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BIOHYDROXYLATION OF TERPENES IN MAMMALS

T. S. SANTHANAKRISHNAN
Bush Boake Allen (India) Ltd., Madras-600 016, India

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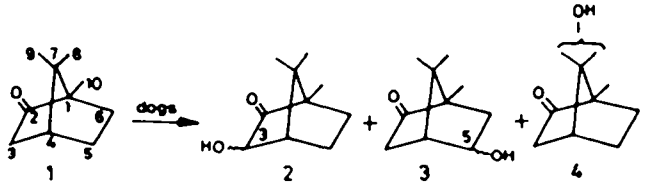
INTRODUCTION

Biohydroxylation is one aspect of the applications of enzymes to synthetic organic chemistry as they allow regio-, stereo-, and even enantio- selective functionalisation of non-activated carbons. Such reactions have been found to be of industrial value. The use of microbial hydroxylations to introduce new functional groups on non-activated C atoms has led to final proof of the structure of some terpenoids, e.g. guaioxide and its isomers. Yet another aspect of the study has been the investigation of detoxication mechanisms in mammals. These metabolic studies include allylic oxidations, epoxidations, stereoselective gem-dimethyl hydroxylations, cleavage of a conjugated double bond by epoxidation and regioselective oxidations—some of which are not found commonly in chemical reactions. It is interesting to compare the metabolism of terpenoids in mammals with that in microorganisms with regard to enzymatic systems. This review covers the work done so far on the hydroxylation of terpenes by biochemical means in mammals.

MONOTERPENES

In many countries, plants or their oils and extracts have been used in medicine.[1] Medicinal plants with essential oils find a place in many pharmacopeias today. Turpentine oil, containing in particular α-pinene, β-pinene, 3-carene, and myrcene, although irritating to the skin, is used as a rubefacient and a liniment and has choleretic activity. Other monoterpenoids have expectorant (e.g. 1,8-cineole, bornyl acetate and phellandrenes) or diuretic (diosphenol and terpinen-4-ol) properties. In addition some sesquiterpenoids have been used for their reported anticancer, analeptic, antibiotic and anthelmintic properties. Thus the metabolic studies of crude drugs containing potentially toxic terpenoids may have significant implications for human toxicology.

As early as in 1877, Wiedemann[2] isolated a glucoside derivative in the urine of dogs fed with food containing camphor. Two years later, Schmiedeberg and Meyer[3] fed dogs with food containing (+)-camphor (**1**) and, by analysing their urine after acid hydrolysis, showed the formation of "hydroxycamphors". In 1935, Asahina and Ishidate[4] repeated the feeding experiments with (+)-camphor (**1**) on dogs and were able to identify the "hydroxy-camphors" as 3-hydroxycamphor (**2**), 5-hydroxycamphor (**3**), and *cis* and *trans*-π-hydroxycamphors (**4**). Shimamoto[5] also carried out



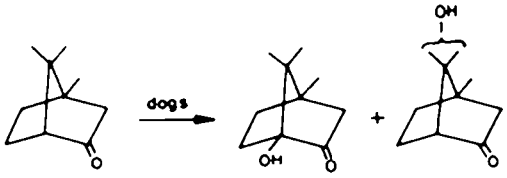
1 2 3 4

the feeding of dogs with food containing (+) -camphor and reported the formation of the hydroxycamphors as follows: 3-hydroxycamphor 15%, 5-hydroxycamphor 55% and *cis* and *trans* π-hydroxycamphors 20%, the configurations of the OH group remaining undefined.

Reinartz *et al.*[6] isolated a small amount of π-apo-camphor-7-aldehyde apart from 5-hydroxycamphor from the urine of dogs fed on camphor. For the first time both racemic[7] and optically active[8] (−) -camphor were fed to dogs and it was shown that hydroxycamphors were the product of metabolism in both cases.

Shimamoto[5] also used rabbits for his studies and fed them with (+) -camphor. He obtained from their urine, after hydrolysis, 5-hydroxycamphor as the major product and 3-hydroxycamphor as minor product. Thus, irrespective of the mammals used in the case of (+)-, (−)- or (±)-camphor, the hydroxylation appears to take place at C-3, C-5 and/or π-position, with C-5 hydroxylation predominating.

There is a single report on epi-camphor (**5**), by Reinhartz *et al.*,[9] who report the formation of 4-hydroxy-epi-camphor (**6**) as the major product with a small amount of π-hydroxy-epi-camphor (**7**) in the urine of dogs.

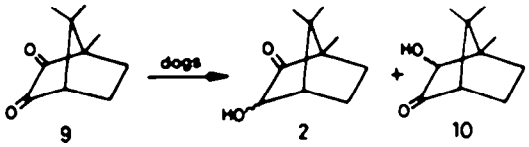


5 6 7

Reports on the metabolism of camphane-2,5-dione (**8**) in dogs by Reinhartz *et al.*[10] were confusing, but Ishidate[11] reinvestigated it and isolated 5-hydroxycamphor (**3**) as the only metabolite after the hydrolysis of the conjugated glucuronide from the urine of the dogs. The formation of this derivative involved only the reduction of one of the two keto groups.

