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# Isolation of 9-Hydroxy-10*E*,12*Z*-octadecadienoic Acid, an Inhibitor of Fat Accumulation from *Valeriana fauriei*

Keiji YUKI,[1] Mariko IKEDA,[1] Kenji MIYAMOTO,[1,†] Osamu OHNO,[2] Kaoru YAMADA,[3] and Daisuke UEMURA[3,†]

[1]*Department of Biosciences and Informatics, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[2]*Department of Chemistry, Keio University, 3-14-1 Hiyoshi, Yokohama 223-8522, Japan*
[3]*Department of Chemistry, Kanagawa University, 2946 Tsuchiya, Hiratsuka 256-1293, Japan*

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**An EtOH extract of *Valeriana fauriei* was found to exhibit potent inhibition of fat accumulation against 3T3-L1 murine adipocytes. After performing several chromatographic steps, we successfully isolated the conjugated linoleic acid derivative, 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE). Synthesized 9-HODE and its analogs showed inhibitory activity against fat accumulation.**

**Key words:** *Valeriana fauriei*; 3T3-L1 murine adipocytes; inhibitor; fat accumulation

Such lifestyle-related diseases as cancer, cardiovascular disease, hypertension, hyperlipidemia, and diabetes are rapidly growing epidemics in developed countries, and obesity is one of the causes.[1)] The Ministry of Health, Labor and Welfare in Japan has reported increasing obesity rate in the population. Although such approaches for anti-obesity as low-calorie foods have been proposed, we have focused on inhibitors of fat accumulation, because they could be applied to the development of anti-obesity drugs.

Our previous study screened the fat accumulation inhibitors from various sources. We found that the mushroom, *Coriolus versicolor*, and plant, *Valeriana fauriei*, potently inhibited fat accumulation against 3T3-L1 murine adipocytes. The highly *N*-methylated cyclic heptapeptide, (−)-ternatin, was successfully isolated from *C. versicolor* as a novel inhibitor of fat accumulation.[2–4)] *V. fauriei* is a Chinese herbal medicine and is used to treat hysteroepilepsy and cardiac palpitations. These effects have been mediated by such terpenoid glycosides as kessoglycol diacetate and kessoglycol $\beta$-monoacetate from *V. fauriei*.[5,6)] A novel iridoid glycoside and a sesquiterpenoid have been isolated from *V. fauriei* and showed NGF-potentiating activity.[7)] However, there are no reports on the inhibition of fat accumulation. We report in this study the isolation and determination of fat accumulation inhibitors from *V. fauriei*.

To isolate the fat accumulation inhibitors, we used the assay system with 3T3-L1 murine adipocytes as described previously.[2)] LabAssay™ Triglyceride (Wako Pure Chemical Industries) was used to determine the amount of triglyceride in the 3T3-L1 cells, and Cell Counting Kit-8 (Dojindo Laboratories) was used to determine the cell viability. Both the fat accumulation (FA) and cell viability (CV) rates were determined by dividing the absorbance value of a sample by the absorbance of the control which had been exclusively treated with a vehicle. An FA rate of 50% and CV rate of 50% are respectively presented as the $EC_{50}$ and $IC_{50}$ values.

The rhizomes and roots of *V. fauriei* (2 kg) purchased from Tochimoto Tenkaido Co. (Japan) were extracted with 80% aqueous ethanol over 2 weeks. The concentrated extract (125 g) was partitioned between ethyl acetate and water, and the ethyl acetate layer was partitioned between 90% aqueous methanol and hexane. Since the 90% aqueous methanol layer showed inhibitory effects on fat accumulation (66% FA rate and 93% CV rate at a 100 μg/mL conc.), the methanol fraction was separated by ODS column chromatography with stepwise elution by 70% aqueous MeOH to MeOH. The 80% aqueous MeOH fraction (73% FA rate and 93% CV rate at a 50 μg/mL conc.) was separated by silica-gel column chromatography with stepwise elution by $CHCl_3$/MeOH (19/1, 9/1, and 2/1). The 9/1 $CHCl_3$/MeOH fraction (68% FA rate and 94% CV rate at a 50 μg/mL conc.) showed four spots on the TLC plate [$R_f = 0.63$, 0.70, 0.73, and 0.80 ($CHCl_3$/MeOH = 9 : 1)]. These spots were separated by preparative TLC ($CHCl_3$/MeOH = 9 : 1) to afford an active compound (0.6 mg; 50% FA rate and 81% CV rate at a 20 μg/mL conc.) with an $R_f$ value of 0.70. To determine the structure, this active compound was analyzed by $^1$H-NMR (300 MHz, $CDCl_3$), using high-resolution electrospray ionization mass spectrometry (HR-ESIMS) with a Waters LCT Premier XE and ESIMS with a Brucker Esquire 3000 Plus in the negative ESI mode. Characteristic $^1$H-NMR peaks of the active compound are shown in the experimental section. Its molecular formula was determined to be $C_{18}H_{32}O_3$ by HR-ESIMS ($m/z$ 295.2262 $[M-H]^-$, as calculated for $C_{18}H_{31}O_3$, 295.2273). The ESI mass spectrum showed major peaks at $m/z$ 295 ($[M-H]^-$), and an MS/MS analysis gave

