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Synthesis of a series of 3,4-methanoarginines as side-chain conformationally restricted analogues of arginine

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ABSTRACT

A series of optically active stereoisomers of 3,4-methanoarginine (**1**–**4** and ***ent*-1**–***ent*-4**) with *trans*/*cis*, D/L, and *syn*/*anti* stereochemical diversity, the side-chains of which were restricted in various special arrangements, was designed as biologically useful arginine mimetics. These conformationally restricted arginine analogues were synthesized effectively by using a series of chiral 3,4-methanoamino acid equivalents (**7**–**10** and ***ent*-7**–***ent*-10**) as the key synthetic units. Their biological evaluation with three isoforms of nitric oxide synthase showed that *trans*-3,4-methano-L-*syn*-arginine (**2**) was a good substrate, having close potency to L-arginine, and isoforms selectivities were also similar to those of L-arginine.

1. Introduction

The conformational restriction of backbone or side-chains of biologically active peptides to the bioactive conformation can be a promising approach in the development of small useful peptidomimetics.[1] A cyclopropane is very effective for restricting the conformations of a molecule because of its rigid and small structural properties. In fact, conformationally restricted analogues of various amino acids containing a cyclopropane, which have improved the biological potency and selectivity of lead molecules depending on the conformations, are known.[2] Therefore, the development of useful synthetic methods of conformationally restricted amino acid analogues is important for peptidomimetics studies.

Based on the characteristic conformational restriction of cyclopropanes, we devised a stereochemical diversity-oriented conformational restriction strategy for compounds that bind selectively to a target protein.[3–5] In this strategy, a series of cyclopropane-based conformationally restricted analogues of a conformationally flexible lead compound, which has stereochemical diversity, is designed and synthesized, and the compounds that selectively bind to the target protein can be identified. In fact, we have successfully developed useful compounds by this strategy, which have high affinity for target proteins such as histamine receptor subtypes or the proteasome.[3–7] Thus, we applied this strategy to arginine to design and synthesize a series of cyclopropane-based conformationally restricted arginine analogues with stereochemical diversity. These analogues are of importance in medicinal chemistry, for example, as potentially selective ligands for the isoforms of nitric oxide synthase (NOS, EC 1.14.13.39) and also as side-chain conformationally restricted amino acid mimetics of biologically active peptides.

The cyclopropane-based conformationally restricted arginine analogues have been reported,[8,9] which are two isomers of 3,4-methaonoarginines, that is, *trans*-3,4-methano-L-*anti*- and -*syn*-arginines (**1** and **2**), shown in Figure 1, of which the carboxylic group is oriented *anti* or *syn* to the cyclopropane moiety, respectively. We decided to synthesize all of the stereoisomeric L- and D-3,4-methanoarginine analogues including known **1** and **2**, which have *trans*/*cis*, D/L, and *syn*/*anti* stereochemical diversity (**1**–**4** and ***ent*-1**–***ent*-4**, Fig. 1).

To prepare various cyclopropane derivatives effectively, we have designed chiral cyclopropane units, which are composed of four stereoisomeric cyclopropanes, **5** and **6**, and their enantiomers, ***ent*-5** and ***ent*-6**, bearing two adjacent carbon substituents in a *trans* or *cis* relationship (Fig. 2).[3,5] These units are generally useful for synthesizing a series of optically active stereoisomeric cyclopropane derivatives having an asymmetric *trans*- or *cis*-cyclopropane structure.[3–7,10,11]

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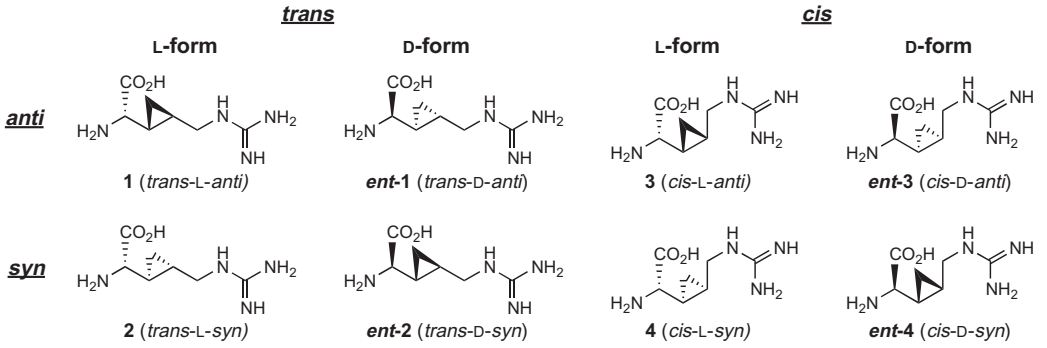


Figure 1. A series of optically active cyclopropane-based conformationally restricted analogues of L- and D-arginine with stereochemical diversity.

