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Isolation of coniferyl esters from *Capsicum baccatum* L., and their enzymatic preparation and agonist activity for TRPV1

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Abstract

Coniferyl esters—capsiconiate and dihydrocapsiconiate—were isolated from the fruits of the pepper, *Capsicum baccatum* L. var. *praetermissum*. Their structures were determined by spectroscopic methods to be coniferyl (*E*)-8-methyl-6-nonenoate (capsiconiate) and coniferyl 8-methylnonanoate (dihydrocapsiconiate). This finding was further confirmed by the lipase-catalyzed condensation of coniferyl alcohol with its corresponding fatty acid derivative. The agonist activity of the esters for transient receptor potential vanilloid 1 (TRPV1) was evaluated by conducting an analysis of the intracellular calcium concentrations in TRPV1-expressing HEK293 cells. The EC_{50} values of capsiconiate and dihydrocapsiconiate were 3.2 and 4.2 μM, respectively.

Keywords: *Capsicum baccatum* L. var. *praetermissum*; Solanaceae; Coniferyl ester; Capsiconiate; Dihydrocapsiconiate; Spectroscopic analysis; Lipase-catalyzed preparation; Transient receptor potential vanilloid 1 (TRPV1); Calcium influx

1. Introduction

Hot red pepper (*Capsicum*) is widely used as a spicy seasoning and in medicinal materials worldwide. The pungency of the fruits of red pepper is caused by a lipophilic alkaloid capsaicin **4** (Fig. 1) and its analogs, termed capsaicinoids. The fundamental structure of capsaicinoids is that of an acid amide of vanillylamine with a fatty acid. Numerous reports on the chemical, biological, and physiological properties of capsaicinoids have been published; in particular, studies on the use of capsaicinoids as a medicinal target have increased tremendously since the cloning of a capsaicin receptor from mammalian sensory nerves (Caterina et al., 1997). Nowadays, the capsaicin receptor is termed as transient receptor potential vanilloid type 1 (TRPV1); it is a calcium permeable, non-selective cation channel. The activation of TRPV1 is considered to contribute to the various physiological activities of capsaicin (Szallasi, 2002).

We previously reported a novel compound group named capsinoids as an ingredient of the fruits of a non-pungent cultivar of *Capsicum annuum* L., 'CH-19 Sweet' (Kobata et al., 1998). The basic structure of capsinoids comprises an ester of vanillyl alcohol with a fatty acid, and the chemical structure of capsiate (**3**)—the major component of capsinoids—is as shown in Fig. 1. Interestingly, it was found that the TRPV1 agonist potential of capsiate was comparable to that of capsaicin despite its pungency being considerably lower than that of capsaicin (Iida et al., 2003). Recently, several reports on the TRPV1 related activities of capsiate (**3**) such as enhancement of thermogenesis and endurance capacity were published (Ohnuki et al., 2001; Haramizu et al., 2006). Furthermore, the potencies of cap-

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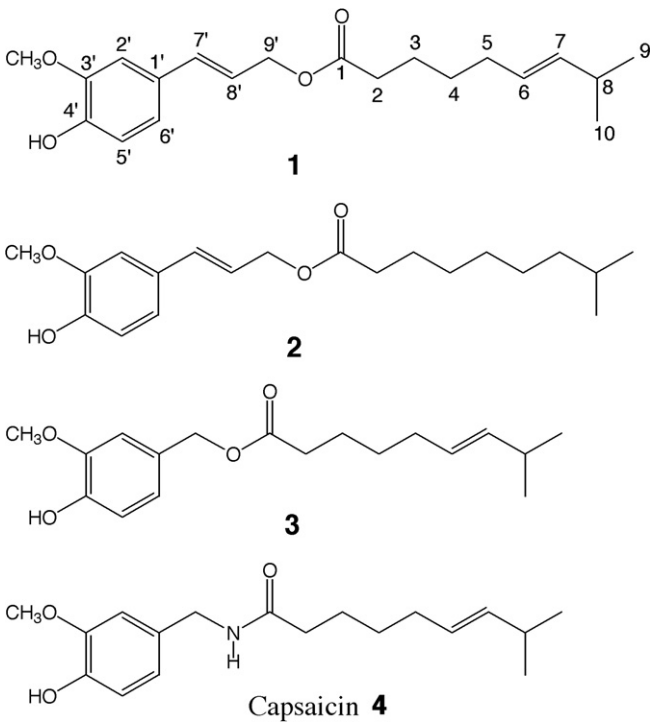

