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Research Articles

Angewandte
Chemie
International Edition
www.angewandte.org

Isotopic Labeling

Controlled Tetradeuteration of Straight-Chain Fatty Acids: Synthesis, Application, and Insight into the Metabolism of Oxidized Linoleic Acid

Ayako Watanabe+, Kotaro Hama+, Kohei Watanabe, Yuko Fujiwara, Kazuaki Yokoyama, Shigeo Murata, and Ryo Takita**

Abstract: We describe a concise and reliable protocol for the precisely controlled tetradeuteration of straight-chain fatty acids (FAs) at the α- and β-positions that is generally applicable to a variety of FAs, including *trans*-FAs, polyunsaturated FAs (PUFAs), and their oxidized derivatives. The precisely controlled introduction of four deuterium atoms into the FAs enables their persistent and quantitative tracking by LC-MS/MS analysis based on their molecular structures. In addition, the phosphatidylcholine (PC) species prepared from the tetradeuterated FAs thus obtained give a diagnostic peak, namely, a phosphocholine fragment that contains deuterium, in the LC-MS/MS analysis. With these features, the metabolism of a representative oxidized linoleic acid, that is, hydroxyoctadecadienoic acid (HODE), was investigated, leading to the identification of acyltransferases that transfer the acyl moiety derived from HODE to lysophosphatidylcholine.

Introduction

Straight-chain fatty acids (FAs) possess rather simple molecular structures that consist of a terminal carboxyl functionality and a long alkyl chain. The variable parameters usually lie in the long alkyl chains, and include the chain length, the number and configuration of C=C double bonds, the presence or absence of oxygen, as well as some other metabolism-derived functionalities. Despite their relatively basic structures, these FAs play a variety of significant roles in biological systems.[1] The formation of lipids is tremendously varied with regard to the structures of the acids and linkers, their combinations, and the site-selectivity, thus providing lipids with distinct properties for e.g., membranes, signal transduction, and energy storage. While the significance of straight-chain FAs and complex lipids that contain fatty-acyl moieties as biomolecules is widely recognized, the study of the relationships between their biological activity and molecular structures has not progressed to the same degree. One important reason for this are the difficulties associated with tracking and visualizing FAs and lipids.[2]

The structures of FAs and lipids vary in particular due to the wide variety of metabolic pathways, which can produce a broad spectrum of molecular structures.[3] For example, hydroxyoctadecadienoic acid (HODE, Scheme 1a) is produced from linoleic acid through an oxidation and reduction process, and elicit proinflammatory responses in cardiovascular disorders.[4] Complex lipids, such as phospholipids, that contain HODE also exert significant biological effects; high-density lipoproteins enriched in phosphatidylcholine (PC) species that contain HODE have been found to inhibit platelet aggregation.[5] Additionally, increased concentrations of PC species that contain oxidized FAs have been identified in patients with breast cancer and Kawasaki disease.[6] These facts highlight the significance of determining the synthetic mechanism of PC species that contain HODE. However, precisely tracking HODE derivatives in vitro and in vivo is non-trivial, given that HODE is endogenously produced from linoleic acid, which is abundantly present in cells and biological fluids such as sera.

The deuteration of bioactive molecules plays a pivotal role in identifying their molecular mechanisms in e.g., metabolism, pharmacokinetics, and physiological events, as they can serve as molecular probes due to the strong C–D bond as well as their distinct molecular weight.[7] They not only provide their characteristic properties as bioactive molecules, but also are of use in a range of chemical analyses, including NMR and Raman spectroscopy as well as mass spectrometry. During the past decade, several direct deuteration (hydrogen-isotope exchange, HIE) methods for FAs have been reported.[8] For example, Sajiki and co-

[*] Dr. A. Watanabe,+ Prof. K. Watanabe, Prof. S. Murata, Prof. R. Takita
One-stop Sharing Facility Center for Future Drug Discoveries,
Graduate School of Pharmaceutical Sciences, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
E-mail: takita@mol.f.u-tokyo.ac.jp

Prof. K. Hama,+ Prof. Y. Fujiwara, Prof. K. Yokoyama
Faculty of Pharma-Sciences, Teikyo University
2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605 (Japan)
E-mail: khama@pharm.teikyo-u.ac.jp

Prof. K. Hama+
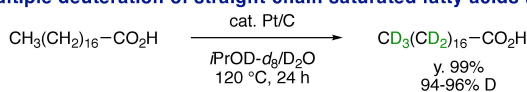Advanced Comprehensive Research Organization (ACRO)
Teikyo University (Japan)

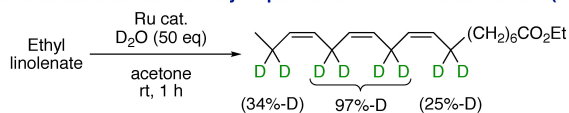[+] These authors contributed equally to this work.

a) Structural variation of straight-chain fatty acids and their lipids
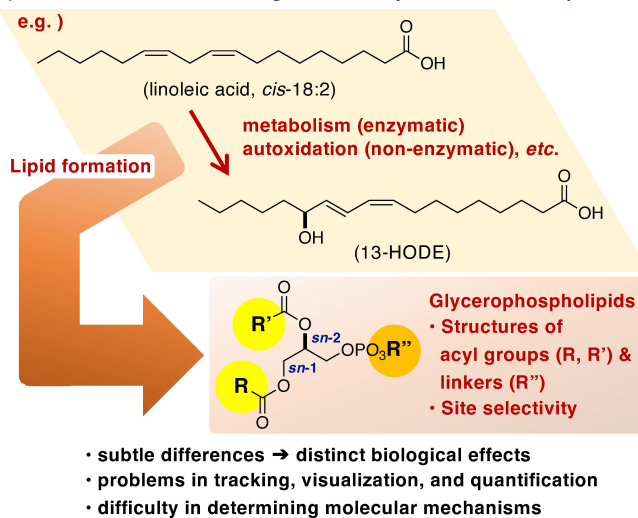
b) Multiple deuteration of straight-chain saturated fatty acids (ref. 8c)

