The Biosynthesis of Sesquiterpene Lactones in Chicory (*Cichorium intybus* L.) Roots

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
prof. dr. ir. L. Speelman
in het openbaar te verdedigen
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The Biosynthesis of Sesquiterpene Lactones in Chicory (*Cichorium intybus* L.) Roots

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1. De detectie van germacreen B als belangrijkst 'nevenproduct' roept de vraag op of het δ–selineen synthase uit *Abies grandis* gezien de gebruikte assay-methode niet beter een germacreen B synthase genoemd had kunnen worden.
   Steele et al., J Biol Chem 273: 2078-2089 (1998). Dit proefschrift Hoofdstuk 2 en 5.

2. De specificiteit van een 'specifieke inhibitor' valt nogal eens tegen.
   Dit proefschrift Hoofdstuk 3.

3. Het gebruik van hexaan of pentaan als oplosmiddel voor het substraat bij reacties gekatalyseerd door een cytochroom P450 enzym gaat voorbij aan het feit dat het hierbij een membraangebonden enzym betreft.
   Bijvoorbeeld: *Funk and Croteau,* Arch Biochem Biophys 308: 258-266 (1994). *Lupien et al.,* Arch Biochem Biophys 386: 181-192 (1999). Dit proefschrift Hoofdstuk 6.

4. Het gen MAM1 dat codeert voor een actief 2-(2'-methylthio)ethylmalaat synthase lijkt in het verkeerde ecotype van Arabidopsis aanwezig te zijn.
   Kroyman et al., Plant Physiol 127: 1-12 (2001).

5. De absorptie bij 590 nm van een aceton extract zoals beschreven in de "Deutscher Arzneimittel Codex" is geen goede maat voor de anti-depressieve werking van een *Hypericum perforatum* (Sint-Janskruid) preparaat.
   Wagner en Bladt, J Geriatr Psychiatry Neurol 7: S65-S68 (1994).

6. Het pleiten voor volledig Engelstalig universitair onderwijs in Nederland staat in scherp contrast tot de verbeten gevoerde strijd voor een Nederlandstalige universiteit bij onze Zuiderburen.

7. De roep van managers om een projectnummer overstemt te vaak de inhoud van het wetenschappelijk onderzoek.

8. Het verdrinken van problemen heeft weinig zin als ze in de loop van de tijd hebben leren zwemmen.
   Tom Manders (beter bekend als Dorus)

Stellingen behorend bij het proefschrift: The Biosynthesis of Sesquiterpene Lactones in Chicory (*Cichorium intybus* L.) Roots.

Jan-Willem de Kraker, 20 november 2001

Voorwoord — Preface

De afgelopen vijf jaar was het voor mij witlof, dag in dag uit. Waarschijnlijk is dit ook de reden dat ik al ruim 3 jaar geen witlof meer kan ruiken, laat staan eet. Als je dan ongeacht waar je in Europa bent overal in de wegberm uitsluitend blauwe bloemen ziet opduiken, is het wel goed en verstandig er eens een punt achter te zetten. Hoewel het eind van dit promotie-onderzoek door al het 'wachtgeldgedoe' en bijbehorende 'dode mussen' wel erg abrupt en weinig chique kwam, kan ik me nu met goed gevoel weer eens op wat anders storten.

Dat anders blijft dus wel onderzoek, want het genot van een beetje prutsen en prullen met plantenprakjes is gebleven. Het uitzoeken van hoe die bitterstoffen nu in elkaar gezet worden door die witlofwortel, viel het best te vergelijken met het oplossen van een legpuzzel. Hoewel sommige puzzelstukjes er ruim een jaar over deden, kwam ik toch stap voor stap verder met die puzzel. Uiteindelijk kwam die ook nog verder af dan dat ik ooit had durven hopen, een èchte lactonring zomaar uit het niets. Ondanks die grote PUNT die nu gezet gaat worden, zou ik stiekem nog wel eens willen kijken hoe alle gevonden biosynthesestapjes uiteindelijk uitkomen op die bitterstoffen van witlof.

Het belangrijkste ingrediënt voor dit proefschrift lijkt misschien die witlofwortels, maar zonder collega's en vrienden sta je nergens. Het aandragen van ideeën of begrijpen van wat ik zoal uitspookte werd weliswaar moeilijker naarmate het AIO-project vorderde, desalniettemin was er altijd een gewillig oor op momenten dat het echt even niet meer ging. Het belang van zomaar leuteren over koetjes en kalfjes wordt sowieso te vaak over het hoofd gezien.

Allereerst wil ik mijn promotor Aede de Groot bedanken, voor wie ik misschien wel erg ver verstopt zat daar ergens onderdaan de berg. Je hebt me er in ieder geval heerlijk m'n gang laten gaan, maar tegelijkertijd wist je bij het schrijven van een artikel en later het proefschrift toch weer net de kernpunten aan te geven op plaatsen waar deze dreigden onder te sneeuwen. Ook de gedachtewisseling hoe je nu zo'n proefschrift in elkaar zet was erg nuttig, hoewel het kort-maar-krachtig principe misschien nog niet overal even goed tot zijn recht is gekomen.

De belangrijkste co-factoren waren natuurlijk Maurice Franssen en Harro Bouwmeester (in volstrekt willekeurige volgorde), die beiden op geheel eigen en totaal verschillende wijze invulling aan het project probeerden te geven. Harro is de eeuwige optimist en absolute kampioen "luchtfietsen". Zag ik het weer helemaal niet zitten, dan was er tenminste nog jouw enthousiasme. Bedenk echter Aken en Keulen zijn niet in één dag gebouwd, volgens mij heb ik dan uiteindelijk nog geen kwart van de vrijdagmiddagexperimenten uitgevoerd die jij zoal in je hoofd had. Jammer dat spontaan overleg steeds meer gekanaliseerd werd in werkbesprekingen op momenten dat ik toch echt op het lab wilde staan. Maurice was over het algemeen aangaande experimenten heel wat realistischer, en dat van die door cytochroom P450's gekatalyseerde omzettingen van willekeurige sesquiterpenen bleek uiteindelijk geeneens 'luchtfietserij' te zijn. Ondanks die ietswat pedante nakijksnelheid (mag een AIO ook eens effe bijkomen), wist je me te behoeden voor heel wat Bouwmeesterzinnen (van die kommaconstructen van een half A4'tje). Mijn hantering van het begrip "lactonisering" (handig tòch, vijf reacties één woord) wist je aardig in te perken. Desalniettemin resulteerde de optelsom van vier auteurs volgens een referent van Phytochemistry in een "awkward English". De ster was uiteindelijk voor Maurice, maar de AIO werd gekaapt door Harro.

Zonder mensen die verstand hebben van apparaten en lab gaat natuurlijk alles mis. Maurice Konings bedankt voor het inleidende eerste jaar en tolereren van de chaos die door "die studenten van de LU" nu eenmaal wordt gemaakt (waar gehakt wordt, vallen spaanders). Als ongewild komisch duo op vrijdagmiddag met Harro was je onvergetelijk (wié had nu ook alweer hóé de buizen genummerd ??). Francel Verstappen was op papier de "strenge" labbeheerder gedurende het laatste anderhalf jaar, maar in de praktijk was mijn rondslingerende afwas altijd nog wat kleiner. Wat heb ik (achteraf) veel plezier beleefd aan onze gemeenschappelijke projectnummerallergie alsook aan de oplossing van het mysterie van de glasmagazijnvretende glasbak uit paviljoen 122. Jij en Jack Davies bedankt voor het in de lucht houden van de GC-MS. Beste mensen van Plantenfysiologie (ex AB-DLO), bibliotheek en celcybernetica (PRI), ik kan echt niet iedereen kwijt in dit voorwoord, maar ik heb jullie hulp, interesse, ideeën en koffie+borrelpraat zeer gewaardeerd.

Op Organische Chemie mag Maarten Posthumus niet worden vergeten, die minutieus zowel de costus-olie alsook die schoenpoets (resinoid) wist te analyseren. Daarnaast was er Bep van Veldhuizen voor het opnemen van NMR-spectra van die zo schaarse milligrammen.

Dankzij het actieve zieltjes winnen door Maurice is er ook bergen werk verzet door studenten. De eerste was meteen twee koppen groter dan ik, en dronk bovendien alleen thee. Co, helaas is dat germacreen B synthase nooit meer opgeschreven, maar jouw ideeën en proto-

collen zitten overal in dit proefschrift verborgen. Je silica-schepjes zijn nog steeds in gebruik, misschien hadden we ze moeten patenteren. Ciska, je wist me elke ochtend weer feilloos op de hoogte te brengen van de laatste trend in welriekende theevariëteiten. Jouw werk is terug te vinden in hoofstuk 3 en 6. Maaike, de eerste proef was direct bingo. Sommige mensen hebben het gewoon, hoewel het schrijven je misschien iets minder makkelijk afging. Hoofdstuk 5 komt voor een groot deel van jou af. Tenslotte Marloes, jij was echt niet te stuiten in al je werklust en enthousiasme. Soms moest er subtiel zelfs een beetje de rem op worden gezet. Met de combinatie van stage en afstudeervak hield je het bijna een jaar vol en leek stilletjes het hele lab (inclusief GC-MS) over te nemen. Wat ik er nog toe deed, was me af en toe een raadsel. Ik hoop dat je als AIO net zo goed je ei kwijt kunt als is gebeurd in (jouw) hoofdstuk 6. Dan was er nog een hele rij slachtoffers van die notoire 'takkenplant' *Artemisia annua*. Officieel hoorden jullie bij Harro, de praktijk was vaak anders. Michiel, Bert (v. Loo), Robert, Vitor, Hester, Lars, en Bert ([Janssen] de uitzondering met zonnige bloemen), jullie hielden het leven in de brouwerij uitstekend op peil.

Van groot belang waren de collega-AIO/OIO's van zowel PRI als Organisch, al was het maar voor de AIO-praat (gedeelde smart is halve smart). Corine en Joke, als medebewoners van de voormalige AIO-kamer zijn jullie zijn waarschijnlijk diegenen die het best weten hoe interactief ik met een computer omga. Ondanks alle ups en downs, was het erg gezellig. Anja en Iris (Kappers), OIO-oma's jullie quote staat: "dat proefschrift komt er toch wel !". Claus, heerlijk dat eeuwig cynisme, maar dat van "simply no job, no future" viel uiteindelijk toch nog wel mee. En tot slot nog een vraag aan Iris (Altug): "Hoe doe je dat toch, elke dag weer het-zonnetje-in-het-lab ? Wat is je geheim ?"

Het AIO-zijn is ook iets wat zich chronisch NIET aan werktijden houdt en maar al te vaak de tijd opslorpt die eigenlijk bedoeld was voor meer wereldse zaken. Debby, Jan, Ton, Inge, Jeroen, Bartel en Willem, alsook andere ex-Rijnsteeg-5C'rs, jullie wisten me nog net te behoeden voor de totale transformatie tot 'super-nerd'. Het onderdeel ontspanning was bij jullie in goede handen: koken+eten, 'terrassen', bioscoop, theater, wandelen, museum, de reservevoorraad wijn/port van een paar straten verderop. Ik hoop dat de afstand naar Thüringen niet te groot zal zijn. Tja, dat "Hotel de Kraker" ergens in het zonnige Zuiden (sol, vino y playa) komt nog wel een keer.

Tenslotte mams, bedankt dat je me altijd gestimuleerd hebt vooral datgene te studeren wat ik zelf wilde. De enige keuze die je me ooit hebt opgedrongen, dat vreselijke Engels, was misschien toch nog niet zo'n slechte.

Although the major part of this preface is in Dutch, some words in English should be added. Clearly, parts of this thesis would not have existed without the collaboration of people from outside the Netherlands, scientists that I —unfortunately— sometimes only know by E-mail. Prof. W.A. König (Hamburg University, Germany) thank you for making me aware of the fact that germacrene A can be detected in GC-measurements as a genuine peak and for the donation of all the different sesquiterpene standards. Dr. B. Maurer (Firmenich SA, Geneva, Switzerland) was so kind to provide a sample elemene alcohol that was, remarkably, still available after its storage in the freezer since 1977. Your fascinating article about all the different sesquiterpenoids present in costus root oil made me realise that the so desperately needed germacrenes were likely to exist in costus roots. These roots with a somewhat peculiar smell could be freshly obtained via the helpful Dr. T. Shibata (Hokkaido Experimental Station for Medicinal Plants, Japan) who had already been cultivating them for several years. Finally, there are the people that were so kind to send me standards of sesquiterpene lactones: Prof. M. Ando (Niigata University, Japan), Dr. Shi Yong Ryu (Korea Research Institute of Chemical Technology, Korea), and Prof. Y. Asakawa (Tokushima Bunri University, Japan). It was a pleasure working together with all of you, despite the distances, and to benefit from your investigations and knowledge.

No por mucho madrugar,

amanece más temprano

Voor papa

De suikerij (Cichorium intybus) is eene plant oorspronkelijkuit onze streken; ze behoort tot de familie der samengesteld-bloemigen (composeeën) en is min of meer voortlevend, doch maar tweejarig in teelt.

Onder de suikerij, die men teelt voor de koffie of de zoogezegde bitterpeeën, onderscheidt men twee voorname soorten :

a) DE BRUINWIJKSCHE, die groote, dikke wortels vormen en waarvan de bladeren zich bijna vlak op den grond openspreiden, getand zijn en eene roode middennerf aanbieden. Op sommige plaatsen noemt men ze angelbladeren ;

b) DE MAAGDEBURGSCHE. Deze vormt ook groote wortels doch de bladeren zijn niet ingesneden, staan bijna recht omhoog en de middennerf is hier wit.

't Is uit deze laatste soort dat de Schaarbeeksche groenten-kweekers, door gedurige verbeteringen, er eindelijk in gelukt zijn het zoogezegde Brusselsch Witloof te bekomen, hetwelk zij nog degelijks trachten te verbeteren om nog meer volmaakte uitslagen te bekomen.

De bleeke bladeren dezer plant worden als groente gebruikt en kunnen zich op twee manieren voordoen :

a) In kroppen zooals de teelt in 't groot voor den handel zich altijd voordoet of

b) In losse bladeren die men soms bij liefhebbers vindt, teelt, die nochtans oneindig minder gedaan wordt dan de eerste.

De teelt in haar eigen is nog al gemakkelijk, maar een der hoofdzaken tot goed gelukken is het goed zaad te beschikken ; dit laat bij beginnelingen gewoonlijk te wenschen.

(De teelt van Witloof in woord en beeld, de Backer P, 1918)

Contents

1. Introduction

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GENERAL

Chicory

The botanical name of chicory *Cichorium intybus* presumably refers to the occurrence of this plant at roadsides (*kio* = go and *chorion* = field) and to its hollow stalk (*tubus* = pipe). The wild form (Fig. 1) (var. *sylvestre*) of this 1.5 m high Asteraceae has spread from the Mediterranean all over the world. It is a perennial that passes the winter in dormancy after senescence of the aerial parts. Most characteristic of chicory are the blue flowers that are only open in the morning and start to bloom two weeks after the longest day. These flowers have appealed to peoples imagination in various ways throughout history. In ancient times the plant was used as a medicine against eye-diseases, poisoning and stomach disorders. The Teutons used it as a magic plant to make themselves inviolable, after which the Catholic Church christianised it into a symbol of Assumption of the Virgin. Nowadays, chicory is cultivated for its tap roots and the etiolated sprouts that develop on these roots in the dark. (Weeda et al., 1991; Vogel et al., 1996).

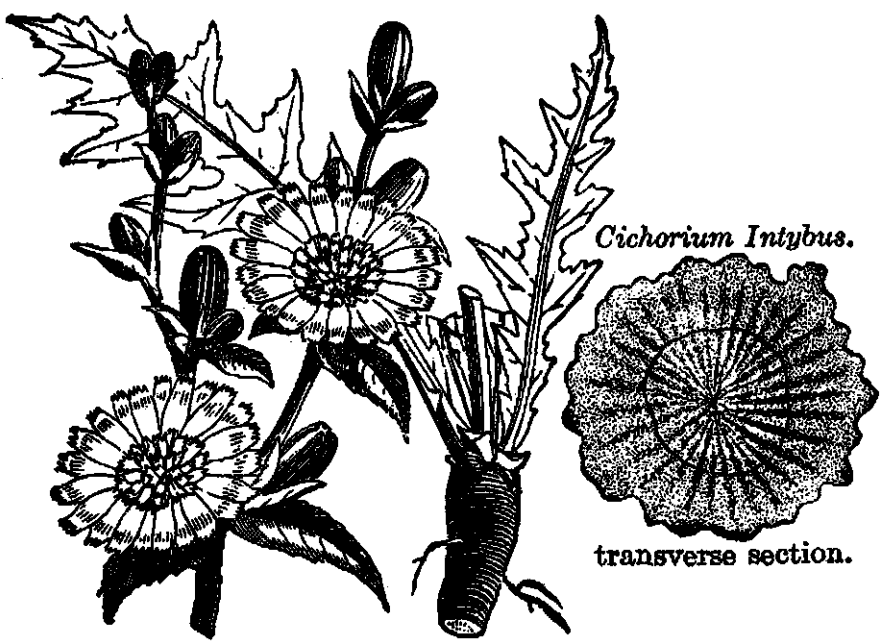

Figure 1. Pen and ink drawing of chicory (source: www.asap.unimelb.edu.au/hstm/data/184.htm).

*Uses of Chicory Roots (*in Dutch *Cichorei)*

From the seventeenth century on, chicory was cultivated for its bitter roots (var. *sativum* DC) that were roasted and used in hot "coffee-like" beverages. Because of warfare and blockades, the prices of genuine coffee from *Coffea arabica* rose dramatically in the eighteenth century and its replacement by roasted chicory became popular, whereas similar attempts with roasted rye and acorns were less successful. Prices of coffee remained high after the death of Napoleon throughout the nineteenth century, and in 1882 in Prussia alone 130 factories were in operation that together processed 200.000 tons of fresh chicory roots. Nowadays this type of industry has mostly disappeared in western Europe, and the last roasting house for chicory in the Netherlands closed in 1968 at Kloosterburen (Vogel et al., 1996; Westerdijk, 2000).

In Belgium cultivation of chicory for the production of coffee substitute was still continued until the eighties of the last century, but at that time it was not profitable any more due to the cheaper production in eastern Europe. An alternative use was sought and found. Together with dahlia (*Dahlia variabilis*) and Jerusalem artichoke (*Heliantus tuberosus*), chicory is one of the few plant species that contains high profitable amounts of inulin in its roots (19% of fresh weight). This reserve carbohydrate is a fructose polymer terminated by a glucose with a polymerisation degree of 2-30. Inulin can be used for the production of fructose and 5-hydroxymethylfurfural (HMF), a building block for the polymer industry, but has also been acknowledged as a dietary fibre because it is not digested by man. In 1992 a factory for the processing of inulin from chicory roots was opened in Roosendaal and the cultivation of this variety of chicory was reintroduced in The Netherlands (Westerdijk,1996; Westerdijk, 2000).

*Belgian Endive (*in Dutch *Witlof)*

A different type of chicory cultivation started around 1850 in the triangle Brussels-Leuven-Mechelen. The winter-carrot like roots of chicory (var. *foliosum* Hegi) were, after harvest in August-September, removed from their foliage and embedded in beds. The roots were covered with hotbed manure and a few weeks later white chicons/sprouts developed. After digging them out, the slightly bitter tasting chicons were traded as Belgian endive. The roots were regarded as a waste product. Export of Belgian endive to Paris started in 1875 and twenty-two years later already amounted up to 1500 tons (Vogel et al., 1996; Kruistum, 1997).

The cultivation of Belgian endive in France and The Netherlands started around the First World War. Ever since, much has changed and Belgian endive is not any more a typical winter vegetable owing to post-harvest storage of the roots in cold warehouses. The roots are no longer forced in hotbeds, but placed upright in movable water-tight trays. These trays are placed in the dark in climate chambers and a nutrient solution is forced through the trays Peters, 1996; Vogel et al., 1996; Kruistum, 1997). The major producers of Belgian endive in the world are France, Belgium and The Netherlands, they accounted for 98% of the 455400 tons produced in Europe in 1991/1992 (Vogel et al., 1996).

Closely related to Belgian endive are endive (*Cichorium endivia* L.) and radicchio (*Cichorium intybus* L. var. *foliosum*), vegetables that also have a slightly bitter taste but are grown in daylight (Vogel et al., 1996; Kruistum, 1997).

Sesquiterpene Lactones

Bitter Taste of Chicory

The bitter taste of chicory is due to the presence of sesquiterpene lactones. The major sesquiterpene lactones of Belgian endive are the guaianolides lactucin (Fig. 2, **1**) 8-deoxylactucin (**2**) and lactucopicrin (**3**), and these compounds also cause the, slight, bitterness of lettuce (*Lactuca sativa*), endive and radicchio (Holzer and Zinke, 1953; Rees et al., 1985; Pyrek 1985; van Beek et al., 1990; Price et al., 1990). Coumarins may to some extent contribute to the bitter taste of chicory as well, whereas it is less certain that the bitter taste of roasted chicory roots is also due to sesquiterpene lactones since these compounds are assumed to be degraded during the roasting process (Leclercq, 1992).

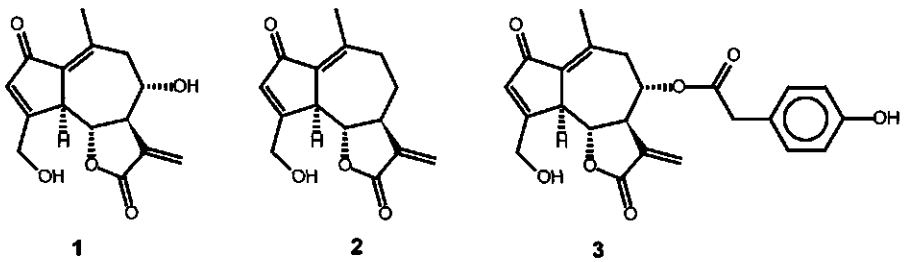

Figure 2. Major sesquiterpene lactones of chicory, the guaianolides lactucin (**1**) 8-deoxylactucin (**2**) and lactucopicrin (**3**).

Small amounts of the dihydro derivatives of these guaianolides, 11(*S*),13-dihydrolactucin (Fig. 3, [**4**]), 11(*S*),13-dihydro-8-deoxylactucin (**5**) and 11(*S*),13-dihydrolactucopicrin (**6**), are

reported as well. Other sesquiterpene lactones known to be present in chicory are the eudesmanolides sonchuside C (**8**) and cichoriolide A (**9**), and the germacranolides sonchuside A (**10**) and cichorioside C (**11**) (Seto et al., 1988; van Beek et al., 1990; Leclercq, 1992). The most bitter tasting sesquiterpene lactones isolated from chicory are 11(*S*),13-dihydrolactucopicrin (**6**) and lactucopicrin (**3**) with threshold concentrations of 0.2 and 0.5 ppm (w/w) respectively, whereas lactucin with a threshold value of 1.7 ppm is the least bitter one (Van Beek et al., 1990; Leclercq 1992).

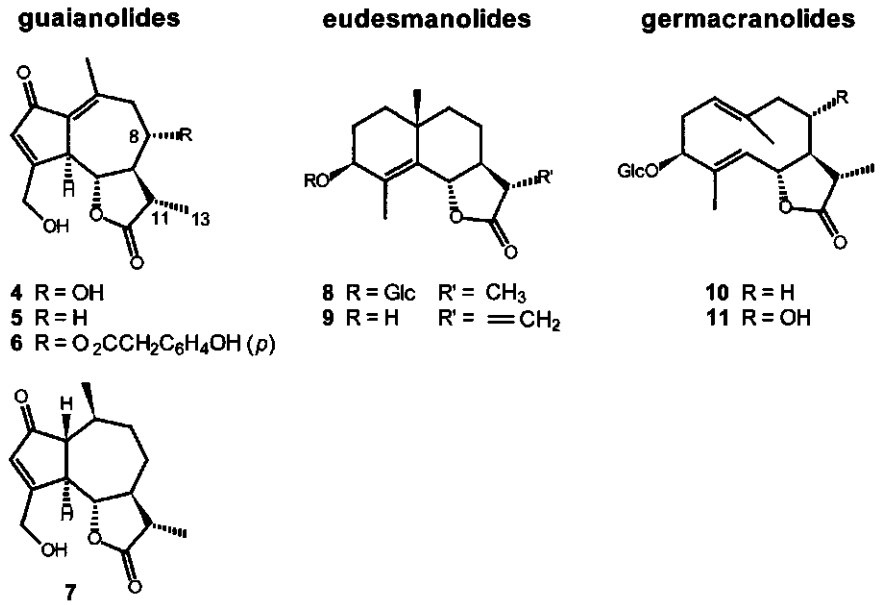

Figure 3. Minor sesquiterpene lactones of chicory: the guaianolides 11(*S*),13-dihydrolactucin (**4**), 11(*S*),13-dihydro-8-deoxylactucin (**5**), 11(*S*),13-dihydrolactucopicrin, (**6**) and cichoralexin (**7**), which is induced by bacterial infection; the eudesmanolides sonchuside C (**8**) and cichoriolide A (**9**), and the germacranolides sonchuside A (**10**) and cichorioside C (**11**).

The defensive function of sesquiterpene lactones in chicory is well illustrated by cichoralexin (**7**), a guaianolide that is only present in chicory when it has been inoculated with the bacterium *Pseudomonas cichorii*. Cichoralexin inhibits completely the conidial germination of *Bipolaris leersiae*. The antifungal activity ascribed to this compound was detected in the plant at the first day of infection and increased to a maximum at the third day after which it declined (Monde et al., 1990). Lactucin (**1**), 8-deoxylactucin (**2**) and lactucopicrin (**3**) occur throughout the plant, but the highest levels of these substances are to be found in those parts of the plant which are of greatest importance at any particular time,

i.e. the most actively growing tissues. Total sesquiterpene lactone levels vary from 0.11 to 0.81% dry weight in the roots and from 0.06 to 0.45% dry weight in the leaves. Together with cichoriin, a coumarin that is present in lower levels, they act as feeding deterrent towards insects (Rees and Harborne, 1985).

The sesquiterpene lactones of chicory are located in a somewhat sticky triterpene-rich latex that might increase their repellence, as the latex prevents some kinds of insect feeders such as lepidopterous leaf miners from burrowing within the leave (Holzer and Zinke, 1953; González, 1977; Rees and Harborne, 1985; Genderen and Schoonhoven, 1996). Latex is the milky content of laticifers, tubular structures within the plant that originate from files of cells that develop cell wall perforations which allow the protoplasmic content of adjacent cells to merge. Laticifers contain the nuclei and organels from the cells they originate from and maintain cellular activity for a period of time after cell wall breakdown (Olson et al., 1969; Vertrees and Mahlberg, 1975; Vertrees and Mahlberg, 1978).

Occurrence and Structures throughout the Plant Kingdom

Sesquiterpene lactones are a major class of plant secondary metabolites and over 4000 different structures have been elucidated (Song et al., 1995). They may constitute up to 5% of the dry weight of the plant and occur mainly in the Asteraceae, the largest of all plant families, but infrequently also occur in other higher plant families and lower plants. Sesquiterpene lactones can be found throughout a plant, but are most commonly located in glandular hairs on leaves and stems of a plant (Fischer, 1991; Seigler, 1998). However, chicory does not have secretory glandular hairs or schizogenous ducts and instead the sesquiterpene lactones are present in the latex as mentioned above (Mabry and Bohlmann 1977; González, 1977; Fischer, 1991).

The large number of skeletal types of sesquiterpenoids in general is contrasted by a relatively small group of different carbocyclic skeletons among the sesquiterpene lactones. Their classification is based on their carbocyclic skeleton in which the suffix "olide" refers to the lactone function. Germacranolides are, by far, the most common type. They generally contain a *trans,trans*-cyclodecadiene system, but helangiolides that have a 1(10)-*trans,* 4-*cis* ring system and melampolides that have a 1(10)-*cis*, 4-*trans* ring system are not uncommon, whereas few *cis, cis*-germacranolides are known. The majority of sesquiterpene lactones are considered to be derived from germacrane precursors, and biomimetic transformations have led to the generally accepted biogenetic pathways such as depicted in figure 4. Either before or —most likely— after formation of the lactone ring, the germacrene precursor undergoes a

variety of cyclisations, ring fissions, and methyl migrations to yield other skeletal types of sesquiterpene lactones. Lactones grouped together in the same vertical column are produced, at least superficially, by the same number in changes of the carbon skeleton present in the germacranolide, even though individual members of a particular class may differ widely in oxidation state at various sites in the molecule (Rodriguez, 1976; Herz, 1977; Fischer et al., 1977; Fischer et al, 1979; Seaman, 1982; Fischer, 1990; Fischer, 1991).

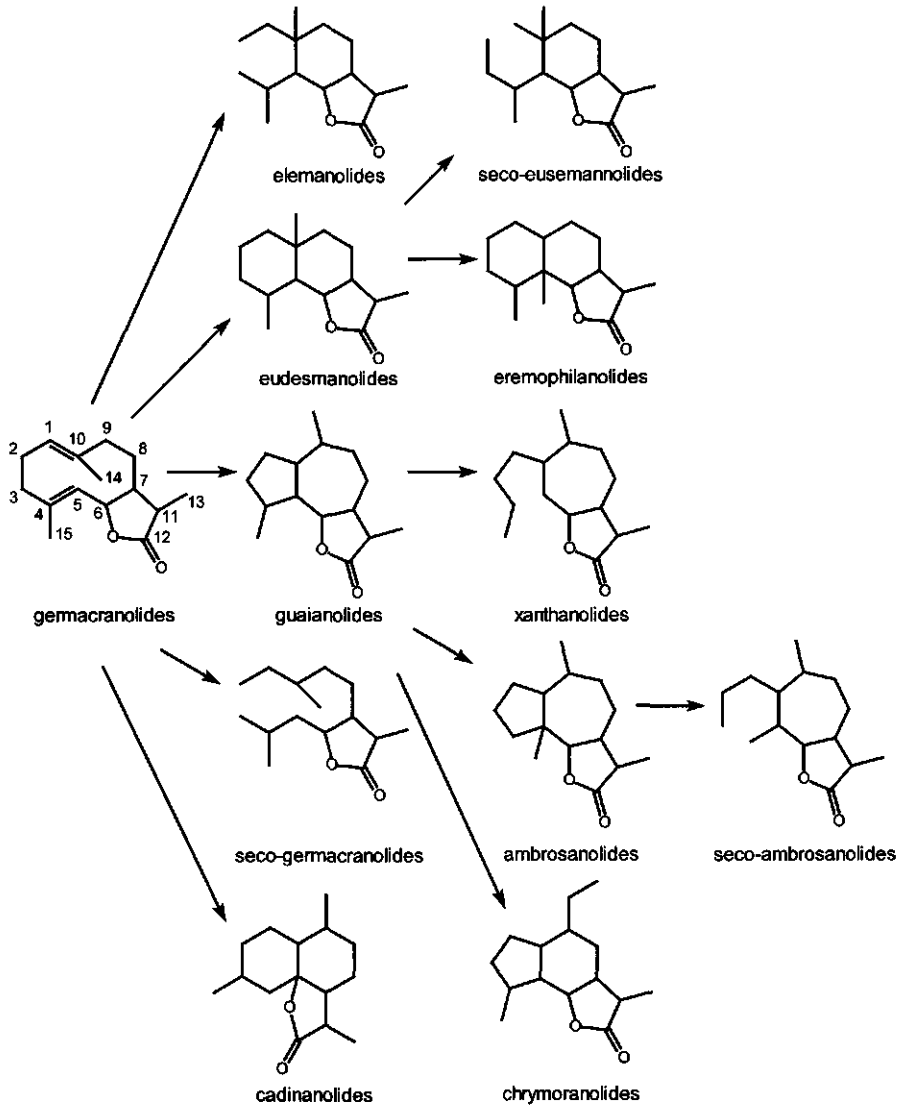

Figure 4. Types and biogenetic relationships of germacranolide-derived 6,7-lactones (after Rodriguez [1976], Herz [1977], and Fischer et al. [1979]).

The deceptive simplicity of the scheme in Figure 4 may also lead to misinterpretation. For example, some of the elemanolides appear to be artefacts formed as the result of the Cope rearrangement of a germacradienolide during work-up of certain plant extracts, notwithstanding the discovery of more complex elemanolides which may indicate the existence of a biological equivalent of the Cope rearrangement. Moreover, the proposed biosynthetic routes to the various classes are based on inference rather than on actual experiments and significant biosynthetic differences may reveal themselves in only minor structural alterations (Herz, 1977).

For simplicity, the smaller group of sesquiterpene lactones that contains a 7,8-lactone ring has not been included in Figure 4, but a similar scheme can be made for these types of compounds. Only few sesquiterpene lactones will not fit in either of these schemes. Sesquiterpene lactones isolated from higher plants contain, without exception, α-methylene-γ-lactone groups in which H-7 is α-oriented. However, certain liverworts (Hepaticae) produce sesquiterpene lactones of the enantiomeric series. A majority of the lactone groups is *trans*-fused to the C_6-C_7 or C_7-C_8 positions of the carbocyclic skeleton, and in many sesquiterpene lactones the exocyclic C_{11}-C_{13} bond is unsaturated (Herz, 1977; Fischer et al., 1977; Fischer et al., 1979; Fischer, 1990; Seigler, 1998).

Biological Activity

Sesquiterpene lactones display a wide variety of activities against numerous types of organisms and play an important role in the defence of plants against pathogens, herbivorous insects and mammals, and in the competition with other plants (Rodriguez et al., 1976; Picman, 1986; Fischer, 1991; Seigler 1998). These biological activities are in general associated with the presence of the C_{11}-C_{13} exocyclic double bond conjugated to the γ-lactone which might conjugate with sulphydryl groups of proteins by a "Michael-type" addition. The presence of a functional group, such as an epoxide, hydroxyl, chlorohydrin, unsaturated ketone or *O*-acyl, adjacent to this exocyclic double bond might further enhance the reactivity of the conjugated lactone toward biological nucleophiles (Kupchan et al., 1970; Rodriguez et al., 1976; Fischer, 1991; Seigler, 1998).

The sensation of bitter taste requires an interaction between the bitter-tasting compound and a receptor cell on the tongue, a process that is not well understood for sesquiterpene lactones yet. A single lactone group in a sesquiterpene seems to suffice for bitter taste. However, the bitter taste of sesquiterpene lactones can also be due to the presence of more or less polar moieties in the structure possibly in combination with lipophilic centres (Leclercq,

1992; Peters, 1996). The C_{11}-C_{13} exocyclic double bond is clearly not involved in bitterness, since 11(*S*),13-dihydrolactucopicrin (**6**) is the strongest bitter tasting sesquiterpene lactone isolated from chicory (van Beek et al., 1990).

Structure activity studies have demonstrated that enzymes containing essential thiol groups, such as phospho-fructokinase, glycogen synthase, DNA polymerase, and thymidylate synthase, are inhibited by sesquiterpene lactones. Moreover, sesquiterpene lactones can directly inhibit DNA synthesis, most likely by interfering with the DNA-template. Both the effect on DNA synthesis and the inhibitory effect on thiol-containing enzymes might explain the cytotoxic and anti-tumour activities of sesquiterpene lactones (Rodriguez et al., 1976; Picman, 1986; Fischer, 1991; Seigler, 1998).

In spite of the reported anti-tumour activities of sesquiterpene lactones and intensive screening of several hundreds of compounds by the U.S. National Cancer Institute, none of them reached clinical testing mainly due to their toxic properties (Fischer, 1990; Fischer, 1991). Currently, the anti-malarial drug artemisinin is the only sesquiterpene lactone used in regular western medicine (Seigler, 1998; Bouwmeester et al., 1999). In addition, parthenolide —a germacranolide 4,5-epoxide— is the supposed active component in commercial preparations of feverfew (*Tanacetum parthenium*) that are available as anti-migraine drug through pharmacies and health stores in England (Robles et al., 1994). Nevertheless, the activity of medicinal plants used in traditional medicine is often attributed to the presence of sesquiterpene lactones. A well-known example is the latex of wild lettuce (*Lactuca virosa*; in Dutch gifsla) that contains high amounts of lactucin (**1**) and was used in cough syrup and as a sedative (Fischer, 1991; Genderen and Schoonhoven, 1998). A reason for the infrequent use of sesquiterpene lactones in medicine might be that, apart from their toxicity, they are infamous for causing allergic contact dermatitis and respiratory allergies. This has become a well known problem to people that work professionally in the cultivation of chicory and to forest-workers who handle the bark of trees that houses the tiny epiphytic liverworts of the genus *Frullania* (Rodriguez et al., 1976; Picman, 1986; Genderen and Schoonhoven, 1998; Seigler, 1998).

BIOSYNTHESIS OF SESQUITERPENE LACTONES

Hypothetical Pathway

Despite the wealth of information on the structural aspects and the biological activities of sesquiterpene lactones, little is known about their biosynthesis. The majority of sesquiterpene lactones are thought to evolve from the germacranolides, and the simplest member of this group, (+)-costunolide, is considered to be the common intermediate of all germacranolide-derived lactones with a 6,7-lactone ring. Various authors have proposed a pathway for the biosynthesis of (+)-costunolide, as depicted in figure 5 (Geissman, 1973; Herz, 1977; Fischer et al., 1979; Fischer, 1990).

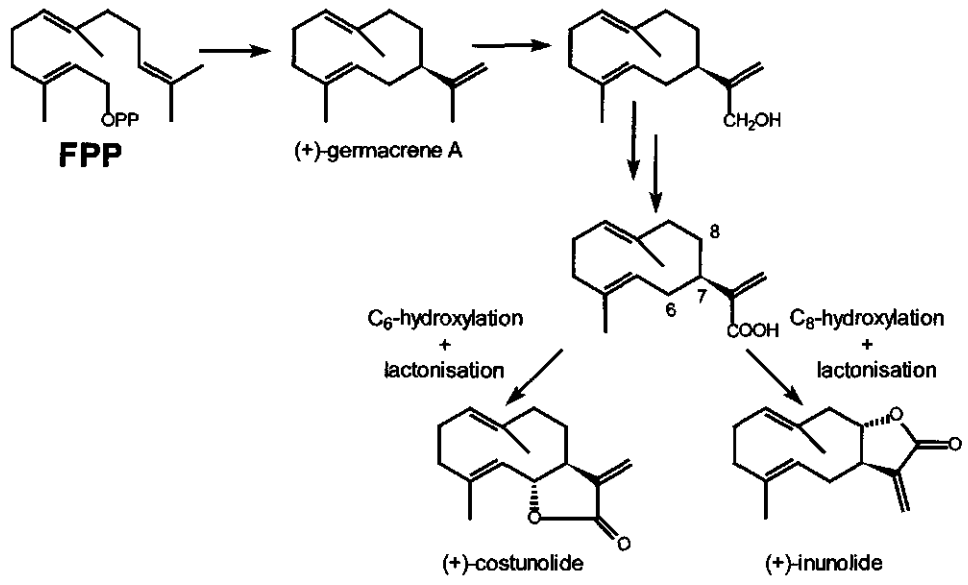

Figure 5. Proposed pathway for biosynthesis of (+)-costunolide and (+)-inunolide (after Fisher et al. [1979]; stereochemistry of (+)-inunolide according to Buckingham [2001]).

In this hypothetical pathway farnesyl diphosphate (FPP), the common precursor of all sesquiterpenoids, is cyclised to the germacrene framework of germacrene A, or alternatively germacrene B (see Fig. 7). Germacrene A would be hydroxylated in the isopropenyl side chain and further oxidised to form germacrene carboxylic acid. Hydroxylation of germacrene carboxylic acid at the C_6-position and subsequent lactonisation would result in formation of (+)-costunolide, whereas hydroxylation at the C_8-position would lead to (+)-inunolide, a

possible intermediate of sesquiterpene lactones with a 7,8-lactone ring. The postulated pathway for (+)-costunolide has mainly been based on the isolation of Cope rearrangement and cyclisation products of the involved germacrene intermediates from *Saussurea lappa* (for details see Chapter 4), a plant whose roots are particularly rich in (+)-costunolide. There are however no direct biochemical data (e.g. isolation of enzyme activities) that support this postulated pathway (Geissman, 1973; Herz, 1977; Fischer, 1979; Seaman, 1982; Fischer, 1990).

Also, it has to be emphasised that neither (+)-costunolide nor the germacrene-related volatile sesquiterpenes of *Saussurea lappa* have ever been reported for the well studied chicory roots (Mabry and Bohlmann, 1977; Sannai et al, 1982; Rees et al., 1982; Leclercq et al., 1992). The only volatile sesquiterpenes present in chicory are found in the headspace of flowers, viz. *trans*-caryophyllene and an unknown sesquiterpene olefin (Dobson, 1991).

Biosynthetic studies with a hairy-root culture of blue-flowered lettuce (*Lactuca floridana)* supplied with ^{13}C-labeled precursors of secondary plant metabolism (acetate and mevalonate) seem to confirm the acetate-mevalonate-FPP-germacradiene pathway for the guaianolide-type sesquiterpene lactones 8-deoxylactucin and 11(*S*),13-dihydrolactucin-8-*O*-acetate (Song et al., 1995). However, the labelling experiment gives no information about the exact sequence of oxidations involved in the biosynthesis of the lactone ring that may as well start at the C_6-position of germacrene A and not in the isopropenyl side chain. Neither does this experiment exclude the possibility that formation of the guaiane framework precedes formation of the lactone ring and that, as a consequence, (+)-costunolide is not at all involved in the biosynthesis of guaianolides.

Studies in the mid-nineties at our laboratory (Piet, 1996) have revealed that the carbon skeleton formed by cyclisation of a germacrene strongly depends on the structure of the concerned germacrene derivative. Through cyclisation of a series of model compounds using homogenised chicory roots as a catalyst, it was shown that eudesmanes are solely formed from germacrenes and germacrene 1,10-epoxides, whereas guaianes are only formed from 4,5-epoxides and 7-hydroxy-1(10),6-cyclodienes (Fig. 6) (Piet et al., 1995; Piet et al., 1996). The involvement of epoxy-germacranolides in formation of guaiane- and eudesmane lactones has often been suggested in literature and is based on biomimetic reactions: 4,5-epoxycostunolide (parthenolide) would lead to guaianolides, whereas 1,10-epoxycostunolide would lead to eudesmanolides (Fischer 1979; Seaman, 1982; Fischer, 1990; Teisseire, 1994; Song et al., 1995).

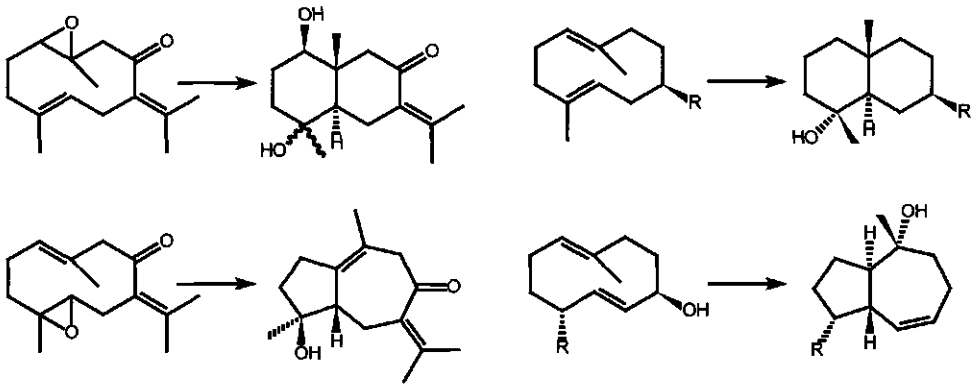

Figure 6. Cyclisation of model compounds by homogenised chicory roots as investigated by Piet (1996).

