

Identification of very long chain unsaturated fatty acids from *Ximenia* oil by atmospheric pressure chemical ionization liquid chromatography–mass spectroscopy

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Abstract

A method is described for the enrichment of very long chain unsaturated fatty acids from total fatty acids of *Ximenia* oil and their identification as picolinyl esters by means of liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC–MS/APCI). The method is based on the use of preparative reversed phase HPLC and their subsequent identification by microbore LC–MS/APCI. The combination of these two techniques was used to identify unusual unsaturated VLCFAs up to tetracontenoic acid. All four positional isomers of tetratriacontenoic acid were also synthesized to unambiguously confirm their structure.
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1. Introduction

Fatty acids with more than 22 carbon atoms (very long chain fatty acids, VLCFA) are found only rarely in nature (Smith, 1970; Řezanka, 1989). Their plant sources include, e.g., oils from seeds of plants from the Oleaceae family, which are also known as a rich source of unsaturated fatty acids, whether acetylenic or monoenoic (Badami and Patil, 1980). The degree of unsaturation of these compounds varies depending on their source, but alkenoic VLCFA generally have the *Z* stereochemistry. Important representatives of this family are plants of the genus *Ximenia*.

Ximenia americana (also known as seaside plum, wild plum, sour plum, wild olive, monkey plum or hog plum) is a spiny, deciduous shrub or small tree growing in southern Africa and South America in low altitude areas, woodland and across grassy savannahs, in coastal areas or on riverbanks. The species is drought resistant. The fruits are similar in shape and size to a plum, dark reddish-brown

to bright orange-scarlet. The edible pulp clings to a stone, which contains an oil-rich, soft cream-colored seed.

Ximenia is a key part of native African medicine. The fruits, rich in vitamin C, form an important component of the diet: eaten raw, made into jams, jellies, or syrup. The seed oil is edible and used in cooking. However, its principal use is as an emollient, hair oil, conditioner and skin softener, in soap manufacture, and as component of lipsticks and lubricants. The roots, bark and leaves are used for medicinal purposes; the bark is used to treat toothache, mouth infections and stomach aches.

The seed fats of *Ximenia caffra*, *X. caffra* var. *natalensis*, and *X. americana* var. *microphylla* were found to contain large proportions of monoenoic acids of the hexacosenoic (ximenic), octacosenoic, and triacontenoic (lumequic) series (Ligthelm et al., 1954). *X. americana* was later recorded to contain octadec-11-en-9-ynoic acid, named ximenynic (santalbic) acid as well as icosenoic-triacontenoic acids, all of which belong to the ω -9 series (Mikolajczak et al., 1963).

Surprisingly, no identification of monoenoic VLCFA was done by those authors who studied *Ximenia* seed oil

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(Rovesti, 1979; Fatope et al., 2000; Eromosele and Eromosele, 2002; Khumalo et al., 2002).

Other seeds have been found to contain monoenoic VLCFA (Rezanka, 1989). Also, 17-hexacosenoic, 21-hexacosenoic, and 23-octacosenoic acids were identified in Chilean hazelnut (*Gevuina avellana*) seed oil (Aitzetmüller, 2004), major 15-tetracosenoic and 17-hexacosenoic acids with minor octacosenoic acid in *Tropaeolum speciosum* (Litchfield, 1970), 15-tetracosenoic, 17-hexacosenoic, and octacosenoic acids in *T. majus* (Carlson and Kleiman, 1993), triacontenoic acid in *Curupira tefeensis* (Spitzer et al., 1990); 8-dotriacontenoic acid with unusual position of a double bond was identified in the plant *Centella asiatica* (Srivastava and Shukla, 1996). Very long chain mono-unsaturated fatty acids, including 19-octacosenoic and 23-dotriacontenoic acids, were identified in the alga *Botryococcus* collected in Lake Shira (Siberia, Russia) (Kalacheva et al., 2002).

In previous papers (Rezanka, 2002; Rezanka and Sigler, 2006) we published a method suitable for enriching mixtures of natural fatty acids. This method is based on RP-HPLC, which could be used not only for enrichment of total fatty acids with VLCFAs but also for separation of individual compounds. The individual components were resolved by mobile phases based on methanol with a small amount of triethylamine in water. A column with phases based on C8/C18 multi-alkyl bonding and exhaustive endcapping minimizes the use of organic modifiers. The technique used for identification in this paper, i.e. APCI, avoids the discrimination against higher homologues, which increases with increasing number of carbons and double bonds (Rezanka, 2000a,b; Rezanka and Votruba, 2005).

Since no monoenoic VLCFA is commercially available, we synthesized all positional isomers of tetratriacontenoic acid, presumed to be present in *Ximenia* oil, for complete confirmation of the structure. A number of different procedures have been described for the synthesis of monoenoic VLCFA. We chose previously described syntheses that make use of the Wittig reaction that produces *Z*-alkenates, and also an alternative method utilizing reactions between ω -iodo esters and complexes formed from methylcopper (1) and Grignard reagents.

Based on our previous experience with semi-preparative RP-HPLC (Rezanka, 2002; Rezanka and Sigler, 2006) we have now used its preparative mode for the rapid enrichment of the VLCFA fraction with minimal losses. The presence of VLCFAs in *Ximenia* oil was demonstrated by a newly developed method, i.e. identification of picolinyl esters of the VLCFAs by means of LC-MS/APCI. The present study describes also the synthesis of four positional isomers of dotriacontenoic acid.

