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# Isolation and diversity-oriented semisynthetic modification of biologically active phenanthrenes from *Juncus* species

## Ph.D. Thesis

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Szeged, Hungary

2021

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## LIST OF PUBLICATIONS RELATED TO THE THESIS

**I.** **Bús Cs**, Kúsz N, Jakab G, Senobar Tahaei SA, Zupkó I, Endrész V, Bogdanov A, Burián K, Csupor-Löffler B, Hohmann J, Vasas A. Phenanthrenes from *Juncus compressus* Jacq. with promising antiproliferative and anti-HSV-2 activities.
*Molecules* **2018**, *23*, 2085-2093. **IF: 3.060**

**II.** **Bús Cs**, Tóth B, Stefkó D, Hohmann J, Vasas A. Family Juncaceae: promising source of biologically active natural phenanthrenes.
*Phytochemistry Reviews*, **2018**, *17*, 833-851. **IF: 4.545**

**III.** **Bús Cs**, Kulmány Á, Kúsz N, Gonda T, Zupkó I, Mándi A, Kurtán T, Tóth B, Hohmann J, Hunyadi A, Vasas A. Oxidized juncuenin B analogues with increased antiproliferative activity on human adherent cell lines: semisynthesis and biological evaluation.
*Journal of Natural Products* **2020**, *83*, 3250-3261. **IF: 3.779***
*(Shared first autorship)*

**IV.** **Bús Cs**, Kúsz N, Kincses A, Szemerédi N, Spengler G, Bakacsy L, Purger D, Berkecz R, Hohmann J, Hunyadi A, Vasas A. Antiproliferative phenanthrenes from *Juncus tenuis*: isolation and diversity-oriented semisynthetic modification.
*Molecules* **2020**, *25*, 5893-5907. **IF: 3.267***

## LIST OF PUBLICATIONS NOT RELATED TO THE THESIS

**I.** Szabados M, **Bús Cs**, Ádok-Sipiczki M, Kónya Z, Kukovecz Á, Sipos P, Pálinkó I. Ultrasound-enhanced milling in the synthesis of phase-pure, highly crystalline ZnAl-layered double hydroxide of low Zn(II) content.
*Particuology*, **2016**, 27, 29-33. **IF: 2.280**

* Impact factor in 2019

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| 1D | one dimensional |
| 2D | two dimensional |
| $^{1}$H NMR | proton nuclear magnetic resonance spectroscopy |
| $^{12}$C NMR | carbon nuclear magnetic resonance spectroscopy |
| CC | column chromatography |
| COSY | correlation spectroscopy |
| δ | chemical shift |
| equiv. | equivalent |
| fr | fraction |
| GF | gel filtration |
| HPLC | high performance liquid chromatography |
| HMBC | heteronuclear multiple-bond correlation |
| HRE(S)IMS | high-resolution electron (spray) ionization mass spectrometry |
| HSQC | heteronuclear single-quantum correlation |
| $IC_{50}$ | half maximal inhibitory concentration |
| JMOD | *J*-modulated spin-echo experiment |
| MPLC | medium pressure liquid chromatography |
| MS | mass spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NMR | nuclear magnetic resonance |
| NOE | nuclear Overhauser-enhancement |
| NOESY | nuclear Overhauser-enhancement spectroscopy |
| NP | normal phase |
| PIFA | [bis(trifluoroacetoxy)iodo]benzene |
| PIDA | (diacetoxyiodo)benzene |
| RPC | rotation planar chromatography |
| RP-HPLC | reverse-phase high performance liquid chromatography |
| SAR | structure-activity relationship |
| TLC | thin-layer chromatography |
| $t_R$ | retention time |
| UV | ultraviolet |
| VLC | vacuum-liquid chromatography |

## 1. INTRODUCTION

Family Juncaceae represents a rather unique position among the angiosperms, consists of eight genera with about 500 species worldwide [1], of which *Juncus* (more than 300 species) is the most important one [2, 3]. *Juncus* species are widespread and present in many parts of both hemispheres from arctic regions to subtropics [4]. These species usually grow in the salty marshes or badly–drained soils under different climatic conditions [5].

Various *Juncus* species are used in traditional Chinese medicine or have economic significance. The dried stem pith of *J. effusus* (''Deng Xin Cao'') is official in the Pharmacopoeia of the People's Republic of China [6] and recommended for the treatment of fidgetiness, insomnia, oliguria or ulceration in the mouth or on the tongue [7]. The whole herb of *J. effusus* and its medulla (Medulla Junci, TCM) are used for the treatment of aphta, traumatical bleeding or pharyngitis [8]. In Egypt, the seeds of *J. rigidus* is applied to treat diarrhoea and diuretic disorders [9]. The aerial parts of *J. effusus* and *J. balticus* and the roots of *J. ensifolius* were the part of healthy food of Canadian indigenous people [10].

*Juncus* plants (e.g. *J. acutus*, *J. arabicus*, *J. rigidus*) have been used to make mats, mattresses, sandals, and baskets throughout Africa [11, 12]. Numerous *Juncus* species are native to South-America, therefore, these plants (e.g. *J. arcticus* var. *andicola*, *J. effusus*) are commonly used in that region for preparation of traditional craftworks [13]. Some species, such as *J. acutus*, *J. rigidus*, and a few *Luzula* species were used in the paper industry [14].

The most characteristic secondary metabolites of *Juncus* species are phenanthrenes. Phenanthrenes form a rather uncommon class of aromatic secondary metabolites which are presumably formed during oxidative coupling of aromatic rings of stilbene precursors [15]. Other biosynthetic pathways of phenanthrenes are also likely, e.g. diterpenoid origin in case of Euphorbiaceae phenanthrenes [16, 17] or alkaloid-like compounds with phenanthrene skeleton from *Cryptocaria crassinervia* (Lauraceae) [18].

Phenanthrenes may be divided into three major groups: mono-, di- and triphenanthrenes. Monophenanthrenes are further divided according to the saturation of bond between C-9 and C-10 (phenanthrenes and dihydrophenanthrenes) (**Fig. 1**), and the number and type of functional groups joining to the skeleton, while di- and triphenanthrenes can be classified by the type of monomers and connection of the phenanthrene units [19].

Phenanthrenes have a limited occurrence; to date approx. 500 compounds were identified from the members of Annonaceae, Aristolochiaceae, Cannabaceae, Combretaceae, Dioscoreaceae, Euphorbiaceae, Juncaceae, Lauraceae, Malpighiaceae, Orchidaceae and Stemonaceae families. The

majority of these metabolites have been identified from plant species belonging to the Orchidaceae and Juncaceae families [20].

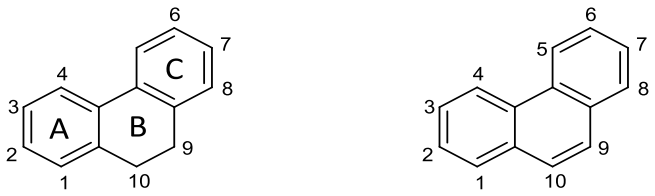



**Figure 1.** Common structure of phenanthrenes (dihydrophenanthrene and phenanthrene)

Phenanthrenes have become the objects of numerous research programs, because of their structural diversity and wide range of pharmacological activities, including antiproliferative, antimicrobial (antibacterial, antiviral, antifungal), antioxidant, anti-inflammatory, spasmolytic and anxiolytic effects.

A research program, started in 2014, in the Department of Pharmacognosy, aims the phytochemical and pharmacological investigations of Juncaceae species native to the Carpathian Basin. The main target of these phytochemical investigations is the isolation, structure determination and biological characterization of phenanthrenes.

The present thesis summarizes the results of the isolation of phenanthrenes from two *Juncus* species, *J. compressus* and *J. tenuis*, and the preparation of semisynthetic derivatives of three naturally occuring phenanthrenes, namely juncuenin B, juncusol and effusol.

## 2. LITERATURE OVERVIEW

### 2.1 Botany of the investigated plant species

Juncaceae plants are usually perennial herbs, but some representatives are annual. These plants are characterised with long, narrow leaves with heathing base, which are sometimes reduced to scales. The hermaphrodyte or protogynous flowers have an actinomorphic symmetry and are usually crowded in monochasial cymes which are sometimes condensed into heads or solitary. *Juncus* species are glabrous, rhizomatous perennials or annuals. Their inflorescence is sympodial, usually many-flowered, the ultimate divisions form cymes which are often contracted into heads. Variation of vegetative characters is great, this subgenera are most easily characterised by the different structure and arrangement of leaves. Juncaceae plants have dehiscent fruits and endospermic seeds [21, 22].

*Juncus* L. is a nearly cosmopolitan genus and counts about 300–315 species [23, 24]. *Juncus* sect. *Steirochloa* Griseb., to which *J. compressus* and *J. tenuis* belongs, is readily distinguished by its perennial life form, singly borne flowers, presence of floral bracteoles, terminal inflorescences and non-septate leaves [25]. It accommodates 35 species that are widely distributed throughout the temperate regions of the world (except South Africa). Its centres of diversity are in North America, temperate South America, and Central Asia [26]. *J. compressus* Jacq., *J. gerardii* Loisel. and *J. squarrosus* L. are more or less widespread native species in Europe. An additional species, *J. tenuis* Willd., originally native to North America, has become a widely naturalised xenophyte in large parts of Europe. According to DAISIE (Delivering Alien Invasive Species Inventories for Europe, 2008) project, it is among the most widespread non-native plant species in Europe [27].

*Juncus compressus* Jacq. has a creeping rhizome or laxly caespitose. The stems of the plant are 10-40 cm high, usually slightly compressed with 0-3 basal sheaths, 1-4 basal and 1-2 upper cauline leaves. The leaves are flat or rarely canaliculate, 5-25 cm long. The inflorescence is usually lax, comprises of 40-60 flowers. The perial segments measure 2-3 mm, they are equal, ovate, obtuse. Stamens have the same lenght as perial segments, and anthers are one-two times longer than the filaments. Seeds measure 0.35-0.5 mm, without appendages. The sheaths are brown to black in colour. The plant is native to Europe and Western Asia [21, 22].

*Juncus tenuis* Wild. is densely caespitose. Inflorescence is small, not diffusely branched and with congested flowers (internodes rarely longer than tepals); the plant is usually smaller than 70 cm; capsules are mostly more than 3 mm long, ellipsoid, only slightly shorter than tepals; longest ultimate branches of the cymes are 10–20 mm long. Leaves are flat, often conspicuosly convolute with long, obtuse, thin and whitish auricles [21, 22]. In its area of origin as well as in Europe, *J. tenuis* is much less demanding and grows in exposed or shaded sites, in soils ranging from sand to clay, under moist or drier conditions [23]. In Europe, it is often confined to dry, sandy and rather acidic soils (tracks or

clearings in woodland, disturbed heaths etc.) but also occurs on river banks and other wet habitats. In recent times, it has been increasingly seen in urban habitats as well (e.g. in cracks of the pavement).

## 2.2. Chemical constituents of *Juncus* species

As the result of several phytochemical investigations of Juncaceae species, numerous secondary metabolites have been isolated including flavonoids, coumarins, terpenes, sterols, phenolic acids, stilbenes, dihydro-dibenzoxepin, carotenoids and phenanthrenes [28].

Flavonoids are often reported from the species of *Juncus* genus. These metabolites were identified in different forms: free flavonoids, *O*- or C-glycosides, glucuronides and their *O*- or C-alkylated analogues. Isoscutellarein pentamethyl ether was isolated from the medulla of *J. effusus*, quercetin and its 3-*O*-rutinoside were identified from the rhizomes of *J. subulatus* and from the aerial parts of *J. acutus* and *J. rigidus* [29]. Apigenin and its analogues, the 7-methyl ether, 7-methyl ether-4'-*O*-glucoside, 7-*O*-glucoside, 4'-*O*-glucoside and 7-*O*-glucuronide were yielded from the aerial parts of *J. acutus* and *J. rigidus* [30]. Apigenin, together with an other flavonoid, luteolin were isolated from the methanolic extract of *J. atratus* and *J. gerardii* [31, 32].

To date, only a few coumarins and coumarinic acid esters were reported from Juncaceae species. Most of the identified coumarins are benzocoumarin derivatives isolated from the aerial parts of *J. acutus*. These benzocoumarins are highly oxidized, and substituents on ring C are identical with those of the phenanthrenes also isolated from the plant. These compounds are biogenetically derived from phenanthrenes [33, 34]. Two coumarinic acid esters were identified from the medulla of *J. effusus* [34].

Terpenes are also relatively rare secondary metabolites of *Juncus* species. Some compounds, namely betulin, betulinaldehyde, phytol, dreminin, *p*-cymen-7-ol acetate, α-cyclogeraniol acetate, *E*-ionone and kaurene were reported from *J. subulatus* [29, 30]. Thymol, pulegone, sabinol and camphor are monoterpene constituents of *J. roemerianus* [35], while effusenone A is a diterpene, isolated from *J. effusus* [36]. Five triterpene glycosides, namely juncoside I–V, were described from the aerial parts of *J. effusus* [37].

Sterols, like β-sitosterol, stigmasta-4-en-3-one, stigmast-4,22-dien-3-one, 5α-spinasterol, stigmasterol, and β-sitosteroyl-β-D-glyceride were isolated from *J. subulatus* and the medulla of *J. effusus* [29–31].

A few members of phenolic acids were isolated from two *Juncus* species. *p*-Coumaric acid, vanillic acid, methyl-*p*-hydroxybenzoate, markhamioside F, canthoside B and caffeic acid-3'-*O*-glucorhamnoside were identified from the medulla of *J. effusus* and aerial parts of *J. acutus* [34, 36].

The presence of dihydrodibenzoxepin-type secondary metabolites is not characteristics of *Juncus* species, two such derivatives were identified from *J. effusus*. Such compounds might originate

in an analogous oxidative coupling with the formation of an ether bridge between the *ortho*-carbons of the aromatic rings [38].

Stilbenes and their derivatives are a quiet rare secondary metabolites in this genus. Only two stilbene glycosides, oxyresveratrol-2-*O*-β-D-glucopyranoside and resveratrol-3',4'-*O*,*O*'-di-β-D-glucopyranoside were isolated from the aerial parts of *J. acutus* [39].

## 2.3. Biosynthesis of phenanthrenes

Phenanthrenes are formed during a specific biosynthetic pathway. These compounds are considered to be derived from stilbenes, a small group of phenylpropanoids characterized by a 1,2-diphenylethylene backbone, since the phenanthrene skeleton could be generated from stilbenes by UV irradiation [40]. All higher plants seem to be able to synthesize malonyl-CoA and CoA-esters of cinnamic acid derivatives, but only few plant species are able to produce stilbenes [41]. Malonyl-CoA and CoA-esters of cinnamic acid derivatives are the precursors of stilbene synthesis, and the stilbene backbone is formed in presence of stilbene synthase. However, stilbene synthase activity is detectable in a small number of plant genera [42].

The biosynthesis of phenanthrenes was investigated using Orchidaceae species, as these species were observed to accumulate dihydrophenanthrenes as phytoalexins by affects of fungal infection and wounding [43].

According to the experimental data it was determined that at least one of the aromatic rings in dihydrophenanthrenes must be originated from phenylpropanoid derivatives. The results of the biosynthetic studies provided evidence for a biosynthetic sequence originating from L-phenylalanine, and leading to 9,10-dihydrophenanthrenes via *m*-coumaric acid, dihydro-*m*-coumaric acid, and bibenzyl (**Fig. 2**) [44]. This condensation can be regarded as the key step in building up the carbon skeleton of 9,10-dihydrophenanthrenes [45].

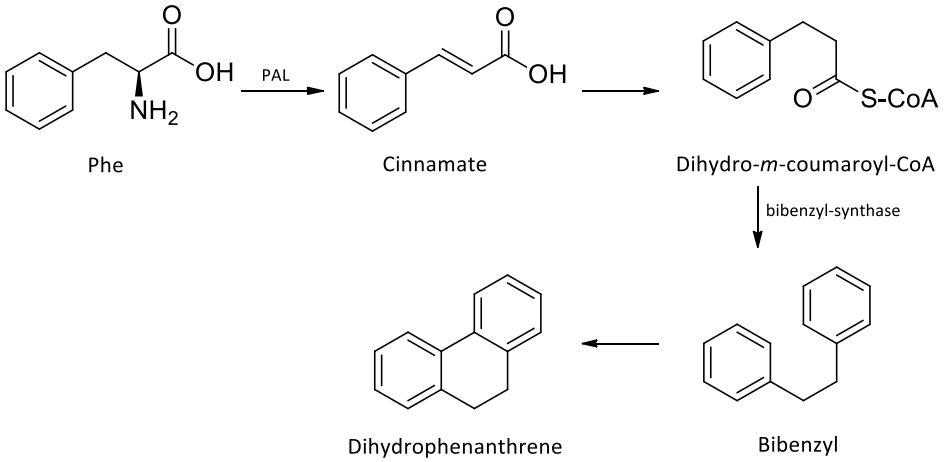



**Figure 2**. Biosynthesis of dihydrophenanthrenes (PAL: phenylalanine ammonia-lyase)

The transformation of dihydro-*m*-coumaroyl-CoA into bibenzyls is catalyzed by bibenzyl synthase [46]. It was also determined that hydroxylation of the aromatic ring should not occur at the stage of stilbenes or phenanthrenes, but rather at the $C_6$-$C_3$ level (phenylpropanoids) [44].

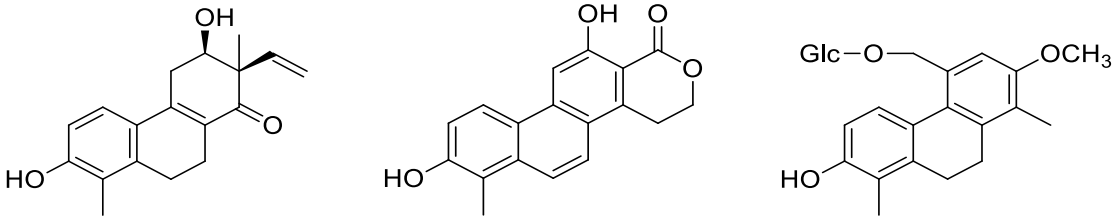
Phenanthrenes are important taxonomic markers because of their limited occurence: vinyl-substituted phenanthrenes have been isolated only from Juncaceae species [47], while prenylated and stilbene-substituted compounds were reported mainly from Orchidaceae species [48].

## 2.4. Phenanthrenes of Juncaceae species

To date, more than one hundred novel phenanthrenes have been isolated from the family Juncaceae, but only from eight *Juncus* and two *Luzula* species (*Juncus acutus, J. atratus*, *J. effusus, J. inflexus, J. maritimus, J. roemerianus, J. setchuensis, J. subulatus, Luzula luzuloides and L. sylvatica*). Almost 20% of the currently known (approx. n = 500) natural phenanthrenes were described from Juncaceae species. Most of these compounds are dihydrophenanthrenes, majority of them are substituted with vinyl group [49–59]. Almost all of the phenanthrenes of the Juncaceae family were isolated from *Juncus* species; four compounds were identified from *L. luzuloides* and eight from *L. sylvatica* [59, 60]. The metabolite profiles of *Luzula* species showed similarities to *Juncus* species.

