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INVESTIGATIONS ON LEAF WAXES III* PENTACYCLIC TRITERPENES, SECO-TRITERPENES AND NON-VOLATILE ALIPHATICS OF FOUR HOYA SPECIES AND FICUS BENJAMINA IN RELATION TO LEAF AGE

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Key word index: *Hoya australis, H. keysii, H. crassipes, H. lacunosa,* Asclepiadaceae, *Ficus benjamina,* Moraceae, leaf epicuticular wax, leaf age, aliphatics, triterpenoids, *seco-nor*-triterpenoids, *seco*-triterpenoids, gas chromatography-mass spectrometry.

SUMMARY

In all the species investigated the leaf epicuticular wax composition was found to be largely dependent on leaf age.

In very young, still expanding, leaves characteristic patterns of aliphatic alcohols and non-volatile aliphatic hydrocarbon homologues were found. The chain-length distributions of the latter differ among the species and seem to be age dependent.

In fully expanded leaves of all the investigated species leaf epicuticular wax synthesis continues during subsequent leaf life: the amount of wax per unit leaf area increases several fold and compounds not found in very young leaves are synthesized. In all the species investigated leaf ageing is accompanied by accumulation of a complex mixture of relatively short-chain aliphatic hydrocarbon homologues.

In fully expanded and older leaves of the four *Hoya* species especially triterpenes and aliphatic hydrocarbons are synthesized. At these stages of leaf life *seco-nor*-triterpenols accumulate in the wax of *H. australis* and *H. crassipes*. These substances are absent in young leaves. In comparable stages of leaf life methyl esters of *seco*-triterpene acids were found in *H. australis, H. lacunosa* and *H. crassipes*. These substances are also absent in young leaves. In *H. keysii,* on the contrary, free triterpenols were found to accumulate during leaf ageing. In the leaf wax of quite a different latex plant, *Ficus benjamina,* in addition to the aliphatics, only small amounts of triterpenols are present.

Several observations have lead to the conclusion that the *seco*-triterpenoids found in the leaf waxes are probably enzymatically formed by the plants themselves, and not photochemically nor by microbial phyllosphere activity.

1. INTRODUCTION

Triterpenes are common plant constituents. They occur within the cell as water soluble compounds in the form of polar metabolites, e.g. bound to sugars as saponins or glycosides. The quantitatively more important lipophylic substances are found mainly in insoluble, accumulated, form. Within the cell they occur as particles composed of complex mixtures of mainly triterpenoid alcohols and

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their fatty acid esters, either in more or less specialized parenchyma cells or, more generally, in laticifers. Lipophylic triterpenoids may also be abundantly present in suberin layers of the bark of many tree species. They also occur in the wax layers that cover the stem, fruit and leaves of many plant species (e.g. HEGNAUER, 1962–1973).

In addition to the usual 3-hydroxy triterpene alcohols also higher oxydized, oxygenated derivatives bearing additional hydroxyl and/or carboxyl groups are found in such layers and waxes.

The identification of ring-opened (*seco*) triterpenoids in waxes indicates that oxydative ring fission is also involved in the formation of leaf wax triterpenes (BAAS & FIGDOR 1978a, b).

Contrary to the stable and species specific composition of the lipids present in the particles of the plant latex system (BAAS et al. 1981, BAAS & NIEMANN 1979), the composition of leaf waxes varies to a great extent with environmental conditions (HULL et al. 1975, HUNT et al. 1976, HULL et al. 1979, GÜNTHARD & WANNER 1982) and leaf age. So temperature (GIESE 1975, HAAS 1977), light (MACEY 1970, GIESE 1975, HUNT et al. 1976), growth region (SMITH & MARTIN-SMITH 1978, ABRAMSON et al. 1973, MANHEIM et al. 1979, HEEMANN et al. 1981, GÜNTHARD & WANNER 1982) and leaf development (HERBIN & ROBINS 1969, STOCKER & WANNER 1975, SCHUCK 1976, BAAS & FIGDOR 1978a, NIEMANN et al. 1980, HAAS 1977, BAKER & HUNT 1981, CRANKSHAW & LANGENHEIM 1981, GÜNTHARD & WANNER 1982) greatly influence both the leaf wax composition and its ultrastructure. From the site of location of the leaf wax constituents in general it has been inferred that the differences in wax composition during leaf development might be important for survival of the plant in its natural habitat, by protecting it from damage by e.g. light, invasion of microorganisms (KOLATTUKUDY et al. 1981, DAVID 1967, lit. ref. from NELSON 1978, CROTEAU & FAGERSON 1971, LAMPARD & CARTER 1973), drought (SCHÖNHERR 1976, SCHÖNHERR & SCHMIDT 1979, SCHÖNHERR et al. 1979, HULL & BLECKMANN 1977, PROKSCH et al. 1981), frost damage (BARBER 1955, lit. ref. from HAMILTON & HAMILTON 1972), and herbivores (CRANKSHAW & LANGENHEIM 1981).

During our work on *Hoya* species (Asclepiadaceae) a ring-opened *seco-nor*-triterpenol was found to accumulate in the wax of old leaves of *Hoya australis* (BAAS & FIGDOR 1978a, b).

Since it is at present widely accepted that the main role of secondary metabolites is in plant-environment interactions (JANZEN 1973), it is tempting to think of a role of these oxydized, ring-opened, terpenoids in the protection of the leaf. So one of the roles of the triterpenoids from plants is believed to be the control of growth and reproduction of fungal pathogens (NES et al. 1982 and references). The water soluble cardenolides from the Asclepiadaceae and Apocynaceae laticifers form another, well documented, example of plant anti-herbivore defence (NELSON et al. 1981).

Investigations on many *seco*-terpenoids in general revealed different kinds of biological activities, such as feeding-deterrents (*seco*-iridoids, KUBO & NAKANISHI 1977, in ROSENTHAL & JANZEN 1979, p. 511) and for example for the

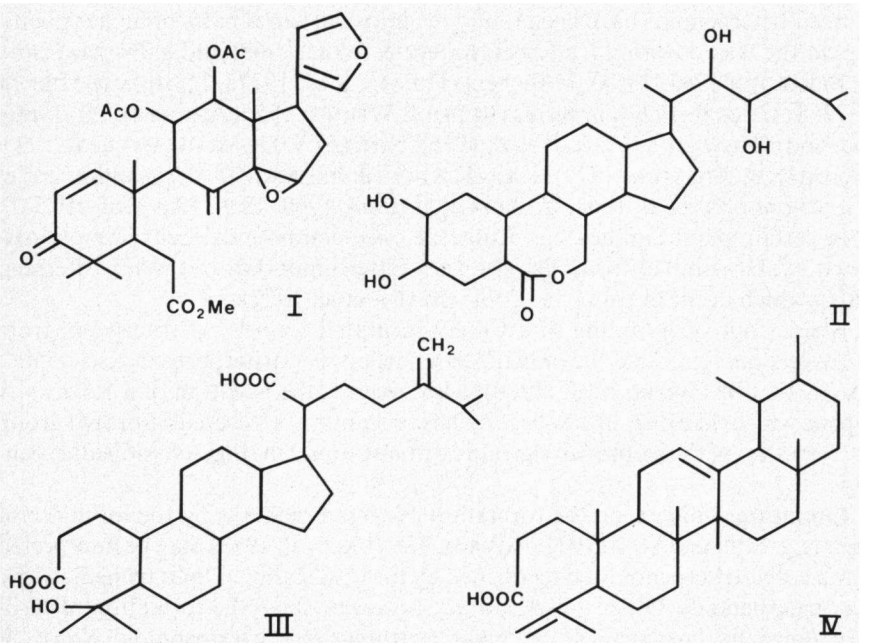

Fig. 1. Typical structures of *seco*-triterpenoids, as reported in literature.
I Toonacilin; an antifeeding bark extractive from *Toona ciliata* (Meliaceae); (KRAUS et al. 1978).
II Brassinolid; a phytohormone from *Brassica napus* (Brassicaceae); (ADAM 1981).
III *seco*-A-Derivative of eburicoic acid; an antibacterial metabolite from the pentacyclic acid produced by the fungus *Glomerella fusarioides*; (FRIED et al. 1965).
IV Roburic acid; resinous exudate of the galls produced by the insect *Cynips mayri* on common oak (*Quercus robur*, Fagaceae); (MANGONI & BELARDINI 1963).

brassinolides (hydroxylated ring-B-*seco*-sterols) as phytohormones (ADAM 1981 and references). The ring-B-*seco* tetranortriterpenoids from the bark of *Toona ciliata* (Meliaceae) have insect antifeeding properties (KRAUS & GRIMMIGER 1980). A very important group of natural compounds with feeding deterrent action is the related *seco*-C-tetranor triterpenoid azadirachtin with its many related compounds isolated from the Indian neem tree, *Azadirachta indica*, and also from the Persic lilac or chinaberry, *Melia azadarach* (SCHMUTTERER et al. 1981, KRAUS & CRAMER 1981).