2. Results and discussion

The RP-HPLC method is very useful for both analytical and preparative purposes. In an analytical mode and in combination with soft ionization mass spectrometry, this method gives excellent results, as has been repeatedly documented in our studies (Rezanka, 2002; Rezanka and Sigler, 2006). As mentioned above, the fatty acids from *Ximenia* oil have been identified and quantified by gas chromatography many times. One wonders, however, why the analyses done in the last century have not been repeated in the last 40

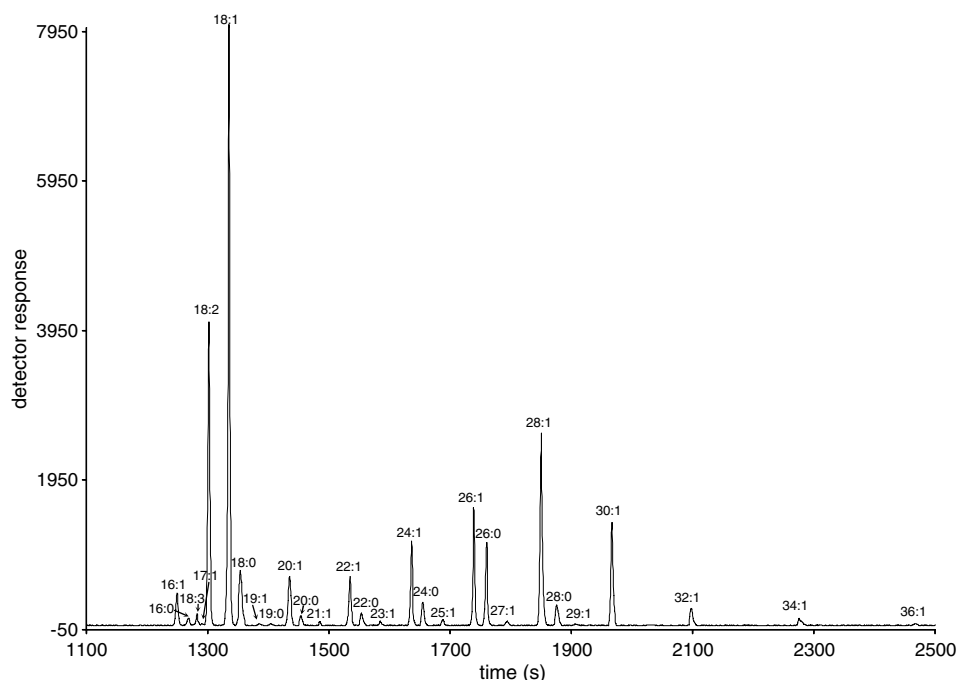


Fig. 1. LC-MS/APCI chromatogram of *Ximenia* oil. Peak identification, see Table 1.

years. This can be explained by the fact that, in recent years, all researchers have concentrated on identifying fatty acids with a triple bond (and/or a hydroxyl) in the molecule, completely neglecting the identification of VLCFA. In view of our long-term research concerning the presence of VLCFA in natural materials, we decided to fill this gap. Long-chain FA having double bond(s) tend to be thermally degraded during GC analysis (Rezanka, 1990; Rezanka and Mares, 1991). The problems associated with the separation and identification of VLCFA by gas chromatography, i.e. the thermal degradation under the conditions used for the elution from a GC column, and with their low proportion in the analyzed materials can be solved by using RP-HPLC, which eliminates the risk of thermal degradation, semipreparative RP-HPLC making it possible to enrich the FA fraction above 28:0. Identification and quantification can then be performed with fatty acid picolinyl esters by using LC-MS/APCI.

Although higher homologues are present in minor amounts, we succeeded in identifying in *Ximenia* oil FA up to tetracontenoic acid, including the position of the double bond. Fig. 1 shows the chromatogram of total FA from *Ximenia* oil up to 36:1 and the data are also given in Table 1. In contrast to previous studies (Rovesti, 1979; Fatope et al., 2000; Eromosele and Eromosele, 2002; Khumalo et al., 2002) we found the oil to contain both odd numbered monoenoic FA and VLCFA with chain length C34 and C36 that have not yet been identified in the oil. The formation of higher homologues of VLCFA drops dramatically with increasing chain elongation but the FA 40:1 is still detectable even though it constitutes only 0.0033% of total fatty acids. The enrichment of the individual fractions with VLCFA enabled us to identify previously unknown homologues. The total ion current was used for quantification, as shown also in Fig. 1. Table 2 shows the percent representation of VLCFA in the enriched complex of fatty acids of *Ximenia* oil. The fatty acids were monounsaturated from 29:1 up to 40:1 and saturated, odd and even numbered with a straight chain.

When analyzing monoenoic acids in the form of picolinyl derivatives, analysis of mass spectra revealed that most of the chromatographic peaks were not homogeneous but were formed by several positional isomers. An example of analysis of positional isomers is given for peak 34:1 of the original mixture, which constituted 0.114% of total fatty acids. Analysis of spectra led to the preliminary conclusion that the peak contains four (cf. Fig. 2) positional isomers of tetratriacontenoic acid in an approximate ratio of 13:7:65:15, their sequence being Δ^5 , Δ^9 , Δ^{25} , and Δ^{27} . The presence of multiple positional isomers of monoenoic acids in natural samples has been described several times (Rezanka, 1989, 2002). An illustrative example is the GC analysis of the 24:1 concentrate from flathead flounder lipids that showed four peaks having different ECL values (Ota et al., 1995). These peaks agreed with 11–24:1, 13–24:1, 15–24:1 and 17–24:1 isomers. The 24:1 isomers were dominated by 15–24:1 (83%), the other isomers being much

