New ACE Inhibitor Designed from Nicotianamine and Its Docking Pose Prediction Using the Gold Program

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Abstract: Hypertension is currently one of the most serious health issues worldwide. Nicotianamine, a non-peptide-type amino acid trimer, is ubiquitously present in higher plants and plays a role as an internal metal transporter. It is known that nicotianamine inhibits ACE activity and that oral treatment with the compound improves hypertension. However, the mode of action remains unclear, due to lack of crystallographic data. Although a structure-activity relationship study of nicotianamine has the potential to uncover the details of the inhibition profile, the azetidine-2-carboxylic acid moiety in nicotianamine has become a critical barrier for further biochemical research due to limited commercial supply and difficulties with structural modification. In this paper, ten nicotianamine analogs without azetidine-2-carboxylic acid moiety were prepared and their inhibition of angiotensin I-converting enzyme was investigated. Among these analogs, a phenylalanine analog, \((2\text{S},3\text{S},3\text{S})\)-N-{3′-(3″-amino-3″-carboxypropylamino)-3′-carboxypropyl}phenylalanine, displayed the most potent activity. The inhibition activity of the compound corresponded to that of captopril. These results suggested a possibility of structural modification of nicotianamine to develop antihypertensive drugs. Molecular docking studies with Gold were also performed to predict the binding poses of nicotianamine and its analog, suggesting that nicotianamine and its analogs combine a plausible allosteric site in an area away from the catalytic site in ACE.

Keywords: Nicotianamine, Structure-Activity-Relationship Study, Angiotensin I-Converting Enzyme Inhibitors, Molecular Docking Study, Allosteric Binding Site

1. Introduction

Hypertension is currently one of the most serious health issues worldwide. Renin-angiotensin system (RAS) is involved in the long-term regulation of blood pressure and volume in the human body and is considered to be one of the key targets for hypertension drugs. Angiotensin I-converting enzyme (ACE) is a RAS component that catalyzes hydrolysis of angiotensin I to generate the vasopressor angiotensin II. Hence, many ACE inhibitors (ACEIs) such as captopril [1-3] have been developed and are being used in hypertension therapy (Figure 1). The inhibition pattern of captopril is verified to be competitive, and the binding features of captopril with ACE have been well-established by X-ray crystallographic studies [4], thereby leading to the development of other antihypertensive drugs [5], such as enalapril, that bind to ACE in a similar manner.

![Figure 1. Structures of nicotianamine and captopril.](image)

Nicotianamine, a non-peptide-type amino acid trimer, is ubiquitously present in higher plants and plays a role as an internal metal transporter [6-9]. Nicotianamine also inhibits ACE activity, and oral treatment with the compound improves hypertension in both spontaneously hypertensive rats (SHR) and Tsukuba hypertensive mice (THM) [10-12].
The synthase genes have already been observed in various plants [13-14], and overexpression of nicotianamine in transgenic plants has also been established [15]. The inhibition pattern was reported to be mixed noncompetitive [16]. Nicotianamine exhibited weak chelating effects for zinc, copper, and cobalt ions, although the role of nicotianamine is as an internal metal transporter in plants.

These data suggest that nicotianamine is an allosteric ACEI, providing an alternate strategy in the development of ACE-targeted therapy. However, the nature of the allosteric site in ACE remains unclear to date, due to lack of crystallographic data. In this case, a structure-activity relationship (SAR) study of nicotianamine has the potential to uncover the details of the inhibition profile, which would lead to novel hypertensive drug development. However, the azetidine-2-carboxylic acid moiety in nicotianamine has become a critical barrier for further biochemical research due to limited commercial supply (L-azetidine 2-carboxylic acid: 34 USD/g, L-phenylalanine: 0.73 mmol) dissolved in MeOH (2.5 mL). The mixture was stirred at room temperature overnight and then was poured into a mixture of saturated aqueous NaHCO₃ (50 mL) and EtOAc (50 mL). After the removal of MeOH in vacuo, the aqueous mixture was extracted with EtOAc (50 mL×3). The combined organic layer was washed with brine (50 mL), dried over Na₂SO₄, and then concentrated in vacuo. Silica gel column chromatography of the residue (hexane: EtOAc = 3:1 → 2:1 → 1:1) yielded 3a (150 mg, 67 %); δ(OD, 9H) s, 1.95 (1H, m), 2.41 (1H, ddd, J = 1.6, 7.0, 9.1, 12.4 Hz), 3.40 (1H, dt, J = 1.6, 9.5 Hz), 3.46 (1H, ddd, J = 7.0, 9.5, 9.7 Hz), 4.05 (1H, d, J = 17.7 Hz), 4.23 (1H, d, J = 17.7 Hz), 4.26 (1H, ddd, J = 9.1, 9.5 Hz), 5.17 (4H, s), 7.29-7.37 (10H, m); HRESIMS: MH⁺, found 457.2330. C₂₂H₂₃NO₉ requires 457.2339.

