Review Letter

TRANSFORMATION OF ORGANIC COMPOUNDS BY MICROBIAL ENZYMES*

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1. Introduction

It is well known that micro-organisms are able to synthesize, transform and degrade substances which possess very complicated structures. The structural formulae of the primary and especially of the secondary metabolites, e.g. of the various antibiotics, demonstrate this statement in an impressive manner. Apart from these products, micro-organisms also produce very active enzyme systems which are capable of catalyzing chemical reactions with foreign substances when the latter are added to a culture solution in which the organism is grown. Ordinarily, these substances are not essential for the growth of the microorganism since sources of carbon and nitrogen are available in the nutrient. However, they can replace a normal nutritional chemical if they are not too toxic and if the concentrations are high enough. The microbial enzymes assume the role of chemical reagents characterized by a high degree of selectivity and stereospecificity. Thus, micro-organisms are frequently in a position to carry out conversions in a single step with high yields, whereas many complicated steps would be required using synthetic organic methods. The oldest of these reactions is perhaps the microbiological oxidation which has been used for centuries in the production of vinegar. This oxidation of ethanol to acetic acid by *Acetobacter aceti* has been investigated thoroughly by Louis Pasteur in 1864 [1]. Up to the middle thirties, microbiological reactions were primarily of academic interest. This situation changed when it was found that L-sorbose, which is required for the synthesis of vitamin C, can be produced commercially from D-sorbitol by oxidation with *Acetobacter suboxydans* [2] (fig. 1).

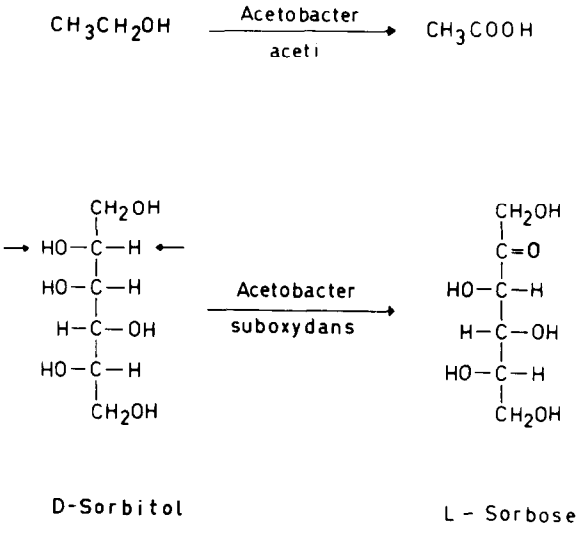


Fig. 1. Oxidation of ethanol to acetic acid and of D-sorbitol to L-sorbose.

Before the second world war the industrial microbiological production of citric acid and L-glutamic acid from D-glucose had been developed. The discovery of penicillin, the streptomycins and other antibiotics after the war led to the unprecedented development of chemical microbiology in both its scientific and industrial aspects. In the course of these investigations not only new mould metabolites, but also numerous types of reactions with microbial enzymes were discovered. They are tabulated in table 1 [3–5] (experimental methods [3–5]). Some types of reactions and their mechanistic aspects are illustrated

* Based on a paper presented at the 9th FEBS Meeting, Budapest, 25–30 August 1974.

Table 1
Types of Microbial Reactions

Oxidation	Acylation
Oxygenation of ⩾CH or ≻C=C≺	Transglycosidation
Dehydrogenation of ≻CHOH	Methylation
Dehydrogenation of ≻CH-CH≺	Condensation
	Cleavage of ⩾C-C⩽
Reduction	Decarboxylation
Hydrogenation of ≻C=O	Dehydration
Hydrogenation of ≻C=C≺	Amination/Deamination
Isomerization	Halogenation
Esterification/Hydrolysis	Phosphorylation

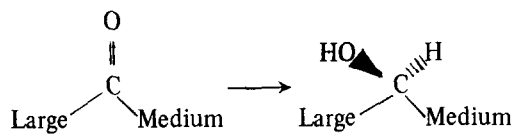
in the following sections by selected examples chosen either from the literature or our own work. A great number of these conversions have reached technical significance.

2. Decarboxylation

The selective and stereospecific decarboxylation of meso, but not of racemic, α,α-diamino pimelic acid, by *Bacillus sphaericus* permits a convenient synthesis of L-lysine using 2-ethoxy-3,4-dihydro-pyrane as starting material (fig. 2) [6]. After separation of L-lysine, the residual D,L-diamino pimelic acid is epimerized by heating with Dowex-50 (H^+-form) to yield a mixture of the D,L and meso acids. The latter is decarboxylated again.

3. Reduction

The reduction of ketones is stereoselective. Optically active alcohols which possess *S*-configuration are obtained in most cases. The reduction of racemic decalones and hexahydro-indanones by *Curvularia falcata* has yielded alcohols with *S*-configuration in 49 out of 50 cases according to the following scheme [7]:



Further examples of stereospecific reductions of bicyclo [2:2:1]heptanes will be discussed below.

Hydrogenation of olefinic double bonds is possible if they are activated by an adjacent oxygen function. This reaction has no preparative significance.

4. Cleavage of amides

The conversion of antibiotics, which are themselves

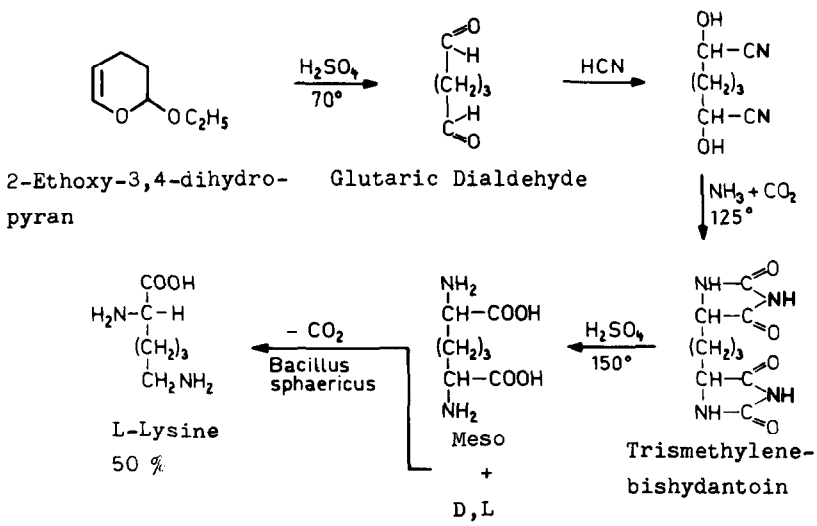


Fig. 2. Stereospecific decarboxylation: Synthesis of L-lysine.

