Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 1529–1535
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Biosynthesis of pyrethrin I in seedlings of *Chrysanthemum cinerariaefolium*

Kazuhiko Matsuda [a,*], Yukio Kikuta [a], Atsushi Haba [a], Koji Nakayama [b], Yoshio Katsuda [b], Akikazu Hatanaka [c], Koichiro Komai [a]

[a] *Department of Applied Biological Chemistry, School of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan*
[b] *Dainihon Jochugiku Co., Ltd., 1-1-11 Daikoku-cho, Toyonaka 561-0827, Japan*
[c] *Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan*

Received 1 July 2004; received in revised form 7 April 2005
Available online 16 June 2005

Abstract

The biosynthetic pathway to natural pyrethrins in *Chrysanthemum cinerariaefolium* seedlings was studied using [1-^{13}C]D-glucose as a precursor, with pyrethrin I isolated using HPLC from a leaf extract. The ^{13}C NMR spectrum of pyrethrin I from the precursor-administered seedlings indicated that the acid moiety was biosynthesized from D-glucose via 2-*C*-methyl-D-erythritol 4-phosphate, whereas the alcohol moiety was possibly biosynthesized from linolenic acid.

Keywords: *Chrysanthemum cinerariaefolium*; Compositae; Pyrethrin; Biosynthesis; Non-mevalonate pathway; Octadecanoid pathway; 1-Deoxy-D-xylulose 5-phosphate; 2-*C*-methyl-D-erythritol 4-phosphate

1. Introduction

Pyrethrins are insecticidal second metabolites of *Chrysanthemum cinerariaefolium* which accumulate in high concentrations in the flowers (Casida, 1973; Morgan and Wilson, 1999). Natural pyrethrins, as well as dried flower powders including them, were previously employed to control household pests, but have been replaced by synthetic pyrethroids since the natural insecticides are inferior in stability, insecticidal efficacy and production costs (Katsuda, 1999). Nevertheless, recent requirements for safe and environmentally friendly pesticides are boosting the use of natural pyrethrins. Thus, a detailed understanding of the biosynthetic pathway to pyrethrins is of potential importance to enhance pyrethrin levels in *C. cinerariaefolium*, e.g., in order to lower production costs and to enable construction of transgenic plants synthesizing pyrethrins.

Pyrethrins that possess an ester linkage are classified into types I and II (Fig. 1, compounds **1**–**6**). The type I esters (**1**–**3**) have a chrysanthemoyl moiety, whereas type II (**4**–**6**) have a pyrethroyl moiety with an additional ester linkage, methoxycarbonyl group. A common structural feature of the acid moieties is the presence of a cyclopropane ring. Rivera et al. (2001) have isolated chrysanthemyl diphosphate synthase (CPPase) and cloned the gene from chrysanthemum flowers. The recombinant CPPase expressed in *E. coli* was able to catalyze cyclopropane ring formation to give chrysanthemyl pyrophosphate (**12**) using two molecules of dimethylally pyrophosphate (DMAPP (**11**)) with the substrate generated by isomerization of isopentenyl pyrophosphate (IPP (**10**)) (Fig. 2). IPP (**10**) is biosynthesized via mevalonate (**8**) in animals, but recent research has shown that IPP (**10**) is also biosynthesized

* Corresponding author. Tel.: +81 742 43 7153; fax: +81 742 43 1445.
E-mail address: kmatsuda@nara.kindai.ac.jp (K. Matsuda).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.05.005

