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ORIGINAL PAPER

Apocynaceae Seed Lipids: Characterization and Occurrence of Isoricinoleic Acid and Triacylglycerol Estolides

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Abstract Isoricinoleic acid (9-hydroxy-*cis*-12-octadecenoic acid; IR) is a potential renewable feedstock for the oleochemical industry, a precursor for the synthesis of antimicrobial compounds and a component of the seed oil of certain plants in the Apocynaceae. For a more detailed survey of this plant family, seeds of 18 species representing different subfamilies were obtained and acyl composition and oil content was determined. IR was observed only in species of the tribes Wrightieae and Nerieae in the Apocynoideae subfamily and is reported for the first time in the seed oil of the desert rose *Adenium obesum* in which it is present at a level of around 26 %. In contrast to previous reports, IR was not found in oil from *Holarrhena* species, *H. antidysenterica* and *H. pubescens,* nor in oil from *Annona squamosa*. To examine the oil structure, samples were analyzed using MALDI-TOF mass spectrometry. This technique proved to be a simple method to demonstrate the occurrence of the estolide 9-acetoxy-*cis*-12-octadecenoic acid in oil from *Nerium oleander* and gave further insight into the distribution of estolides within the oil, revealing the presence of tetra- and penta-acyl-TAG molecules, and molecules containing IR esterified to all three position of glycerol. For other species in which IR was observed, the HFA was found to be a component of seed TAG, but no secondary acylation of the hydroxyl groups was observed.

Keywords Apocynaceae · Seed oil · Isoricinoleic acid · MALDI-TOF MS · *Adenium obesum* · *Nerium oleander* TAG-estolide

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Abbreviations

FAME(s)	Fatty acid methyl ester(s)
HFA(s)	Hydroxy fatty acid(s)
MALDI-TOF MS	Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry
TAG	Triacylglycerol
TAG-estolide	Triacylglycerol estolide

Fatty Acid Nomenclature

$X:Y^{\Delta z}$	Where X is the chain length, Y is the number of double bonds and $^{\Delta z}$ is the double bond position relative to the carboxyl end of the molecule

Triacylglycerol Nomenclature

A:B	Triacylglycerol, where A is the number of carbon atoms in the acyl groups and B is the total number of double bonds
A:B:C	Triacylglycerol containing HFAs, where A in the number of carbon atoms in the acyl groups, B is the total number of double bonds and C is the total number of free hydroxyl groups
A:B:C:D	Triacylglycerol estolide, where A in the total number of carbon atoms in the acyl groups, B is the total number of double bonds, C is the number of free hydroxyl groups and D is the number of secondary ester bonds

Introduction

Higher plants synthesize a wide range of fatty acids with one or more hydroxyl group present on the acyl chain. These hydroxy fatty acids (HFAs) are generally present only in the seed oil and have limited species distribution,

but can be the predominant acyl component of the oil [1]. The HFA isoricinoleic acid (9-hydroxy-*cis*-12-octadecenoic acid, 9-OH 18:1$^{\Delta 12}$, IR) has received considerable attention as a potential renewable feedstock for the oleochemical industry [2–4] and as a precursor for the synthesis of antimicrobial compounds [5]. Identification of natural sustainable sources of IR to serve as feedstocks for industrial applications is therefore of interest. IR was first identified as a component of the seed oil of the poison arrow vine, *Strophanthus sarmentosus* [6], in which it accounted for approximately 7 % of the total fatty acyl groups. This HFA was subsequently reported for additional members of the genus *Strophanthus* at levels of 7–14 % of seed oil fatty acids [7], and for two other members of the Apocynaceae (Dogbane family), oleander (*Nerium oleander* syn. *N. indicum*) and bitter oleander (*Holarrhena antidysenterica*) at levels of 11 and 73 %, respectively [8]. IR has subsequently been identified as the primary acyl component of the seed oil of two *Wrightia* species, *W. tinctoria* (70 %) and *W. coccinea* (76 %) [9], suggesting that the fatty acid may have relatively widespread occurrence in the Apocynaceae. More recently, IR has been reported as a component of the seed oils from plants belonging to diverse families including the Anacardiaceae (*Semecarpus kurzii,* 11 % [10]), Annonaceae (*Annona squamosa*, 10 % [11]), Plantaginaceae (*Plantago* species, 0–13 % [12, 13]), and Scrophulariaceae (*Celsia coromandeliana,* 22 % [14], suggesting that the ability to synthesize IR may have arisen independently on multiple occasions during the evolution of higher plants. In the genus *Plantago*, IR is usually accompanied by the structurally related oxo-fatty acid 9-oxo-*cis*-12-octadecenoic acid (9-oxo 18:1$^{\Delta 12}$, OX) [13]. This fatty acid has not been reported from other species that synthesize IR.

