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SESQUITERPENE BIOSYNTHESIS IN MARITIME PINE NEEDLES

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Abstract—The biosynthesis of sesquiterpene hydrocarbons was studied in maritime pine (*Pinus pinaster*) needles by incorporation of $^{14}CO_2$, [1-^{14}C]acetate and [2-^{14}C]mevalonate. It was shown that the mechanisms of sesquiterpene biosynthesis are different according to the applied tracer. The important role of the acyclic compound, *trans*-β-*farnesene*, before cyclisation processes is discussed.

INTRODUCTION

The compartmentation of synthesis of monoterpene and sesquiterpene hydrocarbons in maritime pine has been described previously [1, 2]. Similarly it has been suggested that in *Mentha* [3, 4] the monoterpenes and sesquiterpenes are synthesized at different sites and that the sesquiterpene site is the less isolated of the two. Furthermore, if the oil glands are the site of biosynthesis of mono- and sesquiterpenes, numerous biosynthetic sites are probably located within the glands themselves [5]. In maritime pine, monoterpene biosynthesis is restricted to young secretory cells located at the base of the leaves but sesquiterpene elaboration does not appear to be limited to specialised structures [6]. As the composition of the oil of leaves or cortical tissues exhibits a large variation between individuals [7], it is absolutely necessary to work on a single clone in order to study the biosynthesis of terpene compounds. Furthermore, in maritime pine, it has been shown that the synthesis of some sesquiterpenes is genetically controlled. This hereditary control is monogenic for longifolene and caryophyllene [8]. The aim of this study was to investigate the biosynthetic patterns leading to sesquiterpene hydrocarbons from three precursors, $^{14}CO_2$, [1-^{14}C]acetate and [2-^{14}C]mevalonate, and to compare their utilisation in the biosynthesis of sesquiterpene hydrocarbons by the needles.

RESULTS AND DISCUSSION

The analysis of the sesquiterpene hydrocarbons of the oil of the leaves of the clone of maritime pine selected for this study is given in Table 1. The main sesquiterpenes of the oil were all cyclic compounds such as caryophyllene, δ- and γ-cadinene, γ-muurolene, germacrene D and humulene whereas the acyclic *trans*-β-farnesene was present in small amount (1% of total hydrocarbons).

The time course labelling (Table 2) of sesquiterpene hydrocarbons from $^{14}CO_2$, [1-^{14}C]acetate and [2-^{14}C]mevalonate showed that the radioactivity incorporated into total sesquiterpenes from MVA after 12 hours was ten times greater than with $^{14}CO_2$. [1-^{14}C]Acetate was almost as efficient a tracer as MVA. However, if

Table 1. Amount and percentage composition of the sesquiterpene hydrocarbons of maritime pine leaves (clone 00 01)

Compounds	μg/g (fr. wt)	%
α-Cubebene	8	0.65
α-Copaene	27	2.24
α-Longipinene	3	0 23
Unknown compound	3	0 23
Longifolene	4	0 31
Caryophyllene	160	12 86
Trans-β-farnesene	14	1.15
γ-Muurolene	44	3.66
α-Humulene	25	2.10
γ-Amorphene	2	0.18
α-Muurolene	5	0.43
Germacrene D	51	4.18
δ-Cadinene	88	7.27
γ-Cadinene	34	2.83
β_1-Cadinene	2	0.15
Calamenene	20	1.71
Total sesquiterpene hydrocarbons	490	40.18
Total monoterpene hydrocarbons	729	59.81

The relative percentages are expressed as a percentage of the total volatile hydrocarbons.

Table 2. Incorporation yields of [^{14}C] substrates into the sesquiterpene hydrocarbons of maritime pine leaves during the time course labelling

	Precursor		
Time (hr)	$^{14}CO_2$	[1-^{14}C]Acetate	[2-^{14}C]Mevalonate
0.5	1×10^{-5}	—	—
3	3×10^{-5}	18×10^{-5}	8×10^{-5}
12	2×10^{-5}	20×10^{-5}	25×10^{-5}