Enzymes of Sesquiterpene Lactone Biosynthesis

Biosynthesis of the Sesquiterpene Framework

The biosynthesis of sesquiterpene olefins from farnesyl diphosphate (FPP) is catalysed by sesquiterpene synthases. Most likely the same holds true for biosynthesis of the sesquiterpene framework present in sesquiterpene lactones, irrespective of the fact whether it involves a germacradiene or not. Since most sesquiterpenes are cyclic and many contain multiple ring structures, these enzymes are also referred to as sesquiterpene cyclases (Cane, 1990; Croteau et al., 2000).

Sesquiterpene synthases have been isolated from both plant and microbial sources, and they catalyse the formation of more than 200 different sesquiterpene skeletons. These enzymes are all moderately lipophilic and may be associated with the ER *in situ*, but are operationally soluble proteins of molecular weights in the range of 40-100 kD. Several sesquiterpene synthases are monomers of 40-60 kD, whereas at least two of them (trichodiene synthase and patchoulol synthase) are homodimers of 40-44 kD. Their pH optimum is generally within one unit of neutrality and they require no cofactor other than a divalent metal ion, Mg^{2+} usually being preferred. The K_m value for the acyclic substrate FPP is generally in the 0.5-10 μM range (Cane, 1990; Alonso and Croteau, 1993; McCaskill and Croteau, 1997).

The enzymatic cyclisation of FPP is initiated by ionisation of the diphosphate ester to generate an allylic carbocation that can be attacked by the distal double bond, yielding the macrocyclic *trans*-germacradienyl- or humulyl-cation (Fig. 7). The carbocation hence formed may undergo additional electrophilic cyclisations and rearrangements, including hydride

shifts and methyl migrations, before the reaction is terminated by deprotonation of the carbocation or the capture of a nucleophile (e.g. water) (Cane, 1990; McCaskill and Croteau, 1997; Croteau et al., 2000). Formation of germacrene A or germacrene B would only require deprotonation of the *trans*-germacradienyl cation (Fig. 7).

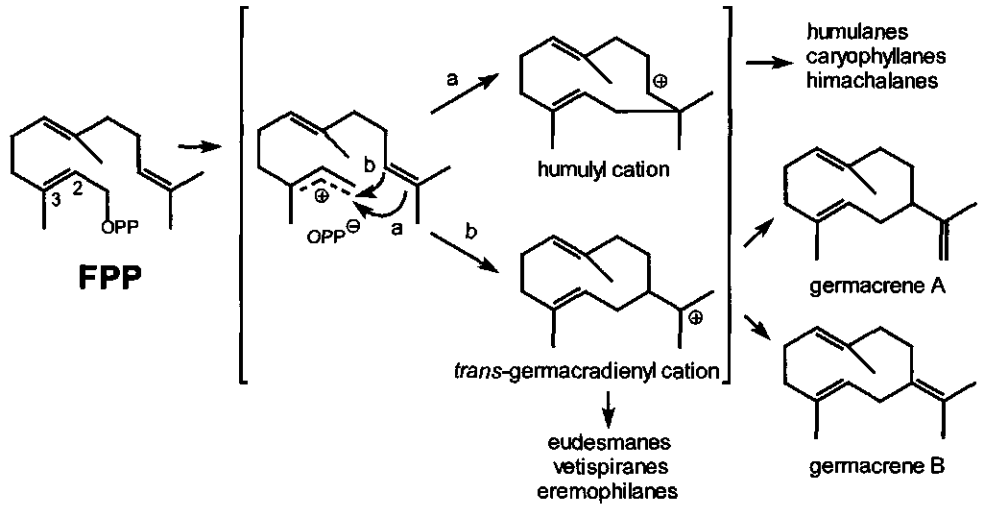

Figure 7. Cyclisation of farnesyl diphosphate (FPP) by sesquiterpene synthases via a humulyl or *trans*-germacradienyl cation.

Nevertheless, formation of many sesquiterpene skeletons is complicated by the fact that the *trans*-geometry of the 2,3-double bond of FPP prevents the formation of six-, seven-, or ten-membered rings with a *cis*-double bond. This stereochemical barrier to direct cyclisation is overcome by initial isomerisation in the catalytic site of the *trans,trans*-farnesyl diphosphate substrate to its corresponding tertiary allylic isomer, nerolidyl diphosphate (NPP). This intermediate has the appropriate reactivity and conformational flexibility to allow, after ionisation of the diphosphate ester, formation of either the *cis*-germacradienyl-, bisabolyl- or daucyl-cation (Fig. 8) (Cane, 1990; Teisseire, 1994; McCaskill and Croteau, 1997).

Whereas some sesquiterpene synthases produce exclusively one sesquiterpene, others may produce more than one sesquiterpene. Those involved in the production of conifer resin for example are capable of individually producing more than 25 different olefins (Croteau et al., 2000).

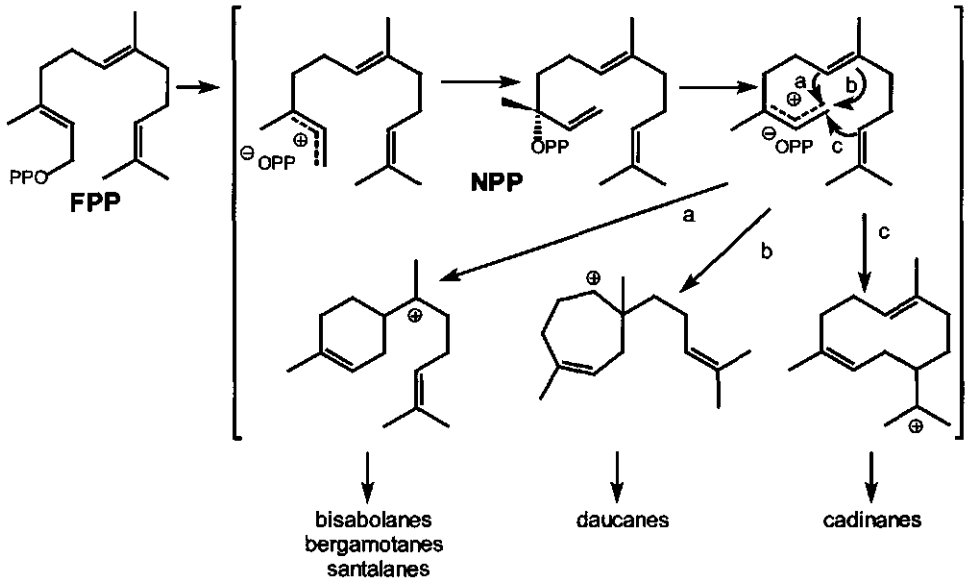

Figure 8. Cyclisation of FPP by sesquiterpene synthases via neryl diphosphate (NPP) to frameworks containing a *cis*-double bond.

Biosynthesis of the Lactone Ring

Biosynthesis of a lactone ring as present in sesquiterpene lactones involves oxidising enzymes, but on the whole little is known about the enzymes in plant secondary metabolism that oxidise sesquiterpenes. The few examples of oxidation of sesquiterpene olefins described in literature (Fig. 9) involve cytochrome P450 enzymes: i.e. the conversion of 5-*epi*-aristolochene into capsidiol in green pepper (*Capsicum annuum*) (Hoshino et al., 1995; Chávez-Moctezuma and Lozoya-Gloria, 1996) and into capsidiol and debneyol in tobacco (*Nicotiana tabacum*) (Whitehead et al., 1989), and the conversion of vetispiradiene into lubimin and related compounds in thornapple (*Datura stramonium*) and potato (*Solanum tuberosum*) (Coolbear and Threlfall, 1985; Whitehead et al., 1990).

Far more is known about the oxidising enzymes of monoterpene biosynthesis, in particular about those involved in the oxidation of limonene to the monoterpene alcohols and ketones which are present in mint oils (e.g. McConkey et al., 2000). Enzymatic hydroxylation of monoterpenes to their corresponding alcohols generally involves cytochrome P450 enzymes, whereas further oxidation to ketones/aldehydes is catalysed by $NAD(P)^+$-dependent dehydrogenases. Further transformations of the basic monoterpene skeleton may involve reduction, isomerisation, and conjugation reactions as well (Croteau et al., 2000).

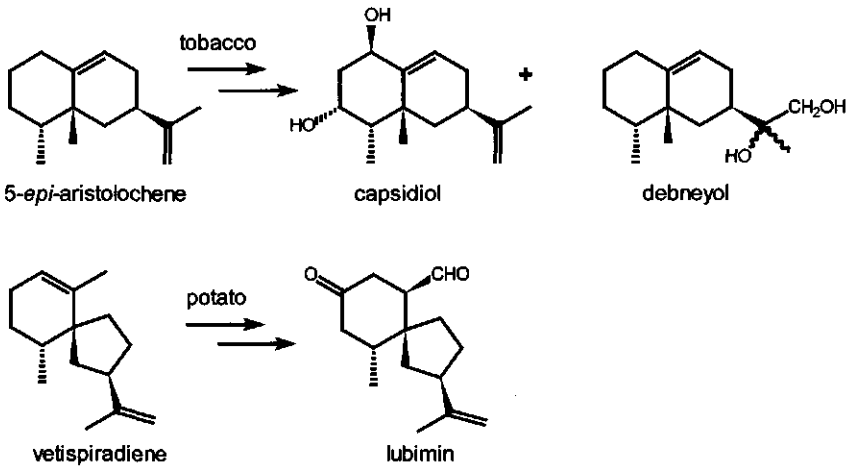

Figure 9. Oxidation of sesquiterpenes in Solanaceae .

The dehydrogenases of monoterpene biosynthesis are rather specific enzymes that oxidise a narrow range of related monoterpene alcohols. They are water soluble enzymes that use either $NADP^+$ and/or NAD^+ as a cofactor, have a K_m-value for the alcohol of 10 to 100 µM, and a molecular mass in the range of 40 to 100 kD. They have an optimum pH that can vary between 8.0 and 11.0, whereas at a more neutral or slightly basic pH some of them may catalyse the reverse reaction in the presence of NAD(P)H (Croteau et al., 1978; Croteau and Felton, 1980; Kjonaas et al., 1985; Ikeda et al., 1991; Croteau and Karp, 1991; Sangwan et al., 1993; Hallahan et al., 1995; McCaskill and Croteau, 1997; Bouwmeester et al., 1998).

Biosynthesis of mono- and sesquiterpene acids in plants has not yet been described. A reason might be that these compounds are less common. The involvement of $NAD(P)^+$-dependent dehydrogenases in the formation of perillic acid from limonene by bacteria (Dhavilakar et al.; 1966) and the conversion of farnesol to farnesoic acid in insect hormone biosynthesis (Baker et al., 1983) suggests the involvement of this type of enzymes in the postulated oxidation of germacrene alcohol to germacrene acid. Biosynthesis of the diterpenoid abietic acid (Fig. 10), a major component of conifer resin, from the corresponding aldehyde involves an NAD^+-dependent aldehyde dehydrogenase, but remarkably the conversion of alcohol into aldehyde is catalysed by a cytochrome P450 enzyme. Moreover, oxidation of kaurene to kaurenoic acid in gibberellin biosynthesis is catalysed exclusively by cytochrome P450 enzymes, even though this conversion resembles that of abietadiene to abietic acid (Funk and Croteau, 1994) .

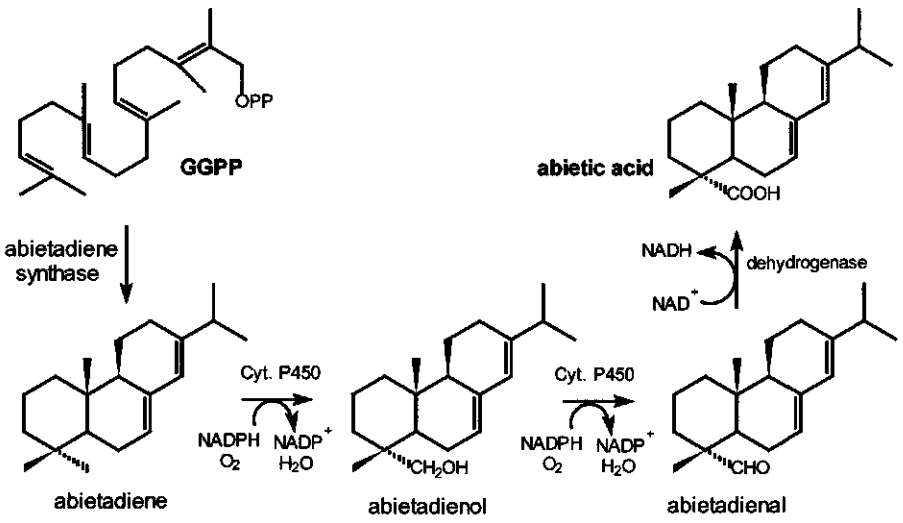

Figure 10. Pathway for biosynthesis of abietic acid from geranylgeranyl diphosphate (GGPP).

In conclusion, for biosynthesis of the lactone ring, literature suggests that the postulated hydroxylations of the germacrene intermediates at the C_6- and C_{12}-position are catalysed by cytochrome P450 enzymes. Though, further oxidation of the hydroxyl group at C_{12} into a carboxylic acid might either completely be catalysed by dehydrogenases or also involve cytochrome P450 enzymes.

Cytochrome P450 Enzymes

Cytochrome P450 enzymes are likely involved in the formation of the lactone ring, but might as well mediate the introduction of other oxygen atoms present in the sesquiterpene lactones of chicory. Cytochrome P450 enzymes are powerful oxidising catalysts that activate molecular oxygen and typically insert one oxygen atom into the substrate, whereas the other oxygen atom is reduced at the expense of NAD(P)H to form water. Because of this insertion of one oxygen atom, they are often named cytochrome P450 monooxygenases. They are however also referred to as mixed-function oxygenases (or sometimes mixed function oxidases), because of their simultaneous catalysis of an oxygenation reaction (incorporation of oxygen) and an oxidase reaction (the reduction of oxygen to water) (West, 1982; Faber, 2000; Halkier, 1996)

$$S + NAD(P)H + H^+ + O_2 \rightarrow S\text{-}O + NADP^+ + H_2O$$

Cytochrome P450 enzymes are hemoproteins of circa 50 kD in which the heme is available for O_2 or CO binding. Binding of CO to a reduced cytochrome P450 heme moiety yields a characteristic absorption band at 450 nm, but also blocks further redox activity of the enzyme. Exposure to blue light (450 nm) causes displacement of CO from the heme, thus CO binding is photoreversible. Blue-light reversible CO-inhibition of enzymatic activity is generally the definitive experiment to demonstrate the involvement of a cytochrome P450 in an enzymatic reaction. The dependence on oxygen and NAD(P)H is also an indication of the involvement of a cytochrome P450 enzyme, but other types of enzymes might also meet these criteria. Additionally, cytochrome P450 activity can be blocked by specific inhibitors such as metyrapone and clotrimazol. The optimum pH is generally in the range of 7.0-8.0 (Estabrook and Werringvloer, 1978; Donaldson and Luster, 1990; Mihaliak et al., 1993).

The Cytochrome P450 Complex

A cytochrome P450 enzyme does not operate on itself, but interacts with other enzymes to obtain electrons for activation of the molecular oxygen bound to its heme group. In eukaryotes, transport of the electrons from NADPH to the cytochrome P450 is mediated by an NADPH:cytochrome P450 reductase that contains FAD and FMN as prosthethic groups. The cytochrome P450's and NADPH:cytochrome P450 reductases are clustered together in the phospholipid matrix of membranes and form the so-called cytochrome P450 complex (Fig. 11). Functional cytochrome P450 complexes of plants are usually obtained from the microsomal fraction, a loosely defined collection of membranous vesicles that sediments between 15,000 and 100,000 *g* and may include membrane vesicles from the breakage of various vesicles such as those of ER, Golgi, plasma membrane, tonoplast, plastid envelopes, nucleus and peroxisomes. Removal of the membrane with phospholipase A disrupts the link between the cytochrome P450 and the reductases and results in the loss of enzyme activity, a process which can be reversed by the addition of new lipids (West, 1980; Donaldson and Luster, 1991; Mihaliak et al., 1993; Halkier, 1996).

Cytochrome P450 processes are often stimulated by the addition of NADH or might even to some extent function with NADH as sole electron donor. This is possible because cytochrome P450 enzymes can also receive electrons from cytochrome b_5, which itself obtains electrons from an NADH:cytochrome b_5 reductase. Both cytochrome b_5 and its reductase are embedded in the phospholipid matrix of the membrane as well (not shown in Fig. 11). In mitochondria and bacteria electron transport from NAD(P)H to cytochrome P450 is somewhat different and mediated by a ferredoxin reductase, a non-heme iron protein.

Instead of cytochrome P450, cytochrome *c* can serve as an alternative electron acceptor for the electrons that are transported via either cytochrome b_5 ore one of the two reductases. As a consequence, cytochrome *c* is a very effective inhibitor of cytochrome P450 enzyme activity in *in vitro* experiments (West, 1980; Donaldson and Luster, 1991; Mihaliak et al., 1993; Halkier, 1996).

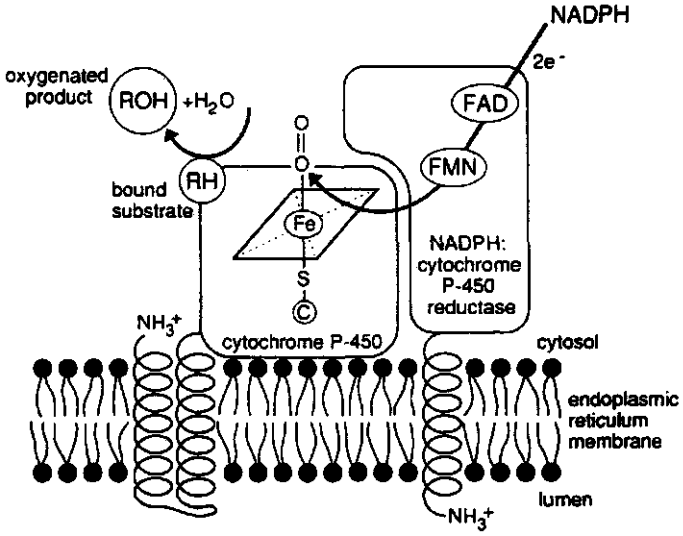

Figure 11. Schematic drawing of the cytochrome P450 complex (taken from Meijer, 1993).

Catalytic Cycle

The catalytic cycle of cytochrome P450 monooxygenases that is depicted in figure 12 has largely been deduced from studies on the camphor 5-exo-hydroxylase from *Pseudomonas putida* that, in contrast with eukaryotic cytochrome P450 enzymes, is cytosolic and has been crystallised. The active site of the cytochrome P450 contains the heme group embedded in a hydrophobic environment. The heme consists of a protoporphyrin IX ring that has a ferric ion bound to the four nitrogen atoms in its centre. The ferric ion can bind two additional ligands which are in cytochrome P450's a cysteinyl thiolate and a molecule of water.

The first step in the catalytic cycle involves binding of the substrate to the active site by displacement of the water molecule, which shifts the iron from a hexa-coordinated state to a penta-coordinated state. This facilitates the reduction of the Fe-atom from the ferric to the ferrous state by an electron delivered from NAD(P)H via the cytochrome P450 reductases. Next, an oxygen molecule is bound to give an cytochrome P450 di-oxygen complex. Delivery of a second electron weakens the O-O bond and allows cleavage of the oxygen

molecule. This cleavage of molecular oxygen is not very well understood. Presumably the cysteinyl thiolate ligand assists by "pushing" electrons leading to a heterolytic cleavage of the O-O bond, which results in formation of the activated oxygen species $[FeO]^{3+}$ and the release of water.

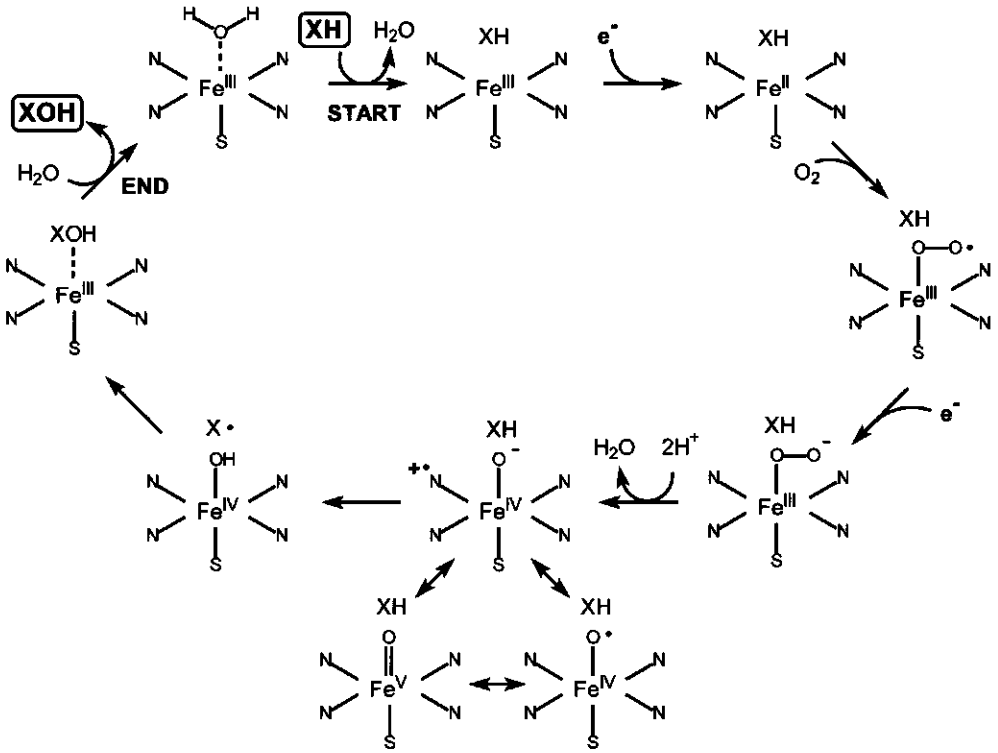

Figure 12. Catalytic cycle for the hydroxylation of substrate XH by a cytochrome P450 (based on schemes by Akhtar and Wright [1991]; Guengerich [1991], Drauz [1995], Halkier [1996], and Faber [2000]).

The activated species of the iron-oxo complex is a radical that may be represented by several resonance forms, e.g. $Por[Fe^{V}=O] \leftrightarrow Por[Fe^{IV}\text{-}O^{\bullet}] \leftrightarrow Por^{+\bullet}[Fe^{IV}=O] \leftrightarrow Por^{+\bullet}[Fe^{III}\text{-}O^{\bullet}]$. When assigning the charge to the iron atom in these structures, it is assumed that the pair of electrons forming the co-ordination bond resides entirely on the ligand and is not shared with the metal. The activated species in the general catalytic cycle of a cytochrome P450 is believed to be the oxo-ion(IV) porphyrin radical cation $Por^{+\bullet}[Fe^{IV}=O]$.

The last steps of the reaction mechanism in which the product is formed are not well understood either. In case of hydroxylation, the activated oxygen species is believed to abstract a hydrogen atom from the substrate yielding a carbon radical and iron-bound hydroxyl radical, followed immediately by radical recombination (oxygen rebound) to form an alcohol. In many cytochrome P450 catalysed reactions the product is however not an alcohol because rearrangement has occurred, and the last steps of the cycle can more generally be described in terms of two steps: abstraction of a hydrogen atom or electron, and oxygen rebound (radical recombination). Expulsion of the product restores the resting state of the enzyme and closes the catalytic cycle (Akhtar and Wright, 1991; Guengerich, 1991; Drauz and Waldmann, 1995; Halkier; 1996; Faber 2000).

Substrate and Product Specificity

Cytochrome P450 enzymes are ubiquitous and participate in numerous reactions in vertebrates, microbes and plants, including hydroxylation, epoxidation, N-, S- and O-demethylation and dealkylation. Under special circumstances they even carry out peroxidase and isomerase functions. Furthermore, in a few cases cytochrome P450 enzymes act as reductases (e.g. dechlorination of tetra) that, after uptake of the first electron, do not bind molecular oxygen and pass the reducing equivalents on to the substrate (West, 1980; Guengerich 1991; Mihaliak et al., 1993; Halkier, 1996; Schuler, 1996).

Insects and vertebrates utilise the mixed function oxygenases in hormone production and to detoxify and eliminate a vast array of xenobiotic products. Microbes employ cytochrome P450 enzymes for instance to catabolise monoterpenes when these compounds are given as a sole carbon source. In plants cytochrome P450 enzymes are involved in numerous metabolic pathways, for instance those of sterols, terpenoids, gibberellins, fatty acids, lignins and isoflavonoids. Cytochrome P450's in plants are also involved in detoxification of xenobiotics. For example, biochemical resistance of plants to herbicides is mediated by a rapid, cytochrome P450 catalysed, conversion of the herbicide to a hydroxylated inactive product, which is subsequently conjugated to carbohydrate moieties in the plant cell wall (Donaldson and Luster 1991; Mihaliak et al, 1993; Schuler, 1996).

Generally cytochrome P450 enzymes involved in detoxification (e.g. in the liver of mammals) have broad substrate specificities, whereas those involved in biosynthesis of endogenous (e.g. steroids and plant secondary products) compounds are considered to have a narrow substrate specificity (West, 1980; Aktar and Wright, 1991; Donaldson and Luster, 1991; Halkier, 1996). Though for plants, it is yet not clear whether the metabolic and

detoxicative cytochrome P450 enzymes represent distinct classes at protein level. Several studies have suggested that some or all of the detoxicative cytochrome P 450's may represent biosynthetic enzymes "moonlighting" as detoxicative enzymes (Schuler, 1996).

The cytochrome P450 monooxygenases involved in hydroxylation of cyclic monoterpenes, such as (+)-sabinene in sage (*Salvia officinalis*) (Karp et al., 1987) and limonene in caraway (*Carum carvi*) (Bouwmeester et al., 1998) and mint-species show a very high substrate specificity with a modest degree of enantioselectivity. Furthermore, these reactions are completely regiospecific and yield only one product. Differences in the essential oils between *Perilla frutescens*, *Mentha spicata* (spearmint) and *Mentha piperita* (peppermint) are in fact due to the presence of different specific limonene hydroxylases (Fig. 13) that exclusively hydroxylate limonene at the C_7-, C_6- or C_3-position, respectively (Karp et al., 1990; Croteau et al., 2000).

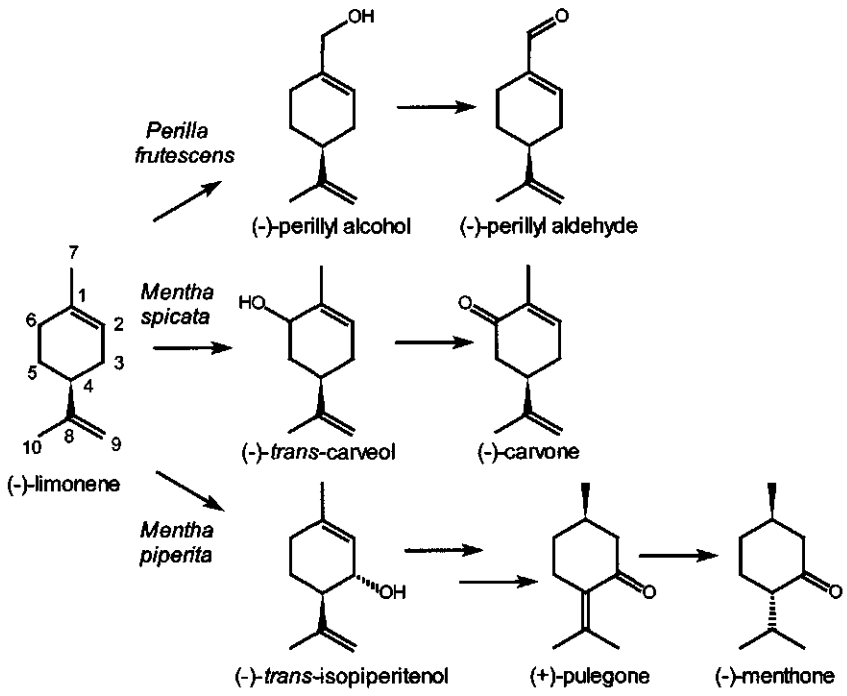

Figure 13. Regioselective hydroxylation of limonene governs the spectrum of monoterpenoids present in mint oils (after Karp et al [1990]).

The substrate binding pocket of cytochrome P450 enzymes is adjacent to the heme and accessible through a long hydrophobic channel. Binding of substrates to cytochrome P450

enzymes appears to be largely determined by hydrophobic interactions. The high substrate specificity as well as the high regio- and stereoselectivity of cytochrome P450 catalysed reactions is thought to be imposed by structural constraints within the binding pocket of the protein. The reaction between the activated heme-bound oxygen and the substrate is determined by the orientation of the substrate relative to the activated oxygen species, the relative reactivities of the oxidisable sites on the substrate, and the degree of mobility within the substrate binding pocket. The latter can be very well illustrated by the camphor 5-exo-hydroxylase of *Pseudomonas putida*. It converts the endogenous substrate camphor into only one product, whereas norcamphor is hydroxylated to form at least three different detectable products. This observation could be related to the looser fit of nor-camphor in the binding site (Halkier, 1996).

Unfortunately, a three-dimensional structure of any eukaryotic cytochrome P450 has not yet been resolved, due to the fact that they are membrane-bound enzymes and thus hard to crystallise. It would give more insight in the structure-function relationship of the active site, but at present the available data concern four cytochrome P450's of bacterial origin that share only 10-20% sequence identity with eukaryotic microsomal cytochrome P450 enzymes (Halkier, 1996).

SCOPE OF THIS THESIS

Sesquiterpene lactones are an important group of plant secondary metabolites, but about their biosynthesis little is known. Roots of chicory (*Cichorium intybus* L.) are a good plant material to study the biosynthesis of these compounds. They are rich in bitter tasting guaianolides, eudesmanolides and germacranolides, whereas they are not very ligneous and do not contain olefins nor green pigments that might hinder the isolation of enzyme activities. Moreover, chicory roots are easy to obtain, because they are cultivated in The Netherlands for the production of Belgian endive. After harvest of the white sprouts, these roots are actually considered a waste product of which 100,000 tons are produced in The Netherlands annually.

For elucidation of the biosynthetic pathway of sesquiterpene lactones the transformation of FPP into a sesquiterpene hydrocarbon (i.e. the involved sesquiterpene synthase) had to be studied first. The major part of this thesis is however primarily focussed on the oxidising enzymes involved in sesquiterpene lactone biosynthesis. So far, relatively little attention has been paid to the oxidising enzymes of plant secondary metabolism. In particular the involved

cytochrome P450's are ill-studied, whereas these enzymes might play an important role in plant defence mechanisms, e.g. the production of phytoalexins in Solanaceae. Although cytochrome P450 monooxygenases involved in biosynthesis are generally considered to be highly substrate specific, it is additionally worthwhile to investigate their possible use as catalysts in organic synthesis. Noteworthy, the regio- and stereoselective introduction of an oxygen group into unactivated organic compounds still remains a largely unresolved challenge to synthetic chemistry.

The first dedicated step in sesquiterpene lactone biosynthesis, cyclisation of farnesyl diphosphate (FPP) to the sesquiterpene backbone, is described in **Chapter 2**. The initial steps in formation of the lactone ring are revealed in **Chapter 3**, i.e. the introduction of a carboxylic acid group by a cytochrome P450 hydroxylase and NAD(P^+)-dependent dehydrogenases. **Chapter 4** concerns the isolation of the germacrene intermediates of sesquiterpene lactone biosynthesis from fresh roots of *Saussurea lappa* Clarke. One of the isolated compounds is germacrene carboxylic acid that is used in **Chapter 5** to investigate the final step(s) in the formation of the lactone ring present in the sesquiterpene lactones of chicory. This chapter discusses also to some extent the subsequent conversions of (+)-costunolide. Studies on the substrate specificity of the isolated oxidising enzymes from chicory are described in **Chapter 6**, in particular those of the cytochrome P450 hydroxylases. Finally, in **Chapter 7** an overview of the established pathway for sesquiterpene lactones is given and discussed.

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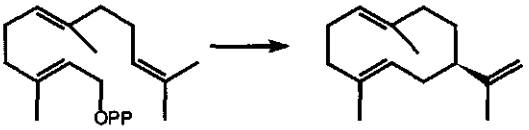
2. (+)-Germacrene A Biosynthesis

The Committed Step in the Biosynthesis of Bitter Sesquiterpene Lactones in Chicory Roots

A adapted version of this chapter has been published in Plant Physiol. (1998) 117: 1381-1392



Abstract: The postulated biosynthesis of sesquiterpene lactones through the mevalonate-FPP-germacradiene pathway has been confirmed by the isolation of a (+)-germacrene A synthase from chicory roots. This sesquiterpene synthase was purified 200-fold using a combination of anion exchange and dye-ligand chromatography. It has a K_m-value of 6.6 μM, an estimated molecular weight of 54 kD, and a (broad) pH-optimum around 6.7. (+)-Germacrene A, the enzymatic product, proved to be much more stable than reported in literature. Its heat-induced Cope rearrangement into (-)-β-elemene was utilised to determine its absolute configuration on an enantioselective GC-column. In sesquiterpene biosynthesis germacrene A has only been reported as an (postulated) enzyme-bound intermediate, which, instead of being released, is subjected to additional cyclisation(s) by the same enzyme that generated it from farnesyl diphosphate (FPP). However in chicory, germacrene A is released from the sesquiterpene synthase. Apparently, the lactone ring is formed before any further cyclisation of the germacrane skeleton occurs.

INTRODUCTION

It is assumed that the guaiane-, eudesmane- and germacrane-type lactones, such as present in chicory, originate from a common germacrane precursor that is formed via the acetate-mevalonate-FPP pathway by a germacrene synthase, an enzyme belonging to the group of sesquiterpene synthases (Geissman, 1973; Herz, 1977; Fischer et al., 1979; Seaman 1982; Fischer, 1990). Whether this common germacrane precursor is transformed into a guaiane skeleton or a eudesmane skeleton would depend on the position of enzyme-mediated epoxidations further down the pathway. A germacrene C_4-C_5-epoxide would lead to a guaiane, whereas a germacrene C_1-C_{10}-epoxide would lead to a eudesmane (Brown et al., 1975; Teisseire, 1994; Piet et al., 1995). For this reason the assumption is that, apart from the oxidising enzymes, two different cyclising enzymes are involved in the biosynthesis of the guaianolides and eudesmanolides: an enzyme that cyclises farnesyl diphosphate (FPP) to a germacrane skeleton, and a separate enzyme that cyclises the germacrane skeleton to a guaiane or eudesmane skeleton (Fig. 1) (Piet et al., 1995; Piet et al., 1996).

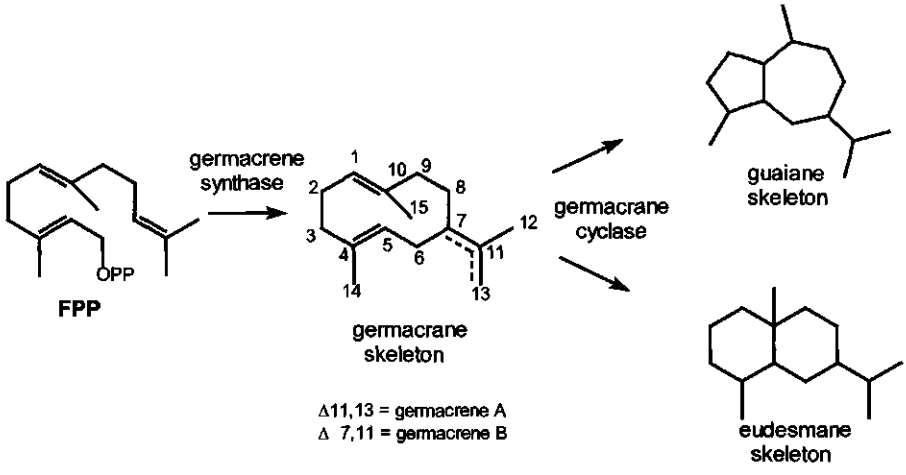


Figure 1. A simplified scheme (without oxidative steps) for the formation of guaiane- and eudesmane-type sesquiterpene lactones involves two cyclising enzymes, a germacrene synthase and a germacrane cyclase. Literature suggests either germacrene A or germacrene B to be the germacrane intermediate.

Biosynthetic studies with a hairy root culture of blue-flowered lettuce (*Lactuca floridana*) supplied with ^{13}C-labelled precursors of secondary plant metabolism (acetate and mevalonate) seem to confirm this acetate-mevalonate-FPP-germacradiene pathway. From the

patterns of ^{13}C-enrichment in the produced guaianolides, Song *et al.* (1995) deduced that the C_{12}-atom and the C_{13}-atom of the germacrane intermediate are chemically not identical; this indicates the formation of either germacrene A (**1**) or germacrene B. Formation of germacrene B would be supported by the existence of C_8-oxygenated sesquiterpene lactones such as lactucin and lactucopicrin, because in germacrene B the C_8-position is activated for allylic oxidations (Fischer, 1990).

Sesquiterpene synthases catalyse the conversion of FPP to over 200 different cyclic skeletons, and a growing number of these enzymes have been isolated and characterised in recent years. cDNA sequences (Cane, 1990; McCaskill, 1996) are available, and protein crystal structures have been published recently (Lesburg et al., 1997; Starks et al., 1997). Although germacrenes, especially germacrene D, are important constituents of many essential oils, until recently no germacrene synthase had been described. Only the biosynthesis of germacrene C by a homogenate of immature seeds of *Kadsura japonica* had been reported (Murikawa et al., 1971), as well as the partial purification of a synthase for β-selinene (Belingheri et al., 1992), a germacrene A related compound, from the outer peels of *Citrofortunella mitis*. In the time span of this PhD period, isolation of a germacrene B synthase (gene) (van der Hoeven et al., 2000) and a germacrene C synthase (gene) (Colby et al., 1998) from tomato (*Lycopersicon esculentum*) as well the isolation of separate (+)-germacrene D and (-)-germacrene D synthases from golden rod (*Solidago canadensis*) (Schmidt et al., 1998) have been described.

A problem in studying the germacrene synthases may be the reported instability of all four known germacrenes. Germacrene A (**1**, Fig. 2) is in particular reported to be susceptible to heat-induced Cope rearrangement toward β–elemene (**2**), even during freezer storage, and to proton induced cyclisation toward α–selinene (**3**) and β-selinene (**4**) on silica gel (Weinheimer, 1970; Bowers et al., 1977; Teisseire, 1994). Germacrene A itself has often been postulated as an intermediate (bound to the sesquiterpene synthase) in the biosynthesis of patchoulol and phytoalexins, such as aristolochene, 5-epi-aristolochene, capsidiol, debneyol, and vetispiradiene (Threlfall et al., 1988; Hohn et al., 1989; Whitehead et al., 1989; Beale, 1990; Cane, 1990; Munck et al., 1990; Cane et al., 1993; Back and Chappell, 1995).

The aim of the investigations described in this chapter is to identify the germacrane intermediate involved in the sesquiterpene lactone biosynthesis of chicory, and to isolate and characterise the sesquiterpene synthase responsible for its formation.

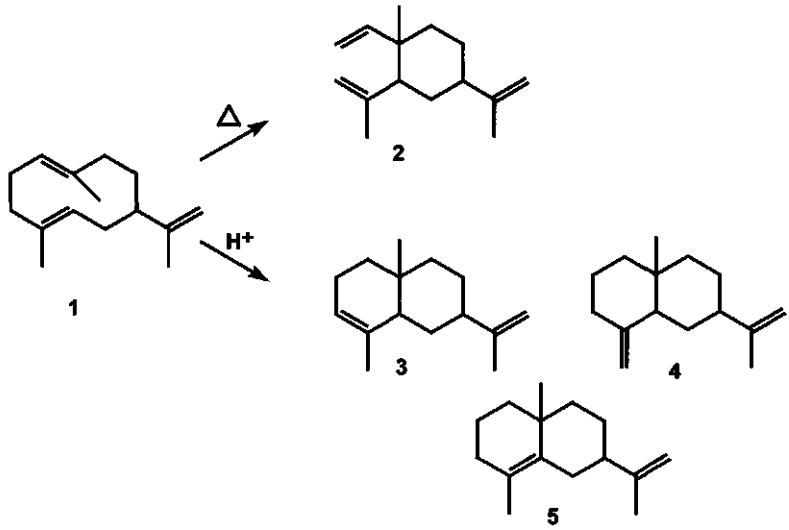


Figure 2. The reported high sensitivity of germacrene A (**1**) to heat and slightly acidic conditions gives Cope rearrangement toward β-elemene (**2**), respectively, cyclisation toward α-selinene (**3**) and β-selinene (**4**). Selina-4,11-diene (**5**) would be another acid-induced cyclisation product.

MATERIAL AND METHODS

Fresh roots of cultivated chicory (*Cichorium intybus* L., cv Focus) were harvested during late summer and obtained from a grower (J. de Mik) in Veenendaal, the Netherlands. Roots of wild chicory were collected in October in the forelands of the Rhine near Wageningen. The chicory roots were cut into small pieces, frozen in liquid nitrogen, and stored at -80°C. Unlabelled FPP was obtained from Sigma in a solution of 70% (v/v) methanol in 10 mM aqueous ammonium hydroxide. The solvent was evaporated *in vacuo* using a Gyrovap GT (Howe, Oxon, UK), and a 10 mM FPP stock solution was prepared with 50% (v/v) ethanol in 200 mM aqueous ammonium bicarbonate. [1(n)-^{3}H]-FPP dissolved in a solution of 50% (v/v) ethanol with 100 mM aqueous ammonium bicarbonate (16.0 Ci mmol^{-1}, 200 μCi mL^{-1}) was purchased from Amersham.

Enzyme Isolation and Assay (assay I)

Fifty grams of frozen root material from either cultivated or wild chicory was homogenised in a blender with 5 g insoluble polyvinylpolypyrrolidone and 80 mL buffer containing 50 mM Mopso (3-[N-Morpholino]-2-hydroxy-propanesulfonic acid) (pH 7.0),

50 mM sodium *meta*-bisulfite, 50 mM ascorbic acid, 10 mM $MgCl_2$, 5 mM DTT, and 20% glycerol (buffer A). The homogenate was transferred to a beaker using another 40 mL buffer A, mixed with 5 g polystyrene resin (Amberlite XAD-4, Serva) and allowed to stand on ice for several minutes. The slurry was filtered through premoistened cheesecloth and centrifuged for 20 min at 20,000*g* at 4°C. The supernatant was centrifuged once more for 90 min at 100,000*g* at 4°C; after that it was desalted with an Econo-Pac 10DG column (Biorad) to 1 mM ascorbic acid in buffer B. This less concentrated buffer B contained 15 mM Mopso (pH 7.0), 10 mM $MgCl_2$, 2 mM DTT, and 10% glycerol. A 1.0-mL aliquot of the desalted supernatant was incubated for 1 h at 30°C with either 50 μM unlabeled FPP or 20 μM [^{3}H]-FPP (50 Ci mol^{-1}), using an overlay of 1 mL of pentane (assay I). As a control, both types of incubations were also performed with supernatant that had been boiled for 5 min. Incubations were stopped by vigorous shaking. For analysis, the pentane phase was filtered through DMCS treated glasswool (Chrompack, Bergen op Zoom, The Netherlands) plugged Pasteur pipette that contained 0.90 g of aluminium oxide (grade III) and a little anhydrous magnesium sulphate. The extraction was repeated with 1 mL of 20% (v/v) ether in pentane, and the aluminium oxide column was washed with this extract and an additional amount of 1.5 mL 20% (v/v) ether in pentane. The combined pentane eluate contained sesquiterpenoid hydrocarbons that do not bind to aluminium oxide under these conditions. The same assay was then twice extracted with 1 mL of ether, and the ether extracts were passed through the same column. At the end of this extraction-filtration procedure, the column was rinsed with 1.5 mL of ether to ensure a complete elution of (oxygenated) products. The separately collected pentane/ether and ether phases were carefully concentrated to approximately 50 μL under a stream of nitrogen. Both the pentane and ether (diethyl ether) were redistilled before use in the filtration-extraction procedure described above.

