# Transformation of α-Pinene Using *Picea abies* Suspension Culture

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α-Pinene, both as the pure enantiomers and as the racemate, was transformed mainly to *trans*-verbenol by treatment with a *Picea abies* suspension cell culture. These reactions were followed by a slow transformation of the verbenol to verbenone, which was not transformed further. *trans*-Pinocarveol, myrtenol, *cis*-verbenol, and α-terpineol were byproducts of intermediate abundance. When subjected to the action of the suspension culture, *cis*-verbenol was not only transformed to verbenone but also isomerized to *trans*-verbenol. The transformation of α-pinene was fast, and the products were detected within one minute. The absolute configuration of the major products corresponded to that of the starting α-pinene enantiomer.

The compounds and mixtures that are most valuable for the flavor and fragrance industry are those that can be classified as "natural".[1] This has to hold for the turpentine that can be collected as a minor byproduct in the thermomechanical pulping (TMP) process. The raw material yielding the turpentine, wood chips from recently cut Norway spruce (*Picea abies*), is subjected to relatively mild conditions, i.e., treatment with steam at a temperature of 120−130 °C and at a pressure of 3−3.5 bar, followed by milling in a refiner. Thus, the wood chips experience only thermal and mechanical treatments, and the turpentine is separated from the chips by concomitant steam distillation. The turpentine formed in this way consists mainly of monoterpene hydrocarbons.[2] Normally it has not been considered to be of higher value than fuel and is indeed often used as such if it is collected at all.

To increase the commercial value of TMP-turpentine, it would be of interest to be able to convert its major monoterpene hydrocarbon constituents to more valuable compounds, e.g., oxygenated ones. To maintain the classification of the resulting compounds as "natural", one must use only mild transformation methods, such as enzymatic and microbial ones. Our purpose with the work described below is to enable the development of such a mild transformation method for TMP-turpentine.

Transformations of the major TMP-turpentine constituent,[2] α-pinene, to verbenols by microorganisms (fungi or bacteria) are reported in the literature,[3−6] but only a few examples of the use of plant cells for the conversion of α-pinene are known.[7,8] To our knowledge, undifferentiated plant cells do not produce terpenes by themselves (see cf. Corbier and Ehret).[7] These cells are, however, able to transform added terpene substrates. Thus, some of us have described a stereoselective oxidation of (*S*)-(−)-*cis*-verbenol to (*S*)-(−)-verbenone using free and immobilized cells of *Solanum aviculare*.[9,10]

When we used TMP-turpentine as a substrate for a *P. abies* suspension culture, preliminary results showed a production of a complicated mixture of oxygenated terpenes, which was difficult to analyze. To simplify the analytical procedures and with α-pinene as the major constituent of the substrate, we decided to use this compound as a model for TMP-turpentine in this biotransformation. Hence, (*R*)-, (*S*)-, and *rac*-α-pinenes were examined as substrates for *P. abies* suspension cultures.

## Results and Discussion

**Transformation of α-Pinene.** (*R*)-, (*S*)-, and *rac*-α-pinenes were treated with a suspension culture of *P. abies*. Transformation of either of the enantiomers of α-pinene gave the same products, albeit with opposite absolute configuration. The major transformation products from each of the enantiomers of α-pinene were the corresponding *cis*- and *trans*-verbenols (Figures 1, 2a−c, Table 1). The transformation of α-pinene was a fast reaction. Thus, the products were detected within 1 min. In 3 h about 35% (relative integrated area GC−MS) of *trans*-verbenol was detected. Longer reaction times led to the formation of verbenone, and this was the major product in the mixture after 8 days (68%, from *rac*-α-pinene) (Figure 3a−c, Table 2).

The data on enantiomeric ratios of the major products, given in Tables 1 and 2, were relative integrated GC−MS areas. Due to low product concentrations, the enantiomeric ratios could not be determined for the products resulting from experiments that were run only for a short time with (*R*)-(+)- or with (*S*)-(−)-α-pinene. The enantioselectivity in the transformations of α-pinenes showed a low preference for (*R*)-α-pinene (Tables 1 and 2).

Reports show that sobrerol is a transformation product of α-pinene,[3,5,11−13] and *Armillariella melles* treatment of α-pinene gives sobrerol as the major product.[5] Some of our experiments also show sobrerol as one of the products (up to 4% in some short-time experiments and between 2 and 18% in some long-term experiments).

**Workup.** Two workup procedures were used and compared:

(1) Extraction of the biotransformation mixture including the biomass, the starting material, and the products with hexane or *tert*-butyl methyl ether (TBME).

(2) (a) Filtration to remove the biomass. (b) Solid-phase extraction of the filtrate by Sep-Pak $C_{18}$ cartridges, followed by elution of the products adsorbed.

Extraction of the biotransformation mixture with hexane or TBME generally gave samples with a lower concentration of products than that obtained using the Sep-Pak cartridges followed by elution. When methanol was used

