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Some aspects of monoterpene biosynthesis

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Abstract. Biosynthesis of terpenes present some special features. Incorporations of precursors are generally low. Degradation of substrates or higher terpenes into smaller units and their reutilisation into lower terpenes cause randomisation of the labels. Unbalanced distribution of the labels from labelled mevalonalactone (MVA) into isopentenyl pyrophosphate and dimethylallyl pyrophosphate moieties and incorporation of amino acids into terpenes are some of the other special features of monoterpene biosynthesis. Bakuchiol, a meroterpene isolated from the plant *Psoralea corylifolia* provides a good model for biosynthetic studies pertaining to some of the above points. Using substrates labelled with suitable isotopes, it has been established that bakuchiol is biosynthesised from one phenylpropane and two MVA units. Incorporation of leucine into bakuchiol has also been observed. Degradation of biosynthesised bakuchiol indicated extensive randomisation of the labels, thus its terpenic part is biosynthesised by the normal mevalonate pathway.

Keywords. Monoterpene biosynthesis; anomalous incorporations; bakuchiol; meroterpene; *Psoralea corylifolia leguminosae.*

1. Introduction

The first specific step in the biosynthesis of terpenoids involves conversion of R-mevalonic acid (MVA) into isopentenyl pyrophosphate (IPP) and 3,3-dimethylallyl pyrophosphate (DMAPP), followed by condensation of IPP and DMAPP giving monoterpenoids. Further analogous condensations with different isoprenoids furnish a variety of terpenoids like the sesqui-, di-, sester-, tri-terpenoids R-MVA is formed from three units of acetate which are mainly derived from carbohydrate and fat metabolisms. In the entire sequence of reactions leading to the biosynthesis of MVA, the reduction of (S)-3-hydroxy-3-methylglutarate is the only non-reversible process. R-MVA is thus the first specific precursor and is particularly useful for studies on biosynthesis of isoprenoids. Though the biosynthesis of isoprenoids in general has been studied extensively and intricate mechanistic and stereochemical aspects elucidated, the details of the biosynthesis of monoterpenes are yet far from clear. Some special features observed in the biosynthesis of lower terpenes and biosynthesis of a meroterpene, bakuchiol (1) are discussed in this presentation.

2. Special features of monoterpene biosynthesis

Incorporation of substrates is usually very low and lies between 0·01 and 0·1% (Banthrope *et al* 1972). Low incorporations have been ascribed due to different factors.

