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ROLE OF ACYCLIC COMPOUNDS IN MONOTERPENE BIOSYNTHESIS IN *PINUS PINASTER*

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Abstract—The biosynthesis of monoterpene hydrocarbons was studied in maritime pine needles by incorporation of $^{14}CO_2$. It was shown that the acyclic terpenes β-myrcene and *trans*-β-ocimene, act as transitory compounds before the biosynthesis of cyclic monoterpenes such as α- and β-pinene. The mechanisms of biosynthesis are genetically controlled.

INTRODUCTION

Feeding experiments with $^{14}CO_2$ have shown [1] that the biosynthesis of monoterpene hydrocarbons (C_{10}) in maritime pine needles is associated with the active secretory cells located at the base of the needles. Moreover, a metabolic role was attributed to trans-β-ocimene in the synthesis of acyclic hydrocarbons [2]. This compound is an acyclic hydrocarbon which is always present in very small amounts in maritime pine needles [2]. In contrast, β-myrcene, another acyclic monoterpene, showed significant variations from tree to tree and it was established that its content is controlled by a single gene. The allele *M* is responsible for a high level of β-myrcene and is dominant to the allele *m* responsible for a low level [3]. The aims of this study were to investigate the behaviour of β-myrcene in the biosynthesis of monoterpene hydrocarbons and to compare the roles of β-myrcene and *trans*-β-ocimene in the C_{10} biosynthetic pathways of different clones of maritime pine.

RESULTS

The incorporation of $^{14}CO_2$ into acyclic monoterpenes was studied for 15 phenotypes of maritime pine characterized either by a high amount of β-myrcene (myrcene-rich) or by a small amount (myrcene-poor). After 10 min exposure to $^{14}CO_2$ (Table 1) myrcene-poor phenotypes showed two kinds of response: either the tracer was only incorporated in β-myrcene or β-myrcene and *trans*-β-ocimene were both radioactive but in different percentages according to the phenotypes. In the myrcene-rich phenotypes, the radioactivity was entirely located in *trans*-β-ocimene.

Pulse-chase experiments with $^{14}CO_2$ were performed with three different phenotypes (Table 2) whose characteristics were: myrcene-rich (clone 00 01), myrcene-poor (clone 01 47) and β-myrcene- and β-pinene-poor (clone 3 43 19 2). In the case of clone 00 01, the *trans*-β-ocimene was highly labelled after the 15 min pulse of $^{14}CO_2$ while other monoterpene hydrocarbons (α- and β-pinene, β-myrcene) were poorly labelled (Fig. 1a). The radioactivity in the *trans*-β-ocimene declined steadily in the chase period and disappeared completely after 6 hr. As has already been demonstrated elsewhere [2], the increase in the amount of radioactivity (cpm/g) in the two pinenes in the chase period corresponded almost exactly to that lost by the *trans*-β-ocimene. In the experiment with clone 01 47, the only acyclic monoterpene labelled after 15 min exposure to $^{14}CO_2$ was β-myrcene (Fig. 1b) which also completely lost its radioactivity after 6 hr whilst that in α- and β-pinene increased. In this poor-myrcene phenotype, β-myrcene has a behaviour similar to that of *trans*-β-ocimene in the myrcene-rich phenotype. The oils from these two phenotypes are rich in β-pinene (Table 2) and this compound was the main cyclic hydrocarbon labelled at the end of the 6 hr chase, particularly in clone 00 01. In the experiment with clone 3 43 19 2 [characterized by a small amount of β-myrcene, a β-pinene concentration lower than 20% and a high α-pinene content (Table 2)], β-myrcene was rapidly and highly labelled from $^{14}CO_2$ but the first pinene elaborated while the radioactivity was lost from β-myrcene was the β-isomer (Fig. 1c). α-Pinene was labelled much more slowly, and its radioactivity exceeded that of β-pinene only 6 hr after the $^{14}CO_2$ pulse. It should be noted that in this phenotype, there was no more radioactivity in β-myrcene after 2 hr. In contrast, in the myrcene-rich phenotype (00 01), the tracer was accumulated in β-myrcene but to a lesser extent than in α- and β-pinene (Fig. 1a). The radioactivity was also weakly accumulated in the other cyclic monoterpene hydrocarbons such as Δ_3-carene or limonene (below 1%) not shown in Fig. 1.

Table 1. Percentage distribution of radiolabel between *trans*-β-ocimene and β-myrcene after 10 min exposure to $^{14}CO_2$

Clones	Phenotypes	*Trans*-β-ocimene	β-Myrcene
Myrcene-rich*			
00 01	$\beta^+M^-C^+L^-O^-$	100	0
13 08	$\beta^+M^+C^+L^-O^-$	100	0
01 42	$\beta^+M^+C^-L^+O^-$	100	0
01 46	$\beta^+M^+C^-L^-O^-$	100	0
43 06	$\beta^+M^+C^-L^-O^-$	100	0
01 60	$\beta^+M^+C^-L^-O^-$	100	0
38 44 12 2	$\beta^-M^+C^-L^-O^-$	100	0
Myrcene-poor†			
31 15	$\beta^+M^-C^+L^-O^-$	95	5
01 33	$\beta^+M^-C^+L^-O^-$	90	10
02 83	$\beta^+M^-C^-L^+O^-$	0	100
13 05	$\beta^+M^-C^+L^-O^-$	95	5
43 01	$\beta^+M^-C^+L^-O^-$	80	20
13 03	$\beta^+M^-C^-L^+O^-$	90	10
01 47	$\beta^+M^-C^-L^-O^-$	0	100
3 43 102	$\beta^-M^-C^-L^-O^-$	80	20

+ or − indicate a high or low level in the hydrocarbon concentration of the needles for β-pinene (β), β-myrcene (M), Δ_3-carene (C), limonene (L) and *trans*-β-ocimene (O).