$$CH_3(CH_2)_{16}-CO_2H \xrightarrow[\substack{iPrOD\text{-}d_8/D_2O \\ 120\,°C,\ 24\,h}]{cat.\ Pt/C} CD_3(CD_2)_{16}-CO_2H$$

y. 99%
94–96% D

c) Deuteration at the bis-allylic positions of PUFA derivatives (ref. 8b)

d) Stepwise synthesis of deuterated γ-linolenic acid (ref. 11)

metabolic tuning
and
Raman imaging
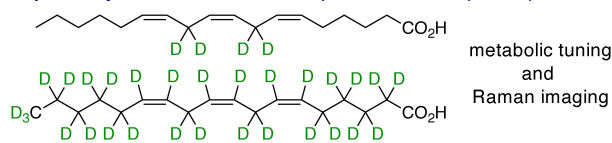
e) Controlled tetradeuteration of fatty acids (this work)
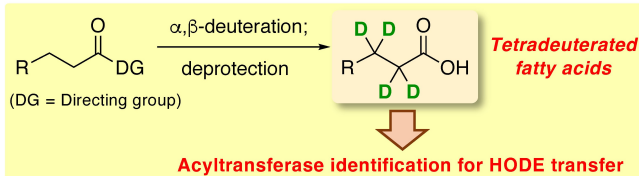
Scheme 1. Structural variation of fatty acids and lipids, as well as preparation of their deuterated derivatives for biological applications.

workers reported the exhaustive deuteration of saturated FAs catalyzed by Pt/C (Scheme 1b).[8c] Vidovic, Shchepinov, and co-workers developed the deuteration of polyunsaturated fatty acids (PUFAs) at the bis-allylic positions of *cis*-olefins using a ruthenium complex (Scheme 1c),[8b] and the presence of such deuterated PUFAs was found to inhibit lipid peroxidation.[9] Very recently, Gemmeren and co-workers reported the direct β-deuteration of α-branched carboxylic acids, which gives moderate to good deuterium incorporation using 1,1,1,3,3,3-hexafluoroisopropanol-d_1 as the deuterium source.[10] In addition, the utility of selectively deuterated straight-chain FA derivatives has been demonstrated by Dodo, Sodeoka, and co-workers, who reported the synthesis of polydeuterated γ-linolenic acid and its

application to biological studies via Raman imaging by taking advantage of the C–D bonds (Scheme 1d).[11]

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis is currently the most powerful technique for the precise analysis of FAs and complex lipids. Stable-isotope-labelled internal standards, which should contain exactly three to five deuterium atoms, are crucial for the accurate quantification in LC-MS/MS analysis, since they are precisely quantified simultaneously with the endogenous target molecules.[7,12] As FAs are metabolized and incorporated into complex lipids through multiple enzymatic processes depending on their structures, FAs labeled with stable isotopes in both a number- and site-selective manner are desirable for an in-depth investigation of the metabolism of FAs and their lipids. Furthermore, given that the fragmentation patterns of lipids in MS/MS analysis have been intensively studied, the diagnostic product ion derived from well-designed deuterated probes can be efficiently used to track the fate of the FAs of interest and is useful in lipidomic studies. Previous HIE methods for FA derivatives incorporated undefined numbers of deuterium atoms depending on the substrate structures.[8] In particular, a general method applicable to straight-chain FAs with a broad range of structural variations, including saturated/unsaturated, *cis*-/*trans*-, and oxidized FAs, would be highly desirable. Herein, we disclose a concise protocol with broad generality for the controlled tetradeuteration of straight-chain FAs. We demonstrate that the d_4-fatty acids thus prepared are effective not only as molecular probes for quantification, but also as diagnostic tools that can be used to study phospholipids via MS/MS analysis. Taking advantage of these features, the metabolism of the HODE, an oxidized form of linoleic acid, was efficiently investigated to elucidate the enzymes responsible for the acyl-transfer events.

Results and Discussion

For the development of a controlled and reliable HIE method for straight-chain FAs, we focused on the following points: (i) the deuteration reaction should be based on the carboxyl functionality (or its derivatives) to achieve a site- and number-selective deuteration process, which should enable broad generality, including applicability to rare and valuable FAs; (ii) low-cost and user-friendly deuterium sources, such as D$_2$O and CH$_3$OD, should be used;[7a] and (iii) an easy reaction setup using catalytic or inexpensive reagents without the need for strictly anhydrous or oxygen-free conditions should be developed. The first point, i.e., the production of uniformly deuterated structures, can be expected to contribute to the production of common diagnostic product ions, which are advantageous for tracking PC species (see below). The latter points in the reaction design are important for its use in interdisciplinary biological and pharmaceutical applications, where high levels of chemical craftsmanship are not necessarily granted.

After screening the reaction conditions for each step (for details, see the Supporting Information), we developed a concise sequential protocol for the controlled tetradeutera-

tion of straight-chain FAs (Scheme 2). Although aliphatic amides are challenging substrates,[7a,13] the use of 8-amino-quinoline amides[8a,14] facilitates the catalytic β-selective deuteration, followed by a K_2CO_3-mediated α-selective deuteration, both of which are carried out with D_2O or CH_3OD as deuterium sources *under atmospheric conditions.* The two-step tetradeuteration conditions were applied to a variety of 8-aminoquinoline amides derived from a range of straight-chain FAs (Table 1). The presence of carbon-carbon double bonds, regardless of their *cis-* or *trans*-configuration, and free- and TBS-protected hydroxy groups was not problematic and the corresponding tetradeuterated products were obtained with a highly selective incorporation of deuterium. Sensitive functional groups such as the diyne functionality in **16a** were also tolerated under these reaction conditions to furnish **16a**-d_4 with a high deuterium content; both the diyne functionality and C–D bonds of **16a**-d_4 are valuable for Raman imaging.

