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ECDYSTEROIDS ISOLATED FROM *SILENE ITALICA* SSP. *NEMORALIS*

PH.D. THESIS

BY

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I dedicate this work to my family.

Silene italica ssp. *nemoralis*

SCIENTIFIC PUBLICATIONS

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VIII. Simon, A., Pongrácz, Z., Tóth, G., Mák, M., Máthé, I., Báthori, M. 2003. A unique ecdysteroid with modified skeleton at C-9 and three accompanying ecdysteroids of *Silene italica* ssp. *nemoralis*. *Journal of Natural Products* in prep.

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1. INTRODUCTION

1.1. Aims of the Study

The study of the ecdysteroid spectrum in *Silene* species belonging to the Caryophyllaceae family has already been in progress by research group of Department of Pharmacognosy in University of Szeged in the last decades.

The main purpose was (a) to find Hungarian plant species rich in ecdysteroids, which can either be cultivated or grow wild. Similarly, there was an effort (b) to isolate and (c) elucidate the structure of new native phytoecdysteroids. On the basis of the earlier studies within *Silene* genus, it was possible (d) to compare not only the main representatives of the ecdysteroid spectra but also concentrations of the individual ecdysteroids.

1.2. Importance of Ecdysteroids

The ecdysteroids represent two classes according to the occurrence in living world: zoo- and phytoecdysteroids. Zooecdysteroids are polyhydroxylated steroid growth hormones in insects, molluscs, and crustaceans. They have pronounced stimulatory effects on growth and proliferation of insect tissues (1, 2). The moulting hormones of all arthropods ecdysone (E) and 20-hydroxyecdysone (20E) (also called β-ecdysone, ecdysterone, crustecdysterone, isoinokosterone, crustecdysone) are the major biologically active ecdysteroids (3).

Research discovered the occurrence of these hormones in plants to have similar chemical structures and biological effects. It soon became apparent that ecdysteroids were rather widespread in plant kingdom (4, 5). Among terrestrial plants over 100 plant families representing ferns, gymnosperms and angiosperms are sources of ecdysteroids (6-8). We know at present more than 312 ecdysteroids (described at the onset of 2000 and available on the web), most of which occur in plants and the number of compounds is still growing (9). Level of ecdysteroids in plants is 1000 fold higher as in insects (up to 3.2 %), but in most species is not more than 0.1 % of the dry weight (10). Five to six percent of all higher plant species accumulate detectable levels of phytoecdysteroids (11, 12), their concentration ranging from 50 ng/g to as much as 30 mg/g dry mass. According to the most accepted hypothesis, the phytoecdysteroid-cocktail is assumed to have a defensive function against insect predators (6). Phytoecdysteroids attract great scientific interest, because of their potential contribution to crop protection strategies (12) and their possible medical uses (see below) (13). The roles of these compounds in plant survival are still to be elucidated.

Phytoecdysteroids would appear to have wide-ranging allelopathic value on mammals with low toxicity (2). Several medicinal and pharmaceutical benefits have also been described to them, involve anabolic, adaptogenic, tonic, roborant, antidepressant, anticholesterolaemic, antihyperlipidaemic, anti-arrhythmic, antiulcerative, antioxidant and antimicrobial immunomodulator. They are used as wound healing, because of strengthen the water-barrier function of skin. Therefore, they have beneficial effect on sexual activity, memory and learning. They reduce the hyperactivation of the vestibular neuron, have curative effect in some form of renal pathology, and act as nephro- and hepatoprotective agents (for review, see 14, 15). The most pronounced effects of them are anabolic and adaptogenic effects, so the other effects mostly balance and improve the state of health. Ecdysteroids are powerful anabolic substances and recently, they have become doping substances, but should not be classified among illegal growth promoters (16). Commercial available ecdysteroids are generally extracted from plants, such as: (a) *Leuzea* (=*Rhaponticum*) *carthamoides* from Eastern Europe countries, where it is cultivated as a remedy in traditional medicine (17); (b) *Pfaffia* (in fact a group of related species; = Brazilian ginseng, Para Toda; = Suma), again a plant used in traditional medicine; (c) *Cyanotis vaga* or *C. arachnoides*, a monocotyledonous plant, extracts of which are used in a large scale also for the synchronisation of spinning in silkworm larvae (15, 18). Given the still limited market now, over 140 different preparations containing ecdysteroids for oral use can be found on the market. Preparations are easily available through Internet (some of them in **Table 1**; ref. 9, 19). These contain crude or semi-purified plant extracts (plant powders, or alcoholic extracts, elixirs) and containing 'pure' 20E or a defined ecdysteroid mixture. Most of them are proposed for use by bodybuilders, but some have been designed for more specific users, or for animals (dogs, horses, meat-producing animals). 20-40 milligrams per kg body weight per day is generally recommended.

The ecdysteroids, like vertebrate steroid hormones, act on nuclear receptors, but they do not cause such side effects in human (e.g. adverse androgenic, antigonadotropic, thymolytic) (20-22). Ecdysteroid receptor (EcR), a nuclear hormone receptor essential to the gene regulation of insect moulting, has been engineered into gene regulation systems (ecdysteroid-controlled gene expression). They are potentially useful induced by phytoecdysteroids (two most active inducers: ponasterone A and muristerone A) to modulate gene expression in a host cell for applications such as gene therapy, large scale production of proteins and antibodies, cell-based high throughput screening-assays, functional genomics and regulation of traits in transgenic organism (23). Comparative comparative molecular field analysis (CoMFA) and quantitative structure-activity relationship (4D-QSAR) analyses are reported for EcR affinity of the natural ecdysteroid ligands (24, 25).

Table 1 Some OTC products containing ecdysteroids.

Ref No	Product Name	Supplier	Source	Supplement facts: Servings Per container/ Serving Size	**20-E and/or Plant Source Amount Per Serving**	Other Ingredients	Directions Serving Size	Recommanded Use
19/1	ACT-SUM™	7th Millenium Nutrition	P	100 Tablets/ 3-4 Tablets	**20 mg 75% Beta-Ecdysteine™ (standardized at 75%)**	–	Take 20 mg of 75 % Beta-Ecdysteine (Sumax-75 %™ with 75 % Beta-Ecdysteine™) per 60-80 pounds of bodyweight per day, divide daily consumption into three or more doses.	adaptogen, leading to lower blood sugar, triglyceride and cholesterol levels, helping the liver resist damage from alcohol or hepatitis, reducing inflammatory responses, and supporting resistance to viruses and bacteria
17	Maralan™	J. Kren Firm (Slovak Firma)	L	No data (Green tea)	**0.08%-0.22% 20E**	–	No data	Stimulant, Removal of Fatigue, Improvement of Physical Condition, Improvement of Digestion, Resistance against Stress, Stimulation of Functions of CNS
19/2	Elite Athlete™	MD Healthline	C	60 Tablets / 2 Tablets	**40 mg *Cyanotis vaga* root extract (standardized to 97% 20E)**	Vit B6 ATP Methoxy-isoflavone	Take 2 tablets three times a day with protein rich meals.	Designed to maximize nitrogen retention and protein synthesis. Provides anti-oxidant protection. Peak performance.
19/3	Horny Goat Weed™	Bodyonics Pinnacle	P v	30, 60 Caps/ 2 Caps	**25 mg *Polypodium* (standardized to 8% 20E)**	*Epimedium grandiflorum, Mucuna pruriens, Lepidium*	Take 2 capsules daily with a meal. For more immediate action, Take 3-4 capsules 90 minutes before activity.	Sexual enhancer (20E actively support the contractile activity of erectile proteins associated with sexual activity)
19/4	REAP= Recovery Enhancing Adaptoganic Potentiator™	Phyto-longevity Inc.	L A	6 Caps / 1-3 Caps	**Contains 60 mg *Leuzea* and *Ajuga* root extract**	Glutamine	Take one to three capsules with before and after exercise for ten to fourteen days and take a ten to fourteen day break before repeating.	Adaptogenic/Anabolic Helps support recovery after physical and mental stress

20E=20-hydroxyecdysone; P=*Pfaffia paniculata*, L.=*Leuzea carthamoides*, C=*Cyanotis vaga*, Pv.=*Polipodium vulgare*, A=*Ajuga turcestenica*; Web adresses and references (17, 19): 19/1. http://www.diabetestea.com/velocity/active.html; 17: Oppletal, 1997; 19/2. http://mdhealthline.com/cgi/sgx/store/web_store.cgi?_id=134.157.163.233&page=elite.html; 19/3. http://www.marigarden.com/hgw/index.html; 19/4. http://www.phytolongevity.com/products/performance/performance.htm.

1.3. Structural Diversity of Phytoecdysteroids

The carbon skeleton of ecdysteroids is termed cyclopentano-perhydro-phenantrene with beta side-chain at C-17, which is the product of biosynthesis through mevalonic acid, cholesterol and related sterols. The whole side chain of the precursor sterols is generally kept in the case of ecdysteroids, and this is the way the C_{27}-C_{29} ecdysteroids are biosynthesised. Cleavage of the side chain gives C_{19}, C_{21} and C_{24} ecdysteroids, whose biosynthesis is similar to that of the class of pregnanes, 5β-androstanes and bile acids. Anellations of the rings are characteristic: C/D is *trans*, and A/B is generally *cis*.

Essential characteristics of ecdysteroids are an α-hydroxyl at position C-14, β-hydroxyl at C-3 and a Δ^7-6-keto chromophore in the B ring. Ecdysteroids are highly hydroxylated with two to eight

hydroxyl-groups (26). **Figure 1** shows the hydroxylation position of phytoecdysteroids. Further possibilities are additional/alternative double bond at one of locations of 4-5, 8-9, 9-11, 12-13, 14-15, 24-25, 25-26 or 24-28. Second oxo group can occur in one of the positions of C-2, C-3, C-12, C-17, C-20 or C-22 (ref. 27).

Natural ecdysteroid derivatives are also considered as ecdysteroids in the definite sense. Ecdysteroid derivatives include the esters (with both organic and inorganic acids), the ethers (with organic alcohols and intra-molecular ones), and glycosides of ecdysteroids. Ecdysteroid glycosides can be mono-glycosides, diglycosides, and triglycosides. Diglycosides and triglycosides have two types. The sugar moiety is linked to two different hydroxyls, or two sugars are linked subsequently. Ecdysteroid glycosides are solely O-glycosides, while ecdysteroid-C-glycoside has not been discovered yet. The above-mentioned diversity of derivatives is a unique characteristic of the phytoecdysteroids, no other steroid group shows such a kaleidoscope of various descendent compounds.

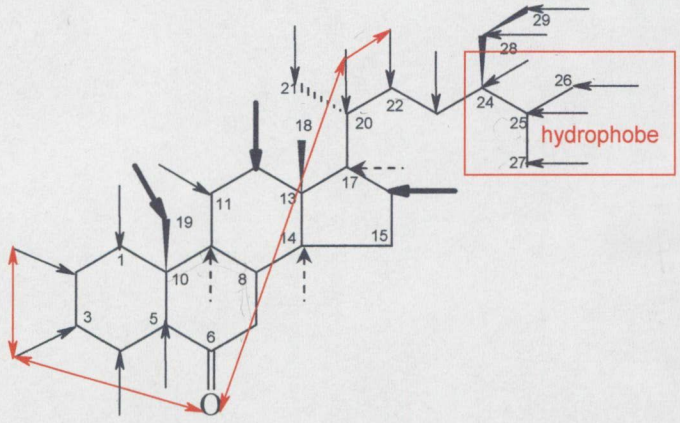

Figure 1 Common structures of ecdysteroids and their diversity. Arrows mark the possible substitution sites on ecdysteroid skeleton. Heavy (wide) arrows show the β position of substituents, three dotted arrows show the α positions at C-9, C-14 (part of the ecdysteroid nucleus) or C-17. The red arrows give the cross-relation matrix (with possible molecular features for hydrogen-bond donation redrawn from Dinan et al., 1999, ref. 24), where the 'active ecdysteroids' might be hypothesized as 2β,3β, 20*R*, 22*R*-tetrahydroxy-5β-cholest-7,8-dien-6-one (=ponasterone A).

Modelling studies are used for phytoecdysteroids to examine the ligand binding to the ecdysteroid receptor (24, 25). The 4D-QSAR techniques additionally permit the prediction of which functional groups on the ecdysteroid are involved in hydrogen bonding to the receptor. These models suggest that the local conformation of C-20–C-22 hydroxyls (in each case the *R*

configuration) seems to be stabilized by a weak hydrogen bond between the two hydroxyls (C-20 as donor), and 6-C=O, 2-OH, 3-OH are also involved in hydrogen-bond donations but unknown the acceptor/donor characteristics. Additionally these models imply a sterically restrictive hydrophobic cylinder around the steroid side-chain (e.g., 24-OH, 25-OH, 26-OH are disfavoured) (see **Figure 1**) (25).

1.4. The *Silene* genus (Caryophyllaceae) from Taxonomic Aspects

Within families ecdysteroid-containing plants form groups of closely cognate species (within certain tribes and/or genera). The families Caryophyllaceae (genera *Silene*, *Lychnis*, *Coccyganthe*), Amaranthaceae (genus *Amaranthus*), Chenopodiaceae (genera *Chenopodium*, *Atriplex*, *Axyris*, *Kochia*), and Asteraceae (genera *Rhaponticum* and *Serratula*) contain the highest number of ecdysteroid-containing species (10).

The *Silene* genus L. (Caryophyllaceae) differs from other genera by having many species that contain ecdysteroids in high amount and a wide variety of them (28). There is no other genus of any other plant family comprising such a large number of known ecdysteroid-containing species (100 different *Silene* species) (12, 29-31). Ecdysteroids have been detected in 70 *Silene* species and in 25 species from other genera, based on investigations performed with 230 species of different genera of Caryophyllaceae (32, 33). Apart from the fact, that it is a well-investigated genus, this is one of the largest genera of the world flora with ca. 700 predominantly perennial species. It is mainly distributed in temperate regions of the northern hemisphere, and has its principal centre of diversity in the Mediterranean and the Middle East area (34).

So far, sixty ecdysteroids have been isolated from various *Silene* species (28). In our laboratory, thirty-two ecdysteroids were isolated from *Silene otites* (L.) Wib., *Silene nutans* L., *Silene tatarica* (L) Pers. and *Silene dioica* (L.) Clairv. Nineteen ecdysteroids were first reported by us in these plants (30, 35-38).

Generic limits in the Caryophyllaceae, and in *Silene* species also, notoriously difficult, have generated considerable taxonomic problems with its genera (34). This lumping trend at the intergeneric level has led up from the multi-level hierarchical classification by Rohrbach (39) to classification by Chowdhuri (40). The first used subgenera and sections, while the latter used only sections (44 sections) and subsections. Chowdhuri expanded the genus, because the previously separated genera, *Lychnis, Silene* and *Melandrium*, were grouped in the single genus *Silene* (34, 40).

A persistent view is that ecdysteroid distribution is erratic and cannot be related to phylogenetic classification. However, recent detailed studies of the Caryophyllaceae revealed, that ecdysteroid-containing species are grouped in sections, genera or even in tribes (41-48). Some sections and groups of the genus *Silene* probably include only ecdysteroid-containing species (e.g., sections *Siphonomorpha*, *Dipterospermae*, *Silene*, *Otites*), whereas others probably comprise only ecdysteroid-negative species (e.g., *Auriculatae*, *Conomorpha*, *Eudianthae*, *Heliospermae*, *Inflatae*)[†] (28).

The frequency of ecdysteroid-containing species is not equal and there does not appear to be a correlation between the frequency of a positive species in a family and the levels of ecdysteroids. In spite of this, it seems to be that phytoecdysteroids may also have chemotaxonomic value (6, 49).

Our earlier studies of 20-hydroxyecdysone distribution in *Silene nutans*: and *S. otites* revealed, that highest content of ecdysteroids was associated with points of active growth (36, 50). In the case of some other perennial *Silene* species, *S. frivaldszkyana* and *S. italica* maximal levels occurred also in the aboveground parts in the second year during the vegetative growth (1.63 % and 1 % of the dry wt.) and budding (1.39 % and 1.37 % of the dry wt., respectively) (28). Our preliminary studies related to *S. italica* ssp. *nemoralis*, *S. nutans* and *S. otites* was justified such ecdysteroid dynamics during vegetation. However, we measured lower levels of 20-hydroxyecdysone (peak concentrations 0.5 % at the stage of flowering in *S. italica* ssp. *nemoralis*, 0.7 % during the vegetative phase in *S. nutans*, and 0.95 % at the stage of flowering and fruiting in *S. otites*) (50). Polypodine B (pB), the second most common ecdysteroid in plants over 20-hydroxyecdysone, can be found in many species of sections *Siphonomorpha* Otth. (6 sp.), *Suffruticosae* Rohrb. (3 sp.) [†]. 2-Deoxy-20-hydroxyecdysone is also a significant ecdysteroid in *Silene* genus, but cannot be related sections (31, 32).

Taxonomic classification of <u>*Silene italica* ssp. *nemoralis*</u>:

Taxa[∞]**:**Division: Angiospermae
Class: Dicotyledonae
Subclass: Archichlamydeae
Order: Centrospermae
Family: Caryophyllaceae
Subfamily: *Silenoideae*
Tribe: *Lychnideae* (*Sileneae**)
Genus: *Silene*

[†]Section designations following Chowdhuri (1957, 40).

[∞]Following classification of Flora Europae (1964, 51) and Hegnauer (1964, 52). *The tribe *Sileneae* is characterized by some authors (Rohrbach, 1869, 39; Oxelman & Lidén, 1995, 53, etc.)

Subgenus and/or Section[#]
Species: *Silene italica* (L.) Pers.
Subspecies: *Silene italica* (L.) Pers. ssp. *nemoralis* (Waldst. & Kit.) Nyman

Section[1]: *Botryosilene* Rohrb. (belonging to the *Silene* subg. *Eusilene*)
Section[2]: *Paniculatae* (Boiss.) Chowdhuri (syn.: *Silene* subg. *Eusilene* sect. *Botryosilene* series *Italicae* Rohrb.)
Section[3]: *Siphonomorpha* Otth. (including sect. *Paniculatae* (Boiss.) Chowdhuri and sect. *Siphonomorpha* (Otth.) Chowdhuri = *Silene* subg. *Eusilene* sect. *Botryosilene* series *Nutantes* Rohrb.)

