Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 5

AN EFFICIENT METHOD FOR FOLLOWING THE ENZYMIC REACTIONS INVOLVED IN CAMPHOR BIOSYNTHESIS IN *CINNAMOMUM CAMPHORA* BY USE OF GC-MS AND REGIOSPECIFICALLY DEUTERIATED SUBSTRATE

**Shinji Ohta, Tohru Yamamitsu and **Takayuki Suga
Department of Chemistry, Faculty of Science, Hiroshima University, Kagamiyama, Higashi-Hiroshima 724, Japan;
*Instrument Center for Chemical Analysis, Hiroshima University, Higashisenda-machi, Naka-ku, Hiroshima 730, Japan

SUMMARY

An efficient method has been developed to follow the enzymic reactions involved in the biosynthesis of camphor in *Cinnamomum camphora* (camphor tree) by use of [5,5-$^2H_2$]geranyl diphosphate as a substrate and GC-MS with selected ion monitoring. Borneol and camphor biosynthesized in the enzymic reactions gave a base peak due to the deuterium-containing ion in its EI mass spectrum. It is possible to detect 1.5 ng of the biosynthesized borneol and camphor per injection into the GC-MS. This method enabled us to differentiate easily the biosynthesized camphor from the endogenous camphor and it is a facile and sensitive technique to determine the amount of the biosynthesized camphor.

Key Words: *Cinnamomum camphora*, Lauraceae, biosynthesis, camphor, borneol, [5,5-$^2H_2$]geranyl diphosphate.

INTRODUCTION

The biosynthesis of camphor, which is a cyclic monoterpenoid with a bornane carbon skeleton, is known to involve the divalent metal ion-dependent cyclization of geranyl diphosphate (GPP) to bornyl diphosphate. This is followed by hydrolysis of the bornyl diphosphate to borneol and the subsequent NADP (or NAD)-dependent oxidation to camphor in sage (1) and tansy (2).

In the course of studies on the biosynthesis of (+)-camphor in

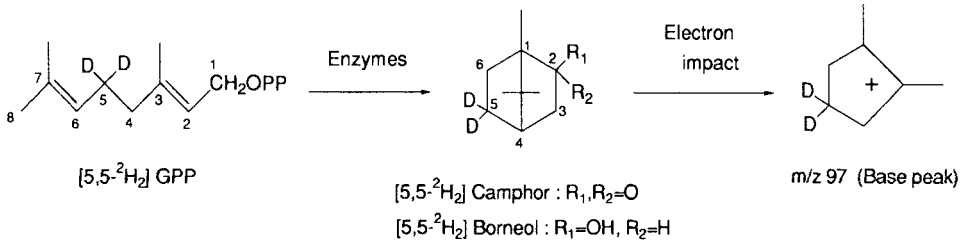
**Author to whom correspondence should be addressed.

0362–4803/92/050397–06$05.00

Received 4 December, 1991
Revised 6 January, 1992

Cinnamomum camphora (camphor tree) (3), we needed a sensitive method to follow the enzymic reactions involved in the biosynthesis of camphor. Thus far, radioactive GPP, e.g. [1-^{3}H]GPP, has been used as the substrate for studies on the biosynthesis of camphor (1,2). However, it is difficult to determine the radioisotopically labelled position of the products and, in addition, special care must be taken in handling the radioactive materials.

For the purpose of identification of products and obtaining structural information, mass spectrometry is most useful. [4-^{2}H]- and [5,5-$^2H_2$]GPP are suitable for such studies since, when subjected to enzymic reactions, they should yield camphor deuteriated at the 6- or 5-positions, respectively (2). The deuteriated camphor species are expected to give a base peak due to the deuterium-containing ion in the EI-MS (4,5) (Scheme 1). Chemical syntheses of [4-^{2}H]- and [5,5-$^2H_2$]GPP have been established and described in our previous report (6). We report here a facile and sensitive method to observe their conversion into camphor by enzymic reactions using GC-MS with selected ion monitoring (SIM) and the deuteriated GPP as substrate.