The following conversion of selectively deuterated 8-aminoquinoline amides into the corresponding esters and carboxylic acids was also examined, where oxidative and harsh conditions should be avoided in the interest of a broadly applicable process. Methyl esters-d_4 are particularly valuable as standards in gas chromatography. We found that the nickel catalysis based on a recent report by Morimoto, Ohshima, and co-workers[15] was effective without strict inert and anhydrous conditions (in non-anhydrous CH_3OD as a solvent in a roughly N_2-purged screw-capped tube). In addition, saponification into the corresponding carboxylic acid was achieved using a commercially available NaOD/D_2O solution to afford linoleic acid-d_4 (**6c**-d_4) in good yield (the use of a NaOH/H_2O solution is also possible with the slight loss of the deuterium content; *cf.* Scheme S3). The generality of these transformations is summarized in Table 2. In addition, we carried out a large-scale demonstration producing more than 1 gram of linoleic acid-d_4 (**6c**-d_4) with this protocol (Scheme S5). The overall process offers an efficient route to a variety of tetradeuterated straight-chain FAs and their methyl esters, *from the corresponding non-labeled fatty acids.* The use of easily available starting materials/reagents, as well as easy reaction set up can be expected to facilitate the use of the produced molecules as molecular probes and standards.

Glycerophospholipids, especially PC species, are the most abundant class of lipids in cellular membranes to form
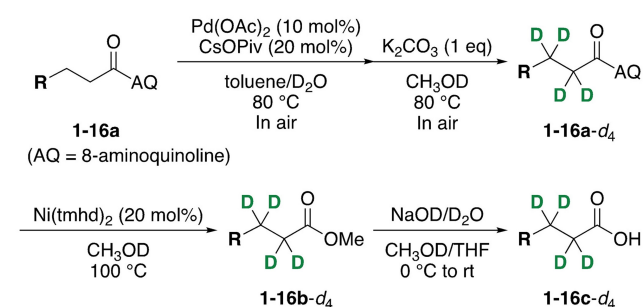
lipid bilayers. Moreover, these phospholipids also function as sources of lipid-signaling molecules with important biological roles.[1] In lipidomic studies, understanding the fragmentation mechanism of glycerophospholipids via collision-induced dissociation (CID) in MS/MS analysis is invaluable. In the case of protonated PC species, the positive charge on the headgroup limits the product ion spectrum almost exclusively to a single peak at $m/z = 184$, which corresponds to phosphocholine, making it thus difficult to understand the fragmentation mechanism. Hsu and Turk proposed a fragmentation mechanism in which the α-hydrogen of one of the acyl groups in the PC should be incorporated in the phosphocholine fragment (Scheme 3a).[16] Several recent reports have supported this mechanism in alkali-metal-mediated PC fragmentation.[17] Very recently, Pagel and co-workers elegantly identified the formation of a protonated five-membered dioxolane fragment from a precursor PC cation using cryogenic IR spectroscopy and experimentally demonstrated the fragmentation mechanism and preferential abstraction of α-hydrogen at the *sn*-2 position relative to the *sn*-1 position.[18] Inspired by these mechanistic considerations, as well as the deuteration experiments by Hsu and Turk,[16a] we planned to use the phosphocholine fragment to track the relevant PC species. Namely, the incorporation of the exogenously added α-deuterated FA into the PC species should give a diagnostic phosphocholine-d_1 peak at $m/z = 185$; this is another rationale for our reaction design to realize α,β-selective deuteration.

An example of the preparation of a deuterated PC species using *trans*-18:2 **7c**-d_4 and lysophosphatidylcholine (LPC) 16:0 (1-palmitoyl-*sn*-glycero-3-phosphocholine, **17**) is presented in Scheme 3b. Under the standard condensation conditions,[19] the corresponding PC **18** (PC 16:0/*trans*-18:2-d_4) was obtained in 50% yield. The obtained **18** was analyzed using MS/MS, and the obtained MS/MS spectrum for the product ion peaks from the parent ion ($m/z = 762.5951$) is shown in Scheme 3c. The major peak A ($m/z = 185.0793$), which was assigned to the mono-deuterated phosphocholine ($C_5H_{14}DNO_4P^+$; calculated exact mass: $m/z = 185.0796$) was observed. Another smaller peak was present in the same region, which corresponds to the phosphocholine fragment ($C_5H_{15}NO_4P^+$; calculated exact mass: $m/z = 184.0733$). Minor peaks were also observed at $m/z = 478$, $m/z = 497$, and $m/z = 506$; these are derived from the dissociation of one of the acyl moieties and have a positive charge on the phosphocholine moiety (for details, see Scheme S4.) For the regioisomeric PC **S4** (PC *trans*-18:2-d_4/16:0) that contains *trans*-18:2-d_4 at the *sn*-1 position, the peak at $m/z = 184$ was preferentially observed (Scheme S5), while the peak at $m/z = 185$ was still observed. This is consistent with the proposed fragmentation mechanism of PC species with CID (Scheme 3a).

Furthermore, the versatility of tetradeuterated PC species in LC-MS/MS analysis was experimentally confirmed in terms of the following two points. (i) They allow the accurate quantification of phospholipids;[7,12] for example, when PC 34:2 ($^{12}C_{42}H_{80}NO_8P$; nominal mass: $m/z = 757$) was analyzed as an endogenous PC species present abundantly

Scheme 2. Tetradeuteration of straight-chain fatty acids.

Table 1: Scope of the tetradeuteration of 8-aminoquinoline amides derived from straight-chain fatty acids.

