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Kinetic Studies on the *Cis–Trans* Isomerization of Farnesol by Liver Alcohol Dehydrogenase

CARMEN CAPELLINI, ATTILIO CORBELLA, PIERLUIGI GARIBOLDI,
AND
GIANCARLO JOMMI

Laboratorio di Chimica Organica, Facoltà di Scienze, Università degli Studi, Via Saldini, 50, 20133 Milano, Italy

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The isomerization of 2-*trans*,6-*trans* farnesol to the 2-*cis*,6-*trans* isomer proceeds through the corresponding aldehydes. The rate of the reaction in the presence of liver alcohol dehydrogenase (LADH) and diaphorase has been compared with the rates of the isomerization of 2-*trans*,6-*trans* farnesal with different catalysts (OH^-, H^+, –SH diaphorase alone, albumin). The results are in keeping with enzymatic catalysis by LADH.

INTRODUCTION

During studies on the biosynthesis of sesquiterpenoids with the picrotoxane skeleton (*1*) we were faced with the problem of the geometrical isomerism of the open chain precursor, namely 2-*trans*,6-*trans* farnesol (I) or 2-*cis*,6-*trans* farnesol (II). The problem is not limited to the aforementioned class but is of general interest. In the review of Parker, Roberts, and Ramage (*2*) on sesquiterpenoid biosynthesis, there is a clear cut separation between the compounds derived from *trans,trans* farnesol and those for which *cis,trans* farnesol is supposed to be the precursor. This assumption is based mainly on the geometrical limitation of a six membered carbocyclic ring to accommodate a *trans* double bond. The group of the so called "sesquiterpenoids derived from 2-*cis*, 6-*trans* farnesol" includes the bicyclic skeletons of cadinanes, muurolanes, amorphanes, bulgaranes, and all the structures derived from these by further cyclization (longifolene, sativene, copaene, etc.) or by cyclization and oxidative rearrangement (helmintosporal, picrotoxanes, etc.).

Up to 1970 (*3*), the stereospecific retention of either of the prochiral hydrogens at C-4 of mevalonic acid was accepted as a discriminating criterion of the geometrical isomerism in an isoprenoid chain: a *trans* double bond follows the retention of the 4-*pro*-R hydrogen, whereas a *cis* double bond derives from the loss of the 4-*pro*-R hydrogen. However, this assumption has been contradicted by recent results for mono (*4*) and sesquiterpenoids (*5*), where the presence of a *cis* double bond is associated with the loss of the 4-*pro*-S hydrogen of mevalonic acid. This finding may be interpreted in one of the two ways suggested by Overton and Roberts (*6*): (a) Two different enzymatic systems with different stereospecificity govern the formation of a *cis* double bond in the coupling of two isoprenic units. (b) The *trans* isomer (e.g., *trans-trans* farnesol) is formed first

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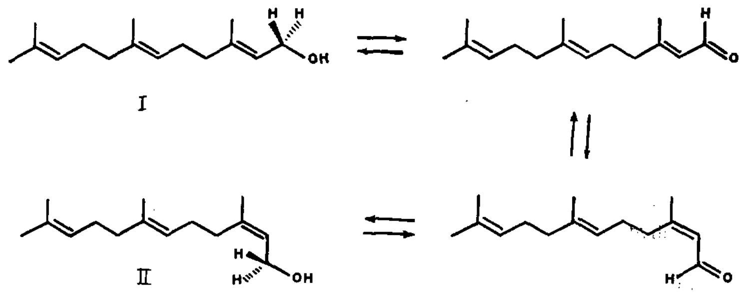
with the normal loss of the 4-*pro*-S hydrogen and is then isomerized to the *cis* one (*cis,trans* farnesol) with retention of the vinyl proton.