Botany: Biennial, with single flowering stem and rather dense panicle; Stems 20-80 cm, pubescent below; basal leaves lanceolate-spathulate, obtuse; Inflorescence lax, with ascending branches usually bearing 3-flowered dichasia; Calyx 1.5-2 cm; glandular-pubescent, teeth are obtuse; Petals usually creamy-white above, reddish or greenish beneath, deeply bifid, coronal scales very small or absent, petal-claw usually glabrous; Capsule 8–10 (–12) mm, ovoid, more or less equalling pubescent carpophore; Seeds ca. 1 mm, reniform. This species is naturalized in South and Central Europe (Carpathian basin), and casual in North Europe (rarely naturalized), however it has not been the subject of a through phytochemical investigation before.

1.5. Ecdysteroid Isolation Procedures

Plants may have thousands of constituents, therefore the difficulties in separating our one particular component can be appreciated. From plant sample to pure compounds, the purification strategy always comprises a multi-step procedure including extraction, preparation of sample and then one or several chromatographic steps, the scale of the operation decreases. Thus the initial stages of a separation procedure involve methods with a high loading capacity and inexpensive stationary phases – these have traditionally been column chromatography with silica gel, alumina, polyamide or other supports, although liquid-liquid methods are widely used. Subsequent steps employ techniques, which require smaller samples – HPLC, for example (54).

Plants usually contain one or a few major ecdysteroids together with a wide array of closely related molecules. The major ecdysteroid sometimes represents up to 1-3 % of the plant's dry mass (6). The minor ecdysteroids are present only at very low concentrations (pg/g to ng/g). In the latter case, large samples (i.e., kg, or even more) have to be processed, whereas for major components a few

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[#]Section designations following : [1]Rohrbach (1868, 39); [2]Chowdhuri (1957, 40); [3]Chater and Walters (1964, Flora Europaea, 51), and Greuter (1995, 34).

grams are enough. Fortunately, 1-2 mg of any pure steroid is usually sufficient to establish its structure using both mass spectrometry (MS) and nuclear magnetic resonance (NMR), thanks to the progress of these spectroscopic methods. It is so important, that the purity should be the highest. Finally, the polarity of the compound and polarity scale among constituents within plant determines the complexity of the procedure and of course, purification methods must be adopted for each specific case. Methods for the isolation and analysis of ecdysteroids are well documented (54, 55). The general strategy is similar that used for other classes of plant molecules, such as other steroids.

1.5.1. Isolation and Prepurification

Extraction of dried, milled material can be performed using a large range of solvents (with ca. 10 % weight/volume), among which alcohols (methanol or ethanol) are the most widely used. After concentration, a second step usually involves a partition between two non-miscible solvents (liquid-liquid extraction). The purpose of such a step is that it can be used whatever the sample size and it can be very efficient in removing polar and non-polar contaminants, if two complementary partition steps are being used. Non-polar components are generally extracted using a non-polar solvent such as hexane, petroleather, chloroform, benzene or ethyl acetate. Polar contaminants are removed from aqueous solutions using butanol and isopropanol. The substitution of butanol extraction by acetone precipitation make the procedure more simple and faster (56). At the third step, chromatographic procedures are used. Large samples are usually purified by column chromatography (CC) on alumina or silica. Alternatively, several counter-current chromatographic (CCC) techniques are used, which are based on the distribution of compounds between organic and water phases: e.g., droplet counter-current chromatography (DCCC). These techniques require the availability of specific equipment. Column chromatography (CC) generally uses normal-phase systems, i.e., a polar stationary phase (alumina, silica: normal-phase column chromatography - NPCC) eluted with organic solvent mixtures of increasing eluting power (e.g., a step-gradient of methanol or ethanol (96 %) in dichloromethane). The irreversible adsorption of certain ecdysteroids to the adsorbent is reduced using a water-containing eluent (ethanol contains some water) (55). Alternatively, reversed-phase systems (reversed-phase column chromatography – RPCC) are sometimes used, such as C_{18}-bonded silica, and the mobile phases are step-gradient of aqueous methanol. In fact, normal-phase systems are more generally used in separation of some ecdysteroids, such as 20E and pB. After one or possibly two such steps, major compounds can usually be considered as satisfactorily pure, especially if they are crystallized from the corresponding fractions. However, in the case of complex mixtures of structurally related compounds, these methods are not efficient enough, and further chromatographic steps are needed.

1.5.2. Final Purification

Thin-layer chromatography (TLC) can be used both to control the purity of isolated compound, and sometimes TLC serves as an additional purification step (preparative TLC). Several visualisation procedures can be used to observe the migration of compounds during analytical experiments. The most usual detection procedure is the vanillin-sulphuric acid spray reagent. TLC on silica plates is an efficient way to separate ecdysteroids. Several efficient reversed-phase TLC (RP-TLC) systems are also available, that use octadecylsilanized (ODS or C_{18})-bonded silica developed with various mixtures. However, HPLC is rather preferred to TLC when minor ecdysteroids are looked for and are isolated. These ecdysteroids are present in trace amount (< 0.001 %), and therefore the sample requirement for TLC can take the majority of their gross amount (57).

High-performance liquid chromatography (HPLC) is more widely used, as it most powerful technique and allows the recovery of pure compounds in the 1-100 mg range. Again, mixtures of related compounds possibly require several HPLC steps using systems of different selectivity. Both reversed-phase (RP) and normal-phase (NP) systems are used. It is generally accepted that co-migration of a given ecdysteroid with the same reference compound in three (at least one RP and one NP) solvent systems can be considered as sufficient evidence for establishing their identity. Of course, in each case it is also important to make sure of the identity spectroscopically (54).

Normal phase systems for HPLC: The systems generally use silica columns. Solvent systems consist of either binary mixtures (directly derived from the solvent systems used for TLC), or ternary and even quaternary mixtures. In practice, ternary systems give better results, regarding both peak symmetry and selectivity. Among them, it seems that mixtures of dichloromethane-isopropanol-water (125:30:2 or 125:40:3) or cyclohexane-isopropanol-water (e.g., 100:40:3) are the most widely used. The water content in these solvents is just below saturation, and is essential for peak symmetry. It allows to perform partition rather than absorption chromatography, and recovery of the injected compounds is very good (54, 55).

Reversed-phase systems for HPLC: They are the most common used, essentially with C_{18}-bonded columns. The most important is its application in separation of main ecdysteroids (20E and E, except pB) from biological samples prior to radioimmunoassay, and this may be achieved with methanol/water mixtures. Aqueous acetonitrile and aqueous isopropanol acetonitrile mixtures are generally preferred (e.g., water-acetonitrile – 77:23, 80:20; water-acetonitrile-isopropanol – 77:16.5:6). Peak tailing is overcome by using 0.1 % trifluoroacetic acid in water. The selectivity of

reversed-phase systems varies with their origin: columns from different manufacturers, elution with different solvents (isocratic or gradient mode) (54).

Detection: Most ecdysteroids show a strong UV absorbance ($\varepsilon \approx 12{,}000$ l mol^{-1} cm^{-1} at 242 nm). This allows the easy and sensitive detection of these compounds in the nanogram range during operation (TLC or HPLC). In addition, UV absorbance spectrum may represent an additional evidence for identity and purity of compound (54).

1.5.3. Structure Elucidation

The identification of the pure ecdysteroids is done by physical (physical state of compound, melting and/or boiling point; optical rotation and/or circular dichroism measurements) and basic spectroscopic methods (ultraviolet-visible spectra, infrared spectra). For all new compounds evaluation of their high-resolution spectral data (mass and NMR spectral data) are required. Compounds in appropriate amount may be subjected to crystallographic measurements. NMR spectroscopy and X-ray diffraction are developed into a highly complex arsenal of instrumentation and methods, which are capable of solving even sophisticated structural problems.

2. MATERIALS and METHODS

2.1. Plant Material

The aerial parts of *Silene italica* (L.) Pers. ssp. *nemoralis* (Waldst. and Kit.) Nyman were collected in May 1997 from the Botanical Garden of Vácrátót (Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót)[‡]. A voucher specimen was deposited at the Department of Pharmacognosy, University of Szeged, Hungary. The collection number was 227.

2.2. General Experimental Procedures

CC: The first and second large-scale column chromatography (NP1 and NP2) used Alumina Brockmann II neutral (Reanal, Budapest, Hungary) as the column material (1st NP column: 1200 g, 300 × 93 mm; 2nd NP column: 69 g, 300 × 20 mm). Elution was carried out using a stepwise gradient of methanol in dichloromethane (1st NP column) or various mixtures of ethanol (96 %) and

‡ The author thanks Prof. Imre Máthé and Vilmos Miklóssy-Vári for plant material.

dichloromethane (2nd NP column). The 3rd column chromatography (NP3) used Silica gel 60 (E. Merck, Darmstadt, Germany) as a column material (10 g, 80 × 18 mm), which was eluted with mixtures of dichloromethane-ethanol (96 %). The further column chromatography was performed on an octadecylsilanized (C_{18}) silica gel (0.06 – 0.2 mm particle size, Chemie Urticon-C-Gel, C-560, 187 g) packed in a 3.5 × 42 cm column (RP1-14), and the whole procedure was carried out at low-pressure drop (less than 1 atm) (reversed-phase low-pressure column chromatography-RPLPCC). Elution was carried out with a stepwise gradient of 30 %, 35 %, 40 %, 45 %, 50 %, 55 %, and 60 % aqueous methanol (1800mL or 300 mL each), and 300 mL (RP1, RP2, RP5-RP7, RP9-RP13) or 50 mL (RP3, RP4, RP8, RP14) of the fractions were collected. The elution used on the fourth RP column was started from 40 % aqueous methanol. The elution on 14th RP column was carried out with a 30-38 % stepwise gradient of methanol-water mixture (300 mL each), and the methanol content of the eluent was increased with 2 % at each step.

TLC: Normal-phase thin-layer chromatography was done on TLC plates 20 × 20 cm Silica gel 60 F_{254} (Merck, Darmstadt, Germany, Cat. No. 1.05554). Mixtures of toluene-acetone-ethanol (96 %)-ammonia (25 %) (100:140:32:9, v/v/v/v) (solvent system I), dichloromethane-methanol-benzene (25:5:3, v/v/v) (s. s. II), ethyl acetate-ethanol (96 %)-water (16:2:1, v/v/v) (s. s. III), dichloromethane-ethanol (96 %) (8:2, v/v) (s. s. IV), ethyl acetate-methanol-ammonia (25 %) (85:10:5, v/v/v) (s. s. V) were used as the mobile phases. Reversed-phase TLC employed TLC plates 20 × 20 cm RP-18 F_{254S} (Merck, Cat. No. 1.05559) with acetonitrile-water also containing trifluoroacetic acid (0.1 %) (25:75 and 35:65, v/v) (s. s. VI and VII), ethyl acetate-formic acid-water (85:10:5, v/v/v) (s. s. VIII), methanol-water (6:4, v/v) (s. s. IX), and tetrahydrofuran-water (45:55, v/v) (s. s. X) as mobile phases. The spots were visualized both by fluorescent quenching at 254 nm and in the case of NP-TLC after spraying with vanillin-sulfuric acid and observing in daylight or at 366 nm. For the preparative forms of the NP-TLC, TLC plates 20 × 20 Silica gel 60 F_{254} (Merck, Cat. No. 1.05715) was used. For the preparative RP-TLC, TLC plates 20 × 20 cm RP-18 F_{254S} (Merck, Cat. No. 115389) was employed. The solvent systems were usually s. s. I and s. s. III for the prep. NP-TLC. UV detection was used to locate the bands as described above. The bands of compounds were scarped after development, and the ecdysteroids were eluted from the stationary phase using methanol.

HPLC: HPLC experiments were performed using Shimadzu LC-10AS Pump, SPD-10A Shimadzu UV-VIS Detector, (a) analytical; NP-HPLC: Zorbax®-SIL column (5 μm, 250 × 4.6 mm i.d.) (DuPont, Paris, France) eluted using either dichloromethane-isopropanol-water (125: 40: 3 and 125:30:2, v/v/v/v) (s. s. XI and XII) or cyclohexane-isopropanol-water (100:40:3, v/v/v) (s. s. XIII) with flow rate of either 0.7 mL/min or 1 mL/min., RP-HPLC: Nucleosil C18 (5 μm, 250 × 4.6 mm

i.d.) (AB&L Jasco, Budapest, Hungary) eluted with water containing trifluoroacetic acid (0.1 %)-acetonitrile (77: 23, v/v) (s. s. XIV) with a flow rate of 1 mL/min; Supelcosil LC-18-DB (3 μm, 150 × 4.6 mm i.d.) (Supelco, Bellefonte, PA) eluted with water containing trifluoroacetic acid (0.1 %)-acetonitrile (80:20 and 83.5:16:5, v/v) (s. s. XV and XVI) with a flow rate of 1 mL/min (b) semipreparative; RP-HPLC: ODS-283 (6 μm, 250 × 9.4 mm i.d) (Chemie Urticon-C-Gel, C-560) eluted with water containing trifluoroacetic acid (0.1 %)-acetonitrile (77:23, v/v) (s. s. XIV) with a flow rate of either 0.7 or 1 mL/min. Chromatographic separations were monitored at 242 nm.

DCCC: An Eyala DCC-A Instrument (Rikakikai, Tokyo, Japan) was used with chlorophorm-methanol-water (65:20:20, v/v/v) (s. s. XVII) 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 **5** (2.2 mg) was subjected to enzymatic hydrolysis with 18 mg of β-glucosidase (Type II from Almonds, EC 3.2.121, Sigma, St. Louis, MO, USA). 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, ca. 56 % of the original ecdysteroid glycoside 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. NP-TLC was used for the identification of sugar. The mobile phase was chloroform-methanol-water (64:50:10, v/v/v), and visualization was carried out with thymole sulfuric acid.

Melting points were measured with a Boetius melting point apparatus (Dresden, Germany).

Spectroscopically measurements: Optical rotation was measured with Perkin-Elmer 341 polarimeter. Circular dichroism was determined using J-720 type Spectropolarimeter of Jasco (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The UV spectra were recorded in methanol using a Shimadzu UV 2101 PC spectrophotometer. FT-IR spectra (KBr) were recorded using a Perkin-Elmer Paragon 1000 PC FT-IR spectrophotometer. ^{1}H, ^{13}C, and 2D (COSY, NOESY, HMBC and HMQC) NMR spectra were taken in methanol-d_4, dimethylsulfoxide-d_6, chloroform-d_1 or pyridine-d_5 using a Bruker Avance DRX-500 spectrometer. In the 1D measurements (^{1}H, ^{13}C, DEPT-135) 64 K data points were used for the FID. The pulse programs of the 2D experiments (gs-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 100 ms), gs-HMBC, NOESY (mixing time = 500 ms), ROESY (compound **11** was recorded in dimethylsulfoxide-d_6, in mixing time = 350 ms) were taken from the Bruker software library. The pulse programs of the ^{1}H, ^{13}C, COSY, HMQC, HMBC, HMQC-TOCSY and NOESY

measurements were taken from the Bruker software library. Chemical shifts are given on the δ-scale and were referenced to the solvents (δ_C = 49.15 and δ_H = 3.31 in MeOH-d_4, δ_C = 77.05 and δ_H = 7.27 in $CHCl_3$-d_1, δ_C = 39.51 and δ_H = 2.51 in DMSO-d_6). In the 1D measurements (^1H, ^{13}C, DEPT-135) 64 K data points were used for the FID. HREIMS were recorded on a Finnigan MAT 95SQ (Finnigan MAT, Bremen, Germany) hybrid tandem mass spectrometer and ESIMS-MS using a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan Ltd., San Jose, CA, USA). X-ray analysis was employed using a CAD-4 diffractometer with graphite monochromated Cu $K\alpha$ or Mo $K\alpha$ radiation. The crystallographic phase problems were solved by direct methods, using the program SHELXS97.

2.3. Reagents and Standard Ecdysteroid Samples

Solvents with analytical-grade were obtained from Reanal (Budapest, Hungary), and solvents with HPLC grade were obtained from Merck (Darmstadt, Germany). Reference ecdysteroids (20-hydroxyecdysone, ecdysone, polypodine B, 2-deoxy-20-hydroxyecdysone, 2-deoxyecdysone, 22-deoxy-20-hydroxyecdysone, 26-hydroxypolypodine B, makisterone C, 24(28)-dehydromakisterone A, integristerone A, 2-deoxyintegristerone A, 22-deoxyintegristerone A) used as standards were available from earlier isolation work and fully characterised in previous studies (35, 58). Their identities and purities were confirmed by NMR and chromatographically by NP- and RP-HPLC.

2.4. Extraction and Isolation

2.4.1. Prepurification

The dried herb (4.5 kg) of *Silene italica* ssp. *nemoralis* was milled and percolated with methanol (47 L) at room temperature. The methanolic extract was evaporated into dryness (626.2 g), dissolved in 1650 mL of methanol, and acetone (1000 mL) was added to the solution. The resulting precipitate was removed by decantation, then rinsed three times, each with 200 mL of methanol-acetone (1:1, v/v). The supernatant and the methanol-acetone solution were combined and evaporated into dryness. The residue (447.6 g) was redissolved in methanol (960 mL), and acetone (1000 mL) was added to the solution. The precipitate was washed twice with 150 mL of methanol-acetone (1:1). The supernatant and the methanol-acetone solutions were combined and taken into dryness. The residue (358.7 g) was dissolved in 50 % aqueous methanol (700 mL) and extracted with benzene three times (1 × 2000 mL, 2 × 1000 mL). The aqueous methanol phase was evaporated to dryness, and the residue (232.6 g) was

dissolved in methanol (400 mL) and adsorbed onto alumina (700 g) using a rotatory evaporator. This was added to the top of a previously packed column of alumina (500 g, NP1) suspended in dichloromethane. After the column was extensively washed and conditioned with dichloromethane (4000 mL), the ecdysteroids were eluted with dichloromethane-methanol (9:1, 8:2, 1:1) (9600, 8000 and 4800 mL, respectively) and 800 mL fractions were collected. The progress of elution was monitored by the use of normal-phase TLC using the solvent systems I, II, III. Preliminary TLC analysis suggested that the fractions 1-7 (eluted with dichloromethane and dichloromethane-methanol 9:1), fractions 8-13 (eluted with dichloromethane-methanol 8:2) and fractions 14-20 (eluted with dichloromethane-methanol 1:1) contained several ecdysteroids, on which further fractionation was carried out.

