Development of a Biocatalytic Platform For the Enantioselective Hydrolysis of γ- and δ-Lactams

Diplomarbeit

Zur Erlangung des akademischen Grades
eines Diplomingenieurs (Dipl.-Ing.)
der Studienrichtung Biotechnologie

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Zusammenfassung

Nicht natürliche γ- und δ-Aminosäuren sind aufgrund ihres Vorkommens als Baustein in der Synthese von biologisch aktiven Verbindungen und ihrer Rolle als Signalmoleküle im Zentralnervensystem wichtige Verbindungen in der pharmazeutischen Industrie. Es wurde bereits eine beträchtliche Anzahl von unterschiedlichen Strategien für die chemische Synthese dieser Aminosäuren in enantiomerenreiner Form entwickelt, wobei sich die meisten auf die Verwendung von teuren und giftigen Katalysatoren und mehrere Syntheseschritte stützen. Eine elegante, einfache und umweltverträgliche Strategie ist die enzymatische Hydrolyse der entsprechenden γ- und δ-Lactame. Bis zu diesem Zeitpunkt sind nur wenige Enzyme, die die Amidbindung dieser stabilen 5- oder 6-Ringe hydrolysieren können, in der Literatur beschrieben und die einzigen bekannten Substrat der γ-Lactamasen sind 2-Azabicyclo[2.2.1]hept-5-en-3-on, auch bekannt als Vince-Lactam, und zwei Derivate dieser Verbindung.

Ziel dieser Arbeit war die Identifizierung neuer γ- und δ-Lactamasen und ihre Verwendung in enantioselektiven hydrolytischen Reaktionen mit verschiedenen Lactam-Substraten. Vier unterschiedliche Enzyme wurden erfolgreich für die Hydrolyse von Vince-Lactam eingesetzt und zeigten dabei enantiokomplementäre Aktivitäten. Einige dieser Enzyme zeigten auch hydrolytische Aktivität an drei Derivaten von Vince-Lactam. Zusätzlich konnten drei Enzyme die perfekte kinetische Racemtrennung von Vince-Lactam, einem *N*-methylierten und einem reduzierten Derivat der Verbindung katalysieren. Drei Derivate des Vince-Lactams wurden von keinem der Enzyme als Substrat akzeptiert.

Zusätzlich wurden 14 unterschiedliche γ-Lactame und vier δ-Lactame für ein Aktivitäts-Screening dieser Enzyme eingesetzt. Leider konnte keine enzymatische Aktivität beobachtet werden.

Basierend auf einer Analyse der bekannten Literatur scheint die Enantiopräferenz der γ-Lactamasen durch ihre Aminosäure-Sequenz und Proteinstruktur vorgegeben zu sein: (+)-γ-Lactamasen scheinen zur Amidase-Signature-Familie zugehörig zu sein und zeigen eine konservierte, charakteristische Sequenz mit einer Ser-Ser-Lys Triade. (-)-γ-Lactamasen hingegen gehören zur α/β-Hydrolase Familie und beinhalten die für Serin-Hydrolasen typische Ser-His-Asp Triade. Diese vorgeschlagene Klassifizierung wird durch die Ergebnisse dieser Arbeit unterstützt.

Abstract

Non-natural γ- and δ-amino acids are important target compounds for the pharmaceutical industry due to their use as building blocks for the synthesis of biologically active compounds and their importance as signaling molecules in the central nervous system. A significant number of different routes for the chemical synthesis of these amino acids in enantiopure form have been developed; however, most of them rely on expensive and toxic catalysts and multiple synthetic steps. An elegant, simple and green route is the enzymatic hydrolysis of the corresponding racemic γ- and δ-lactams. To date, only few enzymes able to hydrolyze the amide bond of these stable 5- or 6-membered rings are described in the literature and the only substrates for γ-lactamases known so far are 2-azabicyclo[2.2.1]hept-5-en-3-one, also known as Vince lactam, and two derivatives thereof.

Aim of this thesis was the identification of new γ- and δ-lactamases and their implementation in enantioselective hydrolytic reactions with various lactam substrates. Four different enzymes were successfully employed for hydrolysis of Vince lactam, thereby showing enantiocomplementary activities. Some of these enzymes also showed activity on three derivatives of Vince lactam. In addition, three enzymes were able to perform the perfect kinetic resolution of Vince lactam, an *N*-methylated and a reduced derivative thereof, respectively. Three derivatives of Vince lactam were not accepted as substrates by any of the enzymes.

In addition, 14 different γ-lactams and four δ-lactams were screened for activity with these enzymes. Unfortunately, no enzymatic activity could be detected.

Based on analysis of the literature, the enantiopreference of γ-lactamases on Vince lactam derivatives appears to be dictated by their amino acid sequence and protein structure: (+)-γ-Lactamases seem to belong to the amidase signature family, displaying a conserved signature sequence and a Ser-Ser-Lys catalytic triad. (-)-γ-Lactamases, on the other hand, are members of the α/β-hydrolase fold family and contain the Ser-His-Asp catalytic triad typical for serin hydrolases. This proposed classification is supported by the results presented in this thesis.

Acknowledgements

First and foremost I want to express my gratitude to Prof. Kurt Faber for the opportunity to write my diploma thesis as member of his group and for his guidance during the course of my work.

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Next, I would like to express my gratitude to all members of the ElkGroup for the wonderful working atmosphere and their valuable support. I want to give a lot of thanks to Michael Tösch, Nikolaus Turrini and Alexander Dennig for their great company and a lot of laughs in the "big lab". As well I want to thank Judith Farnberger and Tamara Reiter for our daily tea/coffee routine in the mornings. I felt honored being part of this group.

My deepest thanks I give to my family. My mother Ingrid and my father Gerhard gave me their everlasting support and always had great trust in me to go my own way. I want to thank them and my brother Thomas for encouraging and motivating me and for staying patient and understanding.

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Last but not least, I would like to thank my friends. Thank you for your encouragement, your understanding, for keeping me grounded and for making me laugh in times of frustration. You once again proved me that I can count on you unconditionally. I can't find words to express my gratitude for having friends like you!

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Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Contents

1. Introduction

1.1. Amino acids - a general introduction

Proteins and peptides play an essential role in nature having both catalytic and structural functions. These activities are directly connected to the very specific fold of each protein, which is the result of interactions between the peptide chain constituted of a combination of 20 (22 respectively, if taking non-canonical amino acids into account) amino acids. These so called "proteinogenic" amino acids are α-amino acids, meaning that their carboxylic acid group and their amino group are both bound to the α-carbon atom. Following this structural classification according to the respective position of their functional groups, β-, γ- and δ-amino acids have been described (Figure 1).

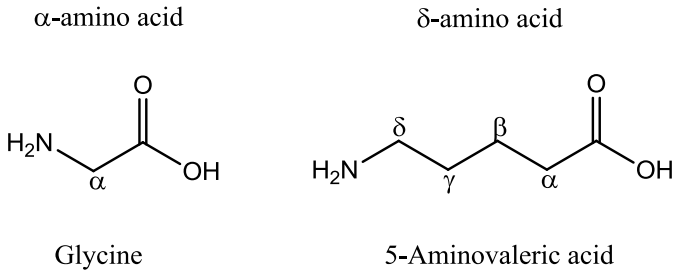
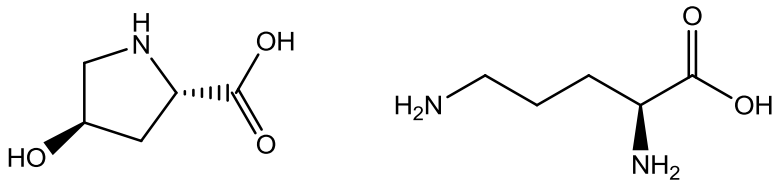


Figure 1: Structure of an α-amino acid (glycine) and a δ-amino acid (5-aminovaleric acid)

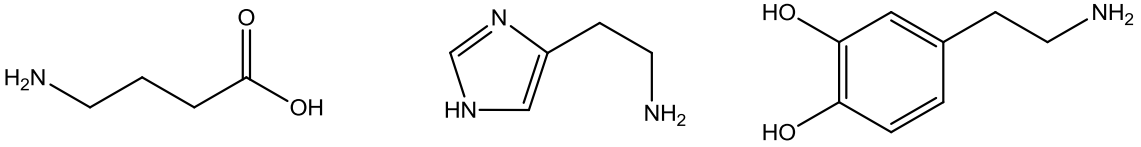
Aside from the proteinogenic α-amino acids, so called non-proteinogenic or non-standard amino acids exist as well. Some of them can be found as building blocks in proteins after posttranslational modifications, for example hydroxyproline in collagene,[1] while others are intermediates in metabolism (for example ornithine in the urea cycle, Figure 2). Also spontaneous rearrangement of aspartic acid or asparagine to the corresponding β-structure has been described in literature. This "ageing-process" of proteins is usually accompanied by loss of function and has been found in plaques of β-amyloid peptides in association with Alzheimer's disease.[2]



Hydroxyproline Ornithine

Figure 2: Structures of hydroxyproline and ornithine

Both proteinogenic and non-proteinogenic amino acids and their derivatives play an important role outside of proteins. Examples are glutamate, dopamine (obtained by hydroxylation/decarboxylation of tyrosine) and γ-aminobutyric acid (GABA), which act as neurotransmitters, or histamine (obtained through decarboxylation of histidine), which mediates allergic reactions (Figure 3).[3]



γ-Aminobutyric acid (GABA) Histamine Dopamine

Figure 3: Examples of biologically active amino acid (γ-aminobutyric acid) and derivatives (histamine and dopamine)

Furthermore, amino acids can serve as building blocks for larger molecules. The α-amino acid glycine, for example, is used in the biosynthesis of the porphyrin structure.[3] The antibiotic polymyxin B_2 contains six molecules of α,γ-diaminobutanoic acid with five of them incorporated as α-amino acid in the peptide chain and the sixth in a "α and γ" fashion (Figure 4).[2]

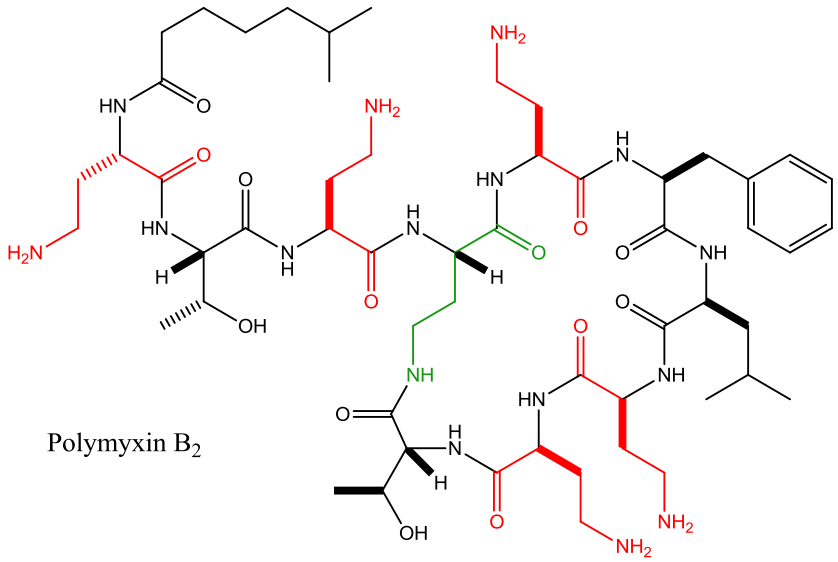
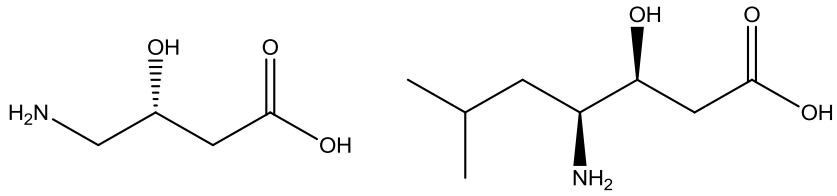


Figure 4: Polymyxin B2. The structure contains six molecules of α,γ-diaminobutanoic acid incorporated as α-amino acid (depicted in red) and in α- and γ-fashion (depicted in green).

Synthesis of non-natural amino acids has gained scientific interest because of their large scope of applications. Incorporation of unnatural amino acids in proteins has been used as tool for investigation and manipulation of protein structure and function.[4] Oligomeric structures – so called foldamers – of those newly synthesized compounds are especially relevant due to their ability to adopt specific conformations, which might give rise to new pharmaceutical and material properties.[5]

1.2. γ- and δ-Amino acids

Most amino acids whose amino group is not located at the α-position have been isolated from naturally occurring peptide antibiotics or alkaloids.[6] The γ-amino acid 4-amino-3-hydroxybutyric acid, for example, is a building block for microsclerodermins, a family of cyclic peptides displaying antitumor and antifungal activity.[7] The β-hydroxy-γ-amino acid (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine) is a component of the hexapeptide antibiotic pepstatine (Figure 5).[8]



(*R*)-4-Amino-3-hydroxybutyric acid Statine

Figure 5: Examples of naturally occurring γ-amino acids

Despite their role as building blocks for larger molecules, γ-amino acids, γ-aminobutyric acid being the most prominent, also serve as important bioactive compounds in the central nervous system.

1.2.1. Biological relevance and application of γ- and δ-amino acids

γ-Aminobutyric acid (GABA) acts as an inhibitory neurotransmitter in the mammalian central nervous system. It binds to specific receptors, which can be divided into two classes: the ionotropic $GABA_A$ and $GABA_A$-ρ and the metabotropic $GABA_B$ receptors. The $GABA_A$ and $GABA_A$-ρ receptors are anion-selective ligand-gated ion channels, which are mainly permeable to chloride ions and to some extent to bicarbonate anions.[9] Upon binding of GABA, the channel opens, which leads to hyperpolarization of the neuron and thus to a reduction of action potential. The $GABA_A$-ρ receptor, previously known as $GABA_C$ receptor, is a subtype of $GABA_A$ receptors consisting only of ρ-subunits.[10] The G-protein coupled $GABA_B$ receptors are linked to potassium channels which leads again to hyperpolarization and inhibition of action potential upon binding of GABA.[11] While the main effect of GABA receptors is inhibition of action, potential excitatory effects on developing neurons have been reported.[12]

GABA is synthesized from glutamamic acid (Glu) by glutamic acid decarboxylase (GAD) in the brain since it is not able to cross the blood-brain barrier. It is degraded by GABA transaminase (GABA-T) by oxidative deamination to succinic semialdehyde (Figure 6).[13]

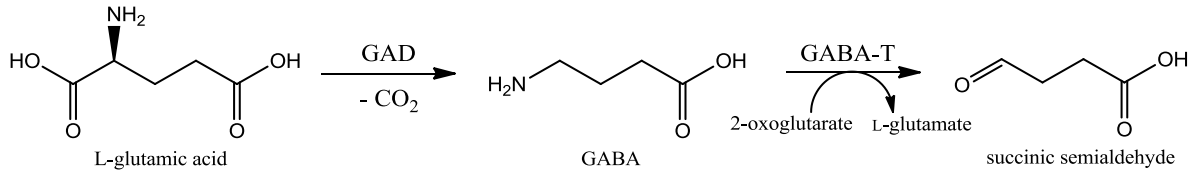


Figure 6: Biosynthesis and degradation of γ-aminobutyric acid (GABA)

Low levels of GABA in the brain can result in convulsions and lead to epilepsy, Parkinson's disease, motion disorders and other neurological diseases as well as depression, pain or alcoholism. The polarity and high flexibility of the structure of GABA prevents the molecule from passing the blood-brain barrier and renders administration of the molecule as drug therapy useless. An approach to increase GABA levels in the brain is the design of analogue structures which are lipophilic and more rigid. Potential targets for therapeutic drugs are either the GABA receptors or the enzyme GABA-T.[13]

Baclofen, (±)-4-amino-3-(*p*-chlorophenyl)-butanoic acid, is selectively recognized by the $GABA_B$ receptor and acts as a muscle relexant. The drug is sold as racemate but only the (*R*)-enantiomer is active. Vigabatrin (4-aminohex-5-enoic acid) is a suicide substrate for GABA-T due to its vinyl moiety and thus increases the level of GABA by prohibiting its degradation. The pharmacologically active enantiomer is the (*S*)-form of the drug. The (*S*)-enantiomer of 3-isobutyl GABA is called Pregabalin and is not recognized by GABA receptors. It acts on calcium channels on the membrane and reduces the release of neurotransmitters and is used to treat neuropathic pain, generalized anxiety disorder and epilepsy (Figure 7).[13, 14]

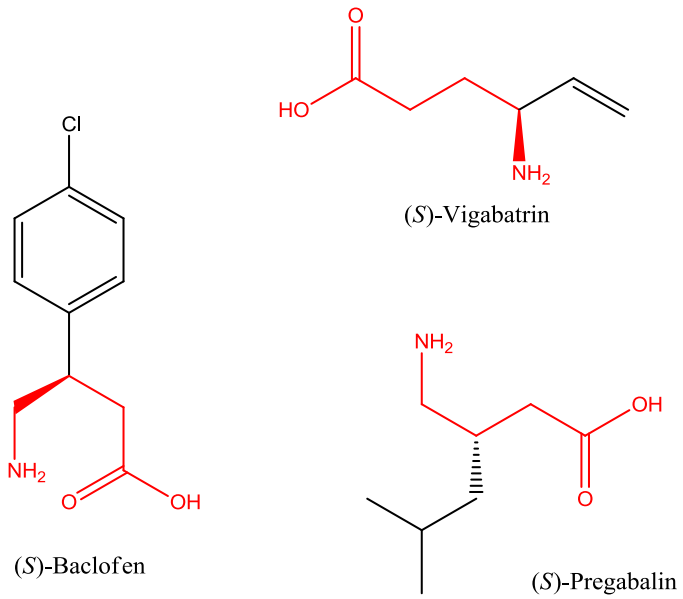


Figure 7: Examples of GABA-analogues. The GABA sub-structure is depicted in red.

An additional field where γ- and δ-amino acids are employed is the synthesis of peptidomimetics and foldamers. The term "foldamer" refers to polymers which adopt a complex and very specific conformation. Proteins or DNA are the prototypes found in nature for this class of substances.[5] It has long been known that incorporation of β- or γ-amino acids in conventional α-peptide chains can lead to the formation of stabilized secondary structures [15] and resistance against hydrolytic cleavage by serum and tissue proteases [16]. However, peptides consisting solely of β- or γ-amino acids are also able to adopt stable secondary structures. Due to the additional carbon atoms in the backbone and the thus generated increase in flexibility of the molecule, more order and stability than in α-peptides can be obtained. The secondary structures like β-sheets, α-helices and turns in β- and γ-peptide chains differ significantly from their α-counterparts. However, it has been shown that these peptides can mimic α-peptidic ligands in peptide-receptor interactions but cannot be cleaved by proteases, thus providing great means for medicinal chemistry.[2]

Another application of synthetic amino acids lies in the field of nanotechnology for the preparation of nanotubes. These tubular structures might find application in catalysis, drug delivery and chemotherapy.[17] Stacking of cyclic peptides is stablized by hydrogen bonds and leads to the formation of so-called self-assembling peptide nanotubes (SPNs). Appropriate modification of the side chains of the amino acids allows functionalization and may produce nanotubes with high selectivity as ion channels or catalysts.[18, 19]

1.2.2. Chemical synthesis of γ-amino acids

Various strategies for the chemical synthesis of γ-amino acids with different substitution patterns have been developed. Thus, the following examples focus on the more general applications. The first methods describe homologation reactions, using chiral α-amino acids as starting materials and transforming them into higher homologues.

1.2.2.1. Double Arndt-Eistert homologation

Starting from a carboxylic acid, a series of chemical reactions named the Arndt-Eistert homologation yields the higher homologue of the acid.

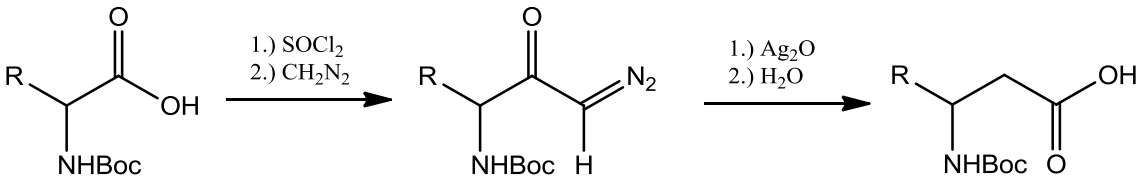


Figure 8: General procedure for Arndt-Eistert homologation [20]

As depicted in Figure 8, homologation of a Boc-protected α-amino acid yields the β-amino acid homologue. Thus, for the γ-homologue a second round of reaction is necessary. While the preparation of β-amino acids in the first round occurs smoothly, yields drop to 40-50% in the first step and to 66-67% in the second step towards γ-amino acids. The overall yield of double Arndt-Eistert homologation is <20%.[20] Furthermore, the acid chlorides of *N*-Boc protected α-amino acids are prone to racemization, thus decreasing the enantiomeric excess of the product. The original Arndt-Einstein homologation uses thionyl chloride for the synthesis of the acid chloride and diazomethane to yield the diazoketone, however any procedure which yields the acid chloride and less harmful and dangerous compounds for diazo-group transfer can be used.

Double Arndt-Eistert homologation of α-amino acids yields γ-amino acids, which are not substituted at the α- and β-position.

1.2.2.2. Wittig olefination

Chain homologation by a Wittig-type olefination reaction starts from α-amino aldehydes. The latter can be prepared either by oxidation of 2-amino alcohols or by reduction of the carboxyl group of the α-amino acid. Figure 9 shows the synthesis starting with reduction of the mixed anhydride or the acyl fluoride of the *N*-protected α-amino acid with $NaBH_4$ to the corresponding alcohol (step a or b, Figure 9), which in turn is oxidized by NaOCl to the aldehyde (step c). Wittig olefination yields the α,β-unsaturated ester, which is converted to the *N*-protected γ-amino acid by hydrogenation.[21]

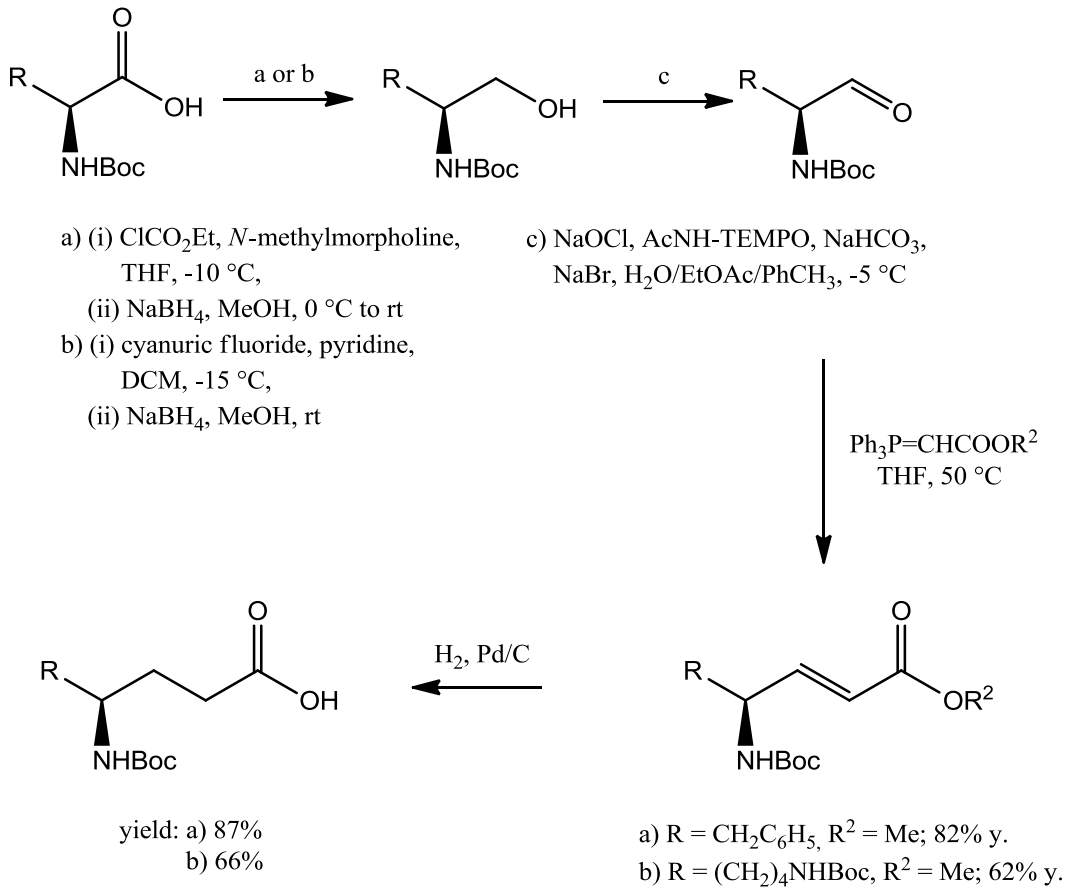


Figure 9: Procedure for homologation of α-amino acids via Wittig olefination [21]

The enantiomeric purity of the final product depends mainly on the conditions used for the preparation of the α-amino aldehyde, prone to racemization, and the Wittig reaction. Oxidation of the amino alcohol by NaOCl/AcNH-TEMPO provided products with high

enantiomeric purity and thus presents an efficient method for the preparation of γ-substituted γ-amino acids with proteinogenic side chains.

1.2.2.4. Examples for enantioselective synthesis of pharmaceutical γ-amino acids

Enantioselective synthesis of (*S*)-pregabalin was also reported using asymmetric hydrogenation of 3-cyano-5-methylhex-3-enoic acid as key step. Several Rh-based catalysts have been described, yielding the corresponding cyano derivative in high yield und enantiomeric excess (Figure 10).

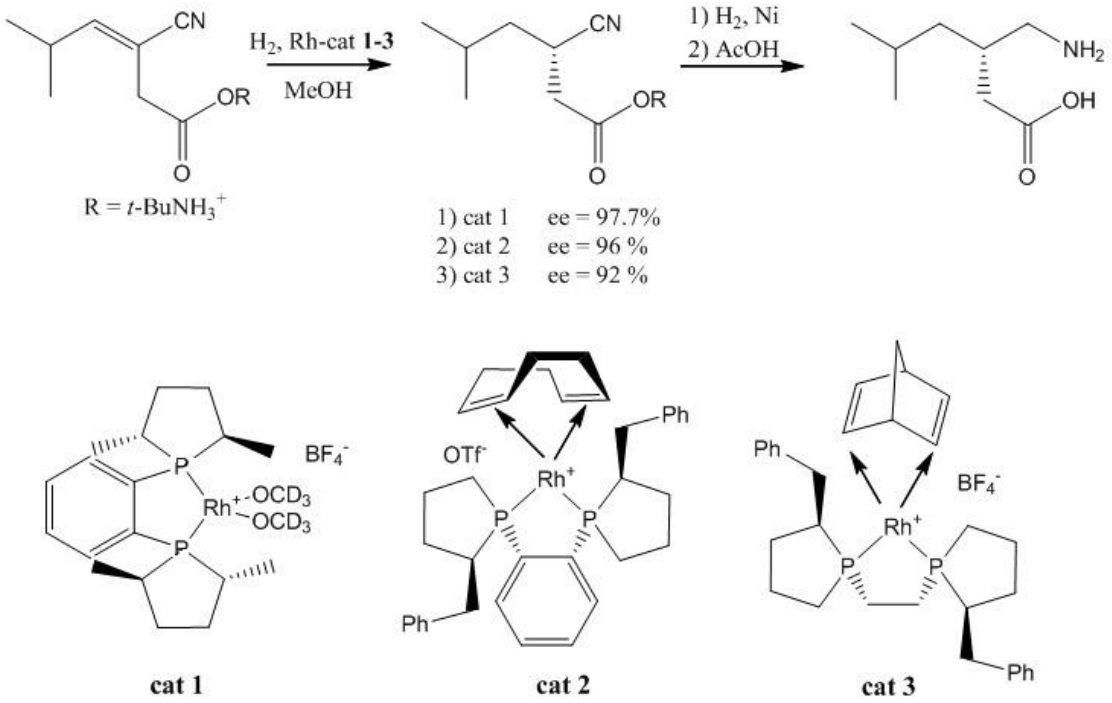


Figure 10: Enantioselective synthesis of (*S*)-pregabalin by asymmetric hydrogenation of 3-cyano-5-methylhex-3-enoic acid and subsequent hydrogenation [22, 23, 24]

The *tert*-butylammonium salt of 3-cyano-5-methylhexanoic acid was obtained by hydrogenation catalyzed by the Rh-based catalysts **1-3** in enantiomeric excess ranging from 92% (cat. 3) to 97.7% (cat 1, Figure 10). Catalytic hydrogenation of the cyano function and treatment with acetic acid provided (*S*)-pregabalin in 61% yield and an enantiomeric excess of 99.8%.[22, 23, 24]

Another important GABA analogue is the β-arylsubstituted γ-amino acid 4-amino-3-(4-chlorophenyl)butanoic acid, sold as racemate under the name baclofen. However, several

strategies for the synthesis of enantiomerically pure (*R*)- and (*S*)-baclofen have been developed. An elegant possibility is to synthesize a β-aryl lactone by intermolecular enantioselective Heck-Matsuda arylation and further transformations to afford (*R*)-baclofen (Figure 11).

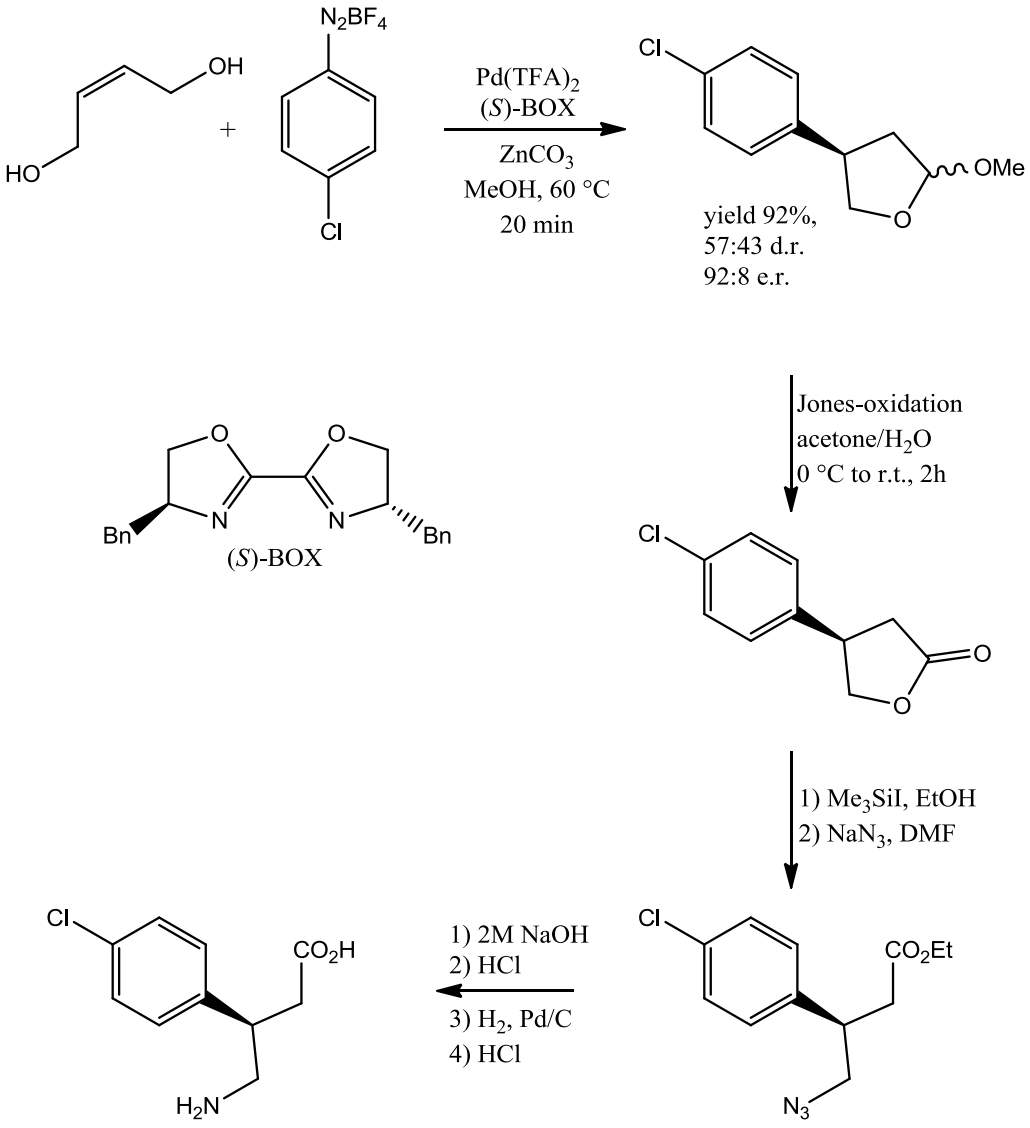


Figure 11: Synthesis of (*R*)-baclofen by enantioselective Heck-Matsuda arylation [25, 26]

The synthesis of the lactone starts with arylation of the *Z*-olefin by an aryldiazonium tetrafluoroborate in the presence of $Pd(TFA)_2$ and a chiral bisoxazoline ligand [(*S*)-BOX, Figure 11]. The diastereomeric *O*-methyl lactols were obtained in high enantiomeric excess (92%) and were directly converted into the corresponding 4-aryl-γ-lactone, thus eliminating the stereocenter of the lactol ring. Lactone opening with iodotrimethylsilane and treatment

with sodium azide gave the azido ester, which can be converted to (*R*)-baclofen by hydrolysis, hydrogenation and treatment with hydrochloric acid.[25, 26]

Another possibility to synthesize (*R*)-baclofen involves Claisen rearrangement of an allylic alcohol with triethyl orthoester in the presence of propionic acid. An overview of the synthesis route is given in Figure 12.