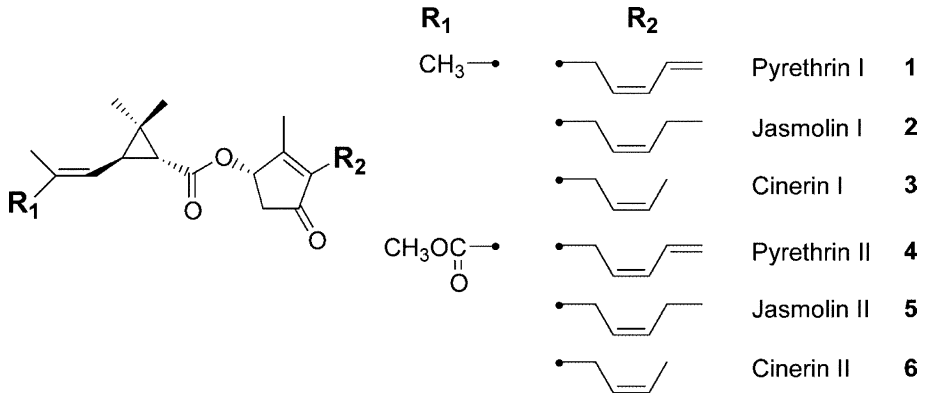

Fig. 1. Chemical structures of natural pyrethrins (**1**–**6**).

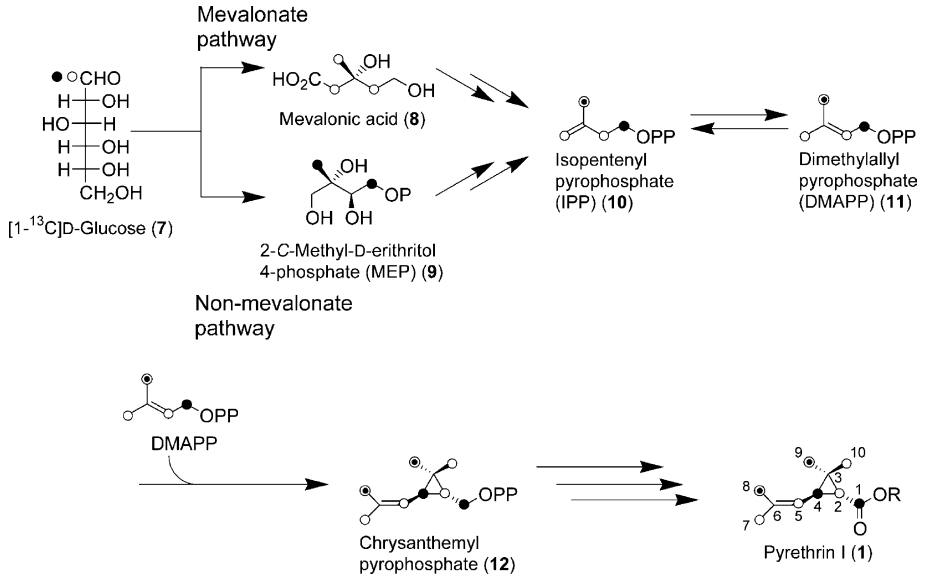

Fig. 2. Biosynthesis of the acid moiety of pyrethrin I (**1**) via mevalonate and non-mevalonate pathways. Open and closed symbols show the ^{13}C label from [1-^{13}C]D-glucose (**7**) in compounds occurring in the mevalonate and non-mevalonate pathways, respectively.

via 1-deoxy-D-xylulose 5-phosphate (DXP) and its metabolite 2-*C*-methyl-D-erythritol 4-phosphate (MEP, **9**) in plants and many eubacteria (Rohmer et al., 1996; Rohmer, 1999; Dubey et al., 2003; Eisenreich et al., 2004) (Fig. 2). Such a "non-mevalonate pathway" or "mevalonate-independent pathway", involving DXP and MEP (**9**) as intermediates, is seen in the plastids, whereas the mevalonate pathway is present in the cytosol (Lichtenthaler, 1999; Rohmer, 1999). Hence, although it has been shown that [^{14}C]mevalonic acid (**8**) was incorporated into the acid moiety of pyrethrins when investigated using the flowers (Crowley et al., 1961, 1962), the non-mevalonate pathway can be involved, as a major pathway in the biosynthesis of the acid moiety, at least in leaves.

