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A lipase inhibitor monoterpene and monoterpene glycosides from *Monarda punctata*

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ABSTRACT

An 80% acetone extract of *Monarda punctata* showed an inhibitory effect on lipase activity in isolated mouse plasma in *vitro* and carvacrol was obtained as the active constituent. It had an IC_{50} value of 4.07 mM *in vitro* and suppressed elevations in blood triacylglycerol levels in olive oil-loaded mice. Furthermore, from the whole plant, 22 compounds were isolated. Six monoterpene glycosides (**3–8**), a flavone glucuronide (**9**), and other known compounds were identified based on the results of spectroscopic analyses.

1. Introduction

Monarda punctata L. (Lamiaceae) is a traditional herbal medicine of North American Indians used as a remedy for colds and a treatment for nausea, vomiting, and rheumatic pains (Chevallier and Nanba, 2000). Previous studies on the ingredients of *M. punctata* have identified essential oils using gas chromatography (Scora, 1965).

Lipase is an enzyme that hydrolyzes triacylglycerols (TGs). The digestion and absorption of natural lipids begins with hydrolysis by pancreatic lipase. The activity of this enzyme greatly affects the metabolism of fat and the concentration of TG in blood (Mu and Porsgaard, 2005). Recently, inhibitors of lipase and lipid absorption have been isolated from natural sources with the aim of preventing and treating metabolic syndrome. These inhibitors include terpenoids (Bitou et al., 1999; Ninomiya et al., 2004; Jang et al., 2008; Kim et al., 2009), triterpene glycosides (Zheng et al., 2004; Xu et al., 2005; Yoshizumi et al., 2006; Morikawa et al., 2009; Sugimoto et al., 2009), flavonoids (Kawaguchi et al., 1997; Hatano et al., 1997; Won et al., 2007), catechins (Yoshikawa et al., 2002; Nakai et al., 2005), stilbenes (Matsuda et al., 2009), chitosan (Ostanina et al., 2007), and phenyl compounds (Raghavendra and Prakash, 2002; Han et al., 2007). Most of these inhibitory activities were examined using porcine pancreatic lipase. On the other hand, we have reported inhibitory effects of natural products on mouse lipase *in vitro* and *in vivo*, namely, rhodionin and rhodiosin in *Rhodiola rosea* (Kobayashi et al., 2008a) and myricitrin and myricetin in *Myrica rubra* (Kobayashi et al., 2008b).

In the present study, a major component of essential oil, carvacrol (**1**), was obtained as a mouse lipase inhibitor, which depressed the elevation of blood TG levels in olive oil-loaded mice, from *M. punctata*. Additionally, six new monoterpene glycosides, monardins A–F (**3–8**) and a new flavone glucuronide, keshonin (**9**), were isolated from the plant together with 14 known compounds (**2**, **10–22**). We describe the structural elucidation of novel compounds and inhibitory effects of several constituents on lipase in isolated mouse plasma.

2. Results and discussion

An 80% acetone extract of *M. punctata* had an inhibitory effect on lipase activity in isolated mouse plasma in *vitro* (Table 1; IC_{50}, 2.0 mg/mL). The extract was dissolved in water and extracted with diethyl ether. An essential oil fraction was obtained from the diethyl ether layer and its extract was found to exhibit an inhibitory effect (Table 1; IC_{50}, 0.18 mg/mL). A major component of the essential oil fraction was identified as carvacrol (**1**) (Han et al., 2005). **1** showed a concentration-dependent inhibition and its IC_{50} value was 4.07 mM (Table 1). This suggested that **1** has the potential to affect lipase activity in the mouse alimentary canal. Then, we evaluated the suppressive effect of **1** on the increase in blood TG levels in olive oil-loaded mice. The TG levels of control mice (olive oil alone, 5 mL/kg, *p.o.*) gradually increased, but **1** (300 mg/kg, *p.o.*) significantly suppressed the increase at 90, 120, and 180 min as shown in Fig. 2. Orlistat (positive control, 10 mg/kg, *p.o.*) likewise depressed the increase in the blood TG level. As the diethyl ether

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Table 1
Inhibitory effects of extracts on lipase in isolated plasma of mice.

Extract	Lipase inhibition (IC_{50})
80% Acetone	2.0 mg/mL
Water	5.6 mg/mL
Ether	0.18 mg/mL
Carvacrol	4.07 mM
Orlistat	0.09 mM

extract exhibited stronger activity, presence of other minor constituents having strong inhibitory activities in the diethyl ether layer are suggested.

The water layer, its extract having showed weak inhibition (Table 1; IC_{50}, 5.6 mg/mL), was passed through a column of HP-20, a porous polymer gel. The 80% methanol eluate was separated by preparative Yamazen cartridge column chromatography (YCCC) and HPLC to afford nine monoterpene glycosides (**2–8**), a flavone glucuronide (**9**), and other known compounds (**10–22**). The compounds **2** and **10–22** were identified based on spectroscopic data as 2-methyl-5-(1-methylethyl)phenyl-β-D-glucopyranoside (**2**) (Shimoda et al., 2006), apigenin 7-*O*-β-D-glucoside (**10**) (Oyama and Kondo, 2004), apigenin 7-*O*-(6-*O*-malonyl-β-D-glucoside) (**11**) (Svehlikova et al., 2004), apigenin 7-*O*-β-D-rutinoside (**12**) (Wang et al., 2003), apiin (**13**) (Yoshikawa et al., 2000), 6″-*O*-malonylapiin (**14**) (Yoshikawa et al., 2000), apigenin 7-*O*-β-D-glucuronide (**15**) (Flamini et al., 2001), luteolin 7-*O*-β-D-glucuronide (**16**) (Vanhoenacker et al., 2002), chrysoriol 7-*O*-β-D-glucuronide (**17**) (Stochmal et al., 2001), scutellarin B (**18**) (Kawasaki et al., 1988), 4-allyl-1-*O*-β-D-glucopyranosyl-2-hydroxybenzene (**19**) (Norr and Wagner, 1992), citrusin C (**20**) (Teng et al., 2005), rosmarinic acid (**21**) (Jayasinghe et al., 2003), and eritrichin (**22**) (Fedoreyev et al., 2005).

The novel monoterpene glycosides (**3–8**) were isolated as amorphous powders with the ^{1}H- and ^{13}C-NMR spectroscopic data (measured in methanol-d_4 at 30 °C) shown in Table 2.

