Promega

Spectrum Compact CE System

Sanger Sequencing and Fragment/STR Analysis

- benchtop 4-capillary electrophoresis instrument for 4-, 5- and 6-dye chemistries and up to 32 samples in a single run
- compatible with a broad range of commercial chemistries for DNA sequencing, forensic STR profiling, MLPA, MSI and many more
- flexible raw data analysis
- prefilled, plug-and-play consumables for maximal flexibility and efficiency
- controlled by the integrated touchscreen or by Remote Access Software
- on-site instrument and software installation, operational and application trainings

www.promega.com/spectrum-compact-system

Spectrum COMPACT CE SYSTEM

Andrea Palyzová[1]
Tomáš Cajthaml[1,2]
Tomáš Řezanka[1]

[1]Institute of Microbiology, The Czech Academy of Sciences, Prague 4, Czech Republic
[2]Institute for Environmental Studies, Faculty of Science, Charles University, Prague 2, Czech Republic

Received October 26, 2020
Revised December 4, 2020
Accepted December 5, 2020

Research Article

Separation of regioisomers and enantiomers of triacylglycerols containing branched fatty acids (iso and/or anteiso)

A combination of two chromatographic and one enzymatic methods was used for identification of the molecular species of triacylglycerols (TAGs) from *Streptomyces avermitilis*. *Streptomyces avermitlis*was cultured on various carbon sources and the ratio of iso- (i-FAs), anteiso- (ai-FAs), and straight-chain- (n-FAs) fatty acids was modified by precursor-directed biosynthesis. Saturated TAGs were separated from other lipids (including TAGs containing unsaturated FAs) using Ag^+ ion cartridges. Analysis of TAGs wereperformed by RP-HPLC/ESI^+ tandem mass spectrometry. Both the synthetically prepared *sn*-TAGs and the natural mixture of TAGmolecular species of wereseparated and identified by tandem MS. The structures of synthetic TAGs werefurther confirmed by pancreatic lipase, which cleaves *sn*-TAGs into *sn*-2-monoacylglycerols. The retention times (*tR*) of the individual regioisomers and enantiomers were found to be depend on the structure of the TAGs. If one branched acyl (iso or anteiso) is present in the TAG molecule, then the elution order is enantiomer (n/n/br), opposite enantiomer (br/n/n), regioisomer (n/br/n). In the case where two branched acyls are in the TAG molecule, the order of the elution is different, that is, br/n/br, n/br/br, br/br/n. In all cases, it was further demonstrated that tandem MS of either synthetically prepared TAGs or TAGs obtained from natural material, that is, n-16:0/ai-15:0/n-16:0 and i-16:0/n-15:0/i-16:0 are identical. Unfortunately, it is not possible to distinguish by ESI^+ tandem MS such TAGs, which differ only in the branching of the acyls. The results of our analyses of TAGs are in good agreement with previously published data in other streptomycetes.

Keywords:
Branched fatty acids / Enantiomeric separation / Enzymatic hydrolysis / RP-HPLC/MS-ESI^+ / Streptomyces / Triacylglycerols DOI 10.1002/elps.202000320

Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Branched chain fatty acids (Fas), that is, iso and anteiso, occur predominantly in Gram-positive bacteria [1]. FAs are biosynthesized by condensation of branched coenzyme A (e.g., isobutyryl, isovaleryl, or 2-methyl butyryl) with malonyl CoA. The genus *Streptomyces* is the largest producer of clinically used antibiotics [2], including antifungal, antiviral, or antiparasitic, etc. drugs. They also contain a very large genome (~ 8 million base pairs), which is about twice as large as other bacteria. The complete genome of many dozens of *Streptomycete* species has been established, see, for example GenBank for *Streptomyces avermitilis* (RefSeq NC_0 03155.5).

FAs analysis was performed on several strains of the genus *Streptomyces*, for example, *S. jeddahensis*, *S. massasporeus*, *S. hawaiiensis*, *S. indiaensis*, *S. luteogriseus*, *S. purpurascens* and the following FAs were identified as major: i-14:0, i-15:0, ai-15:0, i-16:0, n-16:1, n-16:0, ai-17:1, ai-17:0, i-17:0 [3]. The effect of salinity on the relative composition of *S. avermitilis*FAs showed that the majority FAs wereanteiso FAs(ai-Fas) [4].

Triacylglycerols (TAGs) are biosynthesized by various strains of the genus *Streptomyces* [5]. Both TAGs and some antibiotics are biosynthesized from the same precursors, that is, short chain Acyl CoA[6–8]. Intracellular TAGs are accumulated in primary metabolism and they are degraded during

Correspondence: Dr. Tomáš Řezanka, Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic
E-mail: rezanka@biomed.cas.cz

Abbreviations: **ai-FAs**, anteiso fatty acids; **DAGs**, diacylglycerols; **ECN**, effective carbon number; **EI**, electron ionization; **FAs**, fatty acids; **i-FAs**, iso fatty acids; **n-FAs**, straight-chain fatty acids; **PIS**, precursor ion scans; **TAGs**, triacylglycerols; **tR**, retention times

the stationary phase to form precursors necessary for polyketide biosynthesis (e.g., actinorhodin, jadomycin B, oxytetracycline, and avermectin B1). Wang et al. [6] called this strategy dynamic degradation of TAGs, and engineered strains with increased polyketide production.

In various species of the genus *Streptomyces*, the relationship between the content of TAGs and antibiotic biosynthesis was examined, for example, the overproduction of antibiotics in *Streptomyces lividans* [9], the biosynthesis of TAGs in *Streptomyces coelicolor* [10], and neutral lipid (TAG) biosynthesis in *S. avermitilis* [11]. TAGs and phospholipids have been identified in wild strains and mutants of *S. griseus* [12]. The different strains of *Streptomyce*s sp. produced branched FAs in TAGs that reached 30–40%, the structure of TAGs was confirmed by ^{1}H-NMR [13].

In the past, the analysis of molecular species of TAGs from *S. avermitilis* by both capillary GC-MS and desorption chemical ionization (DCI) MS with ammonia as reagent gas was principally performed [14]. The authors identified 10 peaks, many of which contained more than one molecular species. TAGs, for example, C15-C15-C16, C15-C16-C16, and C15-C16-C17 were found as the majority. Unfortunately, the methods used at that time, that is, either GC-MS or DCI-MS, made it impossible to identify individual molecular species. From a further analysis of the FAMEs of these authors, it is quite clear that FAs commonly present in *Streptomycetes*, such as i-14:0, n-14:0, i-15:0, ai-15:0, n-15:0, i-16:0, n-16:0, i-17:0, ai-17:0, n-17:0, etc., were identified.

Based on our previous experiences [15] with analyses of TAGs containing branched FAs, predominantly iso and/or ai-FAs(anteiso FAs), a serial coupling of three UPRP columnswas used. TAGs have been identified in *S. avermitilis* (producer of avermectins) cultivated on a pilot scale by LC-MS in positive ESI mode using high-resolution tandem MS. Chiral phase columns based on tris-3,5-dimethyl-phenylcarbamate modified β-cyclodextrin were used for separation of enantiomers of TAGs.

2 Materials and methods

2.1 Chemicals and standards

For a full description of all chemical compounds, see the Supporting Information.

2.2 Strain and cultivation

The cultivation of the mutant strain *S. avermitilis* C-18 from the collection of the Institute of Microbiology has been described previously [16]. Similarly, as in our previous article [17], cultivation was performed in production medium containing sodium propionate, isobutyrate, and isovalerate as individual precursors at a concentration of 10 g/L. The obtained biomass was after centrifugation lyophilized, and used for extraction of total lipids.

2.3 Isolation and fractionation of lipids

The extraction procedure was based on the method described by Bligh and Dyer [18] with modification as previously described [19]. Briefly, the lyophilized cells were suspended in a dichloromethane-methanol mixture (2:1) for 30 min with stirring, after which DCMand water were added and the DCM-phase was evaporated to dryness under reduced pressure. Total lipids were fractioned by methods described by Yener and van Valenberg [20]. The Discovery® Ag-Ion SPE Tube was washed with 4 mL of acetone and conditioned with 4 mL of hexane. Total lipids from four cultures (control and cultivation with sodium propionate, isobutyrate, and isovalerate) were applied on four tubes and eluted with hexane. The hexane was removed in a stream of N_2 and TAGs were dissolved in the mobile phase (acetonitrile and 2-propanol (99:1, vol/vol)) for further analysis by LC-MS. Analysis of the eluate revealed that it contained only TAGs with saturated FAs, see Table 1Sa (Supporting Information).