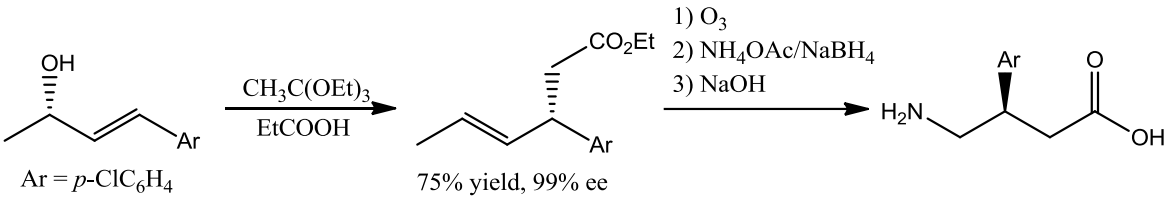


Figure 12: Synthesis of (*R*)-baclofen by Claisen rearrangement, ozonolysis and hydrolysis [27]

The reaction of the allylic alcohol with the orthoester afforded the (*S*)-γ,δ-unsaturated ester in perfect enantiomeric excess and 75% yield. Ozonolysis, followed by treatment with NH_4OAc and hydrolysis with NaOH afforded (*R*)-baclofen in 73% yield.[27]

1.2.3. Chemical synthesis of δ-amino acids

A method for the synthesis of enantiomerically pure 5-amino-2-methylpentanoic acid by hydrolysis of a chiral auxiliary yielding 4-cyano-2-methylbutanoic acid and hydrogenation thereof has been developed (Figure 13).[28]

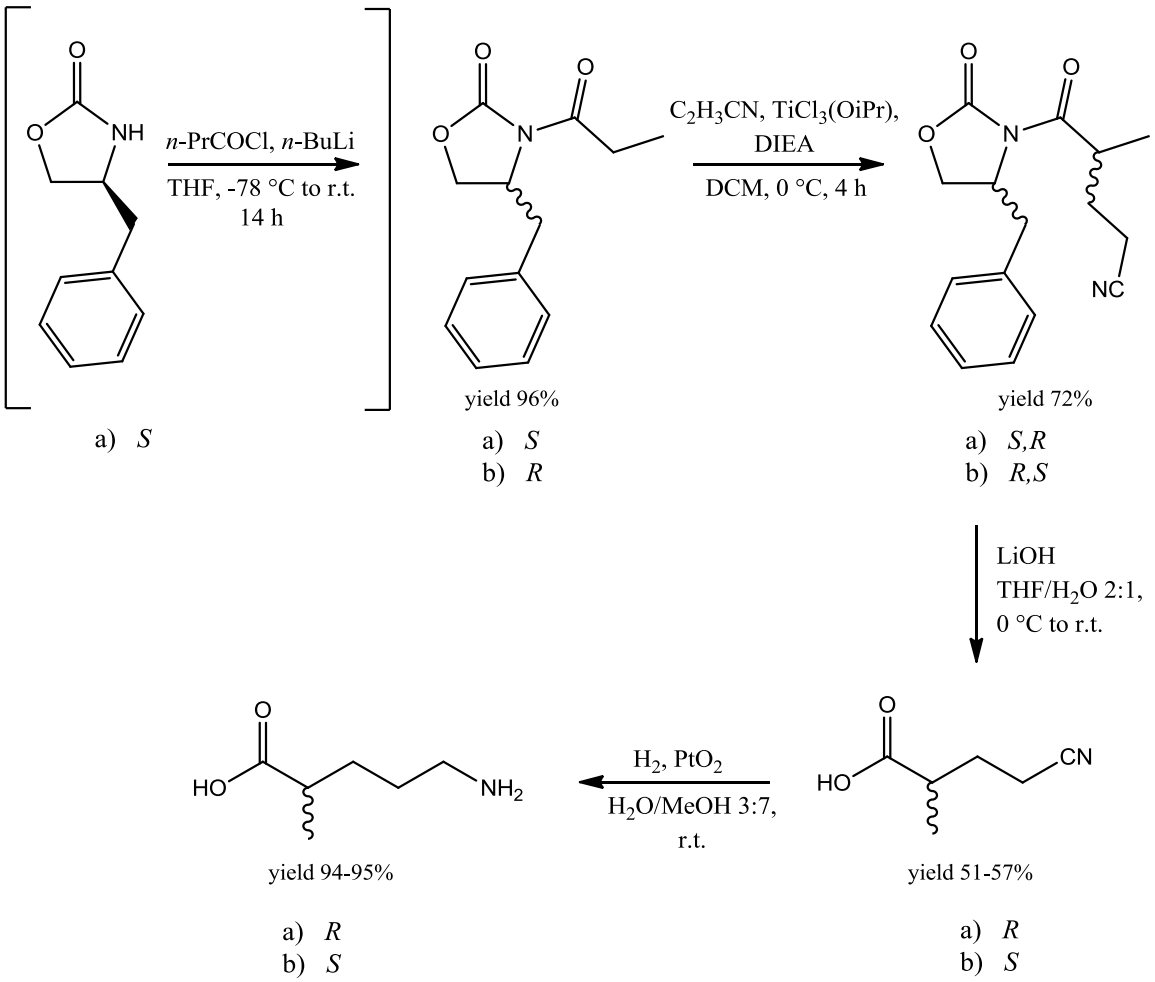


Figure 13: Synthesis of both enantiomers of 5-amino-2-methylpentanoic acid [28]

While (*R*)-4-benzyl-3-propionyl-2-oxazolidinone was commercially available, the (*S*)-enantiomer was prepared by functionalization with propionyl chloride and *n*-butyllithium from (*S*)-4-benzyl-2-oxazolidinone. After functionalization with acetonitrile, the chiral auxiliary was hydrolyzed and the enantiopure 5-amino-2-methylpentanoic acid hydrochloride was isolated after hydrogenation with PtO_2.[28]

Another strategy to synthesize the (*S*)-enantiomer of 5-amino-2-methylpentanoic acid is the utilization of the Evans chiral auxiliary (*S*)-4-benzyl-2-oxazolidinone for stereoselective introduction of the methyl group at the C_α-position (Figure 14).[29]

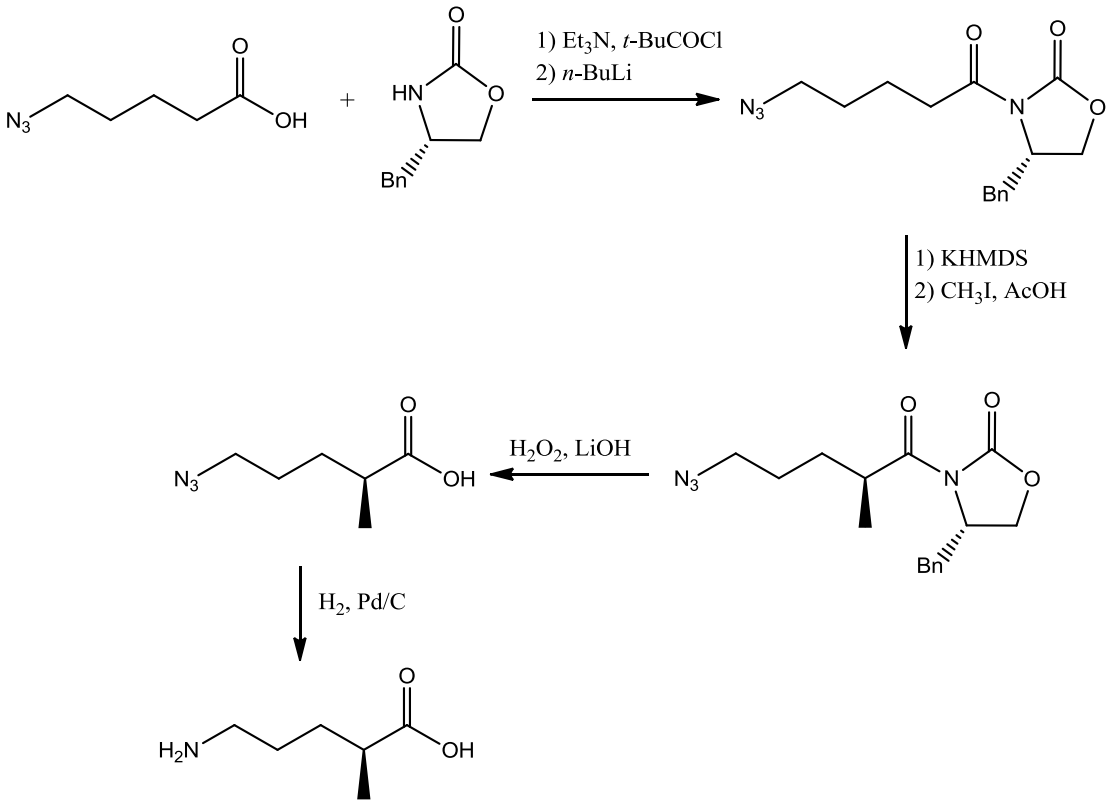


Figure 14: Synthesis of (*S*)-5-amino-2-methylpentanoic acid [29]

1.2.4. Synthesis of amino acids by chemical hydrolysis of lactams

Lactams are cyclic amides and can be converted to the corresponding amino acids by chemical hydrolysis. While chapter 1.3 (p. 26) describes properties and examples of naturally occuring lactams, this chapter focuses on the synthesis of lactams and subsequent hydrolysis.

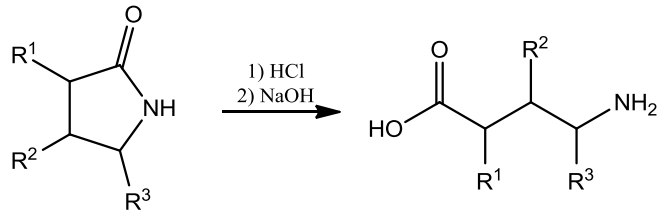


Figure 15: Hydrolysis of γ-lactam

As depicted in Figure 15, hydrolysis of lactams requires concentrated acids and high temperatures. These conditions are on one hand energy consuming and on the other hand

incompatible with sensitive functional groups, which limits the possibilities of potential substituents of the compound. Furthermore, large amounts of salt are generated by neutralization.

One more general possibility for the synthesis of lactams is the Michael addition of enolates to nitroethenes to afford nitro olefins, which can be reduced to the corresponding amines, followed by spontaneous cyclization to the lactams. Up to three stereogenic centers can be included in the final product by using 1,2-disubstituted nitroethenes and α-substitued enolates (Figure 16).

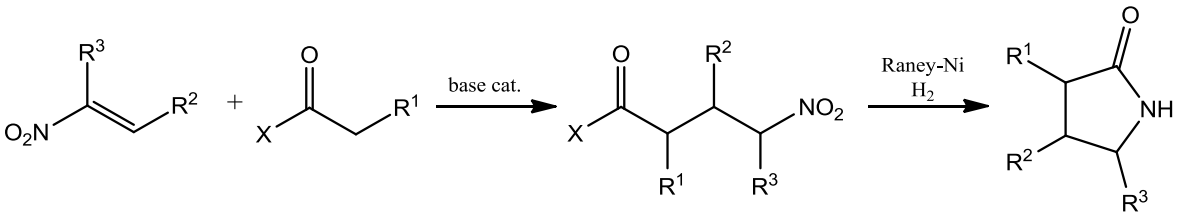


Figure 16: Michael addition of enolates to nitroolefins and subsequent reduction of the nitro group to afford the lactam

Many protocols rely on the use of chiral starting material for the introduction of chirality in the product. As depicted in Figure 17, Michael addition of acyl-oxazolidinones to nitrostyrene affords the corresponding nitro olefin. Both diastereomers are formed in a diastereomeric ratio of >95:5 and can be separated by crystallization.[30, 31, 32]

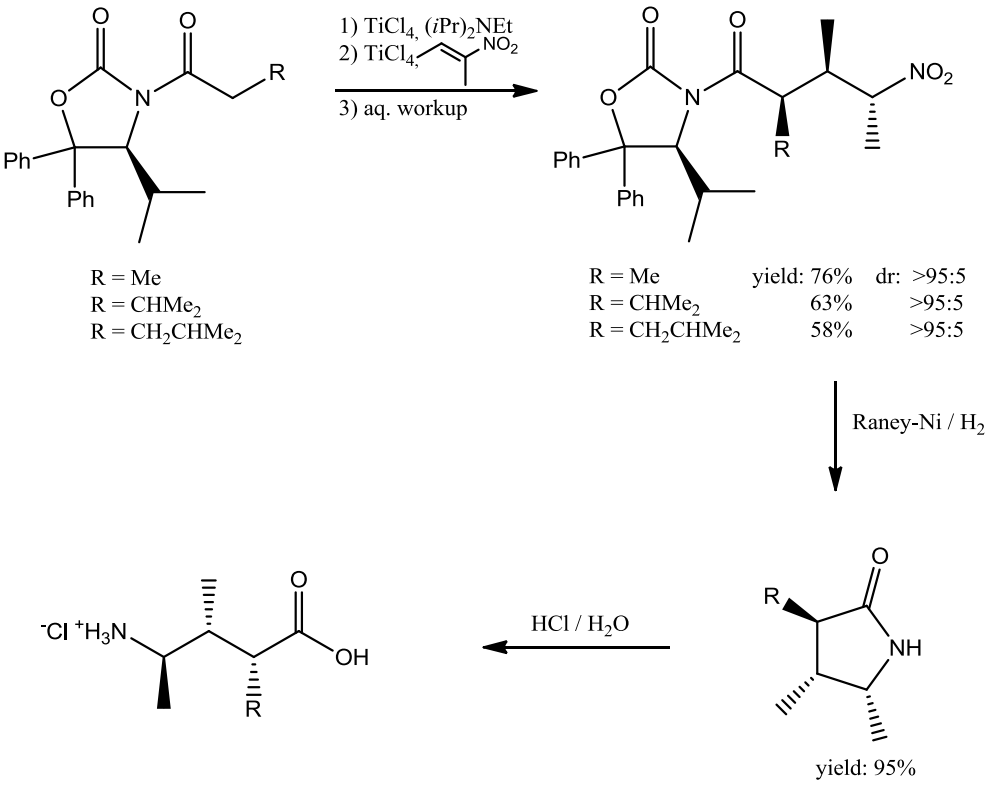


Figure 17: Michael addition of acyl-oxazolidinones to nitrostyrene, reduction and subsequent hydrolysis to yield the γ-amino acid [30, 31, 32]

Another possibility to introduce chirality is the use of chiral catalysts, for example the squaramide-based organocatalyst **2** (Figure 18) for the additon of malonic acid hemithioesters to nitroolefins.

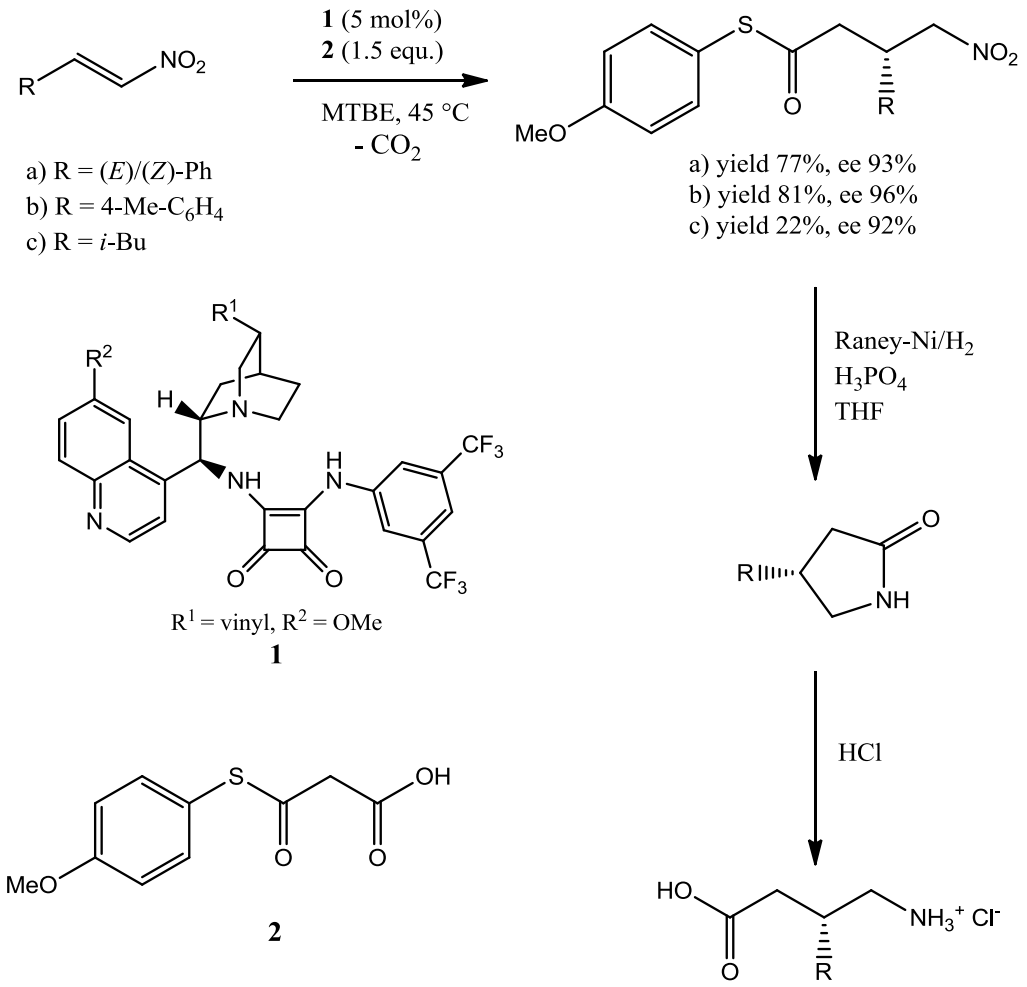


Figure 18: Michael addition of malonic acid hemithioesters to nitroolefins catalyzed by a squaramide-based organocatalyst [33]

The reaction depicted in Figure 18 is mimicking chain elongation of polyketides and fatty acids by enzymatic activation of malonic acid hemithioesters (MAHTs) to generate ester enolates. The Michael addition of MAHT **2** to different nitroolefins yields the Michael adduct in high enantiomeric excess (92-96%) and moderate to good yields (22-81%). Poor conversions can usually be observed in the case of alkyl-substituted nitroolefins. The Michael addition occurs in an enantioconvergent fashion, meaning that *E*/*Z*-olefins always yield the same (*S*)-enantiomer of the Michael adduct. Reduction of the nitro group to the amine is followed by intramolecular cyclization and yields the corresponding γ-lactam, which can be hydrolyzed to afford the β-substituted γ-amino acid.[33]

The reaction presented in Figure 18 can be used for the synthesis of the β-alkyl substituted γ-amino acid (*S*)-3-aminomethyl-5-methylhexanoic acid, also known as (*S*)-baclofen, by addition of a malonic acid hemithioester to *trans*-β-nitro-4-chlorostyrene (Figure 19).

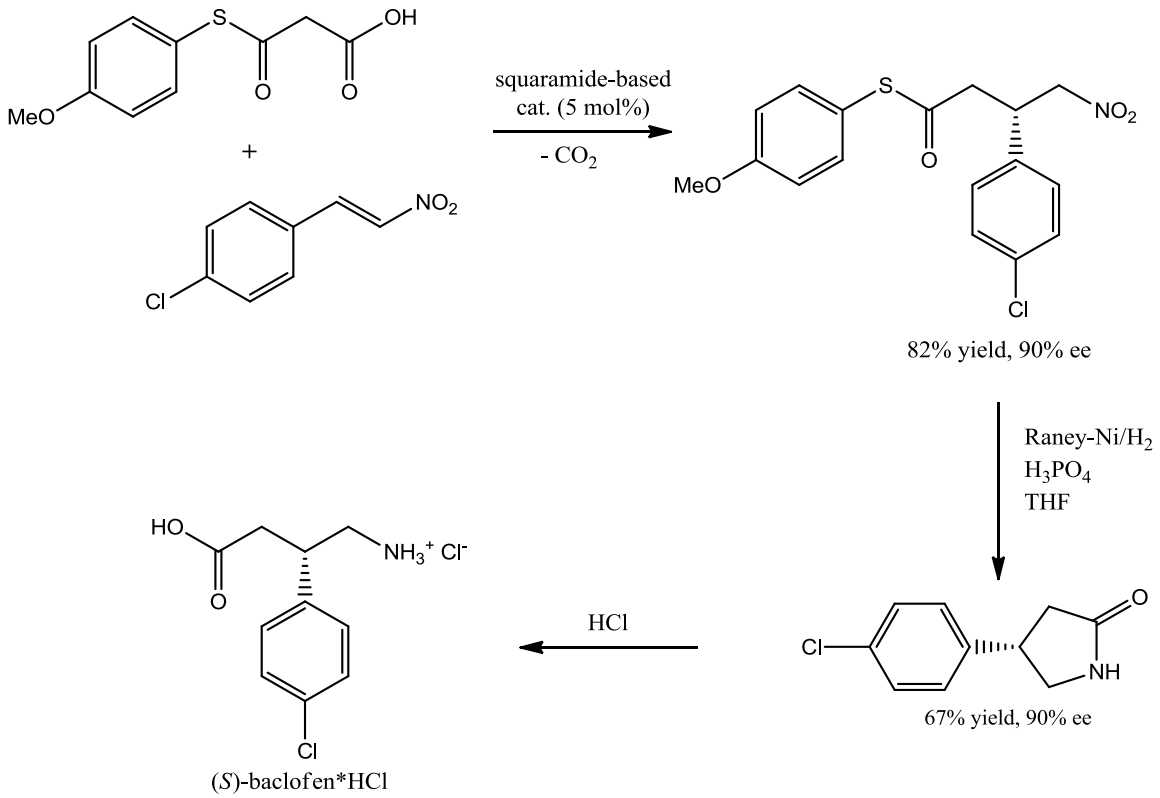


Figure 19: Synthesis of (*S*)-baclofen by Michael addition of malonic acid half thioesters to *trans*-β-nitro-4-chlorostyrene catalyzed by a squaramide-based organocatalyst [33]

The depicted reaction has been run on a 5 g scale and yielded enantiopure (*S*)-baclofen after one single step of recrystallization of the hydrolyzed lactam.[33]

The GABA analogue (*S*)-baclofen can be obtained by synthesis and subsequent hydrolysis of an aryl-substituted γ-lactam, which is prepared from (1*R*)-camphor (Figure 20).

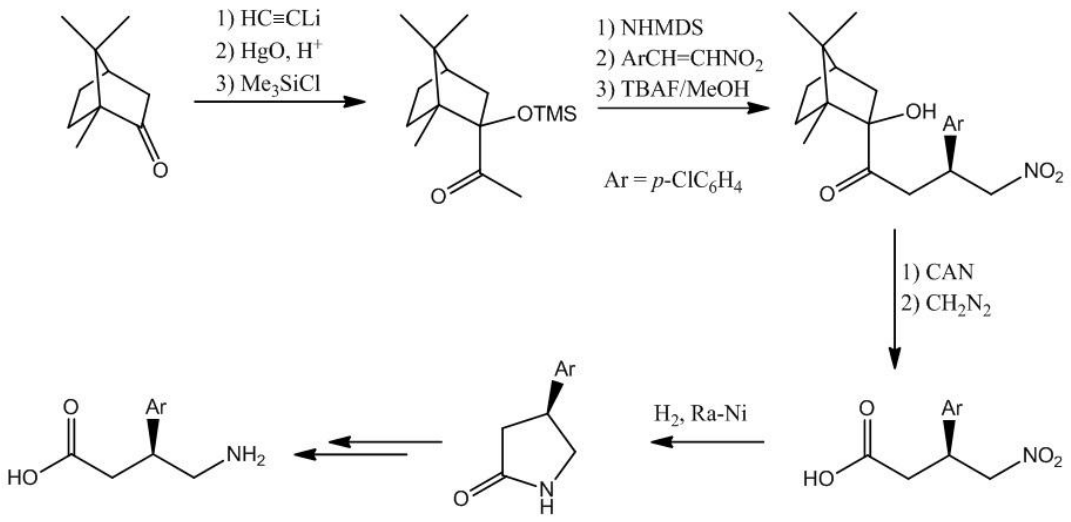


Figure 20: Synthesis of (*S*)-baclofen by hydrolysis of the aryl-substituted γ-lactam prepared from (1*R*)-camphor [34]

Deprotonation of the methyl ketone synthesized from (1*R*)-camphor by sodium bis(trimethylsilyl)amide (NHMDS) yields the sodium enolate, which reacts with the nitrostyrene derivative upon Michael addition. Addition of an excess of cerium ammonium nitrate (CAN) and esterification by diazomethane affords the nitromethyl ester, which is converted to the corresponding lactam by reduction of the nitro group. Hydrolysis and further neutralization yields the corresponding γ-amino acid (*S*)-baclofen.[34]

Among other different synthesis protocols, (*S*)-pregabalin can also be obtained from hydrolysis of a lactam precursor synthesized via a highly stereoselective conjugate-addition of potassium alkenyltrifluoroborates to cyclic α,β-unsaturated carbonyl compounds (Figure 21).

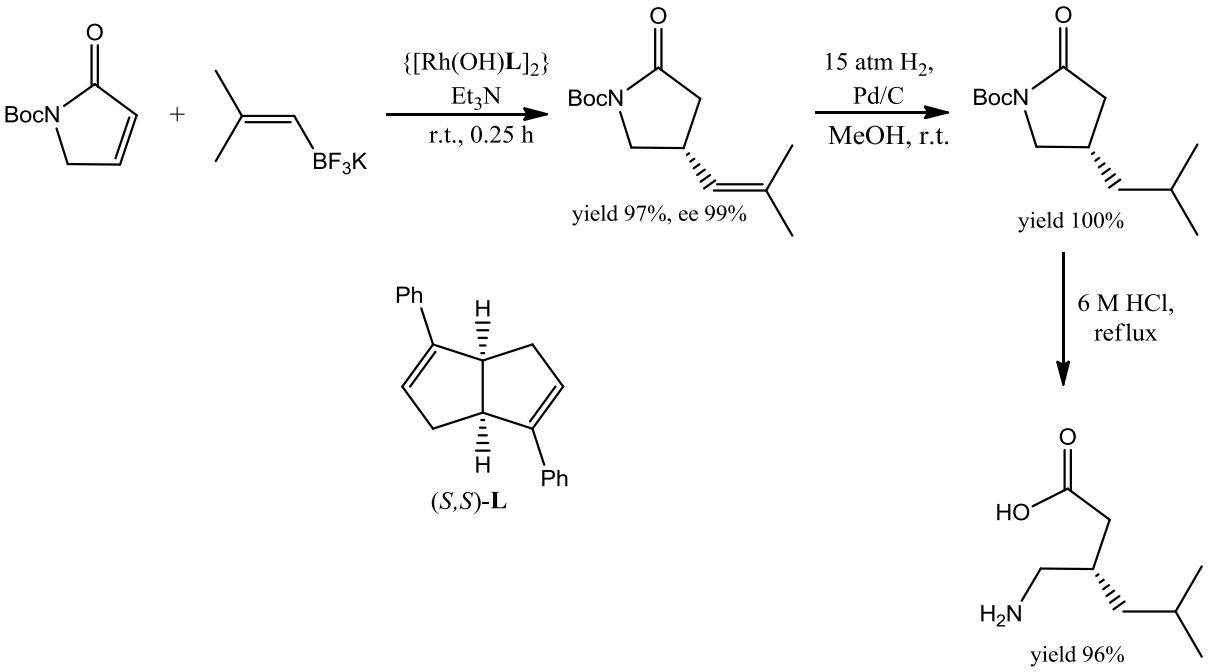


Figure 21: Stereoselective synthesis of (*S*)-pregabalin conjugate-addition of potassium alkenyltrifluoroborates to a cyclic α,β-unsaturated carbonyl compound [35]

The rhodium catalyst used for the synthesis was produced *in situ* from $\{[RhCl(C_2H_{4)2}]_2\}$ and a chiral ligand (**L**, Figure 21). The product of the conjugate-addition was isolated in high yield (97%) and enantiomeric excess (99%) and was subsequently reduced by hydrogenation and was further hydrolyzed to afford (*S*)-pregabalin.[35]

1.2.5. Biocatalytic synthesis of enantiopure γ-amino acids

1.2.5.1. Enzymatic resolution employing acylase

Derivatives of racemic mixtures of γ-amino acids can undergo enzymatic resolution by penicillin acylase from *E. coli*. This enzyme is highly specific for the hydrolysis of the phenylacetyl group and converts the *N*-phenylacetyl derivatives to the corresponding γ-amino acids (Figure 22).

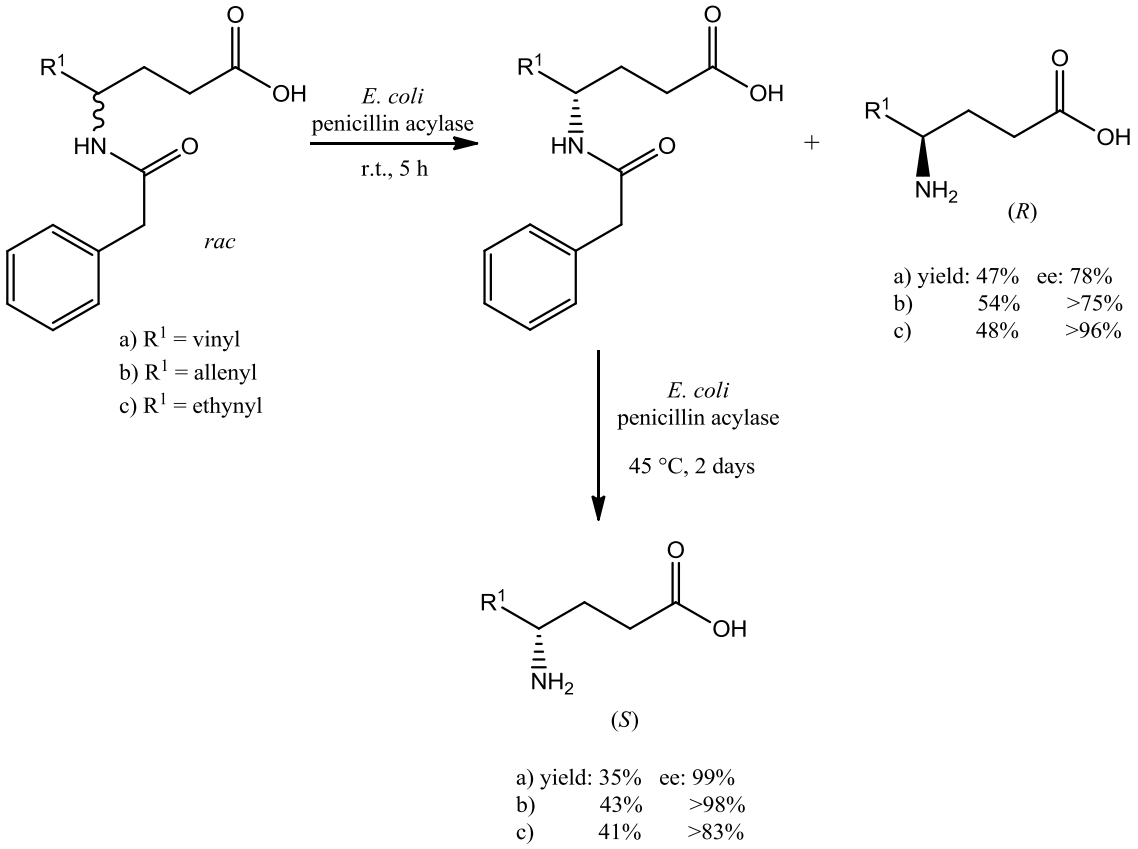


Figure 22: Enzymatic resolution of *N*-phenylacetyl derivatives of γ-amino acids by penicillin acylase [36]

Penicillin acylase first hydrolyzes the (*R*)-enantiomer of the amino acid derivative and leaves the (*S*)-*N*-phenylacetyl enantiomer behind, which can be extracted with dichloromethane and subjected to subsequent hydrolysis with the same enzyme to yield the (*S*)-γ-amino acid.[36]

1.2.5.2. Nitrilase-mediated hydrolysis of γ-amino nitriles

Nitrilases catalyze the enantioselective hydrolysis of nitrile groups and produce the corresponding carboxylic acid as only product released (Figure 23) in contrast to the nitrile hydratase- amidase system, in which the amide produced by the nitrile hydratase is subequently hydrolyzed by the amidase. This single-enzyme system has led to an increase in the interest for nitrilases in carboxylic acid synthesis.[37]

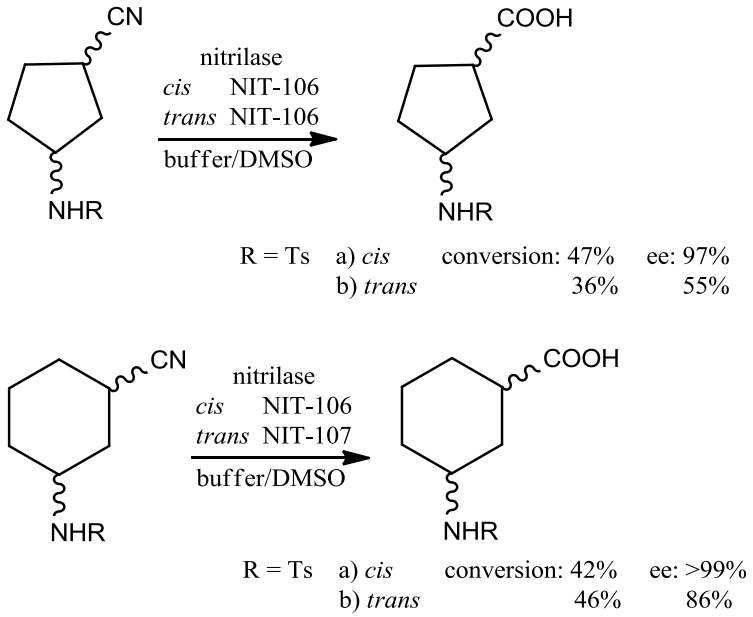
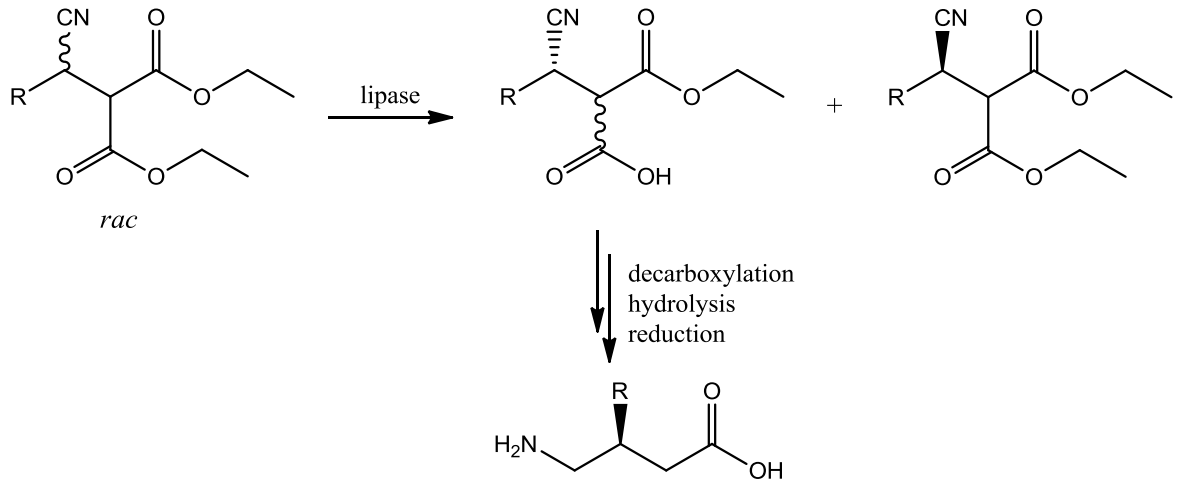


Figure 23: Nitrilase-catalyzed hydrolysis of γ-amino nitriles using nitrilases NIT-106 and NIT-107[37]

1.2.5.3. Chemo-enzymatic synthesis of γ-amino acids

Several chemoenzymatic processes employing different enzymes for the synthesis of chiral precursors of γ-amino acids and corresponding pharmaceutical drugs exist.

One strategy starts with enzymatic resolution of a β-cyanodiester by lipases. The process generates the single (*S*)-enantiomer that can be converted to the corresponding β-substituted γ-amino acid upon decarboxylation, hydrolysis and reduction.



	ee_P	ee_S	c	lipase
a) R = *i*-Pr	91%	>99%	52%	*Mucor miehei*
b) R = *i*-Bu	>99%	95%	47%	*T. lanuginosa*
c) R = Bn	>99%	96%	49%	*Rhizoptus delemar*

Figure 24: Preparation of precursors of γ-amino acids by enzymatic resolution of a β-cyanodiester by lipases. The hydrolysis product was not isolated after extraction and further used for decarboxylation. Ee values correspond to the resolution by lipase. Conversions correspond to decarboxylated products.[38]

As depicted in Figure 24, hydrolysis of the ester at the C-2 center can result in the formation of two diastereomers. Since the chirality at C-2 gets lost during decarboxylation, the diastereoselectivity in the desymmetrization reaction is not relevant; however, all enzymes were highly diastereoselective. It should be noted that this method can be used to synthesize pregabalin (product b).[38]

Similarly to this lipase-based route, hydrolytic enzymes, namely porcine liver esterase and *Candida antarctica* lipase B (CAL-B), can be employed for the desymmetrization of 3-alkylglutaric acid diesters. The reaction scheme and further chemical transformations necessary for the preparation of the corresponding amino acid are depicted in Figure 25.

