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Biosynthesis of 5-alkylresorcinol in rice: incorporation of a putative fatty acid unit in the 5-alkylresorcinol carbon chain

Yoshikatsu Suzuki,* Minoru Kurano, Yasuaki Esumi, Isamu Yamaguchi, and Yoshiharu Doi

RIKEN Institute (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

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Abstract

A previously unspecified "starter" unit in the predicted biosynthesis pathway of 5-alkylresorcinols has now identified as a fatty acid or its equivalent, using an efficient 5-alkylresorcinol production system of etiolated rice seedlings. Feeding saturated, odd-carbon fatty acid ester substrates from C11 to C19 specifically and markedly increased the amount of the corresponding 5-alkylresorcinol homologs with even-carbon chains that are shorter by one carbon than those of the supplied fatty acids. The amount of these homologs depended on substrate concentration. Some of the homologs whose amounts increased had linear carbon chains and the dodecyl homolog was shown to be 5-*n*-dodecylresorcinol. Moreover, the ^{13}C label in the dodecyl homolog that was biosynthesized from the [1-^{13}C]tridecanoate substrate was localized on the C-5 carbon of the resorcinol ring. These results obviously show that the fatty acid unit acts as a direct precursor and forms the side-chain moiety of 5-*n*-alkylresorcinol via the predicted biosynthesis pathway.

Keywords: 5-Alkylresorcinol biosynthesis; Odd-carbon fatty acid; Poly(ethylene glycol); Etiolated rice seedlings

* Corresponding author. Fax: +81-48-462-4667.
E-mail address: yoshi@postman.riken.go.jp (Y. Suzuki).

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1. Introduction

5-Alkylresorcinols (ARs) are found in many different living organisms, such as lower and higher plants, fungi, bacteria, and animals, and show multiple aspects of cellular biochemistry, membrane structure, and physiology [1]. In particular, ARs in cereal grains [2–4] and in their seedlings [5,6], and in mango fruit [7] play a role in the defense system as phytoanticipins against fungal pathogens. ARs are polyketide-derived products and their biosynthesis pathway, route A, is predicted based on 6-methylsalicylic acid biogenesis [8], as shown in Fig. 1. The Aldol-type condensation of malonyl-Coenzyme A (malonyl-CoA) to a pre-existing fatty acid

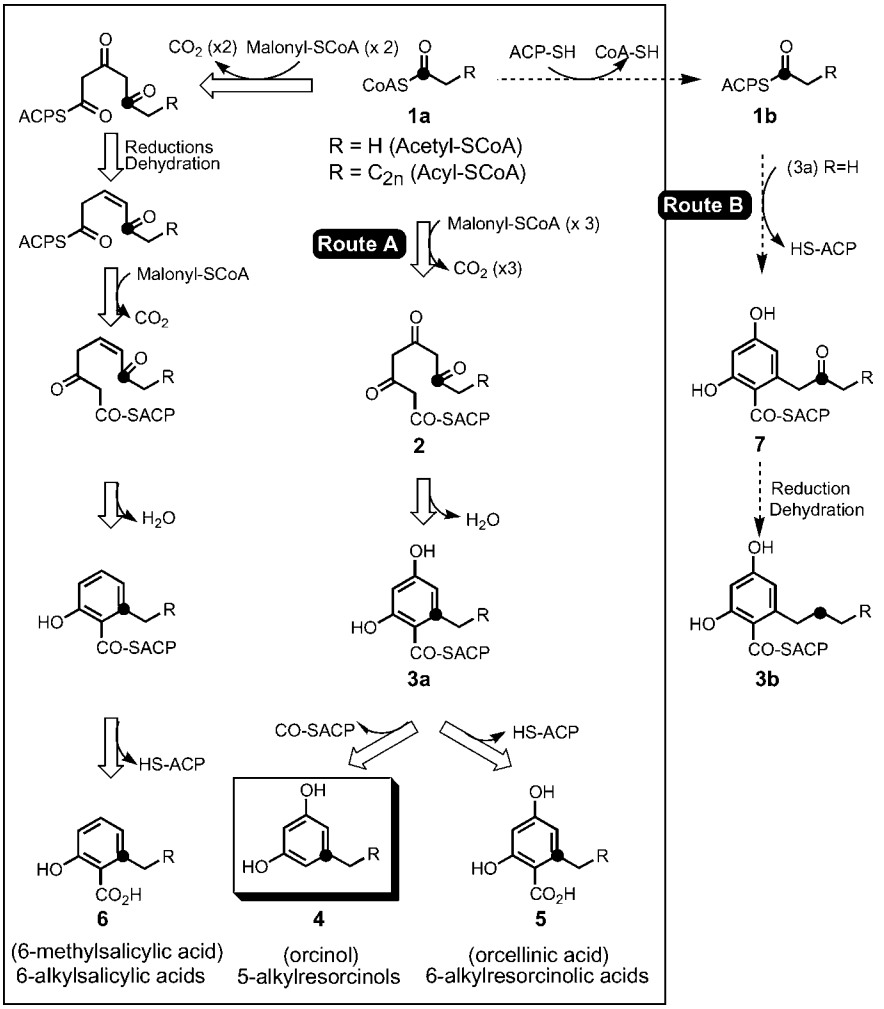

Fig. 1. Predicted biosynthesis pathways of 5-alkylresorcinol and related compounds [1].

unit **1**, the so-called "starter" unit, followed by second and third extensions of malonyl-CoAs to 3-oxoacyl-acyl carrier proteins (3-oxoacyl-ACPs), without the reductive removal of oxygen similarly to fatty acid biosynthesis, yields a polyoxomethylene intermediate **2** in which all the carbonyl oxygens are retained. The Claisen-type ring closure of the acyclic intermediate **2** yields 6-alkylresorcinolic acid **3a**. The concerted liberation of ACP and decarboxylation from **3a** yields 5-alkylresorcinol **4** with an odd-carbon-number chain. Simple ACP liberation from **3a** affords 6-alkylresorcinolic acid **5**. To date, the biosynthesis pathway for short-chain ARs and their reduced analogs (R = H), e.g., namely orcinol, orsellinic acid, and 6-methylsalicylic acid [9–12], has been established through labelling experiments using malonate and acetate where acetyl-CoA is the starter unit.