2.4 Preparation and analysis of methyl and 3-pyridylcarbonyl esters of FAs

Methyl and 3-pyridylcarbonyl esters were prepared from TAGs using standard procedures. A detailed description of the preparation and the GC-MS analysis is provided in the Supporting Information. The mass spectra in the electron ionization (EI) mode were analyzed as previously described [21-25], see also three mass spectra of 3-pyridylcarbonyl C15 esters (Fig. 1S to 3S) in the Supporting Information.

2.5 RP-HPLC/ESI-MS^2

TAGs were analyzed by an LC-MS system containing three RP columns connected in series, see Supporting Information. The mass spectrometer LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high-resolution hybrid mass spectrometer equipped with a liquid chromatograph was used, see also Supporting Information. Run time 540 min, hold for 10, and the composition was returned to the initial conditions over 10 min. Nine cycles had to be performed to obtain sufficient TAGs for chiral HPLC. This instrument was used for tandem mass spectra, multiple precursor ion scans (PIS), and selected ion monitoring (SIM).

2.6 TAG analysis using chiral LC/ESI-MS

The same LC system used in the RP mode was also used for separation in chiral mode. Samples collected from nine runs of RP-HPLC were pooled and evaporated under N_2. TAGs (~1 mg/mL in mobile phase were chromatographically separated on two Astec cyclobond™ I 2000 DMP [3,5-dimethyl-

Table 1. Separation and identification of TAGs from *Streptomyces avermitilis* by RP-LC/MS-ESI

Peak[a]	*tR*	TAG	$[M+NH_4]^+$	ACN[b]	Control	Prop[c]	Isobu[d]	Isoval[e]
1	210.2	i-14:0/i-14:0/i-14:0	740.6763	42	0.0	0.0	3.4	0.0
2	225.3	i-14:0/i-15:0/i-14:0	754.6920	43	0.0	0.0	1.1	0.0
3	230.1	i-14:0/i-14:0/ai-15:0	754.6919	43	1.2	0.0	1.8	0.6
4	242.7	i-14:0/i-16:0/i-14:0	768.7074	44	0.0	0.0	3.6	0.0
5	247.3	i-14:0/i-15:0/ai-15:0	768.7076	44	1.1	0.0	1.0	0.6
6	251.2	i-14:0/ai-15:0/ai-15:0	768.7075	44	4.4	2.1	4.3	2.2
7	255.0	i-14:0/i-14:0/n-16:0	768.7074	44	0.0	0.0	3.2	0.0
8	262.5	i-14:0/i-16:0/i-15:0	782.7230	45	0.0	0.0	5.2	0.0
9	266.1	i-15:0/i-15:0/ai-15:0	782.7232	45	1.1	0.0	0.0	0.0
10	266.8	i-14:0/i-16:0/ai-15:0	782.7231	45	2.9	1.4	2.4	1.5
11	267.2	i-14:0/i-14:0/ai-17:0	782.7232	45	0.0	0.0	0.7	0.0
12	270.3	i-15:0/ai-15:0/ai-15:0	782.7233	45	4.2	2.1	2.0	2.2
13	274.4	ai-15:0/ai-15:0/ai-15:0	782.7230	45	16.0	7.8	7.7	8.2
14	281.0	i-14:0/ai-15:0/n-16:0	782.7231	45	1.7	0.8	1.9	0.9
15	284.5	i-14:0/i-16:0/i-16:0	796.7390	46	1.9	0.9	4.7	1.0
16	285.2	ai-15:0/ai-15:0/n-15:0	782.7231	45	0.0	7.0	0.0	0.0
17	287.3	i-15:0/i-16:0/ai-15:0	796.7388	46	2.7	1.3	2.9	1.4
18	290.1	ai-15:0/n-15:0/n-15:0	782.7232	45	0.0	9.5	0.0	0.0
19	291.0	i-16:0/ai-15:0/ai-15:0	796.7389	46	10.5	5.1	8.3	5.4
20	291.8	i-14:0/ai-17:0/ai-15:0	796.7390	46	1.3	0.0	1.3	0.7
21	293.3	n-15:0/n-15:0/n-15:0	782.7230	45	0.0	11.9	0.0	0.0
22	294.6	i-14:0/i-16:0/n-16:0	796.7389	46	1.1	0.0	1.1	0.0
23	299.2	i-15:0/ai-15:0/n-16:0	796.7391	46	1.6	0.8	0.8	0.8
24	304.4	ai-15:0/ai-15:0/n-16:0	796.7389	46	6.1	3.0	2.9	3.1
25	307.1	i-16:0/n-15:0/n-15:0	796.7390	46	0.0	3.5	0.0	0.0
26	307.9	i-17:0/i-15:0/i-15:0	810.7545	47	0.0	0.0	0.0	16.0
27	308.4	i-14:0/n-16:0/n-16:0	796.7391	46	0.0	0.0	0.7	0.0
28	312.2	i-15:0/i-16:0/i-16:0	810.7545	47	1.8	0.9	2.4	0.9
29	315.0	i-16:0/i-16:0/ai-15:0	810.7544	47	6.8	3.3	6.8	3.5
30	315.5	i-14:0/i-16:0/ai-17:0	810.7546	47	0.8	0.0	1.1	0.0
31	318.7	i-17:0/ai-15:0/ai-15:0	810.7544	47	0.1	0.0	0.0	9.1
32	319.1	i-15:0/ai-15:0/ai-17:0	810.7543	47	1.2	0.0	0.0	0.6
33	323.0	ai-15:0/ai-15:0/ai-17:0	810.7546	47	4.6	2.3	2.2	2.4
34	327.4	i-16:0/i-15:0/n-16:0	810.7545	47	1.0	0.0	1.3	0.0
35	327.7	i-16:0/i-16:0/n-15:0	810.7544	47	0.0	2.5	0.0	0.0
36	331.2	i-16:0/ai-15:0/n-16:0	810.7544	47	4.0	2.0	4.4	2.0
37	338.1	ai-15:0/ai-15:0/n-17:0	810.7546	47	0.0	0.7	0.0	0.0
38	338.8	ai-17:0/n-15:0/n-15:0	810.7545	47	0.0	1.2	0.0	0.0
39	339.3	ai-15:0/n-16:0/n-16:0	810.7546	47	2.3	1.1	1.1	1.2
40	342.1	i-16:0/i-16:0/i-16:0	824.7700	48	4.4	2.2	6.5	2.3
41	345.0	i-15:0/i-16:0/ai-17:0	824.7703	48	0.0	0.0	0.7	0.0
42	347.4	n-17:0/n-15:0/n-15:0	810.7544	47	0.0	6.0	0.0	0.0
43	350.3	i-16:0/ai-17:0/ai-15:0	824.7701	48	3.0	1.5	3.1	1.6
44	355.2	i-16:0/i-16:0/n-16:0	824.7702	48	2.6	1.3	2.8	1.3
45	364.1	ai-15:0/ai-17:0/n-16:0	824.7703	48	1.8	0.9	0.8	0.9
46	368.4	i-16:0/n-16:0/n-16:0	824.7701	48	1.5	0.7	1.5	0.8
47	370.0	i-17:0/i-16:0/i-16:0	838.7859	49	0.0	0.0	0.0	5.3
48	371.2	i-17:0/i-17:0/i-15:0	838.7858	49	0.0	0.0	0.0	7.3
49	372.3	n-16:0/n-16:0/n-16:0	824.7700	48	0.9	0.0	0.0	0.0
50	374.3	i-17:0/i-17:0/ai-15:0	838.7860	49	0.0	0.0	0.0	4.7
51	375.0	i-16:0/i-16:0/ai-17:0	838.7858	49	2.0	1.0	2.1	1.0
52	384.4	ai-15:0/ai-17:0/ai-17:0	838.7857	49	1.3	0.0	0.0	0.7
53	389.2	i-16:0/ai-17:0/n-16:0	838.7859	49	1.2	0.0	1.1	0.6
54	395.3	i-17:0/i-17:0/i-16:0	852.8015	50	0.0	0.0	0.0	2.9
55	402.1	i-16:0/i-16:0/n-17:0	838.7857	49	0.0	3.7	0.0	0.0

(*Continued*)

Table 1. (Continued)

Peak[a]	*tR*	TAG	$[M+NH_4]^+$	ACN[b]	Control	Prop[c]	Isobu[d]	Isoval[e]
56	408.3	n-17:0/n-17:0/n>-15:0	838.7859	49	0.0	7.0	0.0	0.0
57	410.4	i-16:0/ai-17:0/ai-17:0	852.8014	50	0.9	0.0	1.1	0.0
58	434.2	i-17:0/i-17:0/i-17:0	866.8171	51	0.0	0.0	0.0	6.3
59	461.1	n-17:0/n-17:0/n>-17:0	866.8170	51	0.0	4.5	0.0	0.0

[a] See Fig. 1.
[b] ACN = ECN because DB = 0.
[c] Sodium propionate.
[d] Sodium isobutyrate.
[e] Sodium isovalerate.

