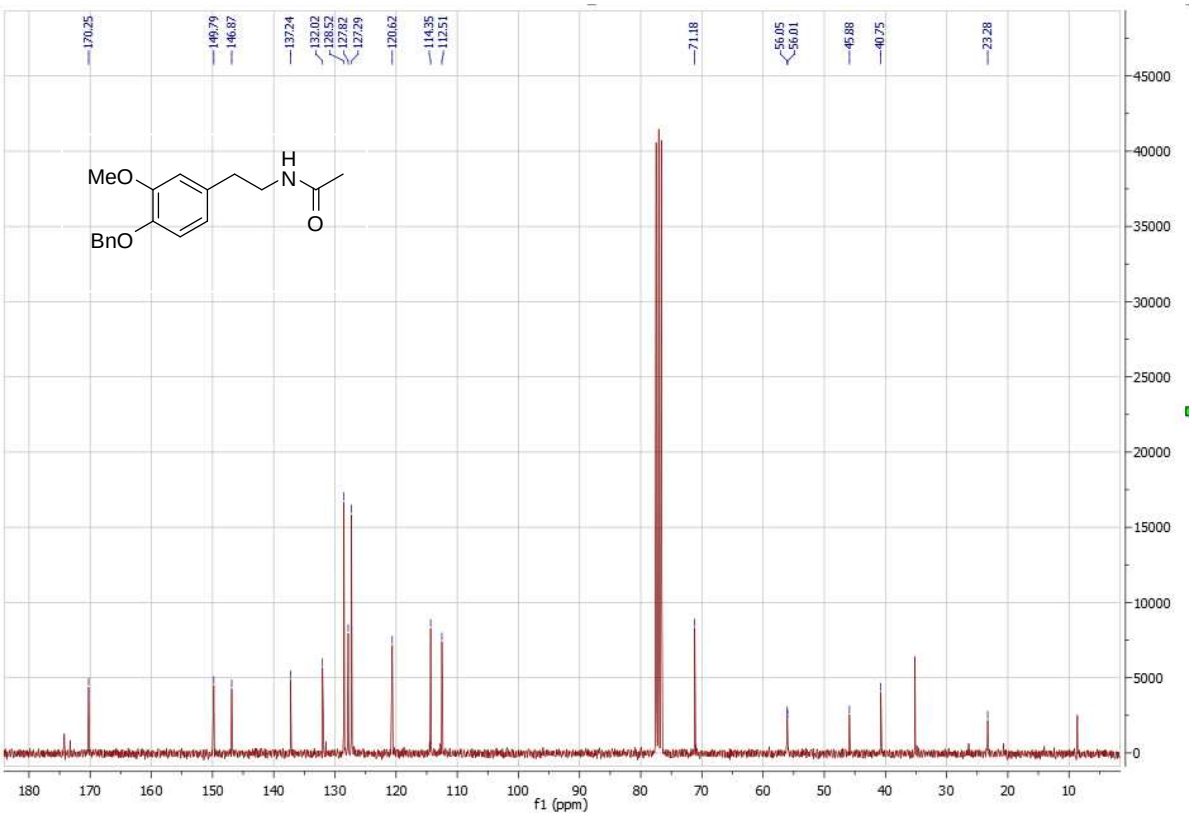

Figure 93: ^{13}C-NMR spectrum of **5c**

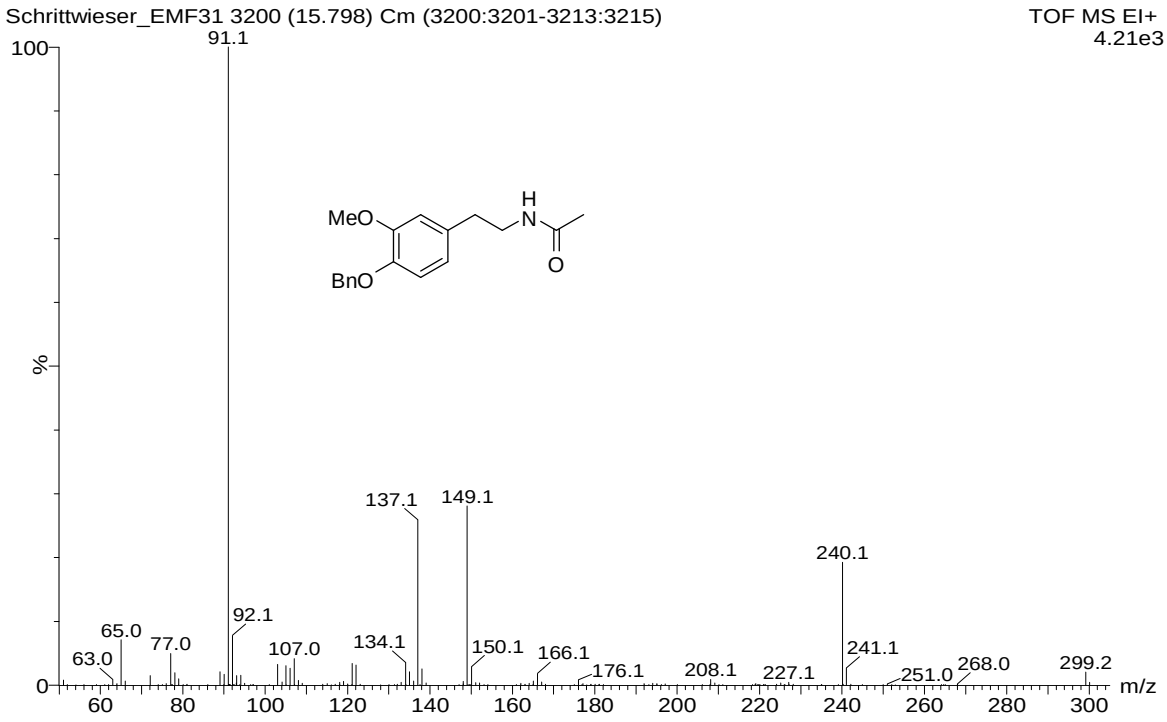

Figure 94: MS spectrum **5c**

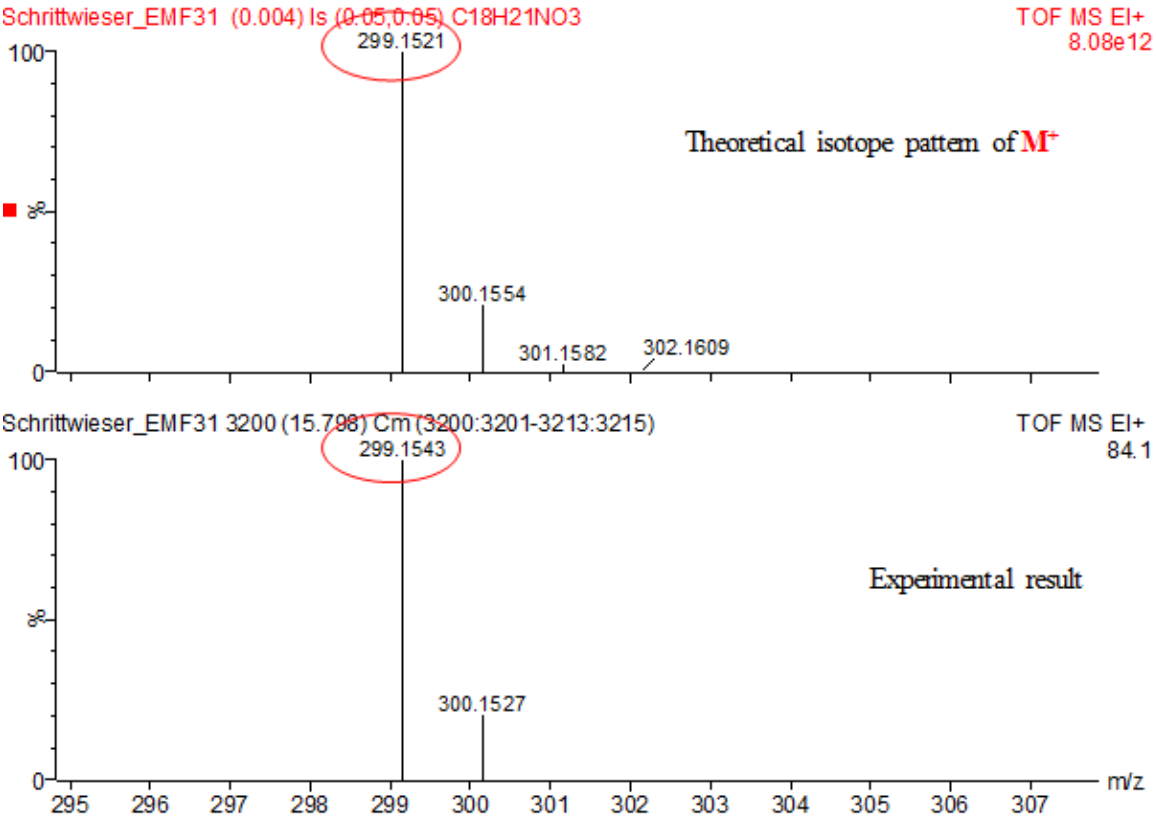

Figure 95: HR-MS spectrum of **5c**

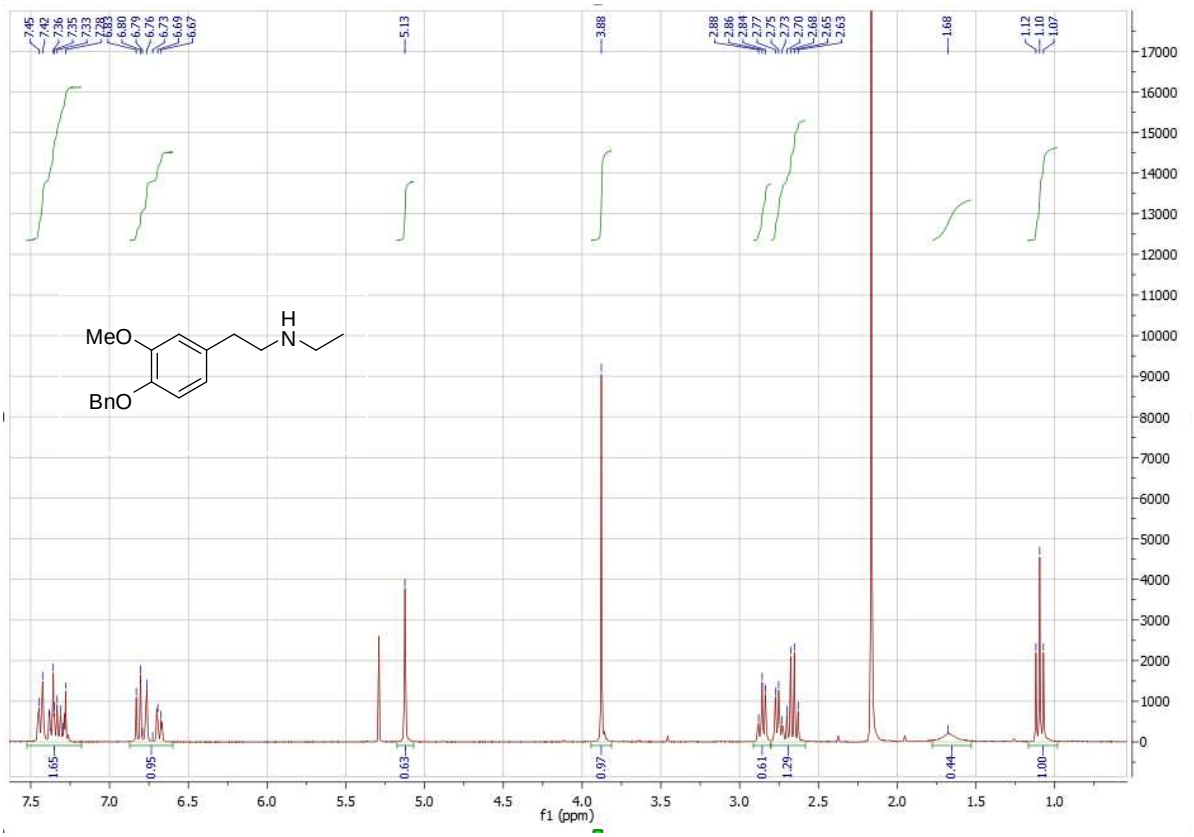

Figure 95: ^{1}H-NMR spectrum of **4c**

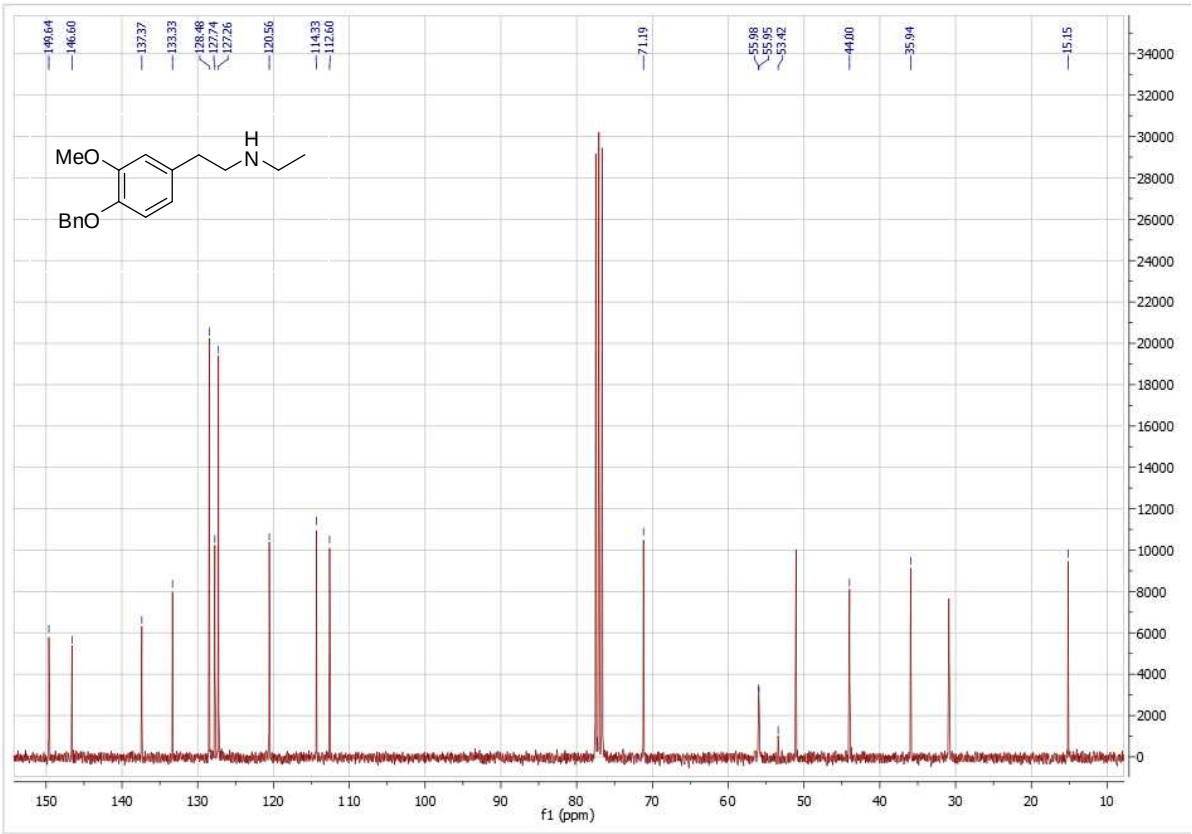

Figure 96: ^{13}C-NMR spectrum of **4c**

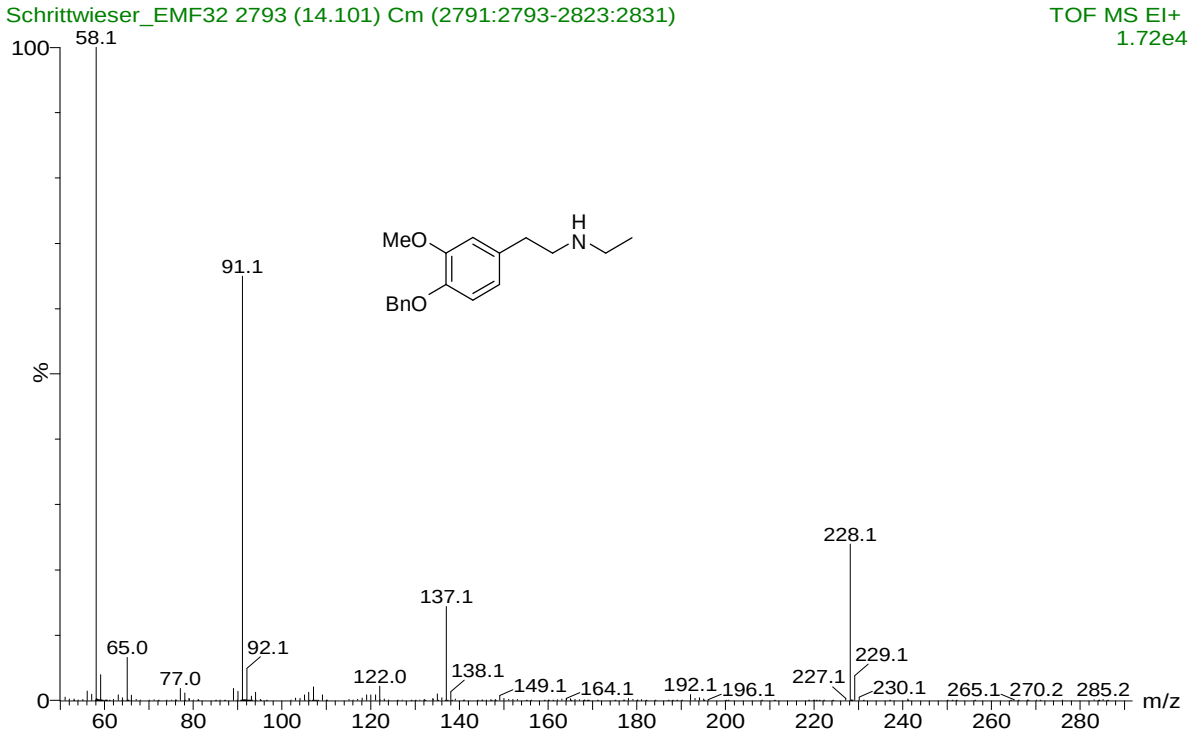

Figure 97:MS spectrum of **4c**

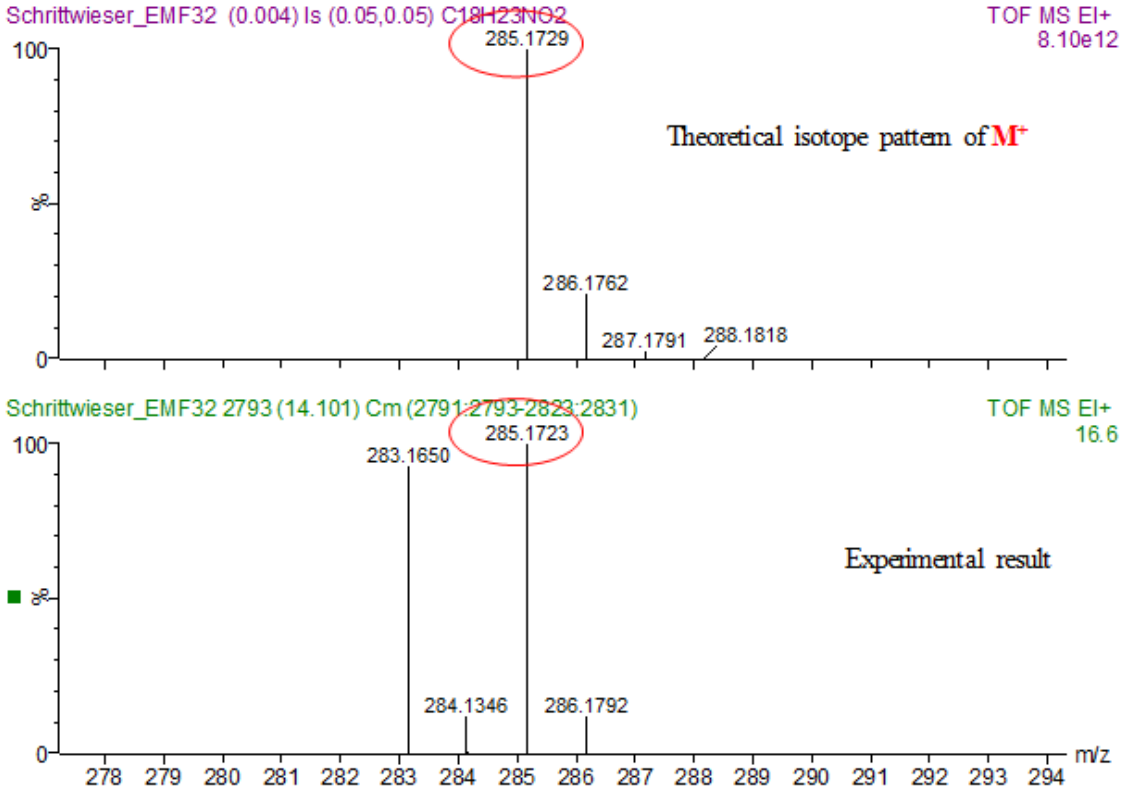

Figure 98: HR-MS spectrum of **4c**