On the other hand, pyrethrolone (**19**), the keto-alcohol moiety of pyrethrin I (**1**), is conceivably generated from *cis*-jasmone (**17**, Fig. 3). Alternatively, it may be generated from 7-hydroxy-jasmonic acid (**18**), which is unsaturated and subsequently decarboxylated to give jasmolone. Since not only *cis*-jasmone (**17**, Koch et al., 1997), but also its upstream compounds 12-oxo-phytodienoic acid (**16**) and jasmonic acid (Yoshihara and Greulich, 1999; Feussner and Wasternack, 2002), are biosynthesized from linolenic acid (**15**), pyrethrolone probably derives from linolenic acid (**15**) as predicted earlier by Crombie (1995). However, no convincing result in support of this prediction has been obtained.

The aim of this study was to examine these hypotheses for pyrethrin biosynthesis in the seedlings of *C. cinerariaefolium*. [1-^{13}C]D-Glucose (**7**) was administered to the seedlings, with pyrethrin I (**1**) isolated thereafter subjected to ^{13}C NMR spectroscopic analysis. The results suggest that the acid moiety is synthesized from MEP (**9**) by the non-mevalonate pathway (Fig. 2), whereas

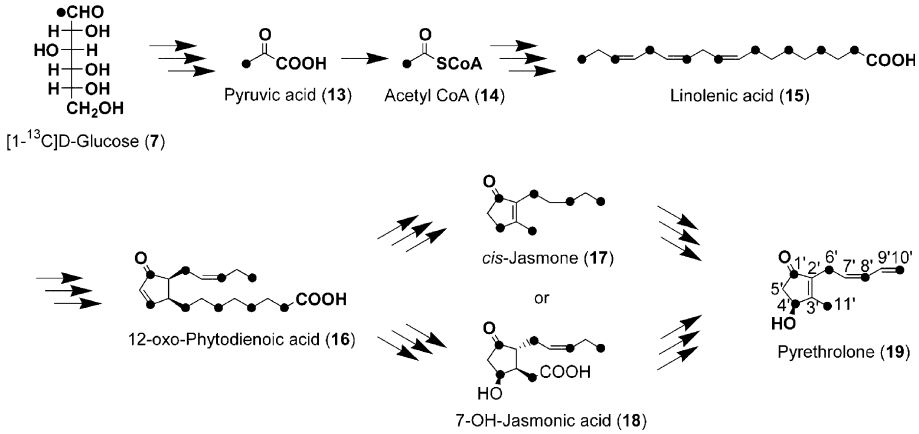

Fig. 3. Biosynthesis of the alcohol moiety of pyrethrin I via linolenic acid (**15**). Closed ^{13}C label from [1-^{13}C]D-glucose (**7**).

the alcohol moiety is likely to stem from linolenic acid (**15**) by the octadecanoid pathway (Fig. 3).

2. Results and discussion

All six pyrethrin members (**1**–**6**) were found to be present in the leaf extract of the *C. cinerariaefolium* seedlings (Fig. 4) as reported elsewhere (Zito and Tio, 1990). This is not surprising because pyrethrins are thought to operate as defense substances (e.g., insect antifeedants). The presence of pyrethins in young seedlings with no flowers demonstrates that the compounds build up in the leaves. Preliminary experiments using 2 months old seedlings revealed that, among natural pyrethrins (**1**–**6**, Fig. 1), pyrethrin I (**1**) was present at the highest concentration. In addition, the highest content of pyrethrin I (**1**) was observed in 5–6 months old seedlings (Fig. 4). Therefore, considering these preliminary findings along with greater leaf amounts compared to those of younger seedlings, 6 months old seedlings were employed for the ^{13}C-administration experiments.