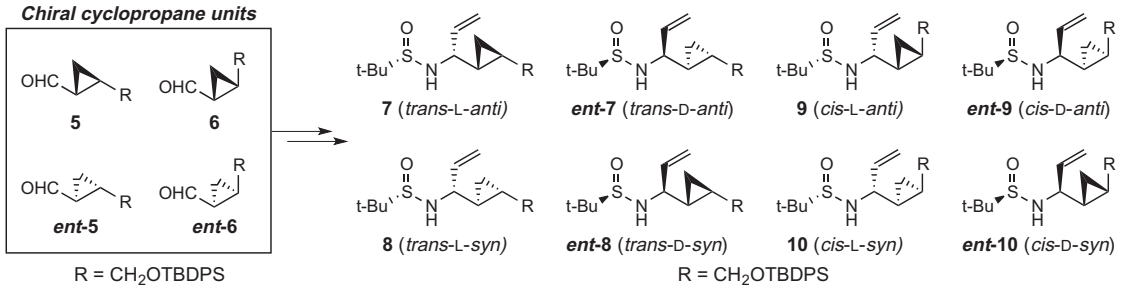


Figure 2. Chiral cyclopropane units and chiral 3,4-methanoamino acid equivalents as the key units for the synthesis of conformationally restricted L- and D-amino acid analogues.

Also, we recently developed an efficient method for the systematic synthesis of a series of 3,4-methanoamino acid equivalents (**7**–**10** and ***ent*-7**–***ent*-10**), shown in Figure 2, with *trans*/*cis*, D/L, and *syn*/*anti* stereochemical diversity from the chiral cyclopropane units **5**, **6**, ***ent*-5**, and ***ent*-6**.[6] This series has an optically active CH_2═CHC∗H(NH–)- moiety in the structures. These compounds can be considered to be equivalents of D- or L-methanoamino acids because a vinyl group is stable under various reaction conditions and can be converted easily to a carboxyl group.[7,12]

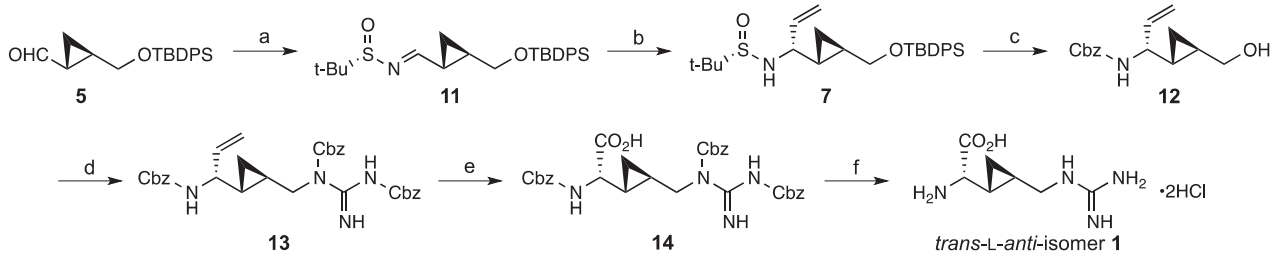
Herein, we report the systematic synthesis of a series of optically active cyclopropane-based conformationally restricted arginine analogues (**1**–**4** and ***ent*-1**–***ent*-4**) using our 3,4-methanoamino acid equivalents as key intermediates.