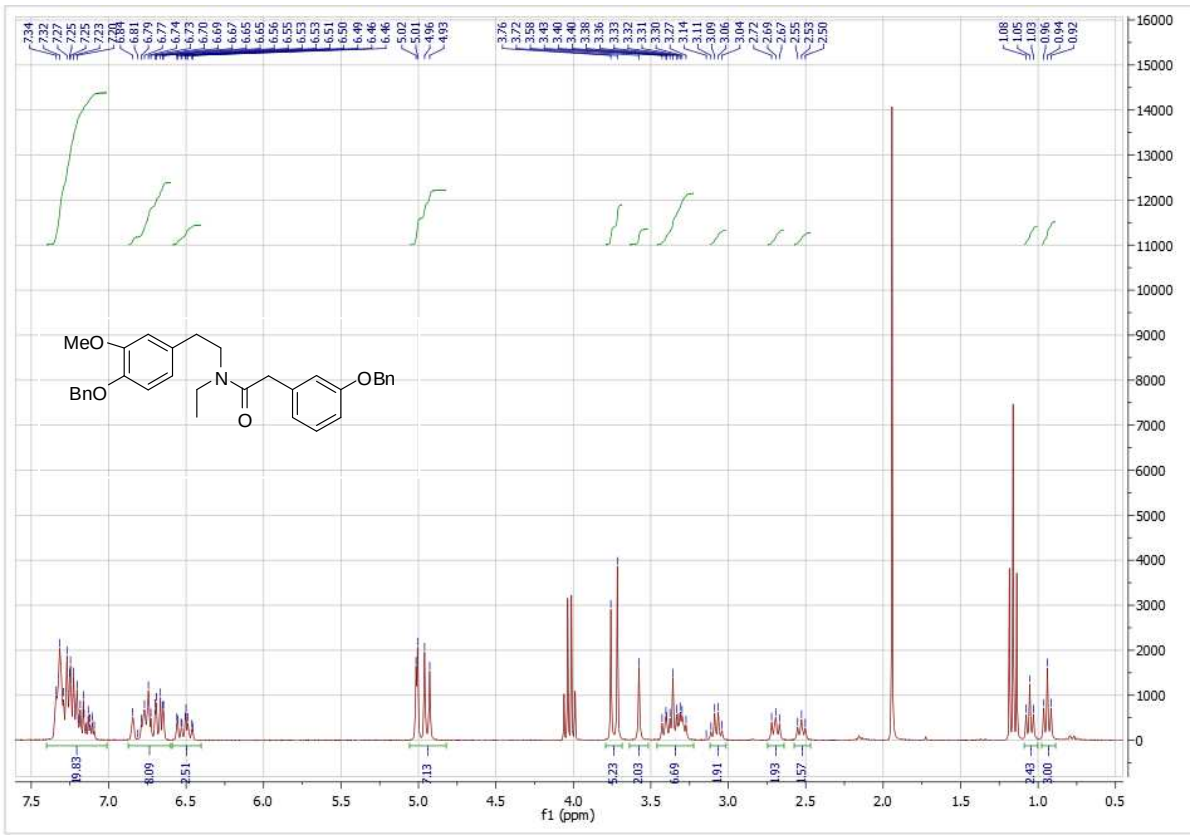

Figure 99: ^{1}H-NMR spectrum of **3c**

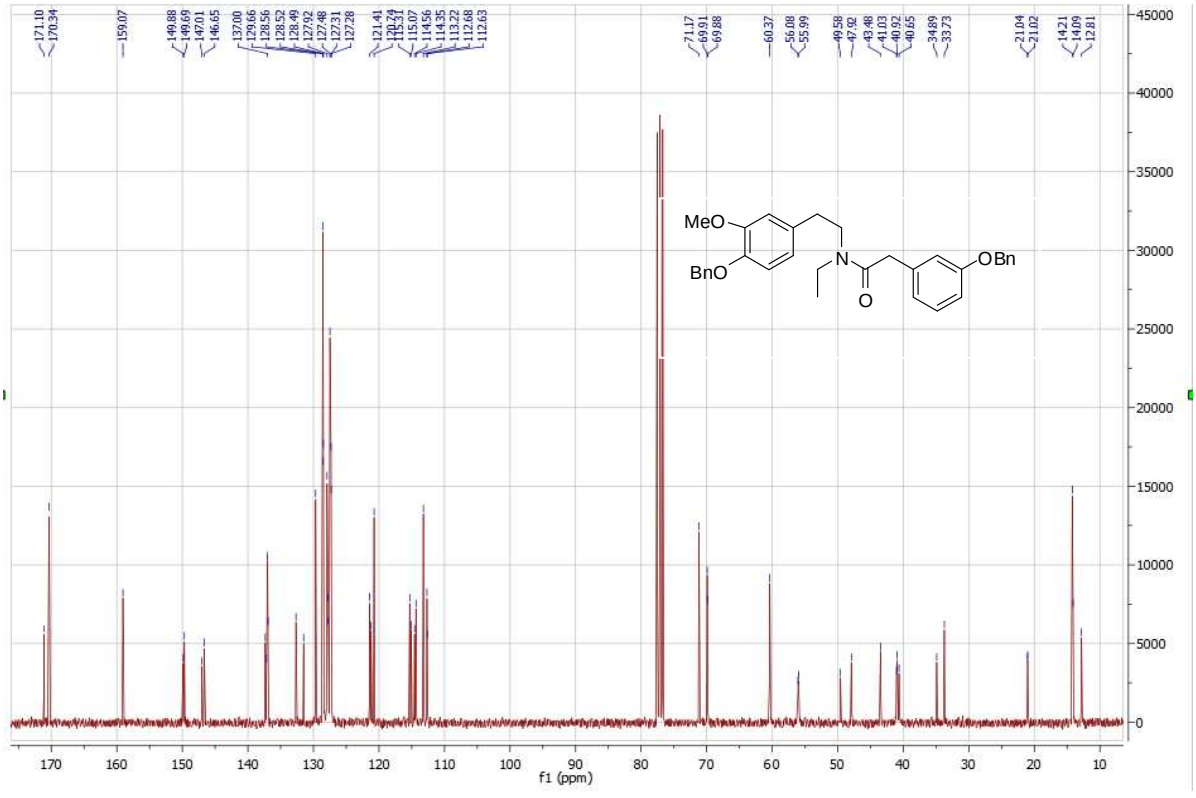

Figure 100: ^{13}C-NMR spectrum of **3c**

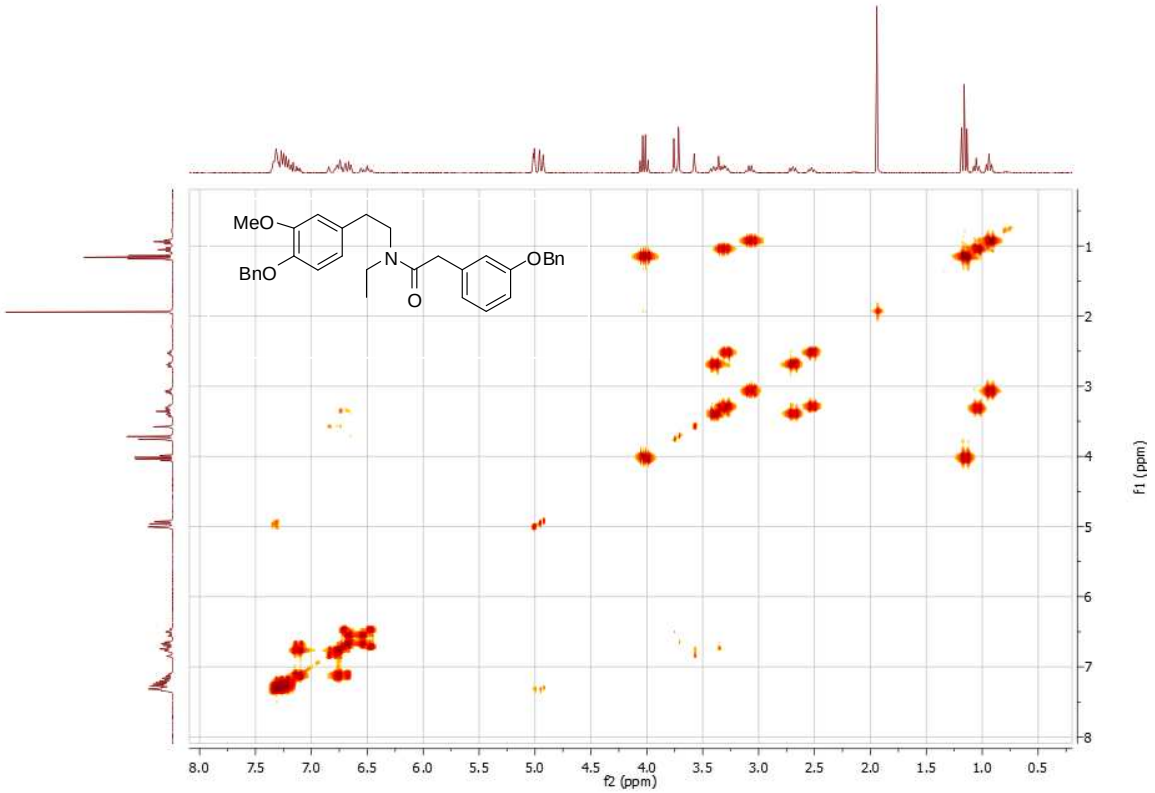

Figure 101: COSY-spectrum of **3c**

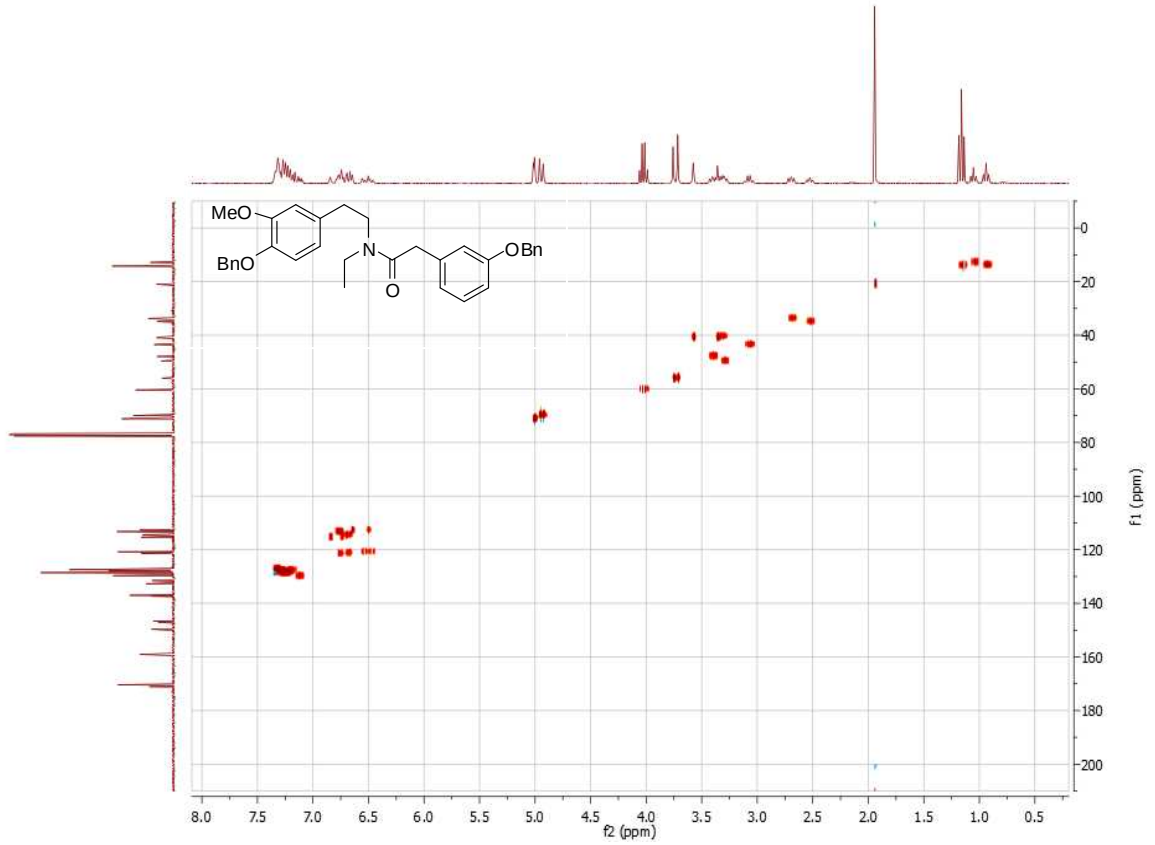

Figure 102: HSQC-spectrum of **3c**

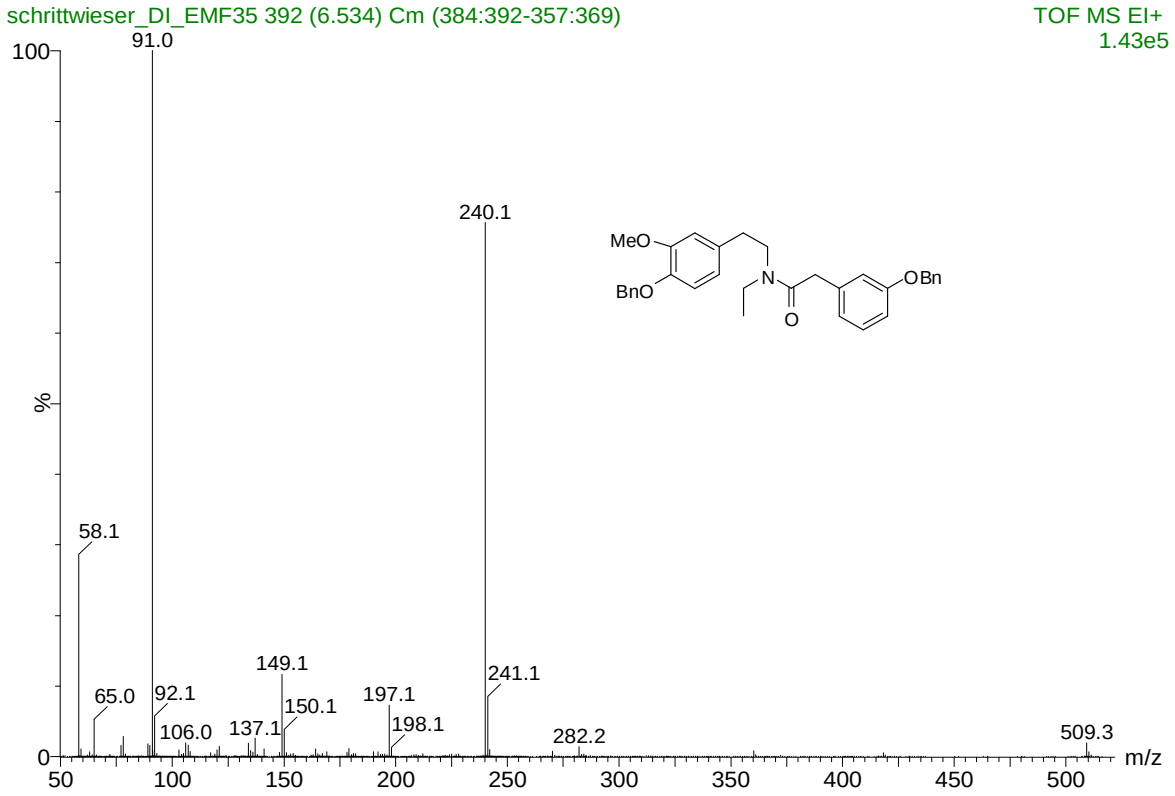

Figure 103: MS spectrum of **3c**

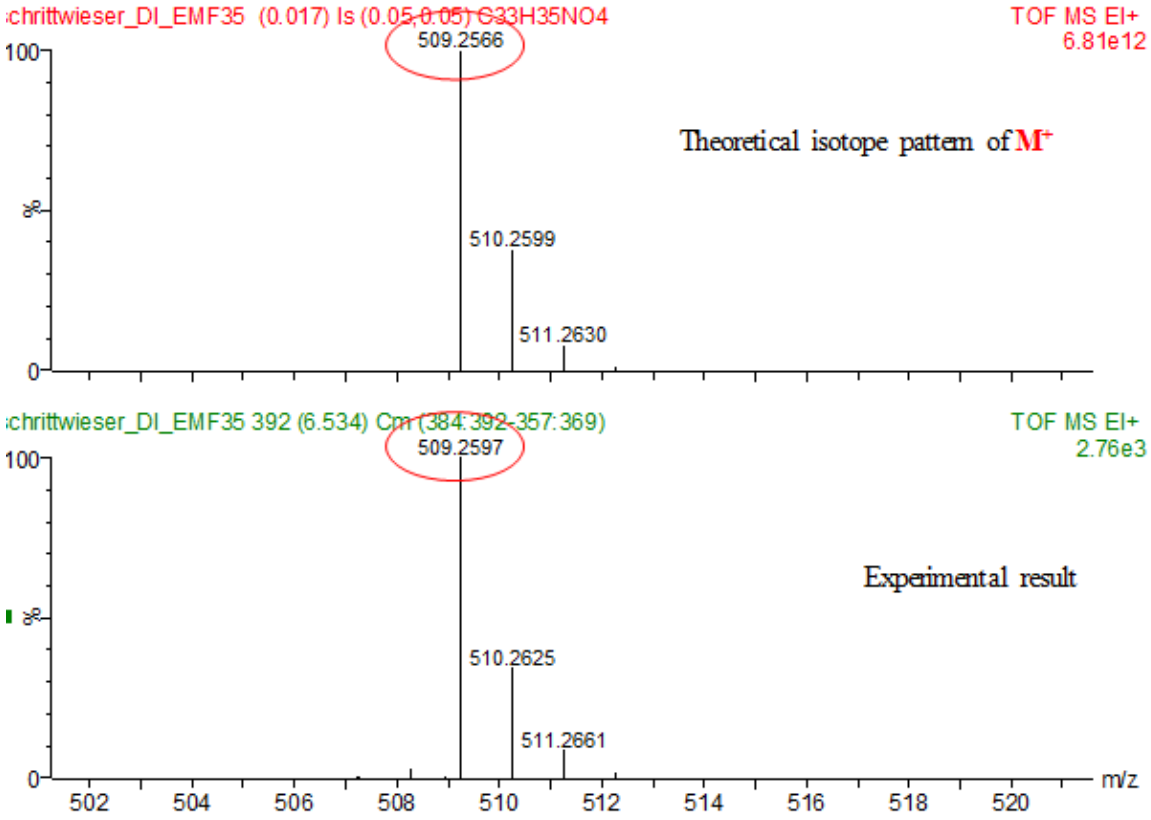

Figure 104: HR-MS spectrum of **3c**

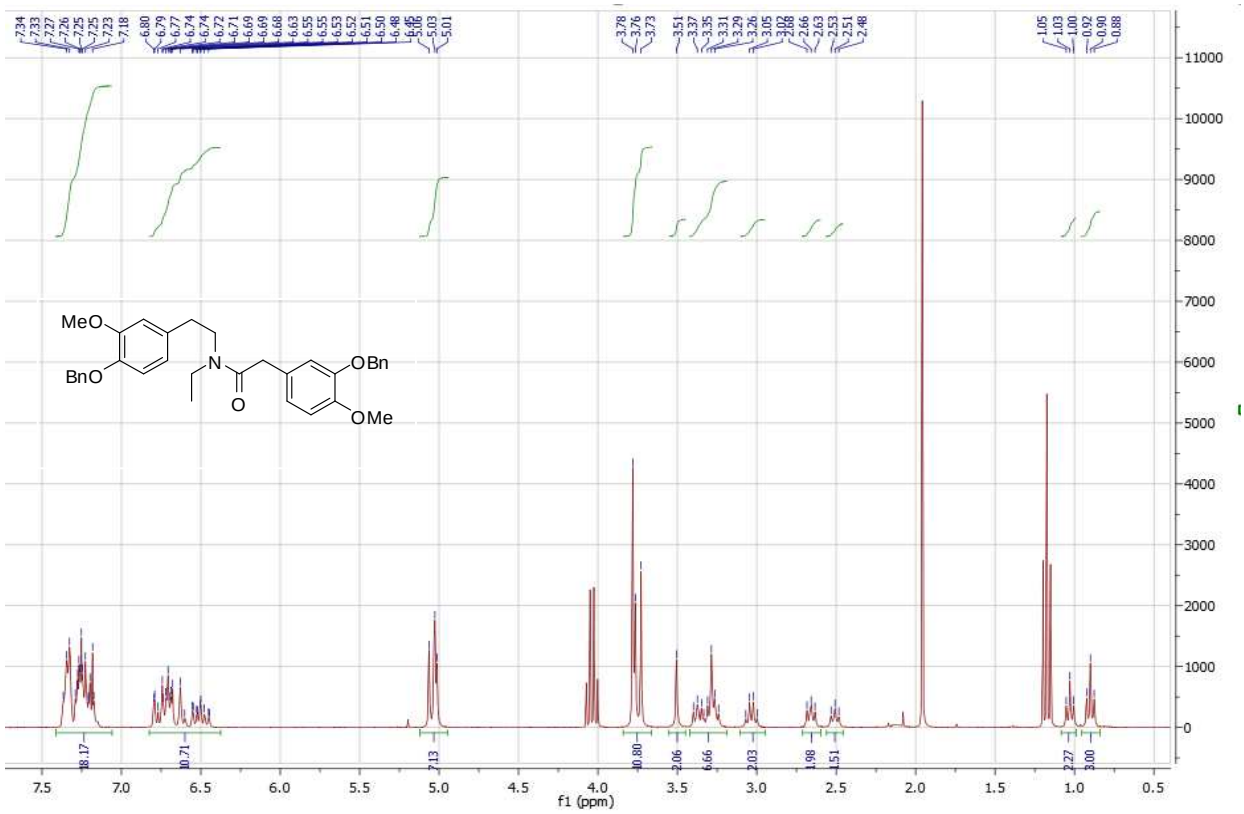

Figure 105: [1]H-NMR spectrum of **3d**

