Biosynthesis of (+)- and (−)-Germacrene D in *Solidago canadensis*: Isolation and Characterization of Two Enantioselective Germacrene D Synthases**

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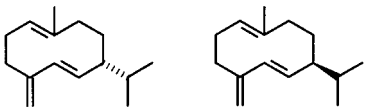
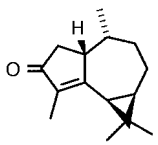
Sesquiterpenes are synthesized by plants, fungi, bacteria, and marine invertebrates.[1] Especially the oxygenated sesquiterpenes are important fragrances,[2] and they also act as antibiotics,[3] insect repellents, and pheromones;[4, 5] they are also produced in response to attack by microorganisms.[6] The biosynthesis of sesquiterpenes is initiated by cyclization of farnesyl diphosphate (FPP) and catalyzed by synthases (cyclases).[7] Most sesquiterpenes are chiral, and plants usually synthesize only one of the two enantiomers. However, sometimes both enantiomers of a sesquiterpene are present in the same plant.[8] Germacrene D is considered an important intermediate in the biosynthesis of sesquiterpenoid compounds.[9] Most higher plants produce only (−)-germacrene D (**1b**), but the essential oils of various *Solidago* species contain similar amounts of both (+)- (**1a**) and (−)-germacrene D (**1b**).[10, 11] Furthermore, germacrene D is not a precursor for oxygenated products in these plants.



1a **1b**

Our aim was to investigate whether one or two synthases are involved in the biosynthesis of **1a** and **1b** in *S. canadensis*. The major constituents of the essential oil of *S. canadensis* are **1a** and **1b**, and in many cases cyclocolorenone (**2**). The enantiomers **1a** and **1b** can be resolved and quantified by enantioselective gas chromatography[12] (Figure 1a).



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For the isolation of the germacrene D synthases, enzyme extracts were prepared and incubated with radiolabeled FPP. Product formation was monitored by radio gas chromatography (Figure 1b–d).

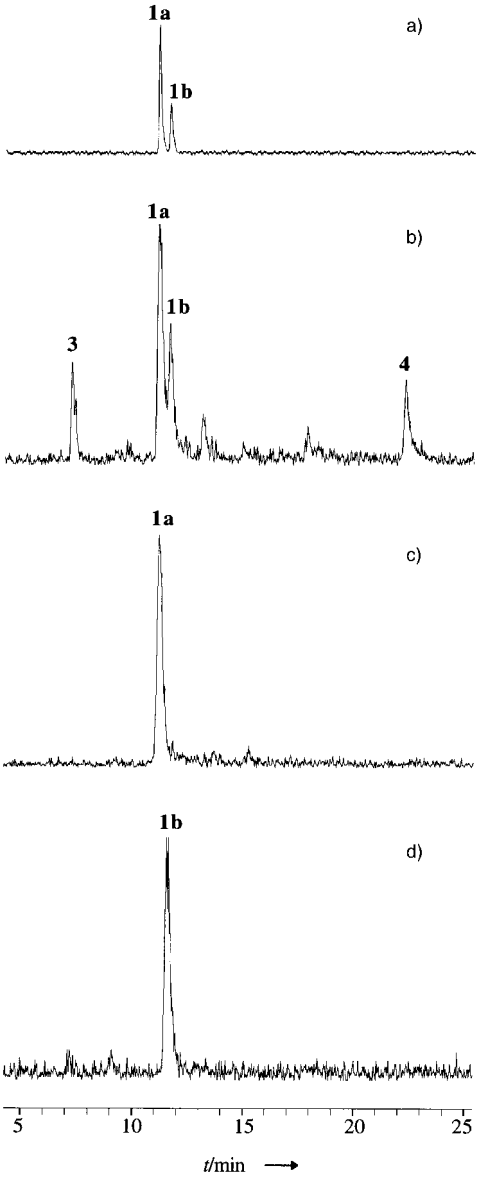
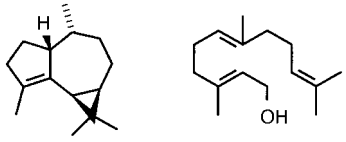


