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Studies towards Reductive Cyclization using Engineered Ene Reductases

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AFFIDAVIT

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Die vorliegende Arbeit wurde unter der Betreuung von Univ.-Prof. Dipl.-Ing. Dr. rer. nat. Rolf Breinbauer in der Zeit von März bis Dezember 2016 im Fachbereich Chemie am Institut für Organische Chemie der Technischen Universität Graz angefertigt.

» Wo damals die Grenzen der Wissenschaft waren, da ist jetzt die Mitte. «

Georg C. Lichtenberg

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Abstract

Asymmetric reductive cyclization reactions represent an interesting access to cyclic structures bearing several stereogenic centers. During this work, enoate reductases were employed in an attempted biocatalytic reductive cyclization.

New substrates suitable for biocatalytic reductive cyclization were synthesized within this work to further investigate the substrate scope of this biocatalytic transformation. The attempted formation of five-membered rings should be facilitated by employing the *gem*-dimethyl effect and ring substitution. The synthesized substrates were investigated in enzymatic transformations.

Kurzfassung

Asymmetrische reduktive Zyklisierung stellt eine interessante Möglichkeit zur Synthese von zyklischen Strukturen dar, welche stereogene Zentren aufweisen. Im Zuge dieser Arbeit wurden Enoat-Reduktasen zur biokatalytischen reduktiven Zyklisierung eingesetzt.

Neue Substrate für die biokatalytische reduktive Zyklisierung wurden im Rahmen dieser Arbeit synthetisiert, wodurch der Substratumfang dieser biokatalytischen Transformation untersucht werden soll. Die angestrebte Bildung von Fünfringen soll durch die Anwendung des geminalen Dimethyleffekts oder Ringsubstitution erleichtert werden. Die synthetisierten Substrate wurden in enzymatischen Transformationen untersucht.

1 Introduction

Complementing classical catalytic methods in organic chemistry, biocatalysts represent an upcoming class of catalysts, particularly when it comes to the preparation of complex organic molecules by the use of enzymes. It is not only possible to control the formation of a stereogenic center, but also to perform transformations in a highly stereo- and regioselective fashion under exceptionally mild reaction conditions. Furthermore, this high selectivity and efficiency makes biocatalysts more environmentally compatible than their chemical counterparts.[1]

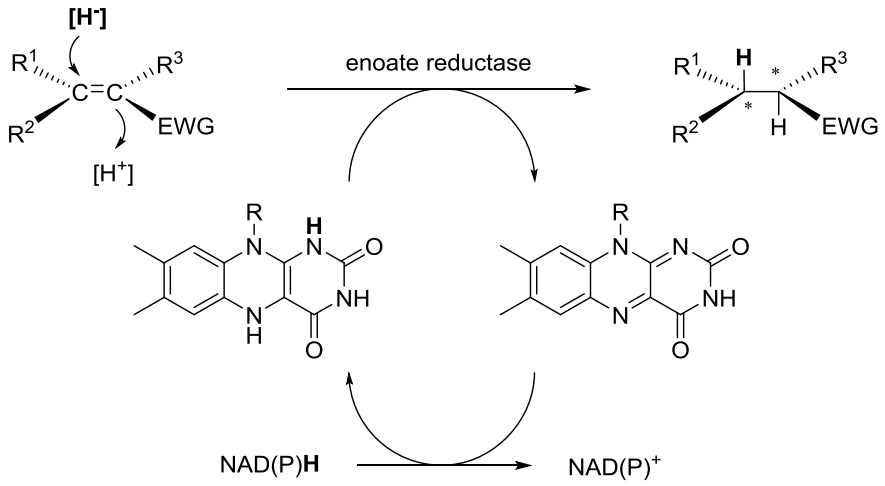
Biocatalysis has experienced continuous progress and three waves of technological progress can be observed during the last century.[2]

During the first wave, already more than 100 years ago, it was recognized, that parts of living cells can be applied for transformations of diverse chemical compounds. One of the early examples was the synthesis of (*R*)-mandelonitrile from benzaldehyde (Rosenthaler, 1908).[2] During the second wave (1980s – 1990s), first protein engineering techniques were used to influence the substrate tolerance of enzymes, thus making the synthesis of uncommon intermediates possible. The range of biocatalytic reactions could be therefore broadened towards production of pharmaceutical precursors, herbicides,[3] cosmetic constituents,[4] and polymer precursors.[5] Adapting the biocatalyst for non-natural substrates was the major challenge during this period. Yet, a third revolutionary wave was started by the establishment of directed evolution in the 1990s.[6–8] Through this methodology it was possible to randomly generate mutants, which were further screened for variants exhibiting improved properties (stability, substrate specificity, enantioselectivity). These major developments now permit the design of a certain enzyme, which perfectly fits for a distinct process. Intelligent design of engineered enzymes can thus even allow for non-natural reaction pathways.[2]

2 Theoretical Background

2.1 Enoate reductases

Asymmetric reduction of C=C double bonds represents a powerful method for the preparation of chiral molecules, as it is accompanied by the formation of up to two new stereogenic centers. Transition-metal catalyzed *cis*-hydrogenation has been brought to remarkable success, not least through the work of Noyori and Knowles, who were rewarded with the 2001 Nobel Prize for Chemistry.[9,10] However, the complementing biocatalytic reduction of activated alkenes by enoate reductases is known to proceed in a *trans*-fashion. The catalytic mechanism has been studied in great extent,[11,12] comprising a 'reductive' and 'oxidative half reaction'. During the 'reductive half reaction', oxidized FMN gets reduced at the expense of NAD(P)H and is therefore capable of donating a hydride to C-β of the activated alkene ('oxidative half reaction'). A proton is finally abstracted from a highly conserved tyrosine residue on the opposite face (Scheme 1).[13] Due to this MICHAEL-type mechanism of hydride addition, non-activated alkenes proved entirely unreactive.[14]

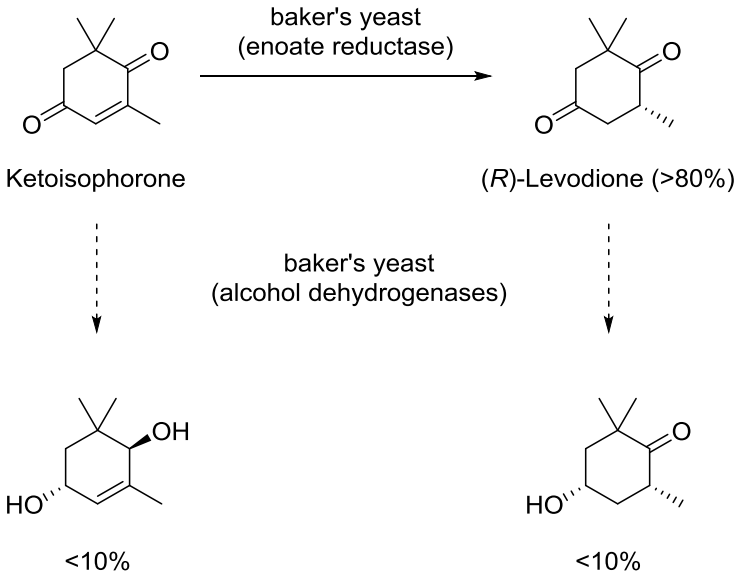


Scheme 1 Asymmetric reduction of C=C double bonds, activated by an electron withdrawing group (EWG = aldehyde, ketone, carboxylic acid, ester, imide, nitro…; R = ribityl phosphate) by enoate reductases.[13,15]

Enoate reductases are ubiquitously appearing in nature, and involved in diverse pathways with the role to reduce activated alkenes. Examples are morphinone reductase (morphine biosynthesis),[16] 12-oxophytodienoic acid reductase (jasmonic acid biosynthesis),[17] and

enoyl reductase (fatty acid biosynthesis).[18] Beside this common reactivity of enoate reductases, also other reactions are found to be catalyzed by ERs, such as reductive cleavage of nitroesters (glycerol trinitrite reductase),[19] reduction of aromatic nitro-groups,[20] or direct reduction of electron-deficient aromatics (e.g. 2,4,6-trinitrotoluene (TNT)) yielding a (non-aromatic) MEISENHEIMER complex.[21] Thus, this remarkable activity towards xenobiotics plays an important role during detoxification processes and oxidative stress response.[22]

The advantages of using whole cells for bioreduction, namely no need for an external cofactor recycling system or extensive protein purification, are narrowed by the loss of chemoselectivity and yield resulting from the variety of other present enzymes. The most prominent unwanted side-reaction is C=O reduction to the corresponding alcohol by ADHs, which leads either to depletion of starting material by formation of allylic alcohols, or depletion of product through over-reduction to the corresponding saturated alcohol.[14,22] However this problem of competing side-reactions has been overcome in many cases, one prominent example being the industrial production of (*R*)-levodione, a chiral intermediate in the synthesis of carotenoids[23] (Scheme 2). The desired diketone species could be obtained in >80 % yield through *in situ* product crystallization, thus preventing additional reduction by *sec*-alcohol dehydrogenase to the corresponding saturated *sec*-alcohol.[24]



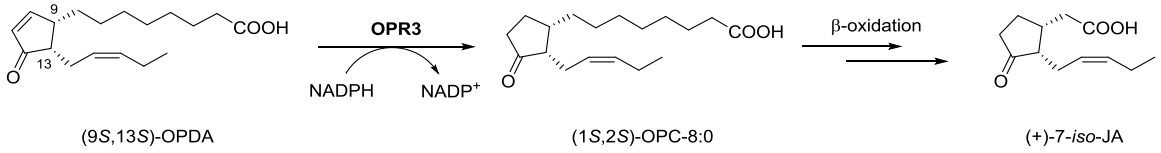
Scheme 2 Asymmetric biocatalytic reduction of ketoisophorone by use of baker's yeast on industrial scale.[22]

Undesired side-reactions encountered during whole-cell bioreduction can be circumvented by the use of isolated enzymes, which is often accompanied by an increase in yield and chemo- and stereoselectivity.[22] The lack of a cofactor recycling system in isolated enzyme preparations is often resolved by the use of 'designer bugs', where the corresponding redox enzyme is coexpressed with the requested ene reductase in the same host. Kataoka *et al*.[25] successfully coexpressed GDH together with OYE from *Candida macedoniensis* in *E.coli* for the purpose of (*R*)-levodione preparation (as discussed above), only to name one example. Compared to the whole-cell reduction, reactivity was increased 100-fold and C=O reduction could be eliminated. It should be also denoted that Hall *et al*. described the potential influence of the used cofactor recycling system on stereoselectivity.[13]

Besides the common substrates known to be accepted by enoate reductases (e.g. α,β-unsaturated carbonyl derivatives), also α,β-unsaturated carboxylic acids (*Chlostridium* spp.),[26] α,β-unsaturated β-substituted γ-lactones (baker's yeast),[27] acid anhydrides (*Marchantia polymorpha*),[28] or thiazolidine-2,4-dione bearing an exocyclic alkene (*Rhodotorula* or *Rhodosporidium* spp.)[29] were reported to be accepted as substrates.

2.1.1 12-Oxophytodienoate reductase (OPR) 3

In 1999, OPR3 was identified as the OPR isoenzyme involved in jasmonate biosynthesis by Schaller.[17] Jasmonates comprise a class of lipid-derived compounds, which are not only relevant in stress response or defense mechanisms but also regulate plant growth and development.[30] As illustrated in Scheme 3, OPR3 catalyzes the reduction of (9*S*,13*S*)-12-oxophytodienoic acid (OPDA) to 3-oxo-2-(2'(*Z*)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), which eventually undergoes β-oxidation to yield jasmonic acid (JA).



Scheme 3 OPR3 catalyzed reduction of (9*S*,13*S*)-OPDA to (1*S*,2*S*)-OPC-8:0.[31,32]

The oxylipin OPDA originates from the plastids, where it is synthesized from α-linolenic acid.[17] In a first step, α-linolenic acid gets oxidized by a lipoxygenase (LOX) to yield the corresponding 13-hydroperoxy species. This hydroperoxide is further transformed into a rather instable allylic epoxide by allene oxide synthase (AOS).[30] In aqueous environment

this allene oxide undergoes either spontaneous hydrolysis (α- and γ-ketol species) or cyclization to *rac*-OPDA.[33] However, when allene oxide cyclase (AOC) is present, mainly one enantiomer ((9*S*,13*S*)-OPDA) is formed.[34] This cyclic intermediate gets further transferred to the peroxisome, where the last steps of JA biosynthesis occur. The cyclopentenone moiety of OPDA gets then reduced by OPR3 to yield OPC-8:0. Subsequently, OPC-8:0 gets activated *via* formation of the corresponding CoA ester before entering β-oxidation, which finally yields jasmonic acid.

Interestingly, OPR3 turned out to be the only member of the OPR isoenzyme family being able to reduce the naturally occurring (9*S*,13*S*)-enantiomer.[17,35] Elucidation of the crystal structure of OPR1 and OPR3 gave new insights regarding the reaction mechanism and strongly differing stereoselectivity of the two isoenzymes.[31,36] OPR3 was found to accept all four isomers of ODPA, which is in sharp contrast to OPR1, which could only reduce non-natural (9*R*,13*R*)-OPDA. In OPR1, entry to the active site is restricted by tyrosine residues (Tyr78 and Tyr246), whereas in OPR3, the active site is better accessible, thus explaining the broader tolerance towards OPDA isomers. Furthermore, to the surprise of Breithaupt *et al*., OPR3 forms self-inhibiting dimers. It was suggested that the equilibrium of mono- and dimer is controlled by reversible phosphorylation and therefore regulates enzyme activity.

2.1.2 YqjM from *Bacillus subtilis*

Since the discovery of yeast OYE in 1932[37] many members of the OYE family were identified. However, their physiological function remains still elusive, one rare exception being OPR3 (see section 2.1.1). YqjM, a protein isolated from *Bacillus subtilis*, was characterized in the last decade and showed remarkable similarities in biochemical properties to yeast OYE, albeit it was the first OYE homolog which has been isolated from bacterial origin.[38]

B. subtilis is an aerobic, Gram-positive bacterium, ubiquitously found in soil and water sources. *Bacillus subtilis* is a well-studied organism with many industrial applications, such as production of hydrolytic enzymes, insecticides, or fine chemicals.[39]

As common throughout the OYE family, YqjM possesses a tightly, but non-covalently bound FMN cofactor and NADPH is the preferred source of reducing equivalents. YqjM is

capable of reducing several xenobiotics (e.g. NG or TNT) as well as other substrates common for OYE family members (e.g. α,β-unsaturated compounds), suggesting involvement of YqjM in oxidative stress response mechanisms. This assumption was supported by the work of Fitzpatrick *et al*., where YqjM expression was found to be highly up-regulated by addition of xenobiotic compounds, especially by TNT, but also by H_2O_2, mimicking oxidative stress.[38] When considering the easily accessible active site, specification to a distinct substrate is unlikely, therefore allowing for a great variety of compounds, which is beneficial in terms of combating general oxidative stress in the cell.[40]

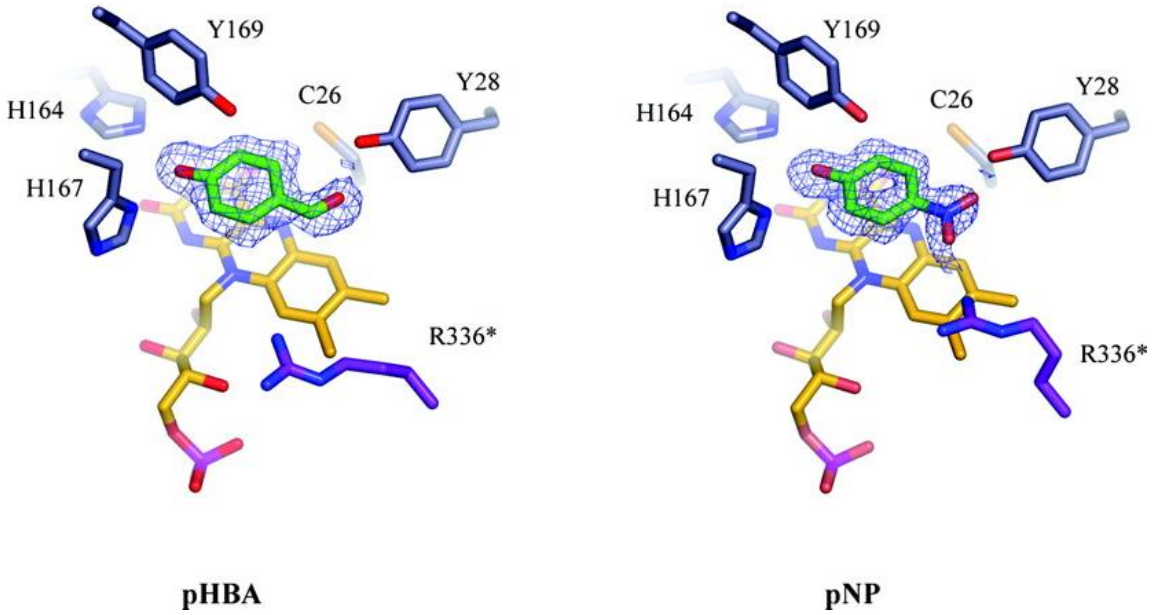


Figure 1 Substrate binding pocket of YqjM – *p*-hydroxybenzaldehyde (pHBA) or *p*-nitrophenol (pNP) (green), FMN cofactor (yellow) and relevant amino acid residues (blue) in the active site of YqjM (Picture taken from Ref. [40]).

In contrast to all other OYE homologs, YqjM is assembled as homotetramer (dimer of active dimers). Elucidation of the crystal structure of YqjM exposed even further structural differences. His164 and His167 are, in analogy to other OYE enzymes, properly positioned to activate substrates upon coordination. Tyr169 serves as proton donor, comparable to Tyr190 in OPR3 active site. In addition to these common residues, Tyr28 turned out to coordinate the aldehyde moiety of pHBA for proper alignment. In the case of pNP, Arg336*, which belongs to the adjacent subunit of the enzyme dimer, forms an extra

H-bond to the nitro moiety (Figure 1). Thus, Tyr28 and Arg336* seem to provide a second binding pocket for some functional group, which is ideally slightly electronegative and positioned *vis-à-vis* to the carbonyl function.[40]

2.2 Five-membered ring systems

2.2.1 Influences on ring formation

The effective molarity (EM) quantitatively describes the ease of ring-formation, as defined in equation (1), where k_{intra} and k_{inter} represent the rate constants for the intramolecular and intermolecular reaction, respectively. It can be also described as intramolecular reactivity with respect to the intrinsic reactivity of the end groups.[41]

$$EM = k_{intra}/k_{inter} \quad (1)$$

For bifunctional molecules, the effective molarity describes also the concentration of substrate, where k_{intra} (cyclization) equals k_{inter} (polymerization).

In a kinetic study performed by Casadei *et al.*, cyclization of a series of diethyl (ω-bromoalkyl)malonates was investigated.[42] They revealed, that formation of five-rings proceeds more than 1400 x faster than four-ring formation or over 800 x faster than formation of six-rings. Only formation of three-membered rings proceeds even faster.[43]

Ruzicka hypothesized, that the ring-strain and plausibility of end-to-end reaction of a linear precursor are factors independent of each other regarding a cyclization reaction.[41,44] Thus, the ring strain should be reflected in the activation energy for ring-formation (enthalpic share) and the probability of end-to-end reaction represents the entropic contribution. This probability diminishes when the chain gets longer, so that the influence of entropy dominates in the formation of larger rings. In terms of the enthalpy of activation, a correlation with the strain energy of the formed ring is not obtained for all ring-sizes.[41]

2.2.1.1 Influence of the ring strain

Already in 1885, Baeyer described that the angles present in a regular pentagon (108°) are very similar to the tetrahedral angle of 109.5°.[45] Resulting from that, the angle strain present in a planar five-ring should be rather low. However, torsional strain is significant, due to the eclipsed conformation of the hydrogens. The torsional strain is released to some

extent through formation of non-planar conformations. The envelope (four carbon atoms coplanar) and half-chair (three carbon atoms coplanar) conformations are only two of many possible geometries, which are similar in energy and rapidly interchange ('pseudorotation').[46,47]

Figure 2 The planar, envelope and half-chair conformations of cyclopentane.[46]

Considering the ring strain of cycloalkanes, six-membered rings should exhibit the highest ease of formation (Table 1). Nevertheless, three- and four-membered rings are also relative easily formed, which is mainly due to the energetically favorable transition states.[42] Galli and Mandolini tried to explain this unusual behavior through reduction of non-bonded interactions, common during S_N2 reactions, in the transition state. This effect seems to be important for three-, four- and five-membered rings, but only marginal for six-membered rings.[41] This might also explain why five-membered rings often form more easily, than the less strained six-membered rings. During the formation of lager, strainless rings, ease of cyclization is solely dependent on entropic contributions.[48]

Table 1 Strain energies for selected cycloalkanes.[49]

Ring size	**Ring strain** [kcal/mol]
3	27.5
4	26.1
5	6.2
6	0.1

2.2.1.2 Thorpe-Ingold vs. reactive-rotamer effect

Generally speaking, the *gem*-dimethyl effect describes the rate acceleration of cyclization reactions, when *gem*-hydrogen atoms are replaced by alkyl groups at the backbone connecting the two reacting centers.[50] It is also commonly referred to as THORPE-INGOLD effect, but this postulation is only one of many hypotheses made towards the origin of the *gem*-dialkyl effect.

Speaking of the THORPE-INGOLD effect it should be properly described as angle change resulting from substitution. In 1915, Thorpe and Ingold suggested that upon exchange of *gem*-hydrogens against alkyl groups the internal angle θ gets compressed due to repulsion of the substituents (see Figure 3, $\theta_1 > \theta_2 > \theta_3$).[51] The alteration of the angle θ brings the reactive groups closer together, thus promoting cyclization. However, the strain relief and stabilization upon alkyl substitution accounts only for the formation of small rings (3- to 4-membered) but can be disregarded during formation of larger rings.[50]

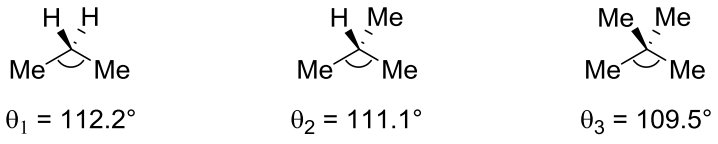


Figure 3 Thorpe-Ingold effect.[50]

In 1960, Bruice *et al*. described another model for explaining the rate enhancement upon introduction of *gem*-dialkyl substituents, the so-called 'reactive-rotamer effect'.[52] They suggested, that rotation about a C-C bond is necessary to approximate the two reactive groups (X, Y) for successful reaction. It is therefore necessary to change the conformation from the most stable *anti*-conformer to the less favored *gauche*-conformer (reactive rotamer). However, when one or two *gem*-hydrogens are exchanged against other substituents (R^1, R^2), the *anti*-conformation is no longer energetically favored over the *gauche*-conformation, so that cyclization is facilitated by the increased population of reactive rotamers (Figure 4).