To our knowledge, however, there are few biosynthesis studies on ARs with a carbon chain longer than five carbons. Fate and Lynn [13] showed that labelled acetate was incorporated into the ring carbons of quinone-type xenognosin and its AR-type derivative indicating that they were derived from a polyketide biosynthesis pathway. However, no detectable incorporation of labelled acetate into their side chains was shown. An alternative hypothetical pathway, route B, has also been considered based on the non-incorporation of a label into their side chains [1]. In this pathway, orsellinic acid-ACP **3a** (R = H) might condense onto a pre-existing fatty acid unit **1b**, affording 6-(2′-oxoalkyl)resorcinolic acid **7**. The reduction of the 2′-oxo group in **7** to a methylene group affords 6-alkylresorcinolic acid **3b**. There is little biochemical precedence for this pathway because an activation mechanism of the methyl group in orsellinic acid-ACP required for attack on the activated fatty acid unit is unknown. Thus, there are two unresolved problems with respect to the 5-alkylresorcinol biosynthesis pathway. First, do pre-existing fatty acids function as starter units as predicted. Second, does malonyl-CoA (route A) or orsellinic acid-ACP **3a** (R = H) (route B) condense onto the starters if pre-existing fatty acids are the starter units. Non-incorporation of acetate into the side carbon chains of xenognosin and its AR-type derivative are presumably due to a dilution of the fatty acids derived from exogenous acetate by endogenous fatty acids. It may also be due to a difference between the organelle and rate of fatty acid biosynthesis and those of ARs formation from fatty acid starters. If such an assumption is correct, feeding odd-carbon fatty acids under higher concentrations may effectively elevate their ratio of incorporation in AR carbon chains because, in general, odd-carbon fatty acids are relatively lower in content than even-carbon fatty acids in plants. In such cases, the produced AR homologs should have even-carbon chains, and not odd-carbon chains.

We have previously developed an efficient AR production system that uses etiolated rice seedlings, which produces a series of AR homologs with odd-carbon side chains of C13, C15, and C17 [6]. In our recent survey of these rice seedlings, a series of even-carbon chain AR homologs as well as odd-carbon ones were detected, although all in trace amounts. Here, we establish the biosynthesis of long chain ARs using our AR production system and show that a fatty acid unit acts as a direct precursor and forms the carbon-chain moiety of ARs via the predicted AR biosynthesis pathway.

2. Results and discussion

2.1. Detection and tentative identification of minor AR homologs

Prior to an AR biosynthesis study, minor AR homologs were detected and identified. We focused on saturated carbon-chain AR homologs. One of the reasons for this is that saturated carbon chains are more amenable to structure identification than unsaturated carbon chains. The fatty acids required for feeding-substrate preparation are also more available for the saturated types.

In the GC-EI-MS analyses of the extracts obtained from the 8-day-old seedlings, we detected several AR homologs. Based on a common, intense fragment ion of m/z 268 and moderately intense molecular ions, five homologs with saturated, even-carbon chains of C10, C12, C14, C16, and C18 (AR10:0, AR12:0, AR14:0, AR16:0, and AR18:0), and three homologs with saturated, odd-carbon chains of C9, C11, and C19 (AR9:0, AR11:0, and AR19:0) were detected. Fig. 2A shows the GC-EI-MS/SIM chromatograms of the silylated rice seedling extracts, monitoring with a fragment ion at m/z 268. The hatched and black peaks represent the minor and major homologs, respectively. As shown in the control entry of Table 1, all the minor homologs were detected in extremely low amounts that ranged from about 0.05% (AR10:0) to 1.5% (AR14:0) of that of AR13:0, a major homolog. Whether or not those minor homologs have linear or branching carbon chains and alkylbenzene-1,3-diol moieties was not determined.

2.2. Feeding experiments of fatty acid ester substrates

Based on a possibility that a high endogenous fatty acid content in plants reduces the incorporation ratio of the supplied fatty acid substrates to ARs, we focused on even-carbon chain AR homologs that should be synthesized from odd-carbon fatty acids. A 6 mM higher substrate concentration was supplied except in the case of fatty acids that were severe germination and growth inhibitors. As substrates for supplying the fatty acids, we chose an ester conjugate of a *n*-fatty acid and monomethoxy poly(ethylene glycol) because it was not possible to prepare millimolar substrate solutions of the free fatty acids or their corresponding salts due to their low solubility. The free acids also caused both germination and growth inhibition, especially the short-chain fatty acids. Poly(ethylene glycol) is used for many synthetic surfactants, is relatively low in toxicity, and tends to be absorbed by living organisms.

AR homolog contents (ng/seedling) in the seedlings treated with ester substrates for 8 days were determined by GC-EI-MS/SIM and the results are shown in Table 1. Substrate concentrations between 0.2 and 0.6 mM were also used for the shorter-chain substrates, e.g., C10, C11, and C12, in order to reduce the germination and growth inhibition. Interestingly, all of the substrate feedings increased only the amounts of the AR homologs that had chain lengths shorter by one carbon than those of supplied fatty acid substrates. If such homologs have 5-*n*-alkylresorcinol structures, these results strongly suggest the direct incorporation of fatty acid units liberated from the substrates to the AR carbon chain moieties via route A, but not

