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Structure and absolute configuration of an unsaturated anteiso fatty acid from *Bacillus megaterium*

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ABSTRACT

Gas chromatography in combination with electron ionization mass spectrometry (GC/EI-MS) was used to determine the fatty acids of a membrane lipid from *Bacillus megaterium*. Special attention was put on the structure and absolute configuration of a monoenoic fatty acid previously described in this sample. GC/EI-MS operated in the selected ion monitoring mode was used to determine twelve fatty acids in the bacterium. Methyl esters were prepared to verify the presence of a 14-methylhexadecenoic acid (a17:1) isomer. The position of the double bond of the a17:1 isomer and four further monoenoic fatty acids was elucidated by means of their picolinyl esters produced by the transesterification of the phospholipid. For the a17:1 isomer, the double bond was located between C-5 and C-6. Silver ion liquid chromatography was used to verify that the double bond was in *cis*-configuration. The bacterial 14-methylhexadec-5-enoic acid (a17:1Δ5) is chiral due to the stereogenic C-14 carbon. Initial enantioselective measurements were carried out with isomers of a17:1Δ5 which were available in form of racemic and (*S*)-enantiopure *cis*- and *trans*-isomers of a17:1Δ12 previously synthesized. The *cis*-a17:1Δ12 enantiomers were partly resolved on a chiral stationary phase coated with 50% heptakis(6-O-*tert.*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin in OV-1701 (β-TBDM). However, resolution of the enantiomers of the *trans*-isomer of a17:1Δ12 failed. Only one peak was also observed for the a17:1Δ5 isomer from *B. megaterium*. Thus, it remained unclear whether the compound a17:1Δ5 was racemic or enantiopure in the sample. To clarify this point, we separated the *cis*-monoenoic fraction from the saturated fatty acids. Then, the monoenoic fraction was hydrogenated in order to transform a17:1Δ5 into 14-methylhexadecanoic acid (a17:0). This chiral fatty acid was known to be sufficiently enantioseparated on the β-TBDM column and was found to be (*S*)-enantiopure in the sample. Hence, these measurements verified that the *B. megaterium* sample contained enantiopure (*S*)-a17:1Δ5.
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1. Introduction

Chirality is recognized as a key feature of life. Many of the essential compounds found in biological systems are chiral, and in most occasions they are rather enantiomerically pure than racemic. For instance, most biogenic amino acids (i.e. the building blocks of proteins) are naturally occurring in L-form while many monosaccharides (i.e. the building blocks of polysaccharides) are in D-form. In this context, the major building blocks of membrane lipids, the fatty acids, form an exception. The key fatty acids are straight chained with up to six double bonds but they have no stereogenic center. Thus, fatty acids can only be chiral if a further substituent is present on the carbon chain. The simplest case for this event is fulfilled by a methyl group on the aliphatic carbon chain. The most relevant chiral fatty acids with this feature bear the methyl group on the penultimate carbon, and these are called anteiso fatty acids (aFAs). The aFAs occur as minor fatty acids in the lipids of ruminants and aquatic organisms with 12-methyltetradecanoic acid (a15:0) and 14-methylhexadecanoic acid (a17:0) being the predominant homologues [1–5]. In contrast, high amounts of aFAs are found in the lipids of various bacteria [6]. Thus, the presence of aFAs in food has been linked with bacterial sources. A few data collected in the 1950s indicated that the chiral aFAs are present in the (*S*)-form in dairy products and shark liver oil [1,7–9]. These results were obtained by cumbersome isolation from several kilograms of one individual sample including hydrogenation followed by repeated crystallization, distillation, column chromatography, and X-ray diffraction measurements [1,7]. Progress in the field was hampered by the lack of suitable chromatographic methods for the enantioseparation of aFAs.

The gas chromatographic enantioseparation of methyl-branched carboxylic acids is becoming increasingly more difficult

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the farther the stereogenic carbon is separated from the polar head group. While α-, β-, and γ-methyl-substituted carboxylic acids of different chain lengths were enantiomerically resolved by different GC approaches [10–12], only one indirect HPLC method [13] and one direct GC method have been successfully applied to partly resolve racemic aFA standards where up to thirteen methylene units lay between the head group and the stereogenic carbon [14]. The direct enantioselective GC method was also used for investigations of the enantioselectivity of aFAs in food samples. These measurements verified a predominance of the (*S*)-enantiomer of a15:0 and a17:0 with an enantiomeric excess (ee) of 86% or higher [14].