Characterization of the seed oil from four species of *Strophanthus* [15], indicated that IR was a component of the seed triacylglycerol (TAG), being found predominantly in the *sn-2* position. Similarly, the oils of *W. tinctoria* and *W. coccinea* were reported to be triacylglycerol oil composed primarily of tri-isorinoleoylglycerol and di-isoricinoleoylglycerol [9]. Examination of the seed oil from *Nerium indicum,* in contrast, revealed that in portions of the lipids, secondary acylation of the hydroxyl group of IR was observed, with the acyl group being identified as acetic acid. The resulting estolide, 9-acetoxy-*cis*-12-octadecenoic acid (AcIR), was excluded from the β-carbon (*sn-2*) of the glycerol backbone [8]. As a potential source of IR for industrial use and as a source of novel acetylated oils, the Apocynaceae clearly merits deeper investigation.

To further characterize the distribution of IR within the Apocynaceae, we examined the seed lipid composition of a small number of species representative of 3 of the 5 subfamilies within the Apocynaceae. We also applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to obtain more detailed information on the acyl-glycerol structure of oils containing IR.

Experimental Procedures

Seed

Seed samples were purchased from the commercial seed companies B & T World Seeds (Paguignan, 34210 Aigues-Vives, France, www.b-and-t-world-seeds.com; B + T), Commerce India (www.agrisources.com; CI), Secret Seeds (Bristol, UK, www.secretseeds.com; SS), Silverhill Seeds and Books, (Kenilworth, South Africa, www.silverhill-seeds.co.za; SSB), or Richters Herbs (Ontario, Canada, www.richters.com; RH) as indicated in Table 1.

Seed Lipid Extraction

Lipids were extracted from dry seeds by crushing in hexane. The hexane extract was transferred to a clean glass tube and centrifuged at 2000*g* for 2 min at room temperature (22 °C) to precipitate solid material. After transfer to a fresh tube, the hexane was evaporated under a stream of nitrogen gas, and lipids were dissolved in a small volume of hexane or chloroform as required.

Gas Chromatography of Fatty Acid Methyl Esters and GC–MS

To determine quantitative acyl composition, seeds were gently crushed and woody pericarp material was removed if present. Four individual samples (20–40 mg) from each species were weighed to determine dry weight and total lipids were transmethylated in situ by refluxing for 16 h at 80 °C in glass tubes containing 2 mL 1 M HCl in methanol, acyl standard (100 μg of 17:0 FAME) and 300 μL of hexane. After cooling, 2 mL of 0.9 % NaCl was added and FAMES were recovered in the hexane phase. To confirm the identity of HFAs and assign the position of the hydroxyl group, FAMEs in hexane were mixed with an equal volume of BSA in pyridine (bis(trimethylsilyl)acetamide/pyridine 1:1 by volume) to convert free hydroxyl groups to their trimethylsilyl-ethers (TMS-FAMEs). Gas chromatography (GC) of FAMES and TMS-FAMES was conducted using an Agilent 6890 N GC equipped with a DB-23 capillary column (0.25 mm × 30 m, 0.25 μM thickness; J&W, Folsom, CA, USA) and a flame ionization detector as described previously [16]. For GC–MS analysis, an Agilent 7890A GC equipped with a 30 m DB-23 capillary column and 5975C mass selective detector (ionization energy of 70 eV, scan rate of 2.2 scans s^{-1}, mass range 40–700 Da) was used.

Table 1 Seed fatty acyl composition and content of 18 plants in the family Apocynaceae

