Isomer-Specific Accumulation of Perfluorooctanesulfonate from (*N*-Ethyl perfluorooctanesulfonamido)ethanol-based Phosphate Diester in Japanese Medaka (*Oryzias latipes*)

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Ⓢ *Supporting Information*

ABSTRACT: While (*N*-ethyl perfluorooctanesulfonamido)-ethanol (FOSE) -based phosphate diester (diSPAP) has been proposed as a candidate precursor of perfluorooctanesulfonate (PFOS), its potential biotransformation to PFOS has not been verified. Metabolism of diSPAP was investigated in Japanese medaka (*Oryzias latipes*) after exposure in water for 10 days, followed by 10 days of depuration. Branched isomers of diSPAP (B-diSPAP) were preferentially enriched in medaka exposed to diSPAP, with the proportion of branched isomers (BF) ranging from 0.56 to 0.80, which was significantly greater than that in the water to which the medaka were exposed (0.36) ($p < 0.001$). This enrichment was due primarily to

preferential uptake of B-diSPAP. PFOS together with perfluorooctanesulfonamide (PFOSA), *N*-ethyl perfluorooctanesulfona-mide (NEtFOSA), 2-(perfluorooctanesulfonamido)acetic acid (FOSAA), NEtFOSAA, FOSE, and NEtFOSE were detected in medaka exposed to diSPAP, which indicated the potential for biotransformation of diSPAP to PFOS via multiple intermediates. Due to preferential metabolism of branched isomers, FOSAA and PFOSA exhibited greater BF values (>0.5) than those of NEtFOSA, NEtFOSAA, and NEtFOSE (<0.2). Such preferential metabolism of branched isomers along the primary pathway of metabolism and preferential accumulation of B-diSPAP led to enrichment of branched PFOS (B-PFOS) in medaka. Enrichment of B-PFOS was greater for 3-, 4-, and 5-perfluoromethyl PFOS (P_3MPFOS, P_4MPFOS, and P_5MPFOS), for which values of BF were 0.58 ± 0.07, 0.62 ± 0.06, and 0.61 ± 0.05 (day 6), respectively; these values are 5.8-, 7.8-, and 6.4-fold greater than those of technical PFOS. This work provides evidence on the isomer-specific accumulation of PFOS from diSPAP and will be helpful to track indirect sources of PFOS in the future.
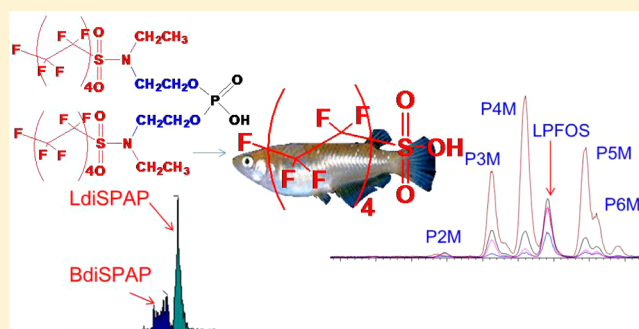
■ INTRODUCTION

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) have received increasing attention due to their global occurrence in environmental media.[1,2] Perfluorooctanesulfonate (PFOS) was added to Appendix B of the persistent organic pollutants (POPs) under the Stockholm Convention in 2009.[3] PFOS, which is the terminal degradation product of a number of chemicals, is one of the most abundant PFASs detected in tissues of wildlife and human serum.[4−7] PFOS can cause a number of adverse effects in animals,[8−10] and human exposure to PFOS has been associated with greater concentrations of cholesterol, lesser weight of fetuses, and impaired thyroid function in epidemiological studies.[11−13] Besides the environmental ubiquity and toxicity, trophic magnification of PFOS in

the food web has been widely reported,[6,14−16] which has been suggested to be partly due to the contribution by the biotransformation of precursors.[6,17,18] Thus, indirect sources of PFOS from precursors complicate our understanding of the fate of PFOS in the environment.

The potential precursors of PFOS are numerous, and chemicals based on perfluorooctanesulfonyl fluoride (POSF) have been proposed as candidate precursors. Of these chemicals, perfluorooctanesulfonamide ethanols (FOSEs) and

perfluorinated sulfonamides such as perfluorooctanesulfona-mide (PFOSA) and *N*-ethyl perfluorooctanesulfonamide (NEtFOSA) have been well accepted as precursors of PFOS,[19] since in vitro liver microsomes and in vivo animal experiment have demonstrated biotransformation from PFOSA, NEtFOSA, and FOSEs into PFOS.[20,21] PFOSA, NEtFOSA, and FOSEs have been detected in different environmental matrices such as air, sewage effluents, human serum, and wildlife,[17,22−24] although concentrations of these chemicals were often several orders of magnitude less than that of PFOS in biota samples.[22,23] Besides the above precursors, (*N*-ethyl perfluorooctanesulfonamido)ethanol-based phosphate diesters (diSPAP) comprise another group of chemicals containing PFOS substructure. diSPAP was introduced in 1974 in food contact paper and packaging, and the sale in 1997 of commercial diSPAP ester formulation FC-807 is the largest quantity of "PFOS equivalents" of all PFOS-containing products produced by 3M Co.[25,26] Despite the phase-out of POSF-based chemicals in 2000, diSPAP is still produced in some places, including several manufacturers in China. A recent study has identified the presence of diSPAP in marine sediment from Vancouver (Canada) at concentrations of 40−200 pg/g dry weight (dw), which is comparable to concentrations of PFOS (71−180 pg/g dw).[25] Thus, biotransformation of diSPAP to PFOS is of concern. While data submitted to the U.S. Environmental Protection Agency (EPA) by the 3M Co. indicated the minor biotransformation of diSPAP to PFOS in rat after a single oral dose,[27] there is no peer-reviewed paper on in vitro or in vivo metabolism of diSPAP to clarify its potential role as PFOS precursor.