Next to the saturated aFAs mentioned above, monoenoic aFAs have also been reported to occur in some *Bacillus* species (*B. megaterium*, *B. cereus*, *B. anthracis*, *B. xanthus*, *B. insolitus*, *B. psychrophilus* and *B. globisporus*), in different myxobacteria (*Stigmatella aurantiaca* and *Myxococcus xanthus*), in *Desulfovibrio desulfuricans*, but also in *Vernix caseosa* and adult human skin lipids, in sponges, as well as in *Brevibacterium fermentans*, a biotechnological producer of amino acids [15–22]. These monoenoic aFAs were shown to have the double bond in different positions and included both *cis*- and *trans*-isomers. However, the chirality aspect of the monoenoic aFAs has not been explored to date.

In this study, we thus focused on the gas chromatographic enantioselective determination of monoenoic aFAs. For this purpose, we studied a membrane phospholipid of *Bacillus megaterium* prepared in the 1990s in which the presence of such a monoenoic fatty acid has been reported to occur in significant amounts [23]. Additional standards were available from synthesized racemic and (*S*)-enantiopure *cis*- and *trans*-isomers of 14-methylhexadec-12-enoic acid (a17:1Δ12) [24].

2. Materials and methods

2.1. Samples, standards, and chemicals

A facultatively thermophilic strain of *B. megaterium* (Ft R32) was grown at 20 °C and the membrane lipid phosphatidylethanolamine was isolated according to Rilfors et al. [23]. The resulting lipid fraction (∼50 mg) dissolved in 1 mL chloroform/methanol (2:1, v/v) was stored frozen at −55 °C until the recent analysis. The *cis*- and *trans*-isomers of a17:1Δ12 (AKA a17:1*n*−5) were synthesized by Thurnhofer and Vetter [24]. In brief, the geometrical isomers of a17:1Δ12 (the racemate and in a parallel reaction the (*S*)-enantiomers) were obtained by a Wittig reaction and separated by silver ion HPLC [24]. All other fatty acids (used for peak identification in the bacterial lipids) were from Larodan (Malmö, Sweden). Tetrahydrofuran (THF) and *n*-hexane (HPLC gradient grade) were from Fluka. Methanolic BF_3 (∼13%) and platinum IV oxide (Adam's catalyst) were from Riedel-de-Haën and Sigma–Aldrich, respectively (all located in Taufkirchen, Germany). Dichloromethane (analytical reagent grade) was from Fisher Scientific (Schwerte, Germany). Technical acetone was distilled prior to use. Potassium *tert*.-butoxide (98%) was from Fisher Scientific (Schwerte, Germany), and 3-hydroxymethylpyridine (for synthesis) was from Merck (Schuchardt, Hohenbrunn, Germany). Celite 545 and Discovery silver ion solid phase extraction cartridges (750 mg/6 mL) were from Supelco (Bellefonte, PA, USA). All gases were from Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

2.2. Preparation of fatty acid methyl esters

FAMEs were prepared using the "DFG Einheitsmethode" as previously described [28]. In brief, 10 μL of the sample solution in chloroform/methanol (2:1, v/v) was evaporated to dryness and the resulting ∼0.5 mg of sample lipids were heated with 0.5 mL 0.5 M potassium hydroxide for 5 min at 80 °C. After cooling on ice, 1 mL boron trifluoride in methanol was added and the solution was heated (80 °C) for further 5 min. After cooling at room temperature, each 2 mL of saturated sodium chloride solution and *n*-hexane was added, and the FAMEs were extracted with the organic phase.

2.3. Separation of saturated and monoenoic fatty acid methyl esters

The separation using silver ion (Ag^+) chromatography according to AOAC [29] was recently described in detail [30]. In brief, pre-conditioned solid phase extraction (SPE) cartridges (750 mg/6 mL) were loaded with 1 mL sample solution in *n*-hexane. Saturated and *trans*-monoenoic FAMEs were eluted with 6 mL *n*-hexane/acetone (96:4) into fraction 1, *cis*-monoenoic FAMEs with 4 mL *n*-hexane/acetone (90:10) into fraction 2, and PUFA with 4 mL acetone into fraction 3. The three individual fractions obtained were separately evaporated to dryness by means of a gentle stream of N_2 and were finally made up with *n*-hexane to 1.0 mL volume.

