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Biological evaluation of novel benzisoxazole derivatives as PPARδ agonists

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ABSTRACT

We discovered novel peroxisome proliferator-activated receptor δ agonists with a characteristic benzisoxazole ring. Compound **5** exhibited potent human PPARδ transactivation activity. Furthermore, it stimulated the differentiation of oligodendrocyte precursor cells in vitro. This indicates that this potential drug may be effective for the treatment of demyelinating disorders such as multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease that suddenly manifests itself in persons in their 20s and 30s, accompanying multiple inflammatory demyelinating disorders in various sites of the central nervous system (CNS).[1–5] Genetic as well as environmental factors are believed to be implicated in the onset of MS. In Europe, the incidence of MS is 50 per 100,000 persons and is particularly high at high latitudes, and the incidence in Japanese individuals was approximately 5-fold lower than that in Caucasians. However, due to rapid environmental changes, the incidence in Japan increased 4- to 5-fold in the last 30 years, and currently, approximately 12,000 Japanese individuals have MS. 'Conventional MS' now occurs at similar frequencies in Japanese and Caucasians, and the peak prevalence rates shifted from persons in their 30s to younger persons in their 20s.

In addition to three existing interferon (IFN)-β products, a synthetic peptide, 'Glatiramer,' comprising major components of myelin basic protein (MBP), which is involved in the onset of MS, was introduced into medical practice in Europe as a therapeutic anti-MS drug, and integrin inhibitors were also introduced. Therefore, annual sales of anti-MS drugs have been rapidly increasing. In addition, new anti-MS drugs are under development worldwide. Although causes of the onset of MS are still unknown, a proposed mechanism is that the immune system destroys myelin sheaths within neurons, which disturbs signal transmission between neurons, leading to multiple lesions in various sites. Reflecting this hypothesis, potential therapeutic drugs targeting disease-related molecules now receive more attention than conventional nonspecific immunotherapy.

Anti-MS drugs can be broadly classified as drugs for symptomatic treatment to alleviate acute symptoms for new-onset or recurrent MS and disease modifiers to relief physical dysfunction. In symptomatic treatment, steroids are widely considered highly effective for MS treatment. However, only IFN-β, glatiramer acetate, and mitoxantrone prevented the advancement of physical dysfunction in clinical studies, and they were approved as disease modifiers in Europe. Therefore, recent development efforts concentrate on disease modifiers. Major disease modifiers under development can be classified into the following three groups: (1) drugs to inhibit or induce nonspecific immune response; (2) drugs to induce immunological tolerance specific to MS antigens; and (3) neuroprotective drugs. As MS is an autoimmune disease targeting the CNS, various immunological inhibitors have been clinically used, although most of them had no significant clinical effect. Because MS is a demyelinating disease, the clinical use of neuroprotective drugs has been investigated for the treatment of MS. As stated before, MS is a disease associated with disorders of myelin and its component, oligodendrocytes. Recent studies clarified that although normal oligodendrocyte precursor cells (OPCs) can be detected in patient brains, they cannot differentiate and regenerate myelin because of unknown reasons. Therefore, if differentiation of precursor cells can be facilitated in patient brains, myelin will be regenerated, which may lead to the restoration of neural function.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function after heterodimerization with other

Abbreviations: MS, Multiple sclerosis; CNS, Central nervous system; OPCs, Oligodendrocyte precursor cells; PPAR, Peroxisome proliferator-activated receptor.

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nuclear receptors, such as retinoid X receptors (RXRs). Three PPAR subtypes, designated as PPARα, PPARδ (β), and PPARγ, have been identified. Recent advances in PPARα and PPARγ research have revealed important roles of these PPARs in lipid and glucose metabolism. However, the functions of PPARδ are not well understood. The availability of PPARδ transgenic mice and the discovery of PPARδ agonists, especially GW-501516[6] and L-165041[7] (Fig. 1), have stimulated PPARδ research, and many reports have shown that PPARδ activation leads to interesting effects such as antiobesity, improved lipid metabolism, and wound healing.[8–12]

PPARδ agonists have been reported to stimulate oligodendrocyte differentiation.[13] Oligodendrocytes are members of the glial cell family and form the central myelin sheath. Any disease of the nervous system in which the myelin sheath of neurons is damaged is considered a demyelinating disorder. The most common demyelinating disorder is MS, which is characterized by demyelination of the CNS. In the CNS tissues of MS patients, OPCs are found to survive the demyelinating damage in MS, but they apparently fail to proliferate and differentiate.[14] Therefore, potent PPARδ agonists, which can stimulate oligodendrocyte differentiation, are expected to be a possible remedy for dysmyelination and demyelinating diseases. Based on these findings, the authors have great interests in compounds with potent PPARδ activity, stimulating us to develop novel compounds. We discovered fibrate derivative compound **3** by HTS and confirmed that this compound with a characteristic benzisoxazole ring has PPARδ transactivation activity. Next, we optimized compound **3** whereby we obtained compound **4**, which has an isopropyl group at the C5 position of the oxazole ring, and confirmed that compound **4** has good selectivity for PPARδ.

After the optimization approach to compound **4**, we discovered compound **5**, which exhibits the strongest PPARδ transactivation activity in this series of compounds. We also successfully created compound **6**,[15] which has a high selectivity for PPARδ (Fig. 2). This paper describes the synthesis method for novel PPARδ agonists with a characteristic benzisoxazole ring and the degree of PPAR transactivation activity and stimulation of oligodendrocyte differentiation by the representative compounds.

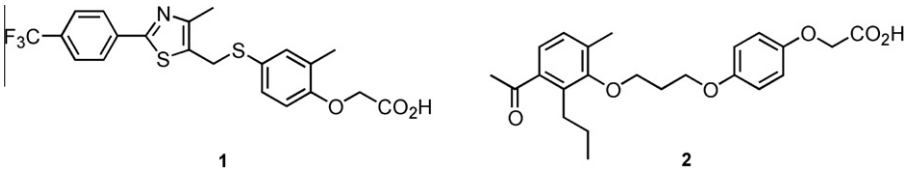
2. Chemistry

The synthesis scheme for the benzisoxazole-ring derivatives is shown below. The outline of the synthesis method for an oxazole derivative, compound **6**, has been reported previously.[15] A similar method was used for synthesizing compounds **3**, **4**, and **7–17**. Thiazole derivatives, compounds **5** and **18**, by using the method given below.

In this paper, we have briefly explained the method of synthesis of compound **5**. Key intermediate **22** was synthesized as shown in Scheme 1. After chlorination of the starting material methyl 4-methyl-3-oxovalerate (**19**) with sulfuryl chloride, the resulting intermediate was treated with 4-(trifluoromethyl)thiobenzamide to obtain a substantial yield of thiazole (**20**). Compound **20** was then treated with sodium bis(2-methoxyethoxy)aluminum hydride to obtain the alcohol (**21**), which was further treated with thionyl chloride to produce 5-chloromethyl-4-isopropyl-2-[4-(trifluoromethyl)phenyl]thiazole (**22**). Compounds **22** and **23a**[15] were condensed using LDA to obtain compound **24**, which was then treated with hydrochloric acid to produce compound **25**. This compound was further treated with sodium nitrite and then hydrolyzed with 75% sulfuric acid to produce compound **26**. Compound **26** was condensed with ethyl 2-bromo-2-methylpropionate in the presence of potassium carbonate in 2-butanone to obtain compound **27**, which was then treated with lithium hydroxide monohydrate in ethanol to obtain compound **5** (Scheme 2).