Identification of Sesquiterpenoid Products

Before the concentrated extracts of the [^{3}H]-FPP incubated assays were analysed by radio-GC, 1 μL of a sesquiterpene standard was added containing (each 1 mg mL^{-1} in pentane) germacrene B (prepared by Dr. D.P. Piet according to Piet et al., 1995), γ-elemene (prepared by Dr. D.P. Piet from germacrene B by heating at 160°C under argon for 16 h), nerolidol, and farnesol. In later experiments this sesquiterpene standard was replaced by either 5 μL of an alkane set (n=7 to n=22, 1 mg mL^{-1} each in pentane) to calculate Kovats' indices, or 5 μL of a liverwort (*Frullania tamarisci*) extract containing germacrene A as one

of its major constituents (Hardt et al., 1995; W.A. König, unpublished results). Radio-GC analysis was performed on a Carlo-Erba 4160 Series gas chromatograph coupled to a RAGA 93 radioactivity detector (5 mL-counting tube; Raytest, Straubenhart, Germany). The GC was equipped with a EconoCap EC-Wax column (30 m × 0.32 mm, d_f 0.25 μm) and programmed at 5°C min^{-1} from 70°C to 210°C using a helium flow rate of 2.7 mL min^{-1}. Samples of 1 μL were injected in the cold on-column mode. The compounds eluting from the column were split in a ratio of 3:1 between the radio-detector and a FID at 210°C. Before entering the radioactivity detector, eluted compounds were quantitatively reduced by addition of hydrogen at 3 mL min^{-1} and passage through a conversion reactor filled with platinum chips at 800°C. After reduction, methane was added as a quenching gas to give a total flow of 36 mL min^{-1} through the counting tube of the radioactivity detector. The incubations with unlabeled FPP were analysed by GC-MS using a HP 5890 Series II gas chromatograph (Hewlett-Packard) equipped with a mass selective detector (model 5927 A, Hewlett-Packard) and a capillary HP-5MS column (30 m x 0.25 mm, d_f 0.25 μm) at a helium flow rate of 0.969 mL min^{-1}. The splitless injection of the 1-μl sample was initially done at an injection port temperature of 210°C, and at 150°C in later experiments because of the sensitivity of germacrenes to high temperatures. After an initial temperature of 55°C for 4 min, the column was programmed at 5°C min^{-1} to 210°C. The mass spectra were recorded at 70 eV scanning from 30 to 250 amu. MS data were compared with those recorded from compounds present in the natural oil of *Mentha mirennea* (β-elemene (**2**); Maat et al., 1992) and the extract of *Frullania tamarisci* (germacrene A [**1**], α-selinene [**3**], β-selinene [**4**], and selina-4,11-diene [**5**]; gift of Prof. W.A. König, Hamburg University).

Determination of the Absolute Configuration of Germacrene A

The absolute configuration of germacrene A (**1**) was determined by means of its Cope rearrangement to β-elemene (**2**), a reaction which occurs with retention of configuration at C_7 (Weinheimer et al., 1970; Takeda 1974; March 1992). The GC-MS was essentially used as described above, but with an injection port temperature of 250°C to induce the rearrangement of enzymatically produced germacrene A. The oven temperature was programmed to 45°C for 4 min followed by a ramp of 2°C min^{-1} to 170°C, and spectra were recorded in the selected ion-monitoring mode (m/z 121, 147 and 189). The apparatus itself was equipped with a 25-m (0.25-mm i.d.) heptakis(6-O-TBDMS-2,3-di-O-methyl)-β-cyclodextrin (50% in OV17) column that is able to separate the enantiomers of racemic

β–elemene (König et al., 1994). A racemic β-elemene standard was isolated from a hydrodistillate of the liverwort *Frullania macrocephalum* (gift of Dr. L. Kraut, University of Saarbrücken; and Prof. W.A. König), and the elution order of its enantiomers was determined with a (-)-β–elemene standard (König et al., 1994). To substantiate correct identification of the β–elemene enantiomer derived from chicory germacrene A, racemic β–elemene was co-injected with enzymatically produced germacrene A at injection port temperatures of 150°C and 250°C.

Sesquiterpene Synthase Assay (assay II) and Protein Determination

For routine determination of germacrene A synthase activity, 10 µL of sample was added to an Eppendorf vial with 90 µL of buffer C (consisting of 0.1% [v/v] Tween 20 in buffer B) and incubated at 30°C with 20 µM [^{3}H]-FPP (50 Ci mol^{-1}). The reaction mixture was overlaid with 1 mL of hexane to trap formed, labelled olefins (assay II). After 30 min the vial was vigorously mixed and cooled to stop the reaction; then it was briefly centrifuged to separate phases. Of the hexane phase, 750 µL was transferred to a new Eppendorf vial containing 40 mg of silica (0.06-0.2 mm) to bind farnesol produced from FPP by phosphohydrolases. After mixing and centrifugation, 450 µL of the hexane layer was removed for scintillation counting in 4.5 mL of Ultima Gold™ cocktail (Packard, Meriden, CT). Protein concentrations were determined using the microassay protocol of the Coomassie Plus Protein Assay (Pierce) and BSA as protein standard. MonoQ fractions containing Tween 20 were desalted to 50 mM ammonium bicarbonate using a HiTrap desalting column and assayed by the Micro BCA Protein Assay (Pierce).

Sesquiterpene Synthase Purification

Cellular extracts and enzyme preparations were kept on ice throughout the purification. The purification was started by making a 100,000*g* supernatant as described above, but with a buffer containing 25 mM Mopso (pH 7.0), 25 mM sodium *meta*-bisulfite, 25 mM ascorbic acid, 10 mM $MgCl_2$, and 2 mM DTT (buffer D). A column (∅ 2.5 cm) of 25 g DEAE (preswollen DE52, Whatman) suspended in 150 mL buffer containing 150 mM Mopso (pH 7.0), 100 mM $MgCl_2$, and 20 mM sodium *meta*-bisulfite was prepared and washed at 1.6 mL min^{-1} with 150 mL 2 mM sodium *meta*-bisulfite in buffer B (buffer E). Seventy-five millilitres of the 100,000*g* supernatant were loaded onto this column and washed with another 100 mL of buffer E to remove unbound proteins. A 100 mL-gradient of 0 to 0.5 M KCl in

buffer E was used to elute sesquiterpene synthase activity. Fractions containing sesquiterpene synthase activity were pooled and desalted to buffer B, after which glycerol was added to a final concentration of 30% (v/v). The enzyme preparation was frozen in liquid nitrogen and stored at -80°C in 1-mL aliquots. One aliquot was tested for the nature of its enzymatic sesquiterpenoid product, whereas the others served as a stock for further purification steps. Prior to the second purification step, various dye resins from a dye resin test kit (no. RDL-9, Sigma) and Red A (Amicon, Beverly, MA) were screened for their affinity for chicory germacrene A synthase. The dye-resin columns were tested according to manufacturer's instructions by applying 180 µL of DEAE purified synthase to each column. Best results were obtained for Reactive Green 5, and a column was prepared (∅ 1.0 cm) of a 5-mL suspension containing 1.5 mg mL^{-1} Reactive Green 5 (Sigma R2257) that had been rinsed twice with an equal volume of buffer B. The column was equilibrated with 15 mL buffer B, and an aliquot of DEAE purified synthase (thawed and warmed up to room temperature) was applied to this column at 0.5 mL min^{-1}. Unbound proteins were washed off the column with buffer B while monitoring the A_{280}; the sesquiterpene synthase was eluted using a one step gradient of 1.5 M KCl in buffer B. Sesquiterpene synthase activity containing fractions were combined, desalted to buffer C with an Econo-Pac 10DG column, and applied to a Mono-Q fast-protein liquid chromatography column (HR5/5, Pharmacia Biotech) previously equilibrated with buffer C. The column was washed with 4 mL of buffer C at 0.75 mL min^{-1}, after which bound proteins were eluted with a 26-mL gradient of 0 to 0.66 M KCl in buffer C. Fractions were assayed, and those containing enzyme activity were tested for the nature of their sesquiterpenoid product(s). The fraction (0.75 mL) containing the highest amount of germacrene A synthase activity was used to determine the M_r and K_m (see below). After each purification step, the purification was visualised by SDS-PAGE using pre-prepared 10% (w/v) polyacrylamide gels (Bio-Rad) according to manufacturer's instructions. Gels were stained using a silver staining kit (Pharmacia Biotech).

K_m, pH Optimum, and M_r Determination

Before determining the K_m of the chicory germacrene A synthase, assay II (with buffer C) was checked for its linearity using MonoQ-purified enzyme. When the enzyme was diluted with an equal amount of buffer C, the assay was linear during the first 40 minutes (r^2 = 0.987) at a concentration of 2 µM FPP. When undiluted MonoQ-purified enzyme was used, enzyme activity was twice as high and linear during 30 minutes (R^2= 0.963) (Fig. 6A). For

the kinetics study, enzyme activity was determined in the range of 0.5 to 80 μM (enzyme diluted twice in buffer C). The pH-optimum was determined with DEAE-purified germacrene A synthase in the pH range of 4.0 to 9.0 using the protocol of assay II and 5 μL enzyme. For pH values of 4.0 to 5.5, 5.5 to 6.5; and 7.5 to 9.0, NaAc, Mes, and Tris-HCl, respectively, were used instead of Mopso. pH experiments were carried out in duplicate and in both the presence and the absence of 0.1% Tween 20. The M_r of the germacrene A synthase from chicory was estimated by exclusion chromatography on Superdex 75 column (HR10/30, Pharmacia Biotech) in Buffer C. The column was calibrated at 0.5 mL min^{-1} with cytochrome *c* (12.4 kD), RNase A (13.7 kD), α-chymotrypsinogen (25.0 kD), ovalbumin (45.0 kD), and bovine serum albumin (BSA) (67.0 kD), all purchased from Sigma. The column was loaded with 200 μL of Mono-Q-purified germacrene A synthase, and fractions of 0.5 mL were assayed for their sesquiterpene synthase activity.

RESULTS

Detection of (+)-Germacrene A Synthase Activity in Chicory Roots

A 100,000*g* supernatant was prepared from both cultivated and wild chicory and incubated with [^{3}H]-FPP. The incubations were extracted subsequently with pentane and ether; the extracts were analysed by radio-GC after being passed over a short aluminium oxide column. The pentane extracts of both types of plant material revealed one radioactive product, which in the ether extracts was accompanied by farnesol, a result of aspecific phosphohydrolase activity. The product peak did not coincide with that of germacrene B nor that of γ-elemene. The unknown product and farnesol were not present when the supernatant was boiled before incubation, and the amount of product was raised after the DEAE purification step in which almost all phosphohydrolase activity was discarded (Fig. 3).

The peak of the unknown product contained a small shoulder peak that became a major peak when silica instead of aluminium oxide was used during the extraction-filtration procedure. A similar effect was observed when somewhat larger amounts of magnesium sulphate were used. GC-MS analyses demonstrated that this shoulder peak consisted of α-selinene (**3**), β-selinene (**4**), and selina-4,11-diene (**5**), the typical acid-induced cyclisation products of germacrene A. The Kovats' index of the enzymatic product was 1737 (EC-Wax column), which matches the value reported for germacrene A (1734; M.H Boelens [1995] database Essential Oil, version 4.1, The Netherlands). The final proof for the identity of the

unknown product was obtained by co-injection of an extract from the liverwort *Frullania tamarisci* that contains germacrene A (Fig. 3).

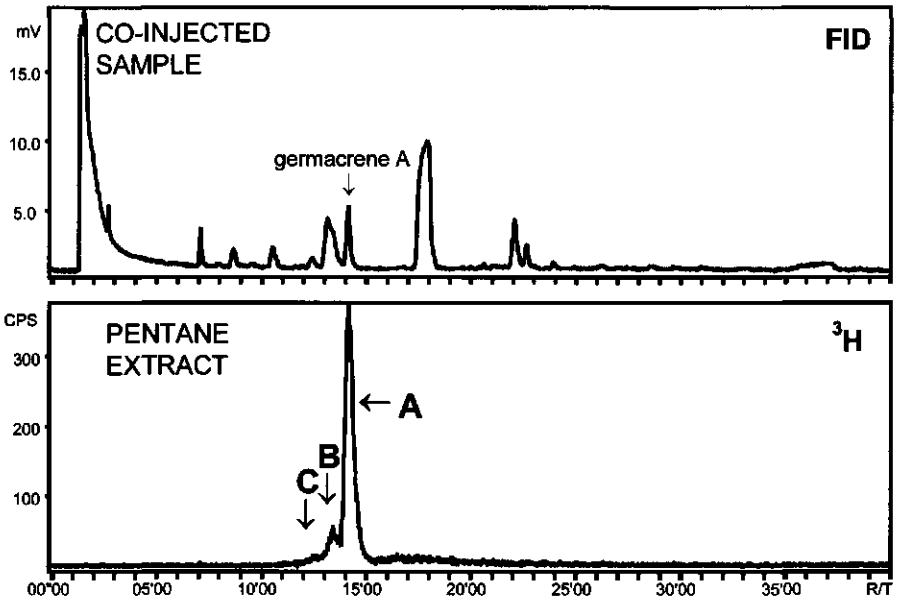


Figure 3. Identification by radio-GC of the unknown product formed by DEAE-purified chicory sesquiterpene synthase. The upper trace represents the FID response to the hydrocarbons of the co-injected liverwort (*Frullania tamarisci*) extract. The lower trace indicates the labelled compounds, extracted from the assay, detected by the radiodetector. The unknown product represented by peak A coincides with that of germacrene A present in the liverwort extract. Peak B and the small peak C were, respectively, identified as α/β-selinene (**3+4**) and selina-4,11-diene (**5**).

Initially, the GC-MS identification of germacrene A (**1**) in both the liverwort extract and the extracts of the enzyme assay was troublesome because germacrene A rearranged into β-elemene (**2**) almost completely during the measurement. This problem was overcome by lowering the GC injection port temperature from 210°C to 150°C. At this lowered injection port temperature, almost no Cope rearrangement of germacrene A occurred, although the germacrene A peak broadened significantly and was preceded by a 'hump' in the baseline.

The Cope rearrangement is a stereospecific reaction that proceeds via a chair-like transition state (March, 1992). Since germacranes also prefer the chair-chair conformation, Cope rearrangement proceeds easily (Takeda, 1974). *E,E*-germacrenes are relatively flexible

molecules, but in the case of a large substituent at C_7, the conformation having the substituent at an equatorial position predominates (Takeda, 1974; Piet et al., 1995). Hence, the two enantiomers of germacrene A (Fig. 4, **1a** and **1b**) will yield two enantiomers of β–elemene upon Cope rearrangement (Fig. 4, **2a** and **2b,** respectively); the diastereomers **2c** and **2d** will hardly be formed because the germacrene conformations **1a'** and **1b'** are energetically unfavourable. This also explains why the β–elemene diastereomers **2c** and **2d** have not been reported in literature (Connolly and Hill, 1991; Buckingham, 1999; see also chapter 4).

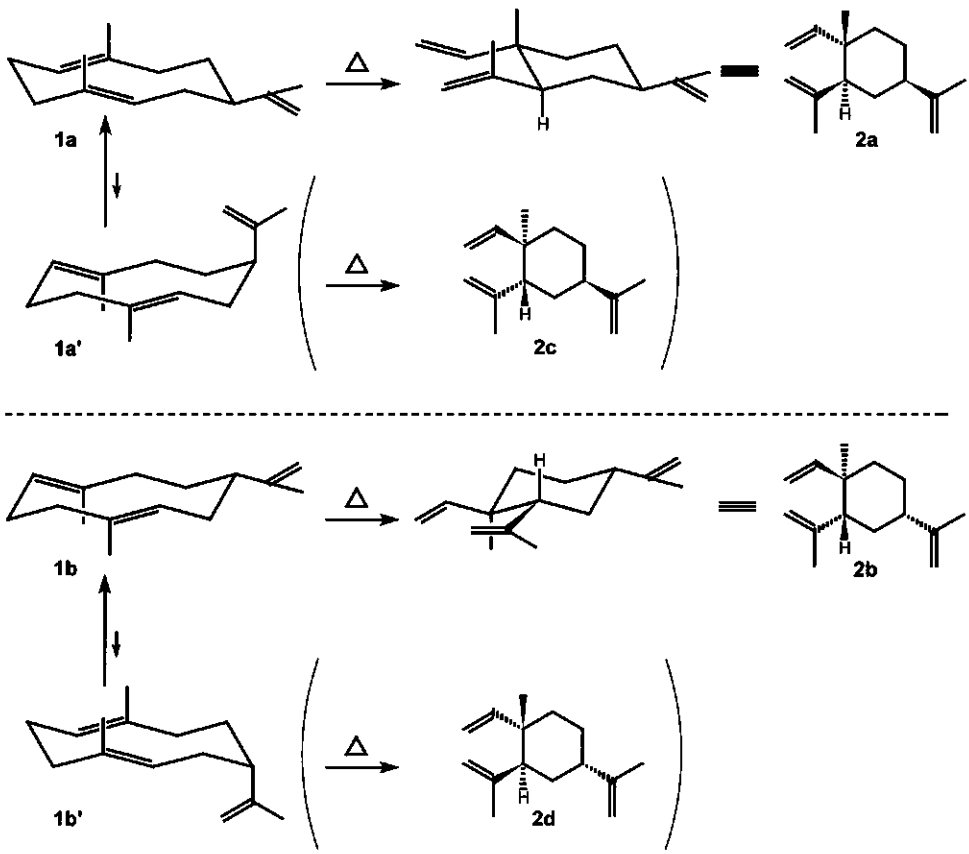


Figure 4. Conformations of the enantiomers of germacrene A ([+]-enantiomer **1a**; [-]-enantiomer **1b**), and their relation to the configuration of the β–elemenes formed by Cope rearrangement.

Using this knowledge, the Cope rearrangement can be used for the determination of the absolute configuration of germacrene A. Enzymatically produced germacrene A was injected on an enantioselective column at an injection port temperature of either 150°C or 250°C.

Whereas a huge peak of germacrene A was visible at 150°C, the germacrene A was almost completely rearranged into (-)-β–elemene at an injection port temperature of 250°C (Fig. 5, A and B). This rearrangement of chicory germacrene A into (-)-β–elemene (and not into (+)-β–elemene) was substantiated by co-injection of the germacrene A with a racemic mixture of β–elemene at an injection port temperature of both 150°C and 250°C (Fig. 5, C and D). The (+)-enantiomer of β-elemene (**2b**) (König et al., 1994; Teisseire, 1994) has the same absolute configuration as (-)-germacrene A (**1b**) as was determined by Weinheimer et al. (1970). Considering present experiments where no trace of (+)-β-elemene was observed and only its counterpart (-)-β-elemene (**2a**) was detected, it is concluded that the germacrene A synthase of chicory produces exclusively (+)-germacrene A (**1a**) (Fig. 4 and Fig. 7).

Purification of the (+)-Germacrene A Synthase from Chicory Roots

A summary of the protocol used in the purification of the chicory (+)-germacrene A synthase and its results are given in table I. Purification was started by preparing a chicory root 100,000*g* supernatant and applying it to a DEAE column. The enzyme activity eluted from the column around 0.2 M KCl. Although the recovery of this first purification step was only 30%, it was very successful in discarding aspecific phosphohydrolase activity. Aliquots of 1 mL from this partially purified germacrene A synthase remained stable for several months in 30% (v/v) glycerol at -80°C, and they served as a stock for all further experiments. Several Dye-ligands were screened for their ability to bind and release the DEAE-purified germacrene A synthase; good results were obtained with Red A (Amicon), Reactive Blue 72, Reactive Red 120, and Reactive Green 5. This seems to be in line with the results of Lanzaster and Croteau (1991) for the Dye-ligands Red A, Blue A, and Green A, and those of Moesta and West (1985) with Red A and Reactive Blue 2. Since Reactive Green 5 gave the best results and since it had been used successfully before in the purification of a trans-β-farnesene synthase from pine needles (Salin et al., 1995), it was chosen as well for the purification of the germacrene A synthase. As shown in Table I, a recovery slightly above 100% and a 9-fold purification were obtained. To ensure a good interaction of the germacrene A synthase with the matrix, it was important to warm the sample (at -80°C stored DEAE-stock; 1 mL) to room temperature before applying it to the Reactive Green 5 column. For enzyme stability the fractions containing enzyme activity required quick desalting into buffer C.

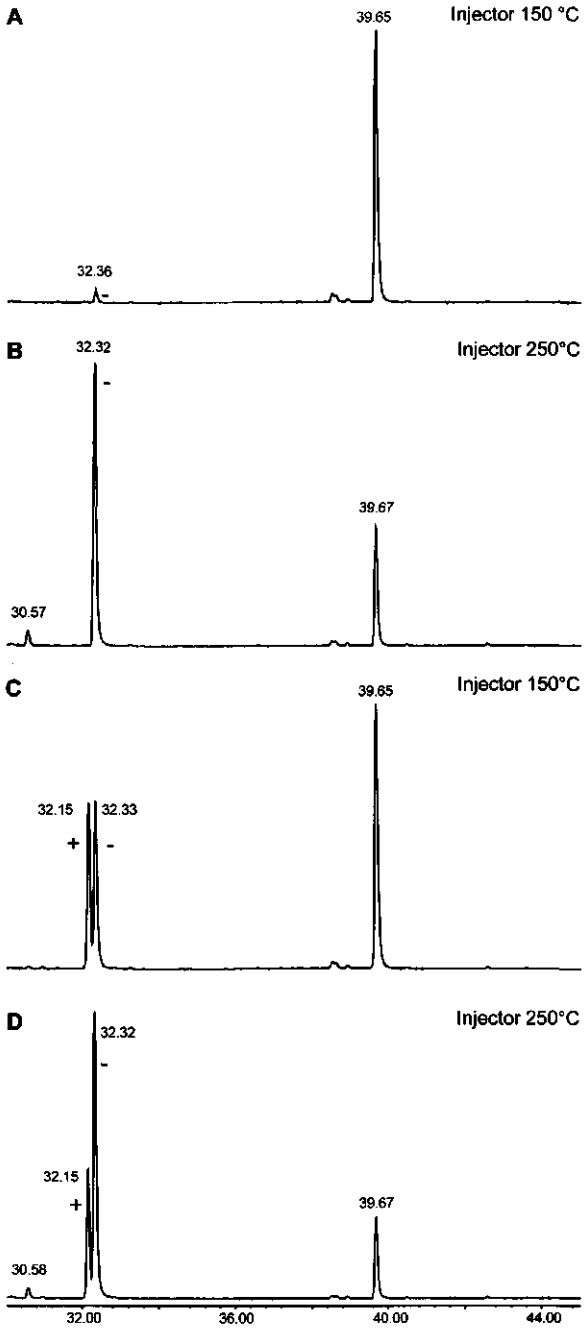


Figure 5. Determination of the absolute configuration of germacrene A using GC-MS equipped with a enantioselective column. Enzymatically (DEAE purified enzyme) produced germacrene A (39.66 min) that is stable at an injection port temperature of 150°C (A) is rearranged into (-)-β-elemene (**2a**) (32.33 min) at an injection port temperature of 250°C (B). Co-injection of the germacrene A with a racemic β–elemene standard at 150°C (C) and 250 °C (D) confirms the identity of its rearrangement product that co-elutes with (-)-β-elemene and is separated from the (+)-enantiomer of β–elemene (**2b**) (32.15 min). A small amount of diastereomeric β–elemene (**2c**) is also detected (30.57 min; see also chapter 4).

For the next purification step a Mono-Q column was used, a common step in the purification of terpene synthases (e.g. Savage et al., 1994). An additional advantage of this method is that it concentrated the enzyme activity, which was diluted over 8 mL after the Dye-ligand purification step. Enzyme activity eluted from the MonoQ column in two fractions of 0.75 mL at 0.15 M KCl with a recovery of 61%. A total purification fold of 201 was obtained. If this last purification step was carried out in the absence of Tween 20, almost no sesquiterpene synthase activity would be detected.

Table I. Purification of the chicory (+)-germacrene A synthase from 75 mL of a 100,000*g* supernatant (≈34 g root material).

Purification Step	Protein	Synthase Activity	Specific Activity	Recovery	Purification Factor
	mg	*nmol h^{-1}*	*nmol^{-1} h^{-1} mg^{-1}*	%	
Crude extract (pH 7.0)	34.2	1073	31.3	100	1.0
DEAE	3.95	321	81.3	30	2.6
Green 5	0.45	338	751	31.5	24
Mono-Q	0.033	206	6242	19.5	201

Radio-GC analysis of the incubated MonoQ purified enzyme fractions showed only germacrene A (and its non-enzymatically produced derivatives). No trace of farnesol or any other sesquiterpene was detected.

SDS-PAGE showed that the purification was not complete, since at least three bands were detected after the last purification step (56 kD, 59 kD, and 62 kD). They were also visible when an excess of iodoacetamide was added to the sample immediately before applying it to gel; so these bands did not originate from keratin skin proteins, a common artefact in the 50 to 70 kD range when using silver staining (Ochs, 1983; Görg et al., 1987).

Characterisation of the (+)-Germacrene A Synthase.

A fitted curve (r^2 = 0.951) of the germacrene A synthase activities versus FPP concentration (between 0.5 and 180 μM FPP) gave rise to a typical hyperbolic saturation curve and yielded a K_m value of 6.6 μM (Fig. 6B). The V_{max} was estimated at 8.10^3 nmol h^{-1} mg^{-1} protein. The DEAE-purified sesquiterpene synthase showed a rather broad peak of activity at approximately pH 6.3 with half-maximal activities at pH 5.1 and pH 7.3 (Fig. 6C). After the last (Mono-Q) purification step, the optimum pH was slightly

higher (6.7). In the absence of 0.1% Tween 20, enzyme activity was reduced 5-fold. This preserving/renaturing effect of Tween 20 on sesquiterpene synthase activity has been described by Lewinsohn *et al.* (1992) and Davis *et al.* (1996). The molecular weight of the (+)-germacrene A synthase was estimated at 54 kD (Fig. 6D) with calibrated gel filtration. This is in line with the results obtained from the protein gel. Recovery of enzyme activity from the gel filtration column was 74%.

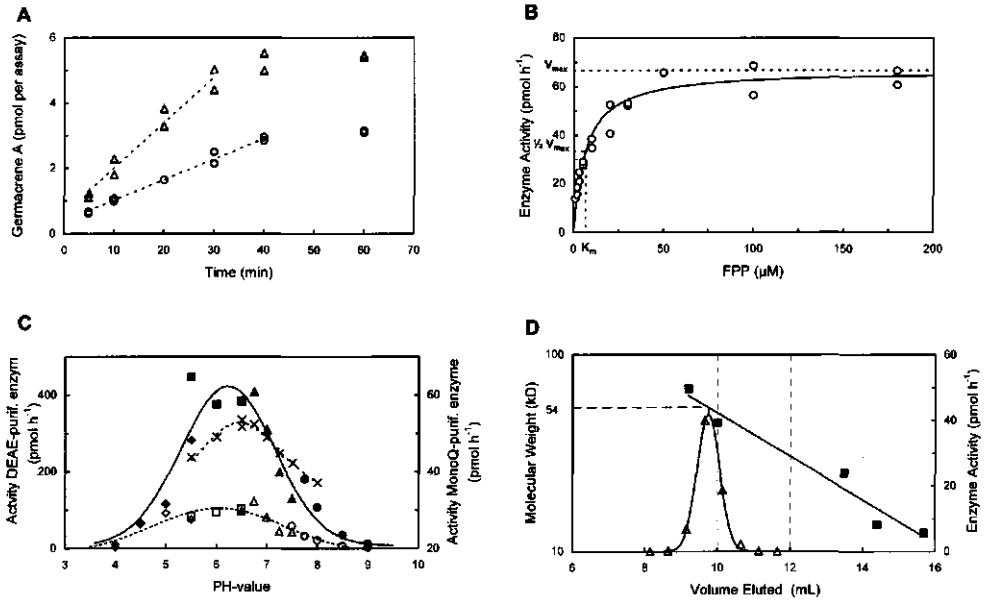


Figure 6. Characterisation of the (+)-germacrene A synthase. A, Linearity of the enzyme assay at 2 µM for undiluted Mono-Q eluent (Δ) and two-fold diluted Mono-Q eluent (O). B, Michaelis-Menten curve featuring a K_m of 6.6 µM and a V_{max} of 66.8 pmol h^{-1}. C, pH curve for DEAE-purified enzyme in the absence (- - -) and presence of 0.1% Tween 20 (——) using NaAc (◊,♦), Mes (□,■), Mopso (Δ,▲), and Tris-HCl (O,●). Also shown is the pH curve for Mono-Q-purified enzyme in the presence of Tween 20 (×). D, Determination of the M_r by calibrated gel filtration. The column is calibrated by measuring the elution volume (■) of cytochrome *c* (12.4 kD), RNase A (13.7 kD), α-chymotrypsinogen (25.0 kD), ovalbumin (45.0 kD), and BSA (67.0 kD). The elution volume of germacrene A synthase activity (Δ) corresponds to an estimated molecular weight of 54 kD.

DISCUSSION

The sole enzymatically produced sesquiterpenoid product in incubations of a 100,000*g* chicory (*Cichorium intybus* L.) root supernatant with FPP was (+)-germacrene A (Fig.7, **1a**). Additional enzymatic cyclisation of this product did not occur. Therefore it can be concluded that this (+)-germacrene A synthase activity, present in both wild and cultivated chicory, represents the first dedicated step in the biosynthesis of bitter compounds in chicory and, more generally, proves the previously proposed mevalonate-FPP-germacradiene pathway in sesquiterpene lactone biosynthesis (Geissman, 1973; Herz, 1977; Fischer et al., 1979; Seaman 1982; Fischer, 1990). Germacrene B, which would be a likely intermediate from a chemical point of view, appears not to be involved in the biosynthesis of C_8-oxygenated guaianolides in chicory.

In line with other enzymes belonging to the group of sesquiterpene synthases, the (+)-germacrene A synthase from chicory operated optimally at approximately pH 6.7 with a rather broad peak of activity. A molecular mass of 54 kD, estimated by calibrated gel filtration, and a K_m value of 6.6 μM were also in the same range as those observed for a number of higher plant sesquiterpene synthases (Croteau and Cane, 1985; Cane, 1990) (Fig. 6).

Germacrene A itself is reported to be a highly unstable compound susceptible to both proton-induced cyclisations and heat-induced Cope rearrangement, and it would be unstable even at -20°C (Fig. 2) (Weinheimer et al., 1970; Bowers et al., 1977; Teisseire 1994). Present experiments show otherwise. When silica and/or too large amounts of $MgSO_4$ were used during the assay extraction-filtration procedure, approximately one-half of the germacrene A was indeed cyclised toward α–selinene (**3**), β–selinene (**4**), and selina-4,11-diene (**5**). The latter compound has not been reported before in this context, but originates from the same intermediate carbocation as the other two selinenes. Using neutral aluminium oxide instead of the slightly acidic silica effectively minimised the non-enzymatic cyclisation, and this cyclisation was further minimised by the use of sodium sulphate instead of magnesium sulphate. Cope rearrangement of germacrene A did not occur, not even during incubations at 30°C overnight.

Cope rearrangement to β–elemene (**2**) can be a problem in GC measurements due to the high temperatures involved. However, reducing the injection port temperature to 150°C greatly diminished Cope rearrangement. If cold on-column injection is applied, no Cope

rearrangement will be observed at all. Rearrangement of germacrene A and germacrene B during GC-MS measurement was also detected by Wichtman and Stahl-Biskup (1987), whereas the influence of the GC injection port temperature on Cope rearrangement was studied for germacrone by Reichardt *et al.* (1989). Nevertheless, high injection port temperatures in combination with enantioselective gas chromatography proved to be very useful in determining the absolute configuration of the germacrene A formed by the isolated enzyme. The heat-induced Cope rearrangement is stereospecific and the chiral centre at C_7 is not involved in this reaction (Weinheimer et al., 1970; Takeda, 1974; March, 1992) (Fig. 4). Since only (-)-β–elemene (**2a**) was obtained and not (+)-β–elemene (**2b**), the enzymatic product can be designated as the (+)-enantiomer of germacrene A (**1a**) (Figs. 4, 5 and 7).

In chicory, just as in the majority of higher plants, sesquiterpene lactones possess an α-methylene-γ-lactone ring in which the proton at the C_7-position of the sesquiterpenoid framework is, without exception, α-oriented (Fischer 1990). Therefore, the absolute configuration of (+)-germacrene A corresponds with its biochemical fate, and the configuration is already determined in the first step of sesquiterpene lactone biosynthesis (Fig. 7).

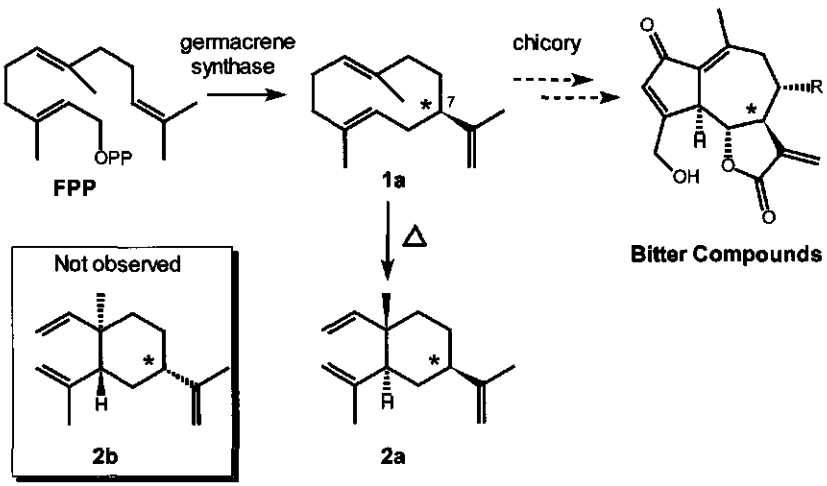


Figure 7. Upon heating, the (+)-germacrene A (**1a**) produced in the enzyme assay rearranges towards (-)-β-elemene (**2a**) preserving the configuration of its chiral centre. Since chicory does not produce (-)-germacrene A, no (+)-β-elemene (**2b**) was observed. The configuration of C_7 in (+)-germacrene A (**1a**) is in accordance with the configuration of the corresponding carbon atom in the sesquiterpene lactones of chicory.

Chappell and co-workers have elucidated the crystal structure of 5-epi-aristolochene synthase and unravelled its enzymatic mechanism, which must be similar to that of vetispiradiene synthase as several constructed epi-aristolochene-vetispiradiene chimeras demonstrated. In a first step FPP is bound to the enzyme and dephosphorylated, generating germacrene A. The germacrene A intermediate is then once more cyclised toward a eudesmane carbocation whose final destination, either epi-aristolochene or vetispiradiene, depends upon the particular active site conformation of the sesquiterpene synthase involved (Back and Chappell, 1996; Starks et al., 1997).

The existence of the germacrene A intermediate has also been revealed in incubations of epi-aristolochene synthase using the anomalous substrate (7*R*)-6,7-dihydrofarnesyl diphosphate instead of FPP. In these experiments the intermediate dihydrogermacrene A is released because it cannot be further cyclised to the eudesmane (Cane and Tsantrizos, 1996). More recently germacrene A has also been found as a minor side product of the germacrene C synthase from cherry tomato (*Lycopersicon esculentum* cv. VFNT) (Colby et al., 1998), and as one of the 34 products produced by the δ-selinene synthase of grand fir (*Abies grandis*) (Steele et al., 1998).

It is generally assumed that the germacrene A intermediate is involved in the biosynthesis of numerous eudesmane- and eremophilane-type sesquiterpenes (Beale, 1990); however, in most cases it is not detected because it remains bound to the sesquiterpene synthase as a carbocation and undergoes additional electrophilic cyclisations and rearrangements, before the final product is released (Cane et al., 1990; Cane and Tsantrizos, 1996; McCaskill and Croteau, 1997; see also chapter 1). Nevertheless, various species such as the liverwort *Frullannia tamarisci* (W.A. König, unpublished results), caraway (*Carum carvi* L.) (Wichtman and Stahl-Biskup, 1987), the gorgonian *Eunice mammosa* (Weinheimer et al., 1970), and the spotted alfalfa aphid (*Therioaphis maculata*) (Bowers et al., 1976), do contain germacrene A and should therefore also contain the corresponding germacrene A synthase.

It is rather peculiar that chicory contains an enzyme that releases germacrene A, whereas the major sesquiterpene lactones contain a guaiane or eudesmane framework. Why is FPP not immediately cyclised, within one enzymatic step, towards a eudesmane or a guaiane by a (hypothetical) eudesmane synthase or guaiane synthase? In other words, why is germacrene A released by the chicory sesquiterpene synthase instead of being subjected to a second cyclisation step in the active site of the enzyme? It seems that in sesquiterpene lactone biosynthesis the formation of the lactone ring by oxidative enzymes precedes any further cyclisation of the germacrene skeleton (Cordell, 1976). Biosynthesis of the guaianolides and

eudesmanolides is assumed to occur via a common germacrene lactone intermediate, i.e. costunolide, and cyclisation to either a guaianolide or a eudesmanolide is apparently mediated by other enzymes than a sesquiterpene synthase.

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3. Biosynthesis of Germacrene A Carboxylic Acid

Demonstration of a Cytochrome P450 (+)-Germacrene A Hydroxylase and NADP$^+$-Dependent Sesquiterpenoid Dehydrogenase(s) Involved in Sesquiterpene Lactone Biosynthesis of Chicory Roots

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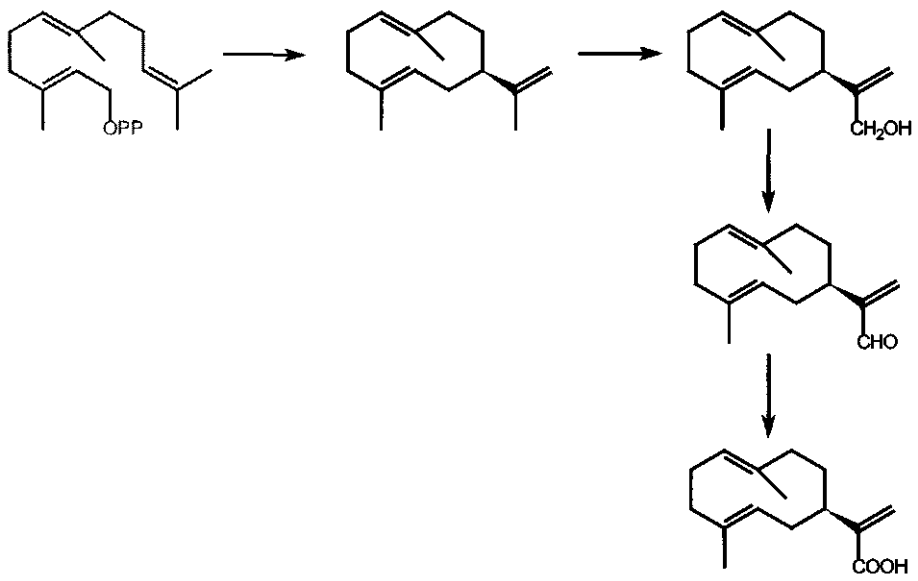

Abstract: The committed step in the biosynthesis of the sesquiterpene lactones present in chicory is catalysed by a (+)-germacrene A synthase. Formation of the lactone ring is the postulated next step in the biosynthesis of the germacrene-derived sesquiterpene lactones. The present study confirms this hypothesis by isolation of enzyme activities from chicory roots that introduce a carboxylic acid function in the isopropenyl group of germacrene A, which is necessary for lactone ring formation. (+)-Germacrene A is hydroxylated to germacra-1(10),4,11(13)-trien-12-ol by a cytochrome P450 enzyme, and is subsequently oxidised to germacra-1(10),4,11(13)-trien-12-oic acid by NADP$^+$-dependent dehydrogenase(s). Both oxidised germacrenes were detected as their Cope rearrangement products elema-1,3,11(13)-trien-12-ol and elema-1,3,11(13)-trien-12-oic acid, respectively. The acid-catalysed cyclisation products of germacra-1(10),4,11(13)-trien-12-ol, i.e. costol, were also observed. The (+)-germacrene A hydroxylase is inhibited by carbon monoxide (blue-light reversible), has an optimum pH at 8.0, and hydroxylates β-elemene with a modest degree of enantioselectivity.

INTRODUCTION

The germacranolides are by far the largest group of naturally occurring sesquiterpene lactones and the majority of sesquiterpene lactones are thought to evolve from this group. The simplest member of the germacranolides (+)-costunolide (Fig. 1, **5**) is generally accepted as the common intermediate of all germacranolide-derived lactones (Geissman, 1973; Herz, 1977; Fischer et al., 1979; Seaman, 1982; Song et al., 1995). (+)-Costunolide was first isolated from costus roots (*Saussurea lappa* Clarke) by Paul et al. (1960) and Somasekar Rao et al. (1960), and has since been reported together with other sesquiterpene lactones in various plant species (Fischer et al., 1979). Among them is lettuce (*Lactuca sativa*), a species that is closely related to chicory and also contains the bitter tasting compounds lactucin and lactucopicrin (Takasugi et al, 1985; Price et al., 1990).

In Chapter 2 it was demonstrated that the sesquiterpenoid skeleton of the sesquiterpene lactones in chicory is formed by a (+)-germacrene A synthase, which cyclises FPP to (+)-germacrene A (Fig. 1, **1**). This (+)-germacrene A is not further transformed into a guaiane or a eudesmane, indicating that functionalisation of the molecule precedes its cyclisation. Studies on the biosynthesis of santonin (Barton et al., 1968) suggested that lactone formation precedes any other oxidation of the sesquiterpenoid ring system (Cordell, 1976), and various authors have proposed a biosynthetic route from (+)-germacrene A (**1**) toward (+)-costunolide (**5**) (Fig. 1) (Geissman, 1973; Herz, 1977; Seaman, 1982; Fischer, 1990; Song et al., 1995). In this hypothetical route (+)-germacrene A (**1**) is hydroxylated to germacra-1(10),4,11(13)-trien-12-ol (**2**), which is further oxidised via germacra-1(10),4,11(13)-trien-12-al (**3**) to germacra-1(10),4,11(13)-trien-12-oic acid (**4**). The germacrene acid is thought to be hydroxylated at the C_6-position and subsequent loss of water should lead to the formation of a lactone ring such as present in (+)-costunolide (**5**).