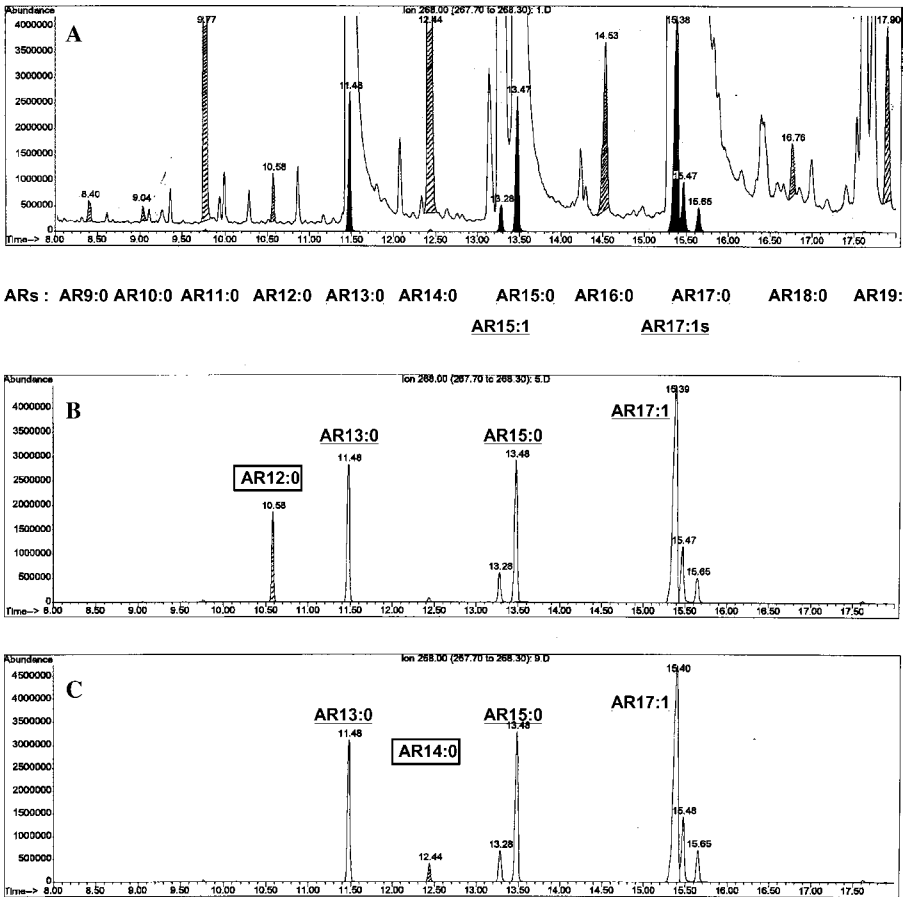

Fig. 2. GC-EI-MS/SIM chromatograms of the silylated rice seedling extracts, monitored with a fragment ion at m/z 268. (A) Extract of non-treated sample (control), and samples treated with 6 mM (B) C13 substrate and (C) C15 substrate for 8 days at 29 °C in darkness. In the chromatogram (A), black peaks show the known major homologs with an odd-number of side-chains of C13, 15, and 17, and hatched peaks show newly detected minor homologs with saturated side-chains of C9, C10, C11, C12, C14, C16, C18, and C19.

route B (Fig. 1). In route B, the AR homologs would have have carbon lengths longer by one carbon than the fatty acid chains of the supplied substrates. While the observed ratios varied, they were at least 3-fold higher than the control. Moreover, they depended on the substrate concentration between 0.2 and 6 mM.

In Table 1, the ratios for the AR10:0 and AR12:0 homologs were surprisingly high, about 100- and 4500-fold greater than the control, respectively. In particular, the level of AR12:0 (about 4500 ng/seedling) was comparable to that of the major homolog, AR13:0 (about 7700 ng/seedling) (Fig. 2B) using 6 mM of C13-substrate feedings. AR14:0 production also increased to a fairly high level (about 1200 ng/seedling) using 6 mM of C15-substrate feedings although its increased ratio was 10-fold higher than

Table 1
AR homolog content of seedlings treated with fatty acid ester substrates

Substrate (mM)	AR homolog content (ng/seedling)														
	Control	C10		C11		C12		C13		C15		C17		C19	
		0.2	0.6	0.2	0.6	0.6	2	2	6	2	6	2	6	2	6
AR 9:0	**1**	**3**	**14**	1	3	1	5	4	3	3	3	3	3	3	2
AR10:0	**0.4**	1	1	**5**	**39**	1	5	8	13	8	15	5	5	7	4
AR11:0	60	69	82	53	130	**192**	**310**	103	106	61	86	97	82	89	78
AR12:0	1	1	1	1	3	1	2	**934**	**4454**	3	9	3	2	2	2
AR13:0	7672	7387	8144	5776	11188	7312	8062	9348	7994	6992	9270	10246	8678	9303	8122
AR14:0	110	126	144	86	217	126	160	221	262	**301**	**1176**	223	181	191	184
AR15:1	1670	2110	2395	1329	3561	1903	1991	2430	1892	1840	2384	2656	2283	2711	2449
AR15:0	9065	8916	9335	6645	12737	8662	9781	11332	9790	8504	11601	12587	10569	11376	9779
AR16:0	10	15	16	10	27	15	16	28	29	17	34	**94**	**161**	24	20
AR17:1	19820	19158	20380	14957	25988	18677	21278	24819	21670	18932	25674	2648 6	22503	24071	20508
AR17:1	3730	4013	4528	2746	6139	3781	4128	5129	3911	3590	4904	5116	4343	5124	4336
AR17:0	1710	1837	1792	1157	2837	1699	1859	2457	1771	1445	2460	2602	1917	2433	1946
AR18:0	2	5	4	4	7	4	3	5	3	3	5	7	4	**10**	**9**
AR19:0	9	16	14	9	26	14	13	21	15	12	23	25	18	24	22
Total amount	43880	43656	46851	32780	62900	42388	47613	56840	51920	41710	57640	60150	50750	55370	47460

Data in boldface indicate products expected from route A (Fig. 1).

the control. Shorter-chain substrates, namely C10, C11, and C12, also gave high increased ratios despite the low substrate concentrations of 0.2–0.6 mM. Interestingly, the supply of an odd-chain fatty acid did not change the relative compositions of major, odd-carbon chain AR homologs, AR13:0, AR15:0, and AR17:1 (Fig. 2).

The effect of substrate supply on the production of the major homologs was also examined. Feeding the tetradecanoate substrate C14 under the same conditions described above efficiently increased AR13:0 production (Table 2). The increase was about 2000 ng/seedling and was almost the same order as that of AR12:0 (about 4500 ng/seedling, Table 1) although the ratio was only about 25% higher than the control (about 8000 ng/seedling). Therefore, the major homolog as well as the minor homologs were found to increase with an increase in substrate supply.