The *seco*-A derivative of the possibly biologically inactive triterpene acid eburicoic acid, produced from this acid by the fungus *Glomerella fusarioides*, shows antibacterial properties (FRIED et al. 1965), while synthetic *seco*-derivatives of betulinic acid were found to be active against *Bacillus subtilis* (KLINOT et al. 1972).

A non-specific physical function of *seco*-triterpenes on plant galls is suggested by MONACO et al. (1973) who tried to explain their occurrence as a need of the gall to produce a resinous exudate.

Fig. 1 shows some typical structures of *seco*-triterpenoids, as reported in literature.

Seco-triterpenoids have been found in almost all aerial parts of higher plants, e.g. in the wax covering fruit peel (LANTZ & WOLF 1968) and galls (MANGONI & BELARDINI 1963, 1964), in flowers (HIRATA et al. 1977a, b; SUGA & HIRATA 1979, SUGA et al. 1977), in resins (MILLS & WERNER 1955, ARIGONI et al. 1960), in wood (CROWLEY 1962, RAO et al. 1975), bark (AOYAGI, MORIYAMA et al. 1973, PRADHAN & KHASTGIR 1973), stem (KIKUCHI & YOSHIKOSHI 1972) and in leaves (GOVINDACHARI et al. 1968, CHOPRA et al. 1968, 1969, 1970; HUI et al. 1975).

In certain plant families, e.g. Rutaceae, *seco*-compounds occur in more oxidized (water-soluble) form, like the very bitter limonoids (tetra-*nor*-triterpenoids), which occur in fruits and roots (MAIER et al. 1977).

Besides our own finding of a C4(29)-saturated *seco*-A *nor*-triterpenol from *Hoya australis* leaf wax, the only other report on *seco*-triterpenes in Asclepiadaceae is that of CASTRO et al. (1980), who describe the isolation of a 3,4-*seco*-A lupene which, like our substance, also has the unusual saturated isopropyl group at C_5 instead of the isopropenylgroup normally found in ring-A fissioned terpenoids.

Literature evidence on the formation of *seco*-terpenoids by the influence of light (e.g. SHIRASAKI et al 1977; AOYAGI, TSUYUKI et al. 1973) may be interpreted as wax *seco*-triterpenoids being formed by the absorbtion of light by pentacyclic wax triterpenoids. Other investigations, however, show the formation of *seco*-terpenoids by enzymes of the plant (tetra-*nor*-*seco*-triterpenoids, NICOL & CHANDLER 1978, HASEGAWA & HOAGLAND 1977; *seco*-beyerene diterpenoids, GHISALBERTI et al. 1978; *seco*-iridoid glucosides, INOUYE et al. 1976 and references). Microbial formation of *seco*-terpenes has also been described (HASEGAWA et al. 1974).

In order to evaluate whether the *seco*-derivatives as are found in the epicuticular wax of old leaves of *Hoya australis*, are general leaf wax metabolites, waxes of young and old leaves of *Ficus benjamina* and some *Hoya* species were investigated for these compounds. In addition preliminary information on the non-terpenoid aliphatic wax-compounds is given.

2. MATERIAL AND METHODS

Plants, originating from different botanical gardens (BAAS et al. 1981), were raised under greenhouse conditions in pot-culture or on water culture. Voucher specimen were deposited at the Institute for Systematic Botany of the University of Utrecht.

Leaves were cut with a razor, weighed, washed with tap water, and the surface wax was extracted by dipping the leaves in a mixture of chloroform/methanol (2:1 v/v) for 20 seconds. The eluate was filtered and dried on a rotary evaporator. The amount of total wax was determined gravimetrically and is expressed as $\mu g.cm^{-2}$ leaf area. The latter was determined by weighing single leaf projections onto paper.

Column chromatography. Total leaf wax from up to ten leaves was ultrasonically suspended in light petrol (b.p. 40–60) and fractionated on a column of

1 cm inner diameter, filled with 5 g of alumina oxide (Brockman grade III, Merck). Elution with 50 ml light petrol (b.p. 40–60), 10% diethyl ether in light petrol (25 ml), 30% diethyl ether in light petrol (50 ml) and finally with pure diethyl ether, in this order afforded an apolar fraction A_0 with hydrocarbons and apolar esters, fraction A_{10} with more polar esters, fraction A_{30} in which the alcohols elute and finally a polar fraction (A_{100}). To each fraction 100 μg of 5 α-cholestane was added as an internal standard.

Thin layer chromatography (TLC). This was used to control the alumina oxide column separation and in some cases also for total leaf wax fractionation. The eluent used was a mixture of cyclohexane/ethylacetate (9:1 v/v). Plates were developed twice. Free and esterified triterpenes were detected by spraying the plates with a mixture of chlorosulphonic acid and glacial acetic acid (2:1 v/v) followed by heating for some minutes at 105 °C.

Gaschromatography (GC). Column and TLC fractions were analysed by isothermal gaschromatography on a Becker type 420 gaschromatograph equipped with a glass column, 4 mm inner diameter, filled with 3% SE-30 coated onto Varaport-80 (Varian). Oven temperature used was 250 °C. The eluting compounds were detected with a flame detector and amounts estimated relative to the added 5 α-cholestane referent with an electronic integrator (LDS-40). No respons factors were used. The amount and percentual composition of the fractions given is based on the compounds eluting after the internal standard only (RRT > 1.0), unless stated otherwise.

Gaschromatography-mass spectrometry (GC-MS). This was done on a Hewlett-Packard type 5992A GC-MS combination fitted with a 25 m fused silica flexible capillary wall-coated open tubular column, inside diameter 0.34 mm, coated with 0.12 μm CP Sil 5 liquid phase (Chrompack). Samples to be analysed were dissolved in hexane and injected splitless at 60 °C column temperature. During analysis the oven temperature was programmed between 250 and 280 °C at 5 °C/min. Helium-flow through the column was 2–4 ml/min depending on the column temperature, at 20 psi column pressure. 5 æ-Cholestane was used again as the internal standard. Selected ion monitoring (SIM) of ring-fissioned *seco-nor*-triterpenoids was done by tracing their molecular ion (m/e 414). The same technique was used to visualize homologous series or individual compounds in the often complex wax fractions.

3. RESULTS

TLC-control of the alumina oxide fractions of the leaf wax showed triterpene positive spots for the free alcohol fractions (A_{30}) and in most cases also for the less polar fractions A_0 and A_{10} indicating the presence of esterified triterpenols (*table 1*).

GC-analysis of the column fractions containing the free triterpenols (A_{30}) shows a remarkable difference in leaf wax alcohol composition between young and old leaves of the same plant species (*table 2*). Differences in the composition of fractions A_0 and A_{10} are generally great between both types of leaves of the

Table 1. Triterpene-positive spots of TLC-separated alumina oxide fractions of leaf total waxes.

Plant	Fraction			
	A_0	A_{10}	A_{30}	A_{100}
Hoya lacunosa				
young leaf	R_f 0.76 (+++) R_f 0.86 (++)		R_f 0.35	
old leaf	R_f 0.77 (+++) R_f 0.90 (+)	R_f 0.36 R_f 0.44	R_f 0.26 R_f 0.30 R_f 0.35	
Hoya australis				
young leaf		R_f 0.77 R_f 0.83 (+)	R_f 0.19 R_f 0.33	
old leaf	R_f 0.84 R_f 0.93	R_f 0.76 R_f 0.84	R_f 0.33 (+)*	R_f 0.12 (+)
Hoya crassipes				
young leaf	R_f 0.86 (++) R_f 0.95 (+)	R_f 0.88 (++)	R_f 0.23 R_f 0.38	
old leaf	R_f 0.88 (++)	R_f 0.86 (+)	R_f 0.23 R_f 0.40	
Hoya keysii				
young leaf			R_f 0.22 (+) R_f 0.35	
old leaf	R_f 0.94	R_f 0.86	R_f 0.21 (+) R_f 0.36 (+++)	
Ficus benjamina				
young leaf	R_f 0.90		R_f 0.20 R_f 0.35	
old leaf	R_f 0.88	R_f 0.77 R_f 0.86	R_f 0.33 R_f 0.41	

Referents: triterpene acetates: R_f 0.76; triterpenols R_f 0.35; sterols R_f 0.21

*double spot.

same plant, although resemblance is found among the composition of leaves of comparable age of the different plants. Analytical results are presented below for the individual plant species.

3.1. *Hoya lacunosa* Blume

TLC showed the presence of two groups of esterified triterpenes in the A_0 fractions of the wax of both young and old leaves. One group cochromatographed with β-amyrinacetate referent (R_f 0.76), the second one had a slightly higher R_f-value of about 0.88.

