

Preparative-Scale Chromatography of Ecdysteroids of *Serratula wolffii* Andrae

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Abstract

Numerous ecdysteroids are isolated from the herb of *Serratula wolffii* Andrae, a cultivated plant. The isolation procedure includes a variety of low-pressure liquid chromatography, thin-layer chromatography (TLC), gel chromatography, and high-performance liquid chromatography (HPLC) methods. The progress of separation is monitored by TLC, and the final proof of purity is carried out by HPLC. The isolation process involves the removal of proteins, flavonoids, chlorophylls, other sterines, etc. The purification also includes the separation of the target ecdysteroids from each other. Isolation of the pure compounds requires 2–8 chromatographic steps. The consecutive steps are based on the different physicochemical properties of the ecdysteroids. In some cases, a special peak-cut method employing a flush of dichloromethane into the dichloromethane–isopropanol–water mobile phase is used. This flush of dichloromethane leads to an almost perfect separation of otherwise unresolved peaks. Two ecdysteroids, 25-hydroxydacryhainansterone and 14-epi-20-hydroxyecdysone, are identified as natural products for the first time. The structure–chiroptical relationships for some ecdysteroids are also discussed.

Introduction

Ecdysteroids represent a group of steroids containing a sterane skeleton (cyclopentanoperhydrophenanthrene) with a conjugated 7-en-6-one structural element. They are highly hydroxylated compounds, with a minimum of three and a maximum of eight hydroxy groups. The main structural variety is formed by the conjugation of the hydroxy groups with sulphate, acetate, coumarate, and glycosylation with sugars, etc., an acetone formation, the presence of an additional double bond at various positions, and the presence of an additional oxo group. There are three groups of steroid hormones: (i) ecdysteroids, whose hormonal activity is restricted to arthropods, but a group of which also exert specific effects on vertebrates (1); (ii) verte-

brate hormones, such as corticoids, androgens, progestagens, and estrogens, etc., which are found in all living organisms (humans, other mammals, insects, plants, etc.); and (iii) brassinoids, which act on the growth and development of plants.

The first ecdysteroid (ecdysone, 25 mg) was isolated from about 500 kg of silkworm pupae (*Bombyx mori*) in 1954 by Butenandt and Karlson (2). In 1963, Karlson et al. devised a large-scale isolation method, which resulted in 250 mg of ecdysone from 1000 kg of *Bombyx mori* (3). This isolation process included several steps of extraction, solvent–solvent partition, and also chromatography (separation on an alumina column) and counter-current distribution. The final step was crystallization to produce pure ecdysone. 20-Hydroxyecdysone was also isolated from silkworm and crayfish (4). The plants were later found to contain larger amounts of ecdysteroids and an even wider variety of ecdysteroid structures than those in the insects [currently 260 phytoecdysteroids, but only around 100 zoo-ecdysteroids are known (5)]. Some of the phytoecdysteroids are common to insects.

The substantial ecdysteroid contents of certain plants allow the use of a simple separation procedure for the main ecdysteroids. The separation pathway was optimized earlier (6,7). The isolation of ecdysteroids may be monitored in two ways: using their UV absorbance to employ an on-line UV detector during their high-performance liquid chromatography (HPLC) separation and using their thin-layer chromatography (TLC) separation to utilize multiple detection, with one of them being based on their absorbance at 242 nm (6,7). Ecdysteroids in low concentrations have been successfully monitored by using displacement TLC, a method introduced by Horváth and Kalász (8–11). The enormous concentrating power of the displacement train allows the enrichment of ecdysteroids and also their specific differentiation.

The isolation of 22 ecdysteroids from the herb of *Serratula wolffii* are reported in this paper. They included several known, previously identified compounds and two new ecdysteroids. The determination of the structures of the new ecdysteroids and the CD spectra of nine compounds are also reported. Some structure–chiroptical relationships are established on the basis of the molecular ellipticity and molar absorption values.

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Materials and Methods

Plant material

The aerial parts of *Serratula wolffii* were collected in July 2001 from Herencsény, Hungary. A voucher specimen (collection number S94) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

TLC and Normal-phase TLC

Normal-phase (NP)-TLC was performed on silica plates, 20 × 20 cm (Silica gel 60F₂₅₄) (E. Merck, Darmstadt, Germany) with the following developing solvents: dichloromethane–ethanol (96%) (8:2, v/v); ethyl acetate–methanol–ammonia (25%) (85:10:5, v/v/v); toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v/v/v); dichloromethane–methanol–benzene (25:5:3, v/v/v); and ethyl acetate–ethanol–water (16:2:1, v/v/v).

Reversed-phase TLC

Whatman KC₁₈F, 20 × 20 cm, TLC plates (Whatman, Clifton, NJ) were used with the following developing solvents: methanol–water (4:6, v/v); acetonitrile–water (35:65, v/v); acetonitrile–water–0.1 % trifluoroacetic acid (35:65, v/v); and tetrahydrofuran–water (45:55, v/v).

Cyano silica (CN) TLC, 10 × 20 cm CN-HP-TLC plates (CN F₂₅₄, 5–7 μm) (E. Merck) were employed with the following developing solvents: *n*-hexane–acetone (6:4, v/v); and acetonitrile–water (2:8, v/v). The spots were visualized both by fluorescent quenching at 254 nm and also after spraying with vanillin-sulfuric acid and observing them in daylight or at 366 nm.

HPLC and NP-HPLC

A Zorbax SIL (DuPont, Wilmington, DE), 5 μm, 250 × 4.6-mm i.d. column (for analytical purposes) and a 250 × 9.4-mm i.d. column (for preparative purposes) were used with the following mobile phases: dichloromethane–isopropanol–water (125:50:5, v/v/v); dichloromethane–isopropanol–water (125:40:3, v/v/v); dichloromethane–isopropanol–water (125:30:2, v/v/v); dichloromethane–isopropanol–water (125:25:2, v/v/v); and cyclohexane–isopropanol–water (100:40:3, v/v/v).

Reversed-phase HPLC stationary phases

A Zorbax-ODS (DuPont, Wilmington, DE), 5 μm, 250 × 4.6-mm i.d. column and an Agilent Zorbax SB-C18 (Agilent Technologies, Palo Alto), 5 μm, 250 × 4.6-mm i.d. column were used with the acetonitrile–water (23:77, v/v) mobile phase. The flow rate was usually 1 or 0.7 mL/min in analytical HPLC, and 2 or 4 mL/min in preparative HPLC.

