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Heterologous expression and functional analysis of cytochrome P450s that are involved in the biosynthesis of defensive compounds in the leaf beetle *Phaedon cochleariae*

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List of abbreviations

3-NPA	3-nitropropionic acid
°C	degrees Celsius
ATP	adenosine triphosphate
bp	base pair
C480	P450 protein, candidate for conversion of β-alanine
C573	CPR, coexpressed with C573
C7758	P450 protein, candidate for conversion of geraniol
cDNA	complementary DNA
CPR	cytochrome P450 reductase
CYP	cytochrome P450
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
g	gram
GC-MS	gas chromatography–mass spectrometry
h	hour
HPLC-MS	high-performance liquid chromatography–mass spectrometry
k	kilo
kb	kilo base pairs
Da	Dalton
l	litre
A	ampere
min	minute
m	milli
mRNA	messenger RNA (Ribonucleic acid)
m/z	mass-to-charge ratio
M	molar
µ	micro
n	nano
NADPH	nicotinamide adenine dinucleotide phosphate
-OH	-hydroxy
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
rpm	rounds per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfat
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TAE	tris, actetate, EDTA
TBS	tris-buffered saline
V	voltage

4. Abstract

Phaedon cochleariae developed two chemical defensive tactics to defend itself from predators. One is based on the toxic 3-nitropropionic acid (3-NPA), which is stored as isoxazolin-5-one derivative and can be found in every life stage of *P. cochleariae*, while the other tactic relies on the volatile deterrent chrysomelidial, which is secreted by the larvae' glandular reservoir upon disturbance. Previous studies have shown two P450s (C7758 and C480) are involved in the biosynthetic pathways of these two compounds, with C7758 related to the production of chrysomelidial while C480 participating in the biosynthesis of isoxazolin-5-one and its 3-NPA derivate. In this study, those two P450s as well as a cytochrome P450 reductase (CPR) were subcloned into mammal expression vector and heterologous expressed in HEK293T cells. The microsomes containing corresponding recombinant proteins were isolated, and their catalytic function against presumable substrates were analysed. Enzymatic analysis showed C7758 converted geraniol to 8-OH-gernaiol and C480 showed no activity to free β-alanine.

5. Zusammenfassung

Phaedon cochleariae hat zwei chemische Verteidigungstaktiken entwickelt und sich vor Prädatoren zu verteidigen. Eines basiert auf der toxischen 3-Nitropropionsäure, welches als Isoxazolin-5-one Derivat in jedem Entwicklungsstadium von *P. cochleariae* gefunden werden kann, während die andere Taktik auf das flüchtige, abschreckende Chrysomelidial beruht, welches durch Beunruhigung von der Larve aus den glandulären Reservoiren sekretiert wird. Vorrangehende Studien zeigten zwei P450-Enzyme (C7758 und C480) auf, welche in die biosynthetischen Stoffwechselwegen dieser zwei Komponenten involviert sind. C7758 wird dabei der Produktion von Chrysomelidial zugeordnet, während C480 an der Biosynthese von Isoxazolin-5-one und deren Derivate der 3-Nitropropionsäure teilnimmt. In dieser Arbeit wurden diese zwei P450-Enzymen sowie eine NADPH-Cytochrom-P450 Oxidoreduktase (CPR) in einen Säugetier-Expressionsvektoren kloniert und heterolog in HEK-293T-Zellen exprimiert. Die Mikrosomen, welche die zugehörigen rekombinanten Proteine beinhalten, wurden isoliert und deren katalytische Funktionen auf vermutete Substrate geprüft. Enzymatische Untersuchungen zeigten, dass C7758 Geraniol zu 8-OH-Geraniol umwandelt und C480 keine Aktivität in Anwesenheit von freien β-Alanin aufweist.

6. Introduction

Of all described animal species about 25% belong to the order Coleoptera. Beetles are ubiquitous and extremely diverse. They can be divided into the four suborders: Adephaga, Archostemata, Myxophaga and Polyphaga. About 85% of the beetle species belong to the suborder Polyphaga. The species of this suborder vary greatly in size, shape, habit and habitat, and are mostly found on leaves and flowers[1].

The leaf beetle family Chrysomelidae in the suborder Polyphaga consist of at least 35000 species, which vary greatly in size and color. Its distribution reaches all over the world (except Antarctica) and some species are known to be the "world's worst economic pests", while others are used as biological control agents against weeds[2].

Phaedon cochleariae belongs to the family Chrysomelidae. Their distribution includes most of the northern hemisphere. It is known as a pest on different cruciferous crops, in particular watercress, with all the active stages feeding on the same host[3].

To defend itself from predators *P. cochleariae* uses two different chemical defensive tactics. One involves toxic component (3-nitropropanonic acid) that is found in all life stages, while the other depends on the volatile defensive compound (chrysomelidial) that is stored in the glanular' glandular reservoirs.

3.1. Deterring defensive strategy: Chrysomelidial

Juvenile *P. cochleariae* secrete volatile defensive compounds in droplets through the eversion of nine pairs of dorsal glandular reservoirs, located on abdominal and thoracal segments[4], when disturbed[5]. The content of the droplets has an antimicrobial effect[6] and a repellent effect on predators like arthropods and birds[7]. Major components of volatile defensive compounds are monoterpene iridoids, like chrysomelidial[7].

The biosynthesis of chrysomelidial begins with isopentenyl diphosphate and dimethylallyl diphosphate, which derive from the mevalonate pathway[8]. It takes place in the fat body[8]. The isoprenyl diphosphate synthetase 1 (IDS1) catalyzes the allkylation of isopentenyl diphosphate and dimethylallyl diphosphate into geranyl diphosphate[8, 9]. Geranyl diphosphate is converted into geraniol and subsequently into 8-hydroxygeraniol. Until now no enzymes were described for those two reaction steps. A phosphatase and a P450 protein are promising candidates for the dephosphorylation of geranyl diphosphate and the ω-hydroxylation of geraniol[8]. A glycosylation of 8-hydroxygeraniol is required for the transport from the fat body through the hemolymph to the glandular reservoir[10]. A glandular β-glucosidase converts the 8-hydroxygeraniol-O-β-D-glucoside back into 8-hydroxygeraniol, which is afterwards oxidized into 8-

oxogeranial by 8-hydroxygeraniol oxidoreductase[8, 10]. The final step is the cyclization of 8-oxogeranial to chrysomelidial (Fig. 1)[8].

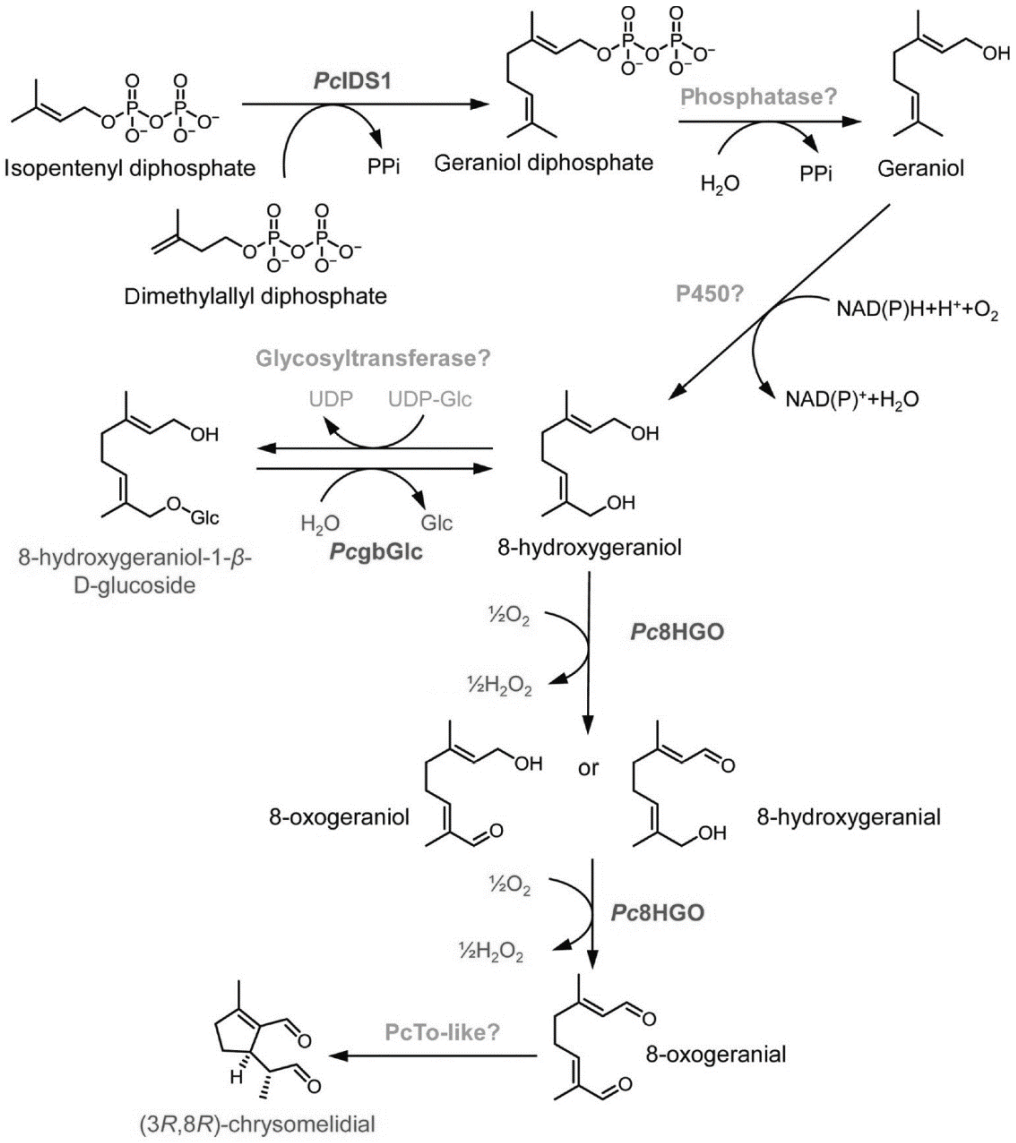


Figure 2: Proposed biosynthesis of chrysomelidial in *P. cochleariae*

3.2. Toxic defensive strategy: Isoxazolin-5-one and its 3-nitropropionic acid derivatives

The 3-nitropropinate (3-NPA) ester of isoxazolin-5-one can be found in the hemolymph or yolk (for eggs) of every life stage and act as a defensive secretion in adults' exocrine glands, that are located laterally on the elytra and the pronotum of beetles[11, 12] .

