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MURICADIENIN, MURIDIENINS AND CHATENAYTRIENINS, THE EARLY PRECURSORS OF ANNONACEOUS ACETOGENINS*

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IN HONOUR OF THE RETIREMENT OF PROFESSOR ANDRÉ CAVÉ

Key Word Index—*Annona muricata*; *A. nutans*; Annonaceae; acetogenins; oxidative degradation; enzymatic oxidation; tris-unsaturated fatty acid derivatives; biogenetic pathway.

Abstract—Chatenaytrienins-1,-2 and -3, muridienins-3 and -4 and muricadienin were characterized by tandem mass spectrometry (MS/MS) in a mixture of natural precursors of annonaceous acetogenins from *Annona muricata*. Chatenaytrienin-1, -2, -3 and -4 were then isolated from *A. nutans* and fully characterized by spectroscopic methods (NMR, MS) and by chemical and enzymatic oxidative processes. Isolation of these trienes confirmed the postulated biosynthetic pathway leading to the acetogenins.

INTRODUCTION

Acetogenins of Annonaceae have been isolated so far only from the archaic Annonaceae family [1–5]. The biological activities of these compounds are quite interesting, particularly as antitumour agents, and as antiparasitic, pesticidal and immunosuppressant compounds [2]. The mechanism of action is still not completely understood, but both the complexation with metal ions and inhibition, even at every low concentration (ED_{50} 10^{-12} μg/ml), of complex I in mitochondria, as well as NADH oxidase on carcinogenic cell membranes have been postulated to account for the antitumour effect [3–5]. So far, over 250 acetogenins have been isolated from 30 different species of Annonaceae (among 2500 known species). They have in common a very long alkyl chain (32 or 34 carbon atoms) substituted by oxygenated groups (tetrahydrofuran, epoxide, hydroxyl,...) and are terminated by an α,β-unsaturated γ-methyl γ-lactone.

We have isolated the first acetogenins without a tetrahydrofuran ring [6–17], particularly epoxy-ene compounds, which led us to postulate the natural occurrence of a *bis*-unsaturated precursor, muricadienin [8]. Soon after, other acetogenins without any THF rings were isolated by several groups [18–21]. Recently [22], we isolated, from the roots of *Annona muricata*, muridienin-1 and -2, the first *bis*-unsaturated precursors of monotetrahydrofuran (type A) acetogenins of Annonaceae (see Refs 1 and 2 for classification of the natural acetogenins), which are positional isomers of the hypothetical muricadienin [8]. Encouraged by these results, we looked for the natural precursors of the *bis*-tetrahydrofuran acetogenins. We now report on the successful isolation and characterization of the *tris*-unsaturated natural precursors chatenaytrienin-1, -2, -3 and -4 (**1**–**4**) together with the new *bis*-unsaturated isolates (**5**–**7**).

RESULTS AND DISCUSSION

Liquid–liquid partition of the methanolic extract of the roots of *A. muricata* with dichloromethane,

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† These results form part of the PhD theses of C.G. and S.R.