Classical column chromatography

The different stationary phases used, arranged in ascending order based on the column number, were as follows: column 1, Silica gel 60, 1010 × 55-mm i.d. (E. Merck); column 2, Polyamide SC6, 300 × 25-mm i.d. (Woelm, Eschwege, Germany); column 3, Silica gel 60 GF-254 used for TLC, 65 × 30-mm i.d. (Reanal, Budapest, Hungary); column 4, Silica gel 60 GF-254 used for TLC, 65 × 34-mm i.d. (Reanal); columns 5 and 6, Sephadex LH-

20, 560 × 20-mm i.d. (Pharmacia Fine Chemicals AB, Uppsala, Sweden); column 7, Sephadex LH-20, 570 × 10-mm i.d. (Pharmacia Fine Chemicals AB); columns 8 and 9, Sephadex LH-20, 560 × 20-mm i.d., (Pharmacia Fine Chemicals AB); column 10, Aluminium oxide Brockman II neutral, 170 × 15-mm i.d., (Reanal); column 11, Kovasil C18, 450 × 30-mm i.d. (Chemie Ueticon, Ueticon, Switzerland); column 12, Aluminium oxide Brockman II neutral, 400 × 45-mm i.d. (Reanal); column 13, Kovasil C18, 450 × 35-mm i.d. (Chemie Ueticon, Zurich, Switzerland); and column 14, Aluminium oxide Brockman II neutral, 255 × 35-mm i.d. (Reanal).

Extraction and purification of the crude extract

The dried herb (2 kg) of *Serratula wolffii* was milled and percolated with methanol (20 L) at ambient temperature. The methanolic extract was evaporated to dryness (232.9 g) and dissolved in methanol (1250 mL), and acetone (600 mL) was added to the solution. The resulting precipitate was removed by decantation, then rinsed three times, each with 150 mL of methanol–acetone (1:1, v/v). The supernatant and the methanol–acetone solutions were combined and evaporated to dryness. The residue (166.9 g) was redissolved in methanol (700 mL), and acetone (700 mL) was added to the solution. The precipitate was washed twice with 150 mL of methanol–acetone (1:1, v/v). The supernatant and the methanol–acetone solutions were combined and evaporated to dryness. The residue (124.5 g) was dissolved in 50% aqueous methanol (500 mL) and extracted four times with *n*-hexane (4 × 1000 mL). The aqueous methanolic phase was evaporated to dryness, and the residue (108 g) was dissolved in methanol (100 mL) and adsorbed onto a silica gel (200 g) using a rotatory evaporator. This was added to the top of a previously packed column of silica (1000 g, column 1) suspended in dichloromethane. After the column had been extensively washed and conditioned with dichloromethane (4.8 L), the ecdysteroids were eluted with dichloromethane–methanol (9:1, 85:15, 8:2, 7:3, and 1:1 v/v) (8, 7.2, 7.2, 3.2, and 4 L, respectively) and 800-mL fractions were collected. The progress of the elution was monitored by the use of NP-TLC, using developing solvents 3, 4, and 5.

Isolation of ecdysteroids

Fractions 27–30 from column 1, eluted with dichloromethane–methanol (9:1 and 85:15, v/v), were combined and evaporated to dryness. The dried residue (4.3 g) was dissolved in 5 mL of methanol. The solution was mixed with 9 g of polyamide and taken to dryness by rotatory evaporation. The sample was adsorbed onto polyamide and packed in a G3 glass filter (Pyrex, Nemours, France) (column 2). Elution was carried out with water, 25% and 50% aqueous methanol, and pure methanol (100 mL each), and 100-mL fractions were collected. The fractions eluted with water and 25% aqueous methanol were combined and evaporated to dryness. The dry residue (3.5 g) was dissolved in methanol (5 mL) and adsorbed onto silica (10 g), which was then added to the top of a previously packed column of silica (35 g), and suspended in ethyl acetate–methanol–water (85:10:5, v/v/v; column 3). Vacuum column chromatography was used with isocratic elution (ethyl acetate–methanol–water, 85:10:5, v/v/v), and 14 fractions (25-mL each) were collected. Fractions

3–6 were subjected to repeated crystallization in ethyl acetate–methanol (2:1, v/v) to yield compound 1 (427 mg). The mother liquid and the dry residue of fractions 1–2 and 7–11 (2.25 g) were combined and separated by vacuum column chromatography on silica (40 g) (column 4). The components were eluted with a stepwise gradient of dichloromethane, dichloromethane–ethanol (98:2, 95:5, and 9:1, v/v), and methanol (250, 75, 175, 1025, and 75 mL, respectively), and 25-mL fractions were collected. Fractions 22–25 (0.43 g), eluted with dichloromethane–ethanol (9:1, v/v), were combined and fractionated by gel chromatography on a Sephadex LH-20 (24 g), using ethyl acetate–methanol–water (16:2:1, v/v/v) as the eluent (column 5). Two milliliter fractions were collected. Fractions 36–48 (0.24 g) were combined and separated by preparative TLC using TLC system 2 (NP-TLC₂) (all systems are described in Figure 1). The final purification of the ecdysteroid obtained by TLC was carried out by NP-HPLC using HPLC system 3 (NP-HPLC₃), resulting in compound 2 (5 mg). Fractions 35–39 from column 4 (0.38 g) were also combined, and they were fractionated on a Sephadex LH-20 (column 6) in the same way as fractions 22–25 from column 4. Fractions 32–40 from column 6 (0.158 g) were further separated by Sephadex gel chromatography (13 g) (column 7), using ethyl acetate–methanol (2:1, v/v) as the eluent, and 1-mL fractions were collected. Fractions 11–16 (0.037 g) were finally purified by using preparative NP-TLC₂ and NP-HPLC₃ to yield compound 3 (7.2 mg). Fractions 51–60 (0.18 g) from column 6 were fractionated by preparative NP-TLC₂ and NP-HPLC₂ to obtain compound 4 (18 mg). Fractions 40–46 (0.51 g) from column 4 were purified by repeated use of Sephadex gel chromatography (columns 8 and 9) in the same way as fractions 35–39 from column 4. First, ethyl acetate–ethanol–water (16:2:1, v/v/v) was used, and 2-mL fractions were collected. Fractions 22–25 (0.08 g) from column 8 were further separated by Sephadex gel chromatography (column 9), and the ecdysteroids were eluted with ethyl acetate–methanol (2:1, v/v), collecting 2-mL fractions. Fractions 6–10 (0.04 g) were purified by using NP-HPLC₃ to yield compound 5 (13 mg). Fractions 29–38 from column 8 (0.179 g) were separated on the same Sephadex column (column 8), and the ecdysteroids were eluted with ethyl acetate–ethanol–water (16:2:1, v/v/v), collecting 2-mL fractions. Fractions 45–63 (0.08 g) from this column were purified by NP-TLC₂ and NP-HPLC₃ to yield compound 6 (24 mg).

Fractions 47–63 from column 4 were combined and evaporated to dryness. The dry residue (0.63 g) was dissolved in methanol and adsorbed onto 2 g of alumina. This was added to the top of a previously packed column of alumina (18 g) and suspended in chloroform (column 10). The ecdysteroids were eluted from the alumina with a stepwise gradient of chloroform–methanol (95:5 and 9:1, v/v, 370 and 1160 mL, respectively), and 10-mL fractions were collected. Fractions 44–138 (0.26 g), eluted with chloroform–methanol (9:1, v/v), were combined and subjected to vacuum column chromatography on octadecyl silica (180 g, column 11). Elution was carried out with a stepwise gradient of 30% to 60% aqueous methanol (250 mL in each step), and 50-mL fractions were collected. The ratio of methanol to water was increased by 5% in each step. Fractions 11–13, eluted with 40% aqueous methanol, produced compound 7 (2.2 mg). Fractions 23–28 (0.13 g), eluted with 50% and 55%

aqueous methanol, were further purified by NP-HPLC₂ to obtain compound 8 (28 mg).