Entry	Xa	Fatty acid	Product	Yield[c] [%]	α-Deuteration [%]	β-Deuteration [%]
1	**1a**	palmitic acid (16:0)		81	96	98
2	**2a**	stearic acid (18:0)		65	96	93
3	**3a**	9-hydroxystearic acid		71	98	96
4	**4a**	oleic acid (*cis*-18:1)		78	97	97
5	**5a**	elaidic acid (*trans*-18:1)		76	97	96
6	**6a**	linoleic acid (*cis*-18:2)		78	96	97
7	**7a**	linolelaidic acid (*trans*-18:2)		75	95	97
8	**8a**	(9*Z*,11*E*)-conjugated linoleic acid (CLA)		71	96	98
9	**9a**	ricinoleic acid		53	95	96
10	**10a**	13-HODE		64	93	94
11	**11a**	TBS-13-HODE		69	97	97
12	**12a**	linolenic acid (*cis*-18:3)		68	97	95
13[a]	**13a**	arachidonic acid (*cis*-20:4)		67	95	88
14[a]	**14a**	EPA (*cis*-20:5)		91	92	85
15	**15a**	erucic acid (*cis*-22:1)		83	97	97
16	**16a**			60	97[b]	96

[a] The first step was carried out using 1 equiv of Pd(OAc)$_2$ and 2 equiv of CsOPiv at 80 °C for 1 h. [b] The second step was carried out twice to achieve a high deuteration content. [c] The major side reaction seemed to be the partial hydrolysis of amide functionality in both the first and second steps.

Table 2: Scope of the conversion of tetradeuterated 8-aminoquinoline amides into the corresponding methyl esters and carboxylic acids.

Entry	Xa-d_4	Methyl ester Xb-d_4	Yield [%]	Carboxylic acid Xc-d_4	Yield [%]
1	1a-d_4	1b-d_4 (95%-D / 96%-D)	66	1c-d_4 (95%-D / 96%-D)	82
2	2a-d_4	2b-d_4 (96%-D / 97%-D)	75	2c-d_4 (>94%-D / 96%-D)	88
3	3a-d_4	3b-d_4 (>93%-D / 97%-D)	58	3c-d_4 (93%-D / 97%-D)	95
4	4a-d_4	4b-d_4 (96%-D / 97%-D)	87	4c-d_4 (96%-D / 97%-D)	71
5	5a-d_4	5b-d_4 (N.D. / 96%-D)	85	5c-d_4 (95%-D / 96%-D)	90
6	6a-d_4	6b-d_4 (N.D. / 97%-D)	85	6c-d_4 (94%-D / 96%-D)	90
7	7a-d_4	7b-d_4 (N.D. / 97%-D)	86	7c-d_4 (94%-D / 96%-D)	73
8	8a-d_4	8b-d_4 (N.D. / 96%-D)	70	8c-d_4 (98%-D / 96%-D)	97
9	9a-d_4	9b-d_4 (N.D. / 95%-D)	61	9c-d_4 (93%-D / 95%-D)	89
10	10a-d_4	10b-d_4 (N.D. / 94%-D)	60	10c-d_4 (N.D. / 94%-D)	84
11	11a-d_4	11b-d_4 (N.D. / 97%-D)	75	11c-d_4 (96%-D / 97%-D)	94

Table 2: (Continued)

R–CH(D)(D)–C(=O)–NH–(quinolin-8-yl), **1-16a**-d_4 → [a] Ni(tmhd)$_2$ (20 mol%), CH$_3$OD, 100 °C, 16 h → R–CH(D)(D)–C(=O)–OMe, **1-16b**-d_4 → [b] NaOD/D$_2$O, CH$_3$OD/THF, 0 °C to rt → R–CH(D)(D)–C(=O)–OH, **1-16c**-d_4

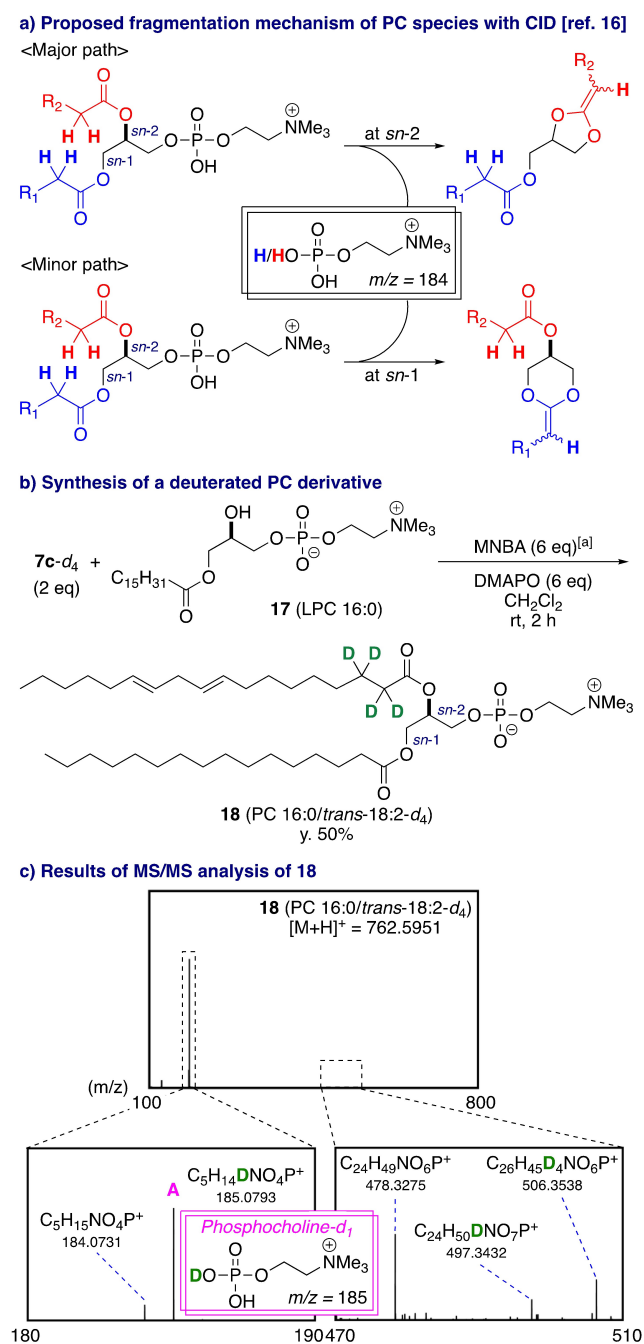| Entry	Xa-d_4	Methyl ester Xb-d_4	Yield [%]	Carboxylic acid Xc-d_4	Yield [%]
12[c]	**12a**-d_4	**12b**-d_4 N.D. / 96%-D	72	**12c**-d_4 95%-D / 96%-D	89
13[c]	**13a**-d_4	**13b**-d_4 91%-D / 96%-D	75	**13c**-d_4 91%-D / 96%-D	93
14[c]	**14a**-d_4	**14b**-d_4 91%-D / 96%-D	47	**14c**-d_4 91%-D / 94%-D	96
15	**15a**-d_4	**15b**-d_4 N.D. / 96%-D	83	**15c**-d_4 97%-D / 97%-D	95
16	**16a**-d_4	**16b**-d_4 96%-D / 97%-D	80	**16c**-d_4 96%-D / 97%-D	>99