Monardin A (**3**) was deduced to have the molecular formula $C_{19}H_{26}O_9$ based on HRFABMS (*m/z* 399.1649, calcd for $C_{19}H_{27}O_9$, 399.1655). Its ^{1}H- and ^{13}C-NMR spectra were similar to those of **2** except for the presence of a 6-acylated glucopyranose moiety in **3**. The ^{1}H-NMR spectrum showed ABX-type aromatic protons at δ 7.02 (1H, d, J = 9.0 Hz), 6.80 (1H, dd, J = 9.0, 2.0 Hz) and 6.96 (1H, br s). Two doublet methyl [δ 1.22 (6H, d, J = 7.0 Hz), H-9, 10] and methine [δ 2.84 (1H, m), H-8] protons showed the presence of a $(CH_3)_2$–CH– spin system. These signals and a singlet methyl proton [δ 2.22 (3H, s)] suggested the presence of a carvacrol moiety as an aglycone. The anomeric proton at δ 4.86 (1H, overlapped, H-1′), ^{1}H–^{1}H COSY and ^{13}C-NMR spectra showed the presence of a 6-acylated glucopyranosyl moiety. The sugar analysis showed that it was D-glucose (Tanaka et al., 2007). The anomeric configuration of the D-glucosyl moiety was determined to be β from the chemical shifts in the ^{13}C-NMR spectrum (Kasai et al., 1979). The H-6′ resonances at δ 4.25 (1H, dd, J = 12.0, 6.0 Hz) and 4.54 (1H, dd, J = 12.0, 2.0 Hz) were shifted downfield relative to those of **2**. The two carbonyl carbons at δ 168.6 and 170.0, the methylene carbon at δ 41.9, and the corresponding methylene proton at δ 3.35 (s) suggested the presence of a malonyl moiety. In the HMBC spectrum, the H-6′ signals were long-range coupled with δ 168.6 (C-7′), and the H-1′ signal was long-range coupled with δ 157.0 (C-2). Hence, the structure of **3** was determined as shown in Fig. 1.

Monardin B (**4**) was deduced to have the molecular formula $C_{28}H_{38}O_{17}$ by HRFABMS (*m/z* 647.2166, calcd for $C_{28}H_{39}O_{17}$, 647.2187). Its ^{1}H- and ^{13}C-NMR spectra were similar to those of **3** except for the presence of another 6-acylated glucopyranose moiety in **3**. The anomeric protons at δ 5.07 (1H, overlapped, H-1′) and 4.72 (1H, d, J = 7.5 Hz, H-1″), and the ^{1}H–^{1}H COSY, ^{13}C-NMR and HMBC spectra showed the presence of two 6-malonated β-D-glucopyranose moieties. The missing malonyl methylene proton and carbon signals in CD_3OD were confirmed by ^{1}H- and ^{13}C-NMR spectra in DMSO-d_6 at δ_H 3.19, δ_H 3.22, δ_C 41.7, and δ_C 42.2. The H-2′ resonance at δ 3.76 (1H, dd, J = 9.0, 7.5 Hz) and the C-2′ (δ 83.2) carbon signals were shifted downfield relative to the resonances of **3**. In the HMBC spectrum, the H-1″ signal was long-range coupled with C-2′. Hence, the structure of **3** was formulated as shown in Fig. 1.

Monardin C (**5**) had the molecular formula $C_{16}H_{24}O_7$ according to HRFABMS (*m/z* 351.1412, calcd for $C_{16}H_{24}O_7Na$, 351.1419). Its ^{1}H- and ^{13}C-NMR spectra were similar to those of **2** except for the presence of a methylene group in **5** instead of a methyl group in **2**. The ^{1}H-NMR spectrum showed oxymethylene proton signals at δ 4.53 (1H, d, J = 12.5 Hz, H-7) and δ 4.81 (1H, d, J = 12.5 Hz, H-7). These signals were long-range coupled with C-1, 2 and 6 in the HMBC spectrum. They indicated that **5** possesses a $-CH_2OH$ moiety instead of the methyl group of **2**. Hence, the structure of **5** was formulated as depicted in Fig. 1.

The molecular formula $C_{19}H_{26}O_{10}$ for monardin D (**6**) was deduced by HRFABMS (*m/z* 437.1431, calcd for $C_{19}H_{26}O_{10}Na$, 437.1431). In the ^{1}H- and ^{13}C-NMR spectra of **6**, the H-6′ resonance at δ 4.26 (1H, dd, J = 12.0, 6.5 Hz) and 4.56 (1H, dd, J = 12.0, 2.0 Hz) were shifted downfield relative to those of **5**, and the two carbonyl carbons at δ 168.6 and 170.0 suggested the presence of a malonyl moiety. In the HMBC spectrum, the H-6′ signals were long-range coupled with δ 168.6 (C-7′). Hence, **6** was the malonate derivative of **5**.

The ^{1}H- and ^{13}C-NMR spectra (Table 2) suggested that aglycone moieties of monardin E (**7**) and monardin F (**8**) were *p*-menth-3-ene-1,2-diol, similar to those of (1*R*,2*R*)-*p*-menth-3-en-1,2-diol 2-*O*-β-D-glucopyranoside (Kitajima et al., 2004). The molecular formulas $C_{16}H_{28}O_7$ for **7** and $C_{19}H_{30}O_{10}$ for **8** were determined (see Section 4). For **7**, the anomeric proton at δ 4.71 (1H, d, J = 7.5 Hz, H-1′) and the coupling constants of the sugar protons in the ^{1}H-NMR spectrum, ^{13}C-NMR spectrum and sugar analysis suggested the presence of an β-D-allopyranosyl unit. In the HMBC spectrum, the H-1′ signal was long-range coupled with δ 86.6 (C-2) and δ 4.08 (1H, overlapped, H-2) was long-range coupled with δ 102.3 (C-1′). The relative configuration was determined from the ^{1}H-NMR and NOE spectra recorded in pyridine-d_5 (Fig. 3 and Section 4), and ^{13}C-NMR spectra recorded in $CDCl_3$ (Table 3). In the NOE spectrum, a methyl proton at δ 1.48 (3H, s, H-7) was correlated with a C-5ax proton at δ 2.00 (1H, overlapped), a C-6 eq proton at δ 1.85–1.98 (overlapped), and other methyl protons at δ 0.87 (3H, d, J = 6.6 Hz, H-10) and 0.88 (3H, d, J = 6.6 Hz, H-9), which showed the methyl was axial. An oxygenated methine proton at δ 4.47 (br s, H-2) was correlated with a C-6ax proton at δ 1.85–1.94 (overlapped) in the NOE spectrum. The decoupling ^{1}H-NMR spectra on irradiation around δ 2.00–2.07 showed a coupling constant between H-2 and H-3 of J = 1.5 Hz, which suggested a *trans*-diol conformation (Suga et al., 1968; Kitajima et al., 2004). Moreover, ^{1}H- and ^{13}C-NMR ($CDCl_3$, Table 3) signals of the aglycone moiety were partly superimposable with those of a (1*R*,2*R*)-3-*p*-menth-3-ene-1,2-diol (Abraham et al., 1986; Jefford et al., 1989). Hence, **7** was *rel*-(1*R*,2*R*)-*p*-menth-3-ene-1,2-diol 1-*O*-β-D-allopyranoside. Although the absolute configuration of **7** was not determined, it was deduced by application of the ^{13}C-NMR glycosidation shifts rule of a secondary alcohol (Seo et al., 1978). The paper indicated that the method can be extended to all glycopyranosides including β-D-allopyranoside having an equatorial hydroxyl group at C-2′ of a sugar moiety. The glycosidation shift for the anomeric carbon (C-1′) [$\Delta\delta = \delta$ (alcoholic glycoside) $- \delta$ (methylglycoside)] was $\Delta\delta$ −0.7 ppm (CD_3OD, Li et al., 2003), and the glycosidation shifts for the aglycone moiety [$\Delta\delta = \delta$ (alcoholic glycoside) $- \delta$ (alcohol)] were shown in Fig. 3 (pyridine-d_5) and Table 3