It has been reported that biomagnification factors (BMFs) of branched PFOS isomers (B-PFOS) are greater than those of linear PFOS (L-PFOS), as exemplified by the BMF values of 1.5 for L-PFOS and 6.4 for B-PFOS in lake trout.[16] Since results of studies conducted under laboratory conditions have demonstrated that bioaccumulation potential and half-life of B-PFOS in rainbow trout were both lesser than that of L-PFOS,[28] it is not easy to explain the relatively great biomagnification of B-PFOS in wildlife. Preferential metabolism from branched isomers of precursors to B-PFOS has been proposed as a potential explanation for their great BMF values, such as enrichment of 5-perfluoromethyl PFOS (P$_5$M-PFOS) in rats exposed to PFOSA.[29] Since diSPAP is produced from POSF, the same raw material for producing PFOS by electrochemical fluorination (ECF) manufacturing process, the isomeric composition of diSPAP is expected to be ∼30% branched.[30] Thus, investigation on isomer-specific biotransformation of diSPAP to PFOS would be helpful to clarify the environmental behaviors of PFOS isomers and to track indirect sources of PFOS in wildlife.

The current study investigated the uptake and depuration of diSPAP in the animal model Japanese medaka (*Oryzias latipes*; medaka) during exposure in water for 10 days, followed by a 10-day depuration phase. Pathways of metabolism of diSPAP were investigated by monitoring 10 potential products of transformation, including PFOS, three FOSAs, three (perfluorooctanesulfonamido)acetates (FOSAAs), and three FOSEs. Isomers of diSPAP and its metabolites were also analyzed to systematically elucidate isomer-specific biotransformation.

■ MATERIALS AND METHODS

Chemicals and Reagents. Standards including NEtFOSE, NMeFOSE, FOSE, NEtFOSA, NMeFOSA, PFOSA, 2-(perfluorooctanesulfonamido)acetic acid (FOSAA), NEtFO-SAA, NMeFOSAA, and PFOS were obtained from Wellington Laboratories Inc. (Guelph, Ontario, Canada). Stable isotope-labeled standard [1,2,3,4-^{13}C$_4$]PFOS, NEtFOSA-d_5, and NEtFOSAA-d_5 were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). The technical product of diSPAP, FC-807, was purchased from Henxin Co. in China and then was purified by HPLC as described in the Supporting Information. The purified diSPAP was characterized by ultraperformance liquid chromatography−quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS). NMR spectra (^{19}F NMR) were taken on a Bruker Avance III (1H, 400 MHz) spectrometer. The potential products (NEtFOSE, NMeFOSE, FOSE, NEtFOSA, NMeFOSA, PFOSA, FOSAA, NEtFOSAA, NMeFOSAA, and PFOS) of diSPAP in medaka were not detected in the purified diSPAP. Details of HPLC purification methods and NMR and UPLC−Q-TOF MS analytical results are described in the Supporting Information. Tetrabutylammo-nium hydrogen sulfonate (TBAS) was purchased from Sigma (St. Louis, MO). Oasis WAX (6 cm^3, 150 mg) solid-phase extraction (SPE) cartridges were purchased from Waters (Milford, MA); methanol (HPLC-grade) and diethyl ether (HPLC-grade) were purchased from Fisher Chemical Co. Water obtained by a Milli-Q synthesis water purification system (Millipore, Bedford, MA) was used throughout the study.

Medaka Rearing and Sampling. Female, orange-red strain medaka, 2 months old with body weight 210−310 mg and body length 18−23 mm, were from brood stock maintained in the College of Urban and Environmental Sciences, Peking University. The flow-through system with a 4-fold volume of water flowing through every 24 h was used in this study as described in our previous paper.[31] Water used in the experiment was filtered through activated carbon and had a hardness of 81.1 ± 1.2 mg/L CaCO$_3$, pH of 7.8 ± 0.3, dissolved oxygen of 7.8 ± 0.3 mg/L, and was maintained at 25 ± 1 °C. Medaka were kept under a constant 16/8 h light/dark photoperiod and fed with live brine shrimp (*Artemia nauplii*) twice a day. Medaka were allowed to acclimate for 1 week prior to exposure to diSPAP. One tank contained control medaka (*n* = 20), while two tanks contained medaka (*n* = 20 in each tank) exposed to waterborne diSPAP. The initial loading of medaka in the tanks was ∼0.4 g/L.

Stock solution of 500 mg of diSPAP/L was prepared in methanol. The ratio of chemical-stock solution/water was 0.005% (v/v). During the 10-day uptake phase, medaka were exposed to diSPAP at 25 µg/L in water, followed by a depuration period of 10 days during which medaka were maintained in clean water. Fish were sampled on days 0, 2, 4, 6, 8, and 10 during the exposure (uptake) period and days 12, 14, 16, 18, and 20 during the depuration period, and the medaka were sampled in triplicate (*n* = 3) at each time point before feeding. Samples were stored at −20 °C until identification and quantification of target analytes. Samples of water were collected each day during the 20-day experiment and diluted 10-fold with methanol prior to identification and quantification by UPLC-MS/MS. The method detection limit (MDL) for diSPAP in water, based on peak-to-peak noise of the baseline near the analyte peak on a minimal value of signal-to-noise of 3, was 1.7 µg/L.

To investigate the metabolism of NEtFOSA and NEtFOSAA, Japanese medaka were exposed to 1 μg of NEtFOSAA/L and to 25 μg NEtFOSA/L, respectively. After 10 days' exposure, medaka (n = 3) were collected for quantification by UPLC-MS-MS.

