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Effects of amide constituents from pepper on adipogenesis in 3T3-L1 cells

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Abstract—Several amide constituents (piperlonguminine and retrofractamides A, B, and C) from the fruit of *Piper chaba* promoted adipogenesis of 3T3-L1 cells. Among them, retrofractamide A was the most active and significantly increased the amount of adiponectin released into the medium and the uptake of 2-deoxyglucose into the cells. Retrofractamide A also increased mRNA levels of adiponectin, peroxisome proliferator-activated receptor γ2 (PPARγ2), glucose transporter 4 (GLUT4), and insulin receptor substrate 1 (IRS-1), but did not act as a PPARγ agonist different from troglitazone.

Type 2 diabetes is closely associated with other metabolic disorders, such as hypertension, cardiovascular diseases, and atherosclerosis and its incidence is increasing worldwide. Insulin resistance is an important marker for developing type 2 diabetes. The roles of life-style changes and weight loss in preventing diabetes have been proven in clinical trials. Several oral hypoglycemic agents and the antiobese drug orlistat have been shown to significantly decrease progression to diabetes. However, more effective and safe medicines are required.[1]

Thiazolidinedione-type compounds (TZDs) such as pioglitazone are potent insulin sensitizers and currently used clinically to treat type 2 diabetes. TZDs were originally identified based on their antihyperglycemic activity, but they are also able to improve other abnormalities associated with type 2 diabetes, such as hyperlipidemia, atherosclerosis, hypertension, and chronic inflammation. TZDs are potent ligands of peroxisome proliferator-activated receptor γ (PPARγ) and improve insulin sensitivity by increasing the levels of adiponectin, an important adipocytokine associated with insulin sensitivity in adipose tissue, and by decreasing free fatty acid (FFA) and inflammatory factor TNF-α levels in diabetic subjects and animal models in vivo and in adipocytes such as 3T3-L1 cells in vitro.[2–4] It is well established that PPARγ agonists such as TZDs promote the adipogenesis of 3T3-L1 cells, and so the cells have been used for the development of anti-diabetic compounds.[5]

Recently, spice-derived compounds (e.g., allyl isothiocyanate, zingerone, and curcumin) were reported to inhibit the cellular production of proinflammatory mediators such as TNF-α and nitric oxide markedly, and significantly inhibited the release of monocyte chemoattractant protein-1 (MCP-1) from 3T3-L1 adipocytes.[6] Furthermore, capsaicin, a well-known transient receptor potential vanilloid type-1 (TRPV1) agonist, was reported to prevent adipogenesis in stimulated 3T3-L1 cells,[7,8] but it induced an up-regulation of adiponectin expression.[8] On the other hand, effects of piperine (**4**), which was reported as a TRPV1 agonist,[9] and related amide constituents on 3T3-L1 cells have not been examined yet.

In our previous study,[10] we reported the isolation and chemical elucidation of new amide constituents named piperchabamides A (**1**, 0.0029% from the dried fruit), B (**8**, 0.0041%), C (**10**, 0.0032%), D (**14**, 0.0037%), and E (0.0083%) from the fruit of *Piper chaba* Hunter (syn. *Piper retrofractum* Vahl) together with known amide constituents [piperanine (**3**, 0.42%), piperine (**4**, 2.84%), piperoleine B (**5**, 0.0082%), pipernonaline (**6**, 0.47%), dehydropipernonaline (**7**, 0.078%), piperundecalidiene (**9**, 0.017%), piperlonguminine (**11**, 0.22%),

Keywords: *Piper chaba*; Amide constituents; Retrofractamide A; 3T3-L1 cells; Adipogenesis; Adiponectin.
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retrofractamides A (**13**, 0.0075%), B (**15**, 0.049%), and C (**12**, 0.036%), guineensine (**16**, 0.081%), brachystamide B (**17**, 0.0083%), *N*-isobutyl-(2*E*,4*E*)-decadienamide (**18**, 0.018%), *N*-isobutyl-(2*E*,4*E*)-dodecadienamide (**19**, 0.0034%), *N*-isobutyl-(2*E*,4*E*)-octadecadienamide (**20**, 0.14%), *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicosatrienamide (**21**, 0.18%), and dihydropiperlonguminine (0.015%)] and a known aromatic constituent [methyl piperate (**2**, 0.11%)] (Fig. 1).