Fractions 31–35 from column 1 were combined. The dry residue (13.6 g) was dissolved in 10 mL of methanol and adsorbed onto 68 g of alumina, which was added to the top of 400 g of alumina previously packed into a column (column 12). A stepwise gradient elution was carried out with 9:1, 85:15, 8:2, and 7:3, v/v, mixtures of dichloromethane–ethanol (11.1, 4.1, 1.7, and 1.8 L, respectively), and 100-mL fractions were collected. Fractions 24–30 (0.67 g), eluted with dichloromethane–ethanol (9:1, v/v), were separated by using preparative NP-TLC₅, which gave three well-defined zones. The ecdysteroids of these zones were further purified by NP-HPLC₅ to obtain compounds 9 (3 mg) and 10 (0.7 mg) from the first zone, compounds 11 (1.7 mg) and 12 (2.7 mg) from the second zone, and compound 13 (0.7 mg) from the third zone.

Fractions 31–90 from column 12 were subjected to repeated crystallization in ethyl acetate–methanol (2:1, v/v) to yield compound 14 (1.22 g). Fractions 91–190 from column 12 (0.67 g) were separated using reversed-phase (RP) vacuum column chromatography on 180 g of octadecyl silica (column 13) as previously described, and 50-mL fractions were collected. Fractions 15–17 (3 mg), eluted with 45% aqueous methanol, were separated by NP-HPLC₂ to produce compounds 14 (5.7 mg), 15 (0.7 mg), and 16 (1.6 mg). Fractions 21–23 (85 mg), eluted with 50% aqueous methanol, were also separated by NP-HPLC₂, and five compounds were isolated: compounds 8 (0.6 mg) and 14 (11.7

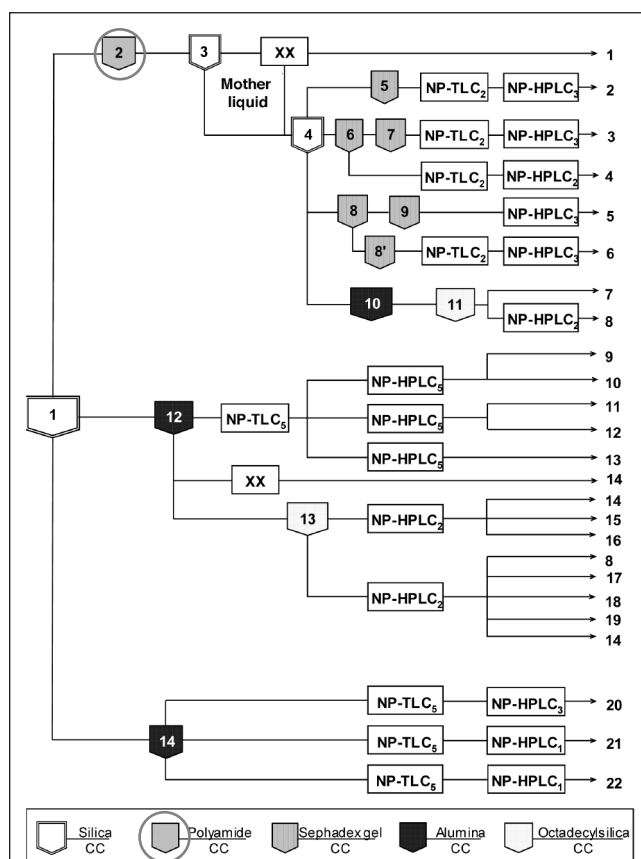


Figure 1. The scheme of the ecdysteroid isolation from the prepurified extract. The numbers in subscripts denote the solvent systems used.

mg) again, as well as compound 17 (8.4 mg), compound 18 (1.8 mg), and compound 19 (9.8 mg).

Fractions 41–46 (9.1 g) from column 1, eluted with dichloromethane–methanol (7:3 and 1:1, v/v), were adsorbed onto 45 g of alumina, which was packed on the top of a column of 270 g of alumina (column 14). The gradient elution was carried out with chloroform–ethanol (9:1, 8:2, 7:3, 65:35, and 6:4, v/v, 1700, 1800, 4900, 8900, and 2900 mL, respectively) and 100-mL fractions were collected. Fractions 42–60 (0.36 g), eluted with chloroform–ethanol (7:3, v/v), were combined and further fractionated using preparative NP-TLC₅. The final purification was carried out by NP-HPLC₃ to yield compound 20 (9.9 mg). Fractions 91–96 (0.11 g), eluted with chloroform–ethanol (65:35, v/v), were purified by NP-TLC₅ and NP-HPLC₁, and compound 21 (10 mg) was isolated. Fractions 97–108 (0.23 g) from column 14, eluted with chloroform–ethanol (65:35, v/v), were also purified by NP-TLC₅ and NP-HPLC₁ to give compound 22 (11 mg). Figure 1 shows the procedure of the ecdysteroid isolation.

Physicochemical characterization and structure determination of the isolated compounds

The circular dichroism (CD) and UV spectra were measured with a Jasco J-720 Spectropolarimeter (Japan Spectroscopic, Tokyo, Japan).

High-resolution electrospray ionization (ESI) and fast atom bombardment (FAB) mass spectrometry (MS) results were recorded on a Finnigan MAT 95SQ (Finnigan MAT, Bremen, Germany) hybrid tandem MS, and ESI-MS–MS were determined with a Finnigan TSQ 7000 tandem MS (Finnigan, San Jose, CA). One-dimensional (¹H, ¹³C, DEPT-135) and two-dimensional [correlated spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), HMQC-total correlated spectroscopy (TOCSY), and heteronuclear multiple bond correlation (HMBC)] NMR spectra of ecdysteroids were taken in methanol-d₄ using a Shigemi sample tube on a Bruker Avance DRX-500 spectrometer (Bruker, Ettlinger, Germany).

Results

The ecdysteroids of the herb of *Serratula wolfii* Andrae were subjected to exhaustive extraction with methanol in a percolator at a solvent–plant ratio of 10:1. The crude extract was then subjected to pre-purification using fractionated precipitation and solvent–solvent distribution. These two consecutive purification steps removed the overwhelming majority of both the polar and the apolar contaminants. The purification was monitored by TLC.