2.4. Hydrogenation of unsaturated fatty acid methyl esters

In a 50-mL two-necked flask, FAMEs and 2 mg of platinum IV oxide were diluted with ∼2 mL dry THF. The solution was stirred for 1 h in an H_2 atmosphere, maintained with an H_2-filled balloon on the one side and a tight glass stopper on the other neck of the flask [31]. The hydrogenated derivatives were filtered through celite 545 to remove the catalyst, before the THF was evaporated and made up with *n*-hexane for GC/EI-MS measurements.

2.5. Preparation of fatty acid picolinyl esters

Approximately 0.5 mg of phospholipids isolated from *B. megaterium* were diluted in 1 mL dry dichloromethane. Then, 0.1 mL potassium *tert*.-butoxide in THF (20%) and 0.2 mL 3-hydroxymethylpyridine was added and the solution was mixed and heated to 40 °C which was held for 30 min. After cooling to room temperature, 2 mL saturated NaCl solution was added and the picolinyl esters were extracted three times with 2 mL *n*-hexane. The volume of the *n*-hexane phase was adjusted to 1 mL and analyzed by GC/EI-MS [32–34].

2.6. Gas chromatography interfaced with electron ionization mass spectrometry (GC/EI-MS)

Enantioselective measurements were performed with an HP GCD plus system equipped with an HP 6890 autosampler (Hewlett-Packard, Waldbronn, Germany). One microliter of standard/sample solution was splitless injected (split opened after 2 min). The injector and transfer line temperatures were set to 250 and 280 °C, respectively. Helium (purity 99.9990%) was used as the carrier gas at a constant flow rate of 0.9 mL/min. The ionization energy was 70 eV, and the temperature of the ion source was set at 165 °C. For GC/EI-MS in the selected ion monitoring (SIM) mode, nine fragment ions (*m/z* 74, *m/z* 87, *m/z* 81, and *m/z* 79 for all FAMEs [25] along with five specific ones, i.e. *m/z* 284 (M^+ for 17:0 ME isomers), *m/z* 256 (M^+ for 15:0 ME isomers), *m/z* 282 (M^+ for 17:1 ME isomers), *m/z* 250 ($[M-32]^+$ for 17:1 ME isomers), and *m/z* 208 ($[M-74]^+$ for 17:1 ME isomers) were recorded throughout the run. A solvent delay of 100 min was applied.

Enantioseparations were performed with a 30 m × 0.25 mm i.d. column coated with 50% heptakis(6-*O*-*tert*.-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (β-TBDM) in OV-1701 [14]. The chiral stationary phase β-TBDM was synthesized according to Dietrich et al. [26]. A purity of >99% was determined by TLC (toluene/2-propanol 7:2) and high-temperature gas chromatogra-

phy as described previously [27]. A column of the same type was previously used for the enantioseparation of (saturated) aFAs [14]. The GC oven program was as follows: after 1 min at 60 °C, the temperature was ramped at 10 °C/min to 115 °C (hold time 400 min), and finally ramped at 1 °C/min to 137 °C (hold time 221 min).

Achiral fatty acid methyl ester analyses were carried out with an HP 5890 gas chromatograph coupled to an HP 5971 mass selective detector (MSD) (Hewlett-Packard, Waldbronn, Germany). Injector-, transfer line- and ion source temperatures were set at 250, 280, and 175 °C, respectively. A 60 m × 0.25 mm i.d. fused silica capillary column coated with 0.2 μm 100% cyanopropyl polysiloxane (SP 2331, Supelco) was installed in the GC oven. The GC oven temperature program started at 60 °C (held 1 min). It was then ramped with 7 °C/min to 180 °C (hold time 2 min), thereafter with 3 °C/min to 200 °C (hold time 2 min), and finally with 3 °C/min to 220 °C (held for 10 min). The total run time was 45.5 min. Helium (see above) was used as carrier gas with a constant flow rate at 1 mL/min. For GC/EI-MS-SIM measurements a solvent delay of 8 min was applied and the four fragment ions *m*/*z* 74 and *m*/*z* 87 [25] for saturated and monounsaturated FAMEs, *m*/*z* 81 and *m*/*z* 79 for dienoic and polyenoic FAMEs were recorded throughout the run. In the GC/EI-MS full scan mode, *m*/*z* 50–450 was recorded with a rate of 1.8 scans per seconds.