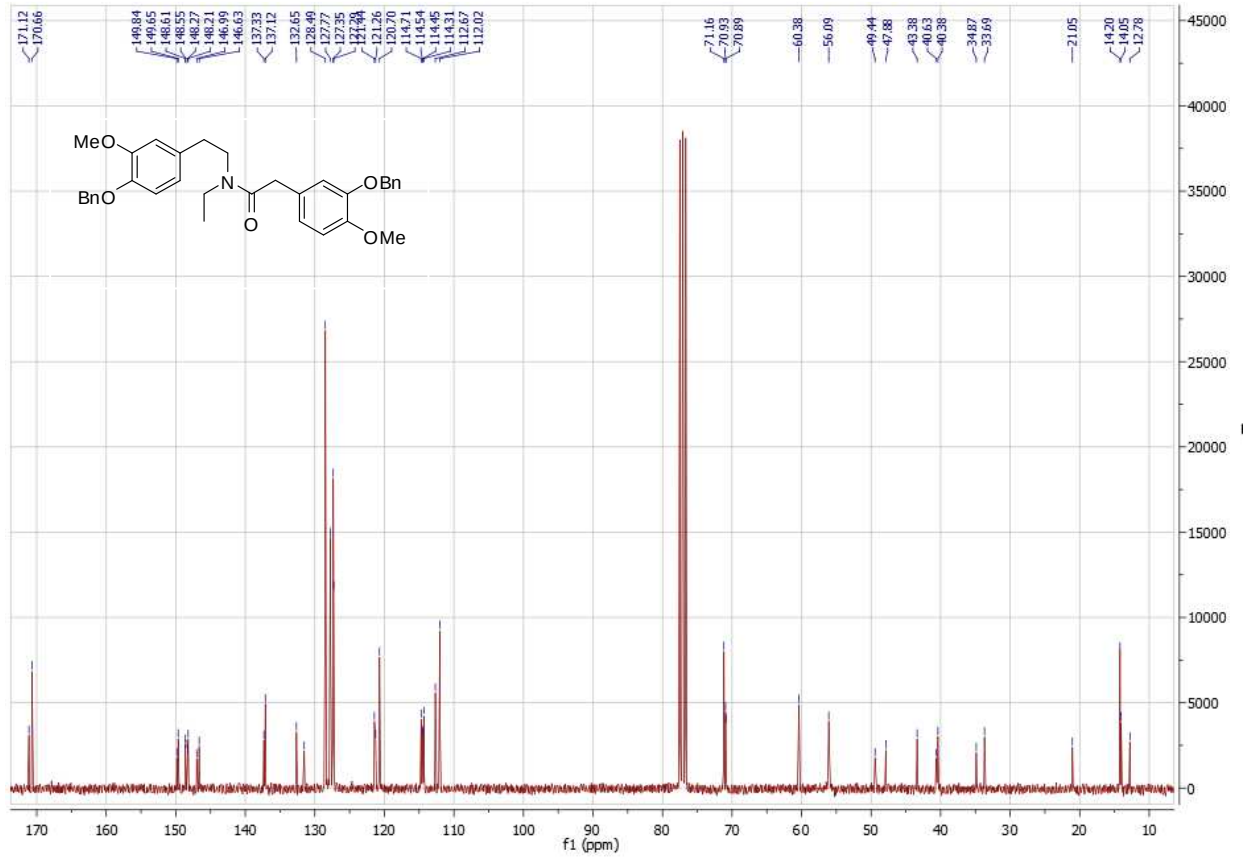

Figure 106: [13]C-NMR spectrum of **3d**

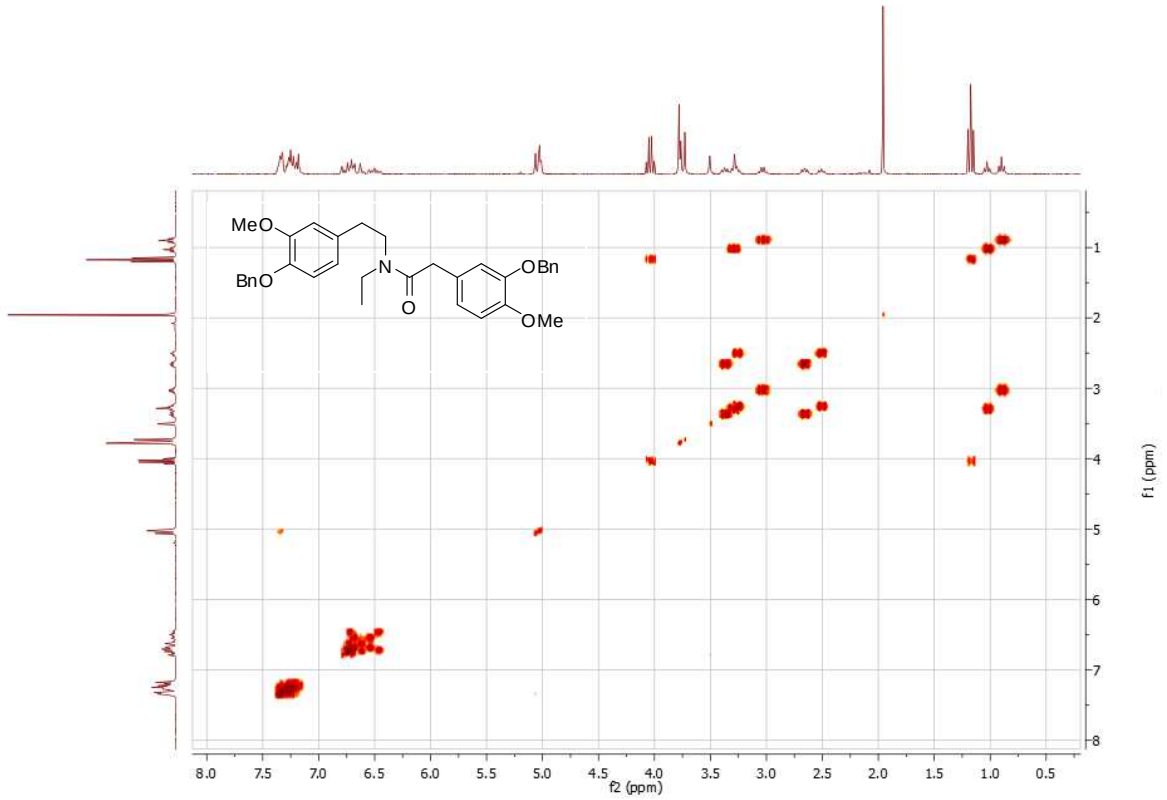

Figure 107: COSY-spectrum of **3d**

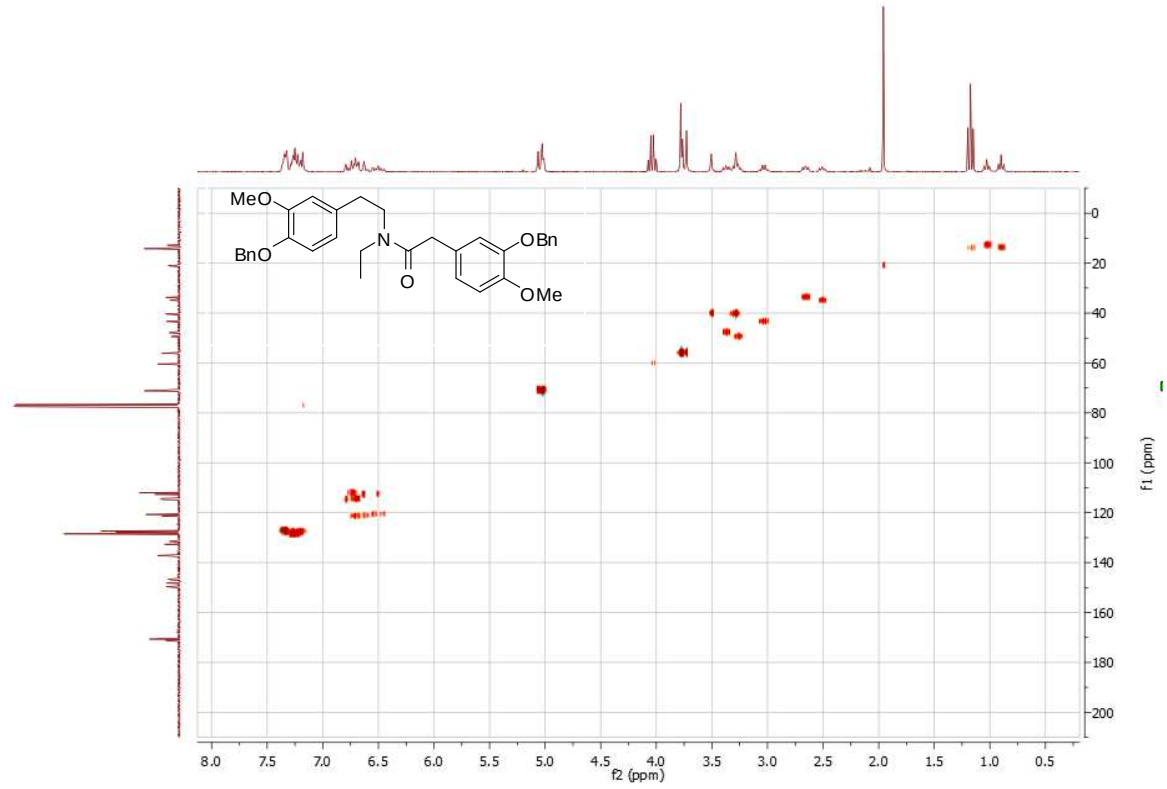

Figure 107: HSQC-spectrum of **3d**

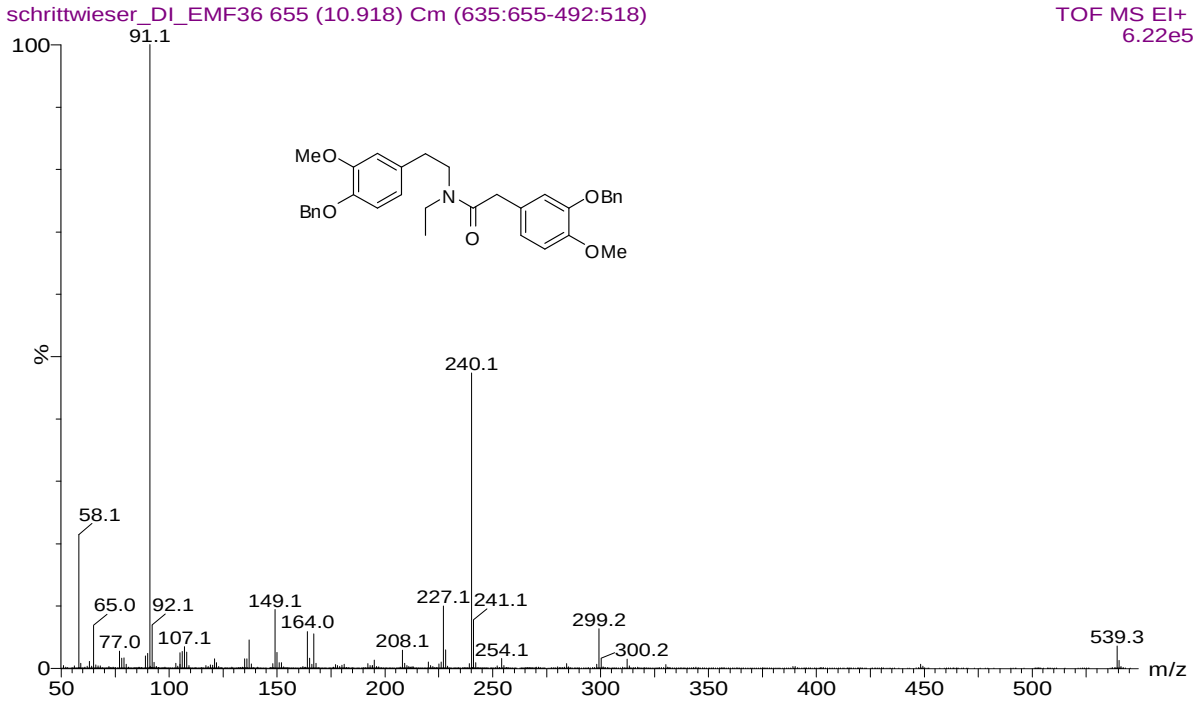

Figure 108: MS spectrum of **3d**

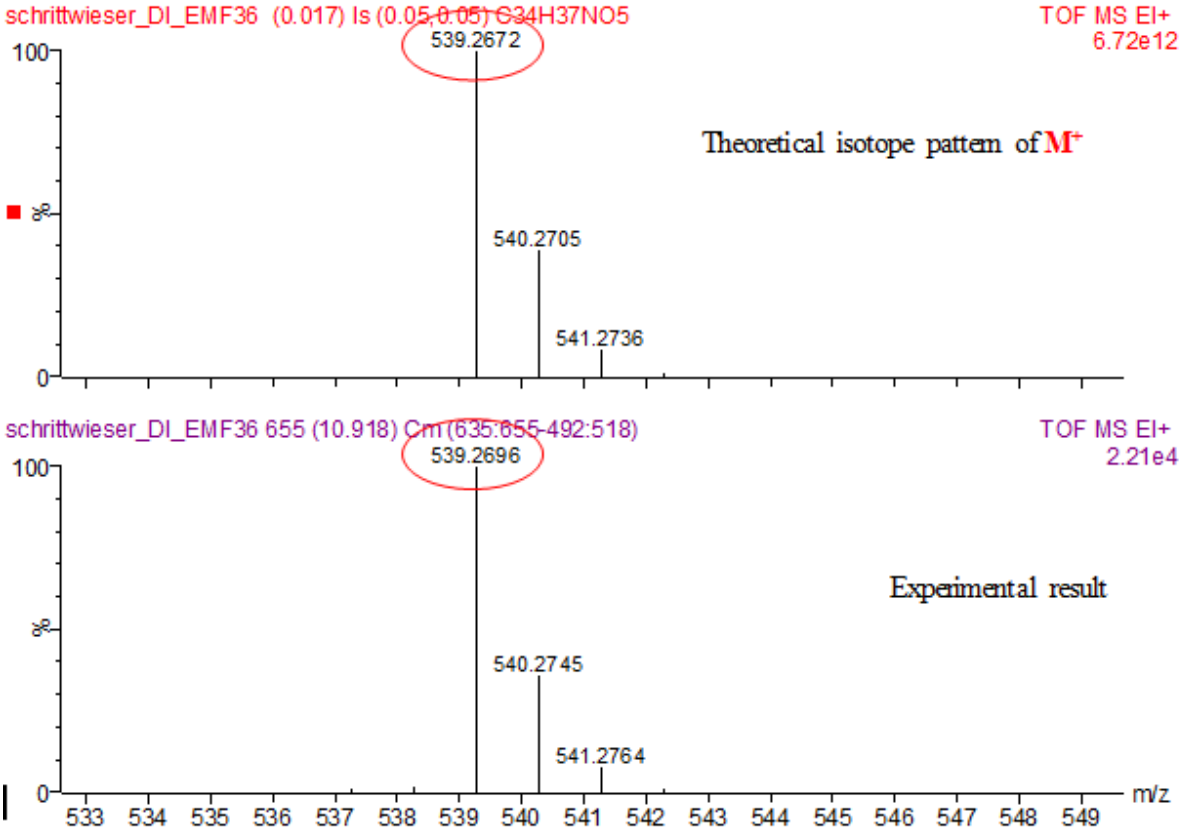

Figure 109: HR-MS spectrum of **3d**

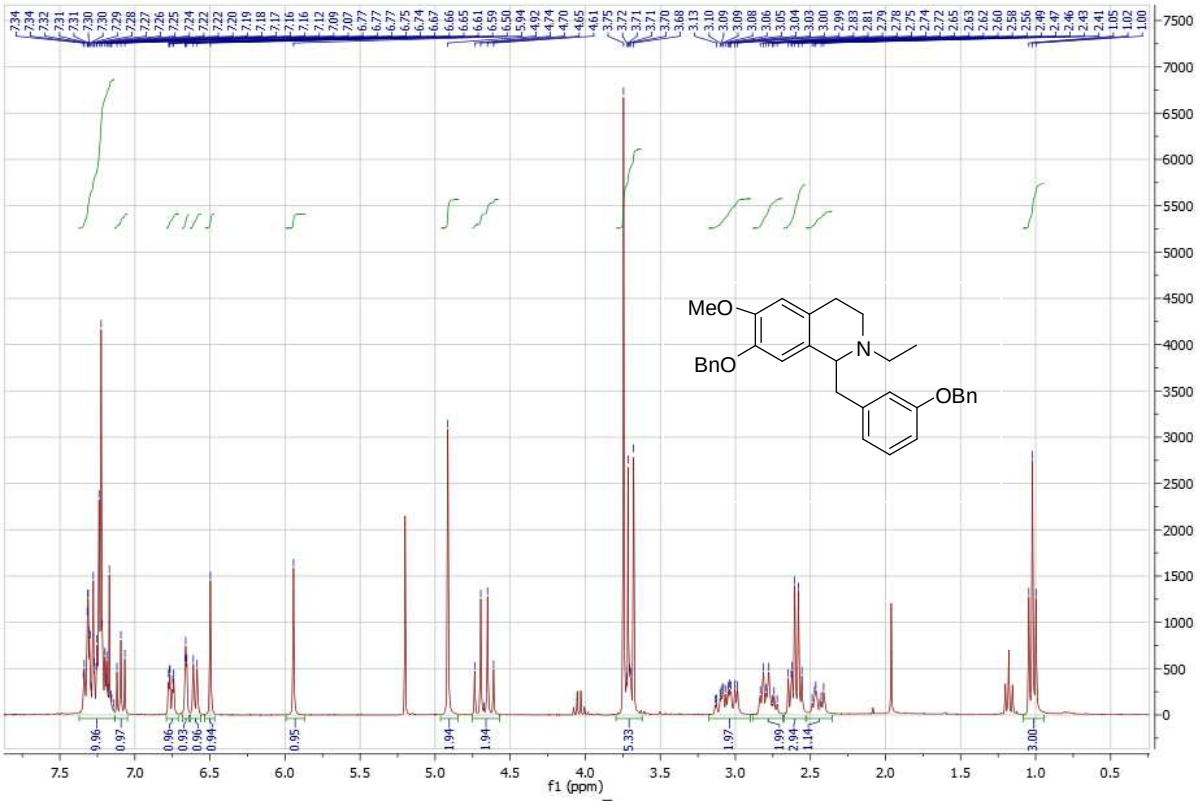

Figure 110: ^{1}H-NMR spectrum of **2c**

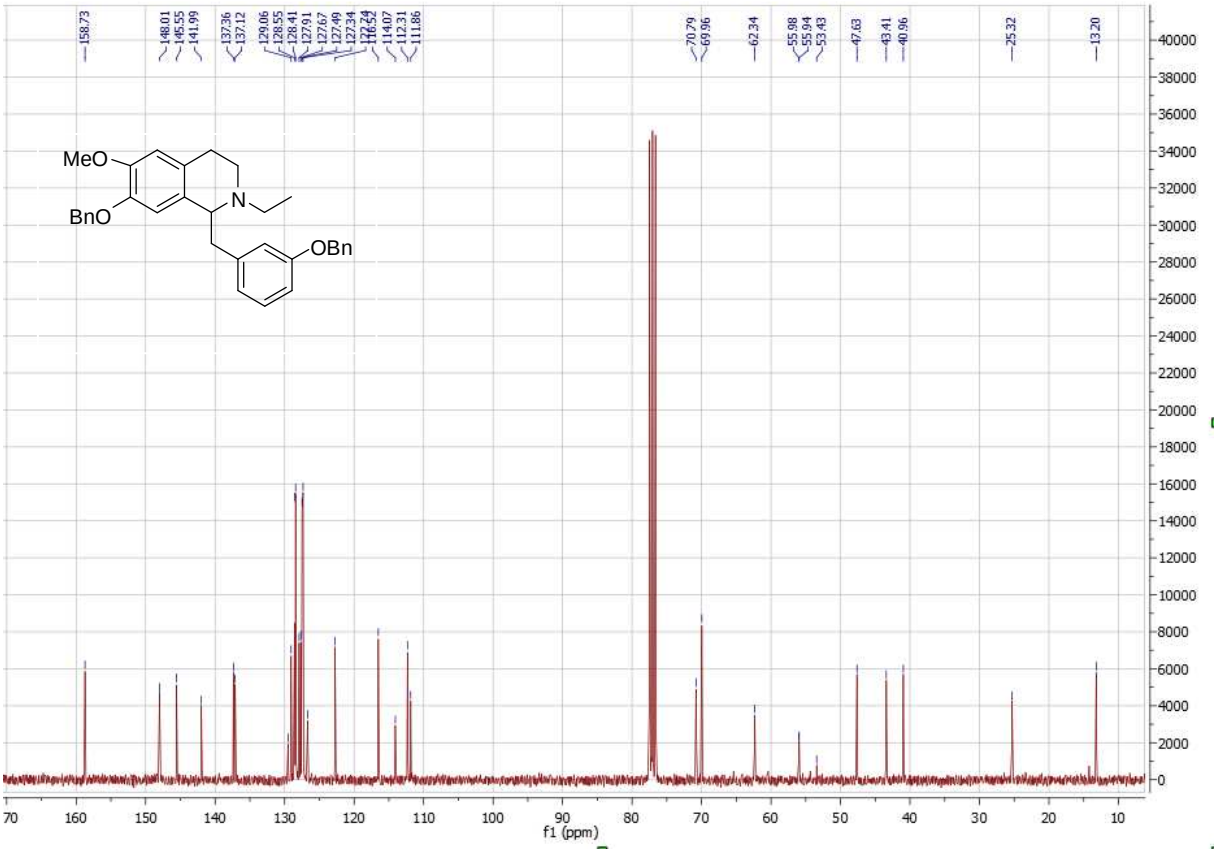

Figure 111: ^{13}C-NMR spectrum of **2c**

