Phytochemistry, 1967, Vol. 6, pp. 1107 to 1110 Pergamon Press Ltd. Printed in England

SHORT COMMUNICATION

BIOSYNTHESIS OF STEVIOL FROM (−)-KAURENE

RAYMOND D. BENNETT, ELLEN RUTH LIEBER and ERICH HEFTMANN

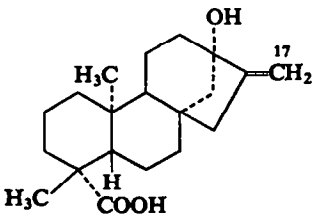
Western Regional Research Laboratory,* Albany, California, and
Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.

(*Received* 8 *February* 1967)

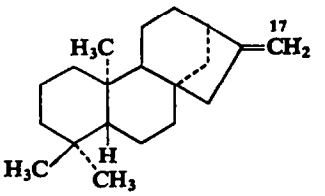
Abstract—(−)-Kaurene-17-^{14}C was administered to the leaves of a *Stevia rebaudiana* plant, and radioactive steviol was isolated by extraction, hydrolysis, and chromatography. Its radiochemical purity was demonstrated by dilution with carrier material, recrystallization to constant specific activity, conversion to the methyl ester, and recrystallization. The steviol methyl ester was degraded to establish that essentially all of the radioactivity was located at the 17-carbon atom.

INTRODUCTION

THE diterpene steviol (I) is found in the leaves of *Stevia rebaudiana* in the form of the glycoside stevioside. It is related both structurally and in biological activity[1] to the gibberellins, and therefore its biosynthesis in this plant is of considerable interest. Previously we demonstrated that radioactive acetate, but not mevalonic acid, is incorporated into steviol.[2] (−)-Kaurene (II) is known to be a precursor of gibberellins in the fungus *Fusarium moniliforme*,[3] and the same fungus converts steviol to a gibberellin-like substance.[4] We have now found that (−)-kaurene-17-^{14}C is converted to steviol in *S. rebaudiana*.



(I) Steviol



(II) (−)-Kaurene

RESULTS

(−)-Kaurene-17-^{14}C was applied twice weekly for 5 weeks to the leaves of a *Stevia rebaudiana* plant, which was then extracted with ethanol. Most of the radioactivity in the extract was removed by leaching the residue from evaporation of the ethanol with ether. This left a residue consisting largely of glycosides, which was subjected to enzymatic hydrolysis.[2] A steviol-containing acidic fraction was isolated and purified by preparative thin-layer

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Work conducted under a cooperative agreement with the California Institute of Technology.

[1] M. RUDDAT, A. LANG and E. MOSETTIG, *Naturwissenschaften* **50**, 23 (1963).
[2] M. RUDDAT, E. HEFTMANN and A. LANG, *Arch. Biochem. Biophys.* **110**, 496 (1965).
[3] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).
[4] M. RUDDAT, E. HEFTMANN and A. LANG, *Arch. Biochem. Biophys.* **111**, 187 (1965).

chromatography (TLC). Carrier steviol was added, and crystallization to constant specific activity was carried out as shown in Table 1. Steviol methyl ester was then prepared, and, after recrystallization, it had the same molar specific activity as steviol (Table 1). Finally, the 17-methylene group was removed by oxidation, giving the keto ester III, which was essentially devoid of radioactivity (Table 1).

TABLE 1. RECRYSTALLIZATION OF STEVIOL AND ITS DERIVATIVES*

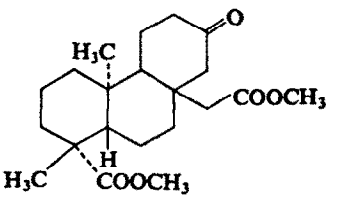
Compound	Solvent used for crystallizations	Counts/min/μM†
Steviol		8·40 ± 0·44
	Acetone–methanol	6·57 ± 0·35
	Acetone–methanol	6·65 ± 0·35
	Benzene–methanol	6·57 ± 0·35
Steviol methyl ester	Methanol–water	6·51 ± 0·35
Keto ester III	Hexane	0·37 ± 0·20

* 0·2-mg portions were plated from chloroform or benzene solutions on ringed planchets over an area of 12·7 cm² and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent and background was 2 counts/min.

