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Hydroxylation of sesquiterpenes by enzymes from chicory (*Cichorium intybus* L.) roots

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Abstract—A microsomal enzyme preparation of chicory roots catalyses the hydroxylation of 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 any of the substrates is confined to one or, in a few cases, two sesquiterpene alcohols. In addition, the conversion of (+)-valencene into nootkatone through β-nootkatol was observed. The involvement of (+)-germacrene A hydroxylase (a cytochrome P450 enzyme) and other enzymes of sesquiterpene lactone biosynthesis in these reactions is discussed.

1. Introduction

Recent investigations at our laboratories have identified a biochemical pathway in chicory (*Cichorium intybus* L.) roots that leads to the formation of (+)-costunolide from farnesyl diphosphate (FPP) (Scheme 1).[1–3] (+)-Costunolide is the most elementary structure of a germacrane sesquiterpene lactone and the precursor of the germacrane, eudesmane and guaiane lactones present in chicory. Moreover, it is the postulated key intermediate in the formation of the majority of sesquiterpene lactones found in plants.[4,5] Biosynthesis of sesquiterpene lactones in chicory roots involves various types of enzymes including microsomal (i.e. membrane bound) cytochrome P450 enzymes. These enzymes activate molecular oxygen and regioselectively insert one oxygen atom into the substrate at an allylic position.[2,3] Such a cytochrome P450 enzyme is the (+)-germacrene A hydroxylase (Scheme 1, step II) that hydroxylates (+)-germacrene A (**5**) to germacra-1(10),4,11(13)-trien-12-ol (**17**).[2] Interestingly, the (+)-germacrene A hydroxylase of chicory has been demonstrated to hydroxylate both enantiomers of β-elemene (**4**, structure in Table 1) as well,[2] in contrast to the common idea that cytochrome P450 hydroxylases of plant terpenoid biosynthesis have a narrow substrate specificity.[6,7]

Regio and stereoselective introduction of a hydroxyl group into an unactivated organic compound or at an allylic position such as catalysed by the (+)-germacrene A hydroxylase is still a challenge in synthetic organic chemistry.[8] On the whole, the lack of specificity and the occurrence of undesired side reactions are a major drawback in the use of organic chemical methods for hydroxylation. However, hydroxylation of terpenes is of importance to the flavour and fragrance industry in the search for new production methods and new compounds.[8,9] Hence, it was investigated whether the microsomal fraction of a chicory root extract, containing the (+)-germacrene A hydroxylase, is capable of converting other sesquiterpene olefins in addition to the reported hydroxylation of β-elemene (**4**).[2]

2. Results

2.1. Conversion of terpenes

Sixteen different terpenes were tested as possible substrates for the microsomal cytochrome P450 hydroxylase(s) present in chicory roots. GC–MS analysis showed that most of the tested sesquiterpenes were hydroxylated and that their molecular mass was correspondingly raised by 16 amu. Without the addition of NADPH the substrates were not hydroxylated, or only in negligible amounts. The accepted substrates (**1**–**11**) and their products (**12**–**25**) are depicted in Table 1. (−)-α-Cubebene and (−)-α-gurjunene, i.e. compounds without an isopropenyl or isopropylidene group, were not converted, neither was germacrone. Some conversion of the tested monoterpenes (−)-limonene and

Keywords: plant; enzyme; hydroxylation; sesquiterpene; bioconversion; nootkatone; cytochrome P450; dehydrogenase.

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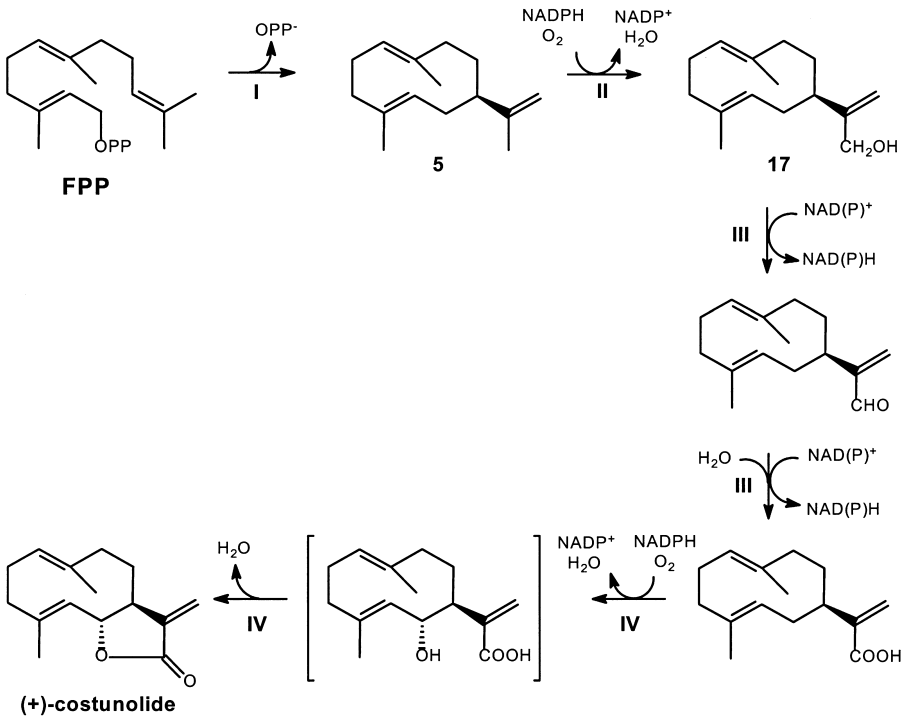


Scheme 1. Biopathway for the formation of (+)-costunolide from farnesyl diphosphate (FPP) in chicory roots, involving: (I) germacrene A synthase; (II) (+)-germacrene A hydroxylase; (III) dehydrogenases; and (IV) (+)-costunolide synthase. (+)-Germacrene A hydroxylase (II) catalyses the hydroxylation of (+)-germacrene A (**5**) to germacra-1(10),4,11(13)-trien-12-ol (**17**).