2.2.2. (3’S)-N-(3”-Benzoxycarbonyl-3’-Tert-Butoxycarbonylaminopropyl)Glycine Benzyl Ester (3a)

To a stirring solution of aldehyde 1 (150 mg, 0.49 mmol) and toluenesulfonfum salt of glycine benzyl ester (2a, 247 mg, 0.73 mmol) dissolved in MeOH (2.5 mL) was added sodium cyanoborohydride (46 mg, 0.73 mmol). The mixture was stirred vigorously under a H₂ atmosphere at room temperature overnight and then was poured into a mixture of saturated aqueous NaHCO₃ (50 mL) and EtOAc (50 mL). After the removal of MeOH in vacuo, the aqueous mixture was extracted with EtOAc (50 mL×3). The combined organic layer was washed with brine (50 mL), dried over Na₂SO₄, and then concentrated in vacuo.

Silica gel column chromatography of the residue (hexane: EtOAc = 3:1 → 2:1 → 1:1) yielded 3a (150 mg, 67 %); δ(OD, 9H) s, 1.95 (1H, m), 2.41 (1H, ddd, J = 1.6, 7.0, 9.1, 12.4 Hz), 3.40 (1H, dt, J = 1.6, 9.5 Hz), 3.46 (1H, ddd, J = 7.0, 9.5, 9.7 Hz), 4.05 (1H, d, J = 17.7 Hz), 4.23 (1H, d, J = 17.7 Hz), 4.26 (1H, ddd, J = 9.1, 9.5 Hz), 5.17 (4H, s), 7.29-7.37 (10H, m); HRESIMS: MH⁺, found 457.2330. C₂₂H₂₃NO₉ requires 457.2339.

2.2.3. (3’S)-N-(3”-Amino-3’-Carboxy-Propyl)Glycine (4a)

Amine 3a (28.3 mg, 0.062 mmol) was dissolved in trifluoroacetic acid (1 mL) and the mixture was left to stand at room temperature for 1 h and then was concentrated in vacuo. To a stirred solution of the residue in a mixture of EtOH (2 mL) and H₂O (0.5 mL) was added a catalytic amount of 10 % Pd-C at room temperature. The mixture was stirred vigorously under a H₂ atmosphere at room temperature for 20 min and then filtered through a pad of celite. The filtrate was concentrated in vacuo to yield 5a (11
mg) quantitatively; δH (D2O) 2.06 (1H, m), 2.53 (1H, m), 3.42-3.52 (2H, m), 3.97 (1H, dd, J = 5.5, 7.4 Hz), 4.07-4.16 (2H, m); HRESIMS: MH+, found 177.0883. C6H11N2O4 requires 177.0875.

Other compounds (4b-3e, 6a-6e) were prepared in a similar way (2.2.2. and 2.2.3.) with corresponding amino acids counter parts (2a-2c).

2.2.4. Spectral Data for Other Analogs

(i) (2S,3'S)-N-(3′-amino-3′-carboxypropyl)proline (4b)

δH (D2O) 1.87 (1H, m), 1.97-2.08 (3H, m), 2.15 (1H, m), 2.21 (1H, m), 2.38 (1H, m), 3.07 (1H, m), 3.25 (1H, m), 3.44 (1H, m), 3.68 (1H, m), 3.89 (1H, m), 3.99 (1H, m); HRESIMS: MH+, found 217.1190. C6H11N2O4 requires 217.1188.