Extraction and Analysis. All target chemicals were extracted from homogenates of whole medaka into methanol as described in a previous study.[32] In brief, homogenates of approximately 0.25 g wet weight (ww) were transferred to a 15 mL polypropylene (PP) centrifuge tubes and were spiked with 80 μL of internal standard containing 100 μg/L each of [$^{13}C_4$]PFOS, NEtFOSA-d_5, and NEtFOSAA-d_5, and then 3 mL of methanol was added for extraction. After vigorous shaking for 20 min, sonication for 20 min, and centrifugation at 1538g for 10 min, 0.2 mL of the supernatant was used directly for analysis of diSPAP and its transformation products by UPLC-MS/MS, and the residual 2.8 mL of extract was used to quantify isomers of PFOS by the in-port derivatization GC-MS with some modifications of a previously described method.[33] In brief, the residual 2.8 mL methanol extract was diluted with 12 mL of ultrapure water and then was loaded on WAX cartridges that had been conditioned by 6 mL of methanol and 6 mL of ultrapure water. After the cartridges were rinsed with 4 mL of ultrapure water and 4 mL of methanol, 4 mL of methanol containing 0.5% NH_4OH was used to elute PFOS from the WAX cartridge. The eluate was dried under a gentle stream of nitrogen and redissolved in 0.2 mL of derivatization solution containing TBAS in diethyl ether, and then PFOS isomers in extracts were analyzed by GC-MS.

Instrumental Analysis. Identification and quantification of isomers of diSPAP and its metabolites except for PFOS were analyzed by a Waters Acquity UPLC system (Waters, Milford, MA) with a Waters Micromass Quattro Premier XE (triple-quadrupole) detector operated in negative ionization electrospray mode. Separation of diSPAP, FOSAs, NMeFOSAA, and NEtFOSAA isomers was achieved with a Waters Acquity UPLC ethylene bridged hybrid (BEH) fluorophenyl column (1.7 μm; 2.1 mm ×150 mm) (Figure S3, Supporting Information).[34] Injection volume was 5 μL. Methanol (phase A) and 5 mM ammonium acetate (phase B) were used as the mobile phases. Initially 20% A was increased to 50% in 2 min, then increased to 58% at 12 min, then to 66% at 20 min, a further increase to 75% over 1 min and then to 80% at 33 min, then increased to 100% over 3 min and kept for 2 min, followed by a decrease to initial conditions of 20% A and held for 3 min to allow for equilibration. The flow rate was 0.18 mL/min. The column and sample room temperatures were maintained at 40 and 10 °C, respectively. For analyzing isomers of FOSAA and FOSEs, an Acquity UPLC BEH C18 column was used to achieve good peak shape and instrument sensitivities as shown in Figure S4 in Supporting Information. Data were acquired in multiple reaction monitoring (MRM) mode (Table S1, Supporting Information), and the optimized parameters were described as follows: source temperature, 110 °C; desolvation temperature, 350 °C; capillary voltage, 2.50 kV; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h; and multiplier, 650 V.

Identification and quantification of PFOS isomers were performed on a gas chromatography−electron capture negative ionization mass spectrometer (GC-ENCI-MS) (Shimadzu QP 2010 plus, Japan). Chromatographic separation was achieved on a DB-5MS capillary column (30 cm × 0.25 mm × 0.1 μm film thickness; J&W Scientific). A splitless injector was used, and the injector was held at 300 °C. Injection volume was 1 μL.

The temperature program increased from 40 °C (2 min) to 300 °C (5 min) at a rate of 30 °C/min. The temperature of the transfer line and the ion source temperature were maintained at 280 and 220 °C, respectively. The carrier gas was helium at a constant flow rate of 4 mL/min. Data acquisition was conducted in selected ion monitoring mode.

Quantification and Quality Assurance/Quality Control. Linear isomers of chemicals were quantified by using corresponding commercially obtained standards. As shown in Figures S3 and S4 (Supporting Information), the branched isomers for all target chemicals except for NEtFOSA and NEtFOSE were detected in exposed medaka. Since only linear isomers for chemicals except for PFOS can be obtained commercially, qualification of branched isomers for the chemicals except for PFOS was conducted by detecting different MRM transitions and comparison with samples of unexposed medaka. Thus, the exact structure of each compound remained unclear, so the peaks of branched isomers were expressed as B1, B2, etc. according to their order of retention times. Quantification of branched isomers except for PFOS was accomplished by use of their corresponding linear isomers as the , as conducted in previous studies.[16,29,34] Surrogate standards were used to automatically correct for losses of analyte during extraction or preparation of samples, and to compensate for variations in responses of the instrument from injection to injection. Concentrations of individual PFOS isomers in tissues of medaka were quantified relative to [$^{13}C_4$]PFOS; isomers of NEtFOSE, PFOSA, NEtFOSA, NMeFOSA, FOSE, and NMeFOSE were quantified relative to NEtFOSA-d_5; and isomers of FOSAA, NMeFOSAA, and NEtFOSAA were quantified relative to NEtFOSAA-d_5. To avoid sample contamination, all equipment was rinsed regularly with methanol. One distilled water blank was incorporated in the analytical procedures for every batch of samples. Recoveries of all target PFASs from tissues of medaka were calculated to assess the accuracy of the method, and the relative standard deviation (RSD) was used to evaluate precision. Recoveries (n = 3) were calculated by spiking standards into samples of medaka or tissues. The spiked concentrations were similar to concentrations of chemicals in samples of exposed medaka. These were 1000 ng/g ww for diSPAP; 50 ng/g ww for PFOS, PFOSA, and NEtFOSE; 500 ng/g ww for NEtFOSAA; and 5 ng/g ww for NEtFOSA, NMeFOSA, FOSAA, NMeFOSAA, FOSE, and NMeFOSE. Method detection limits (MDLs) were calculated on the basis of peak-to-peak noise of the baseline near the peak of the analyte obtained by analyzing medaka exposed to diSPAP and on a minimal value of signal-to-noise of 3. Recoveries of chemicals ranged from 82% ± 13% (NMeFOSA) to 102% ± 12% (FOSAA), and the MDLs ranged from 0.4 ng/g ww (FOSAA) to 29 ng/g ww (diSPAP) (Table S2, Supporting Information). Recoveries of surrogate standards in all samples were 92% ± 5%, 85% ± 7%, and 98% ± 4% for [$^{13}C_4$]PFOS, NEtFOSA-d_5, and NEtFOSAA-d_5, respectively.