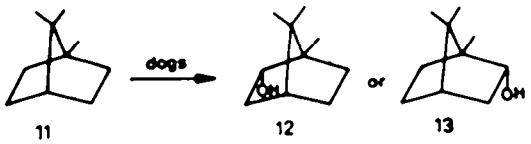
8 3

Camphorquinone (**9**) has been found by Reinhartz and Zanke[12] to be reduced to a mixture of 3-hydroxycamphor (**2**) and 2-hydroxy-epi-camphor (**10**) in dogs.



9 2 10

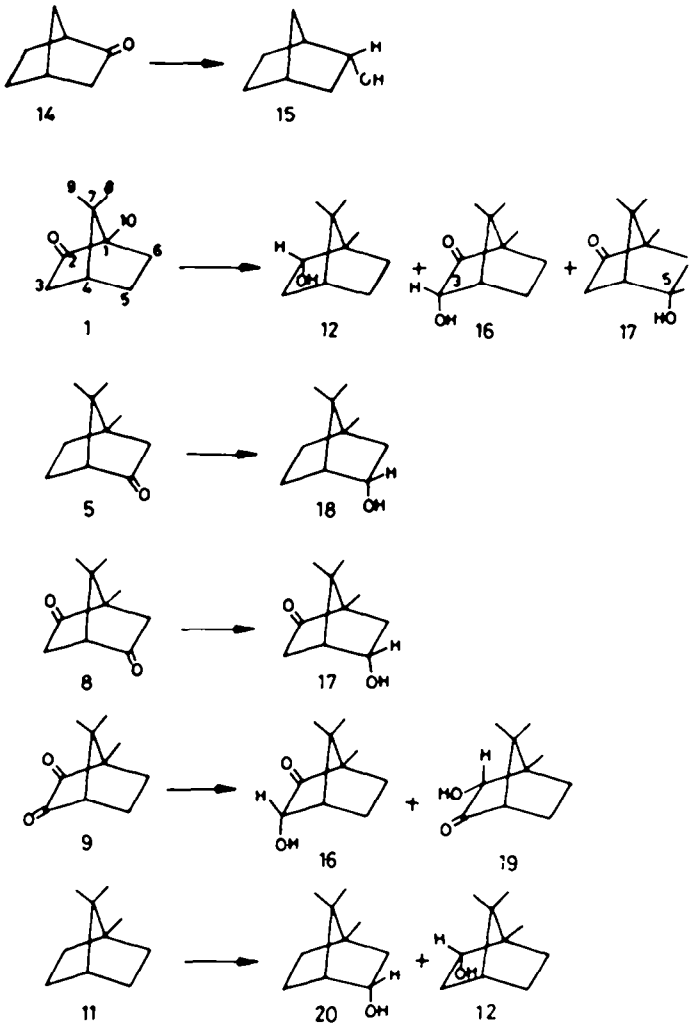
The metabolism of camphane (**11**) was studied by Hamalainen[13] in dogs; he isolated from the urine a mixture of (+)- and (−)-borneols (**12, 13**), as the corresponding glucuronides due to hydroxylation at C-2 or C-6.



11 12 13

Robertson and Hussain[14] reinvestigated in 1969 the metabolism in rabbits of camphor and related compounds such as (±)-nor-camphor (**14**), (+)-camphor (**1**), (−) camphor, (+) -epi-camphor (**5**), (±)-camphorquinone (**9**), (±)-camphane -2,5 dione (**8**) and camphane (**11**) in a systematic manner.

(±) Norcamphor (**14**) was reduced to *endo*-norborneol (**15**) whereas (+)-camphor (**1**), contrary to expectation, was reduced to (+)-borneol (**12**) as well as being hydroxylated to (+)-3-*endo*-hydroxycamphor (**16**) and (+)-5-*endo*-hydroxycamphor (**17**), 5-*endo*-hydroxycamphor being the major product. (+) Epicamphor (**5**) was found to be reduced mainly to (+)-epi-borneol (**18**). (±)-Camphorquinone (**9**) yielded 3-*endo*-hydroxycamphor (**16**) and 2-*endo*-hydroxy-epi-camphor (**19**), the former being the major metabolite. (±)-Camphane-2,5-dione (**8**) was reduced to 5-*endo*-hydroxycamphor (**17**) while camphane (**11**) was hydroxylated to (±)-borneol (**12**) and epi-borneol (**20**), the latter predominating.



Hydroxylation. (+)-and (−)-Camphors get hydroxylated to the corresponding 5-*endo*-hydroxycamphor and 3-*endo*-hydroxycamphor, the former predominating in each case. Similarly in other cases the secondary alcohols formed are *endo* and not *exo* derivatives. In general, alicyclic compounds are either specifically hydroxylated, as found often in steroids, or randomly hydroxylated as seen in methylcyclohexane.

King, Mason and Morrison[16] refer in their discussion of enzyme induction by 17-ketosteroids to the existence of progesterone-induced ring cleavage enzymes for (+)-camphor. The similarity in the structures of D-ring of steroids and camphor was considered (Fig 1).

Fig. 1.

It is thus possible that steroid hydroxylases might be involved in the oxygenation of camphor and camphane. If this were so, there should have been an attack at a specific site leading to the formation of only one alcohol (or a pair of epimers).