When been ingested by a predator, the ester bond between isoxazolin-5-one-glucoside and 3-NPA is cleaved, releasing the neurotoxic 3-NPA. Due to its structural resemblance to succinic acid 3-NPA can bind to the catalytic center of the succinate

dehydrogenase, an enzyme found in complex II in the mitochondrial membrane[11, 13]. The covalent interaction of 3-NPA with the catalytic center of succinate dehydrogenase leads to irreversible inhibition of the citric acid cycle[7] and the electron transport chain[14], which are two major energy producing pathways in most aerobic organisms. Therefore, 3-NPA leads to neurodegeneration with effects mostly involving dysfunctional movement comparable to the symptoms of Huntington's disease[11]. Until now, no biological function for isoxazolin-5-one-glucoside was observed, whereas other derivates of isoxazolin-5-one have antifungal properties[11].

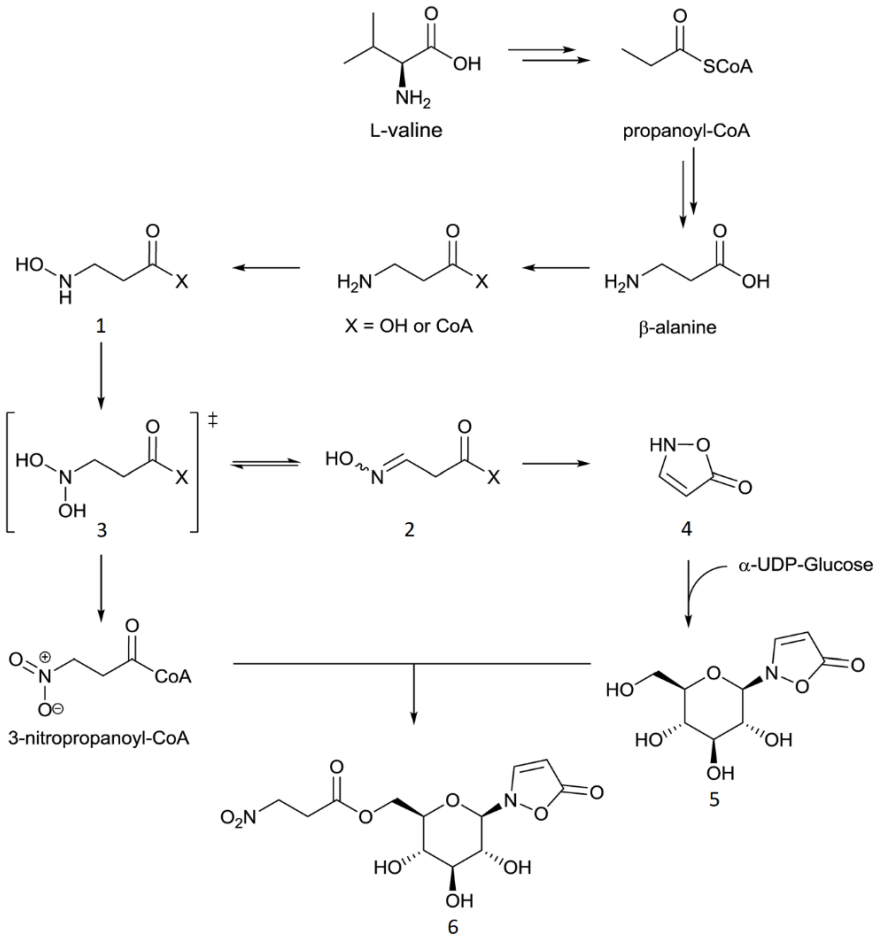


Figure 2: Proposed biosynthetic pathway for Isoxazolin-5-one-glucoside and its 3-NPA ester

The proposed biosynthetic pathway for these compounds is as follows. First the oxidation of the amino acid precursors, e.g. valine, leads to propanoyl-CoA, which subsequently is converted into β-alanine[15]. Then the consecutive oxidation of the amino group of β-alanine results in (N-hydroxyamino)-propanic acid and the corresponding oxime, (N-hydroxyimino)-propanic acid or 3-nitropropanoly-CoA[15]. Afterwards (N-hydroxyimino)-propanic acid is cyclized to 3-isoxazolin-5-one, which reacts further with α-UDP-Glucose to from isoxazolin-5-one-glucoside[11]. In the final

step, isoxazolin-5-one-glucoside is esterified with 3-nitropropanoly-CoA under the consumption of ATP (Fig. 2)[11].

3.3. Cytrochrome P450

Cytochrome P450 (CYP) are unspecific monooxygenases found every class of organisms[16]. CYPs are membranes proteins, mostly found in the endoplasmic reticulum in invertebrates[17]. They are mostly involved in biosynthetic and degenerative pathways of secondary metabolites like alkaloids, bile acids, eicosanoids, fatty acids, steroid hormones, terpenoids, vitamin D3 and xenobiotics[17, 18]. A general reaction scheme for a CYP metabolited reaction is:

$RH + O_2 + NAD(P)H \rightarrow ROH + H_2O + NAD(P)^+$ [19]

In general, the reaction cycle is as follows. First, the substrate binds proximal to the active site of the heme sitting in the center of the CYP, replacing the resting water[20]. The conformation of the enzyme changes leading to the transfer of an electron from NAD(P)H via the cytochrome P450 reductase (CPR), which reduces the iron from a ferric to a ferrous state[21, 22]. Molecular oxygen (O_2) binds to the heme, resulting in a ferrous dioxygen adduct. Afterwards, a second electron is transferred from NAD(P)H and a peroxoiron(III)complex is formed[21]. The peroxo group is protonated twice, cleaving the bond between the oxygens and releasing a water molecule (H_2O) and forming the highly reactive compound I, an iron-(IV) porphyrin cation radical[20]. The remaining iron-oxo complex transfers the second oxygen to the substrate. The hydroxylated substrate dissociates and the P450 returns to its original state[21, 22].

3.4. Aim of study

Prelimary work has shown that two *P. cochleariae* cytochrome P450, C7758 and C480, are involved in the biosynthetic pathways of the two defensive lines mentioned above. C7758 participates in the biosynthesis of chrysomelidial and C480 is involved in the biosynthesis of isoxazolin-5-one and its 3-NPA derivate. This study focuses on the functional expression and characterization of the two cytochrome P450s.

4. Methods

4.1. Generating recombinant plasmids

4.1.1. RNA isolation

The RNA was isolated using the RNAqueous® Kit (Thermo Fisher Scientific), following the manufacture instructions. The concentration of the isolated RNA was measured with Nanodrop (Thermo Fisher Scientific), and the samples were stored at -80 °C until use.

4.1.2. Reverse Transcription of the RNA

The cDNA was synthesized using SuperScript™ III Reverse Transcriptase (invitrogene) and following the manufacture instructions. A 20 µl system was used with 400 ng RNA as template. The concentration of the synthetized cDNA was measured, and the samples were stored at -20 °C until use.

4.1.3. Producing blunt end PCR products

To amplify the C573 ORF and generate blunt end PCR products, Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used for the PCR. To facilitate the detection of the recombinant protein, two reverse primers, one with and one without stop codon, were used.

The final reaction volume was 10 µl.

Table 1. Primers

Primer	Sequence (5' → 3')
CpC573_pc_F	CACCATGGATGAGACAGAAGCGACC
CpC573_pIB_R(+)	CTAACTCCACACATCAGCAGAATATC
CpC573_pc_R(-)	ACTCCACACATCAGCAGAATATCG
BGH Reverse primer (ThermoFisher Scientific)	TAGAAGGCACAGTCGAGG

Table 2. PCR Mastermix and program

Master Mix for 1 sample:

5xHF buffer	1 µl
dNTP (10 mM)	0.2 µl
CpC573 pcDNA F	0.3 µl
CpC573 pcDNA R(+)/R(-)	0.3 µl
cDNA	0.3 µl
Phusion polymerase	0.1 µl
ddH_2O	7.8 µl

PCR program:

98 °C	2 min
98 °C	10 s
67 °C	20 s
72 °C	1 min 50 s
72 °C	5 min

Gel electrophoresis was used to insure the success of amplification. A 1 % agarose gel was prepared with TAE buffer and MidoriGreen was used for staining of nucleic acids. 5 µl of the

PCR product was mixed with 1 µl 6xDNA loading dye and filled into the gel. 5 µL GeneRuler 1kb plus DNA Ladder ready-to-use were used as marker. The gel run for 30 min at 130 V and 120 mA. The results of the electrophoresis were visualized under a UV transilluminator. The estimated size for the C573 insert was 2040 bp. The concentration of the PCR product was measured.