The two possible biosynthetic pathways for the acid moiety of pyrethrin I (**1**) are illustrated in Fig. 2. A key step in the biosynthesis is the cyclopropanation catalyzed by CPPase. According to the reaction mechanism proposed for CPPase (Rivera et al., 2001), C-2, 5, 7–10 in pyrethrin I (see Figs. 2 and 5 for these numbering of the carbons) are labeled if mevalonate (**8**) is the intermediate, whereas C-1, 4, 8 and 9 are labeled if MEP (**9**) is the intermediate (Fig. 2). The ^{13}C NMR spectrum (Fig. 5) of pyrethrin I (**1**) isolated from the precursor-treated seedlings indicated that ^{13}C was preferentially incorporated into the C-1, 4, 8 and 9 positions (Fig. 5 and Table 1),

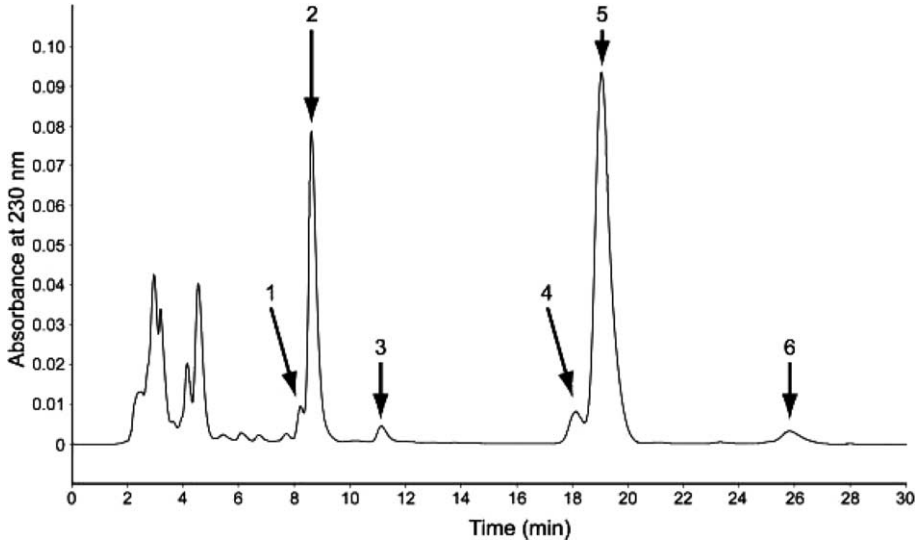

Fig. 4. High-performance liquid chromatogram (HPLC) of natural pyrethrins contained in a leaf extract of 6 months old seedlings of *C. cinerariaefolium* (see Section 3). The arrows 1–6 indicate the peaks of natural pyrethrins (peak 1, cinerin II (**6**); peak 2, pyrethrin II (**3**); peak 3, jasmolin II (**4**); peak 4, cinerin I (**3**); peak 5, pyrethrin I (**1**); peak jasmolin I (**2**)).