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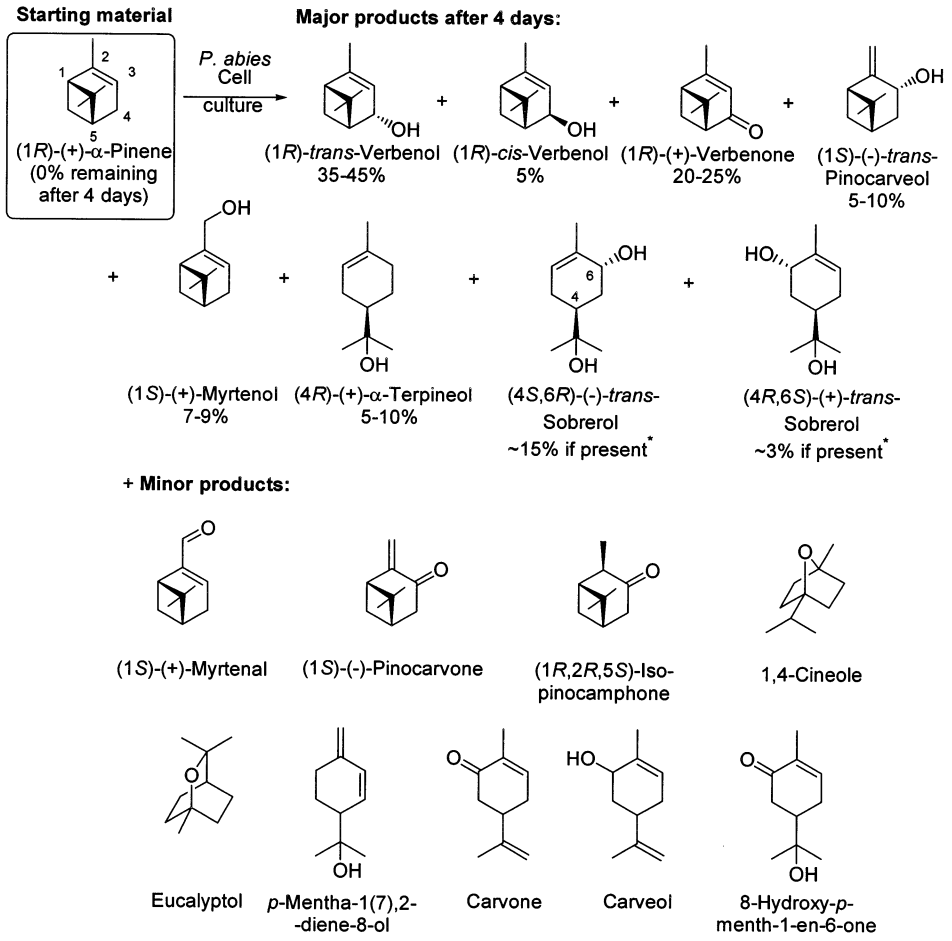



**Figure 1.** Biotransformation products of (*R*)-α-pinene after 4 days. Transformations of (*S*)-α-pinene gave a similar distribution of products but with the opposite stereochemistry. Sobrerol was not present in all samples.

**Table 1.** Enantiomeric Compositions of the Transformation Mixture in Short-Time Experiments[a]

| | components | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| time | α-pinene (1*R*/1*S*) | *trans*-verbenol (1*R*/1*S*) | *cis*-verbenol (1*R*/1*S*) | verbenone (1*R*/1*S*) | *trans*-pinocarveo1 (1*S*/1*R*)[b] | myrtenol (1*R*/1*S*) | α-terpineol (4*R*/4*S*) | *trans*-sobrerol (4*S*/4*R*) |
| 0 min | 53/47 | | | | | | | |
| 1 min | 46/54 | 66/34 | absent | 64/36 | absent | absent | absent | 69/31 |
| 0.5 h | 49/51 | 71/29 | 67/33 | 71/29 | 61/39 | *d* | >99/<1[c] | absent |
| 1 h | 49/51 | 70/30 | 70/30 | 67/33 | 59/41 | *d* | *d* | absent |
| 3 h | 48/52 | 69/31 | 83/17[c] | 68/32 | 65/35 | 38/62 [c] | *d* | absent |
| 24 h | 43/57 | 69/31 | 69/31 | 59/41 | 79/21 | 35/65 | *d* | absent |

[a] Substrate: *rac*-α-pinene. Mean values from 2 to 3 parallel experiments. The enantiomeric ratio of the products of the short-time experiments of (*R*)-(+)- and (*S*)-(−)-α-pinene were not shown since their concentrations were too low to allow an accurate determination by GC. [b] (*S*)-*trans*-Pinocarveol corresponds to (*R*)-α-pinene. [c] Only one sample. [d] The enantiomeric ratio of the product could not be accurately determined due to low concentrations of the product.

as the eluent of the products, these were found to contain large amounts of contaminants from the medium. The exchange of the eluent methanol for TBME did not change the quantity of terpenes. The use of TBME as eluent decreased the amount of interfering contaminants from the nutrient medium. In addition, TBME reduced another disadvantage associated with the use of methanol: the solubility of water being lower in TBME than in methanol. The use of TBME extended the lifetime of the water-sensitive chiral GC columns that we employed for our analyses. The TBME samples so obtained were qualitatively equivalent to the ones resulting from direct extraction of the biotransformation mixture with hexane.

***P. abies* Suspension Culture without Substrate.** Under normal reaction conditions and in the absence of a terpene source, no terpenoids were produced by the suspension culture. However, straight-chain hydrocarbons of various lengths (C10−C16) as well as straight-chain, branched, and unsaturated ones of higher chain lengths (C>16) were found in the suspension. The stress-related product methyl salicylate was also detected in the suspension culture. Ethyl benzoate was found in some suspension culture samples, including all samples collected more than 0.5 h after addition of the terpene substrate.

**Autoxidation.** As early as 1960 the autoxidation of α-pinene was described in the literature. A report by Bhattacharyya et al.[11] described the identification of verbenone, verbenol, and sobrerol among the autoxidation products of α-pinene.

We used internal standards in order to allow quantitative evaluations of the extent of autoxidation. Thus, 1-adamantol was added to the *P. abies* suspension culture or to the nutrient medium together with the substrate, and adamantane was added after the workup of the products.

