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CHROMATOGRAPHY OF TANNINS

IV[a]. SEPARATION OF LABILE OLIGOMERIC HYDROLYSABLE TANNINS AND RELATED POLYPHENOLS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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SUMMARY

Using centrifugal partition chromatography for the separation and purification of oligomeric hydrolysable tannins extracted from *Heterocentron roseum*, a labile trimer, nobotanin J, which was readily decomposed into a monomer and dimer(s) on a gel column chromatographic solid support, and a more polar tetramer, nobotanin K, were efficiently purified by normal- and reversed-phase developments using the solvent system *n*-butanol–*n*-propanol–water (4:1:5, v/v/v). Licochalcone B, a polyphenol having tannin-like activity, was also separated from other components with related structures in Sinkiang licorice extract by centrifugal partition chromatography with normal-phase development using chloroform–methanol–water (7:13:8, v/v/v) as the solvent system.

INTRODUCTION

Recently found activities of tannins, such as antitumour[1–3] and antiperoxidation[4,5], which are largely dependent on the structure of the tannin, have increased the need for the isolation of each component in tannin mixtures obtained from plants, as such tannins are frequently present in foods and drinks and also in medicinal plants.

In general, the separation and purification of these tannins from plant extracts can be achieved by a combination of droplet counter-current chromatography with column chromatography over an appropriate solid support such as vinyl polymer resins, *e.g.*, Toyopearl HW-40 and MCI-gel CHP-20P, and dextran gel (Sephadex LH-20)[6], and sometimes with high-performance liquid chromatography (HPLC) on a preparative scale in the final stage of purification. However, as tannins are often present as mixtures of labile polyphenols with large molecules, which are easily adsorbed on various substances, column chromatography of tannins and related polyphenols using solid supports is often problematic owing to adsorption, and some-

[a] For Part III, see T. Hatano, T. Yoshida and T. Okuda, *J. Chromatogr.*, 435 (1988) 285.

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times hydrolysis, on the solid support. Preparative HPLC is often accompanied by sample loss and deterioration and contamination of the column.

The application of centrifugal partition chromatography to the preparative fractionation of hydrolysable tannins was reported in a previous paper[6]. This technique has the advantages over other techniques of chromatography, particularly on the preparative scale, of absence of a solid support, which causes losses of tannins by adsorption, and of a shorter development time[7]. It also has the advantage of requiring only a small volume of the developing solvent, which shortens the time of evaporation of the solvent from each fraction. The absence of a solid support is particularly favourable for the separation of tannins with large molecules, such as the oligomeric hydrolysable tannins which are often adsorbed strongly on a solid support.

However, most of the examples described in our previous paper[6] involved the separation of tannins in the crude extracts from plants into several fractions containing two or more tannins or related polyphenols, except for one example in which the normal-phase development of a fraction obtained by reversed-phase development led to further purification of each tannin. Final purification of the components in each fraction was mostly carried out by gel column chromatography.

We now present the results of the application of centrifugal partition chromatography to the purification of labile oligomeric hydrolysable tannins extracted from *Heterocentron roseum*, which are hardly purified by gel column chromatography because it is readily hydrolysed during the development. The separation of bioactive polyphenols of other types, extracted from Sinkiang licorice, by centrifugal partition chromatography is also described.

EXPERIMENTAL

Apparatus

A Model L-90 centrifugal partition chromatograph (Sanki Engineering, Nagaokakyo, Kyoto, Japan), consisting of a centrifuge with twelve column cartridges, each containing a polyfluoroethylene resin block (150 mm × 40 mm × 40 mm), was used. Each resin block has 50 holes which act as the separation columns (40 mm × 3 mm), connected to each other by fine resin tubes. The internal volume of each cartridge is *ca.* 15 ml[7]. The sample solution and the solvents were pumped into the columns, rotating at 700 rpm, with a Model CPC-LBP-II pump (Sanki). A Model CPC-UVM-I UV absorbance monitor, equipped with a cell of light path 0.2 mm (Sanki), was used at 254 nm, and fractions were collected with a Model SF-160 K fraction collector (Toyo Kagaku Sangyo, Tokyo, Japan).