Data Treatment and Statistical Analyses. The depuration rate constant (K_d) was calculated by fitting the concentration in the depuration phase to the first-order model:

$$\ln(C_{medaka}) = K_d t + C_0 \qquad (1)$$

where C_{medaka} is the growth-corrected whole-body concentration of diSPAP, t is the depuration time (days), and C_0 is the regression intercept. Depuration half-life ($t_{1/2}$) was then calculated as
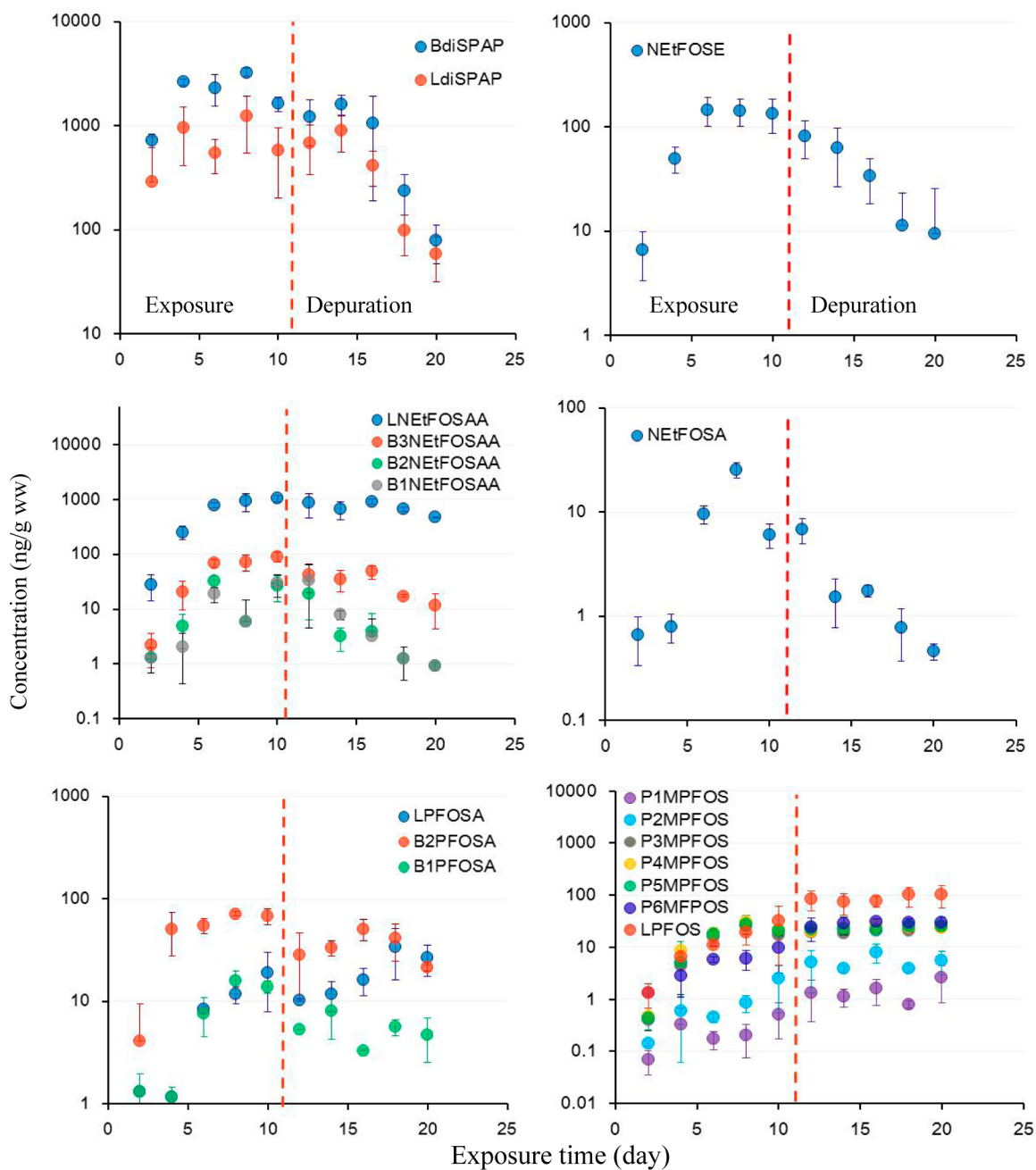
Figure 1. Growth-corrected whole-medaka concentrations of diSPAP and its metabolites during uptake phase and depuration phase. Each data point represents the arithmetic mean concentration of triplicate sampling at each time point. The error bar represents the standard deviation.

$$t_{1/2} = \ln(2)/K_d \tag{2}$$

Uptake rate constant (K_u) was determined by using linear regression to fit the whole-medaka concentration in the exposure phase based on K_d value:

$$C_{medaka} = (K_u C_w / K_d)[1 - \exp(-K_d t)] \tag{3}$$

where C_w is the diSPAP concentration in exposure water.