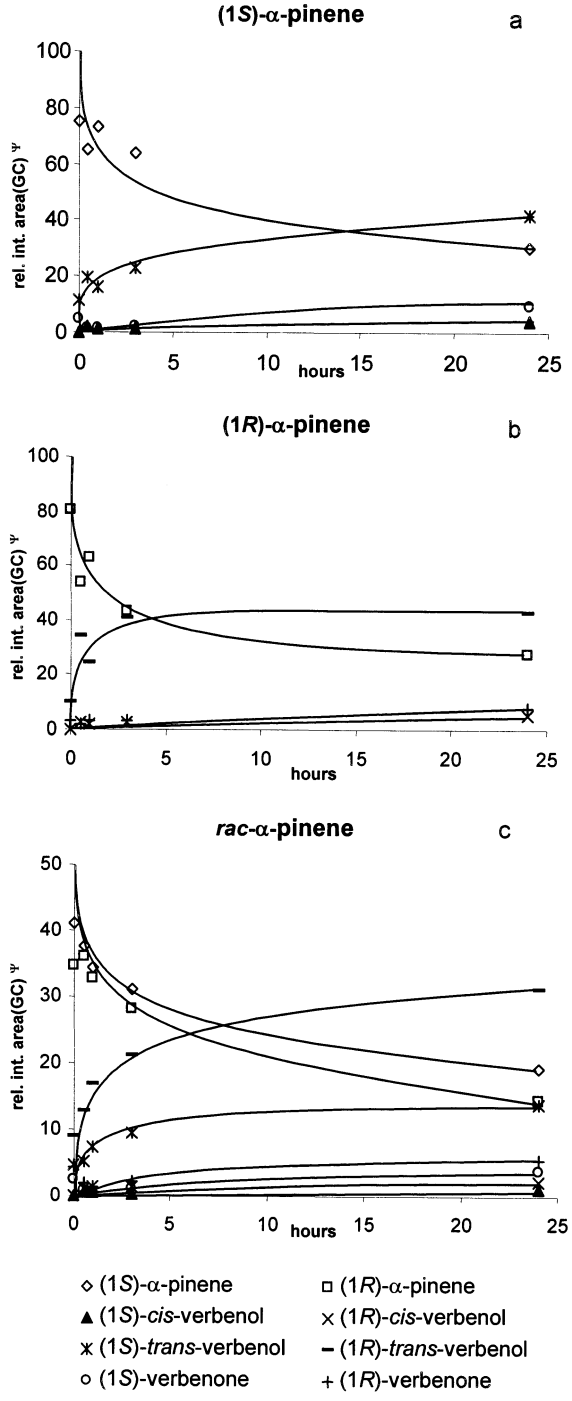



**Figure 2.** Conversion of α-pinene into verbenols and verbenone versus time in short-time experiments. Ψ = Integrated GC area of peak relative to total integrated area in percent.

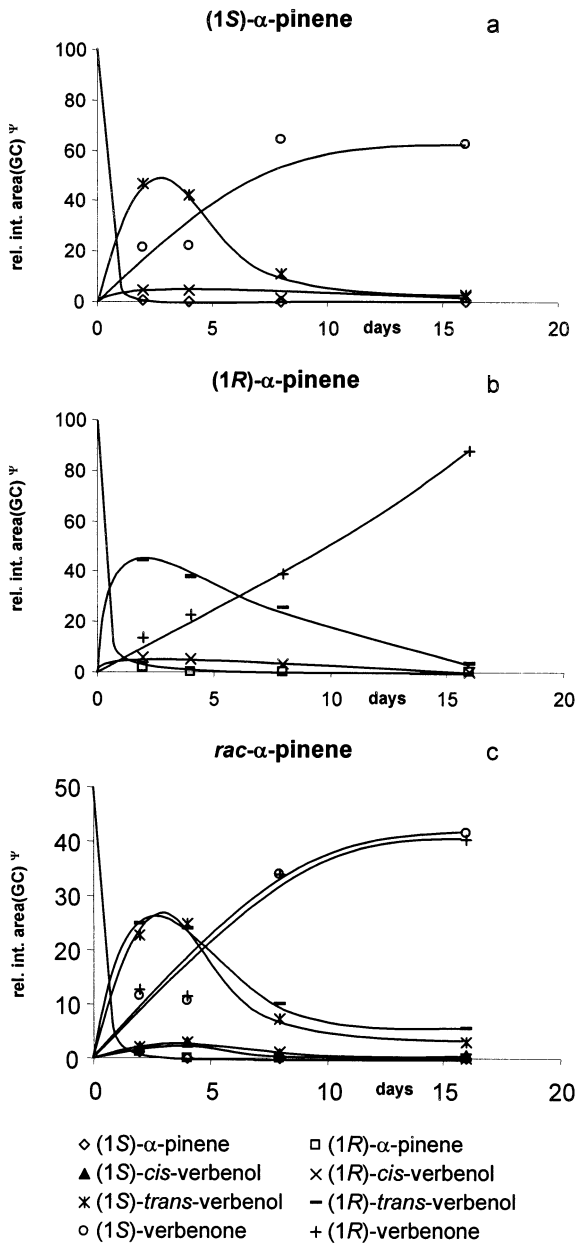



**Figure 3.** Conversion of α-pinene into verbenols and verbenone versus time in long-time experiments. Ψ = Integrated GC area of peak relative to total integrated area in percent.

No autoxidation products were formed when (*R*)-α-pinene was added to pure water. When (*R*)-α-pinene or *rac*-α-pinene was added to the nutrient medium under sterile conditions, a small amount of autoxidation products was formed. However, this amount was much smaller than the amount of products produced from the same substrates by the suspension culture under the same conditions and time. Suspension culture and α-pinene [20 $\mu$L (16.4 mg)] gave 0.04 mg of *trans*-verbenol after 24 h, whereas the nutrient medium and α-pinene gave only 0.005 mg. Thus, the amount of *trans*-verbenol produced by the suspension culture was 8 times that in the nutrient medium. In addition, the suspension culture and the nutrient medium treatments gave different ratios of products (Table 3). The relative amount of *trans*-verbenol was higher in the suspension culture. The enantiomeric compositions of the products did not differ between the experiments with suspension culture and those with the nutrient medium.