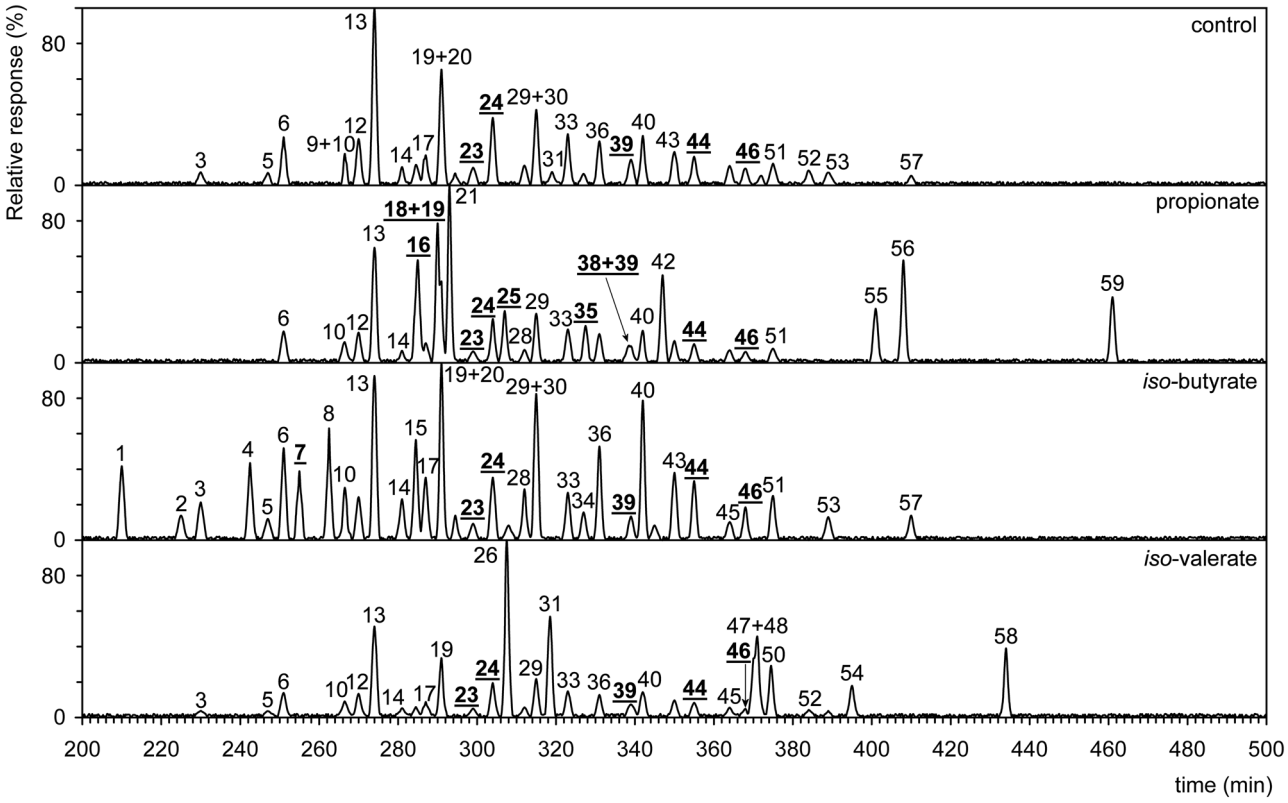

Figure 1. Reversed phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP–LC/ESI–MS) chromatogram from 200 to 500 min of total ion current (TIC) of TAGs from the four cultivations of *Streptomyces avermitilis*. For identification of the peaks, see Table 1. The TIC was filtered and indicates the types of $[DAG]^+$, ions, that is, ions resulting from loss of neutral RCOOH + NH_3 from $[M+NH_4]^+$ in the interval from 450 to 650 Da. The $[DAG]^+$ type ion was always the base peak.

phenyl-carbamate modified β-cyclodextrin] chiral LC columns (5 μm, 25 cm × 4.6 mm) (Sigma-Aldrich, Ltd.) connected in series. The mobile phase was a gradient introduced at 93% A and 7% B at 0 min to 50% A and 50% B over 160 min, and subsequently, isocratically maintained for 10 min, where A is hexane and B is a hexane,propan-2-ol(97:3% v/v) mixture [26,27]. The flow rate, column temperature, and injection volume were 0.4 mL/min, 24°C, and 10 μL, respectively. The total analysis time was 185 min, that is, 160 min gradient of chiral HPLC, 10 min hold, and 15 min recycle time. Other conditions were the same as those used for RP-LC.

2.7 Pancreatic lipase hydrolysis, that is, determination of FAs at the *sn*-2 position

Collected peaks that were eluted at different intervals, see Table 1, pancreatic lipase, 150 μL Tris buffer, 25 μL sodium cholate (1 g/L), and 10 μL calcium chloride (2.2%) were mixed in a vial, heated at 40°C for 1 min, and stirred for 2 min in a Vortex mixer. After cooling to 20°C, 50 μL of 6 M HCl and 0.2 mL of diethyl ether were added. The reaction mixture was extracted with 0.2 mL diethyl ether three times. The combined ether extracts were separated by TLC (silica gel with fluorescent indicator on 20 × 20 cm TLC plates), mobile phase

Table 2. Synthesis standards of TAGs and other properties

tR (min)[a]	Starting compound	Reaction of DAG with FA	FA_1	FA_2	FA_3	DAG_1	Abundance	DAG_2	Abundance	ECN	Optical purity (%)
104.12	n>-16:0/n>-16:0-PC	i-16:0	n>-16:0	n>-16:0	i-16:0	551.5034	100	-		48	92
113.59	n>-16:0/OH-PC	2x i-16:0	n>-16:0	i-16:0	i-16:0	551.5034	100	-		48	84
113.77	n>-16:0/n>-16:0-PC	ai-15:0	n>-16:0	n>-16:0	ai-15:0	537.4877	100	551.5034	76	47	93
120.63	n>-15:0/n>-15:0-PC	ai-17:0	n-15:0	n-15:0	ai-17:0	523.4721	73	551.5034	100	47	91
119.98	n>-16:0/ai-15:0-PC	ai-15:0	n>-16:0	ai-15:0	ai-15:0	523.4721	72	537.4877	100	46	94
121.71	n>-15:0/OH-PC	2x i-16:0	n>-15:0	i-16:0	i-16:0	537.4877	100	551.5034	74	47	87
128.64	n>-16:0/i-15:0-PC	i-15:0	n>-16:0	i-15:0	i-15:0	523.4721	100	537.4877	72	46	94
128.66	n>-15:0/n>-15:0-PC	i-16:0	n>-15:0	n>-15:0	i-16:0	523.4721	71	537.4877	100	46	87
129.05	n>-16:0/i-15:0-PC	ai-15:0	n>-16:0	i-15:0	ai-15:0	523.4721	100	537.4877	73	46	93
129.42	n>-16:0/ai-15:0-PC	i-15:0	n>-16:0	ai-15:0	i-15:0	523.4721	100	537.4877	72	46	94
141.12	n>-15:0/n>-15:0-PC	ai-15:0	n>-15:0	n>-15:0	ai-15:0	523.4721	100	-		45	93
141.24	n>-15:0/OH-PC	2x ai-15:0	n>-15:0	ai-15:0	ai-15:0	523.4721	100	-		45	86
142.83	n>-16:0/OH-PC	2x i-14:0	n>-16:0	i-14:0	i-14:0	495.4408	77	523.4721	100	44	91

[a] Chiral column.

hexane/diethyl ether/acetic acid (60:39:1, v/v/v). FFAs and *sn*-2 MAGs were scraped off and were extracted from silica with diethyl ether and evaporated to dryness. *sn*-2 MAGs were transesterified to FAMEs as described previously [28]. Briefly, FAMEs were prepared from FFAs by means of methanolic BF_3[29], see again in Supporting Information.

