Triterpenoids in Epicuticular Waxes of Three European *Euphorbia* Species

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Dedicated to Professor Lothar Jaenicke on the occasion of his 65th birthday

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The epicuticular leaf waxes from *E. characias, E. nicaeensis* and *E. peplus* were found to contain pentacyclic triterpenoids in great amounts in addition to the common lipid wax constituents: alkanes, wax esters, aldehydes, primary alcohols and fatty acids. The gross amount of triterpenoids were triterpenols, however, their acetates and fatty acid esters as well as the corresponding ketones were found, too. Distinctive variations in the occurrence of 13 triterpenoids α- and β-amyrin, lupeol, germanicol, α-fernenol, simiarenol, β-amyrinone, lupenone, lupeol acetate, lupeol esters, α- and β-amyrin esters and α-fernenol esters were observed. Therefore all *Euphorbia* species showed very species specific compositions concerning these triterpenoids.

Introduction

Most of the European members of the family *Euphorbiaceae* are monoecious herbs or small shrubs. *E. characias* L. (section esula) is a densely tomentose, glaucous, perennial shrub, sometimes with biennial stems up to 180 cm and 13–30 (–40) axillary rays. It preponderantly grows on dry, fairly open grounds in mediterranean regions. *E. nicaeensis* All. (section paralias) is a glaucous, often reddish-suffused perennial shrub up to 80 cm, with 0–10 (–20) axillary rays. It also prefers dry and open grounds and is widespread through the whole parts of south, central and east Europe. In contrast to these two species *E. peplus* L. (section cymatospermum) is a glabrous annual weed up to 40 cm, with two or more branches from the base and with 0–3 axillary rays. It is a weed of cultivated ground spread through the most of Europe northwards to 65° N [1].

All *Euphorbia* species produce a characteristic milky-white chemically rich latex which contains various biologically active components [2–8] and a number of tetra- and pentacyclic triterpenoids, partly in considerable quantities [9–14], with various physiological functions and therapeutical properties [15]. The analysis of wax constituents from several *Euphorbia* species showed unusually high amounts of triterpenoids in the surface waxes, too [16, 17]. These substances, resulting from the isoprene metabolism, were found in addition to the homologous series of the common wax lipids: alkanes, wax esters, aldehydes, primary alcohols and fatty acids. For this reason it was useful to confirm and extend the study of these waxes by complete analyses of triterpenoids. This paper summarizes the triterpenoid composition of *E. characias, E. nicaeensis* and *E. peplus*.

Materials and Methods

Plant material was cultivated from seeds in the gardens of the Botanical Institute, University of Cologne under identical environmental and horticultural conditions. Stems were cut in autumn and extracted immediately twice by short time immersion in distilled chloroform. Care must be taken that no latex contaminates the extract. The wax extracts were filtered, evaporated to dryness, weighed and subsequently redissolved in hexane. Waxes were fractionated by CC on silica gel 60, 0.063–0.02 mm (Merck, Darmstadt). Elution was with solvents of

Abbreviations: CC, column chromatography; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; LPLC, low pressure liquid chromatography; TLC, thin-layer chromatography.

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increasing polarity (pentane, 2-chloropropane, methanol) as carried out previously [16, 18, 19, 20]. The columns finally were eluted with chloroform. Single triterpenoid classes were further separated by LPLC on silica gel 60, 0.04–0.063 mm (Merck, Darmstadt) using toluene as eluent for the 2-chloropropane fractions of the initial CC, which contained triterpenol esters and -acetates. The methanol fractions from the first CC, containing triterpenols and triterpenones, were eluted with dichloromethane. In both cases the flow rate was 1.5 ml/min and the collection volume was 5 ml/fraction. Individual triterpenoids and also triterpenoid mixtures from LPLC separation were examined by chemical reactions (ethanolysis, acetylation, reduction, oxidation), TLC, GC, GC-MS and comparison with authentic samples as described recently [18, 21, 22].

TLC: Silica gel 60 precoated plates (Merck, Darmstadt) with the solvents toluene (Rf_1); CH_2Cl_2/EtOAc 24:1, appropriate for polar components (Rf_2); $AgNO_3$ impregnated plates, CH_2Cl_2/EtOAc 24:1 (Rf_3); spray reagents used: a) bromothymol-blue; b) carbazole for the selective detection of triterpenoids [23].

GC: Hewlett Packard 5710, equipped with FID and integrator 3380 S, fused silica column 10 m OV-1 CB, temperature program 180–280 °C, 4 °C/min.

GC-MS: Finnigan-MAT 4510, 70 eV, EI, fused silica column 15 m DB-1.

