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Discovery, structure–activity relationship study, and oral analgesic efficacy of cyproheptadine derivatives possessing N-type calcium channel inhibitory activity

Takashi Yamamoto, Seiji Niwa, Satoshi Iwayama, Hajime Koganei, Shin-ichi Fujita, Tomoko Takeda, Morikazu Kito, Yukitsugu Ono, Yuki Saitou, Akira Takahara, Seinosuke Iwata, Hiroshi Yamamoto and Masataka Shoji*

Pharmaceutical Research Laboratory, Ajinomoto company Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Japan

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Abstract—Antiallergic drug cyproheptadine (Cyp) is known to have inhibitory activities for L-type calcium channels in addition to histamine and serotonin receptors. Since we found that Cyp had an inhibitory activity against N-type calcium channel, Cyp was optimized to obtain more selective N-type calcium channel blocker with analgesic action. As a consequence of the optimization, we found **13** with potent N-type calcium channel inhibitory activity which had lower inhibitory activities against L-type calcium channel, histamine (H_1), and serotonin ($5\text{-}HT_{2A}$) receptors than those of Cyp. **13** showed an oral analgesic activity in rat formalin-induced pain model.

1. Introduction

Neuronal functions, such as neurotransmitter release and neuronal cell excitation, are regulated by voltage-dependent calcium channels (VDCCs), which are classified into at least five subtypes (L: Ca_V1, P/Q: $Ca_V2.1$, N: $Ca_V2.2$, R: $Ca_V2.3$, and T: Ca_V3) based on their pharmacological and biophysical properties.[1] Among them, the N-type channel has been shown to play a significant role in the pathophysiological processes of stroke and neuropathic pain[2] in addition to physiological regulation. Indeed, Ziconotide (25-residue peptide, molecular weight 2639, SNX-111, Prialt), which is a synthetic version of ω-conotoxin MVIIA as a neuron-specific N-type calcium channel blocker, has been proven to have non-addictive analgesic efficacy in clinical trials via intrathecal administration. Moreover, tolerance to Ziconotide is not detected[3] as observed by opiates. Now, Ziconotide is on market for the treatment of severe chronic pain. However, due to its restricted administrative route, different types of small molecules with N-type calcium channel inhibitory activity have been explored[4] to obtain orally available drugs.

Recently, some of the classical L-type calcium channel blockers were found to have a potent inhibitory activity against the N-type as well.[5] Flunarizine (**1**) is one of such kind of non-specific calcium channel blockers.[5a] Interestingly, some of the newly found N-type calcium channel blockers, PD0176078 (**2**),[4a] emopamil derivative (**3**)[2c], and 4-aminopiperidine derivative (**4**),[6] have the similar pharmacophore as flunarizine, in which a nitrogen atom locates at four atoms distance from a diphenylmethyl moiety. We presumed that this structure is a key for N-type calcium channel inhibitory activity and noticed that cyproheptadine (Cyp: **5**), which is an orally active antiallergic drug on market, has this pharmacophore (Chart 1).

From this point of view, we assumed that Cyp could have N-type calcium channel inhibitory activity with the potential to show analgesic efficacy. Then, we started the research to discover Cyp derived analgesics with potent inhibitory activity for N-type calcium channels. However, Cyp was also reported to have the potent antagonistic activity against L-type calcium channel using whole-cell

Keywords: N-type calcium channel; Analgesic activity; Structure–activity relationship study; Cyproheptadine.
* Corresponding author. Tel.: +81 44 210 5826; fax: +81 44 210 5876; e-mail: masataka_shouji@ajinomoto.com