In the course of searching for anti-diabetic constituents from natural medicines,[11] we found that several amide constituents from the fruit of *P. chaba*, which is used as a spice in southeast Asia, promoted the accumulation

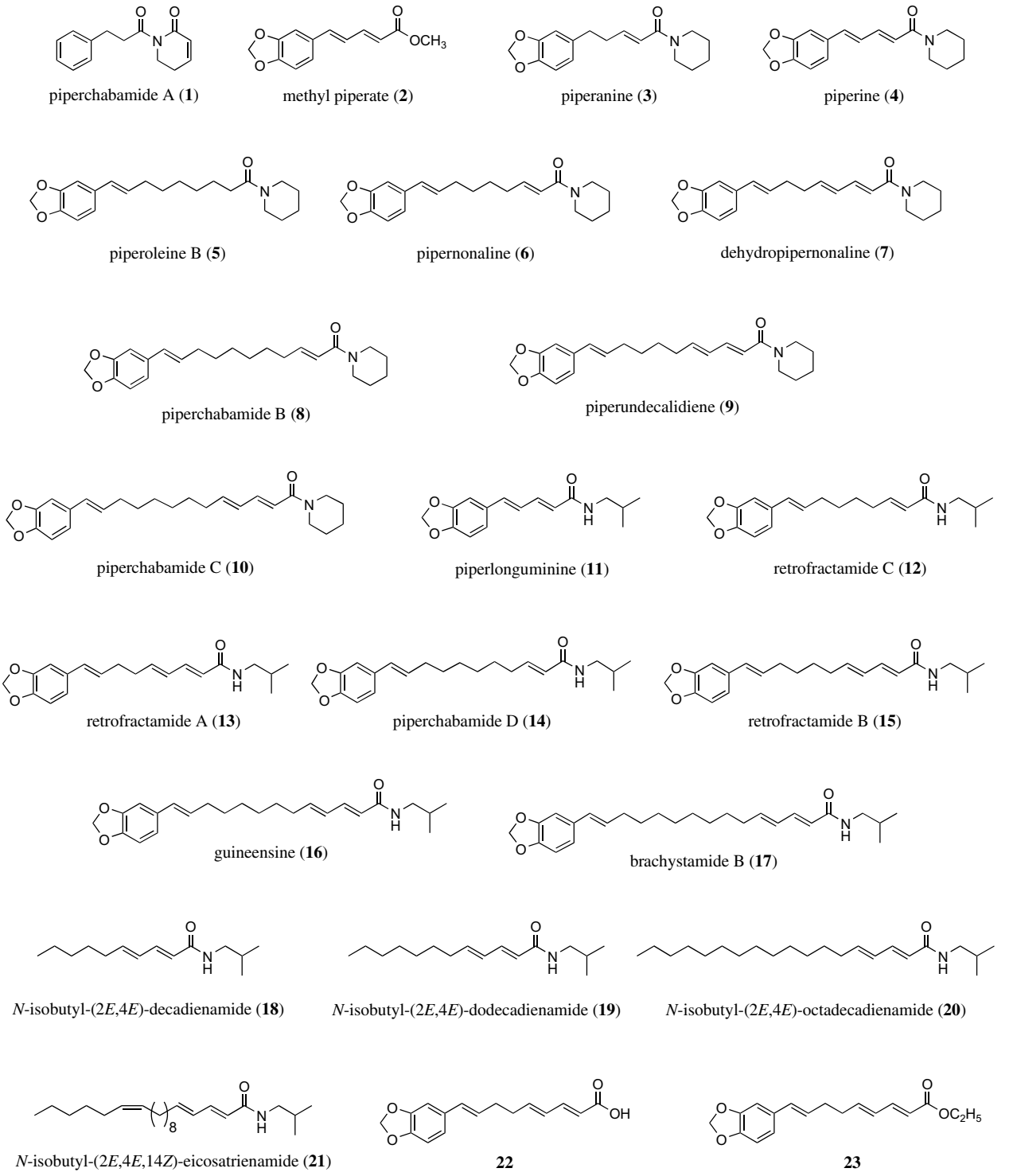

Figure 1. Chemical structures of isolated constituents (**1**–**21**) from the fruit *of Piper chaba* and synthetic compounds (**22** and **23**).

Table 1. Effects of constituents (**1**–**21**) from the fruit of *Piper chaba* and synthetic compounds (**22** and **23**) on TG levels in 3T3-L1 cells

Concn (μM)→	Increase in TG levels (%)				
	0	1	3	10	30
Piperchabamide A (**1**)	0.0 ± 2.0	10.2 ± 2.7	14.0 ± 3.9*	8.9 ± 1.0	−5.0 ± 4.5
Methyl piperate (**2**)	0.0 ± 4.1	5.2 ± 5.5	17.8 ± 3.5*	25.2 ± 4.9**	17.1 ± 2.9*
Piperanine (**3**)	0.0 ± 2.6	20.4 ± 6.4	19.1 ± 8.4	13.1 ± 2.1	8.3 ± 2.2
Piperine (**4**)	0.0 ± 4.6	−1.2 ± 3.3	−3.9 ± 2.3	−14.2 ± 1.3	−1.2 ± 3.0
Piperoleine B (**5**)	0.0 ± 3.8	−2.8 ± 7.1	30.3 ± 5.7**	20.9 ± 4.7*	9.4 ± 3.8
Pipernonaline (**6**)	0.0 ± 2.0	1.0 ± 4.6	5.4 ± 3.9	10.5 ± 5.6	−61.2 ± 2.4**,[a]
Dehydropipernoline (**7**)	0.0 ± 4.5	0.5 ± 0.8	1.8 ± 3.8	1.5 ± 1.0	−19.9 ± 1.2**,[a]
Piperchabamide B (**8**)	0.0 ± 5.8	−14.1 ± 3.4	−2.2 ± 1.0	−3.5 ± 1.6	−30.3 ± 0.6**,[a]
Piperundecalidiene (**9**)	0.0 ± 3.0	−13.0 ± 3.4	− 7.3 ± 6.1	7.3 ± 1.0	−60.8 ± 3.6**,[a]