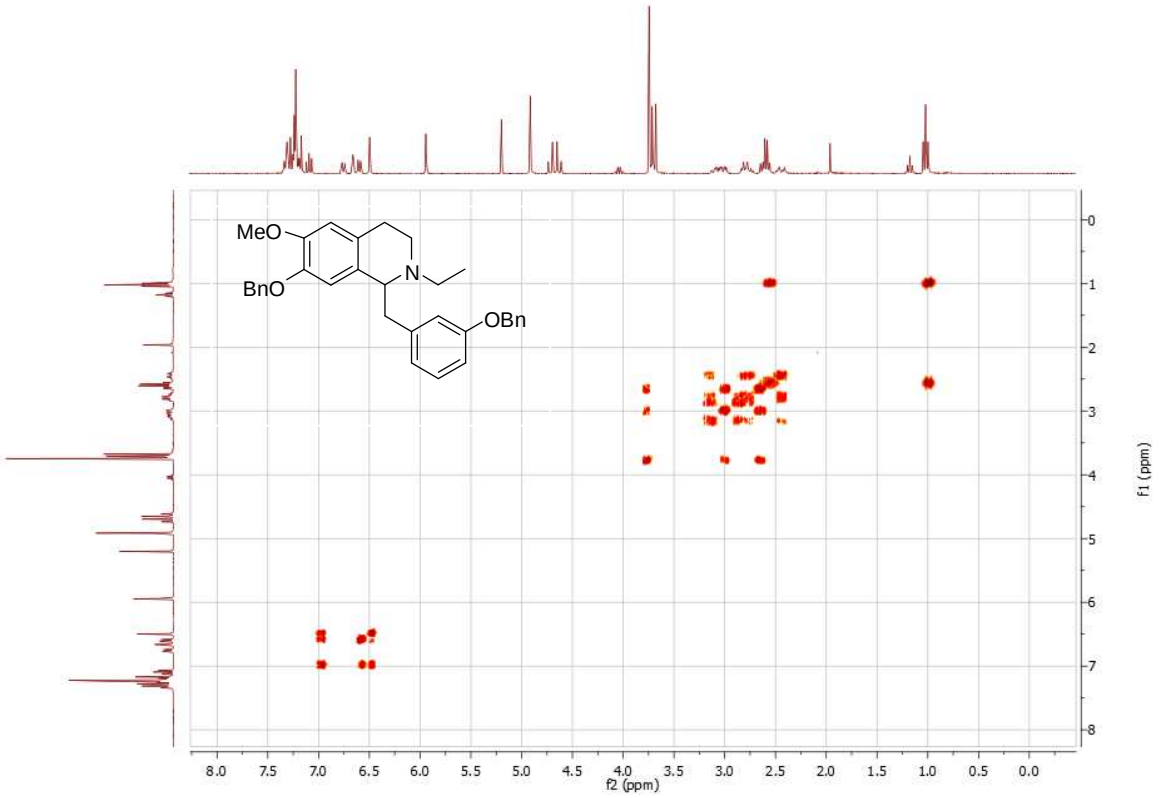

Figure 112: COSY-spectrum of **2c**

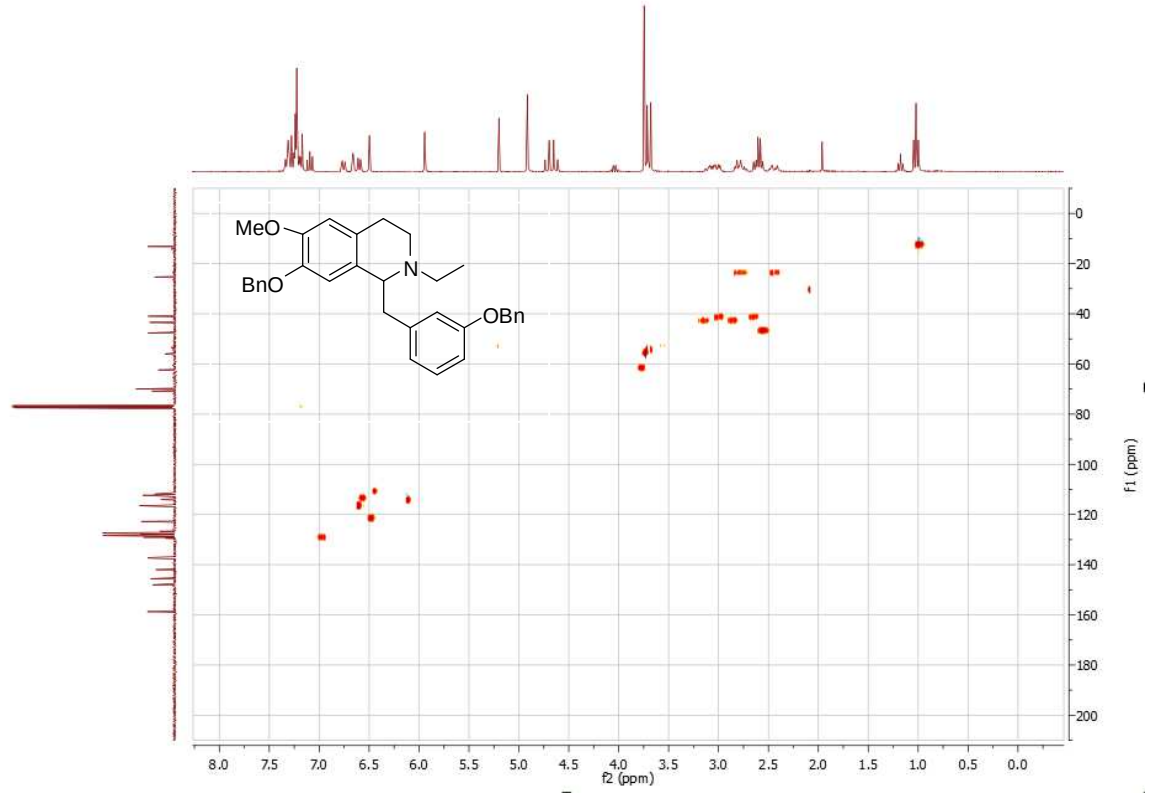

Figure 113: HSQC-spectrum of **2c**

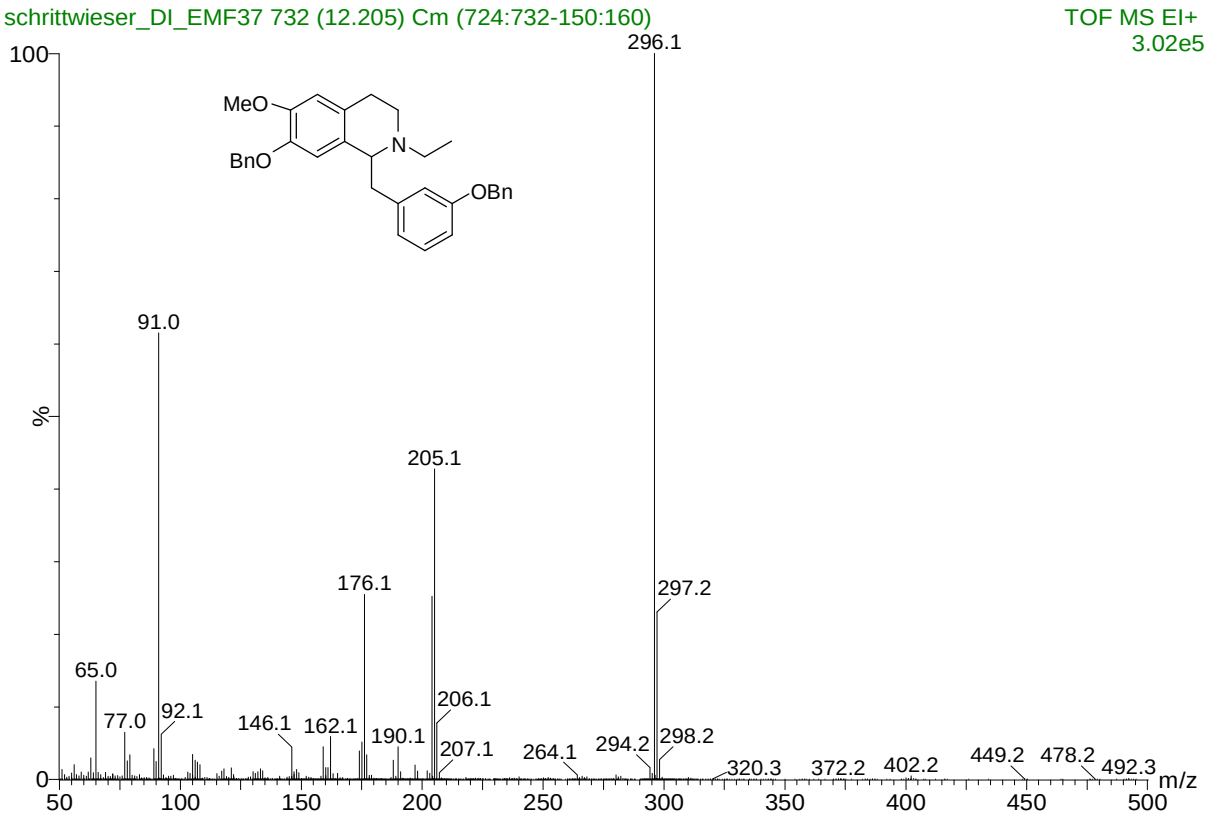

Figure 114: MS spectrum of **2c**

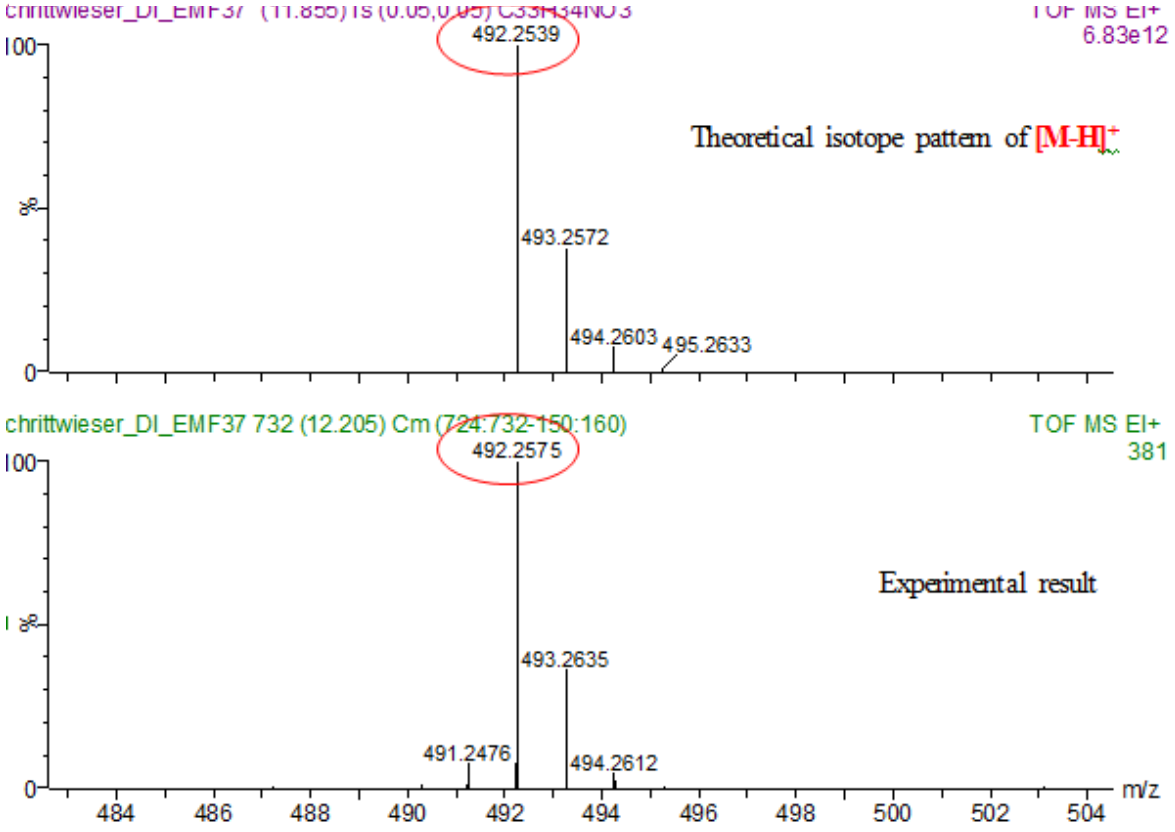

Figure 115: HR-MS spectrum of **2c**

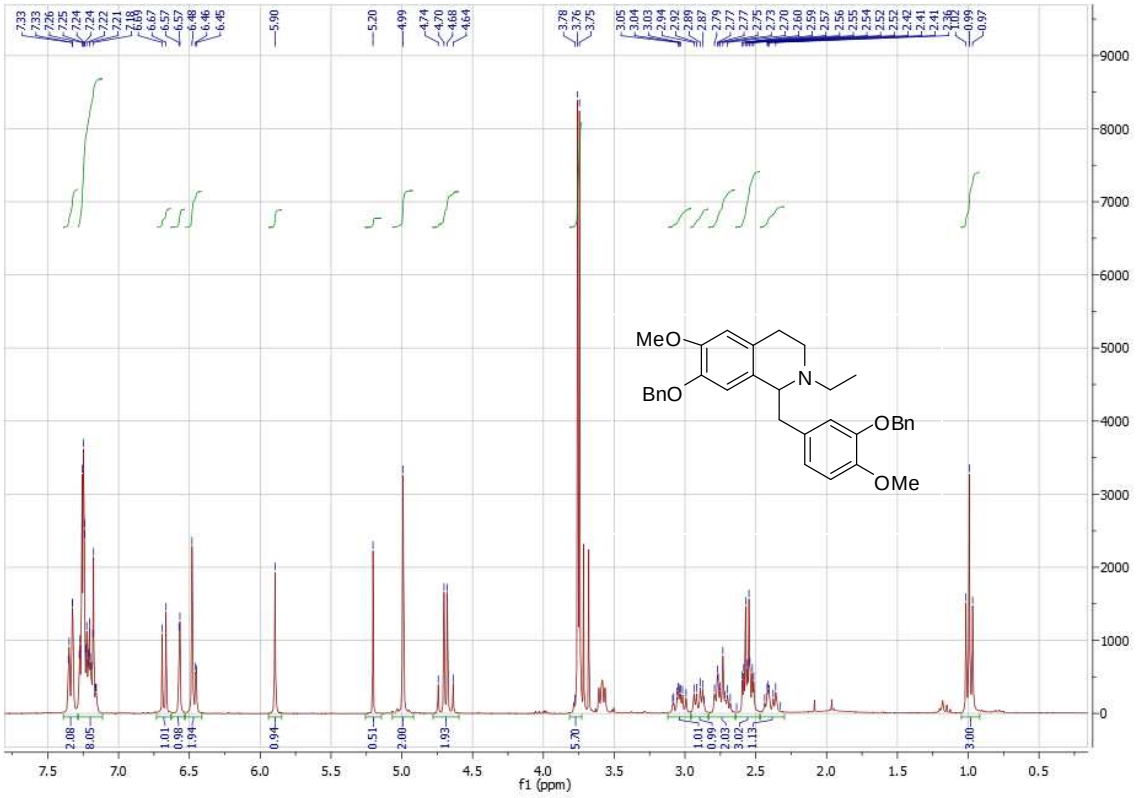

Figure 116: ^{1}H-NMR spectrum of **2d**

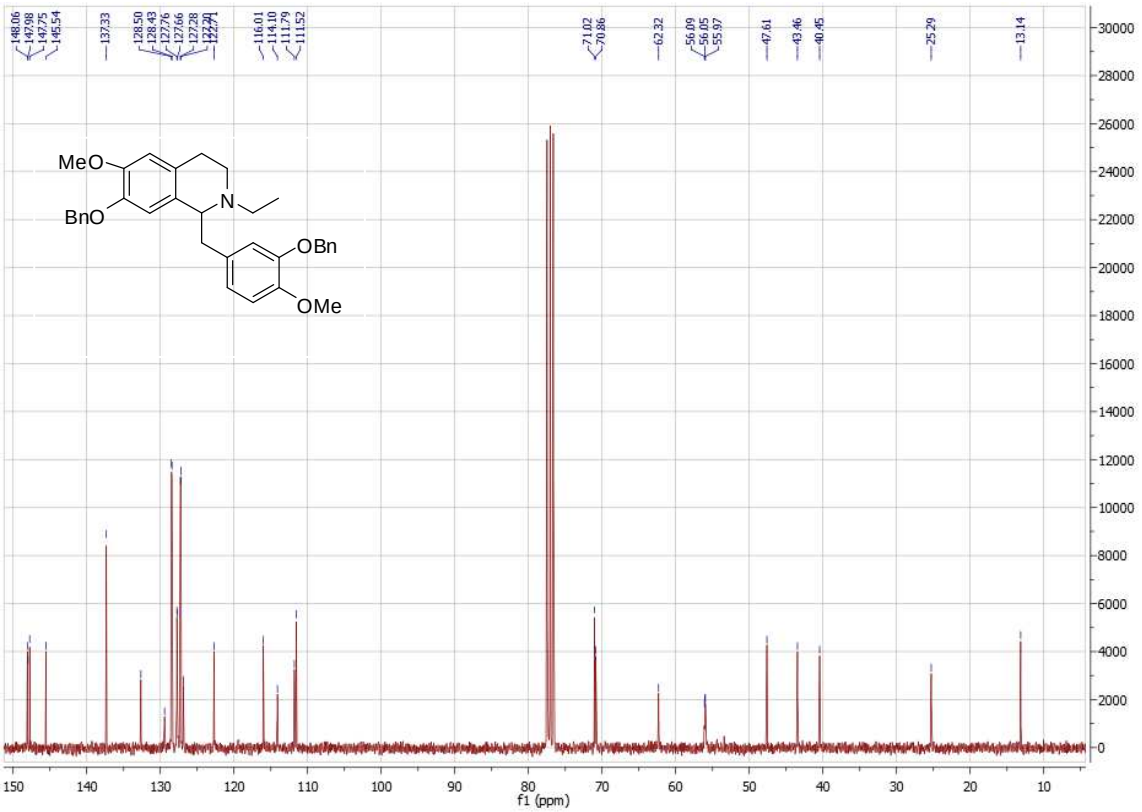

Figure 117: ^{13}C-NMR spectrum of **2d**

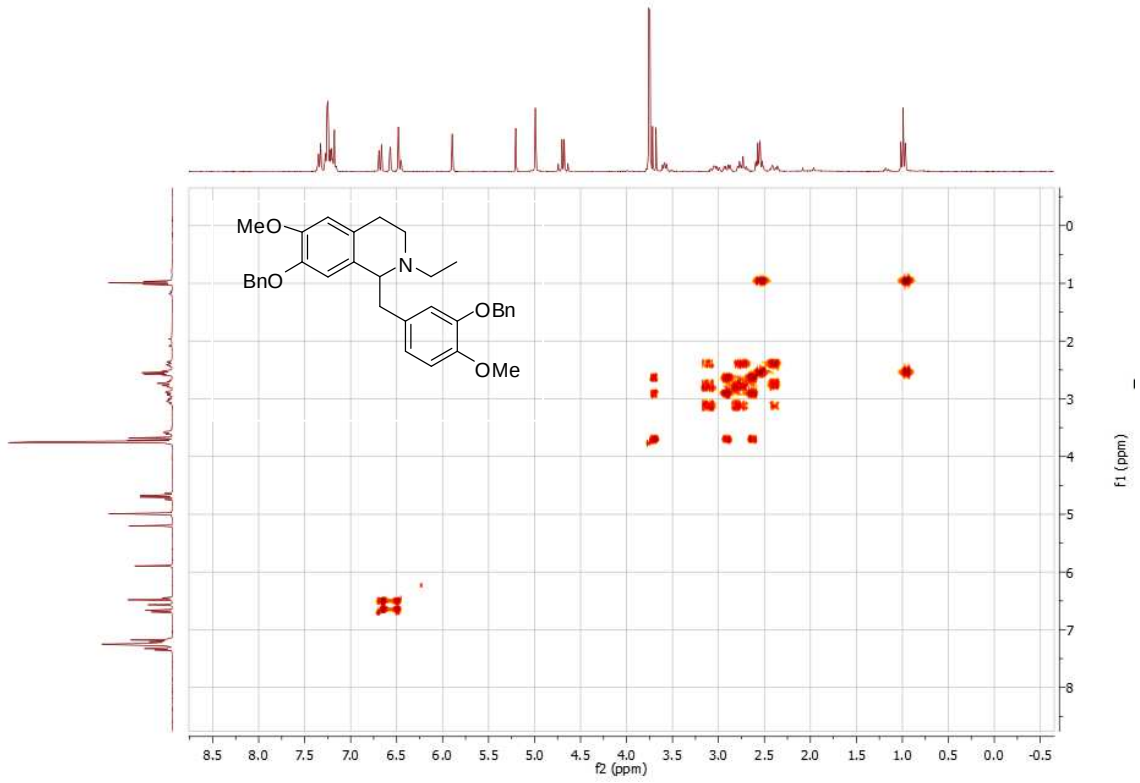

Figure 118: COSY-spectrum of **2d**

