Journal of Chromatography, 257 (1983) 157–161
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,422

Note

Efficient isolation of phytoecdysones from *Ajuga* plants by high-performance liquid chromatography and droplet counter-current chromatography

ISAO KUBO*, JAMES A. KLOCKE, IRAJ GANJIAN, NOBUTAKA ICHIKAWA and TAKESHI MATSUMOTO

Division of Entomology and Parasitology, College of Natural Resources, University of California, Berkeley, CA 94720 (U.S.A.)

(Received October 4th, 1982)

Since the isolation of the first insect molting hormone from a plant, *Podocarpus nakai* (Podocarpaceae)[1], a number of phytoecdysones have been found in plants[2]. These phytoecdysones have significant physiological effects in insects and have therefore been suggested to play a role in the defense of plants from insect attack[3]. Our recent findings[4] that two phytoecdysones, β-ecdysone and cyasterone, isolated from an East African medicinal plant, *Ajuga remota* (Labiatae), exhibit insect ecdysis inhibitory activity against two species of lepidopterous agricultural pests strongly support this hypothesis. This has led to our investigation of several *Ajuga* species for phytoecdysones.

High-performance liquid chromatography (HPLC) is an excellent method for the analysis of phytoecdysones[5], especially as it is non-destructive, so that natural products can be isolated. We have accordingly used this technique to identify the phytoecdysones in three species of *Ajuga*. However, a disadvantage of this method is that the substantial amount of phytoecdysones needed for our bioassay of insect ecdysis inhibitory activity in the *Ajuga* plants is not readily isolated.

Droplet counter-current chromatography (DCCC)[6], on the other hand, is an especially efficient method for the preparative separation of polar compounds such as the phytoecdysones. Thus, with DCCC, while requiring only small volumes of solvent, we have been able to separate rapidly and non-destructively and recover fully more than 100 mg of each of the bioactive *Ajuga* phytoecdysones from each 0.5–3 g injection. Thus, HPLC and DCCC tend to complement each other.

This paper describes both the phytoecdysone constituency of the foliage of three *Ajuga* plants, *A. remota*, *A. reptans* and *A. chameacistus*, as determined by HPLC analysis, and also the efficient isolation of the *A. remota* phytoecdysones by DCCC.

MATERIALS AND METHODS

Plants

The three species of *Ajuga* analyzed for phytoecdysone content, with the local-

0021-9673/83/0000–0000/$03.00

ities and dates of collection, were *A. remota* (Nairobi, Kenya, August 1980), *A. reptans* (Texas, U.S.A., July 1981) and *A. chameacistus* (Tehran, Iran, July 1980).

HPLC

HPLC was carried out with a DuPont Model 850 liquid chromatograph on a pre-packed reversed-phase DuPont Zorbax ODS (particle size 5–6 μm) stainless-steel column (25 cm × 4.6 mm I.D.). This column was protected with a Whatman stainless-steel pre-column (7 cm × 2.1 mm I.D.) packed with pellicular Whatman Co:Pell ODS. Effluents were detected with a DuPont variable-wavelength UV spectrophotometer and micro-flow cell at 254 nm.

The samples were dissolved in methanol and injected into the column using a Rheodyne rotary valve 7120 syringe-loading injector. The methanol extracts of the leaves of *Ajuga*, from different geographical origins, were prepared and partially purified prior to the HPLC injection. In each instance the methanol extract was partitioned between ethyl acetate and water; the residue that was obtained from the evaporation of the organic layer was further purified by the addition of diethyl ether. The materials that were not soluble in diethyl ether were separated by filtration. This semi-purified solid was dissolved in methanol and the solution was injected into the HPLC system without further purification. The solvent system consisted of water–acetonitrile–methanol (82:18:2.25) at a flow-rate of 1.5 ml/min and an average pressure of 110–112 bar. In each instance a methanol solution of analytically pure phytoecdysones was co-injected with the plant extract as internal standards. HPLC solvents were of high purity (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

DCCC

DCCC was performed on a Model DCC-300-G2 (Tokyo Rikakikai Co., Tokyo, Japan) with 300 glass columns (400 × 2 mm I.D.). The DCCC solvent system of chloroform–methanol–water (13:7:4) was chosen empirically by prescreening the crude ethyl acetate extract of the *A. remota* roots on thin-layer chromatographic (TLC) plates (Macherey, Nagel & Co., Düren, G.F.R., Polygram Sil G/UV 254)[7]. By using the organic layer of this system as eluent, the R_F values of cyasterone, ajugasterone C and β-ecdysone were determined to be 0.37, 0.30, and 0.22, respectively. We therefore chose the upper phase as the mobile phase in our DCCC system.

