Synthesis and Activity of a Metabolite of (*S*)-6-Amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4-(1*H*,3*H*)-pyrimidinedione (CX-659S)

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CX-659S (1) [(*S*)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4-(1*H*,3*H*)-pyrimidinedione], has been developed as a new type anti-inflammatory agent for the treatment of dermatitis. The structure of a major metabolite of CX-659S was determined as (*S*)-6-amino-5-[2-hydroxy-2-methyl-4-(2,4,5-trimethyl-3,6-dioxo-1,4-cyclohexadienyl)butanamide]-3-methyl-1-phenyl-2,4-(1*H*,3*H*)-pyrimidinedione (2) by direct comparison with the synthesized authentic compound. The anti-inflammatory activity of 2 was equipotent with that of 1 on the contact hypersensitivity reaction (CHR) induced by picryl chloride (PC) in mice, suggesting that compound 2 contributes, at least in part, to the anti-inflammatory activity of CX-659S.

Key words CX-659S; metabolite; CHR; ROS; antioxidative activity

We have recently reported a novel pyrimidine derivative ((*S*)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4-(1*H*,3*H*)-pyrimidinedione ((**1**), CX-659S, Fig. 1), which showed anti-inflammatory activities against both acute inflammation induced by irritants and delayed-type hypersensitivity in various animal models.[1—3] The chemical structure of **1** is quite different from those of typical non-steroidal anti-inflammatory agents, steroid and immunosuppressant, and thus is of interest. In the course of our pharmacokinetic study on **1**, a metabolite was commonly observed in the plasma when **1** was administered to animals. According to the analysis of the metabolite of **1**, the *m*/*z* on liquid chromatography-mass spectrometry (LC-MS) of it was larger than that of **1** by 16. On the other hand, a number of α-tocopherol metabolites have been described. Among them, α-tocopheronic acid, which results from ring opening of the chroman moiety was characterized in the urine of animals and man.[4] On the basis of the above evidence, we considered that compound **1** (exact mass=464.2), having a tocopherol-related chroman moiety, would be converted into the corresponding quinone **2** (exact mass=480.2) under oxidative conditions. But this possibility needed to be confirmed by direct comparison of this metabolite with the synthesized authentic sample. In this present study, we prepared **2** to confirm the structure of this metabolite, and additionally, evaluated its anti-inflammatory activity.

Compound **2** was prepared by 2 synthetic methods, outlined in Chart 1. Cerium (IV) ammonium nitrate (CAN) is a reagent that oxidizes hydroquinone derivatives into their corresponding quinones, however, direct oxidation of **1** by CAN gave **2** in very poor yield (15%) along with many by-products. Therefore, chroman carboxylic acid, *S*-(−)-**3**, was first oxidized by CAN into the corresponding quinone, *S*-(+)-**4**,[5] with retention of configuration. The resulted *S*-(+)-**4** was then reacted with **5** using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) to give *S*-(+)-**2** in moderate yield. Other reagents for oxidation of **1** were also evaluated, and *S*-(+)-**2** was obtained in excellent yield when iodine was used.

The above mentioned metabolite of **1** in guinea pig plasma was identified to be **2** by LC/MS/MS comparison with the authentic compound. Figure 2 shows a typical reverse phase (RP)-HPLC elution profile of plasma samples after administration of CX-659S to dogs. The main peak corresponding to the metabolite of CX-659S was eluted with a retention time characteristic of authentic **2** at 8.8 min.