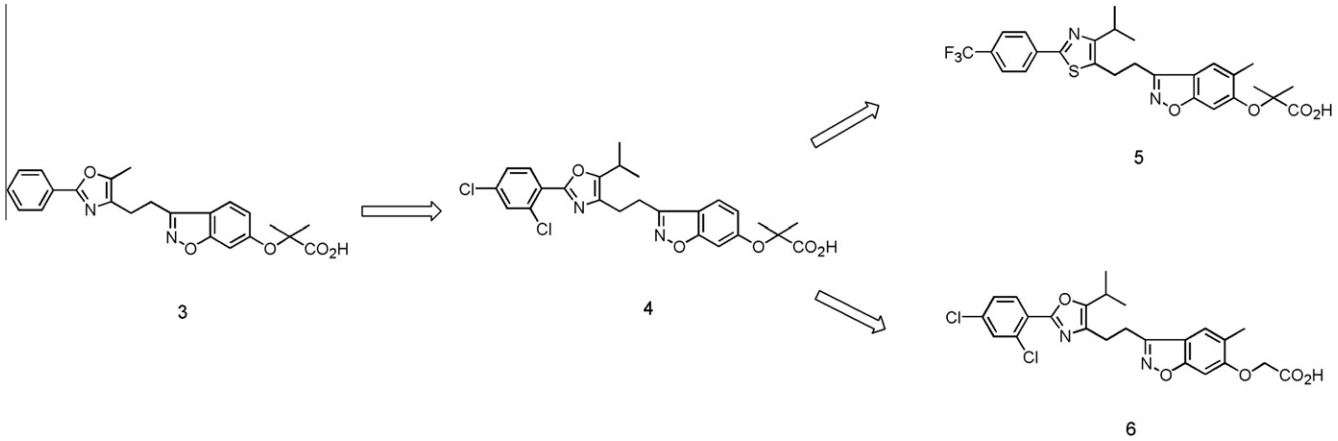
3. Results and discussion

The human PPAR transactivation activity of test compounds was evaluated by the following method. The mammalian expression vectors used were pSG5-GAL4-hPPARα, pSG5-GAL4-hPPARγ,



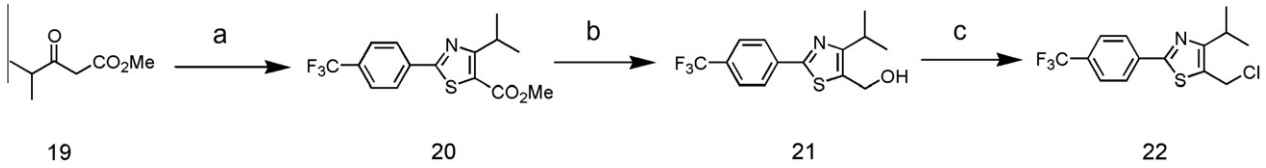
1 2

Figure 1. Structures of representative PPARδ agonists (1: GW-501516 and 2: L-165041).

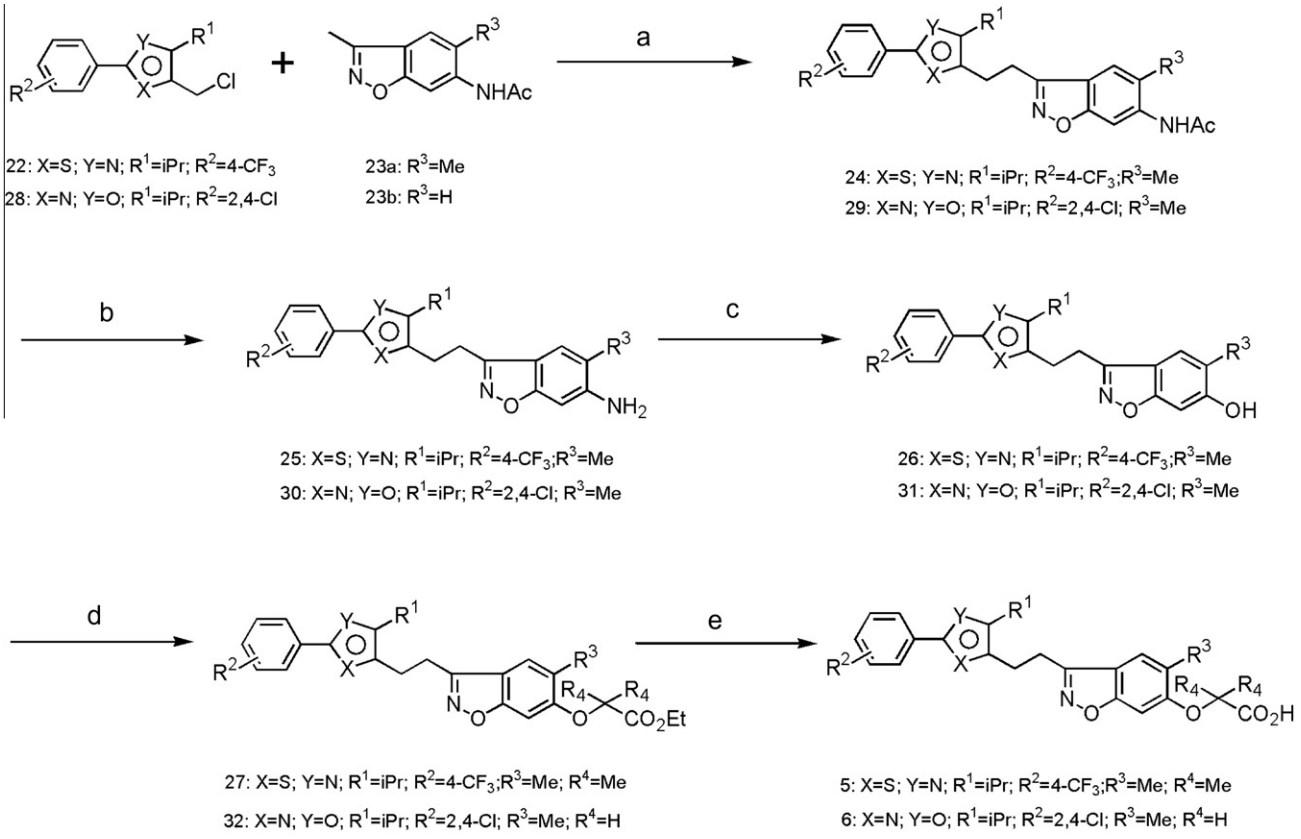


3 4 5 6

Figure 2. Lead 3 and optimization process.



Scheme 1. Synthesis of compound **22**. Reagents and conditions: (a) (1) SO_2Cl_2, 0 °C; (2) 4-trifluoromethylthiobenzamide, EtOH, reflux, 71%; (b) $NaAlH_2(OCH_2CH_2OCH_3)_2$, toluene, 0 °C, 69%; (c) $SOCl_2$, Ph-H, 100%.

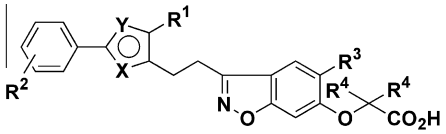


Scheme 2. Synthesis of target compounds. Reagents and conditions: (a) LDA, THF, −78 °C; (b) 3 M HCl, AcOH, reflux; (c) (1) $NaNO_2$, 25% H_2SO_4, 0 °C; (2) 75% H_2SO_4, 120 °C; (d) ethyl 2-bromo-2-methylpropionate, K_2CO_3, 2-butanone, room temperature; or ethyl 2-bromoacetate, K_2CO_3, acetone, room temperature; (e) LiOH monohydrate, EtOH, H_2O, reflux.

and pSG5-GAL4-hPPARδ, which express the ligand-binding domains of human PPARα, PPARγ, and PPARδ, respectively; each of these vectors was fused to the yeast transcription factor GAL4 DNA binding domain.[16] Each receptor expression vector and the UASx4-TK-LUC[17] reporter plasmid were cotransfected into CV-1 cells (kidney fibroblasts isolated from an African green monkey). After the addition of compounds (0.001–100 μM), CV-1 cells were incubated for 40 h, and luciferase activity was measured. The PPARα, PPARγ, and PPARδ transactivation activity of the test compounds was calculated relative to the luciferase activity induced by 1 μM GW-590735[18] (PPARα selective agonist), 10 μM rosiglitazone (PPARγ selective agonist), and 0.1 μM GW-501516 (PPARδ selective agonist), respectively (Table 1).