Table 3 shows the free fatty acid content of substrate-treated rice seedlings. In non-substrate supply (designated control), the amounts of odd-carbon fatty acids were relatively lower than those of even-carbon ones, as expected. Among the even-carbon fatty acids, FA16 was the most predominant. Three fatty acids, namely FA10, FA11, and FA19, were not detected. In the seedlings treated with the substrates, only free fatty acids that can be derived from the supplied substrates markedly increased and this increase depended on substrate concentration. These results showed that free fatty acids are released from the supplied substrates by hydrolysis. In addition, the amount of fatty acid decreased as the number of carbons decreased. The relationships between the amount of liberated fatty acid and carbon-chain length suggest that the liberated fatty acid content does not directly influence the corresponding homolog production. For example, AR12:0 production is extremely high

Table 2
AR homolog content of seedlings treated with C14 substrate

Substrate	AR homolog content (ng/seedling)			
	Control	SD[a]	C14[b]	SD
AR 9:0	0.5	0.1	1.6	0.4
AR10:0	0.3	0.1	0.5	0.1
AR11:0	66	2	131	17
AR12:0	1	0.4	28	1
AR13:0	**8085**	**326**	**10367**	**716**
AR14:0	107	5	143	5
AR15:1	1893	55	1794	76
AR15:0	9506	475	9091	539
AR16:0	10	0	14	1
AR17:1	23420	924	22337	1109
AR17:1	4105	124	3711	212
AR17:0	2005	135	1768	199
AR18:0	3	0.04	2	0.5
AR19:0	11	1	15	2
Total amount	49212	1957	49820	2010

Data in boldface indicate product expected from route A (Fig. 1).
[a] SD; standard deviation ($n = 3$).
[b] Treatment with 6 mM tetradecanoate ester substrate.

Table 3
Free fatty acid content of substrate-treated rice seedlings

Free fatty acid	Free fatty acid content (μg/seedling)		
	Substrate	Control	Substrate supply
FA10	C10	ND	ND (0.2 mM), ND (0.6 mM)
FA11	C11	ND	ND (0.2 mM), ND (0.6 mM)
FA12	C12	0.11	0.1 (0.6 mM), 0.31 (2 mM)
FA13	C13	0.12	0.11 (2 mM), 0.7 (6 mM)
FA14	C14	0.68	NE
FA15	C15	0.41	4.90 (2 mM), 31.85 (6 mM)
FA16	C16	3.53	NE
FA17	C17	0.13	26.7 (2 mM), 107 (6 mM)
FA18:1	C18:1	1.53	NE
FA18	C18	0.91	NE
FA19	C19	ND	45.5 (2 mM), 90.4 (6 mM)

ND, not detected; NE, not examined.

despite a free-FA13 content lower than the contents of the other odd-carbon free fatty acids, namely FA15, FA17, and FA19. Interestingly, free-FA10 and -FA11 were not detected even when substrate was supplied.

2.3. Structure determination of minor AR homologs

We attempted to isolate the minor homologs for which the quantities increased with substrate supply by preparative HPLC to determine their structures. The elution of the extract with solvent A (see Section 3) gave a mixture of each homolog (AR11:0, AR12:0, AR14:0, and AR16:0) and the free fatty acid liberated from the supplied substrate. These enriched homologs, without further purification, were subjected to a negative ion collision-induced-dissociation mass spectrometry (CID-MS) analysis to determine the carbon chain structures [14]. Fig. 3 shows the CID-MS spectra of the four homologs together with that of AR13:0. The fragment patterns for the four homologs (spectra A, B, E, and F) were quite similar to that of AR13:0 (spectrum D) and spaced by 14 mass unit, showing that the four homologs examined have linear carbon-chain structures. Due to the extremely low amounts, the carbon chain structures for the other homologs (AR9:0, AR10:0, and AR18:0) were not determined.

In order to prove the presence of an alkylbenzene-1,3-diol structure in the minor homologs, the AR12:0 homolog, for which production was the highest with substrate supply, was isolated in pure form by re-preparative HPLC (solvent B, see Section 3) of the AR12:0 homolog fraction containing only FA13 as the impurities. The ^{1}H-NMR and proton-noise-decoupled ^{13}C-NMR (^{13}C{^{1}H}-NMR) spectra are consistent with the presence of an alkylbenzene-1,3-diol structure. Fig. 4B shows the ^{1}H-NMR spectrum of the AR12:0 homolog. The triplet at δ 6.17 ppm, doublet at δ 6.24 ppm, and triplet at δ 2.48 ppm are identical with those found for AR13:0 (data not shown) and indicated the presence of an alkylbenzene-1,3-diol structure. The ^{13}C{^{1}H}-NMR spectrum (Fig. 5B) also supported this assignment (see Section 3).