Actually, the isomerization of the farnesol *trans* double bond to *cis* has been verified in some biological systems, including cell-free systems from Andrographis paniculata (*7*), Orange flavedo (*8*), and Trichotecium roseum (*9*). In these systems the isomerization occurs after oxidation of the allylic alochol to the α,β-unsaturated aldehyde. A direct isomerization of geraniol to nerol, with no intermediacy of the aldehydes, has been observed with cell-free extracts of peppermint and carrots (*10*).



The interconversion of *cis,trans*- and *trans,trans*-farnesol has also been reported (*7*) to occur in the presence of an isolated enzyme, liver alcohol dehydrogenase (LADH) and in this case the reaction is obviously mediated by the aldehydes. The aim of this work was to establish whether the enzyme simply catalyzes the red-ox equilibrium, or if it intervenes also in the isomerization of the conjugated double bond of *cis,trans*-farnesal and *trans,trans*-farnesal.

MATERIALS AND METHODS

LADH (EC 1.1.1.1) (10 mg/ml) and diaphorase (EC 1.6.4.3) (5 mg/ml) were obtained from Boehringer, Mannheim, as water suspension and dialyzed against 300 volumes of 0.1 *M* phosphate buffer for 15 hr at 4°C. The enzymes were used within 48 hr after the dialysis. The activity for LADH was 2.5 U/mg and for diaphorase 200 U/mg (with lipoic amide). NADH and NAD^+ were purchased from the same company as crystalline compounds. Bovine serum albumin was obtained from Merck.

A commercial farnesol mixture (10 g) was separated on a silica gel column (1200 g) using Pet.ether–AcOEt (9:1, v:v) as eluting solvent. 2-*trans*,6-*trans* farnesol, distilled before incubation, contained 3% of the 2-*cis*,6-*trans* isomer. Oxidation of 2-*trans*, 6-*trans* farnesol with MnO_2 in hexane according to the method described (*11*) for nerol afforded the corresponding aldehyde. 2-*trans*,6-*trans* farnesal was distilled immediately before use and was contaminated by 8% of the 2-*cis*,6-*trans* isomer.

The quantitative analysis of the isomeric mixtures of farnesols and farnesals obtained from incubation as well as the control of starting materials were done by glc on a dual column FID instrument using N_2 (40 ml/min) as carrier gas; oven temperature 180°C. Glass columns (2 m × 3 mm) were packed with 10% Carbowax 20 *M* on Chromosorb;

retention times for *cis,trans*- and *trans,trans*-farnesal, *cis,-trans* and *trans,trans*-farnesol were 7, 8.2, 9.5, and 10.8 min, respectively.

Incubation Conditions

All the incubations were done at 35°C with magnetic stirring. The experiments can be divided into two groups: (a) incubations in homogeneous phase, and (b) incubations in heterogeneous phase.

The experiments of group (a) were carried out by dissolving 10 mg of 2-*trans*,6-*trans*-farnesal in 5 ml of 1:1 (v:v) mixture of water buffer (pH 2–10) and an organic solvent (dioxane, CH_3CN, DMF). One-half-milliliter samples were taken at appropriate intervals, diluted with 2.5 ml of water and extracted with 0.5 ml of hexane; 1 μl of the hexane solution was injected in the glc column.

The buffer-organic solvent mixture were left 48 hr before addition of farnesal. During this time the pH changed slightly until it reached a stable value (unchanged after two weeks); pH values reported for these mixtures are apparent, as measured by the glass electrode in 50% aqueous medium. Water buffers were 0.1 *M* phthalate (pH 2.4–5.8), 0.1 *M* phosphate (pH 5.8–8) and 0.1 *M* borax (pH 8–10.8).

