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Short synthesis of labeled and unlabeled 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid as metabolic probes for biosynthetic studies on diatoms

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Abstract

We describe a short synthesis of the unusual polyunsaturated 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid found in marine and fresh water diatoms. Using a one pot reductive bis-Wittig-olefination, the homoconjugated tetraene backbone of the fatty acid can be generated from easy available precursors. Reductive olefination allows the non-statistical dissymmetrisation of a symmetrical bis-Wittig salt as key synthon. This short sequence was also applied to the generation of the corresponding 9,10-[$^2H_2$] labeled fatty acid. If administered to cell fragments of *Thalassiosira rotula* 9,10-[$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid is transformed oxidatively to the aldehyde 1,2-[$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal which is involved in the chemical defense of this alga. Using the synthetic standard it could be shown that the C16:4 ω1 fatty acid is released upon wounding of *T. rotula* cells. The synthesis with the labeled bis-Wittig salt is of general use and can also be applied for the fast generation of other internally labeled functionalized and non-functionalized polyunsaturated fatty acids. To our knowledge this represents the first synthesis of 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid.

Keywords: One-pot synthesis; bis-Wittig reaction; Homoconjugated tetraenes; Deuterium labeled; Unsaturated fatty acids; Reductive olefination

1. Introduction

6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (C16:4 ω1) has initially been detected in fish oil (Silk and Hahn, 1954) and was later found as an important constituent in phytoplankton samples (Klenk and Eberhagen, 1962). It is a common fatty acid in diatoms and, can therefore, be used as a trophic marker in the marine food chain (Ackman and Tocher, 1968; Dunstan et al., 1994). Recently, this fatty acid was also identified from fresh water diatom biofilms as toxic principle against the anostracan grazer *Thammocephalus platyurus* (Jüttner, 2001). Despite this importance as yet no synthesis of this unusual fatty acid was reported. This is at least partially due to the fact that the synthesis of homoconjugated tetraene systems, like it is found in C16:4 ω1 requires long sequences involving numerous coupling and protection steps (see

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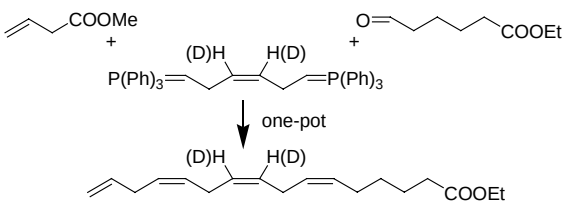
Durand et al., 2000 for a review). Here we show that a one-pot bis-Wittig sequence, recently established in our lab for the production of homoconjugated dienes and trienes (Pohnert and Boland, 2000; Pohnert et al., 1999) can be extended for the generation of the homoconjugated tetraenoic acid C16:4 ω1 (Scheme 1).

Besides the direct toxicity of free polyunsaturated fatty acids, also unsaturated aldehydes derived from fatty acids are discussed to play a key role in diatom defense (Ianora et al., 2003; Miralto et al., 1999). After cell disruption these unicellular algae release a complex bouquet of saturated and unsaturated aldehydes (Pohnert, 2000). Among these, the α,β,γ,δ-unsaturated aldehydes act antiproliferatively against the eggs of herbivorous copepods and sea urchins (Adolph et al., 2003; d'Ippolito et al., 2002b; Miralto et al., 1999). Most of the identified unsaturated aldehydes are derived from the lipoxygenase-mediated transformation of eicosanoic fatty acids (Pohnert and Boland, 2002), but recently, 2*E*,4*E*/*Z*-octadienal was reported as an exception to be derived of ω4-hexadecatrienoic acid (d'Ippolito et al., 2003). d'Ippolito et al. suggested C16:4 ω1 to be a possible precursor of the unusual metabolite 2*E*,4*E*/*Z*,7-octatrienal but experimental evidence is still lacking. Our synthetic concept of bis-Wittig olefination can be exploited for the generation of position specific labeled metabolic probes that allowed to show that 9,10-[$^2H_2$]-C16:4 ω1 is the precursor of the unusual metabolite 1,2-[$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal.