Only small amounts of free triterpenol were present in the A_{30}-fractions, mainly in that of wax of old leaves (*table 3*).

Table 2.* Qualitative composition of the compounds identified in the alumina oxide separated leaf wax fractions (non-volatile compounds with RRT > 1.0 only).

Species	Fraction						
	total wax ($\mu g.cm^{-2}$)	mg	A_0 ("apolar") compounds	mg	A_{10} compounds	mg	A_{30} ("alcohol") compounds
H. lacunosa							
young	5.4	0.44	*HC*, SAM, Ac	0.03	n.i.	0.02	*OH*
old	32.2	3.60	HC(tr), SAM, Ac	0.08	*TAM*?	0.04	*n.i.*, OH
H. australis							
young	2.3	0.48	HC, *Yac*	0.02	Ac?	0.19	OH(tr), YOH
old	14.0	1.52	*HC*, SAM	0.03	Ac, LCE**	2.25	*SN*, OH
H. crassipes							
"young"	12.0	1.43	*HC*, SAM	0.07	n.i.	0.17	*SN*, OH
old	6.1	1.91	*HC*, SAM	0.05	n.i.	0.31	*SN*, OH
H. keysii							
young	5.7	0.30	*HC*	0.03	*Yac*, Ac	0.17	*YOH*, SN
old	11.4	0.52	*HC*	0.02	Ac?	2.45	*OH*, ON(tr)
F. benjamina							
young	0.60	0.08	n.i.	0.02	n.i.	0.03	*YOH*, OH?
old	10.4	1.11	*HC*	0.07	n.i.	0.09	*OH*, ON(tr)

*Abbreviations used are: HC = aliphatic hydrocarbon, YOH = aliphatic alcohol, Yac = acetylated aliphatic alcohol, OH = triterpenol, ON = triterpenon, Ac = triterpene acetate, LCE = long chain ester of triterpenol, SN = *seco-nor*-triterpenol, SAM = *seco*-triterpene acid methyl ester, TAM = triterpene acid methyl ester, tr = trace, n.i. = not identified.
Main type of compounds in the fraction in italics.
**Detected as free triterpenol after saponification.

Table 3. Total of GC-detectable compounds with RRT > 1.0 in the alumina-oxide separated fractions of the total leaf wax of young and old leaves of *Hoya lacunosa*.

Fraction	Young leaves (32; 91 cm^2)			Old leaves (17; 116 cm^2)		
	amount	%	$\mu g.cm^{-2}$	amount	%	$\mu g.cm^{-2}$
Total wax	2.7 mg[2]	100	29.6	17.7 mg[2]	100	152.5
A_0	0.44 mg	20,1		3.60 mg	15.9	
A_{10}	0.03 mg	1.4		0.08 mg	0.4	
A_{30}	0.02 mg	0.9		0.04 mg	0.2	
	0.49 mg[1]	22.4	6.6	3.72 mg[1]	16.5	25.2

[1]Gaschromatographically determined, RRT > 1.0.
[2]Gravimetrically determined.

Young leaves

Fraction A_0 (433 μg): GC-analysis of the saponified and unsaponified fractions reveals their complex composition. At least three types of compounds are present in the unsaponified fraction (*table 4*):

Table 4. GC and GC/MS analysis of the apolar fraction A_0 of leaf wax of *Hoya lacunosa* before and after saponification.

Young leaves

Unsaponified (0.4 mg)					Saponified (0.5 mg)				
RRT	%	M^+	b.p.	compound	RRT	%	M^+	b.p.	compound
1.16	4.8	408	57	$C_{29}H_{60}$	1.16	10.9	408	57	$C_{29}H_{60}$
1.49	4.7	422	57	$C_{30}H_{62}$	1.48	7.3	422	57	$C_{30}H_{62}$
*1.91	22.5	436	57	$C_{31}H_{64}$	1.91	35.8	436	57	$C_{31}H_{64}$
2.18	19.9	456	245	$C_{31}H_{52}O_2$**					
*2.47	17.6	456	57	$C_{31}H_{52}O_2$/ $C_{32}H_{66}$	2.45	6.1	450	57	$C_{32}H_{66}$
					*2.92	2.9	426	57/218	β-OH/$C_{33}H_{68}$
3.15	9.9	464	57	$C_{33}H_{68}$	*3.11	2.9	426	57/218	α-OH/$C_{33}H_{68}$
3.67	3.0	468	218	β-Ac					
*3.99	14.8	468/ 478	218/ 57	α-Ac					
					4.06	2.7	478	57	$C_{34}H_{70}$
5.26	2.7	492	57	$C_{35}H_{72}$	5.23	5.3	492	57	$C_{35}H_{72}$

Old leaves[1]

Unsaponified (3.6 mg)					Saponified (0.48 mg)				
1.15	0.5				1.15	+			
1.47	0.5				1.47	+			
1.91	2.6		57		1.90	22.9	414	57	
2.18	83.2	456	245	$C_{31}H_{52}O_2$					
2.54	6.9	456	55	$C_{31}H_{52}O_2$	2.46	2.8	436	57	
					2.82	7.2			triterpenol
					3.08	53.6	426	218	α-OH
3.16	1.0								
3.69	0.6			trit.acet.					
3.99	5.3		218	α-Ac					
4.43	0.4								
5.20	1.0				5.23	7.2			

*Mixture.

**_Seco_-triterpene acid methyl ester.

[1] In addition a broad peak of a mixture of quantitative important aliphates elutes before the referent (*fig. 2*).

- a homologous series of hydrocarbons: RRT 1.14($C_{29}H_{60}$) to RRT 5.52($C_{35}H_{72}$), with $C_{31}H_{64}$ and $C_{33}H_{68}$ as main compounds.
- esters at RRT 2.18 and 2.45 (main compounds), very probably *seco*-A-triterpene acid methylesters (see MS data below).
- triterpene acetates at RRT 3.67 and 3.99.

GC-MS analysis gave M^+ of 456 and characteristic Retro-Diels-Alder (RDA) fragments of the pentacyclic triterpenoids for the compounds at RRT 2.18 and

RRT 2.45. They disappear from the GC-chromatogram upon saponification without the liberation of a long-chain alcohol. Their mass fragmentation pattern is characteristic for 3,4-A-*seco*-triterpenoids, and comparable with those found for *seco*-A-triterpene acid methyl esters (APLIN & COX 1975). Information on composition and structural features of these compounds will be given in a subsequent paper.

The mass spectra of the triterpene acetates at RRT 3.67 and RRT 3.99 correspond to those of β-amyrin acetate and α-amyrin acetate. No germanicol acetate could be detected by SIM-analysis at m/e 204.

Fraction A_{10} (27 μg): Only trace amounts of compounds were present in this fraction. They have not been analysed.

Fraction A_{30} (20 μg): SIM-analysis revealed the presence of trace amounts of β-amyrin, eluting as a front shoulder of the mixed peak at RRT 3.07 by tracing the fragment m/e 218. The latter peak consisted in part of α-amyrin. Taraxerol eluted as a single peak as the first triterpenol at RRT 2.69. The mass spectrum is in agreement with that given for taraxerol by DESHMANE & SUCK DEV (1971) and by DJERASSI et al. (1962).

Old leaves

Fraction A_0 (3600 μg): From GC-analysis of the unsaponified and saponified fraction it was evident that this apolar fraction was almost entirely composed of triterpenoid *seco*-acid methyl esters eluting at RRT 2.18 and RRT about 2.50 (*table 4, fig. 2*). Of the compounds eluting after cholestane, these compounds account for more than 90% of the leaf total wax (*table 3*). The increase in mass of the GC-detectable total wax compounds per cm^2 in ageing leaves (5.4 μg.cm^{-2} for young leaves to 32 μg.cm^{-2} for old ones) is apparently mainly due to an accumulation of these *seco*-acid methyl esters.

Fraction A_{10} (80 μg): This minor polar ester fraction had a main compound eluting at RRT about 5 as a tailing peak. GC-MS analysis showed it to have a molecular weight of 470, a base peak at m/e 219 with no other important fragments in the higher region. Because of its chromatographic behaviour it is supposed to be a triterpenoid acid methyl ester (referent methylursolate also eluted as a tailing peak at a RRT in the same region) and possibly related to the diosphenolic triterpene acids derived from compounds isolated by BRIESKORN & KRAUSE (1974) from *Melissa officinalis*.

Fraction A_{30} (40 μg): Only trace amounts were present in this fraction. Taraxerol, eluting as a single peak at R_f 2.70 was the only compound that could be identified.

3.2. *Hoya australis* R.Br. ex Traill.

In the alumina oxide separated wax fractions of young leaves TLC showed the presence of only traces of free triterpenol, besides two types of triterpene ester eluting in fraction A_{10} (*table 1*).

