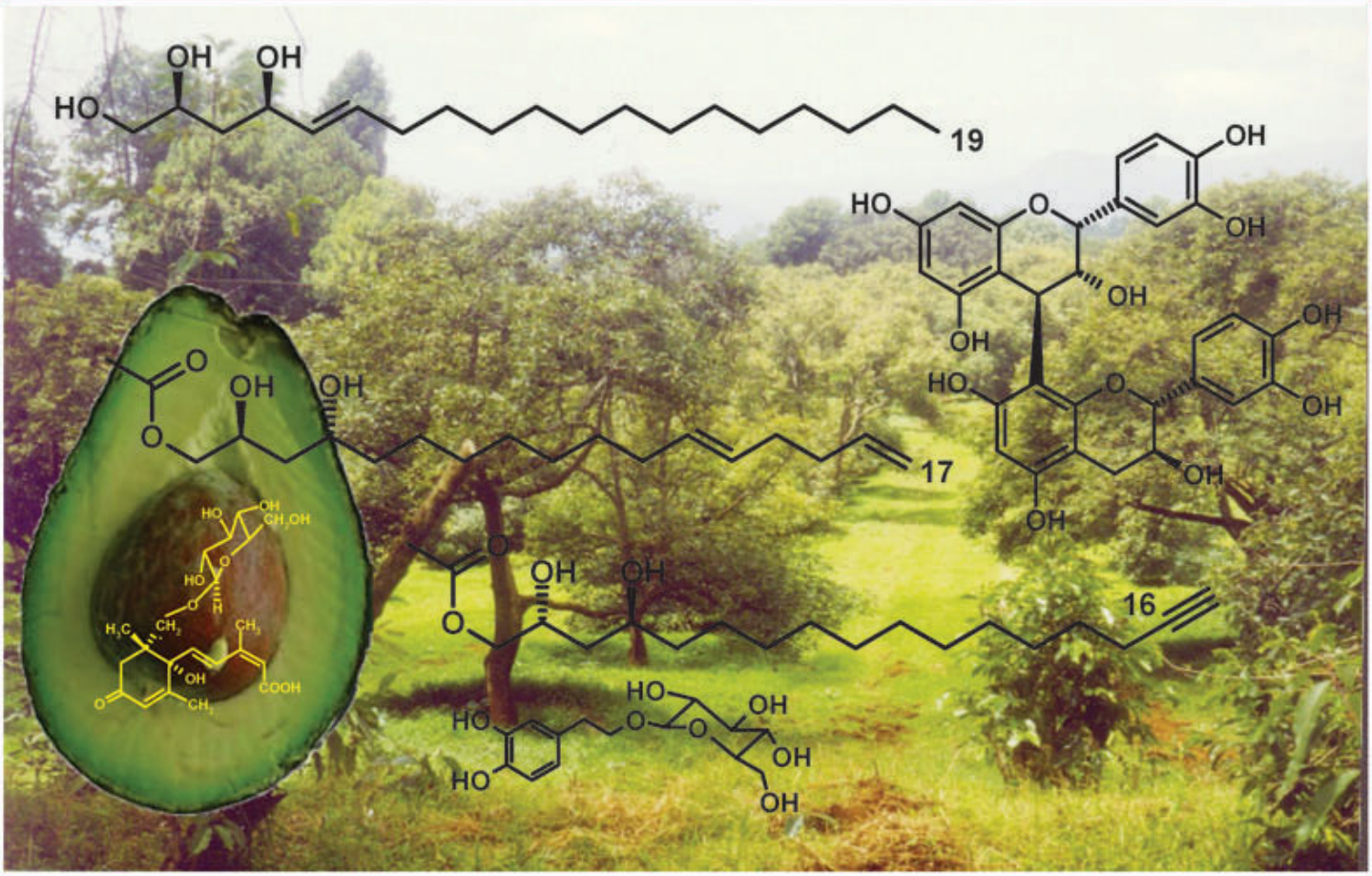
María del Refugio Ramos-Jerz

Phytochemical analysis of avocado seeds (*Persea americana* Mill., c.v. *Hass*)



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Phytochemical analysis of avocado seeds

(*Persea americana* Mill., c.v. *Hass*)

Von der Fakultät für Lebenswissenschaften

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Ramos M. del R., Jerz, G., Villanueva, S., Winterhalter, P. Bioactive lipids isolated from avocado seeds (*Persea americana* Mill., Lauraceae) by HSCCC. Lebensmittelchemie 60, 4, 84 (2006).

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Ramos, M. del R., Jerz, G., Waibel, R., Hensel, A., Winterhalter, P. Bioactive proanthocyanidine from avocado seeds (*Persea americana* Mill., cv. *Hass*, Lauraceae). (Poster). Botanikertagung, Braunschweig (2004), p. 360.

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To my parents

To Ana-Victoria and Gerold

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CONTENT Page

ABBREVIATIONS

a ..... Slope on the straight line

ACN..... Acetonitrile

Å..... Angstrom

ABA ..... Abscisic acid

Abs ..... Absorbance

ABTS ..... 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

$ABTS^{\bullet+}$: ..... Free stable radical cation of the ABTS

Ac ..... Ethyl Acetate

ACC..... Acetyl Coenzyme A carboxylase

ADV ..... Aujeszky's disease virus

AD3 ..... adenovirus type 3

Amu: ..... Atom mass unit

ANOVA..... Variance Analysis

AOAC ..... Association of Official Analytical Chemists

ARP ..... Antiradical power

ASTM: ..... American Society for Testing and Materials

A-549..... Human lung tumour cell line

A-498..... Kidney carcinoma cell line

b ..... Intercept on the straight line

BHA..... *tert*-butylated hydroxyanisole

BHT 2,6-di-*tert*-butyl-4-methylphenol

C or CH_2Cl_2 ..... dichloromethane

^oC ..... Celsius grade

CC ..... Column chromatography

CCC: ..... Countercurrent Chromatography

Chl ..... Chlorogenic acid

CD ..... Circular Dichroism spectroscopy

$CDCl_3$ ..... Deuterated chloroform

CD_3CN ..... Deuterated acetonitrile

CD_3OD ..... Deuterated methanol

cf. ..... Confer

CIATEJ ..... "Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco"

cm ..... Centimeter

CoA ..... Coenzyme A

CO_2 ..... Carbon dioxide

d ..... Doublet

D ..... Proanthocyanidin dimers

Da ..... Dalton

DAD ..... Diode array detector

DCI-MS ..... Desorption Chemical Ionization Mass Spectrometry

D_2O ..... Deuterated water

1-D ..... First dimensional NMR measurement

2-D ..... Second dimensional NMR measurement

DPPH ..... 2, 2 -Diphenyl-1-picrylhydrazyl

dw ..... Dry weight

Exp. ..... Experimental

$E_{(1\%)}$ ..... Extinction coefficient at 1% of concentration

EC_{50} ..... Median Effective Concentration

EI-MS ..... Electron Ionization Mass Spectrometry

Eq. ..... Equation

ESI/MS	Electron Spray Ionization Mass Spectrometry
eV	Electron-Volt
FAOSTAT	Food and Agriculture Organization of the United Nations. Statistics Division
FID	Flame ionization detector
Fig.	Figure
g	Gram
GC	Gas Chromatography
GC-EI/MS	Electron ionization mass spectrometry coupled to gas chromatography
Glc	Glucose
h	Hours
HaCat:	Cells Human adult, low Calcium medium, high Temperature Keratinocytes (cell line)
HDO	In part deuterated water
HPLC:	High Pressure Liquid Chromatography
HSCCC	High-speed Countercurrent Chromatography
HSV-1	herpes simplex virus type 1
HTG	Hydroxytyrosol glucoside
HT-29	Human colon tumour cell line
Hz	Hertz
ID	Internal diameter
Int.	Intensity
J	Coupling constant
kg	Kilogram
K60	Kieselgel 60
L	Liter

l	Column length (cm)
LD_{50}	Median Lethal Dose
lit.	Literature reported value
log	Logarithmical value
“logit”	Logarithmical unit of transformed data
M	Proanthocyanidin monomers
MA	Melon aphid
MCF-7	Human breast tumour cell line
mg	Milligram
MHz	Megahertz
min	Minutes
mL	Milliliter
ML	Mosquito larvae
mmol/L	Millimol per Liter
mM	Milli-Molar
MPLC	Medium Pressure Liquid Chromatography
MSTFA	N-methyl-N-(trimethylsilyl)trifluoro acetamide
MTPA	α-methoxy-α-trifluoro-methylphenylaceto-esters
MW	Methanol-water
MXN	Mexican money unit: pesos
m/z	mass-to-charge ratio
n	Repetitions in the experimental design
n.d.	Not detected
neg	Negative value
nm	Nanometer
ns	Not sensitive

nt ..... Not tested

nv ..... Not reported value

Obs..... Observed value

p ..... Pressure (bar)

fp ..... Further purification

PaCa-2 ..... Pancreatic carcinoma cell line

PC-3 ..... Prostate adenocarcinoma cell line

PE ..... Petrol ether

pos. ..... Positive

p.p. ..... *Per* procurationem

ppm: ..... Parts *per* million

“probit”: ..... Probability unit of transformed data

PVA ..... Polyvinyl acetal

Q ..... Quinic acid

r ..... Correlation coefficient in the straight line

p.a. ..... Analytical reactant grade

RC ..... Rest of the column in the preparative separation

rDPPH$^{\bullet}$ ..... No. radicals of DPPH$^{\bullet}$ reduced/ molecule antioxidant

R_f ..... Ratio of front

RI..... Kóvats’ retention Index

RP ..... Reversed Phase

rpm: ..... Revolutions *per* minute

RSCe..... Transformed scavenging capacity of extracts relative to ascorbic acid

RSCs ..... Transformed scavenging capacity of standards relative to ascorbic acid

R_t	Retention time
"S"	Sigmoid form curve
s	Singlet
(s)	Supercritical conditions
SAGAR	"Secretaría de agricultura, ganadería y desarrollo rural"
SCe	Scavenging capacity of extracts
SCs	Scavenging capacity of standards
SD	Standard deviation
SFE	Supercritical Fluid Extraction
SPME	Solid Phase Micro Extraction
Std.	Standard
Sv	stoichiometric value
syn.	synonymous
2SSM:	Two spotted spider mite
t	Triplet
t	Temperature
T	Proanthocyanidin trimers
TAA	Total Antioxidant Activity
TAE	Tannic acid equivalents
TBME	tert-Butyl methyl ether
TEAC	Trolox Equivalent Antioxidant Capacity
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
Trolox:	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TU	"Technische Universität"

U ..... Unknown

UDP ..... Uridin diphosphate

UK ..... United Kingdom

USA ..... United States of America

$US: ..... United State money units: Dollar

UV ..... Ultraviolet

v ..... Volume

vs ..... Versus

(z) ..... “logit” value

2^{5-1} ..... Factorial fractionated experimental design

δ ..... Chemical shift (ppm)

% ..... Relation of percentage

Δ ..... Difference of

$[\alpha]_D$ ..... Optical rotation on deuterium lamp

λ ..... Wavelength (nm)

θ ..... Molar ellipticity symbol

µg ..... Microgram

µm ..... micrometer

µM: ..... Micro-Molar solution

$[\Phi]$ ..... Optical rotary dispersion

1. INTRODUCTION

1.1. Objectives of phytochemical analysis

During thousands of years of development of human cultures the use of medicinal plants with curing properties influenced people around the world. For European people, active plant species were precious and an important reason for settlement and trade (Rätsch, 1997).

The ethnomedicine covers healthcare systems that include beliefs and practices related to diseases and health, which are products of indigenous development and are not explicitly derived from a conceptual framework of modern medicine (Iwu and Wootton, 2002).

The indigenous groups have used curing plants as their personal phytomedical remedies and also for spiritual reasons. The herbal medicines are a readily available resource for primary health care (Ankli et al., 2002). Nowadays, Mexico is still a country where ethnomedicine is very popular and socially of high importance. People of the tribes Tepehuan (cf. Fig. 1-1), Mayan, Huichol and other Mexican folk cultures believe in the direct relation between herbal activity and the healers in contact with God.

Figure 1-1. Tepehuan ethnomedicine (unknown Tepehuan artist: “El curandero tepehuano está bendiciendo las ofrendas para los dioses”. The healer is blessing the gifts for God).

During the acquisition of phytomedicinal knowledge of healing, many people died by ingestion of overdosed preparations made of extracts using bioactive plants containing toxic ingredients.

Many cultures tried to preserve their acquired phytomedical knowledge in illustrated or written form, e.g. on stone tablets or later in pharmacopoeias.

Through the close relationship between people and plants some scientific disciplines were born. The pharmacologists evaluate the benefits and risks of using potentially active medicinal plants, botanists classify plants according to morphological and chemotaxonomical data, and chemists elucidate the structures of molecules responsible for activity (Balick and Cox, 1997).

The phytochemistry was born first as part of the pharmacognosy with the aim of isolation of plant substances and their systematic structural elucidation. The discovery of substances having biological activities started with the industrial development in the second half of the 19th century. The morphine alkaloid was first isolated by F.W. Sertürner. The principles of the antibiotic action of penicillin (not of plant origin but from fungus) was discovered in 1928 by Sir A. Fleming. Both

discoveries may serve as good examples for the beginning of the phytochemistry area (Nuhn, 1997).

The growing possibilities using chromatography and spectroscopy in phytochemical analysis made it possible to find new bioactive natural products.

Ingredients used in the ethnomedical remedies provide attractive templates for the development of new pharmaceutical products. Nowadays, there is an enormous natural product pool containing substances of well known bioactivities and scientists have the opportunity to investigate structure-activity relationships and mechanisms of action.

Synthetic variation of the natural lead-structures have led in various cases to the discovery of more active compounds being possible new drugs of the future and therapeutic models in the treatment of so far incurable diseases.

Another way the ethnomedical preparations possess self vital healing energy and can be developed into dietary supplements and phytomedicines with defined characteristics but with different pharmacodynamic properties as the pharmaceuticals containing isolated single chemical compounds.

In the tropical rain forests there are still many plant species waiting for a thorough phytochemical investigation. This natural pharmacy is endangered by fastly moving destruction of the vital biosystems by emerging uncontrolled timber logging for wood production and generation of new farm land. Time seems to run out for many species and making needs to awake and stop the elimination of natural resources.

Avocado fruits (*Persea americana* Mill.) are a very popular and recognized healthy fruit (Knight, 2002) cultivated in many subtropical and tropical areas of the world. The origin of the plant is Southern Mexico (Hegi, 1958; Rodríguez-Suppo, 1982; Ochse et al., 1986; De Luna and Flores, 1994). Although avocado grows in wild forms, the variety *Hass* is preferably cultivated in Mexico due to a higher content of fruit flesh and superior sensory attributes (Ramos et al., 2004a).

After industrial fruit processing, avocado seed material is generally disposed, although it could be a potential source for food supplements and for medicinal products. It is noteworthy that ethnopharmacology of the Aztec and Maya cultures (cf. Fig. 1-2) used decocts of avocado seeds as a potent agent to treat mycotic

and parasitic infections. They are used also against diabetes (Lozoya and Lozoya, 1982; Kunow, 2003). Additionally, local anesthetic effects of avocado seed preparations are known to decrease muscle pain. The seed is also used as anti-inflammatory drug (Argueta-Villamar et al., 1994; Cabrera, 1996).

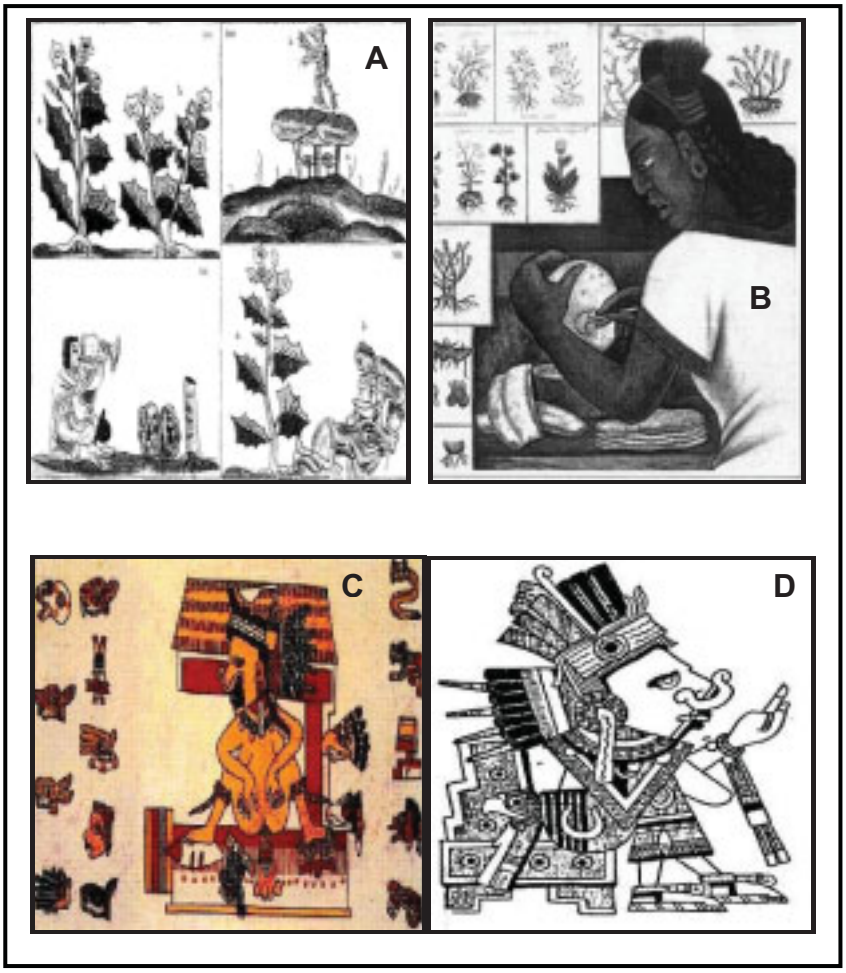


Figure 1-2. Aztecan ethnomedicine. **A**. Some used plants ("peyote") (Rätsch, 1998). **B**. Ancient Aztec healers and some of the medicinal plants they used (Balick and Cox, 1996). **C** and **D**. Aztecan medicine and fertility goodess (Codex Borgia, 68; http://www.urologiaaldia.com/imagenes/aztecas/23.jpg; http://fis.ucalgary.ca/aval/321/Mexico.html).

1.2. Current knowledge about avocados

Previous phytochemical studies of avocado seeds identified various classes of natural products, such as phytosterols, triterpenes (Werman et al., 1990; Lozano et al., 1993), fatty acids with olefinic, and acetylenic bonds (Kashman et al., 1969),

furanoic acids (Farines et al., 1995), dimers of flavanols (Geissman and Dittmar, 1965), and oligomeric proanthocyanidins (Valeri and Gimeno, 1953; Thompson et al., 1972).

Different *in vivo* assays with animals (Werman et al., 1989, Werman et al., 1991; Oelrichs et al., 1995; Alvizouri et al., 2003), and humans (Carranza et al. 1995) as well as in *vitro* assays with virus (de Almeida et al., 1998), bacteria (Sugiyama et al., 1982), enzymes (Hashimura et al., 2001), fungus (Domergue et al., 2000), insects (Rodríguez-Saona et al., 2000), and cells (Henrotin et al., 1998; Kim et al., 2000a, Kim et al., 2000b) have been used to test the biological activities from avocado tissues (fruit, leaves, seeds, and bark). Variable results have been obtained depending on the system used.

1.2.1. Known biological activities from avocado (*Persea americana* Mill.).

The avocado leaves' aqueous extract can be very toxic for horses but not for cows, while the lipophilic extracts are toxic (Oelrichs et al., 1995). In case of humans the aqueous extracts are reported to have positive influences on human health (Adeyemi et al., 2002). Some lipids isolated from the avocado fruit have shown a selective activity against human prostate adenocarcinoma (Oberlies et al., 1998).

Details about the known biological activities from Persea *americana* will be described below and the structures of the involved substances responsible for these activities will be explained on pages 28 to 31.

1.2.2. Pharmacological and toxicological profiles

From avocado fruit four principal groups of lipophilic substances are known: acetylenic, vinylic, trihydroxylated and furanoic compounds. The last group are called avocatins (Farines et al., 1995) or avocadofurans (Rodríguez-Saona et al., 2000), and the rest are called acetogenins. In the polar fraction proanthocyanidins have been identified (Valeri and Gimeno, 1953; Geissman and Diettmar, 1965; Thompson et al., 1972).

Some of the known biological activities from avocado tissue extracts are known as rooting promoters, exhibiting insecticidal-, antifungal- and antimicrobial-activities.

Also cholesterol lowering effects, enzyme inhibiton, anti-inflammatory, analgesic, antitumor, vasorelaxant and hypotensive activities were described (Adeboye et al., 1999).

An excellent avocado rooting promoter is 16-heptadecyne-1, 2, 4-triol (**56**) (cf. Fig. 2-13, p. 29) in its (2*R*, 4*R*) natural form (Becker et al., 1990).

A strong suppressive effect has been shown for the liver toxin D-galactosamine, in rats liver system by the acetogenins persenone A [(5*E*,12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-5,2,15-trienyl-acetate] and persin [(2R,12Z,15Z)-2-hydroxy-4-oxoheneicosa-12,15-dienyl-acetate] (Kawagishi et al., 2001). The last one also showed a high insecticidal (Rodríguez-Saona et al., 1998 and 1999) and antifungal activitiy (Bull and Carman, 1994; Ardi et al., 1998; Domergue et al., 2000).

Antifungal and antibacterial activities have also been shown by 1-acetoxy-2, 4-dihydroxy-n-heptadec-16-ene (**60**) that is a growth inhibitor (Bittner et al., 1971). Its trihydroxy homologue showed antibacterial activity (Sugiyama et al., 1982). Similar compounds with antifungal activity possessing (2*S*, 4*S*) configuration (cf. Fig 2-14, p. 31) have been identified by Domergue et al. (2000).

Adikaram et al., (1992) suggested that the acetylenic partial structures in some of these compounds seem to be the active side and reason for the fungicidal and insecticidal activity. In this study, both persin (**66**) and persenone A (**64**) were tested. The only difference (cf. general chapter Fig. 2-14 p. 31) between both compounds is an additional double bond conjugated with a keto group in case of persenone A (the less active) and therefore it remains unclear which chemical group is responsible for the observed activities.

With regard to its nutritional value, avocado fruit is one of the richest in the content of unsaturated fatty acids and people consuming a special avocado based diet showed lower cholesterol levels. Also beneficial skin healing effects may be due of the positive influence of this kind of fatty acids (Carranza et al. 1995).

The enzyme acetyl-CoA carboxylase (ACC), which is a key enzyme in fatty acid biosynthesis, is strongly inhibited by persenone A, persin and as well by (2*R*,4*R*)-1-acetoxy-2,4-dihydroxy-heptadec-16-ene (**61**) and (2*R*,4*R*)-1-acetoxy-2,4-dihydroxy-heptadec-16-yne (**58a**) (cf. Fig. 2-14 p. 31) found in the fruits and also in the seeds. It seems to be likely that the ACC inhibition is not related to the

unsaturated terminal bond (in the case of vinylic and acetylenic groups) but to the chain-length. It has been also proposed that the non-toxic ACC-inhibiting substances could be a beneficial tool to suppress fat accumulation and hence to avoid obesity (Hashimura et al., 2001).

In our present study on avocado seed, the brine shrimp assay (*Artemia salina* L.) revealed for the lipid fractions high toxicity values (cf. p. 206). For the further use of these components, e.g. therapy of obesity, it has to be assured that there is no toxicity related to these inhibitors of ACC enzymes.

Against the human prostatic carcinoma (PC-3), the compounds 1, 2, 4-trihydroxy-nonadecane (**54**), 1, 2, 4-trihydroxy-heptadec-16-ene (**55**) and 1, 2, 4-trihydroxy-heptadec-16-yne (**56**) (cf. Fig. 2-13, p. 29) had shown high activity and good selectivity. According to their structure-activity-relationship, the compounds with terminal alkyne groups are more potent than the terminal alkene. A terminal alkane is less active than all other substances under investigation (Oberlies et al., 1998).

Persenone A and persenone B could be possible agents to prevent inflammation-associated diseases including cancer by suppression of the expression of the inducible form of the enzymes (i.e. nitric oxide synthase and cyclooxygenase in macrophages) implicated in this biochemical processes (Kim et al., 2000a and 2000b).

The osteoarthritis is an inflammatory process and some of the used therapies are the administration of drugs for symptomatic relief; drugs that stimulate production of cartilage matrix components; and drugs that inhibit cartilage destruction. Henrotin et al. (1998) reported about the effects of avocado/soybean oils mixture acting as potent inhibitors of substances responsible for inflammation processes in human articular chondrocytes (i.e. neutral protease activity, proinflammatory cytokines and prostaglandine-E2). Furanoic compounds from the unsaponifiable avocado fruit-oil are considered to be responsible for such anti-inflammatory activities. Their structures are described later (cf. Fig. 2-12 p. 28).

For chronic diseases such as hepatic fibrosis and lung fibrosis, the activity of the enzyme lysyl oxidase is increased accompanied with collagen accumulation. The unsaponifiable avocado fruit oil components are able to inhibit this enzyme.

Therefore it could be considered to be used in the treatment of disorders of connective tissue (Werman et al., 1990).

Anti-inflammatory and analgesic activities have also been shown by the aqueous extracts from the avocado leaves (Adeyemi et al., 2002) as well as a strong inhibition on herpes simplex virus type 1 (HSV-1), Aujeszky's disease virus (ADV) and adenovirus type 3 (AD3) (de Almeida et al., 1998).

The leaves of *Persea americana* have been studied as source for vasorelaxant activity (Adeboye et al., 1999). Hence the aqueous extracts from the leaves could be considered also for treatment of high blood pressure (Owolabi et al., 2005).

With regard to the toxicological profiles, avocado fruits and their fruit oils are considered to be safe (Werman et al., 1990), whereas the unsaponifiable oils from avocado seeds are potentially hepatotoxic (Valeri and Gimeno, 1953; Werman et al., 1989; Werman et al., 1991; Werman et al., 1996). The natures of the substances responsible for this activity are unknown.

Persin (in its *R*-configuration) was responsible for the mammary cell necrosis in lactating mice (Oelrichs et al., 1995; Carman et al., 1995). This activity was also tested with derivatives in which the acetyloxy group at C1 was substituted with a butoxy or phenoxy group by changing the ester linkage for an ether linkage, or replacing the ketone group at C4 with a hydroxyl group. It was found that only persin was active (Oelrichs et al., 1995; Oelrichs et al., in Garland et al., 1998).

It would be interesting to know which of these chemical groups are responsible for this activity because structurally related substances (i.e. with the same functional groups such as persin but not having a longer extension of the carbon chain) were not active. A combination of the chain-length and substitution pattern could therefore be important factors for this activity (Hashimura et al., 2001).

More details about the chemical structure of these substances will be discussed in the general chapter (cf. p. 27).

1.3. General objectives

The avocado is a tropical fruit. Mexico continues to be the most important producing nation, accounting for nearly 40 percent of total world production (http://www.fao.org/docrep/006/y5143e/y5143e1a.htm). The production amounts

to more than 700.000 tons/year (SAGAR, 1996) from which only 3-5% is industrialized. As an important fruit crop for cultivation and its high value for the daily alimentation in Mexico, there is a huge waste stream due to over-excess in production and quick decomposition of ripened fruit under tropical temperatures.

We have started a joint project between the avocado producers and education institutions, such as the “Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ)” in Guadalajara, Mexico as well as the Technical University of Braunschweig (TU-Braunschweig), Germany. Important part of the studies at TU Braunschweig will be the systematic screening of avocado seeds, preparative isolation and structure elucidation of natural products and search for alternative uses of this fruit material.

In Mexico, the research efforts are focussed on the conservation of the avocado fruit and its products.

The avocado seed is approximately 15-16% of fruit weight (Ramos, 1999). After the avocado industrialization approximately 3000 tons of seed materials are resulting and at the moment they are disposed in landfills without further use.

Actually we have described some of the biological activities of *Persea americana* as well as the known involved substances.

The aim of this project was to extract selectively the avocados seeds (*Hass* var.) by using different extraction methods with solvents as well as modern technologies i.e. by applying CO_2 under supercritical conditions (SFE), in order to gain wide spectra of the chemical groups present in these seeds for investigation of structure-activity relationships.

Some of the obtained avocado extracts and isolated substances could have an application in pharmaceutical, food and cosmetic industries.

Aims of the phytochemical analysis on seeds of *Persea americana*:

1. - Bioassay-guided isolation on crude extracts and bioactive substances from the avocado seeds extracts obtained by solvent extraction of different polarities (methanol, dichloromethane, ethyl acetate, petrol ether and methanol-water mixtures) as well as alternative methods (solvent extraction with ultrasound and supercritical-fluid extraction with CO_2) was done by using antioxidant testsystems (TEAC, DPPH) and the brine-shrimp (*Artemia salina* L.) assay.

2. - For the large scale preparative separation of bioactive substances from crude avocado extracts high-speed countercurrent chromatography (HSCCC) in combination with multiple-step column chromatography (normal-, reversed-phase and size-exclusion chromatography), and also preparative HPLC for final purification was applied.

3. - Structure elucidation of isolated compounds was achieved by means of spectroscopical methods such as 1D-NMR (^{1}H, ^{13}C, *diff*NOe), 2D-NMR (^{1}H/^{1}H COSY, HMQC and HMBC), UV- and circulardichroism spectroscopy (CD). Mass spectrometry was applied by GC-EI/MS for molecular weight determinations as well as direct EI-MS (70eV), DCI-MS (isobutene), and HPLC-ESI-MS/MS experiments in positive and negative mode.

4. - Application of crude extracts and purified substances in different biological assays such as antioxidant capacity (TEAC, DPPH), and toxicity ('brine-shrimp' (*Artemia salina*) lethality assay), with the aim to explain existing structure-activity relationships. Connecting activity of pure substances and extracts will be the basis for application of industrial avocado seed extract in nutraceutical or cosmetical products.

2. GENERAL

The following chapter will cover the morphological, chemotaxonomical, and botanical data of species of the plant family *Lauraceae* as well as the general analytical concepts of the techniques applied for this research on *Persea americana* seeds.

2.1. Morphological data of the Lauraceae family

The avocado is a fruit with a pear form. It's scientific name is *Persea americana* Mill., (Ochse et al., 1986) from Lauraceae's family (cf. Fig. 2-1).

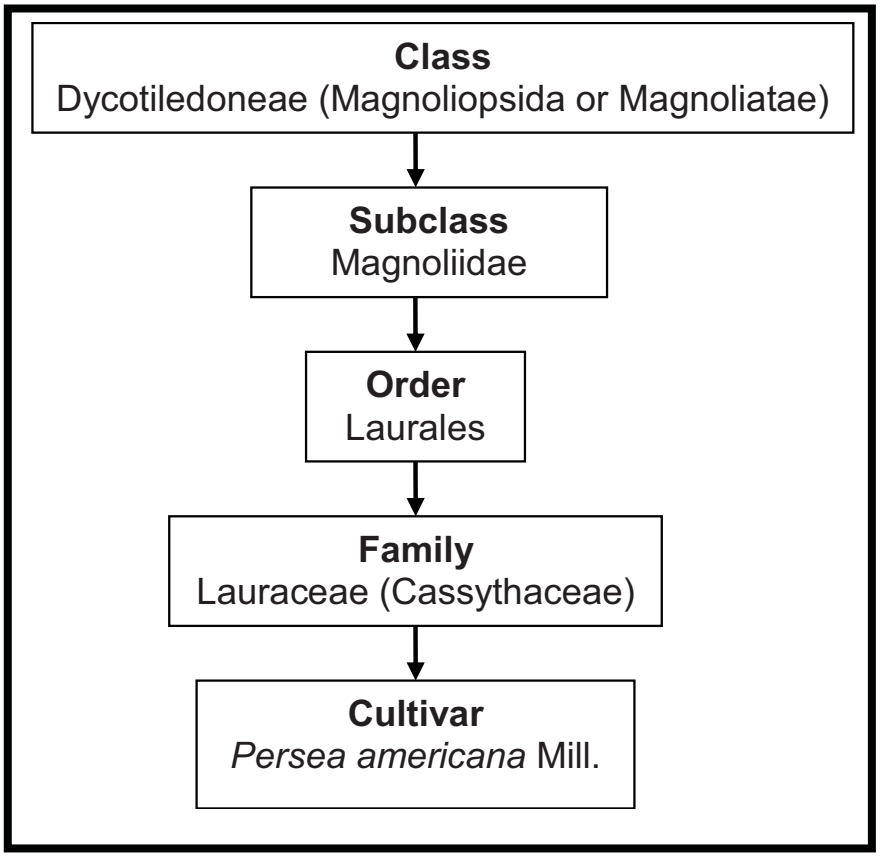


Figure 2-1. Botanical classification of *Persea americana* Mill.

The order Laurales consists of 8 families with about 2500 different species. They are characterized to have essential oil cells, perigynous flowers with a well developed perianth of free and distinct petals, inaperturate or biaperturate pollen, and unilacunar nodes. Most of them are woody plants (Cronquist, 1988; Baltisberger, 2003).

In the order Laurales, the family Lauraceae with about 2000 species is by far the largest group, including those plants whose seeds are produced in an enclosed ovulary or carpel, a specialized organ of reproduction. The carpel may go on to develop into a fruit, for which the plants are classified also in angiosperm. For the higher plants, seeds are the next generation, and serve as the primary means by which individuals of a species are dispersed across the landscape (http://www.fact-index.com/l/la/lauraceae.html).

These plants are trees or shrubs almost always green with aromatic bark. They grow in tropical and subtropical countries. Some of these species are edible, and possess important nutritional factors. Some species are used as medicinal plants.

Among the best known genera with commercially important species are *Cinnamomum* (cinnamon and camphor), *Laurus* (laurel leaves), *Lindera* (spice bush), *Sassafras* (sassafras) and *Persea* (avocado fruit) (Hegi, 1958; Sitte at al., 2002; Baltisberger, 2003).

2.2. The cultivar *Persea americana*

2.2.1. Historical development of cultivation of *Persea americana* Mill.

The origin of the Avocado plant is Southern Mexico (Hegi, 1958, Rodríguez-Suppo, 1982; Ochse et al., 1986, De Luna and Flores, 1994). Since the 16th Century the plant was shipped to the West Indies and various countries of the tropical and subtropical world. Many areas did not provide suitable environmental conditions for growing avocado fruits of acceptable quality.

Probably in the early 17th century, Spaniards took the avocado plant to Chile. In Hawaii first avocados were planted in the year 1825. Introduction from Mexico to Florida occurred in 1833 and to California in the year 1871. In 1892, avocado plants reached India, and first trees were planted in the region what today is Israel in 1908. In the last years, New Zealand had started a program to extend commercial avocado production. Regarding quantity and quality, Mexico is still the world leading avocado fruit producer.

2.2.2. Description of *Persea americana* Mill.

Avocado trees grow up 9 m, with a trunk of 30 to 60 cm in diameter. It may be short and spreading with branches beginning close to the ground (cf. Fig. 2-2).

The avocado fruit is a berry shaped like a pear with a mesocarp and endocarp (cf. Fig. 2-3) and contains only a single seed (Hegi, 1958; Barrientos-Priego et al., 1996; Sitte et al., 2002). The seed is composed of a testa where most of the polyphenolic substances are localized. The cotyledons have the highest quantity of carbohydrates. Also the embryo – similar to the cotyledons – contains high contents of carbohydrates and triglycerides (García-Fajardo et al., 1999) (cf. Fig. 2-4).

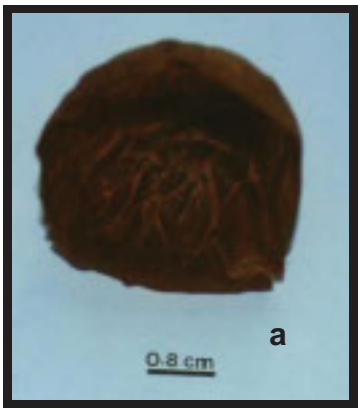
Most of the existing trivial names for the avocado fruit are derived from the native languages of Mexican indigenous cultures. The language Nahuatl formed the words ‘aocatl’ or ‘ahuacatl‘ for the avocado. In South America it is also named ‘palta‘ whereas worldwide the name “avocado” is used.

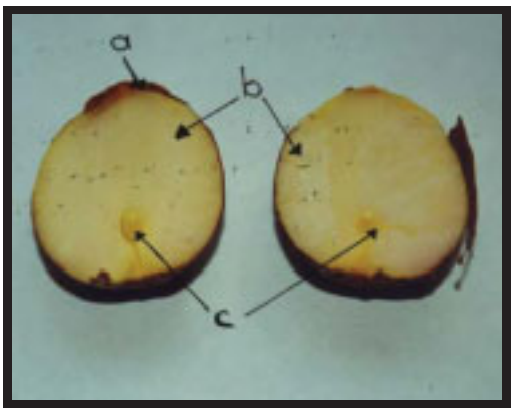
The avocado tree begins fruit production at an average age of 8-10 years and from there prolongation of fruit production is approximately 10-20 years. It is classified in three main racial groups: the Mexican, Guatemalan, and West Indian (Rodríguez-Suppo, 1982) avocados. Many other varieties are derived from these races. The *Hass* and *Fuerte* varieties are the most common in the world. *Hass* is derived from the Guatemalan race.

Figure 2-2. Extensive cultivation of avocado fruits on a farm in Michoacán, Mexico. (Photo: M. del R. Ramos-Jerz).

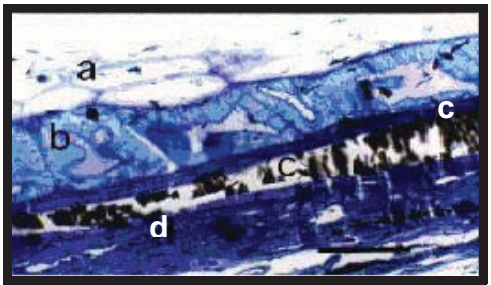
Figure 2-3. *Persea americana* Mill. fruits, c.v. *Hass*. (Photo: M. del R. Ramos-Jerz).

Avocado seeds from c.v. *Hass* (line = 2 cm)

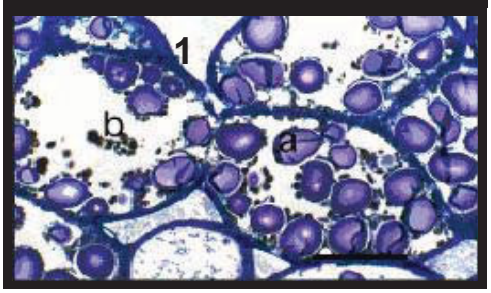


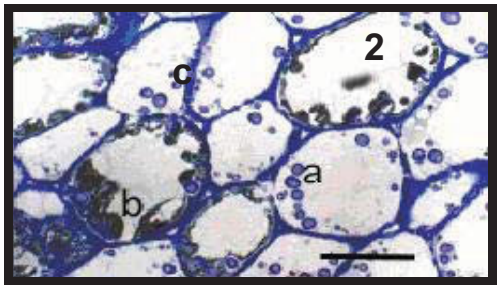


Avocado seeds from c.v. *Hass*. Testa (a), Cotyledons (b), and Embryo (c).



Microscopical cut of testa
Endocarp (a)
Sclerenchyma (b)
Tannine cells (c)
Parenchym (d)





Microscopical cut of Cotyledons (1) and Embryo (2). Starch grains (a), fat cells (b) and cellular wall (c).

Figure 2-4. Microscopical investigation of avocado seeds. Line= 0.1 mm, cut: 1µm, "Spurr" resins inclusion, staining-agent: toluidin blue (Ramos, 1999).

2.2.3. Biological activities from *Persea americana*

The biological activities are numerous. Avocado fruit extracts are reported to possess a strong suppressive effect on the powerful liver toxin D-galactosamine (Kawagishi et al., 2001).

Insecticidal- (Rodríguez-Saona et al., 1998; Rodríguez-Saona et al., 1999), antibacterial- (Sugiyama et al., 1982), as well as antifungical- (Bull et al., 1994; Domergue et al., 2000) activities have also been reported.

Consumption of avocado fruit may reduce cholesterol levels. The cholesterol lowering and skin healing effects may be due to the high content of unsaturated fatty acids (Carranza et al., 1995).

Avocado fruit extracts have anti-inflammatory activity through inhibition of the neutral protease activity, pro-inflammatory cytokines and prostaglandine-E2 (Henrotin et al., 1998).

Extracts of the avocado leaves have also shown analgesic effects (Adeyemi et al., 2002) and also strong inhibiting effects against herpes simplex virus type 1 (HSV-1), Aujeszky's disease virus (ADV) and adenovirus type 3 (AD3) (de Almeida et al., 1998) were observed. The aqueous extracts from the leaves of *Persea americana* showed besides anti-inflammatory also antidiuretic activities (Adeyemi et al., 2002).

The lipophilic substances isolated from the fruit flesh - persenone A and persenone B - could be potential agents for prevention of inflammation-associated diseases including cancer (Kim et al., 2000a and 2000b). In-vitro results have shown that these compounds are selective in their action against the human prostate carcinoma (PC-3). Investigations with regard to a possible structure-activity relationship revealed that a terminal alkyne group is more potent than the terminal alkene group. Lowest activity was found for a terminal saturated chain-ending (Oberlies et al., 1998).

In animal feeding experiments, the avocado seed oil was acting as a hepatotoxic agent (Werman et al., 1989, Werman et al., 1991) and caused abnormalities in lipid metabolism in growing rats (Valeri and Gimeno, 1953; Werman et al., 1996). The oil also inhibited lysyl oxidase activity (Werman et al., 1990). The foliage (variety: *Fuerte*, *Nabal*, and *Hass*) was lethal to rabbits and a Venezuelan avocado

variety was toxic for mice and caused mastitis (Valeri and Gimeno, 1953; Burger et al., 1994; Sani et al., 1994).

2.2.4. Chemotaxonomical aspects

The existing phytochemical researches on different plants of the Lauraceae family (including Persea) were of high importance for verification of the botanical classification (Hegnauer, 1966). Phytochemical results indicate that this plant family is phylogenitically old. Some typical substance classes are in common (e.g. furano-sesquiterpenes) with other families such as the Compositae (Gottlieb, 1972).

Typical chemical structures found in plants of the Lauraceae family belong to the following groups: essential oils (mono- and sesquiterpenes), diterpenes, triterpenes, saponines, alkaloids, phenylpropanoids (lignans and neolignans), polyphenols and flavonoids, carbohydrates, phytosterols, waxes, fatty acids, fatty acid derivatives, carotenoids, and abscisic acid derivatives.

Extensive reviews about the chemotaxonomical data collected for the family Lauraceae have been published by Hegnauer (1966, 1980), and Gottlieb (1972).

With regard to chemotaxonomical data, the attention in this work will be focused on *Persea* genus especially *P. americana* (also called *P. gratissima*).

2.2.4.1. Essential oils: Mono- and Sesquiterpenes

Monoterpenoic substances are a widely distributed group of chemicals in plants. Sabinene has been reported in P. *tolimanensis* (Scora and Ahmed, 1993).

Sesquiterpenes - in contrast to monoterpenes - occur only in lower concentrations in the Lauraceae family, but caryophyllene appears as principal constituent in several *Persea* species (Gottlieb, 1972). Other sesquiterpenes such as machikusanol (**1**), γ-eudesmol (**2**), carissone (**3**), and γ-selinene (**4**) have been reported in *P. japonica* (Wang, 1996) (cf. Fig. 2-5).

Volatile compounds isolated from *Persea americana* are estragol (also called methyl-chavicol or iso-anethol), chavicol, eugenol, methyl-eugenol, β-caryophyllene, anethol, farnesen, α-humulene, hexanal, camphene, sabinene, β-

myrcene, α-phellandrene, α-terpinene, limonene, 1,8-cineole, β-ocimene, γ-terpinene, D-limonene, D-carvone, *cis*-3-hexen-1-ol, germacrene d (**5**), α- and β-cubebene, α-copaene, α-selinene, α- and δ-cadinene, as well as monoterpenes α- and β-pinene, cymol, bergamotene, bisabolene, and α-farnesene (Montes et al., 1981; MeriÇli et al., 1992; King and Knight, 1992; Sagrero-Nieves and Bartley, 1995; Sinyinda et al., 1998). The quantity of each of these substances is a good parameter to differentiate between *Persea* species (Hegnauer, 1980).

The principal difference between avocado leaves is the content of estragol and caryophyllene. Leaves are containing high amounts of estragol and high hepato-carcinogenic effects are documented (Adeyemi et al., 2002).

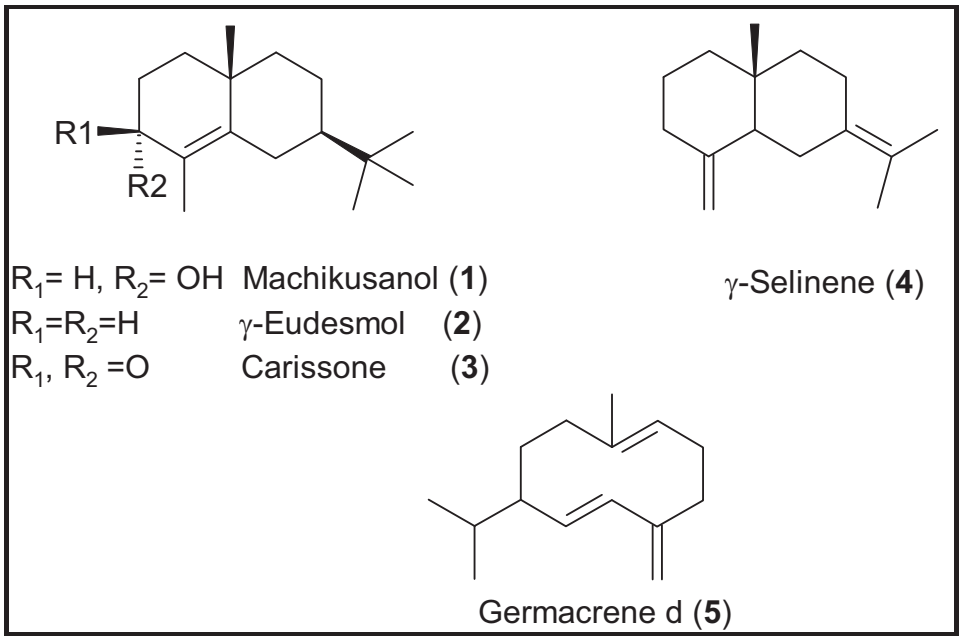


Figure 2-5. Sesquiterpenes from *Persea* species

2.2.4.2. Diterpenes, triterpenes, and saponines in the Lauraceae plant family

Persea indica is a natural source for various diterpenes with a highly complex substitution pattern, such as ryanodol (**6**) (González-Coloma et al.,1993), indicol, vignaticol, perseanol (**7**) (Fraga et al., 1997), ryanodane, isoryanodane cinnzeylanone (**8**), ryanodol 14-monoacetate and epi-cinnzeylanol (González-Coloma et al., 1996). Some of these compounds showed antifeedant and insecticidal potential (Fraga et al., 1997; González-Coloma et al., 1999; Fraga et al., 2001).

A saponine component, hexanorcucurbitacin-type glucopyranoside called “perseapricoside A” (2, 3, 16-trihydroxy-4, 4, 9, 14-tetramethyl-19-norpregn-5-ene-11, 20-dione) (**9**) has been reported as a bitter principle of *Persea mexicana*. In ethnomedicine the extract is used as an aperitive (Ohsaki et al., 1990) (cf. Fig. 2-6).

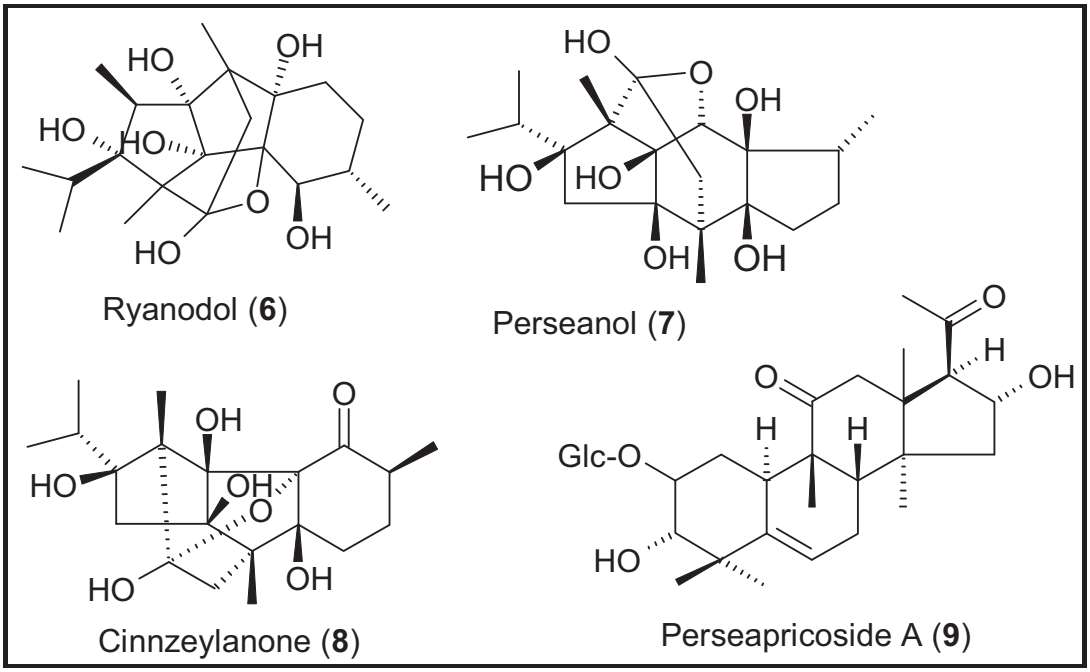


Figure 2-6. Diterpenes, triterpenes, and saponines in the genus *Persea.*

2.2.4.3. Alkaloid components

Perseae is able to produce only benzyl-tetrahydro-isoquinolines (type I) (**10**) alkaloids while the Cryptocaryeae (also from the Lauraceae family) is able to produce almost all kind of alkaloids through secondary modifications on the benzyl-tetrahydro-isoquinoline precursors. This results in quite distinctive array types [pleurospermin (**11**) and cryptopleurine (**12**)] (Gottlieb, 1972). Isoboldine (**13**) and corytuberine (**14**) have been reported in *Persea japonica* (Wang et al., 1996) (cf. Fig. 2-7).

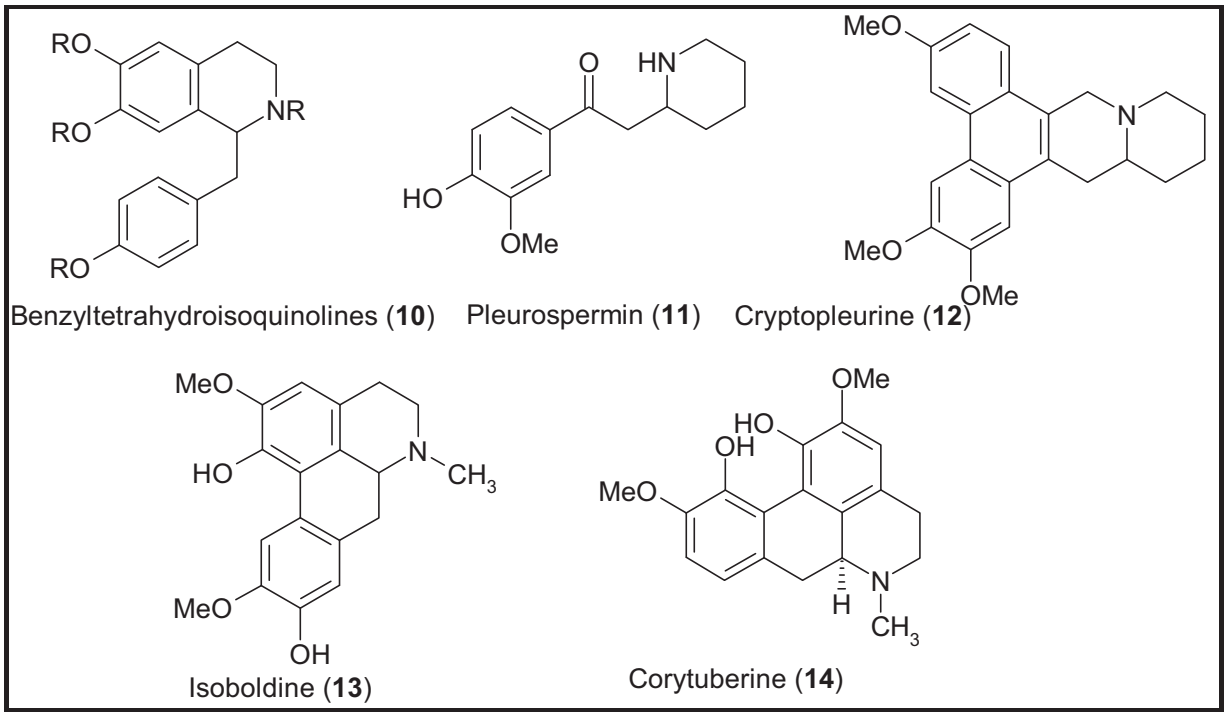


Figure 2-7. Occurrence of alkaloids in the Lauraceae family

2.2.4.4. Lignans and neolignans (Arylpropanoid compounds)

The presence of the chemical class of arylpropanoids seems to be a substitute for the alkaloids in the Lauraceae family. They are principally found in *Cinnamomeae* and Cryptocaryeae (Gottlieb, 1972; Ishige et al., 1991) and are classified in three groups: Cinnamic acid derivatives, lignans and neolignans. The lignans (phenylpropan dimers) are widespread in gymnosperms and angiosperms and achieve a high level of oxidation in the neolignans which appear to be largely restricted in distribution to the Lauraceae and some allied woody families (Waterman and Mole, 1994). Significant cytotoxic activities against cancer cell lines have been reported for the identified neolignanes [obovatinal (**15**), perseals A (**16**), B (**17**), C (**18**) and D (**19**) as well as obovatifol (**20**) and obovaten (**21**)] from *Persea obovatifolia* (Tsai et al., 1996 and 1998).

The lignan (+)-*epi*-syringaresinol (**22**) was isolated from *Persea japonica* (Wang et al., 1996) and lingueresinol (**23**) (the *epi* isomer of lyoniresinol) has been isolated from *Persea lingue* which is used in the traditional medicine for the treatment of dysentery, leucorrhoea, endometritis and some kind of tumours (Sepúlveda-Boza et al., 1990) (cf. Fig. 2-8).

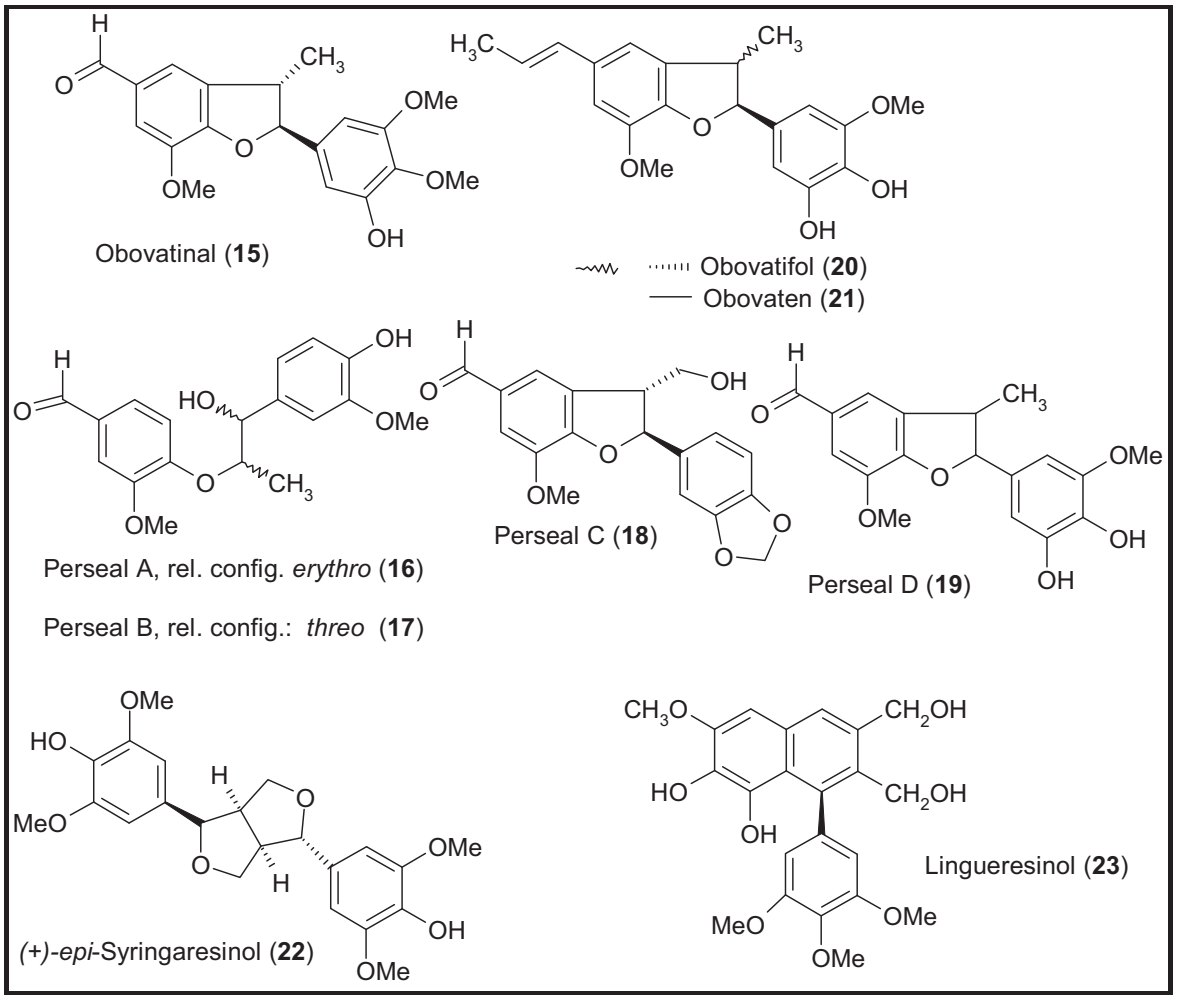


Figure 2-8. Phenylpropanoids from the genus *Persea.*

2.2.4.5. Polyphenols and related derivatives

Cinnamomum and *Lindera* (*umbellata*) species are particularly used in the ethnomedicine of Eastern Asia as antipyretic medicine. They are rich in catechin monomethylether (**24**) and dimethyl-ethers from penta-hydroxy-1,3-diarylpropan-2-ol (**25**) (Morimoto et al., 1985). *Persea cordata* is used to cure infections. A basic phytochemical analysis confirmed the presence of terpenes, steroids and phenolics. However, so far no pure substances have been isolated or identified (Schlemper et al., 2001). The same is true for *P*ersea *laevigata* which revealed higher antifungal activity. Again the substances responsible for this activity are not known yet (Freixa et al., 1998).

Golan et al. (1977) have reported that most of the flavonoids in avocado fruit exhibit substitution of the hydroxyl group (OH) at position C5 (phloroglucinol type A-ring).

It is well-known that proanthocyanidins, isoflavones, and chlorogenic acid are present in different tissues of the avocado plant (Valeri and Gimeno, 1953; Geissman and Dittmar, 1965; Brown, 1972; Ramírez-Martínez and Luh, 1973; Bate-Smith, 1975; Nose and Fujino, 1982; Ardi et al., 1998; Adeyemi et al., 2002).

The known phenolics from *Persea americana* have been determined in most cases by hydrolytic assays. This chemical degradation resulted in the detection of 4-hydroxy-benzoic acid (p-hydroxy-benzoic), 2,3-dihydroxy-benzoic acid (o-pyrocatechuic acid), 2,4-dihydroxy-benzoic acid (β-resorcylic acid), 2,6-dihydroxy-benzoic acid (γ-resorcylic acid), 3,4-dihydroxy-benzoic acid (protocatechuic acid), 3,5-dihydroxy-benzoic acid (α-resorcylic acid), 3,4,5-trihydroxy-benzoic acid (gallic acid), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid), 2-hydroxy-cinnamic acid (o-coumaric acid), 3-hydroxy-cinnamic acid (m-coumaric acid), 4-hydroxy-cinnamic acid (p-coumaric acid), 3,4-dihydroxy-cinnamic acid (caffeic acid), 4-hydroxy-3-methoxy-cinnamic acid (ferulic acid), 4-hydroxy-3,5-dimethoxy-cinnamic acid (sinapic acid) (Torres et al., 1987) as well as p-coumaroyl-quinic acid, and caffeoyl-quinic acid, 4-methyl-catechol, and trans-cinnamic acid (Ramírez-Martínez and Luh, 1973; Golan et al., 1977).

Numerous proanthocyanidins ranging from (+)-catechin (**96**) (cf. Fig. 3-81, p.163) and (-)-epicatechin (**26**) to highly polymeric substances have been reported in avocado seeds. They have been classified in three groups: the ether soluble catechins, the ethyl acetate extractable mixtures (oligomers) and the water soluble but not extractable polymers (Bate-Smith, 1975; Geismann and Dittmar, 1965). On the epicarp l-epicatechin (**27**) was found (Nose and Fujino, 1982).

Several proanthocyanidins with A-type linkage, which were obtained partly as a mixture (Jacques et al., 1974), were documented, they have trivial names such as cinnamonol D1 (**28**) and D2 (**29**), the cinnamtannins A2 (**30**), A3 (**31**) and A4 (**32**) (Morimoto et al., 1986) and tetramers cinnamtannin B1 (**33**) and D1 (**34**) (Hegnauer, 1980) (cf. Fig. 2-9).

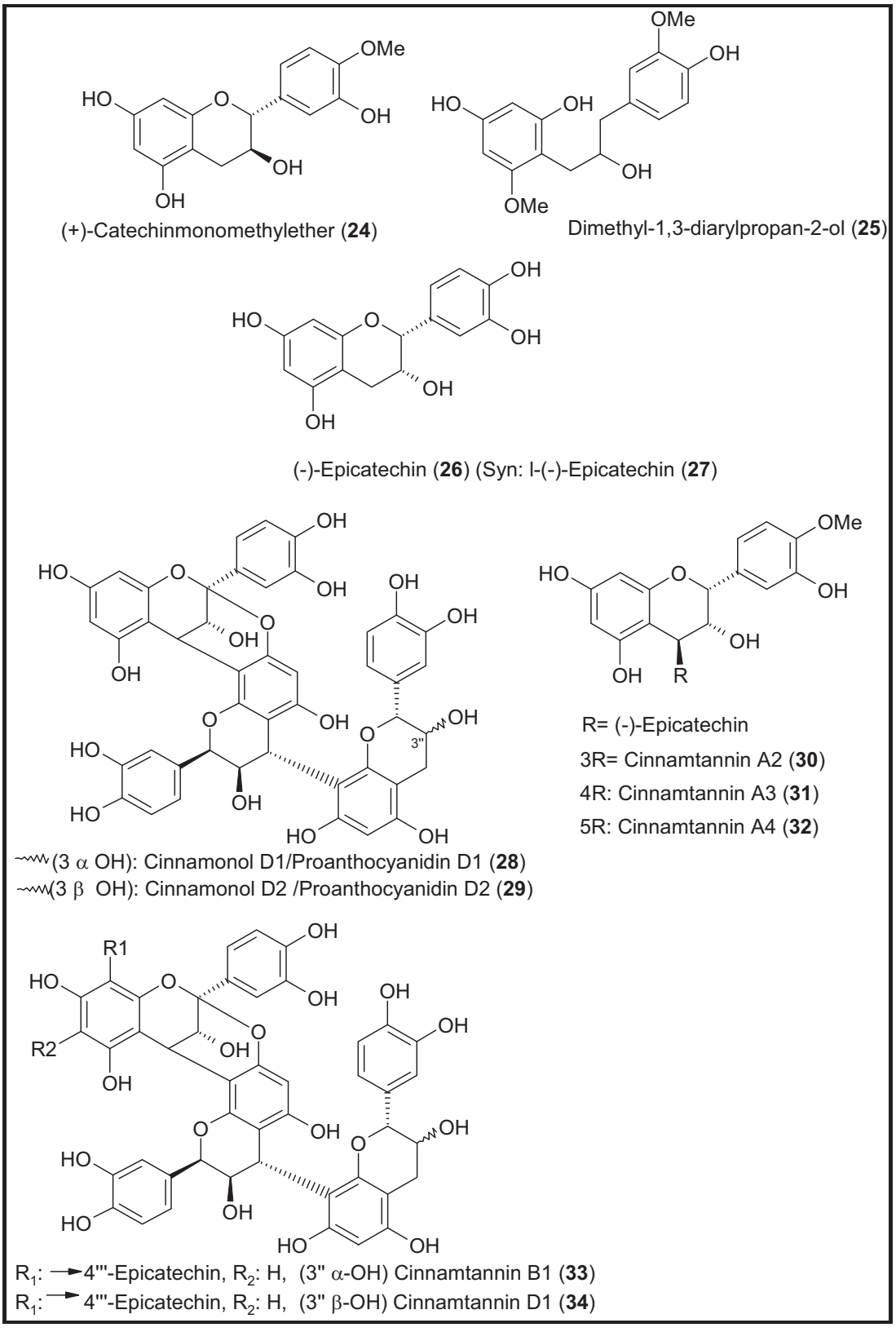


Figure 2-9. Flavonols, chalcone and proanthocyanidin structures from the Lauraceae family.

2.2.4.5.1. Flavonoids

The flavonoids present one of the most common, widely distributed and characteristic group of phenolic plant metabolites. In the formation of all flavonoids, a key central metabolite intermediate is the chalcone structure or its isomeric flavanone (Haslam, 1998). Besides chalcones and flavanones, flavonols and leucoanthocyanidins (both flavonoids) are also considered to be of primitive chemical character in the evolutionary scale of the plants (Gottlieb, 1972). The flavonoids of the Lauraceae family are common members of these classes: apigenin, astragalin (kaempferol-3-*O*-glucoside) (**35**), coumarin, C-glycosyl-flavone [orientin, isoorientin (**36**), vitexin (**37**), isovitexin (**38**)], kaempferol, afzein (kaempferol 3-*O*-α-L-rhamnopyranoside), luteolin, luteolin 7-glucoside, quercetin, quercetin 3-*O*-β-glucopyranoside, quercetin 3-*O*- α-D-arabinopyranoside, quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside), scopoletin, as well as chlorogenic acid (Hegnauer, 1980; MeriÇli et al., 1992; de Almeida et al., 1998; Adeyemi et al., 2002).

In the flowers of *Persea americana,* 3-*O-trans*-p-coumaroyl-kaempferol, quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin), and isorhamnetin 3-*O*-glucoside have been reported (Kruthiventi and Krishnaswamy, 2000) (cf. Fig. 2-10).

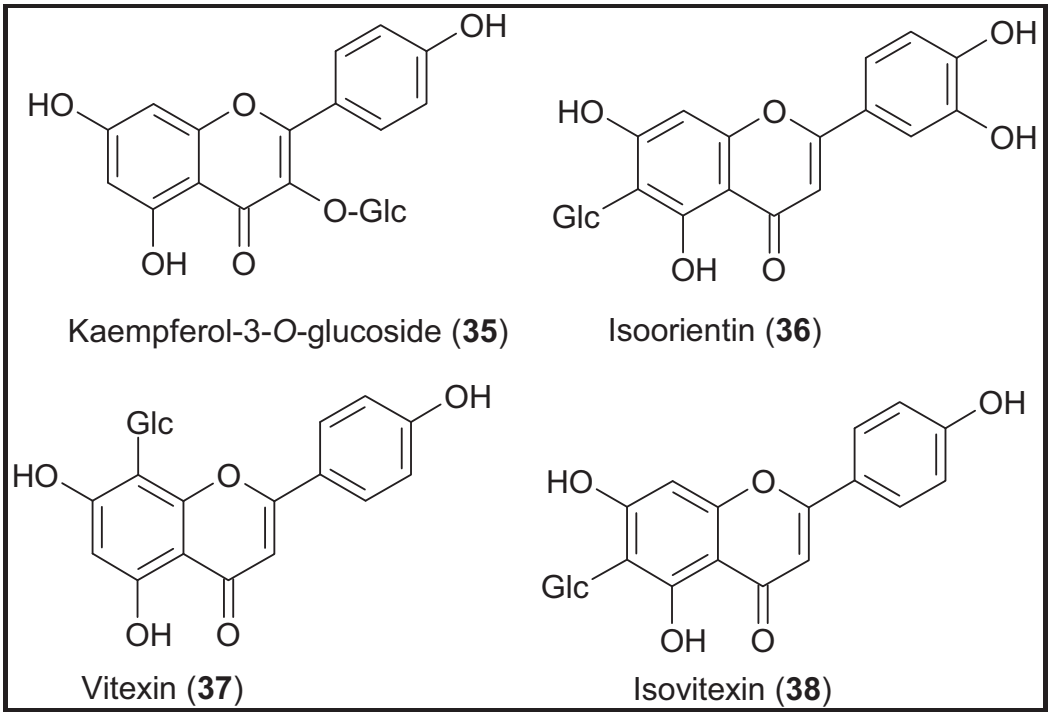


Figure 2-10. Flavonoid O- and C-glucosides from the genus *Persea.*

2.2.4.6. Sugars and substances that contain sugars

In general, the fruit of *Persea* contains the sugar-alcohol D-glycero-D-galacto-heptitol (*syn*.: perseitol, perseit) (**39**), volemitol, and in addition also D-talo-heptulose (**40**), the aldose D-glycero-D-galacto-heptose (**41**), erythro-octulose, D-glycero-L-galacto-octulose (**42**), D-erytro-D-galacto-octitol, D-glycero-D-manno-octulose (**43**), and D-erythro-L-galacto-nonulose (**44**) (Charlson and Richtmyer, 1959 and 1960; Sephton and Richtmyer, 1966; Richtmyer, 1970).

D-manno-heptulose (**45**) has not only been isolated from avocado fruit (Altiniğne and Özer, 1991), it also seems to be a major photosynthetic product in the leaves (Bean et al., 1962) (cf. Fig. 2-11). This sugar is able to inhibit the insulin secretion. Therefore consumption of avocado fruits might be beneficial for treatment against hypoglycaemic disease (Simon et al., 1972).

From the bark of the avocado tree two polysaccharides (arabino-xylans) composed of *L*-arabinose and *D*-xylose sugar units were isolated. The first arabino-xylan has a highly branched structure consisting of a (1→4)-linkages in the β-D-xylan backbone in which each of the D-xylopyranosyl residues is substituted at *O*-2 and *O*-3 with L-arabinofuranosyl-(1→3)-L-arabinofuranosyl and L-arabinofuranosyl groups, respectively. The second arabino-xylan is essentially a linear (1→4)-linked β-D-xylan with limited branches at *O*-2 or *O*-3 consisting of L-arabinofuranosyl groups. Traces of galactose and glucose were also present (Gowda et al., 1988).

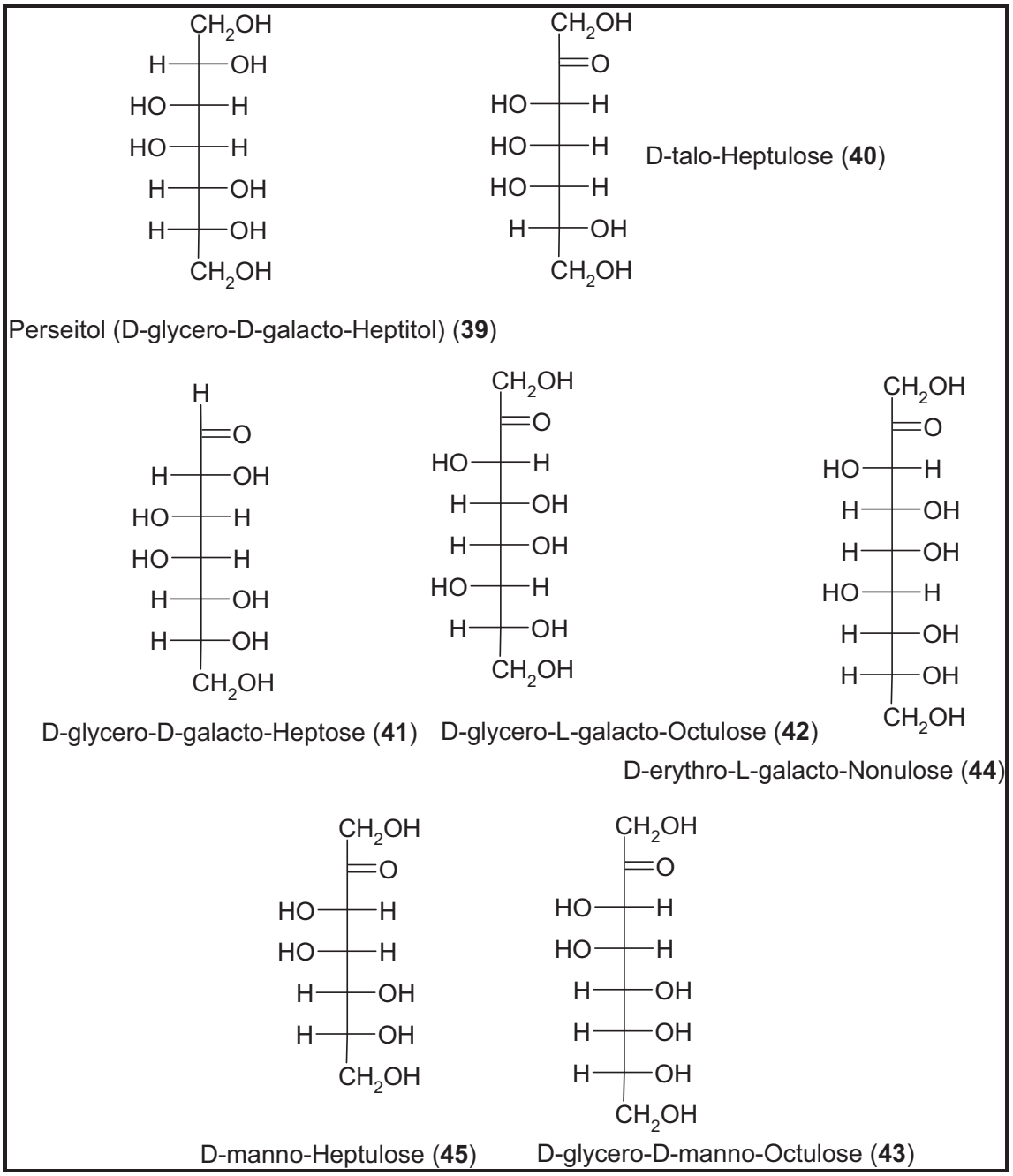


Figure 2-11. Sugar components from the genus *Persea.*

2.2.4.7. Phytosterines and Waxes

The profiles for alkanes and wax materials from *Persea* have been described. The structures range from C_{23} to C_{35} carbon atoms (Hegnauer, 1980).

As phytosterols, β-sitosterol, stigmasterol, β-sitosteryl glucoside, and stigmasteryl glucoside have been reported by Wang (1996).

2.2.4.8. Carotenoids

Gross et al. (1973) published a quantitative study about the carotenoid content of fruits, peels and leaves from *Persea americana*. The carotenoids include carotenes (α, β , ζ, γ, and hydroxy-α), cryptoxanthin, α-citraurin, lutein, *cis*-lutein, iso-lutein, violaxanthin, chrysanthemaxanthins [a, b and c], zeaxanthin, antheraxanthin, luteoxanthin, auroxanthin, neochrome, and neoxanthins (α and β). In avocado oil, chlorophyll pigments have been reported by Werman and Neeman (1986b).

2.2.4.9. Fatty acids and acetogenins with terminal alkene and alkyne groups

The presence of high quantities of fatty acids in Lauraceae seeds is not a characteristic attribute of the family. Many fruits accumulate oils in the pericarp tissue (Hegnauer, 1966) and compared to other seed oils, the pericarp contains only minor proportions of lauric acid, while oleic acid predominates (Ortíz-Moreno et al., 2003). The fatty acids profile of avocado seed is composed by caprylic, heptanoic, caprylic, pelargonic, lauric, myristic, undecanedioic, hexadecenoic, palmitic, linoleic, oleic, stearic, linolenic, arachidonic (Ramos, 1999) and palmitoleic acid (Werman and Neeman, 1986a; Werman and Neeman, 1987). The seeds contain only 1-2% of fat as was corroborated in this work (cf. Table 3-1 p. 49).

The unsaponifiable partition of this oil is composed of long-chain acetogenins with furanyl compounds (Werman et al., 1990), squalen, polyols, tocopherols, and sterols (β-sitosterol) (Henrotin et al., 1998).

Substances from avocado material with a furan moiety were named “avocatins” or “avocadofurans”. Frega et al. (1991) reported from avocado oil - recovered from the fruit flesh - a cyclic keto compound **46**. Counting from the chain endings (in positions of the double bonds) **46**, **52** and **53** are identical to linoleic acid. Farines et al. (1995) reported nine substances of this structural class for avocado fruits with an α-mono-substituted furan cycle (cf. Fig. 2-12 compounds **47** - **53**). Structural differences exist in chain-length, and number of double bonds. Particularly, some structures (**49**, **51**, and **53**) have a double bond in conjugation to the furan moiety.

For avocado seed material, 2-(tridec-12-ene)-furan (**47**) (*syn*. Avocadenofuran) and 2-(tridec-12-yne)-furan (**48**) (*syn.* Avocadynofuran) also have been reported by Kashman et al. (1969).

The pharmacological activity as lysyl oxidase inhibitor (Werman et al., 1990) of the unsaponifiable part of the avocado lipids might be related to this natural product class (Farines et al., 1995). Insecticidal properties were reported and beside the length of the side chain also the double bond in the ring system of the avocadofurans is strongly determining the activity (Rodríguez-Saona et al., 2000).

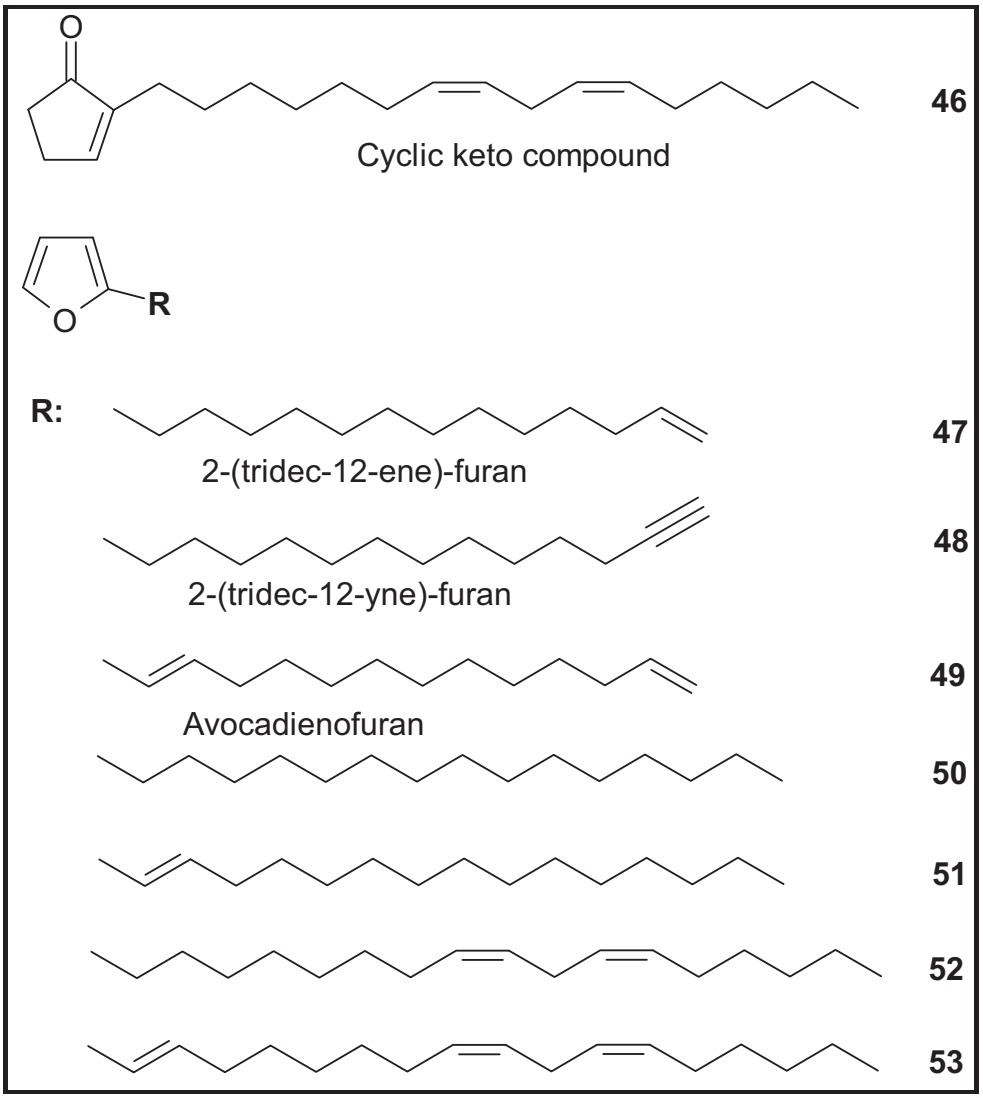


Figure 2-12. Non-saponifiable compounds from the avocado fruit flesh.

Other classes of compounds identified from the fresh avocado fruit pulp are composed of triols (cf. Fig. 2-13, compounds **54** to **56**), attached to saturated, unsaturated or polyunsaturated linear aliphatic chains (Kashman et al., 1969; Néeman et al., 1970; Brown, 1972; Werman et al., 1990; Adikaram et al., 1992; Sugiyama et al., 1982). Compounds **55** (*syn*. Avocadene) and **56** (*syn.* Avocadyne) have also been reported in the peels of immature avocado fruits (Adikaram et al., 1992) showing antifungal activities against plants fungus.

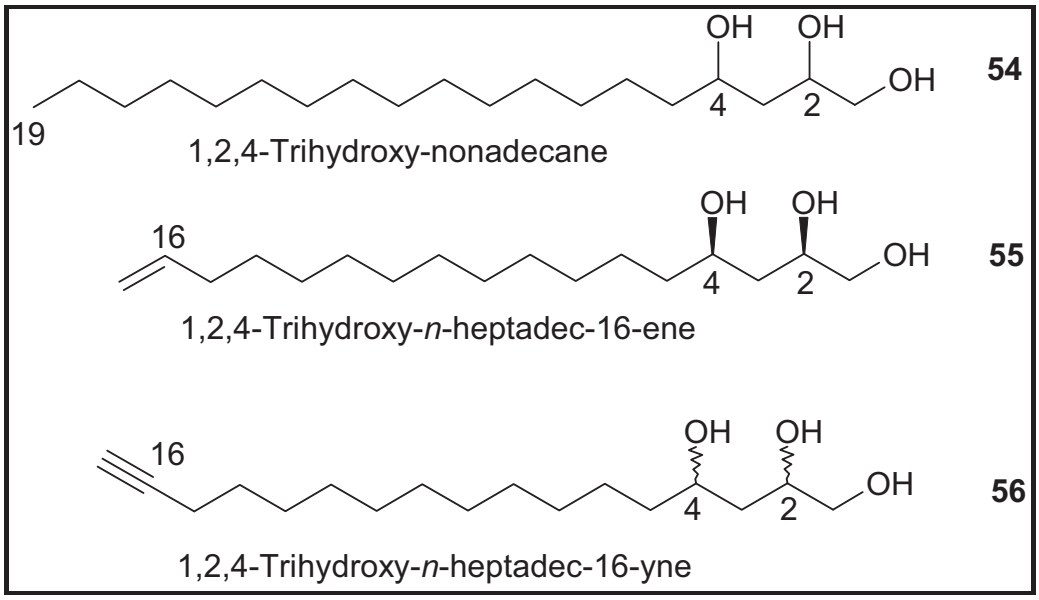
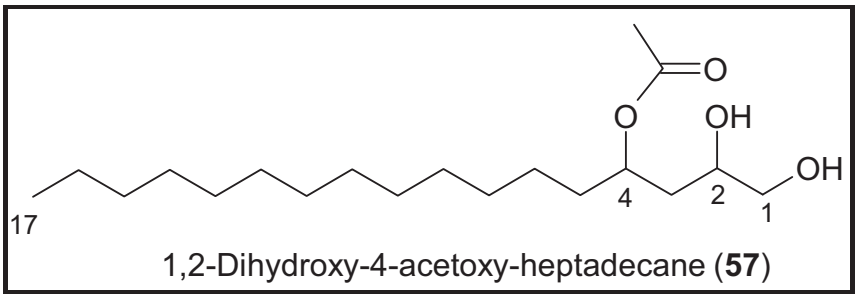


Figure 2-13. Long-chain trihydroxy-compounds from avocado fruit.

Sugiyama et al. (1982) reported a *R*-configuration at C-2 and C-4 for 1,2,4-trihydroxyheptadec-16-ene (**55**). The only difference between the (2S,4S) enantiomer was the value of optical rotation $[\alpha]_D$. The IR and NMR-data were as expected completely identical.

Three aliphatic compounds with an unusual C_{17} carbon-chain - each one having a terminal acetylenic bond - appeared to be the main constituents responsible for the unpleasant “bitter” type sensorial properties of certain avocado fruits (Brown, 1972). Some of them are identical to earlier reported compounds by Kashman et al. (1970) such as 1, 2-dihydroxy-4-acetoxy-heptadecane (**57**).



1,2-Dihydroxy-4-acetoxy-heptadecane (**57**)

A variety of hydroxylated compounds and the related acetates ranging from C_{19} to C_{23} atoms (in general called acetogenins) have been also identified in fruits and avocado seeds (Kashman et al., 1969 and 1970; Adikaram et al., 1992; Oberlies et al., 1998; Domergue et al., 2000; Hashimura et al., 2001; Kawagishi et al., 2001) (cf. Fig. 2-14 compounds **58** to **67**). Compound **58** (*syn*. Avocadyne acetate) has the same absolute configuration at C-2 and C-4 (Alves et al., 1970; Kashman et al., 1970) and some of these substances show antifungal activity "*in-vitro*" (Adikaram et al., 1992).

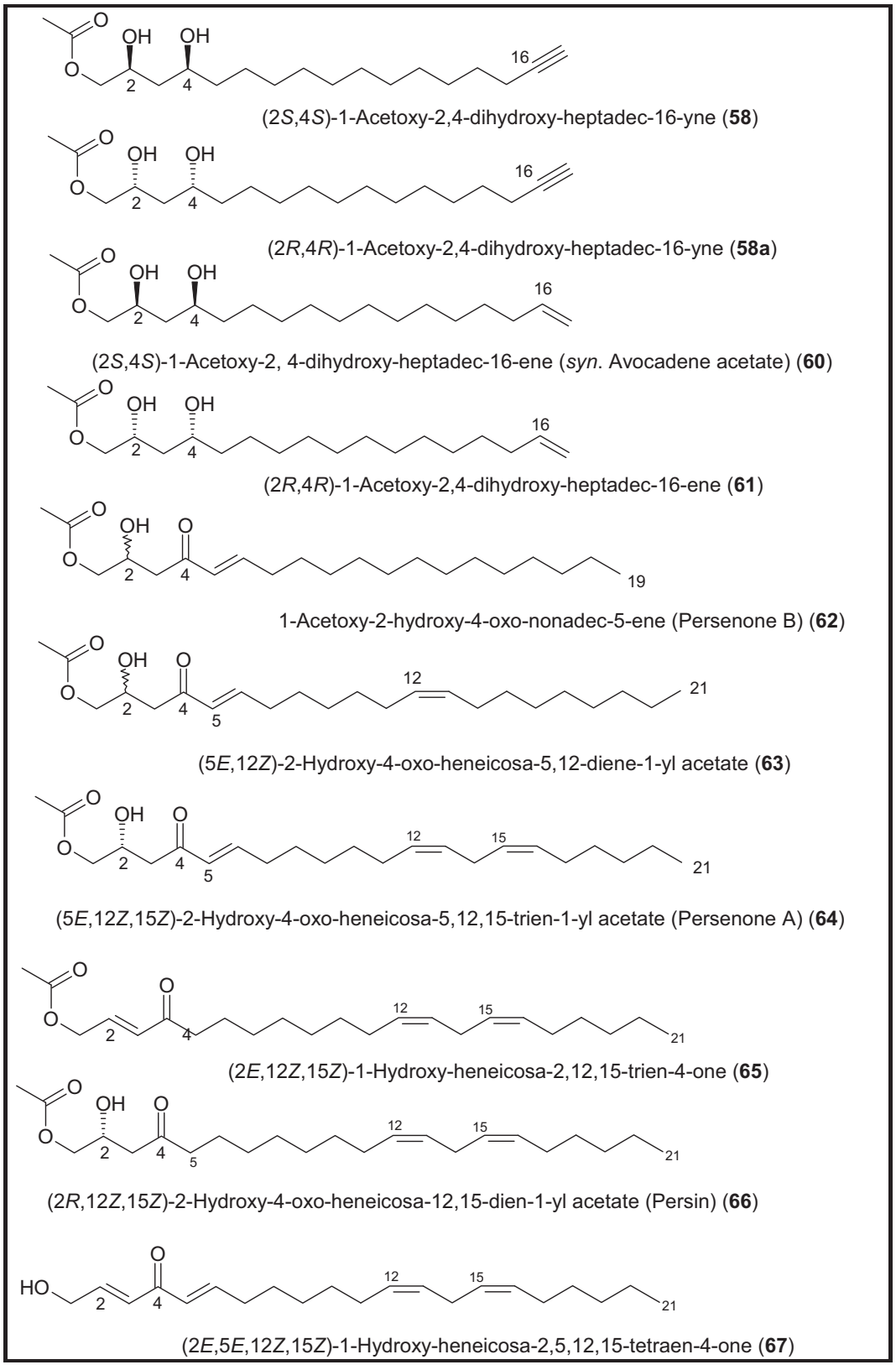
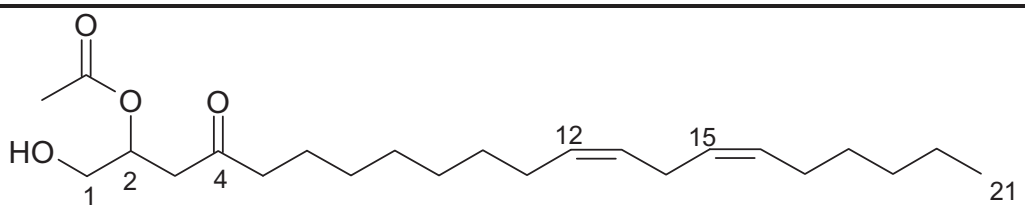


Figure 2-14. Acetogenins from the genus *Persea.*

Persin (**66**) with *S*-configuration at C2 was isolated from avocado leaves (Bull et al., 1994; Carman et al., 1995). **66** has a close structural relationship to the monoglyceride of linoleic acid (i.e. position and (*Z*, *Z*)-stereochemistry of the two double bonds) (Carman et al., 1995). Interestingly, only the *R*-enantiomer of persin is biologically active and caused necrosis on the acinar epithelium of the lactating mammary gland and the myocardium (Oelrichs et al., 1995).

Isopersin (**68**) tends to isomerize to persin (**66**). Both compounds are labile in acidic solutions being transformable to alkyl furan structures (**52**) that are known to have insecticidal activity (Rodríguez-Saona et al., 1998 and 1999).



(12*Z*,15*Z*)-1-Hydroxy-4-oxo-heneicosa-12,15-dien-2-yl acetate (Isopersin) (**68**)

The acetogenins (avocadofurans, avocadenofurans and avocadynofurans) have been found also in *Elodea canadensis* (Hydrocharitaceae) (Previtera et al., 1985), *Persea indica* (Fraga et al., 1996) and *Persea tolimanensis* (Scora and Ahmed, 1993).

These compounds are “biologically active aliphatic acetogenins” (Hegnauer, 1980) and most of them had shown significant cytotoxicity ‘*in-vitro’* as well as antitumor activity (Oelrichs et al., 1995).

A patent for the extraction of avocadofurans including polyhydroxylated compounds from avocado fruits for applications in the field of therapy, cosmetics and food had been assigned by Broutin et al. (2003).

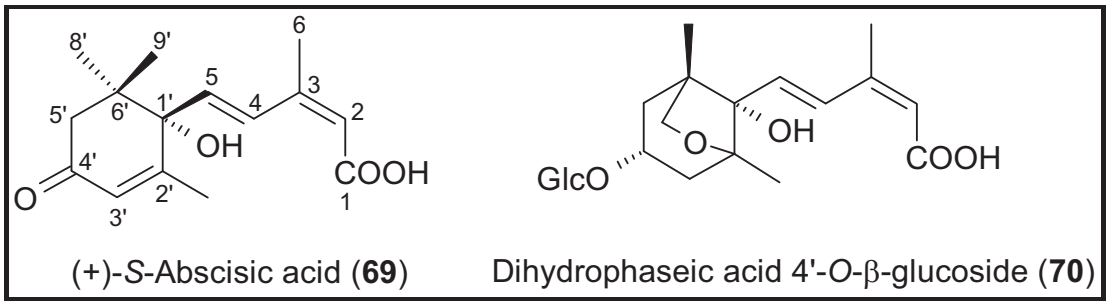
2.2.4.10. Abscisic acid and derivatives

Abscisic acid (ABA) (**69**) is a phytohormone with multiple functions. ABA has function as stress hormone when there is not enough water causing stoma closuring (by influencing the ions-transport mechanisms) and allot dormancy. The

discard of leaves, flowers and fruits is a normal process in the plants, which is called “abscission” (abortion) (Brummitt, 1992; Krochko, 1998).

The tissue that incorporates labeled mevalonate into ABA with the highest yield is ripening avocado fruit mesocarp, in which permeability barriers weaken during maturation. It may be significant that the fruit giving the highest yield of labeled ABA is the one that has the most intense and rapid climacteric (Addicott, 1983)

Only one glycoside-derivate of ABA, the dihydrophaseic acid 4’-*O*-β-glucoside (**70**) has been reported from the fruits of *Persea americana* (Hirai and Koshimizu, 1983) (cf. Fig. 2-15).



(+)-*S*-Abscisic acid (**69**) Dihydrophaseic acid 4'-*O*-β-glucoside (**70**)

Figure 2-15. Known abscisic acid derivatives from *Persea.*

2.2.4.11. Diterpenes, δ-Lactones and miscellaneous compounds

Additional compounds that have been isolated are biogenous amines such as dopamine and serotonin (known as mammalian neurotransmitters) (Golan et al., 1977), a protein classified as calmodulin (Le Roux and Dubery, 1989) and tocopherols (Lozano et al., 1993).

From *Persea indica* minor concentrated diterpenes have been reported: anhydrocinnzeylanone (**71**), garajonone (**72**), and 2,3-didehydrocinnzeylanone (**73**) (Fraga et al., 2001; González-Coloma et al., 1993), ryanodane diterpenes: i.e. ryanodol 14-monoacetate (**74**) and *epi*-cinnzeylanol (**75**) (González-Coloma et al., 1996) (cf. Fig. 2-16).

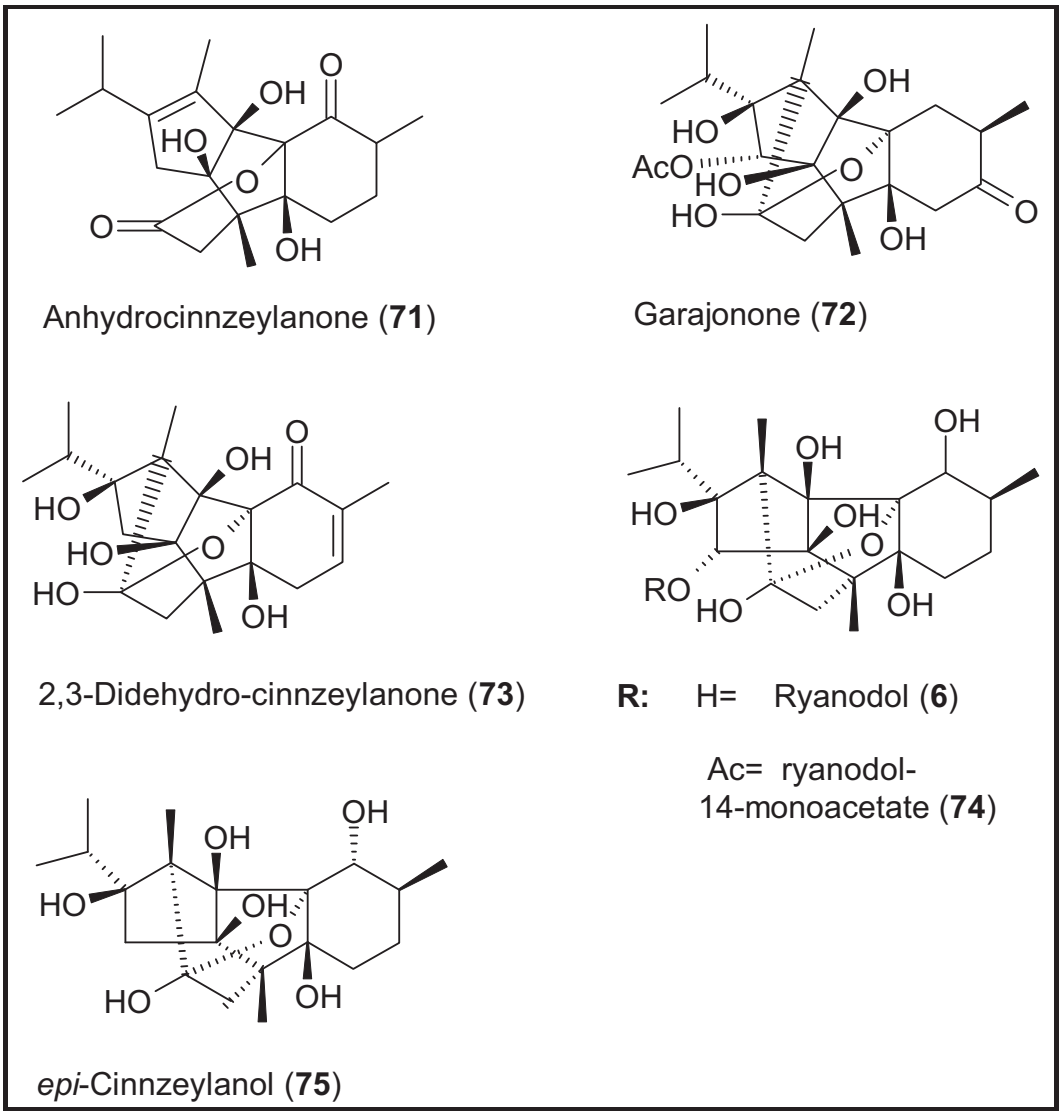


Figure 2-16. Minor concentrated diterpenes from *Persea indica*.

