EurJOC
European Journal of Organic Chemistry

A Journal of
ChemPubSoc Europe

Accepted Article

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To be cited as: *Eur. J. Org. Chem.* 10.1002/ejoc.201601302

Link to VoR: http://dx.doi.org/10.1002/ejoc.201601302

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FULL PAPER

Synthesis of Nontoxic Fluorous Sphingolipids as Molecular Probes of Exogenous Metabolic Studies for Rapid Enrichment by FSPE

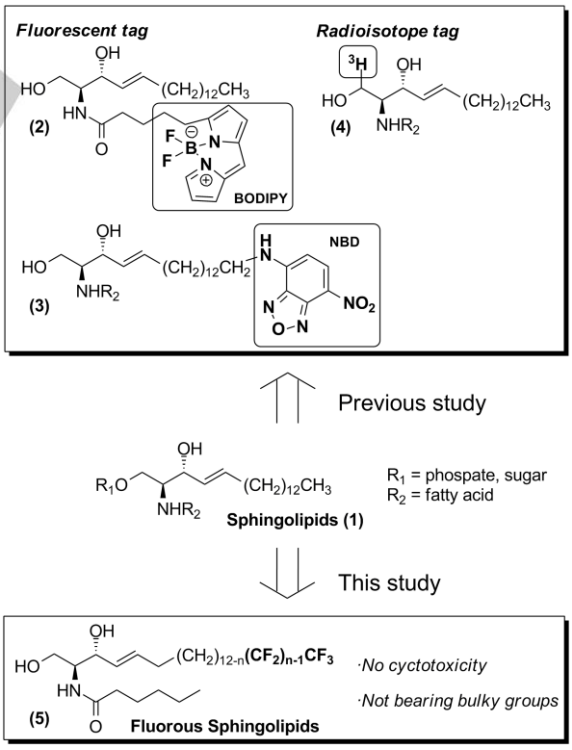
Shota Saito,[a][‡] Yuta Murai,[b][‡] Seigo Usuki,[b] Masafumi Yoshida,[a] Mostafa A.S. Hammam,[b] Susumu Mitsutake,[b] Kohei Yuyama,[b] Yasuyuki Igarashi,[b] and Kenji Monde[b]*

Abstract: Fluorous solid phase extraction (FSPE) is one of the useful techniques for efficient selective enrichment of fluorous compounds from nonfluoros molecules. Sphingolipids and their metabolites, which are ubiquitous building blocks of eukaryotic and prokaryotic cell membranes, play crucial roles such as signaling molecules. However, details of the functions and metabolic mechanisms of exogenous sphingolipids have remained unknown compared with that of endogenous. To better understand the veiled roles, chemical probes with appropriate biological and physicochemical properties are needed. In this study, we designed and synthesized novel fluorous sphingolipids to reveal their roles. Furthermore, we confirmed that they could be efficiently and rapidly separated from normal sphingolipids by FSPE and that they scarcely exhibited cytotoxic activities as what normal sphingolipids did at the same dose. Also, we demonstrated that these fluorinated ceramides could be metabolic substrates utilizing sphingomyelin synthase 2 (SMS2) showing their potential for further biological studies.

Introduction

Sphingolipids (SLs), which have common backbone sphingoid bases of sphingosine, sphinganine and phytosphingosine, are ubiquitous membrane components of all eukaryotic and prokaryotic cells.[1] SLs have been reported to they play crucial roles in many physiological processes, and their metabolites are well known as bioactive compounds called lipid mediators that regulate cell growth[2], apoptosis[3], migration, angiogenesis and immune responses.[4] In addition, it has recently reported that they are expected to be new pharmaceutical targets against metabolic syndrome and Alzheimer's disease.[5] Although these mechanisms of endogenous SLs have gradually been elucidated by mutations or knockouts in genes that encode for enzymes involved in a specific metabolic step, details of the metabolic fate of exogenous SLs are scarce. Recently, there have been a few studies in which the function and metabolic mechanism of exogenous SLs were investigated by using chemical probes bearing a fluorescence tag[6] or using radioisotope labeling.[7] (Fig.1) Those strategies are useful but have some problems such as loss of original biological activity and affinity for the target biomolecules or the requirement of special equipment. On the other hand, recently Haberkant and Schults et al. has reported that achievement for analysis of protein-sphingolipid interactions by using photoreactive and clickable analog of sphingosine.[8]

The use of fluorine chemistry involving trifluoromethyl groups or perfluoroalkyl chains (fluorous compounds) with biological properties is a well-known new strategy for application to medicinal chemistry.[9] In addition, substitution of hydrogen by fluorine cause scarcely steric effects despite their different van der Waals radii.[10] Recently, applications of fluorous molecules to various biological techniques have been increasing. For instance, it has been applied for fluorous metabolomics[11], fluorous microarrays[12], fluorous delivery systems[13], and fluorous photo cross-linker.[14] Moreover, fluorous compounds can be rapidly and efficiently separated from non-fluorous compounds with selective retention on fluorous silica gel.[15] Separation of fluorous peptides[16], oligosaccharides[17], and oligonucleotides[18] from non-fluorous counterparts was also performed by fluorous solid phase extraction (FSPE).



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Supporting information for this article are available on the WWW under http://**********.

Fig. 1. Structures of sphingolipids labeled with a fluorescent tag (2), (3) and a radioisotope tag (4) in previous studies and labeled with a fluorous tag (5) in this study.

