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THE OCCURRENCE OF LANOSTEROL AND 24-METHYLENELANOST-8-EN-3β-OL IN LEAVES OF *SYMPHORICARPUS ALBUS*

GUNTER WILLUHN, IRMGARD MERFORT and UWE MATTHIESEN*

Institut fur Pharmazeutische Biologie der Universitat Dusseldorf, *Institut fur Physiologische Chemie II der Universitat Dusseldorf, Dusseldorf, West Germany

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Abstract—From the leaves of *Symphoricarpus albus* the triterpene alcohols lanosterol and 24-methylenelanost-8-en-3β-ol in addition to cycloartenol, 24-methylenecycloartanol, butyrospermol, lupeol, α- and β-amyrin, and bauerenol, the 4α-methylsterols gramisterol, obtusifoliol, cycloeucalenol, citrostadienol, 31-norlanosterol, 31-norcycloartenol, 31-norlanost-8-en-3β-ol and lophenol were identified Free sterols, and sterols bound in the form of esters, glucosides and acylated glucosides, were isolated and identified as a mixture of cholesterol, campesterol, 24-methylenecholesterol, stigmasterol, sitosterol, isofucosterol and clerosterol The total sterol contents and the amounts of the different forms of sterols were determined The triterpene acids ursolic acid, oleanolic acid, and 2α-hydroxyursolic acid were isolated The possible use of lanosterol, clerosterol, the pentacyclic triterpene alcohols and the acids as taxonomic markers within the Caprifoliaceae is discussed Additionally the aliphatic compounds such as fatty acids, alkanes, primary and secondary alcohols were analysed

INTRODUCTION

Symphoricarpus albus belongs to the chemically little investigated family of the Caprifoliaceae of which phylogenetic unity and, therefore, classification in the plant system is controversial [1–5] Knowledge of the lipid composition may help to clarify these disagreements Therefore we have analysed the triterpene alcohol and acid, 4α-methylsterol, sterol, fatty acid, alkane and primary and secondary alcohol constituents of *S albus*

RESULTS AND DISCUSSION

Triterpene alcohols

From the triterpene alcohol fraction the following tetracyclic triterpenes were identified by TLC, GC and GC/MS cycloartenol (**1**), lanosterol (**2**), 24-methylenecycloartanol (**3**), 24-methylenelanost-8-en-3β-ol (**4**), butyrospermol (**5**) and probably lanosta-7,24-dien-3β-ol (**6**) In addition to these substances, a further triterpene alcohol, with an unsaturated side chain and two double bonds in the ring system, was isolated All these tetracyclic triterpenes were found in free and esterified form

From the group of pentacyclic triterpene alcohols lupeol (**7**), α- and β-amyrin (**8**, **9**), and a compound derived from α- or β-amyrin (28-nor-urs-12-en-3β-ol or 28-nor-olean-12-en-3β-ol, **10**) were identified They mostly occurred in the free form Moreover, bauerenol (**11**) and four unidentified compounds were found in the esterified fraction Based on the mass spectral fragmentation pattern, one of them must be a taraxerene derivative with a methyl group at C-19 and C-20 Another one has a hopane or an arborane skeleton Further investigations were not possible because of the very small amounts The compositions of the triterpene alcohols are listed in Table 1

The most significant feature of the triterpenes of *S albus* is the occurrence of lanosterol and 24-methylenelanost-8-en-3β-ol which, up to now, have been found only in four families (Euphorbiaceae, Solanaceae, Brassicaceae and Sapotaceae) [6–15] In these families the two Δ^8-lanostane triterpenes were found in the latex or in the seeds This is the first reported identification of lanosterol and 24-methylenelanost-8-en-3β-ol in leaves The co-occurence of the Δ^8-triterpenes with the corresponding 9β,19-cyclo-isomers, cycloartenol and 24-methylenecycloartanol, suggests that the enzymatic opening of the 9β,19-cyclopropane ring may not only occur at the 4α-methylsterol level [16] but also at the 4,4-dimethylsterol level in this plant [8] Another possibility is the occurrence of two different enzymes for squalene cyclization, one to yield lanosterol and another for cycloartenol [17] The occurence of these Δ^8-triterpenes may be important for comparative chemotaxonomic studies of the Caprifoliaceae Itoh *et al* [15] have shown that not all plants are able to form lanosterol Therefore, it may be possible to conclude relationships between species, genera and tribes from the occurence or absence of lanosterol The same is valid for butyrospermol and the pentacyclic triterpenes with different ring systems