**Transformation of Verbenols and Verbenone.** Biotransformations of *cis*-verbenol into verbenone using plant cells (*Nicotiana tabacum* L. and *Cannabis sativa* L.) have been reported earlier,[14,15] but the formation of *trans*-verbenol has been observed only after biotransformation using *S. aviculare* plant cells.[9]

We found that *cis*-verbenol, when added to the suspension culture, was transformed into *trans*-verbenol and verbenone (Table 4a, Figure 4). However, when *trans*-verbenol was used as the substrate, it was very slowly transformed into *cis*-verbenol and verbenone (Table 4b). Thus, the equilibrium between *cis*- and *trans*-verbenol seemed to be characterized by a dominating concentration of *trans*-verbenol. When verbenone was added as the sole substrate to the suspension culture, only trace amounts of

**Table 2.** Enantiomeric Compositions of the Transformation Mixture in Long-Time Experiments[d]

| Substrate: *rac*-α-Pinene | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | components | | | | | | | |
| time | α-pinene (1*R*/1*S*) | *trans*-verbenol (1*R*/1*S*) | *cis*-verbenol (1*R*/1*S*) | verbenone (1*R*/1*S*) | *trans*-pinocarveo1 (1*S*/1*R*)[a] | myrtenol (1*R*/1*S*) | α-terpineol (4*R*/4*S*) | *trans*-sobrerol (4*S*/4*R*) |
| 0 | 53/47 | | | | | | | |
| 2 days | 52/48 | 52/48 | 51/49 | 53/47 | 73/27 | 46/54 | *c* | 37/63 |
| 4 days | absent | 49/51 | 51/49 | 53/47 | 63/36 | 48/52 | *c* | 45/55[b] |
| 8 days | absent | 59/41 | 62/37 | 50/50 | >99/<1 | 30/70 | *c* | 64/36[b] |
| 16 days | absent | 79/21[b] | absent | 49/51 | absent | 31/69[b] | 49/51[b] | absent |
| Substrate: (1*R*)-(+)-α-Pinene | | | | | | | | |
| | components | | | | | | | |
| time | α-pinene (1*R*/1*S*) | *trans*-verbenol (1*R*/1*S*) | *cis*-verbenol (1*R*/1*S*) | verbenone (1*R*/1*S*) | *trans*-pinocarveo1 (1*S*/1*R*)[a] | myrtenol (1*R*/1*S*) | α-terpineol (4*R*/4*S*) | *trans*-sobrerol (4*S*/4*R*) |
| 0 | 99/1 | | | | | | | |
| 2 days | *c* | 94/6 | 94/6 | 92/8 | 91/9[b] | 8/92 | *c* | 85/15 |
| 4 days | absent | 95/5[c] | 95/5[b] | 93/7[b] | *c* | *c* | *c* | 85/15[b] |
| 8 days | absent | 94/6 | *c* | 93/7 | 90/10[b] | *c* | *c* | 71/29 |
| 16 days | absent | *c* | absent | 94/6 | *c* | *c* | *c* | absent |
| Substrate: (1*S*)-(−)-α-Pinene | | | | | | | | |
| | components | | | | | | | |
| time | α-pinene (1*R*/1*S*) | *trans*-verbenol (1*R*/1*S*) | *cis*-verbenol (1*R*/1*S*) | verbenone (1*R*/1*S*) | *trans*-pinocarveo1 (1*S*/1*R*)[a] | myrtenol (1*R*/1*S*) | α-terpineol (4*R*/4*S*) | *trans*-sobrerol (4*S*/4*R*)[b] |
| 0 | 5/95 | | | | | | | |
| 2 days | *c* | 7/93 | 7/93[b] | 7/93 | 22/78 | 88/12[b] | *c* | 14/86[b] |
| 4 days | absent | 6/94 | *c* | 7/93 | 21/79 | 90/10 | 14/86[b] | 16/84[b] |
| 8 days | absent | 12/88 | *c* | 5/95 | absent | *c* | *c* | 20/80[b] |
| 16 days | absent | *c* | *c* | 6/94 | 27/73[b] | 93/7 | 10/90[b] | *c* |

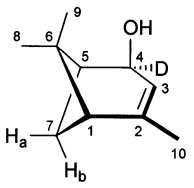
[a] (*S*)-*trans*-Pinocarveol corresponds to (*R*)-α-pinene. [b] Only one sample. [c] The enantiomeric ratio of the product could not be accurately determined due to low concentrations of the product. [d] Mean values from 2 to 3 parallel experiments.

**Table 3.** Product Ratio of *trans*-Verbenol, *cis*-Verbenol, and Verbenone by Transformation of (*R*)-α-Pinene in *P. abies* Suspension Culture or in Nutrient Medium

| | components (relative ratio) | | |
|---|---|---|---|
| experiment | *trans*-verbenol | *cis*-verbenol | verbenone |
| suspension culture, 0.5 h | 13.8 | | 1 |
| suspension culture, 24 h | 14.2 | 1.7 | 1 |
| nutrient medium, 0.5 h | 2.2 | 1 | 1.6 |
| nutrient medium, 24 h | 1.2 | 1 | 2.0 |

*cis*- and *trans*-verbenol were detected (Table 4c). The transformation of verbenol occurred during the first day only, and after that time, the proportions of the constituents remained constant.

An obvious pathway for the verbenol interconversion observed would be oxidation to verbenone, followed by reduction to a verbenol. However, verbenone was not converted into verbenols. We observed a similar situation in our previous work with *S. aviculare* immobilized cells.[9,10] To investigate the suspension culture mediated interconversion observed between *cis*- and *trans*-verbenol, we prepared the deuterium-labeled (1*S*)-(4$^2$H)-*cis*-verbenol.




