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DIHYDRONYCTANTHIC ACID METHYL ESTER AND OTHER 3,4-*SECO*-PENTACYCLIC TRITERPENOIDS FROM *HOYA LACUNOSA*

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Key Word Index—*Hoya lacunosa*; Asclepiadaceae; leaf wax; 3,4-*seco*-pentacyclic triterpenoid acid methyl ester; dihydronyctanthic acid methyl ester.

Abstract—From the wax of old leaves of *Hoya lacunosa* was isolated a mixture of 4(23)-saturated *seco*-ring-A triterpene acid methyl esters. One of these compounds was identified as the β-amyrin derivative 5-isopropyl-10(2-methoxycarbonylethyl)-des-A-olean-12-en (dihydronyctanthic acid methyl ester), while another compound was a taraxerol derivative 5-isopropyl-10(2-methoxycarbonylethyl)-des-A-olean-14-en. Two other constituents were probably derived from α-amyrin and taraxasterol, respectively.

INTRODUCTION

Hoya species are, with a few exceptions, evergreen, xeromorphic climbing tropical epiphytes [1]. Investigations on the composition and biosynthesis of triterpenes of the leaf cuticular waxes revealed a considerable change in the relative amounts and composition of the triterpenes during leaf development and subsequent ageing [2–4]. Pentacyclic and ring-opened (*seco*-)triterpenoids, not present in the wax of very young leaves, become a major part of the wax of the very old green leaves of all species investigated thus far.

In the surface wax of the old leaves of *Hoya lacunosa*, a number of hitherto unknown *seco*-triterpene acid methyl esters were found to accumulate. This paper describes the structure analysis of two of them.

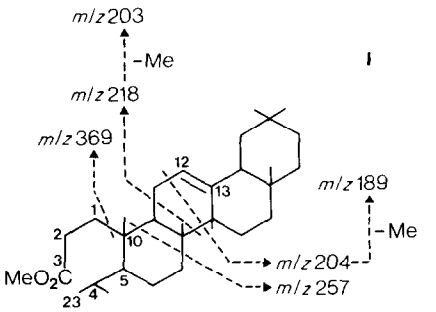
RESULTS AND DISCUSSION

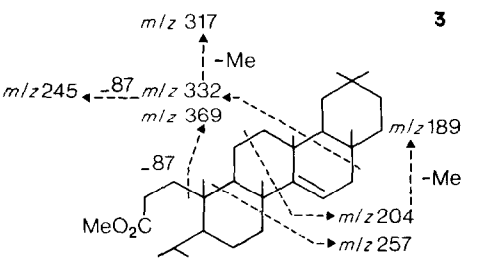
The total leaf wax (28.4 mg) from 65 old green leaves (single surface 241 cm^2, fresh wt 43 g, dry wt 3.6 g) was separated on an alumina column. The first fractions to be eluted with petrol which were triterpene-positive by a TLC-spot test contained a total of 4.9 mg of triterpene material as determined by GC analysis. GC showed three peaks in each of the fractions: minor ones at retention times relative to cholestane of 2.01 and 2.52, and a main peak at RR_t around 2.20. The RR_t of the latter changed slightly among the fractions, indicating that this peak had a heterogeneous composition.

Mass spectra of the compounds in the mixture were determined by GC/MS analysis and the elemental composition of specific fragments was obtained by high resolution GC/MS. All compounds in the mixture proved to be isomers with a mass peak at *m/z* 456 ($C_{31}H_{52}O_2$) and common fragments at *m/z* 441 $[C_{30}H_{49}O_2]^+$, 369 $[C_{27}H_{45}]^+$, 257 $[C_{19}H_{29}]^+$, 218 $[C_{16}H_{26}]^+$, 203 $[C_{15}H_{23}]^+$ and 189 $[C_{14}H_{21}]^+$. As these compounds were destroyed by saponification of the extracts without the liberation of a long-chain alcohol, the compounds were assumed to be triterpenoid esters. Upon methylation with diazomethane, the original compounds were recovered again. From this result they were concluded to be triterpenoid acid methyl esters. However, their mass spectra could not be related to those of known triterpenoid acids with the carboxylic acid function at C-17.

Their common fragment at *m/z* 369 $[M-87]^+$ is characteristic for *seco*-A-triterpenoid acid methyl esters [5] and it is formed by the loss of C-1, C-2 and C-3. Fragment *m/z* 425, although too weak to be determined by high resolution mass spectrometry, was probably formed by the loss of methoxyl. Fragment *m/z* 257 ($C_{19}H_{29}$), formed by loss of rings A and B, indicated that in this fragment, derived from rings C, D and E, a double bond was present as otherwise fragment *m/z* 259 would be found [6].