Fig. 1. Chemical structures of capsiconiate (**1**), dihydrocapsiconiate (**2**), capsiate (**3**) and capsaicin (**4**).

sinoids in apoptosis induction (Macho et al., 2003a) and as anticancer (Macho et al., 2003b), antioxidant (Rosa et al., 2002), immunosuppressive, and anti-inflammatory (Sancho et al., 2002) agents were reported. Therefore, "non-pungent esters" such as capsinoids and "amides" such as capsaicinoids will probably be attractive targets for pharmaceutical studies.

In the course of the analyses of capsaicinoids and capsinoids in several varieties of pepper, we discovered the existence of two unknown compounds **1** and **2** in a kind of variety of *Capsicum baccatum*. In the present paper, we have described the isolation of **1** and **2** from the fruits of *C. baccatum* L. var. *praetermissum*, and the structural determination of these compounds was performed by spectroscopic methods such as HR-FABMS, IR, UV, and ^{1}H and ^{13}C NMR spectroscopy. Furthermore, the structure of **1** and **2** was confirmed by their enzymatic preparation and analyses. Finally, the agonist potency of **1** and **2** for TRPV1 was evaluated by measuring the intracellular calcium concentration in TRPV1-expressing mammalian cells.

2. Results and discussion

2.1. Structural determination of capsiconiate (1) and dihydrocapsiconiate (2)

The IR spectrum of **1** was similar to that of capsiate (**3**) (Kobata et al., 1998). Therefore, the structure of **1** was considered to be closely related to that of **3**. The coupling constants and patterns of the three aromatic protons in the ^{1}H NMR spectrum of **1** indicated presence of the typical 1-, 2-, and 4-substituted phenyl group. A methoxy group attached to the phenyl group was observed in the ^{1}H and ^{13}C NMR spectra. In the ^{1}H NMR spectroscopy data, characteristic signals for an isopropyl group were observed. The presence of an ethylenic moiety of *trans* configuration was also indicated from the ^{1}H NMR data. Thus, the NMR data of **1** was extremely similar to those of **3** (Kobata et al., 1998). These results suggested that the structure of **1** contains a partial structure of **3**.

The molecular formula of **1** was estimated by the HR-FABMS measurement to be $C_{20}H_{28}O_4$. This molecular formula indicated an additional C_2H_2 group in **3**. The ^{13}C NMR spectrum of **1** also suggested the presence of the additional two olefinic carbons when compared to that of **3**. The ^{1}H NMR spectrum of **1** exhibited 2 olefinic methine protons at δ 6.57 *d* and 6.14 *dt*; the coupling constant 15.6 Hz indicated the trans configuration of the protons. One of the olefinic methine protons (δ 6.14) was coupled with the protons of an oxygen-attached methylene group observed at δ 4.71. On the other hand, the former methine was considered to be neighboring an aromatic ring because its chemical shift values as observed in the ^{1}H and ^{13}C NMR spectroscopy were high. These ^{1}H NMR signals that indicated the typical AMX_2 pattern suggested the existence of the moiety, Ar–CH=CH–CH_2–O–, in the structure of **1**. Therefore, a part of the structure of **1** was considered to be an ester of 3-(4-hydroxy-3-methoxyphenyl)-2(*E*)-propenyl, i.e., a coniferyl ester. The existence of the coniferyl group in **1** was supported by UV absorption at 297 nm.

From the spectroscopic data, we estimated the structure of **1** to be coniferyl (*E*)-8-methyl-6-nonenoate as shown in Fig. 1.