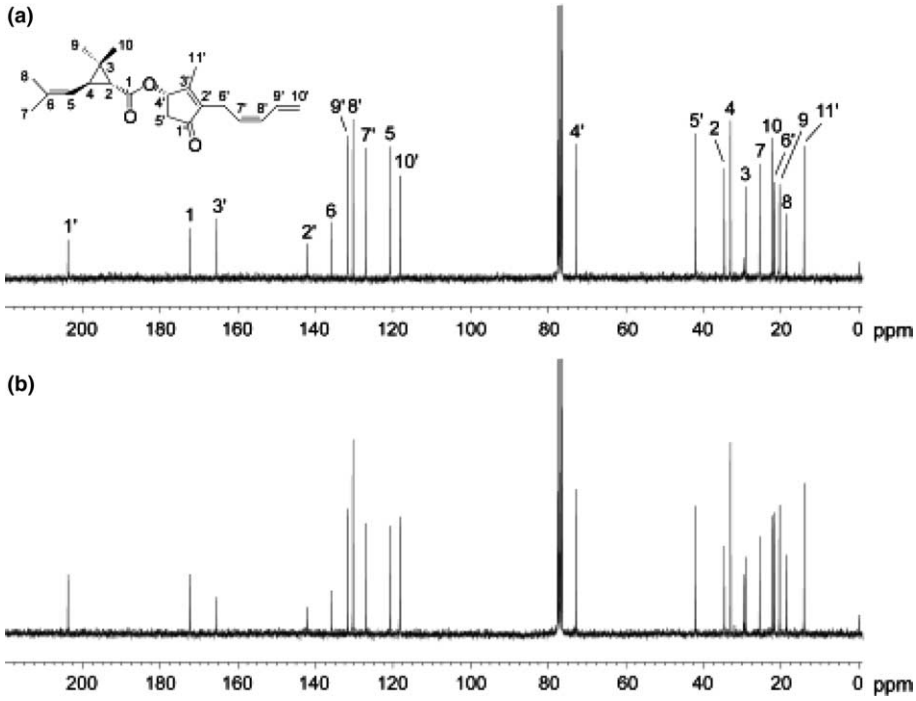

Fig. 5. ^{13}C NMR spectra of control (a) and ^{13}C-labeled (b) pyrethrin I (**1**).

Table 1
^{13}C isotopic abundance of pyrethrin I (**1**)

Carbon atom[a]	δ (ppm)	^{13}C abundance[b]
Acid moiety		
1	172.3	1.28
2	34.5	0.81
3	29.1	0.85
4	33.0	1.23
5	120.8	0.81
6	135.9	0.79
7	25.6	0.84
8	18.5	1.24
9	20.4	1.33
10	22.1	0.86
Alcohol moiety		
1′	203.8	1.36
2′	142.0	0.76
3′	165.4	0.66
4′	72.9	1.10
5′	42.0	0.90
6′	21.7	1.24
7′	126.9	0.83
8′	130.4	1.21
9′	131.6	0.86
10′	118.3	1.18
11′	14.1	1.15

[a] The carbon numbers in the acid and alcohol moieties are shown in Figs. 2 and 3, respectively.
[b] Abundance of each ^{13}C signal of the labeled pyrethrin I was represented as the ratio to that of the corresponding carbon of the unlabeled one.

suggesting that the mevalonate-independent pathway was predominantly involved in the biosynthesis of the acid moiety. It has been shown that labeling at C-3 as well as C-1 and C-4 in DMAPP (**11**), which results in labeling at C-3 and C-6 in pyrethrin I (**1**), could occur by generation of [2-^{13}C]pyruvic acid from [3-^{13}C]pyruvic acid by tricarboxylic acid cycle reactions combined with phosphoenol pyruvic carboxykinase and pyruvic kinase reactions (Hirai et al., 2000). Furthermore, labeling at C-7 and C-10 in pyrethrin I (**1**) might also be caused by imperfect stereocontrol of the DMAPP/IPP isomerase reaction. However, it was difficult to detect significant labeling at C-3, C-6, C-7 and C-10 in pyrethrin I (Fig. 5 and Table 1), excluding marked generation of [2-^{13}C] pyruvic acid in the administration experiment.

The earlier reports that [^{14}C]mevalonic acid (**8**) was incorporated into the flower-derived pyrethrins (Crowley et al., 1961, 1962) appear to present a contradiction to our findings. However, the material employed by Crowley et al. differs from that used in the present study. In addition, even if the acid moiety is biosynthesized by the non-mevalonate pathway in the flowers, mevalonic acid (**8**) could be incorporated to give the acid moiety as the result of cross talk between the mevalonate and non-mevalonate pathways (Arigoni et al., 1997; Adam et al., 1998; Hemmerlin et al., 2003). The low incorporation ratio of [^{14}C]mevalonic acid (**8**) into pyrethrin I (**1**) observed by Crowley et al. (1961, 1962) may reflect such cross talk. In any way, it will be of interest in future to investigate which pathway is mainly involved in the biosynthesis of the acid moiety in the flowers.