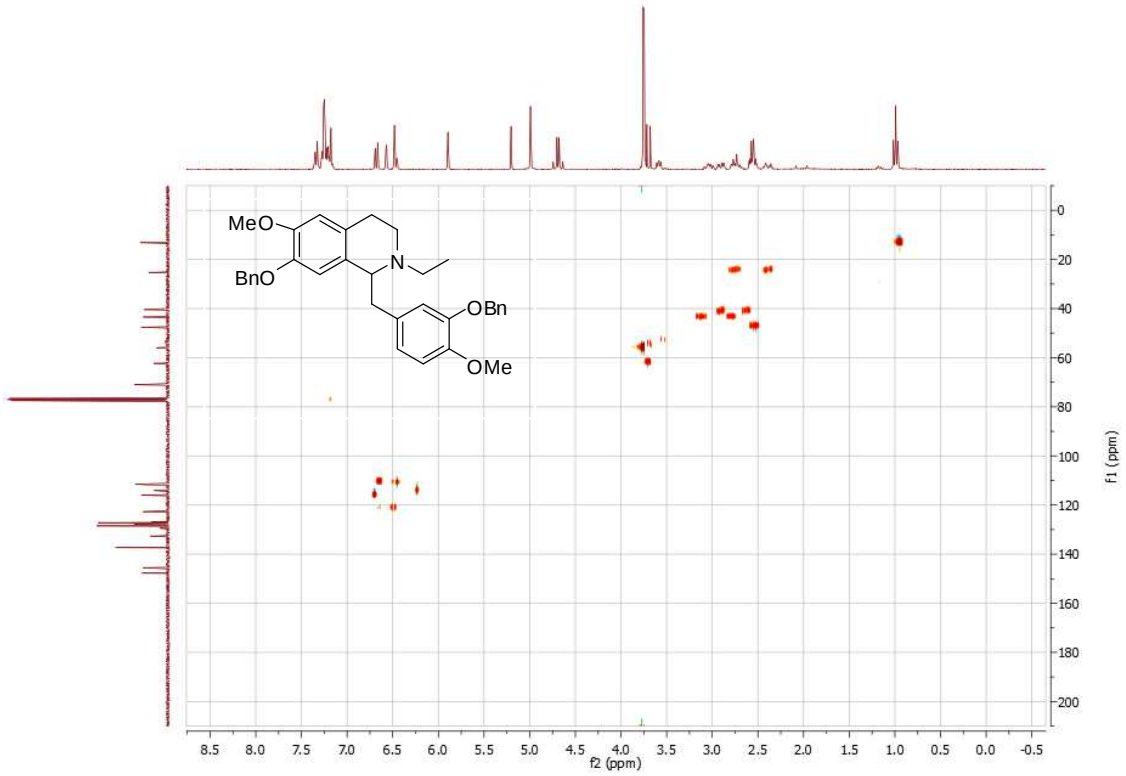

Figure 119: HSQC-spectrum of **2d**

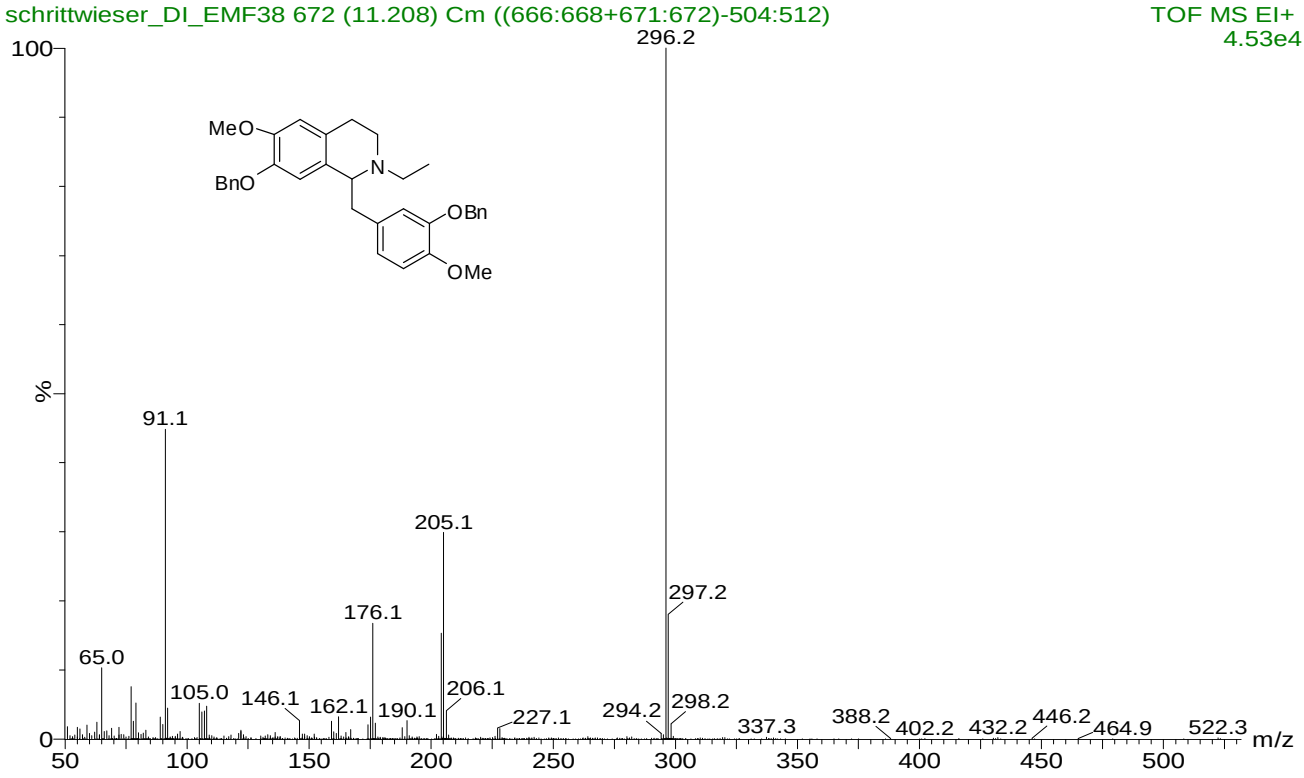

Figure 120: MS spectrum of **2d**

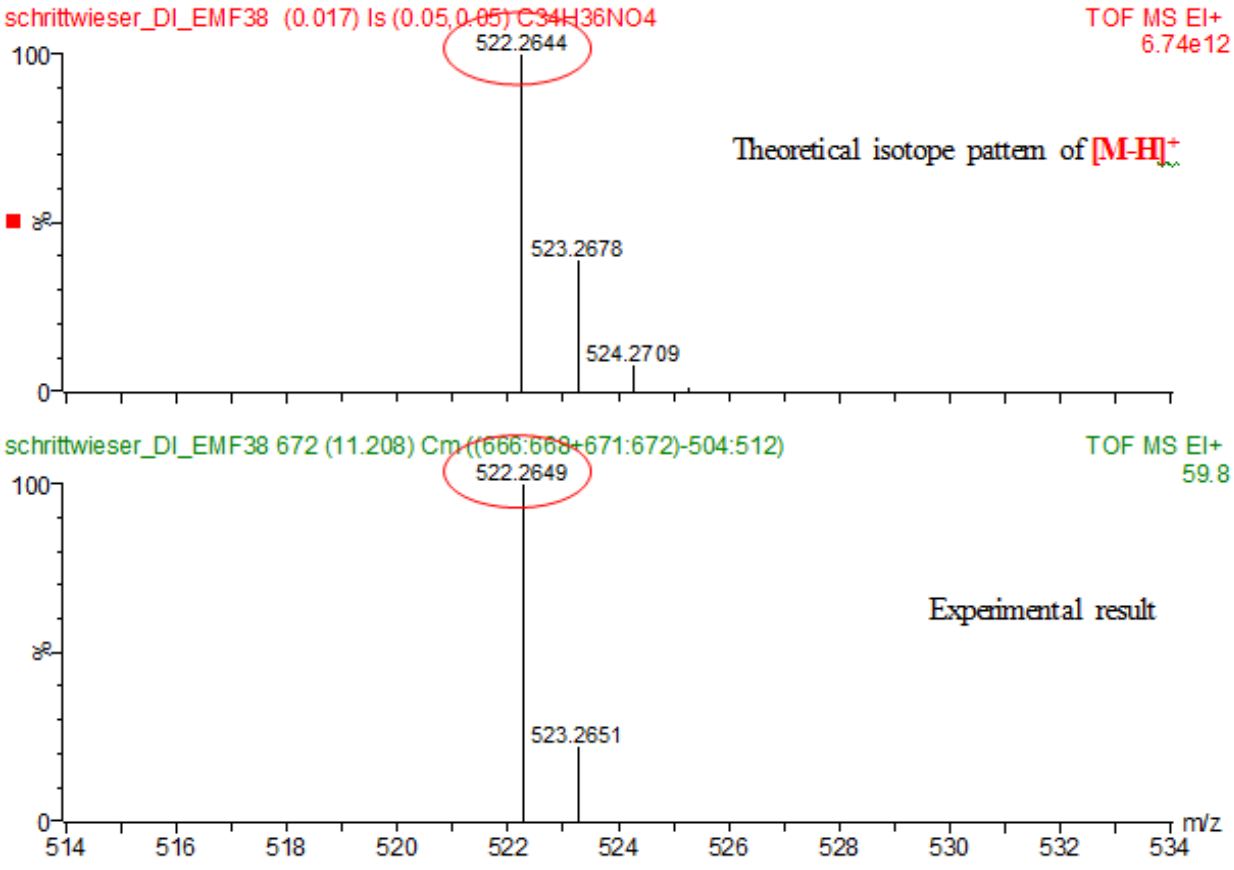

Figure 121: HR-MS spectrum of **2d**

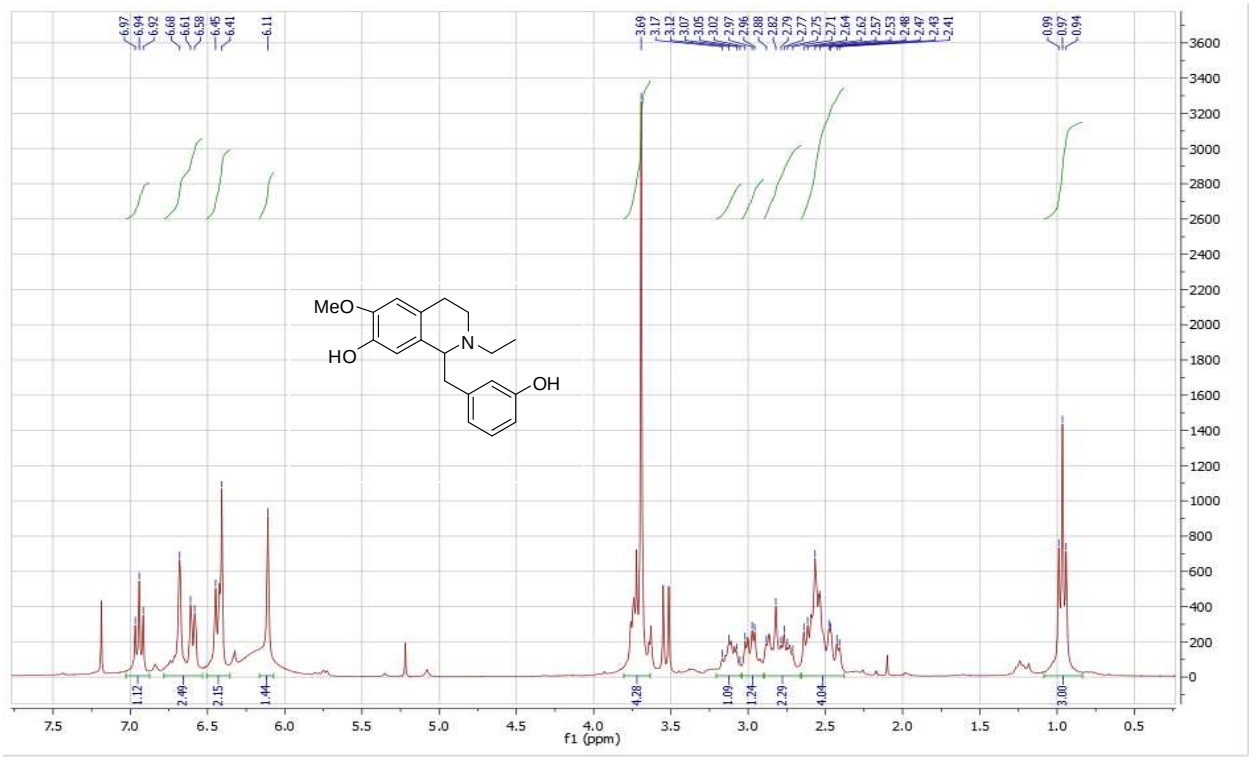

Figure 122: ^{1}H-NMR spectrum of **1c**

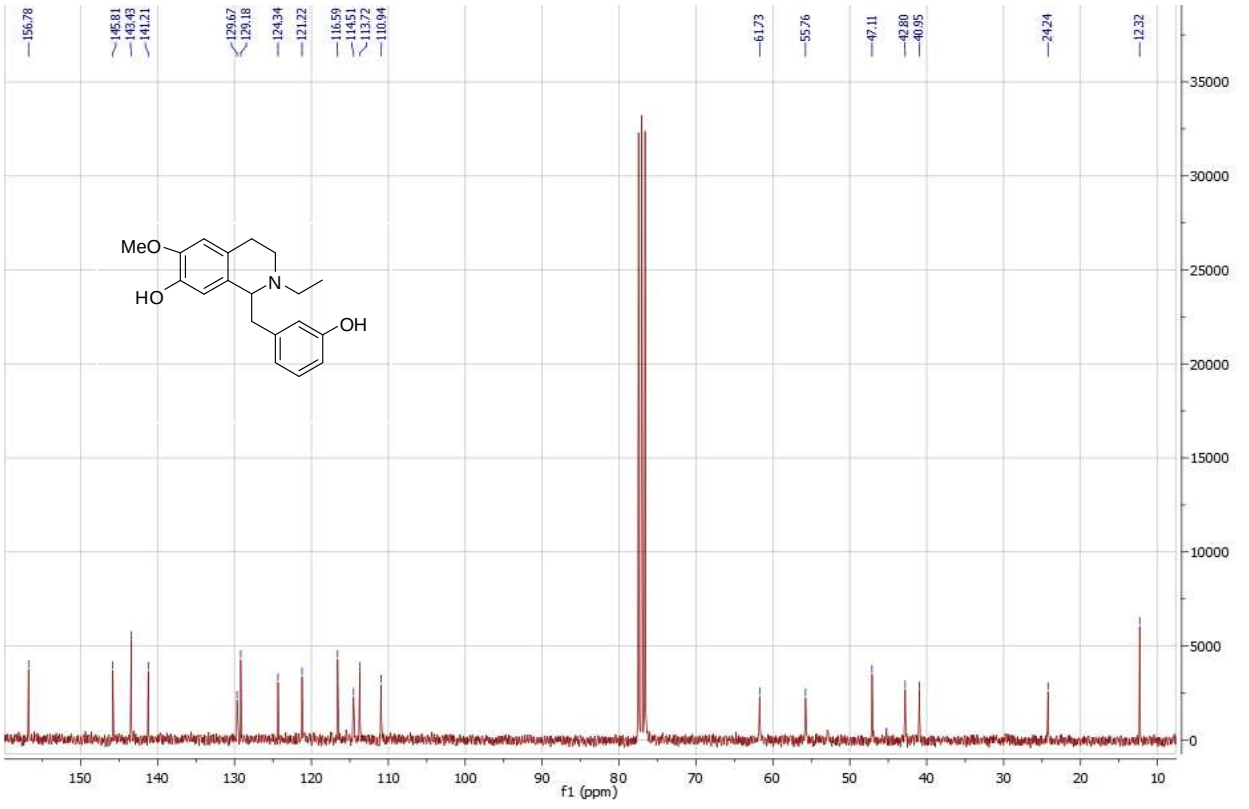

Figure 123: ^{13}C-NMR spectrum of **1c**

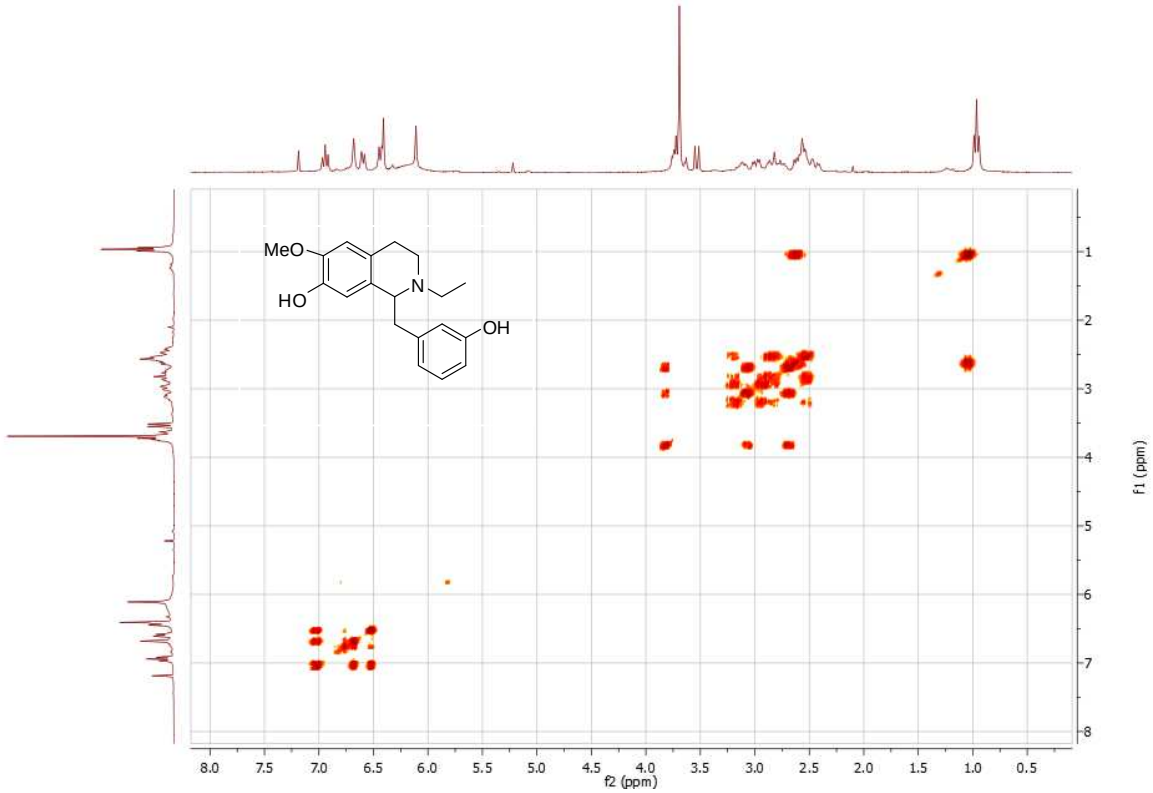

Figure 124: COSY-spectrum of **1c**

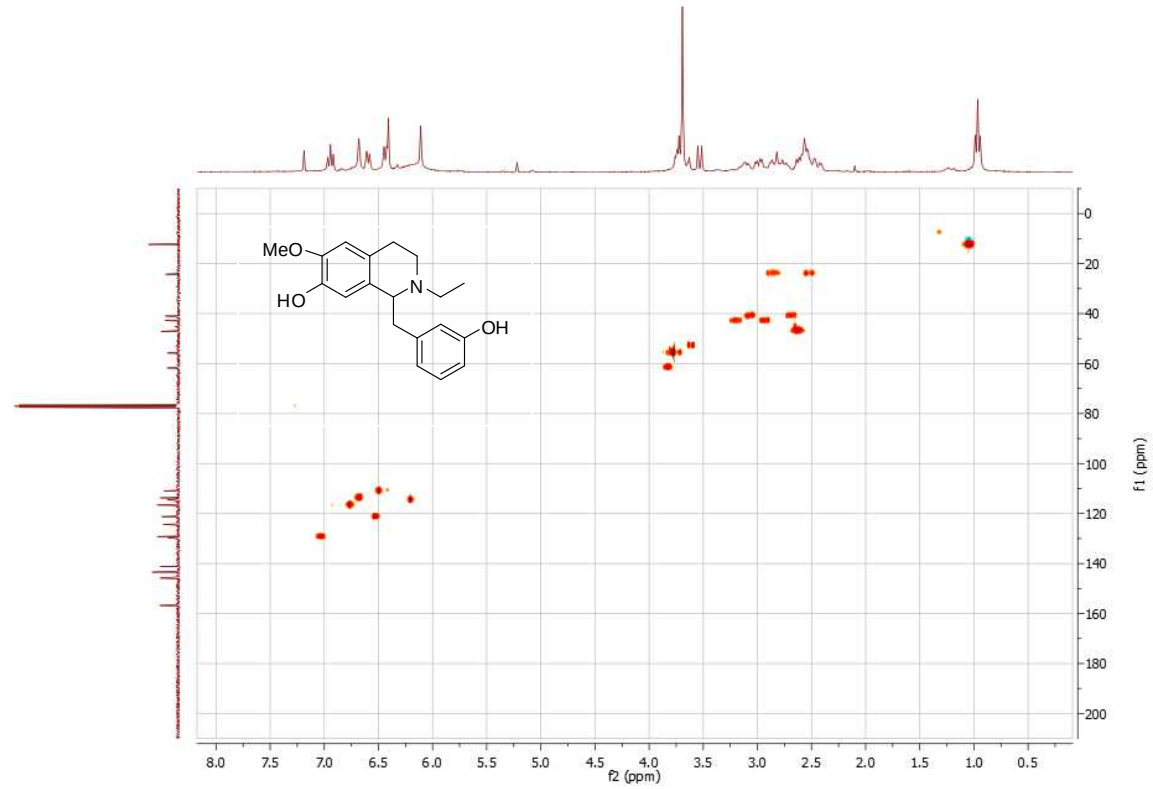

Figure 125: HSQC-spectrum of **1c**

