Isolation of a New Member of the Ecdysteroid Glycoside Family: 2-Deoxy-20-hydroxyecdysone 22-*O*-β-D-Glucopyranoside

Mária Báthori[1,*], Zita Pongrácz[1], Gábor Tóth[2], András Simon[2], Lili Kandra[3], Zoltán Kele[4], and **Róbert Ohmacht[5]**

[1]Department of Pharmacognosy, University of Szeged, H-6720 Szeged, Eötvös utca 6, Hungary; [2]Institute for General and Analytical Chemistry, Technical University of Budapest, Budapest, Hungary; [3]Institute of Biochemistry, University of Debrecen, Debrecen, Hungary; [4]Department of Medical Chemistry, University of Szeged, Szeged, Hungary; and [5]Institute of Medicinal Chemistry and Biochemistry, University of Pécs, Pécs, Hungary

Abstract

A new ecdysteroid glycoside, 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside, is isolated from the herb *Silene italica ssp. nemoralis* (Waldst. and Kit.) Nyman. The compound is purified with multistep chromatography, such as classical column chromatography on alumina and droplet countercurrent distribution. Also, it is expanded using twice low-pressure reversed-phase liquid column chromatography. Chromatography in four steps results in the purified 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside. Two other ecdysteroids have also been separated, including the formerly identified integristerone A and 24(28)-dehydromakisterone A.

Introduction

Ecdysteroids are widely distributed both in invertebrates and plants, and they regulate the development of insects and certain other invertebrates. Ecdysteroids possess a series of pharmacological effects on vertebrates such as increasing the protein synthesis (1,2), decreasing the cholesterol level (3), and potentiating the effect of insulin (4). Ecdysteroids also normalize hyperglycemia (5), have hepatoprotective action (6), and influence sexual activity (7). Ecdysteroids are the important constituents in a number of health-improvement preparations and also in tonics (1,6). Ecdysteroid-containing preparations are commercialized and widely used to improve physical and mental conditions, increase protein incorporation, and multiply stress resistance (1,6). Certain sources in the literature indicate ecdysteroids as chemopreventive agents (8).

The occurrence of ecdysteroids has also been verified in both human beings and animals. Mammalian ecdysteroids originates from vegetables of alimentary supply. Various ecdysteroids have been detected at the subnanomole level in mammalian serum and urine (9).

Various plants are useful sources for ecdysteroids and ecdysteroid-containing preparations. The isolation procedure of ecdysteroids utilizes fractionated crystallization, liquid–liquid partition, solid-phase extraction, low-pressure liquid column chromatography, preparative thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) (10,11). The usual requirement for the structural elucidation of ecdysteroids is a purity over 90%.

This study deals with the isolation of one new minor ecdysteroid glucoside and two known ecdysteroids from the herb *Silene italica ssp. nemoralis* (Waldst. and Kit.) Nyman (Caryophyllaceae). Also, an account is given on their structural elucidation.

Experimental

Plant material, extraction, and prepurification of the crude extract

The ecdysteroids of the herb *Silene italica ssp. nemoralis* were extracted and isolated as described in literature (11). Briefly, the dried herb was milled and percolated with methanol at ambient temperature. The extract was taken to dryness and dissolved in methanol. Acetone was added and the precipitate was removed and rinsed three times with a methanol–acetone mixture. The supernatants and the methanol–acetone solution were combined and evaporated to dryness. The residue was redissolved in methanol, and further acetone was added to precipitate and remove the contaminants. The supernatant and the methanol–acetone solution were combined, diluted with water, and extracted with benzene three times. The aqueous methanol phase was concentrated and adsorbed onto aluminum oxide and packed into a column (1st column). The ecdysteroid of interest

* Author to whom correspondence should be addressed: email bathori@pharma.szote.u-szeged.hu.

was eluted with 9:1 and 8:2 mixtures of dichloromethane–methanol (fractions 1–7 and 8–13, respectively).

Fractions 1–7 were evaporated to dryness. The dried residue (2.97 g) was dissolved in 6 mL of methanol. The methanol solution was mixed with 9 g of aluminum oxide and taken to dryness by rotary evaporation. The sample adsorbed on aluminum oxide was packed on the top of a column containing 60 g of aluminum oxide (2nd column). Ecdysteroids were stepwise eluted with dichloromethane and 98:2, 95:5, and 9:1 dichloromethane–ethanol (175, 375, 550, and 550 mL, respectively), and 25-mL fractions were collected.