Picolinyl esters were determined with GC/MS system consisting of an HP 6890 gas chromatograph coupled to an HP 5972 mass selective detector (MSD) using the same conditions as shown above. An HP5-ms column (30 m × 0.25 mm i.d. × 0.25 μm d_f, J&W Scientific, Agilent) was installed in the GC oven which was heated with the following oven temperature program: after injection at 60 °C (hold 1 min), the temperature was raised at 10 °C/min to 180 °C and then directly at 5 °C/min to 320 °C, which was held for 10 min (total run time 51 min). Analyses were performed in the full scan mode (*m*/*z* 50–500).

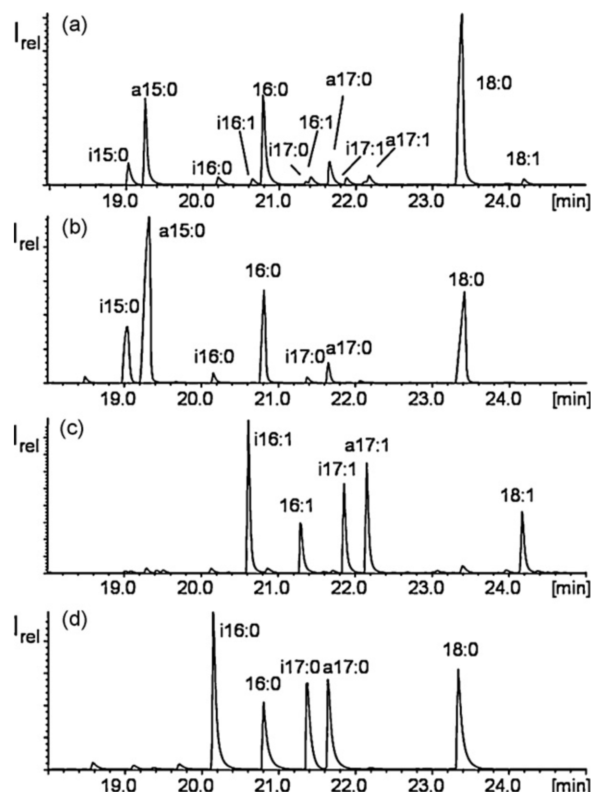

Fig. 1. GC/EI-MS-SIM chromatograms (*m*/*z* 74, SP 2331 column) of (a) the methyl esters of fatty acids from *Bacillus megaterium*, their fractionation by silver ion liquid chromatography into (b) saturated fatty acids (fraction 1) and (c) *cis*-monoenoic acids (fraction 2), as well as the saturated fatty acids obtained by hydrogenation of fraction 2 initially containing the *cis*-monoenoic fatty acids.

3. Results and discussion

3.1. Fatty acids in B. megaterium

In addition to the dominant peaks of 16:0 and 18:0, the GC/EI-MS chromatogram of *B. megaterium* contained five saturated branched-chain fatty acids (i15:0, a15:0, i16:0, i17:0, and a17:0) along with five monoenoic fatty acids, three of which were tentatively identified as monomethyl-branched fatty acids (Fig. 1a). Previous analyses by Rilfors et al. [23] indicated the presence of two monoenoic isomers – tentatively identified as a17:1 and i17:1 isomers – in this sample. The ratio of the fragment ions monitored by GC/EI-MS-SIM supported this assignment [30]. Moreover, the corresponding saturated branched-chain fatty acids eluted prior to the 17:1 isomers and the gap in the retention time between the two monoenoic fatty acids and between the (saturated) i17:0 and a17:0 was virtually identical (Fig. 1a). Therefore, it was conceivable that the two monoenoic fatty acids had the double bond in the same position and that the first eluting compound was an i17:1 and that the second eluting peak was an a17:1 isomer. However, neither the positions of double bonds nor the enantiomeric distribution of the compounds in the sample were known nor could they be derived from the mass spectrometric data of the methyl esters. It is important to note that the potential a17:1 isomer is chiral unless the double bond is located on C-14.