Some δ-lactones with a six-membered ring [majoranolide (**76**), majorenolide (**77**) and majorynolide (**78**)] isolated from *Persea major* showed antitumor-activities against human tumor cell-lines ("*in-vitro*") [lung (A-549), breast (MCF-7) and colon (HT-29) carcinoma]. Also pesticide activity against mosquito larvae (ML), two spotted spider mite (2SSM) and melon aphid (MA) was reported (Ma et al., 1989 and 1990) (cf. Fig. 2-17).

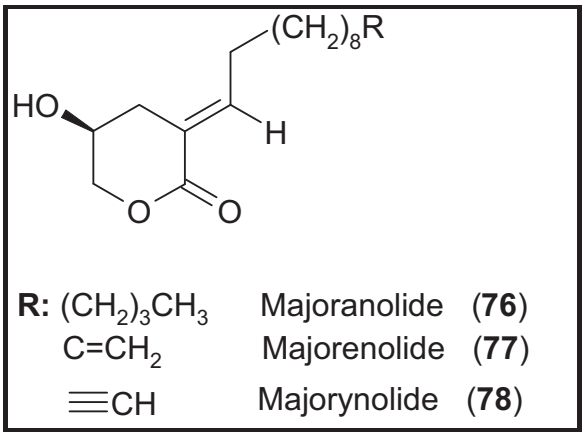


Figure 2-17. δ-Lactones from *Persea major.*

2.2.5. Economical importance of avocado cultivation in Mexico.

In Mexico, people prefer avocados of medium weights (200 to 300 g) with aromatic sensorial attributes of the fruit flesh oil. For international export, the most important commercial varieties are *Fuerte*, *Hass*, and *Nabal*. Origin of the variety *Hass* is the Guatemalan type. This variety is widely cultivated due to its high quality (Ibar, 1979).

Worldwide commercial expansion of avocado cultivation started 50 years ago in Californian, Florida, and Israel. The most important countries for export are Israel, South Africa, Cameroon, and Spain. Mexico exports principally to USA and part of Europe.

The economical importance of the plant led recently to an expansion of cultivation with higher annual production rates not only in Mexico but also in other countries like Israel, USA, and Spain.

Importing countries (France, UK, Germany, and Belgium) have increasing avocados consumption and marketing. The importation in the case of USA increased from approximately 13,300 tons in 1990 to 155,600 tons in 2004 (http://faostat.fao.org/site/343/DesktopDefault.aspx?PageID=343) (cf. Fig. 2-18).

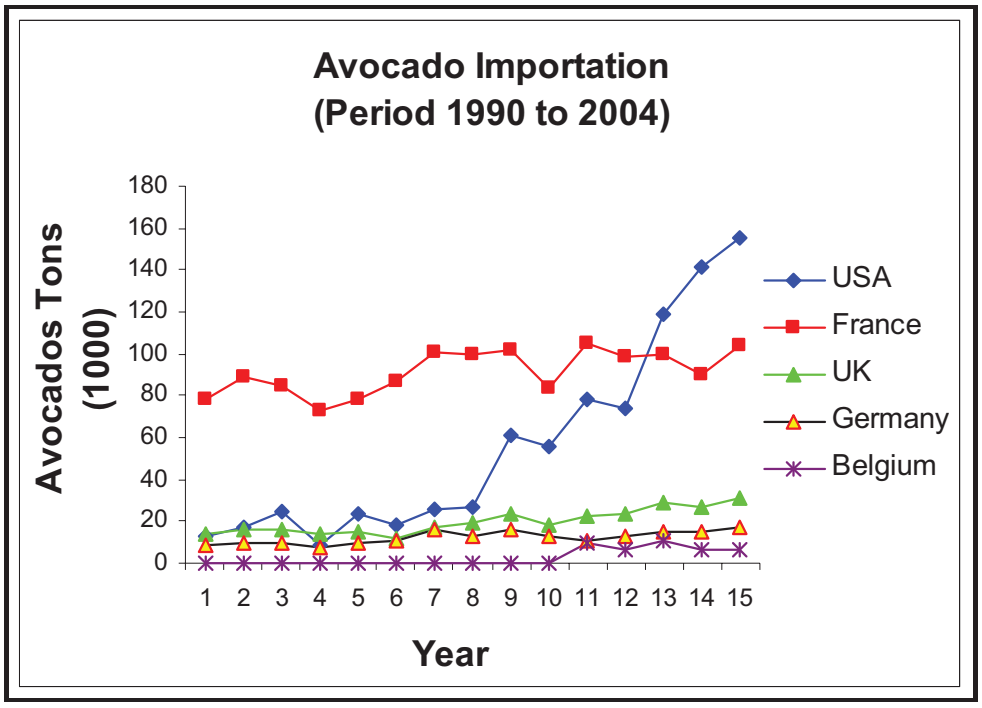


Figure 2-18. Principal avocados importation countries (Period from 1990 to 2004) (Data origin: FAOSTAT, 2006).

Mexico is the most important avocado producer worldwide (cf. Fig. 2-19) with a production of more than 700,000 tons per year (http://faostat.fao.org/site/340/DesktopDefault.aspx?PageID=340). Moreover, it also consumes the highest amount of avocados worldwide (http://faostat.fao.org/site/346/DesktopDefault.aspx?PageID=346) (cf. Fig. 2-20).

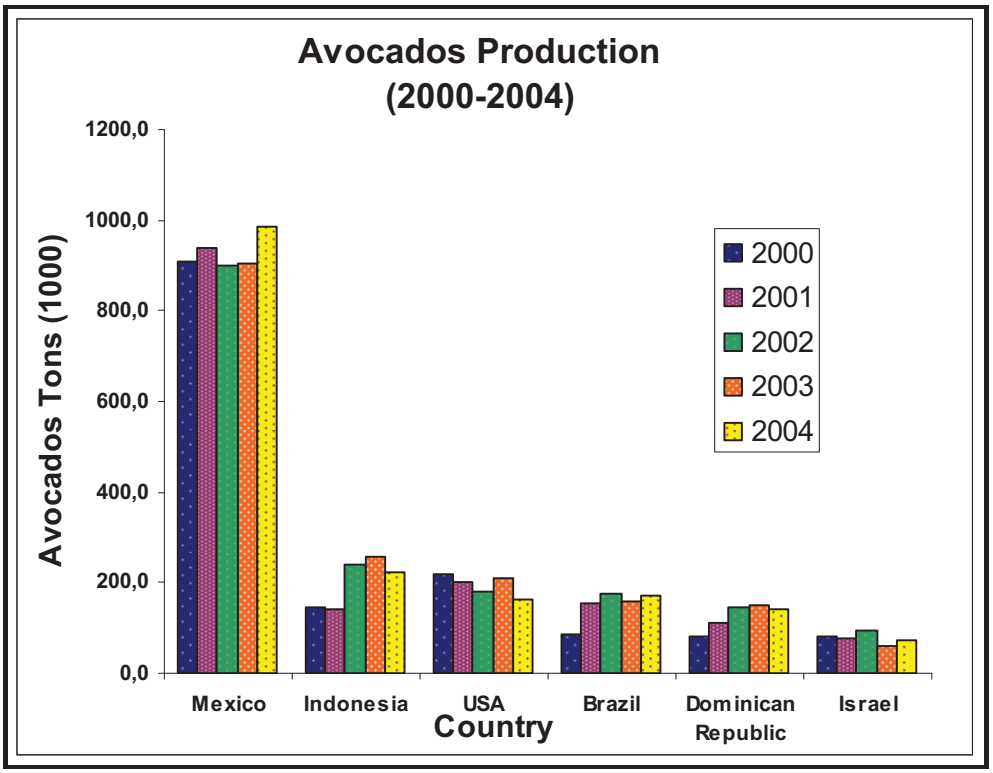


Figure 2-19 Avocado production: Period 2000-2004 (Data origin: FAOSTAT, 2006).

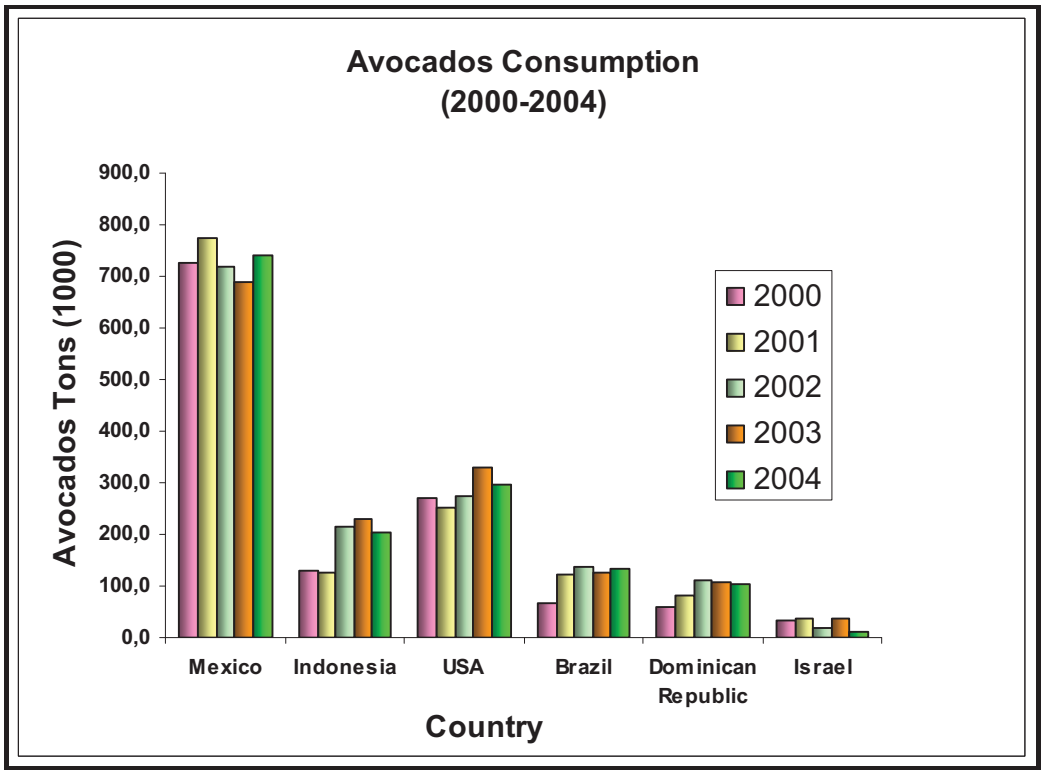


Figure 2-20. Avocado consumption: Period 2000-2004 (Data origin: FAOSTAT, 2006).

In Mexico the State of “Michoacán” is the principal avocado producer (cf. green area on Fig. 2-21). Economically, avocado fruit is a very important crop for Mexico. More than 40.000 employees are working in the cultivation and fruit processing areas generating a value of approx. 90 million $US (approx. 1 billion MXN pesos) per year (De Luna and Flores, 1994; SAGAR, 1996).

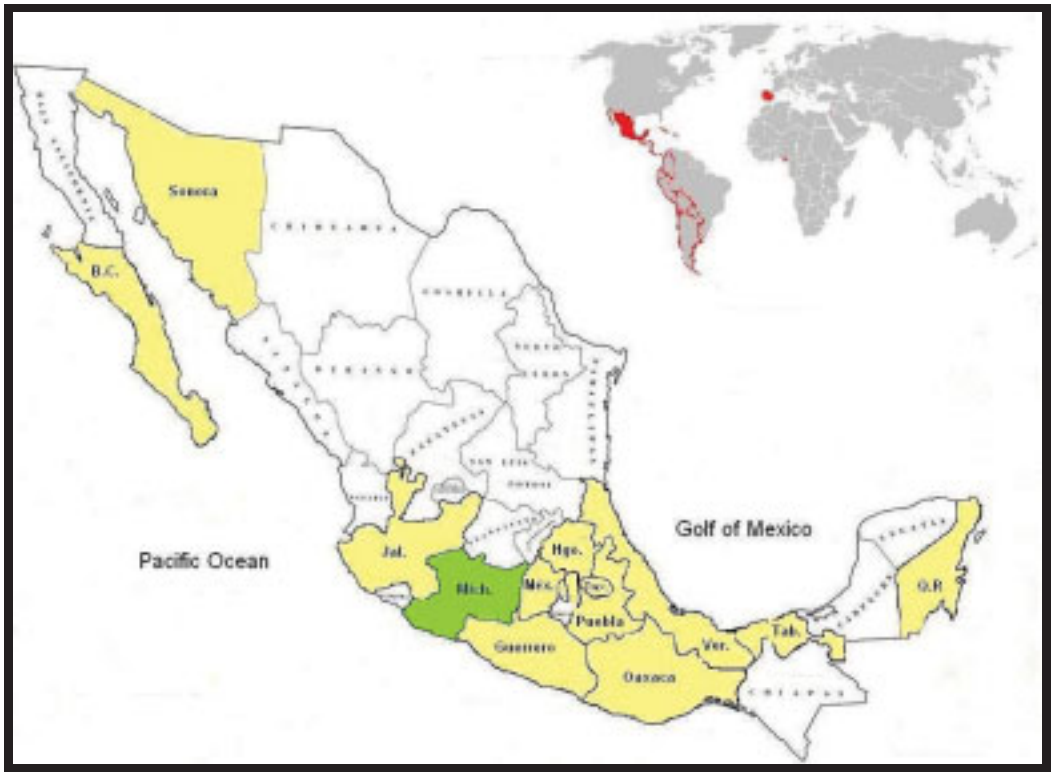


Figure 2-21. Principal avocado production zones in Mexico.

The oil content of avocado fruits ranges between 5 to 30% (of dry weight). A substantial amount of the harvested avocados are processed for recovery of valuable avocado fruit flesh oils for cosmetics, e.g. skin care products. So far, the residual seed is not used for oil production waste material. For the *Hass* variety, the seed represents almost 15% of the total fruit weight (Ramos, 1999). The commercial processing of avocados for recovery of fruit flesh (inclusive oil production) generates more than 3000 tons of avocado seeds per year. In the areas where the avocado seed materials are dumped or composted, germination capacity of other plant species appears to be significantly decreased or inhibited. Therefore, avocado seeds might contain natural products acting as natural herbicides. The already known bioactive ryanodines with insecticide activity were

mentioned (cf. p. 34). Hence, avocado seeds are still a manifold pool for natural products just waiting for extraction and recovery of useful 'by-products'. In combination with the existing ethnopharmacological knowledge of the Aztec people (cf. p. 3) avocado seeds could also be a promising source for development of new pharmaceutical drugs.

The aim of our study was to conduct a thorough phytochemical investigation of avocado seed constituents (variety *Hass*) as well as correlating the isolated bioactive substances with the knowledge of Mexican ethnomedicine which used avocado seed decocts e.g. for treatment of skin diseases (Argueta-Villamar et al., 1994; Cabrera, 1996).

2. 3. Extraction processes, experimental design, and selective extraction

During processing of avocado seeds there are various factors (temperature, solvent type, contact time etc.) that influence the extraction yield and the amounts of extracted components.

Polyphenols are sensible to oxygen and partly to light, and temperature. According to their chemical structures, they are easily extractable with polar solvents such as methanol, ethyl acetate and also solvent-water mixtures. For extraction of lipophilic polyphenols, acetone or dichlormethane can be used.

2.3.1. Extraction methodologies

The extraction yield of target compounds is highly influenced by the polarity of the solvents, and the extraction methodologies (ultrasonication, and CO_2-extraction under supercritical conditions).

By using solvents, a selective extraction could be induced and by increase of the temperature the yield could be also influenced (unfortunately some substances are labile at these conditions). Supercritical fluid extraction (SFE) is a technique that exploits the unique properties of the solvents above their critical values to extract soluble components from a mixture. This method offers the advantage of higher yields and higher quality. SFE can be operated under a wide range of conditions in order to increase the selectivity (Rizvi et al., 1986b). An SFE-system consists of a

solvent compressor (high pressure pump), an extraction vessel, a temperature/pressure control system and a separator (cf. Fig. 2-22).

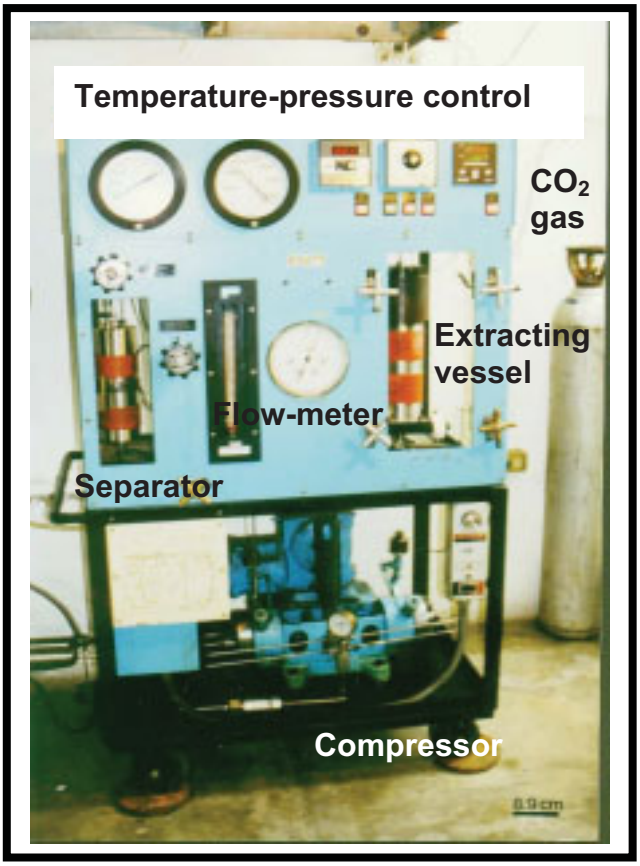


Figure 2-22. A supercritical fluid extractor (Newport Scientific Inc., equipment Model 46-19345, USA).

A gas is reaching the supercritical fluid state at a temperature above its critical point (i.e. CO_2: p= 73.8 bar, temp. = 31.06^{o} Celsius). Fluids exhibit physicochemical properties intermediate between those of liquids and gases which enhance its role as a solvent (Rizvi et al., 1986a and 1986b). The phase-diagram of CO_2 and the area of supercritical conditions are shown in Figure 2-23.

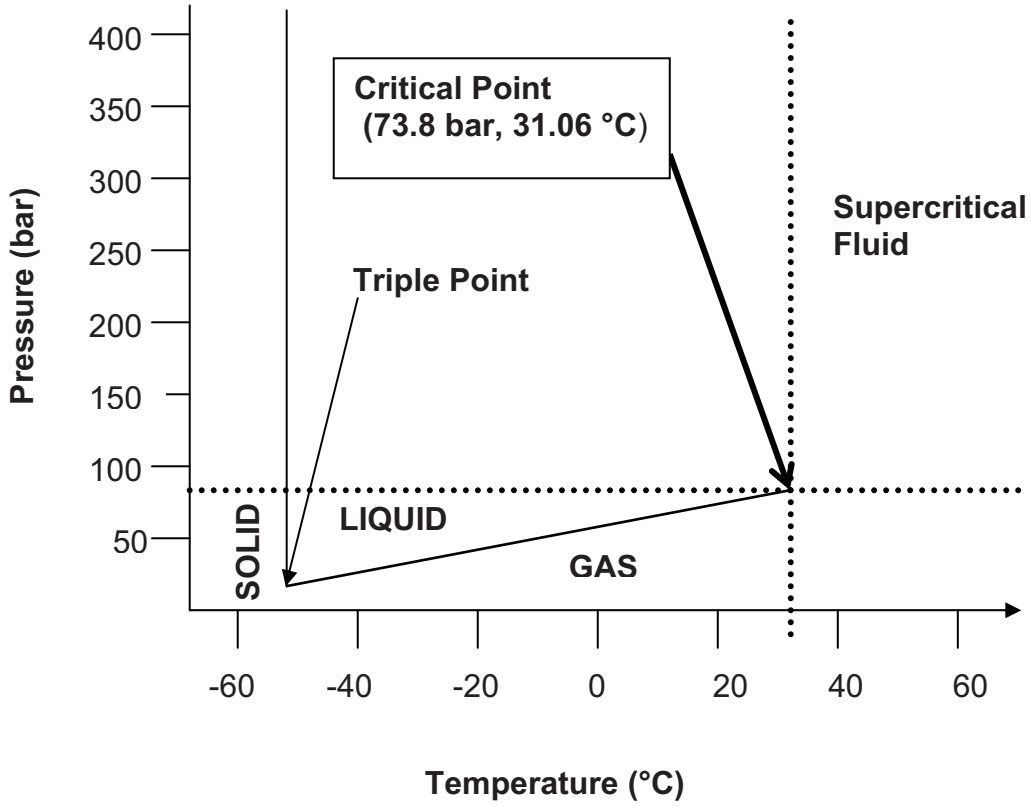


Figure 2-23. Carbon dioxide phase-diagram for supercritical conditions.

The history, principles and applications of this method in food technology are reviewed in the literature by different authors (Rizvi et al., 1986a; Rizvi et al., 1986b; Chandra and Nair, 1996; Shen et al., 1996; Roy et al., 1996; Rostagno et al., 2002).

From all the fluids used, CO_2 has the advantages that it's not toxic, not flammable, non corrosive, relatively cheap, and easily available. It also has a low critical temperature. Some advantages for the extraction of plants are the moderate temperature (40-60°C) which enables extraction of heat-labile materials.

Both extraction methods, solvents and SFE, have been applied in this research to obtain avocado seeds extracts. In the case of solvents we obtained petroleum ether extract; methanol extract, ethyl acetate extract, and aqueous extract (cf. materials and methods Fig. 5-2 p. 224).

The highest yield was obtained by using methanol (9.1%) (cf. Table 3-4 and Fig. 3-4 in results p.p. 54 and 58). Considerable differences were observed between

petroleum ether and supercritical fluid CO_2 extraction yield (1.7 and 3.4%, respectively).

2.3.2. Extraction of volatile compounds by using solid phase micro extraction (SPME)

In recent years, the solid phase micro extraction (SPME) technique has been used for qualitative and quantitative analysis of volatiles in different raw materials as well as the determination of benzene, ethyl benzene and other compounds in ground water.

SPME is a novel approach to the analysis of volatiles. As advantages the elimination of solvents in the sample preparation can be seen. Besides, it is a fast method. It can be used for liquids, gas or solid samples. This technique is based on chemically modified fused-silica fibers. The diameter of these fibers varies between 0.05 and 1.0 mm. The SPME analysis requires two steps. The first is to extract analytes from a sample matrix and the second is to desorb those analytes directly into a GC column for further analysis.

Details of this method have been published (Arthur et al., 1992; Chen and Pawliszyn, 1995).

2.3.2.1. Determination of Kóvats' Index (RI)

The retention time is a useful parameter to record and order in GC analysis data. The Kóvats retention index (RI) provides a valuable tool to document the retention data regardless column length or operating temperature. The basis of the method is assigning to the normal hydrocarbons (straight-chain) an index of 100 times the number of carbon atoms in the molecule. The retention index of a compound is then calculated by difference of the retention indices of the normal alkanes eluting before and after the substance (cf. p. 227).

2.4. The High- Speed Countercurrent chromatography (HSCCC) technique

The counter-current chromatography (CCC) technique is based on liquid-liquid partitioning effects. Since no solid support is used adsorbing effects on stationary phase material and artifact formation are eliminated (Ito and Conway, 1996).

Two immiscible liquid phases are used. The first is the mobile phase and the second is the stationary phase. The CCC “column” is usually a continuous open tube coiled on a spool which is rotated in a centrifuge. The centrifugal field holds the liquid stationary phase tightly so that the mobile phase can be pushed through it (Berthod, 1991) (cf. Fig. 2-24).

Figure 2-24. A preparative triple-coil HSCCC equipment (Pharma Tech Research Comp., U.S.A.).

The universal features of chromatographic theory relevant to CCC as well as the principal types of CCC apparatusses and applications have been summarized by Conway (1991). Some authors have suggested guidelines for the selection of the right solvents (Abbott and Kleiman, 1991; Oka et al., 1991) and reviews about applications of HSCCC in natural products chemistry have been published (Okuda et al., 1986; Schaufelberger, 1991; Degenhardt et al., 2000)

There are many variations of the CCC technique. The high-speed countercurrent chromatography (HSCCC) however is the most widely used.

2.5. Screening of biological activities

The screening of biological activities is important in each step of the phytochemical analysis. At the beginning it helps to identify the fractions that provide a certain activity and at the end it will show which substances in the fractions are responsible for such an activity. However it is also possible that there are synergisms or antagonism between the substances present in a complex matrix. The kinds of biological assay are variable and depend on the activity that is in the focus of the study.

In the present study it was important to test the antioxidant activity as well as the possible toxicity. Questions that have been addressed were the follows:

1. – Is there really a reason to use avocado seeds as antioxidant in food by the Mexican people?

2. - Which kind of avocado seeds extracts possess a high antioxidant capacity?

3. - Can the avocado seeds extracts be used by the food industry as antioxidant instead of the known synthetic antioxidants normally used for food conservation?

4. – Can the avocado seeds extracts be used as antioxidant by other industries, such as e.g. the cosmetic industry?

5. – In case of toxicity one has to find out, which avocado seeds extracts are toxic.

According to these results it will be important to decide which kind of avocado seeds extracts are suitable for the use in the food industry. In case of other kind of assays (antibacterial and fungicide) a likely use might be in the medicine because of these properties.

2.5.1. Antioxidant activity

Free radicals are known to be responsible for lipid oxidation (Wang et al., 1999) therefore the addition of antioxidants to prevent the oxidation has a huge potential. Synthetic antioxidants such as butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA) have been dominant since their introduction because they are very effective (Dapkevicius et al., 1998).

A number of assays have been introduced for the measurement of the total antioxidant activity of food extracts and pure compounds (Brand-Williams et al., 1995; Hopia et al., 1996; Dapkevicius et al., 1998; Donovan et al., 1998; Re at al., 1999; Schwarz et al., 2001; Ley, 2001). Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a certain period (Hogg et al., 1961; Brand-Williams et al., 1995).

Two methods use the stable radical, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS). These methods will be named as “TEAC (Trolox equivalent antioxidant capacity)” along this research. They involve the direct production of the free radical cation $ABTS^{\bullet+}$ through the reaction between ABTS and potassium persulfate in case of the “modified TEAC” method (Re et al., 1999; Dorman et al., 2000). In the “original TEAC” assay (Miller et al., 1993), the activation of metmyoglobin (peroxidase) was performed with hydrogen peroxide (H_2O_2) in presence of ABTS to produce the radical.

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), which is a synthetic analogue of vitamin E (α-tocopherol) with enhanced water solubility, is used for calibration.

The unit is the TEAC value which is defined as the concentration (in mmol/L) of Trolox having the equivalent antioxidant capacity to a 1.0 mmol/L solution of the substance under investigation. The derivation of a TEAC value provides a method for comparison of antioxidant activity among groups of drugs and chemicals, provided that they are water soluble (Miller et al., 1993). Only in the case of complex mixtures such as e.g. plant extracts, results are reported as total antioxidant activity (TAA) in mmol Trolox per g or kg extract.

Another method uses 2,2-Diphenyl-1-picrylhydrazyl or $DPPH^{\bullet+}$ (Brand-Williams et al., 1995; Moon and Terao, 1998; Yan et al., 1999; Wang et al., 1999; Ley, 2001). The reaction between DPPH and a large number of phenols is of second-order and the antioxidant activity of these phenols is increased with alkyl groups in the aromatic ring, principally in (*p*) – position. Substituents in both (*o*)-positions may cause sterical hindrance to the hydrogen-abstraction reaction (McGowan et al., 1959; Hogg et al., 1961). The antioxidant activity in this case is reported as the quantity of an antioxidant that is required to reduce the radical $DPPH^{\bullet+}$ to a 50%

concentration of the original value and this is called median effective concentration (EC_{50}).

By monitoring the decrease of the absorbance at 734 nm (TEAC-Method) or at 514 nm (DPPH-Method) one can follow the reduction of the radical by these methods.

In the case of the DPPH method, the evolution of the reaction kinetics depends on the nature of the antioxidant being tested. There are three types of kinetics observed:

a) Rapid kinetics: Substances which reach the steady state in less than 1 minute.

b) Intermediate kinetics: The steady state is reached in approximately 15 minutes.

c) Slow kinetics: Substances that reach the steady state within 1 to 6 hours with a hyperbolic curve.

The remaining amount of DPPH (%) at the steady state vs. the concentration of the analyte is used to calculate the scavenging effect (antiradical efficiency) as amount of antioxidant necessary to reduce the initial quantity of DPPH to 50% (EC_{50} expressed as mmol Substance/mmol DPPH). The antiradical activity is not calculated as EC_{100} because there are some compounds (i.e. coumaric acid and vanillin) that never react with more than 75% of initial DPPH regardless time and concentration. Another important factor to calculate the “antiradical efficiency” to avoid errors when the kinetic is slow, therefore it should be analyzed at the steady state (Brand-Williams et al., 1995).

At present the increasing interest in employing antioxidants from natural sources is due to the consumer’s preference for natural ingredients. Many different plant extracts have been studied with variations in the antioxidant activity depending on extraction techniques and solvents used (Hopia et al., 1996; Dapkevicius et al., 1998; Schwarz et al., 2001). The results depend furthermore on the assay used to determine the antioxidant capacity (Huang et al., 1996).

Moreover it has been demonstrated that antioxidants from fruits and vegetables have activity against a wide range of diseases (Cao et al., 1996; Aruoma, 1996; Aruoma et al., 1996; Donovan et al., 1998; Oberlies et al., 1998; De Bruyne et al.,

1999). Each population has their own plants which are used against different illnesses.

The avocado seed (*Persea americana* Mill) has an important role as antioxidant and is used in Mexico to extend the shelf life of some foods.

2.5.2. Brine shrimp (*Artemia salina* L.) cytotoxicity assay

The 'brine shrimp'-assay, which uses the salt-water crustacean *Artemia salina* Leach is a versatile screening assay to monitor occurrence of cytotoxic agents in natural product extracts. This assay is not very specific, but certain components such as e.g. acetylenic fatty acids showed activities and also strong '*in-vitro*'-antitumor properties. Hence this system is eligible for a rough pre-screening of potentially bioactive plant extracts and to perform a 'bio-assay guided' fractionation and isolation of active lead-structures. It is possible to monitor general toxic activity as well as pharmacological activities in plant extracts (Carballo et al., 2002) as a cost effective method. The method is rapid, reliable, inexpensive and convenient (Meyer et al., 1982).

Results of this test are given in LD_{50} values (in µg/mL of active compounds and extracts) in the brine medium (Meyer et al., 1982; Padmaja et al., 2002; Wanyoike et al., 2004). Some authors recommend biological data transformation to "probit" (Finney, 1964) or to "logit" (Hafner et al., 1977) methods. The presented LD_{50} values are according to the best correlation coefficient (r) obtained by using the original data or by the "logit" or "probit" transformation. The used reference standard was podophyllotoxin.

3. RESULTS AND DISCUSSION

The results of the investigations on avocado seeds (*Persea americana* Mill., c.v. *Hass*) are presented in the following order:

3.1. Nutritional analysis of the **complete avocado seeds** (% water, proteins, fat, fiber, minerals).

3.2. Volatile compounds obtained from fresh **complete avocado seeds** by solid phase micro-extraction (SPME).

3.3. Extraction yield and total polyphenolics (by Folin-Ciocalteu method) of **avocado seeds** extracts obtained by two experimental (fractionated factorial 2^{5-1}, and one factor experimental design).

3.4. Fractionation and isolation by CC, HSCCC, as well as preparative HPLC, and structure elucidation by means of 1D-NMR (^{1}H, ^{13}C, DEPT135), 2D-NMR (HMQC, HMBC, NOE, COSY), ESI-LC-MS (MS, MS^{n}), and GC-MS (CE and EI), of compounds from the different partitions: petrol ether (PE), ethyl acetate (Ac), and methanol-water (MW) from the methanolic extract of **avocado cotyledons.**

3.5. Bioactivity tests: scavenging capacity by three different methods [original TEAC (by Miller et al., 1993), modified TEAC (by Re et al., 1999) and DPPH method (by Brand-Williams et al., 1995)] as well as the brine-shrimp assay (by Meyer et al., 1982) on avocado cotyledon extracts and isolated substances.

3.1. Nutritional composition of avocado seeds

The knowledge about the nutrient composition of the plant is important for possible applications in food, pharmaceutical and/or cosmetic industries.

The nutritional analysis of three varieties of avocado seeds (*Hass*, *Fuerte* and *Criollo*) was carried out in order to have a comparison.

Almost all nutrients (cf. Table 3-1) were higher in the *Hass* variety than in the other two tested varieties, except the amounts of cyanic compounds and arsenic. These antinutrients were present in smaller amounts in the *Hass* variety which can be considered as an advantage due to their toxicological properties.

Table 3-1. Nutritional analysis of three different avocado varieties

Determination (AOAC, 1990)	Avocado seed variety		
	Hass	***Fuerte***	***Criollo***
Water[1]	19.96	62.31	58.31
Protein[1]	3.76	1.18	1.61
Fat[1]	2.10	1.05	0.51
Ash[1]	1.88	1.10	1.86
Fiber[1]	6.20	5.34	5.19
Carbohydrates[1]	66.10	29.02	32.52
Vitamin C[2]	< 1.0	< 1.0	< 1.0
Iron[3]	19.90	6.95	5.35
Copper[3]	6.94	3.44	1.94
Zinc[3]	12.40	6.10	3.85
Arsenic[3]	< 0.50	1.32	< 0.50
Cobalt[3]	< 1.0	< 1.0	< 1.0
Mercury[3]	< 0.5	< 0.5	< 0.5
Cyanic compounds	< 0.02	< 0.02	< 0.02

[1](%), [2](mg/100 g), [3](ppm)

These results confirm a preliminary microscopical study (not presented here) of the avocado seeds which indicated a high content of carbohydrates in the cotyledons while the testa had more polyphenols.

In some towns in México, the avocado seed meal (waste from the avocado oil production in Michoacan, Mexico) it is used to feed cows and pigs.

It is important to look for antinutritional factors to assure the safe use of avocado seeds for feeding animals. According to the preliminary results in the nutritional analysis it seems to be likely that avocado seeds mixed with others feed formula could well be used for these purposes.

3.2. Volatile compounds of Avocado seeds (*Persea americana* Mill., c.v. *Hass*)

The volatile constituents of three avocado seeds varieties (*Criollo*, *Fuerte* and *Hass*) have been determined for the first time by means of solid phase micro extraction (SPME) enrichment. Two different fibers (polydimethylsiloxane and carbowax) were used.

After sample extraction, the fibers were desorbed in a GC-MS equiped with a HP-1 capillary column (for conditions cf. materials and methods p. 226).

The highest number of components was detected with the polydimethylsiloxane fiber that is a special fiber for the analysis of volatile substances. *Criollo* variety had the highest quantity of compounds (cf. Table 3-2).

Table 3-2. Number of volatile compounds detected with the tested fibers

Variety	Fiber	
	Polydimethylsiloxane	Carbowax
Criollo	30	10
Fuerte	22	12
Hass	16	11

3.2.1. Kóvats' Index (RI).

Kóvats retention indices of the analyzed compounds (cf. Table 3-3) were estimated according to literature data (Willard et al., 1986; King and Knight, 1992; Baugh, 1993; Sagrero-Nieves and Bartley, 1995; Sinyinda et al., 1998).

Table 3-3. Volatile compounds from three different avocado seeds (*Persea americana* Mill.) cultivars (*Criollo*, *Fuerte* and *Hass*) by SPME method

R_t [min]	Compound[4]	Obs RI	Literature RI values				relative area (%)		
			[5]	[6]	[7]	[8]	*Criollo*	*Fuerte*	*Hass*
13.62	α-Pinene*	866	938	932	nv	935	27.5 (1.46)	4.1 (0.30)	7.5 (8.38)
15.37	β-Pinene*	949	984	977	nv	980	24.6 (0.31)	2.6 (0.12)	3.4 (4.20)
15.54	Myrcene	957	991	987	nv	995	n.d.	**6.7** (1.13)	n.d.
17.53	δ-4 Carene	1040	nv	nv	nv	nv	n.d.	**5.8** (1.76)	n.d.
17.45	Limonene	1037	1023	nv	1032	1032	5.8 (0.40)	n.d.	1.2 (0.93)
18.03	γ-Terpinene	1059	1048	1088	nv	1057	n.d.	**5.3** (2.46)	n.d.
19.80	α-Terpinolene	1124	nv	nv	nv	1081	0.4 (0.17)	0.84 (0.14)	n.d.
20.11	Linalool	1135	1083		nv	1087	**1.8** (0.07)	n.d.	n.d.
21.27	β-Ocimene	1173	1040	1044	nv	1044	n.d.	**5.3** (1.57)	n.d.
23.31	Estragol	1236	1224	1208	nv	nv	**2.0** (0.07)	n.d.	n.d.
23.64	n-Octyl acetate	1246	nv	nv	nv	nv	**0.3** (10)	n.d.	n.d.
26.10	(-)Bornyl acetate	1314	nv	nv	nv	1285	**0.2** (0.18)	n.d.	n.d.
27.28	4-Allylphenyl acetate	1345	nv	nv	nv	nv	**0.1** (0.03)	n.d.	n.d.
28.18	α-Cubebene	1367	1380	nv	1356	nv	3.79 (2.02)	12.6 (3.75)	0.9(0.03)
28.36	Geranyl acetate	1371	nv	nv	nv	1362	**2.6** (0.01)	n.d.	n.d.
28.80	α-Ylangene	1382	nv	nv	nv	nv	n.d.	0.5 (0.06)	0.8 (0.30)
28.80	Cyclosativene	1382	nv	nv	nv	nv	**0.3** (0.07)	n.d.	n.d.
28.92	α-Copaene*	1385	1390	nv	1383	1369	2.9 (1.43)	4.2 (0.55)	1.6 (0.08)
29.17	β-Cubebene	1391	1380	1354	1391	nv	0.4 (0.15)	2.5 (0.38)	n.d.
29.65	γ-Caryophyllene	1402	nv	nv	nv	nv	**0.05** (0.02)	n.d.	n.d.
29.98	β-Caryophyllene	1410	1439	1437	1426	1406	3.7 (0.59)	n.d.	3.8 (2.46)
30.73	β-Selinene	1427	nv	nv	nv	nv	**0.4** (0.10)	n.d.	n.d.
30.90	γ-Muurolene	1431	nv	nv	nv	1470	0.05 (0.01)	0.7 (0.03)	0.7 (0.26)
31.30	Germacrene d*	1439	nv	nv	nv	1470	0.4 (0.03)	11.3 (1.05)	14.8 (4.94)
31.74	α-Amorphene	1449	nv	nv	nv	nv	n.d.	1.7 (0.06)	n.d.
31.92	γ-Cadinene	1453	nv	nv	nv	nv	0.05 (0.01)	n.d.	1.4 (0.46)
32.04	δ-Cadinene*	1455	1511	nv	nv	nv	0.5 (0.16)	3.9 (0.87)	2.8 (0.89
32.33	Cadina 1,4-diene	1462	nv	nv	nv	nv	**0.1** (0.03)	n.d.	n.d.
32.44	α-Muurolene	1464	nv	nv	nv	nv	n.d.	0.7 (0.19)	0.6 (0.24)
33.37	Caryophyllene oxide	1483	1573		1573	1559	0.05 (0.02)	n.d.	n.d.

Obs. Observed value. Bold value: only present in this variety; underlined value: highest percentage; *Common substance in all of the three varieties; n.d.= Not detected; [4]Identification was achieved by correlation (more than 95%) with GCMS library. For GC-MS conditions cf. methodology (p. 226). Compounds are listed according to the elution order on HP-1 column, and values (areas %) represent averages of minimal two determinations which SD value is in parenthesis (SD value). RI= Retention index on non-polar HP-1 column. nv= not reported; [5]Sagrero-Nieves and Bartley, 1995; [6]King and Knight, 1992; [7]Syninda et al., 1998; [8]Baratta et al., 1998

Sesquiterpenes were the principal constituents in all of the three varieties and the common substances signed as (*) were α-copaene, germacrene d, δ-cadinene, α- and β-pinene (cf. Table 3-3). The last two compounds were present in highest concentration in the *Criollo* variety (27.5 and 24.6%, respectively).

Linalool, n-octyl acetate, (-) bornyl acetate, 4-allylphenyl acetate, geranyl acetate, cyclosativene, γ-caryophyllene, β-selinene, cadina-1, 4-diene and estragol were only present in the *Criollo* variety.

Estragol has been tested by Marcus and Lichtenstein (1979) as insecticide with good results. It may allow protection from some insects and therefore confer an advantage to cultivars in which it is present.

Myrcene, δ-4-carene, γ-terpinene, and β-ocimene were only present in seeds of the *Fuerte* variety, which had the highest concentration of α–cubebene (12.6 %) and α-copaene (4.16%).

The amount of some substances seems to be especially high in the *Hass* variety, such as γ-cadinene (1.4%), α-ylangene (0.8%), and germacrene d (14.8%). Germacrene d together with other volatile compounds is also present in oregano (*Origanum vulgare*) oils, which is antimicrobial and antioxidant active (Baratta et al., 1998). Germacrene d could therefore play an important role in the assessed (Valeri and Gimeno, 1953) antimicrobial activity of avocado seeds.

Estragol, anethol and methyl eugenol are specific for the leaves of all Mexican cultivars and hybrids. Its absence is typical for the Guatemalan and West Indian cultivars (King und Knight 1992; Scora and Ahmed, 1993).

The fact that these substances were not found in the seeds of the *Hass* variety, confirms that the *Hass* variety belongs to the Guatemalan race as reported before (Bergh and Scora, 1973).

From the three tested varieties, the *Criollo* variety is the richest in volatile substances and due to the presence of estragol it bears a potential to be used for insecticidal purposes.

Since volatile compounds are very low in the *Hass* variety, the high content of germacrene d may offer a possibility to use the volatiles of this variety for antimicrobial purposes.

3.3. Extraction yield and total phenolics (by Folin-Ciocalteu method) on complete avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts.

3.3.1. Extraction yield on factorial fractionated (2^{5-1}) experimental design

The extraction yield and the composition of the extract are influenced by more than one factor in all processes.

Some polyphenols are very sensible to light and temperature. According to their chemical structure, they are easily extractable with polar solvents such as methanol, ethyl acetate and acetone.

In the present case, it was attempted to obtain high yield of polyphenols by applying a factorial fractionated experimental design. This kind of experiment is especially useful to get information around a process.

In the case of avocado seeds, extraction yield and total phenolics were the response factors. The solvents, material:solvent ratios, temperature etc. were the factors to be studied.

The yield was quantified by difference in weight after solvent evaporation. The total polyphenolics were determined according to Singleton and Rossi (1965). Conditions and results of this experiment are given in Table 3-4.

Table 3-4. A 2^{5-1} experimental design for the study of avocado seeds (*Persea americana* Mill., c.v. *Hass*) extraction

Experiment (Nr.)	[9]Extraction conditions	[10]Extraction yield	Total polyphenolics in avocados (% TAE)	
			[10]Extracts	[11]Seeds([10])
1	Ac/1:10/25/0/50	1.5	8.26	1239.5 (0.12)
2	Ac /1:10/40/0/70	1.0	**19.14**	1913.9 (0.19)
3	Ac /1:5/25/500/50	0.6	**14.11**	846.7 (0.08)
4	MeOH/1:10/25/0/50	7.0	8.84	6188.0 (0.62)
5	Ac /1:5/40/500/70	2.0	9.10	1820.5 (0.18)
6	**MeOH**/1:10/40/500/70	**8.9**	7.22	6426.1 (0.64)
7	**MeOH**/1:10/40/0/**50**	**7.7**	11.92	9178.3 (**0.92**)
8	Ac /1:10/25/500/70	1.9	**17.87**	3395.7 (0.34)
9	Ac /1:5/25/0/70	0.9	9.72	874.8 (0.09)
10	MeOH/1:5/40/0/70	5.8	11.75	6814.3 (0.68)
11	**MeOH**/1:5/40/500/**50**	**7.7**	11.17	8601.2 (0.**86**)
12	MeOH/1:5/25/500/70	6.9	10.63	7337.9 (0.73)
13	Ac /1:5/40/0/50	2.1	6.30	1322.9 (0.13)
14	**MeOH**/1:10/25/500/**50**	**9.1**	9.80	8920.5 (0.**89**)
15	MeOH/1:5/25/0/50	5.0	**13.46**	6728.9 (0.67)
16	Ac /1:10/40/500/50	2.5	8.61	2152.6 (0.22)

[9]solvent/material:solvent rate/temperature (°C)/ agitation (rpm)/Ultrasonic potency (Volts). Ac= ethyl acetate, MeOH= Methanol,[10] % dry weight, [11] mg /kg.

Experiments 14, 6, 7 and 11 (all of them with methanol) gave the highest yields (9.1, 8.9, 7.7, and 7.7 % respectively) (cf. Fig. 3-1).

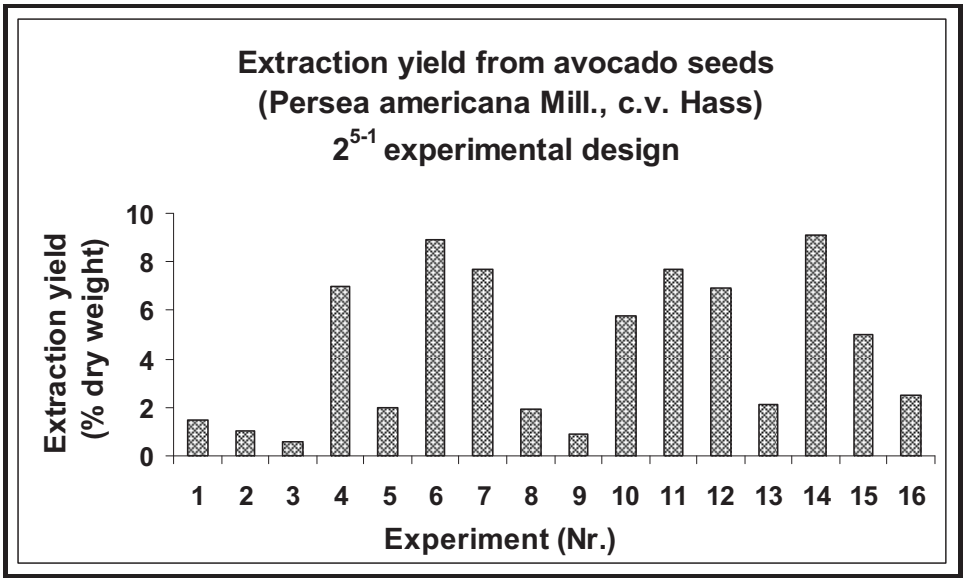


Figure 3-1. Extraction yield on the 2^{5-1} experimental design.

The statistical analysis of this experiment showed that the solvent-effect (cf. Table 3-5) has the most significant influence on the yield and on the total phenolics. The temperature (factor C) was definitive not a relevant factor, and the ultrasonic potency (factor E) had a negative influence on both response factors.

Table 3-5. Effects of factors studied in the statistical analysis

Factor to study	Effects (on yield)	Effects (on % total phenolics)
Solvent (**A**)	5.7	0.582
Rate (**B**)	1.075	0.065
Temperature (**C**)	**0.600**	**0.035**
Agitation (**D**)	1.075	0.065
Ultrasonic potency (**E**)	**-0.225**	**-0.052**

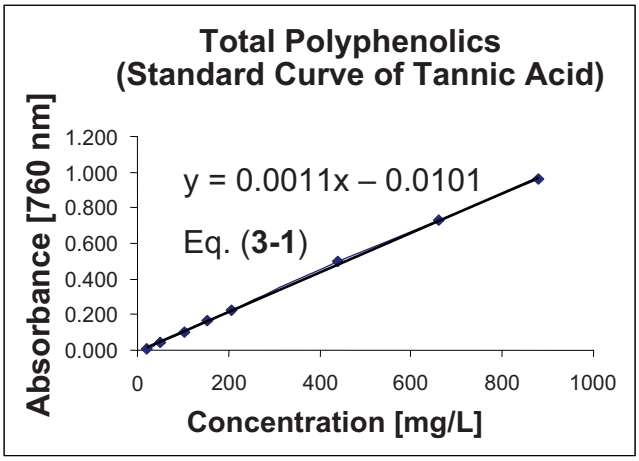
As factors C and E were irrelevant, it was decided to eliminate them for the ANOVA analysis. According to this analysis (cf. Table 3-6) it was corroborated that solvent (factor A), material/solvent rate (factor B) and agitation (factor D) were of relevant influence for extraction yield and polyphenol content. Hence in the successive work, these conditions (solvent, material/solvent, and agitation) were used to extract avocado seeds.

Table 3-6. ANOVA to 95% of confidence

Factor	P-value (% yield)	P-value (% total phenolics)
Solvent (**A**)	0.0063	0.0058
Material/Solvent ratio (**B**)	0.1410	0.2825
Agitation (**D**)	0.1410	0.2825
R^2	0.9888	0.9894

3.3.2. Total polyphenolics

Tannic acid was chosen as standard for the determination of total polyphenolics. Eight different concentrations were prepared for the standard curve (for conditions cf. p. 227).



Tannic acid concentration [mg/L]	Abs.
20.52	0.009
51.30	0.041
102.60	0.100
153.90	0.165
205.20	0.221
439.60	0.497
659.40	0.730
879.20	0.960
(r)= 0.9996	
Intercept (b) = -0.0101	
Slope (a) = 0.0011	

Figure 3-2. Tannic acid standard curve.

The measured absorbances were plotted (absorbance vs tannic acid concentration) to get the regression equation (Eq. 3-1) (cf. Fig. 3-2).

By using Eq. 3-1, the total phenolics in 16 different extracts were determined as tannic acid equivalents (mg/L) and converted as % of phenolics (% TAE) (cf. p.p. 227-228).

Experiments 7, 11, and 14 yielded the highest quantity of phenolics (0.92, 0.86 and 0.89% respectively) (cf. Fig. 3-3).

The use of methanol and 70 volts of ultrasonic potency reduces the percentage of total phenolics. One explanation can be seen in a possible polymerization reaction.

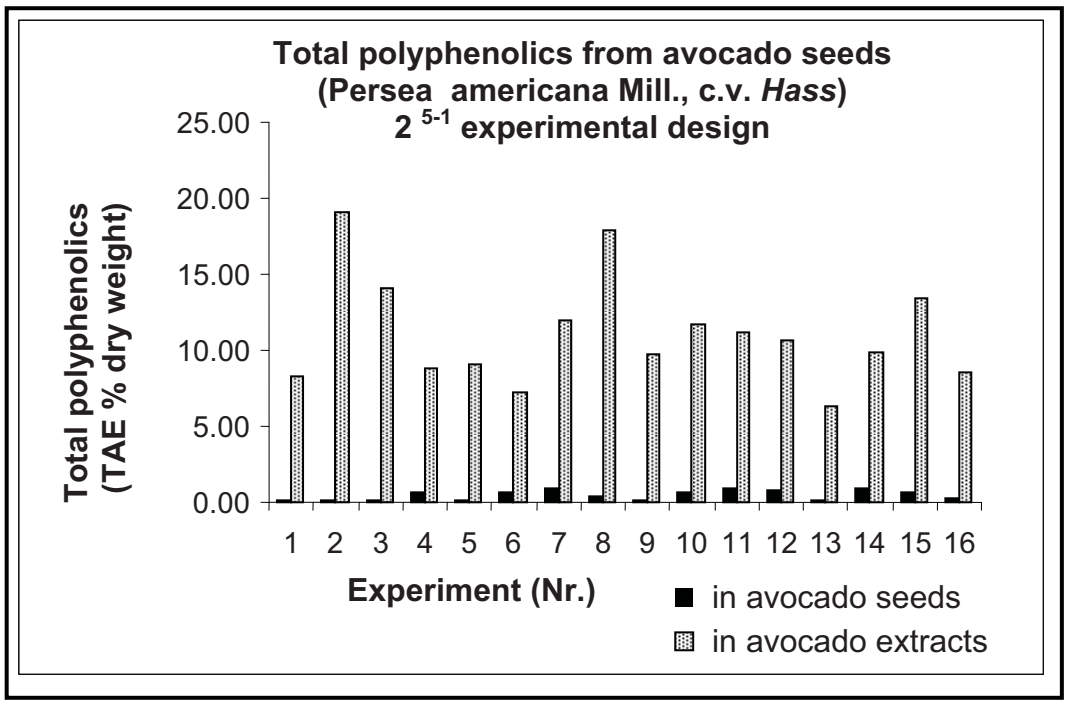


Figure 3-3. Total phenolics in avocado seeds and extracts from 2^{5-1} experimental design.

Temperature and ultrasound potency were again not important factors for the rate of total polyphenol extraction (cf. Table 3-2).

With regard to content of polyphenols in the extracts, it has to be pointed out that with ethyl acetate extracts (Ac) (experiments number 2, 3 and 8) the highest activities (19.1, 14.1 and 17.9, respectively) have been obtained.

With methanol the highest yield of polyphenols is obtained because the high extractions yield (higher than with ethyl acetate). Nevertheless the ethyl acetate extracts have been shown higher concentration of polyphenolic substances.

The total phenols in the avocado seeds is then relative to the extraction yield (% dry weight).

In order to clarify this observation a one factor experiment design was applied using 3 solvents: methanol, petroleum ether and ethyl acetate. The experimental conditions were the same (material solvent ratio 1:10 and 500 rpm of agitation) (cf. under p. 222-223).

3.3.3. Extraction yield in the one factor experimental design

The highest yield and polyphenolics content by using methanol as solvent were confirmed with a unifactorial experimental design (Fig. 3-4).

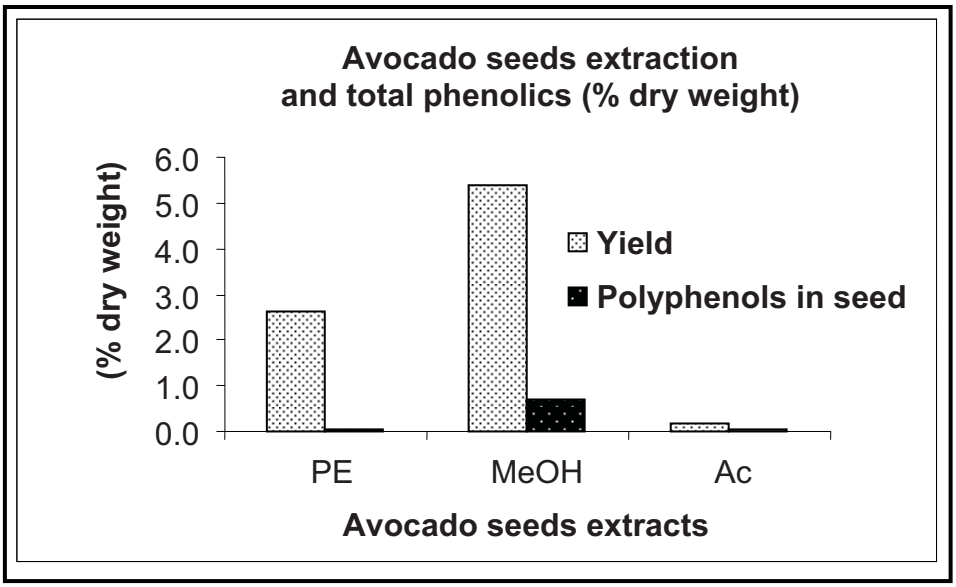


Figure 3-4. Avocado seeds extraction yields

We concluded again that from the tested solvents methanol is the best to extract the polyphenolics from avocado seeds.

The total phenolic compounds can vary in different species of the same genus, in the same species at different times of year, and in the same tissue at different stages of growth.

Torres et al., (1987) have determined the total phenols in avocado cotyledons. Unfortunately we could not make any comparison because these results were presented as mg/g “fresh weight equivalents in gallic acid or chlorogenic acid” (Golan et al., 1977) whereas we used “equivalents of tannic acid in dry weight” (TAE).

Based on these results it was decided to undertake further extractions with methanol to gain a higher yield followed by a partition with petroleum ether (PE), dichloromethane (C), ethyl acetate (Ac), and methanol:water (MW) to get a target pre-fractionation of the methanolic extract.

The ABA derivatives described below (cf. p. 120) were isolated from this complete avocado seeds methanolic extract.

3.4. Fractionation, isolation (CC, HSCCC, preparative HPLC) and structure elucidation (1D-NMR, 2D-NMR, ESI-LC-MS, GC-MS), of compounds from avocado seeds (*Persea americana* Mill., c.v. *Hass*).

During our phytochemical investigation of the seeds of *Persea americana* var. *Hass*, we isolated various components with both lipophilic and also with very polar characteristics. In the thesis, description of structure elucidation of isolated natural product classes will follow in the order of decreasing lipophilicity sweeping from hydrophobic acetogenins to hydrophilic proanthocyanidins.

3.4.1. Lipophilic substances from PE partition of avocado seeds

In the general chapter (cf. p. 27), a comprehensive overview is presented regarding already known lipophilic substances from different tissues of the avocado fruit (fruit flesh, seeds and leaves). Principally, lipophilic fractions of avocado plant material consist of highly complex mixtures containing acetylenic, furanoic and trihydroxylated long chain hydrocarbons with similar substitution pattern.

This chapter will cover the preparative fractionation, isolation and complete structure elucidation of lipophilic substances from extracts of avocado seeds. Characteristically all of these substances are constituted of a long aliphatic chain (C_{17} to C_{21}) and seem to be products from fatty acid biosynthesis (Kashman et al., 1970; Kawagishi et al., 2001). In this work we identified acetylenic-, vinylic-, trihydroxy- and furanoic compounds. To the best of our knowledge, some of these components have novel substitution pattern.

The complete structural characterization has been made by using modern spectroscopic methods 1D- (^{1}H-, ^{13}C-, DEPT135) and 2D-NMR (HMQC, HMBC, ^{1}H/^{1}H-COSY) experiments as well as LC-ESI-MS/MS, EI-MS (70eV) and CI-MS (reactant gas: isobutane or ammonia).

3.4.1.1. Acetylenic substances

In the lipophilic extracts of avocado seed material (fruit flesh and seeds), various compounds with terminal alkyne group have already been identified (Kashman et al., 1969; Oelrichs et al., 1995; Rodríguez- Saona et al., 1998; ; Kim et al., 2000b; Domergue et al., 2000; Kawagishi et al., 2001). Details about this class of compounds as well as structures are given in the general chapter (p. 31).

During our investigation of the petrol ether partition (PE) of the avocado seeds a '*bio-assay guided fractionation'* procedure was performed using the brine-shrimp-assay (*Artemia salina*) (Meyer et al., 1982). These crustacean organisms are very sensitive to cytotoxic agents and were used as 'biosensors' for detection of potentially bioactive compounds in the avocado extracts. During fractionation of the avocado partitions and crude extracts we discovered that in particular the lipophilc petrolether-soluble partition and as well the supercritical fluid extracts of avocado seeds showed significant cytotoxic effects in this bio-assay. Later we were able to verify that the cytotoxic activity in the lipophil extracts is in principal related to the lipids having a terminal acetylene substitution, a furan ring and a long carbon chain (cf. p.p. 206).

The straight forward chromatographic separation into pure substances was hampered due to the complexity of the investigated mixtures. Structural variations regarding the number of double bonds and their location in the aliphatic chain caused minimal differences in the polarities and the chromatographic behavior. Hence, for the isolation of lipophil avocado seed constituents it was necessary to combine sequentially different chromatography techniques, such as step-gradient '*middle-pressure chromatography'* on silica gel (MPLC), '*high-speed countercurrent chromatography*' (HSCCC) and preparative C18-reversed phase HPLC. A short description about the HSCCC method was already mentioned before in the general chapter (cf. p. 43).

Figure 3-5 shows the TLC-monitoring of the recovered fractions from the first MPLC of the petrol ether partition (cf. purified fraction 8.1 Table 5-3 p. 246) on silica gel. The RP-18 TLC plate is demonstrating the complexity of the investigated extract.

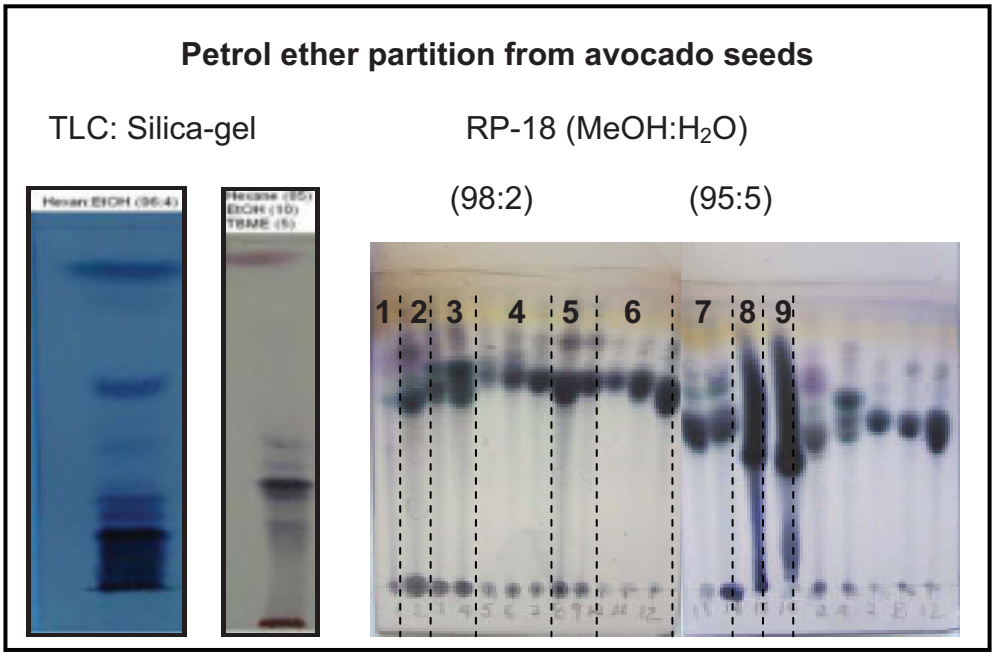


Figure 3-5. Left side: SiO_2-TLC of the petrol ether partition from avocado showing the complexity of the mixture. Right side: Step-gradient '*middle-pressure chromatography'* (MPLC) on silica gel of the same material. For better recognition of separation, the recovered fractions were separated on a RP18-TLC and visualized with spray reagent anisaldehyde-sulfuric acid (Stahl, 1967).

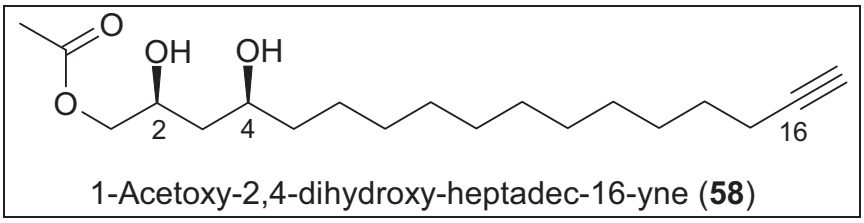
In this study compounds were identified having acetylene and acetoxy functions as terminal groups in the molecule. In this classification are substances such as **58**, **78, 79**, **81** and **82,** which were newly isolated from avocado seeds as well as the known substances (2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-yne (Kashman et al., 1969, Domergue et al., 2000) and (2*R*,4*R*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-yne (**58a**) (Hashimura et al., 2001) from the avocado flesh.

Compounds **78**, **79** and **82** were isolated for the first time from the avocado seeds and are new natural products.

As already mentioned the substances from avocado seed show minimal differences in the constitution such as location of double bounds, number of hydroxyl groups, or replacement of a hydroxyl group by a keto group (cf. Fig. 3-16, p. 75).

3.4.1.1.1. 1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**)

The lipophilic petrol ether partition recovered from the methanolic avocado seed extract was fractionated by means of HSCCC and MPLC as described in Table 5-3 (cf. p.p. 246 and 247). The separations resulted in 85.5 mg of colorless crystals of 1-acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**) (cf. materials and methods p. 251). The structural assignment was deduced from the 1D- and 2D-NMR experiments.



1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**)

For a molecular weight determination within the natural product class of avocado acetogenins and lipids, different ionization techniques were utilized for MS-detection. During EI-MS (70eV) of **58**, a strong fragmentation occurred indicating a long aliphatic chain, but the molecular ion signal could not be ascertained. However, the generated EI-MS fragments from the α-fragmentations of the hydroxylic groups helped to elucidate the constitution of the identified compounds.

LC-ESI/MS analysis of **58** in positive ionization mode detected a pseudomolecular ion peak at *m/z* 349 $[M+Na]^+$ (cf. Fig. 3-6). The soft ionization by CI-MS (with reactant gas isobutene) showed the molecular ion signal at *m/z* 327 $[M+H]^+$ and clearly confirmed a molecular weight of Mr 326 for substance **58**.

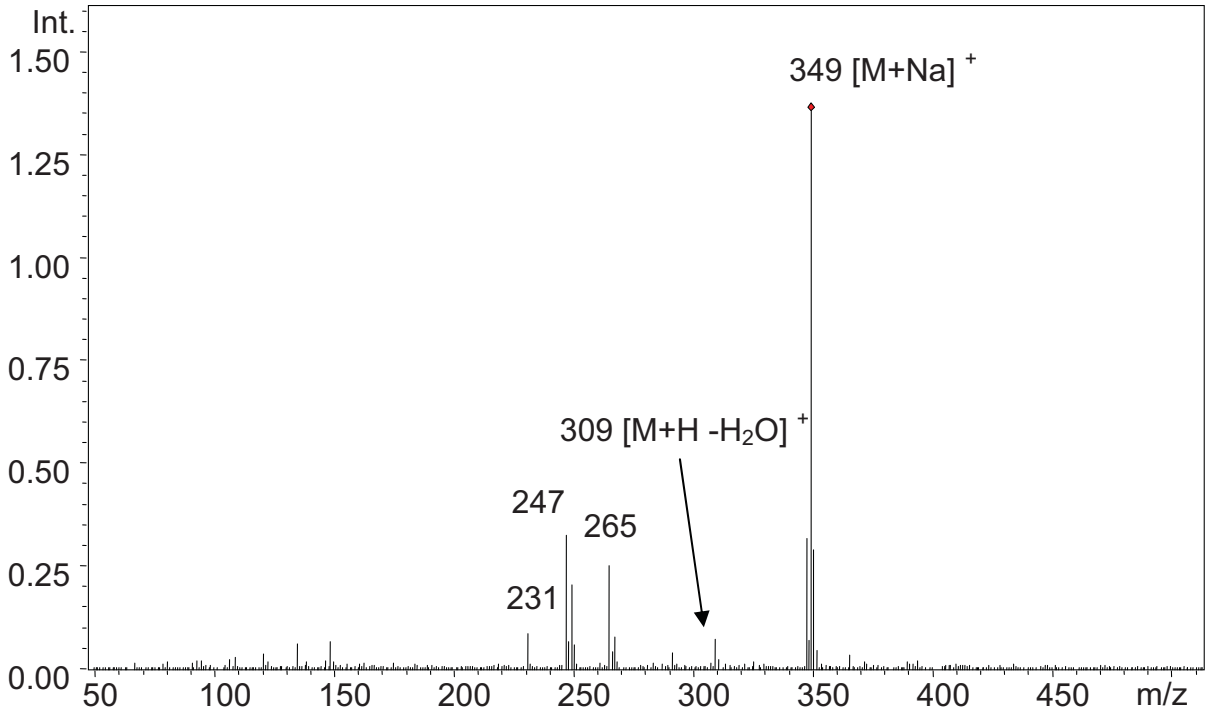


Figure 3-6. LC-ESI-MS (pos. ion mode) of 1-Acetoxy-2,4,-dihydroxy-heptadec-16-yne (**58**) (Intensity: x 10^7).

The existence of two hydroxy groups in structure **58** was supported by the CI-MS data with ion fragments indicating the loss of water at *m/z* 309 $[M+H-H_2O]^+$ and *m/z* 291 $[M+H-2H_2O]^+$. Cleavage of the acetate function is explained by the signal *m/z* 231 $[M+H-CH_3COOH-2H_2O]^+$ (cf. Fig. 3-7). The possible mechanism of fragmentation is presented in figures 3-8 and 3-9.

In the EI-MS spectrum (70eV), the fragmentation pattern of hydroxylated hydrocarbon structures with a chain length greater than C_6 is characterized by the bond cleavage carbon to carbon atom in α-position to the oxygen atom. Nevertheless, a prominent ion peak can usually also be found at M-18 due to the loss of water as a typical mechanism in the ionization of alcohols (Silverstein et al., 1991).

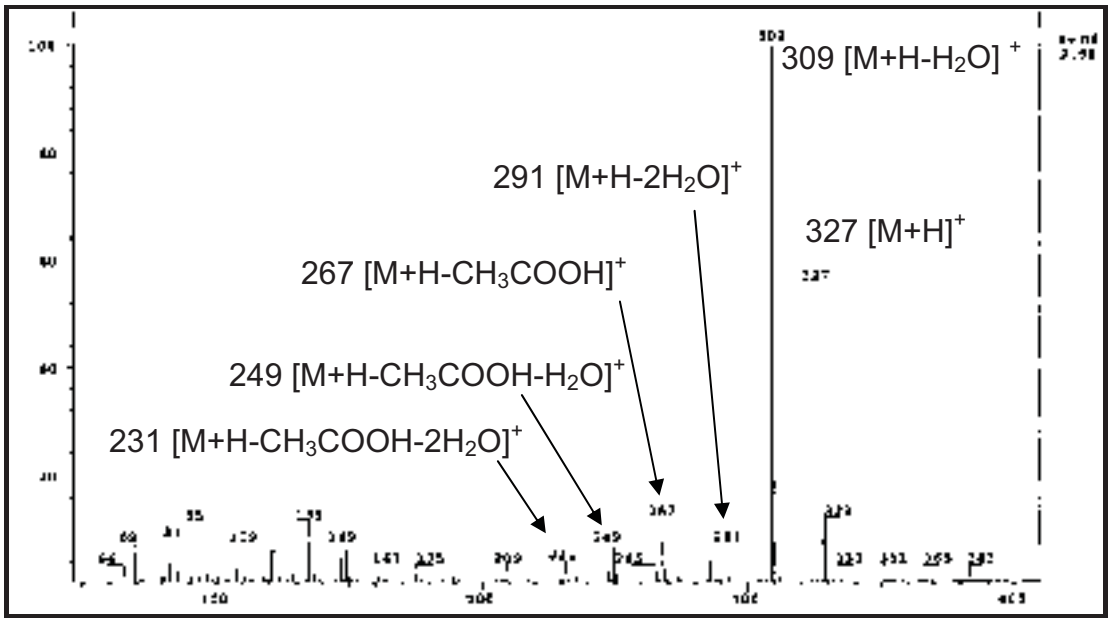


Figure 3-7. CI-MS of **58** (isobutane).

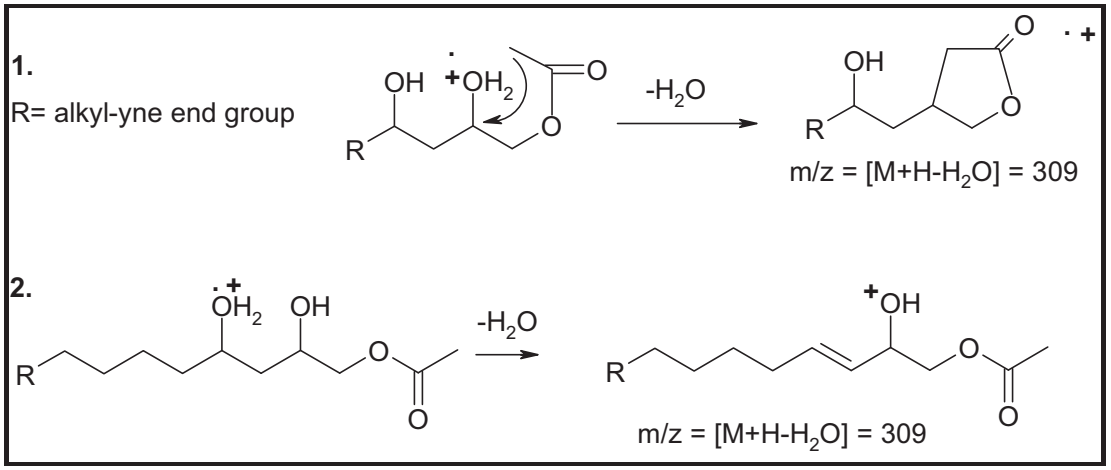


Figure 3-8. Possible cleavages of water in hydroxy-acetylenic compounds from avocado seeds.

The EI-MS spectra of **58** showed the structurally relevant fragmentations in α-position of the two hydroxyl groups with fragments at *m/z* 103, *m/z* 147 (fragments in direction to the terminal alkyne group) and *m/z* 209, *m/z* 253 and *m/z* 265 (fragments in direction to acetate group). Together with the information of the molecular weight of Mr 326, these fragment signals clearly determined the carbons C-2 and C-4 as positions of hydroxylation (cf. Fig. 3-9).

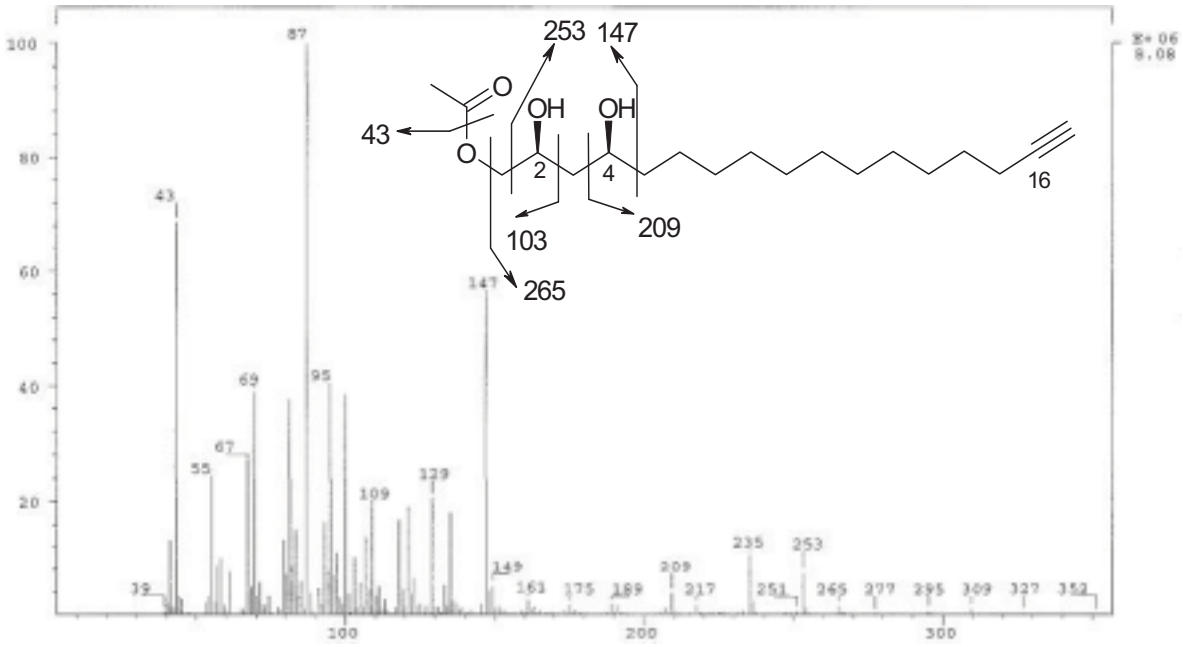


Figure 3-9. EI-MS spectra (70 eV) and characteristic α-fragmentations of 1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**).

The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) of 1-acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**) showed characteristic signals revealing the presence of three carbinol groups (δ 3.88, 4.09 and 4.12 ppm) (cf. materials and methods p.p. 251 for similar compounds), and the singlet resonance at δ 2.09 ppm (3H) for the methyl function of an acetate group. The existence of a terminal acetylene group was indicated by a triplet resonance (1H, δ 1.92 ppm, *J*= 2.6 Hz) of a single proton showing a long-range coupling (^{4}J-^{1}H/^{1}H) over the alkine bond to the methylene group in α–position at δ 2.18 ppm (2H, *d t,* J_1= 2.6, J_2= 6.9 Hz). This is corroborated by the missing methyl resonance at δ= 0.89 ppm (3H, *t*,) normally observed for the chain end in linear aliphatic systems.

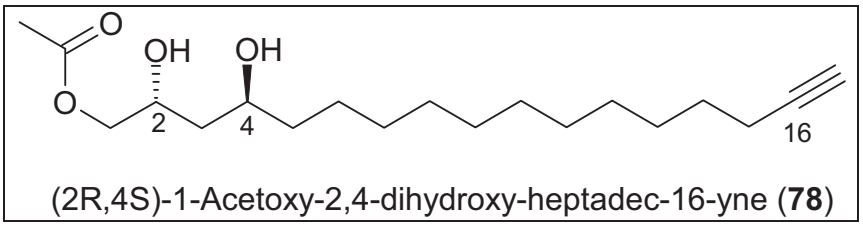
The ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) showed the characteristic resonances of the terminal alkine at δ 68.3 and 85.1 ppm, the strongly shielded α-methylene carbon at δ 18.7 ppm, and three carbinol signals (δ 68.9, 71.1, 72.7 ppm). The acetate group appeared with two resonances at δ 21.2 and 171.5 ppm. All aliphatic signals (δ 28.9 to 38.6 ppm) corroborated a long aliphatic chain (cf. Table 3-7, p. 83).

Assignments of C- and H-atoms were made according to direct ^{1}J C-H correlation and COLOC experiments ($^{2,3}J$ C-H, long-range correlation with direct carbon detection).

For 1-acetoxy-2,4-dihydroxy-heptadec-16-yne from avocado fruit flesh Domergue et al. (2000) determined the stereochemistry to be (2*S*, 4S). Due to the complete accordance of ^{13}C-NMR and ^{1}H-NMR data, we assumed to have the same (2*S*, 4S)-stereochemistry for substance (**58**) isolated from avocado seeds.

3.4.1.1.2. (2*R*, 4*S*)-1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**78**)

Compound **78** (approx. 132 mg) was recovered as white powder from the petrol ether partition fractionated as described in separation 35 (cf. Table 5-3 p. 248). The structural assignment of **78** was deduced from ^{1}H-, ^{13}C-, and DEPT135 NMR data in comparison with reference data.



(2R,4S)-1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**78**)

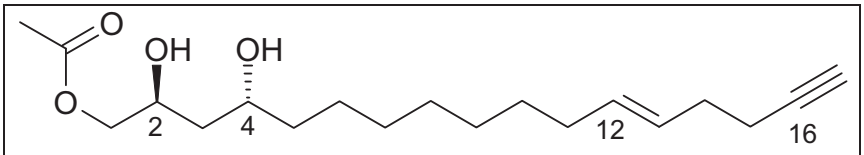
The molecular weight of compound **78** was determined by EI-MS and LC-ESI/MS and was identical to **58** (Mr 326 Da). The LC-ESI/MS analysis showed for **58** a retention time of 10.6 min and for compound **78** a faster elution at R_t 10.0 min (cf. conditions p. 252). The slight shift in retention time values is likely to be due to different absolute configurations of the hydroxyl groups. Compound **78** was therefore assumed to be a diasteromeric compound to substance **58**.

The ^{13}C-NMR ($CDCl_3$, 75.6 MHz) revealed only three differences in chemical shift values between compounds **58** and **78**. In case of compound **78** the values for C-1, C-2 and C-3 are localized at higher field (cf. Table 3-7, p. 83). The opposite stereochemistry of **78** at position C-2 could explain the different chemical shift values of the attached carbon atoms C-1 and C-3 (δ 66.84 and 34.81 ppm,

respectively). Substance **78** appeared to be an epimer of **58** regarding position C-2 (cf. Fig. 3-16).

3.4.1.1.3. 1-Acetoxy-2,4-dihydroxy-heptadec-12-en-16-yne (**79**)

From the lipophilic petrol ether extract of the avocado seed cotyledon we recovered by combination of middle-pressure-chromatography (MPLC) on silica gel and high-speed countercurrent chromatography (HSCCC) 3 mg of substance **79** as colorless oil with a characteristic sweet odor (cf. Table 5-3, separation 32, p. 247). The proposed structure for substance **79** is:



1-Acetoxy-2,4-dihydroxy-heptadec-12-en-16-yne (**79**)

ESI-MS measurements for **79** via syringe pump resulted in a strong signal at m/z 323 $[M-H]^-$ (negative ionization mode). The quasimolecular ion adduct in positive mode at *m/z* 347 $[M+Na]^+$ (cf. Fig. 3-10) confirmed the molecular mass of 324 Da. Also the CI-MS data (reactant gas: isobutane) confirmed the molecular weight of **79** by showing a signal for proton addition at *m/z* 325 $[M+H]^+$. The ion fragment at *m/z* 307 indicated the loss of water (Δ*m/z* 18). The abundant ion signal at *m/z* 265 is generated due the cleavage of the acetate function $[325-CH_3COOH]^+$ (cf. Fig. 3-11 and 3-12). The CI-MS is also giving a strong fragment at *m/z* 147 caused by α-fragmentation at the bond C-4/C-5 thus indicating one position of hydroxylation.

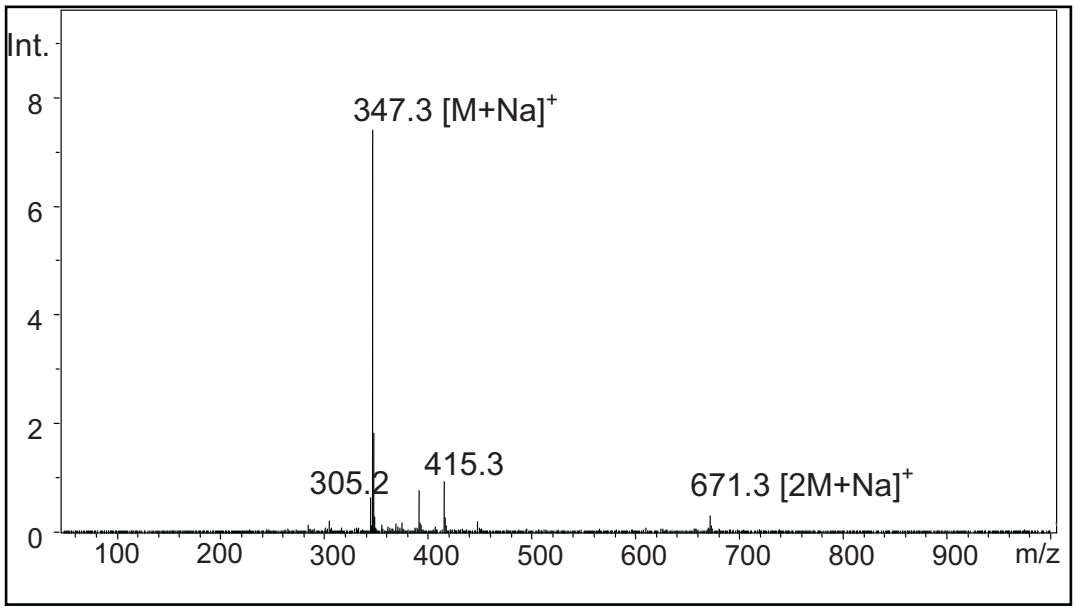


Figure 3-10. ESI-MS (pos. mode) of substance **79** (Intensity: x10^6).

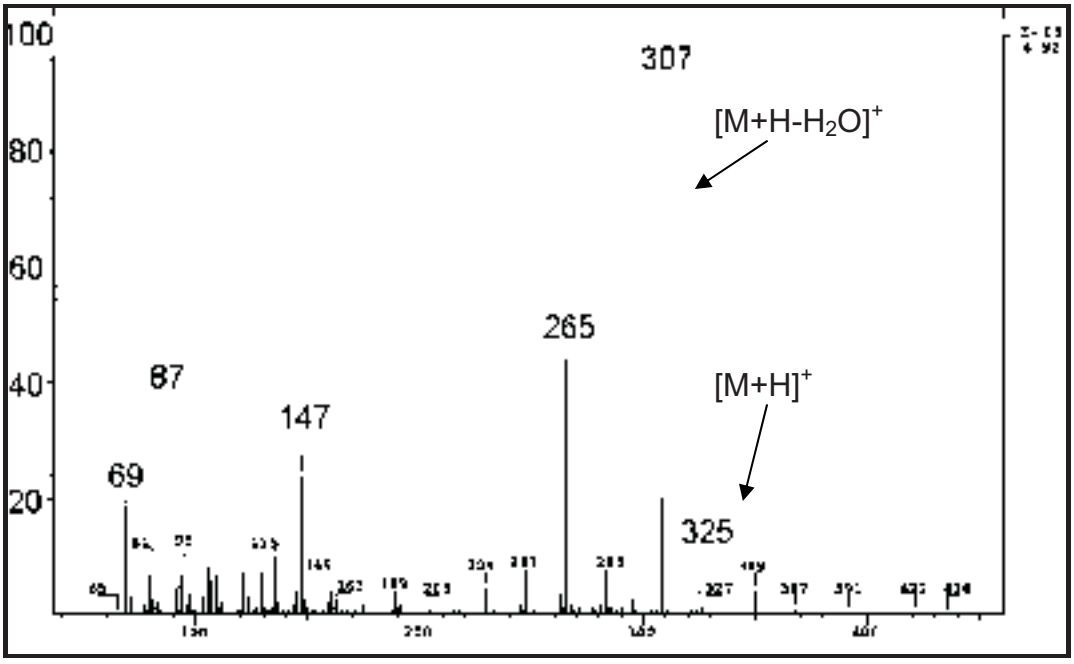


Figure 3-11. CI-MS (isobutane) of 1-acetoxy-2,4-dihydroxy-heptadec-12-en-16-yne (**79**).

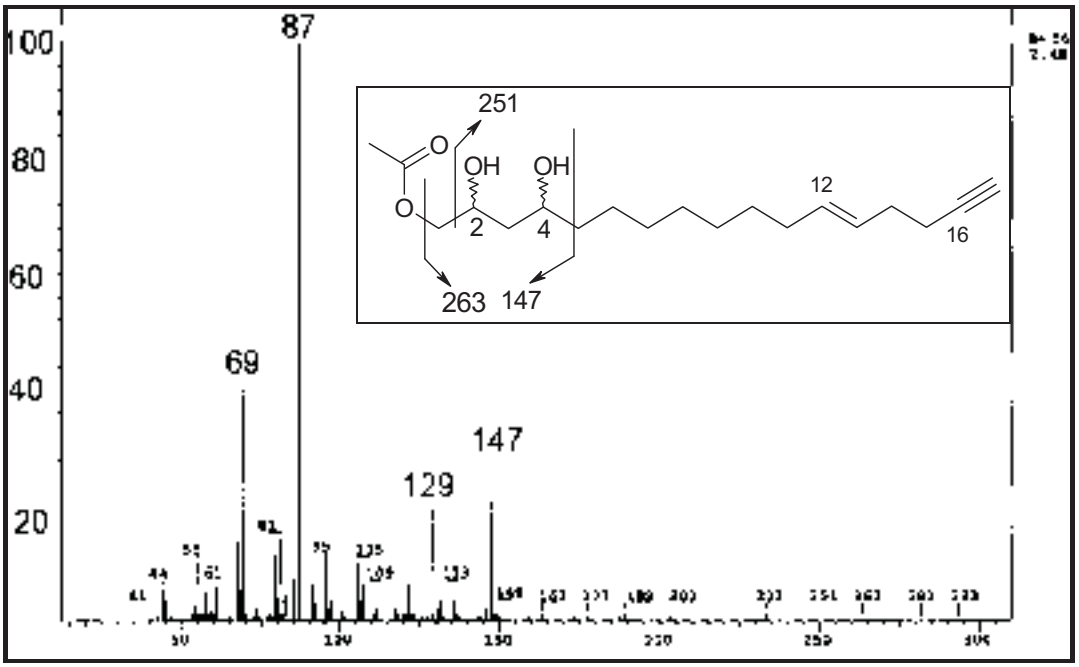


Figure 3-12. EI-MS (70 eV) of 1-acetoxy-2, 4-dihydroxy-heptadec-12-en-16-yne (**79**) showing relevant α-fragmentations.

The EI-MS spectra of **79** showed the structurally relevant fragmentations in α-positions of the two hydroxyl groups with the α-fragmentation product at *m/z* 147 (fragment in direction to acetate group). The fragments in direction to the terminal acetylene group are *m/z* 251 and *m/z* 263 $[M\text{-}H\text{-}CH_3COOH]^+$ having two mass units less than the observed for substance **58**. Fragment ion m/z 263 is most likely a product from a McLafferty rearrangement reaction (McLafferty and Turecěk, 1993). These observations are indicating an additional double bond in the aliphatic chain of substance **79** (cf. Fig. 3-12).

The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) of **79** (cf. Fig. 3-13) showed the common signals of the acetylenic group described before (cf. substance **58**).

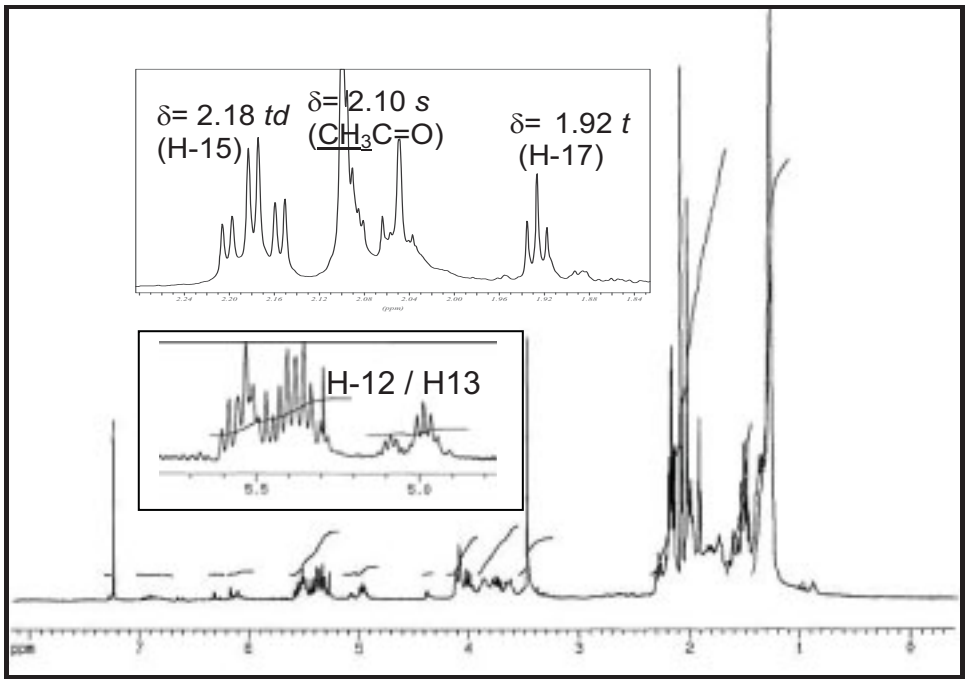


Figure 3-13. ^{1}H-NMR spectra ($CDCl_3$, 300 MHz) of compound **79** showing the characteristical signals for the acetylenic bond as well as the olefinic signals.

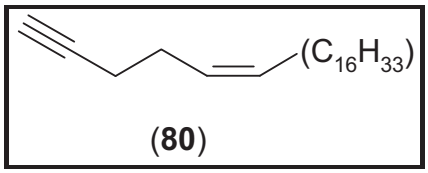
In order to achieve a better resolution of important carbinol proton signals, sample **79** was measured again in a mixture of $CDCl_3$/ CD_3OD (7:3) using the better spectral dispersion of a 600 MHz spectrometer. The addition of deuterated methanol significantly improved the quality of the ^{1}H-NMR signals.

Interestingly, addition of CD_3OD caused that the proton signal H-17 of the terminal acetylene group at δ 1.92 ppm disappeared from the spectrum. A possible explanation is that protons belonging to terminal acetylene groups do have CH-acidity and are interchangeable with deuterium liberated from protic solvents such as CD_3OD (H/ D interchange) (Günther, 1992). Furthermore the presence of two olefinic resonances at δ 5.41 and δ 5.50 ppm with a typical coupling constant *J*=15 Hz corroborated a *trans*-configured double bond. The signals between δ 1.28-1.50 ppm integrating for ~ 20 H atoms indicated a long aliphatic chain.

The ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) presented also typical signals of long chain acetylenic compounds. Natural compounds related to the biosynthetic pathway of unsaturated fatty acids have the double bonds at positions C-6, C-9, C-12 or C-15 such as the avocado constituents persenone A (**64**) and persin (**66**) (Oelrichs et al., 1995). Important $^{2,3}J$ HC-long-range correlations (HMBC)

($CDCl_3$+CD_3OD, 7:3, 600 MHz) of **79** located the double bond at position C-12/ C-13 in the aliphatic chain of this substance. The key correlations for this assignment were $^{2,3}J$ HC correlations from H-15 to C-13, furthermore the correlations between the olefinic signals H-13 to C-14, C-11 and C-12 as well as H-12 to C-14, C-11 and C-13 (cf. Fig. 3-14).

Also the synthetic product docos-5-en-1-yne (**80**) (Hollowood et al., 2003) is in good agreement with NMR-data of substance **79** with regard to the double bond position.



(**80**)

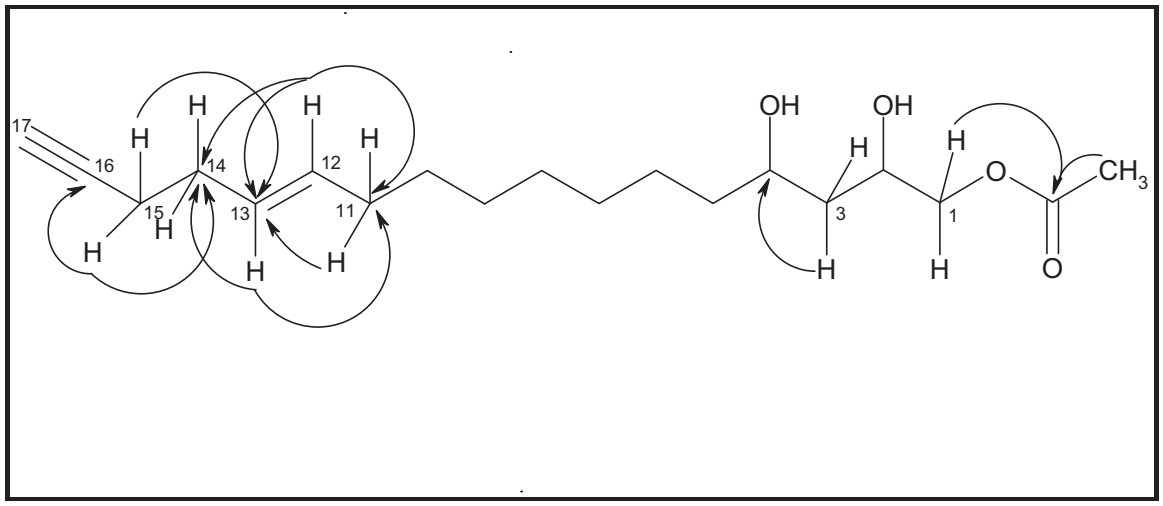


Figure 3-14. Important HMBC correlations in structure **79**.

Compound **79** has two chiral centers at positions C-2 and C-4. Two already known natural products (2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-yne (**58**), (2*R*,4*R*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-yne (**58a**) isolated from avocado fruits (Kashman et al., 1969, Kashman et al., 1970; Domergue et al., 2000; Hashimura et al., 2001) show similar NMR data by comparing the ^{13}C data of compounds **58** and **79** (cf. Table 3-7, p. 83), a stereochemistry of (2S, 4R) could be deduced for compound **79**.

The differentiation of enantiomeric pairs such as a 2*S*, 4*S*- and 2*R*,4*R* is not possible by simple NMR experiments alone due to the magnetic equivalence of

protons. In comparison to the diasteromers, (*2S*,4*R*) and (2*R*,4*S*), only slight differences in respect to their ^{1}H NMR-data (Sugiyama et al., 1982; Hashimura et al., 2001) are observable.

Kashman et al. (1970) determined the configuration of **58** by fixing the two hydroxyl groups at C-2 and C-4 through acetonide ring-closure. He concluded that both OH groups should have the same “absolute” configuration (that in fact is only a “relative” configuration!). The optical rotation values for **58** (2S, 4S) were reported to be $[\alpha]_D$ -2.7° ($CHCl_3$) (Domergue et al., 2000) and for **58a** (2R,4R) $[\alpha]_D$ -18° ($CHCl_3$) (Hashimura et al., 2001). In respect to these small rotational values our measurement with only 3 mg sample amount did not allow an unambiguous elucidation of the stereochemistry at C-2/ C-4.

NOE irradiation experiments might help to determine the relative stereochemistry of C-4 and C-2 of substance **79**.

For elucidation of the absolute stereochemistry of the carbinols in substance **79**, the 'Exciton Coupled Chirality CD' method by Harada and Nakanishi (1972) and Gonnella and Nakanishi (1982) can be applied. This method introduces strong chromophors by derivatization of the two carbinol function with p-bromobenzoic acid chloride (cf. Fig. 3-15). The stereochemical orientation of the p-bromobenzoate functional groups is generating characteristic COTTON-effects (CD-curve) determining the absolute stereochemistry of the chiral positions C-2 and C-4 of **79**.