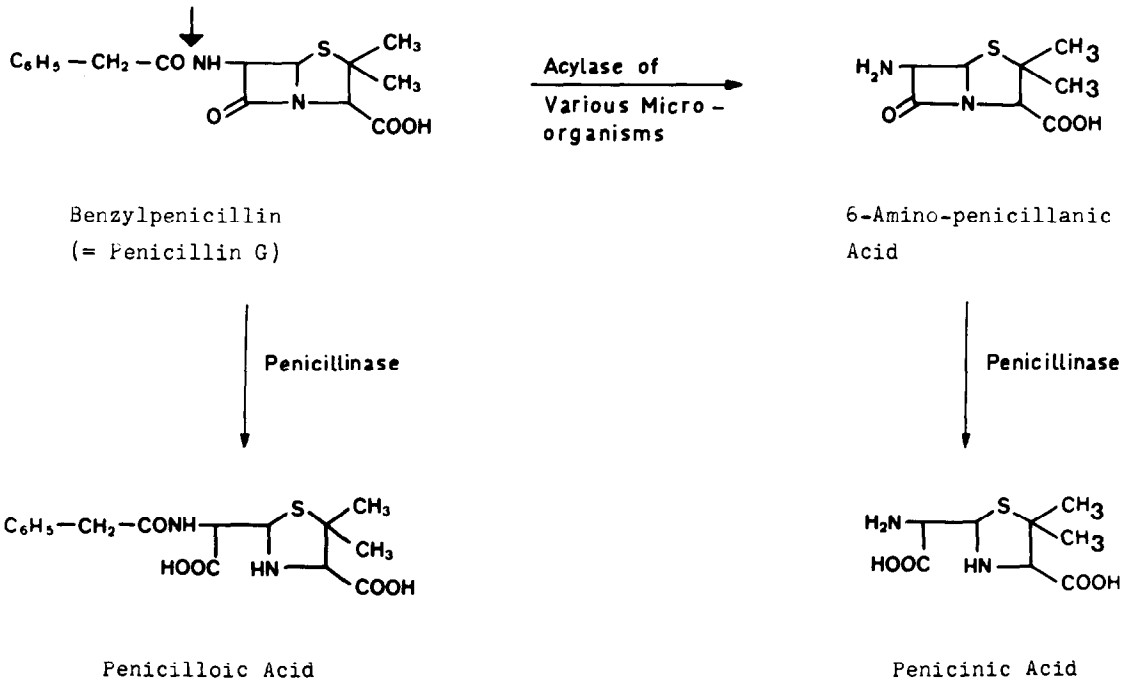


Fig. 3. Microbial transformation of benzylpenicillin (Penicillin G).

produced by micro-organisms, by enzymes of other micro-organisms has proved to be a significant discovery [8,9]. An important example with industrial relevance is the selective cleavage of the acyclic amide bond of the natural penicillins by acylases from Actinomycetes, fungi, yeasts and bacteria. This offers a productive method for preparing 6-amino-penicillanic acid from readily accessible penicillin G (fig. 3). In contrast to penicillinases, these acylases leave the β-lactam ring untouched [10,11]. The products obtained after opening of the β-lactam ring have lost their biological activity.

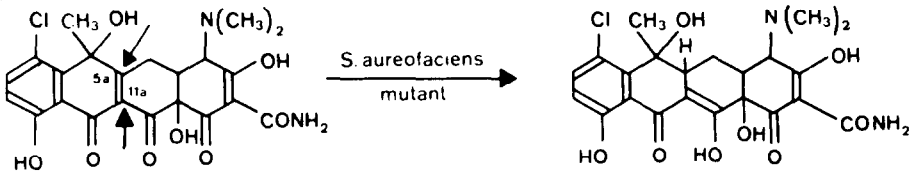
The amides of lysergic and isolysergic acid are hydrolyzed to the corresponding carboxylic acids in good yield by *Claviceps purpurea* [12].

5. Oxidation

The oxidation of primary alcohols to aldehydes and carboxylic acids is known, but of less practical significance than the selective oxidation of a hydroxyl group in polyhydroxy compounds. Selective oxidation of steroidal secondary hydroxyl groups to ketones represents an important application. Some of these alcohol dehydrogenases have been isolated in crystalline form and are the most studied enzymes. The reactions which they catalyse, are reversible and require NAD^+ or $NADP^+$ as hydrogen transfer agents [4]. The practical application of this reaction has been improved considerably by the development of the 'bioreactor' technique.

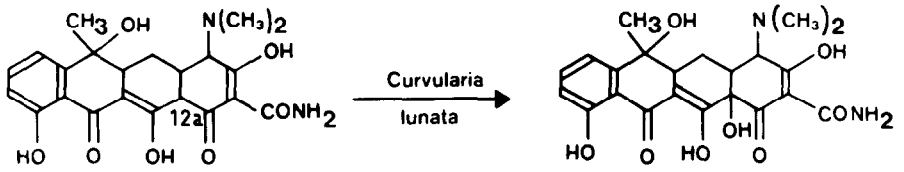
The introduction of a hydroxyl group by the direct replacement of a hydrogen atom of a $\geq$C-H group with retention of configuration has proved to be one of the most interesting microbiological conversions [14]. It can replace numerous complicated chemical operations by a single incubation under mild conditions. Such hydroxylations not only take place on activated carbon atoms, e.g. in allylic positions, as shown by the hydroxylation of 12α-deoxytetracycline to tetracycline by *Curvularia lunata* [8,9,15] (fig. 4), but also on non-activated primary, secondary and tertiary carbon atoms. (Fig. 4 also shows an example of a microbiological hydrogenation of an antibiotic by incubation with a mutant of the producing strain of *Streptomyces*

1) Reduction



7-Chloro-5a-(11a)-dehydro-tetracycline

2) Oxygenation



12a-Deoxy-tetracycline

Tetracycline

Fig. 4. Microbial transformation of tetracyclines.

aureofaciens [16]). This 'functionalization of unactivated carbon' has become most important in steroid chemistry. Today hydroxylating microorganisms are known for the oxygenation of almost all positions of the steroid molecule [17–19]. These microbial oxygenases exhibit remarkable substrate specificity. Our own interest was concerned with the relationship between the structure of different types of substrates and the reactivity of the microbial enzymes in order to gain a more fundamental appreciation of the geometry of microbiological hydroxylations. For this comparison growing cultures of *Fusarium lini* were used. Fig. 5 shows that 15α-hydroxylation takes place in the androstane and pregnane series, which are characterized by C/D-trans-fusion. The presence of a hydroxyl group at C-14 does not inhibit the hydroxylation of the adjacent carbon atom [20,21]. However, in substrates with C/D-cis-fusion, such as in cardenolides and bufadienolides, 12β-hydroxylation took place instead of 15α-hydroxylation [22–27]. The replacement of the 14-hydroxy group by a 14,15β-epoxy group did not

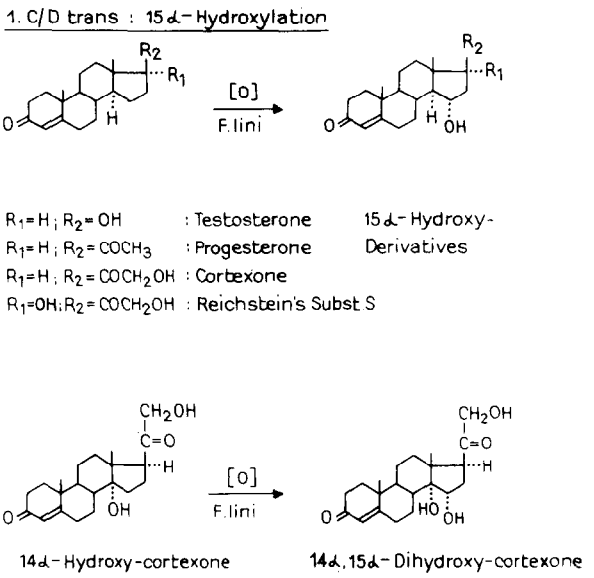


Fig. 5. Substrate specificity in the hydroxylation by *Fusarium lini:* C/D-trans-fusion.