3.2. Structure of the unsaturated anteiso fatty acid and other monoenoic fatty acids

The locations of the double bond and the methyl-branching were determined by means of the picolinyl esters (see Section 2.5.). The mass spectrum of the picolinyl ester of the potential a17:1 showed the molecular ion at *m*/*z* 359 which verified the presence of one double bond equivalent on a C_{17}-fatty acid (Fig. 2). Fragment ions separated by 14 u starting from the $[M-15]^+$ at *m*/*z* 344 were found until *m*/*z* 204 (Fig. 2). The gap of 28 u between *m*/*z* 330 and *m*/*z* 302 verified the anteiso nature of this fatty acid. While *m*/*z* 190 was missing (in favor of the low abundant *m*/*z* 192), the next fragment ion following at lower mass was *m*/*z* 178. The latter mass differs from *m*/*z* 204 by 26 u which is clear indication for a double bond between C-5 and C-6 (Fig. 2). According to Christie, the two fragment ions following at higher mass of the double bond are abundant (i.e. *m*/*z* 218 and *m*/*z* 232), and for isomers with the double bond close to the head group the higher mass clearly dom-

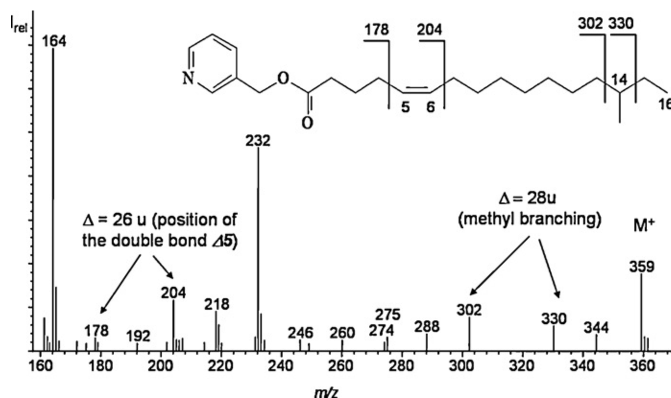

Fig. 2. GC/EI-MS full scan mass spectrum of the picolinyl ester of 14-methylhexadec-5-enoic acid with inserted structure.

inates [35,36]. In fact, *m*/*z* 232 was very abundant (Fig. 2), and this feature is also found for 18:1Δ5, 20:1Δ5, and 22:1Δ5 [36]. Thus, the structure of the monoenoic aFA was established to be a17:1Δ5 or a17:1*n*−12 (see structure inserted in Fig. 2). Suutari and Laakso reported the presence of Δ5- and Δ9-isomers in *Bacillus subtillis* and *Streptomyces griseus*, respectively [37]. Although the authors did not detect these a17:1 isomers in a sample isolated from *B. megaterium* their reports on a17:1Δ5 in related samples supported our findings. Verification of the geometry of the double bond was achieved by silver ion chromatography in SPE cartridges (see Section 2.3.). Using this technique, saturated fatty acids and *trans*-fatty acids are eluted into a first fraction and *cis*-monoenoic fatty acids in the second. This experiment demonstrated that all saturated compounds were found in fraction 1 (Fig. 1b) whereas a17:1Δ5 along with four further abundant monoenoic fatty acids eluted into fraction 2 (Fig. 1c) and thus were *cis*-fatty acids.

In addition, the GC/EI-MS full scan mass spectra of the picolinyl esters allowed to determine the exact structure of the second branched-chain fatty acid to be i17:1(Δ5), which confirms the prediction made from the GC retention time (see above). Furthermore, we identified i16:1(Δ5), 16:1(Δ7), and 18:1(Δ9) by means of their picolinyl esters. The three Δ5-fatty acids were previously described in the phospholipid acyl chains from *B. insolitus*, *B. psychrophilus*, *B. globisporus* and from the sponge *Polymastia penicillus* (or rather from bacteria associated to the sponge) [20,22]. However, a17:1Δ5 remained the only chiral monoenoic fatty acid in the sample.