However, germacrenes are notoriously unstable compounds, susceptible to proton-induced cyclisations and heat induced (e.g. steam distillation, GC-analysis) Cope rearrangement (Takeda, 1974; Bohlman et al., 1983; Reichardt et al., 1988; Teisseire, 1994; Chapters 2 and 4). Until their isolation from costus roots as described in Chapter 4, none of the intermediates between (+)-germacrene A and (+)-costunolide had ever been reported, apart from germacra-1(10),4,11(13)-trien-12-al (**3**) that was isolated with greatest difficulty from *Vernonia glabra* and could not be separated from its cyclisation product costal (**13**) (Bohlman et al, 1983). Most likely as a result of this instability, the hypothetical biosynthetic route for (+)-costunolide has merely been based on the isolation from costus roots of the Cope

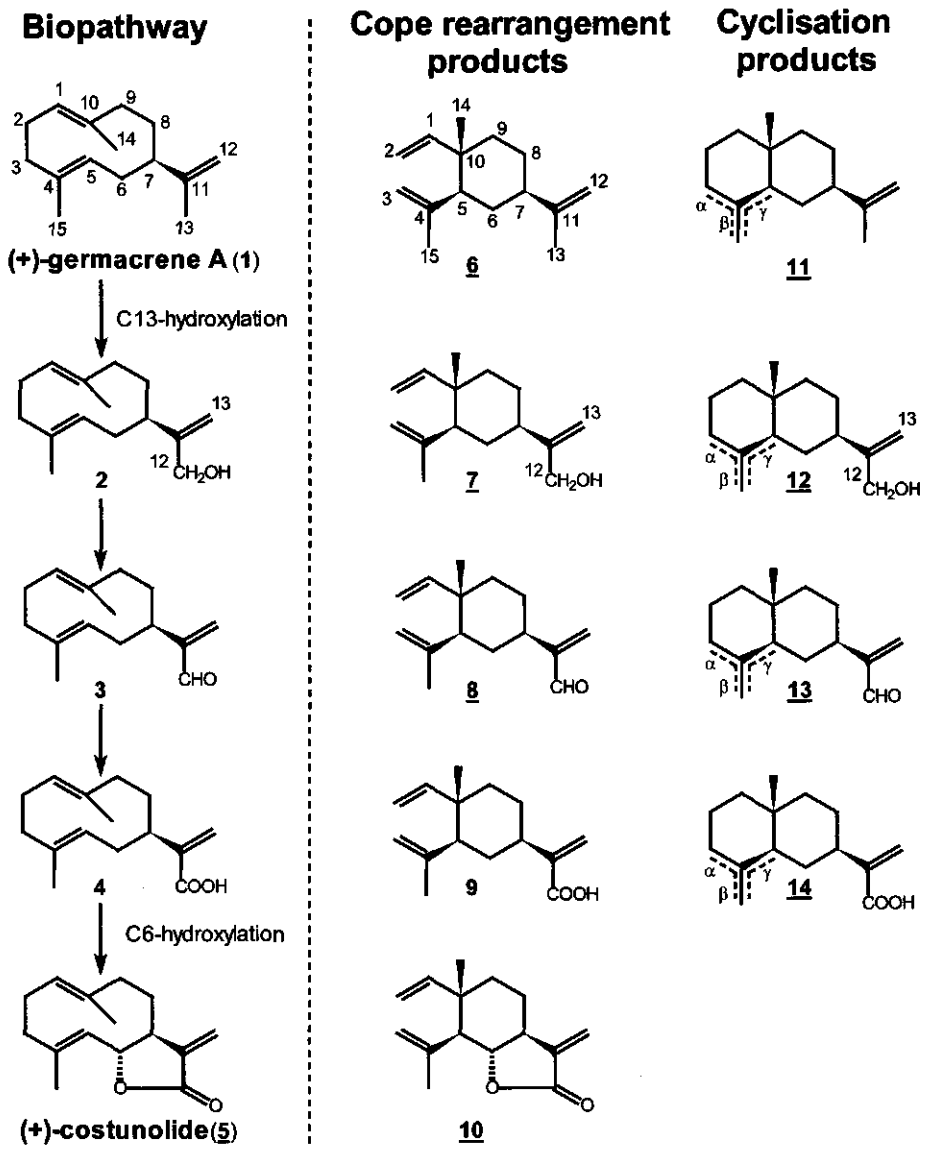

Figure 1. Proposed biosynthetic route from (+)-germacrene A (**1**) to (+)-costunolide (**5**) via germacra-1(10),4,11(13)-trien-12-ol (**2**), germacra-1(10),4,11(13)-trien-12-al (**3**), and germacra-1(10),4,11(13)-trien-12-oic acid (**4**). At the right side of the dotted line, compounds are shown that can be formed from these unstable germacrenes: the heat induced Cope rearrangement products (-)-β-elemene (**6**), (-)-elema-1,3,11(13)-trien-12-ol (**7**), (-)-elema-1,3,11(13)-trien-12-al (**8**), elema-1,3,11(13)-trien-12-oic acid (**9**), and dehydro-saussurea lactone (**10**); and the acid induced cyclisation products selinene (**11**) (γ-selinene is usually named selina-4,11-diene), costol (**12**), costal (**13**), and costic acid (**14**). Compounds with underlined numbers have all been identified in costus roots; (+)-germacrene A (**1**) and germacra-1(10),4,11(13)-trien-12-al (**3**) were isolated from other plant species. Note that after hydroxylation the numbering of carbon atoms 12 and 13 is inverted.

rearrangement products (-)-elema-1,3,11(13)-trien-12-ol (**7**) and (-)-elema-1,3,11(13)-trien-12-al (**8**), and the proton-induced cyclisation products costol (**12**), costal (**13**) and costic acid (**14**) (Bawdekar and Kelkar, 1965; Bawdekar et al., 1967; Maurer and Grieder, 1977). Besides the reported (+)-germacrene A synthase of Chapter 2, thus far no other enzyme has been isolated that might be involved in this proposed pathway from FPP to (+)-costunolide.

The aim of the research presented in this chapter is to find enzymes in chicory that are involved in oxidation of the isopropenyl group of germacrene A, and thus to investigate the initial steps in the formation of the lactone ring, as present in (+)-costunolide and other germacrene-derived sesquiterpene lactones.

MATERIALS AND METHODS

Materials

Fresh roots of cultivated chicory (*Cichorium intybus* L., cv Focus) harvested during late summer were obtained from a grower in Veenendaal, the Netherlands. The chicory roots were cut into small pieces, frozen in liquid nitrogen, and stored at -80°C. An FPP solution was obtained from Sigma and concentrated to 10 μM (Chapter 2); [1(n)-^{3}H]FPP (16.0 Ci mmol^{-1}, 200 μCi mL^{-1}) was obtained from Amersham. *trans,trans*-Farnesol, *cis*-nerolidol, and *trans*-caryophyllene were purchased from Fluka. (±)-β-Elemene and (-)-β-elemene (**6**) were a gift from Prof. Dr. W.A. König (Hamburg University, Germany). (-)-Elema-1,3,11(13)-trien-12-ol (**7**) was a gift from Dr. B. Maurer (Firmenich SA, Geneva, Switzerland). Spectra and retention times of costol (**12**) were recorded from costus root oil (Pierre Chauvet SA, Seillans, France; Maurer and Grieder, 1977). Ether (diethyl ether) and pentane were redistilled before use.

Preparation of Elema-1,3,11(13)-trien-12-oic acid (8)

(-)-Elema-1,3,11(13)-trien-12-al (**8**) necessary for the synthesis of elema-1,3,11(13)-trien-12-oic acid was isolated from 5 g of costus root oil that had been stirred twice for 6 h with 10 g of MnO_2 (Merck) in 100 mL of pentane. The aldehydes and ketones were extracted from the oil with 3 g of Girard P reagent and were separated by column chromatography as described by Maurer and Grieder (1977). It yielded 13 mg (-)-elema-1,3,11(13)-trien-12-al (**8**), 15 mg β-costal (**13**), 22 mg of a α/γ-costal mixture (**13**), and smaller amounts of

trans-bergamota-2,12-dienal and ionone. To obtain elema-1,3,11(13)-trien-12-oic acid (**9**), 2 mg of the corresponding aldehyde was dissolved in 1.2 mL of *tert*-butyl alcohol and 0.3 mL of 2-methyl-2-butene to which 0.5 mL of a solution of sodium dihydrogen phosphate and sodium chlorite (0.1 g mL^{-1} each) was carefully added (Balkrishna et al, 1981). The mixture was vigorously shaken overnight at room temperature. Volatile components were removed under vacuum, and the residue was dissolved in 1.5 mL of demineralised water and 20 µL of 2-methyl-2-butene. On ice, the aqueous layer was acidified with 20 µl of 5 M HCl to pH 3 and quickly extracted with four portions of 1.5 mL of ether. The combined ether layers were washed with 1 mL of cold demineralised water and dried with $MgSO_4$. After evaporation of the ether, elema-1,3,11(13)-trien-12-oic acid (**9**) was obtained as a white powder (1.5 mg). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.04 (s, 3H, Me_{14}); δ 1.2-1.7 (m, 6H); δ 1.74 (br s, 3H, Me_{15}); δ 2.11 (dd, 1H, H_5, $J_{5,6}$ = 3.4 Hz, $J_{5,6'}$ = 13 Hz); δ 2.54 (dddd, 1H, H_7, $J_{6,7} = J_{7,8}$ = 3 Hz, $J_{6',7} = J_{7,8'}$ = 12 Hz); δ 4.60 (br s, 1H, H_3); δ 4.80 (m, 1H, H_3'); δ 4.92 (dd, 1H, H_2, $J_{1,2}$ = 10 Hz, $J_{2,2'}$ = 1.4 Hz); δ 4.96 (dd, 1H, $H_{2'}$, $J_{1,2'}$ = 18 Hz, $J_{2,2'}$ = 1.4 Hz); δ 5.71 (s, 1H, H_{13}); δ 5.85 (dd, H_1, $J_{1,2}$ = 10 Hz, $J_{1,2'}$ = 18 Hz); δ 6.33 (s, 1H, $H_{13'}$). ^{13}C-NMR (100 MHz, DEPT, $CDCl_3$) δ 17.0 (q), δ 25.3 (q), δ 27.6 (t), δ 30.1 (s), δ 33.7 (t), δ 39.8 (d), δ 40.1 (t), δ 52.9 (d), δ 110.5 (t), δ 112.7 (t), δ 125.2 (t), δ 145.0 (s), δ 147.8 (s), δ 150.4 (d), δ 171.5 (s). EIMS *m/z* (rel int) M^+ 234 (1), $[M\text{-}Me]^+$ 219 (7), 81 (100), 67 (50), 79 (47), 41 (43), 68 (40), 91 (38), 53 (34), 93 (29), 105 (29), 55 (26), 77 (26), 107 (26), 121 (23), 119 (19), 69 (17), 177 (15).

Enzyme Isolation and Assay for (+)-Germacrene A Hydroxylase Activity

A cell-free extract of chicory roots was prepared that contains both the sesquiterpene synthase (germacrene A synthase) and the microsomal bound cytochrome P450 enzymes (e.g. sesquiterpene hydroxylases), similar to the approach of Threlfall and Whitehead (1988) that had demonstrated hydroxylation of 5-epi-aristolochene in tobacco (Whitehead et al, 1989). Twenty-five grams of frozen root material was homogenised in a Sorvall Omni-mixer (Newtown, CT, USA) with 2.5 g of insoluble polyvinylpolypyrrolidone and 40 mL of buffer containing 50 mM Tris (pH 7.5), 50 mM sodium *meta*-bisulfite, 50 mM ascorbic acid, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2.5 mM EDTA (disodium), 5 µM FAD, 5 µM FMN, and 20% (v/v) glycerol (buffer A). The slurry was filtered through pre-moistened cheesecloth with an additional 10 mL of buffer and centrifuged for 20 min at 20,000*g* at 4°C. The supernatant was filtered through rough glass wool and desalted with an Econo-Pac 10DG column (Biorad) to buffer B. This buffer contained 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 1 mM ascorbic acid, 5 µM FAD, 5 µM FMN, 10% (v/v) glycerol, and 6 mM

sodium orthovanadate to suppress phosphohydrolase activity (Croteau and Karp, 1979). A 1-mL aliquot of the desalted supernatant was incubated with 20 μM [^{3}H]FPP (50 Ci mol^{-1}) for 1 h at 30°C in the absence or presence of an NADPH-regenerating system. The NADPH-regenerating system consisted of 1 mM NADPH, 5 mM glucose-6-phosphate, and 1.2 IU glucose-6-phosphate dehydrogenase (all from Sigma).

After incubation, the assay mixture was extracted twice with 1 mL of 20% (v/v) ether in pentane, and the organic phase was filtered through a glasswool-plugged (dimethyl chlorosilane-treated glasswool; Chrompack) Pasteur pipette that contained 0.90 g aluminium oxide (grade III) and a little anhydrous $MgSO_4$. The column was washed with 1.5 mL ether and the extract was carefully concentrated to approximately 50 μl under a stream of nitrogen. Samples of 1 μL were analysed by radio-GC and GC-MS. Radio-GC analyses was achieved by cold on column injection as described in Chapter 2, using a final oven temperature of 240°C. Signals of the FID and radioactivity detector were synchronised with [^{3}H]farnesol (a gift of dr. E. Wallaart, University of Groningen). GC-MS analysis was essentially carried out as described in Chapter 2 at an injection port temperature of 250°C and an oven programmed for 4 min at 55°C followed by a ramp of 5°C to 280°C.

Assay for Sesquiterpenoid Dehydrogenase Activities

The involvement of dehydrogenases in the oxidation of germacra-1(10),4,11(13)-trien-12-ol (**2**) was initially investigated with (-)-elema-1,3,11(13)-trien-12-ol (**7**). A 20,000*g* supernatant was prepared as described above, but $MgCl_2$, EDTA, and flavins were omitted from buffer A. The supernatant was desalted to buffer C containing 25 mM glycine (pH 10.0), 10% (v/v) glycerol and 2 mM DTT (Bouwmeester et al., 1998). Aliquots of 1 mL were incubated at 30°C with 100 μM of substrate and 1 mM NAD^+, 1 mM $NADP^+$, or no cofactor. Boiled controls were included. After 90 min, 20 μM *cis*-nerolidol was added as internal standard and each incubation was acidified with 20 μL of 5M HCl. The incubation mixture was extracted three times with 1 mL of ether, and the combined ether layers were filtered through a Pasteur pipette that contained 0.45 g silica and a little anhydrous $MgSO_4$. The pipette was rinsed with 1 mL of ether and the extract was concentrated to approximately 150 μL and analysed by GC-MS. Experiments were repeated with 200 μM of (-)-elema-1,3,11(13)-trien-12-al (**8**).

[^{3}H]Germacra-1(10),4,11(13)-trien-12-ol (**2**) was produced in (+)-germacrene A hydroxylase assays that consisted of 1.5 mL of supernatant and 50 μM [^{3}H]FPP (40 Ci mol^{-1}). The pentane-ether extracts of two of such assays were combined, concentrated to

approximately 10 μL, and added to one dehydrogenase assay. After incubation, the reaction mixture was carefully acidified with 0.5 M HCl to pH 3 and extracted with ether. The extract was concentrated to approximately 50 μL and analysed by radio-GC and GC-MS.

Characterisation of the (+)-Germacrene A Hydroxylase

(+)-Germacrene A (**1**) necessary for characterisation of the (+)-germacrene A hydroxylase was produced in 1.5 mL enzyme assays consisting of 150,000*g* supernatant, 50 μM FPP (Chapter 2), and an additional 6 mM sodium orthovanadate. The pentane-ether extracts of several of these incubations were combined and the volume was carefully reduced under a flow of nitrogen. The concentration of (+)-germacrene A was estimated by comparison with a 10 μM *trans*-caryophyllene solution using GC-MS; in general, one incubation of 50 μM FPP with 150,000*g* supernatant would give sufficient substrate for one hydroxylase experiment (approx. 10 μM germacrene A).

The optimum pH of the (+)-germacrene A hydroxylase was determined by desalting the 20,000*g* supernatant to buffer B without $MgCl_2$, in which 25 mM Tris was replaced by 50 mM Bis-Tris of pH 6.5 to 9.0 in 0.5-unit increments. After 30 min of incubation, 5 μM of *cis*-nerolidol was added as internal standard and the incubation mixtures were extracted and analysed by GC-MS. Areas of the product peak were compared to those of *cis*-nerolidol.

Inhibition of the (+)-germacrene A hydroxylase by established cytochrome P450 inhibitors was demonstrated with microsomal pellets. They were prepared by centrifugation of 8 mL of 20,000*g* supernatant (without $MgCl_2$) at 150,000*g* and stored at - 80°C under argon. Before use the pellets were pottered in 2 mL of buffer B without $MgCl_2$, combined, and divided into 1-mL aliquots. Inhibitors were dissolved in ethanol: clotrimazole (10 mM), miconazole (10 mM), aminobenzotriazole (10 mM), and metyrapone (100 mM); cytochrome *c* (10 mM) was dissolved in buffer. Ten microliters of one of these solutions was added to 1 mL of enzyme preparation 15 min prior to the addition of (+)-germacrene A and NADPH-regenerating system, after which the incubations were continued for 1 hour. Inhibitory effects were expressed relative to a control for the solvent, and each inhibitor was tested in duplicate.

For demonstration of blue-light reversible CO-inhibition of (+)-germacrene A hydroxylase activity, a mixture of 80% CO and 20% O_2 was prepared from pure CO and O_2. In 4.5-mL septum-capped vials, 50 μM FPP and an NADPH-regenerating system were added to 1 mL of 20,000*g* supernatant that had been desalted to buffer B devoid of flavins. Reaction mixtures were slowly bubbled with 50 mL of the gas mixture via a needle inserted through the (vented) septum. CO-treated vials and non-CO-treated control vials were incubated on a rotary shaker

in a climate chamber at 30°C in blue light or protected from the light by aluminium foil. Blue light was obtained by passing a beam of a 100 W H44GS-100 mercury lamp (Sylvania, Winchester, KY, USA; ballast number 1A024, Grainger, Morton Grove, IL, USA) through a TLC chamber (width 7 cm) filled with a 10% $CuSO_4$ solution (Karp et al., 1987). After 1 h of incubation, 5 μM *cis*-nerolidol was added as internal standard and the assays were extracted and analysed by GC-MS. Each treatment was done in triplicate. In similar experiments the effects of an Argon atmosphere, NADH, and the absence of flavins were tested on (+)-germacrene A hydroxylase activity.

Enantioselectivity of the (+)-Germacrene A Hydroxylase

The enantioselectivity of the (+)-germacrene A hydroxylase was investigated with (-)-β-elemene (**6**) and (±)-β-elemene, as no source of (-)-germacrene A was available. Aliquots of 1 mL of resuspended microsomal pellet were incubated with 20 μM β-elemene in the presence or absence of an NADPH-regenerating system. Extracts of the enzyme assay were analysed on an enantioselective column (Chapter 2) programmed at 45° for 4 min, a ramp of 2°C min^{-1} to 170°C, and a final time of 10 min; spectra were recorded in Scan mode and Selected Ion Monitoring Mode (m/z 119, 121, 145, 147 and 189). In a separate experiment, 100 μM of (-)-β-elemene was added to the standard germacrene A hydroxylase assay to demonstrate whether it competed as a substrate with [^{3}H]germacrene A. Production of [^{3}H]germacratrien-12-ol was measured by radio-CG and compared with that of incubations where only 10 μL of ethanol (the solvent of β-elemene) had been added.

RESULTS

Demonstration of (+)-Germacrene A Hydroxylase Activity

Radio-GC analysis of the pentane-ether extract from the incubation of a 20,000*g* chicory root supernatant with [^{3}H]FPP revealed a peak of germacrene A, as a result of the (+)-germacrene A synthase present in the supernatant (Fig. 2A). In the presence of NADPH, 60% of the (+)-germacrene A was converted into a more polar compound that eluted from the GC-column at a higher temperature (Fig. 2B). In both incubations a small amount of farnesol was also formed due to non-specific phosphohydrolases, but it was efficiently reduced by the addition of 6 mM sodium orthovanadate to the assay buffer (Croteau and Karp, 1979).

Farnesol formation decreased even further in the presence of an NADPH-regenerating system, possibly because glucose-6-phosphate (1 mM) acts as a competitive inhibitor on the phosphohydrolases.

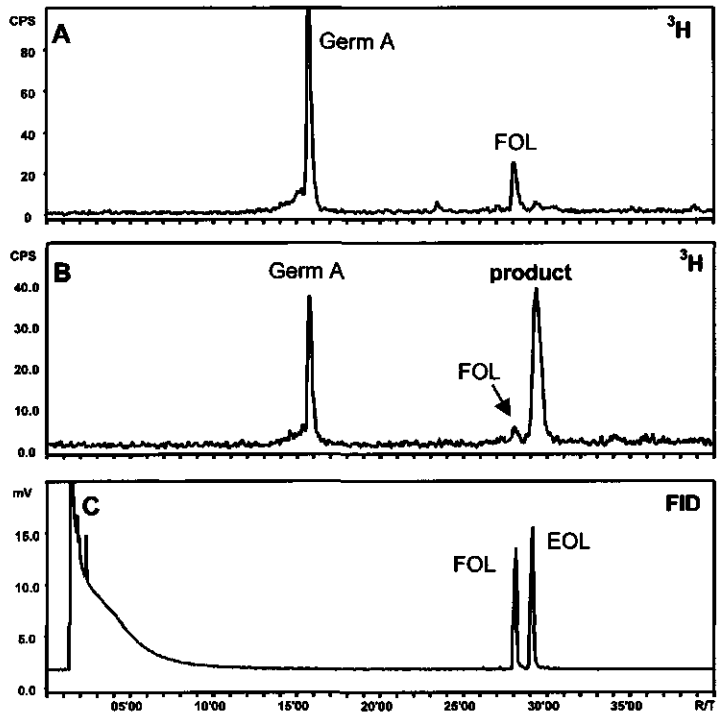

Figure 2. Radio-GC analyses of the products formed in incubations of a 20,000*g* supernatant from chicory roots with [^{3}H]FPP in the absence (A) or presence (B) of a NADPH-regenerating system. In the presence of NADPH the produced [^{3}H]germacrene A (Germ A) is converted into a more polar product. Panel C shows the response of the FID to a standard solution of *trans,trans*-farnesol (FOL) and (-)-elema-1,3,11(13)-trien-12-ol (EOL), injected under the same GC conditions.

In GC-MS measurements at an injection port temperature of 250°C, germacrene A was detected as its Cope rearrangement product β-elemene (**6**). The unknown product was displayed as a sharp peak with the same retention time and mass spectrum as (-)-elema-1,3,11(13)-trien-12-ol (**7**), the Cope rearrangement product of germacra-1(10),4,11(13)-trien-12-ol (**2**). Lowering the injection port temperature to 150°C yielded the slightly fronting peak of germacrene A (Chapter 2), but the peak of elema-1,3,11(13)-trien-12-ol was replaced by a broad "hump" in the baseline that started at the position of elema-1,3,11(13)-trien-12-ol and stretched over a two minutes period. If the germacrene alcohol is not rearranged in the injection port, it apparently rearranges during migration through the

GC-column (HP5-MS) to the faster migrating elema-1,3,11(13)-trien-12-ol and is observed as a broad 'peak' similar to the one described for 7-hydroxygermacrene (Stahl, 1984). Germacra-1(10),4,11(13)-trien-12-ol is also expected to rearrange on the column of the radio-GC (cold on column injection), but was nevertheless detected as a somewhat broadened peak that co-elutes with a standard of elema-1,3,11(13)-trien-12-ol (Fig. 2B+C). It appears that on the Carbowax column of the radio-GC there is only little difference in retention time between the elemene and germacrene alcohol.

Considerable amounts of costol (**12**) were detected when, during the extraction-filtration procedure, aluminium oxide was replaced by silica gel and larger amounts of $MgSO_4$ were used (data not shown). Costol is the acid induced cyclisation product of germacra-1(10),4,11(13)-trien-12-ol, just as costal (**13**) is derived from germacra-1(10),4,11(13)-trien-12-al (**3**) (Bohlman et al., 1983; Chapter 4). Its observation confirms the conclusion that (+)-germacrene A is enzymatically converted into the somewhat unstable germacra-1(10),4,11(13)-trien-12-ol. Further oxidation of the germacrene alcohol was not observed in any of the (+)-germacrene A hydroxylase assays.

Sesquiterpenoid Dehydrogenase Activities

Incubations of (-)-Elema-1,3,11(13)-trien-12-ol (7) and (-)-Elema-1,3,11(13)-trien-12-al (8)

(-)-Elema-1,3,11(13)-trien-12-ol (**7**) (100 μM) was converted into elema-1,3,11(13)-trien-12-oic acid (**9**) by a 20,000*g* chicory root supernatant in the presence of $NADP^+$ at pH 10 (Fig. 3B+D). This reaction did not take place in the presence of NAD^+ or without cofactor (Fig. 3A). After centrifugation at 200,000*g*, dehydrogenase activity was retained in the supernatant, showing that it originates from soluble enzyme(s). Elema-1,3,11(13)-trien-12-al (**8**) was only detected in minute quantities, which might be explained by the chemical reactivity of the isopropenal group. Elema-1,3,11(13)-trien-12-al added in 10 μM concentration to a solution of 0.5 mg/mL bovine serum albumin (a protein concentration similar to the supernatant) or 2 mM DTT was only extractable for 20 to 50% in comparison with equal amounts of elematrien-12-al that had been added to demineralised water. DTT could, however, not be omitted from the assay buffer, since no dehydrogenase activity was detected in its absence, which is consistent with the literature about dehydrogenases (Kjonaas et al., 1985; Ikeda et al., 1991).

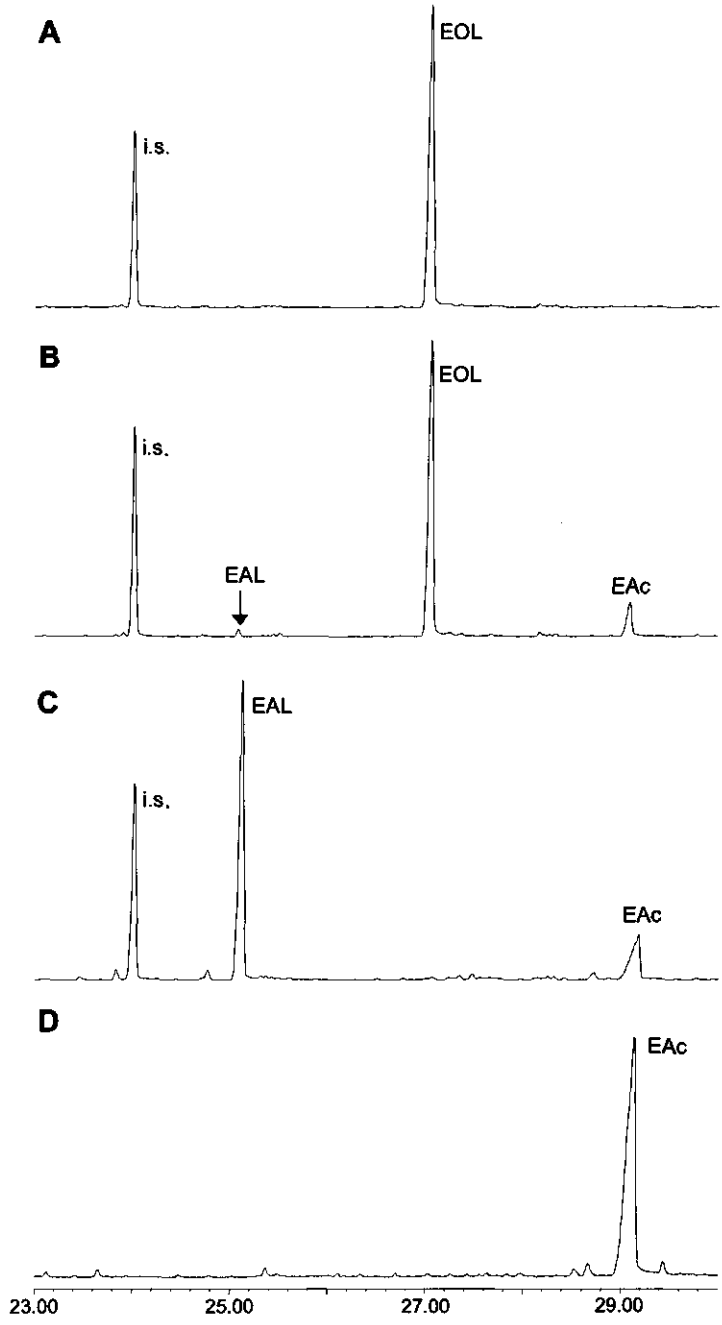

Figure 3. Identification by GC-MS of the products formed by a 20,000*g* supernatant from chicory roots incubated with (-)-elema-1,3,11(13)-trien-12-ol (**7**) (EOL) in the absence (A) or presence of $NADP^+$ (B); or with (-)-elema-1,3,11(13)-trien-12-al (**8**) (EAL) and NAD^+ (C). The produced elema-1,3,11(13)-trien-12-oic acid (EAc) (**9**) has the same retention time as the synthesised standard (D).

(-)-Elema-1,3,11(13)-trien-12-al (**8**) (200 μM) was oxidised to elema-1,3,11(13)-trien-12-oic acid most effectively in the presence of NAD^+ (Fig. 3C), whereas a smaller aldehyde dehydrogenase activity (≤15 % of maximum activity) was detected in the presence of $NADP^+$ or in the absence of any cofactor. Boiled controls showed no conversion at all. Enzyme activity without cofactor has also been observed for the abietadienal dehydrogenase of grand fir (Funk and Croteau, 1994).

Incubation of [^{3}H]Germacra-1(10),4,11(13)-trien-12-ol (2)

Incubation of [^{3}H]germacra-1(10),4,11(13)-trien-12-ol (**2**) with 20,000*g* supernatant and $NADP^+$ showed a new broadened peak in the [^{3}H]trace of the radio-GC, whose front co-elutes with a standard of elema-1,3,11(13)-trien-12-oic acid (**9**) (Fig. 4). During GC-MS analysis a small peak was detected with the mass spectrum and retention time of elema-1,3,11(13)-trien-12-oic acid. This peak should result from Cope rearrangement of germacra-1(10),4,11(13)-trien-12-oic acid (**4**) in the GC-MS injection port. Formation of the germacrene/elemene aldehyde was not detected.

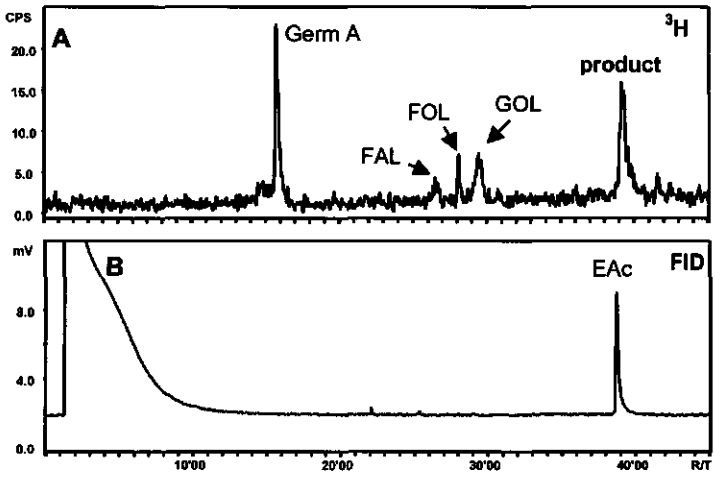

Figure 4. A, Radio-GC analyses of the products formed in an incubation of 20,000*g* supernatant with $NADP^+$ and a pentane-ether extract from the (+)-germacrene A hydroxylase assay of Figure 2B, containing [^{3}H]germacra-1(10),4,11(13)-trien-12-ol (**2**; GOL) and smaller amounts of [^{3}H]germacrene A (**1**; Germ A) and [^{3}H]farnesol (FOL). A more polar product is formed together with a minute amount of [^{3}H]-farnesal (FAL). Trace B shows the response of the FID to a standard solution of elema-1,3,11(13)-trien-12-oic acid (**9**; EAc).

Characterisation of the (+)-Germacrene A Hydroxylase

Hydroxylation of (+)-germacrene A was optimal at a pH of 8.0 with 60% of maximal enzyme activity at pH 7.5 and 9.0 (no difference in activity between Bis-Tris and Tris). The reaction required NADPH. NADH was less effective as a reductant (Table I). The combination of both cofactors showed an additive effect, which is not uncommon for plant cytochrome P450 systems and is believed to result from the participation of both NADH:cytochrome *b* reductase and NADPH:cytochrome P450 reductase in electron transfer to cytochrome P450 (West, 1980; Funk and Croteau, 1994). Flushing the reaction mixture for 1.5 min with Argon prior to incubation caused a 69% decrease of enzyme activity because of O_2 depletion. An assay buffer without flavins (FAD and FMN) gave 18% loss of hydroxylase activity, however, omitting these flavins from the extraction buffer resulted in a loss of more than 70% in enzyme activity (data not shown).

Table I. Requirements for (+)-germacrene A hydroxylase activity

Addition[a]	Percentage enzyme activity[b] ± SD
None	3 ± 0.5
1 mM NADPH	100 ± 9.8
1 mM NADH	30 ± 4.7
1 mM NADPH + 1mM NADH	120 ± 11
1 mM NADPH – flavins	82 ± 4.8
1 mM NADPH + argon atmosphere	31 ± 5.1

[a]Flavins were present during incubation, unless otherwise mentioned; NAD(P)H was regenerated during incubation

[b]100% enzyme activity corresponds to an elematriene-12-ol peak size of 0.96 × internal standard (5 nmol *cis*-nerolidol in each assay).

These results and the observation that the enzyme activity resided in the 150,000*g* pellet support the involvement of a cytochrome P450 enzyme in the hydroxylation of (+)-germacrene A. This was confirmed by the effect of cytochrome P450 inhibitors on (+)-germacrene A hydroxylase activity: cytochrome *c* (100 μM) caused 97% inhibition; miconazole (100 μM) caused 30% inhibition; aminobenzotriazole (100 μM) 26% inhibition; metyrapone (1 mM) caused 23% inhibition; and clotrimazole (100 μM) caused 16% inhibition. Somewhat unexpectedly, all of them except cytochrome *c* could inhibit (+)-germacrene A synthase activity as well.

The strongest proof for the involvement of a cytochrome P450 enzyme is blue-light reversible inhibition of enzyme activity by CO (Table II) (West, 1980; Mihaliak et al., 1993). An atmosphere of 80% CO + 20% O_2 inhibits (+)-germacrene A hydroxylase by 69%. The corresponding decrease of the elema-1,3,11(13)-trien-12-ol peak was accompanied by an increase in the GC-MS-peak size of β-elemene, because the production of (+)-germacrene A from FPP was not affected by CO. The inhibitory effect of CO on (+)-germacrene A hydroxylase activity could be convincingly reversed by blue light (450 nm).

Table II. Carbon monoxide inhibition and blue-light reversal of (+)-germacrene A hydroxylase activity

Assay conditions[a]	Percentage enzyme activity[b] ± SD
Air (≈ 80% N_2 + 20% O_2)	
Dark	100 ± 2.9
450 nm light	92 ± 6.7
80% CO + 20% O_2	
Dark	31 ± 1.5
450 nm light	50 ± 6.7

[a]1 mM NADPH-regenerating system was present in all incubations, but no flavins were added.

[b]100% enzyme activity corresponds to an elematriene-12-ol peak size of 1.03 × internal standard (5 nmol *cis*-nerolidol in each assay

Enantioselectivity of the (+)-Germacrene A Hydroxylase

A microsomal preparation from chicory roots was able to convert β-elemene (**6**) into elema-1,3,11(13)-trien-12-ol (**7**). Addition of 100 μM β-elemene to the (+)-germacrene A hydroxylase assay reduced the conversion of [^{3}H]germacrene A into [^{3}H]germacratrien-12-ol with 37%. This substrate competition shows that both hydroxylations are most likely catalysed by the same enzyme, so β-elemene can be used to investigate the enantioselectivity of the (+)-germacrene A hydroxylase. The amount of (+)-β-elemene hydroxylated was only two times less than that of (-)-β-elemene (Fig. 5), the structural analogue of (+)-germacrene A. This indicates a modest enantioselectivity of the (+)-germacrene A hydroxylase.

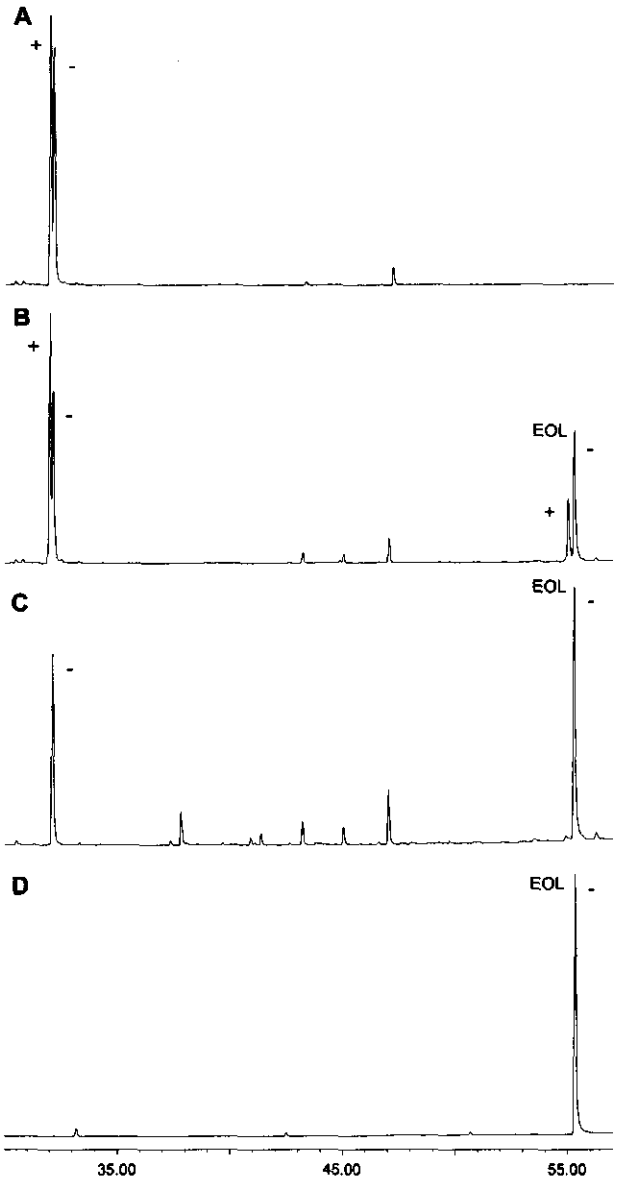

Figure 5. Determination of the stereochemical preference of the (+)-germacrene A hydroxylase using GC-MS equipped with an enantioselective column in selected ion monitoring-mode (m/z 119,121, 145, 147, 189). A, Incubation of (±)-β-elemene in the absence of NADPH (blank). B, Incubation of (±)-β-elemene in the presence of NADPH, resulting in a mixture of elema-1,3,11(13)-trien-12-ol (7) enantiomers (EOL). C, Incubation of (-)-β-elemene giving (-)-elema-1,3,11(13)-trien-12-ol. D, Standard of (-)-elema-1,3,11(13)-trien-12-ol (EOL).

DISCUSSION

The present results have established the pathway for biosynthesis in chicory roots of germacra-1(10),4,11(13)-trien-12-oic acid (**4**), a compound en route to the germacrene-derived sesquiterpene lactones (Fig. 6). The reported oxidation of the (+)-germacrene A isopropenyl group supports the hypothesis that formation of the lactone ring precedes any cyclisation or other oxidation of the germacrene framework (Cordell, 1976). The results presented are in line with the proposed pathway for costunolide (**5**), a germacranolide that is structurally related to (+)-germacrene A (Geissman, 1973; Herz, 1977; Fischer et al., 1979; Seaman, 1982; Song et al., 1995).

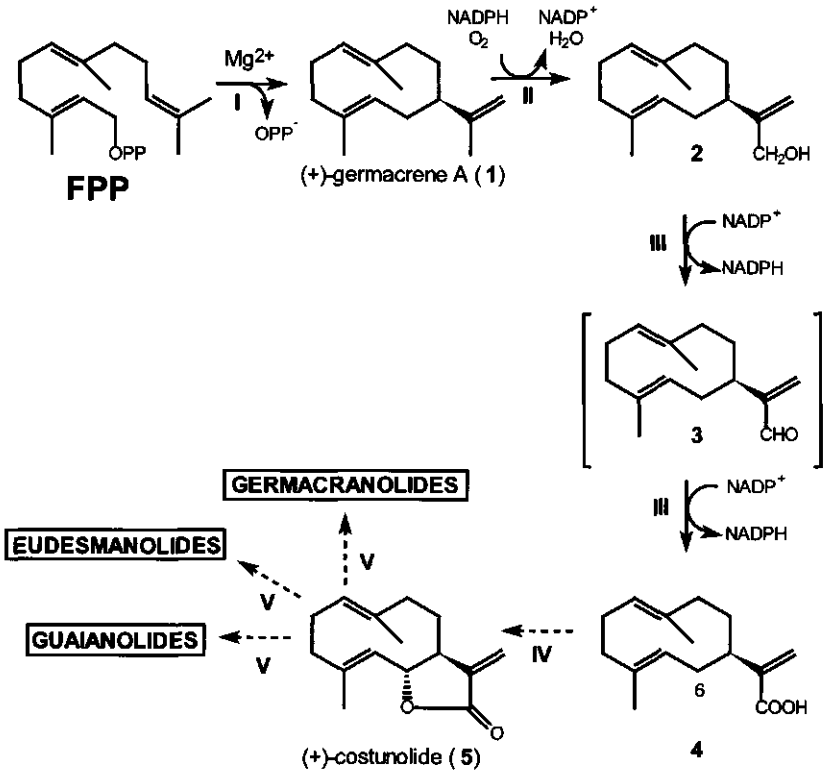

Figure 6. Proposed pathway for the germacrene derived sesquiterpene lactones present in chicory. I, cyclisation of FPP to (+)-germacrene A (**1**) by a sesquiterpene synthase. II, Hydroxylation of the isopropenyl group by (+)-germacrene A hydroxylase, a cytochrome P450 enzyme. III, Oxidation of germacratrien-12-ol (**2**) to germacratrien-12-oic acid (**4**) by $NADP^+$-dependent dehydrogenase(s) via the intermediate germacratrien-12-al (**3**). IV, The postulated (Ch. 5) hydroxylation at the C_6-position of germacratrien-12-oic acid and subsequent lactonisation to (+)-costunolide (**5**). V, Postulated formation of guaiane, eudesmane, and germacrane lactones.

The biosynthesis of germacrene-derived sesquiterpene lactones (Fig. 6) starts with the cyclisation of FPP into (+)-germacrene A (**1**) by a sesquiterpene cyclase (reaction I; Chapter 2). In the next step (reaction II), the isopropenyl group of (+)-germacrene A (**1**) is subjected to a hydroxylation reaction resulting in the formation of germacra-1(10),4,11(13)-trien-12-ol (**2**). This sesquiterpene alcohol is visible as its Cope rearrangement product (-)-elema-1,3,11(13)-trien-12-ol (**7**) in GC-MS measurements at high injection port temperatures, and it is detected as costol (**12**) when extracted under acidic conditions. The involvement of a cytochrome P450 enzyme in this hydroxylation reaction is clearly demonstrated by its NADPH and O_2 dependence, the blue-light reversible inhibition by CO, and by the inhibitory effect of various established cytochrome P450 inhibitors (West, 1980; Mihaliak et al., 1993).