Fractions 51–53 were combined and taken into dryness. The residue (0.3 g) was dissolved in 5 mL of methanol and adsorbed on 0.9 g silica. A total of 9 g silica was packed into a column, and the silica with the adsorbed sample was topped over the sorbent (3rd column). Stepwise gradient elution was carried out using 98:2, 95:5, 92:8, and 9:1 mixtures of dichloromethane–ethanol (100, 700, 400, and 200 mL, respectively). Fractions 167–220 were combined and taken into dryness. The residue (0.17 g) was separated using preparative TLC with solvent system no. 1. The TLC separation resulted in two major bands with ecdysteroid content. One of them was further purified to give compound 1 (0.023 g). The final purification of compound 1 was accomplished by normal-phase (NP) HPLC using solvent system no. 4 to prepare pure compound 1 (8 mg).

Fractions 8–13 from the first alumina column were combined and taken into dryness. The residue (24.79 g) was used to isolate 20-hydroxyecdysone by crystallization in ethyl acetate–methanol (2:1, v/v). One part of the mother liquid (2.1 g dry residue) was fractionated by droplet countercurrent chromatography (DCCC) using system no. 7. The DCCC fractionation was carried out in three parallel experiments. DCCC fractions 188–226 were combined, evaporated to dryness, and the residue (1.8 g) was dissolved in 5 mL of 30% aqueous methanol and further purified using reversed-phase (RP) liquid chromatography (4th low-pressure column chromatography). RP-liquid chromatography was carried out in two parallel procedures on 180 g of an end-capped octadecyl silica column. Elution from the column was carried out with a stepwise gradient of 30%, 35%, 40%, 45%, 50%, 55%, and 60% aqueous methanol (300 mL each), and 50 mL of the fractions were collected. Fractions 9–15 included compound 2, and fractions 25–31 included compound 3. Fractions 9–15 were combined and evaporated, and the dry material (0.69 g) was dissolved in 3 mL of 30% aqueous methanol and purified again on the same low-pressure RP column (4th column). Elution was carried out using a stepwise gradient as described previously. Fractions 8–14 were collected, and after evaporation the residue (0.27 g) was crystallized from methanol to get pure compound 2 (0.18 g).

The fractions containing compound 3 from the first RP column (fractions 25–31) were evaporated, and the residue (76 mg) was dissolved in 5 mL of 40% aqueous methanol and separated on the previous C18 silica column (4th column). Aqueous methanol with concentrations of 40%, 45%, 50%, 55%, and 60% (300 mL each) was used as the eluent, and 50 mL fractions were collected. Fractions 17–20 were combined (19 mg dry residue) and crystallized from methanol, yielding 11 mg of pure compound 3.

Low-pressure liquid chromatography

The first isolation (1st column) used Alumina Brockmann II neutral (Reanal, Budapest, Hungary) as the column material (300 × 93 mm). Elution was carried out using a stepwise gradient of methanol in dichloromethane. The second isolation (300 × 20 mm) was also carried out on Alumina Brockmann II neutral, and mixtures of dichloromethane and ethanol (96%) were used as the eluent. Silica gel 60 (E. Merck, Darmstadt, Germany) was used to pack the 3rd column (80 × 18 mm), which was eluted with various mixtures of dichloromethane–ethanol (96%). The fourth isolation was performed on an endcapped C18 silica (0.06–0.2-mm particle size) (Chemie Urticon–C-Gel, C-560) packed in a 420- × 35-mm column. Elution was carried out with a stepwise gradient of methanol and water.

TLC

TLC was performed on silica plates (20- × 20-cm silica gel 60 F_{254}) (E. Merck). Mixtures of toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v/v/v) (system no. 1), chloroform–methanol–benzene (25:5:3, v/v/v) (system no. 2), or ethyl acetate–ethanol–water (16:2:1, v/v/v) (system no. 3) were used as the mobile phases. The spots were visualized both by fluorescent quenching at 254 nm and also after spraying with vanillin–sulfuric acid and then observing in daylight or at 366 nm.

HPLC

For NP-HPLC a Zorbax-SIL column (5 µm, 250- × 4.6-mm i.d.) (DuPont, Paris, France) eluted with dichloromethane–isopropanol–water (125:40:3, v/v/v) (system no. 4) (1 mL/min) was used. RP-HPLC separation was carried out on an octadecyl silica SUPELCOSIL LC-18-DB (3 µm) stationary phase (Supelco, Bellefonte, PA) packed in a 150- × 4.6-mm stainless steel column. The mobile phase was either acetonitrile–water (20:80, v/v) (system no. 5) or acetonitrile–water (16.5:83.5, v/v) (system no. 6) with a flow rate of 1 mL/min.

DCCC separation

An Eyela DCC-A Instrument (Rikakikai, Tokyo, Japan) was used with chloroform–methanol–water (65:20:20, v/v/v) (system no. 7) as the solvent system. The sample (0.7 g) was dissolved in 3 mL of the upper phase. A 20-mL/h flow rate was applied and 7-mL fractions were collected. The descending mode of operation was used, and the separation was repeated twice.