(+)-limonene did occur, but in such small amounts that it was not investigated further.

The conversion rates of the various reactions listed in Table 1 are expressed relatively to the hydroxylation of β-selinene (**10**) that is set to 100% and corresponds to a β-costol (**22**) peak-size of 6.3× the internal standard (2.5 nmol *cis*-nerolidol in each assay). The reason to take β-selinene as a reference instead of the natural substrate (+)-germacrene A was because of the instability of the latter (see below) and the fact that the two compounds are converted at nearly the same rate. Approximately 30% of the added 50 nmol of β-selinene (**10**) was hydroxylated in the time span taken (1 h). Not all of the formed hydroxylation products could be identified, since reference samples or mass spectra of the anticipated sesquiterpene alcohols were not always available.

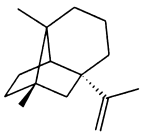
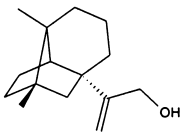
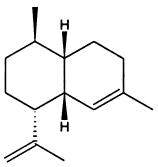
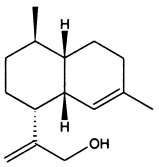
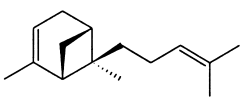
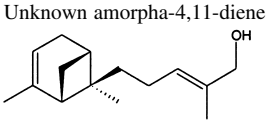
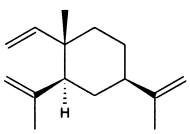
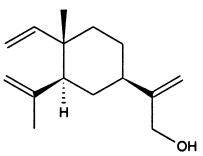
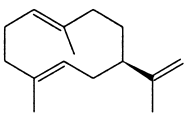
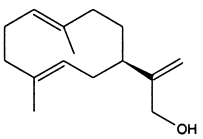
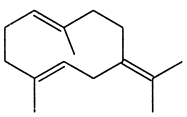
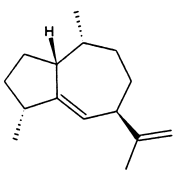
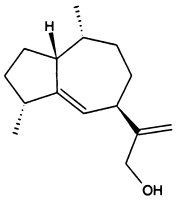
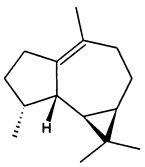
It is clear from the table that many sesquiterpenes are accepted by the chicory cytochrome P450 hydroxylase(s). Some compounds, like amorpha-4,11-diene (**2**), (−)-β-elemene (**4**), (+)-γ-gurjunene (**7**) and β-selinene (**10**), are converted with an efficiency that is quite close to that of the natural substrate (+)-germacrene A (**5**), although their structures are sometimes quite different. It is noteworthy that in the case of (−)-β-elemene only the isopropenyl group is hydroxylated that is positionally equivalent to that in (+)-germacrene A.

Incubation of amorpha-4,11-diene (**2**) yielded two alcohols. The major product was identified as amorpha-(4,11)-diene-12-ol ([**13**], Kovats' index [KI] 1761) which results from hydroxylation of the isopropenyl group. The structure of the earlier eluting alcohol **14** (KI 1650) could not be established.

Incubation of (−)-α-*trans*-bergamotene (**3**) yielded (*E*)-*trans*-bergamota-2,12-dien-14-ol ([**15**], KI 1720). This was verified by stirring the pentane–ether extract of the incubation with a spatula of MnO_2 overnight which yielded a compound with the same retention time and mass spectrum as that of (*E*)-*trans*-bergamota-2,12-dien-14-al, which is present in costus root oil.[10]

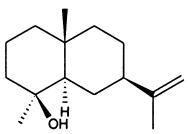
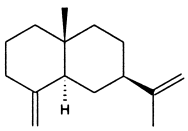
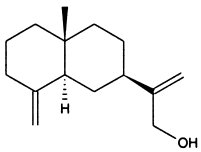
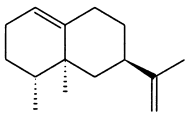
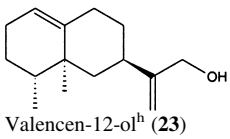
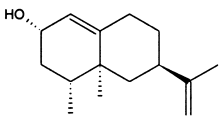
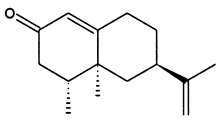
Incubation of (+)-germacrene A (**5**) yielded the expected germacra-1(10),4,11-trien-12-ol (**17**) because of the present (+)-germacrene A hydroxylase,[2] but the subsequent oxidation products of **17**, germacra-1(10),4,11-trien-12-al and germacra-1(10),4,11-trien-12-oic acid, were also clearly detected (approximately 15% of the total amount of products). This indicates that the microsomal pellet was not completely devoid of active dehydrogenases, and apparently the produced amount of alcohol **17** was sufficient to enable the subsequent reactions to germacrene acid (Scheme 1, step III).[2] Furthermore, the dehydrogenases were active despite the rather low pH of 7.5 and the presence of an NADPH-regenerating system that is expected to reduce most of the $NADP^+$ present. In the incubations with β-selinene (**10**) and β-elemene (**4**) only minute quantities of the corresponding aldehyde and/or acid were measured. This was one of the reasons why the conversion of β-selinene (**10**) was set at 100% and not that of the natural substrate **5**. The major reason to do so was because of difficulties in the analysis of the product. At low concentrations and high injection port temperatures, **17** undergoes complete Cope rearrangement into the corresponding elemene alcohol **16**.[2,11] In the present case, with much higher concentrations of **17**, the Cope rearrangement was incomplete leading to broad peaks that could not be quantified reliably.

