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Non-Saponifiable Lipid Composition of Four Salt-Secretor and Non-Secretor Mangrove Species from North Sumatra, Indonesia

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Abstract

Non-saponifiable lipid (NSL) of the fresh leaves and roots from two salt-secretor mangrove species, namely *Aegiceras corniculatum* (L.) Blanco and *Avicennia alba* Bl. and two non-secretor mangroves, i.e. *Acrostichum aureum* L. and *Excoecaria agallocha* L. was analyzed with special emphasize to triterpenoids and phytosterols. Identification of the triterpenoids and phytosterols was confirmed by comparison of their retention time on the GC column with those of authentic standards and on the interpretation of GC-MS spectra. Triterpenoids and phytosterols comprised the major proportion of NSL. The triterpenoids and phytosterols mainly consisted of 7 and 4 compounds. Triterpenoids were the largest constituent of *Ac. aureum* and *Ae. corniculatum* leaves and roots, and *E. agallocha* roots. In contrast to these triterpenoids-rich species, phytosterols were relatively dominant in the roots of *Av. alba*. The species of *Av. alba* and *E. agallocha* in the leaves were distinguished from the others in that both species contained a larger quantity of phytol. *Ae. corniculatum* contained a large amount of betulin and α-amyrin in the roots, as well as lupeol in the roots of *Av. alba*. The diversity in the NSL composition noted with mangrove species in both the leaves and roots suggested that NSL of mangrove leaves and roots can be used as chemotaxonomical character to differentiate species.

Abstrak

Komposisi Lipid Tak Tersabunkan dari Empat Spesies Mangrove Sekresi dan Non-Sekresi, Sumatera Utara, Indonesia. Senyawa lipid tak tersabunkan (*non-saponifiable lipid*/NSL) dari daun dan akar dua spesies mangrove sekresi, yaitu *Aegiceras corniculatum* (L.) Blanco dan *Avicennia alba* Bl, dua spesies mangrove non-sekresi, yaitu *Acrostichum aureum* L. dan *Excoecaria agallocha* L dianalisis dengan penekanan khusus pada triterpenoid dan fitosterol. Identifikasi triterpenoid dan fitosterol dikonfirmasi dengan perbandingan waktu retensi pada kolom GC dengan standar otentik dan interpretasi spectrum GC-MS. Triterpenoid dan fitosterol merupakan proporsi utama NSL. Triterpenoid dan fitosterol masing-masing terdiri dari 7 dan 4 senyawa. Triterpenoid merupakan konstituen terbesar dari spesies *Ac. aureum* dan *Ae. corniculatum* di jaringan daun dan akar, dan di akar spesies *E. agallocha*. Berbeda dengan spesies tersebut yang kaya kandungan triterpenoid, senyawa fitosterol relatif dominan dalam akar *Av. alba*. Spesies *Av. alba* dan *E. agallocha* di daun dibedakan dari spesies yang lain bahwa kedua spesies tersebut mengandung jumlah yang lebih besar dari senyawa fitol. Spesies *Ae. corniculatum* mengandung sejumlah besar konten betulin dan α-amyrin di akar, serta lupeol di akar spesies *Av. alba*. Keragaman dalam komposisi NSL tercatat dengan jenis mangrove untuk kedua jaringan daun dan akar, studi ini menyarankan bahwa komposisi NSL pada daun dan akar tumbuhan mangrove dapat digunakan sebagai karakter kemotaksonomi untuk membedakan spesies.

Keywords: leaf, mangrove, phytosterol, triterpenoid, root

1. Introduction

Mangrove plants are halophytes that are distributed in the intertidal zone of tropical or sub tropical regions. Mangrove plants have long been well known as a source of phytochemical compounds or biologically active compounds [1]. Because lipid comprises a significant proportion of carbon output from mangroves [2-3], knowledge of the lipid composition of mangrove promisingly contributes to estimating the sources and accumulation rates of sedimentary organic matter. Non-saponifiable lipid (NSL) basically denotes simple lipid

fraction except for fatty acid (saponifiable lipid) after alkaline hydrolysis of the total lipids, and contains sterols, long-chain alcohols and alkanes. In general, NSL represents a more stable lipid fraction than the saponifiable lipid fraction (fatty acids), and its resistance to microbial degradation has been considered to be a relatively important factor in controlling the diagenetic pathways [4-5].