Table 2
NMR spectroscopic data (CD_3OD) for compounds **3**–**8**.

Position	**3**			**4**			**5**			**6**			**7**			**8**		
	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	δ_H (*J* in Hz)	δ_C	HMBC (H–C)
1		126.6			126.0			129.7			129.8			72.3			72.4	
2		157.0			156.7			157.4			157.2		4.08, Overlapped	86.6	1, 3, 4, 1′	4.04, m	87.7	3, 1′
3	6.96, br s	115.2	2, 4, 5, 8	6.92, *d* (1.5)	114.0	2, 4, 5, 8	7.11, *d* (1.0)	115.7	2, 4, 5, 8	7.06, *d* (2.0)	115.8	2, 5, 8	5.40, br s	121.4	1, 5, 8	5.39, br s	121.4	1, 5, 8
4		149.0			149.2			151.7			151.6			146.5			146.8	
5	6.80, *dd* (9.0, 2.0)	121.4	1, 3, 6, 8	6.79, *dd* (7.5, 1.5)	121.1	1, 3, 6, 8	6.91, *dd* (7.5, 1.0)	121.8	1, 3, 6, 8	6.93, *dd* (8.0, 2.0)	121.8	3, 6, 8	2.07, m	25.9	1, 4, 6, 8	2.07, m	25.9	6
6	7.02, *d* (9.0)	131.5	1, 4, 5, 7	7.03, *d* (7.5)	131.6	2, 4, 5, 7	7.24, *d* (7.5)	130.1	2, 4, 5, 7	7.23, *d* (8.0)	130.0	2, 4, 5, 7	1.68, m	35.4	1, 4, 5, 7	1.69, m	35.5	4, 5, 7
7	2.22, s	16.1	1, 2, 6	2.22, s	16.4	1, 2, 6	4.53, *d* (12.5)	60.9	1, 2, 6	4.52, *d* (12.5)	61.0	1, 2, 6	1.14, s	21.0	1, 2, 6	1.10, s	21.1	1, 2, 6
							4.81, *d* (12.5)		1, 2, 6	4.73, *d* (12.5)		1, 2, 6						
8	2.84, m	35.1	3, 4, 5, 9, 10	2.84, m	35.3	3, 4, 5, 9, 10	2.88, m	35.4	3, 4, 5, 9, 10	2.89, m	35.3	3, 4, 5, 9, 10	2.22, m	35.5	3, 4, 5, 9, 10	2.22, m	35.5	3, 5, 9, 10
9	1.22, *d* (7.0)	24.5	4, 8, 10	1.22, *d* (6.5)	24.6	4, 8, 10	1.24, *d* (6.5)	24.4	4, 8, 10	1.24, *d* (7.0)	24.5	4, 8, 10	1.02, *d* (6.5)	21.7	4, 8, 10	1.02, *d* (7.0)	21.7	4, 8, 10
10	1.22, *d* (7.0)	24.5	4, 8, 9	1.22, *d* (6.5)	24.7	4, 8, 9	1.24, *d* (6.5)	24.4	4, 8, 9	1.24, *d* (7.0)	24.5	4, 8, 9	1.02, *d* (6.5)	21.8	4, 8, 9	1.02, *d* (7.0)	21.7	4, 8, 9
1′	4.86, Overlapped	103.1	2, 2″	5.07, *d* (7.5)	100.7	2	4.71, *d* (7.5)	103.7	2	4.84, *d* (7.5)	103.7	2, 3′	4.71, *d* (7.5)	102.3	2	4.73, *d* (8.0)	102.4	2
2′	3.49, Overlapped	74.9		3.76, *dd* (7.5, 9.0)	83.2		3.49, Overlapped	75.2		3.51, *dd* (7.5, 9.0)	75.0		3.37, *dd* (7.5, 3.0)	72.2		3.39, *dd* (8.0, 3.5)	72.2	
3′	3.49, Overlapped	77.9		3.66–3.73, Overlapped	77.9		3.40–3.50, Overlapped	78.1		3.47, *dd* (9.0, 9.0)	77.7	4′, 5′	4.06, *dd* (3.0, 3.0)	73.1		4.07, m	73.1	
4′	3.38, Overlapped	71.5		3.66–3.73, Overlapped	71.3	6′	3.40–3.50, Overlapped	69.2		3.38, *dd* (9.0, 9.0)	71.5	3′, 6′	3.47, *dd* (9.5, 3.0)	69.2		3.50, *dd* (9.5, 3.0)	69.2	
5′	3.63, m	75.3		3.44, *dd* (9.0, 9.0)	75.2	6′	3.40–3.50, Overlapped	78.4		3.65, m	75.5	6′	3.75, m	75.6		3.99, m	75.7	
6′	4.25, *dd* (12.0, 6.0)	65.6	7′	4.24, *dd* (11.5, 6.5)	65.6	7′	3.74, *dd* (12.0, 5.0)	62.7		4.26, *dd* (12.0, 6.5)	65.6	7′	3.62, *dd* (11.5, 6.5)	63.2		4.17, *dd* (12.0, 7.5)	66.3	7′
	4.54, *dd* (12.0, 2.0)			4.54, *dd* (11.5, 2.0)		7′	3.90, *dd* (12.0, 2.0)			4.56, *dd* (12.0, 2.0)		7′	3.87, *dd* (11.5, 2.0)			4.56, *dd* (12.0, 2.5)		7′
7′		168.6			168.6						168.6						168.3	
8′	3.35 (s)	41.9	7′, 9′	Missing	Missing					3.38, Overlapped	41.9					Missing	Missing	
9′		170.0			170.1						170.0						170.2	
9′-OMe																		
1″				4.72, *d* (7.5)	105.3	2′												
2″				3.27, Overlapped	76.1													
3″				3.35–3.41, Overlapped	77.7													
4″				3.35–3.41, Overlapped	71.2	6″												
5″				3.35–3.41, Overlapped	75.5	6″												
6″				4.14, Overlapped	65.3	7″												
7″					168.6													
8″				Missing	Missing													
9″					170.1													