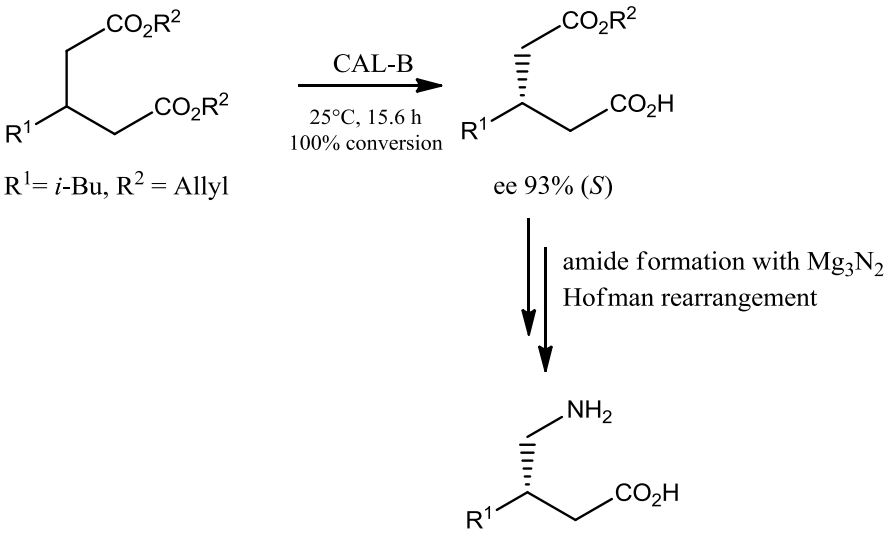
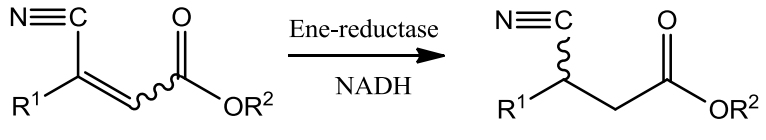


Figure 25: Preparation of precursors of γ-amino acids by desymmetrization of 3-alkylglutaric acid diesters employing lipases [39]

While full conversions of diethylesters with different residues on R^1 and R^2 were achieved in most cases, smaller residues than the allyl-group on R^2 resulted in a reduced enantiomeric excess. The desymmetrization shown in Figure 25 has been used for the synthesis of a precursor of (*S*)-pregabalin, which can be prepared by conversion of the ester to the corresponding amide, subsequent recrystallization and Hofmann rearrangement, to yield the enantiopure product.[39]

Another synthesis route involves the asymmetric bioreduction of β-cyanoacrylates by ene-reductases, thus improving process efficiency by increasing the maximal yield from 50% associated with kinetic resolutions to 100%.



	OYE2	NCR
1) (*E*) R^1 = Me, R^2 = Et	c. >99%, ee >99% (*S*)	c. 45%, ee 97% (*S*)
2) (*Z*) R^1 = Me, R^2 = Et	c. 16%, ee >99% (*R*)	c. 94%, ee >99% (*S*)

Figure 26: Preparation of precursors of γ-amino acids by asymmetric bioreduction using ene-reductases [40]

Regarding the formation of both enantiomers, it was observed that (*E*)-β-cyanoacrylic acid esters were converted to the (*S*)-product while the (*R*)-products could be obtained from the (*Z*)-analogues (substrate-stereocontrol). Conversions dropped with increasing sizes of R^1 and R^2. Similarly to the example presented in Figure 24, the corresponding γ-amino acid can be prepared by hydrolysis of the ester moiety and reduction of the cyano group.[40]

In addition to the synthetic strategy of (*R*)-baclofen introduced in chapter 1.2.2.4 (Figure 11, p. 10), (*R*)-*p*-chlorophenyl lactone can also be produced by a microbiologically mediated Baeyer-Villiger oxidation (Figure 27).

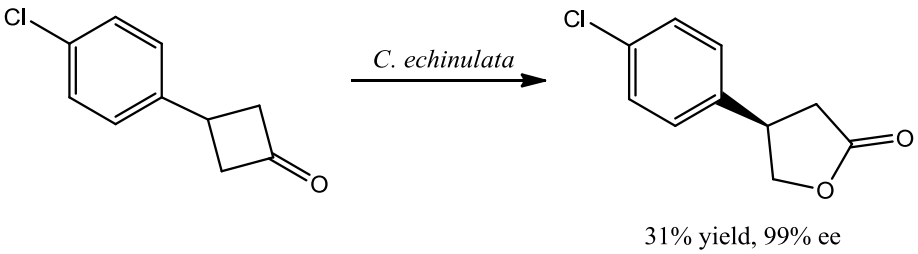


Figure 27: Preparation of precursors of γ-amino acids by microbiological Bayer-Villiger oxidation [41]

Enzymatic oxidation of 3-(*p*-chlorophenyl)cyclobutanone by *Cunninghamella echinulata* NRLL 3655 afforded the lactone in 31% yield and perfect enantiomeric excess. (*R*)-Baclofen can be synthesized by further transformations in analogy to the example presented in Figure 11 (p. 10).[41]

1.2.6. Enzymatic synthesis of δ-amino acids

The synthesis of δ-amino acid 5-aminovaleric acid employing L-lysine oxidase is depicted in Figure 28.

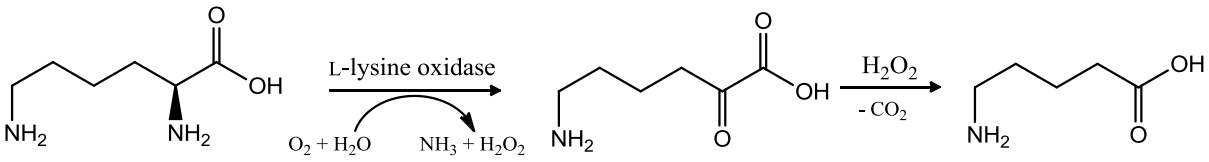
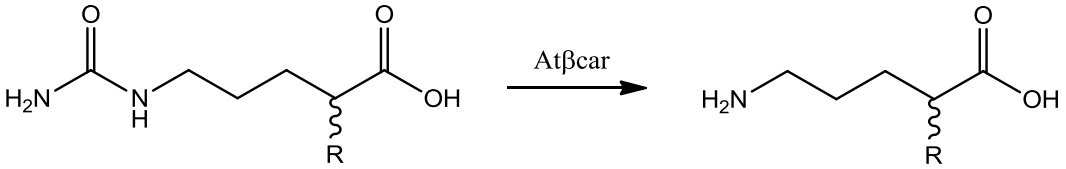


Figure 28: Synthesis of 5-aminovaleric acid employing L-lysine oxidase [42]

The flavin-dependent enzyme L-lysine oxidase oxidizes the α-carbon atom of L-lysine using molecular oxygen. 6-Amino-2-ketocaproic acid is produced and further converted to 5-aminovaleric acid via oxidative decarboxylation by hydrogen peroxide, which is formed as by-product during the enzymatic oxidation. Removal of H_2O_2 by catalase leads to intramolecular cyclization and the formation of Δ^1-piperidine-2-carboxylate.[42]

N-Carbamoyl-β-alanine amidohydrolase from *Agrobacterium tumefaciens* (Atβcar) is able to hydrolyze different *N*-carbamoyl-, *N*-acetyl- and *N*-formyl-amino acids to produce the corresponding amino acids. Figure 29 shows an example for the formation of α-substituted δ-amino acids from the *N*-carbamoyl derivative.



R = NH_2 *N*-Carbamoyl-D/L-ornithine
R = H *N*-Carbamoyl-5-aminopentanoate

Figure 29: Synthesis of α-substituted δ-amino acids by Atβcar [43, 44]

The enzyme Atβcar hydrolyzes the L-enantiomer of *N*-carbamoyl-ornithine 6-7 times faster than the D-enantiomer, thus displaying enantioselectivity for these δ-amino acid precursors.[43, 44]

1.3. Lactamases

Lactams – the term being a combination of the words lactone and amide - are cyclic amides with an 1-azacycloalkan-2-one structure or analogues.[45] Greek letters as prefixes indicate the number of atoms in the cyclic system apart from the carboxyl moiety and the nitrogen heteroatom. A β-lactam, for example, represents a 4-membered ring and a γ-lactam a 5-membered cyclic system (Figure 30).

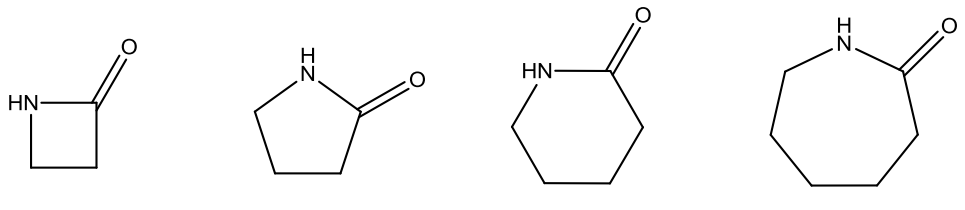


Figure 30: General classification of lactams

The most prominent members of the lactam-family are the β-lactams due to their frequent occurrence as conserved structure in various antibiotics, for example penicillins or cephalosporins. Larger lactams, like γ- and δ-lactams, occur in nature as structural motifs in alkaloids and oxindole derivatives. Examples are brevianamide A, a fluorescent metabolite isolated from *Penicillium brevicompactum* [46], salinosporamide A, containing a γ-lactam-β-lactone bicyclic structure and displaying anticancer activity [47], or horsfiline, an oxindole alkaloid isolated from *Horsfieldia superba* [48] (Figure 31).

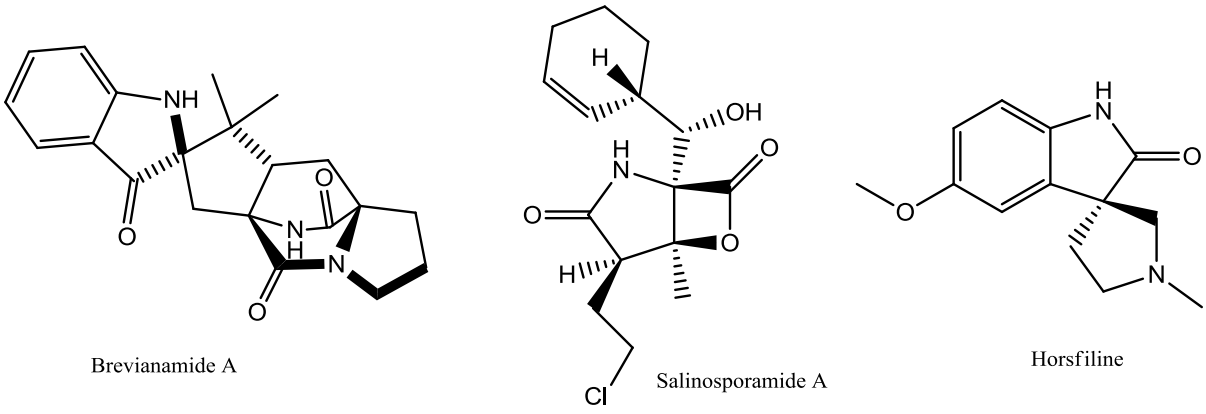


Figure 31: γ- and δ-Lactams as structural motifs in brevianamide A, salinosporamide A and horsfiline [46, 47, 48]

Of special importance is the high chemical stability of the amide bond, which arises from its partial double bond character due to resonance effects (Figure 32).[49] This stabilization might also be a reason, why Nature built proteins as polyamides instead of polyesters. Additionally, the cyclic structure gives lactams of a certain size more chemical stability than linear structures, thus preventing conventional proteases from hydrolyzing the amide bond.[50]

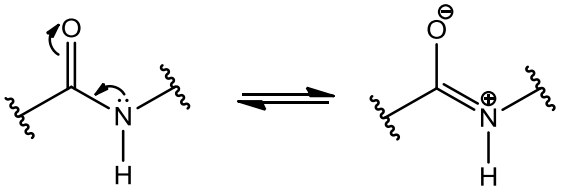


Figure 32: Partial double bond character of amide bonds

The ring strain, which is a result of compressed or expanded angle values compared to their optimal value, is counteracting the stabilizing resonance effect. For instance, cyclohexane has no or very little ring strain, while in smaller cyclic systems ring strain is indirectly proportional to ring size.[51] Thus β-lactams are easier hydrolyzed than γ- or even δ-lactams, posing a problem in clinical research since it renders bacteria resistant against the commonly used β-lactam antibiotics.

In addition to hydrolysis by chemical means, which was discussed in chapter 1.2.4 (p. 13), the amide bond of lactams can also be hydrolyzed by enzymes, for example amidases, that cleave bonds of simple amides, and peptidases, which cleave peptide bonds of peptides and proteins. Despite the significant amount of studies regarding β-lactamase activity, only a few surveys about the enzymatic hydrolysis of γ- or δ-lactams have been published.

The only substrate reported so far in enzymatic hydrolysis of γ-lactams is (±)-2-azabicyclo[2.2.1]hept-5-en-3-one (Figure 33), also known as *rac*-Vince lactam, and few derivatives thereof. The double bond in the second 5-membered ring of the lactam allows different chemical modifications such as epoxidation, fluorination, hydroxylation, etc.[52]

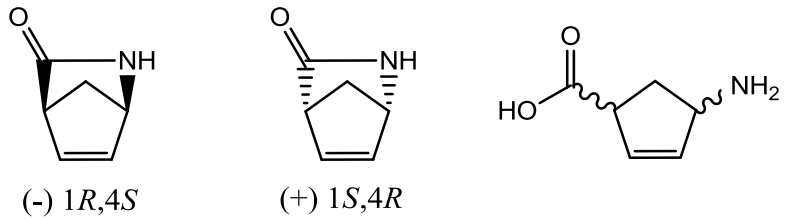


Figure 33: Structure of Vince lactam enantiomers and the hydrolysis product of Vince lactam

The hydrolysis product of Vince lactam - *cis*-4-aminocyclopent-2-ene carboxylic acid - does not only provide a template for the synthesis of conformationally restricted γ-amino acids but also for the preparation of carbocyclic nucleosides, since it provides a cyclopentane ring structure. Carbocyclic nucleosides are nucleosides where the oxygen atom of the sugar moiety is replaced by a carbon atom, giving rise to compounds with antiviral or anticancer activity. They are stabile against hydrolysis by nucleases [53], which leads to a greater bioavailability of the compounds.[54] Examples are abacavir, the commercially available form of the active agent carbovir, which mimics the structure of 2'-deoxyguanoside and inhibits HIV reverse transcriptase [55], or carbocyclic oxanosine, an anti-HIV-agent (Figure 34).

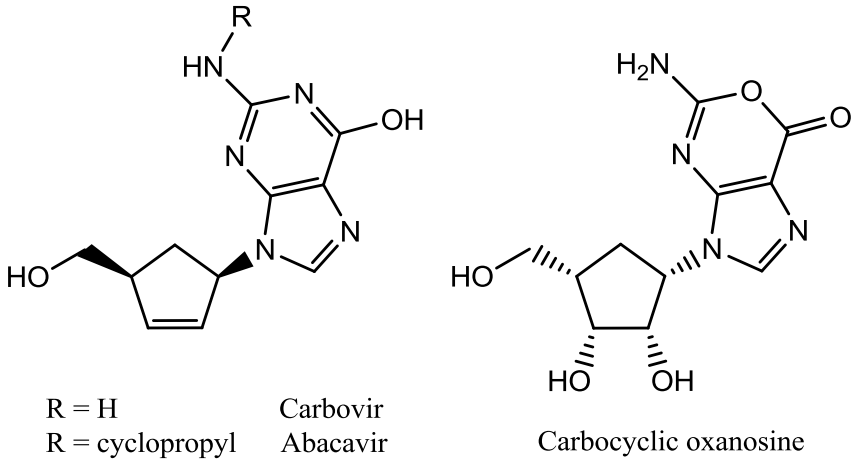


Figure 34: Carbocyclic nucleosides derived from hydrolized Vince lactam

As indicated in Figure 34, synthesis of these pharmaceutical compounds requires enantiopure starting material or powerful asymmetric synthesis routes. Strategies for the production of enantiomerically enriched preparations of Vince lactam include chiral synthesis as well as resolution of the racemate by preferential crystallisation or kinetic resolution.

Attempts of asymmetric synthesis of Vince lactam enantiomers – Diels-Alder reactions either with aluminium and titanium chiral Lewis acids as catalysts or chiral sulfonyl chlorides to introduce chirality – did not provide the compound in good yields and acceptable enantiomeric excess yet.[52] Preferential crystallization of one enantiomer cannot be applied because of the oscillating manner of crystallization, which means that one enantiomer crystallizes until it reaches a certain treashold and the other enantiomer starts to crystallize, thus leading to low ee values.[56]

1.3.1. Kinetic resolution of Vince lactam [57]

The term "kinetic resolution" describes the selective transformation of one substrate enantiomer over the other in a racemic mixture by a given catalyst. The enantiomeric excess of the unreacted starting material rises during the reaction and reaches - in an ideal case - its maximum (ee >99%) while the preferred enantiomer is completely depleted at a conversion of 50%. Potential catalysts for kinetic resolution are chiral, chemical catalysts or enzymes. In biocatalytic kinetic resolutions, the enzyme enantiopreference is the result of a better fit of one enantiomer into the enzyme's active site, leading to a faster reaction.

Enzymes which are able to hydrolyze the amide bond of Vince lactam have been classified as γ-lactamases. Based on their preference for (+)-2-azabicyclo[2.2.1]hept-5-en-3-one or the opposite (-)-enantiomer, enzymes are categorized as (+)- or (-)-γ-lactamases respectively (Figure 35).

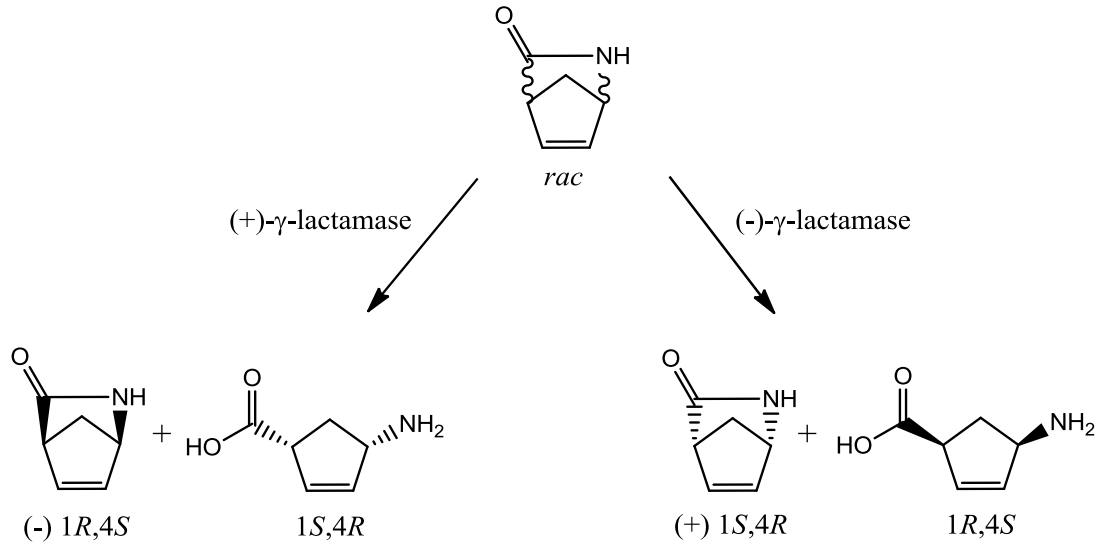


Figure 35: Enzymatic kinetic resolution of *rac*-Vince lactam

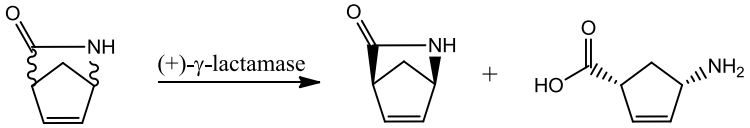
One example of a chemical approach to the resolution of *rac*-Vince lactam is the regioselective hydroarylation of the double bond, which leads to so-called "parallel kinetic resolution" because both enantiomers are transformed into different products.[58]

One major drawback of kinetic resolution is the maximal yield of 50% of enantiopure product. However, in the case of Vince lactam, the "unwanted" enantiomer can be used for further synthesis, because valuable products of both enantiomers exist (examples can be seen in Figure 34, p. 28).

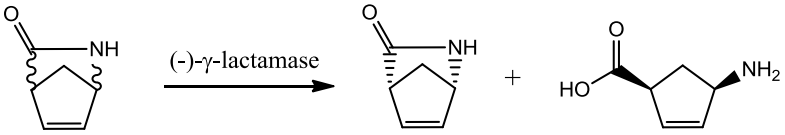
1.3.1.1. Enzymatic kinetic resolution of Vince lactam

Previous studies focused mainly on the enzymatic hydrolysis of unsubstituted Vince lactam. Several enantiocomplementary γ-lactamase activities have been identified and are summarized in Table 1.

Table 1: Enzymatic kinetic resolution of Vince lactam



Entry	Enzyme	Organism	Reference
1	ENZA20	*Pseudomonas solanacearum*	[54]
2	ENZA22	*Pseudomonas fluorescens*	[54]
3		*Comamonas acidovorans*	[59]
4	(amidase)	*Sulfolobus solfataricus* MT4	[59]
5		*Bradyrhizobium japonicum* USDA 6	[55]



Entry	Enzyme	Organism	Reference
6	ENZA1	*Rhodococcus* sp.	[54]
7	ENZA25	*Aureobacterium* sp.	[54]
8	PFEI	*Pseudomonas fluorescens*	[60]
9	Mhg	*Microbacterium hydrocarboxydans*	[61]
10		*Aureobacterium sp.*	[62]
11	lipase PS	*Pseudomonas cepacia*	[63]
12	lipase AK	*Pseudomonas fluorescens*	[63]
13	PPL	pig	[63]
14	CAL-A, CAL-B	*Candida antarctica*	[63]

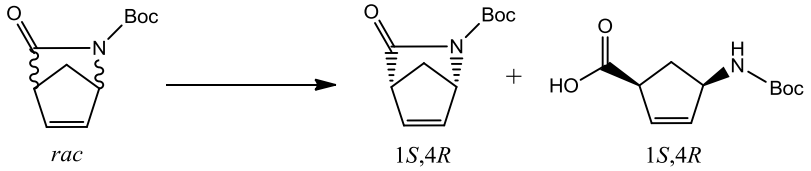
First studies were carried out employing whole cells of *Rhodococcus* sp., *Aureobacterium* sp., *Pseudomonas solanacearum* and *Pseudomonas fluorescens* for the hydrolysis. The enantioselectivity values of the whole cell resolutions with *Aureobacterium* sp. (ENZA25) and *Pseudomonas fluorescens* (ENZA22) were 76 and 94 respectively, however after purification and immobilization of the (-)-γ-lactamase ENZA25 the E-value of the whole process increased (E >200). Due to stability issues ENZA22 could not be isolated.[54] The first (+)-γ-lactamase to be successfully isolated, cloned and overexpressed in *E. coli* was the enzyme from *Comamonas acidovorans*, which showed perfect kinetic resolution of *rac*-Vince lactam (E>200) and high sequence similarity to a formamidase and an acetamidase.[59] Amidase from *Sulfolobus solfataricus* is an interesting candidate for industrial processes, since it originates from a thermophilic archaeon and thus promises high stability.[59] The lactamase from *Bradyrhizobium japonicum* was discovered by rational genome mining: a BLAST search using (+)-γ-lactamase from *Sulfolobus solfataricus* as template identified the unnamed protein, which could hydrolyze (+)-Vince lactam after cloning in *E. coli*.[55] An enzyme with promiscuous bromoperoxidase activity from *Aureobacterium* sp., which displayed hydrolytic activity on (-)-Vince lactam, was crystallized and used for elucidation of the reaction mechanism.[62]

As described in Table 1, lactamase activity was also found in several known hydrolytic enzyme preparations. Unlike lactamases, lipases are commercially available and already used on industrial scale. However, reaction rates seemed to be low and as it is the case in all lipase bioconversions, organic solvents are needed, which can be seen as problem in a green process.[63] Furthermore, esterase I from *Pseudomonas fluorescens* (PFEI) displayed promiscuous hydrolytic activity on (-)-Vince lactam.[60]

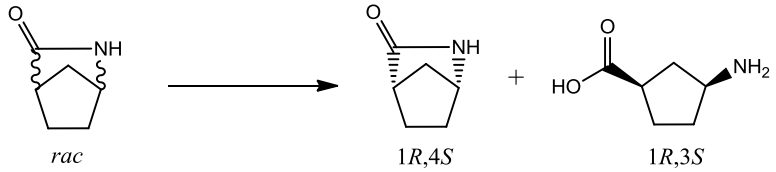
1.3.1.2. Enzymatic kinetic resolution of Vince lactam derivatives

In addition to kinetic resolutions of Vince lactam the activities of an immobilized preparation of CAL-B on *N*-protected and reduced Vince lactam and of PFEI on the saturated derivative were tested.[63, 60] Positive results are summarized in Table 2.

Table 2: Enzymatic kinetic resolution of Vince lactam derivatives



Entry		Organism	Reference
1	CAL-B	*Candida antarctica*	[63]



Entry		Organism	Reference
2	CAL-B	*Candida antarctica*	[63]

Although the hydrolysis of both Vince lactam derivatives by CAL-B was slower than the reaction with unsubstituted compound, the enzyme showed conserved enantiopreference (E >200) and the unreacted substrate could be obtained with high enantiomeric excess (>99% for reduced, >96% for *N*-protected Vince lactam).[63] *Pseudomonas fluorescens* esterase I (PFEI) did not show activity on the reduced Vince lactam derivative.[60]

1.3.2. (-)-γ-Lactamases

The enzyme isolated from *Aureobacterium* sp. displaying (-)-γ-lactamase activity on Vince lactam belongs to the α/β-hydrolase fold family.[62] This tertiary fold is adopted by diverse hydrolytic enzymes, which do not share significant sequence similarity and have diverged from a common ancestor. Enzymes which share the α/β-hydrolase fold include lipases, proteases, esterases, dehalogenases, peroxidases and epoxide hydrolases. These enzymes consist of a mostly parallel, 8-stranded β-sheet which is surrounded by α-helices at both sides, one β-strand only is antiparallel (Figure 36). The catalytic residues usually consist of a highly conserved triad: a nucleophile (Ser, Cys or Asp), an acidic residue (Asp or Glu) and a

conserved histidine residue. The nucleophile is located in the "nucleophile elbow", a sharp turn, which ensures easy approach by the substrate and the hydrolytic water molecule. It also directs the substrate to the oxyanion hole, made of two backbone nitrogen atoms, which stabilizes the negatively charged transition state.[64]

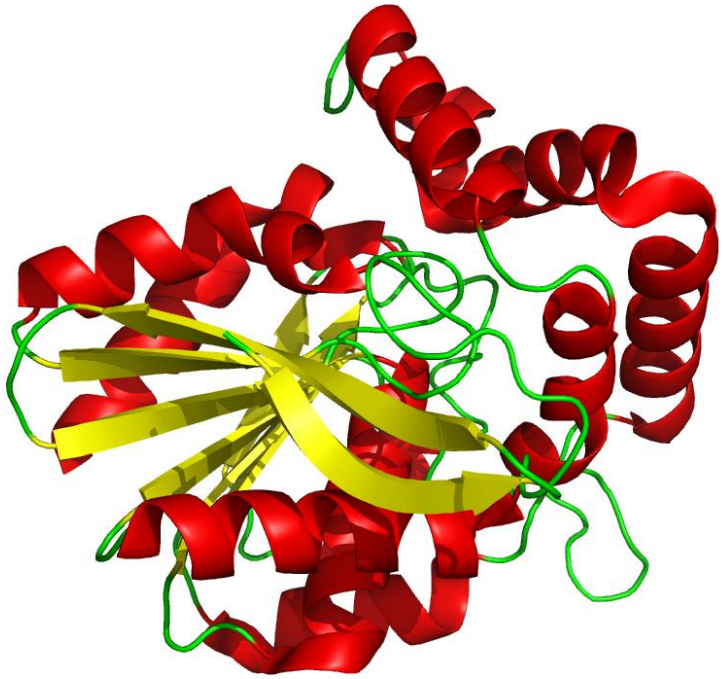


Figure 36: Protein structure of the (-)-γ-lactamase from *Aureobacterium sp.* (PDB code 1HKH). The α-helices are depicted in red, β-sheets in yellow and loop regions in green. The figure was drawn using PyMol [65]

The mechanism for the hydrolysis of (-)-Vince lactam was proposed for *Aureobacterium* γ-lactamase and follows the classical steps in α/β-hydrolase fold enzyme-catalyzed reactions (Figure 37):

i. The carbonyl oxygen of Vince lactam binds to the oxyanion hole, which is formed by Tyr32 and Met99. Deprotonation of Ser98 by His259 yields a nucleophile, which attacks the activated carbonyl group.

ii. A tetrahedral intermediate is formed. The latter collapses upon donation of a proton from His259 to the ring nitrogen and an acyl-enzyme complex is formed.

iii. A molecule of water is deprotonated by His259 and attacks the complex. The second tetrahedral intermediate is formed.

iv. The hydrolyzed product is released by reprotonation of Ser98 by His259.[62]

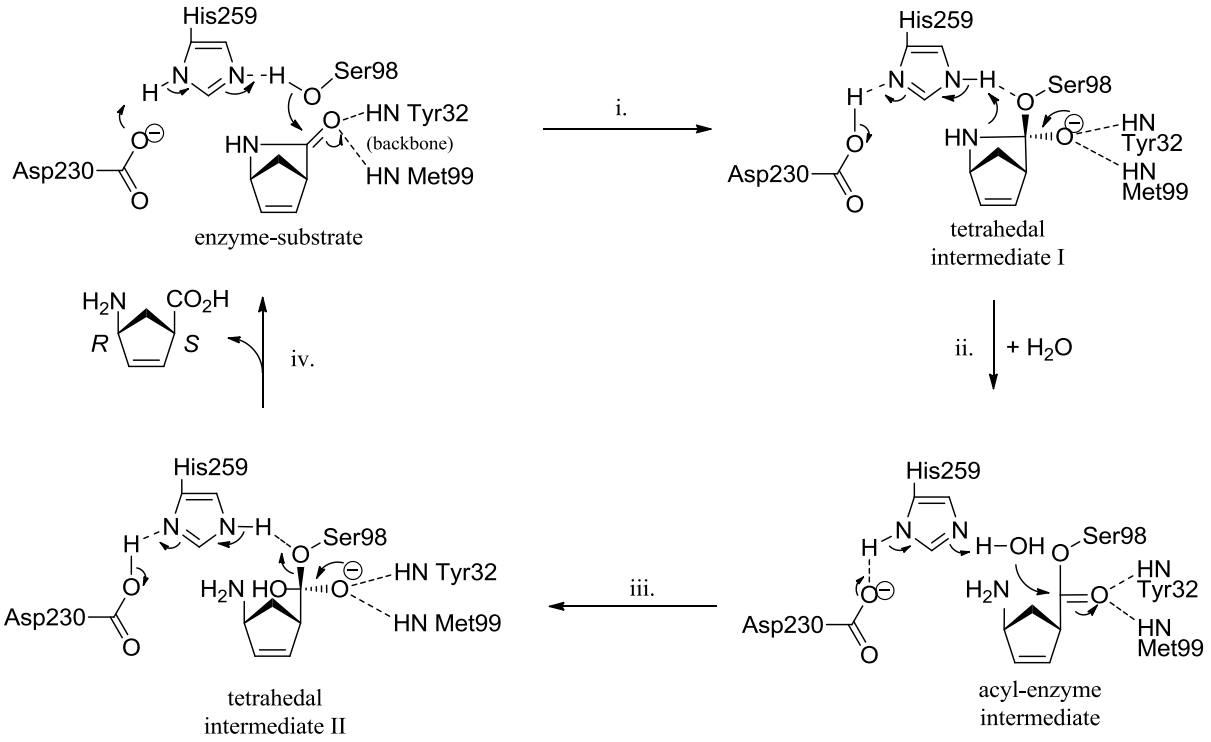


Figure 37: Proposed mechanism for the hydrolysis of (-)-Vince lactam by *Aureobacterium* sp. γ-lactamase [62]

1.3.3. (+)-γ-Lactamases

Alignment of the amino acid sequences of the (+)-γ-lactamases of *Sulfolobus solfataricus* and *Bradyrhizobium japonicum* reveals a sequence identity of 49% and the presence of several conserved regions (Figure 38), such as the catalytic triad and the so called amidase signature sequence (AS sequence).

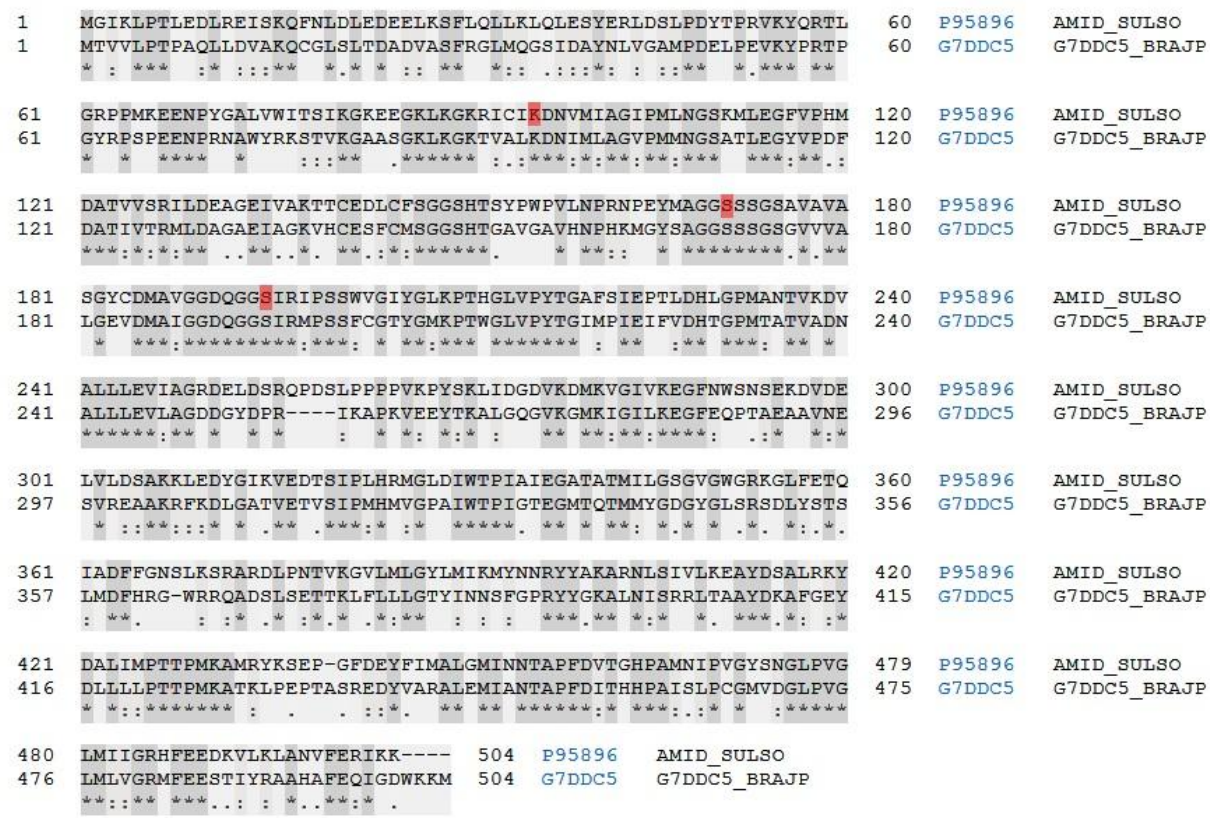


Figure 38: Sequence alignment of (+)-lactamases from *Sulfolobus solfataricus* (AMID-SULSO, #P95896) and *Bradyrhizobium japonicum* (G7DDC5_BRAJP, #G7DDC5). Active site residues are depicted in red. The AS sequence (GGSSSGS) lies within the highly conserved block surrounding Ser171 (AMID-SULSO numbering). The alignment was generated using Clustal Omega.[66]

Members of the "amidase signature family" (AS family) are defined by the amidase signature sequence, a conserved stretch of amino acids which has been identified by primary structure analysis of several amidases. A strictly conserved block consisting of GGSSSGS is located in this approximately 130 residues long consensus sequence.[67,68] The first serine of this motif is part of the catalytic triad Ser-Ser-Lys (Figure 39), which is absolutely conserved in the AS family proteins.