Plant species	Source of seed	Oil content (%)	Fatty acid (%)													
			16:0	16:1 $^{\Delta 9}$	16:2$^{\Delta 9,12}$	18:0	18:1$^{\Delta 9}$	18:1$^{\Delta 11}$	18:2$^{\Delta 9,12}$	18:3$^{\Delta 9,12,15}$	20:0	20:1$^{\Delta 11}$	22:0	24:0	26:0	IR
Subfamily Apocynoideae, tribe Apocynaceae																
Apocynum cannabinum (dogbane)	B + T	ND	6.6 ± 0.2	0.3 ± 0.1		3.7 ± 0.1	19.2 ± 0.4	0.9 ± 0.0	58.5 ± 0.5	8.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.0	0.7 ± 0.0			
Subfamily Apocynoideae, tribe Malouetieae																
Holarrhena antidysenterica (kutaj)	CI	22.3 ± 3.4	7.7 ± 0.6			6.5 ± 0.8	9.8 ± 1.4	0.4 ± 0.1	37.1 ± 6.1	36.8 ± 6.2	0.7 ± 0.1	0.7 ± 0.2	0.2 ± 0.1			
Holarrhena pubescens	B + T	23.5 ± 4.6	11.4 ± 1.0	0.2 ± 0.2		7.3 ± 0.3	11.9 ± 2.4	0.5 ± 0.0	46.7 ± 0.8	19.4 ± 2.9	1.4 ± 0.1	0.2 ± 0.0	0.8 ± 0.1	0.3 ± 0.0		
Mascarenhasia spp (unidentified species)	B + T	ND	10.7 ± 0.1	0.1 ± 0.1		7.2 ± 0.3	10.5 ± 0.6	0.5 ± 0.1	66.7 ± 0.1	2.1 ± 0.2	1.2 ± 0.1	0.8 ± 0.1	0.3 ± 0.0			
Pachypodium lamerei (Madagascar palm)	B + T	36.4 ± 4.3	26.2 ± 1.3	0.5 ± 0.1		11.2 ± 1.7	38.9 ± 1.7	1.3 ± 0.3	18.5 ± 3.2	0.3 ± 0.1	1.7 ± 0.3	0.1 ± 0.0	0.6 ± 0.1	06 ± 0.1		
Pachypodium rosulatum (elephant's foot plant)	B + T	ND	29.1 ± 2.0			10.3 ± 0.8	27.1 ± 0.3	0.5 ± 0.1	29.0 ± 2.4	1.0 ± 0.1	1.5 ± 0.1		0.6 ± 0.0	0.7 ± 0.0		
Subfamily Apocynoides, tribe Mesechiteae																
Mandevilla laxa	B + T	11.3 ± 0.8	14.6 ± 0.3			4.5 ± 0.3	7.9 ± 0.7	0.2 ± 0.1	68.2 ± 0.9	3.6 ± 0.5	0.7 ± 0.0	0.2 ± 0.2				
Subfamily Apocynoideae, tribe Nerieae																
Adenium obesum (desert rose)	B + T	35.6 ± 1.3	13.0 ± 0.6			17.3 ± 3.0	26.6 ± 4.3	0.3 ± 0.1	15.0 ± 1.4	0.2 ± 0.0	1.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0		25.5 ± 4.5
Nerium oleander	B + T	17.7 ± 2.9[a]	10.4 ± 0.6			5.0 ± 0.5	18.9 ± 1.1	0.7 ± 0.0	53.9 ± 0.7	1.0 ± 0.1	0.5 ± 0.1	0.3 ± 0.1				9.2 ± 1.0
Strophanthus speciosus	SSB	ND	12.4 ± 0.2	0.1 ± 0.0		6.7 ± 0.2	14.1 ± 2.2	0.6 ± 0.0	58.7 ± 1.8	0.4 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.8 ± 0.1	4.8 ± 0.1
Subfamily Apocynoideae, tribe Wrighteae																
Wrightia natalensis	B + T	25.9 ± 2.4	9.3 ± 0.6			2.4 ± 0.1	9.7 ± 1.6	1.0 ± 0.1	14.6 ± 0.5	2.0 ± 0.3	0.4 ± 0.0		0.3 ± 0.0	0.3 ± 0.0		60.0 ± 1.4
Subfamily Asclepiadoideae, tribe Asclepiadeae																
Asclepias incarnata (swamp milkweed)	SS	15.0 ± 1.9	6.0 ± 0.2	11.0 ± 0.4	1.4 ± 0.0	2.2 ± 0.1	16.0 ± 0.2	16.5 ± 0.7	43.9 ± 0.8	1.9 ± 0.2	0.5 ± 0.0	0.6 ± 0.0[c]				

Table 1 continued

Plant species	Source of seed	Oil content (%)	Fatty acid (%)													
			16:0	16:1 $^{\Delta 9}$	16:2$^{\Delta 9,12}$	18:0	18:1$^{\Delta 9}$	18:1$^{\Delta 11}$	18:2$^{\Delta 9,12}$	18:3$^{\Delta 9,12,15}$	20:0	20:1$^{\Delta 11}$	22:0	24:0	26:0	IR
Asclepias tuberosa (butterfly-flower)	B + T	27.1 ± 1.4	8.3 ± 0.4	12.3 ± 0.7	1.9 ± 0.2	2.7 ± 0.2	14.8 ± 1.9	21.5 ± 0.5	35.8 ± 2.3	1.5 ± 0.1	0.4 ± 0.0	0.7 ± 0.1[c]				
Subfamily Asclepiadoideae, tribe Ceropegieae																
Stapelia gigantea	B + T	27.6 ± 2.2	22.4 ± 0.7	0.8 ± 0.1		6.6 ± 0.3	20.2 ± 0.4	2.6 ± 0.2	43.2 ± 0.8	0.2 ± 0.0	1.4 ± 0.1	0.3 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.9 ± 0.2	
Subfamily Rauvolfioideae, tribe Plumerieae																
Thevetia peruviana (yellow oleander)[b]	B + T	43.2 ± 2.3	22.7 ± 1.3	0.2 ± 0.0		6.4 ± 0.2	41.7 ± 5.5	0.6 ± 0.1	25.8 ± 6.3		1.4 ± 0.2	0.2 ± 0.0	0.7 ± 0.0	0.2 ± 0.0		
Subfamily Rauvolfioideae, tribe Tabermontantaneae																
Tabernaemontana ventricosa (forest toad tree)	SSB	ND	15.5 ± 0.7	0.1 ± 0.1		7.7 ± 0.2	60.1 ± 2.3	0.8 ± 0.0	14.9 ± 1.6	0.2 ± 0.0	0.7 ± 0.0					
Subfamily Rauvolfioideae, tribe Vinceae																
Catharanthus roseus	B + T	32.8 ± 0.8	14.1 ± 0.2	0.1 ± 0.0		8.4 ± 0.3	63.6 ± 0.2	0.6 ± 0.0	11.9 ± 0.4	0.4 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0			
Rauvolfia serpentina (serpent wood)[b]	RH	40.0 ± 6.5	18.5 ± 0.5	0.1 ± 0.0		6.3 ± 0.6	323.3 ± 0.6	0.6 ± 0.1	40.9 ± 0.4	0.20.0	0.7 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.1		