Figure 1. Gas chromatograms on a 25 m fused silica capillary column with heptakis(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (50 wt % in OV 1701); temperature program: 116°C for 14 min, heating to 170°C at 15°C min^{-1}, 20 min at 170°C; flame ionization (a) or radioactivity detector (b–d). a) Separation of a (+)/(−)-germacrene D standard (obtained by preparative GLC from the essential oil of *S. canadensis*); b) Incubation products of the crude enzyme extract (assay 2); c) [^{3}H](+)-germacrene D as the incubation product of the purified (+)-germacrene D synthase (assay 2); d) [^{3}H](−)-[^{3}H]germacrene D as the incubation product of the purified (−)-germacrene D synthase (assay 2).

Incubation of the crude enzyme extract with (*E*,*E*)-[^{3}H]FPP gave four major products: enantiomerically pure (−)-α-gurjunene (**3**), **1a**, **1b**, and (*E*,*E*)-farnesol (**4**) (Figure 1b). Since (−)-α-gurjunene occurs only in small amounts in



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[**] This work was supported by a grant from the Fonds der Chemischen Industrie.

  1433-7851/98/3710-1400 $ 17.50+.50/0

essential oils of *S. canadensis*, the high (−)-α-gurjunene content of the crude enzyme extract suggests that it is the in vivo precursor of **2**. The formation of farnesol is due to the high phosphohydrolase activity of the crude enzyme extract.

The enzymes were isolated by ion-exchange chromatography (DEAE-52), dye–ligand chromatography (Red 120), and fast protein liquid chromatography (FPLC) on a MonoQ column. The final purification step yielded two active fractions. On incubation with [^{3}H]FPP, one fraction produced only **1a** (Figure 1c), while the other produced only **1b** (Figure 1d). Both enzymes were purified by a factor of 123 (Table 1). These results prove that the biosynthesis of **1a** and **1b** in *S. canadensis* is controlled by two enantioselective synthases. For the first time it was shown that

Table 1. Isolation of the (+)- and (−)-germacrene D synthases.

Fractionation step	Total protein [mg]	Total activity [nmol h^{-1}]	Specific activity [nmol h^{-1} mg^{-1}]	Yield [%]	Purification
crude enzyme extract	63.2	834.1	13.20	100	1.0
DEAE-52 eluate	7.1	714.9	100.7	86	7.63
Dye Red 120	1.5	226.9	151.3	27	11.5
MonoQ					
(+)-germacrene D synthase	0.04	64.7	1618	7.8	123
(−)-germacrene D synthase	0.06	97.3	1622	12	123

one plant species contains two enzymes for the biosynthesis of sesquiterpene enantiomers. Previously the involvement of separate enzymes in the formation of enantiomers has only been demonstrated in the biosynthesis of monoterpenes.[13]

Analytical SDS-PAGE gel electrophoresis showed the molecular mass of both synthases to be 51 kDa. The native molecular mass of both synthases as determined by gel filtration[14] was also 51 kDa. Thus, both synthases appear to be monomers. The optimum pH for (+)-germacrene D synthase activity was 7.0, and for (−)-germacrene D synthase 7.1. The K_M values for (*E*,*E*)-[^{3}H]FPP were 9.97 μM for (+)-germacrene D synthase and 4.24 μM for (−)-germacrene D synthase. The results are comparable to those reported for other sesquiterpene synthases.[15]

The cyclization mechanisms of the two synthases are still unknown. One hypothesis[10] is that (*E*,*E*)-FPP is cyclized to a germacrenyl cation, which can undergo a 1,3-hydride shift to give (**1b**) or a double 1,2-hydride shift to form **1a**. Other authors[16] demonstrated that (2*Z*,6*E*)-FPP is a substrate for the biosynthesis of **1a** in the liverwort *Heteroscyphus planus*. We have now shown that in *S. canadensis*, (*E*,*E*)-FPP is the substrate for the formation of both **1a** and **1b**.