Piperchabamide C (**10**)	0.0 ± 3.5	9.4 ± 1.8	17.6 ± 5.2*	−10.2 ± 7.0	−11.0 ± 5.1
Piperlonguminine (**11**)	0.0 ± 7.8	19.5 ± 2.3*	28.1 ± 5.9**	31.8 ± 3.5**	20.4 ± 4.2*
Retrofractamide C (**12**)	0.0 ± 2.9	9.7 ± 1.6**	15.0 ± 1.8**	22.2 ± 1.6**	15.4 ± 1.4**
Retrofractamide A (**13**)	0.0 ± 1.9	25.1 ± 3.6**	27.3 ± 3.0**	27.8 ± 1.4**	33.3 ± 1.6**
Piperchabamide D (**14**)	0.0 ± 4.8	1.7 ± 1.7	8.0 ± 2.5	8.5 ± 6.5	4.7 ± 5.5
Retrofractamide B (**15**)	0.0 ± 3.3	18.8 ± 3.3**	29.9 ± 1.3**	28.4 ± 3.0**	27.6 ± 3.1**
Guineensine (**16**)	0.0 ± 1.3	1.5 ± 1.5	9.7 ± 3.5	0.8 ± 3.4	2.0 ± 1.3
Brachystamide B (**17**)	0.0 ± 2.5	11.1 ± 3.4*	18.5 ± 2.3**	16.8 ± 2.5**	13.7 ± 3.2**
N-Isobutyl-(2*E*,4*E*)-decadienamide (**18**)	0.0 ± 3.8	4.4 ± 3.0	18.2 ± 1.1**	10.0 ± 3.4	6.2 ± 1.9
N-Isobutyl-(2*E*,4*E*)-dodecadienamide (**19**)	0.0 ± 2.1	10.1 ± 2.1	16.3 ± 3.4**	11.1 ± 5.4	15.3 ± 2.3**
N-Isobutyl-(2*E*,4*E*)-octadecadienamide (**20**)	0.0 ± 2.8	14.1 ± 2.9	3.3 ± 3.4	19.6 ± 7.7**	20.6 ± 2.5**
N-Isobutyl-(2*E*,4*E*,14*Z*)-eicosatrienamide (**21**)	0.0 ± 1.2	−3.0 ± 1.5	−7.2 ± 1.9*	7.2 ± 2.1*	20.2 ± 2.2**
22	0.0 ± 2.4	−3.9 ± 4.0	1.5 ± 2.3	3.9 ± 3.2	−7.3 ± 1.0
23	0.0 ± 2.6	2.9 ± 1.4	2.9 ± 7.0	1.2 ± 3.1	8.7 ± 3.9
Troglitazone	0.0 ± 1.6	33.8 ± 1.6**	40.5 ± 1.6**	39.6 ± 1.8**	—