Mangroves are also unique in that these plants have cellular mechanisms to tolerate high salinity [6]. Our previous studies shed some light on the triterpenoid content and gene expression of triterpenoid synthase in salt stressed and re-adapted mangrove plants [7-11]. These studies suggested another physiological significance of triterpenoids in mangrove trees. According to their morphological characteristics in salt management, mangrove plants fall into two major groups [12]. The first group is the salt-secretor species that have either salt glands or salt hairs to remove excess salt. *Aegiceras corniculatum* and *Avicennia alba* have salt glands in leaves and are salt secretors. The second is the non-secretor species, exemplified by *Acrostichum aureum* and *Excoecaria agallocha*, that do not have such morphological features for the excretion of excess salt [6,12]. *Ac. aureum, Ae. corniculatum, Av. alba* and *E. agallocha* are common mangrove species in North Sumatra, Indonesia and are considered to be representative of each group. It thus became important to analyze the triterpenoid compositions of mangroves. This study sheds light on the non-saponifiable lipid compositions of mangroves with special emphasize on the triterpenoid and phytosterol compositions.

2. Methods

Sample collection. Leaves and roots of four species of mangroves existing in Pulau Sembilan, Langkat, North Sumatra were collected in April 2012: *Acrostichum aureum* L., *Aegiceras corniculatum* (L.) Blanco, *Avicennia alba*, and *Excoecaria agallocha* L.

Isolation of lipid. The leaves (3-4 leaves) or roots (3 to 4 g wet weight, respectively) were first ground in liquid nitrogen, and extracted with 25 times volume of chloroform-methanol (2:1 by volume) (CM21). The cell wall debris insoluble to CM21 was removed by filtration through No. 2 filter paper (Advantec, Tokyo, Japan), and the resultant extract was partially purified for lipid analysis as described in our previous paper [13]. The extract was concentrated to dryness, and the lipid weight was measured gravimetrically. Total lipid content was expressed as tissue weight (mg/g tissue).

Analysis of non-saponifiable lipid (NSL). The lipid extract containing 1 mg of total lipid was concentrated to dryness with nitrogen stream, saponified at 90 ºC for 10 mins with 20% KOH in 50% ethanol. The NSL was extracted with hexane by vigorous mixing, and its weight was measured gravimetrically. The NSL was analyzed by gas chromatograph (GC 2014, Shimadzu, Kyoto, Japan) or gas chromatograph-mass spectrometer (GC-MS QP 2010, Shimadzu). An Rtx-1 (0.25 mm ID × 15 m, Restek, Bellefonte, PA, USA) column was used, and the column temperature was programmed as isothermal 300 ºC for 15 mins. The carrier gas was helium with a flow rate of 0.70 mL/min (20 cm/s), the temperatures for the injector and detector were 300 ºC and 300 ºC, respectively. NSL content was also expressed on the basis of fresh tissue weight (mg/g tissue) or total lipid weight (mg/mg total lipid).

Identification of chemical structure. The chemical structures of NSL were mainly identified by comparison of their retention time on the GC column with those of authentic standards, and by interpretation of the mass spectrum. Authentic standards of β-amyrin, α-amyrin, lupeol, lupenone and cycloartenol were purchased from Extrasynthese (Genay, France). β-Sitosterol, stigmasterol and campesterol were purchased from Tama Biochemical (Tokyo, Japan), and cholesterol from Wako Pure Chemical Industries (Osaka, Japan).

Ionization of the samples was by electron impact (EI) at 70 eV to estimate chemical structure, or by chemical ionization with methane as a reaction gas to determine molecular weight. The similarity search of the spectrum was carried out with the mass-spectrum library (NIST 147 and 27, Shimadzu). In the case where the authentic standards were commercially not available, the identification was essentially based on the similarity of the mass spectrum with that in the spectrum database.

3. Results and Discussion

Results. The chemical structures for triterpenoids identified in the mangrove leaves and roots (*Ac. aureum, Ae. corniculatum, Av. alba* and *E. agallocha*) are presented in Figure 1.