Authentic samples: α-amyrin, β-amyrin, lupeol, lupenone, lupeol acetate (Roth, Karlsruhe); germanicol, simiarenol, α-fernenol (Dr. A. P. Tulloch, Prairie Regional Laboratory, Saskatoon, Canada).

Results

Epicuticular waxes from *E. characias, E. nicaeensis* and *E. peplus* contained the common long chain and saturated lipid components *n*-alkanes, wax esters, aldehydes, primary alcohols and fatty acids. With exception of small deviations, yields and compositions were in good agreement with those previously observed [16, 17]. Similar patterns were found for other *Euphorbia* species [18, 19, 24]. The analytical method used in an earlier investigation [16] was less capable of estimating the amount of primary alcohols exactly. For this reason their quantity had to be corrected (Table I).

Pentacyclic triterpenoids were present in high amounts in all the surface waxes (18–43%) of the *Euphorbia* species studied. These triterpenoids mainly appeared as triterpenols whereas triterpenones, triterpenol acetates and triterpenol esters were present in smaller amounts (Tables I and IV).

Plant epicuticular waxes frequently contain triterpenoids but they are usually described as minor components [25]. In this investigation, however, triterpenoids and primary alcohols (26–31%) were the dominating major components. In *E. characias* triterpenoids and alcohols together even reached 69% of the total wax (*E. nicaeensis* 62%; *E. peplus* 45%). The highest amounts could be noted in

Table I. Composition and yield of epicuticular leaf waxes from three *Euphorbia* species*.

Components	*E. characias*	*E. nicaeensis*	*E. peplus*	Rf_1	Rf_2
n-Alkanes	14.5	3.0	5.9	0.71	–
Wax esters	9.1	6.1	12.6	0.65	–
Aldehydes	3.0	4.5	1.3	0.42	0.79
pr. Alcohols	26.1	31.3	26.5	0.06	0.30
Fatty acids	1.1	4.0	7.4	0.01	0.15
Triterpenol esters	3.0	0.7	1.8	0.67	–
Triterpenol acetates	+	–	–	0.30	0.66
Triterpenones	16.2	–	+	0.20	0.55
Triterpenols	23.4	18.3	16.2	0.06+	0.30
Unidentified	2.1	11.7	5.0		
Lost on column	1.5	20.4	23.3		
Yield (% dry wt)	1.1	0.7	1.3		

* In wt % of crude wax.
+ Except simiarenol; Rf values see Table II.

Table II. Characterization and identification of triterpenoids from waxes of three *Euphorbia* species.

Components	TLC Rf_1	Rf_2	Rf_3	Carbazole	GC rrt*	MS M^+
Triterpenol acetates						
Lupeol acetate	0.30	0.65	0.44	+	1.130	468
Triterpenones						
β-Amyrinone	0.20	0.55	0.52	+	0.979	424
Lupenone	0.20	0.55	0.40	+	1.024	424
Triterpenols						
β-Amyrin	0.06	0.30	0.30	+	1.000	426
Germanicol	0.06	0.30	0.31	+	1.013	426
α-Amyrin	0.06	0.30	0.30	+	1.041	426
Lupeol	0.06	0.30	0.19	+	1.043	426
α-Fernenol	0.06	0.30	0.30	+	1.095	426
Simiarenol	0.13	0.41	0.03	+	1.147	426

* Relative retention time (10 m OV 1 CB, β-Amyrin = 1.000).

E. aphylla wax which accounted for 73% of both, which is in fact nearly three fourth of the crude wax [19].

Triterpenols

In *Euphorbia* surface waxes mostly mixtures of different triterpenols were found accompanied with primary alcohols in the methanol fraction (CC). Individual triterpenols could be isolated by LPLC with dichloromethane as eluent. They were identified by a positive carbazole colour reaction, their individual Rf values on $AgNO_3$ impregnated silica gel plates (Rf_3) and their relative retention times (rrt) by GC analysis in comparison with authentic samples, using a capillary column (Table II). Furthermore GC-MS analysis proved to be an excellent method for the identification of triterpenoids. The mass spectra of all triterpenols showed a molecular ion at *m/z* 426, corresponding to $C_{30}H_{50}O$. Additionally all spectra showed significant fragment ions and individual fragmentation patterns. By the aid of these spectra the identification of triterpenols succeeded definitively.