The mass spectra of compounds **1** and **2** (RR_t 2.01 and





the back shoulder of RR_t 2.20 of the first eluting fractions) had a base peak at m/z 218, characteristic of Δ^{12}-oleanenes and Δ^{12}-ursenes [7]. Modifications of ring A do not affect the general fragmentation pattern by the retro-Diels–Alder (RDA) cleavage, but only promote additional cleavage, leading to the loss of ring A to give the characteristic *seco*-fragments [8]. The two compounds thus belong to this skeleton type.

The mass spectrum of the natural compound 3,4-*seco*-olean-(4,23),12-dien-3-oic acid methyl ester (methyl nyctanthate), which shows a close resemblance to that of compound **1**, has the characteristic RDA-fragments of the parent compound, the pentacyclic triterpenol β-amyrin, plus the additional *seco*-A-fragments [5]. However, the molecular ion and fragment $[M-87]^+$ were found at 2 amu lower (m/z 454 and 367, respectively). Therefore compound **1** is concluded to be the 4(23)-saturated analogue, 5-isopropyl-10(2-methoxycarbonylethyl)-des-A-olean-12-en(dihydronyctanthic acid methyl ester). Compound **2**, by analogy, will be the methylated 4(23)-saturated form of roburic acid, the urs-12-en isomer [9].

Compound **3**, which appeared as the front shoulder of the GC peak at RR_t 2.20 in the first fractions, eluted as a single peak in the later fractions from which the fragment at m/z 218 had largely disappeared from the mass spectrum. From the mass spectral data it was concluded that this compound was the 4(23)-saturated *seco*-derivative of the Δ^{14}-oleanene, taraxerol, and it had the structure 5-isopropyl-10(2-methoxycarbonylethyl)-des-A-olean-14-en. The pentacyclic triterpenol, taraxerol, was the main free triterpenol of *Hoya lacunosa* leaf wax [4] and it was eluted in the more polar fractions of the extract. Table 1 shows the corresponding mass fragments of the pentacyclic alcohol and those from the derived *seco*-acid, compound **3**.

In the mass spectrum of compound **4**, the last eluting GC peak at RR_t 2.52, RDA-fragments in the m/z 200 region were absent. The double bond in the ring skeleton was thus probably located externally, e.g. in ring E. This fact, and a high molecular mass fragment ion, pointed to a derivative of ψ-taraxasterol or its isomer taraxasterol [10]. This was also indicated by the relatively long GC retention time which was also found for the free pentacyclic alcohol. Because of the lack of RDA-fragments in the mass spectrum, more analytical data are necessary for final conclusions regarding the structure of this compound.

From the literature, formation of acids by ring cleavage of cycloalkanones upon irradiation with UV light is a well-known process [11]. Both photolysis (under the exclusion of oxygen) and photo-oxidation in the presence of oxygen of triterpenoid 3-ketones in solution cause ring fission giving the 4(23)-saturated or the 4(23)-unsaturated 3,4-*seco* acids, respectively.

The structure of nyctanthic acid (4(23)-unsaturated) was deduced by photolysis of β-amyrone to give dihydronyctanthic acid [12, 13]. Carman [14] and Hirota *et al.* [15] prepared dihydrocanaric acid from lupanone. These reactions were carried out in the presence of water, which results in the formation of the free carboxylic acids by the addition of water. In oxygen-free pure methanol, the solvent is added instead to give the methyl esters of the dihydro acids [16–19].

By analogy, we prepared the methyl ester of dihydronyctanthic acid in a one-step conversion by photolysis of β-amyrone in methanol under a nitrogen atmosphere. Irradiation was carried out with a shielded UV-lamp giving light with a wavelength of 300 nm and higher (absorption maximum of β-amyrone, $\lambda_{max}^{MeOH} = 287.5$ nm). The reaction was followed by gas chromatographic analysis of the mixture.

After 4.5 hr of irradiation 18.7% of the β-amyrone was left. Two photo-products were formed: the main compound, **A** (yield 63.8%) with a RR_t 2.01, and a minor photo-product, **B** (yield 17.5%) which eluted at RR_t 1.82. Prolonged irradiation did not change the ratio of the two compounds formed.

The mass spectrum of the main compound **A** was identical to that of the dihydronyctantic acid methyl ester isolated from the leaf wax. The molecular ion was at m/z 456, the characteristic *seco*-A fragment was at m/z 369 and the dominant RDA-fragments of the Δ^{12}-oleanene skeleton were at m/z 218, 203 and 189, with identical relative abundances. The identical mass spectra and co-chromatography on GC of the synthetic reference compound and the leaf wax compound confirmed the latter to be dihydronyctanthic acid methyl ester.