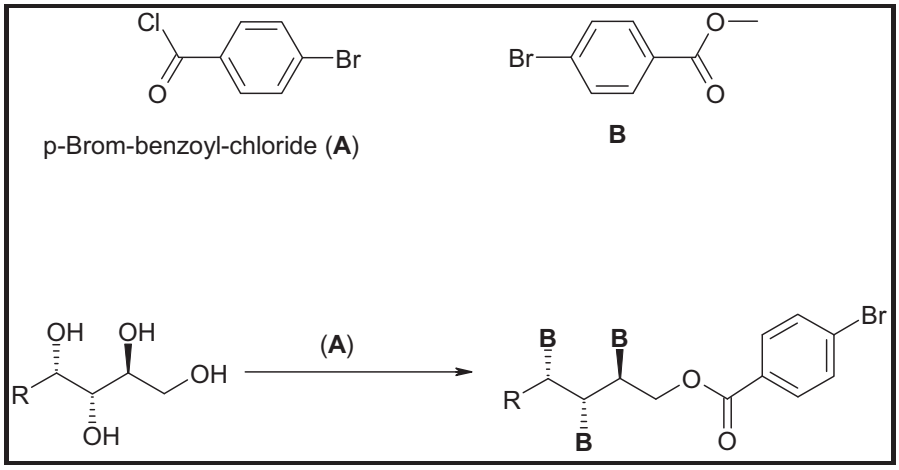


Figure 3-15. Reaction example of a polyol substance introducing the chromophor *p*-bromobenzoic acid.

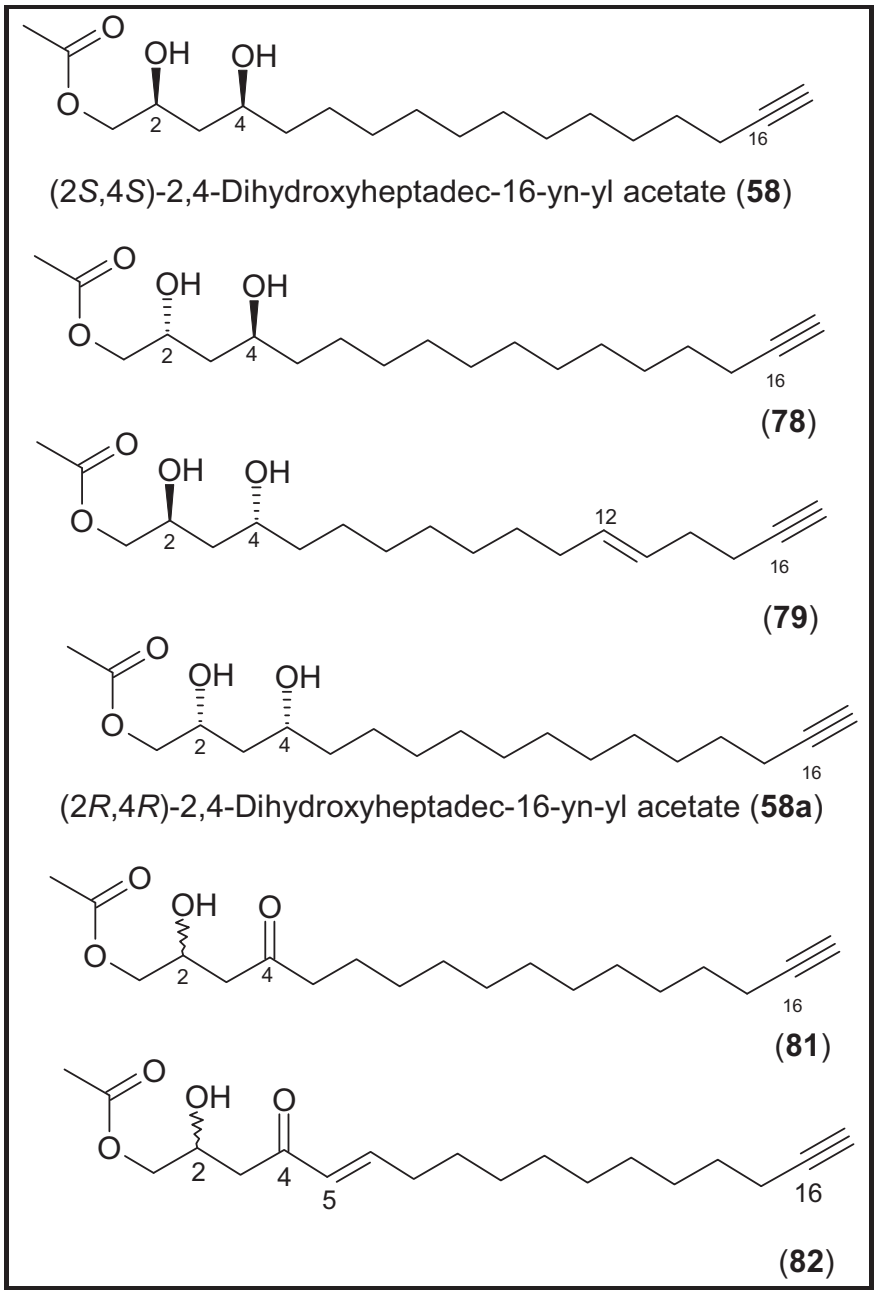
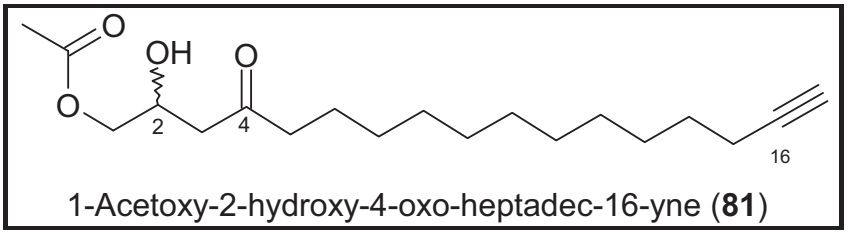


Figure 3-16. Structures of acetylenic compounds isolated from avocado seeds in the frame of this study (except compound **58a**).

The complete ^{1}H-NMR data of **79** are given in materials and methods (cf. p. 253). To the best of our knowledge compound **79** is a new natural product of avocado seeds.

3.4.1.1.4. 1-Acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (**81**)

From the purification number 33 (8.1.9b.3.4.) (cf. Table 5-3 p. 247) of the petrol ether partition approximately 10 mg of substance **81** have been recovered as white crystals.



1-Acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (**81**)

In the investigation by LC-ESI/MS (cf. conditions p. 254), substance **81** eluted at R_t 10.2 min. The LC-ESI/MS spectra showed the molecular ion as sodium adduct at *m/z* 347 $[M+Na]^+$ related to a molecular weight of Mr 324. Hence, the Mr of Persin (**66**) is two mass units less than **58** and **78**.

The fragment ion in the ESI-MS at m/z 265 $[M-60+H]^+$ is the cleavage of an acetate unit while the fragment at *m/z* 247 is the following loss of a molecule water. This was indicating the presence of acetate and only one hydroxyl group in the molecule in comparison to structures **58** and **78** (Fig. 3-17).

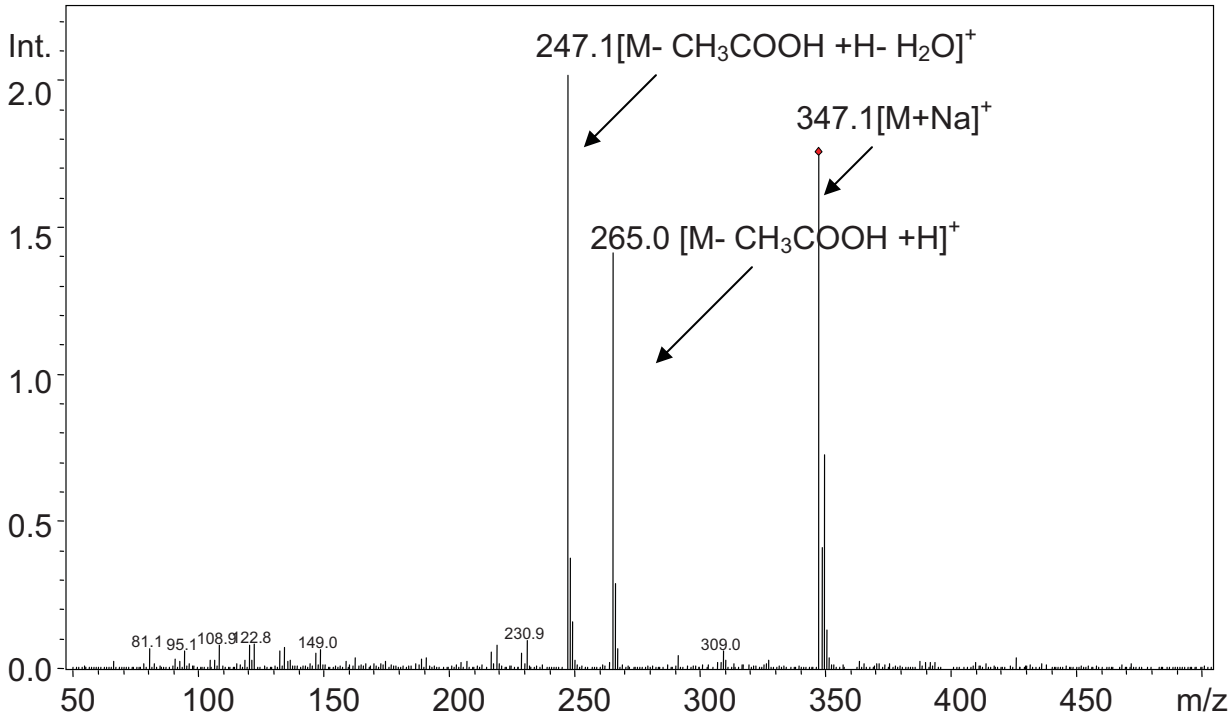


Figure 3-17. LC-ESI-MS (positive mode) of compound **81** (Intensity: x10^7).

Important ions for **81** from the EI-MS were *m/z* 145 (fragmentation to the acetate end) and *m/z* 207 (fragmentation to the acetylene end) indicating a keto group in position C-4. In comparison to substance **58** with a hydroxyl group at C-4, a mass difference of 2 amu was observed.

The ^{1}H NMR ($CDCl_3$, 360 MHz) (cf. Figure 3-18) showed a doublet (2H) at δ 2.60 ppm (*J*= 8 Hz) attributed to the H-3 protons, and a triplet at δ 2.44 ppm (*J*= 7.5 Hz) of the protons located at C-5. For substance **81**, the ^{1}H NMR only detected three complex carbinol proton signals (H-1a, δ 4.05 and H-1b, δ 4.00 and H-2 at 4.11 ppm) occurring instead of four separated resonances observed for the acetoxy-2,4-dihydroxy derivate **58**.

The other proton signals were in good accordance with the already described acetylene components **58**, **78**, and **79** having the characteristic triplet signal (1H) of the terminal acetylene proton at δ 1.93 ppm. (cf. materials and methods p.p. 251-254).

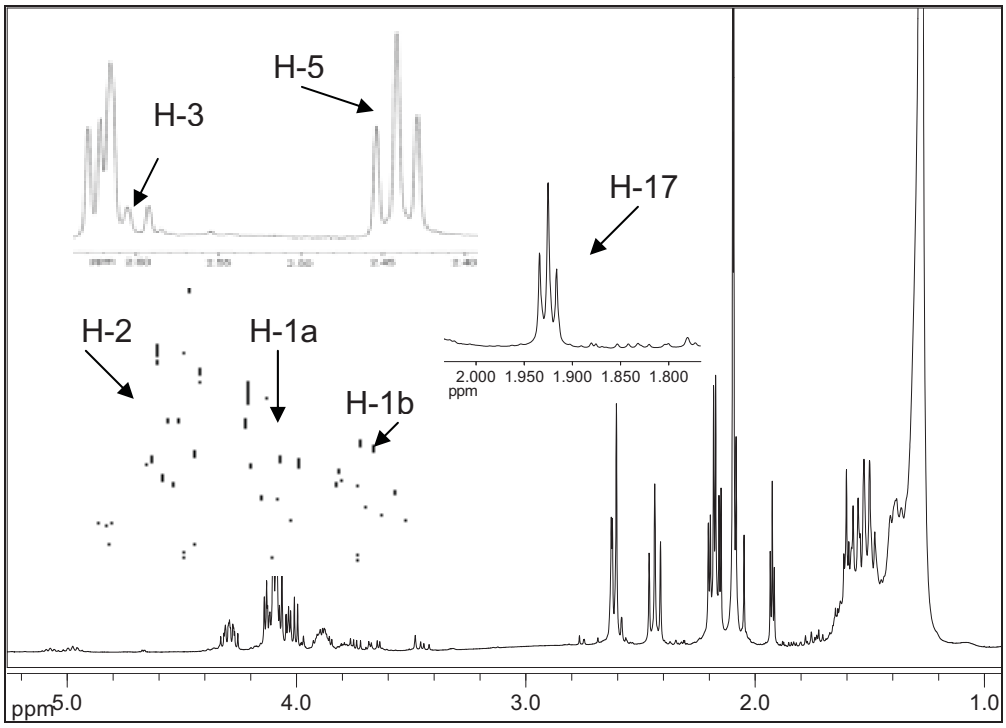


Figure 3-18. ^{1}H-NMR spectrum of compound **81**.

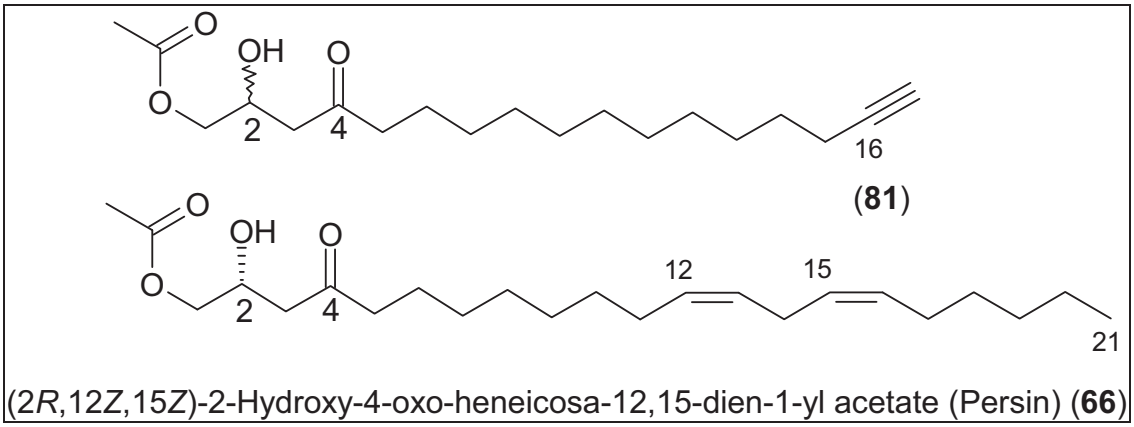
The ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) of **81** shows in respect to the already known and described substances, only four differences in δ-shift values at the positions C-2 to C-5. The most important is at C-4 (δ 211.14 ppm) corresponding to a keto group while the signals around C-2, C-3, and C-5 are moved to lower field (cf. Table 3-7, p. 83).

The NMR data for compound **81** are identical to 1-Acetoxy-2-hydroxy-4-oxo-heptadec-16-yne isolated from the avocado fruit (Kashman et al., 1969; Domergue, 2000).

Compound (**81**) has a stereochemical centre at C-2. Reference ^{13}C-NMR data regarding positions C-1 to C-5 of a structural analogue – the persin (**66**) - isolated from the avocado leaves (Oelrichs et al., 1995) is showing very similar data as obtained for 1-acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (**81**).

The stereochemistry at C-2 of (**66**) was determined by synthesis (Bull and Carman, 1994; MacLeod and Schäffeler, 1995) to be *R*. The Mosher method (Dale et al., 1969, Dale and Mosher, 1973; Ohtani et al., 1991) could be a potential tool to determine the absolute stereochemistry of (**81**) at C-2 via NMR chemical shift

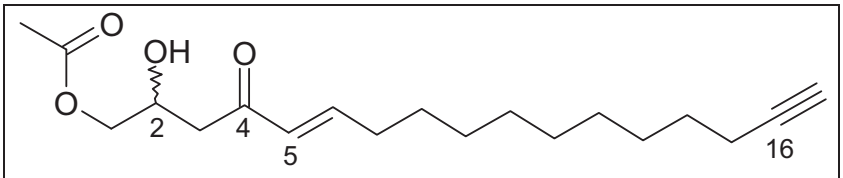
changes induced by derivatization to diastereomeric α-methoxy- α-trifluoro-methylphenylacetate esters (MTPA).



(2*R*,12*Z*,15*Z*)-2-Hydroxy-4-oxo-heneicosa-12,15-dien-1-yl acetate (Persin) (**66**)

3.4.1.1.5. 1-Acetoxy-2-hydroxy-4-oxo-heptadec-5-en-16-yne (**82**)

Approximately 7 mg of compound **82** were obtained as colorless oil from the petrol ether separations number 32 and 33 fractions 8.1.9b.2.6 and 8.1.9b.3.3, respectively (cf. Table 5-3, p. 247).



1-Acetoxy-2-hydroxy-4-oxo-heptadec-5-en-16-yne (**82**)

The LC-ESI/MS spectra showed a sodium adduct $[M+Na]^+$ (positive mode) at *m/z* 345 and a corresponding ion at *m/z* 321 $[M-H]^-$ in the negative mode indicating a molecular weight Mr of 322 amu. As for **81**, compound **82** shows the presence of a hydroxyl function which was confirmed by the loss of one water molecule leading to fragment ion *m/z* 245 in the same LC-ESI/MS spectrum (cf. Fig. 3-19).

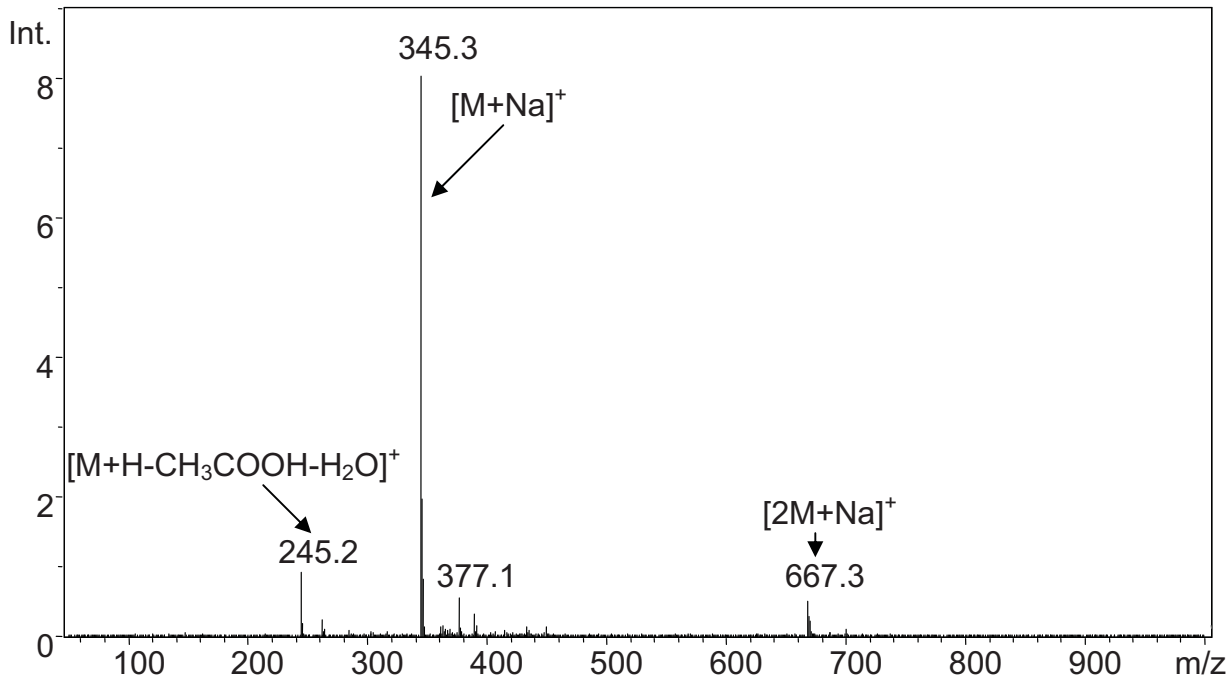


Figure 3-19. LC-ESI-MS of compound **82** (Intensity: x 10^6).

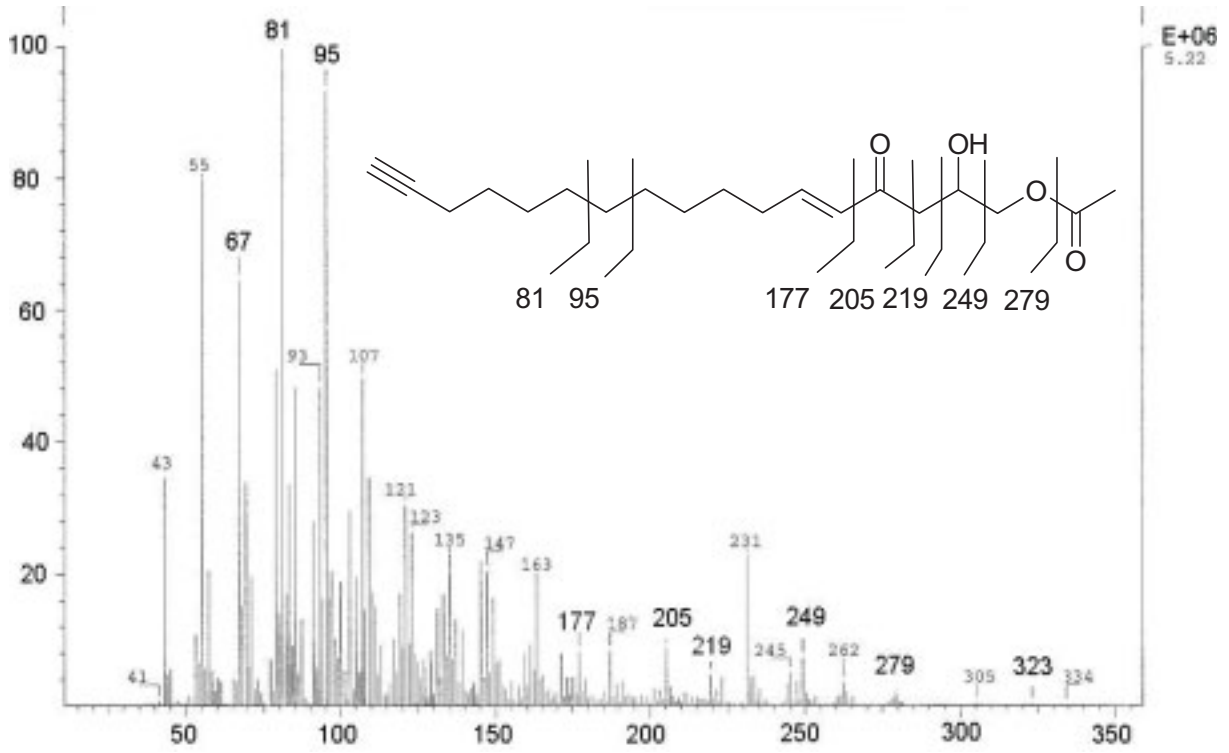


Figure 3-20. EI-MS (70 eV) of compound **82**.

The EI-MS spectrum from compound **82** (cf. Fig. 3-20) showed fragments that confirmed the localization of the double bond (*m/z* 177) as well as the presence of one hydroxyl group (*m/z* 219 and m/z 249) and one keto function (*m/z* 205 and 219).

The ^{1}H NMR ($CDCl_3$, 300 MHz) of **82** showed a doublet (2H) at δ 2.77 ppm attributed to the H-3 protons. In comparison to structure **66,** the resonances were down-field shifted by a value of $\Delta\delta$ 0.18 ppm. Again only three complex proton signals were present. Two of the carbinol signals were assigned to the H-1 protons (δ 4.10 and δ 4.17 ppm) and the resonance for H-2 (δ 4.35 ppm). The new signals occurring in respect to compound **81** were two double triplets from the olefinic protons at δ 6.12 and δ 6.85-6.95 ppm localized at C-5 and C-6, respectively. The neighbouring keto group at C-4 with the deshielding effect is shifting the resonances to the lower field. The *trans* double bond configuration was recognized by the coupling constant of *J*=16 Hz. The typical signals of the protons in α-position of the alkyne and the H-17 were also present in **82**.

In respect to **81**, the ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) of **82** exhibited only differences at positions C-3 to C-7 due to the presence of a double bond at C-5/ C-6 with olefinic carbon chemical shifts at δ 130.7 and δ 149.7 ppm, respectively. Interestingly, the resonance of the keto group at C-4 was strongly shifted to higher field (δ 199.9 ppm) in comparison to compound **81**. This clearly indicated that the double bond system is in conjugation with the keto group (cf. Table 3-7, p. 83).

Furthermore, ^{1}H- and ^{13}C-NMR data as well as $^{1}H/^{1}H$-COSY (600 MHz, $CDCl_3$ - CD_3OD 7:3) experiments were helpful for localization of the double bond in the structure. $^{1}H/^{1}H$-correlation signals between the olefinic protons (H-5/ H-6) and their neighbouring protons (H-7/ H-8) as well as the correlation between proton H-3 to the carbinol proton H-2 (cf. Fig. 3-21) and $^{2,3}J$ HC-long-range correlations confirmed our structure proposal (cf. description of compound **79** p. 68).

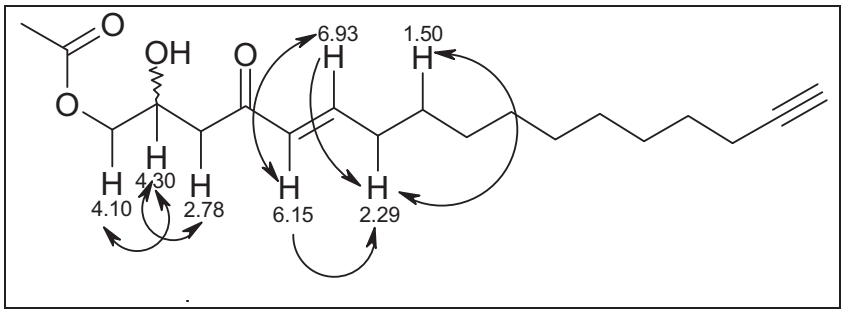


Figure 3-21. Structure relevant correlations in the $^{1}H/^{1}H$-COSY spectrum of compound **82**.

All NMR-data of compound **82** are showing for the 1-acetoxy-2-hydroxy-4-oxo-5-ene partial structure very similar resonances as for the known avocado fruit constituents persenone A (**64**) (Kim et al., 2000a and b; Domergue et al., 2000, Kawagishi et al., 2001) and persenone B (**62**) (Kim et al., 2000b). Our isolated compound from avocado seeds is characterized by a shorter carbon chain (C-19) than the mentioned persenones (C-23 and C-21, respectively) (cf. Fig 3-22). The configuration at C-2 for persenone A was reported to be 2*R* (Domergue et al., 2000) having an optical rotation value of $[\alpha]_D$ + 11.7.

To the best of our knowledge compound **82** is a new natural product present in avocado seeds.

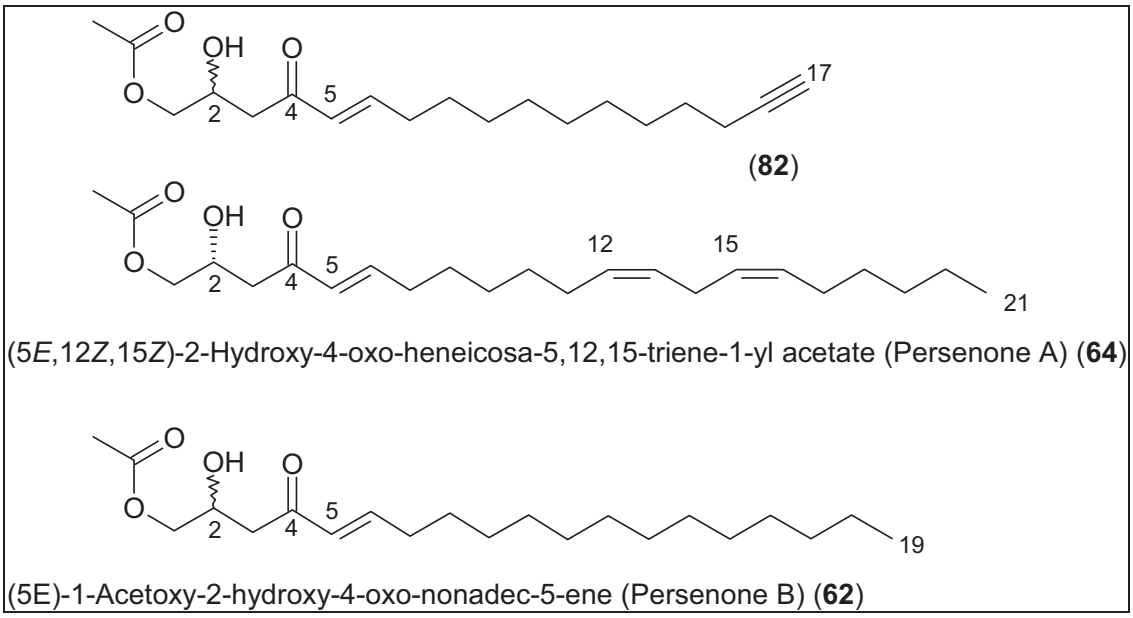


Figure 3-22. Differences in length chain between compound **82** and Persenones A/B.

Table 3-7. ^{13}C-NMR data of acetylenic compounds **58**, **78**, **79**, **81** and **82** from avocado seeds ($CDCl_3$, 75.5 MHz, δ= [ppm])

C-Atom	**58**[12]	**78**[12]	**79**[13]	**81**[13]	**82**[14]
1	68.9	66.84	68.03	67.64	67.71
2	71.1	70.91	70.42	66.48	66.64
3	39.6	34.81	41.48	45.59	42.79
4	72.7	72.60	71.39	211.14	199.92
5	38.6	38.21	38.80	44.04	130.66
6	25.6	25.52	29.03	23.95	149.73
7	29.9	29.9	28.72	29.93	32.88
8	29.9	29.9	29.29	29.89	28.36
9	29.8	29.8	29.22	-	29.77
10	29.7	29.7	29.10	-	29.68
11	29.6	29.6	29.37	29.79	29.64
12	29.4	29.4	134.59	29.43	29.43
13	29.1	29.05	124.86	29.10	29.05
14	28.9	28.81	28.48	28.86	28.83
15	18.7	18.69	18.39	18.75	18.75
16	85.1	85.08	84.74	85.13	85.07
17	68.3	68.82	69.85	68.37	68.42
CH_3CO	21.2	21.59	20.83	21.14	21.18
CH_3CO	171.5	171.51	171.09	171.28	171.3

Superscripts indicate the molecular weights of the avocado seed lipids: Mr [12] 326, [13] 324, [14] 322 Da. The ^{13}C-chemical shifts relevant for the differentiation of the components are underlined.

Persin (**66**) had already shown a strong inhibition of spore germination of *Colletotrichum gloeosporioides*, the later being responsible for fruit-rot causing the loss of great amounts of avocado fruit in production. Persin is also inhibiting the vegetative growth of the same organism and acts as an antifungal agent. Substituted as a monoglyceride, the compound is supposed to be cardiotoxic and could have interference with triglyceride metabolism (Bull and Carman, 1994).

The biological activities of this lipophilic substance class from avocado seeds will be described in more detail in the corresponding chapter (cf. p. 202).

3.4.1.2. Lipophilic compounds with vinyl-function

The petrol ether partition (PE) from the avocado seeds treated by liquid-liquid extraction (cf. separation 25 Table 5-3 p. 245) was separated on preparative scale (8.4 g) by 'step-gradient' middle-pressure chromatography (MPLC) on silica gel (cf. separation number 26 Table 5-3, p. 246). In this way 10 fractions were obtained (8.1.1. to 8.1.10.) (cf. Table 5-3, p. 246). Fractionation was done on the basis of UV-absorbance (λ=226 nm) and RP18-TLC with visualization by anisaldehyde-reagent (Stahl et al., 1967) (Fig. 3-23).

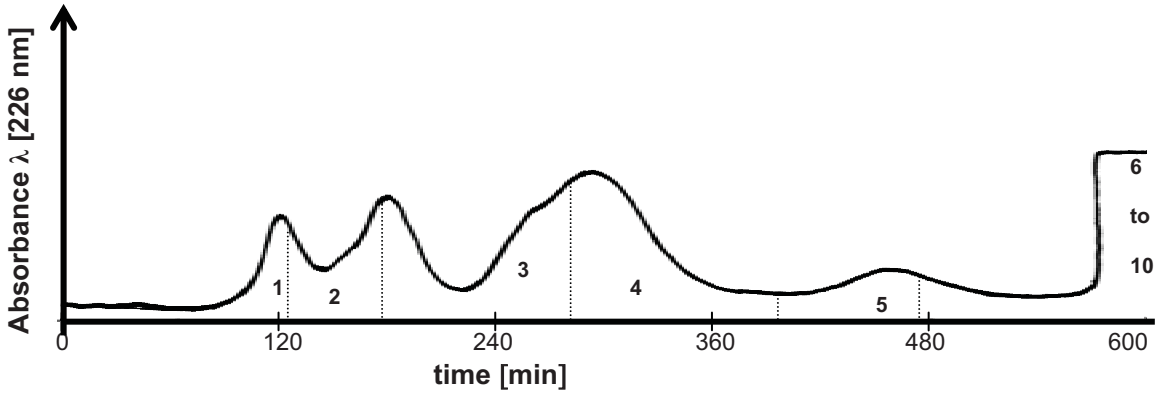


Figure 3-23. MPLC-chromatogram of the petrol ether partition from avocado seeds recorded at λ 226 nm [Silica gel K_{60} column: 5 cm ID x 46 cm. Gradient: PE (16.7h), Hexan:EtOH (95:5 v:v) (8.3 h)].

From the MPLC separation, fractions 8 (8.1.8.) and 9 (8.1.9.) contained most of the sample amount (3 and 1.6 g respectively). All of the other fractions (8.1.1. until 8.1.7) resulted only in 296 mg. Hence, separation of 8.1.9 was done by preparative HPLC on RP-18 (8.1.9a) (cf. separation 30 Table 5-3 p. 246) obtaining **58** in pure form. A separation by 'high-speed countercurrent chromatography' (HSCCC) (8.1.9b) was performed (cf. separation 31 Table 5-3 p. 247) with 1 g of 8.1.9., obtaining 10 additional fractions (8.1.9b.1 to 8.1.9b.10.) (cf. Fig. 3-24).

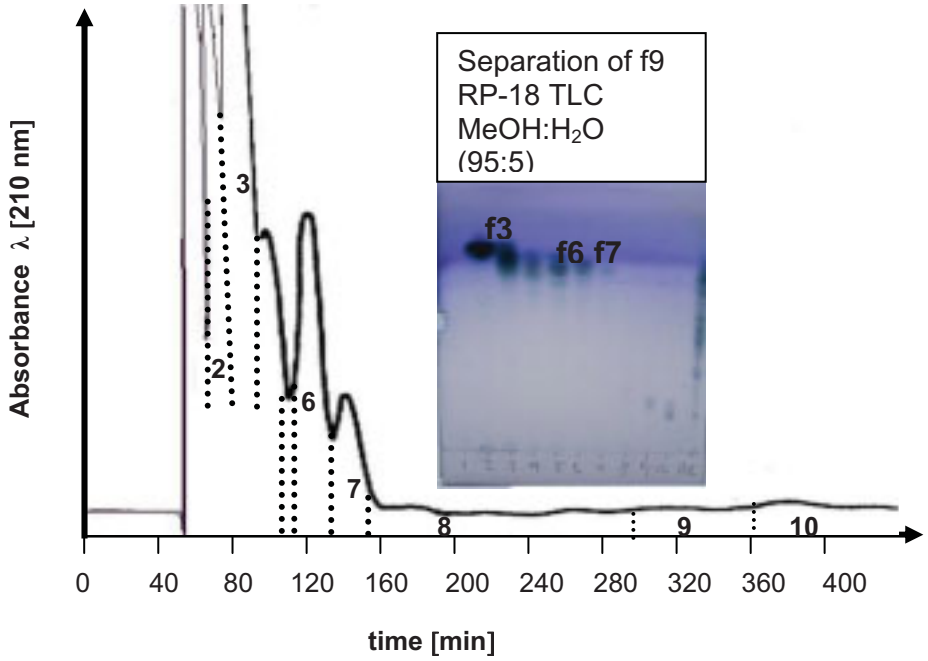


Figure 3-24. HSCCC separation of the petrol ether partition fraction 8.1.9b. (cf. MPLC-separation of petrol ether partition). Solvent system n-hexane – Ac- H_2O (6:5:1, v: v: v), flow rate: 3 ml min^{-1}. UV-trace: λ=210 nm, rotational speed: 900 rpm; mobile phase: lower aqueous phase; elution mode: head to tail.

Although 8.1.9b.6. (45 mg) and 8.1.9b.7. (28 mg) seemed to be almost pure on the RP18-TLC (cf. Fig. 3-24) it turned out to be still very complex mixtures by LC-ESI/MS analysis.

Also 8.1.9b.3. fraction was a complex mixture. This was clearly demonstrated by LC-ESI/MS analysis (pos. mode) (cf. TLC, Fig. 3-25). In this case, the sample amount was high enough for further purification. The whole quantity of 8.1.9b.3. (323 mg) was used for purification by RP18-MPLC resulting in the already described 1-acetoxy-16-alkyne-compounds **58**, **81**, **82** (cf. Fig. 3-25, p. 87 and p.p. 63, 76 and 79) and as well in compound **60** (cf. p. 88) and **86** (cf. p.103).

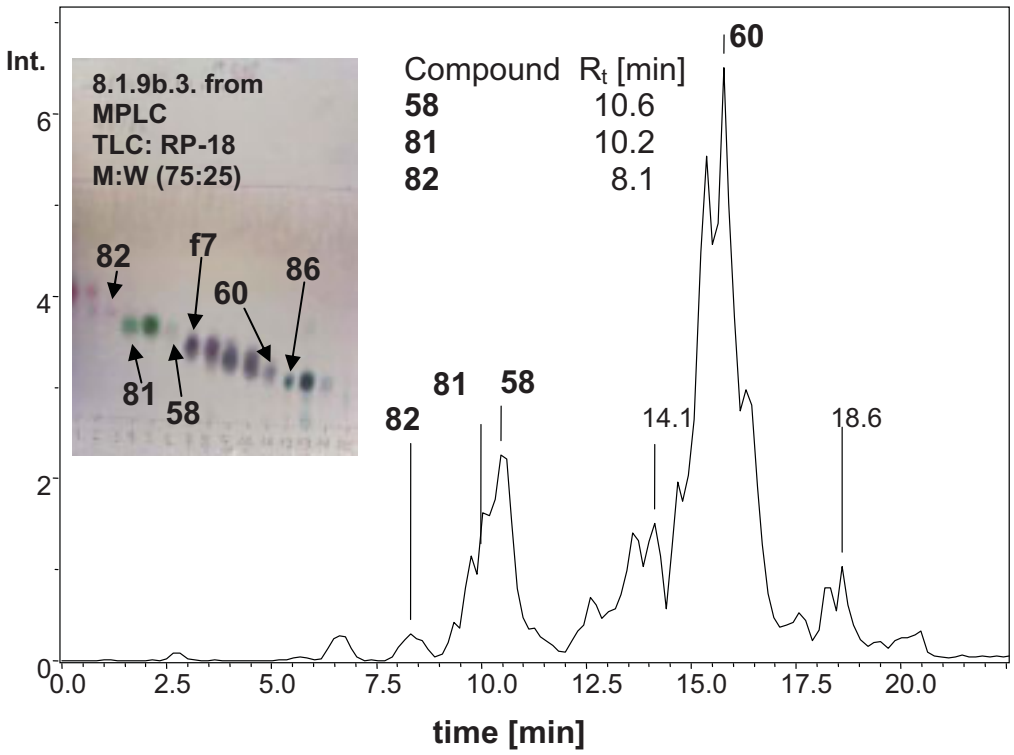


Figure 3-25. LC-ESI/MS (pos. mode) of fraction 8.1.9b.3. (Intensity x 10^7). [Column ODS Beckman 250 x 4.6 mm. A= 0.1M CH_3COONH_4 in H_2O; B: 0.1M CH_3COONH_4 in MeOH. Gradient: A (20, 15, 0, 0, 20), B (80, 85, 100, 100, 80), t (0, 7, 20, 55, 60 min). Flow: 0.8 mL min^{-1}, λ 225 nm. $[M+Na]^+$: **58** *m/z* 349, **81** *m/z* 347, **82** *m/z* 345, and **60** *m/z* 351.

Fraction f7 (8.1.9b.3.7.) (25 mg) from this last MPLC-separation was purified by preparative HPLC (cf. materials and methods Table 5-3 p. 247) resulting in compounds **83**, **84** and **85** (cf. Fig. 3-26).

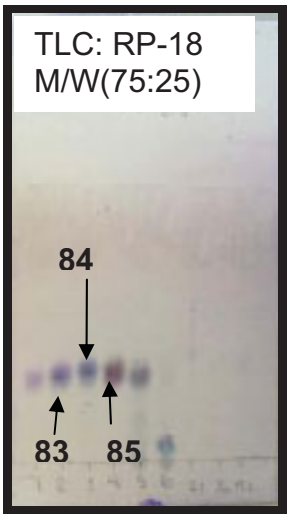
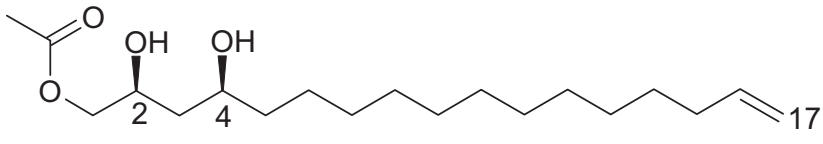


Figure 3-26. RP-18 TLC screening for purity control of fractions recovered by preparative HPLC separation of 8.1.9.3.7.

All of the compounds **60**, **61**, **83**, **84**, **85** have as common structural element a terminal vinyl-group (exomethylene group). A biosynthetic relation to the terminal alkynes (**58**, **81** and **82**) seems therefore to be likely. From the avocado seed material (var. *Hass*) we were able to isolate a variety of substances containing the rare structural element of a terminal double bond. Detailed structure elucidation will be given in the following chapter.

3.4.1.2.1. 1-Acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**60**)

Compound **60** (approx. 74 mg) was obtained as colourless oil (isolation procedure described on p.p. 247 and 248). The proposed structure for **60,** deduced from NMR- and MS-data, is the 1-acetoxy-2, 4-dihydroxy-*n*-heptadec-16-ene:



1-Acetoxy-2, 4-dihydroxy-*n*-heptadec-16-ene (**60**)

In principle, the only difference between compound **58** and **60** is the presence of a vinyl terminal group instead of an acetylenic terminal group. LC-ESI/MS analysis of **60** resulted in a retention time of 15.4 min while **58** had a much shorter R_t of 10.6 min (cf. conditions p. 251). The stronger affinity of the exo-methylene substance (**60**) to RP18 phase material compared to the terminal alkyne (**58**) is in correlation with a higher lipophilicity of **60** and longer retention in the liquid stationary phase during HSCCC-separation (later elution of exomethylene components). It seems to be that in this special case the HSCCC technique is an excellent tool for fractionation/ separation of substances on preparative scale having minimal structural differences (separation of olefins and alkynes).

The ESI-MS data (pos. mode) of **60** exhibited the pseudomolecular ions $[M+Na]^+$ at m/z 351 and a $[2M+Na]^+$ signal at m/z 679 (cf. Fig. 3-27) while the CI-MS (cf. Fig. 3-28) spectra showed the proton adduct at m/z 329 $[M+H]^+$. Following *'in-sito'*-silylation of **60** with MSTFA-reagent (cf. conditions p. 236), the ESI-MS (pos. mode) detected a *m/z* at 495 of $[M-2H+2Si(CH_3)_3+Na]^+$ (spectrum not shown here) caused by the up-take of two TMS-groups indicating the presence of two hydroxyl groups and confirming a molecular weight of Mr 328 amu.

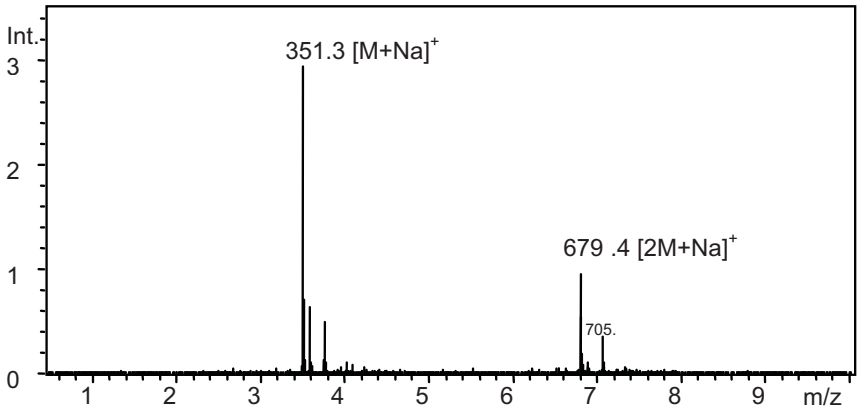


Figure 3-27. ESI-MS (positive mode) of **60** (Intensity: $x10^6$).

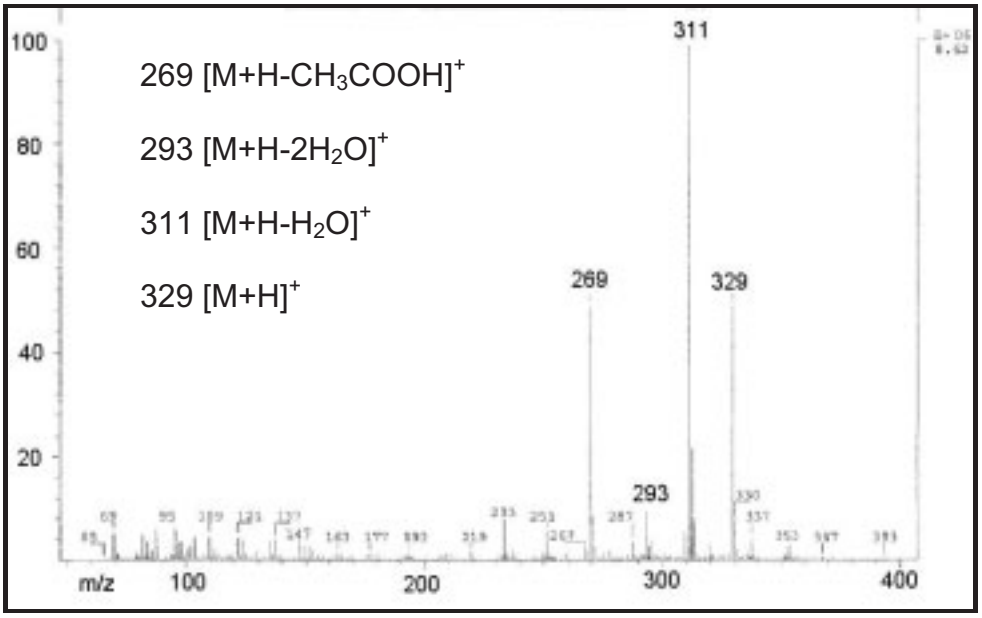


Figure 3-28. CI-MS (isobutane) of **60.**

The EI-MS (70eV) spectra showed characteristic ion fragments for a 1-acetoxy-2,4-dihydroxy-substitution at m/z 147, m/z 103 and *m/z* 237 (cf. Fig. 3-29). Values for the fragment signals at *m/z* 255 $[M\text{-}CH_3COOCH_2^{\cdot}]^+$ and *m/z* 211 $[M\text{-}CH_3COOCH_2CHOHCH_2]^+$ were two mass units higher than observed for the terminal-alkyne **58** assuring the terminal vinylic group as well as the presence of an acetate group in **60**. The mechanism for fragment formation was already described for compound **58** (cf. p. 65).

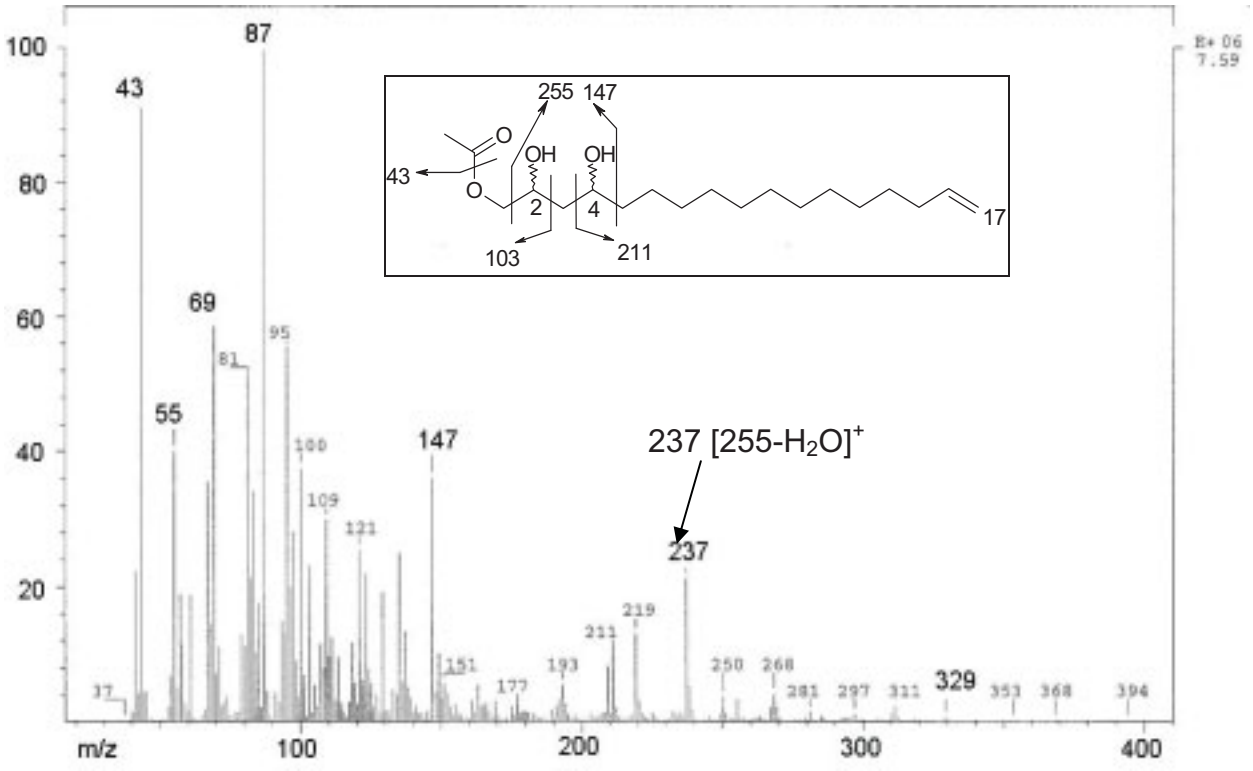


Figure 3-29. EI-MS (70eV) of **60.**

From the viewpoint of spectroscopy, the ^{1}H-NMR (300 MHz, $CDCl_3$) (cf. Fig. 3-30) of **60** had shown an interesting coupling scheme for the three olefinic protons of the exomethylene-group: H-16 resulted in a noticeable superposed *ddt*-signal at δ 5.80 ppm (1H, J_1=17, J_2=10, J_3= 6.5 Hz) caused by the couplings to the '*trans*' configured H-17b, to the '*cis*'-configured H-17a and to the protons of the methylene group -C<u>H</u>$_2$-15. The proton of H-17a and H-17b are magnetically not equivalent and occur at slightly different δ-values in the spectrum. The orientation of H-17a at δ 4.89 ppm (1H, *ddt*, J_1= 10, J_2=J_3=1.5 Hz) and of H-17b at δ 4.94 ppm (1H, *ddt*, J_1= 17, J_2= J_3= 1.5 Hz) in respect to H-16 can easily be distinguished by the '*cis*'- (10 Hz) and '*trans*'- (17 Hz) coupling constants (cf. Fig 3-30). The small coupling constants of J=1.5 Hz are caused by the geminal couplings of the two protons at C-17, and the allylic couplings to the protons of -C<u>H</u>$_2$-15. The resolution is depending on the used NMR-solvents, improvement for a better visibility of the small coupling constants was achieved by measurements in a mixture of $CDCl_3$/ CD_3OD 7: 3 (v/v).

^{1}H-NMR key-signals for immediate differentiation between avocado seed structures with terminal double bond or terminal alkyne are the typical

exomethylene couplings and the one-proton triplet resonance at δ 1.95 ppm (1H, H-17) of the triple bond substitution at C-16/C-17, respectively (cf. substance **58**, p. 63).

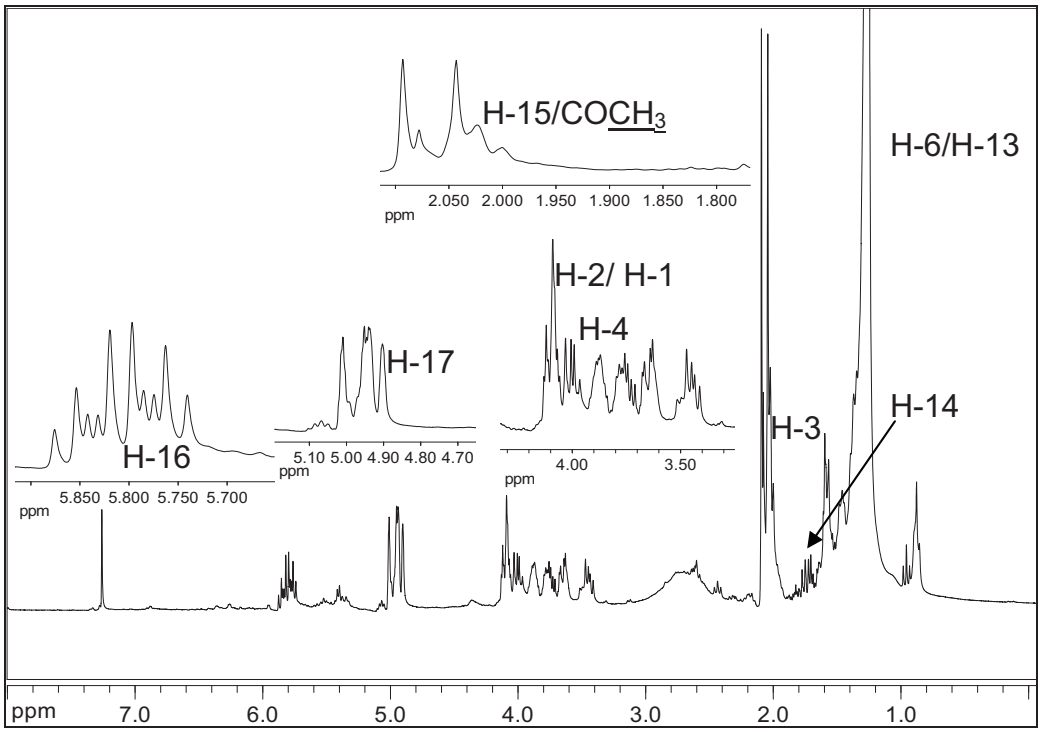


Figure 3-30. ^{1}H-NMR spectra of **60** (300 MHz, $CDCl_3$).

^{13}C- and DEPT135 NMR experiments revealed the presence of a long hydrocarbon chain (signals 25.5 to 34.12 ppm), three carbinols with chemical shifts at δ 68.88, 70.21 and 72.73 ppm, and a down-field shifted methyl group (δ 21.63 ppm) from an acetate group (δ 171.5 ppm). The characteristic exomethylene resonances were also present at δ 139.57 (CH) and δ 114.34 ppm (CH_2) (cf. Fig. 3-31). Except for the ^{13}C-signals of the chain end (C-16/C-17), all observed resonances are in good accordance to NMR-data of compound **58**.

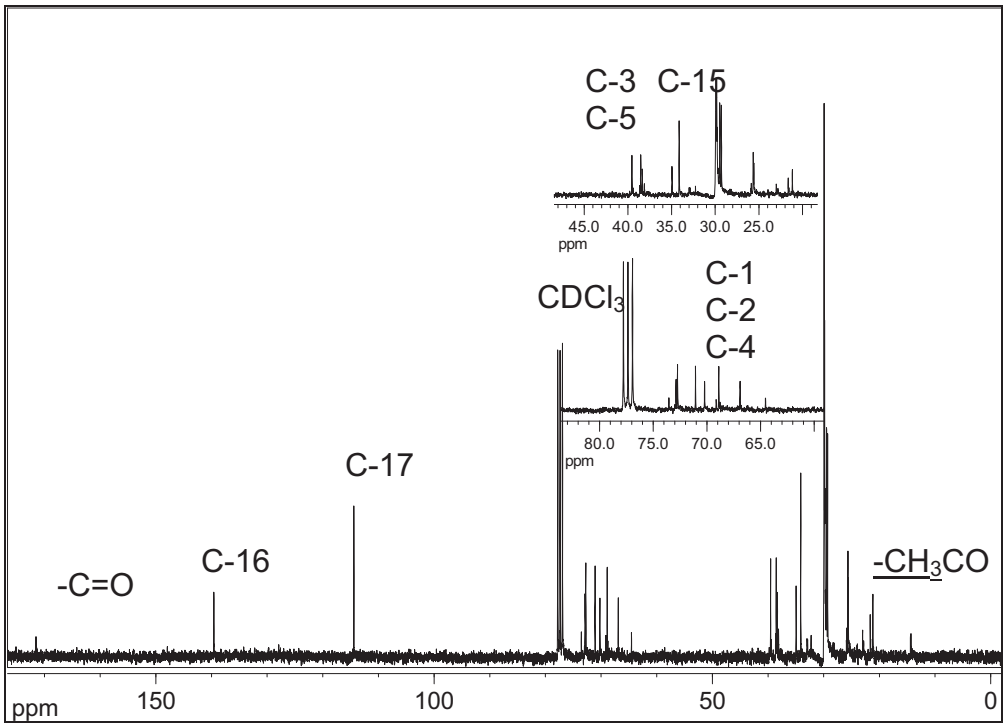
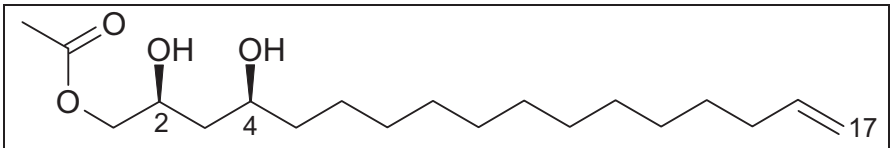
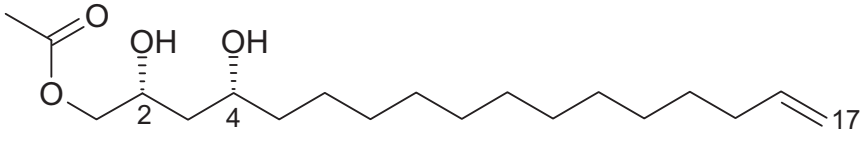


Figure 3-31. ^{13}C-NMR spectrum of **60** (75.5 MHz, $CDCl_3$).

Compound **60** had the same NMR data as the known substance 1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene identified in the oil from avocado fruit flesh (Kashman et al., 1969; Domergue et al., 2000) with an optical rotation of $[\alpha]_D$ -2.5. The chiral centers were reported by Domergue et al. (2000) to be (2*S*, 4*S*) for **60** and by Hashimura et al. (2001) to be (2*R*, 4*R*) for the enantiomer **61**.



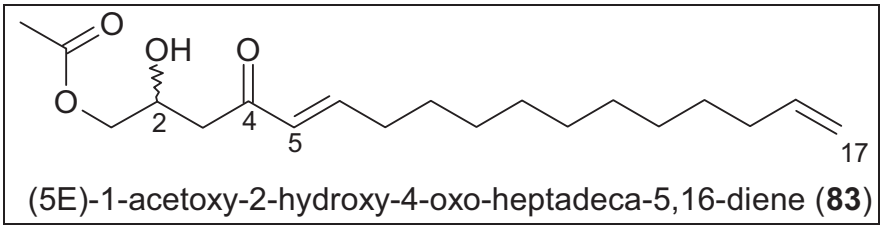
(2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**60**)



(2*R*,4*R*)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**61**)

3.4.1.2.2. (5*E*)-1-Acetoxy-2-hydroxy-4-oxo-heptadeca-5,16-diene (**83**).

2 mg of compound **83** were obtained as colorless oil (isolation procedure described on p. 247). ^{1}H-, ^{13}C- and DEPT135 NMR as well as MS-data guided us to the following structural proposal:



(5E)-1-acetoxy-2-hydroxy-4-oxo-heptadeca-5,16-diene (**83**)

The quasimolecular ion signal at m/*z* 347 $[M+Na]^+$ and the MS-generated dimeric adduct at m/*z* 671 $[2M+Na]^+$ in the positive (ESI-MS) suggested a molecular weight of Mr 324 amu (cf. Fig. 3-32). By comparing the molecular weights, we see for **83** a mass of 4 amu less than for substance **60** confirming the structural change to the C-4 oxo-function and the existence of an additional double bond in the chain.

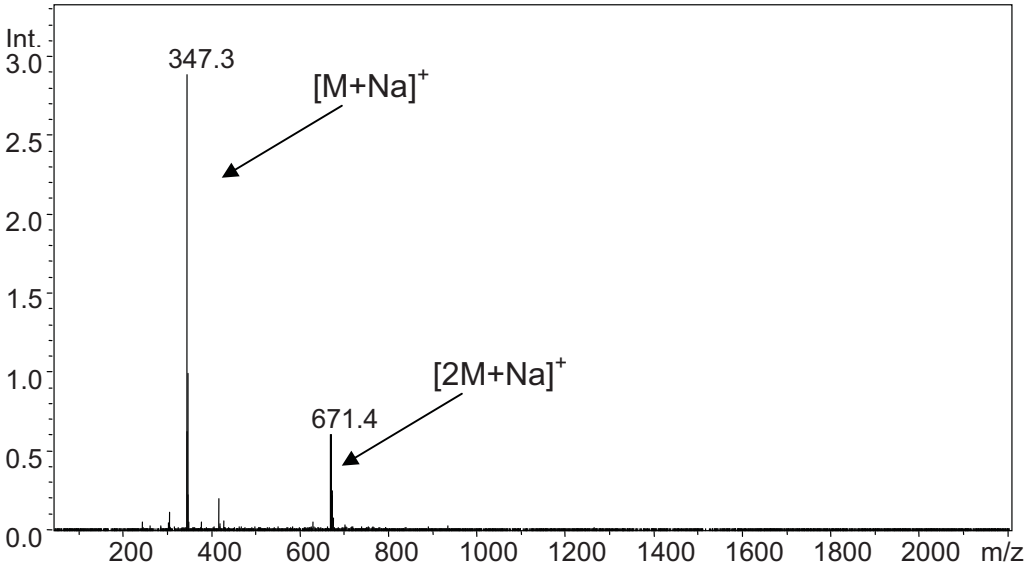


Figure 3-32. ESI-MS spectrum of **83** (positive mode, Intensity: x10^7).

The ^{1}H-NMR (cf. Fig. 3-33) detected for compound **83** the unique vinyl-resonances already described for **60**. In addition, signals of a newly observed double bond system occurred at δ 6.12 ppm (1H, *dt*, J_1=16, J_2=1.5 Hz, H-5) and δ 6.89 ppm (1H, *dt*, J_1= 16, J_2= 6.9 Hz, H-6). The defined coupling pattern and the significant down-field shifts indicated a α, β-unsaturated ketone partial structure, already observed for the terminal alkyne structure **82.** In this structure only two carbinol groups (δ 4.12 and δ 4.34 ppm) were present.

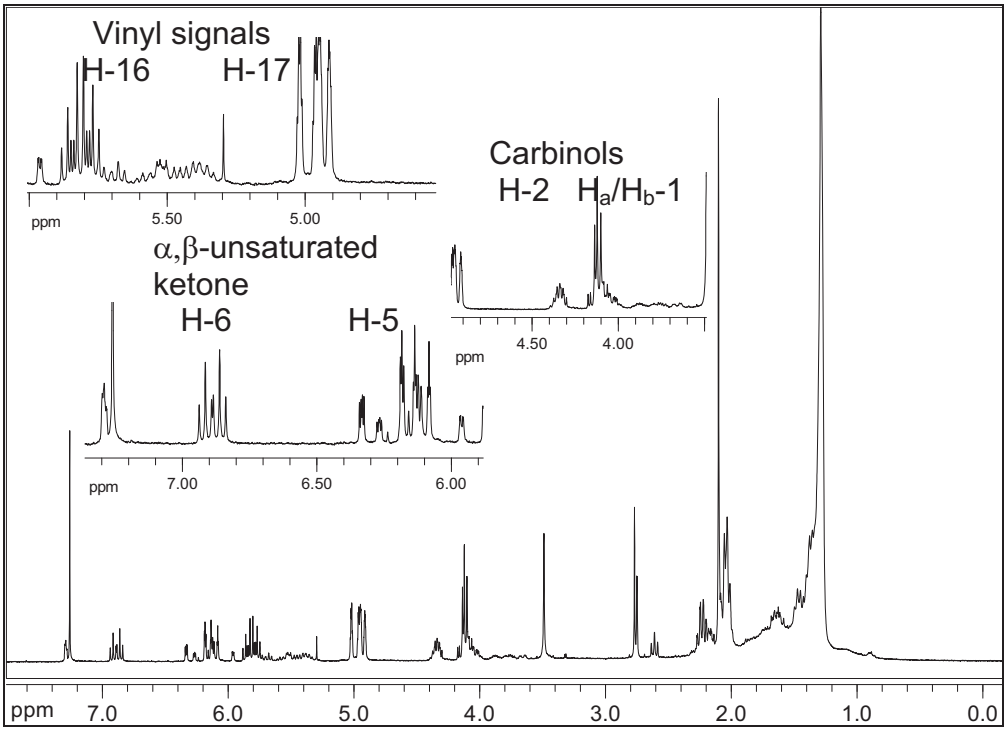


Figure 3-33. ^{1}H-NMR of **83** ($CDCl_3$, 300 MHz).

The ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) showed 19 carbon atoms (cf. Table 3-8, p. 101). Important signals for the identification of this substance were the terminal double bond at δ 114.45 and 139.57 ppm, the confirmation of only two carbinols groups (δ 68.86 and δ 70.35 ppm), the presence of a α,β–unsaturated keto-partial structure (δ 199.93 ppm, δ 130.70 ppm, and δ 149.77 ppm). The shifted resonance of the methylene C-3 (δ 40.14 ppm), C-7 (δ 32.99 ppm) and C-8 (δ 32.90 ppm) confirmed the presence of the keto-group at C-4. The correct assignments of the carbon nature of the detected ^{13}C-resonances were verified by a DEPT135 experiment.

(5*E*)-1-Acetoxy-2-hydroxy-4-oxo-heptadeca-5,16-diene (**83**) containing 19 C atoms is a structural analogue to persenone B (21 C atoms) (**62**) and persenone A (**64**) (23 C atoms) which were already isolated from the avocado fruit flesh (Kim et al., 2000b; Domergue et al., 2000). Substance **83** is the vinylic analogue of the acetylenic compound **82** (cf. p. 79).

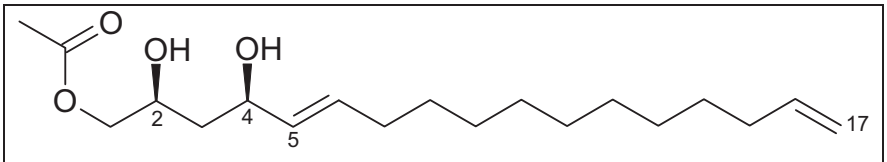
The stereochemistry at C-2 of persenone A (cf. formula p. 31) was determined to be *R* with a optical rotation value of $[\alpha]_D$ +11.7° (Domergue et al., 2000).

In this case, explicit determination of the absolute stereochemistry at C-2 in **83** is possible using the ^{1}H-NMR determination of the synthesized diasteromeric Mosher esters (Dale et al., 1969), but 2 mg sample amount were not sufficient to carry out the necessary two synthesis steps with S-MTPA and R-MTPA.

As result of our literature survey, (5*E*)-1-acetoxy-2-hydroxy-4-oxo-heptadeca-5, 16-diene (**83**) is a novel natural product. In correlation to the already known compounds from avocado materials (*Persea americana*), we named the structure **83** as persenone C.

3.4.1.2.3. (5*E*)-1-Acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**)

5 mg of (5*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**) were recovered as colorless oil from the petrol ether partition (8.1.) (Isolation procedure was described on p.p. 246 to 247). All assembled spectroscopic data (NMR and MS) guided us to the structural proposal of structure **84**:



(5*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**)

The LC-ESI/ MS spectrum has shown the quasimolecular ion at 349 *m/z* $[M+Na]^+$ and *m/z* 675 $[2M+Na]^+$ resulting in a Mr of 326 Da (cf. Fig. 3-34), two mass units more than observed for substance **83**.

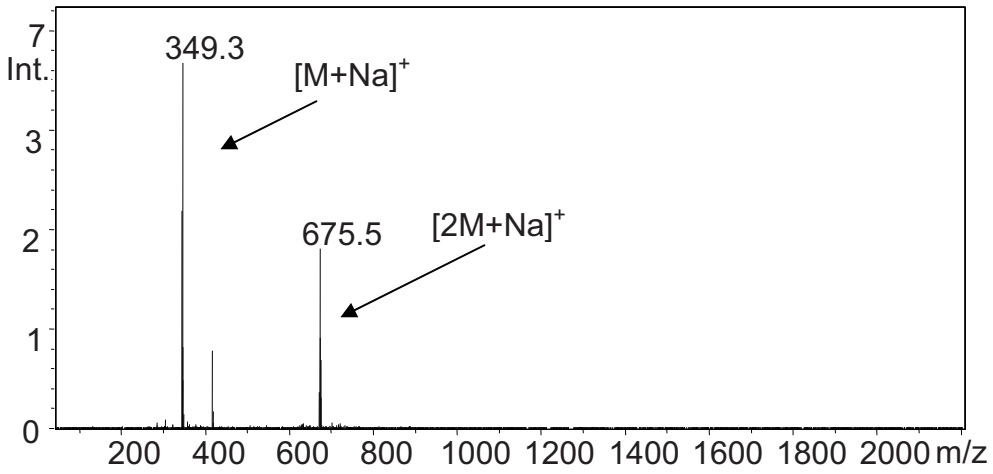


Figure 3-34. ESI-MS of compound **84** (positive mode. Intensity: x 10^7)

The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) of compound **84** (cf. Fig. 3-35) revealed new signals in the olefinic region of the spectrum. As major difference to the α,β-unsaturated keto-partial structure of **83** we observed signals at δ 5.47 ppm (1H, J= 17 Hz, H-5) and δ 5.74 ppm (1H, J= 17Hz, H-6) respectively, indicating a new 2,4-dihydroxy-5-ene moiety. The double bond signals of the allylic alcohol were not so strongly deshielded as recognized for the double bond in conjugation to the keto-function of **83** (δ 6.12 and 6.89 ppm, respectively). The presence of the typical exomethylene double bond signals and four complex carbinol signals were completing the basis for the structure proposal of **84**.

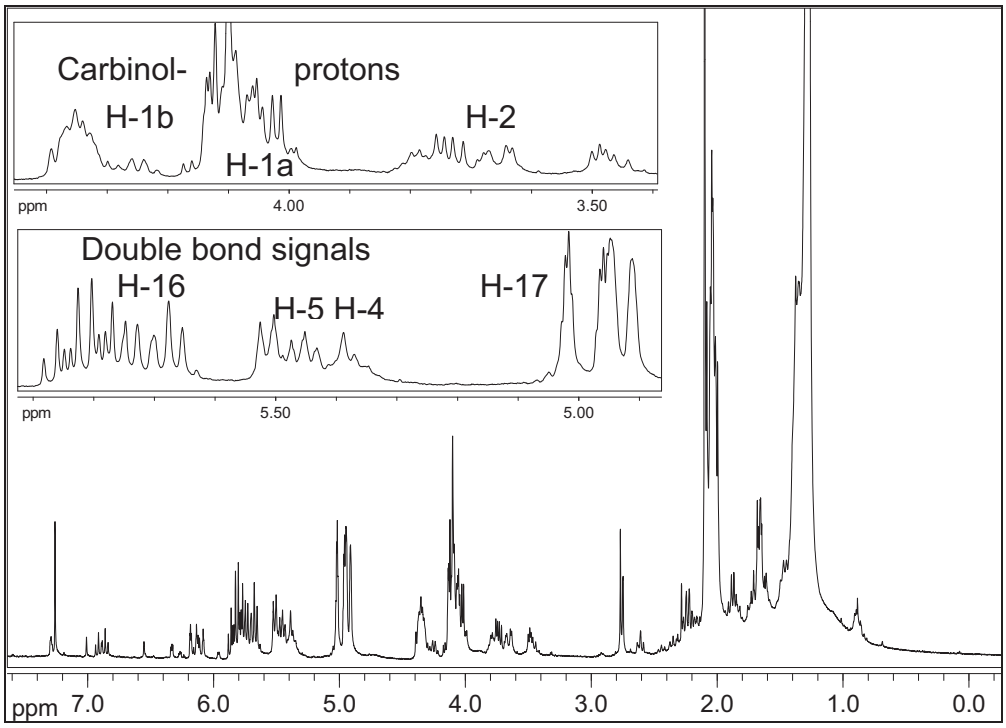


Figure 3-35. ^{1}H-NMR of compound **84** ($CDCl_3$, 300 MHz).

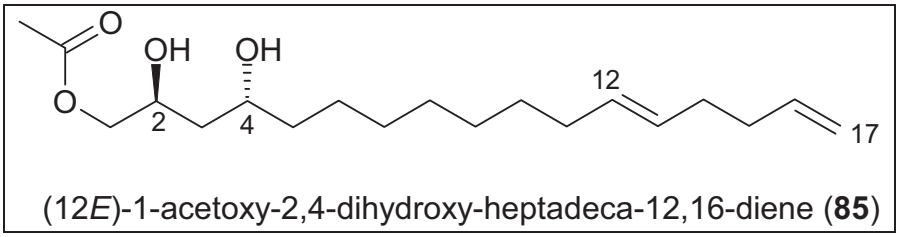
The ^{13}C-NMR and DEPT135 experiments ($CDCl_3$, 75.5 MHz) showed three carbinol groups at δ 68.87 (CH_2), 70.34 (CH) and 73.35 ppm (CH), the terminal vinyl group (δ 139.58 and 114.46 ppm), and the double bond carbons (δ 130.71 and 135.77 ppm). The location at C-5/ C-6 was confirmed by the significant change of chemical shifts of the adjacent carbons C-3, C-4 and C-7 (δ 40.15, 73.35 and 32.46, respectively) (cf. Table 3-8, p. 101). All data are demonstrating the novel 2,4-dihydroxy-5-ene partial structure in the avocado lipid (5*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**).

Comparing ^{1}H- and ^{13}C-NMR data of **84** with literature NMR-data, they are partly similar with persenone B (**63**) (Kim et al., 2000) and 1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**60**) having a (2S,4S)-configuration (Domergue et al., 2000). As the ^{13}C-NMR chemical shifts at C-2 and C-4 of **84** are very similar to **60** (data also in $CDCl_3$) we propose the absolute stereochemistry of **84** to be (2*S*, 4*S*).

To the best of our knowledge the (5*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**) is a new natural product isolated from avocado seeds.

3.4.1.2.4. (12*E*)-1-Acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**)

3 mg of (12*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**) were gained from petrol ether partition (8.1.) as colorless oil (isolation procedure described on p.p. 246 and 247). The proposed structure is:



(12*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**)

The ESI-MS spectrum (pos. mode) detected a quasimolecular ion at *m/z* 349 $[M+Na]^+$, resulting in a Mr of 326 Da, and showing the identical molecular weight as obtained for substance **84**.

The resonances detected in the ^{1}H-NMR spectrum of **85** (cf. Fig. 3-36) and of compound **79** (cf. Fig. 3-13, p. 71) were beside the determining areas for the exomethylene and the alkyne group almost identical. In consequence location of the double bond (C-12/C-13) in the chain and also stereochemistries of C-2 and C-4 (2*S*, 4*R*) are in good accordance with the findings for structure **79**. In the coupling scheme of H-12 and H-13 (5.55 and 5.40 ppm) we perceived a *dt* coupling pattern (J_1=17, J_2=6.5 Hz) indicating a '*trans*'-configuration of the double bond.

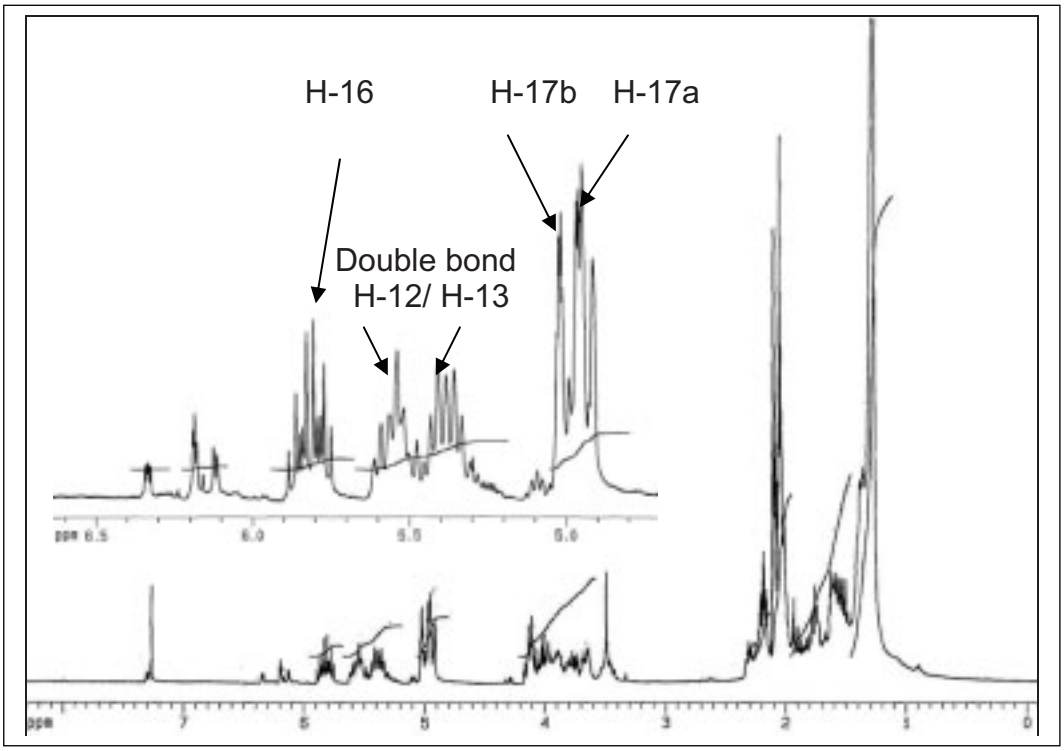


Figure 3-36. ^{1}H-NMR of compound **85** ($CDCl_3$, 300 MHz).

The ^{13}C-NMR spectrum of **85** ($CDCl_3$, 75.5 MHz) detected 19 carbon atoms: The only differences to compound **79** were positions C-15 to C-17 having the exomethylene group instead of the alkyne substitution (cf. Table 3-8, p. 101).

From the consistency of ^{13}C-NMR data of carbon resonances between the positions C-2 until C-5 we assumed that the stereochemistry of **85** is also a (2*S*, 4*S*)-configuration as published for substance **60** by Domergue et al. (2000).

The comprehensive literature survey in respect to (12*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**) showed that this avocado constituent is also not known as a natural product.

In Table 3-8 and 3-9, the δ-value chemical shift differences (^{1}H- and ^{13}C-NMR) of the avocado seed constituents with terminal double-bond are presented.

The listed order is starting from the simple to the more prominent shift differences in respect to the reference compound **60**.

Table 3-8. ^{13}C-NMR data of acetylenic compounds **60**, **60**[16], **83, 84,** and **85** from avocado seeds ($CDCl_3$, 75.5 MHz, δ [ppm]).

C-Atom	**60**[15, 16]	**60**[15]	**83**[13]	**84**[12]	**85**[12]
1	68.51	68.88	68.86	68.87	68.82
2	70.75	70.21	70.35	70.34	70.78
3	38.98	38.5	40.14	40.15	41.71
4	72.49	72.73	199.93	73.35	71.76
5	38.18	38.21	130.70	130.71	38.43
6	25.34	25.57	149.77	135.77	28.87
7	28.98	29.29	32.99	32.46	29.45
8	29.18	29.47	32.90	29.45	29.11
9	29.54	29.65	29.93	29.88	29.53
10	29.62	29.77	29.83	29.83	29.29
11	29.62	29.82	29.70	29.49	32.93
12	29.62	29.86	29.61	29.81	135.87
13	29.54	29.91	29.53	29.53	125.18
14	29.18	29.29	29.29	29.32	29.47
15	33.85	34.12	34.13	34.15	34.14
16	139.14	139.57	139.57	139.58	139.57
17	114.01	114.41	114.45	114.46	114.46
$\underline{CH_3}CO$	20.96	21.63	21.18	21.19	21.63
$CH_3\underline{CO}$	171.09	171.52	nd	171.46	171.4

Superscripts indicate the molecular weights of the avocado seed lipids: Mr: Mr: [15]328, [13]324, [12]326. [16]**60** = (2S,4S)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (Domergue et al., 2000). The ^{13}C-chemical shifts relevant for the differentiation of the components are underlined, nd= not detected.

Table 3-9. ^{1}H -NMR data of compounds **60**, **60**[16], **79**, **83**, **84**, **and 85** from avocado seeds ($CDCl_3$, 300 MHz, δ [ppm]).

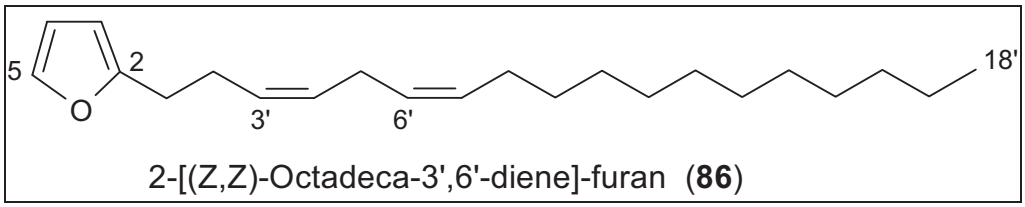
H-Atom	**60**[15,16]	**60**[15]	**79**[13]	**83**[13]	**84**[12]	**85**[12]
1a	4.11 *m*	4.08 *sbr*	4.08 *m*	4.12 *m*	4.12 *m*	4.12 *m*
1b	4.00 *m*	3.99 *dd*	3.78 *m*	4.12 ***m***	4.35 *m*	3.78 *m*
2	4.11 *m*	4.15 *m*	4.02 *m*	4.34 *m*	3.80 *m*	4.02 *m*
3	1.60 *m*	1.58 *m*	1.50 *dd*	2.77 *d*	1.60 *m*	1.58 *m*
4	3.89 *m*	3.90 *m*	3.65 *m*	-	5.35 *m*	3.90 *m*
5	1.47 *m*	1.37 *m*	1.28 *sbr*	6.12 *dt*	5.47 *m*	1.28 *m*
6	1.27 *m*	1.25 *m*	1.28 *sbr*	6.89 *dt*	5.74 *m*	1.28 *m*
7	1.27 *m*	1.25 m	1.28 *sbr*	2.23 *m*	1.30-1.35 *m*	1.28 *m*
8	1.27 *m*	1.25 *m*	1.28 *sbr*	1.64 *m*	1.30-1.35 *m*	1.28 *m*
9	1.27 *m*	1.25 *m*	1.28 *sbr*	1.28 *m*	1.30-1.35 *m*	1.28 *m*
10	1.27 *m*	1.25 *m*	1.28 *sbr*	1.28 *m*	1.30-1.35 *m*	1.28 *m*
11	1.27 *m*	1.25 *m*	1.28 *sbr*	1.28 *m*	1.30-1.35 *m*	1.28 *m*
12	1.27 *m*	1.25 *m*	5.28-5.60	1.28 *m*	1.30-1.35 *m*	5.55 *dt*
13	1.27 *m*	1.25 *m*	4.98-5,10	1.28 *m*	1.30-1.35 *m*	5.40 *dt*
14	1.37 *m*	1.73 *m*	1.28 *sbr*	1.28 *m*	1.30-1.35 *m*	1.28 *m*
15	2.04 *br q*	2.03 *t*	2.18 dt	2.03 *m*	2.03 *m*	2.05 *m*
16	5.82 *ddt*	5.80 *ddt*	-	5.81 *dq*	5.81 *m*	5.82 *dq*
17a	4.93 *ddt*	4.89 *ddt*	1.92 t	4.95 *m*	4.91-4.95 *m*	4.95 *m*
17b	4.99 *ddt*	4.94 *ddt*	-	5.01 *m*	4.91-4.95 *m*	5.02 *sbr*
CH_3CO	2.09 *s*	2.08 *s*	2.10 *s*	2.10 *s*	2.10 *s*	2.10 *s*

Superscripts indicate the molecular weights of the avocado seed lipids: Mr: [15]328, [13]324, [12]326. [16]**60** = (2S,4S)-1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (Domergue et al., 2000). The ^{1}H-NMR chemical shifts relevant for the differentiation of the components are underlined.

3.4.1.3. Furan compounds

3.4.1.3.1. 2-[(*Z*,*Z*)-Octadeca-3',6'-diene]-furan **(86)**

From the 8.1.9b.3 separation on RP-18-MPLC (cf. Table 5-3. p. 247) 3 mg of substance **86** were obtained as colorless oil. ^{1}H-, ^{13}C- and DEPT135 NMR as well as MS-data guided us to the structural proposal of 2-[(*Z*, *Z*)-octadeca-3',6'-diene]-furan (**86**).



2-[(Z,Z)-Octadeca-3',6'-diene]-furan (**86**)

LC-ESI-MS analysis (pos. mode) showed no signal and also addition of ammonium acetate (0.1M) to the HPLC-solvents was not able to ionize compound **86** under the tested MS-conditions (cf. materials and methods p. 259). Only CI-MS measurement (reactant gas: isobutane) (Fig. 3-37) yielded the quasimolecular proton adduct at *m/z* 317 $[M+H]^+$ indicating a molecular weight of 316 Da.

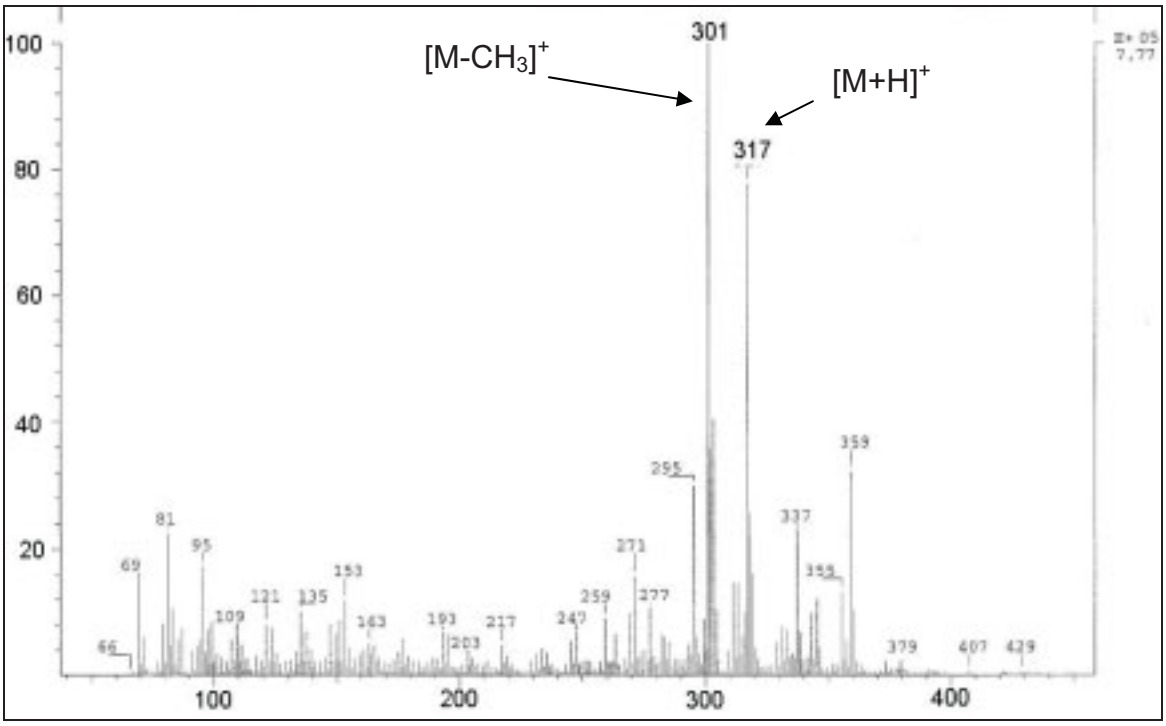


Figure 3-37. CI-MS (isobutane) of 2-[(*Z,Z*)-octadeca-3',6'-diene]-furan (**86**).

The EI-MS (70eV) of the alkyl-furan compound 2-[(*Z,Z*)-octadeca-3',6'-diene]-furan (**86**) resulted in a molecular ion signal *m/z* 316 $[M]^+$ of very low abundance. However the fragmentation pattern of **86** revealed relevant EI-MS cleavages of the carbon-chain and helped us to elucidate the constitution – including location of double bonds. Assuming charge stabilization in the hetero-aromatic system, the compound seemed to fragment from the terminal C-18 end in direction to the furanoic system (Fig. 3-38). The cleavage of a methylene group is clearly indicated by a $\Delta m/z$ 14 amu mass-difference between adjacent fragment ions. The double bond locations were recognized by strong allylic fragment ions: for C-3'/C-4' (*m/z* 81 and *m/z* 135) and C-6'/C-7' (*m/z* 121 and *m/z* 175), respectively.

The fragment ion at *m/z* 81 (base peak) is the cleavage in α-position of the furan nucleus. Signal *m/z* 95 was assigned to the ion of the $[\text{furanyl-CH}_2\text{-CH}_2]^+$ moiety (Farines et al., 1995). This result determined that one of the double bonds in **86** is located at C-3'/C-4' and not in β–position in respect to the oxygen function as reported for already known furan constituents from avocado fruits (Farines et al., 1995; Fraga and Terrero, 1996).

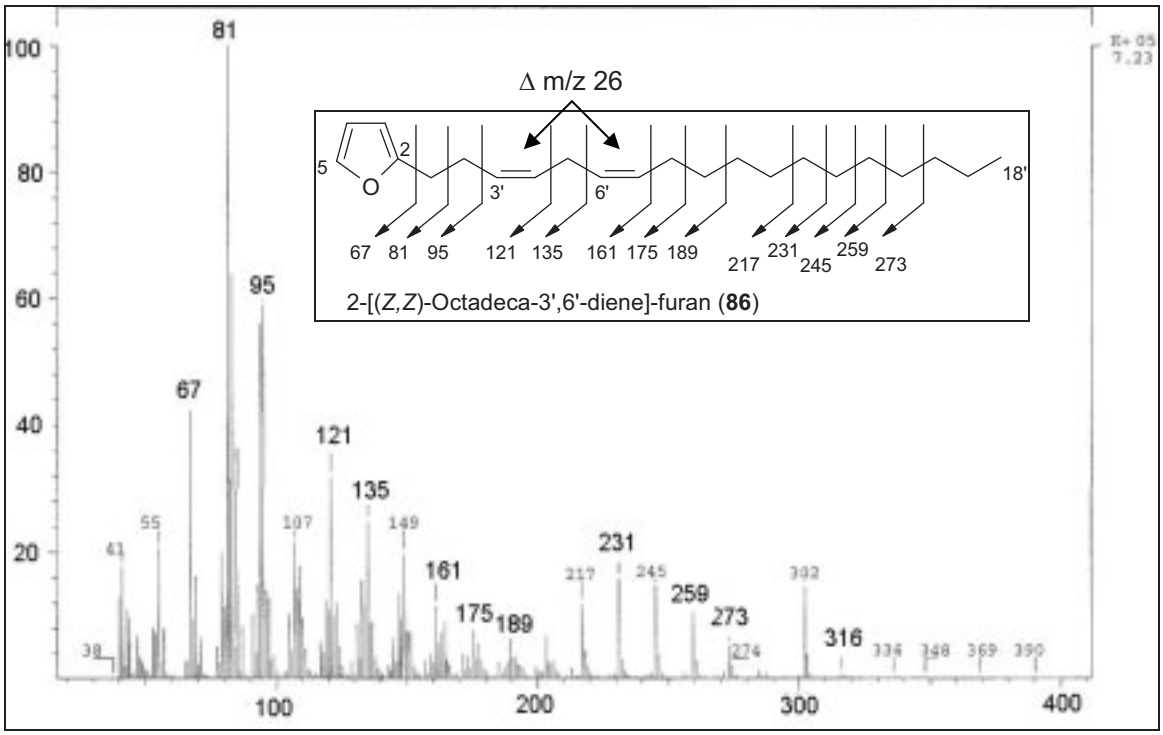


Figure 3-38. EI-MS (70eV) of compound **86** including structure relevant cleavage products.

The ^{1}H-NMR ($CDCl_3$, 300 MHz) (Fig. 3-39) showed the presence of three characteristic olefinic signals (δ 5.90, 6.28 and 7.28 ppm for H-3, H-4, and H-5, respectively) of a furan ring substituted in α-position to the oxygen function (Kashman et al., 1969; Farines et al., 1995; Fraga and Terrero, 1996). All protons of the furan ring displayed ^{1}H/^{1}H-coupling constants within the range of 1.0-1.5 Hz confirming a C-2 substitution (Fraga et al., 1996; Farines et al., 1995). The presence of a triplet resonance at δ 2.6 ppm (2H, *J*= 7.5 Hz) was assigned to the protons of C-1', the adjacent α–methylene group of the aromatic nucleus (Farines et al., 1995). The triplet resonance at δ 2.78 ppm (2H, *J*= 6.5 Hz) represented the methylene group (C-5') acting as bridge between both double bonds.

The superimposed multiplet signal at δ 5.35 ppm (4H) was clearly assigned to the pair of two double bond protons H-3'/ H-4', and H-6'/ H-7' all having a *cis*-configuration (coupling constant <12 Hz).

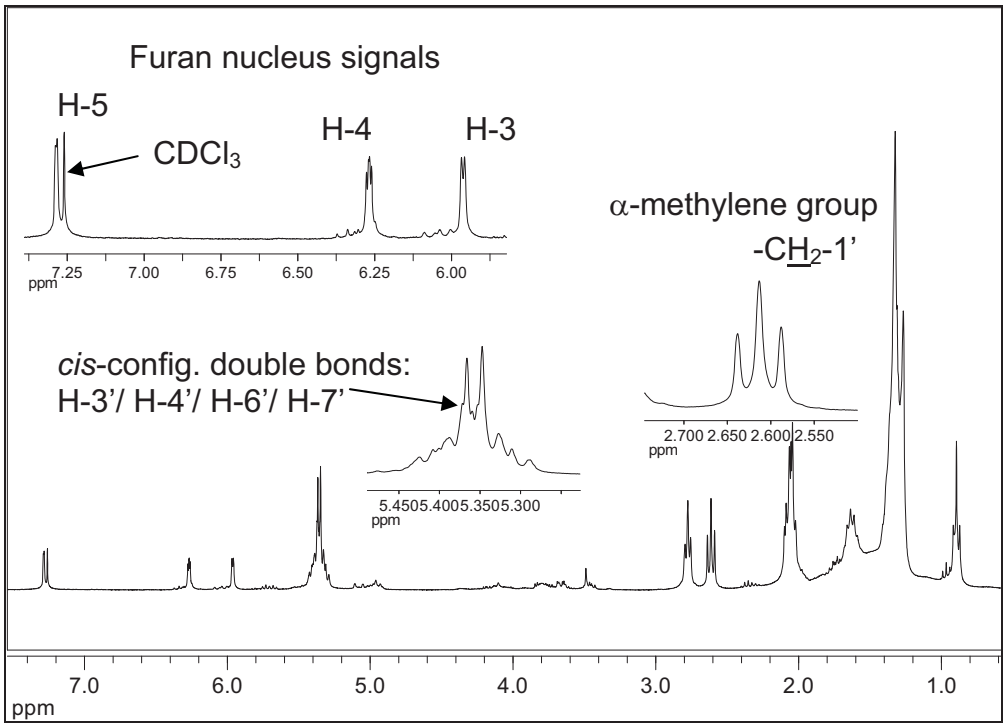


Figure 3-39. ^{1}H-NMR ($CDCl_3$, 300 MHz) of 2-[(*Z*,*Z*)-octadeca-3',6'-diene]-furan (**86**).

The ^{13}C-NMR spectrum of **86** (cf. Fig. 3-40) detected 21 carbon atoms including typical resonances of a furan nucleus (Fraga and Terrero, 1996) at δ 104.87 (C-4), 110.37 (C-3), and 140.97 ppm (C-5). Only C-2 that should appear at δ= 152 ppm was not detected. The existence of two isolated double bond systems in a long aliphatic chain - comparable to the constitution of linoleic acid – was confirmed by four ^{13}C-resonances (CH) appearing at δ 128.32 (C-3'), 130.47 (C-4'), 128.41 (C-6') and 130.58 ppm (C-7'). The characteristic signal at δ 26.02 ppm (C-5') was related to the methylene bridge (Oelrichs et al., 1995) connecting these two double bonds. As already shown by ^{1}H-NMR data, both double bonds were in a *cis*-configuration. As expected were the chemical shift values of the methylene C-atoms in α-positions located in the upper field of the spectrum at δ 26.02 (C-5'), and δ 27.58 (C-8'). In contrast, α-methylene C-resonances of a similar double bond system in '*trans'*-configuration would be visible by characteristic δ-values of approx. ~31 ppm (Hungerford et al., 1998). The signal at δ 14.38 ppm assured a terminal methyl group at C-18'. A DEPT 135 experiment verified the assigned carbon nature of the detected ^{13}C-resonances in **86**.