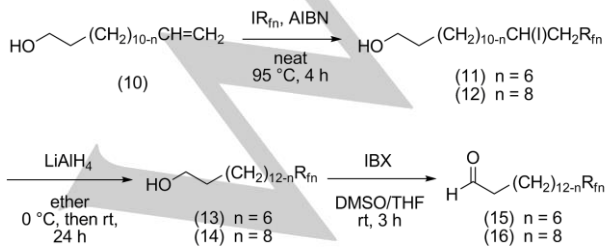
Therefore, fluorous SLs (5) are useful for investigating the functions and metabolic mechanisms of exogenous SLs. Here, we describe the efficient synthesis of enantiopure D-*erythro* fluorous SLs and sphinganine as the upstream precursor of SLs. Furthermore, we also show that they scarcely exhibited cytotoxicity and that they were efficiently separated from mixed nonfluorous SLs by FSPE.

Results and Discussion

An important point in our synthesis of fluorous SLs and sphinganine is the installation of perfluorinated aliphatic chains (fluorous) instead of alkyl chains. There has only been a report on the synthesis of fluorous $C_{11,12,13}$-sphingosine mimics[8e], and the synthesis of fluorous C_{18}-sphingosine, SLs, and sphinganine has not been reported so far. Additionally, their synthesis using olefin metathesis remains a challenge because of poor yield, small-scale reaction, and requirement of precious catalysts. To solve these problems, *Horner-Wadsworth-Emmons* (HWE) olefination is the most useful method to construct a sphingoid base skeleton with inexpensive reagents. D-*Erythro* fluorous SLs and sphinganine were synthesized as reported by Inazu et al. and Koskinen et al.[19] with slight modification.

As shown in Scheme 1, our synthesis started with commercially available L-serine, and this was sequentially protected by Me ester for the carboxylic acid group and Boc for the amino group to obtain Boc-(*S*)-Ser-OMe (7) in a quantitative yield. Subsequently, the alcohol moiety and carbamate group reacted with 2,2-dimethoxypropane under an acidic condition to afford oxazolidine (8) in excellent yield (97%). Compound (8) was transformed to the corresponding β-ketophosphonate (9) by treatment with excess lithium dimethyl methylphosphonate at -78 °C (75%).

On the other hand, perfluorinated aliphatic chains (fluorous) were synthesized from commercially available homoallylic alcohol (10). Compound (10) was first subjected to radical addition with iodide perfluoroalkyls (11, 12) by the radical initiator AIBN under a neat condition, and this was followed by reductive deiodination by $LiAlH_4$[20] instead of Bu_3SnH under a mild condition to obtain perfluoroalkyl alcohols (13, 14) in two sequential steps in high yield (80%). Additionally, Ni-catalyzed reductive deiodination reported by Hájek et al. [21] could be also useful for these perfluoroalkyl alcohols. Finally, perfluoroalkyl aldehydes (15, 16) were created by oxidation of those alcohols with IBX in DMSO/THF at room temperature under a mild condition. (Scheme 1)

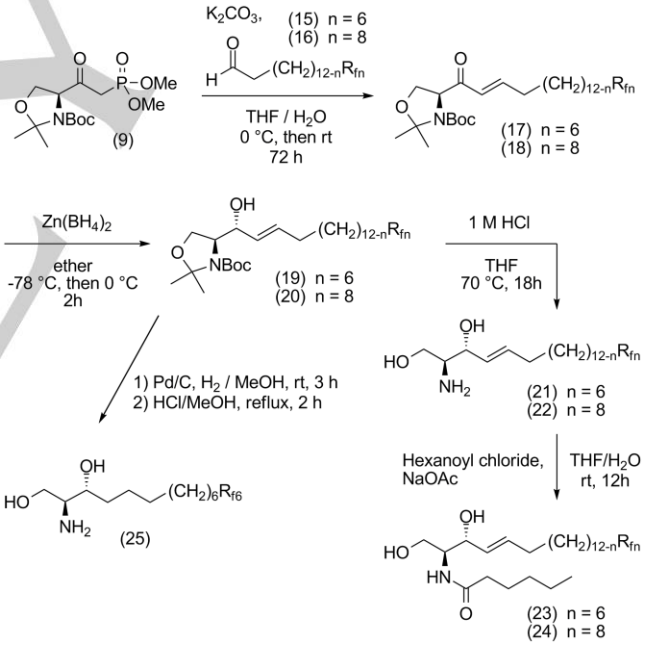


R_{fn} = $(CF_2)_{n-1}CF_3$; ***Perfluorinated aliphatic chains* (*Fluorous*)**

Scheme 1. Synthesis of perfluoroalkyl aldehydes (15 and 16).

Next, we applied compound (9) and compounds (15, 16) to the synthesis of D-*erythro* fluorous C_{18}-sphingosine, which is one of the essential components of SLs. Trans-selective double bond formation with β-ketophosphonate (9) and perfluoroalkyl aldehydes (15, 16) were performed by modified HWE olefination with K_2CO_3 in THF/H_2O to obtain oxazolidine-3-keto fluorous sphingosines (17, 18) in good yield (80%). Subsequently, compound (17, 18) were subjected to diastereoselective reduction by using $Zn(BH_4)_2$ at -78 °C and then at 0 °C to obtain oxazolidine D-*erythro* fluorous C_{18}-sphingosines (19, 20) in over 90% d.e., due to the high chelating ability of the Zn cation.[22] The desired D-*erythro* fluorous SLs (23, 24) were synthesized in high yield (90%) by deprotecting the oxazolidine moiety in compounds (21, 22) with 1 M HCl / THF under reflux followed by acylation of the amino group with hexanoyl chloride.