The minor photoproduct **B** (17.5%), molecular ion at m/z 424, was concluded to be a *seco*-alcohol (characteristic *seco*-fragments at m/z 365, 409 $[M-Me]^+$ and 391 $[M-Me-H_2O]^+$) and thus possessed two more double bonds than its precursor β-amyrone which has the same MW. They were probably located externally in the molecule as the same RDA-fragments (m/z 218, 203 and 189) were still dominant, though with a higher relative abundance for m/z 203. Upon acetylation of the reaction

Table 1. Comparison of the mass spectral fragments of taraxerol and the derived 4(23)-saturated *seco*-acid methyl ester from the wax of old green leaves of *Hoya lacunosa*

m/z	Taraxerol		*Seco*-acid methyl ester		
426	$C_{30}H_{50}O$	$[M]^+$	456.3986	$C_{31}H_{52}O_2$	$[M]^+$
411	$C_{29}H_{47}O$	$[M-Me]^+$	441.3771	$C_{30}H_{49}O_2$	$[M-Me]^+$
			369.3521	$C_{27}H_{45}$	$[M-87]^+$
302	$C_{21}H_{34}O$	$[M-\text{ring E}]^+$	332.2710	$C_{22}H_{36}O_2$	$[M-\text{ring E}]^+$
278	$C_{20}H_{31}O$	$[302-Me]^+$	317.2503	$C_{21}H_{33}O_2$	$[332-Me]^+$
			257.2242	$C_{19}H_{29}$	$[M-\text{ring A, B}]^+$
			245.2262	$C_{18}H_{29}$	$[332-87]^+$
218	$C_{16}H_{26}$	[ring D, E, part C]$^+$	218.2026	$C_{16}H_{26}$	[ring D, E, part C]$^+$
204	$C_{15}H_{24}$	[ring D, E, part C]$^+$	204.1880	$C_{15}H_{24}$	[ring D, E, part C]$^+$
189	$C_{14}H_{21}$	$[204-Me]^+$	189.1638	$C_{14}H_{21}$	$[204-Me]^+$

mixture compound **B** afforded a monoacetate ($[M]^+$ at *m/z* 464) which on GC eluted at the back shoulder of the main (unchanged) photo-product, dihydronyctanthic acid methyl ester. This was confirmed by single ion monitoring of the characteristic fragments at *m/z* 464 and 365. The structure of compound **B** has not been investigated further.

The *seco*-acid methyl ester isomers isolated from the leaf wax of *Hoya lacunosa* are new 4(23)-saturated *seco*-triterpenoids. Related compounds have been found earlier in plant material. The 4(23)-saturated 3,4-*seco*-lup-20(29)-en-3-oic acid methyl ester has been obtained from *Caralluma buchardii*, which also belongs to the Asclepiadaceae [6], and the free saturated *seco*-dammarane acids, casasequic acid and donanic acid, were isolated from *Cistus bourgeanus* (Cistaceae) [18].

The *seco*-pentacyclic acid methyl esters isolated from the leaf wax of *H. lacunosa*, but also present in the wax of other *Hoya* species, e.g. *H. crassipes* (syn. *H. diversifolia*) [4], may be intermediates in a biogenetic route to the C-1 hydroxylated *seco*-nortriterpenols isolated from *H. australis* and *H. crassipes* leaf wax. The structure of the β-amyrin-derived nortriterpenol has recently been elucidated [20] (Scheme 1).

Free and methylated 4(23)-saturated triterpenoid 3,4 *seco*-acids have also been isolated from recent and fossil sediments [19, 21]. Although their formation by photolysis of the leaf ketones prior to incorporation into the sediment is not excluded, they are thought to be photomimetic products formed by micro-organisms during the early stages of maturation of sediments of continental influence. They would be produced from pentacyclic precursors of plant origin through biochemical reactions leading to the corresponding 3-ketones prior to degradation [19]. However, the responsible micro-organisms have not been isolated or identified [22].

Our isolation of these *seco*-acid methyl esters in quantitatively important amounts from the leaf waxes of *Hoya* species now offers an alternative formation mechanism. They may be produced by the plant itself rather than photomimetically during diagenesis of the sediment or by the action of soil micro-organisms (cf. ref. [23], p. 85).

The further degraded fossil A-ring cleaved triterpenoids are suggested to be formed also during diagenesis with the *seco*-acids as intermediates via a C-1 hydroxylated compound [21]. If this biogenetic proposal is correct, at least some steps leading to ring-A degradation can be accomplished by the plant *in vivo* (Scheme 1). In this respect it is important to note that C-1 hydroxylation is not uncommon in the plant kingdom, e.g. it is a common feature of the limonins which are C_{26}-nortriterpenoids found in the Rutaceae and Meliaceae [24].