Enzymatic hydrolysis

Compound 2 (2.2 mg) was used for enzymatic hydrolysis with 18 mg of β-glucosidase (Type II from Almonds, EC 3.2.121, Sigma, St. Louis, MO). The mixture was incubated at 37°C for 96 h in an acetic acid–sodium acetate buffer (5 mL at pH 5.2). The progress of the hydrolysis was monitored using TLC and HPLC, and approximately 56% of the original ecdysteroid glucoside was hydrolyzed. The reaction mixture was diluted with water and extracted using 5- × 5-mL ethyl acetate. The ethyl acetate extracts were combined, concentrated under vacuum, and separated on a Sephadex LH-20 column with recycling. Methanol was used as the mobile phase, and 0.9 mg of aglycone was purified. Its MS spectrum and TLC were identical to that of the standard 2-deoxy-20-hydroxyecdysone.

Using TLC, the sugar fraction was found to contain only D-glucose. TLC silica plates (silica gel 60 F_{254}) (E. Merck) were used. The mobile phase was chloroform–methanol–water (64:50:10, v/v/v), and the visualization was carried out with thymole sulfuric acid.

Results

Figures 1 and 2 show the scheme of isolation. Certain polar and apolar contaminants from the methanolic extract of the herb *Silene italica ssp. nemoralis* were removed using fractionated precipitation and solvent–solvent distribution. The prepurified extract was subjected to column chromatography on alumina. The column chromatography was repeated on alumina and also on silica. Preparative NP-TLC and NP-HPLC resulted in the pure compound 1. The first low-pressure column chromatography gave fractions that contained an excess of 20-hydroxyecdysone (24.79 g). These fractions were used to isolate pure 20-hydroxyecdysone by crystallization (14.3 g). One-fifth of the crystallization mother liquid was subjected to DCCC fractionation. Selected DCCC fractions were further purified repeatedly using low-pres-

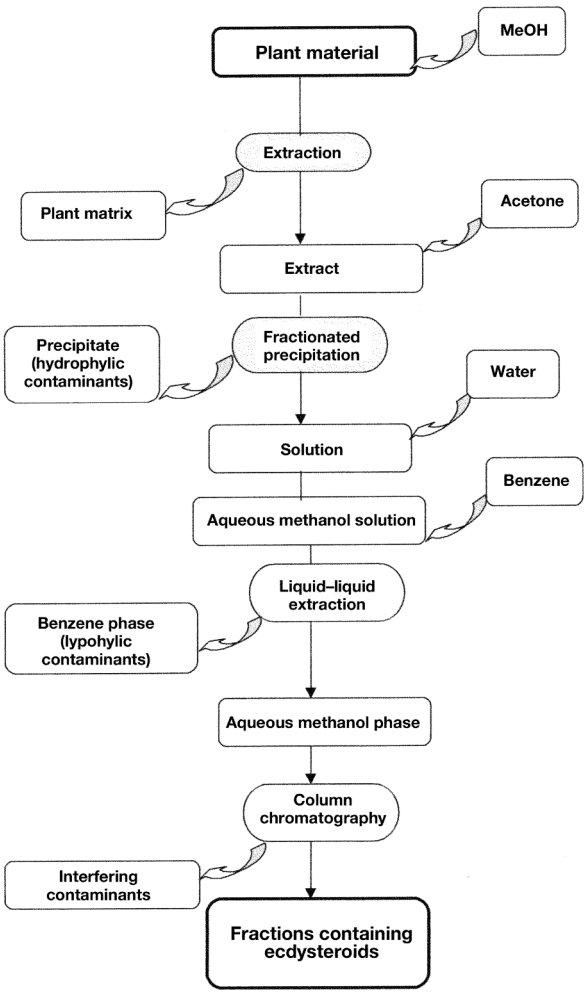

Figure 1. Flowsheet of the multistep cleanup of ecdysteroids from the dried plant *Silene italica ssp. nemoralis*.