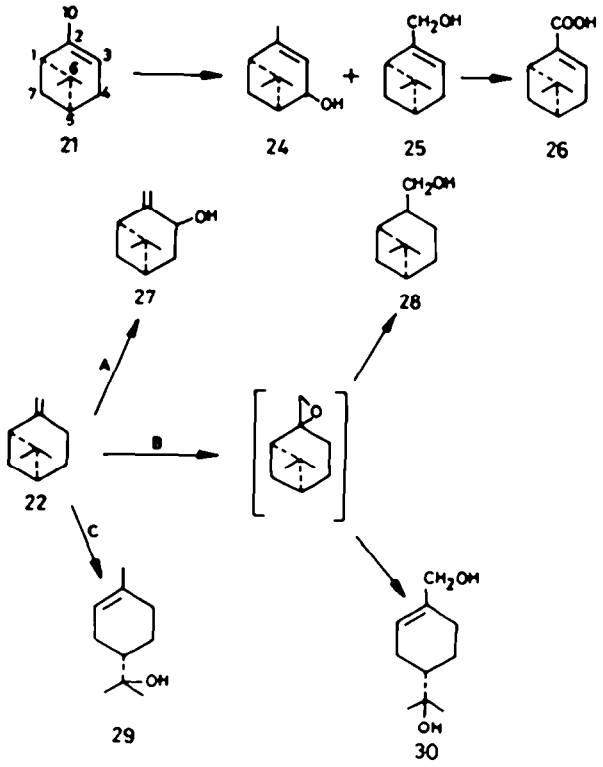
Whatever be the mechanism of hydroxylation, the products were all *endo* alcohols—which may be due only to steric control. The formation of more of the hydroxylated metabolites over that produced by reduction has been explained by Robertson and Hussain as due to the mammal's body finding hydroxylation the easier process—a moot point indeed.

The other monoterpenoids that have been biotransformed in mammals are (+)-, (−)-, and (±)-α-pinenes **(21)**, (−)-β-pinene **(22)**, (−)-*cis*-pinane **(23)**, (+)-3-carene **(40)**, (−)-*cis*-carane **(51)**, myrcene **(55)**, and *p*-cymene **(66)**.

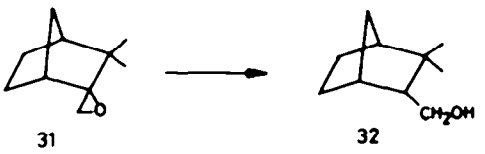
Asakawa and his group[17] carried out the above biotransformations to determine the relationships between the structures of the metabolites and the structures of the natural monoterpenoid precursors. They used rabbits for their studies. This is a follow-up of Ourisson's[18] work on the sesquiterpene patchoulol, which is being described later on in the section Sesquiterpenoids.

(−)-β-Pinene **(22)** gave predominantly 10-pinanol **(28)** and 1-*p*-menthene-7,8-diol **(30)** and judging from their yields (39% and 37% respectively) route B involving an epoxide formation of the *endo*-methylene group has been preferred. In support, Ishida's[19] work on the metabolism of camphene in rabbits has been cited. Ishida[20] obtained camphanol **(32)** by feeding camphane epoxide **(31)** to rabbits.

(−)α-Pinene **(21)** gave essentially 99% pure (−)-*trans*-verbenol **(24)** whereas (+)- and (±)-α-pinene gave respectively 67% and 68% verbenols. This finding would mean that the biotransformation of (−)-α-pinene in rabbits is highly efficient in the preparation of (−)-*trans*-verbenol **(24)**. In addition the relation between the administered α-pinenes and the resultant verbenols suggests the stereoselective hydroxylation of α-pinene. Here, two minor metabolites of α-pinene, the two allylic products myrtenol **(25)** and myrtenic acid **(26)**, have been obtained.



Scheme 1. Metabolism of (−)α- and (−)β-pinenes.

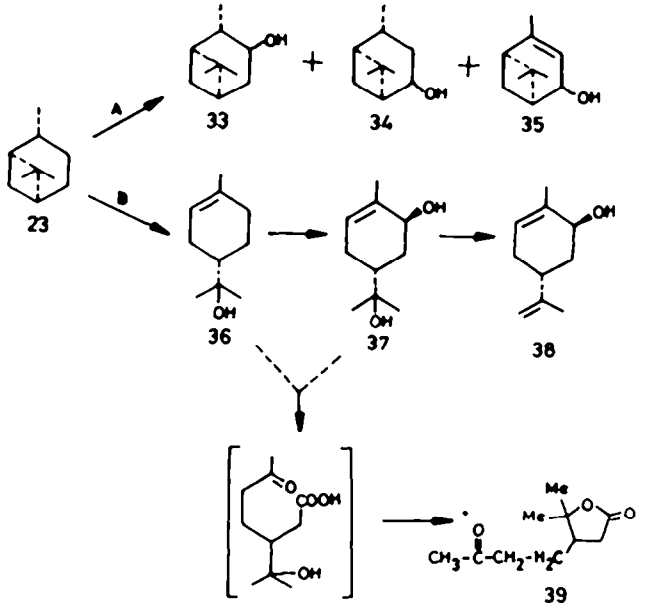


31 32

(−)-*cis*-Pinane (**23**) interestingly gave α-terpineol (**36**) as the main product besides 3-pinanol (**33**), 4-pinanol (**34**), *trans*-carveol (**38**), and *trans*-sobrerol (**37**). Asakawa *et al.* have postulated two possible routes; route A involves a simple hydroxylation at secondary C atoms whereas in route B, ring cleavage of the four-membered ring and hydration have been involved. Route B has been preferred since the major product is α-terpineol (**36**).