Table 1
Total fatty acids from *Ximenia* oil

Acid	Ratio of positional isomers in % ^a				Total %
	<i>n</i> – 7	<i>n</i> – 9	Δ^9	Δ^5	
16:1	7	93	0	0	1.88
16:0					0.43
18:3 <i>n</i> – 3	–	–	–	–	0.42
17:1	5	95	0	0	0.09
18:2 <i>n</i> – 6	–	–	–	–	21.34
18:1	6	94 ^b	0	0	32.51
18:0					2.54
19:1	6	94	0	0	0.11
19:0					0.12
20:1	5	95	0	0	0.57
20:0					0.23
21:1	7	93	0	0	2.43
22:1	6	94	0	0	2.27
22:0					0.67
23:1	5	95	0	0	0.18
24:1	8	92	0	0	4.68
24:0					1.16
25:1	8	92	0	0	0.32
26:1	10	90	0	0	6.77
26:0					3.84
27:1	9	91	0	0	0.27
28:1	10	90	0	0	9.68
28:0					0.97
29:1	11	89	0	0	0.096
30:1	12	85	3	0	5.355
30:0 ^c					0.023
31:1	14	84	2	0	0.054
32:1	15	80	5	0	0.891
32:0 ^c					0.033
33:1 ^c	14	80	6	0	0.027
33:0 ^c					0.003
34:1	15	65	7	13	0.114
34:0 ^c	17	62	8	13	0.017
35:1 ^c	15	58	9	18	0.014
36:1	12	61	10	17	0.032
37:1 ^c	16	57	8	19	0.012
38:1 ^c	19	52	11	18	0.013
40:1 ^c	7	93	0	0	0.003

^a The ratio of positional isomers, determined solely from the detector response to each one of the compounds and from the mass spectra of the respective picolinyl esters.

^b In this case *n* – 9 and Δ^9 positional isomer are identical, and represent oleic acid.

^c Determined only after sample enrichment, see Section 3.

less conspicuous. In addition, six positional isomers of 20:1 acid and five positional isomers of 22:1 acid were found. Chemical ionization tandem mass spectrometry was used to isolate FA from different sources and localize the position of the double bonds in their molecules; among these FA were, e.g., 15–24:1 and 17–24:1 acids and also their lower homologues (Michaud et al., 2002).

We synthesized esters 1–4 to confirm the structure of all four presumed positional isomers. Methyl (Z)-tetratriacont-27-enoate (1) and methyl (Z)-tetratriacont-25-enoate (2) were synthesized as depicted in Schemes 1 and 2, respectively. The key reaction in both cases was the Grignard reaction performed in the presence of cuprous iodide. When synthesizing acid 1, we did not succeed in finding an

available supplier of ω -bromostearic acid (**11**) and had to synthesize it according to the literature (Kling et al., 1993) from two commercially available compounds (**7** and **9**), again by using Grignard reaction in the presence of cuprous iodide. The subsequent reaction step, i.e. the joining of two long chains (compounds **6** and **12**) was analogous to that described above. In the case of acid **2**, both long chain compounds (i.e. **13** and **14**) were commercially available.

A different route (Scheme 3), based essentially on the study of Hahn et al. (1988), was followed for the synthesis of methyl (*Z*)-tetratriacont-9-enoate (**3**) and (*Z*)-tetratriacont-5-enoate (**4**). The key step for the synthesis of both esters (Scheme 3) was Wittig reaction of nonacosanal or pentacosanal with triphenylphosphonium bromides (**22a** or **23a**, respectively), affording the *Z*-configured esters **3** and **4**. Since the compounds having suitable position of (*Z*) double bond and a chain length of 25 and 29 carbon atoms are not commercially available, we used a different reaction sequence illustrated in Scheme 3. Both aldehydes needed for the Wittig reaction were prepared by a reduction of commercially available acids. ω -Bromo acids were also purchased (9-bromononanoic acid (**21**) from Matrix Scientific, Columbia, USA).

As seen from Tables 2–5, the spectra of all four positional isomers are virtually identical and, based on analysis of the spectra, we can only state that positional isomers cannot be distinguished solely by NMR. The isomeric purity of the esters **1–4** was determined using ^{13}C NMR spectroscopy (Table 2). Allylic carbons of straight chain *Z*-alkenes give rise to NMR signals in the range δ 27.2–27.4, while those of the corresponding *E*-alkenes appear in the range δ 32.6–32.9.

As we published earlier, the APCI mass spectra of the picolinyl esters of straight chain saturated fatty acids contain ions from the lower part of mass spectra in minor concentrations – see also Fig. 3. An entirely different situation arises in the M^+ region, which features two abundant ions $[\text{M}-1]^+$ and $[\text{M}+1]^+$ including the generation of adducts with the mobile phase such as $[\text{M}+40]^+$, $(\text{M} + \text{C}_2\text{H}_2\text{N})$ and $[\text{M}+54]^+$ ($\text{M} + \text{C}_3\text{H}_4\text{N}$). In all investigated VLCFA spectra, the ion $[\text{M}+\text{H}]^+$ is the most abundant one. Scheme 4

With monoenoic derivatives (34:1, see Fig. 3) the detection revealed also a minority group of ions with m/z 93, 109, 151 and 164. The same spectrum features ions that differ from others by a value of 14 Daltons, as well as other