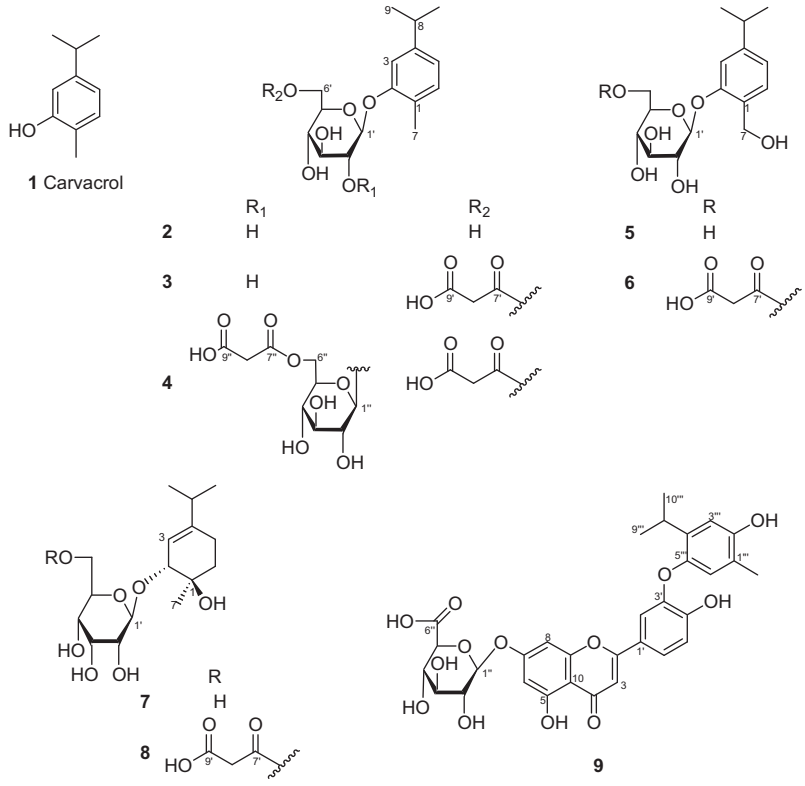

Fig. 1. Structures of compounds **1**–**9**.

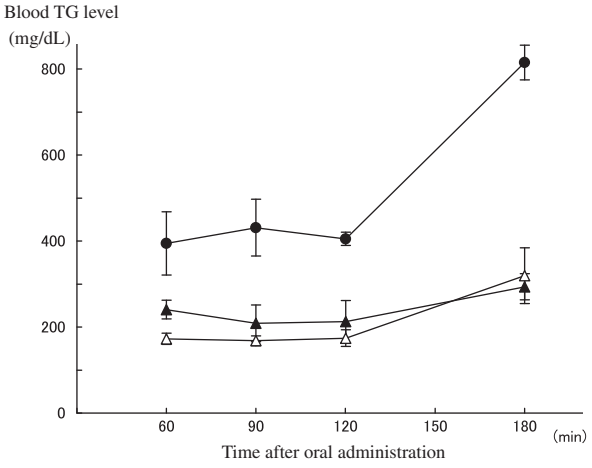

Fig. 2. Variation in blood triacylglycerols levels in olive oil-loaded mouse plasma after the oral administration of carvacrol. ●, olive oil alone (5 mL/kg, *p.o.*); ▲, orlistat (10 mg/kg, *p.o.*) and olive oil (5 mL/kg, *p.o.*); △, carvacrol (300 mg/kg, *p.o.*) and olive oil (5 mL/kg, *p.o.*). All points represent the mean for four mice per group. Vertical lines show the standard error of the mean. Significantly different from the control, $^{*}p < 0.05$ and $^{**}p < 0.01$.

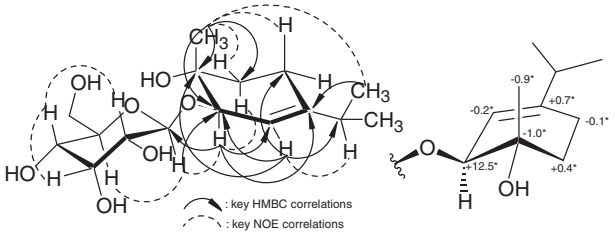

Fig. 3. Key HMBC and NOE correlations for the relative configuration of **7** in pyridine-d_5 and "Sterically Hindered Case II" for **7** in glycosidation shifts rules[*] The glycosidation shifts in pyridine-d_5 ($\Delta\delta$ in ppm).

Table 3
^{13}C-NMR Spectroscopic Data ($CDCl_3$) for *p*-Menth-3-ene-1,2-diols, **7**, and **7a**.

Position	*cys*[a,b] δ_C	*trans*[b,c] δ_C	**7** (**7**-*trans*[b,c]) δ_C	**7a**[d] (**7a**-*trans*[b,c]) δ_C
1	70.1	72.7	71.7 (−1.0)	71.0 (−1.7)
2	71.8	74.8	87.0 (12.2)	89.0 (14.2)
3	119.7	121.0	119.8 (−1.2)	119.7 (−1.3)
4	147.8	145.0	146.2 (1.2)	146.2 (1.2)
5	24.4	25.0	24.9 (−0.1)	24.9 (−0.1)
6	32.4	34.0	34.1 (0.1)	33.9 (−0.1)
7	24.4	21.3	20.5 (−0.8)	20.5 (−0.8)
8	34.5	34.0	34.2 (0.2)	34.1 (0.1)
9	21.4	21.2	21.5 (0.3)	21.4 (0.2)
10	21.3	20.1	21.2 (1.1)	21.1 (1.0)

[a] (1*R*,2*S*)-3-*p*-menth-3-ene-1,2-diol.
[b] Reported value (Abraham et al., 1986).
[c] (1*R*,2*R*)-3-*p*-menth-3-ene-1,2-diol.
[d] Tetra-*O*-acetylated **7**.

