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Biosynthesis of the dimethylallyl moiety of glabrol in *Glycyrrhiza glabra* hairy root cultures via a non-mevalonate pathway

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Abstract

Incorporation of [1-^{13}C]glucose indicates that the biosynthesis of the hemiterpene moiety of glabrol, the main prenylated flavanone in the hairy root cultures of *Glycyrrhiza glabra*, proceeds via a glyceraldehyde/pyruvate non-mevalonate pathway. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Many hemiterpenoid compounds, prenylated flavonoids (Barron and Ibrahim, 1996; Nomura and Fukai, 1998), coumarins (Murray, 1989, 1997; Estevez-Braum and Gonzalez, 1997), quinoline alkaloids (Grundon, 1988; Michael, 1997a,b, 1998), etc., have been isolated as natural products. Isopentenyl diphosphate (IPP), probably via dimethylallyl diphosphate (DMAPP), is the precursor of the prenyl moieties of these compounds. In the past, the mevalonate pathway has been accepted as the general biosynthetic route leading to IPP. Recently, an alternative mevalonate-independent route to IPP was discovered in eubacteria (Rohmer et al., 1993, 1996) and green algae (Schwender et al., 1996). This alternative metabolic route to IPP was later found to be widespread in higher plants. Monoterpenes (Eisenreich et al., 1997) and diterpenoids (Eisenreich et al., 1996; Knöss et al., 1997) are synthesized by the mevalonate-independent route. This route is also operative in the formation of other chloroplast isoprenoids such as carotenoids, phytol and plastoquinone (Lichtenthaler et al., 1997; Disch et al., 1998).

Flavonoids are found as free, prenylated and glycosylated compounds in nature (Harborne and Mabry, 1982; Harborne, 1994). We have already reported the isolation of prenylflavonoids from *Glycyrrhiza glabra* hairy root cultures (Asada et al., 1998). Glabrol (**1**)), having two prenyl moieties, is the main flavanone in the hairy roots. Hano et al. reported the biosynthesis of the hemiterpene moieties of a prenylchalcone, chalcomoracin, in *Morus alba* cell culture (Hano et al., 1994). They demonstrated that in the pair of three acetate units composing the two hemiterpene moieties of chalcomoracin, the starter acetate unit is of glycolytic origin, while the second and third acetate units come from the pentose cycle. These results prompted us to elucidate the origin of the dimethylallyl moiety of glabrol (**1**). Administration of [1-^{13}C]glucose was carried out, and it was found that the dimethylallyl moiety of glabrol (**1**) was synthesized via the non-mevalonate pathway.

2. Results and discussion

Two-week-old hairy root cultures of *G. glabra* were administered [1-^{13}C]glucose and incubated for a further 2 weeks. The hairy roots were harvested and extracted as described in the Experimental section. The ethyl acetate extract was subjected to silica gel column chromatography and further purified by normal-phase HPLC to give ^{13}C-labeled glabrol (**1**). The ^{13}C NMR spectra of the natural abundance and the enriched samples were measured under identical conditions. The relative

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^{13}C abundance of individual carbon atoms was calculated from the integrals of the labeled sample by comparison with the natural abundance sample. The values were referenced to 1.1% for the carbon with the lowest ^{13}C enrichment.

IPP can be provided by two different biosynthetic routes: either via the acetate/mevalonate pathway, or via the glyceraldehyde phosphate/pyruvate pathway. These two pathways are easily differentiated by incorporation of [1-^{13}C]glucose and analysis of the labeling patterns found in the derived isoprenoids. The labeling pattern of DMAPP is explained on the basis of the metabolism of glucose. The label from [1-^{13}C]glucose is converted to C-3 of triose phosphate/pyruvate and C-2 of acetyl CoA by glycolysis. DMAPP arising from three C-2 labeled acetyl CoA molecules via the mevalonate pathway should be labeled at C-2, C-4 and C-5 of DMAPP. On the other hand, DMAPP derived from C-3 labeled pyruvate and glyceraldehyde 3-phosphate via the alternative pathway should be labeled at C-1 and C-5.