It has been reported that reactive oxygen species (ROS) participate in the process of inflammation in various tissues including skin,[6] and compound **1** is considered to have anti-inflammatory effects on the contact hypersensitivity reaction (CHR) in mice by topical administration due to its potent radical scavenging activities against the hydroxyl radical and peroxynitrite and to its inhibitory effects on lipid peroxida-

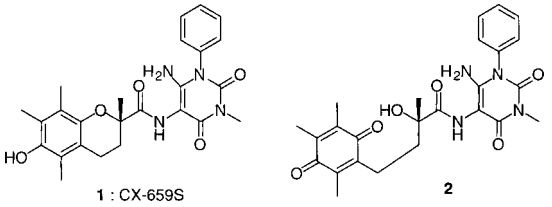
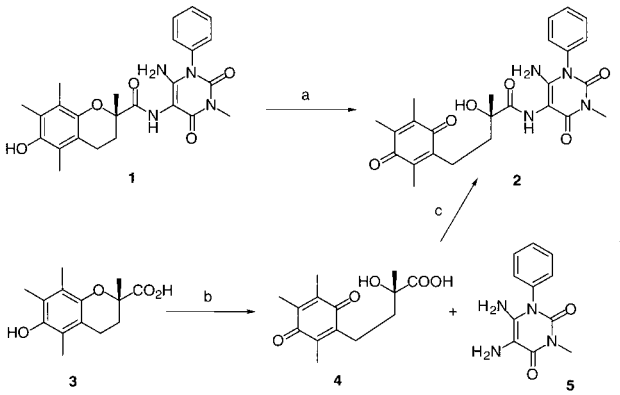


Fig. 1. Structures of **1** (CX-659S) and **2**



Reagents and conditions: (a) I_2, $NaHCO_3$, CH_2Cl_2-H_2O; (b) CAN, CH_3CN-H_2O; (c) EDC·HCl, HOBt, DMF

Chart 1. Synthesis of the Quinone Derivative, **2**

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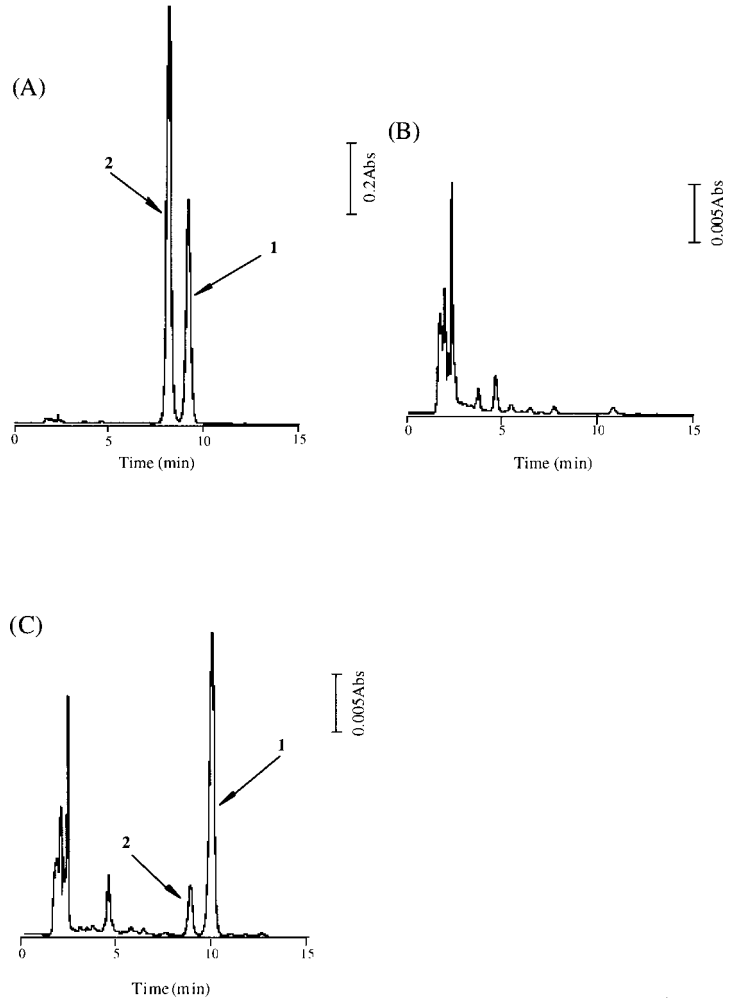

Fig. 2. Representative HPL Chromatograms of the Plasma Samples
(A) standard, (B) blank plasma, (C) plasma after subcutaneous administration of **1**.