The conversion of germacra-1(10),4,11(13)-trien-12-ol (**2**) into germacra-1(10),4,11(13)-trien-12-oic acid (**4**) (Fig. 6; reaction(s) III) is catalysed by water-soluble pyridine nucleotide dependent dehydrogenase(s). This enzyme activity oxidises germacrene alcohol (**2**) as well as (-)-elema-1,3,11(13)-trien-12-ol (**7**) to their corresponding acids (**4** and **9**) solely in the presence of $NADP^+$. Because two hydrides have to be abstracted to obtain the acid, presumably the stoichiometry is 2 $NADP^+$ for each molecule of germacrene/elemene acid formed. Since a crude root extract was used it is uncertain whether formation of germacra-1(10),4,11(13)-trien-12-oic acid (**4**) from the germacrene alcohol is catalysed by one or more dehydrogenase(s), and whether oxidation occurs via germacra-1(10),4,11(13)-trien-12-al (**3**) or within one enzymatic step. The corresponding aldehyde was not detected in incubations with the germacrene alcohol and only in trace amounts in incubations with the elemene alcohol. A study of the oxidation of perilla alcohol to perillic acid by bacterial extracts (Dhavalikar et al., 1966) could not demonstrate the presence of the expected intermediate aldehyde either. Isopropenal side chains are sensitive towards nucleophilic attack and both proteins and DTT were demonstrated to "bind" elema-1,3,11(13)-trien-12-al (**8**). It could be a reason why these aldehydes are not or hardly detectable in enzyme assays. Despite all of that, enzymatic oxidation of elema-1,3,11(13)-trien-12-al (**8**) to the elemene acid (**9**) could be established with the chicory root supernatant, but strikingly NAD^+ was preferred over $NADP^+$ and some conversion occurred also in the absence of any cofactor.

Neither the isolation of germacra-1(10),4,11(13)-trien-12-oic acid (**4**) nor that of elema-1,3,11(13)-trien-12-oic acid (**9**) from a natural source have been reported in literature (Buckingham, 1999). Both compounds are however present in fresh costus roots and in costus resinoid, respectively (Chapter 4). In general, cyclic mono- and sesquiterpenoid acids

are rather uncommon in plants (Bauer et al., 1990) and little is known about their biosynthesis.

The established pathway for germacrene A carboxylic acid resembles the biodegradation of limonene via perillic acid in bacteria (Dhavalikar et al., 1966) and the biosynthesis of monoterpenoid aldehydes/ketones in plants (e.g. McConkey, 2000), but with regard to the dehydrogenases it differs from the diterpenoid pathways of kaurenoic- and abietic acid (Funk and Croteau, 1994). It is assumed that in the biosynthesis of the sesquiterpene artemisinin by *Artemisia annua* oxidation of the isopropenyl group of amorphadiene is catalysed in the same way as the oxidation of (+)-germacrene A to germacrene acid (Bouwmeester et al., 1999).

Cytochrome P450 enzymes involved in plant terpenoid secondary metabolism are known to be rather substrate specific (West, 1980; Mihaliak et al., 1993). Nevertheless, β-elemene (**6**) is hydroxylated by the (+)-germacrene A hydroxylase of chicory and competitively inhibits the hydroxylation of (+)-germacrene A, probably because of the strong similarity in three-dimensional structure between (+)-germacrene A and β-elemene (Fig. 7). β-Elemene contains two isopropenyl groups, but its hydroxylation occurs regioselectively and only elema-1,3,11(13)-trien-12-ol (**7**) is formed. When the C_{13}-containing isopropenyl groups of the β-elemene enantiomers are depicted in the same way (Fig. 7), there are no evident differences at the site of hydroxylation and consequently it is not unexpected that the enzyme only shows a modest degree of enantioselectivity toward these enantiomers.

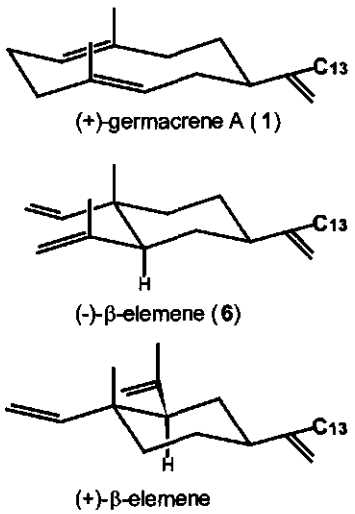

Figure 7. Three-dimensional structures of (+)-germacrene A, (-)-β-elemene, and (+)-β-elemene demonstrating the resemblance of these compounds at the site of hydroxylation (C_{13}-position).

A similar degree of enantioselectivity is also observed for the hydroxylation of limonene in peppermint (C_3), spearmint (C_6) and perilla (C_7) (Karp et al., 1990), although the limonene C_6-hydroxylase in caraway has a 10-fold preference for the (+)-enantiomer (Bouwmeester et al., 1998). The modest enantioselectivity of the (+)-germacrene A hydroxylase has no influence on the stereochemistry of the sesquiterpene lactones present in chicory, as in vivo only (+)-germacrene A is offered to the enzyme.

In general, all guaianolides, eudesmanolides, and germacranolides are thought to originate from (+)-costunolide (**5**) (Fischer, 1990), and the same holds for the bitter sesquiterpene lactones of chicory (Fig. 6; reaction V). Formation of (+)-costunolide from germacrene acid (**4**) involves a C_6-hydroxylation, after which formation of the lactone ring can be completed (reaction IV). This reaction is investigated in Chapter 5.

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4. Germacrenes from Fresh Costus Roots

Isolation of the Germacrene Intermediates of Sesquiterpene Lactone Biosynthesis from *Saussurea lappa* Clarke

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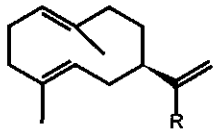


1 R = CH_3
2 R = CH_2OH
3 R = CHO
4 R = COOH

Abstract: Four germacrenes, in previous chapters shown to be intermediates in sesquiterpene lactone biosynthesis, were isolated from fresh costus roots (*Saussurea lappa*). The structures of (+)-germacrene A (**1**), germacra-1(10),4,11(13)-trien-12-ol (**2**), germacra-1(10),4,11(13)-trien-12-al (**3**), and germacra-1(10),4,11(13)-trien-12-oic acid (**4**) were deduced by a combination of spectral data and chemical transformations. Heating of these compounds yields (-)-β-elemene (**6**), (-)-elema-1,3,11(13)-trien-12-ol (**7**), (-)-elema-1,3,11(13)-trien-12-al (**8**), and elema-1,3,11(13)-trien-12-oic acid (**9**) respectively, in addition to small amounts of their diastereomers. Acid induced cyclisation of the germacrenes yields selinene (**10**), costol (**11**), costal (**12**), and costic acid (**13**) respectively. It is highly probable that the elemenes reported in literature as constituent of costus root oil are artefacts.

INTRODUCTION

Saussurea lappa Clarke is a member of the Asteraceae indigenous to parts of India and Pakistan where it grows in the Himalayas at 2500-3500 m elevation (Fig. 1). The dried 4-5 year old roots of this plant are known as costus roots and have a reputation for their medicinal properties as well as their fragrance. Attempts are made to cultivate this plants since it has become an endangered species due to ruthless collection from forest areas (Paul et al., 1960; Hatakeyama et al., 1990; Ayaz, 1996).

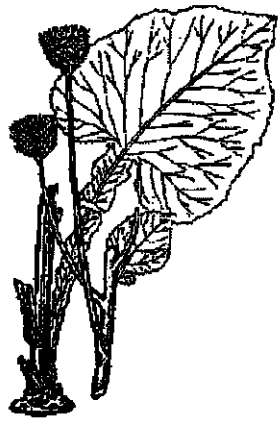
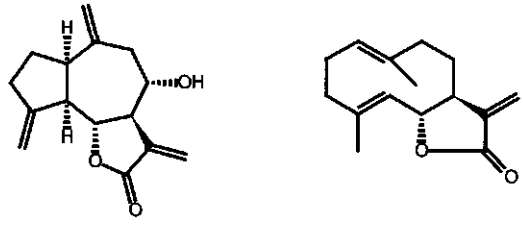


Figure 1. Pen and ink drawing of *Saussurea lappa* Clarke (source: www.fao.org).

Various studies have shown that costus roots are extremely rich in sesquiterpene lactones (3% of fresh weight), of which the most important are dehydrocostus lactone and (+)-costunolide (Fig. 2) (Somasekar Rao et al., 1960; Paul et al., 1960). The latter is the structurally simplest sesquiterpene lactone and generally accepted as the common intermediate in the biosynthesis of most sesquiterpene lactones including those present in chicory (Herz, 1977; Fischer, 1990a).



(-)-dehydrocostus lactone (+)-costunolide

Figure 2. The major sesquiterpene lactones of costus roots, (-)-dehydrocostus lactone and (+)-costunolide.

Chapter 2 and 3 support the theory that costunolide is formed from farnesyl diphosphate (FPP) via germacrenes **1-4** (Fig. 3). However, further investigations concerning the pathway for sesquiterpene lactones in chicory were hampered by the lack of a source of these germacrene intermediates **1-4**, in particular germacrene acid (**4**). Hitherto only the isolation of (+)-germacrene A (**1**) from various plant species (e.g. Wichtman and Stahl-Biskup, 1987) and the partial purification of germacra-1(10),4,11(13)-trien-12-al (**3**) from *Vernonia glabra* (Steetz) (Bohlman et al., 1983) have been described. A reason for the scarcity of reports on these germacrenes might be their notorious instability. They are susceptible to proton-induced cyclisations and to heat induced (e.g. steam distillation, GC-analysis) Cope rearrangement yielding the eudesmane or elemene framework, respectively (Fig. 3) (Southwell; 1970; Takeda, 1974; Wichtman and Stahl-Biskup, 1987; Teisseire, 1994; Minnaard, 1997; Chapter 2 and 3).

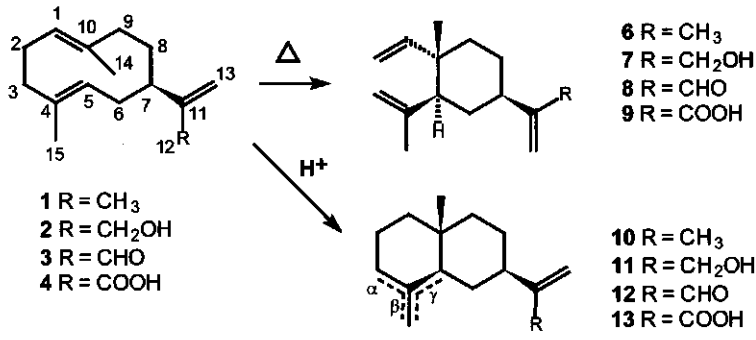


Figure 3. The sought-after germacrene intermediates of sesquiterpene lactone biosynthesis (+)-germacrene A (**1**), germacra-1(10),4,11(13)-trien-12-ol (**2**), germacra-1(10),4,11(13)-trien-12-al (**3**), and germacra-1(10),4,11(13)-trien-12-oic acid (**4**). Heat induced Cope-rearrangement of **1-4** yields (-)-β-elemene (**6**), (-)-elema-1,3,11(13)-trien-12-ol (**7**), (-)-elema-1,3,11(13)-trien-12-al (**8**), and elema-1,3,11(13)-trien-12-oic acid (**9**); acidic conditions are expected to give selinene (**10**), costol (**11**), costal (**12**), and costic acid (**13**).

The presence of β-elemene (**6**), (-)-elema-1,3,11(13)-trien-12-ol (**7**), and (-)-elema-1,3,11(13)-trien-12-al (**8**) in commercially available costus root oil catches attention (Klein and Thömel, 1976; Maurer and Grieder, 1977). Since this oil is obtained by steam distillation and elemenes are generally considered to be heat-induced artefacts, it can be assumed that costus roots originally contain the corresponding germacrenes **1-3**. The reported isolation of costic acid (**13**) from costus roots also suggests the presence of germacrene acid (**4**)

(Bawdekar and Kelkar, 1965), since HCl was used in the isolation procedure which might have initiated the cyclisation of germacrene acid.

This chapter describes the analysis of (fresh) costus root material(s) for the presence of germacrenes **1-4**, and the development of a mild procedure for the isolation of these compounds so that they can be used for further studies on sesquiterpene lactone biosynthesis.

EXPERIMENTAL

General Experimental Procedures

^{1}H NMR and ^{13}C NMR: recorded at 400 MHz with C_6D_6 as solvent and TMS as internal standard. GC-MS: HP 5890 series II gas chromatograph and HP 5972A Mass Selective Detector (70 eV), equipped with a capillary HP5-MS column (30 m × 0.25 mm, film thickness of 0.25 μm) at a helium flow rate of 0.969 mL min^{-1}, programmed at 55°C for 4 min followed by a ramp of 5°C min^{-1} to 280°C, and a standard injection port temperature of 250°C. Column chromatography: Silica gel flash (Janssen, 0.035-0.07 mm, pore 6 nm; column width 1.8 cm) (Still et al., 1978). 5% $AgNO_3$/Silica gel was prepared according to Maurer and Grieder (1977). TLC: Silica gel (Merck) 60 F254, eluent EtOAc-hexane (1:4); spray of 5 mL *p*-anisaldehyde in 5 mL H_2SO_4 and 95 mL EtOH, 1-2 min heating at 120°C.

Plant Material

Seeds of *Saussurea lappa* Clarke were introduced from Hortus Botanicus Vilr, Moscow (USSR) to the fields of Hokkaido Experimental Station for Medicinal Plants (Japan) in 1981 (Accession No. 9678-81) (Hatakeyama et al., 1989). Five-year old roots were harvested in august 2000 under supervision of Dr. T. Shibata and transported by courier to The Netherlands. Upon arrival they were cut into pieces, powdered in liquid N_2 with a Waring blender and stored at -80°C. Dried costus roots were obtained via internet from gaines.com (El Cajon CA, USA) and ginseng4less.com (Petaluma CA, USA).

Chemicals and Reference Compounds

Costus resinoid and costus oil were obtained from Pierre Chauvet SA (Seillans, France). Costunolide and dehydrocostus lactone were isolated from costus resinoid according to Fischer et al. (1990b) making use of the described TLC-spot colour (Asakawa et al., 1981),

and recrystallised in pentane; NMR data were identical to those reported in literature (Kuroda et al., 1987; Takahashi, et al., 1987). Standards of (-)-elema-1,3,11(13)-trien-12-al (**8**) (NMR in C_6D_6 did not markedly differ from those recorded in $CDCl_3$ [Maurer and Grieder, 1977; Bohlman et al., 1983]), elema-1,3,11(13)-trien-12-oic acid (**9**) and costal (**12**) were isolated/synthesised as previously described in Chapter 3. A mixture of α- and γ-costic acid (**13**) was synthesised from a mixture of α- and γ-costal using $NaClO_3$ (Chapter 3). (-)-Elema-1,3,11(13)-trien-12-ol (**7**) was a gift of Dr. B. Maurer (Firmenich SA, Geneva, Switzerland), standards of (+)-germacrene A (**1**) and (-)-β-elemene (**6**) were a gift of Prof. W. A. König (Hamburg University, Germany).

Screening of Crude Materials for the Presence of Germacrene A (1)

Samples of dried costus roots were powdered in liquid N_2, and 0.2 g of powdered root was shaken in 3 mL Et_2O and centrifuged after 5 min. The Et_2O was quickly passed through a Pasteur pipette filled with 0.4 g silica gel. A spatula tip of costus resinoid and a droplet of costus oil were also dissolved in 3 mL of Et_2O and passed over silica gel. The Et_2O extracts were screened at an injection port temperature of 150°C for a germacrene A (**1**) peak that should be replaced by a peak of β-elemene (**6**) at an injection port temperature of 250°(Chapter 2).

Isolation of Germacrenes 1-3

Powdered fresh costus root (300 g) was extracted twice with 500 mL of Et_2O at 6°C; the Et_2O extract was filtered and its volume reduced to 75 mL by rotary evaporation at room temperature. The concentrate was extracted with 5% aq. Na_2CO_3 (3 × 50 mL). The aq. Na_2CO_3 layers were combined and stored at 4°C. The Et_2O layer was washed with brine, dried with Na_2SO_4, and the solvent removed yielding 2.8 g of a yellow-brown solid. Lactones (1.5 g) were partially removed by dissolving the residue in pentane and cooling at –20°C, after which the crystallised lactones were filtered off and the solvent was removed *in vacuo*. This procedure was repeated twice. The obtained oil was dissolved in 15 mL of pentane and extracted with 20% aq. $AgNO_3$ (4 × 10 mL). The germacrenes were liberated by complexing the Ag^+ ions with 10 mL of aq. NH_3 (25%) ammonia that was carefully added while cooling on ice, after which the water layer was extracted with pentane (3 × 50 mL) (Southwell, 1970). The pentane layers were washed with brine and dried with Na_2SO_4 yielding 100 mg of a yellow oil. This oil was chromatographed with 350 mL of pentane-CH_2Cl_2 (3:7) after which

the column was eluted with CH_2Cl_2. Fractions of 10 mL were collected and monitored by GC-MS and TLC; costunolide and dehydrocostus lactone could easily be distinguished by their greenish-blue (R_f 0.30) and blue spots (R_f 0.35) respectively. After removal of aplotaxene (a major constituent of costus root oil [Klein and Thömel, 1976; Maurer and Grieder, 1977]) by a second silver extraction (4 × 1.5 mL 20% aq. $AgNO_3$, etc.), fractions 2+3 yielded 4 mg of germacrene A (**1**) with an impurity of 4% of humulene. Fractions 19-33 yielded, after a second silver extraction, 1.5 mg germacratrien-12-al (**3**). Fractions 40-64 contained a mixture of costunolide, dehydrocostus lactone, β-costol (**11**), hedycaryol and germacrene alcohol (**2**). Attempts to separate the latter from β-costol and costunolide by column chromatography on silica gel using pentane-CH_2Cl_2 (1:9) or Et_2O-pentane (1:4) failed. Column chromatography on 5% $AgNO_3$-silica gel (10 gram) was more successful; costol and subsequently costunolide were eluted with Et_2O (400 mL), whereas germacratrien-12-ol (2 mg, of which ≈ 30% elematrien-12-ol) was eluted with 150 mL MeOH-Et_2O (1:9).

Isolation of Germacra-1(10),4,11(13)-trien-12-oic Acid (4)

The aq. $NaCO_3$ layers (150 mL) were carefully acidified on ice to pH 6 with 5M HCl and extracted with Et_2O (4 × 60 mL). The Et_2O layers were washed with brine, dried with Na_2SO_4, concentrated to 15 mL, and extracted with 20% aq. $AgNO_3$ (4 × 10 mL). The Ag^+ ions were precipitated with 35 mL 2M NaCl liberating the germacrenes, and the remaining solution was extracted with Et_2O (4 × 50 mL). After washing with brine and drying with Na_2SO_4, 20 mg of a yellow solid was obtained that mainly contained germacratrien-12-oic acid (**4**), costunolide and dehydrocostus lactone. Column chromatography with EtOAc-pentane (1:4; 350 mL) removed the lactones after which the germacrene acid was eluted with 100 mL EtOAc. The last minor impurities were removed by column chromatography with Et_2O-pentane (1:4; 400 mL), and 1.5 mg of germacratrien-12-oic acid was obtained.

Acid induced Cyclisation of Germacrenes

A small amount (≈ 10 nmol) of the germacrenes (**1-4**) was dissolved in 2 mL of CH_2Cl_2 and a crystal of *p*-toluenesulphonic acid was added (Minnaard, 1997). After 7 min at room temperature, the solution was washed with 1 mL of $NaHCO_3$ (satd.) and passed through a Pasteur pipette filled with 0.25 g $MgSO_4$. The solvent was reduced to 100 µl under a stream of N_2 and the sample was analysed by GC-MS. In case of germacratrien-12-oic acid (**4**) $NaHCO_3$ was replaced by NaH_2PO_4. The experiments were repeated with the elemenes **6-9**, which did not cyclise.

Compound Characterisation

Germacrene A (1), 4 mg

Colourless oil, purple TLC-spot R_f 0.75, R_I 1511, EIMS *m/z* (rel int) M^+ 204 (8), $[M\text{-}Me]^+$ 189 (37), 93 (100), 67 (99), 68 (94), 107 (79), 81 (77), 41 (68), 79 (67), 105 (59), 91 (57), 53 (54), 147 (47), 119 (43), 121 (41), 55 (41), 161 (36), 133 (36). Cope rearrangement to **6**, R_I 1396, EIMS *m/z* (rel int) M^+204 (1), $[M\text{-}Me]^+$ 189 (28), 93 (100), 81 (96), 67 (83), 68 (76), 41(67), 79 (64), 107 (63), 53 (51), 91 (48), 147 (45), 121 (42), 105 (42), 55 (41), 161 (31), 119 (31), 133 (29); and diastereomer of **6**, R_I 1388, EIMS *m/z* (rel int) M^+ 204 (1), $[M\text{-}Me]^+$ 189 (23), 93 (100), 81 (78), 67 (61), 68 (58), 41 (52), 79 (51), 107 (50), 91 (41), 121 (35), 161 (34), 55 (33), 53 (32), 105 (31), 119 (27), 133 (23), 147 (21). Cyclisation yields **10**, R_I 1479 (γ), 1491 (β) and 1500 (α) (MS-data are comparable with those of Maurer and Grieder [1977] and were used in combination with the R_I's reported by Adams [1995] to determine the elution order).

Germacra-1(10),4,11(13)-trien-12-ol (2), 2 mg, mixture with 7 (≈30%)

Colourless/slightly yellow oil, pink/purple TLC-spot R_f 0.29, R_I 1673 to ± 1806. Cope rearrangement to **7**, R_I 1673 EIMS *m/z* (rel int) M^+ 220 (1), $[M\text{-}Me]^+$ 205 (4), 79 (100), 81 (94), 41 (91), 91 (90), 67 (86), 93 (85), 55 (69), 105 (66), 53(60), 119 (58), 77 (55), 68 (53), 189 (42), 121 (37), 133 (35), 145 (33), 163 (25), 161 (25); and diastereomer of **7**, R_I 1657, EIMS *m/z* (rel int) M^+ 220 (1), $[M\text{-}Me]^+$ 205 (3), 81 (100), 41 (94), 79 (92), 93 (78), 67 (78), 91 (71), 69 (70), 55 (63), 105 (55), 119 (50), 77 (46), 53 (45), 161 (28), 133 (24), 145 (18), 189 (15). Cyclisation yields **11**, R_I 1755 (γ), 1775 (β) and 1781 (α) (MS-data are comparable with those of Maurer and Grieder [1977] and used to determine elution order).

Germacra-1(10),4,11(13)-trien-12-al (3), 1.5 mg

Colourless/slightly yellow oil with strong mossy odour, pink spot R_f 0.58, R_I 1585 to ± 1715. ^1H NMR δ 9.35 (s) 9.37 (*s*), 9.45 (*s*) and 9.47 (*s*) (ratio 1:1:0.01:0.01, 1H, H-12); 5.77 (*br s*) and 5.72 (*s*) (ratio 1:1, 1H, H-13); 5.36 (*br s*) and 5.33 (*s*) (ratio 1:1, 1H, H'-13); 5.22 (*br s*, 1H, H-1); 4.84 (*br d*, J = 13.3 Hz) and 4.62 (*br d*, J = 10.3 Hz) (ratio 1:1, 1H, H-5) 2.75-2.55 (*br m*, 1H, H-7). Cope rearrangement to **8**, R_I 1585 EIMS *m/z* (rel int) M^+ 218 (1), $[M\text{-}Me]^+$ 203 (12), 81 (100), 67 (70), 79 (68), 41 (63), 91 (56), 53 (50), 93 (46), 95 (38), 77 (38), 55 (37), 107 (37), 105 (35), 119 (27), 161 (25), 121 (24); and diastereomer of **8**, R_I 1570 EIMS *m/z* (rel int) M^+ 218 (ND), $[M\text{-}Me]^+$ 203 (8), 81 (100), 79 (64), 67 (60), 41 (57), 91 (48), 93 (43), 95 (41), 53 (40), 77 (36), 105 (36), 107 (35), 68 (34), 55 (34), 119 (25), 121

(22), 175 (22). Cyclisation yields **12**, R_I 1673 (γ), 1692 (β) and 1695 (α) (MS-data are comparable with those of Maurer and Grieder [1977] and used to determine elution order).

Germacra-1(10),4,11(13)-trien-12-oic acid (4), 1.5 mg

White crystals, blue TLC-spot (note: **9** gives pink spot) R_f 0.18, R_I 1771 to ± 1845. ^{1}H NMR δ 6.44-6.37 (*m*) and 6.36 (*s*) (ratio 1:1, 1H, H-13); 5.44 (*br m*) and 5.38 (*s*) (ratio 1:1, 1H, H'-13); 5.30-5.15 (*br s*, 1H, H-1); 4.85 (*br m*) and 4.63 (*br d*, J = 10.6 Hz) (ratio 1:1, 1H, H-5); 2.75-2.55 (*br m*, 1H, H-7). Cope rearrangement to **9**, R_I 1771 EIMS *m/z* (rel int) M^+ 234 (1), $[M\text{-}Me]^+$ 219 (7), 81 (100), 67 (50), 79 (47), 41 (43), 68 (40), 91 (38), 53 (34), 93 (29), 105 (29), 55 (26), 77 (26), 107 (26), 121 (23), 119 (19), 69 (17), 177 (15); and diastereomer of **9**, R_I 1750 EIMS *m/z* (rel int) M^+ 234 (ND), $[M\text{-}Me]^+$ 219 (4), 81 (100), 79 (67), 41 (58), 67 (56), 107 (55), 91 (49), 93 (49), 53 (41), 55 (40), 68 (39), 105 (38), 121 (35), 162 (33), 147 (26), 119 (25), 163 (25). Cyclisation yields **13**; R_I 1854 (γ), EIMS *m/z* (rel int) M^+ 234 (26), $[M\text{-}Me]^+$ 219 (100), 91 (57), 41 (35), 79 (35), 81 (34), 105 (34), 147 (34), 107 (32), 93 (31), 55 (30), 77 (30), 173 (25); R_I 1876 (β), EIMS *m/z* (rel int) M^+ 234 (25), $[M\text{-}Me]^+$ 219 (67), 91 (100), 79 (95), 93 (85), 41 (78), 121 (78), 77 (63), 81 (63), 55 (61), 67 (59), 105 (55), 107 (55), 53 (45), and R_I 1879 (α), EIMS *m/z* (rel int) M^+ 234 (27), $[M\text{-}Me]^+$ 219 (100), 91 (57), 79 (46), 41 (38), 81 (37), 107 (36), 105 (35), 93 (35), 55 (29), 205 (26) (elution order of isomers presumed to be the same as for **10-12**).

RESULTS AND DISCUSSION

Et_2O extracts of commercially available dried costus roots, costus resinoid and costus root oil were screened on GC-MS for the presence of germacrene A (**1**). This germacrene can be measured as a clear peak at a GC-injection port temperature of 150°C. The more polar germacrenes **2-4** cannot be measured as a real peak (Chapter 2 and 3), because they will rearrange during migration through the GC-column to their faster migrating elemenes and will be observed as a "broad hump" in the baseline such as described for hedycaryol and 7-hydroxygermacrene (Stahl, 1984; Chapter 2 and 3). Yet, in all of these extracts no germacrene A but only its Cope-rearrangement product β-elemene (**6**) could be detected. It was concluded that the germacrene A originally present in these materials had been exposed to heat and that the commercially available materials are not suitable for the isolation of germacrenes. Interestingly, in the resinoid and dried roots the previously unreported elema-

1,3,11(13)-trien-12-oic acid (**9**) was detected in addition to (-)-elema-1,3,11(13)-trien-12-ol (**7**) and (-)-elema-1,3,11(13)-trien-12-al (**8**).

In contrast, the Et_2O extract of fresh costus roots did contain germacrene A (**1**), whereas no (-)-β-elemene (**6**) was observed. At the applied GC-injection port temperature of 150° C, also the foreseen "broad humps" of the oxygenated germacrenes **2-4** were visible in the GC-chromatogram instead of sharp peaks of the oxygenated elemenes **7-9**.

In order to isolate germacrenes **1-4**, 300 g of fresh costus roots was powdered in liquid N_2 and extracted with Et_2O. The organic extract was shaken with aq. Na_2CO_3 to remove germacrene acid (**4**) and other acidic/polar compounds; the lactones (mainly costunolide and dehydrocostus lactone, 1.5 g) were partially removed by crystallisation from pentane (Somasekar Rao et al., 1960). Germacrenes **1-3** could be selectively extracted from the remaining pentane with aq. $AgNO_3$ (Minnaard, 1997; Southwell, 1970), because of the complex formation between the Ag^+-ions and the diene-ring system present in the germacrenes. Subsequent flash column chromatography of the obtained crude germacrene-mixture in combination with a second $AgNO_3$ extraction yielded: 4 mg germacrene A (**1**), 1.5 mg germacrene aldehyde (**3**); and a mixture of germacrene alcohol (**2**) with β-costol (**11**), hedycaryol, costunolide, and dehydrocostus lactone. The isolated germacrene A contained a small impurity of 4% humulene (determined by GC-MS) that is extracted with $AgNO_3$ as well due to the presence of a cyclic diene moiety. Purification of the germacrene alcohol (**2**) was more elaborate because β-costol (**10**) with its two adjacent exocyclic double bonds is also extracted with $AgNO_3$, whereas α- and γ-costol are not. Furthermore the separation of germacrene alcohol from costunolide was not very successful on silica gel. Argentation chromatography yielded 3 mg of germacrene alcohol (**2**) that was, however, unlike the isolated germacrenes **1**, **3**, and **4**, contaminated with elematrien-12-ol (**7**) (approx. 30%).

The germacrene acid (**4**) containing alkaline (Na_2CO_3) extracts were carefully brought to pH 6 and extracted with Et_2O. As expected from the protocol of Bawdekar and Kelkar (1967), the sesquiterpenoid acids did not dissolve in aq. $NaHCO_3$ and acidification to a lower pH was not necessary, thus preventing the undesired cyclisation of germacrene acid to costic acid (**13**). Extraction with aq. $AgNO_3$ and column chromatography yielded 1.5 mg of germacrene acid (**4**).

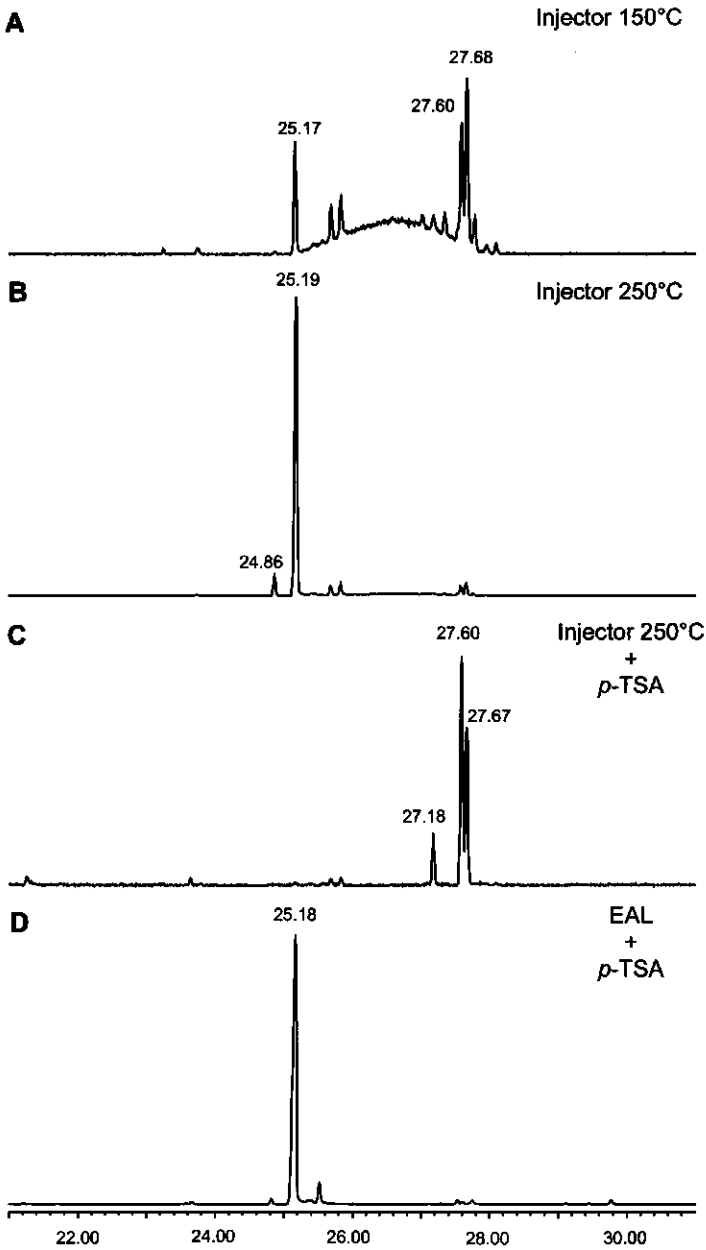


Figure 4. GC-MS analyses of the germacrene aldehyde (**3**) at an injection port temperature of 150°C (A) yields a broad hump that starts at the position of elematrien-12-al (**12**) (25.17 min). Injection at a temperature of 250°C (B) gives almost complete rearrangement of **3** to elematrien-12-al (**12**) (25.19), whereas a small amount of diastereomeric elemene aldehyde can also be detected (24.86). In the presence of *p*-toluenesulfonic acid (C) the germacrene aldehyde (**3**) is completely converted into costal (**8**): γ-costal (27.18 min), β-costal (27.60 min), and α-costal (26.67 min). The elemene aldehyde (**12**) is not cyclised in the presence of *p*-toluenesulfonic acid (D).

GC-analysis of germacrene aldehyde (**3**) at a GC-injection port temperature of 150°C results in a "broad hump" (Fig. 4A) that displays the mass spectrum of elematrien-12-al (**8**) over its entire range. At a GC-injection port temperature of 250°C a sharp peak of elematrien-12-al is visible instead (Fig. 4B), because the germacrene aldehyde is immediately rearranged in the GC-injection port and not during its migration through the GC-column. In the presence of *p*-toluenesulfonic acid the germacrene aldehyde is completely cyclised to the three possible costal isomers (**12**) (Fig. 4C). This indicates that the sample is free of elemene aldehyde, because the elemene aldehyde will not cyclise to costal (Fig. 4D). ^{1}H-NMR of the germacrene aldehyde confirmed that it did not contain any of its Cope rearrangement product, since a double doublet at δ 5.85 of C_2=C_1H-C_{10} typical for the elemene aldehyde was clearly missing. Unfortunately, in solution many germacrenes show broadened NMR signals or even multiple NMR signals, due to the existence of different conformations of the ten-membered ring (Fig. 5) (Takeda, 1974; Minnaard, 1997). This hinders a complete interpretation of the NMR data. Most strikingly is the presence of two aldehyde proton signals of similar height at δ 9.37 and δ 9.35 that cannot be due to coupling with a proton at C_{13}, accompanied by two very small signals at δ 9.47 and δ 9.45 (ratio 1:1:0.01:0.01).

The existence of different conformations for germacrenes also explains the formation of a small amount of diastereomeric elematrien-12-al during the Cope rearrangement of germacrene aldehyde (Fig. 4B). Cope rearrangement is a stereospecific reaction that preferably proceeds via a chair-like transition state (March, 1992), via either the UU or DD conformation of the germacrene (Fig. 5). However, the UU conformation with the isopropenal group in the equatorial position predominates and consequently the diastereomer of **8** will be formed only in low yield (Takeda, 1974; Piet et al., 1995; Minnaard, 1997). Small amounts of diastereomeric elemenes were also detected for the GC-injection port induced Cope rearrangement of germacrene A, germacratrien-12-ol and germacratrien-12-oic acid. These diastereomers have a mass spectrum very similar to their true elemene and typically constitute only 2-7 % of the total amount of Cope rearrangement products formed. Minute amounts of β-elemene diastereomer were also detected in the experiments of Chapter 2. The structure of the elematrien-12-ol diastereomer has tentatively been assigned by Maurer and Grieder (1977).

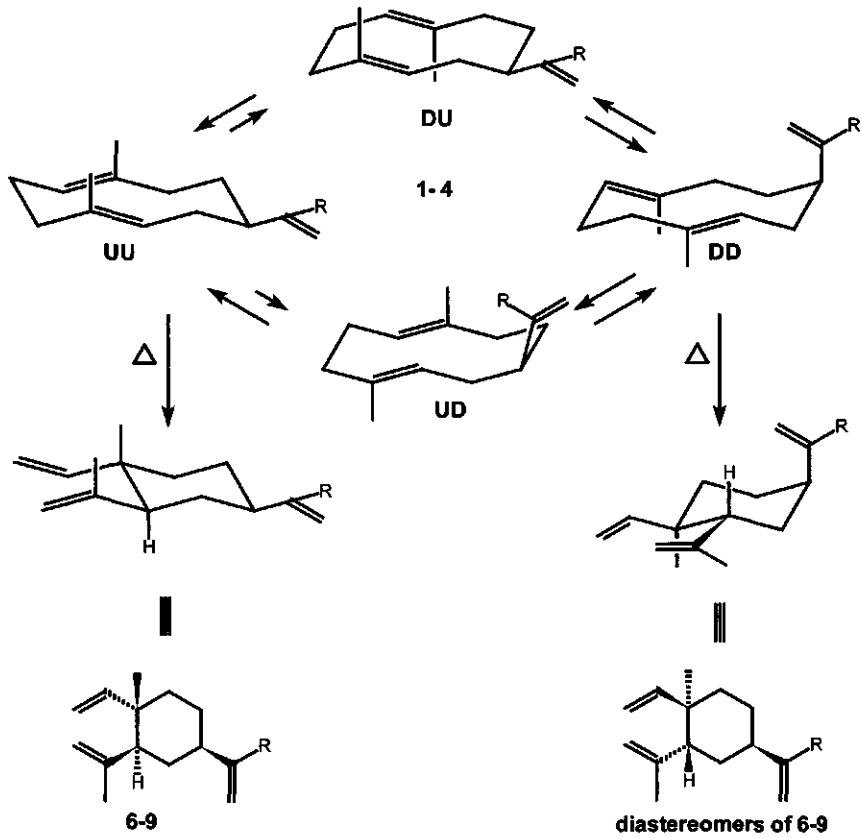


Figure 5. Conformations of germacrenes **1-4** denoted as UU, UD, DU, and DD. U (up) and D (down) refer to the orientation of the C_{14} and C_{15} methyl groups. Both conformations UU and DD will easily undergo Cope rearrangement, however, the UU conformation is predominant and will yield elemenes **6-9**, whereas the diastereomeric elemenes that originate from the DD conformation will hardly be formed.

When the injection port temperature of the GC was varied between 150°C and 250°C, germacrene alcohol (**2**) and germacrene acid (**4**) showed chromatogram patterns similar to that of germacrene aldehyde (**3**). In the presence of *p*-toluenesulfonic acid both germacrene A (**1**) and germacrene acid (**4**) were completely cyclised into their eudesmanes (**10** and **13**, respectively). However after acid treatment, the GC-chromatogram of the germacrene alcohol (**2**) still showed a peak of elematrien-12-ol (**7**), indicating that the isolated sample of germacrene alcohol is actually a mixture of germacrene- and elemene alcohol. This was confirmed by ^{1}H-NMR that showed a double doublet (J=18 and 10 Hz) at δ 5.81 typical for elematrien-12-ol (Maurer and Grieder, 1977). It is not known whether the presence of elematrien-12-ol is caused by an intrinsic higher instability of the germacrene alcohol and/or

by the more difficult and time-consuming purification of this compound. Although less likely, it cannot be ruled out that the elematrien-12-ol was already present in the crude plant material. In this respect it is remarkable that the germacratrien-12-ol produced in an enzyme assay from germacrene A (Chapter 3) was free of any elematrien-12-ol and could be fully cyclised into costol (**11**), notwithstanding the fact that the germacratrien-12-ol had been exposed to 30°C, a temperature unlikely to occur in costus roots that are underground.

The ^{1}H NMR of germacrene acid (**4**) was free of the double doublet at δ 5.85 from elematrien-12-oic acid (**9**) (Chapter 3), but once again few signals could be assigned. Similar observations were made by Zdero et al. (1991) for 3α- and 3β-acetoxygermacra-1(10)4,11(13)-trien-12-oic acid whose structures could only be resolved after Cope-rearrangement to their corresponding elemene derivatives.

The isolation under mild conditions from fresh costus roots of (+)-germacrene A (**1**), germacra-1(10),4,11(13)-trien-12-al (**3**), and germacra-1(10),4,11(13)-trien-12-oic acid (**4**) clearly demonstrates that their reported elemene derivatives (**6-9**) are indeed heat-induced artefacts formed during drying of the roots or manufacturing of the costus resinoid/oil, as the literature on germacrenes suggests (e.g. Bohlmann et al., 1983; Wichtman and Stahl-Biskup, 1987; Teisseire et al., 1994). Very similar results have for example been reported for the Australian shrub *Phebalium ozothamnoides* F. Muell whose main essential oil component is elemol, whereas the petroleum extract of macerated leaves yields hedycaryol instead (Southwell, 1970). Only in the case of germacra-1(10),4,11(13)-trien-12-ol (**2**) it cannot completely be ruled out that a small quantity of its corresponding elemene is already present in fresh costus roots. Apart from the presence of less volatile compounds like fatty acids, the presence of elemenes instead of germacrenes was the only significant difference between an Et_2O-extract from fresh costus roots and the commercial costus root oil that was thoroughly described by Maurer and Grieder (1977). It is less certain whether the eudesmanes **10-13** have to be regarded as artefacts as well. Although they can be formed from germacrenes under acidic conditions, maybe during storage in the plant —an enzyme specifically producing β-selinene from FPP has been described for *Citronella mitis* fruits (Belingheri et al., 1992). It cannot be ruled out that costus roots also contain such an enzyme and the eudesmanes present are genuine enzymatic products.

The presence of germacrenes **1-4** together with vast amounts of costunolide in costus roots supports the pathway for costunolide as described in Chapter 3. The isolated germacrene acid (**4**) is used in Chapter 5 to investigate the formation of costunolide during sesquiterpene lactone biosynthesis in chicory.

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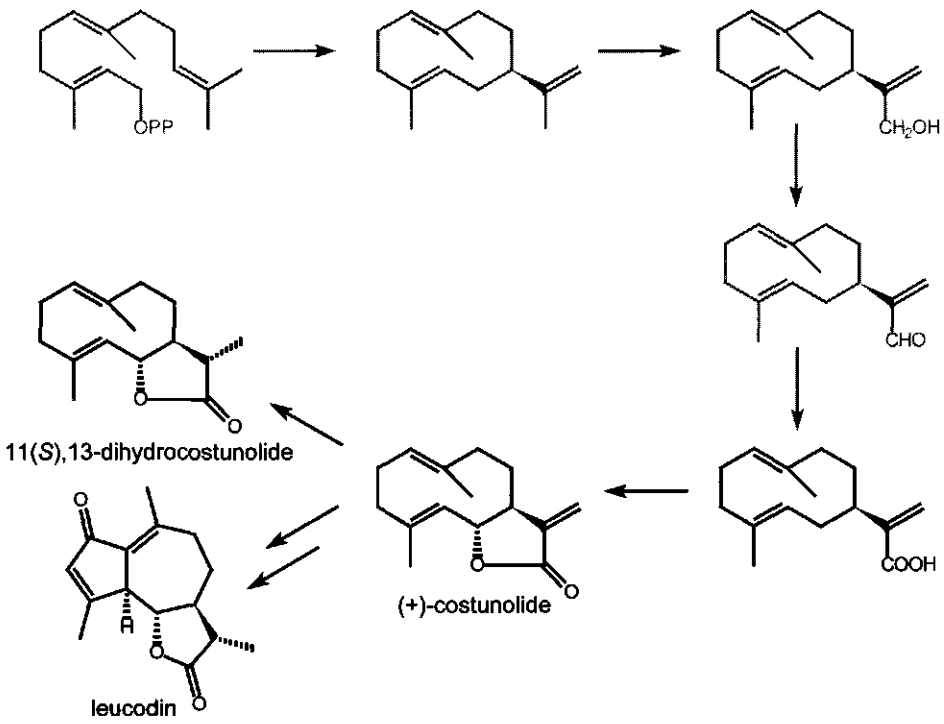
5. Biosynthesis of (+)-Costunolide, 11(*S*),13-Dihydrocostunolide and Leucodin

Demonstration of Cytochrome P450 catalysed Formation of the Lactone Ring Present in Sesquiterpene Lactones of Chicory



Abstract: Chicory roots contain an enzyme that converts germacra-1(10),4,11(13)-trien-12-oic acid into (+)-costunolide in the presence of NADPH. This (+)-costunolide synthase catalyses the last step in the formation of the lactone ring present in sesquiterpene lactones. Incubation of the germacrene acid in the presence of $^{18}O_2$ resulted in the incorporation of one atom of ^{18}O. Hence, formation of the lactone ring is likely to happen via C_6-hydroxylation of the germacrene acid and subsequent attack of this hydroxyl group at the C_{12}-atom of the carboxyl group. Blue-light reversible CO inhibition and experiments with cytochrome P450 inhibitors demonstrated that the (+)-costunolide synthase is a cytochrome P450 enzyme. In addition, enzymatic conversion of (+)-costunolide into 11(*S*),13-dihydrocostunolide and leucodin was detected. The first mentioned reaction involves an enoate reductase, whereas the formation of leucodin from (+)-costunolide probably involves more than one enzyme, including a cytochrome P450 enzyme.