Murine 3T3-L1 cells [Cell No. IFO50416 obtained from Health Science Research Resources Bank (Osaka, Japan)] (5.0×10^4 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS were seeded into the 48-well multiplate. After 24 h, the differentiation was induced by changing the medium to a differentiation medium [DMEM (high glucose) supplemented with 10% FCS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin]. After 72 h, the differentiation medium was replaced with a maintenance medium [DMEM (high glucose) supplemented with 10% FCS and 5 μg/mL insulin]. After 4 days (on day 8), the medium was removed and H_2O (200 μL/well) was added to each well, and then the cells were sonicated. The triglyceride (TG) level in the sonicate was determined by a commercial kit (Triglyceride E-test Wako, Wako Chemical Industries). Test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM of % increase in TG levels ($n = 4$). Significantly different from the control group,*$p < 0.05$, **$p < 0.01$.[13]

[a] Cytotoxic effect was observed. Troglitazone was used as a reference compound.

of triglyceride (TG) in 3T3-L1 cells. This letter describes effects of the amide constituents from *P. chaba* on adipogenesis in 3T3-L1 cells together with several structural requirements for the activity and mode of action of retrofractamide A (**13**).

Among the amide constituents, piperlonguminine (**11**), retrofractamides A (**13**), B (**15**), and C (**12**), and brachystamide B (**17**) significantly enhanced the accumulation of TG levels at 1–30 μM as shown in Table 1. The marked reduction in TG levels observed on treatment with **6**–**9** at a higher concentration (30 μM) may depend on cytotoxic effects, since the cells tended to detach from the surface of the culture plate at the high concentration. However, piperine (**4**) at 1–30 μM did not significantly inhibited the accumulation of TG in the present conditions, although capsaicin was reported to reduce the adipogenesis of 3T3-L1 cells.[7,8] In addition, methyl

Table 2. Primers for mouse adiponectin, PPARγ2, GLUT4, IRS-1, and β-actin

Adiponectin[14]	Sense	5′-AAGGACAAGGCCGTTCTCT-3′
	Antisense	5′-TATGGGTAGTTGCAGTCAGTTGG-3′
PPARγ2[15]	Sense	5′-GGTGAAACTCTGGGAGATTC-3′
	Antisense	5′-CAACCATTGGGTCAGCTCTTG-3′
GLUT4[16]	Sense	5′-CCTGAGAGCCCCAGATACCTCTAC-3′
	Antisense	5′-GTCGTCCAGCTCGTTCTACTAAG-3′
IRS-1[14]	Sense	5′-GCTCTAGTGCTTCCGTGTCC-3′
	Antisense	5′-GTTGCCACCCCTAGACAAAA-3′
β-Actin[17]	Sense	5′-ATGGGTCAGAAGGACTCCTACG-3′
	Antisense	5′-AGTGGTACGACCAGAGGCATAC-3′