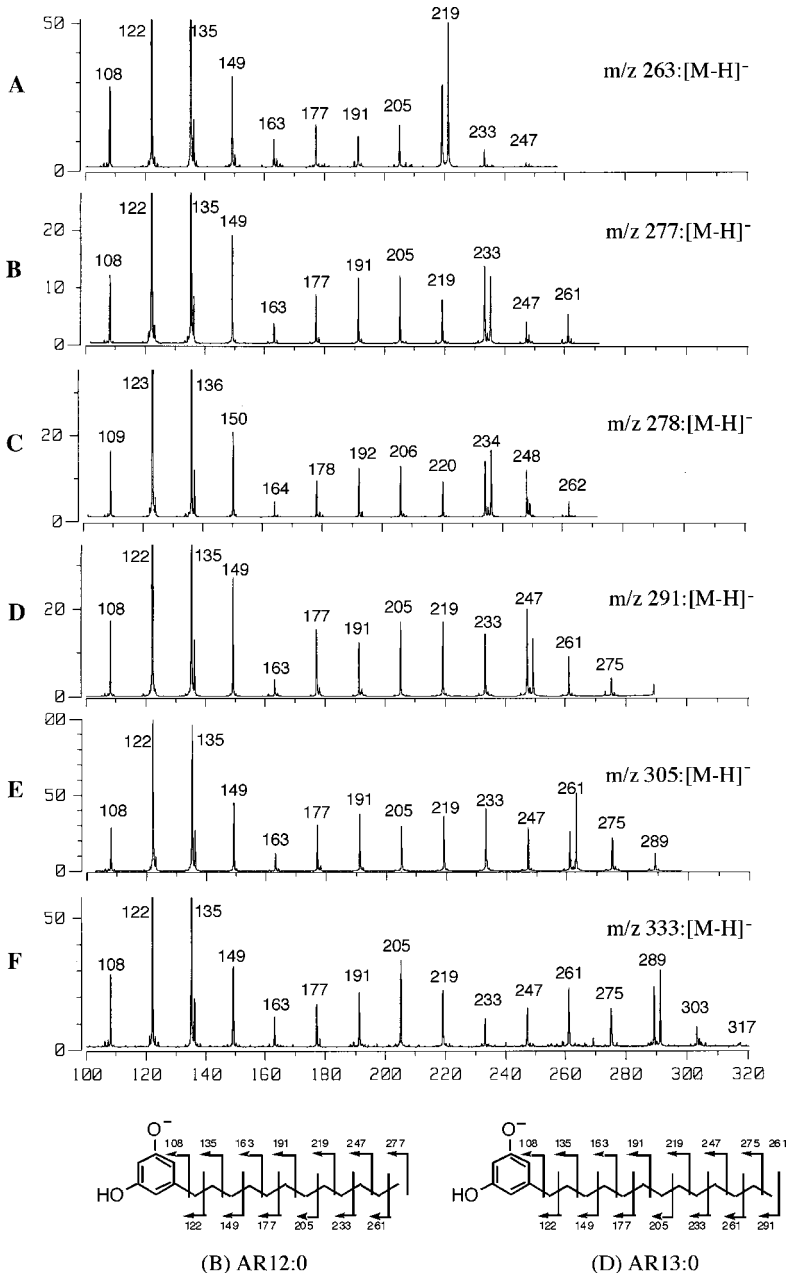

Fig. 3. Negative-ion FAB-CID-MS spectra of minor AR homologs. Fractions, (A) AR11:0, (B) AR12:0, (C) [5-^{13}C]AR12:0, (E), AR14:0, (F) AR16:0, which were purified by preparative HPLC from root extracts treated with each corresponding fatty acid ester substrate, and (D) AR13:0.

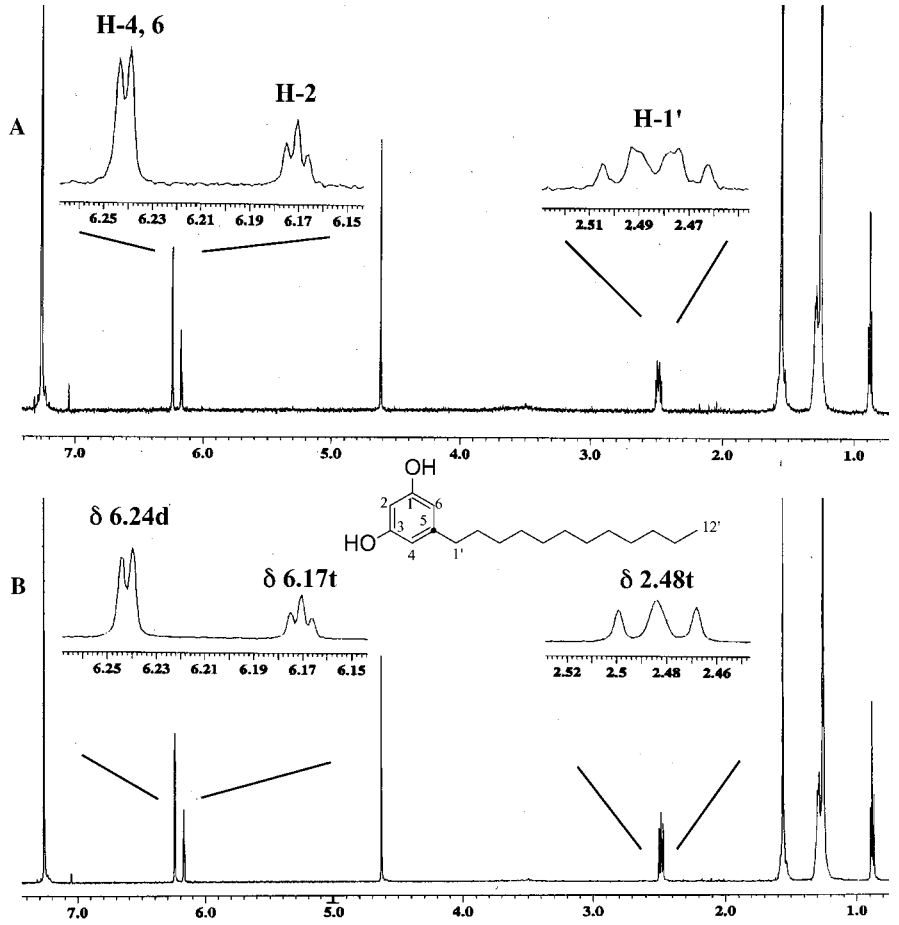

Fig. 4. ^{1}H-NMR spectra (500 MHz, $CDCl_3$) of AR12:0s. (A) The spectrum of the [5-^{13}C]AR12:0 and (B) the spectrum of AR12:0 which was isolated by preparative HPLC from extracts treated with 6 mM [1-^{13}C]C13 substrate or non-labelled C13 substrate for 8 days at 29 °C in darkness.

The results of CID-MS and NMR analyses showed that the structure of AR12:0 is 5-*n*-dodecylresorcinol.

2.4. Biosynthesis of ^{13}C-labelled AR12:0 using [1-^{13}C]tridecanoate substrate ([1-^{13}C]C13)

[1-^{13}C]FA13 as a labelled fatty acid was prepared from [1-^{13}C]acetic acid and 1-bromoundecane [15]. The spectral data of [1-^{13}C]FA13 are consistent with the expected structure including the ^{13}C label. For example, in the ^{13}C{^{1}H}-NMR spectrum (see Section 3), the ^{13}C label was detected as an intense singlet at the C-1 carboxylate carbon (δ 179.3 ppm). Moreover, carbons of C-2, C-3, and C-4 were observed as doublets that were due to [^{13}C–^{13}C]-coupling with an [1-^{13}C]-atom. These data showed

