Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 1111–1124
Bioorganic & Medicinal Chemistry

Imine derivatives as new potent and selective CB_2 cannabinoid receptor agonists with an analgesic action

Hiroshi Ohta,[a,*] Tomoko Ishizaka,[a] Makoto Tatsuzuki,[a] Mitsukane Yoshinaga,[a] Izumi Iida,[b] Tomomi Yamaguchi,[c] Yasumitsu Tomishima,[c] Nobuko Futaki,[c] Yoshihisa Toda[c] and Shuji Saito[a]

[a]*Medicinal Chemistry Laboratories, Taisho Pharmaceutical Co., Ltd, 1-403 Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[b]*Drug Safety and Pharmacokinetics Laboratories, Taisho Pharmaceutical Co., Ltd, 1-403 Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[c]*Molecular Function and Pharmacology Laboratories, Taisho Pharmaceutical Co., Ltd, 1-403 Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*

Received 10 September 2007; revised 26 October 2007; accepted 28 October 2007
Available online 1 November 2007

Abstract—In this study, a novel series of CB_2 receptor agonist imine derivatives, **1**–**6**, was synthesized and evaluated for activity against the CB_2 receptor. In a previous paper we reported the synthesis and SARs of thiazole derivative **1**, a potent CB_2 receptor agonist, but we had not assessed chemical modifications of the 5-membered heteroring of **1**. In the present study, we therefore tried chemically modifying the 5-membered heteroring of **1** in an attempt to further improve binding affinity for the CB_2 receptor. In the course of making the structural modifications, we discovered that a novel pyrazole derivative **6b** (CBS0550) had high affinity for the CB_2 receptor (IC_{50} = 2.9 nM, EC_{50} = 1.8 nM, E_{max} = 85%), high selectivity for CB_2 (CB_1 IC_{50}/CB_2 IC_{50} = 1400), and good physicochemical properties (solubility in water: 5.9 mg/100 mL at 25 °C). Oral administration of **6b** to rats at a dose of 10 mg/kg resulted in significant plasma concentrations, and orally administered compound **6b** significantly reversed mechanical hyperalgesia in the Randall–Selitto model of inflammatory pain in rats.

1. Introduction

Although *Cannabis sativa* L. (marijuana) has a long history of use for medicinal and recreational purposes, more knowledge about marijuana has been acquired in the last three decades than the entire last century. Delta[9]-tetrahydrocannabinol (delta[9]-THC), which contains tricyclic structures derived from the benzopyrane, has been isolated from marijuana as its major psychoactive component.[1] Cannabinoids, whose definition has now been extended to include THC analogues, have a wide range of biological properties, including analgesic, anti-inflammatory, antiemetic, anticonvulsive, and anticancer properties.[2] However, therapeutic use of cannabinoids has been limited because of the difficulty of separating their beneficial and psychotropic activities. The discovery and identification of two distinct cannabinoid receptors, the CB_1 receptor[3] and the CB_2 receptor,[4] stimulated a rebirth of interest in the medicinal chemistry of the cannabinoids. The CB_1 receptor is abundant in the CNS and is present to a lesser extent in other tissues, and the central effects of the cannabinoids are related to the CB_1 receptor. The CB_2 receptor, on the other hand, is found wholly in the periphery and is primarily associated with cells of the immune system.[5–7] Recently, however, the CB_2 receptor has also been found in CNS tissue.[8] Both receptors are members of the G-protein-coupled superfamily of receptors, and they share 68% homology with one another at the transmembrane level and 44% homology overall.[4] Although the development of highly selective CB_2 receptor ligands is important to exploration of some of the physiological effects of cannabinoids, such as their immunosuppressive, anti-inflammatory, and antinociceptive effects, a few compounds that are selective for the CB_2 receptor, such as aminopyrimidine[9] (GW842166X, CB_2

Keywords: CB_2 agonist; Imine; Inflammatory; Pain; Analgesic; CB_2.
* Corresponding author. Tel.: +81 48 663 1111; fax: +81 48 652 7254; e-mail: hiroshi.ohta@po.rd.taisho.co.jp

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.10.087

EC_{50} = 50 nM, CB_1 EC_{50} > 30,000 nM), 3-carbonylindole[10] (A-796260, CB_2 IC_{50} = 0.77 nM, CB_1 IC_{50} = 330 nM), and aminoalkylindole[11] (AM1241, CB_2 K_i = 3.4 nM, CB_1 K_i = 280 nM), are known (Fig. 1).

In this paper, we report the synthesis and SARs of imine derivatives **1**–**6**, which are novel CB_2 receptor ligands. In a previous paper we described the evaluation of thiazole derivative **1** (IC_{50} = 13 nM, EC_{50} = 10 nM, E_{max} = 91%, CB_1 IC_{50}/CB_2 IC_{50} = 270) and its analogue as potent and selective CB_2 agonists,[12] but we had not assessed chemical modifications of thiazole ring of **1**. In the present study, we tried chemically modifying the heteroring of **1** in attempt to further improve binding affinity and selectivity for the CB_2 receptor.

2. Chemistry

Scheme 1 illustrates the synthesis of the 1,3,4-thiadiazole derivative (**2**). 2-Amino-5-*tert*-butyl-1,3,4-thiadiazole (**7**), a commercially available compound (Aldrich Chemical Company, Inc.), was treated with trifluoroacetic anhydride to obtain **8**, with a 91% yield. The 3-position of the thiadiazole ring of **8** was alkylated, followed by deprotection under basic conditions, producing the intermediate **10**. Acylation of intermediate **10** by treatment with 3-trifluoromethylbenzoyl chloride and Et_3N converted it to 1,3,4-thiadiazole (**2**), with a 49% yield.

Scheme 2 shows the synthesis of the oxazole derivative (**3**). 2-Amino-4-methyl-1,3-oxazole (**12**) was obtained by reaction between hydroxyacetone (**11**) and cyanamide, with a 48% yield.[13] Friedel–Crafts acylation at the 5-position of the oxazole ring was then performed to obtain **13**,[14] and protection of the amino group in the 2-position of oxazole **13** was achieved with a Boc group. Compound **14** was treated with MeLi to obtain **15**, with a 90% yield. Reduction and deprotection of **15** with Et_3SiH under acidic conditions yielded 2-amino-4-methyl-5-(1-methylethyl)-1,3-oxazole **16**. Amino oxazole **16** was converted to oxazole derivative **3** by the same procedure as from **7** to **2**.

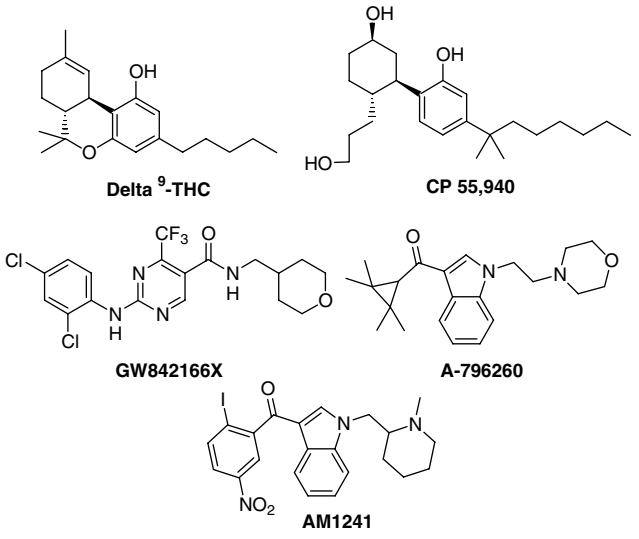


Figure 1. Structure of Delta[9]-THC, CP 55,940, GW842166X, A-796260, and AM1241.

Scheme 3 illustrates the synthesis of the isothiazole derivative (**4**). 3-Amino-5-*tert*-butylisothiazole **22** was obtained from pinacolone **20** by Hackler's procedure.[15] (2*Z*)-3-chloro-4,4-dimethylpent-2-enenitrile (**21**) was prepared from pinacolone **20** in a one-pot procedure, with a 100% yield, and ring closure of **21** resulted in formation of the 3-aminoisothiazole **22**. Aminoisothiazole **22** was converted to the isothiazole derivative **4** by the same procedure as from **7** to **2**.

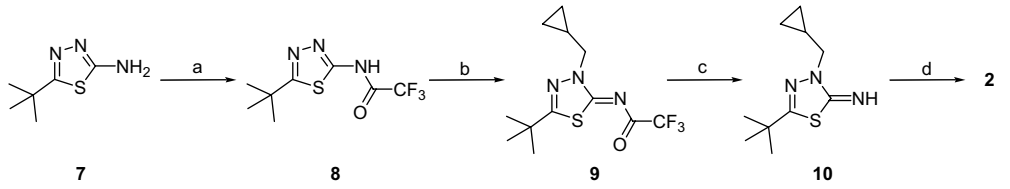
Scheme 4 shows the synthesis of the isoxazole derivative (**5**). Aminoisoxazole **26**, a commercially available compound (Wako Pure Chemical Industries, Ltd), was converted to isoxazole derivative **5** by the same procedure as from **7** to **2**.

Scheme 5 illustrates the preparation of pyrazole derivatives **6a**–**f**. Cyclopropylmethyl alcohol **30** was treated with MsCl and Et_3N to yield mesylate **31**. Mesylate **31** was transformed into alkylhydrazine **32** by treatment with hydrazine, with a 53% yield. Cyclization of **32** by treatment with pivaloylacetonitrile yielded aminopyrazole **33**, with a 42% yield. Aminopyrazole **33** was treated with trifluoroacetic anhydride and pyridine to obtain **34**, with a 100% yield. The 1-position of the pyrazole ring of **34** was alkylated, followed by deprotection under basic conditions, producing the intermediate **36**. The intermediate **36** was easily transformed into pyrazole derivatives **6a**–**f** in one step by acylation of the intermediate **36** by treatment with the corresponding aryl carbonyl chlorides and Et_3N.

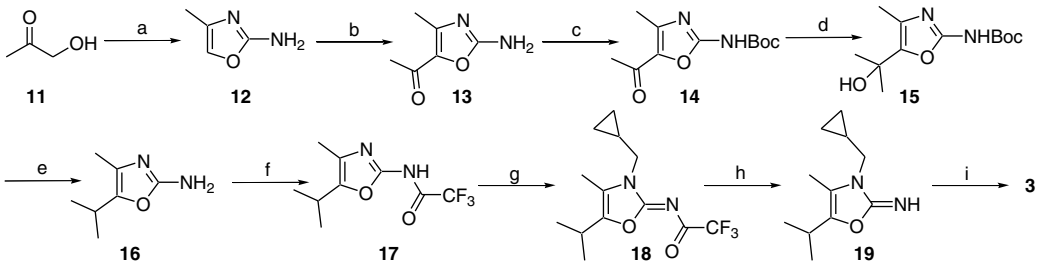
Scheme 6 shows the preparation of the pyrazole derivatives **6g**–**j**. 2-Alkyl-3-amino-5-*tert*-butylpyrazoles **40g**–**j** were synthesized from the corresponding alcohols, **37g**–**j**, by the same procedure as from **30** to **33**. Acylation of **40g**–**j** was performed by treatment with 2-fluoro-3-trifluoromethylbenzoyl chloride and Et_3N. Finally, methylation at the 1-position of the pyrazole ring **41g**–**j** yielded **6g**–**j**.