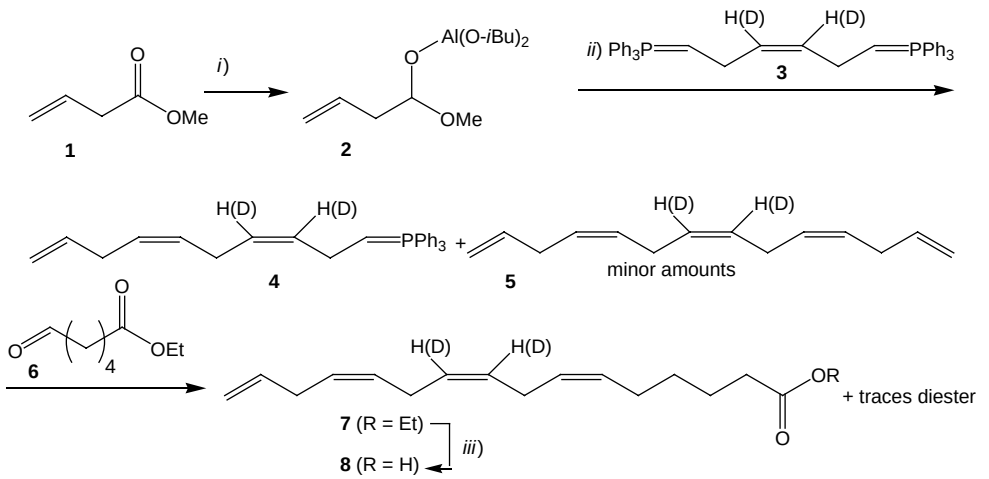
2. Results and discussion

Based on a previously introduced synthesis of homoconjugated dienes and trienes we developed a short synthesis of C16:4 ω1. Using the symmetrical bis-Wittig salt **11** (Pohnert and Boland, 2000), which was prepared from the mono THP-protected (*Z*)-hex-3-ene-1,6-diol (**9**) the homoconjugated tetraene was generated in a sequential one pot procedure. To avoid the statistical distribution of wanted unsymmetrical and unwanted symmetrical side-products arising out of the reaction of the symmetrical bis-ylide **3**, dissymmetrisation was required. This can be achieved by generating the first aldehyde equivalent in situ through diisobutyl aluminium hydride (DIBALH) reduction of the corresponding ester.



Scheme 1. Schematic representation of the formation of homoconjugated tetraenes using the bis-Wittig reaction.

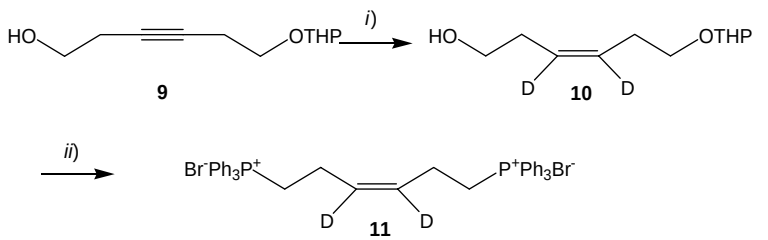
We selected the commercial available methyl 3-butenoate (**1**) as first aldehyde equivalent which also delivers one homoconjugated double bond. This was reduced by slowly adding pre-cooled DIBALH at −78 °C (Scheme 2). Meanwhile the bis-Wittig salt **11** was deprotonated to the bis-ylide **3** with 2.2 equivalents of $KN(SiMe_3)_2$. The aluminate reaction mixture was then transferred directly via a pre-cooled cannula to the bis-ylide and the mixture was allowed to warm slowly to 0 °C. During this procedure the aluminum-complex **2** decomposes and releases slowly but-3-enal that reacts preferentially with the more reactive bis-ylide **3** compared to the mono-ylide **4**. The reaction mixture was re-cooled to −30 °C and 1.2 equivalents of the second aldehyde equivalent, ethyl 6-oxo-hexanoate **6** were added. After warming to room temperature the reaction mixture was worked up as described in the material and methods section to give ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**) in 24% yield. The symmetrical by-products were detected in minor amounts of 1:0.35:0.05 (**7**:(4*Z*,7*Z*,10*Z*)-tetradeca-1,4,7,10,13-pentaene (**5**):(6*Z*,9*Z*,12*Z*)-diethyl octadeca-6,9,12-triendioate (GC-MS)). ^{13}C NMR analysis showed that the newly generated double bonds of **7** were of >95% *Z*-geometry, and the double bond geometry of the bis-Wittig-salt **11** (>98% *Z*) was retained during the reaction. This method for the generation of homoconjugated tetraenes allows thus the fast generation of highly unsaturated products from simple starting materials in a one-pot reaction. Compared to other known routes to related highly unsaturated products which are often based on sequential alkyne coupling reactions followed by a final reduction step of the generated polyyne the present approach reduces the amount of reaction steps and use of protecting groups to a minimum.