[a] Ni(tmhd)$_2$: nickel bis(2,2,6,6-tetramethyl-3,5-heptanedionate). [b] A NaOD/D$_2$O solution is commercially available from Sigma–Aldrich. [c] 50 mol% of Ni(tmhd)$_2$ was used.

in human platelets, the endogenous PC 34:2 was rarely observed through an MRM channel set with $m/z = 762$ as the Q1 value, confirming that quantification of tetradeuterated compounds should be unaffected by endogenous target molecules. The representative chromatograms and quantitative data obtained through each MRM channel are listed in Figure S1a–c. (ii) PC species that contain a deuterated fatty acyl moiety were efficiently monitored in human platelets treated with **6c**-d_4 or **7c**-d_4, while they were barely observed in control platelets when selecting $m/z = 185$ as the Q3 value (Figure S1c–g). Therefore, this peak can be used as a diagnostic peak for the selective tracking of PC species of interest in the LC-MS/MS analysis, as demonstrated in the following section.

Given the efficient tracking with tetradeuterated derivatives, we planned to investigate the metabolism of linoleic acid, a most abundant PUFA in cells, which is metabolized in a variety of ways, including oxidation. Platelets as a cell-based model of lipid metabolism were activated by thrombin immediately after treatment with **6c**-d_4, before the PC species were comprehensively analyzed using LC-MS/MS (Figure S2a). We found that **6c**-d_4 was efficiently transferred to PC species upon activation, and notably, the amounts of

five PC species that contain HODE-d_4, an oxidized form of **6c**-d_4, were elevated by a factor of 8–30 (Figure S2b,c and S3). The amount of PC 16:0-HODE-d_4 was not significantly altered in the presence of lipoxygenase inhibitors MK591 and ML351 (5-lipoxygenase and 15-lipoxygenase inhibitors, respectively), suggesting that other enzymes or autoxidation are involved in the synthesis of PC that contain HODE (Figure S2d). We then examined the possibility that PCs that contain HODE are directly produced by oxidation of PCs that contain linoleic acid. The intracellular amount of PC 16:0-18:2-d_4 was significantly higher in platelets treated with PC 16:0/*cis*-18:2-d_4 (**19**) than that with **6c**-d_4 (Figure 1b), while the amount of PC 16:0-HODE-d_4 was not significantly elevated upon activation in platelets treated with **19** (Figure 1a). These results show that linoleic acid is first converted into HODE, before it is transferred into LPC in activated platelets.

Since little is known about the enzymes responsible for the oxidized fatty acyl-transfer events, we conducted a siRNA-based screening using a HODE-d_4 (**10c**-d_4) synthesized by the present protocol (Figure S4b). Among the 18 lysophospholipid acyltransferases (LPLAT) tested, 17 genes were expressed in the HEK293T cells used in this study

a) Proposed fragmentation mechanism of PC species with CID [ref. 16]

b) Synthesis of a deuterated PC derivative

18 (PC 16:0/*trans*-18:2-d_4)
y. 50%

c) Results of MS/MS analysis of 18

Scheme 3. Fragmentation mechanism of PC species as well as synthesis and MS/MS analysis of deuterated PC derivative **18** (PC 16:0/*trans*-18:2-d_4). [a] MNBA: 2-methyl-6-nitrobenzoic anhydride; DMAPO: 4-(dimethylamino)pyridine *N*-oxide.

(Figure S4a and S4c). We found that the synthesis of PCs that contain **10c**-d_4 was significantly affected by the suppression of the 1-acylglycerol-3-phosphate O-acyltransferase family 6 (AGPAT6) expression (Figure 1c). We then prepared AGPAT6 knockout (KO) cells (Figure S5a) and confirmed that the amount of PC 16:0-HODE-d_4 was significantly attenuated without decrease of other endogenous PC species in the AGPAT6 KO cells (Figure 1d and S5b). AGPAT6 does not exert lysophospholipid-acyltrans-