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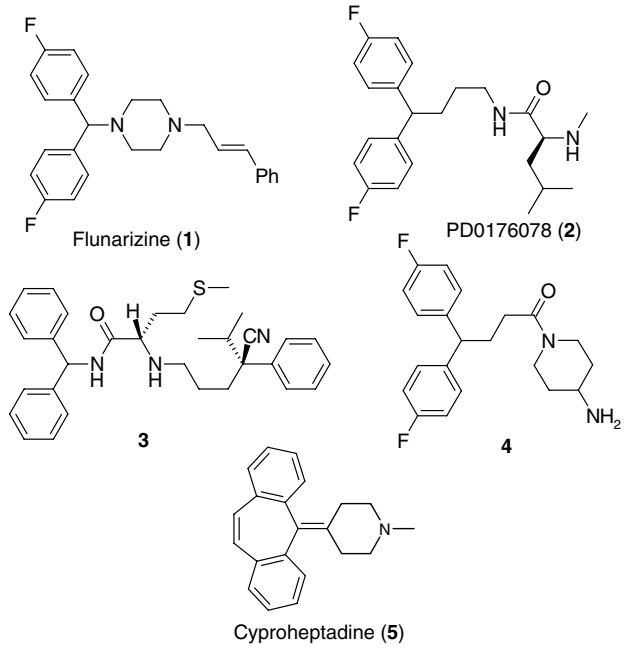


Chart 1. Structures of flunarizine (**1**), PD0176078 (**2**), reported N-type calcium channel blockers **3**–**4**, and cyproheptadine (**5**).

patch-clamp technique.[7] The strong inhibition on cardiovascular L-type channel invokes several side effects on cardiovascular system including hypotensive action. Moreover, although the inhibition of neuronal L-type calcium channel is suggested to contribute analgesic activities,[8] we proved that the inhibitory activity for N-type calcium channels is more essential than that for L-type to show the analgesic efficacy.[9] Therefore, the lower activity against L-type channel might lead to lesser influence on the cardiovascular systems. Furthermore, Cyp was also reported to have strong antagonistic activities for histamine (H_1) and serotonin ($5\text{-}HT_{2A}$) receptors, which were not necessary for a novel analgesic drug because of the expected side effects.[10]

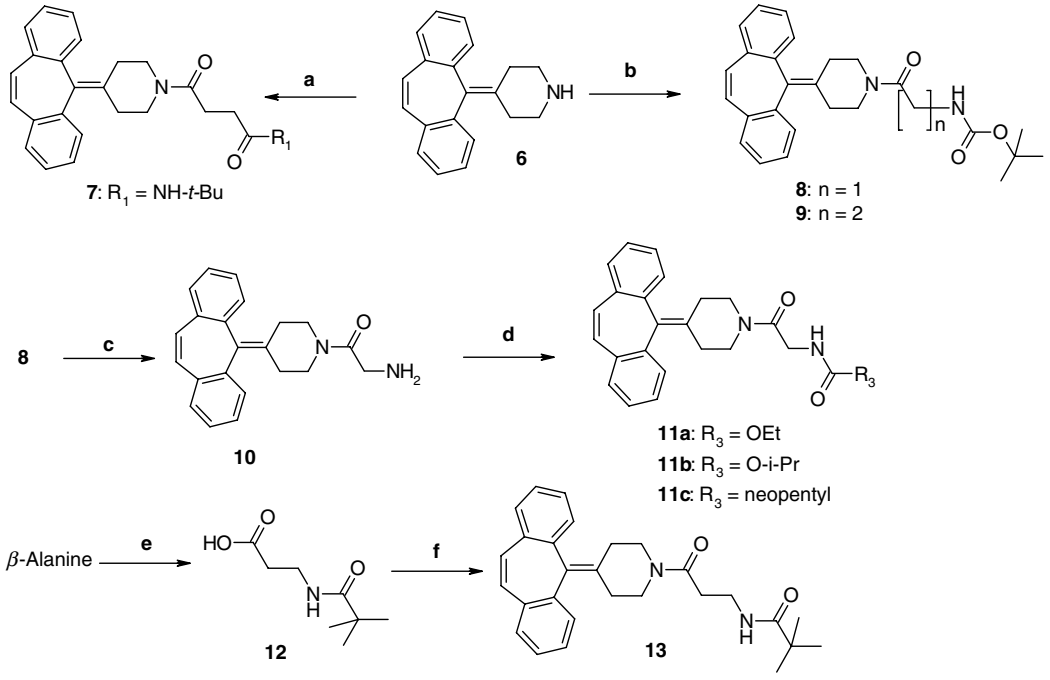
Here, we would like to report the structure–activity relationship studies of Cyp derived analgesics with N-type calcium channel inhibitory activity which have reduced inhibitory activities against L-type calcium channel, H_1 and $5\text{-}HT_{2A}$ receptors compared to Cyp.