Scheme 2. Preparation of 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**). (i) DIBAL-H, (ii) from **11** by deprotonation with 2.2 equivalents of $KN(SiMe_3)_2$, (iii) $LiOH/THF/H_2O$.

Another advantage of this short sequence is that isotope labels can be flexibly introduced into specific parts of the target molecule. We reasoned that the deuterated bis-Wittig salt would be useful for the introduction of internal labels in the homoconjugated polyene segment. The labeled bis-Wittig salt was generated after a modified procedure reported for the generation of the unlabeled analogue (Pohnert and Boland, 2000). Mono THP-protected hex-3-yne-1,6 diol (**9**) (Eya et al., 1990) was reduced with D_2 using in situ generated P-2-nickel as catalyst (Brown and Ahuja, 1973). The resulting mono THP-protected [3,4-$^2H_2$]-(3*Z*)-hex-3-ene-1,6-diol (**10**) was transformed directly without deprotection into the labeled bis-Wittig **11** salt using Br_2/PPh_3 (Scheme 3). Compared to the published procedure (Pohnert and Boland, 2000) this direct transformation bears the advantage that the work-up of the polar intermediate hex-3-ene-1,6-diol can be avoided, but lower yields using the mono-protected diol have to be considered. The labeled bis-Wittig salt **11** was transformed as described above to ethyl [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate in 27% yield.

The labeled bis-Wittig salt **11** can be obtained in large batches and stored for a prolonged time. Since it can be also employed to flexibly generate other metabolic probes where internal label of (functionalized) unsaturated fatty acids is required, it provides a useful synthetic tool. The tolerance of aldehydes of varying chain lengths and degree of unsaturation and of functional groups now extends the synthetic approach beyond the generation of homoconjugated trienes. Established syntheses of labeled homoconjugated polyenes involve often the deuteration of corresponding polyynes (see, e.g. d'Ippolito et al., 2003).



Scheme 3. Preparation of [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**). (i) P2-Ni/D_2, (ii) 1: Br_2, PPh_3; 2: PPh_3 reflux.

This results in uniformly labeled double bonds, which often make mass spectrometric investigations difficult. In contrast, our synthesis provides the advantage, that labels can be introduced in selected internal positions of the homoconjugated polyene.

The availability of [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**) allowed to investigate whether this fatty acid is a precursor for defensive metabolites in the diatom *Thalassiosira rotula*. This diatom is known to produce a variety of α,β,γ,δ-unsaturated aldehydes upon cell disruption, that contribute to its chemical defense (Adolph et al., 2003; d'Ippolito et al., 2002a; Miralto et al., 1999; Pohnert, 2000; Pohnert et al., 2002). In this alga 2*E*,4*Z*-decadienal and 2*E*,4*Z*,7*Z*-decatrienal are derived from the lipoxygenase-mediated transformation of arachidonic and eicosapentaenoic acid, respectively (Pohnert, 2002). To verify if shorter chain length fatty acids are transformed to α,β,γ,δ-unsaturated aldehydes as well, we applied [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid **8** to mechanically wounded *T. rotula*. Transformation products were enriched during the first 10 min after wounding using solid phase microextraction (SPME) and products were monitored with GC-MS (Fig. 1).