In the wax of old leaves, in addition, a less polar ester was detected in fraction A_0. In fraction A_{100} in which the polar compounds are present, significant

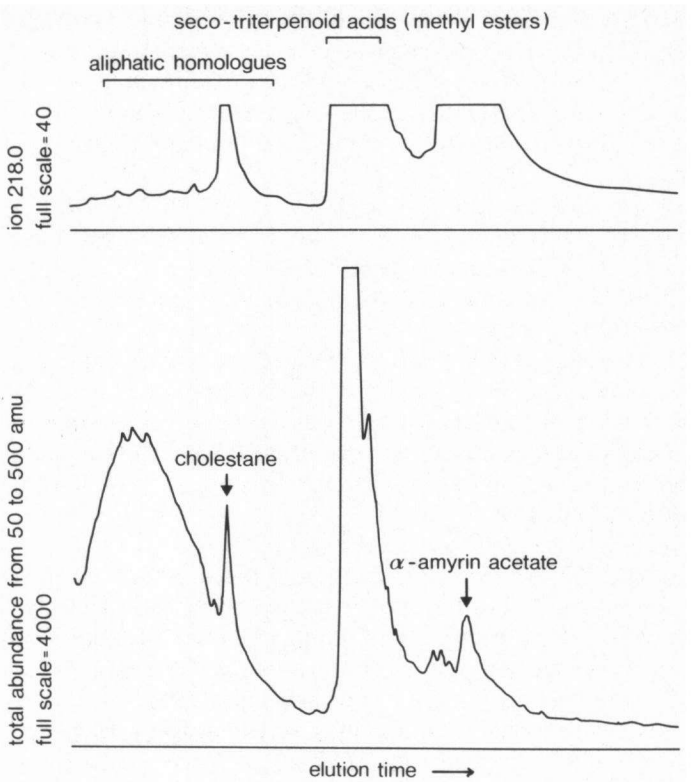

Fig. 2. GC-MS analysis of the unsaponified apolar lipids (fraction A_0) from the epicuticular wax layer of old leaves of *Hoya lacunosa*. Upper trace: selected ion monitoring of triterpenoids with the characteristic fragment m/e 218; lower trace: total ion monitoring of fragments m/e 50–m/e 500..

amounts of triterpenes, possibly acids, were detected. In these leaves free triterpenols were the main constituents (fraction A_{30}).

Young leaves

Fraction A_0 (480 μg): Two types of co-chromatographing homologues constitute the main part of this fraction: aliphatic hydrocarbons and acetates of aliphatic alcohols (Y_1ac, Y_2ac and Y_3ac). The latter are characteristic for the surface wax of very young leaves (see the other investigated species, *table 2*). They elute at RRT 1.51, 1.95 and 2.52 respectively. Molecular weights of the free alcohols liberated after saponification are 364, 378 and 392 respectively which correspond with bruto formulae $C_{25}H_{48}O$, $C_{26}H_{50}O$ and $C_{27}H_{52}O$, They elute at RRT 1.16, 1.51 and 1.94 (*table 7*). In analogy with *H. lacunosa* and *H. crassipes* (below) the presence of a *seco*-A triterpene acid methyl ester in the peak at RRT 1.95 cannot be excluded.

Table 5. GC and GC-MS analysis of the free alcohol fraction (A_{30}) of leaf epicuticular wax of young and old leaves of *Hoya lacunosa*.

Young leaves (0.02 mg)					Old leaves (0.04 mg)				
RRT	%	M^+	b.p.	compound	RRT	%	M^+	b.p.	compound
1.49	+	402?	56						
1.88	58	395?	57		1.92	25	395?		
					2.33	15			
2.69	16.5	426	204	taraxerol	2.70	3.5	426	204	taraxerol
3.07	21	426*	218	α-amyrin*	3.08	17	466	57	
					3.40	40	466	57 (466?)	
3.55	57								

*β-amyrin as front shoulder (SIM evidence), an aliphate elutes just before α-amyrin.

Table 6. GC-detectable compounds (RRT > 1.0) in the alumina oxide separated fractions of the total leaf wax of young and old leaves of *Hoya australis*.

Young leaves (16; 300 cm^2)				Old leaves (6; 254 cm^2)			
fraction	amount	$\mu g.cm^{-2}$	%	fraction	amount	$\mu g.cm^{-2}$	%
total wax	4 mg	13.3	100	total wax	24.6 mg	96.8	100
A_0	0.48		12.0	A_0	1.52*		6.2
A_{10}	0.02		0.5	A_{10}	0.03**		0.1
A_{30}	0.19		4.8	A_{30}	2.25		9.2
	0.69	2.3	17.3%		3.80	14.0	14.5%

*Plus hydrocarbon homologues eluting before the cholestane referent.
**Plus non-volatile esters of triterpenes and of aliphatic alcohols.

Fraction A_{10} (18 μg): Only minute amounts of saponifiable compounds were eluting at RRT 3.69 and 4.02 (RRT after saponification 2.90 and 3.30, co-chromatographing with β-amyrin and lupeol/α-amyrin). TLC diagnosis of the unsaponified fraction pointed to triterpene acetates (*table 1*). An ester eluted at RRT 0.51 (compare *H. crassipes*). A series of aliphatic alcohols with molecular weight ranging from M^+ 310 to M^+ 424, main compound M^+ 352($C_{24}H_{48}O$?), liberated upon saponification also eluted mainly before the cholestane referent.

Fraction A_{30} (192 μg): Trace amounts of free triterpenols eluted at RRT 2.90 and 3.30, probably β-amyrin and α-amyrin or lupeol (BAAS & FIGDOR 1978a). The elution pattern of the main compounds in this fraction was similar to that of the alcohols, liberated upon saponification from the acetates in fraction A_0: RRT 1.17 (M^+ 364; 54%) and RRT 1.96 (M^+ 392; 36%). The bruto formulae of these compounds may be $C_{25}H_{48}O$ and $C_{27}H_{50}O$.

Old leaves

Fraction A_0 (1521 μg): From *table 7* it follows that this fraction consisted mainly of homologues of saturated hydrocarbons. The chain length of the main

Table 7. GC and GC/MS analysis of the apolar fraction A_0 of *Hoya australis* leaf wax, before and after saponification.

Young leaves									
Unsaponified (480 μg, 16 leaves)					Saponified (275 μg)				
RRT	%	M^+	b.p.	probable compound	RRT	%	M^+	b.p.	compound
1.17	1.5	n.d.	n.d.	$C_{29}H_{60}$	1.16*	59.9	364/408	57	$C_{25}H_{48}O$? (Y_1OH)/C_{29}
1.51	54.5	n.d.	n.d.	Y_1Ac	1.51*	1.8	453/378	57	$C_{26}H_{50}O$? (Y_2OH)
1.95*	6.3	n.d.	n.d.	$C_{31}H_{64}/Y_2Ac$	1.94*	34.5	392/436	57	$C_{27}H_{52}O$? (Y_3OH)/$C_{31}H_{64}$ $C_{31}H_{64}$
2.25	31.9	n.d.	n.d.	Y_3Ac					
3.25	1.9	n.d.	n.d.	$C_{33}H_{68}$	3.22	3.8		57	$C_{33}H_{68}$/triterpenol
4.25	4.0	n.d.	n.d.						

Old leaves									
Unsaponified (1520 μg; 6 leaves)					Saponified (1250 μg)				
1.17	2.7	n.d.	n.d.	$C_{29}H_{60}$	1.17	6.1	408	57	$C_{29}H_{60}$
1.49*	17.5	n.d.	n.d.	ester/$C_{30}H_{62}$	1.50	2.7	422	57	$C_{30}H_{62}$
1.94	36.8	n.d.	n.d.	$C_{31}H_{64}$	1.93	48.6	436	57	$C_{31}H_{64}$
2.50	12.6	n.d.	n.d.	SAM[2]/$C_{32}H_{66}$	2.49	8.5	450	57	$C_{32}H_{66}$
3.21	21.9	n.d.	n.d.	$C_{33}H_{68}$	3.20	26.4	464	57	$C_{33}H_{68}$
4.14	3.6	n.d.	n.d.	$C_{34}H_{70}$	4.12	2.2	478	57	$C_{34}H_{70}$
5.32	4.7	n.d.	n.d.	$C_{35}H_{72}$	5.31*	5.5	492	57	$C_{35}H_{72}$[1]

*Mixture

[1] Plus compound with fragments m/e 207 and m/e 281?

[2] *Seco*-triterpene acid methylester.

component was C_{31} (for young leaves C_{29}). An unidentified saponifiable compound co-eluted with $C_{32}H_{66}$ at RRT 2.50. In analogy with *H. crassipes* and *H. lacunosa* this might be a triterpene *seco*-A-acid methyl ester. As was found for *H. lacunosa* a quantitatively important mixture of aliphatic homologues eluted as a broad peak before cholestane. A highest fragment of m/e 414 was found for its main component.