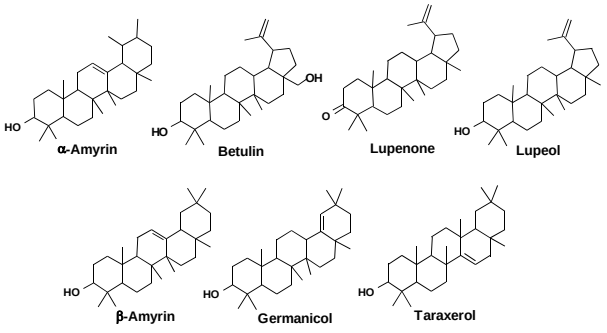

Figure 1. Chemical Structure of Triterpenoids Present in Mangrove Leaves and Roots. Identifications of Triterpenoids were based on GC and GC-MS Analysis as Described in the Methods

The triterpenoids largely fall into three types: lupanes (lupeol, lupenone, betulin); oleananes (β-amyrin, germanicol, taraxerol); an ursane (α-amyrin). α-amyrin, β-amyrin, lupeol, lupenone and betulin were identified by comparison of their retention time on the GC column and mass spectra with those of authentic standards. Identification of chemical structures for germanicol and taraxerol was verified by comparing their retention times and MS spectra with those in our previous report [14].

Figure 2 illustrated the chemical structures of phytosterol present in the mangroves leaves and roots. Campesterol, stigmasterol, β-sitosterol and cycloartenol were identified by use of authentic references as described above; comparison of retention time on GC analysis and mass spectra.

Table 1 shows the total and NSL content of four species of mangrove leaves. Total lipid content of the mangrove leaves ranged from 1.8 to 7.6 mg/g tissues with an average of 5.5 mg/g. The average was almost similar and half of those reported for Okinawan mangroves, 6.0 mg/g, mangroves of India, 11.9 mg/g, and [13,15]. NSL content in the leaves was the lowest for *Ac. aureum*, and the highest for *Ae. corniculatum*. NSL comprised 3 to 17% of total lipids, and the average for four species was 10%. The lipid content of mangrove roots was lower than mangrove leaves with an average of 1.9 mg/g (Table 1). *E. agallocha* had the lowest lipid content in the roots. NSL content in the roots was the lowest for *Ae. corniculatum*, and the highest for *E. agallocha*. NSL in the roots comprised 4 to 26% of total lipids, and the average for four species was 10%.

Table 2 summarized the NSL composition of four mangrove leaves. A variation of compounds was prominent in the composition of NSL. Triterpenoids were the largest constituent of *Ac. aureum* and *Ae. corniculatum*. The leaf species of *Av. alba* and *E. agallocha* were distinguished from the others in that both species contained a larger quantity of phytol. Phytol was probably derived from chlorophyll that was present in the NSL fractions of three species. Phytol is widely distributed as a constituent of chlorophyll in a number of green plants [16-17]. Squalene, an intermediate in isoprenoid biosynthesis, was also detectable with substantial quantities in all species.

The NSL composition of mangrove roots was displayed in Table 3. Diversity within the species was also notable in the NSL composition of root. Likewise, in the *Ac. aureum* and *Ae. corniculatum* leaves, phytosterol and triterpenoids comprised the largest proportion of NSL. It is interesting to note that four mangrove species were abundant in triterpenoid. *Ae. corniculatum* contained a large amount of betulin and α-amyrin in the roots, as well as lupeol in the roots of *Av. alba*. The root of *Av. alba* showed a higher concentration of β-sitosterol compared with other species.

Discussion. The present study described the NSL composition of four salt secretors and non-secretors of North Sumatran mangrove leaves and roots for the first time. Special reference was to the triterpenoid compositions because these compounds were implicated in functioning as a self-protecting agent against salt stress [7-11]. The diversity was noted in the compositions of triterpenoids depending on the mangrove

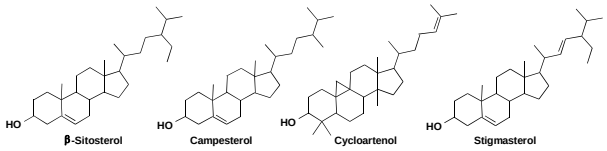

Figure 2. Chemical Structure of Phytosterols Present in Mangrove Leaves and Roots. Identifications of the Chemical Structures were based on GC and GC-MS Analysis as Described in the Methods

Table 1. Total Lipid and NSL Contents of Four Species of Mangrove Leaves and Roots