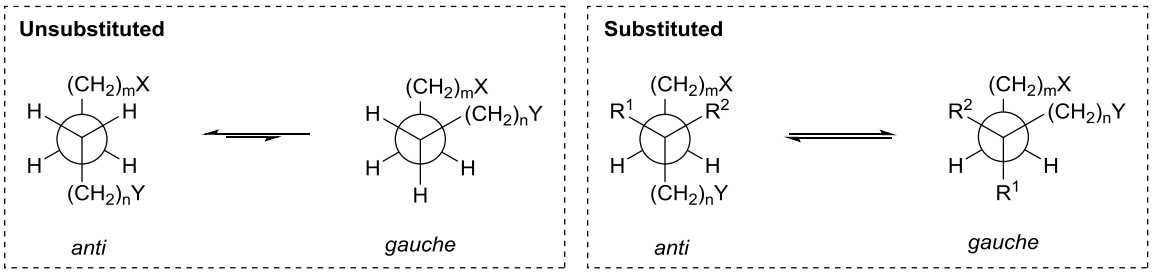
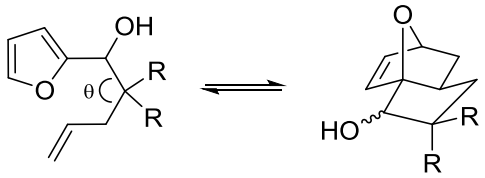


Figure 4 Reactive-rotamer effect.[50]

The relevance of the THORPE-INGOLD effect and reactive-rotamer effect is still under debate. However, an intramolecular DIELS-ALDER reaction served as model reaction during investigations towards the influence of both effects. Sternbach *et al*. noted, that substitution

was necessary for successful reaction and that heteroatom-bearing substituents have an additional beneficial effect (Scheme 4). However, X-ray structure analysis exposed that the internal angle θ, as well as the opposite angle, both comprised more than 109.5°. This result obviously disqualified the THORPE-INGOLD effect, and suggested the major influence of the reactive-rotamer effect.[53] These findings were also emphasized by the work of Jung *et al*.[54] The authors further proposed, that angle compression has only minor influence on the *gem*-dialkyl effect, particularly during formation of five-membered rings.



R = -H, -Me, $-CH_2(CH_2)_3CH_2-$, $-CH_2CH_2CH_3$, $-S(CH_2)_3S-$, $-SCH_2CH_3$, $-OCH_2CH_3$

Scheme 4 Influence of the substituents - Model-reaction investigated by Sternbach *et al*.[53]

2.2.2 Baldwin's rules

Baldwin published general rules for ring-closing reactions in 1976.[55] Based on empirical data as well as on stereochemical considerations, Baldwin proposed 'favored' and 'disfavored' types of ring-closure, depending on the nature of the attacked carbon atom (*tet* – tetrahedral (sp^3), *trig* – trigonal (sp^2), *dig* – diagonal (sp)). Additionally, he distinguished if the broken bond was inside (*endo*) or outside (*exo*) of the formed ring. The favored and disfavored cyclization reactions for five-membered rings are shown in Figure 5.

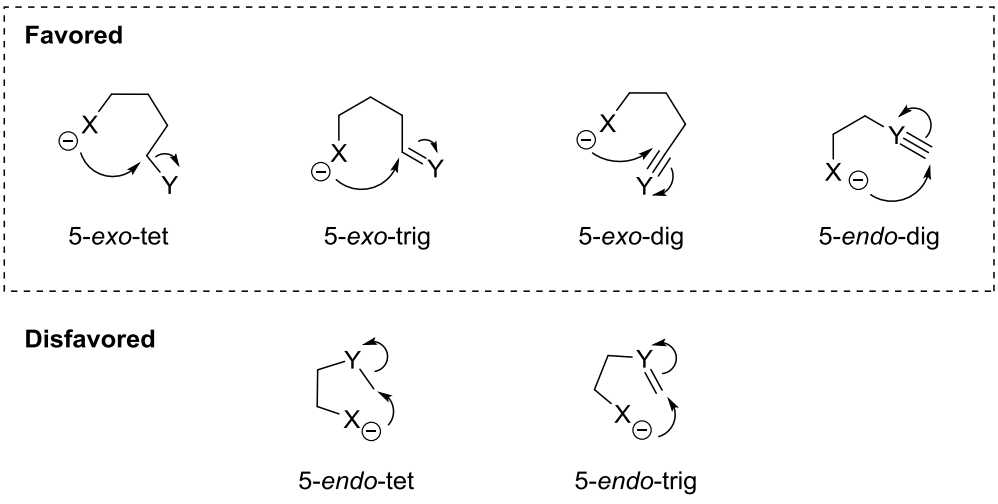


Figure 5 Favored and disfavored ring-closing reactions for five-membered rings.[55]

Depending on the hybridization of the attacked C-atom, the transition states have to fulfill distinct stereochemical requirements for successful cyclization. In the case of a S_N2 reaction (*exo*-tet) a 180° angle of attack is necessary for the nucleophile to interact with the C-Y σ^*-orbital. This type of attack proceeds via well-known WALDEN inversion. In the case of attack at a carbonyl group (sp^2, *trig*), Bürgi and Dunitz suggested a mandatory angle of 109°, known as Bürgi-Dunitz trajectory, necessary for interaction with the C=Y π^*-orbital.[56] For *endo*-dig type ring-closures, Baldwin suggested an acute angle of attack of 60°, which was later redefined by Alabugin and Gilmore to an angle of 120°.[57]

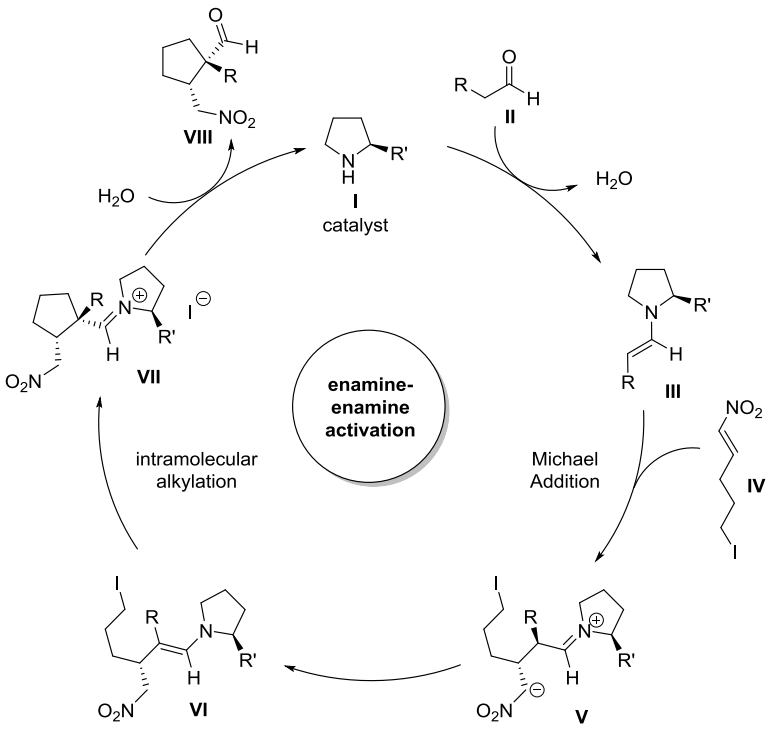
2.2.3 Selected chemical methods for five-membered ring formation

2.2.3.1 Organocatalytic asymmetric domino reaction

In 2008, Enders *et al*. reported an organocatalytic domino MICHAEL addition/α-alkylation between aliphatic aldehydes and nitroolefins *via* an enamine-enamine activation pattern. The highly stereoselective process delivers cyclic γ-nitroaldehydes bearing two stereogenic centers.[58]

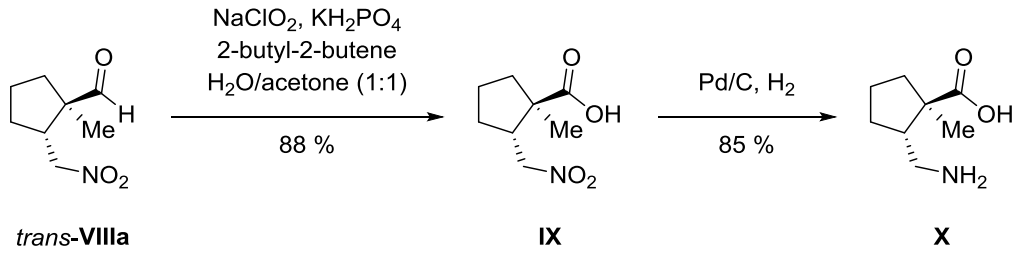
Diphenylprolinol silyl ether **I** proved to be a suitable chiral catalyst. Under optimized conditions (20 mol% catalyst **I**, 5 eq aldehyde, 100 mol% $PhCO_2H$ in DMSO at RT) various aliphatic aldehydes were tested. For all investigated aldehydes, the *cis*-diastereomer was predominately formed, except from propionaldehyde (moderately *trans*-selective). The desired cyclic γ-nitroaldehydes were formed in excellent enantiomeric excess of 93-97 % *ee*.

The proposed catalytic cycle for the formation of cyclic γ-nitroaldehydes **VIII** is shown in Scheme 5. In a first reaction step, aldehyde **II** gets activated through enamine formation. The activated aldehyde **III** undergoes MICHAEL addition with nitroolefin **IV**. After proton transfer, the second enamine species **VI** is generated, which reacts in an intramolecular nucleophilic substitution to yield a cyclic structure **VII**. The desired cyclic aldehyde **VIII** is then released by hydrolysis which furthermore recovers the catalyst.



Scheme 5 Proposed catalytic cycle for the domino reaction (R' = C-(OTMS)Ph_2).[58]

It was also possible to transform the cyclic γ-nitroaldehyde *trans*-**VIIIa** into the cyclic γ-amino acid **X** in two steps *via* PINNICK oxidation and reduction of the nitro moiety in good yields (75 %, Scheme 6). Such γ-amino acids might be interesting pharmaceutically active compounds (e.g. γ-aminobutyric acid (GABA) – inhibitory neurotransmitter).[58,59]

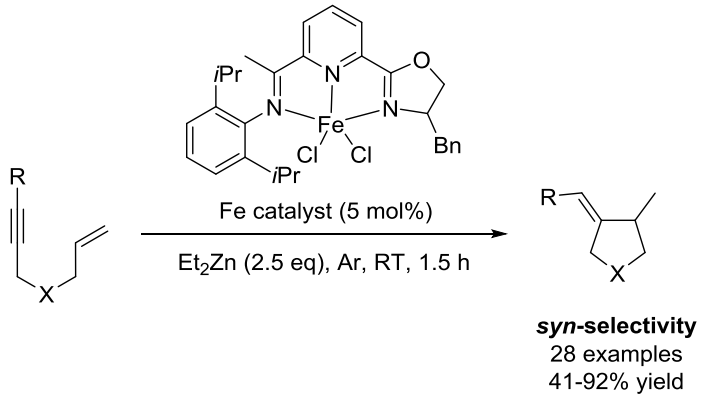


Scheme 6 Follow-up reaction – two-step synthesis of γ-amino acid **X** from the domino product *trans*-**VIIIa**.[58]

2.2.3.2 Reductive cyclization of 1,6-enynes catalyzed by Fe-complexes

Xi *et al*. studied the reductive cyclization of 1,6-enynes by employing an iron-catalyst, bearing an oxazoline iminopyridine ligand.[60] Already many examples were reported, where palladium, rhodium or ruthenium catalysts were used.[61–63] Fürstner was one of only few, who reported Fe-catalysts in the cyclization of 1,6-enynes.[64] However, a broad

spectrum of functional groups were either not tolerated or not investigated during earlier research. The catalytic system presented by Xi *et al*. consists of the enyne, the iron-pre-catalyst and Et_2Zn as reductant and is therefore particularly practicable (Scheme 7).



Scheme 7 Reductive cyclization of 1,6-enynes catalyzed by a Fe-complex (X = O, NR, $C(CO_2Et)_2$, CH_2O; R = H, alkyl, aryl, heteroaryl).[60]

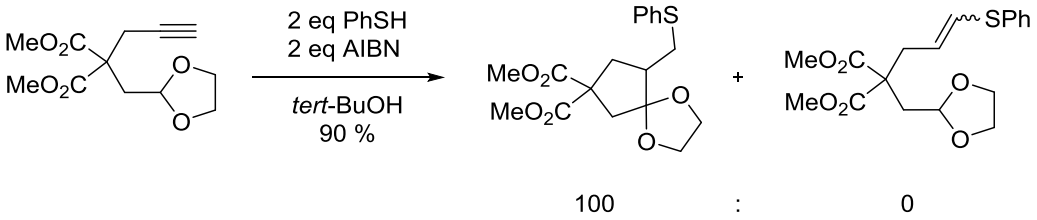
1,6-Enynes carrying oxygen-, nitrogen-, or carbon-moieties were accepted during the reaction, yielding tetrahydrofuran-, pyrrolidine-, or cyclopentane-derivatives, respectively. Under the considerably mild reaction conditions also a large variety of functional groups was tolerated (e.g. -OH, -CN, $-CF_3$, halogens, ketone, ester, amines, amides, imines). They further reported the first example of an asymmetric Fe-catalyzed reductive cyclization of a 1,6-enyne, albeit with rather low enantiomeric excess (39 % *ee*).[60]

2.2.3.3 Thiophenol-mediated hydrogen abstraction and radical cyclization

Alkenyl radicals undergoing cyclization to yield five-membered rings were described in 1967 by Heiba *et al*.[65] and thoroughly investigated by Curran.[66,67] The alkenyl radical is derived from the reaction of the bromine-species with a stannyl radical. 1,5-Hydrogen atom abstraction is followed by cyclization of the translocated radical (*via* 5-*exo*-trig). Subsequent reduction with Sn-hydride yields cyclopentane derived structures. However, formation of reduced, uncyclized product in significant amounts and tin contamination display serious drawbacks of this strategy.

For these reasons, Beaufils *et al*. reported a tin-free variant.[68] Radical addition of thiophenol (PhSH) to terminal alkynes gave the desired alkenyl radicals, which were further undergoing cyclization, yielding cyclopentane derived compounds (Scheme 8). Interestingly, only the formation of the cyclic compound was observed for a whole series

of substrates. With this thiophenol-mediated approach it was even possible to generate a non-stabilized *prim*-alkyl radical of dimethyl 2-*tert*-butyl-2-propagylmalonate, which yielded the corresponding cyclic product in 48 % yield.



Scheme 8 Investigated reaction in the domino hydrogen abstraction-cyclization.[68]

Compared to the classical tin-hydride approach, the use of toxic Bu_3SnH is prevented in this thiophenol-mediated methodology. Moreover, the so generated cyclopentane derivatives carry a thiophenyl moiety, which provides interesting possibilities for further transformations.[68]

3 Aim of this Thesis

Biocatalytic asymmetric C=C reduction is an interesting tool for the formation of two new stereogenic centers. Enzymes capable of catalyzing such type of reactions are NAD(P)H dependent enoate reductases, members of the 'old yellow enzyme' (OYE) family.[22] For successful reduction, an activated double bond is necessary (e.g α,β-unsaturated carboxylic acids, -esters, -ketones or –aldehydes).[69]

As the OPR isoenzymes and YqjM both belong to the OYE family, the binding mode of the substrate is also very similar. In Figure 6, the crucial amino acid residues of OPR1 and OPR3 are depicted. The binding situation in the active site of YqjM is comparable.[40] The His-residues are capable of activating the C=C double-bond by coordinating the carbonyl functionality *via* hydrogen bonds. The hydride gets delivered from the tightly bound reduced FMN cofactor (N(5)) to C-β, followed by subsequent protonation of C-α by the Tyr-residue from the opposite face.[31]

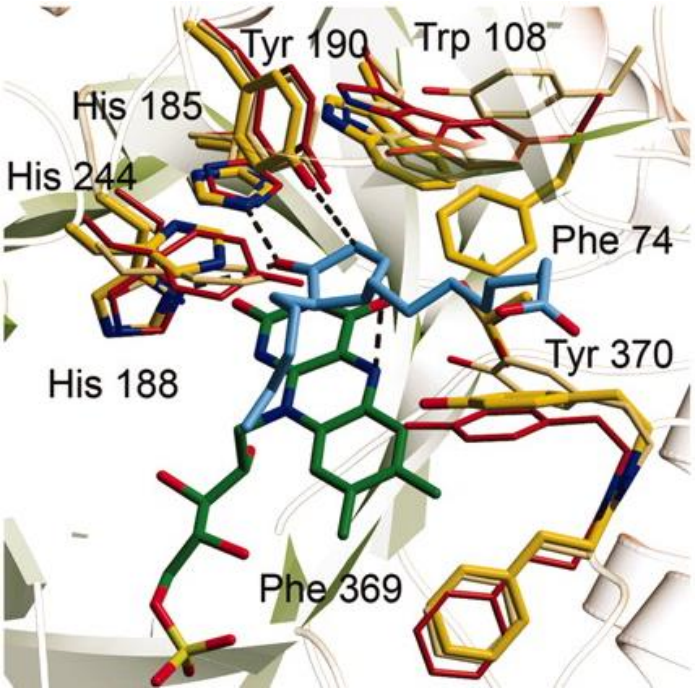
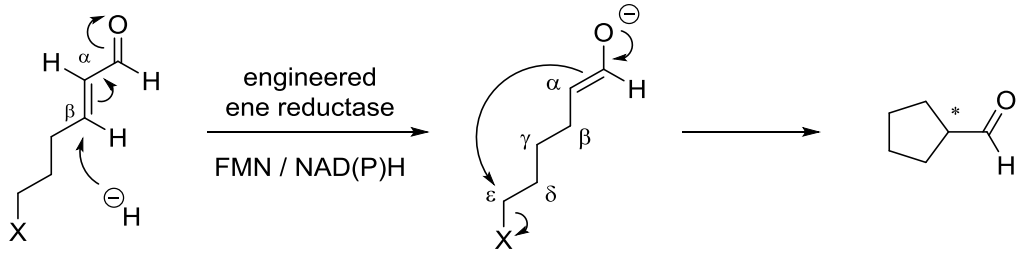


Figure 6 Substrate-binding pockets of OPR3 (yellow) and OPR1 (red). The FMN of OPR3 (green) and the OPR1 substrate 9R,13R-OPDA (blue) are shown to visualize the crucial interactions for catalysis (Picture taken from Ref. [31]).

The here employed rationale was previously introduced and applied by this working group, especially by Kathrin Heckenbichler. It proposes that through exchange of the proton-donating amino acid residue (Tyr) by an aprotic amino acid, it should be possible to suppress reprotonation and to drive the reacting system towards a cyclization reaction.

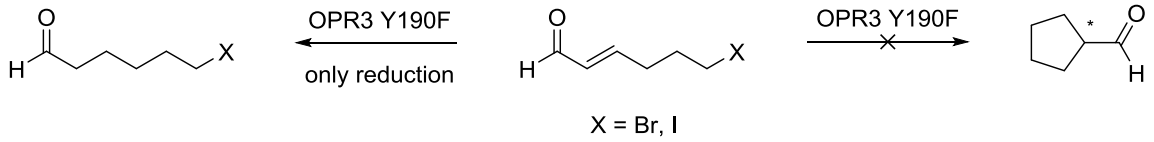
The proposed reaction mechanism suggests that the hydride [H^-] derived from the reduced FMN cofactor attacks C-β, whereupon enolization of the α,β-unsaturated system occurs. Instead of the subsequent protonation step by the tyrosine residue, the enolate should rather attack the intramolecular electrophilic carbon atom C-ε (see Scheme 9).



Scheme 9 Proposed reaction mechanism for reductive cyclization (X = Hal, OTf).

For this purpose, three enzyme variants and the corresponding wild type enzymes (OPR3 WT, YqjM WT) had been expressed and purified by Kathrin Heckenbichler. The 'critical' Tyr residue was exchanged by Phe or Trp in the case of OPR3 (OPR3 Y190F, OPR3 Y190W), and by Phe in the case of YqjM (YqjM Y169F).

In preliminary studies by Kathrin Heckenbichler[70] it could be shown, that the model substrate for five-ring formation, (*E*)-6-bromo-hex-2-enal, did not undergo cyclization, but only reduction. Even by enhancing the electrophilicity of C-ε, through exchange of bromine against iodine, the cyclization product was not observed (see Scheme 10).



Scheme 10 Preliminary studies towards reductive cyclization of (*E*)-6-bromo- and (*E*)-6-iodo-hex-2-enal.

The aim of this thesis was to synthesize more sophisticated structures which should facilitate ring closure, either by alteration of the conformational angle (*gem*-dimethyl effect), or by restriction of the molecules flexibility through attachment to a ring system (see Figure 7).

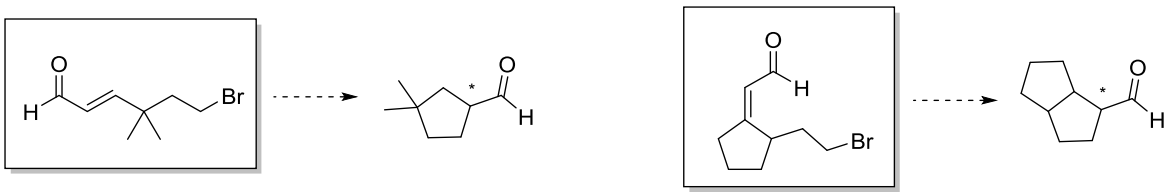


Figure 7 Substrates for reductive cyclization experiments.

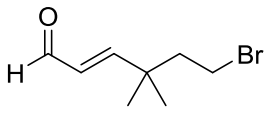
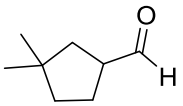
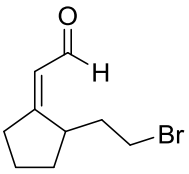
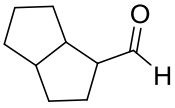
4 Results and Discussion

4.1 Substrate scope

The envisioned substrates for reductive cyclization should feature a reactive aldehyde moiety on the one hand, and bromine as leaving group on the other hand. Bromine and chlorine derivatives proved applicable, whereas the even more reactive iodinated and triflated compounds were often difficult to handle and prone to decomposition. In direct comparison, the bromine derivative would be preferred over the chlorine derivative in terms of enhanced reactivity.

Besides these general requirements, additional substituents were installed at the synthesized substrates, in order to facilitate ring closure. This was achieved by attachment of a dimethyl moiety at C-γ, to utilize the *gem*-dimethyl effect for enhancement of ring formation. Installation of another ring system should reduce the flexibility of the molecule and bring the involved moieties in close proximity (Table 2).

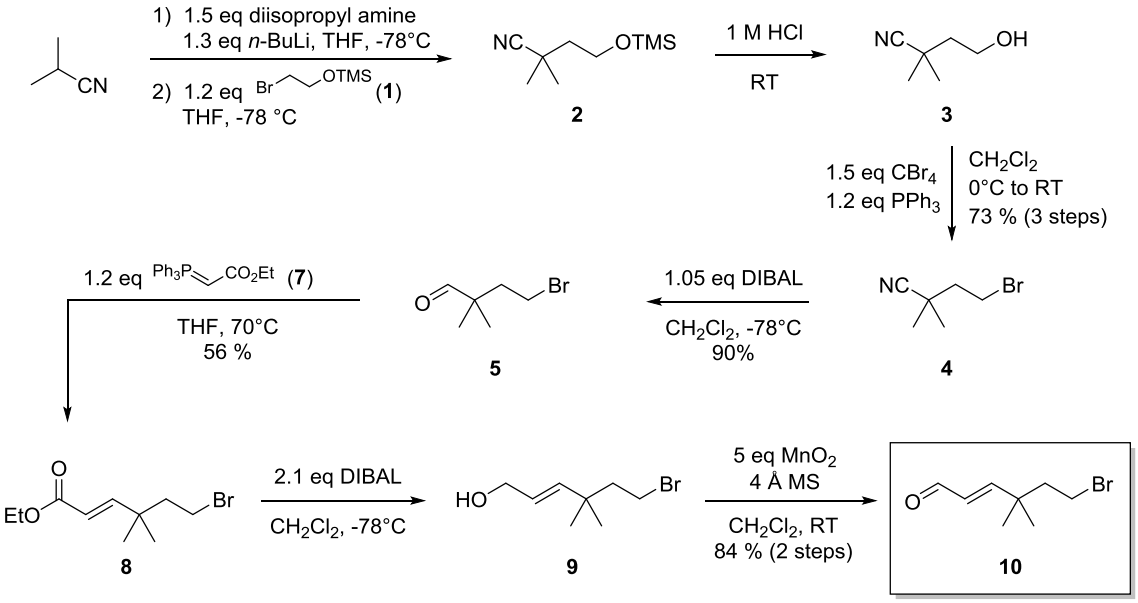
Table 2 Attempted substrates and expected cyclization products.