Scheme 1

RESULTS AND DISCUSSION

[5,5-$^2H_2$]GPP was selected as the substrate for the biosynthesis of camphor rather than [4-^{2}H]GPP, because of the higher deuterium enrichment of [5,5-$^2H_2$]GPP (6). Following our method (6), [5,5-$^2H_2$]GPP was synthesized from 3-methyl-2-butenoic acid with the acetoacetic ester synthesis of [4,4-$^2H_2$]-6-methyl-5-hepten-2-one as the key step. The deuterium enrichment of [5,5-$^2H_2$]GPP was 99%.

A soluble enzyme system was prepared from the leaves of *C. camphora*. The leaves of *C. camphora* were homogenized in 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer

Table 1. Mass spectral data of dideuteriated borneol and camphor biosynthesized from [5,5-$^2H_2$]GPP

Entry no.	Compound	*m/z* (rel. int.)
1	*Dideuteriated camphor	154(8,$[M]^+$), 152(23), 139(1,$[M\text{-}Me]^+$), 137(4), 112(2,$[C_8H_{12}D_2]^+$), 111(10,$[C_8H_{11}D_2]^+$), 110 (13), 109(46),97(34,$[C_7H_9D_2]^+$), 95(100), 83 (67,$[C_6H_7D_2]^+$ and $[C_6H_{11}]^+$), 81(85)
2	Authentic camphor	152(22,$[M]^+$), 137(4,$[M\text{-}Me]^+$), 111(1), 110(10, $[C_8H_{14}]^+$), 109(31,$[C_8H_{13}]^+$), 97(4), 95(100, $[C_7H_{11}]^+$), 83(34,$[C_6H_{11}]^+$), 81(79,$[C_6H_9]^+$)
3	Dideuteriated borneol	156(0.4,$[M]^+$), 141(6,$[M\text{-}Me]^+$), 138(2,$[M\text{-}H_2O]^+$), 112(11,$[C_8H_{12}D_2]^+$), 97(100,$[C_7H_9D_2]^+$)
4	Authentic borneol	154(0.4,$[M]^+$), 139(6,$[M\text{-}Me]^+$), 136(5,$[M\text{-}H_2O]^+$), 110(15,$[C_8H_{14}]^+$), 95(100,$[C_7H_{11}]^+$)

*Also contains the endogenous camphor.

(pH 7.5) in the presence of $MgCl_2$, polyvinylpolypyrrolidone (PVPP), sucrose and dithiothreitol. The presence of PVPP and dithiothreitol served to minimize interference from endogenous phenolic materials (7). The homogenate was filtered, and the filtrate was centrifuged at 18000 × g. Since ammonium sulfate fractionation of the supernatant resulted in a loss of the activities of the enzymes, the supernatant was directly chromatographed on a Sephadex G-25 column using 50 mM TES-NaOH buffer (pH 7.0) containing dithiothreitol and $MgCl_2$ as eluant to give a protein fraction. The protein fraction was used as the soluble enzyme system for the biosynthesis of bornane derivatives.

[5,5-$^2H_2$]GPP was incubated with the soluble enzyme system in the presence of 2 mM NADP. The reaction products were immediately subjected to GC, GC-MS, and GC-HR MS, without prior treatments. The GC analysis showed peaks at retention times 9.8 and 14.4 min. The peaks were identical in retention time with those of authentic camphor and borneol, respectively.

The mass spectral data of the product corresponding to the peak at 9.8 min in the GC analysis and authentic camphor are given in Table 1 (Entry no. 1 and 2, respectively). According to the literature (4), the prominent

peaks in the mass spectrum of authentic camphor were assigned as given in Table 1. The mass spectrum of the deuteriated camphor showed a molecular ion peak at *m/z* 154. The fragment ion peaks at *m/z* 139, 112, 111, 97 (Found: 97.0886. $C_7H_9D_2$ requires 97.0986), and 83 correspond to those at *m/z* 137, 110, 109, 95 (base peak), and 81 of authentic camphor, respectively. The ion peaks appearing at *m/z* 152, 137, 110, 109, 95, and 81 may be ascribed to the ions resulting from endogenous camphor. Thus, the formation of [5,5-$^2H_2$]camphor was confirmed. The deuterium enrichment of the dideuteriated camphor was found to be 24%.