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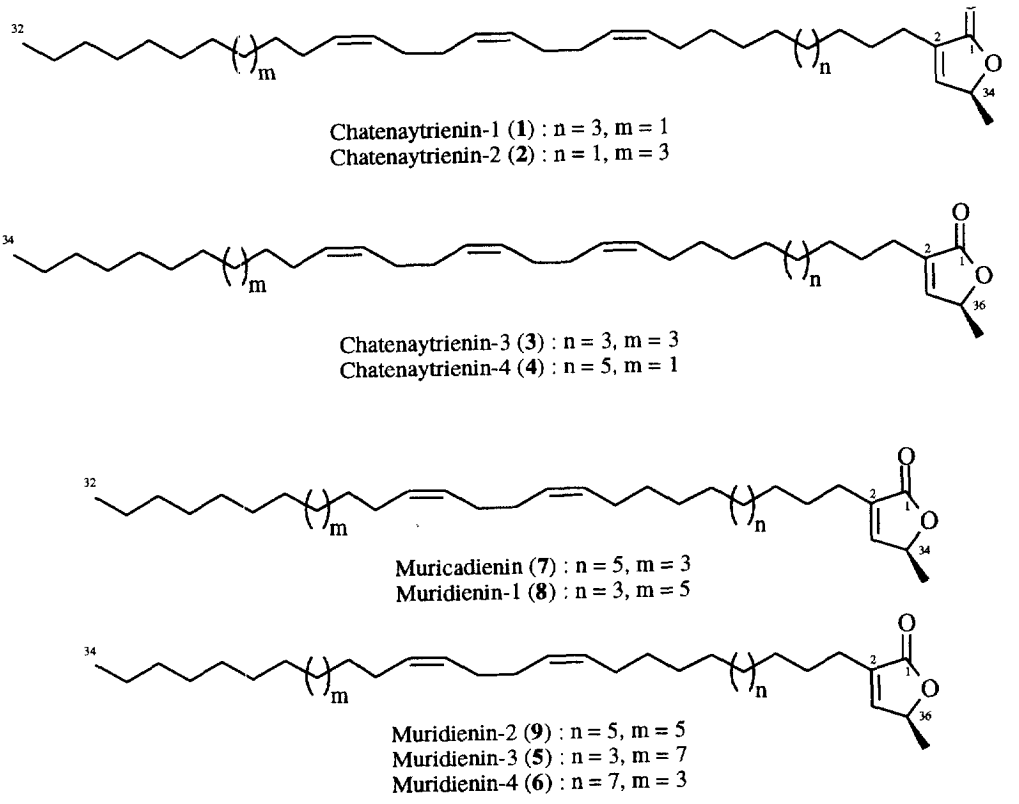
** Dedication: On the occasion of Pr. André Cavé's retirement, the authors and all the members of the Pharmacognosy Department, together with those who spent a part of their life working in the Laboratory, express their gratitude for his permanent interest in Natural Compounds chemistry and for his international leading position in the field of alkaloids and acetogenins from the Annonaceae.



followed by HPLC, led to three main fractions 1–3, each of which displayed identical spectroscopic data (e.g. IR, UV and NMR). The ^{1}H NMR spectra revealed the sole presence of compounds possessing a long alkyl chain ended by the α,β-unsaturated γ-methyl γ-lactone along with several ethylenic protons appearing around δ 5.4 (Fig. 1). These fractions were therefore studied in the search for the hypothetical triene.

The CIMS spectrum of fraction 1 showed a single peak at m/z 513, corresponding to the $[M+H]^+$ ion of an acetogenin with the molecular formula $C_{35}H_{60}O_2$, and in agreement with the presence of six unsaturations. Since the terminal lactone bears three unsaturations, we were dealing with the first *tris*-unsaturated precursor(s). LSI–MS/MS spectroscopy was then performed in order to confirm the presence of the triene, and to locate the double bonds on the long alkyl chain [16, 23]. Typical charge remote fragmentations were observed for the high energy collision-induced dissociation (CID) spectrum of the $[M+Li]^+$ ions, showing the "fingerprints" of two trienes chatenaytrienin-1 and -2 (**1**, **2**), both possessing the expected $\Delta^{n,n+4,n+8}$ pattern. Indeed, the relative intensity of the peaks due to the fragmentations at the allylic positions allowed us to locate unambiguously the triene systems of both compounds [16, 23]; the fragmentation pattern for lithiated **1** is given in Fig. 2. The three double bonds were found to be located between C-13–C-14, C-17–C-18 and C-21–C-22 for **1**, and between C-11–C-12, C-15–C-16 and C-19–C-20 for **2**. Because the specific rotation of this fraction has a positive sign, we postulated that the stereogenic centre of both products has the *S* configuration. This was confirmed later by an enzymatic method (see below).

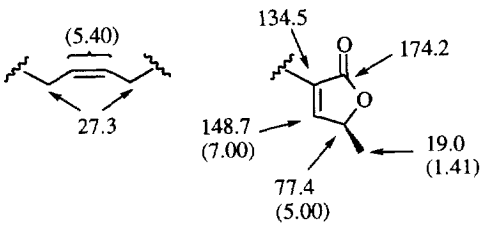


Fig. 1. ^{1}H (in brackets) and ^{13}C NMR data of fractions 1–3 from *A. muricata*.

Fraction 2 was then investigated by mass spectrometry. The CIMS spectrum displayed two intense peaks at m/z 515 and 541, in accordance with the molecular formula $C_{35}H_{62}O_2$ and $C_{37}H_{64}O_2$, respectively. The LSI–MS/MS spectrum of the $[M+Li]^+$ ions showed that the C_{35} compound corresponded to a mixture of two $\Delta^{n,n+4}$ dienes, the known muridienin-1 (**8**) [22] and the expected muricadienin (**7**) [8], the natural precursor of solamin, with double bonds located between C-15–C-16 and C-19–C-20. The homologous C_{37} is a new $\Delta^{n,n+4,n+8}$ triene, chatenaytrienin-3 (**3**), with three double bonds linking C-13–C-14, C-17–C-18 and C-21–C-22.