The first chromatographic step was carried out on a silica column packed with coarse particles (0.06–200 μm), which resulted in fractions combined into three groups. The first group contained certain (approximately 11) ecdysteroids, but an excess of flavonoids were also eluted here as contaminants. The flavonoids were removed by solid-phase extraction on polyamide using a homemade set-up. Ecdysteroids were eluted using plain

water and water with 25% aqueous methanol, but flavonoids remained adsorbed on the polyamide. The adsorption-elution processes were double-checked by consecutive elution with an increasing ratio of methanol, but 25% methanol always resulted in the perfect elution of all the present ecdysteroids. Further removal of the non-ecdysteroid contaminants was achieved by the repeated use of a silica gel column with medium particles (12–15 μm). The second silica column chromatography was carried out according to the conditions of vacuum chromatography. The stationary phase to load ratio was 13:1. In certain cases, the crystallization of an ecdysteroid (pure 1) was observed.

The other ecdysteroids were then further separated by vacuum chromatography on a silica gel, resulting in four subgroups. The first two subgroups were separately subjected to three or four additional chromatographic purifications, that is, to separation on a Sephadex LH-20 column or to repeated Sephadex LH-20

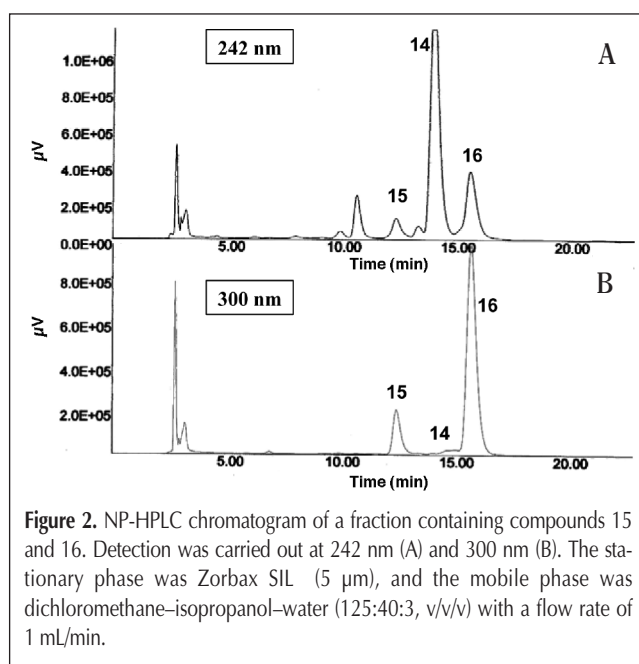


Figure 2. NP-HPLC chromatogram of a fraction containing compounds 15 and 16. Detection was carried out at 242 nm (A) and 300 nm (B). The stationary phase was Zorbax SIL (5 μm), and the mobile phase was dichloromethane–isopropanol–water (125:40:3, v/v/v) with a flow rate of 1 mL/min.

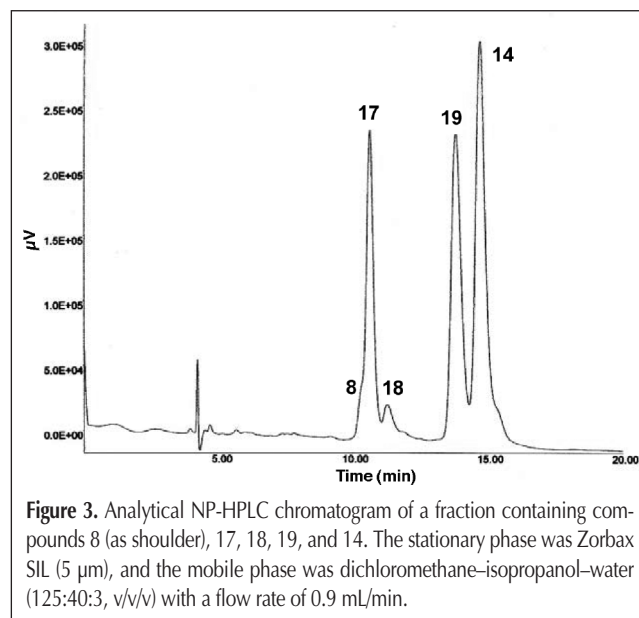


Figure 3. Analytical NP-HPLC chromatogram of a fraction containing compounds 8 (as shoulder), 17, 18, 19, and 14. The stationary phase was Zorbax SIL (5 μm), and the mobile phase was dichloromethane–isopropanol–water (125:40:3, v/v/v) with a flow rate of 0.9 mL/min.

column chromatography and preparative TLC on silica, as well as to NP-HPLC. Compound 2 was separated from the other ecdysteroids on a silica column, and the proceeding three steps removed only the contaminants. The two ecdysteroids in the second subgroup, compounds 3 and 4, were resolved from each other by Sephadex LH-20 column chromatography. Additional Sephadex LH-20 column chromatography, TLC, and HPLC removed the contaminants. Similar separation was carried out for the fractions combined in the third subgroup. However, column chromatography of this fraction on a Sephadex LH-20 differentiated two ecdysteroids (5 and 6), and further chromatographic steps removed the contaminants.

The fourth subgroup of silica fractions was subjected to alumina column chromatography to remove the non-ecdysteroid contaminants. Later, vacuum column chromatography on octadecyl silica was carried out to separate the two ecdysteroids present here. Compound 7 was sufficiently pure after this separation, whereas compound 8 (containing fractions) had to be further purified by using NP-HPLC (silica). As a result, eight ecdysteroids were isolated from the first fractionation group from the initial silica gel column.

The second fractionation group from the initial silica gel column was subjected to separation on alumina to remove the

contaminants. The column chromatography yielded three subgroups. The real separations of the ecdysteroids of the first subgroup were employed by preparative TLC on a silica stationary phase and by NP-HPLC. In this way, five ecdysteroids were obtained (9, 10, 11, 12, and 13). The second subgroup from the alumina column chromatography contained an abundant amount of compound 14, which was purified by crystallization. The ecdysteroids of the third subgroup from the alumina column were purified by vacuum column chromatography on octadecyl silica, resulting in two ecdysteroid-containing fractions. The ecdysteroids of these fractions were resolved by NP-HPLC. The detection and isolation of compounds 15 and 16, containing two double bonds, was greatly facilitated by their double detection (Figure 2). Detection at 242 nm was characteristic of the vast majority of ecdysteroids. Ecdysteroids with the cumulated conjugation of the oxo group and the two double bonds have an additional possibility of monitoring at approximately 300 nm. The absorbance at 300 nm was not only specific, but also higher than that at 242 nm. The other fraction con-

