Pergamon

PII: S0031-9422(97)00450-0

Phytochemistry, Vol. 48, No. 1, pp. 89–92, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

DISTRIBUTION OF GINKGOLIDES AND TERPENOID BIOSYNTHETIC ACTIVITY IN *GINKGO BILOBA*

DANIELLE JULIE CARRIER*, TERIS A. VAN BEEK†, ROBERT VAN DER HEIJDEN‡ and ROBERT VERPOORTE‡

Department of Agricultural and Bioresource Engineering, University of Saskatchewan, 57 Campus Drive, Saskatoon, SK, Canada S7N 5A9; † Phytochemical Section, Laboratory of Organic Chemistry, Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands; ‡ Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

(*Received in revised form* 7 *May* 1997)

Key Word Index—*Ginkgo biloba*; Ginkgoaceae; enzymatic activity; biosynthesis; secondary metabolism; feeding experiments; terpene trilactones; ginkgolides; bilobalide.

Abstract—The terpene trilactone content (bilobalide and ginkgolides) of extracts prepared from leaves of terminal buds, rosettes and side branches, from stem and root bark, and from root and root meristems of three-year-old *Ginkgo biloba* plants was determined. The aerial parts were relatively rich in bilobalide while ginkgolides were the major constituents of the underground parts. The formation of farnesyl and geranylgeranyl pyrophosphate was monitored by incubating cell-free extracts prepared from the corresponding plant parts with [1-^{14}C]isopentenyl pyrophosphate. Extracts prepared from leaves of the terminal buds displayed terpenoid biosynthetic activity, suggesting that terpene trilactone synthesis might occur in actively growing tissues.

INTRODUCTION

The *Ginkgo biloba* tree is the sole representative of the Ginkgoales order [1] and its origins trace back to the Jurassic period. Parts of this unique tree have been used as a phytotherapeutic agent since at least 1300 AD [2, 3]. This tree produces a wide range of secondary metabolites [4], among which, the terpene trilactones ginkgolide A, B, C, J and M (further abbreviated as G-A, G-B, G-C, G-J and G-M, respectively), and bilobalide are the most characteristic. The sesquiterpene bilobalide is the major terpene trilactone in the leaves [5]. It has also been detected as a minor component in roots [T. A. van Beek, unpublished]. Ginkgolides are diterpenes and have been detected in both root bark and leaves [6–8]. They have also been detected in cell cultures [8–10]. Their structures were elucidated in the sixties [11–15], and have been confirmed to be terpenoid in nature [16]. The precise biosynthetic pathway from geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP) to these compound has yet to be elucidated, and their ecological significance remains unclear.

The fact that bilobalide and ginkgolide are terpenoids can be utilized to examine the site and time of synthesis within the tree. If bilobalide and ginkgolides present in leaves and roots of the tree are continuously synthesized, then the corresponding tree parts should display FPP synthase [EC 2.5.1.1, EC 2.5.1.10] and GGPP synthase [EC 2.5.1.1, EC 2.5.1.10, EC 2.5.1.30] activity. In this work, an attempt was made to correlate FPP synthase and GGPP synthase biosynthetic activity with the presence of ginkgolides and bilobalide in roots, leaves and barks of two *G. biloba* plants.

RESULTS AND DISCUSSION

Terpene trilactone content

The ginkgolide and bilobalide concentration of *G. biloba* roots, leaves and barks is shown in Table 1. Leaves from the terminal buds, rosettes and side branches yielded the highest terpene trilactone concentration, with bilobalide accounting for more than one third of the terpene content. Of the ginkgolides contained in these samples, G-A occurred at the highest concentration, followed by relatively equal amounts of G-B and G-C, G-J being detected as a minor component. These results are similar to those of others [6, 8]. Ginkgolides were also detected in stem bark extracts at about half the concentration of that detected in leaves from the terminal buds, rosettes and side branches. Bilobalide concentration was similar to the G-J concentration in these extracts. The under-

* Author to whom correspondence should be addressed.

Table 1. Bilobalide, G-A, G-B, G-C and G-J and total terpene content with standard deviations of various parts obtained from 3-year-old *G. biloba* plants. Contents are expressed as mg terpene g^{-1} dry wt.