Table 1. Conversion of sesquiterpenes by a microsomal pellet of chicory in the presence of NADPH

Substrate		Product(s)		Relative Conversion[a]
Structure	KI	Structure	KI[b]	
	1409		1688	1.5±0.1
Alloisolongifolene (**1**)		Alloisolongifolene alcohol* (**12**)		
	1482		1761	74.0±2.8
Amorpha-4,11-diene (**2**)		Amorpha-4,11-diene-12-ol* (**13**)		
		Unknown amorpha-4,11-diene alcohol (**14**)	1650	18.7±1.2
	1440		1720	13.5±0.2
(−)-α-*trans*-Bergamotene (**3**)		(*E*)-*trans*-Bergamota-2,12-dien-14-ol[c] (**15**)		
	1397		1673	56.2±2.8
(−)-β-Elemene (**4**)		(−)-Elema-1,3,11(13)-trien-12-ol* (**16**)		
	1512[d]		1673–1806[d]	110.0±5.1[e]
(+)-Germacrene A (**5**)		Germacra-1(10),4,11(13)-trien-12-ol* (**17**)		
	1566[d]	Germacrene B alcohols (**18**)	1694 and 1700[f]	3.1±0.1, 8.6±0.8
Germacrene B (**6**)				
	1479		1760	54.9±1.5
(+)-γ-Gurjunene (**7**)		5,11(13)-Guaiadiene-12-ol[g] (**19**)		
	1504	Unknown ledene alcohol (**20**)	1787	7.2±0.4
(+)-Ledene (**8**)				

(*continued on next page*)

Table 1 (*continued*)

Substrate		Product(s)		Relative Conversion[a]
Structure	KI	Structure	KI[b]	
 Neointermedeol (**9**)	1631	Unknown neointermedeol alcohol (**21**)	1909	6.9±2.4
 (+)-β-Selinene (**10**)	1492	 (+)-β-Costol* (**22**)	1778	100±4.5
 (+)-Valencene (**11**)	1500	 Valencen-12-ol[h] (**23**)	1777	2.6±0.1
		 β-Nootkatol* (**24**)	1722	5.1±0.2
		 Nootkatone* (**25**)	1820	24.4±1.1

Alcohols marked with an asterisk were identified using reference standards.

[a] 100% conversion corresponds to the hydroxylation rate of β-selinene (**10**) that yields a β-costol (**22**) peak size of 6.3×the internal standard (2.5 nmol *cis*-nerolidol in each assay).

[b] Kovats' indices were determined on an HP5 column.

[c] Identification based on the MnO_2 catalysed oxidation into (*E*)-*trans*-bergamota-2,12-dien-14-al.

[d] The Kovats' indices of these germacrenes are determined at an injection port temperature of 150°C; quantitative measurements were done on the basis of their Cope-rearrangement products measured at an injection port temperature of 250°C.

[e] This conversion rate is a summation of the peaks of elematrien-12-ol, costol, elematrien-12-al, and elematrien-12-oic acid.

[f] Kovats' indices were determined at an injection port temperature of 250°C and presumably belong to the Cope-rearrangement products.

[g] Identification is based on the fact that the formed alcohol is oxidisable by MnO_2.

[h] The mass spectrum of the detected compound was identical to the mass spectrum of authentic valencen-12-ol.

GC–MS analysis of the incubation of germacrene B (**6**) yielded two products with an almost identical mass spectrum and retention time (KI 1694 and 1700). Possibly, both methyl groups present in the isopropenyl side chain of **6** are hydroxylated, yielding both isomers of germacrene B alcohol **18**. Hydroxylation at other positions is unlikely, because such hydroxylations were also not observed in germacrene A (**5**). The products were measured as their Cope-rearrangement products (presumably γ-elemene alcohols). Lowering the injection port temperature from 250 to 150°C yielded faint broadened peaks due to on-column Cope-rearrangement of **18**.[11,12]

Incubation of (+)-γ-gurjunene (**7**) yielded an unknown sesquiterpene alcohol (KI 1760). Overnight stirring of the pentane–ether extract of the enzyme assay with MnO_2 resulted in the complete conversion of the alcohol product into an aldehyde or ketone ([M^+] 218). Because MnO_2 is specific for α,β-unsaturated alcohols[13] and the only other available allylic position is at a tertiary carbon atom, the biochemical hydroxylation must have occurred in the isopropenyl group of **7** yielding (1*S*,4*S*,7*R*,10*R*)-5,11(13)-guaiadiene-12-ol (**19**).

2.2. Conversion of (+)-valencene (11) and the participation of dehydrogenases

Incubation of (+)-valencene (**11**) with a microsomal pellet from chicory roots and NADPH yielded only a trace of the expected valencen-12-ol (**23**) ([−]-2-[2*R*]-2-[1,2,3,4,6,7,8,8a-octahydro-8α, 8aβ-dimethyl-2α-naphthal-