3.3. Enantiomer separation of the monoenoic anteiso fatty acids

To the best of our knowledge, the gas chromatographic enantioseparation of monoenoic aFAs with 15 carbons and more has not been achieved. Recently, the partial enantioseparation of the (racemic) methyl esters of the saturated a15:0, a16:0, and a17:0 was reported on a β-TBDM phase [14]. However, it could not be excluded that this type of column will not resolve the methyl esters of monoenoic anteiso fatty acids. Only the presence of two peaks in a sample would provide analytical proof for this scenario. Unfortuntely, initial measurements using the chiral β-TBDM column resulted in one peak. Thus, these measurements could neither provide insights into the capability of the GC column to resolve the enantiomers of monoenoic aFAs, nor would such a measurement be suitable to assign the absolute configuration in the case of a pure enantiomer. Clarification of these points would have required the availability of a racemic standard of a17:1Δ5 which was not at our disposal.

For this reason, we first explored the enantioseparation of synthesized standards of *cis*- and *trans*-a17:1Δ12 which were at hand due to our previous synthesis [24]. The synthetic *cis*- and *trans*-a17:1Δ12 standards eluted almost 1 min after the bacterial a17:1Δ5 from the column coated with nonchiral 100% cyanopropyl polysiloxane which is in agreement with an increased GC retention time on polar GC columns with subsequent shifts of the double bonds towards the tail end.

While the enantiomers of racemic *cis*-a17:1Δ12 were sufficiently resolved at 137 °C isothermal (corresponding with a run time of almost 10 h, Fig. 3a), the racemate of the *trans*-isomer produced only one peak in the gas chromatogram. The pure (*S*)-*cis*-a17:1Δ12 gave one peak as expected (Fig. 3b). However, due to the long GC run times required, minor shifts in the retention times did not unequivocally verify the elution order. Spikes of low amounts of the racemate into the enantiopure (*S*)-a17:1Δ12 verified that the (*R*)-enantiomer eluted prior to the (*S*)-enantiomer (Fig. 3c). However, the peak width of the (*S*)-enantiomer and the racemate of *trans*-a17:1Δ12 was virtually the same which supported the fact that only the *cis*-enantiomer was partly resolved on the β-TBDM column.

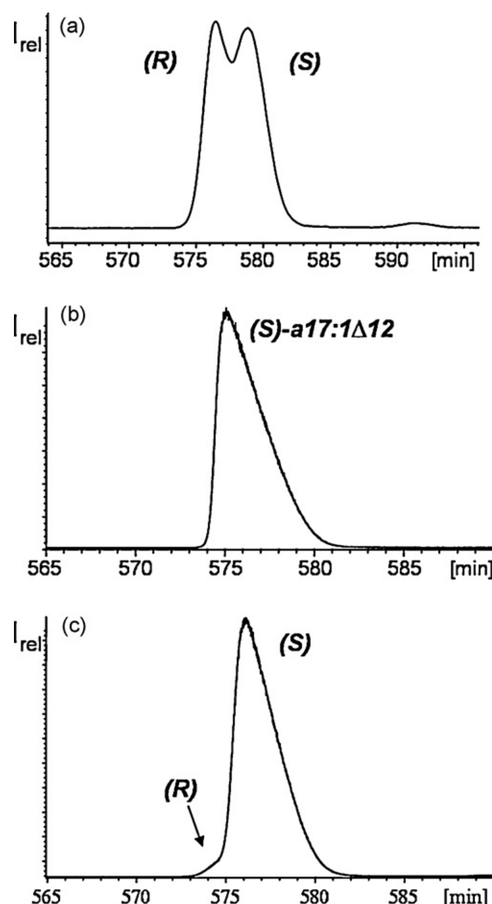

Fig. 3. GC/EI-MS chromatograms (*m*/*z* 74, β-TBDM column) of the enantioseparation of synthesized standards of (a) racemic a17:1Δ12, (b) enantiopure (*S*)-a17:1Δ12 and (c) (*S*)-a17:1Δ12 spiked with low amounts of the racemate.

The separation of the synthetic *cis*-isomer but not the *trans*-isomer supported the hypothesis that the *cis*-isomer of a17:1Δ5 could be enantiopure in the sample but did not proof this. While it remained unknown to this point whether the a17:1Δ12 could be resolved on the β-TBDM column, it was known from our previous study that a17:0 can be partly separated with the (*R*)-enantiomer eluting prior to the (*S*)-enantiomer. The a17:0 was found to be (*S*)-enantiopure in *B. megaterium* (Fig. 4). Enantiopurity of a15:0 and a17:0 in *B. subtilis* has been predicted by Kaneda from the incubation with selective substrates [6,38]. The peak of a17:1Δ5 produced a peak width similarly to i17:1Δ5 (Fig. 4).