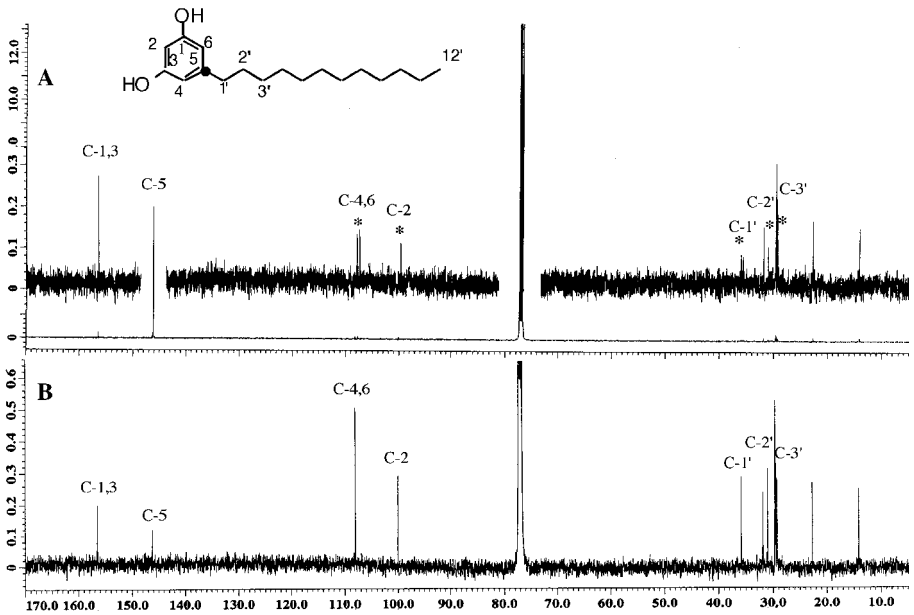

Fig. 5. (A) The spectrum of [5-^{13}C]AR12:0 and (B) the spectrum of the non-labelled AR12:0. The peaks indicated by the (*) show the doublets due to a ^{13}C–^{13}C coupling.

that the ^{13}C-atom is localized on the C-1 carbon. The labelled substrate from [1-^{13}C]FA13 was prepared in the same manner described for non-labelled substrates.

Feeding the ^{13}C-labelled substrate followed by solvent extraction and subsequent isolation of AR12:0 were carried out in the same manner described for the non-labelled AR12:0. The structure of the ^{13}C-labelled AR12:0 was determined by comparing the spectral data between the labelled and non-labelled AR12:0 homologs. In their GC-EI-MS (data not shown) and CID-MS spectra (Figs. 3B and C), the molecular and fragment ions in the labelled AR12:0 increased by one mass unit compared with those in the non-labelled AR12:0. These shifts clearly shows that the ^{13}C label is localized in resorcinol-ring carbons. The ^{13}C{^{1}H}-NMR data (Fig. 5) demonstrated the ^{13}C label to be the C-5 carbon because the label was detected as an intense singlet at δ 146.2 ppm, which is assigned to the C-5 carbon. Moreover, several doublets due to [^{13}C–^{13}C]-coupling with the [5-^{13}C]-atom were observed in carbons of C-2 and C-4, 6 and of C-1′, C-2′, and C-3′. The ^{1}H-NMR spectra (Fig. 4) further supported the assigned ^{13}C-atom position. Only the coupling patterns of the H-1′ protons at δ 2.48 ppm differed. There is a triplet of doublets for the labelled AR12:0 and a triplet for the non-labelled AR12:0. The additional coupling observed for the labelled AR12:0 is due to [^{1}H–^{13}C] coupling with the [5-^{13}C]-atom. The same coupling pattern was also observed in the H-2 protons of the synthetic [1-^{13}C]FA13 (see Section 3). Thus, it has been shown that [1-^{13}C]tridecanoate unit liberated from the supplied substrate, without any carbon–carbon fissions such as

fatty acid β-oxidation, is directly incorporated into the [5-^{13}C]AR12:0 carbon chain via the predicted AR biosynthesis pathway. Hence, a fatty acid or its equivalent is likely a starter unit (precursor) in long-chain-AR biosynthesis.

2.5. Conclusions

We have established here the biosynthesis pathway of long-chain ARs in rice seedlings. The putative starter unit in long-chain-AR biosynthesis is a fatty acid or its equivalent and the pathway is the route A shown in Fig. 1. These studies were made possible by the following developments. First, rice seeds without ARs were used but ARs were eventually produced in their etiolated seedlings. Second, it was found that the AR12:0 homolog content increased markedly with substrate supply because this high production ratio made it possible to isolate the non-labelled AR12:0 homolog and labelled one with higher incorporation of ^{13}C-label. This enabled us to use CID-MS and NMR analysis to provide evidence for the labelled AR12:0 structure.

Hesk et al. [16] reported the biosynthesis of anacardic acids, long-chain 6-alkylsalicylic acids (6), from fatty acid methyl esters in geranium (*Pelargonium xhortorum*). They found that [1-^{14}C]fatty acids are predominantly incorporated into the corresponding anacardic acid homologs expected from the predicted long-chain 6-alkylsalicylic acid biosynthesis pathway (Fig. 1). Although the labelling patterns of these homologs were not determined, these data seem to suggest that pre-existing fatty acids are precursors for the long-chain 6-alkylsalicylic acids.

It is noteworthy that only the AR12:0 homolog was produced in higer quantities when substrate was supplied. This is probably due to substrate specificity for the malonyl-CoA condensation to acyl-CoA starters and for the subsequent reactions. Under conditions where substrate was not supplied, a relationship exists between chain length and degree of chain-unsaturation in ARs (Fig. 2A). Saturated chains are the major component in relatively shorter-chain homologs of AR13:0 and AR15:0, while unsaturated ones are the major component in relatively longer-chain homologs of AR17:1s. On the other hand, the amount of free fatty acids varies (Table 3). Consequently, the production of the major, odd-carbon chain homologs does not directly reflect free fatty acid content. For example, the levels of AR13:0 and AR15:0 are almost identical although the FA16 level is 5-fold higher than the FA14 level. Such a result was also observed in the seedlings treated with the substrates. Therefore, it seems that substrate specificity, but not the amount of free fatty acids as a starter unit, determines AR production levels.

In the course of this study, a series of minor AR homologs with an even-carbon chain as well as an odd-carbon chain were also found. In barley and rye [3,17], the occurrence of such even-carbon chain AR homolog has been reported together with odd-carbon chain ones. However, their identification is poorly documented because they were determined based only on GC-MS data. A complete structural identification of the AR12:0 homolog in this study is the first report on the occurrence of even-carbon chain ARs. In addition to AR12:0, all the minor homologs found here are

also thought to have alkylbenzene-1,3-diol and linear carbon-chain structures because the same increases in the production of the ARs specific to the supplied substrates as that of AR12:0 were observed.

3. Experimental

3.1. Plant materials and experiments

Rice seeds (*Oryza sativa* L. cv. RD-25) were surface-sterilized with 70% ethanol (v/v) for 3 min, washed thoroughly with tap water, and then soaked in tap water at 29 °C for 2 days in darkness. Twenty of these soaked seeds were germinated in vermiculite filled with distilled water or an aqueous substrate solution (50 mL) in 100-mL glass beakers. After 8 days of incubation at 29 °C in darkness, these seedlings were used for extraction of ARs and free fatty acids.