With special attention to the two substituent groups on the oxazole ring, optimization approach was conducted with compound **3** (PPARδ transactivation activity EC_{50}: 14 μM) as a seed compound. First, the methyl group at the C5 position of the oxazole ring was replaced with an isopropyl or hexyl group, and it was confirmed that the resulting compounds exhibited higher PPARδ activation than the other compounds. Substitution of the methyl group with a *tert*-butyl group decreased the PPARδ transactivation activity. Further, we examined the effect of substitution on the benzene ring at the C2 position of the oxazole ring. The substitution of a chloro or hydroxyl group at the C2 position of the benzene ring led to higher PPARδ activation than the substitution of a hydrogen or trifluoromethyl group. The introduction of an *n*-butyl or *tert*-butyl substituent at the C4 position of the benzene ring increased not only PPARδ but also PPARα and γ activation. Finally, compound **4**, with chloro groups at the C2 and the C4 positions of the benzene ring, was shown to exhibit good PPARδ transactivation activity and good δ selectivity. Furthermore, compound **6** having a higher δ selectivity than compound **4** was successfully created by converting the fibrate structure of compound **4** to the acetic acid structure. Compound **18**, a fibrate derivative that was confirmed to exhibit strong PPARδ transactivation activity, was created by another conversion of the oxazole ring of compound **4** to a thiazole ring. Compound **5**, which was derived by substituting a methyl group at the C5 position of the benzisoxazole ring of compound **18**, exhibited the most powerful PPARδ transactivation activity (EC_{50} = 0.011 μM) among the benzisoxazole derivatives.

Table 1
Peroxisome proliferator-activated receptor (PPAR) transactivation activity of compound **3**–**18** and representative PPAR agonists as assessed by a cell-based transactivation assay, using GAL4 DBD-hPPAR LBD



Compound	X	Y	R^1	R^2	R^3	R^4	Transactivation activity EC_{50} (μM)		
							PPARα	PPARγ	PPARδ
3	N	O	Me	H	H	Me	12.9	20.8	14.9
7	N	O	Et	H	H	Me	14.7	25.1	6.7
8	N	O	Pr	H	H	Me	13.2	19.1	9.8
9	N	O	Pr	2-Cl	H	Me	13.9	26.4	5.4
10	N	O	*i*Pr	2-Cl	H	Me	34.1	31.1	1.3
11	N	O	*tert*-Bu	2-Cl	H	Me	>10	>10	>10
12	N	O	Hexyl	2-Cl	H	Me	2.3	5.9	2.0
13	N	O	*i*Pr	2-OH	H	Me	10.4	14.6	0.9
14	N	O	*i*Pr	2-CF_3	H	Me	>10	>10	>10
15	N	O	*i*Pr	4-*tert*-Bu	H	Me	2.9	1.0	1.2
16	N	O	*i*Pr	4-Bu	H	Me	3.3	2.6	0.7
4	N	O	*i*Pr	2,4-Cl	H	Me	8.6	26.3	0.1
17	N	O	*i*Pr	2,6-Cl	H	Me	>10	>10	>10
18	S	N	*i*Pr	4-CF_3	H	Me	0.83	5.1	0.021
5	S	N	*i*Pr	4-CF_3	Me	Me	0.20	0.91	0.011
6	N	O	*i*Pr	2,4-Cl	Me	H	>10	>10	0.025
GW590735							0.010	>10	2.6
Rosiglitazone							>10	0.10	>10
KRP-297							0.40	0.90	>10
GW501516							0.99	4.1	0.0017

This research was conducted on the basis of the forecast that if the PPARδ agonist stimulated the differentiation of adult OPCs inside the brains of patients with MS, they could regenerate myelin. With these adult OPCs as the target cells, the OPCs of the brain of a rat fetus were used as the model. As the OPCs differentiate into mature oligodendrocytes in the cell lineage, they specifically develop proteins associated with this process. Among the proteins, O1 and MBP were selected as the differentiation markers. Within the cell lineage, these proteins exist in the relatively late stage; in other words, they reach the mature oligodendrocyte stage just before myelin is formed in the immature oligodendrocyte. For this evaluation, O1 was mainly used. The evaluation methods and an outline of the results are described below.

In vitro tests were conducted to evaluate the stimulation of oligodendrocyte differentiation by new compounds and reference drugs by using OPCs. OPCs were prepared from primary mixed cell cultures of the fetal brain cortex of Wistar rats,[19] and the OPC-rich fraction was prepared using the Percoll gradient separation technique. OPCs were treated with test compounds after 4 days of culture. The differentiation-stimulating effect was evaluated by an immunostaining method. Compound was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the medium was 0.01%. Each dissolved compound was added to a culture medium at a concentration of 0.01 or 0.1 μM. For control groups, 0.01% DMSO was added to a culture medium. The cells on the cover glass were incubated for 3 days at 37 °C with 5% CO_2. The cells were immunostained using anti-oligodendrocyte marker O1 antibody and placed on a glass slide. After the slide images of the specimens taken randomly were processed, the areas of stained cells were measured. Regarding the mean area of the control group as 100%, the mean area of the treated groups was shown as relative value. The degree of differentiation was determined based on the increase in the number of stained cells, increased diameter of the stained cells, and membrane sheet formation.

Figure 3a shows the stimulation of oligodendrocyte differentiation by T_3 (0.1 μM) and GW-501516 (0.1 μM). For T_3, most of the area was stained red (O1 stain) such that we could see the progression in differentiation.[20] The T_3-treated cells showed a much greater degree of differentiation than the control. For GW-501516, too, a clear and more substantial progression of differentiation could be observed as compared to the control group, although the degree was less than that observed with T_3. Figure

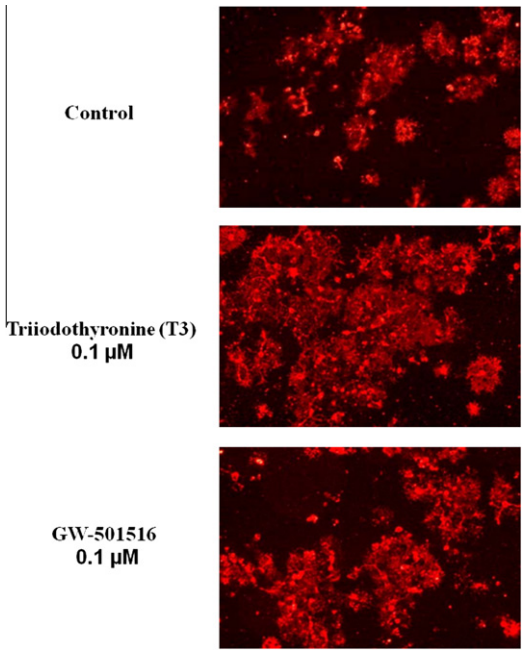


Figure 3a. Oligodendrocyte differentiation-stimulating effect of T_3 and GW-501516. Seven-day-old primary oligodendrocyte cultures were immunostained with anti-O1 antibody. T_3 and GW were added to the cultures 3 days before fixation. In the cultures treated with T_3 and GW, increase in the number of stained cells, membrane sheet formation, and increase in cell diameters were observed.