4α-Methylsterols

From the 4α-methylsterol fraction gramisterol (**12**), obtusifoliol (**13**), cycloeucalenol (**14**), citrostadienol (**15**), 31-norlanosterol (**16**), 31-norcycloartenol (**17**), 31-norlanost-8-en-3β-ol (**18**) and lophenol (**19**) were identified in free and esterified form by TLC, GC and GC/MS These 4α-methylsterols are intermediates in sterol biosynthesis Compounds **16**–**18** are especially interesting because they are considered to be intermediates in the

Table 1 Approximate composition (%) of the free and esterified triterpene alcohols of the leaves from *S albus*

TLC-zone R_f	Triterpene (acetylated)	RR_t*	Composition (%)† Esterified 0·02% from dry wt	Free 0·01% from dry wt
	24-Methylenecycloartanol	1·69	4	2
0·60	Lupeol	1·57	tr	1
	24-Methylenelanost-8-en-3β-ol	1·42	1	2
0·68	Butyrospermol	1·38	14	tr
0·71	Lanosta-7,24-dien-3β-ol	1·56	5	tr
	Unidentified tetracyclic triterpene	1·26	1	tr
0·78	Cycloartenol	1·52	55	3
	Lanosterol	1·28	15	3
	Unidentified pentacyclic triterpene	1·29	tr	—
	Unidentified pentacyclic triterpene	1·35	tr	23
	β-Amyrin	1·37	?	
0·86	Taraxerene derivative	1·47	1	—
	α-Amyrin	1·52	—	15
	28-Nor-urs-12-en-3β-ol or 28-nor-olean-12-en-3β-ol	1·52	—	51
	Hopane or arborane derivative	1·56	1	—
	Bauerenol	1·81	tr	—

*RR_t determined on an OV-17 column, relative to sitosterol
†Determined by GC peak area

biosynthesis of cholesterol [18] Heintz and Benveniste [16] supposed that they are ubiquitous in the plant kingdom Up to now they have not been found as often as the other mentioned 4α-methylsterols

Sterols

Free and esterified sterols of the petrol extract were identified by TLC, GC, and GC/MS as a mixture of sitosterol (**20**), stigmasterol (**21**), campesterol (**22**), cholesterol (**23**), isofucosterol [**24** (= 24-ethyl-cholesta-5, *Z*-24(28)-dien-3β-ol)] clerosterol [**25** (= 24β-ethyl-cholesta-5,25(27)-dien-3β-ol)] and 24-methylenecholesterol (**26**) Compound **20** was the main component

An authentic sample of sterol **25** was not available and its identification was based on the TLC, GC and GC/MS data reported in the lit [19–24] The C-24 configuration is left in doubt in this study Clerosterol, found in *Momordica charantia* [19] and *Kalanchoe daigremontiana* [22], has been demonstrated to have the 24β-ethyl configuration The identified sterols are common in higher plants except for clerosterol (**25**) which, therefore, may be useful in future for taxonomic studies of the Caprifoliaceae

From the defatted acetone extract sterylglycosides and acyl sterylglycosides were isolated Alkaline hydrolysis of the acyl sterylglycosides yielded palmitic acid which was identified by GC after methylation The resulting sterylglycosides and the original sterylglycosides were acid hydrolysed In both cases glucose was identified as the only sugar by TLC and GC of the alditol acetate [25] By GC analysis the aglycones of the sterylglucosides and the acyl sterylglycosides were found to be mainly sitosterol (**20**) besides small amounts of **21**–**24** Thus the sterylglycosides were identified as glucosides and the acyl sterylglycosides as palmitic esters of these glucosides

The total sterol contents and the amounts of the different forms of sterols were determined by photometry The total amount of sterol (0·13% dry wt) did not differ from that found in other plants [26–29], 39% of the sterols occurred in the free form, 30% were esterified and 31% were glucosidic bound Compared with other plants [26, 27] there seem to exist remarkable differences in the amounts of these different forms of sterols

Fatty acids

After methylation the fatty acids were identified by GC as a mixture of saturated acids with carbon numbers 12 and 14–24 as well as the unsaturated acids palmitoleic, oleic and linoleic acid Palmitic (52%), stearic (7%) and oleic acids (20%) were dominant