Table 2
 ^1H NMR spectral data of the methyl esters **1–4**

	CH_3	CH_2	3- H_2	$-\text{CH}_2-\text{C}=\text{}$	CH_2COO	OCH_3	$-\text{CH}=\text{}$
1	0.89, <i>t</i> , 6.2	1.23 <i>m</i>	1.62, <i>m</i>	2.02, <i>m</i>	2.29, <i>t</i> , 7.3	3.64, <i>s</i>	5.35, <i>m</i>
2	0.89, <i>t</i> , 6.3	1.24 <i>m</i>	1.61, <i>m</i>	2.01, <i>m</i>	2.30, <i>t</i> , 7.2	3.65, <i>s</i>	5.34, <i>m</i>
3	0.88, <i>t</i> , 6.5	1.25 <i>m</i>	1.61, <i>m</i>	2.00, <i>m</i>	2.31, <i>t</i> , 7.4	3.64, <i>s</i>	5.36, <i>m</i>
4	0.88, <i>t</i> , 6.4	1.24 <i>m</i>	1.60, <i>m</i>	2.03, <i>m</i>	2.32, <i>t</i> , 7.3	3.65, <i>s</i>	5.34, <i>m</i>

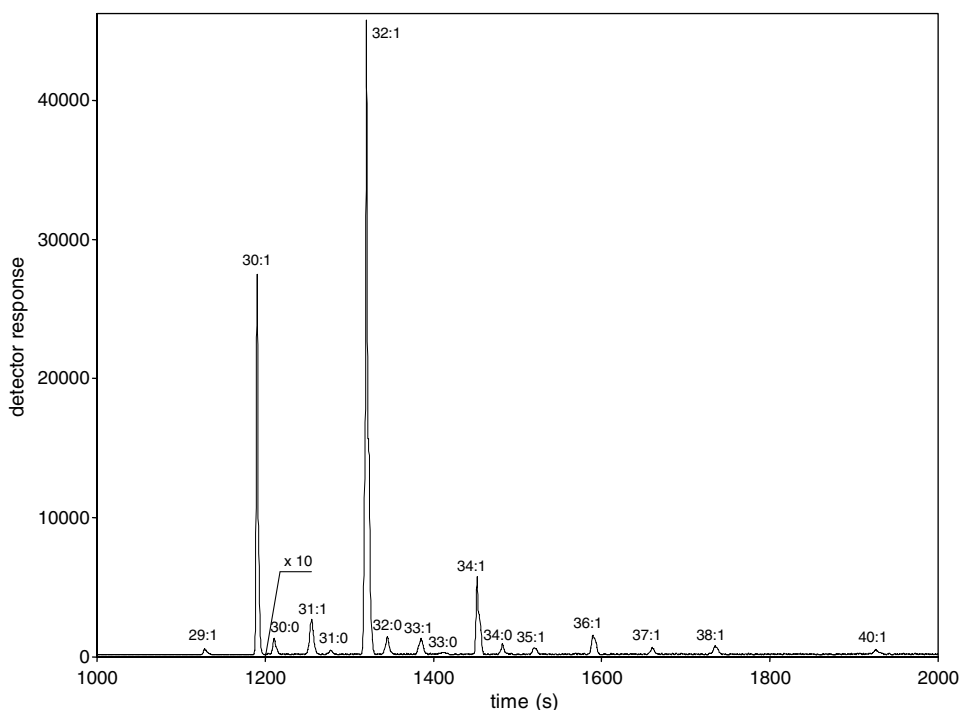
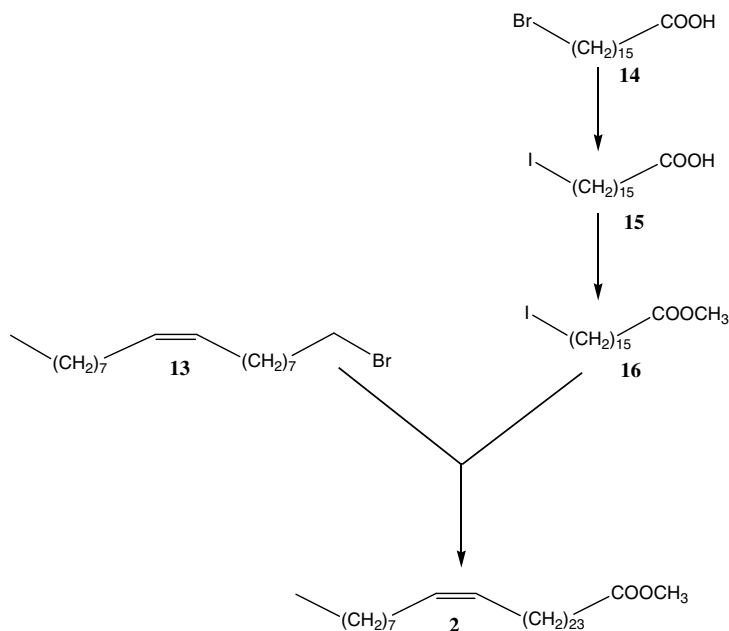
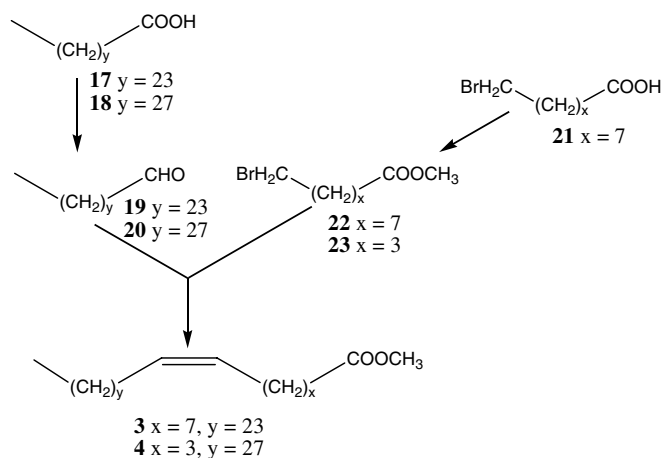


Fig. 2. LC-MS/APCI chromatogram of enriched fraction after preparative RP-HPLC. Peak identification, see Table 1.



Scheme 2.



