Lipids (2010) 45:743–756
DOI 10.1007/s11745-010-3447-7

METHODS

RP-HPLC/MS-APCI Analysis of Branched Chain TAG Prepared by Precursor-Directed Biosynthesis with *Rhodococcus erythropolis*

Olga Schreiberová · Tereza Krulikovská · Karel Sigler · Alena Čejková · Tomáš Řezanka

Received: 26 May 2010 / Accepted: 29 June 2010 / Published online: 16 July 2010

Abstract Reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (RP-HPLC/MS-APCI) was used to analyze both synthetic triacylglycerols (TAG) having 1–3 branched fatty acids (FA) in the molecule, and natural TAG prepared by precursor directed biosynthesis from valine, leucine and isoleucine and the corresponding branched short-chain acids in cultivations of *Rhodococcus erythropolis*. The technique made it possible to identify and quantify TAG differing in a single branched-chain FA. Altogether 11 TAG were synthesized, out of which 8 were synthesized stereospecifically. Branched- and straight-chain-TAG were separated and identified while TAG differing only in *iso* or *anteiso* FA could not be separated. The APCI mass spectra of *iso*-, *anteiso*- and straight-chain TAG were completely identical. The natural material was found to contain 19 TAG having at least one branched FA. Cultivation on six different substrates showed, apart from the presumed and common incorporation of precursors to *iso*-even, *iso*-odd and *anteiso* FA, also some unusual features such as an increase in the content of odd-FA after the addition of Val (attributed to catabolism of Val to propionate) or the appearance of branched monounsaturated FA. The two-sample paired *t* test, when applied to the TAG, showed that only the pair Val and isobutyrate differ in incorporation into FA—see, e.g. proportions of M/M/O and brM/brM/O (1.2:1.2 and 1.9:1.2, respectively). Also, incorporation of Val (isobutyrate) yielded only TAG having two branched FA in the molecule, whereas Leu and Ile (isovalerate and 2-methylbutyrate) gave only TAG with a single branched FA in the molecule.

Keywords *Rhodococcus erythropolis* · RP-HPLC/MS-APCI · Branched chain triacylglycerols · Precursor directed biosynthesis

Abbreviations

GC-MS	Gas chromatography–mass spectrometry.
LC-MS	Liquid chromatography–mass spectrometry
RP-HPLC/MS-APCI	Reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry
FA	Fatty acid(s)
TAG	Triacylglycerol(s)
DAG	Diacylglycerol(s)
MAG	Monoacylglycerol(s)
BrFA	Branched (i or ai) FA
BrTAG	Branched (i or ai) TAG
i-Bu	Isobutyric acid
i-Va	Isovaleric acid
2-MeBu	2-Methylbutyric acid
Val	Valine
Leu	Leucine
Ile	Isoleucine
DMAP	4-Dimethylaminopyridine
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
ACN	Acetonitrile

O. Schreiberová · T. Krulikovská · A. Čejková
Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague, Czech Republic

K. Sigler · T. Řezanka (✉)
Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic
e-mail: rezanka@biomed.cas.cz

iPrOH	2-Propanol
i-14:0 (iM)	12-Methyltridecanoic acid
14:0 (M)	Tetradecanoic acid
i-15:0 (iX)	13-Methyltetradecanoic acid
ai-15:0 (aiX)	12-Methyltetradecanoic acid
15:0 (X)	Pentadecanoic acid
i-16:0 (iP)	14-Methylpentadecanoic acid
16:0 (P)	Hexadecanoic acid
i-16:1 (iPo)	14-Methylpentadecenoic acid
16:1 (Po)	Hexadecenoic acid
i-17:0 (iMa)	15-Methylhexadecanoic acid
ai-17:0 (aiMa)	14-Methylhexadecanoic acid
17:0 (Ma)	Heptadecanoic acid
i-17:1 (iMo)	15-Methylhexadecenoic acid
ai-17:1 (aiMo)	14-Methylhexadecenoic acid
17:1 (Mo)	Heptadecenoic acid
i-18:0 (iS)	16-Methyloctadecanoic acid
18:0 (S)	Octadecanoic acid
i-18:1 (iO)	16-Methyloctadecenoic acid
18:1 (O)	Octadecenoic acid

Introduction

Many prokaryotic microorganisms such as *Mycobacterium* sp., *Nocardia* sp., *Micromonospora* sp., *Dietzia* sp., *Gordonia* sp., *Streptomyces* sp. and *Rhodococcus* sp. accumulate large amounts of lipids in lipid bodies in the cells and mycelia [1, 2]. In the genus *Rhodococcus*, the information about the structure of lipids in the cytoplasmic lipid bodies, in particular triacylglycerols (TAG), is rather scarce [3, 4].

As a carbon source, the genus *Rhodococcus* can utilize very unusual compounds such as hexadecane [5] or phenyldecane that gives rise to triacylglycerol in which one fatty acid was replaced by a phenyldecanoic acid residue [6], or 1-chloro-, l-bromo- and 1-iodohexadecane that ultimately yield ω-halogen fatty acids (FA) [7]. Furthermore, alkanes can be directly oxidized and activated to the respective acyl-CoA thioesters and incorporated into TAG [3, 8]. The composition of the TAG is known to vary strongly when the cells are cultivated on propionic acid, which is activated to propionyl-CoA, resulting in a much higher fraction of odd-numbered FA (over 95% total FA) [8]. With valerate addition the proportions of odd-chain FA can reach nearly 85% total FA.

With other bacteria, the addition of branched amino acids, i.e. Val, Leu or Ile, or their metabolites such as isobutyric (i-Bu), isovaleric (i-Va) or 2-methylbutyric (2-MeBu) acids into the culture medium induces the production of increased amounts of branched FA enriched with the given branched-FA depending on the starter unit (addition of Val produces even-*iso*-FA, addition of Leu odd-*iso*-FA, addition of Ile odd-ai-FA). This fact has been noted many times in myxobacteria [9], in *Nonomuraea* sp. [10], *Propionibacterium freudenreichii* [11], *Staphylococcus xylosus* [12] or *S. carnosus* [13].

Current studies have shown that branched chain FA, predominantly i-15:0 (*iso*-pentadecanoic) and ai-15:0 (*anteiso*-pentadecanoic) inhibit the growth of various cancer cell lines both in vitro and in vivo [14, 15]. Other experiments have documented that their activity decreases with an increase or decrease in chain-length from i-16:0 (isopalmitic) [16]. This effect is explained as being due to cancer cells being more dependent on fatty acid biosynthesis than healthy cells.

As shown in our earlier study of *Rhodococcus* lipids [17], LC–MS analysis of odd-chain TAG is still largely unexplored, mostly due to the scarcity of sources with odd-chain TAG. The situation with branched-chain TAG from natural sources is even worse. To our knowledge only two sources of branched TAG have so far been identified, both of them of bacterial origin. Metz et al. [18] used GC–MS to separate and identify intact TAG from *Streptomyces avermitilis* containing odd- and even-chain FA including *iso*- and *anteiso*-FA. Another source of branched TAG is milk; branched FA contained in it are largely derived from bacteria leaving the rumen [14, 15]. With one exception [19], even here branched TAG have not been separated from straight-chain TAG. All other studies [20, 21] mention only odd-chain TAG, although branched-TAG also have to be present, as seen from the analysis of FA in butter [19, 21]. Figure 1 in the paper by Marai et al. [21] clearly shows the presence, between peaks 48:0 (PPP) and 49:0 (PPMa), of two unidentified peaks that could belong to TAG 49:0 having branched margaric acids (i.e. i-17:0 and/or ai-17:0). Only Myher et al. [19] have given the TCN (theoretical carbon number) for branched TAG 0.38 less than for straight-chain TAG; to our knowledge this is thus the only paper that has explored the LC–MS of branched TAG.

Based on our previous experience with the cultivation of *Rhodococcus* [22] and with LC–MS of lipids [23, 24] we used here precursor directed biosynthesis to prepare branched TAG, which were qualitatively and quantitatively analyzed by using synthetic standards.

Experimental

Microbial Material

Rhodococcus erythropolis CCM 2595 obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) was subjected to a 6-month

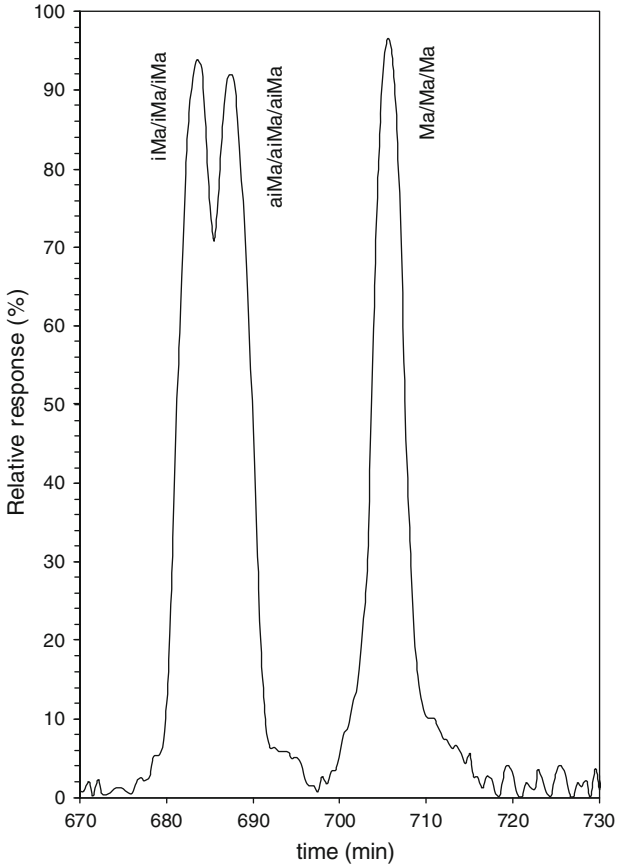

Fig. 1 RP-HPLC/APCI-MS chromatogram of three synthetic TAG (Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa)

physiological adaptation to phenol. The strain reached a high growth rate in the presence of succinate (10 g/L) used as the sole carbon source. Cells were grown in the basic mineral medium (g/L: KH_2PO_4 0.17; $MnCl_2{\cdot}4H_2O$ 0.001; K_2HPO_4 0.13; $CaCl_2{\cdot}2H_2O$ 0.00026; $(NH_4)_2SO_4$ 0.71; $FeSO_4{\cdot}7H_2O$ 0.0006; $MgCl_2{\cdot}6H_2O$ 0.34; $Na_2MoO_4{\cdot}2H_2O$ 0.002; pH was adjusted to 7.0) containing alternatively amino acids or short carboxylic acids (sodium salts) as the carbon source (valine, leucine, isoleucine, isovalerate, isobutyrate, 2-methyl butyrate—each 3 g/L) and 1 g/L of succinate as cosubstrate. The cultivations were performed on a rotary shaker at 100 rpm and 30 °C.