The mixture was then subjected to oxidative degradation with ruthenium chloride in the presence of

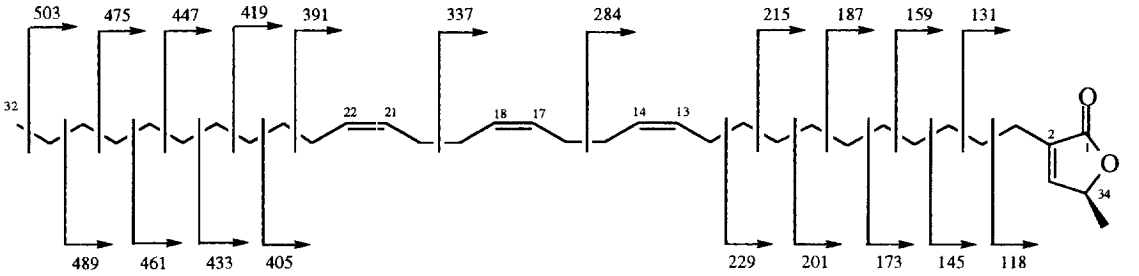


Fig. 2. LSI–MS/MS of chatenaytrienin-1 (precursor ion: $[M+Li]^+$, m/z 519).

periodic acid [24] prior to enzymatic incubation with L- and D-lactate dehydrogenase and nicotinamide-adenine-dinucleotide (NAD) [25]. Since NADH was detected only in the L-LDH incubation medium, the presence of L-lactic acid could then be deduced, and thus the absolute configuration (*S*) for the stereogenic centre of each molecule of the mixture.

Mass spectrometry analyses of fraction 3 (HRCIMS, LSI–MS/MS) revealed the presence of a mixture of three isomeric $\Delta^{n,n+4}$ dienes with the molecular formula $C_{37}H_{66}O_2$, namely the known muridienin-2 (**9**) [22] with the double bonds located at $\Delta^{15,19}$, and the new muridienin-3 and -4 (**5**, **6**), with the double bonds at $\Delta^{13,17}$ and $\Delta^{17,21}$, respectively. As above, enzymatic oxidation was performed on the mixture in order to determine the absolute configuration of lactic acid formed after chemical degradation [25]. Since we could prove the sole presence of the L-isomer, we conclude that the three muridienins (**5**, **6** and **9**) have *S* configuration.

In addition to the above studies, we were also working on a methanolic extract of the root bark of *A. nutans*, a species which to our knowledge, has not been examined before. The methanolic extract was partitioned between H_2O and cyclohexane. The cyclohexane fraction was then purified by column chromatography on silica gel and finally by HPLC to give fractions 7.4 and 7.5. The CIMS spectrum of fraction 7.4 showed a single peak at m/z 513, corresponding to the $[M+H]^+$ ion of an acetogenin with the molecular formula $C_{35}H_{60}O_2$. The spectroscopic data showed identical ^1H and ^{13}C NMR spectra as those of chatenaytrienins (**1**–**3**) isolated from *A. muricata*. The ^1H NMR spectrum displayed the typical resonances for the lactone ring at δ 6.97 (H-33), 4.99 (H-34), and 1.40 (CH_3-35), and a multiplet between δ 5.36–5.41 integrating for six ethylenic protons. In the ^{13}C NMR spectrum, the corresponding signals appeared at δ 174.2 (C-1), 148.7 (C-33), 134.5 (C-2), 77.4 (C-34) and 19.2 (C-35) with three signals at δ 129.1, 129.6 and 130.4 correlated, in the HMQC spectrum, with the ethylenic protons around δ 5.4. Three signals of allylic protons appeared at δ 2.08, 2.03 and 2.26 integrating for eight, four and two protons, respectively. In the ^1H-^1H COSY NMR spectrum, the signal at δ 2.08 correlated only with the ethylenic protons at δ 5.4, whereas the signal at δ 2.03 was correlated to both the ethylenic and the homoallylic protons. This is in accordance with a triene system with the double bonds separated by two methylenes. The signal at δ 2.26 (H-3) was correlated with the vinylic proton of the lactone at δ 4.99. The *Z* configuration for the three double bonds was determined by measuring the ^3J coupling constant (^3J = 9.3 Hz) of the vinylic protons after irradiation at δ 2.08. This was in full agreement with the ^{13}C NMR chemical shifts of the allylic methylene carbon atoms at σ 27.3 (for an *E* configuration the allylic carbon atoms should appear at δ 32) [26]. The location of the $\Delta^{n,n+4,n+8}$ triene pattern, and the absolute configuration of the single asymmetric centre of the molecule remained to be established. For this purpose, a chemical degradative process followed by GC analysis was used. Oxidative degradation of the compound was performed by treatment with a catalytic amount of ruthenium chloride in the presence of periodic acid in the ternary mixture of CCl_4–MeCN–H_2O [24]. All the double bonds were cleaved, affording the expected carboxylic acids (Fig. 3).