2. Chemistry

The synthesis of Cyp derivatives **7**–**10** and **11a**–**c** is described in Scheme 1. Succinic acid derivative (**7**) and *N*-*tert*-butoxycarbonyl-amino acid derivatives (**8** and **9**) were obtained via a coupling condition using desmethylcyproheptadine **6**[11] in the presence of EDC and DMAP. The *t*-butoxycarbonyl group of **8** was removed under acidic conditions to yield **10**, and subsequent condensation with appropriate acyl chloride or alkyl chloroformate gave amide or carbamate compounds **11a-c**. *N*-pivaloyl-β-alanine **12** was synthesized from β-alanine and pivaloyl chloride. Subsequent coupling with **6** yielded **13** by the same coupling method as described for compounds **7**–**9**.

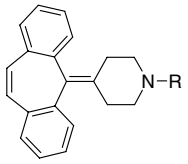
3. Pharmacological studies

N-type calcium channel inhibitory activities of the compounds were determined using fluorescence based Ca^{2+}-flux assay in IMR-32 human neuroblastoma cells.



Scheme 1. Reagents: (a) *N*-*tert*-butyl-succinamic acid, EDC, TEA, DMAP, CH_2Cl_2; (b) *N*-Boc-amino acids, EDC, TEA, DMAP, CH_2Cl_2; (c) 4N HCl/1,4-dioxane, CH_2Cl_2; (d) acyl chloride or alkyl chloroformate, TEA, 1,4-dioxane, CH_2Cl_2; (e) pivaloyl chloride, 1N NaOH, Et_2O; (f) **6**, EDC, TEA, DMAP, CH_2Cl_2.

Table 1. Activity table of cyproheptadine (**5**) and its derivatives

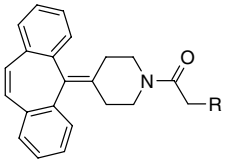


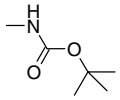
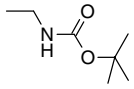
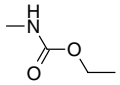
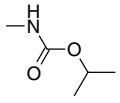
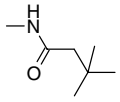
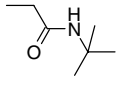
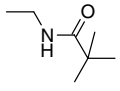
Compound	R	Inhibitory activities against calcium channels[a]		Relative binding affinity data[b] on human 5-HT_{2A}		Relative binding affinity data[b] on guinea-pig cerebellum H_1	
		N-type (IC_{50}, μM)	L-type (IC_{50}, μM)	Inhibition % at 10 nM	Inhibition % at 100 nM	Inhibition % at 10 nM	Inhibition % at 100 nM
Cyproheptadine (**5**)	Me	1.1	0.089	4.5 nM (IC_{50})		10.5 nM (IC_{50})	
6	H	6.2	0.86	26%	80%	13%	58%
10	$COCH_2NH_2$	7.6	1.3	<10%	14%	<10%	<10%
8	$COCH_2NHCO_2$-*t*-Bu	4.8	0.98	<10%	<10%	<10%	<10%

[a] The inhibitory activities against N-type and L-type calcium channels were measured by calcium influx using IMR-32 cells and Magnus method, respectively.

[b] The relative binding affinity (RBA) data were based on human 5-HT_{2A} and guinea-pig cerebellum H_1 receptors using [^{3}H]ketanserin and [^{3}H]pyrilamine as radioligands.

Table 2. The inhibitory activities of cyproheptadine derivatives[a]



Compound	R	N-type (IC_{50}, μM)	L-type (IC_{50}, μM)
8		4.8	0.98
9		4.6	0.24
11a		4.5	2.6
11b		7.5	1.0
11c		3.9	0.91
7		3.7	0.43
13		3.2	0.47

[a] The inhibitory activities against N-type and L-type calcium channels were measured by calcium influx us ing IMR-32 cells and Magnus method, respectively.