The cells were collected at the end of exponential phase (97 h) by centrifugation (10 min, 9,050*g*), washed twice with physiological solution and lyophilized (dry biomass yield depending on the C-sources: 353 mg/L—valine, 438 mg/L—leucine, 663 mg/L—isoleucine, 690 mg/L—isovalerate, 583 mg/L—isobutyrate, 645 mg/L—2-methyl butyrate, 890 mg/L—succinate).

Standards and Isolation

Acetonitrile, 2-propanol, hexane, dichloromethane, glycerol, 4-dimethylaminopyridine (DMAP), *N*,*N*′-dicyclohexyl-carbodiimide (DCC), and standards of glyceryl tripalmitate and glyceryl triheptadecanoate were purchased from Sigma-Aldrich (Prague, CR). The mono- and diacylglycerols were purchased from Larodan Fine Chemicals, Malmo, Sweden.

Preparation of Mixed FA Composition TAG

The esterification of glycerol with acids has been described previously [25]. Briefly, a rapidly stirred suspension of 1,3-diacylglycerol or 1,2-diacylglycerol (10 μmol), the acid (11 μmol), and a catalytic amount of 4-dimethylamino-pyridine (2 μmol) in anhydrous CH_2Cl_2 (5 mL) was treated with *N*,*N*′-dicyclohexyl-carbodiimide (12 μmol). The resulting mixture was stirred under N_2 for 24 h at room temperature, diluted with CH_2Cl_2, and filtered to remove precipitated 1,3-dicyclohexyl urea. The filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine and dried ($MgSO_4$). The solvent was removed in vacuo, and the remaining residue was purified by TLC (silica gel H, developed in hexane/diethyl ether/acetic acid (70:30:1, by vol.). The yields of appropriate TAG are in Table 1. The purity (see also Table 1) of individual synthesized TAG was determined by RP-HPLC, for the conditions see below.

Preparation of Single FA Composition TAG

The TAG were prepared as described above, only the ratios of FA and glycerol were different, i.e. for 10 μmol of FA, 3 μmol of glycerol, 4 μmol of 4-dimethylaminopyridine, and 36 μmol of *N*,*N*′-dicyclohexyl-carbodiimide was used. Further separation and purification was identical, see above. The yields are in Table 1.

The lyophilized cells were mixed with 10 mL of hexane and the mixture was stirred for 15 min. The cells were filtered off, hexane was evaporated and the oil samples

Table 1 Synthesis of TAG

Alcohol (1 μmol)	Acid/μmol	TAG	Yield (%)	Purity (%)
Glycerol	Ma/3.15	Ma/Ma/Ma	92	≥99
Glycerol	iMa/3.15	iMa/iMa/iMa	90	≥99
Glycerol	aiMa/3.15	aiMa/aiMa/aiMa	87	≥99
1-Monopalmitin	Ma/2.10	P/Ma/Ma	75	≥99
1-Monopalmitin	iMa/2.10	P/iMa/iMa	73	≥99
1-Monopalmitin	aiMa/2.10	P/aiMa/aiMa	76	≥99
1,2-Dipalmitin	Ma/1.05	P/P/Ma	69	>98
1,2-Dipalmitin	iMa/1.05	P/P/iMa	68	>98
1,2-Dipalmitin	aiMa/1.05	P/P/aiMa	70	>98
1,3-Dipalmitin	Ma/1.05	P/Ma/P	68	>98
1,3-Diheptadecanoin	P/1.05	Ma/P/Ma	64	>97

were dissolved in an acetonitrile-2-propanol-hexane mixture (1:1:1, v/v/v), which was injected on the column.

FAME Analysis

The TAG (10 mg) were saponified overnight in 10% KOH–MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing FA, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2. GC–MS of fatty acid methyl ester (FAME) mixture was done on a Finnigan 1020 B in EI mode. Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. The structures of FAME were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAME (Supelco, Prague).

RP-HPLC/MS-APCI

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5-μm particle size, 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 390 °C, capillary heater temperature 260 °C, corona current 7 μA, sheath gas—high-purity nitrogen, pressure 0.45 MPa, and auxiliary gas (also nitrogen) flow rate 15 mL/min]. Positively charged ions with *m/z* 200–1,000 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.35 mL/min) was introduced into the APCI source without any splitting. TAG were separated using a gradient solvent program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, v/v); linear from 5 to 120 min ACN/iPrOH 30:70, v/v); held until 30 min; the composition was returned to the initial conditions over 10 min. Isocratic separation by the mixture ACN/iPrOH 65:35, v/v) was used for resolution of the regioisomers. A peak threshold of 0.07% intensity was applied to the mass spectra.

The saturated synthesized TAG (Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa) were dissolved in ACN/iPrOH mixture (99:1, v/v) to concentrations of 1, 5, 10, 50, and 100 μg/mL. All calibration curves were measured using a 10 μL injection volume of solutions in three repeated analyses and the average peak areas were used for the construction of calibration curves. For reliable quantitation, concentrations of individual TAG in analyzed samples have to be in a given interval (after a suitable dilution). The limits of detection at S/N = 3 were determined with the injection volume 10 μL and averaged as 1 μg/mL for saturated TAG. RFs are expressed relative to Ma/Ma/Ma, which is set to 1.00. For MS-APCI *a* and *b* values are coefficients of the linear calibration dependence $y = ax + b$ and RFs are calculated as $RF = a_{Ma/Ma/Ma}/a_{TAG}$, because *b* values can be neglected. Here r^2 is the value of coefficient of determination, *y* corresponds to the peak areas and *x* is the concentration in μg/mL.

Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows XP applications/operating software.

Results and Discussion

As seen in Table 2, FA from *Rhodococcus* comprise not only odd-chain FA [17], but also branched-chain FA that

Table 2 Fatty acid compositions (%) of seven cultivations of *R. erythropolis* at 30 °C with different carbon sources [succinate, Val, Leu, Ile, isobutyric (i-Bu), isovaleric (i-Va) and 2-methyl-butyric (2-MeBu) acid] as determined by GC–MS

	Succinate	Val	i-Bu	Leu	i-Va	Ile	2-MeBu
i-14:0 (iM)	–[a]	8.1	6.7	–	–	–	–
14:0 (M)	9.8	6.3	5.9	4.5	5.3	3.8	4.4
i-15:0 (iX)	–	–	–	14.8	14.2	–	–
ai-15:0 (aiX)	–	–	–	–	–	19.1	20.4
15:0 (X)	8.2	13.5	14.2	11.6	10.8	7.6	6.7
i-16:0 (iP)	–	18.2	17.6	–	–	–	–
16:0 (P)	19.3	12.1	13.0	16.3	15.7	11.2	10.8
i-16:1 (iPo)		0.3	0.2	–	–	–	–
16:1 (Po)	18.5	13.1	14.1	13.1	14.5	10.9	11.3
i-17:0 (iMa)	–	–	–	19.5	20.2	–	–
ai-17:0 (aiMa)	–	–	–	–	–	21.3	19.7
17:0 (Ma)	4.9	9.3	8.8	3.4	4.1	4.2	4.6
i-17:1 (iMo)	–	–	–	0.7	0.6	–	–
ai-17:1 (aiMo)	–	–	–	–	–	0.6	0.5
17:1 (Mo)	9.2	6.7	7.1	5.4	4.8	5.7	6.2
i-18:0 (iS)	–	0.6	0.5	–	–	–	–
18:0 (S)	3.1	1.8	1.6	0.9	0.6	2.8	2.9
i-18:1 (iO)	–	0.4	0.5	–	–	–	–
18:1 (O)	27.0	9.6	9.8	9.8	9.2	12.8	12.5

[a] Minority fatty acids up to 0.1% of total fatty acids were omitted

were produced by precursor directed biosynthesis from branched chain precursors. In view of the scarcity of data on branched chain TAG (see above) we decided to synthesize a series of standards (see Table 1) that were used to verify the basic chromatographic and mass spectral characteristics. The standards were synthesized by conventional procedures, as simply as possible and with the highest yield. We followed the procedure described by Lie Ken Jie and Lam [26] who synthesized TAG of AAB, ABA, BBA and BAB types. Commercially available glycerol, monoacyl- and diacylglycerols (1-palmitin, 1,2-dipalmitin, 1,3-dipalmitin and 1,3-diheptadecanoin) yielded a total of 11 TAG by mild organic synthesis performed at 25 °C for 24 h under catalysis with dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (see "Experimental").