3.2. Extraction of ARs and free fatty acids from rice seedlings

The extraction of ARs and free fatty acids from seedlings was essentially performed as reported by Lynn et al. [13]. The roots of the 8-day-old seedlings (about 0.6 g fresh weight/10 seedlings) were dipped in 10% MeOH/$CHCl_3$ (v/v, 50 mL/10–20 seedlings) for 3 min. The filtered extract was concentrated in vacuo and the residue was subjected to GC-EI-MS analysis and preparative HPLC.

3.3. General

^{1}H- and $^{13}C\{^1H\}$-NMR spectra were recorded in $CDCl_3$ with tetramethylsilane as an internal standard using 300 MHz Bruker and JEOL GSX-500 spectrometers. FAB-MS and FAB-CID-MS were measured on a JEOL JMS-HX/HX110A four-sector (EBEB) tandem mass spectrometer equipped with an array detector. The product ion was scanned manually with a B/E constant and subjected to collision. The second-generation product ions formed by CID were measured using the second mass spectrometer. FAB was generated using xenon as the primary beam with an energy of 6 keV and an ion acceleration voltage of 10 kV. High-energy CID was performed by introducing helium as the collision gas until the intensity of the precursor ion was reduced to 1/3 of the initial value. *m*-Nitrobenzoic acid was used as the matrix.

3.4. Analysis of AR homologs and free fatty acids by GC-EI-MS and GC-EI-MS/SIM

An amount of extract equivalent to 0.1 seedlings and *m-n*-pentadecylphenol (200 ng) as an external standard were placed in a glass microvial for GC and the solvent was evaporated. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (50 μL), a trimethylsilylating agent, was added, and the mixture was heated at 70 °C for 30 min. One microliter of the derivatized sample was loaded on a gas

chromatograph HP 6890 series coupled to a mass spectrometer HP 5973 at 70 eV with a gas flow rate of 1 mL/min of He. A DB-5MS column (ϕ 0.25 mm × 30 m, 0.25 μm film thickness, G&L Science) was used and the column oven temperature was programmed as follows: 90 °C for 1 min, increasing at 30 °/min up to 180 °C, and at 5 °C/min up to 300 °C, and 300 °C for 10 min. GC-EI-MS, R_t min: 8.40 (M^+ 380, AR9:0), 9.04 (M^+ 394, AR10:0), 9.77 (M^+ 408, AR11:0), 10.58 (M^+ 422, AR12:0), 11.48 (M^+ 436, AR13:0), 12.44 (M^+ 450, AR14:0), 13.28 (M^+ 462, AR15:1), 13.47 (M^+ 464, AR15:0), 14.53 (M^+ 478, AR16:0), 15.38 and 15.47 (M^+ 490, AR17:1s), 15.65 (M^+ 492, AR17:0), 16.76 (M^+ 520, AR18:0), 17.90 (M^+ 520, AR19:0). AR15:1 and AR17:1s represent ARs with a mono-unsaturated carbon chain, 5-(8′-pentadecenyl), 5-(8′-heptadecenyl), and 5-(10′-heptadecenyl)resorcinol, respectively.

AR homolog content was quantified according to the method described previously [6] with small modifications by GC-EI-MS/SIM using a fragment ion m/z 268 that is characteristic for 5-alkylresorcinol homologs. *n*-Pentadecylresorcinol (AR15:0, Aldrich) was used for calibration. Free fatty acid content was quantified by GC-EI-MS/SIM using each $[M\text{-}CH_3]^+$ ion of the silylated fatty acids. Saturated fatty acids from FA10 to FA19 and cis-9-octadecenoic acid (FA18:1) were used for calibration.

3.5. Preparation of fatty acid-monomethoxy poly(ethylene glycol) ester substrates

A mixture of fatty acid (4 mM), monomethoxy poly(ethylene glycol) (Mn 350, 3 mM), and camphor sulphuric acid (250 mg) was refluxed for 18 h with the azeotropic removal of water. The mixture was cooled on ice and saturated $NaHCO_3$ solution was added. The solvent layer was washed with saturated NaCl solution and dried over anhydrous $MgSO_4$. The solvent was evaporated and the residue was chromatographed on a silica gel column. Elution with 5% MeOH/$CHCl_3$ (v/v) afforded an ester in a quantitative yield.

3.6. Synthesis of [1-^{13}C]tridecanoic acid ([1-^{13}C]FA13)

[1-^{13}C]FA13 was synthesized as described by Pfeffer et al. [15]. 1.6 M *n*-butyllithium (11.2 mL) was added to a solution of diisopropylamine (1.81 g) in dry tetrahydrofuran (15 mL) with stirring under N_2 at a rate necessary to keep the temperature below 0 °C. Subsequently, [1-^{13}C]acetic acid (500 mg, 99 atom % of ^{13}C, Aldrich) was added at 0 °C. After a 15 min period, hexamethyphosphoramide (4 mL) was added. The solution was stirred for 20 min and 1-bromoundecane (2.06 g) was added at 0 °C. After 2-h stirring, saturated NH_4Cl solution was added at 0 °C. The aqueous layer was extracted with *n*-hexane. The combined organic layers were washed three times with 1 N hydrochloric acid, followed by a saturated NaCl solution. The organic layer was dried and evaporated. The residue was chromatographed on a silica gel column with 1% MeOH/CH_2Cl_2 to give [1-^{13}C]FA13 (1.3 g).

FAB-MS: m/z 214.1 $(M\text{-}H)^-$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 0.88t (3H, $J = 6.7$ Hz, H-13), 1.26m (18H), 1.63m* (2H, H-3), 2.35td* (2H, $J = 7.3$ Hz,

$^{1}J_{H-C} = 7.4$, H-2). $^{13}C\{^{1}H\}$-NMR (75 MHz, $CDCl_3$) δ ppm: 14.1, 22.7, 24.7d* ($^{2}J_{C-C} = 1.5$ Hz, C-3), 29.0d* ($^{3}J_{C-C} = 3.5$ Hz, C-4), 29.2, 29.3, 29.4, 29.5, 29.6 (2×), 31.9, 33.9d* ($^{1}J_{C-C} = 54.9$ Hz, C-2), 179.3 (C-1). The splitting (*) shows the ^{1}H–^{13}C (J_{C-H}) and ^{13}C–^{13}C (J_{C-C}) couplings.

