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STUDIES IN TERPENOID BIOSYNTHESIS—II.[1]

THE BIOSYNTHESIS OF STEVIOL

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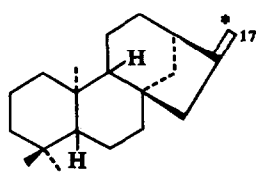
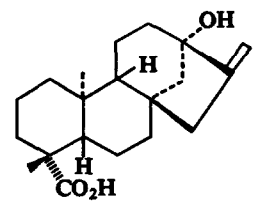
Abstract—The biosynthesis of steviol (II) in *Stevia rebaudiana* is shown to proceed from mevalonic acid through (−)-kaurene (I) and (−)-kaur-16-en-19-oic acid (III). Radiochemical evidence is presented for the formation of (−)-kaurene by *S. rebaudiana*.

(−)-KAURENE (I) has been shown[2,3] to act as a specific precursor of gibberellic acid. At that time we suggested[4] that it was also the logical precursor for a number of tetracyclic diterpenes including steviol (II). In this paper we present our evidence for the biosynthesis of steviol. Recently two publications on this biosynthesis have appeared. In the first[5] radioactive acetate but not mevalonic acid was shown to be incorporated into steviol whilst in the second[6] (−)-kaurene was shown to act as a precursor of steviol.



(I)



(II)

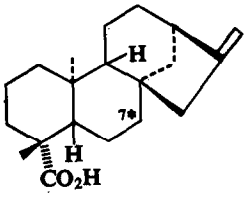
DL-Sodium mevalonate-2-^{14}C was fed to cut stems of young *Stevia rebaudiana* plants for a period of 5 days. The plants were then harvested, dried and extracted with light petroleum. This crude extract was diluted with inactive carrier (−)-kaurene and chromatographed on alumina. The (−)-kaurene was purified as its hydrochloride which was crystallized to constant activity. This showed an incorporation of 0·83 per cent and hence (−)-kaurene was being produced by the plant. Since the isolation of (−)-kaurene has not been reported from *S. rebaudiana*, experiments designed to demonstrate a biosynthesis rather than a transformation must establish the *in situ* formation of (−)-kaurene. Mevalonic acid had previously been shown[3] to act as a precursor of (−)-kaurene in *Echinocystis macrocarpa*.

The residual plant material was then re-extracted with ethanol. This extract was hydrolysed with a pectinase from *Aspergillus niger* and the steviol purified by preparative

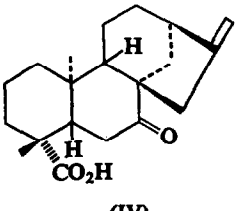
[1] First part, B. ACHILLADELIS and J. R. HANSON, *Phytochem.*, previous communication.
[2] B. E. CROSS, R. H. B. GALT and J. R. HANSON, *J. Chem. Soc.* 295 (1964).
[3] J. E. GRAEBE, D. T. DENNIS, C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **240**, 1847 (1965).
[4] J. R. HANSON, *J. Chem. Soc.* 5061 (1963).
[5] M. RUDDAT, E. HEFTMANN and A. LANG, *Arch. Biochem. Biophys.* **110**, 496 (1965).
[6] R. D. BENNETT, E. R. LIEBER and E. HEFTMANN, *Phytochem.* **6**, 1107 (1967).

TLC in two solvent systems. The steviol which was diluted and crystallized to constant activity, showed a low incorporation (0·03 per cent). To eliminate the possibility of a contaminant, the steviol was converted to isosteviol which retained the radioactivity. This contrasts with earlier negative mevalonate results which, as the authors admit, have at present an elusive explanation. In this connexion it is of interest to note the very different level of activity in the (−)-kaurene and stevioside fractions.

17-^{14}C-(−)-Kaurene was prepared,[2] solubilized in Tween 80 and fed hydroponically to growing *S. rebaudiana* shoots for 5 days. Isolation of the steviol as before showed an incorporation of 0·02 per cent. The purity of the product was established by conversion to isosteviol which retained the activity. Bennett *et al.*[6] obtained a better incorporation by a different feeding method.



(III)



(IV)

(−)-Kaur-16-en-19-oic acid (III) has been shown[7] to act as an intermediate in gibberellin biosynthesis and it therefore seemed a likely intermediate in the biosynthesis of steviol. The acid has been prepared[8] from 7-hydroxykaurenolide by a route which permitted its labelling. Reduction of the 7-keto-acid (IV) with hydrazine hydrate in the presence of T_2O gave 7-$^3H_2$-(−)-kaur-16-en-19-oic acid (III). This acid was fed hydroponically to twenty shoots of *S. rebaudiana*. After 5 days the steviol was isolated and carefully purified. It showed an incorporation of 0·17 per cent. The radioactivity was retained on conversion to isosteviol. Thus there is a stepwise sequence in the biosynthesis of steviol through (−)-kaurene and (−)-kaur-16-en-19-oic acid in which bridgehead hydroxylation takes place as the last stage.