(1*S*)-(4$^2$H)-*cis*-verbenol

**Table 4.** Biotransformation of Intermediates and Products; Relative Percentages According to GC Integration (enantiomeric proportions *R*/*S* in parentheses)

| a. Transformation of *cis*-Verbenol | | | |
|---|---|---|---|
| time | *cis*-verbenol | *trans*-verbenol | verbenone |
| 0 (standard) | 97 (5/95) | 1.4[a] | 0.3[a] |
| 1 day | 57 (3/97) | 38 (6/94) | 4 (36/64) |
| 4 days | 13 (4/96) | 78 (4/96) | 8 (29/71) |
| 7 days | 10 (3/97) | 74 (3/97) | 15 (23/77) |
| b. Transformation of *trans*-Verbenol | | | |
| time | *cis*-verbenol | *trans*-verbenol | verbenone |
| 0 (standard) | 5 (21/79) | 89 (25/75) | 6 (26/74) |
| 1 day | 11 (20/80) | 80 (24/76) | 7 (24/76) |
| 4 days | 12 (23/77) | 68 (24/76) | 19 (34/66) |
| 7 days | 13 (21/79) | 71 (23/77) | 15 (35/65) |
| c. Transformation of Verbenone | | | |
| time | *cis*-verbenol | *trans*-verbenol | verbenone |
| 0 (standard) | 0 | 0 | 100 (22/78) |
| 1 day | 0.2[a] | 1.7[a] | 96 (22/78) |
| 4 days | 0.2[a] | 1.8 (12/88) | 97 (25/75) |
| 7 days | 0.2[a] | 1.7[a] | 98 (23/77) |

[a] The low content did not allow an accurate determination of the enantiomeric composition.

Under normal suspension culture conditions, this was isomerized to *trans*-verbenol with retention of the 4-deuterium. This result and the fact that only very small amounts of verbenols were formed, when verbenone was used as the substrate of the suspension culture, led us to the conclusion that the observed isomerization of *cis*-verbenol into *trans*-verbenol did not proceed via verbenone but tentatively via an enzyme-bound allylic cation, formed formally via loss of $H_2O$ from a protonated *cis*-verbenol (or of −OH from cis-verbenol) (Figure 5).

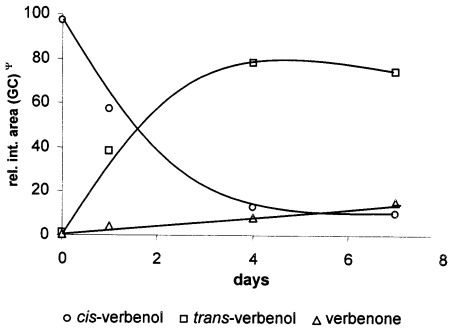



**Figure 4.** Biotransformation of *cis*-verbenol using a *P. abies* suspension culture. Ψ = Integrated GC area of peak relative to total integrated area in percent.

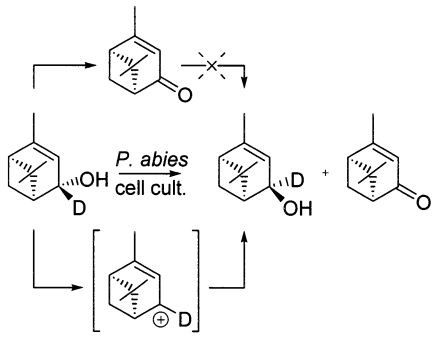



**Figure 5.** Tentative mechanism of the biotransformation of (1*S*)-(4²H)-*cis*-verbenol into *trans*-verbenol.

**Table 5.** Relative Amounts [GC−MS (*β*-dex) integrated areas] of Products after Transformation of *cis*-Verbenol after 24 h in Nutrient Medium Only or by *P. abies* Suspension Culture[a]

| substance | *cis*-verbenol, nutrient medium | *cis*-verbenol, suspension culture | (4²H)-*cis*-verbenol, suspension culture |
|---|---|---|---|
| *cis*-verbenol | 86.0 (97.4) | 20.8 | 12.7 (99.6) |
| *trans*-verbenol | 12.2 (0.66) | 72.1 | 84.5 (−) |
| verbenone | 0.2 (−) | | − (−) |

[a] Numbers in parentheses show the purity of the starting substrate.

In the presence of the nutrient medium, *cis*-verbenol was also transformed into *trans*-verbenol to some extent, although much more slowly than in the suspension culture (Table 5). After 24 h the relative amount of *cis*-verbenol was still 86% in the nutrient medium and in the suspension culture it was 21%.

*trans*- and *cis*-verbenols have frequently been reported as insect semiochemicals, and as such they have often found practical use in the monitoring or mass trapping of bark beetles. For example, *trans*-verbenol has been used, together with *exo*-brevicomin, for trapping the mountain pine beetle, *Dendroctonus ponderosae*.[16] On the other hand *trans*-verbenol has been shown to inhibit aggregation and induce dispersal behavior in the pine engraver, *Ips pini*.[17,18] *cis*-Verbenol together with methyl butenol have been found to be essential for the attraction of *Ips typographus*.[19]

Although others performing biotransformations of α-pinene by plant cell cultures found enantioselectivity in their reactions,[8] the transformations of α-pinene by *P. abies* suspension cultures showed little or no enantioselectivity. The reactions with *P. abies* suspension cultures seemed to be nonspecific. The transformations of α-pinene probably proceeded via a radical mechanism. Peroxidases might be involved in the transformation by the *P. abies* suspension culture.

## Experimental Section

**Solvents and Chemicals.** *tert*-Butyl methyl ether (TBME) (99.8%), (1*R*)-(+)-α-pinene (96%, 98% ee), and (1*S*)-(−)-α-pinene (98%, 98% ee) were purchased from Aldrich. *rac*-α-Pinene was obtained by mixing equal amounts of the two enantiomers. (1*S*,4*S*,5*S*)-*cis*-Verbenol (two *cis*-verbenol preparations were used, one with 97%, 90% ee for experiments in Table 4, and the other with 97.4%, 91% ee for experiments in Table 5) and (1*R*,4*S*,5*R*)-*trans*-verbenol (89.4%, 52% ee) were available at the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic). (*S*)-Verbenone (96%, 56% ee) was obtained from Aldrich. The chemical purities and the ee's of the substrates were determined by gas chromatography on a DB-WAX and an HP Chiral column (20% permethylated *β*-cyclodextrin), respectively. The following compounds were used as references for the terpenoid products (GC−MS analyses): (1*R*,4*S*,5*R*)-*trans*-pinocarveol (89.4%, 52% ee), (1*R*)-(−)-myrtenol (92%, 91% ee), carvone, and carveol were purchased from Sigma-Aldrich Sweden AB. (4*S*)-(−)-α-Terpineol (98%) was bought from Fluka. Isopinocamphone was prepared according to a published procedure.[20] Pinocarvone was prepared with modifications of published procedures[21−23] (see below in (1*S*)-Pinocarvone). Racemic *trans*-sobrerol (purchased from Sigma-Aldrich) was resolved by transesterification of vinyl acetate by *Psudomonas cepacia* lipase (PCL-PS "Amano") bought from Amano International Enzyme Co. and was analyzed on the *β*-dex 120 GC column, all according to Bovara et al.[24] In this way, the absolute configurations of the two enantiomers of *trans*-sobrerol were determined.