3. Results and discussion

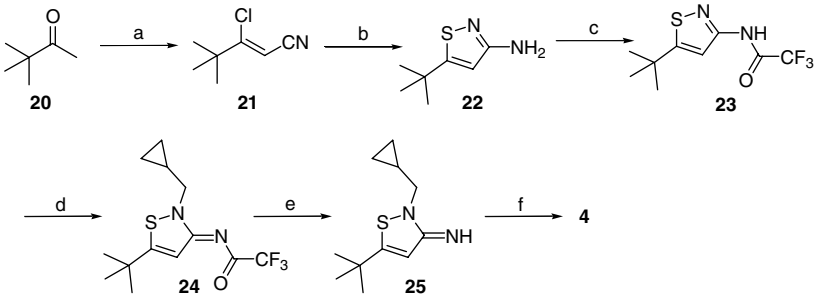
The affinity of imine derivatives **1**–**5**, **6a**–**j** and CP 55,940,[16] a nonselective cannabinoid receptor agonist for CB_2 receptor, was evaluated on the basis of [^{3}H]CP 55,940 binding to membranes of Chinese hamster ovary (CHO) cells expressing the human CB_2 receptor, and the affinity of compounds **1**, **6b**–**e**, **6g**–**j** and CP 55,940 for the CB_1 receptor was evaluated on the basis of [^{3}H]CP 55,940 binding to membranes of CHO cells expressing the human CB_1 receptor. The agonist activities of **1**, **6b**–**e**, **6g**–**j** and CP 55,940 were evaluated by using a functional assay based on [^{35}S]GTPγS binding to membranes of CHO cells expressing the human CB_2 receptor. E_{max} was compared to the maximal response to CP 55,940, a full agonist of the human CB_2 receptor.



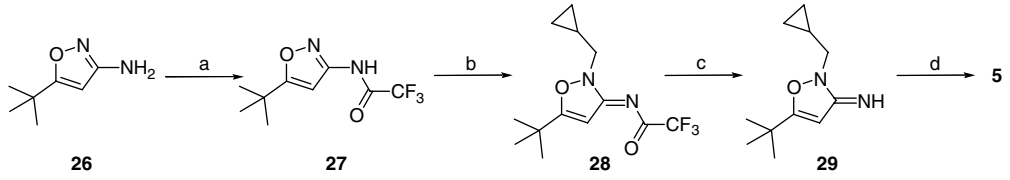
Scheme 1. Reagents and conditions: (a) $(CF_3CO)_2O$, toluene, rt, 91%; (b) cyclopropylmethylbromide, NaI, K_2CO_3, DMF, rt, 91%; (c) 2 M NaOH, THF, 65 °C, 99%; (d) 3-trifluoromethylbenzoyl chloride, Et_3N, $CHCl_3$, rt, 49%.



Scheme 2. Reagents and conditions: (a) NH_2CN, H_2O, 45 °C, 48%; (b) Ac_2O, $AlCl_3$, $CHCl_3$, 0 °C, 10%; (c) Boc_2O, DMAP, THF, 60 °C, 22%; (d) MeLi, THF, −78 °C, 90%; (e) Et_3SiH, TFA, rt, 91%; (f) $(CF_3CO)_2O$, pyridine, $CHCl_3$, rt, 82%; (g) cyclopropylmethylbromide, NaI, NaH, DMF, 60 °C, 10%; (h) 2 M NaOH, MeOH, rt, 100%; (i) 3-trifluoromethylbenzoyl chloride, Et_3N, $CHCl_3$, rt, 22%.

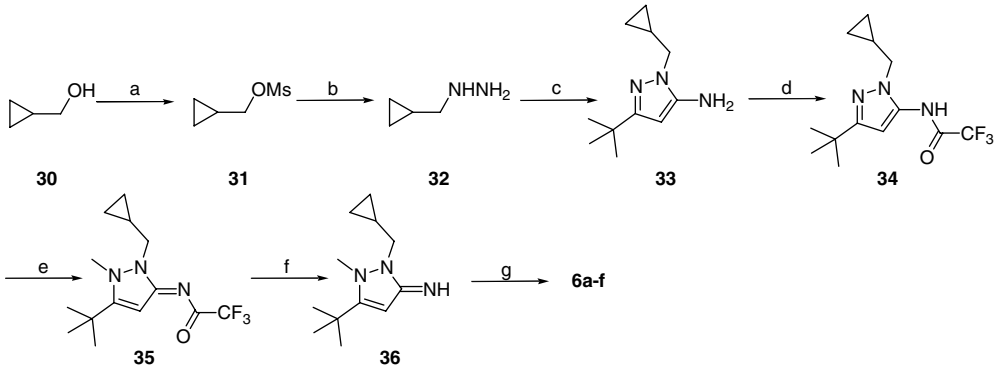


Scheme 3. Reagents and conditions: (a) $POCl_3$, dichloroethane, $(H_2NOH)_2H_2SO_4$, DMF, 85 °C, 100%; (b) (i) NaS; 1 M HCl, EtOH, rt; (ii) NH_3aq, NaClO, rt, 9%; (c) $(CF_3CO)_2O$, pyridine, $CHCl_3$, rt, 82%; (d) cyclopropylmethylbromide, NaI, NaH, DMF, 60 °C, 17%; (e) K_2CO_3, MeOH, H_2O, rt, 95%; (f) 3-trifluoromethylbenzoyl chloride, Et_3N, $CHCl_3$, rt, 44%.

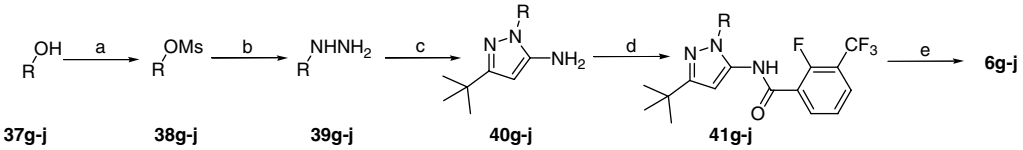


Scheme 4. Reagents and conditions: (a) $(CF_3CO)_2O$, pyridine, $CHCl_3$, rt, 98%; (b) cyclopropylmethylbromide, NaI, NaH, DMF, rt, 11%; (c) K_2CO_3, MeOH, H_2O, rt, 100%; (d) 3-trifluoromethylbenzoyl chloride, Et_3N, $CHCl_3$, rt, 52%.

In a previous paper we reported the synthesis and SARs of thiazole derivative **1** and its analogue, potent CB_2 receptor agonists.[12] However, we had not assessed chemical modifications of the 5-membered heteroring of **1**. Since we thought that the 5-membered heteroring acted as a linker and that fine adjustment of the size of heteroring would affect affinity for the CB_2 receptor, we tried chemically modifying the 5-membered heteroring of **1** in attempt to further improve binding affinity for the CB_2 receptor. A previous SAR study of thiazole derivatives suggested (1) that introduction of a cyclopropylmethyl group at the 3-position of the thiazole ring was suitable to obtain a high affinity for the CB_2 receptor, (2) that the presence of a bulky lipophilic group like *tert*-butyl at the 5-position of the thiazole ring was important to obtaining high affinity for the CB_2 receptor, and (3) that a 3-trifluoromethylphenyl amide yielded high affinity and selectivity for the CB_2 receptor. As shown in Table 1, we assessed 5-membered heteroring derivatives which had a cyclopropylmethyl group, a *tert*-butyl group, and a 3-trifluoromethylphenyl amide at corresponding positions, respectively. We synthesized 5-isoproryl oxazole instead of 5-*tert*-butyl oxazole because of the difficulty of synthesizing. The oxazole (**3**),



Scheme 5. Reagents and conditions: (a) MsCl, Et_3N, $CHCl_3$, rt, 96%; (b) $NH_2NH_2H_2O$, EtOH, rt, 53%; (c) pivaloylacetonitrile, EtOH, reflux, 42%; (d) $(CF_3CO)_2O$, pyridine, $CHCl_3$, rt, 100%; (e) Me_2SO_4, $NaHCO_3$, toluene, 80 °C, 14%; (f) 2 M NaOH, MeOH, rt, 100%; (g) ArCOCl, Et_3N, $CHCl_3$, rt.



Scheme 6. Reagents and conditions: (a) MsCl, Et_3N, $CHCl_3$, rt; (b) $NH_2NH_2H_2O$, EtOH, rt; (c) pivaloylacetonitrile, EtOH, reflux; (d) 2-fluoro-3-trifluoromethylbenzoyl chloride, Et_3N, $CHCl_3$, rt; (e) Me_2SO_4, $NaHCO_3$, toluene, 80 °C.

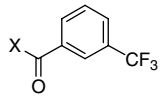
isothiazole (**4**), isoxazole (**5**), and pyrazole (**6a**) derivatives had good affinity for the CB_2 receptor (**3**: IC_{50} = 24 nM, **4**: IC_{50} = 9.5 nM, **5**: IC_{50} = 46 nM, and **6a**: IC_{50} = 51 nM), but the thiadiazole derivative (**2**) exhibited lower affinity for the CB_2 receptor than thiazole derivative (**1** vs **2**). These findings suggested that CB_2 binding affinity increased as the size of the 5-membered heteroring increased (ring size: **6a** < **3**, **5** < **1**, **4**), but that too large heteroring was unsuitable for obtaining high affinity for the CB_2 receptor. The 5-membered heteroring acts as a linker, and fine adjustment of the size of the heteroring affected affinity for the CB_2 receptor.