The ^{13}C NMR spectrum of glabrol (**1**) derived from [1-^{13}C]glucose showed clear increases of the signals of C-1″, C-1‴, C-5″ and C-5‴ in the dimethylallyl groups (Table 1). The significant incorporation of the label into these carbons is explained by the non-mevalonate pathway. As shown in Fig. 1, incorporation of C-2, C-2′ and C-6′ in **1** comes from *p*-coumaroyl CoA based on the shikimic acid pathway where phenylalanine derives from phosphoenol pyruvate (PEP) and erythrose 4-phosphate. Incorporation of C-6, C-8 and C-10 arises from malonyl CoA that is produced from acetyl CoA. The carbon atoms at C-4″ and C-4‴ showed ^{13}C enrichments of 3.2, slightly above the enrichment of the other unlabeled carbon atoms (C-2″ and C-2‴). This phenomenon was observed in similar feeding experiments and it may be attributed to some imperfect stereocontrol in the DMAPP–IPP isomerase reaction (Arigoni et al., 1997; Li et al., 1998).

Stanjek et al. reported that deuterium labeled 1-deoxy-D-xylulose (DOX) was incorporated into the prenyl moiety of furanocoumarin in *Apium graveolens* (Stanjek et al., 1999). That report is the first example of the participation of DOX-derived DMAPP in aromatic ring prenylation. This is the first report that the dimethylallyl moiety of prenylated flavonoid, glabrol (**1**) is synthesized via a non-mevalonate pathway.

Table 1
^{13}C Abundance in glabrol (**1**) after feeding of [1-^{13}C]glucose

Carbon	δ[a]	Abundance	Carbon	δ	Abundance
C-4	191.0	2.2	C-8	116.5	**6.8**
C-7	162.2	1.5	C-5′	115.6	1.9
C-9	161.9	1.3	C-10	115.5	**6.5**
C-4′	155.9	3.0	C-6	110.4	**7.6**
C-3‴	132.6	1.3	C-2	80.5	**9.1**
C-3″, 1′	131.6	1.1	C-3	44.6	1.1
C-2′	128.8	**8.0**[b]	C-1‴	29.1	**7.9**
C-2′, 3′	128.8	1.9	C-4″, 4‴	25.9	3.5
C-5	126.3	1.4	C-1″	22.8	**7.7**
C-6′	125.9	**8.2**	C-5‴	18.0	**8.7**
C-2‴	123.5	1.1	C-5″	17.9	**9.1**
C-2″	123.2	1.4			

[a] Referenced to the acetone-d_6 centreline at 29.8 ppm.
[b] The numbers in bold type represent significant ^{13}C incorporation from [1-^{13}C]glucose.

3. Experimental

3.1. General

D-[1-^{13}C]Glucose (99% isotopic abundance) was purchased from Isotec Inc., USA. The ^{1}H and ^{13}C-NMR spectra were measured with a Varian XL-400 spectrometer. For HPLC, a Waters model 510 HPLC system was used. Column chromatography was carried out using Wako-gel C-200. TLC was conducted on Kieselgel $60F_{254}$ plates (Merck). ^{1}H-decoupled ^{13}C NMR spectra of natural abundance and ^{13}C-labeled samples were measured under identical conditions (100 MHz; 25°C; repetition time 5.00 s; 60° pulse angle) using standard Varian software.

3.2. Culture conditions of Glycyrrhiza glabra hairy root cultures

Hairy roots of *G. glabra* were cultured on woody plant hormone-free liquid media containing 3% sucrose.

3.3. Administration of [1-^{13}C]glucose into Glycyrrhiza glabra hairy root cultures

The hairy roots were subcultured on woody plant basal medium at 25°C in the dark at 50 rpm on rotary shaker at 4 week intervals. [1-^{13}C]glucose (3 g) dissolved in distilled water was administered to three Erlenmeyer flasks containing the hairy roots cultured for 2 weeks on woody plant liquid medium (200 ml per flask).