2.4.2. Further Purification of Ecdysteroid Containing Fractions

Fractions 1-7 were combined and evaporated into dryness. The dried residue (2.97 g) was dissolved in 6 mL of methanol. The methanol solution was mixed with 9 g of aluminium oxide and taken to dryness by rotatory evaporation. The sample absorbed on aluminium oxide was packed on the top of a column containing 60 g of aluminium oxide (NP2). The preparation of the column was done as detailed above, and a gradient elution was performed using dichloromethane and dichloromethane-ethanol (96 %) 98:2, 95:5, and 9:1 (175, 375, 550, and 550 mL, respectively), and 25 mL fractions were collected.

Fractions 51-53 from the 2nd alumina column were combined. The dry 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 (NP3). Stepwise gradient elution was carried out using 98:2, 95:5, 92:8, and 9:1 mixtures of dichloromethane-ethanol (96 %) (100, 700, 400, and 200 mL, respectively) and 5 mL fractions were collected. Fractions 20-58 was subjected to repeated crystallization in ethyl acetate-methanol (2:1) to yield compound **2** (15 mg). Fractions 167-220 were combined and taken into dryness. The residue (0.17 g) was separated using preparative NP-TLC (pNP-TLC1) with s. s. I. The TLC separation resulted in two major bands with ecdysteroid content. The apolar of them (R_F = 0.35 – 0.38, 0.023 g) was further purified by NP-HPLC (s. s. XI) to give compound **1** (8 mg) and compound **12** (10 mg). Compound **10** was also detected in small amount.

Fractions 54-66 from the 2nd alumina column were combined and taken into dryness. The residue (0.017 g) was further purified by preparative NP-TLC (pNP-TLC2) (s. s. I).

Bands of R_F = 0.30 and 0.36 were separately scarped and eluted with methanol (the dried residues were 0.0065 and 0.003 g, respectively). Compound **7** was found in both bands determined by HPLC. Both eluted samples were further purified by NP- and RP-HPLC (s. s. XI and XIV), which yielded three ecdysteroid components in both cases, among which two were identified and termed compounds **6** and **7**.

Fractions 8-13 from the first alumina column (24.79 g dry residue) were used to isolate compound **14** (20-hydroxyecdysone, 15.3 g) by repeated crystallization in ethyl acetate-methanol (2:1). One part of mother liquid (2.1 g dry residue) was fractionated by droplet counter-current column chromatography (DCCC) using s. s. XVII in descending mode. From fractions 57-82 (55 mg), compound **18** (polypodine B) was crystallized in ethyl acetate-methanol (3:1). DCCC fractions 40-56 and fractions 15-50 from the 2nd alumina column were combined and taken into dryness. The residue (1.3 g) was subjected to reversed-phase low-pressure column chromatography (RPLPCC) (RP1). Elution was carried out with stepwise gradient of 30 %, 35 %, 40 %, 45 %, 50 %, 55 %, and 60 % aqueous methanol (1800mL each), and 300 mL of the fractions were collected. Fractions 30-37 (78 mg dry residue) were crystallized in ethyl acetate-methanol (2:1, v/v) to give compound **3** (50 mg). The mother liquid (28 mg dry residue) was further purified by NP-HPLC (s.s. XI) with a flow rate of 0.7 mL/min to yield three HPLC fractions: the first peak (**9**; 5 mg), the second peak (**17**, 7 mg) and the third peak (**3**; 15 mg). Fractions 38-42 from 1st RP column were combined and evaporated, and the residue (27 mg) was separated using NP-HPLC with s. s. XI to yield **9** (5.7 mg), and peak 2 with two ecdysteroid content. After peak 2 was eluted, compound **3** was crystallized from the solvent system. The final purification of mother liquid of peak 2 (7 mg) by NP-HPLC (s. s. XI) gave compound **3** (2 mg) and compound **19** (3 mg). All isolated compound **9** (10.7 mg) from 1st RP column was combined and final purified with RP-HPLC (s. s. XIV) to prepare pure compound (8 mg).

DCCC fractions 188-226 were combined, evaporated to dryness, and the residue (1.8 g) was dissolved in 5 mL 30 % aqueous methanol and further purified using RPLPCC (RP2). The separation method was carried out on RP column with aqueous methanol mixtures, as described at RP1. Fractions 9-15, fractions 25-31 and fractions 32-33 were further purified. The dry material (0.69 g) of combined fractions 9-15 was dissolved in 3 mL of 30 % aqueous methanol and purified again on RP column (RP3). Elution was carried out using the same procedure, but 50 mL of the fractions were collected. Fractions 8-14 were collected, and after evaporation the residue (0.27 g) was crystallized from methanol to get pure compound **16** (0.18 g). Fractions 25-31 from the 2nd RP column were evaporated, and the residue (76 mg) was dissolved in 5 mL of 40 % aqueous methanol and separated on RP column (RP4). The elution stepwise was started from

40 % aqueous methanol, and 50 mL of the fractions were collected. Fractions 17-20 were combined (19 mg dry residue) and crystallized from methanol, yielding 11 mg of pure compound **5**. The collected fractions 32-33 from the 2nd RP column were evaporated, and the residue (26 mg) was further purified by preparative NP-TLC (pNP-TLC3) (s. s. III). The scarped band at $100 \times R_F$ =36 yielded compound **8** (16 mg).

The crystallized part of fractions 8-13 from the first alumina column was subjected to crystallization again from ethyl acetate-methanol (3:1, v/v) to give pure 20E (1.3 g). The mother liquid (1.9 g dry residue) was applied to RPLPCC (RP5) and eluted with a 30-60 % stepwise gradient of aqueous methanol (1800 mL each). Fractions 6-10 were combined and evaporated (0.068 g dry residue) and was subjected to preparative TLC (pNP-TLC4) (s. s. III). The TLC separation resulted in two major bands, one of them was identified with 20E (0.05 g). The other band (6 mg) was further purified by normal-phase (NP) HPLC with s. s. XI to prepare pure compound **11** (2 mg).

The remaining part of mother liquid of fractions 8-13 (5.5 g) from the first alumina column was subjected to RPLPCC in two procedures (RP6-7). The fractions were collected and used to crystallize a great amount of compounds **3, 12** and **18** (**3**, 0.45 g; **12**, 0.22 g; **18**, 0.35 g).

Fractions 12-16 from the 1st RP column and fractions 11-15 from the 5th RP column were combined (0.1 g dry residue) and subjected to RPLPCC (RP8). Fractions 12-17 was subjected to crystallization to obtain compound **6** from methanol (41.4 mg). Fractions 17-19 from the 1st RP column and fractions 16-18 from the 5th RP column were separated using preparative TLC (pNP-TLC5) with s. s. III. From the dry residue, (0.13 g) three major bands were identified with ecdysteroid content. One of them was final purified by NP-HPLC (s. s. XI) to yield pure compound **13** (10 mg), which was crystallized from aqueous methanol. The other two bands was identified as compounds **6** and **10** (5-5 mg).

Fractions 19-20 from the 5th RP column were combined (1.01 g dry residue) and after crystallization in ethyl acetate-methanol (3:1, v/v) (crystallized 20E with small amount of compound **7**) the mother liquid (0.19 g dry residue) was further purified by preparative NP-TLC (pNP-TLC6) (s. s. III). Three major bands were separated. The broad band was 20E. The other bands (1st: 14 mg and 2nd: 63 mg) were further purified by NP-HPLC using s. s. XI to yield pure compound **10** (4.5 mg) and crude compound **12**. Compound **10** was crystallized from aqueous methanol. The crude compound **12** was separated by NP-HPLC (s. s. XI) to yield compounds **1** and **12** (**1**, 5 mg; **12**, 20mg). Fractions 26-36 (dry residue 49 mg) from the 5th RP column were subjected to NP-HPLC (s. s. XI) to get pure compounds **3** and **4**. From fractions 37-42 compound **4** was crystallized (40 mg).

Fractions 14-20 from the first alumina column (dry residue 12.6 g) were fractionated by RPLPCC in five parts (RP9-13) with a 30-60 % stepwise gradient of aqueous methanol. The combined fractions 11-14 (dry residue 0.82 g) purified again on the same RP column (RP14), but elution was carried out with a 30-38 % stepwise gradient of methanol-water mixture (300 mL each). The methanol content of the eluent was increased with 2 % at each step. The dry residue (0.19 g) of fractions 13-16 was subjected to preparative NP-TLC (pNP-TLC7) (s. s. III). The band at $R_F = 0.18$ was further purified by preparative RP-TLC (s. s. VI) (pRP-TLC) to get pure compound **15** (8 mg).

3. RESULTS

3.1. Isolation of Ecdysteroids from *Silene italica* ssp. *nemoralis*

Figure 2 shows the HPLC chromatogramm of the crude extract.

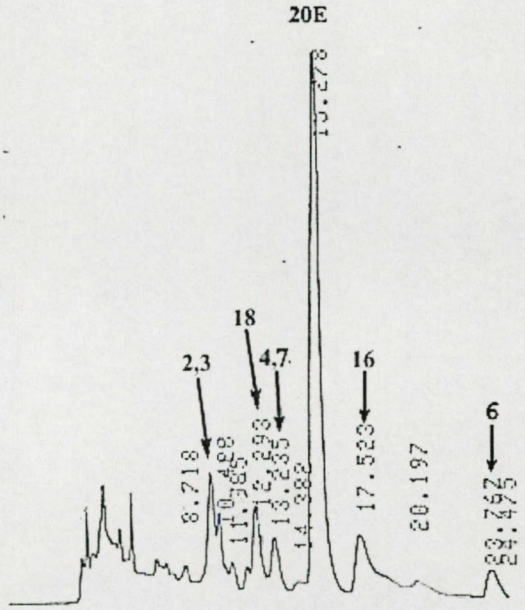


Figure 2 HPLC chromatogramm of the fresh plant material extract. Stationary phase: Zorbax®-SIL column with solvent system XI (flow rate 1 mL/min). The peaks represent compounds **2** (2-deoxyecdysone), **3** (2-deoxy-20-hydroxyecdysone), **18** (polypodine B), **4** (22-deoxy-20-hydroxyecdysone), **7** (5-beta-2-deoxyintegristerone A), 20E, **16** (integristerone A) and **6** (5-alpha-2-deoxyintegristerone A).

The isolation used a particular combination of chromatographic and related separation methods is given in **Figures 3/a, 3/b**. The crude extract was subjected to prepurification. Two consecutive precipitation steps removed the overwhelming majority of polar ballast compounds with acetone. The majority of the apolar contaminating components were removed by solvent-solvent distribution using benzene. The prepurified extract was subjected to column chromatography on alumina, which was a production-scale separation, because of 200 g of the sample. 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 several step-gradient elutions were employed.

To separate apolar ecdysteroids (fractions 1-7 from the 1st NP column), column chromatography once more on alumina and thereafter on silica were employed. In both cases, stepwise gradient elution was used but with various mixtures of dichloromethane-ethanol (96 %) as mobile phases. In the first case, three gradient steps were applied, which were slightly changed such as straight dichloromethane, dichloromethane-ethanol (96 %) 98:2, 95:5, and 9:1, respectively. The sample to stationary phase ratio was about 1:20. On the silica column, the elution system was the same, the stepwise gradient was widen with only one step (92:8) and the sample to sorbent ratio was 1:30.

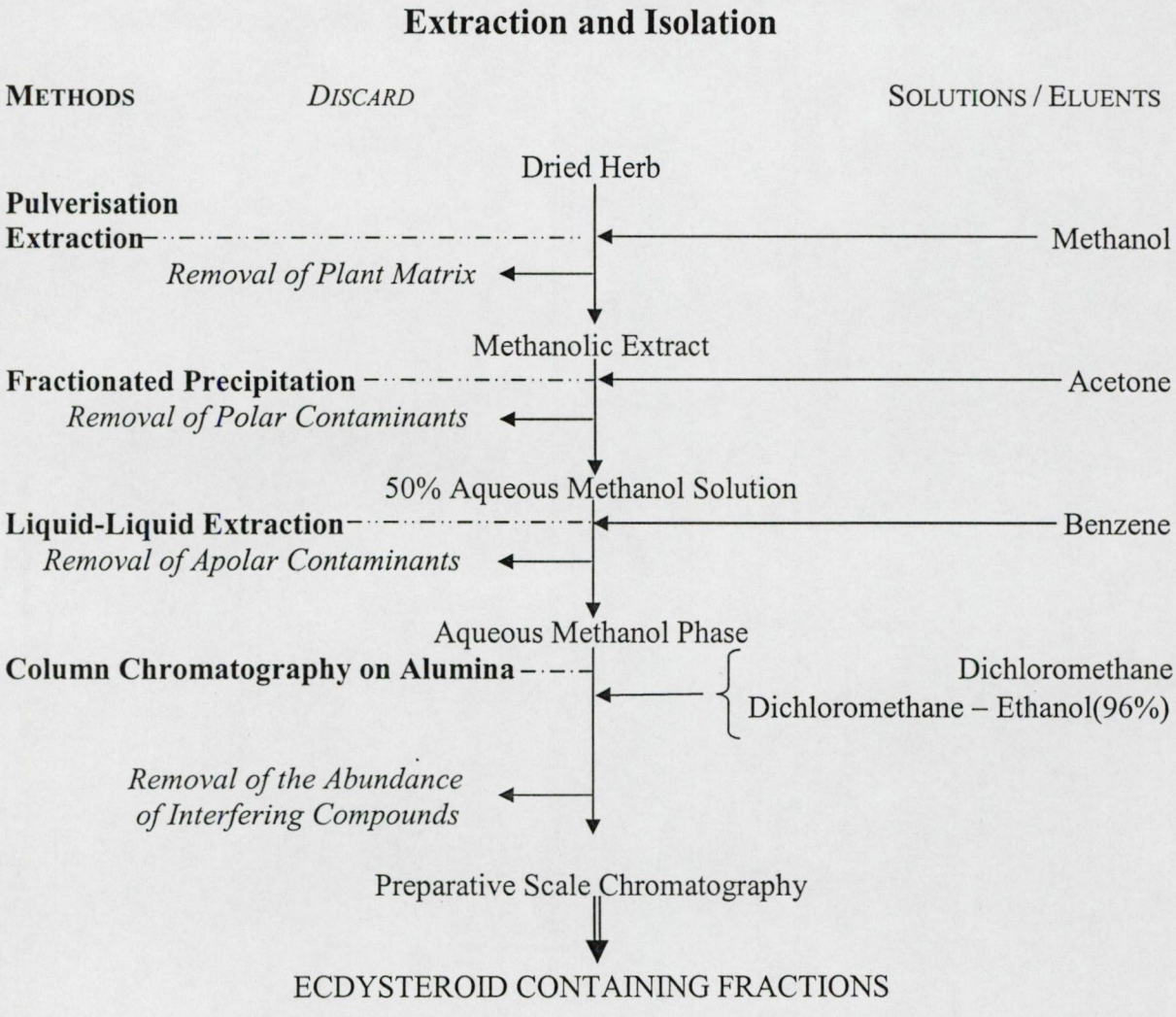


Figure 3/a General procedure for the isolation of ecdysteroids from *Silene italica* ssp. *nemoralis*: (a) Extraction and prepurification of the crude extract.

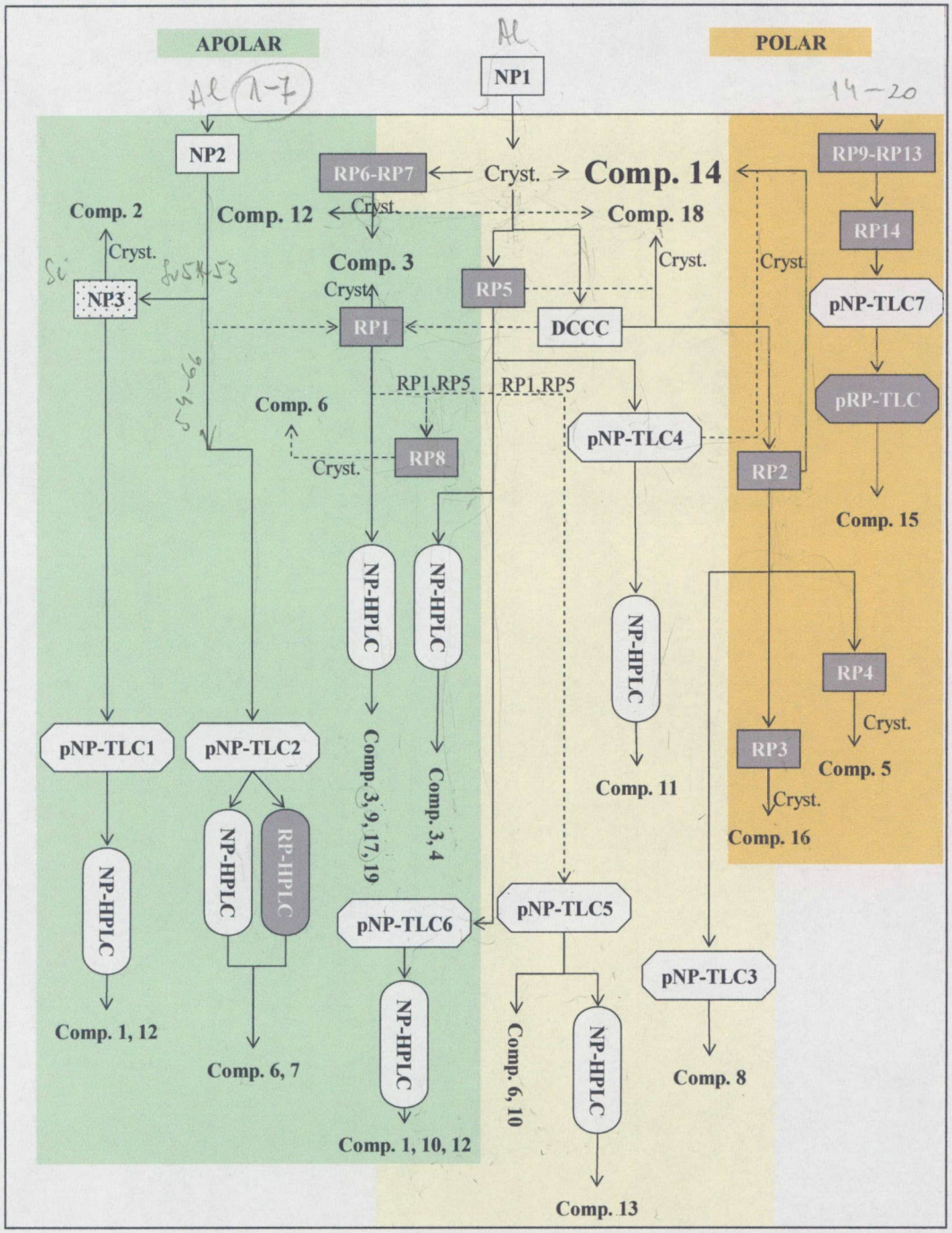


Figure 3/b General procedure for the isolation of ecdysteroids from *Silene italica* ssp. *nemoralis*: (b) Separation of ecdysteroids using preparative scale chromatography. The initial step was adsorption, followed by efficient and selective reversed-phase column chromatography using low-pressure operation; (c) Purification was sometimes completed by the use of HPLC and preparative TLC.