The most common substituents of monophenanthrenes are methyl, methoxy and hydroxy groups. Almost all of the monophenanthrenes are substituted with a methyl group at C-1, with exception of e.g. jinflexin B; this compound contains an oxymethylene group at this position [61]. Methyl substitution is also common at C-6, C-7 and C-8. Methoxy and hydroxy groups are located mainly at C-2 and C-7 [36]. Only a few compounds are substituted at C-3, and it is exclusively a hydroxy group [62]. The structural particularity of Juncaceae phenanthrenes is the vinyl substitution, and this moiety was described to be connected exclusively on ring C, at positions C-5, C-6 or C-8 [56, 63].

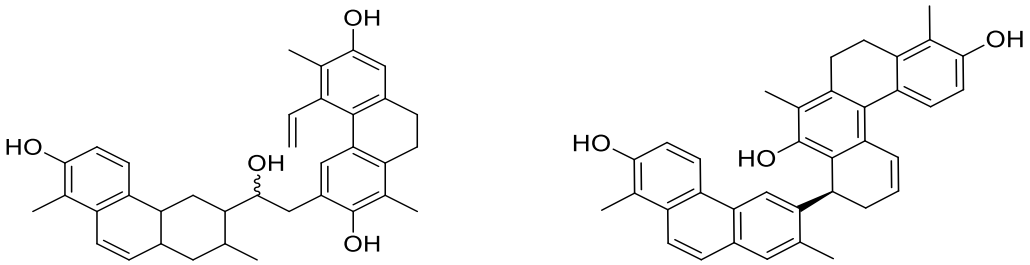
From Juncaceae family homosubstituted and C-9 and C-10 substituted phenanthrenes are not known. Only four monophenanthrenes are containing a carbonyl group in the molecule. Besides carbonyl group, these compounds are substituted only with methyl and hydroxy groups with the exception of jinflexin C (**Fig. 3**); not only its core is unusual, but also the presence of a methyl and a vinyl group at the same carbon (C-7) is unique [56]. In case of dehydrojuncuenin C, the carbonyl group comprises a six-membered lactone ring [63]. All the five formyl group-containing phenanthrenes were isolated from *J. effusus* [8, 61]. Only one compound, juncuenin E is substituted with an amide group [54]. Phenanthrene glycosides are rare in the family Juncaceae. From *J. effusus* five glycosides have been isolated (effusides I–V) [64] (**Fig. 3**).




jinflexin C dehydrojuncuenin C effuside I

**Figure 3.** Some examples of monophenanthrenes isolated from Juncaceae species

Phenanthrenoid dimers have been isolated rarely from Juncaceae species so far. Based on the dimerization pattern between two monomers, two types could be classified. One features a cage-like carbon framework resulted in a hepta- or octacyclic ring system [65], and the other is characterized by a single C–C' linkage between two monomers [63], such as the reported 3–3', 7–7', 8–3', 8–8', and 8–11' linkages [66, 67]. Diphenanthrenes have been reported from *J. acutus* with unusual hepta-and octacyclic structures [67]. Jinflexin D is a dimer with an unprecedented heptacyclic ring system, which may be considered to derive by the coupling of dehydrojuncuenin A, with 2,7-dihydroxy-1,8-dimethyl-5-vinyl-9,10-dihydrophenanthrene through their vinyl groups, forming a unique structure (**Fig. 4**) [53]. In case of dijuncuenins A and B, linkages occur between a C-2 substituent and C-3' or C-8', respectively (**Fig. 4**). The isolated diphenanthrenes are substituted with vinyl, methyl, carbonyl, and hydroxy groups [68]. There are a variety of possible linkage patterns between two monomers. Therefore, additional novel dimeric phenanthrenoids are to be expected.




dijuncuenin A jinflexin D

**Figure 4.** Phenanthrene dimers isolated from Juncaceae species

In cases of asymmetric carbon atom containing phenanthrenes, the absolute configurations were determined by chiral HPLC analysis and calculations based on their ECD spectra. Jinflexin A was determined to be a racemic mixture. The absolute configuration of jinflexin C, juncuenin D and luzulin A was described to be (6*R,*7*R*, 8a*S*, and 1*S*, respectively), the chiral HPLC analysis showed 80, 4, and 25% enantiomeric excess respectively, derived from the *R*-, and *S*-enantiomers [56, 61]. Jinflexin D is

the only chiral dimeric compound, 9% enantiomeric excess was measured, derived from the (6*S*) enantiomer [56].

### 2.5. Chemical constituents of *J. compressus* and *J. tenuis*

From the ethanolic extract of *J. tenuis* luteolin 7-*O*-glycoside and chlorogenic acid were identified by comparing their retention factor values and colour with standards [69].

Extensive phytochemical investigations of *J. compressus* and *J. tenuis* have not been reported yet.

### 2.6. Synthetic phenanthrenoid derivatives with biological activities

The common phenanthrene structure is a polycyclic aromatic hydrocarbon (PAH) type material. The traditional synthesis of PAHs, including phenanthrenes, is rather complicated, includes several sequential reaction steps [70, 71]. As several natural phenanthrenes possessed important biological activities, numerous synthetic methods were developed for more effective phenanthrene derivatives [72]. One of these methods based on the ring-closing metathesis (RCM), which means the metathetic interaction of two alkenes, two alkynes, or an alkene with an alkyne to furnish carbo- or heterocycles with one internal unsaturated bond [73].

As an efficient synthetic route to obtain 9-acyl phenanthrenes, the intramolecular alkyne-carbonyl metathesis of 2'-alkynyl-biphenyl-2-carbaldehydes in presence of catalytic amount of $FeCl_3$ was reported [74].

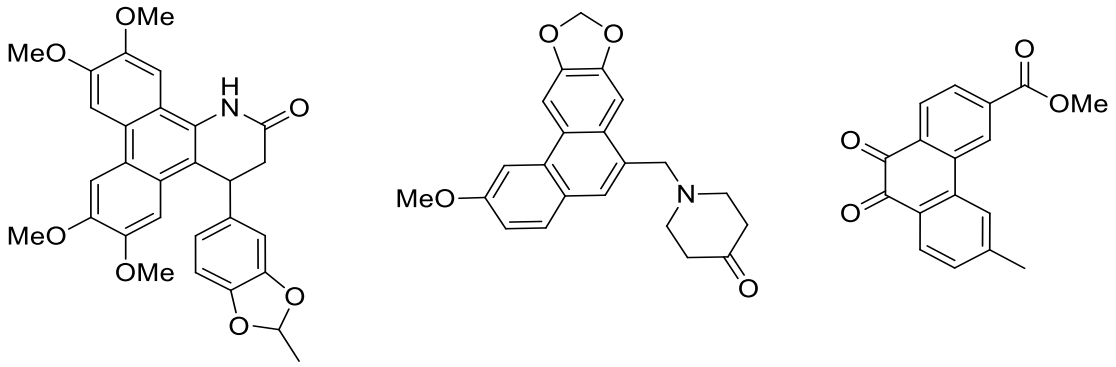
Several types of synthetic phenanthrenes, including heterocyclic compounds and dimers, are possible to obtain during oxidative cyclization of stilbenes. This reaction pathway is also called Mallory-reaction [75]. The *cis/trans* isomerization of stilbenes occurs rapidly during this iodine-catalyzed reaction, as a consequence compositions of *cis*- and *trans*-stilbenes give the same products, but only the *cis*-isomer is capable of further cyclization [76]. The resulting dihydrophenanthrene can be trapped by oxidation to form a phenanthrene, or by elimination gives the suitable substituent in the *ortho*-position on one of the aromatics [77].

Not only formation of the phenanthrene skeleton has attracted considerable attention, but also several synthetic phenanthrenes and their analogues were reported to have considerable biological, especially cytotoxic, antiproliferative and antiviral activities [78]. One type of such synthetic phenanthrene derivatives are the phenanthrene fused dihydrodibenzo-quinolines [79]. These compounds were described to be derived from 2,3,6,7-tetramethoxyphenanthren-9-amine during reactions with substituted aldehydes in EtOH. A such compound, namely 4-(benzo[d][1,3]dioxol-5-yl)-6,7,10,11-tetramethoxy-3,4-dihydrodibenzo[f,h]quinolin-2(1*H*)-one (**Fig. 5/a**) was reported to have broad range of activities on different type of cancer cell lines. Moreover, the dose dependent apoptosis

induced by this compound in A549 lung cell line ($IC_{50}$ value 3.2 μM) is connected to ROS generation [80].

Another investigated and promising group of synthetic compounds are phenanthrene-based tylophorine analogues [81]. These products are considered to be analogues of tylophorine, a naturally occurring alkaloid, which was described to show considerable cytotoxic effects in antitumor screening experiments [82]. The molecules share the tricyclic phenanthrene core structure, but have only one, rather than two methylene linkages to a fourth, rather than fifth pyrrolidine or piperidine D ring. The analogue, *N*-(2,3-methylenedioxy-6-methoxyphenanthr-9-ylmethyl)-4'-piperidone (**Fig. 5**/**b**) was measured to have 0.1 μM $IC_{50}$ value on both A549 and DU-145 (prostate) cell lines. The inhibitory activity of this compound was connected to the inhibition of the activation of Akt and NF-κB signaling pathway in tumor cells [83].

One of the widest class of clinically approved antitumor agents are quinones [84]. Synthetic phenanthroquinones are also one type of investigated products as possibly new antitumor agents. These compounds could be easily formed from the corresponding phenanthrenes by oxidation using chromium trioxide in glacial acetic acid. Beside the obtained and examined *o*-quinones, 8-methyl-9,10-phenanthrenequinone-3-carboxylate had a considerable $IC_{50}$ values 2.8 and 1.0 μM on Hep-2 (epithelial) and Caco-2 (colon) adherent cell lines, respectively (**Fig. 5**/**c**) [85].




a b c

**Figure 5**. Synthetic phenanthrene derivatives with anticancer effects: a dihydro-dibenzo-quinoline (a), a tylophorine analogue (b), and a phenanthraquinone (c)

Comparing the abovementioned structures, presence of a carbonyl group or a quinoidal structure in the molecule can be observed. The cytotoxicity of quinoidal compounds is explained on the basis of many mechanisms including intercalation, inhibition of DNA and RNA, breaking of DNA strands, alteration of cell membrane functions, and free radical mediated alkylation [86, 87].

These experiments resulted in numerous synthetic phenanthrenoid derivatives. Nevertheless, besides preparation and pharmacological investigation of new compounds natural phenanthrenes are

also worthy for investigation. Confusarin is a natural phenanthrene, which was isolated from different plant species, and showed antiproliferative and anti-inflammatory activities [88]. The total synthesis of this compound was carried out starting from gallic acid and benzyl bromide by using stilbene intermediates, which are able to be entailed in free-radical cyclization to provide the phenanthrene skeleton. [89].

## 2.5. Biological activities of Juncaceae phenanthrenes

Natural phenanthrenes have become the objects of numerous research programs, not only because of their structural diversity, but these compounds possess a wide range of pharmacological activities, including antiproliferative, antimicrobial (antibacterial, antiviral, antifungal), antioxidant, anti-inflammatory, spasmolytic and anxiolytic effects [90–94].

The pharmacological activities of phenanhtrenes, isolated from Juncaceae species, are discussed below.

### *2.5.1 Antiproliferative activity*

The inhibitory effect of effusol, juncusol, juncuenin B, dehydrojuncuenin B and juncuenin D was determined on a mouse hippocampal neuroblastoma cell line at 10, 30 and 100 μM for 24 h, by the MTT assay; the assay resulted in 9.9, 25.4, 13.6, 8.4 and 23.7% inhibition values at 100 μM, respectively, and the compounds caused the destruction of neuronal integrity [95].

The antiproliferative activity of dehydroeffusol was investigated on two metastatic cancer cell lines [SGC-7901 (human gastric carcinoma) and AGS (human caucasian gastric adenocarcinoma)] using the Alamar blue assay. The compound inhibited dose dependently (12–48 μM) the gastric cancer cell mediated vasculogenic mimicry on SGC-7901 cells. It also decreased the VE-cadherin expression and exposure, and suppressed the MMP2 protease expression and activity. Dehydroeffusol inhibited the gastric cancer cell adhesion, migration, and invasion effectively without significant acute toxicity [96]. This compound was also observed to inhibit the gastric cell growth and the tumorigenicity through inducing tumor suppressive ER stress responses, and concurrently diminishing tumor adaptive ER responses [96].

The cytotoxicity of juncusol was determined on HeLa cells. The flow cytometric cell-cycle analysis showed that juncusol treatment for 24 h increased the cell population in the G2/M and sub-G1 phases. It also showed pro-apoptotic properties through the presence of active caspase-3, 8, and 9 in HeLa cells, suggesting that juncusol cause cell death by apoptosis induction. Furthermore, this compound inhibited the tubulin polymerization *in vitro* [97].

The screening of phenanthrenes on different adherent cell lines indicated that the presence of a hydroxy group at C-7 is necessary for cytotoxic activity. Vinyl substitution at C-5, and an unsaturated C-9–C-10 bond increased this type of activity [98].

Among the dimeric compounds, effususin B possessed cytotoxic activity against several cell lines [SHSY-5Y ($IC_{50}$ 32.6 μM), HepG2 ($IC_{50}$ 12.9 μM), HeLa ($IC_{50}$ 25.1 μM), MCF-7 ($IC_{50}$ 12.5 μM) and SMMC-7721 ($IC_{50}$ 13.6 μM)] compared to paclitaxel as a positive control [(100 μM (SHSY-5Y), 0.09 μM (SMMC-7721), 36.8 μM (HepG2), 25.9 μM (HeLa), 28.6 μM (MCF-7), and 0.09 μM (SMMC-7721)], in the CCK-8 assay. Effususin A displayed moderate activity on all tested cell lines, while effususins C and D were found to be inactive [99].

### *2.5.2. Antimicrobial activity*

Dehydroeffusol and juncusol were tested against methicillin-susceptible and resistant *Staphylococcus aureus* (MSSA and MRSA), *Bacillus subtilis* and *Candida albicans* in normal (dark) and UVA irradiated conditions [MIC values were 1.6 (UV) and 25.0 μg/mL (dark) (MSSA), 1.6 (UV) and 25.0 μg/mL (dark) (MRSA), 3.1 (UV) and 12.5 μg/mL (dark) (*B. subtilis*), and 1.6 (UV) and 25.0 μg/mL (dark) (*C. albicans*) in case of dehydroeffusol, and 12.5 (UV) and 25.0 μg/mL (dark) (MSSA), 25.0 (UV) and 25.0 μg/mL (dark) (MRSA), 12.5 (UV) and 12.5 μg/ mL (dark) (*B. subtilis*), and 12.5 (UV) and 50.0 μg/mL (dark) (*C. albicans*) in case of juncusol], using gentamicin (MIC values were 0.6 μg/mL for each bacteria, in both condition), methicillin (MIC values 1.6 μg/mL for MSSA, 500.0 μg/mL for MRSA, and 31.25 μg/mL for *B. subtilis*, in both conditions) and nystatin [MIC values [10.0 (UV) and 2.5 μg/mL (dark), in case of *C. albicans*] as positive controls [100].

Furthermore, juncusol have been tested on several other bacterial and fungal strains. *Bacillus* species were inhibited at all concentrations, while *Plannococcus* species were inhibited only at the highest concentration. *Pseudomonas* species, *Mycobacterium smegmatis*, *Enterobacter aerogenes* and *Escherichia coli* were not inhibited at any of the concentrations used [101].

Effusol was proven to be active (MIC 19 μg/ mL, $IC_{50}$ 9.98 μg/mL, respectively) against the wheat pathogen fungi *Zymoseptoria tritici* [102]. The monophenanthrenes juncusol, dehyrojuncuenin B, juncuenin D and jinflexin B, isolated from *J. inflexus*, showed antibacterial activity against MRSA with MIC values of 94 μM, 95 μM, 44 μM, and 338 μM, respectively [56].

### *2.5.3. Anti-inflammatory activity*

The anti-inflammatory properties of juncutol was evaluated by an assay involving the inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells. Juncutol at 10 μM showed pro-antiinflammatory activity (protein expression 11.2%). Dehydrojuncusol and its dimer were also assayed by the same procedure. According to the immunoblot analysis, the inhibitory effect of

dehydrojuncusol on iNOS protein expression (59.0%) decreased dramatically (protein expression > 88.0%) using the dimeric form of this compound [53].

In an other investigation, effususin B inhibited the LPS-induced NO production on RAW 264.7 cells (7.4 μM), while the other three investigated dimers, effususins A, C and D were less active [103].

The anti-inflammatory effects of juncusol ($IC_{50}$ 3.1 μM), juncuenin B ($IC_{50}$ 4.9 μM), and dehydrojuncuenin B ($IC_{50}$ 3.2 μM), isolated from *L. luzuloides*, were determined in a superoxide anion generation assay. Juncuenin B also inhibited the elastase release on human neutrophils in response to fMLP/CB activation, with an $IC_{50}$ of 5.5 μM, which was comparable to that of the positive control [LY294002 (4.8 μM)]. Juncuenin D and luzulin A were inactive in both assays [61].

### *2.5.4. Antioxidant activity*

The antioxidant capacity of 8,8'-bidehydrojuncusol was measured by an ABTS radical cation decolorization assay, and it was observed that the diphenanthrene possessed free radical scavenging activity (85.2%). This effect was comparable to the value of the positive control ascorbic acid (88.7%) [104]. The dose-dependent cellular protective effect of dehydroeffusol was proven by the use of reactive oxygen induced photohemolysis assay ($\tau_{50}$/control $\tau_{50}$ = 5.3 at 10 μM) on human neutrophils. Dehydroeffusol showed 11.3% penetration rate into the erythrocyte membrane (it can affect the antioxidant activity), and it had a high antioxidant effect because of its strong ROS scavenging activity [the $OSC_{50}$ of dehydroeffusol and L-ascorbic acid was 1.7, and 0.41 μM, respectively [105].

### *2.5.5. Spasmolytic activity*

Dehydroeffusol inhibited KCl-, Bay-K8644-, pilocarpine-, and histamine-induced smooth muscle spasms. However, at high doses (30–90 μM), it provoked contractions on the isolated rat jejunum. Therefore, it is suspected that dehydroeffusol may have antagonist activity on L-type $Ca^{2+}$ channels, with agonistic properties at high concentrations [106].

### *2.5.6. Anxiolytic activity*

Dehydroeffusol reduced anxiety in animal studies [elevated plus-maze test: time mice stayed on open arms (OT) = 56.54 s, entries into open arms (OE) > 6; and hole-board test (head dips > 80)] at 5 mg/ kg, without change in motor function [107]. The anxiolytic properties of effusol and juncusol were determined on mice, at 2.5, 5 and 10 mg/kg, by using the same tests (OT = 58.8 s, OE = 6.6 for effusol and OT = 75.5 s, OE = 7.8 for juncusol, respectively) [108].

## 3. AIMS OF THE STUDY

Juncaceae species are a considerable sources of bioactive secondary metabolites, including phenanthrenes. As mentioned above, these rare compounds are a promising group of plant metabolites with wide-range structural and pharmacological attributes. Furthermore, synthetic and semisynthetic phenanthrenes have also been described with considerable pharmacological effects. According to these aspects, the objects of this PhD-work were:

- Isolation and structure determination of phenanthrenes from *Juncus compressus* Jacq. and *Juncus tenuis* Willd.
- *In vitro* pharmacological evaluation of the isolated compounds, and according to the experimental data determination of structure-activity-relationships.
- According to the abovementioned characteristics of quinoidal molecules, preparing oxidised semisynthetic derivatives from the isolated phenanthrenes.
- Antiproliferative investigation of the prepared compounds and comparison of data with those of the starting materials.
- Determination of structure-activity relationships based on the results of pharmacological evaluations and structure informations.