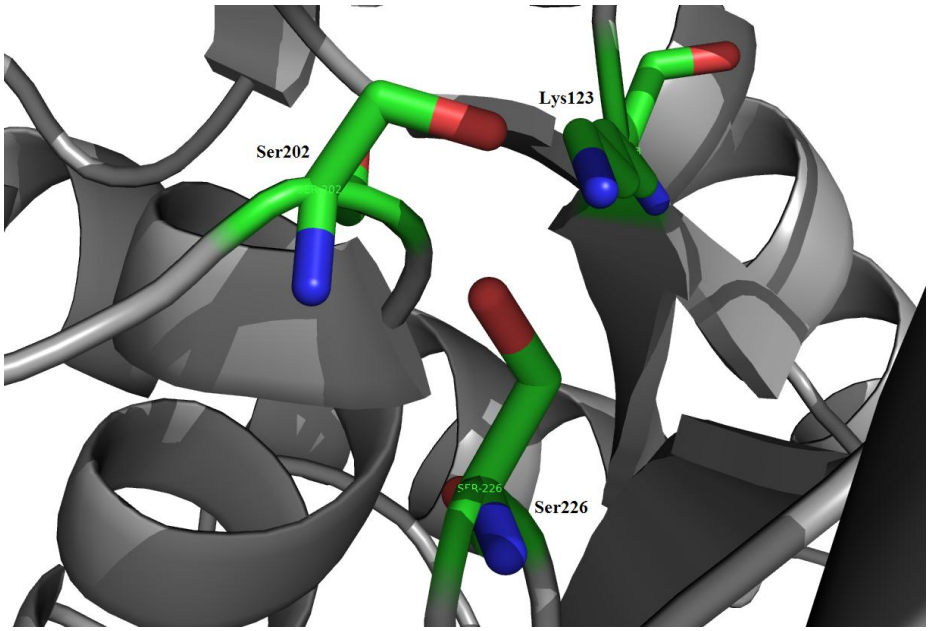


Figure 39: Active site of the AS family enzyme peptide amidase Pam from *Stenotrophomonas maltophilia* (PDB code 1M22) with catalytic triad. The figure was generated using PyMol.[65]

While the crystal structure of several members of the AS family (peptide amidase, Pam; fatty acid amide hydrolase, FAAH) has already been solved, no structural data is available for the (+)-γ-lactamases of *Sulfolobus solfataricus*. A homology model suggested, that the catalytic residues are correctly positioned for amide bond hydrolysis and revealed that the oxyanion hole is likely formed by the backbone amide nitrogen atoms of Gln192 and Gly193.[69]

The proposed mechanism for the hydrolysis of (+)-Vince lactam by γ-lactamase from *Sulfolobus solfataricus* is based on the analysis of a homology model (Figure 40) [69]:

i. The carbonyl group is activated by hydrogen bonds of the oxygen atom with the backbone amide nitrogen atoms of Gly193 and Gln192. Activation of Ser195 occurs *via* a hydrogen bond network with Ser171 and Lys96. The nucleophile attacks the carbonyl group.

ii. A tetrahedral intermediate is formed. The intermediate collapses following donation of a proton to the nitrogen atom of the lactam. An acyl-enzyme complex is formed.

iii. A molecule of water is deprotonated by Lys96 and attacks the acyl-enzyme complex. The second tetrahedral intermediate is formed.

iv. The hydrolyzed product is released upon reprotonation of Ser195.

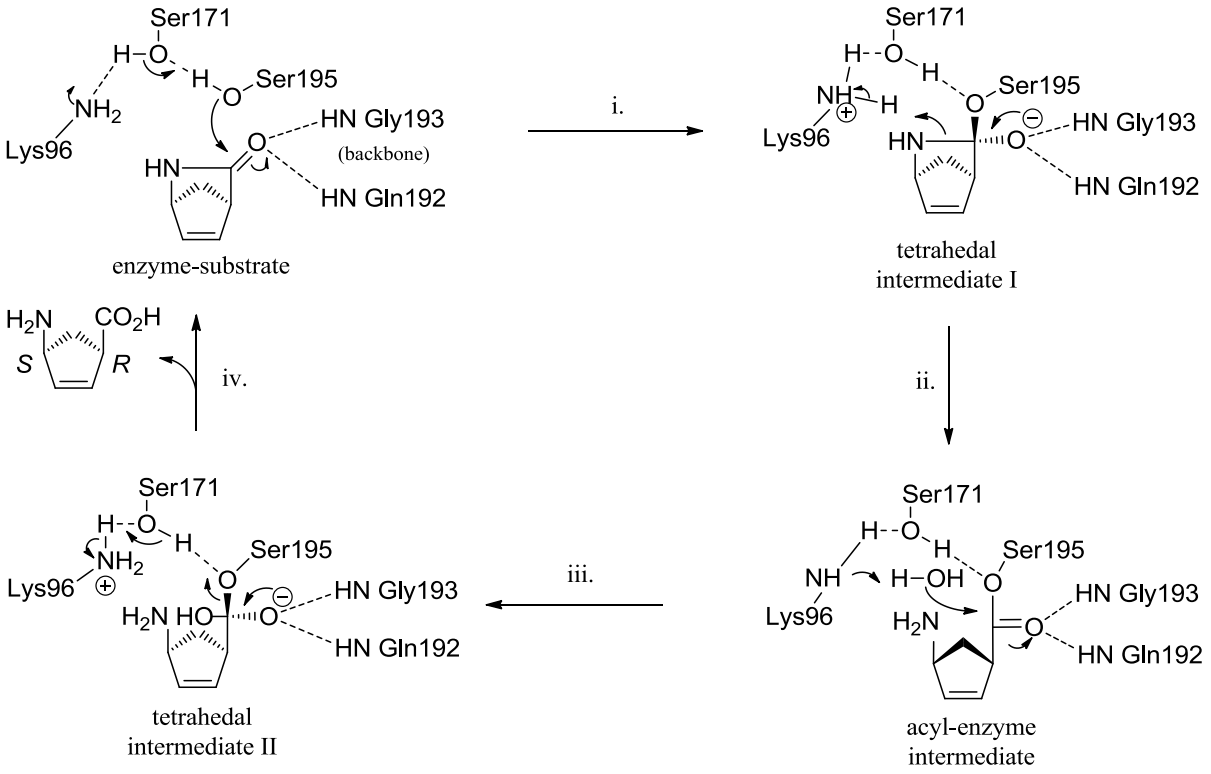


Figure 40: Proposed mechanism for the hydrolysis of (+)-Vince lactam by γ-lactamase from *Sulfolobus solfataricus* based on a homology model built on crystal structure of peptide amidase Pam from *Stenotrophomonas maltophilia*

1.3.4. Proposed classification of γ-lactamases [57]

Based on the observations from protein structure analysis and related enantiopreference for Vince lactam enantiomers, a classification for γ-lactamases can be proposed:

i. Enzymes which belong to the class of serine hydrolases, thus sharing the conserved catalytic triad Ser-His-Asp and the oxyanion hole, display selective catalytic activity on (-)-Vince lactam.

ii. (+)-γ-Lactamases are members of the amidase signature family. They possess the amidase signature sequence GGSS(S/G)GS and the catalytic triad consisting of Ser-Ser-Lys.

2. Research objectives

2.1. Aim of this thesis

The aim of this thesis is the identification of new γ- and δ-lactamase activities. The strategy relies on the investigation of various enzymes (in purified form) with known or suspected amidase activity. Of importance is the development of biocatalysts with high enantioselectivity allowing perfect kinetic resolution. Substrates of interest include derivatives of 2-aza-bicyclo[2.2.1]hepten-3-one (Vince lactam), a synthon used in the preparation of GABA analogues and carbocyclic nucleosides.

2.2. The quest for new γ- and δ-lactamases

While searching for an enzyme candidate in order to identify new lactamases, two major considerations were crucial: the enzyme should display hydrolytic activity on amide bonds and be able to accept bulky substrates to allow 5- and 6-membered lactam rings with different substituents to fit into the active site. An enzyme meeting both requirements was found in NfpolyA - a polyamide hydrolyzing amidase isolated from a soil bacterium.

Polyamidase **NfpolyA** from *Nocardia farcinica* (#Q5YVW2) was identified from a screening of microbial hydrolytic activity on polyamides as part of a study dedicated to improving nylon hydrophilicity by enzymatic surface modifications. Functional groups can be introduced on the polymer surface *via* the amino groups formed by hydrolysis of amide bonds. NfpolyA consists of 4 subunits and has a molecular weight of 190 kDa in its native form. The enzyme is able to hydrolyze various amides and esters and is classified as aryl acylamidase due to its activity on *p*-nitrophenyl butyrate. The enzyme belongs to the AS family displaying both the conserved signature sequence as well as the catalytic Ser-Ser-Lys triad.[70, 71] A sequence identity of 98% with ω-octalactam hydrolase from *Rhodococcus* sp. Oct1, active on ω-octalactam, ω-laurolactam but also o-nitroacetanilide [72], strongly suggested that NfpolyA could hydrolyze lactam rings.

Several searches for homologous proteins were conducted using BLAST [73] and PDBeFold [74]. An amidase from *Rhodococcus globerulus* and malonamidase E2 from *Bradyrhizobium japonicum* showed sequence similarities of 31% and 36%, respectively, with NfpolyA and

were selected for activity screenings. Figure 41 shows a sequence alignment of these enzymes. Chloroperoxidase T from *Streptomyces aureofaciens* on the other hand showed structural similarity to the (-)-γ-lactamase from *Aureobacterium* sp. (chapter 1.3.2, p. 33).

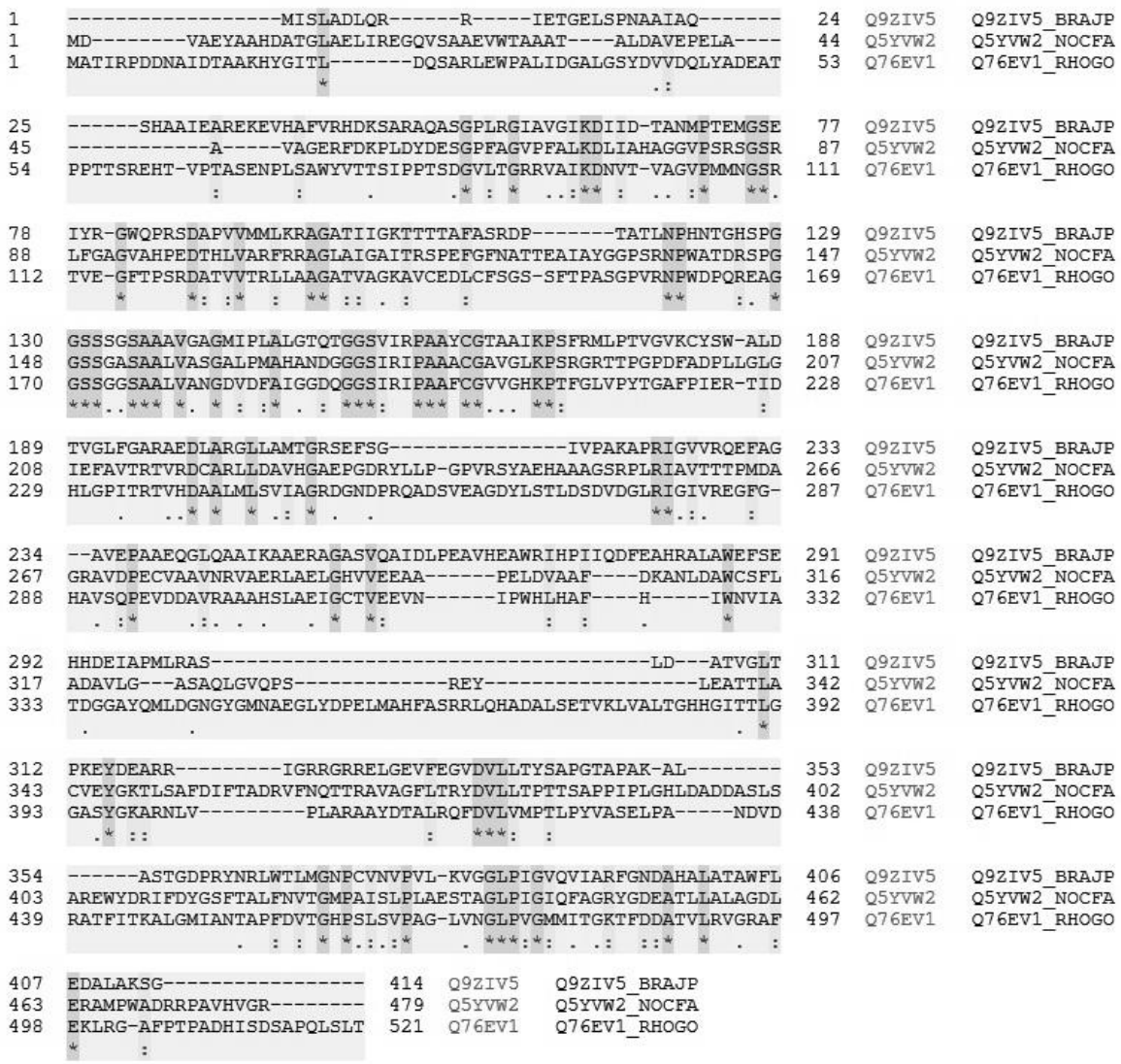


Figure 41: Sequence alignment of malonamidase E2 from *Bradyrhizobium japonicum* (Q9ZIV5-BRAJP), polyamidase from *Nocardia farcinica* (Q5YVW2-NOCFA) and amidase from *Rhodococcus globerulus* (Q76EV1-RHOGO). The alignment was generated using Clustal Omega.[66]

The amidase **AMI** from *Rhodococcus globerulus* (#Q76EV1) is encoded in a gene cluster responsible for alkylaldoxime metabolism. The other two enzymes responsible for aldoxime catabolism are aldoxime dehydratase, which converts the aldoxime into the corresponding nitrile, and nitrile hydratase, which hydrolyzes the nitrile to the corresponding amide. The amide is in turn hydrolyzed to the carboxylic acid by the amidase.[75] The amidase is 521 residues long and has a molecular weight of 54.6 kDa.

Malonamidase **MAE2** from *Bradyrhizobium japonicum* (#Q9ZIV5) is a 414 amino acid long, dimeric protein with a molecular weight of 43.6 kDa. It is highly specific for malonamate, which is hydrolyzed to malonate. The enzyme did not show activity with substrate analogues like malonate, succinate, acetate, succinamate, malonamide or acetamide.[76] Malonamidase belongs to the AS family and is the first member whose crystal structure has been solved (pdb code: 1OCK). The structure revealed a mixed α/β fold constituted of 12 α-helices surrounding a central β-sheet composed of 11 β-strands and differs from any known protein fold (Figure 42). The conserved AS sequence residues compose mainly the core of the protein while the upper half of the active site is built from non-conserved residues. Thus it can be concluded that substrate recognition of each individual protein of this class is achieved by substitution of these non-conserved amino acids.[77]

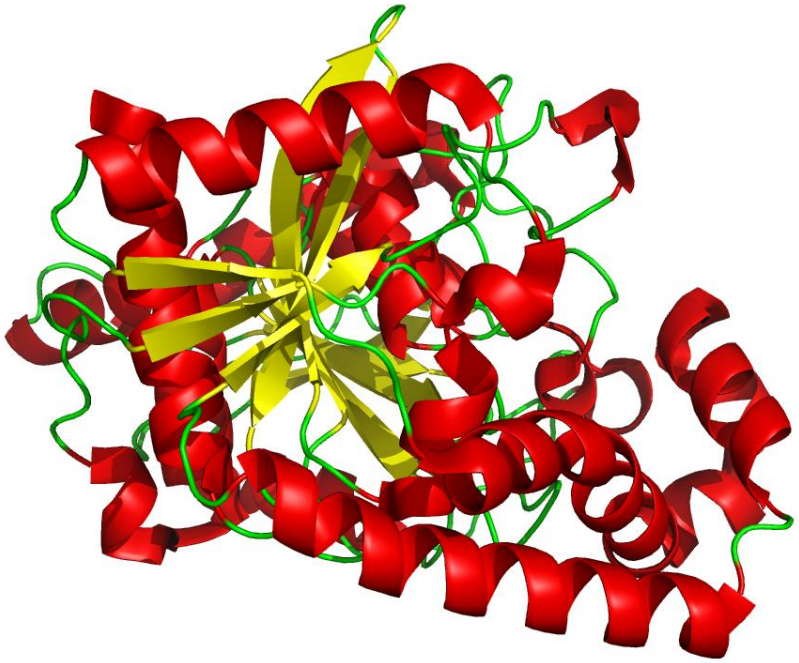


Figure 42: Fold of the amidase signature protein malonamidase E2 from *Bradyrhizobium japonicum* (PDB code: 1OCK). α-Helices are depicted in red, β-sheets in yellow and loop regions in green respectively. The figure was generated using PyMol.[65]

Structural data confirmed that MAE2 contains a Ser-*cis*Ser-Lys triad with Ser155 as reactive nucleophile. Ser131 was found in the unusual *cis*-conformation, which appeared essential for the formation of hydrogen bonds with the other active site residues. The residues Ser131 and Lys62 polarize the nucleophile Ser155.[78] Furthermore, an arginine at position 158 seems to stabilize the transition state oxyanion by neutralizing the negative charge of the substrate and is involved in substrate recognition.[79]

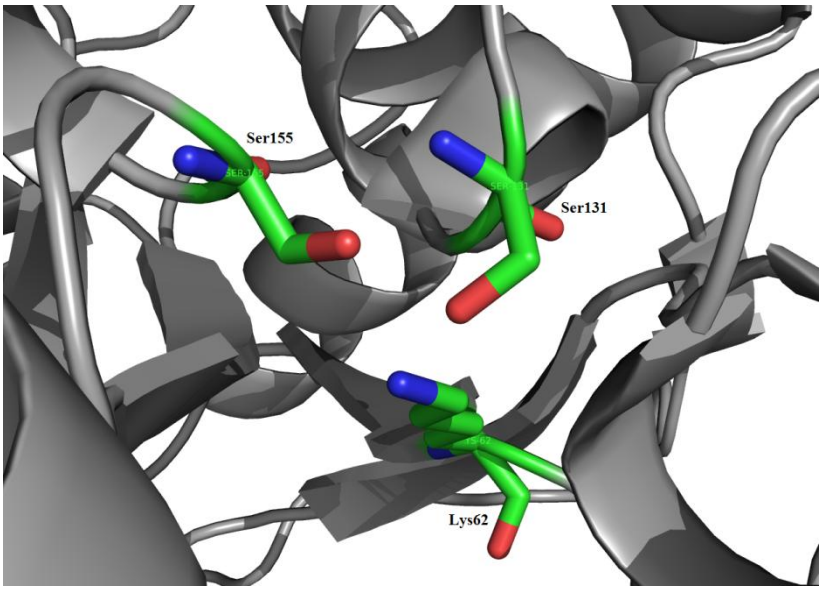


Figure 43: Active site of malonamidase E2 from *Bradyrhizobium japonicum* (PDB code: 1OCK) with catalytic triad. The figure was generated using PyMol.[65]

Chloroperoxidase **CPO-T** from *Streptomyces aureofaciens* (#O31168) belongs to the class of cofactor-free haloperoxidases. These enzymes mediate the halogenation of organic compounds by hypohalous acid, which is generated by oxidation of halide ions by hydrogen peroxide. The nomenclature is based on the most electronegative halide that the enzyme is able to utilize, thus chloroperoxidase can oxidize chloride, bromide and iodide.[80] Cofactor-free haloperoxidases belong to the class of α/β-hydrolases and display the catalytic triad Ser-Asp-His characteristic of serine hydrolases.[81] The catalytic triad catalyzes the formation of a peroxoacid in analogy to the mechanism of serine hydrolases: by nucleophilic attack of Ser on the carboxyl group of an organic acid a tetrahedral intermediate is formed. By elimination of a water molecule the intermediate forms an acyl-enzyme complex. Hydrolysis of the complex by nucleophilic attack of hydrogen peroxide forms the peroxo acid. The formation of the hypohalous acid and the actual halogenation of organic compounds are not catalyzed by the enzyme.[82]

Cyclic imide hydrolase **CIH** from *Pseudomonas putida* (#Q4JG22) is a member of the cyclic amidohydrolase family.[83] The imidase is composed of four identical subunits with a weight of 36 kDa each and contains one atom of zinc per subunit. The tightly bound zinc atom is required for the enzymatic activity. CIH showed hydrolytic activity on hydantoin, dihydrouracil, succinimide and maleimide.[84, 85]

In addition to the already mentioned enzymes, several hydrolases from *Nocardia brasiliensis*, *Thermus thermophilus*, *Clostridium hathewayi* and *Clostridium botulinum* were tested for lactamase activity on three different substrates. Available data regarding these enzymes are summarized in Table 3:

Table 3: Overview on data regarding hydrolases from *Nocardia brasiliensis, Thermus thermophilus, Clostridium hathewayi* and *Clostridum botulinum*. The asterisk after the length of the protein indicates the presence of a signal peptide in front of the actual coding sequence.

Name	Enzyme	Organism	Accession #	Length
1_Nb	secreted hydrolase	*Nocardia brasiliensis*	-	303 aa
2_Nb	secreted esterase	*Nocardia brasiliensis*	K0EQD2	345 aa
3_Nb	secreted lipase	*Nocardia brasiliensis*	K0EWE4	244 aa
Tth_5	secreted hydrolase	*Thermus thermophilus*	H9ZRI9	306 aa*
Tth_6	carboxylesterase	*Thermus thermophilus*	H9ZP60	513 aa*
Est_5	carboxylesterase	*Clostridium hathewayi*	D3AU79	414 aa
Est_7	secreted lipase	*Clostridium botulinum*	A5I3I2	429 aa*
Est_8	secreted lipase	*Clostridium botulinum*	A5I055	452 aa*

Several of these enzymes were active in the degradation of aromatic polymers, which (together with the observation of lactamase activity of polyamidase NfpolyA) suggested that they could potentially show lactamase activity. *Nocardia brasiliensis* and *Thermus thermophilus* are both thermophilic organisms and thus provide enzymes with good thermostability in biocatalytic processes, which makes them good candidates for industrial applications.

2.3. Substrates

The substrates tested included derivatives of 2-aza-bicyclo[2.2.1]hepten-3-one (Vince lactam), a synthon used in the preparation of GABA analogues and carbocyclic nucleosides. A short overview of Vince lactam (**1a**) and derivatives therof – a saturated (**2a**), a *N*-methylated (**3a**), a reduced and *N*-methylated (**4a**) derivative and compounds derived by dihydroxylation (**5a**), dihydroxylation and protection (**6a**) and epoxidation of the C=C double bond (**7a**) – is given in Figure 44, their synthesis is described in chapter 5.6.

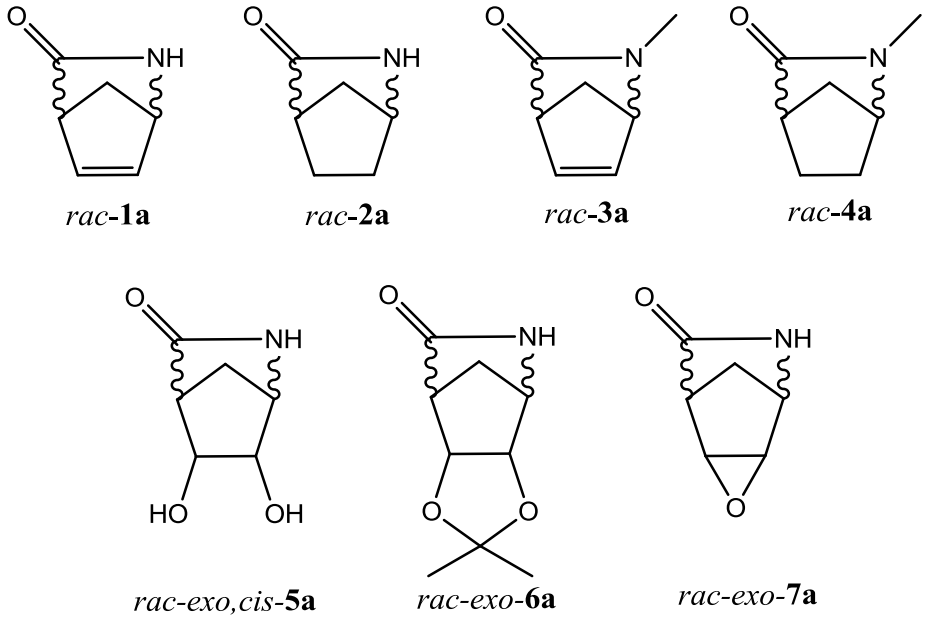


Figure 44: Vince lactam and derivatives used as substrates in lactamase activity screening

Additionally, several γ- and δ-lactams, succinimide and maleimide derivatives, phthalimide and hydantion were tested. Structures are summed up in Figure 45.

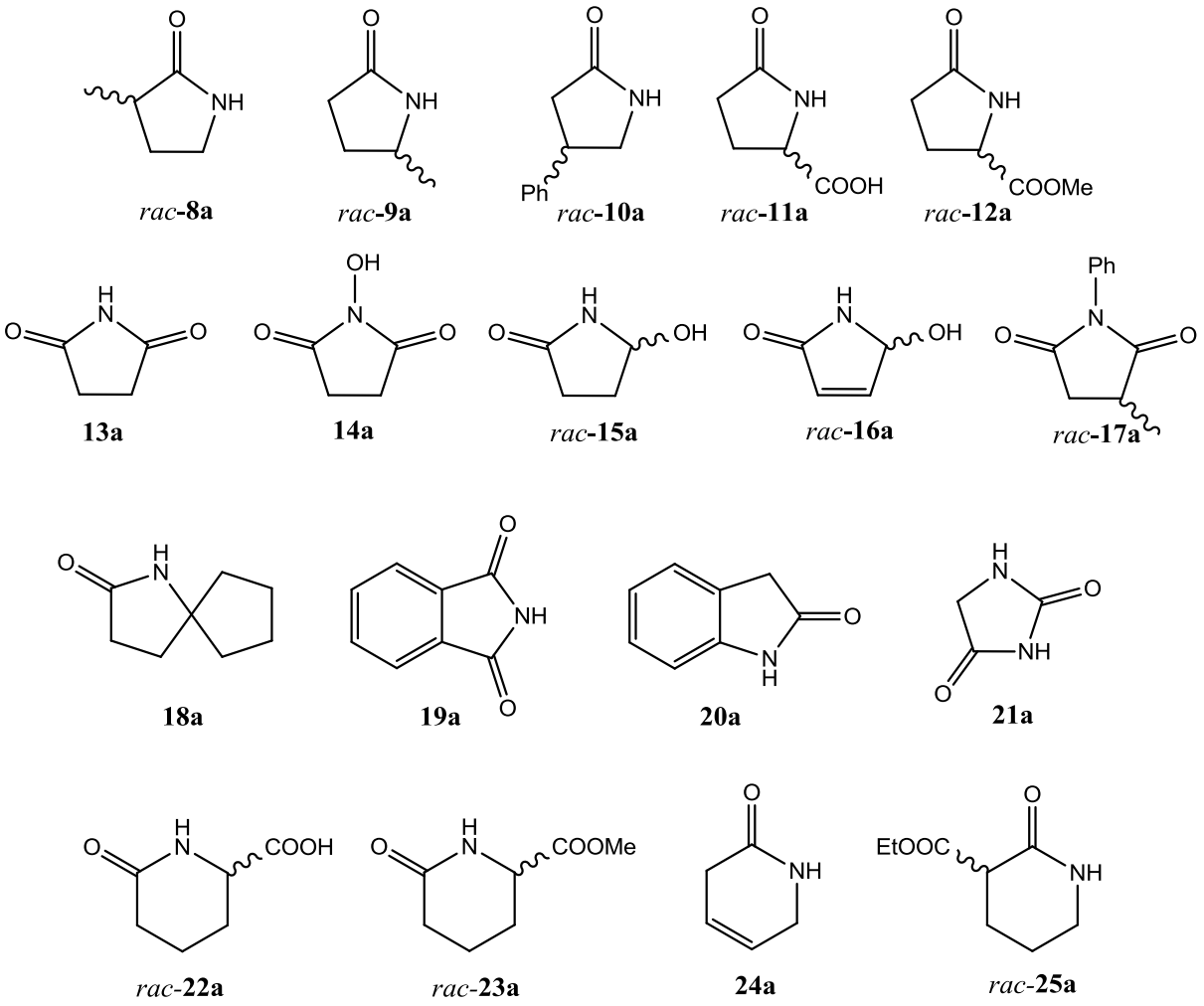


Figure 45: γ- and δ-Lactams (8a-12a, 22a-25a), succinimide and maleimide derivatives (13a-17a), phthalimide (19a), oxindole (20a) and hydantoin (21a)

3. Results and discussion

Several enzymes from different organisms were investigated in pure form regarding their γ- and δ-lactamase activity on γ- and δ-lactam-type substrates (Figure 46). Substrates included succinimide and maleimide derivatives, phthalimide, hydantion and derivatives of the bicyclic lactam 2-azabicyclo[2.2.1]hept-5-en-3-one (Vince lactam). Structures of all substrates tested are given in Figure 44 (p. 44) and Figure 45 (p. 45).

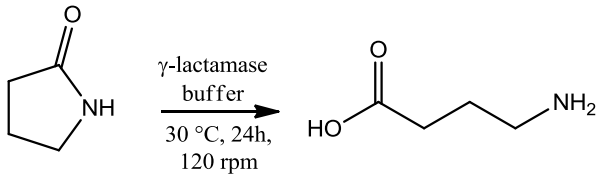


Figure 46: Biohydrolysis of lactams exemplified by γ-butyrolactam

The results of the bioconversions will be discussed in the following chapter. Control reactions (reaction in buffer with no enzyme present) did not show formation of the corresponding products.

In addition, eight esterases, which displayed hydrolytic activity on aromatic polymers, were screened for lactamase activity using substrates **1a**, **19a**, and **26a**. The results will be discussed separately in chapter 3.4.

All samples were analyzed on GC/MS after derivatization, except **6a**, **15a** and **16a**, where samples were analyzed on LC/MS.

3.1. Vince lactam and derivatives

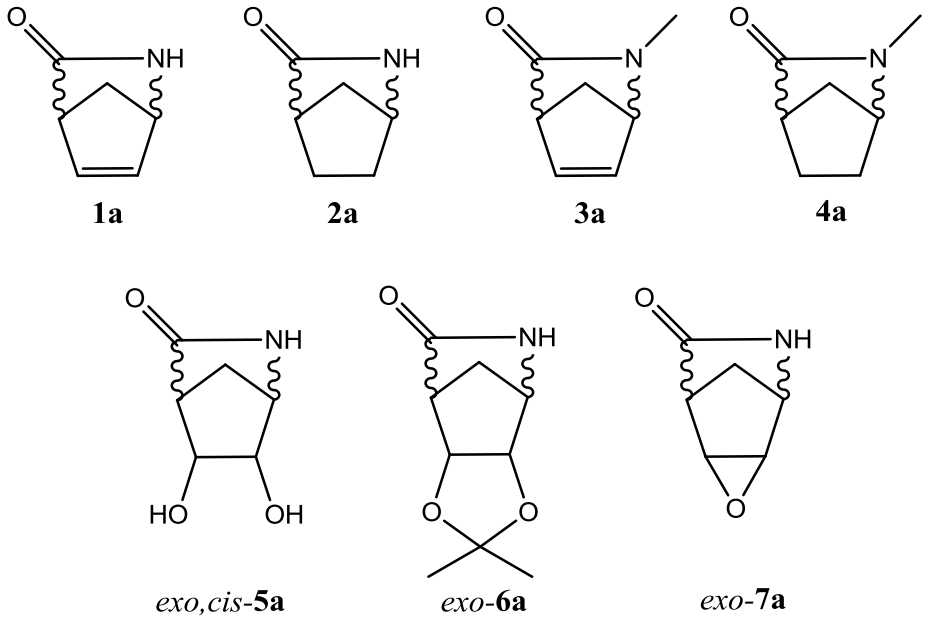


Figure 47: Vince lactam derivatives used in lactamase activity screening

Unsubstituted *rac*-Vince lactam (*rac*-**1a**, Figure 47) and – to some extent – its (-)-enantiomer [(1*R*,4*S*)-**1a**, (-)-Vince lactam] was converted by all enzymes. The best results were achieved by CIH with perfect kinetic resolution of the racemic starting material and practically full conversion of (-)-Vince lactam (E >200). *Rac*-**1a** was hydrolyzed by AMI and CPO-T with high conversions (76 and 66%, respectively) but poor enantioselectivities (E = 8 and 15, respectively). NfpolyA hydrolyzed *rac*-**1a** with good conversion and enantioselectivity (conv. 45% and ee_P 96%). MAE2 was not very active on *rac*-Vince lactam (conv. 5%), but displayed perfect enantioselectivity (E >200). The structures of substrate and product enantiomers are depicted in Figure 48. The results are given in Table 4.

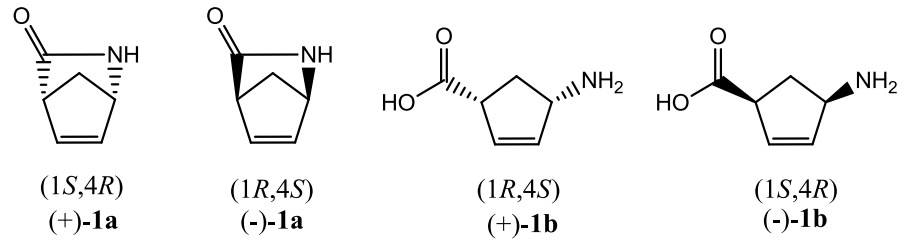


Figure 48: Substrate and product enantiomers of Vince lactam 1a

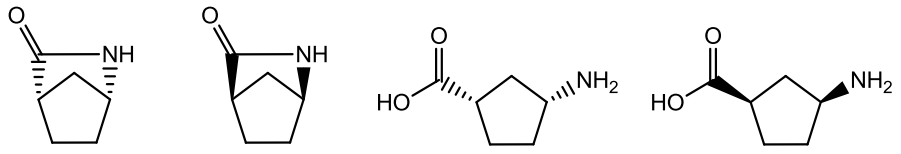
Table 4: Biohydrolysis of Vince lactam 1a with pure enzymes[a]

	***rac*-1a**				**(-)-1a**
	conv.[b] [%]	ee_S [c] [%]	ee_P [%]	E[b]	conv. [%]
NfpolyA	45	77 (1*R*,4*S*)	96 (1*R*,4*S*)	115	2
AMI	76	>99 (1*R*,4*S*)	32 (1*R*,4*S*)	8	53
CPO-T	66	>99 (1*S*,4*R*)	52 (1*S*,4*R*)	15	>99
MAE2	5	5 (1*S*,4*R*)	>99 (1*S*,4*R*)	>200	4
CIH	50	>99 (1*S*,4*R*)	>99 (1*S*,4*R*)	>200	>99

[a] Reactions were performed in 0.8 mL phosphate buffer (50 mM, pH 7.5) with 10 mM substrate and 100 µg enzyme. [b] Conversion and E-values were calculated from ee-values (chapter 5.4.). [c] ee values were determined after double derivatization (chapter 5.3.)

Enantiocomplementary activities on Vince lactam were obtained: NfpolyA and AMI hydrolyzed the (1*S*,4*R*)-enantiomer and thus displayed (+)-lactamase activity. CPO-T, MAE2 and CIH were (-)-selective. Interestingly, malonamidase seems to prefer the (1*R*,4*S*)-enantiomer although the enzyme belongs to the amidase signature family and was expected to be a (+)-γ-lactamase according to our classification. However, in contrast to α/β-hydrolase fold enzymes CPO-T and CIH, conversion of enantiopure (-)-**1a** is very low and is in the same range as the conversion of *rac*-**1a**. This unexpected result may be attributed to the particular substrate recognition of MAE 2, which is achieved by the non-conserved amino acids of the upper half of the active site.

The highest conversion of (±)-2-azabicyclo[2.2.1]heptan-3-one (*rac*-**2a**) was achieved by CPO-T. The enzyme allowed perfect kinetic resolution of the substrate with preference for the (1*S*,4*R*)-enantiomer (E >200), which was further confirmed by full conversion of (-)-**2a**. *Rac*-**2a** as well as (-)-**2a** was not converted by MAE2, which was already poorly active with **1a** (conversions of 5% and 4% respectively, Table 5). CIH displayed very low activity level (conversion <1%). NfpolyA and AMI showed moderate conversions (6% and 21% respectively) of *rac*-**2a**, but high enantioselectivities (ee_P >99%). The structures of substrate and product enantiomers are given in Figure 49. All results are summarized in Table 5.