The HR-FABMS analysis elucidated the molecular formula of **2** to be $C_{20}H_{30}O_4$, which indicated the dihydrogenation of **1**. The IR and UV spectra of **2** were similar to those of **1**. The NMR spectrum of **2** was also similar to that of **1**. However, 2 aliphatic methylene carbons (δ 27.2 and 38.9) instead of the 2 olefinic methine carbons detected in **1** (C-6 and C-7) were observed in the ^{13}C NMR spectrum. Further, the ^{1}H NMR spectrum of **2** exhibited none of the olefinic proton signals observed in **1**. These results suggested the structure of **2** to be a 6,7-dihydro derivative of **1**, that is, coniferyl 8-methylnonanoate, as shown in Fig. 1.

In order to confirm the structures of **1** and **2**, they were enzymatically prepared by the lipase-catalyzed esterification or transesterification of corresponding fatty acid derivatives with coniferyl alcohol. The transesterification of methyl (*E*)-8-methyl-6-nonenoate derived from the methanolysis of capsaicin with coniferyl alcohol yielded a compound with the estimated structure of **1** in 13.1% yield. The esterification of 8-methylnonanoic acid with coniferyl alcohol yielded a compound with the estimated structure of **2** in 22.5% yield. The spectral data of the enzymatically prepared compounds completely coincided with those of the naturally occurring compounds mentioned above. There-

fore, the structures of **1** and **2** were confirmed as coniferyl (*E*)-8-methyl-6-nonenoate and coniferyl 8-methylnonanoate, respectively (Fig. 1).

The novel compounds **1** and **2** were named capsiconiate and dihydrocapsiconiate, respectively. Based on their yield, the concentration/content of these compounds in dry fruits was estimated to be ca. 450 μg/g for **1** and 180 μg/g for **2**. In the case of HPLC analysis of the oleoresin of the *C. baccatum*, the contents of **1**, **2**, capsiate (**3**), capsaicin, and dihydrocapsaicin were estimated to be 1060, 620, 110, 970, and 420 μg/g for dry fruits, respectively.

We previously reported the enzymatic preparation of the capsinoid **3** (capsiate) by lipase-catalyzed esterification of vanillyl alcohol with corresponding fatty acid derivatives in an organic solvent (Kobata et al., 2002). Various lipases can catalyze the esterification by strictly selecting an aliphatic hydroxy group and not a phenolic hydroxy group in a vanillyl moiety. Among them, Novozym 435—an immobilized *Candida antarctica* lipase—possessed the highest activity, particularly, under non-water conditions. This condition was suitable for the preparation of a water-unstable product such as capsiate (**3**). Therefore, we applied the condition to the preparation of the coniferyl esters **1** and **2** in this study. As expected, Novozym 435 yielded coniferyl esters of corresponding fatty acids under the non-aqueous condition. However, the yields were low (13.1% for **1** and 22.5% for **2**). These low yields might be due to the unstability of coniferyl esters as compared with capsinoids. Further investigation to improve the yield of the enzymatic preparation of these compounds is now in progress.

The present paper is the first report on coniferyl esters isolated from *Capsicum* plants. Only a few other reports have revealed coniferyl esters of longer chain acyl groups such as palmitoyl (C16:0), stearoyl (C18:0), and oleoyl (C18:1) in the *Opopanax* and *Platycodon* plants (Appendino et al., 2004; Lee et al., 2004). Therefore, we are interested in researching the novel "coniferyl ester" compound group with respect to its biosynthesis and biological and physiological activity.

2.2. Agonist potency of *1* and *2* for TRPV1

The burning sensation caused by capsaicin is triggered by the activation of a membrane protein, namely, TRPV1, in the sensory nerves (Caterina et al., 1997). The agonist activity of several types of capsaicin analogs with respect to this non-selective calcium permeable cation channel has been investigated (Walpole et al., 1993; Appendino et al., 2002). In the present study, we evaluated the agonist potency of two new compounds capsiconiate (**1**) and dihydrocapsiconiate (**2**) for TRPV1-expressing HEK293 cells by measuring the $[Ca^{2+}]_i$ by using the fluorescent calcium indicator Fura-2. Fig. 2 shows the dose-response curves for capsaicin (**4**), capsiate (**3**), **1**, and **2**. Capsaicin (**4**) evoked the increase of $[Ca^{2+}]_i$ at EC_{50} (0.082 μM), and the maximum increase was recorded at a dose exceeding