## 4. MATERIALS AND METHODS

### 4.1. General

Different chromatographic techniques were applied in the course of preparative work. Column chromatography (CC) was performed on polyamide (MP Biomedicals), and normal phase vacuum liquid chromatography (VLC) on silica gel (Kieselgel 60 $GF_{254}$, 15 µm, Merck). Medium pressure liquid chromatography (MPLC) was processed with a Combi Flash $Rf^+$ Lumen instrument (Teledyne Isco), rotation planar chromatography (RPC) by a Chromatotron instrument (Model 8924, Harrison Research). Sephadex LH-20 (25-100 µm, Pharmacia Fine Chemicals) was used for gel filtration (GF). Each separation steps were monitored by thin layer chromatography (TLC) on Kieselgel $60F_{254}$ silica plates obtained from Merck (Merck, Germany), and examined under UV light at 254 and 366 nm and at daylight after spraying with vanillin-sulfuric acid reagent and heating at 120 °C for 5 min.

All of the solvents were of at least analytical grade (VWR Ltd, Szeged, Hungary).

High performance liquid chromatography (HPLC) system was comprised of a Waters 600 controller, Waters 600 pump, and Waters 2998 photodiode array detector. The data were acquired and processed with the Empower software. The reaction mixtures and the processed fractions were investigated by HPLC on a Jasco instrument equipped with an MD-2010 Plus PDA detector (Jasco Analytical Instruments, Japan) in a detection range of 210-400 nm, with an Agilent Eclipse XDB C8 column (4.6 × 150 mm, 5 µm) (Agilent Technologies, Inc., USA). MeCN-$H_2O$ solvent system was used as eluent. Racemic mixtures were separated to pure enantiomers on a Lux amylose-1 column (5 µm, 250 × 21.2 mm) (Phenomenex, USA).

The structures of the compounds were elucidated by spectroscopic methods. NMR spectra were recorded in $CD_3OD$ or $CDCl_3$ on a BrukerAvance DRX 500 spectrometer at 500 MHz ($^1H$) and 125 MHz ($^{13}C$). The signals of the deuterated solvents were taken as references. The chemical shift values ($\delta$) were given in ppm and coupling constants ($J$) are in Hz. Two-dimensional (2D) experiments were performed with a standard Bruker software. In the $^1H$-$^1H$ COSY, HSQC and HMBC experiments, gradient-enhanced versions were applied.

High resolution MS spectra were acquired on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer equipped with ESI ion source in positive ionization mode. The data were acquired and processed with the MassLynx software.

For the preparation of the semisynthetic derivatives hypervalent iodine reagents, [bis(trifluoroacetoxy)iodo]benzene (PIFA) and (diacetoxyiodo)benzene (PIDA) were used as oxidizing agents (Sigma-Aldrich, Sweden).

The absolute configuration of the pure stereoisomers was determined using ECD analysis and TDDFT-ECD calculations. Mixed torsional/low-mode conformational searches were carried out by

means of the Macromodel 10.8.011 software using the MMFF with an implicit solvent model for $CHCl_3$ applying a 21 kJ/mol energy window [109]. Geometry reoptimizations of the resultant conformers [B3LYP/6-31+G(d,p) level in vacuo and ωB97X/TZVP with PCM solvent model for MeCN], TDDFT ECD and DFT-NMR calculations were performed with Gaussian 09 [110]. For ECD using various functionals (B3LYP, BH&HLYP, CAM-B3LYP, PBE0) and the TZVP basis set were applied with the same solvent model as in the preceding DFT optimization step. ECD spectra were generated as the sum of Gaussians with 3000 $cm^{-1}$ half-height widths, using dipole-velocity computed rotational strengths. NMR calculations were performed at the mPW1PW91/6-311+G(2d,p) level [111]. Boltzmann distributions were estimated from the B3LYP and the ωB97X energies. The MOLEKEL program was used for visualization of the results [112].

The antiproliferative activity and tumour cell selectivity of the compounds were measured by standard MTT assay. Tumorous cell lines MCF-7, T47D (human breast), HeLa (human cervical), A2780 (human ovarian) and NIH/3T3 (mouse embryonic fibroblast) were provided by European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK); SiHa and C33A (human cervical) cell lines were purchased from LGC Standards GmbH (Wesel, Germany). The cisplatin resistant human ovarian cancer cell line A2780cis (ECACC European Collection of Authentical Cell Culture) was purchased from Merck KGaA (Darmstadt, Germany). MRC-5 (human embryonal lung), KCR and HTB-26 (human breast) fibroblast cell lines were purchased from LGC Promochem (Teddington, UK). All chemicals and materials used for the antiproliferative assays, if otherwise not specified, were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary).

### 4.2. Plant material

The whole plant of *Juncus compressus* Jacq. were collected in June 2014, near Gyula (GPS coordinates are 46°35′46.19′′N, 21°10′18.97′′E). Aerial parts of *J. tenuis* Willd. were collected in the flowering period in the Botanical Garden of the University of Szeged in June 2019. The corresponding voucher specimens (No. 876 and 889) have been deposited at the Herbarium of the Department of Pharmacognosy, University of Szeged. The plant materials were dried at room temperature.

### 4.3. Juncuenin B

Juncuenin B was isolated previously from the methanolic extract of *Juncus inflexus* using chromatographic methods (VLC on silica gel, and gel chromatography on Sephadex LH-20) and was characterised by our group. *J. inflexus* contains this compound in approximately in 0.043% amount [56].

### 4.4. Isolation of compounds from *J. compressus*

The air-dried, whole plant of *J. compressus* (2.2 kg) was ground and percolated with methanol (150 L) at room temperature. The crude methanol extract was concentrated under reduced pressure (323.7 g). The residue was dissolved in 50% methanol and subjected to solvent–solvent partitioning with dichloromethane ($CH_2Cl_2$) (6 L) and ethyl acetate (EtOAc) (3 L), respectively (**Fig. 6**). After evaporation, the $CH_2Cl_2$ fraction (96.4 g) was chromatographed on a polyamide column with mixtures of MeOH–$H_2O$ (1:1 and 4:1 (10 L and 32 L, respectively), each eluent was collected as a fraction). The fraction obtained from the polyamide column with MeOH–$H_2O$ (4:1) (27.5 g) was further chromatographed by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH (from 98:2:0 to 5:5:1 (1000 mL/eluent); volume of each fraction was 100 mL) to yield 28 major fractions (I–XXVIII). Fraction IX (1.3 g) was subjected to VLC on silica gel with *n*-hexane–diethyl ether solvent system (from 1:0 to 0:1) to obtain five sub-fractions (the volume of the collected fractions was 25 mL). From sub-fraction IX/4 compound **1** (5.7 mg) was purified by Sephadex LH-20 column chromatography eluting with $CHCl_3$–MeOH (1:1). Fraction X (1.3 g) was fractionated by MPLC, using *n*-hexane–EtOAc gradient system (from 1:0 to 0:1), to yield 13 sub-fractions (the volume of the collected fractions was 25 mL). Sub-fraction X/6 was purified by gel filtration, applying $CHCl_3$–MeOH (1:1) as eluent to yield compound **2** (3.9 mg). Fraction XII (389 mg) was further chromatographed by RPC on silica gel using *n*-hexane–acetone–MeOH gradient system (from 8:2:0 to 0:0:1; volume of the collected fractions was 15 mL) to obtain 4 sub-fractions. Sub-fraction XII/3 was subjected to column chromatography over Sephadex LH-20 gel, eluting with $CHCl_3$–MeOH (1:1). Sub-fraction XII/3/a was purified by NP-HPLC with a Zorbax Sil (5 µm, 9.4 × 250 mm) column under isocratic conditions, using cyclohexane–EtOAc (8:2) as eluent, at a flow rate of 3.5 mL/min to yield compound **7** ($t_R$ = 6.0 min, 9.7 mg). Fraction XIII (2 g) was rechromatographed on silica gel, using *n*-hexane–EtOAc–MeOH gradient system (from 95:5:0 to 1:1:1) as mobile phase to get 6 sub-fractions (the volume of the collected fractions was 15 mL). Subfraction XIII/2 was subjected to Sephadex LH-20 gel chromatography (MeOH–$CHCl_3$ 1:1) to obtain three fractions, from which XIII/2/1 resulted in compound **5** (8.0 mg) after purifying by NP-HPLC (using Zorbax Sil (5 µm, 9.4 × 250 mm) column and cyclohexane–EtOAc (85:15) as an isocratic eluent system at a flow rate of 3 mL/min, $t_R$ = 8.2 min). Sub-fraction XIII/3 was subjected to column chromatography over Sephadex LH-20 gel (MeOH–$CHCl_3$ 1:1) to gain compound **3** (112 mg). The remaining fraction was purified by RP-HPLC applying a Zorbax octadecylsilane (ODS) (5 µm, 9.4 × 250 mm) column and MeOH–$H_2O$ isocratic system (7:3, flow rate 3.5 mL/min) and compound **4** ($t_R$ = 8.8 min, 4.5 mg) was isolated. Fraction XIII/4 was further separated by gel filtration, using MeOH–$CHCl_3$ (1:1) mixture as mobile phase to obtain three sub-fractions. Sub-fraction XIII/4/2 was purified by RP-HPLC using a Zorbax ODS (5 µm, 9.4 × 250 mm) column, and MeOH–$H_2O$ (3:2) (flow rate 3.5 mL/min) to yield compound **9** ($t_R$ = 10.0

min, 4.3 mg). Fraction XV (124 mg) was subjected to column chromatography over Sephadex LH-20 gel, eluting with MeOH–$CHCl_3$ (1:1) solvent system, and 3 sub-fractions were obtained, from which XV/2 provided compound **6** (3.6 mg) after purification by RP-HPLC (Zorbax ODS (5 µm, 9.4 × 250 mm) with isocratic mobile phase (MeOH–$H_2O$ 85:15); 3.5 mL/min flow rate; $t_R$ = 4.9 min). Fraction XX (183 mg) was separated by Sephadex LH-20 gel chromatography (MeOH–$CHCl_3$ 1:1) to yield three sub-fractions. Sub-fraction XX/3 was further purified by NP-HPLC using a Zorbax Sil (5 µm, 9.4 × 250 mm) column and cyclohexane–EtOAc (85:15) isocratic system, at a flow rate of 3.5 mL/min to afford compound **8** ($t_R$ = 5.5 min, 4.5 mg). Fraction XXVIII (2 g) was further separated by RP-MPLC (MeOH–$H_2O$ from 0:1 to 1:0) to get nine sub-fractions (volume of collected fractions was 25 mL). Sub-fractions XXVIII/4-7 were further purified by Sephadex LH-20 ($CHCl_3$–MeOH 1:1) gel chromatography, and compounds **10** (23.3 mg) and **11** (18.5 mg) were isolated (**Fig. 6**).

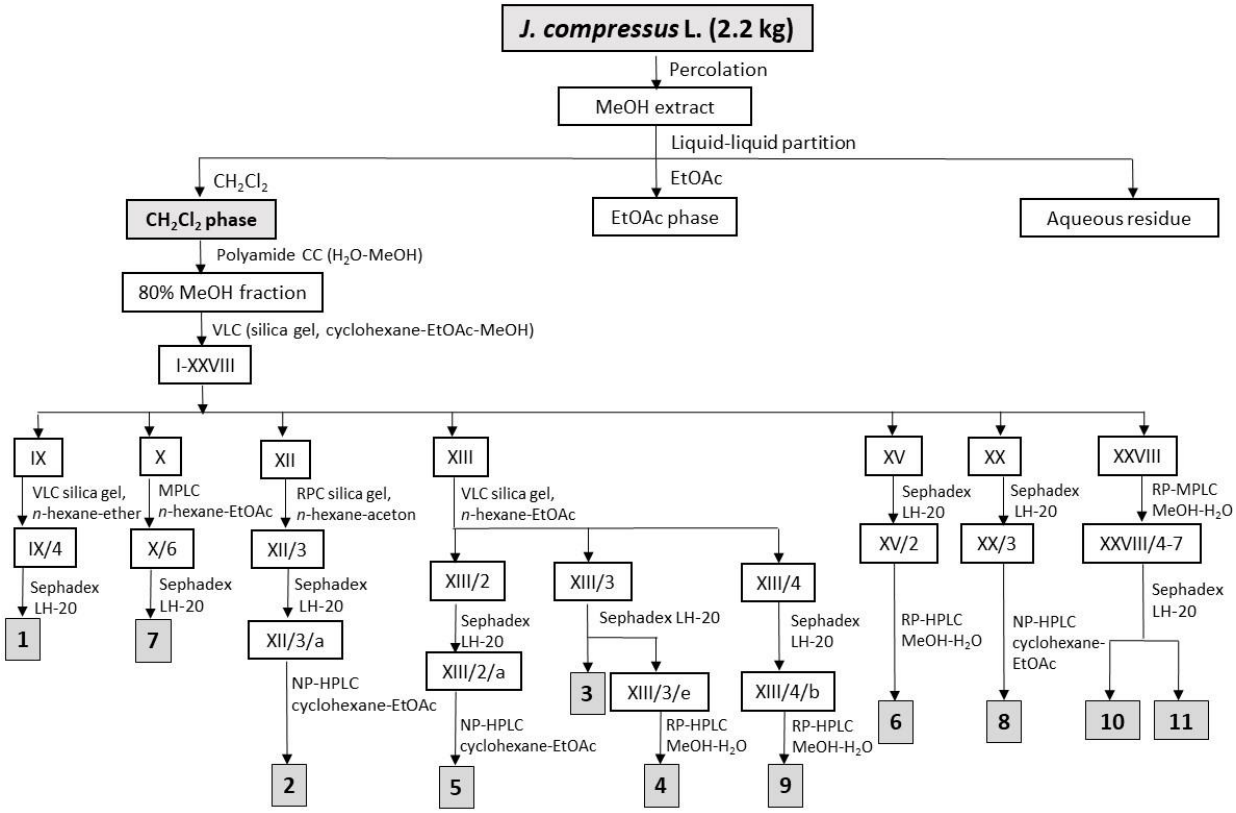



**Figure 6**. Isolation of the compounds of *J. compressus*

### 4.5. Isolation of compounds from *J. tenuis*

The air-dried, whole plant of *J. tenuis* (1.68 kg) was ground and percolated with methanol (50 L) at room temperature. The crude MeOH extract was concentrated under reduced pressure (130.0 g), the residue was dissolved in 50% methanol, and subjected to solvent–solvent partitioning with *n*-hexane (1.5 L) and $CH_2Cl_2$ (3 L), respectively. After evaporation, the $CH_2Cl_2$ fraction (22.5 g) was

chromatographed by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH [from 98:2:0 to 5:5:1 (1000 mL/eluent); volume of each fractions was 100 mL] to yield 3 major fractions (I–III) (**Fig. 7**).

Fraction I (650 mg) was separated by flash chromatography, on normal-phase silica gel with *n*-hexane–EtOAc gradient (linear gradient from 96:4 to 5:95) solvent system, to obtain 2 subfractions (the volume of the collected fractions was 25 mL). Subfraction 1/2 was re-chromatographed on reversed-phase flash chromatography, with $H_2O$–MeOH gradient (linear gradient from 8:2 to 0:1) elution, and compounds **5** (176 mg) and **3** (207 mg) were obtained. Fraction III (270 mg) was further chromatographed by RPC, on silica gel, using *n*-hexane–acetone gradient system (from 9:1 to 0:1, volume of the collected fractions was 15 mL), to obtain 2 subfractions. Subfraction III/2 was purified by RP-HPLC, applying a Zorbax ODS (5 µm, 9.4 × 250 mm) column and MeOH–$H_2O$ gradient system (linear gradient from 25:75 to 94:6) as eluent (flow rate 3.0 mL/min), and compound **12** ($t_R$ = 24.3 min, 4.5 mg) was isolated (**Fig. 7**).

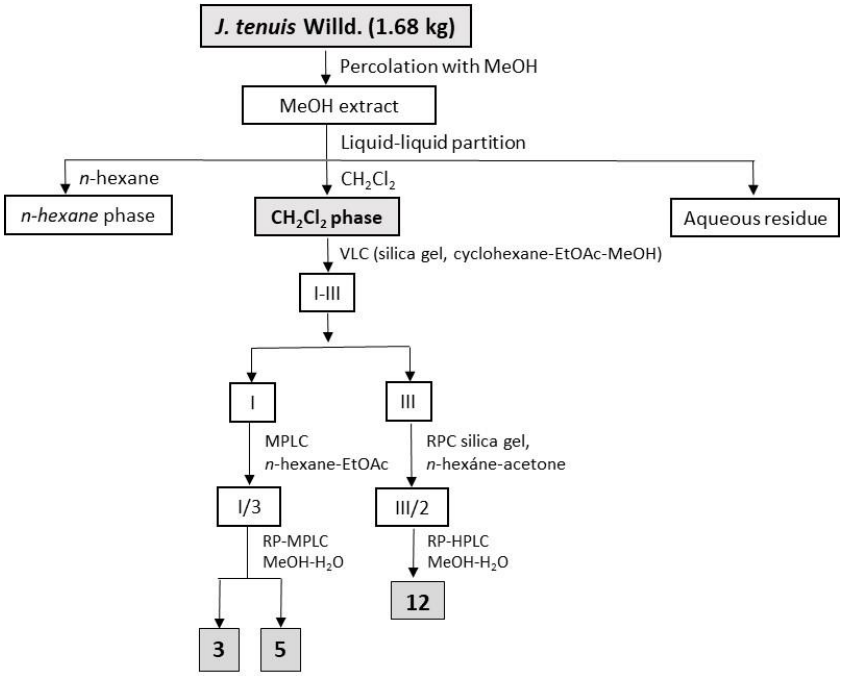



**Figure 7**. Isolation of the compounds of *J. tenuis*

## 4.6. Preparation of the semisynthetic derivatives of juncuenin B

Eleven racemic and enantiomerically pure chiral semisynthetic derivatives (**13**–**23**) (**Fig. 16**) have been prepared from juncuenin B in five transformations (I-V) by hypervalent iodine(III) reagents using a diversity-oriented approach. PIFA and PIDA were used as oxidants (PIFA in processes I-IV, PIDA in process V), under different conditions, in MeCN–MeOH (process I), MeOH (II), EtOH (III), *n*-BuOH (IV, V). PIFA and PIDA are well known reagents for the oxidation of phenols leading to the formation of

quinone-type products [113]. For processing the reactions 50 or 100 mg starting material was dissolved at a concentration of 1 mg/mL, and 2 equiv reagent was added. Each reaction mixture was stirred for 30 min at room temperature (reaction process I at 0 °C). Following the oxidation, the mixture of products were subjected to solid-phase extraction on silica to remove the remaining oxidizing agent and the oxidation sideproducts. The purification process was followed by MPLC and HPLC (**Fig. 8**).