INTRODUCTION

(+)-Costunolide is the structurally simplest germacranolide and considered to be the parent compound of all germacrene-derived sesquiterpene lactones, among them the guaianolides, eudesmanolides and germacranolides of chicory. Previous chapters have made it clear that this compound is formed from FPP through (+)-germacrene A, germacra-1(10),4,11(13)-trien-12-ol, and germacra-1(10),4,11(13)-trien-12-oic acid (**1**). The germacrene acid is thought to be hydroxylated at the C_6-position by a cytochrome P450 enzyme after which lactonisation yields (+)-costunolide (**2**) (Fig. 1) (Geissman, 1973; Fischer et al., 1979; Fischer, 1990). Since germacrene acid was isolated in useful quantities from fresh costus roots (Chapter 4), it is now possible to investigate this last step in formation of the lactone ring of sesquiterpene lactones in detail.

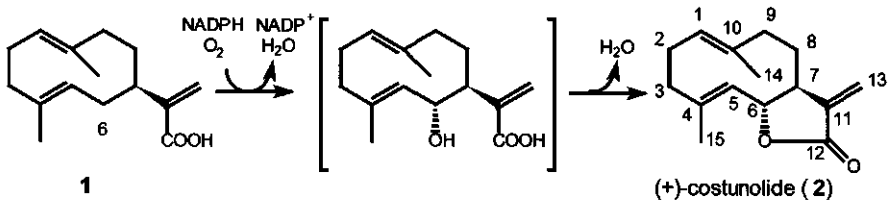


Figure 1. Postulated cytochrome P450 catalysed conversion of germacra-1(10),4,11(13)-trien-12-oic acid (**1**) into (+)-costunolide (**2**) via hydroxylation and subsequent lactonisation.

(+)-Costunolide is considered to be a branching point in the biosynthesis of sesquiterpene lactones from where pathways for the formation of guaianolides, eudesmanolides and germacranolides divide. It has been postulated by various authors that cyclisation of (+)-costunolide to either guaianolides or eudesmanolides is mediated by C_4-C_5 epoxidation or C_1-C_{10} epoxidation respectively (Brown et al., 1975; Fischer 1990; Teisseire, 1994; Piet et al., 1995). Alternatively, the possibility of a C_3-hydroxylation of the germacrenolide for formation of a guaianolide has been suggested (Piet et al., 1996).

This chapter will mainly focus on the formation of (+)-costunolide (**2**) from germacrene acid (**1**), but also some attention is paid to the subsequent conversions of (+)-costunolide.

MATERIAL AND METHODS

Materials

Fresh roots of cultivated chicory (*Cichorium intybus* L., cv Focus) were harvested during late summer and obtained from a grower (J. de Mik) in Veenendaal, the Netherlands. The chicory roots were cut into small pieces, frozen in liquid nitrogen and stored at –80°C. Germacra-1(10),4,11(13)-trien-12-oic acid (**1**), (+)-costunolide (**2**), and dehydrocostus lactone (Fig. 2, **6**) were isolated from costus roots as described in Chapter 4. Synthesis of a mixture of α- and γ-costic acid (**3**) is also described in Chapter 4, whereas the synthesis of elema-1,3,11(13)-trien-12-oic acid (**4**) is described in Chapter 3. The germacrene acid, costic acid and elemene acid were dissolved in *tert*-butyl methyl ether at 15 mM concentrations. (+)-Costunolide, dehydrocostus lactone and parthenolide (**5**; purchased from Sigma) were dissolved in ethanol at 10 mM concentrations. A sample of leucodin (**10**) was kindly provided both by Prof. M. Ando (Niigata University, Japan; Ando et al., 1994) and Dr. Shi Yong Ryu (Korea Research Institute of Chemical Technology, Yusung, Taejon, Korea), who also provided its C_{11}-epimer achillin (Ho et al., 1998). 11,13-Dihydro-dehydrocostus lactone (mokko lactone) was a gift of Prof. Y. Asakawa (Tokushima Bunri University, Japan). *cis*-Nerolidol was purchased from Fluka; ether (diethyl ether) and pentane were redistilled before use.

A GC-standard of 11(*S*),13-dihydro-costunolide (**8**) was prepared from 2 mg of (+)-costunolide (**2**) that was dissolved in 1.5 mL of ethyl acetate and stirred with 1.5 mg of $NaBH_4$ at 0°C, a procedure described for the reduction of the C_{11}-C_{13} exocyclic double bond of various sesquiterpene lactones (Asakawa et al., 1980; Asakawa, 1982; Seto et al., 1988). After 45 minutes the reaction was stopped by the addition of 1% HAc and an extra 2 ml of ethyl acetate. The organic phase was filtered through a glass-wool plugged (dimethyl chlorosilane-treated glasswool; Chrompack) Pasteur pipette that contained 0.45 g of silica and a little anhydrous $MgSO_4$. GC-MS analysis of the filtrate showed that half of the (+)-costunolide was converted into 11(*S*),13-dihydro-costunolide, whereas no trace of its C_{11}-epimer was detected.

Enzyme Isolation and Assay for (+)-Costunolide Synthase Activity

A cell free extract of chicory roots was prepared from the frozen material in the same way as described for the isolation of the germacrene A hydroxylase (Chapter 3), but $MgCl_2$ was omitted from the extraction buffer. The prepared 20,000*g* supernatant was desalted with an

Econo-Pac 10 DG column (Biorad) to an assay buffer containing 25 mM Tris (pH 7.5), 1 mM ascorbic acid, 5 μM FAD, 5 μM FMN and 10 % (v/v) glycerol. DTT was omitted from the assay buffer, because the SH-groups present in DTT might undergo a Michael-type addition to the C_{11}-C_{13} exocyclic double bond of (+)-costunolide (Kupchan et al., 1970). A 1-ml aliquot of the desalted supernatant was incubated in the presence of 45 μM germacrene acid (**1**) and a 1 mM NADPH-regenerating system, which consists of 1 mM NADPH, 5 mM glucose-6-phosphate, and 1.2 IU glucose-6-phosphate dehydrogenase (all from Sigma). Incubations were also done with boiled desalted supernatant and in the absence of NADPH. The experiments were repeated with elema-1,3,11(13)-trien-12-oic acid (Fig. 2, **4**) and with a mixture of α- and γ-costic acid (**3**) which may serve as substrate analogues for the germacrene acid (**1**). After 1h of incubation at 30°C, the reactions were stopped by storage in the freezer at –20°C.

The incubations were extracted thrice with 1 mL of 20% (v/v) ether in pentane, after the addition of 5 μL of a 0.2 mM *cis*-nerolidol solution in ethanol that serves as internal standard. The organic phase was filtered through a glass-wool plugged Pasteur pipette that contained 0.45 g of silica and a little anhydrous $MgSO_4$. The column was washed with 1.5 ml of ether and the extract was carefully concentrated to approximately 50 μL under a stream of nitrogen. Samples of 2 μL were analysed by GC-MS using an injection port temperature of 320°C to provoke Cope rearrangement of (+)-costunolide (**2**). Mass spectra were recorded at 70 eV scanning from 35 to 300 atomic mass units; the GC oven temperature was programmed as described in Chapter 2.

Characterisation of (+)-Costunolide Synthase Activity

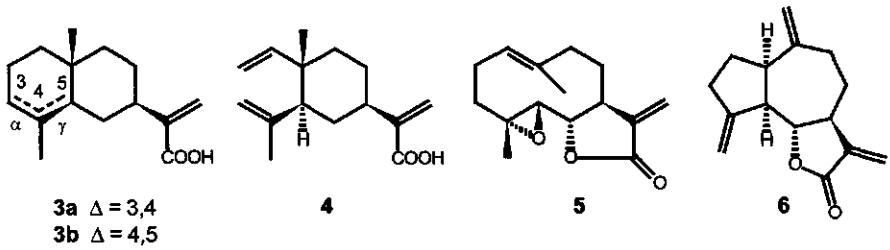
To determine whether the formation of (+)-costunolide (**2**) from germacrene acid (**1**) was catalysed by a cytochrome P450-enzyme, the effect of various established cytochrome P450-inhibitors (cytochrome *c*, metyrapone, clotrimazole, miconazole, and amino-benzotriazole) on this reaction was tested, as well as the effect of CO or an argon atmosphere. The cofactor dependence was also investigated, i.e. $NAD(P)^+$ or NADH was added instead of NADPH. Experiments were carried out in a similar manner as described for the germacrene A hydroxylase in Chapter 3, using a 20,000*g* supernatant and 5 μL of 0.2 mM *cis*-nerolidol as internal standard. Blue-light reversal of CO inhibition was investigated with gas mixtures of 10% O_2 + 90% N_2 (blank) and 10% O_2 + 90% CO.

The origin of the oxygen incorporated in the lactone ring of (+)-costunolide was investigated with $^{18}O_2$ (99% pure; Icon Services, Mt. Marlon, NY, USA). One millilitre of

incubation mixture in a (vented) septum-capped 4.5-mL vial was first bubbled with nitrogen to remove air and subsequently bubbled with $^{18}O_2$. The mass spectra of the compounds produced were compared with those formed in the standard enzyme assays under air.

Investigation of subsequent Conversions of (+)-Costunolide

Incubations as described for the germacrene acid (**1**) were also done with (+)-costunolide (**2**) (30 μM) to test whether any further enzymatic conversion of this compound would occur. Parthenolide (20 μM) (Fig. 2, **5**) was incubated as well, since it might be an intermediate in the formation of guaianolides. Dehydrocostus lactone (20 μM) (**6**) was incubated to investigate the reduction of the C_{11}-C_{13} exocyclic double bond in sesquiterpene lactones of chicory. The effect of established cytochrome P450 inhibitors and CO on the conversion of (+)-costunolide was studied, just as the effect of various pyridine nucleotide cofactors and an argon atmosphere.



3a Δ = 3,4
3b Δ = 4,5 **4** **5** **6**

Figure 2. Various substrates that were also incubated with the chicory supernatant and NADPH: a mixture of α-costic acid (**3a**) and γ-costic acid (**3b**), elema-1,3,11(13)-trien-12-oic acid (**4**), parthenolide (**5**), and dehydrocostus lactone (**6**).

RESULTS

Conversion of Germacrene Acid into Sesquiterpene Lactones

GC-MS analyses of the pentane-ether extract from the incubation of a 20,000*g* chicory root supernatant with germacra-1(10),4,11(13)-trien-12-oic acid (**1**) and NADPH revealed three products that were not detected in incubations without NADPH or in incubations with boiled supernatant (Fig. 3 A + B). The major peak co-eluted with a standard of (+)-costunolide (**2**) that at an injection port temperature of 320°C is detected as its Cope rearrangement product dehydrosaussurea lactone (**7**) (Fig. 3C). The substrate germacrene acid is likewise Cope rearranged to elemene acid (**5**) and diastereomeric elemene acid (Chapter 4).

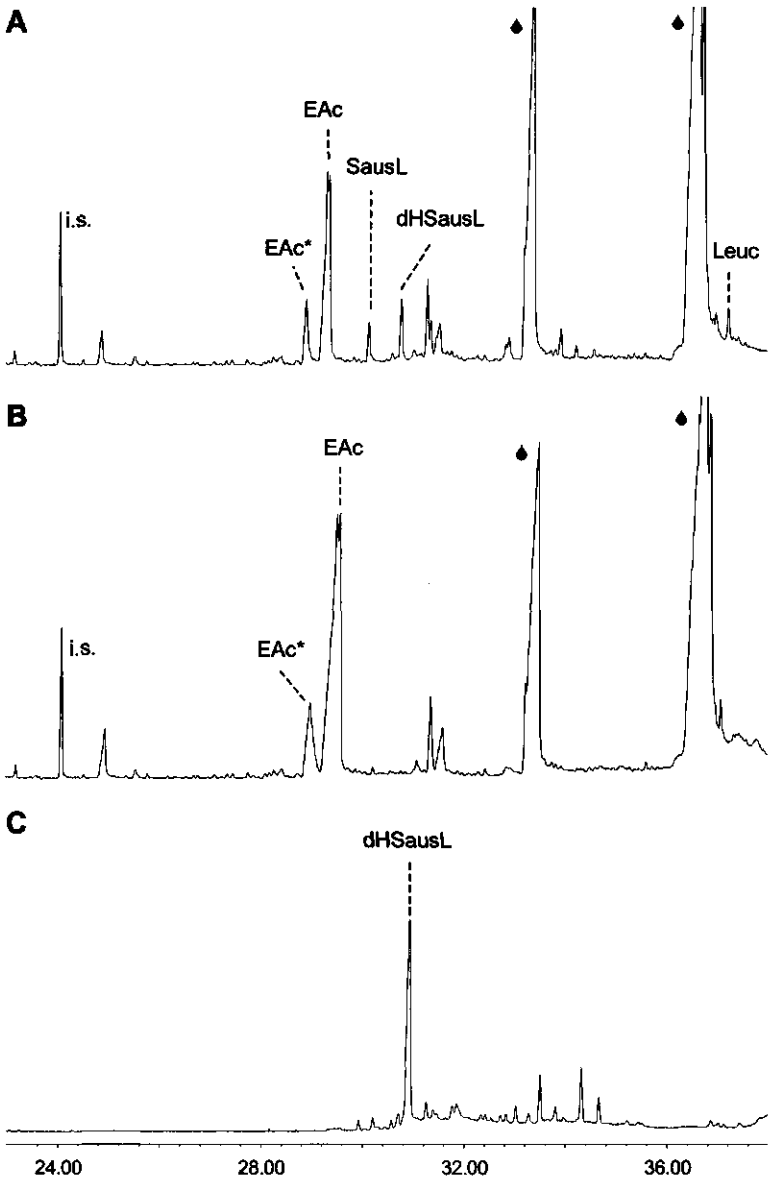


Figure 3. **A**, GC-MS analyses of the products formed in the incubation of a 20,000*g* supernatant from chicory roots with NADPH and germacra-1(10),4,11(13)-trien-12-oic acid (**1**) displays peaks of dehydrosaussurea lactone (dHSausL [**7**]), saussurea lactone (SausL [**9**]) and leucodin (Leuc [**10**]). **B**, These products are not observed in the absence of NADPH. Germacra-1(10),4,11(13)-trien-12-oic acid (**1**) is observed as elema-1,3,11(13)-trien-12-oic acid (EAc) plus its diastereomer (EAc*); the internal standard (i.s.) is 1 nmol *cis*-nerolidol. The huge fronting peaks (♦) are fatty acids (palmitic- and linoleic acid). **C**, A standard of 0.5 mM costunolide (**2**) in ethanol yields a tailing peak of dehydrosaussurea lactone (dHSausL [**7**]).

The two other products were identified as 11(*S*),13-dihydrocostunolide (**8**) that is Cope rearranged into saussurea lactone (**9**), and leucodin (**10**) (Fig. 4). Both 11(*S*),13-dihydrocostunolide and leucodin are enzymatically formed from (+)-costunolide, since they also appeared in incubations of (+)-costunolide with the 20,000g supernatant and NADPH. It cannot be excluded that even more products are formed during the incubation of germacrene acid, because higher oxygenated sesquiterpene lactones are likely not volatile enough for detection by gas chromatography. Furthermore, the presence of fatty acids in chicory extracts (Sannai et al., 1982) complicates the GC-analysis, because they yield big peaks (♦) under which smaller product peaks may "disappear".

Conversion of the substrate analogues α- and γ-costic acid (**3**) and elema-1,3,11(13)-trien-12-oic acid (**4**) was not detected.

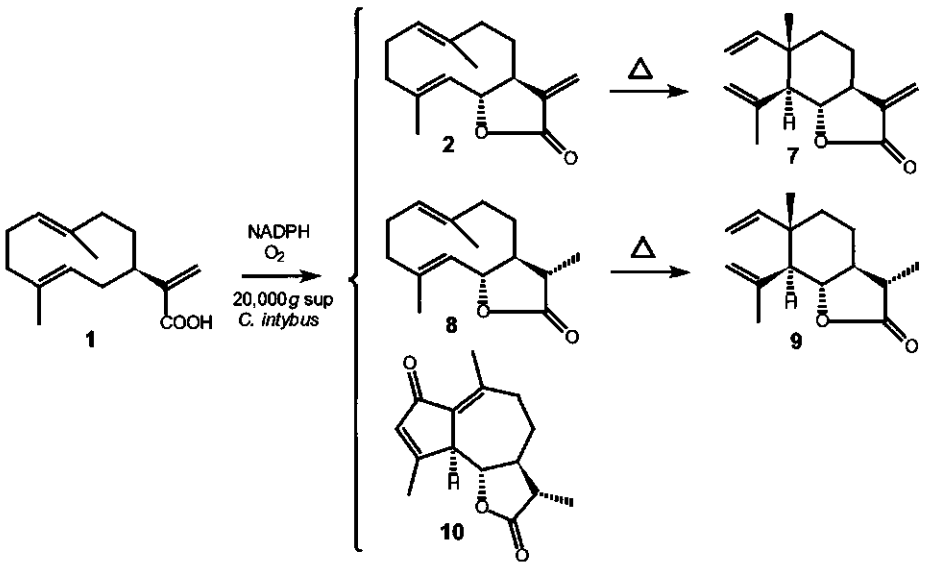


Figure 4. Products formed from germacra-1(10),4,11(13)-trien-12-oic acid (**1**) in the presence of NADPH and oxygen by a chicory 20,000*g* supernatant. Leucodin (**10**) is detected as such, whereas costunolide (**2**) and 11,13-dihydrocostunolide (**8**) are detected as their Cope rearrangement products dehydrosaussurea lactone (**7**) and saussurea lactone (**9**), respectively.

Characterisation of the (+)-Costunolide Synthase

For characterisation of the (+)-costunolide synthase the response of the GC-MS to different concentrations of (+)-costunolide should preferably be linear. Yet, at the used injection port temperature of 320°C, the GC-trace of costunolide (Fig. 3C) does not show a

of dehydrosaussurea lactone (**7**) with the intensity of the products peaks strongly suggested the formation of other products that are not detected by GC. The enzyme activity that catalyses the reduction of the 11(*S*),13-exocyclic double bond of (+)-costunolide was not capable of doing the same with dehydrocostus lactone (**6**), i.e. did not yield 11(*S*),13-dihydro-dehydrocostus lactone. Nevertheless, incubation of dehydrocostus lactone in the presence of NADPH and a chicory root extract gave an new product that had a shorter retention time, the same molecular mass, and exhibited a very similar mass spectrum as dehydrocostus lactone. It is probably the result of a double bond isomerisation somewhere in the molecule. Incubation of parthenolide (**5**) did not give leucodin (**10**), but conversion of parthenolide into other not-measurable sesquiterpene lactones cannot be excluded. Parthenolide itself disintegrates upon GC-analysis in a number of compounds and for this reason no quantitative determination could be done of the amount of parthenolide present after incubation.

To test which type(s) of enzymes might catalyse the formation of 11(*S*),13-dihydrocostunolide (**8**) and leucodin (**10**), the pyridine nucleotide cofactors were varied (Table II). Formation of both compounds was dependent on NADPH, but a part of the (+)-costunolide reductase activity was retained in the absence of any cofactor.

Table II. Pyridine nucleotide cofactor dependency of 11(*S*),13-dihydrocostunolide and leucodin biosynthesis from (+)-costunolide.

Pyridine Nucleotide Cofactor	Percentage Product Formation ± SD	
	Dihydrocostunolide (**8**)	Leucodin (**10**)
1 mM NADPH	[a]100 ± 3	[a]100 ± 10
None	15 ± 1	0 ± 0
1 mM NADH	14 ± 2	0 ± 0
1 mM NADPH + 1 mM NADH	107 ± 4	65 ± 19
1 mM $NADP^+$ + 1 mM NAD^+	18 ± 3	0 ± 0

[a]100% product formation corresponds to a saussurea lactone and leucodin peak size of 1.34 and 0.42 × internal standard (1 nmol *cis*-nerolidol) respectively.

Table III shows that formation of leucodin from (+)-costunolide is dependent on oxygen, whereas the formation of 11(*S*),13-dihydrocostunolide is not. Formation of leucodin was strongly inhibited by CO, which suggests the involvement of a cytochrome P450 enzyme Accordingly the reaction was also inhibited by all of the tested cytochrome P450 inhibitors except amino-benzotriazole.

Interestingly the measured amount of 11(*S*),13-dihydrocostunolide was raised in the presence of carbon monoxide or the absence of oxygen. This indicates that in these incubations at least a part of the successive (hypothetical) conversion of 11(*S*),13-dihydrocostunolide to higher oxygenated sesquiterpene lactones is inhibited and that some of the involved reactions are possibly cytochrome P450 catalysed. Somewhat contradictory, the detected amount of 11(*S*),13-dihydrocostunolide was hardly effected or even lowered when cytochrome P450-inhibitors had been added to the incubations. Perhaps each individual inhibitor effects only some of the succesive cytochrome P450 catalysed reactions, whereas CO and cytochrome *c* inhibit all cytochrome P450 catalysed reactions. It cannot be excluded that the inhibitors themselves influence the formation of 11(*S*),13-dihydrocostunolide as well, since they were also able to inhibit the (+)-germacrene A synthase (Chapter 3).

Table III. Inhibition of 11(*S*),13-dihydrocostunolide and leucodin biosynthesis from (+)-costunolide.

Assay Conditions[a]	Percentage Product Formation ± SD	
	Dihydrocostunolide (**8**)	Leucodin (**10**)
Air (≈80% N_2 + 20% O_2)	[b]100 ± 9	[b]100 ± 33
80% CO + 20 % O_2	140 ± 4	8 ± 1
Argon	134 ± 6	0 ± 0
Control	[c]100 ± 3	[c]100 ± 10
Cytochrome *c* (100 μM)	105 ± 5	0 ± 0
DMSO (1%) (solvent control)	[d]100 ± 5	[d]100 ± 10
Metyrapone (100 μM)	87 ± 11	70 ± 16
Clotrimazole (10 μM)	119 ± 9	0 ± 0
Miconazole (10 μM)	100 ± 5	0 ± 0
Aminobenzotriazole (10 μM)	75 ± 8	86 ± 33

[a]All incubations were carried out in the presence of 1 mM NADPH-regenerating system and flavins.

[b]100 % product formation corresponds to a saussurea lactone and a leucodin peak size of 1.63 and 0.34 × internal standard (1 nmol *cis*-nerolidol) respectively.

[c]100 % product formation corresponds to a saussurea lactone and a leucodin peak size of 1.34 and 0.42 × internal standard (1 nmol *cis*-nerolidol) respectively.

[d]100% product formation corresponds to a saussurea lactone and a leucodin peak size of 1.43 and 0.18 × internal standard (1 nmol *cis*-nerolidol) respectively.

Incorporation of Oxygen-18

Incubation of germacrene acid (**1**) in the presence $^{18}O_2$ led to the incorporation of one atom of ^{18}O into (+)-costunolide (**2**). As a result the ion peak in the mass spectrum of dehydrosaussurea lactone (**7**) was shifted from 232 to 234 amu (Fig 6). Similar changes were observed in the mass spectrum of saussurea lactone (**9**), the Cope rearrangement product of 11(*S*),13-dihydrocostunolide (**8**) (data not shown). The ion peak of this compound was shifted from 234 to 236 while the $[M\text{-}Me]^+$ peak was shifted from 219 to 221 amu.

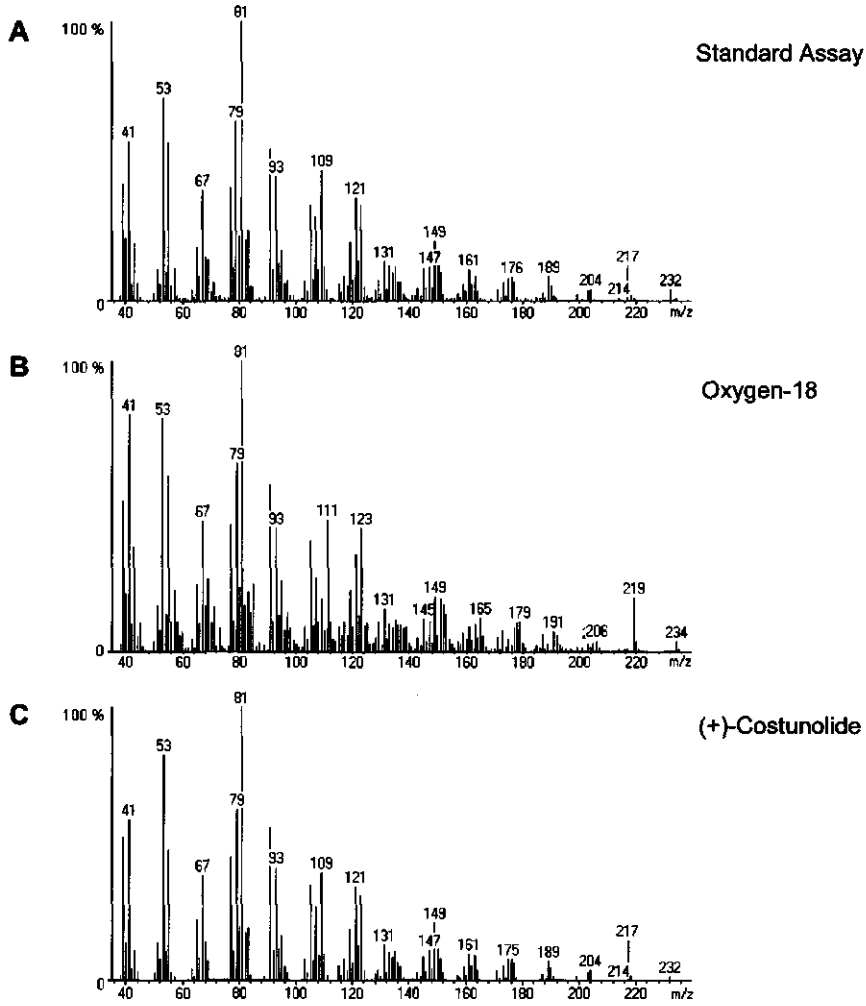


Figure 6. Mass spectra of dehydrosaussurea lactone (**7**) originating from (+)-costunolide (**2**) that has been produced from germacrene acid (**1**) under standard assay conditions (A), in the presence of $^{18}O_2$ (B), or that originates from a standard of (+)-costunolide (C).

The mass spectra of leucodin (**10**) showed the incorporation of two atoms of ^{18}O since the mass of the ion peak was shifted with 4 units from 246 to 250 amu (Fig. 7). Unfortunately, in the enzyme assay the GC-peak of leucodin is superpositioned on the tailing peak of linoleic acid (Fig. 3A), which obscures the mass spectrum of leucodin particular in the lower mass range.

In general, bubbling of the enzyme assay with $^{18}O_2$ did not have any effect on the detected amounts of enzymatic products, i.e. does not effect the enzyme activities.

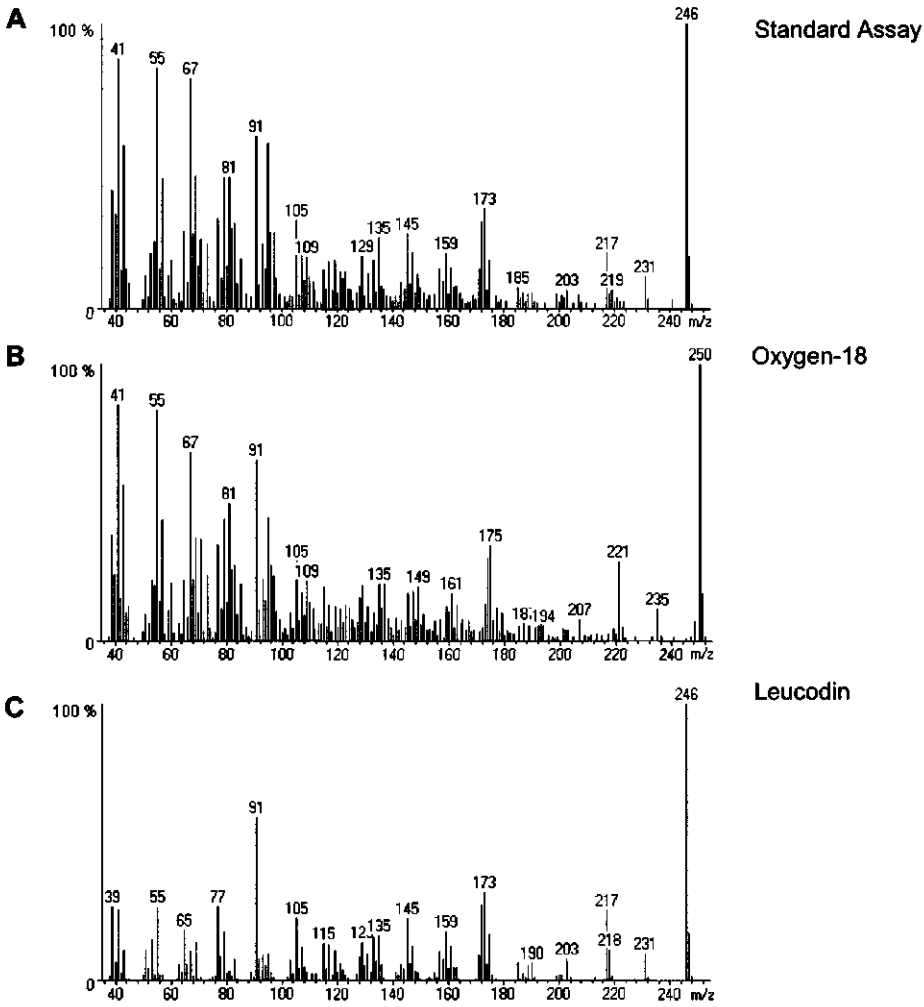


Figure 7. Mass spectra of leucodin (**10**) produced from germacrene acid (**1**) in an enzyme assay under standard conditions (A) or in the presence of $^{18}O_2$ (B). Panel C shows the mass spectrum of the leucodin standard (C).

DISCUSSION

The present results show that chicory roots contain an enzyme that converts germacra-1(10),4,11(13)-trien-12-oic acid (**1**) into (+)-costunolide (**2**), yielding the lactone ring present in sesquiterpene lactones. This step is the final proof for the postulated pathway from farnesyl diphosphate (FPP) through (+)-germacrene A, germacra-1(10),4,11(13)-trien-12-ol, and germacra-1(10),4,11(13)-trien-12-oic acid (**1**) to the germacrene-derived sesquiterpene lactones (Geissman, 1973; Fischer et al., 1979; Fischer, 1990).

The (+)-costunolide synthase is a cytochrome P450 enzyme that is dependent on NADPH and O_2 and is accordingly inhibited by various established cytochrome P450 inhibitors. Blue-light reversible CO inhibition of the (+)-costunolide synthase could be demonstrated as well, although the results are somewhat obscured by subsequent enzymatic conversions of (+)-costunolide. Incubation of the germacrene acid in the presence of $^{18}O_2$ showed the incorporation of one atom of ^{18}O into (+)-costunolide, another typical feature of cytochrome P-450 enzymes (West, 1980; Mihaliak et al., 1993). This incorporation of one atom oxygen also validates that germacrene acid is first hydroxylated at the C_6-position (Fischer et al., 1979) after which this hydroxyl group attacks, possibly enzyme-mediated, the carboxyl group at C_{12} (Fig. 8). After lactonisation the oxygen isotope is incorporated in the lactone ring.

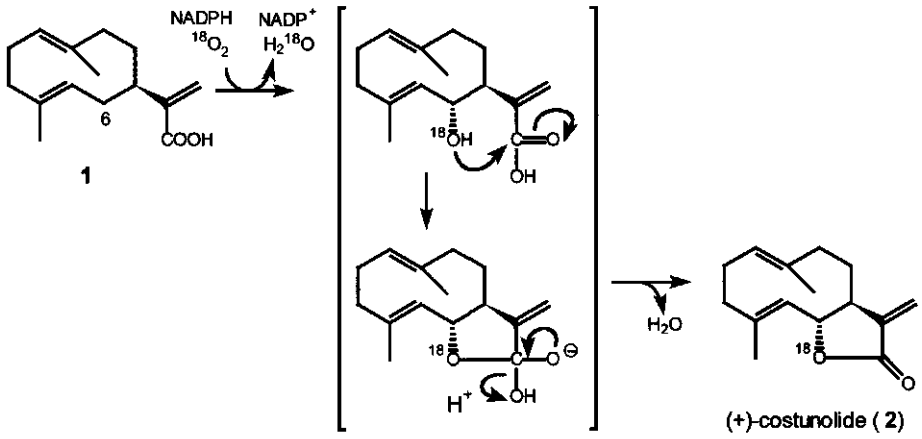


Figure 8. Mechanism for the enzyme catalysed formation of (+)-costunolide (**2**) from germacrene acid (**1**) that results in the incorporation of one atom of ^{18}O from $^{18}O_2$.

The (+)-costunolide synthase is not capable of converting the substrate analogues α-costic acid (Fig. 2, **3a**), or elema-1,3,11(13)-trien-12-oic acid (**4**) which is not unexpected, because

the C_6-position is not allylic. However, γ-costic acid (**3b**) —in which the C_6-position is allylic— was not converted either, so apparently the geometry of the cyclodecadiene ring system is also required for the reaction catalysed by the (+)-costunolide synthase.

In incubations with NADPH and the 20,000*g* supernatant of chicory roots, (+)-costunolide (**2**) is subsequently converted into 11(*S*),13-dihydrocostunolide (**8**) and leucodin (**10**) (Fig. 9). Formation of 11(*S*),13-dihydrocostunolide is not dependent upon oxygen, but is strongly enhanced in the presence of NADPH whereas some enzyme activity (15%) is retained in its absence. The reduction of the C_{11}-C_{13} exocyclic double bond is comparable to the type of reactions catalysed by enoate reductases. This is a group of iron-sulphur flavoproteins that are involved in fatty acid biosynthesis and can be found in many micro-organisms such as *Clostridia* and baker's yeast, but they have also been observed in plant cell cultures such as that of tobacco. They catalyse the reduction of double bonds that are 'activated' by an electron-withdrawing substituent, like the olefinic bond of α,β-unsaturated carboxylic acids and esters, under anaerobic conditions in the presence of reducing agents (generally NADH) (Holland, 1992; Faber, 2000). Similar to the stereoselective reactions catalysed by enoate reductases of micro-organisms, reduction of the C_{11}-C_{13} exocyclic double bond of (+)-costunolide yields only one isomer, i.e. the 11(*S*),13-stereoisomer of dihydrocostunolide. This compound has the same stereochemistry that is present in the 11,13-dihydro sesquiterpene lactones of chicory (Seto et al., 1988; van Beek et al., 1990). Notwithstanding, some plant species do contain C_{11}-epimers, like achillin which is the 11(*R*)-epimer of leucodin (Martínez et al., 1988; Ho et al., 1998), indicating that a stereoselective enzyme that synthesises these C_{11}-epimers should also exist. The enzyme exhibits at least some substrate specificity as the C_{11}-C_{13} exocyclic double bond of dehydrocostus lactone (**6**) was not reduced.

The detected formation of leucodin (**10**) proves that guaianolides originate from (+)-costunolide. This conversion of (+)-costunolide into leucodin most likely involves more than one enzyme, but it was not investigated whether leucodin originates from 11(*S*),13-dihydrocostunolide (**8**). Parthenolide (**5**) is not involved in leucodin biosynthesis, still it cannot be excluded that 11(*S*),13-dihydroparthenolide is. Various authors have suggested that either such a 4,5-epoxide or a C_3-hydroxyl group is necessary to direct the cyclisation of (+)-costunolide towards a guaiane framework (Brown et al., 1975; Fischer 1990; Teisseire, 1994; Piet et al., 1995; Piet et al., 1996).

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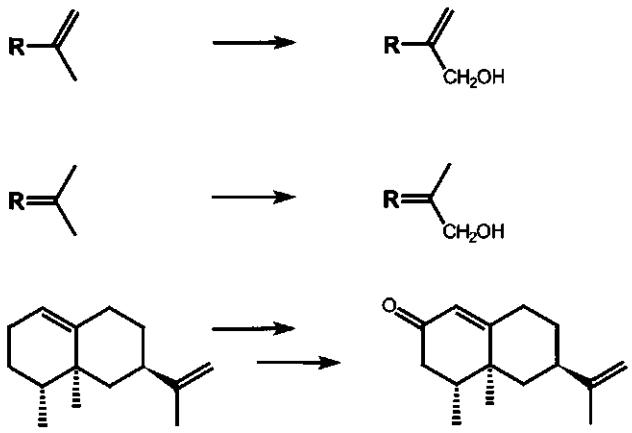
6. Hydroxylations of Sesquiterpenes by Enzymes from Chicory Roots

Substrate Specificity of Oxidising Enzymes Involved in Chicory Sesquiterpene Lactone Biosynthesis



Abstract: A microsomal pellet of chicory roots is able to hydroxylate various sesquiterpene olefins in the presence of NADPH. Most of these hydroxylations take place at an isopropenyl or isopropylidene group. The number of products obtained from a certain substrate is confined to one or, in a few cases, two sesquiterpene alcohols. These reactions are most likely catalysed by the (+)-germacrene A hydroxylase, an enzyme involved in sesquiterpene lactone biosynthesis, thus opposing the general idea that cytochrome P450 enzymes of plant secondary metabolism have a narrow substrate specificity. The established conversion of (+)-valencene into nootkatone does presumably not involve the (+)-germacrene A hydroxylase and is catalysed by other enzymes of sesquiterpene lactone biosynthesis. The reaction proceeds via β-nootkatol and involves both a hydroxylating enzyme and $NAD(P)^+$-dependent dehydrogenases.

evaporation of the ether, 140 mg of a crude oil was obtained that besides β-nootkatol contained 4% of α-nootkatol. After flash chromatography of 50 mg of this crude oil on silica with ether-pentane (2:1), fractions devoid of any trace of α-nootkatol were pooled yielding 3.6 mg of β-nootkatol. ^{1}H NMR (400 MHz, C_6D_6) δ 0.83 (*d*, 3H, Me_{14}, J = 3 Hz), δ 0.95 (*s*, 3H, Me_{15}), δ 0.96-1.67 (*m*, 7H) δ 1.75 (*s*, 3H, Me_{13}) δ 1.93 (*dt*, 1H, H_3, J = 12.7 and 2.7 Hz), δ 2.07 (*ddd*, 1H, H_9, J = 14.0, 2.6 and 1.6 Hz) δ 2.20-2.33 (*m*, 2H, H_7 and $H_{9'}$) δ 4.19-4.26 (*m*, 1H, H_2), δ 4.89 (*br s*, 2H, H_{12}), δ 5.44 (*br d*, 1H, H_1, J = 1.7 Hz). ^{13}C NMR (100 MHz, DEPT, C_6D_6) δ 15.7 (q), δ 18.4 (q), δ 21.1 (q), δ 32.8 (t), δ 33.4 (t), δ 37.8 (t), δ 38.5 (s), δ 39.8 (d), δ 41.3 (d), δ 45.1 (t), δ 68.1 (d), δ 109.2 (t), 125.9 (d), δ 144.9 (s), δ 150.2 (s). Mass spectra are described at the end of this chapter in the appendix.

Alloisolongifolene alcohol was prepared from alloisolongifolene via its corresponding aldehyde. This aldehyde was prepared by (over)oxidation of alloisolongifolene with selenium dioxide, making use of the fact that in the structure of alloisolongifolene only one allylic position is available for selenium dioxide mediated hydroxylation. To a solution of 77 mg SeO_2 and 68 mg salicylic acid in 30 mL of CH_2Cl_2, 100 mg alloisolongifolene was added. Stirring at room temperature turned the reaction mixture yellow and a red solid precipitated in the first few hours. The reaction was monitored by GC-MS along with TLC and was stopped by the addition of 60 mL demineralised water after 2.5 days. The reaction mixture was extracted with 45 mL of ether, and the organic phase was subsequently washed with 30 mL brine. The red precipitate remained in the aqueous phase during extraction. After drying and evaporation, the organic phase yielded 214 mg of a crude solid that after flash chromatography on silica with pentane-CH_2Cl_2 (3:1) yielded 31 mg of alloisolongifolene aldehyde, a strong odorous compound (cedar-wood like). ^{1}H-NMR (200 MHz, C_6D_6) δ 0.71 (*s*, 3H, Me) δ 0.90 (*s*, 3H, Me), δ 0.93-2.04 (*m*, 13H), δ 5.42 and 5.73 (2 × *s*, 2H, CH_2=C), δ 9.35 (*s*, 1H, CH=O). ^{13}C-NMR (50 MHz, C_6D_6) δ 15.0 (q), δ 19.6 (q), δ 20.5 (t), δ 22.2 (t), δ 32.3 (t), δ 36.3 (t), δ 38.7 (t), δ 45.1 (t), δ 46.1 (s), δ 47.1 (s), δ 47.7 (s), δ 50.8 (d), δ 133.5 (t), δ 157.0 (s), δ 193.9 (d). Fifteen milligrams of the aldehyde was added to a solution of 1.8 mg $LiAlH_4$ in 0.5 mL of ether. The grey suspension was stirred for 17.5 hours at room temperature and stirred for an additional half hour after the careful addition of one spatula of $Na_2SO_4 \cdot 10H_2O$. One and a half millilitres of demineralised water was added to the mixture, which was then thrice extracted with 1 mL of ether. The ether was passed through a glasswool-plugged Pasteur pipette filled with silica and a spatula tip of $MgSO_4$. The solvent was evaporated, yielding 15 mg of alloisolongifolene alcohol (approx. 95 % pure). ^{1}H-NMR (200 MHz, C_6D_6) δ 0.77 (*s*, 3H, Me), δ 0.91 (s, 3H, Me), 0.96-1.63, δ 3.96 (*m*, 2H, CH_2-

[OH]), δ 4.95 and 5.30 (2 × *dt*, 2H, CH_2=C, J = 1.5 and 1.5 Hz; J = 1.5 and 1.9 Hz respectively). ^{13}C NMR (50 MHz, C_6D_6) δ 15.1 (q), δ 19.7 (q), δ 20.7 (t), δ 22.3 (t), δ 32.4 (t), δ 37.5 (t), δ 38.7 (t), δ 44.0 (t), δ 46.1 (s), δ 47.8 (s), δ 48.2 (s), δ 51.6 (d), δ 63.4 (t), δ 107.9 (t), δ 155.9 (s).