**Analysis: General Procedure.** The general procedure of analysis was used unless otherwise stated. The GC−MS analyses were made on a gas chromatography−mass selective detector (GC: Hewlett-Packard 6890, inj temp 200 °C, split/splitless injector, MS: Hewlett-Packard 5973). Helium (0.7 mL/min) was used as carrier gas. A chiral column, *β*-dex 120 (30 m × 0.25 mm i.d., film thickness 0.25 *μ*m), was used in the GC, temperature program: 60 °C (3 min)−3°/min−80 °C−2°/min−200 °C (10 min). The MS source temperature was 200 °C. The response factors of the various monoterpenes were neglected unless otherwise noted. The amount of each product was then described as the GC−MS integration area of that product, divided by the GC−MS integration area of the total amounts of products or in relation to an internal standard. Adamantane was used as the internal standard for the quantitative analysis. The response factors were determined according to the following:

Response factor of compound X = (weight of internal standard/area of internal standard) × (area of compound X/weight of compound X). The response factors were determined to be as follows: α-pinene (0.83), *cis*-verbenol (0.40), *trans*-verbenol (0.40), verbenone (0.75).

**Identification of Products.** The identification of the products was performed by GC−MS analysis described above (see section: Analysis: General Procedure). The products from the biotransformations were identified by comparisons of their retention times and mass spectra with those of the reference substances and by the use of the MS library Wiley 275.

**Cultivation of Suspension Culture.** *P. abies* embryogenic cultures were induced from immature zygotic embryos and maintained on sterile media, solidified with 0.75% (w/v) agar (Sigma) according to Gupta and Durzan.[25] The maintenance medium was supplemented with 5 *μ*M 2,4-dichlorophenoxyacetic acid (2,4-D), 2 *μ*M kinetin, 2 *μ*M 6-benzylaminopurin (BAP) (purchased from Sigma), and 30 g/L sucrose (purchased from Lachema, C.R.). The pH was adjusted to 5.8 ± 0.05 before autoclaving.

The suspension cultures were initiated from the embryogenic culture. The same supplemented maintenance medium as that described above (excluding agar) was used as the nutrient medium. The suspension cultures were kept on rotary shakers at 100 rpm in 250 mL Erlenmeyer flasks at 24 °C in darkness. The cultures were subcultivated every 7 days.

**Biotransformation: A General Procedure.** Each substrate (20 μL) was added to a *P. abies* suspension culture (1 week old, 100 mL) in an Erlenmeyer flask under sterile conditions. The flasks were sealed with aluminum foil and kept in the dark on a rotary shaker at 24 °C for different time intervals (1 min to 16 days). The selected best workup procedure (see below) was filtration, first through a filter paper (Munktell's density 204, Grycksbo, Sweden) and thereafter through a Sep-Pak C-18 cartridge (Waters, Milford). The sugar and salts originating from the nutrient medium were removed from each cartridge with 10 mL of distilled water, whereupon the products were eluted with 2 or 3 mL of TBME.

**Evaluation of Workup Procedures.** Four workup procedures were evaluated. After the biotransformation of 20 or 40 μL of (*R*)-α-pinene (in duplicates), the biotransformation mixture (including the biomass, remaining terpene substrate, and terpenoid products) was filtered through a filter paper, and the filtrate was split into two flasks. Two 2 mL portions were withdrawn, one portion from each flask. One portion was extracted with 400 μL of hexane and one with the same amount of TBME. The extracts were dried with $MgSO_4$. The remaining content of each of the two flasks (~23 mL) was filtered through a Sep-Pak cartridge and washed with 10 mL of distilled water. Thereafter, the compounds adsorbed on the cartridge were eluted with 3 mL of methanol or with 3 mL of TBME, respectively. The resulting samples were analyzed by GC−MS (β-Dex 120 column) and compared.

**Biotransformation of α-Pinene.** *rac*-α-Pinene [(*R*)/(*S*) 53/47], (*R*)-α-pinene, and (*S*)-α-pinene were used as substrates. The experiments were performed in duplicates or triplicates. The biotransformation mixtures were worked up after 1 min, 30 min, 1 h, 24 h, and 2, 4, 8, or 16 days. The average volume of the filtrate was 60 mL. The Sep-Pak was eluted with 2 mL of TBME.

For a quantitative evaluation of the mass balance in some experiments, about 2 mg (the exact weight was determined by weighing) of adamantol was added to the suspension culture as an internal standard and 2 mg of adamantane was added after workup.

**Control Experiments.** A suspension culture with no substrate added was treated in the same way as above. Its workup was performed after 0 (5 parallel samples), 3, 7, and 15 days. The samples were analyzed by GC−MS (β-Dex).

**Autoxidation in Nutrient Medium.** A 20 μL sample of α-pinene (racemic α-pinene (*R*/*S*) 53/47 or (*R*)-α-pinene) or 30 mg of (1*S*)-*cis*-verbenol was added to 100 mL of nutrient medium in an Erlenmeyer flask, 1−10 mg of adamantol (determined by weighing) was added as an internal standard, and 1−5 mg of adamantane was added after workup. Otherwise, the experimental details were the same as in the general procedure for suspension cultures. The samples containing *rac*-α-pinene or *cis*-verbenol as a substrate (in duplicates) were worked up after 0.5 and 24 h. Samples containing (*R*)-α-pinene (triplicates) were worked up after 0.5 and 24 h. Some of the nutrient medium samples were not filtered. However, this turned out to be necessary in order for the nutrient medium samples to be comparable with the suspension culture samples, since the adamantane showed a tendency to stick onto the filter paper.