The principal triterpenols of the three *Euphorbia* waxes examined were β-amyrin, α-amyrin and lupeol (Table IV). The waxes of *E. characias* and *E. nicaeensis* only contained these three triterpene alcohols. In *E. characias* lupeol was the major component, whereas α-amyrin was prominent in *E. nicaeensis* (Table IV). In *E. peplus* only small

Table III. Characteristic mass fragments of triterpenoids isolated from waxes of three *Euphorbia* species*.

Components	Characteristic *m/z* (relative intensity)
β-Amyrin	426 (2), 411 (1), 257 (1), 243 (0.5), 218 (100), 2033 (40), 189 (15)
α-Amyrin	426 (1), 411 (0.5), 257 (1), 243 (0.5), 218 (70), 203 (20), 189 (20)
Lupeol	426 (3), 411 (0.5), 315 (1.5), 257 (2), 218 (20), 207 (30), 189 (35)
Germanicol	426 (2), 411 (1), 281 (0.5), 231 (4), 204 (50), 189 (50), 177 (70)
α-Fernenol	426 (0.5), 411 (1), 273 (0.5), 259 (40), 241 (18), 229/191 (1)
Simiarenol	426 (1), 408/393/286 (0.5), 274 (50), 259 (40), 245 (5), 231 (15) 205/204/189 (10), 175/161 (15), 152 (60), 134 (100)
β-Amyrinone	424 (3), 409 (1), 257 (1), 243 (0.5), 218 (100), 203 (22), 189 (18)
α-Amyrinone	424 (3), 409 (1), 257 (1), 243 (0.5), 218 (75), 203 (20), 189 (20)
Lupenone	424 (0.6), 409/257 (0.3), 313 (1), 218 (5), 205 (20), 189 (10)

* First peak is M^+.

Table IV. Composition of triterpenoids from leaf waxes of three *Euphorbia* species*.

Components	*E. characias*	*E. nicaeensis*	*E. peplus*
Triterpenol esters			
β-Amyrin esters	–	–	10
α-Amyrin esters	17	100	–
Lupeol esters	40	–	60
Fernenol esters	–	–	30
Unidentified 1	22		
Unidentified 2	13		
Unidentified 3	8		
	100	100	100
Triterpenol acetates			
Lupeol acetate	< 1	–	–
Triterpenones			
β-Amyrinone	1	–	< 1
Lupenone	37	–	–
Triterpenols			
β-Amyrin	2	10	< 1
Germanicol	+	–	–
α-Amyrin	< 1	60	–
Lupeol	59	30	16
α-Fernenol	–	–	18
Simiarenol	–	–	63
Unidentified	–	–	3
	100	100	100

* Relative amounts.

amounts of lupeol and β-amyrin were found. β-Amyrin, α-amyrin and lupeol also were major triterpenols in other *Euphorbia* species [18, 19, 24]. They all showed a reddish brown carbazole reaction. The presence of a free alcohol group was proven by a positive acetylation.

β-Amyrin and α-amyrin structurally are identical with exception of the position of one methyl substituent in ring E of the molecule (Fig. 1). For that reason their chromatographic behaviour on normal and $AgNO_3$ impregnated silica gel plates was quite the same, whereas a gaschromatographic separation

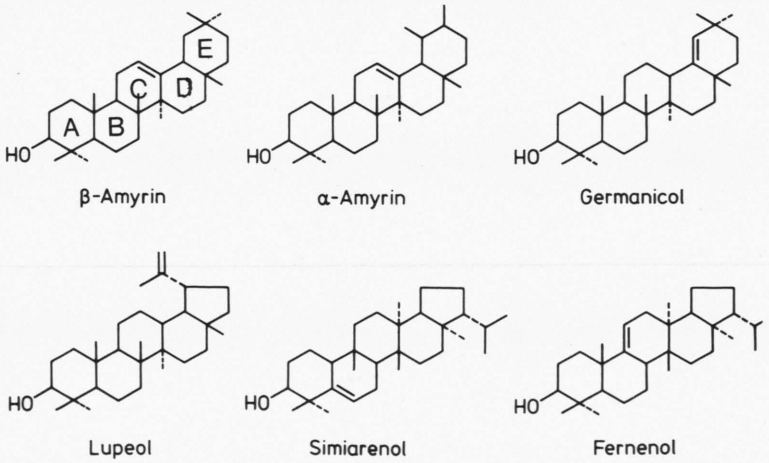

Fig. 1. Structure of triterpenols from epicuticular waxes of *Euphorbia* species.

of both was possible very well (β-amyrin rrt 1.000, α-amyrin 1.041).