4.1.4. TOPO cloning

The fresh PCR product was ligated into a pcDNA™3.1D/V5-His-TOPO® vector (invitrogene) according to the manufacture instructions. For the cloning reaction a ratio for 1:2 (vector: PCR product) was used. The calculated volume of PCR product and 0.5 µl salt solution were added into a tube and filled up with ddH_2O to a total volume of 1.8 µl. Finally, 0.2 µl vector was added and the solution incubated for 30 min at room temperature.

Transformation

The generated vector was transformed into chemical competent *E. coli* to multiply the vector. The whole ligation solution was carefully added into 25 µl of half frozen One Shot® TOP10 Competent Cells (Invitrogen) and incubated 30 min on ice. Then they were heat shocked in a water bath at 42 °C for 30 s and afterward incubated on ice for 2 min. 250 µl of S.O.C. medium that has been pre-warmed to room temperature was added, and the solution was incubated at 37 °C and 350 rpm for 1 h. 100 µl and 150 µl of the solution were spread on two pre-warmed plates containing 50 µg/ml carbenicillin and incubated over night at 37°C.

4.1.5. Colony PCR

Colony PCR

To select the positive colonies, colony PCR was done wit the combination of a gene specific primer and a primer from the vector. The colonies are picked with a pipette tip and put into the master mix for 5 min at RT.

Table 3. PCR Mastermix and program

Master Mix for 1 sample:

5xGoTaq Buffer	1 µl
dNTP (10 mM)	0.2 µl
CpC573 pcDNA F	0.3 µl
BGH Reverse Primer	0.3 µl
GoTaq G2 DNA Polymerase	0.1 µl
ddH_2O	7.8 µl

PCR program:

95 °C	10 min
95 °C	30 s
50 °C	30 s
72 °C	2 min 30 s
72 °C	5 min

The size of the PCR products was checked in a 1 % agarose gel was prepared with TAE buffer and MidoriGreen was used (1:25000 dilution) for staining. 5 of the PCR product was loaded onto the gel. 5 µl GeneRuler 1kb plus DNA Ladder ready-to-use were used as marker. The gel run for 30 min at 130 V and 120 mA. The estimated size for the C573 insert was approximately 2200 bp.

Sequencing

The successful insertion of the target gene in the positive colonies was further checked by sequencing. To prepare the samples, the isolated plasmids were diluted to 50-100 ng/µl. 15 µl were filled into a safe lock tube and 2 µl primer (10 µM) were added. The samples were sent to Eurofins Genomics for sequencing.

4.1.6. Plasmid isolation

To isolate plasmids, the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) was used and the protocol was described as follows. Single, positive colonies from the colony PCR were used to inoculate 3 mLlof LB medium with carbenicillin and incubated overnight at 37 °C and 225 rpm. 1 ml was used to make a glycerol stock with 190 µl 50 % glycerol and stored at -80 °C. The remaining 2 ml were transferred into a 2 ml tube and centrifuged at 1200 g for 1 min. The supernatant was discarded, and the pellet was resuspended in 200 µL Resuspension Solution. 200 µl Lysis Solution were added, the tube was inverted 6-8 times and incubated for 5 min on ice. 350 µl Binding/Neutralisation Solution were added, the tube was inverted 4-6 times and centrifuged at 12000 g for 10 min. The tube was placed on ice for 5-10 min and the supernatant transferred into a new tube and centrifuged at 12000 g for 10 min (to wash). The supernatant was transferred into a new tube containing 800 µL isopropanol. The tube was inverted a few times and placed on ice for 5-10 min. The tube was centrifuged at max. speed for 15 min, the supernatant was discarded and 500 µl 70 % ethanol added. The tube was inverted and centrifuged at max. speed for 10 min. The wash step with ethanol was repeated once and the supernatant carefully and thoroughly removed. The pellet was air-dried for 10-15 min and resuspended in 40 µl nuclease free water.

The concentration was measured, and the plasmid was stored at -20 °C.

4.2. Expression and analysis of P450

4.2.1. Cell line and cultivation

HEK 293 T cells origin from the human embryonic kidney were used in this study.

Cultivation

HEK 293 T cells were cultivated at 37 °C and 5 vol% CO_2. When confluency was reached the medium was removed and the cells were washed with 2 ml 1xPBS. The PBS was removed, and 2 ml trypsin were added and incubated for 1-2 min under the clean bench. The cells were resuspended in 4 ml medium, transferred into a falcon and centrifuged 125 g for 5 min. The supernatant was removed, and the pellet was resuspended in 10 ml medium and 2 ml were transferred into a new falcon. 15 ml medium were added, and the cells were mixed thoroughly and transferred into a new T75 flask and incubated until reaching confluency again.

4.2.2. Transfection - Electroporation

For the transfection, the SF Cell Line Kit (Amaxa) was used. The following protocol is for one T75 cell flask.

For the transfection reagent 1,5 µg plasmid was added to 16,4 µl solution SF and 3,6 µl supplement.

All centrifugation steps were at 800 g for 3 min

The old medium was removed, and the cells were resuspended in 10 ml prewarmed fresh DMEM medium and transferred into a falcon. The cells were centrifuged, and the supernatant was removed. The cell pellet was resuspended in 1 ml PBS centrifuged and the supernatant was removed. 10 µl pellet were resuspended in the transfection reagent and filled into the electroporation cuvette. The cells were electroporated with the program CM 130 afterwards 140 µl prewarmed RMPI were directly added. The cells rested for 10 min at 37 °C and were resuspended and transferred into a new T75 flask with 20 ml prewarmed DMEM medium and incubated at 37 °C for 2 days.

4.2.3. Microsome isolation

The following protocol is for one 6 well plate:

All centrifugation steps were carried out at 4 °C and the samples were kept on ice.

The cells were resuspended in their medium, transferred into 50 ml falcons and centrifuged at 1850 g for 10 min. The medium was discarded, and the cells were washed twice with first 2 and then 1 ml 1xPBS (centrifugation at 1850 g for 10 min). The pellet was resuspended in 750

µl hypotonic buffer, kept on ice for 20 min and checked under the microscope after 10 min. The cells were homogenized with a potter (3x20 strokes with 1 min incubation on ice between each 'repetition'). 750 µl sucrose buffer were added and the suspension was centrifuged for 10 min at 1200 g using a swing-out rotor. The supernatant was transferred into a new tube and the homogenization repeated with the pellet. The resulting supernatant was added to the first supernatant and centrifuged at 10000 g for 10 min. The supernatant was transferred into a polycarbonate tube and centrifuged at 100000 g for 10 min. The supernatant was discarded and the pellet (containing the microsomes) was resuspended in 200 µl resuspension buffer with a potter. The microsomes were stored at -80 °C.

4.2.4. Detection of recombinant protein

SDS-PAGE and Western Blot

Samples were taken during the microsome preparation. 5 µl of the samples were mixed with 1 µl 5x protein loading buffer, heated for 5 min at 70 °C and loaded onto an SDS-polyacrylamid gel. The chamber was filled with SDS running buffer and run for 30 min at 200 V. The gel was assembled between filter paper on top of the membrane and the proteins were transferred from the gel to the membrane with 25 V in 7 min. The membrane was blocked with 5% non-fat milk powder in TBST buffer for 1 h at RT under continual shaking and then incubated with the anti-V5 antibody (in 0.25% non-fat milk powder in TBST, 1:5000) over night at 4 °C under continual shaking. The membrane was washed three times with TBST buffer for 10 min under continual shaking. The membrane was incubated with hydrogen peroxid substrate and enhancer form SuperSignal West Femto Trial Kit (Thermo Scientific) for 1 min. The excess reagent was drained, and the membrane was placed into a development folder. An auto radiographic film was laid on top and exposed for 10 sec to 5 min. Afterward, the film was placed in development solution, ddH_20 and fixing solution, each for 10 sec.

4.2.5. Activity test

The enzymatic assay was conducted in a 100 µl volume system in phosphate buffer (pH7.4). 25 ug microsomes were used with the presence of 100 µM substrate and 1 mM NADPH. Sample without NADPH was used as negative control and sample with microsomes containing only C573 was used as empty control. All sample were incubated at 30° C for 1 hour.
The samples containing β-alanine were prepared for the HPLC/MS as followed. The tubes were centrifuged at max. speed for 10 min and the supernatant was transferred into fitting glass vials.

For the samples with geraniol as substrate, 100 µl DCM were added, the tubes were vortexed for 10 s and centrifuged for 1 min at 10000 g. The lower/ heavier layer was transferred into glass tubes and dried under a stream of nitrogen. 30 µl MFSTA were added and the samples

were vortexed and incubated at 70 °C for 30 min. The samples were dried again and 100 µL. The solution was transferred into new fitting glass tubes and the samples were measured at the GC/MS.