(**7a**: tetra-*O*-acetylglycoside, $CDCl_3$). The NMR data of (1*R*,2*R*)-3-*p*-menth-3-ene-1,2-diol recorded in pyridine-d_5 and in $CDCl_3$ were cited from the literatures (Kitajima et al., 2004; Abraham et al., 1986), respectively. Shift values [$\Delta\delta$ −1.0 ppm (C-1), $\Delta\delta$ + 12.5 ppm (C-2), and $\Delta\delta$ −0.2 ppm (C-3) in pyridine-d_5 and $\Delta\delta$ −1.7 ppm (C-1), $\Delta\delta$ + 14.2 ppm (C-2), and $\Delta\delta$ −1.3 ppm (C-3) in $CDCl_3$ (**7a**)] suggested that "Sterically Hindered Case II" discussed in the paper (Seo et al., 1978) was applicable to **7** and **7a**, which shows that C-2 is *S* configuration. However, the $\Delta\delta$ (C-3) values were larger than those for C-1, and parameters of shifts rule for this allyl alcohol type glycoside had not been established except for iridoid glycosides (Tagawa and Murai, 1997). Therefore, it was not possible to confirm the absolute stereochemistry of **7** from only the shifts rule. The

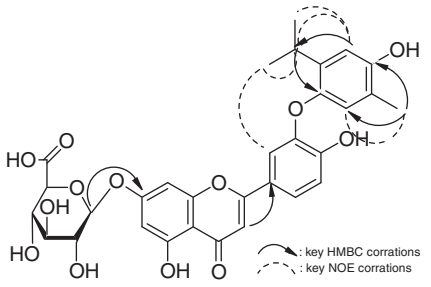

Fig. 4. Key HMBC and NOE correlations for **9** in DMSO-d_6.

^{1}H- and ^{13}C-NMR spectra of **8** were similar to those of **7** except for the presence of a 6-acylated allopyranose moiety in **8** instead of an allopyranose in **7**. The H-6′ resonance at δ 4.17 (1H, dd, J = 12.0, 7.5 Hz) and 4.56 (1H, dd, J = 12.0, 2.5 Hz) were shifted downfield relative to those of **7**. The two carbonyl carbons at δ 168.3 and 170.2 suggested the presence of a malonyl moiety. The missing malonyl methylene proton and carbon signals in CD_3OD were confirmed by ^{1}H- and ^{13}C-NMR spectra in DMSO-d_6 at δ_H 3.51 and δ_C 40.7. In the HMBC spectrum, the H-6′ signals were long-range coupled with δ 168.3 (C-7′). Hence, **8** was a malonyl derivative of **7**.

Keshonin (**9**) was an amorphous powder with ^{1}H- and ^{13}C-NMR spectroscopic data (measured in DMSO-d_6, 30 °C) assigned as shown in the Section 4. On the basis of HRFABMS, the molecular formula $C_{31}H_{30}O_{13}$ was determined (*m/z* 611.1757, calcd for $C_{31}H_{31}O_{13}$, 611.1764). The ^{1}H- and ^{13}C-NMR spectra of **9** showed a flavone glycoside moiety and a monoterpenoid moiety. The aromatic carbons δ 94.4 (C-8), 99.4 (C-6), 103.5 (C-3), 105.3 (C-10), 115.9 (C-2′), 117.1 (C-5′), 121.4 (C-1′), 122.5 (C-6′), 146.2 (C-3′), 151.9 (C-4′), 156.7 (C-9), 161.1 (C-4), 162.4 (C-7), and 163.7 (C-2) and the carbonyl carbon (δ 181.7) of a flavone moiety were partly superimposable with those of a 7,3′-substituted luteoline skeleton (Lu and Foo, 2000). The anomeric proton at δ 5.14 (1H, d, J = 6.5 Hz, H-1″), and ^{1}H–^{1}H COSY and ^{13}C-NMR spectra showed the presence of glucuronic acid unit. The sugar analysis (Tanaka et al., 2007) and the coupling constant of the anomeric proton indicated a β-D-glucuronic acid. In the HMBC spectrum (Fig. 4), the H-1″ signal was long-range coupled with δ 162.4 (C-7). Accordingly, the flavoneglycoside unit was 3′-*O*-substituted luteoline 7-β-D-glucuronic acid. The ^{1}H- and ^{13}C-NMR spectra of **9** suggested the presence of a 5‴-*O*-substituted thymoquinol moiety as compared to zataroside-A or B (Ali et al., 1999). In the NOE spectrum (Fig. 4), the H-9‴ and -10‴ methyl signals (δ 1.16, 3H, d, J = 7.0 Hz; δ 1.18, 3H, d, J = 7.0 Hz) were correlated with the H-2′ signal (δ 7.24, 1H, d, J = 2.0 Hz). Hence, the structure of **9** was formulated as shown in Fig. 1.

The mouse lipase inhibitory activities of monoterpene glycosides (**2**–**4**, **6** and **7**) were evaluated but none of the compounds showed significant activity. However, it is expected that their hydrolysis influences lipase activity in the alimentary canal of mice after conversion to aglycons by enterobacilli.

3. Conclusion

From whole plant of *M. punctata*, a lipase inhibitor: carvacrol (**1**), six new monoterpene glycosides (**3**–**9**) and a new flavone glycoside (**9**) were isolated. **1** had an IC_{50} value of 4.07 mM for lipase in isolated mouse plasma *in vitro* and suppressed the postprandial elevation of blood TG concentrations in mice *in vivo*. Compounds **2**–**4** were glucosides of carvacrol, whereas **7** and **8** were monoterpene allopyranosides. The sugar moieties of **3**, **4**, **6**, and **8** were malonated, and **9** was a complex of the flavone glucuronide and the monoterpene moiety.