[a] Not accounting for acetate

[b] Woody pericarp removed

[c] Mostly 20:1$^{\Delta 13}$

ND = not determined

1H-MAS-NMR

Data were collected using an 8.46 T (360 MHz ^{1}H frequency) Bruker Avance NMR spectrometer. To analyze intact seeds of *N. oleander*, seed hairs were pulled off and seed samples (approx. 20 mg) were packed with glass beads into the rotor of a 7-mm outer diameter double-resonance magic-angle spinning probe. Samples were spun at a rate of 3.0 kHz with an acquisition time of 204.8 ms and temperature of 301 K.

Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

Seed lipids were examined by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in positive ion mode as described previously [17], using 2,5-dihydroxybenzoic acid (DHB) prepared in the presence of 20 mM NaCl as matrix to ensure that sodiated adducts ($[M + Na]^{+}$) were the predominant ions.

Results and Discussion

Seed Acyl Composition

Species chosen for this work were selected by availability of seed from reputable seed companies, with species representing ten tribes from three of the five subfamilies of the Apocynaceae [18, 19]. Seed acyl composition has been previously reported for some of the species in this study, but in many cases reports give conflicting data. For example, the seed oil fatty acid database ([20], www.sofa.mri.bund.de) lists 5 references reporting the acyl composition of seed oil from *Holarrhena antidysenterica*, with no concensus on composition for this species. For this reason, we chose to conduct our own analysis, rather than relying on reports in the literature.

The majority of species contained only common fatty acids as acyl components of their seed oil (Table 1). The HFA IR was exclusively observed in members of the subfamily Apocynoideae, tribe Wrightieae, being present in *Nerium oleander*, *Wrightia natalensis*, *Strophanthus speciosus* and *Adenium obesum*. The identity of IR was confirmed for each of these species by GC–MS of FAMEs and of TMS-FAMEs. Mass spectra of the TMS-IR-FAMEs (not shown) matched those reported previously for IR [13, 21], with prominent fragment ions at *m/z* 227 and *m/z* 259 arising from α-cleavage at either side of the OTMS group on carbon 9. The position of the double bond between C12 and C13 has previously been confirmed for IR from *N. oleander* by oxidative cleavage [8]. It has also been suggested that the ion at *m/z* 124 seen in the mass spectrum of TMS-IR-FAMES corresponds to ionized 1,3 nonadiene, a fragment from the methyl end of IR resulting from cleavage α- to the OTMS group, placing the double bond between carbons 12 and 13 [22]. The acyl composition of seeds of *W. natalensis* and *S. speciosus* (syn. *S. capensis*) have not been reported previously, and to the best of our knowledge this is also the first record of the occurrence of IR in the genus *Adenium*.

In a previous study, 9-oxo-*cis*-12-octadecenoic acid accompanied IR as an acyl component of the seed oil from members of the genus *Plantago* [12, 13]. This oxo-fatty acid was not abundant in the Apocynaceae species and was only observed as a minor component (<0.1 %) of the seed lipids of *W. natalensis*. No evidence of 9-hydroxy-octadecanoic acid (9-OH 18:0) was found in the oil from the mature seeds of any species examined. This saturated fatty acid has been implicated as a precursor in the biosynthesis of IR in *Wrightia* species [9] and may be restricted to developing seed, material which we did not have available for analysis.

HFAs were not observed in the seed oil of the two *Holarrhena* samples, or in any other members of the subfamily Apocynoideae, tribe Malouetieae, that we examined. These results suggest that the previous high level of IR reported from *H. antidysenterica* [8] may be a result of misidentification of species, as suggested earlier [23]. The seeds of *H. antidysenterica* and those of *Wrightia* species are similar in appearance. Although *H. antidysenterica* is considered a synonym for *H. pubescens* [24], the acyl composition of the seeds in our study was very different, with oil from *H. antidysenterica* showing a higher degree of unsaturation with a linolenic acid ($18{:}3^{\Delta 9,12,15}$) content of 38 % compared to 19 % for *H. pubescens*. Whether this is due to environmental effects, seed maturity, varietal differences or errors in species identification is unknown. Seeds from both samples had similar oil content at 22–23 %. Seed kernels from *Annona squamosa* (sugar apple) have previously been reported to be a source of IR at close to 10 % of total seed fatty acids [11]. More recent studies have failed to identify IR in this species with oleic ($18{:}1^{\Delta 9}$, 40 %) and linoleic ($18{:}2^{\Delta 9,12}$, 29 %) as the dominant acyl components and an oil content of 24 % [25]. In our analysis of seed kernels from *A. squamata* and the closely related *A. cherimola*, no evidence of IR was observed with oil content and acyl composition of *A. squamata* matching that reported by Hotti and Hebbal [25]. Seed lipids from the two *Pachypodium* species examined were high in saturated fatty acids at 40 % for *P. lamerei* and 42 % for *P. rosulatum*. In both species, the predominant saturated fatty acid was palmitic acid at 26 and 29 %, respectively.