Fraction A_{10} (31 μg): Apart from the ester eluting at RRT 0.52, the two other detected compounds had RRT 3.33 and 3.75 respectively. They were probably triterpene acetates (TLC evidence, *table 1*, RRT 0.76). The second group of triterpene esters found on TLC (RRT 0.84) was not detectable by GC at 250 °C since they contained long-chain fatty acid esters. Their presence was responsible for the increase in triterpenol found upon saponification of this fraction (*table 8*).

Main triterpenes found in the saponified fraction were β-amyrin (RRT 2.85; 75.5%) and α-amyrin which eluted in a composed peak (14.1%). At RRT 3.69 an as yet unidentified compound eluted.

Table 8. GC analysis of the compounds eluting at RRT > 1.0 in fraction A_{10} of wax of old leaves of *Hoya australis*.

Unsaponified (31 μg)			Saponified (205 μg)				
RRT	%		RRT	%	M^+	b.p.	compound
1.34	11	not identified					
1.51	27	not identified	1.50	4.1		n.i.	
3.33	34	not identified	2.85	75.5	426	218	β-amyrin
			3.10*	14.1	426	218	α-amyrin*
3.75	3	not identified	3.69	5.9		n.i.	

*Double peak.

Table 9. GC and GC/MS analysis of the compounds eluting after cholestane in fraction A_{30} of wax of old leaves of *Hoya australis*.

RRT	%	M^+	b.p.	compound
1.16	7		n.i.	
1.51*	2	412?	n.i.	
1.80	52	414	195	SN-1[1]
1.94	20.5	414	218	SN-2
2.43	3.5	414	55	SN-3
2.83*	2	426	218	β-amyrin/SN-4
3.13*	13	426	218	α-amyrin/lupeol
3.68	+		n.i.	

[1] *Seco-nor*-triterpenol.
*Double peak.

Also liberated upon saponification were two series of homologous alcohols. They constituted the main part of the saponified fraction. Volatility of the main compounds was comparable to that of cholestane. Homologues in one series had M^+ 310 to M^+ 422 and were also found in the young leaves. Their bruto formulae possibly are $C_{21}H_{42}O$ to $C_{29}H_{58}O$.

Fraction A_{30} (2254 μg): This fraction is almost fully composed of intact and ring-A degraded (*seco*-A *nor*-A) triterpenols, which thus appear to accumulate in the wax of old leaves. Besides three major *seco-nor* derivatives, two minor ones may be present, co-eluting with β-amyrin, as shown by SIM analysis tracing the molecular ion m/e 414 (*table 9*).

It proved to be possible to separate the *seco-nor*-triterpenols and the intact triterpenols by TLC with the usual eluent. The *seco-nor*-triterpenols had a RRT 0.40, the pentacyclic triterpenols had RRT 0.35. *Fig. 4a* and *b* show a GC-MS analysis of the isolated TLC bands.

3.3. *Hoya crassipes* Turcz.

No really young leaves were present at the time of the experiment. Therefore, a comparison has only been made between very old and younger leaves.

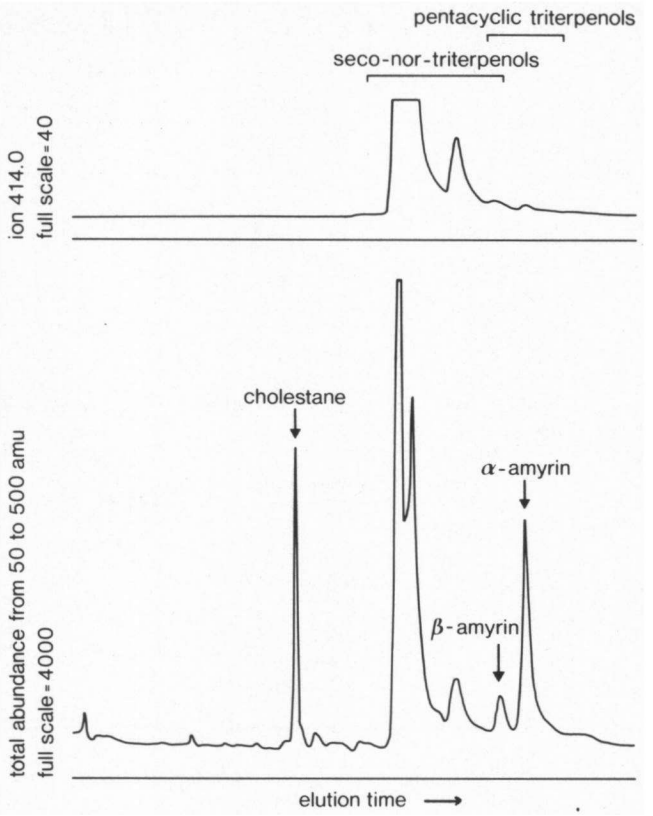

Fig. 3. GC-MS analysis of the free alcohol fraction A_{30} from epicuticular wax of old leaves of *Hoya australis*. Upper trace: registration of *seco-nor*-triterpenols by selected ion monitoring of their molecular ion m/e 414; lower trace: total ion current (m/e 50–m/e 500).

The main triterpenoids found in the leaf waxes were detected in the ester fractions. They are less polar than triterpene acetates (*table 1, TLC*).

Compared with *H. australis*, the final size of *H. crassipes* leaves is possibly much more dependent on growth conditions. This may account for the high amount of surface wax on the younger leaves, which probably did not attain the size that normally corresponds to their age. The young leaves used in this particular experiment show a rather "old" pattern for their wax composition.

Young leaves

Fraction A_0 (1434 μg, *table 11*): In this fraction hydrocarbons were already present. Carbon numbers ranged from C_{29} to C_{35} with $C_{31}H_{64}$ as main compound. The ester also present had RRT 2.10 and M^+ 465 and belongs to the class of ring-A-fissioned triterpene acid methyl esters already encountered in *H. lacunosa*.

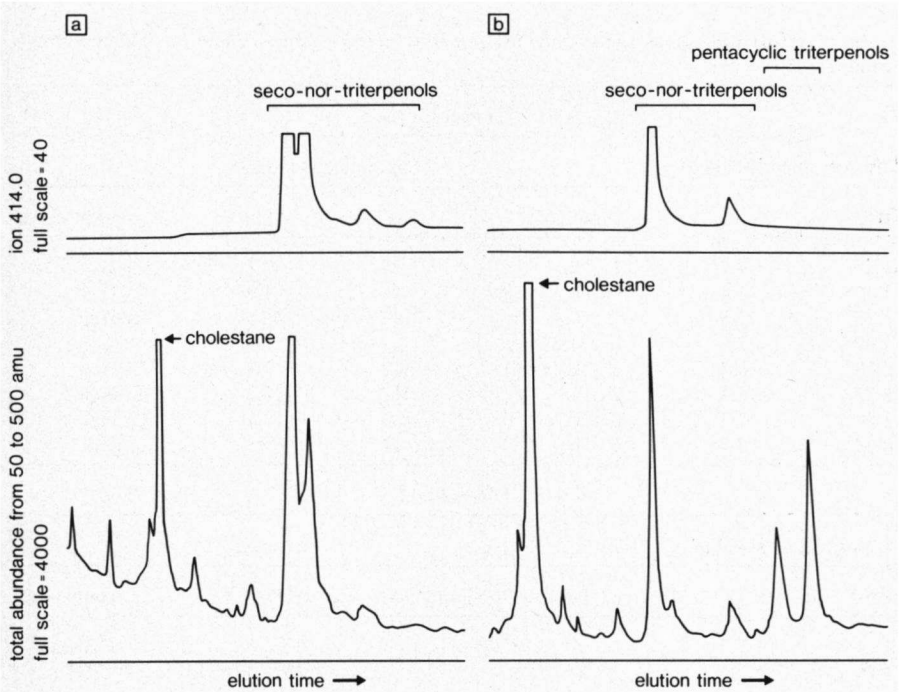

Fig. 4. GC-MS analysis of the free triterpenol fractions, isolated and partly separated by TLC, from the epicuticular wax of old leaves of *Hoya australis*. Fig. 4a: upper part of the TLC-band (R_f0.37); fig. 4b: lower part of the TLC-band (R_f0.34).
The *seco-nor*-triterpenols, found mainly in the upper part of the TLC-band are traced by selected ion monitoring of their molecular ion (m/e 414, upper trace). The lower trace represents the total ion current (m/e 50 to m/e 500). Most of the pentacyclic triterpenes are found in the lower part of the TLC-band.