2.8 Synthesis of TAGs

Phosphatidylcholines and/or lysophosphatidylcholines (Table 2) were treated with phospholipase C (Type I from *Clostridiumperfringens* (*C. welchii*)), as described by Christie [28]. A detailed description of the preparation methods is provided in the Supporting Information. Three synthetic standards, marked in Fig. 3 (see later) as A, B, and C, were separated by chiral chromatography and it was found that the desired TAGs, prepared by synthesis, is at least 92% pure, see also our previous articles[26,30].

The esterification of glycerol derivatives with acids has been described previously [31]. Briefly, diacyl- and/or monoacylglycerol(s) were reacted with free acid(s) in the presence of 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) to yield the corresponding TAG(s) (Table 2).

2.9 Statistics

The statistical analysis was performed using the IBM SPSS Statistics (Statistical Package for the Social Sciences; IBM Corp, 2013) Statistics software version 26 (IBM® Corporation, Armonk, NY, USA), to explore the metabolic relationships between different sources of carbons. Statistic significant differences between molecular species of TAGs were computed by ANOVA (analysis of variance) or by ALSCAL (principal component analysis (PCA)). Three

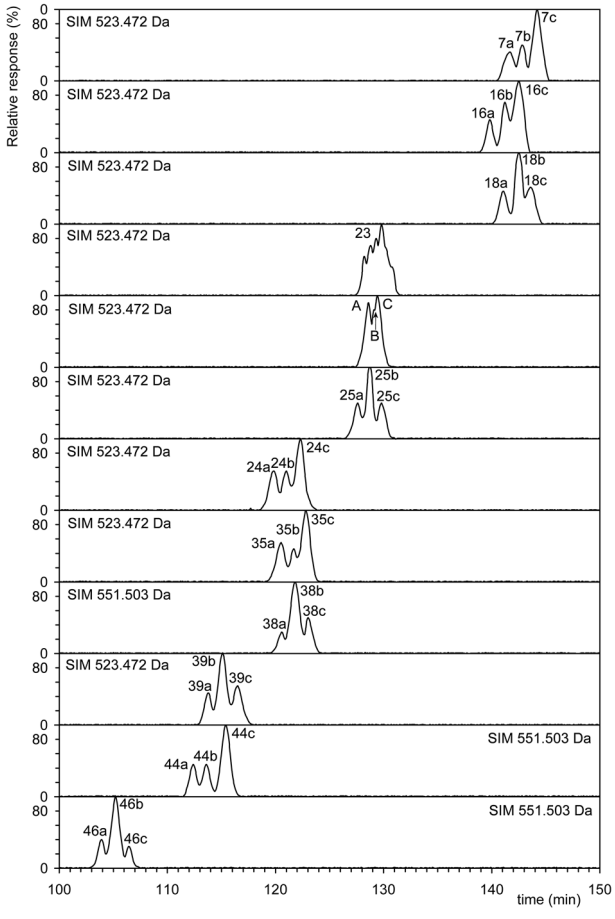

Figure 3. Chiral separation of natural TAGs, that is, enantiomers and a regioisomers, see Table 3. The synthesized enantiomers are marked **bold** in Table 3 or see also Table 2. The TIC was filtered and indicates the type of $[DAG]^+$ ions, that is, ions resulting loss of neutral RCOOH + NH_3 from $[M+NH_4]^+$ in the interval from 450 to 650 Da. The $[DAG]^+$ type ion was always the base peak.

replicates ($n = 3$) of each analysis were used. Data were presented as means ± SD.

3 Results and discussion

Supporting information Table 1Sa shows the results of the analysis of FAMEs by GC-MS. They were identified as the majority depending on the added precursor, especially straight and also branched saturated FAs. The structure of the three major FAs (i-15:0, ai-15:0, and n-15:0) was proved to be 3-pyridylcarbonyl (formerly called picolinyl) esters, see Figs. 1S to 3S (Supporting Information). The mass spectra of branched 3-pyridylcarbonyl esters (i- or ai-) are very similar to the spectra of 3-pyridylcarbonyl ester of n-15:0. The only exception is the gap of 28 Da, which represents the loss of the penultimate and/or ante-penultimate carbon atom and the CH_2 groups attached to them. Supporting information Table 1Sb shows the FA content, where unsaturated TAGs were removed after fractionation on Ag^+ ion cartridges. TAGs containing one or more unsaturated FAs were removed according to the article [20], because in the preliminary analysis they greatly complicated the separation of either straight or branched saturated chain of TAGs.

3.1 Preparation of standards

The preparation of standards is described in detail both in the Experimental part and especially in the Supporting Information. Standards were prepared by hydrolysis of commercially available phosphatidylcholines with phospholipase C to give diacylglycerols (DAGs) and these were further esterified with the appropriate FAs. Optical purity and other characteristics such as *tR*, *m/z* value of DAGs ions, abundance of these ions or ECN (effective carbon number) are given in Table 2. Another method used to identify relevant molecular species of TAGs was to use the ability of *S. avermitilis* to utilize various sources of carbons [16,17]. *Streptomycetes* are able to biosynthesize both primary (e.g., FAs) and secondary (e.g., antibiotics) metabolites using precursor direct biosynthesis. As can be seen from Supporting information Table 1Sa, the addition of sodium propionate increased the content of odd FAs, for example, n-15:0 from 3.3 to 31.2% of total FAs. In TAGs, this increase was even more pronounced, with n-15:0/n-15:0/n-15:0 not being identified at all in the three cultures (control, with Na isobutyrate, and Na isovalerate), whereas in the culture with Na propionate the content was of 15% of total TAGs. By this way, that is, by a dramatic change in the abundance of TAGs, we were able to identify total of 56 molecular species of fully saturated TAGs, see Table 1.

3.2 RP-HPLC of saturated TAGs

The production of total FA in lipids of *Streptomycetes* has been widely studied, see the articles mentioned in the introduction. In contrast, the composition of TAGs, that is, the identification of molecular species of TAGs in *Streptomycetes*, has received much less attention. Unlike dozens of vegetable oils or some animal fats for which such TAGs identification have been performed [26,31], only a few articles [14,32] have addressed the identification of molecular species of TAGs in the genus *Streptomyces*.

Preliminary identification, which we have already successfully applied several times [33–35], and which is based on the elution order of TAGs by ECN (ECN = ACN-2xDB (ACN = acyl carbon number; DB = number of double bonds)) was not successful. We assume that one of the reasons is the very high structural similarity of acyls chain in the TAGs. By combining three HPLC columns connected in series, it was finally possible to separate dozens of molecular species, which in many cases differed only in the branching of the acyls. We used Luna Omega columns (see Supporting Information) which the manufacturer claims the efficiency is 350 000 plates/meter (see manufacturer's literature). Using tripalmitin ($tR = 372.3$ min) as an external standard, an efficiency of ∼327 000 plates/meter has been determined.

Separation of TAGs by RP usually does not cause problems if ECN are different. The separation i-14:0/i-14:0/i-14:0 and i-14:0/i-15:0/i-14:0, where the ECN is 42 and 43, respectively, proceeded without problems. As can be seen from the Table 1 and also from Fig. 1, the separation lasted almost 6 h. Further, TAGs that are very structurally similar (they had the same ECN) were separated and subsequently identified. An example is the pair n-15:0/n-15:0/ai-15:0 versus n-15:0/ai-15:0/ai-15:0, which differ in only FA in the position *sn*-2 (straight versus branched chains). This observation was confirmed by the characteristics obtained using synthetically prepared standards. If two TAGs have the same m.w. and the same type of acyls, for example, two molecular species i-16:0/i-16:0/ai-15:0 and i-14:0/i-16:0/ai-17:0 then the identification was based on the $[DAG]^+$ ions (neutral loss of RCOOH + NH_3 from $[M + NH_4]^+$). Peaks 29 and 30 were only partially separated, see Fig 1. However, because ions of type $[DAG]^+$ have different values of *m/z*, that is, 551.5034 Da for $[i\text{-}16:0/i\text{-}16:0]^+$ and 523.4721 Da for $[i\text{-}14:0/i\text{-}16:0]^+$ it was possible to identify both TAGs. In the case where the two TAGs had identical compositions of acyls, see peaks 31 and 32 in Fig. 1 and in Table 1, the difference in the abundance depending on the cultivation (carbon sources) was used. Peak 31 (i-17:0/ai-15:0/ai-15:0) is over biosynthesized in the cultivation with Na valerate as carbon source. The excess of the TAG (i-15:0/ai-15:0/ai-17:0), that is, peak 32 was found in the control culture. The change in the peak 31: peak 32 ratio from 0.1 : 1.2 to 9.1 : 0.6, that is, more than two orders of magnitude lies in the different biosynthesis of these TAGs, which depends on the carbon source, preferentially biosynthesize odd iso FAs.