3.4. Extraction and isolation of glabrol (1)

After an additional 2 weeks of culture, the hairy roots were harvested. The fresh hairy roots (76 g) were extracted with MeOH, whose solvent removal under reduced pressure gave the MeOH extract. The extract was partitioned between EtOAc and H_2O, and the EtOAc solubles were evaporated under reduced pressure to yield the EtOAc extract (480 mg). This was then subjected to silica gel chromatography and eluted with $CHCl_3$ to give eight fractions. Fr. 4 was purified by repeated normal-phase HPLC (hexane–$CHCl_3$–EtOH = 70:30:2) to give ^{13}C-labeled glabrol (**1**) (4.3 mg). The purity of ^{13}C-labeled

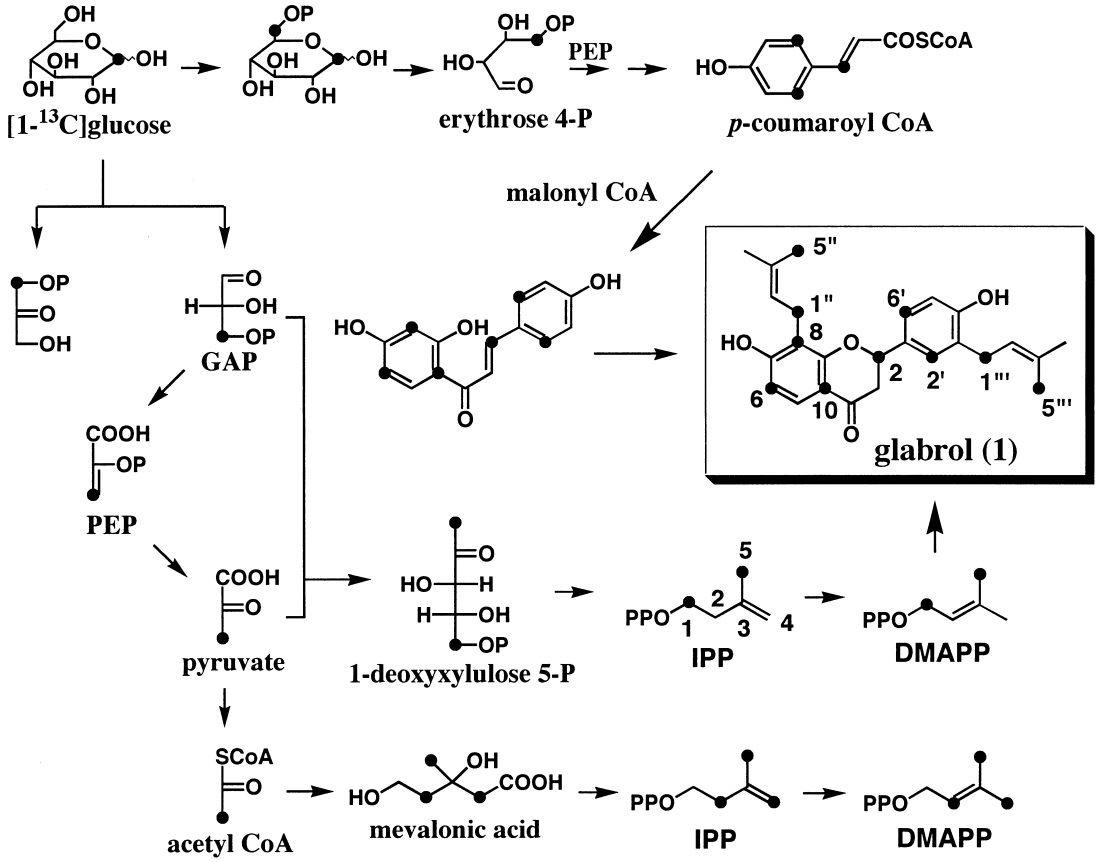

Fig. 1. Expected labelling patterns of glabrol (**1**) via the non-mevalonate and mevalonate pathways.

glabrol (**1**) was confirmed by TLC and ^{1}H and ^{13}C NMR spectra.

3.5. Glabrol (*1*)

^{1}H NMR (400 MHz, acetone-d_6) δ: 1.63 (3H, *d*, $J=1.0$ Hz, 5″-CH_3), 1.65 (3H, *d*, $J=1.0$ Hz, 4″-CH_3), 1.73 (6H, *br s*, 4‴ and 5‴-CH_3), 2.70 (1H, *dd*, $J=16.5$, 3.0 Hz, 3-H_β), 3.00 (1H, *dd*, $J=16.5$, 13.0 Hz, 3-H_α), 3.34 (2H, *d*, $J=7.0$ Hz, 1″-H), 3.37 (2H, *d*, $J=7.0$ Hz, 1‴-H), 5.26 (1H, *m*, 2″-H), 5.38 (1H, *m*, 2‴-H), 5.43 (1H, *dd*, $J=13.0$, 3.0 Hz, 2-H), 6.62 (1H, *d*, $J=8.5$ Hz, 6-H), 6.90 (1H, *d*, $J=8.5$ Hz, 5′-H), 7.23 (1H, *dd*, $J=8.5$, 2.5 Hz, 6′-H), 7.34 (1H, *d*, $J=2.5$ Hz, 2′-H), 7.59 (1H, *d*, $J=8.5$ Hz, 5-H). For ^{13}C NMR assignments and intensities, see Table 1.