Plant part	Bilobalide	G-A	G-B	G-C	G-J	Total terpene content
Terminal bud	0.22±0.04	0.18±0.01	0.09±0.02	0.09±0.02	0.03±0.00	0.60±0.02
Side branch	0.16±0.06	0.13±0.01	0.06±0.00	0.07±0.01	0.01±0.01	0.42±0.07
Rosette	0.27±0.12	0.19±0.04	0.08±0.01	0.08±0.00	0.02±0.01	0.63±0.08
Root meristem	0.00±0.00	0.13±0.01	0.05±0.01	0.06±0.01	0.03±0.01	0.27±0.01
Root	0.02±0.02	0.13±0.07	0.05±0.02	0.05±0.02	0.02±0.01	0.26±0.14
Root bark	0.02±0.02	0.13±0.07	0.05±0.02	0.05±0.02	0.02±0.01	0.26±0.14
Stem bark	0.02±0.02	0.13±0.07	0.05±0.02	0.05±0.02	0.02±0.01	0.26±0.14

ground parts of the plant, consisting of roots, root meristems and root bark, yielded less terpenes than leaves from terminal buds, rosettes and side branches. The extracts prepared from underground parts yielded in decreasing order of concentration G-A, G-C and G-B, and about equal amounts of G-J and bilobalide. This terpene distribution pattern is similar to that reported by [6], with bilobalide being practically absent from underground parts, whereas it is the major constituent in aerial parts. This is in contrast to G-C which is the most abundant ginkgolide in root bark of gingko trees with trunks exceeding 30 cm in diameter [17]. The age of the plants extracted in this study could account for the differences in the ginkgolide concentrations.

Formation of terpenoid products

The formation of FPP and GGPP, which are most likely the terpenoid precursors of bilobalide and ginkgolides [16], was monitored by the incorporation of [1-^{14}C]isopentenyl pyrophosphate ([1-^{14}C]IPP) by cell-free extracts. These extracts were prepared from leaves of terminal buds, rosettes and side branches, from stem and root bark, and from root and root meristems. FPP and GGPP were detected as the corresponding alcohols: farnesol and geranylgeraniol, respectively. The results from all the cell-free extracts incubations are presented in Table 2. Stem bark, root and root meristem cell-free extract preparations did incorporate the labelled substrate into terpenoid product(s). Crude enzyme solutions prepared from either leaves of the terminal buds or the side branches incorporated [1-^{14}C]IPP into FPP and GGPP. However, rosette leaf cell-free extracts incubated with [1-^{14}C]IPP did not yield terpenoid products.

Table 2. Formation of radioactive terpenoid products, monitored by radio-HPLC as the corresponding alcohols (farnesol and geranylgeraniol), from [1-^{14}C]isopentenyl pyrophosphate in cell free extracts prepared from various parts of 3-year-old *G. biloba* plants.

Plant part	Radioactivity (cpm/mg protein ± SD) IPOH	GGOH	FOH
Terminal bud	416±96	164±57	32±11
Side branch	717±210	57±12	33±15
Rosette	748±284	0±0	0±0
Root meristem	717±35	12±17	0±0
Root	788±153	24±34	0±0
Root bark	526±195	22±12	186±78
Stem bark	532±50	0±0	73±38

Possible sites for the biosynthesis of terpene trilactones

Cell-free extracts prepared from root bark and from leaves of terminal buds and side branches incorporated some [1-^{14}C]IPP into FPP and GGPP. Extracts from the terminal bud region possessed both a high terpene content and high terpenoid biosynthetic activity. The detection of labelled geranylgeraniol in this extract indicated that GGPP synthase activity was present. The production of GGPP in these growing tissues may be necessary for the biosynthesis, of among others, gibberellic acid, carotenoids, the phytol chain of chlorophyll and ginkgolides. Similarly, FPP synthase activity, detected by the formation of labelled farnesol, may be necessary for the synthesis of bilobalide and sterol derived constituents. Interestingly, the concentration of terpene trilactones in ginkgo leaves has been documented to increase throughout the summer season [6–8]. In this work, rosette leaves contained high concentrations of terpene trilactones, but yielded crude enzyme preparations incapable of incorporating the labelled substrate. Results obtained with *Capsicum* [18] have shown that the activity of GGPP synthase and its mRNA expression varied as a function of plant development. The high ginkgolide concentration in rosette leaves can be explained by the following or a combination of the following: (a) synthesized elsewhere in the plant and transported, (b) synthesized when the tissues were actively growing and then sequestered or (c) obtained from the catabolism of other moieties also derived from GGPP.