(ii) (2S,3'S)-N-(3′-amino-3′-carboxypropyl)phenylalanine (4c)

δH (D2O) 1.86 (1H, m), 1.97 (1H, m), 2.05 (1H, m), 2.32 (1H, m), 2.95 (1H, m), 3.06 (1H, m), 3.95 (1H, m), 4.08 (1H, dd, J = 5.1, 8.9 Hz), 7.14-7.35 (5H, m); HRESIMS: MH+, found 267.1353. C13H19N2O4 requires 267.1345.

(iii) (2S,3'S)-N-(3′-amino-3′-carboxypropyl)glutamic acid (4d)

δH (D2O) 1.94 (1H, m), 2.00-2.10 (2H, m), 2.13 (1H, m), 2.30-2.41 (2H, m), 3.05-3.14 (2H, m), 3.55 (1H, t, J = 6.2 Hz), 3.72 (1H, dd, J = 5.3, 7.6 Hz); HRESIMS: MH+, found 249.1090. C9H17N2O5 requires 249.1087.

(iv) (2S,3'S)-N-(3′-amino-3′-carboxypropyl)leucine (4e)

δH (D2O) 0.80 (3H, d, J = 6.6 Hz), 0.86 (3H, d, J = 6.6 Hz), 1.52-1.67 (4H, m), 2.16 (1H, m), 3.01 (1H, m), 3.12 (1H, m), 3.59 (1H, m), 3.77 (1H, dd, J = 5.7, 7.4 Hz); HRESIMS: MH+, found 233.1511. C9H17N2O4 requires 233.1501.

(v) (3′S,3′S)-N-(3′-amino-3′-carboxypropylamino)-3′-carboxypropyl]glycine (6a)

δH (D2O) 1.95-2.06 (4H, m), 2.90-3.25 (4H, m), 3.71 (1H, m), 3.89 (1H, dd, J = 6.0, 7.2 Hz), 4.07-4.16 (2H, m); HRESIMS: MH+, found 278.1340. C10H20N3O5 requires 278.1352.

(vi) (2S,3'S,3'S)-N′-[3′-[(3′-amino-3′-carboxypropyl)amino]-3′-carboxypropyl]proline (6b)

δH (D2O) 1.87-2.38 (8H, m), 2.98-3.12 (2H, m), 3.44 (1H, m), 3.68 (1H, m), 3.71 (1H, m), 3.89 (1H, dd, J = 5.5, 7.0 Hz), 3.99 (1H, m); HRESIMS: MH+, found 318.1653. C13H23N3O5 requires 318.1665.

(vii) (2S,3'S,3'S)-N′-[3′-[(3′-amino-3′-carboxypropyl)amino]-3′-carboxypropyl]phenylalanine (6c)

δH (D2O) 1.86-2.30 (6H, m), 2.95-3.20 (4H, m), 3.71 (1H, t, J = 6.2 Hz), 3.95 (1H, m), 4.08 (1H, dd, J = 5.1, 8.9 Hz), 7.14-7.35 (5H, m); HRESIMS: MH+, found 368.1830. C17H26N4O requires 368.1822.

2.3. ACE Inhibition Assay Protocol [18]

To a test tube an appropriate amount of sample solution was added, and the solvent was evaporated in vacuo. Forty µL of ACE (5 mU) solution in 20 mM phosphate buffer (pH 8.3) was added to the test tube, and the mixture was preincubated for 5 min at 37°C. The enzymatic reaction was initiated by adding 25 µL of 13.0 mM HHL in 250 mM sodium borate buffer (pH 8.3). After incubation for 1 h at 37°C, the reaction was quenched by adding 12.5 µL of 2 M aqueous HCl. After the addition of 0.2 M phosphate buffer (pH 8.3, 875 µL), cyanuric chloride (3%) in 1,4-dioxane (400 µL) was added to the mixture with vigorous stirring. The enzyme activity in the resulting solution was evaluated by its absorbance at 382 nm. The negative control run was identical to the above procedure without the inhibitor. Captopril was used as the positive control. A unit of inhibitory activity was defined as an amount of inhibitor needed to inhibit 50% of the ACE activity (IC50 value).

2.4. Docking Studies

Docking calculations were performed with Gold 5.2 software. The protein data in the docking studies was obtained from Protein Databank (PDB code 1UZF). The energy minimization of ligands (nicotianamine and its analog 6c) was performed using MM2 force field with Spartan’08 software. Default parameters are used in all docking experiments and GoldScore was used as a scoring function.