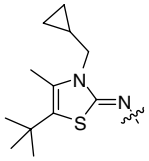
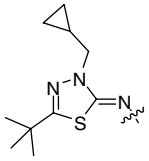
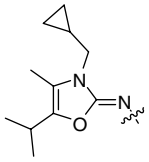
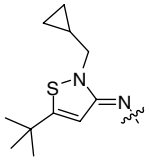
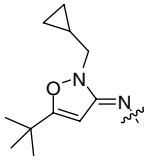
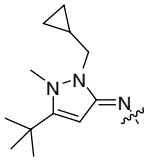
Compounds **1**–**5** are neutral lipophilic molecules and have very low solubility in water (solubility in water **1**: <0.01 mg/100 mL at 25 °C). By contrast, pyrazole derivative **6a** is basic molecule and will probably have good solubility in water. We tried assessing its analogues in attempt to discover compounds with high affinity for the CB_2 receptor and good physicochemical properties, using **6a** as lead compound. Since a previous SAR study of thiazole derivatives suggested that the 2-substituted-5-trifluoromethylphenyl or 2-substituted-3-trifluoromethylphenyl amide exhibited high affinity for the CB_2 receptor,[12] we examined 2,3- or 2,5-disubstituted phenyl compounds **6a**–**f** (Table 2). The 2,3-disubstituted phenyl compounds (**6b**–**d**) exhibited higher CB_2 binding affinity than 2,5-disubstituted phenyl compounds (**6e**–**f**), and compounds containing electron-withdrawing groups, such as fluorine and chlorine, had higher affinity than compounds containing electron-donating groups, such as methyl group (**6b**–**c** vs **6d**, **6e** vs **6f**). 2-Fluoro-3-trifluoromethylphenyl compound **6b** exhibited particularly high affinity and selectivity for the CB_2 receptor and had good solubility in water (solubility in water **6b**: 5.9 mg/100 mL at 25 °C). These findings suggested that the electron density on the benzene ring affects the interaction between the CB_2 receptor and its ligand. Next modification focused on the cyclopropylmethyl group at the 2-position of the pyrazole ring. Compounds that contained a cycloalkyl group larger than a cyclopropylmethyl group exhibited high CB_2 binding affinity (**6g**–**h**). Alkoxyalkyl groups, which were suitable functional groups at the 3-position of thiazole derivatives for obtaining high affinity for the CB_2 receptor,[12] were evaluated. Compounds **6i**–**j** exhibited good affinity for the CB_2 receptor, the same as compound **6b**, but they exhibited lower selectivity for the CB_2 receptor than **6b**, and compounds **6h** and **6j** were partial agonists (**6h**: E_{max} = 67%, **6j**: E_{max} = 76%). The results of structural transformations indicated that the most suitable functional group at this site for selectivity and agonist activity was a cyclopropylmethyl group (**6b**).

Compound **6b**, which showed high affinity and selectivity for the CB_2 receptor and had good solubility in water, exhibited good metabolic stability in human and rat liver microsomes (human metabolic stability 92% and rat metabolic stability 86%).[17] we therefore evaluated plasma levels of **6b**. Table 3 shows the pharmacokinetic parameters of **6b** following oral administration in rats at a dose of 10 mg/kg.

The maximum plasma concentration (C_{max}) of 545 ng/mL was reached 1.67 h after the oral administration of **6b** at a dose of 10 mg/kg. The C_{max} of **6b** was about twofold higher than that of thiazole derivative **1**,[12] and the corresponding $AUC_{0-24\ h}$ value was 2160 ng h/mL. The oral administration of **6b** at a dose of 10 mg/kg (po) in rats resulted in significant plasma concentration.

Table 1. Pharmacological profile of imine derivatives



Compound	X	Binding affinity IC_{50} (nM)		Agonist activity	
		CB_2	CB_1	CB_2 EC_{50} (nM)	E_{max}[a] (%)
1		13	3500	10	91
2		300	—	—	—
3		24	—	—	—
4		9.5	—	—	—
5		46	—	—	—
6a		51	—	—	—

[a] Compared to maximal response of CP 55,940, a full agonist of human CB_2 receptor.

Compound **6b** also showed high affinity for the rat CB_2 receptor, displacing [^{3}H]CP 55,940 binding to rat spleen membranes with an IC_{50} value of 1.4 nM, and thus **6b** did not show species differences in affinity for the CB_2 receptor. Thus, the analgesic effect of compound **6b** was assessed in the Randall–Selitto test in rats (Fig. 2).[18] Oral administration of **6b** at 10 mg/kg and 30 mg/kg dose-dependently and significantly reversed mechanical hyperalgesia in the Randall–Selitto test at 1 h after administration, and the antinociceptive effect lasted at least 3 h after administration. These results indicate that compound **6b** has an analgesic effect in rats.

4. Conclusions

In this paper, we reported the synthesis and SARs of novel CB_2 receptor agonists. The SAR study showed that the 5-membered heteroring acted as a linker, and that the functional groups at the 2-position in the pyrazole ring and the Ar group in the amide greatly affected affinity and selectivity for the CB_2 receptor. It was also found (1) that CB_2 binding affinity increased as the size of 5-membered heteroring increased, but that too large size of a heteroring was unsuitable for achieving high affinity for the CB_2 receptor, (2) that introduction of a cyclopropylmethyl group at the 2-position of the pyrazole ring is important to obtaining high selectivity for the CB_2 receptor, and (3) that a 2-substituted-3-trifluoromethylphenyl amide yielded high affinity for the CB_2 receptor. These studies led to compound **6b** (CBS0550), which showed combine good potency for the CB_2 receptor and excellent pharmacokinetics in rats. Furthermore, when administered orally, compound **6b** significantly reversed mechanical hyperalgesia in the Randall–Selitto model of inflammatory pain in rats. We think that CBS0550 may be useful for exploring the function of the CB_2 receptor and for the treatment of pain.

5. Experimental

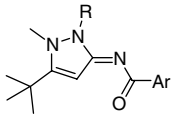
5.1. Chemistry

Melting points were determined on a Mettler FP-61 or a Yanaco MP-500D melting point apparatus. NMR spectra were recorded at 200, 300 or 600 MHz using a Varian Instruments Gemini 2000, a Varian Instruments INOVA 300 or a JEOL ECA600 with tetramethylsilane as an internal standard. Electron impact (EI) mass spectra and Chemical ionization (CI) were taken on a Micromass Platform GCI mass spectrometer. Electrospray ionization (ESI) mass spectra were taken on a Micromass Platform LC mass spectrometer. Elemental analyses were performed on EA2400 elemental analyzers, and the results were within 0.4% of calculated values. Reactions were monitored by TLC analysis using Merck silica gel 60F-254 thin-layer plates. Column chromatography was carried out on silica gel Wako Pure Chemical C-200 and NH silica gel Fuji Silicia chromatorex DM1020.

5.1.1. *N*-(5-*tert*-Butyl-1,3,4-thiadiazol-2-yl)-2,2,2-trifluoroacetamide (8). To a mixture of **7** (3.0 g, 19 mmol) and toluene (15 mL) was added trifluoroacetic anhydride (4.8 g, 23 mmol) with ice-cooling and the mixture was stirred for 1 h. To the reaction mixture was added water and the mixture was extracted with ethyl acetate. The organic layer was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 7:1) to give 4.4 g (yield; 91%) of **8** as colorless powder: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.48 (s, 9H); MS(ESI) *m*/*z* 254 (M+H).

5.1.2. *N*-[5-*tert*-Butyl-3-(cyclopropylmethyl)-1,3,4-thiadiazol-2(3*H*)-ylidene]-2,2,2-trifluoroacetamide (9). To a mixture of **8** (1.0 g, 4.0 mmol), potassium carbonate

Table 2. Pharmacological profile of pyrazole derivatives



Compound	Ar	R	Binding affinity IC_{50} (nM)		Agonist activity	
			CB_2	CB_1	CB_2 EC_{50} (nM)	E_{max}[a] (%)
6a	3-CF_3–Ph	CH_2-(cyclopropyl)	51	—	—	—
6b	2-F-3-CF_3–Ph	CH_2-(cyclopropyl)	2.9	4000	1.8	85
6c	2-Cl-3-CF_3–Ph	CH_2-(cyclopropyl)	3.7	2200	1.5	82
6d	2-Me-3-CF_3–Ph	CH_2-(cyclopropyl)	11	1300	4.4	73
6e	2-F-5-CF_3–Ph	CH_2-(cyclopropyl)	23	3700	8.8	90
6f	2-Me-5-CF_3–Ph	CH_2-(cyclopropyl)	88	—	—	—
6g	2-F-3-CF_3–Ph	CH_2-(cyclobutyl)	1.7	940	0.32	96
6h	2-F-3-CF_3–Ph	CH_2-(cyclopentyl)	1.1	810	0.79	67
6i	2-F-3-CF_3–Ph	C_2H_4–OEt	3.7	870	1.1	82
6j	2-F-3-CF_3–Ph	C_2H_4–O^{i}Pr	2.6	760	0.85	76
CP 55,940			0.82	2.1	0.86	100

[a] Compared to maximal response of CP 55,940, a full agonist of human CB_2 receptor.

Table 3. Pharmacokinetic parameters of **6b** following oral administration to rats at a dose of 10 mg/kg

Compound	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	$t_{1/2}$ (h)	$AUC_{0-8\ h}$ (ng h/mL)
6b	10	1.67 ± 0.58	545 ± 170	2.47 ± 0.88	2160 ± 293

Each value represents the mean ± SD of three animals.

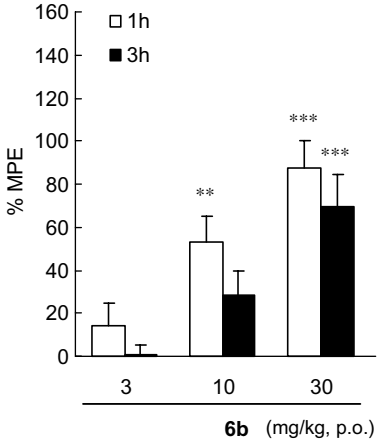


Figure 2. Effect of **6b** (3, 10, and 30 mg/kg, po) in the Randall–Selitto model of inflammatory pain in rats. Data are expressed as means ± SEM of seven animals. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle-treated animals (Dunnett's test). %MPE = (nociceptive threshold post drug − nociceptive threshold control)/(nociceptive threshold baseline − nociceptive threshold control) × 100.

(0.66 g, 4.8 mmol), sodium iodide (0.060 g, 0.40 mmol), and DMF (10 mL) was added cyclopropylmethyl bromide (0.64 g, 4.7 mmol) and the mixture was stirred for overnight. To the reaction mixture was added 2 M HCl and the mixture was extracted with ethyl acetate. The organic layer was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 6:1) to give 1.1 g (yield; 91%) of **9** as colorless oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.42–0.69 (m, 4H), 1.24–1.42 (m, 1H), 1.43 (s, 9H), 4.25 (d, J = 7.5 Hz, 2H); MS(ESI) *m*/*z* 308 (M+H).

5.1.3. 5-*tert*-Butyl-3-(cyclopropylmethyl)-1,3,4-thiadiazol-2(3*H*)-imine (10). To a mixture of **8** (1.1 g, 3.6 mmol) and THF (10 mL) was added 2 M NaOH (5.0 mL, 10 mmol), and the mixture was stirred at 65 °C for 1 h. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 7:1) to give 0.75 g (yield; 99%) of **9** as colorless oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.30–0.59 (m, 4H), 1.26 (s, 9H), 1.16–1.37 (m, 1H), 3.73 (d, J = 7.0 Hz, 2H); MS(ESI) *m*/*z* 212 (M+H).

