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Note

Analytical droplet counter-current chromatography isolation of 20-hydroxyecdysone from *Vitex thyrsiflora* (Verbenaceae)

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Droplet counter-current chromatography (DCCC) has proved a very useful tool for preparative-scale separations of highly polar compounds[1]. Water-soluble compounds can be easily separated in gram amounts[2,3].

In our continuing search for alternative insect-control agents on natural products[4,5], we have recently examined the root bark of the West African medicinal plant *Vitex madiensis* (Verbenaceae)[3]. The crude methanol extract proved active in our insect artificial-diet feeding assays[4,6] and subsequently yielded the phytoecdysteroids 20-hydroxyecdysone and ajugasterone C in very large amounts. Phytoecdysteroids have significant physiological effects in insects and have therefore been suggested to play a role in the defence of plants from insect attack[7]. The extremely large yields from the root bark decided us to investigate the closely related *Vitex thyrsiflora* (Verbenaceae), which is also from West Africa and is used there in the treatment of stomach complaints, sexual sterility and wounds.

A preliminary investigation of the methanol extracts of the fruits and leaves by thin-layer chromatography (TLC) showed neither 20-hydroxyecdysone nor ajugasterone C to be present in a detectable amount. Although concentional high-performance liquid chromatography (HPLC) techniques can be used very effectively to detect low concentrations of ecdysteroids, laborious preliminary purification steps are necessary to ensure that all the components in the mixture are compatible with the particular column and solvent system used. This step also serves to preconcentrate the sample, which is necessary since the inherent low sample capacity of HPLC can place low-percentage components below the detection limits. However, in a high-sample-capacity system such as (DCCC)[1,8] a large enough sample can be applied, so that even low percentages of phytoecdysteroids will be present in sufficiently large enough amounts and, due to inherent low solvent consumption, in sufficiently high concentrations to be readily detected. Also, the problems of column-sample incompatibility found in most solid-support chromatographic systems are not encountered. Hence, DCCC was employed for the detection and isolation of phytoecdysteroids in the methanol extract of the fresh fruits and of the leaves of *V. thyrsiflora*.

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EXPERIMENTAL

Plant material

Fruits and leaves of *V. thyrsiflora* were collected in Cameroon in 1981. Fresh fruits (250 g) were extracted with methanol to give 30 g of extract. Fresh leaves (500 g) were extracted with ethyl acetate to give 17.5 g of extract.

DCCC separation

A 2.05-g portion of the crude methanol extract of fresh fruits of *V. thyrsiflora* was dissolved in 10 ml of the mobile phase and injected into the 10-ml sample loop of a DCCC-300-G2 (Tokyo Rikakikai, Tokyo, Japan) equipped with 300 (400 × 2 mm I.D.) glass columns. The chloroform–methanol–water (13:7:4, v/v) eluent, in the ascending mode, was chosen empirically by pre-screening authentic 20-hydroxyecdysone (previously isolated from *V. madiensis*[3] on a TLC plate, Polygram Sil G/UV 254, Macherey-Nagel, Düren, F.R.G.). A flow-rate of 2.1 ml/h was used and the eluates were collected in 2.1-ml fractions. The fractions were monitored by UV detection at 254 nm (Single path monitor UV-1, Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and by TLC, with use of a vanillin–sulphuric acid–ethanol (3 g:1.5 ml:100 ml) spray reagent. A 12-mg amount of 20-hydroxyecdysone was obtained and identified by direct comparison with an authentic sample[3]. The DCCC separation is shown in Fig. 1.

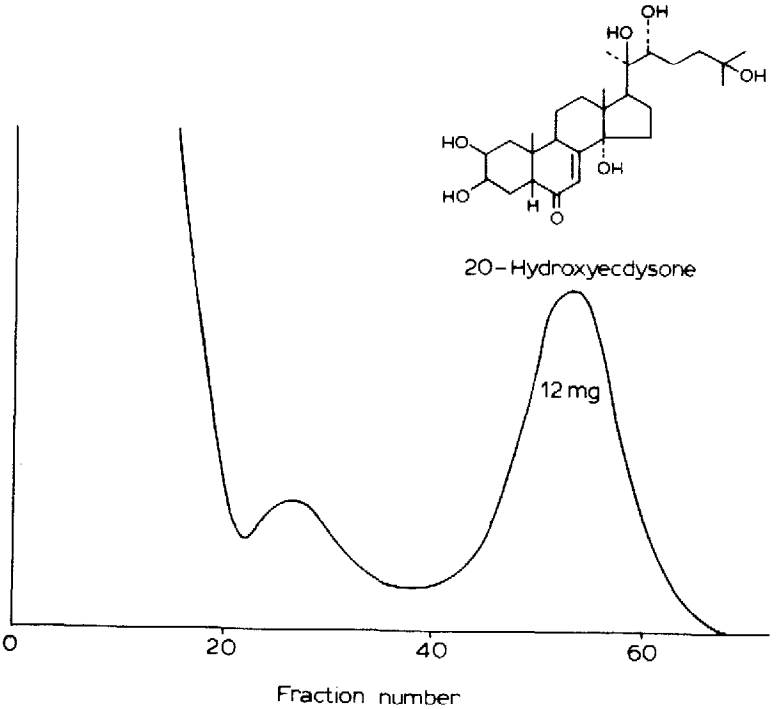

Fig. 1. DCCC of the methanol extract of *V. thyrsiflora* (2.05 g) with chloroform–methanol–water (13:7:4, v/v) as eluents by the ascending method; 2.1 ml per fraction; detection at 254 nm.

RESULTS AND DISCUSSION

A chloroform–methanol–water (13:7:4, v/v) solvent system was used as eluent in our DCCC separation of the methanol extract of the fruits in the ascending mode. The eluates were collected in 2.1-ml fractions, and the fractions were monitored by UV detection at 254 nm. Fig. 1 shows the weight of the eluate per fraction, and shows baseline separation. A 12-mg sample of 20-hydroxyecdysone was isolated from a 2.05-g injection of the methanol extract to give a yield of 0.07% (7000 ppm) of 20-hydroxyecdysone in the fresh fruits, without any preliminary purification steps.

Although the root bark of *V. madiensis* contains 20-hydroxyecdysone and ajugasterone C in a 3:1 ratio, no ajugasterone C could be found in the fruits studied here. Also, when the above procedure was repeated with fresh leaves of *V. thyrsiflora*, neither 20-hydroxyecdysone nor ajugasterone C could be found.

The detection limit for this separation is much lower than 12 mg, and 1 mg can be readily detected. The ratio of 1 mg to the 2 g of injected sample is comparable with the 10-ng HPLC detection limit of compound (I)[9] in an analytical-scale injection of 0.02 mg. However, such a direct injection of the crude methanol extract would, of course, result in permanent column contamination due to the wide range of compounds present.

The present success achieved in the direct isolation of only 700 ppm of 20-hydroxyecdysone by using DCCC should be directly applicable to the isolation of 20-hydroxyecdysone in relatively small amounts from insect sources. This has proved to be quite difficult in the past.

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