HPLC and GC MS

The possible conversion of β-alanine was analysed by HPLC and mass spectrometry in the APCI mode. The separation was achieved on an Agilent HP1100 HPLC-system equipped with the Purospher® Star RP 18 endcapped (5 µm) (Merck KGaA) connected on a Finnigan LTQ (Thermo Scientific™) mass spectrometer. The components in the sample were separated by gradient elution. The elution started with 3 % solvent B (acetonitrile and 0.1 % formic acid) and stay for 5 min at 3 %. Afterwards it increased to 10 % solvent B from minute 5 to minute 10 and then to 80 % in the following minute. Solvent B decreased from minute 11 to minute 12 to 3 % again and stayed on that level to minute 20. Solvent A consisted of water and 0.1 % formic acid.

For GC/MS a TRACE 1310 gas chromatograph (Thermo Scientific) was linked to an ISQ LT single quadrupole mass spectrometer (Thermo Scientific). The carrier gas was helium with a flow rate of 1.5 mL/min. The mode of mass spectra was electron ionization. The oven temperature setting is as follows: 50 °C (2 min), increase (10 °C/min) to 280 °C, increase (30° C/min) to 310 °C. The MS transfer line temperature was set at 280 °C and the ion source temperature at 250 °C. The injection volume was 1 µl.

5. Results

5.1. Generating Recombinant Plasmids

5.1.1. Creating inserts

To verify the size and purity of the amplified PCR products, 5 µl of the samples were analysed with gel electrophoresis (Fig. 4). Both samples showed a length of approximately 2000 bp and no unspecific fragments. Since the size of the C573 insert is 2040 bp and no contamination with unspecific fragments were detected, the rest of the PCR products was used for the TOPO cloning reaction.

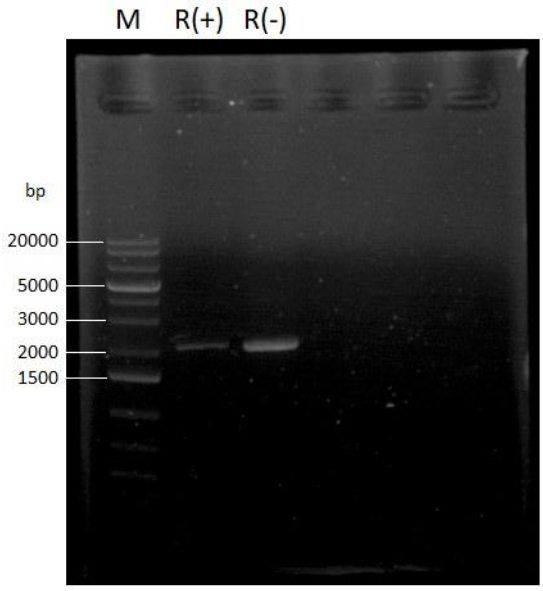


Figure 3: 1% agarose gel of the amplified PCR products for C573 with R(+) and without R(-) stop codon. M: GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific), unit is base pairs (bp).

5.1.2. Positive colonies selection

The inserts were cloned into a pcDNA3.1 TOPO vector and transfected into chemical competent *E. coli*. The success of the transformation was verified with colony PCR colonies and gel electrophoresis (Fig. 5). Four out of 16 selected colonies of the *E. coli* that were transformed with pcDNA3.1 C573 R(+) and three out of 30 selected colonies transformed with pcDNA3.1 C573 R(-) showed a band at approximately 2200 bp (Fig. 6 and 7). Each colony showing a band at 2200 bp had a second lighter and unspecific band at approximately 2000 bp. Plasmids were isolated from the *E. coli* colonies giving a signal in the gel electrophoresis and sent for sequencing. The sequencing results showed identical nucleotide sequence to the transcript from the transcriptome database, which indicated C573 has been successfully sub-cloned into the expression vector.

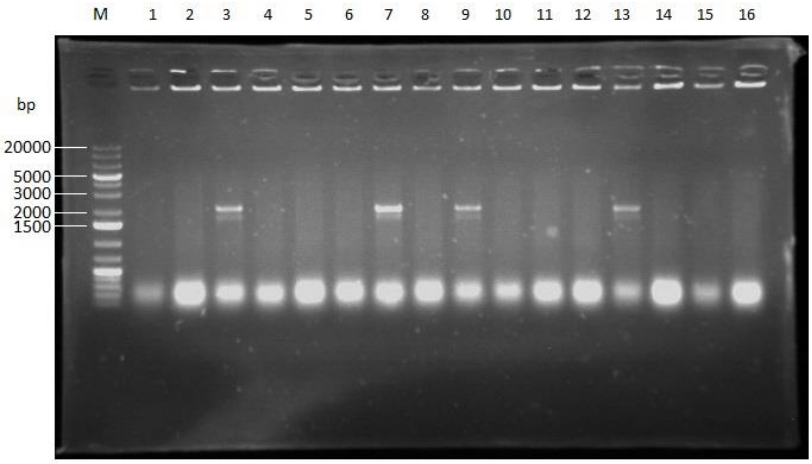


Figure 4: 1% agarose gel of the *E. coli* colonies 1-16 with the vector pcDNA3.1 C573 R(+). M: GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific), unit is base pairs (bp).

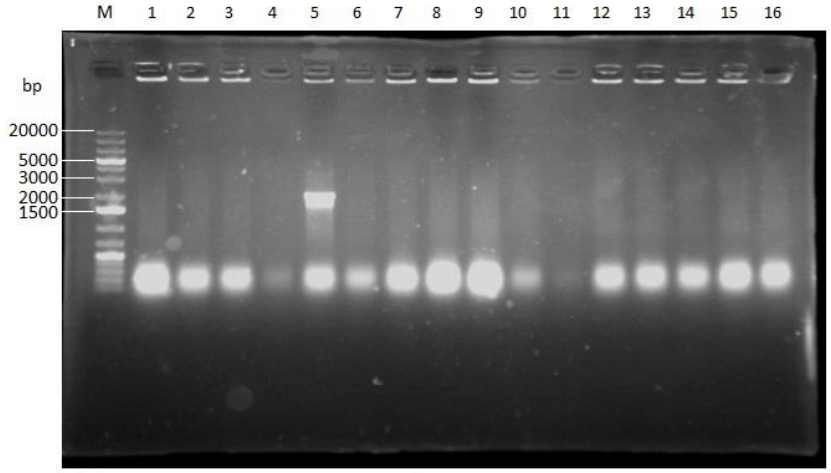


Figure 5: 1% agarose gel of the *E. coli* colonies 1-16 with the vector pcDNA3.1 C573 R(-). Colonies 1 to 16. M: GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific), unit is base pairs (bp).

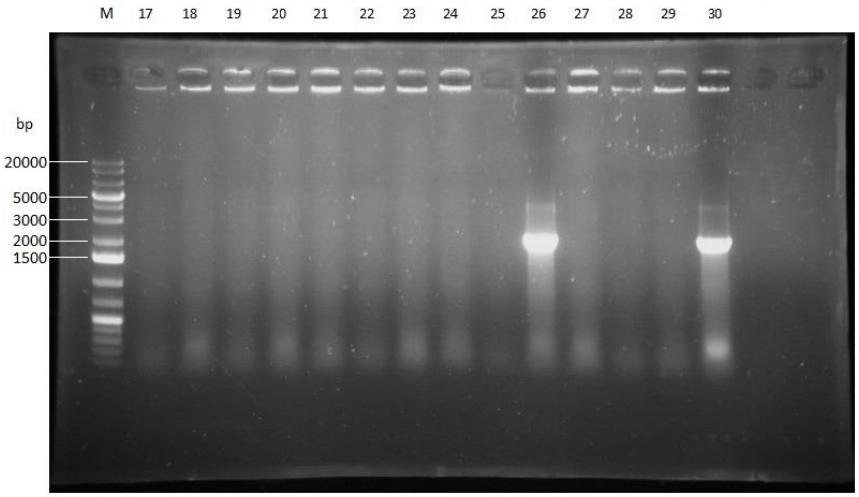


Figure 6: 1% agarose gel of the *E. coli* colonies 1-16 with the vector pcDNA3.1 C573 R(-). Colonies 17 to 30. M: GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Scientific), unit is base pairs (bp).

5.2. Heterologous Expression

5.2.1. Transfection in HEK 293 T cells

To track the transfection efficiency and expression of the proteins, cells transfected with an YFP vector were used as a control group.

As shown in Fig. 8, 48 h after infection, 53 % cells exhibited green light under fluorescence microscopy, indicating the transfection was successful and the cells expressed the protein.

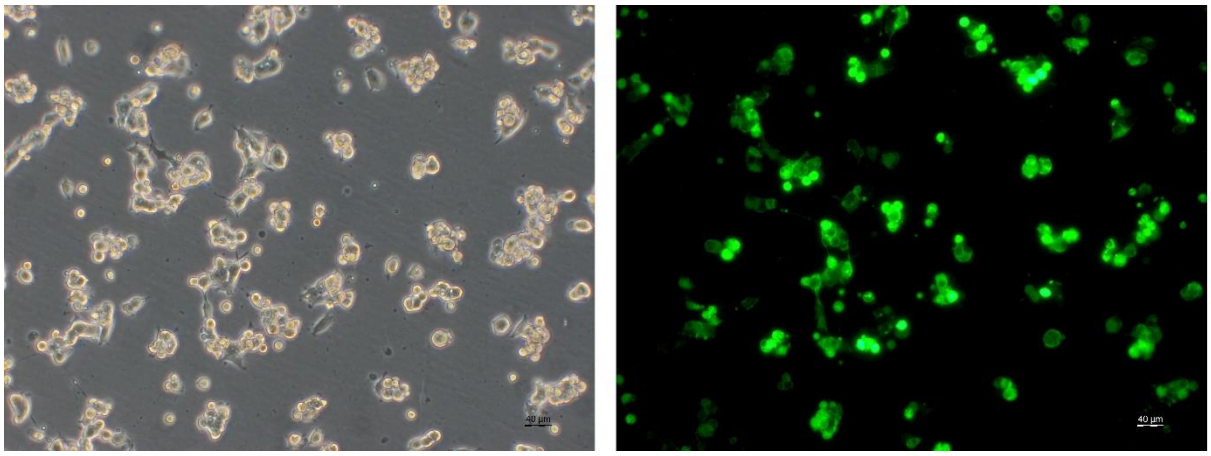


Figure 7: YFP transfected HEK 293 T cells. Transmitted light microscopy (left) and fluorescence microscopy (right), 48 h after transfection.