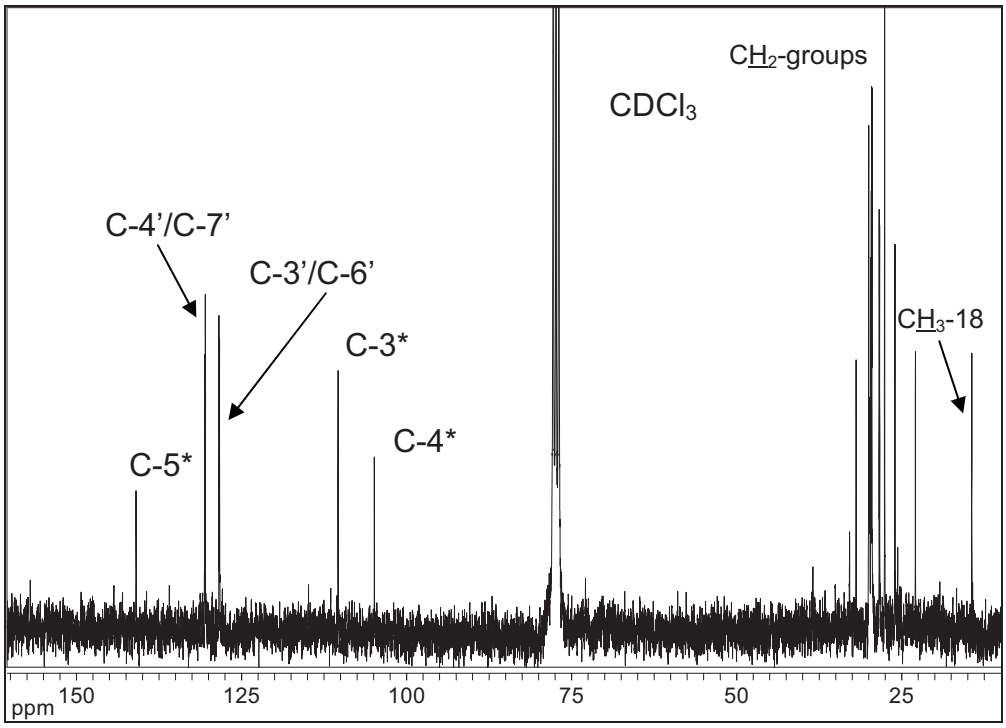


Figure 3-40. ^{13}C-NMR ($CDCl_3$, 75.5 MHz) of **86** (*furan signals).

The substance class of alkyl-furanoic components (*syn.:* avocado-furans) – so far solely recovered from avocado fruit flesh had shown significant insecticidal activity. Previous, structure-activity-relationship studies had shown that the furan ring and also the chain length play an important role in the observed toxicity against insects (Rodríguez-Saona et al., 1999; Rodríguez-Saona et al., 2000).

Various alkyl-furanoic compounds were recovered from *Persea indica* (fruit flesh) (Fraga and Terrero, 1996; Rodríguez-Saona et al., 2000), and also from *Persea americana* oil (Farines et al., 1995). Substances isolated from avocado seed material were named as “avocatins” (Kashman et al., 1969). Chain-length of all reported furanoic substances was between 13 and 18 carbon atoms.

For details about “avocatin” compounds and their differing substitution pattern, see general chapter pages 27 to 32.

To the best of our knowledge the alkyl-furan substance 2-[(*Z, Z*)-octadeca-3',6'-diene]-furan (**86**) is a new natural product isolated from avocado seed material (var. *Hass*).

3.4.1.4. Trihydroxylated lipids from avocado seeds

The 8.1.10 fraction (cf. Table 5-3 p. 248) (312 mg) recovered from the first SiO_2-MPLC fractionation of the avocado-seed petrol ether partition (cf. Separation 26, p. 246) was further separated by a 'step-gradient' RP-18 MPLC procedure (cf. Table 5-3 separation 35, p. 248). As chromatographic result (fractions 8.1.10.1 to 8.1.10.10.) some of the newly recovered fractions (cf. Fig. 3-41) already contained pure components: (2*R*,4*S*)-1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**78**) (cf. acetylenic compounds p.p. 67) and 1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**60**) (vinylic compounds p.p. 88) and **54**, **87**, and **88** that will be described here as the trihydroxylated structures from the lipophilic extracts of avocado seeds.

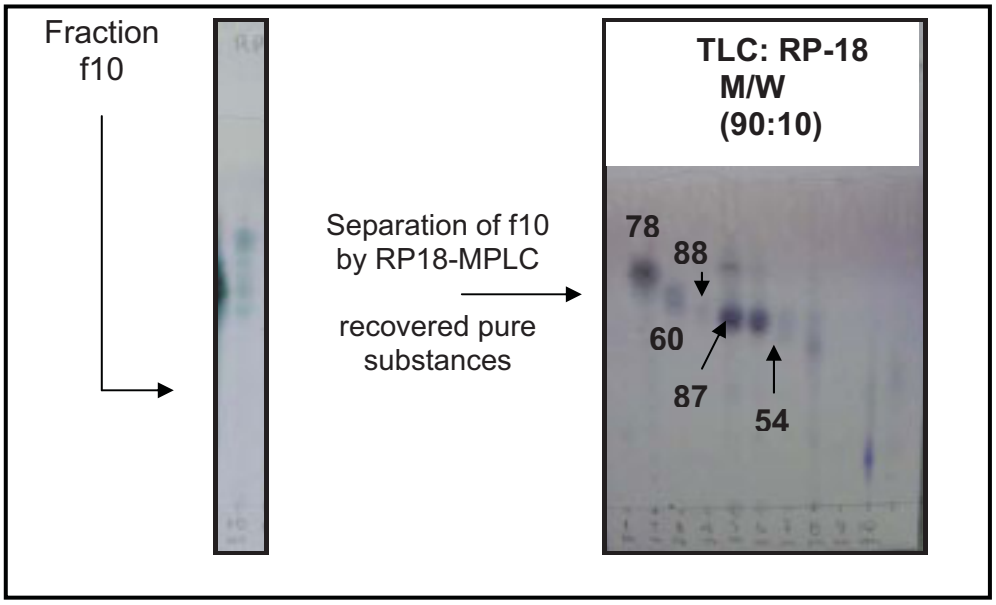


Figure 3-41 Fractions and pure substances (**78**, **60, 54**, **87**, and **88**) obtained after RP18-MPLC separation of fraction **f10** (8.1.10.) (cf. p. 248). TLC: RP-18 (M/ W = 90:10 v:v).

The LC-ESI-MS analysis of fraction 8.1.10. (cf. Fig. 3-42) detected signals of the trihydroxy-compounds later recovered in pure form by RP18-MPLC. Compound **54** showed a quasimolecular ion signal $[M+Na]^+$ at *m/z* 339 at a retention time of 20.5 minutes. The signals for the substances **87** and **88** were both sodium adducts $[M+Na]^+$ at *m/z* 337 with retention times of 18.9 min and 17.8 min, respectively. Compound **54** is saturated and seemed to be more lipophilic than the structures containing one double bond in the structure.

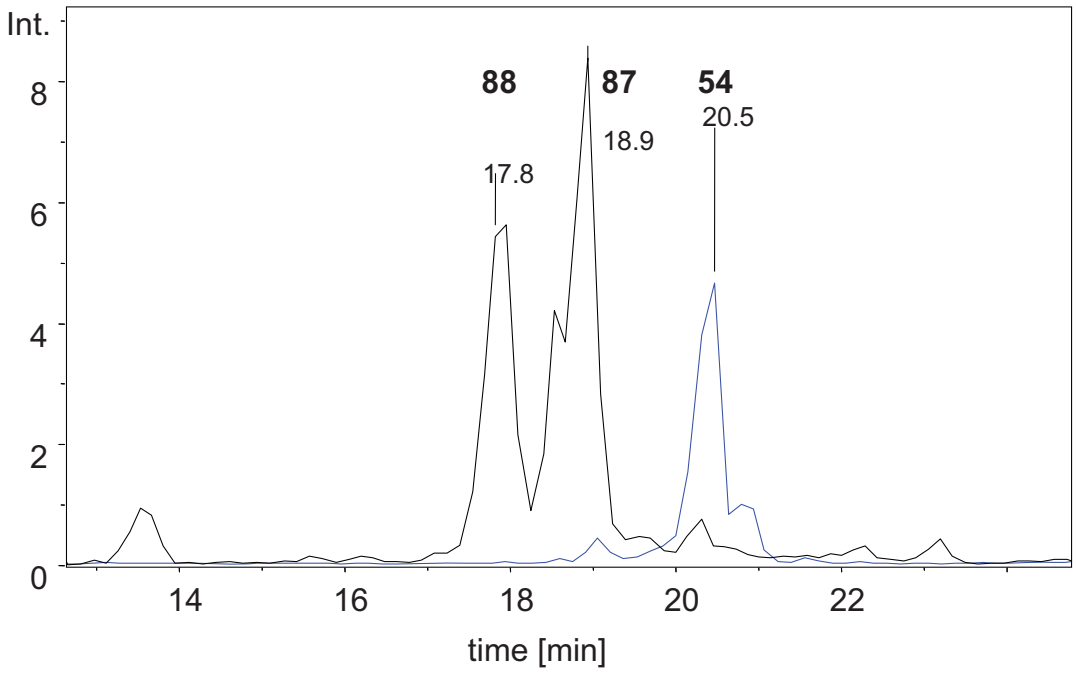
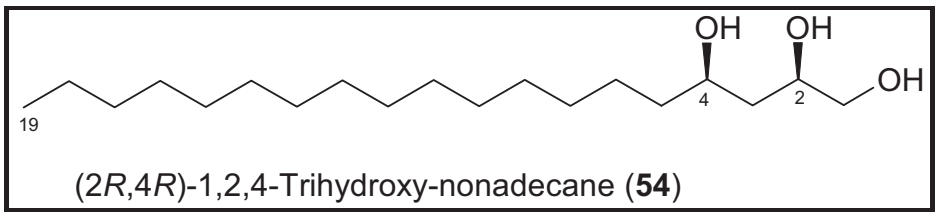


Figure 3-42 LC-ESI/ MS analysis of fraction 8.1.10. (cf. p. 248) detecting trihydroxylated substances **54** ([M+Na]$^+$, *m/z* 339), **87** ([M+Na]$^+$, *m/z* 337), and **88**, ([M+Na]$^+$, *m/z* 337) [column: ODS-Beckman 4.6 x 250 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH), gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %), flow rate 0.8 mL min^{-1}. Intensity= x 10^7].

3.4.1.4.1 (2*R*, 4*R*)-1, 2, 4-Trihydroxy-nonadecane (**54**)

21 mg of white crystals of (2*R*, 4*R*)-1, 2, 4-trihydroxy-nonadecane (**54**) were recovered from fraction 8.1.10. as described above. The structural assignment of **54** was deduced from ^{1}H-, ^{13}C-, DEPT135 and 2D-NMR data (HMQC, HMBC) and the molecular weight determination by ESI-MS (pos. mode).



(2*R*,4*R*)-1,2,4-Trihydroxy-nonadecane (**54**)

The ESI-MS spectrum of the purified substance **54** (cf. Fig. 3-43) showed two ions of pseudomolecular sodium adducts at *m/z* 339 [M+Na]$^+$ and [2M+Na]$^+$ at *m/z* 655 leading to the molecular weight Mr of 316 Da.

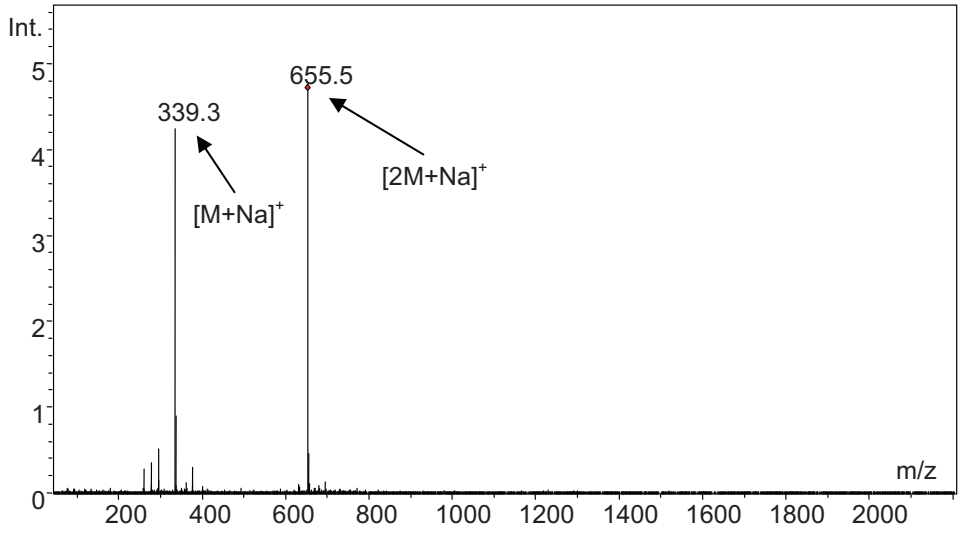


Figure 3-43 ESI-LC/ MS (pos. mode) of compound **54** (gradient LC-ESI/ MS details cf. materials and methods p.p. 259) (Intensity x 10^7).

The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) (cf. Fig. 3-44) of **54** revealed the presence of one methyl group (δ 0.88, t, 3H), a long aliphatic hydrocarbon chain (δ 1.20 -1.40 m), and three carbinol groups in the molecule (δ 3.49, 3.63, 3.92, and 4.15 ppm). The chemical shifts at δ 3.49 and 3.63 ppm were of the primary alcohol function at C-1 giving two distinct NMR-signals due to their magnetic inequality caused by the chiral environment of C-2. The protons H-1a and H-1b showed a typical, large geminal coupling constant (*J*=10, 15Hz). The couplings to the most downfield shifted broad carbinol signal H-2 (δ 4.15 ppm, m, 1H) were confirmed by cross-peaks observed in a ^{1}H/^{1}H-COSY experiment.

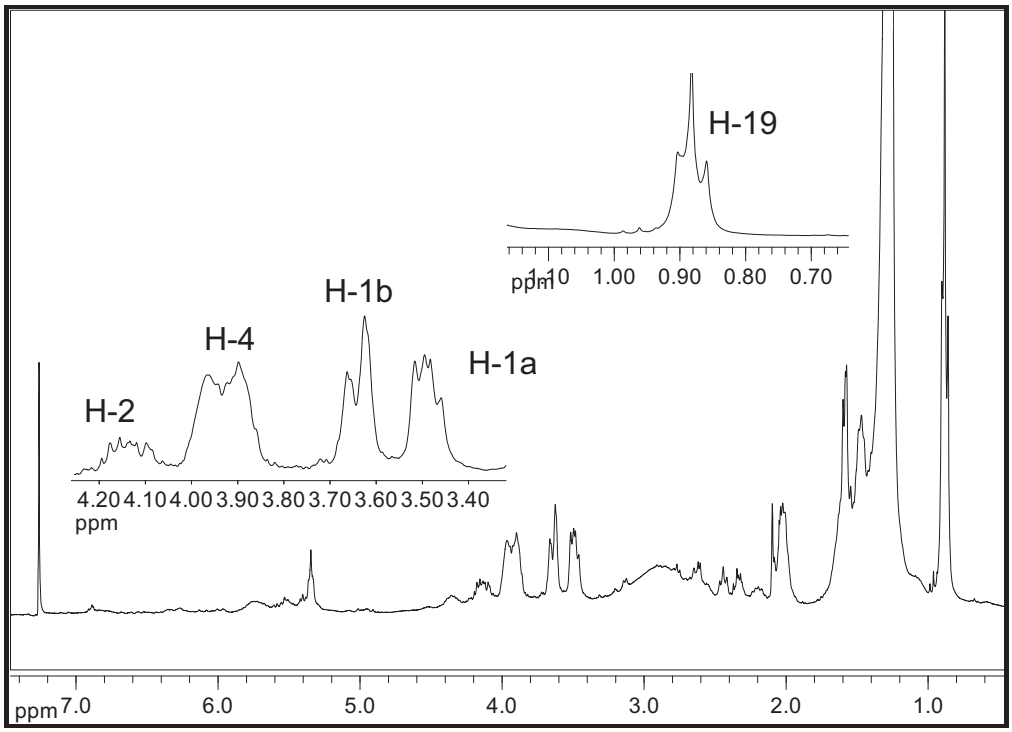


Figure 3-44 ^{1}H-NMR spectrum of compound **54** ($CDCl_3$, 300 MHz).

The ^{13}C-NMR spectrum ($CDCl_3$, 75.5 MHz) of **54** showed some overlapped signals in the methylene region. Signals including three carbinols C-1, C-2 and C-4 at δ 67.19 (CH_2), 72.97 (CH) and 72.79 ppm (CH), respectively (cf. Fig. 3-45). Confirmation of the carbon nature of the terminal CH_3 (δ 14.42 ppm) and all methylene groups (δ 23.03 to 39.45 ppm) of the aliphatic chain was achieved by a DEPT 135 experiment. This experiment also verified that no quarternary signals were present in structure **54**.

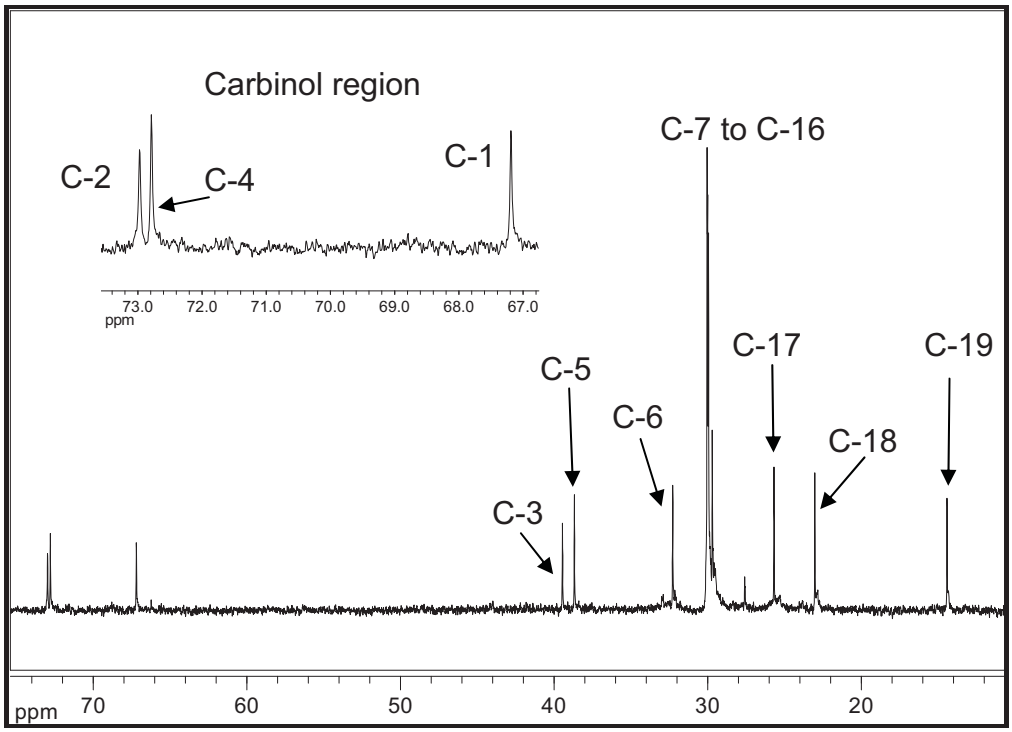


Figure 3-45 ^{13}C-NMR spectrum of compound **54** ($CDCl_3$, 75.5 MHz).

Structure relevant proton resonances were clearly assigned to the corresponding carbon atoms according to the observed cross-signals in the ^1J HC-correlation (HMQC) (cf. Fig. 3-46). Confirmation of proton couplings was done by a ^{1}H-^{1}H-COSY experiment (data not shown).

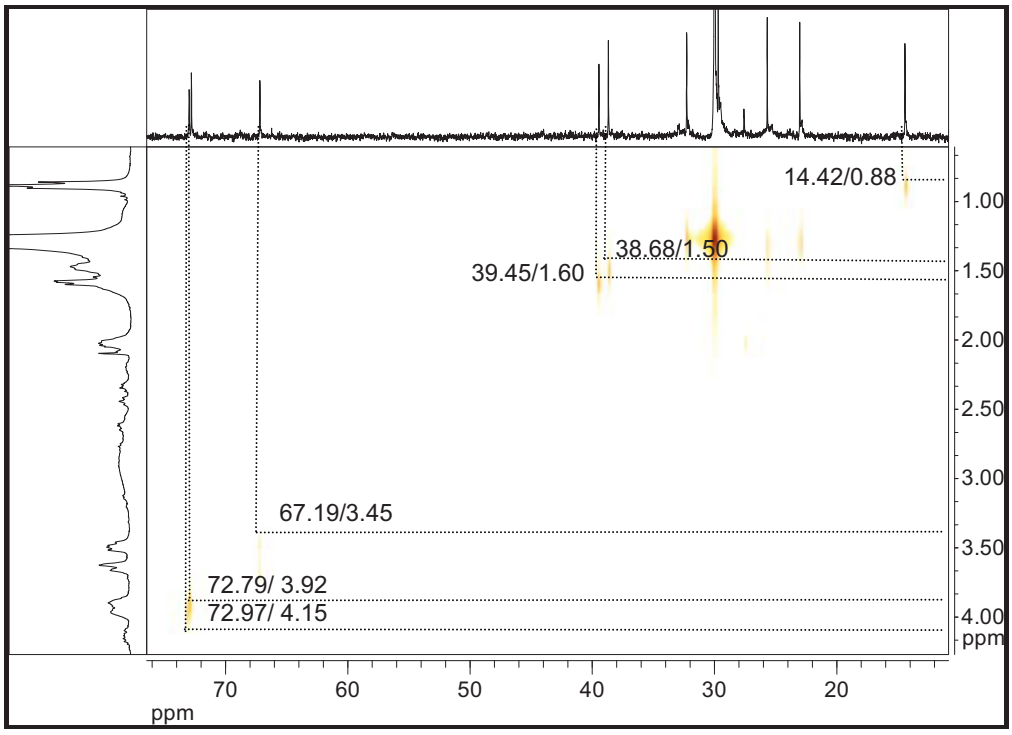
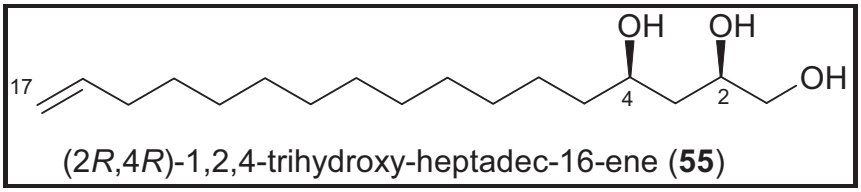


Figure 3-46 ^{1}J-HC correlation (HMQC) of compound **54** ($CDCl_3$, 300 MHz).

For substance 1, 2, 4-trihydroxy-nonadecane (**54**) possessing two chiral carbinol centers (C-2 and C-4), two possible enantiomeric pairs might be present. Oberlies et al. (1998) performed for 1,2,4-trihydroxy-heptadec-16-ene (**55**) the intramolecular ring-closure using the reagent formaldehyde and clarified the relative stereochemistry of C-2 and C-4 to be in a *cis*-configuration. Oberlies et al. recovered **55** from avocado fruit flesh. It was also found before by Adikaram et al. (1992) in the peel of immature avocado fruits.

Much earlier, Sugiyama et al. (1982) elucidated the absolute stereochemistry to be a (2*R*,4*R*)-configuration for the structure 1, 2, 4-trihydroxy-heptadec-16-ene (**55**) (by chemical synthesis of all possible stereoisomers). In accordance to the principal NMR-data of the carbinol region published for **55**, the observed chemical shifts in the ^{1}H- and ^{13}C-NMR of **54** suggested also a (2*R*,4*R*)-configuration.



(2*R*,4*R*)-1,2,4-trihydroxy-heptadec-16-ene (**55**)

In this study, (2*R*, 4*R*)-1, 2, 4-trihydroxy-nonadecane (**54**) was isolated for the first time from avocado seeds (*P. americana,* var. *Hass*). Before **54** had been identified only as constituent from avocado fruit flesh (var. *Lulu*). All spectroscopic assignments of **54** were in excellent agreement with the reference data of Oberlies et al. (1998).

Substance **54** was tested against six human tumor cell lines [lung carcinoma (A-549), mammary adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), kidney carcinoma (A-498), pancreatic carcinoma (PaCa-2) and prostate adenocarcinoma (PC-3)] showing moderate *'in-vitro'* activity and also demonstrated selectivity against the human prostate carcinoma (PC-3) cells (Oberlies et al., 1998).

3.4.1.4.2. (5*E*)-1, 2, 4-Trihydroxy-5-nonadecene (**87**) and

3.4.1.4.3. (5Z)-1, 2, 4-Trihydroxy-5 -nonadecene (**88**)

The RP18-MPLC separation (cf. Table 5-3 separation 35 p. 248) of fraction f10 (8.1.10) resulted in the pure compounds **87** (20 mg) and **88** (7 mg). Both compounds were obtained as white crystals. Interestingly, they were detected in the LC-ESI-MS analysis of f10 (cf. Fig. 3-47) and in purified form by direct syringe pump ESI-MS measurement with an identical quasimolecular sodium ion signal at m/z 337 $[M+Na]^+$ and at *m/z* 651 $[2M+Na]^+$, only differing in the HPLC retention time values. With a resulting molecular weight of Mr 314 Da, we suggested that the only constitutional difference of **87** and **88** could be the existence of one additional double bond located in the hydrocarbon chain in comparison to the completely saturated substance **54**.

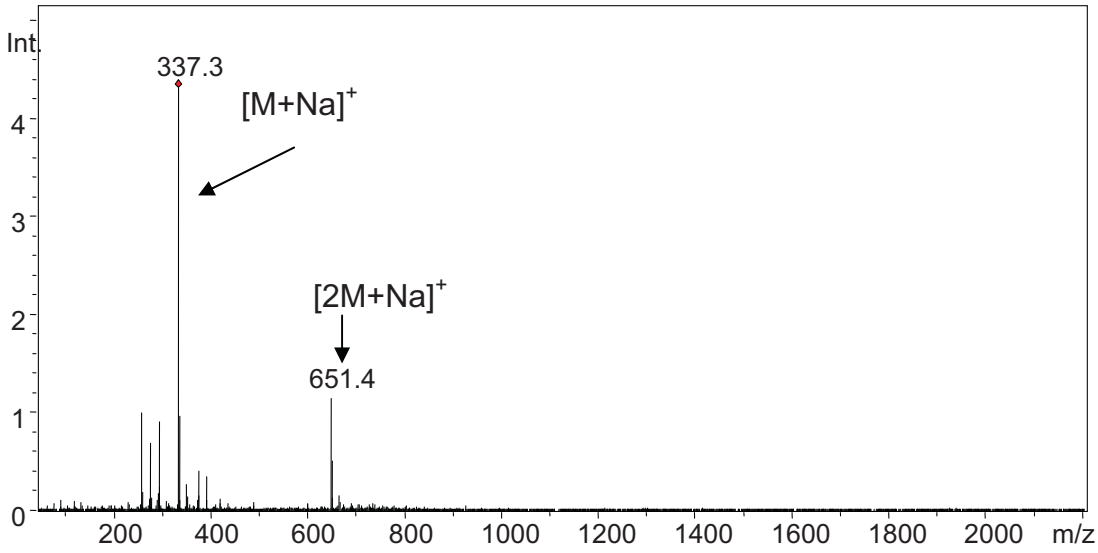
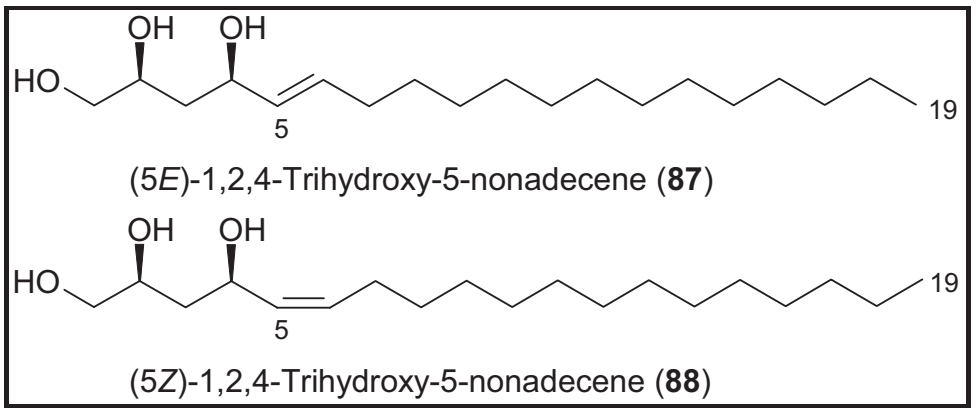


Figure 3-47 ESI-MS of compounds **87** and **88**. (Int. x 10^7)

The proposed chemical structures for both compounds are:



^{1}H-NMR ($CDCl_3$, 300 MHz), of both compounds **87** and **88** showed almost the same signals. Comparing the ^{1}H-NMR data of compound **54** with the ppm-values of **87** and **88**, additional resonances occurred at δ 2.00 - 2.20 ppm which were assigned as methylene-protons located in α-position to a double bond. Furthermore the olefinic regions of the ^{1}H-NMR (cf. Table 3-11) showed at lower field the double bond proton signals at δ 5.40 ppm in **87** (*J*= 15 Hz) demonstrating a *trans*-configured double bond. In the case of substance **88**, a *cis*-configured olefinic bond (*J*= 10 Hz) was recognized.

For a better spectroscopic resolution, compound **87** was re-measured in a mixture of $CDCl_3$:CD_3OD (7:3, v/v) with a 600 MHz-spectrometer resulting in better

resolved proton signals (Table 3-11). The use of this solvent mixture is helping to break possible micelle formation of the amphiphilic compounds normally causing strong signal broadening in the ^{1}H-NMR. Proton connectivities in structure **87** were assigned by a ^{1}H/^{1}H-COSY (spectrum not shown here).

The ^{13}C-NMR spectra ($CDCl_3$, 75.5 MHz) for both compounds (**87** and **88**) showed significant differences (cf. Table 3-10). The chemical shift values at δ 129.77 and 132.25 ppm for **88** were indicating a non symmetrically substituted *cis* double bond, while the chemical shifts for a *trans* configuration are occurring at ppm-values higher than δ 133 ppm. This was observed for the olefinic resonances in **87** (135.79 ppm) indicating a *trans*-configuration.

The position of the double bond in **87** was confirmed by a $^{2,3}J$ HC-long-correlation experiment (cf. Fig. 3.48) to be located at position C-5.

For elucidation of the exact location of the double bond in **88**, it will be highly important to measure an EI-MS and HMBC.

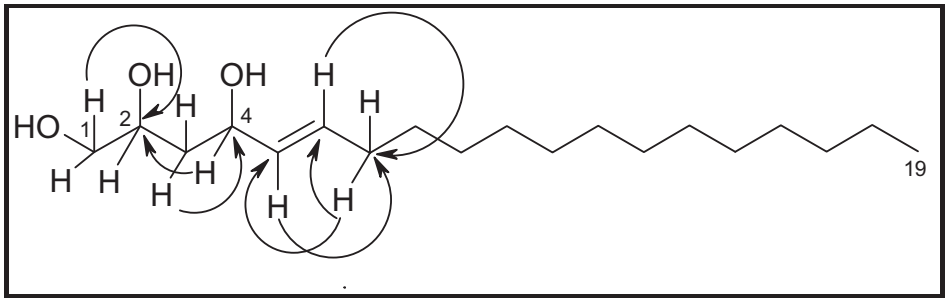


Figure 3-48 Important HC-long-range correlations in the HMBC of **87** identified the 1,2,4-trihydroxy-partial structure and double bond position at C-5/ C-6.

For compound **55** isolated from unripe avocado fruit, a moderate activity and selectivity against the human prostate carcinoma cell-line PC-3 (Oberlies et al., 1998) was detected. The same substance from the avocado seeds had also shown antifungal (Adikaram et al., 1992) and antibacterial (Sugiyama et al., 1982) activities. For the structurally analogous components **54**, **87**, and **88** from avocado seeds, similar biological activities can be expected.

To the best of our knowledge, the avocado lipids **87** and **88** are new natural products isolated from the seed material of *Persea americana* (c.v. Hass).

Investigations regarding the cytotoxic activity of different avocado seed lipid fractions i.e. against the salt water shrimp *Artemia salina* L. (brine shrimp-assay) will be described later in the chapter on evaluation biological activities (cf. p. 202).

All structures isolated from avocado seed material do have amphiphilic substitution pattern, e.g. a trihydroxylated polar 'head' and a lipophilic end ('tail') observed for **54**, **87**, and **88**. Hence, the avocado lipids are acting as typical emulsifiers and might potentially be able to penetrate or incorporate membranes.

Most of the known avocado lipids seem to be products of a very typical biosynthetical pathway generating structures with hydrocarbon-backbones consisting of an uneven number of C-atoms. Several pathways were already proposed and will be discussed in more detail in the biosynthesis-chapter (cf. p. 208).

Table 3-10. ^{13}C-NMR comparison data of **54**, **87** and **88** from avocado seeds ($CDCl_3$, 75.5 MHz, δ= [ppm])

Position	**55**[f]	**54**[g]	**87**[h]	**88**[h]
1	66.82	67.19	67.10	67.19
2	72.62	72.97	72.73	72.93
3	38.99	39.45	39.42	39.48
4	72.55	72.79	71.67	72.85
5	38.34	38.68	125.25	129.77
6	29.59 to 25.31	32.28	135.79	132.25
7	29.59 to 25.31	29.95	32.26	32.28
8	29.59 to 25.31	29.70	29.69	
9	29.59 to 25.31	30.00	29.53	29.93
10	29.59 to 25.31	29.84	29.97	
11	29.59 to 25.31	29.59	29.56	29.54
12	29.59 to 25.31	29.87	29.87	32.91
13	29.59 to 25.31	29.84	29.84	
14	29.59 to 25.31	30.00	30.02	32.28
15	33.83	27.59	27.57	
16	139.27	29.69	29.79	29.70
17	114.09	25.68	25.70	25.94
18	-	23.03	22.90	25.67
19	-	14.42	14.33	14.35

Superscripts indicate the molecular weights [Da]: Mr [f] 286 ((2*R*,4*R*)-1,2,4-trihydroxy-heptadec-16-ene by Oberlies et al., 1998), [g] 316, and [h] 314. The ^{13}C-chemical shifts relevant for the differentiation of the components are underlined.

Table 3-11. ^{1}H -NMR-comparison data of **54**, **87** and **88** from avocado seeds ($CDCl_3$, 300 MHz, for **87** measured at 600 MHz using $CDCl_3$:CD_3OD (7:3), δ= [ppm]).

Position	**55**[f]	**54**[g]	**87**[h]	**88**[h]
1a	3.428	3.49 *dd*	3.46 *dd*	3.50 *dd*
1b	3.620	3.63 *dd*	3.54 *dd*	3.65 *dd*
2	3.964	4.15 *m*	3.86 *m*	3.93 *m*
3	1.560	1.60 *m*	1.64 *m*	1.28-1.50 *m*
4	3.964	3.92 *m*	3.81 *m*	4.12 *m*
5	1.560	1.50 *m*	5.50 *m* (*J*=7, 15 Hz)	5.42 *dd* (*J*=5.3,10.3 Hz)
6	1.270 s*br* (H-6 to H-14)	1.20-1.40 *sbr* (H-6 to H-18)	5.40 *m* (*J*= 15 Hz)	4.36 *m*
7			2.18 *dt*	2.0 *m*
8			1.27-1.50 *m* (H-8 to H-18)	1.28-1.60 *m* (H-8 to H-18)
15	2.010	2.0 *m*		
16	5.82			
17_{cis}	4.93			
17_{trans}	4.99			
18	-	1.20-1.40 *sbr* (H-6 H18)	1.27-1.50 *m* (H-8 to H-18)	1.28-1.50 *m* (H-8 to H-18)
19	-	0.88 *t*	0.88 *dd*	0.88 t

Superscripts indicate the molecular weights [Da]: Mr [f] 286 ((2*R*,4*R*)-1,2,4-trihydroxy-heptadec-16-ene by Oberlies et al., 1998), [g] 316, and [h] 314. The ^{1}H-chemical shifts relevant for the differentiation of the components are underlined.

3.4.2. Hydrophilic substances

3.4.2.1. Glucosylated abscisic acid (ABA) derivatives

Abscisic acid (ABA) and some of its structurally related derivatives are important phytohormones in regulation of seed development controlling desiccation tolerance, storage product deposition, and dormancy (Krochko et al., 1998).

Our phytochemical investigation of avocado seed material (*Persea americana* Mill., Lauraceae) resulted in the isolation of two glucosylated abscisic acid derivatives. One of these was not known as a natural product and can be regarded as a potential '*missing link*' in abscisic acid metabolism in plants.

1600 g of **complete** avocado seeds were extracted as described in the experimental section (cf. Fig. 5-2 p. 225). Approximately 6 g of the methanolic crude extract (1) was fractionated by four preparative high-speed countercurrent chromatography (HSCCC) experiments of high reproducibility yielding fractions 1.1 to 1.9. (cf. Fig. 3-49). The recovered amounts of the combined fraction ranged from 11 mg to 3716 mg (cf. Table 5-3 separation 1 p. 242). Due to the high tannin content of the investigated crude extract, strong emulsifying effects were observed with different biphasic solvent systems. For the investigated extract, the maximum sample load for a successful HSCCC separation was limited to 3.2 g per injection.

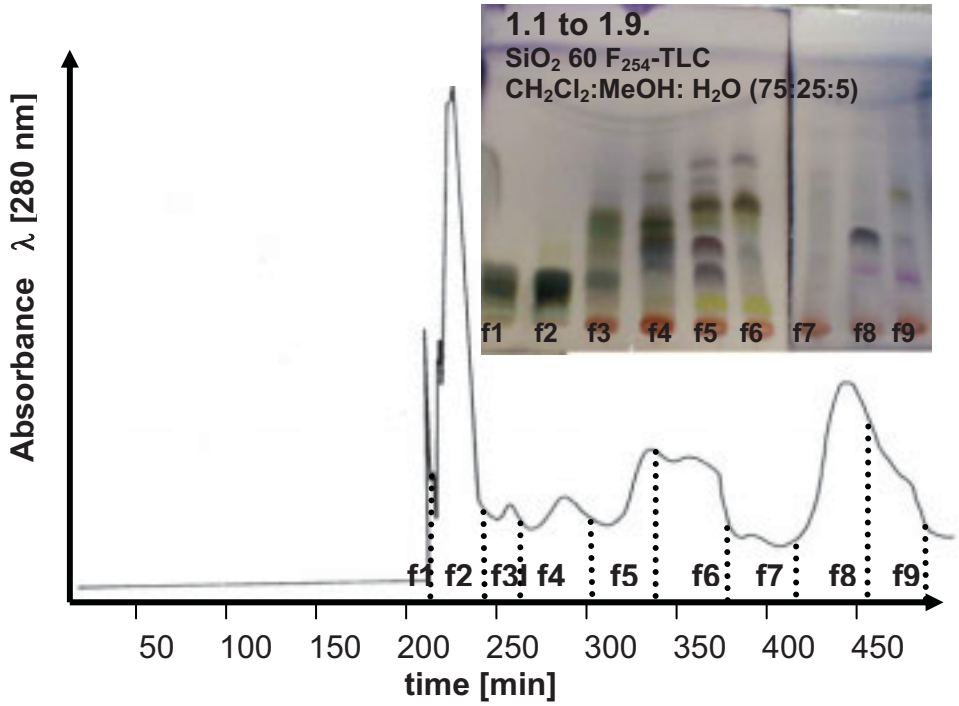
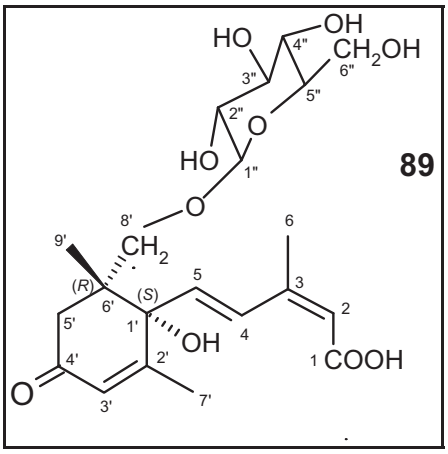


Figure 3-49. HSCCC-separation of the crude methanolic extract of avocado seeds. Biphasic solvent system: *t*-BME- *n*-BuOH - ACN - H_2O (1:3:1:5, v:v:v:v), flow rate: 2.5 mL min^{-1}. UV-trace: λ=280 nm, rotation speed: 1000 rpm; mobile phase: lower aqueous phase; elution mode: '*head to tail*'.

3.4.2.1.1. (1'*S*,6'*R*)-*8'*-Hydroxyabscisic acid β-D-glucoside (**89**).

The late eluting HSCCC-fractions 1.8. and 1.9. (400 - 450 min) were combined (47 mg) as fraction 1.89 due to results of TLC-visualization, and purified by column chromatography (CC) on lipophilic organic resin Sephadex LH-20® (1.5 cm i.d. x 70 cm). Elution with MeOH resulted in 2 mg of pure (1'*S*,6'*R*)-*8'*-hydroxyabscisic acid β-D-glucoside (**89**) which was obtained as an ambar gum, $[\alpha]_D$ +196.3 ^{o} (*c* 0.13, MeOH).



HPLC-ESI-MS (pos. mode) analysis of **89** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 465 and the corresponding ion signal $[M-H]^-$ at *m/z* 441 (neg. ESI) at R_t 27.4 min determining the molecular weight to be 442 amu. Further ESI-MS/MS fragmentation of ion signal *m/z* 441 resulted in three abundant diagnostic ions: *m/z* 397 suggested a decarboxylation reaction (Δ*m/z* 44), ion *m/z* 330 was attributed to an α-fragmentation with cleavage of the olefinic side chain of the abscisic acid backbone. The loss of a hexose unit is indicated by the fragment ion *m/z* 161 (cf. Fig. 3-50).

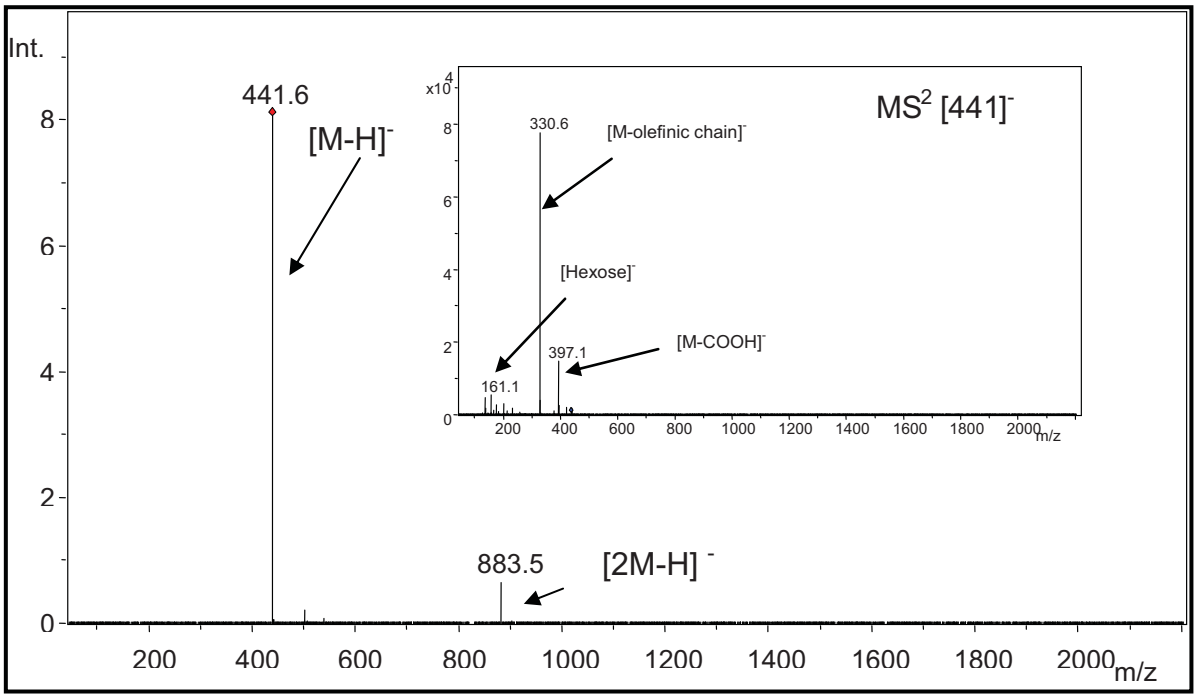


Figure 3-50. HPLC-ESI-MS (neg. mode) of **89** (gradient details cf. p. 263) (Intensity x 10^5).

The ^{1}H NMR spectrum of **89** (300 MHz, CD_3OD) (cf. Fig. 3-51) contained abundant singlet signals of three methyl groups [δ 1.08 (3H, *s*; C<u>H</u>$_3$-9'), 1.93 (3H, *s*; C<u>H</u>$_3$-7'), 2.01 (3H, *s*; C<u>H</u>$_3$-6)], four methylene signals [δ 2.41 (1H, *d*, *J*=17 Hz; H-5'a), 2.66 (1H, *d*, *J*=17 Hz; H-5'b), 3.62 (1H, *d*, *J*=10 Hz, H-8'a), 3.97 (1H, *d*, *J*=10 Hz; H-8'b)], and four signals of double bond protons [δ 5.78 (1H, s_{br}; H-2), 5.94 (1H, s_{br}; H-3'), 6.15 (1H, *d*, *J*=16.5 Hz; H-5) and 7.75 (1H, *d*, *J*=16.5 Hz; H-4)]. In the aliphatic region, coupling constants of *J*=17 Hz elucidated two geminal protons. Two olefinic resonances with a *J*=16 Hz coupling confirmed a *trans*-configured double bond, whereas two remaining double bond singlet signals had no direct coupling partners. The complex oxymethine region integrated for six protons which belong to a glucose unit [δ 3.15 (1H, *dd*, J_1=8.0 Hz, J_2=8.5 Hz; H-2''), 3.24 (1H, m_{br}, H-4''), 3.27 (1H, *m,* H-3"), 3.66 (1H, *dd*, J_1=5.0 Hz, J_2=10.0 Hz; H-6"b), 3.85 (1H, *dd*, J_1=2.0 Hz, J_2=10.0 Hz; H-6"a)], and resonance δ 4.16 (1H, *d*, *J*=8.0 Hz; H-1'') was assigned to the anomeric glucose proton, with a diaxially coupling constant of a β-glucosidic linkage. Signal of H-5" is overlapped by the methanol signal at δ 3.30 ppm.

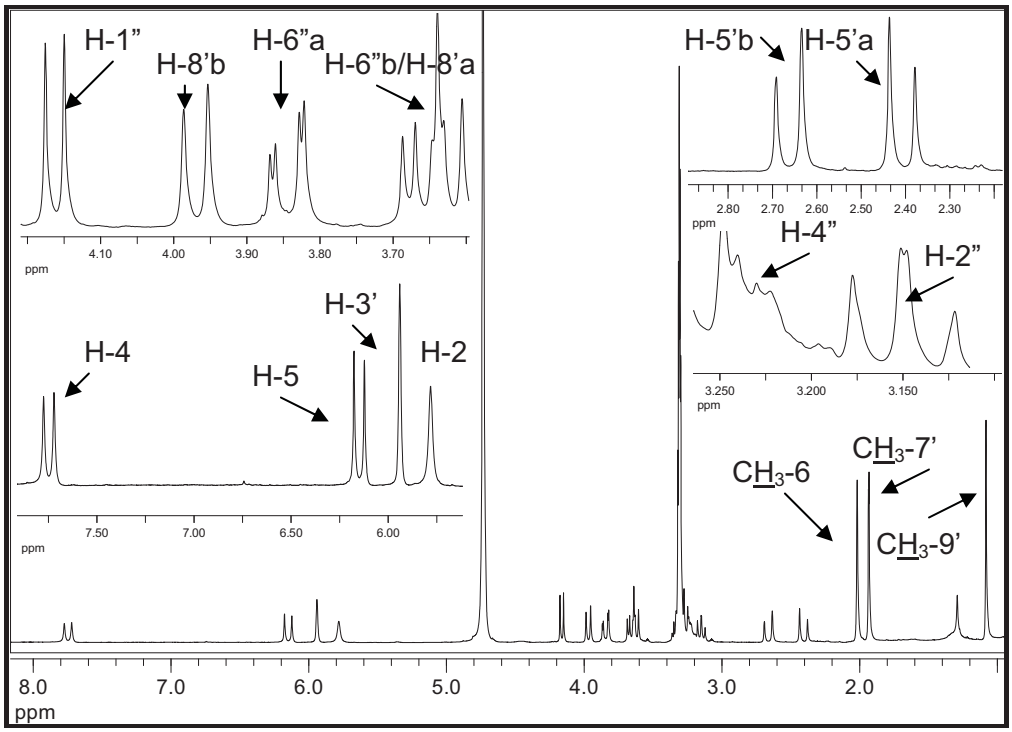


Figure 3-51. ^{1}H-NMR of compound **89** (300 MHz, CD_3OD).

The ^{13}C NMR spectrum (cf. Fig. 3-52) revealed 20 carbon resonances, consisting of three methyl, three methylene, nine methine-groups, and five quaternary C-atoms. Overall data suggested a glucosylated abscisic acid derivate. In comparison to abscisic acid (Milborrow, 1975a), substance **89** revealed a hydroxymethylene instead of four methyl groups, which was proposed to be the linkage to the glucose moiety. The quarternary nature of hydroxyl group C-1’ (δ 80.0 ppm) was verified by a DEPT135 experiment.

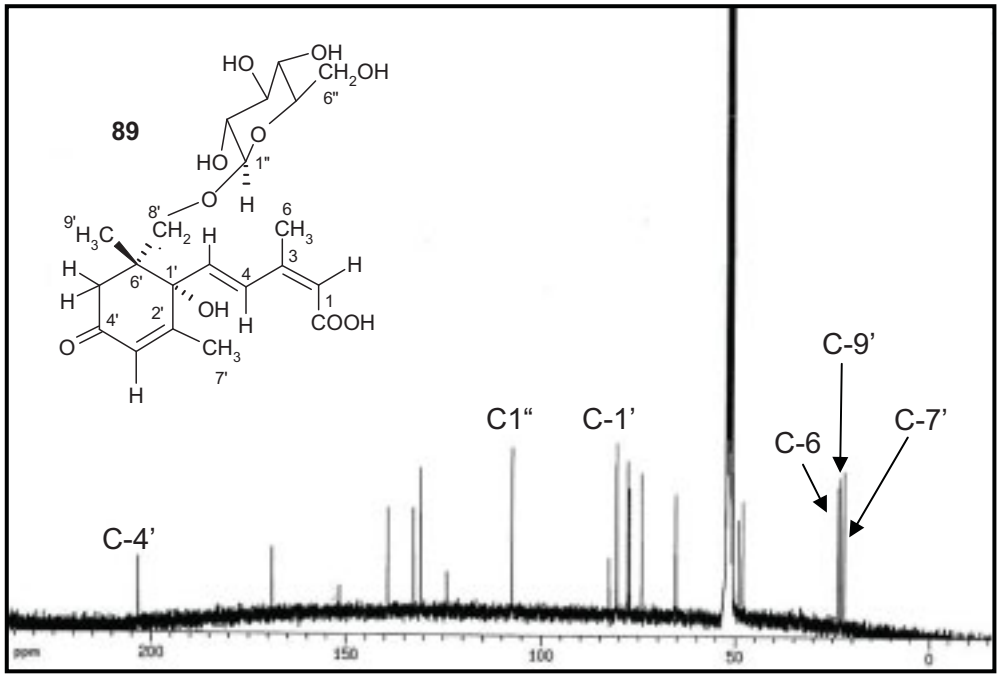


Figure 3-52. ^{13}C-NMR spectrum of compound **89** (75.5 MHz, CD_3OD).

The constitution of structure **89** was unambiguously confirmed by heteronuclear correlation experiments such as ^{1}J-^{1}H{^{13}C}- and $^{2,3}J$-^{1}H{^{13}C}-COSY. All long-range correlations are summarized in Table 3-12 (p. 129), and structurally relevant correlations of the HMBC spectrum are presented in Figure 3-53. The $^{2,3}J$-CH cross peak from the anomeric sugar proton H-1” (δ_H 4.16) elucidated the glucosidic linkage to the hydroxymethylene position C-8’ (δ_C 74.4). Several abundant cross signals corroborated the location of C-8’ (H-9’, H-5’a, H-5’b, H-1’’) (cf. Fig. 3-53).

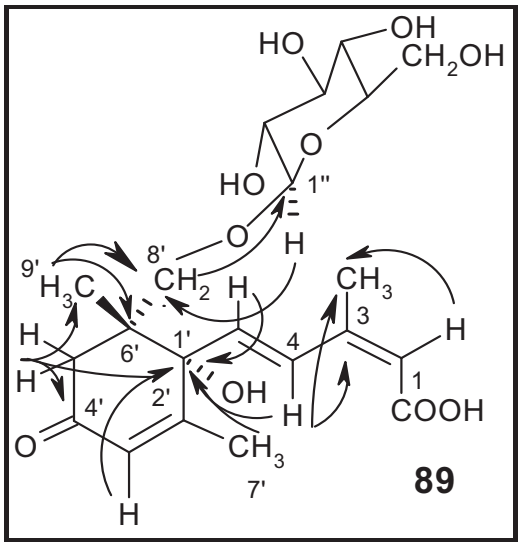


Figure 3-53. Structure relevant $^{2,3}J$-$^{1}H\{^{13}C\}$ - correlations in the HMBC of compound **89**.

Absolute stereochemistry of two chiral carbons at C-1'*S*, and C-6'*R* in the aglycone of **89** were derived by circulardichroism spectroscopy (CD), and multiple *diff*-NOe experiments (in MeOH-d_4).

The CD-curve of **89** showed two intense cotton effects at λ 233 nm and λ 266 nm (cf. Fig. 3-54), and elucidated 1'*S*-configuration for the tertiary hydroxyl function. This well known chiroptical effect for abscisic acid derivates was the key information for determining stereochemistry at the second chiral position C-6' (Ohloff et al., 1973; Hirai et al., 1986). Since steric flexibility of cyclohexenone rings is limited, such as for other abscisic acid derivates (Todoroki and Hirai, 2000), only two main conformers – envelope or inverted envelope type - are possible. Our results of multiple *diff*-NOE experiments on substance **89** are in agreement with NOE experiments reported for abscisic acid derivates (Milborrow, 1975b), and corroborate a predominant envelope-type conformer with the axially oriented side chain.

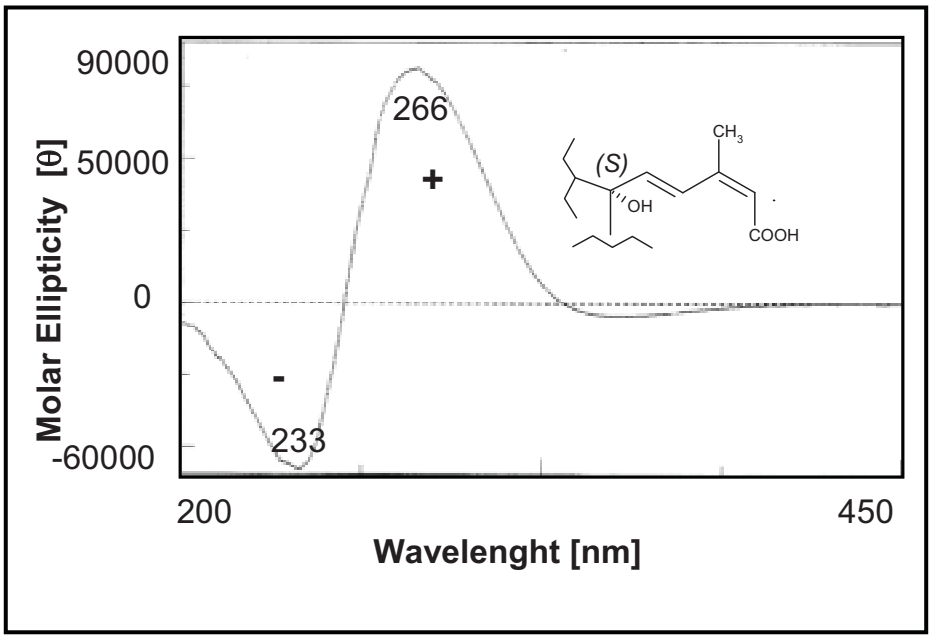


Figure 3-54. Recorded CD-curve and cotton effects for the *S*-configured chromophore of (1'*S*, 6'*R*)-8'-Hydroxyabscisic acid β-D-glucoside (**89**). (CD was recorded on a Jasco spectropolarimeter type J-715 at 21 °C. Scan range: λ=200-450 nm, cell length: 0.1 cm. Used solvent: MeOH).

Observation of strong NOE resonances (cf. Fig. 3-55) between the side chain double bond proton H-5 (δ 6.15), protons of methyl group CH_3-9' (δ 1.08), and also H-5'a (δ 2.41), clearly indicated that all interfering protons are located on the same side of the cyclohexene plane. This implies an equatorial orientation for CH_3-9', and the olefinic side chain in axial orientation, hence the hydroxymethylene β-D-glucose moiety is located on the opposite side of the ring system, and is also axially oriented.

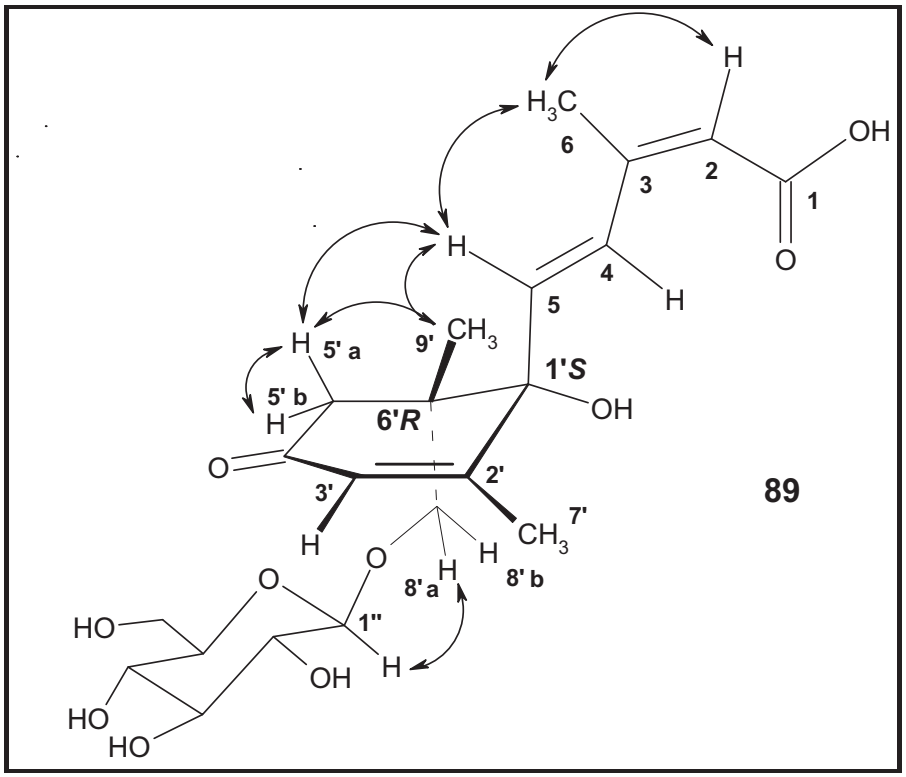


Figure 3-55. Important NO-effects determining the stereochemistry of carbon 6'*R* in respect to the tertiary hydroxyl function at carbon 1'*S* of structure **89**.

Relative stereochemical data gained by NOE-irradiation in combination with the absolute configuration at carbon 1'*S*, unequivocally determined an *R*-configuration for the second chiral position C-6'.

Confirmation for the stereochemistry at C-6' was obtained by selective irradiation of H-5'b (δ 2.66) which resulted in a NOE resonance to H-5'a (δ 2.41 ppm) but not in a through-space interaction to CH_3-9'.

The double bond configurations in the olefinic side chain were visible by selective NOE enhancements: *trans* configuration of Δ4-bond was observed by a significant NO effect between H-5 (δ 6.15 ppm) and CH_3-6 (δ 2.01 ppm), also irradiation of CH_3-6 (δ 2.01 ppm) resulted in a NOE for H-2 (δ 5.78 ppm) showing a *trans*-configuration of double bond Δ2. Also glucosidation at C-8' was recognized by abundant NOE resonances between H-8a' (δ 3.62 ppm) and the anomeric proton (δ 4.16 ppm) in vice-versa direction (cf. Fig. 3-55).

To the best of our knowledge the 8'-*O*-β-D-glucoside of (1'S,6'R)-8'-hydroxyabscisic acid isolated from avocado seed material was not yet known as a natural product.

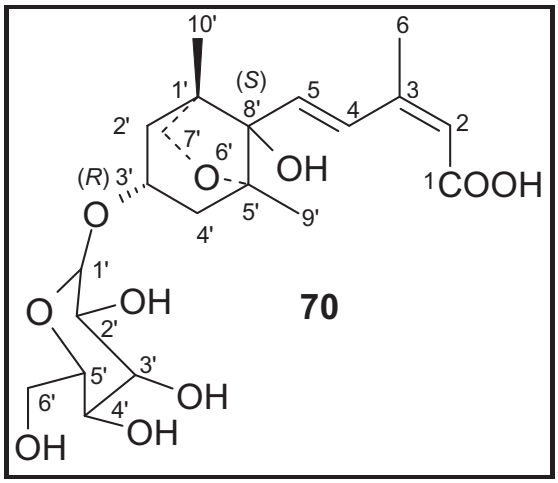
Table 3-12. ^{1}H-, *diff*-NOE, ^{13}C-, DEPT-NMR, and $^{2,3}J$-HC long-range correlations for compound **89** (δ [ppm]; J [Hz]) in MeOH-d_4.

Position	^{1}H	*diff*-NOE	^{13}C	DEPT	$^{2,3}J$-HC correlation
1			n.d.	n.d.	
2	5.78 *s* br	C<u>H</u>$_3$-6	121.2	CH	C-4, C-6
3			150.0	C	
4	7.75 *d* (16.5)	n.d.	129.6	CH	C-1', C-3, C-6
5	6.15 *d* (16.5)	C<u>H</u>$_3$-6, H-5'$_a$, C<u>H</u>$_3$-9'	136.3	CH	C-1', C-2', C-3, C-4
6	2.01 *s*	H-2, H-5	20.8	CH_3	C-2, C-3, C-4
1'			80.0	C	
2'			166.3	C	
3'	5.94 *s* br	C<u>H</u>$_3$-7'	127.9	CH	C-1', C-4', C-5', C-7'
4'			200.5	C	
5'$_a$	2.41 *d* (17.0)	CH_3-9', H-5, H-5'$_b$	45.2	CH_2	C-1', C-4', C-6', C-8', C-9'
5'$_b$	2.66 *d* (17.0)	H-5'$_a$			C-1', C-3', C-4', C-6', C-8', C-9'
6'			46.6	C	
7'	1.93 *s*	H-3'	19.3	CH_3	C-1', C-2', C-3'
8'$_a$	3.62 *d* (10.0)	H-1'', H-8'$_b$, C<u>H</u>$_3$-9'	74.4	CH_2	C-1'', C-5', C-6', C-9'
8'$_b$	3.97 *d* (10.0)	H-8'$_a$, C<u>H</u>$_3$-9'			C-1', C-1'', C-5', C-6', C-9'
9'	1.08 *s*	H-5, H-8'$_b$, H-5'$_a$	20.0	CH_3	C-1', C-5', C-6', C-8'
1"	4.16 *d* (8.0)	H-8'$_a$	104.4	CH	C-8'
2"	3.15 *dd* (8.5, 8.0)		74.9	CH	C-1'', C-4''
3"	3.27 *		71.3	CH	
4"	3.24 *m* br		77.8	CH	C-3''
5"	3.30 *		77.7	CH	
6"$_b$	3.66 *dd* (10.0, 5.0)		62.5	CH_2	
6"$_a$	3.85 *dd* (10.0, 2.0)				

*signal overlapped by MeOH-d_4 signal, n.d. not detected.

3.4.2.1.2. *(1'R,3'R,5'R,8'S)-epi*-Dihydrophaseic acid β-D- glucoside (**70**)

56 mg of HSCCC fraction 1.5. was rechromatographed on Sephadex LH-20 (cf. details on Table 5-3 separation 2 p. 242). Fraction 1.5.2. was then subjected to silica gel 60 column chromatography (cf. Table 5-3 separation 3 p. 242), and finally fraction 1.5.2.6. was purified on PVA 500 (cf. Table 5-3 separation 4 p. 242) yielding 2.4 mg of *epi*-dihydrophaseic acid-β-D-glucoside (**70**) as a brown transparent gum, $[\alpha]_D$ -5.0 (c 0.16, MeOH). Compound **70** was identified as constituent in avocado flesh (Hirai and Koshimizu, 1983). It was isolated for the first time in this research from avocado seeds.



70

ESI-MS in positive mode revealed a quasimolecular ion $[M+Na]^+$ at *m/z* 467, and LC-ESI-MS (neg. mode) resulted in a $[M-H]^-$ signal at *m/z* 443 (cf. Fig. 3-56), respectively. Therefore the resulting M_r is 444 amu for compound **70**, 2 amu higher than for substance **89**. ESI-MS/MS of ion *m/z* 443 yielded two abundant ions: the formal loss of 18 mass units to fragment *m/z* 425 was correlated to a dehydration process, and due to the structure of **89**, ion *m/z* 237 is related to the cleavage of a glucose unit, and simultaneous decarboxylation.

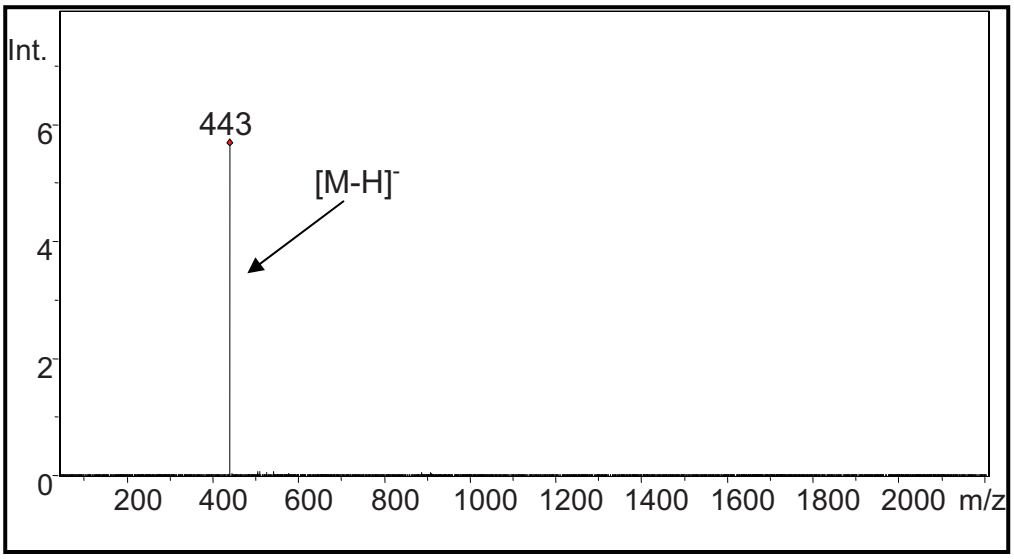


Figure 3-56. ESI-MS (neg. mode) of **70** (conditions cf. Fig. 3-50) (Intensity: $x10^5$).

In comparison to the ^{1}H NMR of substance **89**, the aliphatic region of **70** appeared highly complex, and several signals in the carbinol region were overlapped.

In detail, the ^{1}H spectrum in MeOH-d_4 (cf. Figure 3-57) indicated three singlet resonances [δ 0.90 (3H, *s* ; C$\underline{H}_3$-10'), 1.15 (3H, *s*; C$\underline{H}_3$-9'), and 2.10 (3H, *s*; C$\underline{H}_3$-6)], five methylene protons [δ 1.78 (1H, *dd*, J_1=12 Hz, J_2=10 Hz; H-2'ax), 1.90 (1H, *dd*, *J*=12 Hz; H-2'eq), 2.18 (2H, m_{br}; H-4'), 3.63 (1H, m_{br}; H-6'')], and 3.78 (2H, *d*, *J*=11 Hz; H-7'a/H-3"), six carbinol protons, 3.10 (1H, *dd*, J_1=7 Hz, J_2=8 Hz; H-2''), 3.25 (1H, m_{br}; H-4''), 3.27 (1H, m_{br}; H-5''), 3.85 (1H, *d*, *J*=10 Hz; H-6"), 4.23 (1H, m_{br}; H-3')], and the anomeric signal δ 4.30 (1H, *d*, *J*=8 Hz; H-1'') indicative for a β-glucosidic linkage.

Only three olefinic resonances [δ 5.70 (1H, s_{br}; H-2), 6.45 (1H, *d*, *J*=16 Hz; H-5), and 7.90 (1H, *d*, *J*=16 Hz; H-4)] appeared, proposing a similar substitution pattern, and double bond configuration as elucidated for the side chain moiety of **89**, Interestingly, the olefinic proton δ 5.94 of the cyclohexene ring system **89** was missing in **70**, hence a ring closure to a phaseic acid derivate was assumed.

Formal numbering of the carbon ring skeleton of phaseic acid derivate **70** is different to abscisic acids (Addicott, 1983), defining the chiral tertiary hydroxyl group to be C-8' instead of carbon 1' in structure **89**.

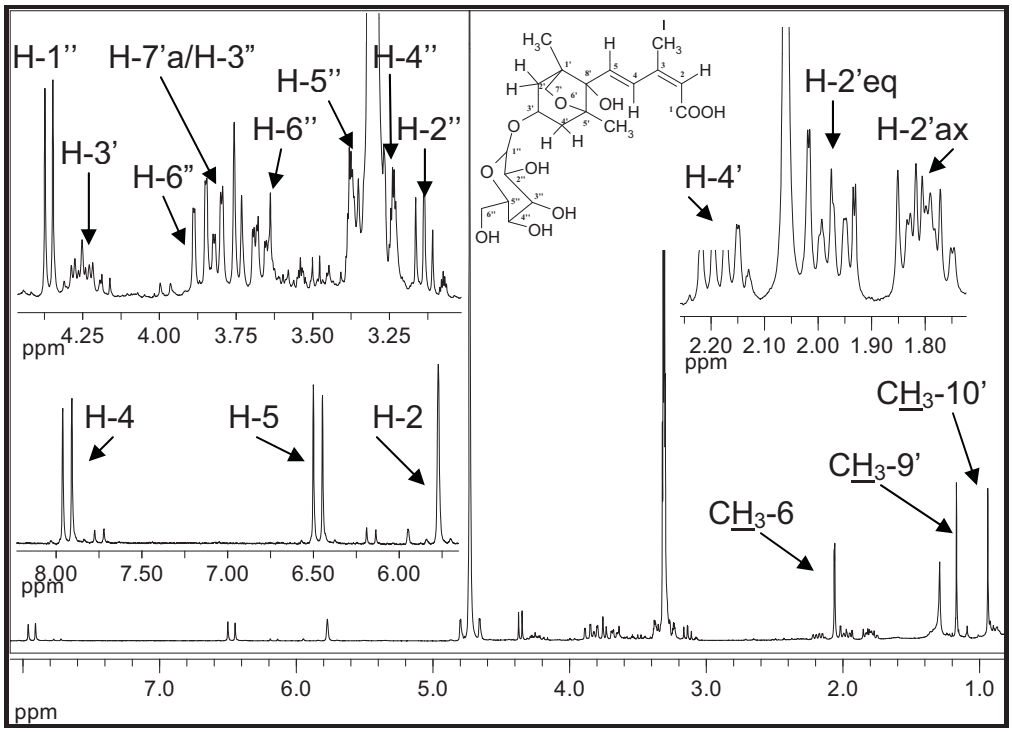


Figure 3-57. ^{1}H-NMR of compound **70** (300 MHz, CD_3OD).

^{13}C NMR data of compound **70** (cf. Fig. 3-58) revealed 20 carbon signals: three methyl, four methylene, nine methine, and four quarternary signals, but like in the case of previous studies (Champavier et al., 1999) the carboxylic function was not detected. Beside the tertiary hydroxyl group (δ_C 83.2) similar to structure **89**, a ^{13}C-DEPT135 experiment revealed the quarternary nature of a second tertiary oxymethine function (δ_C 87.6), and also an additional hydroxymethylene carbon (δ_C 77.2).

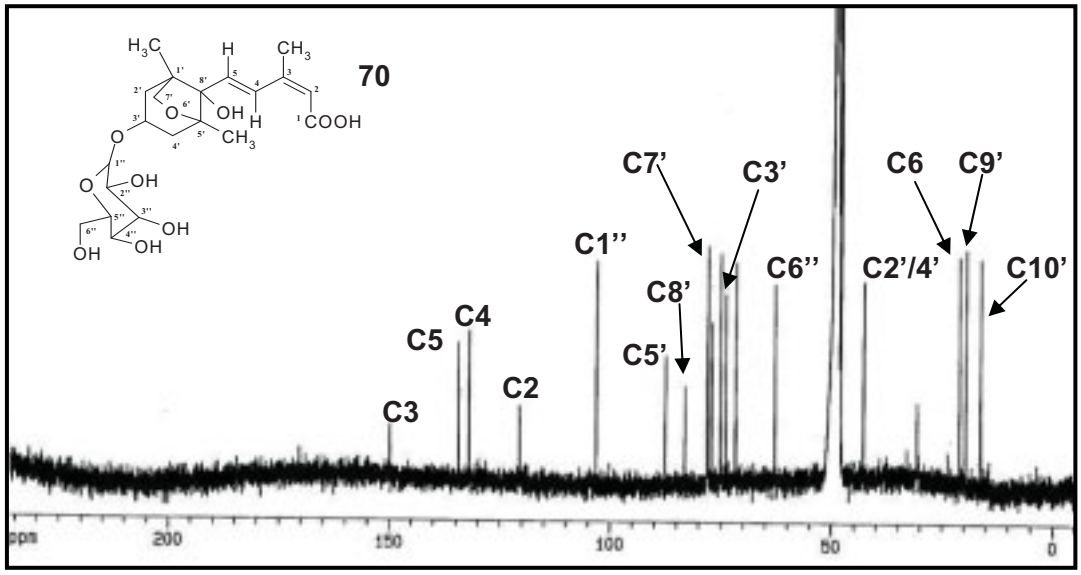


Figure 3-58. ^{13}C NMR spectrum of compound **70** (75.5 MHz, CD_3OD).

The glucosidic linkage was recognized via a strong ^{2,3}J-CH cross signal (cf. Fig. 3-59) from the anomeric sugar proton (δ_H 4.30) to the C-3' (δ_C 74.0). Intense ^{2,3}J-CH-long-range cross signals proved the intramolecular ether ring formation in the dihydrophaseic acid structure: protons of CH_2-7' (δ_H 3.78) correlated to C-1' (δ_C 49.3), C-8' (δ_C 83.2 ppm), and most important to C-5' (δ_C 87.6 ppm).

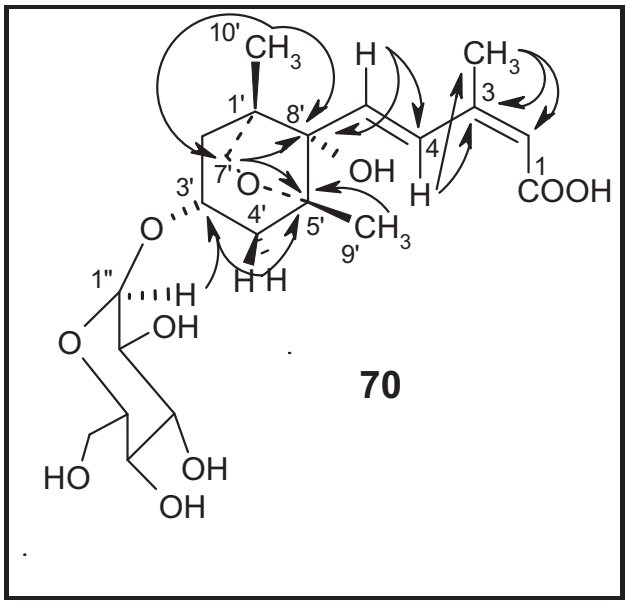


Figure 3-59. Structure relevant long-range correlations in the HMBC of compound **69.**

In good accordance to chiroptical reference data of abscisic acid derivates (Hirai et al., 1986), the CD-curve of *epi*-dihydrophaseic acid glucoside (**70**) (Fig. 3-60) showed two characteristic cotton effects (λ 235 and λ 272 nm), which were similar to the CD-effects of **89**, and clearly assigned a 8'S-configuration.

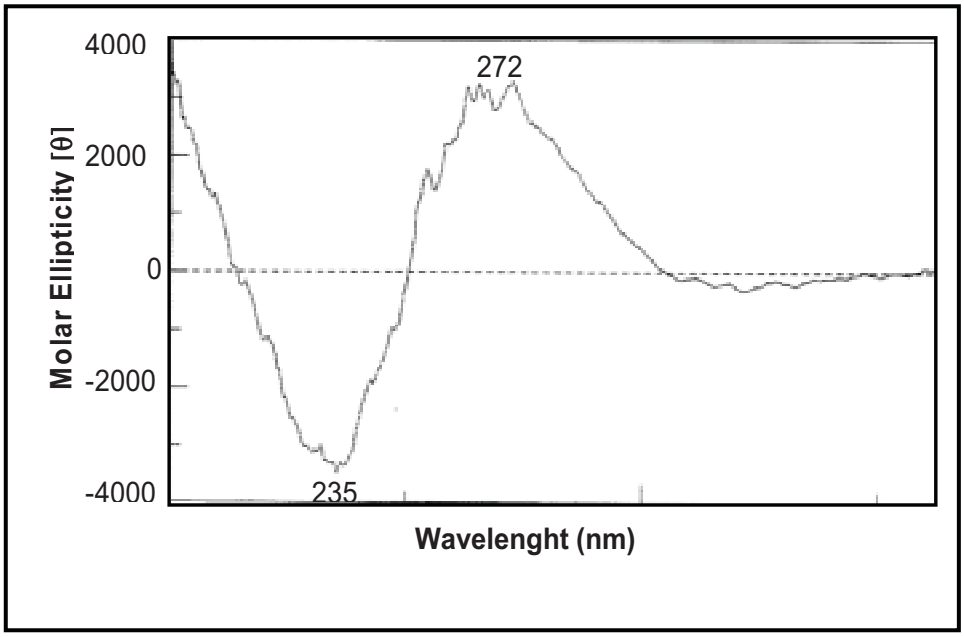


Figure 3-60. CD-curves and cotton effects for (1'*R*, 3'*R*, 5'*R*, 8'*S*)-*epi*-dihydrophaseic acid β-D-glucoside (**70**) (cf. CD-Exp.-details in Fig. 3-54).

From a biosynthetic viewpoint, 8'-hydroxyabscisic acid β-D-glucoside (**89**) is a potential precursor of *epi*-dihydrophaseic acid and phaseic acid (Balsevich et al., 1994; Hirai and Koshimizu, 1983). Hence stereochemistry of the formed ether bridge in **70** should directly correlate to the likely precursor substance **89**. As a steric assumption for a successful ether ring formation in system **70**, hydroxymethylene carbon C-7' has to be in axial orientation of the cyclohexane plane (Fig. 3-61). Ring closure is causing a partly rigid cyclohexane system, where H-2ax' and H-4ax' are preferably in axial orientation.

Selective NOE irradiation (in MeOH-d_4, 25 °C) on axial proton H-2'ax (δ 1.78) resulted in strong NO effects to H-4'ax (δ 2.18) and the olefinic proton H-5 (δ 6.45), clearly indicating that these interfering protons are located on the same side of the cyclohexane plane (Fig. 3-61). Equatorial orientation of CH_3-9' (δ 1.15) was shown by NO enhancement of the olefinic proton H-5. There are no other stereochemical

configurations which are eligible for the observed NO effects. Therefore the olefinic side chain has to be in axial orientation as well, and ether bridge CH_2-7' to C-5' is in trans-location, on the opposite side of the ring plane.

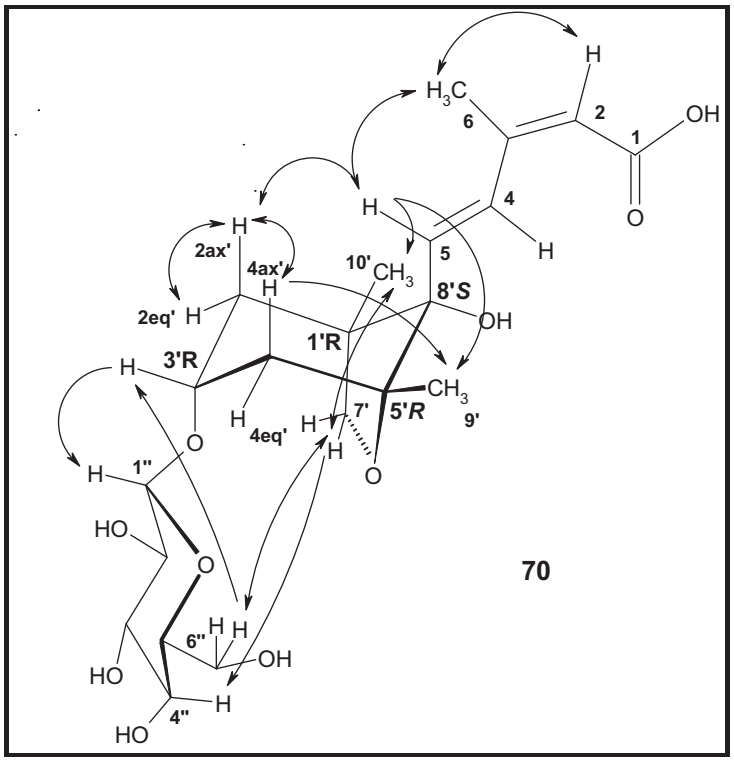


Figure 3-61. Structure relevant NOE-resonances in compound **70** determining the absolute configuration of carbons 1'*R*,3'*R*, and 5'*R* in respect to the tertiary (8'*S*)-hydroxyl function, and a $^4C_1$-conformation of the glucose moiety.

A significant NO effect from the glucose proton H-6a'' (δ_H 3.63) to protons of the ether bridge CH_2-7' (δ_H 3.78) - and in vice-versa direction also to H-4'' (δ_H 3.25) – elucidates that the β-D-glucose unit and the ether ring are on the same side of cyclohexane plane. Interestingly, the observed NO effects are only possible if the glucose cyclohexane ring is in a $^4C_1$-conformation.

All NO effects observed for the olefinic side chain elucidated that both double bonds are *trans*-configured, similar to substance **89**.

Combining all results of the *diff*-NOE experiments with the known absolute configuration of carbon 8'*S*, elucidate the stereogenic C-atoms to be 1'*R*, 3'*R*, and 5'*R*.

The determined configurations of **89** and **70** are in line with a likely common biosynthetic route between these components (cf. Biosynthesis chapter p. 212).

Beside substance **70**, we screened for the glucoside of dihydrophaseic acid, which is the C-3'-epimer. But ESI-LC/MS analysis of the methanolic crude avocado seed extract, co-elution experiments, and selective ion extraction of *m/z* 443 detected solely substance **70**. This result implies that in avocado seeds the conversion of phaseic acid to the *epi*-dihydrophaseic acid might be a stereoselective oxidoreductase reaction.

All NMR-data from compound **70** are summarized in Table 3-13.

Table 3-13. ^{1}H-, *diff*-NOE, ^{13}C-, DEPT-NMR, and $^{2,3}J$-HC long-range correlations for compound **70** (δ [ppm]; J [Hz]) in MeOH-d_4.

Atom	^{1}H	*diff*-NOE	^{13}C	DEPT	$^{2,3}J$-HC correlation
1			n.d.	n.d.	
2	5.70 *sbr*	C$\underline{H}_3$-6	120.5	CH	C-4, C-6
3			149.9	C	
4	7.90 *d* (16.0)	C$\underline{H}_3$-9', C$\underline{H}_3$-10'	132.1	CH	C-2, C-3, C-6, C-8'
5	6.45 *d* (16.0)	C$\underline{H}_3$-6, C$\underline{H}_3$-9', C$\underline{H}_3$-10', H-2'ax	134.4	CH	C-3, C-4, C-8'
6	2.10 *s*	H-2, H-5	21.1	CH_3	C-2, C-3, C-4
1'			49.3	C	
2'$_{ax}$	1.78 *dd* (12, 10)	H-2'eq, H-4'ax, H-5	42.90	CH_2	
2'$_{eq}$	1.90 *dd* (12)	H-2'ax			C-3'
3'	4.23 *m* $_{br}$	H-1''	74.0	CH	
4'$_{ax, eq}$	2.18 *m* $_{br}$	C$\underline{H}_3$-9', H-2'ax	42.95	CH_2	C-2', C-3', C-5', C-8'
5'			87.6	C	
7'	3.78 *d* (11)	H-4'', C$\underline{H}_3$-10'	77.2	CH_2	C-2', C-5', C-8'
8'			83.2	C	
9'	1.15 *s*		19.7	CH_3	C-4', C-5'
10'	0.90 *s*	C$\underline{H}_2$-7	16.3	CH_3	C-2', C-7', C-8'
1"	4.30 *d* (8.0)		103.1	CH	C-3'
2"	3.10 *dd* (7, 8)		75.1	CH	C-1''
3"	3.78 *d* (11)		78.0	CH	
4"	3.25 *		71.7	CH	
5"	3.27 *		78.1	CH	
6"$_a$	3.63 *m* $_{br}$	C$\underline{H}_2$-7	62.8	CH_2	
6"$_b$	3.85 *d* (10.0)				

* signal overlapped by MeOH-d_4 signal, n.d. not detected

As chemical proof of the structure as well as the postulated immediate ring-closure reaction to phaseic acid (Barton and Nakanishi, 1999), the novel glucoside **89** was treated with Rohapect (Röhm) enzyme (Fig. 3-62). The direct investigation by LC-ESI-MS (neg. mode) of the enzymatic incubation showed the presence of phaseic acid at R_t 29.2 min $[M-H]^-$ at *m/z* 279.

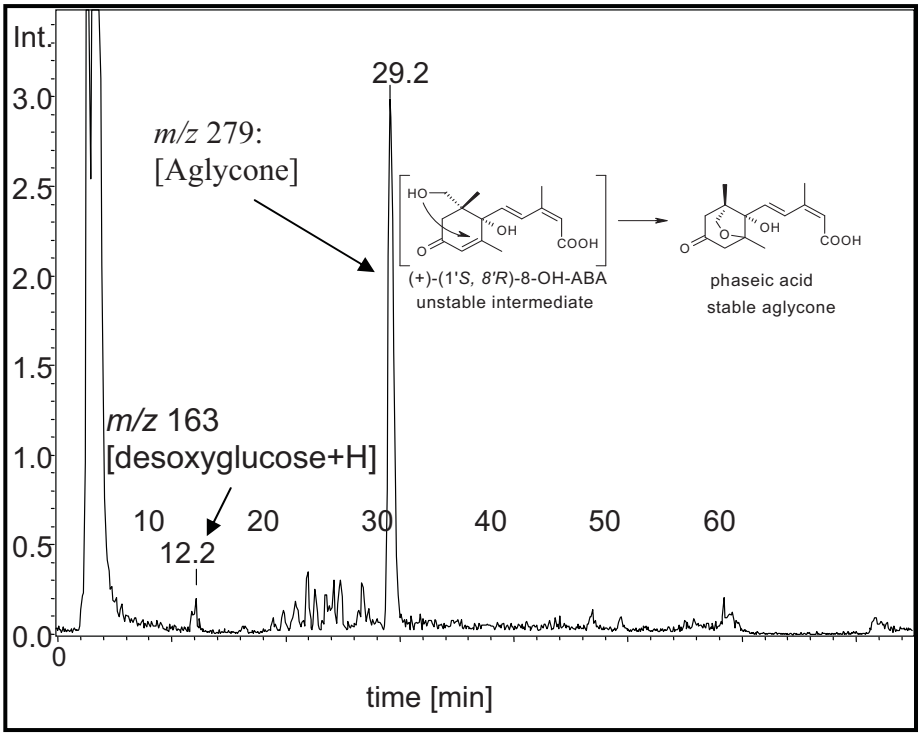
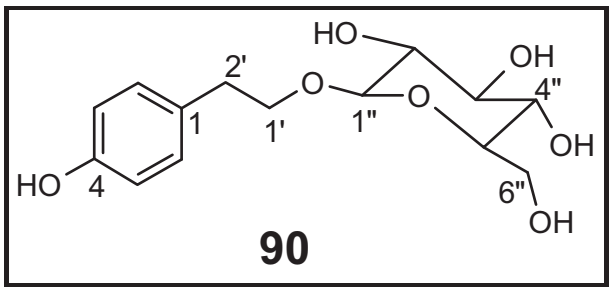


Figure 3-62. LC-ESI-MS (neg. mode) analysis of the enzymatic incubation of **89** with Rohapect as β-D-glucosidase activity (cf. Details p. 236 and 239). (Int. x 10^4).

3.4.2.2. Tyrosol derivatives

3.4.2.2.1. Tyrosol-1'-β-D-O-glucoside (**90**) (Salidroside)

Fractions 1.5.4.1., 1.6.4., 1.6.5., and 5.3.2. (cf. Table 5-3 separations 5, 6, and 18 p.p. 242 and 244), yielded 59 mg of compound **90** as ambar gum. The molecular structure was elucidated as:



90

The ESI-MS spectrum of **90** (neg. mode) (cf. Fig. 3-64) revealed quasimolecular ion signals $[M-H]^-$ at *m/z* 299 and $[2M-H]^-$ at *m/z* 599 resulting in a molecular weight of 300 Da.

ESI-MS^2 fragmentation of the MS-generated dimer *m/z* 599 yielded a $[M-H]^-$ signal at *m/z* 299. Subsequent ESI-MS^3 fragmentation of signal *m/z* 299 resulted in five abundant ions (Fig. 3-64): *m/z* 179 for the glucose unit, *m/z* 161 for a desoxy-glucose and m/z 143 was interpreted to be the successive cleavage of water from the desoxy-glucose unit. Ions at *m/z* 119 and *m/z* 101 are related to the aglycone partial structure.

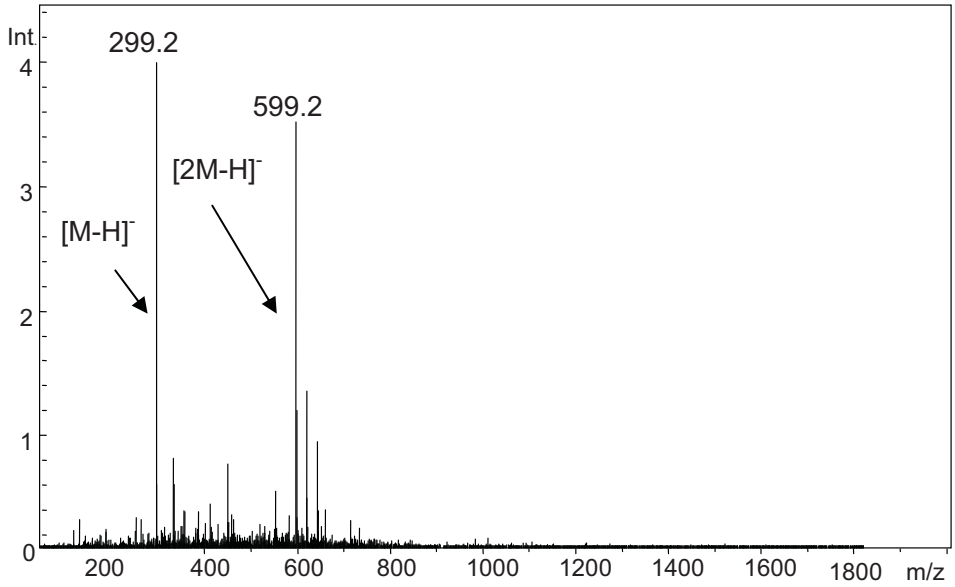


Figure 3-63. ESI-MS (neg. mode) of compound **90** (Intensity: x 10^4).

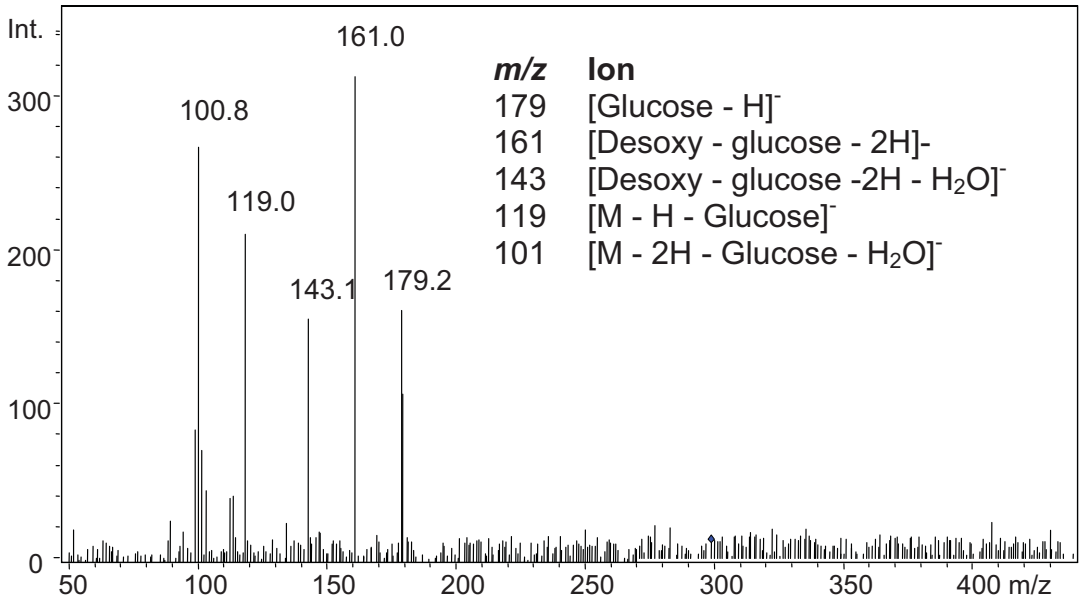


Figure 3-64. ESI-MS^3 fragmentation of MS^2-signal *m/z* 299 of compound **90**.

The ^{1}H-NMR (300 MHz, CD_3OD) of compound **90** (cf. Fig. 3-65) showed for the tyrosol moiety the characteristic signals: δ 2.83 ppm (2H, t, *J* 7.4 Hz, H-2'), δ 3.67 (H-1'a) overlapped by H-6"a, δ 4.03 ppm (H-1'b), and for the glucose partial structure five carbinol signals, δ 3.18 (H-2"), δ 3.26 (H-4"), and δ 3.35 (H-3"), δ 3.85 ppm (H-5"). The anomeric signal at δ 4.29 (H-1") with a coupling constant of *J*= 7.7 Hz is indicative for a β-glucosidic linkage.

In the aromatic region, solely an AA'-BB'-system occurred, indicating that this part of the molecule is a symmetrical substituted benzene system with signals at δ 6.69 (H-2/ H-6) and δ 7.06 ppm (H-3/ H-5).

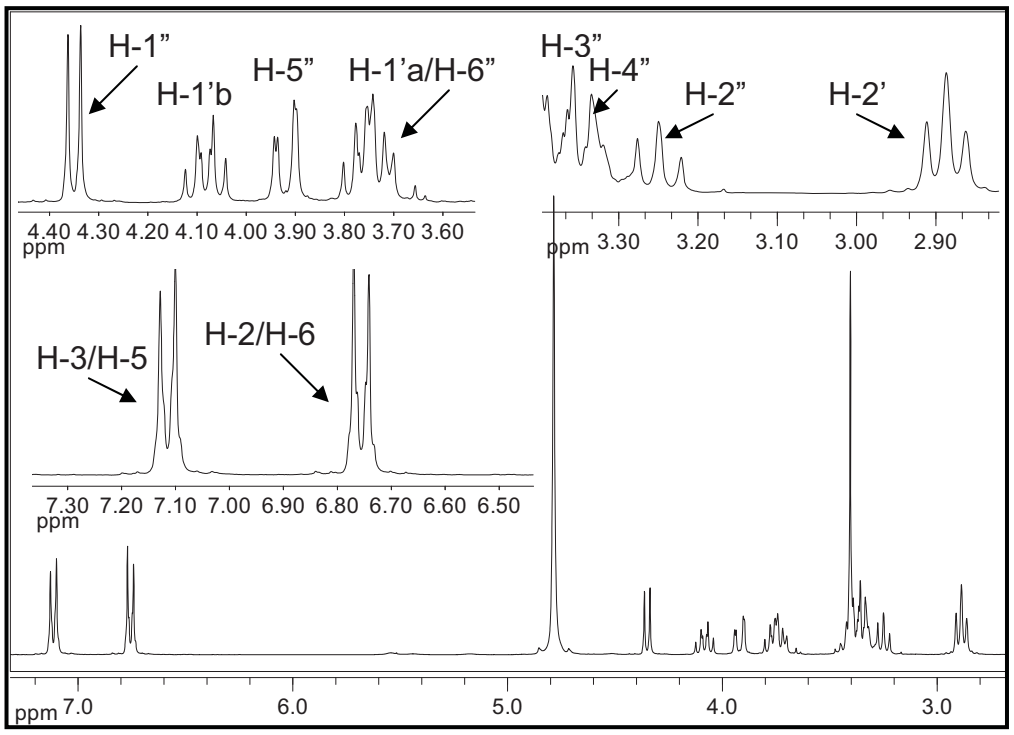


Figure 3-65. ^{1}H-NMR of compound **90** (300 MHz, CD_3OD).