Species	Tissue	Total lipid/tissue (mg/g)	NSL/tissue (mg/g)	NSL/Total lipid (mg/mg)
Non-secretor species				
Ac. aureum	Leaves	7.58	0.22	0.03
	Roots	1.54	0.12	0.26
E. agalloca	Leaves	1.76	0.29	0.17
	Roots	1.20	0.21	0.18
Secretor species				
Ae. corniculatum	Leaves	7.33	0.37	0.05
	Roots	1.90	0.07	0.04
Av. alba	Leaves	5.18	0.27	0.05
	Roots	2.78	0.14	0.05

Table 2. Nonsaponifiable Lipid Composition (%) of Four Mangrove Leaves

Lipid component	Non-secretor species		Secretor species	
	Ac. aureum	*E. agallocha*	*Av. alba*	*Ae. corniculatum*
(Phytosterols)	(34.1)	(10.8)	(6.0)	(34.0)
Campesterol	3.6 ± 1.7		4.0 ± 0.0	13.9 ± 2.8
Stigmasterol	9.4 ± 3.4	2.2 ± 1.4	2.0 ± 0.0	8.2 ± 2.1
β-Sitosterol	16.1 ± 5.2	2.7 ± 0.5		7.1 ± 3.2
Cycloartenol	5.0 ± 0.0	5.9 ± 3.1		4.8 ± 0.0
(Triterpenoids)	(40.5)	(8.4)	(14.1)	(52.6)
Taraxerol	4 ± 3.1			5.4 ± 1.4
β-Amyrin	11.3 ± 5.7		2.1 ± 0.2	10.2 ± 2.7
Germanicol	7.3 ± 0.7			5.8 ± 0.0
Lupenone	9 ± 0.8			7.2 ± 5.2
Betulin				4.6 ± 4.8
Lupeol	3.7 ± 0.1	8.4 ± 4.4	5.7 ± 1.5	10.5 ± 4.8
α-Amyrin	5.2 ± 0.0		6.3 ± 3.1	8.9 ± 3.1
Cholesterol	5.1 ± 1.5		3.3 ± 0.8	7.2 ± 2.2
Phytol	16.5 ± 0.0	74.5 ± 6.7	71.4 ± 4.9	
Squalene	3.9 ± 1.5	3.6 ± 1.7	5.2 ± 1.2	6.2 ± 0.5

Data are mean of triplicate analyzes ± SE

Table 3. Nonsaponifiable Lipid Composition (%) of Four Mangrove Roots

Lipid component	Non-secretor species		Secretor species	
	Ac. aureum	*E. agallocha*	*Av. alba*	*Ae. corniculatum*
(Phytosterols)	(38.8)	(37.0)	(51.3)	(38.8)
Campesterol	11.6 ± 1.8	11.7 ± 2.0	8.3 ± 3.4	12.1 ± 3.1
Stigmasterol	11.6 ± 0.0	13.2 ± 3.4	15.2 ± 5.8	15.2 ± 3.1
β-Sitosterol	11.5 ± 0.0	6.8 ± 2.4	27.8 ± 0.6	11.5 ± 2.4
Cycloartenol	4.2 ± 0.5	5.3 ± 1.6		
(Triterpenoids)	(57.9)	(60.9)	(46.3)	(53.1)
Taraxerol	4.0 ± 0.0	12.5 ± 2.9		
β-Amyrin	9.2 ± 2.1	10.7 ± 1.1	6.8 ± 0.5	
Germanicol	8.3 ± 2.9	9.6 ± 2.5	6.9 ± 1.9	
Lupenone	4.0 ± 0.0	7.2 ± 2.4		
Betulin	3.8 ± 0.1	10.8 ± 1.9	10.1 ± 2.8	31.0 ± 2.7
Lupeol	14.7 ± 2.1	6.2 ± 1.6	22.5 ± 6.7	
α-Amyrin	13.9 ± 3.3	3.9 ± 1.3		22.1 ± 6.5
Squalene	3.3 ± 0.2	2.1 ± 0.6	2.5 ± 0.2	8.2 ± 1.5

Data are mean of triplicate analyzes ± SE

species (Tables 2 and 3). This observation was in agreement with the previous reports [2-3,13] the leaves of mangroves are chemotaxonomically distinguishable on the basis of their marker component.