5.3. Analysing P450

5.3.1. Western Blot

The samples taken during microsome isolation were analysed with Western Blot (see Fig. 9).

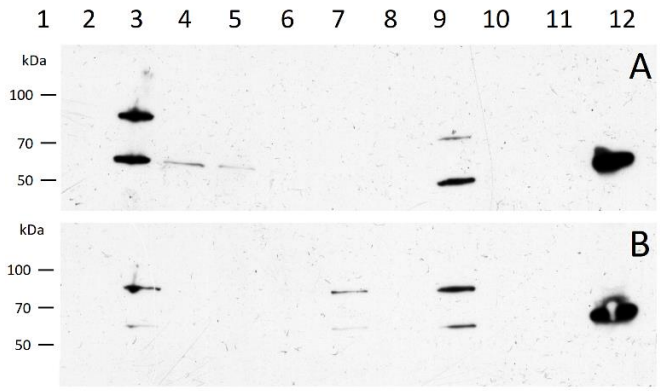


Figure 8: Western Blot of samples taken during the microsome preparation for C7758 R-13 (**A**) and C480 R-3 (**B**). 1, Marker; 2, harvested cell in PBS; 3, cells after 1. pottering; 4, cells after 1. centrifugation at 1200 g; 5, cells after 2. centrifugation at 1200 g; 6, supernatant after centrifugation at 10000 g; 7, pellet after centrifugation at 10000 g; 8, supernatant after ultra-centrifugation; 9, microsome; 12, positive control.

Two bands were detected with a size of approximately 62 kDa and 82 kDa, which is in consistent with the predicted size of P450 (57 kDa) and CPR (76,9 kDa) with the plus of V5 epitope His Tag (about 5 kDa). The strongest signals were observed in the samples after the first pottering (3) and the microsomes (9).

A P450 (C7758), expressed in the beginning of this study and a size of approximately 59.4 kDa, was used as a positive control (12).

5.3.2. Activity tests

5.3.2.1. Conversion of geraniol by C7758

The internal standard for geraniol showed a peak at 12.32 min and the internal standard for 8-OH-geraniol showed a peak at 16.33 min (Fig 8). The sample containing C7758, C573, geraniol as substrate and NADPH showed no peak at 12.32 and a peak at 16.33 indicating that all geraniol was converted into 8-OH-gernaiol. In absence of NAPDH or C7758 the geraniol was not converted.

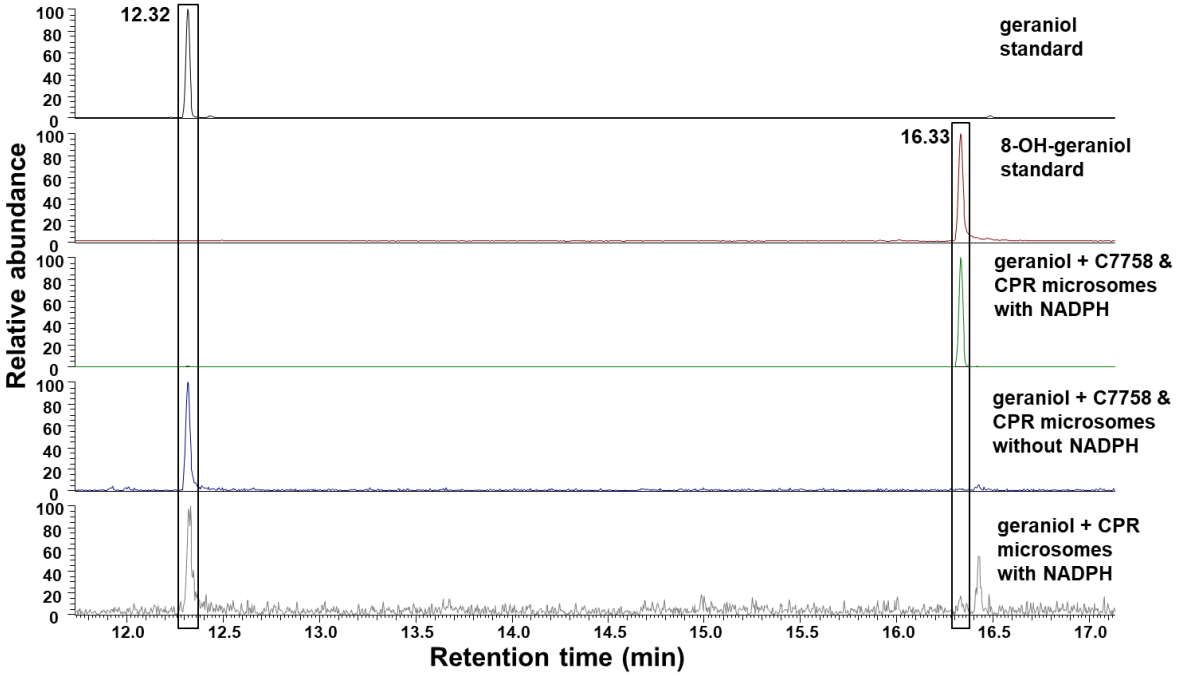


Figure 9: Chromatogram for C7758 or the conversion of geraniol.

5.3.2.2. Conversion of β-alanine by C480

The internal standards showed peaks for β-alanine (2.13 min) and 3-NPA (7.87 min) (Fig. 11). Neither the peak of the substrate nor the peak of the product was found in the samples with the microsomes. The corresponding peaks in the mass spectrum of

the internal standards are 90 m/z for β-alanine and 118 m/z for 3-NPA. Those peaks were not found in the samples containing the proteins (Fig. 12).

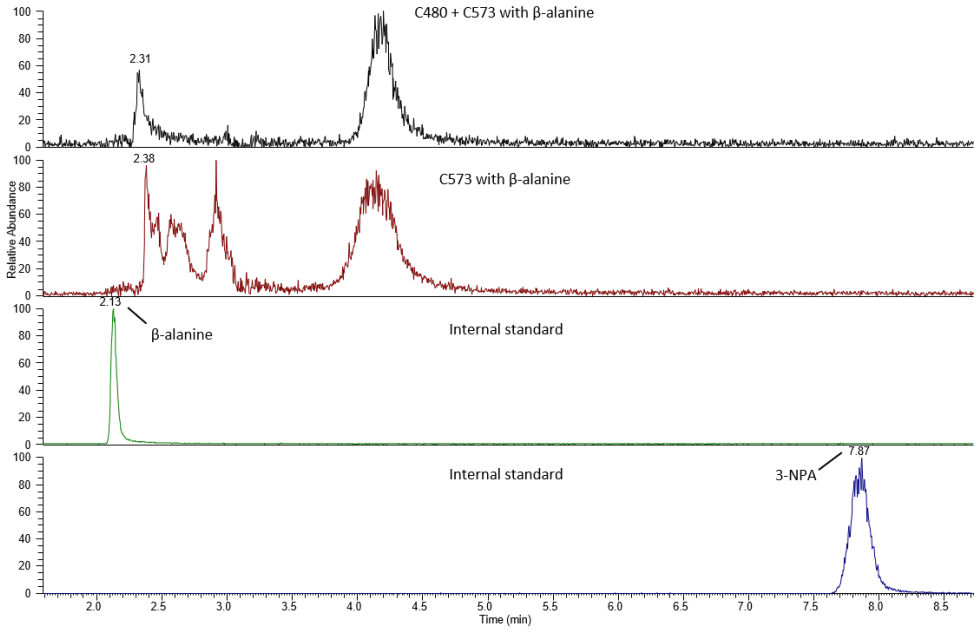


Figure 10: Chromatogram for the activity test of C480 or the conversion of β-alanine.