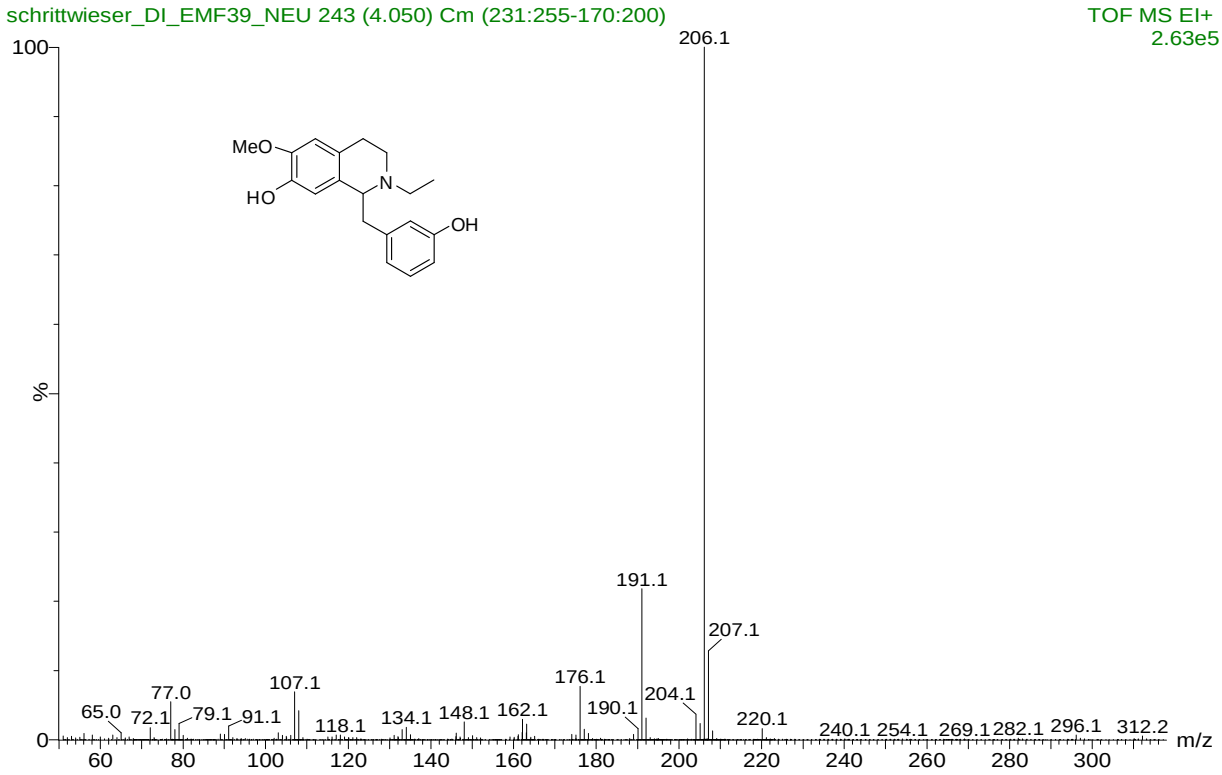

Figure 126: MS spectrum of **1c**

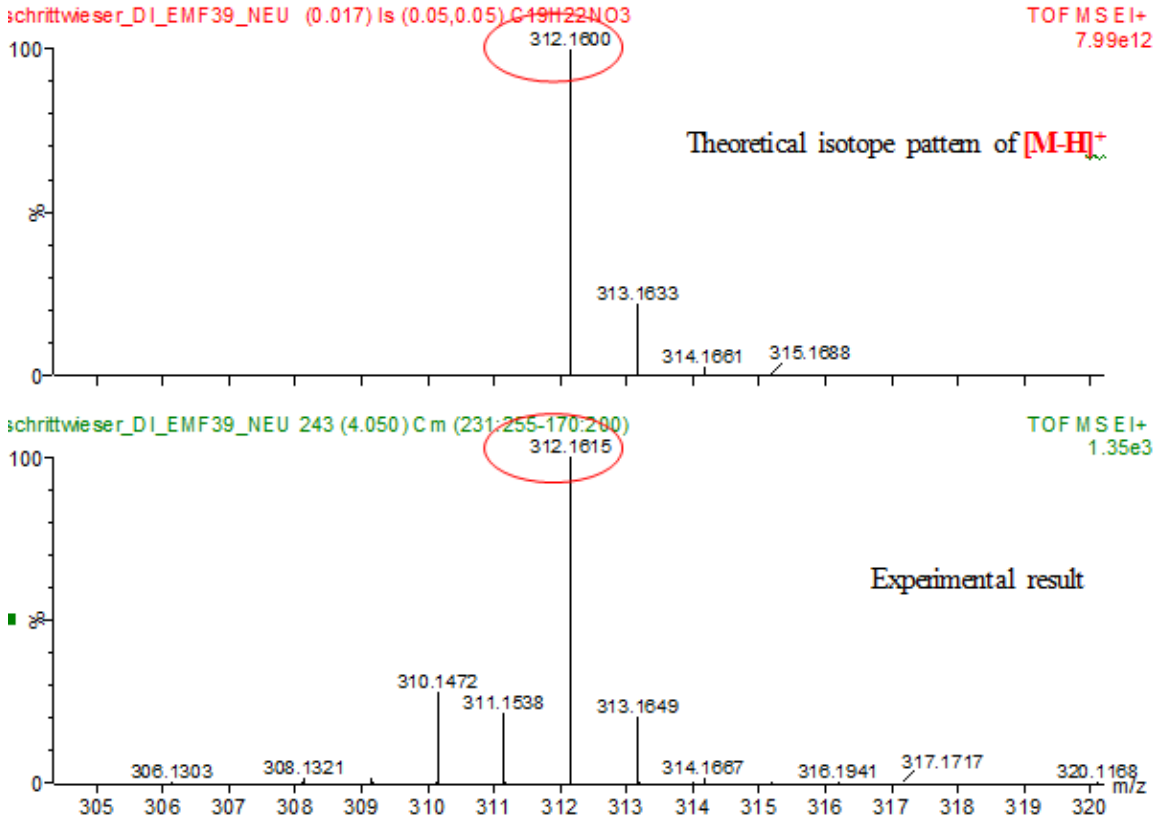

Figure 127: HR-MS spectrum of **1c**

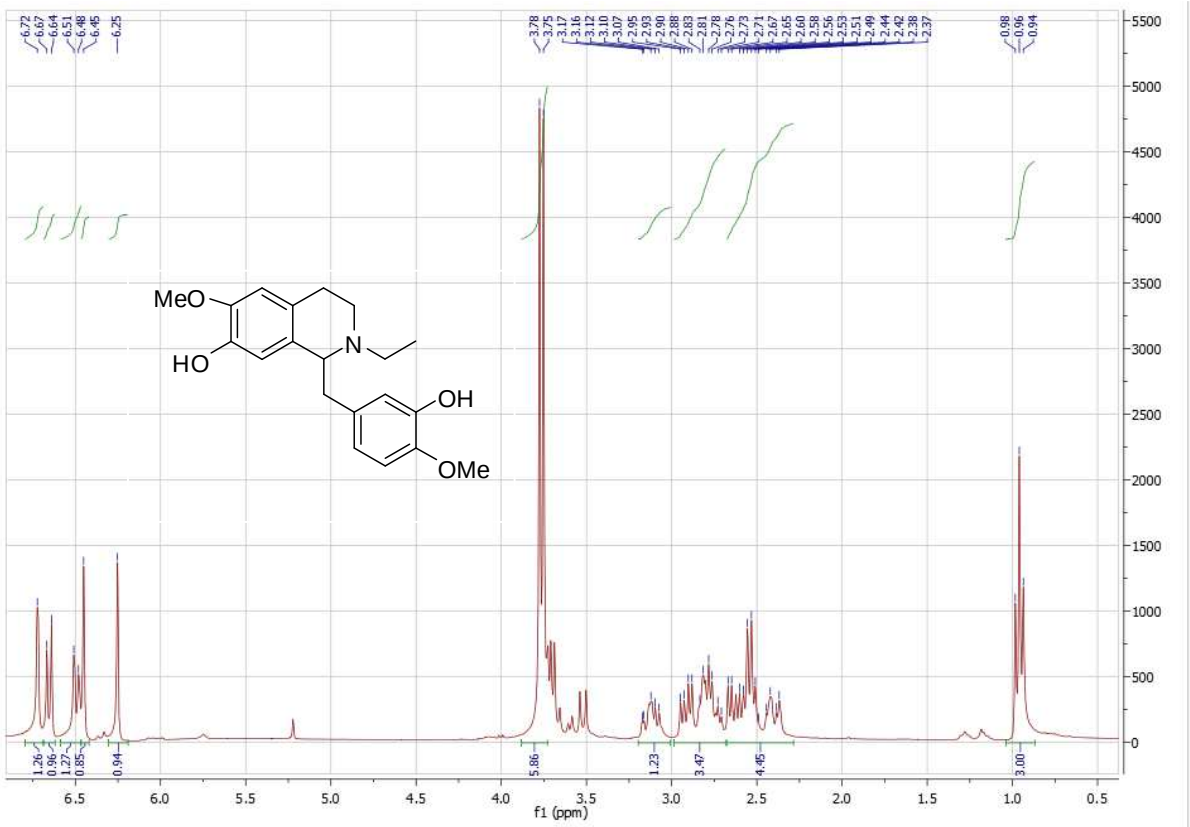

Figure 128: ^{1}H-NMR spectrum of **1d**

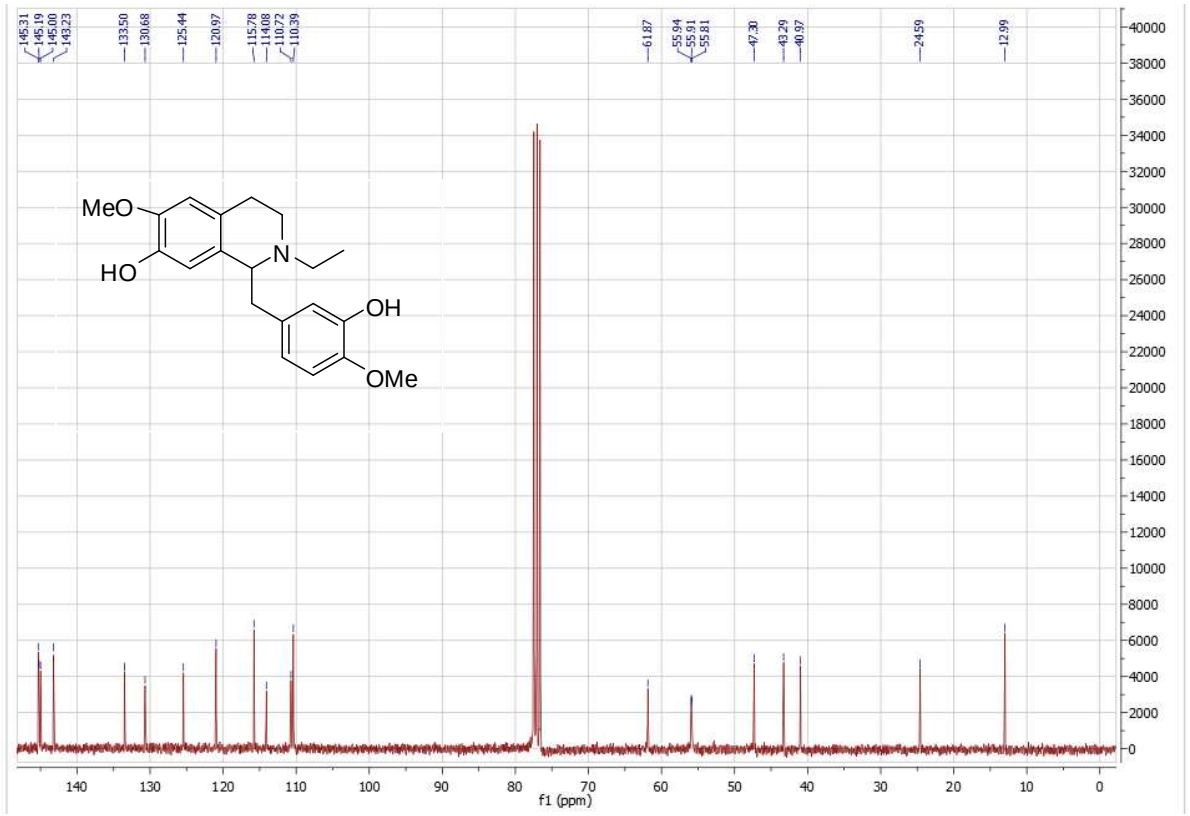

Figure 129: ^{13}C-NMR spectrum of **1d**

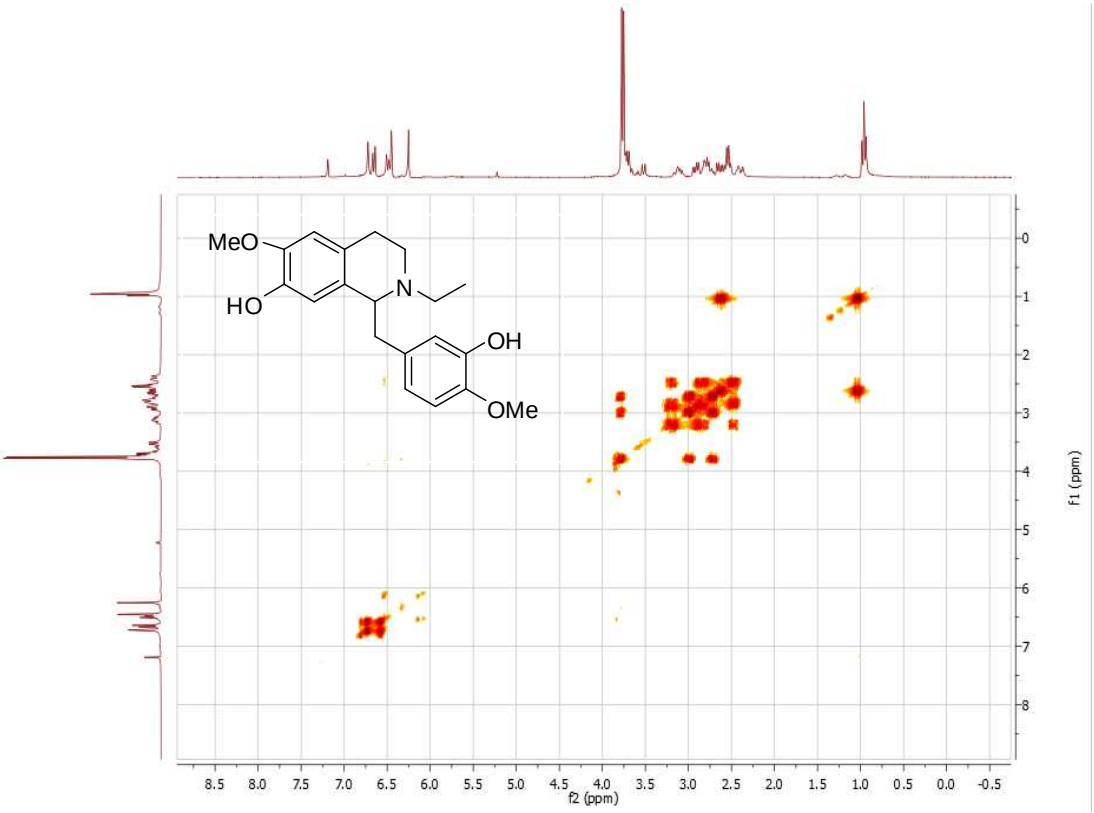

Figure 130: COSY-spectrum of **1d**

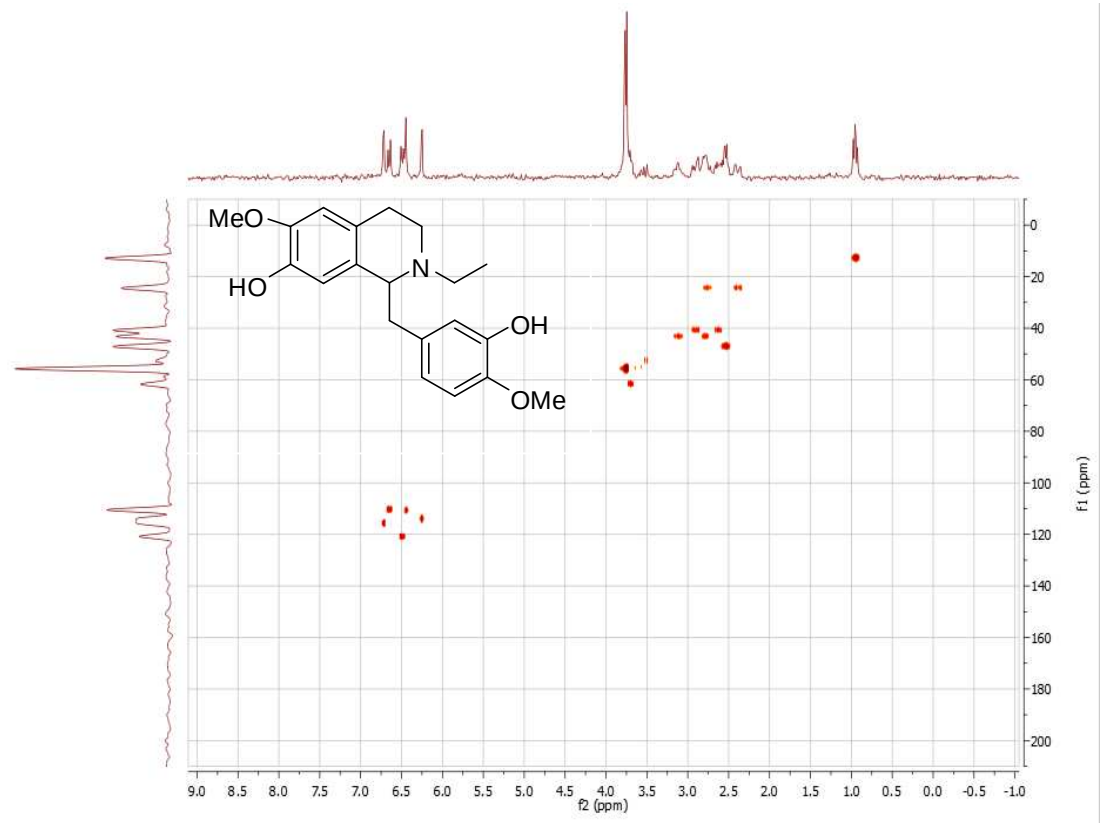

Figure 131: HSQC-spectrum of **1d**

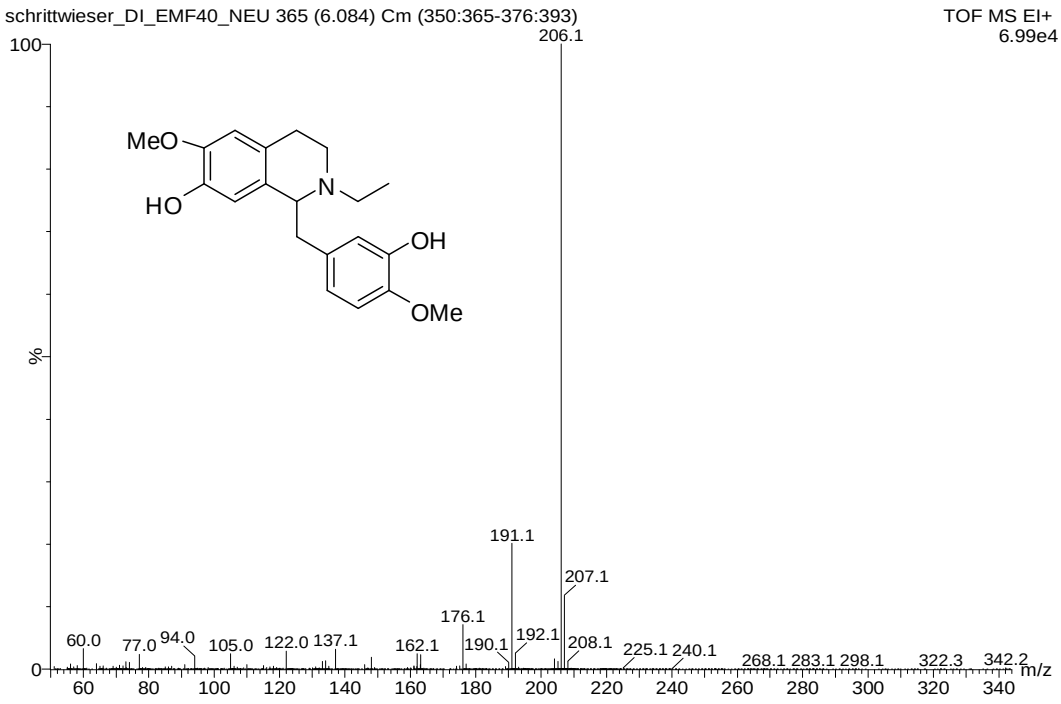

Figure 132: MS spectrum of **1d**