To verify the chirality aspect of a17:1Δ5 we took advantage of the *cis*-monoenoic fraction obtained after silver ion liquid chro-

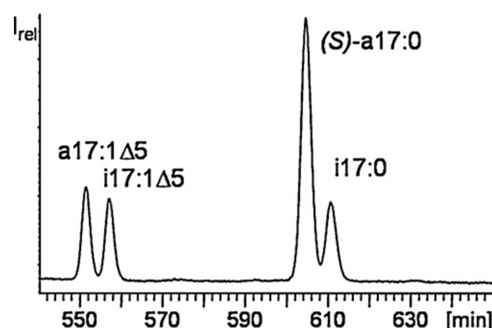

Fig. 4. Partial GC/EI-MS-SIM chromatogram (*m*/*z* 74) of the enantioselective analysis of the methyl esters of branched-chain C_{17}-fatty acids from *B. megaterium* on β-TBDM.

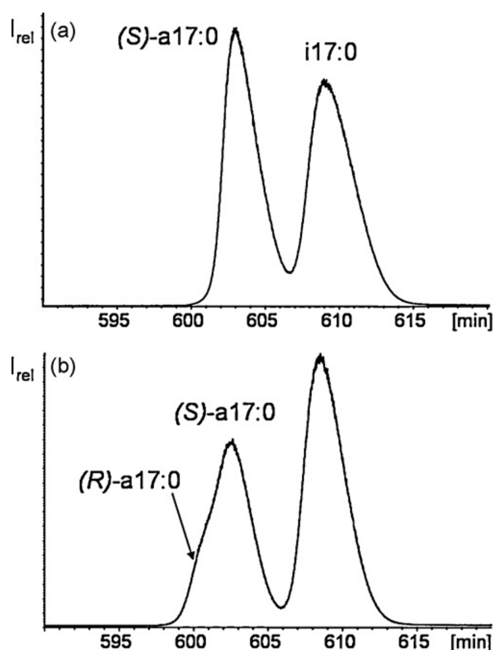

Fig. 5. Partial GC/EI-MS-SIM chromatograms (*m*/*z* 74, β-TBDM) of (top) the enantioselective analysis of the methyl esters of a17:0 (and i17:0) obtained after hydrogenation of the corresponding monoenoic branched-chain fatty acids from *B. megaterium* as well as (bottom) the same sample spiked with a racemic a17:0 standard.

matography (Fig. 1c). Fig. 1b and c demonstrates that a17:0 and other saturated fatty acids were effectively separated from the *cis*-monoenoic fraction. In the next step we hydrogenated the double bonds of the fatty acids present in the *cis*-monoenoic fraction (Fig. 1d). As can be seen from Fig. 1c and d, the five monoenoic *cis*-fatty acids were transformed into the five corresponding saturated analogues i16:0, 16:0, i17:0, a17:0, and 18:0. Consequently, a17:1Δ5 was transferred into a17:0. Finally, the enantioselectivity of a17:0 (produced by the hydrogenation of a17:1Δ5) was determined on the β-TBDM column. The resulting gas chromatogram showed one peak at the retention time of (*S*)-a17:0 (Fig. 5, top). Spikes of low amounts of racemic a17:0 (and i17:0) into the *B. megaterium* sample verified that only the (*S*)-enantiopure a17:0 was present in this bacterial sample (Fig. 5, bottom). Since the hydrogenation does neither affect the configuration of the stereogenic carbon nor does it alter the priority rule of substituents, the configuration of the chiral center of the precursor (i.e. the non-hydrogenated monoenoic aFA obtained from (*S*)-a17:0) is also sinister. Hence, it is proven that the absolute configuration of the monoenoic fatty acid in *B. megaterium* is (*S*)-a17:1Δ5*cis*.

Although the presence of (*S*)-a17:1Δ5*cis* together with (*S*)-a17:0 was not unexpected, the definite analytical proof finally provided in this study was still missing. The combination of transformation of the unknown monoenoic aFA from the sample into the saturated aFA turned out to be the clue to the successful structure assignment in the field of aFAs. The a17:0 and the a17:1Δ5-fatty acids remain the longest aFAs enantioseparated to date by enantioselective gas chromatography.

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