4. Experimental

4.1. General

Optical rotations were measured on a Jasco P-2300 polarimeter. UV spectra were recorded on a Shimadzu MPS-2450. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Jeol JNM-AL400 FT-NMR spectrometer, and chemical shifts were given as δ values with TMS as an internal standard at 30 °C. Inverse-detected heteronuclear correlations were measured using HMQC (optimized for $^1J_{C-H}$ = 145 Hz) and HMBC (optimized for $^nJ_{C-H}$ = 8 Hz) pulse sequences with a pulsed field gradient. HRFABMS data were obtained on a Jeol JMS700 mass spectrometer, using a *m*-nitrobenzyl alcohol or a glycerol matrix. YCCC and HPLC were performed on a Jasco 2089 with UV at 210 nm, using the following columns: Ultra Pack ODS-SM-50C-M [Yamazen, 37 × 100 mm; mobile phase, MeOH–0.2%TFA in H_2O (35:65) → (65:35)], ODS-100V [Tosoh, 20 × 250 mm; mobile phase, CH_3CN–0.2%TFA in H_2O (20:80) → (35:65)], Cosmosil 5C_{18}–AR II column [Nacalai Tesque, 20 × 250 mm; mobile phase, CH_3CN–0.2%TFA in H_2O (25:75)], Capcell-Pak Ph [Shiseido, 20 × 250 mm; solvent, CH_3CN–H_2O (20:80) or (25:75)] and Mightysil RP-18 GP [Kanto Chemical, 10 × 250 mm; solvent, acetonitrile–water (25:75)]. Olive oil, 5,5′-dithiobis(2-nitrobenzoic acid), and phenylmethylsulfonyl fluoride were purchased from Nacalai Tesque Inc. (Kyoto, Japan), while 2,3-dimer-capto-1-propanol tributyrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Orlistat was obtained from Roshe Products Ltd. (Auckland, NZ). Carvacrol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

4.2. Plant material

M. punctata L. was collected in July 2008 in Sendai City, Japan. The young plants were purchased from and identified by Tamagawa engei, Nagano, Japan. A voucher specimen is deposited at the herbarium of Tohoku Pharmaceutical University, No. 20080716.

4.3. Extraction and isolation

Powdered whole plants (640 g) of *M. punctata* were extracted with acetone–H_2O (80:20) (7 L) twice at 50 °C for 3 h. The extract (75.6 g) was suspended in H_2O (1.5 L), and extracted with Et_2O (3 × 1 L). The ether extract (17.5 g) was suspended in EtOH–H_2O (8:2) (1.5 L), and extracted with hexane (3 × 1 L). The hexane layer extract (7.0 g) was passed through a silica gel column (Wakogel C-200, Wako Pure Chemical Industries Ltd., Osaka, Japan, 30 g) yielding 14 fractions, one of which, eluted with $CHCl_3$-MeOH (99:1) was an essential oil fraction whose major component was **1** (3.4 g). The H_2O layer extract (50.5 g) was a red-brown syrup. It was dissolved again in H_2O, and the aqueous solution was passed through a porous polymer gel column (Mitsubishi Diaion HP-20, Mitsubishi Chemical Corporation, Tokyo, Japan, 60 × 300 mm) and eluted with H_2O, MeOH–H_2O (80:20) and MeOH. The MeOH–H_2O (80:20) eluate (10.2 g) was subjected to on a reversed-phase CC using ODS (Cosmosil 140C_{18}-OPN, Nacalai Tesque, Osaka, Japan, 150 g) and eluted with 20%, 30%, 40%, 50%, 60%, 80% MeOH in H_2O, and MeOH (fractions 1A–1G). Fraction 1B (2.19 g) was subjected to YCCC and HPLC, yielding compounds **5** (6.8 mg), **6** (10.0 mg), **7** (15.8 mg), **12** (1.0 mg), **13** (297.1 mg), **16** (22.1 mg), **18** (31.0 mg), **19** (1.0 mg), **20** (2.0 mg) and **21** (12.4 mg). Fraction 1C (1.54 g) was subjected to YCCC and HPLC, yielding compounds **2** (10.5 mg), **4** (4.9 mg), **8** (4.3 mg), **10** (29.4 mg), **11** (19.9 mg), **14** (33.3 mg), **15** (38.3 mg), **17** (4.6 mg) and **22** (18.9 mg). Fraction 1D (750 mg) was subjected to YCCC and HPLC, yielding compounds **3** (13.4 mg) and **9** (2.8 mg).

4.4. Compound **1**

Pale yellow oil; EI-MS *m/z* 150; ^{1}H-NMR ($CDCl_3$) δ 6.71 (1H, d, J = 7.5 Hz), 6.70 (1H, dd, J = 7.5, 1.0 Hz), 6.62 (1H, br s), 5.40 (1H, br s), 2.77 (1H, sep, J = 7.0 Hz) 2.19 (3H, s), 1.18 (6H, d, J = 7.0 Hz); ^{13}C-NMR ($CDCl_3$) δ 153.5, 148.3, 130.8, 121.1, 118.7, 113.1, 33.6, 23.9, 15.3.

4.5. Compound **3**

Colorless amorphous powder; $[\alpha]_D^{22}$ −16.1 (*c* 0.41, MeOH); HRFABMS *m/z* 399.1649 $[M+H]^+$ (calcd for $C_{19}H_{27}O_9$, 399.1655); for ^{1}H-NMR and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 2.

4.6. Compound **4**

Colorless amorphous powder; $[\alpha]_D^{22}$ −18.5 (*c* 0.43, MeOH); HRFABMS *m/z* 647.2166 $[M+H]^+$ (calcd for $C_{28}H_{39}O_{17}$, 647.2187); for ^{1}H-NMR and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 2. ^{1}H-NMR signals of methylene (DMSO-d_6) δ 3.19, 3.22; ^{13}C-NMR signals of methylene (DMSO-d_6) δ 41.7, 42.2.

4.7. Compound **5**

Colorless amorphous powder; $[\alpha]_D^{25}$ −4.9 (*c* 0.90, MeOH); HRFABMS *m/z* 351.1412 $[M+Na]^+$ (calcd for $C_{16}H_{24}O_7Na$, 351.1419); for ^{1}H-NMR and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 2.

4.8. Compound **6**

Colorless amorphous powder; $[\alpha]_D^{25}$ −6.7 (*c* 0.95, MeOH); HRFABMS *m/z* 437.1431 $[M+Na]^+$ (calcd for $C_{19}H_{26}O_{10}Na$, 437.1423); for ^{1}H-NMR and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 2.