The ^{13}C-NMR (75.5 MHz, CD_3OD) (cf. Fig. 3-66) showed six carbinol signals of a glucose unit [δ 78.2 (C-5"), 78.0 (C-3"), 75.2 (C-2"), 71.7 (C-4"), 62.8 (C-6") and the anomeric signal at δ 104.3 ppm (C-1")]. The two methylene groups of the tyrosol moiety were clearly distinguished from the glucose signals by a DEPT135 [δ 36.4 ppm (C-2') and δ 72.1 ppm (C-1')]. Glucosidation at C-1' was established according to results of a $^{2,3}J$-CH long-range correlation (COLOC) (cf. Fig. 3-67) and literature data (Baderschneider, 2000) (cf. Material and methods p. 265-266).

^{13}C-signals of higher intensity at δ 130.8 and δ 116.1 ppm are related to the AA'-BB'-system constituted of C-2/ C-6 and C-3/ C-5, respectively. The low-field shifted quarternary signal at δ 156.8 ppm of the phenolic function C-4 was corroborated by a DEPT135 experiment.

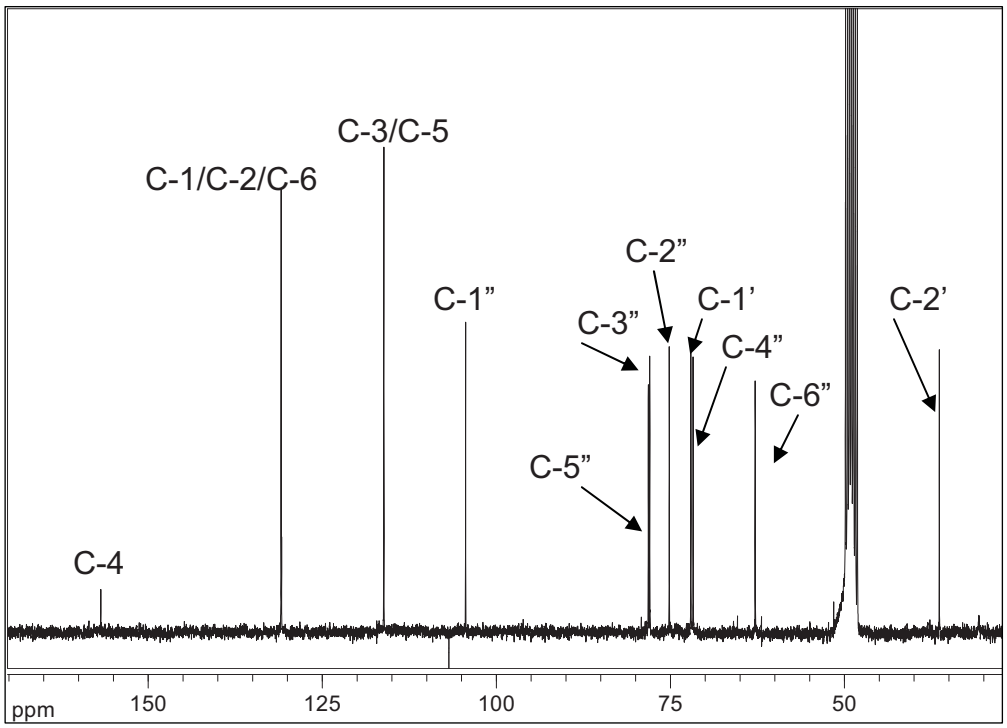


Figure 3-66. ^{13}C-NMR of compound **90** (75.5 MHz, CD_3OD).

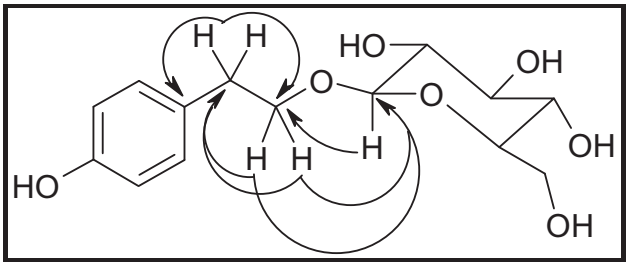


Figure 3-67. Important $^{2,3}J$-CH COLOC correlations in salidroside (**90**).

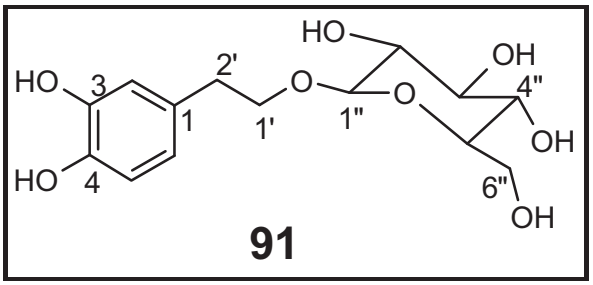
^{1}H- and ^{13}C-NMR data of compound **90** are in complete accordance with reference data reported by Landtag et al. (2002) for tyrosol-*O*-β-D-glucopyranoside.

Tyrosol is one of the major phenolic components in olive oil (Visioli and Galli, 1998). Tyrosol glucoside is also called salidroside, one of the active components of the arctic root *Rhodiola rosea* (Landtag et al., 2002). The diverse biological activities of **90** including anti-oxidant, anti-inflammatory, anti-atherogenic, and cardioprotective effects were reviewed by Obied et al. (2005).

3.4.2.2.2. 3-Hydroxy-tyrosol-1'-O-β-D-glucoside (**91**)

The methanol-water partition (5.4 g) from the avocado seed cotyledons was separated by preparative HSCCC (cf. Fig. 3-68) resulting in 6 fractions (10.1. to 10.6.) (cf. Table 5-3 separation 39, p. 248). Further separation of fraction 10.5., by size-exclusion CC (cf. Table 5-3 separation 46 p. 249) and preparative RP-18 HPLC of fraction 10.5.45., (cf. Table 5-3 separation 49 p. 250) yielded 3 mg of compound **91** as a pink amber powder. **91** was also gained (2 mg) from the ethyl acetate partition, separated by column chromatography on silica gel (cf. Table 5-3 separation 19 p. 245). Fraction 10.6. contained only tannin compounds (not shown here).

The chemical structure of the isolated compound was determined to be:



91

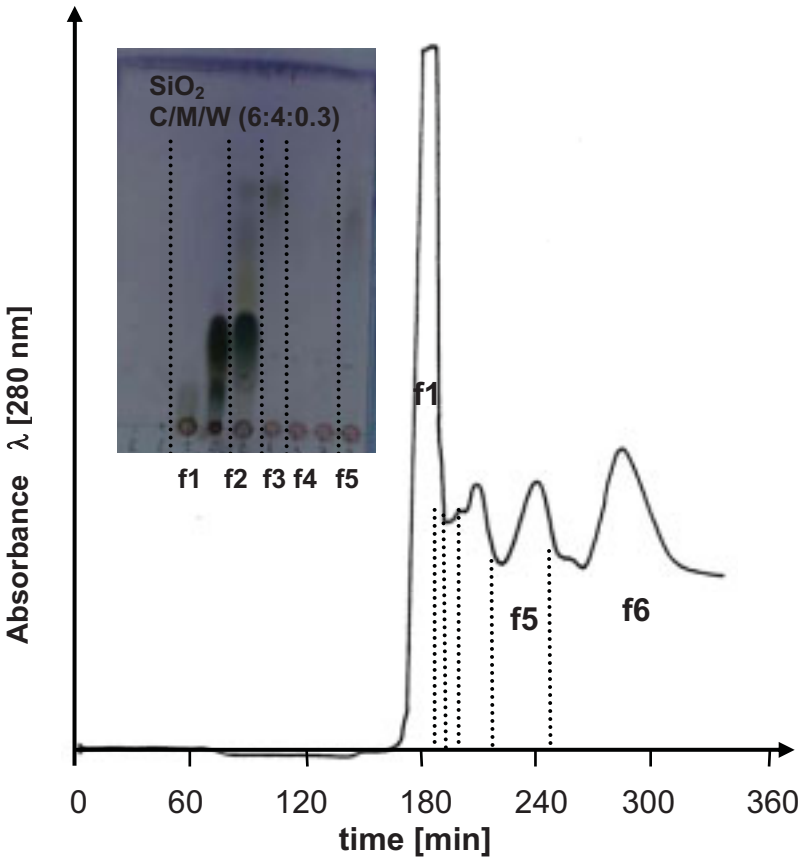


Figure 3-68. HSCCC-separation of the M:W partition recovered from avocado seed cotyledons material (cf. Material and methods separation p. 248). Biphasic solvent system: *tert.*-butylmethylether - *n*-BuOH - ACN - H_2O (1:3:1:5, v:v:v:v), flow rate: 3.0 mL min^{-1}. UV-trace: λ=280 nm, rotational speed: 1000 rpm; mobile phase: lower aqueous phase; elution mode: *'head-to-tail'*. Peak f5 generated **91**.

LC-ESI-MS analysis (pos. mode) showed two strong quasimolecular ions at *m/z* 339 $[M+Na]^+$ and *m/z* 655 $[2M+Na]^+$, while in negative polarity (cf. Fig. 3-69) the most abundant ions were at *m/z* 315 $[M-H]^-$, and *m/z* 631 $[2M-H]^-$ corroborating a molecular weight of 316 Da for compound **91**. The MS/MS-fragmentation of signal *m/z* 315 resulted in diagnostic fragments at *m/z* 179 $[Glucose-H]^-$, *m/z* 153 for the phenolic aglycone $[M-Desoxy-glucose]^-$, and *m/z* 135 $[M-2H-Glucose]^-$. The ion at *m/z* 119 is related to the aglycone partial structure. The increase of molecular weight Δ16 amu (in comparison to **90**), suggested for **91** a tyrosol structure with an additional OH-group.

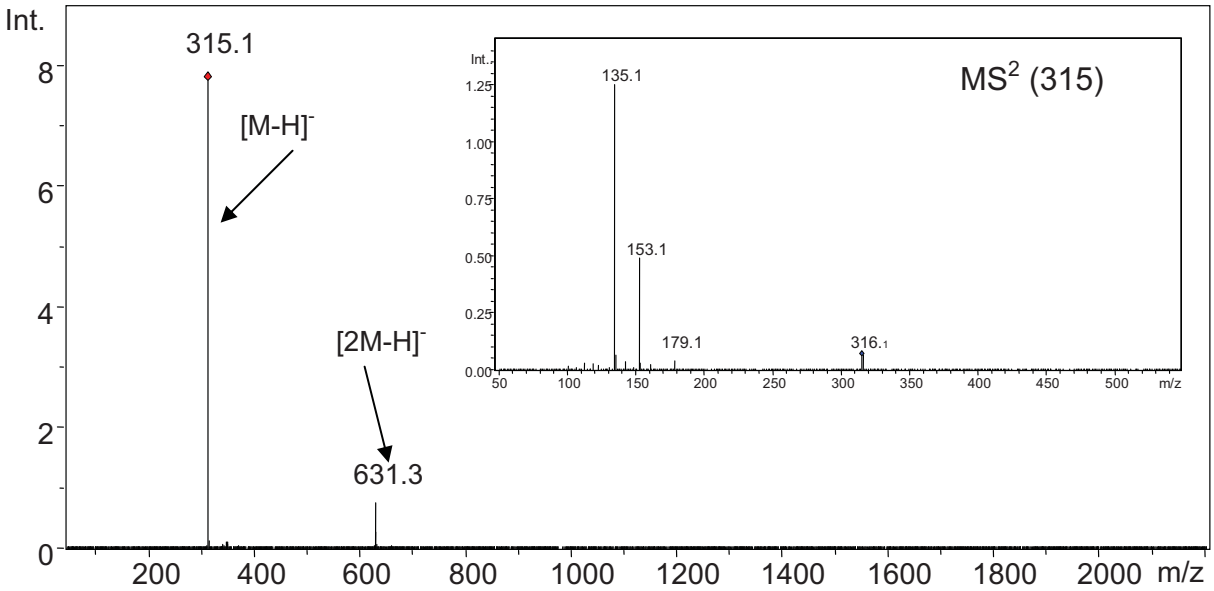


Figure 3-69. LC-ESI-MS and MS/MS (neg. mode) of compound **91** (conditions cf. p. 267) (Intensity x 10^5).

Compound **91** showed more complex NMR spectra as **90** due to the asymmetrical molecule.

The ^{1}H-NMR (300 MHz, CD_3OD) spectrum (cf. Fig. 3-70) of **91** revealed for the carbinol region similar resonances and coupling pattern as observed in structure **90**: e.g. δ 3.66 and 3.84 ppm for the magnetically non-equivalent protons H-6"b/ H-6"a, and H-1'b/ H-1'a at δ 3.68 and 4.01 ppm, respectively. The anomeric signal at δ 4.27 (H-1") with a coupling constant of *J*= 7.7 Hz indicated a β-glucosidic linkage. By direct comparison with resonances in **90** a differing appearance of the aromatic region was observed for **91** indicating a tri-substituted aromatic system represented by three signals at δ 6.54 (H-6 J_1=2, J_2=8 Hz), δ 6.64 (H-2) and δ 6.67 ppm (H-5, *J*= 2.6 Hz). These assignments were verified by a ^{1}H/^{1}H-COSY experiment (cf. Table 3-14, p. 147).

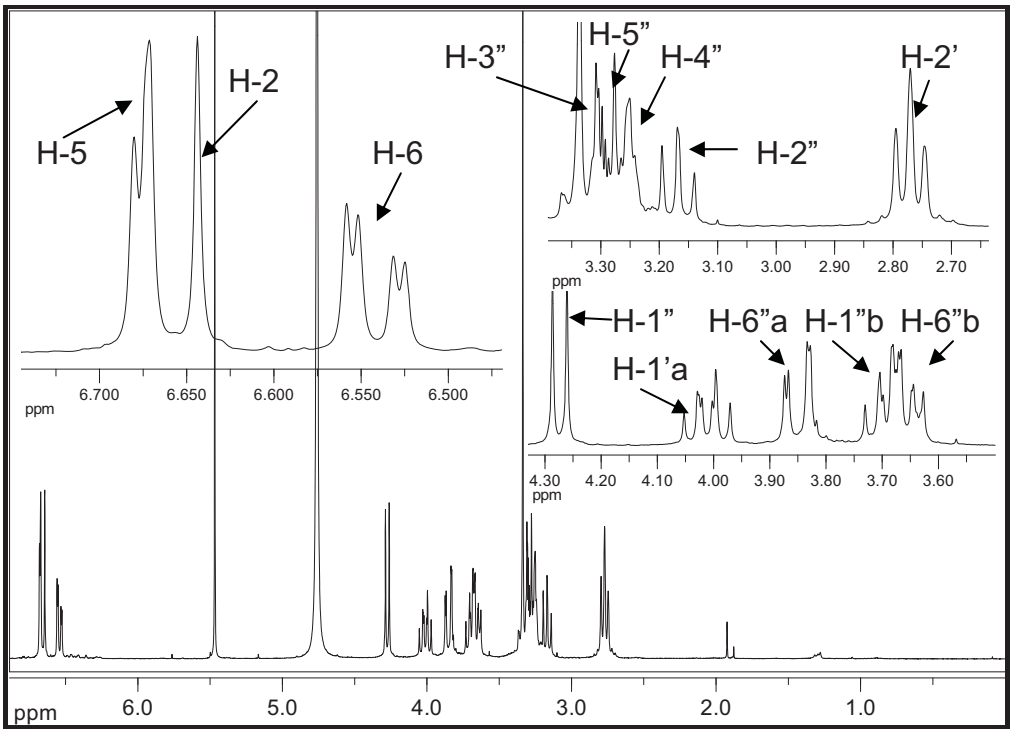


Figure 3-70. ^{1}H-NMR of compound **91** (300 MHz, CD_3OD).

The ^{13}C-NMR spectrum (75.5 MHz, CD_3OD) (cf. Fig. 3-71) showed the characteristical carbinol signals of a glucose unit: δ 78.1 (C-5”), 78.0 (C-3”), 75.2 (C-2”), 71.7 (C-4”), 62.8 (C-6”), and δ 104.4 ppm (C-1”). Identical methylene resonances at δ 36.59 (C-2’) ppm and 72.05 ppm (C-1’) - as observed for **90** – defined a 1’-oxyethyl-substitution in **91**. The quarternary nature of three signals at δ 131.6 (C-1), 144.6 (C-4) and 146.1 (C-3) ppm was confirmed by a DEPT 135 experiment, and corroborated a tri-substituted aromatic system with two phenolic functions in **91**.

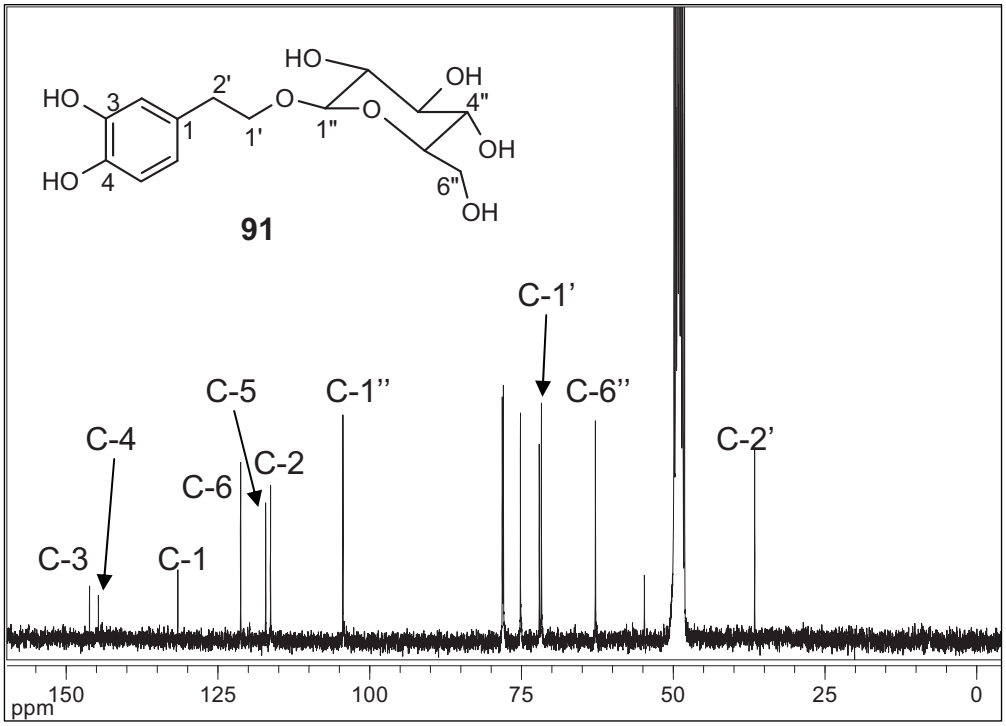


Figure 3-71. ^{13}C-NMR of compound **91** (75.5 MHz, CD_3OD).

In the HMBC-experiment, long-range correlation signals from H-1' to C-1" and in *vice-versa* direction H-1" to C-1' elucidated the glucose moiety to be connected to C-1' of the hydroxytyrosol unit (cf. Fig. 3-72).

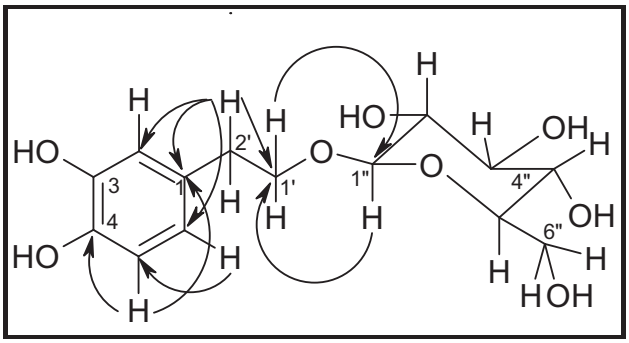
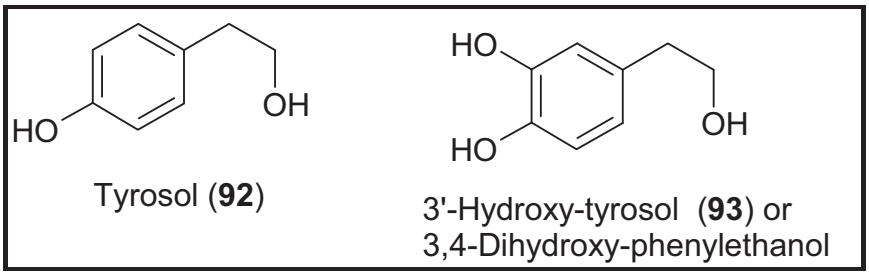


Figure 3-72. Structure relevant $^{2,3}J$ CH long-correlations (HMBC) in structure **91**.

Table 3-14. ^{1}H-, ^{13}C-, DEPT-NMR, and $^{2,3}J$-HC long-range correlations for compound **91** (δ [ppm]; J [Hz]) in CD_3OD).

atom	^{1}H	^{13}C	^{1}H/^{1}H-COSY	$^{2,3}J$-CH
1	-	131.6	-	-
2	6.64 (1H, *s*)	116.3	-	C-1, C-4
3	-	146.1	-	-
4	-	144.7	-	-
5	6.67 (1H, *dd*, J_1=2, J_2= 8 Hz)	117.3	H-2'	C-1, C-4
6	6.54 (1H, dd, J_1=2, J_2= 8 Hz)	121.2	H-2'	C-2', C-5, C-4
1'a	4.01 (1H, *dt*)	72.1	H-1'b; H-2'	C-1"
1'b	3.68 (1H, *m*)		H-1'a; H-2'	-
2'	2.77 (2H, *t*, *J*= 7.3 Hz)	36.6	H-6"a; H-1'b; H-1'a; H-6; H-5	C-1', C-6, C-2, C-1
1''	4.27 (1H, *d*, *J*= 7.7 Hz)	104.4	H-2"	C-1'
2''	3.17 (1H, *dd*, *J*= 7.9, 8.8 Hz)	75.2	H-1"; H-3"; H-5"; H-4"	C-1"
3''	3.30 (1H, *m*),	77.9	-	C-4"
4''	3.25 (1H, *m*),	71.7	H-6"b; H-1'b; H-6"a	-
5''	3.28 (1H, *m*)	78.1	H-1'b; H-6"a	-
6"a	3.66 (1H, *m*)	62.8	H-6"b	C-5"
6"b	3.84 (1H,*dd*, J_1= 2, J_2= 12 Hz)		H-6"a	-

Tyrosol (**92**) and 3'-hydroxy-tyrosol (**93**) are two of the most common phenolic components in olive oil (*Olea europaea* L.). There are various publications and reviews dealing with the origin (Amiot et al., 1989; Uccella, 2003), synthesis (Capasso et al., 1999; Bouaziz and Sayadi, 2003; Soler-Rivas et al., 2000), antioxidant activity (Visioli and Galli, 1998; Medina et al., 2002; Aparicio et al., 1999; Trujillo et al., 2006), biological activities (Obied et al., 2005), and metabolism (Manna et al., 2000; Manna et al., 2005; D'Angelo et al., 2001; Mateos et al., 2005) of this compounds mostly related to substance **93**. Aspects of biosynthesis and biological activities will be discussed later (cf. p. 213 and Table 3-20 p. 196).



Tyrosol (**92**)

3'-Hydroxy-tyrosol (**93**) or
3,4-Dihydroxy-phenylethanol

Both compounds tyrosol and hydroxyl-tyrosol have also been isolated from red and white Italian wines (Di Tommaso et al., 1998). In case of wines, 3'-hydroxy-tyrosol could be formed by a hydroxylation step at the aromatic tyrosol-ring, generated by fermentative processes of yeasts (Soleas et al., 1997).

Deglucosidation of compounds **90** and **91** might enzymatically generate tyrosol (**92**) and hydroxyl-tyrosol (**93**) having a potential utilization as antioxidant for either pharmaceutical and cosmetic preparations as well as ingredients in "functional foods".

To the best of our knowledge both phenolic structures **90** and **91** were not isolated before from avocado fruits or seeds.

3.4.2.3. Proanthocyanidins

Avocado seeds (*Persea americana* Mill. cv *Hass*) collected in Michoacán México (year 2000) were immediately frozen and gently lyophilized. The dried cotyledons (approx. 2 kg) were separated from the testa and kept frozen (-18°C) until extraction. After grinding of the material, defatting with *n*-hexane and subsequent methanolic extraction, crude extracts of testa and cotyledons were recovered and further solvent partitioning of the polar methanolic crude extract of cotyledons resulted in the ethyl acetate partition (cf. Fig. 5-3 extraction scheme p. 225).

HPLC-ESI-MS analyses (neg. mode) of the methanol-water partitions from testa and cotyledons were carried out. Both plant matrices were found to have the same compounds, although the relative concentration is higher in cotyledons. There are two exceptions: signals at 29.7 min (m/z 441) and 31. 8 min (*m/z* 425) are higher in the testa (cf. Fig. 3-73). Some of the compounds were isolated and identified already in the course of this research as quinic acid (*m/z* 191 $[M-H]^-$) verified by comparison with authentic standard material (not presented here), chlorogenic acid (*m/z* 353 $[M-H]^-$), 3-hydroxy-tyrosol-1'-*ß*-D-*O*-glucoside (*m/z* 315 $[M-H]^-$) (**91**) (cf. p. 142), proanthocyanidin monomers (*m/z* 289 $[M-H]^-$), regular dimers (*m/z* 577 $[M-H]^-$, *m/z* 579 $[M-H]^-$) and A-type-linked trimers (*m/z* 863 $[M-H]^-$), as well as abscisic acid derivatives (*m/z* 441 $[M-H]^-$) (**89**) (cf. p. 121).

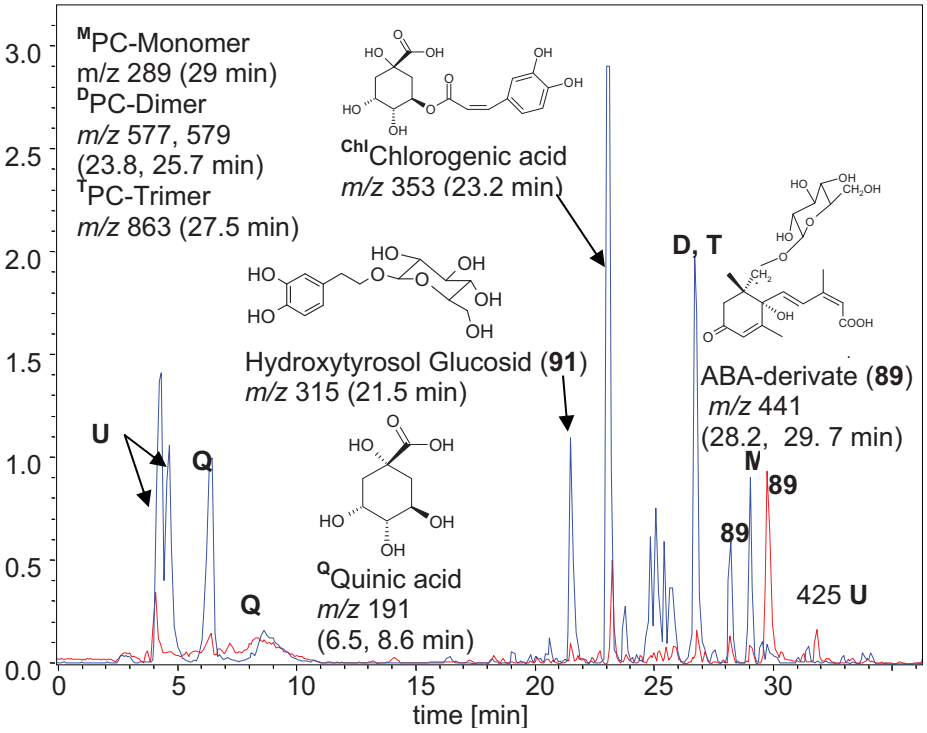


Figure 3-73. LC-ESI-MS (neg. mode) of M:W extracts from avocado seed cotyledons (blue line) and testa (red line). U: Unknown. (Column: Phenomenex RP-18, details cf. p. 238) (Int. x 10^6).

The objective in this part of the study was to isolate oligomeric proanthocyanidins, present in the avocado seed cotyledons, by means of '*high speed countercurrent chromatography*' (HSCCC) and preparative HPLC. The recovery of sufficient sample material is important to perform a complete structural characterization by 1D-/2D-NMR experiments.

Furthermore the methanol-water partition showed in the HaCat assay (not shown in this work) the highest activity. The ethyl acetate partition was chosen because of its high antioxidant activity (by TEAC method) (3983 mmol Trolox/kg extract also as 73% reported) (cf. p. 194, Fig. 3-101 p. 195, and Table 3-20 p. 196) relative to ascorbic acid activity (100%) (cf. Table 3-22). On the other hand the brine shrimp assay showed only very low toxicity (>1000) (cf. Fig. 3-104 p. 203, and Table 3-25 p. 206).

The further fractionation yielded principally proanthocyanidins. This class of compounds that is also referred as non-hydrolizable tannins is widely distributed in the plant kingdom. Proanthocyanidins are present as monomers, oligomers and

polymers. It has been reported that they have physiological (Anderson et al., 2004) as well as various biological activities (Ito et al., 2000; Kolodziej et al., 1999; Takechi et al., 1985), such as e.g. antioxidant (Shahat et al., 2002) and insulin like-biological activities (Anderson et al., 2004).

Methodology for the extraction of the cotyledons was described in the standard protocol (cf. p. 225). Although the methanol-water extract and the ethyl acetate partition from cotyledons were analyzed by two different columns (cf. Fig. 3-74), it was obvious by HPLC-ESI-MS that the ethyl acetate partition contained the proanthocyanidin dimers and trimers in the highest concentration (cf. Fig. 3-74).

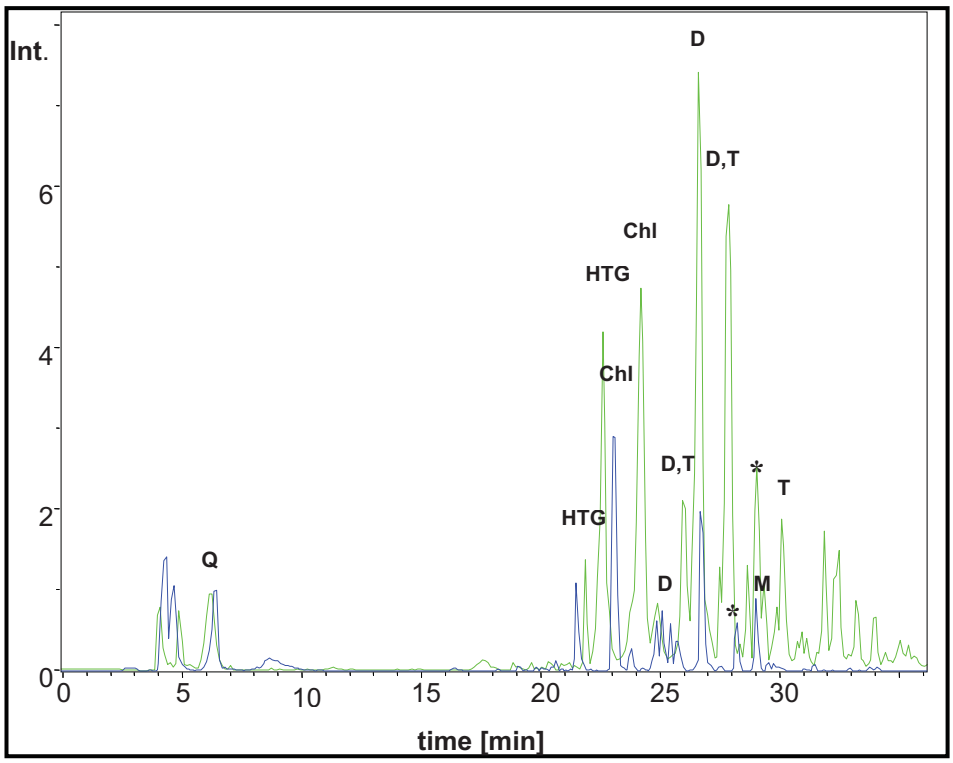


Figure 3-74. HPLC-ESI-MS (neg.mode) of avocado cotyledons M:W extract ([ß]blue line) and Ac partition ([#]green line) (Int. x 10^6). [Q: quinic acid, Chl: Chlorogenic acid, HTG: Hydroxytyrosol glucosid, Proanthocyanidins (M: Monomers, D: Dimers, T: Trimers), ***89**]. [Conditions: A: H_2O, B: ACN. Flow: 0.7 mL min^{-1}. Columns AQ RP-18 250 mm x 4.6 mm. Gradient: [ß]Phenomenex t (0,10,40,45,50 min), A (97,97,40,97,97), B (3,3,60,3,3) and [#]Prontosil t (0,10,40,55,65,75 min), A (97,97,40,0,0,97), B (3,3,60,100,100,3)].

Approximately 2000 mg of the ethyl acetate partition was separated by means of '*high-speed countercurrent chromatography*' (HSCCC) using the biphasic solvent system n-hexane – Ac – MeOH – H_2O (1 : 54 : 1 : 54, v,v,v,v). This system was applied due to the good results of partioning studies of the crude extract. The decision for combining the fractions was done on the basis of UV detection (λ 210 nm) and TLC-monitoring of the fraction tubes. The preparative separation yielded 7 principal fractions 6.1. to 6.7., (cf. Fig. 3-75) with amounts ranging from 17 mg to 769 mg (cf. Table 5-3 separation 21 p. 245). Fraction 6.6. (95 mg) resulted the monomer (-)-epicatechin (**26**) (with an antioxidative activity of 50 % relative to ascorbic acid (cf. Table 3-22 p. 199) in pure form. The highest quantity was found in fractions 6.1. (769 mg) and 6.3. (469 mg). The sample amount applicable to the HSCCC system was limited (2000 mg) due to strong emulsifying effects of the proanthocyanidin structures in the investigated two-phase system.

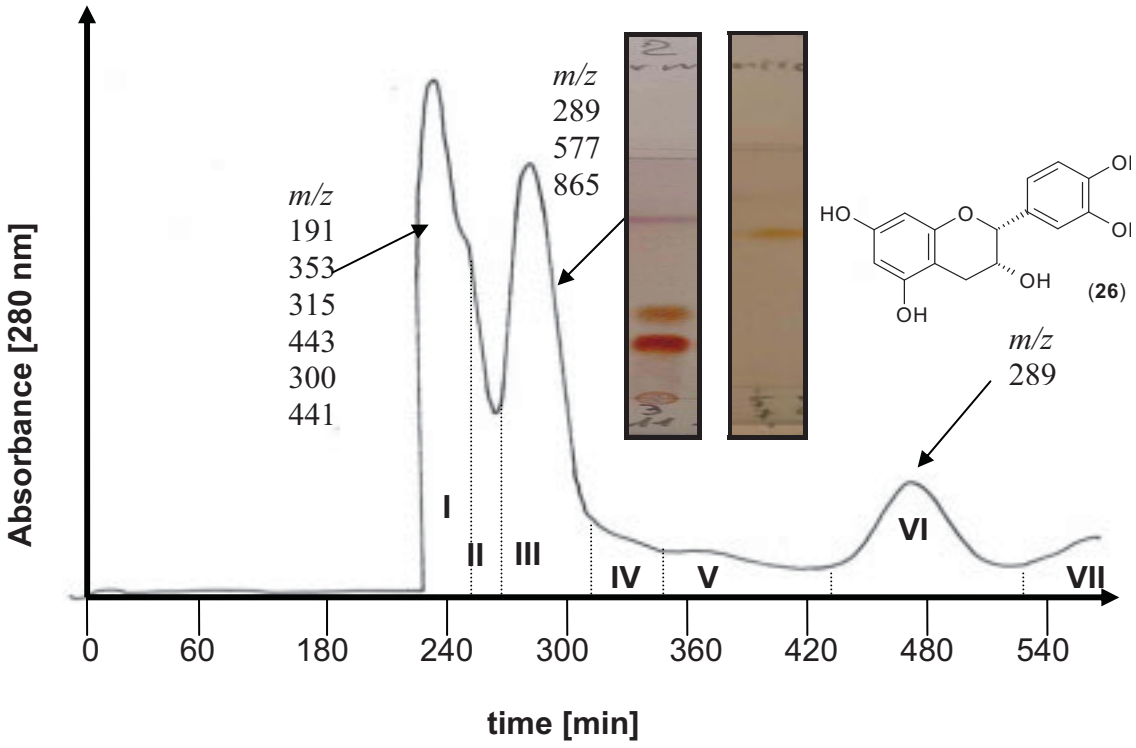


Figure 3-75. HSCCC-separation of 2 g of the ethyl acetate partition from avocado seed cotyledons. Biphasic solvent system: *n*-hexane – Ac-MeOH - H_2O (1: 54: 1: 54, v: v: v: v), flow rate: 3.0 mL min^{-1}. UV-trace: λ=280 nm, rotational speed: 800 rpm; mobile phase: lower aqueous phase; elution mode: *'head-to-tail'*. (TLC: solvent system S5, cf. p. 239).

In the reverse HPLC analysis, (+)-catechin and its oligomers eluted before (-)-epicatechin and its derivates (Bartolomé et al., 1996). Dimers and trimers with a terminal (+)-catechin unit also eluted earlier than the proanthocyanidins analogues with a terminal (-)-epicatechin unit (Santos-Buelga et al., 1995 in Rohr, 1999).

In case of HSCCC, the elution order depends on the composition of mobile phase as well as the polarity of the compounds. In the present case the mobile phase (heavy phase) is very polar and it was expected that the polar compounds elute first.

Reasons for the earlier elution of dimers and trimers in respect to epicatechin, could be an emulsion effect between these oligomers and the most polar compounds (included in signals I and II) due to bridge proton interactions.

Fraction 6.1 contains also the proanthocyanidin polymers, which were not ionisable under the tested HPLC-MS conditions. They were only notable on the

UV_{280} spectra by a broad signal (spectra not shown here) and on the TLC as orange spot remaining at the start point.

The HPLC-ESI-MS (negative mode) (spectra not presented) of fraction 6.1. showed signals $[M-H]^-$ at *m/z* 191 (quinic acid), *m/z* 353 (chlorogenic acid), as well as the already identified compounds *m/z* 315 (3-Hydroxy-tyrosol-1'-β-D-O-glucoside), *m/z* 299 (tyrosol-1'-β-D-O-glucoside) (cf. p. 138), *m/z* 441 [(1'*S*, 6'*R*)-*8'*-hydroxyabscisic acid β-D-glucoside], *m/z* 443 [*(1'R,3'R,5'R,8'S)-epi*-dihydrophaseic acid β-D- glucoside] (cf. p. 130). HPLC-ESI-MS of fraction 6.3. (not presented) showed that this fraction contains the highest quantity of dimeric and trimeric proanthocyanidins. The antioxidative assay revealed that this fraction (469 mg) is the most active (56.6% relative to ascorbic acid activity) (cf. Table 3-20 and 3-22 p.p. 196, 199). Separation by means of preparative RP18-HPLC (cf. Fig. 3-76) resulted in 9 main fractions 6.3.1., to 6.3.9., (cf. Table 5-3 separation 22 p. 245). Two substances in fraction 6.3.4. were identified as the dimeric proanthocyanidin B1 (**94**) and proanthocyanidin B2 (**95**), whereas fractions 6.3.5. and 6.3.6. were pure A-type-linked trimers A2-(+)-catechin (**29**), and A2-(-)-epicatechin (**28**), respectively (cf. Fig. 3-76).

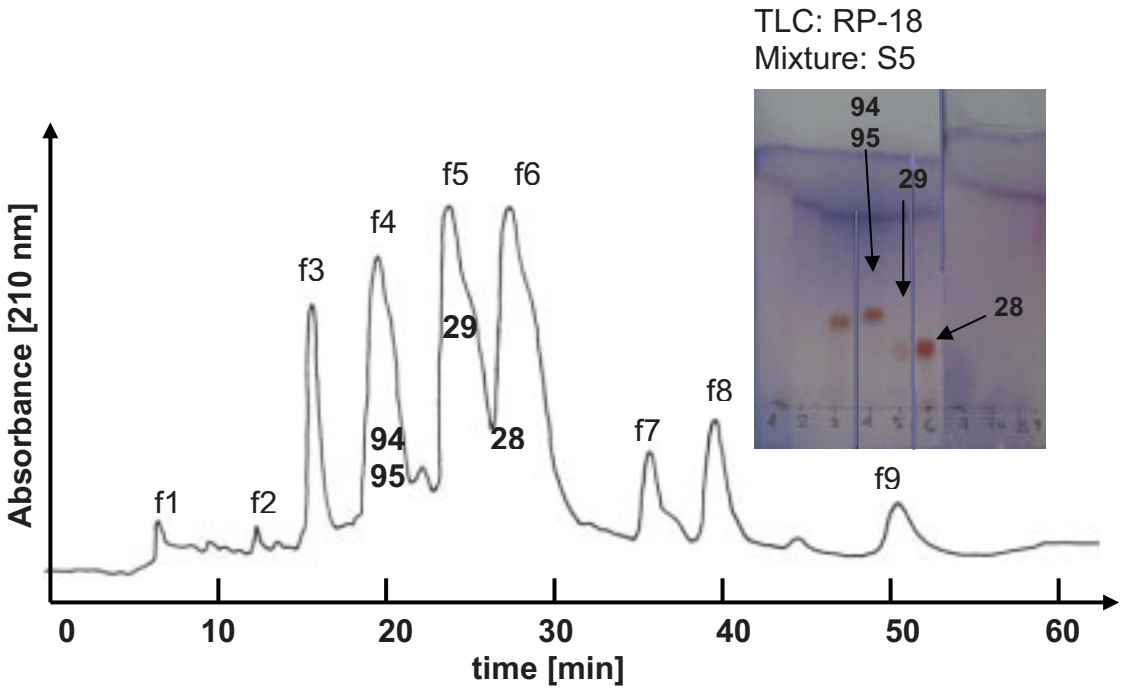


Figure 3-76. Preparative separation by RP18-HPLC of proanthocyanidin fraction 6.3. from HSCCC-separation (avocado seed cotyledons Ac partition). [Prontosil AQ 120-C18, 5.0 µm, 250 x 16 mm, isocratic: ACN/ H_2O (17: 83, v:v). Flow rate 5.0 mL min $^{-1}$, λ= 210 nm].

The structures of the isolated compounds were fully elucidated by spectroscopic methods such as ESI-MS, 1D- and 2D-NMR experiments as well as by chemical degradation using the thiolysis reaction.

The HPLC-MS analysis showed that fractions 6.3.3., as well as 6.3.7. to 6.3.9 contained tetrameric proanthocyanidins (*m/z* 1151 to 1197 $[M-H]^-$) but they were not pure. Also the quantity of these fractions was not high enough (cf. Table 5-3 p. 245) for further NMR-analysis.

For structural characterization of proanthocyanidins – in almost all cases – a combination of different spectroscopical techniques is neccesary. For molecular mass determination, liquid chromatography-electrospray-MS (HPLC-ESI-MS) is a versatile technique. Most frequently the negative ion mode is used for the detection of monomers (epicatechin, catechin, etc.) and oligomeric structures (proanthocyanidin A- and B-type). For the investigation of the constitution of molecules, including the elucidation of interflavanoid connections, different 1D- and 2D-NMR experiments (Shoji et al., 2003) are the methods of choice. The use

of circular dichroism spectroscopy (CD) finally gives more insight into stereochemical properties of the structures.

Some difficulties may arise during structure elucidation in determination of the interflavan connections: upper and lower flavonol units are more frequently connected by 4β→8 interflavanoid bonds than by 4β→6 linkages.

The most important NMR-signals for proanthocyanidin identification are positions C-2, C-3 and C-4. Restricted rotation of the monomers around the interflavanoid bond is causing atropisomerism. Generation of the peracetates for NMR-spectroscopy is reported to lower this effect (Porter et al., 1982).

Atropisomerism depends on solvent and temperature and is not always predictable. It depends also on stereochemistry of the intermolecular linkage between the 3-flavan-ol units, α-configuration (proanthocyanindin B-3 and B-4) or β-configuration (proanthocyanidin B-1 and B-2) causing steric interferences (Haslam, 1998) as well as the involucred carbon atoms in the interflavan linkage (4→8) or (4→6) (Nonaka et al., 1982).

In case of the linked (4→8) proanthocyanidins broad signals in the ^{1}H-NMR spectrums are obtained. These are due to a slower rotation of the aromatic ring caused by steric interactions between the B-rings. On the contrary, in case of proanthocyanidins with (4→6) interflavan linkage as such proanthocyanidin B-5, there is a faster rotation resulting in sharp signals in the ^{1}H-NMR spectrum (Nonaka et al., 1982). Thus the atropisomerism effects are important for structure elucidation of proanthocyanidins.

Another very common method for the identification of the monomer units in an oligomer or polymeric proanthocyanidin is the chemical degradation by acid catalyzed thiolysis with the nucleophil benzylmercaptane (*syn.* toluene-α-thiol) (Furuichi et al., 1986; Nonaka et al., 1982).

In our investigations combination of HPLC-ESI-MS, 1D-/ 2D-NMR, CD spectroscopy, thiolysis degradation and peracetate derivatization led to the full characterization of procyanidins.

Another general but very important aspect is the nomenclature of proanthocyanidin. They are complex structures due to the existence of different stereochemical configurations at positions C-2, C-3 and C-4 of the flavanol units.

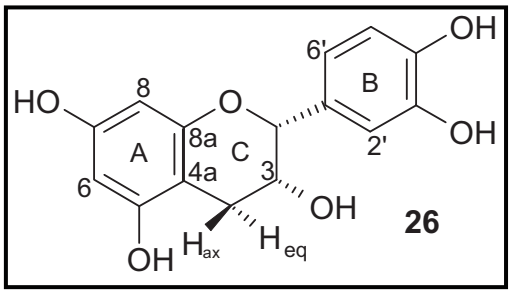
Hemingway et al. (1982) proposed basic rules naming proanthocyanidin oligomers in analogous manner to oligo- and polysaccharides. Thus C-4 in proanthocyanidins is the equivalent to C-1 of sugars due to the inter linkages bonds between more than two units.

Porter et al. (1982) and Hemingway et al. (1982) named the flavonols as upper (U), middle (M) or lower (L) units, in the proanthocyanidin structure, while Shoji et al. (2003) name this units as C, B and A, respectively. The last system might cause confusion because normally the nomenclature of each flavan-3-ol unit is specified as C, B and A for their ring systems (cf. **26**).

In this work, we will use the nomenclature suggested by Porter et al. (1982). In a slight modification specifying the flavan-3-ol rings as A, B, C, D, E, F. Lou et al. (1999) and Hatano et al. (2002) allocated the letters A, B and C for the top unit, further D, E, F for the middle unit and G, H, I for the bottom unit, in this case defining the ring systems of a trimeric proanthocyanidin. For higher oligomers with more flavonol units the system will continue in alphabetical order.

3.4.2.3.1. (-)-Epicatechin (**26**)

95 mg of epicatechin (**26**) were recovered as a white and slightly pink powder. (-)-Epicatechin revealed orange-red colouring properties with anisaldehyde spray-reagent (Stahl et. al, 1967) indicating a flavonoid structure.



26

HPLC-ESI-MS (neg. mode) analysis of **26** (cf. Fig. 3-77) in comparison with an authentic standard of (-)-epicatechin showed the signal $[M-H]^-$ at *m/z* 289 identical with the data of authentic epicatechin (HPLC-retention time: 6.5 min).

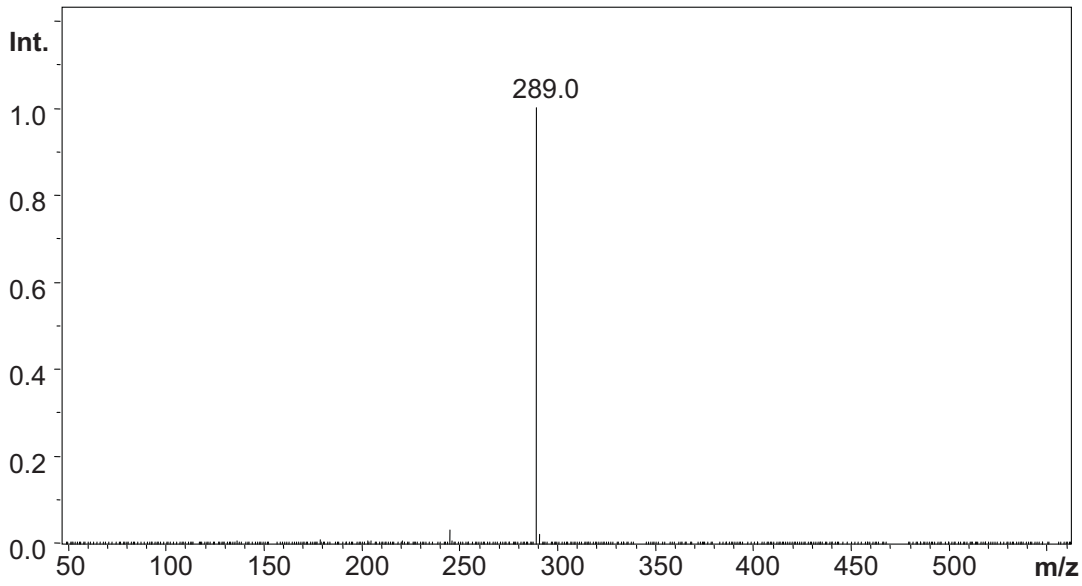


Figure 3-77. HPLC-ESI-MS (neg. mode) of **26** (Intensity: x 10^7) [Column: Zorbax C-18 SB 150 x 4.6 mm I.D. and precolumn 3.5 µm. Solvent A: 2.5% CH_3COOH in H_2O and solvent B is a mixture of ACN and solvent A (80:20, v/v). Flow rate: 0.8 mL min^{-1}, wavelength λ= 280 nm].

The ^{1}H-NMR spectrum (300 MHz, CD_3OD) of (-)-epicatechin (**26**) (cf. Fig. 3-78) exhibited three characteristic regions of a flavan-3-ol system: two characteristic aromatic protons with meta-couplings [*J* = 2.0 Hz] at δ 5.92 (H-6) and δ 5.95 (H-8) indicated a 5, 7-dihydroxylated flavonoid A-ring system. Two proton signals at δ 2.73 (*dd*, J_1= 14 Hz, J_2= 3 Hz) and at δ 2.89 ppm (*dd*, J_1=12 Hz, J_2=4.5 Hz) were related to the C-4 methylene group with a large geminal coupling constant in the B-ring moiety. The coupling partner H-3 appeared as multiplett at δ4.18 in addition to the broad singlet H-2 resonance at δ4.82 ppm. The ^{1}H-NMR data (d values and coupling constants) already suggested the stereochemistry of epicatechin at C-2 and C-3.

Three proton signals at δ 6.76 ppm (H-5'), 6.80 ppm (H-6') and 6.98 ppm (H-2') confirmed a tri-substituted B-ring in substance **26** (cf. Material and methods p. 268).

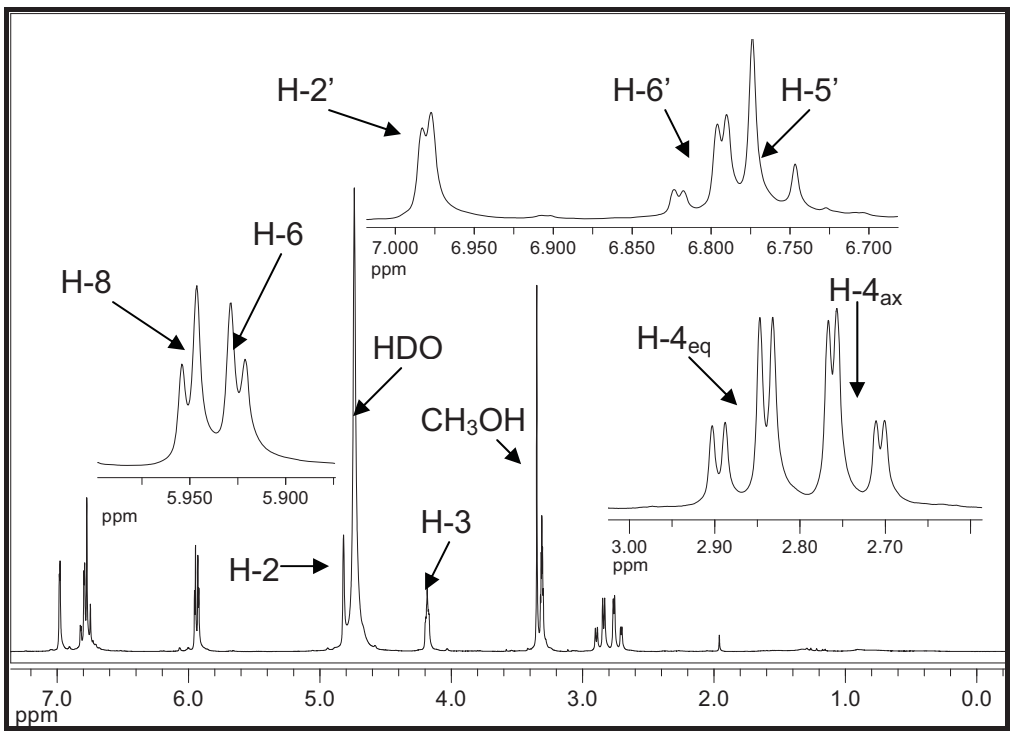


Figure 3-78. ^{1}H-NMR spectrum of **26** (300 MHz, CD_3OD).

The ^{13}C-NMR (75.5 MHz, CD_3OD) spectrum of **26** (cf. Fig. 3-79) showed 15 carbon atoms. By a DEPT135 experiment, seven quaternary C-atoms [δ= 100.15 ppm (C-4a), 132.3 (C-1'), 145.78 (C-3'), 145.95 (C-4'), 157.36 (C-5), 157.68 (C-8a), and 157.97 (C-7) ppm] were assigned, seven methine groups [δ 67.49 (C-3), 79.89 (C-2), 95.95 (C-6), 96.49 (C-8), 115.36 (C-2'), 115.94 (5'), 119.44 (C-6')] and a single methylene signal at δ 29.21 pm (C-4).

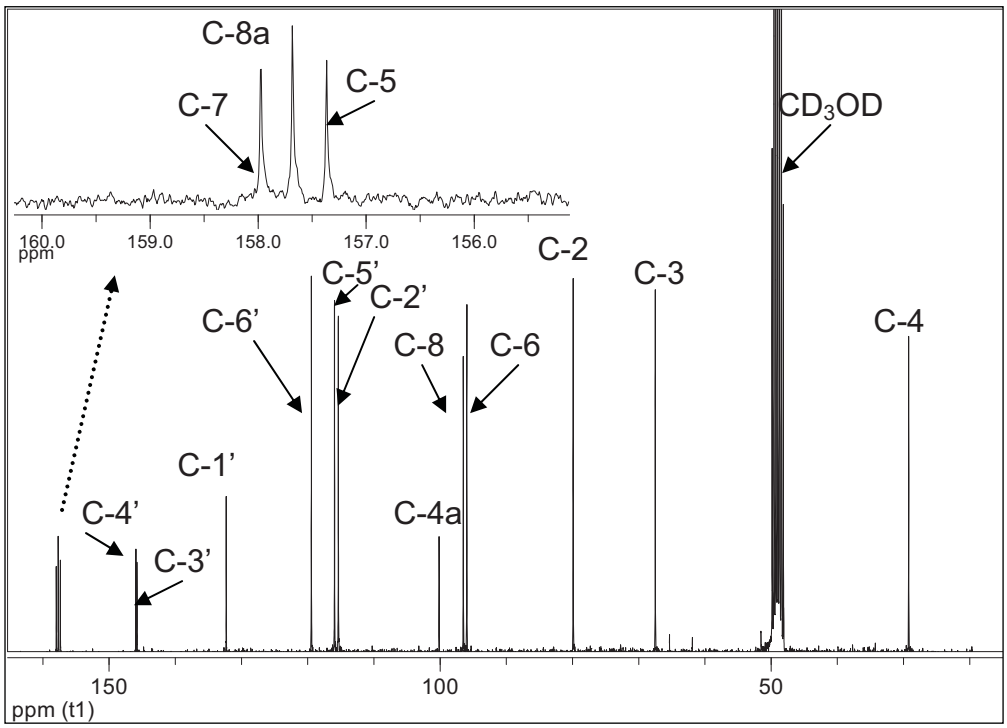
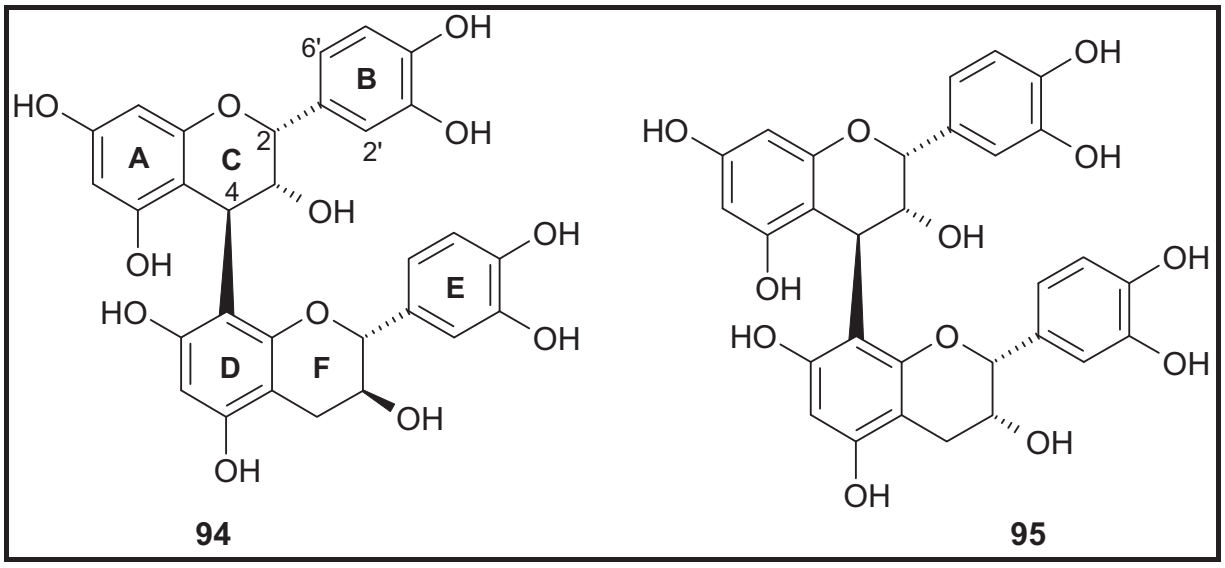


Figure 3-79. ^{13}C-NMR spectrum of compound **26** (75.5 MHz, CD_3OD).

^{1}H- and ^{13}C-NMR data of compound **26** were in excellent accordance to literature data (Höhn, 1998; Nonaka et al., 1982) identifying the flavan-3-ol monomer (-)-epicatechin.

3.4.2.3.2. Epicatechin (4β→8)-catechin (*syn*: Proanthocyanidin B-1) (**94**) and **3.4.2.3.3.** Epicatechin (4β→8)-epicatechin (*syn*: Proanthocyanidin B-2) (**95**)

16 mg of fraction 6.3.4. resulted in a mixture of compounds **94** and **95** as a brilliant grey-white powder. This fraction was identified by LC-ESI-MS (neg. mode) as proanthocyanidin dimers with retention times of 2.0 min and 5.4 min, respectively (cf. Fig 3-80). The ion signal $[M-H]^-$ at *m/z* 577 indicated only one connecting interflavan bond (type B). The ESI-MS spectrum (pos. mode) (not presented here) of the peracetate of **94** und **95** showed a quasimolecular ion signal $[M+Na]^+$ at *m/z* 1023 confirming the molecular weigth of Mr 578 Da.



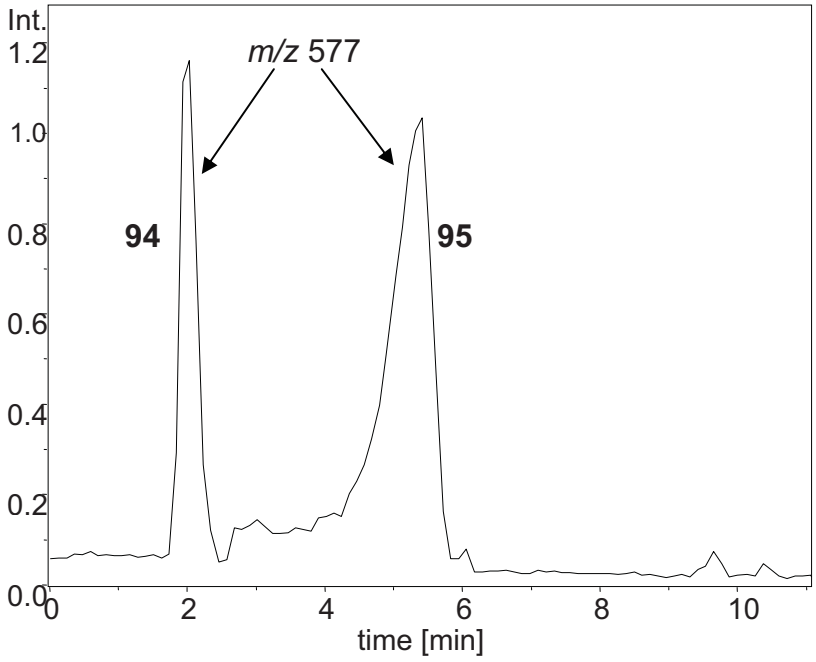


Figure 3-80. LC-ESI-MS (neg. mode) resulting in *m/z* 577 $[M-H]^-$ for proanthocyanidin dimers **94** and **95** (Int. x 10^6). [Column: Zorbax C-18 SB 150 x 4.6 mm I.D. Precolumn 3.5 µm; λ= 280 nm. Gradient: A= 2.5% CH_3COOH in H_2O, B= ACN: A (80:20). Flow rate: 0.8 mL min^{-1}].

^{1}H-NMR (300 MHz, CD_3OD) spectra of **94** and **95** appeared very similar in the mixture, consequently it was not possible to differentiate and identify the linked flavonol units solely by data derived from the ^{1}H-spectrum. Nevertheless, in both

compounds detection of the broad signals H-2, H-3, (rings C and F) were visible as well as the resonances for the terminal unit (H-4 $_{ax}$ and H-4 $_{eq}$) (cf. Table 3-15, p. 181).

Resonances at δ 4.65 and 4.60 ppm were assigned to the proton positions H-4/ ring C in the compounds **94** and **95,** respectively. For both compounds the broad singlet signals of the methine position H-4 suggested the presence of a 4→8 interflavan linkage with β–configuration (Nonaka et al., 1982). Interestingly, the interflavanoid linkage is causing a significant down-field shift for H-2 (ring C). In the case of dimers such as B-1 (epi-4β → 8-cat) and B-2 (epi-4β → 8-epi) resonances at δ 5.10 ppm and δ 4.87 ppm are observed.

The remaining ^{1}H-signals appeared similar to the proton data of (-)-epicatechin (**26**) (^{1}H-NMR-data: cf. Table 3-15, p. 181).

A better differentiation of **94** and **95** is possible by ^{13}C-NMR data (75.5 MHz, CD_3OD) detecting the epimeric flavonol units in the terminal part of the dimers. Compound **94** revealed the presence of one (+)-catechin unit in its structure with the characteristic resonance of C-2 (δ 81.8 ppm), whereas compound **95** was composed only of (-)-epicatechin units recognized by two C-2 resonances at δ 77.1 and 79.9 ppm (Porter et al., 1982) (cf. Table 3-16, p. 182). Identification of upper and terminal units was corroborated by thiolysis reaction.

All of the ^{1}H-NMR (300 MHz, CD_3OD) and ^{13}C-NMR (75.5 MHz, CD_3OD) data of compounds **94** and **95** were in good agreement with published data (Shoji et al., 2003) for proanthocyanidins B-1 and B-2, respectively.

To ensure the chemical identification of the flavonol units occurring in both dimers, acidic catalyzed thiolysis was performed for the mixture of dimers (conditions cf. p. 235).

In case of compound **94**, thiolysis liberated free (+)-catechin (**96**) which was detected by HPLC-ESI-MS confirming this as terminal flavan-3-ol unit. Substance **95** resulted in monomeric (-)-epicatechin (**26**) as terminal unit. The more lipophilic (-)-epicatechin-benzylmercaptan derivative (**97**) eluted later at retention time of R_t 20.6 min (cf. Fig. 3-81).

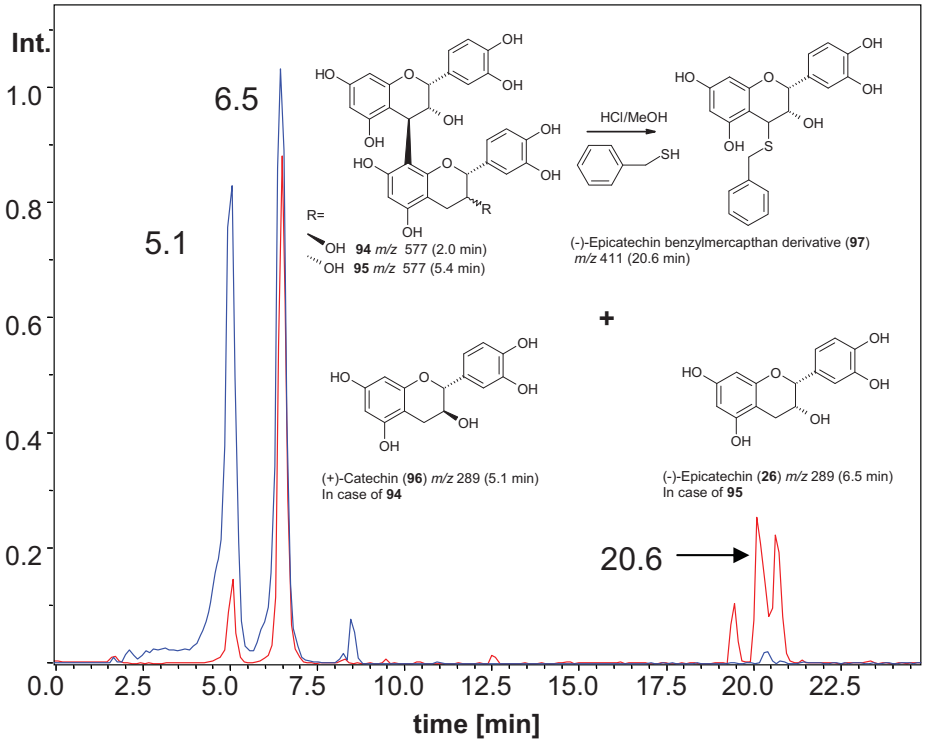


Figure 3-81. LC-ESI-MS (neg. mode) of the thiolysis-degradation products of **94** and **95** (red ion trace) as well as standards catechin and epicatechin (blue ion trace) (Column: Zorbax C-18 SB 150 x 4.6 mm I.D. Precolumn 3.5 µm; λ= 280 nm. Gradient of: A= 2.5% CH_3COOH in H_2O, B= ACN: A (80:20). Flow rate: 0.8 mL min^{-1}). (Intensity x 10^7).

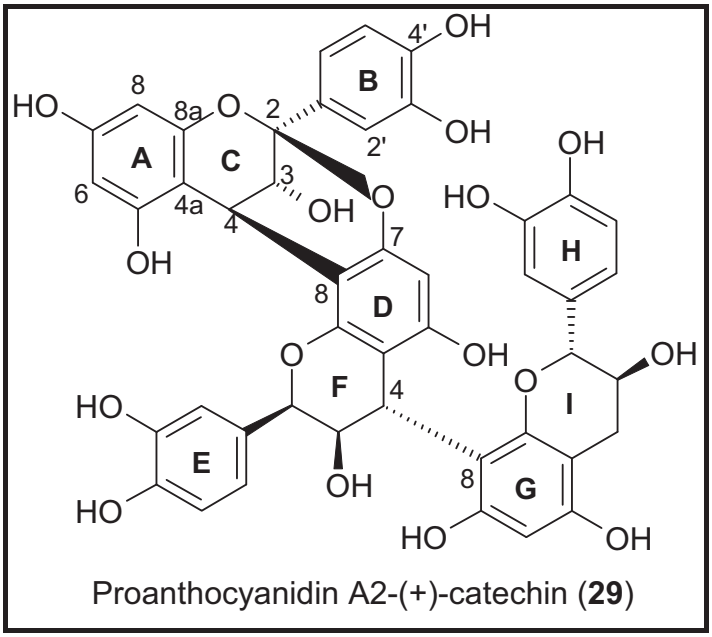
Spectroscopical data (MS, NMR) and thiolysis degradation experiments confirmed the structures of proanthocyanidins B-1 (**94**) and B-2 (**95**), respectively, which were already reported as major components in avocado fruit flesh, leaves and seed material (Thompson et al., 1972).

3.4.2.3.4. A-type proanthocyanidins

3.4.2.3.4.1. Epicatechin [4β→8, 2β→*O*→7]-epicatechin-[4β→8]-catechin (*syn*: Proanthocyanidin A2-(+)-catechin) (**29**)

Separation of the ethylacetate partition by HSCCC and preparative HPLC resulted in the trimeric proanthocyanidin **29** (fraction 6.3.5., cf. p. 245) which was obtained

as an amorphous grey powder (41 mg). All spectroscopical data (ESI-MS, 1D-/ 2D-NMR, CD, $[\alpha]_D$) led to the proposition of the following structure:



Proanthocyanidin A2-(+)-catechin (**29**)

The signal $[M-H]^-$ of high abundance at *m/z* 863 (ESI neg.) and the corresponding ion adduct $[M-Na]^+$ at *m/z* 887 (ESI pos.) verified the molecular weight Mr of 864 Da. This suggested a proanthocyanidin trimer solely constituted of epicatechin or catechin monomer units.

In negative ESI-mode, the MS^2-experiment of the base peak *m/z* 863 resulted in a characteristic fragment ion of proanthocyanidins at *m/z* 289 and one abundant ion signal at *m/z* 573 for a dimeric partial structure indicating the loss of a single flavan-3-ol unit.

Normally the interflavan bonds in proanthocyanidins are located between the C-ring (flavonol ring) of the upper unit and the D-ring (phenolic ring) of the middle or terminal unit. Therefore ^{1}H-NMR-signals of H-2, H-3 and H-4 are relevant data for structure elucidation of proanthocyanidins including the interflavanoic connectivities. The presence or absence of the H-2 resonance δ 4.82 ppm (ring C) gives information about the existence of an additional interflavan 2→7 bond (A-type proanthocyanidin) (cf. Fig. 3-82).

The ^{1}H-NMR spectrum (CD_3CN, 300 MHz) of **29** (cf. Fig. 3-82) revealed two methylene proton signals at δ 2.35 and δ 2.94 ppm in the terminal flavonol unit of the A-type trimer (H-4_{ax}/I and H-4_{eq}/I). Proton signals at δ 4.35 and 3.85 ppm were assigned to H-4/F and H-4/C.

H-3/I, H-3/F and H-3/C were identified as broad singlets at δ 3.70, 4.05 and 3.35 ppm respectively. Singlet signals at δ 3.90 and 5.42 ppm were assigned to H-2 for ring-I and the F-ring, respectively. The absence of H-2 signal in the C-ring [for a B-type proanthocyanidin at δ 4.95-5.05 ppm, (broad singulet)] indicated the presence of an interflavan linkage to C-2 (Hatano et al., 2002), in addition to the interflavan C4$\rightarrow$C8 bond.

The aromatic signals in the ^{1}H-NMR signals were related to H-6, H-8, H-2' and H-6' (δ 5.80 to 7.30 ppm) of the ring systems A, B, D, E, G, H (cf. Table 3-15, p. 181 and materials and methods p. 271). In conclusion, ^{1}H-NMR and ESI-MS data indicate an A-type proanthocyanidin. All assignments discussed for proton data were corroborated by 2D-heteronuclear correlation experiments (HMQC, HMBC).

The ^{1}H-NMR reference data for similar compounds as **29** were given in *d*-DMSO at 150°C (Nonaka et al., 1982) or in *d*-aceton at 40°C (Hatano et al., 2002) in order to reduce the presence of rotamers.

In this work all the NMR spectra of proanthocyanidins were measured in d-MeCN because of a better stability in case of long-time experiments (HMBC, HMQC).

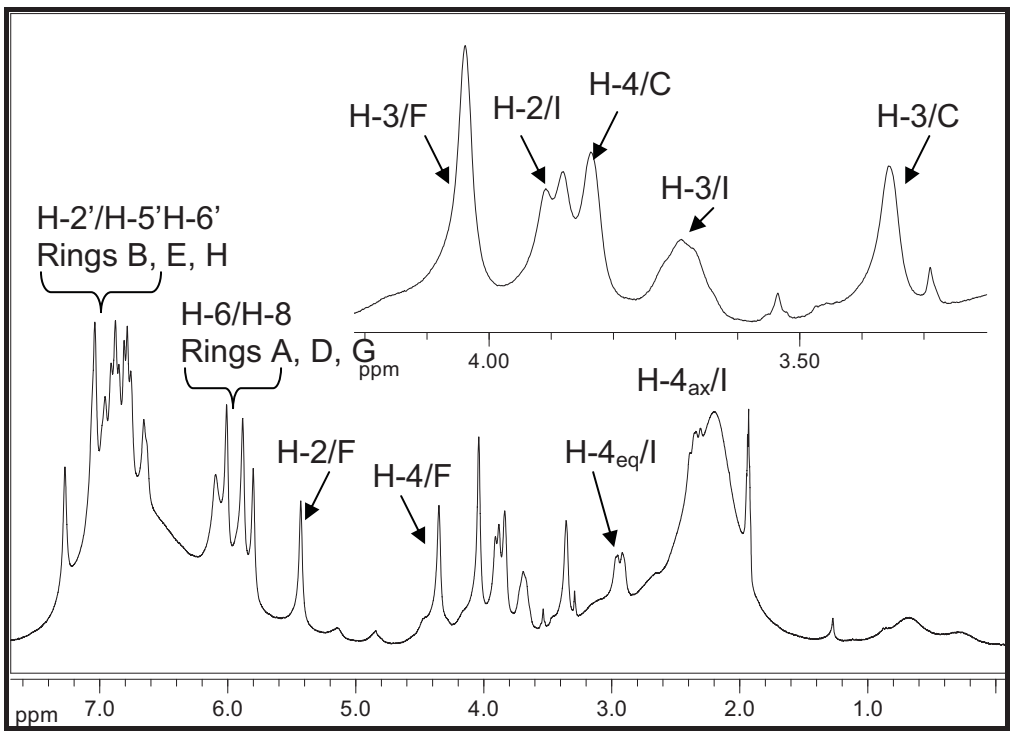


Figure 3-82. ^{1}H-NMR spectrum of **29** (CD_3CN, 300 MHz).

The ^{13}C-NMR spectrum detected 45 carbon atoms consisting of one methylene (C-4/C) at δ 28.3 ppm, nineteen methine-groups, and twenty-five quaternary C-atoms (cf. Table 3-16) determined by a DEPT135 experiment. The most important and characteristic signal for the A-Type linkage was assigned to a single acetal carbon signal for C-2/C at δ 104.7 ppm (Lou et al., 1999).

The ^{13}C-NMR spectrum in combination with the correlation signals in the HMBC (cf. Fig. 3-83) revealed that the terminal unit is constituted of catechin. Literature data (Fletcher et al., 1977; Furuichi et al., 1986) reported that signals around δ 79 ppm are related to the C-2 of (-)-epicatechin whereas resonances above δ 81 ppm are indicative for the C-2 of a (+)-catechin. Overall 1D-NMR data already led to the structure of a trimeric A-type proanthocyanidin composed of two (-)-epicatechin (δ 104.7 and δ 78.0 ppm) units, and one (+)-catechin unit (δ 83.1 ppm)

Through HMQC experiments assignments for all protons to the corresponding carbon atoms in **29** were acquired and presented in Figure 3-83.

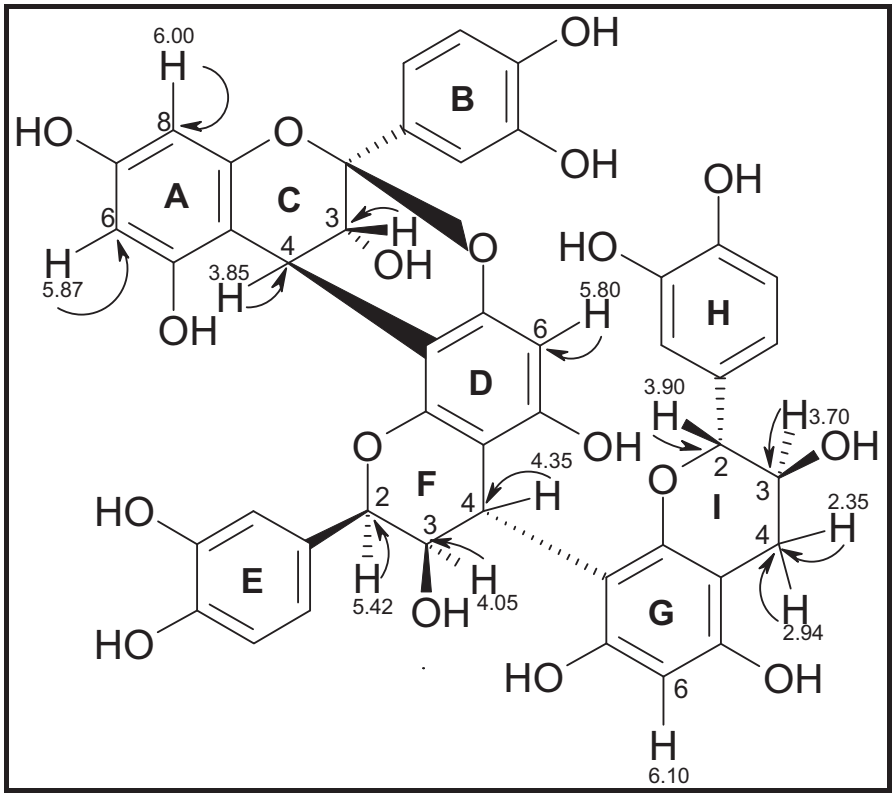


Figure 3-83. Structurally relevant ^{1}J HC-correlations (HMQC) in the A-type trimer **29**.

All observed two- and three-bond HC-connectivities (HMBC) confirmed the postulated structure of the proanthocyanidin A2-(+)-catechin (**29**) and significant long-range HC-cross signals are presented in Figure 3-84.

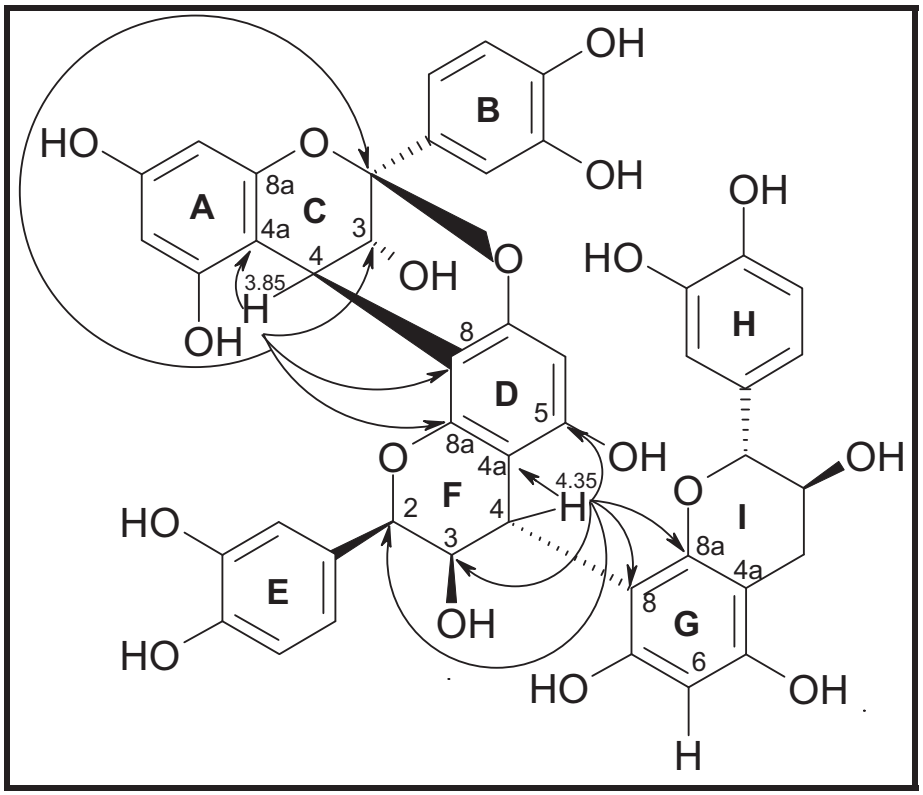


Figure 3-84. Structure determining $^{2,3}J$ HC-correlations (HMBC) in the A-type trimer proanthocyanidin A2-(+)-catechin (**29**).

Most important correlations elucidated the linkages between all three flavanol units. In the HMBC correlation signals from proton H-4/C (δ 3.85 ppm) to [C-8/D (δ 99.8 ppm), and C-8a/D (δ 156.7 ppm)] afforded the connectivity between the upper and middle flavonol units.

Similarily, H-4/F ((δ 4.35 ppm) of the middle unit showed connectivities to C-3/F (δ 71.2 ppm) and to the terminal unit [C-8/G (δ 108.3 ppm), and C-8a/G (δ 155.4 ppm)]. This confirmed that both of the interflavan bonds are (4→8) linkages (cf. Figure 3-85). All long-range HC-correlations are summarized in materials and methods (cf. p. 272).

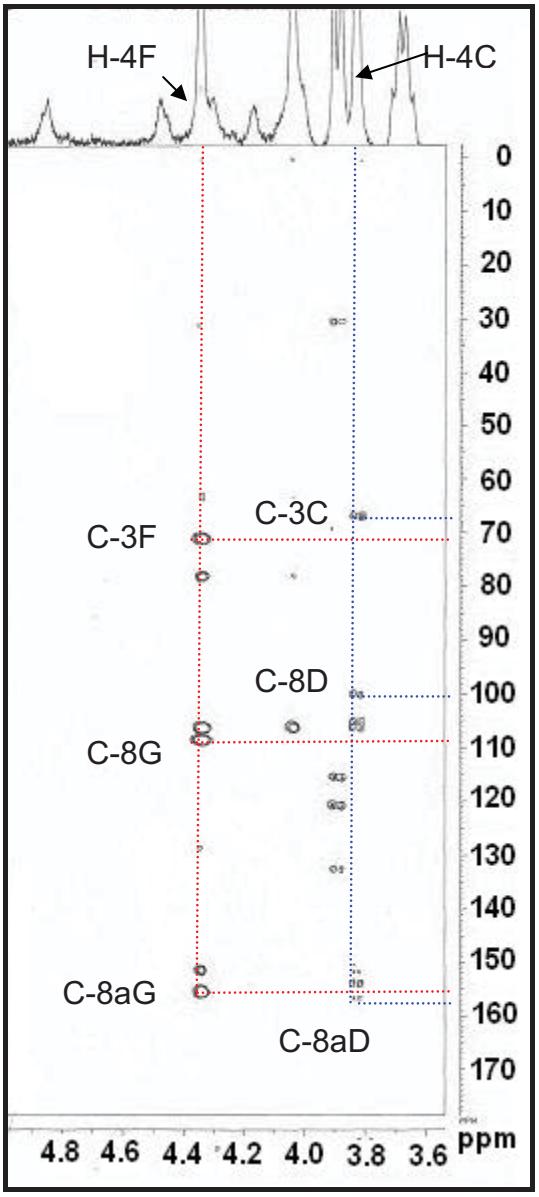


Figure 3-85. Relevant $^{2,3}J$-$^{1}H\{^{13}C\}$ – correlation signals (HMBC) determining the connectivities in the A-type trimer **29** (H-4C to C-3C, C-8D and C-8aD; H-4F to C-3F, C-8G, and C-8aG).

The acid catalyzed thiolytic degradation was performed for structural confirmation of structure **29**. This thiolysis reaction using benzylmercaptane (*syn.* toluene-α-thiol) was applied in modification of the method described by Rigaud et al. (1991) (cf. Materials and methods p. 235).

LC-ESI-MS analysis of the standards catechin and epicatechin appeared with ion signals at *m/z* 289 $[M-H]^-$ (Rt: 4.8 min and 6.2 min, respectively) and the original trimer **29** was detected at *m/z* 863 $[M-H]^-$ (5.6 min).

After thiolysis the cleavage product (+)-catechin *m/z* 289 $[M-H]^-$ with the retention time at 4.8 min was detected, identifying this flavonol as terminal unit of the trimer. During the reaction with the nucleophil the terminal units are liberated without benzylmercaptan-addition. In addition the novel ion signal at *m/z* 697 was detected. The molecular mass matched for the proanthocyanidin-A2 benzylmercaptane derivative (**99**) at *m/z* 697 $[M-H]^-$ (22.5 min) (cf. Fig. 3-86). Although, proanthocyanidin-A2 is resistant to degradation under thiolysis conditions due to the A-type linkage (Thompson et al., 1972), it was also reported (Jacques et al., 1974) that the acidic degradation of proanthocyanidin-A2 resulted in minor products such as (-)-epicatechin (**26**) and the red colored flavylium cation cyanidin (**98**). These signals were observed at *m/z* 289 $[M-H]^-$ (6.2 min) and *m/z* 287 $[M-H]^-$ (20.3 min), respectively.

The ion signal of the residual reagent benzylmercaptane was expected at *m/z* 123 $[M-H]^-$ but was not detected under the applied LC-ESI-MS conditions. However the UV-trace at λ 280 nm clearly detected the reagent at Rt 30.17 min. This experiment corroborated the proposed structure of **29** as the A-type linked proanthocyanidin A2-(+)-catechin.

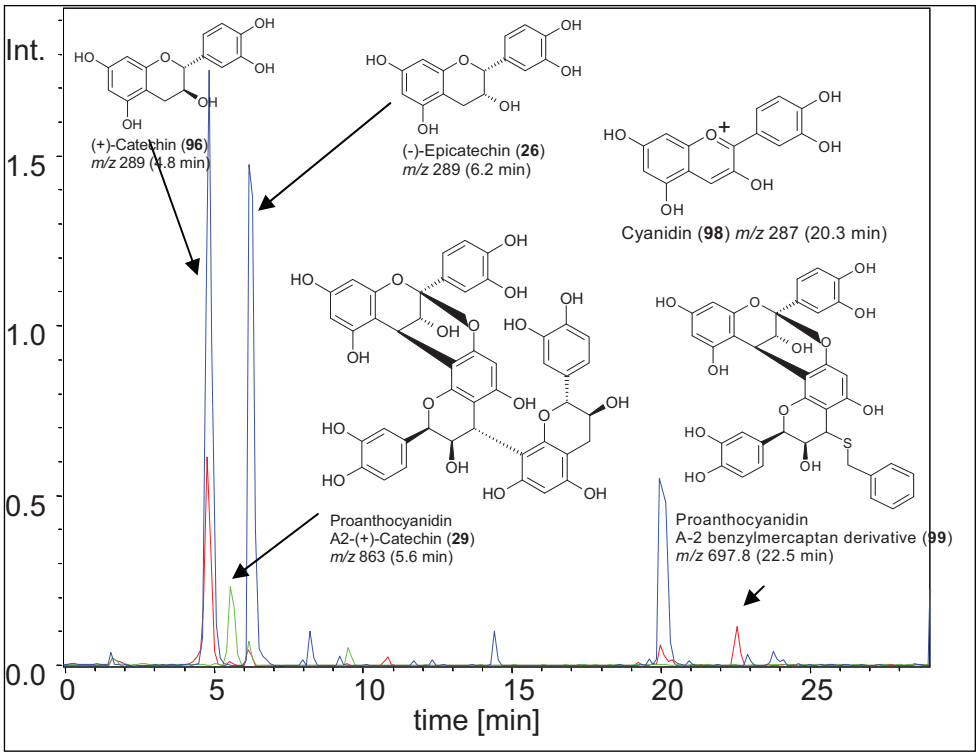


Figure 3-86. LC-ESI-MS (neg. mode) analysis of **29** (green ion trace) and its degradation products (red ion trace) compared versus (+)-catechin and (-)-epicatechin standards (blue ion trace). (Column: Zorbax C18-SB 150 x 4.6 mm. Solvents: A (CH_3COOH 2.5 % in H_2O), B [ACN: Solvent A (80:20)]. (Intensity x10^6).

By means of circulardichroism spectroscopy (CD) the stereochemical orientation of the interflavan bonds (C4→C8) can be determined. A negative cotton effect (around λ= 228 nm) indicates α-orientation (Hatano et al., 2002). In contrary, a positive cotton effect at short wavelengths (λ= 221 nm) in the CD-curve allows assignment of the absolute configuration at C-4 as *R*, meaning a 2β, 4β-configuration (Lou et al., 1999). The CD spectra of proanthocyanidins are largely determined by the interaction of the A-ring chromophores. This effect facilitates the direct assignment of the absolute configuration at C-4 and thus the interflavanoid linkage (Hemingway et al., 1982).

The specific optical rotation of **29** was determined as $[\alpha]_D$ +95^o (*c*=0.1, MeOH) and the absolute stereochemistry of chiral carbon at C-4/C of **29** was derived as *R* by circulardichroism spectroscopy (CD) and strong amplitudes of cotton effect were obtained as molar ellipticity values: $[\theta]_{229}$ + 158437, $[\theta]_{271}$ - 13265 (MeOH) (cf. Fig. 3-87).

The intense Cotton effect at λ 230 nm in **29** confirmed the stereochemistry at C-4/C as *R* having a 4β-configuration according to literature data (Hemingway et al., 1982). For the C-4/F ring, we assume also a 4β-configuration due to a similar chromophoric system.

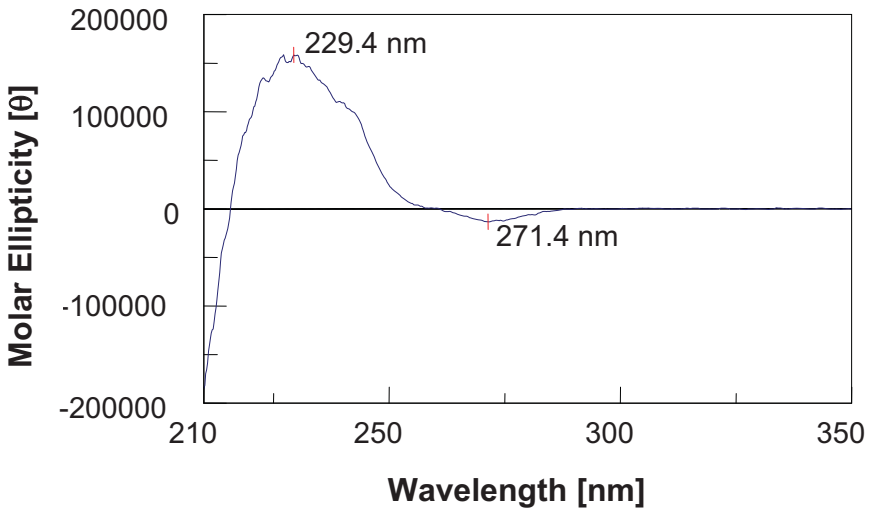


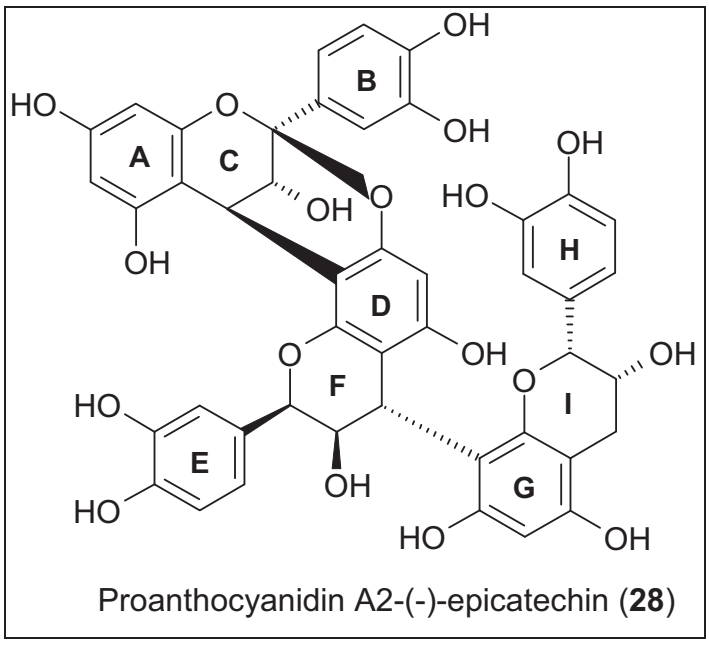
Figure 3-87. CD-curve (in MeOH) and cotton effects observed for proanthocyanidin A2-catechin (**29**) indicating the absolute stereochemistries for C-2 and C-4 as *R*-configurations (for details cf. text and materials and methods p. 239).

Based on spectroscopic data, 1D- (^{1}H-, ^{13}C-, DEPT) and 2D-NMR including heteronuclear correlation experiments (HMQC, HMBC), chemical degradation experiments (thiolysis) and circulardichroism spectroscopy, compound **29** was fully characterized and identified as proanthocyanidin A2-(+)-catechin.

Compound **29** was for the first time identified in avocado seeds by Thompson et al. (1972) and later better described by Jacques et al. (1974). From *Cinnamon* compound **29** was isolated by Anderson et al. (2004).

3.4.2.3.4.2. Epicatechin-[4β→8, 2β→*O*→7]-epicatechin-[4β→8]-epicatechin (*syn*: Proanthocyanidin A2-(-)-epicatechin) (**28**)

In the same separation sequence of **29**, 43 mg of compound **28** was recovered from the ethylacetate-partition by means of HSCCC and then final isolation by preparative HPLC as a white-grey powder (cf. p. 245). All spectroscopic data (ESI-MS, 1D-/ 2D-NMR) led to the assumption of the following structure:



Proanthocyanidin A2-(-)-epicatechin (**28**)

LC-ESI-MS (neg. mode) analysis of **28** using identical conditions detected also the ion signal of a trimer at *m/z* 863 $[M-H]^-$ at R_t 6.15 min in contrast to substance **29** at R_t 5.6 min. The ESI-MS^2-experiment resulted in identical fragment ions as observed for **29**.

Thiolysis reaction on **28** liberated the degradation product epicatechin as terminal flavonol unit (identified by *m/z* 289 $[M-H]^-$ and the specific retention time value of authentic epicatechin R_t= 6.28 min). The ion-signal *m/z* 697 $[M-H]^-$ (at R_t 22.55 min in the Zorbax column) was related to the epicatechin-A2 benzylmercaptane adduct (at R_t 39.1 min in the Prontosil column) already detected in the degradation of **29**.

In this case thiolysis reaction was not complete so LC-ESI-MS detected residual trimer molecules at *m/z* 863.8 $[M-H]^-$ (R_t 27.2 min). The benzylmercaptane reagent was solely detected by the UV-trace at R_t 30.01 min and did not ionize under the tested ESI-MS (neg. mode) conditions.

The principal degradation products of the thiolysis reactions revealed important structural data – especially for the identification of the terminal units of the two trimeric A-type proanthocyanidins **28** and **29**.

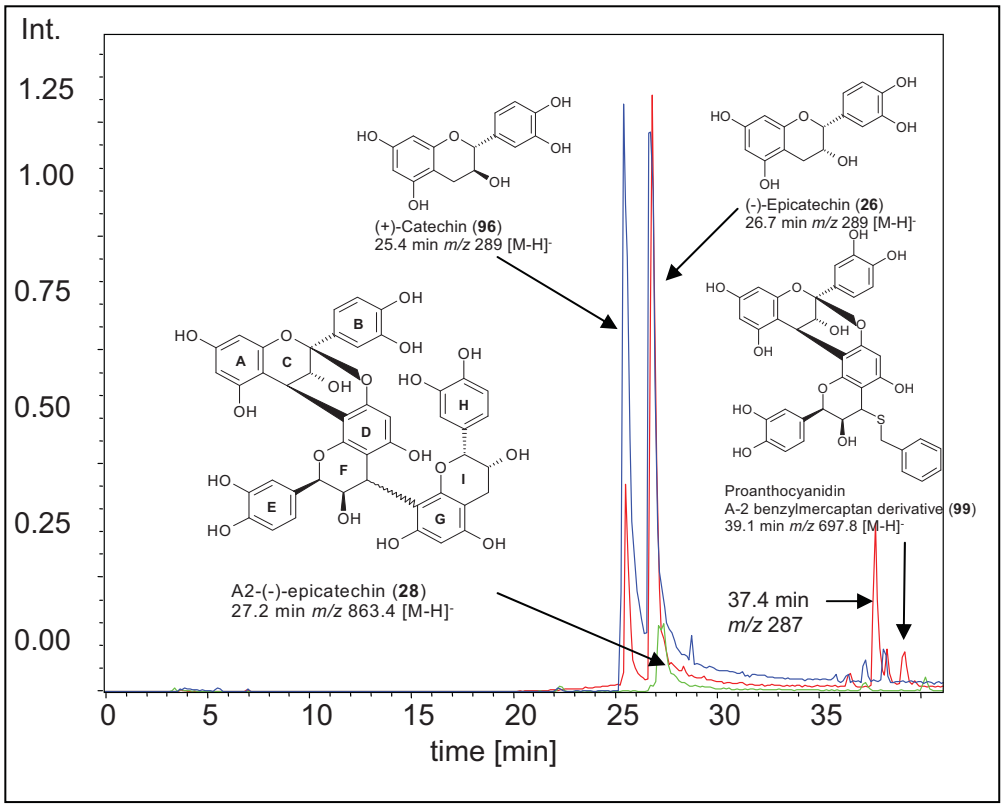


Figure 3-88. LC-ESI-MS analysis (neg. mode) of **28** (green ion trace) and its thiolysis degradation products (red ion trace) compared versus (+)-catechin (R_t: 25.4 min) and (-)-epicatechin (R_t: 26.7 min) standards (blue ion trace). [Column: Prontosil C18 Aq 250 x 4.6 mm x 5µm. Solvents: A: H_2O, B= ACN. Gradient: t (0, 10, 40, 55, 65, 75 min), A (97, 97, 40, 0, 0, 97), B (3, 3, 60, 100, 100, 3). Flow rate = 0.7 mL min^{-1}. (Intensity x 10^6).