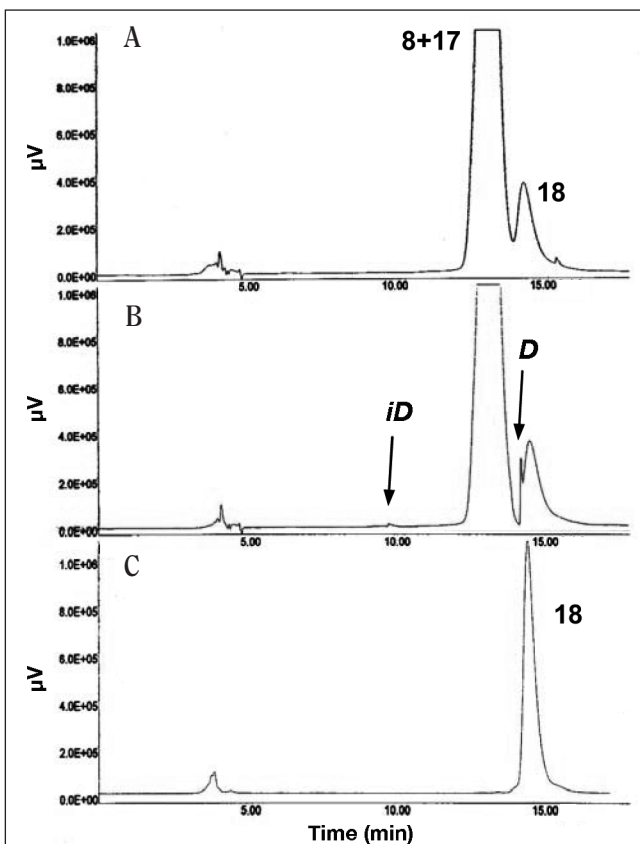


Figure 4. Preparative NP-HPLC separation of compound 18 from compounds 17 and 8 by the peak-cut method without dichloromethane flush (A) and with dichloromethane flush (B), and the chromatogram of pure compound 18 (C). In the preparative HPLC separation, compounds 8 and 17 eluted together. The stationary phase was Zorbax SIL (5 μm), and the mobile phase was dichloromethane–isopropanol–water (125:40:3, v/v/v) with a flow rate of 0.7 mL/min. The peak marked with D is the eluted peak of the dichloromethane. The peak marked with iD is its injection time.

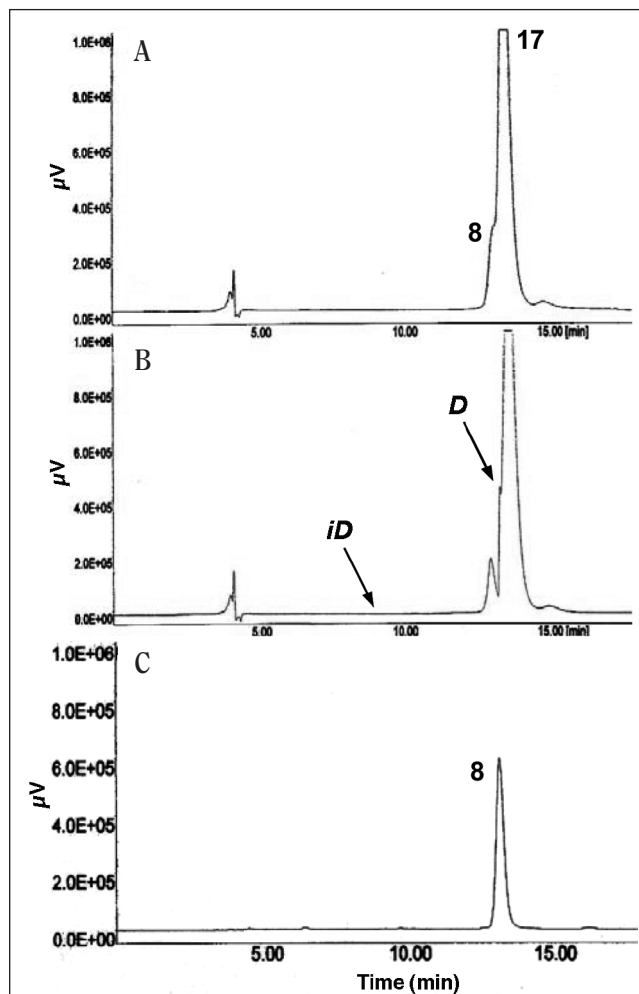


Figure 5. Analytical NP-HPLC separation of compounds 8 and 17 by the peak-cut method without dichloromethane flush (A), with dichloromethane flush (B), and the chromatogram of pure compound 8 (C). The stationary phase was Zorbax SIL (5 μm), and the mobile phase was dichloromethane–isopropanol–water (125:40:3, v/v/v) with a flow rate of 0.7 mL/min. The peak marked with D is the eluted peak of the dichloromethane. The peak marked with iD is its injection time.

tained five ecdysteroids: compounds 8, 17, 18, 19, and 14 (Figure 3). Three of the compounds (8, 17, and 18) produced coeluting peaks. They were separated by NP-HPLC on silica using the newly established peak-cut method.

The peak-cut method served to separate overlapping peaks. One operation improved the separation of two components. When preparative HPLC separation was used on a silica stationary phase with dichloromethane–isopropanol–water (125:40:3, v/v/v), compound 8 was eluted together with compound 17, and compound 17 produced a peak overlapping with compound 18 (Figure 4A). First, these latter two ecdysteroids were separated. Injecting a flush of dichloromethane improved the separation, and, thereby, the peak of compound 18 separated adequately from the peaks of compounds 17 and 8 (Figure 4B). However, a further operational step was necessary to remove compound 8 from the leading part of the peak of compound 17 (Figure 5) using a lower load.

The third fractionation group from the initial silica gel column contained three ecdysteroids (20, 21, and 22). These were separated by using column chromatography on alumina, which was followed by their individual purification through preparative TLC and HPLC on silica. This was the preferred mode of separation of other ecdysteroids with 6 and 7 hydroxy groups. Tables I and II report the chromatographic characteristics of the isolated ecdysteroids.

Elucidation of structures of the newly isolated ecdysteroids

Comparison of the physicochemical properties and spectral data with authentic compounds allowed the identification of

compounds 1–15, 17, and 19–22. The spectral data of compound 18 was compared with the synthetic 14-*epi*-20-hydroxyecdysone. The data were in good agreement with each other (12). The newly isolated ecdysteroid, compound 16, had $(\alpha)_D^{29-17^\circ}$ (c, 0.5), and its UV spectrum in methanol produced a peak with λ_{\max} (log e) at 299 nm (3.33). The mass spectrum revealed major ions at m/z 479 [M+H]⁺, 461.6 [M+H–H₂O]⁺, 443 [M+H–2H₂O]⁺, 427 [M+H–3H₂O]⁺, and 345 [M–C₂₀–C₂₇]⁺. The molecular weight of m/z 478 was determined by FAB-MS. This was 16 m/z higher than compound 3, therefore making compound 16 a mono-hydroxylated derivative of compound 3. The fragment ions of FAB-MS furnished evidence that compound 16 bears the additional OH group on the side-chain of compound 3.