The crude mixture of compounds so obtained was analysed by GC after trimethylsilylation. Undecanoic acid was identified by comparison with an authentic sample. Both the presence of this carboxylic acid in the reaction mixture, and the molecular formula, allowed us to determine without ambiguity the position of the last double bond between C-21 and C-22. Therefore, the two other double bonds were located between C-13–C-14 and C-17–C-18. Two other fragments were characterized as succinic acid and dodecanedioic acid, by comparison with authentic samples (Fig. 4). However, minor carboxylic acids, such as tridecanoic acid, were also detected. This is in full agreement with the presence of two compounds: the major one (82%) identified as chatenaytrienin-1 (**1**) and the minor one (18%) identified as chatenaytrienin-2 (**2**). LSI–MS/MS analysis of fraction 7.4 confirmed the present of the two compounds based on the typical allylic fragmentations shown in Fig. 2.

Two aliquots of the crude reaction mixture were then separately incubated with L- and D-lactate dehydrogenase and NAD [25]. Since NADH was only detected in the L-LDH incubation medium, the presence of L-lactic acid could then be deduced, and thus the *S* configuration for C-34 of both **1** and **2**.

$$R^1\text{-}CH{=}CH\text{-}R^2 \xrightarrow[CCl_4/CH_3CN/H_2O]{H_5IO_6/RuCl_3} R^1\text{—}COOH + HOOC\text{—}R^2$$

Fig. 3. Oxidative degradation of unsaturated acetogenins.

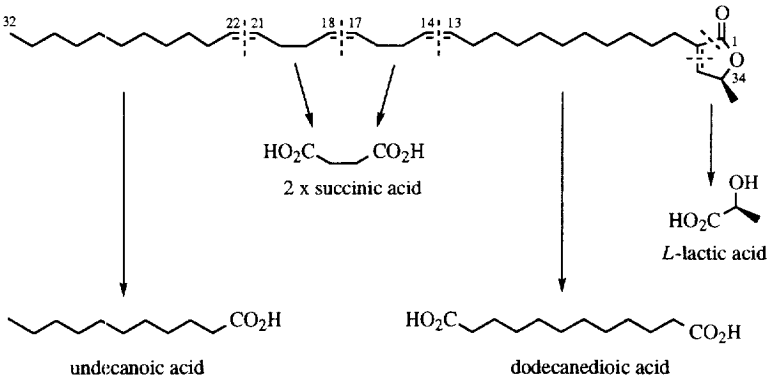


Fig. 4. Carboxylic acid derivatives of chatenaytrienin-1 (tridecanoic acid is obtained from chatenaytrienine-2).

Fraction 7.5 isolated from *A. nutans* was also studied by mass spectrometry, ^1H and ^{13}C NMR, as well as by degradative methods as described above. All these data indicated that this fraction contained chatenaytrienin-3 (**3**) as a major product (83%), and chatenaytrienin-4 (**4**) as a minor one (17%), with the triene system at $\Delta^{15,19,23}$. The absolute configuration of the lactone ring of both compounds was then determined by the enzymatic procedure as *S*.

In conclusion, we succeeded in the rapid and efficient characterization of the first natural triene acetogenins in several fractions of *A. muricata* by using LSI–MS/MS. We were also able to separate and purify chatenaytrienins-1, 2, 3 and -4 (**1–4**) from *A. nutans*, and to determine their absolute configurations along with the position of the double bonds after chemical degradation. This alternative method allows one to quantify polyunsaturated compounds in a mixture of isomeric precursors of acetogenins. These triene precursors are the proof that the biogenic pathway of the adjacent *bis*-tetrahydrofuran acetogenins is similar to the biological synthesis of the *mono*-tetrahydrofuran acetogenins, and implies oxidation of an unsaturated precursor followed by a ring expansion.