Solvent

The solvent systems used were those previously found to be widely applicable for the separation and purification of tannins and related polyphenols by droplet counter-current chromatography[6] and also by centrifugal partition chromatography[6], with good efficiency and without causing hydrolysis. Solvent A was *n*-butanol–*n*-propanol–water (4:1:5, v/v/v). The lower layer was used as the stationary phase for the normal-phase development, and was pumped into the column prior to loading of the sample solution. The upper layer was used as the stationary phase for the

reversed-phase development. Solvent B was chloroform–methanol–water (7:13:8, v/v/v). The upper layer was used as the stationary phase for the normal-phase development.

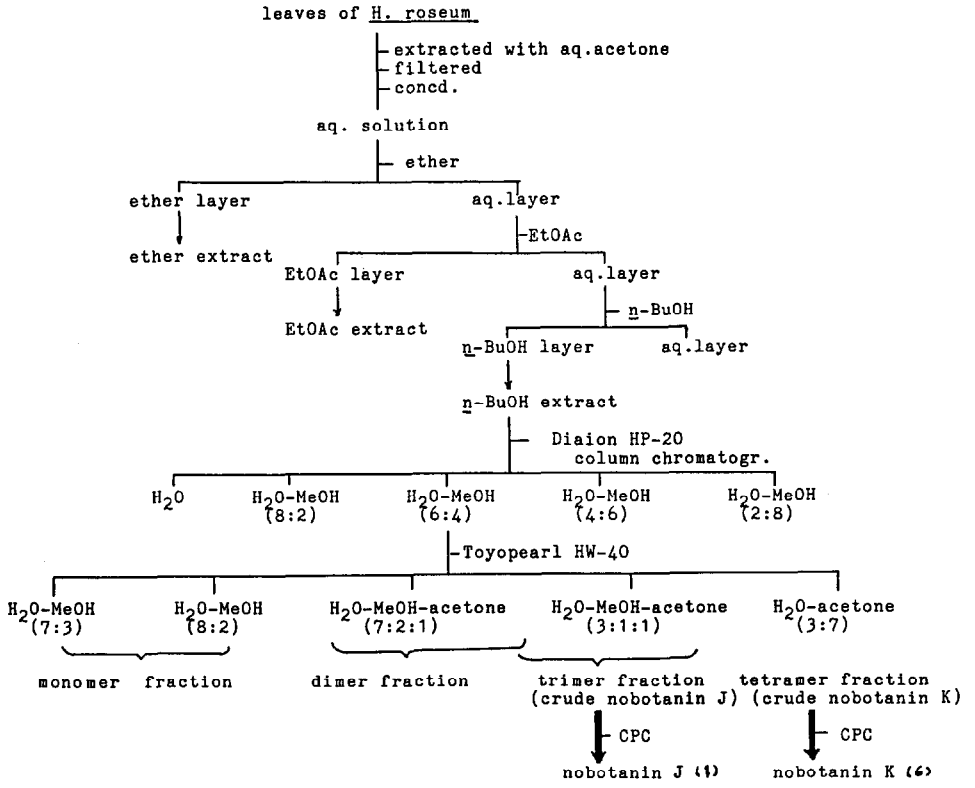
Analysis of fractions

Every second or third fraction was monitored, after evaporation of the solvent, by HPLC with UV detection at 280 nm, and also with diode-array UV detection. The apparatus consisted of an LC-6A system (Shimadzu, Kyoto, Japan) equipped with a YMC A312 (ODS) column (150 × 6 mm I.D.; Yamamura-kagaku, Kyoto, Japan), an SPD-6A UV monitor (Shimadzu) and an MCPD-350PC, System II diode array detector (Otsuka Electronics, Hirakata, Osaka, Japan). The column was kept at 40°C in an oven and was eluted with the solvent system (a) 0.05 *M* H_3PO_4–0.05 *M* KH_2PO_4–ethanol–ethyl acetate (40:40:15:5, v/v), (b) acetonitrile–water–acetic acid (35:60:5, v/v/v) or (c) methanol–water–acetic acid (60:35:5, v/v/v). Normal-phase HPLC was performed on a column (150 × 4 mm I.D.) of Develosil 60-5 (high-porosity silica) (Nomura Chemical, Seto, Aichi, Japan), using hexane–methanol–tetrahydrofuran–formic acid (60:45:15:1, v/v) containing oxalic acid (500 mg in 1.2 l) as eluent.