**Autoxidation in Water.** (*R*)-(+)-α-Pinene (20 μL) was added to 100 mL of sterile water in an Erlenmeyer flask. The experiments were performed in duplicates. The flask was sealed by aluminum foil and put on a rotary shaker at room temperature (24 °C). The samples were worked up after 1 day. They were allowed to pass through a Sep-Pak cartridge, and the cartridge was eluted with 2 mL of TBME. The eluate was analyzed by GC−MS (CP-SIL 8 CB, 30 m, 0.25 mm i.d., 0.25 μm film thickness, Chrompack).

**Biotransformation of Verbenols and Verbenone.** (*S*)-*cis*-Verbenol (20 mg), (*R*)-*trans*-verbenol (20 μL), or (*S*)-verbenone (20 μL) were used as substrates for the *P. abies* suspension culture. The time of transformation was 1 day, 4 days, or 7 days.

**Analyses of Autoxidation Products in Water.** The analyses to detect autoxidation products in water were performed on the Varian Saturn ITD mass spectrometer, column CP-SIL 8 CB, 30 m × 0.25 mm i.d., film thickness 0.25 μm, Chrompack, temperature program 50(4)−10−120(0)−5−210-(15).

**Analyses of the Products from Transformation of Verbenols and Verbenone.** Quantification of the analyses of the transformation of verbenols and verbenone was performed on the GC HP 5890A, FID; column DB-WAX (30 m × 0.25 mm, film thickness 0.25 μm), temperature program 50-(4)−10−120(0)−5−210(15); $H_2$ flow 1.96 mL/min; split.

The enantiomeric compositions of the products of transformation of verbenols and verbenone were analyzed on the GC HP 6850, FID, column β-dex 120 (30 m × 0.25 mm i.d., film thickness 0.25 μm) Agilent Technologies, temperature program 40(1)−50−60(13)−10−120(7)−10−150(20).

**Biotransformation of D-Labeled (1*S*)-*cis*-Verbenol.** (1*S*)-*cis*-verbenol (30 mg) labeled with deuterium (position 4) was used as the substrate in biotransformations following the general procedure. The flasks were worked up after 0 (1 min), 1 day, and 3 days. Some of the verbenol was not dissolved in 1 min but remained on the filter. The SepPak was eluted with 3 mL of TBME into a vial. A small amount of water on the bottom of the vial was removed with a Pasteur pipet.

**(1*S*)-(4$^2$H)-*cis*-Verbenol.** D-Labeled (1*S*)-*cis*-verbenol was synthesized following previously published procedures.[26,27] A solution of 1.965 g of (1*S*)-verbenone in dry ether (20 mL) was added dropwise to a stirred suspension of $LiAlD_4$ (0.326 g) in dry ether (50 mL). The suspension was cooled with a mixture of ice and sodium chloride. When the addition of verbenone was completed, the mixture was stirred for 1 h under ice-cooling. Water (0.27 mL) was then carefully added to the stirred and cooled mixture. Sodium hydroxide (0.27 mL, 5 M) and another portion of water (0.81 mL) were added to the mixture. The mixture was stirred and cooled for another hour and then filtered through a glass filter funnel with Celite. The filtrate was dried overnight with potassium carbonate. The drying agent was removed with a glass filter funnel, and the solvent was evaporated on a rotary evaporator. The yield was 1.563 g (79.5%) based on verbenone.

The product was purified through recrystallization in pentane. Some of the *trans*-verbenol formed in the reaction was still present and was separated from the *cis*-verbenol by medium-pressure chromatography through silica gel (60 μm, Merck) using cyclohexane and ethyl acetate as eluents (gradient elution). The following GC−MS (β-Dex 120 column) and spectroscopic data of the purified deuterated *cis*-verbenol were obtained: chemical purity 99.6%, (*S*)/(*R*) 84.0/15.6. $^1$H NMR ($CDCl_3$, 250 MHz): δ 5.37 (m, $J_{H1-H3}$ = 1.2, $J_{H3-H5}$ = 1.9, $J_{H3-H10}$ = 1.7 Hz, H-3), 2.45 (1H, ddd, $J_{H1-Ha7}$ = 5.3, $J_{H5-Ha7}$ = 6.2, $J_{Ha7-Hb7}$ = 8.9 Hz, $H_a$-7), 2.29 (ddd, $J_{H1-H5}$ = 5.8, $J_{H3-H5}$ = 1.9, $J_{H5-Ha7}$ = 6.2 Hz, H-5), 1.97 (ddd, $J_{H1-H3}$ = 1.2, $J_{H1-H5}$ = 5.8, $J_{H1-Ha7}$ = 5.3 Hz, H-1), 1.72 (3H, d, $J_{H3-H10}$ = 1.7 Hz, H-10), 1.65 (s, O*H*), 1.35 (3H, s, H-8), 1.31 (d, $J_{Ha7-Hb7}$ = 8.9 Hz, $H_b$-7), 1.08 (3H, s, H-9). The shifts and coupling constants are somewhat different from the one reported for nonlabeled *cis*-verbenol.[28] The $^{13}$C NMR shifts obtained are in good accordance with the shifts of the nonlabeled *cis*-verbenol previously described.[29] The only difference is that there is a triplet instead of a singlet at 73.1 ppm due to the deuterium in position 4. MS, *m*/*z* (%): 153 $[M]^+$ (0.3), 138 (13.1), 120 (34.0), 110 (70.1), 95 (100.0), 82 (38.6), 69 (24.4), 59 (65.4), 41 (53.9).