Apolar and polar characteristics of compounds were given in comparison with 20E. Abbreviations: NP 1-3 = NP columns; RP 1-8 = RP columns; Cryst. = crystallization; Comp. = compound(s).

To separate polar ecdysteroids (from fractions 8-13 and 14-20), DCCC and reversed-phase low-pressure column chromatography (RPLPCC on C_{18}) were used, which gave fractions enriched in various ecdysteroid compounds. The highest loading at RPLPCC was 1:100 (sample to sorbent), and stepwise gradient elution was carried out using aqueous methanol (30 %-60 %). Fractions 8-13 from the first alumina column was subjected to RPLPCC, resulted some fractions with an excess of 20-hydroxyecdysone (compound **14**, 24.79 g). These fractions were used to isolate pure 20-hydroxyecdysone by repeated crystallization (15.3 g). One part of the crystallization mother liquid was subjected to DCCC fractionation. Selected DCCC fractions and the other part of mother liquid were further purified using repeated low-pressure RP column (RP1-RP8) chromatography to obtain compounds **1-19**.

Figure 4 shows the simplified scheme of RP analysis obtained after NP column chromatography. Cells of the table show those individual compounds, which were detected in sufficient amount with monitoring the separation by TLC (**Figure 5**). The fractions enriched in certain compounds are marked by encircled area in **Figure 4**.

			1st NPCC on alumina							
	Fr.No		1-7		8-13					14-20
	CH_2Cl_2									
	CH_2Cl_2-MeOH		9:1	9:1						
					8:2	8:2	8:2	8:2	8:2	
										1:1
	Fr. No	aqu.MeOH								
	1-6	30%						10		
	7	35%						10		
R	8	35%						10		
P	9	35%	6	6				10	16	
L	10	35%	6	6				10	16	
P	11	35%	6	6				10	16	15
C	12	35%	6	6				18	16	15
C	13	40%	6	6				18	16	15
	14	40%	6	6			14	18	16	15
	15	40%	6	6			14	18	16	
	16	40%	6	6			14	13		
	17	40%	6	6			14	13		
	18	40%					14	13		
	19	45%					14	11		
	20	45%				7	14	11		
	21	45%	12	12	12	7	14	18		
	22	45%	12	12	12	7	14	18	5	
	23	45%	12	12	12	7	14	18	5	
	24	45%	12	12	12	7	14	18	5	
	25	50%	1	1	12	7	14	18	5	
	26	50%	1	1	12		14	18	5	
	27	50%			12		14	8	5	
	28	50%					14	8	5	
	29	50%					14	8	5	
	30	50%					14	8	5	
	31	55%						8	5	
	32	55%						8		
	33	55%		3				8		
	34	55%	2	3	17	4				
	35	55%	2	3	17	4				
	36	55%	2	3	17	4	9	19		
	37-42	60%	2	3	17	4	9	19		

Figure 4 Reversed-phase separation (RPLPCC on C_{18}) of fractions containing ecdysteroids obtained from the 1st NPCC on alumina. For abbreviations see section "Abbreviations".

The consecutive use of NP and RP column chromatography gave adequate separation also for minor ecdysteroids. **Figure 5** shows the TLC analysis of fractions obtained by RPLPCC.

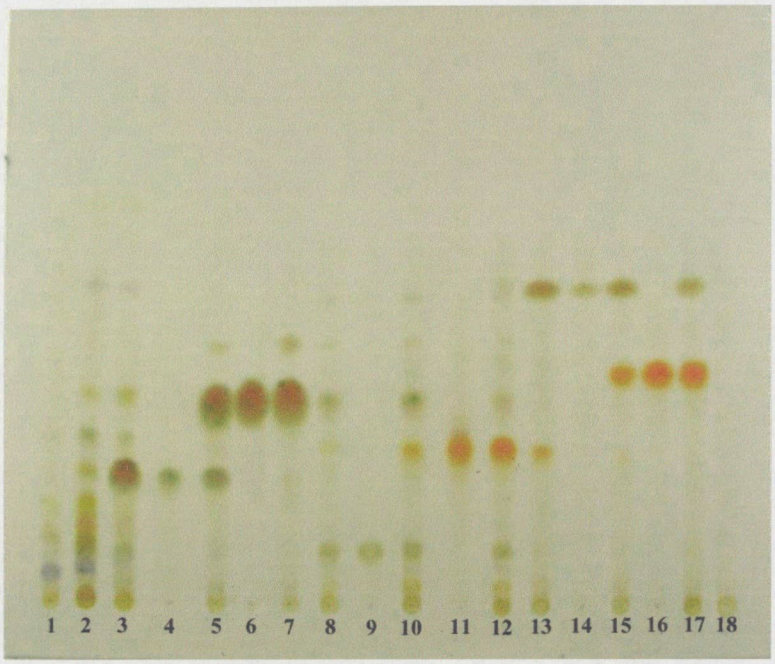


Figure 5 TLC analysis of fractions obtained by RPLPCC (fractions of RP2). The stationary phase was TLC silica F_{254} 20 × 20 cm, and the mobile phase was s.s. I. The spots were visualized after spraying with vanillin-sulphuric acid (UV detection at 366 nm).

The samples were loaded as: (1) fractions 1-6, (2) fr. 7-9, (3) fr. 10-13 (containing comp. **16**), (4) *IntegristeroneA*, (5) fr. 14-15 cont. **14**), (6) *20E*, (7) fr. 16-23 (cont. **14**) (8) fr. 24-26 (cont. **5**), (9) *2-deoxy-20E 22glucoside*, (10) fr. 27-28 (cont. **5**, **14**), (11) *22-deoxyintegristerone A*, (12) fr. 29-31 (cont. **8**, **14**) (13) fr. 32-33 (cont. **8**, **3**), (14) *2-deoxy20E*, (15) fr. 34-35 (cont. **3**, **4**), (16) *22-deoxy20E*, (17) fr. 36-38 (cont. **3**, **4**), (18) fr. 39-42.

In addition to 20-hydroxyecdysone, compounds **2**, **3**, **4**, **5**, **6**, **10**, **12**, **13**, **16**, **18** were crystallized from the adequate RPLPCC fraction to yield pure compounds. Other compounds were usually further purified first on normal-phase preparative TLC (pNP-TLC) giving ecdysteroid containing bands. After the separated bands have been scarped and eluted from the sorbent, they were analysed by TLC using the same developing systems as in the case of preparative TLC (s. s. III in most cases). Hence, further purification was done using both reversed-phase HPLC and normal-phase HPLC, whereas ecdysteroids were separated more selectively by NP-HPLC. RP-HPLC has been used for monitoring the purity of compounds (see **Figure 6**). **Figure 6** gives an example, where the normal-phase HPLC of a band of TLC fractions resulted in pure compounds **1, 10** and **12.** The entire purification was monitored by TLC and consecutive use of NP- and RP-HPLC.

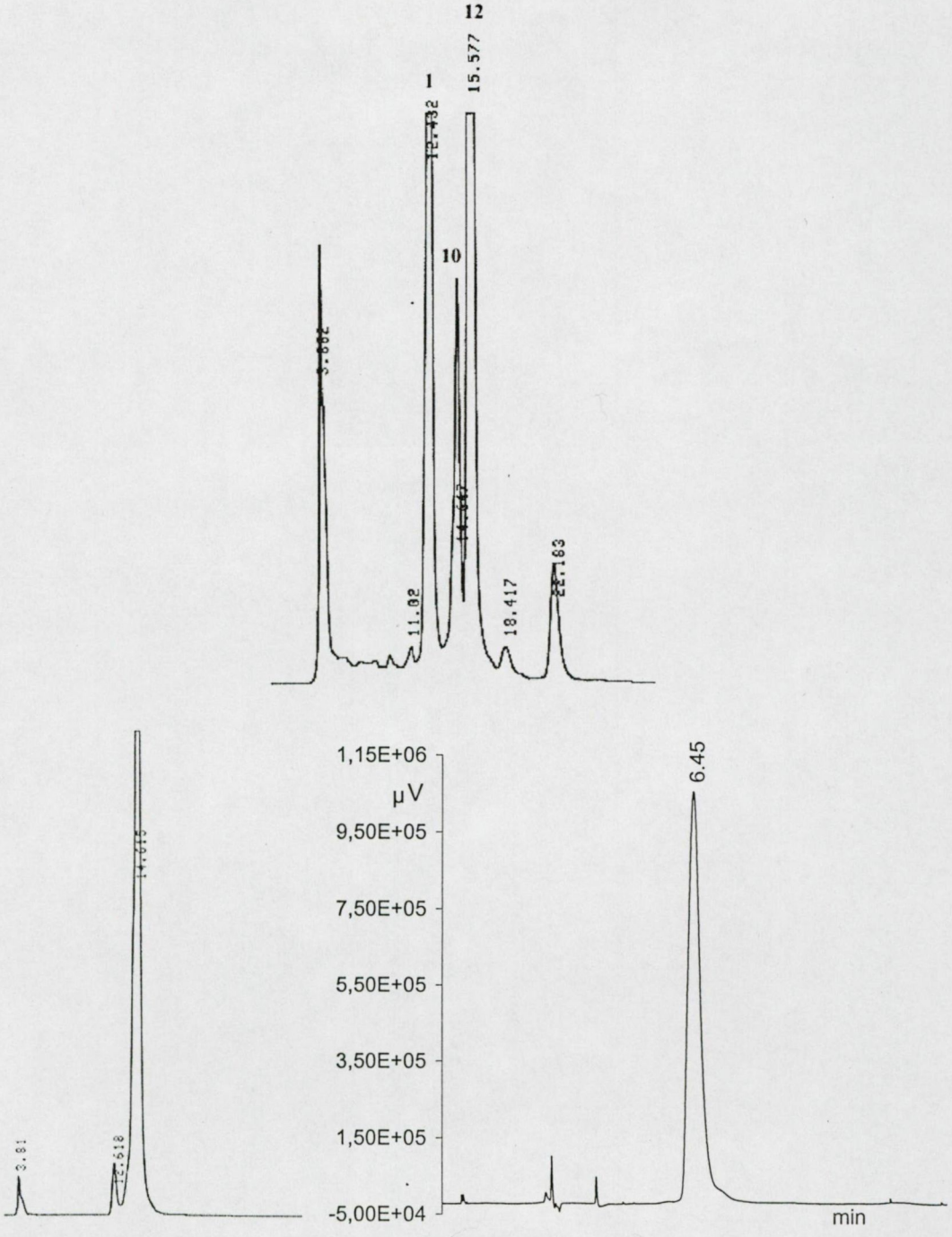


Figure 6 Analytical separation of fractions containing 24(28)-dehydromakisterone A (**1**), 9α,20-dihydroxyecdysone (**10**) and ecdysone (**12**) (at the top), and the NP- and RP-HPLC curve of the pure 9α,20-dihydroxyecdysone (**10**) after preparative NP-HPLC purification (at the bottom). NP chromatography was carried out on Zorbax®-SIL (5µm)(left), and the reversed-phase chromatography on Nucleosil C18 (5 µm) (right), respectively. Column dimensions: 250 × 4.6 mm i.d.. The mobile phases were dichloromethane-isopropanol-water (125:40:3, v/v/v) (0.7 mL/min flow rate) in the case of NP-HPLC and acetonitrile-water containing trifluoroacetic acid (0.1%) (77:23, v/v) (1 mL/min flow rate) for reversed-phase chromatography.

3.2. Compound Characterization

The known compounds were characterised by direct comparison of their physical and spectroscopic characteristics published in the literature (2, 5). They were also identified by co-chromatography with pure reference ecdysteroids using NP- and/or RP-TLC and also NP- and/or RP-HPLC. In addition to chromatography, all compounds were characterised by different spectroscopic methods. UV, IR, CD, NMR and MS have been utilized to identify ecdysteroids. Wherein, MS and NMR provided the basic information on the structure of compounds. In the course of structural elucidation of compounds, MS and NMR spectral data were usually evaluated in comparison with the main ecdysteroid, 20-hydroxyecdysone. The final structure elucidation of crystallized compounds can be X-ray diffraction.

3.2.1. Chromatographic Identification

In the case of TLC, ten mobile phases with different selectivity were used. The stationary phase was either silica gel (NP-TLC) with a fluorescent indicator or octadecyl silica with a fluorescent indicator. During NP-TLC analysis triple detections were employed: (a) under UV light at 254 nm to locate dark spots; (b) after spraying with vanillin/sulfuric acid reagent, and detection under UV light at 366 nm according to usually blue fluorescent spots, and (c) under day-light (usually olive green spots within a short time). If a 22-hydroxyl group (compounds **4** and **8**) was absent, an orange colour could be seen under UV light and also by day-light. We observed canary-yellow or orange colour at 9-hydroxyecdysteroids (**10, 11**) under day-light. In the case of HPLC, mainly two NP-HPLC systems (s. s. XI, and XIII) and one or two RP-HPLC systems (s. s. XIV) have been used for purification and for purity control. **Table 2** and **Table 3** show their TLC characteristics, and **Table 4** gives that of the HPLC characteristics of compounds in various mobile phases.

Table 2 Colour of phytoecdysteroids after spraying with vanillin-sulfuric acid.

	Colour after vanillin-sulfuric acid			Colour after vanillin-sulfuric acid	
Comp.	Under UV (366 nm)	Under daylight	Comp.	Under UV (366 nm)	Under daylight
1	violet	yellow	**11**	violet	orange
2	violet	yellow	**12**	violet	yellow
3	violet	green	**13**	violet	green
4	orange	orange	**14**	violet	green
5	violet	green	**15**	violet	pink
6	violet	green	**16**	violet	green
7	violet	green	**17**	violet	green-brown
8	orange	orange	**18**	violet	green
9	violet	green	**19**	violet	green
10	violet	canary-yellow			

Table 3 TLC retention factors for isolated phytoecdysteroids (PE)

Comp.	Retention factor × 100									
	NP-TLC					RP-TLC				
	s.s.I	s.s.II	s.s.III	s.s.IV	s.s.V	s.s.VI	s.s.VII	s.s.VIII	s.s.IX	s.s.X
1	40	43	55	39	n.d.	n.d.	37	57	38	53
2	55	51	58	54	49	19	18	71	21	42
3	49	46	53	43	42	28	29	59	31	52
4	32	34	42	26	23	n.d.	30	46	34	54
5	7	14	17	10	7	n.d.	42	16	46	71
6	30	21	32	16	24	n.d.	53	35	58	73
7	36	38	39	32	21	n.d.	44	40	44	58
8	18	25	36	22	16	n.d.	37	30	40	61
9	46	56	58	60	n.d.	n.d.	28	61	28	47
10	37	38	47	39	n.d.	n.d.	46	45	46	65
11	29	38	37.5	n.d.	n.d.	n.d.	n.d.	n.d.	34	n.d.
12	35	38	41	33	27	34	34	47	36	58
13	32	38	38	28	23	n.d.	50	n.d.	49	65
14	29	28	37	24	21	47	47	35	47	66
15	13	12	18	7	n.d.	59	60	14	57	82
16	16	24	29	18	15	n.d.	53	20	53	71
17	41	46	55	50	n.d.	n.d.	28	60	30	41
18	25	35	38	32	20	47	46	35	48	65
19	44	60	59	55	n.d.	n.d.	17	67	18	34

Comp = compound, s. s.=solvent system, n.d.=no data observed; s.s. I: toluene-acetone-ethanol (96%)-ammonia (25%) (100 :140: 32: 9, v/v/v/v), s.s. II: dichloromethane-methanol-benzene (25: 5: 3, v/v/v), s.s. III: ethyl acetate-ethanol (96%)-water (16: 2: 1, v/v/v), s.s. IV: dichloromethane-ethanol (96%) (8:2, v/v), s.s. V: ethyl acetate-methanol-ammonia (25%) (85:10:5, v/v/v), s.s. VI: acetonitrile-water with trifluoroacetic acid (0.1%) (25:75, v/v), s.s. VII: acetonitrile-water with trifluoroacetic acid (0.1%) (35:65, v/v), s.s. VIII: ethyl acetate-formic acid-water (85:10:5, v/v/v), s.s. IX: methanol-water (6:4, v/v), s.s. X: tetrahydrofuran-water (45:55, v/v).

Table 4 HPLC retention times for isolated phytoecdysteroids.