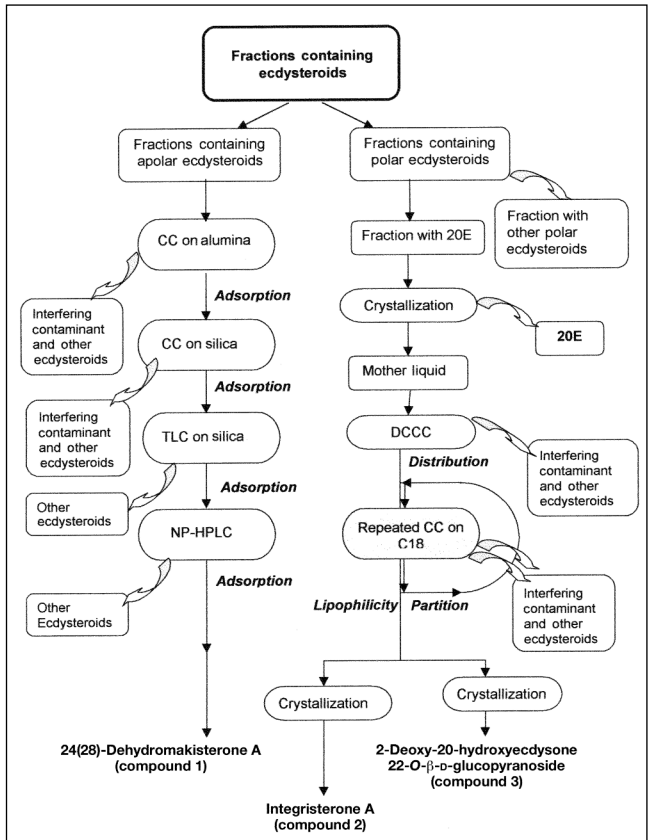

Figure 2. Essential steps of the isolation of plant ecdysteroids from the prepurified extract.

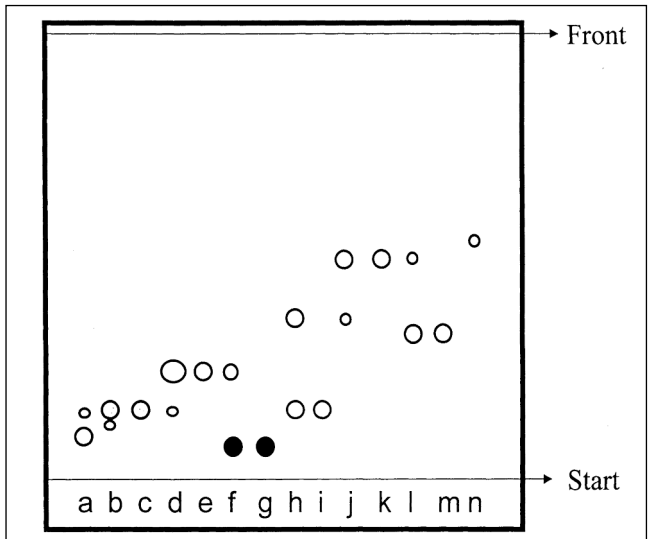

Figure 3. TLC analysis of fractions obtained from the 1st low-pressure RP column chromatography. The stationary phase was TLC silica F254 20 × 20 cm, and the mobile phase was toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v/v/v). The samples were loaded as: (a) fractions 1–8, (b) fractions 9–15 (containing compound 2), (c) integristerone A (pure standard), (d) fractions 16–24, (e) 20-hydroxyecdysone (pure standard), (f) fractions 25–31 (containing compound 3), (g) purified glucoside (2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside), (h) fractions 32–33, (i) 22-deoxy-integristerone A (pure standard), (j) fractions 34–35, (k) 2-deoxy-20-hydroxyecdysone (pure standard), (l) fractions 36–40, (m) 22-deoxy-20-hydroxyecdysone (pure standard), and (n) fractions 41–42.

sure RP column chromatography to obtain compounds 2 and 3. Stepwise gradient elution was carried out in each step with a 5% increase of the methanol content. Figure 3 shows the thin-layer chromatogram of the fractions obtained from the first low-pressure column chromatography and that of the standards. The elution of compounds 2 and 3 took place using 35–40% and 50–55% aqueous methanol, respectively. After crystallization the purity of the isolated ecdysteroids was over 90%. Figure 4 shows the HPLC of the fraction containing compound 3 (loaded on line f of Figure 3) after the first RP column chromatography.

Both TLC and HPLC monitored the entire process of purification. Tables I and II show the TLC and HPLC characteristic of compounds 1–3.

Compound 1 has already been encountered with 24(28)-dehydromakisterone A previously isolated from *Spinacia oleracea* (Chenopodiaceae) (12). Compound 1 showed the same chromatographic characteristic as the reference 24(28)-dehydromakisterone A. The molecular weight was determined by mass spectrometry (MS)–MS (492 amu), and prominent ions were formed at *m/z* 515 $(M+Na)^+$, *m/z* 497 $(M–H_2O+Na)^+$, and *m/z* 479 $(M–2H_2O+Na)^+$. The tetracyclic part of compound 1 was identical to that of 20-hydroxyecdysone (*m/z* 363), which proves the existence of C24 methylene. The nuclear magnetic resonance (NMR) spectra verified the chemical structure of 24(28)-dehydromakisterone A.