5.1.4. *N*-[5-*tert*-Butyl-3-(cyclopropylmethyl)-1,3,4-thiadiazol-2(3*H*)-ylidene]-3-(trifluoromethyl)benzamide (2). To a mixture of **10** (0.10 g, 0.47 mmol), triethylamine (0.027 g, 0.47 mmol), and $CHCl_3$ (1.0 mL) was added 3-trifluoromethylbenzoyl chloride (0.098 g, 0.47 mmol) and the mixture was stirred for 1 h. To the reaction mixture was added 2 M NaOH and the mixture was extracted with $CHCl_3$. The organic layer was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 2:1) to give 0.090 g (yield; 49%) of **2** as colorless oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.50–0.68 (m, 4H), 1.36–1.52 (m, 1H), 1.44 (s, 9H), 4.32 (d, J = 7.5 Hz, 2H), 7.57 (t, J = 7.7 Hz, 1H), 7.75 (d, J = 7.7 Hz, 1H), 8.47 (d, J = 7.7 Hz, 1H), 8.57 (s, 1H); MS(ESI) *m*/*z* 384 (M+H); Anal. Calcd for $C_{18}H_{20}F_3N_3OS$: C, 56.38; H, 5.26; N, 10.96. Found: C, 56.39; H, 5.24; N, 10.72.

5.1.5. 2-Amino-4-methyl-1,3-oxazole (12). To a mixture of **11** (180 g, 2.4 mol) and H_2O (390 mL) was added cyanamide (100 g, 2.4 mol) and the mixture was stirred at 45 °C for 30 min. To the reaction mixture was added 2 M NaOH and the mixture was extracted with Et_2O. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 110 g (yield; 48%) of **12** as yellow oil: ^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 2.03 (s, 3H), 4.52 (br s, 2H), 6.82–6.97 (m, 1H); MS(ESI) *m*/*z* 99 (M+H).

5.1.6. 1-(2-Amino-4-methyl-1,3-oxazol-5-yl)ethanone (13). To a mixture of **12** (50 g, 0.51 mol), aluminum chloride (140 g, 1.0 mol), and $CHCl_3$ (1.0l) was added dropwise acetic anhydride (52 g, 0.51 mol) in $CHCl_3$ (500 mL) with ice-cooling and the mixture was stirred for 6 h. To the reaction mixture was added saturated aqueous $NaHCO_3$ with ice-cooling and the precipitate was filtered off through Celite pad, the filtrate was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated in vacuo, and washed with Et_2O to give 5.7 g (yield; 10%) of **13** as yellow solid: ^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 2.36 (s, 3H), 2.41 (s, 3H), 5.02 (br s, 2H); MS(ESI) *m*/*z* 141 (M+H).

5.1.7. *tert*-Butyl (5-acetyl-4-methyl-1,3-oxazol-2-yl)carbamate (14). To a mixture of **13** (3.6 g, 26 mmol), dimethylaminopyridine (0.31 g, 2.6 mmol), and THF (200 mL) was added di-*tert*-butyldicarbonate (32 g, 150 mmol) and the mixture was stirred at 60 °C for 30 min. The reaction mixture was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:1) to give colorless solid. The solid was washed with IPE to give 1.4 g (yield; 22%) of **14** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.55 (s, 9H), 2.45 (s, 3H), 2.49 (s, 3H), 8.98 (br s, 1H); MS(ESI) *m*/*z* 239 (M−H).

5.1.8. *tert*-butyl [5-(1-Hydroxy-1-methylethyl)-4-methyl-1,3-oxazol-2-yl]carbamate (15). To a mixture of **14** (1.4 g, 5.7 mmol) and THF (30 mL) was added dropwise MeLi (1.0 M Et_2O solution, 13 mL, 13 mmol) at −78 °C and the mixture was stirred for 1 h. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:1) to give 1.3 g (yield; 90%) of **15** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.52 (s, 9H), 1.60 (s, 6H), 2.24 (s, 3H); MS(ESI) *m*/*z* 257 (M+H).

5.1.9. 2-Amino-4-methyl-5-(1-methylethyl)-1,3-oxazole (16). To a mixture of **15** (1.3 g, 5.1 mmol) and TFA (15 mL) was added triethylsilane (1.2 g, 7.7 mmol) and the mixture was stirred for 2.5 h. To the reaction mixture was added saturated aqueous $NaHCO_3$ and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: $CHCl_3$/MeOH = 10:1) to give 0.65 g (yield; 91%) of **15** as pale yellow solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.18 (d, J = 7.0 Hz, 6H), 1.97 (s, 3H), 2.87 (quin, J = 7.0 Hz, 1H), 4.34 (br s, 2H); MS(ESI) *m*/*z* 141 (M+H).

5.1.10. 2,2,2-Trifluoro-*N*-[4-methyl-5-(1-methylethyl)-1,3-oxazol-2-yl]acetamide (17). To a mixture of **16** (0.65 g, 4.6 mmol), pyridine (0.40 g, 5.1 mmol), and $CHCl_3$ (10 mL) was added trifluoroacetic anhydride (1.1 g, 5.1 mmol) with ice-cooling and the mixture was stirred at room temperature for 1 h. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and washed with hexane and IPE to give 0.90 g (yield; 82%) of **15** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.27 (d, J = 7.0 Hz, 6H), 2.17 (s, 3H), 2.96 (quin, J = 7.0 Hz, 1H); MS(ESI) *m*/*z* 237 (M+H).

5.1.11. *N*-[3-(Cyclopropylmethyl)-4-methyl-5-(1-methylethyl)-1,3-oxazol-2(3*H*)-ylidene]-2,2,2-trifluoroacetamide (18). To a mixture of **17** (0.77 g, 3.3 mmol) and sodium iodide (0.049 g 0.33 mmol) in DMF (15 mL) was added sodium hydride (0.13 g, 3.3 mmol), and the mixture was stirred for 5 min. To the reaction mixture was added cyclopropylmethyl bromide (0.66 g, 4.9 mmol), and the reaction mixture was stirred at 60 °C for 2 h. To the reaction mixture was added H_2O, and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: ethyl acetate) to give 0.090 g (yield; 10%) of **18** as brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.38–0.66 (m, 4H), 1.04–1.22 (m, 1H), 1.30 (d, J = 7.0 Hz, 6H), 2.15 (s, 3H), 2.84–3.03 (m, 1H), 3.68 (d, J = 7.0 Hz, 2H); MS(ESI) *m*/*z* 291 (M+H).

5.1.12. 3-(Cyclopropylmethyl)-4-methyl-5-(1-methylethyl)-1,3-oxazol-2(3*H*)-imine (19). To a mixture of **18** (0.090 g, 0.31 mmol) and MeOH (2.0 mL) was added 2 M NaOH (2.0 mL, 2.0 mmol) and the mixture was stirred for 3 h. The reaction mixture was evaporated in vacuo and extracted with Et_2O. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 0.062 g (yield; 100%) of **19** as yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.26–0.62 (m, 4H), 1.05–1.27 (m, 1H), 1.16 (d, J = 7.0 Hz, 6H), 1.93 (s, 3H), 2.60–2.83 (m, 1H), 3.39 (d, J = 6.6 Hz, 2H); MS(ESI) *m*/*z* 195 (M+H).

5.1.13. *N*-[3-(Cyclopropylmethyl)-4-methyl-5-(1-methylethyl)-1,3-oxazol-2(3*H*)-ylidene]-3-(trifluoromethyl)benzamide (3). To a mixture of **19** (0.020 g, 0.10 mmol), triethylamine (0.011 g, 0.11 mmol), and $CHCl_3$ (2.0 mL) was added 3-trifluoromethylbenzoyl chloride (0.023 g, 0.47 mmol) with ice-cooling and the mixture was stirred at room temperature for overnight. To the reaction mixture was added saturated aqueous $NaHCO_3$ and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 0.0080 g (yield; 22%) of **3** as colorless solid: mp 65–67 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.42–0.68 (m, 4H), 1.14–1.36 (m, 1H),

1.31 (d, J = 7.0 Hz, 6H), 2.14 (s, 3H), 2.86–3.05 (m, 1H), 3.74 (d, J = 7.0 Hz, 2H), 7.44–7.57 (m, 1H), 7.64–7.73 (m, 1H), 8.37–8.44 (m, 1H), 8.47–8.55 (m, 1H); MS(ESI) m/z 367 (M+H); Anal. Calcd for $C_{19}H_{21}F_3N_2O_2$: C, 62.29; H, 5.78; N, 7.65. Found: C, 62.17; H, 5.78; N, 7.58.

5.1.14. (2*Z*)-3-Chloro-4,4-dimethylpent-2-enenitrile (21). $POCl_3$ (74 g, 480 mmol) was added dropwise to DMF (38 g, 520 mmol) with ice-cooling. To the reaction mixture was added pinacolone (20 g, 200 mmol) in 1,2-dichloroethane (32 mL) and the reaction mixture was stirred at 75 °C for 4 h. To the reaction mixture was added hydroxylamine sulfate (79 g, 480 mmol) in 1,2-dichloroethane (150 mL) and the reaction mixture was stirred at 85 °C for 1 h. The mixture was partitioned between H_2O and hexane. The separated organic phase was dried over sodium sulfate and evaporated in vacuo to give 29 g (yield; 100%) of **21** as yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.25 (s, 9H), 5.56 (s, 1H); MS(ESI) m/z 144 (M+H).

5.1.15. 3-Amino-5-*tert*-butylisothiazole (22). To a mixture of **21** (12 g, 80 mmol) and EtOH (63 mL) were added dropwise sodium sulfide (6.5 g, 83 mmol) and 1 M HCl (84 mL, 84 mmol) with ice-cooling and the mixture was stirred at room temperature for 2 h. To the reaction mixture was added dropwise NH_2Cl, which was prepared from 24% NH_3 (50 mL) and 5.3% NaClO (120 mL), with ice-cooling and the reaction mixture was stirred at room temperature for overnight. To the reaction mixture was added 6 M HCl with ice-cooling and the mixture was washed with Et_2O. The water phase was added into 6 M NaOH with ice-cooling and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 1.2 g (yield; 9%) of **22** as yellow solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.36 (s, 9H), 4.28 (br s, 2H), 6.28 (s, 1H); MS(ESI) m/z 157 (M+H).

5.1.16. *N*-(5-*tert*-Butylisothiazol-3-yl)-2,2,2-trifluoroacetamide (23). To a mixture of **22** (0.60 g, 3.8 mmol), pyridine (0.33 g, 4.2 mmol), and $CHCl_3$ (6.0 mL) was added trifluoroacetic anhydride (0.89 g, 4.2 mmol) and the mixture was stirred for 30 min. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 0.92 g (yield; 82%) of **23** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.44 (s, 9H), 7.67 (s, 1H), 9.61 (br s, 1H); MS(ESI) m/z 253 (M+H).