*Genotype *MM* or *Mm*.

†Genotype *mm*.

Table 2. Percentages of monoterpene hydrocarbons of the needles of three clones of maritime pine representing three different phenotypes

Compounds	Clones and phenotypes		
	00 01 $\beta^+M^+C^+L$	01 47 $\beta^+M^-C^-L^-$	3 43 19 2 $\beta^-M^-C^+L$
α-Pinene	21.7	44.7	57.0
Camphene	0.6	0.3	6.9
β-Pinene	45.3	51.3	17.3
Δ_3-Carene	13.3	0	10.7
β-Myrcene	11.6	1.0	1.3
Limonene	1.9	1.5	3.7
β-Phellandrene	1.1	0.3	1.1
trans-β-Ocimene	2.2	0.7	0.3
Terpinolene	2.0	0	1.3
Total	99.7	99.8	99.6

The phenotypes are defined in Table 1.

DISCUSSION

In maritime pine needles, *trans*-β-ocimene is not the only acyclic hydrocarbon acting as a metabolic form in monoterpene biosynthesis. Thus β-myrcene has the same role in trees with the genotype *mm*. In the scheme proposed in Fig. 2, different steps lead to the formation of α-pinene (**11**) and β-pinene (**10**). The pathways from geranyl, neryl or even linaloyl pyrophosphate have as intermediates the acyclic carbonium ions **1–4** and **4'**, however, the alcohols could be involved as well. Nevertheless, in our experiments, we have never observed the presence of labelled C_{10} alcohols. From **3** the cyclic ion **5** could be irreversibly formed and α- and β-pinene would be directly synthesized [4–8] via **6**. In this situation, α- and β-pinene would be irreversibly accumulated. The fact that radioactivity accumulates first in *trans*-β-ocimene (**9**) or in β-myrcene (**8**) and then disappears gradually shows that these acyclic hydrocarbons are easily metabolized in pine needles. These results suggest that the carbonium ions **2** and **4–4'** are first capable of leading to the biosynthesis of β-myrcene or *trans*-β-ocimene. These mechanisms could be reversible. Addition of a proton to β-myrcene or *trans*-β-oci-

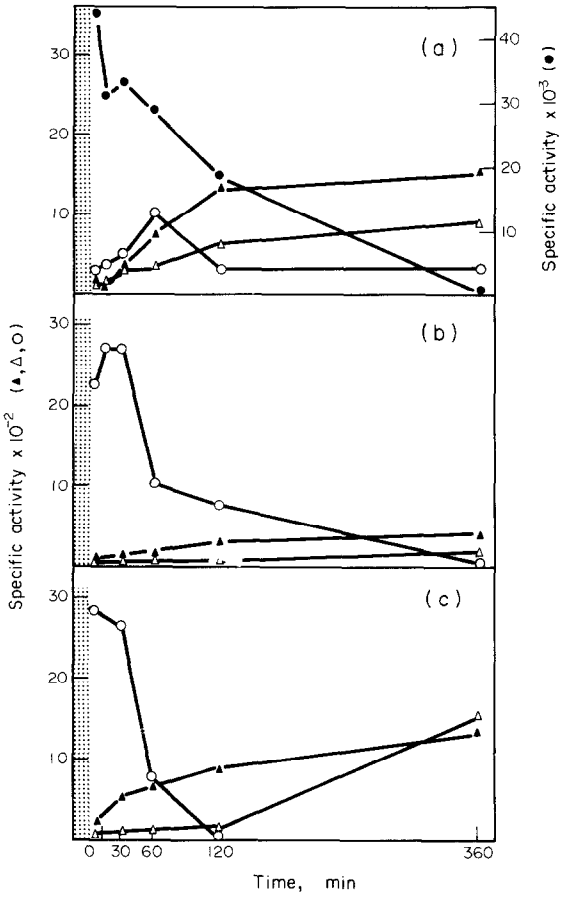

Fig. 1. Time course of the labelling of the main monoterpene hydrocarbons after 15 min of exposure to $^{14}CO_2$ (dotted area) followed by 6 hr $^{14}CO_2$. (a) Clone 00 01; (b) clone 01 47; (c) clone 3 43 19 2. The phenotypes are given in Table 2. (●) *trans*-β-Ocimene; (○) β-myrcene; (△) α-pinene; (▲) β-pinene.