The amyrins showed very characteristic mass spectra which unambiguously indicated a *retro*-Diels-Alder cleavage of ring C with a double bond in position Δ 12. Thus these compounds exhibited a strong peak *m/z* 218 corresponding to this process [26–28] as well as prominent peaks *m/z* 203 (218-Me) and 189. Their identical ring skeleton also resulted in nearly identical mass spectra with exception of the intensities of the three fragments mentioned above. β-Amyrin had very distinguished relative intensities {218(100), 203(40), 189(15)} for these fragments. In contrast to β-amyrin the spectrum of α-amyrin showed a somewhat lower intensity for *m/z* 218 and nearly the same intensities for *m/z* 203 and *m/z* 189 (Table III).

Characteristic mass fragments for lupeol were *m/z* 315, 218, 207 and 189, *m/z* 207 (30%) being the main fragment ion. Lupeol (rrt 1.043) and α-amyrin (rrt 1.041) had very similar relative retention times so that no complete separation was achieved by GC. Nevertheless the presence of both compounds in *E. characias* and *E. nicaeensis* was confirmed by their characteristic mass spectra (see Table III). Additionally lupeol showed a quite lower Rf_3 value (Rf_3 0.19) than α-amyrin (Rf_3 0.30), according to an exocyclic positioned double bond in Δ 20(30) (see Fig. 1).

Furthermore in *E. characias* wax germanicol was found as a minor component and the wax from *E. peplus* contained α-fernenol and simiarenol, the latter one being predominant, whereas α-fernenol appeared in amounts comparable to lupeol. Another triterpenol, not yet identified, could be isolated in traces from this species (Table IV).

Germanicol belongs to the oleanen group with a double bond in position Δ 18 of ring E (Fig. 1). Methyl and hydroxyl substitutions are identical to those of β-amyrin. The generally similar chemical structure of both triterpenols leads to identical Rf-values in TLC (Table II). Also the difference in GC retention times was but small. In contrast, the mass spectra of Δ 18-oleanenes were quite different from those of Δ 12-oleanenes, due to the position of the double bond. The mass spectrum of germanicol exhibited a *m/z* 177 peak, representing a $C_{13}H_{21}^+$ ion accompanied by two further important fragments with slightly lower intensities, namely the ions *m/z* 189 and 204 (see Table III) [26].

α-Fernenol belongs to the fern-9(11)-ene group (Fig. 1). The mass spectrum showed a prominent peak at *m/z* 259 and two minor peaks at *m/z* 241 and 273. The general fragmentation pattern, especially the very pronounced appearance of *m/z* 259, followed by *m/z* 241 suggested the molecular structure of fernenol [29]. α- and β-fernenol have identical mass spectra but can be distinguished by the relative GC retention times [30].

Simiarenol is a Δ 8 unsaturated triterpenol with an isopropyl group in position C_{21} (Fig. 1). Characteristic fragment ions in its mass spectrum were *m/z* 274(50), 259(40), 241(18) and 231(15), due to the easy loss of the isopropyl group (C_3H_7) from ion *m/z* 274 (Table III). In addition, simiarenol showed Rf-values (Rf_1 0.13, Rf_3 0.03) significantly deviating from the other triterpenols mentioned above (Rf_1 0.06, Rf_3 0.30 and 0.19) (Table II). The presence of all triterpenols found in the three surface waxes could be confirmed by comparison of the mass spectra with those of authentic samples. Germanicol, fernenol and simiarenol mainly were observed in waxes of panicoid and eragrostoid grasses [30–32]. Fernenol was present in *E. lathyris* epicuticular waxes, too [22], and simiarenol also was found in waxes from *E. lathyris* [22] and *E. aphylla* [19].

Triterpenol esters

All epicuticular leaf waxes from *Euphorbia* species contained triterpenols esterified with homologous series of long chain fatty acids [18, 19]. They showed the same colour reaction with carbazole as determined for free triterpenols. In some cases, depending on the chain length of the combined fatty acids, these triterpenol esters could be separated from wax esters by TLC [18]. Ethanolysis of the esters yielded the free triterpenols and fatty acid ethyl esters. The triterpenols were identified in the same manner as discussed above (TLC, GC, GC-MS) (Table IV). *E. characias* wax contained α-amyrin esters, lupeol esters and further esters with not yet identified alcohols. In *E. nicaeensis* wax only α-amyrin esters were found. *E. peplus* wax contained β-amyrin esters, lupeol esters and α-fernenol esters but no simiarenol esters; this alcohol represented the dominating component in the free triterpenol mixture of this species (Table IV). Simiarenol was present in *E. aphylla* wax, too [19] and also in this wax it was not found esterified. Comparison of free and combined triter-

penols analyzed in this and other investigations about epicuticular waxes in the genus *Euphorbia* [18, 19, 22], as well as in panicoid and eragrostoid grass waxes [29, 30], indicated, that β-amyrin, α-amyrin and lupeol may be selectively and predominantly esterified.