The labeled fatty acid was transformed with high efficiency, resulting in strongly increased levels of [1,2-$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal (**12**). The specific labeling of the fatty acid combined with the analysis of the fragmentation pattern of the mass spectrum allowed to show, that the intact C9–C16-terminus of the fatty acid is incorporated into [1,2-$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal without loss of the label at C9. This product formation is in accordance with a lipoxygenase/lyase mechanism in which molecular oxygen is introduced in 9-position of the fatty acid, followed by the action of a lyase that releases 2*E*,4*E*/*Z*,7-octatrienal (**12**) and a second yet unidentified C8-fragment (Fig. 1). The diatom *T. rotula* has thus the ability to transform both, polyunsaturated C20 and C16 fatty acids. In contrast, *Skeletonema costatum* which was shown to produce octadienal from 6*Z*,9*Z*,12*Z*-hecodecatrienoic acid lacks the activity for the formation of C10 aldehydes (d'Ippolito et al., 2002a; d'Ippolito et al., 2003). Since fatty acid addition results in a pronounced increase of labeled metabolites, the substrate availability seems to determine if C10- or C8-unsaturated aldehydes are preferentially generated as wound activated defense by *T. rotula*. This is also reflected by the fact that intact *T. rotula*, which does not contain any 2*E*,4*E*/*Z*,7-octatrienal (**12**) lacks also free C16:4 ω1 (Fig. 1C). Only upon wounding of the alga C16:4 ω1 is released from lipids and is available as precursor for the unsaturated aldehyde (Fig. 1D). The wound activated production of 2*E*,4*E*/*Z*,7-octatrienal (**12**) as well as of the unsaturated C10 aldehydes (Pohnert, 2002) is thus controlled through the release of free fatty acids upon cell disruption.

3. Materials and methods

3.1. General remarks

Reactions were performed under Ar. Solvents were dried according to standard methods. ^1H and ^{13}C NMR: Bruker Avance DRX 500 or AV 400 spectrometer. Chemical shifts of ^1H and ^{13}C NMR are given in ppm (δ) downfield relative to TMS. GC-MS: Finnigan Trace MS equipped with an Alltech EC5 column (i.d.: 0.25 mm, 0.25 μm film thickness, 15 m), Helium as carrier. HR-MS: Micromass MasSpec (Micromass, Manchester, UK). Preparative column chromatography was performed on Florisil (Sigma), or SiO_2 (ICN) 32–63, 60 Å. Reagents and solvents were purchased from Aldrich, Merck and Fluka.

3.1.1. Ethyl 6*Z*,9*Z*,12*Z*,15-Hexadecatetraenoate (7)

3.1.1.1. Preparation of the bis-ylide. 0.5 g (0.65 mmol) (*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) was suspended in 25 ml THF and cooled to −78 °C before addition of 1.4 ml of 28 ml a 0.5 M solution $KN(SiMe_3)_2$ (1.4 mmol) in hexane. The suspension was warmed to −20 °C until no further deepening of the dark red color could be observed (20 min). The solution was re-cooled to −78 °C.

3.1.1.2. Preparation of the aluminate. To a cold (−78 °C) solution of methyl but-2-enoate (**1**) (65 mg, 0.65 mmol) in ether (3 ml), pre-cooled (−78 °C) DIBALH (0.65 ml of a 1 M solution in hexanes, 0.65 mmol) was added drop wise.