The crude ethyl acetate extract (2.5 g per injection) was dissolved in a 1:1 mixture of the mobile and stationary phases and injected directly into the DCCC apparatus using a 10-ml sample chamber. The flow-rate was 4.5 ml/h and the eluents were collected in 4.5-ml fractions. Fractions were monitored by TLC (Sil G/UV 254) with chloroform–methanol (5:1) as the solvent system. Detection of the compounds on the TLC plates was accomplished by UV spectroscopy (Model UVSL-58 Mineralight lamp, 254 nm) and using vanillin–sulfuric acid–ethanol (3 g: 1.5 ml: 100 ml) as a spray reagent.

RESULTS AND DISCUSSION

Simple partitioning of crude methanolic extracts of the *Ajuga* foliage prior to HPLC injection was sufficient to give efficient and rapid separations of phytoecdysones. This is illustrated with the three phytoecdysones detected in *A. remota* (Fig. 1),

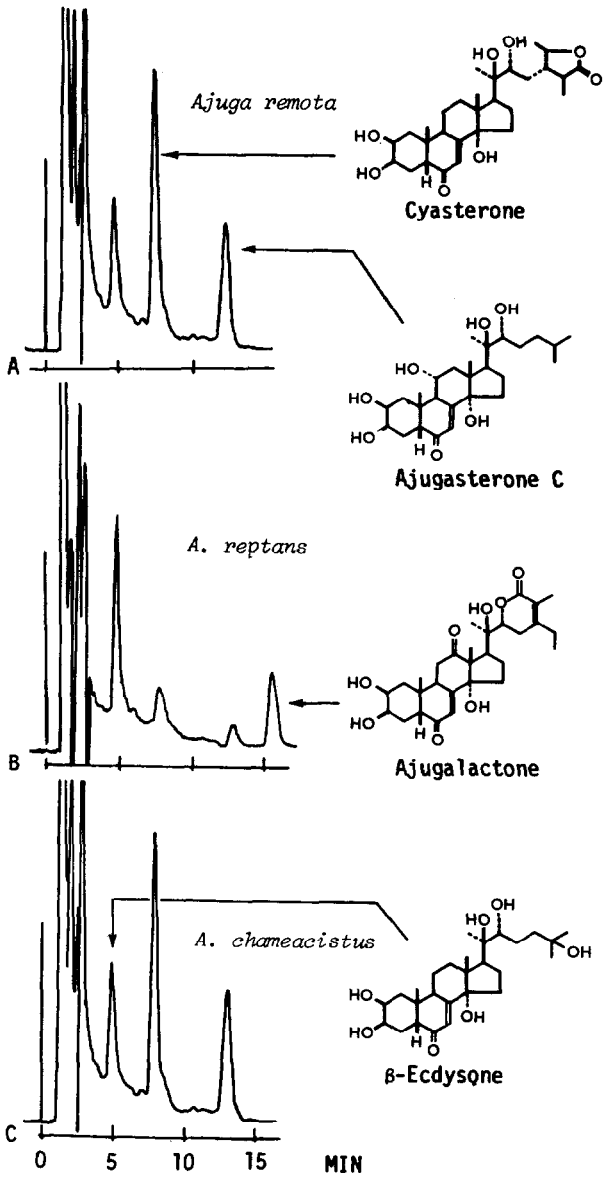

Fig. 1. C_{18} reversed-phase HPLC separation of the ethyl acetate extract of *Ajuga* plants. Mobile phase: water–acetonitrile–methanol (82:18:2.25). Flow-rate: 1.5 ml/min. Detection at 254 nm.

including β-ecdysone, which eluted first with a retention time of 4.7 min, and cyasterone and ajugasterone C, which eluted at 7.5 and 12.0 min, respectively. Utilizing the same conditions β-ecdysone and cyasterone were also detected in *A. chameacistus*, while β-ecdysone and ajugalactone (retention time 15.3 min) were detected in *A. reptans*. Of these three *Ajuga* species, the phytoecdysones of both *A. reptans* and *A. chameacistus* have not been reported previously.

The optimal mobile phase, in terms of rapid resolution, was water–acetonitrile–methanol (82:18:2.25). We expect this mixture to be applicable to phytoecdysone analysis in other materials. For example, we have found it to be satisfactory for the identification of ecdysones in insect tissue (the detailed data will be published elsewhere). If interference is encountered, the retention times of the phytoecdysones can easily be increased or decreased by increasing of decreasing, respectively, the percentage of acetonitrile in the mixture.

Detection of the phytoecdysones is facilitated by their α,β-unsaturated ketone moiety, which absorbs strongly (ε = 12,000) at 242 nm.