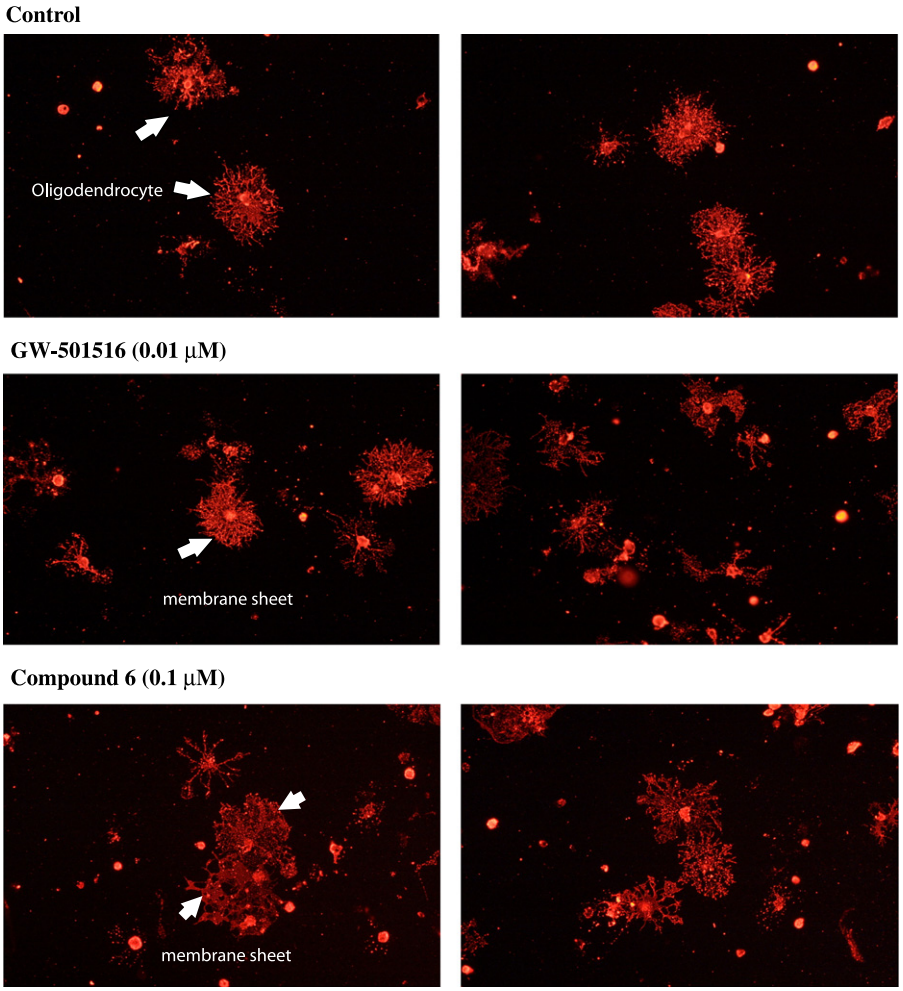


Figure 3b. Oligodendrocyte differentiation-stimulating effect of GW-501516 and compound **6**. Seven-day-old primary oligodendrocyte cultures were immunostained with anti-O1 antibody. GW-501516 and Compound **6** were added to the cultures 3 days before fixation. In the cultures treated with GW and compound **6**, increase in the number of stained cells, membrane sheet formation, and increase in cell diameters were observed.

3b shows a parallel comparison between compound **6** (0.1 μM) and GW-501516 (0.01 μM) for the stimulation of oligodendrocyte differentiation. With both compounds, substantial membrane sheet formation was observed. Moreover, the number of stained cells and the proportion of the stained area were also substantial for both compounds. Figure 4 shows the stimulation of oligodendrocyte differentiation by compound **5**, which exhibits strong PPARδ transactivation activity. The stained area with compound **5** at a concentration of 0.01 μM was double that of the control group. Membrane sheet formation was also observed. To reconfirm that each compound stimulated oligodendrocyte differentiation, double staining was conducted by using myelin basic protein (MBP) in addition to O1. Table 2 shows the stimulation of oligodendrocyte differentiation by the evaluated compounds, including the findings of the control group. We confirmed that compounds **5** and **6** and GW-501516, which exhibit strong PPARδ transactivation activity, stimulated oligodendrocyte differentiation. Moreover, we confirmed that KRP-297, which exhibits weak PPARδ transactivation activity, showed extremely weak stimulation of oligodendrocyte differentiation.

4. Conclusion

We discovered novel PPARδ agonists with a characteristic benzisoxazole ring. Representative compounds **5** and **6** exhibited potent human PPARδ transactivation activity. Moreover, we confirmed that compounds **5** and **6** potently stimulate oligodendrocyte differentiation. Therefore, we believe that these compounds may be effective for the treatment of demyelinating diseases such as MS.

5. Experimental

5.1. Chemistry

5.1.1. General procedures

Analytical samples were homogeneous as confirmed by TLC, and the spectroscopic results were consistent with the assigned strctures. ^{1}H NMR was performed on a JEOL JNM-A400 spectrometer by using deuterated chloroform ($CDCl_3$) and deuterated DMSO (DMSO-d_6) as the solvent. Fast atom bombardment mass spectra (FAB-MS, HR-MS) were obtained on a JEOL JMS-SX102A spectrometer. IRs were measured using a HORIBA FT-720 spectrometer. Column chromatography was performed on silica gel [Wako gel C-300 or C-400]. Thin layer chromatography was performed on silica gel (Merck TLC). The following abbreviations for solvents and reagents are used: ethanol (EtOH), isopropyl alcohol (IPA), tetrahydrofuran (THF), diethylether (Et_2O), ethyl acetate (EtOAc), *N*,*N*-dimethylformamide (DMF), acetic acid (AcOH), sulfuryl chloride (SO_2Cl_2), thionyl chloride ($SOCl_2$), and lithium diisopropylamide (LDA).

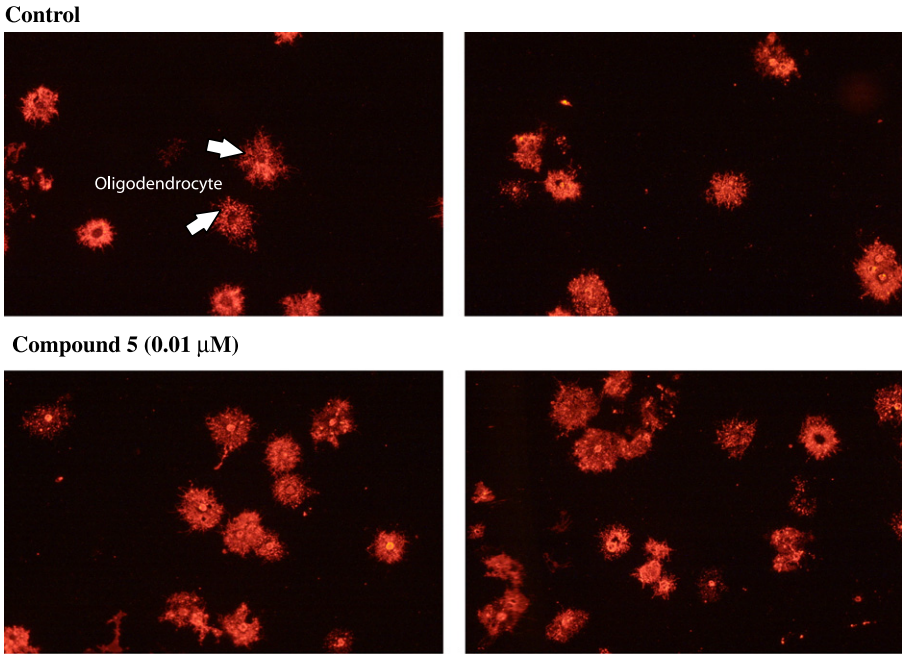


Figure 4. Oligodendrocyte differentiation-stimulating effect of compound **5**. Seven-day-old primary oligodendrocyte cultures were immunostained with anti-O1 antibody. Compound 5 was added to the cultures 3 days before fixation. In the cultures treated with compound **5**, increase in the number of stained cells, membrane sheet formation, and increase in cell diameters were observed.