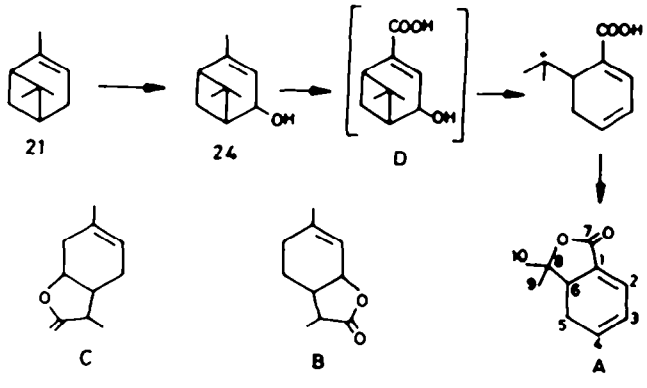
It is interesting to observe that the koala-bear[21] when fed with *Eucalyptus punctata* containing α- and β-pinenes, cineole and *p*-cymene, gave lactones (A, B, C) as the major metabolites. Later studies revealed that one of the lactones was formed from 4-hydroxymyrtenic acid (D). Hence Flynn and Southwell[22] suggested that myrtenic acid might be a metabolite of α-pinene in rabbits. However in the study with rabbits this was not detected even in GLC-mass spectrometry and so Asakawa concluded that it was a species-specific metabolite of α-pinene in mammals.

It is also interesting to note here that from the biological activity point of view, mammals (rabbits) and insects (bark beetles) show convergence in the metabolism of monoterpenoids:



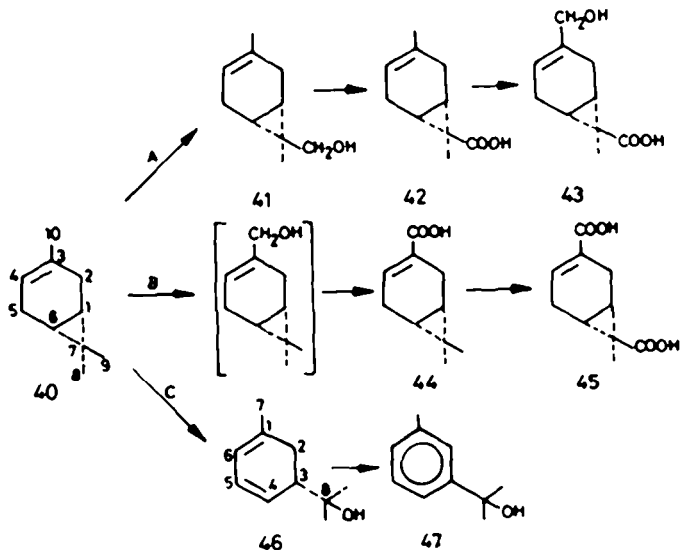
23 33 34 35 36 37 38 39

Scheme 2. Metabolism of ( ) *cis*-pinane in rabbit.



21 24 D C B A

Scheme 3. Metabolites found in koala urine and formation of lactone A.

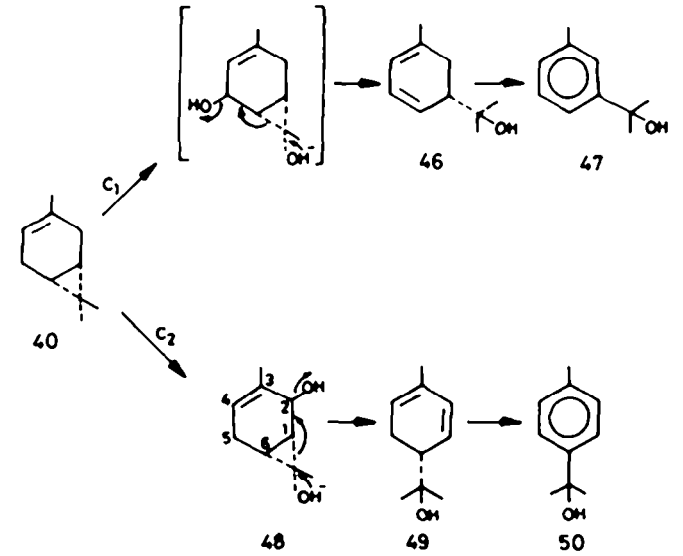


Scheme 4.

verbenol and pinocarveol have been reported as oxidation products of α- and β-pinenes in the bark beetle, *Dendroctonus frontalis*.[23]