Substrate	Expected cyclization product
 **10**	
 **15**	

4.2 Synthesis of Substrates

4.2.1 (*E*)-6-Bromo-4,4-dimethylhex-2-enal (10)

(*E*)-6-Bromo-4,4-dimethylhex-2-enal (**10**) was synthesized in seven steps according to the sequence shown in Scheme 11.



Scheme 11 Synthesis of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**).

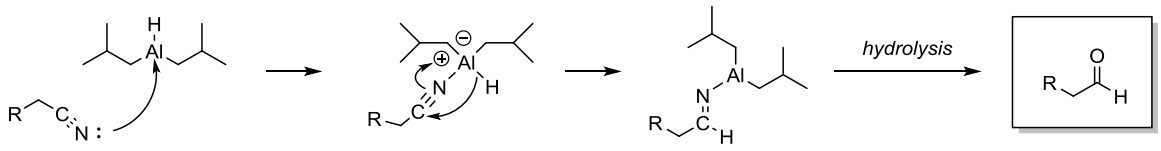
TMS-protected 2-bromoethanol **1** was prepared following a procedure from Kelly *et al*, which was adapted to our needs.[71] TMS-Cl (1.3 eq) was added to a solution of 2-bromo ethanol in CH_2Cl_2, Et_3N (2 eq) and DMAP (0.1 eq) at 0 °C. After 2 h completion was indicated by GC-FID. Standard aqueous workup was extended by additional treatment of the organic layer with 10 w% $CuSO_4$ solution, leading to complexation of the contained Et_3N and DMAP. This procedure was repeated until no more complexed amines precipitated. Further purification by flash chromatography, as indicated in literature, proved unnecessary.

Synthesis of compound **2** and **3** followed a procedure published by Coldham[72] Isobutyronitrile was deprotonated with LDA at -78 °C and subsequent addition of the previously synthesized TMS-protected 2-bromoethanol **1** led to the desired product 2,2-dimethyl-4-((trimethylsilyl)oxy)butanenitrile (**2**). After standard aqueous workup, GC-FID and NMR measurements indicated no further impurities, making the described purification

by column chromatography again obsolete. The deprotection step was realized by simply stirring the crude product **2** in aqueous HCl (1M) at RT for less than 1 h. Extraction of alcohol **3** proved to be challenging, as the obtained product was readily water-soluble. This problem was solved by saturation of the aqueous phase with NaCl before extraction. The crude product again did not require further purification.

Coldham *et al*. proceeded then with chlorination of the hydroxy moiety followed by reduction of the nitrile to the corresponding aldehyde. Instead of chlorine, we decided to introduce more reactive bromine by a simple APPEL reaction.[73] Alcohol **3** and CBr_4 (1.5 eq) were dissolved in CH_2Cl_2 and a solution of PPh_3 (1.2 eq) in CH_2Cl_2 was added at 0 °C. At this stage, for the first time in the reaction sequence, column chromatography was mandatory due to the formed triphenylphosphine oxide. Apart from that, no side products were observed during the APPEL reaction. 4-Bromo-2,2-dimethylbutanenitrile (**4**) could be isolated in satisfying 73 % yield over three steps.

Reduction of nitrile **4** turned out to be a particularly challenging task, not in terms of the reaction itself but concerning the workup. The reaction proceeded within minutes in a spot-to-spot fashion. It is thinkable, that by addition of excess DIBAL-H full reduction to the amine occurs. For that reason only a slight excess (1.05 eq) of DIBAL-H was used, although literature suggests, that the second reduction step is hardly ever observed.[74] The workup procedure as described by Coldham, namely quenching with HCl, failed due to formation of a colorless sludgy mixture, which could not be subjected to extraction. Also the use of saturated Rochelle salt solution for quenching the reaction was unsuccessful. After isolation, GC-FID measurement was inconclusive and the recorded ^{1}H-NMR spectrum did not show the desired aldehydic proton in the area of around 9 ppm, but a vast number of signals, which could not be assigned to either the starting material or the desired product. Considering the mechanism of DIBAL-H reduction of a nitrile (Scheme 12), the formed imine intermediate, which is stable only at low temperature, gets hydrolyzed during aqueous workup, releasing the corresponding aldehyde.



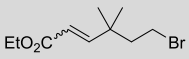
Scheme 12 Proposed mechanism for reducing a nitrile with DIBAL-H.

As workup with Rochelle salt solution was successfully applied for the reduction of esters, we speculated, that even more acidic conditions are needed to hydrolyze the formed imine intermediate during reduction of nitriles. Indeed, workup with tartaric acid solution (25 w%) was successful. As aldehydes represent very reactive and sensitive intermediates, compound **5** was prepared on the same day as needed for the next reaction step and long-term storage was avoided. Aldehyde **5** could be isolated in 90 % yield.

The next step in the reaction sequence was the olefination of the prepared aldehyde **5**. For this purpose two different types of olefination reactions were screened. The WITTIG reaction was performed by simply adding the readily prepared WITTIG-ylen **7** to a solution of aldehyde **5** in THF and subsequent heating under reflux (see Table 3, entry 1). In this first attempt, the desired ethyl ester (*E*)-**8** was obtained in 48 % yield after 39 h, although full conversion was not reached. The WITTIG-ylen **7** was prepared in two steps starting from 2-bromoacetate and triphenylphosphine. WITTIG-salt **6** was then deprotonated by addition of aqueous NaOH in H_2O and toluene. A crude yellow oil resulted from extraction. The desired product **7** was precipitated from this solution by addition of cyclohexane. The isolated colorless powder proved to be stable upon storage in the freezer. In parallel to the WITTIG reaction, HORNER-WADSWORTH-EMMONS (HWE) reaction was tested towards olefination of aldehyde **5** (see Table 3, entry 2). Triethyl phosphonoacetate (1.2 eq) in dry THF was deprotonated by use of potassium *tert*-butoxide (1.2 eq) at 0 °C. The crude, freshly prepared aldehyde **5** was again added at 0 °C before the cloudy mixture was heated to 70 °C. After 39 h under reflux, almost full conversion (97 %) was indicated by GC-FID. Subsequent aqueous work-up and column chromatography yielded the desired product in only 14 % (*E*). The reason for such low yield remained unclear, therefore the WITTIG reaction remained the method of choice for preparation of the α,β-unsaturated ethyl ester **8**. Yet, when it came to scale-up (see Table 3, entry 3), the reaction proceeded much slower and was stopped after 7 d (86 % conversion). Nevertheless, the desired ethyl (*E*)-6-bromo-4,4-dimethylhex-2-enoate (**8**) could be isolated in 56 % yield after column

chromatography. For both, the WITTIG and the HORNER-WADSWORTH-EMMONS reaction only the formation of one double-bond isomer was observed. According to the KARPLUS relation,[75] a vicinal coupling constant $^3J_{HH}$ of 15.9 Hz, as it was observed during ^{1}H-NMR experiments (doublets at 6.87 and 5.74 ppm), would correspond to a dihedral angle of about 180° and thus to the configuration present in the (*E*)-isomer.

Table 3 Screened conditions for the WITTIG reaction for synthesis of **8**.

Entry	Conditions	Scale	Base	Conv. [%][a]	 *E*:*Z* [a]	Isolated yield [%]
1	1.2 eq **7** THF, 70 °C	1 mmol	-	88 (39 h)	>99:1	48 (*E*)
2	1.2 eq **A** 0 °C then 70 °C	1 mmol	1.2 eq KO*t*Bu	97 (39 h)	>99:1	14 (*E*)
3	1.2 eq **7** THF, 70 °C	22 mmol	-	86 (7 d)	>99:1	56 (*E*)

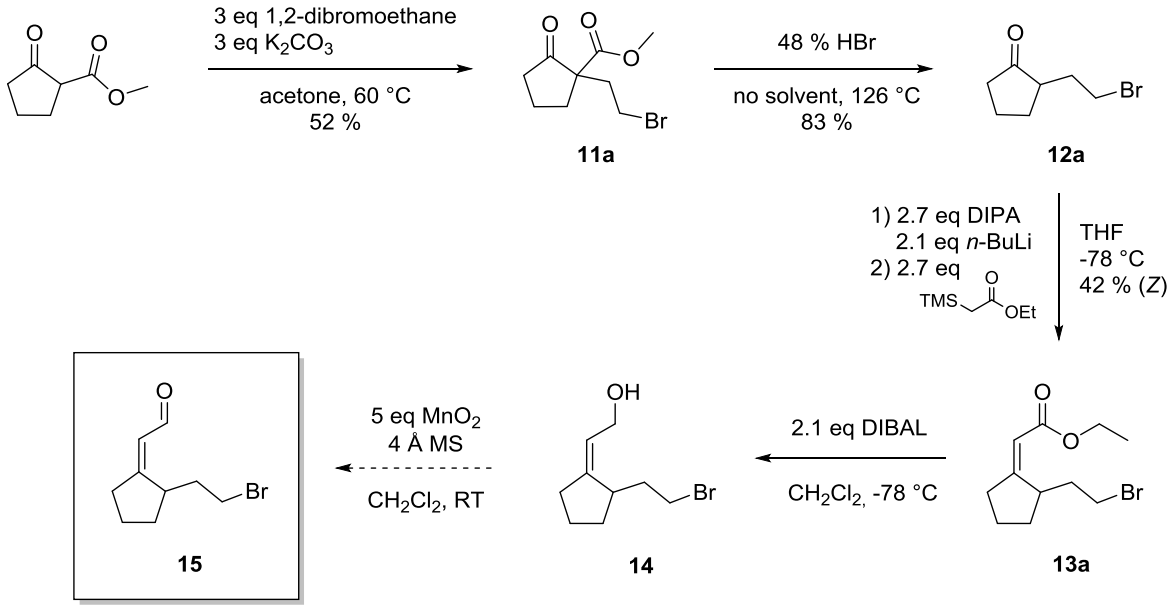
[a] Monitored with GC-FID.

The last two steps of the reaction sequence were carried out in a consecutive fashion, directly followed by the enzymatic screening due to instability of the prepared compounds. As the allylic alcohol **9**, as well as the allylic aldehyde **10** are prone to undergo light induced isomerization of the double bond, this two-step sequence was performed under exclusion of light. To a solution of ethyl ester **8** in dry CH_2Cl_2, DIBAL-H (2.1 eq) was slowly added at -78 °C. Typically after some minutes, quantitative conversion was detected by TLC and GC-FID. The reaction mixture was then quenched by addition of saturated Rochelle salt solution at -78 °C and was then allowed to warm to RT. Vigorous stirring was essential for facilitating phase separation of the formed emulsion, which, nonetheless, took several hours. Separation of the organic phase and extraction still proved to be difficult in some cases, so that the inseparable mixture was dried with Na_2SO_4 as a last resort. The obtained colorless and clear organic layer was directly transferred into a flame-dried reaction vessel equipped with 4 Å molecular sieves. After addition of activated MnO_2 (5 eq) the heterogeneous mixture was stirred overnight at RT. Quantitative conversion was again detected by either GC-FID or TLC (aldehyde only poorly stains with $KMnO_4$). The molecular sieves and MnO_2 were separated by simple filtration through a short pad of silica, which was rinsed with CH_2Cl_2. After evaporation of the solvent under reduced

pressure the obtained (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**) could be isolated in 84 % yield over the last two steps and was kept under a slight stream of N_2 until it was used for the enzymatic reaction.

4.2.2 (*Z*)-2-(2-(2-Bromoethyl)cyclopentylidene)acetaldehyde (15)

In Scheme 13 the attempted synthesis of (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene) acetaldehyde (**15**) over five steps is shown.



Scheme 13 Synthesis of (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetaldehyde (**15**).

Synthesis of methyl 1-(2-bromoethyl)-2-oxocyclopentane-1-carboxylate (**11a**) and 2-(2-bromoethyl)cyclopentan-1-one (**12a**) were prepared according to a procedure from Heimann[76] In a first reaction step, alkylation of methyl 2-oxocyclopentan-1-carboxylate was achieved by heating the substrate under reflux together with excess 1,2-dibromoethane and K_2CO_3 in acetone. In contrast to the suggestion in literature, acetone was not dried over 3 Å molecular sieves, but was only distilled on a rotary evaporator prior to use. When dried over molecular sieves one should be particularly careful, since self-condensation of acetone is promoted under these slightly acidic conditions, especially during extended storage times.[77] For the purpose at hand, distilled acetone turned out to be sufficient. Also a shorter reaction time of 5.5 h was enough to reach full conversion. The heterogeneous reaction mixture was then filtrated and concentrated, before excess 1,2-dibromoethane was removed by bulb-to-bulb distillation (10 mbar, 60 °C). After column chromatography, the

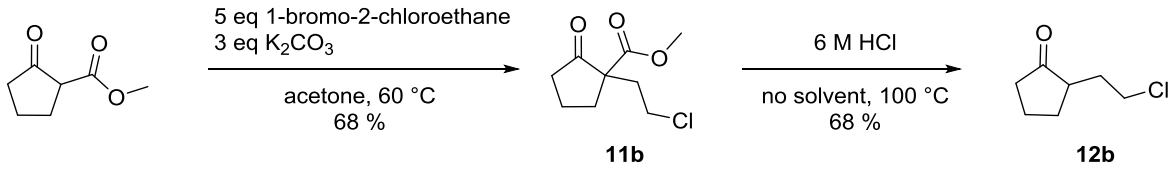
desired product **11a** was isolated in 52 % yield. Surprisingly after purification the obtained 1-(2-bromoethyl)-2-oxocyclopentane-1-carboxylate (**11a**) formed colorless crystals with a melting point between 37 °C and 39 °C, in contrast to descriptions in literature. As the formation of a second product was observed on TLC and also on GC-FID (in almost equal amounts), it was partly isolated during column chromatography and several NMR experiments (^{1}H-, ^{13}C-NMR, H,H-COSY, HSQC) were performed in order to elucidate the structure of the byproduct. However, this attempt turned out to be unsuccessful, as presumably a cyclopentane-derived compound was formed. The CH_2 groups give a vast number of signals, which overlap in the proton NMR spectrum and make the interpretation process nearly impossible. The same problem was also present during interpretation of the obtained spectra of compound **11a**, but in agreement with the data given in literature, there was no doubt, that the desired compound had been isolated.

The decarboxalkylation of compound **11a** was again performed under the conditions described by Heimann *et al*. After addition of hydrobromic acid (48 %), the mixture was heated under reflux, until CO_2 evolution ceased, which was a reliable sign for full conversion. The reaction mixture was diluted with H_2O before it was neutralized to a pH of 7 by addition of $NaHCO_3$. The aqueous layer was further extracted with Et_2O and washed with saturated $NaHCO_3$ solution and brine. After column chromatography, 2-(2-bromoethyl)cyclopentan-1-one (**12a**) could be isolated in 83 % yield as yellowish oil. During the first attempts to isolate desired compound **12a**, the crude brown oil was additionally filtrated through a short plug of basic alox in order to remove potential acid traces from the rather harsh reaction conditions. Remainders of acid would be particularly problematic during the following olefination step (e.g. HORNER-WADSWORTH-EMMONS reaction), as it would lead to reprotonation of the prepared phosphonate carbanion reagent. However, some kind of decomposition was indicated by GC-FID after the filtration procedure. The nature of this species remained unclear until it was observed again during an attempted WITTIG reaction (see Table 4, entry 2) as the only formed product. It was then isolated from the reaction mixture by bulb-to-bulb distillation (7 mbar, 65 °C) and confirmed by NMR experiments as spiro[2.4]heptan-4-one. A strong hint was the vanished signal during APT experiments for the only CH group present in the desired compound **12a** and the distinctive "roof effect" of the two neighboring CH_2 groups of the cyclopropane moiety. To conclude, 2-(2-bromoethyl)cyclopentan-1-one (**12a**) proved

instable not only towards basic conditions but also towards elevated temperatures. Based on this knowledge, all conditions applied during further experiments were adapted to these constraints.

The olefination of compound **12a** turned out as a major challenge in this synthetic route. Several attempts were needed until the desired α,β-unsaturated ester **13a** could be finally isolated in sufficient amounts. Progress was complicated by the fact that formation of spiro[2.4]heptan-4-one remained unnoticed during several experiments, which mainly led to unsatisfying yields.

As the WITTIG reaction was successfully applied for the synthesis of ethyl (*E*)-6-bromo-4,4-dimethylhex-2-enoate (**8**), it was also tried first for the synthesis of compound **13a** (see Table 4) in the awareness of ketones being significantly less reactive than aldehydes. This was confirmed during the first experiment (Table 4, entry 1), as no conversion could be detected after heating under reflux in THF for 18 h. Conversion was only monitored by TLC, so that potential formation of spiro[2.4]heptan-4-one could not be observed. When switching to elevated temperatures (180 °C/toluene in pressure tube) full conversion was achieved in 19 h, but an undesired byproduct was formed, which was identified as spiro[2.4]heptan-4-one, as already discussed above (Table 4, entry 2). Moreover, the corresponding chlorine derivative of compound **12a** (**12b**) was synthesized (see Scheme 14) and was also tested for the WITTIG reaction, hoping that it might be less prone towards enolization, which would result in the formation of spiro[2.4]heptan-4-one. Nevertheless, the chlorine derivative (**12b**) was not only reacting significantly slower (74 % conversion after 17 h), but also considerable amounts of the undesired spiro-byproduct were formed (Table 4, entry 3).

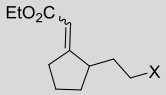


Scheme 14 Synthesis of 2-(2-chloroethyl)cyclopentan-1-one (**12b**).

2-(2-Chloroethyl)cyclopentan-1-one (**12b**) was synthesized in two steps according to the synthesis of the corresponding bromine derivatives **11a** and **12a**.

Methyl 2-oxocyclopentane-1-carboxylate was alkylated by reaction with 1-bromo-2-chloroethane (5 eq). in the presence of K_2CO_3 (3 eq). After extended heating under reflux in acetone for 3 d full conversion was detected by GC-FID and methyl 1-(2-chloroethyl)-2-oxocyclopentane-1-carboxylate (**11b**) could be isolated in 68 % yield after column chromatography. Decarboxalkylation was performed by heating under reflux in 6 M HCl (90 min) and 2-(2-chloroethyl)cyclopentan-1-one (**12b**) could be isolated after column chromatography in 68 % yield.

Table 4 Screened conditions for the WITTIG reaction for synthesis of **13ab**.

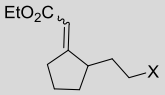
Entry	**X**	**Conditions**	**Conv.**			
			[%]	[%][c]	*E*:*Z*[c]	[%][c]
1	Br	1.2 eq **7** THF, 70 °C	n.d.[a]	-	-	-
2	Br	1.2 eq **7** toluene, 180 °C[b]	100 (19 h)[c]	-	-	100
3	Cl	1.2 eq **7** toluene, 150 °C[b]	74 (17 h)[c]	14	3.7:1	60

[a]Monitored only by TLC. [b] Performed in a pressure tube. [c] Monitored with GC-FID.

In literature, several articles for the olefination of cyclopentanone *via* HWE reaction have been published,[78–81] which suggested that this would also be a suitable method for olefination of ketones **12a** or **12b**. Reaction conditions were chosen considering the previously observed sensitivity of these compounds towards basic conditions. In a first experiment (Table 5, entry 1), excess triethyl phosphonoacetate (2.0 eq) was deprotonated with KO*t*Bu (1.1 eq) at 0 °C. A solution of ketone **12a** was then added at 0 °C and the mixture was subsequently heated to 70 °C. After 19 h the starting material was converted in 76 %, but mainly spiro[2.4]heptan-4-one was formed (61 %). When the chlorinated derivative **12b** was used under the same reaction conditions (Table 5, entry 2), it was converted much slower (25 % after 1 d) and again, mainly the spiro-byproduct was formed (14 %). When the reaction temperature was kept at 0 °C after addition of the ketone, it could be observed that only small amounts (10 %) of the byproduct were formed after 1.5 d (Table 5, entry 3). When the base was exchanged against NaH, ethyl ester (*E*)-**13a** could be isolated in 28 % yield after column chromatography. Lower temperatures

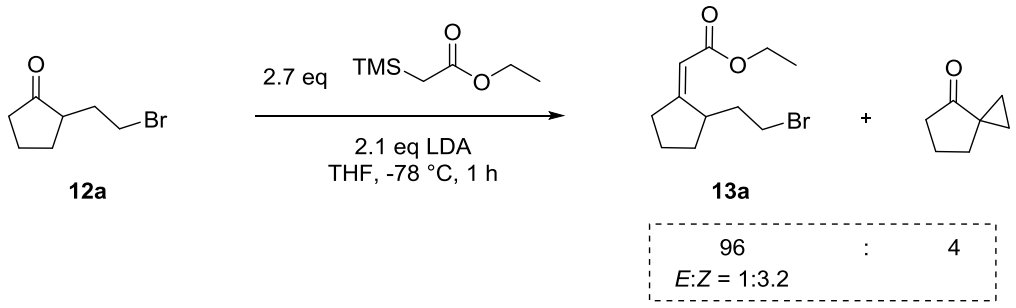
of -10 °C did not have a beneficial effect on the extent of byproduct formation (Table 5, entry 4).

Table 5 Screened conditions for the HORNER-WADSWORTH-EMMONS (HWE) reaction for synthesis of **13ab**.

Entry	X	Conditions	Base	Conv.				Isolated yield
				[%][a]	[%][a]	*E*:*Z*[a]	[%][a]	[%]
1	Br	2.0 eq **A**[b] 0 °C then 70 °C	1.1 eq KO*t*Bu	76 (19 h)	15	2.8:1	61	-
2	Cl	2.0 eq **A**[b] 0 °C then 70 °C	1.1 eq KO*t*Bu	25 (1 d)	11	n.a.	14	-
3	Br	2.0 eq **A**[b] 0 °C	1.2 eq NaH	82 (1.5 d)	72	11.2:1	10	28 (*E*)
4	Br	1.5 eq **A**[b] -10 °C	1.3 eq NaH	85 (2 d)	77	10:1	8	-

[a] Monitored with GC-MS. [b] A = triethyl phosphonoacetate.

Finally, the desired product could be synthesized using PETERSON olefination (Scheme 15), following a procedure from Evans *et al*., which was successfully and selectively applied during the synthesis of (+)-Miyakolide.[82] LDA was freshly prepared from diisopropylamine (2.7 eq) and *n*-BuLi (2.1 eq) at -78 °C. After 15 min, ethyl 2-(trimethylsilyl)acetate (2.7 eq) was added and the resulting solution was stirred at -78 °C for 1 h. In a second reaction vessel, a solution of ketone **12a** in dry THF was prepared which was also cooled to -78 °C. The solution of the previously prepared α-silyl carbanion was added to the ketone solution at -78 °C *via* cannula. This 'reversed' addition, compared to the performed HWE experiments, should prevent excess reactive species in the reaction mixture. After 1 h at -78 °C quantitative conversion was detected by GC-MS. The reaction was quenched at -78 °C by addition of saturated NH_4Cl solution and the desired α,β-unsaturated ethyl ester was isolated in 42 % pure (*Z*)-isomer. Column chromatography could not entirely separate (*E*)- and (*Z*)-isomers, so that also some mixed fractions were obtained, which were isolated separately. The formation of (*Z*)-isomer was confirmed by NOESY experiment, as standard determination *via* the vicinal coupling constant was not possible in this case due to the quaternary carbon at one end of the double bond.



Scheme 15 Synthesis of (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetate (**13a**). Product and *E*/*Z* ratio were determined by GC-MS.