Table 10. GC-detectable compounds (RRT > 1.0) of the alumina oxide separated fractions of the leaf waxes of *Hoya crassipes*.

Young leaves (9; 142 cm^2)				Old leaves (5; 373 cm^2)		
fraction	amount	μg.cm^{-2}	%	amount	μg.cm^{-2}	%
total wax	17.7 mg	127	100	9.0 mg	24	100
A_0	1.43		8.1	1.91		21.2
A_{10}	0.07		0.4	0.05		0.6
A_{30}	0.17		1.0	0.31		3.5
	1.67	12	9.5		6.1	25.3

Table 11. GC analysis of the apolar fraction A_0 of *Hoya crassipes* leaf wax before and after saponification.

Younger leaves					
Unsaponified (1430 μg)			Saponified (1170 μg)		
RRT	%	compound	RRT	%	compound
1.13	1.7	$C_{29}H_{60}$	1.15	3.1	$C_{29}H_{60}$
1.45*	3.4	$C_{30}H_{62}$ + ester	1.47	1.9	$C_{30}H_{62}$
1.85	33.6	$C_{31}H_{64}$	1.89	46.5	$C_{31}H_{64}$
2.09	27.9	ester (SAM[1])			
2.36	8.3	$C_{32}H_{66}$/SAM	2.42	7.7	$C_{32}H_{66}$
3.02	18.4	$C_{33}H_{68}$	3.11*	25.2	$C_{33}H_{68}$
3.87	2.7	$C_{34}H_{70}$	4.00	2.1	$C_{34}H_{70}$
4.93	3.7	$C_{35}H_{72}$	5.13	5.3	$C_{35}H_{72}$

Old leaves**							
Unsaponified (1907 μg)					Saponified		
RRT	%	M^+	b.p.	compound	RRT	%	compound
1.14	1.5	408	57	$C_{29}H_{60}$	1.15	1.3	$C_{29}H_{60}$
1.45	2.2		n.i.		1.47	1.6	$C_{30}H_{62}$
1.56	0.6		n.i.	ester			
1.84*	29.1	436	57	$C_{31}H_{64}$*	1.89	48.6	$C_{31}H_{64}$
2.10	33.2	456	57	SAM, high M^+			
2.37	8.3	450	57	$C_{32}H_{66}$	2.42	8.7	$C_{32}H_{66}$
3.02	18.2	464	57	$C_{33}H_{68}$	3.10	30.4	$C_{33}H_{68}$
3.88	2.9		n.d.	$C_{34}H_{70}$	3.97	2.4	$C_{34}H_{70}$
4.95	3.9		n.d.	$C_{35}H_{72}$	5.10	6.7	$C_{35}H_{72}$
					6.53	+	

**Two series of quantitative important aliphatic hydrocarbon homologues with two mass units difference, co-elute before cholestane. Mass at the main compound about 390.
[1] *Seco*-triterpene acid methyl ester.

Fraction A_{10} (73 μg): Of this fraction only one main compound eluted after cholestane; RRT 2.16 (76%). Minor compounds were present with RRT 2.98 and 3.84. A second main peak eluted before the referent at RRT 0.52.

Fraction A_{30} (174 μg): Besides free pentacyclic triterpenols also a *seco-nor*-triterpenol (RRT 1.81, M^+ 414, identical to the corresponding compound in the wax of old leaves of *H. australis*) is present in fraction A_{30}. *H. crassipes* thus contains both types of *seco*-triterpenes in its leaf wax: methylesters of *seco*-A triterpenoid acids (SAM, fraction A_0) and the free *seco*-A-*nor*-triterpenols (SN, fraction A_{30}).

Old leaves

Fraction A_0 (1907 μg): As was found for the younger leaves, old leaves contain the series of hydrocarbon homologues with the odd carbon numbers predomin-

Table 12. GC and GC-MS analysis of the free alcohol fraction A_{30} of leaf epicuticular wax of relatively young and old leaves of *Hoya crassipes*.

Younger leaves					Old leaves				
RRT	%	M^+	b.p.	compound	RRT	%	M^+	b.p.	compound
1.16	6.7		n.d.		1.16	12.3		n.d.	
1.50	2.1		n.d.		1.51	3.3		n.d	
1.81	18.5	414	195	SN 1[1]	1.84	fr.sh. in RRT 1.93			SN 1
1.93	30.0	n.d.		ibid. 2 (SIM ev.)	1.93	62.7	414	218	SN 2
2.47	2.0	n.d.		ibid. 3 (SIM ev.)	2.49	+			SN 3?
2.83	4.9	426	218	β-amyrin	2.83	+	426	218	β-amyrin?
3.13	27.1	426	218	triterpenol	3.20*	18.6	426	218	triterpenol
					3.72	3.0		n.d.	

*Double peak.
[1]SN = *seco-nor*-triterpenol.

Table 13. GC-detectable compounds eluting after cholestane in the on alumina oxide separated fractions of total wax of young and old leaves of *Hoya keysii*.

Young leaves (7; 95.5 cm^2)				Old leaves (5; 253.5 cm^2)		
fraction	amount	$\mu g.cm^{-2}$	%	amount	$\mu g.cm^{-2}$	%
total wax	3.7 mg	42	100	23.6 mg	95	100
A_0	0.30		8.0	0.52		2.2
A_{10}	0.03		0.9	0.02		0.1
A_{30}	0.17		4.7	2.45		10.4
	0.50 mg	5.7	13.6%	2.99	11.4	12.7%

Table 14. GC and GC-MS analysis of the alumina oxide leaf wax fractions from the young leaves of *Hoya keysii*.

Fraction A_0			A_{10}			A_{30}				
RRT	%	probable compound	RRT	%	probable compound	RRT	%	M^+	b.p.	probable compound
1.14	2.6		1.52	50.7	$C_{25}H_{48}OAc$ (Y_1Ac)	1.15	51.0	364	57	$C_{25}H_{38}O$ (Y_1)
1.44	46.0	series of	1.96	4.1	$C_{26}H_{50}OAc$ (Y_2Ac)	1.51	4.2	n.i.		$C_{26}H_{50}O$ (Y_2)
1.84	10.3	homologues	2.53	33.3	$C_{27}H_{52}OAc$ (Y_3Ac)	1.93	37.6	392	57	$C_{27}H_{52}O$ (Y_3)
2.37	33.5	aliphates	3.72	8.9	trit.ac.	2.88	2.5	414	218	*seco-nor*-triterpenol
3.01	2.5		4.13	3.3	trit.ac.	3.26	2.7			n.i.
3.89	5.1					3.67	2.0			n.i.

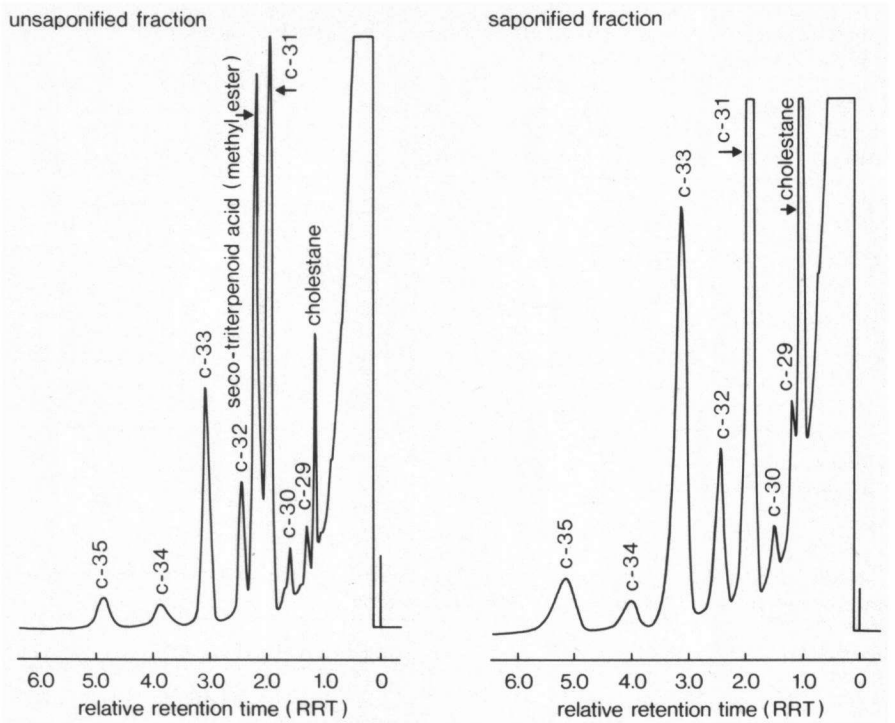

Fig. 5. GC analysis of the apolar lipid fraction A_0 from the epicuticular wax of old leaves of *Hoya crassipes* before (left) and after saponification (right).

ating (*table 11, fig. 5*). The ratio C-33 versus C-31 has increased compared to the younger leaf. In front of the cholestane referent a broad peak of short-chain hydrocarbons eluted. A third type of compounds in this fraction is a mixture of methylesters of *seco*-A-triterpene acids (SAM) with a mass fragmentation pattern comparable with those found in the wax of old leaves of *H. lacunosa*. In general, the composition of this fraction A_0 resembles that of *H. australis*. The only difference is the identity of the main *seco*-triterpene acid methylester (*H. australis*: RRT 2.50, co-eluting with $C_{32}H_{66}$; *H. crassipes*: RRT 2.10, eluted as a single peak).

