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Structure–Activity Study and Analgesic Efficacy of Amino Acid Derivatives as N-Type Calcium Channel Blockers

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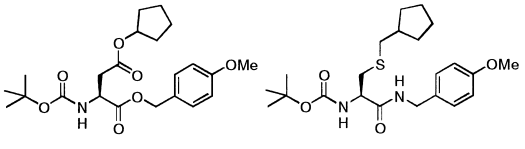
Abstract—The synthesis and structure–activity relationship (SAR) study of a novel series of N-type calcium channel blockers are described. L-Cysteine derivative **2a** was found to be a potent and selective N-type calcium channel blocker with IC_{50} 0.63 μM on IMR-32 assay. Compound **2a** showed analgesic efficacy in the rat formalin-induced pain model by intrathecal and oral administration. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Six types of pharmacologically and/or biophysically distinct voltage-dependent calcium channels (T, L, N, P, Q, and R) have been described in neurons. Among these calcium channels, the N, P, Q, and R-type channels have all been shown to play key roles in neurotransmitter release.[1] N-Type calcium channels are located at presynaptic terminals throughout neurons and directly mediate spinal transmission of pain signals from the peripheral to the central nervous system. ω-Conotoxin MVIIA, a 25-amino acid peptide, is a selective blocker of N-type calcium channels which shows analgesic activity when administered intrathecally.[2] Over the last decade, synthetic efforts have focused on small-molecule, non-peptide N-type calcium channel blockers for analgesia or neuroprotection, since clinical observations were reported for ω-conotoxin MVIIA.[2] A number of small-molecule, non-peptide blockers of N-type calcium channels have been reported, some of which have been shown to be active in analgesic models.[3,4] However, most of these compounds also block the other calcium channel subtypes including L-type channels. Although blocking of neuronal L-type calcium channels shows neuroprotective effects,[5] inhibition of cardiac L-type calcium channels probably causes a decrease of blood pressure.

In the course of screening of our compound library, *N*-(*t*-butoxycarbonyl)-L-aspartic acid derivative **1a** was identified as an initial lead compound for a new series of N-type calcium channel blockers, which inhibited calcium influx into IMR-32 cells with an IC_{50} of 3.4 μM.[6] The inhibitory activity of compound **1a** for N-type calcium channel was also confirmed by electrophysiological study using IMR-32 cells (34% inhibition at 10 μM, $n=3$).[7] Here, we report the synthesis and structure–activity study of N-type calcium channel blockers that include the *N*-(*t*-butoxycarbonyl) L-amino acid moiety as a structural motif.

The compounds were evaluated for inhibitory activity against both N-type and L-type calcium channels focusing on selectivity to reduce cardiovascular side effects due to blocking of L-type calcium channels (Fig. 1).



1a 2a

Figure 1.

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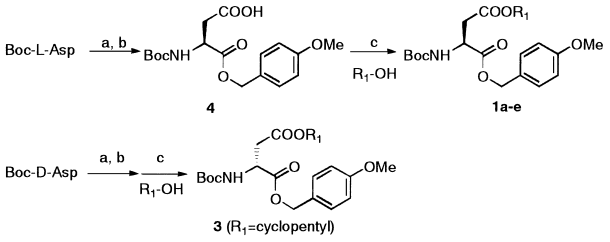
Chemistry

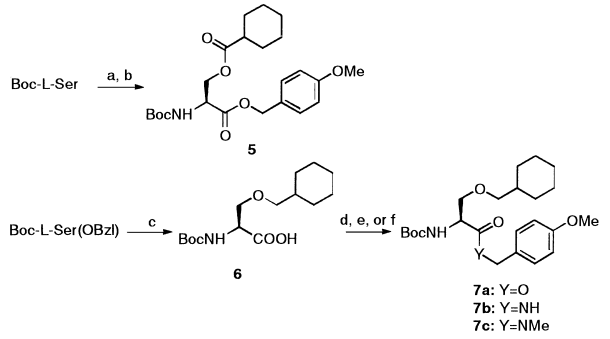
The synthesis of aspartic acid ester (**1** and **3**) was carried out by sequential esterification as shown in Scheme 1. Half ester **4** was obtained from Boc-L-Asp anhydride and esterification of **4** with the appropriate alcohol in the presence of EDC and DMAP gave Boc-L-Asp diester **1**. Compound **3**, an enantiomer of **1a**, was synthesized from Boc-D-Asp by the same method as described for compound **1a**.[10]