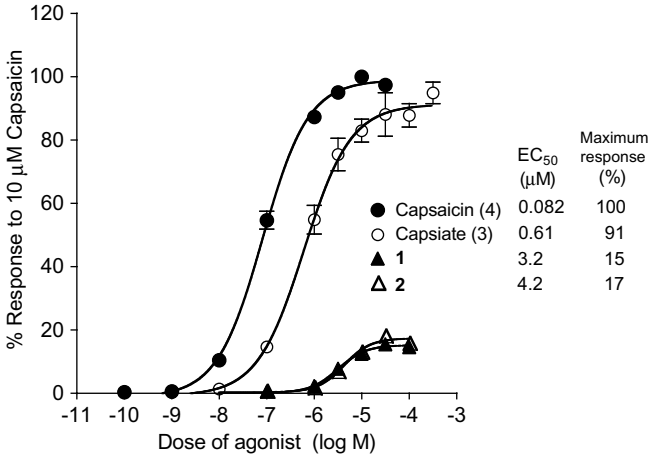

Fig. 2. Dose-response curves for agonists and intracellular calcium concentration of TRPV1-expressing HEK293 cells. The *y* axis represents % response to $\Delta[Ca^{2+}]_i$ caused by 10 μM capsaicin. Agonists: capsaicin (**4**) ($n = 10$); capsiate (**3**) ($n = 5$–6); capsiconiate (**1**) ($n = 3$); dihydrocapsiconiate (**2**) ($n = 3$). Each maximum response is the percentage of the response to 10 μM capsaicin.

1 μM capsaicin. The efficiency of **3** in TRPV1 activation was $EC_{50} = 0.61$ μM, and the maximum $[Ca^{2+}]_i$ increase was observed at a dose greater than 30 μM of **3**. This increase was 91% comparable to that of capsaicin (**4**). The agonist activities of capsaicin (**4**) and **3** were in agreement with the measurement of membrane current by whole-cell patch clamp method (Iida et al., 2003). On the other hand, the agonist potencies of **1** and **2** for TRPV1 were $EC_{50} = 3.2$ μM and 4.2 μM, respectively, and the maximum responses obtained were approximately 16% comparable with that of capsaicin. The responses generated by 10 μM of **1** and **2** were completely abolished by 100 μM capsazepine (CPZ), an antagonist for TRPV1 (Fig. 3). Further, no response was observed in non-trans-

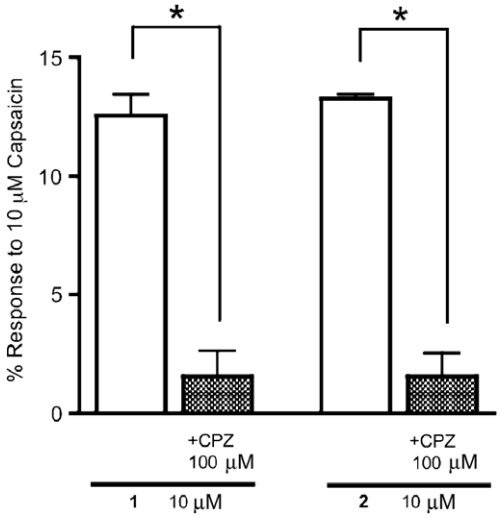

Fig. 3. Effects of the TRPV1 inhibitor capsazepine (CPZ) on the responses of TRPV1-expressing HEK293 cells to capsiconiate (**1**) and dihydrocapsiconiate (**2**). $n = 3$, $^*P < 0.01$ (unpaired *t* test).

fected HEK293 cells at any dose of all these agonists (data not shown).