An MS–MS analysis of compound 2 gave fragment ions characteristic for integristerone A, which were *m/z* 503 $(M+Li)^+$, *m/z* 485 $(M–H_2O+Li)^+$, *m/z* 467 $(M–2H_2O+Li)^+$, *m/z* 449 $(M–3H_2O+Li)^+$, and *m/z* 361 $(M–C_{22–27}–H_2O)^+$. Integristerone A was previously isolated from *Silene otites* (Caryophyllaceae) (13). Both the HPLC and TLC characteristics of compound 2 and integristerone A were the same (co-chromatography of integristerone A and with reference to NP- and RP-HPLC and NP-TLC using solvent system nos. 4 and 5 and nos. 1–3, respectively). The NMR data of compound 2 corresponded with that of the standard integristerone A.

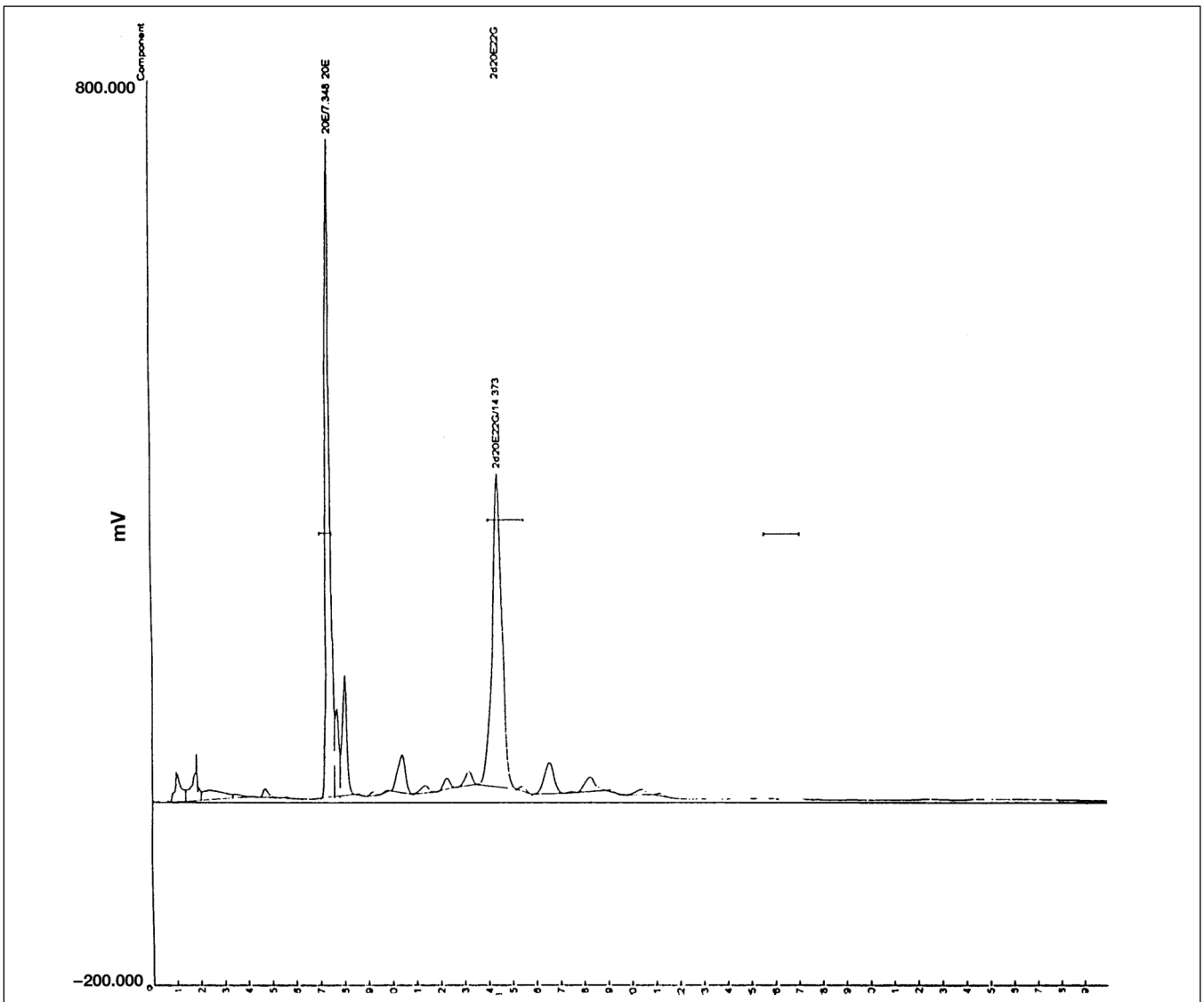

Figure 4. Analytical separation of fractions containing compound 3 (2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside). RP chromatography was performed on a SUPELCOSIL LC-18-DB (3 µm) stationary phase packed into a 150- × 4.6-mm stainless steel column. The mobile phase was acetonitrile–water (16.5:83.5, v/v) with a flow rate of 1 mL/min.