L-Serine ester **5** was synthesized from Boc-L-Ser by reaction with 4-methoxybenzyl chloride and then with acid chloride as shown in Scheme 2. L-Serine ether **7** was obtained from commercially available Boc-L-Ser (OBzl) by catalytic hydrogenation of the benzene ring using rhodium on alumina and subsequent condensation of carboxylic acid **6** with the appropriate alcohol or amine (Scheme 2).

A similar method was applied for the ether formation of L-homoserine (L-Hse) ether **10** by reaction with cyclohexene-3-bromide and Boc-L-Hse. Subsequent catalytic reduction of compound **8** and condensation with 4-methoxybenzylamine gave the Boc-L-Hse ether **10** (Scheme 3). Sulfide bond formation was carried out by reaction of L-cysteine with halide or methanesulfonate yielding compounds **11**, which were easily derived to compounds **2** by the usual method (Scheme 4).



Scheme 1. Reagents: (a) Ac_2O; (b) 4-methoxybenzyl alcohol, DCHA, THF–Et_2O; (c) EDC, DMPA, CH_2Cl_2.

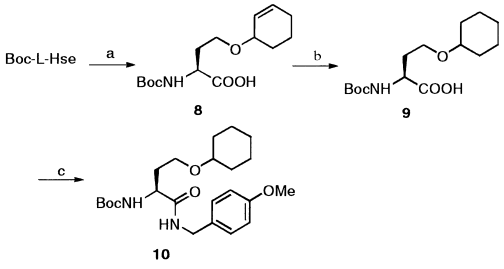


Scheme 2. Reagents: (a) 4-methoxybenzyl chloride, K_2CO_3, NaI, DMF; (b) cyclohexanecarbonyl chloride, Et_3N, CH_2Cl_2; (c) Rh–Al_2O_3, H_2, *i*-PrOH; (d) 4-methoxybenzyl alcohol, EDC, HOBt, DMF; (e) 4-methoxybenzylamine, EDC, HOBt, DMF; (f) 4-methoxybenzylmethylamine, EDC, HOBt, DMF.

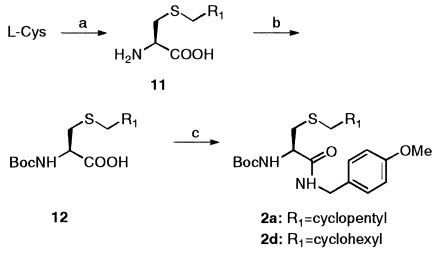
Biological Results and Discussion

The initial lead compound **1a** had moderate N-type calcium channel blocking activity (IC_{50} of 3.4 μM; IMR-32 assay[6,9]) and selectivity over L-type calcium channels (IC_{50} of 15 μM; in AtT-20 assay,[8,9] selectivity ratio L/N = 4.4). Compound **3**, the enantiomer of **1a** derived from D-Asp, showed slightly higher activity in IMR-32 assay (IC_{50} = 2.0 μM). However, inhibitory activity for L-type calcium channels in AtT-20 assay was increased by 5-fold (IC_{50} = 2.9 μM, L/N = 1.5). Due to lack of selectivity of the D-Asp derivative **3**, all of the following compounds were synthesized in optically active forms, which were derived from the corresponding L-amino acids.[10]