Table 1. Inhibitory Effects of Compounds **1** and **2** on PC-Induced CHR in Mice

Compound	Dose (mg/ear)	Inhibition[a)] (%)
1	0.03	21
	0.1	35
	0.3	69*
2	0.03	−9
	0.1	42
	0.3	70*
Prednisolone	0.1	85**

a) Percent inhibition was calculated from the values of percent responses of drug-treated and control groups ($n=6$). $*p<0.05$, $**p<0.01$ *versus* control (Dunnet's test).

tion.[3)] However, compound **2** had no anti-oxidative activities, and the IC_{50} value against lipid peroxidation was over 100 μM (data not shown). Therefore, we expected that compound **2**, an oxidized product of **1**, would show a weaker or lesser activity on the CHR in mice. However, contrary to our expectation, compound **2** also suppressed the CHR in mice by topical administration, and the potency of **2** was equipotent to that of **1** (Table 1), suggesting that compound **2** may have an action mechanism other than the anti-oxidative activity to inhibit the CHR.

In conclusion, we identified a metabolite structure of CX-659S, a new type of anti-inflammatory compound, and examined its biological activity on the CHR in mice. As a result, the potency of **2** was found to be equipotent to that of **1** in this model. These findings suggest that this metabolite contributes, at least in part, to the anti-inflammatory activities of CX-659S *in vivo*. An investigation on the action mechanisms of CX-659S and its metabolite is now in progress.

Experimental

General All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were measured with a BÜCHI 535 melting point apparatus and were uncorrected. Proton NMR spectra were recorded on a JEOL GSX270 FT NMR spectrometer. Chemical shifts were given in parts per million (ppm) using tetramethylsilane as the internal standard for spectra obtained in DMSO-d_6 and $CDCl_3$. TOF MS (time-of-flight mass spectrometry) were recorded on a Kompact MALDI 3 V4.0.0 spectrometer. Optical rotation was recorded on a DIP-370 spectrometer. Elemental analyses were performed at the Toray Research Center. Wakogel C-200 (Wako; 70—150 mm) was used for column chromatography. Monitoring of reactions was carried out by using Merck 60 F_{254} silica gel, glass-supported TLC plates, and visualization with UV light (254 and 365 nm).

(*S*)-(+)-6-Amino-5-[2-hydroxy-2-methyl-4-(2,4,5-trimethyl-3,6-dioxo-1,4-cyclohexadienyl)butanamide-3-methyl-1-phenyl-2,4-(1*H*,3*H*)-pyrimidinedione (2) A solution of **1** (13.94 g, 30 mmol) in CH_2Cl_2 (200 ml) and $NaHCO_3$ (6.30 g, 75 mmol) in water (150 ml) were mixed and agitated vigor-

ously. Iodine (8.38 g, 33 mmol) in CH_2Cl_2 (200 ml) was added to the mixture dropwisely for 3 h under ice-cooling. $Na_2S_2O_3$ (6.3 g, 75 mmol) was then added to the mixture to reduce the surplus iodine. The organic layer was separated and washed with water and brine, dried over $MgSO_4$, and concentrated to give **2** (14.12 g, 98%) as an orange foam. mp 125—127 °C (dec.). Optical rotation $[\alpha]_D^{25}$ 10.5° (c=1, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.54 (3H, s), 1.68—1.72 (1H, m), 1.96 (3H, s), 1.99 (3H, s), 2.01 (3H, s), 2.46—2.48 (1H, m), 2.65—2.69 (1H, m), 3.36 (3H, s), 3.89 (2H, s), 5.32 (2H, s), 7.37—7.40 (2H, m), 7.56—7.62 (3H, m), 8.55 (1H, br s). MS (TOF) m/z 481 $(M+H)^+$. *Anal.* Calcd for $C_{25}H_{28}N_4O_6 \cdot 0.5H_2O$: C, 61.34; H, 5.87; N, 11.45. Found: C, 61.37; H, 5.97; N, 11.35.