Our initial interest in phytoecdysones arose as a result of the biological activity we observed in *A. remota* extracts containing them. We subsequently decided to conduct more extensive biological studies which required several hundred milligrams each of the *A. remota* phytoecdysones. Although the qualitative capacity of HPLC easily led to phytoecdysone separation and detection, our quantitative requirement for phytoecdysones led to our use of DCCC.

DCCC, an all-liquid separation technique, is particularly useful for the separation of polar constituents, such as the phytoecdysones (Fig. 2). We were able to fractionate 2.5-g samples of an ethyl acetate extract of *A. remota* roots into over 200 mg of the three phytoecdysones, including 40 mg of β-ecdysone, 47 mg of ajugasterone C and 137 mg of cyasterone, with the use of only 400 ml of solvent. None of the injected sample was lost as both the mobile and the stationary phase were fully recovered.

The ascending mode (lighter mobile phase) was utilized in our DCCC separation of the phytoecdysones, as the more polar mobile phase (less polar stationary phase) is optimal for the separation of highly polar solutes such as the phytoecdysones.

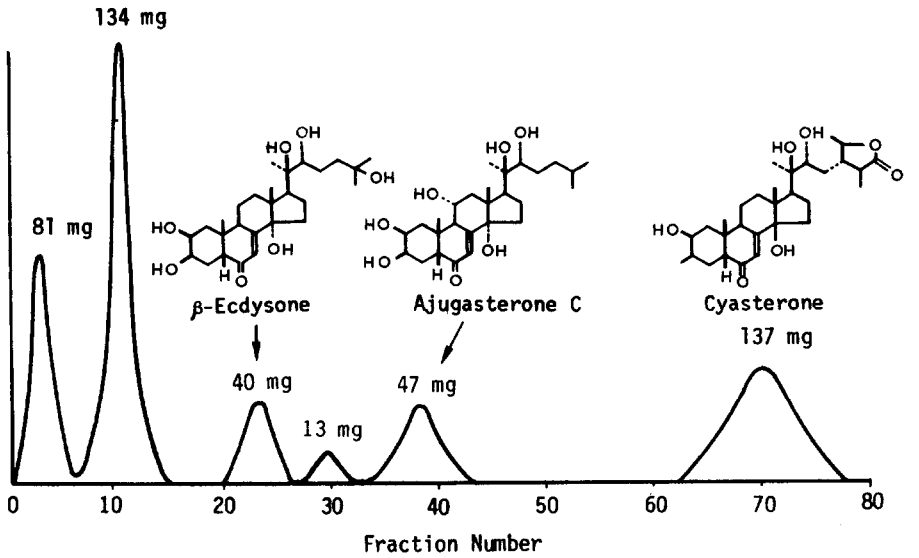

Fig. 2. DCCC separation of the ethyl acetate extract of *Ajuga remota* (2.5 g) with chloroform–methanol–water (13:7:4) by the ascending method.

The order of elution of the *A. remota* phytoecdysones on DCCC differed from the order on C_{18} reversed-phase HPLC columns. Thus, on DCCC, β-ecdysone eluted first, followed by ajugasterone C and finally cyasterone.

In conclusion, the qualitative analysis of the ecdysone content of both plant and animal materials is best accomplished with HPLC, while whereas for the quantitative recovery of these ecdysones DCCC is more suitable.

ACKNOWLEDGEMENTS

We are grateful to Takasago Perfumery Company for the purchase of the DCC-300-G2 instrument. We are also thankful to Dr. J. C. James for helpful suggestions, Professor T. Takemoto and Professor F. Camps for the authentic phytoecdysones and Professor J. Aynehchi and Mr. C. Allenson for *A. chameacistus* and *A. reptans* collections, respectively.

REFERENCES

1 K. Nakanishi, M. Koreeda, S. Sasaki, M. L. Chang and H. Y. Hsu, *Chem. Commun.*, (1966) 915.
2 C. Hetru and D. H. S. Horn, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 13.
3 C. G. Jones and R. D. Firn, *J. Chem. Ecol.*, 4 (1978) 117.
4 I. Kubo, J. A. Klocke and S. Asano, *Agr. Biol. Chem.*, 45 (1981) 1925; *J. Insect Physiol.*, in press.
5 M. Koreeda and B. A. Teicher, in R. B. Turner (Editor), *Analytical Biochemistry of Insects*, Elsevier, Amsterdam, 1977, p. 207.
6 K. Hostettmann, M. Hostettmann-Kaldas and K. Nakanishi, *J. Chromatogr.*, 170 (1979) 355.
7 K. Hostettmann, *Planta Med.*, 39 (1980) 1 and references cited therein.