Comp.	Retention time (min)					
	NP-HPLC			RP-HPLC		
	s.s.XI	s.s.XII	s.s.XIII	s.s.XIV	s.s.XV	s.s.XVI
1	8.9	n.d.	19	[c]6.8	[b]6.7	[b]17.3
2	8.4	n.d.	10	[c]26	n.d.	n.d.
3	8.3	12.7	13.3	[c]9.96	[b]9.9	[b]26.2
4	13	19.7	18	[c]10.1	n.d.	n.d.
5	28	n.d.	36.7	n.d.	[b]5.2	[b]14.6
6	23	n.d.	28	[a]4.1	n.d.	n.d.
7	13	n.d.	19	[a]5.9	n.d.	n.d.
8	n.d.	n.d.	21.5	[a]9.54	n.d.	n.d.
9	7.1	8.7	9.7	[c]7.7	n.d.	n.d.
10	10.5	16	18.7	[a]6.45	n.d.	n.d.
11	11.5	n.d.	32	[a]6	n.d.	n.d.
12	12	n.d.	16.4	[a]10.1	n.d.	n.d.
13	12.3	n.d.	24	[c]3.7	n.d.	n.d.
14	15.6	23.8	22	[c]4.2	[b]3.6	[b]7.3
15	n.d.	n.d.	42.3	[c]5.33	n.d.	n.d.
16	18	n.d.	29	[c]3.5	[b]3.1	[b]6.1
17	7.8	10.5	13	[c]15.6	n.d.	n.d.
18	12	n.d.	22.4	[c]4.4	n.d.	n.d.
19	5.9	7.5	10.6	[c]23.3	n.d.	n.d.

NP-HPLC: Zorbax®-SIL column (5 μm, 250 × 4.6 mm i.d.), s.s. XI: dichloromethane-isopropanol-water (125: 40: 3, v/v/v/v), s.s. XII: dichloromethane-isopropanol-water (125: 30: 2, v/v/v/v), s.s. XIII: cyclohexane-isopropanol-water (100:40:3, v/v/v), with flow rate 1 mL/min. RP-HPLC: [a]Nucleosil C18 (5 μm, 250 × 4.6 mm i.d.), [b]Supelcosil LC-18-DB (3 μm, 150 × 4.6 mm i.d.), [c]ODS-283 (6 μm, 250 × 9.4 mm i.d), s.s. XIV: water with trifluoroacetic acid (0.1%)-acetonitrile (77: 23, v/v), s.s. XV: water with trifluoroacetic acid (0.1%)-acetonitrile (80:20, v/v), s.s. XVI: water with trifluoroacetic acid (0.1%)-acetonitrile (83.5:16:5, v/v) with flow rate 1 mL/min.

3.2.2. Physical properties, UV, IR and CD characteristics of compounds

IR and UV spectra rendered the characteristic information on the 7-en-6-one chromophore. Typically, the ecdysteroids were characterised by a strong UV absorption spectrum with a maximum at 242 nm (log ϵ = 4.08). The structural differences between compounds were minor, and distant from the chromophore, they were essentially indistinguishable from each other (59). Infrared spectroscopy gave C=O band at 1640 cm^{-1}, and C=C gives a signal at 1612 cm^{-1}. In the IR spectrum, a wide absorption band at 3300-3500 cm^{-1} verifies the presence of hydroxyl groups. The fingerprint regions showed some differences between compounds enabling them to be distinguished from each other (35, 38, 59).

Table 5 shows physical and spectroscopic data for the isolated compounds, such as melting point, results of optical rotation and circular dichroism measurements, IR and UV spectroscopic data.

Table 5 Physical, UV, IR and CD data of isolated ecdysteroids

C	M.P (°C)	$[\alpha]_D^{20}$ (*c*; MeOH)	IR (KBr) ν_{max} (cm^{-1})	UV (EtOH) λ_{max} nm (log ε)	CD (MeOH) molecular ellipticity [θ]
1	244-246	$[\alpha]_D^{20}$ + 54.4 ± 2° (*c* 0.37)	3300-3500 (OH), 1665 (cyclohexenone)	245 (4.15)	n.d.
2	231-232	$[\alpha]_D^{20}$ +92.5 ± 2° (*c* 1.5)	3450 (OH), 1650 (cyclohexenone)	244 (4.111)	$[\theta]_{229.4}$ + 11236, $[\theta]_{256.4}$ – 4161, $[\theta]_{330.6}$ + 5891
3	250-252	$[\alpha]_D^{22}$ +82 ± 2° (*c* 0.5)	3450 (OH), 1645 (cyclohexenone)	243 (4.083)	$[\theta]_{230.4}$ + 6827, $[\theta]_{257}$ – 4085, $[\theta]_{330.2}$ + 3796
4	241-242	$[\alpha]_D^{20}$ + 80.9 ± 2° (*c* 0.42)	3400-3430 (OH), 1710, 1647 (cyclohexenone)	244 (4.03)	$[\theta]_{223.4}$ + 11545, $[\theta]_{253.6}$ – 17150, $[\theta]_{330.2}$ + 6929
5	253-255	$[\alpha]_D^{26}$ + 92.7° (*c* 0.47)	3400 (OH), 1707, 1640 (cyclohexenone)	239 (4.095)	n.d.
6	n.d.	$[\alpha]_D^{26}$ + 4° (*c* 0.1)	3400 (OH), 1650 (cyclohexenone)	242 (4.13)	$[\theta]_{245.4}$ – 27567, $[\theta]_{332}$ + 12324
7	n.d.	$[\alpha]_D^{26}$ + 70° (*c* 0.05)	3400 (OH), 1650 (cyclohexenone)	240 (4.08)	$[\theta]_{243.8}$ + 20227, $[\theta]_{331.4}$ + 2380
8	n.d.	$[\alpha]_D^{26}$ + 80° (*c* 0.1)	3300-3430 (OH), 1645 (cyclohexenone)	242 (4.105)	n.d.

C	M.P (°C)	$[\alpha]_D^{20}$ (*c*; MeOH)	IR (KBr) ν_{max} (cm^{-1})	UV (EtOH) λ_{max} nm (log ε)	CD (MeOH) molecular ellipticity [θ]
9	n.d.	n.d.	3450 (OH), 1650 (cyclohexenone)	242 (4.01), 206 (3.69)	$[\theta]_{227.2}$ + 10358, $[\theta]_{257.4}$ – 4875, $[\theta]_{330.4}$ + 4424
10	269-271	$[\alpha]_D^{24}$ - 10 (*c* 0.05)	3380,3460 (OH), 1660 (cyclohexenone)	228 nm (3.87), 242 (4.1)	$[\theta]_{234.8}$ – 10374, $[\theta]_{339.4}$ + 1836
11	n.d.	$[\alpha]_D^{26}$ + 14 (*c* 0.1)	3400-3450 (OH), 1660 (cyclohexenone)	206 (1.8), 243 (3.89)	n.d.
12	237-239	$[\alpha]_D^{20}$ + 63.6 ° (*c* 0.83)	3333 (OH), 1657 (cyclohexenone)	242 (4.093)	n.d.
13	278	$[\alpha]_D^{20}$ + 55.5 ° (*c* 0.54)	3400 (OH), 1655 (cyclohexenone)	242 (n.d.)	$[\theta]_{248.8}$ – 27011, $[\theta]_{333.2}$ + 9457
14	241-242.5	$[\alpha]_D^{20}$ + 58.9 ± 2° (*c* 0.3)	3500 (OH), 1645 (cyclohexenone)	240 (4.103)	$[\theta]_{224}$ + 10047, $[\theta]_{254}$ – 12883, $[\theta]_{330}$ + 5697
15	n.d.	$[\alpha]_D^{26}$ + 29.2 ° (*c* 0.5)	n.d.	242 (4.11)	n.d.
16	246-248	$[\alpha]_D^{22}$ + 36.2 ± 2° (*c* 0.32)	3400 (OH), 1730, 1655 (cyclohexenone)	245 (4.15),	n.d.
17	258-259	n.d.	3400-3470 (OH) 1643-1650 (cyclohexenone)	243-244 (1.146)	n.d.
18	254-257	$[\alpha]_D^{26}$ + 94.2 ± 2° (*c* 0.05)	3400 (OH), 1673 (cyclohexenone)	243 (4.11), 317 (2.07)	$[\theta]_{227}$ + 22830, $[\theta]_{256.2}$ – 15930, $[\theta]_{329.2}$ + 9757
19	245-250	$[\alpha]_D^{20}$ + 65.0 ° (*c* 0.18; $CHCl_3$)	3600 (sharp, free OH), 3400 (broad, bonded OH), 1664	243 (4.04)	n.d.

C = comp.; n.d. = no data observed

3.2.3. Mass Spectrometry

For determination of molecular mass, chemical ionization mass spectra (CIMS) and electrospray-ionization mass spectra (ESIMS) were used. Electron-impact mass spectra (EIMS) gave molecular ions that are of low intensity (30). Mass spectra of ecdysteroids characterized by the appearance of numerous signals differing from each other by the loss of water (18 units) from the polyhydroxylated ecdysteroids. Moreover, the majority of ecdysteroids suffered side-chain cleavage; the splitting occurred between C-20 and C-22, and C-17 and C-20. Their mass spectra were characterized by mass numbers, which depend on the degrees of hydroxylation of the side-chain and nucleus. Fragmentation resulted in two major series derived from the nucleus or side-chain, as shown in **Table 6**. Further cleavage occurred between either C-22 and C-23, C-23 and C-24, or C-24 and C-25. In the D ring, either C-13–C-17 or C-14–C-15 fragmentation took place (35).

Table 6 The molecular mass and fragment ions of compounds **1-19**.

Comp.: M.W.	MS	MS fragments *m/z* (relative intensity %)
1: 492	EIMS	474 $[M\text{-}H_2O]^+$ (3), 456 (11), 438 (15), 363 (66), 345 (100), 327 (47), 309 (13), 300 (26), 173 (17), 113 $[C_{22}–C_{27}]^+$ (37), 111 (55), 97 (52), 95 (63), 93 (35), 83 (91), 71 (62), 69 (62).
2: 448	EIMS	448 $[M]^+$, 430, 412, 361, 332, 314, 284, 99, 81.
3: 464	EIMS	428 $[M\text{-}2\ H_2O]^+$ (0.5), 397 (2), 347 $[M\text{-}C_{22}–C_{27}]^+$ (9), 284 $[M\text{-}C_{20}–C_{27}\text{-}H_2O]^+$ (6), 99 (17), 81 (34), 43 (100).
4: 464	EIMS	464 $[M]^+$ (0.3), 446 (1.4), 431 (0.8), 428 $[M\text{-}2\ H_2O]^+$ (35), 418 (1), 413 (16), 410 (28), 400 (2.5), 395 (12), 385 (0.7), 343 (5), 327 (54), 325 (20), 320 (20), 309 (10), 302, 301 (19), 300 (35), 299 (27), 250 (30), 249 (15), 145 (20), 127 (97), 109 (100), 81 (20), 69 (54).
5: 626	ESIMS	633 $[M+Li]^+$ (35), 615 $[M\text{-}H_2O+Li]^+$ (58), 471 [M-glucose+Li]$^+$ (68), 453 [M-glucose-H_2O+Li]$^+$ (100), 435 [M-glucose-$2H_2O$+Li]$^+$ (25), 417 [M-glucose-$3H_2O$+Li]$^+$ (20), 329 [M-glucose-H_2O-$C_{22}–C_{27}$+Li]$^+$ (16), 301 (5), 279 (1), 247 (15), 223 (4), 97 (10), 87 (8), 65 (21).
6: 480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 380, 363, 347, 345.
	ESIMS	487 $[M+Li]^+$ (35), 469 $[M\text{-}H_2O+Li]^+$ (52), 451 $[M\text{-}2H_2O+Li]^+$ (100), 433 $[M\text{-}3H_2O+Li]^+$ (62), 415 $[M\text{-}4H_2O+Li]^+$ (<1), 377 (2), 301 (2), 279 (1), 247 (20), 223 (3), 97 (100), 87 (23), 65 (22).
7: 480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 380, 363, 347, 345.
8: 480	CIMS	498 $[M+H+NH_3]^+$, 481 $(M+H]^+$, 463, 445, 427, 409, 391, 380, 363, 347.
9: 480	ESIMS	983 $[2M+Na]^+$ (7), 963 (2), 855 (2), 611 (2), 503 $[M+Na]^+$ (22), 481 $[M+H]^+$ (100), 463 $[M\text{-}H_2O+H]^+$ (4), 391 (3), 301 (3), 279 (6), 247 (18), 223 (18), 181 (5), 97 (2), 87 (4), 65 (85).
10: 496	EIMS	460 $[M\text{-}2H_2O]^+$ (2), 442 $M\text{-}3H_2O]^+$ (4), 424 $M\text{-}4H_2O]^+$ (2), 410 (4), 392 (4), 379 $[M\text{-}C_{22}–C_{27}]^+$ (1), 374 (6), 361 (59), 343 (43), 325 (19), 316 (1), 301 (94), 283 (96), 250(54), 232(28), 211(40), 161 $[C_{20}–C_{27}]^+$ (22), 143 (46), 125 (40), 99 $[C_{22}–C_{27}]^+$ (62), 81 (100), 69 (63).
	HREIMS	460,5998 $[M\text{-}2H_2O]^+$ (calcd. for $C_{27}H_{44}O_8$, 460.5962).
	ESIMS	503 $[M+Li]^+$ (26), 485 $[M\text{-}H_2O+Li]^+$ (40), 467 $[M\text{-}2H_2O+Li]^+$ (85), 449 $[M\text{-}3H_2O+Li]^+$ (60), 431 $[M\text{-}4H_2O+Li]^+$ (5), 379 (4), 335 (2), 321 (2), 223 (1), 181 (8), 150 (4), 122 (32), 97 (4), 87 (2), 65 (2).
11: 496	ESIMS	497 $[M+H]^+$ (24), 478 $[M\text{-}H_2O]^+$ (14), 460 $[M\text{-}2H_2O]^+$ (22), 442 $[M\text{-}3H_2O]^+$ (8), 424 $[M\text{-}4H_2O]^+$ (19), 410 (5), 392 (11), 361 (22), 325 (38), 316 (43), 301 (100), 283 (81), 250 (57).
12: 464	EIMS	446 $[M\text{-}H_2O]^+$ (3), 428 $[M\text{-}2H_2O]^+$ (29), 413 (2), 410 $[M\text{-}3H_2O]^+$ (3), 359 (4), 348 (8), 330 (15), 300 (23), 99 $[C_{22}–C_{27}]^+$ (100), 81 (61).
13: 480	ESIMS	503 $[M+Na]^+$ (2), 463 $[M\text{-}H_2O+H]^+$ (18), 445 $[M\text{-}2H_2O+H]^+$ (10), 413 (1), 391 (2), 301 (2), 279 (1), 247 (20), 223 (3), 97 (100), 87 (23), 65 (22).
14: 480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 380, 363, 347, 345, 329.
	EIMS	480 $[M]^+$ (<1), 462 $[M\text{-}H_2O]^+$ (1), 444$[M\text{-}2H_2O]^+$ (1), 429 (3), 426 $[M\text{-}3H_2O]^+$ (12), 411 (2), 408 $[M\text{-}4H_2O]^+$ (3), 393 (1), 363 $[M\text{-}C_{22}–C_{27}]^+$ (7), 346 (11), 345 $[M\text{-}H_2O\text{-}C_{22}–C_{27}]^+$ (30), 344 (26), 328 (17), 327 (19), 300 (13), 145 (8), 143 $[C_{20}–C_{27}\text{-}H_2O]^+$ (8), 99 $[C_{22}–C_{27}]^+$ (100), 81 $[C_{22}–C_{27}\text{-}H_2O]^+$ (27).
	ESIMS	503 $[M+Na]^+$ (20), 481$[M+H]^+$ (50), 463 $[M\text{-}H_2O+H]^+$ (100), 445 $[M\text{-}2H_2O+H]^+$ (10), 413 (1), 391 (2), 301 (2), 279 (1), 251 (6), 247 (20), 223 (3), 215 (2), 119 (5), 97 (100), 87 (23), 65 (22).
15: 512	CIMS	513 $[M+H]^+$, 495, 477, 459, 441, 396, 379 $[M+H\text{-}C_{22}–C_{27}]^+$, 363, 361, 345.
16: 496	EIMS	478 $[M\text{-}H_2O]^+$ (3), 460 (4), 445 (10), 442 (18), 427 (11), 409 (7), 391 (4), 379 (73), 374 (5), 368 (17), 361 (74), 343 (100), 325 (43), 316 (17), 309 (7), 283 (41), 143 (60), 135 (61), 99 (35), 81 (34).
17: 508	EIMS	508 $[M]^+$, 493, 490, 475, 472, 457, 454, 439, 363, 345, 189, 171, 145 $[M\text{-}363]^+$, 127, 109.
18: 496	EIMS	478 $[M\text{-}H_2O]^+$, 460, 442, 424, 361, 360, 344, 343, 99, 81.
19: 462	EIMS	462 $[M]^+$, 405 (1), 363 (19), 345 (50), 327 (15), 99 $[C_{22}–C_{27}]^+$ (60), 81 (54), 43 (100).

M.W. = molecular weight; CIMS = chemical-ionization MS; EIMS = electron-impact MS; HREIMS = high-resolution electron-impact MS; ESIMS = electrospray-ionization MS

Compounds **6-9** and **13-14** gave very close MS spectra with prominent ions at *m/z* 498 $[M+H+NH_3]^+$, 481 $[M+H]^+$ and 463 $[M+H-H_2O]^+$ (CIMS was performed). These data are consistent with a molecular weight (M.W.) of 480 Da, identical with that of 20-hydroxyecdysone. The mass spectra of compounds **10, 11, 15, 16, 18** indicated the presence of additional OH groups (on the nucleus or both on the nucleus and on the side chain), or the lack of OH group(s) at compounds **2-5, 12, 19** with respect to 20-hydroxyecdysone.