The ¹H and ¹³C chemical shifts of compound 16 are given in Table III. For the signal assignment, the five methyl signals appearing as singlets in the ¹H-NMR spectrum were identified first. The characteristic correlations were utilized in the assignment. The identification of the geminal Me-26 and Me-27 groups were straightforward, owing to their mutual HMBC correlation, whereas Me-21 correlated with two OH substituted carbon atoms exhibiting strong deshielding (~ 77–78 ppm). The differentiation between H₃-19 and H₃-18 atoms of the angular methyl groups was achieved considering the coupling of the latter with C-17, which also coupled to H₃-21. In accordance with a 6-oxo- $\Delta^{7,8}$ -moiety, H-7 olefinic hydrogen (5.75 ppm) correlated with C-5, C-9, and C-14 carbon atoms. The high ¹³C chemical shift value of C-10 (136.3 ppm) and the correlations of the other olefinic hydrogen (6.29 ppm) to C-8, C-10, and C-13 in the HMBC spectrum of compound 16 justified the presence of a $\Delta^{9,11}$ double

Table I. The TLC Characteristics of the Isolated Ecdysteroids

Ecdysteroid	Color after spraying with sulfuric acid reagent		Retention Factor × 100										
			NP-TLC					RP-TLC				CN-TLC	
	UV (366 nm)	Daylight	1	2	3	4	5	6	7	8	9	10	11
1	Violet	Turquoise	32	20	25	35	38	48	55	49	66	33	50
2	Violet	Yellow	–	59	–	58	–	–	–	–	–	–	–
3	Red	Brown	48	56	55	63	71	10	21	–	34	50	15
4	Violet	Turquoise	–	–	–	49	59	–	–	–	–	–	–
5	Violet	Turquoise	–	38	–	49	–	–	–	–	–	–	–
6	Violet	Brown	50	49	41	40	57	26	33	30	45	30	27
7	Turquoise	Red	–	–	21	30	40	–	–	–	–	–	–
8	Dark red	Dark red	35	–	34	35	45	29	34	–	47	35	24
9	Violet	Brown	–	–	–	–	37	–	–	–	–	–	–
10	Orange	Orange	26	23	30	34	47	30	34	32	58	31	31
11	Violet	Turquoise	28	23	32	38	38	49	54	50	65	21	46
12	Violet	Purple	–	22	31	38	40	57	49	41	–	28	37
13	Violet	Light yellow	33	27	35	40	41	33	38	35	61	30	30
14	Violet	Turquoise	24	21	27	30	37	47	56	46	66	26	48
15	Red	Red	26	–	–	25	–	57	54	–	68	21	51
16	Red	Red	21	–	27	28	37	52	54	–	68	24	46
17	Violet	Green	32	–	37	37	43	39	41	–	50	33	23
18	Violet	Turquoise	–	–	–	35	42	–	–	–	–	–	–
19	Violet	Turquoise	–	–	27	30	38	–	–	–	–	–	–
20	Violet	Turquoise	–	–	–	–	30	–	–	–	–	–	–
21	Dark red	Dark red	10	–	12	14	19	99	68	–	77	8	62
22	Violet	Brown	–	–	–	–	20	–	–	–	–	–	–

bond. The hydrogen atoms of ring-A formed a common spin-system, which was analyzed by the $^1\text{H}, ^1\text{H}$ -COSY, and HMQC-TOCSY experiments. The assignments of rings-C and -D, as well as the side-chain attached to C-17 were obtained in an analogous way. The H-19/H₂-1 and H-19/H-5 correlations in the NOESY spectrum of compound 16 proved *cis* type junction of rings-A/B. The H_b-12/H-19, H_b-12/H-21, and H_a-12/H-17 cross-peaks verified the *trans* type junction of rings-C/D. Figure 6 depicts the structures with the trivial names and the numbering of the isolated ecdysteroids.

Chiroptical analysis of ecdysteroids

In ecdysteroids with 6-oxy- $\Delta^{7,8}$ -moiety, the conjugated enone grouping was inherently chiral, and the torsion angle of this grouping was of great importance for the sign of CD bands (13). The main ecdysteroid, compound 14, and other ecdysteroids, 1, 7–8, 10–11, 18, 21, etc. bear a positive Cotton effect in the $n \rightarrow \pi^*$ electronic transition range at a 328–333 nm wavelength. The $\pi \rightarrow \pi^*$ electron transition explains that a sharp negative Cotton effect curve can be seen at the majority of ecdysteroids 1, 7–8, 10–11, 14, 18, and 21 at a 247–257 nm wavelength. The deviating CD curves of ecdysteroids will be interpreted later.

Compound 3, with a 7,9(11)-dien-6-one structure, caused a bathochromic shift of the Cotton effect curve ($\lambda_{\text{max}} = 345$ nm). The second double bond in the 9 position caused the extension of the conjugation and the maximum of the negative Cotton effect

curve (because of $\pi \rightarrow \pi^*$ transmission), which was shifted to the longer wavelengths with 46 nm. The change in the UV spectrum is of the same extent. The ecdysteroid with a pregnane skeleton, compound 7, which did not contain a long side chain, had a chirally perturbed saturated ketone chromophore in the 20 position. Its positive Cotton effect at 289 nm could easily be separated from the CD signal, having a maximum at 332 nm because of the unsaturated ketone chromophore $n \rightarrow \pi^*$ transition.

The maximum of the Cotton effect in the $\pi \rightarrow \pi^*$ electron transmission had a hypsochromic shift with 6–7 nm at the 5 α -ecdysteroids (compound 11). The intensity of this band increased in a great extent. The intensity of a short wavelength band at 221 nm decreased compared to the 5 β -ecdysteroids. The CD spectrum was characteristic of compound 18. This ecdysteroid had a β -hydroxy substituent in the 14 position. The intensity of the R-band decreased markedly. The Cotton effect, because of $\pi \rightarrow \pi^*$ electron transition, had a hypsochromic shift, and the band at short wavelengths could not be measured. The chiroptical data of the isolated ecdysteroids are given in Table IV.

Discussion

Serratula wolffi Andrae belongs to Asteraceae, a family with numerous species rich in ecdysteroids (14–36). Accordingly, the determination of the ecdysteroid profile of *Serratula wolffii* Andrae was a worthwhile goal. There were two distinct ways to screen the profile. One was the direct analysis of the extract, and the other was to isolate the ecdysteroids giving the profile (37–40). Direct HPLC was not possible because of the lack of an adequately specific determination for each of the individual 260 ecdysteroids. The effects of saponoids, flavonoids, and other phenolic compounds could have disturbed the analytical methods when TLC, HPLC, or even HPLC–MS was employed. Isolation of the various ecdysteroids was a reliable method of establishing the ecdysteroid spectrum (37). Moreover, the physicochemical characteristics of the pure ecdysteroids isolated could be checked.

Twenty-two ecdysteroids were isolated from *Serratula wolffii* Andrae. The main ecdysteroid (compound 14) was purified in the simplest way: by using two-column chromatography to yield fractions pure enough for its crystallization. Three chromatographic steps were needed to isolate compound 1, the most frequently biosynthesized phytoecdysteroid after compound 14. The isolation procedure from 2 kg of the dried herb *Serratula wolffii* resulted in 1.2 g of compound 14 and 0.4 g of compound 1 in pure form. The overall recovery of these purifications was as high as 30% to 40%.