The effluent was monitored by UV absorption at 280 nm. The purity of the isolated components was also confirmed by TLC on Kieselgel PF_{254} (Merck) developed with chloroform–water–acetic acid (155:33:12, v/v/v), and by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy using a Varian VXR 500 instrument (500 MHz for ^1H and 126 MHz for ^{13}C) at the SC NMR Laboratory of Okayama University.

Preparation and fractionation of plant extracts

Fresh leaves (2.1 kg) of *Heterocentron roseum* were extracted with acetone–water (7:3, v/v) and filtered. After removal of the acetone by distillation, the aqueous solution was succesively extracted with diethyl ether, ethyl acetate and *n*-butanol saturated with water. The *n*-butanol extract (8.1 g) was chromatographed on a column (33 cm × 3 cm I.D.) of Diaion HP-20 polystyrene resin (Mitsubishi Chemical Industries, Tokyo, Japan) by stepwise elution with water with an increasing amount of methanol. The eluate with 40% methanol (3.6 g) was then rechromatographed on a column (42 cm × 2.2 cm I.D.) of Toyopearl HW-40 high-porosity vinyl polymer (coarse grade) (TOSOH, Tokyo, Japan) by stepwise elution with methanol–water (7:3, v/v; 1.4 l) → methanol–water (8:2, v/v; 4 l) → methanol–water–acetone (7:2:1, v/v/v; 1 l) → methanol–water–acetone (3:1:1, v/v/v; 0.7 l) → water–acetone (3:7, v/v; 0.5 l). Fractions of 7–10 ml were collected with a Model SF-100G fraction collector (Toyo Kagaku Sangyo) and every third fraction was monitored by UV absorption at 280 nm. The crude trimer nobotanin J (**1**) (1.1 g) was obtained from the fraction eluted with methanol–water–acetone (3:1:1, v/v/v). The 70% aqueous acetone eluate yielded a mixture of tetramers (650 mg) containing nobotanin K (**6**) as the main component (Scheme 1). The crude nobotanin J was dissolved in the lower layer of solvent system A (20 ml) and was subjected to centrifugal partition chromatography with development with the upper layer of the same solvent system (normal-phase development) at a rate of 1.0 ml/min. A portion (410 mg) of the tetramer fraction, which is more polar than the trimers, was purified by centrifugal partition chromato-



Scheme 1. MeOH = methanol, BuOH = butanol, EtOAc = ethyl acetate.

graphy with reversed-phase development with solvent system A to yield nobotanin K (**6**) (112 mg).

The aqueous acetone extract of Sinkiang licorice (licorice roots from the Sinkiang region of China) was partitioned between water and diethyl ether. The aqueous layer was further extracted with ethyl acetate and then *n*-butanol saturated with water. A portion (3 g) of the ether extract was fractionated by centrifugal partition chromatography with normal-phase development with solvent system B, collecting 10-g portions of the elute. Crude licochalcone B obtained on evaporation of fraction IV (Fig. 4) was further chromatographed over Kieselgel 60 (Merck) using chloroform–methanol (98:2, v/v) to isolate pure licochalcone B (**7**) (122 mg) and 7,4′-dihydroxyflavone (**8**) (6 mg). The eluate from fraction II (Fig. 4) was similarly subjected to a further fractionation on a Kieselgel 60 column using the same solvent, to give licochalcone A (**9**) (112 mg).

The purities of these compounds were confirmed by TLC (Kieselgel PF_{254}) and reversed-phase HPLC (solvent b or c).