The experiments of group (b) were carried out by dissolving 2 mg of 2-*trans*,6-*trans* farnesol or 2-*trans*,6-*trans* farnesal and 4 mg of Tween 80 in 2 ml of acetone; substitution of Tween 80 with Tween 60 did not alter the kinetic data. The solvent was evaporated with a stream of nitrogen, and the residue was taken up into 1 ml of 0.1 *M* phosphate buffer (pH 8) containing NADH (0.340 mg), NAD^+ (0.340 mg), EDTA (0.660 mg), albumin (1.32 mg). After addition of 0.4 ml of LADH solution and 0.2 ml of diaphorase solution the mixture was magnetically stirred and 0.1-ml aliquots were withdrawn at proper intervals.

In the experiments where some of the components of the complete system were omitted, the remaining species were kept at the same concentration described above. In the isomerization experiments at different pH, the mixture of farnesal (2 mg) and Tween (4 mg) was taken up into 1 ml of the appropriate buffer.

RESULTS

Simon (*12*) has reported a useful method for stereospecific labeling of large amounts of primary alcohols by means of deuterated or tritiated water, thus avoiding the use of the very expensive deuterated or tritiated NADH as label donor. The method is based on the coupling of two enzymes, yeast alcohol dehydrogenase and diaphorase and requires only catalytic amounts of NADH and NAD^+. Overton (*6*) has applied this reaction to prepare farnesols stereospecifically labelled at C-1, using liver alcohol dehydrogenase as the dehydrogenase from yeast does not operate on isoprenic alcohols.

We have repeated the reaction using both 2-*trans*,6-*trans* farnesol and 2-*trans*, 6-*trans* farnesal, following the evolution of product during 24 hr. The results are reported in Fig. 1(a) and 1(b), respectively, and are expressed in percentage of the indicated compound of the total amount of C-15 compounds (alcohols and aldehydes).

The reaction with 2-*trans*,6-*trans* farnesol was performed also in a N_2 atmosphere, and the results are summarized in Fig. 1(c). Comparison of the data of Fig. 1(a) and 1(c) clearly shows that the amount of aldehyde formed is much lower when operating with exclusion of O_2.

In a further experiment, 2-*trans*,6-*trans* farnesal was incubated in the presence of LADH, with cofactors, albumin and EDTA, but excluding diaphorase (Fig. 1(d)). After 24 hr about 1 % of 2-*trans*,6-*trans* and 4 % of 2-*cis*,6-*trans* farnesol were present.

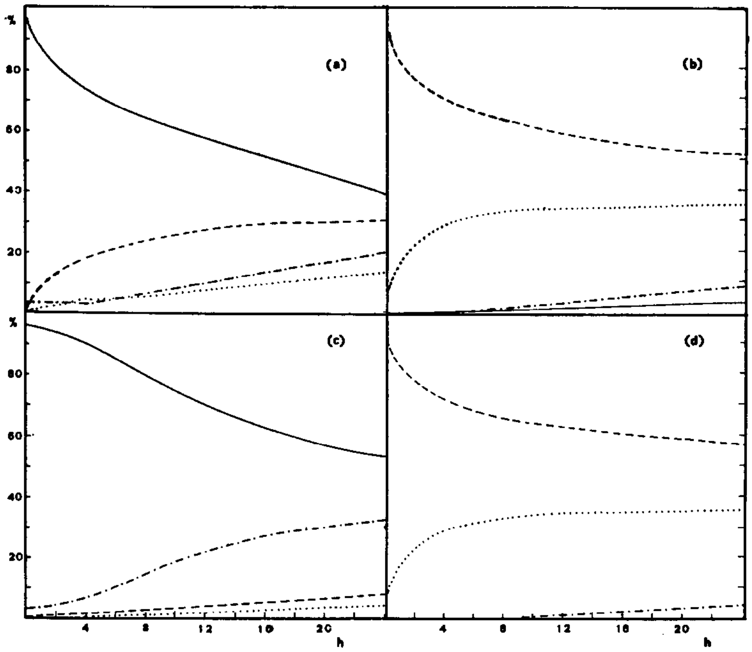
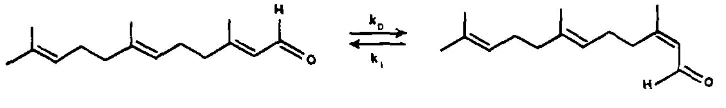