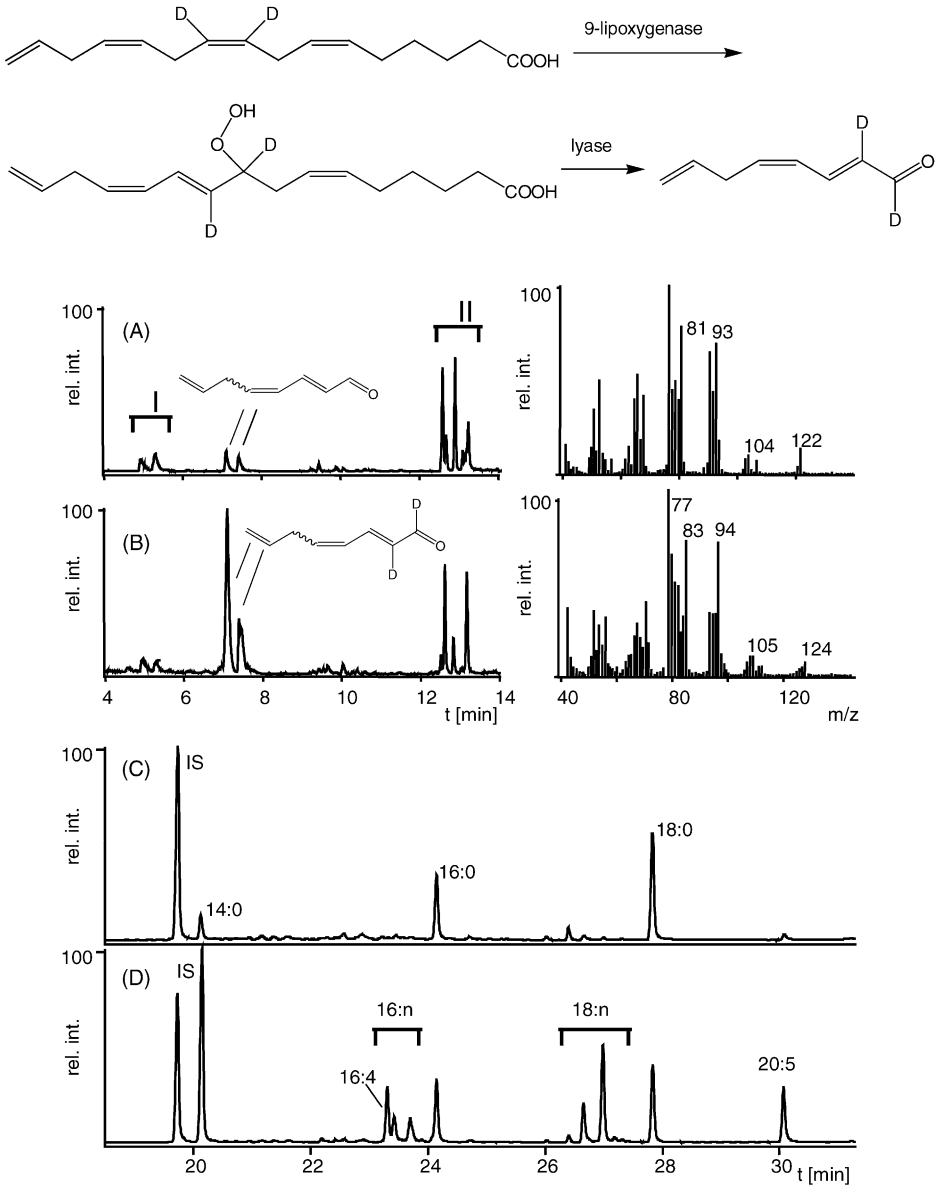


Fig. 1. Transformation of [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**) by suspensions of damaged *T. rotula* in seawater. Above: Mechanistic suggestion for the lipoxygenase-mediated transformation of [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**). (A) Gas chromatogram of SPME extracted volatiles from damaged *T. rotula* (I: 2*E*,4*E*/*Z*-heptadienal; II: 2*E*,4*E*/*Z*-decadienal and 2*E*,4*E*/*Z*,7*Z*-decatrienal, 2*E*,4*E*/*Z*-octadienal at RT, 7.4 min) and EI/MS of 2*E*,4*E*/*Z*,7-octatrienal (**12**). (B) as (A) after addition of 100 μg [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**). (C) Free fatty acids in intact *T. rotula* IS: [$^2H_{27}$]-myristic acid. (D) Free fatty acids in wounded *T. rotula*.