Table 2
Evaluation of the stimulatory effect of compound **5**, **6** and reference compounds on the differentitation of OPCs

Compound	Concentration of compound		
	0.01 μM	0.03 μM	0.1 μM
5	Active	Active	Active
6	NT[a]	NT	Active
GW-501516	Active	Active	Active
KRP-297	NT	NT	Inactive
T_3	NT	Active	Active

[a] Not tested.

5.1.2. Methyl 4-isopropyl-2-[4-(trifluoromethyl) phenyl]thiazole-5-carboxylate (20)

Methyl 4-methyl-3-oxovalerate (**19**) (28.1 g, 195 mmol) was cooled to 5 °C, and to this solution, SO_2Cl_2 (15.7 mL, 195 mmol) was added dropwise over 45 min, stirred for 30 min, and allowed to room temperature. To this solution, 4-(trifluoromethyl)thiobenzamide (30.8 g, 150 mmol) in EtOH (15 mL) was added and refluxed for 2 h. The reaction mixture was cooled, and to this solution, IPA was added. The insoluble material was filtered and washed with IPA. Water was added to the filtrates, which were stirred at room temperature for 1 h. The precipitated product was filtered and washed with a solution of IPA/H_2O = 1:2 (50 mL) and dried under a vacuum. The crude product was recrystallized from IPA/H_2O to give the target compound as a pale brown crystal (34.9 g, yield 71%).

^{1}H NHR (400 MHz, $CDCl_3$) δ: 1.35 (d, 6H, J = 7 Hz), 3.90 (s, 3H), 3.15 (m, 1H), 7.70 (d, 2H, J = 8 Hz), 8.10 (d, 2H, J = 8 Hz).

5.1.3. 4-Isopropyl-2-[4-(trifluoromethyl)phenyl]thiazole-5-methanol (21)

To the solution of methyl 4-isopropyl-2-[4-(trifluoromethyl) phenyl]thiazole-5-carboxylate (**20**) (30.0 g, 91.1 mmol) in dry toluene (300 mL) was added dropwise sodium bis(2-methoxyethoxy) aluminum hydride toluene solution (39.6 g, 137 mmol) at 0 °C. After 2 h, the reaction mixture was acidified with HCl aq and extracted with EtOAc. The organic layer was washed with water and brine and then dried over Na_2SO_4. The solvent was evaporated to give the crude product, which was recrystallized from EtOAc–hexane to produce the target compound as a pale yellow solid (19.0 g, yield 69%).

^{1}H NHR (400 MHz, $CDCl_3$) δ: 1.35 (d, 6H, J = 7 Hz), 1.82 (t, 1H, J = 6 Hz), 3.15 (m, 1H), 4.87 (d, 2H, J = 6 Hz), 7.67 (d, 2H, J = 8 Hz), 8.04 (d, 2H, J = 8 Hz). FAB-MS (m/z): 302 (M+1).

5.1.4. 5-Chloromethyl-4-isopropyl-2-[4-(trifluoromethyl) phenyl]thiazole (22)

To a stirred solution of 4-isopropyl-2-[4-(trifluoromethyl) phenyl]thiazole-5-methanol (**21**) (10.3 g, 34.2 mmol) in benzene (100 mL) was added $SOCl_2$ (3.84 ml, 52.3 mmol) for 30 min at 0 °C. The mixture was stirred at room temperature for 20 h, and then the solvent was removed in vacuo. The residue was dried in vacuo to give the target compound as a brown solid (yield 100%).

^{1}H NHR (400 MHz, $CDCl_3$) δ: 1.36 (d, 6H, J = 7 Hz), 3.18 (m, 1H), 4.71 (s, 2H), 7.68 (d, 2H, J = 8 Hz), 8.02 (d, 2H, J = 8 Hz).

5.1.5. 6-Acetamido-3-[2-[4-isopropyl-2-[4-(trifluoromethyl) phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazole (24)

6-Acetamido-3,5-dimethyl-1,2-benzisoxazole (**23a**)[15] (9.18 g, 45.0 mmol) was dissolved in dry THF (315 mL). To the solution, 2 M LDA solution (53.0 mL, 106 mmol) was added dropwise for 40 min at −78 °C under a nitrogen atmosphere, and the mixture was stirred for 15 min at −78 °C, after which a THF solution (100 mL) of 5-chloromethyl-4-isopropyl-2-[4-(trifluoromethyl) phenyl]thiazole (**22**) (14.39 g, 45.0 mmol) was added dropwise for 45 min. The mixture was stirred for 1 h under the same conditions and warmed slowly to room temperature. A saturated aqueous ammonium chloride solution and EtOAc was added to the reaction mixture. The organic layer was washed with water and brine and dried over Na_2SO_4. After the organic solvent was removed under reduced pressure, the residue was purified by column chromatography on silica gel with hexane/EtOAc (1:1) to give the target compound as pale yellow crystals (7.40 g, yield 34%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (d, 6H, J = 7 Hz), 2.26 (br s, 3H), 2.32 (s, 3H), 3.04 (m, 1H), 3.26 (dd, 2H, J = 6 Hz, 8 Hz), 3.37 (dd, 2H, J = 6 Hz, 8 Hz), 7.12 (br s, 1H), 7.65 (d, 2H, J = 8 Hz), 7.99 (d, 2H, J = 8 Hz), 8.40 (br s, 1H).

5.1.6. 6-Amino-3-[2-[4-isopropyl-2-[4-(trifluoromethyl) phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazole(25)

6-Acetamido-3-[2-[4-isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazole (**24**) (18.0 g, 36.9 mmol) was suspended in 4 M HCl (360 mL) and AcOH (180 mL). The suspension was heated under reflux for 24 h. Then reaction mixture was cooled to room temperature, poured into ice-cold water, and neutralized with 10 M NaOH aq. After EtOAc was added to the mixture, the organic layer was washed with brine and dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude crystal in the residue was filtered and washed with hexane to give the target compound as pale brown crystals (16.8 g, yield 100%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.25 (d, 6H, J = 7 Hz), 2.21 (s, 3H), 3.05 (m, 1H), 3.21 (dd, 2H, J = 6 Hz, 9 Hz), 3.35 (dd, 2H, J = 6 Hz, 9 Hz), 4.01 (br s, 2H), 6.75 (s, 1H), 7.14 (s, 1H), 7.64 (d, 2H, J = 8 Hz), 8.02(d, 2H, J = 8 Hz).

5.1.7. 6-Hydroxy-3-[2-[4-isopropyl-2-[4-(trifluoromethyl) phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazole (26)

6-Amino-3-[2-[4-isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thi azolyl]ethyl]-5-methyl-1,2-benzisoxazole (**25**) (15.4 g, 34.6 mmol) was suspended in 25% H_2SO_4 (170 mL). An aqueous sodium nitrite (3.10 g, 45.0 mmol) was added to the suspension while it was cooled with ice. After stirring for 20 min under the same conditions, the mixture was added dropwise to 75% H_2SO_4 and then heated to 130 °C. The mixture was refluxed for 3 h under the same conditions, cooled to room temperature, and poured into ice-cold water. After EtOAc was added to the mixture, the organic layer was dried over Na_2SO_4. After the solvent was removed under reduced pressure, the crude crystal in the residue was filtered and washed with hexane to give the target compound as pale brown crystals (8.36 g, yield 54%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.24 (d, 6H, J = 7 Hz), 2.30 (s, 3H), 3.04 (m, 1H), 3.2–3.4 (m, 4H), 5.31 (s, 1H), 6.93 (s, 1H), 7.22 (s, 1H), 7.65 (d, 2H, J = 9 Hz), 8.00 (d, 2H, J = 9 Hz).