RESULTS AND DISCUSSION

Separation of labile and highly polar oligomeric hydrolysable tannins

Heterocentron roseum, a tropical plant belonging to Melastomataceae, is rich in oligomeric hydrolysable tannins, including dimers, trimers and tetramers. Although five dimers (nobotanin B, F, G, H and I)[8–10] and a trimer (nobotanin E)[10] were isolated by column chromatography on a vinyl polymer resin (Toyopearl HW-40), another trimer, nobotanin J (**1**), which is more labile than the others, could not be purified even by repeated chromatography on the resin column developed with water–methanol–acetone of various proportions. Substantial contamination by monomers and dimers, which should include those produced by the partial hydrolysis of **1** on the solid support during the development, was always observed. This hydrolysis was particularly extensive when the column chromatography was carried out on Sephadex LH-20, resulting in conversion of more than 70% of nobotanin J into a mixture of a monomer and dimers, although a small amount of pure nobotanin J was isolated by this column chromatography. In order to minimize this undesirable hydrolysis during the purification procedure, application of centrifugal partition chromatography, which can be performed rapidly in the absence of the solid support, was attempted.

The crude nobotanin J (**1**) (1.1 g) (purity *ca.* 72% when calculated from peak areas in HPLC), which was obtained from column chromatography over Toyopearl HW-40 (coarse grade) (Scheme 1), was finally purified by centrifugal partition chromatography in the normal-phase mode using solvent system A, to yield nobotanin J (154 mg) composed of fraction III, as shown in Fig. 1. The nobotanin J thus obtained was pure enough to exhibit a single peak in normal- and reversed-phase HPLC, and showed ^{1}H and ^{13}C NMR peaks almost exclusively assignable to the given structure (**1**). The HPLC trace of the residue (510 mg) obtained by evaporation of fraction II still showed small peaks ascribable to **2** and **3** (88% purity for **1**). Fraction I (122 mg)

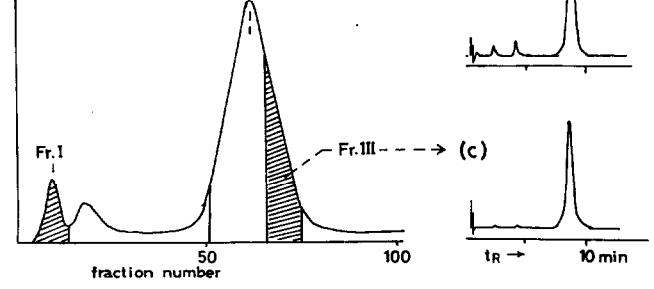
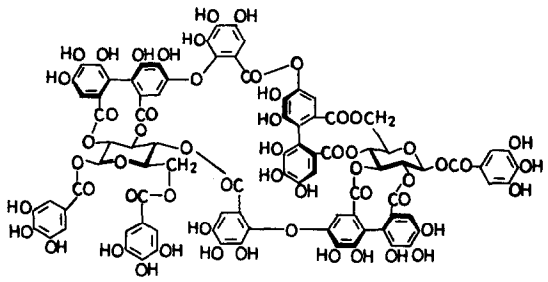


Fig. 1. Centrifugal partition chromatogram of crude nobotanin J from *n*-butanol extract of *H. roseum*, and HPLC fractions of II and III. (a) Centrifugal partition chromatogram (normal-phase development with solvent A); (b) HPLC (normal phase) of fraction II; (c) HPLC (normal phase) of fraction III. HPLC conditions: column, Develosil 60-5; solvent, hexane–methanol–tetrahydrofuran–formic acid (60:45:15:1) containing oxalic acid (500 mg in 1.2 l); detection, 280 nm; 0.04 a.u.f.s.