(*S*)-(+)-2-Hydroxy-2-methyl-4-(3,4,6-trimethyl-2,5-dioxocyclohexa-1,3-dienyl)-butanoic Acid (4) To an ice-cooled solution of **3** (5 g, 20 mmol) in CH_3CN/H_2O (40 ml/20 ml) was added dropwisely a solution of CAN (32.9 g, 60 mmol) in H_2O (36 ml), and the mixture was then stirred for 30 min at room temperature. After partitioning with CH_2Cl_2 (100 ml) and water (100 ml), and dried over $MgSO_4$. The solvent was concentrated, and the residue was purified by column chromatography on silica gel (hexane : ethyl acetate=7 : 1) to give **4** (4.53 g, 85%) as a white solid. Optical rotation $[\alpha]_D^{25}$ 21.8° (c=1, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.52 (3H, s), 1.73—1.83 (1H, m), 1.90—1.96 (1H, m), 2.00 (6H, s), 2.03 (3H, s), 2.44—2.54 (1H, m), 2.63—2.72 (1H, m). MS (TOF) m/z 251 $(M+H)^+$.

Condensation of 4 and 5 To a mixture of **4** (293 mg, 1.1 mmol) and **5** (232 mg, 1 mmol) in *N*,*N*-dimethylformamide (DMF) (10 ml) were added HOBt (149 mg, 1.1 mmol) and EDC·HCl (211 mg, 1.1 mmol), and the mixture was then stirred for 8 h at room temperature. Next, the solvent was concentrated and partitioned with CH_2Cl_2 (20 ml) and 5% $NaHCO_3$ solution (20 ml). The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. Finally, the crude product was recrystallized from ether/ethyl acetate to give **2** (300 mg, 63%).

Pharmacokinetic Study Male beagle dogs (9 months of age, 9.5—11.2 kg) were obtained from Ridglan Research Farms Inc. (Wisconsin, U.S.A.). Compound **1** dissolved in 0.5% propyleneglycol was administered subcutaneously to the dogs. Blood samples (4 ml) were taken from a jugular vein, and the serum was obtained by centrifugation at 1630 ***g*** for 10 min at 4 °C, and kept at −20 °C until drug analysis. The concentrations of **1** and **2** in the serum were assayed by an HPLC method. Briefly, 1 ml of serum was extracted with 5 ml of diethylether, and centrifuged at 724 ***g*** for 5 min at 4 °C. The aqueous layer was extracted with the same volume of diethylether again. The combined organic layers were concentrated and dissolved in 200 μl of H_2O/MeOH (1 : 1, v/v), and a portion (100 μl) was injected into the chromatograph. Chromatographic separations were achieved on a Shimadzu LC-10A HPLC (Shimadzu, Japan) using a Capcell Pak C18 SG120 (150×4.6 mm, Shiseido, Japan). The mobile phase consisted of acetonitrile : water (7 : 3, v/v), with the pH adjusted to 5.0 by 0.05 M ammonium acetate. The flow rate of the mobile phase was maintained at 1.0 ml/min, and the effluent was monitored at a UV detection wavelength of 267 nm.

Picryl Chloride-Induced Contact Hypersensitivity Reaction PC-induced CHR was assessed by the method of Asherson and Ptak.[7] Male ICR mice were sensitized by applying 100 μl of 7% (w/v) PC solution in acetone to the shaved abdomen. Seven days later, the mice were challenged by applying 20 μl of 1% (w/v) PC solution in acetone to the left ear. The ear thickness was measured with a digital thickness gauge before and 24 h after the challenge, and the difference in thickness was calculated. Test compounds were dissolved in acetone and were administered immediately after the challenge.

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