3.2.4. NMR Spectroscopy and X-ray diffraction

The number of C, CH, CH_2 and CH_3 fragments in the molecule was identified from the ^{13}C NMR, DEPT and HMQC spectra. The number of connecting oxygen atoms was established from the ^{13}C chemical shifts (> 60 ppm). Methyl groups was utilized as a starting point for the determination of structure, because they are singlets and show strong two- and three-bonds correlations in the HMBC spectrum. With knowledge of these HMBC correlations and the NMR data of the ecdysteroid skeleton, methyl groups were unambiguously assigned to 18, 19, 21, 26 and 27 positions (H-26 and H-27 protons assigned corresponding carbon atoms, and H-18 and H-21 protons reached C-17). Protons of sp^2 carbon atoms gave correlations to C-5, C-9 and C-14 in the HMBC spectra, which proved 7, 8 double bonds in all compounds. Using the COSY and HMBC correlations of the separated or only partially overlapped ^1H signals (mainly in HC-O area) we could assemble the skeleton, and attached the side chain to the skeleton. The structure was supported by HMQC-TOCSY measurements. The NOESY correlations provided informations about stereochemistry of rings and orientation of substituents connecting to the skeleton. It was important to determine the anellation of A/B rings, which can be *cis* or *trans*. NOESY correlations between H-9/H-2, H-9/H-4 and H-19/H-5 proved that rings A and B are *cis* fused. However, NOESY signals correlating H-9 with H-1 and H-5, and H-19 with H-4 were due to A/B *trans*-ring junction (shown in **Figure 7**). In addition, the configuration of C-20 (*R*) or C-22 (*S*) could be determined using NOESY responses (see **Figure 8**). To avoid the strong steric interactions between Me-18/Me-21 and Me-18/OH-20 groups in the preferred conformation around C-17—C-20 bond were the H-17 and C-22 bonds *synplanar*. The diastereomeric discrimination (pro-*R* and pro-*S*) of the geminal methyl groups in position 26 and 27 and the H-24 protons were not possible.

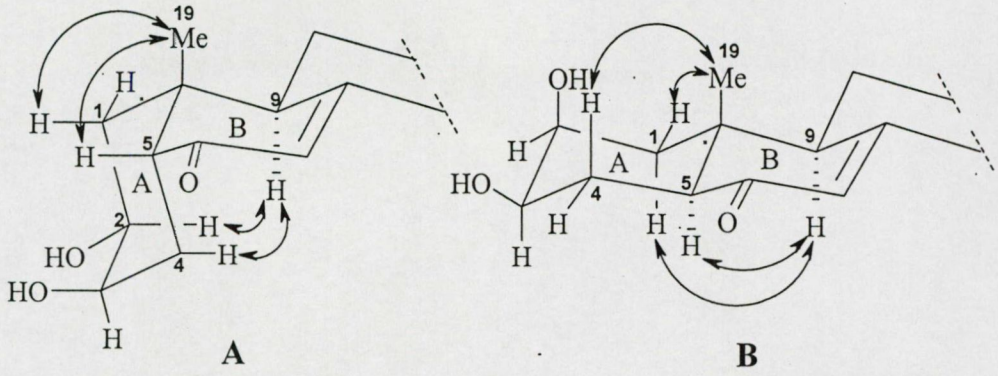


Figure 7 Selected NOESY correlations for partial structures of compounds **14** and **13** (**A** (A/B *cis*) and **B** (A/B *trans*), respectively). Double arrows indicate the characteristic NOESY correlations.

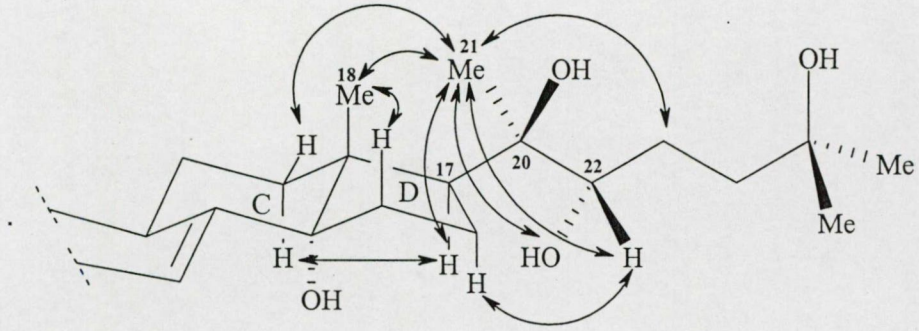


Figure 8 NOESY correlations for side chain of C_{27} ecdysteroids. Double arrows indicate the characteristic NOESY correlations around C-17–C-20–C-22.

Specific structural modifications in the ^{1}H- and ^{13}C-NMR spectra relative to ecdysone and 20-hydroxyecdysone were used for structure assignements of compounds (30). From the one-(see **Table 7** at the end of this section) and two-dimensional ^{1}H- and ^{13}C-NMR data, it was clear, that the steroid nucleus of the isolated compounds is 'classical' with respect to the presence of the 3β-OH, the 14α-OH functionalities, and in the most cases the 2β-OH (30).

NMR spectra of compounds **1-19** showed the typical features as described below in points (a)-(k). From these considerations, the structures could be established. Compounds were compared with 20-hydroxyecdysone (**14**), a characteristic ecdysteroid. Structural differences compared to 20E are in bold type in Table of **Figure 9**.

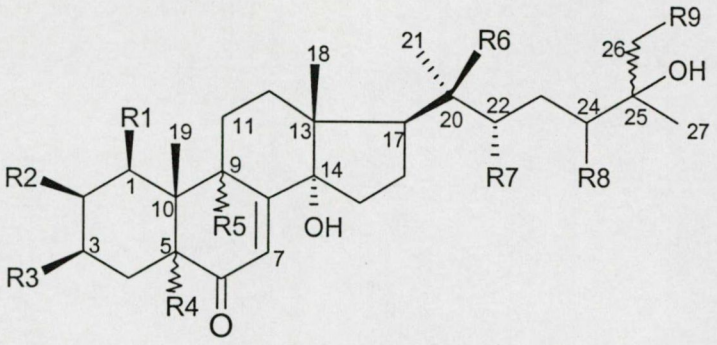
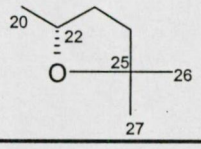


Figure 9 Structure of ecdysteroids isolated from *Silene italica* ssp. *nemoralis*

Comp.	R1	R2	R3	R4	R5	R6	R7	R8	R9
1	H	OH	OH	H	H	OH	OH	**=CH$_2$**	H
2	H	**H**	OH	H	H	**H**	OH	H	H
3	H	**H**	OH	H	H	OH	OH	H	H
4	H	OH	OH	H	H	OH	**H**	H	H
5*	H	**H**	OH	H	H	OH	**O-glc**	H	H
6*	**OH**	**H**	OH	**H (α)**	H	OH	OH	H	H
7	**OH**	**H**	OH	H	H	OH	OH	H	H
8	**OH**	OH	OH	H	H	OH	**H**	H	H
9*	H	**H**	OH	**OH**	H	OH	OH	H	H
10*	H	OH	OH	H	**OH (α)**	OH	OH	H	H
11*	H	OH	OH	H	**OH (β)**	OH	OH	H	H
12	H	OH	OH	H	H	**H**	OH	H	H
13	H	OH	OH	**H (α)**	H	OH	OH	H	H
14	H	OH	OH	H	H	OH	OH	H	H
15	H	OH	OH	**OH**	H	OH	OH	H	**OH**
16	**OH**	OH	OH	H	H	OH	OH	H	H
17	H	OH	OH	H	H	OH	OH	**C$_2$H$_5$**	H
18	H	OH	OH	**OH**	H	OH	OH	H	H
19	H	OH	OH	H	H	OH			

Comp = compounds; R1-R9 = functional groups; glc = glucose; *new phytoecdysteroids. Structural differences compared to 20E are given in bold type.

Compounds: name (synonym name)

1: 24(28)-dehydromakisterone A
2: 2-deoxyecdysone
3: 2-deoxy-20-hydroxyecdysone
4: 22-deoxy-20-hydroxyecdysone (taxisterone)
5: 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside
6: 5α-2-deoxyintegristerone A
7: 2-deoxyintegristerone A
8: 22-deoxyintegristerone A
9: 2-deoxypolypodine B
10: 9α,20-dihydroxyecdysone
11: 9β,20-dihydroxyecdysone
12: ecdysone
13: 5α-20-hydroxyecdysone
14: 20-hydroxyecdysone (ecdysterone)
15: 26-hydroxypolypodine B
16: integristerone A
17: makisterone C
18: polypodine B
19: shidasterone (stachysterone D)

(a) Compounds of the ecdysone versus 20-hydroxyecdysone series could be easily distinguished by the H-21 signal, which undergoes changes both in its chemical shift and in shape: a singlet for 20-OH compounds, a doublet in 20-deoxycompounds.

(b) 2-Deoxyecdysteroids shared the main features already known for 2-deoxyecdysone (**2**) and 2-deoxy-20-hydroxyecdysone (**3**), i.e., the lack of the H-2 signal in the hydroxymethine signal zone, broadening of the H-3 signal ($w_{1/2}$ ≈12 Hz) and correlation of this signal in the upfield part of the ^{1}H-^{1}H COSY spectrum with four signals (H_{ax}-2, H_{eq}-2, H_{ax}-4, H_{eq}-4) could be observed with respect to the appropriate signal in 20-hydroxyecdysone (**14**) or ecdysone (**12**). In NMR spectrum of compound **7** a broadening of the signals were observed at 20° in D_2O, which by contrast become sharp at 80°. The broadening of the signals at 20° could be due to a slow conformational equilibrium of the A ring, as a result of hydrogen bonding of 1β-OH and 3β-OH (30).

(c) 22-Deoxyecdysteroids showed specific characteristics similar to those of 22-deoxy-20-hydroxyecdysone (**4**): the lack of the H-22 and C-22 signals in the hydroxymethine zone, downfield shift of C-21 (+ 5.4 ppm in CD_3OD), two additional signals at H-22 (22a and 22b).

(d) 1β-Hydroxyecdysteroids were characterised by: the appearance of a new signal in the hydroxymethyl zone ($w_{1/2}$ ≈7 Hz, broad); the H_{ax}-2 signal appearing as a narrow triplet ($J_{1,2} = J_{2,3}$ = 3 Hz); a downfield shift of the H_{eq}-3 signal (+ 0.09 ppm); a strong downfield shift of the H-5 signal (+ 0.23 ppm) which resulted from the axial interaction between 1β-OH and H-5; a strong upfield shift of the C-19 signal (− 4.3-4.4 ppm in the case of H-5β; − 7.9 ppm in the case of H-5α) with respect to the appropriate signal in 20-hydroxyecdysone (**14**) or 5α-20-hydroxyecdysone.

(e) 5β-Hydroxyecdysteroids showed the disappearance of the H-5 signal, a modification of the H_{ax}-2 and H_{eq}-3 signals which became nearly isochronous and gave a complex multiplet at 3.94-3.99 ppm in CD_3OD, signal broadening at 4.15-4.26 ppm in C_5D_5N, a large downfield shift of the H_{ax}-1 signal (resulting from the axial interaction between 5β-OH and H_{ax}-1, as observed in the 2D COSY experiments), and a large downfield shift of the H_{ax}-4 and H_{eq}-4 signals.

(f) 9-Hydroxyecdysteroids showed high chemical shift at δ ca. 72-76 ppm (^{13}C NMR) in accord with the OH substitution in position C-9.

(g) 26-Hydroxyecdysteroid were characterised by the appearance of a hydroxymethyl signal (two if the diastereoisomers 25*R*, 25*S* are present) as a singlet at δ = 3.35 ppm and the loss of the H-26 signal, an upfield shift of the H-27 signal (− 0.05 ppm in CD_3OD).

(h) 5α-Ecdysteroids (A/B *cis* fused) have already been characterised by their NOESY correlations, see above.

(i) The presence of a sugar moiety on 22-OH was evident from ^{1}H NMR peaks in the region δ 3.3-4.4 ppm and from ^{13}C NMR spectrum, where six additional oxygenated carbon signals (related to a hexose moiety) were observed in the region δ 62.3-105.4 ppm. The C-22 signal (δ 89.6) was more deshielded (+ 11.1 ppm with respect to the 20-hydroxyecdysone, **14**) and thus suggested the attachment of the sugar unit at C-22, a conclusion conformed by the correlations H-1'→ C-22 and H-22→ C-1' observed in the HMBC spectrum, and from the medium NOE between H-1' and H-22.

(j) C_{28} and C_{29} ecdysteroids usually contain one- or two alkyl group at C-24 (e.g. C_{28}: makisterone A [24*R*], C_{29}: makisterone C (**17**)). The alkyl group may be unsaturated (methenyl: 24(28)-dehydromakisterone A (**1**) or ethenyl). Their NMR spectra of these compounds were characterized by the appearance of either 28-Me (110.4 ppm at C-28; 4.96 and 5.14 ppm at H-28a, b, as in **1**) or 28- and 29-Me signals (1.14, 1.53 at H-28a, b; 1.01 at H-29 as in **17**).

(k) A furan ring was generated by cyclization of a parent C_{27} skeletal side chain through dehydration of C-22 and C-25 hydroxyls. In this case, the most significantly shifted were C-22 and C-25 which moved downfield 7.2 and 10.5 ppm, respectively, from 20-hydroxyecdysone. These shifts were those expected as the two hydroxyls on the two carbons in 20-hydroxyecdysone were dehydrated to form an ether linkage (C-22-O-C-25). The connectivities within the five-membered ring were further confirmed by NOESY signals, such as correlating H-22 with H-26 existed during cyclization of side chain, therefore proton at C-25 had to be in the vicinity of H-22.

9β,20-Dihydroxyecdysone (**11**) will be discussed below in detail, and **Table 8/a and b** (at the end of this section) gives 2D NMR data in addition to ^{1}H and ^{13}C chemical shifts.

COMPOUND **11**: The planar formula of this compound was identified as described above for the 9α,20-dihydroxyecdysone (**10**). However, the stereochemical elucidation displayed a major problem: ^{13}C chemical shifts showed considerable differences, especially at C-4, C-6, C-8, C-11, C-12, C-13, C-18, and C-19 in comparison with 9α,20-dihydroxyecdysone (**10**) (see Δδ in **Table 8/a, b**). We first considered, that these differences derived from a *trans* A/B ring junction in addition to an α-positioned 9-OH group. In this case, the hydroxyl group at C-2 should be α, and the A ring is a twisted-boat or turn on the other chair forms. However, the H-19/H-5 NOESY correlation proved the *cis* A/B ring junction, notwithstanding small coupling constants emerged that H-5 is equatorial. This fact was inconsistent with the structure **B**, where A/B was cis fused and H-5 was axial (depicted in **Figure 7**). Studying of all possible conformations the assignment of hydroxyl group position at C-3 was not possible. For the determination of hydroxyl substituents at C-2, C-3, and C-9, the sample was dissolved in dimethylsulfoxide-d_6. Sharp signals were observed for two hydroxyl groups identified by HMBC correlations at C-9 and C-14. 9-OH correlated with H-5, H-18, and H-

19, which proved its β-position. This assignation verified, that ring C has twisted-boat configuration, which was confirmed by multiplicity of H-11α (in methanol H-11α was partially overlapped with H-4α, therefore it showed dd instead of dt). We concluded that compound **11** is 9β,20-dihydroxyecdysone.

Table 7 ^{1}H (500 MHz) and ^{13}C (125 MHz) chemical shifts of compounds **1-19** (MeOH-d_4, $CHCl_3$-d_1, DMSO-d_6, or C_5D_5N-d_5) (d in ppm).

		1*		**2**		**3**	
No.		^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C	^{1}H (mult; *J* [Hz]) CD_3OD
1	α	37.4	1.80 (d)	29.48			
	β		1.43 (t; 12.8)				
2	α	68.8	3.84 (dt; 11.8, 3.7)	29.07			
3	α	68.6	3.95 (q; 2.5)	64.06	3.98 (m, $w_{1/2}$=12)		3.96 (m, $w_{1/2}$=12)
4	α	32.9	1.71	33.07			
	β		1.76				
5	β	51.9	2.38	51.61	2.41 (dd, 12, 4)		2.38 (dd, 12, 5)
6		206.6	–	203.2	–		–
7		122.3	5.82 (d; 2.2)	121.3	5.96 (d, 2)		5.80 (d, 2.5)
8		168.1	–	166.01	–		–
9	α	35.2	3.16 (ddd)	34.4	3.20 (m, $w_{1/2}$=22)		3.20 (m, $w_{1/2}$=22)
10		39.4	–	36.96	–		–
11	α	21.6	1.81	21			
	β		1.70				
12	α	32.6	2.14 (t)	31.7			
	β		1.89 (d)				2.13 (ddd, 13, 13, 5)
13		48.8	–	48	–		–
14		85.4	–	84.01	–		–
15	α	31.9	1.60 (dd; 12.2, 9.6)	31.7			
	β		1.98				
16	α	21.6	1.81	25.5			
	β		2.02				
17	α	50.6	2.42 (t, 9.5)	48.32	2.02 (m)		2.39 (m)
18	β	18.1	0.90 (s)	15.8	0.74 (s)		0.89 (s)
19	β	24.5	0.97 (s)	24.3	0.89 (s)		0.96 (s)
20		77.9	–	42.99			–
21		21.1	1.23 (s)	13.6	0.94 (d, 6.5)		1.19 (s)
22		78.1	3.60 (d; 10.7)	73.9	3.69 (m, $w_{1/2}$=16)		3.33 (dd, 11, 2)
23	a	34.7	2.14 (t)	26.7			
	b		2.39 (d)				
24	a	155.4		42.46			
	b						
25		73.7	–	69.6	–		–
26		30.3	1.32 (s)	29.98	1.24 (s)		1.19 (s)
27		29.8	1.37 (s)	30.2	1.24 (s)		1.20 (s)

Multiplicity of signals: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad signal; $w_{1/2}$, width at half-height in Hertz; δ in ppm.*Additional chemical shifts of compound **1**: ^{1}H: δ = 4.96 (s; H-28a), 5.14 (s; H-28b); ^{13}C: δ = 110.5 (C-28).