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References

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. Proceedings of the National Academy of Sciences USA 94, 10600–10605.

Asada, Y., Li, W., Yoshikawa, T., 1998. Isoprenylated flavonoids from hairy root cultures of *Glycyrrhiza glabra*. Phytochemistry 47, 389–392.

Barron, D., Ibrahim, R.K., 1996. Isoprenylated flavonoids — a survey. Phytochemistry 43, 921–982.

Disch, A., Hemmerlin, A., Bach, T.J., Rohmer, M., 1998. Mevalonate-derived isopentenyl diphosphate is the biosynthetic precursor of ubiquinone prenyl side chain in tobacco BY-2 cells. Biochemistry Journal 331, 615–621.

Eisenreich, W., Menhard, B., Hylands, P.J., Zenk, M.H., Bacher, A., 1996. Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin. Proceedings of the National Academy of Sciences USA 93, 6431–6436.

Eisenreich, W., Sagner, S., Zenk, M.H., Bacher, A., 1997. Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Letters 38, 3389–3892.

Estevez-Braum, A., Gonzalez, A.G., 1997. Coumarins. Natural Product Reports 14, 465–475.

Grundon, M.F., 1988. Quinoline, quinazoline, and acridone alkaloids. Natural Product Reports 5, 293–307.

Hano, Y., Ayukawa, A., Nomura, T., Ueda, S., 1994. Origin of the acetate units composing the hemiterpene moieties of chalcomoracin in *Morus alba* cell cultures. Journal of the American Chemical Society 116, 4189–4193.

Harborne, J.B. (Ed.), 1994. The Flavonoids: Advances in Research Since 1986. Chapman & Hall, London.

Harborne, J.B., Mabry, T.J. (Eds.), 1982. The Flavonoids: Advances in Research. Chapman & Hall, London.

Knöss, W., Reuter, B., Zapp, J., 1997. Biosynthesis of the labdane diterpene marrubiin in *Marrubium vulgare* via a non-mevalonate pathway. Biochemistry Journal 326, 449–454.

Li, S., Hennig, S., Heide, L., 1998. Biosynthesis of the dimethylallyl moiety of novobiocin via a non-mevalonate pathway. Tetrahedron Letters 39, 2717–2720.

Lichtenthaler, H.K., Schwender, J., Disch, A., Rohmer, M., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Letters 400, 271–274.

Michael, J.P., 1997a. Quinoline, quinazoline and acridone alkaloids. Natural Product Reports 14, 11–20.

Michael, J.P., 1997b. Quinoline, quinazoline and acridone alkaloids. Natural Product Reports 14, 605–618.

Michael, J.P., 1998. Quinoline, quinazoline and acridone alkaloids. Natural Product Reports 15, 595–606.

Murray, R.D.H., 1989. Coumarins. Natural Product Reports 6, 591–624.

Murray, R.D.H., 1997. Naturally occurring plant coumarins. In: Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, C. (Eds.), Fortschrite der Chemie Organischer Naturstoffe, 72. Springer-Verlag, New York, pp. 1–119.

Nomura, T., Fukai, T., 1998. Phenolic constituents of licorice (*Glycyrrhiza* species). In: Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, C. (Eds.), Fortschritte der Chemie Organischer Naturstoffe, 73. Springer-Verlag, New York, pp. 1–140.

Rohmer, M., Knani, M., Simonin, P., Sutter, B., Sahm, H., 1993. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochemistry Journal 295, 517–524.

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. Journal of the American Chemical Society 118, 2564–2566.

Schwender, J., Seemann, M., Lichtenthaler, H.K., Rohmer, M., 1996. Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. Biochemistry Journal 316, 73–80.

Stanjek, V., Piel, J., Boland, W., 1999. Biosynthesis of furanocoumarins: mevalonate-independent prenylation of umbelliferone in *Apium graveolens* (Apiaceae). Phytochemistry 50, 1141–1145.