3. Results and Discussion

Nicotianamine is a non-peptide-type amino acid trimer that includes an azetidine-2-carboxylic acid moiety as its structural feature. Determining whether the trimeric structure and the azetidine-2-carboxylic acid moiety are essential for ACEI activity would help define the mode of action of nicotianamine and aid in the development of a new type of hypertensive drug. To this end, dimeric analogs (for examining the necessity of the trimeric structure) and trimeric analogs (including other amino acid moieties instead of azetidine-2-carboxylic acid) were prepared. Five amino acids with chemically different residues were selected as follows: i) glycine without a residue, ii) L-proline with a cyclic residue, iii) L-phenylalanine with an aromatic residue, iv) L-glutamic acid with a negatively-charged residue, and v)
L-leucine with an aliphatic bulky residue. Several practical protocols for nicotianamine synthesis are described in the literature. These are categorized based on key reactions as follows: (i) reductive amination of intermediate protected aldehydes [19-21], (ii) reduction of amide bonds via thioamide [22], and (iii) nucleophilic substitution of iodine by an amine [23]. One of our purposes was to establish the necessity of the azetidine-2-carboxylic acid moiety for ACEI activity. The moiety should be incorporated at the latter step in the synthetic scheme. In this context, the reductive amination protocol by Ofune and coworkers was the most adequate for our purposes and was adapted for our analogs’ syntheses.

Aldehyde 1 [20] prepared from commercially available L-homoserine lactone was coupled with glycine benzyl ester 2a via a reductive amination procedure (sodium cyanoborohydride, MeOH, room temperature) to give the desired 3a in 67% yield (Figure 2). All protecting groups in 3a were then removed under mild conditions (i) CF₃COOH (TFA), room temperature; (ii) H₂, Pd-C (10 %), EtOH-H₂O, room temperature) to afford 4a quantitatively. The other analogs 4b-4e were prepared in the same manner using L-proline benzyl ester (2b), L-phenylalanine benzyl ester (2c), L-glutamic acid α,γ-dibenzyl ester (2d), and L-leucine benzyl ester (2e), respectively. Aldehyde 5 was also prepared via a reductive amination of 1 with L-homoserine lactone in the same manner as described above, and the coupling reaction between 5 and 2a was achieved under reductive amination conditions. The successive removal of all protecting groups gave the desired 6a in 19% yield in three steps. Analogs 6b-6e were also prepared in the same manner.

The ACEI activities of ten analogs (4a-4e, 6a-6e), together with nicotianamine and captopril, were examined (Table 1). None of five dimeric analogs 4a-4e exhibited detectable ACEI activities (IC₅₀ >50 mg/L), suggesting that the trimeric structure is essential for ACEI activity. Indeed, all trimeric analogs (6a-e) exhibited potent ACEI activities. In other words, the azetidine-2-carboxylic acid moiety is verified not to be essential for activity. The glycine analog 6a without a residue and the proline analog 6b with a pyrrolidin as its residue exhibited three-fold higher potency (IC₅₀ = 0.15 mg/L) than that of nicotianamine (IC₅₀ = 0.50 mg/L). The glutamic acid analog 6d (a negative-charged residue) and the leucine analog 6e (a bulky aliphatic residue) exhibited four-fold weaker activity (IC₅₀ = 2.0 mg/L) than that of nicotianamine, respectively. The bulky and negatively-charged residues would be inadequate for activity. Interestingly, the phenylalanine analog 6c, which possessed an aromatic residue, exhibited the most potent activity among the analogs prepared in this study. Its activity (IC₅₀ = 0.050 mg/mL) corresponded to that of captopril (IC₅₀ = 0.035 mg/mL) and was ten-fold greater than that of nicotianamine.