Table 7 Continued

No.		4		5*		6	
		^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD
1	α	38.4		29.5	1.61 (d; HMQC)	78.3	3.655 (dd; 11.8, 4.3)
	β				1.45 (t; HMQC)		
2	α	67	3.90 (m; $w_{1/2}$=21)	[h]	[h]	41.8	1.96
	β						1.47
3	α	68.7	4.07 (m; $w_{1/2}$=8)	65.5	4.03 (br s)	68.4	3.59 (tt; 11.5, 4.6)
4	α	34		33.2	1.56 (d)[h]	30.8	2.055 (dm)
	β				1.79		1.34 (q; 12.5)
5	β	51	2.34 (t like)	52.3	2.41 (dd; 12.5, 4.1)	52.3	
	α						2.31 (dd; 12.5, 3.35)
6		202	–	207.7	–	202.8	–
7		121.5	5.97 (d; 2.5)	121.9	5.83 (d; 2.4)	123.0	5.84 (d; 2.7)
8		164.8	–	169.1	–	166.9	–
9	α	33.6	3.10 (m, $w_{1/2}$=22)	34.3	3.20 (br)	48.1	2.955 (ddd; 11.7, 7, 2.6)
10		38.4	–	37.7	–	44.5	–
11	α	21.4		21.4	1.73	25.1	2.42 (dm)
	β				2.00		1.69
12	α	31.5		32.7	2.07 (td; 13.0, 4.8)	33.3	2.115 (td; 13.1, 4.8)
	β				1.87 (d)		1.77
13		46.9	–	49.2	–	48.8	–
14		84.8	–	85.7	–	85.6	–
15	α	30.9		31.6	1.62 (t)	32.3	1.535 (dd; 10.5, 9.6)
	β				2.00 (d)		1.97
16	α	29.7		21.4	1.73	21.4	1.71
	β				2.00		1.96
17	α	52.2	2.31 (m)	51.0	2.34 (t; 8.8)	50.8	2.38 (dd; 9.9, 8.1)
18	β	17.5	0.82 (s)	18.2	0.87 (s)	18.4	0.895 (s)
19	β	23.8	0.99 (s)	24.4	0.95 (s)	7.9	0.84 (s)
20		75	–	77.9	–	78.1	–
21		26.5	1.30 (s)	22.4	1.24 (s)	21.1	1.184 (s)
22		44.3		89.6	3.50 (d; 9.9)	78.6	3.325 (dd;[h], 1.7
23	a	18.7		27.6	1.54 (q; HMQC)	27.5	1.29
	b				1.75 (t; 1D TOCSY)		1.67
24	a	44.7		41.0	1.46 (td; 12.7, 3.9)	42.6	1.42
	b				2.01 (t; HMQC)		1.80 (td; 12, 4.8)
25		71	–	71.8	–	71.5	–
26		29.3	1.22 (s)	29.1	1.20 (s)	29.1	1.19
27		29.5	1.22 (s)	29.5	1.21 (s)	29.8	1.204

t like: triplet-like structure (because H_{ax}-4 and H_{eq}-4 are isochronous). [h]Indicates signal overlap. *Chemical shifts of glucose in **5**: ^{1}H: δ = 4.38 (d; *J*=7.8 Hz; H-1'), 3.29 (t; *J*=8.8 Hz; H-2'), 3.42 (t; *J*=9.0; H-3'), 3.36 (H-4' and H-5'), 3.70 (dd; $J_{5'6'}$=12.0, $J_{4'6'}$=4.3; H-6'a), 3.88 (d; 12.0; H-6'b); ^{13}C: δ = 105.4 (C-1'), 75.4 (C-2'), 77.8 (C-3'), 77.9 (C-4'), 71.3 (C-5'), 62.3 (C-6').

Table 7 Continued

No.		7		8		9*	
		^{13}C	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C $CDCl_3$	^{1}H (mult; *J* [Hz]) $CDCl_3$
1	α		3.68 (m br; 20°C; dd, 4, 8, 80°C)	76.6	3.82 (s; br)	24.5	1.49
	β						1.88
2	α		1.91	68.6	3.87 (t; 3.0)	28.7	1.88
	β		1.42				1.88
3	α		3.70 (m br; 20°C); 3.87 (m br; 80°C)	71.1	4.04 (s; br)	65.5	4.055 (m)
4	α			33.7	1.76	36.4	1.90
	β				1.81		1.67
5	β		2.76 (m br; 20°C; dd, 4, 8, 80°C)	46.9	2.606 (dd; 12.7, 4.6)	79.4	–
6			–	205.8	–	201.3	–
7			5.94 (d; 2.5, 80°C)	122.3	5.84 (d; 2.4)	118.8	5.97 (d; 2.8)
8			–	167.5	–	166.9	–
9	α		3.23 (m br; 20°C); 3.19 (m; $w_{1/2}$=22, 80°C)	35.8	3.08 (t; 9.2)	36.8	3.21 (ddd; 11.3, 7.4, 2.8)
10			–	43.9	–	41.9	–
11	α			22.0	1.69	21.1	1.71
	β				1.74		1.74
12	α			32.5	2.09 (td; 12.8, 5.4)	31.3	2.10
	β				1.83		1.89 (d; HMQC)
13			–	48.2	–	47.6	–
14			–	85.5	–	84.8	–
15	α			31.8	1.61 (ddd; , 9.6)	31.9	1.53
	β				1.97 (dd; 11.5, 4.0)[h]		2.075
16	α			22.0	1.88	20.4	1.77
	β				1.92		2.08
17	α		2.36 (br; 20°C); 2.34 (t; 8.5, 80°C)	53.6	2.33 (t; 9.1)	49.0	2.36 (t; 9)
18	β		0.85 (s)	18.3	0.85 (s)	17.5	0.90 (s)
19	β		1.10 (s)	20.2	1.08 (s)	16.7	0.88 (s)
20			–	76.1	–	76.8	–
21			1.22 (s)	26.6	1.27 (s)	20.8	1.23 (s)
22	a		3.43 (dd; 11, 2, 80°C)	46.0	1.38	76.6	3.46 (d; ~10.5)
	b				1.51 (t; 8.5)		
23	a			20.3	1.40	26.1	1.40
	b				1.43		1.67-9
24	a			45.6	1.43	40.8	1.63
	b				1.43		1.75
25			–	71.6	–	71.0	–
26			1.23 (s)	29.3	1.19 (s)	29.4	1.27 (s)
27			1.24 (s)	29.5	1.19 (s)	30.1	1.28 (s)

[h]Indicates signal overlap. * Additional ^{1}H chemical shifts in compound **9**: δ = 1.88 (H-2β), 4.61 (d; 9.6; COSY to 4.055; 3-OH), 4.31 (s; 5-OH).

Table 7* Continued

No.		10		12		13	
		^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD
1	α	37.6	2.06 (d)	37.3	1.78	43.9	1.55 (dd; 14.5, 3.1)
	β		1.38 (t)		1.43		2.09 (dd; 14.4, 3.3)
2	α	69.7	4.45 (dt; 10.4, 3.9)	68.8	3.83 (ddd; 12, 3, 3)	70.4	3.96 (q; 3.0)
3	α	69.8	3.96 (d; 2.2)	68.6	3.94 (ddd; 3, 3, 3)	72.8	3.585 (ddd; 12.0, 4.4, 3.3)
4	α	36.3	2.44 (td; 13.2, 2.4)	32.8	1.75	24.8	1.90
	β		1.74 (d)		1.65		1.75 (q; HMQC)
5	β	51.4	2.56 (dd; 13.4, 3.9)	51.8	2.38 (dd; 12, 5)	55.4	
	α						2.39 (dd; 12.2, 3.4)
6		206.7	–	203.1	–	203.2	–
7		123.8	5.85 (s)	121.9	5.81 (d; 2.5)	123.7	5.84 (d; 2.8)
8		162.6	–	167.5	–	166.4	–
9	α	76.6	–	35.4	3.14 (m; $w_{1/2}$=22)	48.3	2.72 (ddd; 11.4, 7.5, 2.8)
10		43.0	–	39.3	–	39.3	–
11	α	30.9	1.95	21.6	1.78	21.7	1.77
	β		2.04		1.65		1.67 (q; HMQC)
12	α	29.6	2.19 (td; 13.1, 4.6)	32.1	1.7-1.8	32.5	2.12 (t; HMQC)
	β		1.83 (d)		2.1 (ddd; 13, 13, 5)		1.84 (d; HMQC)
13		49.1	–	48.3	–	48.6	–
14		87.2	–	85.1	–	85.1	–
15	α	31.6	1.67	32.1	2.00	31.9	1.60
	β		1.98		1.53		1.97
16	α	21.4	1.71	27	1.98	21.5	1.77
	β		2.01		1.48		1.97
17	α	50.4	2.45	48.8	2.01	50.7	2.36 (d; 8.4)
18	β	18.5	0.89 (s)	16.2	0.73 (s)	18.1	0.88 (s)
19	β	29.6	1.01 (s)	24.4	0.97 (s)	15.8	1.01 (s)
20		78.0	–	43.4	1.72	78.1	–
21		21.2	1.22 (s)	13.3	0.95 (d; 7.5)	21.1	1.19 (s)
22		78.6	3.32 (dd)	75.3	3.59 (m; $w_{1/2}$=16)	78.5	3.33 (d)
23	a	27.5	1.31 (q)	25.4	1.30	27.4	1.32
	b		1.66 (t)		1.60		1.66
24	a	42.5	1.44 (td; 11.6, 4.2)	42.2	2.26	42.5	1.435 (t; 12.3, 4.6)
	b		1.81 (t)		1.79		1.80 (t; HMQC)
25		71.4	–	71.4	–	71.4	–
26		29.1	1.19 (s)	29.4	1.37 (s)	29.1	1.19
27		29.9	1.205 (s)	29.3	1.37 (s)	29.7	1.20

* ^{1}H chemical shifts of compound 11 will be given in **Table 8/a**.

Table 7 Continued

No.		**14** ^{13}C CD_3OD	**14** ^{1}H (mult; *J* [Hz]) CD_3OD	**15** ^{13}C	**15** ^{1}H (mult; *J* [Hz]) CD_3OD	**16** ^{13}C CD_3OD	**16** ^{1}H (mult; *J* [Hz]) CD_3OD
1	α	37.5	1.80			76.5	3.82 (br s)
	β		1.43 (dd; 13.4, 12.0)				
2	α	68.8	3.84 (ddd; 12.1, 4.3, 3.2)		3.94 (m)	68.6	3.875 (t; 3.0)
3	α	68.6	3.95 (q; 2.9)		3.97 (m)	71.1	4.04 (br s)
4	α	33.0	1.72			33.6	1.77
	β		1.76				1.82
5	β	51.9	2.38 (d; 13.7)		–	46.9	2.607 (dd; 12.7, 4.6)
6		206.5	–		–	205.7	–
7		122.2	5.81 (d; 2.7)		5.84 (d; 2.5)	122.3	5.835 (d; 2.5)
8		168.1	–		–	167.4	–
9	α	35.2	3.15 (ddd; 11.3, 7.2, 2.)		3.14 (m; $w_{1/2}$=22)	35.7	3.084 (t;)
10		39.4	–		–	43.9	–
11	α	21.6	1.72			22.0	1.71
	β		1.80				1.76
12	α	32.6	2.135 (td; 13.0, 5.0)			32.6	2.11 (td; 12.8, 5.3)
	β		1.88 (ddd; 12.9, 4.9, 2.1)				1.87 (d)
13		48.8	–		–	48.7	–
14		85.3	–		–	85.2	–
15	α	31.9	1.59			31.9	1.59 (dd; 10.5, 9.1)
	β		1.97				1.98
16	α	21.6	1.69			21.5	1.73
	β		1.98				1.98
17	α	50.6	2.39 (dd; 12.1)		2.35 (m)	20.7	2.39 (t; 8.8)
18	β	18.1	0.89 (s)		0.88 (s)	18.1	0.905 (s)
19	β	24.5	0.97 (s)		0.9 (s)	20.1	1.08 (s)
20		78.0	–		–	78.0	–
21		21.2	1.20 (s)		1.2 (s)	21.1	1.19(s)
22		78.5	3.325 (dd; 11, 1.8)		3.33 (d br; 10)	78.5	3.34 (d)
23	a	27.5	1.285 (dtd; 13.3, 11.1, 4.7)			27.5	1.29 (dtd; 13.0, 11.3, 4.8)
	b		1.66				1.66 (t)
24	a	42.5	1.44 (dd; 13.4, 11.6)			42.5	1.434 (ddd; 13.1, 11.7, 4.1)
	b		1.80				1.82
25		71.4	–		–	71.4	–
26		29.1	1.19		3.35 (s; 26-CH_2OH)	29.1	1.19 (s)
27		29.8	1.204		1.14, 1.15 (s; 27-Me) (two isomers 25*R* and 25*S*)	29.8	1.20 (s)

Table 7 Continued

No.		**17***		**18**		**19**	
	^{13}C	^{1}H (mult; *J* [Hz]) CD_3OD	^{13}C C_5D_5N	^{1}H (mult; *J* [Hz]) C_5D_5N	^{13}C) CD_3OD	^{1}H (mult; *J* [Hz]) CD_3OD	
1	α	1.79	34.9	1.78	37.5	1.427 (t; 12.8)	
	β	1.43		1.68		1.81	
2	α	3.84 (m; $w_{1/2}$=22)	68.1	4.26 (br dt; 11.4, 4)	68.8	3.83	
3	α	3.94 (m; $w_{1/2}$=8)	69.9	4.15 (br s)	68.7	3.95	
4	α	1.75	36.1	2.08		1.72[q]	
	β	1.65		1.75		1.78[q]	
5	β	2.37 (dd; 12, 5)	79.9	–	51.9	2.38 (dd; 12.0, 4.8)	
6		–	201	–		–	
7		5.80 (d; 2.5)	119.9	6.25 (d; 2.2)	123.5	5.81 (d; 2.2)	
8		–	167	–		–	
9	α	3.16 (m; $w_{1/2}$=22)	38.4	3.62 (br t; 8.6)	35.4	3.14 (d; 11.4)	
10		–	44.8	–	39.4	–	
11	α	1.79	22.1	1.80	21.9	1.81	
	β	1.66		1.70		1.68	
12	α	1.87	32.2	1.88	32.5	2.146 (td; 13.2, 4.8)	
	β	2.13		2.54 (dt; 12.6, 4.6)		1.86	
13		–	48.2	–	48.5	–	
14		–	84.1	–	85.4	–	
15	α	2.00	31.8	2.00	31.9	1.61[q]	
	β	1.55		1.60		1.97[q]	
16	α	2.00	21.4	2.43	21.9	2.02[q]	
	β	1.77		1.75		[q]	
17	α	2.39 (m)	50.1	2.96 (t; 9)	52.0	2.365 (t; 9.1)	
18	β	0.89 (s)	18	1.19 (s)	18.3	0.846 (s)	
19	β	0.96 (s)	17.3	1.13 (s)	24.6	0.96 (s)	
20		–	76.9	–	77.2	–	
21		1.20 (s)	21.7	1.57 (s)	20.85	1.21 (s)	
22		3.42	77.7	3.84 (br d; 9.2)	85.7	3.92 (t)	
23	a	1.43	27.6	1.28	28.6	1.75	
	b	1.55		1.66		1.89	
24	a	1.45	42.3	1.75-1.8	39.8	1.75	
	b			1.45			
25		–	69.7	–	81.9	–	
26		1.21 (s)	30.1	1.35 (s)	28.5	1.24 (s)	
27		1.10 (s)	30.2	1.35 (s)	29.1	1.25 (s)	

*Additional ^{1}H chemical shifts in compound **17**: δ = 1.14, 1.53 (28-CH_2), 1.01 (t, 6.5; 29-Me).

[q] α/β orientation can be inverted.

Table 8/a ^{1}H and ^{13}C chemical shifts and $J(^{1}H, ^{1}H)$ couplings (in Hz), characteristic ^{13}C, ^{1}H long-range correlations (HMBC) and spatial proximities (NOESYof **11** measured in MeOH-d_4 and DMSO-d_6 (d in ppm).