The ^{1}H-NMR spectrum (CD_3CN + D_2O, 300 MHz) of **28** (cf. Fig. 3-89) revealed two methylene proton signals of double doublets at δ 2.68 und δ 2.75 ppm with a coupling constant of *J*= 4.2 Hz assigned to H-4_{ax}I and H-4_{eq}I, respectively. These

resonances are characteristic δ-values for the two H-4 atoms of the terminal flavonol unit.

The absence of the H-2 signal in the C-ring of **28** indicated in analogy to substance **29** the presence of an interflavan linkage at C-2 (Hatano et al., 2002); in addition to the interflavan C4→C8 bond. An A-type partial structure in the trimer with linkage between the upper and the middle unit was strongly suggested (cf. Table 3-15 p. 181). There was also a strong accumulance of aromatic proton signals of the different A- and B-rings around δ 6.5-7.5 ppm. Addition of D_2O-traces significantly improved the spectral quality due to H/D-exchange of the hydroxyl-protons but also causing an intense HDO signal in the area δ 2.2-3.0 ppm.

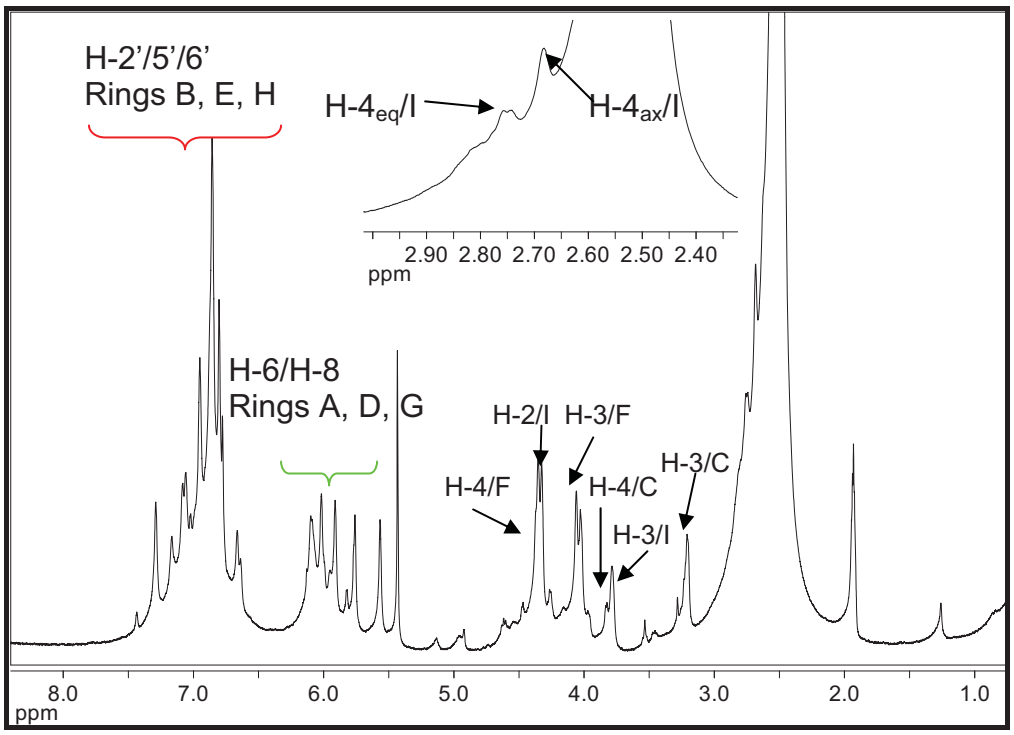


Figure 3-89. ^{1}H-NMR spectrum of **28** (CD_3CN + D_2O, 300 MHz).

^1J-CH connectivities and proton assignments were deduced by a HMQC experiment (cf. Fig. 3-90). H-3/I, H-3/F and H-3/C were identified at δ 3.78, 4.04 and 3.21 ppm, respectively.

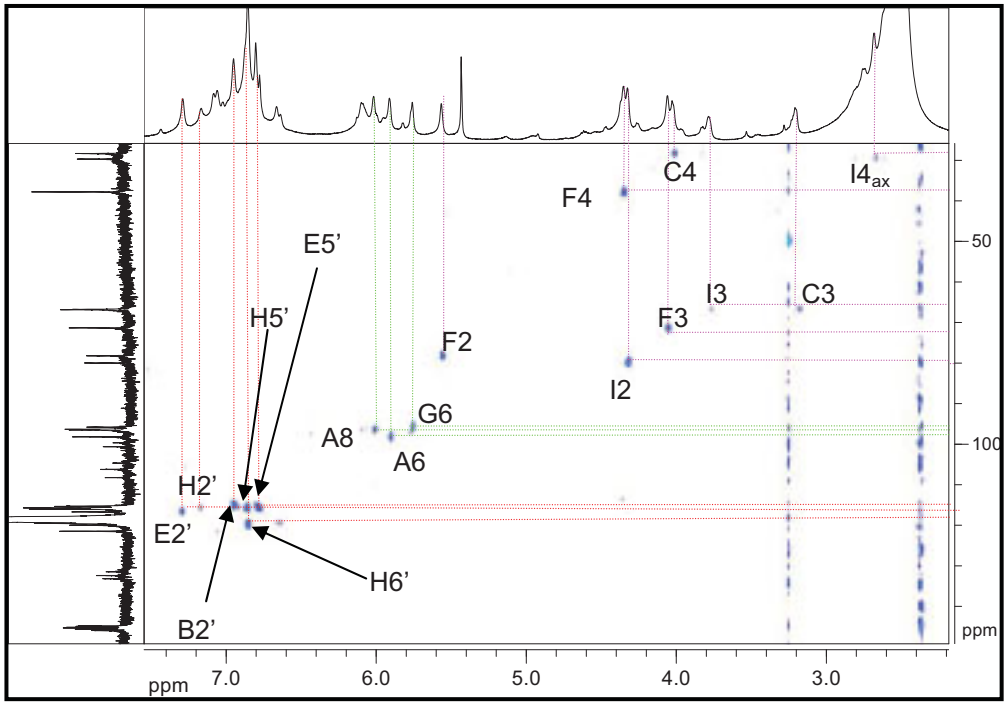


Figure 3-90. Proton assignments by HMQC of **28** (CD_3CN + D_2O, 300 MHz). Nomenclature for HC-relation (letter for ring system, number for position) [Pink lines: rings C, F and I. Green lines: rings A, D and G. Red lines: B, E and H rings. (cf. Materials and methods for details p. 273).

As difference to compound **29** (δ 3.90 ppm), the trimer **28** showed for the H-2 resonance of the I-ring in the terminal unit (catechin) a slightly up-field shift (δ4.31 ppm). Chemical shift values in δ[ppm] of **28** are given in Table 3-15.

The ^{13}C-NMR (75.5 MHz, CD_3CN) spectrum of **28** displayed 45 carbon signals. Thirty-two resonances were well defined but five of them were partly overlayed. Among of them, six quaternary C-atoms were present at small abundances (cf. Fig. 3-91). Indication for epicatechin units are the C-2 signals, with C-2/F (δ 78.2 ppm) and C-2/I (δ79.9 ppm), respectively. Epicatechin has chemical shift values for C-2 below δ 80 ppm (Porter et al., 1982; Furuichi et al., 1986; Fletcher et al., 1977).

On the other hand, the double-linked A-type proanthocyanidin structure in the trimer **28** was also demonstrated by the characteristic acetal carbon at δ 104.7 ppm as described before for other proanthocyanidins A-type analogues (Lou et al., 1999).

Detailed ^{13}C-NMR data of **28** are presented in Table 3-16.

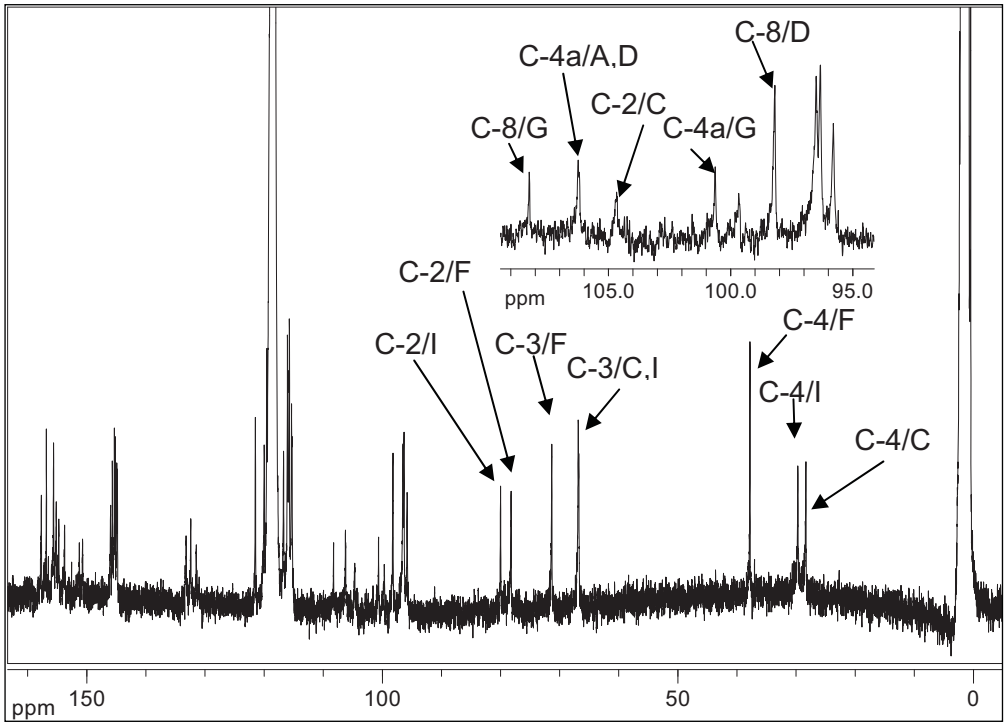


Figure 3-91. ^{13}C-NMR spectrum of compound **28** (75.5 MHz, CD_3CN).

Through a $^{2,3}J$-HC long correlation experiment (HMBC) of **28**, the chemical constitution and correct assignments of C- and H-atoms in the molecule were assured. The most important correlation signals are H-4/C (δ 3.90 ppm) to C-8/D (δ 99.6 ppm) as well as H-3/F (δ 4.04 ppm) and H-4/F (δ 4.34 ppm) to C-8/G (δ 108.2 ppm) determining the interflavan connections between the three flavan-3-ol units (cf. Fig. 3-92). More details about these correlations are given on p. 274.

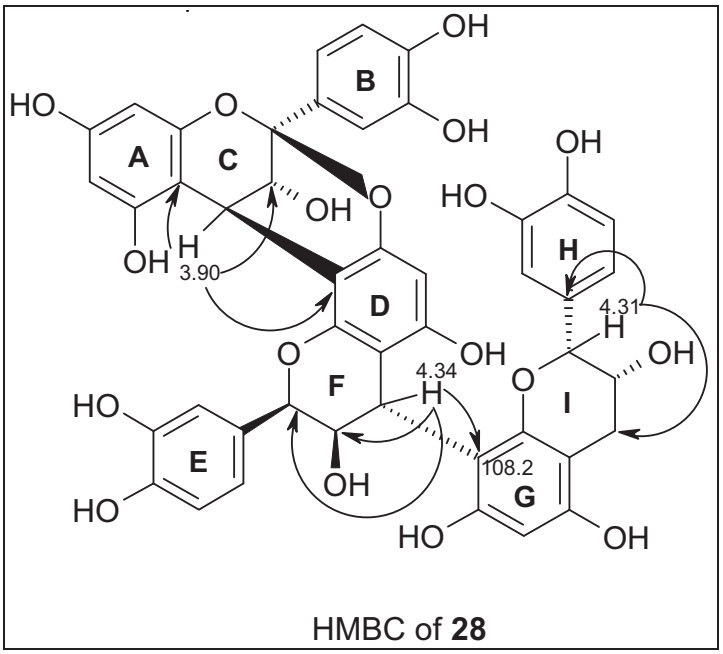


Figure 3-92. Important $^{2,3}J$-CH long-correlations (HMBC) in proanthocyanidin A2-(-)-epicatechin (**28**) ($CD_3CN + D_2O$).

The absolute stereochemistry of the chiral carbon at C-4/C of **28** was determined to be 4*R* due to significant Cotton-effects in the circulardichroism curve (CD). Measurement in methanol resulted in an intense Cotton effect ($[\theta]_{229}$ + 153204, $[\theta]_{272}$ - 14898) near to 230 nm (cf. Fig. 3-93) indicating a 4β-configuration in ring C (Hemingway et al., 1982). Interference of the π-electronic systems between upper and middle unit are in **28** and **29** identical and corroborate similar CD-effects. Interestingly, the appearance of the CD-curve is not influenced by the terminal units with catechin or epicatechin. Epimeric positions of epicatechin and catechin at C-3 in ring-I are without any effect to the CD-appearance in the UV-region of measurement. The epimers - catechin and epicatechin - can be distinguished by opposite cotton effects at λ 207 nm (Höhn et. al, 1998). Nevertheless for the C-4/F ring we assume also a 4β-configuration of the interflavanoic bond to C-8/G due to interaction of similar chromophoric system. For substance **28** we observed a lower value for the specific optical rotation $[\alpha]_D$ +59° (*c*=0.1, MeOH) than for **29** ($[\alpha]_D$ +95°) caused by the exchange of catechin against epicatechin in the terminal unit.

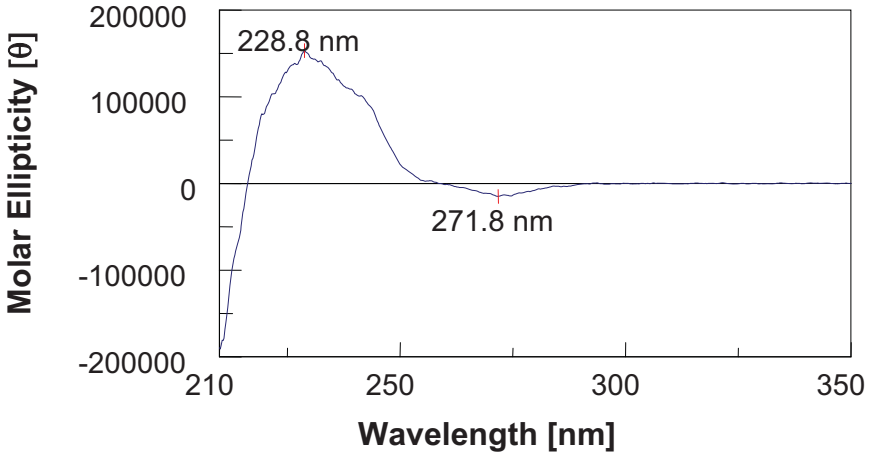


Figure 3-93. CD-curves and cotton effects (in MeOH) observed for proanthocyanidin A2-epicatechin (**28**) indicating for a C-4 and C-3 *R*-configurations (details cf. text and materials and methods p. 239).

Based on spectroscopic data, i.e. ^{1}H-, ^{13}C-, DEPT-NMR, including heteronuclear correlation experiments (HMQC and HMBC), as well as chemical degradation (thiolysis) and circulardichroism spectroscopy (CD), compound **28** was fully characterized and identified as proanthocyanidin A2-(-)-epicatechin.

The trimeric proanthocyanidins **29** and **28** were identified before in avocado seeds (Thompson et al., 1972; Jacques et al., 1974) as an inseparable mixture and were reported as proanthocyanidin D1 and D2, respectively. Until now, a thorough spectroscopical investigation of these compounds was missing.

In the case of avocado seed material, we were able to develop an effective two-step separation procedure combining preparative HSCCC and preparative HPLC to recover **29** and **28** in pure form.

In the frame of this work the antioxidative capacity, possible toxicity as well as the influence on the regeneration of human keratinocytes in the HaCat-Assay of compounds **28** and **29** were tested.

Both compounds **29** and **28** were highly active in the TEAC-assay with an activity of 2.9 and 2.8 mmol Trolox/mmol substance respectively (cf. Table 3-20 p. 196). They were not toxic (cf. Table 3-25 p. 206).

About the HaCAt-assay (Ramos et al., 2004b) (not shown here) both compounds had a big influence on the regeneration of human keratinocytes and exhibited only week toxicity.

Table 3-15. ^{1}H-NMR data of compounds **26, 94 and 95** in CD_3OD, **29** in CD_3CN and **28** in CD_3CN+ D_2O (δ [ppm]; *J* [Hz]).

Ring	No.	26	94	95	A2-catechin (**29**)	A2-epicatechin (**28**)
	2	4.82	5.10	4.87	-	-
C	3	4.18	4.09	3.90	3.35	3.21
	4	2.73_{ax} /2.89_{eq}	4.65	4.60	3.85	3.90
A	6	5.92	5.96	5.81	5.87	5.91
	8	5.95	5.94	5.86	6.00	6.02
	2′	6.98	6.67	6.80	6.98	6.95
B	5′	6.76	6.68	6.63	6.77	6.66
	6′	6.80	6.78	6.45	7.08	7.17
	2	-	4.80	4.60	5.42	5.43
F	3	-	4.47	4.24	4.05	4.04
	4	-	2.68_{ax} /2.87_{eq}	2.77_{ax} /$2,90_{eq}$	4.35	4.34
D	6	-	5.89	5.94	5.80	5.56
	2′	-	6.11	7.10	7.28	7.29
E	5′	-	6.68	6.73	6.77	6.80
	6′	-	6.89	6.86	7.08	7.06
	2	-	-	-	3.90	4.31
I	3	-	-	-	3.70	3.78
	4	-	-	-	2.35_{ax}/2.94_{eq}	2.68_{ax}/2.75_{eq}
G	6	-	-	-	6.10	5.76
	2′	-	-	-	6.90	7.17
H	5′	-	-	-	6.80	6.86
	6′	-	-	-	6.67	6.86

Table 3-16. ^{13}C-NMR data of compounds **26, 94 and 95** in CD_3OD and **29** and **28** in CD_3CN (δ [ppm]; *J* [Hz]).

Ring	No.	26	94	95	29	28
	2	79.9	77.14	77.14	104.7	104.7
C	3	67.5	73.54	73.54	66.9	66.8
	4	29.2	37.20	37.20	28.3	28.3
	4a	100.1	101.58	101.58	106.0	106.2
	5	157.4	157.81	157.40	157.6	157.7
A	6	95.9	96.22	96.22	98.2	98.2
	7	158	157.40	157.81	155.2	155.6
	8	96.5	96.22	96.22	96.3	96.3
	8a	157.7	158.56	157.40	153.8	153.7
	1′	132.3	132.74	132.62	132.3	131.5
	2′	115.4	114.88	115.33	115.4	115.3
B	3′	145.8	145.9	145.92	144.8	145.9
	4′	145.9	145.66	145.66	144.9	145.7
	5’	115.9	115.33	116.10	116.0	116.0
	6′	119.4	119.2	119.41	118.5	118.3
	2	-	81.83	79.95	78.0	78.2
F	3	-	67.82	67.04	71.2	71.3
	4	-	29.2	30.06	37.8	37.8
	4a	-	100.66	99.63	106.6	106.4
	5	-	155.90	156.42	151.3	152.6
D	6	-	96.56	96.56	95.8	95.8
	7	-	156.40	155.90	155.0	155.2
	8	-	106.80	107.14	99.8	99.6
	8a	-	155.42	155.42	156.7	156.8
	1′	-	132.33	132.11	131.3	132.4
	2′	-	113.9	115.33	116.6	116.7
E	3′	-	146.09	145.99	145.2	144.8
	4′	-	145.66	145.66	145.6	145.3
	5′	-	115.99	115.99	115.4	115.7
	6′	-	121.20	120.26	121.4	121.4
	2	-	-	-	83.1	79.9
I	3	-	-	-	69.2	66.7
	4	-	-	-	30.2	29.7
	4a	-	-	-	102.4	100.6
	5	-	-	-	150.7	157.9
G	6	-	-	-	96.5	96.5
	7	-	-	-	154.9	156.5
	8	-	-	-	108.3	108.2
	8a	-	-	-	155.4	154.7
	1′	-	-	-	132.5	132.3
	2′	-	-	-	115.7	115.8
H	3′	-	-	-	145.4	145.2
	4′	-	-	-	145.3	144.9
	5′	-	-	-	116.0	115.7
	6′	-	-	-	120.5	119.4

3.4.2.3.5. LC-MS of active fractions from gel chromatography (Sephadex LH-20)

For comparing the effectiveness of two large-scale preparative techniques, the proanthocyanidins from the ethyl acetate partition (avocado seed cotyledons), were fractionated by means of HSCCC (cf. p. 153) and size exclusion gel chromatography on Sephadex LH-20 (methanol as eluent) (cf. Table 5-3 separation 16 p. 244). The separation with Sephadex LH-20 resulted in 22 fractions (5.1. to 5.22.) and the sample-fractions (5.21., 5.18. and 5.8.) displayed highest activities in the antioxidant-screening-assay TEAC (cf. Table 3-24, p. 202). The latter fractions were analyzed by LC-ESI-MS (cf. Fig. 3-94) for characterization of single components. All fractions contained polymeric proanthocyanidins which could not be ionized under the tested LC-ESI-MS conditions (neg. mode). Trimers and dimers were clearly detected with high ion abundances. In Figure 3-94 the results of three LC-ESI-MS chromatographic runs were presented in the order of decreasing antioxidant activity.

The concentration of trimeric proanthocyanidins was much higher in the most antioxidant-active fraction (5.21. A) compared to the other two fractions 5.18. (B) and 5.8. (C). The lowest active fraction (5.8. C) consisted principally of dimers and possibly of glucosylated monomeric derivates of flavan-3-ol units.

The LC-ESI-MS and UV-HPLC analysis (280 nm) corroborated that active fractions in the TEAC-assay are possibly consisting of proanthocyanidins of a high polymerization grade. Their antioxidant activities might be even higher than for monomeric flavonols.

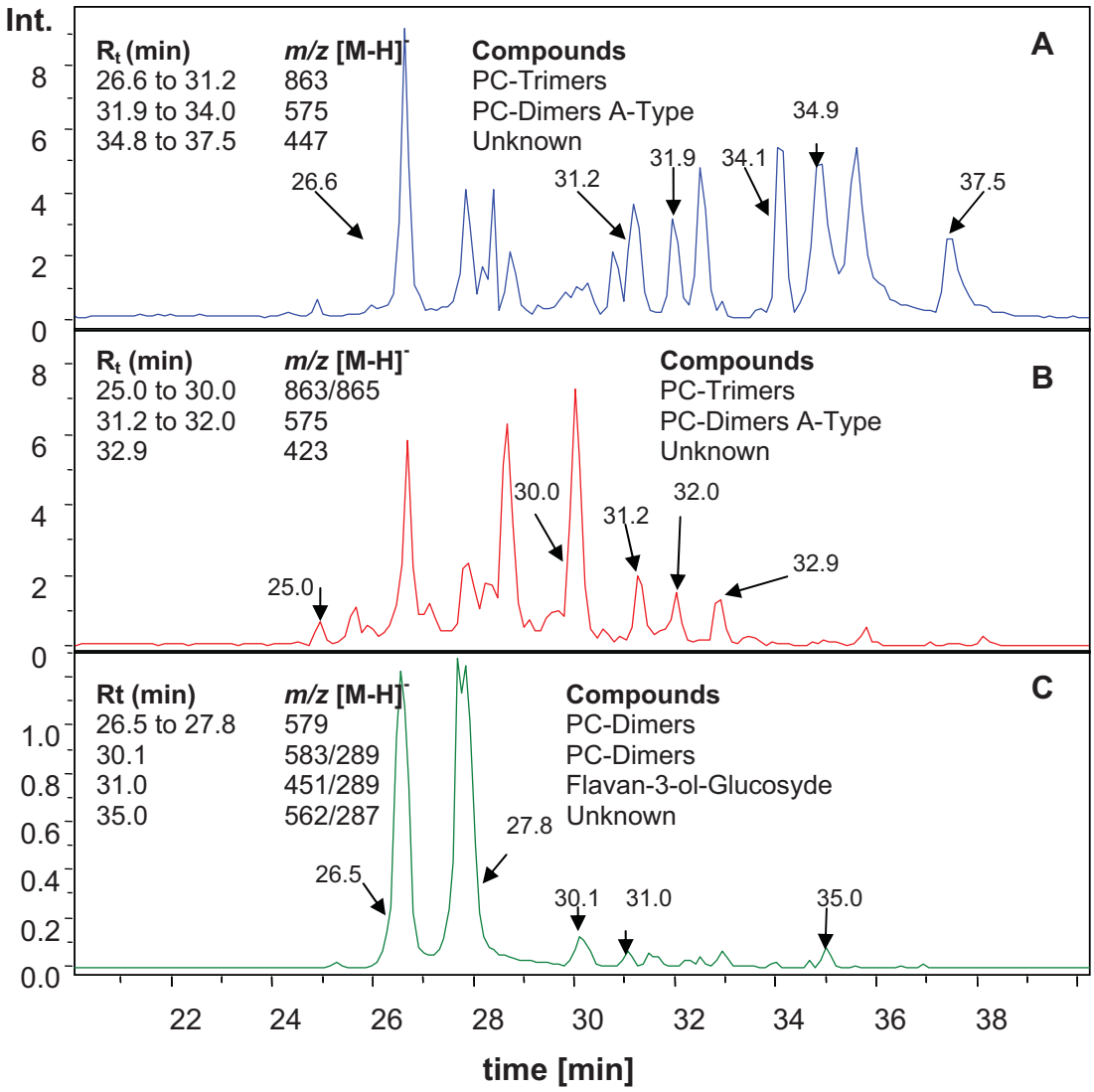


Figure 3-94. LC-ESI-MS (neg. mode) of fractions 5.21. (A), 5.18. (B) and 5.8. (C) from Sephadex LH-20 separation of the Ac partition of avocado seed cotyledons. (Column Prontosil C18 Aq - 4.6 mm x 250 mm. Solvent A (H_2O), Solvent B (ACN). Gradient: t (0, 10, 40, 55, 65, 75 min), A (97, 97, 40, 0, 0, 97 %), B (3, 3, 60, 100, 100, 3 %), Flow rate= 0.7 mL min^{-1}. (Intensity x 10^5 for run A and B and x 10^7 for C).

Preliminary results were achieved for polymeric fractions and will be continued to be studied in future work. Knowledge about the exact chemical composition of the oligomeric and polymers of proanthocyanidins are interesting aspects to assess biological active fractions. Interestingly, some of the avocado seed fractions we observed were more active than some of the commercial antioxidants used in the industry, such as BHA and BHT (cf. Table 3-22 p. 199, and Table 3-24 p. 202).

In comparing the two techniques, i.e. HSCCC and size exclusion chromatography, it can be assumed that the countercurrent technique is more

efficient and gentle in isolating the instable non-hydrolyzable tannins. Short separation times (8 hours) and also relatively high sample loads in HSCCC in comparison to one week separation time with the column chromatography using gel material are the most important advantages. It also needs to be taken into consideration that extended contact of proanthocyanidins to the eluent methanol might cause degradation. Break-down of proanthocyanidin substance material was clearly observed during extended NMR measurements for ^{13}C-NMR spectra with deuterated methanol.

3.5. Bioactivity assays

It is known that the biological response is dose dependent. This relation is represented almost always as a “dose-response curve” which has a sigmoid (“S”) form. This form is generated because at lower dose the response is very low or there isn’t any. As the dose increases, also the response increases until it reaches a plateau, the point in the curve in which the response keeps constant although the dose increases (Gennaro, 1987).

This kind of curve is the basis of the biometry. “Transformation” of the curve using the logarithm of the variable results in a straight line. The transformed data allow a better calculation of the equation.

The dose could be the concentration of a defined reactant, microorganism, or defined enzyme, etc. The response could be absorbance, lethality, growth, inhibition or presence of a defined activity, etc.

Results of some of the applied biological assays (in vitro), such as toxicity by the brine-shrimp assay as well as antioxidative activity, are summarized in this form and presented as “median lethal dose (LD_{50})”, lethality rate (%), “median effective concentration (EC_{50})”, or equivalents in respect to a standard.

3.5.1. Screening of antioxidant activity by TEAC and DPPH methods

A review about antioxidant activity can be found on page 44.

The DPPH method is extensively used (Brand-Williams et al., 1995; Tsimogiannis and Oreopoulon, 2006; Ley, 2001) in order to study the reaction mechanisms of pure compounds. Tsimogiannis and Oreopoulon (2006) investigated different flavonoid substitution pattern. They concluded that the C-ring and the catechol ring (OH at positions 3’, 4’) have a big influence on the reaction of flavonoids with the free DPPH radical.

In this chapter, the results obtained for some standards will be discussed and compared with literature data (Brand-Williams et al., 1995).

Up to now there is no knowledge about the substances that are responsible for the antioxidative activity of avocado seeds.

In the frame of this study, the antioxidant capacity of avocado seed extracts was assessed in vitro by using three different methods ("original" TEAC, "modified TEAC" and DPPH) (cf. Materials and methods p. 228).

Furthermore it was planned to compare the antioxidant capacity of avocado seeds extracts against the activity of synthetic antioxidants used by the industry. Ascorbic acid, ß-carotene, *tert*-butyl hydroxyanisol (BHA), 2,6-di-*tert*-butyl-4-methylphenol (BHT), and α-tocopherol were used as standards (cf. Fig. 3-95).

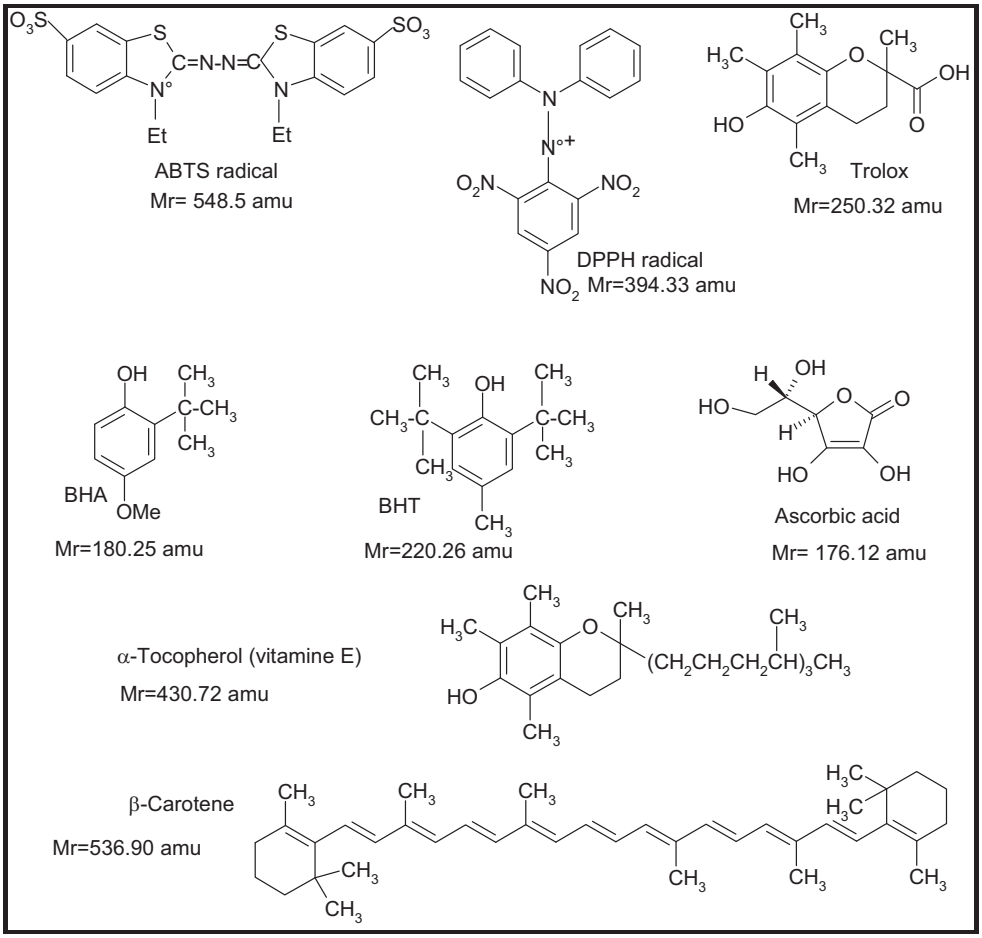


Figure 3-95. Radicals and antioxidants used on the scavenging capacity test.

3.5.1.1. Antioxidant activity of the used standards in the different test systems

Due to their solubility not all standards could be tested in all test systems. In case of the "original" TEAC method (Miller et al., 1993) one requirement is that the substance must be soluble in water.

As these methods are colorimetric (disappearing of absorbance or decolorization), the UV/VIS absorbance of the substance is an important factor. The substances should not interfere with the colour of the assay solutions, as in case of β-carotene.

The antioxidant capacity of BHA and BHT were tested in two solvents (dichloromethane and methanol) to explore the influence of different solvents on the antioxidant activity. Results are presented in Table 3-17. No significant influence was observed. With the TEAC assay BHA was at least two times more active than BHT, may be because sterical hindrance in the last one (cf. Fig. 3-95). With the DPPH system, we could not see a really difference between both antioxidants (Table 3-17).

β-Carotene showed the highest antioxidant capacity in the TEAC assay, while the DPPH system was not sensitive for this compound. It seems that due to its colour there are interferences with the test system.

Ascorbic acid and α-tocopherol showed nearly the same activity in all three tested systems (cf. Table 3-17).

Table 3-17. Antioxidant capacity of standards evaluated with three radical scavenging methods

	[20]TEAC				[21]DPPH			
Compound	**Modified[22]**		**Original[23]**		**[24]EC_{50}**		**[25]ARP**	
	obs	**lit[22]**	**obs**	**lit[22, 23]**	**obs**	**lit[21]**	**obs**	**lit[21]**
Ascorbic acid	0.96±0.02	1.05±0.02	0.65±0.05	0.99±0.04	0.22±0.03	0.27	4.55	3.70
α-Tocopherol	0.93±0.001	0.97±0.06	0.70±0.04	0.97±0.01	0.24±0.01	-	4.17	-
ß-Carotene	3.23±0.08	2.57±0.03	nt	1.90±0.01	ns	-	-	-
BHA[26]	1.20±0.03	-	nt	-	0.19±0.04	-	5.32	-
BHA[27]	1.23±0.01	-	1.01±0.03*	-	0.21±0.02	0.24	4.76	4.17
BHT[26]	0.27±0.01	-	nt	-	0.30±0.02	-	3.28	-
BHT[27]	0.34±0.002	-	0.53±0.02*	-	0.23±0.01	0.24	4.35	4.20

ns= not sensitive. *= Dissolved in ethanol and diluted in water, nt= not tested. Data expressed as means of at least two determinations ± SD. [22]Re et al. (1999), [23]Miller et al. (1993), [21]Brand-Williams et al. (1995).[20]mmol Trolox/mmol Std., [24] mmol Std/ mmol DPPH, [25] mmol DPPH/ mmol Substance, [26] in CH_2Cl_2, [27] in MeOH. obs= observed value, lit= literature value

The DPPH radical has a deep purple colour and an absorbance at 514 nm. A linear regression curve of absorbance against DPPH concentration in methanol (mM DPPH) is presented in Figure 3-96. The equation describing the linear regression is y= 10.366 x -0.0371 (Eq. 3-2). By using equation 3-2, the concentration of free radical DPPH was estimated at various reaction times.

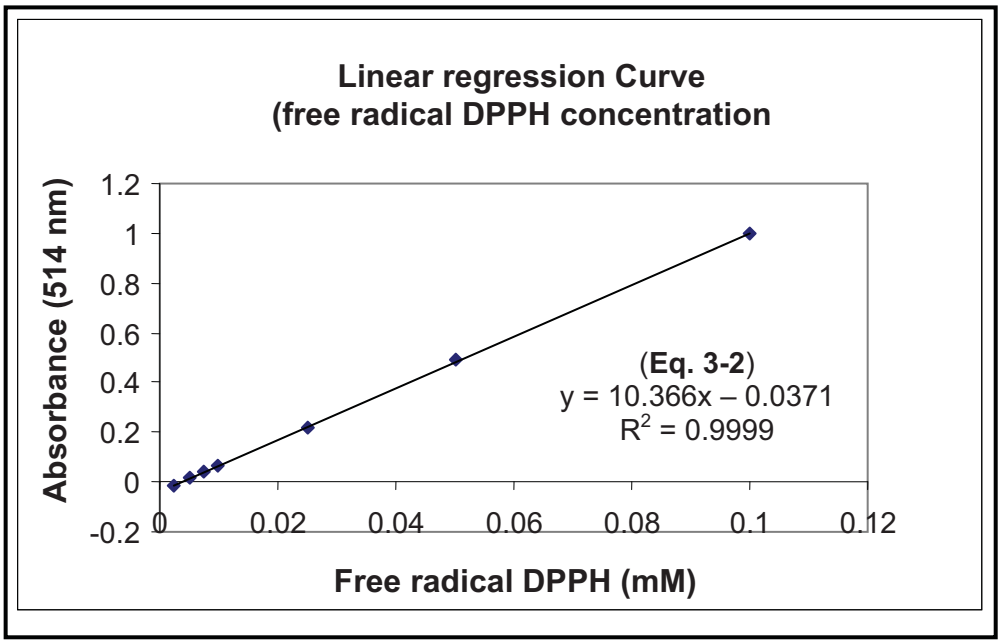


Figure 3-96. Concentration-Response curve of DPPH free radical.

In case of DPPH method, reaction kinetics depend on the nature of the antioxidant being tested. There are three types of kinetics observable:

a) Rapid kinetics as in case of ascorbic acid which reach the steady state in less than 1 minute.

b) Intermediate reactions (example α-tocopherol) which reached the steady state in 15 minutes.

c) Slow kinetics within 1 to 6 hours (examples: BHA and BHT), the steady state is reached in 120 minutes.

The kinetics of the reaction between the DPPH radical and standards or extracts was plotted at different concentrations as absorbance at 514 nm vs. time of reaction. Methanol, ethanol, dichloromethane or a mixture of ethanol and water

were used as negative control. Figure 3-97 shows the kinetic behaviour of the standards.

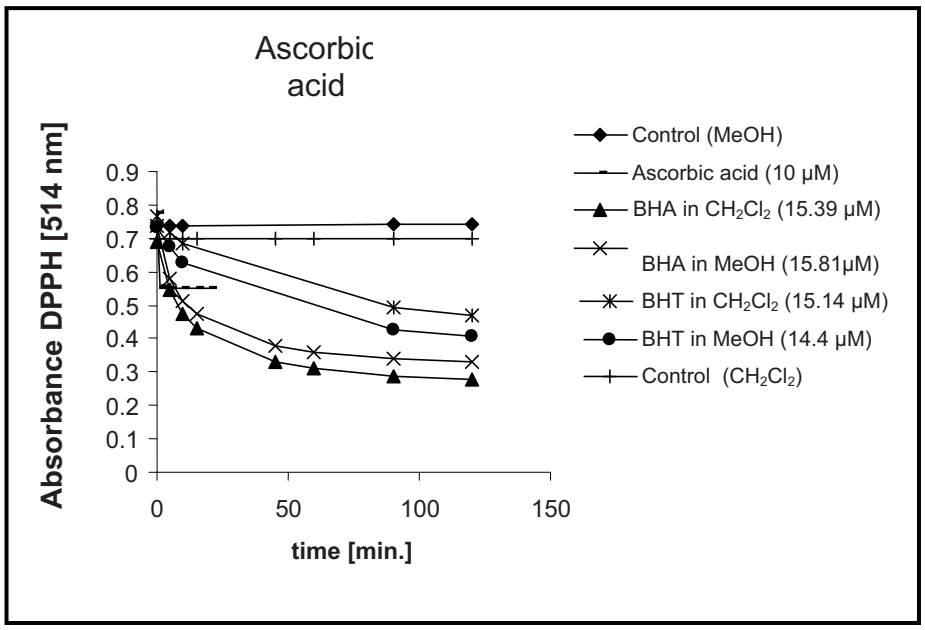


Figure 3-97. Reaction kinetics for the standards in the DPPH assay.

The remaining DPPH (%) at steady state vs concentration of substance was used to calculate the effective concentration (antiradical efficiency) as amount of antioxidant necessary to reduce the initial quantity of DPPH to 50% (EC_{50} mmol substance/mmol DPPH) (cf. Fig. 3-98).

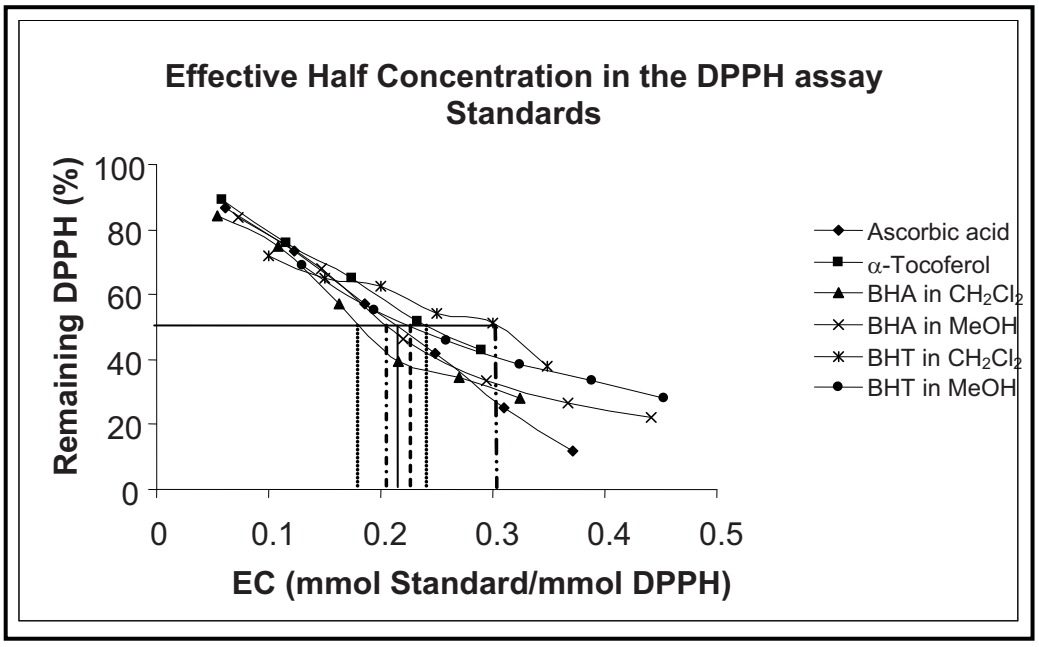


Figure 3-98. Effective Concentration of the standards in the DPPH assay.

Parameters as antiradical power (ARP), stoichiometric value (EC_{100}) as well as number of reduced DPPH radicals (rDPPH$^{\bullet}$) (details cf. p. 231) were also estimated to facilitate the comparison with reported literature data (Brand-Williams et al., 1995) in case of pure compounds. At higher values of ARP and rDPPH$^{\bullet}$, the efficiency of the antioxidant compound is consequently also high.

In case of the standards BHA dissolved in dichloromethane was the most efficient compound with an ARP of 5.3 (cf. Table 3-17, p. 188) and an rDPPH of 2.6 (cf. Table 3-18).

Table 3-18. Stoichiometry of standards in the DPPH assay

Compound	EC_{100}		rDPPH°		g rDDPH°/g Substance	RSC
	obs	[21]lit	obs	[21]lit		
Ascorbic acid	0.4	0.54	2.27	1.85	5.09	100.0
α-Tocopherol	0.5	-	2.08	-	1.91	37.5
BHA[26]	0.4	0.38	2.63	2.63	5.76	113.1
BHA[27]	0.4	-	2.38	-	5.21	102.4
BHT[26]	0.6	0.38	1.67	2.63	2.98	58.6
BHT[27]	0.5		2.17		3.89	76.4

EC_{100}= Stoichiometric value. rDPPH$^{\bullet}$ = No. of radicals of DPPH$^{\bullet}$ reduced/ molecule antioxidant. g reduced of DPPH/ g substance. [21]Brand-Williams et al. (1995). [26] in CH_2Cl_2, [27] in MeOH. RSC= % scavenging capacity relative to ascorbic acid, obs = observed value.

Nevertheless, BHA was the most active compound in all scavenging capacity assays. For comparison of the activity of standards, extracts and isolated compounds ascorbic acid has been used because it is a frequent "natural" compound with a relative high antioxidant capacity (EC_{50}= 0.22).

While in the DPPH assay there were no significant differences between BHA and ascorbic acid (cf. Table 3-17), results of the "TEAC" assay showed higher activity for BHA (125.19 in methanol and 122.14% in dichloromethane) than ascorbic acid (100%). The activity of BHT (57.65 in dichloromethane and 76.45% in methanol) were always smaller than the activity of ascorbic acid (cf. Fig. 3-99, Table 3-18, p. 191, and Table 3-22, p. 199).

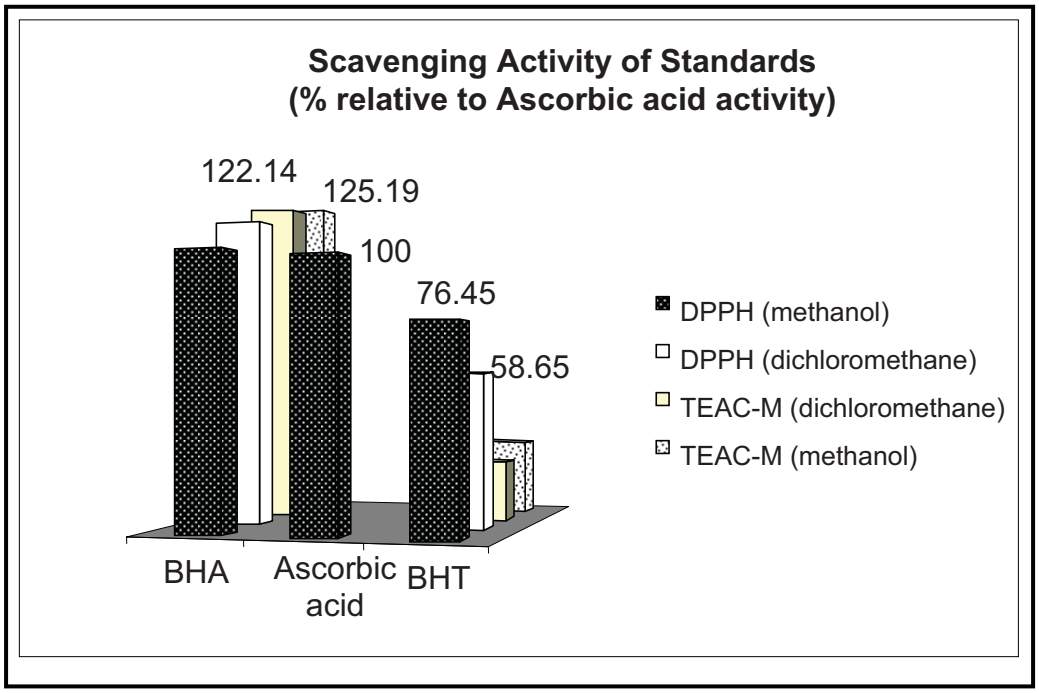


Figure 3-99. Scavenging activity of BHA, BHT and ascorbic acid by "modified TEAC" and DPPH assays.

3.5.1.2. Antioxidant activity of extracts from avocado (*Persea americana* Mill c.v. *Hass*) seeds

The scavenging capacity of extracts (cf. Table 3-20, p. 196) and fractions from avocado seeds were presented as total antioxidative activity (TAA) because they

are mixtures of compounds and a direct equivalent Trolox can not calculated in the "TEAC method". In case of isolated compounds (**90**, **26**, **94**, **95**, **29** and **28**), the results are presented both in mmol Trolox /kg (cf. Fig. 3-101) and in mM Trolox/mM substance (cf. Table 3-20).

In the DPPH assay the effective half concentration (EC_{50}) is presented as kg extract necessary to neutralize 1 mmol DPPH while ARP results are given in mmol DPPH/kg extract (cf. Table 3-19).

The highest antioxidant activity was expected for the ethyl acetate extract (Ac), because of its high content of polyphenolic compounds (19.1%) (cf. p. 54). Surprisingly, the methanolic extract showed the highest antioxidant capacity (cf. Table 3-19).

Obviously the phenolic compounds present in the methanolic extract, e.g. polymers of proanthocyanidins, are more active than the phenolics present in the ethyl acetate extract (cf. Fig. 3-100).

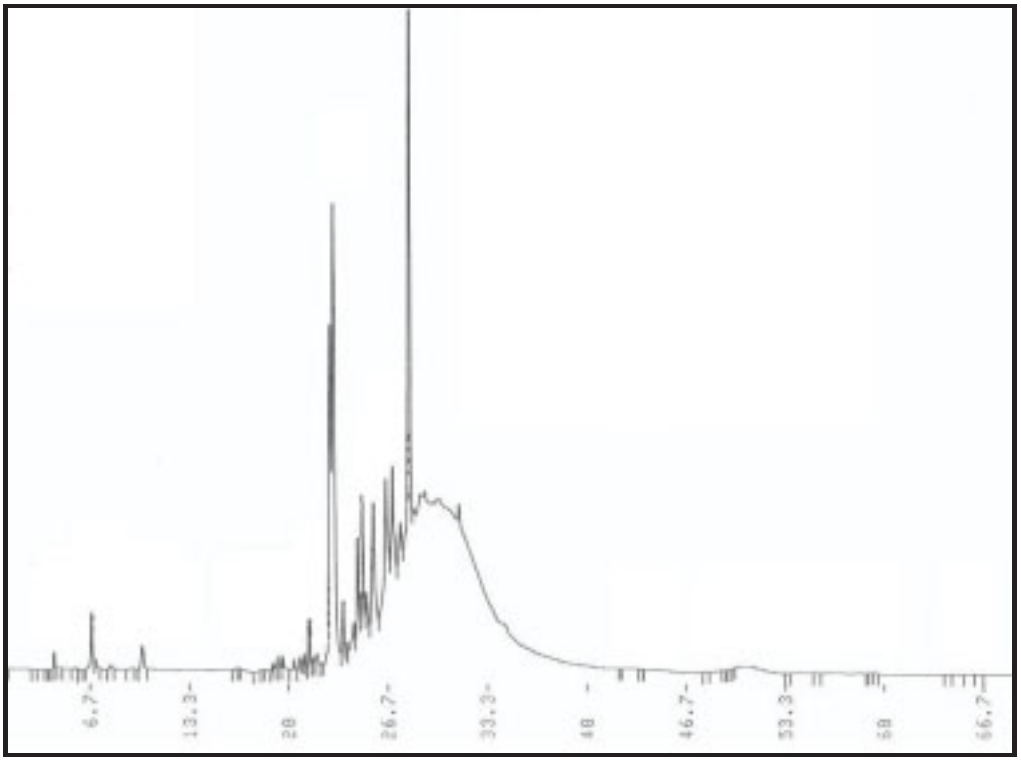


Figure 3-100. UV-HPLC spectrum of the methanolic extract of avocado seeds. [Column: Phenomenex AQUA C-18 250 mm x 4.6 mm, 125 Å. Flow rate: 0.7 mL min^{-1}. λ= 210 nm. A: H_2O, B: ACN, t (0,10,40,45,50 min), A (97,97,40,97,97), B (3,3,60,3,3).].

Continuing with the TEAC assay results, the methanolic extract (MeOH) showed the highest scavenging capacity from all tested avocado seeds extracts, while the petrol ether (PE) extract exhibited the lowest antioxidant activity (cf. Fig. 3-101 and Table 3-19). As the CO_2 extract was almost inactive it was not longer considered for further examinations.

Due to its highest antioxidant capacity (390.5 mmol Trolox /kg), the methanolic extract was considered for further fractionations through different solvents: petrol ether (PE), dichlorometane (C), ethyl acetate (Ac) and methanol:water (MW).

From these partitions the Ac extract showed the highest antioxidant capacity (3983 mmol Trolox/kg) (cf. Table 3-20), and was further separated by HSCCC (cf. p.p.153). The most active fraction 6.3 (3083 mmol Trolox /kg) was obtained and further separated by preparative HPLC (cf. p. 155). Isolated flavan-3-ol compounds were **26**, **94**, **95**, **29** and **28**. Compound **26** (monomer) was less active (2718.6 mmol Trolox/kg) than the mixture of compounds **94** and **95** (dimers) (3205.7 mmolTrolox/kg), and the latter were less active than compounds **29** and **28** (3298.6 and 3225.4 mmol Trolox/kg, respectively) (trimers) (cf. Fig. 101).

For comparison purposes, the results for the pure compounds were presented in mmol Trolox/kg in Figure 3-101 and as mM Trolox/mM substance in Table 3-20.

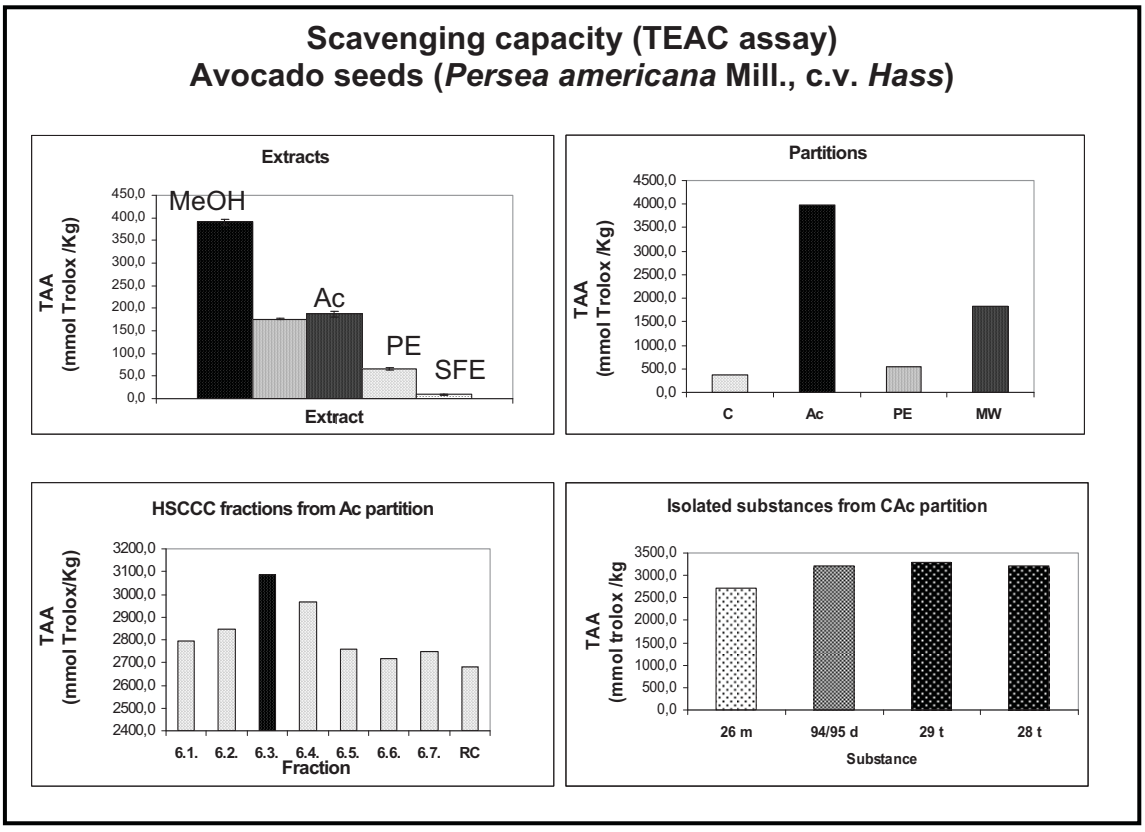


Figure 3-101. Scavenging capacity of avocado seeds by the TEAC assay. C= dichloromethane, Ac= ethyl acetate, PE= petrol ether, MW= methanol:water.

These results support the hypothesis that polymeric and oligomeric proanthocyanidins are more active than monomers (Ito et al., 2000), but their specific and selective activity is not yet clear.

Table 3-19. Antioxidant capacity of avocado seeds extracts using three different assays

TAA (mmol Trolox/kg extract dw)			**[21]DPPH**	
Extract	**[22]modified**	**[23]original**	**EC_{50}** (kg dw/mmol DPPH)	**[25]ARP**
MeOH	390.5 ± 6.9	329.32±14.33	0.00049±0.01	2040.8
H_2O	176.5 ± 0.20	147.32±5.17	0.0012±0.01	833.33
Ac	187.1± 6.0	184.48±7.16	0.00104±0.003	961.54
PE	66.5 ± 3.0	nt	0.002±0.06	400.00
CO_2(s)	9.5± 1.0	nt	nt	nt

dw=dry weight. [25]mmol DPPH neutralized /kg. Extract dw. [22]by Re et al. (1999). [23]by Miller et al. (1993). [21]by Brand-Williams et al. (1995), nt= not tested, (s)= supercritical conditions.

Table 3-20. Antioxidant capacity of partitions, fractions and isolated compounds from avocado seeds cotyledons using the modified TEAC assay

Fraction	[22]TAA (mmol Trolox/kg extract)
CH_2Cl_2 (C)	366.1± 8.4
Ac (6)	**3983.2± 120.0**
PE	541.4± 22.8
MW	1819± 43.6
6.1.	2795.4± 33.6
6.2.	2846.2± 22.9
6.3.	**3083.1± 28.5**
6.4.	2967.8± 13.6
6.5.	2760.4± 67.4
6.6.	2718.6± 215.9
6.7.	2745.9± 41.7
6.RC	2682.1± 70.8
90*	0.5 ±0.03
26*	0.8 ±0.1
94/95*	1.8 ±0,1
29*	2.9 ± 0.1
28*	2.8 ± 0.1

[22] by Re et al. (1999) method

* mM Trolox/mM Substance

In the case of the DPPH method, the extracts showed only slow reaction kinetics which was between 110-170 minutes (cf. Fig. 3-102).

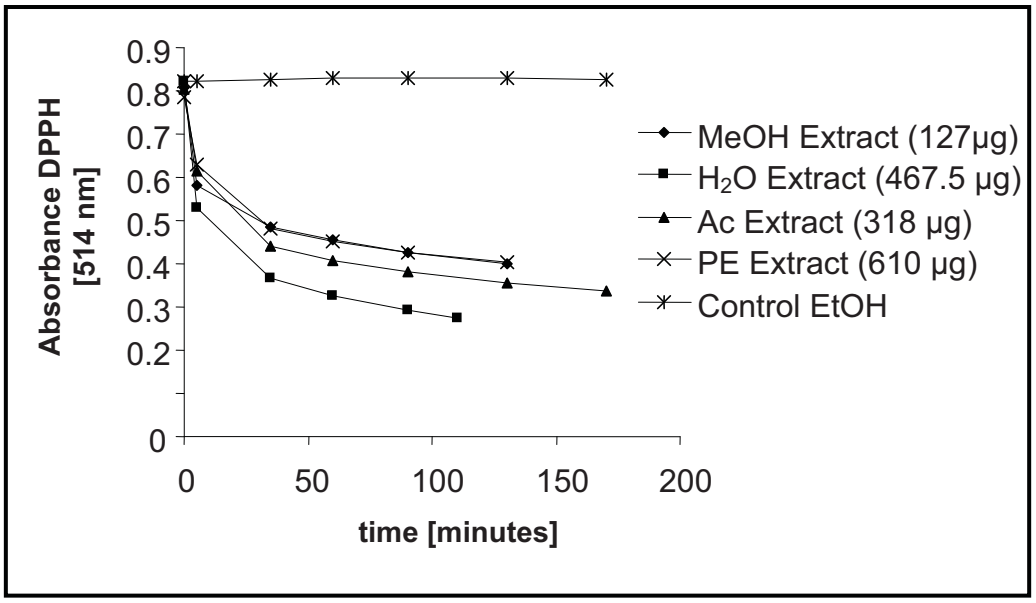


Figure 3-102. Reaction kinetics for the avocado seeds (*Persea americana* Mill., c.v. *Hass*) extracts in the DPPH assay. (Only one concentration is shown).

The EC_{50} extract values were in the range of 0.00049 and 0.0025 (cf. Fig. 3-103) (expressed as kg/mmol DPPH), and the respective ARP values between 2040.8 and 400 (Table 3-19). The corresponding regression equations are presented in Table 3-21. The methanolic extract had the highest ARP value (2040.8) (cf. Table 3-19).

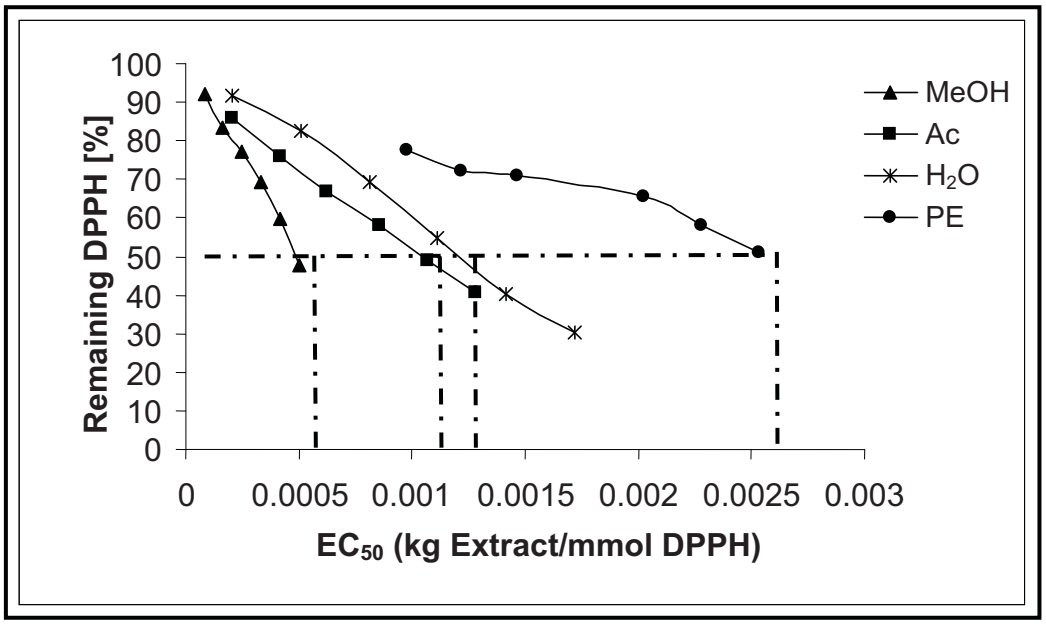


Figure 3-103. Effective concentration in the DPPH assay of avocado seeds extracts.

Table 3-21. Linear regression equations for EC_{50} estimation in the DPPH assay

Extract	Linear regression	Regression coefficient
MeOH	y = -101805x + 101.12	0.9942
H_2O	y = -42387x + 102.29	0.9976
Ac	y = -41789x + 93.774	0.9992
PE	y = -15437x + 92.88	0.9702

It appears that the methanolic extract is more active than the standards because of its high ARP value (2040.8). Nevertheless, it must be noted that the ARP units for extracts are presented in mmol DPPH/kg extract, while for the standards they were presented in mmol DPPH/ mmol substance.

Because a direct comparison between the antioxidant capacities of both groups is difficult, it was decided to estimate the equivalent in g for all standards and extracts in all methods (TEAC and DPPH). The scavenging capacities were estimated as ratio between results of standard or extract and results of ascorbic acid (cf. Material and methods p.p. 228-231) and reported finally as percentage of scavenging capacity relative to the activity of ascorbic acid (100%) (cf. Tables 3-22 and 3-23).

Table 3-22. Comparison of scavenging capacity of avocado seed extracts and standards relative to ascorbic acid in the TEAC assay

Compound	[22]TEAC assay (SCs)		[23]TEAC assay (SCs)	
Pure substances	**g Trolox/g substance**	**RSCs**	**g Trolox/g substance**	**RSCs**
Ascorbic acid	1.36	100	0.92	100
α-Tocopherol	0.56	41	0.41	44.0
ß-Carotene	1.51	110.4	NT	NT
[26]BHA	1.67	122.1	NT	NT
[27]BHA	1.71	125.2	1.40	152.8
[26]BHT	0.31	22.5	NT	NT
[27]BHT	0.39	28.3	0.60	65.2
90	0.43	31.2	nt	nt
26	0.69	50.6	nt	nt
94/95	0.78	57.2	nt	nt
29	0.84	61.3	nt	nt
28	0.8	59.2	nt	nt
Extracts	**(SCe)**	**RSCe**	**(SCe)**	**RSCe**
MeOH	0.1	7.2	0.08	8.9
H_2O	0.04	3.2	0.04	4.0
Ac	0.05	3.4	0.05	5.0
PE	0.02	1.2	nt	nt
CO_2(s)	0.002	0.2	nt	nt
Partitions				
CH_2Cl_2 (C)	0.09	6.7	nt	nt
Ac (6)	1.00	73.1	nt	nt
PE	0.14	9.9	nt	nt
MW	0.46	33.4	nt	nt
HSCCC fractions	**from Ac**			
6.1.	0.70	51.3	nt	nt
6.2.	0.71	52.2	nt	nt
6.3.	0.77	56.6	nt	nt
6.4.	0.74	54.4	nt	nt
6.5.	0.69	50.6	nt	nt
6.6.	0.68	49.9	nt	nt
6.7.	0.69	50.4	nt	nt
RC	0.67	49.2	nt	nt

[26]in CH_2Cl_2, [27]in MeOH, nt = not tested. SCs= Scavenging capacity of standards. SCe= Scavenging capacity of extracts. RSCs= % Scavenging capacity of standards relative to ascorbic acid. RSCe= % Scavenging capacity of extracts relative to ascorbic acid. [22]method by Re et al. (1999), [23]method by Miller et al. (1993).

Table 3-23. Antioxidant capacity of standards and avocado seeds extracts relative to ascorbic acid activity in the DPPH assay.

Compound	Sv (EC_{100})		rDPPH$^{\bullet}$		g/g	RSCs
Standard	**obs**	**[21]lit**	**obs**	**[21]lit**	**obs**	**obs**
Ascorbic acid	0.4	0.54	2.27	1.85	5.09	100.0
α-Tocopherol	0.5	-	2.08	-	1.91	37.5
[26]BHA	0.4	-	2.63	-	5.76	113.1
[27]BHA	0.4	0.38	2.38	2.63	5.21	102.4
[26]BHT	0.6	-	1.67	-	2.98	58.6
[27]BHT	0.5	0.38	2.17	2.63	3.89	76.4
Extract						**RSCe**
MeOH	0.0010	-	1000	-	0.4	7.7
H_2O	0.0025	-	406.5	-	0.2	3.2
Ac	0.0021	-	476.2	-	0.2	3.7
PE	0.0056	-	179.8	-	0.1	1.4

Sv= stoichiometric value. g/g = g rDPPH$^{\bullet}$/ g substance. RSCs= % Scavenging capacity of standards relative to ascorbic acid. [26]in CH_2Cl_2, [27]in MeOH. [21]Brand-Williams et al. (1995), obs= observed value, lit= literature value.

BHA and BHT were tested in two different solvents, a polar (methanol) and a non polar (dichloromethane). In all the three tested methods no significant differences in the scavenging capacity were observed. BHA was the most efficient radical scavenger.

β-Carotene was insensitive in the DPPH assay, but in the modified TEAC assay, it was more active than ascorbic acid.

In general there were more variations in the results of the DPPH assay when dichloromethane was used as solvent. This may depend on the time of reaction and the volatility of the solvent. The slow reaction causes a long analysis time (120 min), hence there was evaporation of the solvent. This might be one reason for the variations in the absorbance measured.

The TEAC assay by Miller et al. (1993) is not applicable to lipophilic substances but in this study it was demonstrated that the TEAC assay by Re et al. (1999) as well as the DPPH assay by Brand-Williams et al. (1995) were applicable to test the scavenging capacity of polar as well as lipophilic compounds.

In the following the DPPH assay was no longer used to test the fractions and isolated substances from avocado seeds because it was shown that the method

by Re et al. (1999) will give comparable results. This method is also easier and faster than the DPPH assay.

Obviously, the antioxidant activity depends not only on the reaction mechanisms but also on the compounds present in the extracts. Synergism and antagonism are also important factors influencing the scavenging capacity of extracts.

The presence of COH or derivates from COOH groups in the substances from avocado seeds (cf. p.p. 63 and 88), as well as the presence of monophenols causes just a poor reaction between both avocado seed extracts and radicals.

According to these results we have decided to continue our research with the methanolic extract in order to know which substances were present. The ABA derivatives compounds **89** and **70** were direct isolated from the methanolic extract (cf. p.p. 120).

The Ac partition from the methanolic extract had the highest scavenging capacity (73%) compared to ascorbic acid (cf. Table 3-22). In the brine shrimp assay this partition was non toxic (>1000) (cf. p.p. 206), therefore it could be used as an alternative antioxidant in the food industry.

The same Ac extract was also fractionated by means of gel chromatography (Sephadex LH-20) resulting in 22 fractions. The first eight fractions seem to be a mixture of polyphenols and sugars (not shown here). Fraction 5.9. to fraction 5.22. contain proanthocyanidins. All these fractions were tested in the TEAC assay, revealing fraction 5.21. as the most active (223%). Further work could be the identification of the compounds present there because almost all of them had a high scavenging capacity (cf. Table 3-24).

Table 3-24. TEAC assay on Sephadex fractions from the Ac partition of avocado seeds

Fraction	[22]TAA (mmol Trolox/kg)	g Trolox/g fraction	RSCe
5.1.	365.6±39.9	0.09	6.7
5.2.	285.4±34.8	0.07	5.3
5.4.	756.3±47.8	0.19	13.9
5.5.	1817.1±69.1	0.45	33.4
5.6.	2724.6±81.0	0.68	50.1
5.7.	3109.8±96.3	0.78	57.2
5.8.	7982.1±482.1	2.00	**146.9**
5.9.	2588.7±100.1	0.65	47.6
5.10.	5034.6± 54.9	1.26	92.7
5.11.	3541.5± 45.3	0.89	65.2
5.12.	5208.4± 116.4	1.30	95.9
5.13.	3993.8± 142.5	1.00	73.5
5.14.	6976.3± 1.6	1.75	128.4
5.15.	3914.3± 12.7	0.98	72.0
5.16.	4114.4± 42.8	1.03	75.7
5.17.	5299.1± 102.2	1.33	97.5
5.18.	9705.6± 117.2	2.43	**178.6**
5.19.	5698.4± 19.0	1.43	104.9
5.20.	6409.6± 194.8	1.60	118.0
5.21.	12123.6± 131.5	3.03	**223.1**
5.22.	6676.1± 179.0	1.67	122.9
5.RC	2623.0± 28.5	0.66	48.3

RSCe = % Scavenging capacity of extracts relative to ascorbic acid. [22]method by Re et al. (1999).

The HSCCC technique proved to be quickly and efficient for the fractionation and isolation of proanthocyanidins. Nevertheless after HSCCC, a second step might be required, such as gel chromatography on Sephadex LH-20 for further fractionation, and finally, preparative HPLC for the isolation of pure compounds.

3.5.2. Brine–shrimp test

The brine-shrimp (*Artemia salina*) assay has been extensively used to monitor the general toxic activity of plant extracts (Meyer et al., 1982; Wanyoike et al., 2004; Padmaja et al., 2002; Anderson et al., 1991) or marine natural products (Carballo et al., 2002), as a simple and inexpensive method.

The toxicity of different lipophilic (C, EP, MW, CO_2, 8.1.9b.2., 8.1.9b.3., cf. p. 247) and polar (Ac, 10.1. cf. p. 248, 10.6. cf. p. 250, **29**, **28**) partitions, fractions and

pure substances from avocado seeds (cotyledons and complete seeds) as well as the standards catechin and epicatechin were evaluated in the brine shrimp assay.

Independent of the tested concentration, the dichloromethane (C), supercritical CO_2, and petrol ether (PE) partitions were almost as toxic as the control (podophyllotoxin) or even more toxic in case of the tested fractions (8.1.9b.2., and 8.1.9b.3.) (cf. Fig. 3-104).

The polar partitions (Ac, MW) as well as the isolated (**29**, **28**) substances and standards (catechin and epicatechin) were only a little or practically non toxic (cf. Fig. 3-104).

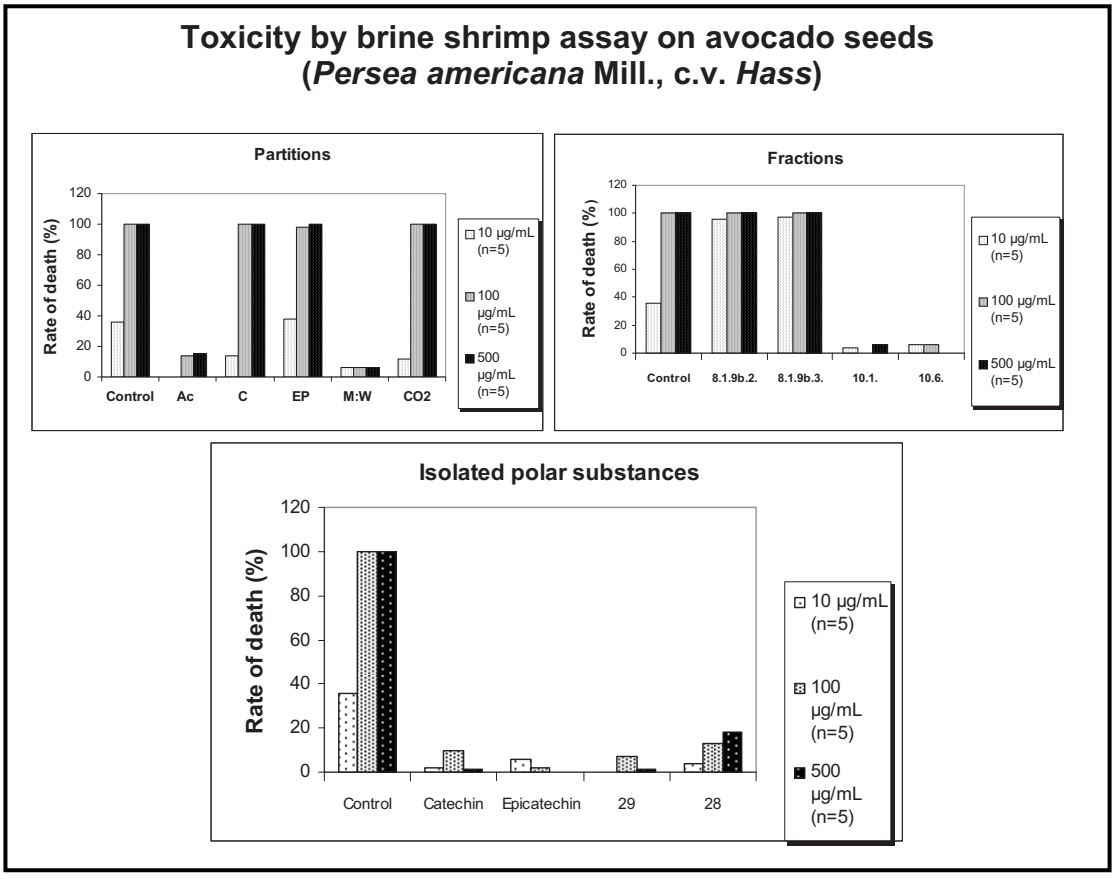


Figure 3-104. Toxicity in the brine shrimps assay (avocado seed extracts, fractions and isolated substances).

The toxicity is normally reported as median lethal dose (LD_{50}) (Meyer et al., 1982; Anderson et al., 1991; Padmaja et al., 2002; Wanyoike et al., 2004).

The median lethal dose can be determined from the concentration-response curve (cf. Material and methods p.p. 233-235).

Sometimes a non-linear relationship is found between the extent of the response (% death) and the dose (concentration) of the tested compounds resulting in a sigmoid curve. Nevertheless in almost all of the biological analysis the percentage of the maximally attainable response (in this case rate of death) on the ordinate is plotted against the concentration on the abscissa in logarithmic scale. This usually yields a straight line in the dose-response curve (Gennaro, 1987), but sometimes also a sigmoid curve is obtained.

In these cases different kind of mathematical transformations of the response are suggested as “probit” (probability unit) (Finney, 1964; Gennaro, 1987) or as “logit” (Hafner et al., 1977) to get a linear regression.

In case of a “probit” transformation, it is necessary to convert the response to “probit values” which are derivated from standard deviation units in a table of the normal probability curve. A response of 50% is equivalent to the left side of the area under the curve of normal probability and a standard deviation (SD) with a zero value. As the probit values are gained by addition of 5 to the standard deviation unities, a response of 50% is direct equivalent to 5 (Finney et al., 1964).

In case of “logit” transformation, the responses are converted in values called logit (z) (for details cf. p. 234).

One advantage of these “transformation data methods” is that LD_{50} can be calculated directly from a linear regression curve with a better regression coefficient than in case when the original data are used.

When using the probit method, the results should finally be expressed by median effective dose in the original units (in this case µg/mL) and the rate of death (%) and not in the probit values (Finney, 1964).

We have calculated the LD_{50} with all of the three mentioned methods (original data, probit and logit transformations) (cf. Material and methods p.p. 234-235) but only one of them is presented for each tested substance or partition according to their best correlation coefficient (r) (cf. Table 3-25).

The concentration-response curve of the positive control (podophyllotoxin) is presented here resulting in a LD_{50} of 9.9 (µg/mL) (cf. Fig. 3-105).

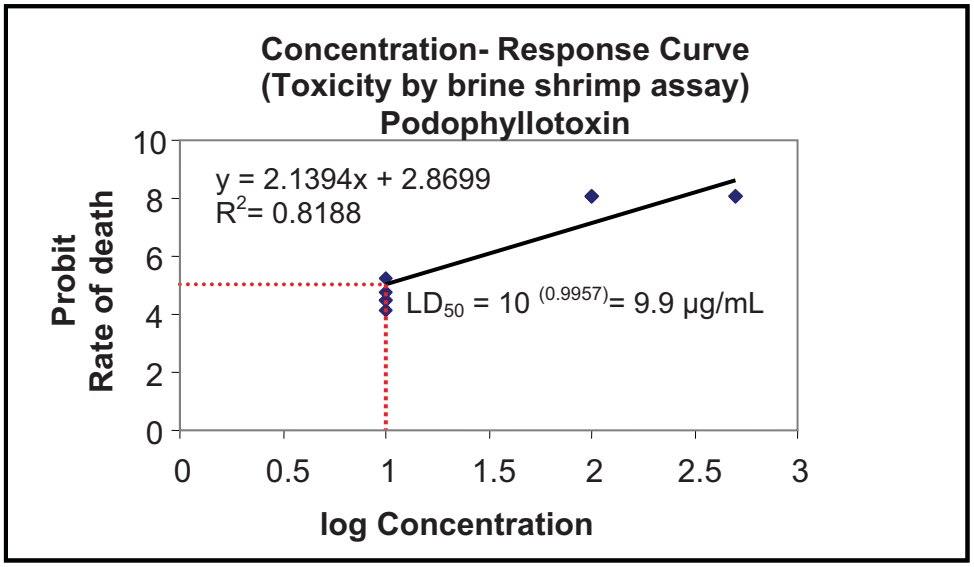


Figure 3-105. Concentration-Response Curve of Podophyllotoxin

Not all tested substances were toxic in the brine shrimp assay. LD_{50} values higher than 1000 µg/ml were considered as non toxic, whereas values smaller than the control LD_{50} (9.9 µg/mL) are considered as being very toxic.

The lipophilic partitions (dichloromethane, petrol ether and supercritical CO_2) and lipophilic fractions were the most toxic (cf. Table 3-25). The petrol ether partition as well as its fractions (8.1.9b.2., and 8.1.9b.3.) were even more toxic than the control, while the polar partitions (ethyl acetate, and methanol:water), fractions (10.1. and 10.6.) as well as the polar isolated compounds (**29** and **28**) were not active.

As the regression coefficients for some of the polar substances were poor, this system was obviously not suitable to test this kind of compounds. Still they were clearly non toxic in this brine shrimp assay.

Polyphenols, especially proanthocyanidins, have been shown by *in vitro* and *in vivo* data to influence cell physiology (Kashiwada et al., 1990; Freixa et al., 1998). Concerning detailed structure-activity-relations there is evidence that oligomeric proanthocyanidins are more active than monomers (Ito et al., 2000), but their specific and selective activity is not yet clear. In the cytotoxicity assay by Deters et al. (2001) polar substances as proanthocyanidins A2-(+)-catechin (**29**) and A2-(-)-epicatechin (**28**) showed a relative low cytotoxic activity (Ramos et al., 2004b).

Table 3-25. Results of the brine shrimp bioassay (partitions, fractions and isolated substances from avocado seed)

Substance	Rate of death (%) at 24 h			[28]LD_{50}	SD	r
	[28]**10**	[28]**100**	[28]**500**			
[29]Podophyllotoxin	36	100	100	9.9 (81-99.8)	1.7	0.90
Ac	0	14	15	>1000 (4.5-14.8)	9.2	0.70
C	14	100	100	12.4 (81-99.8)	1.9	0.90
PE	38	98	100	6.2 (88-99.8)	1.5	0.89
MW	6	6	6,4	>1000 (9-10)	0.2	0.41
CO_2	12	100	100	13.5 (78-99.8)	2.0	0.90
8.1.9b.2.	96	100	100	1.5E-18 (96.7-100.6)	3.5	0.51
8.1.9b.3.	97,5	100	100	1.0E-30 (97.6-100.8)	2.9	0.39
10.1.	4	0	6,4	>1000 (10-14)	0,2	0.41
10.6.	6	6	-2	NV	NV	NV
Catechin	2	10	1	>1000	-	-
Epicatechin	6	2	0	>1000 (12-19)	0.3	0.24
29	0	7	1	>1000 (0.1-5.3)	4.7	0.42
28	4	13	18	>1000 (5.8-14.8)	8.1	0.78

n = 5 repetitions. [28]= Concentration (µg/mL), 95% confidence limits in parentheses. [29]positive control. NV= no value

Some of the identified constituents in the petrol ether partition were already described as trihydroxy (**54**, **87**, and **88**) (cf. p.p. 108), acetylenic (cf. p. 61), vinylic (cf. p. 88) and furan (cf. p. 103) compounds.

Fraction 8.1.9b.2. was composed of acetylenic compounds **79** and **82** and **58**, whereas fraction 8.1.9b.3. (included 8.1.9b.3.7. fp) was composed of a mixture of acetylenic (**58**, **81**), vinylic (**60**, **83**, **84**, **85)** and furan (**86**) (cf. p. 247), as well as other unidentified compounds. Nevertheless both fractions contain acetylenic components, the fraction 8.1.9b.3. was more active than 8.1.9b.2. (cf. Table 3-25), ma be because of synergetic effects.

3.5.3. Summary of biological assays

According to the results of these assays (cf. p. 192) some answers can be given for the mentioned questions (cf. p. 44).

The use of avocado seeds as antioxidant in some dishes is beneficial because the testa, which is in contact with the food, contains the major polyphenols of the avocado seeds (Ramos, 1999).

As the methanolic extract exhibited the highest scavenging capacity (cf. p. 195), this solvent or even better ethanol is suitable to extract the polyphenolic compounds from avocado seeds.

Nevertheless, compared to the common antioxidants (ascorbic acid, BHT, BHA) used in the industry, the avocado seeds methanolic extract was not so active. Still it could be considered as an alternative to avoid the use of synthetic compounds (BHT and BHA).

The proanthocyanidins in the ethyl acetate and methanolic extracts are not only responsible for the antioxidant activity (cf. p. 193) but they also provide skin regenerating properties (Ramos et al., 2004b). Hence both extracts could be interesting for the cosmetic industry.

The petrol ether extract as well as the petrol ether partition of the avocado seeds showed low antioxidant effects in the TEAC-assay (1.2 or 9.9 %, respectively) in comparison to ascorbic acid (100%) (cf. Table 3-22).

Noteworthy, the toxicity (LD_{50}) of the petrol ether partition containing a highly complex mixture of compounds was stronger (6.2 µg/mL) than the control podophyllotoxin used as reference for measurement of cytotoxicity in the 'brine-shrimp' assay (9.9 µg/mL) (cf. Table 3-25). The tested lipophilic compounds were of high cytotoxic activity.

For the isolated and tested structures, structure-activity relationships were observed for the biological activities monitored by the TEAC antioxidative assay and the 'brine shrimp' assay.

In the case of antioxidative activities, the trimeric A-type substituted proanthocyanidins (**28** and **29**) revealed a much stronger antioxidant capacity than the dimers (**94** and **95**) and the flavanol monomer epicatechin (**26**).

In the monitoring of cytoxicity in the 'brine-shrimp' assay, the presence of the acetoxy group in the lipophilic substances (**58**, **78**, **79**, **81**, **82**, **60**, **83**, **84**, and **85**) was important for activity. Unfortunately about the trihydroxy compounds (**54**, **87**, **88**) we could not make any concrete conclusions.

The stereochemistry (2*S*,4*S*) of compounds **58**, **60** and **84** contained in fraction 8.9b.3 seems to be also important for a high toxicity.

4. Biosynthetic pathways of the isolated compounds from avocado seeds (*Persea americana* Mill., c.v. *Hass*).

4.1. Biosynthetic pathway of acetogenins

Biosynthetic pathway of acetogenins is closely related to fatty acid biosynthesis. The metabolic fate of either dietary or '*de novo'* synthesized fatty acids depends on the balance of a series of enzymatic reactions, which are as follows: chain elongation, desaturation, incorporation into complex lipids, and β-oxidation. The product of each of these reactions then becomes a potential substrate for one of the other pathways to finally result in phospholipids, glycolipids and triacylglycerols. Further elongation and the introduction of double bonds are carried out by different enzyme systems (Kawaguchi et al., 1999).

Bu'Lock (1965), Kashman et al. (1970) and Alves et al. (1970) suggested that acetogenins with terminal ethylene and acetylene groups belong to a class of fatty acid derivatives with an uneven number of carbon atoms. Origins of these compounds are most likely fatty acids subjected to dehydration and desaturation reactions resulting in the biosynthetically interesting terminal vinylic and acetylenic end groups.

However replacement of the acetyl-CoA starter group by other acyl-CoA groups (propionyl-CoA), with chain-extension by malonyl-CoA, has been elucidated as pathway of formation for such fatty acids (Bu'Lock, 1965; Kawaguchi et al., 1999). In case of the C_{17} compounds from avocado, Kashman et al. (1970) suggested that the likely origin is a C_{18}-precursor which is loosing one carbon atom resulting in the formation of a terminal vinylic or acetylene group.

According to Kashman et al. (1970) the pathway of formation of terminal acetylenic and vinylic bonds involves five steps (cf. Fig. 4-1):

Step I. Dehydration and formation of a double bond.

Step II. Oxidation of the terminal methyl group to give an alcohol/aldehyde (**Step III**).

Step IV. Further oxidization of the aldehyde moiety generating a carboxylic acid and following in **Step V** decarboxylation with the loss of a carbon atom resulting in C_{17} compounds with a vinylic terminal group.

In case of the terminal acetylenic group the steps are similar – except for step I where a dehydration followed by a dehydrogenation gives rise to an acetylenic compound. The rest of the steps are identical as described above.

Bu'Lock (1965) suggested that the origin of acetylene groups is the result of desaturation of fatty acids (red arrow in Fig. 4.1), while Alves et al. (1970) proposed for the class of avocatins several changes in the biosynthetic steps (cf. Fig. 4-1). In theory, the direct desaturation of double bonds from fatty acids (marked as red arrows) is the most probable way for the generation of alkines (Bohlmann, 1967).

In the case of the synthesis of acetylenic fatty acids more recent studies by Kohn et al. (1994) confirmed the acetylenic pathway assuming the structural relationship between acetylenes and linoleic or linolenic acid. The enzymes that catalyze such reactions are desaturases (more specifically "acetylenases"), they were characterized by Lee et al. (1998).

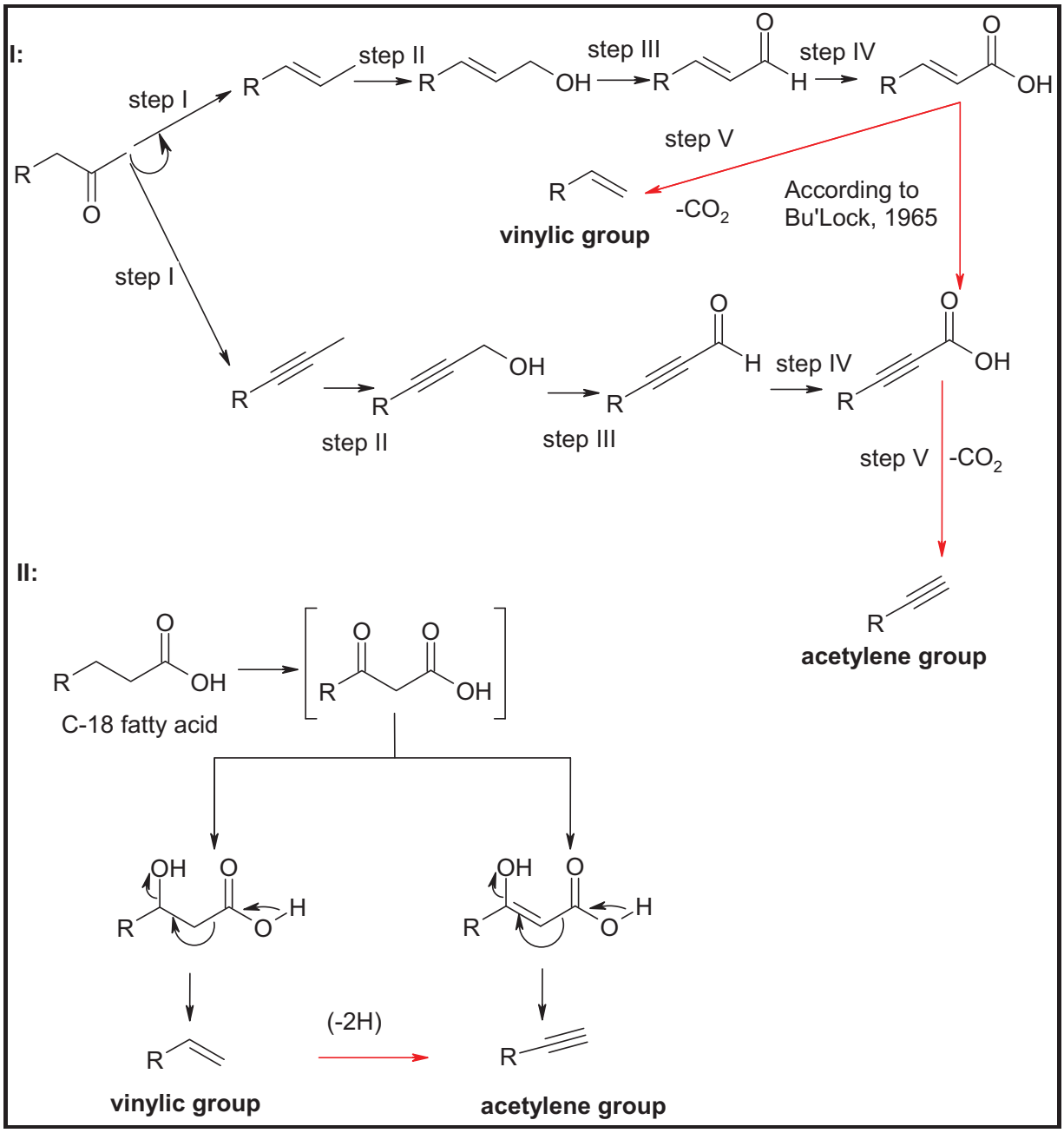


Figure 4-1. Possible biosynthetic pathways for generation of vinylic and acetylenic C_{17} compounds. **I:** According to Kashman et al. (1970), **II:** According to Alves et al. (1970).

The terminal oxygenated compounds from avocado were also considered as intermediates of furan compounds (Alves et al.,1970). Oxygenated terminal compounds called rubrenolides (cf. Fig. 4-2) and similar to trihydroxy- and furan compounds from *Persea americana* were found in *Nectandra rubra* by Cavalcanti-Franca et al. (1971).

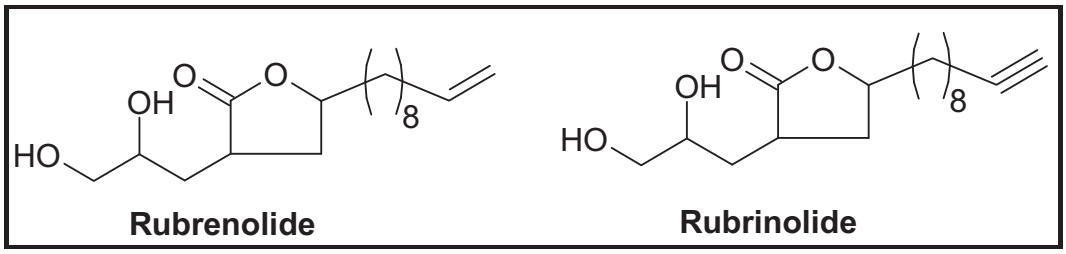


Figure 4-2. Rubrenolides from *Nectandra rubra* (Cavalcanti-Franca et al., 1971)

Gottlieb (1972) suggested that such oxygenated terminal compounds may not be linked to the polyketide pathway and proposed the pathway presented in Fig. 4-3.

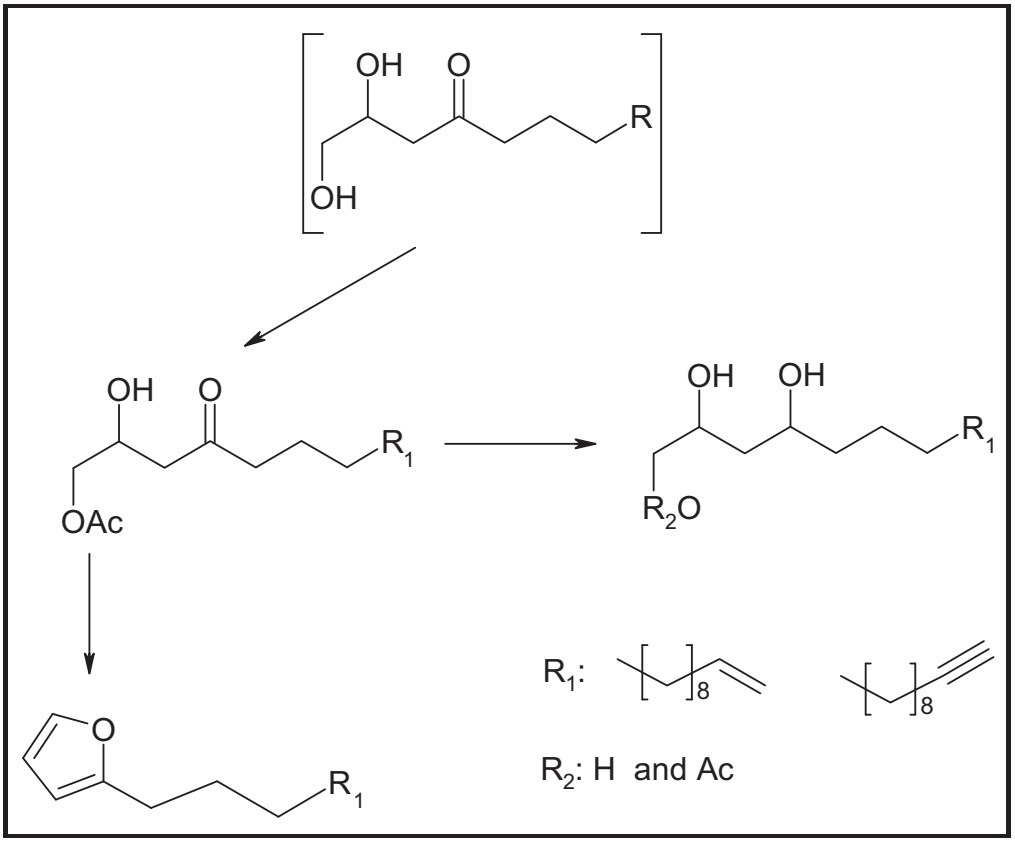


Figure 4-3. Possible biosynthetic pathway for oxygenated terminal groups from *Persea americana* (Gottlieb, 1972).

Another theory was proposed by Carman and Duffield (1995) suggesting that Persin (**66**) might be biosynthesized in the avocado fruit or seed from the monoglyceride of linoleic acid by inclusion of oxygen. Kawagishi et al. (2001) proposed that compounds **64** and **66** (cf. Fig. 2-14 p. 31), both with a terminal oxygenation, may be biosynthesized via a Claisen-type condensation between

oleic or linoleic acid and a three carbon units such as pyruvate. So far no enzymes are known that catalyze such a reaction.

4.2. Biosynthesis of Abscisic acid (ABA)-derivatives

The main metabolic pathway for natural ABA is via oxidation to C-8' hydroxyabscisic acid and ring-closure to dihydrophaseic acid (**101**) and its epimer (*epi*-dihydrophaseic acid), then to natural phaseic acid (**100**) (Balsevich et al., 1994; Hirai and Koshimizu, 1983). From a biosynthetic viewpoint, 8'-hydroxy-abscisic acid β-D-glucoside (**89**) is a 'glucose-protected intermediate' and potential precursor of these compounds. Hence stereochemistry of the formed ether bridge in **70** should directly correlate to the likely precursor substance **89**. As a steric assumption for a successful ether ring formation in system **70**, hydroxymethylene carbon C-7' has to be in axial orientation of the cyclohexane plane (cf. Fig. 3-61, p. 135). Ring closure is causing a partly rigid cyclohexane system, where H-2ax' and H-4ax' are preferably in axial orientation (Ramos et al., 2004a).