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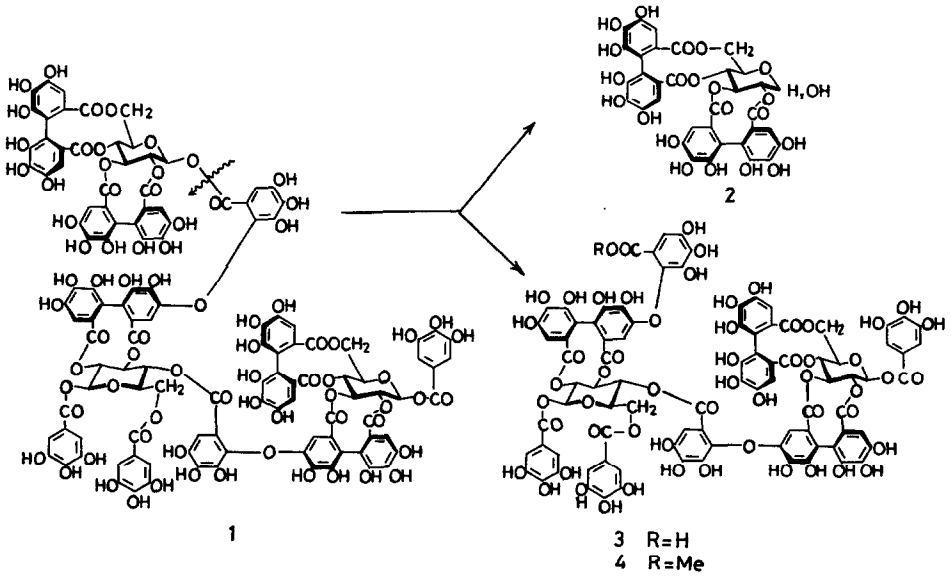
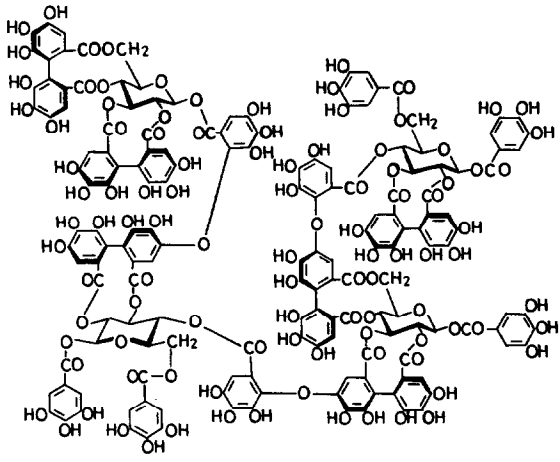
was found to contain a dimeric hydrolysable tannin whose structure was elucidated to be **5** (nobotanin I[10]) based on chemical and spectral evidence. The chemical lability of nobotanin J (**1**) in solution was shown by its decomposition into a mixture of a monomer, pedunculagin (**2**)[11], and a dimer (**3**), which occurred on keeping it in aqueous solution at 37°C for 22 days. Methanolysis of nobotanin J (**1**) at room temperature for 30 days similarly afforded **2** and the methyl ester (**4**) of **3** (Scheme 2).

Nobotanin K (**6**)[12], a tetrameric hydrolysable tannin, which is more polar than the trimers such as nobotanin E[10] and J (**1**), was strongly adsorbed on the solid support. Although the fraction containing **6**, which was the final eluate from column chromatography on Toyopearl HW-40, exhibited a single peak on the normal-phase HPLC, reversed-phase HPLC showed that it is contaminated by the other com-



1 2 3 R=H 4 R=Me

Scheme 2.



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pounds (Fig. 2), which were shown by the NMR spectra to be tetramers with related structures. These tetramers were also efficiently separated from each other by centrifugal partition chromatography with reversed-phase development employing solvent system A. The centrifugal partition chromatogram and high-performance liquid chromatogram of each fraction are shown in Fig. 3, and nobotanin K (**6**) (112 mg) was obtained from fraction II.