Triterpenones

Triterpenones were present in two of the three *Euphorbia* species studied. The compositions are shown in Table IV. In this case they appeared together with triterpenols in the methanol fraction after CC (silica gel 60, 0.063–0.02 mm) and were isolated by LPLC (silica gel 60, 0.04–0.063 mm). Triterpenones showed a positive carbazole colour reaction and were established by reduction with $NaBH_4$ to the corresponding alcohols. Individual components were identified definitely by their relative retention times in comparison with authentic samples and especially by their MS spectra. β-Amyrinone and lupenone generally showed the same fragmentation patterns like the corresponding alcohols but according to their keto function, connected with the loss of two hydrogen atoms, the molecular ions shifted from *m/z* 426 to *m/z* 424. Additionally the characteristic peak *m/z* 315, occurring in lupeol, spectrum, shifted to *m/z* 313 in the lupenone spectrum indicating, that this fragment comprises ring A of the molecule (Table III; Fig. 1).

E. peplus wax contained β-amyrinone in a very low concentration. A similar low concentration for β-amyrinone was observed in *E. characias* wax, whereas lupenone, likewise present in this species, reached a considerably higher content (16.2% of the wax), comparable to the triterpenols (Tables I and IV). *E. nicaeensis* wax was very unusual in containing no triterpenones. Previously in the leaf wax of *E. dendroides* β-amyrinone and lupenone were found in remarkable quantities (13% of wax) [18] whereas *E. aphylla* wax contained β-amyrinone, α-amyrinone and lupenone (2% of the wax) [19].

Triterpenol acetates

Lupeol acetate was the single triterpenol acetate observed in the three waxes. It occurred exclusively in the 2-chloropropane fraction from *E. characias* wax in a very small amount. Identification was carried out as described above (TLC, GC, GC-MS) (see Table II). The MS fragmentation pattern showed the same characteristic ions for lupeol but the molecular ion shifted to *m/z* 468 corresponding to the acetate group. From several triterpenols observed in *E. esula* epicuticular wax only lupeol has been acetylated, too [24]. Acetylated α-amyrin, β-amyrin and lupeol in remarkable amounts were found in *E. aphylla* wax [19].

Discussion

The analysis of triterpenoid constituents of *Euphorbiaceae* has received attention since early analyses suggested their potential availability as chemotaxonomic indicators [33–35]. Furthermore several *Euphorbia* species were examined for their economic utilization as alternative energy sources in the production of liquid fuels [36–38]. With regard to these facts we continued our analyses in epicuticular waxes of *Euphorbiaceae*. The present study of three species from the genus *Euphorbia* agrees with the conclusion observed previously [16–19]. With minor variations, yields and compositions of common lipid components of epicuticular waxes are identical. Dramatic differences were observed concerning yields and occurrences of triterpenoids which showed very species specific compositions. All the species examined had a relatively high triterpenoid content from which triterpenols constituted the largest group. Additionally triterpenones, triterpenol esters and a triterpenol acetate were present. With exception of *E. characias* (triterpenones 16.2%) the latter components appeared in low amounts. The proportions of individual triterpenoids varied considerably. α-Amyrin, prominent as alcohol and ester in *E. nicaeensis* was a minor component in *E. characias* and absent in *E. peplus*. Lupeol, which formed the major ester in *E. characias* and *E. peplus* and the major alcohol and ketone in *E. characias* was a minor component in *E. nicaeensis*. Simiarenol only was prominent in *E. peplus* but appeared not as ester or ketone in this species whereas α-fernenol, likewise only present in *E. peplus* but in much lower amounts appeared esterified in remarkable amounts. *E. characias* contained a triterpenol acetate (lupeol acetate) and germanicol. *E. nicaeensis* differed from the others in having no triterpenones. α- and β-amyrin as well as lupeol are not uncommon constituents in plant waxes [38]. In contrast α-fernenol, simiarenol and germanicol are not frequently men-

tioned as wax constituents. The results shown here for the epicuticular waxes are not in agreement with the triterpenoids described to be present in the latex. In *E. characias* and *E. lathyris* various lanosterols, euphol, cycloartenol and 24-methyl cycloartenol are the latex triterpenoids [13], whereas for *E. peplus* latex β-amyrin and lupeol are described [34]. These results confirm the assumption that the latex system behaves as a metabolically autonomous system in the plant [39].

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