We have confirmed that the IMR model was about 1000 times less sensitive than the Magnus or in vivo model for detecting each calcium channel blocking action.[12] Inhibitory activities against L-type calcium channel were estimated from the effects on high K^+-induced contraction in rat thoracic aorta ring by Magnus technique (Tables 1,2).[9,13] The radioligand-receptor binding assay for human 5-HT_{2A} and guinea-pig cerebellum H_1 was conducted at Cerep (www.cerep.com) using [^{3}H]ketanserin and [^{3}H]pyrilamine, respectively (Tables 1 and 3).[14] The analgesic effect of the compound was evaluated in a rat formalin-induced pain model.[9,15] The test compound was suspended in 0.5% tragacanth solution and administered orally at a dosage of 3 mg/kg. The cumulative flinching response in phase II (10–60 min after injection of formalin) was calculated for each rat (Table 3).

4. Results and discussion

We began our study by testing the potency of Cyp against N-type calcium channel and found that Cyp blocked the channel with the IC_{50} value of 1.1 μM (Table 1). According to our previous results, this activity seems to be enough to show the analgesic effect in vivo.[9] However, its inhibitory activity against L-type calcium channel was shown to be high enough to affect the cardiovascular system (IC_{50} = 0.089 μM).[7] Therefore, the direction of our structure optimization on Cyp should be to decrease its L-type activity maintaining the efficacy against N-type calcium channel. Interestingly, desmethylcyproheptadine (**6**) had micromolar IC_{50} value against N-type calcium channel with a 10-fold lower activity against L-type calcium channel compared to Cyp (IC_{50} = 6.2 and 0.86 μM, respectively). This small modification on the critical nitrogen atom in piperidine ring led to the decreased antagonistic activities for both H_1 and 5-HT_{2A} receptors than those of Cyp. Moreover, the activities of *N*-glycyl Cyp **10**, whose nitrogen atom in piperidine ring was acylated, showed negligible activities for both 5-HT_{2A} and H_1 receptors. On the other hand, its activities against both N- and L-type calcium chan-

Table 3. The biological activity table of **13**

Compound	Relative binding affinity data[a] on human 5-HT_{2A}		Relative binding affinity data[a] on guinea-pig cerebellum H_1		Rat formalin test (3 mg/kg, po)[b]	
	Inhibition % at 10 nM	Inhibition % at 100 nM	Inhibition % at 10 nM	Inhibition % at 100 nM	Number of flinches	Inhibition %
13	<10%	<10%	<10%	<10%	65 ± 15 (n = 5)	44%

[a] The relative binding affinity (RBA) data were based on human 5-HT_{2A} and guinea-pig cerebellum H_1 receptors using [^{3}H]ketanserin and [^{3}H]pyrilamine as radioligands.

[b] Inhibitory activity in rat formalin test is shown as the number of flinches in phase II pain responses following footpad injection of formalin in rat. Number of flinches represents the mean ± SEM. Inhibition % is given by the control (116 ± 8, n = 22) and test compound.

nels were slightly lower than those of **6** (IC_{50} = 7.6 and 1.3 μM, respectively). These results illustrated the fact that the strength of positive charge on the piperidine nitrogen had great influences on the activities for both 5-HT_{2A} and H_1 receptors, but fewer effects on those for N- and L-type calcium channels. Finally, *N*-*tert*-butoxycarbonyl-glycyl derivative **8**, which had no charged nitrogen atom at the terminal of the molecule as well as in piperidine ring, had more potent activity against N-type calcium channel (IC_{50} = 4.8 μM) than that of **10** with 11-fold lower activities for L-type channel (IC_{50} = 0.98 μM) than that of Cyp. **8** also showed no affinity for both 5-HT_{2A} and H_1 receptors. According to this result, we chose **8** as the lead compound and started the further structural optimizations on *tert*-butoxycarbonyl-glycyl moiety without the charge on both of the nitrogen atoms (Table 2).