The other ecdysteroids were purified by an optimum combination of separation methods. Repeated adsorption chromatography using a column or a planar technique (or both) and gel chromatography on Sephadex LH-20 were utilized, followed by NP-HPLC. The gel chromatography on Sephadex LH-20 could be substituted by vacuum RP column chromatography. The ecdysteroids could be separated by gel chromatography on the basis of the differences in their molecular masses, and the Sephadex LH-

Table II. HPLC Retention Time (min) of the Isolated Ecdysteroids

Ecdysteroid	NP-HPLC			RP-HPLC
	2	4	5	
1	12	22.4		4.4
2	ND*	5.7	8.0	
3	6.0	9.2	8.4	25.5
4	14.7			7.6
5		16.1	9.2	
6	7.8	15.8		15.6
7	10.7		22.3	3.7
8	10.5	33.3		
9		53.9	23.6	
10	13	28	15.2	10.1
11	12.3	33	24	3.7
12	12.4	27.0	17.0	5.3
13	12	24.4		10.1
14	14.7 [†]	41	21	4.2
	15.6			
15	12.5			3.45
16	15.7		26.1	3.5
17	10.6 [‡]		14.9	7.4
18	11.3 [‡]		22.1	
19	13.8 [‡]		21.9	
20	23		25.6	
21	26.5		36	
22	37.5 [‡]		47.6	3.8

* ND = Not determined.
[†] Flow rate 0.9 mL/min.
[‡] dichloromethane–isopropanol–water (125:50:5, v/v/v).

20 gel worked as a weak reversed phase as well. For these reasons, ecdysteroids with very small differences in molecular mass could be separated. Moreover, the solubilities of ecdysteroids made it possible to dissolve them in solutions concentrated enough for the direct loading onto a chromatographic system, while the low-pressure RP column chromatography was operated by drying the sample on octadecyl silica and transferring it to the top of the column.

The final purification generally required NP-HPLC. In certain cases, it was the fifth step in the use of silica, but the very first use of fine-particle silica packed into an HPLC column, that permitted the separation to be carried out under high pressure. The exception was compound 7, which was sufficiently pure without this final NP-HPLC. Naturally, altogether six isolation and chromatographic steps resulted in the purification of compound 7. A particular procedure was employed to improve the separation of compound 17 from compound 18 (Figure 4) and from compound 8 (Figure 5). This method is called peak-cut by solvent. The HPLC separation was done by using silica gel as the stationary phase and dichloromethane–isopropanol–water as the mobile phase. The method included isocratic elution with a flush of dichloromethane.

This method used for the separation of compounds 17 and 18 has been illustrated. For preparative-scale separation (given in Figure 4), 100 μ L of dichloromethane was injected after the load. The elution time of dichloromethane and the local minimum between the two overlapping ecdysteroid peaks of the sample was determined before the separation. The difference between these

data gave the appropriate dichloromethane injection time. Preliminary calculation led to the result that the dichloromethane flush reached the moving zones of compounds 17 and 18 (Figure 4A) just before the end of the HPLC column, and this dichloromethane flush was inserted between the peaks of these two ecdysteroids. For preparative separation, the load was 4 μ g/ μ L, which means a gross load of 400 μ g.

The tentative mechanism of this peak-cut was as follows. Dichloromethane was the least polar component of the mobile phase. The conditions allowed its migration with minimal retention. When dichloromethane reached the minimum between the zones of compounds 17 and 18 (Figure 4A), compound 17 was before the dichloromethane; therefore, compound 17 could be eluted without any delay. However, dichloromethane overran the zone of compound 18, and a micro-portion of the mobile phase was richer in dichloromethane. As the dichloromethane-rich mobile phase induced a higher retention, the elution of compound 18 was slightly delayed (Figure 4B). However, it was only a very small portion of the mobile phase that generated an increased retention time, and it did not influence the overall shape of the peak of compound 18. As the intention of this study was the separation of this ecdysteroid pair (compounds 17 and 18), together with the parallel separation of two other ecdysteroids, compounds 19 and 14 (Figure 3), changing the isocratic mobile phase was not preferable. The mechanism of the procedure means a real isocratic elution with a micro flip-flop change in the polarity of the mobile phase.

Only six ecdysteroids with two double bonds (conjugated to

Table III. The ^1H and ^{13}C Chemical Shifts of the Compound 16 (in Methanol d_4 , δ in ppm)

16									
No.		^{13}C	^1H	$m; J^*$ (Hz)	No.	^{13}C	^1H	$m; J^*$ (Hz)	
1	α	37.5	2.08	dd † ; 13.5, 4.0	14		84.6	–	–
	β		1.70	dd † ; 13.5, 11.9	15	α	31.6	1.79	
2	α	68.9	3.72	ddd ‡ ; 11.9, 3.9, 3.1		β		1.95	
3	α	68.4	3.85	q § ; 2.8	16	α	22.0	1.80	
4	α	35.9	1.60			β		1.99	
	β		1.76	dt ** ; 14.0, 3.9	17	α	50.7	2.50	t † ; 8.9
5	β	51.7	2.45	dd † ; 12.6, 3.7	18	β	18.3	0.90	s ‡
6		NS §§			19	β	31.6	1.11	s ‡
7		119.4	5.75	d; 1.0	20		78.0	–	–
8		156.7	–	–	21		21.0	1.211	s ‡
9	(α)	136.3	–	–	22		78.6	3.34	overlapped
10		41.1	–	–	23	α	27.5	1.31	
11	(α)	134.0	6.29	dt ** ; 6.6, 2.0		β		1.64	
	β				24	α	42.5	1.44	ddd ‡ ; 13.6, 11.5, 4.3
12	α	39.2	2.74	dd † ; 17.6, 2.5		β		1.81	
	β		2.42	dd † ; 18.0, 6.8	25		71.6	–	–
13		48.1	–	–	26		29.1	1.19	s ‡
					27		29.8	1.206	s ‡

* $m; J$ = multiplicity; coupling constant.
 † dd = doublet of doublet.
 ‡ ddd = doublet of doublet of doublet.
 § q = quartet.
 ** dt = doublet of triplet.
 † t = triplet.
 ‡ s = singlet
 §§ NS = no signal measured.

each other and to the oxo group) were known earlier (5). Two additional natural ecdysteroids containing 7,9(11)-dien-6-one structural elements were recently discovered, which were named

herkesterone 15 (21) and 25-hydroxydacryhainansterone 16. The conjugation of two double bonds and an oxo group drastically changed their UV spectrum (41). The maximum of their UV

absorbance was shifted to approximately 300 nm (298.6 nm for herkesterone 15 and 399.2 nm for 25-hydroxydacryhainansterone 16). Monitoring the chromatogram at 300 nm permitted their specific detection, and a virtual separation of compounds 15 and 16 was achieved when these ecdysteroids were traced in multi-component samples. Utilizing the specific monitoring of compounds 15 and 16 (and other unknown ecdysteroids with 2 double bonds) opened up new possibilities for their detection in other plants.