Enzymatic hydrolysis of compound 3 yielded 2-deoxy-20-hydroxyecdysone, which was compared with an authentic sample previously isolated from *Silene otites* (Caryophyllaceae) (13). The MS data of aglycone were the same as that of 2-deoxy-20-hydroxyecdysone, as previously reported (14). The aglycone was also co-chromatographed with the reference compound with NP- and RP-HPLC. The hydrolysis yielded a sugar (D-glucose) identified by TLC. The electrospray MS spectrum of compound 3 showed a quasi molecular ion at *m/z* 633 $(M+Li)^+$, indicating a glucoside composed of 2-deoxy-20-hydroxyecdysone. The characteristic fragment ions were formed from the intact parent compound and also from the parent compound with the loss of the sugar and waters. The MS–MS spectrum gave thereby *m/z* 615 $(M–H_2O+Li)^+$, *m/z* 471 $(M–glucose+Li)^+$, *m/z* 453 $(M–glucose–H_2O+Li)^+$, *m/z* 435 $(M–glucose–2H_2O+Li)^+$, and *m/z* 417 $(M–glucose–3H_2O+Li)^+$. The ion of *m/z* 329 corresponded with the water elimination together with a break of the C20–C22 bond. The NMR spectrum definitively established the 22-glucosidation.

Table I. R_F* × 100 Values Characterizing 24(28)-Dehyromakisterone A (Compound 1), Integristerone A (Compound 2), 2-Deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside (Compound 3), and Aglycone of Compound 3 (2-Deoxy-20-hydroxyecdysone) in Three Mobile Phases (Solvent System Nos. 1–3)†

Compound	R_F × 100 in the solvent systems			Color after vanillin–sulfuric acid	
	No. 1	No. 2	No. 3	Under UV (366 nm)	Under daylight
24(28)-Dehydromakisterone A	37	30	46	violet	yellow
2-Deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside	7	13	19	violet	green
Integristerone A	16	24	29	violet	green
2-Deoxy-20-hydroxyecdysone	49	46	53	violet	green
20-Hydroxyecdysone	27	28	37	violet	green

* R_F, TLC retention factor.
† Data are compared with that of 20-hydroxyecdysone.

Table II. HPLC Retention Times of Peaks Belonging to 24(28)-Dehydromakisterone A (Compound 1), Integristerone A (Compound 2), 2-Deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside (Compound 3), and Aglycone of Compound 3 (2-Deoxy-20-hydroxyecdysone) in Various Mobile Phases*

Compound	Retention times (min) using the solvent systems		
	No. 4	No. 5	No. 6
24(28)-Dehydromakisterone A	8.9	6.7	17.3
2-Deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside	28	5.2	14.6
Integristerone A	18.5	3.1	6.1
2-Deoxy-20-hydroxyecdysone	8.3	9.9	26.2
20-Hydroxyecdysone	15.6	3.6	7.3

* The stationary phases were Zorbax-SIL using solvent system no. 4 (dichloromethane–isopropanol–water, 125:40:3, v/v/v) and Supelco C18 using mobile phase no. 5 (acetonitrile–water, 20:80, v/v) and no. 6 (acetonitrile–water, 16.5:83.5, v/v). The data are compared with that of 20-hydroxyecdysone.