Seeds from *Asclepias syriaca* (common milkweed) in the family Apocynaceae are known to contain the unusual

n-7 monounsaturated fatty acids (where *n* = the location of the double bond relative to the methyl carbon) palmitoleic (hexadeca-*cis*-9-enoic acid) and vaccenic acid (octadeca-*cis*-11-enoic acid) as components of seed triacylglycerol [26, 27]. We observed these fatty acids in two additional species, *A. incarnata* and *A. tuberosa*, with *A. tuberosa* oil containing nearly 36 % *n-7* fatty acids in total. We did not detect IR or any other oxygenated fatty acids in the oil from these species.

Characterization of Oils by MALDI-TOF MS and MALDI-TOF MS/MS

Previous structural studies of the seed oil of *N. indicum* reported the presence of the estolide 9-acetoxy-*cis*-12-octadecenoic acid (AcIR) esterified to one α-carbon of glycerol [8]. As the study was conducted using a combination of TLC, GC/MS and digestion with pancreatic lipase, we chose to examine the oil from *N. oleander* in greater detail using ^{1}H magic angle spinning NMR (^{1}H MAS-NMR). Due to the limited amount of seed material available, we applied the technique to intact seeds, packed into the rotor with glass beads, a technique applied previously to identify TAG-estolides in intact sclerotia of the fungus *Claviceps purpurea* and intact seeds of *Lesquerella* species [28]. The NMR spectrum (Fig. 1) showed a resonance pattern typical for an oilseed [28] and revealed signals at 3.570 and 4.900 ppm, close to the characteristic chemical shifts reported previously for the C12 proton of ricinoleate (–***CH****OH*– at 3.554 ppm) and the C12 proton of ricinoleic-estolides (–***CH****OR*– at 4.856 ppm) [28]. As the NMR data strongly suggested the presence of TAG-estolides and isoricinoleic acyl groups with no secondary acylation, we examined oil extracted from *N. oleander* by MALDI-TOF mass spectrometry to obtain additional structural information. In parallel, we also analyzed the oil from the previously uncharacterized species *A. obesum*. MALDI-TOF MS, coupled with GC to determine acyl composition, is a well-established technique for the structural characterization of plant oils and enables the rapid identification and characterization of unusual components such as TAG-estolides without the need for upstream separation [28, 29].

MALDI-TOF mass spectra obtained from oil from the two species are shown in Fig. 2. Due to the inclusion of NaCl in the matrix, lipids are detected as sodium adducts, with *m/z* values corresponding to monoisotopic mass $[M+Na]^{+}$. For *Nerium* oil, the dominant molecular ions were grouped in the range 879.7–1039.7 *m/z* (Fig. 2a). As seed acyl composition was primarily 18-carbon (C18) and 16-carbon (C16) fatty acids (Table 1), the mass distribution suggested the presence of lipids with structure more complex than TAG. MALDI-TOF MS/MS spectra were collected for the most intense ion from

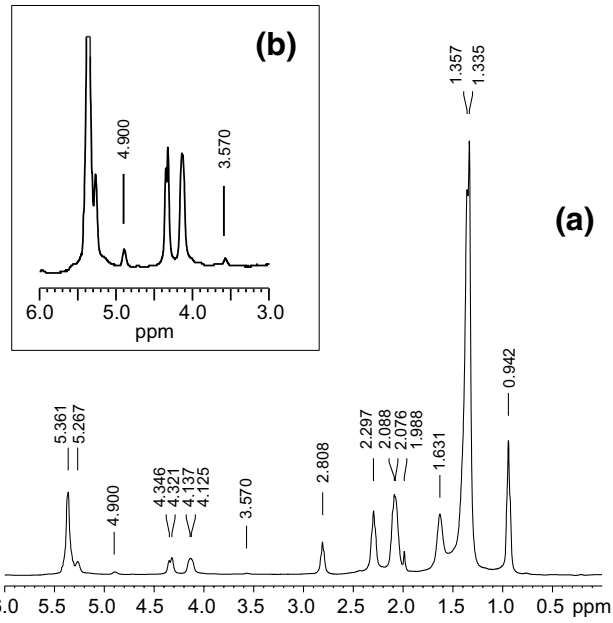

Fig. 1 ^{1}H MAS-NMR spectrum **a** obtained from intact seed of *N. oleander*, **b** enlargement of spectrum in the range of 3.0–6.0 ppm