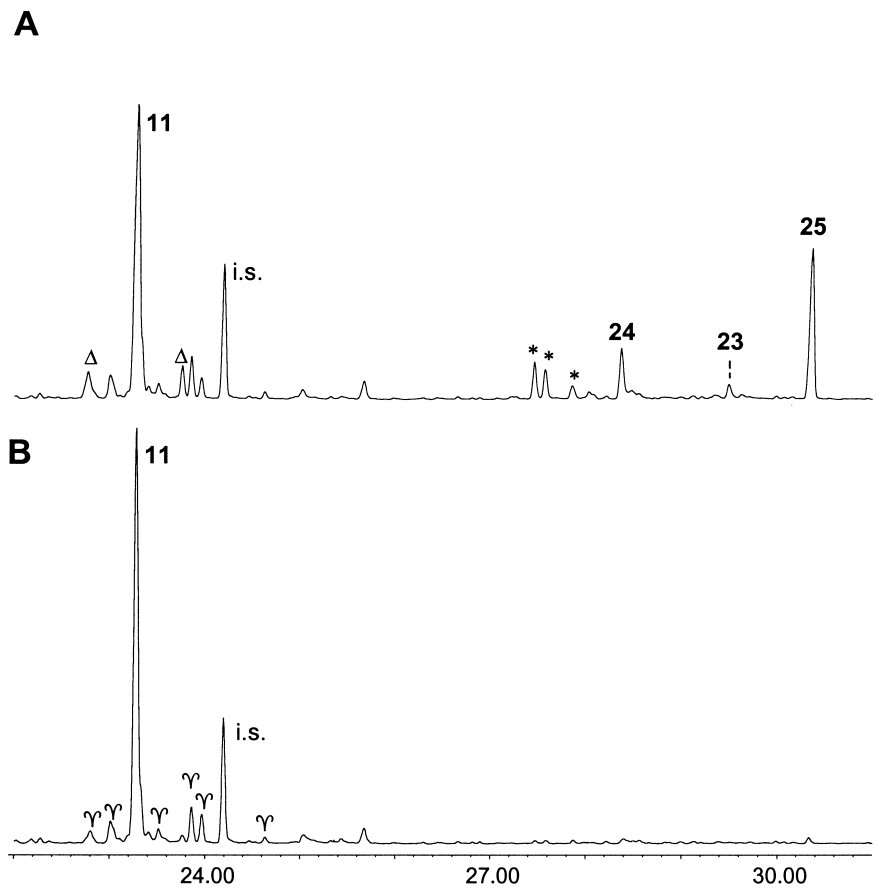
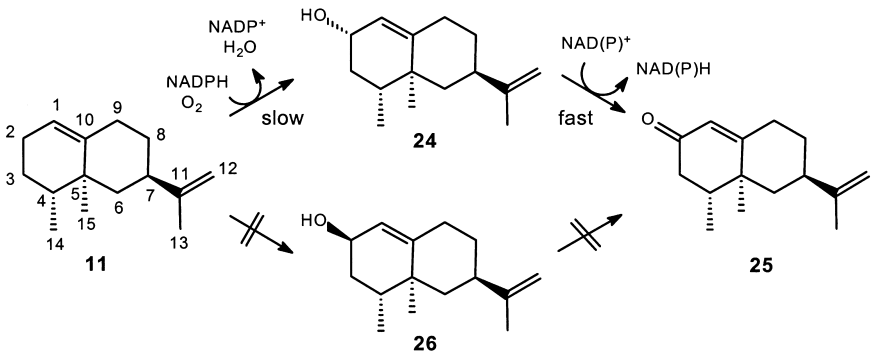


Figure 1. GC–MS analysis of the incubation of (+)-valencene (**11**) with a microsomal preparation of chicory roots. Incubation in the presence of NADPH (panel A) yields nootkatone (**25**), β-nootkatol (**24**) and valencen-12-ol (**23**); these products are absent if NADPH is omitted from the enzyme assay (panel B). Peaks marked with Δ are GC-induced dehydration products of β-nootkatol ($[M]^+$ 202). Peaks labelled with an asterisk are sesquiterpene alcohols that presumably originate from the enzymatic conversion of sesquiterpene impurities (marked with γ in panel B) present in the commercial sample of (+)-valencene (**11**).

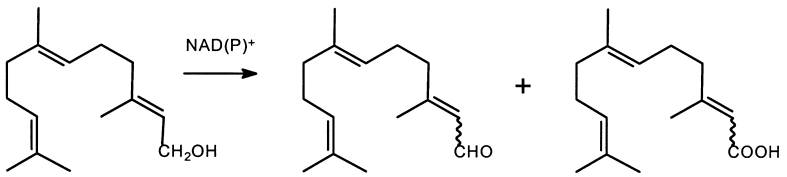


Scheme 2. Conversion of (+)-valencene (**11**) to nootkatone (**25**) proceeds via β-nootkatol (**24**) and not via α-nootkatol (**26**).

enyl]-2-propen-1-ol) (Fig. 1 and Table 1). Its mass spectrum matches exactly that of the (+)-enantiomer of **23**.[14‡] However, the major product from the incubation of **11** was nootkatone (**25**), whereas a smaller quantity of β-nootkatol (**24**) was detected as well (Fig. 1).

We assumed that this β-nootkatol (**24**) is an intermediate in the formation of nootkatone (**25**). Therefore, incubations of 100 μM **24** with a 150,000*g* supernatant of a chicory root enzyme extract were performed essentially as described for the conversion of (−)-elema-1,3,11(13)-trien-12-ol (**16**) and germacra-1(10),4,11(13)-trien-12-ol (**17**) into their corresponding aldehydes and acids that is catalysed by $NAD(P)^+$-dependent dehydrogenases.[2] During incubation, more than 90% of **24** was converted into nootkatone (**25**) 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 negligible conversion of **24**. At pH 7.5 enzyme activity was slightly

‡ The mass spectrum was kindly provided by Dr R. Näf of Firmenich SA (Geneva, Switzerland).



27 28 29

Scheme 3. Conversion of *trans*,*trans*-farnesol (**27**) to farnesal (**28**) and farnesoic acid (**29**) by a 150,000*g* supernatant of chicory roots in the presence of $NAD(P)^+$.