Scheme 3.

was irradiated for a further 15 min and then cooled to -10°C . The resulting precipitate was filtered off and recrystallized from light petroleum to give the title bromide (**11**) as colorless crystals (77 mg, 59%), m.p. $36\text{--}37^{\circ}\text{C}$; ^1H NMR δ 1.45 (30H, *m*, CH_2), 2.28 (2H, *m*, CH_2COO), 3.38 (2H, *t*, $J = 6.5$, CH_2Br), 3.65 (3H, *s*, OCH_3); EIMS (m/z) 378 $[\text{M}]^+$, 376 $[\text{M}]^+$, 346, 344, 296, 264, 199, 143, 87, 74 (base peak).

Methyl 18-iodooctadecanoate (**12**). This compound, prepared from the bromo ester (**11**) (75 mg, 0.20 mmol) as described for the synthesis of the iodide (**8**), was obtained as colorless crystals after recrystallization from methanol (59 mg, 70%), m.p. $44\text{--}45^{\circ}\text{C}$; ^1H NMR δ 1.3–1.5 (26H, *m*, CH_2), 2.30 (2H, *m*, CH_2COO), 3.23 (2H, *t*, $J = 6.4$, CH_2I), 3.68 (3H, *s*, OCH_3); EIMS (m/z) 424 $[\text{M}]^+$, 392, 293, 265, 247, 155.

Table 3
 ^{13}C NMR spectral data of the methyl esters **1–4**

	ω -1 C	ω -2 C	C-3	$-\text{CH}_2-\text{C}=\text{CH}-$	CH_2	ω -3 C	C-2	OCH_3	$-\text{CH}=\text{CH}-$	C-1
1	14.3	22.8	25.0	27.2	29.0–30.0	32.0	34.0	51.6	129.9	174.5
2	14.4	22.9	25.1	27.2	29.0–30.0	32.1	33.9	51.5	129.8	174.5
3	14.2	22.7	24.9	27.1	29.0–30.0	31.9	34.1	51.5	130.0	174.4
4	14.3	22.8	24.9	27.0	29.0–30.0	32.0	34.0	51.6	130.1	174.3

Table 4
 ^1H NMR spectral data of the picolinyl esters **1a–4a**

	CH_3	CH_2	CH_2CON	NCH_2	$-\text{CH}=\text{CH}-$	H-5'	H-4'	H-6'	H-2'
1a	0.91, <i>t</i> , 6.8	1.20–1.44, <i>m</i>	2.23, <i>m</i>	5.10, <i>m</i>	5.30, <i>m</i>	7.14, <i>m</i>	7.50, <i>m</i>	8.35, <i>m</i>	8.40, <i>m</i>
2a	0.90, <i>t</i> , 6.8	1.21–1.43, <i>m</i>	2.21, <i>m</i>	5.09, <i>m</i>	5.31, <i>m</i>	7.15, <i>m</i>	7.51, <i>m</i>	8.37, <i>m</i>	8.41, <i>m</i>
3a	0.89, <i>t</i> , 6.8	1.20–1.47, <i>m</i>	2.22, <i>m</i>	5.08, <i>m</i>	5.30, <i>m</i>	7.14, <i>m</i>	7.49, <i>m</i>	8.35, <i>m</i>	8.39, <i>m</i>
4a	0.90, <i>t</i> , 6.8	1.22–1.44, <i>m</i>	2.21, <i>m</i>	5.09, <i>m</i>	5.29, <i>m</i>	7.16, <i>m</i>	7.51, <i>m</i>	8.36, <i>m</i>	8.41, <i>m</i>

Table 5
¹³C NMR spectral data of the picolinyl esters **1a–4a**

	ω -1	ω -2	C-3	CH ₂ -C=	CH ₂	ω -3	C-2	-CH=	C-1	NCH ₂	H-5'	H-4'	H-6'	H-2'	H-3'
1a	13.2	19.9	24.6	27.2	28.9–29.7	32.0	34.4	129.9	174.1	65.4	124.5	135.8	147.8	147.3	136.4
2a	13.0	19.8	24.6	27.2	28.9–29.7	32.1	34.3	129.8	174.2	65.5	124.6	135.9	148.1	147.3	136.3
3a	13.0	20.0	24.7	27.1	28.9–29.7	31.9	34.3	130.0	174.2	65.6	124.4	135.8	148.0	147.4	136.5
4a	13.1	20.0	24.8	27.0	28.9–29.7	31.9	34.2	130.1	174.1	65.5	124.5	135.7	147.9	147.5	136.4

Methyl (*Z*)-tetratriacont-27-enoate (**1**). A solution of methyllithium in ether (1.6 mol/l; 0.25 ml, 0.4 mmol) was added slowly to a suspension of cuprous iodide (76 mg, 0.4 mmol) in tetrahydrofuran (5 ml), while the temperature was maintained between -60 and -78 °C. The resultant mixture was stirred at -78 °C for 1 h after which it was slowly allowed to warm to 0 °C, whereupon a bright yellow suspension formed. The mixture was immediately cooled to -78 °C and a solution of magnesium bromide [formed by the addition of bromide (**6**) (85 mg, 0.28 mmol) to magnesium (24.3 mg, 1 mmol) in tetrahydrofuran (5 ml), under an atmosphere of nitrogen] was added, while the temperature was maintained below -60 °C. The mixture thus obtained was stirred at -78 °C for 1 h and then allowed to warm to 0 – 10 °C, whereupon a distinct purple coloration appeared. The mixture was then cooled to -78 °C and a solution of the ω -iodo ester (**12**) (58 mg, 0.14 mmol) in tetrahydrofuran (15 ml) was added, while the temperature was maintained below -60 °C. That mixture was stirred at -78 °C for 1 h and then allowed to warm to room temperature whereupon it was stirred for 2 h, before being poured into saturated aqueous ammonium chloride (20 ml). The layers that formed were separated and the aqueous layer was extracted with ether. The organic layer and the ether extracts were combined and the mixture was washed with brine, dried, and concentrated under pressure. Chromatography of the residual oil on silica gel, with ether-light petroleum as eluent gave the title ester (**1**), was obtained as a colorless wax (8.6 mg, 12%), m.p. 46 – 47 °C; IR (cm^{-1}) 3008, 2924, 2852, 1742, 1650, 1468, 1262, 1172, 1116 and 1014; EIMS (m/z) 521 [$\text{M}+1$]⁺, 520 [M]⁺, 489, 488, 446, 404, 143, 125, 111, 97, 83, 74, 69, 57, 55 and 43; HREIMS m/z 520.5218 [M]⁺, calc. for m/z 520.5219 [$\text{C}_{35}\text{H}_{68}\text{O}_2$]⁺; ¹H and ¹³C NMR data are shown in [Tables 3 and 4](#).