Fraction A_{10} (50 μg): The main peak eluting in this minor fraction was the unidentified compound with RRT 2.16 which was also found in the leaf wax of the younger leaves.

Fraction A_{30} (314 μg): Main compound in this fraction was a *seco-nor*-triterpenol (SN) with RRT 2.10 and a mass fragmentation pattern identical to the corresponding compound of the wax of old leaves of *H. australis* (*fig. 6*). There, however, the main compound was β-amyrin-derived (BAAS & FIGDOR 1978a,

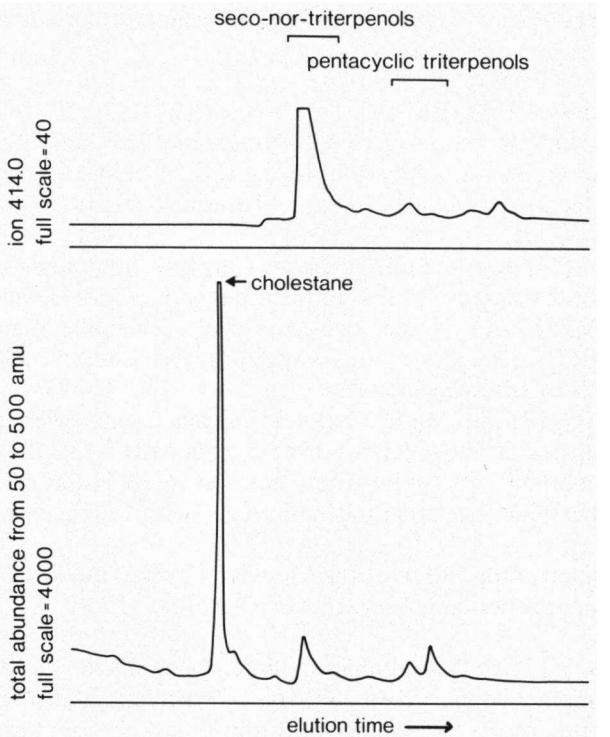

Fig. 6. GC-MS analysis of the free alcohol fraction A_{30} from epicuticular wax of old leaves of *Hoya crassipes*. Upper trace: selected ion monitoring of the *seco-nor*-triterpenols by tracing their molecular ion (m/e 414); lower trace: total ion current (m/e 50 to m/e 500).

Table 15. GC and GC-MS analysis of the free alcohol fraction A_{30} of wax of old leaves of *Hoya keysii*.

RRT	%	M^+	b.p.	compound
1.16	0.1		not determined	
1.92	0.3		not determined	
2.42	0.1		not determined	
2.84	25.0	426	218	β-amyrin
3.07*	21.3	424	218	front: triterpenon
		426	218	back: α-amyrin
3.67	52.3	426	247	isobauerenol ?
4.32	0.8	440?		C-31-triterpenol ?

*Double peak.

b) and had RRT 1.80. The same compound was present in the wax of old leaves of *H. crassipes* visible as a shoulder in the main *seco* compound peak (*table 12*). As mentioned above, this peak is the main *seco-nor*-triterpenol of the wax

of young leaves of *H. crassipes*. Other, less important, peaks eluted in the triterpenol region.

3.4. *Hoya keysii* F.M. Bailey

In the wax of young leaves only free sterols and minute amounts of free triterpenol were found on TLC-examination. In the wax of old leaves, however, large amounts of free triterpenol are present. No esterified triterpenoids were found.

Young leaves (table 14)

Fraction A_0 (297 μg): contained a series of aliphatic homologues with a distribution pattern that resembled that of the other species investigated.

Fraction A_{10} (33 μg): Besides two main peaks, probable Y-acetates (RRT 1.52; 51%; RRT 2.52; 33%) minor compounds were found at RRT 1.96, 3.72 and 4.13, possibly triterpene acetates.

Fraction A_{30} (173 μg): Main compounds in this fraction were the major Y-compounds as free alcohols, RRT 1.15 (51%)) and RRT 1.93 (37.5%). Among the minor compounds a *seco-nor*-triterpenol was found at RRT 2.88 (M^+ 414, b.p. 218). Other minor peaks eluting in this region may be free pentacyclic triterpenols.

The percentual composition of the Y-acetates in fraction A_{10} was very close to that of the corresponding free alcohols in A_{30}.

Old leaves.

Fraction A_0 (517 μg): Only aliphatic hydrocarbons eluted after the cholestane referent, the main compound being $C_{31}H_{64}$. The percentual distribution of this series has shifted to the next higher retention time when compared to that of the young leaves. On saponification an alcohol appeared at RRT 2.84, probably originating from a triterpene long-chain acid ester.

Fraction A_{10} (23 μg): One peak was present at RRT 3.42 in the region where the triterpene acetates elute. The unknown ester with RRT 0.52 found for the young leaves of *H. crassipes* and *H. australis* was present too.

Fraction A_{30} (2450 μg): This fraction, by far the most important one of the wax of the old leaves, contained almost exclusively triterpenes (*table 15, fig. 7*).

Contrary to the other *Hoya* species investigated, the triterpenes accumulating in ageing *H. keysii* leaf wax apparently retain their pentacyclic skeleton. A second distinction is the occurrence of significant amounts of a triterpenon in this fraction. The identity of the main compound has as yet not been elucidated (base peak 247, molecular ion 426). The only triterpene showing this base peak found in the available literature is a dihydroxy triterpene acid methyl ester, isolated from grape cuticle (BLOSCZYK 1979). This compound, however, has a molecular ion of 486.

Isobauerenol, identified earlier as one of the major free triterpenols of *Hoya bella* latex (BAAS & NIEMANN 1979) has similar GC-chromatographic properties as the main triterpenol of the wax of old leaves of *Hoya keysii*. It has a molecular ion of 426 and the same mass-fragmentation pattern. Relative intensities given (THEUMAN & COMIN 1969), however, differ somewhat from our finding. It is

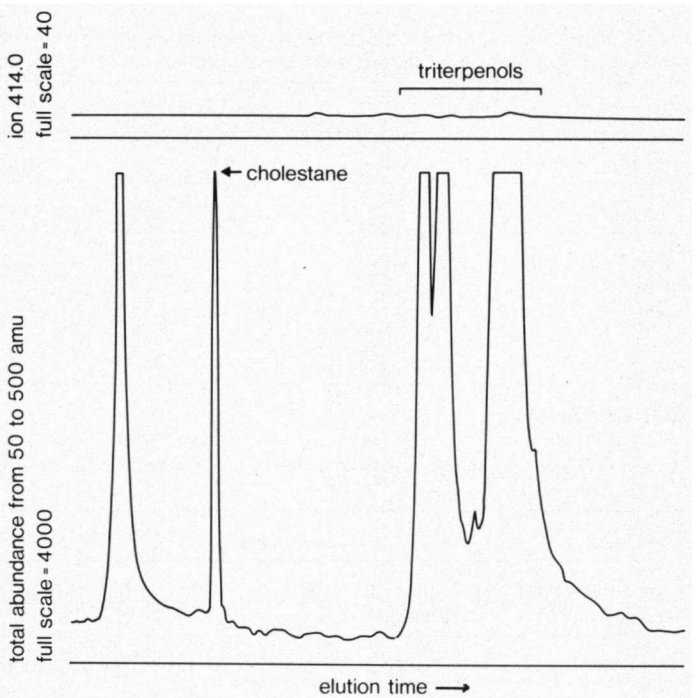

Fig. 7. GC-MS analysis of the free triterpenol fraction A_{30} from the epicuticular wax of old leaves of *Hoya keysii*. Upper trace: selected ion monitoring of the *seco-nor*-triterpenols by tracing their molecular ion (m/e 414); lower trace: total ion current (m/e 50 to m/e 500).

thus likely that the wax triterpenol of *H. keysii* is either isobauerenol or one of its isomers.

3.5. *Ficus benjamina* L.

By TLC only very little free triterpenol (R_f 0.33) was detectable in the wax of young leaves. In the wax of old leaves free triterpenols are present in higher amounts. A minor spot co-chromatographed with triterpene acetate referent (R_f 0.73) while a hardly detectable spot of a less polar triterpene ester chromatographed at R_f 0.86 (*table 1*).