5.1.17. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)isothiazol-3(2*H*)-ylidene]-2,2,2-trifluoroacetamide (24). To a mixture of **23** (0.92 g, 3.7 mmol) and sodium iodide (0.055 g 0.37 mmol) in DMF (5.0 mL) was added sodium hydride (0.15 g, 3.7 mmol), and the mixture was stirred for 5 min. To the reaction mixture was added cyclopropylmethyl bromide (0.74 g, 5.5 mmol), and the reaction mixture was stirred at 60 °C for 4 h. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with ethyl acetate. The organic layer was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 4:1) to give 0.19 g (yield; 17%) of **24** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.44–0.82 (m, 4H), 1.08–1.36 (m, 1H), 1.43 (s, 9H), 4.01 (d, J = 7.5 Hz, 2H), 7.76 (s, 1H); MS(ESI) m/z 307 (M+H).

5.1.18. 5-*tert*-Butyl-2-(cyclopropylmethyl)isothiazol-3(2*H*)-imine (25). To a mixture of **24** (0.19 g, 0.62 mmol), MeOH (5.0 mL), and H_2O (2.0 mL) was added potassium carbonate (0.26 g, 1.7 mmol) and the mixture was stirred for 1 h. The mixture was partitioned between 2 M NaOH and $CHCl_3$. The separated organic phase was dried over sodium sulfate and evaporated in vacuo to give 0.12 g (yield; 95%) of **25** as colorless oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.27–0.74 (m, 4H), 0.99–1.24 (m, 1H), 1.29 (s, 9H), 3.45 (d, J = 7.5 Hz, 2H), 5.85 (s, 1H); MS(ESI) m/z 211 (M+H).

5.1.19. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)isothiazol-3(2*H*)-ylidene]-3-(trifluoromethyl)benzamide (4). To a mixture of **25** (0.040 g, 0.19 mmol), triethylamine (0.019 g, 0.19 mmol), and $CHCl_3$ (1.0 mL) was added 3-trifluoromethylbenzoyl chloride (0.040 g, 0.19 mmol) and the mixture was stirred for 1 h. To the reaction mixture was added H_2O and the mixture was extracted with $CHCl_3$. The organic layer was washed with 2 M NaOH and 2 M HCl, dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 0.032 g (yield; 44%) of **4** as colorless solid: mp 54–55 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.44–0.58 (m, 2H), 0.68–0.83 (m, 2H), 1.21–1.38 (m, 1H), 1.43 (s, 9H), 4.05 (d, J = 7.5 Hz, 2H), 7.52 (t, J = 7.5 Hz, 1H), 7.69 (d, J = 7.5 Hz, 1H), 7.86 (s, 1H), 8.46 (d, J = 7.5 Hz, 1H), 8.57 (s, 1H); MS(ESI) m/z 383 (M+H); Anal. Calcd for $C_{19}H_{21}F_3N_2OS$: C, 59.67; H, 5.53; N, 7.32. Found: C, 59.75; H, 5.55; N, 7.16.

5.1.20. *N*-(5-*tert*-Butylisoxazol-3-yl)-2,2,2-trifluoroacetamide (27). To a mixture of **26** (5.0 g, 36 mmol), pyridine (3.1 g, 39 mmol), and $CHCl_3$ (80 mL) was added trifluoroacetic anhydride (8.3 g, 439 mmol) and the mixture was stirred for 30 min. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 8.2 g (yield; 98%) of **27** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δppm 1.37 (s, 9H), 6.70 (s, 1H), 9.75 (br s, 1H); MS(ESI) m/z 235 (M−H).

5.1.21. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)isoxazol-3(2*H*)-ylidene]-2,2,2-trifluoroacetamide (28). To a mixture of **27** (5.0 g, 21 mmol) and sodium iodide (0.32 g 2.1 mmol) in DMF (45 mL) was added sodium hydride (0.85 g, 21 mmol), and the mixture was stirred for 20 min. To the reaction mixture was added cyclopropylmethyl bromide (4.3 g, 32 mmol), and the reaction mixture was stirred for 4 days. To the reaction mixture was added H_2O and the mixture was extracted with ethyl acetate. The

organic layer was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 0.68 g (yield; 11%) of **28** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.40–0.70 (m, 4H), 1.14–1.32 (m, 1H), 1.36 (s, 9H), 4.13 (d, J = 7.0 Hz, 2H), 7.07 (s, 1H); MS(ESI) *m*/*z* 291 (M+H).

5.1.22. 5-*tert*-Butyl-2-(cyclopropylmethyl)isoxazol-3(2*H*)-imine (29). To a mixture of **28** (0.15 g, 0.52 mmol), MeOH (5.0 mL), and H_2O (2.0 mL) was added potassium carbonate (0.23 g, 1.6 mmol) and the mixture was stirred for 30 min. The mixture was partitioned between 2 M NaOH and Et_2O. The separated organic phase was dried over sodium sulfate and evaporated in vacuo to give 0.12 g (yield; 100%) of **25** as yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.25–0.61 (m, 4H), 1.04–1.21 (m, 1H), 1.24 (s, 9H), 3.56 (d, J = 7.0 Hz, 2H), 5.30 (s, 1H); MS(ESI) *m*/*z* 195 (M+H).

5.1.23. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)isoxazol-3(2*H*)-ylidene]-3-(trifluoromethyl)benzamide (5). To a mixture of **29** (0.040 g, 0.21 mmol), triethylamine (0.023 g, 0.23 mmol), and $CHCl_3$ (2.0 mL) was added 3-trifluoromethylbenzoyl chloride (0.048 g, 0.23 mmol) and the mixture was stirred for 1 h. To the reaction mixture was added saturated aqueous $NaHCO_3$ and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 0.040 g (yield; 52%) of **5** as pale yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.41–0.73 (m, 4H), 1.17–1.41 (m, 1H), 1.36 (s, 9H), 4.17 (d, J = 7.0 Hz, 2H), 7.17 (s, 1H), 7.46–7.57 (m, 1H), 7.65–7.73 (m, 1H), 8.38–8.46 (m, 1H), 8.48–8.57 (m, 1H); MS(ESI) *m*/*z* 367 (M+H); Anal. Calcd for $C_{19}H_{21}F_3N_2O_2$: C, 62.29; H, 5.78; N, 7.65. Found: C, 62.03; H, 5.80; N, 7.67.

5.1.24. Cyclopropylmethyl methanesulfonate (31). To a mixture of **30** (50 g, 690 mmol), triethylamine (0.023 g, 900 mmol), and $CHCl_3$ (200 mL) was added dropwise methanesulfonyl chloride (100 g, 900 mmol) with ice-cooling and the mixture was stirred at room temperature for 3 h. To the reaction mixture was added H_2O and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 100 g (yield; 96%) of **31** as brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.31–0.78 (m, 4H), 1.11–1.37 (m, 1H), 3.03 (s, 3H), 4.09 (d, J = 7.5 Hz, 2H); MS(ESI) *m*/*z* 151 (M+H).

5.1.25. (Cyclopropylmethyl)hydrazine (32). To a mixture of **31** (100 g, 660 mmol) and EtOH (200 mL) was added hydrazine hydrate (200 g, 4000 mmol) with ice-cooling and the mixture was stirred at room temperature for overnight. The reaction mixture was evaporated in vacuo and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 30 g (yield; 53%) of **32** as yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.09–0.23 (m, 2H), 0.43–0.61 (m, 2H), 0.82–1.05 (m, 1H), 2.64 (d, J = 6.6 Hz, 2H); MS(ESI) *m*/*z* 87 (M+H).

5.1.26. 5-Amino-3-*tert*-Butyl-1-(cyclopropylmethyl)-1*H*-pyrazole (33). A mixture of **32** (30 g, 350 mmol) and pivaloylacetonitrile (44 g, 350 mmol) in EtOH (230 mL) was heated at reflux for 5 h. The reaction mixture was evaporated in vacuo and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:1) to give 22 g (yield; 42%) of **33** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.27–0.39 (m, 2H), 0.48–0.62 (m, 2H), 1.10–1.31 (m, 1H), 1.26 (s, 9H), 3.42 (br s, 2H), 3.86 (d, J = 6.2 Hz, 2H), 5.42 (s, 1H); MS(ESI) *m*/*z* 194 (M+H).

5.1.27. *N*-[3-*tert*-Butyl-1-(cyclopropylmethyl)-1*H*-pyrazol-5-yl]-2,2,2-trifluoroacetamide (34). To a mixture of **33** (1.0 g, 5.2 mmol), pyridine (0.41 g, 5.2 mmol), and $CHCl_3$ (10 mL) was added dropwise trifluoroacetic anhydride (1.10 g, 5.17 mmol) with ice-cooling and the mixture was stirred at room temperature for 2 h. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 1.6 g (yield; 100%) of **34** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.28–0.45 (m, 2H), 0.62–0.77 (m, 2H), 1.03–1.30 (m, 1H), 1.29 (s, 9H), 3.96 (d, J = 6.6 Hz, 2H), 6.37 (s, 0H), 8.10 (br s, 1H); MS(ESI) *m*/*z* 290 (M+H).

5.1.28. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2,2,2-trifluoroacetamide (35). To a mixture of **34** (1.6 g, 5.2 mmol), sodium hydrogen carbonate (1.3 g, 16 mmol), and toluene (30 mL) was added dimethyl sulfate (2.0 g, 16 mmol) and the mixture was stirred at 80 °C for overnight. To the reaction mixture was added H_2O and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: $CHCl_3$/MeOH = 3:1) to give 0.72 g (yield; 14%) of **35** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δppm 0.50–0.65 (m, 4H), 0.90–1.18 (m, 1H), 1.43 (s, 9H), 3.90 (s, 3H), 4.21 (d, J = 7.0 Hz, 2H), 6.94 (s, 1H); MS(ESI) *m*/*z* 304 (M+H).

5.1.29. 5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-imine (36). To a mixture of **35** (0.21 g, 0.69 mmol) and MeOH (5.0 mL) was added between 2 M NaOH (2.5 mL, 5.0 mmol) and the mixture was stirred at 70 °C for 4 h. The mixture was partitioned 2 M NaOH and $CHCl_3$, and separated organic phase was dried over sodium sulfate and evaporated in vacuo to give 0.17 g (yield; 100%) of **36** as yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.26–0.58 (m, 4H), 0.93–1.18 (m, 1H), 1.29 (s, 9H), 3.16 (s, 3H), 3.62 (d, J = 6.6 Hz, 2H), 5.29 (br s, 1H); MS(ESI) *m*/*z* 208 (M+H).