In principle, the *tR* of fully saturated TAGs with branched and unbranched acyls and having the same ECN increases in the order i/i/i, i/i/ai, and i/ai/ai, etc. In conclusion, by combining several factors, such as the preparation of synthetic standards, enrichment of specific TAGs by precursor direct biosynthesis, a combination of RP-HPLC on three columns

connected in series and finally using tandem MS (i.e., different *m/z* values for $[DAG]^+$ ions), it was possible to separate and mainly identify dozens of saturated TAGs containing branched acyls.

The separation of TAGs containing only saturated FAs and having identical ECNs is a relatively large problem. Mammalian milk or butter contain saturated and also branched FAs. The presence of branched chain FAs in milk has been associated with bacterial sources. Especially rumen bacteria were said to be responsible for the high branched-chain FAs (BCFA) content in ruminant milk [36]. Separation of TAGs from these sources has been described many times. An example is Chen et al. [37], where TAGs from human milk from different regions of China were analysed, unfortunately for saturated TAGs, see their Table 1, neither *tR* nor other retention characteristics are given. Although in the article by Liu et al. [38] several thousand molecular species of TAGs from bovine milks were analyzed and identified, branched TAGs are not mentioned in this article. In a very extensive publication by Kalo et al. [39], TAGs from butterfat were analyzed by HPLC-ESI. Although the number of identified molecular species has reached many hundreds, branched TAGs are not identified in this article. Butterfat TAGs with saturated FAs, for example, 19:0/16:0/16:0 and 18:0/17:0/16:0 can be separated [39]. On the contrary, the molecular species 19:0/16:0/16:0 and 19:0/14:0/18:0 or 18:0/17:0/16:0 and 18:0/15:0/18:0, respectively were not separated [39]. Further the values of *m/z* for ions of type $[DAG]^+$, for example, for 18:0/14:0 and 16:0/16:0 are identical, which complicates the identification.

In article of Lísa et al. [40], where animal fats (e.g., deer, sheep, mouflon, boar, etc.) were analyzed, it was found that TAGs with branched acyl(s) have a lower *tR* on the RP than unbranched TAGs (e.g., br-SSS = 85.1 min versus SSS = 86.2 min). Residues of TAGs were analyzed by HPLC-ESI-Q-TOF in archaeological samples of ceramic vessels and odd saturated TAGs were identified [41], unfortunately no chromatographic data are given in the article again. Almost 20 years ago, Neff et al. [42] separated saturated TAGs from coconut oil. Pairs of TAGs, for example, n-16:0/n-16:0/n-14:0 versus n-14:0/n-14:0/n-18:0 were separated, other pairs of TAGs (n-16:0/n-16:0/n-12:0 and n-14:0/n-14:0/n-16:0 or n-12:0/n-12:0/n-16:0 and n-14:0/n-14:0/n-12:0) were not separated.

In the first article, the Rezanka et al. [43], saturated TAGs having identical ECNs were separated, among many others. An example of an RP-HPLC/MS-APCI analysis of odd chain TAGs from *Rhodococcus erythropolis* was the separation of two pairs of TAGs, for example, n-16:0/n-16:0/n-16:0 versus n-14:0/n-16:0/n-18:0 and n-16:0/n-16:0/n-18:0 versus n-14:0/n-18:0/n-18:0. In another article [15], TAGs were again separated from the same microorganism when branched TAGs were prepared by precursor direct biosynthesis by culturing *R. erythropolis* with different substrates (Val, Leu, Ile, etc.). Separation of TAGs with branched and/or unbranched acyls was not a problem, except that *tR* was unusually high. Standards i-17:0/i-17:0/i-17:0, ai-17:0/ai-17:0/ai-17:0, and n-17:0/n-17:0/n-17:0 were separated, similarly to,for example, two molecular species (n-16:0/n-17:0/n-17:0 versus n-16:0/br-17:0/br-17:0); these are shown in Figs. 5, 6 and 8, 9 in Schreiberova et al. [15]. The fact, later confirmed, and also manifested itself is that the mass spectra of TAGs differing only in branched and unbranched acyls are identical. In conclusion, the separation of both individual molecular species of saturated TAGs, including regioisomers on RP-HPLC, was unsuccessful. Therefore, we proceeded in the next step to separate the TAGs on a chiral stationary phase column.

3.3 Tandem MS of saturated TAGs

In the positive tandem ESI-MS of TAGs, one of the majority peaks is ammonium adducts, that is, ions of the $[M+NH_4]^+$ type. Furthermore, $[M+H\text{-}RCOOH]^+$ fragments (also called $[DAG]^+$ ions) were identified as base peaks. These ions ($[DAG]^+$) were also used to identify regioisomers of molecular species, see below. The probable description of individual ions obtained by tandem MS is showed for some molecular species that have been synthetically prepared standards as shown in Table 2. Figure 2 shows the mass spectra of four TAGs containing branched acyls. Unfortunately, the fact that the molecular species of TAGs containing acyls with the same number of carbon atoms but differing in branching give the same tandem MS was again confirmed. The only way to distinguish them is to separate them using HPLC with sufficient efficiency, see also previously published results [44].

The validity of the rule that "the loss of FA in the *sn*-2 position is less energetically favorable compared to the *sn*-1 and *sn*-3 positions" [44] was also confirmed in fully saturated TAGs and thus it was possible to identify individual regioisomers of branched TAGs, see below.

3.4 Separation on a chiral column

Figure 3 and also Table 3 shows the characteristics of the separated regioisomers and enantiomers. The peaks marked in Fig. 1 (bold and underlined numbers) were collected and further separated on two chiral columns connected in series. With one exception, that is, peak 23, the collected peaks from RP-HPLC were always separated and identified. Identification was performed both on the basis of tandem MS (regioisomers) and on the identity of the *tR* of one of the enantiomers with the *tR* of the synthesized standard, see 13 molecular species described in Table 3. Furthermore, symmetric molecular species were hydrolyzed by pancreatic lipase after collection of peak. This lipase hydrolytically cleaves TAGs to form 2-monoacylglycerols (MAGs) and free FAs [28]. This confirmed the structure of the appropriate TAGs in addition to the tandem MS. Only peak No. 23 (RP-HPLC) unfortunately failed to separate. According to the rule [44], the number of TAGs ($x = 3^3$) could contain up to 27 regioisomers and enantiomers.

It is seen from Table 3 and also from Fig. 3 that the elution order of individual molecular species varies considerably depending on the number of branched acyls in TAG molecule. If exactly one branched acyl is present in TAG,