**(1*S*)-Pinocarvone.** The title compound was synthesized from (1*S*)-(−)-β-pinene via *trans*-pinocarveol by modifications of published procedures.[21−23] Thus *tert*-butyl hydroperoxide (6.0 mL 70%) was added dropwise to a stirred mixture of β-pinene (5.0 mL), distilled water (5.0 mL), and $SeO_2$ (149 mg). After addition the mixture was maintained at 40 °C for 4 h. After addition of water (10 mL) the solution was extracted with 3 × 20 mL of diethyl ether. The combined ether extracts were washed with HCl (aq, 0.1 M, 20 mL), $Na_2CO_3$ (aq, 10%, 20 mL), brine (20 mL), and water (20 mL). After drying ($MgSO_4$) and filtration, the solvent was evaporated to give an oil (5.61 g). The product was purified through flash column chromatography on silica gel (146 g, Fluka 60 Mesh) using gradient elution with increasing concentration of ethyl acetate in

cyclohexane. The crude *trans*-pinocarveol was oxidized in two batches (one 1.0 g and one 3.0 g) by dropwise addition to a suspension of 15 equiv of $MnO_2$ (15 equiv) in dichloromethane (25 mL/g starting material). After reaction for 6 h the solution was filtered with Celite. The two filtrates were combined and concentrated to give (1*S*)-pinocarvone (2.9 g).

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## References and Notes

(1) Berger, R. G. *Aroma Biotechnology*; Springer-Verlag: Berlin, 1995; pp 4, 36.
(2) Lindmark, M.; Sjödin, K.; Högberg, H.-E. *Fine chemicals from turpentine. Chemical analyses of turpentine and press water from the thermomechanical pulping process.* SCA Report No. F2384, 1998.
(3) Agrawal, R.; Deepika, N.-U.-A.; Joseph, R. *Biotechnol. Bioeng.* **1999**, *63*, 249−252.
(4) Busmann, D.; Berger, R. G. In *Trends in Flavour Research*; Maarse, H., van der Heij, D. G., Eds.; Elsevier Science B.V.: Amsterdam, 1994; pp 503−507.
(5) Draczyńska-Lusiak, B.; Siewiński, A. *J. Basic Microbiol.* **1989**, *29*, 269−275.
(6) Hagedorn, S.; Kaphammer, B. *Annu. Rev. Microbiol.* **1994**, *48*, 773−800.
(7) Corbier, B.; Ehret, C. In *Flavors and Fragrances: A World Perspective;* Proceedings 10th International Congress of Essential Oils, Fragrances and Flavors, November 16−20, Washington, DC.; Lawrence, R. M., Mookherjee, B. D., Willis, B. J. Eds.; Elsevier Science Publishers B.V.: Amsterdam, 1986; pp 731−741.
(8) Hirata, T.; Ikeda, Y.; Izumi, S.; Shimoda, K.; Hamada, H.; Kawamura, T. *Phytochemistry* **1994**, *37*, 401−403.
(9) Vaněk, T.; Macek, T.; Stránský, K.; Ubik, K. *Biotechnol. Tech.* **1989**, *3*, 411−414.
(10) Vaněk, T.; Valterová, I.; Pospísilová, R.; Vaisar, T. *Biotechnol. Tech.* **1994**, *8*, 289−294.
(11) Bhattacharyya, P. K.; Prema, B. R.; Kulkarni, B. D.; Pradhan, S. K. *Nature* **1960**, *187*, 689−690.
(12) Rama Devi, J.; Bhattacharyya, P. K. *J. Indian Chem. Soc.* **1978**, *LV*, 1131−1137.
(13) Wright, S. J.; Caunt, P.; Carter, D.; Baker, P. B. *Appl. Microbiol. Biotechnol.* **1986**, *23*, 224−227.
(14) Hamada, H. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 869−878.
(15) Takeya, K.; Itokawa, H. *Chem. Pharm. Bull.* **1977**, *25*, 1947−1951.
(16) Lindgren, B. S.; Borden, J. H. *Can. J. For. Res.* **1993**, *23*, 286−290.
(17) Miller, D. R. *Can. Entomol.* **2000**, *132*, 789−797.
(18) Pureswaran, D. S.; Gries, R.; Borden, J. H.; Pirce, H. D., Jr. *Chemoecology* **2000**, *10*, 153−168.
(19) Schlyter, F.; Birgersson, G.; Byers, J. A.; Löfqvist, J.; Bergström, G. *J. Chem. Ecol.* **1987**, *13*, 701−716.
(20) Kwong, H.-L.; Wong, W.-L.; Lee, W.-S.; Cheng, L.-S.; Wong, W.-T. *Tetrahedron: Asymmetry* **2001**, *12*, 2683−2694.
(21) Mitchell, P. W. D. (International Flavors and Fragrances Inc.). Patent No. FR2009498, 1970.
(22) Lempers, H. E. B.; Ripollès i Garcia, A.; Sheldon, R. A. *J. Org. Chem.* **1998**, *63*, 1408−1413.
(23) Garsky, V.; Koster, D. F.; Arnold, R. T. *J. Am. Chem. Soc.* **1974**, *96*, 4207−4210.
(24) Bovara, R.; Carrea, G.; Ferrara, L.; Riva, S. *Tetrahedron: Asymmetry* **1991**, *2*, 931−938.
(25) Gupta, P. K.; Durzan, D. J. *Bio-Technology* **1986**, *4*, 643−645.
(26) Mechoulam, R.; Braun, P.; Gaoni, Y. *J. Am. Chem. Soc.* **1972**, *94*, 6159−6165.
(27) Mori, K.; Mizumachi, N.; Matsui, M. *Agric. Biol. Chem.* **1976**, *40*, 1611−1615.
(28) Badjah-Hadj-Ahmed, A. Y.; Meklati, B. Y.; Waton, H.; Pham, Q. T. *Magn. Reson. Chem.* **1992**, *30*, 807−816.
(29) Coxon, J. M.; Hydes, G. J.; Steel, P. J. *J. Chem. Soc., Perkin Trans. 2* **1984**, 1351−1355.

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