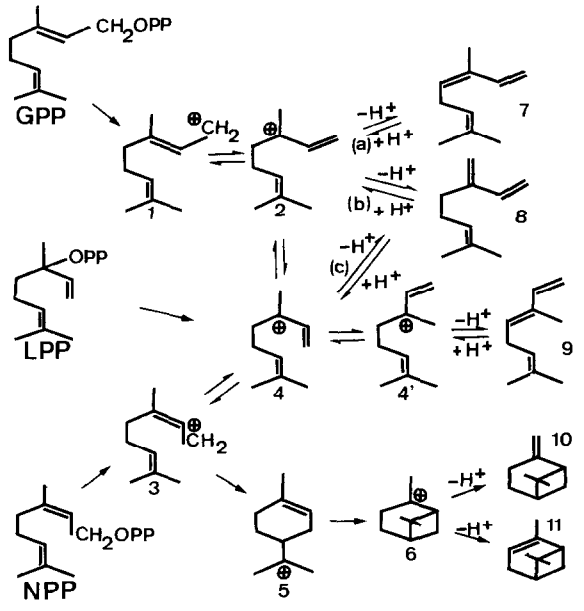

Fig. 2. Probable mechanisms of biosynthesis of monoterpene hydrocarbons from geranyl pyrophosphate (GPP), neryl pyrophosphate (NPP) or linaloyl pyrophosphate (LPP) by the means of intermediate carbocations. Step (a) leading to *cis*-β-ocimene (**7**) never occurs in maritime pine needles. Steps (b) and (c) leading to β-myrcene (**8**) are reversible only in genotype *mm* and are irreversible in genotypes *Mm* and *MM*. The step leading to *trans*-β-ocimene (**9**) is always reversible.

mene would give rise again to ion **4** and to the above process of formation of α- and β-pinene. In maritime pine needles, the mechanism is always reversible for *trans*-β-ocimene, for β-myrcene it occurs only in myrcene-poor phenotypes. In myrcene-rich phenotypes, the reactions (b) or (c) from ions **2** or **4** induce the irreversible formation of β-myrcene. In these cases, genetic control prevents the addition of a proton. Therefore, β-myrcene is accumulated with the cyclic hydrocarbons in the resin ducts after their synthesis by the active secretory cells located at the base of the needles [1].

Another theoretical possibility for the synthesis of α- and β-pinene is that they could be formed from *cis*-β-ocimene (**7**) as proposed by Ruzicka [4], reaction (a) in Fig. 2. This hypothesis was not verified in our experiments in which we never observed the synthesis of *cis*-β-ocimene from $^{14}CO_2$.

The results demonstrate clearly the physiological role of acyclic hydrocarbons as transitory products in the synthesis of cyclic compounds in pine needles. The formation of acyclic hydrocarbons, which depends on the phenotype, appears to be bound to the elimination of a proton occurring preferentially on a given form of carbonium ion. This specificity and the reversibility allowing the conversion from the acyclic hydrocarbon to the carbonium ion could be connected with a genetic origin [9–13] and would be explained by binding of these ions with specific enzymatic sites [7].

EXPERIMENTAL

The phenotypes of maritime pine (*P. pinaster* Ait) were located at the Station d'Amélioration des Arbres Forestiers, INRA, Pierroton-Cestas, France. The expts were performed in June–July (the growing period of young needles), 1978–1980 and were repeated three times a year. Incorporations of $^{14}CO_2$ (200 μCi by assay) were made on young shoots according to the method described in ref. [1].

At the end of the expts, the needles (25 g) were quickly cut into small pieces so that they fell directly into glass stoppered conical flasks containing 35 ml pentane. The pentane extracts were dried (Na_2SO_4) and reduced to a small vol. (5 ml) under vacuum. The hydrocarbons were separated from the oxygenated compounds on Kieselgel 60 (Merck) columns [1].

Radioactive measurements and radio-GC of the hydrocarbon fractions were carried out following the methods described in refs. [1, 14].

The composition of the oil of maritime pine needles has already been described [15]. The coincidence of the radioactivity with the cyclic and acyclic hydrocarbons observed in radio-GC was checked after fractionation of radioactive extracts plus 4 g of a mixture of hydrocarbons extracted from pine needles. The fractionation was carried out on a $AgNO_3$–Kieselgel 60 (1:9) column (15 × 800 mm) packed in pentane. The column was eluted first with 300 ml pentane, then with 200 ml each of 1%, 2%, 5%, 10%, 15%, 25% and 50% Et_2O–pentane and Et_2O. Fractions of 10 ml each were collected. The fractions were assayed for radioactivity and the radioactive fractions analysed by radio-GC. The labelled compounds were purified by prep. GC: 10% polypropylene glycol sebacate (150 × 0.63 cm); temp. programme 70–210° at 4°/min; N_2 50 ml/min; injector temp. 140°; detector 230°. The purified compounds were identified by IR and GC/MS. α-Pinene was recovered from the $AgNO_3$–Kieselgel column in the fraction eluted with pentane–Et_2O (99:1) and (49:1), β-pinene with (49:1) and (19:1). β-Myrcene and *trans*-β-ocimene were obtained in the fraction eluted with pentane–Et_2O (17:3).

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