D-*erythro* fluorous C_{18}-sphinganine (25) was also obtained in moderate yield (60%) from compound (19) by reduction of the alkene moiety with Pd/C in the presence of H_2 atmosphere followed by deprotection of the oxazolidine group under an acidic condition. (Scheme 2)



Scheme 2. Synthesis of D-*erythro* fluorous SLs (23, 24) and sphinganine (25).

Subsequently, we evaluated the cytotoxicity of D-*erythro* fluorous SLs (23, 24) and sphinganine (25) towards NIH3T3 cells and B16 cells. Cell viability was measured by using a cell counting kit-8 (CCK-8).[23] As shown in Fig. 2, all of the fluorous compounds exhibited comparable cytotoxicity towards the cells as what normal SLs and sphinganine did at the same dose (positive controls). These results indicate that fluorous SLs (23, 24) and sphinganine (25) are expected to be useful as metabolic

substrates and can assure their potential for further biological studies.

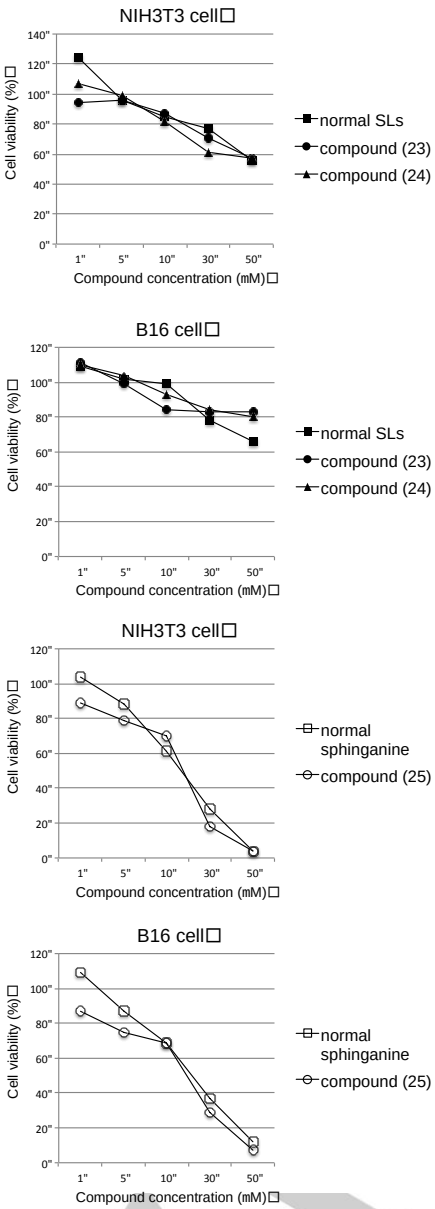


Fig. 2. Viability assays of NIH3T3 cells and B16 cells exposed to fluorous compounds (23), (24), (25) and control normal SLs and sphinganine.

Next, fluorous SLs (23, 24) were subjected to analytical separation by using fluorous LC-MS as shown in Fig. 3. Compounds (23, 24) and normal SLs (control) were mixed, and the mixture was injected on a commercial fluofix-II 120E analytical column.[24] Firstly, normal SLs (control) emerged at 1.4 min, and then fluorous SLs were eluted smoothly in order of their fluorous content from (23): $R_{f6} = C_6F_{13}$ (3.9 min) through (24): $R_{f8} = C_8F_{17}$ (4.3 min). In Fig. 3, these results indicated that rapid and easy separation of fluorous SLs from non-fluorous SLs by using FSPE as well as the possibility of separating fluorous SLs bearing different fluorous tags of different lengths.

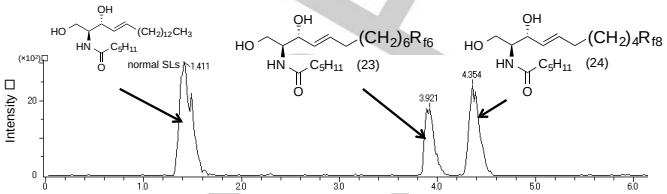
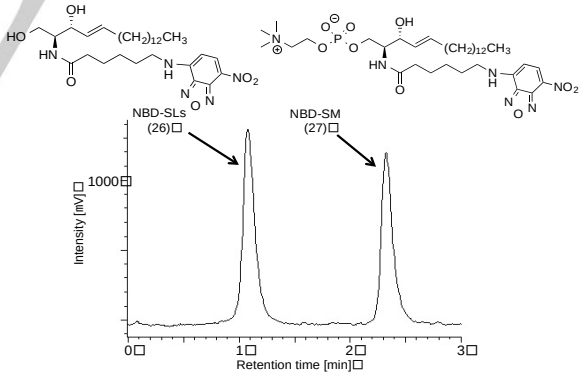


Fig. 3. Fluorous LC-MS condition: the mobile phase comprised solvent (A) MeCN/H_2O/formic acid (20:80:0.1) and solvent (B) MeCN/*i*-PrOH/formic acid (20:80:0.1) was performed in linear gradient program: 50% (B) 0.0-1.2 min., 50%→80% (B) 1.2-3.0 min., 80% (B) 3.0–4.2 min., 80→90% (B) 4.2-6.0 min., flow: 0.3 mL/min.) .