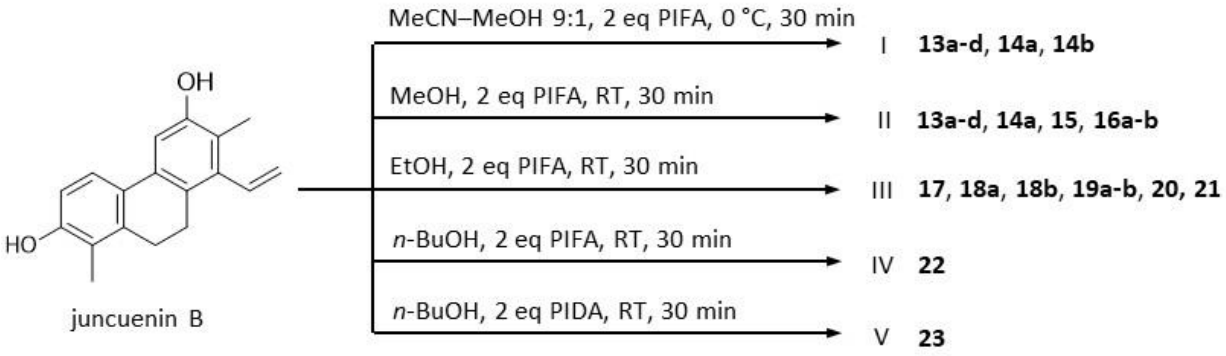



**Figure 8**. Preparation of the semisynthetic derivatives of juncuenin B

From subfr. I/1 compounds **13a**-**d** ($t_R$=6.3 min) were purified on RP-HPLC using MeCN–$H_2O$ (1:1) as eluent (3 mL/min flow rate). Fraction I/2 was also purified by RP-HPLC, MeCN–$H_2O$ (3:2) (3 mL/min flow rate) was applied as eluent to gain compounds **14a** ($t_R$=8.5 min) and **14b** ($t_R$=10.4 min). Fr. II/1, after being chromatographed by RP-HPLC applying MeCN–$H_2O$ (1:1) (flow rate 3 mL/min) as mobile phase, compounds **13a**-**d** ($t_R$=6.3 min) and **14b** ($t_R$=10.1 min) were obtained. Fraction II/2 and II/3 were separated under the same conditions as II/1. From fraction II/2 compounds **13a**-**d** and **15** ($t_R$=9.6 min); from II/3 compounds **13a**-**d** and **16a**-**b** ($t_R$=13.0 min) were isolated. Fr. III/1 gave compound **17** ($t_R$=11.0 min) after purification by HPLC with MeCN–$H_2O$ (1:1) as mobile phase (flow rate 3 mL/min). Fraction III/2 was rechromatographed by HPLC, applying MeCN–$H_2O$ gradient system (flow rate 3 mL/min) as eluent to yield compounds **18a** ($t_R$=9.5 min) and **18b** ($t_R$=10.7 min). Fraction III/4 was found to be pure and determined as compounds **19a**-**b**. Fraction III/6 was subjected to HPLC using MeCN–$H_2O$ (1:1) (flow rate 3 mL/min) as mobile phase and gained compounds **19a**-**b** ($t_R$=14.3 min) and **20** ($t_R$= 10.0 min). Fraction III/7 was purified by RP-HPLC with MeCN–$H_2O$ (1:1) as eluent to gain compound **21** ($t_R$=7.5 min). Subfr. IV/1 was determined as compound **22**. Subfr. V/2 was subjected to HPLC, eluting with MeCN–$H_2O$ (3:2) (flow rate 3 mL/min) as mobile phase and compound **23** ($t_R$= 11.0 min) was isolated.

### 4.7. Preparation of the semisynthetic derivatives of juncusol and effusol

For processing the reactions of juncusol and effusol, PIFA was used as oxidizing agent in different reaction media. 50 mg starting material was dissolved at a concentration of 1 mg/mL, and 2 equiv reagent was added. Each reaction mixture was stirred for 30 min at room temperature. MeCN–MeOH 9:1 (V and VII) and MeCN–EtOH 9:1 (VI and VIII) were chosen as solvents. The reaction mixtures were

fractionated by MPLC with *n*-hexane–EtOAc gradient solvent system. The final purification steps were processed on HPLC with an Agilent Eclipse XDB C-8 column (9.4x250 mm, 5µm), using MeOH–$H_2O$ 3:2 and 1:1 mobile phases with 3.0 mL/min flow rate (**Fig. 9**).

From reaction VI, compound **24** was isolated after purification. From Fr. VII, two compounds (**25a** and **25b**) were yielded after separation with RP-HPLC (MeOH–$H_2O$ 3:2 solvent system, $t_R$= 15.3 and 17.3 min, respectively). From reaction mixture VIII, after the purification process compound **26** was obtained. After separation of reaction mixture IX, two subfractions were gained, from which IX/1 was subjected to RP-HPLC (with MeOH–$H_2O$ 1:1 eluent) and two compounds (**27a** and **27b**) were isolated ($t_R$= 23.0 and 25.3 min, respectively).

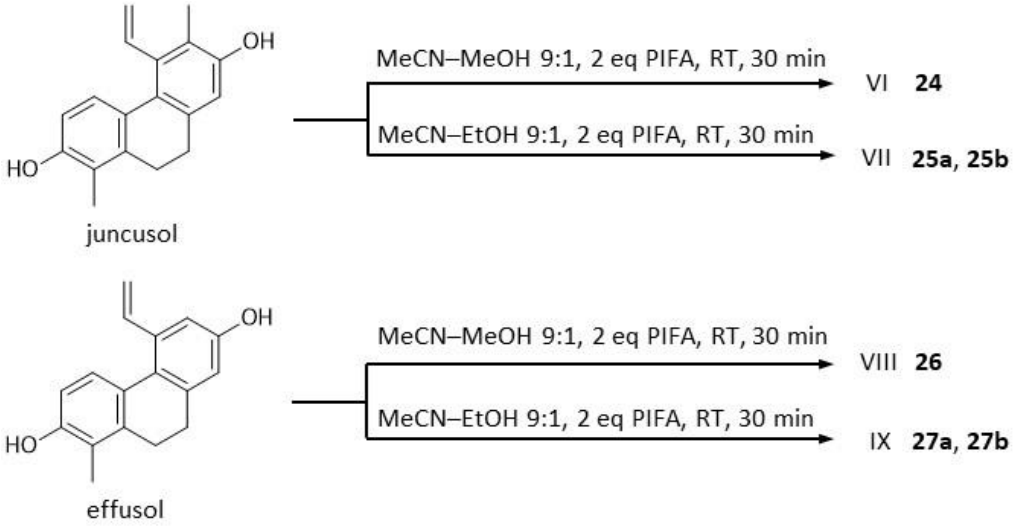



**Figure 9**. Preparation of the semisynthetic derivatives of juncusol and effusol

## 4.8. Separation of racemic mixtures

As racemic mixtures of compounds **13a**-**d**, **16a**-**b** and **19a**-**b**, among the derivatives of juncuenin B, exerted substantial antiproliferative activities (see in section **RESULTS**), these compounds were separated to pure enantiomers to further characterise their biological attributes. For obtaining pure enantiomers, a chiral HPLC method was used with a Lux amylose-1 column (5 µm, 250 × 21.2 mm), eluting with cyclohexane–isopropanol 95:5 (**13a**-**d**) and 4:1 (**16a**-**b**, **19a**-**b**) mixtures (15 mL/min). Compound **13a** was recorded at $t_R$=13.6 min, **13b** at $t_R$=17.4 min, **13c** at $t_R$=21.6 min and **13d** $t_R$=23.6 min, respectively (**Fig. 10**). The retention times of **16a** and **16b** were $t_R$=5.5 min and 10.3 min, while in case of **19a** and **19b** $t_R$=4.7 and 9.1 min were recorded, respectively.

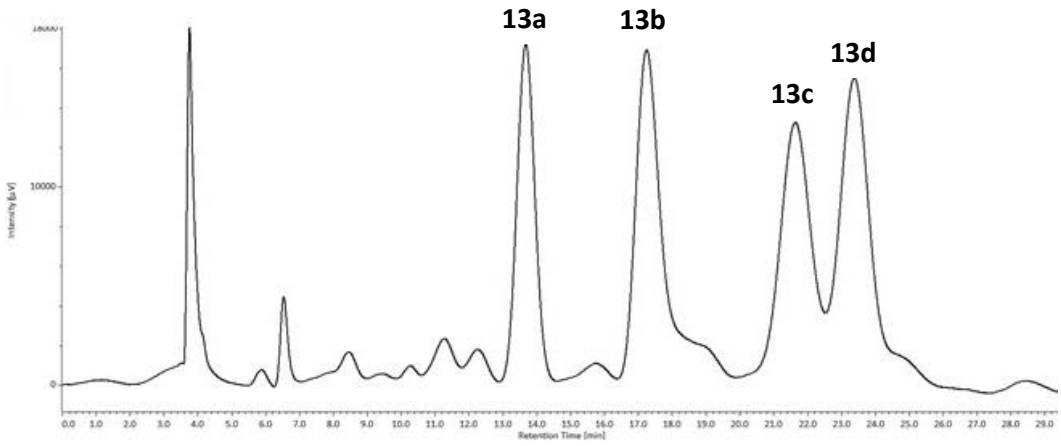



**Figure 10**. HPLC chromatogram of **13a**-**d**

### 4.9. Determination of the antiproliferative activity

Growth-inhibition properties of the isolated natural compounds and their semisynthetic analogues were determined by two methods of standard MTT assay. The first one was applied for determination of antiproliferative activity of phenanthrenes of *J. compressus* and the semisynthetic derivatives of juncuenin B. During the measurement, all cell lines were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% penicillin–streptomycin–amphotericin B mixture, and were stored in humidified air containing 5% $CO_2$ at 37 °C. All cell types were seeded into 96–well plates at a density of 5000 with exception of C33a which was seeded at a density of 10 000 and treated by increasing concentrations (0.1–30 µM) of the drug candidates for 72h under cell culturing conditions. After the incubation, 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to samples for 4 h and precipitated blue formazan crystals were dissolved by dimethyl sulfoxide (DMSO). Absorbance values of the samples were measured at 545 nm by microplate reader (Stat Fax-2100, Awareness Technologies Inc., Palm City, FL, USA) and untreated cells were used as control. Normalised sigmoidal dose-response curves were fitted to the determined data and the $IC_{50}$ values were calculated by GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as a reference agent in the same concentration range.

The other method was carried out for the investigation of compounds isolated from *J. tenuis* and the semisynthetic derivatives of juncusol and effusol. For this measurement, the adherent cells were cultured in 96-well flat-bottomed microtiter plates, using EMEM supplemented with 10% heat-inactivated fetal bovine serum or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, respectively. The density of the cells was adjusted to $6 \times 10^3$ cells in 100 µL per well, the cells were seeded for 24 h at 37 °C, with 5% $CO_2$, and then the medium was removed from the plates, and fresh medium (100 µL per well) was added to the cells. The effects of increasing concentrations of

compounds on cell proliferation were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in the appropriate medium; the dilutions of compounds were performed in separate plates and then added to the cells. The starting concentration of the compounds was 100 µM, and two-fold serial dilution was performed (concentration range was 100–0.19 µM). The culture plates were incubated at 37 °C for 72 h; at the end of the incubation period, 20 µL of MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a stock solution of 5 mg/mL) was added to each well. After incubation at 37 °C for 4 h, 100 µL of sodium dodecyl sulfate (SDS) (Sigma) solution (10% in 0.01 M HCl) was added to each well, and the plates were further incubated, at 37 °C, overnight. Cell growth was determined by measuring the optical density (OD) at 540/630 nm, with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Mean $IC_{50}$ values were obtained by best-fitting the dose-dependent inhibition curves in GraphPadPrism5 program (GraphPad Software version 5.00 for Windows, San Diego, CA, USA) from four parallel experiments for each cell line.

## 5. RESULTS

### 5.1. Phytochemical investigation of *J. compressus* and *J. tenuis*

Dried, whole plant material of *J. compressus* (2.2 kg) was ground and extracted with methanol at room temperature. After concentration, the extract was dissolved in 50% aqueous methanol, and solvent–solvent partition was performed with $CH_2Cl_2$ and EtOAc. The $CH_2Cl_2$ phase was separated and purified with the combination of different chromatographic methods (CC, VLC, gel filtration, RPC, MPLC, and HPLC) to afford 11 compounds (**1**–**11**) (**Fig. 15**).

The plant material of *J. tenuis* (1.68 kg) was ground and percolated with methanol at room temperature. The extract was dried under vacuo, dissolved in 50% aqueous methanol, and partitioned with *n*-hexane and $CH_2Cl_2$. The purification of the $CH_2Cl_2$ phase was performed by using different chromatographic methods (VLC, RPC, MPLC, and HPLC) and 3 compounds (**3**, **5** and **12**) were identified (**Fig. 15**)

The structure of the compounds was elucidated by extensive spectroscopic analysis, using one- ($^1H$, $^{13}C$ NMR, and JMOD) and two-dimensional NMR ($^1H$-$^1H$ COSY, HSQC, HMBC, NOESY) spectroscopy, HRESIMS and comparison of the spectral data with literature data.

Compound **1** was obtained as an amorphous solid. Its HRESIMS provided the molecular formula $C_{19}H_{20}O_2$, through the presence of a peak at *m/z* 281.1532 $[M+H]^+$ (calcd. for $C_{19}H_{21}O_2$, 281.1573). The $^1H$ NMR spectrum (**Table 1**) showed signals of two *ortho*-coupled aromatic protons ($\delta_H$ 6.71 d and 7.62 d), one aromatic proton as a singlet ($\delta_H$ 6.67), two methyls ($\delta_H$ 2.29 and 2.22), two methylenes ($\delta_H$ 2.64 and 2.70), a vinylic system at $\delta_H$ 6.78, 5.52, and 5.25 (C-12, C-13), and signal of a methoxy group ($\delta_H$ 3.85).

In the $^1H$ NMR spectrum, two methylene signals ($H_2$-9, $H_2$-10) indicated this compound to be a 9,10-dihydrophenanthrene derivative (**Fig. 11**). In the JMOD spectrum, the presence of 19 carbon signals was detected (**Table 1**). In the $^1H$-$^1H$ COSY spectrum, correlations were observed between $\delta_H$ 6.71 d and $\delta_H$ 7.62 d (H-3–H-4), $\delta_H$ 2.64 m and $\delta_H$ 2.70 m (H-9–H-10), and $\delta_H$ 6.78 dd and $\delta_H$ 5.52 d, 5.25 d (H-12–H-13). The methoxy group was placed to C-2 as confirmed by HMBC correlations of the $OCH_3$ ($\delta_H$ 3.85) with C-2 ($\delta_C$ 156.2) (**Fig. 12**). The location of the methyl groups was also concluded from the HMBC spectrum, as proton signal at $\delta_H$ 2.22 ($CH_3$-11) showed correlations with $\delta_C$ 122.7 (C-1), 139.5 (C-1a), and 156.2 (C-2), whereas signal at $\delta_H$ 2.29 ($H_3$-14) was found to be in correlation with $\delta_C$ 120.6 (C-6), 137.1 (C-5) and 152.5 (C-7). The position of the vinyl group was verified according to the HMBC correlation between $\delta_H$ 5.52 d, and 5.25 d and $\delta_C$ 137.1 ( $CH_2$-13–C-5).

**Table 1**. NMR spectroscopic data of compressin A (**1**) [500 MHz ($^1$H), 125 MHz ($^{13}$C), $\delta$ in ppm, $CDCl_3$]

| Atom | $\delta_H$ (*J* in Hz) | $\delta_C$, type | HMBC (H→C) |
|---|---|---|---|
| 1 | | 122.7, C | |
| 1a | | 139.5, C | |
| 2 | | 156.2, C | |
| 3 | 6.71, d (8.6) | 106.9, CH | 1, 2, 4a |
| 4 | 7.62, d (8.6) | 128.3, CH | 1a, 2, 5a |
| 4a | | 127.5, C | |
| 5 | | 137.1, C | |
| 5a | | 127.6, C | |
| 6 | | 120.6, C | |
| 7 | | 152.5, C | |
| 8 | 6.67,s | 113.2, CH | 5a, 6, 7, 9 |
| 8a | | 137.9, C | |
| 9 | 2.64, m (2H) | 30.5, $CH_2$ | 1a, 5a, 8, 8a |
| 10 | 2.70, m (2H) | 25.9, $CH_2$ | 1, 1a, 4a, 8a |
| 11 | 2.22, s | 11.9, $CH_3$ | 1, 1a, 2 |
| 12 | 6.78, dd (17.9, 11.4) | 137.8, CH | 5, 5a, 6 |
| 13 | 5.52, d (11.4) | 119.8, $CH_2$ | |
| | 5.25, d (18.0) | | |
| 14 | 2.29, s | 13.4, $CH_3$ | 5, 6, 7 |
| $OCH_3$ | 3.85, s | 55.7, $CH_3$ | 2 |

The NOESY correlations further confirmed the structure of the compound **1**. Overhauser effects were detected between H-3/H-4, H-3/$OCH_3$-2, $OCH_3$-2/$H_3$-11, H-8/H-9, and $CH_3$-14/H-12. All of the above evidence confirmed the planar structure of **1** named as compressin A.

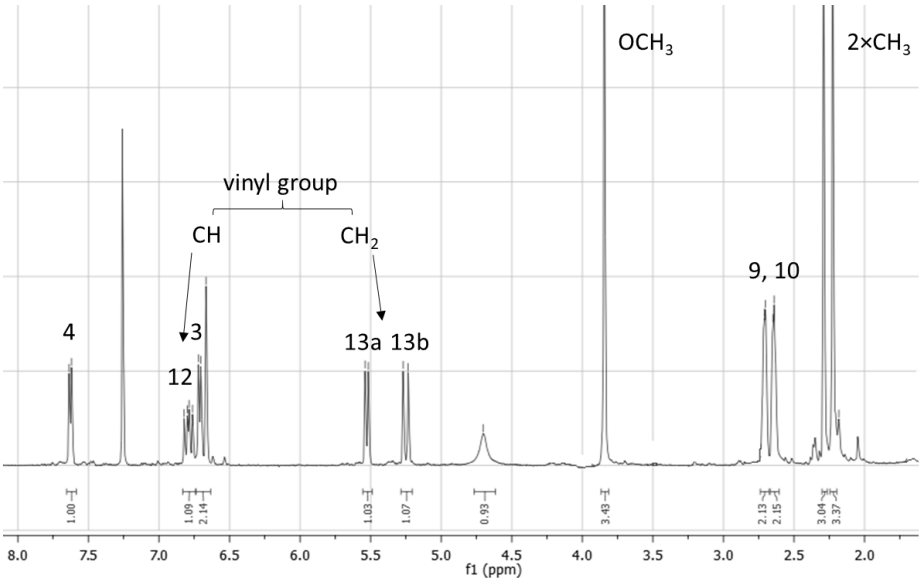



**Figure 11.** $^1$H NMR spectrum of compressin A (**1**)

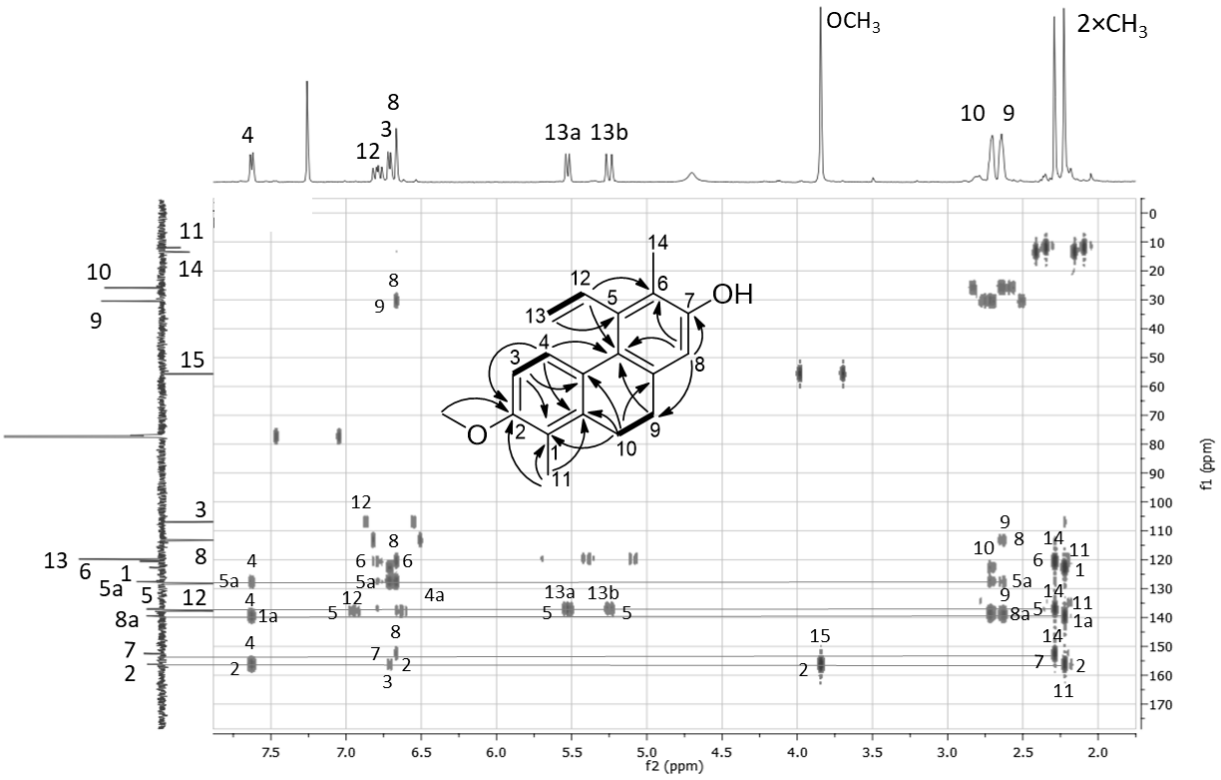



**Figure 12.** HMBC spectrum, and $^{1}$H–$^{1}$H COSY (**—**) and HMBC (H→C) correlations of compressin A (**1**)

Compound **2** was obtained as an amorphous solid. In the $^{1}$H NMR spectrum signals of two methyls ($\delta_H$ 2.28 and 2.27), two singlets of aromatic protons ($\delta_H$ 7.66 and 6.66 s), a vinyl group ($\delta_H$ 6.77 dd/$\delta_H$ 5.57 d and 5.25 d), and two hydroxyl groups ($\delta_H$ 5.56 s and 4.73 s) were observed (**Table 2**). The presence of protons at $\delta_H$ 2.67 m and 2.62 m (2 × 2H) indicated this compound to be a dihydrophenanthrene according to the saturated C-9–C-10 bond in the B ring.