The relative fraction of individual branched isomer to linear isomer (BF) was calculated as

$$BF = \frac{M_{branch}}{M_{branch} + M_{linear}} \tag{4}$$

where M_{branch} indicates the concentration for one branched isomer and M_{linear} is that for linear isomers.

For those results that were less than the MDL, half of the MDL was assigned to avoid missing values in the statistical analyses. All data analyses such as linear regression were performed with Matlab (version 2008b). Statistical significance was defined as $p < 0.05$ (SPSS 15.0).

■ RESULTS AND DISCUSSION

Uptake and Depuration of diSPAP. No mortality occurred in either dosed or control populations. No statistically significant increase ($p = 0.60$) in weight was observed during the experimental period, with an overall growth rate of 0.005 g/ day. During the uptake phase, concentrations were 18 ± 2.1 μg/L for linear diSPAP (L-diSPAP) and 10 ± 1.1 μg/L for branched diSPAP (B-diSPAP), while those during the depuration phase were all less than the MDL (<1.7 μg/L).
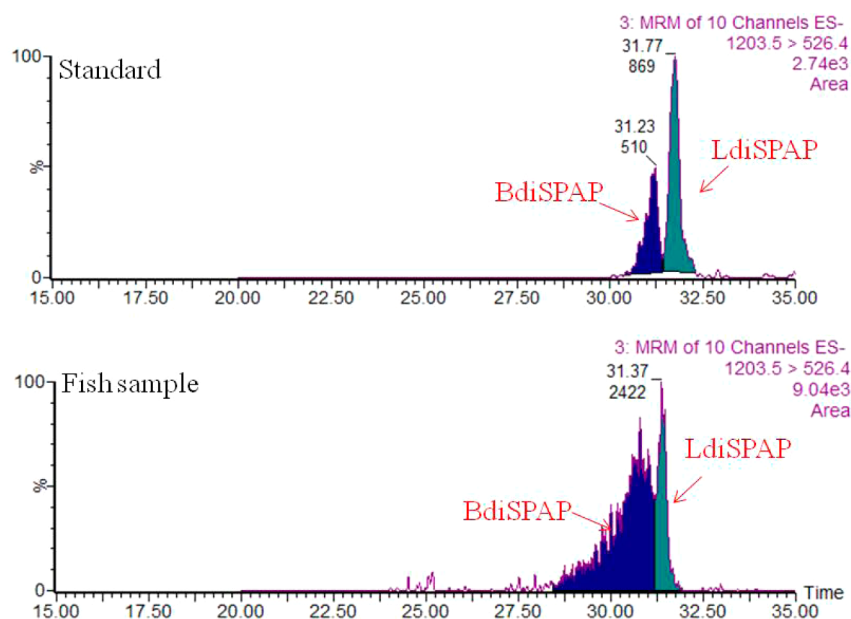
Figure 2. Typical UPLC-MS/MS chromatograms of diSPAP isomers in Japanese medaka exposed to diSPAP and in diSPAP standard (10 μg/L).

**Table 1. Depuration Rate Constant, Depuration Half-Life, and Uptake Rate Constant for Isomers of diSPAP and Its Metabolites**

analyte	K_d (day^{-1})	elimination rate[a] (day^{-1})	$t_{1/2}$ (day)	K_u (L/kg)
L-diSPAP	0.26 ± 0.08		2.7 ± 0.83	6.9 ± 8.6
B-diSPAP	0.29 ± 0.08		2.4 ± 0.66	36 ± 38
NEtFOSE		0.25 ± 0.03	2.8 ± 0.33	
NEtFOSA		0.28 ± 0.04	2.5 ± 0.36	
L-PFOSA		−0.08 ± 0.05		
B$_1$-PFOSA		0.06 ± 0.04	12 ± 10	
B$_2$-PFOSA		0.09 ± 0.05	7.7 ± 5.5	
L-NEtFOSAA		0.06 ± 0.02	12 ± 4	
B$_1$-NEtFOSAA		0.18 ± 0.04	3.9 ± 5.5	
B$_2$-NEtFOSAA		0.36 ± 0.05	1.9 ± 0.42	
B$_3$-NEtFOSAA		0.40 ± 0.05	1.7 ± 0.21	
B-FOSAA		0.20 ± 0.04	3.5 ± 0.69	
L-FOSAA		0.02 ± 0.03		
P$_1$MPFOS		−0.14 ± 0.07		
P$_2$MPFOS		−0.24 ± 0.23		
P$_3$MPFOS		−0.60 ± 0.14		
P$_4$MPFOS		−0.34 ± 0.20		
P$_5$MPFOS		−0.44 ± 0.15		
P$_6$MPFOS		−0.09 ± 0.04		
LPFOS		−0.09 ± 0.04		

[a]Elimination rates of metabolites were dependent on depuration rates of chemicals and transformation from upstream chemicals.