Finally, we examined whether compound 24 is available as the substrate of sphingomyelin synthase 2 (SMS2), which exists in lipid microdomains and partially associates with the fatty acid transporter CD36/FAT.[24] Sphingomyelin (SM) has particularly important roles in the formation of microdomains of the plasma membrane involved in many cellular processes. The utility as a substrate of fluorous SLs were measured by cell-based method[26] using synthetic fluorescent normal and fluorous SLs attached NBD (26, 28). Since this method has been recently established and it is quite facile and reliable to confirm whether compound 24 becomes the substrate of SMS2 or not, we adopted it. The metabolite assay revealed that synthetic compound (28) could be metabolized to fluorous sphingomyelin (29).



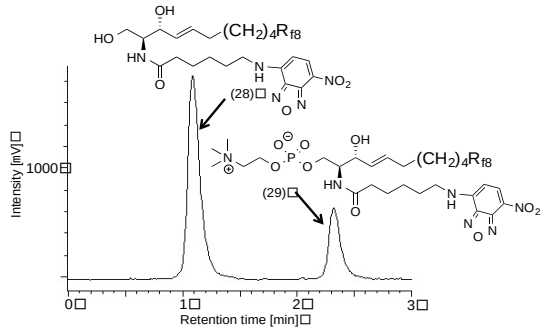


Fig. 4. LC-MS condition: YMC-Pack Diol-120-NP (4.0 x 50mm), 2-propanol/*n*-hexane/H_2O = 52:40:8, flow rate 1 mL/min, fluorescent detector set to excitation and emission wavelength of 470nm and 530nm.

Conclusions

In summary, we have described an efficient pathway for the synthesis of perfluoroalkyl (fluorous) C_{18}-sphingosine, SLs, and sphinganine based on *Horner-Wadsworth-Emmons* olefination. A cytotoxicity activity test by the CCK-8 assay showed that the prepared compounds scarcely exhibited cytotoxicity towards the cells as did normal SLs and sphinganine. In addition, they could be readily and rapidly separated from non-fluorous SLs by FSPE. Furthermore, we proved that in our synthesis, the fluorous SLs could be chemical probes to elucidate those physiological processes.

Experimental Section

9,9,10,10,11,11,12,12,13,13,14,14,14-tridecafluoro-1-tetradecanol (13)

7-octen-1-ol (1.70 g, 13.3 mmol), tridecafluoro-1-iodon-hexane (6.50 g, 14.6 mmol), and AIBN (218 mg, 1.33 mmol) were stirred for 4 h at 95 °C. The reaction was cooled to room temperature and dissolved in dry Et_2O (50 mL). To the reaction mixture was added $LiAlH_4$ (1.00 g, 26.4 mmol) in dry Et_2O (5 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 24 h. The mixture was cooled to 0 °C again, then quenched with water (1 mL), NaOH (15 % w/v; 1 mL). The mixture was filtered through Celite® and concentrated. The residue was purified by FluoroFlash® solid-phase extraction (MeOH/H_2O, 3:1 to acetone) and SiO_2 column chromatography (*n*-hexane/EtOAc = 9:1 to 7:1 to 4:1) to afford **13** as a white amorphous solid (4.75 g, 80 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.64 (q, *J* = 5.9 Hz, 2 H, C*H*$_2$OH), 2.10-1.99 (m, 2 H, CF_2CH_2), 1.63–1.55 (m, 4 H, C*H*$_2$CH$_2$OH, $CF_2CH_2CH_2$), 1.46 (t, *J* = 5.3 Hz, 1 H, OH), 1.38–1.35 (m, 8 H,$CF_2CH_2CH_2CH_2CH_2CH_2$) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 62.93, 32.69, 30.86 (t, $^2J_{C,F}$ = 22.4 Hz, CF_2CH_2), 29.16, 29.14, 29.01, 25.64, 20.06 ppm. HRMS (ESI): calcd. for $C_{14}H_{17}F_{13}ONa$ $[M+Na]^+$ 471.0970, found 471.0967.

7,7,8,8,9,9,10,10,11,11,12,12,13,13,14,14,14-heptadecafluoro-1-tetradecanol (14)

Treatment of 5-hexen-1-ol (1.10 g, 11.0 mmol) and heptadecafluoro-1-iodooctane (6.0 g, 11.0 mmol) was carried out as described above to afford **14** (4.52 g, 79 %) as a white amorphous solid. Analytical data were identical to those reported in the literature.[27]

9,9,10,10,11,11,12,12,13,13,14,14,14-tridecafluorotetradecanal (15)

Perfluoroalkyl alcohol **13** (2.24 g, 5.00 mmol) and IBX (2.80 g, 10.0 mmol) in DMSO (20 mL) and THF (20 mL) were stirred at room temperature for 3 h. The THF was removed on a rotary evaporator, added Et_2O/water, then the insoluble material was removed by filteration. The filtrate was extracted with Et_2O. The organic phase was washed with saturated $NaHCO_3$ and brine and dried with $MgSO_4$, and the solvents were evaporated. The residue was purified with SiO_2 column chromatography (*n*-hexane/EtOAc = 20:1) to afford **15** as a clear liquid (1.72 g, 77 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.77 (t, *J*= 1.6 Hz, 1H, C*H*O), 2.44 (dt, *J* = 7.3, 1.6 Hz, 2H, C*H*$_2$CHO), 2.10–2.00 (m, 2H, CF_2CH_2), 1.66–1.59 (m, 4H, $CF_2CH_2CH_2$, C*H*$_2$CH$_2$CHO), 1.41–1.35 (m, 6H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$CH$_2$CHO) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ = 202.54 (*C*HO), 43.81, 30.85 (t, $^2J_{C,F}$= 22.4 Hz, CF_2CH_2), 29.16, 29.14, 29.01, 21.92, 20.05 ppm. HRMS (EI): calcd. for $[M+H]^+$ $C_{14}H_{17}F_{13}O$ 446.0915, found 446.0916.