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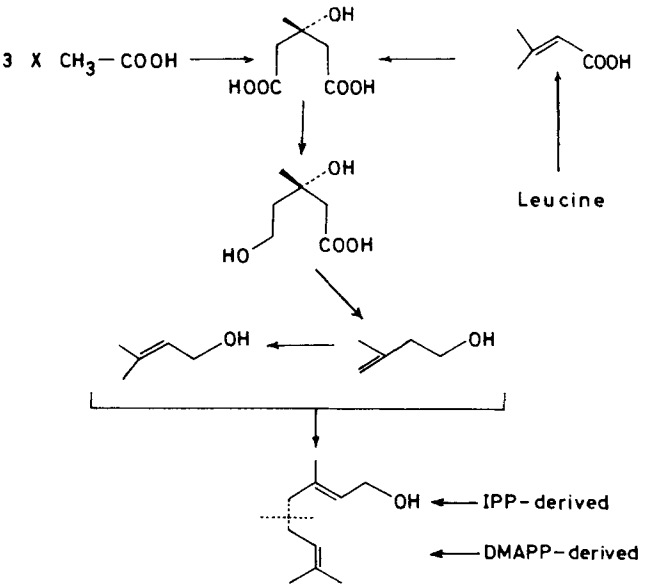
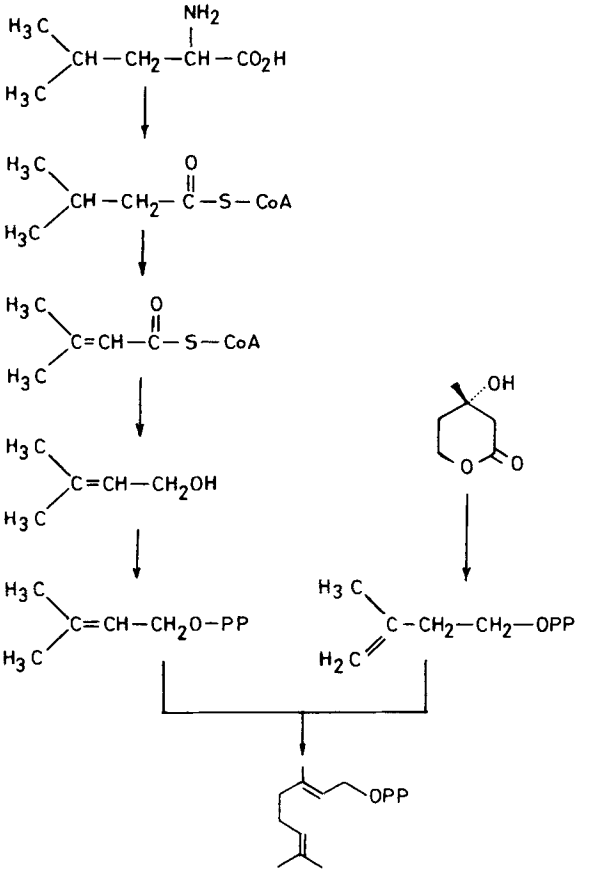
Extensive randomisation of the labels have been observed in many cases resulting in anomalous incorporation. There are instances where the labels from MVA have been found in the non-terpenoid part of the molecule. Thus in the case of sabinyl acetate biosynthesised from [2-^{14}C]-MVA in *Juniperus sabina* (Loomis 1967), 90% of the label was located in the ester moiety. The other reason for anomalous incorporations could be the reutilisation of smaller units formed by the degradation of higher terpenoids. There may be more than one metabolic route for a particular substrate. For example, it has been found that in rats, MVA gets incorporated into fatty acids. Experiments have proved that in this case, MVA is metabolised into acetate units which are then utilised in the biosynthesis of fatty acids (Edmon and Popjack 1974). The presence of biologically inactive enantiomers can also cause anomalous incorporations. S-MVA, a biologically inactive enantiomer, for instance, can be metabolised into smaller units, which may enter the general metabolic pool and result in non-specific incorporations of the labels.

Another unusual phenomenon in the biosynthesis of monoterpenes is the unequal distribution of labels from labelled MVA into the IPP and DMAPP moieties (scheme 1). The label from MVA predominantly goes to the IPP moiety which is in accordance with the mevalonate pathway while the amount of label in the DMAPP derived moiety was considerably lower (Suga and Shishibori 1972). Several explanations have been put forward. One of them is the possibility of the existence of two different metabolic pools. One of the pools is sparsely filled with IPP, while the other pool is well filled with DMAPP and irreversibly bound to protein. Therefore, there is differential dilution of these products on treatment with exogenous substrates (Hanson 1977). IPP condenses with the bound DMAPP and thus give rise to unequal labelling. When terpenoid synthesis is rapid or metabolic periods are longer, both the pools are equally filled and therefore symmetrically labelled products are observed in most of the higher terpenoids.

Incorporation of certain amino acids such as leucine or valine into monoterpenes, present yet another special feature of monoterpene biosynthesis in higher plants (Tange



Scheme 1



Scheme 2

1981). Experiments using leucine and valine as substrates in *Cinnamomum camphora* and *Pelargonium roseum* show predominant incorporation into the DMAPP moiety (64%), while this moiety when derived from MVA contained less than 32% of the incorporated tracer (Tange 1981). It has therefore been inferred that DMAPP is biosynthesised from leucine (scheme 2). A minor pathway to MVA biosynthesis *via* amino acids is known. If the monoterpenoids are biosynthesised from amino acids *via* MVA, the distribution pattern of the labels in the biosynthesised monoterpenoid in that case would be similar to that from MVA. The pattern of distribution of the labels in the biosynthesised products studied by Tange (1981) shows that these amino acids are not incorporated *via* MVA. Therefore it has been suggested that the DMAPP moiety in some of the monoterpenoids is biosynthesised by an alternate route, as shown in scheme 2. It may, however, be noted that the incorporation of these substrates was very low. In fact in some cases, (Banthrope *et al* 1972), there was no significant incorporation of leucine and valine into monoterpenes.