4.9. Compound **7**

Colorless amorphous powder; $[\alpha]_D^{25}$ −26.0 (*c* 0.12, MeOH); HRFABMS *m/z* 333.1907 $[M+H]^+$ (calcd for $C_{16}H_{29}O_7$, 333.1914); for ^{1}H-NMR and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 2. ^{1}H-NMR (pyridine-d_5) δ 4.47 (1H, br s, H-2), 5.57 (1H, br s, H-3), 2.00 (overlapped, H-5ax), 2.05 (1H, m, H-5 eq), 1.85–1.94 (overlapped, H-6ax), 1.85–1.98 (overlapped, H-6 eq), 1.48 (3H, s, H-7), 2.00 (overlapped, H-8), 0.88 (3H, d, J = 6.6 Hz, H-9), 0.87 (3H, d, J = 6.6 Hz, H-10), 5.05 (1H, d, J = 8.4 Hz, H-1′), 4.07 (1H, dd, J = 8.4, 3.0 Hz, H-2′), 4.76 (1H, dd, J = 3.0, 3.0 Hz, H-3′), 4.16 (1H, dd, J = 9.6, 3.0 Hz, H-4′), 4.58 (1H, m, H-5′), 4.30 (1H, dd, J = 11.4, 7.2 Hz, H-6′), 4.61 (1H, dd, J = 11.4, 2.4 Hz, H-6′); ^{13}C-NMR (pyridine-d_5) δ 70.8 (C-1), 87.3 (C-2), 121.7 (C-3), 145.0 (C-4), 25.4 (C-5), 35.3 (C-6), 21.8 (C-7), 34.3 (C-8), 21.2 (C-9), 21.5 (C-10), 103.3 (C-1′), 72.2 (C-2′), 73.1 (C-3′), 69.6 (C-4′), 76.3 (C-5′), 63.1 (C-6′). ^{1}H-NMR ($CDCl_3$) δ 5.25 (1H, s), 4.73 (1H, br d, J = 6.5 Hz), 4.24 (1H, br s), 4.11 (1H, s), 3.45–4.00 (overlapped), 2.22 (1H, m), 2.04 (2H, br s), 1.71 (2H, m), 1.13 (3H, s), 1.00 (6H, d, J = 6.5 Hz); ^{13}C-NMR ($CDCl_3$), δ 101.3, 74.3, 71.0, 70.8, 68.1, 62.5, and Table 3.

4.10. Compound ***7a***

^{1}H-NMR ($CDCl_3$) δ 5.68 (1H, br s), 5.10 (1H, s), 4.92 (3H, overlapped), 4.30 (1H, br d, J = 12.0), 4.18 (1H, dd, J = 9.5, 7.5), 4.05–4.10 (2H, overlapped), 2.21 (1H, m), 2.15 (3H, s), 2.11 (3H, s), 2.06 (3H, s), 2.06 (2H, overlapped), 2.01 (3H, s), 1.73 (1H, m), 1.68 (1H, m), 1.10 (3H, s), 1.00 (3H, d, J = 7.0 Hz), 1.00 (3H, d, J = 7.0 Hz); ^{13}C-NMR ($CDCl_3$), δ 170.7, 169.7, 169.1, 169.0, 100.2, 70.4, 69.2, 68.6, 66.7, 62.7, 20.7, 20.7, 20.7, 20.6, and Table 3.

4.11. Compound **8**

Colorless amorphous powder; $[\alpha]_D^{22}$ −21.7 (*c* 0.23, MeOH); HRFABMS *m/z* 441.1726 $[M+Na]^+$ (calcd for $C_{19}H_{30}O_{10}Na$, 441.1736); for ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see Table 2. ^{1}H-NMR signal of methylene (DMSO-d_6) δ 3.51; ^{13}C-NMR signal of methylene (DMSO-d_6) δ 40.7.

4.12. Compound **9**

Colorless amorphous powder; $[\alpha]_D^{24}$ −6.9 (*c* 0.29, MeOH); UV (MeOH) λ_{max} nm (log ε): 202 (4.92), 253 (4.40), 269 (4.39), 345 (4.42); HRFABMS *m/z* 611.1757 $[M+H]^+$ (calcd for $C_{31}H_{31}O_{13}$, 611.1764); ^{1}H-NMR (DMSO-d_6) δ 6.70 (1H, s, H-3), 6.45 (1H, d, J = 2.0 Hz, H-6), 6.62 (1H, d, J = 2.0 Hz, H-8), 7.24 (1H, d, J = 2.0 Hz, H-2′), 7.07 (1H, d, J = 8.5 Hz, H-5′), 7.68 (1H, dd, J = 8.5, 2.0 Hz, H-6′), 5.14 (1H, d, J = 6.5 Hz, H-1″), 3.2–3.4 (3H, overlapped, H-2″, 3″ and 4″), 3.89 (1H, d, J = 8.0 Hz, H-5″), 6.80 (1H, s, H-3‴), 6.52 (1H, s, H-6‴), 2.03 (3H, s, H-7‴), 3.12 (1H, m, H-8‴), 1.16 (3H, d, J = 7.0 Hz, H-9‴), 1.18 (3H, d, J = 7.0 Hz, H-10‴); ^{13}C-NMR (DMSO-d_6) δ 163.7 (C-2), 103.5 (C-3), 181.7 (C-4), 161.1 (C-5), 99.4 (C-6), 162.4 (C-7), 94.4 (C-8), 156.7 (C-9), 105.3 (C-10), 121.4 (C-1′), 115.9 (C-2′), 146.2 (C-3′), 151.9 (C-4′), 117.1 (C-5′), 122.5 (C-6′), 99.4 (C-1″), 72.7 (C-2″), 75.8 (C-3″), 71.3 (C-4″), 75.0 (C-5″), 170.1 (C-6″), 122.5 (C-1‴), 151.9 (C-2‴), 112.6 (C-3‴), 136.8 (C-4‴), 144.8 (C-5‴), 120.2 (C-6‴), 15.6 (C-7‴), 26.1 (C-8‴), 23.1 (C-9‴), 22.9 (C-10‴).

4.13. Tetra-O-acetylation of sugar moiety of **7**

Compound **7** (5.0 mg) was dissolved in pyridine (1 mL) and stirred with Ac_2O (200 μL) for 5 h at 90 °C to yield compound **7a** (5.5 mg).