5.1.30. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-3-(trifluoromethyl)benzamide (6a). To a mixture of **36** (0.017 g, 0.082 mmol), triethylamine (0.0091 g, 0.090 mmol), and $CHCl_3$ (1.0 mL) was added 3-trifluoromethylbenzoyl chloride (0.019 g, 0.0090 mmol) and the mixture was stirred for 1 h. The reaction mixture was evaporated in vacuo

and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:10) to give 0.010 g (yield; 32%) of **6a** as colorless solid: mp 97–99 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.51–0.69 (m, 4H), 1.00–1.29 (m, 1H), 1.45 (s, 9H), 3.85 (s, 3H), 4.30 (d, J = 7.0 Hz, 2H), 7.10 (s, 1H), 7.40–7.55 (m, 1H), 7.56–7.68 (m, 1H), 8.35–8.47 (m, 1H), 8.48–8.58 (m, 1H); MS(ESI) m/z 380 (M+H); Anal. Calcd for $C_{20}H_{24}F_3N_3O{\cdot}1/4H_2O$: C, 62.57; H, 6.43; N, 10.94. Found: C, 62.90; H, 6.36; N, 10.89.

5.1.31. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-fluoro-3-(trifluoromethyl)benzamide (6b). As colorless solid: mp 104–106 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.51–0.65 (m, 4H), 1.01–1.19 (m, 1H), 1.45 (s, 9H), 3.86 (s, 3H), 4.26 (d, J = 7.0 Hz, 2H), 7.07 (s, 1H), 7.13–7.25 (m, 1H), 7.49–7.63 (m, 1H), 8.07–8.22 (m, 1H); MS(ESI) m/z 398 (M+H); Anal. Calcd for $C_{20}H_{23}F_4N_3O$: C, 60.44; H, 5.83; N, 10.57. Found: C, 60.48; H, 5.74; N, 10.59.

5.1.32. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-chloro-3-(trifluoromethyl)benzamide (6c). As colorless solid: mp 145–146 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.47–0.62 (m, 4H), 1.00–1.16 (m, 1H), 1.45 (s, 9H), 3.85 (s, 3H), 4.21 (d, J = 7.0 Hz, 2H), 7.07 (s, 1H), 7.27–7.37 (m, 1H), 7.57–7.64 (m, 1H), 7.69–7.77 (m, 1H); MS(ESI) m/z 414 (M+H); Anal. Calcd for $C_{20}H_{23}ClF_3N_3O$: C, 58.04; H, 5.60; N, 10.15. Found: C, 58.08; H, 5.51; N, 10.20.

5.1.33. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-methyl-3-(trifluoromethyl)benzamide (6d). As colorless solid: mp 90-91 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.43–0.65 (m, 4H), 0.98–1.16 (m, 1H), 1.45 (s, 9H), 2.59–2.66 (m, 3H), 3.83 (s, 3H), 4.20 (d, J = 7.0 Hz, 2H), 7.06 (s, 1H), 7.17–7.28 (m, 1H), 7.51–7.58 (m, 1H), 7.68–7.77 (m, 1H); MS(ESI) m/z 394 (M+H); Anal. Calcd for $C_{21}H_{26}F_3N_3O{\cdot}1/4H_2O$: C, 63.38; H, 6.71; N, 10.56. Found: C, 63.24; H, 6.60; N, 10.54.

5.1.34. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-fluoro-5-(trifluoromethyl)benzamide (6e). As colorless solid: mp 124–126 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.53–0.65 (m, 4H), 1.03–1.18 (m, 1H), 1.45 (s, 9H), 3.86 (s, 3H), 4.27 (d, J = 7.0 Hz, 2H), 7.06–7.19 (m, 1H), 7.10 (s, 1H), 7.50–7.60 (m, 1H), 8.27–8.35 (m, 1H); MS(ESI) m/z 398 (M+H); Anal. Calcd for $C_{20}H_{23}F_4N_3O{\cdot}1/2H_2O$: C, 59.11; H, 5.95; N, 10.34. Found: C, 58.96; H, 5.74; N, 10.26.

5.1.35. *N*-[5-*tert*-Butyl-2-(cyclopropylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-methyl-5-(trifluoromethyl)benzamide (6f). As colorless solid: mp 156–157 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 0.50–0.66 (m, 4H), 1.02–1.17 (m, 1H), 1.45 (s, 9H), 2.68 (s, 3H), 3.84 (s, 3H), 4.24 (d, J = 6.6 Hz, 2H), 7.06 (s, 1H), 7.21–7.29 (m, 1H), 7.40–7.47 (m, 1H), 8.13–8.17 (m, 1H); MS(ESI) m/z 394 (M+H); Anal. Calcd for $C_{21}H_{26}F_3N_3O{\cdot}1/2H_2O$: C, 62.67; H, 6.67; N, 10.44. Found: C, 62.42; H, 6.64; N, 10.43.

5.1.36. Cyclobutylmethyl methanesulfonate (38g). To a mixture of **37g** (13 g, 150 mmol), triethylamine (20 g, 200 mmol), and $CHCl_3$ (44 mL) was added dropwise methanesulfonyl chloride (23 g, 200 mmol) with ice-cooling and the mixture was stirred at room temperature for 2 h. To the reaction mixture was added H_2O and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 27 g (yield; 100%) of **38g** as brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.72–2.23 (m, 6H), 2.60–2.84 (m, 1H), 3.01 (s, 3H), 4.19 (d, J = 6.6 Hz, 2H); MS(CI) m/z 165 (M+H).

5.1.37. (Cyclobutylmethyl)hydrazine (39g). To a mixture of **38g** (27 g, 150 mmol) and EtOH (45 mL) was added hydrazine hydrate (46 g, 920 mmol) and the mixture was stirred for over night. The reaction mixture was evaporated in vacuo and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate and evaporated in vacuo to give 20 g (yield; 100%) of **39g** as yellow oil: ^{1}H NMR (600 MHz, chloroform-*d*) δ ppm 1.61–1.73 (m, 2H), 1.79–1.98 (m, 2H), 2.00–2.12 (m, 2H), 2.40–2.55 (m, 1H), 2.79 (d, J = 7.3 Hz, 2H); MS(ESI) m/z 99 (M−H).

5.1.38. 5-Amino-3-*tert*-Butyl-1-(cyclobutylmethyl)-1*H*-pyrazole (40g). A mixture of **39g** (20 g, 150 mmol) and pivaloylacetonitrile (19 g, 150 mmol) in EtOH (110 mL) was heated at reflux for 2.5 h. The reaction mixture was evaporated in vacuo, purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:1), and washed with hexane and ethyl acetate to give 12 g (yield; 37%) of **40g** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.25 (s, 9H), 1.71–2.13 (m, 6H), 2.67–2.88 (m, 1H), 3.91 (d, J = 6.6 Hz, 2H), 5.40 (s, 1H); MS(ESI) m/z 208 (M+H).

5.1.39. *N*-[3-*tert*-Butyl-1-(cyclobutylmethyl)-1*H*-pyrazol-5-yl]-2-fluoro-3-(trifluoromethyl)benzamide (41g). To a mixture of **40g** (0.40 g, 1.9 mmol), triethylamine (0.22 g, 2.1 mmol), and $CHCl_3$ (8.0 mL) was added 2-fluoro-3-trifluoromethylbenzoyl chloride (0.48 g, 2.1 mmol) and the mixture was stirred for overnight. To the reaction mixture was added saturated aqueous NH_4Cl and the mixture was extracted with $CHCl_3$. The organic layer was dried over sodium sulfate, evaporated in vacuo, and purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 3:1) to give 0.61 g (yield; 79%) of **41g** as colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.31 (s, 9H), 1.71–2.14 (m, 6H), 2.70–2.92 (m, 1H), 4.04 (d, J = 7.0 Hz, 2H), 6.35 (s, 1H), 7.41–7.54 (m, 1H), 7.79–7.92 (m, 1H), 8.10–8.28 (m, 1H), 8.34–8.47 (m, 1H); MS(ESI) m/z 398 (M+H).

5.1.40. *N*-[5-*tert*-Butyl-2-(cyclobutylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-fluoro-3-(trifluoromethyl)benzamide (6g). To a mixture of **41g** (0.61 g, 1.5 mmol), sodium hydrogen carbonate (0.38 g, 4.6 mmol), and toluene (6.0 mL) was added dimethyl sulfate (0.58 g, 4.6 mmol) and the mixture was stirred at

80 °C for overnight. To the reaction mixture were added H_2O and saturated aqueous $NaHCO_3$, and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated in vacuo, purified by silica gel column chromatography (eluent: hexane/ethyl acetate = 1:20), and washed with hexane and ethyl acetate to give 0.070 g (yield; 11%) of **6g** as colorless solid: mp 102–103 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.42 (s, 9H), 1.83–2.11 (m, 6H), 2.55–2.77 (m, 1H) 3.74 (s, 3H), 4.38 (d, J = 7.0 Hz, 2H), 7.07 (s, 1H), 7.15–7.26 (m, 1H), 7.52–7.62 (m, 1H), 8.12–8.25 (m, 1H); MS(ESI) *m*/*z* 412 (M+H); Anal. Calcd for $C_{21}H_{25}F_4N_3O$: C, 61.30; H, 6.12; N, 10.21. Found: C, 61.35; H, 6.03; N, 10.24.

5.1.41. Cyclopentylmethyl methanesulfonate (38h). As brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.15–1.44 (m, 2H), 1.48–1.92 (m, 6H), 2.18–2.42 (m, 1H), 3.01 (s, 3H), 4.11 (d, J = 7.0 Hz, 2H).

5.1.42. (Cyclopentylmethyl)hydrazine (39h). As yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.04–1.31 (m, 2H), 1.42–1.88 (m, 6H), 1.90–2.15 (m, 1H), 2.72 (d, J = 7.0 Hz, 2H), 3.04 (br s, 3H); MS(ESI) *m*/*z* 115 (M+H).

5.1.43. 5-Amino-3-*tert*-Butyl-1-(cyclopentylmethyl)-1*H*-pyrazole (40h). As colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.14–1.36 (m, 2H), 1.25 (s, 9H), 1.42–1.79 (m, 6H), 2.24–2.51 (m, 1H), 3.36 (br s, 2H), 3.82 (d, J = 7.5 Hz, 2H), 5.40 (s, 1H); MS(ESI) *m*/*z* 222 (M+H).

5.1.44. *N*-[3-*tert*-Butyl-1-(cyclopentylmethyl)-1*H*-pyrazol-5-yl]-2-fluoro-3-(trifluoromethyl)benzamide (41h). As pale brown solid: ^{1}H NMR (600 MHz, chloroform-*d*) δ ppm 1.18–1.31 (m, 2H), 1.30 (s, 9H), 1.49–1.74 (m, 6H), 2.35–2.46 (m, 1H), 3.95 (d, J = 7.3 Hz, 2H), 6.34 (s, 1H), 7.42–7.48 (m, 1H), 7.80–7.86 (m, 1H), 8.14–8.26 (m, 1H), 8.35–8.41 (m, 1H); MS(ESI) *m*/*z* 412 (M+H).