5.1.8. Ethyl 2-[3-[2-[4-isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazol-6-yloxy]-2-methylpropionate (27)

6-Hydroxy-3-[2-[4-isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazole (**26**) (8.36 g, 18.7 mmol), ethyl 2-bromo-2-methylpropionate (14.6 g, 74.8 mmol), and K_2CO_3 (10.3 g, 74.8 mmol) were suspended in 2-butanone (5.0 mL). The suspension was refluxed for 45 h and cooled to room temperature. The mixture was poured into ice-cold water and added to EtOAc. The organic layer was washed with brine and dried over Na_2SO_4. After the solvent was removed, the crude in the residue was purified by column chromatography on silica gel with hexane/EtOAc (10:1–5:1) to give the target compound as a pale yellow oil (8.85 g, yield 85%)

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.23 (d, 6H, J = 7 Hz), 1.24 (t, 3H, J = 7 Hz),1.67 (s, 6H), 2.26 (s, 3H), 3.02 (m, 1H), 3.2–3.4 (m, 4H), 4.25 (q, 2H, J = 7 Hz), 6.77 (s, 1H), 7.25 (s, 1H), 7.65 (d, 2H, J = 9 Hz), 8.00 (d, 2H, J = 9 Hz).

5.1.9. 2-[3-[2-[4-Isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thi azolyl]ethyl]-5-methyl-1,2-benzisoxazol-6-yloxy]-2-methylpro pionic acid (5)

Ethyl 2-[3-[2-[4-isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]ethyl]-5-methyl-1,2-benzisoxazol-6-yloxy]-2-methylpropionate (**27**) (8.85 g, 15.8 mmol) was suspended in an EtOH (180 mL)-H_2O (45 mL). Lithium hydroxide monohydrate (1.33 g, 31.6 mmol) was added to the solution, and the mixture was refluxed for 2 h. Ice was added to the reaction mixture. The mixture was acidified with 3 M HCl and extracted with EtOAc. The organic layer was washed with brine and dried over Na_2SO_4. After the solvent was removed, the residue was crystallized in EtOAc (9 mL)-hexane (90 mL). Pale yellow crystal was recrystallized from EtOH–H_2O. The crystals were filtered, washed with water, air-dried overnight, and further dried in vacuo to give the target compound as a white powder (7.90 g, yield 94%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.23 (d, 6H, J = 7 Hz), 1.72 (s, 6H), 2.28 (s, 3H), 3.02 (m, 1H), 3.2–3.4 (m, 4H), 6.93 (s, 1H), 7.25 (s, 1H), 7.65 (d, 2H, J = 9 Hz), 8.00 (d, 2H, J = 9 Hz). Mp (dec) 166–168 °C.

IR (KBr) cm^{-1}: 3000, 1720, 1620, 1520, 1450, 1370, 1320, 1280, 1160, 1120, 1060, 850, 820. FAB-MS (m/z): 533 (M+1). HR-MS calcd for $C_{27}H_{28}F_3N_2O_4S$ 533.17219, $[M+H]^+$, found 533.17182.

5.1.10. 6-Acetamido-3-[2-[2-(2,4-dichlorophenyl)-4-isopropyl-4-oxazolyl]ethyl]-5-methyl-1,2-benzisoxazole (29)

The titled compound was synthesized from 6-acetamido-3,5-dimethyl-1,2-benzisoxazole (**23a**)[15] and 4-chloromethyl-2-(2, -dichlorophenyl)-5-isopropyloxazole (**28**)[15] in the same manner as that described for **24**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.10 (6H, d, J = 7 Hz), 2.24 (3H, br s), 2.26(3H, s), 2.92 (1H, m), 3.05 (2H, t, J = 7 Hz), 3.33 (2H, t, J = 7 Hz), 7.16 (1H, br s), 7.28 (1H, s), 7.32 (1H, dd, 1H, J = 2 Hz, 9 Hz), 7.51 (1H, d, J = 2 Hz), 7.91 (1H, d, 1 J = 9 Hz), 8.34 (1H, br s).

5.1.11. 6-Amino-3-[2-[2-(2,4-dichlorophenyl)-4-isopropyl-4-oxazolyl]ethyl]-5-methyl-1,2-benzisoxazole (30)

The titled compound was synthesized in the same manner as that described for **25**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.10 (6H, d, J = 7 Hz), 2.14 (3H, s), 2.92 (1H, m), 3.03 (2H, t, J = 7 Hz), 3.27 (2H, t, J = 7 Hz), 3.97 (2H, br s), 6.72 (1H, s), 7.13 (1H, s), 7.32 (1H, dd, J = 2 Hz, 8 Hz), 7.51 (1H, d, J = 2 Hz), 7.92 (1H, d, 1 J = 8 Hz), 8.34 (1H, br s).

5.1.12. 3-[2-[2-(2,4-Dichlorophenyl)-4-isopropyl-4-oxazolyl]ethyl]-6-hydroxy-5-methyl-1,2-benzisoxazole (31)

The titled compound was synthesized in the same manner as that described for **26**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.11 (6H, d, J = 7 Hz), 2.22 (3H, s), 2.92 (1H, m), 3.05 (2H, t, J = 7 Hz), 3.29 (2H, t, J = 7 Hz), 6.15 (1H, br s), 6.15(1H, br s), 6.88 (1H, s), 7.19 (1H, s), 7.32 (1H, dd, J = 2 Hz, J = 9 Hz), 7.51 (1H, d, J = 2 Hz), 7.90 (1H, d, J = 9 Hz).

5.1.13. Ethyl [3-[2-[2-(2,4-dichlorophenyl)-4-isopropyl-4-oxazolyl]ethyl]-5-methyl-1,2-benzisoxazol-6-yloxy]acetate (32)

The titled compound was synthesized in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.11 (6H, d, J = 7 Hz), 1.30 (3H, t, J = 7 Hz),2.23 (3H, s), 2.91 (1H, m), 3.04 (2H, t, J = 7 Hz), 3.31 (2H, t, J = 7 Hz), 4.28 (2H, q, J = 7 Hz), 4.69 (2H, s), 6.80 (1H, s), 7.24 (1H, s), 7.33 (1H, dd, J = 2 Hz, J = 8 Hz), 7.51 (1H, d, J = 2 Hz), 7.91 (1H, d, J = 9 Hz).

5.1.14. [3-[2-[2-(2,4-Dichlorophenyl)-4-isopropyl-4-oxazolyl] ethyl]-5-methyl-1,2-benzisoxazol-6-yloxy]acetic acid (6)

The titled compound was synthesized in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ:1.12 (d, 6H, J = 7 Hz), 2.24 (s, 3H), 2.9–3.0 (m, 1H), 3.04 (dd, 2H, J = 7 Hz, 8 Hz), 3.27 (dd, 2H, J = 7 Hz, 8 Hz), 4.74 (s, 2H), 6.82 (s, 1H), 7.20 (s, 1H), 7.33 (dd, 1H, J = 2 Hz, 9 Hz), 7.52 (d, 1H, J = 2 Hz), 7.88 (d, 1H, J = 9 Hz). Mp (dec) 182–184 °C. HR-MS calcd for $C_{24}H_{22}Cl_2N_2O_5$: 488.0906 $[M]^+$, found 488.0885.

5.1.15. 2-[3-[2-[5-Methyl-2-phenyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (3)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-5-methyl-2-phenyloxazole in the same manner as that described for **5**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.77 (s, 6H), 2.19 (s, 3H), 2.96 (t, 2H, J = 7 Hz), 3.25(t, 2H, J = 7 Hz), 6.86(dd, 1H, J = 2 Hz, J = 9 Hz), 6.96 (d, 1H, J = 2 Hz), 7.45–7.50 (m, 3H), 7.71 (d, 1H, J = 9 Hz), 7.85–7.95 (m, 2H), 13.2 (br s, 1H). FAB-MS (m/z): 407 (M+1).