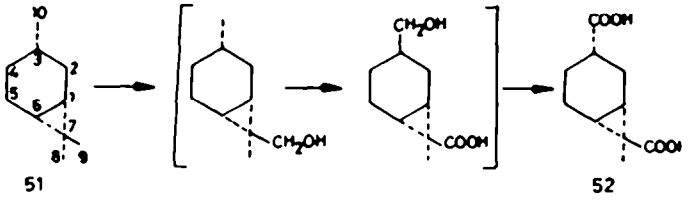
(+)-3-Carene **(40)** was found to be stereoselectively hydroxylated and oxidized, yielding *m*-mentha-4,6-dien-8-ol **(46)** as the main urinary neutral metabolite along with its aromatized relative, *m*-cymen-8-ol **(47)**. The formation of 3-caren-9-ol **(41)** has been explained by the role of the cyclopropane ring, similar to a double bond. Asakawa isolated also a few acidic metabolites and suggested three different metabolic routes (Scheme 4).

In route C, two alternative metabolic pathways (C_1 and C_2) have been proposed involving the *endo*-cyclic allylic hydroxylation. The C_1 pathway involves a 1,3-rearrangement of the OH group in the intermediate 3-carene-5-ol whereas in C_2 pathway, formation of 3-carene-2-ol followed by a rearrangement to dienol and an aromatic alcohol has been suggested. But neither the dienol nor the aromatic alcohol has been detected. Hence pathway C_2 is ruled out and C_1 preferred. This means that C-5 in 3-carene is considered to be more easily hydroxylated than C-2 by the enzymatic systems of the rabbit (Scheme 5).



Scheme 5.

The saturated hydrocarbon carane (**51**) was next studied by Asakawa *et al.*[17] Here the C-9 and C-10 Me groups were hydroxylated and the oxidation of the gem-dimethyl group was found to take place stereoselectively.



51 52

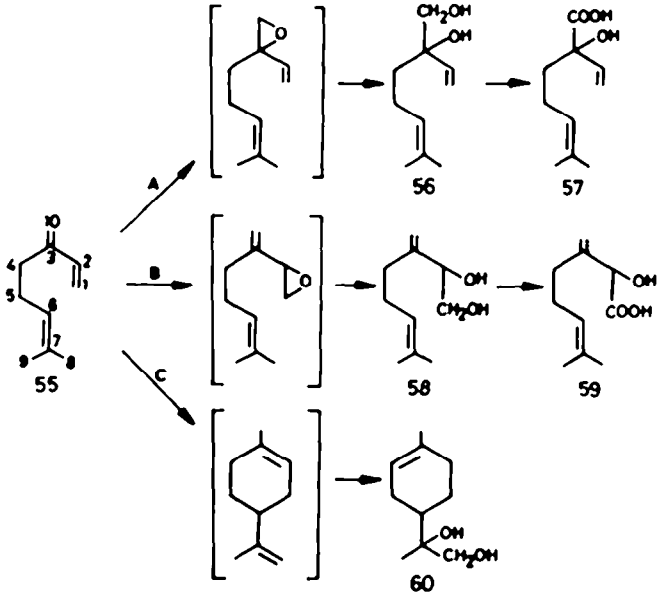
Thus carane-9, 10-dicarboxylic acid (**52**) was isolated. As a neutral metabolite, they obtained 1,4,4-trimethylcycloheptane-1-ol (**54**). Its formation has been explained as a metabolite of 1,1,4-trimethylcycloheptane (**53**) which was reported as a main reduction product of 3-carene by Cocker *et al.*[24] under special hydrogenation conditions and could have been present in the supposedly pure carane fed to the rabbits.

53 54

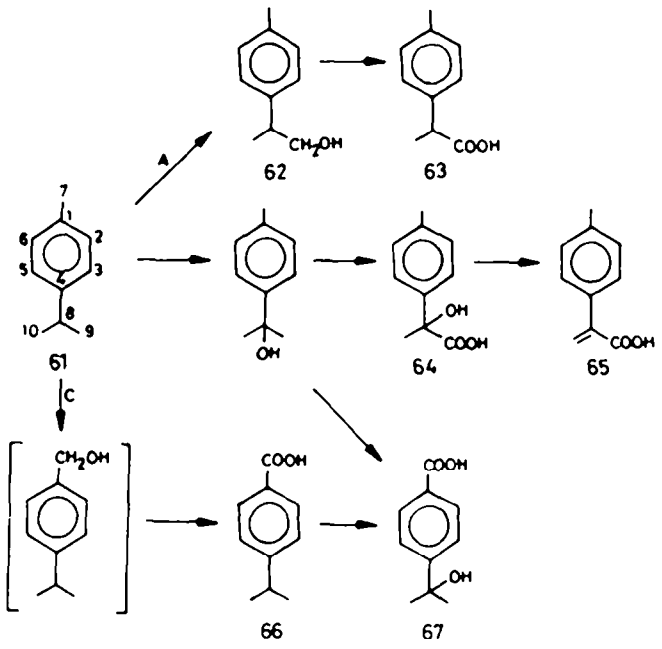
The metabolism of the monocyclic terpene myrcene (**55**) in rabbits was also studied by Asakawa *et al.*[17] The obtained myrcene-3(10)-glycol (**56**), 2-hydroxymyrcene-1-carboxylic acid (**59**) and myrcene-1,2-glycol (**58**). The formation of the two glycols has been explained involving an epoxide as an intermediate (Scheme 6). The yield of the 3,10-glycol was greater than that of the 1,2-glycol. The diols were found to be racemic.