Syntheses of allylic alcohol **14** and allylic aldehyde **15** were again performed under exclusion of light to prevent isomerization of the double bond. α,β-Unsaturated ester **13a** was dissolved in dry CH_2Cl_2 and the resulting colorless solution was cooled to -78 °C. DIBAL-H (2.1 eq) was slowly added to the cooled solution. As quantitative conversion was still not detected (GC-FID) after 1 h at -78 °C, another 0.2 eq of DIBAL-H were added. After additional 30 min, quantitative conversion was finally observed. The reaction mixture was then quenched by addition of saturated Rochelle salt solution and was vigorously stirred for several hours to achieve separation of the phases. The mixture was then simply dried over Na_2SO_4 following the protocol used in the synthesis of allylic alcohol **9**. Again, DIBAL-H reduction was characterized by a clean course of the reaction, as only one single peak was observed in the recorded GC-FID spectrum. For oxidation, the obtained colorless organic layer was transferred into a flame-dried reaction vessel which was equipped with 4 Å molecular sieves. As described earlier, 5 eq activated MnO_2 were added to the solution. After stirring overnight at RT, quantitative conversion was not yet detected by GC-FID, however, a vast number of peaks were observed. Nonetheless, the reaction mixture was stirred for another day, and the peak assigned to the starting material completely vanished. Yet, the origin and nature of formed byproducts remained obscure, as there were never any issues observed during these well-proven reaction conditions. The crude product obtained from silica filtration was then subjected to column chromatography under exclusion of light. One fraction appeared to be clean on TLC and was therefore used for NMR measurements even though the GC-MS spectrum still showed a multitude of peaks. Possible decomposition processes in the inlet of GC-MS could be feasible, however, the obtained NMR spectra also showed some major impurities. It appears that besides side-product formation, also *E*/*Z*-isomerization was an issue, as a second pronounced set of

peaks was visible in the obtained NMR spectra. As the amount of material obtained from the single 'clean' fraction was fairly sufficient for NMR experiments, enzyme screening was abandoned at this point. To conclude, the obtained fraction from column chromatography obviously contained the desired product as indicated by NMR and GC-MS and could be therefore assigned to the distinct spot observed on TLC. The reason for failure of the oxidation step is still unclear. The remaining amount of (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetate (**13a**) was not sufficient for further screenings towards other oxidation methods or conditions, which will be subject of further investigations in the near future.

4.3 Enzyme screenings

4.3.1 Screening of (*E*)-6-bromo-4,4-dimethylhex-2-enal (10)

Enzyme screenings of substrate **10** were performed using five enzymes. Besides the OPR3 and YqjM wild type enzymes three enzyme variants were used, namely OPR3 Y190F, OPR3 Y190W and YqjM Y169F, where the proton-donating Tyr-residue was exchanged by Phe or Trp respectively.

Conversions for substrate **10** are given in Table 6 as obtained from GC-FID measurements using 1,2-DME as internal standard. The presented results are mean values of triple-determinations. (*E*)-6-Bromo-4,4-dimethylhex-2-enal (**10**) was fairly well converted by all enzymes, even full conversion was observed with OPR3 WT, OPR3 Y190F and YqjM WT.

Table 6 Results of the enzymatic screening of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**).

Enzyme	Conversion [%]	t_R = 5.1 min	t_R = 2.9 min
OPR3 WT	> 99	> 95	< 5
OPR3 Y190F	> 99	95	5
OPR3 Y190W	78	95	5
YqjM WT	> 99	> 95	< 5
YqjM Y169F	79	93	7

The main product, which was observed, had a retention time t_R = 5.1 min. It is suggested that, due to the only slightly shifted retention time compared to the starting material (t_R = 5.3 min), this peak comprises the saturated analog of the starting material. However formation of 6-bromo-4,4-dimethylhexanal cannot be confirmed, as no reference material was synthesized. Besides this main product, only one further peak showed up after the enzyme reaction (t_R = 2.9 min). However, as shown in Table 6, this product is formed in rather small amounts of around 5 %, or even lower. Again, formation of desired 3,3-dimethylcyclopentane-1-carbaldehyde cannot be confirmed, albeit the corresponding mass of 126.1 $g \cdot mol^{-1}$ was found during GC-MS measurements. But due to the small percentage this result is quite uncertain and formation of 3,3-dimethylcyclopentane-1-carbaldehyde must not be automatically assigned to the GC-FID peak at 2.9 min. To get a rough estimation of the expectable range of retention time, cyclopentanecarbaldehyde was also injected in GC-FID and was found to have a retention time of 2.6 min. To definitely confirm formation of 3,3-dimethylcyclopentane-1-carbaldehyde, it will be a future task to synthesize suitable reference material.

It should also be denoted, that during the first attempt of enzyme screening, which was only performed under moderate light-protected conditions, the double-bond considerably underwent isomerization (~ 30 %). The *E*/*Z*-mixture was nonetheless subjected to the enzyme reaction and interestingly, the (*Z*)-isomer was not converted at all or rather much slower than the (*E*)-isomer. Yet, it is still unclear, during which reaction step – reduction, oxidation or enzyme reaction – isomerization occurred, however, it is reported in literature, that FMN is capable of catalyzing double-bond isomerization.[83] Therefore, all reaction steps starting from the reduction were carried out with reasonable care towards light protection.

5 Summary and Outlook

5.1 Summary

The aim of this thesis was to synthesize substrates for reductive cyclizations catalyzed by engineered ene-reductases to yield five-membered ring systems. As it could be shown in a preliminary work,[70] model substrate (*E*)-6-bromohex-2-enal did not undergo cyclization, but only reduction to 6-bromohexanal. The tendency to ring formation should therefore be enhanced by introduction of a dimethyl group (*gem*-dimethyl effect) or by attachment to another ring system (restriction of flexibility).

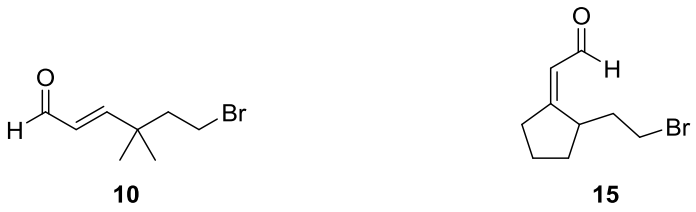
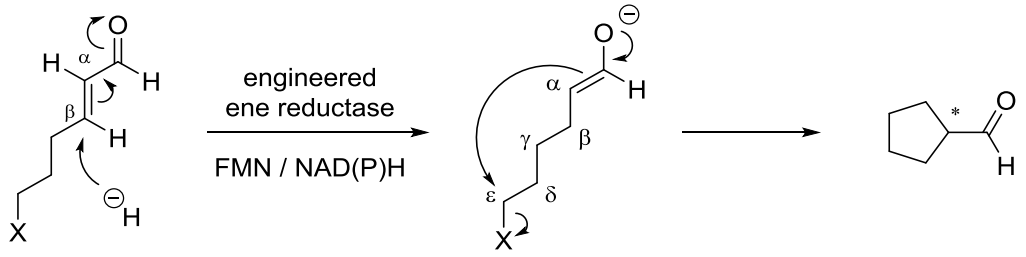


Figure 8 Attempted substrates for biocatalytic screening reactions.

The synthesized substrates should be used for enzymatic screenings towards reductive cyclization by using OPR and YqjM WT enzymes and three enzyme variants (OPR3 Y190F, OPR3 Y190W, YqjM Y169F), where the potential proton-donating Tyr residue is exchanged against Phe or Trp. The reaction should therefore follow the proposed mechanism illustrated in Scheme 16.

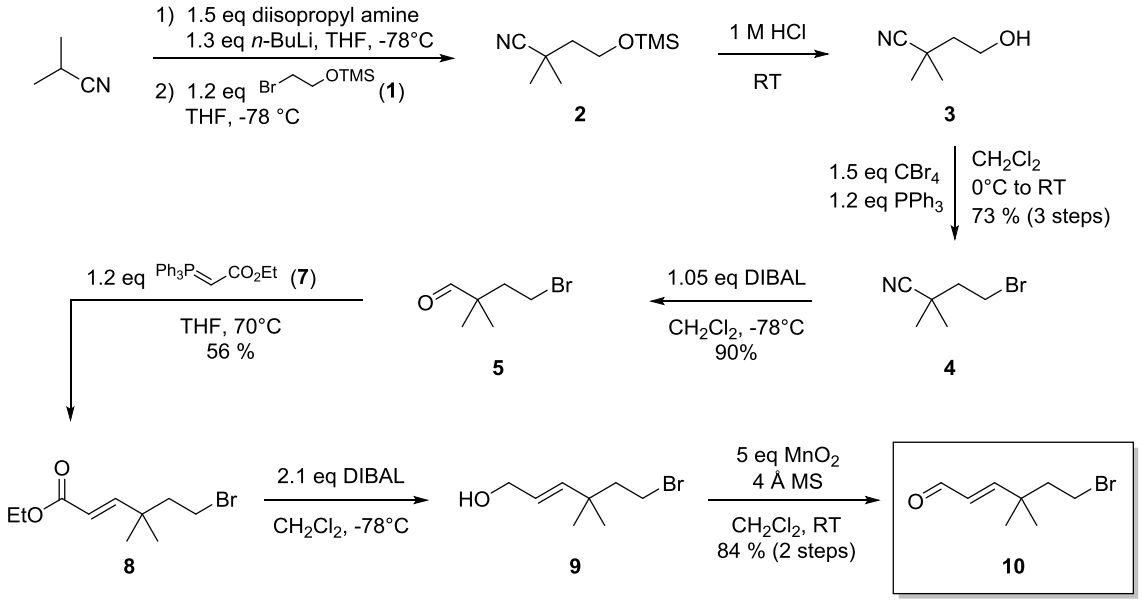


Scheme 16 Proposed reaction mechanism for reductive cyclization (X = Hal, OTf).

After donation of the FMN-derived hydride to C-β, the subsequently formed enolate should rather attack the internal electrophilic C-ε and reprotonation ought to be suppressed through replacement of the Tyr residue. Upon cyclization, one new stereogenic center is generated.

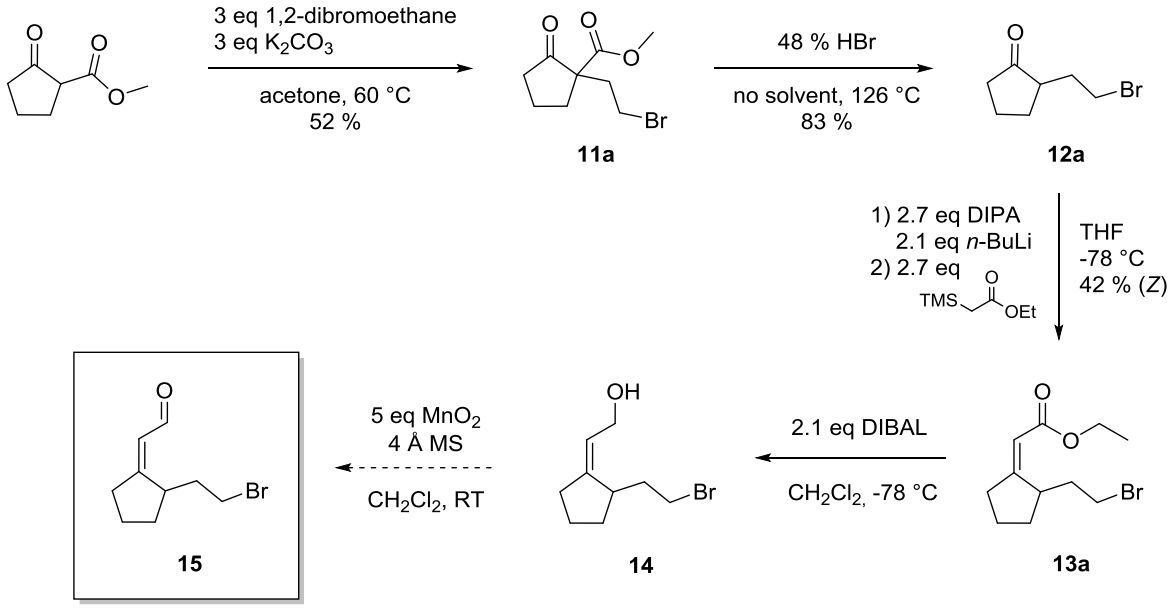
Substrate **10**, (*E*)-6-bromo-4,4-dimethylhex-2-enal, could be successfully synthesized in seven steps with an overall yield of 31 %. No major difficulties were met during the synthetic sequence, however, the multi-stage synthesis itself turned out to be the major drawback.

Enzymatic conversion of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**) gave mainly the assumed reduced product instead of the desired cyclized aldehyde for all tested enzymes with high conversions (≥ 78 %).



Scheme 17 Synthesis of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**).

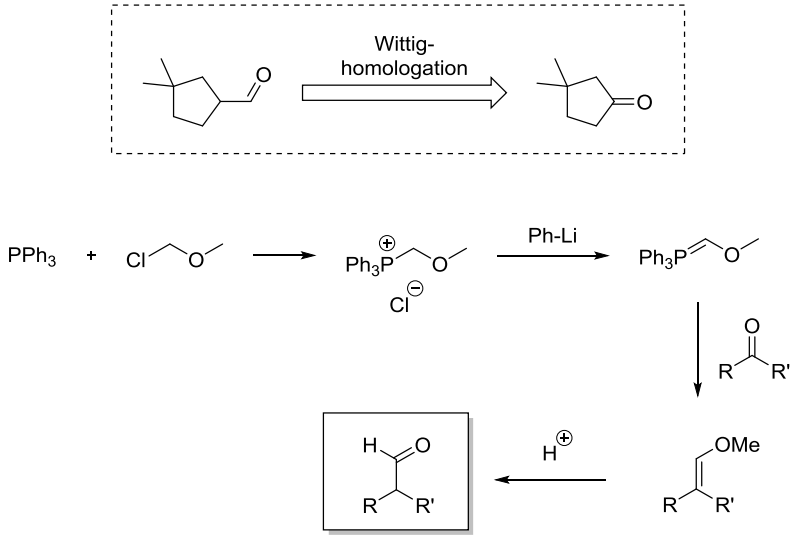
(*Z*)-2-(2-(2-Bromoethyl)cyclopentylidene)acetaldehyde (**15**) was attempted to be synthesized in five steps (Scheme 18). Olefination of 2-(2-bromoethyl)cyclopentan-1-one (**12a**) proved to be a major challenge. However, (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene) acetate (**13a**) was finally accessible *via* PETERSON olefination. Yet, the oxidation of the allylic alcohol (**14**) caused difficulties and it was therefore not possible to finish the synthesis of **15** at this point.



Scheme 18 Attempted Synthesis of (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetaldehyde (**15**).

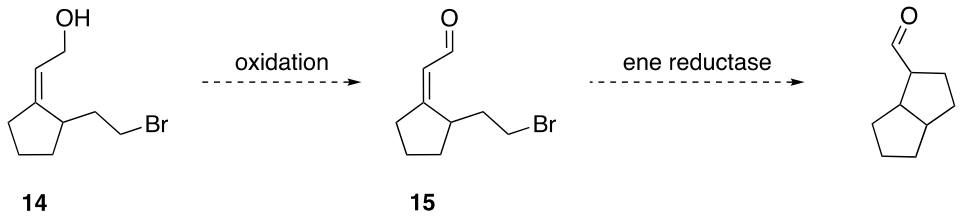
5.2 Outlook

To confirm the possible formation of 3,3-dimethylcyclopentane-1-carbaldehyde, synthesis of this compound remains a major future task. As shown in Scheme 19, synthesis of 3,3-dimethylcyclopentane-1-carbaldehyde should be achieved by WITTIG-homologation of 3,3-dimethylcyclopentan-1-one using methodology introduced by S. Levine.[84] Unfortunately this ketone precursor is commercially inaccessible and therefore has to be synthesized in addition.



Scheme 19 Suggested synthetic approach towards 3,3-dimethylcyclopentane-1-carbaldehyde.

Due to time restriction and deficiency of (*Z*)-2-(2-(2-bromoethyl) cyclopentylidene) acetate (**13a**), the oxidation process could not be investigated further within this work. However, screening and optimization of the oxidation reaction will be subject of future work (Scheme 20). It is of substantial interest if other oxidation agents (e.g. pyridinium chlorochromate (PCC)) would cleanly yield the desired α,β-unsaturated aldehyde **15** or if the substrate itself is prone to decomposition.



Scheme 20 Remaining reaction step towards synthesis of **15** and enzyme screening.

Enzymatic screening of α,β-unsaturated aldehyde **15** can be hopefully carried out in the near future, giving another interesting insight into the enzyme catalyzed reductive cyclization.

6 Experimental Section

6.1 General Aspects, Materials and Methods

The contents of this chapter were taken and adapted from Ref. [85] and [86].

All chemicals were purchased from Sigma Aldrich, Alfa Aesar, Acros Organics, Fluka, TCI Chemicals, and abcr. The reagents were used without further purification, unless stated otherwise.

For reactions under inert conditions, standard Schlenk techniques were applied. For this purpose, the reaction vessel was evacuated, flame dried and purged with inert gas (N_2 or Ar) for a total of three times. All chemicals were added under N_2 or argon counter flow, purposing exclusion of moisture and oxygen.

6.1.1 Solvents

6.1.1.1 Dry Solvents

Dichloromethane (DCM): DCM, stabilized with EtOH, was first dried over P_4O_{10}, distilled and then heated under reflux over CaH_2 and distilled into a brown glass bottle (1 L) where it was stored over 4 Å molecular sieves under argon.

Diisopropylamine (DIPA): DIPA was heated under reflux over CaH_2 and distilled into a brown glass bottle (1 L) where it was stored over 4 Å molecular sieves under argon.

Tetrahydrofuran (THF): THF was dried at reflux temperature under argon atmosphere over sodium and benzophenone until ketyl radical indicated dryness by the appearance of a deep blue color. Dry THF was distilled and stored over 4 Å molecular sieves in a brown glass bottle (1 L) under argon atmosphere.

Toluene: Dry Toluene was prepared by filtering through an alox-column (Pure Solv by Innovative Technology) and it was stored over 4 Å molecular sieves in brown glass bottles (1 L) under argon.

6.1.1.2 Other Solvents and Solutions

For reactions, which could be performed without inert conditions, as well as for work up and further purification procedures, the following solvents were used:

Acetone: Acetone used for reactions, was distilled on a rotary evaporator prior to use.

Ammonium chloride (NH_4Cl): NH_4Cl was added to distilled water until saturation was obtained (~38 g per 100 mL).

Brine: NaCl was added to distilled water until saturation was obtained (~36 g per 100 mL).

Copper(II) sulfate (10 w%): $CuSO_4 \cdot 5\ H_2O$ was dissolved in distilled H_2O to reach a final concentration of 10 w% $CuSO_4$.

Cyclohexane (CH): Cyclohexane (≥ 99.99 %) was purchased from Fisher Scientific in 5 L plastic bottles and used as received.

Dichloromethane (DCM): DCM was bought from Fisher Chemicals in 5 L plastic bottles and was used as received.

Diethyl ether (Et_2O): Et_2O was purchased from VWR in 25 L metal cans and distilled in a rotary evaporator prior to use to remove the stabilizers. Et_2O was stored over KOH in brown glass bottles to bind formed peroxides.

1,2-Dimethoxyethane (1,2-DME): 1,2-DME was purchased from Sigma Aldrich (≥ 99 %) in 1 L glass bottles and used as received.

***N,N*-Dimethylformamide (DMF):** DMF was purchased from Roth (≥ 99.8 %) in 2.5 L plastic bottles and used as received.

Ethyl acetate (EA): Ethyl acetate (≥ 99.99 %) was purchased from Fisher Scientific in 5 L plastic bottles and used without further purification.

Methyl *tert*-butyl ether (MTBE): Methyl *tert*-butyl ether was purchased from Sigma Aldrich (≥ 99 %) in 1 L glass bottles and used as received.

***n*-Pentane:** Pentane was purchased from Roth in 25 L metal cans and distilled in a rotary evaporator prior to use.

Rochelle salt: Rochelle salt ($KNaC_4H_4O_6 \cdot 4\ H_2O$) was added to distilled water until saturation was obtained.

Sodium bicarbonate ($NaHCO_3$): Sodium bicarbonate was added to distilled water until saturation was obtained (~10 g per 100 mL).

Tartaric acid (25 w%): Tartaric acid ($C_4H_6O_6$) was dissolved in distilled water to reach a final concentration of 25 w%.

Tetrahydrofuran (THF): THF was distilled on a rotary evaporator to remove the stabilizers and stored over KOH in brown glass bottles to bind formed peroxides.

Toluene: Toluene was purchased from Fisher Scientific in 5 L plastic bottles and used without further purification.

6.1.2 Analytical Methods

6.1.2.1 Nuclear Magnetic Resonance Spectrometry (NMR)

All standard NMR spectra (^1H, ^{13}C, APT, H,H-COSY, HSQC) were recorded with a Bruker Avance III 300 MHz FT NMR spectrometer (300.36 MHz (^1H), 75.53 MHz (^{13}C)) with auto sampler. NOESY experiments were recorded with a Varian (Agilent) INOVA 500 MHz spectrometer (Agilent Technologies) using VNMRJ 2.2D software (499.98 MHz (^1H), 125.69 MHz (^{13}C)). Chemical shifts δ [ppm] are referenced to residual protonated solvent signals as internal standard (DMSO-d_6: δ = 2.50 ppm (^1H); 39.52 ppm (^{13}C); $CDCl_3$: δ = 7.26 ppm (^1H); 77.16 ppm (^{13}C)).[87] Signal multiplicities are abbreviated as s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), q (quadruplet), m (multiplet). Signals of quaternary C-atoms were abbreviated as C_q. Several 2D-experiments (H,H-COSY, HSQC, NOESY) as well as APT experiments were used for resonance assignment. Chemical shifts are given in ppm (parts per million) and the coupling constants in Hz (Hertz). All ^{13}C experiments were ^1H decoupled for enhancing sensitivity and clarity of the obtained spectra.

6.1.2.2 Thin Layer Chromatography (TLC)

For thin layer chromatography TLC plates from Merck (silica gel 60 F_{254} aluminum sheets, 20 x 20 cm) were used. For detection, UV-light (λ = 254 nm) and/or different staining reagents were used, followed by treatment with hot-air stream. The R_f values calculated for each compound, as well as the eluent composition are given in each procedure.

$KMnO_4$: 1.5 g $KMnO_4$ and 10 g K_2CO_3 were dissolved in 200 mL water and 1.25 mL 10 w% NaOH were added.

6.1.2.3 Flash Column Chromatography

For preparative flash column chromatography, silica gel from Acros Organics was used (silica gel for chromatography 0.035 - 0.070 nm, 60 Å, nitrogen flushed). The amount of silica gel used, was typically between the 50 to 100 fold amount of crude product. The column diameter was chosen to reach a filling level between 15 and 25 cm. Fraction size was typically 25 – 30 % of the silica gel volume. An eluent composition was used, so that the R_f value of the desired product was between 0.2 and 0.3. Sticky crude products were first dissolved in a small amount of DCM and the 1.5 fold amount of silica gel was added, before the solvent was removed using a rotary evaporator.

6.1.2.4 Gas Chromatography with mass selective detection (GC-MS)

GC-MS measurements were performed on an Agilent Technologies 7890A (G3440A) GC system equipped with an Agilent Technologies J&W GC-column HP-5MS ((5%-phenyl) methylpolysiloxane; 30 m x 250 µm x 0.25 µm) at a constant He flow rate (He 5.0) as carrier gas. The samples were injected in split mode, using an Agilent Technologies 7683 Series auto sampler and an Agilent Technologies 7683B Series injector. The GC was coupled to a 5975C inert mass sensitive detector with triple-axis detector (MSD, EI, 70 eV; transfer line: 300 °C, MS source: 240 °C; MS quad: 180 °C). Temperature program:

MT_50_S: 50 °C 1 min, ramp: 40 °C min^{-1} linear to 300 °C, 300 °C 5 min, solvent delay: 3.5 min.