After being stirred for 10 min (GC control) the cold (−78 °C) aluminate **2** was transferred quickly via a pre-cooled cannula to the bis-ylide reaction mixture. The mixture was allowed to warm to RT over a period of 60 min, and stirring was continued for 30 min before re-cooling to −78 °C. Then, a solution of ethyl 6-oxo-hexanoate (**6**) (110 mg, 0.7 mmol) in 5 ml THF was added. The mixture was allowed to

reach room temperature and stirred for 30 min. Hydrolysis with HCl (2 N), extraction with ether, drying over Na_2SO_4, and flash chromatography on silica gel gave ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**) in 24% yield. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.25$ (t, $J = 7.05$ Hz, 3H, O–CH_2CH_3); 1.38 (quint., $J = 7.7$ Hz, 2H, H–C3); 1.6 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt., $J = 7.4$, 7.2 Hz, 2H, H–C5); 2.3 (t, $J = 7.5$ Hz, 2H, H–C2); 2.8 (m, 6H, C8, C11, C14); 4.1 (quart., $J = 7.1$ Hz, 2H, O–CH_2CH_3); 5.0 (dquart., $J = 10.0$ Hz, 1.6, 1H, C16); 5.05 (dquart., $J = 17.2$, 1.7 Hz, 1H, C16); 5.3–5.4 (m, 6H, C6, C7, C9, C10, C12, C13), 5.8 (m, 1H, C15). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 14.66$, 25.02, 25.97, 26.03, 27.28, 29.49, 31.92, 34.66, 60.6, 115.15, 127.46, 128.39, 128.51, 128.76, 129.37, 130.1, 137.14, 174.11. The signals at 131.06 and 129.1 were used to determine the configurational purity of the product. EI-MS (70 eV): 276 (0.7), 247 (1), 235 (3), 208 (5), 189 (7), 147 (20), 133 (31), 119 (27), 105 (45), 91 (60), 79 (100), 67 (40).

*3.1.2. [3,4-$^2H_2$]-(E)-1-(Tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**)*

2.5 g (10.1 mmol) $Ni(Ac)_4{\cdot}(H_2O)_4$ are suspended in 81 ml ethanol (95%) and the reaction vessel was filled with deuterium. The suspension was treated with 382 mg (10.1 mmol) $NaBH_4$ in 10 ml ethanol. After 1 min at RT 2 ml ethylenediamine (30.3 mmol) are added. To the catalyst mixture 16 g (80.7 mmol) 1-(tetrahydro-2H-pyran-2-yloxy)hex-3-yn-6-ol (**9**) was added and stirred over night under D_2 atmosphere. The reaction mixture was filtered over celite and the filtrate was diluted with water. After extraction with Et_2O, drying over Na_2SO_4, removal of the solvent and column chromatography on silica gel [3,4-$^2H_2$]-(*Z*)-1-(tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**) was obtained in 82.1% yield. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.4$–1.65 (m, 5H, H–C2′, 2H–C3′, 2H–C4′); 1.75–1.85 (m, 1H, H–C2′); 2.24 (t, $J = 6.42$ Hz, 2H, H–C5); 2.32 (t, $J = 6.79$ Hz, 2H, H–C2); 3.63–3.82 (m, 2H, H–C6); 3.28–3.45 (m, 2H, C1); 3.45–3.52 (m, 2H, H–C5′); 3.38 (s, 1H, –OH) 4.55 (t, $J = 3.21$ Hz, 1H, C1′). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 20.3$, 26.7, 29.0, 31.7, 32.3, 62.1, 62.6, 67.6, 99.2, (127.9, 128.2, 128.3, 128.4, 128.5, 128.7, 128.8, C3, C4). EI-MS (70 eV): 202 (0.1), 172 (1), 101 (8), 85 (100), 82 (10), 67 (7), 57 (11).