5.1.45. *N*-[5-*tert*-Butyl-2-(cyclopentylmethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-fluoro-3-(trifluoromethyl)benzamide (6h). As colorless solid: mp 140.5–141.5 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.37–1.80 (m, 8H), 1.39–1.49 (m, 9H), 2.18–2.40 (m, 1H), 3.77 (s, 3H), 4.27 (d, J = 7.47 Hz, 2H), 7.08 (s, 1H), 7.20 (t, J = 7.69 Hz, 1H), 7.50–7.63 (m, 1H), 8.06–8.24 (m, 1H); MS(ESI) *m*/*z* 426 (M+H); Anal. Calcd for $C_{22}H_{27}F_4N_3O$: C, 62.11; H, 6.40; N, 9.88. Found: C, 61.98; H, 6.35; N, 9.86.

5.1.46. 2-Ethoxyethyl methanesulfonate (38i). As brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.22 (t, J = 7.0 Hz, 3H), 3.07 (s, 3H), 3.56 (q, J = 7.0 Hz, 2H), 3.71 (t, J = 4.4 Hz, 2H), 4.37 (t, J = 4.4 Hz, 2H); MS(ESI) *m*/*z* 169 (M+H).

5.1.47. (2-Ethoxyethyl)hydrazine (39i). As yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.21 (t, J = 7.0 Hz, 3H), 3.39–3.69 (m, 6H), 3.87 (br s, 3H); MS(ESI) *m*/*z* 105 (M+H).

5.1.48. 5-Amino-3-*tert*-Butyl-1-(2-ethoxyethyl)-1*H*-pyrazole (40i). As yellow solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.17 (t, J = 7.0 Hz, 3H), 1.26 (s, 9H), 3.47 (q, J = 7.0 Hz, 2H), 3.68 (t, J = 4.8 Hz, 2H), 4.04 (br s, 2H), 4.13 (t, J = 4.8 Hz, 2H), 5.35 (s, 1H); MS(ESI) *m*/*z* 212 (M+H).

5.1.49. *N*-[3-*tert*-Butyl-1-(2-ethoxyethyl)-1*H*-pyrazol-5-yl]-2-fluoro-3-(trifluoromethyl)benzamide (41i). As brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.03 (t, J = 7.0 Hz, 3H), 1.33 (s, 9H), 3.52 (q, J = 7.0 Hz, 2H), 3.77 (t, J = 4.8 Hz, 2H), 4.31 (t, J = 4.8 Hz, 2H), 6.55 (s, 1H), 7.35–7.48 (m, 1H), 7.74–7.86 (m, 1H), 8.20–8.35 (m, 1H), 9.82–9.97 (m, 1H); MS(ESI) *m*/*z* 402 (M+H).

5.1.50. *N*-[5-*tert*-Butyl-2-(2-ethoxyethyl)-1-methyl-1,2-dihydro-3*H*-pyrazol-3-ylidene]-2-fluoro-3-(trifluoromethyl)-benzamide (6i). As colorless solid: mp 95–96 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.12 (t, J = 7.0 Hz, 3H), 1.43 (s, 9H), 3.43 (q, J = 7.0 Hz, 2H), 3.73 (t, J = 4.8 Hz, 2H), 3.89 (s, 3H), 4.47 (t, J = 4.8 Hz, 2H), 7.02 (s, 1H), 7.14–7.25 (m, 1H), 7.51–7.61 (m, 1H), 8.10–8.23 (m, 1H); MS(ESI) *m*/*z* 416 (M+H); Anal. Calcd for $C_{20}H_{25}F_4N_3O_2$: C, 57.82; H, 6.07; N, 10.11. Found: C, 57.84; H, 5.96; N, 10.10.

5.1.51. 2-(1-Methylethoxy)ethyl methanesulfonate (38j). As brown oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.18 (d, J = 6.2 Hz, 6H), 3.07 (s, 3H), 3.58–3.74 (m, 3H), 4.32–4.42 (m, 2H); MS(CI) *m*/*z* 183 (M+H).

5.1.52. [2-(1-Methylethoxy)ethyl]hydrazine (39j). As yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.16 (d, J = 6.2 Hz, 6H), 2.94 (t, J = 4.8 Hz, 1H), 3.17 (br s, 3H), 3.49–3.68 (m, 3H); MS(ESI) *m*/*z* 117 (M−H).

5.1.53. 5-Amino-3-*tert*-Butyl-1-[2-(1-methylethoxy)ethyl]-1*H*-pyrazole (40j). As colorless solid: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.11 (d, J = 6.2 Hz, 6H), 1.25 (s, 9H), 3.47–3.63 (m, 1H), 3.70 (t, J = 4.8 Hz, 2H), 4.07 (br s, 2H), 4.12 (t, J = 4.8 Hz, 2H), 5.34 (s, 1H); MS(CI) *m*/*z* 226 (M+H).

5.1.54. *N*-{3-*tert*-Butyl-1-[2-(1-methylethoxy)ethyl]-1*H*-pyrazol-5-yl}-2-fluoro-3-(trifluoromethyl)benzamide (41j). As yellow oil: ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.00 (d, J = 6.2 Hz, 6H), 1.33 (s, 9H), 3.48–3.69 (m, 1H), 3.76 (t, J = 4.4 Hz, 2H), 4.32 (t, J = 4.4 Hz, 2H), 6.55 (s, 1H), 7.35–7.50 (m, 1H), 7.74–7.87 (m, 1H), 8.20–8.33 (m, 1H), 9.83–9.99 (m, 1H); MS(ESI) *m*/*z* 416 (M+H).

5.1.55. *N*-{5-*tert*-Butyl-1-methyl-2-[2-(1-methylethoxy)-ethyl]-1,2-dihydro-3*H*-pyrazol-3-ylidene}-2-fluoro-3-(trifluoromethyl)benzamide (6j). As colorless solid: mp 80–81 °C; ^{1}H NMR (200 MHz, chloroform-*d*) δ ppm 1.06 (d, J = 6.2 Hz, 6H), 1.43 (s, 9H), 3.42–3.57 (m, 1H), 3.73 (t, J = 4.8 Hz, 2H), 3.90 (s, 3H), 4.46 (t, J = 4.8 Hz, 2H), 7.03 (s, 1H), 7.13–7.25 (m, 1H), 7.51–7.62 (m, 1H), 8.12–8.24 (m, 1H); MS(ESI) *m*/*z* 430 (M+H); Anal. Calcd for $C_{21}H_{27}F_4N_3O$: C, 58.73; H, 6.34; N, 9.78. Found: C, 58.65; H, 6.23; N, 9.76.

5.2. Solubility

An excess amount of each compound was added to water and shaken on a shaker (model SA31, Yamato Kagaku) at room temperature for 24 h. The suspension was centrifuged for 10 min at 25 °C, 11,000 rpm with a centrifugal separator (model CF 15R, Hitachi). The supernatant was diluted with 50% aqueous acetonitrile solution, and the concentrations were measured by HPLC. The HPLC analysis was performed with a Shimadzu HPLC system composed of a LC-10AD, SPD-10AV, and SIL-10A. The conditions for HPLC were: mobile phase, 0.1% aqueous phosphoric acid solution/acetonitrile = 7:3; flow rate, 1.0 mL/min; column, reversed-phase (Capcell Pak UG120, 4.6 mm I.D. × 150 mm; Shiseido) at 40 °C; and detection wavelength, 230 nm.

5.3. In vitro pharmacological studies

5.3.1. Cell culture and preparation of human CB_1 receptor and human CB_2 receptor membranes. Cells expressing human CB_1 (hCB_1) receptor or human CB_2 (hCB_2) receptor were generated[19] and grown in alpha modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 600 μg/mL G418. Cells expressing hCB_1 receptor or hCB_2 receptor were collected by centrifugation and used immediately or stored at −80 °C. Cell pellets were suspended in homogenization buffer (10 mM Tris–HCl, 5 mM EDTA, and 3 mM EGTA, pH 7.6) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) and homogenized. Intact cells and nuclei were removed by centrifugation (270*g* for 5 min at 4 °C). The supernatant was centrifuged at 100,000*g* for 30 min at 4 °C, and the pellet was suspended in a Gly-Gly buffer (20 mM glycylglycine, 1 mM $MgCl_2$, and 250 mM sucrose, pH 7.2) and stored at −80 °C. Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA), with bovine serum albumin (BSA) as a standard.

5.3.2. Preparation of rat spleen membranes. Spleens from male Sprague–Dawley rats (160–200 g, Charles River, Yokohama, Japan) were dissected and maintained in ice-cold homogenization buffer (10 mM Tris–HCl, 5 mM EDTA, and 3 mM EGTA, pH 7.6) containing protease inhibitor cocktail. After removing the connective tissue, the total tissue weight was determined. The tissue was then homogenized and centrifuged at 270*g* for 10 min at 4 °C, and after centrifuging the supernatant at 100,000*g* for 20 min at 4 °C, the pellet was washed with the buffer by homogenization and centrifugation again. The final pellet was resuspended in assay buffer (50 mM Tris–HCl, 5 mM EDTA, and 5 mM $MgCl_2$, pH 7.4), and protein concentration was determined with BCA protein assay reagent and with BSA as a standard.

5.3.3. [^{3}H]CP 55,940 binding to recombinant human cannabinoid receptors. Displacement of [^{3}H]CP 55,940 (specific radioactivity, 5942.0 GBq/mmol; PerkinElmer Life Sciences, Boston, MA, USA) as measured in membranes of CHO cells expressing hCB_1 or hCB_2. Prepared membranes of CHO cells expressing hCB_1 or hCB_2 were suspended in assay buffer (50 mM Tris–HCl, 2.5 mM EDTA, and 5 mM $MgCl_2$, pH 7.4) containing 0.2% BSA. Membranes at a protein concentration of 7 μg/well (hCB_1) or 2.5 μg/well (hCB_2) were incubated with various concentrations of test compounds and [^{3}H]CP 55,940 (2.1 nM for hCB_1, 0.82 nM for hCB_2) at 25 °C for 120 min. The reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.1% poly-L-lysine (Sigma–Aldrich, St. Louis, MO, USA). The filter plates were washed three times with the assay buffer containing 0.1% BSA, using a UniFliter96 harvester (Packard Instruments, Meriden, CT, USA). Microscinti 0 scintillator (Packard Instruments, Meriden, CT, USA) was added, and filter-bound activity was counted in a Topcount™ scintillation counter (Packard Instruments, Meriden, CT, USA). Nonspecific binding was determined in the presence of 2 μM CP 55,940. Specific binding was determined by subtracting nonspecific binding from total binding.