† 90 per cent confidence level.

DISCUSSION

The results presented here demonstrate that (−)-kaurene may act as a precursor of steviol in *Stevia rebaudiana*. Because the per cent incorporation, based upon isolated steviol, was low (*ca.* 0·3 per cent), it was important to establish that the conversion had occurred without prior degradation of the radioactive kaurene in the plant. This was accomplished by conversion of steviol methyl ester to the keto ester (III), which lacks only the 17-carbon atom. Mosettig *et al.*[5] used ozone or osmium tetroxide followed by sodium periodate to effect this conversion, but we found the permanganate-periodate reagent of Lemieux and von Rudloff[6] to be more convenient. When the radioactive steviol methyl ester was subjected to this reaction, according to the procedure of Wall and Serota,[7] the keto ester (III) obtained had lost almost all of the original radioactivity. This demonstrates that the steviol was labelled only in the 17-position, and therefore was made directly from (−)-kaurene-17-^{14}C.



(III)

[5] E. MOSETTIG, U. BEGLINGER, F. DOLDER, H. LICHTI, P. QUITT and J. A. WATERS, *J. Am. Chem. Soc.* **85**, 2305 (1963).

[6] R. U. LEMIEUX and E. VON RUDLOFF, *Can. J. Chem.* **33**, 1701 (1955).

[7] M. E. WALL and S. SEROTA, *J. Org. Chem.* **24**, 741 (1959).

This result is consistent with previous indications that steviol biosynthesis is very similar to that of gibberellins. The growth retardant AMO-1618 is known to inhibit not only the biosynthesis of gibberellins, both in *Fusarium moniliforme*[8] and in higher plants,[9] but also that of steviol in *S. rebaudiana*.[10] Furthermore, it has been shown that AMO-1618 acts specifically to block the cyclization of *trans*-geranylgeranyl pyrophosphate to (−)-kaurene.[11] Thus, the present evidence that steviol is biosynthesized from (−)-kaurene supports the hypothesis[12] that the latter is a precursor of gibberellins in higher plants.

When (−)-kaurene-17-^{14}C was administered to *Pharbitis nil* seedlings, only 10–20 per cent of the radioactivity could be recovered by extraction on the following day.[13] This seemed to indicate that the plant was rapidly degrading the kaurene with loss of the 17-methylene group, perhaps as CO_2. In the present work, however, about half of the original radioactivity was recovered after an administration period of 5 weeks. This striking difference in kaurene metabolism may be due to the difference in species and/or the ages of the plants.

EXPERIMENTAL

Thin-layer chromatographic techniques were as described previously.[14] All chromatograms were run on Silica Gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* Zones were located on preparative plates by spraying with bromthymol blue solution and were eluted with acetone–acetic acid (99:1). Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details). Melting points were taken on a Kofler block and are corrected.

A *Stevia rebaudiana* Bert. plant was grown from a cutting for about one year. (−)-Kaurene-17-^{14}C ($1{\cdot}0 \times 10^5$ counts/min), prepared earlier,[13] was then applied to several leaves of the plant by the technique previously described.[15] Two such treatments were given per week until ten doses had been administered. Three days after the final treatment, the plant was cut off above the soil line, frozen in liquid nitrogen, and lyophilized. The dried material (50 g) was homogenized in a blender with three 1250-ml portions of absolute ethanol. The homogenates were filtered, combined, and evaporated to dryness in a rotary evaporator. The residue was repeatedly leached with anhydrous ether to remove lipid-soluble material. The combined ethereal solutions contained $4{\cdot}4 \times 10^5$ counts/min.