Table 2. Effect of the addition of 50 μM (+)-germacrene A (**5**) or 50 μM (+)-α-cubebene upon hydroxylation

Substrate	Product[a]	Inhibition (%)±SD	
		germacrene A (**5**)	α-cubebene
Alloisolongifolene (**1**)	Alloisolongifolene alcohol (**12**)	100.0	100.0
Amorpha-4,11-diene (**2**)	Amorpha-4,11-diene-12-ol (**13**)	84.9±0.1	−9.2±2.5
	Unknown amorpha-4,11-diene alcohol (**14**)	84.9±1.0	−2.3±1.5
α-*trans*-Bergamotene (**3**)	(*E*)-*trans*-Bergamota-2,12-dien-14-ol (**15**)	89.1±1.8	30.1±4.5
(−)-β-Elemene (**4**)	(−)-Elema-1,3,11(13)-trien-12-ol (**16**)	90.4±1.2	19.5±1.8
Germacrene B (**6**)	Germacrene B alcohol (KI 1694) (**18**)	100.0	32.6±3.6
	Germacrene B alcohol (KI 1700) (**18**)	100.0	27.3±10
(+)-γ-Gurjunene (**7**)	5,11(13)-Guaiadiene-12-ol (**19**)	88.0±0.9	-5.6±0.2
(+)-Ledene (**8**)	Unknown ledene alcohol (**20**)	100.0	35.5±1.9
Neointermedeol (**9**)	Unknown neointermedeol alcohol (**21**)	67.5±1.9	3.4±4.5
(+)-β-Selinene (**10**)	(+)-β-Costol (**22**)	60.3±3.4	0.4±2.0
(+)-Valencene (**11**)	Valencen-12-ol (**23**)	100.0	100
	Nootkatone (**25**)	90.2±2.5	8.7±2.5

[a] The amount of sesquiterpene alcohol produced in control incubations is set to 100% and is comparable with those of Table 1.

reduced, and a 150,000*g* pellet yielded three times less dehydrogenase activity (after correction for dilution of protein) than its corresponding supernatant that contains the operationally soluble enzymes. Incubation of a mixture of α and β-nootkatol (produced by chemical synthesis from **24**) showed that α-nootkatol (**26**) was hardly converted while β-nootkatol (**24**) was converted to the same extent as described above (Scheme 2).

To obtain more information about the substrate specificity of the dehydrogenase(s) present in chicory roots, incubations were also performed with 100 μM *trans*,*trans*-farnesol (**27**) and the 150,000*g* supernatant in the presence of either NAD^+ or $NADP^+$ (Scheme 3). About 60% of *trans*,*trans*-farnesol (**27**) was converted into a mixture of more or less equal amounts of *trans*,*trans*-farnesal and *cis*,*trans*-farnesal (**28**), whereas small amounts of farnesoic acid (**29**) were observed as well, predominately the *cis*,*trans*-isomer. The obtained amount of **28** and **29** 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 **27** into **28** was observed at pH 10, decreasing to 30% of the maximum enzyme activity at pH 7.0.

2.3. Competitive inhibition experiments

The microsomal pellets that were used in the experiments do not exclusively contain (+)-germacrene A hydroxylase, but also other membrane bound enzymes that are present in chicory roots. Hence, some of the conversions described in Table 1 might be catalysed by other oxidising enzymes than the (+)-germacrene A hydroxylase. To investigate this, incubations of substrates **1**–**4** and **6**–**11** were performed in the presence of an equal concentration (50 μM) of (+)-germacrene A (**5**). It was expected that if the (+)-germacrene A hydroxylase is involved in these reactions, the conversions would be competitively 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, to test the effect of the addition of an arbitrary sesquiterpene olefin on enzyme activity.

The results presented in Table 2 show that all enzymatic hydroxylations were inhibited to about 90% by the addition of (+)-germacrene A (**5**), except for (+)-β-selinene (**10**) and neointermedeol (**9**) whose hydroxylation was inhibited by 60 and 68%, respectively. The relatively small inhibition of β-selinene (**10**) hydroxylation agrees with the observation that it is nearly as well hydroxylated as (+)-germacrene A (**5**) (Table 1). Hydroxylation of the sesquiterpenes was not dramatically influenced by the addition of (−)-α-cubebene, except for the formation of alloisolongifolene alcohol (**12**) and valencen-12-ol (**23**) which already under normal assay conditions are only formed in small quantities.

2.4. 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 (**7**) were prepared in hexane, pentane, isopropanol, ethanol, and DMSO, and 5 μL of these solutions was added to the individual incubation mixtures. On the basis of the results presented in Table 3, ethanol was chosen

Table 3. Effect of the solvent used for the substrate (+)-γ-gurjunene (**7**) 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

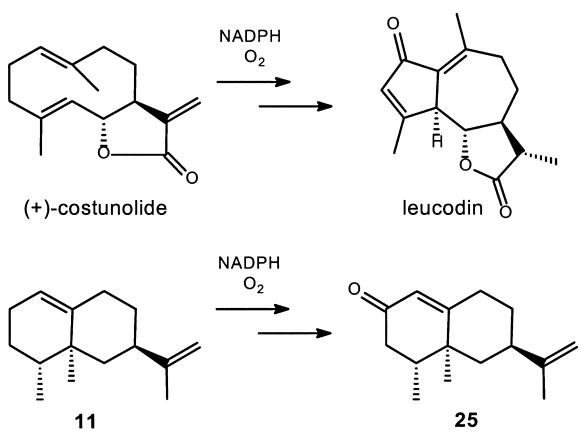
[a] Peak height of 5,11(13)-guaiadiene-12-ol (**19**) relative to the internal standard (5 nmol *cis*-nerolidol).

as solvent for the substrates in all experiments, instead of the more commonly used pentane.[6]

3. Discussion

In the presence of NADPH, a microsomal enzyme preparation from chicory roots is able to catalyse the hydroxylation of a range of sesquiterpene olefins that are exogenous to the plant (Table 1). Most of these hydroxylations take place at an isopropenyl or isopropylidene group, yielding in some cases sesquiterpene alcohols that have not previously been described, e.g. alloisolongifolene alcohol (**12**) and amorpha-4,11-dien-12-ol (**13**). The novelty of the formed sesquiterpene alcohols in some cases hampered their identification. The substrates for hydroxylation preferably do not contain any polar group: neointermedeol (**9**) is 15-fold less efficiently hydroxylated than β-selinene (**10**), and germacrone is not hydroxylated in contrast to germacrene B (**6**). The size of the substrate is also of importance as the hydroxylation of limonene, a monoterpene, hardly occurred. Whereas in general one alcohol was formed, amorpha-4,11-diene (**2**) and (+)-valencene (**11**) each yielded two distinct sesquiterpene alcohols.