ferase (LPLAT) activity, but glycerol-3-phosphate-acyltransferase (GPAT) activity, which produces a lysophosphatidic acid (LPA).[20] Consistent with this previously reported result, we found that the amount of endogenous LPA species was critically reduced in AGPAT6 KO HEK293T cells (Figure 1e). It should also be noted here that significant amounts of LPC 16:0 and LPC 18:1 were still observed, albeit that their quantities were somewhat reduced (Figure 1f), indicating that these LPC species can be expected to be produced by other acyltransferases with GPAT activity, the degradation of PC species, and/or supplied from fetal bovine serum in the culture medium. Moreover, we examined the enzymatic activity in vitro (Figure S4d) and confirmed that AGPAT6 directly transfers 16:0-d_{31}-CoA into glycerol-3-phosphate (G3P) but is unable to transfer HODE-d_4-CoA into G3P (Figure 1g). The lack of LPLAT activity between HODE-d_4-CoA and LPC 16:0 was also corroborated (Figure 1h). Our findings thus far include the following: (i) AGPAT6 plays a pivotal role for the synthesis of PCs that contain HODE in HEK293T cells, (ii) its high GPAT activity to produce LPA 16:0 should be of significance, and (iii) other enzyme(s) are required for the synthesis of PCs that contain HODE from the precursor LPC species derived from LPA species.

To address the third issue above, i.e., to identify the acyltransferases that transfer HODE-CoA into LPC species, we conducted an siRNA-based screening using AGPAT6 KO cells that express 17 LPLATs (Figure S4c). The amount of PC 16:0-HODE-d_4 was significantly affected by the suppression of the expression of AGPAT7, acyl-CoA: lysophosphatidylcholine acyltransferase 1 (LPCAT1), LPCAT2, acyl-CoA:lysophosphatidylglycerol acyltransferase 1 (LPGAT1), glycerol-3-phosphate acyltransferase 1, mitochondrial (GPAM), or glycerol-3-phosphate acyltransferase 2 (GPAT2) (Figure 2a). Among these, since GPAM and GPAT2 exert GPAT activity similar to that of AGPAT6,[21,22] we examined the LPLAT activity of AGPAT7, LPCAT1, LPCAT2, and LPGAT1 using HODE-d_4-CoA and 1-16:0-LPC. Significant LPLAT enzymatic activity in vitro was identified for AGPAT7, LPCAT1, and LPCAT2, but not for LPGAT1 (Figure 2b). Practically, we conducted a kinetic analysis and calculated the value of the apparent K_m and the maximum velocity (V_{max}) for AGPAT7, LPCAT1, and LPCAT2, where LPCAT1 exerts the highest affinity to HODE-d_4-CoA (Figure 2c,d). Thus, these three enzymes (AGPAT7, LPCAT1, and LPCAT2) were unequivocally identified for the first time as the acyltransferases that transfer HODE-CoA into the *sn*-2 position of 1-16:0-LPC species.

At the same time, we found that complex mechanisms can be expected to be involved in the synthesis of PCs that contain HODE. We prepared AGPAT6 and AGPAT7 double-knockout (DKO) cells and confirmed that the production of PC 16:0-HODE-d_4 was further suppressed in these cells compared to that of AGPAT6 KO cells (Figures 2e and S5c,d). However, when AGPAT7 alone was knocked out, or even when two independent AGPAT7, LPCAT1, and LPCAT2 triple-knockout (TKO) HEK293T cell lines were used, the amount of PC 16:0-HODE-d_4 was
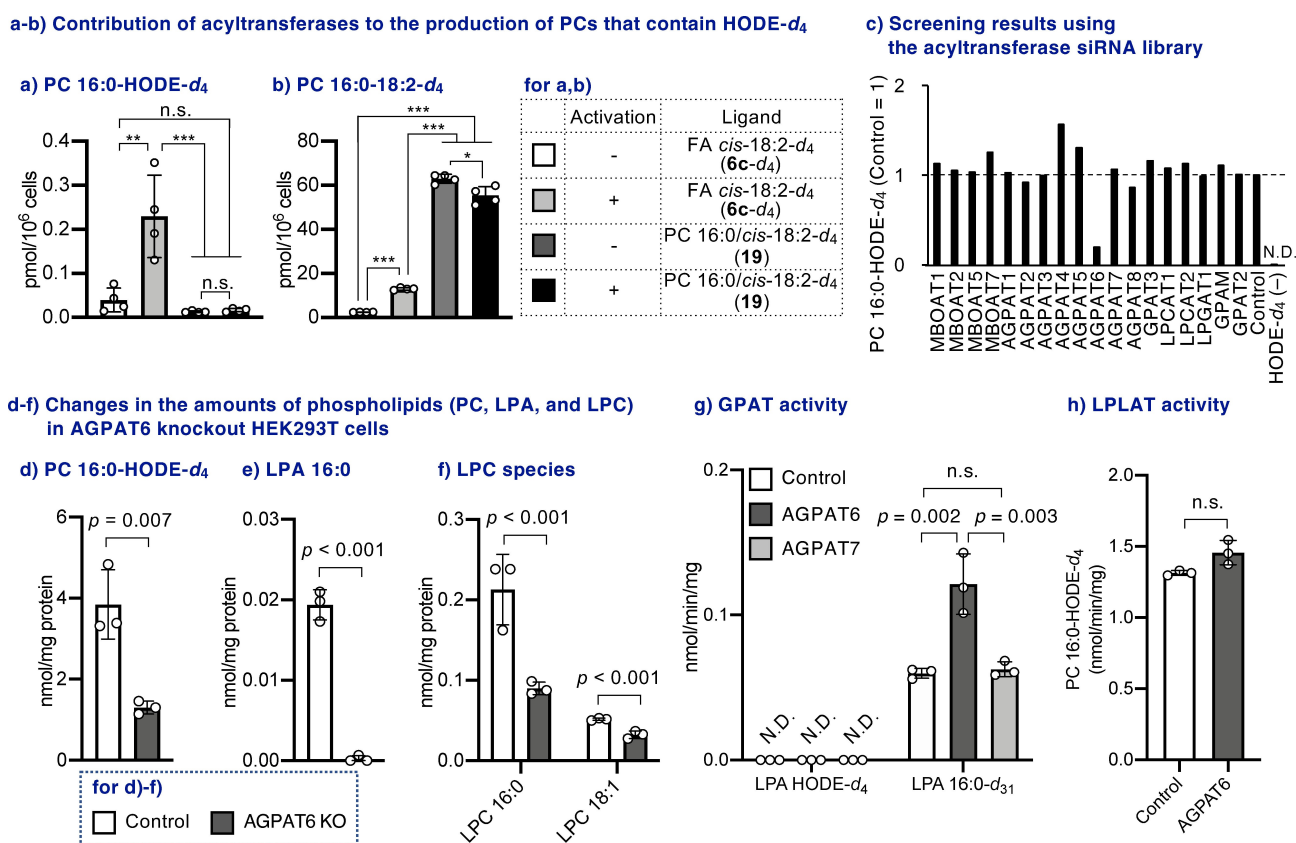
Figure 1. AGPAT6 is essential for the synthesis of PCs that contain HODE in HEK293T cells. a) Amounts of PC 16:0-HODE-d_4 and b) PC 16:0-18:2-d_4 in platelets treated with 18:2-d_4 (**6c**-d_4) or PC 16:0/*cis*-18:2-d_4 (**19**). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. c) Results of the siRNA-based screening for genes involved in the synthesis of PCs that contain HODE. A schematic illustration of the screening is shown in Figure S4b.; N.D.: not detected. d) Amounts of PC 16:0-HODE-d_4 in AGPAT6 KO HEK293T cells. e) Amounts of 1- or 2-16:0-LPA and f) 1- or 2-16:0 (and 18:1)-LPC species in AGPAT6 KO HEK293T cells. g) GPAT activity toward HODE-d_4-CoA and 16:0-d_{31}-CoA. Scheme of the in vitro enzymatic study for HODE- or palmitic acid-transfer activity to G3P is shown in Figure S4d. HODE-d_4-CoA was synthesized by condensation of the corresponding acid chloride with coenzyme A and confirmed by MS/MS analysis (Figure S6). h) LPLAT activity between HODE-d_4-CoA and LPC 16:0. Data represent the mean value $\pm$ SD; statistical analysis was performed using one-way ANOVA followed by the Tukey post hoc test (a, b and g), or Student t-test (d–f and h).