(1*R*,4*S*) **(+)-2a** (1*S*,4*R*) **(-)-2a** (1*S*,3*R*) **(+)-2b** (1*R*,3*S*) **(-)-2b**

Figure 49: Substrate and product enantiomers of reduced Vince lactam 2a

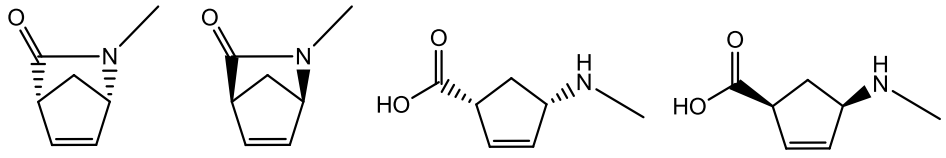
Table 5: Biohydrolysis of reduced Vince lactam 2a with pure enzymes[a]

	***rac*-2a**				**(-)-2a**
	conv. [b] [%]	ee_S [c] [%]	ee_P [%]	E [b]	conv. [%]
NfpolyA	6	6 (1*S*,4*R*)	>99 (1*S*,3*R*)	>200	n.c.
AMI	21	26 (1*S*,4*R*)	>99 (1*S*,3*R*)	>200	n.c.
CPO-T	50	>99 (1*R*,4*S*)	>99 (1*R*,3*S*)	>200	>99
MAE2	n.c.	-	-	-	n.c.
CIH	<1	-	-	-	<1

[a] Reactions were performed in 0.8 mL phosphate buffer (50 mM, pH 7.5) with 10 mM substrate and 100 µg enzyme. [b] Conversion and E-values were calculated from ee-values (chapter 5.4.). [c] ee values were determined after double derivatization (chapter 5.3.). n.c.: no conversion.

In comparison with **1a** (Table 4), all enzymes were less active on compound **2a**. Thus it can be assumed that the C=C double bond is important for enzymatic activity or substrate recognition. However, the enantiopreference of all enzymes was conserved in comparison with substrate **1a**.

(±)-2-Methyl-2-azabicyclo[2.2.1]hept-5-en-3-one (*rac*-**3a**) was not hydrolyzed by MAE2, NfpolyA and CIH and only traces of product could be detected with CPO-T (<1%). Perfect kinetic resolution was achieved with AMI (E >200). The structures of substrate and product enantiomers are depicted in Figure 50. All results are summarized in Table 6.



(1*S*,4*R*) (+)-**3a** | (1*R*,4*S*) (-)-**3a** | (1*R*,4*S*) (+)-**3b** | (1*S*,4*R*) (-)-**3b**

Figure 50: Substrate and product enantiomers of *N*-methylated Vince lactam 3a

Table 6: Biohydrolysis of *N*-methylated Vince lactam 3a with pure enzymes[a]

	***rac*-3a**				**(-)-3a**
	conv. [b] [%]	ee_S [c] [%]	ee_P [%]	E [b]	conv. [%]
NfpolyA	n.c.	-	-	-	<1
AMI	50	>99 (1*R*,4*S*)	>99 (1*R*,4*S*)	>200	n.c.
CPO-T	<1	-	-	-	n.c.
MAE2	n.c.	-	-	-	n.c.
CIH	n.c.	-	-	-	n.c.

[a] Reactions were performed in 0.8 mL phosphate buffer (50 mM, pH 7.5) with 10 mM substrate and 100 µg enzyme. [b] Conversion and E-values were calculated from ee-values (chapter 5.4.). [c] ee values were determined after double derivatization (chapter 5.3.). n.c.: no conversion

AMI showed a much higher activity (conv. 50%, E >200) on *rac*-**3a** compared to *rac*-**2a**. NfpolyA, CPO-T, MAE2 and CIH were not active at all on the substrate, which may be attributed to the methyl group on the nitrogen, which could hinder correct orientation of the substrate in the active site due to sterical hindrance or interruption of H-bonding caused by the substitution.

(±)-2-Methyl-2-azabicyclo[2.2.1]hept-3-one (*rac*-**4a**) combines both derivatizations of substrates **2a** and **3a** and thus was protected at the nitrogen atom by a methyl-group in addition to a saturated ring. No conversion could be detected with any of the enzymes. It can be concluded, that in the case of amidase, enzymatic hydrolysis of the substrate is prevented by the presence of the sterically demanding *N*-protective group in combination with reduction of the double bond.

(±)-*Exo,cis*-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (*rac*-**5a**) was not hydrolyzed by any of the enzymes. This can either be attributed to the steric bulk of the two hydroxyl groups which prevents the substrate from binding in the active site or to some interference with

residues in the enzyme's active site pocket (possibly *via* H-bonds). In order to elucidate this phenomenon, the acetonide derivative was used, yielding (±)-*exo*-2-azabicyclo[2.2.1]heptan-5,6-(dimethylmethylene)dioxy-3-one (*rac*-**6a**). All enzymes were inactive on this compound, which may be attributed to sterical hindrance caused by the protective group.

(±)-*Exo*-7-oxo-3-oxa-6-azatricyclo[3.2.1.0]octane (*rac*-**7a**) was not converted by NfpolyA, MAE2 and CIH and gave full conversion with AMI and CPO-T, indicating a complete loss of enantioselectivity upon epoxidation of Vince lactam. The results are given in Table 7.

Table 7: Biohydrolysis of the epoxide derivative of Vince lactam 7a with pure enzymes[a]

	***rac*-7a**			
	conv. [b] [%]	ee_S [c] [%]	ee_P [%]	E [b]
NfpolyA	n.c.	-	-	-
AMI	>99	n.a.	*rac*	1
CPO-T	>99	n.a.	*rac*	1
MAE2	n.c.	-	-	-
CIH	n.c.	-	-	-

[a] Reactions were performed in 0.8 mL phosphate buffer (50 mM, pH 7.5) with 10 mM substrate and 100 µg enzyme. [b] Conversion and E-values were calculated from ee-values (chapter 5.4.). [c] ee values were determined after double derivatization (chapter 5.3.). n.a.: not applicable, n.c.: no conversion.

Surprisingly, the two enzymes active on *rac*-**7a** belong to different enzyme classes: AMI belongs to the amidase signature family and displayed (+)-γ-lactamase activity on Vince lactam while CPO-T has the α/β-hydrolase fold and is classified as (-)-γ-lactamase, based on its activity on Vince lactam.

3.2. γ-Lactam type compounds: non-substrates

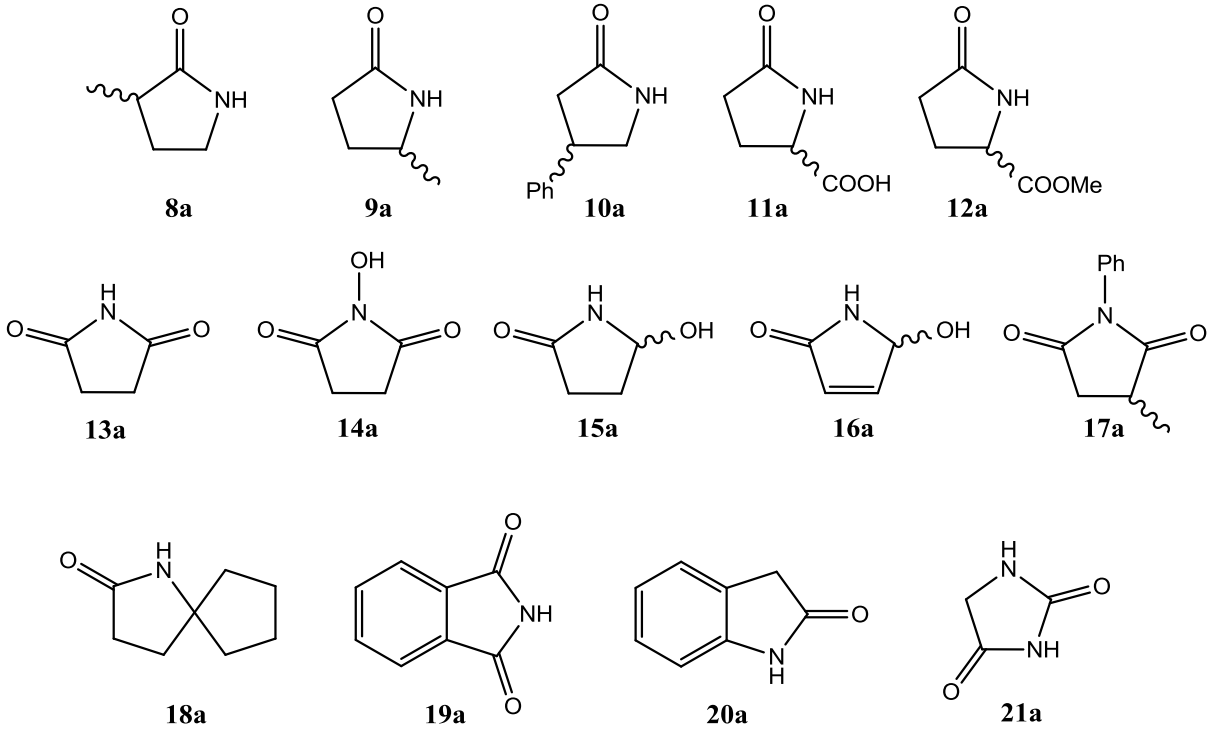


Figure 51: γ-Lactam type compounds used in lactamase activity screening

Several 5-membered ring lactams were tested for hydrolytic activity of all five enzymes. Substrates included derivatives of γ-butyrolactam (**8a-12a**), succinimide and maleimide (**13a-17a**), a spiro compound (**18a**), phthalimide (**19a**), oxindole (**20a**) and hydantoin (**21a**) (Figure 51).

Several of these compounds gave more than one substrate peak upon analysis with GC/MS after derivatization of bioconversion samples. Methylation of the carboxylic acid group of L-2-pyrrolidinone-5-carboxylic acid (**11a**) was expected, however methylation and acetylation reactions occurred on the nitrogen atoms of several substrates (chapter 5.5, p. 61). Since these derivatization products were also present in the control reactions, it was concluded that these reactions were caused by a lack of selectivity of the derivatization methods and not by enzyme-catalyzed reactions.

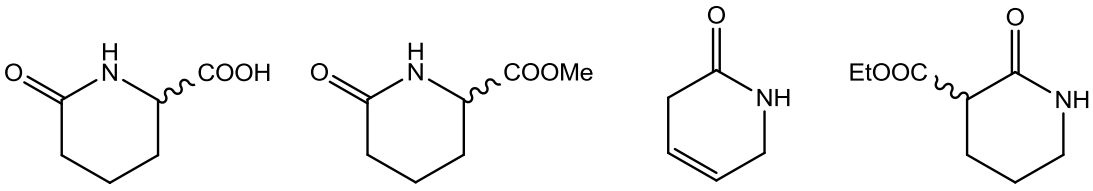
It has already been reported that imidase is active on succinimide (**13a**) [82]. Hydantoin (**21a**), also known to be hydrolyzed by CIH, was used as substrate for spectrophotometrical activity assay.

3-Methyl-1-phenylpyrrolidine-2,5-dione (**17a**), phthalimide (**19a**) and oxindole (**20a**) were not soluble in buffer. Attempts to solubilize the substrates in 10% DMSO were not successful (a precipitate formed upon addition of the stock solution to buffer). Thus, screenings were conducted with the appropriate amount of solid substrate in each sample.

None of the compounds tested (Figure 51) was accepted as substrate by any of the enzymes.

3-Methyl-2-pyrrolidinone (**8a**) and 5-methyl-2-pyrrolidinone (**9a**) provide the γ-lactam core of Vince lactam and both mimic the positions of its substitutions. However, none of them could be hydrolyzed by any of the enzymes. This could either mean that the enzymatic hydrolytic activity on Vince lactam requires a six-membered ring (see chapter 3.3 for details) or that substitution on both positions (1 and 3) is required for acceptance or recognition as substrate.

3.3. δ-Lactams



22a **23a** **24a** **25a**

Figure 52: δ-Lactams used in lactamase activity screening

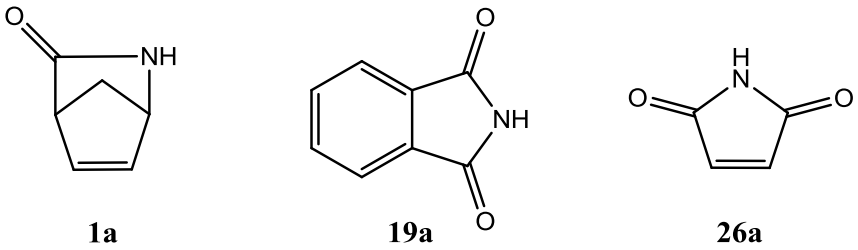
The tested δ-lactams **22a-25a** are given in Figure 52. The derivatization problems that were encountered with γ-lactams were also found with these compounds: esterification occurred as expected in the case of 6-oxopiperidine-2-carboxylic acid (**22a**), only 3,6-dihydropyridin-2(1*H*)-one (**24a**) did not react with the derivatization agents. All peaks from the biotransformations could be detected in the corresponding control reactions too.

None of the δ-lactams presented in Figure 52 was hydrolyzed by the enzymes.

3,6-Dihydropyridin-2(1*H*)-one (**24a**) provides the δ-lactam core of Vince lactam (**1a**) including unsaturation, but lacks the bridging atom between C3 and C6. It can be concluded that this substitution is vital for enzymatic activity. 6-Oxopipecolinic acid (**22a**), the

corresponding methyl ester (**23a**) and 2-oxopiperidinecarboxylic acid ethyl ester (**25a**) lack the double bond but mimic the substitution positions of Vince lactam. However, the carboxylic acid moieties and their esters are both sterically much more demanding than the bridging atom of **1a**. In addition, their electronic contribution cannot be compared.

3.4. Screening of esterases



1a **19a** **26a**

Figure 53: Substrates used for activity screening of esterases

Esterases introduced in chapter 2.2 (p. 39) were tested for lactamase activity with Vince lactam (*rac*-**1a**), phthalimide (**19a**) and maleimide (**26a**) (Figure 53). It was thought that they may possess hydrolytic activities on these substrates as NfpolyA does (at least on Vince lactam), since they were also found active on aromatic polymers. No enzymatic activity could however be detected.[1]

[1] This work was conducted in collaboration with Doris Ribitsch and the research group of Georg Gübitz (ACIB and BOKU Tulln).

4. Conclusion and outlook

Lactamase activity on Vince lactam and derivatives thereof was found in four different enzymes, namely polyamidase NfpolyA from *Nocardia farcinica*, amidase AMI from *Rhodococcus globerulus*, chloroperoxidase CPO-T from *Streptomyces aureofaciens* and cyclic imide hydrolase CIH from *Pseudomonas putida*. Notably, complementary enantiopreferences were identified. Furthermore, racemates of Vince lactam and two derivatives thereof - reduced Vince lactam and the *N*-methylated derivative - were successfully resolved employing imidase, chloroperoxidase and amidase, respectively, in kinetic resolution processes.

In accordance with the proposed classification of γ-lactamases [57], introduced in chapter 1.3.4 (p. 38), all (+)-γ-lactamases identified in this study appear to belong to the amidase signature family, while (-)-γ-lactamase activity can be associated with serine hydrolases of the α/β-hydrolase fold family. These observations were confirmed with (-)-lactamases CIH and CPO-T and (+)-lactamases AMI and NfpolyA. Malonamidase E2, however, displayed a very low hydrolytic activity level on (-)-Vince lactam despite its classification as amidase signature enzyme. However, this enzyme was poorly active and conversion levels never exceeded 5%, indicating that the activity observed might be the result of a poor fit of the substrate into the enzyme's active site.

No lactamase activity could be observed on substrates other than Vince lactam and its derivatives.

Regarding the substrate scope of the lactamases described in this thesis, a few conclusions could be drawn: Enzymatic activity seems to be limited to bicyclic Vince lactam, as no other monocyclic lactam compound was accepted. The presence of the double bond was found to favor hydrolytic activity, while *N*-methylation reduced the activity of most enzymes.

In order to complete the investigation of lactamase substrate scope, *N*-protection with a larger protective group could give further information about the importance of an additional group for substrate recognition, while the role of electronic effects on amide activation could be further studied by introducing an electron withdrawing group.

Lactamase activity screenings of γ-lactamases initially included 6-azatricyclo-[3.2.1.0]-octane-7-one as substrate, which can be obtained by cyclopropanation of the double bond of

Vince lactam. This reaction was attempted *via* two different synthesis routes but did not provide the expected compound. Based on structural similarity to 7-oxo-3-oxa-6-azatricyclo[3.2.1.0]octane, screening of this substrate could offer valuable clues about the effect of the oxygen heteroatom in the cyclopropane ring and further information about the importance of the double bond. Additionally, this would provide access to a range of valuable products.

No true γ-lactamase activity on monocyclic compounds has been identified to date.

As the approach presented in this thesis did only provide enzymes with hydrolytic activity on Vince lactam and derivatives therof, further activity screenings should also include whole-cell bioconversions. A promising technique would be induction using γ- and δ-lactams as sole source of carbon and nitrogen to induce lactamase activity in the microbial strains.

5. Experimental

5.1. General Information

LC/MS measurements were performed using an Agilent Technologies 1260 Infinity system equipped with a 6120 Quadrupole LS/MS detector and a Poroshell 120 EC-C18 column (4.6 x 50 mm).

GC/MS analyses were performed using a HP Agilent Technologies 6890 Series GC system equipped with a 5973 mass selective detector and a 7683 Series injector using a 5% phenyl-methylpolysiloxane capillary column (HP-5 MSI, 30 m, 0.25 mm inner diameter, 0.25 mm film thickness).

GC-FID analyses were performed using a HP Agilent Technologies 7890A GC system equipped with a FID detector and a 7693 Injector/Autosampler. The columns used were:

A) Chirasil Chiraldex DEX-CB (25 m, 0.32 mm inner diameter, 0.25 µm film thickness)
B) Restec Rt-βDEXse (30 m, 0.32 mm inner diameter, 0.25 µm film thickness)

Samples were dried using a GeneVac EZ-2plus HCl compatible centrifugal evaporator.

Optical rotations were measured on a Perkin Elmer 314 polarimeter at a wavelength of 589 nm (Na-line) in a 10 cm-cuvette.

^{1}H- and ^{13}C-NMR spectra were recorded on a 300 Bruker NMR unit.

5.2. General procedure for screenings

Enzymatic reactions were conducted in 1.5 mL Eppendorf tubes. To a solution of 10 mM substrate in phosphate buffer (50 mM, pH 7.5) was added an aliquote of enzyme (final volume 800 µL). In case of water-insoluble substrates 3-methyl-1-phenylpyrrolidine-2,5-dione (**17a**), phthalimide (**19a**) and oxindole (**20a**), the required amount of compound was weighted in an Eppendorf tube and supplemented with phosphate buffer. The enzyme amount was kept at 100 µg in 800 µL phosphate buffer, except for a few exceptions (Table 8):

Table 8: Amounts of protein in samples which did not contain 100 µg

Enzyme	Protein in sample [µg]
1_Nb	50
2_Nb	58
5_Tth	44
6_Tth	40

The reactions were incubated at 30 ºC and 120 rpm for 24 hours, except those run with enzymes from *Nocardia brasiliensis* and *Thermus thermophilus*, which were incubated at 50 °C.

Samples were i) filtered using syringe filters (Rotilabo® PVDF, 0.22 µm pore size) and an aliquot of it diluted with water (1:100, end volume 1 mL) for LC/MS analysis, or ii) concentrated to dryness on SpeedVac in preparation for derivatization before GC analysis.

5.3. General procedure for derivatization

A double derivatization protocol was established for analysis of hydrolysis products on GC, first esterification of the carboxylic acid moiety followed by acetylation of the free amine (Figure 54).

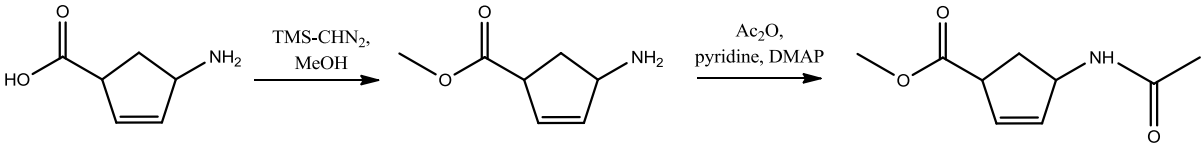


Figure 54: Derivatization procedure shown for hydrolysis product of Vince lactam

Biotransformation samples were dried by using speed vacuum and solubilized in 120 µL MeOH and 200 µL EtOAc. A small amount of Na_2SO_4 was added and the Eppendorf tube was filled with argon. Esterification was carried out by addition of 5 µL of TMS-CHN_2 solution (2 M in diethyl ether, 10 µmol, 1.3 eq. based on maximal theoretical yield) [86] and incubation for 45 min at 25 °C and 450 rpm. For acetylation, Ac_2O (1.5 µL, 16 µmol, 2 eq.), pyridine (0.7 µL, 8.8 µmol, 1.1 eq.) and DMAP (0.1 mg, 0.8 µmol, 0.1 eq.) were subsequently added. The reaction was shaken at 450 rpm and room temperature for 45 min. The reaction was quenched by addition of $KHSO_4$ (200 µL, 0.5 M) and extracted with EtOAc (3 x 200 µL). The combined organic layers were washed with H_2O and dried over Na_2SO_4.

Exceptions from this general procedure were substrates **3a** and **4a**: Since both substrates were volatile, compounds were extracted with EtOAc (4 x 200 µL) before lyophilisation. As the nitrogen atom was already substituted with a methyl group, only the first derivatization step was carried out. After esterification the reaction was diluted with 650 µL EtOAc and transferred into GC-vials.

Investigation of conversion was conducted on GC/MS or LC/MS. Product references were only synthesized in cases where conversion could be observed. In several cases, derivatization yielded more than the expected product(s) which posed the problem of assigning the different peaks in the chromatograms to the corresponding compounds. However, after observing these difficulties with the derivatization method, preparation, derivatization and measurement of the

corresponding products of the substrates seems to be the best way to exclude any uncertainties regarding potential problems with product derivatization.

5.4. Calculations

The enantiomeric excess was calculated after chiral GC-FID measurements using Formula 1. Ee values of substrate and product were calculated separately substituting $area_1$ and $area_2$ with peak areas of base line seperated compound enantiomers.

Formula 1: Calculation of enantiomeric excess

Conversion was calculated from ee values using Formula 2.

Formula 2: Calculation of conversion values from ee values

The enantiomeric ratio [87] was calculated using Formula 3.

Formula 3: Calculation of enantiomeric ratio

5.5. Analytical Methods

LC/MS:

Table 9: Retention times on LC/MS

Compound	Substrate	
	t_R [min]	*m/z*[a]
6a	6.5	184
15a	1.8	102
16a	1.7	100

[a] m/z of single compound peak

Conditions: Injection volume 1 µL, flow rate 0.5 mL/min, H_2O (containing 0.1% formic acid) (A) / CH_3CN (B) used as mobile phase in gradient: 100% A over 2 min, gradually increased to 100% B within 5 min, then 100% A for 5 min.

GC/MS:

Table 10: Retention times of substrates and derivatized substrates on GC/MS

Compound	Substrate		
	t_R [min]	m/z	
4a	5.5	125	Substrate
5a	9.8	143	Substrate
	10.4	143	Substrate
	10.9	143	Substrate
7a	6.9	125	Substrate
8a	4.4	99	Substrate
9a	4.4	99	Substrate
10a	11.9	161	Substrate
11a	7.5	143	Esterified compound

12a	7.6	143	Substrate
	7.9	185	Acetylated compound
	8.3	157	Methylated compound
13a	4.3	113	Methylated compound
	4.6	99	Substrate
14a	5.3	157	Acetylated compound
	5.6	129	-
	6.6	157	-
17a	11.1	189	-
	12.6	221	Substrate
	12.9	221	-
18a	8.3	139	Substrate
19a	8.3	161	Methylated compound
	8.7	147	Substrate
20a	8.0	165	-
	8.5	147	Substrate
21a	6.4	114	Methylated compound
	6.7	156	Methylated compound
22a	9.0	157	Esterified compound
	9.7	171	Esterified + methylated comp.
23a	9.0	157	Substrate
	9.7	171	Methylated compound
24a	5.3	97	Substrate

25a	10.2	171	Substrate
	10.3	185	Methylated compound

Method: 100 °C, hold for 0.5 min, 10°C/min to 300°C, flow: 0.5 mL/min; injector temperature: 250 °C; 3.1 psi H_2

GC-FID:

Table 11: Retention times on GC-FID

Compound	Column	Method	t_R [min]	
			Substrate	Product
1a	A	A	7.84 (-)/8.06 (+)	11.11 (-)/11.52 (+)
2a	A	B	9.17 (-)/9.55 (+)	12.18 (-)/12.42 (+)
3a	B	C	7.93 (-)/8.08 (+)	7.48 (+)/7.62 (-)

Method A: 120 °C, hold for 4 min, 5 °C/min to 137 °C, hold for 2 min, 2 °C/min to 145 °C, 15 °C/min to 180 °C, hold for 2 min; injector temperature: 200 °C, detector temperature: 200 °C, 1 bar He

Method B: 120 °C, hold for 2 min, 5 °C/min to 137 °C, hold for 2 min, 2 °C/min to 145 °C, 15 °C/min to 180 °C, hold for 2 min; injector temperature: 200 °C, detector temperature: 200 °C, 1 bar He

Method C: 100 °C, hold for 2 min, 10 °C/min to 140 °C, 1 °C/min to 145 °C, hold for 2 min, 15 °C/min to 180 °C, hold for 2 min; injector temperature: 230 °C, detector temperature: 230 °C, 1 bar He

5.6. Synthesis

5.6.1. Synthesis of 2-azabicyclo[2.2.1]heptan-3-one

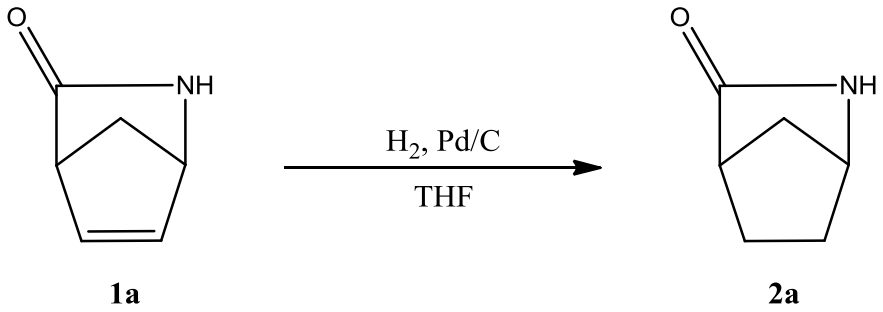


Figure 55: Synthesis of 2-azabicyclo[2.2.1]heptan-3-one

(±)-2-Azabicyclo[2.2.1]heptan-3-one (*rac*-2a): To a solution of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one ***rac*-1a** (200 mg, 1.82 mmol) in THF (5 mL) was added palladium on activated charcoal (10%, 20 mg). The mixture was stirred at room temperature for 13 h under hydrogen atmosphere and filtered through a pad of Celite. The solution was concentrated to yield (±)-2-azabicyclo[2.2.1]heptan-3-one as white solid (120 mg, 60%).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.25 (br. s, 1H, NH), 3.87 (m, 1H, H1), 2.71 (s, 1H, H3), 1.91-1.79 (m, 3H, H5 + H4a), 1.61-1.56 (m, 2H, H5b + H7b), 1.38-1.35 (m, 1H, H7a)

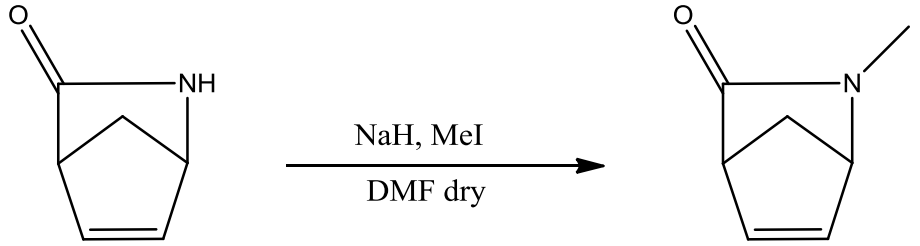
(1*S*,4*R*)-2-Azabicyclo[2.2.1]heptan-3-one [(-)-2a]: To a solution of (-)-2-azabicyclo[2.2.1]hept-5-en-3-one **(-)-1a** (70 mg, 0.64 mmol) in THF (3 mL) was added 10% Pd/C (10 mg). The mixture was stirred at room temperature for 12 h under hydrogen atmosphere, using a balloon. Pd/C was removed by filtration through a pad of Celite and the filtrate was concentrated under reduced pressure. (-)-2-Azabicyclo[2.2.1]heptan-3-one was isolated as a white solid (70 mg, quant.)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.23 (br. s, 1H, NH), 3.87 (m, 1H, H1), 2.71 (s, 1H, H3), 1.91-1.79 (m, 3H, H5 + H4a), 1.61-1.56 (m, 2H, H5b + H7b), 1.38-1.35 (m, 1H, H7a)

^{13}C NMR (100 MHz, D_2O) δ (ppm): 181.40, 55.44, 45.05, 41.38, 30.22, 23.70.

$[\alpha]^{25}_D$ -149 (c 1.2, $CHCl_3$)

5.6.2. Synthesis of 2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one and 2-methyl-2-azabicyclo[2.2.1]hept-3-one [88]



a) **1a**
b) **2a**

a) **3a**
b) **4a**

Figure 56: Synthesis of 2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one and 2-methyl-2-azabicyclo[2.2.1]hept-3-one

(±)-2-Methyl-2-azabicyclo[2.2.1]hept-5-en-3-one (*rac*-3a): To a suspension of NaH (175.8 mg, 7.33 mmol) in anhydrous DMF (4 mL) was added (±)-2-azabicyclo[2.2.1]hept-5-en-3-one ***rac*-1a** (400 mg, 3.67 mmol) under Ar-atmosphere at 0 °C and stirred at room temperature for 1 h. Methyl iodide (1.18 mL, 18.3 mmol) was added at 0 °C and the mixture was stirred at room temperature for 2.5 h. After addition of saturated NH_4Cl solution the mixture was extracted with EtOAc. The organic phase was washed with H_2O, dried with Na_2SO_4 and concentrated. The residue was purified using flash chromatography (eluent: hexane/EtOAc 7:3) yielding (±)-2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one as dark orange oil (289 mg, 64%).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.83 (dd, 1H, H6), 6.63-6.59 (m, 1H, H5), 4.08-4.06 (m, 1H, H1), 3.28 (m, 1H, H4), 2.53 (s, CH3), 2.30-2.26 (m, 1H, H7b), 2.11-2.08 (m, 1H, H7a)

^{13}C NMR (100 MHz, D_2O) δ (ppm): 181.34, 139.14, 138.22, 65.26, 58.24, 53.66, 31.54.

(1*R*,4*S*)-2-Methyl-2-azabicyclo[2.2.1]hept-5-en-3-one [(-)-3a]: To a suspension of NaH (43.9 mg, 1.83 mmol) in anhydrous DMF (2.5 mL) was added (-)-2-azabicyclo[2.2.1]hept-5-en-3-one **(-)-1a** (100 mg, 0.916 mmol) under Ar-atmosphere at 0 °C and stirred at room temperature for 1 h. Methyl iodide (295 µL, 4.58 mmol) was added at 0 °C and the mixture

was stirred at room temperature for 2.5 h. After addition of saturated NH_4Cl solution the mixture was extracted with EtOAc. The organic phase was washed with H_2O, dried with Na_2SO_4 and concentrated. The residue was purified using flash chromatography (eluent: hexane/EtOAc 7:3) yielding (-)-2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one as dark orange oil (40 mg, 70%).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.86 (dd, 1H, H6), 6.66-6.63(m, 1H, H5), 4.11-4.09 (m, 1H, H1), 3.32 (m, 1H, H4), 2.66 (s, CH3), 2.33-2.30 (td, 1H, H7b), 2.14-2.11 (s, 1H, H7a)

^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 181.18, 139.16, 138.24, 65.25, 58.27, 53.68, 31.56.

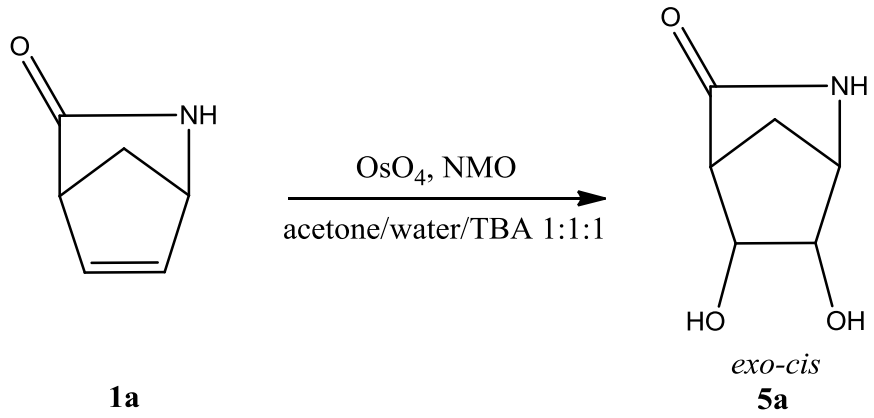
$[\alpha]^{25}_D$ -162 (c 0.875, $CHCl_3$)

(±)-2-Methyl-2-azabicyclo[2.2.1]hept-3-one (*rac*-4a): To a suspension of NaH (43.2 mg, 1.802 mmol) in anhydrous DMF (2.5 mL) was added (±)-2-azabicyclo[2.2.1]heptan-3-one ***rac*-2a** (90 mg, 0.811 mmol) at 0 °C under Ar-atmosphere and stirred at room temperature for 1 h. Methyl iodide (288 µL, 4.505 mmol) was added at 0 °C and the mixture was stirred at room temperature for 2 h. After addition of saturated NH_4Cl solution the mixture was extracted with EtOAc. The organic phase was washed with H_2O, dried with Na_2SO_4 and concentrated. The residue was purified using flash chromatography (eluent: EtOAc/MeOH 9:1) yielding (±)-2-methyl-2-azabicyclo[2.2.1]hept-3-one as yellow oil (30.5 mg, 29 %).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.69 (m, 1H, H1), 3.75 (m, 1H, H4), 2.7 (m, 3H, CH3), 1.86 -1,4 (m, 1H, H7b, 4H, H5 and H6), 1.33 (d, 1H, H7a)

^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 178.65 (C3), 61.46 (C1), 46.61 (C4), 39.81 (C7), 27.38 (CH3), 26.54 (C6), 24.84 (C5)

5.6.3. Synthesis of 5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one [89]



1a **5a**

Figure 57: Synthesis of 5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one

(±)-*exo*-*cis*-5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (*rac*-5a): To a solution of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one ***rac*-1a** (100 mg, 0.917 mmol) in acetone (2 mL), water (2 mL) and *tert*-butyl alcohol (2 mL) was added *N*-methylmorpholine *N*-oxide (354.5 mg, 3.03 mmol) and OsO_4 (42.0 mg, 0.165 mmol) in *tert*-butyl alcohol (1 mL). The mixture was stirred at room temperature for 18 h. The solvents were evaporated and the residue was purified using flash chromatography (eluent: DCM/MeOH 8.5:1.5) yielding (±)-*exo*-*cis*-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one as whitish crystals (80 mg, 61%).

^{1}H NMR (300 MHz, D_2O) δ (ppm): 4.06 (d, 1H, H5), 4.02 (d, 1H, H6), 3.77 (m, 1H, H1), 2.61 (m, 1H, H4), 2.06 (m, 1H, H7).

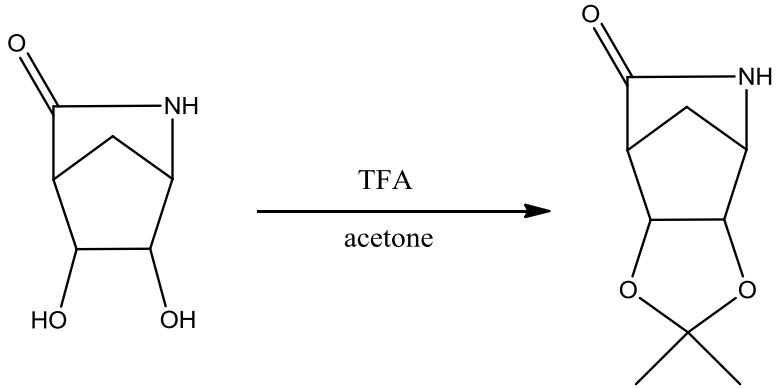
^{13}C NMR (300 MHz, D_2O) δ (ppm): 181.36, 71.05, 67.68, 58.65, 51.2, 35.58.