An initial structure–activity study was started with replacement of the alkyl group on the side-chain ester using the L-Asp skeleton as a structural motif (see Table 1: **1a**–**e**). Methyl ester (**1b**: R = methyl) showed extremely weak activity for N-type calcium channels (IC_{50} = 26 μM) and low selectivity (L/N = 0.5). In the course of modification of ring size on the side-chain ester (**1a** and **1c**–**e**), compound **1c** (R = cyclobutyl), which has a cyclobutyl ring, showed 4-fold decreased N-type inhibitory activity and selectivity. In contrast, compounds **1d** (R = cyclohexyl) and **1e** (R = cycloheptyl) showed similar potency to the initial lead compound **1a**. With respect to the selectivity for N-type calcium channels, cyclohexyl ester was better than cyclopentyl ester. Modification of the substituents on the benzene ring showed no significant influence on the potency (see Table 1: compounds **1f**–**i**). Thus, the compound **1d**, which has 4-methoxy benzyl ester at the α-carbonyl and cyclohexyl ester at the side chain, was selected for further SAR study.

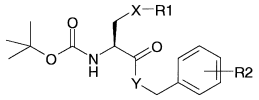


Scheme 3. (a) Cyclohexane-3-bromide, NaH; (b) H_2, Pd/C, EtOH; (c) 4-methoxybenzylamine, EDC, DMAP.



Scheme 4. Reagents: (a) cyclopentylmethyl methanesulfonate or cyclohexylmethyl bromide, 2 N NaOH, EtOH; (b) Boc_2O, 2 N NaOH, EtOH; (c) 4-methoxybenzylamine, EDC, HOBt, DMF.

Table 1. In vitro inhibition of calcium influx in IMR-32 and AtT-20 assays



Compd	X	R1	Y	R2	IC_{50} (μM)[a]		Selectivity ratio (L/N)
					N-type (IMR-32)	L-type (AtT-20)	
1a	CO–O	Cyclopentyl	O	4-OMe	3.4	15	4.4
1b	CO–O	Methyl	O	4-OMe	26	14	0.5
1c	CO–O	Cyclobutyl	O	4-OMe	15	28	1.9
1d	CO–O	Cyclohexyl	O	4-OMe	2.3	16	7.0
1e	CO–O	Cycloheptyl	O	4-OMe	2.6	12	4.6
1f	CO–O	Cyclopentyl	O	2-OMe	5.4	15	2.8
1g	CO–O	Cyclopentyl	O	3-OMe	3.8	12	3.2
1h	CO–O	Cyclopentyl	O	4-Cl	3.3	16	4.8
1i	CO–O	Cyclopentyl	O	4-Br	3.4	5.3	1.6
5	O–CO	Cyclohexyl	O	4-OMe	1.6	10	6.3
7a	O–CH_2	Cyclohexyl	O	4-OMe	1.2	8.4	7.0
7b	O–CH_2	Cyclohexyl	NH	4-OMe	0.95	3.8	4.0
7c	O–CH_2	Cyclohexyl	NMe	4-OMe	1.8	1.4	0.8
10	CH_2–O	Cyclohexyl	NH	4-OMe	4.0	13	3.3
2a	S–CH_2	Cyclopentyl	NH	4-OMe	0.63	3.9	6.2
2b	S–CH_2	Cyclohexyl	NH	4-OMe	0.61	1.7	2.8

Table 2. Activities of selected compounds in electrophysiological study and in vivo (rat) formalin-induced pain model

Compd	Electrophysiology (IMR-32, 10 μM, $n=3$) inhibition %	Formalin test 3 nmol/rat, it	
		Inhibition %	n
1a	34	31	5
7b	38	35	3
2a	45	47	3
2a		43 (100 mg/kg, po)	3
2b	66	36	3

Replacement of the amino acid skeleton was next investigated. L-Ser analogue, the reverse type ester of **1a**, showed increased N-type blocking activity in IMR-32 assay (**5**, $IC_{50}=1.6\,\mu M$). Similar results were observed in L-Ser ether (**7a**, $IC_{50}=1.2\,\mu M$), which had 3-fold improved N-type calcium channel blocking potency than the lead compound **1a**.