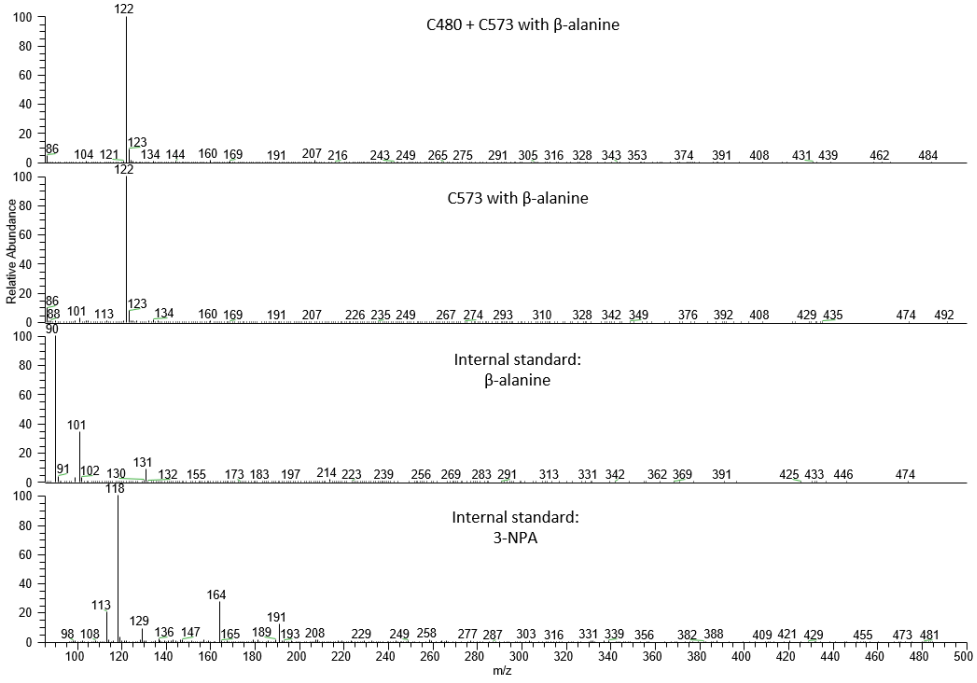


Figure 11: Mass spectrum to Fig. 11. The shown retention times are 2.31 min for C480 + C573 with β-alanine, 2.41 min for C573 with β-alanine, 2.12 min for C573 β-alanine (internal standard) and 7.86 min for 3-NPA (internal standard).

The detection limit of β-alanine was determined to see whether the concentration of β-alanine in the samples for the activity test for C480 was adequate. A peak was detected

in every concentration at 1.86 min for β-alanine dissolved in the phosphate buffer and at 1.75 min for β-alanine solved in methanol (Fig. 13). In the mass spectrum a peak at 90 m/z was detected up to a concentration of 1 µg/ml, although the sample with the concentration of 1 µg/ml showed only a tiny peak (Fig. 14).

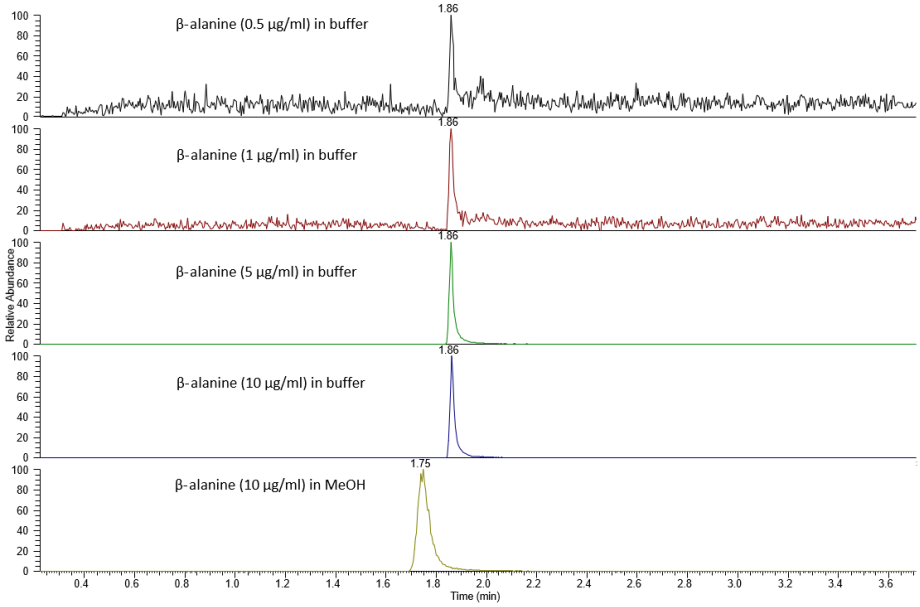


Figure 12: Chromatogram for the β-alanine standards. Different concentration (0,5; 1; 5; 10 µg/ml) in phosphate buffer and 10 µg/ml in methanol.

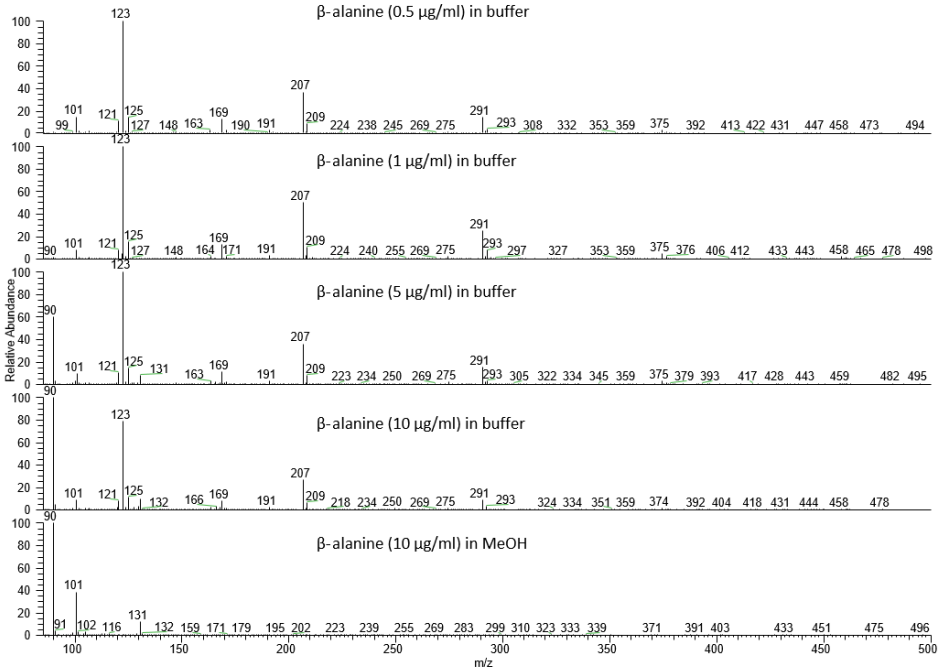


Figure 13: Mass spectrum to Fig. 13. Retention time for the samples in β-alanine is 1.86 min and for β-alanine in methanol 1.73 min.

6. Discussion

The understanding of the biosynthesis of the monoterpenic iridoid chrysomelidial in leaf beetles has been increasing over the past decades, with a special interest in the characterisation of the involved enzymes.

The conversion of geraniol to 8-OH-geraniol in the earlier steps of the biosynthesis of chrysomelidial is a ω-hydroxylation, a reaction type often catalysed by cytochrome P450s[1]. Until now no enzymes involved in the biosynthesis of the 3-NPA ester of isoxazolin-5-one have been described. But the hydroxylation of β-alanine into 3-NPA could, too be catalysed by a CYP. Unpublished know-down studies presented two possible CYP candidates for both biosynthetic pathways.

6.1. Heterologous expression

HEK 293 cells are a highly efficient cell line, capable of processing and folding the expressed proteins in a way most mammalian and non-mammalian proteins required[2]. HEK 293 T, a subline of the HEK 293 cells, carry an additional neomycin resistance gene and the SV40 large T-antigen, which allows the episomal replication of transfected plasmids.

The successful transfection for the proteins with V5 epitope (without stop codon) was observed in figure 7. Since the total amount ratio of the transfected plasmids used for transfection was 4:1 (CYP: CPR), a similar ratio of total expressed proteins amount between CYP and CPR, which can be reflected by the signal strength in the Western Blot was expected. The signal strength between C7758 and C573 showed that pattern, while C573 exhibited a stronger signal than C480. Most reasonable explanation would be a mix-up of the plasmids during transfection. A difference in transfection efficiency could, too, lead to this difference.

The first sample taken during the microsome isolation, containing the transfected cells in PBS, did not show a signal as, expected. This is likely because the heating at 70 °C for 5 min prior the SDS-PAGE did not denature the sample enough to release proteins in the endoplasmic reticulum.

For the supernatant after the centrifugation at 10000 g no signal was detected, even though it was expected. Due to the direct sampling after the centrifugation the sample was most likely take from a layer, that did not contain the proteins. Taking the sample after transferring it to a new tube led to a signal, since the density gradient has been homogenised through this step. Light signals were detected in the samples taken after the first and second centrifugation at 1200 g for C7758, containing whole cells, large cell debris and nuclei, and the pellet of C480 after 10000 g, containing mostly mitochondria,

lysosomes and peroxisomes. The cause for this is most likely a suboptimal homogenization and/or lysis with the hypotonic buffer.

The microsomes fraction showed positive signals, indicating the transfection and expression of the CYP and CPR without the stop codon were successful. Since the experimental procedures for plasmids containing CYP and CPR with the stop codon were conducted in parallel, it was assumed, that the microsome from the cells transfected with plasmids of stop codons contained the recombinant protein as well.

6.2. Activity test

The ability of the P450s to convert a substrate into a product was measured with chromatographic method linked to mass spectrometry. The sample containing C7758, C573 and geraniol as a substrate (see fig. 8) showed peaks where 8-OH-geraniol were expected, indicating that C7758 is the enzyme converting geraniol to 8-OH-geraniol.