4.14. Sugar identification

Each compound [**3** (1.3 mg), **4** (0.5 mg), **5** (0.9 mg), **6** (1.0 mg), **7** (2.3 mg), **8** (1.4 mg), and **9** (0.3 mg)] was refluxed with 7% HCl (1 mL) for 2 h at 60 °C. After cooling, the reaction mixture was passed through an Amberlite IRA400 column, and the eluate was concentrated. The residue was dissolved in pyridine (0.5 mL) and stirred with L-cysteine methyl ester (5 mg) for 1.0 h at 60 °C, and then o-tolyl isothiocyanate (20 μL) was added to the mixture and heated to 60 °C for 1.0 h. The reaction mixture was analyzed by HPLC and detected at 250 nm. Analytical HPLC was performed on a Cosmosil 5C_{18}–AR II column (4.6 x 250 mm) at 20 °C using CH_3CN–0.2%TFA in H_2O (25:75) as mobile phase. Peaks were detected with a Tosoh UV8010 detector. D-Glucose (t_R 17.0 min) was identified as the sugar moieties of **3**–**6** by comparing retention times with those of authentic samples of D-glucose (t_R 17.0 min) and L-glucose (t_R 15.5 min). D-Glucuronic acid (t_R 16.8 min) was identified as the sugar moiety of **9** by comparing retention times with those of authentic samples of D-glucuronic acid (t_R 16.8 min) and L-glucuronic acid (using D-cysteine methyl ester and D-glucuronic acid, t_R 16.1 min) (Tanaka et al., 2007). The D-derivative from D-allose (t_R 34.9 min) was identified as the sugar moieties of **7** and **8** by comparing retention times with that of the authentic sample of D-allose (t_R 34.9 min). After the reaction of L-allose, L-cysteine methyl ester and *o*-tolyl isothiocyanate as above, L-thiohydantoin derivative (t_R 24.5 min) was mainly obtained instead of the expected L-allose derivative.

4.14.1. D-Derivative from D-allose

Colorless amorphous powder; $[\alpha]_D^{22}$ +67.8 (*c* 0.23, MeOH); FAB-MS *m/z* 447 $[M+H]^+$; UV (MeOH) λ_{max} nm (log ε): 240 (5.18); ^{1}H-NMR (400 MHz, CD_3OD) δ 5.89 (1H, br d, H-2), 5.86 (1H, t,

J = 8.0 Hz, H-4), 3.45 (2H, d, J = 8.0 Hz, H-5), 4.05 (1H, dd, J = 8.0, 3.5 Hz, H-6), 4.00 (1H, dd, J = 7.5, 3.5 Hz, H-7), 3.96 (1H, dd, J = 7.5, 7.5 Hz, H-8), 3.82 (1H, m, H-9), 3.65 (1H, dd, J = 11.5, 6.0 Hz, H-10), 3.75 (1H, dd, J = 11.5, 3.5 Hz, H-10), 7.12–7.23 (4H, overlapped, H-3′-H-6′), 2.26 (3H, s, 2′-CH_3), 3.87 (3H, s, OCH_3); ^{13}C-NMR (100 MHz, CD_3OD) δ 68.5 (C-2), 69.2 (C-4), 32.5 (C-5), 79.6 (C-6), 73.9 (C-7), 73.1 (C-8), 74.9 (C-9), 64.2 (C-10), 184.7 (C═S), 127.3, 127.8, 129.2, 131.5, 136.2, 140.1 (C-1′-C-6′), 18.5 (2′-CH_3), 174.2 (C = O), 53.4 (OMe).

4.14.2. L-Thiohydantoin derivative from L-allose

Colorless amorphous powder; $[\alpha]_D^{22}$ +151.4 (c 0.42, MeOH); FAB-MS m/z 415 $[M+H]^+$; UV (MeOH) λ_{max} nm (log ε): 240 (5.03), 276 (5.11); ^{1}H-NMR (400 MHz, pyridine-d_5) δ 7.12 (1H, d, J = 2.0 Hz, H-2), 5.50 (1H, t, J = 8.5 Hz, H-4), 3.38 (1H, dd, J = 10.5, 8.5 Hz, H-5), 3.74 (1H, dd, J = 10.5, 8.5 Hz, H-5), 5.31 (1H, br d, J = 8.0 Hz, H-6), 4.68 (1H, m, H-7), 4.75–4.80 (1H, overlapped, H-8), 4.75–4.80 (1H, overlapped, H-9), 4.38 (1H, dd, J = 10.5, 5.5 Hz, H-10), 4.52 (1H, dd, J = 10.5, 3.0 Hz, H-10), 7.20–7.32 (4H, overlapped, H-3′-H-6′), 2.22 (3H, s, 2′-CH_3); ^{13}C-NMR (100 MHz, pyridine-d_5) δ 69.7 (C-2), 67.8 (C-4), 32.3 (C-5), 75.3 (C-6), 74.7 (C-7), 74.1 (C-8), 75.6 (C-9), 64.8 (C-10), 185.1 (C═S), 127.3, 129.6, 129.9, 131.3, 133.6, 137.4 (C-1′-C-6′), 17.6 (2′-CH_3), 172.4 (C═O).

4.15. Animals

Six-week-old male ddY mice were purchased from Nihon SLC (Hamamatsu, Japan). They were housed in plastic cages with free access to food (until 24 h before use) and water and kept in a room at 25 ± 1 °C, 55 ± 5% humidity with a 12 h light–dark cycle. Experimental procedures were conducted in accordance with the ethical guidelines of Tohoku Pharmaceutical University.

4.16. Assay of lipase activity

Lipase activity was determined using the procedure reported by Kurooka et al. (1977) as described previously (Kobayashi et al., 2004). Cardiac blood was collected from mice with a heparin-treated cylinder and centrifuged to prepare plasma. Each test sample was adjusted to the relevant final reaction concentration in ultrapure water or ethanol (carvacrol and orlistat) and added to the mouse plasma prior to the assay. The inhibitory activity (%) was calculated as follows: $(1\text{-}B/A) \times 100$, where A is the activity of the enzyme in the absence of the test solution and B, that in the presence of the test solution.

4.17. Estimation of blood triacylglycerols

The assay was carried out according to the GPO-DAOS method (Spayd et al., 1978) using a kit (Triglyceride E-test Wako; Wako Pure Chemical Industry, Osaka, Japan) as described previously (Kobayashi et al., 2008a). The administration of samples was also conducted as reported (Kobayashi et al., 2008a) and plasma was prepared from blood collected from the tail of mice using a heparin-treated hematocrit capillary. Each test solution was administered orally to mice at a constant volume of 0.3 mL/30 g body weight.

4.18. Statistics

Statistical analyses were carried out using Student's t-test. Values with $p < 0.05$ were regarded as significant.

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