† To whom correspondence should be addressed. Kenji MIYAMOTO, Fax: +81-45-566-1783; E-mail: kmiyamoto@bio.keio.ac.jp; Daisuke UEMURA, Fax: +81-463-58-9684; E-mail: uemura@kanagawa-u.ac.jp

*Abbreviations*: NGF, nerve growth factor; PPAR$\gamma$, peroxisome proliferator-activated receptor $\gamma$; GPCR, G protein-coupled receptor; FA, fat accumulation; CV, cell viability

ion fragments at $m/z$ 277 ($[(M - H_2O) - H]^-$) and 171 ($[(M - CH_3(CH_2)_4CHCHCHCH_2) - H]^-$). Based on these spectral data, we identified the active compound as the known but unusual fatty acid, 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE, **3**) (Fig. 1). The $^1$H-NMR and MS/MS peaks assigned to the active compound were in good agreement with those reported previously for 9-HODE.[8,9] The enantiomeric excess of isolated 9-HODE was determined by an HPLC analysis after converting to the corresponding methyl ester.[10] The results indicate that the absolute configuration of isolated 9-HODE was the (*R*)-enantiomer (>95% e.e.).

We next synthesized 9-HODE and its analogs by using a reported method.[9] Methyl linoleate (**1**) was added to a mixture of $SeO_2$ and dichloromethane in an argon atmosphere. The reaction mixture was stirred for 24 h at room temperature, before a 10% NaCl solution was added and the mixture extracted with dichloromethane. The extract contained the 9-HODE methyl ester (**4**) and 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE) methyl ester (**6**). These compounds were purified by preparative HPLC and subsequent hydrolysis to afford racemic 9-HODE (**3**) and racemic 13-HODE (**5**), and their inhibitory activity against fat accumulation was evaluated. Figure 2A shows that synthesized 9-HODE significantly inhibited fat accumulation in 3T3-L1 cells ($EC_{50} = 20\,\mu g/mL$, $IC_{50} = 29\,\mu g/mL$). These results are relatively higher than those for (*R*)-9-HODE isolated from *V. fauriei* ($EC_{50} = 40\,\mu g/mL$, $IC_{50} => 40\,\mu g/mL$). The results indicate the stronger activity of the (*S*)-enantiomer than that of the (*R*)-acid. Interestingly, 13-HODE showed slightly greater activity ($EC_{50} = 17\,\mu g/mL$, $IC_{50} = 41\,\mu g/mL$) than that of 9-HODE (Fig. 2B). The methyl esters (**4** and **6**) were also subjected to these assays, but indicated no inhibitory activity (data not shown). These results show that the structure of a conjugated diene with a hydroxyl group at the allylic position and a carboxyl group may be important for the biological activities of 9-HODE. Although the inhibitory activity of 9-HODE and 13-HODE against fat accumulation has not been previously reported, it has been reported that conjugated linoleic acid (CLA, **2**) had such biological activities such as anti-tumor and hypocholesterolemic effects.[11] Interestingly, we found that CLA (**2**) had weak inhibitory activity against fat accumulation ($EC_{50} = 316\,\mu g/mL$, $IC_{50} = 375\,\mu g/mL$) when compared with 9-HODE and 13-HODE.

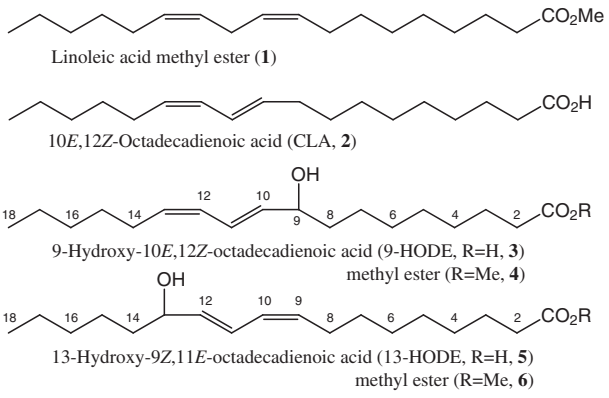


**Fig. 1.** Structures of Linoleic Acid Methyl Ester 1, Conjugated Linoleic Acid 2, Allylic Hydroxylated Derivatives 3 and 5, and Corresponding Methyl Esters 4 and 6.