The formation of uroterpinol (**60**) might involve limonene which could be derived from myrcene under the acidic conditions of the stomach of the rabbit. Surprisingly, no allylic alcohols were detected, although one could have expected allylic hydroxylation to take place at allylic positions C-4, C-5, C-8 and C-9.

Biotransformation of aromatic hydrocarbons has been studied in particular to check their carcinogenic activity. However, *p*-cymene, present in a number of essential oils, had not been studied in mammals. Asakawa's study in rabbits showed the formation of at least seven metabolites (Scheme 7).



Scheme 6. Metabolism of myrcene in rabbit.



Scheme 7. Metabolism of *p*-cymene in rabbit.

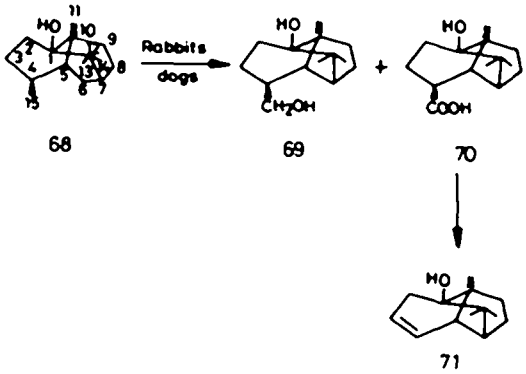
Since intraperitoneal administration gave the same metabolites as oral administration, it was deduced that these were biotransformed not microbiologically by the intestinal flora but through liver enzymatic systems.

Further in the microbial transformation (*Aspergillus niger*) of *p*-cymene, Madhyastha and Bhattacharya[25] found oxidation of the benzylic Me-group to a carboxyl and hydroxylation of aromatic H-atoms taking place. Hence the biotransformations of *p*-cymene in rabbits and by microorganisms are considerably different.

SESQUITERPENOIDS

Very little has been studied in the metabolism of the sesquiterpenoids in mammals. Patchoulol, cedrol, α-santalene, α-santalol, guaioxide, rearranged patchoulene epoxide, and caryophyllene are the few sesquiterpenes which have been studied.

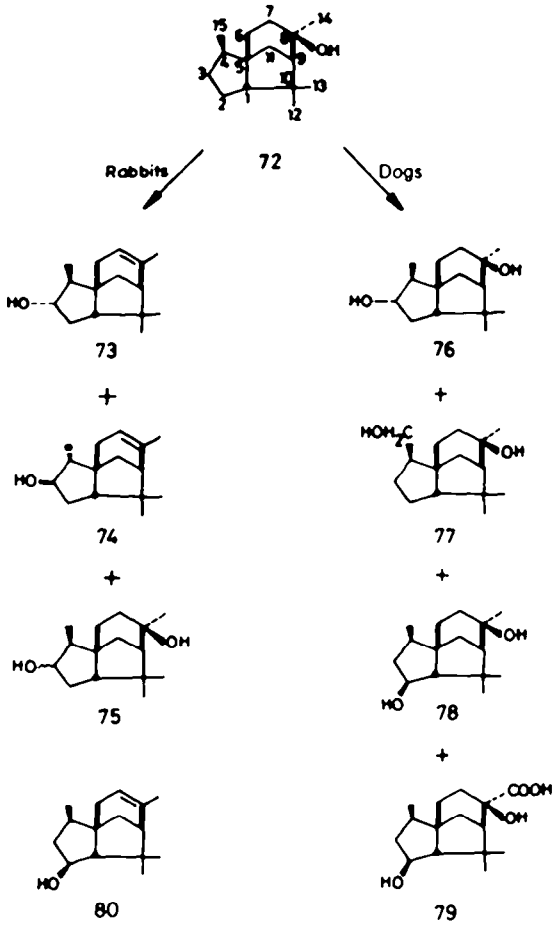
Luu Bang and Ourisson[18] studied the metabolism of patchoulol (**68**), the major sesquiterpene component of patchouli oil, an important essential oil used in perfumery industry. They used both rabbits and dogs to check whether there was any difference in the metabolic pathway in different mammals. In both cases, they obtained a diol (**69**) and an acid-alcohol (**70**) in almost equal amounts. This investigation showed that hydroxylation at a non-activated primary C-atom was possible by this method.



It is interesting to note that Ourisson *et al.* converted the acid to norpatchoulen-11-ol (**71**), the genuine odour of patchouli oil, by chemical means. By labelling studies, it was shown that the liver was the primary site of hydroxylation.