EXPERIMENTAL

General experimental procedures

^1H and ^{13}C NMR: 400 and 100 MHz, respectively, $CDCl_3$; CIMS (NH_4^+): Nermag R 10-10 C spectrometer; LSIMS (matrix: *m*-NBA + LiCl): Kratos NS 80 RF double focusing mass spectrometer under conventional conditions; MS/MS: ZabSpec-T five-sector tandem mass spectrometer (Fisons Instruments, VG organic, Manchester, U.K.); GC: Hewlett Packard 5890 chromatograph, injector at 300°, FID detector at 350° and a HT-5 capillary column (5% phenyl polycarborane–siloxane phase, length 25 m, i.d. 0.22 mm, film thickness 0.1 μm, SGE international, Victoria, Australia) with N_2 as the carrier gas. The average linear gas velocity was 1.4 ml/min at 200°. The temp programme was 100° increasing 5°/min to 270°. HPLC: Waters 590 pump system and a Millipore Waters 484 (Milford, MA, U.S.A.) spectrophotometer. Authentic carboxylic acids were purchased from Aldrich.

Plant material

Roots of *Annona muricata* were collected in Guinea (Conakry) in October 1993. A voucher specimen has been deposited at the Faculty of Medicine and Pharmacy of Conakry. Bark of *A. nutans* were collected in Paraguay.

Extraction and isolation

The dried and powdered roots of *A. muricata* (600 g) were extracted with MeOH. The MeOH extract was then partitioned between H_2O and CH_2Cl_2 to yield 45 g of CH_2Cl_2 extract. This extract was subjected to silica gel CC (silica gel Merck 70–230 Mesh) and eluted with hexane containing increasing amount of EtOAc. The fractions collected were analysed by TLC (silica gel Merck 60 F254), on which basis they were grouped into 17 sets. The solvent of the first fraction was evaporated off. The resulting residue (1.5 g) was subjected to silica gel CC (silica gel Merck 60 H 230–400 Mesh) eluted with C_6H_{14}–EtOAc (18:1). Fractions containing identical products as judged from TLC were combined and purified by reversed phase prep HPLC using a μBondapak C_{18} 10 μm cartridge column (250 × 20 mm), flow rate 9 ml/min, 20 mg/injection, and eluant MeOH–H_2O (99:1). Fraction 1 (2 mg, $[\alpha]_D^{25}$ +9 (MeOH, *ca* 0.11), fraction 2 (24 mg,

$[\alpha]_D^{25}+11$ (MeOH, *ca* 0.1) and fraction 3 (9 mg, $[\alpha]_D^{25}+10$ (MeOH, *ca* 0.1) were thus obtained.

Fraction 1 (*mixture of* **1** *and* **2**). See below.

Fraction 2 [*mixture of chatenaytrienin*-3 (**3**), *muricadienin* (**7**) *and muridienin*-1 (**8**)]. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data were identical with those of muridienin-1 (**8**) [22]; LSIMS of **7**: *m/z* 521 $[M+Li]^+$; MS/MS of the $[7+Li]^+$ ion: 505, 491, 477, 463, 449, 435, 421, 407, 393, 379, 365, 312, 257, 243, 229, 215, 201, 187, 173, 159, 145, 131, 118.

Fraction 3 [*mixture of muridienin*-3 (**5**), *muridienin*-4 (**6**) *and muridienin*-2 (**9**)]. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data were identical with those of muridienin-1 (**8**) [22]; LSIMS of **9**: *m/z* 549 $[M+Li]^+$; MS/MS of the $[9+Li]^+$ ion: *m/z* 533, 519, 505, 491, 477, 463, 449, 435, 421, 407, 393, 379, 365, 312, 257, 243, 229, 215, 201, 187, 173, 159, 145, 131, 118; LSIMS of **5**: *m/z* 549 $[M+Li]^+$; MS/MS of the $[5+Li]^+$ ion: *m/z* 533, 519, 505, 491, 477, 463, 449, 435, 421, 407, 393, 379, 365, 351, 337, 284, 229, 215, 201, 187, 175, 159, 145, 131, 118; LSIMS of **6**: *m/z* 549 $[M+Li]^+$; MS/MS of the $[6+Li]^+$ ion: *m/z* 533, 519, 505, 491, 477, 463, 449, 435, 421, 407, 393, 340, 285, 271, 257, 243, 229, 215, 201, 187, 173, 159, 145, 131, 118.