The HPLC and MS measurement for the conversion of β-alanine was inconclusive (fig. 12). Even the substrate (β-alanine, 8.9 µg/ml) could not be detected in the samples containing the microsomes. An internal standard with a concentration of 0.5 µg/ml was still detectable by HPLC (fig. 13) and the detection limit for the mass spectrometry lay between 1 and 5 µg/ml (fig. 14). Cause for the vanishing of the substrate peak in the samples could be a loss of sensitivity due to problems between matrix and buffer. Another possible reason could be that β-alanine was not converted into 3-NPA, as expected but into another component, which has not been identified. Besides, it should be considered, that there was no actual evidence that the plasmids with stop codon were transfected and expressed successfully, given that they had no tag to detect them, the success of their expression was only based on prediction from the one with tag.

7. Outlook

This study built the base for further studies about P450's function in the biosynthesis of defensive compound in *P. cochleariae*. In the biosynthesis of chrysomelidial, geranyl diphosphate is a second possible substrate for C7758, which still needs to be tested. Since only inconclusive results were achieved in the study of C480 expressed in HEK 293 T cells, attempts to express it in a second cell line (Sf9 cells transfected with baculovirus) look promising.

In addition, the enzymatic kinetics needs to be examined for a complete characterization of those proteins.

8. References

1. Marshall, S. A. *Insects: Their Natural History and Diversity: With a Photographic Guide to Insects of Eastern North America*. (Firefly Books, 2006).
2. *Research on Chrysomelidae. 2*. (Brill, 2009).
3. Hamnett, G. G. An Investigation into the Life-history and Morphology of the Mustard Beetle, Phaedon cochlear& F., on Watercress. *J. Zool.* (1944).
4. Pasteels, J. M., Braekman', J. C. & Daloze, D. CHEMICAL DEFENCE IN CHRYSOMELID LARVAE AND ADULTS. **38,** 1891–1897 (1982).
5. Burse, A. *et al.* Implication of HMGR in homeostasis of sequestered and de novo produced precursors of the iridoid biosynthesis in leaf beetle larvae. *Insect Biochem. Mol. Biol.* (2008). doi:10.1016/j.ibmb.2007.09.006
6. Gross, J., Müller, C., Vilcinskas, A. & Hilker, M. Antimicrobial Activity of Exocrine Glandular Secretions, Hemolymph, and Larval Regurgitate of the Mustard Leaf BeetlePhaedon cochleariae. *J. Invertebr. Pathol.* (1998). doi:10.1006/jipa.1998.4781
7. Pauls, G. *et al.* Two Defensive Lines in Juvenile Leaf Beetles; Esters of 3-nitropropionic Acid in the Hemolymph and Aposematic Warning. *J. Chem. Ecol.* (2016). doi:10.1007/s10886-016-0684-0
8. Burse, A. & Boland, W. Deciphering the route to cyclic monoterpenes in Chrysomelina leaf beetles: Source of new biocatalysts for industrial application? *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* (2017). doi:10.1515/znc-2017-0015
9. Frick, S., Nagel, R. & Schmidt, A. Metal ions control product specificity of isoprenyl diphosphate synthases in the insect terpenoid pathway Histidine protein kinase CorS View project Legume chemistry and the specificity of the pea aphid host races View project. *Artic. Proc. Natl. Acad. Sci.* (2013). doi:10.1073/pnas.1221489110
10. Feld, B. K., Pasteels, J. M. & Boland, W. Phaedon cochleariae and Gastrophysa viridula (Coleoptera: Chrysomelidae) produce defensive iridoid monoterpenes de novo and are able to sequester glycosidically bound terpenoid precursors. *Chemoecology* (2001). doi:10.1007/PL00001851
11. Becker, T. *et al.* Natural Product Reports A tale of four kingdoms – isoxazolin-5-one- and 3- nitropropanoic acid-derived natural products. **34,** 333–462 (2017).
12. Deroe, C. & Pasteels, J. M. Distribution of adult defense glands in chrysomelids (Coleoptera: Chrysomelidae) and its significance in the evolution of defense

mechanisms within the family. *J. Chem. Ecol.* (1982). doi:10.1007/BF00984006

13. Ludolph, A. C., he, F., Spencer, P. S., Hammerstad, J. & Sabri, M. 3-Nitropropionic Acid - Exogenous Animal Neurotoxin and Possible Human Striatal Toxin. *Canadian Journal of Neurological Sciences / Journal Canadien des Sciences Neurologiques* (1991). doi:10.1017/S0317167100032212

14. Flint Beal, M. *et al.* Neurochemical and Histologic Characterization of Striatal Excitotoxic Lesions Produced by the Mitochondrial Toxin 3-Nitropropionic Acid. (1993).

15. Becker, T., Ploss, K. & Boland, W. Biosynthesis of isoxazolin-5-one and 3-nitropropanoic acid containing glucosides in juvenile Chrysomelina. *Org. Biomol. Chem.* (2016). doi:10.1039/C6OB00899B

16. Danielson, P. B. The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. *Curr. Drug Metab.* **3,** (2002).

17. Guzov, V. M., Unnithan, G. C., Chernogolov, A. A. & Feyereisen, R. CYP12A1, a Mitochondrial Cytochrome P450 from the House Fly 1.

18. Gorinova, N., Nedkovska, M. & Atanassov, A. Cytochrome p450 monooxygenases as a tool for metabolizing of herbicides in plants. *Biotechnol. Biotechnol. Equip.* **19,** 105–115 (2005).

19. Guengerich, F. P. Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266,** 10019–10022 (1991).

20. Greule, A., Stok, J. E., De Voss, J. J. & Cryle, M. J. Unrivalled diversity: the many roles and reactions of bacterial cytochromes P450 in secondary metabolism. doi:10.1039/c7np00063d

21. Groves, J. Models and Mechanisms of Cytochrome P450 Action\rCytochrome P450. 1–43 (2005). doi:10.1007/0-387-27447-2_1

22. *Cytochrome P450 Structure, Mechanism, and Biochemistry*. (Kluwer Academic/Plenum Publishers, 2004).

9. Supplementary material

Material

Larvae of leaf beetles

Chrysomela populi was used from own breeding

Table 4. Chemicals

Chemicals	Manufacturer
Ethanol	Carl Roth GmbH & Co. (Karlsruhe)
Isopropanol	Carl Roth GmbH & Co. (Karlsruhe)
Nuclease-Free Water	Thermo Fisher Scientific GmbH (Schwerte)
Glycerol	Carl Roth GmbH & Co. (Karlsruhe)
NADPH-Na_4 (β-Nicotinamide adenine dinucleotide phosphate tetrasodium salt)	Carl Roth GmbH & Co. (Karlsruhe)
Dichloromethane (DCM)	Carl Roth GmbH & Co. (Karlsruhe)
MSTFA (N-Methyl-N-trimethylsilyl-trifluoracetamid)	Macherey-Nagel GmbH & Co. (Düren)
Sodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH & Co. (Karlsruhe)
Potassium dihydrogenphosphate (KH_2PO_4)	Merck KGaA (Darmstadt)
Sodium chloride (NaCl)	Carl Roth GmbH & Co. (Karlsruhe)
Potassium chloride (KCl)	Sigma-Aldrich Co. LLC (St. Louis)
Tris	Carl Roth GmbH & Co. (Karlsruhe)
EDTA	Carl Roth GmbH & Co. (Karlsruhe)
DTT	Thermo Fisher Scientific GmbH (Schwerte)
Sucrose ($C_{12}H_{22}O_{11}$)	Carl Roth GmbH & Co. (Karlsruhe)
Tripotassium phosphate (K_3PO_4)	Sigma-Aldrich Co. LLC (St. Louis, USA)
Tween 20 (10x)	Bio-Rad Laboratories, Inc. (Hercules)

Table 5. Plasmids

Plasmids	Manufacturer
pcDNA™3.1D/V5-His-TOPO®	Invitrogen™ (Darmstadt)

Table 6. Media

Media	Manufacturer
LB-Medium (Lennox)	Carl Roth GmbH & Co. (Karlsruhe)
S.O.C. Medium	Thermo Fisher Scientific GmbH (Schwerte)
DMEM	Thermo Fisher Scientific GmbH (Schwerte)

Table 7. Enzymes

Enzymes	Manufacturer
Phusion Polymerase	Thermo Fisher Scientific GmbH (Schwerte)
GoTaq G2 DNA Polymerase	Promega Corporation (Fitchburg)
SuperScript™ III reverse transcriptase	Invitrogen™ (Darmstadt)

Table 8. Commercial kits

Commercial kits	Manufacturer
RNAqueous™ Total RNA Isolation Kit	Thermo Fisher Scientific GmbH (Schwerte)
RNAqueous™-Micro Total RNA Isolation Kit	Thermo Fisher Scientific GmbH (Schwerte)
SuperScript™ III Reverse Transcriptase	Thermo Fisher Scientific GmbH (Schwerte)
pcDNA™3.1/V5-His TOPO™ TA Expression Kit	Thermo Fisher Scientific GmbH (Schwerte)
GenElute™ Plasmid Miniprep Kit	Sigma-Aldrich Co. LLC (St. Louis)
SF cell line kit	Lonza Group AG (Basel)
SuperSignal West Femto Substrate Trial Kit	Thermo Fisher Scientific GmbH (Schwerte)

Table 9. Standards

Standards	Manufacturer
GeneRuler 1kb plus DNA Ladder ready-to-use	Thermo Fisher Scientific GmbH (Schwerte)
Spectra™ Multicolor Broad Range Protein Ladder	Thermo Fisher Scientific GmbH (Schwerte)

Table 10. Antibody

Antibody	Manufacturer
Anti-V5-HRP antibody	Invitrogen™ (Darmstadt)