First, the elongation of chain length by one carbon atom (**9**) was tested. However, **9** showed no improvement for N-type blocking potency (IC_{50} = 4.6 μM) with higher activity against L-type (IC_{50} = 0.24 μM). The replacement of the *tert*-butylcarbamate moiety of **8** with other alkyl carbamates (**11a** or **11b**) was performed without changing their chain lengths. Interestingly, although ethylcarbamate **11a** had almost equipotent activities against N-type calcium channel to **8**, the activity of isopropylcarbamate **11b** was decreased (IC_{50} = 7.5 μM). Next, the urethane moiety of **8** was replaced by amides (**7**, **11c**, and **13**). All of those derivatives had higher activities for N-type calcium channel than that of **8** and the activity of **13** was the highest among all of the derivatives (IC_{50} = 3.9, 3.7, and 3.2 μM, respectively). Considering the analgesic action in vivo, higher activity for N-type calcium channel is required. As for the activity against L-type, **13** had higher activity (IC_{50} = 0.47 μM) than **8**, but still more than 5 times lower than Cyp. Moreover, the radio binding assay proved that **13** had no affinity for both 5-HT_{2A} and H_1 receptors (Table 3). Therefore, even its N-type activity was still less potent than that of Cyp, we concluded that **13** had the best potential to show an analgesic effect in vivo with fewer side effects. In order to verify its analgesic activity, **13** was tested in rat formalin-induced pain model. As expected, **13** showed promising analgesic activity with 44% inhibition at 3 mg/kg po. (Table 3). In fact, the inhibitory activities for both N-type and L-type calcium channels could affect on the analgesic activity, we might say that the effect of N-type calcium channel was still important, because the superior influence on analgesic activity of N-type to L-type channels was previously reported, and the IC_{50} values coming from IMR-32 assay were underestimated compared to those of Magnus method.[9,12]

In summary, the optimization and structure–activity relationship (SAR) studies on Cyp were performed to find potent analgesics possessing N-type calcium channel inhibitory activity with reduced inhibitory activities against L-type calcium channel, H_1 and 5-HT_{2A} receptors. As a consequence, an *N*-pivaloyl-β-alanyl derivative (**13**) was discovered as the orally active analgesic with effective N-type calcium channel inhibitory activity. Because of the lower inhibitory activities for L-type calcium channel, 5-HT_{2A} and H_1 receptors, **13** is expected to have fewer side effects than Cyp is. Investigations into the further optimizations and pharmacological profiles are in progress and will be reported elsewhere.

5. Experimental

5.1. Chemistry

5.1.1. General. The reagents were purchased commercially and used without further purification. Column chromatography was performed using silica gel (Merck, particle size 0.063–0.200 mm). NMR spectra were recorded on a Varian EM-390 at 300 MHz. Mass spectra (ESI) were measured on JEOL JMS-DX300 instruments. The elemental analysis was carried out on an Elemental Vario EL III analyzer. Where analyses are indicated only by the symbols of the elements, the results obtained were within 0.4% of the theoretical values. Reagent abbreviations: EDC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride; TEA, triethylamine; DMAP, 4-dimethylaminopyridine.

5.1.2. *N*-(*tert*-Butyl)-4-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-4-oxobutanamide (7). 4-(5*H*-Dibenzo [*a*,*d*]cycloheptene-5-ylidene)-1-piperidine (2.00 g, 7.32 mmol), 1.52 g (8.78 mmol) *N*-*tert*-butylsuccinamic acid, 86 mg (0.70 mmol) DMAP, and 2.10 g (11.0 mmol) EDC were dissolved in 1 ml CH_2Cl_2. TEA (1.48 g, 14.6 mmol) was added to the obtained solution, and they were stirred for 2 h. One molar aqueous hydrochloride solution was added to the obtained mixture. After extracting three times with CH_2Cl_2, the organic layer was washed with brine. After drying over anhydrous sodium sulfate, the solvent was evaporated

under reduced pressure, and the residue was purified by the silica gel chromatography (hexane/EtOAc, 2:1–3:7) to obtain the title compound (2.96 g, 94%): ^{1}H NMR ($CDCl_3$) δ: 1.32 (9H, s), 2.08–2.36 (4H, m), 2.41 (2H, m), 2.50–2.71 (2H, m), 2.24–2.96 (2H, m), 3.58 (1H, m), 3.93 (1H, m), 5.77 (1H, br s), 6.92 (2H, s), 7.14–7.38 (8H, m): MS (ESI) 429 $(MH)^+$. Anal. $C_{28}H_{32}N_2O_2$ (C, H, N).