Young leaves

Fraction A_0 (8 μg): Traces of compounds were found at RRT 1.20 and RRT 2.04. Their amounts were too low for identification. Relatively large amounts of an ester at RRT 0.52 probably the one also encountered in the *Hoya* species, was present.

Fraction A_{10} (15 μg): Unidentified compounds eluted at RRT 1.94 (10%), RRT 2.70 (60%) and RRT 2.98 (30%).

Table 16. The GC-detectable compounds, that eluted after the cholestane referent, occurring in the alumina oxide separated fractions of the leaf wax of young and old leaves of *Ficus benjamina*.

Young leaves				Old leaves		
fraction	amount (mg)	%	$\mu g.cm^{-2}$	amount	%	$\mu g.cm^{-2}$
total wax	3.4 mg*	100	46	6.1 mg*	100	50
A_0	0.00	0.0		1.1	18.1	
A_{10}	0.02	0.4		0.07	1.1	
A_{30}	0.03	0.8		0.09	1.5	
		1.2%	0.6 $\mu g.cm^{-2}$		20.7%	10.4 $\mu g.cm^{-2}$

*Gravimetrically determined.

Table 17. GC and GC-MS analysis of various on alumina oxide separated fractions of the wax of old leaves of *Ficus benjamina*.

Fraction A_0 (1107 μg)			A_{10} (67 μg)			A_{30} (92 μg)				
RRT	%	probable compound	RRT	%	probable compound	RRT	%	M^+	b.p.	compound
1.17	3.8	$C_{29}H_{60}$	1.84	4.4	n.i.**	2.70*	69.3	426 426	204 55	taraxerol (front) triterpenol?
1.49	1.3	$C_{30}H_{62}$	2.61	22.5	n.i.	3.06	15.7	426	218	α-amyrin
1.92	46.0	$C_{31}H_{64}$	2.87	24.2	n.i.	3.76*	15.0	426 428	52 128	n.i. mixture
2.47	4.0	$C_{32}H_{66}$	3.86	48.8	n.i.					
3.18	44.9	$C_{33}H_{68}$								

*Mixture.
**n.i. = not identified.

Fraction A_{30} (26.5 μg): Detectable compounds were Y_1 (RRT 1.17; 18.5%), Y_3 (RRT 1.97; 30%) and a mixture of probably triterpenols at RRT 2.91 (51.5%).

Old leaves

Fraction A_0 (1107 μg): The only compounds that eluted after cholestane belonged to the series of hydrocarbon homologues, as was found for the *Hoya* species too. Carbon number 31 was found predominating (*table 17*).

Fraction A_{10} (67 μg): The small amounts of compounds present in this fraction have not been identified (*table 17*). TLC-evidence points to a triterpene acetate nature for the compound with RRT 3.86.

Fraction A_{30} (92 μg): Although quantitatively not very important, the identified compounds in this fraction were triterpenols only. Most of the main peak (RRT 2.70; 69%) consisted of taraxerol.

4. DISCUSSION

Only compounds with volatility less than cholestane have been analysed in these investigations. From the GC-analyses of these compounds three main conclusions have been drawn: (1) The amount of leaf epicuticular wax strongly increases during leaf ageing, simultaneously with (2) a considerable change in composition; (3) the compounds that accumulate in the leaf waxes of the five investigated species are not the same, but, probably dependent upon the species, all belong to triterpene alcohols, *seco-nor*-triterpenols, *seco*triterpene acid methyl esters and hydrocarbons (*table 2*).

In young leaves of some plants studied rather volatile aliphatic alcohols (or their acetates) were found (*H. australis, H. keysii, Ficus benjamina*). They seem to be characteristic for the early stages of leaf development.

Generally, only an increase in the apolar fraction A_0 and the "alcohol"-fraction A_{30} is observed during subsequent leaf life. In *H. lacunosa, H. crassipes, H. australis* and *Ficus benjamina* it is fraction A_0 that increases most. Compounds accumulating are *seco*-triterpene acid methyl esters (*H. lacunosa, H. crassipes* and *H. australis*). In *H. australis, H. crassipes* and *Ficus benjamina* aliphatic hydrocarbons also constitute a considerable part of this fraction in old leaves. In *Ficus* they are the sole compounds found in this fraction. In *Hoya australis* in addition a large increase of fraction A_{30} is observed: *seco-nor*-triterpenols form the major part and the pentacyclic triterpenols constitute the remainder of this fraction of the old leaves. Though quantitatively less important, *seco-nor*-triterpenols were also found in the leaf wax of old leaves of *H. crassipes*.

For *Hoya keysii* leaf wax, only a considerable increase in compounds of the alcohol fraction A_{30} is found at increasing leaf age, pentacyclic triterpenols forming almost all of it in old leaves. *seco*-Triterpenes are not present.

Traces of 3-keto triterpenes were found in fraction A_{30} of old leaves of *Hoya keysii* and *Ficus benjamina*, the two species that do not accumulate detectable amounts of *seco*-triterpenes.

In old leaves of *Hoya lacunosa* traces of a pentacyclic triterpene acid methyl ester are probably present.

Morphologically the results presented may be interpreted in such a way as that young expanding leaves need a well-spreading soft waxy layer (cf. BAAS & FIGDOR 1978a). In this regard the rather volatile alcohols found in the very young leaves may have functional (protective) significance. The increase of their chain length by esterification, thus decreasing their volatility, might be a subsequent mechanism to adjust their physico-chemical properties to a morphologically more stationary stage of epidermal cell life. In older leaves both the triterpenoids and the aliphatic hydrocarbons (with comparable physico-chemical properties) can serve this protective function.

As wax morphology may be largely determined by passive crystallisation of the constituting molecules (HULL et al. 1979 and references) changes in relative amounts of the individual wax components may adapt wax structure to environmental conditions and leaf age. So the chain length of the aliphatic hydrocarbons

go to a higher mean carbon number as the leaves become older (see also HERBIN & ROBINS 1969, BAKER & HUNT 1981).

Triterpenoids also may contribute to the protection of the leaf against abiotic factors. This may be by their physico-chemical effects on wax morphology. Adaptation to natural environment and leaf age may explain the differences found in the triterpenoids of young leaves compared to those of old ones.

The finding that the four xeromorphic *Hoya* species accumulate triterpenes in the wax of their old leaves is well in agreement with the observation that triterpenes are most found in leaves which remain on the plant for some years, and have thick, glaucous wax layers (WOLLENWEBER et al. 1981). So taxerone is found as main compound in the leaf wax of glaucus forms of *Dutleya farinosa,* but is absent in the green forms; β-amyrin acetate is the main compound in the glaucus wax of *D. brittonii,* and only present as traces in the green form (MANHEIM et al. 1979, MANHEIM & MULROY 1978).

A second, perhaps more important reason for the presence of especially *seco*-triterpenoids in the wax of very old leaves may be found in their known role in interactions with the biotic environment (see introduction). Thus herbivory, insect-plant relations and invasion of pathogens may be controlled by the triterpenoids present in the wax of old leaves.

The triterpenones found in the waxes of *H. keysii* and *Ficus benjamina* are oxidation products of the 3-hydroxytriterpenols. They are very probably intermediates in the oxidative ring-fission leading to the *seco*-triterpenoids found in the three other *Hoya* species investigated. This may be either a photochemical- or an enzyme-mediated (e.g. Baeyer-Villiger type) reaction (QUINKERT 1965, DE MAYO 1963). If this oxidation is a photochemical process, then triterpenes may be (also) involved in a protection mechanism against ultraviolet light, since *seco*-triterpenoid synthesis is readily achieved in vitro from triterpenones by irradiation with UV-light (QUINKERT & HEINE 1963). In the leaf wax of *H. keysii,* however, *seco*-triterpenes are not detected; normal pentacyclic triterpenols are accumulating, although this plant is raised under the same environmental conditions as the species that do accumulate *seco*-derivatives. This makes photochemical oxidation of the 3-hydroxy alcohols to the *seco*-compounds less likely. The more so, because all plants were grown in a greenhouse, where solar ultraviolet radiation can be neglected.

Only small amounts of triterpenoids are present in the leaf wax of *Ficus benjamina.* This plant apparently uses aliphatic hydrocarbons as structural compounds. Compared to the leaves of the *Hoya* species its leaves live relatively short. A "defence mechanism" against e.g. pathogens may consequently be less urgent or superfluous.

Like the *Hoya* species, *Ficus benjamina* possesses a latex system. A possible protective function of the triterpenoids against the biotic environment may also be accomplished by oxidized triterpenoids, present in these laticifers. In the case of *Asclepias eriocarpa,* where this aspect has been studied in detail, the latex cardenolides are believed to play a central role in the defence against herbivory (NELSON et al. 1981).

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