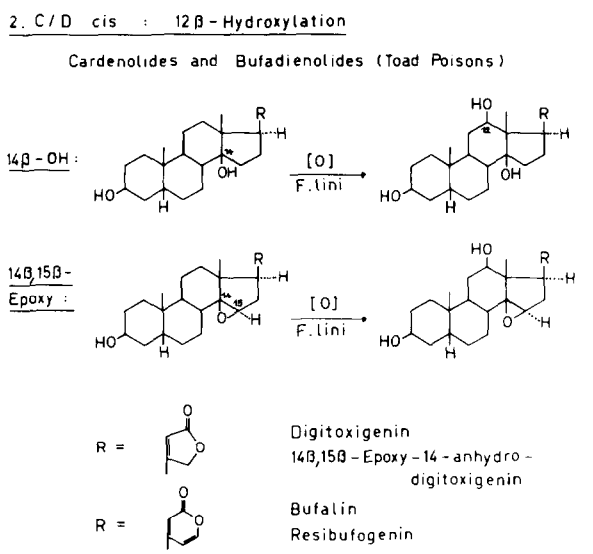


Fig. 6. Substrate specificity in the hydroxylation by *Fusarium lini:* C/D-cis-fusion.

change the position of the hydroxylation (fig. 6).

As shown by fig. 7, the general shape of the two types of molecules is quite different. Whereas the Δ^4-3-ketones with rings C and D trans-fused are almost planar and stretched and the pseudo-equatorial 15α-H atom hardly sterically hindered, the cis-fusion of the rings A/B and C/D twists the molecule considerably on both sides. Thus the 15α-position is strongly hindered. On the other hand, the equatorial 12β-H atom is almost unhindered. The hydroxyl groups introduced are on centres (15α and 12β respectively) about 7–8 Å apart and appear to be at distances from a directing group which are approximately comparable. This interpretation is strongly supported by the results of E. R. H. Jones [28]. He used mono-ketones or keto-alcohols of the androstane series as substrates for cultures of *Calonectria decora.* Whereas the major product of the hydroxylation of 5α-androstan-3-one was 12β,15α-dihydroxy-5α-androstan-3-one, with the 17-ketone as substrate,

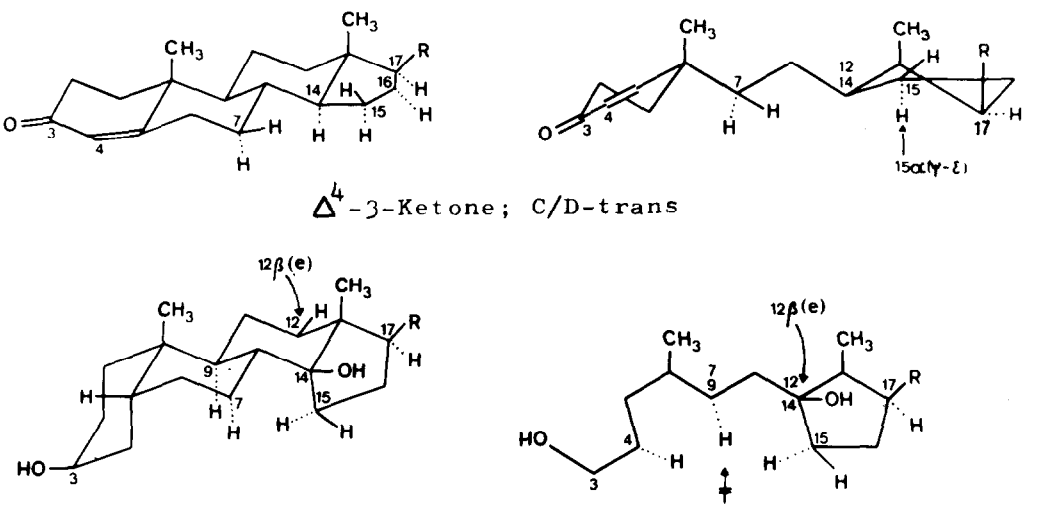


Fig. 7. Conformation of the steroid skeletons. A/B-cis; C/D-cis

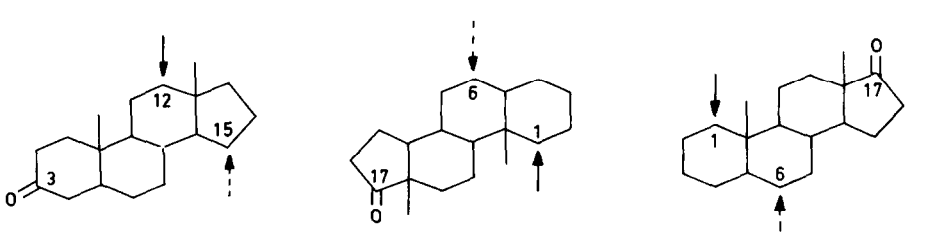


Fig. 8. Geometry of hydroxylation of mono-ketones of the 5α-androstane series by *Calonectria decora.*

dihydroxylation in the 1β,6α-position was observed (fig. 8).

Inspection of the molecular models shows that the hydroxyl groups introduced are equatorial and nearly equivalent as far as the distance (4 Å) from the 'directing' carbonyl group is concerned. Perhaps, because they are more easily able to penetrate the fungal cell walls, dioxygenated substrates were more readily attacked and single hydroxyl groups were introduced. The overall picture which is emerging for these hydroxylations is one in which the dominant polar group becomes associated with a hydrophilic region of the hydroxylating enzyme system and thereby determines the orientation in which the steroid is presented at the complementary hydroxylating hydrophobic sites on the enzyme complex. More precise views can only be formulated when studies with isolated enzyme systems are possible.

Synthetic steroid-like tricyclic compounds were also hydroxylated by the same micro-organism (fig. 9) [28].

We have extended the investigation of microbial systems, which are able to hydroxylate steroids, to

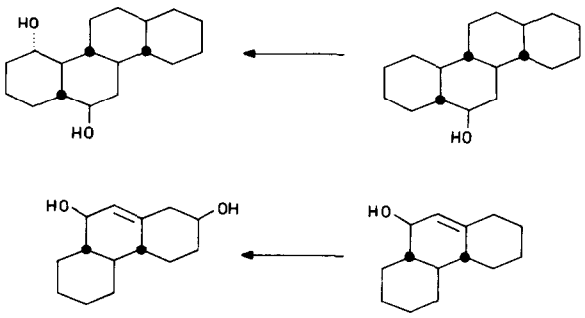


Fig. 9. Hydroxylation of steroid-like synthetic compounds by *Calonectria decora*.