The JMOD spectrum (**Table 2**) showed 18 signals close similar to juncusol [53] and the HRESIMS established a molecular formula $C_{36}H_{34}O_4$ (*m/z* 531.4064 $[M+H]^+$, calcd. for $C_{36}H_{35}O_4$, 531.4607), suggesting the dimeric nature of compound **2**. Moreover, the NMR data of **2** were found to be greatly similar to those of juncusol, except of the replacement of a methine group at C-3 of juncusol by a quaternary carbon ($\delta_C$ 116.4, C-3) in **2**.

In the $^{1}$H-$^{1}$H COSY spectrum two correlations were observed between $\delta_H$ 6.77 dd and 5.57 dd, and 5.25 dd ($CH_2$-13–CH-12, $CH_2$-13'–CH-12'), and between $\delta_H$ 2.67 m and 2.62 m (H-9–H-10, H-9'–H-10') (**Fig. 13**).The vinyl groups according to the HMBC correlation between $\delta_H$ 5.25 (H-13,13') and $\delta_C$ 137.8 are connected to C-5 and C-5'. The position of the methyl groups were also indicated by the HMBC spectrum, where $\delta_H$ 2.28 (H-11,11') was correlated with quaternary carbon atoms $\delta_C$ 122.0 (C-1,1'), 138.6 (C-1a,1a') and 147.6 (C-2,2'), and protons at $\delta_H$ 2.27 s ($H_3$-14) were in correlation with $\delta_C$ 120.7 (C-6,6'), 137.8 (C-5,5') and 152.3 (7,7'), respectively (**Fig. 13**).

**Table 2**. NMR spectroscopic data for compressin B (**7**) [500 MHz ($^1$H), 125 MHz ($^{13}$C), $\delta$ in ppm, $CDCl_3$]

| Atom | $\delta_H$ (*J* in Hz) | $\delta_C$, type | HMBC (H→C) |
|---|---|---|---|
| 1, 1’ | | 122.0, C | |
| 1a, 1a’ | | 138.6, C | |
| 2, 2’ | | 147.6, C | |
| 3, 3’ | | 116.4, C | |
| 4, 4’ | 7.66, s | 127.3, CH | 1a, 1a’, 2, 2’, 3, 3’, 5a, 5a’ |
| 4a, 4a’ | | 128.1, C | |
| 5, 5’ | | 137.8, C | |
| 5a, 5a’ | | 126.3, C | |
| 6, 6’ | | 120.7, C | |
| 7, 7’ | | 152.3, C | |
| 8, 8’ | 6.66, s | 113.2, CH | 5a, 5a’, 6, 6’, 7, 7’, 9, 9’ |
| 8a, 8a’ | | 137.2, C | |
| 9, 9’ | 2.62, m | 30.2, $CH_2$ | 1a, 1a’, 5a 5a’, 8, 8’, 8a, 8a’, 10, 10’ |
| 10, 10’ | 2.67, m | 25.6, $CH_2$ | 1, 1’, 1a, 1a’, 4a, 4a’, 8a, 8a’, 9, 9’ |
| 11, 11’ | 2.28, s | 12.6, $CH_3$ | 1, 1’, 1a, 1a’, 2, 2’ |
| 12, 12’ | 6.77, dd (18.0, 11.4) | 137.1, CH | 13, 13’ |
| 13, 13’ | 5.57, dd (11.4, 1.7) | 120.5, $CH_2$ | 12, 12’ |
| | 5.25, dd (18.0, 1.7) | | |
| 14, 14’ | 2.27, s | 13.3, $CH_3$ | 5, 5’, 6, 6’, 7, 7’ |
| 2-OH, 2’-OH | 5.56, s | | 1, 1’, 2, 2’, 3, 3’ |
| 7-OH, 7-OH’ | 4.73, s | | |

Since compound **2** showed only 18 carbon signals instead of 36 in the JMOD spectrum, it should be a symmetric dimer. From the above findings, **2** was thus proposed to be a dimer composed of two juncusol units. The linkage of the monomeric phenanthrene units was determined to be 3-3’ based on the HMBC correlations detected between $\delta_H$ 7.66 (H-4,4’) and $\delta_C$ 116.4 (C-3,3’) and 147.6 (C-2,2’).

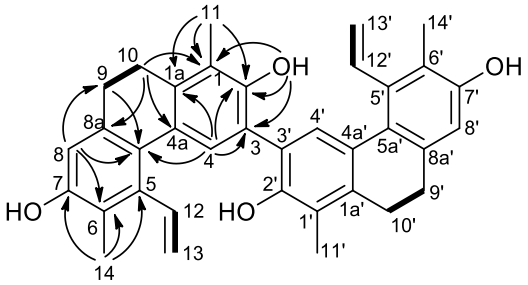



**Figure 13.** Diagnostic COSY (**—**) and HMBC (H→C) correlations of compound **2**

The NOESY correlations confirmed the structure of compound **2**. Overhauser effects were detected between H-4,4’/H-12,12’, H-8,8’/H-9,9’, and $CH_3$-14,14’/H-13,13’ (**Fig. 14**). On the basis of the above findings, the structure of this compound was established as depicted in structural formula **2**, and named as compressin B.

The structure elucidation of all the other compounds was processed using the same method described in the case of compressins A (**1**) and B (**2**) and comparison with literature data (**Fig. 15**).

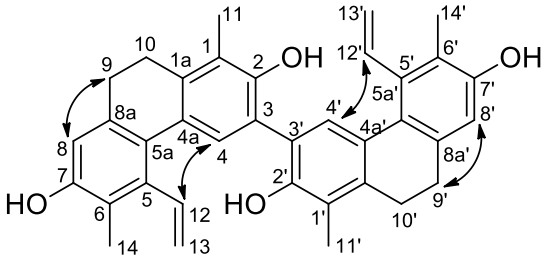
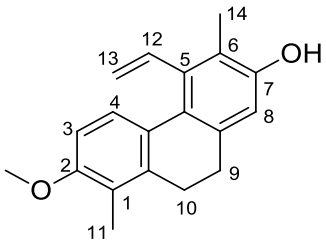
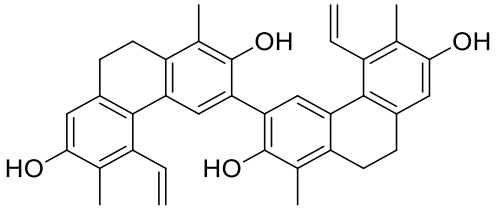


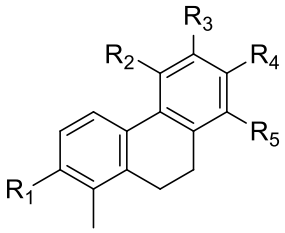
**Figure 14.** NOESY correlations of compound **2**



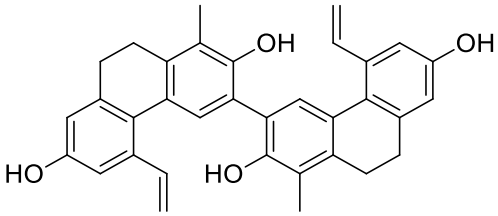
**1**



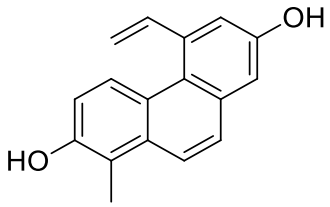
**2**



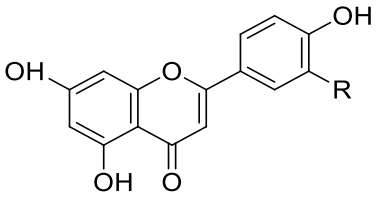
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| **3** | OH | $CH=CH_2$ | H | OH | H |
| **4** | OH | $CH(CH_3)OCH_3$ | H | OH | H |
| **5** | OH | $CH=CH_2$ | $CH_3$ | OH | H |
| **6** | OH | $CH=CH_2$ | H | $CH_2OH$ | H |
| **7** | $OCH_3$ | $CH=CH_2$ | H | OH | H |
| **12** | OH | $CH=CH_2$ | H | OH | $CH_3$ |



**8**



**9**



| | R |
|---|---|
| **10** | H |
| **11** | OH |

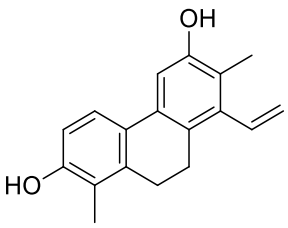
**Figure 15.** Structures of the compounds (**1**–**12**) isolated from *J. compressus* and *J. tenuis*

Compounds **1** and **2** were determined to be new natural metabolites, and were named as compressins A and B. Besides the new compounds five dihydrophenanthrenes [effusol (**3**), effususol (**4**), juncusol (**5**), 2-hydroxy-1-methyl-7-oxymethylene-5-vinyl-9,10-dihydrophenanthrene (**6**), 7-hydroxy-1-methyl-2-methoxy-5-vinyl-9,10-dihidrophenanthrene (**7**)], one phenanthrene [dehydroeffusol (**9**)], one phenanthrene dimer [effususin A (**8**)], and two flavonoids (apigenin and luteolin (**10**, **11**) were also isolated from *J. compressus*. From *J. tenuis* three known phenanthrenes [effusol (**3**), juncusol (**5**) and 2,7-dihydroxy-1,8-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**12**)] were obtained. All compounds were isolated for the first time from the plants.

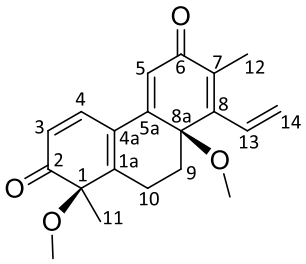
## 5.2. Semisynthetic derivatives of juncuenin B

Eleven semisynthetic derivatives (**13**–**23**) have been prepared from juncuenin B, as starting material during five reaction processes with different reagents, solvent systems, and temperature (**Fig. 16**). Juncuenin B is a naturally occurring dihydrophenanthrene, that was previously isolated from the roots of *J. inflexus*. The purification and separation of reaction mixtures were performed by MPLC and HPLC techniques, their structure determination was based on NMR spectroscopic and mass spectrometric measurements.

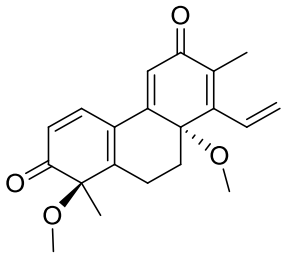
As all these molecules are containing asymmetric carbon atoms, based on the NMR spetroscopic data only the planar structure of the racemic mixtures could be determined.



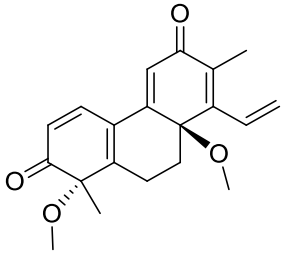

juncuenin B



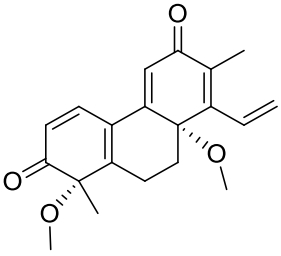

(1*R*,8a*R*)-**13a**



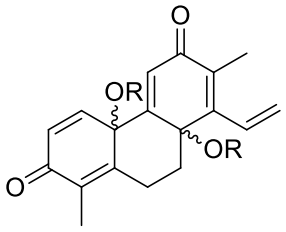

(1*R*,8a*S*)-**13b**



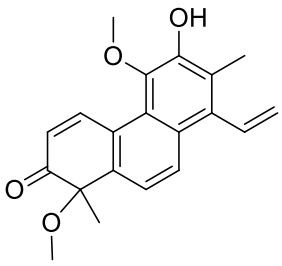

(1*S*,8a*R*)-**13c**



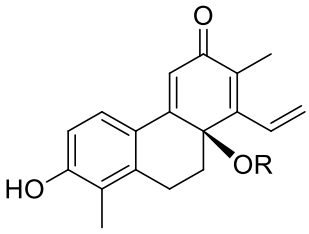

(1*S*,8a*S*)-**13d**



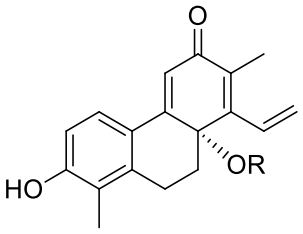

R=$CH_3$ *ent*-**14a**, *ent*-**14b**
R=$C_2H_5$ *rac*-**17**



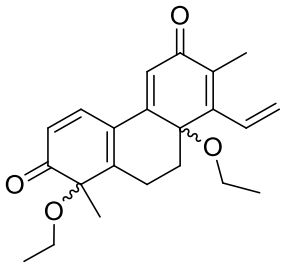
*rac*-**15**



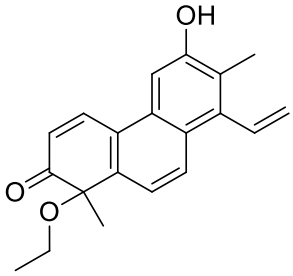
R=$CH_3$ (*R*)-**16a**
R=$C_2H_5$ (*R*)-**19a**



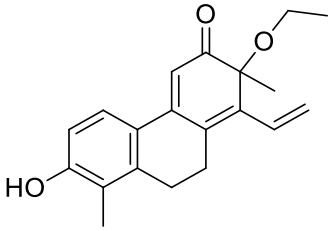
R=$CH_3$ (*S*)-**16b**
R=$C_2H_5$ (*S*)-**19b**



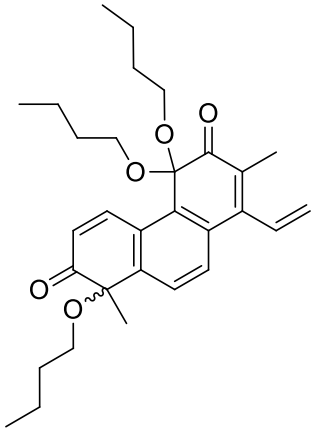
*ent*-**18a**, *ent*-**18b**



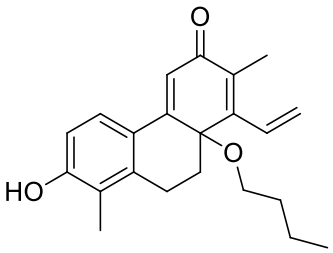
*rac*-**20**



*rac*-**21**



(1*R*,*S*;5*R*,*S*)-**22**



*rac*-**23**

**Figure 16.** Structure of the semisynthetic derivatives of juncuenin B (**13**–**23**)

In the NMR spectra of **13a**-**d**, two sets of signals could be identified that were attributed to two racemic diastereomer pairs, **13a**+**d** and **13b**+**c** (ratio 1:1). The $^{1}$H NMR spectrum of the **13a**-**d** mixture (**Appendix, Tables A1** and **A2**) showed the presence of a pair of isomeric compounds, as some signals were duplicated. The NMR spectra of the mixture of **13b**+**c** were similar to those of **13a**+**d**, only small differences were observed for the $CH_3$O-1, C-10, and C-11 resonances of **13a**+**d** and **13b**+**c**, which suggested that these compounds are structurally related to each other. Some $^{1}$H and $^{13}$C NMR signals were duplicated in the spectra of **13b**+**c**, revealing the presence of a diastereomeric pair as for **13a**+**d**.

To elucidate the relative and absolute configuration of **13a**-**d** the solution TDDFT-ECD method was applied on (1*R*,8a*R*)-**13** and (1*R*,8a*S*)-**13**. The initial Merck Molecular Force Field (MMFF) conformational search resulted in 9 and 9 conformers in a 21 kJ/mol energy window, respectively. These geometries were reoptimized at the ωB97X/TZVP PCM/MeCN level yielding 3 conformers for both stereoisomers. ECD spectra were then computed at various levels and compared to the experimental spectra of **13a**-**d**. Since there were only minor differences in the experimental ECD specta of **13a** vs. **13b** and **13c** vs. **13d**, the ECD was mainly infuenced by one of the two chirality centers. Boltzmann-averaged ECD of both (1*R*,8a*R*)-**13** and (1*R*,8a*S*)-**13** gave acceptable to good agreement with both **13a** and **13b**, therefore for the ECD C-1 has the major effect and the absolute configuration of this stereocenter can be safely determined as (*R*) for the first two (i.e. **13a** and **13b**) and (*S*) for the last two (i.e. **13c** and **13d**) isomers. The shape of the B3LYP/TZVP PCM/MeCN and PBE0/TZVP PCM/MeCN levels resembling better those of the original ones in both cases were closer to **13a** for (1*R*,8a*R*)-**13** and **13b** for (1*R*,8a*S*)-**13**. Although usually both estimation of Boltzmann-distribution and TDDFT-ECD calculations can have a certain amount of error, the better agrement allows tentative assigment of C-8a for **13a** as (*R*) and for **13b** as (*S*). In order to confirm the ECD results, the MMFF conformers were reoptimized at the B3LYP/6-31+G(d,p) level and DFT-NMR calculations were performed at the mPW1PW91/6-311+G(2d,p) level. Unfortunately the chemical shift differences between the experimental values of **13a** and **13b** and the computed ones of (1*R*,8a*R*)-**13** and (1*R*,8a*S*)-**13** were small for most carbons and DP4+ analysis suggested (1*R**,8a*R**) relative configuration for both **13a** and **13b** prohibiting utilization of the DFT-NMR results. Specific optical rotation values were small and the isolated amounts were not sufficient for a VCD study.