Separation of bioactive polyphenols from licorice roots

We have recently found that extracts of licorice roots have tannin-like activity, as judged by the binding ability (astringency) with hemoglobin[13]. The diethyl ether extract from Sinkiang licorice which exhibited significant tannin-like activity [RAG (astringency relative to that of geraniin)[13], 0.18] was separated by centrifugal partition chromatography in the normal-phase mode using solvent system **B** into four fractions (I–IV), among which only fraction IV was found to show considerable

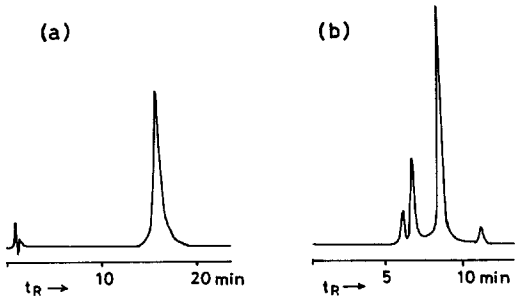

Fig. 2. HPLC of tetramer fraction obtained from column chromatography on Toyopearl HW-40 of *n*-butanol extract of *H. roseum*. (a) Normal-phase HPLC (conditions as in Fig. 1); (b) reversed-phase HPLC [column, YMC A312 (ODS); solvent A; detection, 280 nm, 0.04 a.u.f.s.].

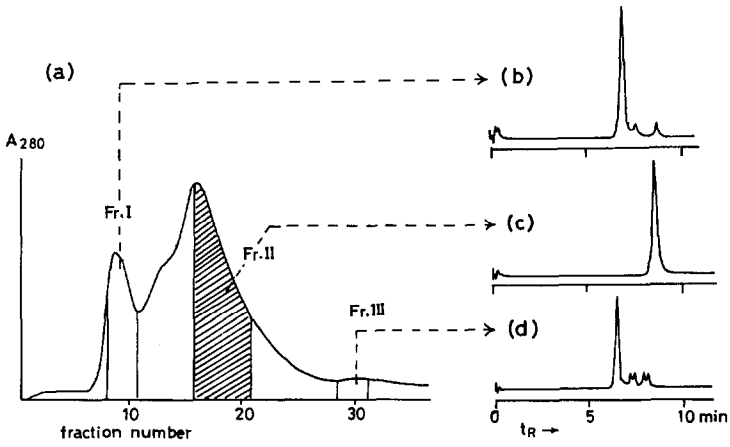

Fig. 3. Centrifugal partition chromatogram of tetramer fraction obtained from Toyopearl HW-40 of *n*-butanol extract of *H. roseum*, and reversed-phase HPLC of each fraction. (a) Centrifugal partition chromatogram (reversed-phase development with solvent A); (b) HPLC of fraction I; (c) HPLC of fraction II; (d) HPLC of fraction III. HPLC conditions as in Fig. 2.

tannic activity[13] (Fig. 4). Subsequent purification of fraction IV by column chromatography over Kieselgel 60 gave the tannin-like component **7** (RAG 0.40) and 7,4'-dihydroxyflavone (**8**), the former of which was characterized as licochalcone B[14]. Similarly licochalcone A (**9**) was obtained from fraction II. Application of centrifugal partition chromatography to the separation of the polyphenols in licorice has therefore been found to be useful in the preliminary separation of these structurally related components on a preparative scale.

Licochalcone A (**9**) and B (**7**) isolated in this way were found to show inhibitory effects on the formation of 5-HETE, 12-HETE, 5,12-diHETE, TXB_2, LTB_4 and LTC_4 in the arachidonate metabolism[15]. Licochalcone A (**9**) also showed inhibitory effects on the cytopathic activity of human immunodeficiency virus[16].

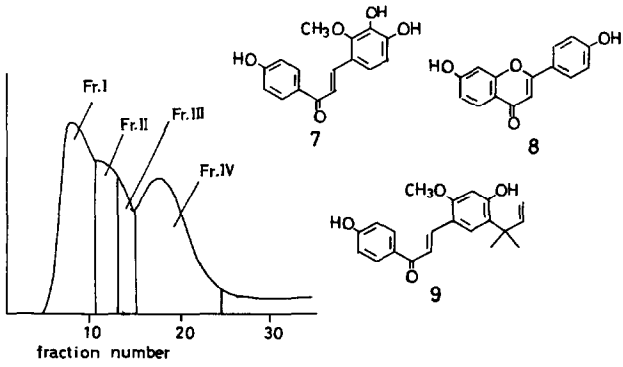

Fig. 4. Centrifugal partition chromatogram of diethyl ether extract of Sinkiang licorice (normal-phase development with solvent B).

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