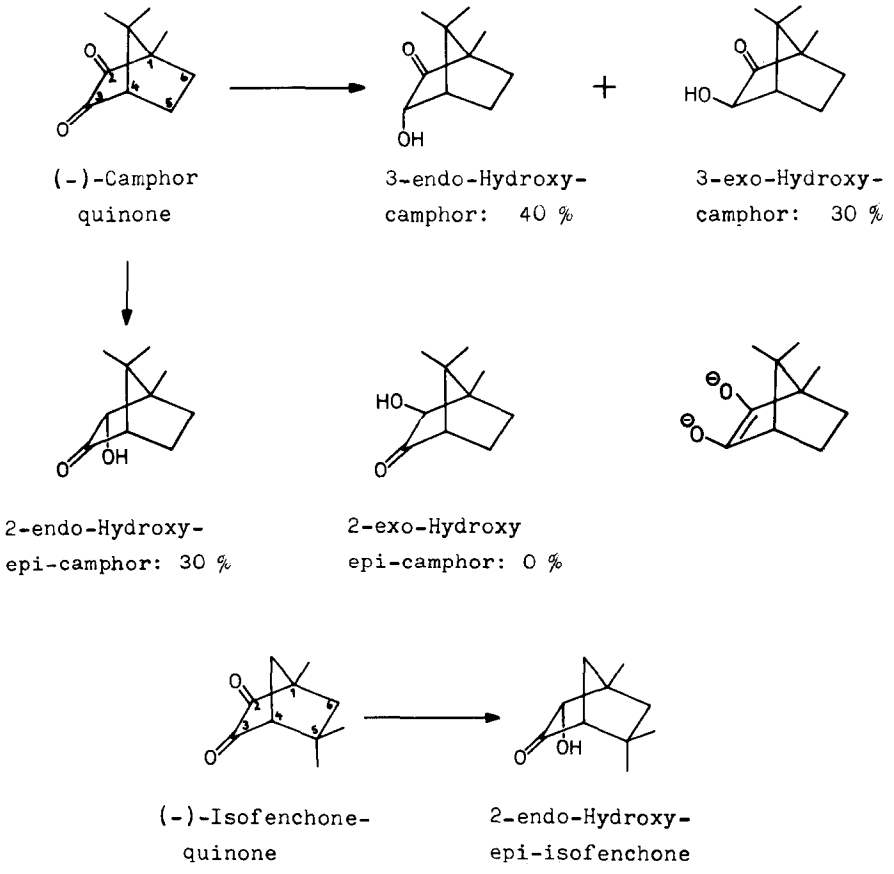


Fig. 10. Microbial transformation of bicyclo[2:2:1]heptanes: Stereospecific reduction of camphorquinone and isofenchonequinone.

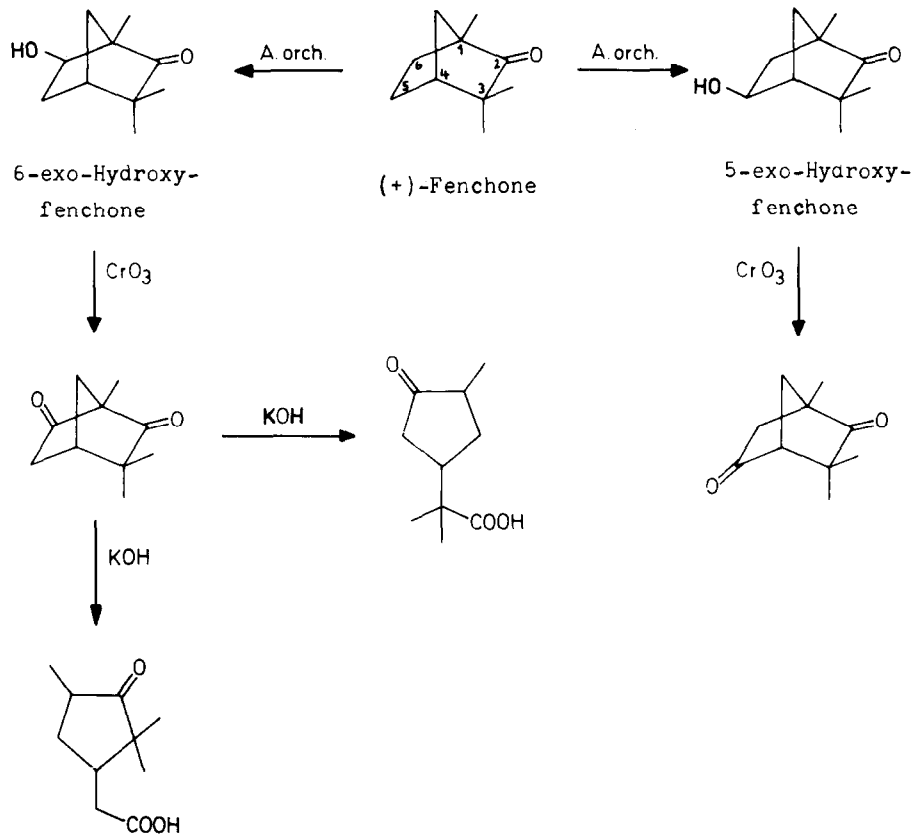


Fig. 11. Microbial transformation of bicyclo[2:2:1]heptanes: Stereospecific hydroxylations of fenchone.

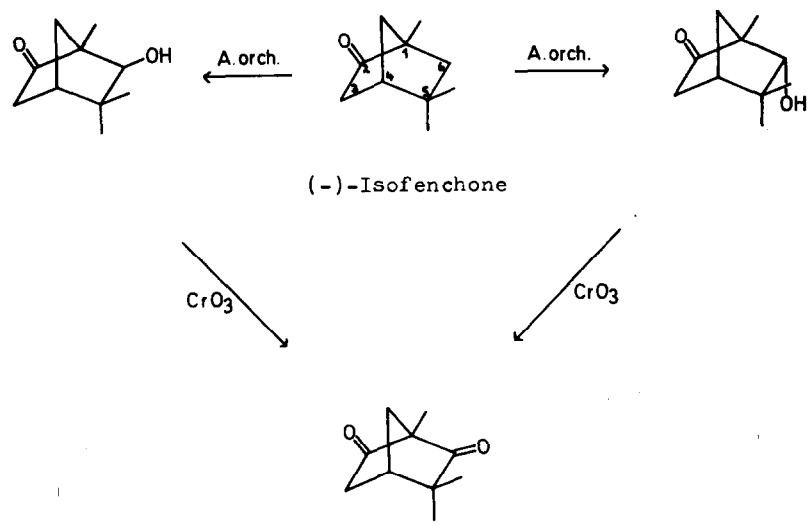


Fig. 12. Microbial transformation of bicyclo[2:2:1]heptanes: Stereospecific hydroxylation of isofenchone.

other types of substrates, such as bicyclic monoterpenes. *Absidia orchidis* monohydroxylates pregnane derivatives in the 6β,7α,11α- or 11β-position. In cardenolides and bufadienolides the hydroxylation occurs mainly in 1β- and 7β-position [29]. - (−)-Camphorquinone was transformed to a mixture consisting of 3-endo-hydroxy-camphor (40%), 3-exo-hydroxy-camphor (30%), and 2-endo-hydroxy-epi-camphor (30%) by an aqueous suspension of the mycelium of *Absidia orchidis* [30] (fig. 10). The fourth possible epimer, 2-exo-hydroxy-epi-camphor, was not formed. Surprisingly the enantiomeric (+)-camphorquinone yielded 3-exo-hydroxy-camphor as single product. The experimental conditions ruled the possibility of a subsequent isomerization out. Camphor was not attacked by *Absidia orchidis*. Thus the reactive ene-diol dianion is not an intermediate as in some chemical reductions. Both (−)- and (+)-isofenchonequinone yielded the same products, i.e. the corresponding 2-endo-hydroxy-epi-isofenchone (fig. 10). Hydroxylation was not observed with either substrate.