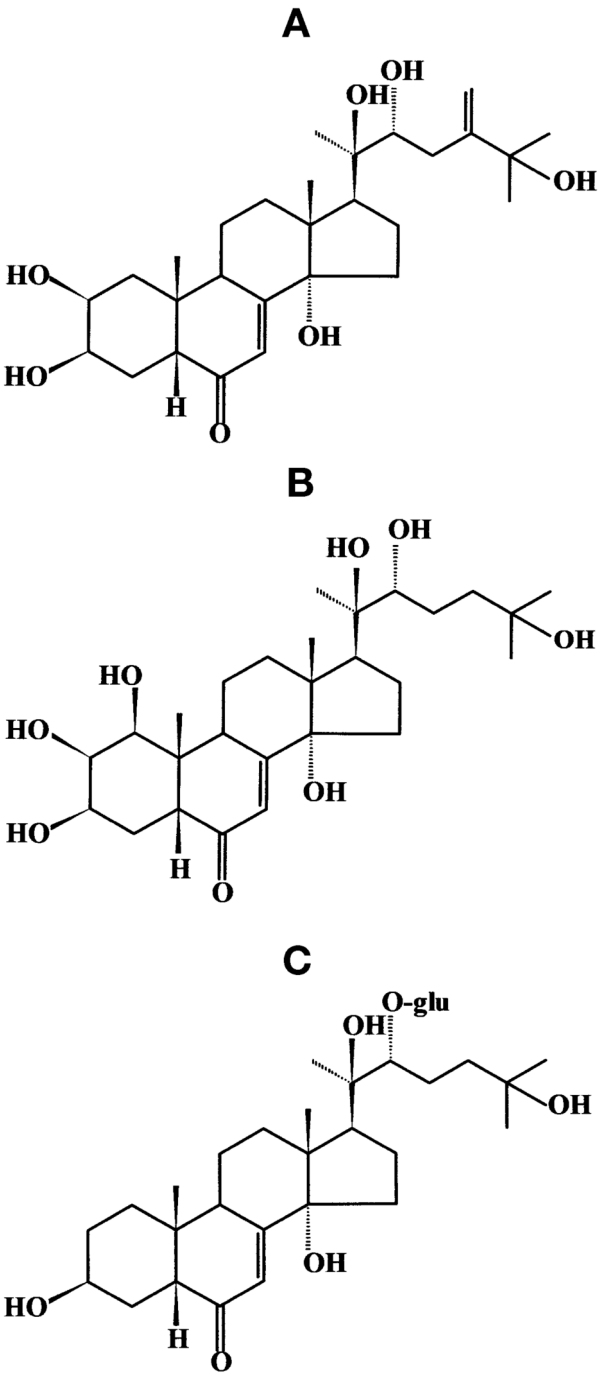

Figure 5. The chemical structure of (A) 24(28)-dehydromakisterone A (compound 1), (B) integristerone A (compound 2), and (C) 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside (compound 3).

Figure 5 shows the chemical structure of the isolated ecdysteroids.

Discussion

The ecdysteroids in *Silene italica ssp. nemoralis* are present in a complex mixture. Seven ecdysteroids have been isolated from this plant (such as 20-hydroxyecdysone, polypodine B, ecdysone, 22-deoxy-20-hydroxyecdysone, 22-deoxy-integristerone A, 2-deoxy-integristerone A, and 5α-2-deoxy-integristerone A) (11). A multistep isolation scheme has been built up including the repeated use of low-pressure RP column chromatography in the last steps. Our intent in using RP low-pressure liquid chromatography was to develop a new method based on different physical–chemical characteristics of ecdysteroids than those used in the earlier steps.

The whole separation process utilized the differences in the solubility, distribution, lipophilicity, and adsorption characteristics of ecdysteroids relative to their contaminants.

The preliminary separation processes were based on precipitation and solvent–solvent distribution. Acetone was added to the methanolic solution, and the hydrophilic contaminants were precipitated. Solvent–solvent distribution between benzene and aqueous methanol removed the contaminants, which were more lipophilic than the ecdysteroids. The load could be high in these purification procedures, because the distribution coefficients depend only moderately on the concentration. At the same time the ecdysteroid content was comprised of several percentages of the whole amount of sample.

Separation at the medium purification stage of compounds 1, 2, and 3 was carried out using adsorption. The high load was also favorable, because the overload of the system could decrease the possible irreversible adsorption of the ecdysteroids. Because 200 g of the sample was a production-scale separation, special procedures were followed. Such an amount could not be separated using either countercurrent distribution or HPLC. The sample-to-sorbent ratio was 1:3 when the sample solution was dried on the sorbent. In order to improve the separation, the load dried on the sorbent was topped onto a similar amount of stationary phase packed in a column, and also a several-step gradient elution was employed. The extents of purification were 10- to 100-fold.

In the further steps of purification of compound 1, adsorption chromatography was employed. The total purification was over 500-fold in the three consecutive chromatographic steps. Purification and identification of compound 1 could be completed using HPLC.

Compounds 2 and 3 were further purified using DCCC and RP chromatography. DCCC essentially decreased the number of components present in the sample because the majority of the trace nonecdysteroid impurities were removed. Minor impurities tend to appear as ghost peaks.

RP material with a 60–200-µm particle size was packed into a column, on which low-pressure column chromatography was performed. From 0.9 g of the sample, the respective amounts of 270 mg and 19 mg of compounds 2 and 3 resulted. The high load of our procedure brings low-pressure RP column chromatography into the limelight. For this, only a minimum of technical setup is required. Both the size of the column and the extent of the load may be easily increased. The sample size of 0.9 g on a 180-g amount of stationary phase could be increased to 5 g using the same column, and the separation would still be satisfactory.

Up to now, approximately 40 ecdysteroid glycosides have been isolated by chromatography. Conjugation of an ecdysteroid with a sugar is more characteristic to plants than animals. The number of the known phytoecdysteroid glycosides is 36, and only 4 ecdysteroid glycosides were detected in animals. Among these phytoecdysteroid glycosides, almost half (sixteen) were discovered from *Silene* species. The sugar moiety of the ecdysteroid glycosides may generally be glucose, galactose, or xylose. The 3-, 22-, and 25-hydroxyls are the most frequent position of the glycosilation in the plants.