(1*R*,4*S*,5*R*,6*S*)-5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one [(-)-5a]: To a solution of (-)-2-azabicyclo[2.2.1]hept-5-en-3-one **(-)-1a** (100 mg, 0.917 mmol) in acetone (2 mL), water (2 mL) and *tert*-butyl alcohol (2 mL) was added *N*-methylmorpholine *N*-oxide (354.5 mg, 3.03 mmol) and OsO_4 (42.0 mg, 0.165 mmol) in *tert*-butyl alcohol (1 mL). The mixture was stirred at room temperature for 3.5 h. The solvents were evaporated and the residue was purified using flash chromatography (eluent: DCM/MeOH 8.5:1.5) yielding (1*R*,4*S*,5*R*,6*S*)-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one as whitish crystals (97mg, 74%).

^{1}H NMR (300 MHz, D_2O) δ (ppm): 4.06 (d, 1H, H5), 4.02 (d, 1H, H6), 3.77 (m, 1H, H1), 2.61 (m, 1H, H4), 2.06 (m, 1H, H7).

^{13}C NMR (300 MHz, D_2O, δ (ppm): 181.36, 71.05, 67.68, 58.65, 51.2, 35.58.

5.6.4. Synthesis of (±)-*exo*-2-azabicyclo[2.2.1]heptan-5,6-(dimethylmethylene)dioxy-3-one (*rac*-6a) [90]



exo-cis **5a** *exo* **6a**

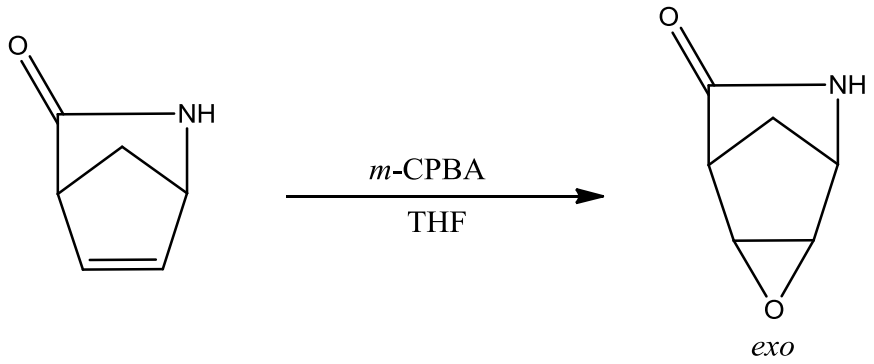
Figure 58: Synthesis of (±)-exo-2-azabicyclo[2.2.1]heptan-5,6-(dimethylmethylene)dioxy-3-one

To a solution of (±)-*exo-cis*-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one ***rac*-5a** (60.3 mg, 0.422 mmol) in acetone (5 mL) was added trifluoroacetic acid (200 µL) and the mixture was stirred at room temperature for 18 h and concentrated to give the crude product. The residue was purified by flash chromatography (eluent: EtOAc/hexane 9:1) to yield (±)-*exo*-2-azabicyclo[2.2.1]heptan-5,6-(dimethylmethylene)dioxy-3-one as white solid (61.2 mg, 79%).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 5.82 (br, s, 1H), 4.56 (d, 1H, H5), 4.42 (d, 1H, H6), 3.79 (m, 1H, H1), 2.74 (m, 1H, H4), 2.17-2.04 (dd, 2H, H7), 1.48 (s, 3H, OCH3), 1.36 (s, 3H, OCH3).

^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 178.07, 114. 23, 81. 63, 77.95, 56.75, 48.74, 36. 38, 25. 75, 24.55.

5.6.5. Synthesis of (±)-*exo*-7-oxo-3-oxa-6-azatricyclo[3.2.1.0]octane (*rac*-7a) [91]



1a **7a**

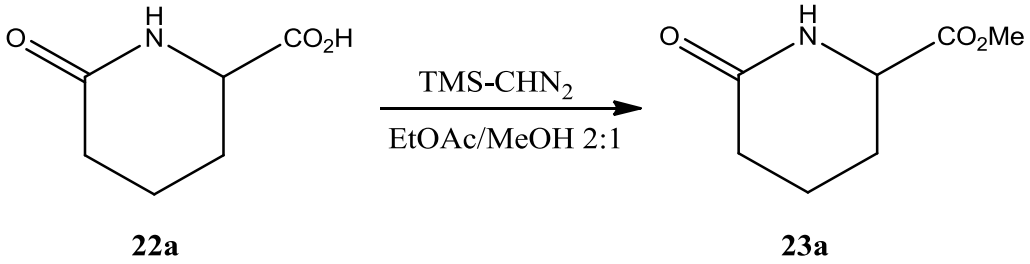
Figure 59: Synthesis of (±)-exo-7-oxo-3-oxa-6-azatricyclo[3.2.1.0]octane

(±)-2-Azabicyclo[2.2.1]hept-5-en-3-one ***rac*-1a** (100 mg, 0.917 mmol) was dissolved in THF (5 mL), *m*-CPBA (1.263 g, 7.32 mmol) was added, and the reaction was stirred at room temperature for 24 h. After this time the solvent was removed under reduced pressure and the solid was dissolved in EtOAC, washed with satured Na_2CO_3, filtered and concentrated under reduced pressure. The white solid obtained was triturated with DCM, filtered, concentrated under reduced pressure then purified by flash chromatography to afford (±)-*exo*-7-oxo-3-oxa-6-azatricyclo[3.2.1.0]octane as white solid (36 mg, 35%).

^{1}H NMR (300 MHz, D_2O) δ (ppm): 6.53 (s, 1H NH), 3.91 (m, 1H, H5), 3.69-3.70 (m, 1H, H4), 3.60-3.58 (m, 1H, H3), 2.91 (m, 1H, H2), 1.89-1.85 (m, 1H, H1b) 1.71-1.67 (m, 1H, H1a).

^{13}C NMR (300 MHz, D_2O) δ (ppm): 180.78, 57.76, 51.61, 47.02, 31.29.

5.6.6. Synthesis of methyl-6-oxopiperidine-2-carboxylate (23a)



22a **23a**

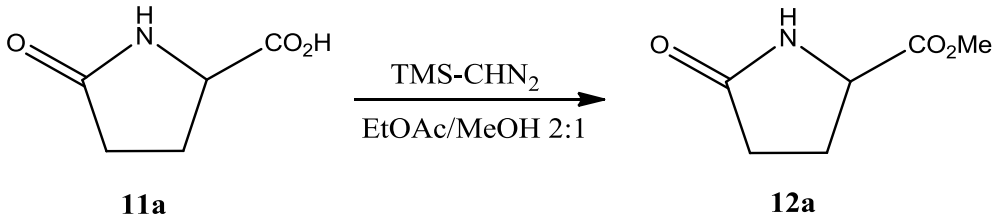
Figure 60: Synthesis of methyl-6-oxopiperidine-2-carboxylate

6-Oxopipecolinic acid **22a** (50 mg, 0.350 mmol) was dissolved in a mixture of MeOH and EtOAc (1:2, 3 mL) and a solution of TMS-CHN_2 in Et_2O (2 M, 352 µL, 0.700 mmol) was added under Ar-atmosphere. The reaction mixture was stirred at room temperature for 16 h. Solvents were removed under reduced pressure and the residue was purified using flash chromatography (eluent: EtOAc) to yield methyl-6-oxopiperidine-2-carboxylate as white solid (39.4 mg, 65 %).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.64 (br s, 1H), 4.06-4.11 (m, 1H, H2), 3.76 (s, 3H, CH3), 2.31 – 2,38 (m, 2H, H5), 2.12 – 2.20 (m, 1H, H3b), 1,78 – 1,93 (m, 3H, H3a and H4).

^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 171.72 (CO), 171.54 (CO), 54. 80 (C2), 52.63 (OCH3), 31.12 (C5), 25.46 (C3), 19,54 (C4)

5.6.7. Synthesis of methyl-2-pyrrolidinon-5-carboxylate (12a)



11a **12a**

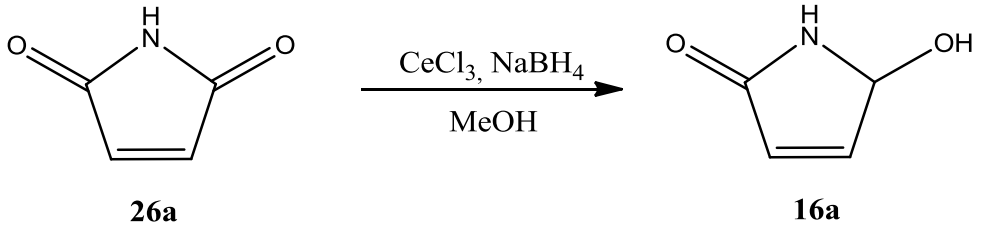
Figure 61: Synthesis of methyl-2-pyrrolidinon-5-carboxylate

L-2-Pyrrolidinon-5-carbonic acid **11a** (50 mg, 0.387 mmol) was dissolved in a mixture of MeOH and EtOAc (1:2, 3 mL) and a solution of TMS-CHN_2 in Et_2O (2 M, 390 µL, 0.774 mmol) was added under Ar-atmosphere. The reaction mixture was stirred at room temperature for 16 h. The solvents were evaporated and the residue was purified by flash chromatography (eluent gradient: EtOAc/hexanes 8:2 to pure EtOAc) yielding methyl-2-pyrrolidinon-5-carboxylate as colourless oil (45 mg, 82%).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.8 (br s, 1H), 4.27-4.22 (m, 1H, H2), 3.75 (s, 3H, CH3), 2.49-2.31 (m, 3H, H3b, H4), 2.26-2.15 (m, 1H, H3a).

^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 178.32, 172.66, 55.38, 52. 68, 29.24, 24.76

5.6.8. Synthesis of 5-hydroxy-1*H*-pyrrol-2(5*H*)-one (16a)



26a **16a**

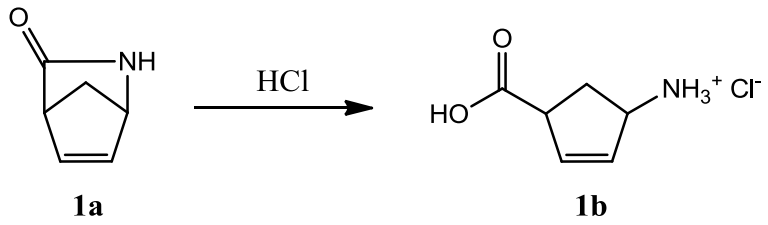
Figure 62: Synthesis of 5-hydroxy-1*H*-pyrrol-2(5*H*)-one

To a solution of maleimide **26a** (100 mg, 1.03 mmol) in MeOH (1 mL) was added cerium(III) chloride heptahydrate (383.7 mg, 1.03 mmol), and the mixture was stirred for 5 min. After the solution was cooled to 0 ºC, $NaBH_4$ (38 mg, 1.03 mmol) was added portion wise, and stirred for 1 h. The reaction mixture was quenched with ice water, and MeOH was removed under reduced pressure. Flash chromatography (eluent DCM/MeOH 9:1) afforded 5-hydroxy-1*H*-pyrrol-2(5*H*)-one (90 mg, 90 %) isolated as a white solid.

^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 7.04 (d, 1H, H4), 6.05 (d, 1H, H3), 5.58 (m, 1H, H5).

5.6.9. Synthesis of reference compounds

Reference compounds were prepared by acidic hydrolysis of the corresponding substrates in concentrated hydrochloric acid:



1a **1b**

Figure 63: Synthesis of 4-amino-2-cyclopentene-1-carboxyclic acid

The substrate was dissolved in 32 % HCl (12 M) and stirred at room temperature for 2-4 h. The preparation of 3-carboxycyclopentanaminium chloride ***rac*-2b** additionally required reflux for 1 h. Reactions mixtures were washed with EtOAc and concentrated in vacuo.

3-Aminocyclopentane-1-carboxylic acid (*rac*-2b) was isolated as white solid from (±)-2-azabicyclo[2.2.1]heptan-3-one (50 mg, 0.45 mmol): yield 59 mg, 79%.

^{1}H NMR (300 MHz, D_2O) δ (ppm): 3.77-3.68 (m, 1H, H4), 3.04-2.94 (m, 1H, H1), 2.43-2.34 (m, 1H, H3a), 2.17-1.74 (m, 5H, H3b + H2+ H5) ppm.

(1*R*,3*S*)-3-Aminocyclopenante-1-carboxylic acid ((-)-2b)) was isolated as white solid from (±)-2-azabicyclo[2.2.1]heptan-3-one (30 mg, 0.27 mmol): yield 35 mg, 79%.

^{1}H NMR (300 MHz, D_2O) δ (ppm): 3.77-3.68 (m, 1H, H4), 3.04-2.94 (m, 1H, H1), 2.43-2.34 (m, 1H, H3a), 2.17-1.74 (m, 5H, H3b + H2+ H5) ppm.

^{13}C NMR (300 MHz, D_2O) δ (ppm): 179.79, 51.39, 42.21, 33.54, 29.74, 27.60.

$[\alpha]^{25}_{D}$ -19 (c 0.875, MeOH)

4-Methylamino-2-cyclopentene-1-carboxylic acid (*rac*-3b) was isolated as yellow solid from (±)-2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one (50 mg, 0,407 mmol): yield 71 mg, 98%.

^{1}H NMR (300 MHz, D_2O) δ (ppm): 6.30-6.27 (m, 1H, H3), 6.02-5.99 (m, 1H, H2), 4.35-4.31 (m, 1H, H4), 3.77-3.73 (m, 1H, H1), 2.71-2.61 (m, 5H, CH3 + H5b), 2.17-2.05 (m, 1H, H5a).

(**1*S*,4*R*)- 4-Methylamino-2-cyclopentene-1-carboxylic acid ((-)-3b)** was isolated as yellow solid from (±)-2-methyl-2-azabicyclo[2.2.1]hept-5-en-3-one (30 mg, 0.24 mmol): yield 35.3 mg, 82%.

^{1}H NMR (300 MHz, D_2O) δ (ppm): 6.30-6.27 (m, 1H, H3), 6.02-5.99 (m, 1H, H2), 4.35-4.31 (m, 1H, H1), 3.78-3.71 (m, 1H, H4), 2.71-2.61 (m, 5H, CH3 + H5b), 2.17-2.09 (m, 1H, H5a)

^{13}C NMR (300 MHz, D_2O) δ (ppm): 177. 16, 137.49, 127.62, 64.15, 49.54, 30.19, 29.32.

$[\alpha]^{25}_{D}$ -86.3 (c 0.9, MeOH).

5.6.10. Unsucessful synthesis attempts: Synthesis of 6-azatricyclo-[3.2.1.0]-octane-7-one

1st strategy:

The first attempt for the synthesis of 6-azatricyclo-[3.2.1.0]-octane-7-one involved cyclopropanation with TMS-CHN_2 [92] and removal of the TMS-group by TBAF as a second step [93]:

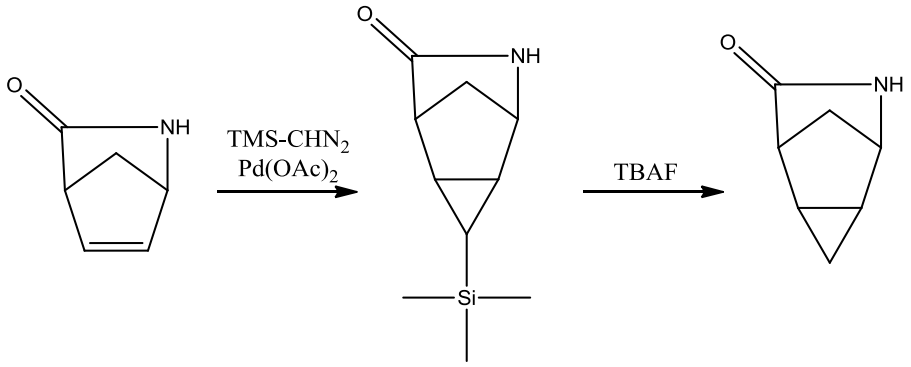


Figure 64: Synthesis of 3-trimethylsilyl-6-azatrizyclo-[3.2.1.0]-octane-7-one

3-Trimethylsilyl-6-azatrizyclo-[3.2.1.0]-octane-7-one: To a cooled suspension of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one (50 mg, 0.459 mmol) and $Pd(OAc)_2$ (5 mg) in dry Et_2O was added TMS-CHN_2 (2 M in ether, 460 µL, 0.920 mmol) dropwise under Ar-atmosphere. After 24 h $Pd(OAc)_2$ (5 mg) and TMS-CHN_2 (2 M in ether, 460 µL, 0.920 mmol) were again added since TLC showed incomplete conversion. The reaction was stopped after 48 h by filtration. GC/MS and NMR measurement of the crude product did not allow conclusions regarding product formation. After flash chromatography (eluent: hexanes/EtOAc 1:1) no product could be detected in any of the fractions by GC/MS or NMR.

2nd strategy: [94, 95]

Due to the pyrophoricity of the zinc reagent the second attempt was carried out in a 2-neck flask equipped with a dropping funnel and dried over night in the heating oven. The flask was evacuated and flamed out using a heat gun and subsequently flooded with argon.

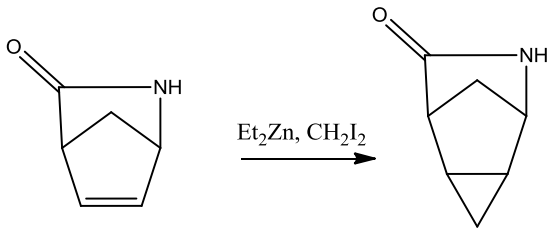


Figure 65: Synthesis of 6-azatricyclo-[3.2.1.0]-octane-7-one

A solution of diethyl zinc (1 mL, 1 mmol) was added dropwise to a cooled solution of (±)-2-azabicyclo[2.2.1]hept-5-en-3-one (50 mg, 0.459 mmol) in dry dichloromethane (2 mL) and stirred for 15 min. Diiodomethane (147 µL, 1.834 mmol) was added rapidly and the reaction was stirred at room temperature. The reaction was stopped after 24 h as TLC did not show the formation of any new spots. The reaction was quenched with saturated NH_4Cl solution, extracted with Et_2O, dried with Na_2SO_4 and concentrated in vacuo. GC/MS and NMR analysis of the crude product did not show any product formation.

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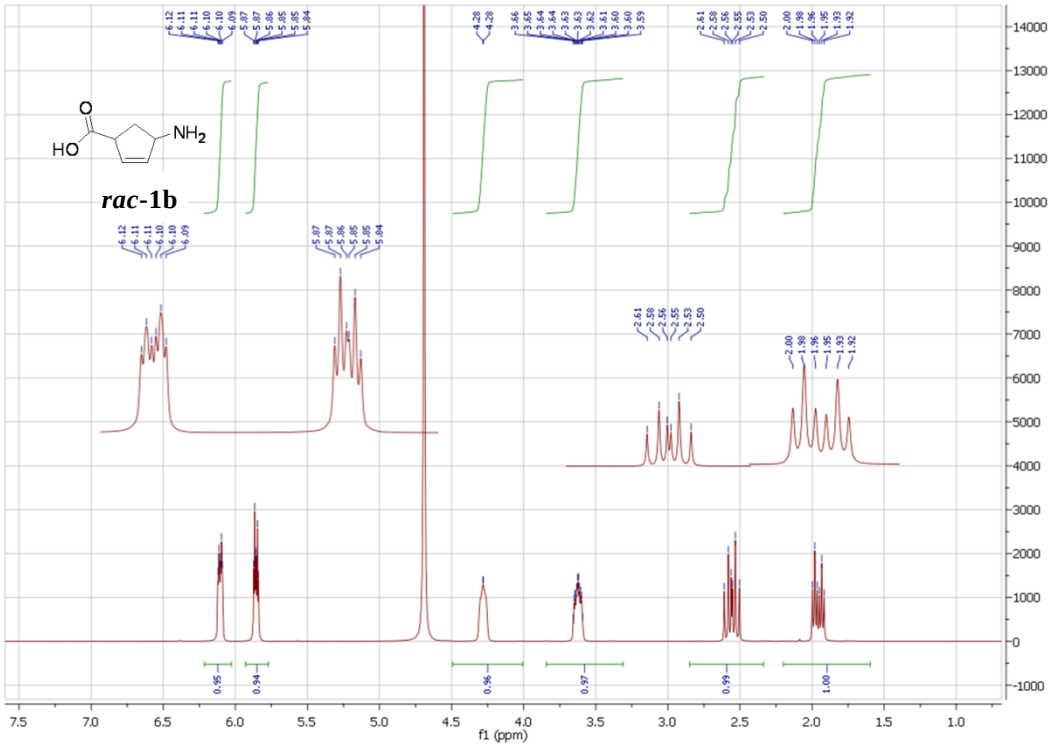
7. Appendix

NMR-Spectra

^{1}H-NMR *rac*-1b



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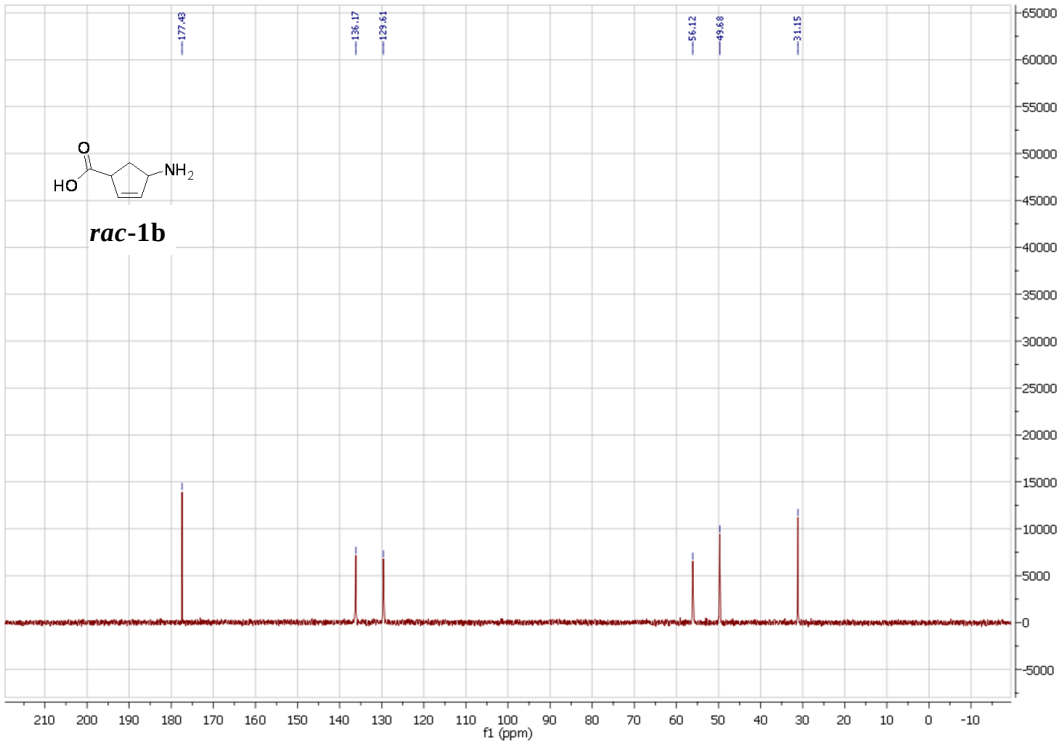

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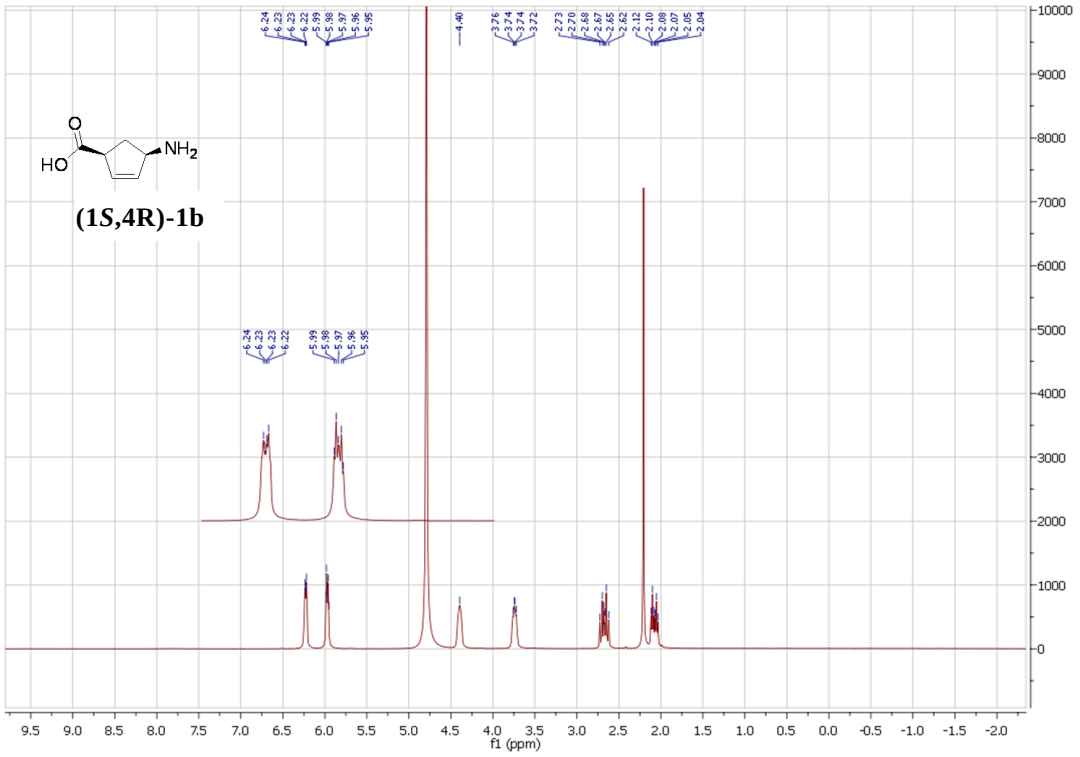

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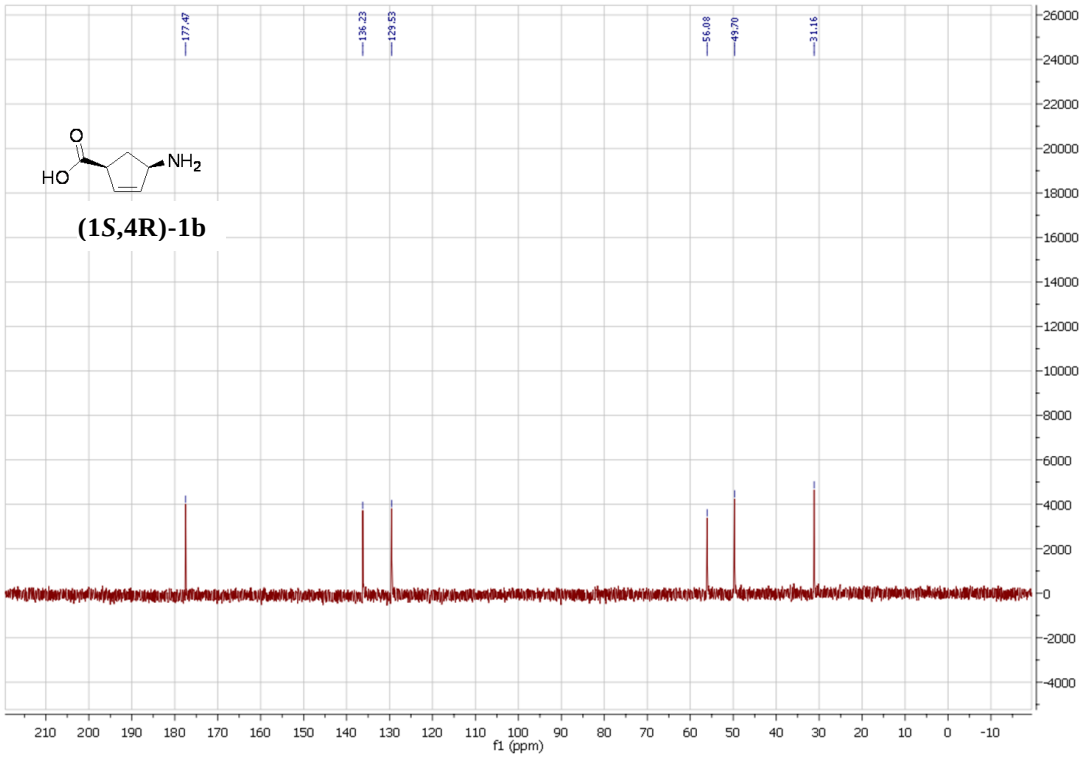

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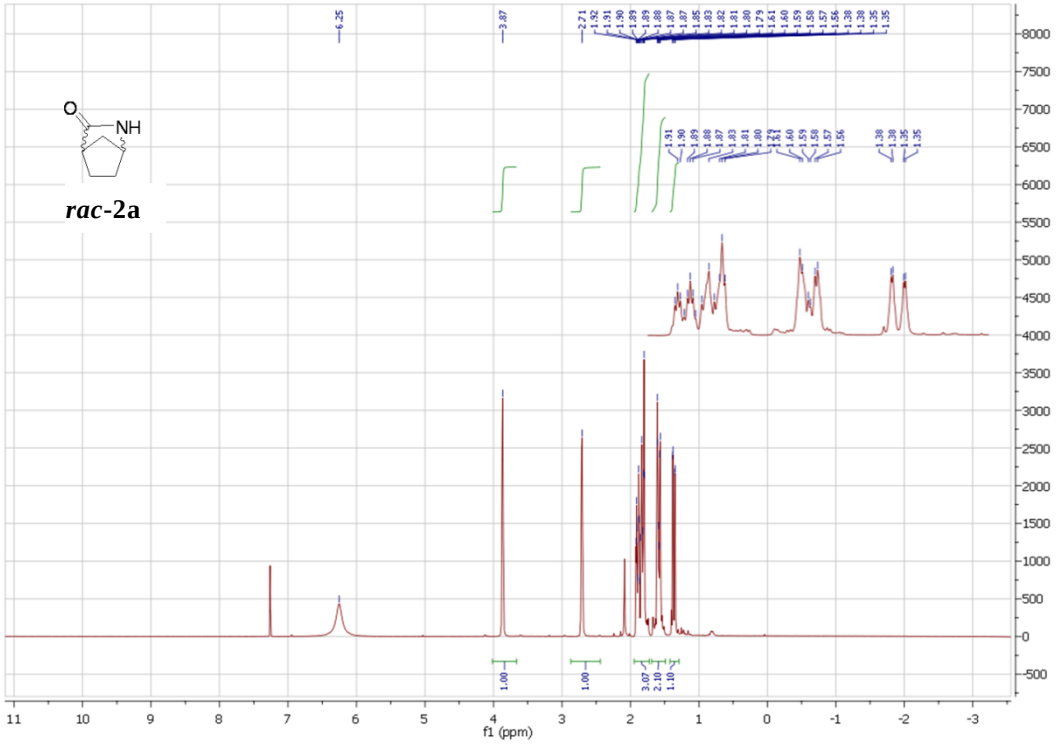

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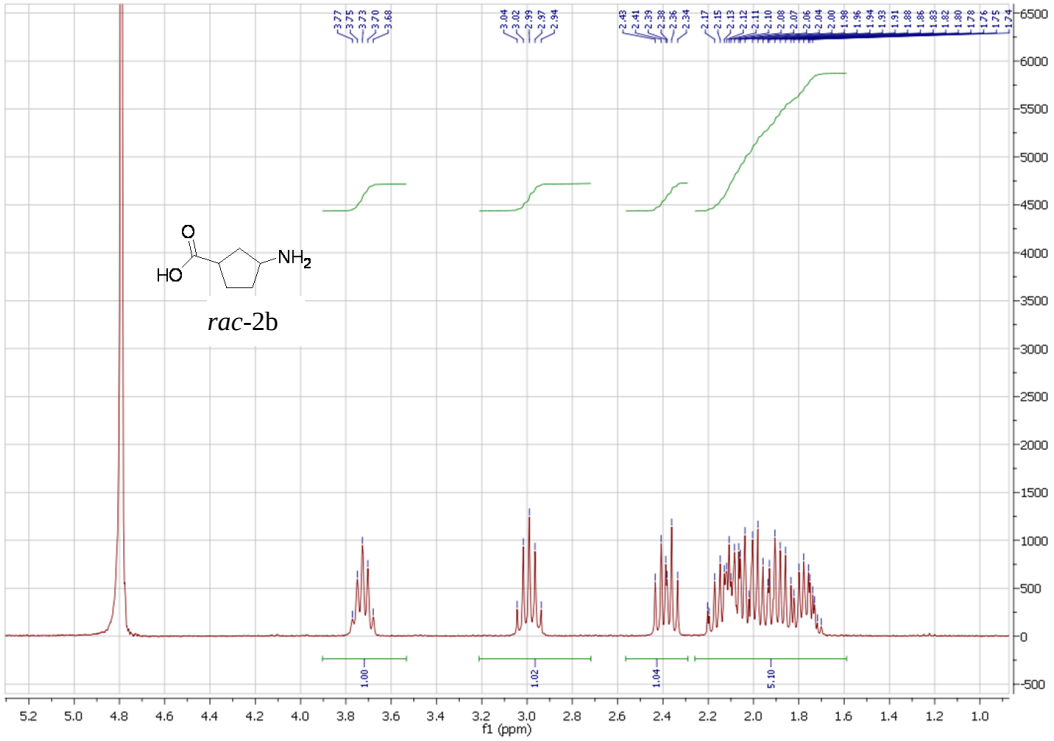

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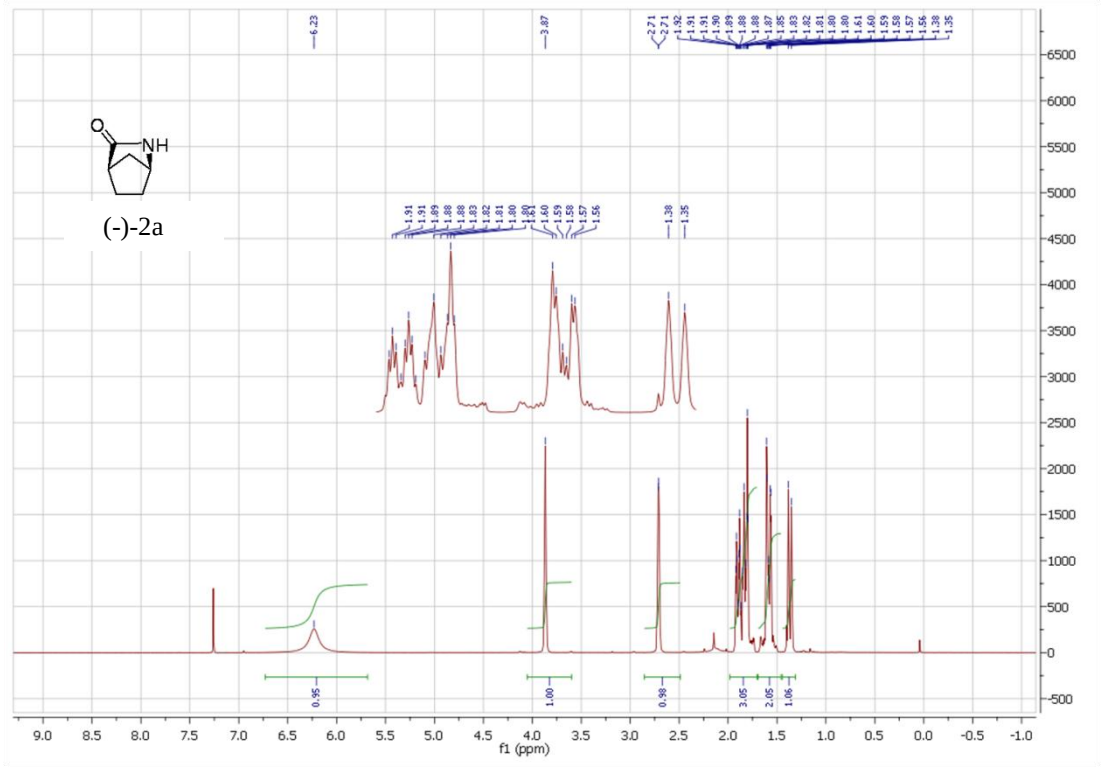