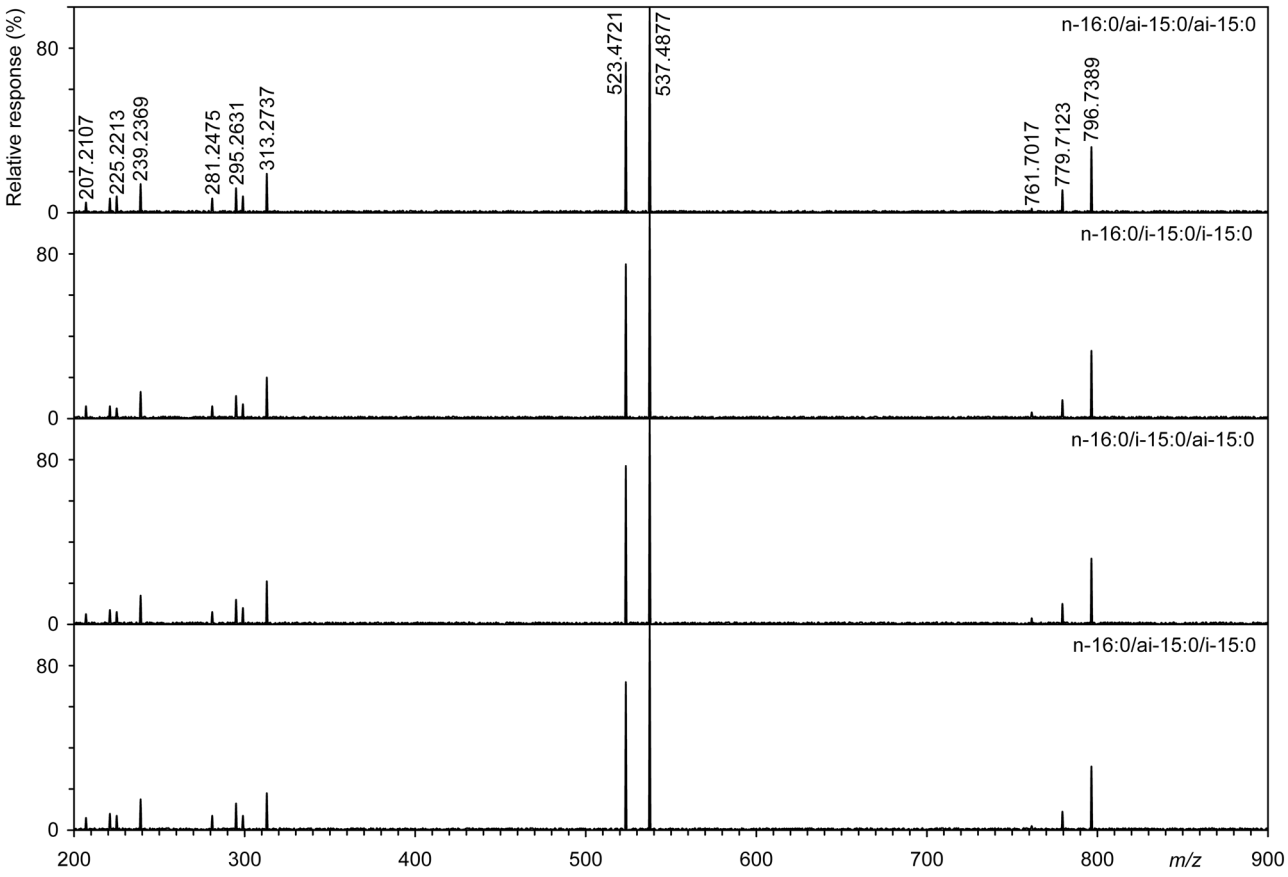

Figure 2. Tandem mass spectra of four synthetic standards, see Table 2. For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and discussion.

and it does not matter whether it is iso- or anteiso-, the order of elution on the chiral column is as follows: enantiomer (n/n/br), enantiomer (br/n/n), regioisomer (n/br/n). A completely different order of elution is in the case of two branched acyls. The elution order is from the chiral column: br/n/br, n/br/br, br/br/n.

This phenomenon is probably caused by two main forces, namely the disruption of hydrophobic interaction and chiral agreement, respectively disagreement. The branched chains disrupt hydrophobic interactions (e.g., Van der Waals forces), so that when the branched acyl chain is bound at the *sn*-1 or *sn*-3 positions of the glycerol backbone, the disruption is maximized and TAG is previously eluted (have lower *tR*). If branched acyls are "hidden or protected" at the *sn*-2 position, then they disrupt hydrophobic forces less and have a higher *tR* (eluted later). In other words, branched = disrupted = less retained, nonbranched = not disrupted = more retained.

This phenomenon is combined with the three-point chiral model [45], where the chiral center of TAG interacts with the carbamate chiral center. Therefore, straight-chain acyls (n-FA) in position *sn*-3 in the TAG are matched to better align with the nonpolar dimethyl phenyl group of stationary phase.

Based on these two effects, several of the following rules can be formulated. (a) If the molecule contains one branched FA, which is bound in the *sn*-2 position and thus protected, then the disruption is minimized and the regioisomer has a longer *tR* (disruption has a greater impact than chiral agreement, respectively disagreement). (b) If the molecule contains one branched FA and in external positions (*sn*-1 or 3), disruption is greater, TAG is less retained and the chiral mismatch enantiomer elutes first and the chiral match enantiomer (n-FA to *sn*-3) elutes as the second, leading to the pattern n/n/br, br/n/n, n/br/n. (c) If the molecule contains two branched FAs and they are in positions *sn*-1 and *sn*-3, the effect of disruption is maximized and the regioisomer elutes first (again, disruption has a greater impact than chiral agreement, respectively disagreement). (d) If the molecule contains two branched FAs and they are in both the *sn*-2 and *sn*-1 or 3 positions, the disruption effect is smaller and the enantiomer with chiral disagreement is less retained and elutes second, and the enantiomer with chiral agreement (n-FA on *sn*-3) is more retained and elutes third in order, see the pattern br/n/br, n/br/br, br/br/n. The behavior of br/n/br and n/br/n can thus be explained using the above principles of the degree of disruption of hydrophobic interaction and chiral agreement for both.

The paper of Nagai et al. [46] describes separation of fully saturated TAGs, such as 16:0/16:0/18:0 or 16:0/18:0/18:0,

Table 3. Separation of TAGs on chiral column

Peak (RP-LC)	tR (min) chiral	FA_1	FA_2	FA_3	Abundance of mol. spec.	DAG_1	%	DAG_2	%	ECN	Confirmed
46	104.12	**n-16:0**[a]	**n-16:0**	**i-16:0**	30	551.5034	100	-		48	-
46	105.14	i-16:0	n-16:0	n-16:0	100	551.5034	100	-		48	-
46	106.46	n-16:0	i-16:0	n-16:0	50	551.5034	100	-		48	lipase
44	112.38	i-16:0	n-16:0	i-16:0	45	551.5034	100	-		48	lipase
44	113.59	**n-16:0**	**i-16:0**	**i-16:0**	45	551.5034	100	-		48	-
44	115.43	i-16:0	i-16:0	n-16:0	100	551.5034	100	-		48	-
39	113.77	**n-16:0**	**n-16:0**	**ai-15:0**	45	537.4877	100	551.5034	76	47	-
39	115.11	ai-15:0	n-16:0	n-16:0	100	537.4877	100	551.5034	74	47	-
39	116.52	n-16:0	ai-15:0	n-16:0	55	537.4877	100	551.5034	31	47	lipase
38	120.63	**n-15:0**	**n-15:0**	**ai-17:0**	50	523.4721	73	551.5034	100	47	-
38	121.78	ai-17:0	n-15:0	n-15:0	100	523.4721	76	551.5034	100	47	-
38	122.99	n-15:0	ai-17:0	n-15:0	55	523.4721	30	551.5034	100	47	lipase
35	120.54	i-16:0	n-15:0	i-16:0	55	537.4877	100	551.5034	33	47	lipase
35	121.71	**n-15:0**	**i-16:0**	**i-16:0**	45	537.4877	100	551.5034	74	47	-
35	122.77	i-16:0	i-16:0	n-15:0	100	537.4877	100	551.5034	72	47	-
24	118.84	ai-15:0	n-16:0	ai-15:0	50	523.4721	36	537.4877	100	46	lipase
24	119.98	**n-16:0**	**ai-15:0**	**ai-15:0**	60	523.4721	72	537.4877	100	46	-
24	121.32	ai-15:0	ai-15:0	n-16:0	100	523.4721	74	537.4877	100	46	-
25	128.66	**n-15:0**	**n-15:0**	**i-16:0**	30	523.4721	71	537.4877	100	46	-
25	129.73	i-16:0	n-15:0	n-15:0	100	523.4721	74	537.4877	100	46	-
25	130.03	n-15:0	i-16:0	n-15:0	50	523.4721	29	537.4877	100	46	lipase
A[b]	128.64	**n-16:0**	**i-15:0**	**i-15:0**	-	523.4721	100	537.4877	72	46	-
B[b]	129.05	**n-16:0**	**i-15:0**	**ai-15:0**	-	523.4721	100	537.4877	73	46	-
C[b]	129.42	**n-16:0**	**ai-15:0**	**i-15:0**	-	523.4721	100	537.4877	72	46	-
23[c]	128.84	15:0	15:0	16:0	-	523.4721	58	537.4877	100	46	-
18	141.12	**n-15:0**	**n-15:0**	**ai-15:0**	45	523.4721	100	-		45	-
18	142.54	ai-15:0	n-15:0	n-15:0	100	523.4721	100	-		45	-
18	143.59	n-15:0	ai-15:0	n-15:0	45	523.4721	100	-		45	lipase
16	139.82	ai-15:0	n-15:0	ai-15:0	45	523.4721	100	-		45	lipase
16	141.24	**n-15:0**	**ai-15:0**	**ai-15:0**	70	523.4721	100	-		45	-
16	142.48	ai-15:0	ai-15:0	n-15:0	100	523.4721	100	-		45	-
7	141.67	i-14:0	n-16:0	i-14:0	40	495.4408	34	523.4721	100	44	lipase
7	142.83	**n-16:0**	**i-14:0**	**i-14:0**	50	495.4408	77	523.4721	100	44	-
7	144.20	i-14:0	i-14:0	n-16:0	100	495.4408	75	523.4721	100	44	-

[a] The synthesized enantiomers are marked **bold**.
[b] Mixture of synthetic standards of TAGs which have not been identified in the natural mixture, see Fig. 3.
[c] The mixture of TAGs was not separated, see Fig. 3.

into individual molecular species (regioisomers or enantiomers) which was not successful. In the article [31], where 16:0/16:0/18:0 was also not separated by repeated HPLC (after five cycles) on a chiral phase (cellulose tris (3,5-dimethylphenylcarbamate)) was published. Other articles, at least to the best of our knowledge, have not been published.