5.3.4. [^{3}H]CP 55,940 binding to rat spleen membranes. Displacement of [^{3}H]CP 55,940 was measured in rat spleen membranes of rat. Prepared rat spleen membranes were suspended in assay buffer (50 mM Tris–HCl, 5 mM EDTA, and 5 mM $MgCl_2$, pH 7.4) containing 0.2% BSA. Membranes at a protein concentration of 100 μg/well were incubated with various concentrations of test compounds and [^{3}H]CP 55,940 (0.57 nM) at 25 °C for 120 min. The reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.1% poly-L-lysine (Sigma–Aldrich, St. Louis, MO, USA), and the filter plates were washed three times with the assay buffer containing 0.1% BSA, using a UniFliter96 harvester (Packard Instruments, Meriden, CT, USA). Microscinti 0 scintillator (Packard Instruments, Meriden, CT, USA) was added, and filter-bound activity was counted in a Topcount™ scintillation counter (Packard Instruments, Meriden, CT, USA). Nonspecific binding was determined in the presence of 2 μM CP 55,940. Specific binding was determined by subtracting nonspecific binding from total binding.

5.3.5. [^{35}S]GTPγS binding assay to recombinant human CB_2 receptors. Prepared membranes of CHO cells expressing hCB_2 were suspended in assay buffer (20 mM Hepes, 100 mM NaCl, 3 mM $MgCl_2$, 0.2% BSA, 3 μM GDP, and 30 μg/mL saponin, pH 7.4). Membranes at a protein concentration of 5 μg/well were pre-incubated with various concentrations of test compounds for 30 min at 30 °C. [^{35}S]GTPγS (specific radioactivity: 46.3 TBq/mmol; PerkinElmer Life Sciences, Boston, MA, USA, 0.1 nM) was then added, and the specimens were incubated for 30 min at 30 °C. The reaction was terminated by rapid filtration under a vacuum through a UniFilter GF/C microplate (PerkinElmer Life Sciences, Boston, MA) presoaked with the assay buffer. The filter plates were washed three times with 0.3 mL of wash buffer (20 mM Hepes buffer, pH 7.4), using a UniFliter96 harvester (Packard Instruments, Meriden, CT, USA). Microscinti 0 scintillator (Packard Instruments, Meriden, CT, USA) was added, and filter-bound

activity was counted in a Topcount™ scintillation counter (Packard Instruments, Meriden, CT, USA). Nonspecific binding was determined in the presence of 10 μM GTPγS. Specific binding was determined by subtracting nonspecific binding from total binding.

5.4. Pharmacokinetics

5.4.1. Metabolic stability. Compound **6b** (CBS0550) was incubated at 37 °C for 15 min with 1.0 mg protein/mL of a human (Xenotech) or rat (Gentest) liver microsomal fraction in 250 mM phosphate buffer containing 69 mM KCl (pH 7.4) at a final compound concentration of 5 μM, in the presence of an NADPH-generating system (2.4 mM $MgCl_2$, 1.4 mM glucose-6-phosphate, 0.18 U glucose-6-phosphate dehydrogenase). The stability was assessed by incubating compounds with microsomes, and then the remaining compounds were analyzed after boiling the microsome reaction mixture. All experiments were performed in triplicate. After incubation, a twofold volume of DMSO was added to the incubation medium, and the tube was vortexed and centrifuged at 2000*g* (4 °C) for 10 min. The supernatant obtained was analyzed with a LC–MS/MS system.

5.4.2. Animals. Male Sprague–Dawley rats (Charles River, Japan) 7–8 weeks old were used in the experiments. All animals were used after acclimation for at least 4 days. The rats were given free access to water and standard laboratory diet (MF, Oriental yeast Co., Japan) during the acclimation period. Environmental conditions were controlled at a relative humidity of 50 ± 20% and temperature of 23 ± 3 °C.

5.4.3. Plasma concentrations. Compound **6b** (CBS0550) was orally administered to male Sprague–Dawley rats (Charles River, Japan). Blood was collected from the tail vein, and plasma was separated by centrifugation (11,200*g*, 4 °C, 3 min) and stored at −80 °C until the bioanalysis. The plasma concentration of **6b** (CBS0550) was determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI). A 50 μL aliquot of plasma was added with 200 μL of organic solvent (acetonitrile/methanol (9:1, v/v)) containing an internal standard and vortex-mixed. After centrifugation, the supernatant (5 μL) obtained was directly injected into a LC–MS/MS system composed of a CTC HTS-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), HP1100 binary pump system (Agilent Technologies, CA, US), and an API 3000 tandem mass spectrometer equipped with a Turbo Ionspray interface (Applied Biosystems, CA, US). Chromatographic separation with linear gradient elution was performed on a Zorbax SB C18 (3.5 μm, 30 mm × 4.6 mm ID, Agilent Technologies) using a 0.1% aqueous solution of formic acid and acetonitrile. The column temperature and mobile phase flow rate were set at 40 °C and 1.0 mL/min, respectively. Approximately 1/5 of the column effluent was directed to the ion source, and multiple reaction monitoring (MRM) with positive ion detection was performed. The lower limit of quantification (LLOQ) for rat plasma was 1 ng/mL.

5.5. In vivo pharmacological studies

5.5.1. Animals. Male Wistar rats (200–220 g, Charles River, Yokohama, Japan) were maintained under a 12-h light/dark cycle (light on 7:00 AM) in a temperature- and humidity-controlled holding room. The animals were given free access to food and water. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in *the Guidelines for Animal Experiments (1987)*.

5.5.2. Randall–Selitto test. The rat model of yeast-induced inflammatory pain was produced by the previously reported method.[18] For induction of inflammation, 0.1 mL of a 20% yeast suspension was injected into the plantar surface of the right hind paw of the rat. The mechanical nociceptive threshold was measured using an algesiometer (Ugo Basile, VA, Italy). The device generated a mechanical force that increased linearly over time, and the force was applied to the dorsal surface of the inflamed hind paw via a cone-shaped stylus with a rounded tip (2 mm^2). The nociceptive threshold was defined as the force (in g) at which the rat withdrew its paw (cut-off force 250 g). The control paw withdrawal threshold was measured two hours after the yeast injection, and the rats were given the indicated doses of **6b** (CBS0550) (3, 10, and 30 mg/kg) or vehicle (5% gum arabic). The paw withdrawal threshold was determined again 1 and 3 h after administration. The effect of the compound was expressed as the percent maximum possible effect (% MPE) calculated as: [(nociceptive threshold after administration of the compound − nociceptive threshold in the control)/(baseline nociceptive threshold − nociceptive threshold in the control) × 100]. There were seven animals per group.

References and notes

1. Gaoni, Y.; Mechoulam, R. *J. Am. Chem. Soc.* **1964**, *86*, 1646.
2. Watson, S. J.; Benson, J. A., Jr.; Joy, J. E. *Arch. Gen. Psychiatry* **2000**, *57*, 547.
3. Devane, W. A.; Dysarz, F. A.; Johnson, M. R.; Melvin, L. S.; Howlett, A. C. *Mol. Pharmacol.* **1988**, *34*, 605.
4. Munro, S.; Thomas, K. L.; Abu-Shaar, M. *Nature* **1993**, *365*, 61.
5. Howlett, A. C.; Barth, F.; Bonner, T. I.; Cabral, G.; Casellas, W. A.; Devane, W. A.; Felder, C. C.; Herekenham, M.; Mackie, K.; Martin, B. R.; Mechoulam, R.; Pertwee, R. G. *Pharmacol. Rev.* **2002**, *54*, 161.
6. Klein, T. W.; Friedman, H.; Specter, S. *J. Neuroimmunol.* **1998**, *83*, 102.
7. Buckley, N. E.; McCoy, K. L.; Mezey, E.; Bonner, T.; Zimmer, A.; Felder, C. C.; Glass, M.; Zimmer, A. *Eur. J. Pharmacol.* **2000**, *396*, 141.
8. Fernandez-Ruiz, J.; Romero, J.; Velasco, G.; Tolon, R. M.; Ramos, J. A.; Guzman, M. *Trends Pharmacol. Sci.* **2007**, *28*, 39.
9. Giblin, G. *Abstracts of Papers*, 229th National Meeting of the American Chemical Society, Sandiego, CA, March 13–17, 2005; Giblin, G. American Chemical Society: Washington, DC, 2005; MEDI 5; Eatherton, A. J.; Giblin, G. M. P.; Green, R. H.; Mitchell, W. L.; Naylor, A.;

Rawlings, D. A.; Slingsby, B. P.; Whittington, A. R. WO2004/018433; Giblin, G. M. P.; O'Shaughnessy, C. T.; Naylor, A.; Mitchell, W. L.; Eatherton, A. J.; Slingsby, B. P.; Rawlings, D. A.; Goldsmith, P.; Brown, A. J.; Haslam, C. P.; Clayton, N. M.; Wilson, A. W.; Chessell, I. P.; Wittington, A. R. *J. Med. Chem.* **2007**, *50*, 2597.

10. Meyer, M. *Abstracts of Papers*, 16th Annual Symposium on the Cannabinoids, Tihany, Hungary, June 24–28, 2006; International Cannabinoid Research Society, Burington,VT, 2006; p 147.
11. Ibrahim, M. M.; Deng, H.; Zvonok, A.; Cockayne, D. A.; Kwan, J.; Mata, H. P.; Vanderah, T. W.; Lai, J.; Porreca, F.; Makriyannis, A.; Malan, T. P., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10529.
12. Ohta, H.; Ishizaka, T.; Tatuzuki, M.; Yoshinaga, M.; Iida, I.; Tomishima, Y.; Toda, Y.; Saito, S. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6299.
13. Crank, G.; Foulis, M. J. *J. Med. Chem.* **1998**, *41*, 1075.
14. Mekonnen, B.; Crank, G. *J. Heterocycl. Chem.* **1997**, *34*, 567.
15. Hackler, R. E.; Burow, K. W.; Kaster, S. V., Jr.; Wickiser, D. I. *J. Heterocycl. Chem.* **1989**, *26*, 1575.
16. Johnson, M. R.; Melvin, L. S., Jr.; U.S. Patent 4,371,720, 1981.
17. In vitro metabolic stability expressed as a residual percentage of parent compound (5 μM) after 15 min.
18. Randall, L. O.; Selitto, J. J. *Arch. Int. Pharmacodyn. Ther.* **1957**, *1*, 409.
19. Shire, D.; Calandra, B.; Rinaldi-Carmona, M.; Oustric, D.; Pessègue, B.; Bonnin-Cabanne, O.; Le Fur, G.; Caput, D.; Ferrara, P. *Biochim. Biophys. Acta* **1996**, *1307*, 132–136.