Root meristems and roots contained a higher terpene content than the root bark, possibly suggesting that terpenes are synthesized in the aerial parts of the plant and unloaded from the phloem sap for storage in the cortex cells. Previous results [17] have shown that, in trees with trunks larger than 30 cm, gink-

golides are mostly found in the roots. The presence of GGPP and FPP synthase activity in root bark could possibly be attributed to epidermal cell division and their requirement of primary terpenoid metabolites. A future experiment where *G. biloba* plants would be girdled, interrupting phloem transport, could possibly further help to establish the site of ginkgolide synthesis.

EXPERIMENTAL

Plant material. Twenty seedlings were purchased from Fopma Nursuries (Boskoop, The Netherlands) in Spring 1991. They were grown in the experimental garden of the Division of Pharmacognosy, Leiden University, Leiden, The Netherlands) under normal seasonal variations in a heavy clay soil. In early spring 1993 they were dug up and potted in individual pots lined with heavy clay and peat. The general height of the plants was 30 to 40 cm. To force a resting period, 15 plants were held at 5° for 28 days. On December 6, 1993 they were moved to a climatic chamber. Conditions: temperature 24°, humidity 90% and 14 hr light/day. They were watered about three times a week and were fertilized twice (22.12.93 and 03.01.94) with N:P:K 4:5:6. On January, 18 1994, the plants were harvested and immediately divided into: terminal buds, side branches, rosette leaves, bark of root and stem as well as root meristems. Cell-free extracts were prepared immediately.

Ginkgolide and bilobalide analysis. The different plant parts were immediately dried after harvesting for 24 hr at 60° in an oven with forced air circulation. Approximately 0.2 g dry plant material was accurately weighed and extracted for 15 min by refluxing in 10% MeOH in H_2O. After filtration over a Büchner funnel, the plant material was extracted a second time under identical conditions. The aq. extracts were pooled and quantitatively transferred to a separatory funnel. After cooling, they were extracted ×3 with 5 ml EtOAc each. The combined EtOAc layers were dried over anhydrous Na_2SO_4 and evapd *in vacuo*. The residue was dissolved and quantitatively transferred to an SPE column (1.2 g silica gel) with a total of 0.70 ml Me_2CO. After drying for 30 min in a vacuum oven at 60°, the SPE column was eluted with 10 ml toluene–Me_2CO (7:3). After evapn of the solvent *in vacuo*, the residue was dissolved in 0.9 ml HPLC solvent plus 0.500 ml benzyl alcohol (int. standard) soln in HPLC solvent. After capping the autosamper vial, the soln was ready for HPLC analysis.

HPLC analysis and quantitation. This was carried out as described in [19]. Response factors were calculated with freshly prepd reference solns. A 100 μl loop was used.

Preparation of the crude extracts. The procedure was carried out at 4°. Two grams of plant parts were homogenized with a mortar and pestle with 6 ml of 100 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 2 mM $MnCl_2$, 20% glycerol, 1% Triton-X-100, 1 g sand and 10% PVPP as reported in [20]. The homogenate was centrifuged at 2330 *g* for 20 min. The supernatant was stored at −80°.

Terpenoid products. The assay was essentially the same as reported in [20], consisting of an incubation of the cell-free extracts (*ca* 125 μg of protein). The reaction was allowed to proceed in a total vol. of 200 μl containing 56 mM of Tris–HCl; pH 7.5, 1.5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 30 mM KF, 1.1 mM DTT, 300 μM DMAPP and 3.7 kBq IPP. Incubation was carried out at 30° for 60 min. The reaction was terminated by the addition of 200 μl of 50 mM Tris–HCl, pH 9, and 10 μl alkaline phosphatase. The Eppendorf tubes were incubated 110 min at 37° and left overnight at room temp. To the tubes, 200 μl of hexane was added before vortexing and centrifugation at 12 000 *g* for 5 min. This procedure was repeated ×3. The total vol. of the obtained organic phase was measured and included in the radioactivity calculations. The terpenoid alcohols were analysed by normal-phase HPLC (10 μl injection in a 20 μl loop, *n*-hexane–*n*-BuOH (125:2) with a flow of 0.5 ml min^{-1}, Shandon 5 μm Hypersil column, UV detection at 210 nm) coupled to a radio-detector. A 500 μl liquid cell with Ultima-flo M flowing at 1.5 ml min^{-1} was used. Protein determinations were carried out according to [21].