7,7,8,8,9,9,10,10,11,11,12,12,13,13,14,14,14-heptadecafluorotetradecanal (16)

Treatment of alcohol **14** (2.60 g, 5.00 mmol) was carried out as described above to afford **16** (2.36 g, 91 %) as a white amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.78 (s, 1H. C*H*O), 2.48 (t, *J* = 7.3 Hz, 2H, C*H*$_2$CHO), 2.13–2.02 (m, 2H, CF_2CH_2), 1.71–1.61 (m, 4H, C*H*$_2$CH$_2$CHO, $CF_2CH_2CH_2$), 1.46–1.40 (m, 2H, $CF_2CH_2CH_2CH_2$) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 201.96, 43.52, 30.67 (t, $^2J_{C,F}$ = 22.3 Hz), 28.55, 21.60, 20.00 (t, $^2J_{C,F}$ = 3.7 Hz). HRMS (APCI): calcd. for $C_{14}H_{10}F_{17}O$ $[M+H]^+$ 517.04657, found 517.04742.

(*S*,*E*)-tert-butyl 2,2-dimethyl-4-(11,11,12,12,13,13,14,14,15,15,16,16,16-tridecafluorohexadec-2-enoyl)oxazolidine-3-carboxylate (17)

Perfluoroalkyl aldehyde **15** (1.68 g, 3.76 mmol) and β-ketophosphonate (1.52 g, 4.33 mmol) were dissolved in THF (15 mL) and water (15 mL) and cooled to 0 °C. K_2CO_3 (1.82 g, 13.2 mmol) was added and the reaction mixture was allowed to warm to room temperature and stirred for 72 h. The reaction mixture was acidified with citric acid solution and extracted with Et_2O. The organic phase was washed with $NaHCO_3$, brine, dried with $MgSO_4$, and solvents were evaporated. The crude product was purified by SiO_2 column chromatography (*n*-hexane/EtOAc = 30:1 to 10:1) to afford **17** as a white amorphous solid (2.01 g, 79 %). ^{1}H NMR (500 MHz, $CDCl_3$, mixture of rotamers): δ = 7.00–6.93 (m, 1H, COCH=C*H*), 6.33–6.23 (m, 1H, COC*H*=CH), 4.70–4.68, 4.51–4.49 (m, 1H, C*H*N), 4.20–4.16, 3.97–3.90 (m, 2H, CHNC*H*$_2$O), 2.27–2.21 (m, 2H, CH=CHC*H*$_2$), 2.07–2.01 (m, 2H, CF_2CH_2), 1.71(s), 1.65 (s), 1.60–1.46 (m), 1.41–1.35 (m) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, mixture of rotamers): δ = 196.67, 149.34, 126.02, 125.25, 95.15, 94.48, 80.84, 80.51, 65.92, 64.25, 63.94, 32.65, 30.88 (t, $^2J_{C,F}$= 22.6 Hz, CF_2CH_2), 29.04, 29.01, 28.97, 28.39, 28.26, 27.95, 27.85, 26.10, 25.28, 25.15, 24.13, 20.12 ppm.

HRMS (ESI): calcd. for $C_{26}H_{34}F_{13}NO_4Na$ $[M+Na]^+$ 694.2178, found 694.2162.

(*S*,*E*)-tert-butyl 2,2-dimethyl-4-(9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-heptadecafluorohexadec-2-enoyl)oxazolidine-3-carboxylate (18)

Treatment of perfluoroalkyl aldehyde **16** (2.85 g, 5.50 mmol) was carried out as described above to afford **18** (3.23 g, 79 %) as a white amorphous solid. 1H NMR (500 MHz, $CDCl_3$, mixture of rotamers): δ = 7.01–6.90 (m, 1H, COCH=C*H*), 6.38–6.23 (m, 1H, COC*H*=CH), 4.68–4.67, 4.50–4.49 (m, 1H, C*H*N), 4.20–4.14, 3.97–3.90 (m, 2 H, CHNCH_2O), 2.28–2.26 (m, 2H, CH=CHCH_2), 2.14–1.97 (m, 2H, CF_2CH_2), 1.71 (s), 1.65 (br. s), 1.63 (br. s), 1.56 (s), 1.52 (br. s), 1.50 (s), 1.43 (br. s), 1.37 (s) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, mixture of rotamers): δ = 196.54, 195.72, 165.02, 152.26, 151.40, 148.62, 126.11, 125.39, 95.11, 94.44, 80.81, 80.46, 65.83, 65.41, 64.24, 63.95, 32.31, 30.73 (t, $^2J_{C,F}$ = 22.4 Hz), 28.61, 28.30, 28.18, 27.65, 27.52, 26.05, 25.23, 25.06, 24.03, 19.95 ppm. HRMS (ESI): calcd. for $C_{26}H_{30}F_{17}NO_4Na$ $[M+Na]^+$ 766.17956, found 766.17950.