Hydroxylations occurring at isopropenyl groups and, less efficiently, at isopropylidene groups of sesquiterpenes are similar to the reaction catalysed by the (+)-germacrene A hydroxylase in the sesquiterpene lactone biosynthesis (Scheme 1, step II).[2] The competitive inhibition of each hydroxylation reaction by (+)-germacrene A (Table 2)



Scheme 4. The putative homology between the formation of leucodin in chicory sesquiterpene lactone biosynthesis and the conversion of (+)-valencene (**11**) to nootkatone (**25**).

indicates that these reactions are indeed catalysed by the (+)-germacrene A hydroxylase. Hydroxylation of various exogenous substrates by one and the same cytochrome P450 enzyme involved in plant secondary metabolism, such as the (+)-germacrene A hydroxylase of chicory, contradicts the general idea that cytochrome P450 enzymes involved in plant secondary metabolism have narrow substrate specificities.[6,7,15–17]

Most intriguing is the unexpected conversion of (+)-valencene (**11**) via β-nootkatol (**24**) into nootkatone (**25**) (Fig. 1 and Scheme 2). To our knowledge, there is not yet any direct proof that in grapefruit, the natural source of **11** and **25**, such a biochemical pathway via β-nootkatol (**24**) exists, although **11** and **24** are known constituents of grapefruit.[18] It is assumed that the formation of nootkatone (**25**) in our experiments is catalysed by the same chicory enzymes that can convert (+)-costunolide into leucodin (Scheme 4).[3] It is reasonable to think that the (+)-germacrene A hydroxylase is not involved in the formation of nootkatone (**25**), nevertheless, formation of **25** was inhibited up to 90% by the addition of (+)-germacrene A (**5**). The inhibitory effect might be due to the structural similarity between (+)-germacrene A (**5**) and (+)-costunolide, but this assumption should be verified by repeating the incubations with (+)-costunolide as a competitor of (+)-valencene (**11**). Notably, the inhibitory effect of (+)-germacrene A (**5**) is not a 'general sesquiterpene effect', since (−)-α-cubebene did hardly have an effect on nootkatone (**25**) formation.

In some cases, further oxidation of the formed alcohols by $NAD(P)^+$-dependent dehydrogenase(s) has been observed (Scheme 4). These dehydrogenases appear to function very efficiently. They are operationally soluble enzymes, but apparently adhere somewhat to the microsomal pellet. The dehydrogenase(s) that are involved in the formation of nootkatone (**25**) have a strong preference for β-nootkatol (**24**) over α-nootkatol (**26**), but on the whole the dehydrogenases present in the 150,000*g* supernatant of a chicory root enzyme extract do not seem to act with a high substrate specificity, since they are also capable of converting *trans*,*trans*-farnesol (**27**) into farnesal (**28**) and farnesoic acid (**29**) (Scheme 3). $NAD(P)^+$-dependent oxidation of **27** to **28** has also been reported for other crude plant enzyme extracts, just as the observed isomerisation of farnesal (**28**).[19,20]

Nootkatone (**25**) 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 satisfactory or the procedure is too laborious.[9,21,22] From that point of view, the established conversion of (+)-valencene into nootkatone by a microsomal pellet of chicory might be of interest, particularly because undesired side products are hardly formed.

More generally, the drawbacks of chemical hydroxylation and the demand for new compounds by the aroma and

fragrance industry have been powerful driving forces in the research on hydroxylation of terpenes by micro-organisms.[8] Although in some cases successful, these microbiological conversions often yield a broad spectrum of products, including epoxides and diols and the oxidations often occur at double bonds.[8,9,23] In contrast, the hydroxylations catalysed by the microsomal pellet of chicory yield mostly one or in a few cases two products and hydroxylation occurs with high regio-selectivity, and as such, they have potential as catalysts in organic chemistry.

4. Experimental

4.1. Substrates

Alloisolongifolene (**1**), (−)-α-gurjunene, (+)-γ-gurjunene (**7**), (+)-ledene (**8**) and (+)-valencene (**11**) were purchased from Fluka. ICN Biomedicals furnished (−)-α-cubebene. (−)-Limonene and (+)-limonene were purchased from Merck and Janssen, respectively. Amorpha-4,11-diene (**2**) was synthesised by Dr B. J. M. Jansen.[24] Germacrone was isolated from the natural oil of *Geranium macrorrhizum* by Dr D. P. Piet, who also synthesised germacrene B (**6**) from this compound.[25] (−)-α-*trans*-Bergamotene (**3**) and (−)-β-elemene (**4**) were isolated by preparative GC from the essential oil of *Conyza canadensis* and of the liverwort *Frullania macrocephalum*, respectively.[26] (+)-Germacrene A (**5**) was isolated from fresh costus roots.[11] Neointermedeol (**9**) was synthesised by Dr R. P. W. Kesselmans.[27] (+)-β-Selinene (**10**) was isolated from celery oil and a gift from Dr T. A. van Beek. Substrates were dissolved at 10 mM concentrations in ethanol.

4.2. Reference compounds of sesquiterpenoid products

β-Nootkatol (**24**) was prepared by reduction of nootkatone (**25**, 190 mg, Fluka) with $LiAlH_4$ (20 mg) in dry ether (5 mL, diethyl ether).[28] After stirring the grey suspension for one night, the reaction was stopped by careful addition of $Na_2SO_4{\cdot}10H_2O$. The mixture was stirred for an additional 30 min, and dried by the addition of $MgSO_4$. The solids were filtered off and the ether was washed with distilled water. After drying and evaporation of the ether, 140 mg of a crude oil was obtained that besides **24** contained 4% of α-nootkatol (**26**). After flash chromatography of 50 mg of this crude oil on silica with ether–pentane (2:1), fractions devoid of any trace of **26** were pooled yielding 3.6 mg of **24** as a viscous colourless oil. ^{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, 2.7 Hz), δ 2.07 (ddd, 1H, H_9, J=14.0, 2.6, 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), δ 125.9 (d), δ 144.9 (s), δ 150.2 (s). HRMS, calcd [M^+]: 220.1827, found 220.1924.