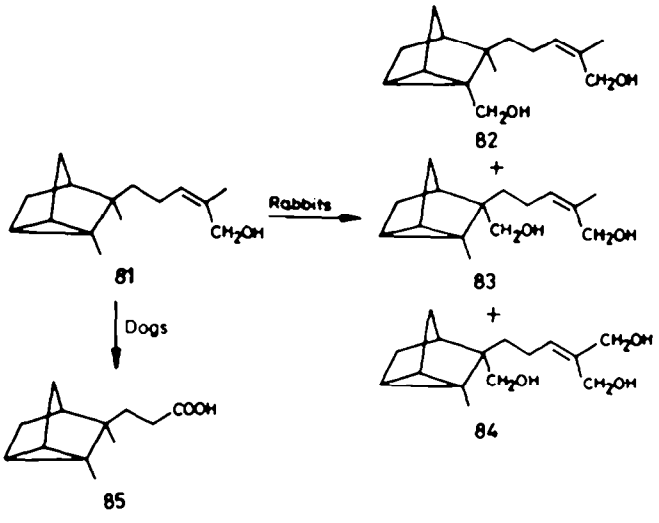
Next Ourisson *et al.*[26,27] studied cedrol (**72**), another important sesquiterpene alcohol present in another useful essential oil, cedarwood oil, for the perfumery industry. Rabbits and dogs were used in this investigation.

From the urine of the rabbits, after acid hydrolysis, they isolated isobiotol (**73**), an unsaturated alcohol, and a diol (**75**). Functionalisation took place by means of hydroxylation at a non-activated saturated C-atom, with or without elimination of the tertiary alcohol.

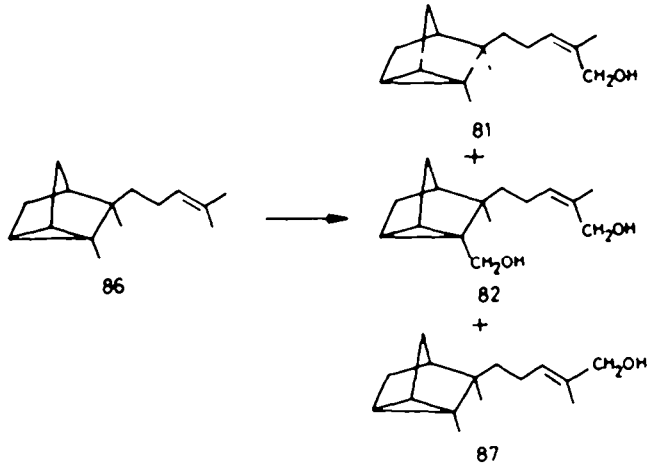


In dogs, the hydroxylation took place at the methylene groups at C-2, and C-3, the secondary Me at C-15 and the tertiary Me at C-14. There is a parallel between the biological hydroxylation of cedrol by animals, by microoganisms, or plants;[28] α-Biotol (**80**) has been isolated from *Biota orientalis* where the major constituent has been reported to be cedrol. It was suggested that in one of the steps in the biotransformation of cedrol to α-biotol the enzymatically hydroxylated derivative (**78**) might intervene. Wang *et al.*[29] have shown that cedrol was hydroxylated by *Aspergillus niger* and that the major metabolite had a structure identical to that of the diol (**76**) obtained in the cedrol feeding experiments with dogs.

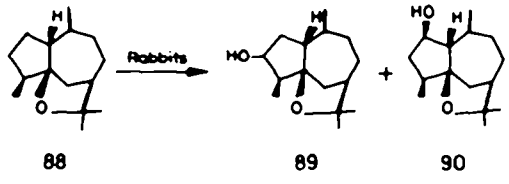
As early as 1902, Hildebrandt[30] studied the biotransformation of santalols in rabbits and isolated a metabolite for which he assigned a molecular formula HO-C_9 H_{16}-COOH. Zundel and Ourisson[31] reinvestigated the metabolism of α-santalol (**81**) in rabbits and dogs and isolated hydroxylated derivatives (**82, 83, 84**) and an acid (**85**) respectively.



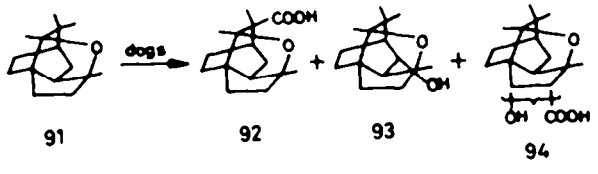
The hydrocarbon α-santalene (**86**), when fed to rabbits gave α-santalol (**81**), the diol (**82**) and an isomer of santalol (**87**).



As mentioned earlier, Ourisson *et al.*[32] have successfully used the hydroxylation with a mammal (rabbit) for introducing a functional group into a di-tertiary ether. Guaoxide (**88**), when fed to rabbits, gave the two hydroxy derivatives (**89, 90**).

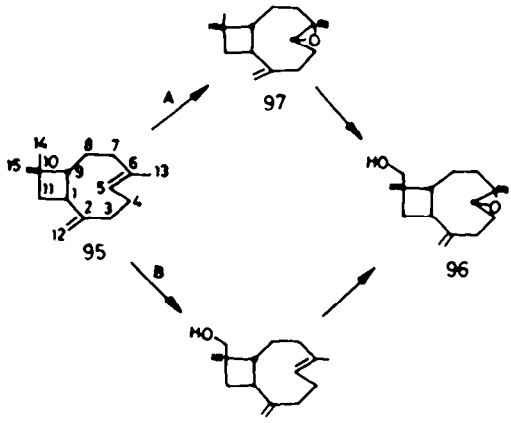


The rearranged β-patchoulene epoxide (**91**), another ditertiary ether, was also fed to dogs and from their urine, Ourisson *et al.*[32] isolated a hydroxy-ether (**93**), an acid (**92**) and a hydroxy-acid (**94**).