*Probably from co-eluting traces of compound **2**.

However, the remarkable preservation of the *seco*-triterpenoids in fossils, e.g. in brown coal [21], makes it unlikely that, if ring-A degradation occurs in the sediments, this would proceed via these compounds as intermediates. The RDA-type mechanism as proposed by Albrecht [25] then seems more likely.

Their resistance against bacterial breakdown during the maturation of the sediments indicates that these leaf wax compounds may provide an effective barrier against attack of the leaves by the microbes of the phyllosphere. Indeed, one of the first natural *seco*-A triterpenoids discovered, the fungal *seco*-A metabolite of the steroid eburicoic acid, has antibacterial properties [26]. Also, various synthetic 3,4-*seco* derivatives of pentacyclic triterpenoids have been shown to inhibit bacterial growth [27, 28, 29].

EXPERIMENTAL

Plants were raised from seed in the greenhouse in pot culture [30].

Extraction. Surface wax from 65 carefully selected, undamaged old leaves was extracted [4] by dipping the leaves in a $CHCl_3$–MeOH mixture.

Column chromatography. The total lipid extract was separated on a column of 10 g alumina (Brockman grade III, Merck). The *seco*-triterpenoid methyl esters were eluted with petrol in fractions 18–34 (fraction vols. 2.5 ml each).

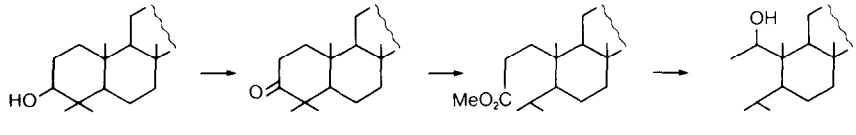
5-*Isopropyl*-10(2-*methoxycarbonylethyl*)*des*-A-*olean*-12-*en* (**1**, *RR*$_t$ 2.01, 5α-cholestane as reference). MS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (18), 441 (8), 425 (1), 369 (5), 281 (1), 271 (1), 257 (9), 251 (6), 245 (9)*, 218 (100), 205 (10), 203 (37), 189 (16).

5-*Isopropyl*-10(2-*methoxycarbonylethyl*)*des*-A-*olean*-14-*en* (**2**, *RR*$_t$ 2.19 front). MS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (22), 441 (15), 425 (2), 369 (5), 332 (10), 317 (18), 289 (4), 277 (2), 271 (6), 257 (15), 245 (100), 231 (17), 218 (23), 205 (23), 204 (86), 203 (12), 189 (25).

Compound **3** (*RR*$_t$ 2.19, back). MS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (27), 441 (11), 425 (2), 369 (38), 332 (3), 317 (3), 289 (7), 277 (8), 257 (13), 251 (15), 245 (38)*, 218 (100), 207 (11), 205 (21), 204 (22)*, 203 (26), 189 (23).

Compound **4** (*RR*$_t$ 2.52). MS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (57), 441 (19), 425 (2), 369 (19), 355 (3), 318 (4), 299 (3), 285 (4), 271 (11), 257 (27), 251 (13), 245 (22), 231 (24), 229 (16), 218 (22), 205 (20), 204 (23), 203 (20), 189 (23), 177 (23).

Photolytic preparation of dihydronyctanthic acid methyl ester from β-amyrone [18, 19]. β-Amyrin was oxidized with chromic acid to yield β-amyrone according to the method described by Lardelli *et al.* [31]. The β-amyrone was dissolved in *ca* 80 ml MeOH and irradiated for 4.5 hr with a water-cooled bare Philips HPK 125W high pressure mercury lamp which was immersed in the soln. The lamp was shielded with jena blue-stripe glass transmitting wavelengths of 300 nm and higher only. Two photoproducts were obtained: dihydronyctanthic acid methyl ester (*RR*$_t$ 2.01, yield 63.8 %), MS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (13), 441 (5), 425 (0.5), 369 (4), 281 (1), 271 (2), 257 (7), 251 (6), 218 (100), 205 (10), 203 (37), 189 (15); and photo-product **B** (*RR*$_t$ 1.82,



Scheme 1. Suggested biogenetic route leading to the *seco*-acid methyl esters and the *seco*-nortriterpenols isolated from the wax of old green leaves of some *Hoya* species.

17.5 %), MS (70 eV) *m/z* (rel. int.): 424 $[M]^+$ (20), 409 (7), 391 (2), 365 (12), 257 (6), 218 (100), 203 (62), 191 (12), 189 (20).

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