Thermal cycling conditions for the PCR were 95 °C for 2 min followed by 40 cycles of 95 °C 30 s, 58 °C 30 s, and 72 °C 30 s.

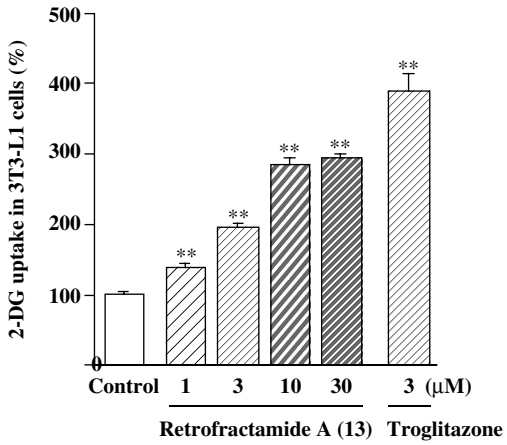

Figure 2. Effect of retrofractamide A (**13**) and troglitazone on 2-deoxyglucose (2-DG) uptake in 3T3-L1 cells. 3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FCS were seeded into the 48-well multiplate. After the differentiation, the medium was replaced with the maintenance medium. After 4 days (on day 8), the cells were incubated in the medium without serum for 4 h, and then washed with Krebs–Ringer phosphate (KRP) buffer. After 20 min incubation in KRP buffer containing insulin (100 nM), 2-deoxyglucose (final concn 0.1 mM) and 2-deoxy-D-(2,6-^{3}H)-glucose (1 μ Ci/mL) were added to each well. After 10 min at 37 °C, the cells were washed with ice-cold PBS to terminate the reaction, and then 1 M NaOH solution (200 μL/well) was added to each well and the cells were incubated for 2 h at 37 °C to lyse them. After neutralization with 1 M HCl, an aliquot was transferred to a vial and the radioactivity was measured using a liquid scintillation counter. Test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Nonspecific uptake was determined in the presence of 20 μM cytochalasin B and was subtracted from the total values. Values represent means ± SEM ($n = 4$). Significantly different from the control group, $^*p < 0.05$, $^{**}p < 0.01$.[13] Troglitazone was used as a reference compound.

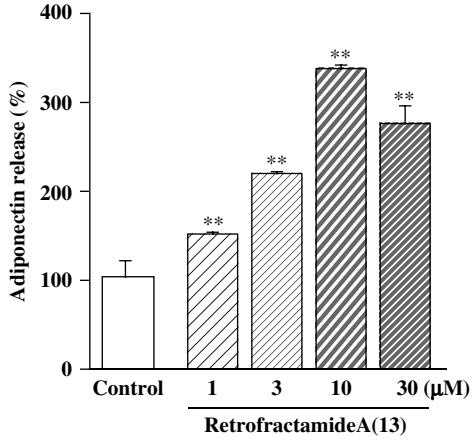

Figure 3. Effect of retrofractamide A (**13**) on release of adiponectin in the medium. The bioassay was performed according to our previous report[11e] with a slight modification. Briefly, 3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FCS were seeded into a 48-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium. After 4 days (on day 8), adiponectin concentrations in the medium were measured using an ELISA kit (R&D systems) according to the manufacturer's instructions. Test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn, was 0.1%). Values represent means ± SEM ($n = 3$). Significantly different from the control group, $^*p < 0.05$, $^{**}p < 0.01$.[13]

piperate (**2**) and synthetic compounds (**22** and **23**)[12] lacking an amide moiety exhibited weak or no activity.