The mass spectral data of the product corresponding to the peak at 14.4 min in the GC analysis, which was biosynthesized from [5,5-$^2H_2$]GPP, and authentic borneol are given in Table 1 (Entry no. 3 and 4, respectively). According to the literature (4,5), the prominent peaks of authentic borneol were assigned as given in Table 1. The mass spectrum of the biosynthesized product showed a molecular ion at *m/z* 156 and the characteristic fragment ions at *m/z* 112 and 97. The ion peak at *m/z* 112 corresponds to the ion peak at *m/z* 110 of authentic borneol, resulting from loss of two carbon atoms at the C-2 and C-3 positions as the enol form of acetaldehyde from the molecular ion (4,5). The ion peak at *m/z* 97 (base peak, Found: 97.0843. $C_7H_9D_2$ requires 97.0986) corresponds to the intense and diagnostically important ion peak at *m/z* 95 of authentic borneol, arising by loss of a methyl group from the *m/z* 110 ion (4,5). The fragment ion peaks at *m/z* 141 and 138 correspond to the ion peaks at *m/z* 139 and 136 of authentic borneol, respectively. Thus, the biosynthesized product was identified as [5,5-$^2H_2$]borneol. The deuterium enrichment of the biosynthesized borneol was found to be 99% by mass spectral analysis.

The base peak at *m/z* 95 due to the dimethylcyclopentenyl ion (4,5) arising from authentic borneol and camphor shifted to *m/z* 97 in the EI mass spectra of the biosynthesized borneol and camphor (Table 1). This enabled us to differentiate easily the biosynthesized borneol and camphor from the endogenous borneol and camphor, respectively, and to determine the amounts of the biosynthesized borneol and camphor by measuring the intensity of the deuterium-containing ion at *m/z* 97 of each compound with GC-SIM. The fragment ion peaks at *m/z* 97 of the biosynthesized borneol and camphor are the most abundant peaks of each mass spectrum. It is possible to detect 1.5 ng of biosynthesized borneol and camphor per injection into the gas chromatograph-mass spectrometer by this method; the signal/noise (S/N) ratio was 10. Advantages of the present method are that conversion of the biosynthesized borneol and camphor into derivatives is not necessary prior to GC-SIM analysis and that it is possible to determine simultaneously the amounts of the biosynthesized borneol and camphor by GC-SIM analysis. The assay with a radioactive GPP as

substrate needs the purification, conversion to a suitable derivative, and determination of radioactivity of the camphor or borneol product (1,2). Consequently, the present method employing a combination of [5,5-$^2H_2$]GPP and GC-SIM analysis, is a facile and sensitive technique for determining the enzymic biosynthesis of borneol and camphor.

EXPERIMENTAL

GC was carried out on a glass column (3 mm × 2 m) packed with 15% DEGS on Chromosorb W (AW-DMCS; 80–100 mesh) at 120°. GC-MS and GC-SIM were carried out on 15% DEGS column (3 mm × 2 m) at 120° by EI mode at 70 eV. High resolution mass spectra were obtained with a Hitachi M-80B GC double-focusing mass spectrometer operating at 70 eV under the same GC conditions as above. The deuterium enrichment of deuteriated compounds was determined by subtracting the natural isotopic abundance from the observed isotopic abundances of the deuteriated compounds as described in the literature (8).

Substrate and plant material. Following the method described in our previous report (6), [5,5-$^2H_2$]GPP was prepared from 3-methyl-2-butenoic acid and the deuterium enrichment of the [5,5-$^2H_2$]GPP was established as 99%. The [†] leaves of *C. camphora*, grown in the campus of Hiroshima University, were collected in summer season.