5.1.16. 2-[3-[2-[2-(2,4-Dichlorophenyl)-5-isopropyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (4)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-(2,4-dichlorophenyl)-5-isopropyloxazole (**28**) in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.13 (6H, d, J = 7 Hz), 1.66 (s, 6H), 2.95 (m, 1H), 3.04 (t, 2H, J = 7 Hz), 3.27 (t, 2H, J = 7 Hz), 6.82 (dd, 1H, J = 2 Hz, 8 Hz), 6.99 (d, 1H, J = 2 Hz), 7.28 (d, 1H, J = 8 Hz), 7.32 (dd, 1H, J = 2 Hz, 8 Hz), 7.51 (d, 1H, J = 2 Hz), 7.87 (d, 1H, J = 8 Hz).

IR (KBr) cm^{-1}: 3635, 2939, 1705, 1618, 1562, 1498, 1466, 1460, 1385, 1381, 1286, 1184, 1147, 1107, 1053, 976, 841, 816, 417. FAB-MS (m/z): 504 (M+1).

5.1.17. 2-[3-[2-[5-Ethyl-2-phenyl-4-oxazolyl]ethyl]-1,2-benziso xazol-6-yloxy]-2-methylpropionic acid (7)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-5-ethyl-2-phenyloxazole in the same manner as that described for **5**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.3 (t, 3H, J = 7 Hz), 1.69 (s, 6H), 2.19 (s, 3H), 2.58 (q, 2H, J = 7 Hz), 3.02 (t, 2H, J = 7 Hz), 3.22(t, 2H, J = 7 Hz), 6.79(dd, 1H, J = 2 Hz, J = 9 Hz), 7.01 (d, 1H, J = 2 Hz), 7.22 (d, 1H, J = 9 Hz), 7.4–7.5 (m, 3H), 7.9–8.0 (m, 2H). FAB-MS (m/z): 421 (M+1).

5.1.18. 2-[3-[2-[2-Phenyl-5-propyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (8)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-phenyl-5-propyloxazole in the same manner as that described for **5**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 0.85 (t, 3H, J = 7 Hz), 1.5–1.7 (m, 2H), 1.64 (s, 6H), 2.55(t, 2H, J = 7 Hz), 3.03 (t, 2H, J = 8 Hz), 3.19(t, 2H, J = 8 Hz), 6.76 (dd, 1H, J = 2 Hz, J = 9 Hz), 7.00 (d, 1H, J = 2 Hz), 7.20 (d, 1H, J = 9 Hz), 7.4–7.5 (m, 3H), 7.9–8.0 (m, 2H). FAB-MS (m/z): 435 (M+1).

5.1.19. 2-[3-[2-[2-(2-Chlorophenyl)-5-propyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (9)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-(2-chlorophenyl)-5-propyloxazole in the same manner as that described for **5**.

^{1}H NMR (DMSO-d_6, 400 MHz) δ: 0.85 (t, 3H, J = 7 Hz), 1.45–1.65 (m, 2H), 1.66 (s, 6H), 2.52 (t, 2H, J = 7 Hz), 3.04 (t, 2H, J = 8 Hz), 3.29 (t, 2H, J = 8 Hz), 4.24 (q, 2H, J = 7 Hz), 6.82 (dd, 1H, J = 2 Hz, J = 9 Hz), 7.00 (d, 1H, J = 2 Hz), 7.3–7.4 (m, 3H), 7.45 (m, 1H), 7.85 (m, 1H). FAB-MS (m/z): 469 (M+1).

5.1.20. 2-[3-[2-[2-(2-Chlorophenyl)-5-isopropyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (10)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-(2-chlorophenyl)-5-isopropyloxazole in the same manner as that described for **5**. Mp (dec) 100–105 °C.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.13 (d, 6H, J = 7 Hz), 1.66 (s, 6H), 2.95 (m, 1H), 3.04 (t, 2H, J = 7 Hz), 3.27 (t, 2H, J = 7 Hz), 6.80 (dd, 1H, J = 2 Hz, 8 Hz), 6.99 (d, 1H, J = 2 Hz), 7.24 (d, 1H, J = 8 Hz), 7.3–7.5 (m, 3H), 7.90 (d, 1H, J = 8 Hz).

IR (KBr) cm^{-1}: 3000, 2950, 2900, 1720, 1700, 1620, 1610, 1560, 1520, 1500, 1475, 1460, 1440, 1380, 1280, 1180, 1140, 1120, 1040, 1020 980, 840, 780, 740. FAB-MS (m/z): 469 (M+1).

5.1.21. 2-[3-[2-[5-*tert*-Butyl-2-(2-chlorophenyl)-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (11)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 5-*tert*-butyl-4-chloromethyl-2-(2-chlorophenyl)oxazole in the same manner as that described for **5**: Mp (dec) 127–129 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.26 (6H, d, J = 7 Hz), 1.65 (s, 6H), 3.17 (t, 2H, J = 7 Hz), 3.31 (t, 2H, J = 7 Hz), 6.81 (dd, 1H, J = 2 Hz, 8 Hz), 7.00 (d, 1H, J = 2 Hz), 7.28 (d, 1H, J = 8 Hz), 7.3–7.4 (m, 2H), 7.93 (m, 1H).

IR (KBr) cm^{-1}: 3855, 3737, 3433, 2978, 2974, 2872, 2347, 1707, 1703, 1620, 1572, 1549, 1522, 1498, 1421, 1383, 1367, 1346, 1284, 1184, 1146, 1055, 982, 837, 816, 768, 744, 739, 609. FAB-MS (m/z): 483 (M+1).

5.1.22. 2-[3-[2-[2-(2-Chlorophenyl)-5-hexyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (12)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-(2-chlorophenyl)-5-hexyloxazole in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.86 (t, 3H, J = 7 Hz), 1.2–1.3 (m, 6H), 1.4–1.6 (m, 2H), 1.66 (s, 6H), 2.55 (t, 2H, J = 7 Hz), 3.02 (t, 2H, J = 8 Hz), 3.25 (t, 2H, J = 8 Hz), 6.80 (dd, 1H, J = 2 Hz, 10 Hz), 6.98 (d, 1H, J = 2 Hz), 7.2–7.5 (m, 4H), 7.90 (dd, 1H, J = 2 Hz, 10 Hz). FAB-MS (m/z): 512 (M+1).

5.1.23. 2-[3-[2-[2-(2-Hydroxyphenyl)-5-isopropyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (13)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-5-isopropyl-2-(2-methoxyphenyl)oxazole in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.16 (d, 6H, J = 7 Hz), 1.66 (s, 6H), 2.95 (m, 1H), 3.06 (t, 2H, J = 7 Hz), 3.31 (t, 2H, J = 7 Hz), 6.8–7.1 (m, 4H), 7.32 (m, 1H), 7.40 (d, 1H, J = 9 Hz), 7.76 (dd, 1H, J = 1 Hz, J = 9 Hz). FAB-MS (m/z): 451 (M+1).

5.1.24. 2-[3-[2-[5-Isopropyl-2-[2-(trifluoromethyl)phenyl]-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (14)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-5-isopropyl-2-[2-(trifluoromethyl)phenyl]oxazole in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.11 (d, 6H, J = 7 Hz), 1.65 (s, 6H), 2.95 (m, 1H), 3.04 (t, 2H, J = 7 Hz), 3.28 (t, 2H, J = 7 Hz), 6.18 (1H, dd, J = 2 Hz, J = 8 Hz), 7.00 (d, 1H, J = 2 Hz), 7.26 (d, 1H, J = 8 Hz), 7.5–7.7 (m, 2H), 7.80 (d, 1H, J = 8 Hz), 8.02 (d, 1H, J = 8 Hz). FAB-MS (m/z): 503 (M+1).