diSPAP was detected in all samples from exposed medaka during both uptake and depuration phases. Concentrations of diSPAP increased rapidly during the exposure phase from 728 ± 109 ng/g ww at day 2 to 2622 ± 207 ww at day 4, and no significantly increasing trend was observed after day 4 (p = 0.43), with concentration of 2302 ± 765 at day 6, 3249 ± 281 ng/g ww at day 8, and 1624 ± 569 ng/g ww at day 10. This result indicated that the concentration of diSPAP reached steady state after 4 days of exposure (Figure 1). Enrichment of B-diSPAP isomers in medaka exposed to diSPAP was unexpectedly observed, although due to their large molecular size and strong retention on column, baseline separation between B-diSPAP and L-diSPAP cannot be achieved (Figure 2). Especially, the peak of B-diSPAP was broader than that of L-diSPAP, possibly due to several branched isomers being contained in the single peak. To determine the concentration
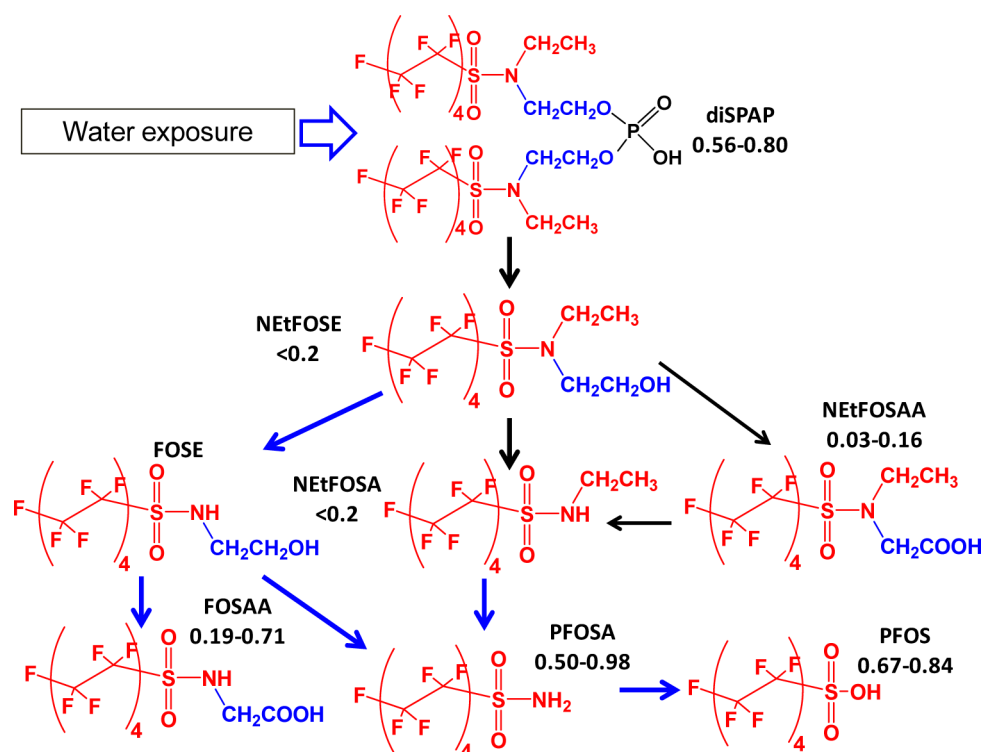
Figure 3. Proposed biotansformation pathways from diSPAP to PFOS in medaka. Blue arrow indicates significant isomer-specific biotransformation. Black arrow indicates that isomer-specific metabolism cannot be observed. Values indicates the fractions of branched isomers (BF) of metabolites.

of B-diSPAP, the partially resolved chromatogram of diSPAP isomers was deconvoluted and integrated with Peakfit software (v4.12, Aspire Software International, Ashburn, VA) as previously described.[18] On the basis of this calculation, the concentration of B-diSPAP in medaka was several times greater than that of L-diSPAP (Figure 1), while the concentration of B-diSPAP in exposure water was 1.8-fold lesser than that of L-diSPAP (Figure S5, Supporting Information). These results indicate that B-diSPAP was more easily accumulated by medaka than L-diSPAP. This phenomenon was different from that of PFOS, which showed preferential enrichment of linear isomers in rat and rainbow trout.[21,28]

Based on log−linear regression analysis (eq 1), depuration rate constants (K_d) for L-diSPAP and B-diSPAP were 0.26 ± 0.08 and 0.29 ± 0.08 day^{-1}, respectively (Table 1), and half-lives for clearance were 2.7 ± 0.83 and 2.4 ± 0.66 days, respectively. Such rates of clearance were higher than that of PFOS in rainbow trout (13 ± 1.8 days),[36] which indicates rapid elimination of diSPAP from medaka. Considering the molecular size of diSPAP, rates of excretion across the gills and in feces would be small, and therefore presumably such rapid elimination rates were due to metabolism to downstream metabolites. In previous studies of metabolism of polyfluor-oalkyl phosphate (PAP), which has a similar chemical structure to diSPAP, dephosphorylation of PAP to fluorotelomer alcohol (FTOH) occurred in the intestine of rat.[37] Minor bio-transformation of diSPAP to PFOS has also been observed in rat after a single oral dose, according to the data submitted to the U.S. EPA by the 3M Co.[27] Therefore, metabolism of diSPAP might also occur in the intestine of medaka, and further studies are warranted. Based on K_d values and eq 2, the rate constant for uptake (K_u) for B-diSPAP was 36 ± 38 L/kg, which is much greater than that of L-diSPAP (6.9 ± 8.6 L/kg). The uptake rate of diSPAP was less than that of PFOS in

rainbow trout (53 L/kg),[36] which could be due to the relatively greater molecular size of diSPAP. Different from diSPAP, the preferential enrichment of L-PFOS in rat and rainbow trout has been thought to be due mainly to more rapid rate of elimination than B-PFOS.[21,28] However, for diSPAP, the elimination rates for the branched and linear isomers were similar. Therefore, differences in rates of uptake are more likely responsible for isomer-specific accumulation for branched diSPAP.