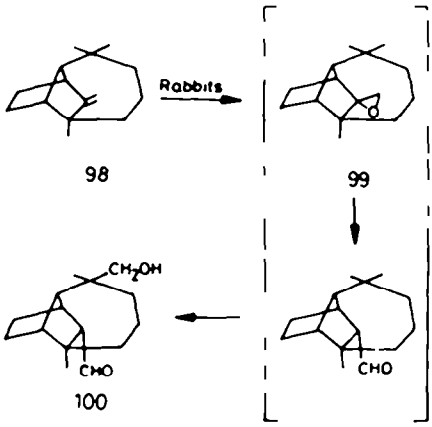


By a judicious use of a combination of biotransformation techniques in mammals and chemical hydroxylation at non-activated C-atoms, Ourisson *et al.*[32] confirmed the postulated structures of the ditertiary ethers, the re-arranged β-patchoulene epoxide and guaioxide.

Very recently Asakawa *et al.*[33] administered to rabbits (−)-caryophyllene (**95**) and isolated from their urine a neutral metabolite, the hydroxy-epoxide (**96**), the hydroxylation taking place on one of the methyls of the gem-dimethyl group in the 4-membered ring. Two metabolic pathways have been considered. Route A was confirmed since when (−)-caryophyllene oxide (**97**) was administered to rabbits, the same hydroxy-epoxide (**96**) was isolated from their urine. Route B remained to be confirmed but route A, according to the author, may be more favourable than B since the epoxide (**96**) was found to be present in some essential oils.[34,35]



Longifolene (**98**) was the next sesquiterpene studied by Asakawa *et al.*[36] Wild rabbits were found to damage the forests in Japan by feeding on the artificially planted young *Chamaecyparis obtusa*, an important tree used commercially in Japan. This cypress contained longifolene as a major sesquiterpene hydrocarbon. Longifolene was administered to rabbits and the metabolites were isolated and analysed. The Japanese workers have now identified the major product as the hydroxy-isolongifolaldehyde (**100**) and have postulated the following route.



The absence of glycols in this instance has been interpreted as due to the lesser stability of the longifolene 7,13-epoxide (**99**).

Thus, the biohydroxylation of the gem-dimethyl group on 3-, 4-, 5-, 6-, and 7-membered rings by mammals has been achieved.

INDUCTION OF OXIDASES IN MAMMALS

During the past decade, various reports[37,38] have appeared concerned with environmental factors which modify drug activity and toxicity in animals. Of the various environmental factors

studied, the inductive activity of DDT, chlordane and related pesticides[39,40] on microsomal enzyme systems that were responsible for example for the modified responsiveness of animals to barbiturates, was found to be dramatic. Hence the constituents of some of the essential oils, widely used in disinfectant sprays and air fresheners as well as in pharmaceutical preparations have come in for detailed metabolic studies in mammals.

In 1966, Ferguson[41] reported a decrease in the hexobarbital and pentobarbital sleeping times in mice reared on red cedarwood chip bedding. In 1967, Vesell[42] explained this marked effect (50% reduction) by a 2- to 3-fold induction of the drug metabolizing cytochrome P-450 dependent enzyme by the terpenes contained in the bedding; this inductive effect was reversed when the animals were switched to hardwood bedding (maple, birch, beech) or when the cedar bedding was extracted with hexane (thus removing the terpenic compounds cedrene and cedrol). Vesell's experiments showed that the concentration of drug-metabolizing enzymes could be significantly affected by the environment of animals, particularly by the presence of inducing substances in the natural habitat.

Jori *et al.*[43,44] found that eucalyptol (1,8-cineole) when given by aerosol for 4 days to rats, decreased the plasma and/or brain levels of amphetamine, zoxayolamine, pentobarbital and aminopyrine, for instance decreasing the duration of sleep induced by pentobarbital. Jori[43] also studied a number of components of essential oils such as menthol, α- and β-pinenes, guaiacol and the oil of *Pinus pumilio* to see whether these affected the metabolism of other drugs in rats. They were able to show that only eucalyptol induced the microsomal enzymes when given by the aerosol route—a kind of administration of drugs particularly used in practical medicine, known to be more effective because of high absorption of these drugs by mucous membranes of the respiratory tract—and this reduced by 50% the sleeping time of 18 hrs pretreated rats. This confirmed the findings of Wade *et al.* who showed that volatile hydrocarbon constituents of cedarwood, such as cedrol and cedrene,[45] were effective inducers of microsomal enzymes by inhalation.

CONCLUSIONS

The results summarized above show that no prediction can yet be made as to the selectivity to be expected from bio-hydroxylation. Thus, it is quite conceivable that, in some cases, metabolism by a mammal would be the simplest route to novel products. For structural work, this has already been used (see Ref. 32), and the first gramme amounts of norpatchoulenol were obtained using rabbits. Potential extensions are obvious, and one can foresee that bio-technological extensions may some day lead to production of some valuable derivatives. Similar studies on diterpenoids will certainly also follow.

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