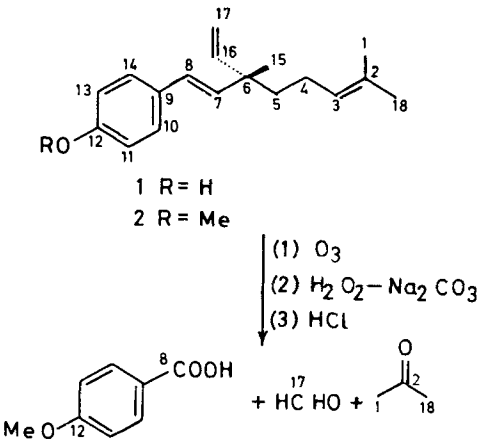
3. Biosynthesis of bakuchiol

In our search for useful bioactive phytochemicals, a screening programme of plants is underway (Progress reports 1974, 1978, 1980). Petroleum ether extracts of *Psoralea*

corylifolia Linn. (N. O. Leguminosae), an important medicinal plant, was found to have insect juvenile hormone activity when tested on the fifth instar nymph of *Dysdercus koenigii* (Indian red cotton bug). Extensive fractionation, monitored by bioassay led to the isolation of the active principle which was characterised as bakuchiol (1) (Joshi *et al* 1974; Mehta *et al* 1973). Inspection of the structure of bakuchiol suggests that ten out of twelve carbon atoms (*i.e.* carbon atoms 1–6 and 15–18) of the side chain are isoprenoid in nature. The aromatic ring with the two-carbon side chain (*i.e.* carbon atoms 7 and 8) may be considered as derived either from a phenylpropane unit or from a polyketide chain. Oxygenation at the 12 position would, however, favour the former possibility. Bakuchiol, therefore, presents an interesting molecule biosynthesised by two major pathways. We have therefore selected this molecule for the biosynthetic studies. In this presentation, some aspects of the biosynthesis of bakuchiol are described.

Though the chemical contents of the seeds of *P. corylifolia* have been examined extensively, not much work has been reported on the other parts of the plant. In the present investigation, the stem, leaves, seeds (both mature and immature) and roots were examined separately for bakuchiol and other components using HPLC. Results of our investigations show that backuchiol is present in all parts of the plant, at all stages of development. Most of the bakuchiol is present in the seeds while the roots contain only traces. Mature plants (8–10 weeks old) were used for the biosynthetic experiments. The optimum time for the harvesting (72 hr) of the treated plants was found by administering sodium[2-^{14}C]-acetate at different time intervals and evaluating the incorporation efficiencies. Bakuchiol (1) was isolated from the ether extract of the whole plant (excluding roots) by repeated preparative layer chromatography and was transformed into its methyl ether (2) by the action of methyl iodide in the presence of sodium hydride. For the location of the labels in the radioactive products, 2 was ozonised and formaldehyde was collected. Anisic acid and acetone were obtained by the oxidative work up of the ozonised product (scheme 3).

Bakuchiol methyl ether (2) prepared from biosynthesised 1 from DL-[U-^{14}C]-phenylalanine on degradation gave radioactive anisic acid which carried 81 % of the radioactivity of the parent compound. Considering the loss of one carbon atom (*i.e.* carbon 7) out of the eight labelled carbon atoms of 2, this amounts to 93 % of expected retention of the radioactivity (table 1). Other degradation products namely,



Scheme 3

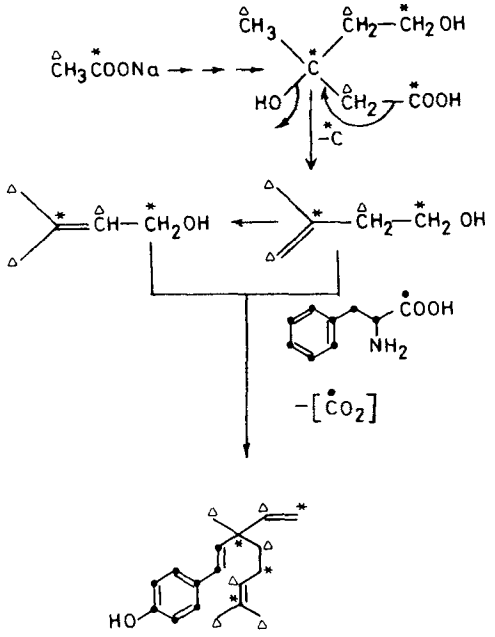
Table 1. Specific activities of bakuchiol, its methyl ether and its degradation products.