Individual TAG were analyzed by RP-HPLC. Figure 1 shows the chromatographic analysis of three synthetic TAG, Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa. The separation of straight-chain TAG from both branched-chain TAG presented no problems while separation of iMa/iMa/iMa from aiMa/aiMa/aiMa was hours-long—a time span unacceptable in the analysis of a natural sample—and succeeded only under isocratic conditions using the mobile phase ACN-iPrOH (65:35, v/v).

Chromatographic separation of branched TAG having two branched FA in the molecule (two i-17:0 or two ai-17:0) from straight-chain counterparts presents no problems—gradient elution provided a base-line separation of P/Ma/Ma from P/aiMa/aiMa or P/Ma/Ma from P/iMa/iMa, as shown in Figs. 2 and 3. Separation of two branched TAG such as P/iMa/iMa and P/aiMa/aiMa could be accomplished only by isocratic elution and only with synthetic TAG. We ascribe this phenomenon to the much higher complexity of the natural mixture that results in a mutual influencing and overlapping of peaks.

In the case of TAG having a single branched FA we succeeded in separating only those TAG that differed in the site of branching. The separation was successful only in the pairs P/P/Ma and P/P/iMa, and P/P/Ma and P/P/aiMa, in both the synthetic and the natural mixture and with both isocratic and gradient elution. The separation P/P/iMa and P/P/aiMa was unsuccessful.

Positional isomers such as P/P/Ma and P/Ma/P or P/Ma/Ma and Ma/P/Ma were partially separated only with isocratic elution (see [27] for positional isomers, i.e. POP and PPO). These two pairs were much more poorly separated than positional isomers in which individual FA differ in two methylene groups, such as P/P/S and P/S/P [17].

The APCI mass spectra of an AAA type TAG are very simple. For instance, trianteisomargarin (aiMa/aiMa/aiMa), exhibits four clusters of ions, i.e. $[M+H]^+$ ($[TAG]^+$, i.e. $[aiMa/aiMa/aiMa]^+$) at *m/z* 849, $[M\text{-}RCOO]^+$ ion ($[DAG]^+$, i.e. $[aiMa/aiMa]^+$) at *m/z* 579 arising by the loss of margarate $[RCOO+58]^+$ called also ($[MAG]^+$, i.e. $[aiMa]^+$) at *m/z* 327, and $[RCO]^+$ ion (FA^+, i.e. $aiMa^+$) at *m/z* 253, see also Table 3 and Fig. 4.

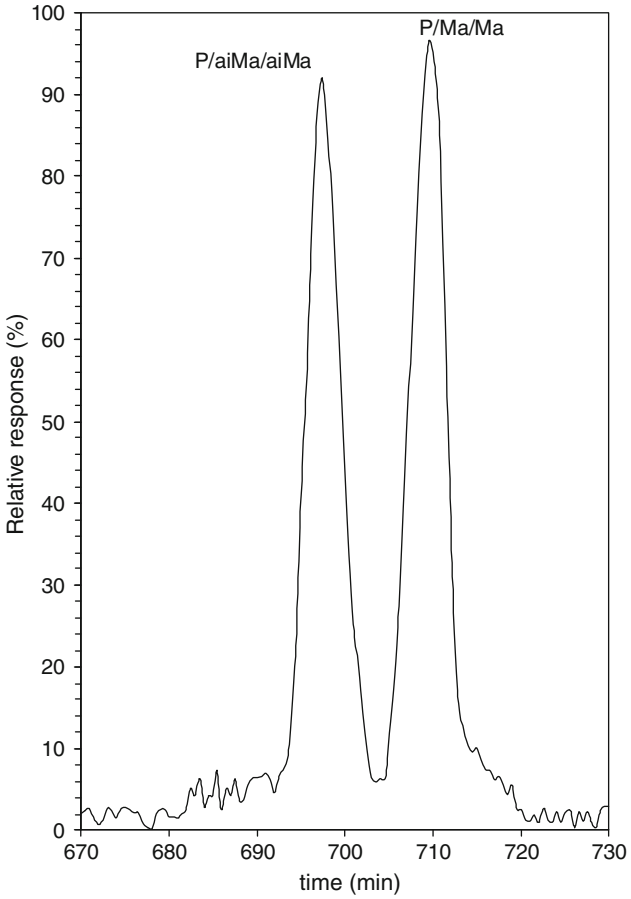

Fig. 2 RP-HPLC/APCI-MS chromatogram of two synthetic TAG (P/Ma/Ma and P/aiMa/aiMa)

APCI mass spectra of all synthetic TAG, i.e. Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa were completely identical. Minute differences in the intensity of ions at *m/z* 41, 43, 55, and 57 can be found only in electron impact mass spectra. These ions arise probably by splitting of the termini of aliphatic chains although Dinh-Nguyen [28] states, based on the comparison with deuterium-labeled compounds, that the situation is more complex. Unfortunately, the HPLC-electron impact connection is not feasible and the determination of all three chain types, i.e. *n-*, *iso-* and *anteiso-* would always require a comparison of natural TAG with a synthetic TAG standard.

The above data clearly show that any combination of branched- and straight-chain acyls in TAG cannot be resolved based on mass spectrum. Even so we performed another experiment with synthetic TAG (P/Ma/Ma and P/aiMa/aiMa, see Figs. 5, 6) that could facilitate the

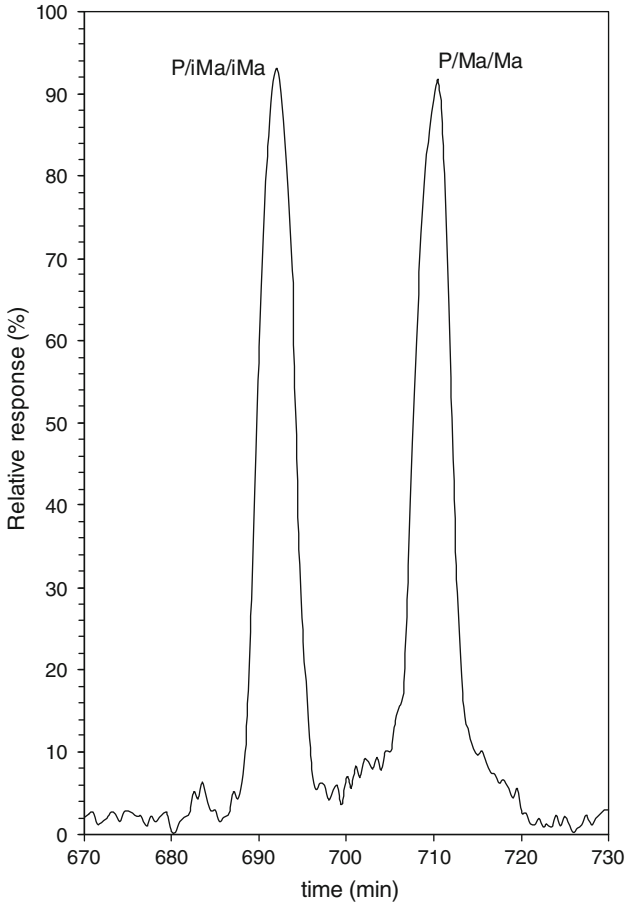

Fig. 3 RP-HPLC/APCI-MS chromatogram of two synthetic TAG (P/Ma/Ma and P/iMa/iMa)

resolution of individual acyls by using MS/MS. However, the MS/MS of the daughter ion at *m/z* 253 ($[RCO]^+$), i.e. ion with the same *m/z* value for all three acyls of all three acids (margaric, *iso*-margaric and *anteiso*-margaric acids) failed to reveal any perceptible change in the intensity of ions in the interval of 35–253 Da (spectrum not shown); this result finally excluded the possibility of distinguishing branched- and straight-chain TAG by MS-APCI.

The mass spectrum of ABB type TAG such as P/aiMa/aiMa (Fig. 6), which contains two FA, exhibits, in addition to $[M+H]^+$ at *m/z* 835, also two acylium ions, with *m/z* at 239 (P^+) and *m/z* 253 ($aiMa^+$), respectively. The spectrum naturally features also ions of the type $[MAG]^+$ at *m/z* 313 ($[P]^+$) and *m/z* 327 ($[aiMa]^+$), respectively. Like with aiMa/aiMa/aiMa, also $[DAG]^+$ ions caused by the loss of margarate at *m/z* 565 and palmitate at *m/z* 579 are present, resulting in ($[P/aiMa]^+$ (type $[AB]^+$) and $[aiMa/aiMa]^+$ (type $[BB]^+$) ions, respectively.

Similarly, the mass spectrum of an ABC type TAG, i.e. TAG containing three different FA, such as P/brMa/S, see Fig. 7, shows three triplets of ions—three $[M\text{-}RCOO]^+$ ions at *m/z* 565, 579, and 593, corresponding to loss of stearate to give $[brMa/P]^+$, loss of margarate to give $[P/S]^+$, and palmitate to give $[brMa/S]^+$, respectively. Another triplet, $[MAG]^+$ at *m/z* 341, 327, and 313 is formed by $[S]^+$, $[brMa]^+$, and $[P]^+$ ions. The last triplet belongs to ions S^+ (*m/z* 267), $brMa^+$ (*m/z* 253), and P^+ (*m/z* 239).