Further investigations are aimed at establishing the cyclization mechanism for both synthases by using deuterium-labeled FPP and ^{2}H NMR spectroscopy[17] and mass spectrometry and at determining the primary structure of both enzymes.

Experimental Section

[^{3}H]FPP was obtained from Amersham (England). During enzyme isolation and preparation of the assays all operations were carried out on ice or at 4°C.

Crude enzyme extract:[18] 15 g of young leaves of an *S. canadensis* plant (collected near Hamburg, Germany) and 1.5 g polyvinylpolypyrrolidone (PVPP, Sigma) were ground in buffer A (25 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (Mopso, Sigma), 25 mM $Na_2S_2O_5$, 10 mM $MgCl_2$, 5 mM dithiothreitol, 25 mM ascorbic acid, 20% (v/v) glycerol, pH 7.0). The extract was carefully stirred with 7.5 g XAD-4 resin and then filtered through cheesecloth. The filtrate was centrifuged at 20000 *g* for 20 min (pellet discarded), and then at 100000 *g* for 90 min. The 100000 *g* supernatant was purified further.

Ion-exchange chromatography on diethylaminoethyl cellulose (DEAE, preswollen DE-52, Whatman):[18] The 100000 *g* supernatant was loaded onto a DEAE-52 column that had been preequilibrated with buffer B (15 mM Mopso, 10 mM $MgCl_2$, 2 mM $Na_2S_2O_5$, 10% (v/v) glycerol, pH 7.0). The column was eluted with a linear gradient of 0.0–0.5 M KCl in buffer B. Fractions were collected and assayed for cyclase activity (assay 1). The active fractions were combined and desalted in buffer C (15 mM Mopso, 10 mM $MgCl_2$, 10% (v/v) glycerol, pH 7.0) on an Econo-Pac 10 DG column (Biorad).

Dye–ligand chromatography:[19] The desalted DEAE-52 eluent was loaded onto a Reactive Red 120 column (Sigma) that had been preequilibrated with buffer C. The column was then eluted with 1.5 M KCl in buffer C. Active fractions were combined and desalted in buffer C.

FPLC on MonoQ:[18b] The desalted Red 120 eluate was loaded onto a MonoQ column (Pharmacia) that had been preequilibrated with buffer C. The column was eluted with a linear gradient of 0.0–0.5 M KCl in buffer C. Both active fractions were used separately for the enzyme characterizations.

Assay 1:[18] 20 μL enzyme preparation was diluted with 80 μL buffer C, and 20 μM [^{3}H]FPP (50 Ci mol^{-1}) was added. The reaction mixture was overlaid with 1 mL of hexane. After incubation for 30 min at 30°C, the reaction was stopped. The hexane phase was mixed with 40 mg of silica gel to remove farnesol, centrifuged, and an aliquot used for liquid scintillation counting.

Assay 2:[18] 1 mL of enzyme preparation was mixed with 20 μM [^{3}H]FPP (50 Ci mol^{-1}), layered with 1 mL of pentane, and incubated for 60 min at 30°C. The reaction mixture was extracted with diethyl ether, and the organic phase was removed and passed through a short column of aluminum oxide. The mixture was slowly concentrated under a stream of N_2 and used for radio-GLC analysis.

Radio-GLC[18a, 20] was performed on a Carlo-Erba 4160 Series gas chromatograph equipped with a RAGA 93 radioactivity detector (Raytest, Straubenhardt, Germany).