16-Iodoheptadecanoic acid (**15**). This compound, prepared from the bromo acid (**14**) (335 mg, 1 mmol) as described for the synthesis of the iodo derivative (**8**), was obtained as colorless crystals (332 mg, 87%), m.p. 71 – 73 °C; EIMS m/z 382 [M]⁺, 364, 143, 125, 97, 83, 55; ¹H NMR δ 1.60 (26H, *m*, CH₂), 2.37 (2H, *m*, CH₂COO), 3.21 (2H, *t*, $J=6.5$, CH₂I).

Methyl 16-iodoheptadecanoate (**16**). Iodo acid (**15**) (325 mg, 0.85 mmol) was added to methanol (20 ml) that had been pretreated with thionyl chloride (1 ml), and the mixture was stirred at room temperature for 4 h. The solvent was then removed under reduced pressure and the residue was dissolved in dichloromethane (10 ml). The resultant solution was washed with saturated aqueous sodium hydrogen carbonate, dried, and concentrated under

reduced pressure. The yield was obtained as colorless crystals after recrystallization from methanol (276 mg, 82%), m.p. 37.5 – 38 °C; EIMS m/z 396 [M]⁺, 364, 259, 226, 208; ¹H NMR δ 1.62 (26 H, *m*, CH₂), 2.31 (2H, *m*, CH₂COO), 3.22 (2H, *t*, $J=6.5$, CH₂I), 3.70 (3H, *s*, OCH₃).

Methyl (*Z*)-tetratriacont-25-enoate (**2**). This compound, prepared from the oleyl bromide (**13**) (450 mg, 1.36 mmol) and the ω -iodo ester **16** (270 mg, 0.68 mmol) as described for the synthesis of the ester (**1**), was obtained as a colorless wax (52 mg, 14.6%), m.p. 45.5 – 46 °C; IR (cm^{-1}) 3004, 2928, 2848, 1742, 1650, 1468, 1172; EIMS m/z 521 [$\text{M}+1$]⁺, 520 [M]⁺, 489, 488, 446, 415, 97, 83, 74 (base peak), 69, 57, 55; HREIMS m/z 520.5217 [M]⁺, calc. for m/z 520.5219 [$\text{C}_{35}\text{H}_{68}\text{O}_2$]⁺; ¹H and ¹³C NMR data are shown in [Tables 2 and 3](#).

Pentacosanal (**19**). An oven-dried, 100-ml flask, fitted with a side arm and a vent adapter connected to a mercury bubbler, was flushed with nitrogen and charged with 122 mg (1 mmol) of 9-borabicyclo[3.3.1]nonane (9-BBN) and pentacosanoic acid (**17**) (383 mg, 1 mmol) ([Chaz et al., 1987](#)). To this mixture was added 1 ml of THF and the slurry was stirred at room temperature until hydrogen evolution ceased. After completing the hydrogen evolution, 2.1 ml of 0.5 M solution of Li 9-BBNH ([Brown et al., 1984](#)) (1.05 mmol, 5% excess) in THF was injected and the reaction mixture was stirred for 1 h at room temperature. The reaction mixture was hydrolyzed with 20 ml of water for 1 h at room temperature. The mixture was then saturated with NaCl and the organic layer was separated, dried, and subjected to crystallization. The yield was colorless crystals (334 mg, 91%), m.p. 55 – 57 °C; IR (cm^{-1}) 2780 (CHO), 1720 (C=O); ¹H NMR δ 0.90 (3H, *t*, $J=6.8$, CH₃), 1.00–1.90 (44H, *m*, CH₂), 2.35 (2H, *t*, $J=7.2$, CH₂CHO), 9.75 (1H, *t*, $J=1.95$, CHO); EIMS m/z 366 [M]⁺, 365, 348, 336, 322. These data are in agreement with the values given by [Marukawa et al. \(2001\)](#).

Nonacosanal (**20**). This compound was prepared from the nonacosanoic acid (**18**) (439 mg, 1 mmol) as described for the synthesis of the aldehyde (**19**). The yield of aldehyde (**20**) as colorless crystals was 90% (381 mg), m.p. 84 – 86 °C; IR (cm^{-1}) 2780 (CHO), 1720 (C=O); ¹H NMR δ 0.91 (3H, *t*, $J=6.8$, CH₃), 1.00–1.90 (52H, *m*, CH₂), 2.35 (2H, *t*, $J=7.2$, CH₂CHO), 9.73 (1H, *t*, $J=1.8$, CHO); EIMS m/z 422 [M]⁺, 421, 404, 392, 378.