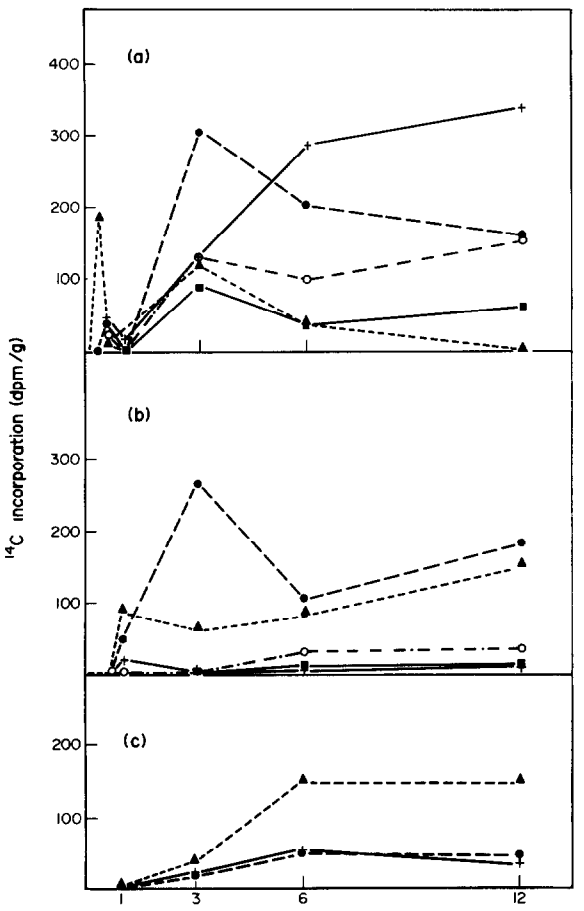

Fig. 1. Time-course of the labelling of the main sesquiterpene hydrocarbons after exposure to $^{14}CO_2$ (a), [1-^{14}C]acetate (b) and [2-^{14}C]mevalonate (c). Each graph represents the average of 9 experiments. (▲) farnesene; (●) caryophyllene; (+) germacrene D + γ-muurolene; (■) cadinene; (○) humulene.

individual sesquiterpene hydrocarbons were considered, their biosynthesis from these three precursors was not the same under our experimental conditions. With $^{14}CO_2$ as precursor (Fig. 1a), a terpenoid was very quickly synthesized, but synthesis of this compound was transitory. A maximum amount of label appeared after only 12 min, then the radioactivity declined up to 12 hr. The compound, which first contained all the radioactivity found in the sesquiterpene hydrocarbons, was identified as *trans*-β-farnesene, an acyclic molecule. The elaboration and the accumulation of cyclic compounds followed the decrease in the amount of *trans*-β-farnesene. This transitory synthesis of one acyclic compound before the formation of cyclic hydrocarbons may be compared with the behaviour of *trans*-β-ocimene or myrcene in the case of monoterpene biosynthesis from $^{14}CO_2$ [9]. As with *trans*-β-ocimene and myrcene, the transitory farnesene was always present in a very small amount in extracts of leaves. With [2-^{14}C]mevalonate as the precursor, the elaboration of *trans*-β-farnesene occurred later after 6 hr (Fig. 1c). Then the amount of label incorporated into this acyclic compound remained steady up to the end of the study. Cyclic compounds were accumulated but always in a small amount. When using [1-^{14}C]acetate (Fig. 1b), it should be noticed that the biosynthetic pathway of sesquiterpene production was intermediary between the synthesis pattern observed from $^{14}CO_2$ and the one from [2-^{14}C]mevalonate. This fact appeared more clearly when pulse experiments were performed. Table 3 shows the specific activity and the relative percentage of radioactivity found in the acyclic compound (*trans*-β-farnesene) on the one hand and in monocyclic (α-humulene and germacrene D), bicyclic (caryophyllene, cadinenes) and tricyclic (α-copaene) sesquiterpenes on the other hand. It should be noted that α-copaene was always synthesized in a very small amount from $^{14}CO_2$ or [1-^{14}C]acetate and it was not elaborated from [2-^{14}C]mevalonate. Three cases were observed. After 6 hr of exposure to $^{14}CO_2$, *trans*-β-farnesene exhibited a low specific activity and only 5.5% of the total of the radioactivity. More than 50% of the label was found in monocyclic sesquiterpenes and nearly

Table 3. Specific activity (dpm/g) and percentage distribution of radiolabel between *trans*-β-farnesene, bicyclic and tricyclic sesquiterpene hydrocarbons during time course labelling after 6 hr of exposure to $^{14}CO_2$, [1-^{14}C]acetate or [2-^{14}C]mevalonate

Time course of labelling (hr)		*trans*-β-Farnesene sp. act.	%	Monocyclic sesquiterpenes sp act	%	Bicyclic and tricyclic sesquiterpenes sp act.	%
$^{14}CO_2$	6+0	5420	5.5	3583	56 5	7173	37 8
	6+6	—	—	2810	68.5	4577	31 4
	6+36	—	—	5660	56.8	12006	43 2
	6+96	—	—	7791	43.8	27467	56.2
	6+192	—	—	9001	38 2	27409	61.7
[1-^{14}C]Acetate	6+0	10569	34.9	489	14.3	2125	50 6
	6+6	14770	34.6	382	7.9	3700	57 4
	6+36	13618	31 2	405	8.1	3505	60 6
	6+96	4878	8.6	413	6.5	6294	84 8
	6+192	2032	1.3	1237	7.1	15883	91.6
[2-^{14}C]Mevalonate	6+0	20054	58.5	328	22.1	652	19 3
	6+6	19376	57.6	246	16.9	838	25.4
	6+36	11463	58.9	296	19.5	545	21 5
	6+96	33739	64.8	497	22 1	665	13.0
	6+192	47831	55 5	1030	27.7	1423	16.8

40% was in bicyclic compounds. At the end of the chase, *trans*-β-farnesene had completely lost its radioactivity; the label declined in monocyclic hydrocarbons and increased in bicyclic sesquiterpenes. With MVA as the precursor, the radioactivity remained equal throughout the chase in *trans*-β-farnesene which contained about 55% of the whole radioactivity measured in sesquiterpenes. The pulse experiment obtained from [1-^{14}C]acetate gave intermediary results. The radioactivity present in *trans*-β-farnesene at the beginning of the pulse showed a subsequent decrease while the radioactivity in bicyclic sesquiterpenes increased.