All NMR characteristics of compound **14b** were similar to that of compound **14a**, indicating that they are diastereomers. The main differences between **14b** and **14a** were found between the $^{13}C$ NMR chemical shifts of C-4a and C-8a and adjacent carbons, suggesting that **14b** and **14a** differ in the configurations of C-4a and C-8a.

The differences between juncuenin B and **15** involved the presence of an aromatic ring B, the oxidation of the phenolic group at C-2 to a carbonyl moiety, and the addition of methoxy groups at C-1 and C-5.

The $^{1}H$ NMR spectrum of the racemic mixture of **16a** and **16b** differ from juncuenin B in the presence of a methoxy group and the carbonyl group at ring C.

The 1D NMR data of compound **17** were found to be similar to those of **14a** and **14b**, except for the presence of two ethoxy groups in the molecule. Relevant HMBC interactions (between $\delta_H$ 0.8, t ($CH_3$) and $\delta_C$ 72.97 (C-4a), and between $\delta_H$ 1.11, t ($CH_3$) and $\delta_C$ 74.53 (C-8a) suggested the side chains to be attached to to the phenanthrene skeleton at C-4a and C-8a.

Compounds **18a** and **18b** were obtained as racemic diastereomers. The difference between compounds **13a**-**d** and compounds **18a**-**b** involved the replacement of the C-1 and C-8a methoxy groups with ethoxy substituents.

Compounds **19a** and **19b** differ from compounds **16a** and **16b** in the presence of an ethoxy group instead of a methoxy group at C-8a.

For the configurational assignments of **19a** and **19b** the abovementioned TDDFT-ECD protocol was applied to (*R*)-**19** and also on the truncated methoxy model compound, (*R*)-**16**. DFT reoptimization of the initial 6 MMFF conformers of (*R*)-**16** yielded 6 conformers above 1% population and Boltzmann-weighted ECD of spectra reproduced well the experimental ECD spectrum of the first-eluting enantiomer of **19a**. Consequently, the absolute configuration of **19a** and **16a**, which was separated under the same conditions, could be elucidated as (*R*) and that of the second-eluting **19b** and **16b** as (*S*).

Interestingly, racemic compound **20** is the only one in which ring C is unchanged comparing to the starting material, juncuenin B. An ethoxy group is attached at C-1, and the phenolic group at C-2 of juncuenin B was oxidized to a carbonyl moiety. Moreover, **20** is an unsaturated compound, containing a $\Delta^{9,10}$ double bond.

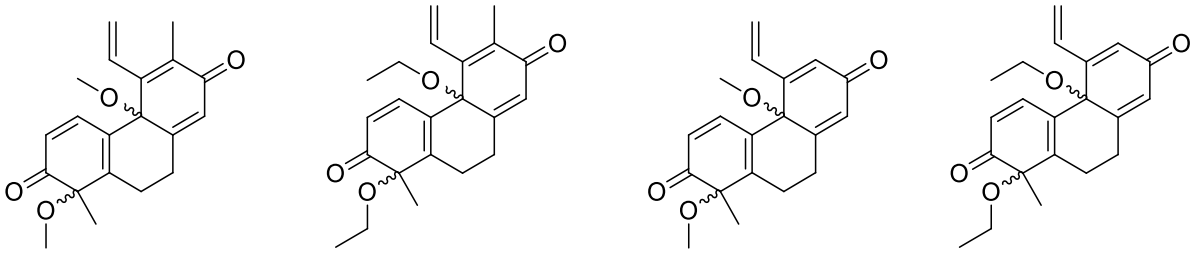
Compound **21** is a unique derivative because an ethoxy group is attached to the phenanthrene skeleton at C-7, as suggested by spectroscopic data evidence. The HMBC correlation of $CH_3$-12 with the oxygen-bearing C-7, as well as the relevant cross peaks H-5/C-7, H-13/C-7, H-5/C-8a and H-13/C-8a indicated the presence of $\Delta^{8,8a}$ double bond instead of a $\Delta^{7,8}$ olefinic bond, to support the above conclusion.

In the case of compound **22**, it was apparent from the 1D NMR spectra that three *n*-butoxy groups were connected to the phenanthrene skeleton. Both the phenolic substituents of juncuenin B were oxidized to carbonyl groups, and a $\Delta^{9,10}$ double bond was also formed. The location of the methyl, vinyl and *n*-butoxy groups were determined by their diagnostic HMBC correlations.

The $^1$H NMR spectrum of the racemic compound **23** displayed signals attributable to an *n*-butoxy moiety. Since the oxidative transformation was performed in a reaction medium containing *n*-BuOH as solvent, and the aromatic ring A and HO-2 remained intact during the reaction, it was anticipated that the *n*-butoxy chain is situated at C-8a. This presumption was corroborated by a strong HMBC cross peak between the oxymethylene protons and C-8a.

## 5.3. Semisynthetic derivatives of juncusol and effusol

Using effusol (**3**) and juncusol (**5**) as starting materials, isolated from *J. tenuis*, four semisynthetic derivatives (**24**–**27**) (**Fig. 17**) have been prepared from four reaction processeses under different reaction conditions. Compounds **24** and **25** are derived from juncusol, while **26** and **27** from effusol. The purification of compounds were performed by MPLC and HPLC techniques.



*rac*-**24** *ent*-**25a**, *ent*-**25b** *rac*-**26** *ent*-**27a**, *ent*-**27b**

**Figure 17.** Structures of semisynthetic derivatives of juncusol and effusol

In the NMR spectra of **24**, two sets of signals could be identified that were attributed to two racemic diastereomers (ratio 1:1). Since this compound is derived from juncusol, similar signals were found in the $^{1}$H NMR spectrum: resonances of two *o*-coupled aromatic protons, two methyls, a vinylic system, two methylene groups and signals of two methoxy groups (**Tables 3** and **4**). Relevant HMBC interactions suggested the methoxy side chains to be attached to the phenanthrene skeleton at C-1 and C-5a. The C-2 and C-7 hydroxy groups of juncusol were oxidized to carbonyl groups, and methoxy-substituted dienone moieties were formed.

Compounds **25a** and **25b** were also obtained as racemic diastereomers, originated from juncusol. The difference between **24** and **25a** and **25b** involved the replacement of the C-1 and C-5a methoxy groups with ethoxy substituents. Differences were found between the $^{13}$C NMR chemical shifts of C-1a in **25a** and **25b**, which indicated the opposite stereochemistry in chiral center C-1 in these compounds (**Tables 3** and **4**).

Compound **26** was also obtained in 1:1 ratio of diastereomers. The differences between effusol and compound **26** involved the oxidation of the phenolic hydroxy groups at C-2 and C-7 to carbonyl moieties and the addition of methoxy groups at C-1 and C-5a.

Compounds **27a** and **27b** differ from compound **26** in the presence of ethoxy side chains at C-1 and C-5a, as it was deduced from the relevant HMBC correlations. Similar to compound **26**, oxidation at C-2 and C-7 to carbonyl groups was observed. The main difference between **27a** and **27b**, detected in the $^{13}$C NMRspectra, was the distinct chemical shifts of C-1, suggesting that the two compounds have the opposite stereochemistry in chiral center C-1.

**Table 3**. $^{1}$H NMR spectroscopic data for compounds **24**–**27** (500 MHz, $CDCl_3$, $\delta$ ppm, $J$ in Hz).

| Atom | 24 | 25a | 25b | 26 | 27a | 27b |
|---|---|---|---|---|---|---|
| 3 | 5.96 dd (10.3, 1.1) | 5.89 d (10.2) | 5.94 d (10.3) | 6.01 dd (10.3, 0.9) | 5. 93 d (10.3) | 5.98 d (10.3) |
| 4 | 6.83 d (10.3) | 6.84 d (10.2) | 6. 80 d (10.3) | 6.98 d (10.3) | 6.96 d (10.3) | 6.94 d (10.3) |
| 8 | 6.29 s | 6.24 s | 6.24 s | 6.28 s | 6.23 s | 6.23 s |
| 9 | 2.89 m, 2.48 m | 2.87 m, 2.51, m | 2.87 m, 2.47 m | 2.89 m, 2.55 m | 2.57 m, 2.91 m | 2.87 m, 2.53 m |
| 10 | 3.00 m, 2.79 ddd (17.4, 8.3, 1.6) | 2.99 m, 2.93 m | 2.99 m, 2.82m | 2.99 m (2H) | 2.99 m, 2.86 m | 2.81 m, 2.47 m |
| 11 | 1.26 s | 1.42 s | 1.25, s | 1.27 s | 1.42 s | 1.27 s |
| 12 | 2.15 s | 2.14 s | 2.14 s | | | |
| 13 | 6. 89 dd (17.8, 10.7) | 6.90 dd (17.7, 11.9) | 6.88 dd (17.7, 11.8) | 6.64 dd (17.7, 11.3) | 6.64 dd (17.7, 11.3) | 6.64 dd (17.7, 11.3) |
| 14 | 6.01 dd (17.8, 1.3), 5.96 dd (10.7, 1.1) | 6.05 d (17.7), 5.67 d (11.9) | 6.03 d (17.7), 5.67 d (11.8) | 6.08 br d (17.7), 5.63 br d (11.3) | 6.10 d (17.7), 5.62 d (11.3) | 6.09 d (17.7), 5.62 d (11.3) |

**24**: $CH_3O$-1: 3.15 s; $CH_3O$-5a: 3.00 s; **25a**: $C_2H_5O$-1: 3.15 dq (14.1, 7.0), 2.73 dq (14.1, 7.0) (C*H*$_2$), 1.16 t (7.0) (C*H*$_3$), $C_2H_5O$-5a: 3.20 dq (14.3, 7.0), 3.04 dq (14.3, 7.0) (C*H*$_2$), 1.12 t (7.0) (C*H*$_3$); **25b**: $C_2H_5O$-1: 3.37 dq (14.2, 7.0), 3.13 dq (14.2, 7.0) (C*H*$_2$), 1.24 t (7.0) (C*H*$_3$); $C_2H_5O$-5a: 3.20 dq (14.1, 7.0), 3.04 dq (14.1, 7.0), 1.11 t (7.0), 6-$CH_3$: 2.14 s; **26**: $CH_3O$-1: 3.15 s; $CH_3O$-5a: 3.06 s; **27a**: $C_2H_5O$-1: 3.18 m, 2.75 m (C*H*$_2$), 1.16 t (7.0) (C*H*$_3$); $C_2H_5O$-5a: 3.24 m, 3.11 m (C*H*$_2$), 1.16 t (7.0) (C*H*$_3$); **27b**: $C_2H_5O$-1: 3.38 m, 3.11 m (C*H*$_2$), 1.24 t (7.0) (C*H*$_3$); $C_2H_5O$-5a: 3.24 m, 3.11 m (C*H*$_2$), 1.15 t (7.0) (C*H*$_3$).

**Table 4**. $^{13}$C NMR spectroscopic data for compounds **24**–**27** (125 MHz, $CDCl_3$, $\delta$ ppm).

| Atom | 24 | 25a | 25b | 26 | 27a | 27b |
|---|---|---|---|---|---|---|
| 1 | 84.7, C | 81.7, C | 84.2, C | 84.5, C | 81.8, C | 84.0, C |
| 1a | 157.6, C | 160.7, C | 158.1, C | 157.7, C | 160.5, C | 153.6,C |
| 2 | 202.6, C | 203.1, C | 202.8, C | 202.3, C | 202.4, C | 202.5,C |
| 3 | 123.7, CH | 123.0, CH | 123.6, CH | 124.4, CH | 123.8, CH | 124.3, CH |
| 4 | 142.2, CH | 142.2, CH | 142.1, CH | 141.1, CH | 141.1, CH | 141.1, CH |
| 4a | 132.4, C | 132.1, C | 132.0, C | 131.8, C | 131.3, C | 131.3, C |
| 5a | 77.5, C | 76.4, C | 76.8, C | 76.6, C | 75.9, C | 76.4, C |
| 5 | 146.1, C | 146.6, C | 146.7, C | 152.7, C | 153.2, C | 153.3, C |
| 6 | 138.4, C | 138.1, C | 137.8, C | 130.5, C | 130.3, CH | 130.0, CH |
| 7 | 184.6, C | 184.8, C | 184.9, C | 184.9, C | 185.1, C | 202.5, C |
| 8 | 126.2, CH | 125.5, CH | 125.8, CH | 126.8, CH | 126.0, CH | 126.3, CH |
| 8a | 160.8, C | 161.6, C | 161.5, C | 161.5, C | 162.3, C | 162.2, C |
| 9 | 25.9, $CH_2$ | 25.8, $CH_2$ | 26.2, $CH_2$ | 26.3, $CH_2$ | 26.2, $CH_2$ | 26.5, $CH_2$ |
| 10 | 24.1, $CH_2$ | 23.2, $CH_2$ | 24.2, $CH_2$ | 25.0, $CH_2$ | 24.0, $CH_2$ | 24.9, $CH_2$ |
| 11 | 25.4, $CH_3$ | 26.4, $CH_3$ | 25.5, $CH_3$ | 25.8, $CH_3$ | 26.5, $CH_3$ | 25.9, $CH_3$ |
| 12 | 11.9, $CH_3$ | 11.8, $CH_3$ | 11.8, $CH_3$ | | | |
| 13 | 132.1, CH | 132.2, CH | 132.3, CH | 134.4, CH | 134.5, CH | 134.5, CH |
| 14 | 125.6, $CH_2$ | 125.3, $CH_2$ | 125.6, $CH_2$ | 123.1, $CH_2$ | 123.1, $CH_2$ | 123.0, $CH_2$ |

**24**: $CH_3O$-1: 55.0; $CH_3O$-5a: 52.8; **25a**: $C_2H_5O$-1: 62.3 (*C*$H_2$), 15.0 (*C*$H_3$); $C_2H_5O$-5a: 60.7 (*C*$H_2$), 15.0 (*C*$H_3$); **25b**: $C_2H_5O$-1: 63.0 (*C*$H_2$), 15.8 (*C*$H_3$), $C_2H_5O$-5a: 60.7 (*C*$H_2$), 15.1 (*C*$H_3$); **26**: $CH_3O$-1: 55.0 (*C*$H_3$), $CH_3O$-5a: 52.9 (*C*$H_3$); **27a**: $C_2H_5O$-1: 62.4 (*C*$H_2$), 15.7 (*C*$H_3$), $C_2H_5O$-5a: 61.0 (*C*$H_2$), 15.2 (*C*$H_3$); **27b**: $C_2H_5O$-1: 63.0 (*C*$H_2$), 15.8 (*C*$H_3$), $C_2H_5O$-5a: 61.0 (*C*$H_2$), 15.2 (*C*$H_3$).

## 6. DISCUSSION

### 6.1. Phenanthrenes from *Juncus compressus* and *Juncus tenuis*

From the methanolic extract of *J. compressus* 11 compounds were isolated, among them 9 phenanthrenes and two flavons. Besides the new compounds, compressin A (**1**) and compressin B (**2**), five dihydrophenanthrenes [effusol (**3**), effususol (**4**), juncusol (**5**), 2-hydroxy-1-methyl-7-oxymethylene-5-vinyl-9,10-dihydrophenanthrene (**6**), 7-hydroxy-1-methyl-2-methoxy-5-vinyl-9,10-dihidrophenanthrene (**7**)], one phenanthrene [dehydroeffusol (**9**)], one phenanthrene dimer [effususin A (**8**)], and two flavons [apigenin (**10**) and luteolin (**11**)] were also isolated from *J. compressus*. Their structures were determined by analysis of MS, 1D and 2D NMR spectra, and by comparison with literature data [50, 62, 63, 94, 95, 114, 115].

All compounds were isolated for the first time from *J. compressus*. It can be observed that the connection of a methyl group at C-1, methoxy-, or hydroxy-substituents on C-2, and vinyl group on C-5 are characteristic features of the isolated phenanthrenes. Effusol (**3**) can be described as the main compound of this plant with regard to the isolation yield. Juncusol (**5**) is a generally occurring phenanthrene as it was isolated from all investigated Juncaceae species. Compound **7** was isolated previously from *J. acutus* and *J. subulatus*, effusol (**3**) from *J. acutus*, *J. effusus*, *J. maritimus*, *J. setchuensis* and *J. subulatus*, effususol (**4**) from *J. effusus*, compound **6** from *J. acutus*, *J. effusus*, and *J. subulatus*, effususin A (**8**) from *J. effusus*, and dehydroeffusol (**9**) from *J. acutus*, *J. effusus* and *J. setchuensis* [28, 52, 56, 61, 62, 95, 102, 103], respectively.

From the methanolic extract of *J. tenuis* three compounds (**3**, **5** and **12**) were isolated. The NMR characteristics of the isolated compounds were compared with literature data [51, 61] and all these compounds were determined to be known natural compounds. All compounds were isolated for the first time from the plant. Juncusol (**5**) and effusol (**3**) can be described also as the main compounds of *J. tenuis*, since considerable amount of both compounds could be isolated from the plant. The third identified compound was 2,7-dihydroxy-1.8-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**12**), which was reported earlier from *J. acutus* and *J. effusus* [51, 99]. Compound **12** was isolated previously from *J. acutus* and showed considerable activity (85% inhibition) against *Agrobacterium tumefaciens* using an in-house assay [51]. This compound was also identified from the ethanolic extract of *J. effusus* during a bioactivity-guided isolation process. However, the compound was not isolated in pure form, but in mixture with juncusol (**5**). The anticancer activity of the mixture was tested using the CCK-8 assay ($IC_{50}$ values 29.6 µM on HeLa and 71.7 µM on MCF-7). Structure-activity relationship results suggest that a vinyl chain at C-5 and the hydroxy group at C-7 are important for antiproliferative activities [99]. As a continuation of pharmacological evaluation of these phenanthrenes, their antiproliferative activity was measured in our study, using the standard MTT assay. The phenanthrenes, isolated from

*J. compressus* were tested against three human tumor cell lines [HeLa and SiHa (cervix adenocarcinoma) and A2780 (ovarian carcinoma)] using cisplatin as a positive control (**Table 5**).