(*S*)-tert-butyl 2,2-dimethyl-4-((*R*,*E*)-11,11,12,12,13,13,14,14,15,15,16,16,16-tridecafluoro-1-hydroxyhexadec-2-enyl)oxazolidine-3-carboxylate (19)

Oxazolidine-3-keto **17** (2.56 g, 3.81 mmol) was dissolved in dry Et_2O (40 mL) and cooled to -78 °C. Freshly prepared zinc borohydride solution in dry Et_2O (0.2 M, 20 mL) was added dropwise. The reaction mixture was allowed to warm to -30 °C and stirred for 1 h, then warm to 0 °C and stirred for 1 h. The reaction was quenched with 1M HCl and extracted with Et_2O. The organic phase was washed with $NaHCO_3$, brine, dried with $MgSO_4$, and solvents were evaporated. The crude product was purified by SiO_2 column chromatography (*n*-hexane/EtOAc = 9:1 to 4:1) to afford **19** as a clear oil (2.27 g, 88 %). 1H NMR (500 MHz, C_6D_6, 75 °C): δ = 5.79–5.73 (m, 1H, CHOHCH=C*H*), 5.55 (dd, *J* = 5.9, 15.45 Hz, 1H, CHOHC*H*=CH), 4.31-4.29 (m, 1H, C*H*OH), 3.96 (br, 1H, C*H*N), 3.81 (br, 1H, CHNCH_2O), 3.72–3.69 (m, 1H, CHNCH_2O), 2.02–1.97 (m, 2H, CH=CHCH_2), 1.85–1.75 (m, 2H, CF_2CH_2), 1.62 (s), 1.47–1.40 (m), 1.40-1.38 (m), 1.32–1.30 (m), 1.20–1.17 (m), 1.12–1.05 (m) ppm. ^{13}C NMR (125 MHz, C_6D_6, 75 °C): δ = HRMS (ESI): calcd. for $C_{26}H_{34}F_{13}NO_4Na$ $[M+Na]^+$ 696.2334, found 696.2326.

(*S*)-tert-butyl 2,2-dimethyl-4-((*R*,*E*)-9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-heptadecafluoro-1-hydroxyhexadec-2-enyl)oxazolidine-3-carboxylate (20)

Treatment of Oxazolidine-3-keto **18** (3.01 g, 4.05 mmol) was carried out as described above to afford **20** (2.66 g, 88 %) as a white amorphous solid. 1H NMR (500 MHz, C_6D_6, 75 °C): δ = 5.76–5.66 (m, 1H, CHOHCH=C*H*), 5.53 (dd, *J* = 15.2, 5.3 Hz, 1H, CHOHC*H*=CH), 4.30 (br. s., 1H, C*H*OH), 3.97 (br. s., 1H), 3.78 (br. s., 1H), 3.73–3.64 (m, 1H), 1.92 (q, *J* = 7.0 Hz, 2H, CH=CHCH_2), 1.86–1.72 (m, 2H, CF_2CH_2), 1.62 (br. s), 1.45 (br. s), 1.42–1.31 (m), 1.20 (quin, *J* = 7.4 Hz, 2H, CH=CHCH$_2$CH_2), 1.13–1.02 (m, 2H, CH=CHCH$_2$CH$_2$CH_2) ppm. ^{13}C NMR (125 MHz, C_6D_6, 75 °C): δ = 165.88, 132.25, 130.98, 95.03, 80.65, 74.24, 65.35, 63.16, 32.69, 31.63 (t, $^2J_{C,F}$ = 22.6 Hz, CF_2CH_2), 29.39, 29.13, 28.78, 27.15, 24.75, 20.71 ppm. HRMS (ESI): calcd. for $C_{26}H_{32}F_{17}NO_4Na$ $[M+Na]^+$ 768.19521, found 768.19528.

(2*S*,3*R*,*E*)-2-amino-13,13,14,14,15,15,16,16,17,17,18,18,18-tridecafluorooctadec-4-ene-1,3-diol (21)

Compound **19** (2.10 g, 3.12 mmol) in THF (50 mL) and 1M HCl (50 mL) was stirred at 80 °C for 18 h. The THF was removed on a rotary evaporator, and then the residue was dissolved in EtOAc. The solution was basified with 1 M NaOH, then it was extracted with EtOAc. The organic phase was washed with brine, dried with Na_2SO_4, and the solvent was evaporated. The crude product was purified by SiO_2 column chromatography ($CHCl_3$/MeOH = 9:1, $CHCl_3$/MeOH/NH_3 aq. = 135:25:4) to afford **21** as a white amorphous solid (1.41 g, 85 %). 1H NMR (500 MHz, CD_3OD): δ = 5.79–5.68 (m, 1H, CH=C*H*), 5.51 (dd, *J* = 15.3, 7.4 Hz, 1H, C*H*=CH), 3.98 (t, *J* = 6.5 Hz, 1H, C*H*OHCH=CH), 3.68 (dd, *J* = 10.8, 4.5 Hz, 1H, CH_2OH), 3.50 (dd, *J* = 11.0, 6.9 Hz, 1H, CH_2OH), 2.76 (td, *J* = 6.3, 4.5 Hz, 1H, C*H*NH_2), 2.22–2.06 (m, 4H, CF_2CH_2, CH=CHCH_2), 1.61 (quin, *J* = 7.6 Hz, 2H, $CF_2CH_2CH_2$), 1.49–1.40 (m, 4H), 1.40–1.32 (m, 4H) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 135.26, 131.04, 75.19, 64.43, 58.18, 33.53, 31.89 (t, $^2J_{C,F}$ = 22.4 Hz, CF_2CH_2), 30.42, 30.40, 30.26, 30.24, 21.44 ppm. HRMS (ESI): calcd. for $C_{18}H_{25}F_{13}NO_2$ $[M+H]^+$ 534.16722, found 534.16730.