These results demonstrate the role of the acyclic hydrocarbons as transitory products in the synthesis of cyclic compounds in pine needles. From $^{14}CO_2$, the only precursor that can be fed at physiological levels, *trans*-β-farnesene was steadily metabolized and the radioactivity was accumulated in cyclic terpenes. The experimental conditions used with MVA and acetate may perturb the metabolic pathway. Consequently, farnesene elaborated from MVA was not metabolized during the chase period and there was no synthesis of cyclic compounds. The elaboration of cyclic sesquiterpenes and the metabolism of *trans*-β-farnesene were obtained from [1-^{14}C]acetate but the processes were very slow.

When *trans*-β-farnesene is metabolized, the nature of the cyclic compounds synthesized is dependent on the precursor used in the experiments. With $^{14}CO_2$, δ-cadinene was elaborated whereas [1-^{14}C]acetate led to γ-cadinene. In all cases, the level of incorporation of the three precursors into sesquiterpenes was low and their specific behaviour emphasizes the multiple factors associated with the biosynthesis of each sesquiterpene hydrocarbons.

EXPERIMENTAL

The clone 00 01 of maritime pine (*Pinus pinaster* Ait) used in this work was located at the Station d'Amélioration des Arbres Forestiers, INRA, Pierroton-Cestas, France.The expts were performed in June–July 1978–1980 and were repeated three times a year. Incorporations of $^{14}CO_2$ (53 mCi/mM) were made on needles (200 μCi by assay) according to the method described in ref [1]. Incorporations of [1-^{14}C]acetate (57 mCi/mmol) and [2-^{14}C]mevalonate (36 mCi/mmol) were performed on leaf pieces (25 μCi by assay) as reported earlier [2].

The methods of preparation and treatment of plant material were described in ref. [9] Hydrocarbons were obtained after fractionation on Kieselgel 60 (Merck) columns [1]. Radioactive measurements and radio-GC were carried out following the methods described in refs [1, 2].

The coincidence of the radioactivity with the cyclic and acyclic sesquiterpene 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 an $AgNO_3$–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%, 20%, 30%, 40%, 50%, 60% and 75% Et_2O–pentane, 200 ml Et_2O and 200 ml MeOH. Fractions of 10 ml each were collected. Each fraction was counted by liquid scintillation spectrometry. The radioactive fractions were analysed by radio-GC. The labelled compounds were purified by preparative GC. 10% polypropylene glycol sebacate (150 × 0.63 cm) column, temp. programmed 70–210° at 4°/min; argon: 50 ml/min; injector temp. 180°, detector 230°. The purified compounds were identified by IR, NMR, GC/MS and checked again by liquid scintillation spectrometry and radio-GC with two different columns: 10% SE 30 (150 × 0.63 cm) and 10% polypropylene glycol sebacate (275 × 0.63 cm). α-Copaene was recovered from the $AgNO_3$–Kieselgel column in the fraction eluted with pentane–Et_2O (9:1), δ-cadinene with the first fractions of the mixture (4.1), γ-muurolene and γ-cadinene with the last fractions (4:1), germacrene D with (3.2), caryophyllene with (3:2) and (1:1), *trans*-β-farnesene with (2.3). Radioactive humulene was obtained with MeOH.

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REFERENCES

1. Bernard-Dagan, C., Carde, J P. and Gleizes, M. (1979) *Can. J. Botany* **57**, 255.
2. Gleizes, M., Pauly, G., Bernard-Dagan, C and Jacques, R. (1980) *Physiol. Plant.* **50**, 16.
3 Croteau, R. and Loomis, W D. (1972) *Phytochemistry* **11**, 1055.
4. Loomis, W. D. and Croteau, R. (1980) in *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds), Vol. 4, *Biochemistry of Terpenoids*, p. 363. Academic Press, New York.
5. Croteau, R., Burbott, A. J and Loomis, W. D (1972) *Phytochemistry* **11**, 2937
6. Bernard-Dagan, C., Pauly, G., Marpeau, A., Gleizes, M., Carde, J. P. and Baradat, P. (1982) *Physiol. Vég.* **20**, 775.
7. Baradat, P., Bernard-Dagan, C and Marpeau, A (1978) in *Biochemical Genetics of Forest Trees* (Rudin, D., ed.) p. 151 Sveriges lantbruksuniversitet, Umea.
8. Marpeau, A., Baradat, P and Bernard-Dagan, C (1975) *Ann. Sc Forest* **32**, 185
9. Gleizes, M., Marpeau, A., Pauly, G. and Bernard-Dagan, C. (1982) *Phytochemistry* **21**, 2641