5.1.3. *tert*-Butyl 2-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxoethylcarbamate (8). 4-(5*H*-Dibenzo [*a*,*d*]cycloheptene-5-ylidene)-1-piperidine, (3.00 g, 10.9 mmol), 2.29 g (13.2 mmol) *N*-*tert*-butoxycarbonylglycine, 3.14 g (16.4 mmol) EDC, and 122 mg (1.00 mmol) DMAP were dissolved in 50 ml CH_2Cl_2. TEA (2.20 g, 21.8 mmol) was added to the obtained solution, and they were stirred overnight. Saturated aqueous sodium hydrogencarbonate solution was added to the obtained mixture. After extracting three times with CH_2Cl_2, the organic layer was washed with brine. After drying over anhydrous sodium sulfate, the solvent was evaporated under reduced pressure, and the residue was purified by the silica gel chromatography (hexane/EtOAc, 4:1–2:1) to obtain the title compound (4.29 g, 94%): ^{1}H NMR ($CDCl_3$) δ: 1.44 (9H, s), 2.15–2.35 (4H, m), 3.02 (2H, m), 3.42 (1H, m), 3.81–4.01 (3H, m), 5.51 (1H, br s), 6.92 (2H, s), 7.15–7.38 (8H, m): MS (ESI) 431 $(MH)^+$. Anal. $C_{27}H_{30}N_2O_3$ (C, H, N).

5.1.4. *tert*-Butyl 3-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-3-oxopropylcarbamate (9). The title compound was prepared from *N*-*tert*-butoxycarbonyl-3-aminopropionic acid and 4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidine as described for **8**: ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 2.12–2.36 (4H, m), 2.86–3.36 (4H, m), 3.42 (1H, m), 3.97 (1H, m), 4.49 (1H, d), 4.76 (1H, m), 5.16 (1H, br s), 6.92 (2H, s), 7.13–7.22 (2H, m), 7.22–7.39 (6H, m): MS (ESI) 445 $(MH)^+$. Anal. $C_{28}H_{32}N_2O_3 \cdot 0.5\ H_2O$ (C, H, N).

5.1.5. 2-[4-(5*H*-Dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxo-ethanamine hydrochloride (10). *tert*-Butyl 2-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxoethylcarbamate, (**8**), (1.40 g, 3.25 mmol) was dissolved in 20 ml of 1,4-dioxane. Twelve microliters of 4 M hydrochloric acid/1,4-dioxane solution was added to the obtained solution, and they were stirred overnight. After the neutralization with 4 M aqueous sodium hydroxide solution, the solvent was evaporated under reduced pressure. Brine was added to the reaction mixture. After the extraction three times with EtOAc, the extract was dried over anhydrous sodium sulfate and then the solvent was evaporated under reduced pressure. The residue was purified by the silica gel chromatography (hexane/EtOAc, 9:1 to EtOAc). The obtained oil was dissolved in 10 ml of a solution of EtOAc/hexane (1:2) and then 2 ml of 4 M hydrochloric acid/1,4-dioxane solution was added to the residue. The resultant precipitates were taken by the filtration, washed with a solution of EtOAc/hexane (1:2), and air-dried. After further drying under reduced pressure, the title compound was obtained (1.15 g, 94%): ^{1}H NMR (DMSO-d_6) δ: 1.97 (2H, m), 2.29 (2H, m), 3.10–2.70 (4H, m), 3.81 (2H, m), 6.96 (2H, s), 7.20–7.44 (8H, m), 8.17(3H, bs): MS (ESI) 415 (MH+DMSO-d_6)$^+$. Anal. $C_{22}H_{23}ClN_2O \cdot 0.9\ H_2O$ (C, H, N).

5.1.6. Ethyl 2-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxoethylcarbamate (11a). Compound **10** (375 mg) was suspended in 3 ml of CH_2Cl_2. TEA (303 mg, 3.00 mmol) was added to the obtained suspension. Then a solution of 130 mg (1.20 mmol) of ethyl chloroformate in 3 ml CH_2Cl_2 was slowly added to the reaction mixture. After stirring overnight, saturated aqueous sodium hydrogencarbonate solution was added thereto. After extracting with EtOAc twice followed by drying under anhydrous sodium sulfate, the solvent was evaporated under reduced pressure. The residue was roughly purified by the silica gel chromatography (CH_2Cl_2/MeOH, 98:2) and then purified by the silica gel chromatography (hexane/EtOAc, 1:2) to obtain the title compound (213 mg, 53%): ^{1}H NMR ($CDCl_3$) δ: 1.24 (3H, t, J = 6.9 Hz), 2.12–2.36 (4H, m), 2.97–3.10 (2H, m), 3.44 (1H, m), 3.86–4.02 (3H, m), 4.13 (2H, q, J = 6.9Hz), 5.65 (1H, br s), 6.92 (2H, s), 7.14–7.20 (2H, m), 7.23–7.38 (6H, m): MS (ESI) 403$(MH)^+$. Anal. $C_{25}H_{26}N_2O_3$ (C, H, N).