(2*S*,3*R*,*E*)-2-amino-11,11,12,12,13,13,14,14,15,15,16,16,17,17,18,18,18-heptadecafluorooctadec-4-ene-1,3-diol (22)

Treatment of compound **20** (1.67 g, 2.24 mmol) was carried out as described above to afford **22** (1.19 g, 88 %) as a white amorphous solid. 1H NMR (500 MHz, CD_3OD): δ = 5.79–5.69 (m. 1H, CHOHCH=C*H*), 5.52 (dd, *J* = 15.4, 7.4 Hz, 1H, CHOHC*H*=CH), 3.99 (t, *J* = 6.6 Hz, 1H, C*H*OH), 3.68 (dd, *J* = 10.6, 4.5 Hz, 1H, CH_2OH), 3.50 (dd, *J* = 11.0, 6.8 Hz, 1H, CH_2OH), 2.77 (td, *J* = 6.4, 4.5 Hz, 1H, C*H*NH_2), 2.23–2.07 (m, 4H, CF_2CH_2, CH=CHCH_2), 1.67–1.57 (m, 2H, $CF_2CH_2CH_2$), 1.52-1.38 (m, 4H) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 134.88, 131.33, 75.18, 64.47, 58.17, 33.28, 31.87 (t, $^2J_{C,F}$ = 21.9 Hz, CF_2CH_2), 30.10, 29.80, 21.31 ppm. HRMS (ESI): calcd. for $C_{18}H_{21}F_{17}NO_2$ $[M+H]^+$ 606.12953, found 606.12981.

***N*-((2*S*,3*R*,*E*)-13,13,14,14,15,15,16,16,17,17,18,18,18-tridecafluoro-1,3-dihydroxyoctadec-4-en-2-yl)hexanamide (23)**

Compound **21** (500 mg, 0.937 mmol) and NaOAc (3.08 g, 37.5 mmol) were dissolved in THF (10 mL) and water (5 mL). Hexanoyl chloride (0.20 mL, 1.46 mmol) was added. The reaction mixture was stirred for 18 h at room temperature. The reaction mixture was extracted with EtOAc, then the organic phase was washed with NaOH (1 M), brine, dried with $MgSO_4$, and solvents were evaporated. The crude product was purified by SiO_2 column chromatography (*n*-hexane/EtOAc = 1:1 to 1:4) to afford **23** (532 mg, 90 %) as a white amorphous solid. 1H NMR (500 MHz, $CDCl_3$): δ = 6.40 (d, *J* = 7.6 Hz, 1H, N*H*), 5.81–5.73 (m, 1H, CHOHCH=C*H*), 5.53 (dd, *J* = 15.4, 6.4 Hz, 1H, CHOHC*H*=CH), 4.28 (d, *J* = 4.5 Hz, 1H, C*H*OH), 3.95–3.86 (m. 2H, C*H*NH, CH_2OH), 3.69 (td, J = 7.1, 3.4 Hz, 1H, CH_2OH), 3.50 (d, *J* = 5.4 Hz, 1H, CHO*H*), 3.43 (dd, *J* = 6.7, 3.8 Hz, 1H, CH2O*H*), 2.22 (t, *J* = 7.9 Hz, 2H, COCH_2), 2.12–1.98 (m, 4H, CF_2CH_2, CH=CHCH_2), 1.68–1.55 (m, 4H), 1.42–1.26 (m, 14 H), 0.90 (t, *J* = 6.9 Hz, 3H,

CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.12, 133.76, 128.97, 74.22, 62.28, 54.62, 36.75, 32.21, 31.40, 30.63 (t, $^2J_{C,F}$ = 22.3 Hz, CF_2CH_2), 29.07, 29.02, 28.95, 25.42, 22.35, 20.07, 13.85 ppm. HRMS (ESI): calcd. for $C_{24}H_{34}F_{13}NO_3Na$ $[M+Na]^+$ 654.22233, found 654.22232.

_N_-((2_S_,3_R_,_E_)-11,11,12,12,13,13,14,14,15,15,16,16,17,17,18,18,18-heptadecafluoro-1,3-dihydroxyoctadec-4-en-2-yl)hexanamide (24)

Treatment of compound **22** (500 mg, 0.826 mmol) was carried out as described above to afford **24** (523 mg, 90 %) as a white amorphous solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 6.39 (d, J = 7.4 Hz, 1H, N*H*), 5.80–5.74 (m, 1H, CHOHCH=C*H*), 5.54 (dd, J = 15.4, 6.4 Hz, 1H, CHOHC*H*=CH), 4.29 (br. s., 1H, C*H*OH), 3.97–3.86 (m, 2H, C*H*NH, CH_2OH), 3.69 (d, J = 9.1 Hz, 1H, CH_2OH), 3.46 (br. s., 1H, CHO*H*), 3.34 (br. s., 1H, CH_2O*H*), 2.22 (t, J = 7.6 Hz, 2H, $COCH_2$), 2.14–1.97 (m, 4H, CF_2CH_2, CH=CHCH_2), 1.62 (m, 4H), 1.37–1.49 (m, 4H), 1.25–1.37 (m, 4H), 0.90 (t, J = 6.9 Hz, 3H, CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.11, 133.25, 129.34, 74.21, 62.27, 54.58, 36.76, 31.98, 31.40, 30.77 (t, $^2J_{C,F}$ = 22.3 Hz, CF_2CH_2), 28.74, 28.62, 25.43, 22.36, 19.97, 13.84 ppm. HRMS (ESI): calcd. for $C_{24}H_{30}F_{17}NO_3Na$ $[M+Na]^+$ 726.18464, found 726.18502.