(+)-Fenchone was hydroxylated by *Absidia orchidis* to 6-exo-hydroxy-fenchone and to 5-exo-hydroxy-fenchone [31] (fig. 11). The structure of the two products was determined by the ^{1}H-NMR-spectra and by CrO_3-oxidation. The 6-hydroxy derivative yielded a β-diketone, which was cleaved by alkali to two cyclopentane carboxylic acids. The diketones obtained from the 5-hydroxy derivative were stable to alkali. Incubation of (−)-fenchone yielded the enantiomeric hydroxylation products in the same ratio.

(−)-Isofenchone, which has a less hindered keto group than fenchone, was transformed by *Absidia orchidis* into the two epimers, 6-endo-hydroxy-isofenchone and 6-exo-hydroxy-isofenchone. CrO_3-oxidation of both gave the same β-diketone (fig. 12) [31]. (+)-Isofenchone gave the corresponding enantiomeric hydroxy derivatives.

These examples demonstrate that non-activated carbon atoms not only of steroidal substrates but also of very different structures are hydroxylated by the same microbial enzymes in a highly regioselective manner. The products obtained are mostly inaccessible by presently available chemical methods.

The hydroxylation of the unnatural compounds diamant-1- and -4-ols to diamantane-1,7-diol and diamantane-1,9-diol by *Rhizopus nigricans* extends this observation. It is interesting to note that the

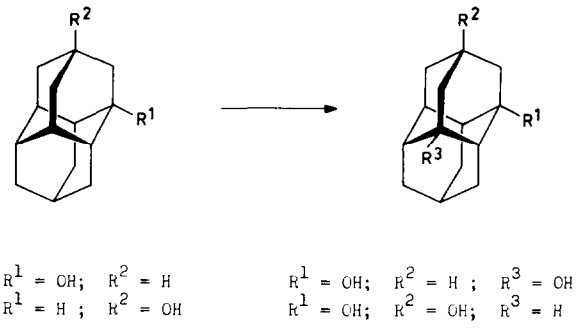


Fig. 13. Hydroxylation of diamantan-1- and -4-ol by *Rhizopus nigricans*.

introduction of the functional groups occurs on the bridgeheads which not attacked by ordinary reagents (fig. 13) [32].

In a similar manner optically active adamantanes can be prepared by *Sporotrichum sulfurescens* [33].

In general nitrogen-containing substrates, either alkaloids or synthetic compounds [5,9], are not hydroxylated very readily, but exceptions of this rule are known, e.g. [34–35].

The hydroxylations proceed with retention of configuration, require molecular oxygen (probably not singlet molecular O_2 [36]) and NADPH. The enzymes have not been isolated yet, but some reactions with resting cell suspensions [37] and cell-free preparations have been reported [38]. The details of the mechanism of the hydroxylation of saturated carbons are still unknown. An electrophilic substitution reaction with OH^+ or an equivalent species can be ruled out. It is possible that cytochrome *P* 450 is involved as in the case of mammalian hydroxylases [39–41]. In the case of the hydroxylations by *Fusarium lini* we have observed that 0.001 M potassium cyanide enhances the consumption of O_2, whereas lower and higher concentrations reduce the O_2-uptake [42].

Aliphatic hydrocarbons (alkanes) are transformed to carboxylic acids by ω-oxidation which is followed by decarboxylation and β-oxidation [5]. Similarly steroids, especially sterols and bile acids, exhibit degradation of the side chain in addition to hydroxylation of the tetracyclic system [43–45].

Aromatic systems (arenes) can also be hydroxylated by microbial enzymes to give phenolic derivatives. These reactions are followed often by the cleavage of

the aromatic ring. In this manner salicylic acid is produced in good yield from naphthalene [46,47].

The mechanism of the microbial oxygenation of arenes is likely to be the same as in mammalian organisms, i.e. based on the NIH-shift with an oxenoid (arene oxide) intermediate [39,48,49]. Chemical models (e.g. the 'Udenfriend-System') for the biological processes have been developed [50].

Mechanistically similar to the hydroxylation of saturated carbon atoms is the epoxidation of alkenes as shown by the stereo-selective conversion of 1,7-octadiene to *R*-(+)-7,8-epoxy-1-octene, to 1,2,7,8-diepoxy-octane and further products by an enzyme system of *Pseudomonas oleovorans* [51,52]. The formation of an optically active product from an inactive starting material is a further example of an asymmetric synthesis.

6. Dehydrogenation

Microbial dehydrogenations have been realized not only with steroids, but also with other types of substrates, such as fatty acids. Stearic acid which was labelled stereospecifically with deuterium or tritium and whose absolute configuration was known, was converted by *Corynebacterium diphteriae* to oleic acid [53, 54] (fig. 14). Hydration (transaddition) of oleic acid by a *Pseudomonas sp.* also occurs stereospecifically, as demonstrated by the conversion of the resulting hydroxy acid to the saturated fatty acid.

The introduction of double bonds in the 1- and 4-position of steroids, a reaction which is technically important for the production of modified antiflammatory corticosteroids, is also a stereospecific reaction. It has been shown that in the dehydrogenation, direct cleavage of the axial 1α- and 2β-hydrogens and of the axial 4α- and 5β-hydrogens takes place [55,56]. A two-step reaction mechanism has been proposed (fig. 15) [55].

The reaction mechanism of the alcohol dehydrogenases is well established for many years and therefore not discussed further.