2.3. Concluding remarks

In this study, we demonstrated that 2 new coniferyl esters—capsiconiate (**1**) and dihydrocapsiconiate (**2**)—possessed agonist activity for TRPV1. However, the activity was weaker than that of capsaicin (**4**) or capsiate (**3**). Earlier work concerning the pungency-structure relationship of capsaicin analogs revealed that an additional methylene group between the vanillyl group and the amide bond of capsaicin (**4**) attenuated the pungency drastically (Kobayashi, 1927). Walpole et al. also reported that the elongation of the carbon chain between the aromatic ring and amide bond of capsaicin attenuated the calcium flux into the dorsal root ganglia neurons; further, attenuation was observed in the compounds possessing double bonds next to the aromatic ring (Walpole et al., 1993). According to our analysis, compounds **1** and **2** had no pungency. Therefore, the low activity of **1** and **2** could be explained on the basis of the structure-activity relationship of the previous works. The instability of **1** and **2** in aqueous solvents may also be responsible for their low activity. Even though the agonist activity of **1** and **2** for TRPV1 was lower than that of capsaicin (**4**), their activity was comparable with that of other naturally occurring compounds such as piperine, gingerol, and capsaicinol (Witte et al., 2002; Iwasaki et al., 2006; Kobata et al., 2006). Furthermore, the presence of the coniferyl ester group indicated the antioxidative potential of these compounds (Lee et al., 2004). Further investigation of the applications of coniferyl esters for practical purposes will therefore be continued.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra (tetramethylsilane used as the internal standard) were recorded on a JEOL a-400 instrument (Tokyo, Japan) at 399.65 and 100.40 MHz, respectively. The IR and UV spectra were recorded on a JASCO FT/IR-550 spectrophotometer (Tokyo, Japan) and a JASCO UVIDEC 660 spectrophotometer, respectively. The HR-FABMS measurements were carried out on a JEOL JMS-700 spectrometer. The intracellular Ca^{2+} concentration was measured on a JASCO CAF-110 fluorospectrophotometer.

Coniferyl alcohol was purchased from Wako Pure Chemical Ind. (Osaka, Japan). Capsaicin and 8-methylnonanoic acid were obtained from Sigma (St. Louis, MO, USA). Methyl (*E*)-8-methyl-6-nonenoate was prepared by the methanolysis of capsaicin (Kobata et al., 2002). Capsiate was prepared using the enzymatic method described in a previous paper (Kobata et al., 2002). Novozym 435 was a kind gift from Novozymes Japan (Chiba, Japan). Fura-2 AM was obtained from Molecular Probes Inc. (Eugene, OR, USA).

3.2. Plant material

The plants of *C. baccatum* L. var. *praetermissum* were grown in the experimental farm of Kyoto University. The fruits of the plants were collected in autumn 2006.

3.3. Isolation of capsiconiate (*1*) and dihydrocapsiconiate (*2*)

The fresh fruits (0.7 kg) of *C. baccatum* L. var. *praetermissum* were lyophilized. The dried fruits (89.7 g) were cut and soaked in *n*-hexane (1.8 L) for 3 weeks. The filtrate was evaporated *in vacuo* to afford an oleoresin (1.54 g). Using an MPLC system (Yamazen Co., Osaka, Japan), the oleoresin was subjected to silica gel cc (UltraPack SI-40C, 37 mm i.d. × 300 mm, Yamazen) with *n*-hexane–EtOAc (9:1) as the elution solvent. The fraction that exhibited significant absorption on UV monitoring at 280 nm was collected and dried by evaporation. The residue (189.9 mg) was filtered through a short column of reversed phase silica gel (35 mm i.d. × 30 mm) with MeOH–water (9:1) containing 0.1% AcOH in order to eliminate materials with higher lipophilic properties. The resulting filtrate was poured into an *n*-hexane–water (1:1) solution in order to extract the desired compounds into the organic layer. The organic layer was dried using Na_2SO_4 and evaporated *in vacuo*. The residue (75.8 mg) was applied to an MPLC system on a reversed phase silica gel column (UltraPack ODS-50B, 26 mm i.d. × 300 mm, Yamazen) and eluted with MeOH–water (3:1) containing 0.1% AcOH. Upon monitoring of the eluent at UV 280 nm, two peaks were revealed indicating the presence of two fractions that were then separated. The separated fractions were transferred into *n*-hexane as described above. The dried fraction of the first peak yielded compound **1** (40.9 mg) and that of the second peak yielded compound **2** (16.4 mg).