5.1.7. Isopropyl 2-[4-(5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxoethylcarbamate (11b). The title compound was prepared from **10** and isopropyl chloroformate as described for **11a**: ^{1}H NMR ($CDCl_3$) δ: 1.23 (6H, d, J = 6.3 Hz), 2.12–2.48 (4H, m), 2.92–3.11 (2H, m), 3.44 (1H, m), 3.83–4.09 (3H, m), 4.90 (1H, m), 5.59 (1H, br s), 6.92 (2H, s), 7.14–7.20 (2H, m), 7.23–7.38 (6H, m): MS (ESI) 417 $(MH)^+$. Anal. $C_{26}H_{28}N_2O_3 \cdot 0.25\ H_2O$ (C, H, N).

5.1.8. *N*-{2-[4-(5*H*-Dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-2-oxoethyl}-3,3-dimethylbutanamide (11c). The title compound was prepared from **10** and 3,3-dimethylbutanoyl chloride as described for **11a**: ^{1}H NMR ($CDCl_3$) δ: 1.03 (9H, s), 2.12 (2H, s), 2.15–2.39 (4H, m), 2.96–3.11 (2H, m), 3.47 (1H, m), 3.88–4.13 (3H, m), 6.49 (1H, br s), 6.92 (2H, s), 7.14–7.21 (2H, m), 7.21–7.41 (6H, m): MS (ESI) 429 $(MH)^+$. Anal. $C_{28}H_{32}N_2O_2 \cdot 0.2\ H_2O$ (C, H, N).

5.1.9. *N*-(2,2-Dimethylpropanoyl)-β-alanine (12). β-Alanine (4.35 g, 48.8 mmol) was dissolved in 49 ml of 1 M aqueous sodium hydroxide solution and 2 ml Et_2O. Pivaloyl chloride (4.90 g, 40.6 mmol) in Et_2O was added to the vigorously stirred solution for 20 min. After stirring for 1 h 15 min, the obtained solution was neutralized with 1 M aqueous hydrochloride solution. The solution was extracted with EtOAc three times. The extract was dried over anhydrous sodium sulfate and then the solvent was evaporated under reduced pressure, the title compound was obtained (5.40 g, 77%): ^{1}H NMR ($CDCl_3$) δ: 1.18 (9H, s), 2.60 (2H, t, J = 6.0Hz), 3.51 (2H, q, J = 6.0 Hz), 6.34 (1H, br s).

5.1.10. *N*-{3-[4-(5*H*-Dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidinyl]-3-oxopropyl}-2,2-dimethylpropanamide (13). [4-(5*H*-Dibenzo[*a*,*d*]cycloheptene-5-ylidene)-1-piperidine (275 mg, 1.01 mmol), 90.0 mg (0.480 mmol) *N*-(2,2-dim-

ethylpropanoyl)-β-alanine, 193 mg (1.01 mmol), EDC and 6 mg (0.05 mmol) of DMAP were dissolved in 3 ml CH_2Cl_2. TEA (152 mg, 1.50 mmol) was added to the obtained solution. After stirring for 3 h, the obtained mixture was purified by the silica gel chromatography (Chromatorex™ NH, Fuji Silysia Chemical LTD., hexane/EtOAc, 89:11–7:3) and then purified by the silica gel chromatography (hexane/EtOAc, 2:3–1:4) to obtain the title compound (147 mg, 72%): ^{1}H NMR ($CDCl_3$) δ: 1.16 (9H, s), 2.11–2.36 (4H, m), 2.38–2.56 (2H, m), 2.94–3.12 (2H, m), 3.44–3.56 (3H, m), 3.92 (1H, m), 6.62 (1H, t), 6.92 (2H, s), 7.13–7.20 (2H, m), 7.22–7.38 (6H, m): MS (ESI) 429 $(MH)^+$. Anal. $C_{28}H_{32}N_2O_2$ (C, H, N).