To evaluate in vivo analgesic efficacy, the ester was converted to amide since the ester function seems to be unstable against metabolic hydrolysis. Conversion to amide was achieved without losing potency in IMR-32 assay (**7b**, $IC_{50}=0.95\,\mu M$) or selectivity (L/N = 4.0). The *N*-methyl analogue **7c** (Y = NMe) was also synthesized for the same purpose. However, this compound was not selective (L/N = 0.8).

In terms of oxygen position on the side chain, interesting results were observed by comparison between L-Ser ether **7b** and L-Hse ether **10**. The calcium channel inhibitory activity of **10** was decreased 4-fold by this minor modification. This observation was similar to the comparison between L-Asp ester **1a** and L-Ser ester **5**. These results also showed that the position of oxygen on the side chain has a significant influence on the inhibitory activity.

Finally, we prepared some L-cysteine analogues, which could be relatively stable against metabolic hydrolysis compared with the initial lead. These compounds were among the most potent analogues in this series (**2a**, $IC_{50}=0.63\,\mu M$ and **2b**, $IC_{50}=0.61\,\mu M$). The inhibitory activity for N-type calcium channels of compound **2a**, the most potent and selective compound, was evaluated by electrophysiological assay. This compound blocked N-type calcium channel current in IMR-32 cells (45% at 10 μM, $n=3$). This compound was also evaluated for analgesic efficacy in the rat formalin-induced pain model.[11] Preliminary results showed that the compound **2a** inhibited paw flinching during the persistent nociceptive phase by intrathecal (47% inhibition at 3 nmol/rat, it, $n=3$) and oral administration (43% inhibition at 100 mg/kg, po, $n=3$) without any significant effect on blood pressure or neurological behavior. Activities of selected compounds in electrophysiological study and in the in vivo formalin test were summarized in Table 2.

In conclusion, the SAR study of a series of *N*-Boc-amino acid derivatives led to the discovery of novel neuronal N-type calcium channel blockers. Compound **2a** was a potent N-type calcium channel blocker and was 6-fold more selective over L-type calcium channels. This compound also showed analgesic efficacy in the rat formalin-induced pain model, and could be a potential lead compound for further modification.

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6. IMR-32 cells were grown as described (Clementi, F.; Cabrini, D.; Gotti, C.; Sher, E. *Eur. J. Neurochem.* **1986**, *47*, 291. Carbone, E.; Sher, E.; Clementi, F. Pflügers Arch. **1990**, *416*, 170) in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin
7. The electrophysiological recordings were performed in the conventional whole-cell configuration under voltage-clamp conditions. Pipettes had a resistance of 3–6 MΩ. Membrane currents were measured using a patch clamp amplifier (Axopatch 2B Axon Instruments). The test compounds were applied using a rapid application method designated as the 'Y-tube method'.
8. (a) Xie, J.; Nagle, G. T.; Childs, G. V.; Ritchie, A. K. *Neuroendocrinology* **1999**, *70*, 1. (b) Mouse pituitary tumor-derived cells (AtT-20/D16v-F2), were grown in Dulbecco's Modified Eagle's medium containing 10% FBS, penicillin-streptomycin
9. $[Ca^{2+}]_I$ was measured in cell suspension. Cell suspension was incubated with 5 mM fura-2/AM for 30 min at 37 °C. Cells were resuspended in Krebs–Ringer HEPES solution, and adjusted to 1.0×10^6 cells/mL (IMR-32), or 2.0×10^6 cells/mL (AtT20/D16v-F2). Fluorescence (λ_{Ex}: 340 and 380 nm, λ_{Em}: 500 nm) was detected with a fluorometer. Cell suspension was incubated with test compound and 10 μM nifedipine (IMR-32) or 3 μM ω-conotoxin MVIIC (AtT-20/D16v-F2) for 360 s before high-K^+ stimulus. To evaluate the inhibitory activities of test compounds, IMR-32 and AtT20/D16v-F2 cells were used for N-type and L-type calcium channels, respectively.
10. No isomerized product was detected by chiral HPLC analysis. **1a**; $[\alpha]_D = -16.0°$ ($c = 1.15$, MeOH). **3**; $[\alpha]_D = +15.1°$ (c 0.92, MeOH).
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