The dried and ground bark of *A. nutans* was extracted with MeOH. 37 g of this methanolic extract were partitioned between H_2O and C_6H_{12}, to yield 3.44 g of cyclohexane extract. This extract was submitted to CC (silica gel, 70–230 Mesh) eluting with C_6H_{12}–CH_2Cl_2 (100:0 to 0:100) then CH_2Cl_2–EtOAc (100:0 to 0:100) gradients, which yielded 40 fractions. Fraction 7 (56 mg) was further separated by CC (silica gel 60H) with CH_2Cl_2 (100%) and led to a fraction containing **1**–**4** (19 mg). These compounds were separated and purified by semi-prep HPLC using a μBondapack C18 prepacked column [10 μm, 25 × 100 mm], eluted with MeOH–H_2O (97:3), flow rate 10 ml/min, UV detection 214 nm. Fraction 7.4 (3.2 mg) and fraction 7.5 (9.1 mg) were thus obtained, containing **1**, **2** and **3**, **4**, respectively.

(5S)-3-(*triacontatrien*-11, 15, 19-*yl*-1)-5-*Methylfuran*-2-5(H)-*one* (*chatenaytrienin*-1, **1**) *with* 18% *chatenaytrienin*-2 (**2**). Oil; UV λ_{max}^{EtOH} nm: 214; ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, *t*, *J* = 6.8 Hz, H-32), 1.26 (32H, *m*, H-5 to H-11, H-24 to H-31), 1.40 (2H, *d*, *J* = 6.8 Hz, H-35), 1.55 (2H, *m*, H-4), 2.03 (4H, *m*, H-12, H-23), 2.08 (8H, *m*, H-15, H-16, H-19, H-20), 2.26 (2H, *t*, H-3), 4.99 (1H, *dq*, *J* = 6.8, 1.7 Hz, H-34), 5.36–5.41 (6H, *m*, H-13, H-14, H-17, H-18, H-21, H-22), 6.97 (1H, *d*, *J* = 1.7, H-33): ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1 (C-32), 19.2 (C-35), 22.6 (C-31), 25.2 (C-3), 27.2–27.4 (C-12, C-15, C-16, C-19, C-20, C-23), 29.1–29.7 (C-4 to C-11, C-24 to C-29), 31.9 (C-30), 77.4 (C-34), 129.1, 129.6 and 130.4 (C-13, C-14, C-17, C-18, C-21, C-22), 134.5 (C-2), 148.7 (C-33), 174.2 (C-1); CIMS (NH_4^+) *m/z* 530 $[M+NH_4]^+$, 513 $[M+H]^+$; MS/MS FAB-Li *m/z* 519 $[M+Li]^+$, 503, 489, 475, 461, 447, 433, 419, 405, 391, 337, 284, 229, 215, 201, 187, 173, 159, 145, 131, 118.

(5S)-3-(*dotriacontatrien*-11, 15, 19-*yl*-1)-5-*Methylfuran*-2-5(H)-*one* (*chatenaytrienin*-3, **3**) *with* 17% *chatenaytrienin*-4 (**4**). Oil; $[\alpha]_D^{20}+25$ ($CHCl_3$, *ca* 0.18); IR ν_{max} cm^{-1}: 2929, 2857, 1761, 1657, 755; UV λ_{max}^{EtOH} nm: 214; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data were identical with those of **1**; CIMS (NH_4^+) *m/z* 558 $[M+NH_4]^+$, 541 $[M+H]^+$; MS/MS FAB-Li *m/z* 547 $[M+Li]^+$, 531, 517, 503, 489, 475, 461, 447, 433, 419, 405, 391, 337, 284, 229, 215, 201, 187, 173, 159, 145, 131, 118.

Fractions 7.4 and 7.5 were submitted separately to oxidative degradation as described in [24] and the crude mixtures were analysed by GC. In both cases, four different peaks with a R_t of 4.05, 8.48, 12.66 and 19.66 min respectively were assigned, by comparison with authentic samples, to succinic, undecanoic, tridecanoic and dodecanedioic acids. Relative amounts of unidecanoic and tridecanoic acids were estimated as 82:18 and 17:83, in the reaction mixtures obtained from fraction 7.4 and 7.5, respectively.

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