Acknowledgements—We wish to thank Mr G.P. Lelyveld for his assistance with the ginkgolide and bilobalide analysis, Mr C. van de Haar for the extraction of many ginkgo samples and Mr W. Snoeijer for the cultivation of the plants.

REFERENCES

1. Engler, A., *Syllabus der Pflanzenfamilien.* 1954, Ed. 12, Band 1.
2. Braquet, P., in *Ginkgolides—Chemistry, Biology, Pharmacology and Clinical Perspectives*, Vol. 1, ed. P. Braquet. J. R. Prous Science Publishers, Barcelona, 1988, p. 15.
3. Michel, P. F. and Hosford, D., in *Ginkgolides—Chemistry, Biology, Pharmacology and Clinical Perspectives*, Vol. 1, ed. P. Braquet. J. R. Prous Science Publishers, Barcelona. 1988, p. 1.
4. Boralle, N., Braquet, P. and Gottlieb, O. R., in *Ginkgolides—Chemistry, Biology, Pharmacology and Clinical Perspectives*, Vol. 1, ed. P. Braquet. J. R. Prous Science Publishers, Barcelona, 1988, p. 9.
5. Nakanishi, K., Habaguchi, H., Nakadaira, Y., Woods, M. C., Maruyama, M., Major, R. T., Alauddin, M., Patel, A. R., Weinges, K. and Bähr, W., *Journal of the American Chemical Society*, 1971, **93**, 3544.
6. Flesch, V., Jacques, M., Cosson, L., Teng, B. P., Petiard, V. and Balz, J. P., *Phytochemistry*, 1992, **31**, 1941.
7. van Beek, T. A. and Lelyveld, G. P., *Planta Medica*, 1992, **58**, 413.

8. Huh, H. and Staba, E. J., *Planta Medica*, 1993, **59**, 232.
9. Carrier, D. J., Chauret, N., Mancini, M., Coulombe, P., Neufeld, R., Weber, M. and Archambault, J., *Plant Cell Reports*, 1991, **10**, 256.
10. Jeon, M. H., Sung, S. H., Jeon, S. H., Huh, H. and Kim, Y. C., *Saengyak Hakhoechi*, 1993, **24**, 304.
11. Maruyama, M., Terahara, A., Itagaki, Y. and Nakanishi, K., *Tetrahedron Letters*, 1967, 299.
12. Maruyama, M., Terahara, A., Itagaki, Y. and Nakanishi, K., *Tetrahedron Letters*, 1967, 303.
13. Maruyama, M., Terahara, A., Nakadaira, Y., Woods, M. C. and Nakanishi, K., *Tetrahedron Letters*, 1967, 309.
14. Maruyama, M., Terahara, A., Nakadaira, Y., Woods, M. C., Takagi, Y. and Nakanishi, K., *Tetrahedron Letters*, 1967, 315.
15. Okabe, K., Yamada, K., Yamamura, S. and Takada, S., *Journal of the Chemical Society*, 1967, 2201.
16. Nakanishi, K. and Habaguchi, K., *Journal of The American Chemical Society*, 1971, **93**, 3546.
17. Nakanishi, K., in *Ginkgolides—Chemistry, Biology, Pharmacology and Clinical Perspectives*, Vol. 1, ed. P. Braquet. J. R. Prous, Barcelona, 1988, p. 27.
18. Kuntz, M., Römer, S., Suire, C., Hugueney, P., Weil, J. H., Schantz, R. and Camara, B., *Plant Journal*, 1992, **2**, 25.
19. van Beek, T. A., Scheeren, H. A., Rantio, T., Melger, W. C. and Lelyveld, G. P., *Journal of Chromatography*, 1981, **543**, 375.
20. Ramos-Valdivia, A. C., Ph.D. Thesis, Leiden University, 1996.
21. Peterson, G., *Analytical Biochemistry*, 1977, **83**, 346.