With regard to the structural requirements of the amide constituents for the activity, all amide structures of **11**–**13**, **15**, and **17** are composed of an isobutyl amine moiety, but **3**–**9** composed of a piperidine ring showed less effects except for **5**. These findings suggested that the amide structure composed of an isobutyl amine moiety was important [**11** (increase at 3 μM: 28.1%) > **2** (17.8%) and **4** (−3.9%); **13** (27.3%) > **7** (1.8%), **22** (1.5%) and **23** (2.9%); **15** (29.9%) > **9** (−7.3%)]. Furthermore, the amide constituents (**18**–**21**) lacking an aromatic ring had weak activities, and the length of the carbon chain between the aromatic ring and amide moiety and a double bond conjugated to the α,β-unsaturated carbonyl group were also suggested to be important {[**13** (increase at 3 μM: 27.3%) and **15** (29.9%) > **16** (9.7%) and **17** (18.5%)], [**13** (27.3%) > **12** (15.0%); **15** (29.9%) > **14** (8.0%)]} (Table 1).

Next, effects of retrofractamide A (**13**) on the uptake of 2-deoxyglucose and the release of adiponectin into the medium were examined, since PPARγ agonists increase the uptake of glucose and production of adiponectin in adipocytes.[5] As a result, **13** concentration-dependently increased the uptake of 2-deoxyglucose like troglitazone and the release of adiponectin into the medium (Figs. 2 and 3). The maximum effect of **13** on the release of adiponectin was observed at 10 μM.

Next, effects of retrofractamide A (**13**) on the gene expressions of adiponectin, PPARγ2, GLUT4, and IRS-1 were examined. As shown in Figure 4, levels of adiponection and GLUT4 mRNA were markedly increased by retrofractamide A (**13**), with a maximum effect on the expression of adiponectin observed at 10 μM similar to that on the release of the protein in the medium. In addition, **13** significantly increased the levels of PPARγ2 mRNA, different from troglitazone, and IRS-1 mRNA at 30 μM. The profile of expression was different from that of troglitazone, a PPARγ agonist. This finding led us to presume that the mechanism of action of **13** was different from that of PPARγ agonists. Therefore, agonistic activities of **13** and **15** were examined using a nuclear receptor cofactor assay system. As a result, EC_{50} value of troglitazone was 2.6 μM in this system, but **13** and **15** showed weak activity as expected (Fig. 5).

In conclusion, several amide constituents [piperlongumininine (**11**) and retrofractamides A (**13**), B (**15**), and C (**12**)] isolated from the fruit of *P. chaba* promoted adipogenesis of 3T3-L1 cells. Among them, **13** was the most active and significantly increased the amount of adiponectin released into the medium and the uptake of 2-deoxyglucose into the cells. Compound **13** also increased mRNA levels of adiponectin, PPARγ2, GLUT4, and IRS-1, but **13** did not act as a PPARγ agonist different from troglitazone. These findings suggest that amide constituents such as retrofractamide A (**13**) are promising seed compounds for the development of anti-diabetic agents. The molecular target of **13** needs to be clarified.