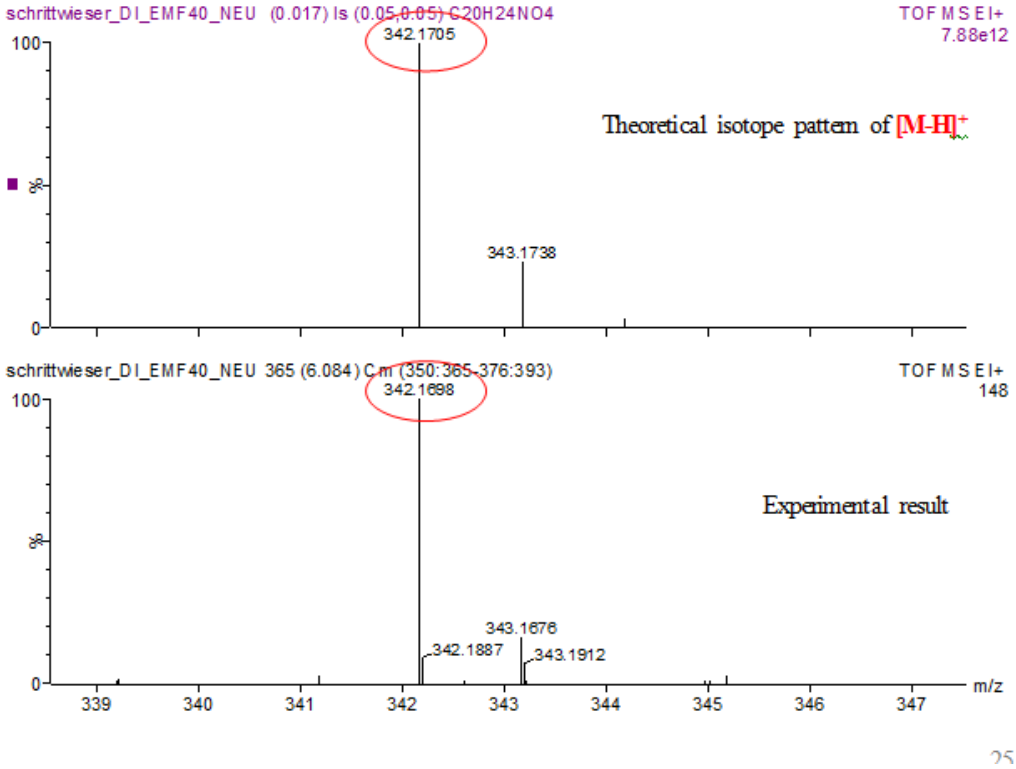

Figure 133: HR-MS spectrum of **1d**

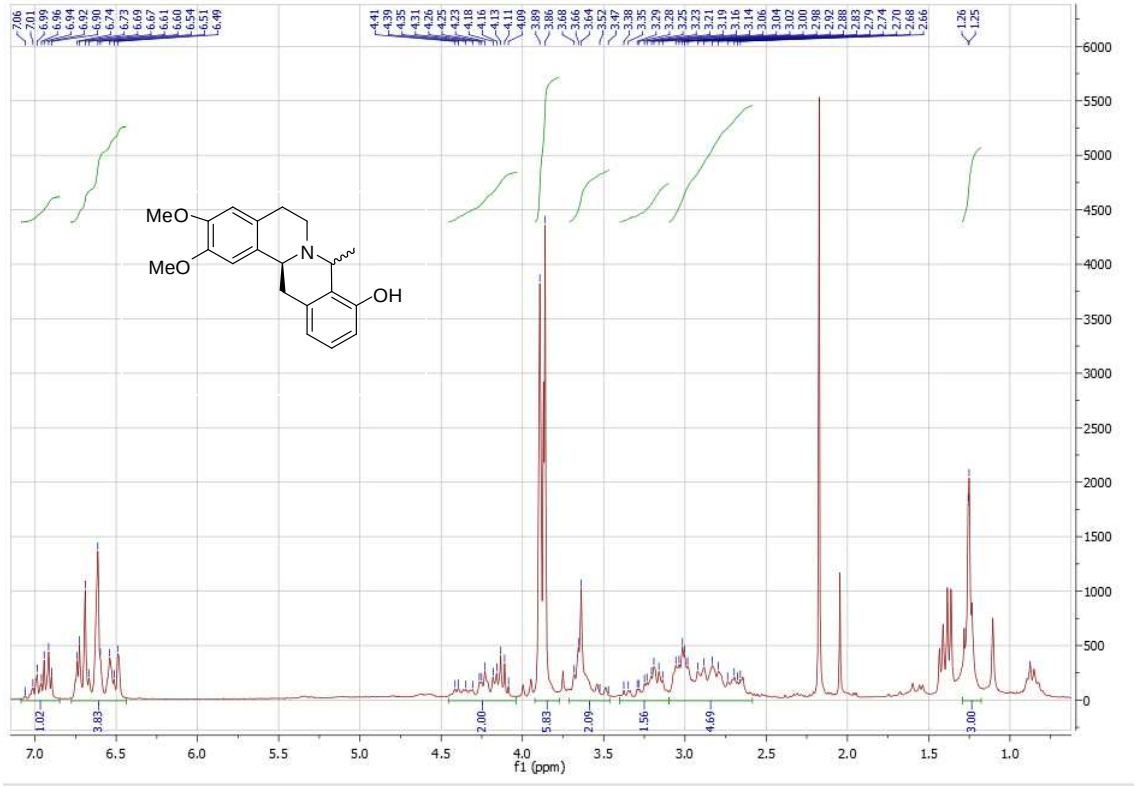

Figure 134: ^{1}H-NMR spectrum of the preparative transformation of substrate **1e** after 0.5 minutes reaction time

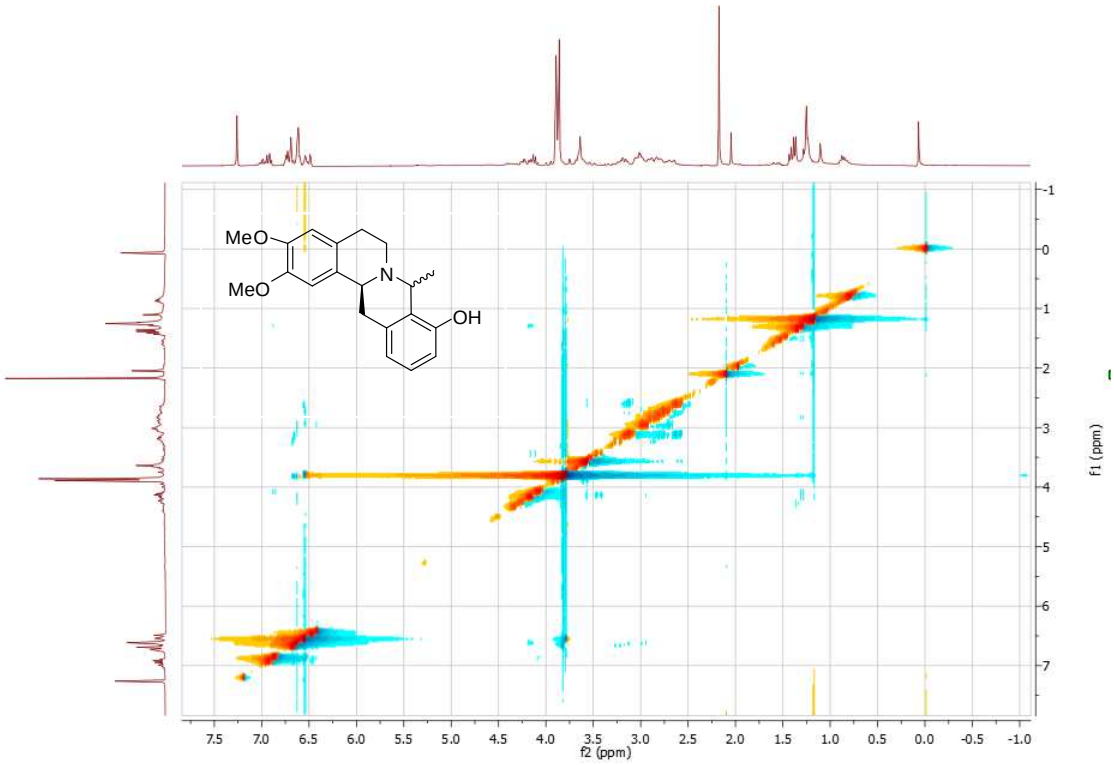

Figure 135: noesy spectrum of the preparative transformation of substrate **1e** after 0.5 minutes reaction time

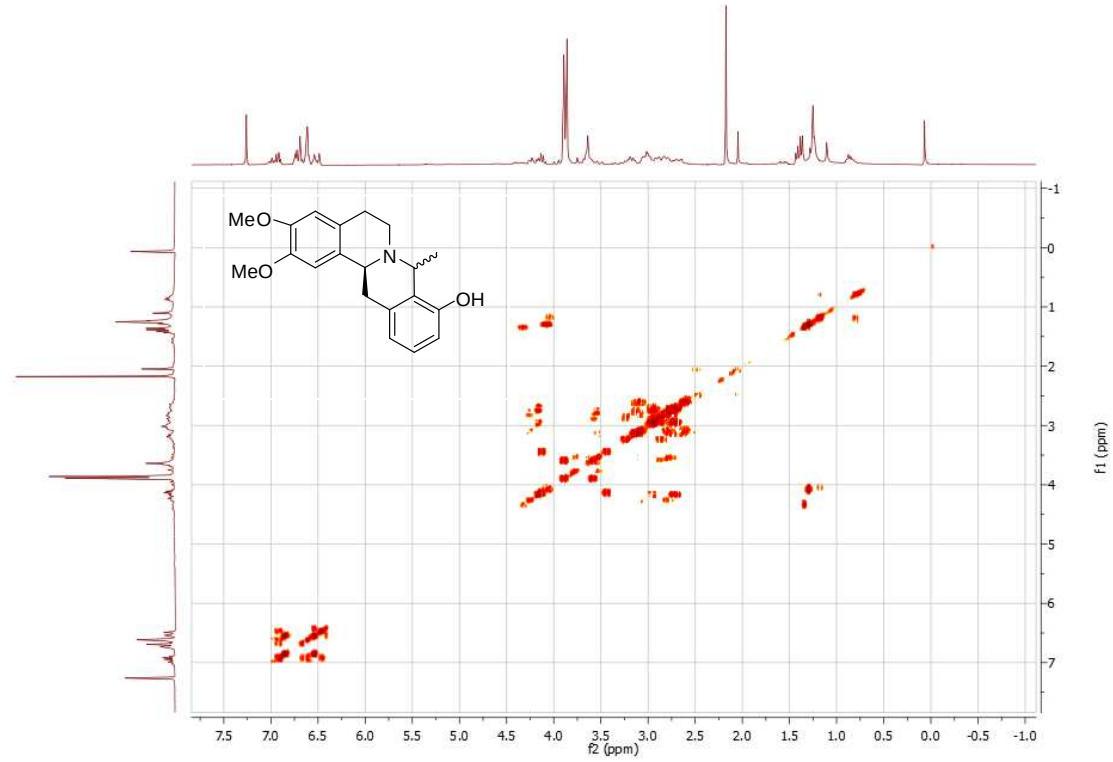

Figure 136: COSY spectrum of the preparative transformation of substrate **1e** after 0.5 minutes reaction time