Recent publications have reported on the ecdysteroid profile of certain *Serratula* species (14–36). Such reports were extremely useful for selecting plants as raw materials for medicinal preparations. Moreover, these accounts permitted the chemo-taxonomical systematization of the plants. The analysis of the ecdysteroid spectrum of *Serratula wolffii* Andrae was discussed by Miladera et al. (19) and Hunyadi et al. (21). This report essentially supplements their findings. Two ecdysteroids, compounds 16 and 18, are newly discovered natural products. At the same time, they have been prepared by synthetic procedures (12,41). The ecdysteroid profile of a species depends on various factors, such as the location of the plant, the soil, the climate, the time of harvesting, etc., thus it depends on the surrounding ecosystem and on the genetic markers of the individual plant (42).

The common occurrence of compound 3 with compound 8, and of compound 16 with compound 21, raised the question of whether compounds 3 and 16 were formed from compounds 8 and 21 by dehydration during the isolation process. The artificial origin of compound 3 was ruled out as it was detected in the extract by TLC and HPLC analyses (results to be published later). This finding led to the assumption of the natural occurrence of compound 16 as well.

Compound 5 could have likewise been an artifact formed from compound 14 on the use of acetone during the pre-purification steps. However, ecdysteroid acetonides have been isolated relatively often from a fair number of plant species (43–47) and accordingly, the verification of the natural occurrence of this compound remains an open question for the present time.

The CD spectra of ecdysteroids furnished information on the structures of these compounds, but only a few data are available in the literature for this field (5). This study has determined the CD and UV spectra of nine com-

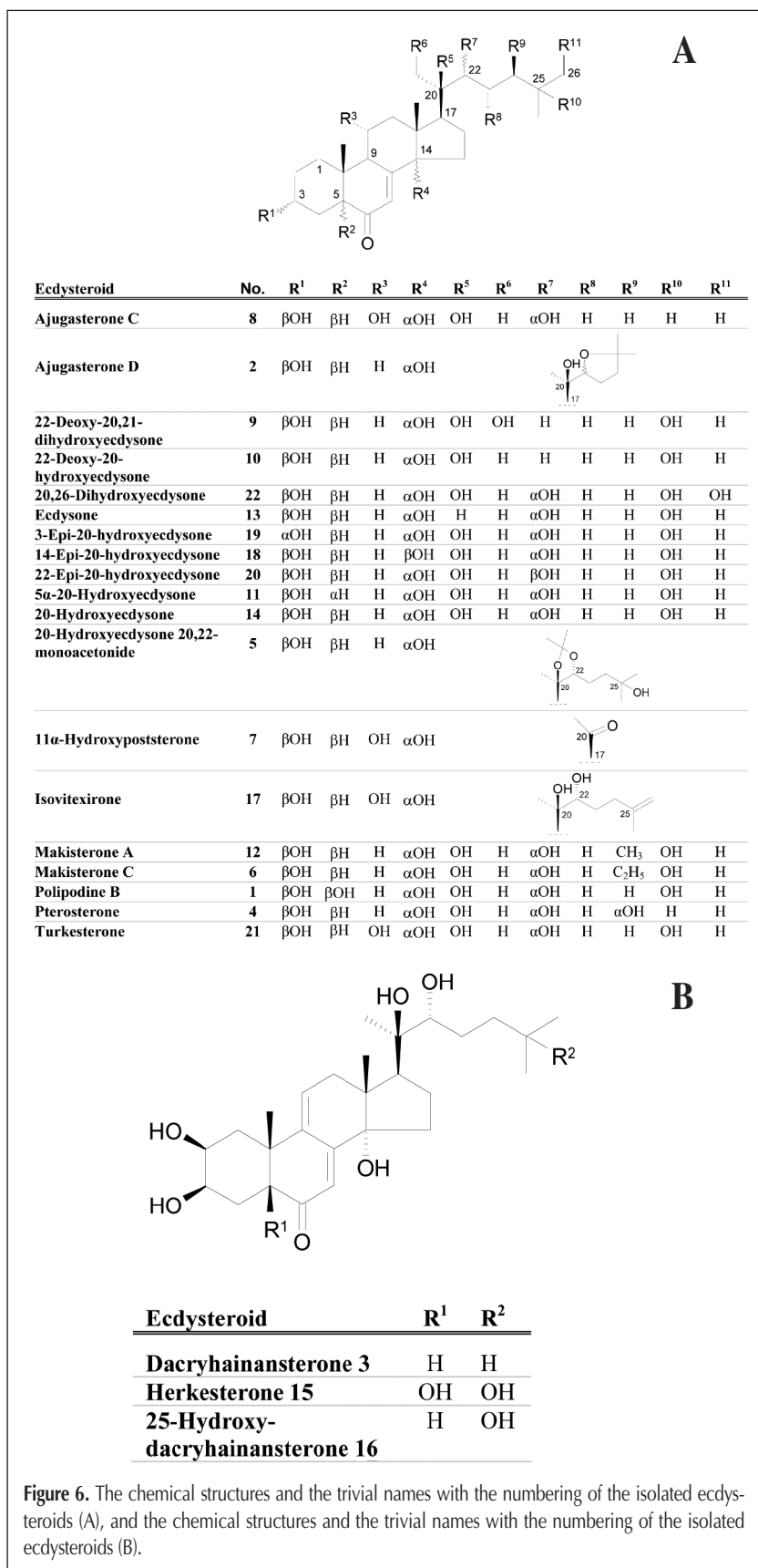


Table IV. Chiroptical Data of Ecdysteroids

Ecdysteroid	Molecular ellipticity					
	λ_1 (nm)	(θ)	λ_2 (nm)	(θ)	λ_3 (nm)	(θ)
1	227	22830	256.2	-15930	329.2	9757
3	231.8	-3424	253.4	3648	345.4	12812
			300.8	-15611		
7	206.2	-5371	251	-3616	289.4	2659
	230.4	1346			331.6	1909
8	207.2	-15117	257	-10059	330.4	5521
	232.4	12330				
10	223.4	11545	253.6	-17150	330.2	6929
11	221	-3757	248.8	-27011	333.2	9457
14	224	10047	254	-12883	330	5697
18			247.4	-9834	328.4	1169
21	208	-6540	257.2	-4525	330.2	2526
	232.6	5602				

pounds. On the basis of the molecular ellipticity and molar absorption values, some structure–chiroptical relationships were established. These results were mainly based on the evaluation of changes in the CD spectrum of the 6-oxo- $\Delta^{7,8}$ chromophore group, depending on its stereochemical environment. In some cases, the electron excitation spectra provide valuable information and promoted an understanding of the relationship between structure and CD spectrum.

Conclusion

Serratula wolffii Andrae, a cultivated plant, is a rich source of ecdysteroids. These ecdysteroids are preferably isolated by means of combined chromatographic methods. The applied elements of the separation steps depend on the nature of the ecdysteroids. The number of steps has to be increased in accordance with the decreasing concentration of the individual ecdysteroid to be isolated. This newly established method, the solvent peak-cut, can be used with ease to improve the separation of the overlapping peaks of isovitexirone 17, 14-epi-20-hydroxyecdysone 18, isovitexirone 17, and of ajugasterone C8. The triple conjugation of the two double bonds and the oxo group permits the specific detection of certain ecdysteroids at 300 nm.

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