each group to determine the acyl composition and structure of these lipid molecular species. Representative spectra are shown in Fig. 3. Spectra showed fragmentation demonstrating the presence of TAGs containing IR moieties and confirmed the presence of AcIR. For example, 5 fragment ions were seen in the MS/MS spectrum from the 919.7 *m/z* ion of *Nerium* oil (Fig. 3a), enabling identification of this ion as the sodium adduct of 54:5:1 (L+L+IR). The fragment ions corresponded to loss of linoleic acid $[M+Na\text{-}\underline{L}COOH]^{+}$ at 639.5 *m/z*, with a low abundance ion corresponding to the loss of sodium salt of linoleic acid $[M+Na\text{-}\underline{L}COONa]^{+}$ at 617.5 *m/z*, or loss of IR $[M+Na\text{-}\underline{IR}COOH]^{+}$ at 621.5 *m/z*, or the sodium salt of IR $[M+Na\text{-}\underline{IR}COONa]^{+}$ at 599.5 *m/z*, accompanied by an ion corresponding to sodiated IR $[IR+Na]^{+}$ at 321.2 *m/z*. Molecular species containing AcIR were clearly identified by two diagnostic ions in the MS/MS spectra (Fig. 3b, c). These corresponded to the sodiated AcIR estolide $[\underline{AcIR}COOH+Na]^{+}$ at 363.3 *m/z* and an $[M+Na\text{-}60]^{+}$ ion resulting from loss of acetic acid (Ac) from the estolide moiety ($[M+Na\text{-}CH_3COOH]^{+}$) . The AcIR estolide was the sole estolide observed indicating that secondary acylation of IR with longer chain fatty acids does not occur in *N. oleander*. The MALDI-TOF MS data indicate that the oil from *N. oleander* seeds is more complex than previously reported, with penta-acyl TAG, containing 2 AcIR estolides, being reported for the first time (Fig. 3c). TAG molecules containing both AcIR and IR were also observed (Fig. 3c). Identities of the most intense ions in the MS spectra from the *Nerium* oil

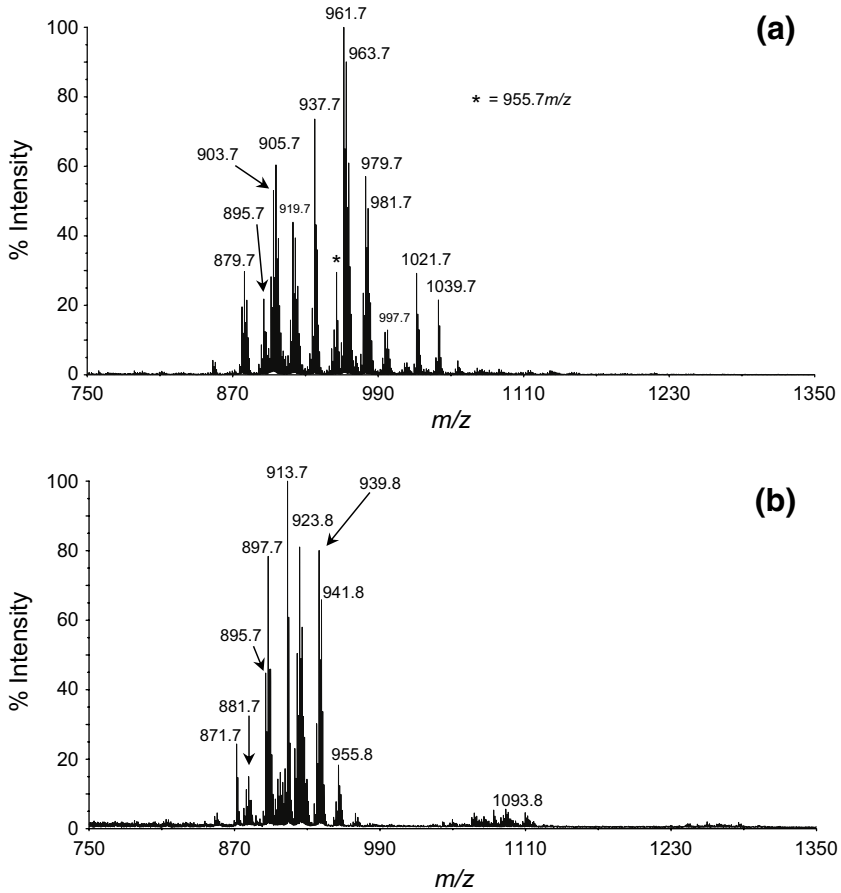

Fig. 2 MALDI-TOF MS spectra of seed lipids from *N. oleander* (**a**) and *A. obesum* (**b**). For each group of signals, only the ions of highest intensity are labeled

sample, as determined from the MS/MS data, are given in Table 2. Adjacent ions differ by the presence or absence of one or more double bonds. Without appropriate standards, the information should be considered qualitative, as the relative signal intensity of TAG molecular species containing estolides, free hydroxyl groups or only common fatty acids is not known. The MALDI-TOF MS/MS technique used in this study did not provide sufficient data to allow the determination of stereospecific position of IR or AcIR within the seed TAG. In this study, the predominant $[FA+Na]^+$ ion observed in the MS/MS spectra of TAG species containing IR was $[IR+Na]^+$ as shown in Fig. 3a, b. Ions corresponding to sodiated common fatty acids with no hydroxyl group, such as linoleic acid $[\underline{L}COOH+Na]^+$ were not obvious. The reason for this observation is unclear perhaps resulting from ion suppression or preferential sodiation of the more polar HFAs.