Alloisolongifolene alcohol (**12**) was prepared from alloisolongifolene (**1**) via its corresponding aldehyde. This aldehyde was prepared by (over)oxidation of **1** with selenium dioxide, making use of the fact that in the structure **1** only one allylic position is available for selenium dioxide mediated hydroxylation. To a solution of SeO_2 (77 mg) and salicylic acid (68 mg) in CH_2Cl_2 (30 mL), **1** (100 mg) was added. Upon stirring at room temperature, the reaction mixture turned 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 demineralised water (60 mL) after 2.5 days. The reaction mixture was extracted with ether (45 mL), and the organic phase was subsequently washed with brine (30 mL). 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 as a yellowish viscous oil. It is a strongly 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). HRMS, calcd [M^+]: 218.1671, found 218.1672.

The aldehyde (15 mg) was added to a solution of $LiAlH_4$ (1.8 mg) in ether (0.5 mL). The grey suspension was stirred for 17.5 h at room temperature and stirred for an additional half hour after the careful addition of $Na_2SO_4{\cdot}10H_2O$ (one spatula). Demineralised water (1.5 mL) was added to the mixture, which was then thrice extracted with ether (1 mL). The ether was passed through a glasswool-plugged (dimethyl chlorosilane-treated, Chrompack) Pasteur pipette filled with silica and a spatula tip of $MgSO_4$. The solvent was evaporated, yielding 15 mg of **12** (approx. 95% pure), as a colourless viscous oil. ^{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, 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). HRMS, calcd [M^+]: 220.1827, found 220.1826.

A mixture of *trans*,*trans* and *cis*,*trans*-farnesal (**28**) was prepared by dissolving *trans*,*trans*-farnesol (**27**) (Sigma) in pentane and stirring with MnO_2. Retention times and mass spectra were identical to two of the four farnesal isomers from Fluka. Oxidation with silver oxide of **28** yielded a mixture of *cis*,*trans* and *trans*,*trans*-farnesoic acid (**29**).[29] Amorpha-4,11-diene-12-ol was prepared from artemisinic acid.[30] (−)-Elema-1,3,11(13)-trien-12-ol (**16**) was kindly provided by Dr B. Maurer (Firmenich SA, Switzerland).[10]

4.3. Incubations with microsomal pellets

Microsomal pellets (150,000–20,000*g*) that contain (+)-germacrene A hydroxylase activity were prepared from deep frozen chicory cubes.[2] The pellets can be easily stored under argon at −80°C. One microsomal pellet corresponds to approximately 4 g of fresh chicory root. Before incubation, ten pellets were homogenised in assay buffer

(30 mL) containing Tris (25 mM, pH 7.5), DTT (2 mM), ascorbic acid (1 mM), FAD (5 μM), FMN (5 μM), and glycerol 10% (v/v). The enzyme suspension was divided into 1 mL aliquots and incubated with substrate solution (5 μL, about 45 μM final concentration) in the presence of a 1 mM NADPH-regenerating system, which consisted of NADPH (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1.2 IU) (all from Sigma). All experiments were done in duplicate. NADPH was omitted from the blank assays. After 60 min the incubations were stopped by storing them in the freezer at −20°C.

cis-Nerolidol (Fluka, 2.5 nmol) was added to each enzyme assay as an internal standard, which was subsequently extracted twice with 1 mL 20% (v/v) ether in pentane. The organic phase was filtered through a glasswool-plugged Pasteur pipette that contained 0.4 g of silica and some 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.

To investigate whether enzymatic hydroxylation of the substrates was catalysed by the (+)-germacrene A hydroxylase, standard incubations of the various substrates (at 50 μM) were carried out in the presence of (+)-germacrene A (**5**, 50 μM) as competitive inhibitor. To control incubations only 5 μL of ethanol was added (i.e. the solvent of the added **5**). To exclude any general negative effect of sesquiterpene olefins on enzyme activity, incubations were also performed with 50 μM of (−)-cubebene instead of **5**.

4.4. GC–MS analysis

GC–MS analysis was performed on a HP 5890 series II gas chromatograph and an 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. Sample (2 μL) was injected at an injection port temperature of 250°C.

4.4.1. Mass spectral data. This part contains in alphabetical order the KI and MS-spectra, EIMS (70 eV), of the enzymatically formed sesquiterpene alcohols and synthesised compounds. Mass spectra of (−)-elema-1,3,11(13)-trien-12-ol (**16**), (−)-elema-1,3,11(13)-trien-12-al, (−)-elema-1,3,11(13)-trien-12-oic acid, and β-costol (**22**) have been reported elsewhere.[10,11]

Alloisolongifolene alcohol (**12**), KI 1688, *m/z*: 220 $[M]^+$ (5), 189 (35), 187 (36), 163 (30), 161 (56), 160 (37), 159 (30), 147 (36), 145 (38), 133 (32), 131 (37), 119 (44), 107 (61), 105 (91), 95 (94), 93 (57), 91 (100), 81 (53), 79 (60), 77 (52), 67 (39), 55 (45), 41 (62).

Alloisolongifolene aldehyde, KI 1601, *m/z*: 218 $[M]^+$ (41), 203 (48), 189 (32), 185 (30), 175 (42), 161 (68), 147 (53), 145 (37), 133 (35), 119 (46), 107 (42), 105 (80), 95 (33), 93 (46), 91 (100), 81 (37), 79 (65), 77 (64), 55 (43), 53 (34), 41 (74), 39 (45).