Pathways of Metabolism of diSPAP. Considering the relatively great clearance rate of diSPAP, potential metabolism pathway of diSPAP in medaka was further investigated. PFOS was detected in extracts of homogenates of medaka that had been exposed to diSPAP, and the concentrations were in the range 3.9 ng/g ww (day 2) to 403 ng/g ww (day 18), dependent on the duration of exposure. This result demonstrated biotransformation of diSPAP to PFOS. Besides PFOS, six other metabolites including PFOSA, NEtFOSA, FOSAA, NEtFOSAA, FOSE, and NEtFOSE were also detected in medaka exposed to diSPAP, which has not been detected in unexposed medaka. Significant trends of greater concentrations as a function of duration were observed for all metabolites, including PFOS ($p = 0.008$), PFOSA ($p = 0.019$), NEtFOSAA ($p = 0.044$), and NEtFOSE ($p = 0.05$), except for NEtFOSA ($p = 0.11$) (Figure 1). Among the six intermediate metabolites of diSPAP, NEtFOSAA was the predominant metabolite with concentrations ranging from 33 to 1196 ng/g ww, followed by FOSAA (10−72 ng/g ww) and PFOSA (6.7−101 ng/g ww). Such a profile of metabolites would be contributed by the difference in their further metabolism as exemplified by the lesser slope of NEtFOSAA in the relationship between concentrations and depuration time compared with FOSAA and PFOSA (Table 1). It should be noted that FOSAA have been detected in wildlife such as Chinese sturgeon and ringed

seal,[22,38] while FOSAA is not commercially produced. Thus, according to the results obtained in the present paper and the relatively great concentrations of diSPAP in marine sediment,[25] the metabolite would be used to track indirect sources (such as diSPAP) of PFOS in field investigation, and the feasibility needs to be further studied.

On the basis of chemical structures of metabolites, NEtFOSE should be the first metabolite in the metabolism pathway of diSPAP (Figure 3). Two studies have reported the metabolism of NEtFOSE in rat and in vitro liver microsomes,[20,39] and it has been identified that NEtFOSE underwent N-dealkylation to form FOSE, then oxidation to form PFOSA, and finally to PFOS (i.e., the NEtFOSE → FOSE → PFOSA → PFOS pathway). In this study, NEtFOSA was detected, indicating that NEtFOSE would be also metabolized to PFOS via a new proposed pathway: NEtFOSE → NEtFOSA → PFOSA → PFOS. Since NEtFOSAA was detected with great abundance, the biotransformation from NEtFOSE to NEtFOSAA would also occur, and PFOS would be also formed via the following pathway: NEtFOSE → NEtFOSAA → NEtFOSA → PFOSA. To clarify this hypothesis, we exposed medaka to NEtFOSAA at 1 μg/L, and PFOS, PFOSA, and NEtFOSA were detected in exposed medaka homogenates, identifying the above hypothesis. It should be noted that the concentrations of PFOS (1.3 ± 0.3 ng/g wm) were less than that of NEtFOSAA (1398 ± 107 ng/g wm), which indicated that this was not the primary pathway of metabolism of diSPAP to PFOS by medaka. Thus, it is proposed that the main biotransformation pathways of diSPAP to PFOS by medaka are (1) diSPAP → NEtFOSE → NEtFOSA → PFOSA → PFOS and (2) diSPAP → NEtFOSE → FOSE → PFOSA → PFOS (Figure 3).

Isomer-Specific Metabolism. Preferential accumulation of B-diSPAP observed in medaka would lead to B-PFOS being more abundant than L-PFOS. Therefore, isomers of PFOS were quantified in diSPAP-exposed medaka. The relative peak intensity of L-PFOS was lesser in homogenates of medaka exposed to diSPAP than that in the technical PFOS standard (Figure 4). This result indicates preferential formation of B-PFOS from diSPAP. Enrichment of B-PFOS isomers was
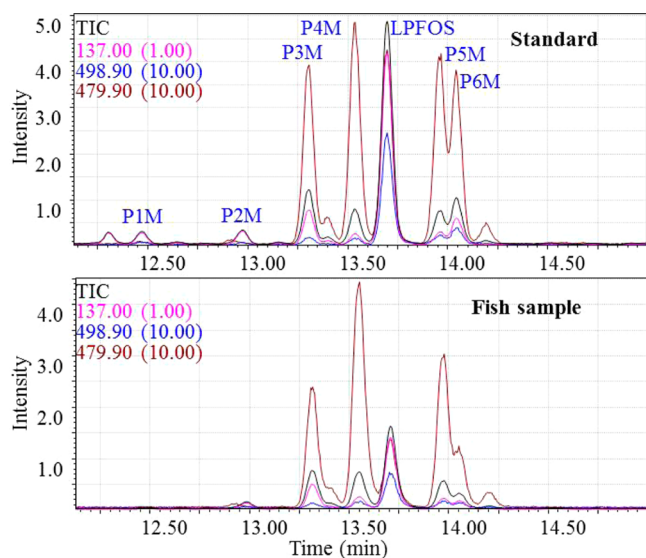
Figure 4. Typical GC-MS chromatograms of PFOS isomers in Japanese medaka exposed to diSPAP and technical PFOS standard (50 μg/L). P$_n$M indicates n-perfluoromethyl PFOS (P$_n$MPFOS).

dependent on the site of the methyl group, and enrichment was greater for P$_{3-5}$MPFOS, the BF values of which were 0.58 ± 0.07, 0.62 ± 0.06, and 0.61 ± 0.05 at day 6, respectively. These BF values were 5.8-, 7.8-, and 6.4-fold greater than those of technical PFOS, respectively (Figure 5). Isomer-specific
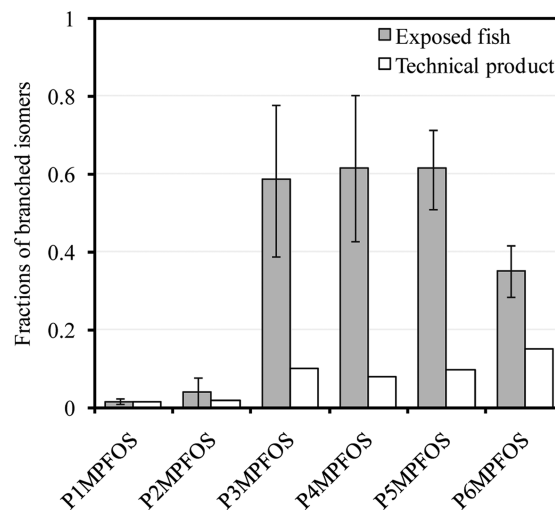
Figure 5. Comparison between fractions of branched isomers (BF) of PFOS isomers in Japanese medaka exposed to diSPAP and technical PFOS standrad.