*3.1.3. [3,4-$^2H_2$]-(Z)-Hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**)*

A suspension of triphenylphosphine (27.3 g, 104 mmol) in benzene (300 ml) was treated at 0 °C with bromine (16.6 g, 104 mmol). After being stirred for 10 min at room temperature, the suspension is recooled to 0 °C and [3,4-$^2H_2$]-(*Z*)-1-(tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**) (10 g, 50 mmol) are added. After additional stirring for 2 h at room temperature, the reaction mixture was poured into petrol ether and triphenylphosphine oxide was filtered off. Crude [3,4-$^2H_2$]-(*Z*)-1,6-dibromo-hex-3-ene that was transferred without further purification into a solution of triphenylphosphine (29 g, 110 mmol) in acetonitrile (200 ml). After reflux for 5 days the mixture was poured in toluene (500 ml) and the resulting bis-Wittig-salt was filtered off. Recrystallization from methanol–ether afforded pure crystals of [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) in 39% yield. ^{1}H NMR ([D_4] MeOH, 400 MHz): $\delta = 2.2$ (m, 4H), 3.41 (m, 4H), 7.98–7.13 (m, 2H), 7.6–7.83 (m, 28H). ^{13}C NMR ([D_4] MeOH, 100 MHz): $\delta = 21.8$ (d, $J = 15.4$ Hz), 22.8 (d, $J = 49.9$ Hz), 119.9 (d, $J = 86.6$ Hz), 139.9 (d, $J = 71.9$ Hz), 131.9 (d, $J = 13.2$ Hz), 135.2 (d, $J = 10.2$ Hz), 136.9 (d, $J = 2.9$ Hz). IR (KBr) ν: 2897, 2857, 2751, 1584, 1482, 1434, 1319, 1111, 992 cm^{-1}.

3.1.4. [9,10-$^2H_2$]-Ethyl 6Z,9Z,12Z,15-hexadecatetraenoate (7)

Prepared as described for the unlabeled **7** from [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) 1 g (1.3 mmol). Yield: 27%. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.24$ (t, $J = 7.05$ Hz, 3H, O–CH_2CH_3); 1.38 (quint., $J = 7.7$ Hz, 2H, H–C3); 1.6 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt., $J = 7.4$, 7.2 Hz, 2H, H–C5); 2.3 (t, $J = 7.5$ Hz, 2H, H–C2); 2.8 (m, 6H, C8, C11, C14); 4.1 (quart., $J = 7.1$ Hz, 2H, O–CH_2CH_3); 5.0 (dd, $J = 10.0$ Hz, 1.6, 1H, C16); 5.05 (dquart., $J = 17.2$ Hz, 1.7, 1H, C16); 5.3–5.4 (m, 4H, C6, C7, C12, C13), 5.7 (m, 1H, C15). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 14.66$, 25.02, 25.97, 26.03, 27.28, 29.49, 31.92, 34.66, 60.6, 115.15, 127.46, 128.51, 129.37, 130.1, 137.14, 174.11. EI-MS (70 eV): 278 (0.8), 249 (1), 237 (3), 210 (7), 191 (9), 149 (40), 135 (49), 121 (56), 107 (71), 92 (90), 80 (100), 67 (87).