Among the glycosides of 2-deoxy-20-hydroxyecdysone, only 3-monoglucoside has been found earlier in the plant kingdom. This study demonstrates the presence and isolation of a new glucoside, 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside, in *Silene italica ssp. nemoralis* (Waldst. and Kit.) Nyman. The glucose is linked at the 22-hydroxyl group of 2-deoxy-20-hydroxyecdysone.

Integristerone A and (24)28-dehydromakisterone A are typical phytoecdysteroids, which have been detected solely in the plant kingdom (14).

(24)28-Dehydromakisterone A possesses a methylene group in the C24 position. The 24-alkyl-ecdysteroids are derived from the appropriate precursor phytosterines (15), which contain an alkyl group at the C24 position. These typical phytosterines occur only in plants; animals cannot synthesize them.

Conclusion

New natural products can be isolated using a combination of various chromatographic methods (10). The proper employment of the chromatographic separations in an appropriate order resulted in compounds that are pure enough for structural elucidation with spectroscopic methods.

Acknowledgments

Thanks are due to the OTKA T032185, T032618, T032657, T035054, and D32830 grants of the Hungarian Ministry of Education (Budapest, Hungary) and to the Bólyai Fellowship given to Dr. András Simon.

References

1. K. Slama and R. Lafont. Insect hormones—Ecdysteroids: their presence and action in vertebrates. *Eur. J. Entomol.* **92:** 355–77 (1995).
2. V.N. Syrov. Mechanism of anabolic action of phytoecdysteroids in mammals. *Biol. Nauk. (Moscow)* **11:** 16–20 (1984).
3. K.D. Catalan, A.M. Martinez, M.D. Aragones, B.G. Miguel,

A. Robles, and J.E. Godoy. Alteration in rat lipid metabolism following ecdysterone treatment. *Comp. Biochem. Physiol. (B)* **81:** 771–75 (1985).
4. T. Yoshida, T. Otaka, M. Uchiyama, and S. Ogawa. Effect of ecdysterone on hyperglycaemia in experimental animals. *Biochem. Pharmacol.* **20:** 3263–68 (1971).
5. D.K. Najmutdinova and Z. Saatov. Lung local defense in experimental *Diabetes mellitus* and the effect of 11,20-dihydroxyecdysone in combination with maninil. *Arch. Insect Biochem. Physiol.* **41:** 144–47 (1999).
6. L. Dinan. Phytoecdysteroids: biological aspects. *Phytochem.* **57:** 325–39 (2001).
7. Y.R. Mirzaev and V.N. Syrov. Effect of phytoecdysteroids on the sexual activity of male rats. *Dokl. Academ. Nauk Respubl. Uzbekistan* **3:** 47–49 (1992).
8. M. Takasaki, H. Tokuda, H. Nishino, and T. Konoshima. Cancer chemopreventative agents (antitumor-promoters) from *Ajuga decumbens. J. Nat. Prod.* **62:** 972–75 (1999).
9. P. Simon and J. Koolman. "Ecdysteroids in Vertebrates: Pharmacological Aspects". In *Ecdysone: From Chemistry to Mode of Action*. J. Koolman, Ed. George Thiene, Stuttgart, Germany, 1989, pp. 254–59.
10. M. Báthori and H. Kalász. Separation methods for phytoecdysteroids. *LC-GC Europe* **14:** 626–33 (2001).
11. M. Báthori, H. Kalász, Z. Pongrácz, I. Máthé, A. Kálmán, and G. Argay. 5-alpha- and 5-beta-2-deoxy-integristerone A, a 5-alpha- and 5-beta-isomer pair of ecdysteroids isolated from the *Silene* genus. *Biomed. Chromatogr.* **16:** 373–78 (2002).
12. M. Báthori, I. Tóth, K. Szendrei, and J. Reisch. Ecdysteroids in *Spinacia oleracea* and *Chenopodium bonus-Henricus*. *Phytochemistry* **21:** 236–38 (1982).
13. M. Báthori, K. Szendrei, and I. Herke. The ecdysteroids of *Silene otites* L. (Wib.). *Herba Hungarica* **25:** 105–17 (1986).
14. R. Lafont and I.D. Wilson. *The Ecdysone Handbook*. 2nd ed. The Chromatographic Society, Nottingham, U.K., 1996.
15. R. Lafont. Phytoecdysteroids in the world flora: diversity, distribution, biosynthesis and evolution. *Russ. J. Plant Physiol.* **45:** 276–95 (1998).

Manuscript accepted June 5, 2002.