FIG. 1. (a) Incubation of 2-*trans*,6-*trans* farnesol (2 mg) with LADH (0.4 ml, 10 U), diaphorase (0.2 ml, 200 U with lipoic amide, phosphate buffer (1 ml, 0.1 *M*, pH 8) containing NADH (0.340 mg), NAD$^+$ (0.340 mg), EDTA (0.660 mg) and albumin (1.32 mg). (——) 2-*trans*,6-*trans* farnesol; (—·—) 2-*cis*,6-*trans* farnesol; (---), 2-*trans*,6-*trans* farnesal; (· · ·), 2-*cis*,6-*trans* farnesal. (b) Incubation of 2-*trans*,6-*trans* farnesal under the same conditions as (a). (c) Incubation of 2-*trans*,6-*trans* farnesol under the same conditions as (a) under N_2 atmosphere. (d) Incubation of 2-*trans*,6-*trans* farnesal: same conditions as (a) with the exclusion of diaphorase.

As the isomerization of a double bond conjugated with an aldehyde also can be effected by a chemical catalysis, both by electrophiles (H^+) or nucleophiles (OH^-, RS^-, etc.), we have examined the possibility that one of these species present in the incubation mixture is responsible of the *cis-trans* isomerization of farnesal. The effect of pH was first investigated treating 2-*trans*,6-*trans* farnesal with different buffers. For pH values between 4 and 10, no appreciable isomerization was noticed after 4 days. At pH 2.4, the reaction was still very slow, with $k_D = 5 \cdot 10^{-5}$ min^{-1} corresponding to an increment of few percent after 24 hr.

To avoid the problems connected with the heterogeneity of the system, we have repeated the above experiments at different pH after addition of an organic solvent. The water suspension of farnesal can be clarified only with high amounts of organic solvent, and our measurements were done using 1 : 1 mixtures of H_2O/CH_3CN, H_2O/DMF, H_2O/Dioxane. The results are reported in Fig. 2.



In a further series of experiments, we have measured the isomerization rate of 2-*trans*,6-*trans* farnesal in the presence of the different species used in the enzymatic reaction. With EDTA, NADH, and NAD^+, no isomerization was observed. The results

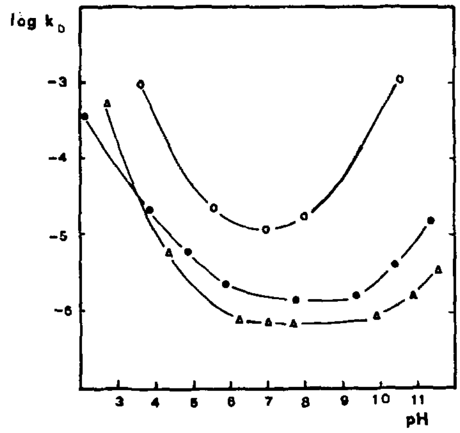


FIG. 2. Isomerization rates of 0.01 *M* solution of 2-*trans*,6-*trans* farnesal in CH_3CN/H_2O (1 : 1) (●), in DMF/H_2O (1 : 1) (○), in dioxane/H_2O (1 : 1) (△) at different pH; apparent pH values as measured with a glass electrode in 50 % aqueous medium.

of the separate incubations of farnesal with diaphorase and albumin are reported in Fig. 3 in comparison with the complete system; the rate of the isomerization in the presence of these two proteins is identical, but much lower than the rate measured for the