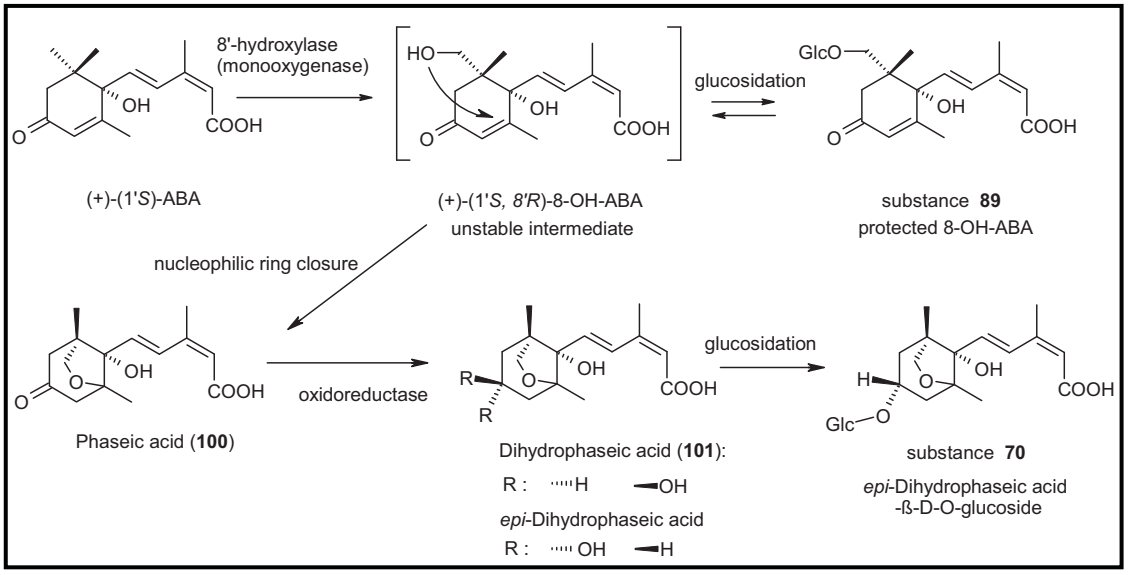


Figure 4-4. Proposed biosynthetical pathway of substances **89** and **70** (Addicott, 1983; Krochko et al., 1998; Ramos et al., 2004a).

The determined configuration of **89** and **70** supports the theory of a biosynthetical linkage between these components (cf. Fig. 4-4). Enzymatic hydroxylation of abscisic acid at C-8' (Krochko et al., 1998) leads to the highly labile intermediate

8'-OH-ABA, which immediately reacts to the bicyclic phaseic acid (Todoroki et al., 2004). This metabolic pathway was assured by feeding experiments with abscisic acid marked with [^{14}C] and [^{18}O] (Addicott, 1983).

In line with these findings is our suggestion that the newly isolated substance **89** (which is protected by glucosidation at C-8') is a missing link in the biosynthetical conversion of hydroxyabscisic acid structures to phaseic acid derivates.

Beside substance **70**, we screened for the glucoside of dihydrophaseic acid, which is the C-3'-epimer. But ESI-LC/ MS analysis of the crude methanolic avocado seed extract, co-elution experiments, and selective ion monitoring of *m/z* 443 detected solely substance **70**. This result implies that in avocado seeds the conversion of phaseic acid to the *epi*-dihydrophaseic acid might be a stereoselective reaction catalized by an oxidoreductase.

4.3. Biosynthetical pathway of tyrosol, hydroxytyrosol and its derivates

In the frame of our studies the β-D-glucosides of tyrosol (**90**) and 3-hydroxytyrosol (**91**) have been isolated from the avocado seeds extracts (cf. p.p.138). The *ß*-D-O-glucoside of tyrosol, named as salidroside (**90**), has been identified in transgenic potatoes (Landtag et al., 2002).

Phenolic compounds are broadly separated in two groups: the flavonoids and the non-flavonoid based structures (Di Tommaso et al., 1998). Non flavonoids are initially synthesized from phenylalanine.

Tyrosol (**92**) and 3-hydroxy-tyrosol (**93**) are the principal constituents of olives (*Olea europaea* L.) (Amiot et al., 1989). Hence, olive oil is containing this antioxidant polyphenol (Visioli et al., 1998; Giovannini et al., 1999; Blekas et al., 2002), as well as waste waters from olive oil processings (Capasso et al., 1999; Medina et al., 2002). Also in red and white wine these compounds have been detected (Di Tommaso et al., 1998; Baderschneider, 2000).

It is known that in case of olive oil hydroxy-tyrosol is formed partly as a result of hydrolysis of oleuropein (**102**) during oil extraction by action of esterases (Amiot et al., 1989). Whereas in wines, tyrosol is generated from tyrosin: Hydroxy-tyrosol could be formed by hydroxylation of the aromatic ring of tyrosol generated during vinification by side activities of the yeasts (Soleas et al., 1997).

The origin of tyrosol and hydroxy-tyrosol in plants is the amino-acid tyrosine and the pathway is shown in cf. Fig. 4-5 (Ucella, 2003; Herrmann, 1995).

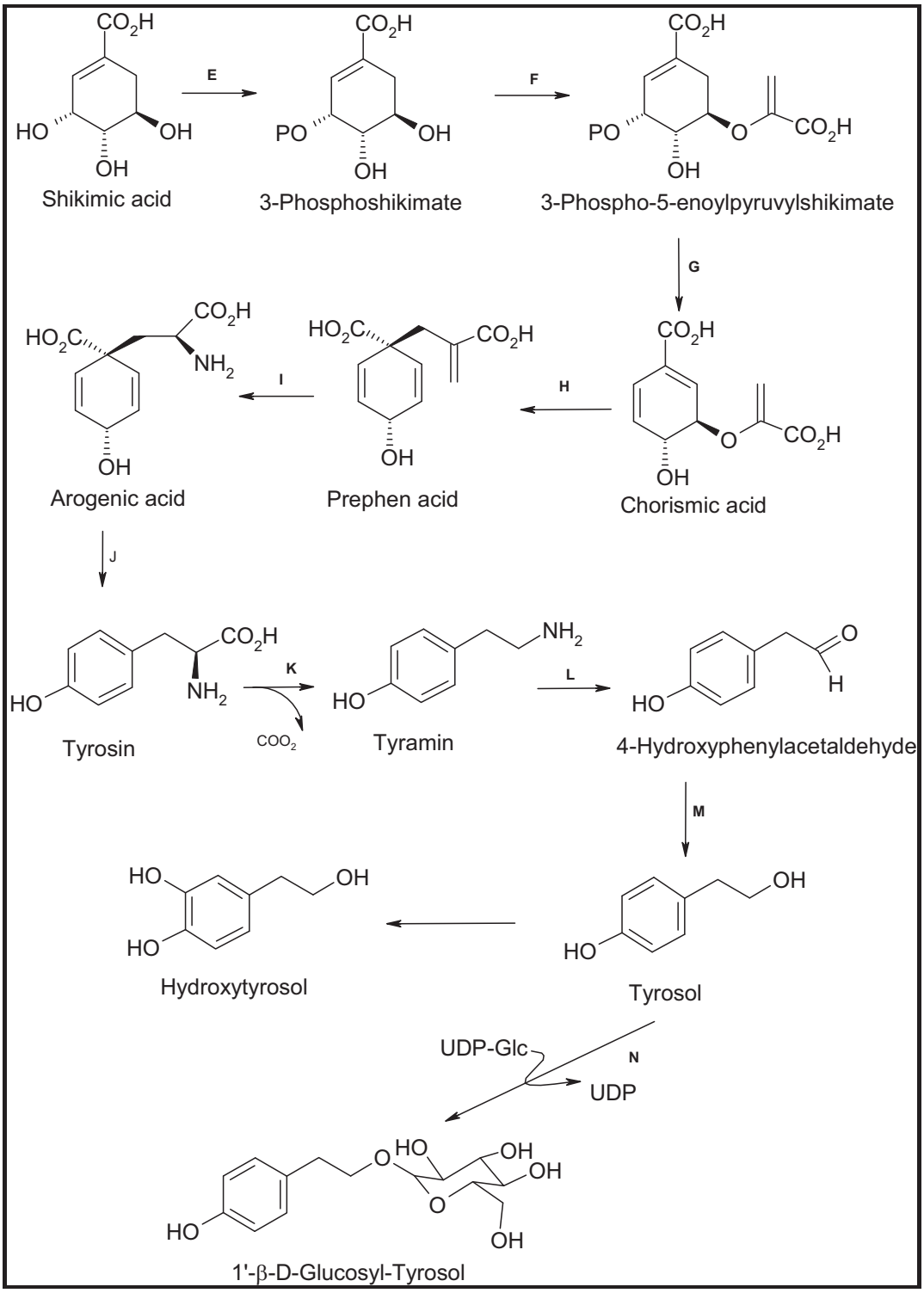


Figure 4-5 Possible biosynthetic pathways of tyrosol-glucoside in the shikimic acid pathway (E: shikimic kinase, F: 5-enol-pyruvyl-shikimic-3-phospate-synthase, G: chorismate syntase, H: chorismate-mutase, I: prephenate-aminotransferase, J: arogenta-dehydrogenase, K: tyrosine-decarboxylase, L: monoamine-oxidase, M: reductase, N: tyrosol-glycosyl-transferase, UDP: uridin diphosphate, Glc: glucose).

It is possible that hydroxytyrosol (**93**) is directly glucosidated with uridin diphosphate glucose (UDP-Glc) (glucoside **91**), similarly also a hydroxylation of the aromatic ring of **90** (cf. Fig. 4-6) is possible.

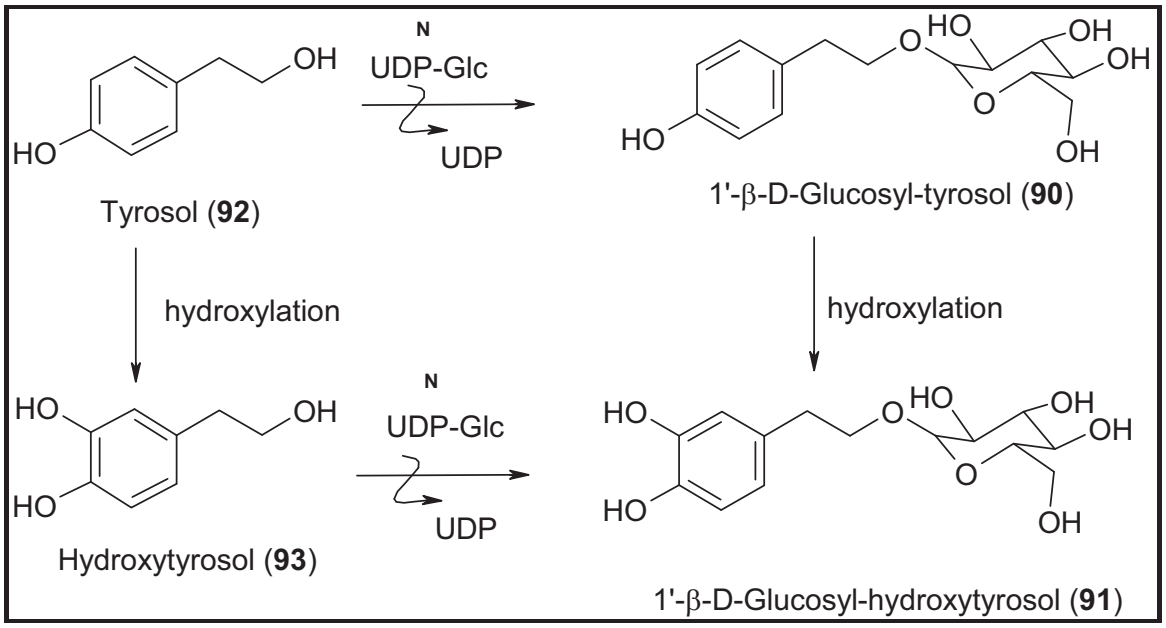


Figure 4-6. Possible biosynthetic pathways of 1'-β-D-Glucosyl-tyrosol (**90**) and 1'-β-D-Glucosyl-hydroxytyrosol (**91**).

4.4. Biosynthesis of Proanthocyanidins

The condensed oligomeric and polymeric non-hydrolyzable tannins are named proanthocyanidins/prodelphinidins and are formed from flavan-3,4-diols. The most common class of proanthocyanidins are those consisting of monomers of catechin, epicatechin, and or epigallocatechin. The biosynthesis of proanthocyanidins has therefore its basis in the biosynthesis of flavan-3-ols.

4.4.1. Biosynthesis of flavan-3-ols

The biosynthesis of flavan-3-ol units starts with malonyl CoA and 4-coumaroyl CoA to give the chalcone narigenin (step I).

In step II the chalcon-naringenin is transformed into the (2*S*)-flavanone naringenin which is later hydroxylated in position 3' (step III). If the hydroxylation occurs as 3'β the (2*R*, 3*R*)-dihydroflavonol (+)-dihydroquercetin or (+)-dihydromyricetin is formed. In case of a 3'α hydroxylation, the (2*R*, 3*S*)-dihydroflavonol (-)-dihydroquercetin or (-)-dihydromyricetin are obtained.

A reductase I acts on the dihydroflavonol to give the (2*R*, 3*S*) - or the (2*R*, 3*R*)-flavan-3, 4-diol respectively (step IV).

Under loss of water, the flavan-3, 4-diol generates a quinone methide (step V). Haslam (1998) suggested that the quinone methide suffers epimerization at position 3 (step VI) to give the (2*R*, 3*R*)-flavan-3-ol (step VII).

However the stereochemistry at the 4-OH function is not certain in the former product. Porter (1994) and Ferreira et al. (1999b) suggested that each quinone methide comes from a β- or α-hydroxylation as described in step III, and generates the corresponding (2*R*, 3*S*)- and (2*R*, 3*R*)-flavan-3-ol (step VII) [(+)-catechin and (-)-epicatechin], respectively (cf. Fig. 4-7).

Stafford and Lester (1984) and Stafford et al. (1985) demonstrated the presence of the two sequential NADPH dependent reductases which catalyse the reduction of (+)-dihydroquercetin to the 2,3-trans-flavan-3,4-cis-diol (leucocyanidin) and then to (+)-catechin.

Lundgren and Theander (1988) isolated the key intermediate (2*R*,3*S*)-dihydroquercetin from *Pinus sylvestris* as ß-D-O-glucoside supporting the pathway from dihydroquercetin to flavan-3-ols.

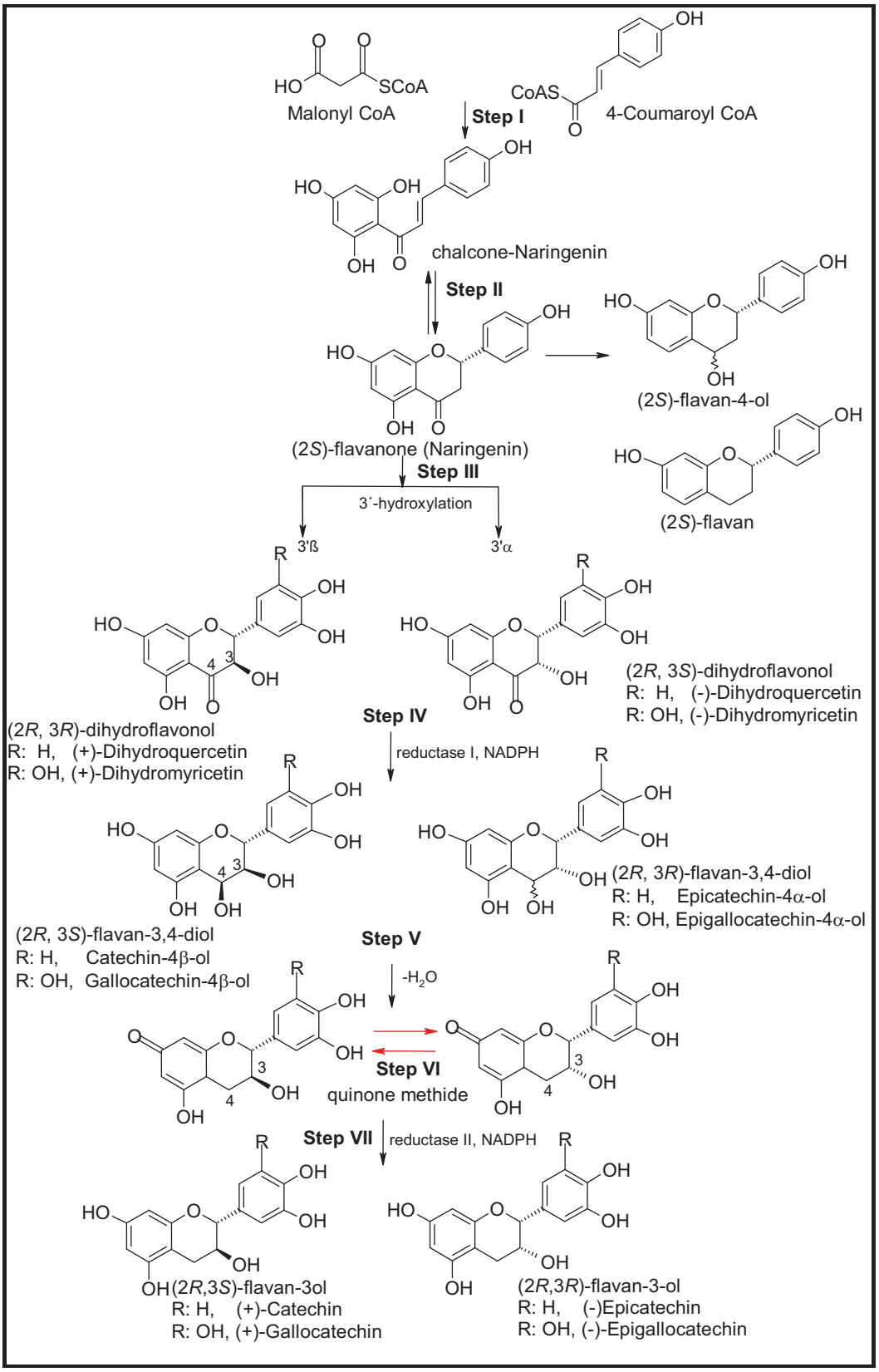


Figure 4-7. Biosynthesis of monomeric precursors to oligomeric proanthocyanidins from dihydroflavonols (Ferreira et al., 1999b; Porter, 1994; Haslam, 1998 in red lines).

4.4.2. Biosynthesis of oligomeric and polymeric proanthocyanidins

It has been suggested that oligomeric proanthocyanidins are formed in a process where the the flavan-3-ol monomer forms in the first step a proanthocyanidin dimer by a stereospecific nucleophilic attack at C-4 of the quinone methide (intermediate in the flavan-3-ols biosynthesis). The dimer then reacts with another quinone methide to generate a trimer and in continuation of this process, chain elongation of proanthocyanidins occurs until a terminal unit is inserted. The majority of the interflavan bonds are connecting the flavonol units over C-4 from the ‘upper unit’ to C-8 of the lower unit. An interflavanoid linkage between C-4 (upper unit) to C-6 (lower unit) is possible but not so prevalent. Then the oligomeric proanthocyanidins generally are originated by the coupling reaction of C-4 (C ring of the upper unit) of an electrophilic flavanyl unit, generated from a flavan-4-ol or a flavan-3,4-diol, to a nucleophilic flavanyl moiety of a flavan-3-ol (Ferreira et al., 1999b). The flavan-3-ol unit contributes to the “terminal unit” and the quinone methide to the “extensions units”. Hence chemical results of these reacting flavan-3-ol units and the quinone methide having different stereochemistry at C-3 and different hydroxyl substitution patterns in the B-ring (Haslam, 1998) lead to highly complex mixtures of oligomeric or polymeric proanthocyanidins (cf. Fig. 4-8 B).

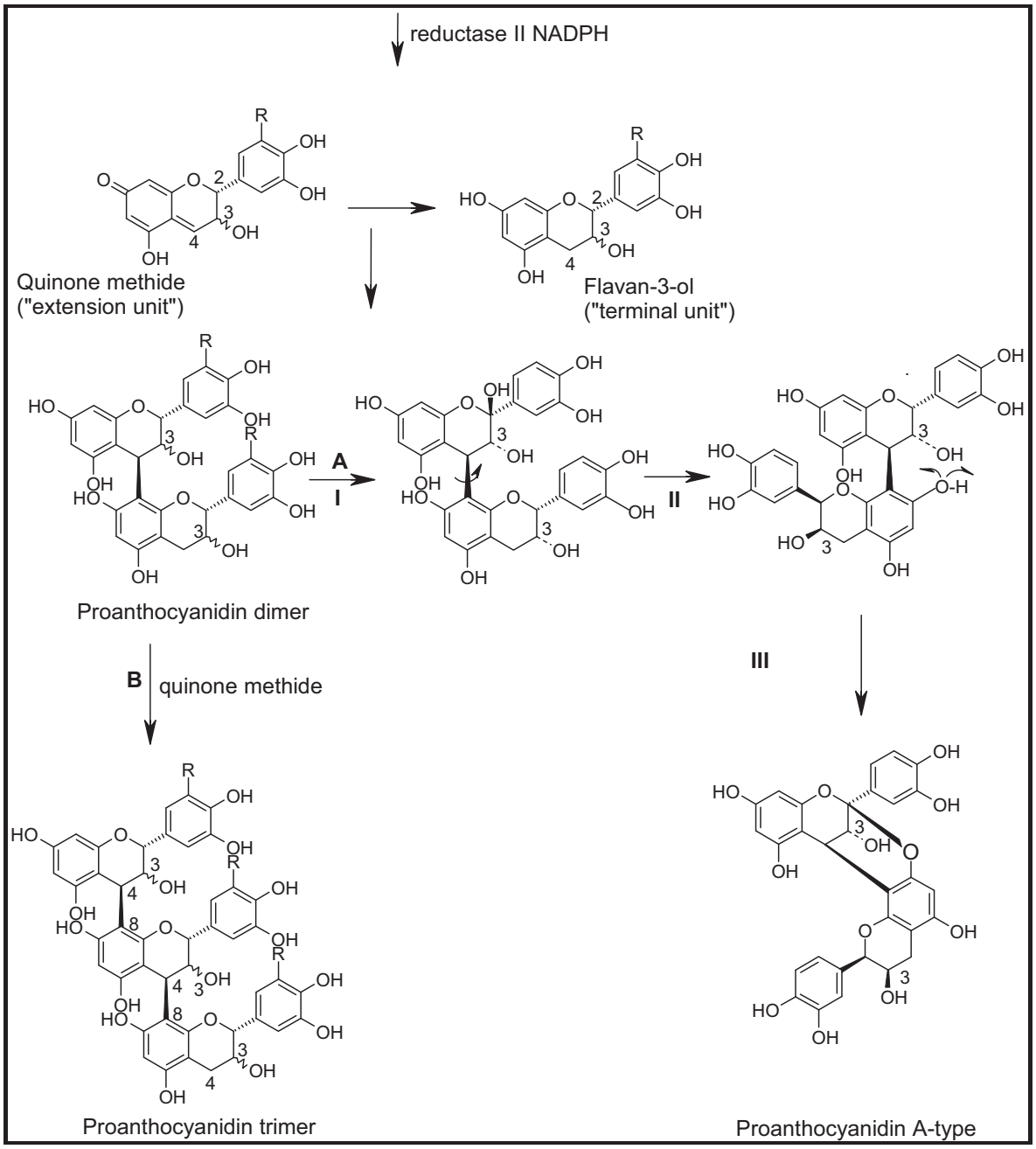


Figure 4-8. Elongation steps (**B**) in the biosynthesis of proanthocyanidin oligomers from flavan-3-ols and quinone methide intermediates (Haslam, 1998). Proposed biosynthetic steps for the conversion of B-type to A-type proanthocyanidins (**A**) starting with hydroxylation of C-2 (step I A), rotation of the lower unit along the C-4/C-8 bond (step II A); radical formation and radical mechanism of ring-closure to the double-linked A-type structure (step III A) (Porter, 1988).

4.4.3. Possible biosynthetic route for the formation of A-type proanthocyanidins

In the case of A-type proanthocyanidins, there are two bonds connecting the flavonol units, one normal linkage (4→8) and the additional one located between C-2 of the upper unit and to O-7 of the lower unit of a dimer or oligomer. Some proanthocyanidins of this class were isolated for the first time from seed shells of horse chestnut (*Aesculus hippocastanum*) by Mayer et al. (1966) and later from avocado seeds (*Persea gratissima*) by Thompson et al. (1972) and Jacques et al. (1974). Recently they have been also isolated from *Ecdysanthera utilis* (Lin et al., 2002), *Cinnamomum* (Anderson et al., 2004) and *Lindera aggregata* (Liu et al., 2005).

The double-linkage in A-type structures creates a rigid structure without possibility for rotation of the flavonol units. Free rotation occurs in the B-type proanthocyanidins and is strongly hampering NMR-analysis.

Probably there is an interconversion between both types of proanthocyanidins mediated by an enzyme leading to hydroxylation at C-2 of the dimer (step I A). In the same way as autoxidation at C-2 takes place, chemical conversion of procyanidins to cyanidine structures occur. This theory suggests a most likely biosynthetic route from B-type to A-type proanthocyanidins (Porter, 1988; Ferreira, 1999a and b) (cf. Fig. 4-8 A).

5. MATERIAL AND METHODS

5.1. Plant material

5.1.1. Seeds of *Persea americana* Mill.

Avocado fruits *Criollo*, *Fuerte* and *Hass* cultivars were collected from trees grown near to Uruapan (Michoacan, Mexico) in summer 2000. The fruits were pitted and the fresh seeds were ground at the moment of volatile analysis by SPME.

Seeds of freshly picked avocados (*Persea americana* Mill. cv. *Hass*) were gently dried in an oven (35-40^{o}C), vacuum sealed and kept at –18°C until extraction.

5.1.2. Cotyledons seed

Avocado seeds (*Persea americana* Mill. cv *Hass*) from Michoacán México, were collected in spring 2002 and gently lyophilized. The cotyledons (approximately 2.5 kg) were separated from the testa and kept at –18°C until extraction.

5.2. Freeze drying (lyophilisation)

A Christ LOC-1m Alpha 2-4 LDC-1M (Osterode, Germany) lyophilization equipment was used.

5.3. Extraction yield

5.3.1. Extraction of complete avocado seeds

5.3.1.1. Solvent extraction

5.3.1.1.1. First Extraction: Experimental design 2^{5-1}

A fractionated factorial experiment (2^{5-1}) was applied for this process. Repetitions were not made and the studied factors were:

a) Solvent type (ethyl acetate, methanol)

b) Material: solvent rate (1:5, 1:10)

c) Temperature (25°C, 40°C)

d) Agitation (0, 500 rpm)

e) Ultrasound potency (50, 70 Volts)

Response factors were:

a) Extraction yield (% dry weight)

b) Total phenolics (expressed as mg phenolics / kg seeds and % TAE dry weight).

Each factor was optimized to gain the highest percentage of extract, content of polyphenols and antioxidant activity.

Knowing the moisture content of the seeds (drying before extraction), the extraction yield was calculated by weight difference after solvent evaporation as percentage (%) dry weight.

Alternatively an aqueous extract was prepared. The extracts were lyophilized and kept frozen (-18°C) until analysis.

The ultrasound equipment was constructed by CIATEJ (cf. Fig. 5-1).

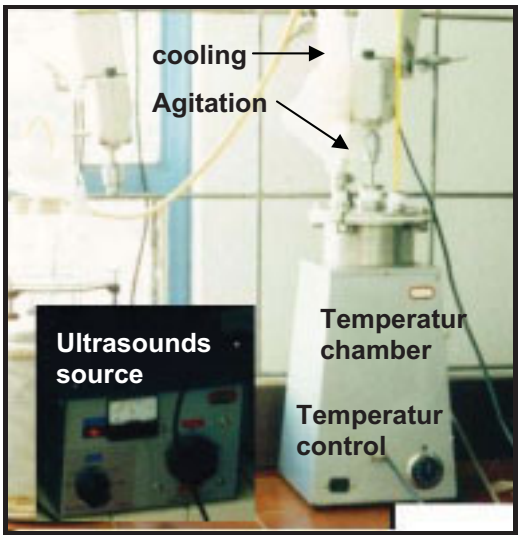


Figure 5-1. Apparatus for solvent extraction under controlled ultrasound conditions and temperature.

5.3.1.1.2. Second Extraction: One factor design

A second extraction design was applied to gain avocado seeds extracts. A one factor design with 4 levels and two repetitions was applied. The factor to study was the kind of solvent (petrol ether, methanol and ethyl acetate). Response factors were the same as above (extraction yield and total polyphenolics).

5.3.1.2. Use of fluids under supercritical conditions (SFE).

300 g of ground avocado seed were extracted by CO_2 under supercritical conditions (207 bars, 50 °C).

The SFE was performed in a Newport Scientific Inc., equipment (Model 46-19345, USA) with a capacity extraction vessel of 0.8 L (cf. Fig. 2-22 p. 40).

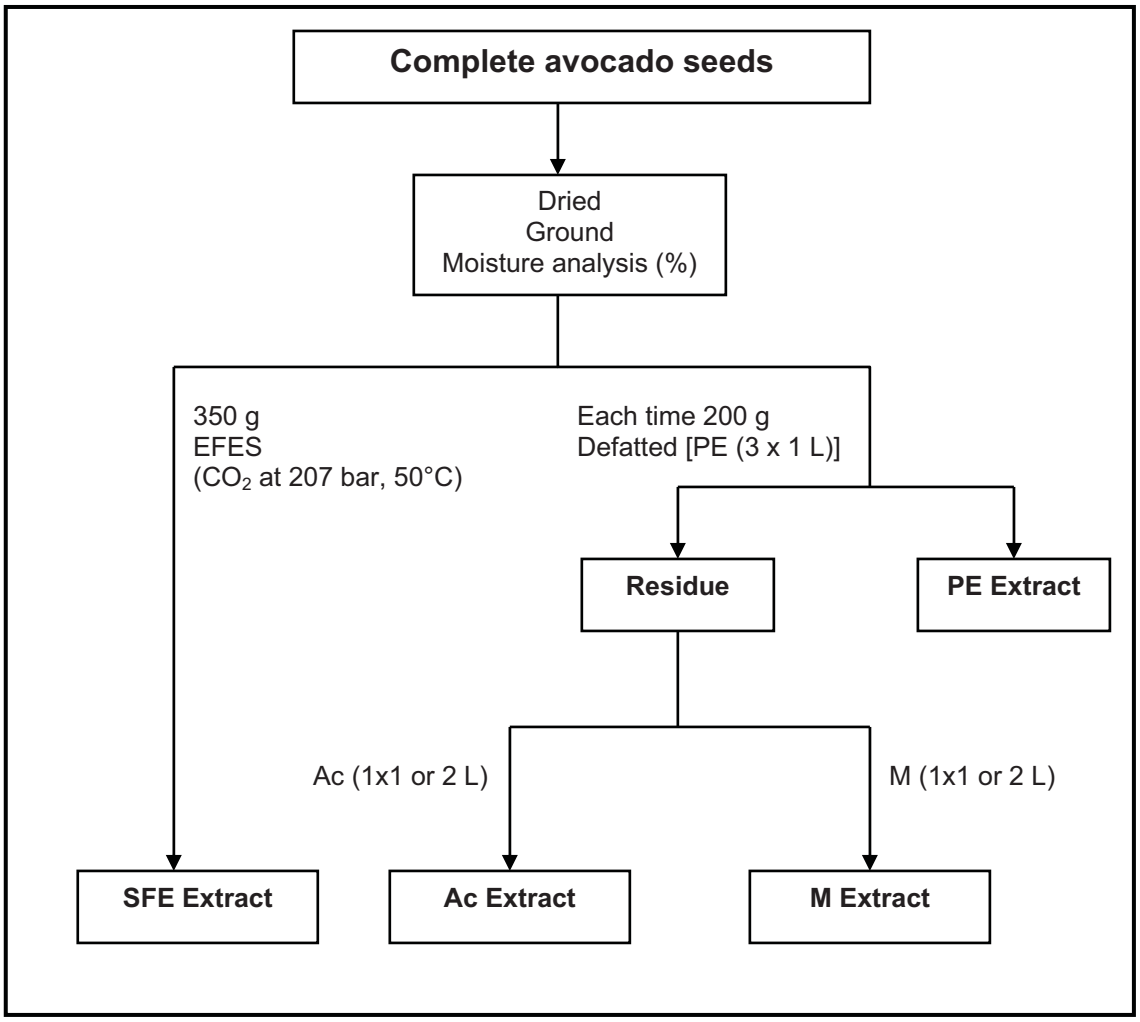


Figure 5-2. Extraction of complete avocado seeds (*Persea americana* Mill., c.v. *Hass*).

5.3.2. Extraction of avocado cotyledons

Avocado seeds were separated in testa and cotyledons. After grinding, the cotyledon powder was defatted with petrol ether and later extracted with methanol. The methanol extract was further extracted with petrol ether (PE), dichloromethane (C), ethyl acetate (Ac) and methanol:water (M:W) (cf. Fig. 5-3).

All these partitions were tested with regards to their scavenging capacity and cytotoxicity (brine-shrimp assays).

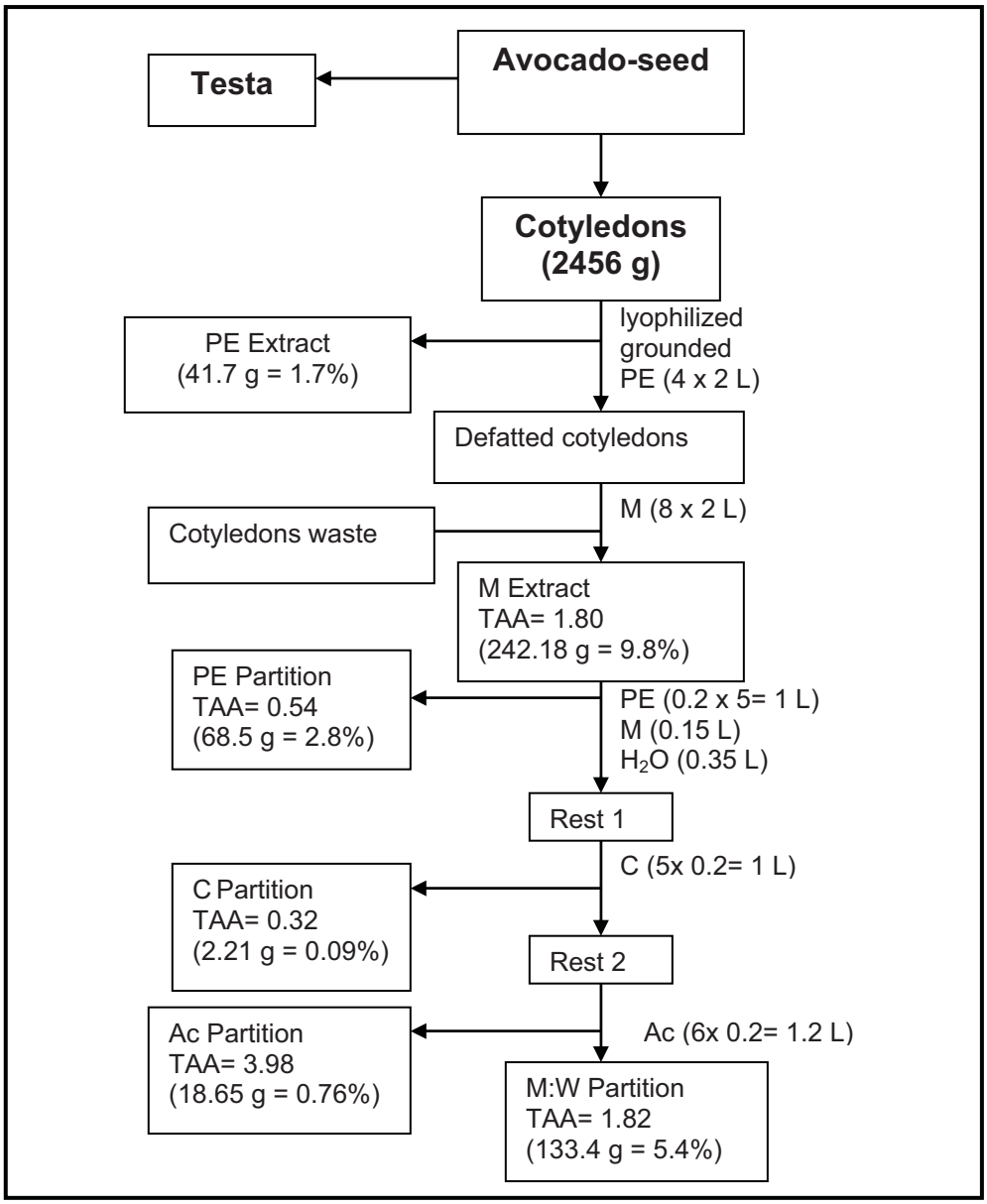


Figure 5-3. Extraction processes for avocado cotyledons

5.4. Methods

5.4.1. Nutritional analysis

Official AOAC methods were used (1999).

5.4.2. Volatile compounds

a) Fibers. Analysis of volatile compounds was achieved by solid phase micro-extraction (SPME). Two classes of fibers were used. **Carbowax**-Divinylbenzene 65 µm (orange) normally used for alcohols and polar substances and **Polydimethylsiloxane**-Divinylbenzene 65 µm (blue) used for volatiles, amines, and nitroaromatic compounds (Supelco Canada).

b) Chromatography: Qualitative analysis was performed on a Hewlett-Packard gas-chromatograph model 5890 series II equipped with a flame ionization detector (FID) and coupled to an integrator. The chromatograph was fitted with a 50 m x 0.2 mm x 0.33 µm dimethylpolysiloxane HP-1 fused silica capillary column. The carrier gas was helium with a flow rate of 1 mL min^{-1}, the injector and detector temperatures were 250°C and 280°C, respectively. The oven temperature was held at 60°C for 0 min, then 3°C min^{-1} to 110°C 0 min and then programmed from 110°C to 260°C at 6°C min^{-1} and maintained during 35 min. Fiber desorption time was 2.5 minutes.

c) Spectrometry: The mass spectra were recorded on a selective quadrupole type Hewlett-Packard detector, model 5972; ionization was obtained by electronic impact under a potential of 70eV, GC conditions were the same as above.

d) Identification of components was based on GC retention times and computer matching with the Wiley Library (300,000 compounds) as well as comparison of fragmentation patterns with those reported in the literature.

e) Sample preparation. Avocado fruits were pitted and the fresh seeds were ground shortly before the analysis.

f) Procedure. Two grams of the powder seeds were put in a vial with a screw cap; 3 mL of phospat buffer (pH 6.5), 0.3 g of chloride sodium as well as a magnet were added. The vial was sealed and stirred for 60 minutes. Adsorption started after this time and continued under agitation for 30 minutes. Each sample was analyzed in duplicate.

5.4.3. The Kóvats Index (RI)

The Kóvats Index (RI) of each compound was calculated according to literature data (Willard et al., 1986; Baugh, P.J., 1993). The linear regression equation 5-1 was used for calculation of RI.

RI= b+ [a (LnRt')] (Eq. 5-1)

With:

RI= retention index

b= intercept of the linear equation

a= slope of the linear equation

Ln Rt'= retention time logarithms

5.4.4. Determination of polyphenolics

Total polyphenolics were determined by the method of Singleton and Rossi (1965).

a) Solutions and chemicals. The Folin-Ciocalteu reagent (Merck, Darmstadt Germany), sodium carbonate solution (20%), and tannic acid (Sigma-Aldrich) for the calibration curve standard (concentration between 20 and 900 mg/L tannic acid).

b) Procedure. Add sample (1 mL) in a 100 mL volumetric flask containing 60-75 mL deionized water. Mix. Add Folin-Ciocalteu reagent (5 mL) and mix again. After 1 min and before 8 min, add the sodium carbonate solution (15 mL), record the time as time zero, and mix again. Make volume up to 100 mL (exactly) with deionized water. Close the flask and mix thoroughly by inverting it several times. After 2 h (to within 1-2-min) record the absorbance of the reactants at 760 nm.

c) Data Analysis. The absorbance of the samples should be put into the linear regression curve to determinate the total phenol content as equivalents of tannic acid (mg phenolics/L). The result is expressed in % (dry weight) of tannic acid equivalents (TAE), in both avocado seeds or in avocado seeds extracts according to equations 5-2 and 5-3.

% TAE avocado seeds= (a) x (b) / ((200-(200 x c)/100) x10) (**Eq. 5-2**)

%TAE avocado seeds extracts = (a) x (b) x (10) / ((d) x e) (**Eq. 5-3**)

With:

a= mg phenolics /L
b= Total volume in L used for extraction
c= Moisture present in the extracted avocado seed (%)
d= g dry seed
e= Yield (%)
200 = g of avocado seeds extracted
10= kg to g conversion
100= to express in %

5.4.5. Biological Test

Plant material is described in part 5.1.

5.4.5.1. Screening of scavenging capacity

5.4.5.1.1. TEAC assay by Miller et al. (1993). Here called the "original TEAC" assay.

a) Chemicals. ß-carotene, *tert* -butylhydroxyanisol (BHA), and 2,6-di-*tert*-butyl-4-methylphenol (BHT), (Buchs, Switzerland). α-Tocopherol and ascorbic acid from Merck (Darmstadt, Germany),

b) Solutions. Phosphate buffer pH 7.4 (221 mg NaH_2PO_4 x H_2O, 1.9192 g Na_2HPO_4 and 8.766 g NaCl were weighed out in a graduated flask of 1L dissolved and filled with H_2O). ABTS-solution [13.7 mg 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt 98% (Sigma Chemical Co., Canada/St. Louis, MO) was weighed out in a 25 mL graduated flask and filled with buffer pH 7.4]. Metmyoglobin solution [6.8 mg Myoglobin (95-100%, Sigma Aldrich, Steinheim) was dissolved in 1mL of buffer. 2.5 mg Potassiumhexacyanoferrate (III) (Merck, Darmstadt, Germany) was dissolved in 10 mL buffer. 1mL this solution was added into the myoglobin solution and stand 5 min. This mixture was separated on a Sephadex G-15 column (40-120 µm, Sigma Aldrich, Steinheim) (L= 20 cm, ID 1 cm) and eluted with buffer. The brown band was collected. The concentration of the metmyoglobin solution was calculated by the extinction of the eluate at 700, 580, 560 and 490 nm

using buffer as blanc. Concentration (µmol/L) was calculated by the formula: C [µmol/L)= 146 x (E_{490}-E_{700})-108 x (E_{560}-E_{700})+ 2.1 x (E_{580}-E_{700}). The solution should be diluted at concentration of 25 µmol/L with buffer]. Solution of H_2O_2 [25.5 µL of H_2O_2 (30% Merck, Darmstadt Germany) into a 10 mL graduated flask and diluted with buffer. 1 mL of this solution was put into a 50 mL graduated flask and diluted to mark with buffer].

b.1) Standard solutions. Ascorbic acid, α-Tocopherol, BHA and BHT standards were dissolved in water or ethanol and diluted with water at 0.1-0.3 mM. 12.516 mg of Trolox was then weighed out into a 5 mL graduated flask (10mM) and filled with ethanol. A Trolox calibration curve was prepared in the range of 0.5 to 2.5 mM.

c) Sample preparation. The methanolic and aqueous extracts from avocado seeds were diluted in a mixture of ethanol-water (concentration between 12 and 26 µg/mL). The extract from ethyl acetate and petrolether were diluted with ethanol and dichloromethane, respectively in a concentration range of 12-26 µg/mL.

d) Procedure. Into plastic cuvettes (1 x 1 x 4.5 cm Hellma, Germany) were transferred 800 µL buffer, 600 µL ABTS-solution,100 µL of each samples, standards or blanc. Then 200 µL of metmyoglobin solution was added into the mixture, stirred and the absorbance was measured versus air at 734 nm (A1). Finally start the reaction by adding 300 µL of H_2O_2 solution, the absorbance was again recorded exactly after 6 min at 734 nm (A2).

e) Data analysis. Cf. 5.4.5.1.2. part e). Determinations were made at least by triplicate.

5.4.5.1.2. TEAC assay by Re et al. (1999). Here called the “modified TEAC” assay.

a) Chemicals. 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt (Sigma Chemical Co., Canada/St. Louis, MO). Potassium persulfate (Riedel de Haën AG, Hannover, Germany). Potassiumhexacyanoferrate (Merck, Darmstadt, Germany). All solvents were of analytical or HPLC grade, ethanol from Carl Roth GmbH + Co (Karlsruhe, Germany), dichloromethane from (Fluka Buchs, Switzerland) and methanol from J.T. Baker (Deventer, Holland). For standards cf. 5.4.5.1.1. part a).

b) Solutions. β-Carotene was dissolved in dichloromethane, BHA and BHT were tested in dichloromethane and methanol at a concentration between 0.1-3.0 mM. α-Tocopherol and ascorbic acid were diluted to 1 mM in ethanol and water, respectively.

b.1) Standard solution. 12.516 mg of Trolox was then weighed out into a 5 mL graduated flask (10mM) and filled with ethanol. A Trolox calibration curve was prepared in a range of 0.5 to 2.5 mM.

c) Sample preparation. Methanolic and aqueous extracts from avocado seeds were diluted in a mixture of ethanol-water. The extracts from ethyl acetate and petrolether were diluted with ethanol and dichloromethane, respectively. Extracts were prepared in a concentration range of 20-100 µg/mL.

d) Procedure. The ABTS work solution should be diluted from the stock solution to give an absorbance of 0.7 at 734 nm. 1 mL of ABTS work solution was transferred into a glass cuvette (0.4 cm x 1 cm x 4.5 cm Hellma, Germany). 10 µL of each sample, standard or blanc were added and stirred. After exactly 6 min the absorbance was registered at 734 nm.

e) Data analysis. The antioxidant activities were calculated as function of absorbance (**y**-axis) and Trolox concentration (mmol/L) (**x**-axis). In case of the standards, they were reported as total equivalent antioxidant capacity (TEAC) in mmol Trolox/mmol Substance. The samples were reported as total antioxidative activity (TAA) in mmol Trolox/ kg extract. Comparison of results between standards and extracts in the TEAC assays were achieved through transformation of the original TEAC (mmol Trolox/mmol standard) or TAA (mmol Trolox /kg extract) units to g Trolox/g standard (SCs) or g Trolox/ g extract (SCe) according to equations 5-4 and 5-5, respectively. The transformed scavenging capacities of extracts and standards relative to ascorbic acid antioxidant capacity (RSCe and RSCs, respectively) were estimated as ratio between results of standard or extract (g/g) and results of ascorbic acid (g/g) as described by equation 5-6. Determinations were made at least by triplicate.

$$SCs\ (g_{Trolox}/g_{substance}) = (mmol\ Trolox \div mmol\ Substance) \times (M_{rTrolox} \div M_{rsubstance}) \quad \textbf{Eq. (5-4)}$$

$$SCe\ (g_{Trolox}/g_{extract}) = (mmol\ Trolox \div kg\ Extract) \times (M_{rTrolox} \div 10^{6}{}_{mg\ factor}) \quad \textbf{Eq. (5-5)}$$

$$RSCe\ or\ RSCs_{\ to\ ascorbic\ acid} = SCe\ or\ SCs \div SCascorbic\ acid \times 100 \quad \textbf{Eq. (5-6)}$$

5.4.5.1.3. DPPH Assay by Brand Williams et al. (1995) and Ley (2001).

a) Chemicals. 2,2 -Diphenyl-1-picrylhydrazyl (DPPH) were from Fluka Chemical Corp. (Buchs, Switzerland). For standards cf. 5.4.5.1.1. part a).

b) Solutions. α-Tocopherol, BHA, BHT and β-Carotene were prepared at final concentrations between 3-34 μM. ß-Carotene was dissolved in dichloromethane and diluted with methanol. BHA and BHT were diluted with dichloromethane or methanol. Ascorbic acid and α-Tocopherol were dissolved in methanol.

b.1) Standard solution. A work DPPH (0.1 mM) methanolic solution was prepared and used for a DPPH calibration curve in the range of 0.0025 to 0.05 mM. A stock ascorbic acid (180 µM) solution was prepared in methanol and used for a calibration curve in the range of 30 to 150 µM.

c) Sample preparation. Avocado extracts were dissolved in ethanol, in a mixture of ethanol:water or in dichloromethane at a final concentration between 7-200 µg/mL.

d) Procedure. 2.5 mL of work DPPH solution (0.1mM) were transferred into a glass cuvette (0.4 cm x 1 cm x 4.5 cm Hellma, Germany) and the absorbance was measured at 514 nm. Add 0.5 mL of each standards, tests solutions at different concentrations or the appropriate control. The absorbance was measured at 514 nm starters at the t=0 and periodically until the steady state was reached. Results were presented as median effective concentration (EC_{50}), in case of pure substances in mmol Standard /mmol DPPH and in case of extracts as kg extract/mmol DPPH. Determinations were made at least by triplicate.

e) Data analysis. DPPH calibration curve was recorded in the range of 0.0025 to 0.05 mM. The initial concentration of DPPH was calculated by equation 5-7,

whereas the residual DPPH concentration was estimated as percentage according to equation 5-8. The effective concentration (Ec) (kg extract/mmol DPPH for extracts or mmol Standard/mmol DPPH) was determined by equation 5-9 and a second response-dose curve was generated by graphing the remanent DPPH (%) vs Ec. From this last response-dose curve the median effective concentration (EC_{50}) was obtained according to equation 5-10. The antiradical power (ARP) is given by equation 5-11. The EC_{100} was calculated by equation 5-12. The inverse (Eq. 5-13) of this theoretical value (EC_{100}) give the number of reduced DPPH$^{\bullet}$ radicals (rDPPH$^{\bullet}$) per antioxidant molecule. By using rDPPH$^{\bullet}$ the scavenging capacity were calculated as g DPPH/ g substance (SCs) or extracts (SCe) according to equations 5-14 and 5-15. The activity of extracts and standards was related to the ascorbic acid activity by equation 5-16.

$\mu M_{initial\ DPPH} = (y - b) \div a$	**Eq (5-7)**
With: y= Abs DPPH solution$_{t=0}$ b= intercept DPPH curve calibration x= µM initial DPPH a= slope DPPH curve calibration	
DPPH Remanent (%)= Abs sample $_{steady\ state}$ ÷ Abs negative control$_{t=0}$	**Eq (5-8)**
Ec = µM Substance or kg extract$_{in\ cell}$ ÷ $\mu M_{initial\ DPPH}$	**Eq. (5-9)**
EC_{50} = (50 – intercept) ÷ slope	**Eq (5-10)**
ARP = EC_{50}^{-1}	**Eq. (5-11)**
EC_{100}= EC_{50} x 2	**Eq. (5-12)**
rDPPH$^{\bullet}$ = EC_{100}^{-1}	**Eq. (5-13)**
SCs ($g_{DPPH}/g_{substance}$) = (rDPPH$^{\bullet}$) x ($Mr_{DPPH} \div Mr_{substance}$)	**Eq (5-14)**
SCe ($g_{DPPH}/g_{extract}$) = (rDPPH$^{\bullet}$) x ($Mr_{DPPH} \div 10^{6}{}_{conversion\ factor\ to\ g}$)	**Eq. (5-15)**
$RSC_{to\ ascorbic\ acid}$ = SCe or SCs ÷ SCascorbic acid x 100	**Eq. (5-16)**

5.4.5.2. Toxicity assay

The brine shrimp (*Artemia salina*) bioassay was performed according to Meyer et al., (1982), Hafner et al. (1977), and Finney (1964) for calculations of lethal dose (LD_{50}) in µg/ml.

a) Chemicals. Methanol, acetone or dichloromethane (P.A. grade) were used as solvents. Podophyllotoxin. Sea NaCl.

b) Solutions/ suspension. Sea salt water (3.2 g of NaCl to 100 mL water), brine shrimps suspension (80 ml of sea water + 50 mg eggs brine shrimps. Incubate at 23°C for 24-48 hrs), yeast suspension (3 mg dry yeast and 5 mL sea water).

b.1) Standard solutions. Podophyllotoxin as positive control [10 µg/µL (A) and 1 µg/µL (B)].

c) Sample preparation. Samples at concentration of 10 µg/µL (A) and 1 µg/µL (B) in acetone, methanol or dichloromethane were prepared. The respective solvent was used as blanc. 5 repetitions were made for each substance to test at 3 different concentrations (10, 100 and 500 µg/mL in cuvette).

d) Procedure**.** Each one filter (12.7 mm ID from Schleicher & Schüll) should be moisten with each diluted solution (30 µL B, 100 µL A and 150 µL A) of sample, blanc or control and stand to dry (cf. Fig. 5-4). Translate 3 mL of sea water to each vial of test, 3 drops of yeast suspension, and 10 shrimps. Translate vials in a water bath, add each filter with the correspondent test substance and incubate at 23°C/ 24 h. After 24 h the alive shrimps were accounted.

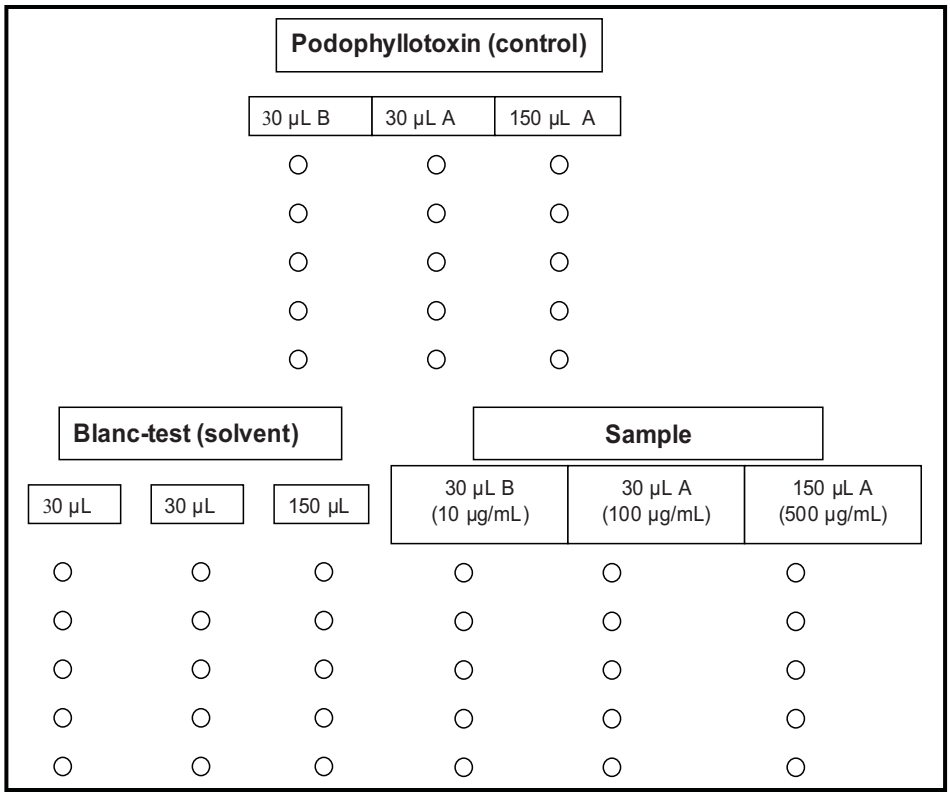


Figure 5-4. Arrangement of filters in the brine shrimp assay.

e) Data analysis. The response (rate of death) was estimated by Eq. 5-17, expressed as percentage and graphed vs log concentration to obtain a dose-response curve. By using equation 5-18 the LD_{50} original data was obtained. In the "probit" transformation, the probit values of the response obtained from literature tables (Gennaro, 1987; Finney et al., 1964) vs. log concentration gave also a LD_{50} according to equation 5-19, whereas in the "logit" transformation (Hafner et al., 1977) the response is transformed to the logit term (z) (Eq. 5-20) and graphed vs. log dose to obtain a dose-response curve, which generates the LD_{50} according to equation 5-21.

Rate of death (%) = [(Substance X-Blind test $_{death\ shrimps}$)]/ Blind test $_{live\ shrimps}$] x 100 **(Eq. 5-17)**

LD_{50} original data = a log x +b **(Eq. 5-18)**

LD_{50} probit transformation data = Antilog (5-b)/a **(Eq. 5-19)**

z= log [y÷(100-y)] **(Eq. 5-20)**

LD_{50} logit transformation data = $10^{(-b/a)}$ **(Eq. 5-21)**

With:

a= slope

b= intercept

x= concentration (µg/ml)

y= rate of death (%)

z= logit term

5.4.6. Standard derivatization

5.4.6.1. Acetylation

The reagents for this reaction were acetanhydride 98-99% (ALDRICH-CHEMIE) and pyridin 99% (J.T. BAKER Chemical).

A mixture of both reagents was made (1:1 v/v) and 1 to 2 mL of the mixture was used for the acetylation of each substance. After 10 hours under agitation at room temperature, the acetylated substance was evaporated under vacuum and then analyzed with LC-MS.

5.4.6.2. Catalyzed acid degradation (thiolysis) modified methods by Scholz et al. (1989) and Rigaud et al. (1991).

The used reagents were benzylmercaptan 99% (FLUKA), HCl (37%). (+)-Catechin, and (-)-Epicatechin (Sigma-Chemical) were used as standards.

125 µL of a methanolic solution of each substance (1mg/mL) to be thiolyzed and 125 µL of benzylmercaptan reactant (5% solution of benzylmercaptan in a solution of 3.3 % HCl in M) were put it in a sealed glass and heated in an oven at 90°C for 3 min. After this time thiolyzed substances were analyzed by LC-MS and HPLC-DAD on a Zorbax column 150 x 4.6 mm. Solvent A (aqueous CH_3COOH 2.5 %), solvent B [ACN: phase A (80:20)]. Gradient: t (0, 35, 40, 45 min), A (85, 25, 0, 85 %), B (15, 75, 100, 15 %). Flow rate=0.8 mL min^{-1}. λ= 280 nm.

5.4.6.3. Trimethylsilylation preparation of TMS-Ethers was made according to the method of Kleiman et al. (1973) and Fluka (1995).

Reagent: MSTFA reagent: N-methyl-N-(trimethylsilyl)trifluoro acetamide (FLUKA).

The samples (0.2-0.5 mg) were put in a 5 mL flask with an excess of MSTFA, stirring overnight at room temperature, the reaction mixture was evaporated with nitrogen gas, and analyzed with LC-MS.

5.4.6.4. Enzymatic hydrolysis with Rohapekt D5L.

Approximately 0.6 mg of the substance was solubilized in 1 mL of buffer (citric acid-Na_2HPO4 pH= 5.5 according to McIllvaine) and 100 µl of Rohapect D5L (Röhm) were added. The flask was sealed, stirring at 37°C for 30 h. The samples were separated in two parts (A and B). Part A was directly analyzed by LC-MS (aglycon and sugar analysis) while part B was further extracted with diethylether (4 x 5 mL). The organic phase was separated and the ether evaporated to dryness. Finally, 1 ml of the same buffer was added prior to LC-MS analysis of the aglycone. LC-MS conditions (neg. mode): Column Prontosil AQ C-18, 250 x 2.0 mm. A: H2O, B: ACN. Gradient t (0, 10, 40, 55, 65, 75 min), A (97, 97, 40, 0, 0, 97), B (3, 3, 60, 100, 100, 3). Flow rate 0.25 mL min^{-1}.

5.5. High-speed countercurrent chromatography (HSCCC)

For preparative high-speed countercurrent chromatography (HSCCC) a model CCC-1000 equipment, Pharma-Tech Research Corp. was used (cf. Fig. 2-24 p. 43).

5.6. Column chromatography (CC)

5.6.1. Columns

Different columns from PHARMACIA, LATEK, and QUICKFIT were used.

5.6.2. Gels (stationary phase)

For permeation chromatography different gels were used. Dextrangel sephadex® LH-20 (PHARMACIA), sephadex G-15 (on antioxidative test TEAC), and Fractogel® PVA 500 (MERCK). The gels were conditioned with solvent before use. The mixtures of solvents are given in Table 5-3.

Flash chromatography on silica gel 60 0.040-0.063 mm (230-400 mesh ASTM) (MERCK) was mainly used for the isolation of lipophilic compounds.

5.6.3. Solvents

Petrolether Rotisolv (F_p 40-60°C) (Carl Roth GmbH). Methanol HPLC, ethyl acetate, n-hexane, acetonitrile, and acetone were from Fisher-Scientific. Dichloromethane was from MWG-Biotech. Nanopure® H_2O. Methanol p.a. was freshly distilled.

5.7. Middle pressure liquid chromatography (MPLC)

MPLC equipment consisted of a middle pressure pump, Büchi glass columns (specifications on Table 5-3), silica gel 60 0.040-0.063 mm (230-400 mesh ASTM) (MERCK), or LiChroprep® RP-18 (0.040-0.063 mm; MERCK). Solvents were distilled before use and mixtures are given in Table 5-3.

5.8. High pressure liquid chromatography (HPLC)

5.8.1. Equipments

5.8.1.1. HPLC diode array detection (DAD)

Jasco PU-980 Intelligent HPLC pump; Jasco DG-980-50 3-line degasser; Jasco LG-980-02 ternary gradient unit and Jasco MD-910 Multi-wavelength detector (DAD).

5.8.1.2. Preparative HPLC

KNAUER HPLC pump 64; 7126 KNAUER injector; KNAUER variable wavelength monitor; recorder LKB Bromma 2210 2 channel recorder and preparative cell 10 mm.

5.8.1.3. HPLC-MS/MS

Hewlett Packard binary gradient pump G1312A and a BRUKER Esquire HPLC-ESI-MS-MS ion trap mass spectrometry system. Detector: L-4000 UV-detector MERCK-HITACHI). SHIMADZU C-R6A CHROMATOPAC integrator.

5.8.2. Columns

5.8.2.1. Analytical columns

- BISCHOFF stainless steel filled with 120-5-C18-AQ, 5µm Prontosil Aqua analytic (250 mm x 2 mm).

- BISCHOFF stainless steel filled with 120 C18, 5µm Prontosil Aqua analytic (250 mm x 4.6 mm). For cotyledons ethyl acetate partition Flow rate: 0.7 mL min^{-1}. A: H_2O, B: ACN, t (0, 10,40,55,65,75 min), A (97,97,40,0,0,97), B (3,3,60,100,100,3)].

- AGILENT TECHNOLOGIES stainless steel Zorbax Aqua filled with SB-C18 rapid resolution, 3.5 µm (150 mm x 4.6 mm).

- PHENOMENEX stainless steel filled with AQ RP-18, 5µm, 125 A° (250mm x 4.6 mm) for LC-ESI-MS of M:W extracts of testa and cotyledons analyses. Flow rate: 0.7 mL min^{-1}. Solvent A (nanopure® water), solvent B (ACN). Gradient: t (0,10,40,45,50 min), A (97,97,40,97,97 %), B (3,3,60,3,3 %).

- ODS-Beckman stainless steel filled with RP-18, 5 µm (250 mm x 4. 6 mm).

5.8.2.2. Preparative columns

- BISCHOFF stainless steel, filled with Prontosil Aqua RP-18, 5 µm (250 mm x 16 mm).

- KNAUER stainless steel, filled with Eurospher 100-C18, 7 µm (250 mm x 16 mm).

- KNAUER stainless steel, filled with ODS-Hypersil, 5 µm (250 mm x 16 mm)

5.9. Thin layer chromatography (TLC)

5.9.1. Stationary phases

- Silica gel 60 F_{254} TLC alufoils 20 x 20 cm (layer 0.2 mm, MERCK).

- Alugram RP-18 W/UV_{254} 20 x 20 cm (layer 0.15 mm; MACHEREY-NAGEL).

- Cellulose F plates with fluorescence indicator (FLUKA).

5.9.2. Detection

- UV at 254 and 366 nm (in connection with fluorescence indicator F_{254})

- Spray reagent anisaldehyd-sulfuric acid according to Stahl (1967). Visualization was done with anisaldehyde- concentrated sulfuric acid- glacial acid (1:2:97), and flash heating (110 ^{o}C) on a hot plate.

- Vanillin reagent (MERCK) for cellulose plates by Waterman and Mole (1994).

5.9.3. Solvent mixtures for TLC

Table 5-1 Solvents mixture codes

Code	Mixture for TLC
S1	C/M/W (75:25:1)
S2	C/M/W(75:25:5)
S3	C/M/W (75:25:10)
S4	C/M/W (75:25:7,5)
S5	Tl/Ace/FA (45:50:10)
S6	6% AA aqueous solution
S7	2-B/S6/W (14:1:5 v:v:v)
S8	M/W (1:1)
S9	M/W (4:6)
S10	Tl/Ace/FA (45:50:12)
S11	M/W (2:8)
S12	M/W (9:1)
S13	M/W (75:25)

For solvents codes cf. Table 5-2.

5.10. Circular dichroism (CD)

CD curves were recorded on a Jasco spectropolarimeter type J-715 at 21°C (scan range: λ=210-350 nm, cell length: 0.1 cm). The standard solvent was methanol. In case where other solvents were used, they are given in the physicochemical description of the respective substance.

5.11. Mass spectrometry (MS)

ESI-LC-MS, and MS^n experiments were performed on a BRUKER Esquire LC-MS-MS ion trap mass spectrometry system.

- For HPLC-ESI-MS-MS analysis, a Hewlett Packard binary gradient pump G1312A was coupled to the ESI-LC-MS system. ESI-MS, negative and positive modes, was used under the following conditions: dry gas nitrogen 9.0 l min^{-1}, nebulizer pressure 40 psi, capillary +3500 V, end plate +3000 V, capillary exit -95 V, skim 1 -25 V, skim 2 -10 V.

- For ESI MS-MS fragmentation studies, samples were introduced via a syringe pump at a flow rate of 240 μl min^{-1}, drying gas nitrogen (7.0 l min^{-1}, 330 ^{o}C), and

nebulizer pressure (5 psi). ESI-MS (negative modes) under capillary +4500 V, end plate +4000 V, cap exit -90 V, cap exit offset -60 V, skim 1 -30 V, skim 2 -10 V; ESI-MS (pos. mode): capillary -4500 V, end plate -4000 V, cap exit +90 V, cap exit offset +60 V, skim 1 +30 V, skim 2 +10 V.

5.12. Nuclear magnetic resonance spectroscopy (NMR)

Depending on the isolated substance, the deuterated solvents were D_2O, CD_3OD, d_6-acetone, CD_3CN, CD_3Cl, CD_2Cl_2, C_5D_5N (pyridine). The chemical shifts are given in δ values (ppm).

^1H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded on a Bruker AMX 300 spectrometer. δ values are relative to those of the solvent signals (δ_H 4.72 ppm for D_2O; δ_H 3.31 and δ_C 49.0 ppm for CD_3OD; δ_H 2.05 and δ_C 30.5-205.1 ppm for d_6-acetone; δ_H 1.93 and δ_C 1.28-118.10 ppm for CD_3CN;δ_H 7.26 and δ_C 77.36 ppm for CD_3Cl; δ_H 7.21, 7.57, 8.72 and δ_C 123.50, 135.50, 149.50 ppm for d-pyridine).

The two-dimensional NMR experiments heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) as well as one-dimensional *diff*-NOE spectra were measured on a Bruker AM 360 (360 MHz for ^1H, and 90.6 MHz for ^{13}C) in MeOH-d_4, CD_3CN or in a mixture of CD_3CN: D_2O.

5.13. Spectrophotometer

A Spectronic 21 DUV for the polyphenolics analysis and a Shimadzu UV-2101 (Shimadzu Co., Japan) for the TEAC and DPPH determinations.

5.14. Abbreviations for solvents

Table 5-2. Solvent code for Table 5-3

Code	Solvent
ACN	Acetonitrile
AA	Acetic acid
Ac	Ethyl acetate
Ace	Acetone
B	n-Buthanol
2-B	2-Buthanol
C	Dichloromethane
$CHCl_3$	Chloroform
$CO_{2\ (s)}$	SFE CO_2 207 bar, 50°C
E	Ethanol
EE	Ethyl ether
PE	Petrol ether
FA	Formic acid
Hx	Hexane
HCl	Hydrochloric acid
M	Methanol
T	tert.-Butyl-methylether
Tl	Toluol
W	Water

5.15. Chromatographical separation

Extracts and partitions of complete avocado seeds and cotyledons were further purified and identified as:

1. Methanolic extract (M) from complete avocado seed by HSCCC

2. CO_2(s) extract from complete avocado seed by HSCCC

3. Petrolether (PE) extract from cotyledons

4. Dichloromethane partition (C) from cotyledons

5. Ethylacetate partition (Ac) from cotyledons on Sephadex LH-20

6. Ethylacetate partition (Ac) from cotyledons by HSCCC

7. Petrolether partition (PE) from cotyledons by HSCCC

8. Petrolether partition (PE) from cotyledons pre-liquid-liquid fractionation and CC

9. Methanol/Water (M:W) partition from cotyledons on Sephadex LH-20

10. Methanol/Water (M:W) partition from cotyledons by HSCCC

A complete separation scheme is given in Table 5-3.

Table 5-3. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
1	1. (6066 mg)	Without (HSCCC)	T/B/ACN/W (1:3:1:5)	3 coils (850 mL)	1 (552) 2 (3716) 3 (94) 4 (84) 5 (118) fp 6 (51) fp 7 (15) 8 (36) fp 9 (11) fp 10 (5) 11 (6) 12 (6)
2	1.5. (56 mg)	Sephadex LH-20 (CC)	M	ID 1.5 cm l 70 cm	1 (6.9) 2 (5.6) fp 3 (2.9) 4 (6.8) fp 5 (4.0) 6 (8.5) 7 (5.2) 8 (6.4) 9 (7.4)
3	1.5.2. (5.6 mg)	SiO_2 (CC)	C/M/W (75:20:1)	ID 1 cm l 15 cm	1 (<1) 2 (<1) 3 (1.5) 4 (<1) 5 (<1) 6 (2.6) fp 7 (1.4)
4	1.5.2.6. (2.6 mg)	PVA (CC)	M	ID 1 cm l 40 cm	1 (2.4) [**70**]
5	1.5.4 (6.8 mg)	SiO_2 (CC)	C/M/W (75:20:3)	ID 1 cm l 15 cm	1 (5.4) [**90**]
6	1.6 (25.8 mg)	PVA	M	ID 1 cm l 40 cm	1 (1.6) 2 (1) 3 (5.1) 4 (5.3) [**90**] 5 (5.5) [**90**] 6 (6.3)
7	1.89 (47 mg)	Sephadex LH-20 (CC)	M	ID 1.5 cm l 70 cm	1(8.8) 2 (15.9) fp 2a (2.2) [**89**] 3 (9.7) 4 (10.8)
8	1.89.2 (15.9 mg)	SiO2 (CC)	C/M/W (75:20:3)	ID 1 cm l 15 cm (not good)	1 (4.8) 2 (6.0) 3 (8.0)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
9	2. (1000 mg)	Without (HSCCC)	HX/E/W (7:4:1)	3 coils (850 mL)	1 (126.8) 2 (31.7) 3 (53.2) 4 (45.3) 5 (7.6) 6 (22.7) 7 (62.6) fp 8 (52.8) 9 (8.5) 10 (74.8) 11 (7.5) 12 (19.4) RC (152.9)
10	2.7. (62.6 mg)	Sephadex LH-20 (CC)	M/C (8:2)	ID 2cm l 86	1 (44.8) 2 (1.7) 3 (1.1) 4 (2.2) 5 (5.2) 6 (0.9)
11	3 (3300 mg)	SiO_2 (CC)	C/M (99:1 24 h) (95:5 24 h)	ID 2 cm l 30cm	1 (524) 2 (96) 3 (116.7) 4 (1388) 5 (219) 6 (915) 7 (251) 8 (62) RC (86)
12	4 (1200 mg)	Sephadex LH-20 (CC)	M	ID 3 cm l 79 cm	1 (208.6) 2 (452.7) fp 3 (230.1) fp 4 (163) 5 (65.1) 6 (16.7) 7 (5.8) 8 (19.3) 9 (1.2) 10 (2.8) 11(3.1)* RC (3.5)
13	4.2. (241 mg)	SiO2 (CC)	C/M (95:5 24 h) (85:15 24 h)	ID 1 cm l 44 cm	1 (7) 2 (70) 3 (88) 4 (19) 5 (6) 6 (5)* 7 (2) 8 (3) 9 (16) 10 (1.7)* 11 (22)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
14	4.3. (70.8 mg)	SiO2 (CC)	C/M (99:1 24 h) (90:10 24 h)	ID 1 cm l 12 cm	1 (4.2) 2 (6.8) 3 (11.8) 4 (9.2) 5 (2.2) 6 (18.2) fp 7 (2.3) 8 (1.9) RC (5.0)
15	4.3.6. (7.4 mg)	Eurospher RP-18 100 (5µm) (HPLC)	M/W (30:70)	ID 4.6 mm l 250 mm (not UV activ) (not good)	1 (<1) 2 (<1) 3 (1.8) 4(2.4)*
16	5 (6700 mg)	Sephadex LH-20 (CC)	M	ID_1 3 cm l_1 79 cm ID_2 5cm l_2 87 cm	1 (438.3) fp 2 (378.4) 3 (154.3) fp 4 (133.3) 5 (135.9) 6 (101.1) 7 (62.2) 8 (260) fp 9 (276.2) 10 (163.6) 11 (52.7) 12 (103) 13 (101) 14 (79.4) 15 (33.7) 16 (116.8) 17 (380.2) 18 (385.6) 19 (365.3) 20 (259) 21 (101.3) 22 (423.2) RC (215.5)
17	5.1 (349 mg)	SiO2 (CC)	C/M/W (75:25:1 72h) (75:25:5 24 h)	ID 2.5 cm l 31 cm	1 (31.1) 2 (40.7) 3 (26.1) 4 (42.7) 5 (34.7) 6 (6) 7 (50.7) RC (89.4)
18	5.3. (128 mg)	SiO2 (CC)	C/M/W (75:25:1 24h) 75:25:5 24 h)	ID 1 cm l 40 cm	1 (12.2) 2 (42.7) [**90**] 3 (7.6) fp 4 (2.3) 5 (5.8) 6 (6.9) 7 (2.6) RC ()

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Abdsorbent (method)	Mobile phase	Column parameter	Fractions (mg)
19	5.3.3. (7.6 mg)	SiO2 (CC)	C/M/W (75:25:1 24h)	ID 1 cm l 12 cm	1 (5) 2 (2) [**91**] 3 (0.5)
20	5.8 (42 mg)	Prontosil AQ RP-18 (HPLC)	ACN/W (20:80)	ID 16 mm l 250 mm	1 (0.3) 2 (1.3) 3 (0.4) 4 (0.7) 5 (0.1) 6 (0.1) 7 (2.6) 8 (1.4) 9 (0.9) 10 (0.1) 11 (3.3) 12 (1.2) 13 (2) 14 (0.4) 15 (0.6) 16 (0.1) 17 (0.5)* RC (19.5)
21	6 (2100 mg)	Without (HSCCC)	Hx/Ac/M/W (1:54:1:54)	3 coils (850 mL)	1 (769.4) 2 (152.9) 3 (469.3) fp 4 (17.5) 5 (50.8) 6 (94.6) [**26**] 7 (18.4) RC (151.5)
22	6.3 (188.8 mg)	Prontosil AQ RP-18 (HPLC)	ACN/W (17:83)	ID 16 mm l 250 mm	1 (1.47) 2 (0.8) 3 (2.7)* 4 (16.4) [**94** and **95**] 5 (41.3) [**29**] 6 (42.6) [**28**] 7 (3.2)* 8 (2.5) 9 (2.2)* RC (6.7)
23	7. (1000 mg)	Without (HSCCC)	M/W (3:4)	3 coils (850 mL)	Not good
24	7a (1000 mg)	Without (HSCCC)	M/H/W (2:4:1)	3 coils (850 mL) (not good)	1 (90.2) 2 (159.1) RC (251.7)
25	8. (25200 mg)	L-L Because Plugging column risk	EP/M (85:75)		1 (14000) fp 2 (7500)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
26	8.1 (8400 mg)	SiO2 (MPLC)	EP (16.7h) Hx/E (95:5) 8.3 h	ID 5 cm l 46 cm	1 (2.2) 2 (18.6) 3 (31.2) fp 4 (43.3) 5 (64.1) fp 6 (24.6)* 7 (12.1) 8 (3323.3) fp 9 (1609.7) fp 10 (312.6) fp RC (1825.5)
27	8.1.3 (31.2 mg)	Eurospher RP-18 (HPLC)	M/ W (90:10 105 min) M (25 min)	ID 16 mm l 250 mm	1 (0.1) 2 (1) 3 (1.2) 4 (0.4)
28	8.1.5 (64 mg)	Eurospher RP-18 (HPLC)	M/W/ min (90:10 0) (90:10 40) (95: 5 40) (100:0 40)	ID 16 mm l 250 mm	1 (2.8) 2 (0.6) 3 (1.7)
29	8.1.8 (561.5 mg)	RP-18 (MPLC)	M/W (80:20 21 h) (90:10 5 h) M (100 9 h)	ID 3 cm l 38 cm	1 (4.7) 2 (2.4)* 3 (2.7)* 4 (9.6)* 5 (24.7)* 6 (70.6) 7 (25.5) 8 (105) 9 (19.1) 10 (79.5) 11 (23.5) 12 (11.3)* 13 (19.3)* 14 (12.1)* 15 (49) 16 (17.3) RC (4.4)
30	8.1.9a (64.8 mg)	RP-18 ODS-Hypersil (HPLC)	M/W (80:20)	ID 16 mm l 250 mm	1 (0.7) 2 (1.1) 3 (8.2) [**58**] 4 (2.0) 5 (6.5)* 6 (0.6) 7 (0.6) 8 (2.3) 9 (-) 10 (0.1) 11 (0.6) 12 (0.7) RC E (9.4) RCAce(1.4)*

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
31	8.1.9b (1000 mg)	Without (HSCCC)	Hx/E/W (6:5:1)	3 coils (850 mL)	1 (20.5) 2 (300.8) fp 3 (323) fp 4 (45.9) 5 (31.7) 6 (44.8) 7 (27.6) 8 (10.2) 9 (11.7) 10 (17.0) RC (21)
32	8.1.9b.2 (287 mg)	RP-18 (MPLC)	M/W (75:25 12 h) (80:20 12 h)	ID 3 cm l 39 cm	1 (2.1) 2 (1.2) 3 (0.8) 4 (0.6) 5 (1.3) 6 (4) [**82**] 7 (2.9) 8 (3.2) [**79**] 9 (2.7) 10 (68.7) [**58**] 11 (115.1) 12 (58.3) 13 (6.8) RC (0.6)
33	8.1.9b.3 (323 mg)	RP-18 (MPLC)	M/W (80:20 12 h) (88:12 12 h)	ID 3 cm l 39 cm	1 (2.1)* 2 (0.9) 3 (3.2) [**82**] 4 (9.7) [**81**] 5 (25.4) 6 (8.6) [**58**] 7 (25.3) fp 8 (13.1) 9 (61.3) 10 (53.9) 11 (42) [**60**] 12 (2.7) [**86**] 13 (8.7) 14 (14) RC (4.5)
34	8.1.9b.3.7 (25.3 mg)	Eurospher RP-18 (HPLC)	M/W (70:30)	ID 16 mm l 250 mm	1 (1.1) 2 (2.1) [**83**] 3 (4.7) [**84**] 4 (3) [**85**] 5 (2) 6 (0.4)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
35	8.1.10 (312.6 mg)	RP-18 (MPLC)	M/W (85:15 4.5 h) (90:10 12 h) (100:0 6 h)	ID 3 cm l 38 cm	1 (10.9) 2 (131.9) [**78**] 3 (31.9) [**60**] 4 (7.1) [**88**] 5 (20.5) [**87**] 6 (10.4) 7 (20.8) [**54**] 8 (12.9) 9 (0.8) 10 (40.5)
36	9. (10000 mg)	Sephadex LH-20 (CC)	M	ID 5 cm l 87.5 cm	1 (3700) fp 2 (600) 3 (1400) 4 (700)
37	9.1 (656 mg)	PVA 500 (CC)	M	ID 1.5 cm L 90 cm	1 (31) 2 (37) fp 3 (462) 4 (10.6) 5 (21.7) RC (25.1)
38	9.1.2 (37 mg)	SiO2 (CC)	C/M/W (75:27:5)	ID 1 cm l 11 cm	1 (1) 2 (1.1) 3 (5)* 4 (2) 5 (4) 6 (2.7) 7 (3.3)
39	10. (5400 mg)	Without (HSCCC)	T/B/ACN/W (1:3:1:5)	3 coils (850 mL)	1 (1729) fp 2 (1440.5) fp 3 (100.2) 4 (87.4) 5 (108.2) fp 6 (221.5) fp RC (743.5)
40	10.1 (1145 mg)	Without (HSCCC)	B/M/W (5:1:6)	3 coils (850 mL)	1 (10.8) 2 (200.3) 3 (785.3) 4 (404.7) 5 (33.9) 6 (3.1) RC (2.3)
41	10.2 (464 mg)	PVA 500 (CC)	M	ID 2.5 cm l 85 cm	1 (6.1) fp 2 (18.9) fp 3 (176.4) fp 4 (155.4) 5 (84.9) 6 (2.6) 7 (1) RC (3)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
42	10.2.12 (25 mg)	Prontosil RP-18 (HPLC)	M/W (10:90)	ID 16 mm l 250 mm	1 (0.1) 2 (4.1) 3 (2.5) 4 (1.9) 5 (1.1) 6 (1.3) 7 (0.8) 8 (1.2) 9 (6.9) RC (1.3)
43	10.2.3. (176.4 mg)	SiO2 (CC)	C/M/W (75:27:5)	ID 2.5cm l 25 cm	1 (2.1) 2 (5.4)* 3 (5.8) 4 (9.1) 5 (11.9)* 6 (21.5) fp 7 (8.1) 8 (47.6) 9 (-) 10 (8.7) fp 11 (-) 12 (-) 13 (2.9) RC (1)
44	10.2.3.6 (21.5 mg)	SiO2 (CC)	C/M/W (75:25:5)	ID 1cm l 11cm	1 (2.1) 2 (1.5) 3 (4.8) 4 (2.2) 5 (2.2) 6 (0.5) 7 (0.9) 8 (0.7) 9 (0.6)
45	10.2.3.10 (9 mg)	SiO2 (CC)	C/M/W (75:25:5)	ID 1cm l 11cm	1 (0.7) 2 (3.1) 3 (0.7)
46	10.5. (108 mg)	PVA 500 (CC)	M	ID 2.5 cm l 85 cm	O (6.8) 1 (8.4) 2 (24.6) fp 3 (23.9) fp 4 (6.8) fp 5 (3.5) fp 6 (3.4) 7 (3.4) 8 (1.8) 9 (4.8) RC (4.4)

Table 5-3 Cont. Chromatographical separation of avocado seeds (*Persea americana* Mill c.v. *Hass*) extracts and partitions.

No.	Purified fraction	Absorbent (method)	Mobile phase	Column parameter	Fractions (mg)
47	10.5.2. (24.6 mg)	Prontosil RP-18 (HPLC)	ACN/W (15:85)	ID 16 mm l 250 mm	1 (0.8) 2 (0.7) 3 (0.3) 4 (1.6) 5 (2.4)* 6 (0.7) 7 (0.3) 8 (1) 9 (0.1)
48	10.5.3. (23.9 mg)	Prontosil RP-18 (HPLC)	ACN/W (15:85)	ID 16 mm l 250 mm	1 (1.7)* 2 (3.6)* 3 (0.1) 4 (-) 5 (0.9) 6 (0.9)* 7 (0.2)
49	10.5.45. (10.3 mg)	Prontosil RP-18 (HPLC)	ACN/W (15:85)	D 16 mm l 250 mm	1 (0.3) 2 (0.9) 3 (3.2) [**91**] 5 (0.2)
50	10.6. (205.7 mg)	PVA 500 (CC)	M	ID 1.5 cm l 42 cm ID_2 2.5 cm l_2 85 cm	1 (13.2) 2 (19) fp 3 (29.5)* 4 (9.3) 5 (38.8) 6 (6) 7 (1.7) 8 (5.8) RC (4.4)
51	10.6.2. (12.7 mg)	SiO2 (CC)	C/M/W (75:32.5:7)	ID 1 cm l 11 cm	1 (3.9) 2 (3.5) 3 (0.8) 4 (0.1) 5 (0.5) 6 (0.9) 7 (0.9)* RC (-)

fp= further purification, * further spectroscopical analysis but not yet identified

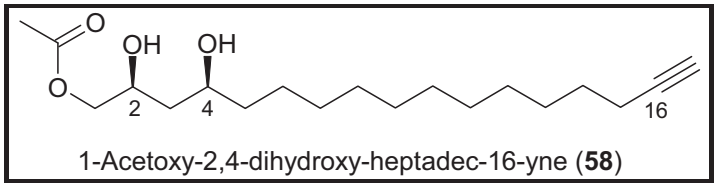
5.16. Physicochemical description of the purified substances

5.16.1. Lipophilic substances

5.16.1.1. Acetylenic compounds

5.16.1.1.1. 1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**) (85.5 mg)

Compound **58** was as white compact fat obtained from fractions 8.1.9a.3.; 8.1.9b.3.6.; and fraction 8.1.9b.2.10, Mr = 326 g/mol.



1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**58**)

TLC: anisaldehyde: blue-green, RP-18 (M/W, 75:25, 90:10)) R_f= (0.48 0.746).

LC- MS (pos) *m/z*: 349 $[M+Na]^+$; $[2M+Na]^+$; MS^2 (349): 287, 166.

CI-MS /Isobutane *m/z*: 327 $[M+H]^+$

EI-MS *m/z*: 67, 81, 95, 43, 103.

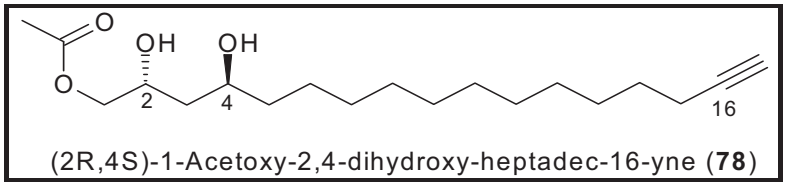
LC-MS: R_t= 10.6 min [ODS-Beckman 4.6 x 250mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient t (0,7,20,55,60 min), A (20,15,0,0,20 %),B (80,85,100,100,80 %). Flow rate= 0.8mL min^{-1}. λ=225 nm].

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.38 (H, *sbr*, H-14), 1.48 (H, *m*, H-5), 1.28 to 1.38 (17H, *m*, H-6 to H-13/H-3), 1.92 (1H, *t*, *J*= 2.65 Hz, H-17), 2.09 (3H, *s*, $\underline{CH_3}CO$), 2.18 (2H, *dt*, *J*= 2.6, 6.9 Hz, H-15), 2.62 (1H, *d*, *J*= 1.48 Hz, OH), 3.88 (H, *m*, H-4), 3.98 (H, *m*, H-1b), 4.09 (4 H, *m*, H-1a), 4.12 (H, *m*, H-2).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 18.7 (C-15), 21.2 ($\underline{CH_3}CO$), 25.6 (C-6), 28.9 (C-14), 29.1 (C-13), 29.4 (C-12), 29.6 (C-11), 29.7 (C-10), 29.8 (C-9), 29.9 (C-7/C-8), 38.6 (C-5), 39.6 (C-3), 68.3 (C-17), 68.9 (C-1), 71.1 (C-2), 72.7 (C-4), 85.1 (C-16), 171.5 ($CH_3\underline{CO}$).

5.16.1.1.2. (2*R*, 4*S*)-1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**78**) (132 mg)

Compound **78** comes from 8.1.10.2 obtained as white crystals, Mr = 326 g/mol.



(2R,4S)-1-Acetoxy-2,4-dihydroxy-heptadec-16-yne (**78**)

TLC: anisaldehyde: blue-grey, R_f= 0.75 (RP-18, S12).

ESI MS (pos) *m/z*: 349 $[M+Na]^+$

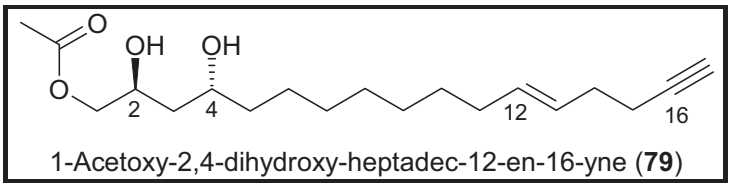
LC-MS: R_t = 10.0 min [Column: ODS-Beckman 4.6 x 250mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH), gradient t (0,7,20,55,60 min), A (20,15,0,0,20 %), B (80,85,100,100,80 %) and flow rate= 0.8mL min^{-1}. λ= 225 nm].

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.29 (H, *sbr*, H-6 to H-13), 1.38 (H, *m*, H-5), 1.53 (H, *m*, H-3/H-14), 1.90 (H, *t*, H-17), 2.02 (H, *m*, $\underline{CH_3}CO$), 2.15 (H, *m*, H-15), 3.40 (H, *m*, H-1b), 3.63 (H, *m*, H-4/H-1a), 4.09 (H, *m*, H-2).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 18.7 (C-15), 21.6 ($\underline{CH_3}CO$), 25.5 (C-6), 28.8 (C-14), 29.1-29.9 (C-7 to C-13), 38.2 (C-5), 34.8 (C-3), 66.8 (C-1), 68.8 (C-17), 70.9 (C-2), 72.6 (C-4), 85.1 (C-16), 171.5 ($CH_3\underline{CO}$).

5.16.1.1.3. 1-Acetoxy-2,4-dihydroxy-heptadec-12-en-16-yne (**79**)

Obtained from fraction 8.1.9b.2.8 (3 mg) as colourless oil (sweet odour), Mr = 324 g/mol.



1-Acetoxy-2,4-dihydroxy-heptadec-12-en-16-yne (**79**)

TLC: Rf= 0.54 (RP-18, S13).

Anisaldehyde: violet

ESI MS (neg) *m/z*: 323 $[M-H]^-$; 647 $[2M-H]^-$.

ESI MS (pos) *m/z*: 347 $[M+Na]^+$; 671 $[2M+Na]^+$; MS^2 (347): 287, 295. MS^2 (671): 346, 391.

CI-MS-isobutan (rel. int.) *m/z*: 325 $[M+H]^+$ (15), 307 $[M+H-H2O]^+$ (100), 265 $[M+H-HOAc]^+$ (43), 247 $[M+H-HOAc-H2O]^+$ (7), 147 (24).

EI-MS *m/z*: 87, 147, 69, 129.

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.28 (H, *sbr*, H-5 to H11, H-14)), 1.92 (H, *t*, *J*=2.6 Hz, H-17), 1.50 (H, *dd*, H-3), 2.10 (H, *s*, $\underline{CH_3}CO$), 2.18 (H, *dt*, H-15), 3.48 (H, *s*, OH), 3.65 (H, *m*, H-4), 3.78 (H, *m*, H-1b), 4.02 (H, *m*, H-2), 4.08 (H, *m*, H-1a), 4.98-5.10 (H, *m*, H-13), 5.28 -5.60 (H, *m*, H-12).

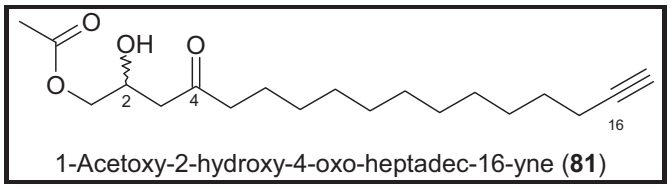
^{1}H-NMR (600 MHz, $CDCl_3$+ CD_3OD, 7:3): δ (ppm) = 1.28 (H, *sbr*, H-5 to H-10), 1.66-1.73 (H, *m*, H-5), 1.55 (H, *m*, H-3), 2.0 (H, *m*, H-11), 2.10 (H, *s*, $\underline{CH_3}CO$), 2.18 (H, *dt*, J= 2.5, 6 Hz, H-15), 2.20 (H, *m*, H-14), 3.55 (H, *dt*, H-OH), 3.64 (H, *dd*, OH), 3.71 (H, *dt*, *J*=3,5, 7 Hz, H-4), 3.83 (H, *dt*, *J*= 3, 3 Hz, H-2), 4.00 (H, *dd*, *J*= 7, 10 Hz, H-1b), 4.05 (H, *dd*, *J*=7, 10 Hz, H-1a), 5.41 (H, *dd*, *J*= 8, 15 Hz H-13), 5.50 (H, *dd*, *J*= 8, 15 Hz, H-12), not detected (H-17).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 18.4 (C-15), 20.8 ($\underline{CH_3}CO$), 28.5 (C-14), 28.7 (C-7), 29.0 (C-6), 29.1 (C-10), 29.2 (C-9), 29.3 (C-8), 29.4 (C-11), 38.8 (C-5), 41.5 (C-3), 68.0 (C-1), 69.8 (C-17), 70.4 (C-2), 71.4 (C-4), 84.7 (C-16), 124.9 (C-13), 134.6 (C-12), 171.1 ($CH_3\underline{CO}$).

^{2,3}*J*-HC (HMBC) ($CDCl_3$+ CD_3OD, 7:3, 600 MHz). δ (ppm). 1.28 H-5 (C-14), 1.28 H-11 (C-13), 1.55 H-3 (C-4), 2.10 $\underline{CH_3}CO$ (C=O), 2.18 H-15 (C-14, C-17, C-16, C-13), 4.05 H-1a (CO), 5.41 H-13 (C-14, C-11, C-12), 5.50 H-12 (C-14, C-11, C-13).

5.16.1.1.4. 1-Acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (**81**) (10 mg)

Obtained from fraction 8.1.9b.3.4, white compact crystals, Mr = 324 g/mol.



1-Acetoxy-2-hydroxy-4-oxo-heptadec-16-yne (**81**)

TLC: R_f= 0.47 (RP-18, S13).

Anisaldehyde: blue-green

LC-MS (pos) *m/z*: 347 $[M+Na]^+$.

EI-MS m*/z*: 67, 81, 95, 43, 103, 145, 207, 309 and 311. Signals at *m/z* 145 and 207 indicative for a keto group on position C-4.

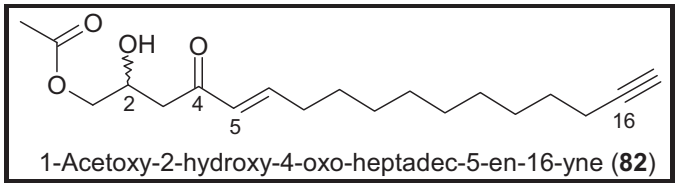
LC-MS: R_t = 10.2 min [Column: ODS-Beckman 4.6 x 250mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH), gradient t (0,7,20,55,60 min), A (20,15,0,0,20 %), B (80,85,100,100,80 %). Flow rate = 0.8mL min^{-1}, λ=225 nm].

^{1}H-NMR (360 MHz, $CDCl_3$): δ (ppm) = 1.27 (H, *sbr*, H-7 to H-13), 1.38 (H, *m*, H-14), 1.52 (H, *q*, *J*= 7 Hz, H-6), 1.93 (H, *t*, *J*= 2.5 Hz, H-17), 2.10 (3H, *s*, $\underline{CH_3}CO$), 2.18 (4H, *dt*, *J*= 2.6, 6.9, 6.9 Hz, H-15), 2.44 (2H, *t*, *J*= 7.5 Hz, H-5), 2.60 (3H, *d*, *J*=8, H-3), 2.62 (H, *m*, OH), 3.45 (H, *m*, OH), 4.00 (1H, *dd*, *J*= 12 H, 7 Hz, H-1b), 4.05 (1H, *dd*, *J*= 11, 6 Hz, H-1a), 4.11 (H, *m*, H-2).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 18.7 (C-15), 21.1 ($\underline{CH_3}CO$), 23.9 (C-6), 28.9 (C-14), 29.1 (C-13), 29.4 (C-12), 29.8 (C-11), 29.9 (C-8), 29.9 (C-7), 44.0 (C-5), 45.6 (C-3), 66.5 (C-2), 67.6 (C-1), 68.4 (C-17), 85.1 (C-16), 171.3 ($CH_3\underline{CO}$), 211.1 (C-4).

5.16.1.1.5. 1-Acetoxy-2-hydroxy-4-oxo-heptadec-5-en-16-yne (**82**) (7 mg)

Obtained from fractions 8.1.9b.2.6 and 8.1.9b.3.3., colourless oil (sweet), Mr = 322 g/mol.



1-Acetoxy-2-hydroxy-4-oxo-heptadec-5-en-16-yne (**82**)

TLC: Rf = 0.59 (RP-18, S13).

Anisaldehyde: blue-violet

ESI MS (neg) *m/z*: 321 $[M-H]^-$; MS^2 (321): 303, 261, 219

LC MS (pos) *m/z*: 345 $[M+Na]^+$; 667 $[2M+Na]^+$; MS^2 (667): 345. MS^2 (345): 125, 243, 285. MS^2 (245): 81, 95, 107, 121, 133, 145, 163, 185, 199, 215, 227 and 243.

CI-MS (Isobutan) *m/z*: 323 $[M+H]^+$ and *m/z* 321, 305, 303, 279, 263, 245, 95, 81.

EI-MS: *m/z* 323 $[M+H]^+$ and *m/z* 43, 55, 67, 81, 95, 107, 109, 121, 123, 135, 137, 147, 163, 177, 187, 205, 219, 231, 245, 249, 262, 279.

LC-MS: R_t = 8.1 min [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.29 (H, *sbr*, H-9 to H-14), 1.50 (H, *dd*, H-8), 1.92 (H, *t*, H-17), 2.10 (3H, *s*, $\underline{CH_3}CO$), 2.18 (H, *dt*, H-15), 2.73 (H, *s*, H-7), 2.77 (2H, *s*, H-3), 3.49 (H, *sbr*, OH), 4.10 (H, *m*, H-1a), 4.17 (H, *m*, H-1b), 4.35 (H, *dd*, H-2), 6.12, (H, *m*, H-5), 6.85-6.95 (H, *m*, H-6).