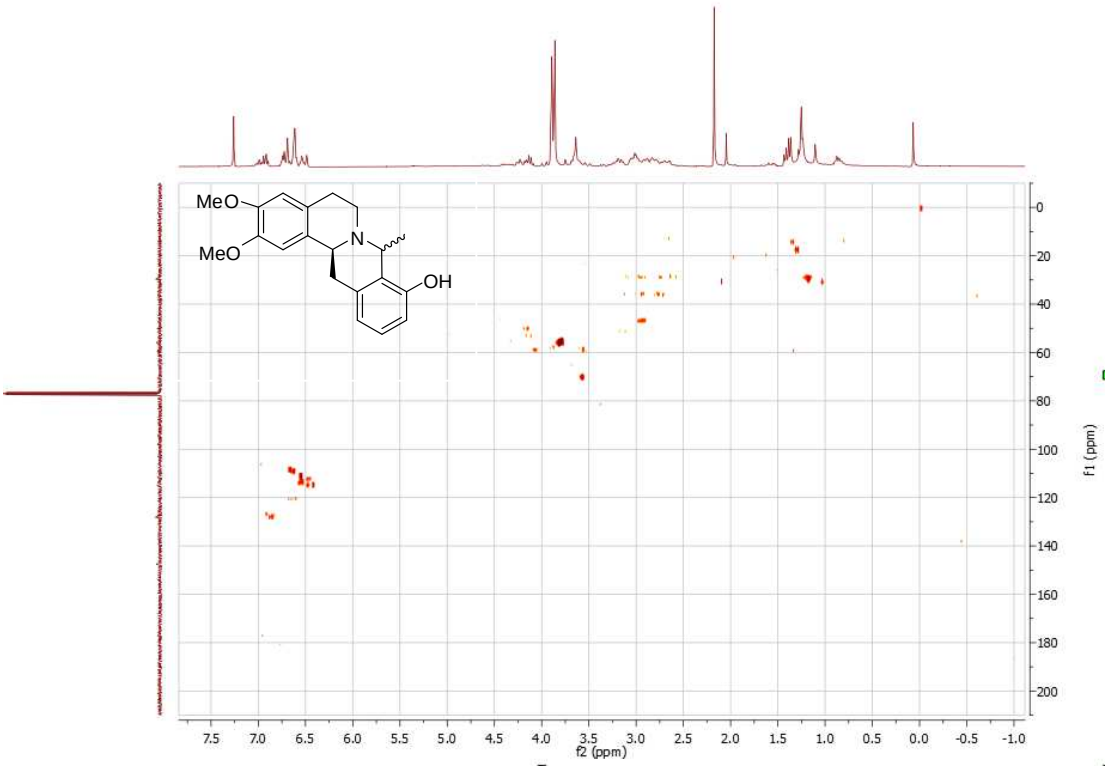

Figure 137: HSQC spectrum of the preparative transformation of substrate **1e** after 0.5 minutes reaction time

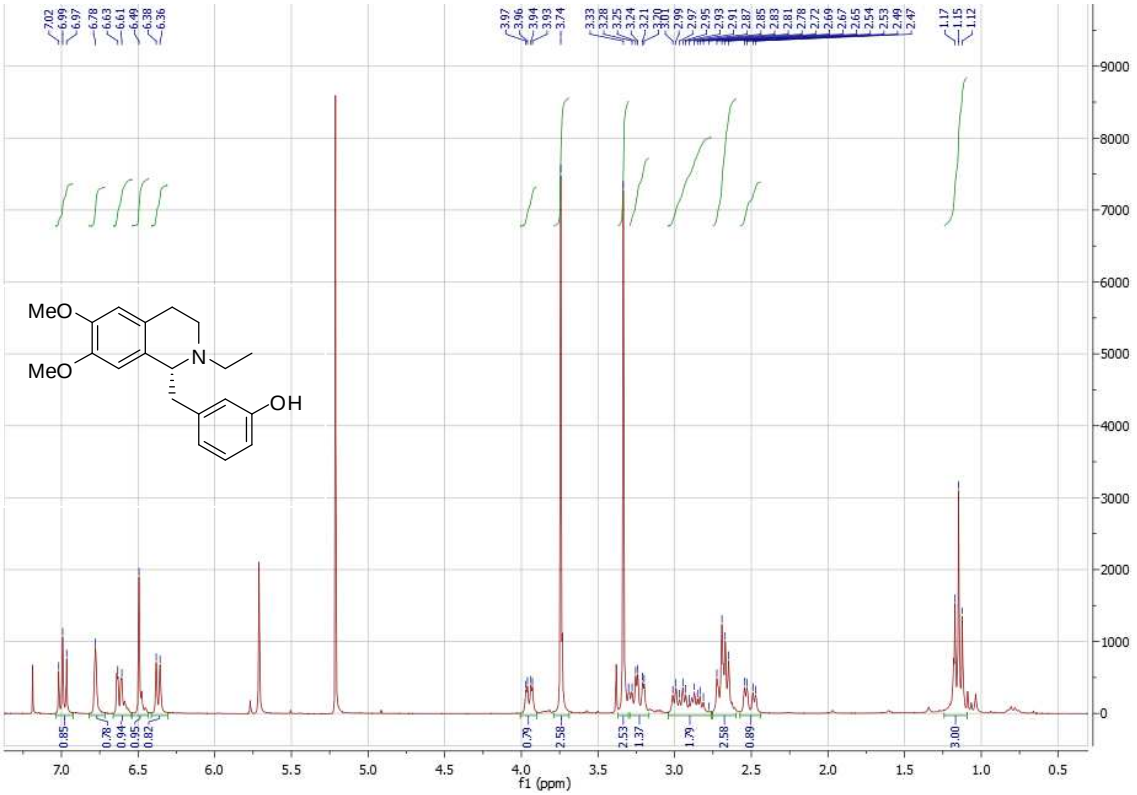

Figure 138: ^{1}H-NMR spectrum of (*R*)-**1e** from the preparative biotransformation after 24 h

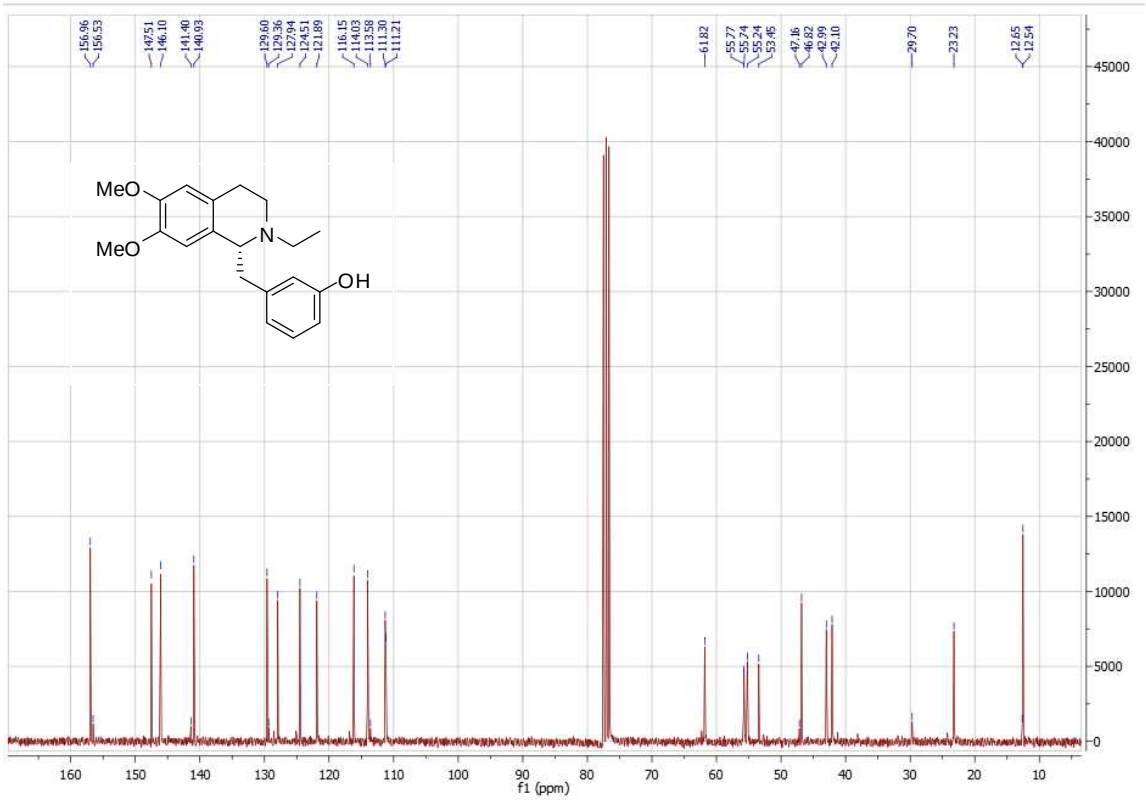

Figure 139: ^{13}C-NMR spectrum of (*R*)-**1e** from the preparative biotransformation after 24 h

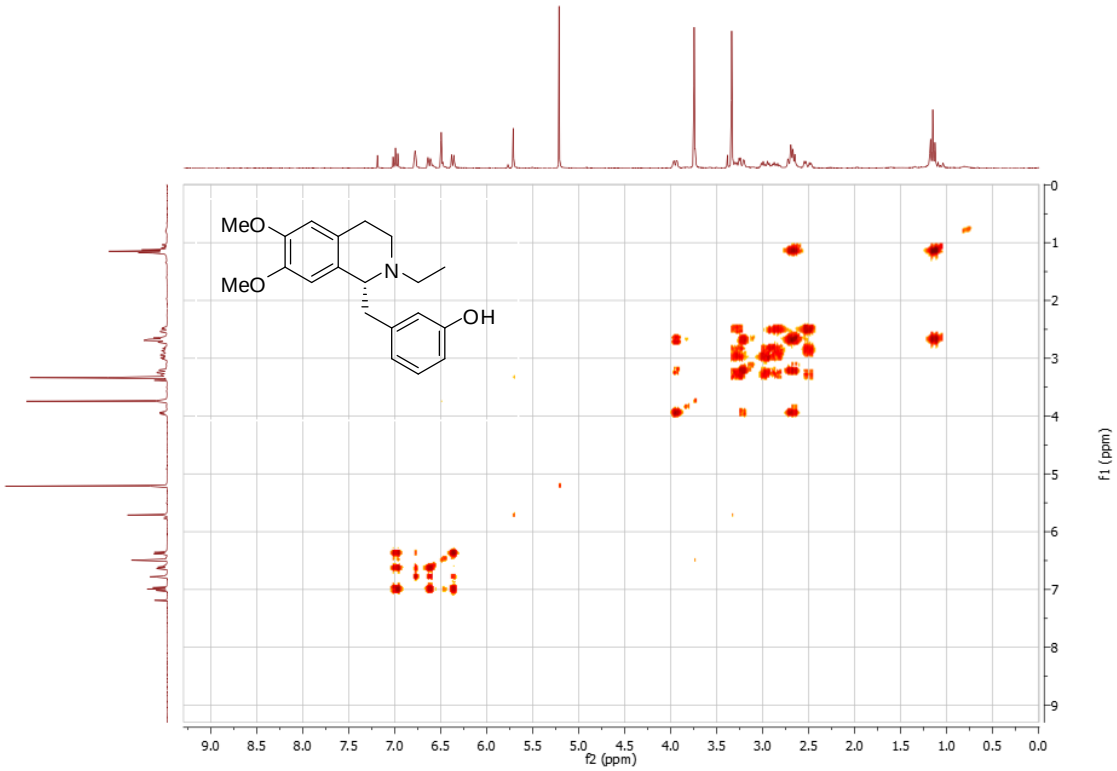

Figure 140: COSY spectrum of (*R*)-**1e** from the preparative biotransformation after 24 h

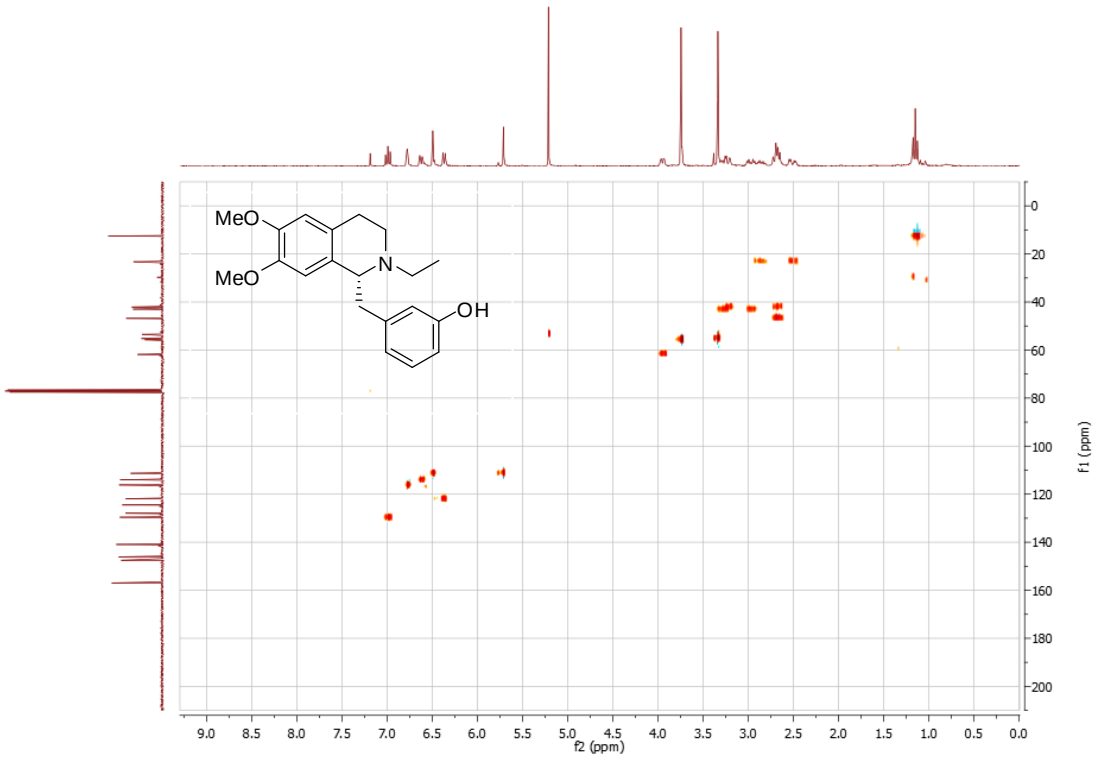

Figure 141: HSQC spectrum of (*R*)-**1e** from the preparative biotransformation after 24 h

CURRICULUM VITAE

PERSONAL INFORMATION

First name/ Surname	Eva Fischereder
Address	13 Schönaugasse, 8010 Graz
Nationality	Austria
Date of birth	27 July 1987
E-mail	fischereder@student.tugraz.at
Mobile	+43 6645545883

EDUCATION

2009 to present	Master degree in chemistry at Graz University of Technology
2006-2009	Bachelor degree in chemistry at University of Graz and Graz University of Technology
2005-2006	Diploma study medicine at Medical University Graz

WORK EXPERIENCE

September – October 2010	Employment: University of Graz – *Teaching assistant* Main responsibilities: Monitoring students in laboratory
July – August 2010	Employment: University of Graz – *Scientific employee* Main responsibilities: Synthesis of Tetrahydroisoquinolines
October 2009 – January 2010	Employment: University of Graz – *Teaching assistant* Main responsibilities: Monitoring students in laboratory
September 2009 to present	Employment: "Kleine Zeitung GmbH & Co KG" – *Promotion assistant* Main responsibilities: Public relation

August-September 2009	Employment: Graz University of Technology – *Scientific employee* Main responsibilities: Development of novel electrode materials for lithium-ion batteries.
May-July 2009	Employment: University of Graz – *Bachelor student* Main responsibilities: Racemisation of α-hydroxy acid amides and synthesis of pantothenic acid derivatives
July-August 2008	Employment: ”Lannacher Pharma GmbH” - *Research assistant* Main responsibilities: Development and production of pharmaceuticals
July-August 2002-2007	Employment: “Seniorenpark Unterpremstätten & HUMANITAS” - *Nurse* Main responsibilities: Caring for elderly people

PERSONAL SKILLS AND COMPETENCES

Languages

Mother tongue	German
English	C1 in understanding and writing - B2 in spoken interactions
Italian	A1 in understanding, writing and spoken interactions
Latin	C1 in writing

Social skills and competences

- Good communication skills gained through my experiences as a teaching assistant
- Flexible, self-reliant as well as the ability to work in a team, working experiences in industry and different working groups at university

Organization skills and competences

Experienced in project and team management gained during my experience as teaching assistant, through seminars and group working activities

Technical skills and competences

Experienced in the field of lithium-ion batteries and the belonging analytical techniques like cyclovoltammetry

Computer skills

- Very good Microsoft office skills (MS Word, MS Excel, MS PowerPoint)
- Very experienced with scientific computer programs (ChemDraw, Scifinder, Beilstein, Isis Draw, BioMol)

Other skills and competences

- Sportive (jogging, squash, swimming, cycling, tennis, surfing)
- Member of Green Peace

Clean driving license

PUPLICATIONS

Publications in peer-reviewed journals:

Deracemization of Benzylisoquinoline Alkaloids Employing Monoamine Oxidase Variants, Joerg H. Schrittwieser, Verena Resch, Bas Groenendaal, Diego Ghislieri, Ian Rowles, Simon C. Willies, Eva-Maria Fischereder, Barbara Grischek, Johann H. Sattler, Wolf-Dieter Lienhart, Nicholas J. Turner, Wolfgang Kroutil

Invited lectures:

Access to novel berbine derivatives employing the berberine bridge enzyme
J. H. Schrittwieser, V. Resch, E.-M. Fischereder, J. H. Sattler, W.-D. Lienhart, S. Wallner, K. Gruber, P. Macheroux, W. Kroutil, *17th Intl. Symposium on Flavins and Flavoproteins*, Berkeley, USA, 24.-29. Juli, 2011.

Biocatalytic approaches to a-chiral *prim*-and *tert*-amines
W. Kroutil, F. G. Mutti, C. S. Fuchs, D. Pressnitz, R. C. Simon, J. H. Schrittwieser, V. Resch, J. H. Sattler, E.-M. Fischereder, A. Orden, P. Macheroux, *14. Österreichische Chemietage 2011*, Linz, Österreich, 26.-29. September, 2011.

Oral communications:

Biocatalytic oxidative enantioselective C-C-formation as the key step in the chemoenzymatic synthesis of novel optically pure berbine alkaloids
J. H. Schrittwieser, V. Resch, E.-M. Fischereder, A. Orden, S. Wallner, P. Macheroux, W. Kroutil, *ISHHC XV International Symposium on Relations between Homogeneous and Heterogeneous Catalysis*, Berlin, Deutschland, 11.-16. September, 2011.

Berberine bridge enzyme: from biochemical studies to preparative-scale kinetic resolution and beyond
J. H. Schrittwieser, V. Resch, J. H. Sattler, W.-D. Lienhart, E.-M. Fischereder, S. Wallner, P. Macheroux, W. Kroutil, *10th International Symposium on Biocatalysis, Biotrans 2011*, Giardini Naxos, Italien, 2.-6. Oktober, 2011.