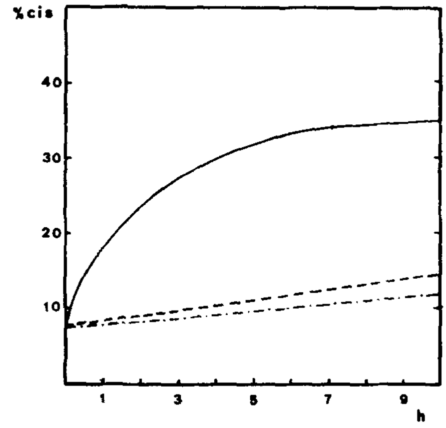


FIG. 3. Isomerization of 2-*trans*,6-*trans* farnesal (2 mg) in 0.1 *M* phosphate buffer (1 ml, pH 8) in the presence of (a) albumin (1.32 mg) or diaphorase (0.2 ml) (— · —), (b) boiled LADH (0.4 ml) (– – –), and (c) with the complete system described for Fig. 1a (——).

system LADH-diaphorase-albumin. In the same Fig. 3, we have reported also the data for isomerization of 2-*trans*,6-*trans* farnesal in the presence of boiled LADH; after this treatment, the red-ox activity of the enzyme is lost, but the protein is still capable of catalyzing isomerization.

Finally, we have examined the influence of different concentrations of a thiol (2-mercaptoethanol) on the isomerization of 2-*trans*,6-*trans* farnesal. The results are shown in Fig. 4.

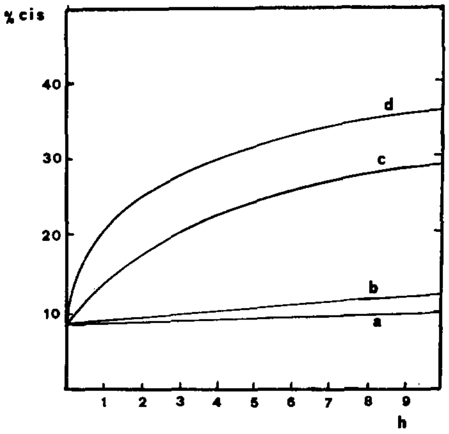


FIG. 4. Isomerization of 2-*trans*,6-*trans* farnesal (10 mg) in 0.1 *M* phosphate buffer (5 ml, pH 8) in the presence of (a) 4 mg, (b) 1.6 mg, (c) 0.16 mg, (d) 0.016 mg of mercaptoethanol.

In Fig. 5, a plot of $\log k_D$ versus p[SH] is reported, and it shows an acceptable degree of linearity in the field of concentrations examined. Of course the values of k_D are calculated on the basis of the variation of the relative percentage of the two isomers with time, and their validity owing to the heterogeneity of the system is strictly limited by the conditions employed.

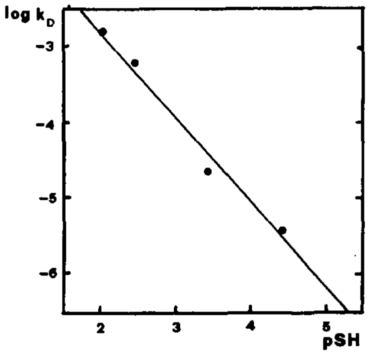


FIG. 5. Isomerization rates of 2-*trans*,6-*trans* farnesal in the presence of mercaptoethanol.

DISCUSSION

The results described above show that a number of different species catalyze the isomerization of farnesal (LADH, albumin, diaphorase, H^+, OH^-, RS^-), but the rate of the reaction varies with the catalyst. The complete system LADH-diaphorase, with cofactors and albumin causes isomerization at a relatively high speed starting either from farnesol and farnesal. As already stated, this is because of the prompt equilibration induced by the enzyme between alcohol and aldehyde. The complexity of the system where three different equilibrium reactions occur (red-ox of the *trans* isomers, *cis,trans* isomerization, red-ox of the *cis* isomers) and its heterogeneity do not allow the calculation of kinetic data of great validity. However, an approximate calculation based on the data from the incubation of farnesal with LADH alone (Fig. 1(d)) leads to an apparent rate constant of 10^{-3} min^{-1}. Under the same conditions the rate of reduction of *trans,trans* farnesal to the alcohol as measured fluorimetrically using the method described by Waller (*13*) is about a thousand times higher.