7. Hydration

The hydration of anhydro-tetracyclines to tetra-

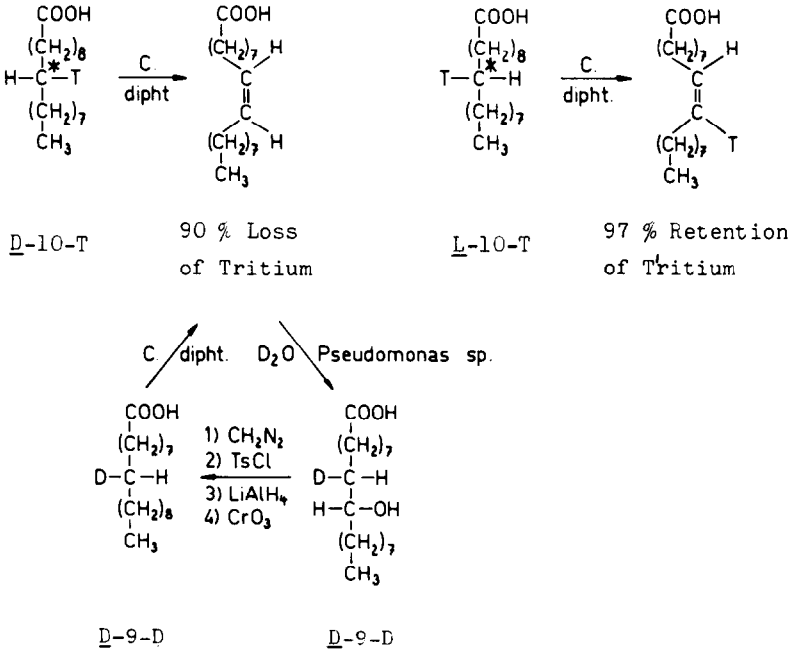


Fig. 14. Stereospecific dehydrogenation of stearic acid to oleic acid by *Corynebacterium diphteriae.*

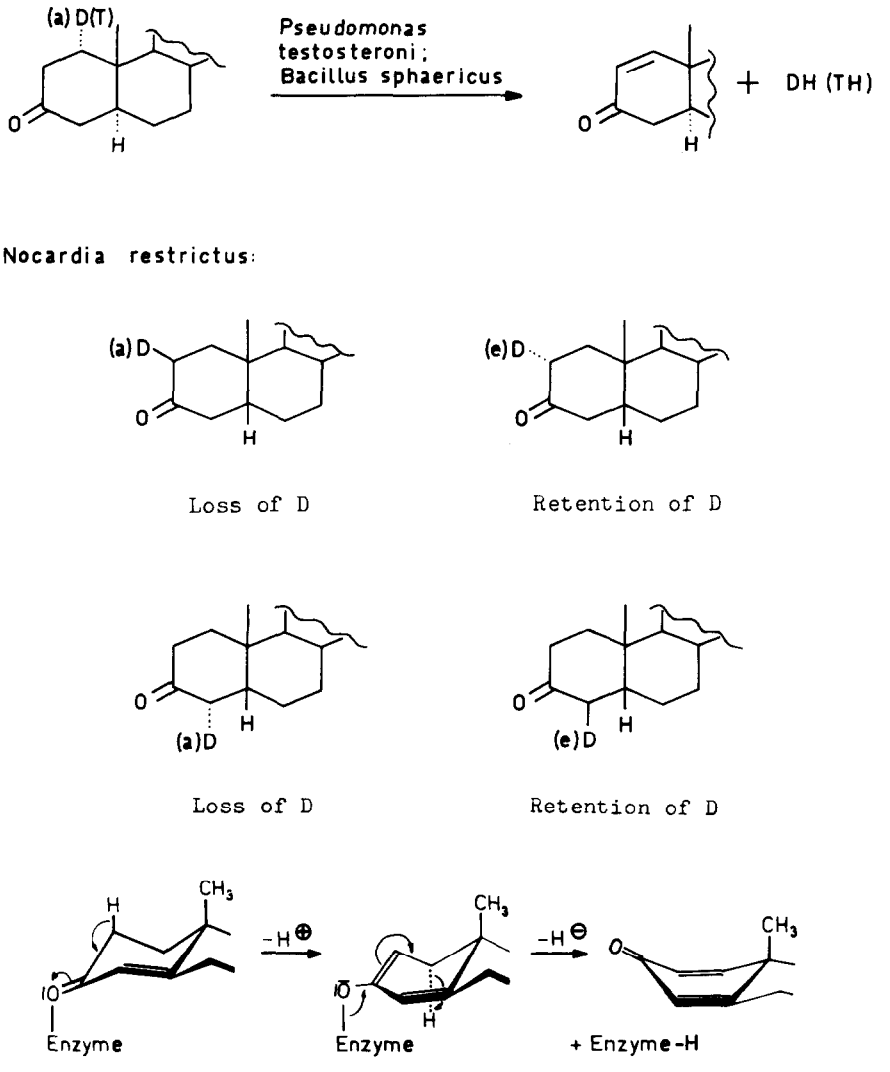


Fig. 15. Stereospecific dehydrogenation of 3-keto-steroids.

cyclines by *Streptococcus aureofaciens* is probably a two-step reaction with hydrogenation preceding subsequent hydroxylation [57,58]. However, the cleavage of the oxiran group of epoxyfarnesol to a glycol is a true hydration reaction whose stereochemistry has been elucidated recently (fig. 16) [59]. The racemic epoxyfarnesol is converted to the same *S*-(−)-10,11-dihydroxy-farnesol.

Both optically active enantiomers also yielded the same dihydroxy-farnesol. It appears that the *R*-(+)-epoxide undergoes trans opening, whereas the process from *S*-(−)-epoxide to the *S*-(−)-glycol is more complex. First the *S*-(−)-epoxide may be trans opened by a backside attack of a nucleophilic centre of an enzyme to form an intermediate complex, which is then converted enzymatically in an S_N2 type reaction to the *S*-(−)-glycol.

8. Isomerization

The Δ^5-3-keto-isomerase is a microbial enzyme

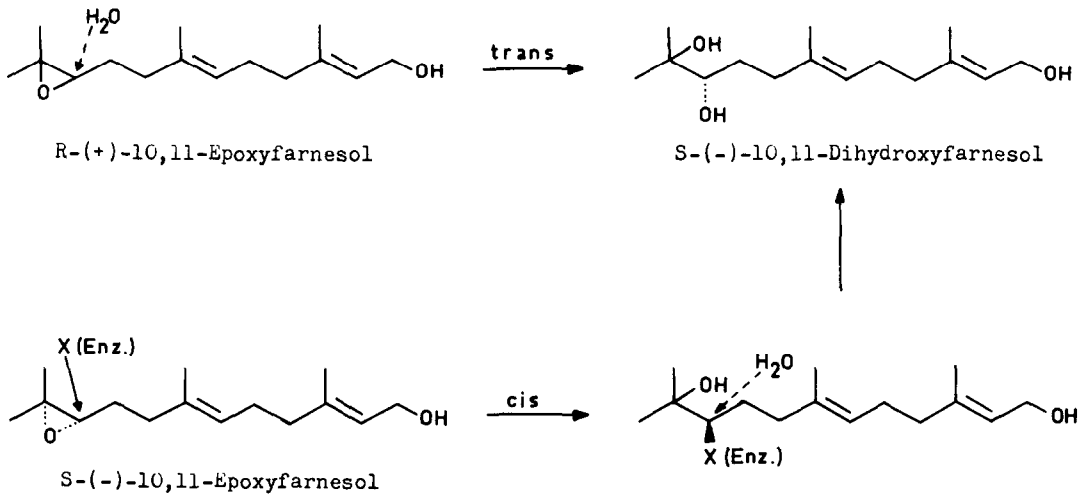


Fig. 16. Trans- and cis-hydration of R-(+)- and S-(−)- epoxyfarnesol by *Helminthosporium sativum.*

Crystals from Pseudomonas testosteroni
Stereospecific Diaxial Proton Transfer from 4β to 6β.