Conversion was calculated *via* surface integration of the peaks. No internal standard was used during GC-MS measurements.

6.1.2.5 Gas Chromatography with flame ionization detector (GC-FID)

GC-FID measurements were performed on an Agilent Technologies 6890N GC system with an Agilent Technologies J&W GC-column DB-17-01 ((14%-cyanopropylphenyl) methylpolysiloxane; 30 m x 250 µm x 0.25 µm). For injection, an Agilent Technologies 7683 Series auto sampler (split mode) was used. Nitrogen 5.0 served as carrier gas and hydrogen 5.0 and air were used for detection together with a flame ionization detector (FID).

Temperature program:

KH_80_30_280: 1 min at 80 °C, ramp 30 °C min^{-1} linear to 280 °C.

6.1.2.6 High Resolution Mass Spectroscopy (HR-MS)

High resolution mass spectroscopy measurements were performed using a "Waters GCT Premier" system after ionization with an EI ionization source (E = 70 eV). The measured compounds were directly injected as solution (DI). All measurements were performed by Ing. Karin Bartl (Institute for Chemistry and Technology of Materials, Graz University of Technology). The reported values for a given species are denoted as mass to charge ratio, accompanied by the corresponding calculated values.

6.1.2.7 Determination of the melting point

Melting points were determined by use of a melting point apparatus "Mel-Temp®" from Electrothermal with integrated microscope attachment. The reported values are uncorrected and represent the mean value of double determination.

6.1.3 General Procedures

6.1.3.1 Titration of alkyllithium reagents

Titration of alkyllithium reagents was carried out in accordance to a method from Kofron.[88] In a flame-dried Schlenk-flask, 250 mg diphenylacetic acid were dissolved in 2.0 mL abs. THF. The alkyllithium solution was added dropwise to the colorless solution under inert conditions until the color change to yellow indicated the equivalence point. A triple determination was performed. Titration was done prior to every use of the alkyllithium reagent.

6.2 Biological section

All used enzymes and enzyme variants were expressed and purified by Kathrin Heckenbichler (Institute of Organic Chemistry, Graz University of Technology).

6.2.1 Generation of enzyme variants by site-directed mutagenesis

For generation of enzyme variants of OPR3 and YqjM, suitable mutagenesis primer were designed and ordered (see Table 7). Plasmids pET21a(*opr3*) and pET21a(*yqjM*) were used as templates. Site-directed mutagenesis was performed according to the manual of

QuikChange Site-directed mutagenesis kit. Plasmids were isolated (from three colonies respectively) after mutagenesis PCR and transformation into *E.coli* Top10 cells. Sequencing was done in order to control the mutations.

Table 7 Enzyme variants and respective mutagenesis primer.

Enzyme variant	Mutagenesis primer 5'-3'
OPR3 Y190F	ccatgg agctcacggt ttcttgattg atcaattctt gaaagatgg
OPR3 Y190W	ccatgg agctcacggt tggttgattg atcaattctt gaaagatgg
YqjM Y169F	c atgcggcgca cggatttta attcatgaat ttttgtctcc gc

6.2.2 Expression and purification of enzyme variants

The respective plasmids were transformed into $CaCl_2$-competent *E.coli* BI21-CodonPlus™-(DE3)-RIL cells (Stratagene). OPR3 WT and the OPR3 variants were expressed with a hexahistidine tag at the C-terminus and purified *via* Ni-NTA affinity chromatography as described by Breithaupt.[31] Expression and purification of the YqjM variant was carried out according to a procedure from Fitzpatrick.[38] For storage, the buffer was changed to 50 mM NaPi, 150 mM NaCl, pH 7.5 (OPR3) or 50 mM TrisHCl, pH 7.5 (YqjM) after purification. Aliquots of the enzymes were stored at 4 °C (OPR3) or -32 °C (YqjM). Enzyme concentration was photometrically estimated (ε = 9500 $L \cdot mol^{-1} \cdot cm^{-1}$) at 466 nm (OPR3) or 455 nm (YqjM).

6.2.3 General procedure for enzyme screenings

Enzyme screenings were performed under exclusion of light in Eppendorf vials (final volume: 0.6 mL). For all experiments a 50 mM NaPi, 150 mM NaCl, pH 7.5 buffer was used. To simplify preparation of the reaction mixtures, a master mix was prepared whereof one aliquot of 300 µL was added to each sample containing a mixture of enzyme in buffer (300 µL).

Composition of the master mix:

Substrate (final concentration: 10 mM, assumed density: ρ = 1 g/mL)

NADH (final concentration: 15 mM)

1,2-DME (internal standard; $V_{internal\ standard} = V_{substrate}$)

DMF (cosolvent, 6 µL per sample)

Buffer (50 mM NaPi, 150 mM NaCl, pH 7.5)

The different enzymes (3 samples for each enzyme) were prepared in Eppendorf vials (final concentration: 5 µM), filled up with buffer to 300 µL and were stored at 4 °C until used. Additionally three blanks were prepared, which contained only 300 µL buffer. The concentration of the enzyme preparations are given in Table 8.

Table 8 Composition of samples for enzyme screening.

Sample	Enzyme concentration [µM]
OPR3 WT	109
OPR3 Y190F	174
OPR3 Y190W	174
YqjM WT	545
YqjM Y169F	279

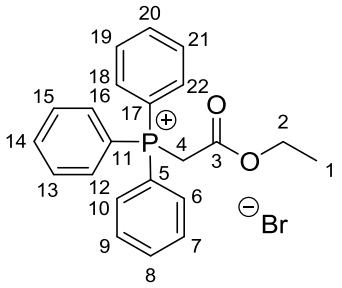
The master mix was prepared and added to the enzyme solutions shortly before the enzyme screening and all samples were thoroughly but carefully mixed. The samples were incubated for 180 min at 25 °C and 300 rpm in an Eppendorf Thermomixer *comfort*. For work-up MTBE (300 µL) was added to each sample which was then intensely mixed and centrifuged for 10 min at 13.000 rpm. The organic layer was then separated and dried over $MgSO_4$. After centrifugation (10 min, 13.000 rpm), the organic layer was transferred into GC glass-vials and all samples were directly measured using GC-FID. The method, which was used, is stated in section 6.1.2.5. Retention times t_R of the internal standard (1,2-DME) and cosolvent (DMF) for the used method are stated in Table 9.

Table 9 Retention time t_R of 1,2-DME and DMF (method: KH_80_30_280).

Compound	Retention time t_R
1,2-DME	1.6 min
DMF	2.4 min

6.3 Experimental Procedures and Analytical Data

6.3.1 Synthesis of (2-ethoxy-2-oxoethyl)triphenylphosphonium bromide (6)



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In a flame-dried 250 mL two-neck round bottom flask with gas inlet 42.15 g (160.7 mmol, 1.1 eq) triphenylphosphine were dissolved in 100 mL dry toluene. 16.2 mL (24.40 g, 146.1 mmol, 1.0 eq) ethyl 2-bromoacetate were added dropwise and the solution was stirred at RT overnight. The precipitated colorless solid was collected by filtration and washed with toluene (3 x 100 mL) and cyclohexane (3 x 100 mL) before it was dried *in vacuo*.

Yield 62.25 g (145.0 mmol, 99 %), colorless powder.

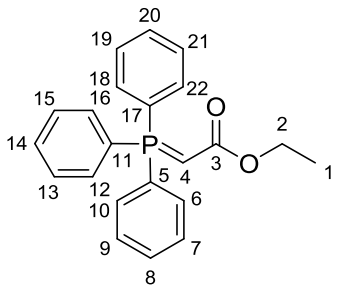
$C_{22}H_{22}BrO_2P$ [429.29]

m.p. 153 °C

^{1}H-NMR (300.36 MHz, DMSO-d_6) δ = 7.94 – 7.73 (m, 15H, H-6, H-7, H-8, H-9, H-10, H-12, H-13, H-14, H-15, H-16, H-18, H-19, H-20, H-21, H-22), 5.35 (d, $^2J_{HP}$ = 14.5 Hz, 2H, H-4), 4.04 (q, $^3J_{HP}$ = 7.1 Hz, 2H, H-2), 0.97 (t, $^3J_{HH}$ = 7.1 Hz, 3H, H-1) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 164.6 (C_q, d, $^2J_{CP}$ = 3.8 Hz, C-3), 135.1 (C^{Ar}, d, $^4J_{CP}$ = 3.0 Hz, C-8, C-14, C-20), 133.7 (C^{Ar}, d, $^3J_{CP}$ = 10.7 Hz, C-7, C-9, C-13, C-15, C-19, C-21), 130.1 (C^{Ar}, d, $^2J_{CP}$ = 13.0 Hz, C-6, C-10, C-12, C-16, C-18, C-22), 118.2 (C_q, d, $^1J_{CP}$ = 88.9 Hz, C-5, C-11, C-17), 62.3 (CH_2, C-2), 29.6 (CH_2, d, $^1J_{CP}$ = 56.0 Hz, C-4), 13.5 (CH_3, C-1) ppm.

6.3.2 Synthesis of ethyl 2-(triphenyl-λ5-phosphaneylidene)acetate (7)



7

In a 1000 mL round bottom flask 58.42 g (135.1 mmol, 1 eq) (2-ethoxy-2-oxoethyl) triphenyl-phosphonium bromide (**6**) were dissolved in a mixture of 350 mL distilled H_2O and 400 mL toluene. An aqueous solution of NaOH (10 %) was added while stirring, until a pH of 10 was reached. The layers were separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 150 mL). The combined organic layers were dried over Na_2SO_4 before the solvent was removed under reduced pressure. The desired product was precipitated from the crude yellow oil by addition of cyclohexane (140 mL). The colorless crystals were collected by filtration, carefully washed with cold cyclohexane (3 x 150 mL), and dried *in vacuo*.

Yield 27.95 g (80.2 mmol, 59 %), colorless powder.

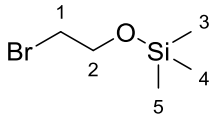
$C_{22}H_{21}O_2P$ [348.38]

m.p. 126-130 °C (lit. 128-130 °C)[89]

^{1}H-NMR (300.36 MHz, DMSO-d_6) δ = 7.64 – 7.57 (m, 15H, H-6, H-7, H-8, H-9, H-10, H-12, H-13, H-14, H- 15, H-16, H-18, H-19, H-20, H-21, H-22), 3.83 (q, $^3J_{HH}$ = 6.9 Hz, 2H, H-2), 2.76 (d, $^2J_{HP}$ = 22.7 Hz, 1H, H-4), 1.10 (t, $^3J_{HH}$ = 7.0 Hz, 3H, H-1) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 170.4 (C_q, d, $^3J_{CP}$ = 14.6 Hz, C-3), 132.5 (C^{Ar}, d, $^4J_{CP}$ = 10.0 Hz, C-8, C-14, C-20), 132.1 (C^{Ar}, d, $^3J_{CP}$ = 9.1 Hz, C-7, C-9, C-13, C-15, C-19, C-21), 128.9 (C^{Ar}, d, $^2J_{CP}$ = 12.0 Hz, C-6, C-10, C-12, C-16, C- 18, C-22), 127.4 (C_q, d, $^1J_{CP}$ = 91.2 Hz, C-5, C-11, C-17), 56.6 (CH_2, C-2), 28.8 (CH, d, $^1J_{CP}$ = 127.9 Hz, C-4), 14.7 (CH_3, C-1) ppm.

6.3.3 Synthesis of (2-bromoethoxy)trimethylsilane (1)



1

4.6 mL (64.0 mmol, 1.0 eq) 2-bromoethanol were dissolved in 200 mL CH_2Cl_2 in a 500 mL round bottom flask before 782.1 mg (6.4 mmol, 0.1 eq) DMAP and 18 mL (128.0 mmol, 2 eq) Et_3N were added. The yellowish solution was cooled to 0 °C and 10.7 mL (83.2 mmol, 1.3 eq) TMS-Cl were added *via* a dropping funnel. After complete addition the cloudy mixture was stirred at RT until quantitative conversion was detected by GC-FID (2 h). The reaction was quenched by addition of 160 mL H_2O and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (2 x 100 mL) and the combined organic layers were washed with 10 % $CuSO_4$ solution until no more precipitation was observed (1 x 100 mL, 3 x 50 mL). The yellowish organic phase was then washed with H_2O (1 x 100 mL) and brine (1 x 100 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure.

The crude product was used without further purification.

Yield 9.91 g (50.3 mmol, 79 %), orange oil.

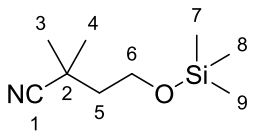
$C_5H_{13}BrOSi$ [197.15]

GC-FID t_R = 2.6 min

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.86 (t, $^3J_{HH}$ = 6.5 Hz, 2H, H-2), 3.40 (t, $^3J_{HH}$ = 6.5 Hz, 2H, H-1), 0.14 (s, 9H, H-3, H-4, H-5) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 63.1 (CH_2, C-2), 33.2 (CH_2, C-1), -0.3 (3 CH_3, C-3, C-4, C-5) ppm.

6.3.4 Synthesis of 2,2-dimethyl-4-((trimethylsilyl)oxy)butanenitrile (2)



2

In a 250 mL flame-dried and nitrogen-flushed round bottom flask with gas inlet 9 mL (61.9 mmol, 1.5 eq) dry diisoproylamine were dissolved in 30 mL dry THF. The slightly yellow solution was cooled to -78 °C (acetone/dry ice cooling bath) and 22 mL (2.43 M in hexanes, 53.6 mmol, 1.3 eq) *n*-butyllithium solution were added dropwise over 30 min. After 2 h a colorless solution of 3.8 mL (41.2 mmol, 1 eq) isobutyronitrile in 15 mL dry THF was slowly added *via* cannula at -78 °C. A solution of 9.76 g **1** (49.5 mmol, 1.2 eq) in 10 mL dry THF was added dropwise at -78 °C to the reaction mixture after 2 h, yielding an intensively yellow colored solution. The reaction mixture was allowed to warm to RT overnight before it was quenched by addition of 150 mL saturated NH_4Cl solution. The phases were separated and the aqueous phase was extracted with Et_2O (3 x 100 mL). The combined organic layers were washed with H_2O (1 x 200 mL) and brine (1 x 200 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure.

The crude product was used without further purification.

Yield 16.28 g (87.9 mmol, 213 %), yellow-orange liquid (contains THF).

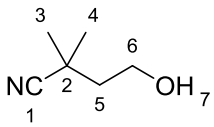
$C_9H_{19}NOSi$ [185.34]

GC-FID t_R = 4.0 min

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.78 (t, $^3J_{HH}$ = 6.8 Hz, 2H, H-6), 1.77 (t, $^3J_{HH}$ = 6.8 Hz, 2H, H-5), 1.37 (s, 6H, H-3, H-4), 0.12 (s, 9H, H-7, H-8, H-9) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 124.9 (C≡N, C-1), 59.3 (CH_2, C-6), 43.0 (CH_2, C-5), 30.9 (C_q, C-2), 27.3 (2 CH_3, C-3, C-4), -0.5 (3 CH_3, C-7, C-8, C-9) ppm.

6.3.5 Synthesis of 4-hydroxy-2,2-dimethylbutanenitrile (3)



3

In a 250 mL round bottom flask 120 mL HCl (1 M) were added to 7.64 g (41.22 mmol, 1 eq) **2** and the mixture was stirred at RT until quantitative conversion was detected by TLC (40 min). The aqueous layer was extracted with CH_2Cl_2 (2 x 120 mL) and subsequently saturated with NaCl. The saturated aqueous phase was again extracted with CH_2Cl_2 (2 x 120 mL) and the combined organic layers were dried over Na_2SO_4. The solvent was removed under reduced pressure.

The crude product was used without further purification.

Yield 10.41 g (92.0 mmol, 223 %), orange liquid (contains THF).

$C_6H_{11}NO$ [113.16]

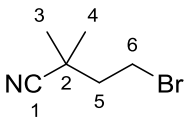
GC-FID t_R = 4.1 min

TLC R_f = 0.18 (CH/EA = 1/1, UV and $KMnO_4$).

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.83 (t, $^3J_{HH}$ = 6.8 Hz, 2H, H-6), 2.20 (bs, 1H, H-7), 1.80 (t, $^3J_{HH}$ = 6.8 Hz, 2H, H-5), 1.37 (s, 6H, H-3, H-4) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 125.1 (C≡N, C-1), 59.4 (CH_2, C-6), 42.9 (CH_2, C-5), 30.7 (C_q, C-2), 27.2 (CH_3, C-3, C-4) ppm.

6.3.6 Synthesis of 4-bromo-2,2-dimethylbutanenitrile (4)



4

In a 250 mL round bottom flask 4.66 g (41.2 mmol, 1 eq) **3** were dissolved in 40 mL CH_2Cl_2. To the orange solution 20.48 g (61.8 mmol, 1.5 eq) CBr_4 were added before it was cooled to 0 °C. 12.96 g (49.4 mmol, 1.2 eq) PPh_3 were dissolved in 50 mL CH_2Cl_2 and the resulting colorless solution was added dropwise to the reaction mixture at 0 °C. The clear, yellow solution was then stirred at RT overnight. Quantitative conversion was detected by TLC (CH/EA = 1/1; UV and $KMnO_4$). The solvent was removed under reduced pressure and the crude brown oil was purified *via* flash chromatography (450 g silica, 21 x 8 cm; CH/EA = 15/1 (fraction 1-7), 10/1 (from fraction 8); fraction size: 250 mL).

Yield 5.29 g (30.0 mmol, 73 %), pale yellow oil.

$C_6H_{10}BrN$ [176.06]

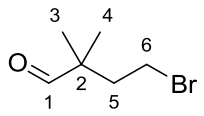
GC-FID t_R = 4.0 min

TLC R_f = 0.50 (CH/EA = 5/1; UV and $KMnO_4$).

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.47 (t, $^3J_{HH}$ = 8.1 Hz, 2H, H-6), 2.12 (t, $^3J_{HH}$ = 8.1 Hz, 2H, H-5), 1.38 (s, 6H, H-3, H-4) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 123.8 (C≡N, C-1), 43.9 (CH_2, C-5), 32.8 (C_q, C-2), 26.6 (2 CH_3, C-3, C-4), 26.5 (CH_2, C-6) ppm.

6.3.7 Synthesis of 4-bromo-2,2-dimethylbutanal (5)



5

In a flame-dried and nitrogen-flushed 250 mL round bottom flask with gas inlet 4.35 g (24.7 mmol, 1 eq) **4** were dissolved in 85 mL dry CH_2Cl_2. The colorless solution was cooled to -78 °C (acetone/dry ice cooling bath). 26 mL (1 M in CH_2Cl_2, 25.9 mmol, 1.05 eq) DIBAL-H solution were slowly added over 45 min, not allowing the temperature to rise above -78 °C. After 15 min of stirring at -78 °C quantitative conversion was detected by GC-FID. The reaction mixture was quenched by addition of 100 mL tartaric acid solution (25 %) at -78 °C and was subsequently allowed to warm to RT under vigorous stirring. When the two phases both became colorless and clear, the organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 120 mL). The combined organic layers were filtrated through a pad of Celite® and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the crude product was directly used for the next reaction step without further purification.

Yield 3.98 g (22.2 mmol, 90 %), pale yellow oil.

$C_6H_{11}BrO$ [179.06]

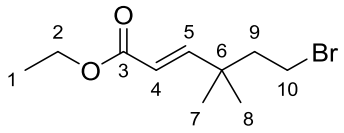
GC-FID t_R = 3.7 min

TLC R_f = 0.54 (CH/EA = 5/1; $KMnO_4$)

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 9.48 (s, 1H, H-1), 3.32 (t, $^3J_{HH}$ = 7.8 Hz, 2H, H-6), 2.12 (t, $^3J_{HH}$ = 7.8 Hz, 2H, H-5), 1.10 (s, 6H, H-3, H-4) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 204.6 (CHO, C-1), 46.6 (C_q, C-2), 40.5 (CH_2, C-5), 27.8 (CH_2, C-6), 21.4 (2 CH_3, C-3, C-4) ppm.

6.3.8 Synthesis of ethyl (*E*)-6-bromo-4,4-dimethylhex-2-enoate (8)



8

In a 500 mL round-bottom flask 3.98 g (22.2 mmol, 1 eq) **5** were dissolved in 220 mL THF. 9.30 g (26.7 mmol, 1.2 eq) WITTIG-ylen **7** were added at RT to the pale yellow solution. The round-bottom flask was equipped with a reflux condenser and the heterogeneous mixture was heated under reflux. After 7 d the reaction was stopped and the solvent was removed under reduced pressure. The crude product was purified *via* flash column chromatography (500 g silica, 22 x 8 cm; CH/EA = 30/1 (fraction 1-9), 20/1 (fraction 10-13), 10/1 (from fraction 14); fraction size: 250 mL).

Yield 3.08 g (12.4 mmol, 56 %), pale yellow oil.

$C_{10}H_{17}BrO_2$ [249.15]

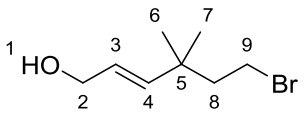
GC-FID t_R = 5.7 min

TLC R_f = 0.70 (CH/EA = 5/1; UV and $KMnO_4$)

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 6.87 (d, $^3J_{HH}$ = 15.9 Hz, 1H, H-5), 5.74 (d, $^3J_{HH}$ = 16.0 Hz, 1H, H-4), 4.19 (q, $^3J_{HH}$ = 7.1 Hz, 2H, H-2), 3.32 – 3.21 (m, 2H, H-10), 2.03 – 1.93 (m, 2H, H-9), 1.29 (t, $^3J_{HH}$ = 7.1 Hz, 3H, H-1), 1.09 (s, 6H, H-7, H-8) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 166.8 (C_q, C-3), 155.9 (CH, C-5), 119.1 (CH, C-4), 60.5 (CH_2, C-2), 45.6 (CH_2, C-9), 38.0 (C_q, C-6), 28.4 (CH_2, C-10), 26.4 (2 CH_3, C-7, C-8), 14.4 (CH_3, C-1) ppm.

6.3.9 Synthesis of (*E*)-6-bromo-4,4-dimethylhex-2-en-1-ol (9)

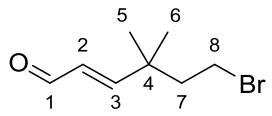


9

Preparation of allylic alcohol **9** was conducted under exclusion of light. In a flame-dried and nitrogen-flushed 100 mL round-bottom flask with gas inlet 1.00 g (4.0 mmol, 1 eq) **8** was dissolved in 18 mL dry CH_2Cl_2. The colorless solution was cooled to -78 °C (acetone/dry ice cooling bath) and 8.5 mL (1 M in CH_2Cl_2, 8.4 mmol, 2.1 eq) DIBAL-H solution were slowly added over 15 min. During addition, the color of the mixture changed to yellow but faded away again after some minutes. After stirring at -78 °C for 25 min, quantitative conversion was detected by TLC (CH/EA = 4/1; UV and $KMnO_4$). The reaction mixture was quenched by addition of 18 mL saturated Rochelle salt solution at -78 °C and was subsequently allowed to warm to RT under vigorous stirring. When complete separation of the phases was achieved the organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (2 x 18 mL). The combined organic layers were washed with brine (1 x 40 mL) and dried over Na_2SO_4. After filtration, the colorless solution was directly used for the next reaction step.