Methyl 9-bromononanoate (**22**). Bromoester (**22**), prepared from the bromoacid (**21**) (286 mg, 1.21 mmol) as described for the synthesis of the ester (**16**), was obtained as a colorless oil (251 mg, 83%). ¹H NMR δ 1.3–1.8

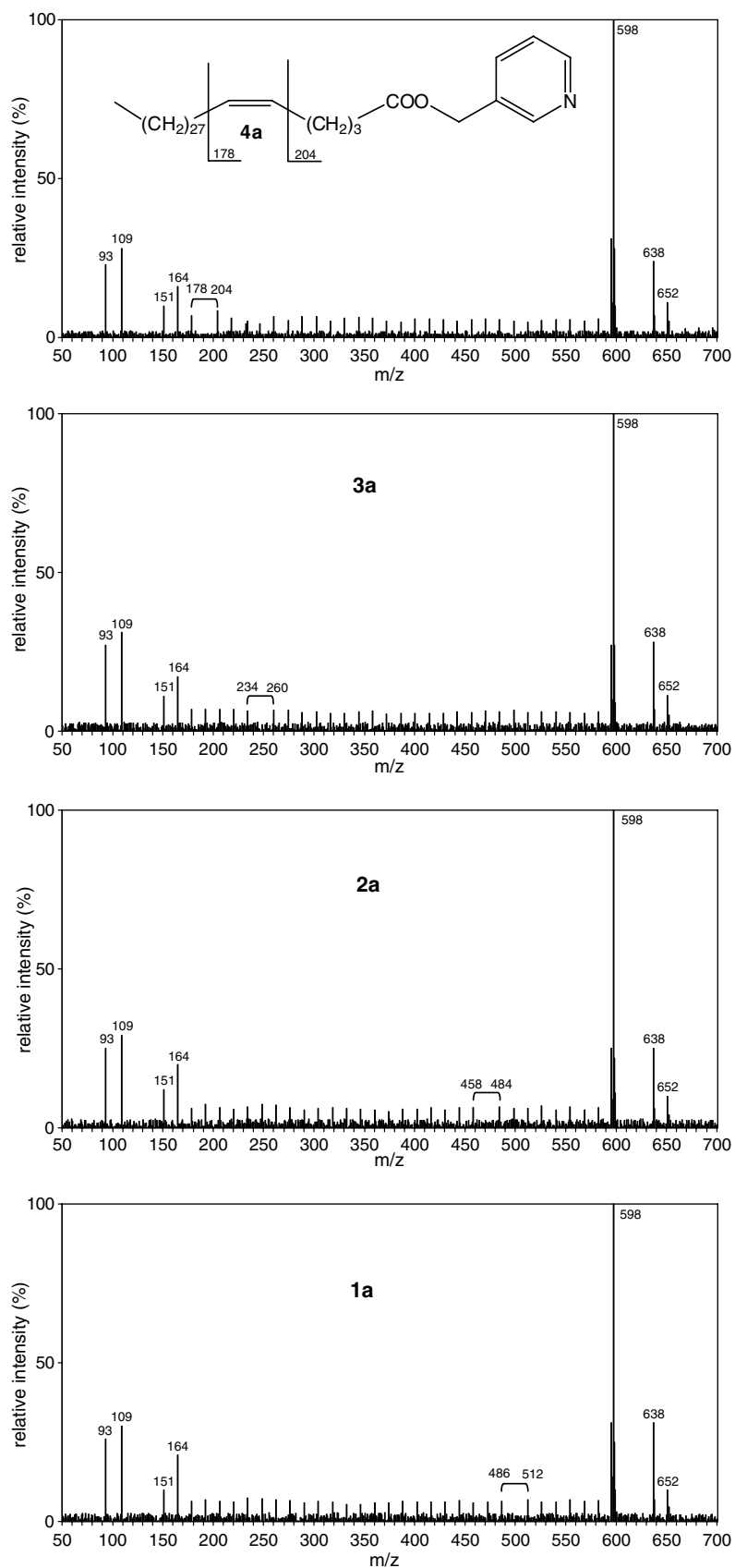
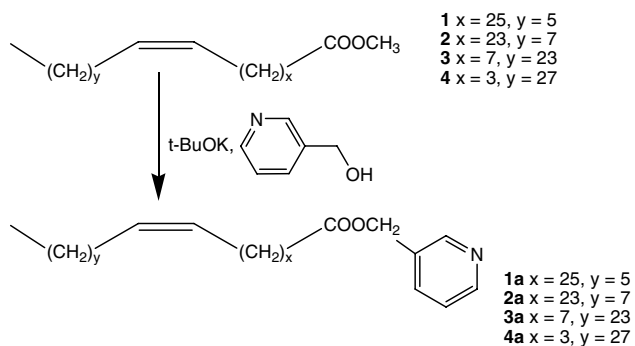


Fig. 3. Mass spectra of picolinyl esters of positional isomers of synthetic 34:1 acid. For explanation of values see the text.



Scheme 4.

(10H, *m*, CH₂), 1.91 (2H, *m*, CH₂CH₂Br) 2.28 (2H, *t*, *J* = 7.4 Hz, CH₂CO), 3.40 (2H, *t*, *J* = 7.3 Hz, CH₂Br), 3.66 (3H, *s*, OCH₃); EIMS (*m/z*) 252 [M]⁺, 250 [M]⁺.

Methyl 5-(triphenylphosphonium bromide) nonanoate (**22a**). A mixture of the bromoester (**22**) (251 mg, 1 mmol), triphenylphosphine (524 mg, 2 mmol) and acetonitrile (5 ml) was heated under reflux for 16 h. The resultant mixture was cooled and concentrated under reduced pressure to afford a colorless solid, which was washed several times with ethyl acetate and then recrystallized from dichloromethane–light petroleum to give the title salt as colorless crystals (251 mg, 55%). HREIMS *m/z* 456.0849 [M]⁺, calc. for [C₂₄H₂₆BrO₂P]⁺ 456.0854.