It has been reported that 9-HODE and 13-HODE showed antitumorial activities.[9] The methyl esters (**4** and **6**) had very weak activity compared with the corresponding acids. Intriguingly, 9-HODE and 13-HODE showed more potent inhibition of fat accumulation and antitumor activity than CLA, although these activities were lost by methyl esterification.

It is crucial for future applications to elucidate the mechanisms for the inhibitory activities of 9-HODE against fat accumulation. 9-HODE has been reported to act as a ligand of both PPAR$\gamma$[12] and G protein-coupled receptor G2A.[13,14] PPAR$\gamma$ is a transcriptional factor belonging to the nuclear receptor super-family and is known to promote the differentiation of adipocytes. Experiments with the heterozygous mouse,[15] inhibitors of PPAR$\gamma$,[16] and Pro12Ala SNP in humans[17] have shown a suppression of obesity by incremental serum leptin levels.[18] However, it has been reported that PPAR$\gamma$ activated by troglitazone, a potent agonist, promoted the differentiation of small adipocytes and the apoptosis of large adipocytes.[19] Troglitazone thus led to the miniaturization of adipocytes. It is likely that 9-HODE may activate PPAR$\gamma$ and miniaturize adipocytes in the same way as troglitazone, leading to decreased fat accumulation. G2A is a stress-inducible G protein-coupled receptor (GPCR) which is known to have such biological functions as those involving inflammatory response, proliferation, and differentiation. It has been reported that 9-HODE acted as a ligand of G2A and released a variety of cytokines.[13,14] However, intra-

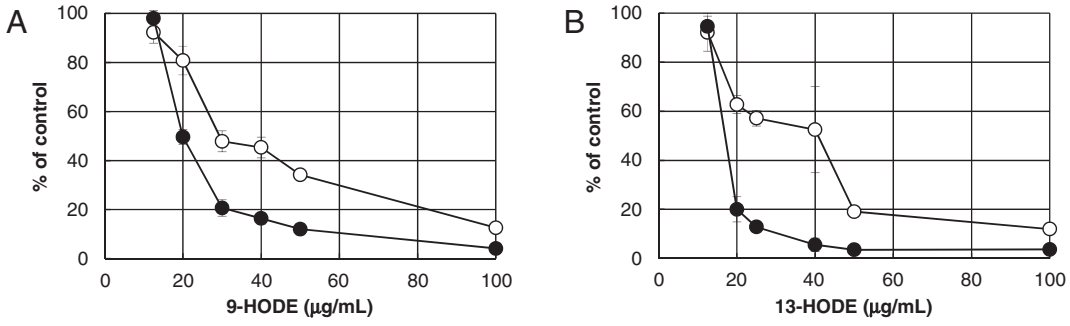


**Fig. 2.** Inhibitory Effects of Fat Accumulation on 3T3-L1 Murine Adipocytes.

3T3-L1 cells were treated with 9-HODE (A), 13-HODE (B), and the vehicle (control) for 1 week during differentiation. The fat accumulation (filled circles) and cell viability (unfilled circles) were then determined. Data are presented as the mean and standard deviation (SD) of four tests and are shown as a percentage of the control value.

cellular signaling mediated by G2A has not been fully elucidated. It is possible that the cytokines released by G2A played a role in the inhibition of fat accumulation after 9-HODE bound to G2A.

We successfully isolated 9-HODE in this study as an inhibitor of fat accumulation and found that 13-HODE had the same effect. The hydroxylated derivatives of conjugated linoleic acid may be potent inhibitors of fat accumulation, making it important to evaluate the activity of other analogs of 9-HODE.

### *Experimental*

*General data.* $^1$H-NMR spectra were recorded with a JeolL JNM AL300 FT NMR spectrometer. 9-HODE (**3**). $^1$H-NMR (300 MHz, $CDCl_3$) $\delta$: 0.89 (3H, t, $J = 6.8$), 1.21–1.59 (18H, m), 2.18 (2H, m), 2.35 (2H, t, $J = 7.5$), 4.15 (1H, m), 5.44 (1H, m), 5.66 (1H, dd, $J = 6.8$, 15.2), 5.97 (1H, dd, $J = 11.2$, 11.2), 6.49 (1H, dd, $J = 11.2$, 15.2).

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