TLC R_f = 0.30 (CH/EA = 4/1; UV and $KMnO_4$)

6.3.10 Synthesis of (*E*)-6-bromo-4,4-dimethylhex-2-enal (10)



10

Preparation of aldehyde **10** was conducted under exclusion of light. A flame-dried and nitrogen-flushed 100 mL three-neck round-bottom-flask with gas inlet was equipped with activated molecular sieves (4 Å) and the filtrated, colorless reaction solution of **9** (theoretical: 831.3 mg, 4.0 mmol, 1 eq). After addition of 1.74 g (20.1 mmol, 5 eq) activated MnO_2, the black, heterogeneous mixture was stirred overnight at RT. Quantitative conversion was detected by TLC (CH/EA = 4/1; UV and $KMnO_4$). The reaction mixture was filtrated through a pad of silica, which was rinsed with CH_2Cl_2 (ca. 900 mL). After removal of the solvent under reduced pressure, a colorless oil was obtained, which was kept under a constant stream of nitrogen and was directly used for the enzymatic assay without further purification.

Yield 690.8 mg (3.4 mmol, 84 %), colorless oil.

$C_8H_{13}BrO$ [205.10]

GC-FID t_R = 5.4 min

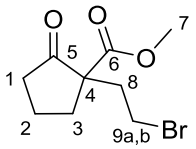
TLC R_f = 0.58 (CH/EA = 4/1; UV and $KMnO_4$)

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 9.53 (d, $^3J_{HH}$ = 7.6 Hz, 1H, H-1), 6.73 (d, $^3J_{HH}$ = 15.9 Hz, 1H, H-3), 6.05 (dd, $^3J_{HH}$ = 15.9 Hz, $^3J_{HH}$ = 7.6 Hz, 1H, H-2), 3.35 – 3.18 (m, 2H, H-8), 2.12 – 1.98 (m, 2H, H-7), 1.14 (s, 6H, H-5, H-6) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 194.1 (CH, C-1), 165.3 (CH, C-3), 130.2 (CH, C-2), 45.4 (CH_2, C-7), 38.6 (C_q, C-4), 27.9 (CH_2, C-8), 26.2 (CH_3, C-5, C-6) ppm.

HRMS (EI) calcd (m/z) for [M^+]: 204.0150; found: 204.0151.

6.3.11 Synthesis of methyl 1-(2-bromoethyl)-2-oxocyclopentane-1-carboxylate (11a)



11a

In a 500 mL two-neck round bottom flask, 4.8 mL (38.7 mmol, 1 eq) methyl 2-oxocyclopentane-1-carboxylate were dissolved in 250 mL distilled acetone. 16.04 g (116.1 mmol, 3 eq) K_2CO_3 were added to the colorless solution followed by 10.0 mL (116.1 mmol, 3 eq) 1,2-dibromoethane. The yellowish, heterogeneous mixture was heated under reflux until quantitative conversion was detected by GC-FID (5.5 h). The suspension was cooled to RT and filtrated through a sintered funnel. The yellow filtrate was concentrated under reduced pressure before excess 1,2-dibromoethane was removed by bulb-to-bulb distillation (~10 mbar, 60 °C). The resulting crude orange-brown oil was purified *via* flash chromatography (500 g silica, 20 x 8 cm; CH/EA = 10/1 (fraction 1-2), 5/1 (from fraction 3); fraction size: 250 mL).

Yield 5.04 g (20.2 mmol, 52 %), colorless crystals.

$C_9H_{13}BrO_3$ [249.10]

GC-FID t_R = 6.3 min

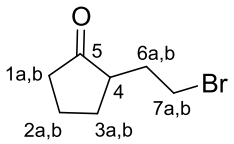
TLC R_f = 0.44 (CH/EA = 4/1; $KMnO_4$)

m.p. 37-39 °C

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.71 (s, 3H, H-7), 3.52 – 3.42 (m, 1H, H-9a), 3.40 – 3.25 (m, 1H, H-9b), 2.56 – 1.90 (m, 8H, H-1, H-2, H-3, H-8) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 213.6 (C_q, C-5), 170.9 (C_q, C-6), 60.3 (C_q, C-4), 52.9 (CH_3, C-7), 37.7 (CH_2, C-1), 37.2 (CH_2, C-8), 33.5 (CH_2, C-3), 27.7 (CH_2, C-9), 19.8 (CH_2, C-2) ppm.

6.3.12 Synthesis of 2-(2-bromoethyl)cyclopentan-1-one (12a)



12a

In a 50 mL round bottom flask, 5.04 g (20.2 mmol, 1 eq) cyclopentanone derivative **11a** and 20 mL HBr (48%) were heated under reflux. After 25 min the evolution of gas ceased indicating full conversion. The previously orange solution changed its color to greenish-black. The reaction solution was diluted with H_2O (20 mL) and solid $NaHCO_3$ was added until a pH of 7 was obtained. The aqueous layer was extracted with Et_2O (3 x 40 mL) and the combined organic layers were washed with saturated $NaHCO_3$ solution (1 x 100 mL) and brine (1 x 100 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude brownish fluid was purified *via* flash chromatography (175 g silica, 20 x 5 cm; *n*-pentane/Et_2O = 7/1 (fraction 1), 5/1 (fraction 2 - 13), 3/1 (from fraction 14); fraction size: 100 mL).

Yield 3.20 g (16.7 mmol, 83 %), yellowish oil.

$C_7H_{11}BrO$ [191.07]

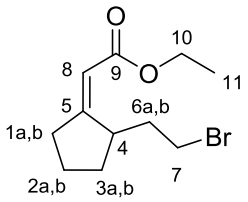
GC-FID t_R = 5.1 min

TLC R_f = 0.46 (CH/EA = 4/1; $KMnO_4$ and UV)

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 3.67 – 3.51 (m, 1H, H-7a), 3.53 – 3.36 (m, 1H, H-7b), 2.47 – 2.18 (m, 4H, H-1a, H-3a, H-4, H-6a), 2.18 – 1.94 (m, 2H, H-1b, H-2a), 1.91 – 1.69 (m, 2H, H-2b, H-6b), 1.59 – 1.40 (m, 1H, H-3b) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 220.1 (C_q, C-5), 47.9 (CH, C-4), 38.0 (CH_2, C-1), 33.0 (CH_2, C-6), 31.9 (CH_2, C-7), 29.5 (CH_2, C-3), 20.8 (CH_2, C-2) ppm.

6.3.13 Synthesis of ethyl (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetate (13a)



13a

In a flame-dried and nitrogen-flushed 250 mL three-neck round-bottom flask, a colorless solution of 2.4 mL (16.8 mmol, 2.7 eq) diisopropylamine in 130 mL dry THF was cooled to -40 °C (acetone/dry ice cooling bath). 5.2 mL (2.51 M in hexanes, 13.2 mmol, 2.1 eq) *n*-BuLi solution were added dropwise over 5 min. The slightly yellowish reaction solution was cooled to -78 °C and after 15 min stirring 3.0 mL (16.8 mmol, 2.7 eq) ethyl 2-(trimethylsilyl)acetate were added dropwise over 5 min. The prepared solution was stirred for 1 h at -78 °C. In a second flame-dried and nitrogen-flushed 250 mL three-neck round-bottom flask 1.20 g (6.3 mmol, 1 eq) ketone **12a** were dissolved in 20 mL dry THF and the resulting colorless solution was also cooled to -78 °C. The solution of deprotonated ethyl 2-(trimethylsilyl)acetate was then slowly added to the solution of **12a** *via* cannula over 1.5 h while keeping both solutions at -78 °C. The cannula was rinsed with dry THF (1 x 5 mL). After stirring for another 1 h at -78 °C quantitative conversion was detected by GC-MS. The reaction mixture was quenched at -78 °C by addition of 30 mL saturated NH_4Cl solution and was then allowed to warm to RT. After separation of the layers, the aqueous phase was extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with brine (1 x 100 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude yellowish oil was purified *via* flash chromatography (250 g silica, 18 x 6 cm; CH/EA = 50/1 (fraction 1 - 7), 40/1 (from fraction 8); fraction size: 150 mL).

Yield 681.2 mg (2.6 mmol, 42 %), pale yellowish oil.

$C_{11}H_{17}BrO_2$ [261.16]

GC-FID t_R = 6.4 min

GC-MS t_R = 5.93 min

m/z = 260.0 (62 %) [M^+], 232.0 (14 %) [M^+-C_2H_5], 215.0 (42 %) [M^+-C_2H_5O], 153.1 (25 %) [M^+-C_2H_4Br], 107.1 (95 %) [$C_2H_4Br^+$], 79.1 (100 %).

TLC R_f = 0.22 (CH/EA = 50/1; $KMnO_4$ and UV)

^{1}H-NMR (300.36 MHz, $CDCl_3$) δ = 5.77 (s, 1H, H-8), 4.16 (q, $^3J_{HH}$ = 7.1 Hz, 2H, H-10), 3.53 – 3.35 (m, 3H, H-4, H-7), 2.56 – 2.30 (m, 2H, H-1a,b), 2.25 – 2.13 (m, 1H, H-6a), 1.85 – 1.61 (m, 5H, H-2a,b, H-6b, H-3a,b), 1.28 (t, $^3J_{HH}$ = 7.1 Hz, 3H, H-11) ppm.

^{13}C-NMR (75.53 MHz, $CDCl_3$) δ = 170.6 (C_q, C-5), 166.3 (C_q, C-9), 112.9 (CH, C-8), 59.8 (CH_2, C-10), 41.9 (CH, C-4), 37.5 (CH_2, C-6), 34.9 (CH_2, C-1), 31.8 (CH_2, C-7), 31.3 (CH_2, C-3), 22.8 (CH_2, C-2), 14.5 (CH_2, C-11) ppm.

HRMS (EI) calcd (m/z) for [M^+]: 260.0412; found: 260.0439.

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8 Abbreviations

Analytical methods:

^{13}C-NMR	carbon NMR
^{1}H-NMR	proton NMR
APT	attached proton test
Ar	aryl
C_q	quaternary carbon
bs	broad singlet
d	doublet
dd	doublet of a doublets
ddd	doublet of doublet of doublets
DI	direct
EI	electron impact ionization
eV	electron volt
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GC-FID	gas chromatography flame ionization detector
H,H-COSY	H,H correlated spectroscopy
HRMS	high resolution mass spectrometry
HSQC	hetero single quantum coherence
Hz	Hertz
J	coupling constant
m	multiplet
m.p.	melting point
m/z	mass to charge ratio
MHz	mega Hertz
NOESY	Nuclear Overhauser effect spectroscopy
ppm	chemical shift
R_f	ratio of fronts
RT	room temperature
s	singlet

t	triplet
TLC	thin layer chromatography
t_R	retention time
UV	ultra violet
δ	chemical shift

Chemical abbreviations:

Ac	acetyl
Bu	butyl
CH	cyclohexane
DCM	dichloromethane
DIBAL-H	diisobutylaluminium hydride
DIPA	diisopropyl amine
DMAP	4-dimethylaminopyridine
DME	dimethoxyethane
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
Et	ethyl
EA	ethyl acetate
EtOH	ethanol
Hal	halide
HWE	HORNER-WADSWORTH-EMMONS
LDA	lithium diisopropylamide
Me	methyl
NG	nitoglycerin
OEt	ethoxy
OMe	methoxy
OTf	triflate
pHBA	*para*-hydroxybenzaldehyde
pNP	*para*-nitrophenol
THF	tetrahydrofuran
TMS-Cl	trimethyl silyl chloride

TNT	2,4,6-trinitrotoluene

Biological abbreviations:

ADH	alcohol dehydrogenase
Arg	arginine
ER	enoate reductase
F	phenylalanine
FMN	flavin mononucleotide
GDH	glucose dehydrogenase
His	histidine
JA	jasmonic acid
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
OPC-8:0	3-oxo- 2(2'[*Z*]-pentenyl)-cyclopentane-1-octanoate
OPDA	9*S*,13*S*-12-oxo-phytodienoate
OPR3	12-oxophytodienoate reductase 3
OYE	old yellow enzyme
Phe	phenylalanine
R	arginine
Trp	tryptophan
Tyr	tyrosine
W	tryptophan
WT	wild type
Y	tyrosine

Others:

Å	angstrom
%	percentage
°C	degree Celsius
µg	microgram
µL	microliter
µmol	micromol

abs.	absolute
cm	centimeter
conc.	concentrated
conv.	conversion
d	days
ee	enantiomeric excess
eq	equivalent
et al.	et alii
g	gram
h	hours
M	molar
mbar	millibar
mg	milligram
min	minute
mL	milliliter
mmol	millimol
nm	nanometer
w%	percentage by weight

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10 Appendix

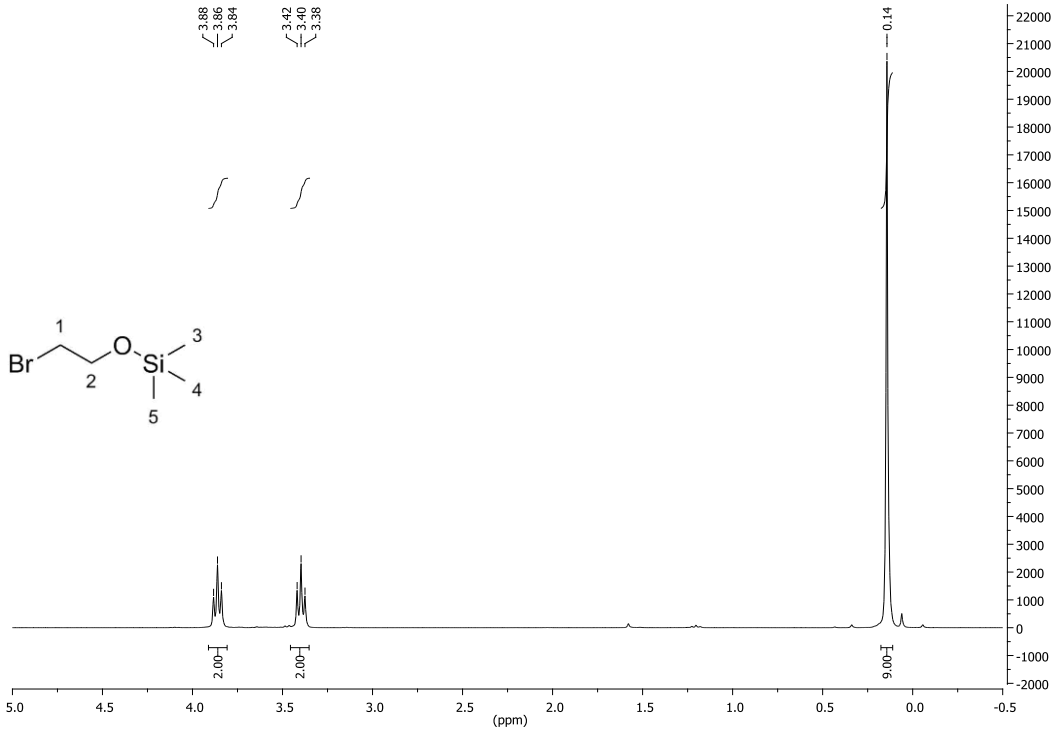

Figure 9 ^{1}H-NMR of (2-bromoethoxy)trimethylsilane (**1**).

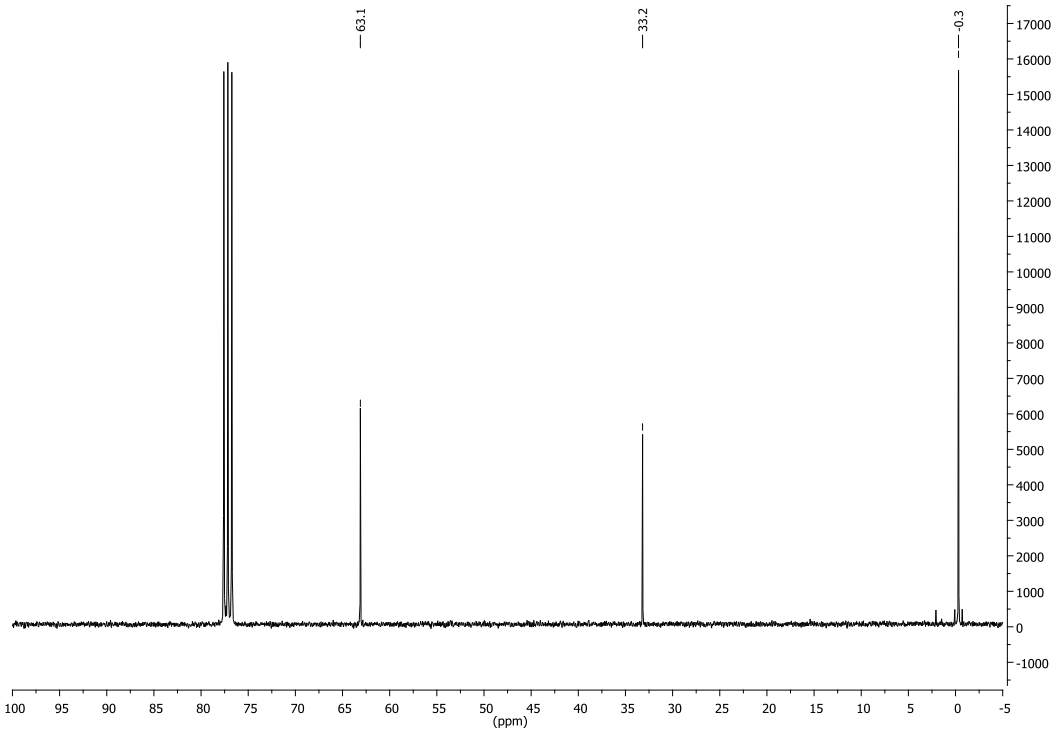

Figure 10 ^{13}C-NMR of (2-bromoethoxy)trimethylsilane (**1**).

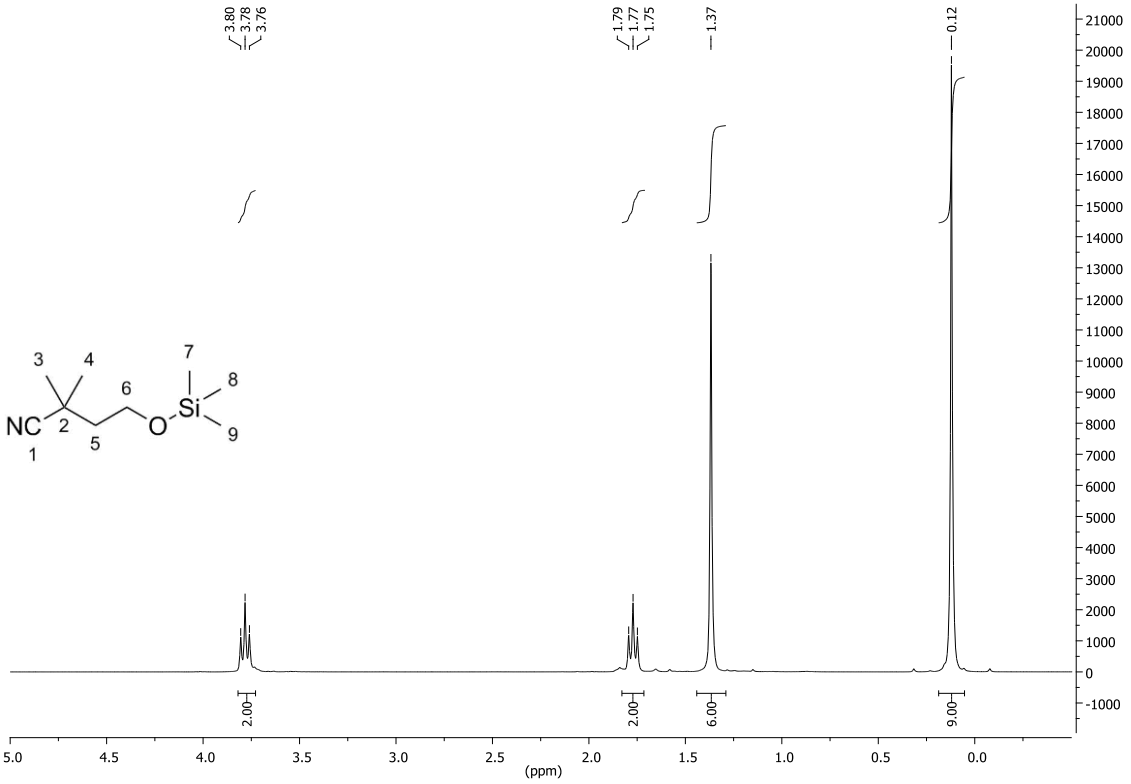

Figure 11 ^{1}H-NMR of 2,2-dimethyl-4-((trimethylsilyl)oxy)butanenitrile (**2**).

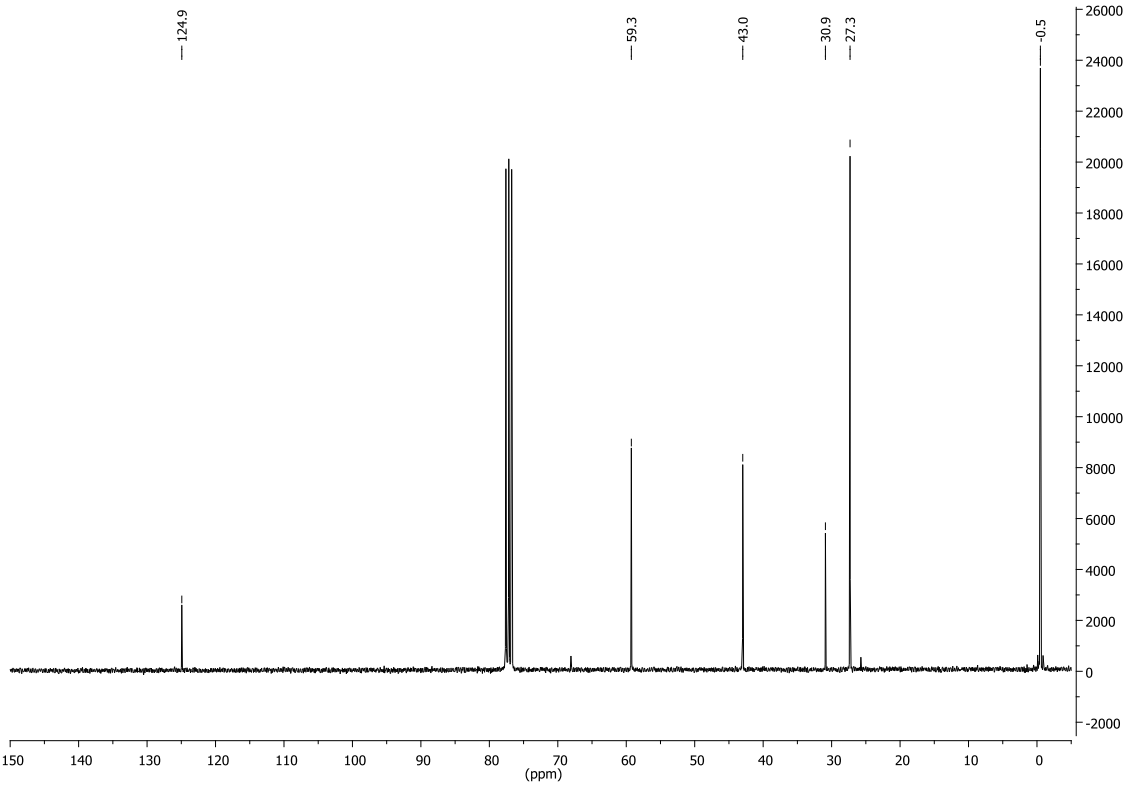

Figure 12 ^{13}C-NMR of 2,2-dimethyl-4-((trimethylsilyl)oxy)butanenitrile (**2**).