Some authors [17, 29–32] observed that the relative intensities of the $[M\text{-}RCOO]^+$ ions, i.e. $[DAG]^+$, could be used to obtain information on the positions of FA within

Table 3 TAG identified in *R. erythropolis*, their retention times (RT), acyl carbon number (ACN), the masses of protonated molecules, and characteristic fragment ions of TAG

Peak no.	TAG	RT[e]	ACN:n	$[M+H]^+$	$[DAG]^+$	*m/z*	$[DAG]^+$	*m/z*	$[DAG]^+$	*m/z*
1	Po/Po/O	50.7	50:3	829	Po/O	575	Po/Po	547	–	–
2	brX/Po/Po	51.0	47:2	789	brX/Po	535	Po/Po	547	–	–
3	Po/Po/Mo	51.2	49:3	815	Po/Mo	561	Po/Po	547	–	–
4	X/Po/Po	51.6	47:2	789	X/Po	535	Po/Po	547	–	–
5	M/Po/O	53.4	48:2	803	Po/O	575	M/Po	521	M/O	549
6	brM/brM/O	53.5	46:1	777	brM/O	549	brM/brM	495	–	–
7	P/Po/Po	53.8	48:2	803	P/Po	549	Po/Po	547	–	–
8	M/P/Po	54.3	46:1	777	P/Po	549	M/P	523	M/Po	521
9	M/M/O	54.7	46:1	777	M/O	549	M/M	495	–	–
10	brM/brM/P	55.1	44:0	751	brM/P	523	brM/brM	495	–	–
11	M/M/P	56.4	44:0	751	M/P	523	M/M	495	–	–
12	Po/Mo/O	58.2	51:3	843	Mo/O	589	Po/Mo	561	Po/O	575
13	P/Po/Mo	58.4	49:2	817	Mo/P	521	Po/Mo	561	Po/P	549
14	Po/O/O	60.9	52:3	857	O/O	603	Po/O	575	–	–
15	M/O/O	61.3	50:2	831	O/O	603	M/O	549	–	–
16	P/Po/O	61.9	50:2	831	Po/O	575	P/Po	549	P/O	577
17	Po/Po/S	62.6	50:2	831	Po/S	577	Po/Po	547	–	–
18	brP/brP/Po	62.9	48:1	805	brP/Po	549	brP/brP	551	–	–
19	M/brP/brP	63.1	46:0	779	brP/brP	551	M/brP	523	–	–

Table 3 continued

Peak no.	TAG	RT[e]	ACN:n	$[M+H]^+$	$[DAG]^+$	*m/z*	$[DAG]^+$	*m/z*	$[DAG]^+$	*m/z*
20	M/P/O	63.5	48:1	805	P/O	577	M/P	523	M/O	549
21	brM/brM/S	63.9	46:0	779	brM/S	551	brM/brM	495	–	–
22	P/P/Po	64.2	48:1	805	P/Po	549	P/P	551	–	–
23	M/P/P	64.7	46:0	779	P/P	551	M/P	523	–	–
24	Mo/Mo/O	65.1	52:3	857	Mo/O	589	Mo/Mo	575	–	–
25	M/M/S	65.2	46:0	779	M/S	551	M/M	495	–	–
26	M/Po/S	65.5	48:1	805	Po/S	577	M/Po	521	M/S	551
27	brX/O/O	65.6	49:1	819	O/O	603	brX/O	563	–	–
28	MoOO	65.9	53:3	871	O/O	603	Mo/O	589	–	–
29	brX/P/O	66.2	51:2	845	P/O	577	brX/P	537	brX/O	563
30	X/O/O	66.3	49:1	819	O/O	603	X/O	563	–	–
31	P/Mo/O	66.7	51:2	845	Mo/O	589	P/Mo	521	P/O	577
32	X/P/O	67.1	51:2	845	P/O	577	X/P	537	X/O	563
33	O/O/O	69.0	54:3	885	O/O	603	–	603	–	–
34	P/O/O	69.5	52:2	859	O/O	603	P/O	577	–	
35	Po/S/O	70.8	52:2	859	S/O	605	Po/S	577	Po/O	575
36	brP/brP/O	70.9	50:1	833	brP/O	577	brP/brP	551	–	–
37	P/P/O	72.2	50:1	833	P/O	577	P/P	551	–	–
38	Po/P/S	72.6	50:1	833	P/S	579	Po/P	549	Po/S	577
39	M/O/S	73.1	50:1	833	O/S	605	M/O	549	M/S	551
40	brMa/O/O	74.5	53:2	873	O/O	603	brMa/O	591	–	–
41	brX/O/S	74.7	51:1	847	O/S	605	brX/O	563	brX/S	565
42	brP/brP/P	74.9	48:0	807	brP/brP	551	brP/P	551	–	–
43	Ma/O/O	75.3	53:2	873	O/O	603	Ma/O	591	–	–
44	X/O/S	75.6	51:1	847	O/S	605	X/O	563	X/S	565
45	P/P/P	76.2	48:0	807	P/P	551	–	–	–	–
46	brMa/O/P	76.3	51:1	847	O/P	577	brMa/O	591	brMa/P	565
47	M/P/S	76.7	48:0	807	P/S	579	M/P	523	M/S	551
48	P/Mo/S	77.1	51:1	805	Mo/S	591	P/Mo	521	P/S	579
49	Ma/O/P	77.2	51:1	847	O/P	577	Ma/O	591	Ma/P	565
50	P/P/brMa	77.2	49:0	821	brMa/P	565	P/P	551	–	–
51	P/P/Ma	78.1	49:0	821	Ma/P	565	P/P	551	–	–
52	Po/S/S	78.2	52:1	861	S/S	607	Po/S	577	–	–
53	O/O/S	79.4	54:2	887	O/S	605	O/O	603	–	–
54	P/O/S	81.4	52:1	861	O/S	605	P/O	577	P/S	579
55	brP/brP/S	84.2	50:0	835	brP/S	579	brP/brP	551	–	–
56	P/P/S	85.5	50:0	835	P/S	579	P/P	551	–	–
57	iMa/iMa/iMa	86.4	51:0	849	iMa/iMa	579	–	–	–	–
58	P/brMa/brMa	85.6	50:0	835	brMa/brMa	579	brMa/P	565	–	–
58	aiMa/aiMa/aiMa	86.6	51:0	849	aiMa/aiMa	579	–	–	–	–
59	M/S/S	86.7	50:0	835	S/S	607	M/S	551	–	–
60	Ma/Ma/P	86.8	50:0	835	Ma/Ma	579	Ma/P	565	–	–
61	brMa/S/O	87.0	53:1	875	S/O	605	brMa/S	593	brMa/O	591
62	Ma/S/O	87.8	53:1	875	S/O	605	Ma/S	593	Ma/O	591
63	P/brMa/S	88.0	51:0	849	brMa/P	565	brMa/S	593	P/S	579
64	Ma/Ma/Ma	88.2	51:0	849	Ma/Ma	579	–	–	–	–
65	Mo/S/S	88.3	53:1	875	S/S	607	Mo/S	591	–	–
66	P/Ma/S	88.9	51:0	849	Ma/P	565	Ma/S	593	P/S	579
67	O/S/S	89.6	54:1	889	S/O	605	S/S	607	–	–
68	P/S/S	93.6	52:0	863	S/P	579	S/S	607	–	–
69	brMa/S/S	96.2	53:0	877	S/S	607	brMa/S	593	–	–
70	Ma/S/S	97.1	53:0	877	S/S	607	Ma/S	593	–	–

ACN acyl carbon number, *n* number of double bond(s), RT^e experimental retention time (min)

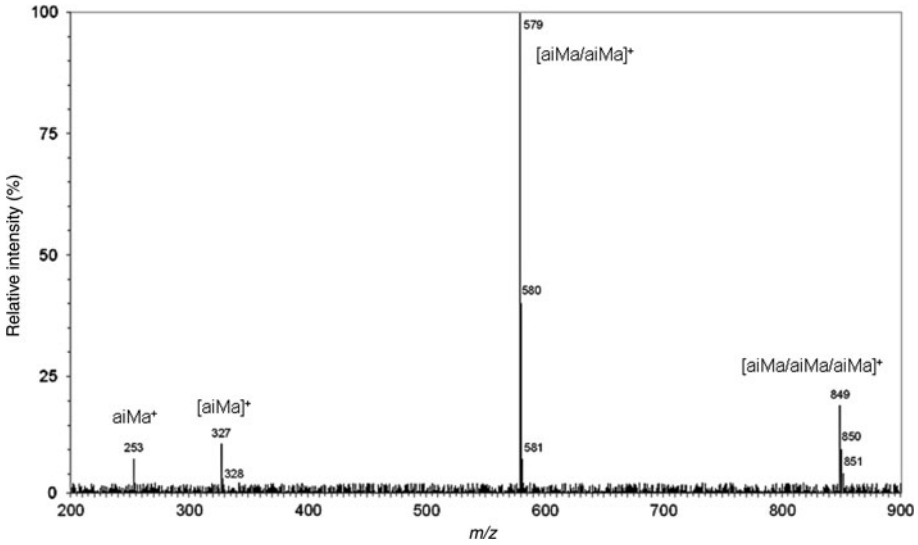

Fig. 4 Mass spectrum (APCI) of aiMa/aiMa/aiMa

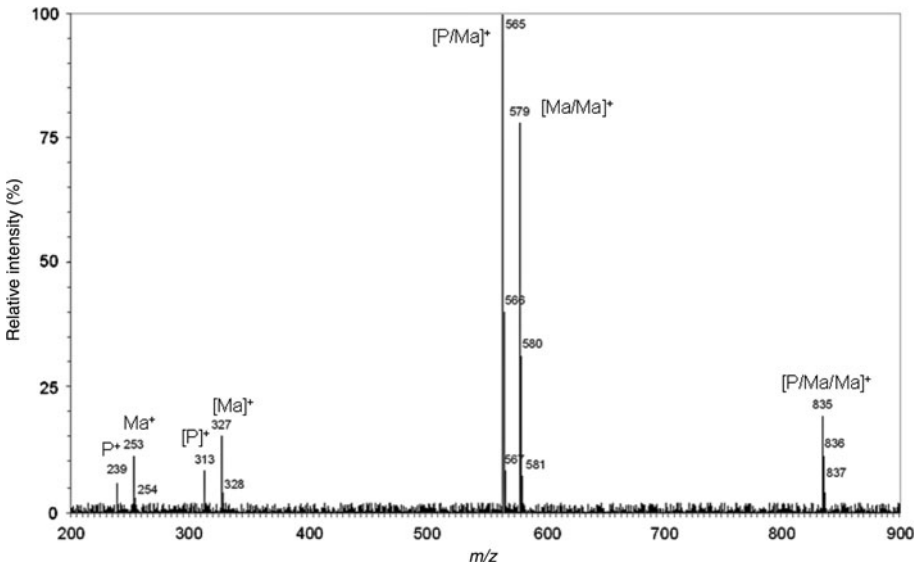

Fig. 5 Mass spectrum (APCI) of molecular species of TAG (i.e. P/Ma/Ma)