Received: December 11, 1997 [Z11251IE]
German version: *Angew. Chem.* **1998**, *110*, 1479–1481

Keywords: enantiomers · enzyme catalysis · gas chromatography · sesquiterpenes

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Combinatorial Solid-Phase Synthesis of Structurally Complex Thiazolylhydantoines**

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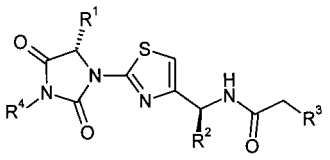
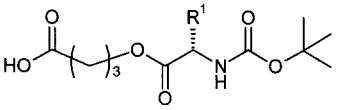
In the past few years combinatorial chemistry[1] and the related solid-phase synthesis[2] of nonpeptide substance libraries have become established as valuable tools in pharmaceutical research for the discovery and optimization of lead compounds. We have developed a synthetic strategy that allows efficient construction by solid-phase synthesis of libraries of structurally complex thiazolylhydantoines of the general formula **1**. At the same time, exploitation of an intrinsic purification principle has led to products with excellent purity after nine synthetic steps and subsequent removal of the target from the polymer support.



1

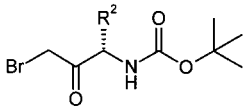
Derivatives **2a – c**,[3] prepared from amino acids with *N*-butoxycarbonyl (Boc) protecting group, served as building blocks for the synthesis of a representative substance library



2a $R^1 = CH_2C_6H_5$

2b $R^1 = CH_2$-cyclo-C_6H_{11}

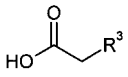
2c $R^1 = CH_2C(O)N(CH_2CH_2)_2O$



3a $R^2 = H$

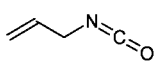
3b $R^2 = CH_3$

3c $R^2 = CH_2CH(CH_3)_2$



4a $R^3 = 4\text{-}FC_6H_4$

4b $R^3 = H_3CC_6H_4O$



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(note the strategic importance of the ester functionality for detachment of the target compounds from the polymer support). Other key components were α-bromoketones **3a – c**[4] derived from *N*-Boc-protected amino acids, carboxylic acids **4a**, **b**, and allyl isocyanate (**5**), which can be seen as a representative of isocyanates in general.

In a first reaction step, building blocks **2a – c** were linked through their free acid functionalities to a benzhydrylamine resin modified with 6-aminohexanoic acid. 1,1,3,3-Tetramethyl-*O*-(2-oxo-1,2-dihydropyridin-1-yl)uronium tetrafluoroborate (TPTU)[5] served as the coupling reagent to give derivatives **7a – c**. After cleavage of the Boc protecting group, free amines **8a – c** were transformed by reaction with allyloxycarbonyl isothiocyanate (**9**)[6] into the thiourea derivatives **10a – c**, which contain the allyloxycarbonyl (Alloc) protecting group. Pd^0 catalyzed removal of the Alloc group[7] yielded thioureas **11a – c** as starting materials for the subsequent construction of 2-aminothiazole templates (Scheme 1).

2-Aminothiazoles **12aa – 12cc** were obtained by the reaction of aliquot portions of resins **11a – c** with α-bromoketones **3a – c**. Free amines **13aa – 13cc** were isolated after removal of the Boc protecting group. Once again, aliquots of resins **13aa – 13cc** were coupled through their amino groups with carboxylic acids **4a**, **b** to give amides **14aaa – 14ccb**. Preliminary experiments in solution had shown that coupling with carboxylic acids takes place exclusively at the primary amino group. Under the chosen reaction conditions the amino group attached to the heterocycle does not react with carboxylic acids. Reaction of compounds **14aaa – 14ccb** with allylisocyanate (**5**) led finally to urea derivatives **15aaa – 15ccb**, which were removed from the polymeric supports by a base-induced cyclization reaction specific to the ester function (the importance of which was alluded to earlier)[8] to give the

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[**] We would like to thank Dr. D. Obrecht and A. Trzeciak for helpful discussions, as well as S. Heulle-Décard and S. Ulsemer for excellent technical assistance.

  1433-7851/98/3710-1402 $ 17.50+.50/0