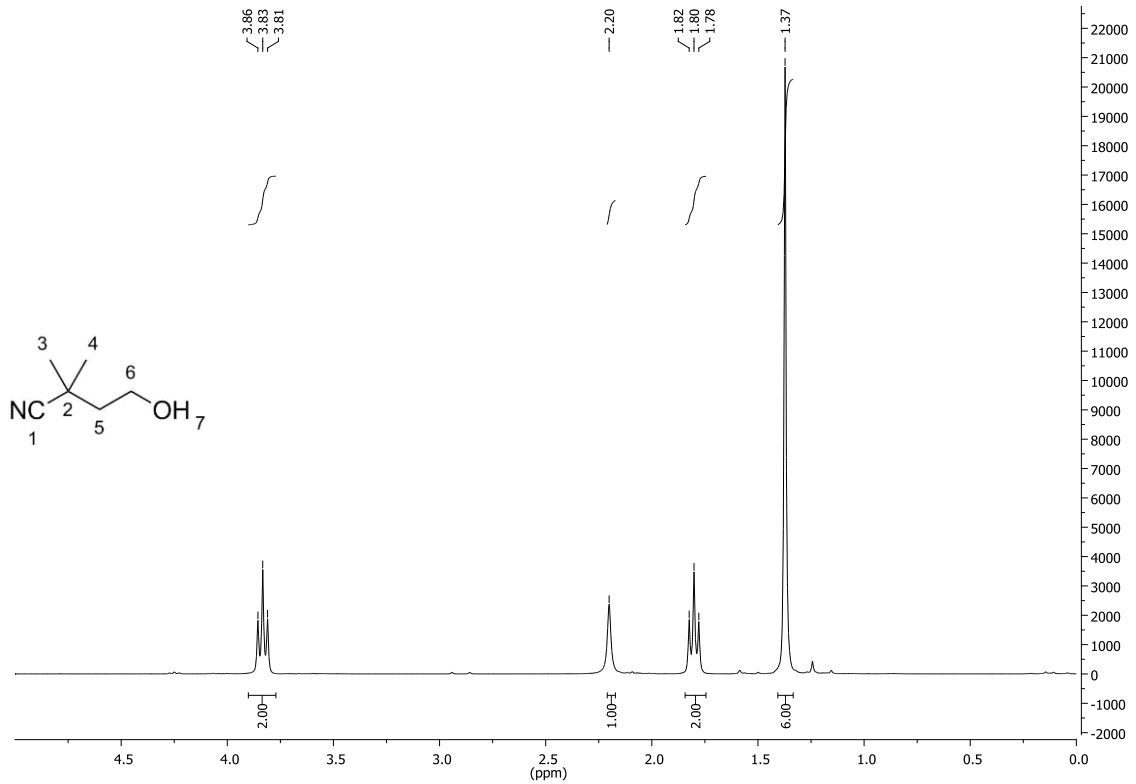

Figure 13 ^{1}H-NMR of 4-hydroxy-2,2-dimethylbutanenitrile (**3**).

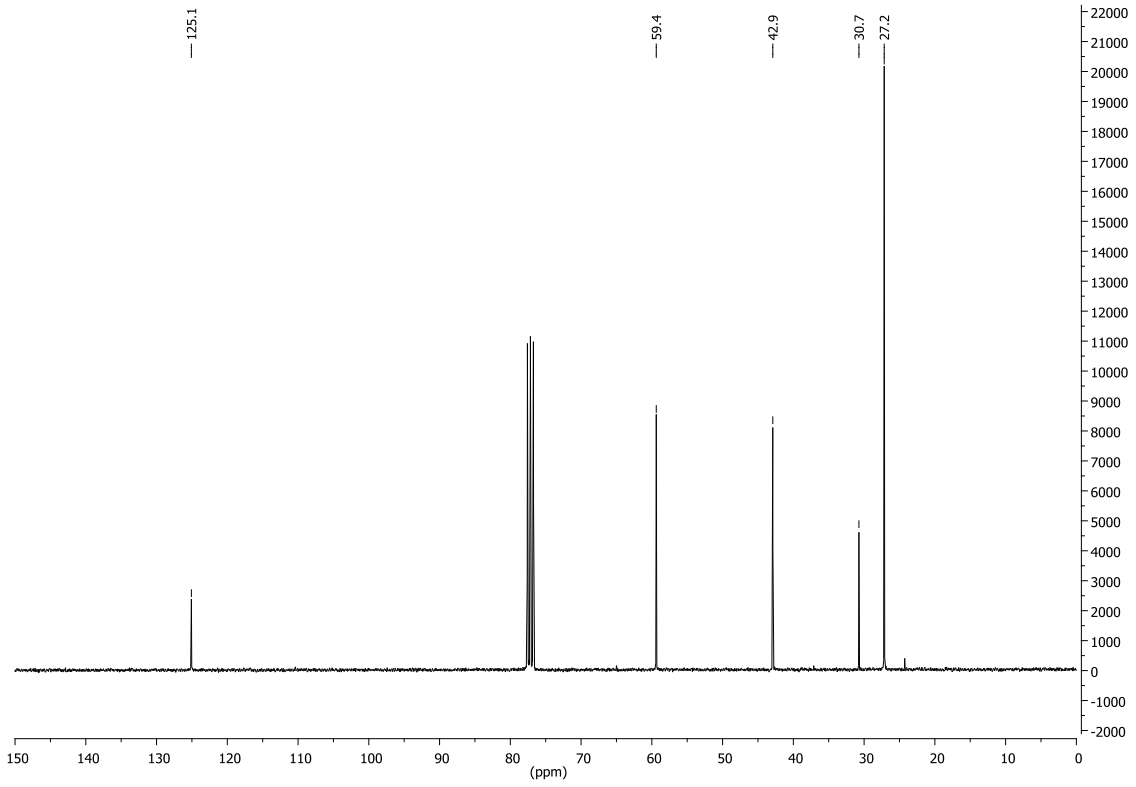

Figure 14 ^{13}C-NMR of 4-hydroxy-2,2-dimethylbutanenitrile (**3**).

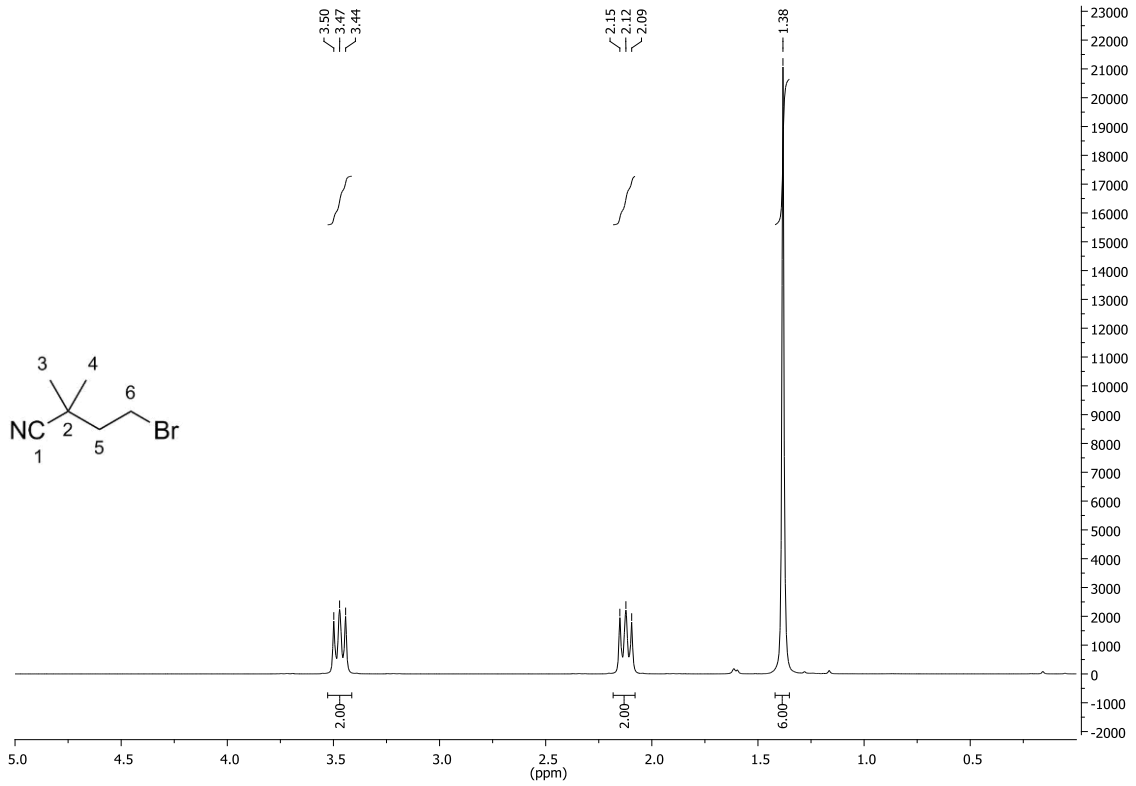

Figure 15 ^{1}H-NMR of 4-bromo-2,2-dimethylbutanenitrile (**4**).

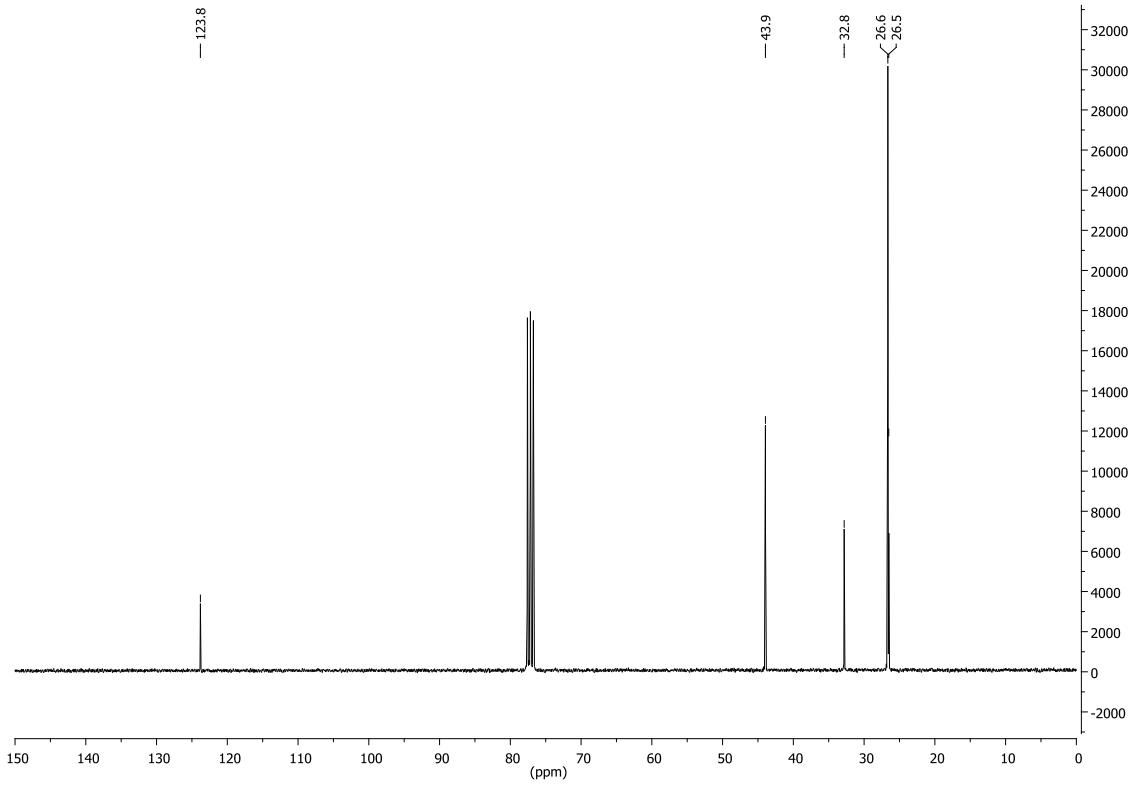

Figure 16 ^{13}C-NMR of 4-bromo-2,2-dimethylbutanenitrile (**4**).

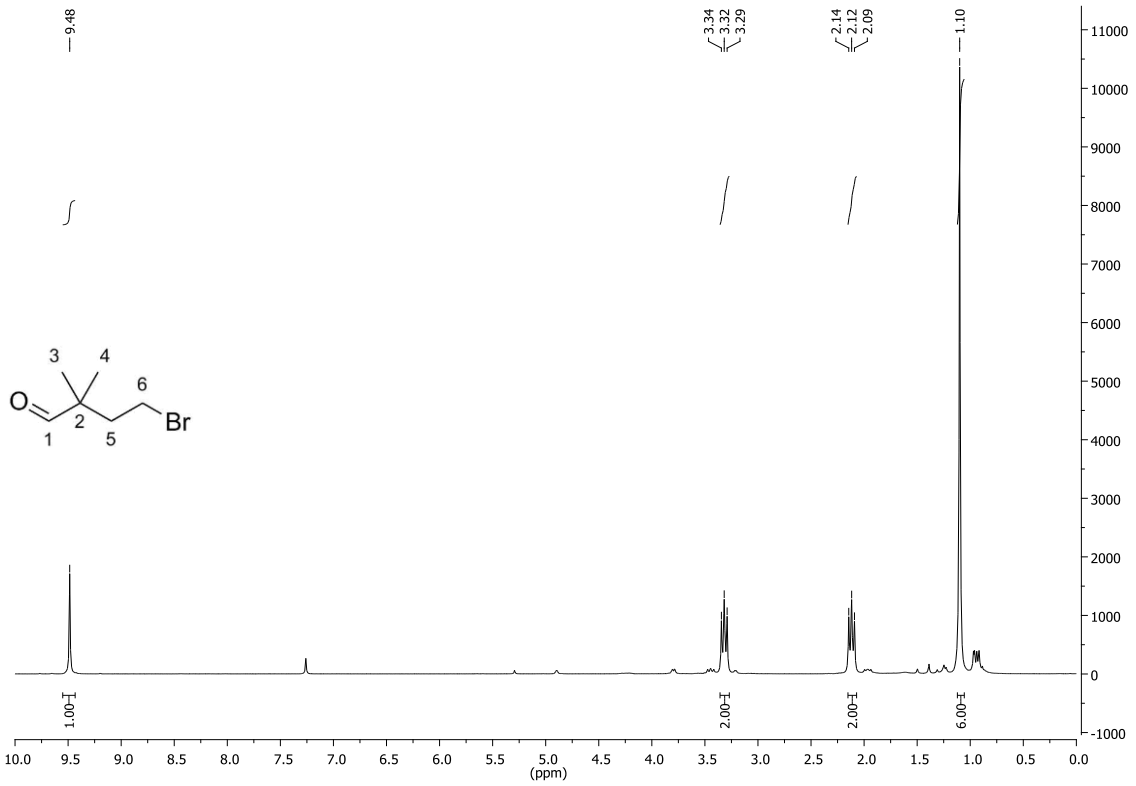

Figure 17 ^{1}H-NMR of 4-bromo-2,2-dimethylbutanal (**5**).

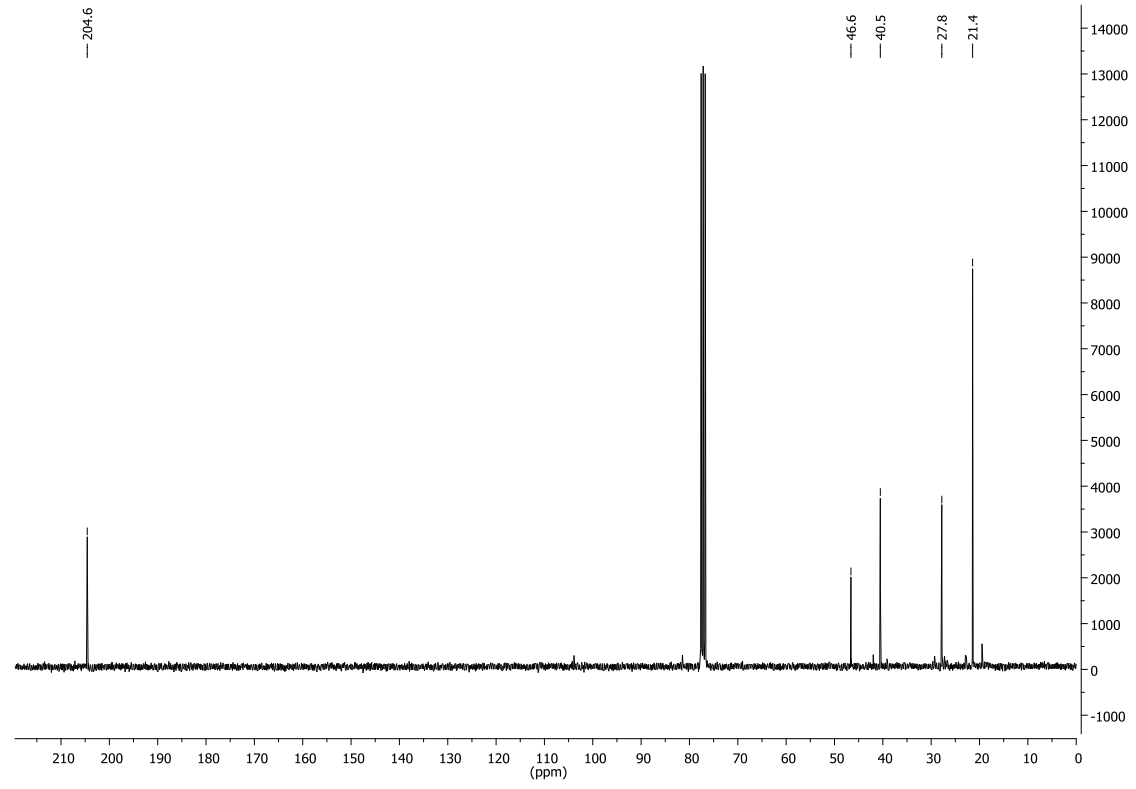

Figure 18 ^{13}C-NMR of 4-bromo-2,2-dimethylbutanal (**5**).

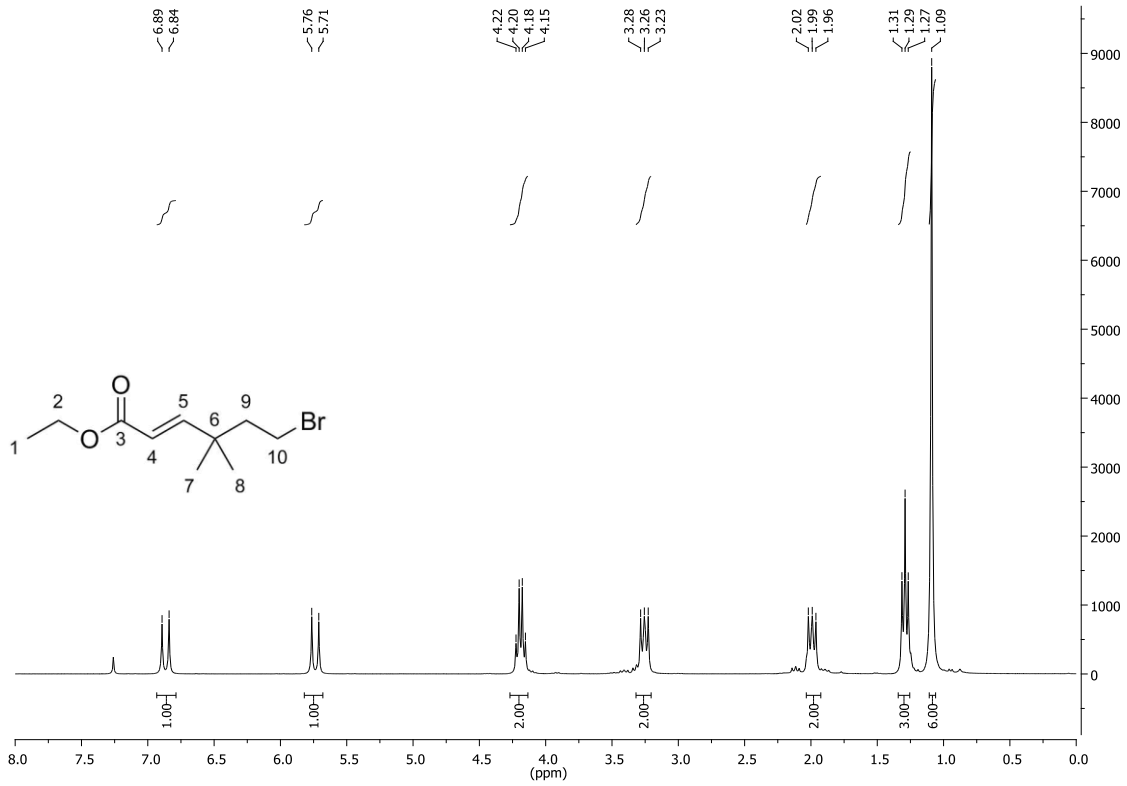

Figure 19 ^{1}H-NMR of ethyl (*E)*-6-bromo-4,4-dimethylhex-2-enoate (**8**).

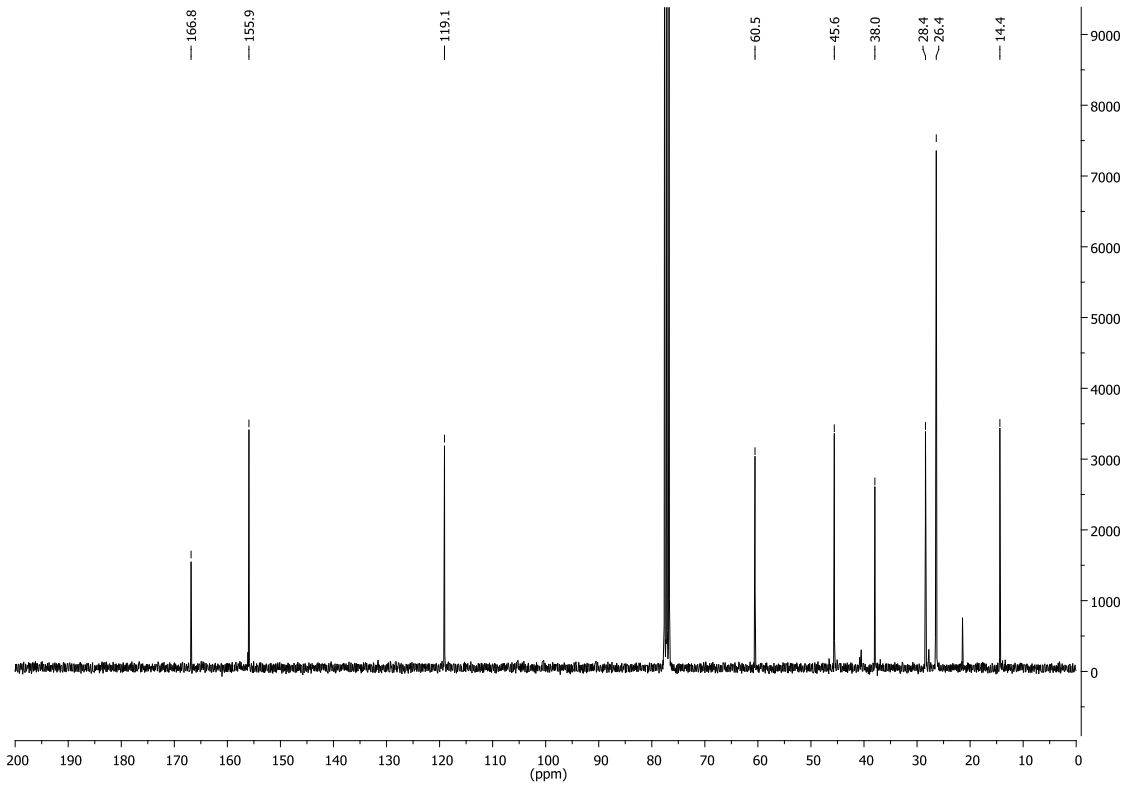

Figure 20 ^{13}C-NMR of ethyl (*E)*-6-bromo-4,4-dimethylhex-2-enoate (**8**).