3.3.1. Capsiconiate (*1*) (coniferyl (*E*)-8-methyl-6-nonenoate)

Colorless oil; UV: λ_{max}^{MeOH} nm (log ε): 297 (3.6), 268 (3.9); IR ν_{max}^{film} cm^{-1}: 3420 (OH), 2960, 2870, 1730 (>C=O), 1600, 1510, 1460, 1430, 1380, 1270, 1160, 1120, 1030, 970, 860, 800; ^{1}H NMR ($CDCl_3$) δ 6.91 (1H, *d*, $J = 1.6$ Hz, H-2′), 6.90 (1H, *dd*, $J = 8.0$, 1.6 Hz, H-6′), 6.86 (1H, *d*, $J = 8.0$ Hz, H-5′), 6.57 (1H, *d*, $J = 15.6$ Hz, H-7′), 6.14 (1H, *dt*, $J = 15.6$, 6.4 Hz, H-8′), 5.38 (1H, *dd*, $J = 15.6$, 5.6 Hz, H-7), 5.32 (1H, *dt*, $J = 15.6$, 6.4 Hz, H-6), 4.71 (2H, *d*, $J = 6.8$ Hz, H-9′), 3.89 (3H, *s*, OCH_3), 2.34 (2H, *t*, $J = 7.2$ Hz, H-2), 2.21 (1H, *oct*, $J = 6.4$ Hz, H-8), 1.99 (2H, *q*, $J = 6.8$ Hz, H-5), 1.65 (2H, *quint*, $J = 7.6$ Hz, H-3), 1.39 (2H, *quint*, $J = 7.6$ Hz, H-4), 0.95 (3H, *d*, $J = 6.8$ Hz, H-9); 0.95 (3H, *d*, $J = 6.8$ Hz, H-10); ^{13}C NMR ($CDCl_3$) δ 173.7 (C-1), 146.6 (C-3′), 145.9 (C-4′), 138.1 (C-7), 134.4 (C-7′), 128.9 (C-1′), 126.5 (C-6), 121.0

(C-8′), 120.6 (C-6′), 114.5 (C-5′), 108.4 (C-2′), 65.1 (C-9′), 55.9 (OCH_3), 34.3 (C-2), 32.1 (C-5), 31.0 (C-8), 29.2 (C-4), 24.5 (C-3), 22.7 (C-9), 22.7 (C-10); HR-FABMS *m/z* 332.2002 (calcd. for $C_{20}H_{28}O_4$ 332.1988).

3.3.2. Dihydrocapsiconiate (**2**) (coniferyl 8-methylnonanoate)

Colorless oil; UV λ_{max}^{MeOH} nm (log ε): 297 (3.6), 268 (3.9); IR ν_{max}^{film} cm^{-1}: 3420 (OH), 2960, 2870, 1730 (>C=O), 1600, 1510, 1460, 1430, 1380, 1270, 1160, 1120, 1030, 970, 860, 800; ^1H NMR ($CDCl_3$) δ 6.91 (1H, *d*, *J* = 1.6 Hz, H-2′), 6.90 (1H, *dd*, *J* = 8.0, 1.6 Hz, H-6′), 6.86 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.57 (1H, *d*, *J* = 15.6 Hz, H-7′), 6.14 (1H, *dt*, *J* = 15.6, 6.4 Hz, H-8′), 5.66 (1H, *s*, OH), 4.71 (2H, *d*, *J* = 6.8 Hz, H-9′), 3.90 (3H, *s*, OCH_3), 2.34 (2H, *t*, *J* = 7.2 Hz, H-2), 1.65 (2H, *quint*, *J* = 7.6 Hz, H-3), 1.50 (1H, *m*, H-8), 1.30 (1H, *m*, H-5), 1.25 (1H, *m*, H-6), 1.25 (1H, *m*, H-4), 1.13 (2H, *q*, *J* = 6.5 Hz, H-7), 0.85 (3H, *d*, *J* = 6.8 Hz, H-9); 0.85 (3H, d, *J* = 6.8 Hz, H-10); ^{13}C NMR ($CDCl_3$) δ 173.7 (C-1), 146.7 (C-3′), 145.9 (C-4′), 134.4 (C-7′), 128.9 (C-1′), 121.0 (C-8′), 120.6 (C-6′), 114.5 (C-5′), 108.4 (C-2′), 65.1 (C-9′), 55.9 (OCH_3), 38.9 (C-7), 34.4 (C-2), 29.5 (C-4), 29.2 (C-5), 27.9 (C-8), 27.2 (C-6), 25.0 (C-3), 22.6 (C-9), 22.6 (C-10); HR-FABMS *m/z* 334.2139 (calcd. for $C_{20}H_{30}O_4$ 334.2144).