Alkanes

GC analysis of the alkanes showed the homologous series of carbon numbers 16–33 in which the odd-carbon number alkanes were dominant, in this case n-C_{29} (65%) and n-C_{31} (29%) [30]

Primary aliphatic alcohols

GC analysis of the free primary aliphatic alcohols revealed the homologous series n-C_{20}–n-C_{30} in which the compounds with even-carbon number dominated, in this case n-C_{20} (10%), n-C_{22} (17%) and n-C_{24} (33%) Esterified primary alcohols were analysed together with

triterpenes after saponification Their composition did not differ from that of the free alcohols

Secondary aliphatic alcohols

Secondary alcohols occurred in the leaves only in the free form as the main components of the petrol-soluble lipids (11%) The secondary nature of the hydroxyl group was established by IR and oxidation to ketones [31] Reduction gave the corresponding paraffins which by GC analysis were mainly identified as *n*-nonacosane (99 5%) The position of the hydroxyl group was determined by mass spectrometry [32] The secondary alcohols were identified as a mixture of nonacosan-10-ol (90%), nonacosan-9-ol (5 8%) and nonacosan-11-ol (3 6%) for the first time in a Caprifoliaceae species The simultaneous main occurrence of *n*-nonacosane as well as the *n*-nonacosanols supported the suggestion that secondary alcohols are formed by hydroxylation of alkanes [33]

Triterpene acids

From the defatted acetone extract ursolic acid, oleanolic acid and 2α-hydroxyursolic acid were isolated The isomeric ursolic acid and oleanolic acid were identified by mp, IR and GC/MS of their methyl esters Their mass spectra differed only in the proportion of the intensity of the fragments at *m/z* 262 and 203 (methyl oleanolate, 1 2, methyl ursolate, 1 1 [34]) The concentrations of these two triterpene acids were determined by GC [27] to be 0 4% on a dry wt basis Ursolic acid predominated (0 22%, cf oleanolic acid 0 18%)

2α-Hydroxyursolic acid was identified by TLC, mp, IR and mass spectral data Whereas oleanolic acid has already been found in the leaves of *S albus* [35], ursolic acid and 2α-hydroxyursolic acid were identified for the first time Ursolic acid and oleanolic acid are also constituents in other Caprifoliaceae species [36–42] Up to now 2α-hydroxyursolic acid has only been identified in *Viburnum* species in this family [39, 41] 20β-Hydroxyursolic acid occurs in flowers of *Sambucus nigra* [43] but we could not detect it in leaves of *S albus* Thus, there seem to exist different routes of hydroxylation to yield the acids derived from amyrin, within the Caprifoliaceae species which may be useful for taxonomic studies

EXPERIMENTAL

Mps are uncorr IR spectra were recorded in KBr MS (70 eV, triterpene alcohols and 4α-methylsterols *m/z* > 150, otherwise *m/z* > 50) were taken with a GC/MS (OV-101 WCOT glass capillary column, 230°, splitless injection, sample vol 0 1–0 5 μl) or with a probe injection GC triterpene alcohols 2 m × 2 5 mm glass column packed with 3% OV-17 (260°, N_2 50 ml/min, RR_t is given relative to sitosterol), 2 m × 2 mm steel column packed with 3% SE-30 (250°, N_2 30 ml/min), 2 m × 3 mm glass column packed with 3% QF-1 (250°, N_2 40 ml/min), 4α-methylsterols 3% OV-17 (270°, N_2 50 ml/min), sterols 3% OV-17 (270°, N_2 30 ml/min), 3% SE-30 (250°, N_2 26 ml/min), 2 m × 3 mm glass column packed with 3% XE-60 (235°, N_2 25 ml/min), RR_t is given in each case relative to cholesteryl acetate, primary alcohols 3% OV-17 (260°, N_2 30 ml/min), alkanes 3% SE-30 (230°, N_2 26 ml/min), secondary alcohols 3% SE-30 (250°, N_2 30 ml/min), fatty acids as Me esters 2 m × 2 mm steel column packed with 10% DEGS on Supelcoport (temp programmed 80–180° at 6°/min, N_2 30 ml/min), glucitol acetate 2 m × 3 mm glass column packed with 3% OV-225 (190°, N_2 40 ml/min), Me esters of triterpene acids 3% OV-17 (280°, N_2 60 ml/min, RR_t relative to cholesterol) Approx compositions of the compounds were based on the GC data