Substrate (specific activity)	Activity administered	%Incorporation in bakuchiol	Bakuchiol dpm/mmol	Bakuchiol methyl ether dpm/mmol	Degradation products of bakuchiol methyl ether		
					Anisic acid	Acetone semicarbazone	Formaldehyde dimedones
L-(U-^{14}C)Phenylalanine (360 mCi/mmol)	$2{\cdot}2 \times 10^8$	0·04	$2{\cdot}97 \times 10^5$				
	$2{\cdot}2 \times 10^8$	0·04	$3{\cdot}07 \times 10^5$				
	$2{\cdot}2 \times 10^8$	0·04	$2{\cdot}29 \times 10^5$	$2{\cdot}28 \times 10^5$	$1{\cdot}86 \times 10^5$	—	—
L-(U-^{14}C)Tyrosine (387 mCi/mmol)	$2{\cdot}2 \times 10^8$	0·005	$4{\cdot}76 \times 10^4$	—	—	—	—
	$2{\cdot}2 \times 10^8$	0·006	$5{\cdot}07 \times 10^4$	—	—	—	—
DL-[4-^{3}H, 1-^{14}C]-Phenylalanine	^{14}C $1{\cdot}95 \times 10^8$	—	$1{\cdot}76 \times 10^4$	—	—	—	—
	^{3}H $2{\cdot}72 \times 10^8$	—	$2{\cdot}01 \times 10^5$	—	—	—	—
	^{14}C:^{3}H 0·72:1	—	0·09:1	—	—	—	—
(U-^{14}C)cinnamic acid (324 mCi/mmol)	$4{\cdot}4 \times 10^8$	0·01	$4{\cdot}25 \times 10^4$	$4{\cdot}51 \times 10^4$	$3{\cdot}25 \times 10^4$	—	—
(3 RS)-[2-^{14}C]MVA (0·92 mCi/mmol)	$8{\cdot}69 \times 10^7$	0·086	$1{\cdot}72 \times 10^5$	$1{\cdot}72 \times 10^5$	$7{\cdot}63 \times 10^3$	$8{\cdot}59 \times 10^4$	—
L-(U-^{14}C)Leucine (132 mCi/mmol)	$2{\cdot}2 \times 10^8$	0·02	$9{\cdot}75 \times 10^4$	$9{\cdot}5 \times 10^4$	$2{\cdot}73 \times 10^4$	$1{\cdot}4 \times 10^4$	$3{\cdot}5 \times 10^3$
	$2{\cdot}2 \times 10^8$	0·02	$8{\cdot}52 \times 10^4$	$8{\cdot}37 \times 10^4$	$2{\cdot}28 \times 10^4$	$1{\cdot}19 \times 10^4$	$3{\cdot}5 \times 10^3$
(1-^{14}C)Sodium acetate (55·55 mCi/mmol)	$2{\cdot}2 \times 10^9$	0·15	$6{\cdot}38 \times 10^6$	$6{\cdot}34 \times 10^6$	$4{\cdot}07 \times 10^3$	$9{\cdot}52 \times 10^5$	$1{\cdot}06 \times 10^6$
(2-^{14}C)Sodium acetate (59·41 mCi/mmol)	$2{\cdot}2 \times 10^9$	0·11	$7{\cdot}08 \times 10^6$	$7{\cdot}37 \times 10^6$	$4{\cdot}86 \times 10^3$	$1{\cdot}06 \times 10^6$	—

formaldehyde and acetone did not contain significant amount of labels. The specific incorporation of phenylalanine into the aromatic ring thus establishes the phenylpropanoid origin of the non-isoprenoid part of 1.