not significantly affected (Figures 2f and S7). The combined results indicate that PC 16:0-HODE can be expected to be synthesized via at least two pathways in HEK293T cells. One is AGPAT6-dependent and responsible for the majority of PC 16:0-HODE production; LPA 16:0 was produced by AGPAT6 and the details of its conversion to PC 16:0-HODE remain to be identified. Another pathway is AGPAT7-, LPCAT1-, and/or LPCAT2-dependent, which should commence with LPC species formed in other ways than the process involving AGPAT6 (Figure 2g).

HODE is a linoleic-acid-derived metabolite generated in situ, and it has previously been challenging to track its molecular transformation and the enzymes involved. For the first time, we identified three enzymes (AGPAT7, LPCAT1, and LPCAT2) as LPLATs that directly transfer HODE-CoA into LPCs (Figure 2a–d). These three enzymes transfer acetyl-CoA, 16:0-CoA, 18:1-CoA, and 22:6-CoA into LPCs, lysophosphatidylethanolamine, or lyso-platelet-activating factor,[23] albeit that the relation to the synthesis of oxidized phospholipid species remains unclear at present. The results discussed herein can be expected to contribute
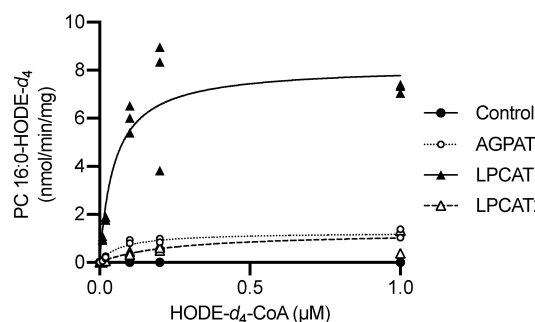
to the understanding of the detailed biological functions of these enzymes. As the present tetradeuteration reaction is applicable to a broad range of oxidized FAs, further explorations based on this strategy will be valuable to understand the metabolic mechanisms of oxidized FAs.

PCs that contain HODE have been implicated in cancer and inflammatory disease,[5,6] and thus, the identification of their synthesis and metabolic derivatization should provide genetic and molecular-biological approaches to clarify the pathological significance of PCs that contain HODE. In this study, we have proposed two pathways in the previous section (Figure 2g). The AGPAT6-dependent pathway, in which 1-acyl-LPA is produced by AGPAT6, should correspond to the de novo phospholipid synthesis pathway (the so-called Kennedy pathway), and this is the major production pathway in HEK293T cells. The other pathway, in which AGPAT7, LPCAT1, and LPCAT2 are responsible for the transfer of HODE-CoA into LPC species, should correspond to the phospholipid-remodeling pathway (the so-called Lands cycle). It should be noted that AGPAT6 produces LPA 16:0 and crucially determines the total
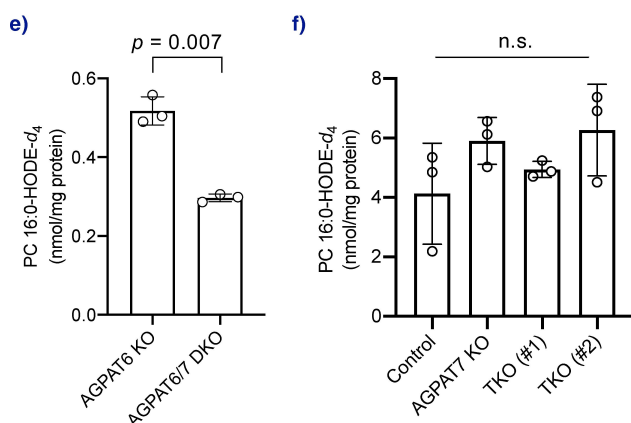
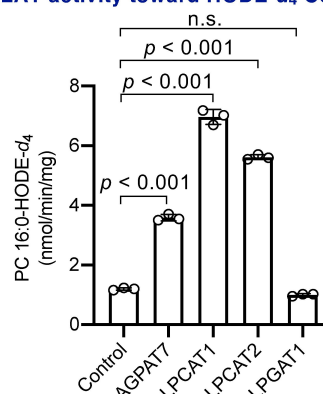## a) Results of siRNA screening with AGPAT6 KO cells