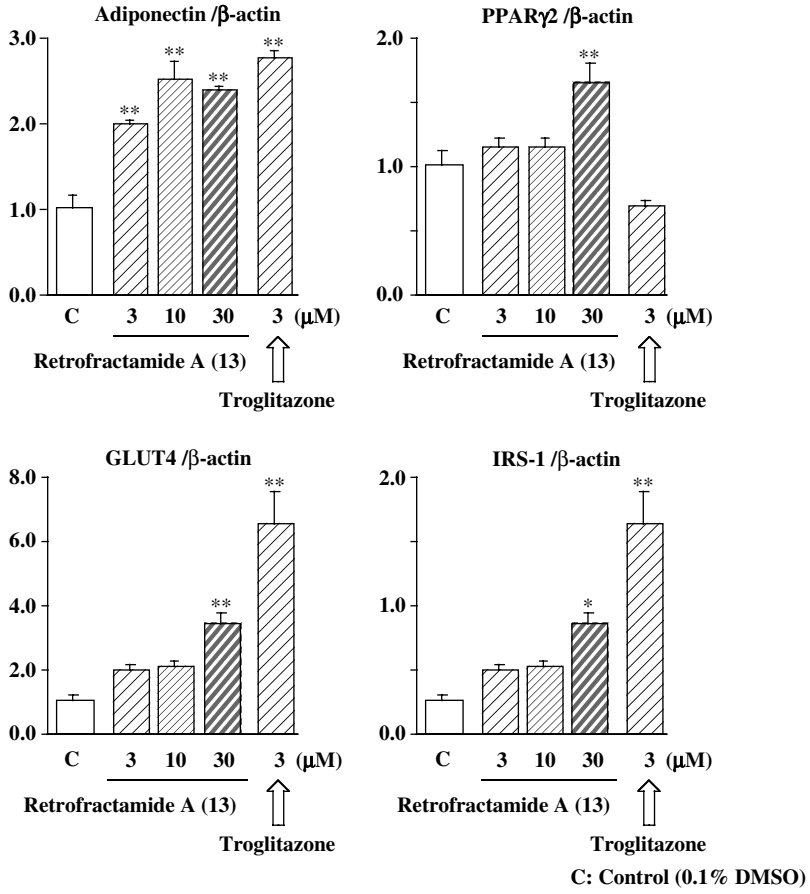

Figure 4. Effect of retrofractamide A (**13**) on gene expressions of adiponectin, PPARγ2, GLUT4, and IRS-1. 3T3-L1 cells (1.0×10^6 cells/well) in DMEM supplemented with 10% FCS were seeded into a 6-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium. After 4 days (on day 8), total RNA was extracted using an RNeasy™ Mini Kit (Qiagen) according to the manufacturer's instructions. The total RNA was reverse transcribed to cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). Then a real-time PCR was carried out on a MiniOpticon real-time machine (Bio-Rad) using the iQ™ SYBR Green Supermix Kit (Bio-Rad). The abundance of each gene product was calculated by relative quantification. In the quantification, values of the target genes were normalized to the β-actin mRNA levels. Thermal cycling conditions for the PCRs were 95 °C for 2 min followed by 40 cycles of 95 °C 30 s, 58 °C 30 s and 72 °C 30 s, then a melting curve analysis from 65 °C to 95 °C, every 0.2 °C. The primer pairs of target genes are shown in Table 2. Test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM ($n = 3$). Significantly different from the control group, $^{*}p < 0.05$, $^{**}p < 0.01$.[13]

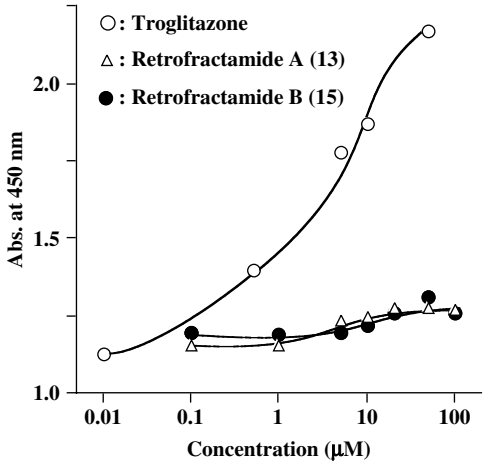

Figure 5. Agonistic activity of retrofractamides A (**13**) and B (**15**) for PPARγ. PPARγ agonistic activity was examined using a nuclear receptor cofactor assay system (EnBio RCAS for PPARγ, EnBioTec Laboratories) according to the manufacturer's instructions. Change of absorbance (450 nm) by troglitazone at 100 μM was calculated as 100% and the EC_{50} value was determined graphically. The experiment was done in duplicate.

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