Loss of the carboxyl group during the biosynthesis of bakuchiol from phenylalanine has been shown by experiments where DL-[1-^{14}C]-phenylalanine was used. Bakuchiol, isolated from the treated plant did not possess any significant radioactivity though expected incorporations into other phenylpropanoids like psoralen and angelicin were observed in the same experiment. Thus, the carboxyl of phenylalanine is lost during the biosynthesis of 1. Further proof for the loss of the carboxyl carbon has been obtained by using [4-^{3}H, 1-^{14}C]-phenylalanine as substrate. Loss of ^{14}C was indicated by the increase in the ratio of ^{3}H/^{14}C in the biosynthesised bakuchiol.

Information about the biosynthesis of the side chain carbon atoms 1–6 and 15–18 could be obtained by using [1-^{14}C]- and [2-^{14}C]-acetates as substrates. The major pathway leading to the biosynthesis of MVA involves the condensation of three acetate units. In the first specific step in the biosynthesis of terpenoids, one carboxyl carbon of MVA is lost during the formation of isopentenyl pyrophosphate. Thus if the side chain of 1 is isoprenoid in nature, the distribution of the labels from [1-^{14}C]- and [2-^{14}C]-acetates will be expected in the positions shown in scheme 4. Bakuchiol methyl ether, obtained from 1 biosynthesised from sodium[1-^{14}C]-acetate, on degradation gave radioactive formaldehyde and acetone. In the corresponding experiment, where sodium[2-^{14}C]-acetate was used as a substrate, formaldehyde did not show significant radioactivity while acetone was labelled with ^{14}C. This pattern of labelling is consistent with the terpenic origin of the side chain (C-atoms 1–6 and 15–18). Quantitative analysis of the results indicate that radioactivities of the degradation products do not



Scheme 4

account for all the activity of the parent molecule. The possibility of a parallel biosynthetic pathway is therefore not ruled out. Experiments in this direction are in progress.

Definite proof of the terpenic origin of the side chain was obtained by using [2-^{14}C]-MVA as a substrate. (±)-[2-^{14}C]-MVA was conveniently prepared by a short synthesis described earlier (Banerji and Kalena 1983). Degradation of 2, obtained from biosynthesised 1, gave acetone containing 45% of the total incorporated radioactivity (expected theoretical incorporation is 50%). This indicates that two units of MVA are involved in the biosynthesis of 1. Anisic acid and formaldehyde, the other isolable degradation products did not show significant labelling. Since radioactive acetone is obtained from the DMAPP derived part of 1, this experiment also establishes that DMAPP derived moiety of 1 is also biosynthesised from MVA and not by the alternate pathway suggested by Tange (1981).

Recently, it has been suggested that bakuchiol could be derived from the thiamine mediated condensation between tyrosine and geranyl pyrophosphate (Risinger *et al* 1981). According to this hypothesis cinnamic acids are not involved in the biosynthesis. Results obtained from our experiments do not agree with this hypothesis since incorporation of labelled tyrosine was much lower when compared to phenylalanine (table 1). Incorporation of labelled cinnamic acid into bakuchiol further rules out the possibility of the above type of biosynthesis.

As has been mentioned earlier, incorporation of amino acids into the DMAPP derived portion of monoterpenes has been shown in *Cinnamomum camphora* and *Pelargonium roseum*. However, the incorporation of the label in both the cases is rather low. In order to examine the possibility of participation of leucine in the biosynthesis of the DMAPP derived part of 1, [U-^{14}C]-L-leucine was fed to the plant. Isolation of radiolabelled bakuchiol indicated the incorporation of leucine (table 1). However, degradation experiments showed that the label from leucine got randomised since anisic acid contained a very significant portion (27%) of the incorporated activity.

4. Conclusion

Though the biosynthesis of higher terpenoids is well understood, details of the biosynthesis of lower terpenes are far from clear and there are some special features. The incorporation of well-known substrates is usually very low. Radomisation of the labels is often seen in monoterpene biosynthesis. Incorporation of leucine and valine into the dimethylallyl moiety of certain monoterpenes suggests that DMAPP is biosynthesised from amino acid by a route which is different from the well-known MVA pathway.

Biosynthesis of bakuchiol has been carried out to study some of the features mentioned above. The monoterpene side chain of bakuchiol is biosynthesised from MVA and there is equal distribution of labels in the IPP and DMAPP moieties. Leucine gets incorporated into bakuchiol with extensive randomisation. The non-terpenic part of bakuchiol arises from the phenylpropane pathway.

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