Fractionation of petrol extract Air-dried and ground leaves of *Symphoricarpus albus* (L) Blake (1 4 kg) were exhaustively extracted with petrol in a Soxhlet apparatus (46 g lipids) The extract (10 g) was fractionated over Al_2O_3 (Woelm, neutral, Act II) with solvents of increasing in polarity (petrol, C_6H_6, EtOAc, MeOH) The extract (9 g) was also separated by dry-CC (Silica Woelm, TSC, Act II) with toluene–petrol (65 35) [44] By repeated CC and prep TLC the following fractions, in addition to fractions with unidentified compounds, were obtained *n*-alkanes (1 4 g), ester compounds (3 76 g), secondary aliphatic alcohols (2 1 g), free triterpene alcohols accompanied by primary aliphatic alcohols (0 21 g), 4α-methylsterols (0 01 g) and sterols (0 33 g) The ester fraction was saponified in refluxing methanolic KOH [45] By repeated Si gel CC of the unsaponifiable lipid (2 34 g) with petrol–EtOAc–toluene (80 15 5), 0 19 g triterpene alcohols together with primary alcohols, 0 05 g 4α-methylsterols, and 0 13 g sterols were obtained The aq soln was extracted (pH 1) with petrol to yield the fatty acids (0 34 g)

Triterpene alcohols The triterpene alcohols from the ester fraction were acetylated and separated into five zones by prep Si gel TLC TLC plates were impregnated with 20% aq $AgNO_3$ soln and developed with *n*-hexane–C_6H_6 (1 1) The zones were extracted and the single components were identified by comparing their GC retention times with those of authentic samples on three different stationary phases and by GC/MS Data were identical with those reported in the lit [12, 46–50] The free triterpene alcohols were separated from primary alcohols via the urea canal inclusion complex [51] After acetylation, further separation and identification was performed as described above Zones 1–4 of the triterpene acetates from the ester fraction corresponded with those of the free triterpenes Zone 1 (R_f 0 60) **3**-, **4**- and **7**- acetate Zone 2 (R_f 0 68) **5**-acetate Zone 3 (R_f 0 71) remained unidentified RR_t 1 26, MS *m/z* (rel int) 466 $[M]^+$ (100), 451 (19), 406 (16), 391 (31), 353 (43), 313 (30) The MS revealed a triterpene with an unsatd side chain and two double bonds in the ring system, RR_t 1 56, MS *m/z* (rel int) 468 $[M]^+$ (35), 453 (100), 408 (4), 393 (92), 355 (9), 301 (24), 297 (9), based on GC and MS data it may be **6**-acetate but further investigations are necessary Zone 4 (R_f 0 78) **1**- and **2**-acetate Zone 5 (R_f 0 86) from the esterified triterpenes components of RR_t 1 29 and 1 35 remain unidentified, component of RR_t 1 47 was a taraxerene derivative, MS *m/z* (rel int) 468 $[M]^+$ (15), 453 (5), 393 (4), 344 (100), 329 (44), 316 (16), 284 (24), 269 (44), 204 (47), MS data differed from those of taraxeryl acetate only in the intensities of *m/z* 344 and 204, component of RR_t 1 56 had a hopane or arborane skeleton, MS *m/z* (rel int) 468 $[M]^+$ (20), 453 (62), 408 (6), 393 (27), 301 (100), 289 (14), 241 (88), 229 (36), 205 (67), component of RR_t 1 81 **11**-acetate, MS *m/z* (rel int) 468 $[M]^+$ (20), 453 (16), 393 (17), 301 (17), 289 (100), 229 (92), 205 (29) Zone 5 from the free acetylated triterpenes **8**-, **9**- and **10**-acetate, MS of **10**-acetate (RR_t 1 52) showed fragments of *m/z* 454, 439, 379, 204 (base peak), 189, the fragment at *m/z* 204 can be derived by a retro-Diels–Alder reaction in ring C which is characteristic for the presence of a Δ^{12} double bond in triterpenes of the α- and β-amyrin class [50]

4α-Methylsterols Separation and identification of the acetylated 4α-methylsterols was performed in the same manner described above Zone 1 (R_f 0 22) **12**-acetate, zone 2 (R_f 0 32) **13**- and **14**-acetate, zone 3 (R_f 0 40) **15**-acetate, zone 4 (R_f 0 51) **16**- and **17**-acetate, zone 5 (R_f 0 65) **18**- and **19**-acetate