With the aldehyde as substrate the rate of isomerization is not influenced by the presence of diaphorase (Fig. 1(b) and 1(d)); the only difference between the two experiments is in the amount of alcohol formed. When LADH is coupled with diaphorase, about 13 % of alcohol (9 % *cis* and 4 % *trans*) is present after 24 hr, whereas with LADH alone only 5 % (4 % *cis* and 1 % *trans*) of aldehyde is reduced. These data can be rationalized by taking into account that in the incubation mixture, the molar percentage of NADH *versus* the aldehyde is about 5 % and that the equilibrium between alcohol and aldehyde is largely in favor of the former at pH 8; the presence of diaphorase increases the amount of alcohol by introducing other red-ox equilibria.

Isomerization of the double bond is slower when the alcohol is used as substrate. Much aldehyde is formed (Fig. 1(a)) when the reaction is run in the presence of oxygen, but the degree of oxidation is distinctly lowered by working in an inert atmosphere. With the aldehyde, *cis,trans* equilibrium is attained after 8–10 hr, whereas the alcohols take about 24 hr to equilibrate. This is easily understandable from the fact that the alcohol must be transformed into the aldehyde in order to undergo isomerization, and that the concentration of the latter is always low.

The experiments at different pH, both in water suspension or in organic solvents, clearly show that the isomerization of the conjugated double bond is subject to acid and base catalysis as one could have easily foreseen. In particular, when the reaction is run in solution, the reaction rates increase significantly at pH values below four and above 10, while catalysis from bases becomes more important in a solvent like DMF, which is known to enhance the reactivity of the anions. However, these results clearly show that reaction rates comparable to those of the enzymatic reaction can be obtained only at pH values far from the physiological range, which enables us to exclude the possibility that in the LADH-diaphorase system, the isomerization of farnesal is simply based on acid–base catalysis.

The kinetic data obtained in the presence of mercaptoethanol unambiguously show that the thiol group is a catalyst for the isomerization of farnesal. This is not surprising, as it is known (*14*) that conjugated double bonds can be isomerized by thiols, most probably through a reversible addition to the β position (*15*). More recently (*16*) the same compounds have been found to catalyze the geometrical isomerization of the isolated double bond of oleic acid. However, also in this case, the concentration of thiol that gives a reaction rate comparable to that of the enzymatic system is very high as compared with the "concentration" of SH groups, which can be calculated from the amount of enzyme used, its molecular weight ($\simeq$84 000) and the number of SH per mole.

The results obtained using diaphorase and albumin separately and using boiled LADH do not exclude the possibility that these proteins contribute to the isomerizing activity. However, the catalytic activity of the full system is so high with respect to the activity of the single proteins that it cannot be accounted for by the sum of the single contributions. In other words, the kinetic data suggest that we are not simply dealing with chemical catalysis in which the proteins play the role of carrier for active groups, but they show that LADH displays enzymatic catalysis also in the isomerization process.

Recent studies (*17*) on the structure of LADH have supported the hypothesis that an SH group of the enzyme is in the proximity of the active site; this fact combined with our experiments with mercaptoethanol suggest the possibility that in LADH a single coordination site can serve for both the red-ox reaction and the isomerization of the double bond, the latter being effected by the adjoining thiol group. The experiment with boiled LADH is in agreement with this interpretation: the red-ox catalysis which implies the stereospecific coordination of the substrate and of the coenzyme (NADH–NAD$^+$) is strictly dependent on the tertiary structure of the enzyme and is completely lost after denaturation. On the other hand, the isomerizing activity is severely lowered after this treatment, suggesting the importance of a proper coordination site also for this reaction; chemical catalysis by the thiol groups in the molecule can account for the residual activity of LADH after denaturation.

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