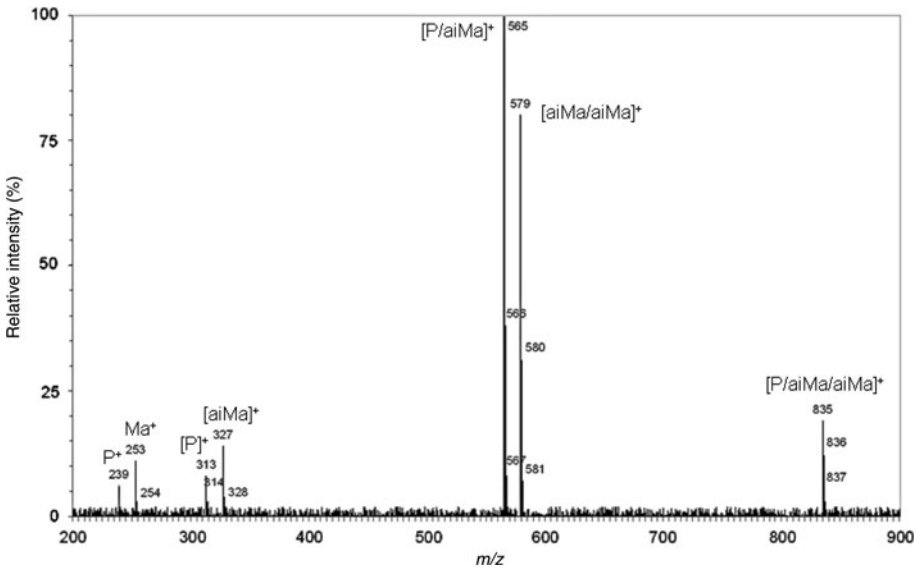

Fig. 6 Mass spectrum (APCI) of molecular species of TAG (i.e. P/aiMa/aiMa)

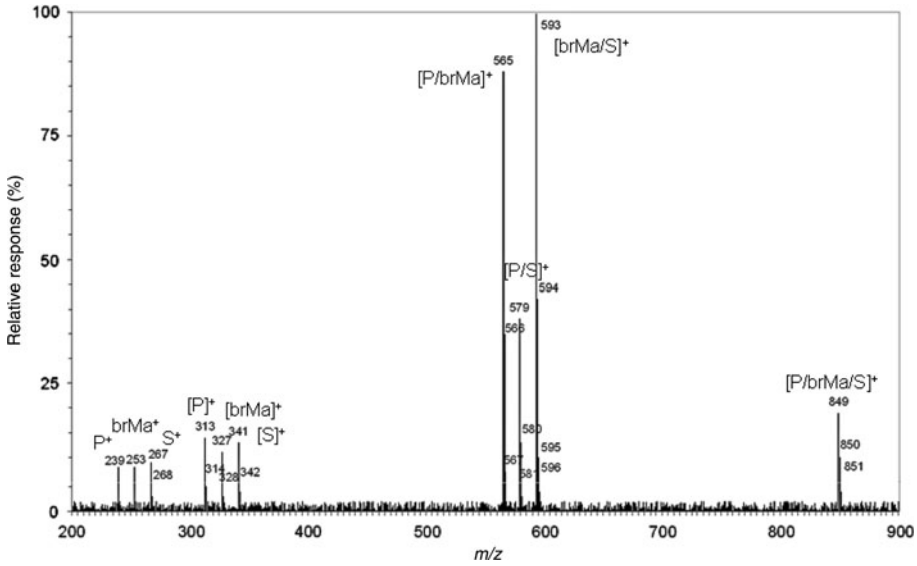

Fig. 7 Mass spectrum (APCI) of P/brMa/S, i.e. molecular species isolated from *Rhodococcus*

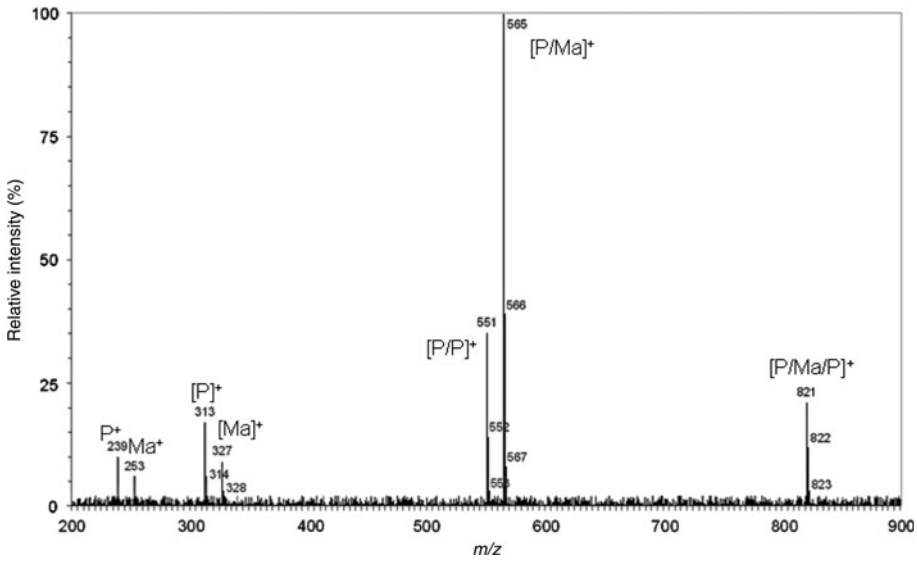

Fig. 8 Mass spectrum (APCI) of molecular species (i.e. P/Ma/P)

the TAG. A rule was formulated about the energetically less favorable loss of FA from position *sn*-2 and formation of $[M\text{-}RCOO]^+$ i.e. 1,3-$[DAG]^+$ ions. The cleavage of these bifunctional TAG gives rise to an isomeric pair of the same $[DAG]^+$ ions, i.e. $[AA]^+$ and $[AB]^+$. The ratio of $[AA]^+$:$[AB]^+$ was found to be lower for the ABA isomer, since formation of the 1,2-isomer of the $[AB]^+$ ion is energetically more favorable than generating the analogous 1,3-$[AB]^+$ ion from the AAB isomer. If these two fragments were energetically equivalent, then the intensity of ions $[AA]^+$ and $[AB]^+$ should be in a 1:2 ratio irrespective of the position of the A or B acyl substitution on the glycerol backbone, which does not occur—see, e.g. Figs. 8 and 9. The positional isomers understandably differed in the intensity of ions types $[P/P]^+$ and $[P/iMa]^+$ since the least abundant $[M\text{-}RCOO]^+$ ion is known to correspond to loss of the FA from the *sn*-2 position.

The $[P/P]^+$:$[P/Ma]^+$ ratio observed for P/Ma/P (Fig. 8) is only 35:100, indicating that the formation of the 1,3-$[PP]^+$ ion is energetically unfavorable. On the other hand, in P/P/iMa (Fig. 9) the $[P/P]^+$:$[P/iMa]^+$ ratio is 80:100 and the rule can thus be used to determine the structure of this positional isomer. This is in agreement with the result of the organic synthesis. Apart from determining the structure of natural compounds the rule can be used for confirming the structure of synthesized standards, since the synthesis may involve racemization or the original diacylglycerols need not contain only pure *sn*-1,2 or *sn*-1,3 positional isomer. Consequently, the two regioisomers show two different spectra.