biomagnification of PFOS was observed in a food web, and P$_{3-5}$MPFOS (M$_1$PFOS) exhibited greatest values for BMF in lake trout, followed by P$_6$MPFOS (M$_2$PFOS) and linear PFOS.[16] Considering the isomer-specific formation of PFOS observed in the present study, the potential indirect sources for PFOS would have been present in Lake Ontario. During exposure phase, values of BF for B-PFOS ranged from 0.76 to 0.84, which were all significantly greater ($p = 0.007$) than those of diSPAP (0.72−0.80). The results indicated that isomer-specific metabolism may be responsible for the enrichment of B-PFOS in addition to preferential accumulation of diSPAP.

To investigate isomer-specific metabolism of diSPAP, isomers of other intermediate metabolites were analyzed by UPLC-MS/MS. Three PFOSA isomers, two FOSAA isomers, and four NEtFOSAA isomers were detected in diSPAP-exposed fish (Figures S3−S5, Supporting Information), and their BF values varied among metabolites. As for NEtFOSE, the first metabolite of diSPAP, no branched isomers were detected in extracts of homogenates of fish, and values of BF were <0.2, which were less than diSPAP. Although three branched isomers of NEtFOSAA were detected, their BF values were limited to 0.023−0.16. Since slopes of accumulation of B-NEtFOSAA during the depuration phase (0.40 ± 0.05, 0.36 ± 0.05, and 0.18 ± 0.04) were 3−7-fold greater than those of L-NEtFOSAA (0.06 ± 0.02) (Figure 1), preferential elimination of B-NEtFOSAA would lead to the preferential accumulation of L-NEtFOSAA. Different from NEtFOSAA, BF values of FOSAA were relatively high and ranged from 0.48 to 0.71 during the exposure phase. Such BF values of FOSAA were higher than its putative precursor NEtFOSE, which cannot be explained by the preferential elimination of L-FOSAA since the clearance of L-FOSAA was obviously slower than those of B-FOSAA (Figure 1). Therefore, preferential metabolism of B-NEtFOSE to B-FOSAA should be responsible for the high BF values of FOSAA and also the low BF values of NEtFOSE. As for the newly

identified pathway NEtFOSE → NEtFOSA → PFOSA → PFOS, similar to the putative metabolite NEtFOSE, no branched NEtFOSA isomers were detected in extracts of homogenates of medaka with BF < 0.2. Preferential metabolism of branched isomers of NEtFOSA may be the potential reason for such a phenomenon, as exemplified in the in vitro system by enzyme and liver microsome.[40] However, it is difficult to deduce the exact reason for enrichment of linear isomers in medaka due to the complex metabolism network of NEtFOSA and lack of information on isomer-specific elimination rates. To further clarify the potential reason, medaka were exposed to 1 μg of technical NEtFOSA/L, and significant enrichment of L-NEtFOSA was observed with BF of 0.04 ± 0.007 compared to exposed NEtFOSA standard (0.17) (Figure S6, Supporting Information), which indicated preferential metabolism of B-NEtFOSA to B-PFOSA and B-PFOS. Corresponding to the results, enrichment of L-PFOS was also observed in exposed medaka samples with BF values of 0.59 ± 0.13, while no enrichment of PFOSA was observed (BF values were 0.04 ± 0.007). This result is not consistent with enrichment of B-PFOSA in medaka exposed to diSPAP, where BF values ranged from 0.50 ± 0.08 to 0.98 ± 0.007 (Figure 3). This result indicates that preferential enrichment of PFOSA was mainly due to biotransformation of FOSE rather than NEtFOSA. Faster elimination was also observed for B-PFOSA than L-PFOSA (Figure 1), which further suggests preferential metabolism of B-PFOSA to B-PFOS.

In conclusion, in addition to preferential accumulation of diSPAP, isomer-specific metabolism of NEtFOSE, NEtFOSA, and PFOSA was partly responsible for the enrichment of B-PFOS in medaka (Figure 3). It should be noted that branched isomers showed preferential metabolism during the main metabolism pathway except for the bioconversion from diSPAP to NEtFOSE. Further studies on metabolism enzymes in Japanese medaka are needed to clarify the molecular mechanism.

Overall, this work provides evidence on the role of diSPAP as precursor of PFOS by evaluating the metabolism of diSPAP to PFOS by medaka. The study reported isomer-specific metabolism and bioaccumulation of diSPAP, which led to preferential formation of B-PFOS. The results presented here will help in understanding the potential contribution of indirect sources to PFOS in field investigations.

ASSOCIATED CONTENT

Ⓢ Supporting Information
Additional text, six figures, and two tables addressing MRM transitions of diSPAP and its metabolites; recoveries and MDLs of perfluorinated compounds in fish samples; ^{19}F spectra of purfied diSPAP; chromatogram and spectra of purified diSPAP standard by UPLC-Q-TOF; chromatograms of isomers of EtFOSAA, PFOA, EtFOSA, and PFOS from BEH fluorophenyl column; chromatograms of EtFOSE and B-FOSAA from BEH C18 column; UPLC-MS/MS chromatogram of diSPAP in culture water sample; and chromatograms of EtFOSA in standard and exposed fish samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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