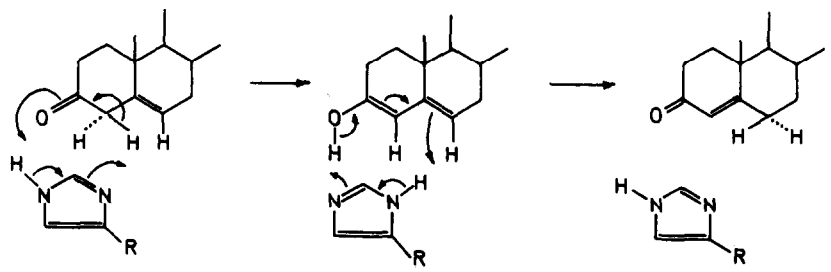


Fig. 17. Δ^5-3-Ketosteroid isomerase.

which has been isolated in crystalline form from *Pseudomonas testosteroni* and studied extensively [60]. Isomerization of a Δ^5-3-ketone to a Δ^4-3-ketone involves a stereospecific diaxial proton transfer from 4β to 6β, whereby the active centre of the enzyme is probably the imidazole ring of histidine (fig. 17).

In the course of biosynthetic studies on the antibiotic complex of the verrucarins and roridins [61] we have discovered that mevalonic acid is isomerized to verrucarinic acid, a building block of verrucarin A (fig. 18). Degradation experiments with preparations of verrucarinic acid which were obtained by the incorporation of [^{14}C]mevalonates and with mevalonates

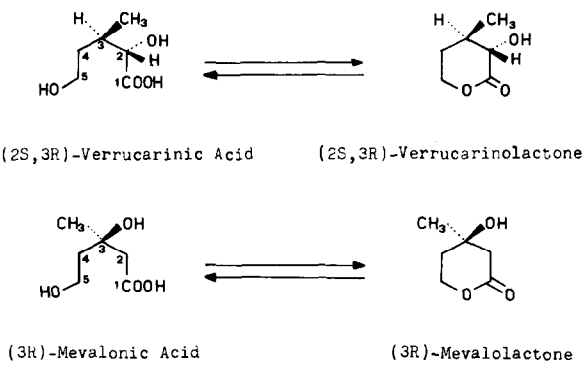


Fig. 18. Structure of verrucarinic acid.

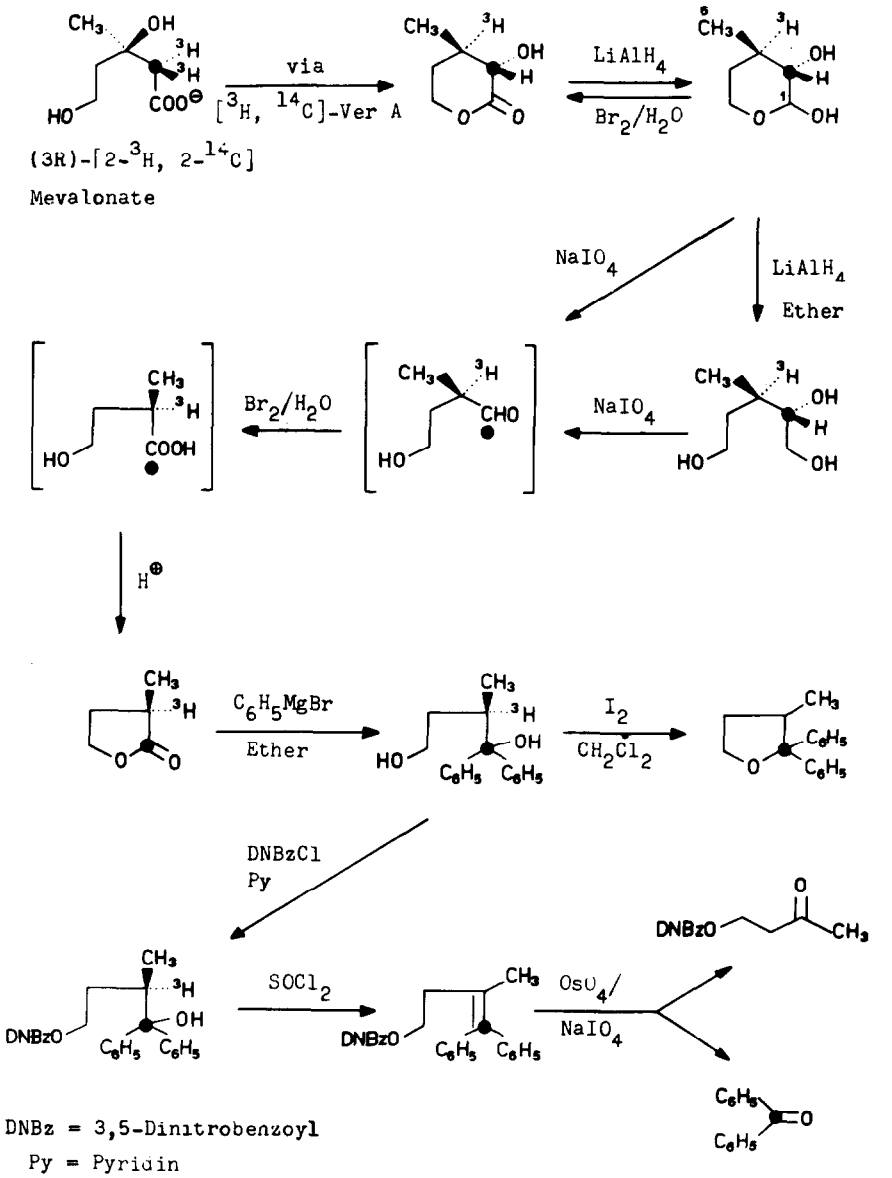


Fig. 19. Incorporation of [2-^{3}H, 2-^{14}C]mevalonate into verrucarinate.

stereospecifically tritiated showed that this transformation occurs with a 1,2 hydrogen shift [62]. It is very likely that analogous reactions will be found not only in biogenetic pathways of secondary mould metabolites, but also in microbial transformation of foreign substrates. Therefore the results are summarized briefly as follows: Degradation of verrucarinolactone derived from [2-^{14}C,2-^{3}H) and 3*R*-[5-^{14}C] mevalonate showed that C-2 of verrucarinic acid is biogenetically identical with C-2 of the isomeric mevalonic acid. The transformation of verrucarinate which was obtained by incorporation of doubly labelled [2-^{3}H, 2-^{14}C]mevalonate to an olefin devoid of tritium activity established the hydrogen shift from C-2 of mevalonate to C-3 of verrucarinate (fig. 19).