To show that even a complex biological material such as *R. erythropolis* biomass can yield mass spectra that make it possible to identify TAG, we present in Figs. 10 and 11 the spectra of brM/brM/O and also brX/P/O. Identification of

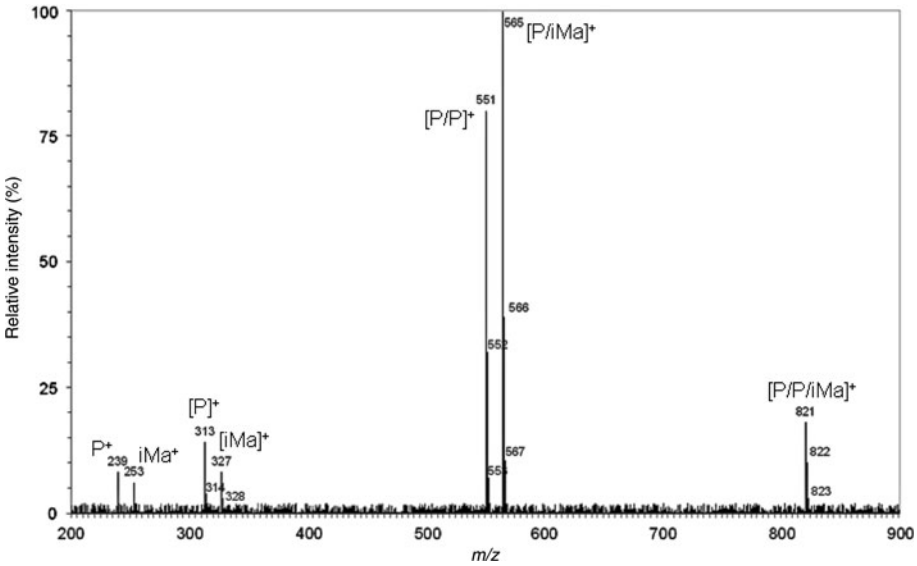

Fig. 9 Mass spectrum (APCI) of molecular species (i.e. P/P/iMa)

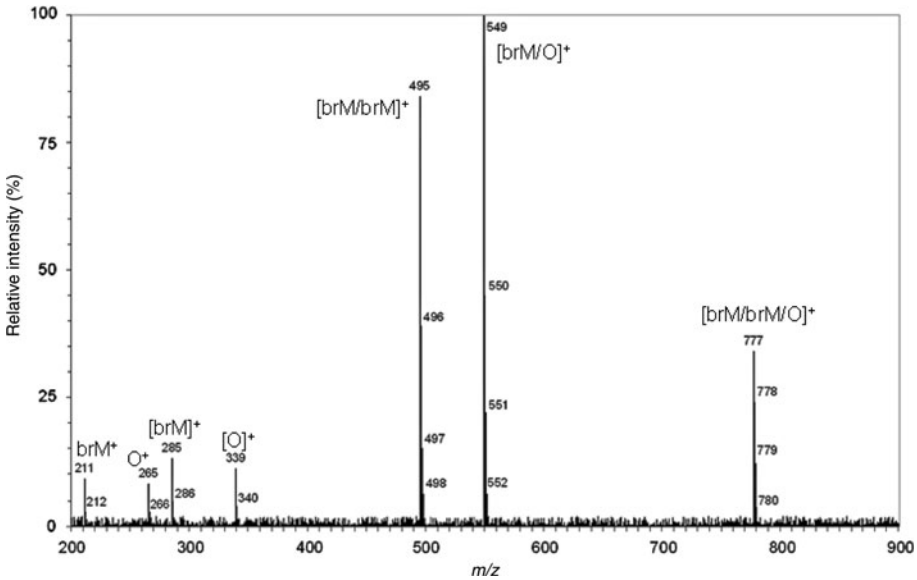

Fig. 10 Mass spectrum (APCI) of brM/brM/O, i.e. molecular species isolated from *Rhodococcus*

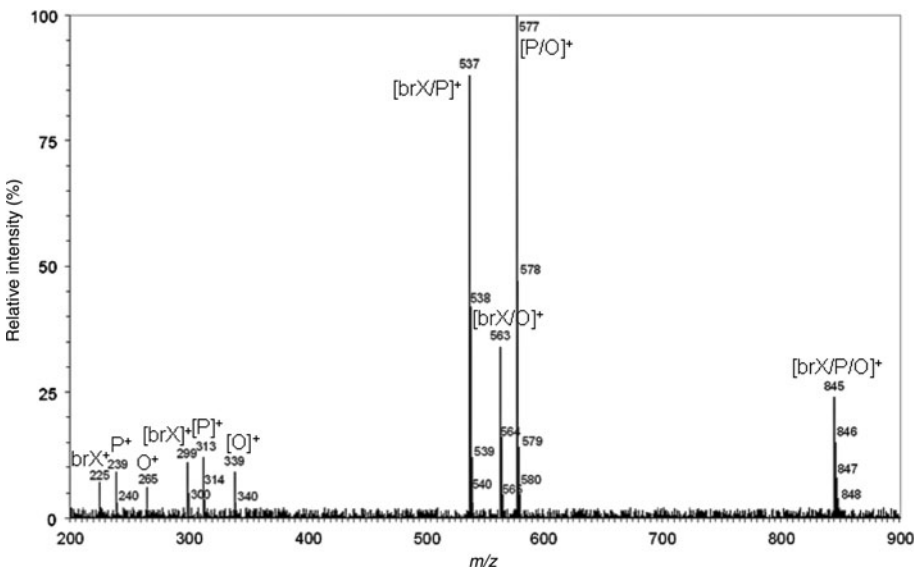

Fig. 11 Mass spectrum (APCI) of brX/P/O, i.e. molecular species isolated from *Rhodococcus*

these and other TAG was performed as described above and given in Table 3.

As expected, and in agreement with literature data, addition of Val and isobutyrate [10, 32] induced the biosynthesis of branched FA. No i- or ai-FA, neither odd- nor even-chain ones, were detected in control cultivation whereas the addition of any of the two precursors caused an increase of i-14:0 from 0 to 8.1% and a rise of i-16:0 from 0 to 18.1% (precursor Val). Precursor directed biosynthesis based on i-Bu showed comparable results, see Table 2. The overall proportion of even-i-FA increased to one quarter of total FA as compared to control (cultivation on succinate as the only carbon and energy source).

Apart from the increase in even-i-FA, an increased content was also noted with odd-straight-chain FA, i.e. 15:0 and 17:0. This is probably due to the fact that one of the possible products of Val (and also isobutyrate) catabolism is propionate, which serves as a starter unit of odd-straight-chain FA. Analogous effect, i.e. increased proportion of odd-straight-chain FA, was found in cultivations of other bacteria [10, 33].

Precursor directed biosynthesis of FA with the use of Leu or i-Va as starter units leads to an increase in odd-i-FA (i-15:0 to 14.8% with Leu and 14.2% with i-Va, i-17:0 to as much as 19.5% with Leu and 20.2% with i-Va). The proportion of these FA rose to 35% of total FA. These results are in agreement with the effect of both starter units described in the literature [12, 34].

The presence of Ile and 2-MeBu in the medium caused an expected increase [11] in ai-FA (ai-15:0 to 19.1% with Ile and 20.4% with 2-MeBu; ai-17:0 to 21.3% with Ile and 19.7% with 2-MeBu). These C-sources had the greatest effect on the FA composition, the content of ai-FA increasing to 40% of total FA.

Following the addition of given precursors, corresponding branched-chain monoenoic FA were identified in amounts of about 0.5% total FA. In contrast to straight-chain monoenoic FA only C17 and C18 branched-chain monoenoic FA were identified. This phenomenon can be explained in two ways. The first possibility is that the appropriate desaturase has a higher affinity for longer chains; this corresponds with the fact that no straight-chain monoenoic C14 and C15 FA were found. The other possibility is that the high melting point of straight-chain FA lowers the membrane fluidity to an extent that forces the bacterium to biosynthesize branched-chain monoenoic FA with a lower melting point in order to enhance membrane fluidity. The melting point of myristic acid je 54 °C, margaric (n-17:0) 61 °C, while that of ai-margaric (ai-17:0) is a mere 37 °C. Hence a change of branching from straight-chain to ai (e.g. C17 fatty acid) the melting point drops by 24 °C, whereas chain shortening of straight-chain from C17 to C14 (i.e. myristic acid) only by 17 °C. Melting point of (*Z*)-15-methyl-hexadec-12-enoic acid (i-17:1) is 21–22 °C. We therefore assume that fluidity change is affected much more by branching than the shortening of FA.

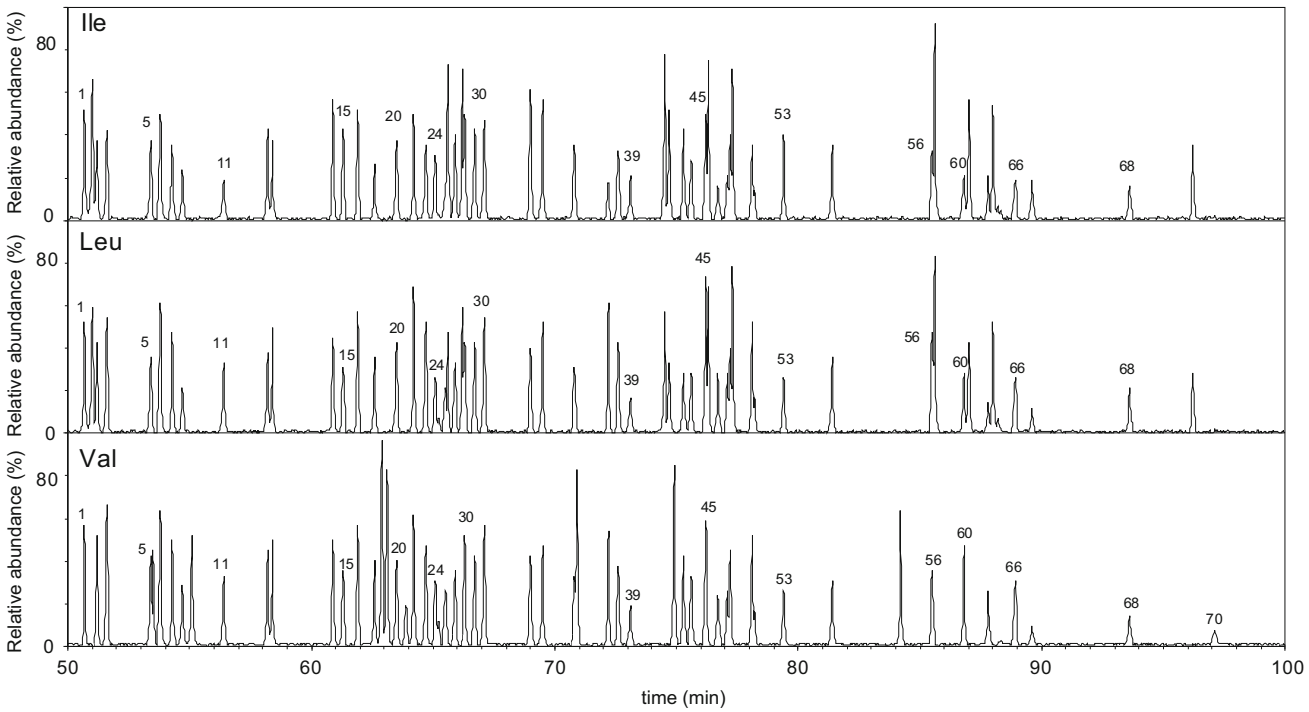

Fig. 12 RP-HPLC/APCI-MS chromatogram of the TAG from *R. erythropolis* cultivations at 30 °C with different carbon sources (Val, Leu, and Ile) as determined by RP-HPLC/MS-APCI

Cultivations in the presence of Leu and Ile and the corresponding short FA (i-Va and 2-MeBu) did not show any catabolism to acetate and propionate. Ile is known to be catabolized to acetate and propionate, Leu to acetoacetate and acetate [35]. In the former case we can assume that the starter units, i.e. acetate and propionate, have the same influence on the biosynthesis of even and odd FA and their contribution is thereby in fact eliminated (1 mol of Leu or i-Va gives rise to 1 mol of acetate and propionate and contributes thereby by the same molar amount to the biosynthesis of even and odd FA).