EXPERIMENTAL

General details are described in Part I. *Stevia rebaudiana* plants were grown from seed generously provided by E. Gasperi (Paraguay). Pectinase from *Aspergillus niger* was purchased from L. Light and Co., and gave a better yield than snail digestive juices in the enzymic hydrolysis of stevioside.

The Feeding of Sodium Mevalonate-2-^{14}C to S. rebaudiana *Plants*

2 N NaOH (3 drops) was added to DL-mevalonic acid-2-^{14}C lactone (0·1 mc) and the solution made up to 10 ml.

Ten cut stems of *S. rebaudiana* (6–8 in. long) were allowed to grow in 1 ml aliquots of the mevalonate solution which was replenished with distilled water as necessary. After 5 days the plant stems were removed, dried and macerated thrice with light petroleum (400 ml). The combined extracts were concentrated, diluted with (−)-kaurene (50 mg) and chromatographed on Al_2O_3. The kaurene-containing fraction was treated with HCl gas in Et_2O and the (−)-kaurene hydrochloride crystallized to constant activity (m.p. 105°; total activity $1{\cdot}83 \times 10^5$ dpm, 0·83 per cent incorporation). The stems were then re-extracted with hot EtOH (300 ml) and the extract evaporated to dryness. This extract was incubated at 35° for 6 days in a citric acid/phosphate buffer at pH 4 (500 ml) in the presence of pectinase (5 g). The steviol was recovered in ether and purified by preparative TLC on silica plates in ethyl acetate:chloroform:acetic acid (8:12:1) and butanol:ammonia (5:1). The steviol was diluted and crystallized to constant activity (m.p. 212–213°; total activity $7{\cdot}3 \times 10^3$ dpm, 0·03 per cent incorporation).

[7] T. A. GEISSMAN, A. J. VERBISCAR, B. O. PHINNEY and G. CRAGG, *Phytochem.* **5**, 933 (1966).
[8] R. H. B. GALT and J. R. HANSON, *Tetrahedron* **22**, 3185 (1966).

Conversion of Steviol to Isosteviol

Steviol (8 mg) in a mixture of ethanol (0·75 ml), water (1·5 ml) and 2 N H_2SO_4 (0·11 ml) was refluxed for 1 hr. The solution was diluted with water and the isosteviol recovered in ether and crystallized to constant activity (m.p. 225°; $7{\cdot}4 \times 10^3$ dpm).

Feeding of 17-^{14}*C-(−)-Kaurene to* S. rebaudiana *Plants*

17-^{14}C-(−)-Kaurene ($3{\cdot}8 \times 10^6$ dpm) (10 mg) was dissolved in light petroleum (10 drops) and dispersed in water (10 ml) with Tween 80 (2 drops/100 ml). Aliquots (1 ml) were fed to ten stems of *S. rebaudiana* plants for 5 days. The stems were dried and extracted with ethanol and the steviol isolated as before. After dilution it was crystallized to constant activity (m.p. 212°; total activity $8{\cdot}02 \times 10^2$ dpm, 0·02 per cent). This was converted to isosteviol as before (m.p. 225°; total activity $7{\cdot}4 \times 10^2$ dpm).

Preparation of 7-$^3H_2$-(−)-*Kaur*-16-*en*-19-*oic Acid*

7-Oxo-(−)-kaur-16-en-19-oic acid (700 mg) in dry diglyme (30 ml) was treated with tritiated water (0·1 C) and $N_2H_4H_2O$ (5 ml) and the temperature raised to 150° over 3 hr and maintained at that temperature for 3 hr. KOH pellets (6·0 g) were added, the water distilled off and the solution refluxed at 230° for 8 hr. The solution was cooled, acidified and extracted with ethyl acetate. Chromatography of the extract on silica gel gave (−)-7-$^3H_2$-kaur-16-en-19-oic acid (332 mg) (m.p. 165–168°; 13,450 dps/mg).

Feeding of 7-$^3H_2$-(−)-*Kaur*-16-*en*-19-*oic Acid to* S. rebaudiana *Plants*

7-$^3H_2$-(−)-Kaur-16-en-19-oic acid (5 mg, total activity $4{\cdot}03 \times 10^6$ dpm) was distributed between twenty *S. rebaudiana* stems as above. After 5 days the plants were dried and extracted with ethanol and the steviol isolated as before. It was crystallized to constant activity (m.p. 212°; total activity $1{\cdot}66 \times 10^4$ dpm, 0·17 per cent). Treatment with acid gave isosteviol (m.p. 224–227°; $1{\cdot}68 \times 10^4$ dpm).

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