Pyrethrolone (**19**) is likely to be generated from *cis*-jasmone (**17**) by hydroxylation of the cyclopentenone

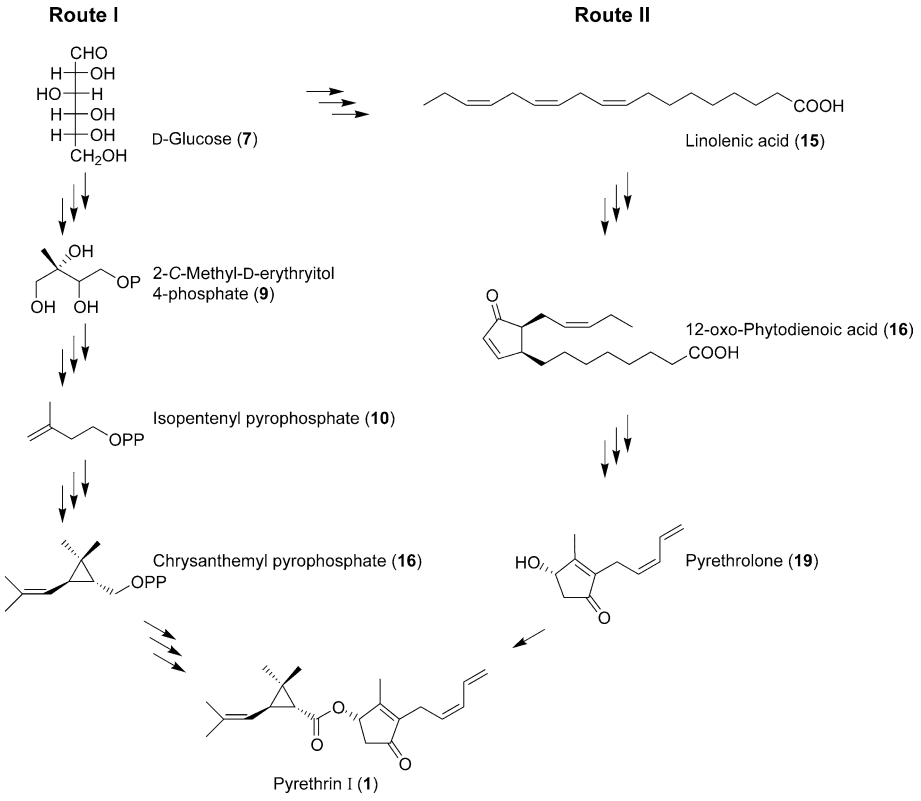

Fig. 6. Non-mevalonate (Route I) and octadecanoid (Route II) pathways involved in biosynthesis of pyrethrin I (**1**) in the seedlings of *Chrysanthemum cinerariefolium*.

ring and unsaturation of its side chain, but it may also occur from 7-hydroxy-jasmonic acid (**18**) by unsaturation and decarboxylation (Fig. 3). Whatever the intermediate, linolenic acid (**15**) is the most possible origin of the alcohol moiety of pyrethrin I. From [1-^{13}C]D-glucose (**7**) administered, C-2 labeled acetyl coenzyme A (**14**) will be obtained via C-3 labeled pyruvic acid (**13**), resulting in generation of linolenic acid (**15**) in which even-numbered carbons are labeled (Fig. 3). If this fatty acid is the origin, then the carbons marked with closed circles in Fig. 3 should be labeled in pyrethrolone (**19**). The ^{13}C NMR spectrum of pyrethrin I (Fig. 5 and Table 1) isolated from the [1-^{13}C]D-glucose-administered seedlings agrees well with this prediction. However, more work is needed to clarify the overall biosynthesis scheme by conducting similar administration experiments using not only linolenic acid (**15**) but also *cis*-jasmone (**17**) and 7-hydroxy-jasmonic acid (**18**), which are labeled at appropriate positions.

In conclusion, we have found for the first time that the acid moiety of pyrethrin I (**1**) is generated via MEP (**9**) in the seedlings of *C. cinerariaefolium*. In addition, the alcohol moiety, pyrethrolone (**19**), was found to likely arise from linolenic acid (**15**). The non-mevalonate pathway reactions, as well as part of the octadecanoid pathway reactions, proceed in the plastids. Therefore, plastids of *C. cinerariaefolium* seedlings seem to play a key role in regulating pyrethrin biosynthesis. The two pathways illustrated in Fig. 6 probably synchronize effectively to ultimately combine the acid and the alcohol moieties. Such cross talk remains to be elucidated in detail to enhance our understanding of the mechanisms underlying pyrethrin biosynthesis.