In the mass spectrum from *A. obesum* oil (Fig. 2b), molecular ions were clustered in the range 870–960 *m/z*, suggesting the presence of TAG molecular species containing HFAs, as seen previously with seed oils from castor bean and *Plantago* species [13, 28]. MS/MS spectra confirmed that IR is a component of seed triacylglycerol and enabled identification of the highest intensity ions (Table 2). No evidence of secondary acylation of IR was observed either with acetate or other fatty acyl groups. The broad cluster of low intensity ions centered at 1093.8 (Fig. 2a) could not be identified. Although *Nerium* and *Adenium* are the only 2 genera in the subtribe Neriinae, within the tribe Nerieae [19], the oils of *N. oleander* and *A. obesum* are clearly different in structure. MALDI-TOF MS spectra obtained for oil from *W. natalensis* and *S. speciosus,* the other 2 species in the study accumulating IR, indicated that these oils were composed of triacylglycerol, with no TAG-estolide present (data not shown).

The pathway of IR biosynthesis in plants remains to be determined. Biochemical studies conducted using developing seeds of *Wrightia* species demonstrated conversion of radiolabeled linoleic acid and oleic acid to IR and 9-OH 18:0, respectively, under both aerobic and anaerobic conditions. These results suggested a mechanism involving hydration of the Δ9 double bond, with linoleic acid as the preferred substrate [30]. Linoleic acid is an abundant component of the seed oil of all the species we examined. The actual lipid substrate for IR biosynthesis is unclear, although IR in *Wrightia* is primarily associated with TAG, not phospholipids [9], TAG is not