*3.1.5. 6Z,9Z,12Z,15-Hexadecatetraenoic acid (**8**)*

The free acid was prepared from the ethyl ester **7** (10 mg, 0.036 mmol) by stirring in 4 ml of a 3:1 mixture THF:H_2O in the presence of LiOH (2.4 mg, 0.1 mmol) over night at room temperature. THF was removed under reduced pressure and pH was adjusted to 1 with dil. HCl. Extraction with Et_2O (3×) and column chromatography on SiO_2 gave **8** in 84% yield. ^{1}H NMR (400 MHz, [D_4] MeOH) $\delta = 1.32$ (quint., $J = 7.4$ Hz, 2H, H–C3); 1.51 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt, $J = 6.6$, 7.5 Hz, 2H, H–C5); 2.2 (t, $J = 7.3$ Hz, 2H, H–C2); 2.72 (m, 4H, C8, C11); 2.83 (m, 2H, C14), 5.0 (dd, $J = 10.2$, 2.0 Hz, 1H, C16); 5.1 (dd, $J = 16.7$, 2.2 Hz, 1H, C16); 5.2–5.4 (m, 4H, C6, C7, C12, C13), 5.7 (m, 1H, C15). ^{13}C NMR (100 MHz, [D_4] MeOH) $\delta = 26.11$, 28.27, 30.26, 30.55, 31.3, 32.88, 35.21, 115.47, 128.42, 129.09, 129.6, 129.75, 130.42, 131.46, 138.31, 177.92.

*3.1.6. [9,10-$^2H_2$]-6Z,9Z,12Z,15-Hexadecatetraenoic acid (**8**)*

[9,10-$^2H_2$]-**8** was prepared as described for **8** from [9,10-$^2H_2$]-ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**) (10 mg, 0.036 mmol) in 82% yield. ^{1}H NMR (400 MHz, [D_4] MeOH) $\delta = 1.31$ (quint., $J = 7.6$ Hz, 2H, H–C3); 1.52 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.0 (dt, $J = 6.5$, 7.4 Hz, 2H, H–C5); 2.2 (t, $J = 7.3$ Hz, 2H, H–C2); 2.7 (m, 6H, C8, C11, C14); 4.9 (d, $J = 10.2$ Hz, 1H, C16); 4.95 (d, $J = 17.0$ Hz, 1H, C16); 5.2–5.4 (m, 6H, C6, C7, C12, C13), 5.8 (m, 1H, C15). ^{13}C NMR (100 MHz, [D_4] MeOH) $\delta = 26.09$, 28.22, 30.26, 30.54, 31.3, 33.87, 35.21, 117.91, 128.41, 129.09, 129.6, 131.46, 138.34, 177.96. EI-MS (70 eV): 250 (0.3), 209 (5), 182 (7), 149 (10), 135 (14), 121 (21), 107 (35), 92 (74), 80 (100), 67 (52).

*3.1.7. Transformation of [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**) by T. rotula*

Twenty microliters of a cultured *T. rotula* in the late logarithmic growth phase (for details see Pohnert, 2002) were concentrated by centrifugation to give 2 ml of a dense cell suspension. The cells were transferred to 5 ml glass vials that can be sealed air tight with a Teflon cap. Ten microliters of a 10 mg ml^{-1} solution of [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**) in MeOH were added and the set up was sonicated in an ice bath with four 80 W, 5 s pulses of a 1000L Sonicator (B. Braun Biotech, Melsungen, Germany). The samples were directly sealed after sonication and a polydimethylsiloxane-coated (100 μm) SPME fiber (Supelco, Bellefonte, PA) was introduced in the headspace over the medium. Extraction was performed for 10 min at room temperature. Evaporation of the volatiles from the fiber was directly performed within the injection port (250 °C) of the GC-MS (*T* program: 50 °C [2 min, splitless] ramped with 10 °C min^{-1} to 200 °C and then with 30 °C min^{-1} to 280 °C). Unsaturated aldehydes were identified as described (Adolph et al., 2003). Free fatty acids in intact and wounded *T. rotula* were determined as described elsewhere (Pohnert, 2002), 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid was identified by co-injection with the synthetic standard; GC-MS (*T* program: 60 °C [2 min, split 1:20] ramped with 5 °C min^{-1} to 300 °C (2 min)).

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