^{1}H-NMR (600 MHz, $CDCl_3$: CD_3OD 7:3): δ (ppm) = 1.30 (H, *sbr*, H-10 to H-14), 1.50 (H, *dd*, H-8), 2.10 (H, *sbr*, $\underline{CH_3}CO$), 2.18 (H, *dt*, H-15), 2.29 (H, *dd*, H-7), 2.78-2.88 (H, *dd*, H-3), 4.10 (H, *m*,H-1b), 4.20 (H, *m*, H-1a), 4.30 (H, *m*, H-2), 6.15 (H, *dt*, *J*= 16, 2Hz, H-5), 6.93 (H, *dt*, *J*=16, 7 Hz, H-6), not detected (H-17).

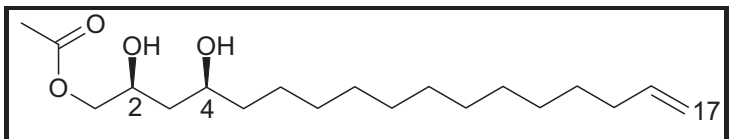
^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 18.7 (C-15), 21.2 ($\underline{CH_3}CO$), 28.4 (C-8), 28.8 (C-14), 29.0 (C-13), 29.4 (C-12), 29.6 (C-11), 29.7 (C-10), 29.8 (C-9), 32.9 (C-7), 42.8 (C-3), 66.6 (C-2), 67.7 (C-1), 68.4 (C-17), 85.1 (C-16), 130.7 (C-5), 149.7 (C-6), 171.3 ($CH_3\underline{CO}$), 199.9 (C-4).

COSY (600 MHz, $CDCl_3$: CD_3OD 7:3): 1.50 H-8 (H-7), 2.29 H-7 (H-8), 2.78-2.88 H-3 (H-2), 4.10 H-1b (H-2), 4.30 H-2 (H-3, H-1b), 6.15 H-5 (H-6, H-7), 6.93 H-6 (H-5, H-7).

5.16.1.2. Lipophilic compounds with vinyl-function

5.16.1.2.1. 1-Acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene (**60**) (74 mg)

Obtained from fractions 8.1.9b.3.11 and 8.1.10.3 as colourless oil, Mr = 328 g/mol.



1-Acetoxy-2, 4-dihydroxy-*n*-heptadec-16-ene (**60**)

TLC: R_f= 0.328, 0.676 (RP-18, S13, S12).

Anisaldehyde: blue-grey.

ESI MS (pos) *m/z*: 351 $[M+Na]^+$; 679 $[2M+Na]^+$

CI-MS /Isobutan *m/z*: 329 $[M+H]^+$

EI-MS: *m/z*: 329 $[M+H]^+$; ions at *m/z* 255 $[CH_3COOCH_2^{\cdot}]$; *m/z* 237 $[CH_3COOCH_2^{\cdot}–H_2O]$, *m/z* 147 $[CH_3COOCH_2CHOHCH2CHOH]$.

LC-MS: R_t = 15.4 min. [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

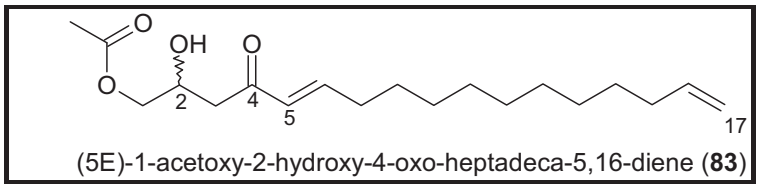
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.25 (H, *m*, H-6 to H-13), 1.37 (H, *m*, H-5), 1.58 (H, *m*, H-3), 1.73 (H, *m*, H-14), 2.03 (H, *t*, H-15), 2.08 (H, *s*, $\underline{CH_3}CO$), 3.45 (H, *m*, OH), 3.90 (H, *m*, H-4), 3.99 (H, *dd*, H-1b), 4.08 (H, *sbr*, H-1a), 4.15 (H, *m*, H-2), 4.89 (H, *ddt*, J_1= 10, J_2=J_3= 1.5 Hz, H-17a),

4.94 (1H, *ddt*, J_1= 17, J_2=J_3= 1.5 Hz, H-17b), 5.80 (1H, *ddt*, J_1= 17, J_2= 10, J_3= 6.5 Hz, H-16).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 21.6 ($\underline{CH_3}CO$), 25.5 (C-6), 29.1 (C-7), 29.3 (C-14), 29.5 (C-8), 29.6 (C-9), 29.8 (C-10), 29.8 (C-11), 29.9 (C-12), 29.9 (C-13), 34.1 (C-15), 38.2 (C-5), 38.5 (C-3), 68.9 (C-1), 70.1 (C-2), 72.7 (C-4), 114.4 (C-17), 139.6 (C-16), 171.5 ($CH_3\underline{CO}$).

5.16.1.2.2. (5*E*)-1-Acetoxy-2-hydroxy-4-oxo-heptadeca-5,16-diene (**83**) (2 mg)

Obtained from fraction 8.1.9b.3.7.2., colourless fat, Mr= 324 g/mol.



(5E)-1-acetoxy-2-hydroxy-4-oxo-heptadeca-5,16-diene (**83**)

TLC: R_f= 0.394 (RP-18, S13)

Anisaldehyde: blue-red

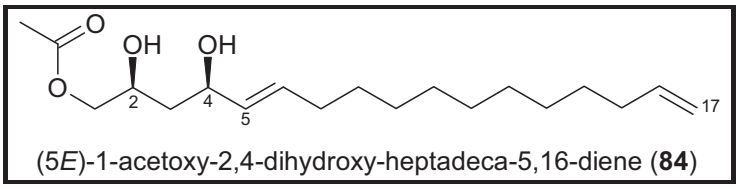
ESI MS (pos) *m/z*: 347 $[M+Na]^+$; 671 $[2M+Na]^+$.

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.28 (H, *m*, H-9 to H-14), 1.64 (H, *m*, H-8), 2.03 (2H, *m*,H-15) 2.10 (3H, *s*, H- $\underline{CH_3}CO$), 2.23 (2H, *m*, H-7), 2.77 (2H, *d*, H-3), 3.49 (1H, *s*, H-OH), 4.12 (2H, *m*, H-1a/H-1b), 4.34 (1H, *m*, H-2), 4.95 (1H, *m*,H-17a), 5.01 (1H, *m*,H-17b), 5.81 (1H, *dq*, H-16), 6.12 (1H, *dt,* J_1= 16*,* 1.5 Hz, H-5), 6.89 (1H, *dt*, *J*= 16, 6.9 Hz, H-6).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 21.2 ($\underline{CH_3}CO$), 29.3 (C-14), 29.5 (C-13), 29.6 (C-12), 29.7 (C-11), 29.8 (C-10), 29.9 (C-9), 32.9 (C-8), 32.99 (C-7), 34.1 (C-15), 40.1 (C-3), 68.9 (C-1), 70.3 (C-2), 114.4 (C-17), 130.7 (C-5), 139.6 (C-16), 149.8 (C-6), 199.9 (C-4), nd ($CH_3\underline{CO}$).

5.16.1.2.3. (5*E*)-1-Acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**) (5 mg)

Obtained from fraction 8.1.9b.3.7.3., colourless fat, Mr = 326 g/mol.



(5*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-5,16-diene (**84**)

TLC: R_f= 0.424 (RP-18, S13).

Anisaldehyde: blue-grey

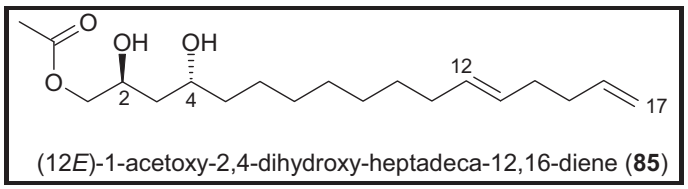
ESI MS (pos) *m/z*: 349 $[M+Na]^+$; and *m/z* 675 $[2M+Na]^+$

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.30-1.35 (H, *m*, H-7 to H-14), 1.60 (H, *m*, H-3), 2.03 (H, *m*, H-15), 2.10 (H, *s*, $\underline{CH_3}CO$), 3.80 (H, *m*, H-2), 4.12 (H, *m*, H-1a), 4.35 (H, *m*, H-1b), 4.91-4.95 (H, *m*, H-17a/H-17b), 5.35 (H, *m*, H-4), 5.47 (H, *m*, H-5), 5.74 (H, *m*, H-6), 5.81 (H, *m*, H-16).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 21.2 ($\underline{CH_3}CO$), 29.3 (C-14), 29.4 (C-8), 29.5 (C-11), 29.5 (C-13), 29.8 (C-12), 29.8 (C-10), 29.9 (C-9), 32.5 (C-7), 34.1 (C-15), 40.1 (C-3), 68.9 (C-1), 70.3 (C-2), 73.3 (C-4), 114.5 (C-17), 130.7 (C-5), 135.8 (C-6), 139.6 (C-16), 171.5 ($CH_3\underline{CO}$).

5.16.1.2.4. (12*E*)-1-Acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**) (3 mg)

Obtained from fraction 8.1.9b.3.7.4. as colourless oil, Mr = 326 g/mol.



(12*E*)-1-acetoxy-2,4-dihydroxy-heptadeca-12,16-diene (**85**)

TLC: R_f= 0.424 (RP-18, S13).

Anisaldehyde: lilac-brown

ESI MS (pos) *m/z*: 349 $[M+Na]^+$; and *m/z* 675 $[2M+Na]^+$

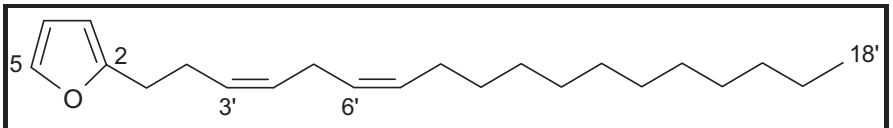
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.28 (H, *m*, H-5 to H-11, H-14), 1.58 (H, *m*, H-3), 2.05 (H, *m*, H-15), 2.10 (H, *s*, $CH_3\underline{CO}$), 3.78 (H, *m*, H-1b), 3.90 (H, *m*, H-4), 4.02 (H, *m*, H-2), 4.12 (H, *m*, H-1a), 4.95 (H, *m*, H-17a), 5.02 (H, *sbr*, H-17b), 5.40 (H, *dt*, *J*= 17, 6.5 Hz, H-13), 5.55 (H, *dt*, *J*= 17, 6.5 Hz, H-12), 5.82 (H, *dq*, H-16).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 21.6 ($\underline{CH_3}CO$), 28.9 (C-6), 29.1 (C-8), 29.3 (C-10), 29.4 (C-7), 29.5 (C-14), 29.5 (C-9), 32.9 (C-11), 34.1 (C-15), 38.4 (C-5), 41.7 (C-3), 68.8 (C-1), 70.8 (C-2), 71.8 (C-4), 125.2 (C-13), 135.9 (C-12), 114.5 (C-17), 139.6 (C-16), 171.4 ($CH_3\underline{CO}$).

5.16.1.3. Furan compounds

5.16.1.3.1. 2-[(*Z*,*Z*)-Octadeca-3',6'-diene)]-furan (**86**) (2.7 mg)

Obtained from fraction 8.1.9b.3.12., as colourless oil, Mr = 316 g/mol.



2-[(Z,Z)-(octadeca-3',6'-diene)]-furan (**86**)

TLC: R_f= 0.30 (RP-18, S13).

Anisaldehyde: blue-grey

CI-MS /Isobutan *m/z*: 317 $[M+H]^+$.

EI-MS: *m/z*: 317 $[M+H]^+$, and *m/z* at 67 [M-furan ring], 81 [M-furan ring-CH_2], 95 [M-furan ring-$2CH_2$], 121 [M-furan ring-$2CH_2$-2CH], 135 [M-furan ring-$2CH_2$-2CH- CH_2], 161 [M-furan ring-$2CH_2$-2CH- CH_2- 2CH].

LC-MS: R_t = 9.4 min. [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

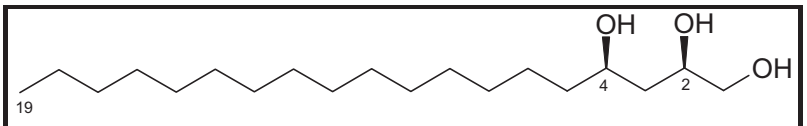
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.89 (H, *t*, H-18'), 1.30 (H, *m*, H-9' to H-17'), 2.08 (H, *m*, H-2'/H-8'), 2.6 (H, *t*, H-1'), 2.78 (H, *t*, H-5'), 5.35 (H, *m*, H-3'/H-4'/ H-6'/H-7'), 5.90 (H, *d*, H-3), 6.28 (H, *dd*, H-4), 7.28 (H, *d*, H-5).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 14.38 (C-18'), 22.91 (C-17'), 25.60 (C-16'), 26.02 (C-5'), 27.58 (C-8'), 28.34 (C-9'), 28.40 (C-10'), 29.51 (C-11'), 29.55 (C-12'), 29.60 (C-13'), 29.71 (C-14'), 29.84 (C-15'), 29.99 (C-2'), 31.90 (C-1'), 104.87 (C-4), 110.37 (C-3), 128.32 (C-3'), 128.41 (C-6'), 130.47 (C-4'), 130.58 (C-7'), 140.97 (C-5), n. d. (C-2).

5.16.1.4. Trihydroxylated compounds

5.16.1.4.1. (2*R*, 4*R*)-1,2,4-Trihydroxy-nonadecane (**54**) (20.8 mg)

Obtained from fraction 8.1.10.7., as white crystals, Mr = 316 g/mol.



(2*R*,4*R*)-1,2,4-Trihydroxy-nonadecane (**54**)

TLC: R_f=0.623 (RP-18, S12)

Anisaldehyde: blue.

LC MS (pos) *m/z*: 339 $[M+Na]^+$; 655 $[2M+Na]^+$.

LC-MS: R_t = 20.5 min [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH) using gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

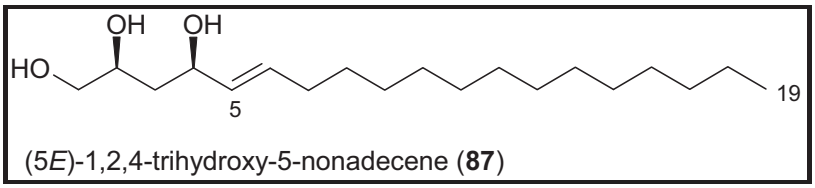
^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.88 (3H, *t*, *J*=6.3 Hz, H-19), 1.20-1.40 (26H, *sbr*, H-6 to H-18), 1.50 (2H, *m*, H-5), 1.60 (2H, *m*, H-3), 2.0 (2H, *m*, H-15), 3.49 (1H, *dd*, *J*=6.7, 10.3 Hz, H-1a), 3.63 (1H, *dd*, *J*=6.8, 15.9 Hz, H-1b), 3.92 (1H, *m*, H-4), 4.15 (1H, *m*, H-2).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 14.42 (C-19), 23.03 (C-18), 25.68 (C-17), 27.59 (C-15), 29.59 (C-11), 29.70 (C-8), 29.79 (C-16), 29.84 (C-10/C-13), 29.87 (C-12), 29.95 (C-7), 30.00 (C-9), 30.04 (C-14), 32.28 (C-6), 38.68 (C-5), 39.45 (C-3), 67.19 (C-1), 72.79 (C-4), 72.97 (C-2).

^{1,2} *J*C-H (HMQC-NMR) (300 MHz, $CDCl_3$): δ (ppm) = 0.88 H-19 (C-19), 1.20-1.40 H-6 (C-6), 1.50 H-5 (C-5), 1.60 H-3 (C-3), 3.49 H-1 (C-1), 3.63 H-1 b (C-1), 3.92 H-4 (C-4), 4.15 H-2 (C-2).

5.16.1.4.2. (5*E*)-1, 2, 4-Trihydroxy-5-nonadecene (**87**) (20.5 mg)

Obtained from fraction 8.1.10.5., as white crystals, Mr = 314 g/mol.



(5*E*)-1,2,4-trihydroxy-5-nonadecene (**87**)

TLC: R_f=0.623 (RP-18, S12)

Anisaldehyde: violet-blue.

LC MS (pos) *m/z*: 337 $[M+Na]^+$; 651 $[2M+Na]^+$.

LC-MS: R_t = 18.9 min [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.88 (H, *t*, H-19), 1.27-1.50 (H, *sbr*, H-8 to H-18), 1.60 (H, *sbr*, H-3), 3.50 (H, *m*, H-1a), 3.65 (H, *m*, H-1b), 3.88 (H, *m*, H-4), 4.38 (H, *m*, H-2), 5.35 (H, *m*, H-6), 5.55 (H, *m*, H-5). Rest of signals were overlapped.

^{1}H-NMR (600 MHz, $CDCl_3$: CD_3OD 7:3): δ (ppm) =0.88 (H, *dd*, *J*=7, 13Hz, H-19), 1.27-1.50 (H, *m*, H-8 to H-18), 1.64 (H, *m*, H-3), 2.18 (H, *dt*, *J*= 7 Hz, H-7), 3.46 (H, *dd*, *J*=6,10Hz, H-1a), 3.54 (H, *dd*, *J*= 3, 10Hz, H-1b), 3.81 (H, *m*, H-4), 3.86 (H, *m*, H-2), 5.40 (H, *m*, *J*= 15 Hz, H-6), 5.50 (H, *dd*, J=7, 15Hz, H-5).

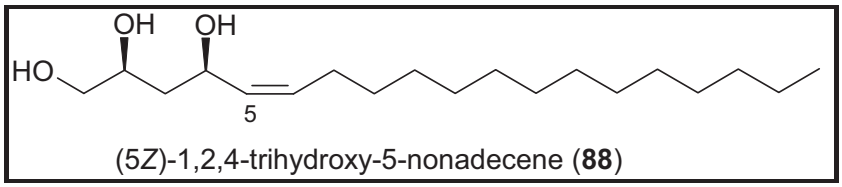
^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 14.3 (C-19), 22.9 (C-18), 25.7 (C-17), 27.6 (C-15), 29.5 (C-9), 29.6 (C-11), 29.7 (C-8), 29.8 (C-16), 29.8 (C-13), 29.9 (C-12), 29.9 (C-10), 30.0 (C-14), 32.3 (C-7), 67.1 (C-1), 39.4 (C-3), 71.7 (C-4), 72.7 (C-2), 125.2 (C-5), 135.8 (C-6).

COSY (600 MHz, $CDCl_3$: CD_3OD 7:3): 3.46 H-1a (H-2), 3.54 (H-2, H-3, H-4), 2.18 H-7 (H-5, H-6), 5.40 H-6 (H-7).

$^{2,3}J$-HC (HMBC) ($CDCl_3$+ CD_3OD, 7:3, 600 MHz). δ (ppm). 3.46 H-1a (C-2), 1.64 H-3 (C-4), 3.81 H-4 (C-2), 5.50 H-5 (C-7), 2.18 H-7 (C-5), 5.40 H-6 (C-7).

5.16.1.4.3. (5Z)-1, 2, 4-Trihydroxy-5-nonadecene (**88**) (7 mg)

Obtained from fraction 8.1.10.4., as white crystals, Mr = 314 g/mol.



(5*Z*)-1,2,4-trihydroxy-5-nonadecene (**88**)

TLC: R_f=0.637 (RP-18, S12)

Anisaldehyde: blue.

LC MS (pos) *m/z*: 337 $[M+Na]^+$; 651 $[2M+Na]^+$.

LC-MS: R_t = 17.8 min [Column ODS Beckman 250 x 4.6 mm. Solvent A (0.1M CH_3COONH_4 in H_2O), solvent B (0.1M CH_3COONH_4 in MeOH). Gradient: t (0, 7, 20, 55, 60 min), A (20, 15, 0, 0, 20 %), B (80, 85, 100, 100, 80 %). Flow rate: 0.8 mL min^{-1}, λ 225 nm].

^{1}H-NMR (300 MHz, $CDCl_3$):δ (ppm) = 0.88 (H, *t*, H-19), 1.28-1.50 (H, *m*, H-3, H-8 to H-18), 2.0 (H, *m*, H-7), 3.50 (H, *dd*, H-1a), 3.65 (H, *dd*, H-1b), 3.93 (H, *m*, H-2), 4.12 (H, *m*, H-4), 4.36 (H, *m*, H-6), 5.42 (H, *dd*, *J*=5.3,10.3 Hz, H-5).

^{13}C-NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 14.3 (C-19), 25.7 (C-18), 25.9 (C-17), 29.5 (C-11), 29.7 (C-16), 29.9 (C-9), 32.3 (C-7/C-14), 32.9 (C-12), 39.5 (C-3), 67.2 (C-1), 72.8 (C-4), 72.9 (C-2), 129.8 (C-5), 132.2 (C-6).

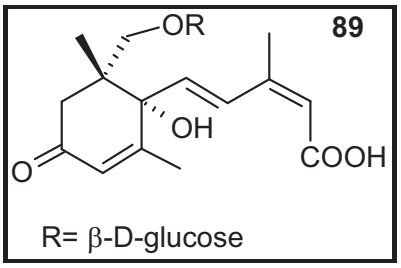
5.16.2. Hydrophilic substances

5.16.2.1. Abscisic acid derivatives

5.16.2.1.1. *(1'S, 6'R)-8'-hydroxyabscisic acid* β-D-glucoside (**89**) (2 mg)

(1'S,6'R,2Z,4Z)-5-[1'-hydroxy-2',6'-dimethyl-6'-hydroxymethyl-4'-oxo-8'-β-D-glucosyl)-cyclohex-2'-en-1'-yl]-3-methyl-penta-2,4-dienoic acid, (1'S, 6'R)-8'-hydroxyabscisic acid β-D-glucoside (**89**)

Obtained from fraction 1.89.2a. as an ambar gum, Mr= 442 g/mol.



TLC: R_f 0.34 (SiO_2, S2), R_f 0.65 (RP-18, S8).

Anisaldehyde: grey-green.

ESI MS (neg) *m/z*: 883 $[2M-H]^-$, 441 $[M-H]^-$;

MS^2 (883): 441, 331; MS^2 (441): 397, 330, 161.

ESI MS (pos) *m/z*: 907 $[2M+Na]^+$, 465 $[M+Na]^+$; MS^2 (907): 465; MS^3 (465): 447, 285.

LC-MS: R_t = 27.4 min [ESI-MS (neg. mode): dry gas nitrogen 9.0 l min^{-1}, nebulizer pressure 40 psi, capillary +3500 V, end plate +3000 V, capillary exit -95 V, skim 1 -25 V, skim 2 -10 V. Column for HPLC-MS was a Prontosil C18 Aqua, 5 µm, 250 x 2.0 mm, flow rate =0.25 mL min^{-1}. Eluents: nanopure® water (solvent A), and ACN (solvent B). Initial conditions of the gradient were 97% A, and 3% B, hold over 10 min, starting a linear gradient in 30 min to 40% A, and 60% B, in 15 min to 0% A, and 100% B for 10 min].

$[\alpha]_D$ +196.5 (*c*=0.13, MeOH);

CD (MeOH): $[\theta]_{266}$ + 81 263, $[\theta]_{233}$ - 57 162.

ORD (MeOH): $[\Phi]_{245}$ -96501, $[\Phi]_{291}$ +41064.

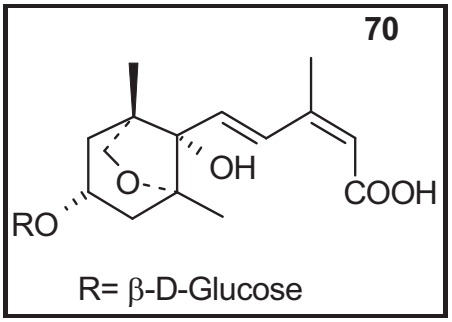
^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) =1.08 (3H, *s*, C<u>H</u>$_3$-9'), 1.93 (3H, *s,* C<u>H</u>$_3$-7'), 2.01 (3H, *s*, C<u>H</u>$_3$-6), 2.41 (1H, *d*, *J*=17 Hz; H-5'a), 2.66 (1H, *d*, *J*=17 Hz; H-5'b), 3.15 (1H, *dd*, J_1=8.0 Hz, J_2=8.5 Hz; H-2''), 3.24 (1H, m_{br}, H-4''), 3.27* (1H, *m,* H-3"), 3.30* (H-5"), 3.62 (1H, *d*, *J*=10 Hz, H-8'a), 3.66 (1H, *dd*, J_1=5.0 Hz, J_2=10.0 Hz, H-6"b), 3.85 (1H, *dd*, J_1=2.0 Hz, J_2=10.0 Hz H-6"a), 3.97 (1H, *d*, *J*=10 Hz, H-8'b), 4.16 (1H, *d*, *J*=8.0 Hz, H-1''), 5.78 (1H, *sbr*, H-2), 5.94 (1H, *sbr,* H-3'), 6.15 (1H, *d*, *J*=16.5 Hz; H-5), 7.75 (1H, *d*, *J*=16.5 Hz; H-4). *signal overlapped by MeOH-d_4 signal.

^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = n.d. (C-1), 19.3 (C-7'), 20.0 (C-9'), 20.8 (C-6), 45.2 (C-5'), 46.6 (C-6'), 62.5 (C-6"), 71.3 (C-3"), 74.4 (C-8'), 74.9 (C-2"), 77.7 (C-5"), 77.8 (C-4"), 80.0 (C-1'), 104.4 (C-1"), 121.2 (C-2), 127.9 (C-3'), 129.6 (C-4), 136.3 (C-5), 150.0 (C-3), 166.3 (C-2'), 200.5 (C-4'). n.d.= not detected.

5.16.2.1.2. (1'R,3'R,5'R,8'S)-*epi*-dihydrophaseic acid-β-D- glucoside (**70**) (2.4 mg)

*(1'R,3'R,5'R,8'S,2Z,*4Z)-5-[(8'-hydroxy-1',5'-dimethyl-3'-β-D-glucosyl-6'-oxabicyclo 3.2.1(oct-8'-yl)]-3-methyl-penta-2,4-dienoic acid, (1'R, 3'R, 5'R, 8'S)-*epi*-dihydrophaseic acid β-D-glucoside (**70**).

Obtained from fraction 1.5.2.6.1. as a brown transparent gum, Mr= 444 g/mol.



TLC: R_f 0.16 (SiO_2, S2); R_f 0.79 (RP-18, S8).

Anisaldehyde: grey-green

$[\alpha]_D$ -5.0 (MeOH *c*= 0.16).

ESI MS (neg) *m/z*: 443 $[M-H]^-$; MS^2 (443): 425, 237.

ESI MS (pos) *m/z*: 467 $[M+Na]^+$; MS^2 (467): 449, 287.

LC-MS: R_t = 22.4 min at the same conditions as for **89**.

CD (MeOH): $[\theta]_{272}$ + 3310, $[\theta]_{235}$ - 3481.

ORD (MeOH): $[\Phi]_{252}$ -4312, $[\Phi]_{294}$ +1575.

^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) = 0.90 (3H, *s*, C<u>H</u>$_3$-10'), 1.15 (3H, *s*, C<u>H</u>$_3$-9'), 1.78 (1H, *dd*, J_1=12 Hz, J_2=10 Hz; H-2'ax), 1.90 (1H, *dd*, *J*=12 Hz; H-2'eq), 2.10 (3H, *s*, C<u>H</u>$_3$-6), 2.18 (2H, m_{br}, H-4'ax,eq), 3.10 (1H, *dd*, J_1=7 Hz, J_2=8 Hz; H-2''), 3.25 (1H, m_{br}, H-4'')*, 3.27 (1H, m_{br}, H-5'')*, 3.63 (1H, m_{br}, H-6''a), 3.78 (2H, *d*, *J*=11 Hz; H-7'a/H-3"), 3.85 (1H, *d*, *J*=10 Hz; H-6"b), 4.23 (1H, m_{br}, H-3'), 4.30 (1H, *d*, *J*=8 Hz; H-1''), 5.70 (1H, *sbr,* H-2), 6.45 (1H, *d*, *J*=16 Hz; H-5), 7.90 (1H, *d*, *J*=16 Hz; H-4). * signal overlapped by MeOH-d_4 signal. n.d. not detected.

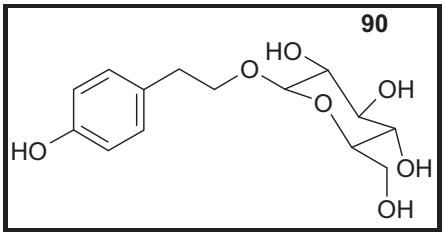
^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = n.d (C-1), 16.3 (C-10'), 19.7 (C-9'), 21.1 (C-6), 42.90 (C-2'), 42.95 (C-4'), 49.3 (C-1'), 62.8 (C-6"), 71.7 (C-4"), 74.0 (C-3'), 75.1 (C-2"), 77.2 (C-7'), 78.0 (C-3"), 78.1 (C-5"), 83.2 (C-8'), 87.6 (C-5'), 103.1 (C-1"), 120.5 (C-2), 132.1 (C-4), 134.4 (C-5), 149.9 (C-3). n.d. not detected.

5.16.2.2. Tyrosol derivatives

5.16.2.2.1. Tyrosol 1'-β-D-glucoside also called 1'- β-D-Glucosyl Tyrosol (**90**) (59 mg)

Obtained as a brown gum from fractions 1.5.4.1.; 1.6.4.; 1.6.5 and 5.3.2., Mr= 300 g/mol.

90



TLC: R_f 0.62 (SiO_2, S2); R_f 0.79 (RP-18, S8).

Anisaldehyde: brown

$[\alpha]_D$ -16.11 (MeOH *c*= 0.17)

ESI MS (neg) *m/z*: 599 $[2M-H]^-$; 299 $[M-H]^-$; MS^2 (599): 299, 178.9; MS^2 (299): 178.9, 119. MS^3 (299): 161,100.8.

ESI MS (pos) *m/z*: 323 $[M+Na]^+$

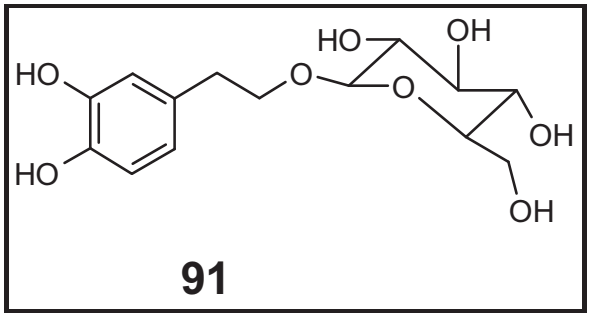
^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) = 2.83 (2H, *t*, J_1= 7.4 Hz, J_2= 7.4 Hz; H-2'), 3.18 (1H, *dd*, J_1= 7.98 Hz, J_2= 0.73 Hz; H-2''), 3.26 (1H, *sbr*; H-4"), 3.35 (1H, *sbr*, H-3''), 3.65 (1H, *dd*, J_1= 7 Hz, J_2= 10 Hz, H-6"a), 3.67 (3H, *dd*, J_1= 7 Hz, J_2= 10 Hz; H-1'a), 3.85 (2H, *m*, H-5''), 4.03 (1H, *dd*, J_1= 7 Hz, J_2= 10 Hz; H-1'b), 4.29 (1H, *d*, J_1= 7.74 Hz; H-1"), 6.69 (2H, *d*, J_1= 8.5 Hz; H-2/H-6), 7.06 (2H, *d*, J_1 =8.5 Hz; H-3/H-5).

^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = 36.38 (C-2'), 62.83 (C-6"), 71.7 (C-4"), 72.1 (C-1'), 75.2 (C-2"), 77.9 (C-3"), 78.2 (C-5"), 104.4 (C-1"), 116.1 (C-3/C-5), 130.8 (C-2/C-6), 130.9 (C-1), 156.8 (C-4).

COLOC-NMR (75.5 MHz, CD_3OD): δ (ppm) = 2.83 H-2' (C-2', C-2, C-1'), 3.18 H-2" (C-2", C-3", C-1"), 3.26 H-4" (C-4", C-3", C-5"), 3.35 H-3" (C-2"), 3.65 H-6"a (C-6", C-4"), 3.67 H-1'a (C-2', C-1', C-1"), 3.85 H-5" (C-6"), 4.03 H-1'b (C-2', C-1', C-1"), 4.29 H-1" (C-4", C-1", C-1'), 6.69 H-2/H-6 (C-2', C-5, C-2), 7.06 H-3/H-5 (C-2', C-5, C-2).

5.16.2.2.2. 3-Hydroxytyrosol 1'-β-D-glycoside (**91**) (5 mg)

Obtained from fractions 5.3.3.2., and 10.5.45.3, Mr= 316 g/mol.



91

TLC: R_f 0.26 (SiO_2, S10), R_f 0.44 (SiO_2, S2), R_f 0.71 (RP-18, S11), R_f 0.86 (RP-18, S8).

Anisaldehyde: light brown-grey

ESI MS (neg) *m/z*: 631 $[2M-H]^-$; 315$[M-H]^-$; MS^2 (631): 315; MS^2 (315): 135, 153, 179, 119.

ESI MS (pos) *m/z*: 655 $[2M+Na]^+$; 339 $[M+Na]^+$; MS^2 (655): 339; MS^2 (339): 185

LC-MS: R_t = 22.2 min [Column: Prontosil C18 Aqua, 5 µm, 250 x 2.0 mm solvent A (H_2O), solvent B (ACN). Gradient: t (0,10,40,55,65,75 min), A (97,97,40,0,0,97 %), B (3,3,60,100,100,3 %). Flow rate: 0.25 mL min^{-1}. λ= 210 nm].

^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) = 2.77 (2H, *t*, J= 7, 7 Hz; H-2'), 3.17 (1H, *dd*, J= 7.9, 8.8 Hz; H-2''), 3.25 (1H, *m*, H-4''), 3.28 (1H, *sbr*, H-5''), 3.30 (1H, *m*, H-3''), 3.66 (1H, *m*, H-6''a), 3.68 (1H, *m*, H-1'b) , 3.84 (1H, *dd*, J= 2, 12 Hz; H-6''b), 4.01 (1H, *dt*, *J*= 5, 9, 15 Hz; H-1'a), 4.27 (1H, *d*, J= 7.7 Hz; H-1''), 6.54 (1H, *dd*, J=2, 8 Hz, H-6), 6.64 (1H, *s*, H-2), 6.67 (1H, *dd*, J= 2, 8 Hz, H-5).

^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = 36.6 (C-2'), 62.8 (C-6″), 71.7 (C-4″), 72.1 (C-1'), 75.2 (C-2''), 77.9 (C-3''), 78.1 (C-5''), 104.4 (C-1''), 116.3 (C-2), 117.3 (C-5), 121.2 (C-6), 131.6 (C-1), 144.7 (C-4), 146.1 (C-3).

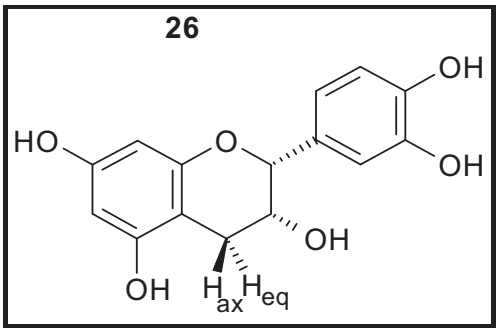
COSY 90-NMR (300 MHz, CD_3OD): δ (ppm) = 2.77 H-2' (H-6"a; H-1'b; H-1'a; H-6; H-5), 3.17 H-2'' (H-1"; H-3"; H-5"; H-4"), 3.25 H-4'' (H-6"b; H-1'b; H-6"a), 3.28 H-5'' (H-1'b; H-6"a), 3.66 H-6''a (H-6"b), 3.68 H-1'b (H-1'a; H-2') 3.84 H-6''b (H-6"a), 4.01 H-1'a (H-1'b; H-2'), 4.27 H-1'' (H-2"), 6.54 H-6 (H-2'), 6.67 H-5 (H-2').

HMBC-NMR (300 MHz, CD_3OD): δ (ppm) = 2.77 H-2' (C-2', C-1'a, C-6, C-2, C-1), 3.17 H-2″ (C-2", C-1"), 3.30 H-3″ (C-4″), 3.66 H-6″a (C-3", C-5", C-6"a), 3.68 H-1'b (C-3", C-5"), 4.01 H-1'a (C-1"), 4.27 H-1″ (C-1'a), 6.54 H-6 (C-2', C-6, C-5, C-4), 6.64 H-2 (C-5, C-6, C-1, C-4), 6.67 H-5 (C-2', C-2, C-1, C-4).

5.16.2.3. Proanthocyanidins

5.16.2.3.1. (-)-Epicatechin (**26**) (95 mg)

Obtained from Fraction 6.6., as a light pink powder, Mr = 290 g/mol.



TLC: R_f= 0.68 (SiO_2, S5), R_f= 0.74 (RP-18, S9), R_f =0.47 (Cellulose F, S6), R_f = 0.60 (Cellulose F, S7).

Anisaldehyde: orange-pink,

Vanillin: pink

$[\alpha]_D$ -36.0 (MeOH *c*= 0.1)

CD (MeOH): $[\theta]_{228}$ - 0.93, $[\theta]_{241}$ +6719, $[\theta]_{254}$ +170, $[\theta]_{282}$ - 1783, $[\theta]_{297}$ - 57.

LC-ESI MS (neg) *m/z*: 289 $[M-H]^-$

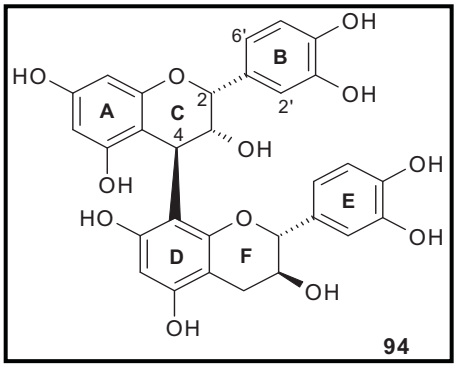
LC-MS: R_t = 6.5 min. (for conditions cf. p. 158)

^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) = 2.73 (H, *dd*, *J*= 3.0, 13.75, H-4axC), 2.89 (H, *dd*, *J*= 4.5, 12.23, H-4eqC), 4.18 (H, *d*, *J*= 1.3, 3.0, H-3C), 4.82 (H, *sbr*, H-2C), 5.92 (H, *d*, *J*= 2.0, H-6A), 5.95 (H, *d*, *J*= 2.0 , H-8A), 6.76 (H, d, *J*= 8, H-5'B), 6.80 (H, *dd*, *J*= 1.78, 6.46, H-6'B), 6.98 (H, *d*, *J*= 1.65, H-2'B).

^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = 29.2 (C-4C), 67.5 (C-3C), 79.9 (C-2C), 95.9 (C-6A), 96.5 (C-8A), 100.1 (C-4aA), 115.4 (C-2'B), 115.9 (C-5'B), 119.4 (C-6'B), 132.3 (C-1'B), 145.8 (C-3'B), 145.9 (C-4'B), 157.4 (C-5A), 157.7 (C-8aA), 158 (C-7A).

5.16.2.3.2. Proanthocyanidin B1 (**94**) (16 mg)

Obtained from fraction 6.3.4., as a brilliant grey-white powder (mixture of **94** and **95**), Mr= 578 g/mol.



TLC: Rf= 0.45 (SiO_2, S5).

Anisaldehyde: orange.

$[\alpha]_D$ -52.0 (MeOH *c*= 0.1).

ESI MS (neg) *m/z*: 577 $[M-H]^-$; MS^2 (577): 289.

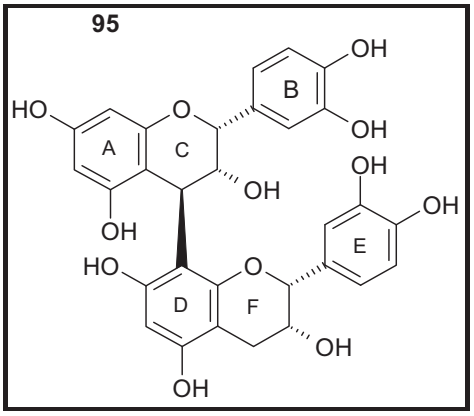
LC-MS: R_t = 2.0 min. (for conditions cf. p.161)

^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) = 2.68 (H, *d*, H-4axF), 2.87 (H, *dd*, H-4eqF), 4.09 (H, *sbr*, H-3C), 4.47 (H, *m*, H-3F), 4.65 (H, *sbr*, H-4C), 4.80 (H, H-2F), 5.10 (H, *sbr*, H-2C), 5.94 (H, *m*, H-8A), 5.96 (H, *sbr*, H-6A), 5.89 (H, *d*, H-6D), 6.11 (H, *sbr*, H-2'E), 6.67 (H, *sbr*, H-2'B), 6.68 (H, *d*, H-5'B/5'E), 6.78 (H, *d*, H-6'B), 6.89 (H, *d*, H-6'E).

^{13}C-NMR (75.5 MHz, CD_3OD): δ (ppm) = 29.2 (C-4F), 37.20 (C-4C), 67.82 (C-3F), 73.54 (C-3C), 77.14 (C-2C), 81.83 (C-2F), 96.22 (C-6A/8A), 96.56 (C-6D), 100.66 (C-4aD), 101.58 (C-4aA), 106.80 (C-8D), 113.9 (C-2'E), 114.88 (C-2'B), 115.33 (C-5'B), 115.99 (C-5'E), 119.2 (C-6'B), 121.20 (C-6'E), 132.33 (C-1'E), 132.74 (C-1'B), 145.66 (C-4'B/4'E), 145.9 (C-3'B), 146.09 (C-3'E), 155.42 (C-8aD), 155.90 (C-5D), 156.40 (C-7D), 157.40 (C-7A), 157.81 (C-5A), 158.56 (C-8aA).

5.16.2.3.3. Proanthocyanidin B2 (**95**)

Obtained from fraction 6.3.4. (16 mg), as a brilliant grey-white powder (mixture of **94** and **95**), Mr= 578 g/mol.



TLC: Rf= 0.45 (SiO_2, S5).

Anisaldehyde: orange.

$[\alpha]_D$ -52.0 (MeOH *c*= 0.1).

ESI MS (neg) *m/z*: 577 $[M-H]^-$; MS^2 (577): 289.

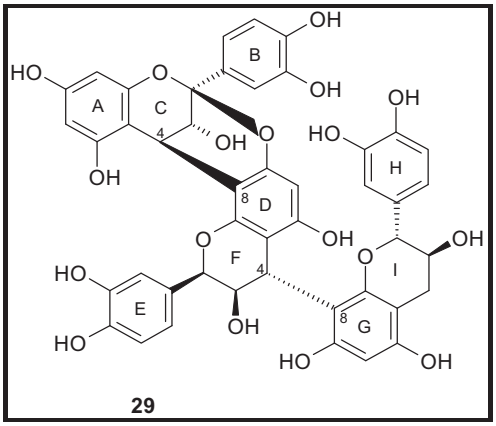
LC-MS: R_t = 5.4 min. (for conditions cf. p.161)

^{1}H-NMR (300 MHz, CD_3OD): cf. Results (Table 3-15 p.181)

^{13}C-NMR (75.5 MHz, CD_3OD): cf. Results (Table 3-16 p.182)

5.16.2.3.4. Epicatechin (4β→8, 2β→*O*→7)- [epicatechin -4β→8]-catechin (**29**)

Obtained from fraction 6.3.5. (41 mg), as an amorphous grey powder, Mr = 864 g/mol.



29

TLC: R_f= 0.35 (SiO_2, S5).

Anisaldehyde: pink.

$[\alpha]_D$ +95 (MeOH *c*=0.1,)

CD (MeOH): $[\theta]_{229}$ + 158437, $[\theta]_{271}$ - 13265

ESI MS (neg) *m/z*: 863 $[M-H]^-$; MS^2 (863): 289, 712, 451, 422, 573; MS^3 (712): 693, 559, 407, 411, 285; MS^3 (411): 285.

ESI MS (pos) *m/z*: 887 $[M+Na]^+$, 451; MS^2 (887): 717, 735.

LC-MS: R_t = 5.63 min [Column: Zorbax 150 x 4.6 mm. Solvent A: CH_3COOH 2.5 % in H_2O, solvent B= ACN:solvent A (80:20). Flow rate= 0.8 mL min^{-1}].

^{1}H-NMR (300 MHz, CD_3CN): δ (ppm) = 2.35 (H, *d*, *J*=11.0, H-4axI), 2.94 (H, *d*, *J*= 11.0, H-4eqI), 3.35 (H, *sbr*, H-3C), 3.70(H, *sbr*, H-3I), 3.85 (H, *sbr*, H-4C), 3.90 (H, *d*, *J*= 8.4,H-2I), 4.05 (H, *sbr*, H-3F), 4.35 (H, *sbr*, H-4F), 5.42 (H, *sbr*, H-2F), 5.80 (H, *s*, H-6D), 5.87 (H, *s*, H-6A), 6.00 (H, *s*, H-8A), 6.10 (H, *s*, H-6G), 6.67 (H, *s*, H-6'H), 6.77 (H, *s*, H-5'B/H-5'E), 6.80 (H, *d*, *J*= 7.4, H-5'H), 6.90 (H, *s*, H-2'H), 6.98 (H, H-2'B), 7.08 (H, *s*, H-6'B), 7.28 (H, *sbr*, H-2'E).

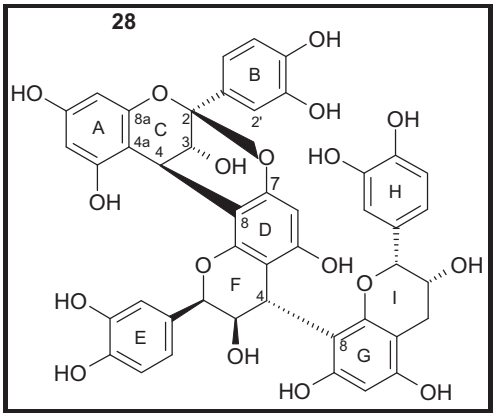
^{13}C-NMR (75.5 MHz, CD_3CN): δ (ppm) = 28.3 (C-4C), 30.2 (C-4I), 32.3 (C-1'B), 37.8 (C-4F), 66.9 (C-3C), 69.2 (C-3I), 71.2 (C-3F), 78.0 (C-2F), 83.1 (C-2I), 95.8 (C-6D), 96.3 (C-8A), 96.5 (C-6G), 98.2 (C-6A), 99.8 (C-8D), 102.4 (C-4aG), 104.7 (C-2C), 106.0 (C-4aA), 106.6 (C-4aD), 108.3 (C-8G), 115.4 (C-5'E/C-2'B), 115.7 (C-2'H), 116.0 (C-5'H/C-5'B), 116.6 (C-2'E), 118.5 (C-6'B), 120.5 (C-6'H), 121.4 (C-6'E), 131.3 (C-1'E), 132.5 (C-1'H), 144.8 (C-3'B), 144.9 (C-4'B), 145.2 (C-3'E), 145.3 (C-4'H), 145.4 (C-3'H), 145.6 (C-4'E), 150.7 (C-5G), 151.3 (C-5D), 153.8 (C-8aA), 154.9 (C-7G), 155.0 (C-7D), 155.2 (C-7A), 155.4 (C-8aG), 156.7 (C-8aD), 157.6 (C-5A).

^{1,2} *J*-HC (HMQC-NMR) (300 MHz, CD_3CN): δ (ppm) = 2.35 H-4Iax (C-4I), 2.94 H-I4eq (C-4I), 3.35 H-3C (C-3C), 3.70 H-3I (C-3I), 3.85 H-4C (C-4C), 3.90 H-2I (C-2I), 4.05 H-3F (C-3F), 4.35 H-4F (C-4F), 5.42 H-2F (C-2F), 5.80 H-6D (C-6D), 5.87 H-6A (C-6A), 6.00 H-8A (C-8A), 6. 10 H-6G (C-6G), 6.67 H-6'H (C-6'H), 6.77 H-5'E (C-5'E), 6.80 H-5'H (C-5'H), 6. 90 H-2'H (C-2'H), 6.98 H-2'B (C-2'B), 7.08 H-6'E (C-6'E), 7.28 H-2'E (C-2'E).

^{2,3} *J*-HC (HMBC-NMR) (300 MHz, CD_3CN): δ (ppm) =2.35 H-4Iax (C-3I, C-4aG), 2.94 H- 4Ieq (C-8aG, C-4aG, C-2I), 3.85 H-4C (C-3C, C-8D, C-4aA, C-8aA, C-8aD), 3.90 H-2I (C-4I, C-1'H, C-2'H), 4.05 H-3F (C-2F, C-4aF), 4.35 H-4F (C-5D, C-4aF, C-2F, C-3F, C-8G, C-8aI), 5.42 H-2F (C-1'E, C-2'E), 5.80 H-6D (C-7D, C-5D, C-4aF), 5.87 H-6A (C-8A), 6.00 H-8A (C-8aA, C-6A), 6.67 H-6'H (C-4'H, C-5'H, C-2I), 6.80 H-5'H (C-4'H, C-1'H), 6.90 H-2'H (C-1'H, C-4'H), 7.08 H-6'E (C-4'E, C-2F), 7.28 H-2'E (C-2F, C-4'E).

5.16.2.3.5. Epicatechin (4β→8, 2β→*O*→7)- [epicatechin -4β→8]-epicatechin (**28**)

Obtained from fraction 6.3.6. (43 mg), as a white-grey brilliant powder, Mr = 864 g/mol.



TLC : R_f= 0.38 (SiO_2, S5).

Anisaldehyde: orange-pink,

$[\alpha]_D$ +59.0 (MeOH *c*= 0.1).

CD (MeOH): $[\theta]_{229}$ + 153204 and $[\theta]_{272}$ - 14898

ESI MS (neg) *m/z*: 864 $[M-H]^-$; MS^2 (864): 711, 573, 451; MS^3 (573): 411; MS^3 (451): 289.

ESI MS (pos) *m/z*: 887 $[M+Na]^+$; MS^2 (887): 717, 555

LC-MS: R_t = 27.2 min. (for conditions cf. p.174)

^{1}H-NMR (300 MHz, CD_3CN+D_2O): cf. Table 3-15, p. 181

^{13}C-NMR (75.5 MHz, CD_3CN): cf. Table 3-16 p.182

^{1,2} *J*-HC (HMQC-NMR) (CD_3CN+D_2O): δ (ppm) = 2.68 H-4I_{ax} (C-4/I), 2.75 H-4I_{eq} (C-4/I), 3.21 H-3C (C-3C), 3.78 H-3I (C-3/I), 3.90 H-4C (C-4C), 4.04 H-3F (C-3F), 4.31 H-2I (C-2I), 4.34 H-4F (C-4F), 5.43 H-2F (C-2F), 5.56 H-6D (C-6D), 5.76 H-6G (C-6G), 5.91 H-6A (C-6A), 6.02 H-8A (C-8A), 6.66 H-5'B (C-5'B), 6.80 H-5'E (C-5'E), 6.86 H-6'H (C-6'H), 6.86 H-5'H (C-5'H), 6.95 H-2'B (C-2'B), 7.06 H-6'E (C-6'E), 7.17 H-2'H (C-2'H), 7.29 H-2'E (C-2'E).

^{2,3} *J*-HC (HMBC-NMR) (CD_3CN+D_2O): δ (ppm) 2.68 H-4I $_{ax}$ (C-3I, C-4AG, C-2I), 2.75 H-4I_{eq} (C-3I, C-2I, C-4aG), 3.21 H-3C (C-2C), 3.90 H-4C (C-3C, C-4aA), 4.04 H-3F (C-4aD), 4.31 H-2I (C-3I, C-1'H, C-4I), 4.34 H-4F (C-3F, C-2F, C-4aD, C-8aD,C-8G), 5.76 H-6G (C-6'G, C-7'G), 5.91 H-6A (C-8A, C-2C, C-5A), 6.02 H-8A (C-8A, C-2C, C-7A, C-5A), 6.66 H-5'B (C-2'B, C-4'B), 6.80 H-5'E (C-6'E, C-1'E, C-3'E), 6.86 H-5'H (C-6'H, C-4'H), 6.86 H-6'H (C-6'H, C-4'H), 6.95 H-2'B (C-6'B, C-4'B), 7.06 H-6'E (C-4'E), 7.17 H-6'B (C-4'B), 7.29 H-2'E (C-2F, C-6'E, C-4'E).

diff-NOE (300 MHz, CD_3CN). 3.21 H-3C (H-3F), 3. 78 H-3I (H-2I), 4.04 H-3F (H-2I, H-3C), 4.31 H-2I (H-3I, H-3F), 5.76 H-6G (H-8A), 6.02 (H-8A to H-6G, H-6A), 7.17 H-2'H (H-2′E), 7.29 H-2'E (H-2′H).

6. SUMMARY

The cultivation of avocado fruits (*Persea americana* Mill.) is expanding around the world. Major producer of this crop is Mexico. In Mexican and African ethnomedicine decocts of avocado seeds are used as a potent remedy against different diseases such as muscle paint, menstruation disturbs and diabetes (Adeboye et al., 1999; Adeyemi et al., 2002).

This was one of the initial points for conducting a thorough phytochemical investigation on avocado seeds with the focus on analysis of extractable natural products in respect to their potential use for pharmaceutical and food applications. During avocado fruit processing, the residual seeds will be deposited as waste material. Aim of the study was to analyze the chemical composition of avocado seeds, including preparative isolation and complete structural characterization of the isolated natural products by spectroscopical tools. Bioactivities of crude extracts and also of purified structures were screened by efficient and relatively inexpensive assays.

During this research on avocado seeds, the implementation of 'high-speed countercurrent chromatography' (HSCCC) technique proved to be a versatile tool for efficient fractionation and isolation of natural products. The combination with other classical separation methods (i.e. size exclusion gelchromatography, preparative HPLC) resulted in the isolation of 22 natural products from avocado seeds. Isolation procedures were guided by using the TEAC-assay (antioxidant capacity) and the 'brine-shrimp'-assay with *Artemia salina* L. (cytotoxic activity) directing to the bioactive principles.

The structure elucidation of the isolated compounds was performed by means of 1D-NMR (^{1}H, ^{13}C, DEPT135, *diff*-NOe), 2D-NMR (^{1}H/^{1}H-COSY, HMQC and HMBC). UV/Vis-spectroscopy and circulardichroism (CD), mass spectrometry (GC-EI/MS, direct EI-MS, DCI-MS, and HPLC-ESI-MS/MS) were also applied. Chemical derivatization such as acetylation, enzymatic hydrolysis and thiolysis reaction were conducted for structural confirmation of complex natural products.

The recovered compounds from avocado seeds ranged in their polarity from extremely polar (i.e. proanthocyanidins) to very lipophilic acetogenins (i.e. persin) (cf. Fig. 6-2 and 6-3).

(1'*S*,6'*R*)-8'-Hydroxyabscisic acid *ß*-D-glucoside (**89**), (1'*R*,3'R,5'*R*,8'S)-epi-dihydrophaseic acid *ß*-D-glucoside (**70**), tyrosol-1'-*ß*-D-O-glucoside (**90**) and 3-hydroxy-tyrosol-1'-*ß*-D-O-glucoside (**91**) were isolated from polar extracts of avocado seeds and were reported here for the first time. The novel abscisic acid derivate **89** is assumed to be a missing link in the biosynthetical conversion of hydroxyabscisic acid structures to phaseic acid derivates (cf. Fig. 6-1). Enzymatic cleavage reaction corroborated the immediate ring-closure of the deglucosylated C-8' of **89** resulting in phaseic acid (cf. p.p. 137).

The investigation of polyphenols revealed that substance **90** showed relatively low activity in the TEAC antioxidative assay (0.5 mmol Trolox/ mmol substance) due to glucosylation. For the liberated aglycone (tyrosol) high antioxidant activities were documented (Visioli et al., 1998; Aparicio et al., 1999; Medina et al., 2002; Trujillo et al., 2006).

The glucosides **90** and **91** can easily be converted by glucosidases to give tyrosol and hydroxytyrosol, respectively, having a potential utilization as natural antioxidants for either pharmaceutical, cosmetic preparations as well as ingredients for "functional foods".

The isolated flavan-3-ol structures (**26**, **94**, **95**, **29** and **28**) are well known and widely distributed in the plant kingdom. In case of the proanthocyanidin A-type trimers **28** and **29,** we were able to isolate them for the first time in pure form from the seeds of *Persea americana* and a complete structural characterization was possible. Compound **28** was also isolated by Nonaka et al. (1983) from *Cinnamomum zeylanicum* and was named as cinnamtannin B1 (Hatano et al., 2002).

Principally, all isolated polar compounds from avocado seeds showed a strong antioxidative activity in the TEAC-assay (cf. Table 3-20 p. 196). For the proanthocyanidins **28** and **29** a more potent activity (2.8 and 2.9 mmol Trolox/mmol substance, respectively) than for the ascorbic acid (0.96 mmol Trolox/mmol substance) was detected, one of the most frequently used water soluble antioxidants in industry.

The investigations concerning the toxicity detected that none of the compounds **26**, **28** and **29** were cytotoxic in the 'brine shrimp'-lethality assay.

Literature data on biological test of compounds **28** and **29** from cinnamon corroborated the insulin-like activity of these substances (Anderson et al., 2004). It is known that Cinnamtannin B1 (**28**) has antitumor activity.

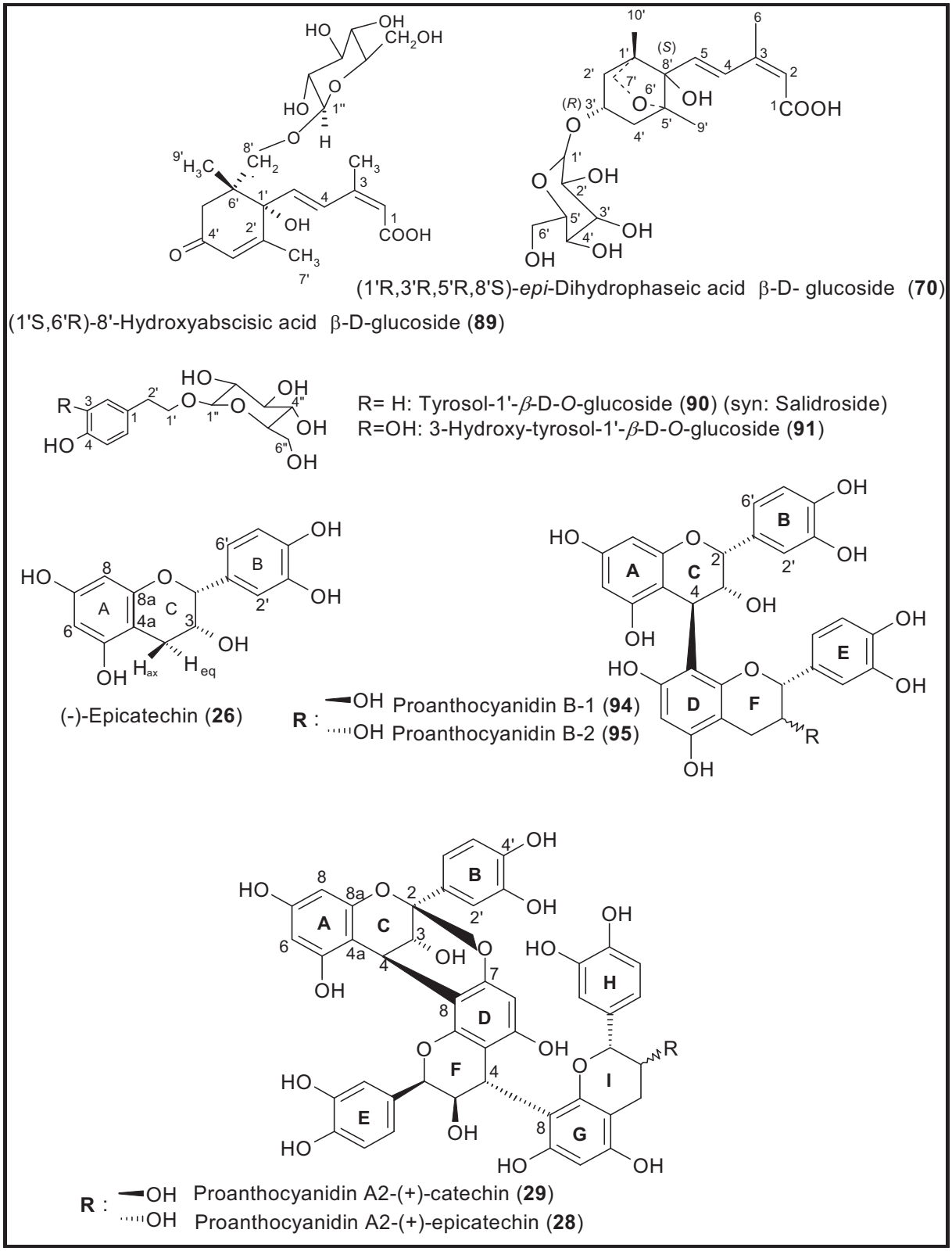


Figure 6-1. Polar natural products isolated from avocado seed material (*Persea americana* Mill., cv. *Hass*).

In the class of lipophilic compounds isolated from avocado seeds, four principal substitutions pattern with acetylenic-, vinylic-, furanoic- and trihydroxy-functional groups were found. Already known compounds of the avocado fruit flesh were the lipophilic substances **58**, **81**, **60**, and **54** (Kashman et al., 1969; Domergue, et al., 2000; Oberlies et al., 1998).

From the avocado seed material, we isolated new lipophilic natural products **78**, **79**, **82** (acetylenic compounds), **83**, **84**, **85** (acetogenins with vinylic function), **86** (furan-substitution), **87** and **88** (trihydroxylation) (cf. Fig. 6-2 and 6-3).

The petrol ether extract as well as the petrol ether partition of the avocado seeds showed low antioxidant effects in the TEAC-assay (1.2 or 9.9 %, respectively) in comparison to ascorbic acid (100%). Consequently, the isolated lipophilic constituents were not of interest for further antioxidative trials.

Noteworthy, the cytotoxicity (LD_{50}) of the petrol ether partition containing a highly complex mixture of compounds was stronger (6.2 µg/mL) than the control podophyllotoxin used as reference for measurement of cytotoxicity in the 'brine-shrimp' assay (9.9 µg/mL).

The fractions of lipophilic polarity such as 8.1.9b.2., contained compounds **79** and **82** and fraction 8.1.9b.3., resulted in the isolated structures **58**, **81**, **60**, **83**, **84**, **85**, and **86** showing a strong cytotoxicity in the assay with LD_{50}-values smaller than for the reference standard Podophyllotoxin (LD_{50} 9.9 µg/mL).

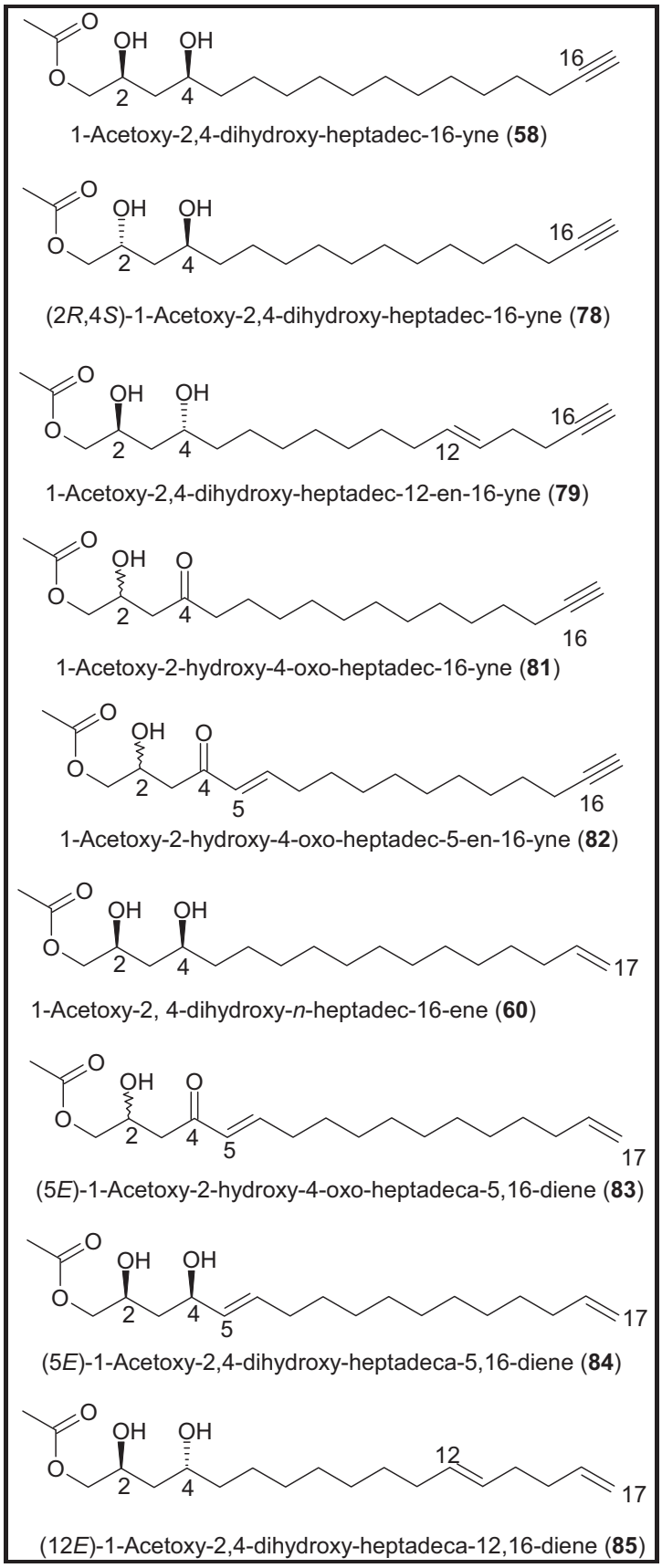


Figure 6-2. Lipophilic compounds with C_{17}-chain isolated from avocado seeds (*Persea americana* Mill., cv. *Hass*).

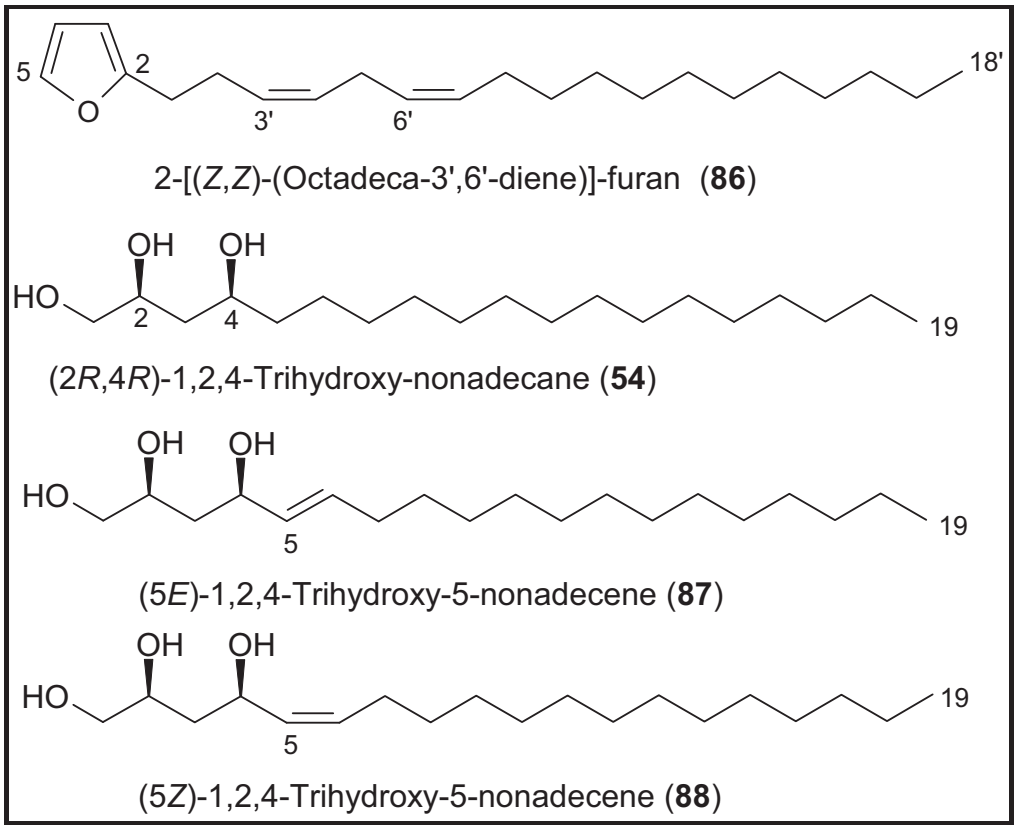


Figure 6-3. Lipophilic constituents with C_{18}- and C_{19}-chain isolated from avocado seeds (*Persea americana* Mill., cv. *Hass*).

For the isolated and tested substances structure-activity relationship were observed for the biological activities monitored by the TEAC antioxidative assay and the 'brine shrimp' assay.

In the case of antioxidative activities, the trimeric A-type substituted proanthocyanidins (**28** and **29**) revealed a much stronger antioxidant capacity than the dimers (**94** and **95**) and the flavanol monomer epicatechin (**26**).

In the monitoring of cytoxicity in the 'brine-shrimp' assay, the presence of the acetoxy group in the lipophilic substances (**58**, **78**, **79**, **81**, **82**, **60**, **83**, **84**, and **85**) was important for a strong activity. For the group of trihydroxy compounds (**54**, **87**, **88**) further studies are in preparation.

The stereochemistry (2*S*,4*S*) of compounds **58**, **60** and **84** contained in fraction 8.1.9b.3 seems to be also important for a high toxicity.

The results of our phytochemical study are coherent with the ethnomedicinal knowledge from the indigenous people of Mexico and other cultures. The use of

avocado seeds (cf. p. 16) for certain diseases are at least in part explainable by the recovered natural products and their known and investigated activities.

Interestingly, the use of avocado seed as antioxidants in some traditional foods and dishes of the Mexican people was proved by the high antioxidative activity of some of the isolated compounds (**26**, **94**, **95**, **28** and **29**).

The radical scavenging capacity of the methanolic extracts of avocado was not as strong as the commonly used food antioxidants in industry (ascorbic acid, BHT, BHA). Nevertheless, they can certainly be considered as an alternative in case of replacement of synthetic compounds such as butylhydroxy-toluene (BHT) and butylhydroxy-anisol (BHA).

Interestingly, substances **94**, **95**, **28** and **29** (recovered from the ethyl acetate partition) demonstrated a higher antioxidant activity than the common synthetic antioxidants.

Natural avocado compounds from the polar extracts seem to be non-toxic, therefore the ethyl acetate extract or its purified compounds could be also used as potent antioxidant formulations by the food industry.

The lipophilic extracts (PE) and fractions were found to be extremely cytotoxic, hence the use in food industry is not appropriate. Evaluation of these compounds against cancer cell lines could result in new bioactive anti-tumor agents.

More research in this field remains to be done in the future for deepening the insights into the potentials of avocado seed natural products.

Further natural compounds from avocado seeds are waiting to be isolated and to be tested in specific bioassays. Avocado seeds already applied in ethnomedicine by the traditional healers of the ancient Aztec cultures in Mexico may provide potential novel drugs of the future.

6. ZUSAMMENFASSUNG

Der Anbau von Avocado-Früchten (*Persea americana* Mill.) expandiert weltweit. Seit langer Zeit ist Mexiko einer der Hauptproduzenten und größten Exporteure.

In der ethnomedizinischen Anwendung wurden in Mexiko und Afrika wässrige Auszüge von Avocado-Kernen vielfältig als wirksame Heilmittel bei verschiedenen Erkrankungen, wie etwa Muskelschmerzen, Menstruations-Beschwerden und Diabetes verwendet (Adeboye et al., 1999; Adeyemi et al., 2002).

Die überlieferten ethnomedizinischen Daten waren einer der Ausgangspunkte für die Durchführung einer phytochemischen Analyse der Avocadosamen. Zielsetzung war es, die extrahierbaren Naturstoffe zu analysieren und Anwendungsmöglichkeiten für den pharmazeutisch-medizinischen sowie den Lebensmittelbereich aufzuzeigen.

Bei der industriellen Verarbeitung von Avocadofrüchten fallen große Mengen an Kern- und Schalenmaterial an, die ohne weitere Anwendung als Abfallstoffe beseitigt werden. Ziel dieser Untersuchung war es, die chemische Zusammensetzung von Avocadosamen eingehend zu analysieren und präparative Isolierungswege für die darin enthaltenen Naturstoffe aufzuzeigen.

Die Isolierung und die Strukturaufklärung der Naturstoffe wurden mittels moderner chromatographischer und spektroskopischer Methoden durchgeführt. Die Bioaktivitäten der Rohextrakte sowie der gewonnenen Reinsubstanzen wurden mit Hilfe schnell durchzuführender Testsysteme überprüft.

Für die Trennung der Inhaltsstoffe aus Avocadosamen hat sich die Gegenstromverteilungs-Chromatographie (*engl.*: '*high-speed countercurrent chromatography*' - HSCCC) als sehr effiziente Methode erwiesen, mit der empfindliche Naturstoffe im Gramm-Maßstab fraktioniert und isoliert werden können. In Kombination mit den klassischen chromatographischen Trennmethoden (z.B. Größenausschluss-Chromatographie, präparative HPLC) konnten insgesamt 22 Naturstoffe aus Avocadosamen isoliert und strukturell charakterisiert werden. Die Isolierungsschritte wurden mittels des 'TEAC-Assays' (Bestimmung der antioxidativen Kapazität) und des '*brine-shrimp'*-Assays mit der Salzwasser Crustaceae *Artemia salina* L. (Bestimmung der zytotoxischen Aktivität) überprüft und führten zur Isolierung einer Reihe bioaktiven Leitsubstanzen.

Die Strukturaufklärung der isolierten Verbindungen erfolgte mittels moderner spektroskopischer Techniken, wie 1D-NMR (^{1}H, ^{13}C, DEPT135, *diff*-NOe), 2D-NMR (^{1}H/^{1}H-COSY, HMQC und HMBC), UV/Vis-Spektroskopie und Circulardichroismus (CD), Massenspektrometrie (GC-EI/MS, Direkteinlass EI-MS, DCI-MS, und HPLC-ESI-MS/MS). Chemische Derivatisierungen, wie Acetylierungen, Thiolyse-Abbau sowie enzymatische Hydrolyse wurden zur Strukturbestätigung der komplexen Naturstoffe verwendet.

Das Polaritätsspektrum der aus den Avocadosamen isolierten Verbindungen erstreckte sich von extrem polar (z.B. Proanthocyanidine, Tyrosol-glycoside) bis hin zu den sehr lipophilen Acetogeninen (z.B. Persin) (siehe Abb. 6-2 und 6-3).

Die *ß*-*O*-Glucoside von (1'*S*,6'*R*)-8'-Hydroxyabscisinsäure (**89**), und (1'*R*,3'R,5'*R*,8'S)-*epi*-Dihydrophaseinsäure (**70**) sowie das Tyrosol-1'-*ß*-D-O-glucosid (**90**) und 3-Hydroxy-tyrosol-1'-*ß*-D-O-glucosid (**91**) entstammen den polaren Extrakten der Avocadosamen und wurden im Zuge dieser Arbeit erstmalig für Avocado beschrieben. Das neue glycosidierte Abscisinsäure-Derivat **89** stellt eine potentielle Vorstufe bei der Umsetzung von Hydroxyabscisinsäure-Strukturen zu Phaseinsäure-Derivaten dar (s. Fig. 6-1). Die enzymatische Deglucosidierung von **89** zum freien Aglykon resultierte im sofortigen Ringschluss zu Phaseinsäure (s. S. 137).

Die Untersuchungen der Polyphenole (TEAC-Assay) zeigten, dass die Substanz **90** nur eine relativ geringe antioxidative Kapazität aufwies (0,5 mmol Trolox/ mmol Substanz), möglicherweise verursacht durch die C-1 Glucosidierung. Das freie Aglykon (Tyrosol) zeigt hingegen eine hohe antioxidative Aktivität (Visioli et al., 1998; Aparicio et al., 1999; Medina et al., 2002; Trujillo et al., 2006).

Die Glucoside **90** und **91** können sehr einfach durch Glucosidasen in Tyrosol und Hydroxytyrosol gespalten werden. Beide Produkte besitzen eine ausgezeichnete Wirkung als natürliche Antioxidantien in pharmazeutischen und kosmetischen Präparaten oder als Zutat in „functional foods".

Bei den isolierten Flavan-3-ol Verbindungen (**26**, **94**, **95**, **29** and **28**) handelt es sich um Proanthocyanidine. Die A-Typ verknüpften Proanthocyanidin Trimere **28** und **29** wurden im Zuge dieser Arbeit erstmalig in reiner Form aus den Samen von *Persea americana* isoliert und einer vollständigen Strukturanalyse unterzogen.

Das A-Typ Trimer **28** wurde zuvor aus *Cinnamomum zeylanicum* (Nonaka et al., 1983) isoliert und als Cinnamtannin B1 benannt (Hatano et al., 2002).

Die Vielzahl der aus Avocadosamen isolierten polaren Verbindungen zeigte eine hohe antioxidative Aktivität im TEAC-Assay (s. Tabelle 3-20 S. 196). Bemerkenswert war die stärkere Aktivität der Proanthocyanidine **28** und **29** (2,8 und 2,9 mmol Trolox/ mmol) verglichen mit Ascorbinsäure (0,96 mmol Trolox/ mmol), eines der am häufigsten eingesetzten wasserlöslichen Antioxidantien in der Lebensmittelverarbeitung.

Für die polyphenolischen Verbindungen **26**, **28** and **29** konnten im 'brine shrimp'-Assay keine zytotoxischen Effekte (>1000 µg/mL) festgestellt werden.

Literaturdaten zu den Proanthocyanidin-Trimeren **28** und **29** aus Zimt ergaben eine insulin-ähnliche Aktivität (Anderson et al., 2004). Für Cinnamtannin B1 (**28**) ist eine Antitumor-Wirkung bekannt.

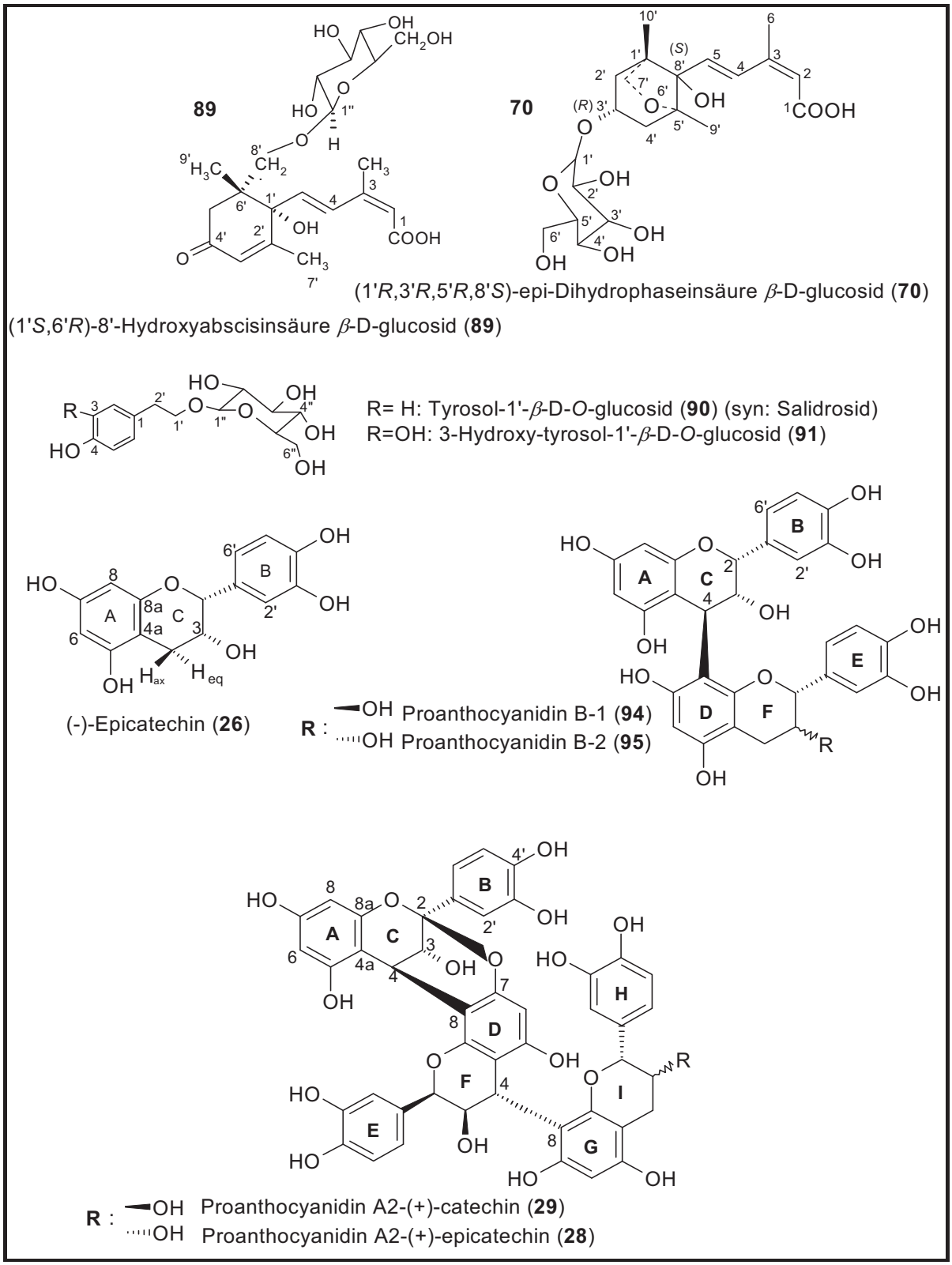


Abb. 6-1. Isolierte polare Naturstoffe aus Avocadosamen (*Persea americana* Mill., cv. *Hass*).

Aus Avocadosamen wurden in der Klasse der fettlöslichen Verbindungen vier hauptsächliche Substitutionsmuster (d.h. mit terminaler Acetylen- und Vinyl-, sowie Furan- und Trihydroxy-Einheit) identifiziert. Literaturbekannte Verbindungen aus dem Fruchtfleisch der Avocado waren die lipophilen Substanzen **58**, **81**, **60** und **54** (Kashman et al., 1969; Domergue,et al., 2000; Oberlies et al., 1998).

Als neue Avocado Inhaltstoffe-Kerne konnten wir die Verbindungen **78**, **79**, **82** mit endständiger Alkinfunktion sowie die Verbindungen **83**, **84**, **85** mit terminaler Vinyl-Funktion identifizieren. Als einziger Naturstoff mit Furan-Substitution wurde **86** gewonnen, neben den Trihydroxy-Verbindungen **87** und **88** (s. Abb. 6-2 und 6-3).

Der Petrolether-Extrakt sowie die Petrolether-Verteilung der Avocadosamen zeigten nur geringe antioxidative Effekte im TEAC-Assay (1,2% und 9,89%) im Vergleich mit Ascorbinsäure. Dafür zeigte die aus einem komplexen Substanzgemisch bestehende Petrolether-Verteilung eine beachtliche Cytotoxizität im '*brine-shrimp*' Assay mit einer LD_{50} von 6,2 µg/mL. Diese Aktivität war stärker als die des verwendeten Referenztoxins Podophyllotoxyn (9,9 µg/mL).

Die lipophile Fraktion 8.1.9b.2. mit Verbindungen **79** und **82** sowie Fraktion 8.1.9b.3. mit Verbindungen **58**, **81**, **60**, **83**, **84**, **85** und **86** zeigten eine beachtliche Cytotoxizität mit geringen LD_{50}-Werten im Vergleich zu Podophyllotoxin (LD_{50}: 9,9 µg/mL).

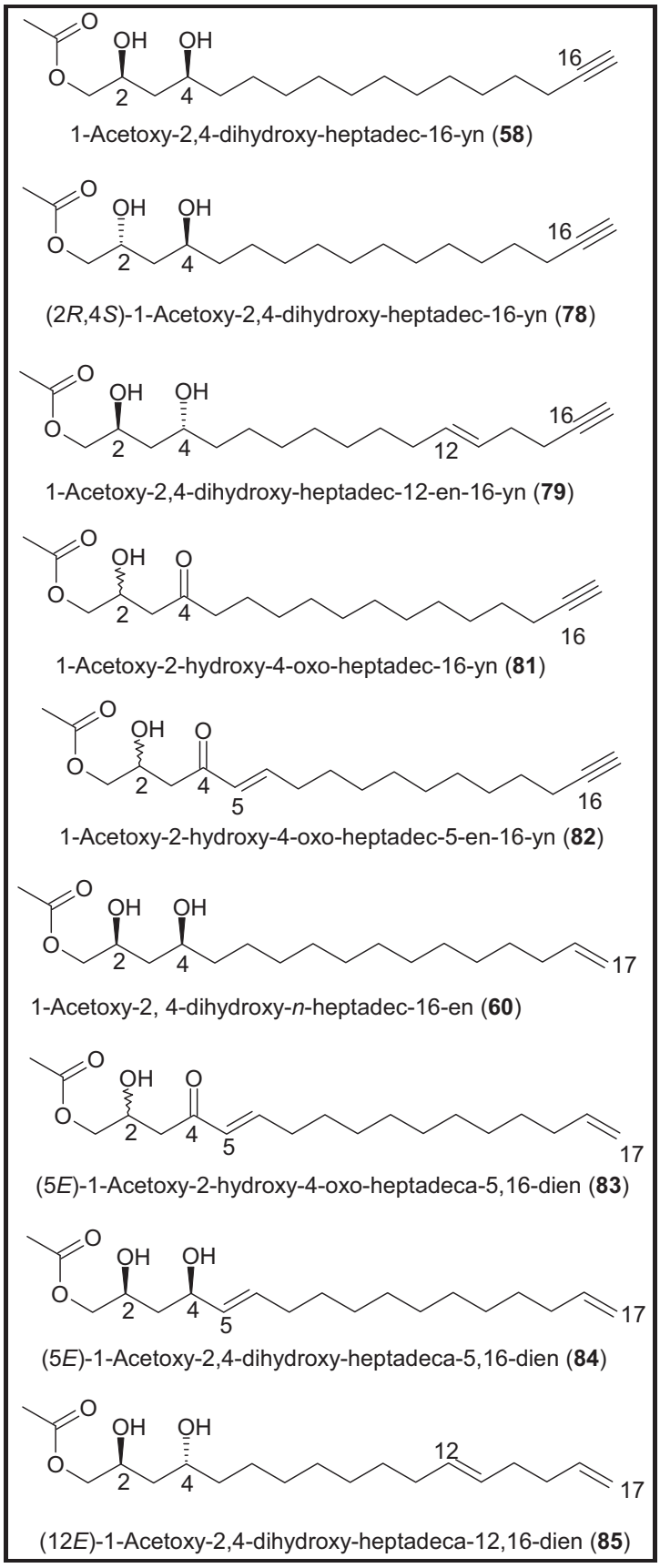


Abb. 6-2. Isolierte lipophile Verbindungen mit C_{17}-Kette aus Avocadosamen (*Persea americana* Mill., cv. *Hass*).

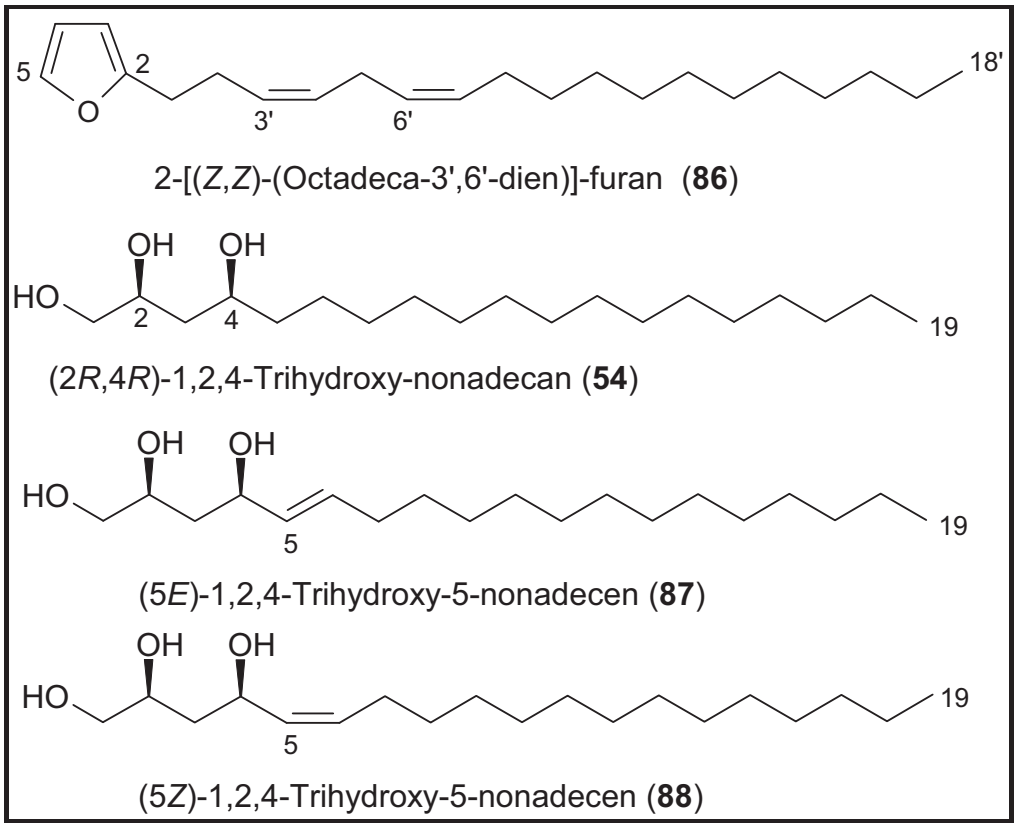


Abb. 6-3. Isolierte lipophile Verbindungen aus Avocadosamen mit eine C_{18}- and C_{19}-Kette.

Für die isolierten Reinverbindungen konnten Struktur-Wirkungsbeziehungen aufgestellt werden (s. unten).

Im TEAC-Modell zeigten die trimeren A-Typ Proanthocyanidine (**28**, **29**) eine höhere antioxidative Aktivitat als dimere Strukturen (**94**, **95**) und das Flavanol-Monomer Epicatechin (**26**). Im Aktivitätsvergleich zwischen **28** und **29** konnte kein nennenswerter Unterschied festgestellt werden.

Die Anwesenheit einer Acetoxy-Substitution in den lipophilen Verbindungen (**58**, **78**, **79**, **81**, **82**, **60**, **83**, **84**, **85**) war eine entscheidende strukturelle Vorgabe für eine starke Cytotoxizität im '*brine-shrimp*' Assay. Im Falle der Trihydroxy-Verbindungen (**54**, **87**, **88**) sind weitere Untersuchungen in Vorbereitung.

Die Stereochemie (2*S*,4*S*) der Hydroxylsubstitutionen in den Verbindungen **58**, **60** sowie **84** scheint für die hohe Cytotoxizität eine wichtige Rolle zu spielen.

Unsere phytochemischen Ergebnisse sind in guter Übereinstimmung mit den ethnomedizinischen Anwendungsformen von Avocado in Mexiko. Die Anwendung von Extrakten aus Avocadosamen (s.S.16) in der Therapie verschiedener

Erkrankungen wird an Hand der isolierten Naturstoffe sowie der hier ermittelten oder schon bekannten Bioaktivitäten erklärbar.

Interessanterweise wurde die Anwendung von Avocadosamen bzw. deren Extrakte als ‚Antioxidationsmittels' in einigen traditionellen mexikanischen Speisen durch die hohe antioxidative Aktivität einiger der isolierten Verbindungen eindeutig bestätigt (**26**, **94**, **95**, **28**, **29**).

Die methanolischen Extrakte der Avocadosamen waren in ihrer Aktivität als Radikalfänger nicht so stark wie die in der Lebensmittelindustrie eingesetzten Antioxidantien (Ascorbinsäure, BHT und BHA). Dennoch können polare Avocadokern-Extrakte als Alternative zu den synthetischen Antioxidanten wie BHT oder BHA verwendet werden.

Die aus der Ethylacetat-Verteilung isolierten Reinsubstanzen **94**, **95**, **28** and **29** zeigten sogar eine höhere antioxidative Aktivität als die genannten synthetischen Antioxidantien. Nach unserem Kenntnisstand sind die von uns beschriebenen antioxidativ wirkenden Polyphenole aus Avocadosamen nicht toxisch. Somit erweist sich gerade der Ethylacetat-Extrakt als eine viel versprechende Quelle für antioxidativ wirkende Polyphenole für den Lebensmittel- und Kosmetikbereich.

Die lipophilen Extrakte (Petrolether Verteilung) und die entsprechenden Fraktionen zeigten eine stark cytotoxische Eigenschaft, so dass eine Anwendung im Lebensmittelbereich nicht denkbar ist. Hingegen ist die Evaluierung dieser Extrakte gegenüber Krebszelllinien eine viel versprechende Ansatzpunkt für mögliche neue Antitumor-Medikamente.

Auch für zukünftige Forschungsprojekte verbleibt ein hohes Potenzial noch weitere neue Naturstoffe aus Avocadofrucht zu isolieren, um sie dann ausgewählten spezifischen Biotests zuzuführen.

Somit lieferte das ethnomedizinische Wissen der Schamanen aus der Azteken-Kultur sowie der traditionellen Heiler Mexikos in der Anwendung von Avocadosamen einen neuen Ansatzpunkt für die Entwicklung zukünftiger Arzneistoffe.

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Curriculum Vitae

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Birthday	April 12th 1968 in Guadalajara, Jalisco, Mexico
Parents	Mr. Margarito Ramos-Cortés, Mechanic
	Mrs. Margarita Godínez-Ramírez
Civil status	Married since March 2003 with Dr. Gerold Jerz
School	1975-1981 Escuela Urbana 89, Guadalajara
	1981-1984 Escuela Secundaria No. 5 Guadalajara
	1984-1987 Escuela Preparatoria No. 3 Guadalajara
School Practical course	1986-1987 "Centro de Detección Oportuna de Cáncer"
	1987 Certificate: High School
University (studies)	1987-1992 Chemistry-Pharmaceutics-Biology in the Faculty of Chemical Sciences in "Centro Universitario de Ciencias Exactas e Ingenierías (CUCEI)". Universidad de Guadalajara.
	Titel: Chemistry-Pharmaceutics-Biology Juli 1992.
Practical course:	Sept. 1990-Nov. 1991
	Microbiological practice at the microbiological Institute Universidad de Guadalajara.
Master studies	1996-1999 CUCEI. Universidad de Guadalajara
	1999 Master in biotechnological processes
Languages	Languages: Spanish, fluently writing and speaking (native language)
	German, fluently speaking (2000 Intensive German course in the Goethe Institute Göttingen, Germany).
	English, fluently writing (school knowledge).
PhD. studies	Since April 2001 Ph.D. student of Prof. Dr. Peter Winterhalter at the „Institut für Lebensmittelchemie an der TU Braunschweig" with a DAAD scholarship (2000-2003).
Employments	1992-1996
Companies	1992-1994 Fa. Control Integral de Calidad S.A de C.V.
	1995 Fa. Sanborns Hnos. S.A. de C.V.

1996 Fa. Control de Calidad Agropecuario S.A. de C.V.
Implementation of methods for the agricultural industry around microbiological analysis on animal foods, water, and seeds. Chemical analysis in raw materials for bakery industries. Statistical analysis and quality control. Development of analytical and extraction methods for plants and phytochemical analysis.

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Eltern	Margarito Ramos-Cortés, Mechaniker Margarita Godínez-Ramírez, Hausfrau
Familienstand	Verheiratet seit März 2003 mit Dr. Gerold Jerz
Schulischer Werdegang	1975-1981 Escuela Urbana 89, Guadalajara 1981-1984 Escuela Secundaria No. 5 Guadalajara 1984-1987 Escuela Preparatoria No. 3 Guadalajara
Praktikum	1986-1987 “Centro de Detección Oportuna de Cáncer”
	1987 Abschluss: Abitur
Studium	1987-1992 Chemie-Pharmazie-Biologie Diplom an der Facultad de Ciencias Químicas im “Centro Universitario de Ciencias Exactas e Ingenierías (CUCEI)”. Universidad de Guadalajara. Abschluss: Diplom Chemie-Pharmazie-Biologie Juli 1992.
Praktikum	Sept. 1990 bis Nov. 1991 Mikrobiologie Praktikum am Mikrobiologischen Institut an der Universidad de Guadalajara.
Masterstudium	1996-1999 CUCEI. Universidad de Guadalajara 1999 Abschluss: Master in Biotechnologischen Verfahren
Sprachkurs	2000 Intensiv-Sprachkurs Deutsch am Goethe-Institut in Göttingen
Promotion	Seit April 2001 Doktorandin bei Herrn Prof. Dr. Peter Winterhalter am Institut für Lebensmittelchemie der TU Braunschweig mit DAAD Stipendium von 2000-2003.
Berufliche Tätigkeit	1992-1996
Firmen	1992-1994 Fa. Control Integral de Calidad S.A de C.V. 1995 Fa. Sanborn Hnos. S.A. de C.V. 1996 Fa. Control de Calidad Agropecuario S.A. de C.V.

	Implementierung von verschiedenen mikrobiologischen Untersuchungsmethoden für Tierfutter, Wasser und Samenkörnern für die Landwirtschaftsindustrie. Chemische Untersuchungen an Rohstoffen für die Bäckerei Industrie und statistische Auswertung zur Qualitätskontrolle. Einführung analytischer Methoden, u.a. Extraktionsmethoden für Pflanzen. Phytochemie.
TU-Braunschweig	Mai 2003-Juni 2004. Wissenschaftliche Mitarbeiterin
	Juli-Oktober 2006. Wissenschaftliche Mitarbeiterin
Elternzeit	Juni 2004 bis Juni 2006