In the case of Ile (2-MeBu) only acetate and no propionate is formed. Because the proportion of even FA is major (more than 75% of total FA), an increase in the content of the precursor, i.e. acetate, has no further effect on the ratio of even and odd FA arising in the biosynthesis.

The results of the cultivation fully supported our assumption that *Rhodococcus* is eminently suitable for precursor directed biosynthesis of branched FA.

Chromatogram of TAG obtained after cultivation of *Rhodococcus* with three amino acids (Val, Leu and Ile) is shown in Fig. 12. Three pairs of mutually metabolically coupled precursors were used - Val and i-Bu, Leu and i-Va, Ile and 2-MeBu. The given amino acid gives rise to corresponding branched FA, which is then incorporated into the molecule of appropriate FA.

Table 4 gives mean (three independent sequential measurements) relative peak areas of 69 TAG from *Rhodococcus* determined in control cultivation on succinate and in individual cultivations using precursor directed biosynthesis with six starter units. It should be stressed that these values were calculated without using response factors, similarly as in our preceding paper in which we also analyzed unusual plant TAG [36]. None of the standards is commercially available and the 11 TAG that we synthesized represent a small fraction (<1/6) of the total possible TAG. Since the other standards are not available, the values are only semi-quantitative. This however does not prevent comparison of the outcomes of individual cultivations because these comparisons involve structurally similar TAG. An extrapolation of the response factors for TAG differing only in the branched chain (e.g. brMa/S/O and Ma/S/O) is unfortunately not feasible since we do not know the differences between straight- and branched chains. Despite this we attempted to quantify the results for Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa, see "Experimental". If a value of 1.00 is arbitrarily allotted to straight-chain TAG (Ma/Ma/Ma), the correction factor for *iso* TAG is 0.97 and for ai-TAG 0.99. In the case of a real sample, and also of several synthetic standards, we have failed to separate TAG differing only in branching (i.e. *iso* and *anteiso*).

Table 4 Relative quantities (%) of TAG identified in *R. erythropolis* cultivations at 30 °C with different carbon sources [succinate, Val, Leu, Ile, isobutyric (i-Bu), isovaleric (i-Va) and 2-methyl-butyric (2-MeBu) acid] as determined by RP-HPLC/MS-APCI

TAG	Succinate	Val	i-Bu	Leu	i-Va	Ile	2-MeBu
Po/Po/O	2.5	2.4	2.7	2.2	2.1	2.2	2.3
brX/Po/Po	–[a]	–	–	2.5	2.2	2.8	3.0
Po/Po/Mo	0.0	2.2	2.4	1.8	2.8	1.6	1.7
X/Po/Po	1.7	2.8	3.1	2.3	2.2	1.8	1.8
M/Po/O	2.0	1.8	1.9	1.5	2.4	1.6	1.7
brM/brM/O	–	1.9	1.2	–	–	–	–
Po/Po/P	2.1	2.7	3.0	2.6	3.4	2.1	2.1
M/Po/P	1.6	2.1	2.2	2.0	2.9	1.5	1.5
M/M/O	1.5	1.2	1.2	0.9	1.6	1.0	1.0
brM/brM/P	–	2.2	1.5	–	–	–	–
M/M/P	1.1	1.4	1.5	1.4	2.1	0.8	0.9
Po/Mo/O	0.8	1.9	2.0	1.6	2.3	1.8	1.8
Po/P/Mo	0.7	2.1	2.3	2.1	2.9	1.6	1.7
Po/O/O	3.8	2.1	2.3	1.9	2.7	2.4	2.4
M/O/O	3.3	1.5	1.5	1.3	1.9	1.8	1.8
Po/P/O	3.4	2.4	2.6	2.4	3.2	2.2	2.3
Po/Po/S	1.2	1.7	1.9	1.5	0.3	1.1	1.4
Po/brP/brP	–	4.1	2.9	–	–	–	–
M/brP/brP	–	3.5	2.1	–	–	–	–
M/P/O	2.9	1.7	1.8	1.8	2.1	1.6	1.6
brM/brM/S	–	0.8	0.4	–	–	–	–
Po/P/P	3.0	2.6	2.9	2.9	3.7	2.1	2.1
M/P/P	2.5	2.0	2.1	2.2	3.0	1.5	1.5
Mo/Mo/O	1.4	1.3	1.4	1.1	1.5	1.3	1.4
M/M/S	0.1	0.5	0.4	0.3	0.0	0.1	0.2
M/Po/S	0.7	1.1	1.2	0.9	0.0	0.7	0.8
brX/O/O	–	–	–	2.0	2.4	3.1	3.2
MoOO	3.2	1.5	1.6	1.4	1.9	1.7	1.9
brX/P/O	–	–	–	2.5	2.9	3.0	3.1
X/O/O	3.1	2.2	2.3	1.8	2.4	2.1	2.0
P/Mo/O	2.9	1.8	1.9	1.8	2.4	1.8	1.8
X/P/O	2.8	2.4	2.6	2.3	2.9	2.0	1.8
O/O/O	5.1	1.8	1.9	1.7	2.3	2.6	2.5
P/O/O	4.7	2.0	2.2	2.2	2.8	2.4	2.4
Po/S/O	2.5	1.4	1.5	1.3	0.4	1.5	1.5
brP/brP/O	–	3.5	2.5	–	–	–	–
P/P/O	4.3	2.3	2.5	2.6	3.3	2.3	2.2
Po/P/S	2.1	1.6	1.8	1.8	0.8	1.4	1.4
M/O/S	1.9	0.8	0.8	0.7	0.1	0.9	0.9
brMa/O/O	–	–	–	2.4	1.8	3.3	3.2
brX/O/S	–	–	–	1.4	0.0	2.2	2.4
brP/brP/P		3.6	2.8	–	–	–	–
Ma/O/O	3.4	1.8	1.8	1.2	1.1	1.8	1.8
X/O/S	0.0	1.4	1.5	1.2	0.8	1.2	1.1
P/P/P	3.9	2.5	2.8	3.1	3.8	2.1	2.1
P/brMa/O	–	–	–	2.9	2.4	3.2	3.0

Table 4 continued

TAG	Succinate	Val	i-Bu	Leu	i-Va	Ile	2-MeBu
M/P/S	1.6	1.0	1.1	1.2	0.4	0.7	0.7
P/Mo/S	0.0	1.1	1.2	1.2	0.2	0.9	0.9
P/Ma/O	3.0	1.9	2.1	1.7	2.4	1.7	1.6
P/P/brMa	–	–	–	3.3	2.9	3.0	2.9
P/P/Ma	–	2.2	2.4	2.2	2.9	1.5	1.5
Po/S/S	1.1	0.7	0.8	0.7	0.6	0.6	0.7
O/O/S	3.7	1.1	1.1	1.1	0.8	1.7	1.6
P/O/S	3.4	1.3	1.4	1.5	0.5	1.5	1.5
brP/brP/S	–	2.7	1.8	–	0.0	–	0.0
P/P/S	3.0	1.5	1.7	2.0	0.4	1.4	1.3
P/brMa/brMa	–	–	–	3.5	2.0	3.9	3.7
M/S/S	0.6	0.1	0.0	0.1	0.1	0.0	0.0
P/Ma/Ma	0.1	2.0	2.0	1.2	2.0	0.9	0.9
brMa/O/S	–	–	–	1.8	0.5	2.4	2.3
Ma/O/S	2.0	1.1	1.0	0.6	0.4	0.9	0.9
P/brMa/S	–	–	–	2.2	1.2	2.3	2.1
Ma/Ma/Ma	–	–	1.6	0.3	1.2	0.3	0.3
Mo/S/S	0.4	0.1	0.1	0.1	0.1	0.2	0.2
P/Ma/S	0.3	1.3	1.3	1.1	0.6	0.8	0.8
O/S/S	2.4	0.4	0.4	0.5	0.3	0.8	0.8
P/S/S	2.0	0.6	0.7	0.9	0.2	0.7	0.6
brMa/S/S	–	–	–	1.2	0.2	1.5	1.4
Ma/S/S	0.2	0.3	0.3	0.1	0.3	0.1	0.0