3.7. Purification of minor AR homologs by HPLC

Preparative HPLC was performed with a reverse phase PEGASIL ODS column (ϕ 10 mm × 250 mm, Senshu Scientific) connected to a SSC system (Senshu Scientific) in the isocratic mode. The solvent system used was H_2O/CH_3CN ((A) 10:90, (B) 25:75 v/v) containing 0.1% trifluoroacetic acid, and the flow rate was 4 mL/min. The substances were monitored by UV absorption at 280 nm. An amount of extract equivalent to 30 seedlings was loaded on the column. R_t (solvent A) min: 7.72 (AR11:0), 9.97 (AR12:0), 12.60 (AR13:0), 16.23 (AR14:0), 21.04 (AR15:0), 28.00 (AR16:0), 37.77 (AR17:0). Five homolog peaks (AR11:0, AR12:0 ([5-^{13}C]AR12:0), AR13:0, AR14:0, AR16:0) were obtained. Only AR13:0 was pure in the ^{1}H-NMR spectrum (data not shown). Re-chromatography of the fraction AR12:0 or [5-^{13}C]AR12:0 by preparative HPLC with solvent B yielded a pure sample (R_t: 24.37 min).

AR12:0 (200 μg). FAB-MS: m/z 277.1 $[M-H]^-$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 0.88t (3H, $J = 6.9$ Hz, H-12′), 1.26m (18H), ca. 1.5m (2H, H-2′, overlapped with solvent), 2.48t (2H, $J = 7.8$ Hz, H-1′), 4.62s (2H, OH), 6.17t (1H, $J = 2.3$ Hz, H-2), 6.24d (2H, $J = 2.3$ Hz, H-4, 6). $^{13}C\{^{1}H\}$-NMR (125 MHz, $CDCl_3$) δ ppm: 156.5 (C-1,3), 146.2 (C-5), 108.0 (C-4, 6), 100.1(C-2), 35.8 (C-1′), 31.9 (C-10′), 31.1 (C-2′), 29.7 × 2, 29.63, 29.59, 29.5, 29.4 (C-4′ to C-9′), 29.3 (C-3′), 22.7 (C-11′), 14.1 (C-12′).

[5-^{13}C]AR12:0 (150 μg). FAB-MS: m/z 278.1 $[M-H]^-$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 0.88t (3H, $J = 6.9$ Hz, H-12′), 1.26m (18H), ca. 1.5m (2H, H-2′, overlapped with solvent), 2.48td* (2H, $J = 7.8$ Hz, $^{1}J_{H-C} = 6.0$ Hz, H-1′), 4.63s (2H, OH), 6.17t (1H, $J = 2.3$ Hz, H-2), 6.24d (2H, $J = 2.3$ Hz, H-4, 6). $^{13}C\{^{1}H\}$-NMR (125 MHz, $CDCl_3$) δ ppm: 156.5 (C-1,3), 146.2 (C-5), 108.0d* ($^{1}J_{C-C} = 59.5$ Hz, C-4, 6), 100.1d* ($^{3}J_{C-C} = 3.8$ Hz, C-2), 35.8d* ($^{1}J_{C-C} = 42.2$ Hz, C-1′), 31.9 (C-10′), 31.1d* ($^{2}J_{C-C} = 42.2$ Hz C-2′), 29.7 × 2, 29.64, 29.6, 29.5, 29.4 (C-4′ to C-9′), 29.3d* ($^{3}J_{C-C} = 3.8$ Hz, C-3′), 22.7 (C-11′), 14.1 (C-12′).

Fractions AR11:0, AR12:0, AR14:0, and AR16:0 were subjected to negative FAB-CID-MS analysis without further purification (See Fig. 3). FAB-MS $[M-H]^-$: m/z 263.1 (AR11:0), m/z 277.1 (AR12:0), m/z 305.1 (AR14:0), m/z 333.1(AR16:0).

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References

[1] A. Kozubek, J.H.P. Tyman, Chem. Rev. 99 (1999) 1–26.
[2] E. Wenkert, E.M. Loeser, S.N. Mahapatra, F. Schenker, E.M. Wilson, J. Org. Chem. 29 (1964) 435–439.
[3] D.E. Briggs, Phytochemistry 13 (1974) 987–996.
[4] S. Gohil, D. Petterson, A.C. Salomonssson, P. Aman, J. Sci. Food Agric. 45 (1988) 43–52.
[5] M.L. Bouillant, C. Jacoud, I. Zanella, J. Favrebonvin, R. Bally, Phytochemistry 35 (1994) 769–771.
[6] Y. Suzuki, Y. Esumi, H. Hyakutake, Y. Kono, A. Sakurai, Phytochemistry 41 (1996) 1485–1489.
[7] M. Cojocaru, S. Droby, E. Glotter, A. Goldman, H.E. Gottlieb, B. Jacoby, D. Prusky, Phytochemistry 25 (1986) 1093–1095.
[8] A.J. Birch, F.W. Donovan, Aust. J. Chem. 6 (1953) 360–368.
[9] P.M. Shoolingin-Jordan, I.D.G. Campuzno, in: D. Barton, K. Nakanishi (Eds.), Comprehensive natural products Chemistry, vol. 1, Pergamon, Amsterdam, 1999, pp. 345–364.
[10] A.J. Birch, R.A. Massey-Westrop, C.P. Moye, Aust. J. Chem. 8 (1955) 540–544.
[11] A.I. Scott, L.C. Beadling, N.H. Georgopapadakou, C.R. Subbarayan, Bioorg. Chem. 3 (1974) 238–248.
[12] J.B. Spencer, P.M. Jordan, J. Chem. Soc., Chem. Commun. (1992) 646–648.
[13] G.D. Fate, D.G. Lynn, J. Am. Chem. Soc. 118 (1996) 11369–11376.
[14] N.J. Jansen, K.B. Tomer, M.L. Gross, J. Am. Chem. Soc. 107 (1985) 1863–1868.
[15] P.E. Pfeffer, L.S. Silbert, J. Org. Chem. 35 (1970) 262–264.
[16] D. Hesk, R. Craig, R.O. Mumma, J. Chem. Ecol. 18 (1992) 1349.
[17] P. Hengtraukul, K. Lorenz, M. Mathias, J. Food Comp. Anal. 4 (1991) 52–57.