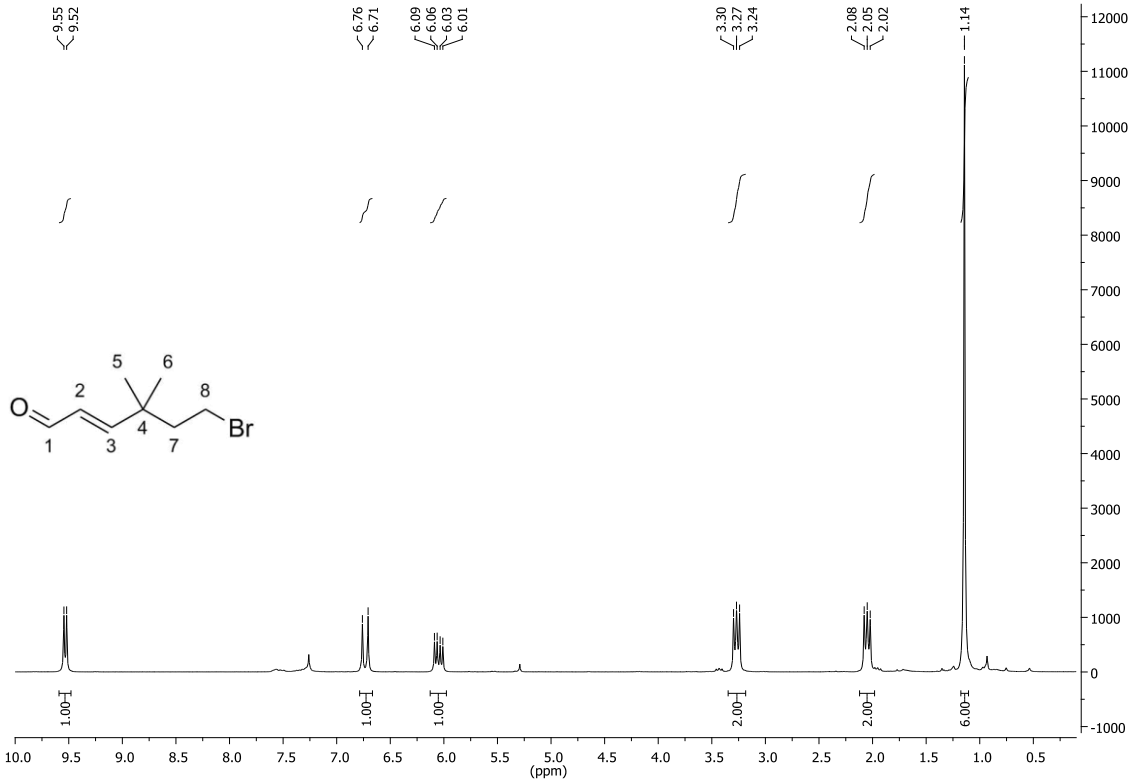

Figure 21 ^{1}H-NMR of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**).

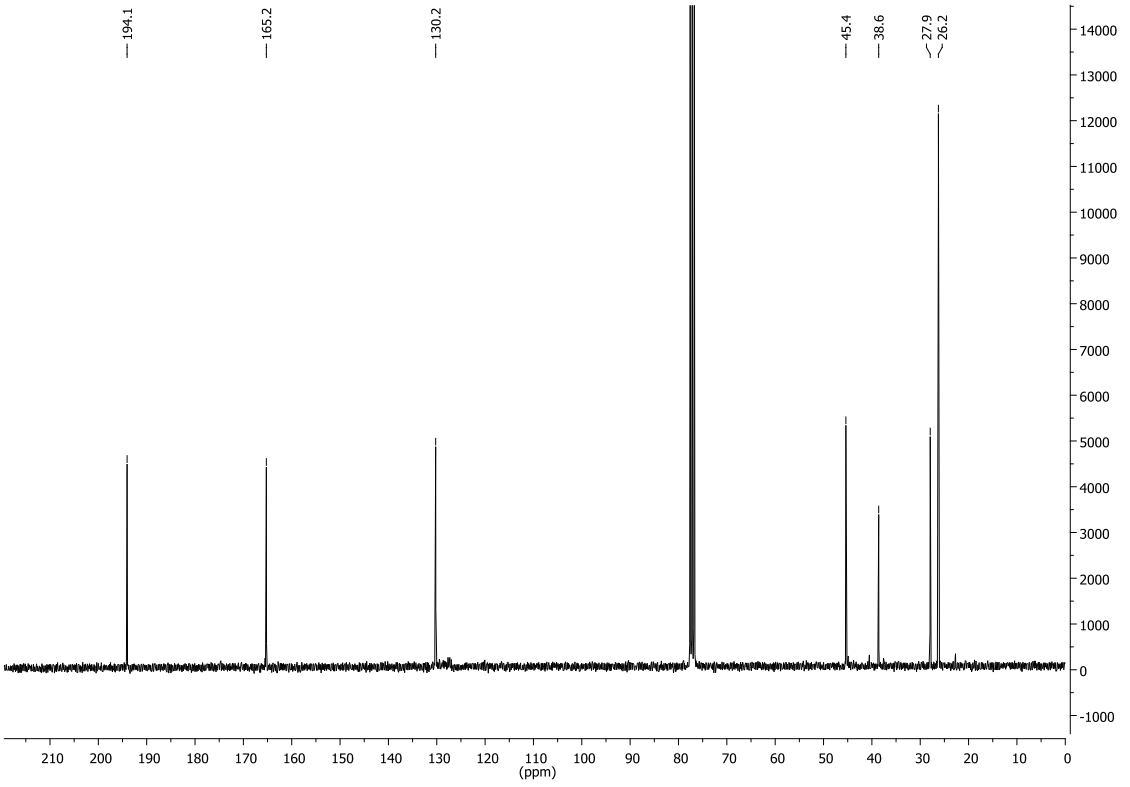

Figure 22 ^{13}C-NMR of (*E*)-6-bromo-4,4-dimethylhex-2-enal (**10**).

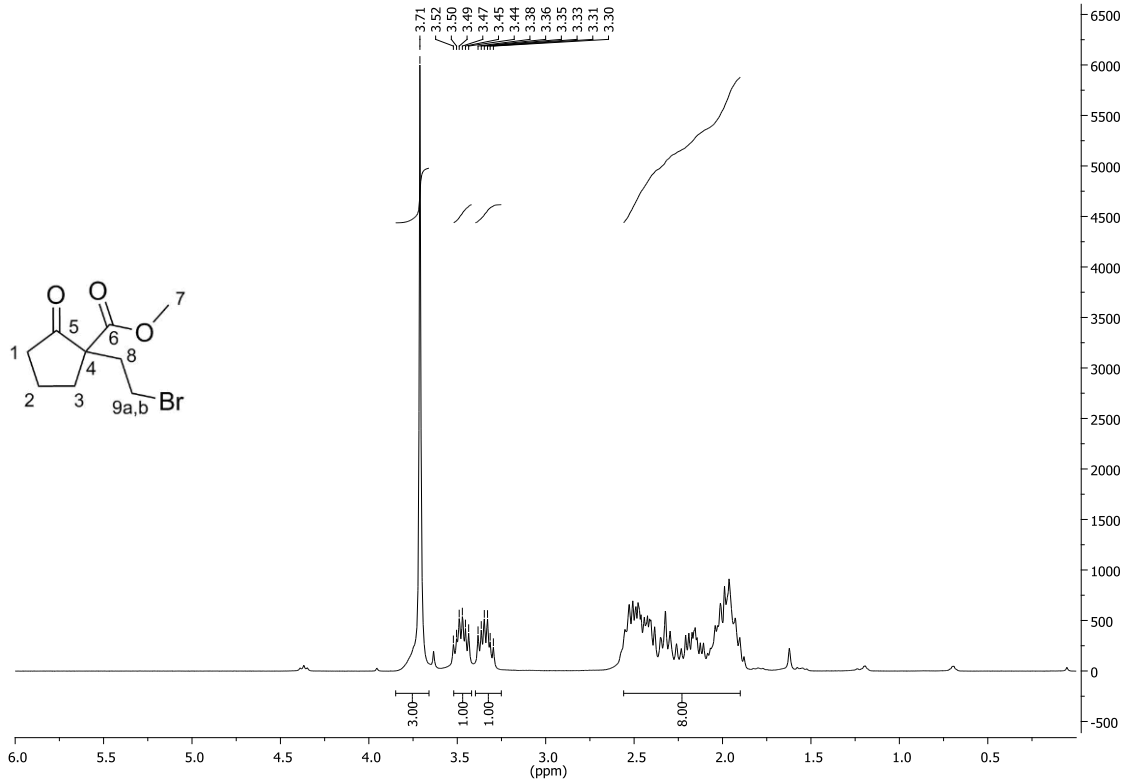

Figure 23 ^{1}H-NMR of methyl 1-(2-bromoethyl)-2-oxocyclopentane-1-carboxylate (**11a**).

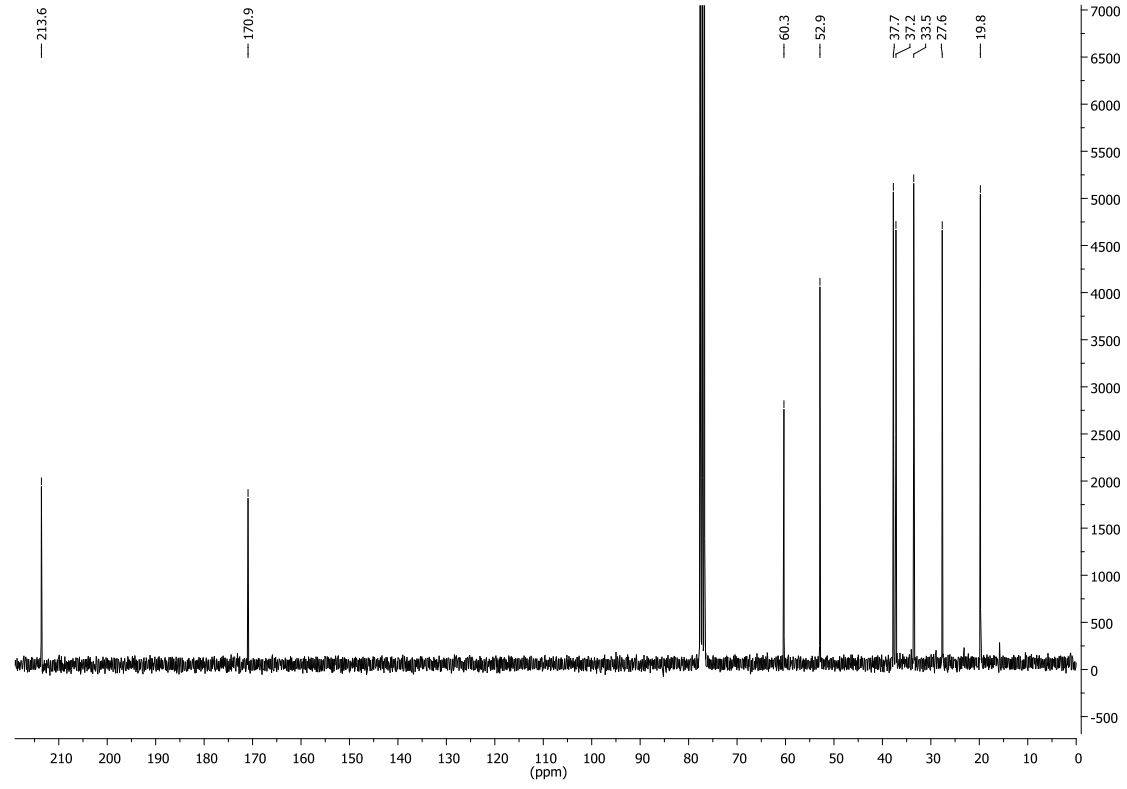

Figure 24 ^{13}C-NMR of methyl 1-(2-bromoethyl)-2-oxocyclopentane-1-carboxylate (**11a**).

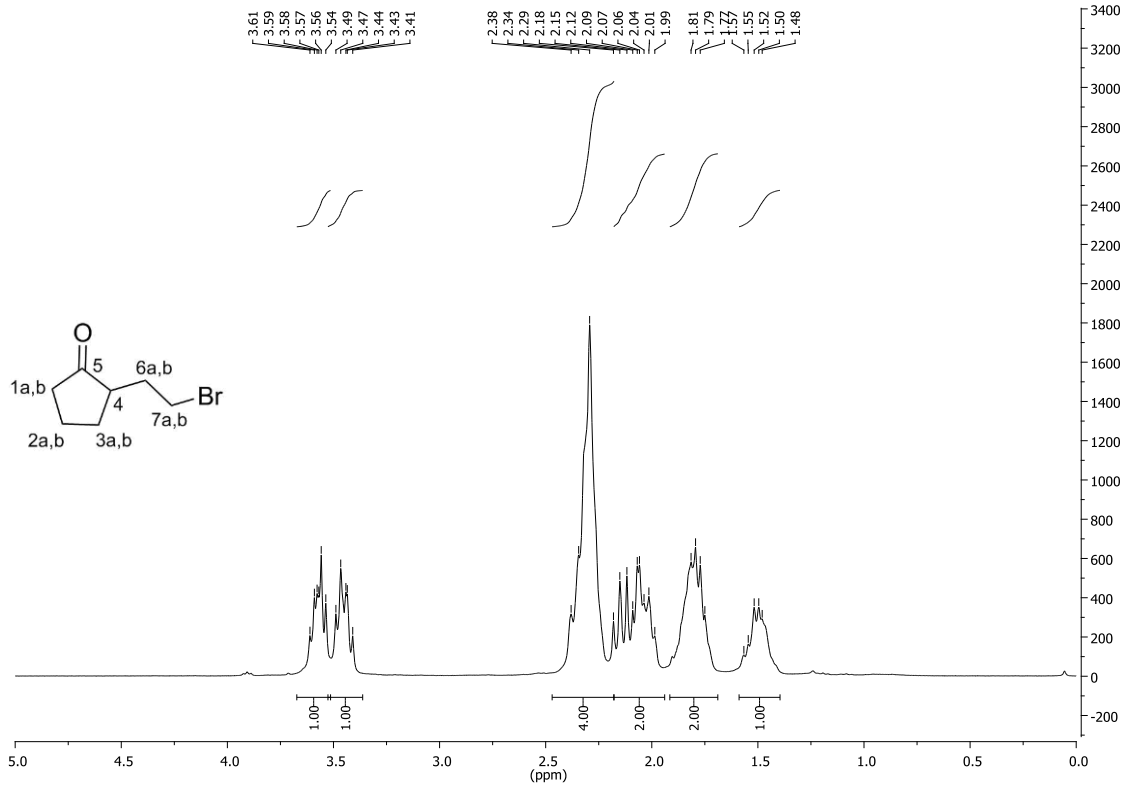

Figure 25 ^{1}H-NMR of 2-(2-bromoethyl)cyclopentan-1-one (**12a**).

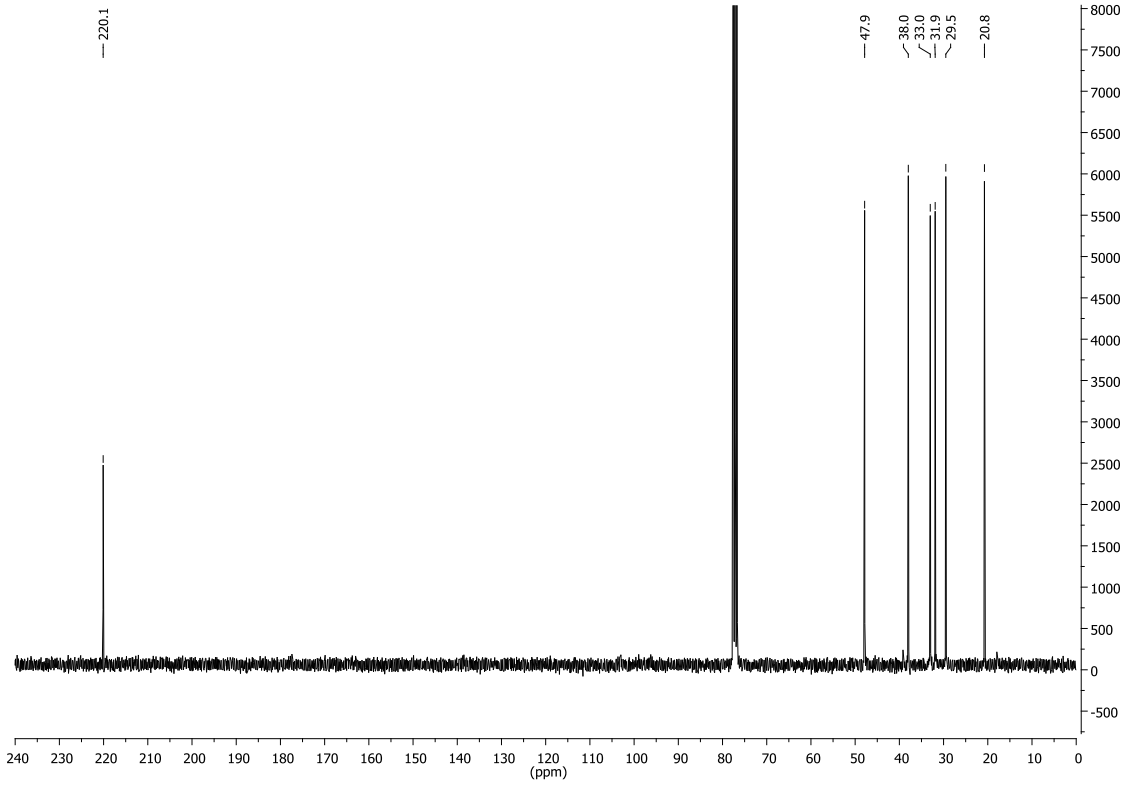

Figure 26 ^{13}C-NMR of 2-(2-bromoethyl)cyclopentan-1-one (**12a**).

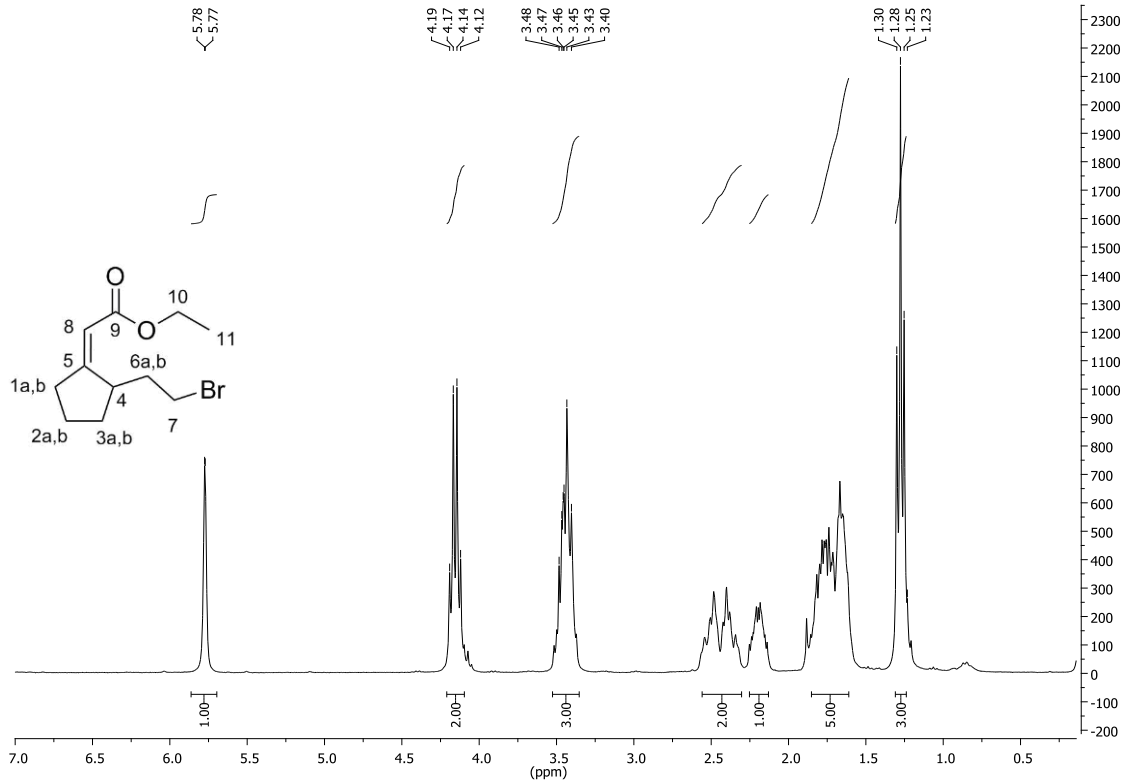

Figure 27 ^{1}H-NMR of ethyl (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetate (**13a**).

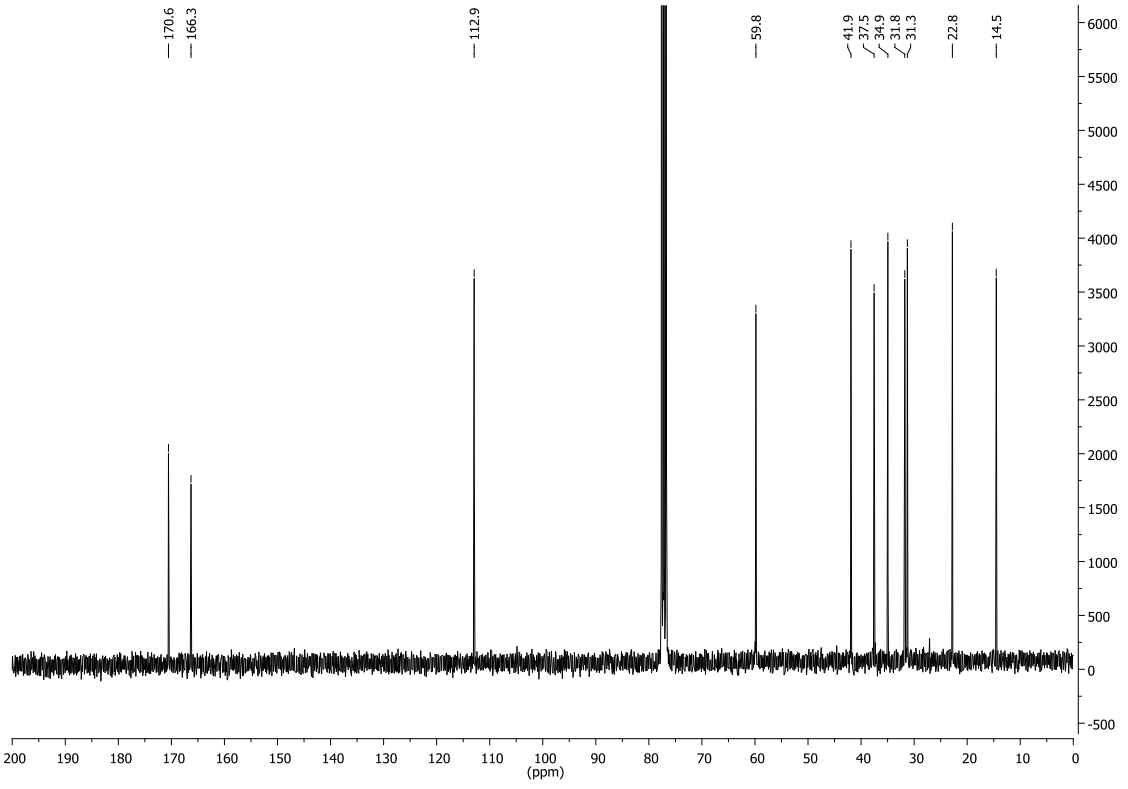

Figure 28 ^{13}C-NMR of ethyl (*Z*)-2-(2-(2-bromoethyl)cyclopentylidene)acetate (**13a**).