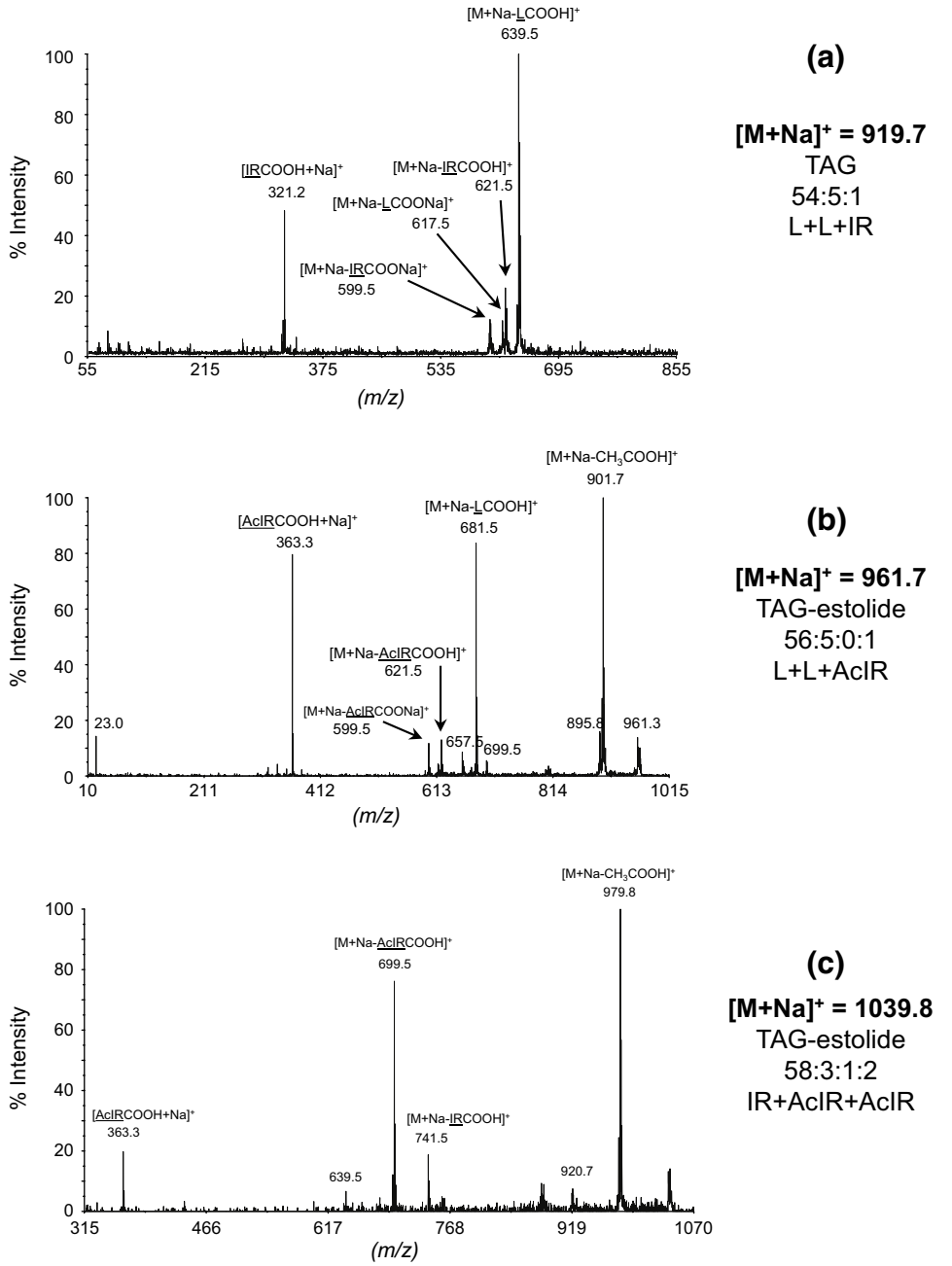

Fig. 3 MALDI-TOF MS/MS spectra of representative TAG and TAG-estolides from *N. oleander*. Diagnostic fragment ions are labeled, **a** TAG containing one HFA (919.7 *m/z* precursor ion), **b** TAG-estolide containing one AcIR (961.7 *m/z* precursor ion), **c** TAG-estolide containing IR and 2 AcIR estolides (1039.8 *m/z* precursor ion)

necessarily the site of synthesis. The castor plant (*Ricinus communis*), for example, synthesizes the HFA ricinoleic acid by desaturation of oleate esterified to the membrane lipid phosphatidylcholine (PC). Efficient removal of the newly formed fatty acid for incorporation into neutral lipids results in low steady state levels of HFA in membrane phospholipids during seed development [31]. Similarly, studies conducted on the TAG-estolide rich exudates of *Petunia hybrida* stigmas identified a cytochrome P450 fatty acyl ω-hydroxylase (CYP86A22) acting on acyl-CoA substrates as an enzyme necessary for the synthesis of the estolide HFA moiety [32]. Further work is required to determine whether esterification of the secondary acyl group of an estolide occurs subsequent to esterification of an HFA to the glycerol backbone of a TAG-estolide, or if the estolide is formed first. Fatty acid estolides not esterified to glycerol have not been reported from seed oils, but have been observed in *Nicotiana tabacum* stigma exudate [33], and are often a major component of the epicuticular waxes of conifers [34]. A genomic approach involving gene discovery in an estolide rich species and validation of encoded enzymatic activity is likely to be the most effective way to elucidate the pathway of seed TAG-estolide biosynthesis.

Table 2 Major TAG molecular species in the seed oil of *Nerium oleander* and *Adenium obesum* as determined by MALDI-TOF MS/MS

Source of seed oil	Mass *m/z* $(M+Na)^+$	TAG class[a]	Acyl composition of dominant TAG species[b]
N. oleander	879.7	52:3	P+O+L
	895.7	52:3:1	P+L+IR
	905.7	54:4	O+O+L
	919.7	54:5:1	L+L+IR
	937.7	54:3:0:1	P+L+AcIR
	955.7	54:2:1:1	P+IR+AcIR
	961.7	56:5:0:1	L+L+AcIR
	979.7	56:4:1:1	L+IR+AcIR
	997.7	56:3:2:1	IR+IR+AcIR
	1021.7	58:4:0:2	L+AcIR+AcIR
	1039.7	58:3:1:2	IR+AcIR+AcIR
A. obesum	871.1	50:1:1	P+P+IR
	881.7	52:2	P+O+O
	897.7	52:2:1	P+O+IR
	913.7	52:2:2	P+IR+IR
	923.8	54:3:1	O+O+IR
	939.8	54:3:2	O+IR+IR
	955.8	54:3:3	IR+IR+IR

P palmitic acid (16:0), *O* oleic acid ($18:1^{\Delta 9}$), *L* linoleic acid ($18:2^{\Delta 9,12}$), *FA* fatty acid

[a] Nomenclature is given in the Abbreviations section

[b] Based on $[M+Na\text{-}FA]^+$, $[FA+Na]^+$ and $[estolide+FA]^+$ ions in MS/MS spectra. Positional isomers cannot be distinguished

Conclusion

Although far from a comprehensive study of the seed lipid composition of the Apocynaceae, this work suggests that IR may be predominantly found in the tribes Wrightieae and Nerieae of the subfamily Apocynoideae, and that it is not present in the seed of *Holarrhena* species. *N. oleander* was the only species examined that produced an oil with secondary acylation (acetylation) of IR. MALDI-TOF mass spectrometry proved to be an effective tool for the identification of the estolide AcIR in this oil with diagnostic ions corresponding to the sodiated estolide $[\underline{AcIR}COOH+Na]^+$ and loss of acetate $[M+Na\text{-}CH_3COOH]^+$ being observed.

As a source of IR enriched oils from the Apocynaceae, *Wrightia* species appear to be the most promising. *W. tinctoria* seed fiber has been shown to have potential in the manufacture of woven and non-woven textiles, and as source of fiber for bio-composites [35].

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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