Preparation of the soluble enzyme system from *C. camphora*. The fresh leaves (65 g) were frozen with liq. N_2, ground in a mortar, and slurried with PVPP (52 g) in 0.1 M TES-NaOH buffer (pH 7.5, 190 ml) containing 0.1 M sucrose, 10 mM dithiothreitol and 5 mM $MgCl_2$. The resulting paste was filtered through three layers of cheesecloth. The filtrate was centrifuged at 18000 × g for 20 min. The supernatant was subjected to gel-filtration on a Sephadex G-25 column with 50 mM TES-NaOH buffer (pH 7.0) containing 1 mM dithiothreitol and 2 mM $MgCl_2$ to give a protein fraction (120 ml; 37 μg protein/ml). All of the operations were carried out at 4°. The protein fraction, after addition of KF (1.3 mmol), was used as the soluble enzyme system for all subsequent experiments.

Biosynthesis of borneol and camphor. To the soluble enzyme system (5 ml; pH 7.0) in a glass stoppered tube, a soln of [5,5-$^2H_2$]GPP (2.0 μmol) in 17 mM TES-NaOH buffer (0.6 ml; pH 7.0) containing 10 mM $MgCl_2$ and 0.3 mM dithiothreitol was added. The mixture was incubated at 30° for 16 hr

---

[†] The essential oil of the leaves contained 72% of camphor and 0.4% of borneol.

in the presence of 2 mM NADP. 28 U (0.2 mg) of alkaline phosphatase (Boehlinger Mannheim GmbH, 108162) was added to the incubation mixture. After incubation at 30° for 1 hr, the whole mixture was extracted with Et_2O (10 ml) containing 3.1 μg 1-dodecanol (int. standard). The Et_2O extract, after drying over Na_2SO_4, was concd to a constant small vol. (20 μl). The concentrate was subjected to GC-MS, GC-HR MS, and GC-SIM. In the GC-SIM analysis, the intensity of the deuterium-containing ion at *m/z* 97 of dideuteriated camphor and the intensity of the ion at *m/z* 83 ($C_6H_{11}^+$) of 1-dodecanol were determined. The amount of the dideuteriated camphor was determined to be 10.7 nmol from the ion intensity ratio of *m/z* 97 to *m/z* 83 by use of a standard curve prepared with authentic camphor and 1-dodecanol. The amount of dideuteriated borneol was determined to be 8.8 nmol in a similar manner.

Acknowledgements: The authors thank the Research Institute for Nuclear Medicine and Biology of Hiroshima University and Professor Osamu Yamamoto (Department of Biology, Faculty of Science, Hiroshima University) for the use of mass spectrometer and Dr. Kiyotaka Munesada of Hiroshima University for measurement of the high resolution mass spectra. The present work was supported in part by Grant-in-Aids for Special Project Research No. 02250227 (1990, T. S.) and No. 03236230 (1991, T. S.) from the Ministry of Education, Science and Culture.

REFERENCES

1. Croteau, R. and Karp, F. – Biochem. Biophys. Res. Commun. 72: 440 (1976).
2. Croteau, R. and Shaskus, J. – Arch. Biochem. Biophys. 236: 535 (1985).
3. Suga, T., Hirata, T., Aoki, T. and Shishibori, T. – Phytochemistry 25: 2769 (1986).
4. Dimmel, D. R. and Wolinsky, J. – J. Org. Chem. 32: 410 (1967).
5. Gloux, J. and Guglielmi, M. – Tetrahedron Letters 2935 (1969).
6. Yamamitsu, T., Ohta, S. and Suga, T. – J. Chem. Soc. Perkin Trans. I 1811 (1989).
7. Loomis, W. D., Lile, J. D., Sandstrom, R. P., and Burbott, A. J. – Phytochemistry 18: 1049 (1979).
8. Biemann, K. – Mass Spectrometry, p. 204. McGraw-Hill, New York (1962).