A mixture of *trans,trans*-farnesal and *cis,trans*-farnesal was prepared by dissolving *trans,trans*-farnesol (Sigma) in pentane and stirring with manganese dioxide. Retention times and mass spectra were identical to those of farnesal from Fluka, which contains all four farnesal isomers. Farnesal was oxidised to a mixture of *cis,trans*- and *trans,trans*-farnesoic acid with silver oxide (Caliezi and Schinz, 1947). (-)-Elema-1,3,11(13)-trien-12-ol was kindly provided by Dr B. Maurer (Firmenich SA, Switzerland) and amorpha-4,11-diene-12-ol was a gift of Dr. H. J. Bouwmeester.

Incubations with Microsomal Pellets

Microsomal pellets that contain (+)-germacrene A hydroxylase activity were prepared from deep frozen chicory cubes as described in Chapter 3. They can easily be stored under argon at –80°C and one microsomal pellet corresponds to approximately 4 grams of fresh chicory root. Before incubation, 10 pellets were pottered in 30 mL of assay buffer containing 25 mM Tris (pH 7.5), 2 mM DTT, 1 mM ascorbic acid, 5 μM FAD, 5 μM FMN, and 10% (v/v) glycerol. The enzyme suspension was divided in 1-mL aliquots and incubated with 5 μL substrate solution in the presence of a 1 mM NADPH-regenerating system, which consisted of 1 mM NADPH, 5 mM glucose-6-phosphate, and 1.2 IU glucose-6-phosphate dehydrogenase (all from Sigma). The initial concentration of the substrate in each assay was 45 μM and all experiments were done in duplicate. To the blank assays no NADPH was added. After 60 minutes the incubations were stopped by storing them at –20°C.

Five nanomols of *cis*-nerolidol (Fluka) was added to each enzyme assay that was extracted twice with 1 mL 20% (v/v) ether in pentane. The organic phase was filtered through a glasswool-plugged (dimethyl chlorosilane-treated, Chrompack) Pasteur pipette that contained 0.4 gram of silica and a little anhydrous $MgSO_4$. The small column was rinsed with 1.5 mL ether and the extract was concentrated to approximately 50 μL under a stream of nitrogen. The concentrated extracts were analysed by GC-MS as described in Chapter 3.

To investigate whether enzymatic hydroxylation of the substrates was catalysed by the (+)-germacrene A hydroxylase, standard incubations of the various substrates (50 μM) were carried out in the presence of 50 μM (+)-germacrene A. To control incubations only 5 μL of ethanol was added (i.e. the solvent of the added [+]-germacrene A). To exclude any general

Germacrene B

GC-MS analyses of the incubation of germacrene B yielded two products with an almost identical mass spectrum and retention time (KI 1694 and 1700). Presumably either of the two methyl groups in the isopropenyl side chain of germacrene B is hydroxylated, yielding very similar products. Hydroxylation at any other position is unlikely, because such a hydroxylation would probably have been observed for (+)-germacrene A as well. The products are measured as their Cope-rearrangement products (γ-elemene alcohols). Lowering the injection port temperature from 250°C to 150°C yielded faint broadened peaks due to on-column Cope-rearrangement of the germacrene alcohols (Chapter 4).

(+)-γ-Gurjunene

Incubation of (+)-γ-gurjunene yielded an unknown sesquiterpene alcohol (KI 1760). Stirring the pentane-ether extract of the enzyme assay with manganese dioxide overnight gave complete conversion into an aldehyde or ketone ([M^+] 218). Because this reagent is specific for α,β-unsaturated alcohols (March, 1992) and the only other available allylic position is at a tertiary carbon atom, the biochemical hydroxylation had most likely occurred in the isopropenyl group of (+)-γ-gurjunene yielding (1*S*,4S,7*R*,10*R*)-5,11(13)-guaiadiene-12-ol.

Neointermedeol

Neointermedeol is presumably hydroxylated in the isopropenyl group like β-selinene, yielding 4β-H-eudesm-11(13)-ene-4,12-diol.

Conversion of (+)-Valencene.

Incubation of (+)-valencene with a microsomal pellet from chicory roots and NADPH yielded (Fig. 2 and Table I) only a trace of the expected valencen-12-ol ([-]-2-[2*R*]-2-[1,2,3,4,6,7,8,8a-octahydro-8α,8aβ-dimethyl-2α-napthalenyl]-2-propen-1-ol). Its mass spectrum matches exactly that of the (+)-enantiomer (compound reported in Näf et al. [1995]). This mass spectrum was kindly provided by Dr. R. Näf of Firmenich SA (Geneva, Switzerland). The major product from the incubation of (+)-valencene was nootkatone, whereas a smaller quantity of β-nootkatol was detected as well.

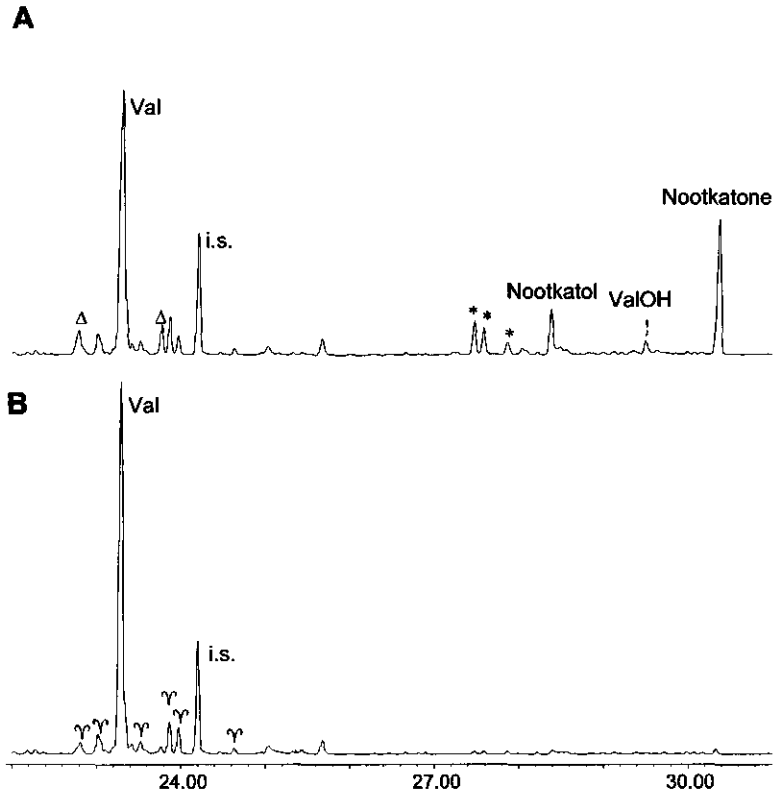


Figure 2. GC-MS analysis of the products formed by a microsomal preparation of chicory roots incubated with (+)-valencene (Val) in the presence (A) or absence (B) of NADPH. Incubation in the presence of NADPH yields nootkatone, β-nootkatol, and valencen-12-ol (ValOH). Peaks marked with Δ are GC-induced dehydration products of β-nootkatol ($[M]^+$ 202). Peaks labelled with an asterisk are sesquiterpene alcohols that presumably origin from the enzymatic conversion of sesquiterpene impurities (marked with ϒ in panel B) present in the purchased sample of (+)-valencene.

It was assumed that this β-nootkatol is an intermediate in the formation of nootkatone. For this reason incubations of 100 μM β-nootkatol were performed at pH 10 with $NAD(P)^+$ and a 150,000*g* supernatant of chicory roots, as described for the conversion of (-)-elema-1,3,11(13)-12-ol and germacra-1(10),4,11(13)-trien-12-ol by $NAD(P)^+$-dependent dehydrogenases in Chapter 3. During incubation the added β-nootkatol was for more than 90% converted into nootkatone in the presence of either 1 mM $NADP^+$ or 1 mM NAD^+. In the absence of these cofactors the conversion still amounted to 25%, whereas the boiled enzyme extract gave no conversion of β-nootkatol. At pH 7.5 enzyme activity was slightly reduced,

and a 150,000*g* pellet yielded three times less dehydrogenase activity than its corresponding supernatant (after correction for dilution of enzyme activity). Incubation of the crude mixture of α- and β-nootkatol showed that α-nootkatol was hardly converted while β-nootkatol was converted to the same extent as described above (Fig. 3).

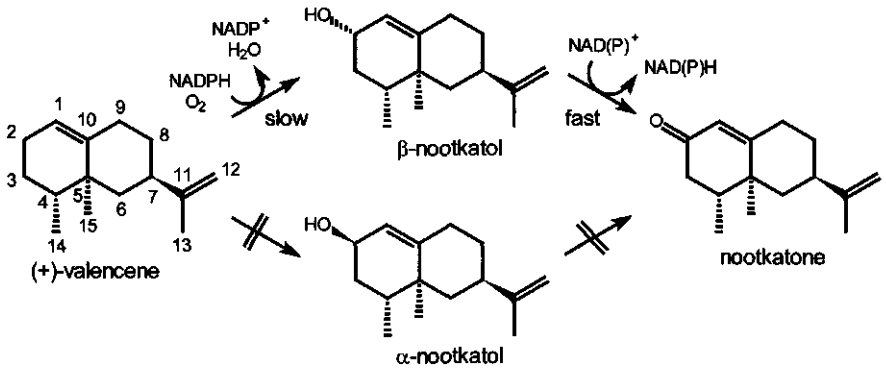


Figure 3. Conversion of (+)-valencene into nootkatone proceed via β-nootkatol and not via α-nootkatol.

To get some more information about the substrate specificity of the dehydrogenase(s) present in chicory roots, incubations have also been performed with 100 μM *trans,trans*-farnesol (Fig. 4) and the 150,000*g* supernatant in the presence of either NAD^+ or $NADP^+$. *trans,trans*-Farnesol was up to 60% converted into a mixture of more or less equal amounts of *trans,trans*-farnesal and *cis,trans*-farnesal, whereas small amounts of farnesoic acid were observed as well, predominately the *cis,trans*-isomer. The obtained amount of products was somewhat higher in the presence of $NADP^+$ than in the presence of NAD^+. Various pH-values were tested between 7.5 and 11.0 using Tris, glycine and CAPS buffers; the highest conversion of farnesol into farnesal was observed at pH 10, decreasing to 30% of maximum enzyme activity at pH 7.0.

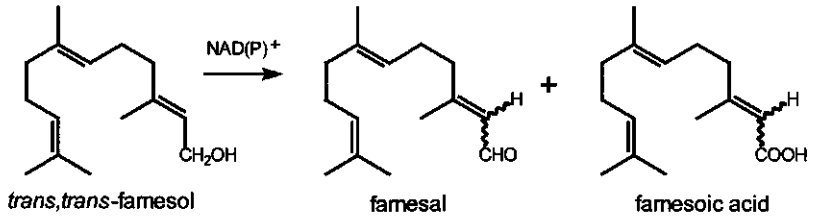


Figure 4. Conversion of *trans,trans*-farnesol into farnesal and farnesoic acid by a 150,000*g* supernatant of chicory roots in the presence of $NAD(P)^+$.

Competitive Inhibition Experiments

The microsomal pellets that were used in the experiments do not exclusively contain the (+)-germacrene A hydroxylase, but also other membrane bound enzymes that are present in chicory roots. Hence, some of the conversions described in Table I might as well be catalysed by other oxidising enzymes than the (+)-germacrene A hydroxylase. To investigate this, incubations of the different substrates were done in the presence of 50 μM (+)-germacrene A. It is to be expected that if the (+)-germacrene A hydroxylase is involved in these reactions, the conversions will be inhibited by the addition of the natural substrate of this enzyme. Incubations were also carried out with 50 μM (-)-α-cubebene instead, a sesquiterpene that is not hydroxylated (Fig. 1), to test the effect of the addition of any arbitrary sesquiterpene olefin on enzyme activity.

Table II. Effect of the addition of 50 M (+)-germacrene A or 50 μM (+)-α-Cubebene upon hydroxylation.

Substrate	Product[a]	Inhibition (%) ± SD	
		(+)-Germacrene A	(-)-α-Cubebene
alloisolongifolene	alloisolongifolene alcohol	100.0	100.0
amorpha-4,11-diene	amorpha-4,11-diene-12-ol	84.9 ± 0.1	- 9.2 ± 2.5
	amorpha-4,11-diene alcohol (KI 1761)	84.9 ± 1.0	- 2.3 ± 1.5
(-)-α-*trans*-bergamotene	(*E*)-*trans*-bergamota-2,12-dien-14-ol	89.2 ± 1.8	30.2 ± 4.5
(-)-β-elemene	(-)-elema-1,3,11(13)-trien-12-ol	90.4 ± 1.2	19.5 ± 1.8
germacrene B	germacrene B alcohol (KI 1694)	100.0	32.6 ± 3.6
	germacrene B alcohol (KI 1700)	100.0	27.3 ± 10
(+)-γ-gurjunene	(+)-γ-gurjunene alcohol	88.0 ± 0.9	- 5.6 ± 0.2
(+)-ledene	ledene alcohol	100.0	35.5 ± 1.9
neointermedeol	4β-H-eudesm-11(13)-ene-4,12-diol	67.5 ± 1.9	3.4 ± 4.5
(+)-β-selinene	(+)-β-costol	60.3 ± 3.4	0.4 ± 2.0
(+)-valencene	valencen-12-ol	100.0	100.0
	nootkatone	90.2 ± 2.5	8.7 ± 2.5

[a]The amount of sesquiterpene alcohol produced in control incubations is set at 100% and is comparable with those of Table I.

The results presented in Table II show that all enzymatic hydroxylations were inhibited to about 90% by the addition of (+)-germacrene A, except for (+)-β-selinene and neointermedeol whose hydroxylation was inhibited by 60% and 68% respectively. The relatively small inhibition of β-selinene hydroxylation agrees with the observation that it is nearly as well hydroxylated as (+)-germacrene A (Table I). Whether the structural relation

between (+)-β-selinene and neointermedeol can explain the small inhibitory effect of (+)-germacrene A upon hydroxylation of the latter is then again dubious, since neointermedeol itself is a poor substrate (Table I). More surprisingly is that the addition of (+)-germacrene A also affected hydroxylations of sesquiterpene alcohols in which no isopropenyl or isopropylidene group is involved, like nootkatone and ledene alcohol.

Hydroxylation of the sesquiterpenes is not dramatically influenced by the addition of (-)-α-cubebene, except for the formation of alloisolongifolene alcohol and valencen-12-ol which already under normal assay conditions are only formed in small quantities.

Effect of Organic Solvents on Enzyme Activity

Before experiments as described in this chapter were started, it was tested which organic solvent could be best used to dissolve the substrate. Stock solutions of 10 mM γ-gurjunene were prepared in hexane, pentane, iso-propanol, ethanol, and DMSO, and 5 μL of these solutions was added to individual incubation mixtures. On the basis of the results presented in Table III ethanol was chosen as solvent for the substrates in all experiments, instead of the commonly used pentane (*e.g.* Karp et al., 1990).

Table III. Effect of the used solvent for (+)-γ-gurjunene on enzyme activity

Solvent	Enzyme Activity[a] ± SD
hexane	< 0.1
pentane	0.47 ± 0.07
iso-propanol	1.76 ± 0.16
ethanol	1.87 ± 0.01
DMSO	1.80 ± 0.09

[a]Peak height of γ-gurjunene alcohol relative to the internal standard (5 nmol *cis*-nerolidol)

DISCUSSION

In the presence of NADPH, a microsomal enzyme preparation from chicory roots is able to hydroxylate a range of sesquiterpene olefins that are exogenous to the plant (Table I). Most of these hydroxylations take place at an isopropenyl group or isopropylidene group, yielding in some cases sesquiterpene alcohols that have not previously been described, e.g. amorpha-4,11-dien-12-ol and alloisolongifolene alcohol. The novelty of the formed sesquiterpene

alcohols hampered their identification in some cases, and the structure assignment of the hydroxylation products of germacrene B, (+)-γ-gurjunene and neointermedeol is tentative or, like for (+)-ledene, impossible. Unfortunately, these compounds were not yet produced in sufficient amount to isolate them for structure elucidation by ^{1}H-NMR. The substrates for hydroxylation preferably do not contain any polar group: neointermedeol is 15-fold less efficiently hydroxylated than β-selinene, and germacrone is not hydroxylated in contrast to germacrene B. The size of the substrate is also of importance as hydroxylation of limonene, a monoterpene, hardly occurred. In the cases of amorpha-4,11-diene and (+)-valencene two distinct sesquiterpene alcohol products were formed.

Hydroxylations occurring at isopropenyl groups and —less efficiently— at isopropylidene groups of sesquiterpenes are similar to the (+)-germacrene A hydroxylase catalysed hydroxylation of the isopropenyl group in (+)-germacrene A. However, the microsomal pellet of chicory roots does not exclusively contain the (+)-germacrene A hydroxylase, but also contains other membrane bound (cytochrome P450) enzymes. Yet, the competitive inhibition of the hydroxylation reactions by (+)-germacrene A (Table II) gives further evidence that these reactions are indeed catalysed by the (+)-germacrene A hydroxylase that is involved in chicory sesquiterpene lactone biosynthesis. Hydroxylation of various exogenous substrates by one and the same cytochrome P450 enzyme involved in plant secondary metabolism contradicts the general idea that enzymes of plant secondary metabolism have narrow substrate specificities (Donaldson and Luster, 1991; Halkier, 1996; Schuler, 1996; Mihaliak et al., 1993; Karp et al., 1990, and Chapter 1).

Hydroxylation to the unknown ledene alcohol and the unknown amorpha-4,11-diene alcohol (KI 1761) cannot have occurred in an isopropenyl/isopropylidene group, nevertheless, these reactions are also inhibited by (+)-germacrene A. Although difficult to understand, this suggests the involvement of the (+)-germacrene A hydroxylase in these reaction as well. Notably, the rate of competitive inhibition by (+)-germacrene A is the same for the formation of amorpha-4,11-diene-12-ol and the unknown amorpha-4,11-diene alcohol.

Most intriguing is the observed conversion of (+)-valencene via β-nootkatol into nootkatone (Figs. 2 and 3). Possibly these reactions are catalysed by the same enzymes that are involved in the biosynthesis of leucodin from (+)-costunolide (Fig. 5; see also Chapter 5). On the basis of its structural resemblance with leucodin, the same may have occurred to (+)-ledene —but this compound was only hydroxylated and not converted into a ketone. It seems likely that the (+)-germacrene A hydroxylase is not involved in formation of nootkatone, nevertheless, its formation was inhibited up to 90% by the addition of

(+)-germacrene A. This inhibitory effect might be due to the structural relationship between (+)-germacrene A and (+)-costunolide, but this assumption should be verified by repeating the incubations with (+)-costunolide as a competitor of (+)-valencene.

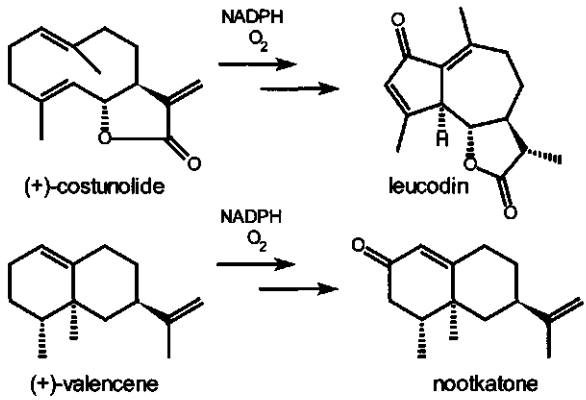


Figure 5. The speculative correspondence between the formation of leucodin in chicory sesquiterpene lactone biosynthesis and the conversion of (+)-valencene into nootkatone.

Formation of nootkatone catalysed by enzymes of chicory proceeds via β-nootkatol that is quickly oxidised to nootkatone by very efficient $NAD(P)^+$-dependent dehydrogenase(s). The involved dehydrogenases are water-soluble, but adhere somewhat to the microsomal pellet just like the (+)-germacrene A alcohol dehydrogenase(s). The dehydrogenase(s) involved in formation of nootkatone have a strong preference for β-nootkatol over α-nootkatol, but on the whole the dehydrogenase(s) present in 150,000*g* supernatant from chicory roots do not seem to act with a high substrate specificity. In addition to the conversion of germacrene alcohol and elemene alcohol/aldehyde (Chapter 3), they are also capable of converting *trans,trans*-farnesol into farnesal and farnesoic acid. $NAD(P)^+$-dependent oxidation of farnesol to farnesal has also been reported for other crude plant extracts, just as the observed isomerisation of farnesal (Chayet et al., 1973; Overton and Roberts, 1974). Whether *in vivo* the isolated dehydrogenase activities are exclusively involved in sesquiterpene lactone biosynthesis or have (also) a different function is unclear. Remarkably, there is not yet any direct proof that in grapefruit, the source of valencene and nootkatone, a biochemical pathway exists for the conversion of (+)-valencene via β-nootkatol into nootkatone (del Río et al., 1992).

Concerning pathways in plant secondary metabolism, the hydroxylation of amorphadiene-4,11-diene in the isopropenyl group is interesting as well. It is likely an important step in

biosynthesis of the anti-malarial drug artemisinin in, but this step has not yet been reported for *Artemisia annua* itself (the source of artemisinin) (Bouwmeester et al., 1999).

Nootkatone is an example of an aromatic substance that is widely used in the food and flavour industry because of its distinctive grapefruit flavour. It is a rather expensive compound and for this reason the possibilities for its (bio)synthesis from the less valuable (+)-valencene have been studied intensively. However, the yields obtained by either chemical or microbiological methods were not satisfying or the procedure is too laborious (Dhavlikar and Albroscheit, 1973; Könst et al., 1975; Lamare and Furstoss, 1990). From that point of view the established conversion of (+)-valencene into nootkatone by a microsomal pellet of chicory might be of interest.

More in general, the drawbacks of chemical hydroxylation and the demand for new compounds by the aroma and fragrance industry have been a powerful driving force in the research on hydroxylation of terpenes by micro-organisms (Faber, 2000). Although in some cases successful, these microbiological conversions often yield a broad spectrum of products, including epoxides and diols; furthermore, the oxidations often occur at double bonds (Lamare and Furstoss, 1990; Drauz and Waldmann, 1995; Faber, 2000). On the other hand, the hydroxylations catalysed by the microsomal pellet of chicory yield mostly one or sometimes two products and they occur with high regiospecificity.

Illustrative is the example of (+)-γ-gurjunene shown in Figure 6: whereas incubation with the microsomal pellet of chicory yields (1*S*,4*S*,7*R*,10*R*)-5,11(13)-guaiadien-12-ol, biotransformation of (+)-γ-gurjunene with the plant pathogenic fungus *Glomerella cingulata* yields (1*S*,4*S*,7*R*,10*R*)-5-guaien-11,12-diol and (1*S*,4*S*,7*R*,10*S*)-5-guaien-10,11,12-triol (Miyazawa et al., 1998). Attempts to prepare alcohols from (+)-γ-gurjunene by chemical means have also been reported (Bombarda et al., 1997). Oxidation of (+)-γ-gurjunene with *m*-chloroperbenzoic acid (MCPBA) in a ratio of 1:2 yields a mixture of two diepoxides which differ in their absolute configuration at C_{11}. Smaller amounts of MPCBA additionally yield the two possible monoepoxides in the reaction mixture. Subsequent reduction of the diepoxides with 2 equivalents of $LiEt_3BH$ gives a mixture of two enediols, an epoxide alcohol, and a dienol, but reduction with 4 equivalents of $LiEt_3BH$ yields only one diol. Clearly, the microsomal pellet from chicory furnishes an alcohol that is different from those produced of (+)-γ-gurjunene by the fungus or by chemical methods.

In conclusion, cytochrome P450 hydroxylases and alcohol dehydrogenases present in *Cichorium intybus* do have potential as catalysts in organic chemistry. However, it should be

stressed that the reactions described in this chapter have been carried out in small vials; more research is needed before these enzymes can be applied on a preparative scale.

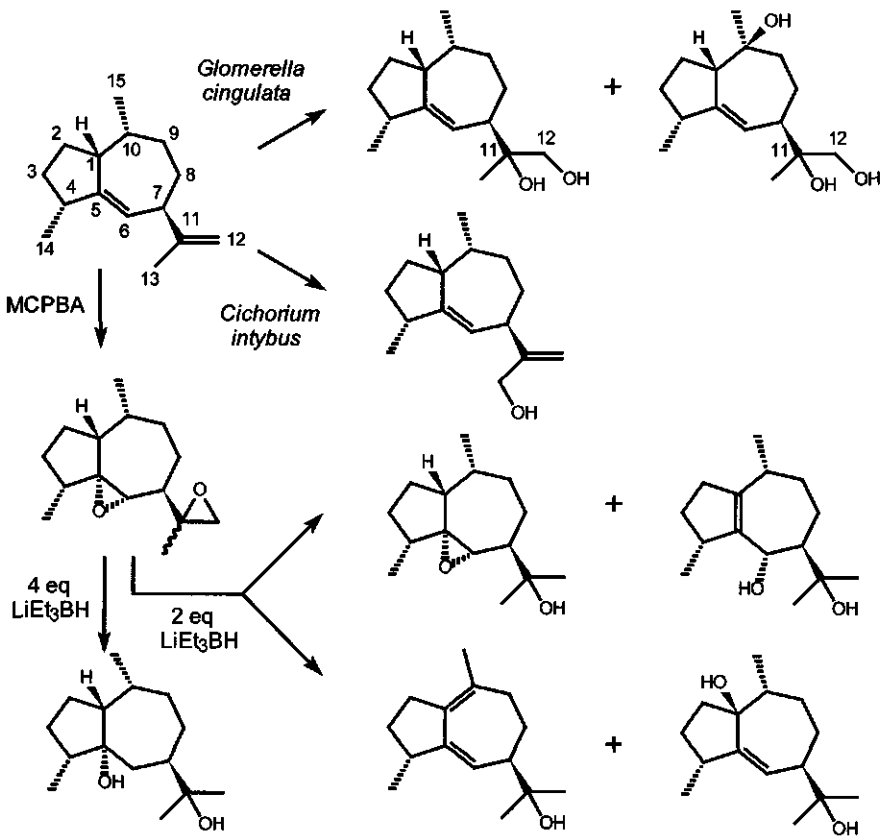


Figure 6. Different ways of converting (+)-γ-gurjunene in various sesquiterpene alcohols. Biotransformation of (+)-γ-gurjunene with a plant pathogenic fungus yields (1*S*,4*S*,7*R*,10*R*)-5-guaien-11,12-diol and (1*S*,4*S*,7*R*,10*S*)-5-guaien-10,11,12-triol; incubation of γ-gurjunene with a microsomal pellet of chicory yields (1*S*,4*S*,7*R*,10*R*)-5,11(13)-guaiadiene-12-ol. Chemical epoxidation of γ-gurjunene with *m*-chloroperbenzoic acid (MCPBA) and subsequent reduction with 2 equivalents $LiEt_3BH$ yields a mixture of alcohols, whereas 4 equivalents of $LiEt_3BH$ results in a single sesquiterpene diol.

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have this stereochemical orientation at C_7 and only a few plant species are reported to contain antipodal sesquiterpene olefins (Kalsi et al, 1979; Suire et al., 1982; Minnaard, 1997). One of these exceptions is *Solidago canadensis* that contains both (+)- and (-)-germacrene D. It was demonstrated that this plant accordingly contains two distinct sesquiterpene synthases, each of them producing exclusively one of the two enantiomers (Schmidt et al, 1999).

Bouwmeester et al. (2001) recently isolated from chicory chicons two different full-length cDNAs encoding distinct (+)-germacrene A synthases that share 72% identity: *CiGASsh* containing a putative open reading frame of 1674 bp (558 amino acids) encoding a protein of 64.4 kD, and *CiGASlo* containing a putative open reading frame of 1749 bp (583 amino acids) encoding a protein of 67.1 kD. Both (+)-germacrene A synthase genes were brought to heterologous expression in *E. coli*. The *CiGASsh* encoded (+)-germacrene A synthase exhibits a K_m of 3.2 μM and an optimum pH of 7.0; the other (*CiGASlo*) exhibits a K_m of 6.9 μM, an optimum pH of 6.8 and seems to function less efficiently. Although both genes are expressed throughout the plant, only the *CiGASlo* encoded (+)-germacrene A synthase was purified in Chapter 2 from chicory roots (K_m of 6.6 μM, optimum pH of 6.7) Apparently the other (+)-germacrene A synthase (*CiGASsh*) was lost during this purification procedure. Bouwmeester et al. (2001) repeated the purification of the (+)-germacrene A synthase activity from a crude plant extract with a different protocol. Subsequent use of Q-Sepharose and Mono-Q anion exchange chromatography yielded indeed the two distinct (+)-germacrene A synthase activities that closely but separately eluted from the Mono-Q column. First eluted the *CiGASsh* encoded protein then the *CiGASlo* encoded one. A sample of the DEAE purified (+)-germacrene A synthase of Chapter 2 was also applied to the Mono-Q column and yielded only one peak of activity at the same position as the *CiGASlo* protein. This shows that the used anion exchanger DEAE, in contrast with Q-Sepharose, does only bind the *CiGASlo* encoded (+)-germacrene A synthase from a crude plant extract, which probably also explains the low recovery of enzyme activity (only 30%) in this first step of the protocol used for purification of the (+)-germacrene A synthase in Chapter 2.

Why chicory does contain two separate germacrene A synthases is unclear. Yet, some regulation also at protein level can be expected. Sesquiterpene synthases play after all an important role in the partitioning of FPP between the formation of isoprenoids essential for cell growth and development (such as sterols, carotenoids and isoprenoid hormones) and the formation of secondary metabolites (Threlfall and Whitehead, 1988; McCaskill and Croteau, 1997). Notably the (+)-germacrene A synthase with the lowest K_m-value (*CiGASsh*), i.e. probably the most efficient one, is preferably expressed in the roots (Bouwmeester et al.,

2001) that are known to contain the highest amounts of sesquiterpene lactones within the chicory plant (Rees and Harborne, 1985).

The sequences of the isolated (+)-germacrene A synthase genes can be used in a technique called gene-silencing to block (+)-germacrene A biosynthesis. This is likely to stop sesquiterpene lactone biosynthesis and may result in the development of less bitter tasting varieties of Belgian endive, a patented idea (Bouwmeester et al., 2000).

(+)-Germacrene A Hydroxylase (II)

Hydroxylation of (+)-germacrene A is catalysed by the (+)-germacrene A hydroxylase, a membrane bound cytochrome P450 enzyme that requires oxygen and NADPH as cofactor (Chapter 3). Incubations with a racemic mixture of the substrate analogue β-elemene demonstrated only a modest degree of enantioselectivity of this enzyme (Chapter 3). More interestingly this enzyme does apparently not exhibit the rather strict substrate specificity that is generally ascribed to the cytochrome P450 enzymes of plant secondary metabolism (Akhtar and Wright, 1991; Donaldson and Luster, 1991; Halkier, 1996) and is able to hydroxylate various other sesquiterpenes (Chapter 6). The presence of less substrate specific biosynthetic cytochrome P450 enzymes in chicory substantiates the idea that some or all of the detoxifying cytochrome P450 enzymes present in plants actually represent biosynthetic enzymes "moonlighting" as detoxifying enzymes (Schuler, 1996).

Pyridine Nucleotide Dependent Sesquiterpenoid Dehydrogenases (III)

The conversion of germacra-1(10),4,11(13)-trien-12-ol into germacra-1(10),4,11(13)-trien-12-oic acid occurs via germacra-1(10),4,11(13)-trien-12-al (Chapter 3). This aldehyde is sensitive towards nucleophilic attack (e.g. DTT present in the enzyme assay) which complicates its detection in enzyme assays, but it was unambiguously observed together with germacra-1(10),4,11(13)-trien-12-oic acid in the experiments described in Chapter 6. Most investigations were done with the substrate analogue (-)-elema-1,3,11(13)-trien-12-ol, because initially the real substrate germacra-1(10),4,11(13)-trien-12-ol was not available. It yields elemene acid only in the presence of $NADP^+$, but the results concerning the cofactor dependence are somewhat ambiguous since oxidation of elema-1,3,11(13)-trien-12-al occurs preferably in the presence of NAD^+. The used enzyme extract probably contains a mixture of $NAD(P)^+$-dehydrogenases and is also capable of converting farnesol into farnesoic acid. In these experiments the intermediate farnesal was clearly observed, presumably because it is less sensitive toward nucleophilic attack. In addition farnesal was formed in the presence of

either NAD^+ or $NADP^+$. This might implicate that the germacrene/elemene aldehyde, and subsequently the acid, are also formed in enzyme assays containing NAD^+, but the produced amount of aldehyde was not sufficient to overcome the problem of aspecific binding to proteins and thiols present in the incubation mixture. Although the dehydrogenases are water-soluble enzymes, they are to some extent also present in the microsomal pellet (↓ 20,000-150,000*g*) and active under conditions (pH 7.5 and high $NADPH/NADP^+$-ratio; Chapter 6) that are more suitable for cytochrome P450 enzymes.

(+)-Costunolide Synthase (IV)

The last step in the formation of (+)-costunolide involves a cytochrome P450 catalysed hydroxylation at the allylic C_6-position of germacra-1(10),4,11(13)-trien-12-oic acid (Chapter 5). The newly formed hydroxyl group then attacks the carbonyl at C_{12} yielding the lactone ring present in (+)-costunolide. It is uncertain whether this process of lactonisation occurs while the germacrene is still in the active site of the (+)-costunolide synthase, or occurs spontaneously once the hydroxylated germacrene acid is released from the enzyme. The (+)-costunolide synthase of chicory cannot convert eudesmane acids into eudesmanolides, even if the C_6-position is allylic as in γ-costic acid.

Germacrene Intermediates

The established pathway of Figure 1 for the biosynthesis of (+)-costunolide in chicory is similar to the one proposed by various authors in the seventies of the last century (Geissman, 1973; Herz, 1977; Fischer et al., 1979). Due to the lack of biochemical data their proposed pathway was entirely based on isolated compounds from the roots of *Saussurea lappa*. These roots —also known as costus roots— contain not only high amounts of (+)-costunolide (Paul et al., 1960), but also eudesmanes whose structures correspond to the putative germacrene intermediates, i.e. selinene, costol, and costic acid (Bawdekar and Kelkar, 1965; Bawdekar et al., 1967). These eudesmanes can be formed from germacrenes under acidic conditions (Teisseire, 1994). Additionally, the Cope rearrangement products of the germacrene intermediates (i.e. β-elemene, elema-1,3,11(13)-trien-12-ol, and elema-1,3,11(13)-trien-12-al) have been isolated from commercially available costus root oil, which is produced by steam distillation (Maurer and Grieder, 1977). The final proof for the existence of the germacrene intermediates of sesquiterpene lactones biosynthesis in costus roots is given in Chapter 4. This chapter describes the isolation of germacrene A, germacra-1(10),4,11(13)-trien-12-al, and the not previously reported germacra-1(10),4,11(13)-trien-12-ol and germacra-

1(10),4,11(13)-trien-12-oic from fresh costus roots. The isolation was successful, as elevated temperatures and acidic conditions were avoided. The occurrence of these germacrenes together with (+)-costunolide is additional support for the pathway established in this thesis, which is depicted in Figure 1.

The reported sensitivity of germacrenes and in particular that of (+)-germacrene A to heat induced Cope rearrangement and acid induced cyclisation reactions (Teisseire, 1994) is in general somewhat exaggerated, e.g. "germacrene A is unstable even in freezer storage over extended periods" (Weinheimer et al., 1970). Only the isolated germacrene alcohol was not free of its corresponding elemene alcohol, indicating that the germacrene alcohol might be somewhat less stable than the other isolated germacrenes. Possibly, small amounts of the elemene alcohol are already present in fresh costus root material, but in general the elemenes present in costus root oil can be considered as heat-induced artefacts. On the other hand, the eudesmanes reported for costus root oil are not necessarily acid induced artefacts and might as well be true secondary metabolites produced by enzymes of *Saussurea lappa*. Unlike what is suggested by Teisseire (1994), germacrene A and its oxygenated products can be purified by flash column chromatography on silica gel and "survive" the performed incubations at 30°C. Yet, the germacrene intermediates of sesquiterpene lactone biosynthesis cannot be measured as distinct peaks in GC-analysis, apart from (+)-germacrene A that has a relative short retention time. The oxygenated derivatives of (+)-germacrene A are Cope rearranged during their migration through the GC-column yielding broad "humps". In order to obtain distinct peaks it is best to rearrange them to their corresponding elemenes directly at the start of the GC-run, using an injection port temperature of at least 250°C. The only serious problems concerning the sensitivity of germacrenes arose during the work-up of the enzyme assays. In some cases up to 50% of the germacrene A or germacrene alcohol was cyclised into selinene, respectively, costol. These problems are apparently caused by the combination of silica gel, magnesium sulphate, and traces of water during the filtration/purification of the pentane-ether extract from the incubation. They could be overcome by replacement of silica gel with aluminium oxide (grade III) and the use of sodium sulphate instead of magnesium sulphate.

(+)-COSTUNOLIDE IN SESQUITERPENE LACTONE BIOSYNTHESIS

Germacranolides, Guaianolides, and Eudesmanolides

The results described in the previous paragraph make it inevitable that (+)-costunolide is the intermediate sesquiterpene lactone in biosynthesis of the guaianolides, eudesmanolides and germacranolides in chicory; it is reasonable to assume that this conclusion is also valid for other plant species that contain these skeletal types of sesquiterpene lactones. Although the used GC-MS techniques are not the appropriate method to measure higher oxygenated sesquiterpene lactones, some information was obtained about the steps between (+)-costunolide and the final products of sesquiterpene lactone biosynthesis in chicory (Chapter 5). Incubations of (+)-costunolide and a 20,000*g* supernatant in the presence of NADPH and oxygen yielded leucodin and 11(*S*),13-dihydrocostunolide (Fig. 2).

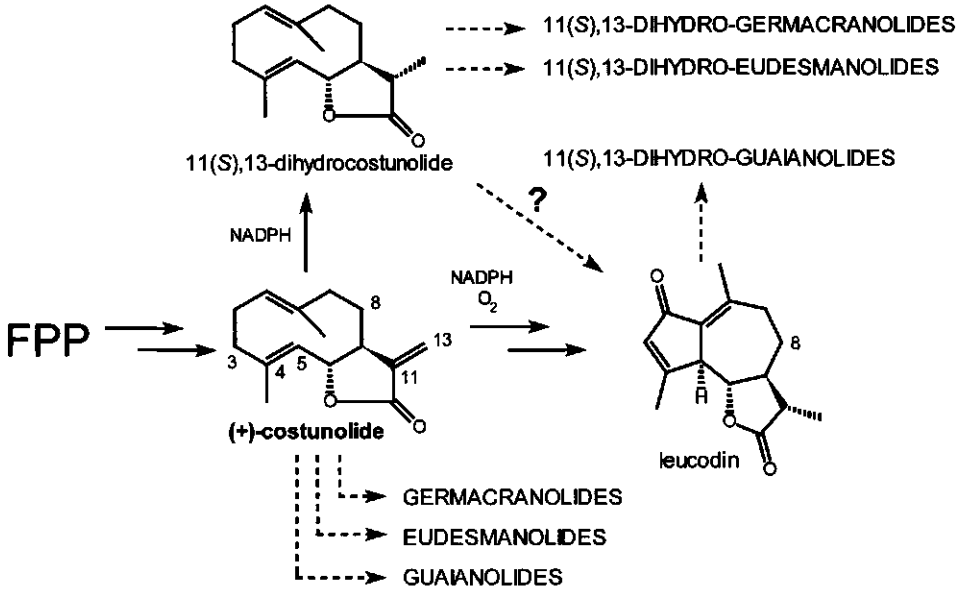

Figure 2. Biosynthesis of 11(*S*),13-dihydrocostunolide and leucodin from (+)-costunolide and their possible role in the biosynthesis of other sesquiterpene lactones.

Formation of leucodin presumably involves a cytochrome P450 enzyme next to other enzymes, whereas formation of 11(*S*),13-dihydrocostunolide does not require oxygen and is catalysed by an enoate reductase (a type of enzyme discussed by Holland [1992] and Faber [2000]). Interestingly, leucodin is only one hydroxylation away from 11(*S*),13-dihydro-8-

deoxylactucin, one of the minor bitter sesquiterpene lactones of chicory (van Beek et al., 1990). Moreover its biosynthesis is solid proof for the hypothesis that guaianolides originate from (+)-costunolide (e.g. Song et al., 1995). 11(*S*),13-Dihydro-costunolide is a reasonable intermediate of all the 11(*S*),13-dihydrosesquiterpene lactones present in chicory (11[*R*]-epimers are not known for chicory), but it was not investigated whether leucodin is formed via 11(*S*),13-dihydrocostunolide. Although the necessity of either 4,5-epoxidation or C_3-hydroxylation for cyclisation of a germacrene to a guaiane framework has often been suggested (Brown et al., 1975; Fischer 1990; Teisseire, 1994; Piet et al., 1995, Piet et al., 1996), parthenolide (i.e. the 4,5-epoxide of [+]-costunolide) is not involved in biosynthesis of leucodin.

An interesting aspect of sesquiterpene lactone biosynthesis in chicory that remains to be elucidated is the formation of the guaianolides and germacranolides that are oxygenated at the C_8-position (lactucin and lactucopicrin). Since germacrene B is not involved, there is seemingly no point during their biosynthesis at which this position is allylic. Whereas all the hydroxylation steps found so far occurred at allylic positions, chicory seemingly also contains an enzyme that hydroxylates at non-allylic positions. Indeed, hydroxylations catalysed by cytochrome P450 enzymes do not necessarily occur at allylic positions as for instance the hydroxylation of kaurene in gibberellin biosynthesis shows (Sponsel, 1995).

Distinct Skeletal Types of *trans*-Fused Sesquiterpene 6,7-Lactones

Although the involvement of (+)-costunolide in biosynthesis of guaianolides and eudesmanolides is evident, it remains to be seen whether (+)-costunolide is also a universal precursor for other types of sesquiterpene lactones with a *trans*-fused C_6,C_7-lactone ring as depicted in Figure 5 of Chapter 1 and postulated by Herz (1977) and Fischer et al. (1979). Formation of guaianolides and eudesmanolides involves a relatively simple cyclisation of a germacradiene framework, whether or not mediated by epoxidation/hydroxylation, but formation of for instance eremophilanolides and ambrosanolides from a germacradienolide involves also the shift of a methyl group (Fig. 3). The only proof for these methyl shifts within a germacradienolide is not based on biochemical data but comes from a few biomimetic chemical reactions (Fischer et al., 1990) that do not always occur under conditions expected to be present in plants. Methyl shifts are on the other hand a common event in reactions catalysed by sesquiterpene synthases (Cane et al., 1990; Croteau et al., 2000). In fact some of the cyclisations of farnesyl diphosphate (FPP), for example aristolochene biosynthesis, occur via a germacradienyl cation that undergoes further methyl

shifts while it is bound in the active site (Cane and Tsantrizos, 1996). Therefore it is not unreasonable to assume that the sesquiterpene backbones present in some of the sesquiterpene lactones are already formed during the cyclisation of FPP by the sesquiterpene synthase and before formation of the lactone ring, notwithstanding the fact that the eudesmane and guaiane framework are formed in a later stage of their biosynthesis.