b) LPLAT activity toward HODE-d_4-CoA
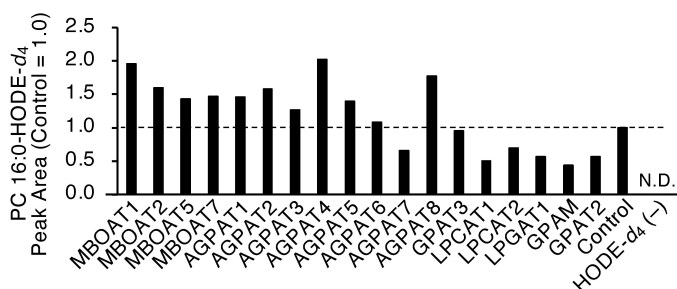
c) Kinetic study for HODE-transfer activity into LPC

d) Apparent K_m and maximum velocity (V_{max})

Enzyme	K_m (nM)	V_{max} (nmol/min/mg)
AGPAT7	71.2	1.25
LPCAT1	49.0	8.17
LPCAT2	237.4	1.26

e,f) Quantifying the change of PC 16:0-HODE-d_4 by the gene knockout

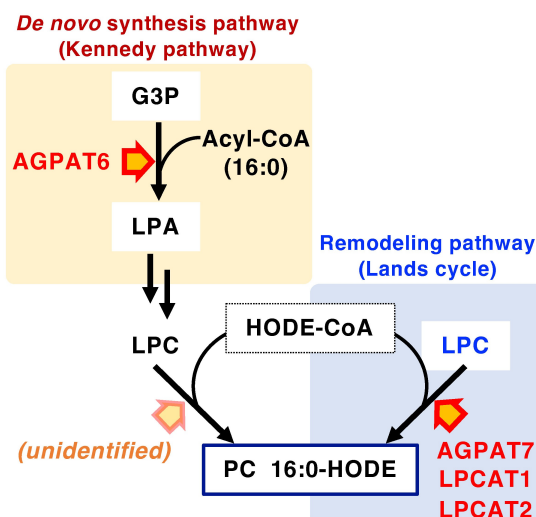## g) Scheme for the synthesis of PC 16:0-HODE

Figure 2. AGPAT7, LPCAT1, and LPCAT2 directly transfer HODE-CoA into LPC. a) Results of the siRNA-based screening for genes responsible for the HODE-transfer activity in AGPAT6 KO HEK293T cells. b) LPLAT activity toward HODE-d_4-CoA. c) Kinetic analysis of the LPLAT activity of AGPAT7, LPCAT1, and LPCAT2. d) Value of the apparent K_m and maximum velocity (V_{max}) for AGPAT7, LPCAT1, and LPCAT2. e) Quantifying PC 16:0-HODE-d_4 in AGPAT6 KO or AGPAT6 and AGPAT7 DKO HEK293T cells. f) Quantifying PC 16:0-HODE-d_4 in one AGPAT7 KO or two independent AGPAT7, LPCAT1, and LPCAT2 TKO (#1 and 2) HEK293T cell lines. g) Proposed mechanism of HODE incorporation into PC species. Data represent the mean value ± SD; statistical analysis was performed using one-way ANOVA, followed by the Dunnett test (vs control) (b), Tukey post hoc test (f), or Student *t*-test (e).

amount of PC 16:0-HODE, at least in HEK293T cells, but not those of other PC species (Figures 1d, e and S5b). Given that several enzymes, including choline phosphotransferases and phospholipases, are necessary to convert LPAs into LPC species, the LPA 16:0 produced by AGPAT6 should be efficiently and selectively used in the following process for the synthesis of PCs that contain HODE. The interaction of these enzymes, including the delocalization in cells,

should be the key to clarifying the entire synthetic landscape of PCs that contain HODE.

Conclusion

We have developed a concise protocol for the controlled tetradeuteration of straight-chain fatty acids (FAs). The keys to this process include its reliable and operationally

straightforward process using inexpensive or catalytic amounts of commercially available reagents, its broad generality for straight-chain FAs, and its highly selective incorporation of deuterium at the α- and β-positions. Not only the FAs with four deuterium atoms, but also the diagnostic peak of monodeuterated phosphocholine obtained from phosphatidylcholines (PCs) that contain these α,β-deuterated acyl moieties are invaluable for comprehensive lipidomic studies to identify metabolic mechanisms via liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Although the tracking of endogenously metabolized FAs has been relatively difficult in the past, the synthetic enzymes responsible for phospholipids that contain a representative oxidized linoleic acid, i.e., hydroxyoctadecadienoic acid (HODE) were determined with our strategy. This selective tetradeuteration method should offer further potential in bioanalytical investigations; for example, the visualization of the metabolic process of each FA and their metabolites in vivo should be feasible by applying both MS imaging and analysis using NMR- or Raman-imaging methodologies.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Acyltransferases · Fatty Acids · Hydroxyoctadecadienoic Acid (HODE) · Phosphatidylcholine · Tetradeuteration

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