5.2. In vitro pharmacology

5.2.1. N-type calcium channel inhibition

5.2.2. Cell culture. Human neuroblastoma cells IMR-32 were obtained from ATCC (American Type Culture Collection) and cultured with the medium of the following composition; a Phenol Red-free Eagle's minimum essential medium containing Earle's salts (GIBCO) supplemented with 2 mM of L-glutamine (GIBCO), 1 mM of sodium pyruvate (pH 6.5) (GIBCO), antibiotic/antimycotic mixture (GIBCO), and 10% fetal calf serum (Cell Culture Technologies). For measurement of intracellular calcium concentration, 3 ml of 1×10^5 cells/ml IMR-32 cells was spread on the glass bottom of a dish (Iwaki Glsss Co., Ltd.) having a diameter of 35 mm which had been treated with poly-L-lysine (SIGMA) and collagen (COLLAGEN VITROGEN 100; Collagen Co.). One day after the culture, 1 mM dibutyl-cAMP and 2.5 μM of 5-bromo-2-deoxyuridine (SIGMA) were added to express N-type calcium channels. After the culture for additional 10–14 days, the cells were subjected to the assay.

5.2.3. Measurement of intracellular calcium concentration. The medium for IMR-32 cells thus prepared was replaced with 1 ml of Phenol Red-free Eagle's minimum essential medium (GIBCO) containing 2.5 μM fura-2/AM (Dojin Kagaku, Co.) and Earle's salts supplement, and the incubation was conducted at 37 °C for 30 min. Then, the medium was replaced with a recording medium (20 mM Hepes–KOH, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 13.8 mM D-glucose). Intracellular calcium concentration was analyzed using a fluorescence microscope (Nikon Corporation) and an image analysis device ARGUS 50 (Hamamatsu Photonics). To prevent the activation of the L-type calcium channels in the differentiated IMR-32 cells, a recording medium containing 1 μM of a selective L-type calcium channel blocker Nifedipine was used throughout the experiment. Then a stimulating medium containing 60 mM KCl (KCl was substituted for equimolar NaCl in the recording medium) was rapidly given by the Y-tube method for 6 s. The change in the intracellular calcium concentration was expressed as the N-type calcium channel activity.[12] Then stimulating medium containing 60 mM KCl was reapplied in the presence of 0.1, 1 or 10 μM of the test compound. The antagonistic activity of each test compound on N-type calcium channel was expressed as a 50% inhibitory concentration (IC_{50}), as previously reported.[7]

5.2.4. L-type calcium channel inhibition. Male Sprague–Dawley rats (7 weeks old) were used. The thoracic aorta was isolated, cleared of adhering periadventitial fat, and cut into rings of 3 mm width. The endothelium was removed by gently rubbing the luminal surface. The ring was mounted in an organ bath filled with warmed (37 °C), oxygenated (95% O_2/5% CO_2) Tyrode's solution (pH 7.4). The ring was equilibrated under resting tension of 2 g for 1 h. Then the ring was incubated in high K^+ solution (containing 50 mM KCl; KCl was substituted for equimolar NaCl in the Tyrode's solution) and then in Tyrode's solution for 45 min each. The solution was replaced with high K^+ solution again. After attaining the maximum contraction reaction, the test compound was cumulatively added at intervals of 90 min to attain concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M. The antagonistic activity of each test compound on L-type calcium channel was expressed as a 50% inhibitory concentration (IC_{50}), as previously reported.[7]

5.3. In vivo pharmacology

5.3.1. Rat formalin test. Male Sprague–Dawley rats were used. One hundred microliters of formalin (2.5%) was subcutaneously injected into the dorsal surface of the left hind paw. Flinching behavior was detected as phase I (0–9 min) and phase II (10–60 min). The cumulative flinching response in phase II was observed for assessing effect of each test compound on hyperalgesia. Test compounds were suspended in 0.5% tragacanth solution and orally administered 3 h before formalin injection.

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