No.		^{13}C CD_3OD	^{1}H (m; J [Hz]) CD_3OD	HMBC ^{13}C partners	NOESY[a]	$\Delta\delta$	^{13}C DMSO	^{1}H (m, J [Hz]) DMSO
1	α	37.2	1.86 (dd, 3.4)[h]		3.88, 3.195	-0.4	35.7	1.74
	β		1.67					1.43 (dt; 14.8, 2.3)
2	α	70.6	3.88 (q; 3.1)		3.195, 1.86, 1.67	+0.9	68.5	3.67 (q; 2.2)
3	α	69.9	3.195 (dt; 12.1, 3.5)		3.88, 2.34, 1.86	+0.1	67.7	2.96 (dt; 12, 3.5)
4	α	26.2	2.34 (dt; 13.6, 3.9)	201.4	3.195, 2.72	**-10.1**	25.3	2.11 (dt; 12.6, 3.5)
	β		2.01					1.79 (td; 12.2, 4.9)
5	β	50.3	2.72 (dt; 4.5, 2.3)		2.34, 2.01, 1.37	-1.1	48.4	2.52
6		204.4	–			**-2.3**	201.2	-
7		124.3	5.82 (s)	85.9, 75.3, 50.3	1.96, 1.73, 1.235 (w)	+0.5	122.0	5.61 (s)
8		166.4	–			**+3.8**	164.5	–
9		75.3	–			-1.3	72.8	–
10		44.2	–			+1.2	42.3	–
11	α	27.0	2.37 (dd; 14.0, 9.8)[h]	166.4		**-3.9**	25.3	2.21 (dt; 13.4, 9.6)
	β		1.69					1.52 (dd; 13.7, 8.7)
12	α	32.9	2.04			**+3.3**	31.4	1.86 (dd; 13.6, 9.6)
	β		1.83					1.67 (dt; 13.8, 8.8)
13		46.2	–			**-2.9**	44.4	–
14		85.9	–	83.4, 44.4		-1.3	83.4	–
15	a	32.3	1.73			+0.7	30.6	1.65
	b		1.97					1.75
16	a	21.8	1.73			+0.4	20.4	1.55
	b		1.99					1.89 (qd; 12, 1.9)
17	α	52.4	2.46 (t; 8.8)[h]		2.03, 1.72, 1.235 (w), 1.21	+2.0	50.3	2.34 (t; 9.1)
18	β	23.4	1.235 (s)	85.9, 52.4, 46.2, 32.9	5.82 (m), 2.46 (w), 1.97-9, 1.83	**+4.9**	22.5	1.13 (s)
19	β	21.6	1.37 (s)	75.3, 50.3, 44.2, 37.2	2.72, 2.01, 1.69	**-8.0**	20.9	1.24 (s)
20		78.1	–			+0.1	75.6	–
21		20.8	1.21 (s)	78.7, 78.1, 52.4	2.46 (s), 2.03, 1.83	-0.4	20.5	1.07 (s)
22		78.7	3.32[h]	78.1, 42.6, 27.6, 20.8	1.73 (w)	+0.1	76.3	3.115 (d; 10.6)
23	a	27.6	1.315			+0.1	26.1	1.15
	b		1.71					1.50
24	a	42.6	1.44 (ddd; 13.0, 11.7, 4.0)		3.32, 1.70, 1.205, 1.19	+0.1	41.4	1.26
	b		1.81					1.66
25		71.5	–			+0.1	68.7	–
26		29.1	1.19	71.5, 42.6, 29.8	1.81, 1.44	0	29.0	1.06 (s)
27		29.8	1.205	71.5, 42.6, 29.1	1.81, 1.70, 1.44	+0.1	30.0	1.09 (s)
9-OH								[$]4.56 (s)
14-OH								[#]4.45 (s)

[h]Indicates signal overlap with the CD_3OD signal. [a]Indicates in NOESY spectrum: w, weak; vw, very weak; s, strong. $\Delta\delta$ show differences between the ^{13}C chemical shifts of compounds **11** and **10** in methanol-d_4. [$] 2D HMBC correlation to C-8 (164.5) and C-11 (25.3); 2D NOESY correlation to H-5β (2.52), H-12β (1.67), H-11β (1.52), H-19β (1.24) and H-18β (1.13); [#] 2D HMBC correlation to C-14 (83.4) and C-13 (44.4); 2D NOESY correlation to H-7 (5.61), H-17α (2.34), H-15α (1.75), H-15β (1.66) and H-16α (1.55).

Table 8/b The individual spin systems (TOCSY) of **11** measured in MeOH-d_4.

No.	^{13}C	HMQC-TOCSY (TOCSY)	No.	^{13}C	HMQC-TOCSY (TOCSY)
1	37.2	3.88	12	32.9	2.38, 1.69
2	70.6	2.72, 1.84, 1.64	15	32.3	2.46
3	69.9	3.88, 2.72, 2.34, 2.01	16	21.8	2.46
4	26.2	3.19, 2.72	17	52.4	1.97, 1.73
5	50.3	2.34, 2.01	22	78.7	1.81, 1.44
7	124.3	–	23	27.6	3.32, 1.80
11	27.0	3.32, 2.04, 1.83	24	42.6	1.68, 1.33

The structure determination of compounds **14, 18** and **6** was completed by X-ray diffraction. **Figure 10** gives the chemical structure of compound **6**.

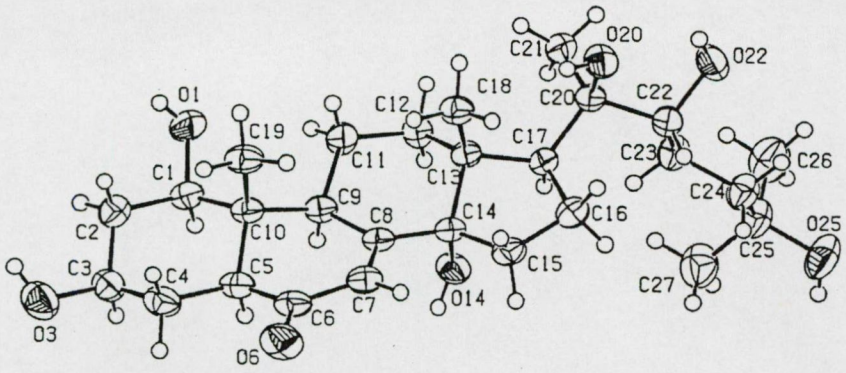


Figure 10 The stereochemical arrangement of 5-alpha-2-deoxyintegristerone A.

4. DISCUSSION

To extend the search for new plant ecdysteroids we have found that *Silene italica* ssp. *nemoralis* (Waldst. and Kit.) Nyman is an excellent plant material with a broad ecdysteroid spectrum (50, 57, 60). Although, the 20-hydroxyecdysone content of this plant (ca. 0.5 % of the dry wt. of herb, ref. 50) is not too large in aerial parts, because concentrations of 20-hydroxyecdysone up to 2-3 % of the dry wt. have been reported for some other plant species (6). *S. italica* ssp. *nemoralis* showed minor compounds typical for *Silene* genera (e.g. 2-deoxy- and 22-deoxy-ecdysteroid derivatives, 5-alpha ecdysteroids, and conjugates with sugar.). However, we found no very high similarities with other *Silene* species in consideration of other minor components; include 9-hydroxy epimers (because they are new type of ecdysteroid derivatives), and hitherto, we were not able to characterised compounds

with side chain-cleavage and/or ester derivatives, which are common ecdysteroids of *Silene* species.

Silene italica ssp *nemoralis* made isolation demanding, because of the diverse chemical structures of ecdysteroids and the relative high levels of 20-hydroxyecdysone. The chromatographic characteristics of ecdysteroids strongly depended on the number and position of the hydroxyl group attached to the steroid skeleton. For example, although polypodine B contains one additional OH-group at C-5 compared to 20-hydroxyecdysone, it resulted in a slight decrease of polarity because of a hydrogen bond between the 6-keto group and OH-group at C-5. This means that polypodine B showed closely similar chromatographic character to 22-deoxy-20-hydroxyecdysone (with 5 hydroxyls) and 20-hydroxyecdysone (with 6 hydroxyls). Their selective separation from 20-hydroxyecdysone was based on the minor difference in the pKa values, which offered a moderate possibility for large-scale separation. These conditions required a multi-step procedure when large-scale throughput was realized (27).

Recent work dealt with the isolation of nineteen ecdysteroids that have been isolated from this plant for the first time, and five novel phytoecdysteroids have been discovered. We utilized our experience gained in the isolation of known and unknown ecdysteroids, as well as the isolation protocols of ecdysteroids published recently to develop the isolation scheme, which is an unusual combination of the established procedures (36). The first target of isolation was the 20-hydroxyecdysone, the major phytoecdysteroid. Without its removal, 20-hydroxyecdysone may contaminate the complete chromatographic pattern. In addition, their separation from the extract opened the way for the isolation of minor ecdysteroids.

At the initial steps of purification, inexpensive methods with high sample load possibilities were used, such as extraction of the plant material using methanol, fractionated precipitation using methanol-acetone and liquid-liquid partition using aqueous methanol-benzene. The extraction with methanol removed the plant support matrix, resulting in an eight-fold enrichment. Fractionated precipitation made the solution free from the polar contaminating compounds (mono-, oligo- and polysaccharides, sugar alcohols and certain proteins). Liquid-liquid extraction removed apolar impurities such as terpene, chlorophylls, certain other steroids, etc. The consecutive operations with fractionated precipitation and liquid-liquid extraction resulted in more than 60 % removal of the contaminants from the crude extract.

The next step was a specific purification on alumina. The method performed a group separation. The alumina column was highly loaded, and it gave four fractions containing

numerous ecdysteroids. The abundant amounts of contaminants were either not eluted, or remained adsorbed on the stationary phase. The high load was also favourable, because the overload of the system could decrease the possible irreversible adsorption of the ecdysteroids. Such an amount could not be separated using either counter-current distribution or HPLC.

Preparative adsorption chromatography was used in the middle part of isolation. The consecutive steps of column and preparative TLC chromatography served the separation of ecdysteroids based on their polarity and enriched certain minor ecdysteroids. The preparative TLC offered a useful alternative preparative method that substituted the column chromatography with smaller sample load. The irreversible adsorption of polar ecdysteroids to the adsorbent was decreased using water-containing (ca. 1 % or less) eluent. In the most cases, we used RP low-pressure column chromatography after column chromatography on alumina to obtain fractions enriched in ecdysteroids. This multistep isolation scheme has been built up including the repeated use of RPLPCC. Our intent in using RPLPCC was to develop a new method based on different physical-chemical characteristics of ecdysteroids than those used in the earlier steps.

Purification and identification of ecdysteroids could be completed using HPLC. Using RP-HPLC followed by normal-phase HPLC, certain fractions resulted in the high purity (97 %) minor compounds. The success of the isolation was based on the difference between the chromatographic processes taking place on the two different stationary phases (reversed-phase and normal-phase). The whole separation process utilized the differences in the solubility, distribution, lipophilicity, and adsorption characteristics of ecdysteroids relative to their contaminants.

20-hydroxyecdysone (**14**) (total yield: ca. 0.5 % of the dry wt. of herb) proved to be the main ecdysteroid of *Silene italica* ssp. *nemoralis*. 2-Deoxy-20-hydroxyecdysone (**3**) ecdysone (**12**) and polypodine B (**18**) have also been isolated in larger amount (0.05-0,01 % of the dry wt.). Minor compounds occurred in relatively low concentrations in plant (in the range of 0.001-0.0001 % of the dry wt.). Some typical phytoecdysteroids were isolated, such as 2-deoxyecdysone (**2**), 22-deoxy-20-hydroxyecdysone (**4**), integristerone A (**16**), 22-deoxyintegristerone A (**8**), 2-deoxyintegristerone A (**7**), makisterone C (**17**), 24(28)-dehydromakisterone A (**1**), 26-hydroxypolypodine B (**15**), which have been detected solely in the plant kingdom (The Ecdysone Handbook, ref. 5, 9). However, especially interesting hydroxylation of 20-hydroxyecdysone at C-9 have not been found yet (61). Structures of the five new ecdysteroids are shown in **Figure 9** (signal marked by "♣" indicate new ecdysteroids). 2-

Deoxypolypodine B was first isolated by us (27, 62, 63). Jayashinghe has been published this compound from *Diploclisia glaucescens* (64).

The majority of isolated ecdysteroids belong to the 5-beta-androstane series, where A/B rings are *cis* fused. The 5-beta form is favoured in ecdysteroids, because the C-2-beta-hydroxyl group causes a steric hindrance with the 19-methyl group (65). However, at 5-alpha-2-deoxyintegristerone A (**6**) and 5-beta-2-deoxyintegristerone A (**7**) the hydroxyl group in C-2 position is absent, therefore the 5-alpha form is allowed to be dominant. A large amount of 5α-2-deoxyintegristerone A was isolated from *Silene italica* ssp. *nemoralis*, thus its crystals could be identified by X-ray diffraction technique to give the final vertification, in addition to 20E and pB (38, 66, 67).

We isolated a new glycoside, 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopiranoside (**5**). However, the conjugation of an ecdysteroid with sugar is more characteristic to plants than we hitherto found in this *Silene* species. Among the glycosides of 2-deoxy-20-hydroxyecdysone, only 3-monoglucoside has been found earlier in plant kingdom (9, 68).

The next rare ecdysteroids, 9α,20- (**10**) and 9β,20-dihydroxyecdysone (**11**) isomer pairs were first isolated from this plant (61, 69). 9α,20-Dihydroxyecdysone was synthesized earlier by Suksamrarn (70). However, there have been only three examples for naturally occurring 9α-ecdysteroids before our work. These three other 9α-ecdysteroids have trans-fused A/B rings, and their oxidation states are lower. They contain only two or three hydroxyl groups. This is the first case on the natural occurrence of ecdysteroid with *cis*-fused A/B ring among the C-9 hydroxylated ecdysteroid. Numerous steroids exhibit cytotoxic activity against hepatoma and HeLa cells. Some of these specifically active steroids can be characterized by 7-en-9α-OH structural elements (71, 72). Among them an ecdysteroid, the 3ß,5α,9α-trihydroxy-ergosta-7,22-dien-6-one shows the highest cytotoxic activity. Considering this finding compounds **10** and **11** seems to be a very promising candidate to the QSAR studies.

5. CONCLUSION

The discovery of new native ecdysteroids may be successful from a representative of *Silene* genus (family Caryophyllaceae), which proved to give the largest number of ecdysteroid-containing species. *Silene italica* ssp. *nemoralis* (section *Siphonomorpha* Otth.) has not been the subject of a through phytochemical investigation before. Hitherto, we have isolated nineteen phytoecdysteroids from this *Silene* species. Five of them were new

phytoecdysteroids, such as 2-deoxypolypodine B, 5α-2-deoxyintegristerone A, 9α,20-dihydroxyecdysone, 9β,20-dihydroxyecdysone, 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside.

This *Silene* species was an appropriate plant material to isolation diversified ecdysteroids and make a comparison between other investigated *Silene* species. The ecdysteroids were distinguished favourably by both their quantitative and their qualitative compositions. The most frequently ecdysteroids were found to be 20-hydroxyecdysone, polypodine B, ecdysone, which are common ecdysteroids in *Silene* species. 2-Deoxy ecdysteroids proved to be characteristic of *Silene italica* ssp. *nemoralis*, thus the occurrence of a new phytoecdysteroid, 5-alpha-2-deoxyintegristerone A in significant amounts was detected. Some plants within sec. *Siphonomorpha* contain also 2-deoxy derivatives in high amount, but considerable conclusion could not be drawn. The most *Silene* species are rich in free ecdysteroids and contain only low levels of hydrolysable ecdysteroid conjugates. Hitherto the 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside was the only ecdysteroid conjugation isolated from *S. italica* ssp. *nemoralis*.

The level of the main ecdysteroid, 20-hydroxyecdysone was too high related to other components. However, minor ecdysteroids occurred in relatively low concentration in *Silene italica* ssp. *nemoralis* as a general rule in plants (e.g. in the range of 0.001-0.0001 % of the dry wt.), which fact complicated the isolation of these structurally similar compounds. Otherwise, the plant contained not only a complex mixture of chemically similar representatives of ecdysteroids, but also a series of other compounds with similar chromatographic behaviour. The proper employment of the chromatographic separations in an appropriate order resulted in compounds that are pure enough for structural elucidation with high-resolution spectroscopic methods (NMR, MS-MS). For isolated compounds, we gave all physical and spectral data, especially some CD, 2D NMR, and X-ray data.

We believe that the discovery of ecdysteroids in this plant may be conducive to our understanding of the connections between the ecdysteroid biosynthesis within *Silene* genus (2-deoxy derivatives may be converted into 20-hydroxyecdysone by hydroxylases in plants) and to give a new source of ecdysteroids for structure-activity studies. The objective of this study is to give an effective combination of purification methods for phytoecdysteroids and their wide application in isolation of a large number of structurally very similar analogues. We used many purification methods, because of the wide polarity of compounds. It was

necessary to employ normal and reversed phase, and chromatographic procedures in repeated steps to separate compounds with similar polarity.

This plant is also a good source of natural ecdysteroid isomer pairs (5α- and 5β-2-deoxyintegristerone A; 9α,20- and 9β,20-dihydroxyecdysone), some of them may be model substances for biological activity measurements. In addition, 9-hydroxyecdysteroids are new natural compounds, having rather unusual structure.

Few of the above compounds isolated from *S. italica* ssp. *nemoralis*, and the many other ecdysteroids recently isolated from plants, may find a direct use in medicine. They may well, as in other areas, act as pointers in the search for new, more active substances, or they may themselves be used as starting material for semi-synthetic compounds, because most of them are available in only minor amounts from plants and hardly available in adequate amounts for research and practical applications.

ABBREVIATIONS

^{13}C NMR ..... carbon- nuclear-magnetic resonance spectroscopy
CD ..... circular dichroism
C_5D_5N ..... pyridine
CC ..... column chromatography
CoMFA ..... comparative molecular field analysis
comp. ..... compounds:
CNS ..... central nervous system
COSY ..... correlation spectroscopy
cryst. ..... crystallization
DCCC ..... droplet counter-current chromatography
DEPT ..... distorsionless enhancement by polarization transfer
DMSO ..... dimethylsulfoxide
20E 20-hydroxyecdysone
E ..... ecdysone
EcR ..... ecdysteroid receptor
EIMS ..... electron-impact mass spectrometry
ESIMS ..... electrospray ionization mass spectrometry
HMBC ..... heteronuclear multiple-bond correlation
HMQC ..... heteronuclear multiple-quantum correlation
^{1}H NMR ..... proton nuclear-magnetic resonance spectroscopy
HPLC ..... high-performance liquid chromatography
HPTLC ..... high-performance thin-layer chromatography
HREIMS ..... high-resolution electron-impact mass spectrometry
IR ..... infrared spectrophotometry
MS: *m/z*, $[M]^+$, MS-MS mass spectrometry: molecular ion, parent ion
MP ..... melting point
M.W. ..... molecular weight
NPCC ..... normal-phase column chromatography
NP-HPLC ..... normal-phase high-performance liquid chromatography
NP-TLC ..... normal-phase thin-layer chromatography
NMR ..... nuclear-magnetic resonance spectroscopy
NOE ..... nuclear Overhauser effect
NOESY ..... nuclear Overhauser enhancement and exchange spectroscopy
ODS ..... octadecylsilanized
pB ..... polypodine B
prep NP-TLC preparative normal-phase thin-layer chromatography
QSAR ..... quantitative structure-activity relationship
RIA ..... radioimmunoassay
R_F ..... retention factor
R_t ..... retention time
RP-HPLC ..... reversed-phase high-performance liquid chromatography
RP-TLC ..... reversed-phase thin-layer chromatography
RPLPCC ..... reversed-phase low-pressure column chromatography
sec. ..... section
s. s. ..... solvent system
sp. ..... species
subg. ..... subgenus
TLC ..... thin-layer chromatography
TOCSY ..... total correlation spectroscopy
UV ..... ultraviolet spectrophotometry
wt. ..... weight

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ANNEX

Papers related to the Ph.D. Thesis