3.5 Tandem MS of regioisomers and enantiomers

The results of the tandem MS of saturated of TAGs analysis are summarized here. As mentioned several times in this article, three ions are always present in the molecular adduct ion region, that is, $[M+NH_4]^+$, $[M+H]^+$, and a very minor $[M+H]^+$ with a loss of H_2O. The base peak of all tandem MS is formed by ions of type $[DAG]^+$, that is, ions arising from the loss of appropriate FA + NH_3 from $[M+NH_4]^+$. Based on the abundances of these ions and also in accordance with a previously published rule [44], which describes that "the loss of FA in the *sn*-2 position is less energetically favorable compared to the *sn*-1 and *sn*-3 positions" it was possible to identify regioisomers, not enantiomers, after separation on a chiral column. In the case of symmetric TAGs, the ratio of abundances of ions of the type $[DAG]^+$ is about 35:100, while in the case of asymmetric TAGs it is about 72:100, see rule previously published [44]. Other ions of the type $[MAG]^+$, that is, ($[RC = O+74]^+$) and ions ($[RC = O]^+$) have low abundance and only confirm the number of carbons in acyls in the TAG, unfortunately not whether the acyls are branched or unbranched.

As in Fig. 2, where the tandem MS of the four synthesized TAGs are shown, and in Figs. 4 and 5 where the tandem MS of several molecular species obtained after separation on a chiral column are shown, it is evident that except for the different abundances of the $[DAG]^+$ ions, the

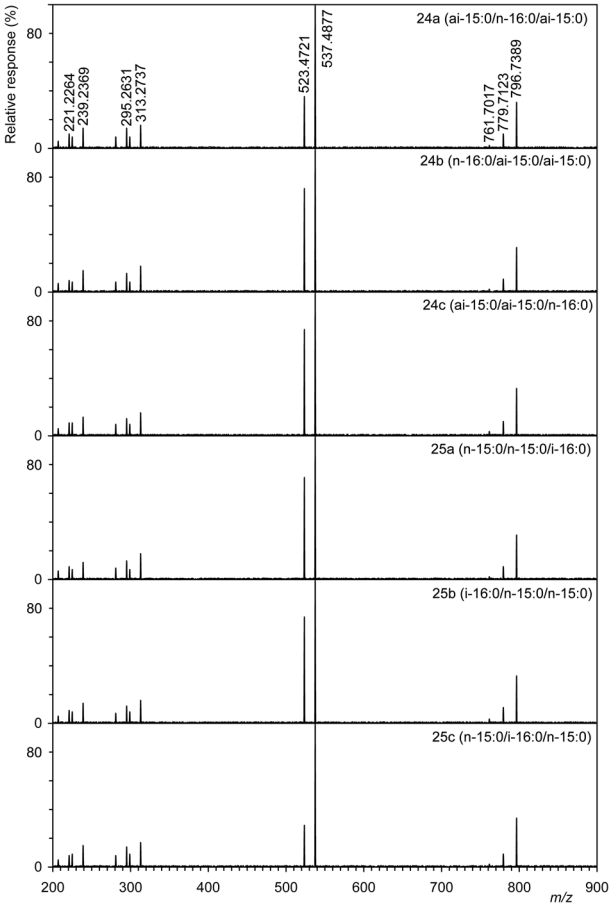

Figure 4. Tandem mass spectra of the natural regioisomers (ai-15:0/n-16:0/ai-15:0) and enantiomers (ai-15:0/ai-15:0/n-16:0 and n-16:0/ai-15:0/ai-15:0), that is, peak 24 from RP-LC and peak 25 (i.e., molecular species n-15:0/n-15:0/i-16:0, i-16:0/n-15:0/n-15:0, and n-15:0/i-16:0/n-15:0).

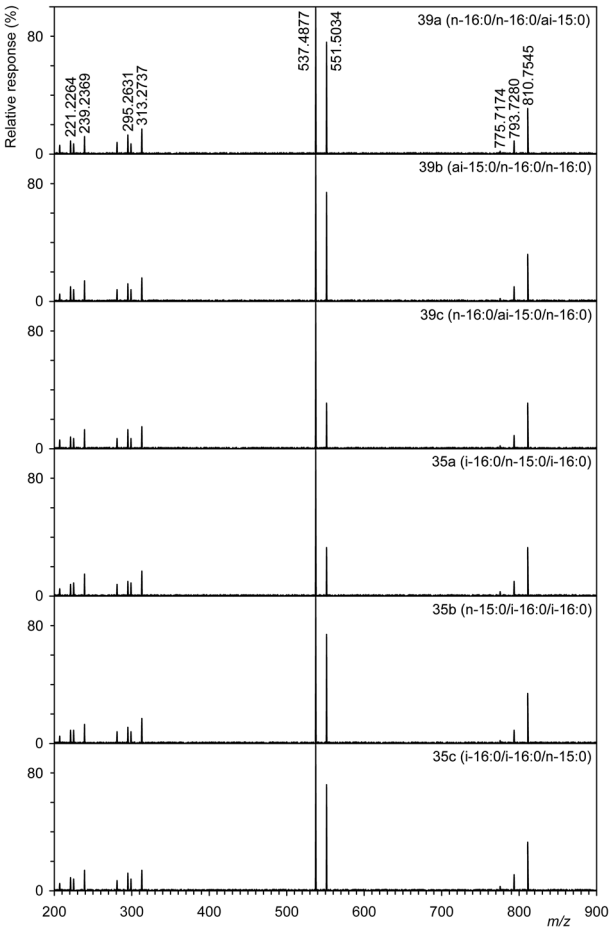

Figure 5. Tandem mass spectra of the natural regioisomers (n-16:0/ai-15:0/n-16:0) and enantiomers (n-16:0/n-16:0/ai-15:0 and ai-15:0/n-16:0/n-16:0), that is, peak 39 from RP-LC and peak 35 (i.e., molecular species i-16:0/n-15:0/i-16:0, i-16:0/i-16:0/n-15:0, and n-15:0/i-16:0/i-16:0).

spectra are almost identical. The differences in the abundances of all ions are not significant and therefore cannot be distinguished between individual molecular species. As an example, we present a tandem MS of such different TAGs as n-16:0/ai-15:0/n-16:0 and i-16:0/n-15:0/i-16:0, where all four FAs are different, see Fig. 2. In conclusion, in accordance with previously published data, branched (iso or anteiso) and unbranched TAGs with any combination of acyls, of course of the same number of carbons, have identical tandem MS. After separation, regioisomers can be distinguished from each other, but not enantiomers.

3.6 TAGs in the genus *Streptomyces*

As mentioned above, only one article has been published (at least to the best of our knowledge) describing the analysis of TAGs in *S. avermitilis* [14]. In this article, lipids were analyzed using a direct probe EI and GLC. Saturated C14-C17 FAs were found to be present in the lipids. A further analysis by capillary GC-MS as well as DCI identified at least 10 TAGs, with many peaks containing more than one molecular species. It can be said that even though the published results of the analyses are more than 40 years old, they confirm the results we found.

The article by Batrakov et al. [32] describes the analysis of tandem MS of TAGs from two *Streptomycetes*, *Actinomyces aureoverticillus* (now *Streptomyces aureoverticillatus*) and *Actinomyces streptomycini* (now *Streptomyces griseus*). As expected, several molecular species were identified by MS, unfortunately it was not possible to distinguish between branched and unbranched TAGs. Saturated TAGs in particular have been identified, for example, in *S. aureoverticillatus* 16:0/16:0/17:0, 16:0/15:0/17:0, 16:0/15:0/14:0, etc.

4 Concluding remarks

Using three independent methods, that is, RP-HPLC-MS, chiral HPLC-MS, and stereospecific hydrolysis by means of pancreatic lipase, the separation and identification of

dozens of molecular species of TAGs were successfully accomplished. Standards of TAGs were synthesized from commercially available phosphatidylcholines containing branched acyls. Unfortunately, based on tandem MS of both synthesized standards of TAGs and TAGs isolated from natural sources, the mass spectra of TAGs having both branched (iso and/or anteiso FA) and straight chain FAs were shown to be identical. Regioisomers and enantiomers could be separated and subsequently identified only after further chromatography on a chiral stationary phase. The elution order of enantiomers and regioisomers depends on the number of branched acyls in the TAG molecule. If one branched acyl is present in TAGs, then the elution order is asymmetric, asymmetric and symmetric molecular species. Using the above methods, it was thus possible to separate and identify molecular species of both regioisomers and enantiomers of branched TAGs. However, these methods of analysis are not limited to bacterial TAGs, but can be used to analyze branched TAGs found in, for example, mammalian milk or depot fats of mammals.