Methyl 5-(triphenylphosphonium bromide) valerate (**23a**) was prepared from the bromoester (**23**) (195 mg, 1 mmol) as described for the synthesis of the bromide (**22a**). It was obtained as colorless crystals (266 mg, 52%). HREIMS *m/z* 512.1481 [M]⁺, calc. for [C₂₈H₃₄BrO₂P]⁺ 512.1479.

Methyl (*Z*)-tetratriacont-9-enoate (**3**). A solution of lithium hexamethyldisilazide, generated by the addition of butyllithium in hexane (1.5 M; 0.34 ml, 0.51 mmol) to hexamethyldisilazane (0.11 ml, 0.51 mmol) in tetrahydrofuran (2 ml) at 0 °C, was added to a suspension of bromide (**22a**) (236 mg, 0.5 mmol) in tetrahydrofuran–hexamethylphosphoramide (4:1; 3 ml) cooled to 0 °C. The resultant orange solution was stirred for 10 min at 0 °C and then cooled to –78 °C when a solution of the aldehyde (**19**) (92 mg, 0.25 mmol) in THF (3 ml) was added to it at that temperature. The mixture was subsequently allowed to warm to 0 °C and was stirred at that temperature for 1 h; it was then poured into saturated aqueous ammonium chloride and extracted with ethyl acetate. The combined organic extracts were washed with water, dried, and concentrated under reduced pressure to give oil, which was chromatographed with hexane as eluent. After reaction with diazomethane the title ester **3** was obtained as colorless crystals after recrystallization from acetone (74 mg, 57%), m.p. 44–46 °C; IR (cm^{–1}) 2995, 2930, 2850, 1734, 1465, 1442, 1175; HREIMS *m/z* 520.5220 [M]⁺, calc. for *m/z* 520.5219 [C₃₅H₆₈O₂]⁺; ¹H and ¹³C NMR data are shown in Tables 2 and 3.

Methyl (*Z*)-tetratriacont-5-enoate (**4**). This compound, prepared from the bromide (**23a**) (215 mg, 0.5 mmol) and aldehyde (**20**) (106 mg, 0.25 mmol) as described for the synthesis of the ester (**4**), was obtained as colorless crystals (74 mg, 57%), m.p. 44–46 °C; IR (cm^{–1}) 2995, 2930, 2850, 1734, 1465, 1442, 1175; HREIMS *m/z* 520.5220 [M]⁺, calc. for *m/z* 520.5219 [C₃₅H₆₈O₂]⁺; ¹H and ¹³C NMR data are shown in Tables 2 and 3.

Picolinyl esters of **1a–4a**. A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 ml, 1.0 M) was added to nicotinyl alcohol (1 ml). After mixing, the appropriate fatty acid methyl ester (~30 mg) in dry dichloromethane (1 ml) was added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

1a–4a as colorless, waxy compounds, LC–MS/APCI: *m/z* 598 [M+H]⁺; HREIMS *m/z* 597.5486–597.5491 C₄₀H₇₁NO₂ [M]⁺, calculated for [M]⁺ 597.5484; for ¹H and ¹³C NMR data see Tables 4 and 5.

3.2. Preparative chromatography

A Chromatospac Prep 100 preparative chromatograph (Jobin-Yvon, Longjumeau, France) with axial compression of the chromatographic bed (61.2 × 8 cm ID) was used for the isolation of the fraction enriched with picolinyl esters. The column efficiency was 6510 theoretical plates, V₀ was 1185 ml and t_R of the picolinyl ester of octacosanoic acid (28:0) was 23.0 min. The mobile phase flow rate was 55 ml/min. The mean particle diameter of the Separon C18 reversed-phase packing (formerly Laboratorní přístroje, Prague, Czechoslovakia) was 15 μm. A Holochrome H/MD variable-wavelength detector (Gilson, France) with a 40-μl flow-through cell was set at 235 nm. All experiments were carried out at room temperature.

The amount of 290 mg picolinyl esters (see below) was dissolved in 15 ml of the mobile phase (injection volume) (methanol–isopropanol–triethylamine, 87:13:0.05). In the time interval 0–35 min the column was eluted with mobile phase that was later replaced with diethyl ether (60 min). The column was again conditioned with mobile phase for 15 min. The fraction up to 23.0 min was discarded and that obtained within the interval 23.0–83 min was used for further analysis. After evaporation of the mobile phase, the total yield of a mixture of fatty acids with the chain longer than 28 carbon atoms was 19.3 mg. These were separated and identified by LC–MS.

3.3. LC–MS/APCI

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett-Packard, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm phase particle, in series. This setup provided us with a high-efficiency column

– approximately ~26,000 plates/250 mm. A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 400 °C, capillary heater temperature 220 °C, corona current 5 μ A, sheath gas – high-purity nitrogen, pressure 0.38 MPa, and auxiliary gas (also nitrogen) flow rate 15 ml/min). Positively charged ions with m/z 70–900 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.37 ml/min) was introduced into the APCI source without any splitting. Fatty acid picolinyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM) and propionitrile (EtCN) as follows: initial ACN/EtCN/DCM (60:30:10, vol/vol/vol); linear from 10 min to 40 min ACN/EtCN/DCM 30:40:30, vol/vol/vol; held until 60.5 min; the composition was returned to the initial conditions over 8 min. A peak threshold of 0.3% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows XP applications/operating software.

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