To study the mechanism of the hydrogen transfer,

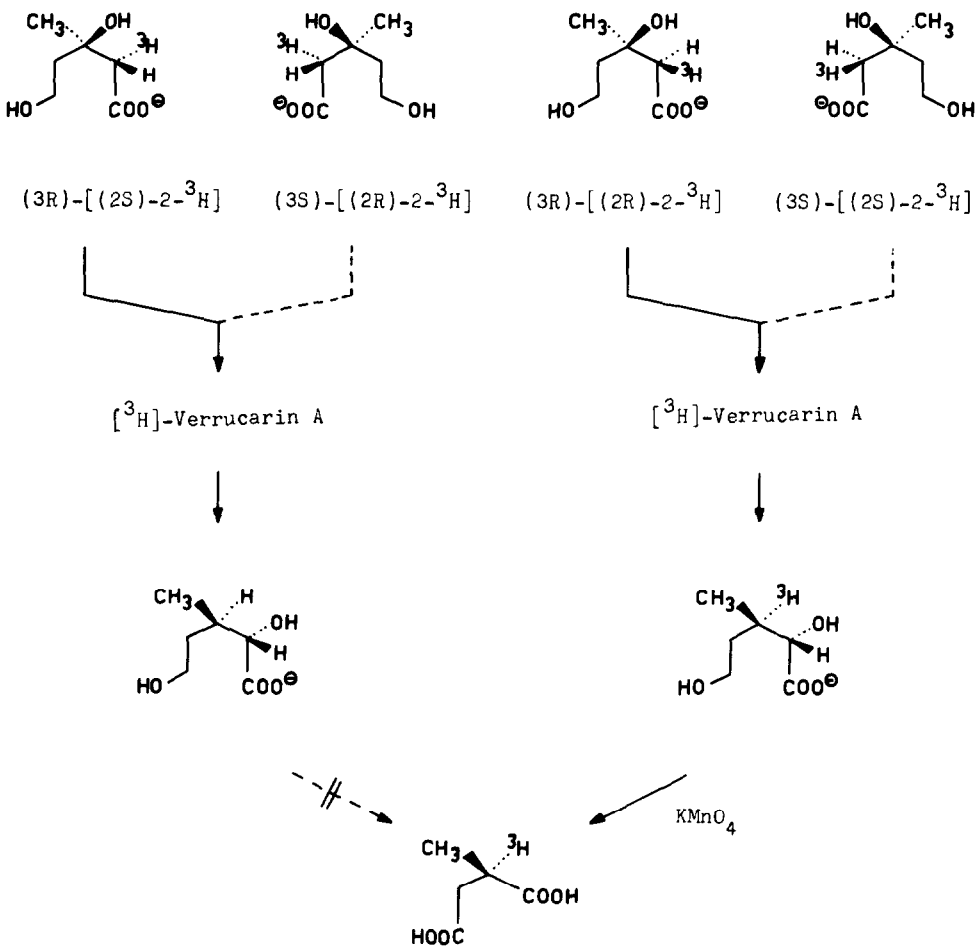


Fig. 20. Incorporation of stereospecifically labelled mevalonates.

the stereospecifically tritriated mevalonates (as indicated in fig. 20) were used for the incorporation into verrucarinic acid. Whereas radio-inactive verrucarinate was isolated after feeding of 3*R*-[2*S*-2-^{3}H]/3*S*-[2*R*-2-^{3}H]mevalonate, the experiment with 3*R*-[2*R*-2-^{3}H]/3*S*-[2*S*-2-^{3}H]mevalonate gave radioactive verrucarinate. From the observation that the natural 3*R*-enantiomer of mevalonate is incorporated in verrucarinate it can be concluded that the 3*R*-[2*R*-2-^{3}H]mevalonate which is transformed to verrucarinate. Consequently it is the 'pro-2*S*' hydrogen atom of mevalonate that is lost in the formation of verrucarinate. The 'pro-2*R*' hydrogen is retained and transferred to C-3 of verrucarinate. A possible mechanism for the biogenetic formation of verrucarinate from mevalonate is outlined in the following scheme (fig. 21). The loss of the 'pro-2*S*' hydrogen H_B agrees with the transelimination of H_2O leading to cis-anhydromevalonate. A formal cis-elimination would yield trans-anhydromevalonate. Epoxidation of the double bond gives rise to two pairs of enantiomeric glycidic acids. Protonation of the 'β-epoxide', cleavage of the C(3)-oxygen bond, and 1,2-hydride shift of the 'pro-2*R*' hydrogen atom H_A leads to the ketone with concurrent inversion of the chiral centre C-3 by analogy with a pinacol rearrangement. Both the cis- and trans-anhydromevalonates yield the same product because of the disappearance of chirality at C-2. The last step in the formation of 2*S*,3*R*-verrucarinate constitutes the stereospecific reduction of 2-dehydro-verrucarinate. The 'α-epoxide' cannot be excluded a

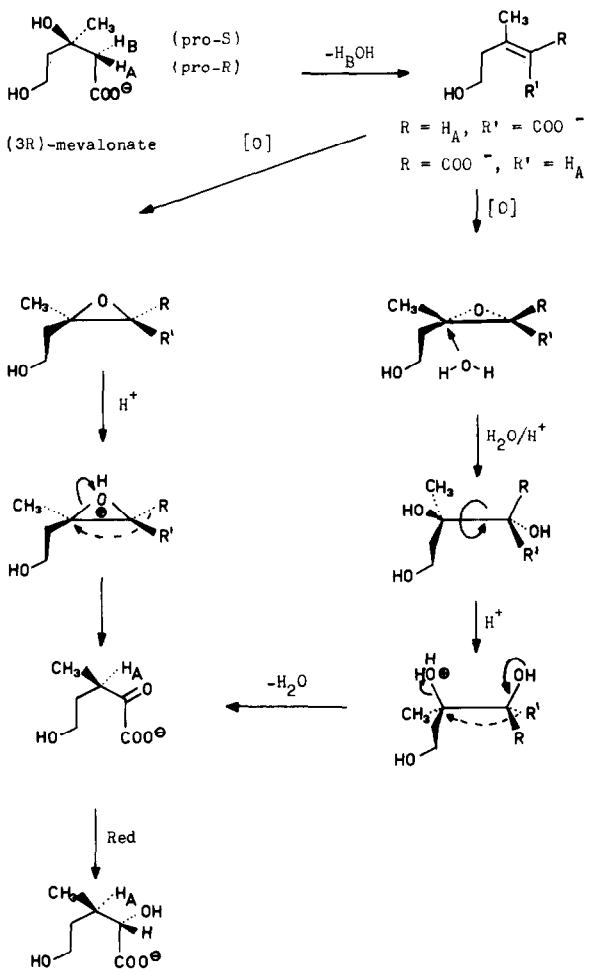


Fig. 21. Mechanism of the biogenetic formation of verrucarinate from mevalonate.

priori as an intermediate. As shown in fig. 21, the same ketone is obtained if a double inversion at C-3 and hydrolytic cleavage of the C(3)-oxygen bond etc. are anticipated. The loss of the 'pro-2*S*' hydrogen in mevalonate appears to preclude a mechanism involving vitamin B_{12} coenzyme.

9. Concluding remarks

The cross-section of current knowledge concerning conversions of organic compounds by microbial enzymes as illustrated by some selected examples shows that considerable success has been achieved. Many useful technical applications, especially for the preparation of drugs [63], are available. Reactions with microorganisms may be interpreted as biological models for metabolic studies in the plant and animal kingdoms. However, many basic problems are still awaiting solution, especially concerning the nature of the enzymes, their mode of action and the detailed reaction mechanisms.

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