3. Experimental

C. cinerariaefolium seedlings were grown in the field for approximately 6 months from May to November of 2001. The roots of twenty seedlings were immersed in 1% (w/v) aqueous solution (200 ml) of [1-^{13}C]D-glucose (**1**, 99%, Cambridge Isotope Laboratories, MA, USA) for seven days under illumination at 25 °C. The leaves of *Chrysanthemum* seedlings (25.3 g) were frozen in liquid N_2 and milled. The resulting powders were divided into five portions and each portion was homogenized in acetone (80 ml/portion, total acetone used: 420 ml including 20 ml acetone for rinsing the glassware) and the combined extract was filtered. An aqueous solution (210 ml) containing 1.25% NH_4Cl and 2.5% phosphoric acid was added to the filtrate and the mixture was placed in an ice-water bath for 30 min. The precipitates were removed by filtration and 4% NaCl aqueous

solution (1000 ml) was added. After removal of acetone under reduced pressure as much as possible, the filtrate was extracted with hexane (3×500 ml) and the combined extract was dried (anhydrous $MgSO_4$). After evaporation of the solvent, the residue was dissolved in CH_3CN (20 ml) with the solution divided equally into five portions. Each portion was added to a Sep-Pak C18-cartridge (Nihon Waters K.K., Tokyo, Japan) with the pyrethrins absorbed eluted from the cartridge with CH_3CN (6 ml). An aliquot (1 μl) of the CH_3CN soluble was subjected to HPLC analysis to confirm the presence of pyrethrin I (**1**) and related components in the extract. The HPLC analysis was conducted using a VP10A system (Shimadzu Co., Kyoto, Japan) equipped with an analytical column (L-column ODS, 150×4.6 mm i.d., Chemical Evaluation and Research Institution, Tokyo, Japan) with a CH_3CN–H_2O mixture (65:35) at a flow rate of 1 ml min^{-1} at room temperature. Natural pyrethrins (**1**–**6**) were detected by monitoring their ultraviolet absorption at 230 nm. An example of HPLC of the leaf extract is shown in Fig. 4. The retention times (min) of pyrethrins are as follows: cinerin II (**6**), 8.2; pyrethrin II (**4**), 8.6; jasmolin II (**5**), 11.1; cinerin I (**3**), 18.1; pyrethrin I (**1**), 19.0; jasmolin I (**2**), 25.8.

The remaining CH_3CN solution of pyrethrins was again concentrated in vacuo, with the residue dissolved in CH_3CN (3 ml). From this solution, pyrethrin I (**1**) was isolated employing the VP10A HPLC system combined with a Shim-Pack PREP-ODS preparative column (250×20 mm i.d., Shimadzu Co., Kyoto, Japan) and a CH_3CN–H_2O mixture (65:35) at a flow rate of 10 ml min^{-1}. The separated pyrethrin I solution was concentrated in vacuo and extracted with diethyl ether (3×100 ml). The Et_2O was dried (anhydrous $MgSO_4$) with the solvent evaporated to give a syrupy residue (8.0 mg) containing pyrethrin I. Unlabeled pyrethrin I was isolated from Pyrethrins Standard (50–60% in isoparaffin) (Wako Pure Chemical Industries, Osaka, Japan) using the same HPLC system, column and solvent system as employed for isolation of labeled pyrethrin I. The ^{13}C NMR spectra of labeled (4.6 mg) and unlabeled pyrethrin I (4.6 mg) in $CDCl_3$ (600 μl) were recorded by the same scan numbers (10,000 scans) using a JEOL JMN EX270 spectrometer at 67.5 MHz with TMS as an internal standard at 25 °C. All ^{13}C NMR signals were assigned according to Crombie et al. (1975).

Acknowledgements

This study was supported in part by the "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2004–2008.

References

Adam, K.-P., Thiel, R., Zapp, J., Becker, H., 1998. Involvement of the mevalonic acid pathway and the glyceraldehyde-pyruvate pathway in terpenoid biosynthesis of the liverworts *Ricciocarpos natans* and *Conocephalum conicum*. Arch. Biochem. Biophys. 354, 181–187.

