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Note

Quantitative determination of phorbic acid by gas–liquid chromatography

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Phorbic acid, (1*R*,3*R*)-1,3-dihydroxypentane 1,3,5-tricarboxylic acid, is a naturally occurring lactone-forming acid[1–5]. So far, no quantitative determination of this acid has been reported, and the purpose of this investigation was to devise a method for its determination that could be used in biosynthetic studies.

Gas–liquid chromatography (GLC) of the ethyl and methyl esters of phorbic acid have been described[4,5]. However, as the esterification of lactone-forming acids usually gives a mixture of esters, resulting in multiple peaks on the gas chromatograms, this method is unsuitable for quantitative determinations. The open-chain trimethylsilyl (TMS) derivatives of lactone-forming acids give single peaks after chromatography[6,7], and these derivatives were therefore used in this work.

EXPERIMENTAL

Chemicals

The dilactophorbic acid used was isolated from *Euphorbia resinifera*[1]. D-Glucaric acid 1,4-lactone (Sigma, St. Louis, Mo., U.S.A.) served as the internal standard.

The silylating reagents were trimethylchlorosilane (pure, Koch-Light, Colnbrook, Great Britain) and 1,1,1,3,3,3-hexamethyldisilazane (Merck, Darmstadt, G.F.R.). Before use, the pyridine (Merck) was distilled and kept over pellets of sodium hydroxide.

Apparatus

A Varian 1400 gas chromatograph with a flame-ionization detector was used. The column was a glass coil (3 m × 6 mm I.D.), filled with 7.5% OV-17 on Gas-Chrom Q (80–100 mesh). The injector temperature was 250° and the detector temperature 300°. The column temperature was kept isothermal at 195°. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min.

Test solutions

The acids were dissolved in water to give solutions of the following concentrations: A, dilactophorbic acid (1 mg/ml) and glucaric acid 1,4-lactone (0.5 mg/ml); B, dilactophorbic acid (0.25 mg/ml); and C, glucaric acid 1,4-lactone (0.25 mg/ml).

Aliquots of these solutions were mixed in such a way that the ratio between dilactophorbic acid and glucaric acid 1,4-lactone varied from 1.33 to 2.25 and the total amount of the acids was about 20 mg.

Preparation of the sodium salts

To the acid mixture was added sodium hydroxide solution (0.1 *N*) to give a pH of 11 (measured with pH meter), and the mixtures were subsequently heated for $\frac{1}{2}$ h at 60°. This procedure was repeated until the pH remained constant at 11. Such a high pH and pH control was necessary as one of the lactone rings of the dilactophorbic acid is relatively stable[7,8]. After evaporation, the sodium salts were dried by repeated evaporation after the addition of benzene.

Preparation of the TMS derivatives

The TMS derivatives were prepared from the dried sodium salts in anhydrous pyridine using the reagents recommended by Raunhardt *et al.*[6].

RESULTS AND DISCUSSION

The retention time of the open-chain TMS derivatives of phorbic acid relative to the corresponding derivative of glucaric acid was 1.92 (Fig. 1). The TMS derivatives of the Krebs cycle acids, prepared as described in this paper, have retention times below 1 relative to the fully silylated glucaric acid. For the identification of

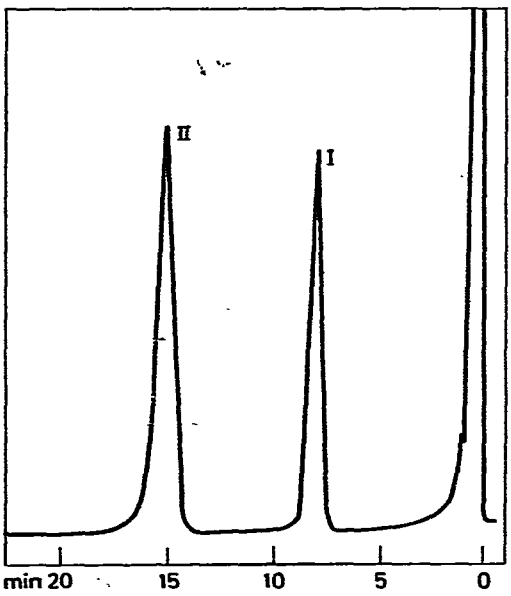

Fig. 1. Gas–liquid chromatogram of the open-chain TMS derivatives of glucaric acid (I) and phorbic acid (II). The weight ratio of dilactophorbic acid to glucaric acid 1,4-lactone before derivatization was 2.

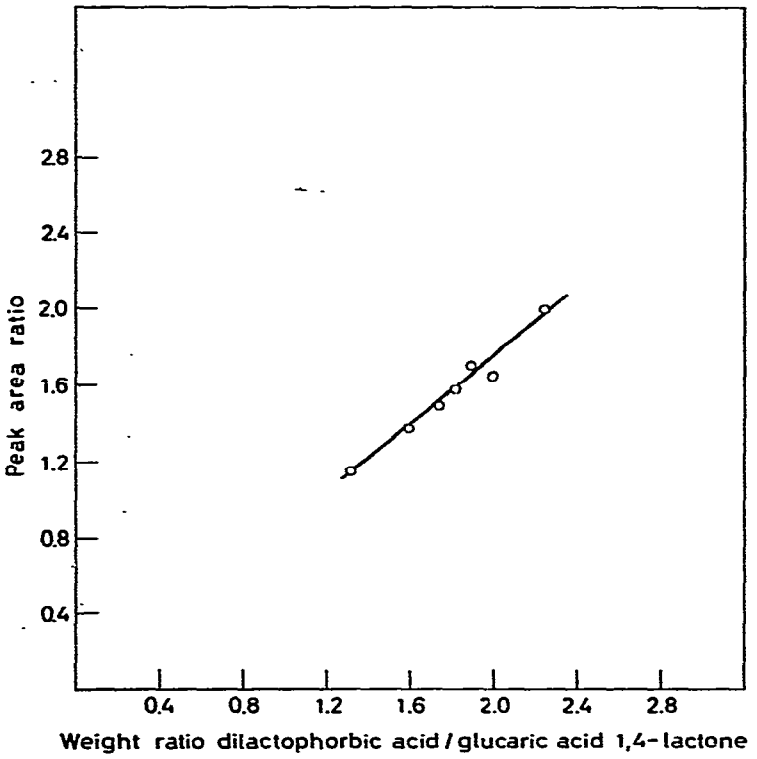

Fig. 2. Calibration graph for the open-chain TMS derivative of phorbic acid with the corresponding derivative of glucaric acid as internal standard.

phorbic acid in plant material, the GLC method outlined here should therefore have certain advantages.

The peak areas were calculated by multiplying the peak heights by the widths at half-height. The ratios of the peak areas of the open-chain TMS derivatives were plotted against the weight ratios of the acids (dilactophorbic acid and glucaric acid 1,4-lactone). The standard deviation of the individual values of the calculated ratios was 0.09. The linearity of the calibration graph (Fig. 2) makes it possible to use the method for quantitative determinations.

Glucaric acid was selected as the internal standard for three reasons: (1) it is a lactone-forming acid like phorbic acid; (2) so far glucaric acid has not been found in plants containing phorbic acid; and (3) in the GLC method described, glucaric acid is well separated from most of the common plant acids.

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