5.1.25. 2-[3-[2-[2-(4-*tert*-Butylphenyl)-5-isopropyl-4-oxazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (15)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 2-(4-*tert*-butylphenyl)-4-chloromethyl-5-isopropyloxazole in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.16 (d, 6H, J = 7 Hz), 1.36 (s, 9H), 1.70 (s, 6H), 2.95 (m, 3H), 3.17 (t, 2H, J = 8 Hz), 6.76 (1H, dd, J = 2 Hz, J = 8 Hz), 7.00 (d, 1H, J = 2 Hz), 7.15 (d, 1H, J = 8 Hz), 7.47 (d, 2H, J = 8 Hz), 7.93 (d, 2H, J = 8 Hz). FAB-MS (m/z): 491 (M+1).

5.1.26. 2-[3-[2-[2-(4-Butylphenyl)-5-isopropyl-4-oxazolyl] ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (16)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 2-(4-butylphenyl)-4-chloromethyl-5-isopropyloxazole in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 0.94 (t, 3H, J = 7 Hz), 1.16 (d, 6H, J = 7 Hz), 1.3–1.5 (m, 2H), 1.6–1.7 (m, 2H), 1.68 (s, 6H), 2.66 (t, 2H), 2.9–3.1 (m, 3H), 3.21 (t, 2H, J = 7 Hz), 6.77 (1H, dd, J = 2 Hz, J = 8 Hz), 7.00 (d, 1H, J = 2 Hz), 7.20 (d, 1H, J = 8 Hz), 7.25 (d, 2H, J = 8 Hz), 7.90 (d, 2H, J = 8 Hz). FAB-MS (m/z): 491 (M+1).

5.1.27. 2-[3-[2-[2-(2,6-Dichlorophenyl)-5-isopropyl-4-oxazolyl] ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (17)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 4-chloromethyl-2-(2,6-dichlorophenyl)-5-isopropyloxazole in the same manner as that described for **5**: MP(dec) 161–163 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.08 (t, 3H, J = 7 Hz), 1.63 (s, 6H), 2.85 (m, 1H), 3.07 (t, 2H, J = 7 Hz), 3.31 (t, 2H, J = 7 Hz), 6.81 (1H, dd, J = 2 Hz, J = 8 Hz), 6.99 (d, 1H, J = 2 Hz), 7.3–7.4 (m, 4H).

IR (KBr) cm^{-1}: 3467, 2976, 2873, 2521, 1722, 1624, 1605, 1560, 1518, 1498, 1468, 1433, 1385, 1383, 1367, 1340, 1321, 1271, 1200, 1176, 1130, 1095, 1041, 974, 933, 883, 839, 793, 771, 750, 600, 569, 482. FAB-MS (m/z): 504 (M+1).

5.1.28. 2-[3-[2-[4-Isopropyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]ethyl]-1,2-benzisoxazol-6-yloxy]-2-methylpropionic acid (18)

The titled compound was introduced from 6-acetamido-3-methyl-1,2-benzisoxazole (**23b**) and 5-chloromethyl-4-isopropyl-2-[4-(trifluoromethyl)phenyl]thiazole (**22**) in the same manner as that described for **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.23 (d, 6H, J = 7 Hz), 1.68 (s, 6H), 3.0–3.1 (m, 1H), 3.1–3.4 (m, 4H), 6.92 (dd, 1H, J = 2 Hz, J = 9 Hz), 7.05 (d, 1H, J = 2 Hz), 7.42 (d, 1H, J = 8 Hz), 7.65 (d, 1H, J = 8 Hz), 8.00 (d, 1H, J = 8 Hz). FAB-MS (m/z): 519 (M+1).

5.2. Pharmacology

5.2.1. Measurement of PPARα, PPARγ, and PPARδ transactivation activity

The PPARα, PPARγ, and PPARδ transactivation activity of each compound was measured in the manner described below.

Plasmid: Receptor expression plasmids (GAL4-hPPARα, LBD GAL4-hPPARγ LBD, GAL4-hPPARδ LBD), a reporter plasmid (UASx4-TK-LUC), and a β-galactosidase expression plasmid (PGAL) similar to that described by Kliewer et al.[21] were used.

Transfection: CV-1 cells were seeded in 24-well culture plates at a density of 2×10^5 cells per well and cultured for 24 h in OPTI-MEM I Reduced Serum Medium (Life Technologies, 500 μL/well) containing 4% fetal bovine serum (FBS). After washing with OPTI-MEM, a transfection mixture (250 μL/well) containing 0.03 μg of GAL4-hPPARδ LBD, 0.25 μg of UASx4-TK-LUC, 0.35 μg of β-GAL, and 2 μL of lipofection reagent, DMRIE-C (Life Technologies) was added. The cells were incubated for 5 h at 37 °C.

Cell treatment by the addition of test compounds: The cells were washed and incubated for 40 h in the presence of each test compound (final concentration was 1×10^{-7} or 1×10^{-6} M).

Measurement of the level of reporter gene expression: The culture medium was removed, and the cells were washed with PBS twice. A solubilizing buffer (100 μL/well) containing 25 mM Tris-PO_4 (pH 7.8), 15% v/v glycerol, 2% CHAPS, 1% lecithin, 1% BSA, 4 mM EGTA (pH 8.0), 8 mM $MgCl_2$, and 1 mM DTT was added. After incubation for 10 min at room temperature, a portion (20 μL) of the solution was transferred into a 96-well plate. Subsequently, 100 μL of luciferase substrate solution (Piccagene: available from Nippon Gene Co., Ltd) was added, and a luminous intensity per 1 s (luciferase activity) was measured using a microluminoreader (Type MLR-100, Corona Electrics Co., Ltd). The luciferase activity of each construct was corrected by the transfection efficiency, which was calculated from the β-galactosidase activity. The assay method of β-galactosidase activity was as follows: A portion (50 μL) of the solubilized sample was transferred into another 96-well plate; 100 μL of 2-nitrophenyl-β-galactopyranoside solution was added and incubated for 5 min at room temperature. Fifty microliters of reaction stopping solution (1 M sodium carbonate solution) was added. Then, the absorbance at 414 nm was measured.

The PPARα, PPARγ, and PPARδ transactivation activity of the test compounds was calculated relative to the luciferase activity induced by 1 μM GW-590735 (PPARα selective agonist), 10 μM rosiglitazone (PPARγ selective agonist), and 0.1 μM GW-501516 (PPARδ selective agonist), respectively.

5.2.2. Oligodendrocyte differentiation-stimulating effects of test compounds

Oligodendrocyte progenitors were isolated from glial cells of the cerebral cortex of fetal Wistar rats (gestational age, 18–19 days). On the day before cell seeding, cover glasses were coated with poly-D-lysine for 1 day. The density of cells was adjusted to 1×10^4 cells/glass and seeded onto the coated cover glasses laid in the dish. Cells were then incubated at 37 °C with 5% CO_2 for 4 days. Drug-containing medium was prepared as follows: drugs were dissolved in DMSO at 1×10^{-1} mol/L, diluted with DMSO to prespecified concentrations, and stored at 4 °C. The stock medium was further diluted 100-fold with medium and added onto 24-well culture plates at 500 μL/well. Plates were covered with cell-seeded cover glasses and incubated at 37 °C with 5% CO_2 for 3 days. The control group was cultured in medium containing 0.01% DMSO only. Afterward, anti-O1 antibody was added onto slide glasses for immunostaining. Cell samples were randomly photographed, and cell-stained area was measured by image processing for each sample. Relative areas to the mean value of the control group (set at 100%) were calculated for each group.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.053.

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