[a] Minority TAGs up to 0.1% of total TAGs were omitted

The two-sample paired *t* test revealed that a significant incorporation of precursors into the molecules of TAG appeared only in the pair Val and i-Bu (*P* value is 0.012). This value of the *t* test shows that at 5% significance level we reject the hypothesis of zero differences. The values for the other two pairs, i.e. Leu and i-Va and Ile and 2-MeBu, are different (*P* value is 0.154 and 0.453, respectively). This feature can be demonstrated in the ensuing pair of TAG, i.e. M/M/O and iM/iM/O. Precursors such as Val or i-Bu cannot give rise to ai-FA. Based on the *t* test we can thus infer that more Val was incorporated in branched TAG than i-Bu, because the ratio of Val:i-Bu incorporation in the former species was 1.2:1.2 while in branched TAG it was 1.9:1.2. Further confirmation of this fact is seen in Table 4. Another feature distinguishing the incorporation of Val (i-Bu) from that of the other two pairs (Leu/i-Va and Ile/2-MeBu) into the molecular species of TAG is the presence of only those molecular species that have two branched FA in the molecule, whereas with Leu and Ile (and the corresponding short-branched acids) the only identified molecular species of TAG were those having only one branched FA in the molecule. Based on the chromatographic behavior of the standards we synthesized we can confirm that the separation and identification of the molecular species of TAG potentially produced by *Rhodococcus* and differing in branched chain FA is feasible. The outcome of such analysis depends understandably on the detection limit, which was in our case 0.1% total TAG. We cannot exclude the possibility that under conditions different from those used in this study, e.g. in stress-inducing culture conditions or in biofilm, the proportions of individual TAG will change. The sum of branched TAG after precursor directed biosynthesis ranged from 21% for the compound with the lowest incorporation, i.e. i-Bu, to 33% for Ile. These data strongly imply that *Rhodococcus* is a good producer of branched chain FA and corresponding TAG and can be successfully used as a biotechnologically important microorganism producing these substances that have considerable pharmacological potential.

In conclusion, RP-HPLC/MS-APCI made it possible to identify and quantify TAG differing in a single branched-chain FA. Branched- and straight-chain-TAG were separated and identified while TAG differing only in *iso*- or *anteiso*-FA could not be separated since their APCI mass spectra were completely identical. Branched TAG having two branched FA in the molecule were successfully separated from straight-chain counterparts by gradient elution. TAG having a single branched FA were separated only if they differed in the site of branching. Positional isomers such as P/P/Ma and P/Ma/P or P/Ma/Ma and Ma/P/Ma were partially separated only with isocratic elution.

Acknowledgments This work was supported by the Institutional Research Concepts AV0Z50200510, projects MSM6046137305 and AROMAGEN—2B08062.

References

1. Waltermann M, Steinbuchel A (2005) Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. J Bacteriol 187:3607–3619
2. Alvarez HM, Steinbuchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376
3. Alvarez HM, Mayer F, Fabritius D, Steinbuchel A (1996) Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. Arch Microbiol 165:377–386
4. Waltermann M, Luftmann H, Baumeister D, Kalscheuer R, Steinbuchel A (2000) *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. Microbiology 146:1143–1149
5. Hernandez MA, Mohn WW, Martinez E, Rost E, Alvarez AF, Alvarez HM (2008) Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. BMC Genomics 9:600
6. Alvarez HM, Luftmann H, Silva RA, Cesari AC, Viale A, Waltermann M, Steinbüchel A (2002) Identification of phenyldecanoic acid as a constituent of triacylglycerols and wax ester produced by *Rhodococcus opacus* PD630. Microbiology 148:1407–1412

7. Hamilton JTG, McRoberts WC, Larkin MJ, Harper DB (1995) Long-chain haloalkanes are incorporated into fatty acids by *Rhodococcus rhodochrous* NCIMB 13064. Microbiology 141:2611–2617
8. Alvarez HM, Kalscheuer R, Steinbuchel A (1997) Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. Fett/Lipid 99:239–246
9. Bode HB, Dickschat JS, Kroppenstedt RM, Schulz S, Muller R (2005) Biosynthesis of *iso*-fatty acids in *Myxobacteria*: *iso*-even fatty acids are derived by alpha-oxidation from *iso*-odd fatty acids. J Am Chem Soc 127:532–533
10. Jovetic S, Feroggio M, Marinelli F, Lancini G (2008) Factors influencing cell fatty acid composition, A40926 antibiotic complex production in *Nonomuraea* sp. ATCC 39727. J Ind Microbiol Biotechnol 35:1131–1138
11. Dherbecourt J, Maillard MB, Catheline D, Thierry A (2008) Production of branched-chain aroma compounds by *Propionibacterium freudenreichii*: links with the biosynthesis of membrane fatty acids. J Appl Microbiol 105:977–985
12. Beck HC (2005) Branched-chain fatty acid biosynthesis in a branched-chain amino acid aminotransferase mutant of *Staphylococcus carnosus*. FEMS Microbiol Lett 243:37–44
13. Beck HC, Hansen AM, Lauritsen FR (2004) Catabolism of leucine to branched-chain fatty acids in *Staphylococcus xylosus*. J Appl Microbiol 96:1185–1193
14. Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM, Dewhurst RJ (2006) Factors affecting odd- and branched-chain fatty acids in milk: a review. Anim Feed Sci Technol 131:389–417
15. Parodi PW (2005) Dairy product consumption and the risk of breast cancer. J Am Coll Nutr 24:556S–568S
16. Wongtangtintharn S, Oku H, Iwasaki H, Toda T (2004) Effect of branched-chain fatty acids on fatty acid biosynthesis of human breast cancer cells. J Nutr Sci Vitaminol 50:137–143
17. Rezanka T, Schreiberova O, Krulikovska T, Masak J, Sigler K (2010) RP-HPLC/MS-APCI analysis of odd-chain TAG from *Rhodococcus erythropolis* including some regioisomers. Chem Phys Lipids 163:373–380
18. Metz PA, Omstead DR, Kaplan L, Liesch JM, Stearns RA, Vandenheuvel WJA (1988) Characterization of a lipid-rich fraction synthesized by *Streptomyces avermitilis*. J Chromatogr A 441:31–44
19. Myher JJ, Kuksis A, Marai L (1993) Identification of the less common isologous short-chain triacylglycerols in the most volatile 2.5% molecular distillate of butter oil. J Am Oil Chem Soc 70:1183–1191
20. Mottram HR, Evershed RP (2001) Elucidation of the composition of bovine milk fat triacylglycerols using high-performance liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry. J Chromatogr A 926:239–253
21. Marai L, Kuksis A, Myher JJ (1994) Reversed-phase liquid chromatography-mass spectrometry of the uncommon triacylglycerol structures generated by randomization of butteroil. J Chromatogr A 672:87–99
22. Cejkova A, Masak J, Jirku V, Vesely M, Patek M, Nesvera J (2005) Potential of *Rhodococcus erythropolis* as a bioremediation organism. World J Microbiol Biotechnol 21:317–321
23. Rezanka T, Mares P (1987) Unusual and very long-chain fatty-acids produced by Basidiomycetes. J Chromatogr 409:390–395
24. Rezanka T (1990) Identification of very long polyenoic acids as picolinyl esters by Ag^{+} ion-exchange high-performance liquid chromatography, reversed-phase high-performance liquid chromatography. J Chromatogr 513:344–348
25. Ziegler FE, Berger GD (1979) A mild method for the esterification of fatty acids. Synth Commun 9:539–543
26. Lie Ken Jie MSF, Lam CC (1995) ^{1}H-Nuclear magnetic resonance spectroscopic studies of saturated, acetylenic and ethylenic triacylglycerols. Chem Phys Lipids 77:155–171
27. Momchilova S, Itabashi Y, Nikolova-Damyanova B, Kuksis A (2006) Regioselective separation of isomeric triacylglycerols by reversed-phase high-performance liquid chromatography: stationary phase and mobile phase effects. J Sep Sci 29:2578–2583
28. Dinh-Nguyen N (1968) Contribution to the study of mass spectrometry m. 3: use of methyl esters of normal long-chain fatty acids labelled with deuterium or C-13. Ark Kemi 28:289–362
29. Mottram HR (2005) Regiospecific analysis of triacylglycerols using high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. In: Byrdwell WC (ed) Modern methods for lipid analysis by liquid chromatography/mass spectrometry and related techniques, AOCS Press, Champaign
30. Byrdwell WC (2005) Qualitative and quantitative analysis of triacylglycerols by atmospheric pressure ionization (APCI & ESI) techniques. In: Byrdwell WC (ed) Modern methods for lipid analysis by liquid chromatography/mass spectrometry and related techniques. AOCS Press, Champaign
31. Fauconnot L, Hau J, Aeschlimann JM, Fay LB, Dionisi F (2004) Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. Rapid Commun Mass Spectrom 18:218–224
32. Leskinen H, Suomela JP, Kallio H (2007) Quantification of triacylglycerol regioisomers in oils and fat using different mass spectrometric and liquid chromatographic methods. Rapid Commun Mass Spectrom 21:2361–2373
33. Dickschat JS, Bode HB, Kroppenstedt RM, Muller R, Schulz S (2005) Biosynthesis of *iso*-fatty acids in myxobacteria. Org Biomol Chem 2824–2831
34. Zhu K, Bayles DO, Xiong A, Jayaswal RK, Wilkinson BJ (2005) Precursor and temperature modulation of fatty acid composition and growth of *Listeria monocytogenes* cold-sensitive mutants with transposon-interrupted branched-chain α-keto acid dehydrogenase. Microbiology 151:615–623
35. Massey LK, Sokatch JR, Conrad RS (1976) Branched-chain amino-acid catabolism in bacteria. Bacteriol Rev 40:42–54
36. Lisa M, Holcapek M, Rezanka T, Kabatova N (2007) HPLC/APCI-MS and GC/FID characterization of 5-olefinic fatty acids in triacylglycerols from conifer seed oils. J Chromatogr 1146:67–77