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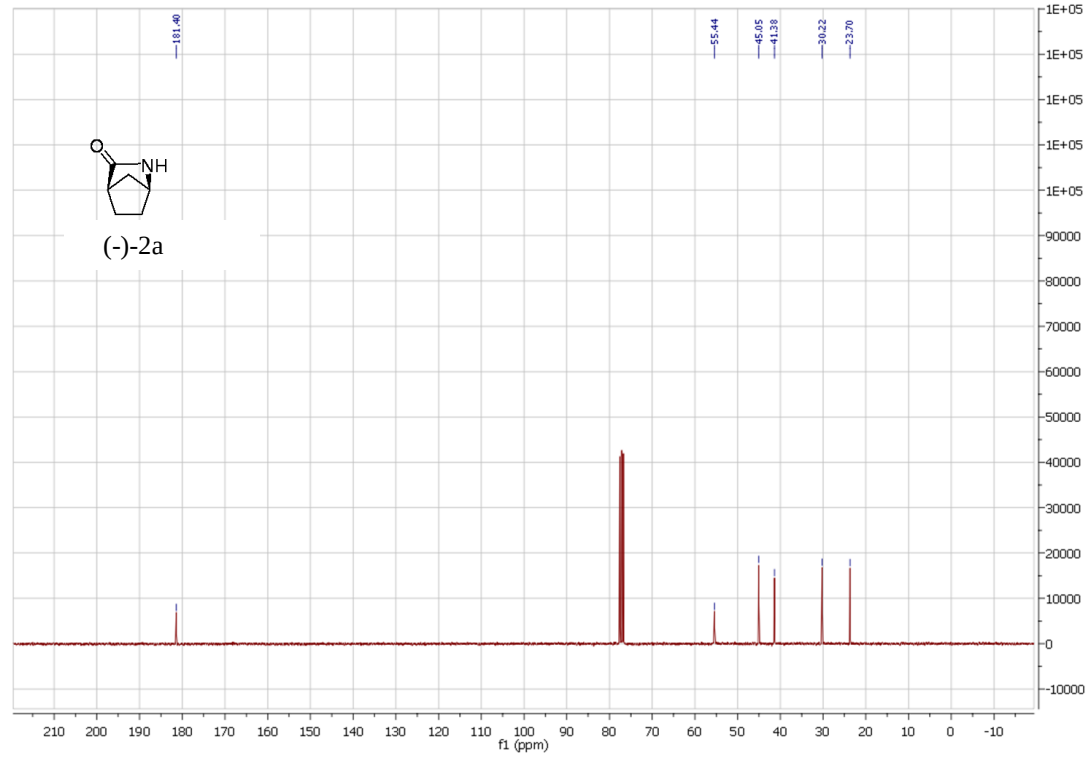

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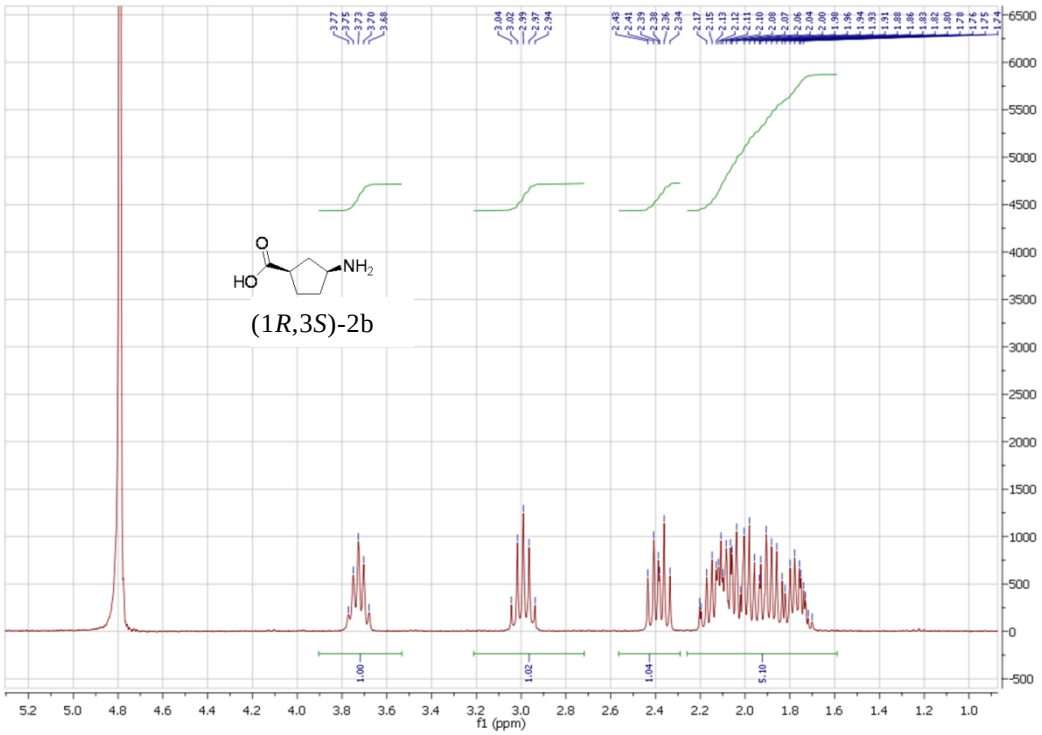

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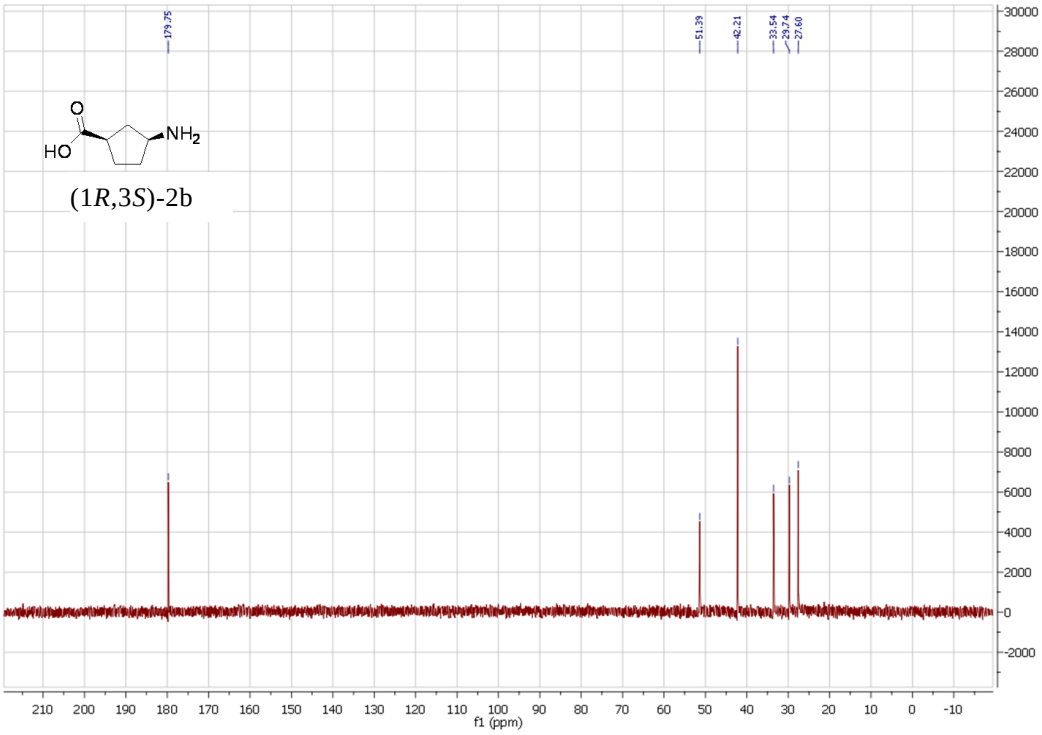

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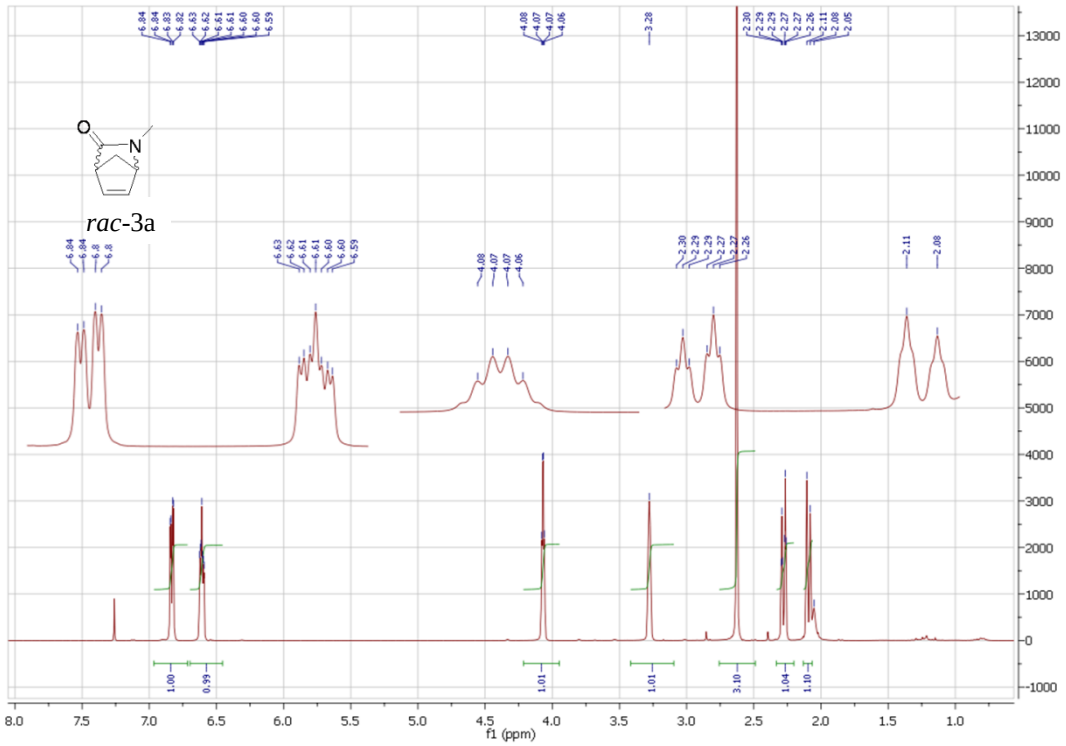

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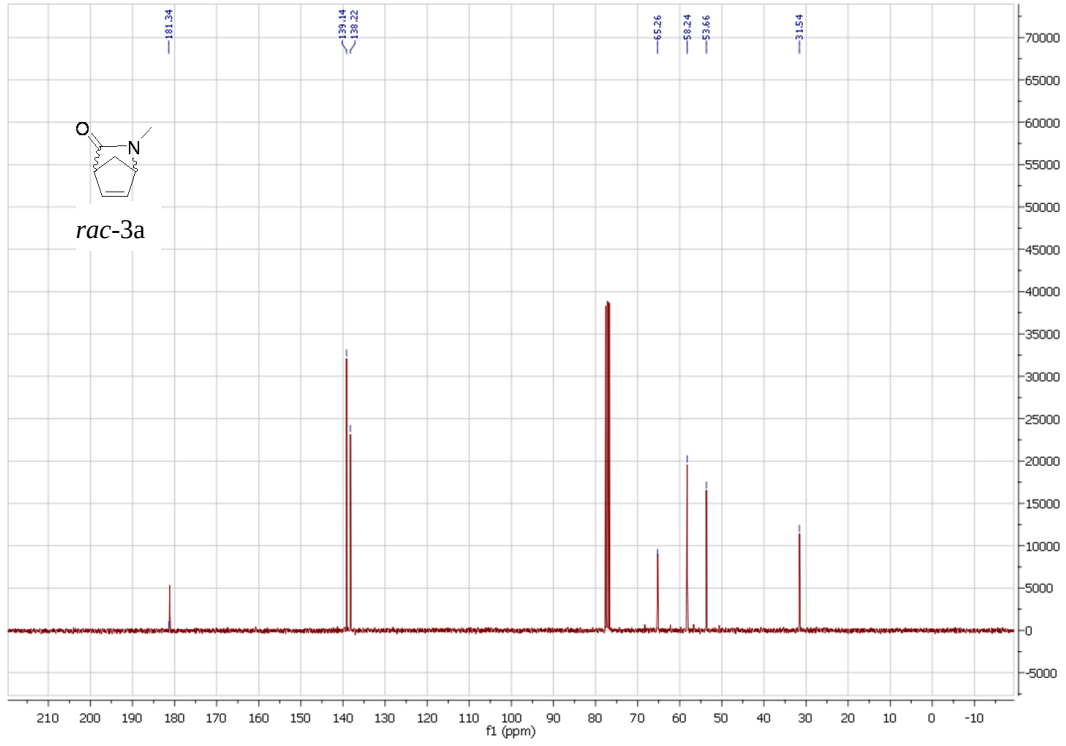

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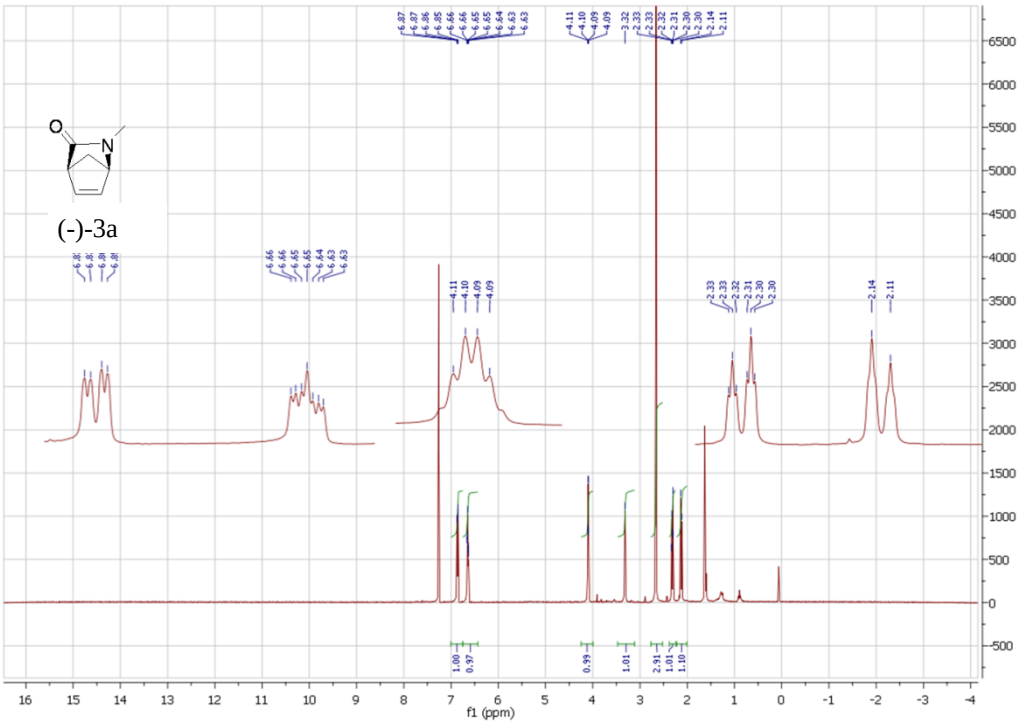

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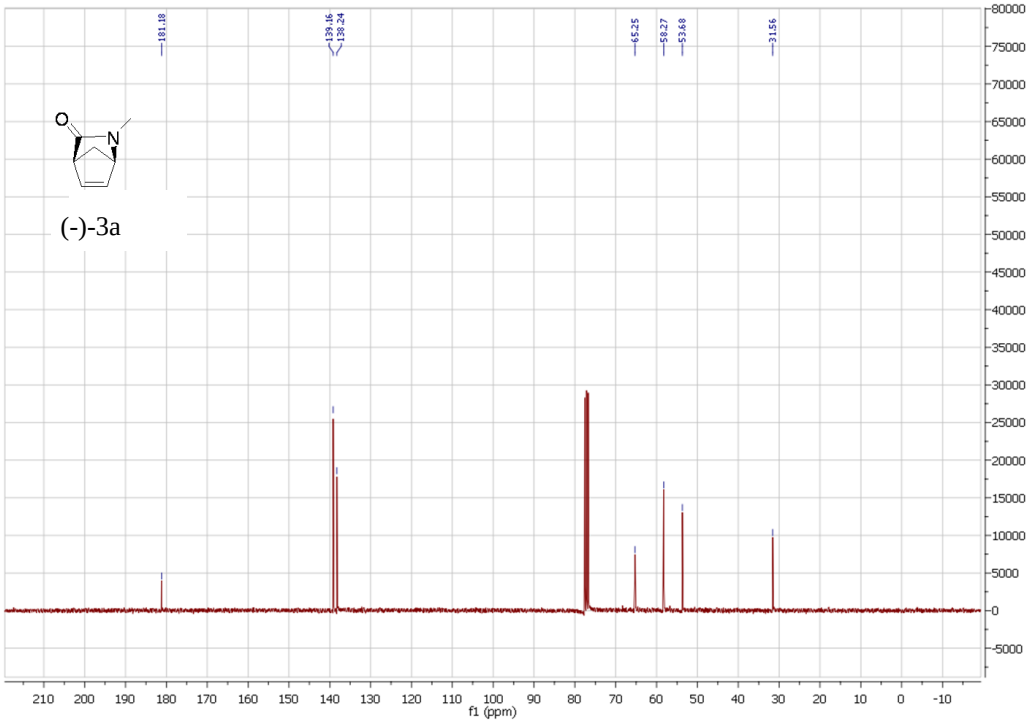

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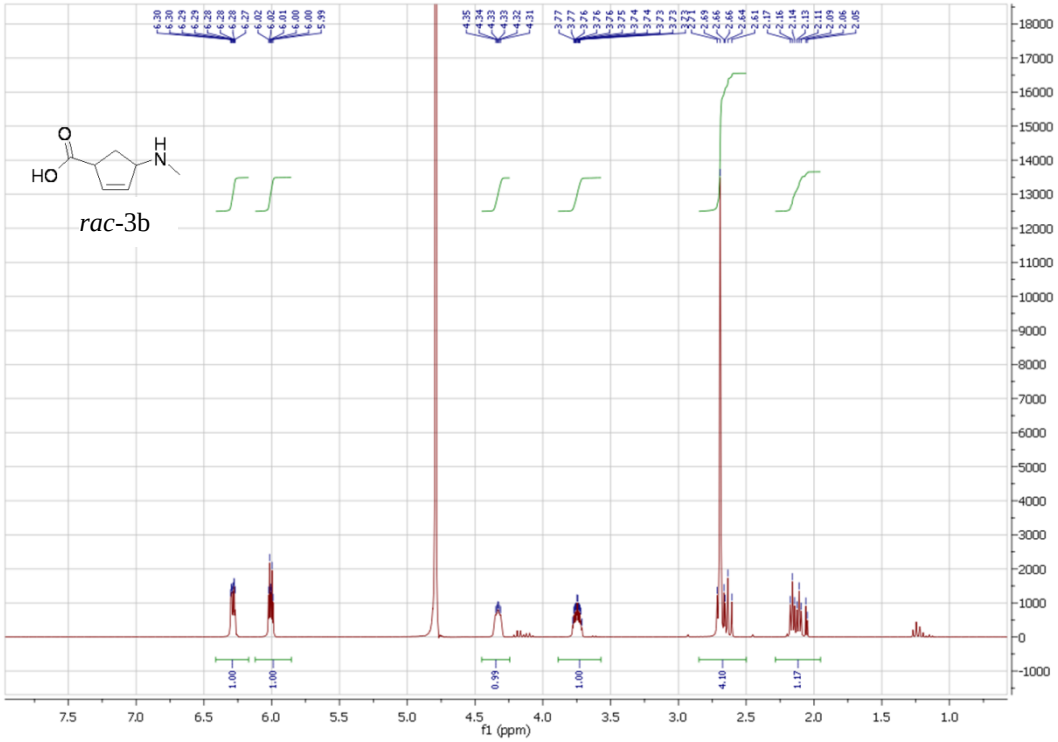

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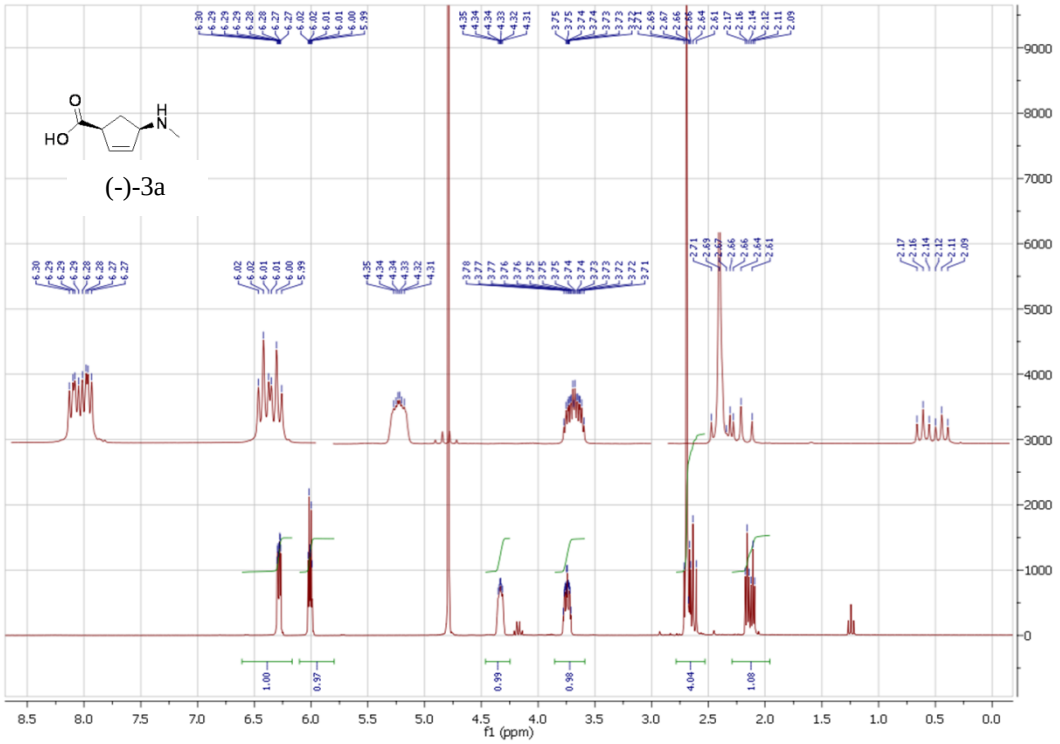

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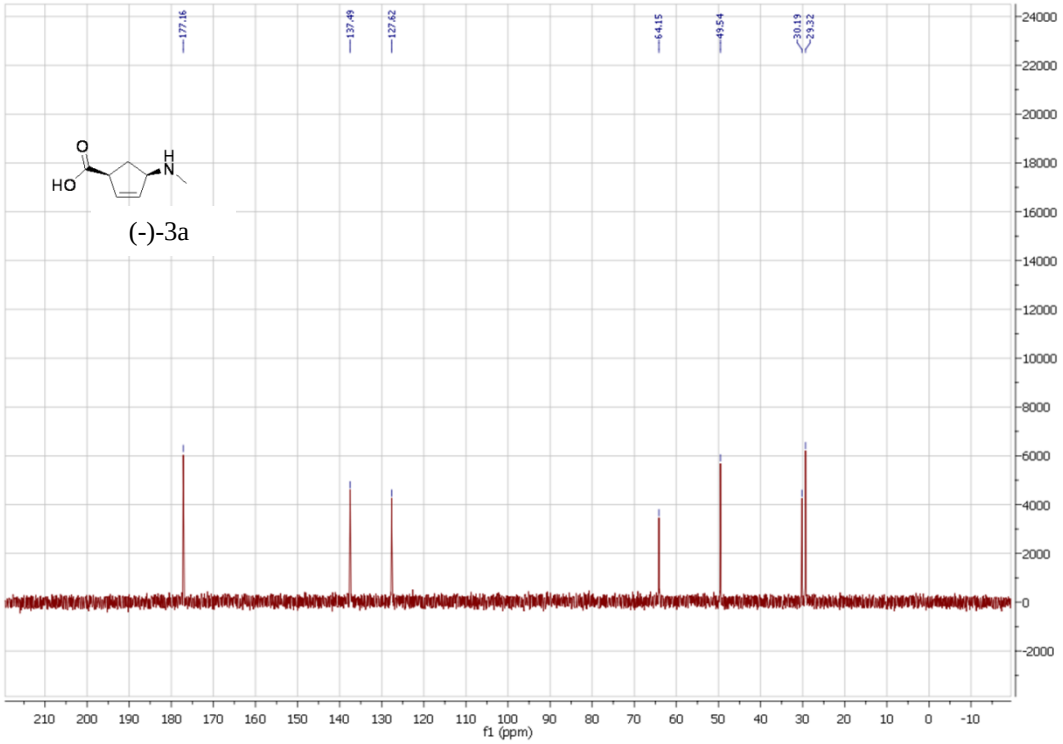
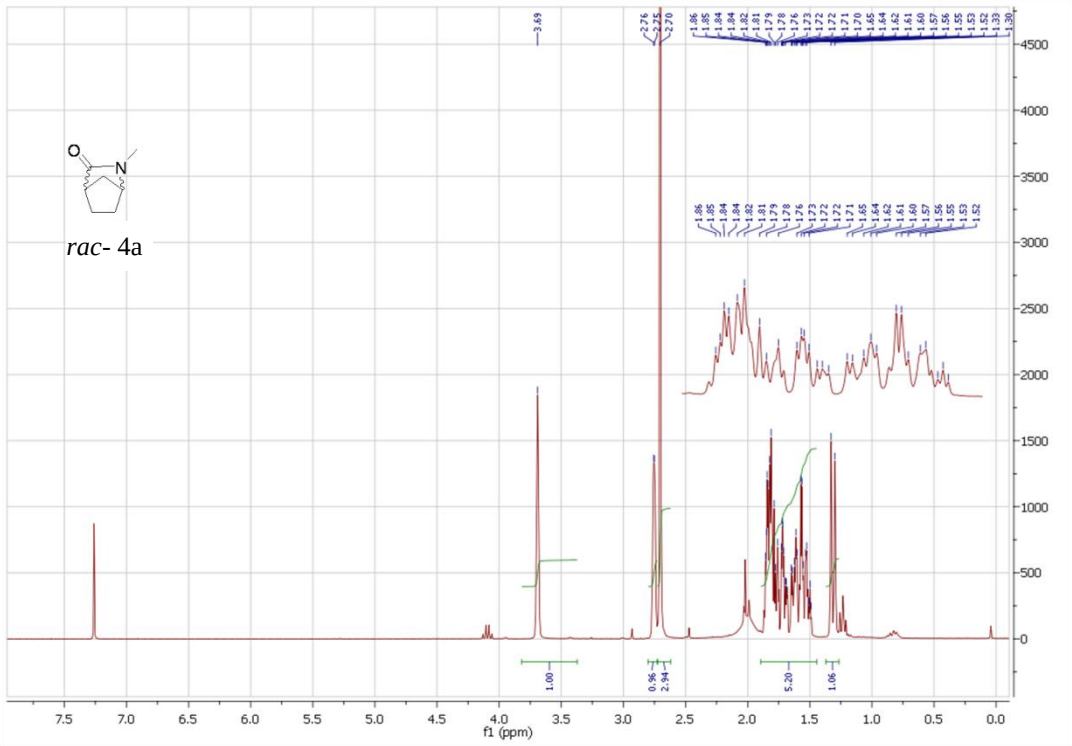


(-)-3a

^{1}H-NMR *rac*-4a



rac- 4a

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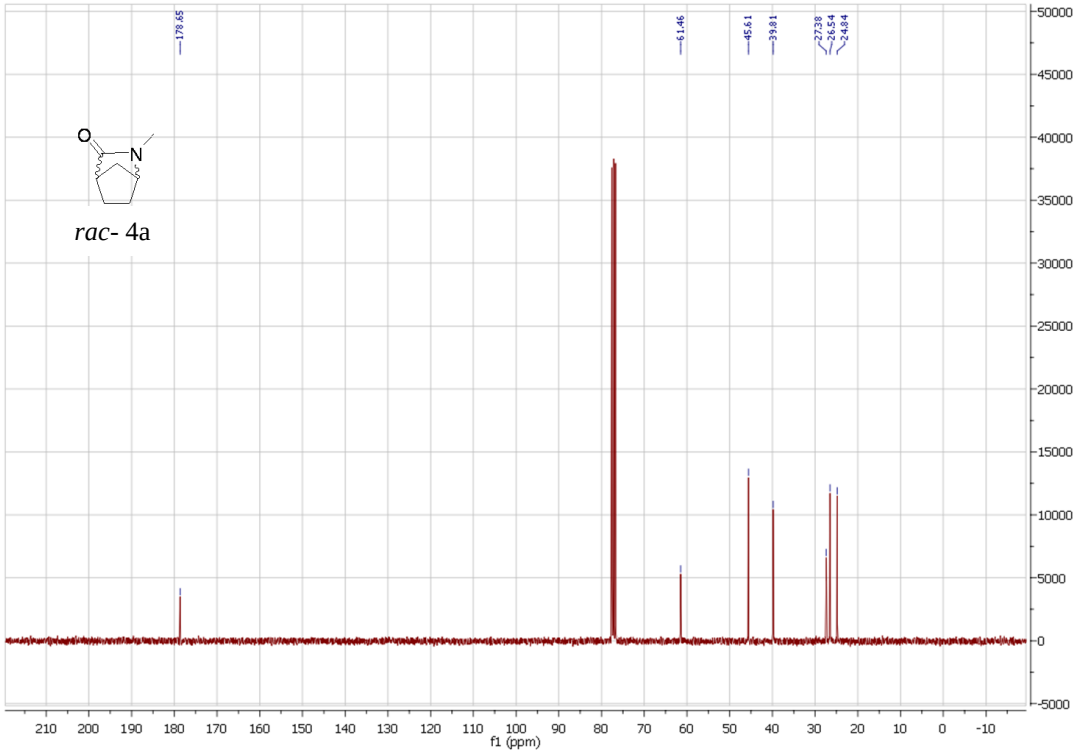

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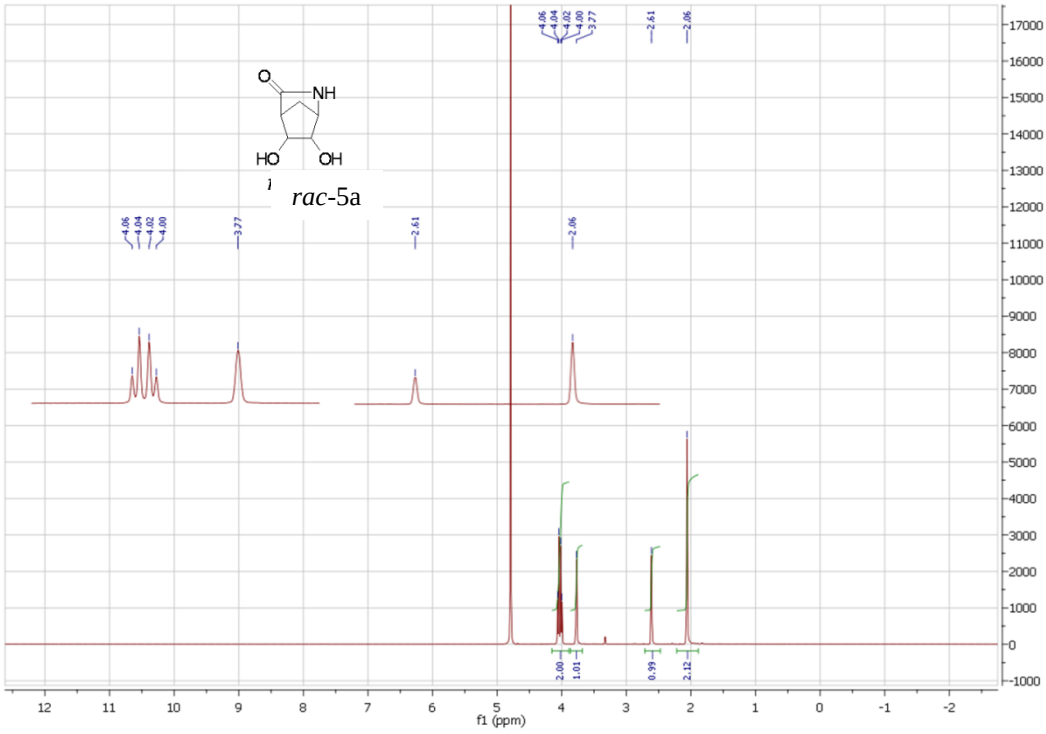
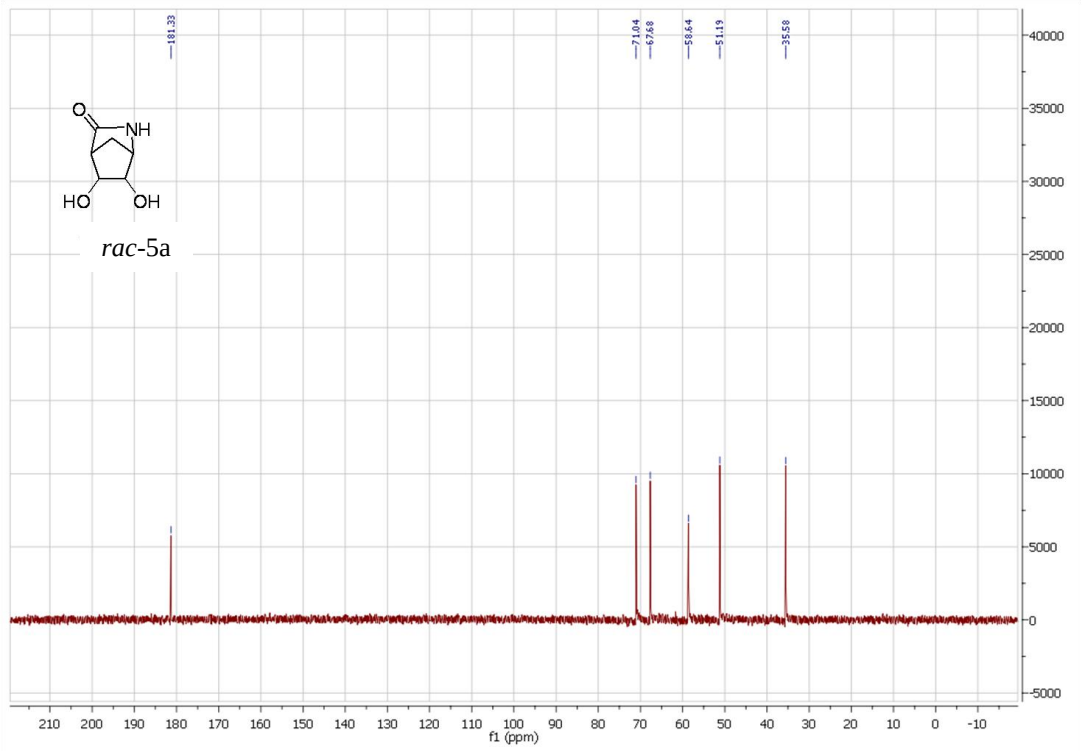


rac-5a

^{13}C-NMR *rac*-5a



rac-5a

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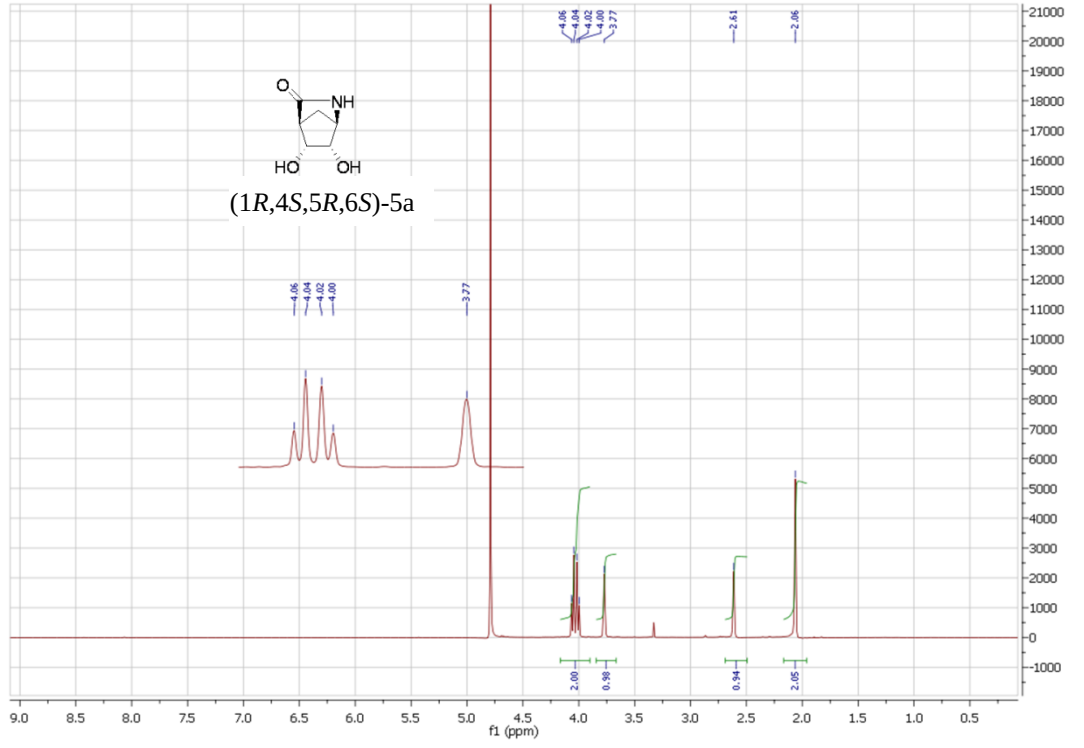