The insoluble residue was taken up in 150 ml of buffer, pH 4 (approximately 0·2 M Na_2HPO_4 and 0·1 M citric acid), and a 60-ml portion of this was incubated with 200 ml of Pectinol 59L† and 1940 ml of buffer for 6 days at 37°. The mixture was subsequently extracted with two 1000-ml portions and two 500-ml portions of benzene. Nitrogen was bubbled through the aqueous layer to remove traces of benzene, and it was reincubated with 200 ml of Pectinol 59L for 6 days. The mixture was extracted with benzene as before, and the extracts were combined with those obtained earlier, dried (Na_2SO_4), filtered, and evaporated. The residue (680 mg) was dissolved in 200 ml of benzene and 8 ml of methanol, and the solution was extracted with three 100-ml portions of 0·1 N NaOH. The extracts were passed through 30 ml of benzene, and the two benzene layers were filtered, combined, and evaporated, giving 88 mg of neutral material ($3{\cdot}4 \times 10^3$ counts/min). The aqueous extracts were combined, acidified to pH 2 with 3 N HCl, and extracted with three 200-ml portions of dichloromethane. The extracts were passed through 20 ml of water, filtered, combined, and evaporated, giving 458 mg of acidic material ($1{\cdot}26 \times 10^4$ counts/min).

TLC indicated that the acidic fraction was a complex mixture containing some steviol. By preparative TLC with butanol–3 N ammonia (5:1) a fraction enriched in steviol was obtained (96 mg, $4{\cdot}21 \times 10^3$ counts/min), and this was rechromatographed with chloroform–ethyl acetate–acetic acid (60:40:1) to give 19·9 mg of almost pure steviol ($1{\cdot}37 \times 10^3$ counts/min). After dilution with 32·0 mg of authentic steviol, this material

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

† Rohm and Haas Co., Philadelphia, Pennsylvania.

[8] M. RUDDAT, E. HEFTMANN and A. LANG, *Naturwissenschaften* **52**, 267 (1965).
[9] B. BALDEV, A. LANG and A. O. AGATEP, *Science* **147**, 155 (1965).
[10] M. RUDDAT, *Nature* **211**, 971 (1966).
[11] D. T. DENNIS, C. D. UPPER and C. A. WEST, *Plant Physiol.* **40**, 948 (1965).
[12] J. E. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **240**, 1847 (1965).
[13] R. D. BENNETT, S.-T. KO and E. HEFTMANN, *Plant Physiol.* **41**, 1360 (1966).
[14] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[15] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

was crystallized to constant specific activity (Table 1). The pure steviol was treated with dizaomethane in ether, and the methyl ester, which showed a single spot corresponding to authentic steviol methyl ester by TLC with dichloromethane–ethyl acetate–acetic acid (80:20:1), was recrystallized as shown in Table 1. The melting point of the crystallized material was 110–112°, and the melting point of a mixture with authentic steviol methyl ester was unchanged (literature m.p. 111–112°).[16]

The pure steviol methyl ester (15·9 mg) was dissolved in 5·0 ml of *t*-butyl alcohol, and a solution of 84 mg of $NaIO_4$, 2·6 mg of $KMnO_4$, and 21 mg of K_2CO_3 in 5·0 ml of water was added. The mixture was shaken vigorously for 1 hr, diluted with 4 ml of water, acidified to pH 2 with 3 N HCl, and extracted with three 8-ml portions of benzene. The extracts were washed with 4 ml of water, filtered, combined, and evaporated. The residue was taken up in 2 ml of ether and treated with an excess of CH_2N_2 in ether for 20 min. Evaporation of the ether gave a residue which was examined by TLC with dichloromethane–methanol (49:1). The major constituent had the same mobility as compound III (R_f 0·53), but minor spots corresponding to steviol methyl ester (R_f 0·38) and more polar material (R_f 0·11) were also observed. By preparative TLC in the same system, 11·1 mg of the material corresponding to compound (III) was isolated. After recrystallization (Table 1) its melting point was 115–116°. The melting point of an authentic sample of compound (III) was 114–115°, as previously reported.[5] A mixture of the two specimens melted at 114–116°.

Attempts to isolate formaldehyde, an expected product of the degradation, as the dimedon derivative were unsuccessful.

Acknowledgements—The authors are indebted to Dr. Manfred Ruddat, Department of Botany, University of Chicago, for a generous gift of steviol, and to Dr. James A. Waters, National Institutes of Health, Bethesda, Maryland, for samples of steviol methyl ester and keto ester (III).

[16] E. MOSETTIG and W. R. NES, *J. Org. Chem.* **20**, 884 (1955).