(2S,3R)-2-amino-13,13,14,14,15,15,16,16,17,17,18,18,18-tridecafluorooctadecane-1,3-diol (25)

To a solution of compound **19** (654 mg, 0.971 mmol) in EtOH (5 mL) was added Pd/C (65 mg) and vigorously stirred under H_2 atmosphere. After 3 h, the solution was filtered by Celite® and the filtrate was evaporated. To this residue was added MeOH (10 mL) and 2 M aqueous HCl solution (0.5 mL) and stirred under reflux. After 2 h, the solvent was evaporated, and residue was purified by SiO_2 column chromatography ($CHCl_3$:MeOH:NH_4OH = 60:10:1) to afford 25 (326 mg, 63 %) as a white amorphous solid. ^1H NMR (500MHz, CD_3OD) δ = 3.73 (dd, J = 4.3, 10.8 Hz, 1H, CH_2OH), 3.53–3.51 (m, 1H, C*H*OH), 3.47 (dd, J = 7.6, 11.1 Hz, 1H, CH_2OH), 2.73–2.70 (m, 1H, C*H*NH_2), 2.19–2.08 (m, 2H, CF2CH_2), 1.63–1.59 (m, 2H, $CF_2CH_2CH_2$), 1.59–1.54 (m, 2H, CHOHCH_2), 1.45–1.35 (m, 16H) ppm. ^{13}C NMR (125 MHz, CD_3OD) δ = 74.17, 64.50, 58.31, 34.54, 31.90 (t, $^2J_{C,F}$ = 22.2 Hz, CF_2CH_2), 30.90, 30.83, 30.66, 30.54, 30.29, 27.18, 21.43 ppm. HRMS (ESI): calcd. for $C_{18}H_{26}F_{13}NO_2$ $[M+H]^+$ 536.1834, found 536.1829.

Construction of cell culture

Cells were cultured in Dulbecco's Modified Eagle's Medium (high glucose, -D6429- with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, obtained from SIGMA) with 10 % fetal bovine serum (obtained from Gibco), 50U/mL penicillin and 50 mg/mL streptomycin (obtained from SIGMA). All cells except for cell viability assay were cultivated in 10-cm dishes with 10mL of medium at 37 °C under a 5% CO_2 humidified atmosphere.

Cell viability assay (Cell Counting Kit-8 assay)

Cell viability assay was conducted as follows; NIH3T3 cell and B16 cell were basically plated and incubated for 24 hours on 96-well plate, which number of cells were 5000. Then, each lipid was dosed in the presence of 0.5 % DMSO. After 24 hours, 10µl of Cell Counting Kit-8 Solution (obtained from Dojindo Molecular Technologies, Inc.) was added. After 4 hours, Absorbance of visible light (wavelength: 450nm) was measured and cell viability was calculated as following formula:

$$Viability\ (\%) = \frac{A_S - A_b}{A_C - A_b} \times 100$$

A_s: Absorbance of sample well, A_c: Absorbance of Negative control well (concentration : 0 µM with cells), A_b: Absorbance of blank well (No lipids and cells, only medium).

Cell-based assay using SMS2

SMS2-expressing cell lysates were prepared according to previous method.[25] Lysates (20 mM Tris-buffer, 100 µL) and compound 26 and 28 (10 µM as total concentration) were incubated for 3 hours at 37°C, then fluorescent lipids were extracted from lysates by the Bligh-Dyer method, and directly applied to HPLC.

Acknowledgements

This work was partially supported by the Innovation COE Project for Future Medicine and Medicinal Research, and Grants in Aid for Scientific Research (24310151 and 15H03111 to K.M. and 15K16552 to Y.M.)

Keywords: perfluoroalkyl chain • fluorous • sphingolipid • lipid mediator • fluorous solid phase extraction

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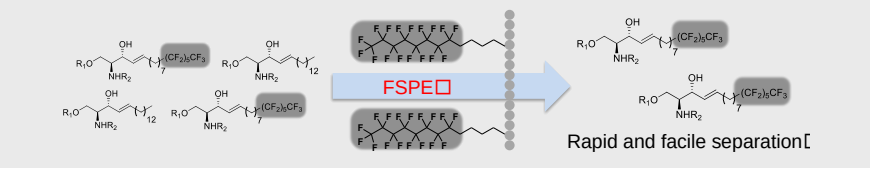

Metabolites of sphingolipids (SLs) are known to act as signaling molecules called lipid mediators to regulate cell behavior. First synthesis of SLs bearing perfluoroalkyl (fluorous) was reported. These derivatives could be applicable to functional analysis of their metabolic mechanisms by FSPE.

*Shota Saito, Yuta Murai, Seigo Usuki, Masafumi Yoshida, Mostafa A.S. Hammam, Susumu Mitsutake, Kohei Yuyama, Yasuyuki Igarashi, and Kenji Monde**

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Title:
Synthesis of Nontoxic Fluorous Sphingolipids as Molecular Probes of Exogenous Metabolic Studies for Rapid Enrichment by FSPE

Key Topic:
Fluorous, Sphingosine