Amorpha-4,11-diene-12-ol (**13**), KI 1761, *m/z*: 220 $[M]^+$ (6), 202 (35), 189 (66), 187 (34), 145 (40), 132 (52), 131 (36), 121 (100), 119 (81), 105 (51), 93 (74), 91 (70), 81 (47), 79 (77), 77 (50), 55 (41), 41 (47).

Unknown amorpha-4,11-diene alcohol (**14**), KI 1650, *m/z*: 220 $[M]^+$(12), 189 (77), 162 (46), 147 (38), 121 (45), 119 (60), 107 (58), 105 (68), 95 (41) 93 (75), 91 (72), 81 (100), 79 (70), 77 (48), 55 (52), 43 (49), 41 (52).

(*E*)-*trans*-bergamota-2,12-dien-14-ol (**15**), KI 1720, *m/z*: 220 $[M]^+$ (<1), 145 (10), 132 (28), 131 (10),121 (11), 120 (14), 119 (75), 107 (29), 105 (22), 95 (10), 94 (15), 93 (100),81 (14), 79 (33), 77 (31), 68 (17), 67 (11), 55 (29), 53 (11), 43 (36), 41 (28), 39 (14).

Germacrene B alcohols (**18**), most likely measured as their corresponding elemene alcohols, KI 1694, *m/z*: 220 $[M]^+$ (<1), 202 (15), 187 (24), 159 (20), 147 (20), 145 (24), 134 (22), 133 (26), 131 (22), 123 (19), 121 (75), 120 (27), 119 (100), 109 (34), 108 (15), 107 (48), 106 (22), 105 (66), 95 (38), 94 (20), 93 (65), 92 (17), 91 (73), 81 (50), 79 (52), 77 (44), 71 (17), 69 (28), 68 (15), 67 (48), 65 (17), 57 (19), 55 (94), 53 (34), 43 (46), 41 (73), 39 (32).

KI 1700, *m/z*: 220 $[M]^+$ (<1), 202 (15), 189 (15), 187 (21), 159 (20), 147 (22), 145 (23), 137 (17), 134 (21), 133 (28), 131 (26), 123 (22), 122 (15), 121 (100), 120 (28), 119 (91), 117 (15), 109 (36), 108 (18), 107 (52), 106 (23), 105 (77), 95 (45), 94 (24), 93 (86), 92 (21), 91 (80), 81 (61), 79 (66), 77 (53), 71 (21), 69 (32), 68 (18), 67 (55), 65 (21), 57 (32), 55 (69), 53 (43), 43 (62), 41 (91), 39 (42).

cis,*trans*-Farnesoic acid[§] (**29**), KI 1791, *m/z*: 236 $[M]^+$ (3), 193 (17), 137 (8), 121 (21), 109 (12), 100 (12), 81 (29), 69 (100), 67 (16), 55 (13), 53 (16), 43 (15), 41 (64), 39 (15).

trans,*trans*-Farnesoic acid[§] (**29**), KI 1824, *m/z*: 236 $[M]^+$ (1), 193 (4), 137 (4), 136 (7), 123 (6), 121 (8), 100 (15), 81 (17), 79 (6), 69 (100), 67 (11), 55 (8), 53 (10), 43 (10), 41 (43), 39 (11).

(+)-γ-Gurjunene alcohol, most likely (1*S*,4*S*,7*R*,10*R*)-5,11(13)-guaiadiene-12-ol (**19**), KI 1760, *m/z*: 220 $[M]^+$ (35), 189 (36), 187 (37), 161 (39), 147 (31), 146 (35), 145 (55), 133 (33), 131 (64), 121 (41), 119 (54), 117 (31), 105 (81), 95 (41), 93 (58), 91 (100), 81 (92), 79 (71), 77 (54), 67 (38), 55 (49), 41 (59).

Unknown neointermedeol alcohol (**21**), KI 1909, *m/z*: 238 $[M]^+$ (<1), 223 (46), 135 (76), 93 (47), 81 (39), 79 (47), 71 (47), 55 (39), 43 (100), 41 (40).

α-Nootkatol (**26**), KI 1710, *m/z*: 220 $[M]^+$ (33), 177 (77), 161 (40), 145 (52), 131 (77), 119 (100), 109 (41), 107 (50), 105 (59), 95 (46), 93 (60), 91 (43), 81 (45), 79 (80), 77 (64), 69 (47), 67 (52), 55 (58), 43 (55), 41 (94), 39 (54).

[§] Elution order of the *cis*,*trans* and *trans*,*trans*-isomer isomer is likely the same as that of the isomers of farnesol and farnesyl acetate (Adams, R. P. Identification of Essential Components by Gas Chromatography Mass Spectroscopy; Allured Publishing Corporation: Carol Stream, 1995).

β-Nootkatol (**24**), KI 1722, *m/z*: 220 $[M]^+$ (56), 177 (100), 145 (31), 135 (51), 131 (43), 123 (38), 121 (91), 119 (81), 109 (42), 107 (66), 105 (62), 95 (49), 93 (69), 91 (87), 81 (39), 79 (60), 77 (56), 69 (45), 67 (51) 55 (62), 53 (42), 43 (52), 41 (100), 39 (55).

Unknown (+)-ledene alcohol (**20**), KI 1787, *m/z*: 220 $[M]^+$ (12), 187 (25), 159 (42), 151 (29), 147 (32), 145 (32), 133 (26), 131 (25), 121 (31), 119 (62), 107 (86), 105 (100), 95 (40), 93 (74), 91 (82), 81 (53), 79 (54), 77 (39), 55 (38), 43 (33), 41 (47).

Valencen-12-ol (**23**), KI 1777, *m/z*: 220 $[M]^+$ (22), 189 (52), 187 (41), 161 (80), 145 (54), 131 (49), 21 (41), 119 (58), 117 (39), 107 (55), 105 (84), 95 (44), 93 (73), 91 (100), 81 (47), 79 (84), 77 (51), 67 (39), 55 (48), 41 (63).

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