Table 1. ACE inhibition activities of nicotianamine and its analogs.

|          | IC₅₀ (mg/L) | IC₅₀ (mg/L) |
|----------|------------|------------|
| nicotianamine | 0.50      | 0.035      |
| 4a       | >50        | 6a         |
| 4b       | >50        | 6b         |
| 4c       | >50        | 6c         |
| 4d       | >50        | 6d         |
| 4e       | >50        | 6e         |

To investigate why analog 6c exhibits higher ACEI activity, docking calculation studies were carried out using
Gold program [24-26]. In recent years, computational methods such as in silico docking studies provide inexpensive and efficient ways for investigating ligand-protein interactions. Docking studies can predict plausible binding features of a ligand-protein complex, and has become a powerful tool in drug discovery processes [27-29]. In current study, a crucial problem is that the features of nicotianamine’s binding site (which is an allosteric site in ACE) remains unclear to date due to the lack of crystallographic data. However, it is expected that a plausible binding site could be predicted using thorough docking calculations. For example, when the ligand-protein affinity is calculated using the parameter that an arbitrary residue is set as the binding site center, a high score should only be counted if the residue is located near the true binding site. On the other hand, if the residue of interest is far away from the true binding site, its affinity score should be low. Additionally, when docking calculations are performed using the parameter that its catalytic site in ACE is occupied with a ligand such as captopril, docking calculations would indicate an allosteric binding site in the protein. Thus, docking studies make it possible to predict the presence of a previously unknown allosteric binding site.

**Figure 3. Nicotianamine binding site prediction with Gold program.** (a) high-scored residues, as results of docking calculations, were highlighted in red (ball and stick; GoldScore > 70). In order to survey an allosteric site candidates, Gold calculations were carried out where captopril (ball and stick) occupied a catalytic site around Zn$^{2+}$ ion (green sphere). (b) Zoom-in of Gold best scored docking pose for nicotianamine-ACE complex. Only residues involved in binding are shown (thin ball and stick), and observed hydrogen bonds are highlighted in yellow lines. (c) Zoom-in of Gold best scored docking pose for 6c-ACE complex. CH-π interaction, in addition to same interactions as nicotianamine, are observed (yellow line).

Docking calculations with nicotianamine were carried out and then all the GoldScores were visualized (Figure 3a). Interestingly, high-scoring residues (shown in red; GoldScore > 70) were concentrated in an area away from the catalytic site, suggesting that this area is a plausible allosteric site in ACE. In top-ranked nicotianamine-ACE complex (Figure 3b), it was observed that one ionic bond (C-1 carboxyl to Lys118, 1.53 Å) and five hydrogen bonds (C-1 carbonyl to 360Tyr, 2.91 Å; C-1’ carbonyl to 92Thr, 2.55 Å; C-1” carbonyl to Tyr51, 1.87 Å; C-2” ammonium to 118Lys, 1.52 Å; and C-2” ammonium to 121ASP, 1.33 Å) interactions. In the docking studies for analog 6c, similar interactions with ACE were observed (Figure 3c). Additionally, an edge-to-face attractive interaction was noted between the phenyl ring in analog 6c and the p-hydroxyphenyl ring in Tyr62 [30]. Specifically, H-5 on the aromatic ring is located at a close-contact perpendicular distance of 2.53 Å above the face of the p-hydroxyphenyl ring in Tyr62, thus suggesting that this additional aromatic interaction is responsible for the high ACEI activity of analog 6c.

Labeling analogs with activity is a powerful molecular tool for determining the binding profile of host-guest complex [31-32]. Analog 6c has the potential to become a valuable tool for additional biochemical study due to its easy application to photoaffinity probes using an azide and a diazirine groups [33-34]. Furthermore, analog 6c would be effective for the treatment of hypertensive disease similar to nicotianamine. Thus, these results imply that (2S,3′S,3″S)-N-\{3′-(3″-amino-3″-carboxypropylamino)-3′-carboxypropyl\}phenylalanine (6c) is a potential ACE inhibitor that can contribute to both the establishment of nicotianamine’s mode of action and novel methods for hypertension therapy. The application of the compound to THM and SHR is currently being studied in our laboratory.

### 4. Conclusion

A phenylalanine analog, (2S,3′S,3″S)-N-{3′-(3″-amino-3″-carboxypropylamino)-3′-carboxypropyl}phenylalanine (6c), was developed as a nicotianamine analog, and exhibited more potent activity (IC$_{50}$ = 0.050 mg/L) than that of nicotianamine. The activity of the analog corresponds to that of captopril, which is one of the most well-known hypertensive drugs. Docking calculations using Gold program suggested the presence of a plausible allosteric binding site of nicotianamine and an edge-to-face attractive interaction between analog 6c and Tyr62.

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