To meet the variations in the chemical structure of triterpenoids as well as phytosterols, the biosynthetic pathway has been shown to be complicated and divergent as shown in Figure 3 [18-21]. The triterpenoids in the mangroves consisting of pentacyclic triterpenoids are biosynthesized via a common precursor 2,3-oxidesqualene, followed by cyclization and backbone rearrangement. The biosynthesis of phytosterol and pentacyclic triterpenoids share the

common pathway up to the branching point of 2,3-oxidesqualene. The cyclization step of 2,3-oxidesqualene was catalyzed by the enzyme oxidesqualene cyclases (OSCs) [18-19]. Several lines of studies have postulated that: 1) Distinct OSC exists for each type of triterpenoids [18,21]; 2) A common enzyme can produce multiple triterpenoids [22-24]. These studies revealed the presence of two types of OSCs in higher plants. One is monofunctional synthases yielding one specific product, as exemplified by β-amyrin synthase or lupeol synthase. The other is multifunctional synthases producing more than one product. In higher plants, OSC family members cycloartenol synthase and lanosterol synthase are responsible for phytosterol biosynthesis, and other OSCs are involved with triterpenoid synthesis.

With respect to North Sumatran mangroves, the dominating OSC in the mangrove tree appeared to vary with the species. This was almost true for all mangrove species, and suggested that the types of OSC in leaf might differ from that in the root even in the same species. Furthermore, this finding suggested that the triterpenoids in the root were biosynthesized in situ, not a translocation of the synthate from the leaves. The occurrence of squalene, the intermediate of isoprenoids biosynthesis, in the root may also support this view.

The greater diversity in NSL composition was in well agreement with the previous results [25-26]. Triterpenoids have been used as a chemotaxonomic marker to monitor the mangrove-derived organic matter because of their constancy during sedimentation and diagenesis [5,27-28]. Triterpenoids are also considered as being a precursor molecule such as α-amyrin, β-amyrin, and taraxerol for older and defunctionalised or aromatized compounds in marine sediments and mangrove ecosystems [29]. On the other hand, the influences of chemotaxonomic rationale were significantly enhanced by using a combination of several different compounds [3].

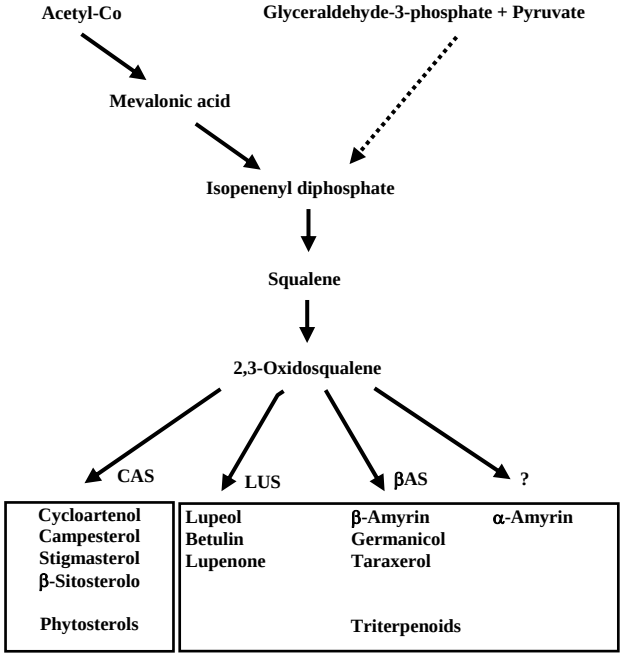

Figure 3. A Working-hypothesis for Triterpenoid and Phytosterol Biosynthesis in Mangrove Plants. CAS, Cycloartenol Synthase; LUS, Lupeol synthase; βAS, β-amyrin Synthase

The phytosterols have been considered to be the membrane components [30], biomarkers for marine and terrigenous organic matter [31-32], while the physiological significance for triterpenoids remains obscure. The higher concentration of triterpenoids in the outer tissue of the root may in part explain their protective role against external stresses including salinity, dryness, bacterial attack or herbivores [7,13]. Our previous studies have shown that salinity increased the triterpenoid concentration in the roots and leaves of salt-secretor and non-secretor mangrove species [7-11].

4. Conclusions

Based on the results, we propose that triterpenoids may function as a plasma membrane component and strengthen the membrane structure to limit the permeation of salt into the cells. This postulate represents a novel biological strategy of plants to confer salinity tolerance, and this scenario appears to merit further investigation. The present-day human-induced rapid environmental changes demand a better understanding of the dynamics of mangrove ecosystem. Thus, the diversity of triterpenoid in the NSL composition of mangrove leaves and roots can be used as chemotaxonomical character to differentiate species.

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