This research was supported by the Center for Geosphere Dynamics (UNCE/SCI/006). The authors thank the prematurely deceased colleague, Dr. Jaroslav Votruba for cultivation mutant strain S. avermitilis in a fermenter.

The authors have declared no conflict of interest.

Data availability statement

Data available in the Supporting Information for the article.

5 References

[1] Kaneda, T., *Microbiol. Rev.* 1991, *55*, 288–302.

[2] Watve, M.G., Tickoo, R., Jog, M.M., Bhole, B.D., *Arch. Microbiol.* 2001, *176*, 386–390.

[3] Röttig, A., Atasayar, E., Meier-Kolthoff, J.P., Spröer, C., Schumann, P., Schauer, J., Steinbüchel, A., *Int. J. Syst. Evol. Microbiol.* 2017, *67*, 1676–1682.

[4] Rezanka, T., Votruba, J., *Folia Microbiol. 43*, 1998, 47–50.

[5] Alvarez, H.M., Steinbüchel, A., *Appl. Microbiol. Biotechnol.* 2002, *60*, 367–376.

[6] Wang, W., Li, S., Li, Z., Zhang, J., Fan, K., Tan, G., Ai, G., Lam, S.M., Shui, G., Yang, Z., Lu, H., Jin, P., Li, Y., Chen, X., Xia, X., Liu, X., Dannelly, H.K., Yang, C., Yang, Y., Zhang, S., Alterovitz, G., Xiang, W., Zhang, L., *Nat. Biotechnol.* 2020, *38*, 76–83.

[7] Hwang, K.S., Kim, H.U., Charusanti, P., Palsson, B.O., Lee, S.Y., *Biotechnol. Adv.* 2014, *32*, 255–268.

[8] Liu, G., Chater, K.F., Chandra, G., Niu, G., Tan, H., *Microbiol. Mol. Biol. Rev.* 2013, *77*, 112–143.

[9] Le Maréchal, P., Decottignies, P., Marchand, C.H., Degrouard, J., Jaillard, D., Dulermo, T., Froissard, M., Smirnov, A., Chapuis, V., Virolle, M.J., *Appl. Environ. Microbiol.* 2013, *79*, 5907–5917.

[10] Arabolaza, A., Rodriguez, E., Altabe, S., Alvarez, H., Gramajo, H., *Appl. Environ. Microbiol.* 2008, *74*, 2573–2582.

[11] Kaddor, C., Biermann, K., Kalscheuer, R., Steinbüchel, A., *Appl. Microbiol. Biotechnol.* 2009, *84*, 143–155.

[12] Gräfe, U., Reinhardt, G., Krebs, D., Roth, M., Bormann, E.J., *Z. Allg. Mikrobiol.* 2007, *22*, 97–106.

[13] Lu, Y., Wang, J., Deng, Z., Wu, H., Deng, Q., Tan, H., Cao, L., *Lett. Appl. Microbiol.* 2013, *57*, 200–205.

[14] Metz, P.A., Omstead, D.R., Kaplan, L., Liesch, J.M., Stearns, R.A., Van-Denheuvel, W.J., *J. Chromatogr. A* 1988, *441*, 31–44.

[15] Schreiberová, O., Krulikovská, T., Sigler, K., Cejková, A., Rezanka, T., *Lipids* 2010, *45*, 743–756.

[16] Rezanka, T., Votruba, J., *Bioprocess Eng.* 1998, *19*, 159–160.

[17] Rezanka, T., Prell, A., Spížek, J., Sígler, K., *J. Antibiot.* 2010, *63*, 524–529.

[18] Bligh, E.G., Dyer, W.J., *Can. J. Biochem. Physiol.* 1959, *37*, 911–917.

[19] Vančura, A., Řezanka, T., Maršálek, J., Vančurová, I., Křišťan, V., Basařová, G., *FEMS Microbiol. Lett.* 1987, *48*, 357–360.

[20] Yener, S., van Valenberg, H.J.F., *Talanta* 2019, *204*, 533–541.

[21] Rezanka, T., Dembitsky V.M., *Tetrahedron* 2001, *57*, 8743–8749.

[22] Rezanka, T., Dembitsky, V.M., *Phytochemistry* 1999, *51*, 963–968.

[23] Dembitsky, V., Rezanka, T., Rozentsvet, O., *Phytochemistry* 1993, *33*, 1015–1019.

[24] Vancura, A., Rezanka, T., Marsalek, J., Melzoch, K., Basarova, G., Kristan, V., *FEMS Microbiol. Lett.* 1988, *49*, 411–415.

[25] Rezanka, T., Podojil, M., *J. Chromatogr. A* 1986, *362*, 399–406.

[26] Rezanka, T., Lukavsky, J., Sigler, K., Nedbalova, L., Vitova, M., *Phytochemistry* 2015, *110*, 37–45.

[27] Rezanka, T., Kolouchová, I., Cejková, A., Cajthaml, T., Sigler, K., *J. Sep. Sci.* 2013, *36*, 3310–3320.

[28] Christie, W.W., Han, X., *Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis*, The Oily Press, Bridgewater, UK 2010.

[29] Temina, M., Rezankova, H., Rezanka, T., Dembitsky, V.M., *Microbiol. Res.* 2007, *162*, 308–321.

[30] Rezanka, T., Nedbalová, L., Sigler, K., *J. Chromatogr. A* 2016, *1467*, 261–269.

[31] Nagai, T., Mizobe, H., Otake, I., Ichioka, K., Kojima, K., Matsumoto, Y., Gotoh, N., Kuroda, I., Wada, S., *J. Chromatogr. A* 2011, *1218*, 2880–2886.

[32] Batrakov, S.G., Sadovskaya, V.L., Rozynov, B.V., Bergelson, L.D., *Chem. Phys. Lipids* 1983, *33*, 331–353.

[33] Rezanka, T., Pádrová, K., Sigler, K., *Anal. Biochem.* 2017, *524*, 3–12.

[34] Rezanka, T., Sigler, K., *Lipids* 2014, *49*, 1251–1260.

[35] Rezanka, T., Lukavský, J., Nedbalová, L., Sigler, K., *Phytochemistry* 2014, *104*, 95–104.

[36] Fievez, V., Colman, E., Castro-Montoya, J.M., Stefanov, I., Vlaeminck, B., *Anim. Feed Sci. Technol.* 2012, *172*, 51–65.

[37] Chen, Y.J., Zhou, X.H., Han, B., Yu, Z., Yi, H.X., Jiang, S.L., Li, Y.Y., Pan, J.C., Zhang, L.W., *J. Dairy Sci.* 2020, *103*, 7761–7774.

[38] Liu, Z., Li, C., Pryce, J., Rochfort, S., *ACS Omega* 2020, *21*, 12573–12582.

[39] Kalo, P., Kemppinen, A., Ollilainen, V., *Lipids* 2009, *44*, 169–195.

[40] Lísa, N., Netušilová, K., Franěk, L., Dvořáková, H., Vrkoslav, V., Holčapek, M., *J. Chromatogr. A* 2011, *1218*, 7499–7510.

[41] Lantos, I., Careaga, V.P., Palamarczuk, V., Aversente, Y., Bonifazi, E., Petrucci, N.S., Maier, M.S., *Archaeol. Anthropol. Sci*. 2020, *12*, 121.

[42] Neff, W.E., Byrdwell, W.C., Steidley, K.R., List, G.R., Snowder G., *J. Liq. Chromatogr. Relat.* 2002, *25*, 985–998.

[43] Rezanka, T., Schreiberová, O., Krulikovská, T., Masák, J., Sigler, K., *Chem. Phys. Lipids* 2010, *163*, 373–380.

[44] Mottram, H.R., in: Byrdwell, W.C. (Ed.), *Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques*, AOCS Press, Champaign 2005.

[45] Pirkle, W.H., Pochapsky, T.C., *Chem. Rev.* 1989, *89*, 347–362.

[46] Nagai, T., Kinoshita, T., Kasamatsu, E., Yoshinaga, K., Mizobe, H., Yoshida, A., Itabashi, Y., Gotoh, N., *J. Oleo Sci.* 2019, *68*, 1019–1026.