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., Zenk, M.H., 1997. Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. Proc. Natl. Acad. Sci. USA 94, 10600–10605.

Casida, J.E., 1973. Pyrethrum: the Natural Insecticides. Academic Press, New York.

Crombie, L., 1995. Chemistry of the pyrethrins. In: Casida, J.E., Quistad, G.B. (Eds.), Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses. Oxford University Press, Oxford, pp. 123–193.

Crombie, L., Pattenden, G., Simmonds, D.J., 1975. Carbon-13 nuclear magnetic resonance spectra of the natural pyrethrins and related compounds. J. Chem. Soc., Perkin Trans. I, 1500–1502.

Crowley, M.P., Inglis, H.S., Snarey, M., Thain, E.M., 1961. Biosynthesis of the pyrethrins. Nature 191, 281–282.

Crowley, M.P., Godin, P.J., Inglis, H.S., Snarey, M., Thain, E.M., 1962. The biosynthesis of the "Pyrethrins": I. The incorporation of ^{14}C-labelled compounds into the flowers of *Chrysanthemum cinerariaefolium* and the biosynthesis of chrysanthemum monocarboxylic acid. Biochim. Biophys. Acta 60, 312–319.

Dubey, V.S., Bhalla, R., Luthra, R., 2003. An overview of the non-mevalonate pathway for terpenoid biosynthesis in plants. J. Biosci. 28, 637–646.

Eisenreich, W., Bacher, A., Arigoni, D., Rohdich, F., 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell. Mol. Life Sci. 61, 1401–1426.

Feussner, I., Wasternack, C., 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. 53, 275–297.

Hemmerlin, A., Hoeffler, J.-F., Meyer, O., Tritsch, D., Kagan, I.A., Grosdemange-Billiard, C., Rohmer, M., Bach, T.J., 2003. Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. J. Biol. Chem. 278, 26666–26676.

Hirai, N., Yoshida, R., Todoroki, Y., Ohigashi, H., 2000. Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. Biosci. Biotechnol. Biochem. 64, 1448–1458.

Katsuda, Y., 1999. Development of and future prospects for pyrethroid chemistry. Pestic. Sci. 55, 775–782.

Koch, T., Bandemer, K., Boland, W., 1997. Biosynthesis of *cis*-jasmone: a pathway for the inactivation and the disposal of the plant stress hormone jasmonic acid to the gas phase?. Helv. Chim. Acta 80, 838–850.

Lichtenthaler, H.K., 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 47–65.

Morgan, E.D., Wilson, I.D., 1999. Insect hormones and insect chemical ecology. In: Barton, D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 8. Pergamon Press, Oxford, pp. 263–375.

Rivera, S.B., Swedlund, B.D., King, G.J., Bell, R.N., Hussey Jr., C.E., Shattuck-Eidens, D.M., Wrobel, W.M., Peiser, G.D., Poulter, C.D., 2001. Chrysanthemyl diphosphate synthase: Isolation of the gene and characterization of the recombinant non-head-to-tail monoterpene synthase from *Chrysanthemum cinerariaefolium*. Proc. Natl. Acad. Sci. USA 98, 4373–4378.

Rohmer, M., 1999. A mevalonate-independent route to isopentenyl diphosphate. In: Barton, D.H.R., Nakanishi, K., Meth-Cohn, O.

(Eds.), Comprehensive Natural Products Chemistry, vol. 2. Pergamon Press, Oxford, pp. 45–67.

Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S., Sahm, H., 1996. Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. J. Am. Chem. Soc. 118, 2564–2566.

Yoshihara, T., Greulich, F., 1999. Biosynthesis of jasmonoids and their functions. In: Barton, D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 1. Pergamon Press, Oxford, pp. 117–138.

Zito, S.W., Tio, C.D., 1990. Constituents of *Chrysanthemum cinerariaefolium* in leaves, regenerated plantlets and callus. Phytochemistry 29, 2533–2534.