**Table 5.** $IC_{50}$ values of the compounds isolated from *J. compressus*

| Compound | Concentration (μM) | Growth inhibition (%) ± SEM [calculated $IC_{50}$ value (μM)] | | |
|---|---|---|---|---|
| | | HeLa | SiHa | A2780 |
| **1** | 10 | 41.7 ± 1.1 | – | 47.6 ± 1.6 |
| | 30 | 93.7 ± 0.4 | 38.6 ± 0.8 | 73.0 ± 0.5 |
| | | **11.3** | | **13.2** |
| **2** | 10 | 92.0 ± 0.4 | – | – |
| | 30 | 92.4 ± 0.2 | 32.8 ± 0.9 | 64.9 ± 2.0 |
| | | **1.9** | | |
| **3** | 10 | 96.4 ± 0.3 | – | – |
| | 30 | 97.8 ± 0.3 | – | 50.5 ± 1.6 |
| | | **3.7** | | |
| **4** | 10 | – | – | 18.3 ± 0.4 |
| | 30 | 30.4 ± 1.9 | 14.2 ± 2.8 | 72.1 ± 1.1 |
| **5** | 10 | 97.7 ± 0.4 | 17.1 ± 2.4 | 18.1 ± 1.3 |
| | 30 | 97.8 ± 0.2 | 29.3 ± 2.0 | 63.4 ± 1.0 |
| | | **1.3** | | |
| **6** | 10 | 96.8 ± 0.3 | | – |
| | 30 | 98.8 ± 0.2 | 10.3 ± 1.5 | – |
| | | **4.2** | | |
| **7** | 10 | 45.4 ± 1.6 | 14.2 ± 1.0 | – |
| | 30 | 92.4 ± 0.2 | 29.5 ± 3.4 | 63.2 ± 2.5 |
| | | **10.7** | | |
| **8** | 10 | – | – | – |
| | 30 | 10.5 ± 0.5 | 14.4 ± 1.5 | 72.7 ± 1.8 |
| **9** | 10 | 75.2 ± 2.5 | – | – |
| | 30 | 96.5 ± 0.3 | 28.7 ± 2.0 | 57.3 ± 2.6 |
| | | **7.8** | | |
| **cisplatin** | 10 | 42.6 ± 2.3 | 88.6 ± 0.5 | 83.6 ± 1.2 |
| | 30 | 99.9 ± 0.3 | 90.2 ± 7.8 | 95.0 ± 0.3 |
| | | **12.4** | **7.8** | **1.3** |

Some structure-activity relationship could be determined based on the pharmacological results. Since **4** is markedly less effective, a bulky substituent (-$CH(CH_3)OCH_3$) instead of vinyl group seems to be disadvantageous. Compound bearing free hydroxy groups (in case of **3**) exerted more pronounced activity than its methyl ether (**7**) or its oxymethylene derivative (**6**). The substantial difference in the activities of the isolated dimers (**2** and **8**) could be attributed to the presence of methyl group at C-6, which is favoured in the monomers, too. The other cell lines (SiHa and A2780) were substantially less sensitive. Although **1**, **4** and **8** exhibited considerable activity against A2780 cells, none of them were comparable to cisplatin. Similarly, none of the presented agents elicited relevant growth inhibition (>40%) against SiHa cells and therefore, $IC_{50}$ values were not determined.

HeLa cell line proved to be the most sensitive to the compounds; the activity of all investigated phenanthrenes was higher than the clinically used reference agent cisplatin.

## 6.2. Semisynthetic derivatives of juncuenin B and their pharmacological investigations

From juncuenin B as a starting material, during a diversity oriented semisynthetic process, 11 (**13**–**23**) phenanthrenoid analogues were prepared and identified. The yields of these reactions were relatively low (1–20%).

The oxidation of juncuenin B with PIFA in water-containing media was previously reported in order to characterise possible, oxidation-related biosynthetic pathways in plants [48]. The aim of this reaction process was to characterise the antiproliferative activity of differently substituted quinoidal compounds with phenanthrene skeleton.

All semisynthesized compounds (**13**-**23**) are chiral, most of them are bearing a *p*-quinol ring, and are substituted with methoxy-, ethoxy-, and butoxy groups. The effect of temperature on production of semisynthetic derivatives may be seen in case of compounds **14a** and **14b**; compound **14a** was obtained only at lower temperature.

From reaction mixture IV, only compound **22** could be identified, which did not show structural analogy with the previously determined ones. As the effect of the incorporation of a longer side-chain into the molecule on the antiproliferative activity was also aimed to be measured, this reaction was repeated with PIDA, as this is a mild oxidizing agent. In this way, compound **23** was obtained.

Compounds **13a**-**d** were eluted together on RP-column, but compounds **18a**-**b**, which are also diastereomer pairs, could be separated by RP-HPLC.

Compounds **13**-**23** were isolated as racemic mixtures. The first step of the comprehensive evaluation of the antiproliferative activity of these compounds was the investigation of all the obtained diastereomers. These compounds were measured on human breast (MCF-7, T47d), cervical (HeLa, SiHa, C33a) and ovarian (A2780) gynecological cell lines with cisplatin as positive control. In order to get some insights into their tumor selectivity, the compounds have also been tested on NIH/3T3 non-tumoral cell line (**Table 6**).

The $IC_{50}$ value of juncuenin-B on MCF-7, C33a, and SiHa cell lines was determined for the first time. According to the structural points of view, it is evident that compounds without a *p*-quinol ring (**15**, **20**, **21**) and compounds with two *p*-quinol moieties (**14a**, **14b**, **17**) did not exert antiproliferative activity. Moreover, these compounds are substituted at C-4a with an alkoxy group.

**Table 6**. $IC_{50}$ values of the most active semisynthetic juncuenin B analogues

| Compound | Calculated $IC_{50}$ values (µM ± SEM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | MCF-7 | T47d | HeLa | SiHa | C33a | A2780 | NIH/3T3 |
| **juncuenin B** | 11.7±1.1 | 28.7±0.7[a] | 2.9±0.5[a] | >30 | 12.7±1.7 | 7.3±1.3[a] | >30 |
| **13a-d** | 11.6±1.8 | 15.0±0.8 | 15.6±0.1 | 20.0±0.9 | 6.7±0.5 | 7.7±1.0 | 17.7±0.7 |
| **16a-b** | 6.5±1.1 | >30 | 1.9±0.4 | >30 | 7.0±1.9 | 9.1±0.5 | 14.9±3.7 |
| **18a** | >30 | 22.9±1.6 | 25.9±0.3 | 29.7±0.2 | 14.1±0.2 | 11.2±0.3 | 24.5±1.9 |
| **19a-b** | 6.2±0.2 | 16.8±1.8 | 5.4±0.1 | >30 | 6.1±0.4 | 5.9±1.1 | >30 |
| **23** | 18.2±0.2 | 30.0±0.5 | >30 | >30 | 19.9±1.3 | 16.5±2.0 | >30 |
| **cisplatin** | 5.8 | 9.8[a] | 12.4[a] | 11.1 | 1.8 | 1.3[a] | 2.7 |

$IC_{50}$ values (µM, mean ± SEM) were determined by MTT assay by treating the cells with each compound (0.1–30 µM) for 72 hours. Data are based on two independent experiments. Values signed with [a] are listed from literature [93].

Based on the preliminary results, the most effective racemic compounds (**13a**-**d**, **16a**-**b** and **18a**-**b**) were separated into pure enantiomers by using chiral HPLC with a Lux-amylose-1 column and cyclohexane–isopropanol as mobile phase. In the second step, the enantiomerically pure compounds (**13a**–**13d**, **16a**, **16b**, **19a** and **19b**) were evaluated for their antiproliferative activity against the same cell lines as used previously (**Table 7**).

**Table 7** The $IC_{50}$ values of the enantiopure semisynthetic juncuenin B analogues

| Compound | Calculated $IC_{50}$ values (µM ± SEM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | MCF-7 | T47d | HeLa | SiHa | C33a | A2780 | NIH/3T3 |
| (1*R*,8a*R*)-**13a** | >30 | >30 | >30 | >30 | 14.8±0.7 | 13.3±1.5 | >30 |
| (1*R*,8a*S*)-**13b** | >30 | >30 | >30 | >30 | 10.2±1.7 | 9.0±1.0 | >30 |
| (1*S*,8a*R*)-**13c** | >30 | 12.5±0.9 | >30 | 28.1±0.1 | 5.3±0.9 | 7.7±0.6 | 21.6±0.4 |
| (1*S*,8a*S*)-**13d** | >30 | >30 | >30 | >30 | 13.0±0.2 | 11.0±0.4 | >30 |
| (8a*R*)-**16a** | 6.2±1.6 | >30 | 0.9±0.4 | >30 | 5.3±0.6 | 8.7±0.8 | 25.8±4.6 |
| (8a*S*)-**16b** | >30 | >30 | >30 | >30 | >30 | >30 | >30 |
| (8a*R*)-**19a** | 20.6±0.3 | 8.8±1.1 | 3.7±0.9 | >30 | 4.9±0.3 | 2.8±0.3 | >30 |
| (8a*S*)-**19b** | >30 | >30 | >30 | >30 | >30 | >30 | >30 |

Among these compounds, the highest activities were recorded for **13c**, **16a** and **19a**, reaching or exceeding the effect of the positive control cisplatin on HeLa and/or T47D cell lines. (*R*)-**16a** was found to be the most promising compound with substantial antiproliferative effects against all tested cell lines except for T47d and SiHa, but it was inactive against the non-tumoral NIH/3T3 cells.

Compounds **16a**, **16b**, **19a**, **19b**, and **23** contain a nonoxidized ring A, while ring C has a *p*-quinol structure substituted with an alkyl ether moiety; **16a** and **16b** are substituted with a methoxy-, **19a**, and **19b** with an ethoxy-, and **23** with an *n*-butoxy group at C-8a, respectively. In the case of these compounds, the effect of the length of the ether chain at C-8a is undefined: on the T47D, C33A, and A2780 cell lines, compound **19a** with an ethoxy group showed the highest inhibition, while its enantiomer (**19b**) had no activity. In case of HeLa cell line, the antiproliferative activity was inversely proportional to the chain length of the alkoxy groups ($IC_{50}$ values **23** > **19a** > **16a**). Compounds **16a** and

**19a** showed an antiproliferative effect on HeLa, C33A, MCF-7, and A2780 cells, which was comparable to or stronger than that of juncuenin B, while their stereoisomers **16b** and **19b** had no relevant influence on the cell proliferation, indicating that (8a*R*) is the preferred absolute configuration of these compounds.

Regarding the pure enantiomers **13a**-**d**, the (1*S*,8a*R*)-configuration (**13c**) was the most beneficial for the antiproliferative effect. The activities of (1*S*,8a*R*)-**13c**, (*R*)-**16a**, and (*R*)-**19a** suggest the preference of the (*R*) configuration for the antiproliferative activity. Since **13a** is less active than **13c**, the (1*S*) configuration seems to be also essential in the case of quinoidal compounds.

It is worth mentioning that several of the semisynthetic compounds demonstrated an altered cell line specificity as compared to their parent compound, juncuenin B. For example, while juncuenin B exerted approximately 2.5-fold stronger activity on MCF-7 than on T47D cells, a similar rate of selectivity in the opposite direction was observed for the enantiopure compound **19a**. While both of these cell lines are estrogen receptor α positive and therefore estrogen-dependent, they show critical differences in their bioenergetic properties [116], as well as in apoptotic events including the activation of different caspases and mitochondrial changes [117]. This selectivity inversion suggests that compounds obtained from juncuenin B were not only more potent than the starting material, but also these compounds likely act on different biochemical targets.

## 6.3. Semisynthetic derivatives of juncusol and effusol and their pharmacological investigations

For the structural modifications of juncusol and effusol the oxidizing agent PIFA was used, and the reactions were processed in methanol-, and ethanol containing solvents at room temperature with 2 equiv amount reagent. During the process that involved 4 reactions, four compounds (**24-27**) were isolated. The yields of these reactions were low (1-15%).

The yielded compounds are racemic mixtures, all of them are containing an *o*-, and *p*-quinol ring, and are substituted with methoxy-, and ethoxy-groups. The most considerable structural attributes of these derivatives is the presence of a side chain at C-5a. Comparing this structure with natural compounds, it may be mentioned as a special case, as neither the naturally occuring phenanthrenes, nor the derivatives of juncuenin B are substitued in this position. This phenomenom may provide further structural informations reflected on antiproliferative activities.

The semisynthesized compounds were measured on human breast (MCF-7, KCR, T47D, and HTB-26), cervical (HeLa), and ovarian (A2780 and A2780cis) cancer cells, and on MRC-5 (human embryonic lung fibroblast) cell line (**Table 8**).

**Table 8.** Antiproliferative activity of isolated compounds from *J. tenuis* and semisynthetic derivatives of juncusol (**5**) and effusol (**3**)

| Compound | Calculated $IC_{50}$ values (μM ± SEM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A2780 | A2780cis | KCR | MCF-7 | HeLa | HTB-26 | T47D | MRC-5 |
| **12** | 23.8±1.3 | 37.1±2.8 | 35.8±1.7 | 37.1±1.1 | 0.5±0.0 | 41.7±3.5 | 25.0±0.4 | 40.9±2.0 |
| **5** | 33.1±3.1 | 30.4±0.4 | 39.3±1.6 | 48.6±3.4 | 2.3±0.7 | 57.0±2.7 | 24.6±1.9 | 60.1±5.1 |
| **3** | 22.3±2.7 | 16.9±4.7 | 24.2±2.1 | 12.9±0.2 | 24.7±0.3 | 22.8±0.2 | 14.2±1.1 | 18.9±4.0 |
| *rac*-**24** | 80.3±3.0 | 88.2±3.1 | >100 | 52.1±4.8 | >100 | 74.3±3.6 | 36.5±1 | >100 |
| *ent*-**25a** | 66.0±4.4 | 62.9±1.7 | >100 | 80.5±3.5 | 94.7±3.4 | 94.3±2.0 | 56.3±3.4 | >100 |
| *ent*-**25b** | 39.4±3.1 | 38.0±4.6 | 44.2±2.6 | 41.0±0.9 | 61.9±0.3 | 45.8±3.2 | 30.3±2.5 | 57.7±0.3 |
| *rac*-**26** | 8.6±0.5 | 10.9±2.2 | 18.9±1.4 | 5.8±0.2 | 12.9±0.4 | 10.9±0.9 | 7.0±1.0 | 12.2±0.2 |
| *ent*-**27a** | 25.2±1.8 | 22.5±0.2 | 23.5±0.8 | 11.7±0.7 | 24.4±0.8 | 16.1±0.2 | 11.6±0.3 | 14.3±0.5 |
| *ent*-**27b** | 22.0±2.0 | 22.1±1.6 | 29.4±0.9 | 10.2±0.1 | 35.0±1.5 | 20.1±1.1 | 14.2±0.6 | 23.4±1.3 |
| **cisplatin** | 3.6±0.3 | 7.3±0.2 | 6.7±0.4 | 1.4±1.1 | 2.3±0.1 | 20.1±0.2 | 5.9±0.1 | 0.6±0.1 |

Determinations were performed by MTT assay, by treating the cells with compounds (100–0.19 μM) for 72 h. Data are based on two independent experiments.

The antiproliferative effects of the natural phenanthrenes of *J. tenuis* (**3**, **5** and **12**) and the semisynthetic products (**24**, **25a**, **25b**, **26**, **27a** and **27b**) were investigated by a standard MTT method. In this investigation, among the natural phenanthrenes, 2,7-dihydroxy-1,8-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**12**) was the most active on all tested cell lines, with the exception of HeLa (**Table 8**). Juncusol (**5**) and effusol (**3**) possessed significant antiproliferative activity on HeLa cells ($IC_{50}$ values 0.5 μM for juncusol, and 2.3 μM for effusol, respectively). The only difference between juncusol and effusol is the presence of a methyl group at C-6 in juncusol.

Among the semisynthetic derivatives, compound **26** was found to be the most promising component with substantial antiproliferative effects against all tested cell lines, except for KCR; the activities were comparable to that of the positive control, cisplatin. Unfortunately, **26** had antiproliferative activity ($IC_{50}$ = 12.2 μM) against the non-tumoral MRC-5 cells, too. This represents a ca. 2.1-fold selectivity against MCF-7 cells, which greatly over-performs the anticancer drug cisplatin that was 2.3-fold more cytotoxic on MRC-5 cells (**Table 8**). Compounds **27a** and **27b** showed marked antiproliferative activity against MCF-7 cells ($IC_{50}$ values 11.7 μM for **27a** and 10.2 μM for **27b**). In the case of 2,7-dihydroxy-1,8-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**12**), $IC_{50}$ value 12.9 μM was detected on MCF-7 cells. In case of effusol derivatives, the semisynthetic compounds (**26, 27a, 27b**) had higher antiproliferative activities than that of the parent compound on all cell lines, except on HeLa. None of the juncusol derivatives exceeded the antiproliferative effects of the parent compound. Although compounds **24** and **25a** did not show an antiproliferative effect against normal (MRC-5) cells at tested concentrations, they possessed very weak activity against the investigated tumor cell lines. Comparing the data of effusol and juncusol derivatives (**24** and **26**), it can be stated that the presence of a methyl group at C-6 in the semisynthetic compounds resulted in a decrease of the toxic effect.

## 7. SUMMARY

In the Department of Pharmacognosy a research program was started in 2014 that targets the isolation of phenanthrenes from Juncaceae species. The present PhD thesis was prepared as a part of this program, and includes the results of phytochemical investigation of two *Juncus* species, *J. compressus* and *J. tenuis*, and the preparation and characterisation of oxidized semisynthetic derivatives of three phenanthrenes, juncuenin B, juncusol and effusol. The structure determination was carried out by spectroscopic analysis, and the antiproliferative activities of the compounds were determined by the MTT assay.

From the dried, whole plant materials using combined chromatographic techniques 12 compounds have been isolated, among them 10 phenanthrenes and 2 flavons. Two components, compressins A and B (**1**, **2**), are new natural products. All of the isolated compounds (**1**-**12**) were detected for the first time from the investigated plants. The chemical constituents of *J. compressus* and *J. tenuis* have not been investigated previously.

Compressin A showed inhibitory activity against A2780 cell line with $IC_{50}$ 13.19 µM, while compressin B inhibited HeLa cells with $IC_{50}$ value 1.86 µM, respectively. Based on our results and literature data, dimerization may increase the antiproliferative activity of phenanthrenes.

15 Semisynthetic phenanthrenoid derivatives of juncuenin B, juncusol, and effusol with wide-range of structural diversity were identified. The pure enantiomers of the most active products of juncuenin B were investigated. Compounds **13c**, **16a** and **19a** were found to be the most effective ones with $IC_{50}$ values 5.3 µM on C33a (**13c**), 0.9 µM on HeLa (**16a**), and 2.8 µM on A2780 (**19a**) cell lines, respectively. According to the pure enantiomers of **16** and **19**, it was evident that (8a*R*) is the preferred configuration for anticancer effects. Moreover, these compounds did not have considerable activity against the non-tumoral NIH/3T3 cell line compared to the positive control cisplatin.

The semisynthetic derivatives of juncusol and effusol possessed an irregular structural attribute as these compounds are substituted at C-5a. Their antiproliferative activites indicate that the presence of this structural moiety does not increase the antiproliferative effect of these quinoidal phenanthrenoid derivatives, as only one derivative, **26** showed considerable $IC_{50}$ values on the tested cell lines compared with its parent compound, effusol.

Our results reveal that Juncaceae species are promising sources of biologically active compounds. Moreover, secondary metabolites of Juncaceae species, especially phenanthrenes, can be regarded as promising starting materials in the search for new, promising bioactive compounds, in consequence of their pharmacological potential, in particular their noteworthy antiproliferative activities. Semisynthetic modification of phenanthrenes not only expanded the chemical space, but also resulted in more effective compounds.

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**ACKNOWLEDGEMENTS**

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Andrea Vasas, for the continuous support of my Ph.D. study, for her patience, motivation, endless energy and enthusiasm. Her guidance helped me in all the time of research and writing of this thesis.

I am very grateful to Prof. Dr. Judit Hohmann, Head of the Department of Pharmacognosy, for the support of my work and the possibility to study in her department.

I am also grateful to Dr. Attila Hunyadi and Tímea Gonda who supported me to work out the topics are connected to semisynthetic processes.

My sincere thanks goes to Dr. István Zupkó, Ágnes Kulmány and Seyyed Ashkan Senobar Tahaei for testing the isolated and prepared compounds for their antitumor activity from multiple aspects.