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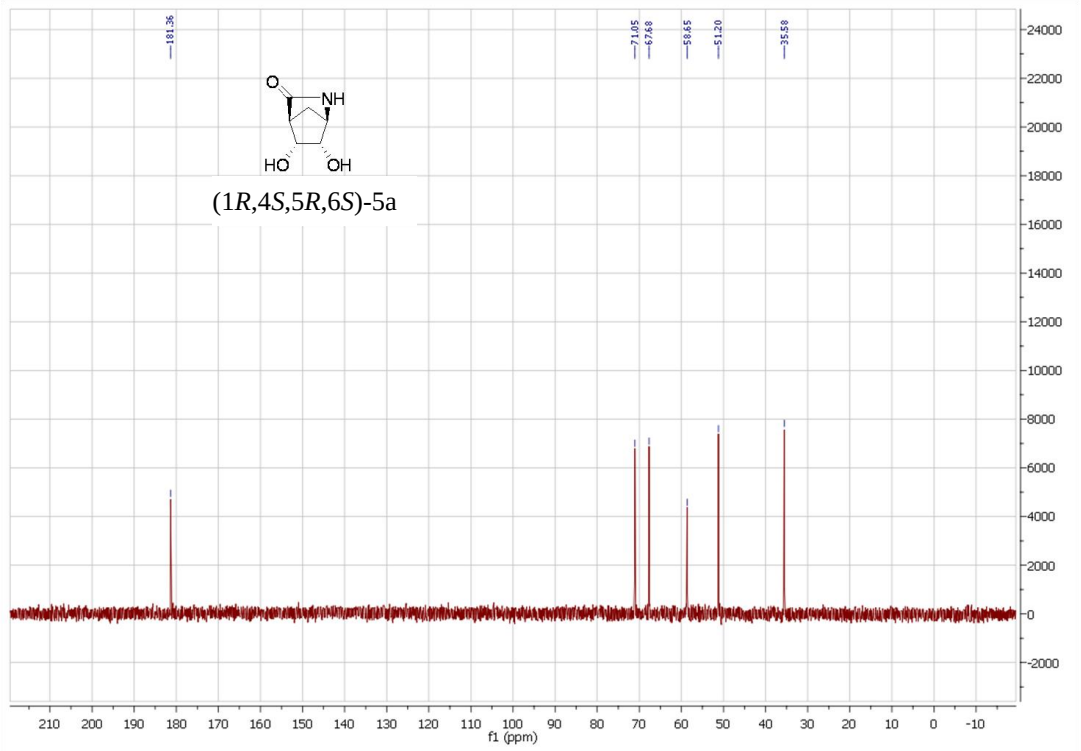

(1*R*,4*S*,5*R*,6*S*)-5a

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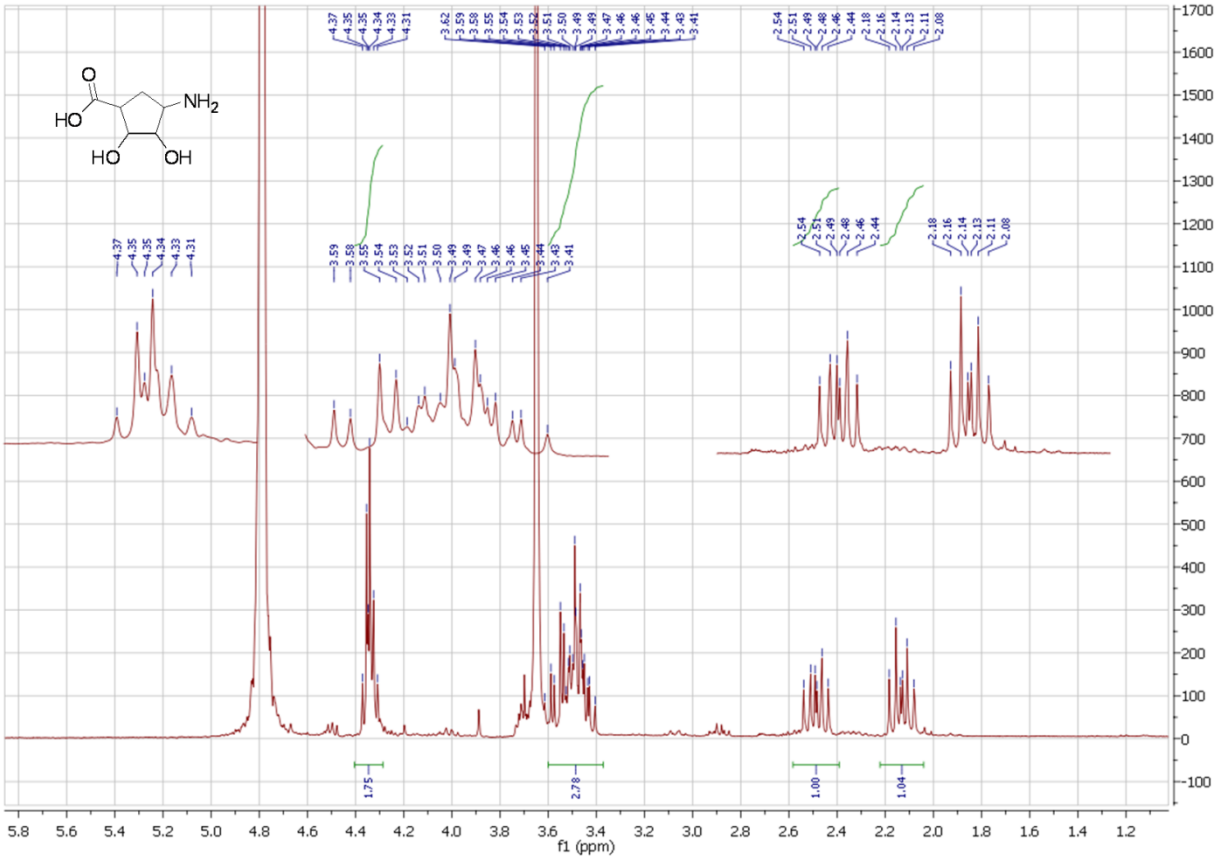

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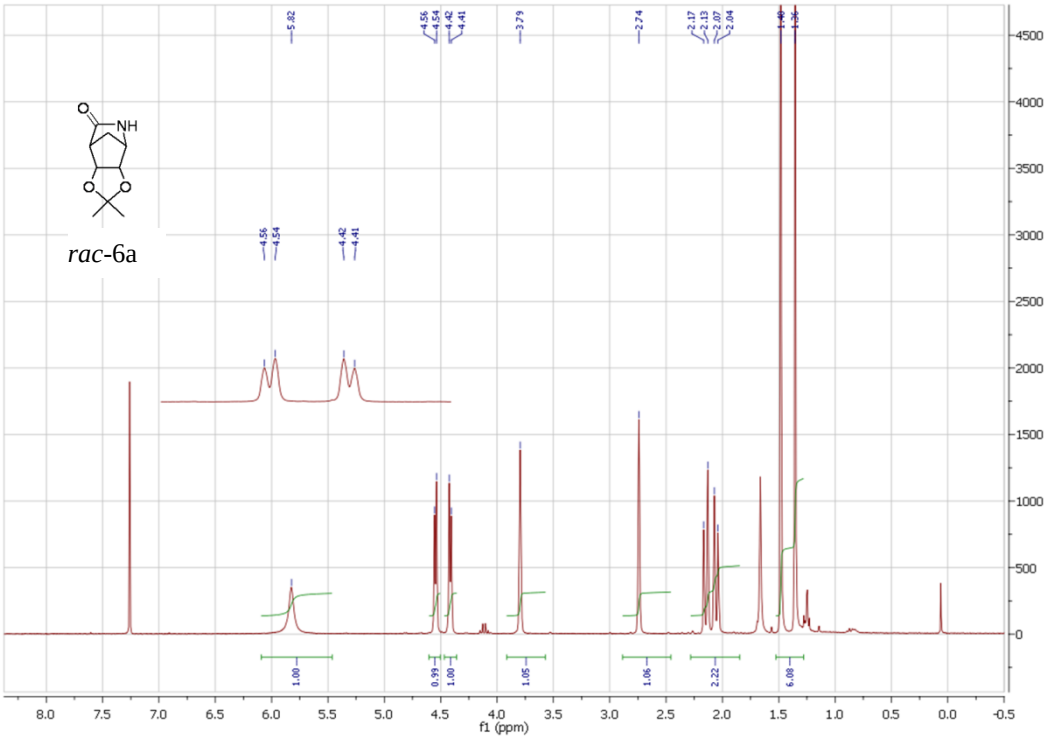

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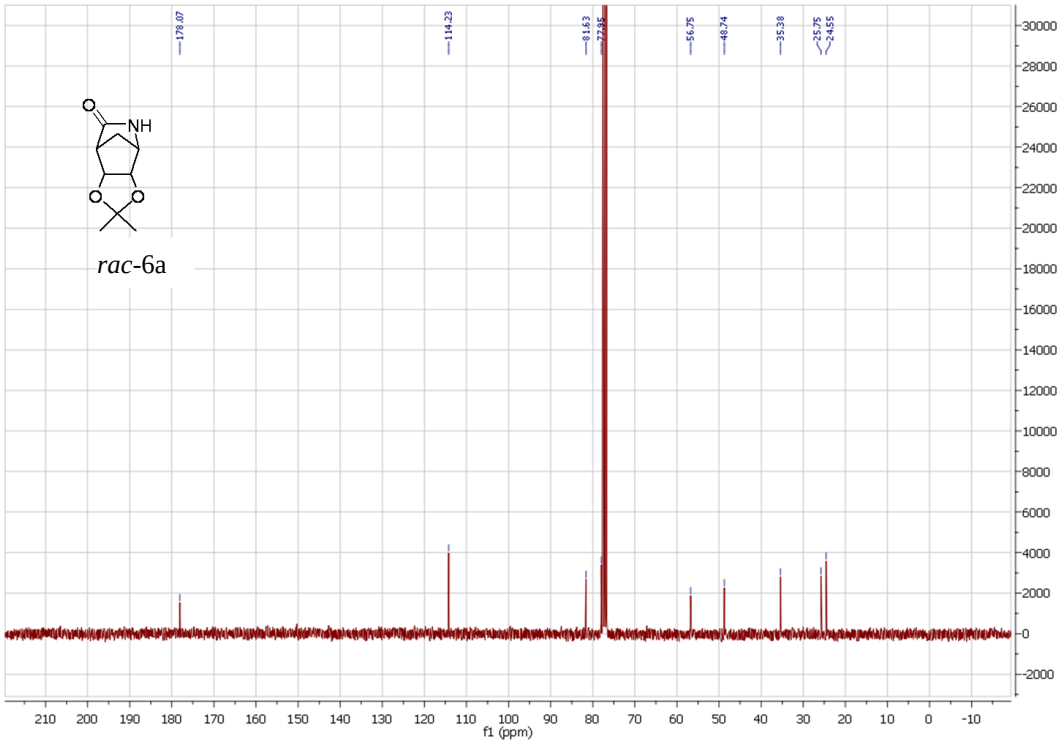

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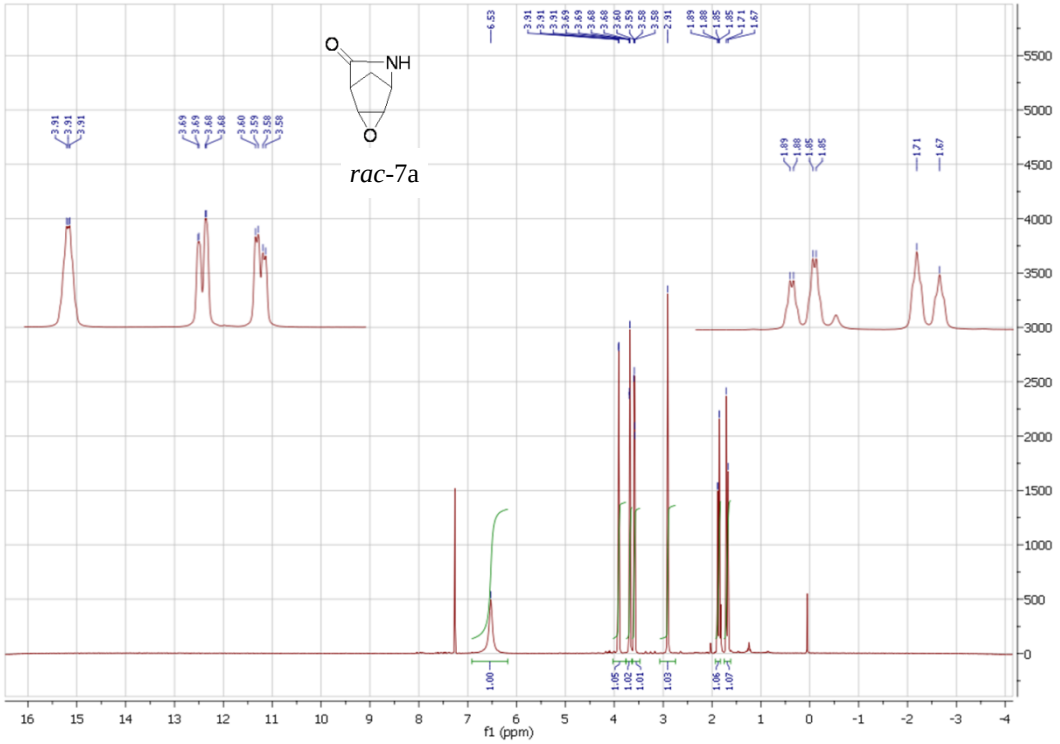

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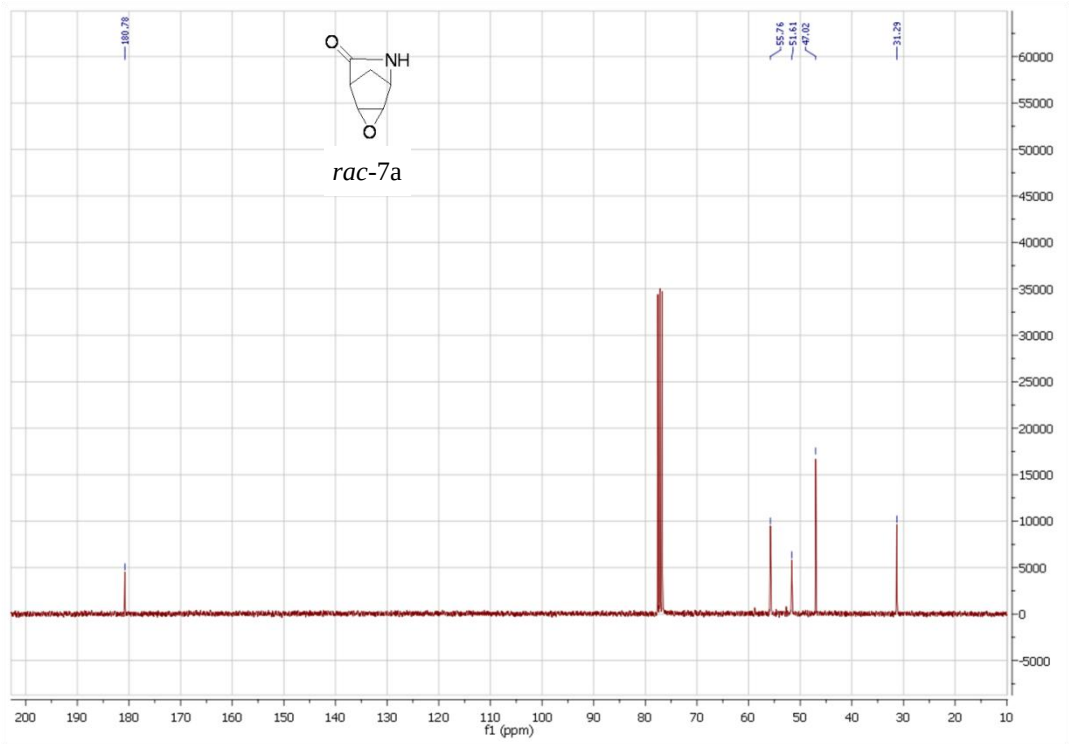

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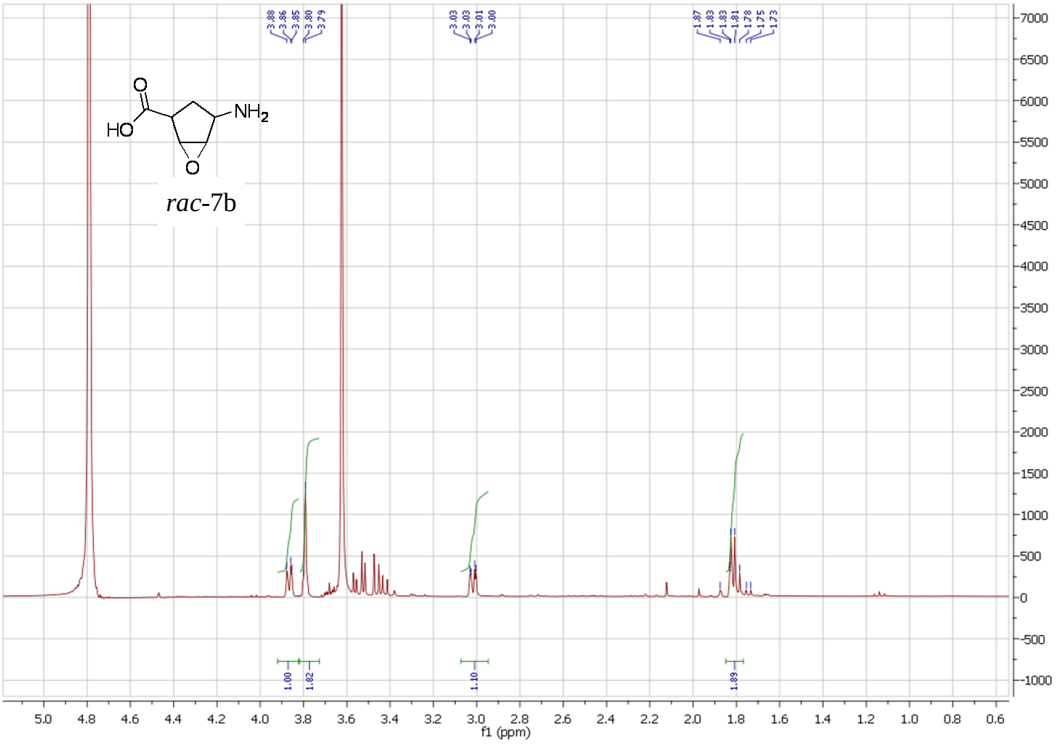

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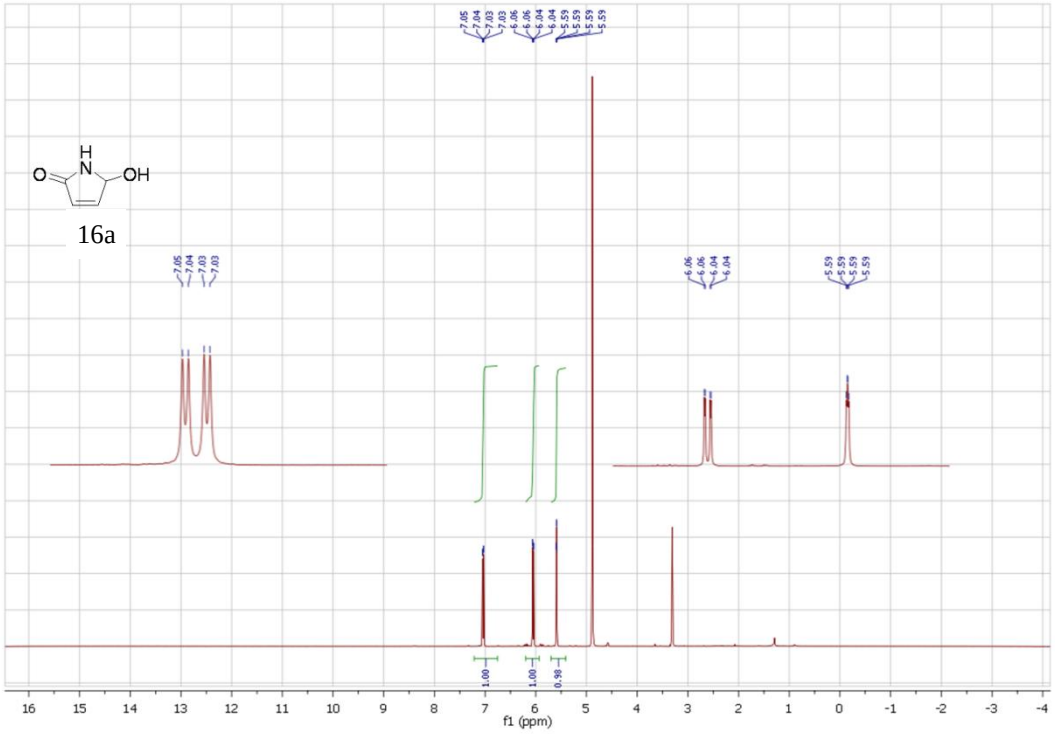

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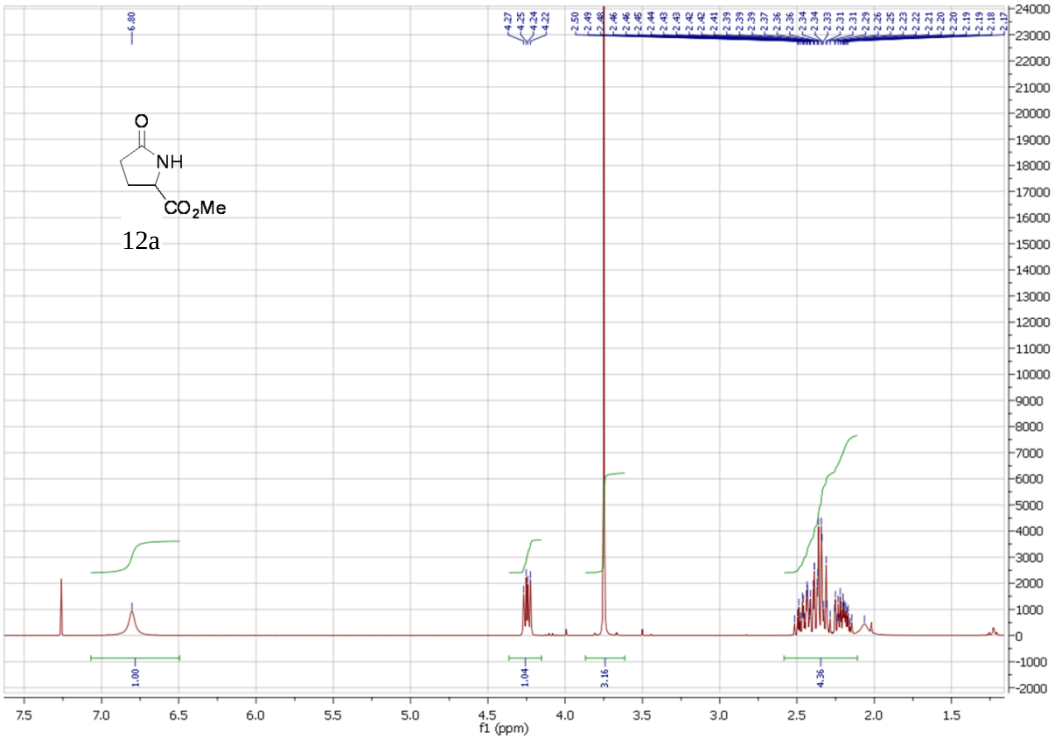

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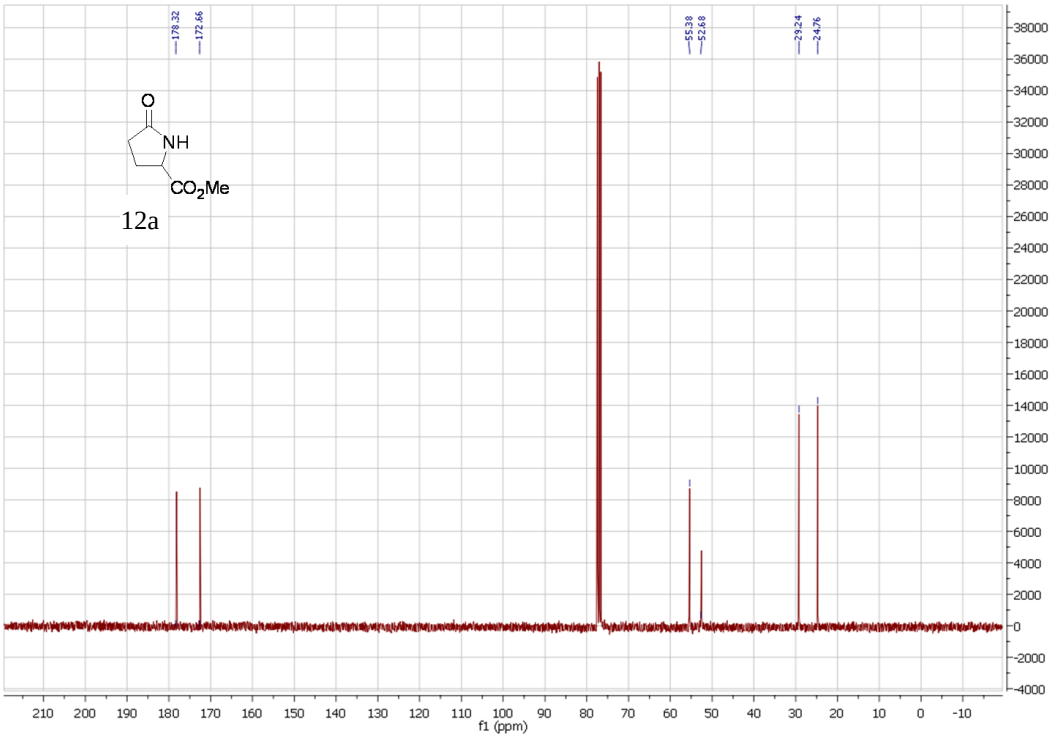

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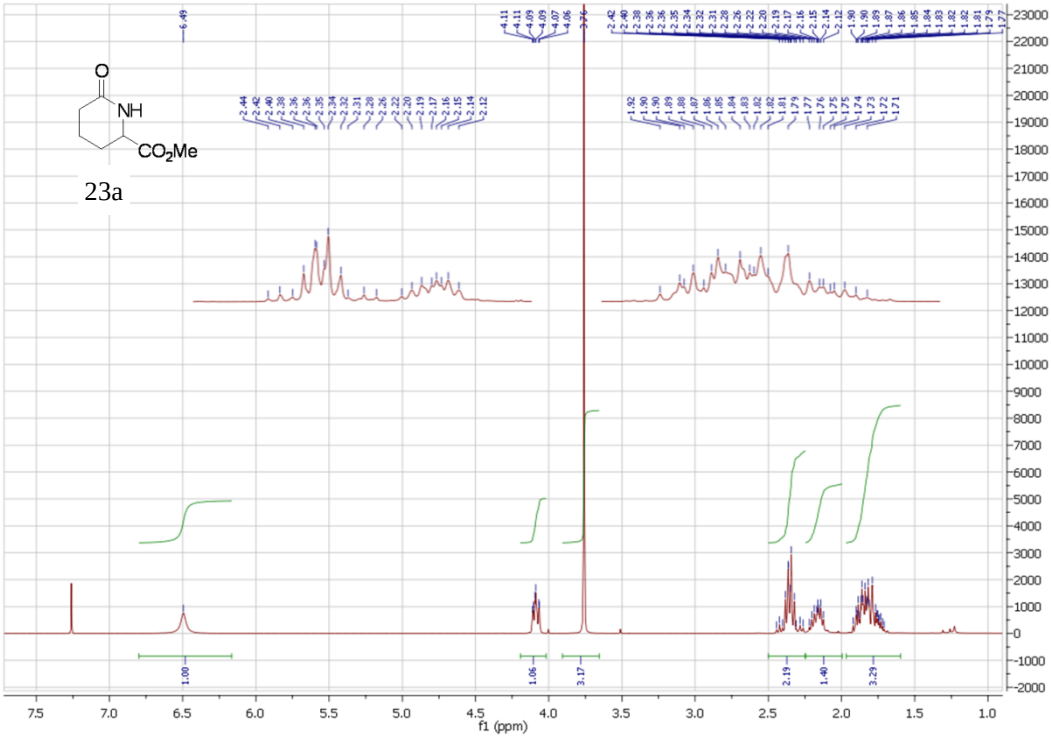

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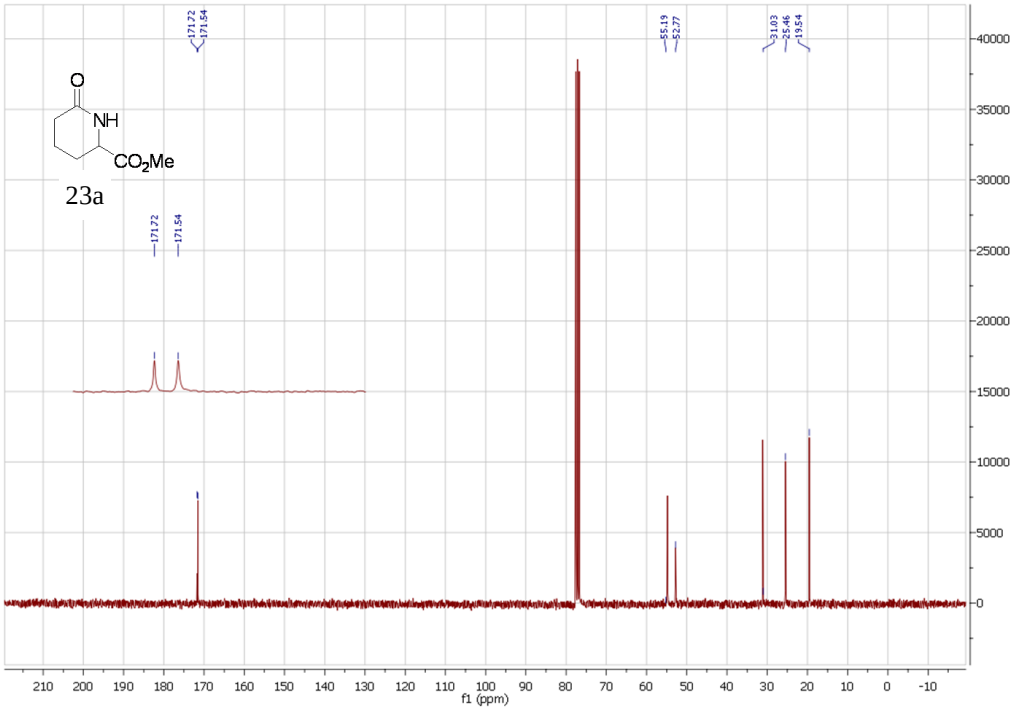

Abbreviations

aa	amino acid(s)
Ac_2O	acetic anhydride
AMI	amidase from *Rhodococcus globerulus*
Asp	aspartic acid
AS sequence	amidase signature sequence
BLAST	basic local alignment search tool
Boc	tert-butoxycarbonyl, protection group
$CeCl_3$	cerium(III) chloride
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CH_2I_2	diiodomethane
CIH	cyclic imide hydrolase from *Pseudomonas putida*
^{13}C NMR	carbon-13 - nuclear magnetic resonance
m-CPBA	*meta*-chloroperoxybenzoic acid
CPO-T	chloroperoxidase T from *Streptomyces aureofaciens*
D_2O	deuterated water
DCM	dichloromethane
DMAP	4-dimethylaminopyridene
DMF	dimethylformamide
E	enantiomeric ratio
ee	enantiomeric excess
Et_2O	diethyl ether
Et_2Zn	diethyl zinc
EtOAc	ethylacetate
G	glycine, 1-letter code
GABA	γ-aminobutyric acid
GC-FID	gas chromatography with flame ionization detector
GC/MS	gas chromatography - mass spectrometry
Gln	glutamine
Gly	glycine
HCl	hydrochloric acid
H_2	hydrogen
His	histidine
^1H NMR	proton/hydrogen-1 - nuclear magnetic resonance
kDa	kilo-Dalton
$KHSO_4$	potassium bisulfate
LC/MS	liquid chromatography - mass spectrometry
m/z	mass-to-charge ratio
MAE2	malonamidase E2 from *Bradyrhizobium japonicum*
MeOH	methanol
Met	methionine
MHz	megahertz
$NaBH_4$	sodium borohydride
NaH	sodium hydride
Na_2SO_4	sodium sulfate
NfpolyA	polyamidase from *Nocardia farcinica*
NH_4Cl	ammonium chloride
NMO	*N*-methylmorpholine *N*-oxide
OsO_4	osmium tetroxide

Pd/C	palladium on activated charcoal
$Pd(OAc)_2$	palladium(II) acetate
rac	racemic
rpm	rounds per minute
S	serine, 1-letter code
Ser	serine
TBA	*tert*-butyl alcohol
TBAF	tetra-*n*-butylammonium fluoride
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
$TMS\text{-}CHN_2$	trimethylsilyl diazomethane
t_R	retention time
Tyr	tyrosine

CURRICULUM VITAE

PERSONAL DATA

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EDUCATION

2011-present	Master Study Biotechnology, TU Graz
	Master thesis: "Development of a biocatalytic platform for the enantioselective hydrolysis of γ- and δ-lactams", Prof. Kurt Faber, Institute of Organic and Bioorganic Chemistry, University Graz
2008-2011	Bachelor Study Chemistry, University Graz and TU Graz
	Bachelor thesis: "Characterization of 3CNE from *Bacteroides thetaiotaomicron*", Prof. Peter Macheroux, Institute of Biochemistry, Graz University of Technology
2004-2008	High School in Deutschlandsberg

EXPERIENCE

Summer 2012	Summer student and project laboratory course "Biocatalysis"
	"Ene-reductases", Prof. Kurt Faber, Institute of Organic and Bioorganic Chemistry, University Graz
Summer 2011	Project laboratory course "Bioanalytics"
	"Characterization of a flavoprotein", Prof. Peter Macheroux, Institute of Biochemistry, Graz University of Technology

LANGUAGE SKILLS

German (native), English (fluent in written and spoken), Latin

PUBLICATIONS

(1) T. Knaus, E. Eger, J. Koop, S. Stipsits, C. L. Kinsland, S. E. Ealick, P. Macheroux, *Reverse structural genomics: An unusual flavin binding site in a putative protease from Bacteroides thetaiotaomicron*, *J. Biol. Chem.* **2012**, *287*, 27490 – 27498

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(3) Z. Assaf, E. Eger, D. Ribitsch, G. Gübitz, M. Hall, K. Faber, Development of a Biocatalytic Platform for the Stereoselective Hydrolysis of γ-Lactams for Pharmaceutical Applications, Poster, 11th International Symposium on Biocatalysis and Biotransformations (Biotrans), July, 21-25, **2013**, Manchester, UK.

(4) Z. Assaf, E. Eger, Z. Vitnik, W. M. F. Fabian, D. Ribitsch, G. M. Guebitz, K. Faber, M. Hall, *Identification and Applications of Enantiocomplementary Lactamases for Vince Lactam Derivatives*, manuscript under revision