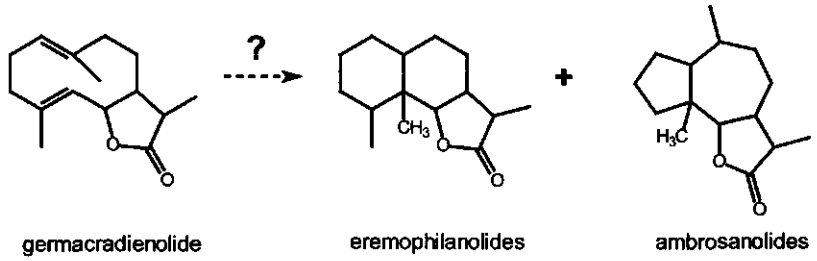

Figure 3. It seems unlikely that eremophilanolides and ambrosanolides are derived from a germacradienolide, because it would involve methyl shifts to obtain the marked methyl groups into their position.

Cadinanolides (in Artemisia annua)

An alternative pathway (Seaman et al., 1982) that does not involve (+)-costunolide can for instance be deduced for cadinanolides such as arteannuin B, *epi*-deoxyarteannuin B (a *cis*-lactone), and deoxyarteannuin B (a *trans*-lactone; not shown). These sesquiterpene lactones occur in *Artemisia annua* (Ranasinghe et al., 1993) (Fig. 4) together with artemisinic acid. This sesquiterpene acid is a reasonable intermediate in the biosynthesis of the cadinanolides, because of its comparable carbon skeleton with a 4,5-*cis*-double bond. Strikingly, the same enzyme preparation of chicory that converts (+)-germacrene A into germacra-1(10),4,11(13)-trien-12-ol can also hydroxylate amorpha-4,11-diene to amorpha-4,11-dien-12-ol (Chapter 6), a putative intermediate *en route* to artemisinic acid (Bouwmeester et al., 1999).

Currently, the biosynthesis of artemisinic acid receives considerable attention, since it is an important intermediate in the formation of artemisinin, an antimalarial drug (Bouwmeester et al., 1999). This sesquiterpene endoperoxide is supposed to be formed non-enzymatically from the 11,13-dihydro derivative of artemisinic acid by photo-oxidation in the presence of chlorophyll and air (Wallaart et al, 1999), but similar experiments with artemisinic acid yielded arteannuin B and *epi*-deoxyarteannuin B (Roth and Acton, 1989). Whether these biomimetic reactions are also valid *in vivo* remains to be seen, and this last step in

cadinanolide biosynthesis might as well be catalysed by a (+)-costunolide synthase-like enzyme that hydroxylates artemisinic acid at the allylic C_6-position.

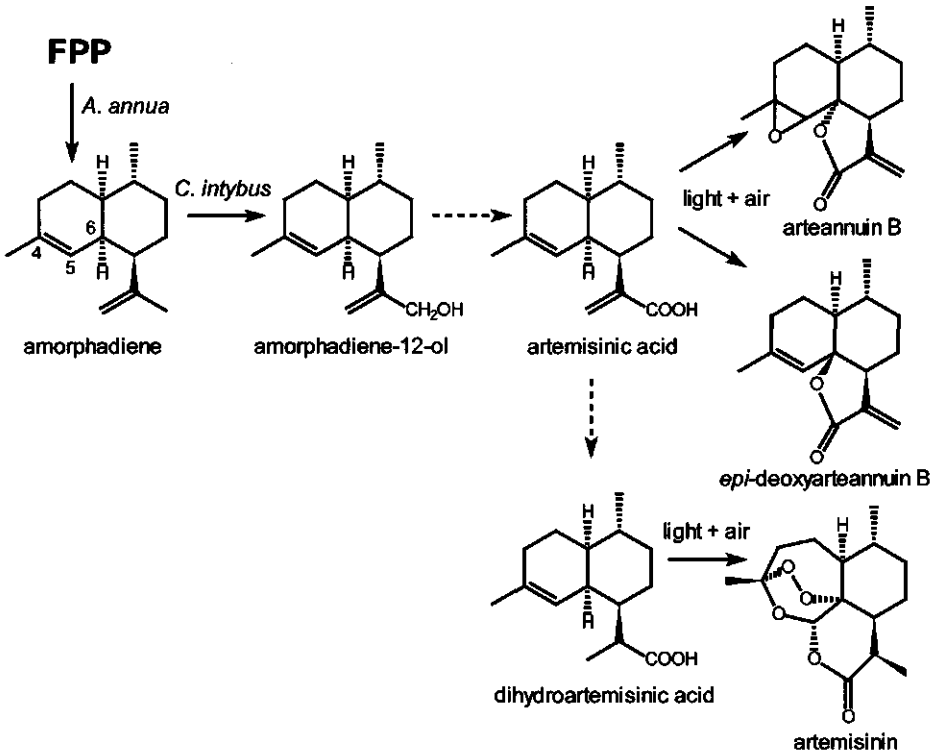

Figure 4. Possible pathway for biosynthesis of the cadinanolides arteannuin B and *epi*-deoxyarteannuin B via artemisinic acid, which is also an intermediate in biosynthesis of artemisinin. Formation of amorphadiene from FPP has been demonstrated for *Artemisia annua* (Bouwmeester et al., 1999); the hydroxylation of amorphadiene can be performed with an enzyme preparation of chicory.

Elemanolides

The occurrence of elemanolides is often discussed, because in general elemene compounds are considered to be artefacts due to Cope rearrangement (Teisseire, 1994). Nonetheless, it was noted that a compound as zinaflorin I (a *cis*-fused 7,8-lactone) in which the 1,2-double bond has undergone epoxidation cannot be regarded as an artefact, nor can an elemanolide as confertiphyllide (Fig. 5). Somewhere during the biosynthesis of these compounds a Cope rearrangement must have occurred, but whether this is a spontaneous or enzyme controlled process is not known (Herz, 1977; Fischer et al., 1979; Seaman, 1982; Fischer 1990). Alternatively the plant could contain an elemene synthase, in spite of the fact

that all elemene olefins reported so far are artefacts and not genuine products of plant secondary metabolism or any (other) enzymatic activity (Teisseire, 1994).

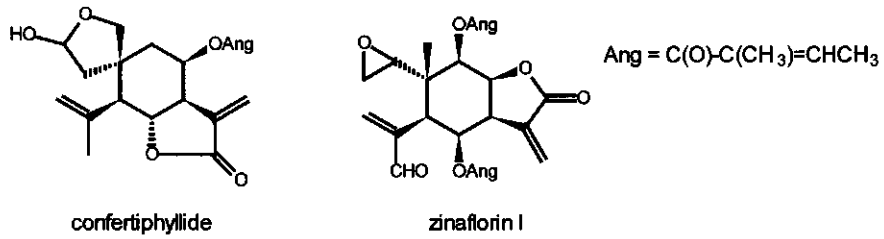

Figure 5. Structures of elemanolides that cannot be regarded as artefacts derived from germacranolides.

During careful isolation of germacrenes from fresh costus roots (Chapter 4) elematrien-12-ol was the only appearing elemene, and possibly this compound is also present when the plant is alive. The elemene alcohol can be converted by the dehydrogenases present in chicory. However, the resulting elemene acid is not converted into an elemanolide by the (+)-costunolide synthase of chicory, probably because the C_6-position is not allylically activated.

Biosynthesis of Stereo-isomeric Sesquiterpene Lactones

Higher plants contain sesquiterpene lactones in which the hydrogen at C_7-position is, without exception, α-oriented. Nevertheless, Hepaticae (liverworts) also contain sesquiterpene lactones in which this hydrogen is β-oriented, whereas *cis*-fused 6,7-lactone rings appear to be more common in these species (Fig. 6) (Asakawa and Heidelberger, 1982; Fischer et al., 1991).

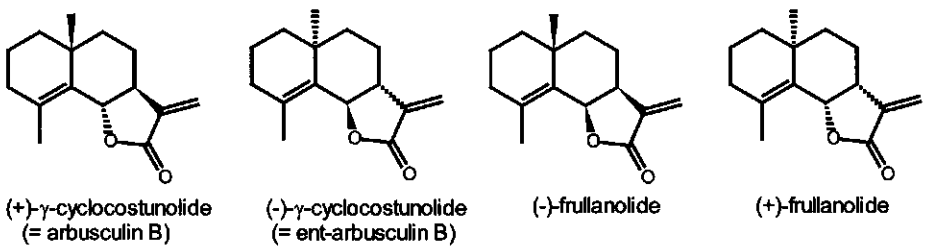

Figure 6. Illustration of the variation in stereochemistry of sesquiterpene lactones found in Hepaticae, (+)-(γ)-cyclocostunolide is the only one also to be found in higher plants.

If these sesquiterpene lactones with a different stereochemical configuration at C_7 are formed in such a way as described for chicory, there will be biosynthesis of (-)-germacrene A in Hepaticae. Indeed it is observed that essential oils of Hepaticae often contain (+)-β-elemene (i.e. the Cope rearrangement product of [-]-germacrene A) next to (-)-β-elemene in varying ratios (König et al, 1994). Presumably liverworts contain separate germacrene A synthases for each enantiomer of germacrene A, alike *Solidago canadensis* that contains a separate synthase for each germacrene D enantiomer (Schmidt et al., 1998). The (putative) costunolide synthase(s) of liverworts should also noticeably differ from that of chicory, for the reason that both *cis*-fused and *trans*-fused lactone rings are present which means that hydroxylation at the C_6-position of germacrene acid does not necessarily occur stereoselective.

TOWARD AN APPLICATION OF ENZYMES FROM CHICORY SESQUITERPENE LACTONE BIOSYNTHESIS IN ORGANIC SYNTHESIS

One of the initial goals of this PhD project was to investigate whether the oxidising enzymes involved in chicory sesquiterpene lactones might be useful as catalysts in organic synthesis. Introduction of a hydroxyl group into an organic substrate such as observed for the (+)-germacrene A hydroxylase can often not be achieved by classical organic chemistry (Faber, 2000; Chapter 6). Furthermore, chicory roots might be a cheap source of oxidising enzymes, because they are regarded as a waste product (100,000 tons each year) of the chicory cultivation.

A microsomal pellet prepared from chicory, which contains the membrane bound enzymes, can hydroxylate various sesquiterpenes that are exogenous to the chicory plant (Chapter 6). Most of these reactions occur at an isopropenyl or isopropylidene group and are presumably catalysed by (+)-germacrene A hydroxylase, despite the common belief that cytochrome P450 enzymes of plant secondary metabolism are rather substrate specific (Donaldson and Luster, 1991; Halkier, 1996; Schuler, 1996). Some of the products are not previously described sesquiterpene alcohols, which may have interesting properties in the field of plant resistance and flavour and fragrance. However, the novelty of these compounds also hampered their identification as they were not produced in sufficient amounts for NMR analyses.

The microsomal pellet does also contain other cytochrome P450 enzymes than the (+)-germacrene A hydroxylase (e.g. (+)-costunolide synthase) and this may explain the few observed hydroxylations of sesquiterpenes that did not take place at an isopropenyl or isopropylidene group. Most eye-catching is the conversion of (+)-valencene into nootkatone (Fig. 7). Whereas the initially expected valencen-12-ol was hardly observed, the unexpected formation of nootkatone was prominent. Its formation proceeds via a cytochrome P450 catalysed formation of β-nootkatol that subsequently is converted efficiently into nootkatone by NAD(P$^+$)-dependent dehydrogenases. Considering the analogy in substrate structure and position where oxidation occurs, these reactions are supposedly also involved in the formation of the guaianolide leucodin from (+)-costunolide.

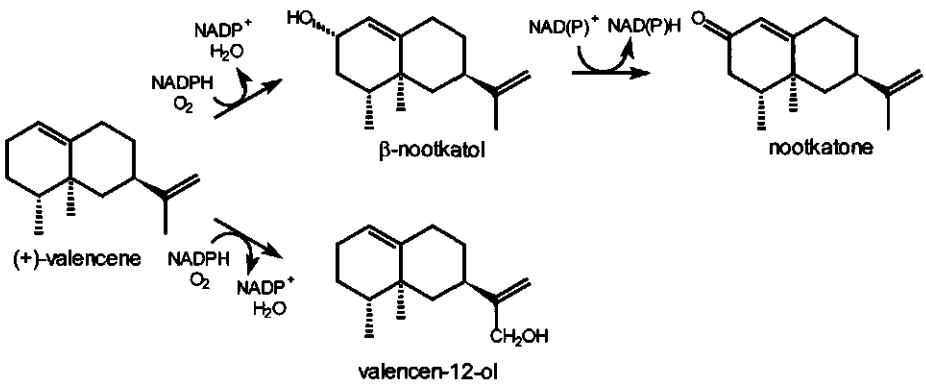

Figure 7. Bioconversion of (+)-valencene by a microsomal pellet of chicory yields nootkatone. Its formation proceeds via β-nootkatol, possibly by the same enzymes that are also involved in leucodin biosynthesis. Valencen-12-ol, which is only produced in low yield, is probably the product of (+)-germacrene A hydroxylase activity.

Nootkatone is a compound with a strong grapefruit odour that is much sought-after by the flavour and fragrance industry. Many attempts have been made to make nootkatone in an economical way from the less valuable (+)-valencene, but the existing chemical and microbiological methods are not satisfying (Könst et al., 1975). The advance of the "chicory catalysed" conversion of (+)-valencene over other methods is that undesired side-products are hardly formed. Nevertheless the production of 1 gram of nootkatone using the protocol described in Chapter 6 would require approximately 1800 kg of chicory roots. Hence, it would be more reasonable to isolate the genes that encode the enzymes involved in biosynthesis of nootkatone —nootkatol in particular— and subsequently try to functionally

overexpress them in yeast (Schuler, 1996). Such expression in yeast would also circumvent the problems concerning cofactor regeneration and the instability of cytochrome P450 enzymes. An example of the successful expression of a terpene hydroxylase in yeast is the geraniol 10-hydroxylase of *Catharanthus roseus* (Collu, 1999). This example and the already achieved isolation of (+)-germacrene A synthase genes from chicory (Bouwmeester et al., 2001) demonstrate that the application of genes and enzymes from chicory is within reach.

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8. Summary

Wild chicory (*Cichorium intybus* L.) is a blue-flowered composite plant that has spread all over the world from the Mediterranean. Sprouts of chicory var. *foliosum* Hegi that are grown in the dark became popular as a vegetable (Belgian endive) halfway through the nineteenth century. Nowadays it is a common crop in Belgium, northern France, and the Netherlands.

The well-known bitter taste of chicory is associated with the presence of sesquiterpene lactones of which the three major ones are the guaianolides lactucin, 8-deoxylactucin, and lactucopicrin (Fig. 1). Additionally, smaller amounts of eudesmanolides and germacranolides are present. The average sesquiterpene lactone content of the wild variety (*sylvestre*) is 0.42% dry weight in the roots and 0.26% in the leaves. The sesquiterpene lactones in chicory act as feeding deterrent toward insects, but may have an antifungal and antibacterial function as well (**Chapter 1**).

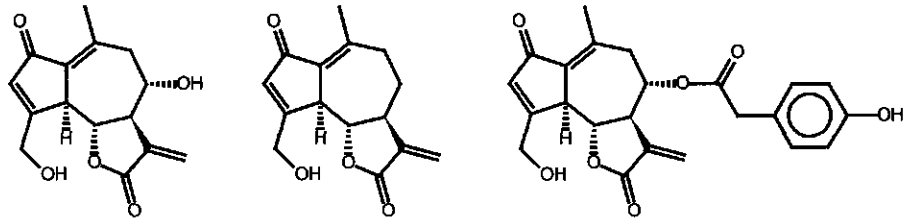


Figure 1. The major sesquiterpene lactones of chicory, all three are guaianolides.

Sesquiterpene lactones are considered as a major class of plant secondary products, which mainly occur in the Asteraceae. Over 4000 different structures are known, but the majority of them has a guaiane, eudesmane, or germacrene framework (i.e. guaianolides, eudesmanolides, germacranolides). Sesquiterpene lactones with such a framework are

thought to originate from (+)-costunolide, the most elementary structure of a germacrene sesquiterpene lactone. In this thesis a pathway for the biosynthesis of (+)-costunolide in chicory roots has been established (Fig. 2).

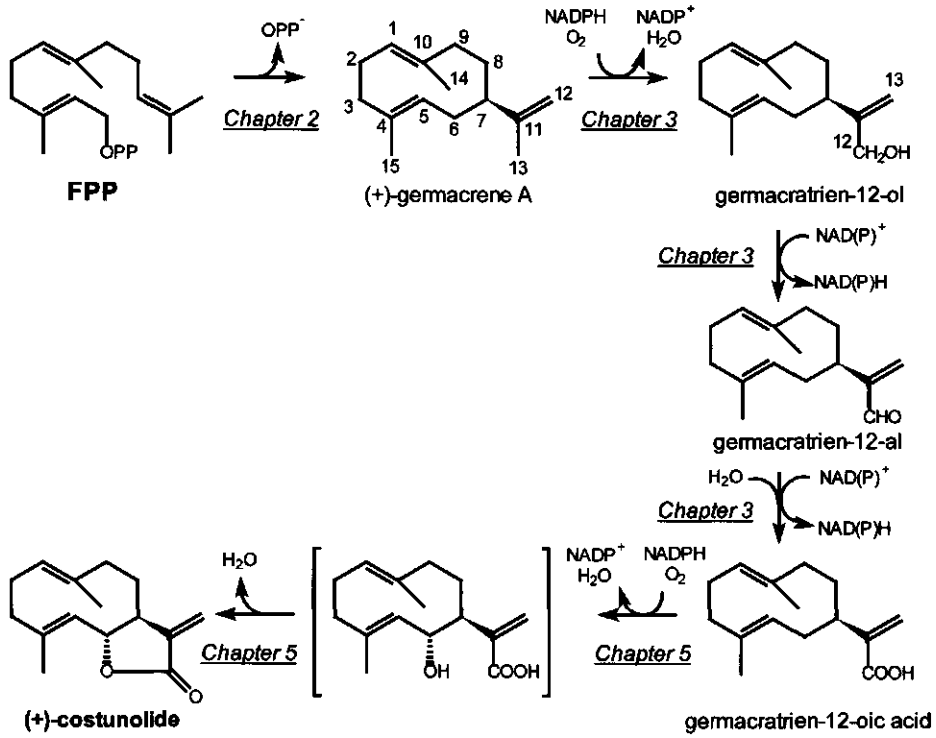


Figure 2. Established pathway for the biosynthesis of (+)-costunolide from farnesyl diphosphate (FPP) in chicory.

The committed step in the biosynthesis of (+)-costunolide is the cyclisation of farnesyl diphosphate (FPP) into (+)-germacrene A (**Chapter 2**). The involved (+)-germacrene A synthase was isolated from chicory roots and purified 200-fold by a combination of anion exchange and dye-ligand chromatography. The isolated enzyme belongs to the group of sesquiterpene synthases, has a K_m-value of 6.6 μM, an estimated molecular weight of 54 kD, and a (broad) pH optimum around 6.7. The recent isolation of genes encoding the (+)-germacrene A synthase of chicory makes it possibly to block this crucial step in sesquiterpene lactone biosynthesis, which may result in new less bitter tasting varieties of Belgian endive.

Formation of the lactone ring involves the introduction of a carboxylic acid function in the isopropenyl group of (+)-germacrene A (**Chapter 3**). It starts with the hydroxylation of (+)-germacrene A to germacra-1(10),4,11(13)-trien-12-ol by the (+)-germacrene A hydroxylase. This cytochrome P450 enzyme is NADPH-dependent, has a pH optimum at 8.0, and is blue-light reversibly inhibited by CO. Germacra-1(10),4,11(13)-trien-12-ol is subsequently oxidised to germacra-1(10),4,11(13)-trien-12-oic acid via germacra-1(10),4,11(13)-trien-12-al by pyridine nucleotide dependent dehydrogenases. Some questions about the exact cofactor dependence of the dehydrogenase catalysed reactions remain, but on the whole the best results were obtained with $NADP^+$.

Conversion of germacra-1(10),4,11(13)-trien-12-oic acid into (+)-costunolide is catalysed by the (+)-costunolide synthase (**Chapter 5**). This enzyme is also a cytochrome P450 enzyme, since it depends upon NADPH and is blue-light reversibly inhibited by CO. Biosynthesis of (+)-costunolide in the presence of $^{18}O_2$ resulted in the incorporation of one atom of ^{18}O. This supports the concept that the lactone ring is formed via a hydroxylation at the C_6-position of the germacrene acid, after which the hydroxyl group attacks the carboxyl group at C_{12}. It is not clear whether the final lactonisation is also mediated by the (+)-costunolide synthase or occurs spontaneously (outside the enzyme).

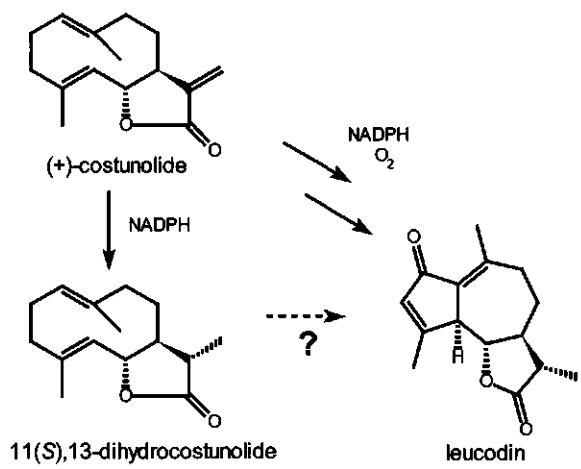


Figure 3. Enzymatic conversion of (+)-costunolide into 11(*S*),13-dihydrocostunolide and leucodin (Chapter 5).

(+)-Costunolide is converted into 11(*S*),13-dihydrocostunolide and leucodin by an enzyme extract from chicory roots in the presence of NADPH and O_2 (Fig. 3). It is to be expected that other sesquiterpene lactones are formed as well in these incubations, but it is unlikely that

they can be detected by the GC-MS method which was used to analyse the enzyme assays. The formation of 11(*S*),13-dihydrocostunolide is catalysed by a stereoselective enoate reductase and does also occur in the absence of O_2. The formation of leucodin involves a cytochrome P450 enzyme and presumably also a dehydrogenase, but it is unclear how cyclisation into the guaiane framework takes place. 11(*S*),13-Dihydrocostunolide is a reasonable intermediate in the biosynthesis of all 11,13-dihydro-sesquiterpene lactones present in chicory, but its involvement in leucodin biosynthesis was not investigated. Notably, leucodin is only one hydroxylation step away from 11(*S*),13-dihydro-8-deoxylactucin, a minor bitter sesquiterpene lactone of chicory.

The germacrene intermediates of sesquiterpene lactone biosynthesis can be isolated from fresh roots of *Saussurea lappa* (costus roots) (**Chapter 4**). The occurrence of these germacrene intermediates along with high amounts of (+)-costunolide and dehydrocostus lactone within one and the same plant is an additional proof for the established pathway depicted in Figure 2. The germacrene compounds are susceptible to proton-induced cyclisations and to heat induced Cope rearrangement yielding eudesmanes and elemenes respectively. However, the isolated germacrenes are not that unstable as often suggested by literature. They remain for instance intact during the enzyme incubations at 30°C. The best way to analyse the oxygenated germacrenes is to let them undergo a Cope rearrangement to their corresponding elemenes immediately at the start of the GC-run. This can be achieved by the use of injection port temperatures of at least 250°C; if this is not done they will generally yield very broad peaks (**Chapter 4**). Cope rearrangement into (-)-β-elemene was used to determine the absolute configuration of the enzymatically produced germacrene A on an enantioselective GC-column (**Chapter 2**).

In the presence of NADPH, a microsomal pellet from chicory roots is able to hydroxylate various sesquiterpene olefins, which are exogenous to the plant (**Chapter 6**). Most of these hydroxylations take place at the allylic position of an isopropenyl or isopropylidene group (Fig. 4). The number of products obtained from a certain substrate is confined to one or in, a few cases, two sesquiterpene alcohols.

Although the microsomal pellet contains various membrane bound enzymes, the majority of hydroxylations is ascribed to the (+)-germacrene A synthase since (+)-germacrene A competitively inhibits their biotransformation. This disputes the common idea that cytochrome P450 enzymes of plant secondary metabolism have a narrow substrate

specificity. The unforeseen hydroxylation of (+)-valencene into β-nootkatol is presumably catalysed by a different cytochrome P450 enzyme, possibly the same that is involved in biosynthesis of leucodin from (+)-costunolide. During incubation β-nootkatol is rapidly oxidised further by $NAD(P)^+$-dependent dehydrogenases into nootkatone, a much sought-after component with a distinctive flavour of grapefruit.

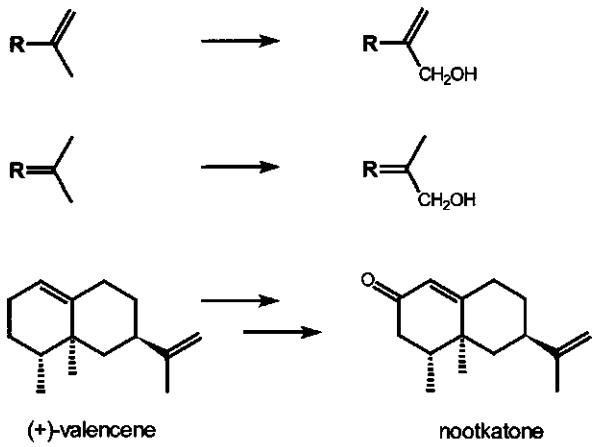


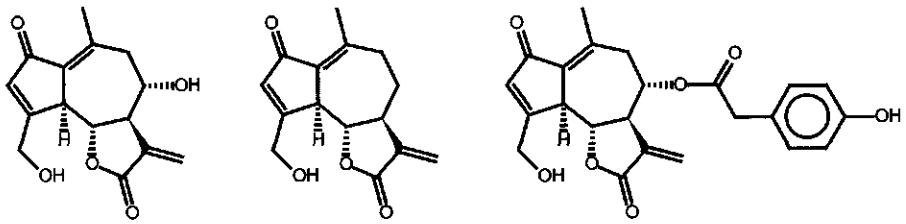
Figure 4. Oxidations of sesquiterpene olefins catalysed by a microsomal pellet from chicory roots in the presence of NADPH.

The achieved regioselective, and in the case of β-nootkatol also stereoselective, introduction of a hydroxyl group into sesquiterpene olefins is often difficult to achieve by organic chemical methods. Nonetheless, the small quantities of oxygenated products obtained are a major drawback in the application of the isolated oxidising enzymes from chicory roots. It would be worthwhile to isolate the genes encoding the involved cytochrome P450 enzymes and to functionally overexpress them in yeast. In this way higher enzymatic activities and larger amounts of possibly interesting products may be expected (**Chapter 7**).

9. Samenvatting

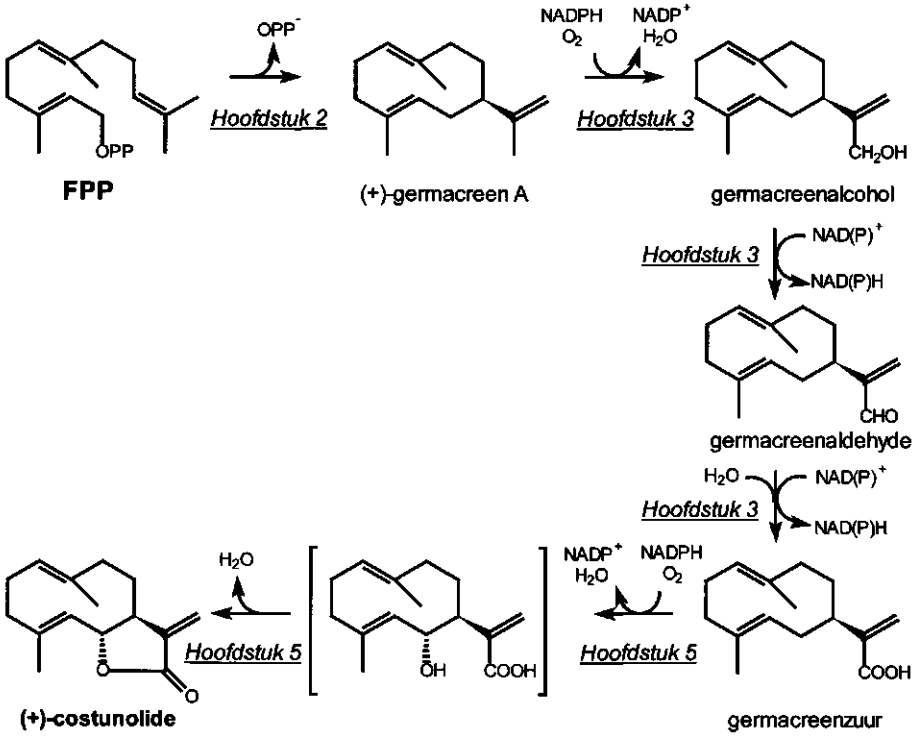
(Iets Vereenvoudigde Nederlandstalige Versie)

Cichorei is een plant met blauwe bloemen die zich vanuit het Middellandse Zeegebied over heel de wereld heeft verspreid. Aanvankelijk werd deze plant geteeld voor de productie van koffiesurrogaat, maar halverwege de negentiende eeuw werden in de omgeving van Brussel de in het donker opgegroeide spruiten van cichorei populair als de groente. Tegenwoordig wordt deze groente als Brussels lof of witlof voornamelijk geteeld in België, noord-Frankrijk en Nederland. Karakteristiek aan witlof is de bittere smaak die veroorzaakt wordt door de aanwezigheid van een groep van verbindingen die bekend staat onder de verzamelnaam sesquiterpeenlactonen. Het gehalte (drooggewicht) aan sesquiterpeenlactonen in wilde cichorei bedraagt gemiddeld in de wortels 0,42% en in de bladeren 0,26%. Ze remmen de vraat door insecten, maar mogelijk geven ze de cichoreiplant ook bescherming tegen ziekten veroorzaakt door schimmels en bacteriën. Sesquiterpeenlactonen vormen een belangrijke groep van secundaire metabolieten (d.w.z. stoffen die niet van direct belang zijn voor de energie en groei van een plant) en op dit moment zijn er meer dan 4000 verschillende structuurformules van sesquiterpeenlactonen bekend. Ze komen voornamelijk voor in de composieten (Asteraceae), de grootste plantenfamilie. De belangrijkste drie sesquiterpeenlactonen uit witlof staan hieronder weergegeven (**Hoofdstuk 1**).



Figuur 1. Structuurformules van de drie belangrijkste sesquiterpeenlactonen in witlof.

Een sesquiterpeenlacton is te herleiden tot een basisskelet bestaande uit twee delen. Een ringvormig sesquiterpeengedeelte van vijftien koolstofatomen (dubbelrings bij de structuren in Fig. 1), en een lactonring die twee zuurstofatomen bevat en die ontstaan is uit de reactie van een hydroxylgroep met een zuurgroep. (+)-Costunolide vertegenwoordigt de meest basale structuur van een sesquiterpeenlacton en een groot deel van de sesquiterpeenlactonen ontstaat vermoedelijk uit deze verbinding. Dit proefschrift gaat over de nog niet eerder beschreven biochemische weg waarlangs (+)-costunolide gevormd wordt (Fig. 2), en de hierbij betrokken enzymen uit witlofwortels.



Figuur 2. De route waarlangs in witlofwortels (+)-costunolide wordt gevormd uit farnesyldifosfaat (FPP).

De eerste stap in de biosynthese van sesquiterpeenlactonen bestaat uit de vorming van het sesquiterpeengedeelte door de cyclisatie van farnesyldifosfaat (FPP) naar (+)-germacreen A. Het hiervoor benodigde farnesyldifosfaat is in de plant ruim voorhanden, omdat het ook als bouwstof dient voor andere typen van verbindingen (o.a. sterolen, carotenen, planthormonen). Het enzym dat deze eerste stap katalyseert, het germacreen A-synthase, is met behulp van (ultra)centrifugatie geïsoleerd uit een pulp van in bufferoplossing vermalen

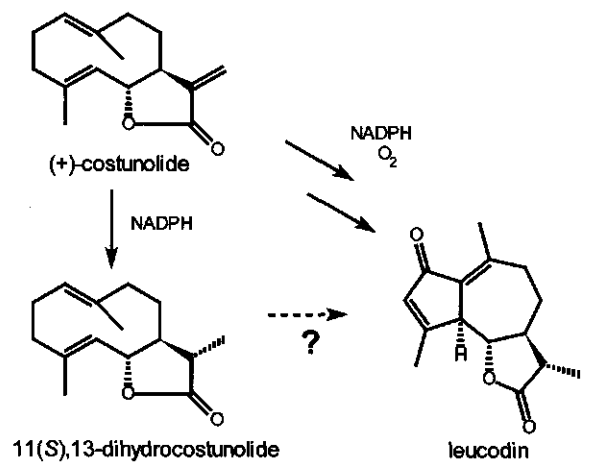
witlofwortel en vervolgens met kolomchromatografie een factor 200 opgezuiverd. Hierna zijn de volgende kenmerken van het germacreen A-synthase bepaald: een molecuulgewicht van 54 kD, een optimaal functioneren bij pH 6.7, en bij een FPP-concentratie van 6.6 μM wordt de helft van de maximale katalysesnelheid bereikt (de K_m-waarde) (**Hoofdstuk 2**).

Voor de vorming van de lactonring dient een zuurgroep in de isopropenylstaart van (+)-germacreen A geïntroduceerd te worden, hetgeen germacreenzuur geeft. Dit gebeurt niet in één keer, maar in een drietal enzymatische stappen (**Hoofdstuk 3**). Allereerst wordt er een hydroxylgroep ingevoerd, waarbij (+)-germacreen A wordt omgezet in germacreenalcohol. Deze reactie wordt gekatalyseerd door het (+)-germacreen A-hydroxylase, een enzym dat behoort tot de groep van cytochroom P450 enzymen. Dit type van enzymen bevat een heemgroep die zuurstof bindt, waarna het zuurstof wordt geactiveerd door de aanvoer van elektronen uit de cofactor NADPH. Tijdens de reactie wordt het zuurstofmolecuul gesplitst en vervolgens worden de zuurstofatomen verdeeld over water en het te vormen alcohol (in dit geval germacreenalcohol). Kenmerkend voor de heemgroep in een cytochroom P450 enzym is dat deze vele malen beter koolmonoxide dan zuurstof bindt. De vorming van germacreenalcohol uit (+)-germacreen A wordt dan ook sterk geremd in de aanwezigheid van koolmonoxide, wat een sterke aanwijzing vormt voor de betrokkenheid van een cytochroom P450 enzym in deze reactie. De binding van koolmonoxide aan de heemgroep kan ongedaan worden gemaakt door belichting van het enzym met blauw-licht, en dit doet de remming van het germacreen A-hydroxylase door koolmonoxide dan ook deels teniet. De omzetting van germacreenalcohol via germacreenaldehyde naar germacreenzuur wordt gekatalyseerd door dehydrogenases. Dit type van enzymen onttrekt elektronen aan het alcohol/aldehyde (oxideren), waarbij NAD^+ of $NADP^+$ wordt omgezet in NADH respectievelijk NADPH.

De voltooiing van de lactonring wordt gekatalyseerd door het (+)-costunolide-synthase dat de noodzakelijke hydroxylgroep naast de isopropenylzuur-staart van het germacreenzuur invoert. Deze hydroxylgroep valt daarna aan op de zuurgroep (mogelijkerwijs gestuurd door het [+]-costunolidesynthase) waarbij de lactonring van (+)-costunolide ontstaat en water vrijkomt (**Hoofdstuk 5**). Dit mechanisme van hydroxylering gevolgd door lactonisering wordt bevestigd door de inbouw van één atoom ^{18}O in (+)-costunolide bij aanwezigheid van de stabiele zuurstofisotoop $^{18}O_2$. De omzetting van germacreenzuur naar (+)-costunolide is zuurstof- en NADPH-afhankelijk, en wordt blauw-licht reversibel geremd door koolmonoxide; het (+)-costunolide synthase is dus een cytochrome P450 enzym.

Voordat de bittere sesquiterpeenlactonen van witlof zijn gevormd, dient (+)-costunolide nog een aantal verdere enzymatische omzettingen te ondergaan. Gekatalyseerd door een

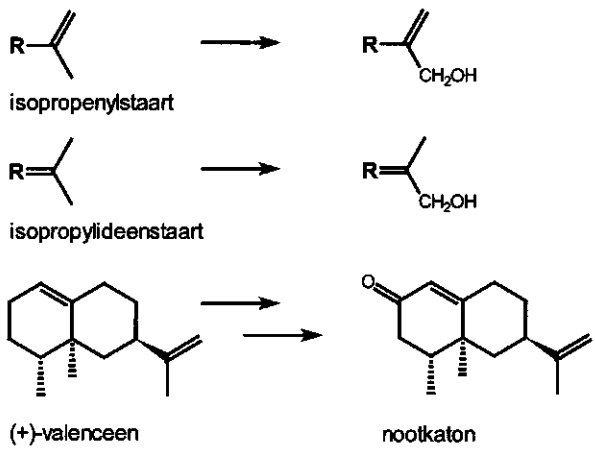
enzymextract uit witlofwortel in aanwezigheid van zuurstof en NADPH zijn er twee van deze vervolgomzettingen van (+)-costunolide waargenomen (**Hoofdstuk 5**): de vorming van 11(*S*),13-dihydrocostunolide en de vorming van leucodin. (Fig. 3). De vorming van 11(*S*),13-dihydrocostunolide wordt gekatalyseerd door een enoaatreductase en vindt ook plaats in de afwezigheid van zuurstof. Bij de vorming van leucodin uit (+)-costunolide zijn meerdere enzymen betrokken, waaronder een cytochroom P450 enzym, en mogelijk vindt de biosynthese plaats via 11(*S*),13-dihydrocostunolide. Opmerkelijk is de gelijkenis tussen leucodin en de sesquiterpeenlactonen uit witlof afgebeeld in Figuur 1; N.B. witlof bevat ook sesquiterpeenlactonen waarin de dubbele band aan de lactonring ontbreekt zoals in leucodin.



Figuur 3. De verder omzetting van (+)-costunolide in 11(*S*),13-dihydrocostunolide en leucodin (Hst. 5).

De germacreenverbindingen betrokken bij de biosynthese van (+)-costunolide kunnen geïsoleerd worden uit de verse wortels van *Saussurea lappa* (costuswortelen) (**Hoofdstuk 4**), een medicinale plant uit de Himalaya. Deze plant bevat ook veel (+)-costunolide, en de aanwezigheid van de afzonderlijke germacrenen samen met (+)-costunolide in één en dezelfde plant vormt een extra bewijs voor de biosyntheseroute uit Figuur 2. De geïsoleerde germacrenen zijn gevoelig voor zuur en hoge temperaturen, wat de niet-enzymatische vorming geeft van eudesmaan/ selineen- respectievelijk elemeenverbindingen (**Hoofdstuk 2** en **4**). Dit heeft weliswaar consequenties voor de analyse van deze verbindingen (gaschromatografie), maar de germacrenen zijn zeker niet zo instabiel als vaak in de literatuur gesuggereerd wordt. Zo kunnen ze bijvoorbeeld tegen de bij de enzymreacties gebruikte temperatuur van 30°C.

Een enzymextract uit witlofwortel kan ook andere sesquiterpenen dan (+)-germacreen A omzetten (**Hoofdstuk 6**). Bij deze enzymatische reacties wordt in de aanwezigheid van NADPH een hydroxylgroep ingevoerd in een isopropenylstaart of isopropylideenstaart (Fig. 4). Het aantal gevormde producten blijft hierbij beperkt tot één of, in een enkel geval, twee sesquiterpeenalcoholen. Het merendeel van deze reacties wordt toegeschreven aan het (+)-germacreen A-hydroxylase. De interessante, onverwachte, omzetting van (+)-valenceen naar nootkaton verloopt eveneens via een alcohol (β-nootkatol), maar wordt waarschijnlijk gekatalyseerd wordt door dezelfde enzymen die ook betrokken zijn bij de vorming leucodin.



Figuur 4. Omzettingen van sesquiterpenen gekatalyseerd door enzymen uit witlof (R= rest v.h. sesquiterpeen).

Nootkaton is een belangrijke verbinding voor de geur- en smaakstoffenindustrie met een kenmerkend grapefruitaroma, maar de bestaande methoden om deze verbinding te synthetiseren geven geen tot tevredenheid stemmende opbrengst. Over het algemeen is het niet eenvoudig om een hydroxylgroep in een verbinding te introduceren zoals de enzymen van witlof dat kunnen. Helaas zijn de enzymen niet in een voldoende mate in de witlofwortel aanwezig om commercieel interessante hoeveelheden van nootkaton en/of de sesquiterpeenalcoholen te produceren. Mogelijk kan dit wel worden bereikt door de genen uit witlof te isoleren die de betrokken enzymen coderen, en deze genen vervolgens tot overexpressie te brengen in bijvoorbeeld gist. Recentelijk zijn al de genen voor het (+)-germacreen A-synthase uit witlof geïsoleerd. Dit met het doel om de germacreen A-biosynthese (d.w.z. de vorming van sesquiterpeenlactonen) in witlof te onderdrukken, wat mogelijk minder bittere witlofvariëteiten geeft.

Curriculum Vitae

Jan-Willem de Kraker werd geboren op 2 april 1970 in Terneuzen (Zeeuws-Vlaanderen), maar al snel werd het weidse polderlandschap vervangen door de bosrijke omgeving van Heeze (Noord-Brabant). In het nabijgelegen Geldrop behaalde hij in 1988 het VWO-diploma, waarna hij de grote rivieren overstak voor de studie Moleculaire Wetenschappen aan de (toen nog zo geheten) Landbouwuniversiteit Wageningen. Binnen deze studie volgde hij de chemisch-biologische oriëntatie met vijfmaands afstudeervakken in de Biochemie en in de Moleculaire Virologie. Tevens werd er in het kader van een Erasmus-project zes maanden zonnig stage gelopen bij het Laboratorium voor Organische Chemie aan de Faculteit voor Farmacie te Barcelona (Spanje). Na het behalen van het doctoraalexamen in 1994, begon hij halverwege 1995 aan het onderzoek naar de biosynthese van bittere sesquiterpeenlactonen in witlofwortels bij het DLO-instituut voor Agrotechnologisch en Bodemvruchtbaarheids-onderzoek (AB-DLO). Dit onder de directe begeleiding van Dr. H.J. Bouwmeester en in nauwe samenwerking met Dr. M.C.R Franssen van het Laboratorium voor Organische Chemie van Wagenigen Universiteit. Dit project kreeg begin 1997 een 'doorstart' in de vorm van het AIO-project "Isolatie, karakterisering en toepassing van oxidatieve enzymen uit witlofwortels" aan het Laboratorium voor Organische Chemie bij Prof. Æ. de Groot. Het onderzoek bleef echter voor een belangrijk deel op het AB-DLO plaatsvinden, dat ondertussen fuseerde met CPRO-DLO en IPO-DLO tot het huidige Plant Research International. Terwijl dit proefschrift langzaam zijn afronding vindt/vond, begon Jan-Willem in november 2001 aan het Max-Planck-Institut für Chemische Ökologie in Jena (Thüringen, Duitsland) aan een onderzoeksproject getiteld: "The methylthioalkylmalate synthases of glucosinolate chain elongation: structure, function and evolution".

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in preparation

The front cover illustrates the cultivation of Belgian Endive in the second part of the nineteenth century.
The back cover shows the different varieties of chicory that were cultivated in Belgium around 1918.

Both illustrations were taken from: De teelt van witloof in woord en beeld, de Backer P (1918), Ed. 2. L'Impremerie moderne, Brussel, 40p.