I am thankful to Gabriella Spengler, Annamária Kincses and Nikoletta Szemerédi for further pharmacological measurements which made these results more diverse.

I would like to extend my special thanks to Dóra Stefkó, Máté Vágvölgyi, Bernadett Kovács and Ibolya Hevérné Herke who taught me numerous important skills, and whose experienced knowledge helped me essentially in my work.

I am thankful to Norbert Kúsz for the NMR investigations, and to Attila Csorba and Róbert Berkecz for the mass spectrometry measurements, and to Prof. Tibor Kurtán and Dr. Attila Mándi for the determination of the absolute configuration.

My thanks are likewise due to all my colleagues in the Department of Pharmacognosy for their support and for providing such a wonderful atmosphere to work in.

Last, but not the least, I would like to thank my family and friends for supporting me spiritually throughout my Ph.D. study and my life in general.

**APPENDIX**

**Table A1**. $^{1}$H NMR spectroscopic data (500 MHz, $\delta$ in ppm, $CDCl_3$) of compounds **13-23** ($\delta_H$ ($J$ in Hz)

| position | **13a+d** | **13b+c** | **14a** | **14b** | **15** | **16a+b** | **17** | **18a** | **18b** | **19a+b** | **20** | **21** | **22** | **23** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $\delta_H$ ($J$ in Hz) | | | | | | | |
| 3 | 6.24, d (10.2) | 6.23, d (10.2) | 6.45, d (9.9) | 6.52, d (10.2) | 6.30, d (10.6) | 6.74, d (8.4) | 6.40, d (9.9) | 6.23, d (10.1) | 6.21, d (10.1) | 6.72, d (8.4) | 6.32, d (10.4) | 6.73, d (8.3) | 6.21, d (10.6) | 6.72, d (8.4) |
| 4 | 7.23, d (10.2)/ 7.25, d (10.2)* | 7.23, d (10.2), /7.25, d (10.2)* | 6.61, d (9.9) | 6.89, d (10.2) | 9.06, d (10.6) | 7.34, d (8.4) | 6.66, d (9.9) | 7.21, d (10.1) | 7.21, d (10.1) | 7.3, d (8.4) | 8.18, d (10.4) | 7.45, d (8.3) | 8.71, d, (10.6) | 7.30, d (8.4) |
| 5 | 6.47, s /6.49, s* | 6.47, s /6.49, s* | 6.84, s | 6.45, s | - | 6.58, s | 6.82, s | 6.43, s | 6.45, s | 6.54, s | 7.49, s | 6.42, s | - | 6.55, s |
| 9 | 1.68, m/1.67, m, 2.63, m* | 1.68, m /1.67, m, 2.63, m* | 1.92, m, 2.45, m | 1.43, m, 2.59, m | 8.19, d (8.8) | 1.76, ddd (14.4, 11.3, 6.5), 2.69, dd (14.4, 5.7) | 1.93, ddd (13.8, 10.0, 4.3), 2.47, m | 1.62, m, 2.62, dd (14.0, 5.5) | 1.66, m, 2.63, dd (13.9, 5.9) | 1.74, ddd (13.9, 11.7, 6.5), 2.68, dd (14.0, 6.0) | 8.15, d (8.8) | 2.71, m* | 7.62, d (8.3) | 1.74, ddd (14.1, 11.6, 6.4), 2.68, dd (14.1, 5.8) |
| 10 | 2.57, dd (20.2, 5.9), 2.83, m | 2.50, dd (20.3, 6.0), 2.85, m | 2.35, m, 2.83, m | 2.67, m, 2.89, m | 7.60, d (8.8) | 2.75, dd (17.3, 6.5), 3.06, m | 2.39, m, 2.87, ddd (13.8, 9.0, 4.3) | 2.53, dd (14.4, 5.9), 2.88, ddd (16.5, 10.6, 5.5) | 2.51, dd (20.4, 6.0), 2.86, ddd (20.2, 10.7, 6.0) | 3.09, m, 2.74, dd (17.3, 6.2) | 7.61, d (8.8) | 2.84, m* | 7.66, d (8.3) | 2.73, dd (17.3, 6.4), 3.09, m |
| 11 | 1.46, s | 1.42, s | 1.97, s | 1.91, s | 1.58, s | 2.16, s | 1.96, s | 1.46, s | 1.43, s | 2.17, s | 1.56, s | 2.22, s | 1.52, s | 2.16, s |
| 12 | 2.08, s | 2.08, s | 2.02, s | 2.00, s | 2.42, s | 2.09, s | 2.01, s | 2.07, s | 2.07, s | 2.09, s | 2.42, s | 1.49, s | 2.04, s | 2.08, s |
| 13 | 6.58, dd (17.8, 12.1) | 6.58, dd (17.8, 12.1) | 6.50, dd (17.9, 12.0) | 6.51, dd (17.9, 11.9) | 6.98, dd (17.9, 11.4) | 6.63, dd (17.9, 11.9) | 6.50, dd (17.8, 11.9) | 6.61, dd (17.9, 11.9) | 6.61, dd (17.9, 12.0) | 6.65, dd (17.9, 11.9) | 7.02, dd (17.7, 11.4) | 6.67, dd (17.8, 11.8) | 6.62, dd (17.9, 11.8) | 6.64, dd (17.9, 11.9) |
| 14 | 5.69 dd (12.1, 1.5), 5.83, dd (17.8, 1.5) | 5.69 dd (12.1, 1.5), 5.83, dd (17.8, 1.5) | 5.63, dd (12.0, 1.4), 5.78, dd (17.9, 1.4) | 5.67, dd (11.9, 1.5), 5.70, dd (17.9, 1.4) | 5.38, dd (17.9, 1.7), 5.82, dd (11.4, 1.7) | 5,66, dd (11.9, 1.7), 5.85, dd (17.9, 1.7) | 5.62, dd (11.9, 1.3), 5.80, dd (17.8, 1.3) | 5.68, dd (11.9, 1.3), 5.87, dd (17.9, 1.3) | 5.68, dd (12.0, 1.5), 5.89, dd (17.9, 1.5) | 5.65, dd (11.9, 1.5), 5.89, dd (17.9, 1.5) | 5.41, dd (17.7. 1.6), 5.84, dd (11.4, 1.6) | 5.55, dd (11.8, 1.3), 5.87, dd (17.8, 1.3) | 5.45, dd (17.9, 1.0), 5.82, d (11.8) | 5.65, dd (11.9, 1.7), 5.89, dd (17.9, 1.7) |

* **13a+d**: 1-$OCH_3$: 3.07, s; 8a-$OCH_3$: 2.92, s/2.93, s* (interchangeable with 1b); **13b+c**: 1-$OCH_3$: 3.12, s; 8a-$OCH_3$: 2.92, s/2.93,s (interchangeable with 1a); **14a**: 4a-$OCH_3$: 3.04, s; 8a-$OCH_3$: 2.72, s; **14b**: 4a-$OCH_3$: 3.10, s; 8a-$OCH_3$: 3.14, s; **15**: 1-$OCH_3$: 3.08, s; 5-$OCH_3$: 3.79, s; **16a+b**: 8a-$OCH_3$: 2.85, s; **17**: 4a-O-$CH_2$-$CH_3$ (interchangeable with 8a): 2.76 dq (7.0, 14.1), 2.96, dq (7.0, 14.1) (C$H_2$), 0.80, t (7.0) (C$H_3$); 8a-$O$-$CH_2$-$CH_3$ (interchangeable with 4a): 3.08 dq (7.0, 14.1), 3.29, dq (7.0, 14.1) (C$H_2$), 1.11, t (7.0) (C$H_3$); **18a**: 1-$O$-$CH_2$-$CH_3$ (interchangeable with 8a): 2.93, m, 3.36 dq (14.3, 7.1) (C$H_2$), 1.21, t (7.0) (C$H_3$); 8a-$O$-$CH_2$-$CH_3$ (interchangeable with 1): 2.91, m, 3.19 dq (14.3, 7.1) (C$H_2$), 1.01, t (7.0) (C$H_3$); **18b**: 1-$O$-$CH_2$-$CH_3$: 3.10, dq (14.2, 7.0), 3.34, dq (14.3, 7.1) (C$H_2$), 1.23, t (7.0) (C$H_3$); 8a-$O$-$CH_2$-$CH_3$: 2.93, dq (14.2, 7.0), 3.17, dq (14.3, 7.1) (C$H_2$), 0.97, t (7.0) (C$H_3$); **19a+b**: 8a-$O$-$CH_2$-$CH_3$: 2.92, m, 3.11, m (C$H_2$), 0.86, t (7.0) (C$H_3$); **20**: 1-$O$-$CH_2$-$CH_3$: 3.00 dq (14.2, 7.0), 3.28 dq (14.2, 7.0) (C$H_2$), 1.20, t (7.0) (C$H_3$); **21**: 7-$O$-$CH_2$-$CH_3$: 3.13, dq (13.9, 7.1), 3.26, dq (14.0, 7.1) (C$H_2$), 1.17, t (7.0) (C$H_3$); **22**: 1-$O$-$(CH_2)_3$-$CH_3$: 3.22, m, 2.92, m (1'-C$H_2$), 1.58, m (2'-C$H_2$), 1.28–1.40, m (3'-C$H_2$), 0.87, t (7.4) (4'-C$H_3$); 5-$O$-$(CH_2)_3$-$CH_3$: 3.14 m, 3.54, m (1"-C$H_2$), 1.47, m (2"-C$H_2$), 1.28–1.40, m (3"-C$H_2$), 0. 81, t (7.5)/0.83, t (7.5) (4"-C$H_3$) (interchangeable with 4'"-C$H_3$); 3.10, m, 3.45, m (1'"-C$H_2$), 1.47, m (2'"-C$H_2$), 1.28–1.40, m (3'"-C$H_2$), 0. 81, t (7.5)/0.83, t (7.5) (4'"-C$H_3$) (interchangeable with 4"-C$H_3$); **23**: 8a-$O$-$(CH_2)_3$-$CH_3$: 2.83, m, 3.07, m (1'-C$H_2$), 1.13–1.23, m (2'-C$H_2$), 0.86–1.03, m (3'-C$H_2$), 0.62, t (7.4) (4'-C$H_3$).

**Table A2**. $^{13}C$ NMR spectroscopic data (125 MHz, $\delta$ in ppm, $CDCl_3$) of compounds **13**-**23**

| position | 13a+d | 13b+c | 14a | 14b | 15 | 16a+b | 17 | 18a | 19b | 19a+b | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $\delta_c$, type | | | | | | | |
| 1 | 83.8, C | 83.1, C | 133.0, C | 132.8, C | 83.5, C | 122.0, C | 132.5, C | 83.2, C | 82.4, C | 121.8, C | 82.5, C | 121.9, C | 81.9, C | 121.8, C |
| 1a | 157.2, C | 157.0, C | 150.9, C | 154.4, C | 145.1, C | 133.9, C | 152.0, C | 157.9, C | 157.5, C | 139.1, C | 144.2, C | 141.0, C | 147.3, C | 139.2, C |
| 2 | 202.6, C | 202.4, C | 185.4, C | 184.7, C | 202.4, C | 155.8, CH | 185.3, C | 202.7, C | 202.5, C | 155.5, C | 202.9, C | 156.3, C | 201.4, C | 155.5, C |
| 3 | 125.9, CH | 125.9, CH | 131.9, CH | 131.8, CH | 124.1, CH | 113.8, CH | 131.2, CH | 125.8, CH | 125.8, CH | 113.7, CH | 124.6, CH | 114.2, CH | 125.1, CH | 113.6, CH |
| 4 | 139.7, CH | 139.7, CH | 144.8, CH | 146.5, CH | 143. 6, CH | 124.6, CH | 145.6, CH | 139.6, CH | 139.6, CH | 124.5, CH | 139.8, CH | 125.7, CH | 142.7, CH | 124.6, CH |
| 4a | 125.4, C | 125.4, C | 73.1, C | 77.3, C | 123.7, C | 125.2, C | 73.0, C | 125.0, C | 125.0, C | 125.5, C | 123.7, C | 126.0, C | 131.0, C | 125.5, C |
| 5 | 123.1, CH | 123.1, CH | 131.0, CH | 128.8, CH | 139.9, C | 122.2, CH | 130.6, CH | 122.6, CH | 122.6, CH | 121.8, CH | 103.3, CH | 117.3, CH | 94.6, C | 121.7, CH |
| 5a | 151.9, C | 151.9, C | 159.6, C | 156.8, C | 122.9, C | 156.7, C | 160.5, C | 152.7, C | 152.9, C | 157.7, C | 130.7, C | 150.6, C | 136.1, C | 157.8, C |
| 6 | 185.9, C | 185.9, C | 186.2, C | 186.1, C | 147.8, C | 186.5, C | 186.3, C | 185.9, C | 186.0, C | 186.6, C | 154.0, C | 202.6, C | 194.4, C | 186.7, C |
| 7 | 135.8, C | 135.8, C | 134.1, C | 133.9, C | 125.2, C | 135.7, C | 133.8, C | 135.2, C | 135.3, C | 135.1, C | 124.4, C | 82.1, C | 130.4, C | 135.1, C |
| 8 | 149.6, C | 149.6, C | 150.2, C | 153.6, C | 134.1, C | 149. 6, C | 150.7, C | 150.2, C | 150.4, C | 150.6, C | 138.2, C | 145.9, C | 147.9, C | 150.5, C |
| 8a | 73.2, C | 73.2, C | 75.1, C | 76.7, C | 127.9, C | 73.8, C | 74.5, C | 72.7, C | 72.7, C | 73.4, C | 127.3, C | 131.6, C | 132.6, C | 73.2, C |
| 9 | 33.1/34.0, $CH_2$* | 33.1/34.0, $CH_2$* | 34.3, $CH_2$ | 41.1, $CH_2$ | 129.7, CH | 34.5, $CH_2$ | 34.1, $CH_2$ | 33.2, $CH_2$ | 34.2, $CH_2$ | 34.8, $CH_2$ | 129.0, CH | 25.8/26.6, $CH_2$* | 129.0, CH | 34.8, $CH_2$ |
| 10 | 21.8, $CH_2$ | 22.4, $CH_2$ | 22.8, $CH_2$ | 22.7, $CH_2$ | 121.3, CH | 23.9, $CH_2$ | 22.9, $CH_2$ | 21.9, $CH_2$ | 22.5, $CH_2$ | 23.9, $CH_2$ | 121.0, CH | 25.8/26.6, $CH_2$* | 127.0, CH | 23.9, $CH_2$ |
| 11 | 28.0, $CH_3$ | 26.6, $CH_3$ | 10.7, $CH_3$ | 10.9, $CH_3$ | 31.1, $CH_3$ | 11.2, $CH_3$ | 10.7, $CH_3$ | 27.2, $CH_3$ | 26.7, $CH_3$ | 11.2, $CH_3$ | 30.9, $CH_3$ | 11.3, $CH_3$ | 31.3, $CH_3$ | 11.2, $CH_3$ |
| 12 | 12.3, $CH_3$ | 12.3, $CH_3$ | 12.0, $CH_3$ | 12.3, $CH_3$ | 13.9, $CH_3$ | 12.3, $CH_3$ | 12.0, $CH_3$ | 12.2, $CH_3$ | 12.3, $CH_3$ | 12.3, $CH_3$ | 13.6, $CH_3$ | 28.2, $CH_3$ | 13.0, $CH_3$ | 12.3, $CH_3$ |
| 13 | 131.7, CH | 131.7, CH | 131.6, CH | 132.0, CH | 133.9, CH | 132.1, CH | 131.8, CH | 131.9, CH | 131.8, CH | 132.2, CH | 134.0, CH | 131.2, CH | 132.9, CH | 132.2, CH |
| 14 | 123.9, $CH_2$ | 123.9, $CH_2$ | 124.0, $CH_2$ | 124.0, $CH_2$ | 122.5, $CH_2$ | 123.3, $CH_2$ | 123.8, $CH_2$ | 123.7, $CH_2$ | 123.8, $CH_2$ | 123.3, $CH_2$ | 122.4, $CH_2$ | 121.7, $CH_2$ | 123.2, $CH_2$ | 123.3, $CH_2$ |

**13a**+**d**: 1-O*C*$H_3$: 51.7; 8a-*O*C$H_3$: 54.7; **13b**+**c**: 1-O*C*$H_3$: 51.7; 8a-*O*C$H_3$: 54.5; **14a**: 4a-O*C*$H_3$: 51.1; 8a-*O*C$H_3$: 52.5; **14b**: 4a-O*C*$H_3$: 51.6; 8a-*O*C$H_3$: 52.9; **15**: 1-O*C*$H_3$: 54.4; 5-O*C*$H_3$: 61.1; **16a**+**b**: 8a-*O*C$H_3$: 51.6; **17**: 4a-O-$CH_2$-$CH_3$ (interchangeable with 8a): 60.2, *C*$H_2$, 14.7, *C*$H_3$; 8a-*O*-$CH_2$-$CH_3$ (interchangeable with 4a): 59.0, *C*$H_2$, 15.8, *C*$H_3$; **18a**: 1-O-$CH_2$-$CH_3$ (interchangeable with 8a): 62.5, *C*$H_2$, 15.7, *C*$H_3$; 8a-*O*-$CH_2$-$CH_3$ (interchangeable with 1): 59.6, *C*$H_2$, 15.5, *C*$H_3$; **18b**: 1-O-$CH_2$-$CH_3$: 62.2, *C*$H_2$, 15.8, *C*$H_3$; 8a-*O*-$CH_2$-$CH_3$: 59.6, *C*$H_2$, 15.6, *C*$H_3$; **19a**+**b**: 8a-*O*-$CH_2$-$CH_3$: 59.5, *C*$H_2$, 15.4 *C*$H_3$; **20**: 1-O-$CH_2$-$CH_3$: 62.2, *C*$H_2$, 15.8, *C*$H_3$; **21**: 7-*O*-$CH_2$-$CH_3$: 61.6, *C*$H_2$, 15.6, *C*$H_3$; **22**: 7-*O*-$(CH_2)_3$-$CH_3$: 66.3 (1'-*C*$H_2$), 32.4 (2'-*C*$H_2$), 19.2/19.3/19.4* (3'-*C*$H_2$) (interchangeable with 3"-*C*$H_2$ and 3'"-*C*$H_2$), 14.1 (4'-*C*$H_3$); 5-*O*-$(CH_2)_3$-$CH_3$: 63.3 (1"-*C*$H_2$), 31.8/31.9* (2"-*C*$H_2$) (interchangeable with 2'"-*C*$H_2$), 19.2/19.3/19.4* (3"-*C*$H_2$) (interchangeable with 3'-*C*$H_2$ and 3'"-*C*$H_2$), 13.8/13.9* (4"-*C*$H_2$) (interchangeable with 4'"-*C*$H_3$); 63.6 (1'"-*C*$H_2$), 31.8/31.9* (2'"-*C*$H_2$) (interchangeable with 2"-*C*$H_2$), 19.2/19.3/19.4* (3'"-*C*$H_2$) (interchangeable with 3'-*C*$H_2$ and 3"-*C*$H_2$), 13.8/13.9* (4'"-*C*$H_2$) (interchangeable with 4"-*C*$H_3$); **23**: 8a-*O*-$(CH_2)_3$-$CH_3$: 63.5 (1'-*C*$H_2$), 31.9 (2'-*C*$H_2$), 19.1 (3'-*C*$H_2$), 13.7 (4'-*C*$H_3$).