Sterols Separation and identification of the sterols was as described above [52–54] Zone 1 (R_f 0 49) **20-**, **21-**, **22-** and **23-**acetate, zone 2 (R_f 0 29) **24-** and **25**-acetate, RR_t of **25**-acetate 1 61 (OV-17), 1 56 (SE-30), 1 55 (XE-60), MS *m/z* (rel int) 394 $[M-AcOH]^+$ (100), 379 (12), 296 (13), 286 (15), 281 (30), 273 (15), 255 (17), 253 (26), 229 (11), 228 (20), 213 (26), 211 (11), zone 3 (R_f 0 14) **26**-acetate

Alkanes Mp 61–63°, IR was identical to those reported [55]

Primary alcohols Mp 57–60°, IR data corresponded to those in the lit [55]

Secondary alcohols Mp 78–80° (Found C, 81 5, H, 14 1 Calc for $C_{29}H_{59}OH$ C, 82, H, 14 2%) IR data were identical with those reported [56] Nonacosan-10-ol MS *m/z* 424 $[M]^+$, 406, 297, 279, 157, nonacosan-9-ol MS *m/z*, 424 $[M]^+$, 406, 311, 293, 143, nonacosan-11-ol MS *m/z* 424 $[M]^+$, 406, 283, 265, 171

Sterylglycosides and acyl sterylglycosides The defatted Me_2CO extract (6 g) was fractionated by prep TLC using $CHCl_3$–MeOH (6 1) as solvent [29] on Si gel (0 75 mm) (sterylglycosides R_f 0 41, acyl sterylglycosides R_f 0 73) The fractions were purified on Si gel 60 columns using $CHCl_3$ with increasing amounts of MeOH (up to 8%)

Sterylglycosides 17 6 mg, mp 260–262° (MeOH), IR ν_{max}^{KBr} cm^{-1} 3400, 2940, 1470, 1380, 1150–1000 [57] The sterylglycosides were hydrolysed in refluxing 2 N HCl for 4 hr The aglycones (sterols) were extracted with petrol, the aq soln yielded the sugar (glucose) TLC of the glucose Kieselguhr G impregnated with Pi buffer, pH 5, *n*-BuOH–Me_2CO–Pi buffer, pH 5 (40 50 1) [58]

Acyl sterylglycosides 11 9mg, mp 130–133° (MeOH), IR showed absorption at 1740 cm^{-1} additionally to those displayed by sterylglycoside [57] Alkaline hydrolysis was as previously described [59], for acid hydrolysis see above for the sterylglycosides

Quantitative determination of the different sterol types was performed by the method reported employing the Liebermann–Burchard reaction [27]

Triterpene acids The defatted Me_2CO extract (12 g) was fractionated twice on Si gel 60 columns by $CHCl_3$, $CHCl_3$–MeOH (99 1), and $CHCl_3$–MeOH (98 2) TLC with toluene–Me_2CO (7 3) [43] ursolic acid and oleanolic acid (R_f 0 60), 2α-hydroxyursolic acid (R_f 0 26), 20β-hydroxyursolic acid (R_f 0 21)

Ursolic acid and oleanolic acid 100 mg, mp 260–263° (EtOH), IR data corresponded to a mixture of authentic ursolic acid and oleanolic acid [60] GC analysis Me ursolate RR_t 3 74, Me oleanolate RR_t 3 24 MS Me ursolate *m/z* (rel int) 470 $[M]^+$ (6), 452 (3), 410 (5), 262 (99), 249 (10), 207 (29), 203 (100), 190 (15), 189 (29), 133 (80), Me oleanolate *m/z* (rel int) 470 $[M]^+$ (6), 452 (2), 410 (4), 262 (59), 249 (6), 207 (13), 203 (100), 190 (13), 189 (26), 133 (32) [37, 50] 2α-Hydroxyursolic acid 84 mg, mp 245–247° (MeOH) (lit [39] 242–245°) IR ν_{max}^{KBr} cm^{-1} 3400, 1690, 1455, 1380, 1270, 1045 (60) MS *m/z* (rel int) 472 $[M]^+$ (2), 454 (2), 248 (100), 235 (3), 223 (9), 203 (46), 190 (4), 189 (8), 133 (21), 60 (12)

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