Table 11. Buffer and supplements

Buffer and supplements	Manufacturer
50xTAE buffer	Carl Roth GmbH & Co. (Karlsruhe)
5xHF buffer	Thermo Fisher Scientific GmbH (Schwerte)
Midori Green Advance	Nippon Genetics Europe (Düren)
5xGoTaq Buffer	Promega Corporation (Fitchburg)
Non-fat skimmed milk powder	Biomol GmbH (Hamburg)
Nuclease free water	Thermo Fisher Scientific GmbH (Schwerte)
oligo $(dT)_{20}$	Invitrogen™ (Darmstadt)
dNTPs (10 mM)	Invitrogen™ (Darmstadt)
DNA Gel Loading Dye (6X)	Thermo Fisher Scientific GmbH (Schwerte)
5X First-Strand Buffer	Invitrogen™ (Darmstadt)
Carestream® Kodak® autoradiography GBX fixer/replenisher	Sigma-Aldrich Co. LLC (St. Louis)
Carestream® Kodak® autoradiography GBX developer/replenisher	Sigma-Aldrich Co. LLC (St. Louis)

Table 12. Equipment

Equipment	Manufacturer
Ultracentrifuge	Beckman Coulter (Brea)
Centrifuge (5424 R, 5417 R)	Eppendorf AG (Hamburg)
Table/Micro centrifuges	Carl Roth GmbH & Co. (Karlsruhe)
Analytical balance	Mettler Toledo (Columbus)
Electrophoresis Power Supply	Pharmacia AG (Uppsala)
Trans-Blot Turbo System	Bio-Rad Laboratories (München)
Electrophoresis Power Supply	Amersham plc (Amersham)
	Biometra (Göttingen)
UV transparent gel trays	Biometra (Göttingen)
Mastercycler	Eppendorf AG (Hamburg)
Shaking incubation cabinet	Satorius AG (Göttingen)
Sterile work bench	Thermo Fisher Scientific GmbH (Schwerte)
Autoclave	Thermo Fisher Scientific GmbH (Schwerte)

BioDocAnalyze (UV light)	Biometra (Göttingen)
Microwave	Panasonic AG (Kadoma)
Dri-block heater	Techne (Stone)
Thermoblock	Eppendorf AG (Hamburg)
NanoDrop One	Thermo Fisher Scientific GmbH (Schwerte)
Shaking device (orbital)	IKA Werke GmbH & Co (Staufen im Breisgau)
Tissue homogenizer	SPEX SamplePrep (Metuchen)
Ultrasonic homogenizers	Bandelin electronic GmbH & Co. KG (Berlin)
Water purification system	Thermo Fisher Scientific GmbH (Schwerte)
pH meter	WTW (Weilheim)
Magnetic stirrers	IKA Werke GmbH & Co (Staufen im Breisgau)
GC-MS	Thermo Fisher Scientific GmbH (Schwerte)
LC-MS	Thermo Fisher Scientific GmbH (Schwerte)

Table 13. Consumption items

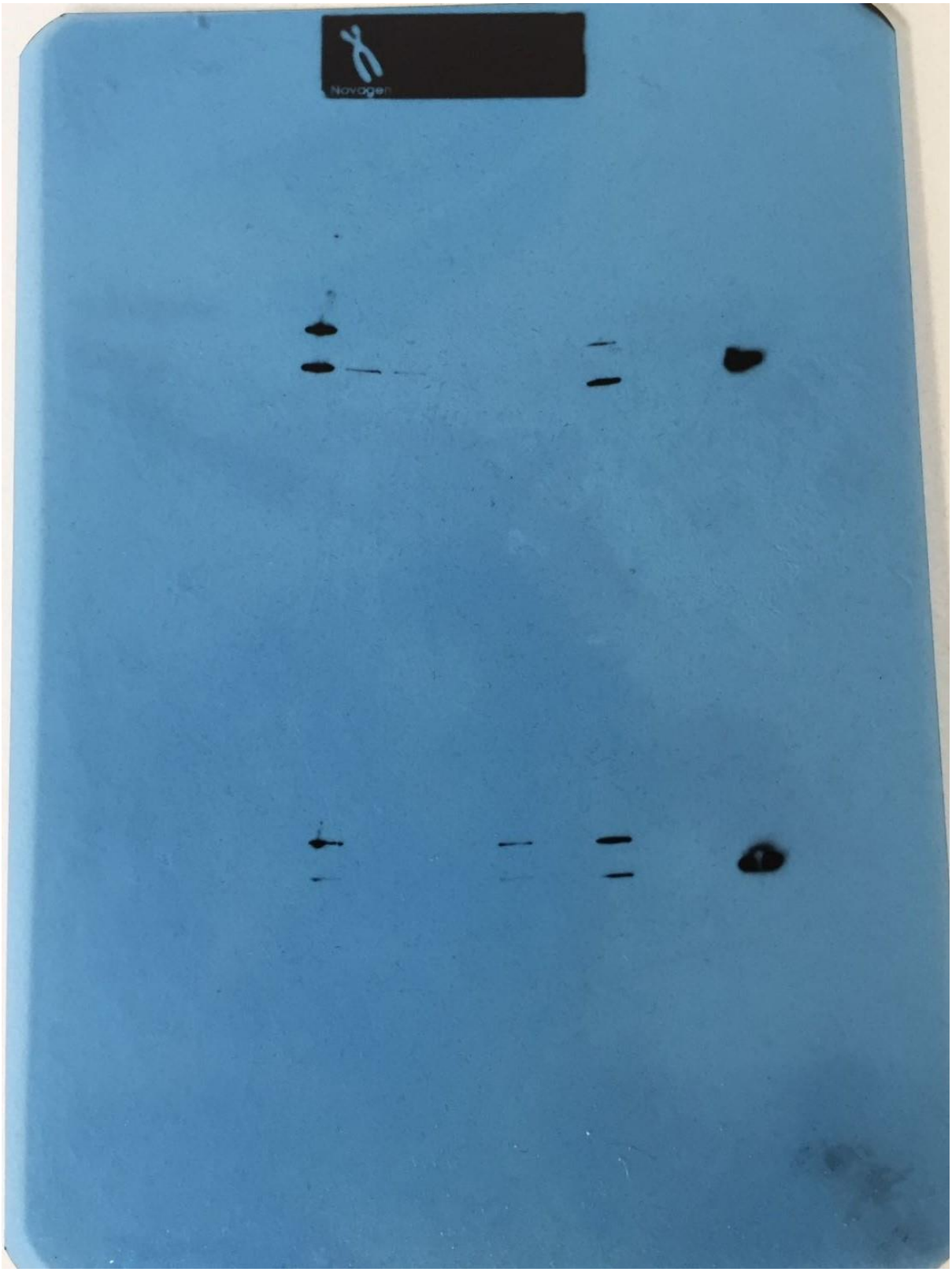
Consumption items	Manufacturer
1,5 mL, 2 mL Eppendorf tubes	Carl Roth GmbH & Co. (Karlsruhe)
15 mL, 50 mL Falcontubes	Carl Roth GmbH & Co. (Karlsruhe)
Thickwall Polycarbonate Tube, 10 mL	Beckman Coulter (Brea)
Petriedishes (94x16 cm^2)	Greiner bio-one
auto radiographic film (CL-XPosure)	Thermo Fisher Scientific GmbH (Schwerte)
Mini Fromat, 0.2 µm PVDF, single application	Bio-Rad Laboratories (München)
Mini-PROTEAN TGX Precast Gels	Bio-Rad Laboratories (München)

Table 14. Buffer

Buffer	Components	Concentration
1xPBS	Na_2HPO_4	10 mM
	KH_2PO_4	1.8 mM
	NaCl	137 mM
	KCl	2.7 mM
Hypotonic buffer	Tris	20 mM
pH 7.5	EDTA	5 mM
	DTT	1 mM
	1xProtease inhibitor	
Sucrose buffer	Tris	20 mM

pH 7.5	EDTA	5 mM
	DTT	1 mM
	Sucrose	500 mM
	1xProtease inhibitor	
Resuspension buffer	K_3PO_4	20 mM
pH 7.4	1xProtease inhibitor	
SDS running buffer	Glycine	192 mM
	Tris	25 mM
	SDS	0,1%
SDS loading dye	Tris	250 mM
	SDS	10%
	Glycerol	30%
	β-Mercaptoethanol	5%
	Bromophenol blue	0.02%
10xTBS buffer	Tris	200 mM
	NaCl	1500 mM
TBST buffer	1xTBS buffer	
	Tween 20	0.05%
Blocking buffer	non-fat skimmed milk powder	5%
	1xTBST	
Antibody Solution	non-fat skimmed milk powder	0.25%
	1xTBST	
	Anti-V5-HRP Antibody	0.02%
LB medium	LB-Medium (Lennox)	7 g
	ddH20	200 mL
	1x Carbenicillin (50 mg/mL)	200 µL

Original Western Blot



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Last, I would like to thank my friends Lena, Sean and Viola and my sister Ann Kathrin for the support, especially during the my time writing the thesis.

11. Declaration of Authenticity

I hereby declare that I have researched and written the Bachelor thesis myself, no passages of text have been taken from third parties or my own examination papers without having been identified as such and that all tools, personal notification and sources used have been indicated in the Bachelor thesis. The persons, who have supported me in selecting and analysing the material and preparing manuscript, are declared in complete.

Jena, 15th October 2018 Sarah Baur