3.4. Enzymatic preparation of capsiconiate (**1**) and dihydrocapsiconiate (**2**)

3.4.1. Preparation of **1**

Two hundred milligrams of coniferyl alcohol (1.1 mmol) and 180 mg of methyl (*E*)-8-methyl-6-nonenoate (1.1 mmol) were dissolved in dry acetone (20 mL). After addition of 600 mg of molecular sieve 4A and 200 mg of Novozym 435, the solution was incubated and agitated at 50 °C for 24 h. To the reaction solution, *n*-hexane (200 mL) and MeOH–water (200 mL, 1:1, v/v) was added, and the mixture was separated into organic and aqueous fractions. The organic fraction was applied to a silica gel column (UltraPack SI-40C, 37 mm i.d. × 300 mm) using the MPLC system with the eluent *n*-hexane–EtOAc (4:1). The fraction containing **1** was further purified on a reversed phase silica gel column (UltraPack ODS-50B, 26 mm i.d. × 300 mm) using the MPLC system with MeOH–water (4:1) containing 0.1% TFA as the eluent. The fraction that produced the major peak upon UV monitoring yielded compound **1** (47.7 mg, 13.1% yield).

3.4.2. Preparation of **2**

One hundred milligrams of coniferyl alcohol (0.55 mmol) and 93 mg of 8-methylnonanoic acid (0.55 mmol) were dissolved in acetone (10 mL). After the addition of 300 mg of the molecular sieve 4A and 110 mg of Novozym 435, the solution was incubated at 50 °C with agitation for 24 h. The same purification method mentioned above was applied to afford compound **2** (41.4 mg, 22.5% yield).

3.5. Measurement of intracellular calcium concentration in TRPV1-expressing HEK293 cells

HEK293 cells stably expressing rat-TRPV1 were obtained as described previously by Morita et al. (2006) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B at 37 °C under 5% CO_2/air. The cells were subcultured every week; the highest passage number used was 35.

The intracellular calcium concentration ($[Ca^{2+}]_i$) in the TRPV1-expressing HEK293 cells and the parent HEK293 cells was recorded by a method described previously by Morita et al. (2006) with a minor modification. The cells were loaded with the cytoplasmic calcium indicator Fura-2 AM (5 μM) in a loading buffer (5.37 mM KCl, 0.441 mM KH_2PO_4, 137 mM NaCl, 0.336 mM $Na_2HPO_4 \cdot 7H_2O$, 5.56 mM glucose, 20 mM HEPES, 1 mM $CaCl_2$, and 0.1% bovine serum albumin at pH 7.4) at 37 °C for 30 min. After two washes with the loading buffer, the cells were suspended at a concentration of 3×10^5 cells/mL with the loading buffer. The suspension was poured into a cuvette placed on a fluorospectrophotometer (CAF-110), and the time-dependent change in fluorescence (excitation wavelength: 340 and 380 nm, emission wavelength: 500 nm) was recorded during magnetic stirring before and after the addition of the test compounds using PowerLab (AD Instruments, NSW, Australia). The ratio of the fluorescence to exitation 340 and 380 nm was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz et al. (1985) and R_{max} and R_{min} were determined using 0.1% Triton X-100 and 5 mM EGTA, respectively. The effective dissociation constant (K_d) for Fura-2 AM at 37 °C was 224 nM. In some experiments, capsazepine (100 μM) was added at least 1 min prior to the addition of the test compounds. Each data value was expressed as a percentage of the response to 10 μM capsaicin. Curve fitting and parameter estimation were carried out using Prism4 (GraphPad Software, San Diego, CA, USA).

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