2. Results and discussion

2.1. Chemistry

The synthesis of *trans*-3,4-methano-L-*anti*-arginine analogue **1** from chiral cyclopropane unit **5** via 3,4-methanoamino acid equivalent **7** as a key intermediate is shown in Scheme 1. Chiral *trans*-cyclopropane aldehyde **5**[3] was treated with (*S*)-*tert*-butanesulfinamide in the presence of $CuSO_4$ to give (*S*)-*tert*-butanesulfinylimine **11** in a high yield. 3,4-Methanoamino acid equivalent **7** was obtained in a very high yield by a highly diastereoselective addition of a vinyl group to imine **11** using a Grignard reaction with CH_2═CHMgBr.[6] The spectral data of **7** was consistent with that of ***ent*-7**, which is the enantiomer of **7** reported previously by us.[6] The removal of the *N*-sulfinyl and the *O*-silyl groups of **7** under acidic conditions, followed by protection of the resulting free amino group with a Cbz group provided alcohol **12**. The Mitsunobu reaction of **12** with *N*,*N*′-bis-Cbz-guanidine[13] gave compound **13** contaminated with an inseparable by-product derived from diisopropyl azodicarboxylate. However, oxidation of the mixture with a mixture of $NaIO_4$/$KMnO_4$/$NaHCO_3$ in aqueous acetone worked well, and pure carboxylic acid **14** was obtained in a good yield for the two steps after silica gel column chromatography. Finally, the Cbz-protecting groups were removed by catalytic hydrogenation to produce *trans*-L-*anti*-isomer **1**.

The other L-arginine analogues, *trans*-L-*syn*-isomer **2**, *cis*-L-*anti*-isomer **3**, and *cis*-L-*syn*-isomer **4**, were synthesized from corresponding chiral cyclopropane units ***ent*-5**, **6**, or ***ent*-6** by the same procedure for the synthesis of compound **1** described above, via corresponding 3,4-methanoamino acid equivalents **8**, **9**, or **10**, respectively. The four D-arginine analogues, *trans*-D-*anti*-isomer ***ent*-1**, *trans*-D-*syn*-isomer ***ent*-2**, *cis*-D-*anti*-isomer ***ent*-3**, and *cis*-D-*syn*-isomer ***ent*-4**, were also synthesized from corresponding



Scheme 1. Reagents and conditions: (a) (*S*)-*t*-$BuSONH_2$, $CuSO_4$, CH_2Cl_2, 94%; (b) CH_2═CHMgBr, toluene, 110 °C, 97%; (c) (i) HCl, AcOEt/MeOH; (ii) Cbz-Cl, Na_2CO_3, aq THF, 75% for two steps; (d) *N*,*N*′-bis-Cbz-guanidine, PPh_3, DIAD, THF, 0 °C to rt; (e) $NaIO_4$, $KMnO_4$, $NaHCO_3$, aq acetone, 68% for two steps; (f) H_2, Pd/C, HCl, EtOH, 48%.

3,4-methanoamino acid equivalents ***ent*-7**, ***ent*-8**, ***ent*-9**, or ***ent*-10**, which were prepared from chiral cyclopropane units ***ent*-5**, **5**, ***ent*-6**, or **6**, respectively, by the procedure using (*R*)-*tert*-butanesulfinamide instead of (*S*)-*tert*-butanesulfinamide described above.[14] These results demonstrate that the series of 3,4-methanoamino acid equivalents, **7**–**10** and ***ent*-7**–***ent*-10**, is useful for the systematic synthesis of conformationally restricted amino acid analogues with stereochemical diversity.

2.2. Evaluation as NOS ligands

NOS is a family of enzymes that catalyzes the production of L-citrulline and nitric oxide (NO) from L-arginine with O_2 and NADPH.[15] The family of NOS consists of three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS).[16] Two of these isoforms, nNOS and eNOS, are constitutively expressed mainly on neurons or vascular endothelial cells, respectively. On the other hand, iNOS is transcriptionally activated by stimulating factors such as cytokines and bacterial lipopolysaccharide in a wide range of cell types. These different NOS isoforms generate NO, which is an important signaling molecule that regulates a variety of physiological functions in the nervous, cardiovascular, and immune systems.[17] Overproduction of NO in these systems induces various pathological states such as stroke,[18] hypertension,[19] and septic shock.[20] Therefore, NOS isoform-selective ligands are useful as biological tools and/or drug leads for NOS isoform-selective therapeutic agents for NO-related diseases.[21]

Thus, to examine the biological activity of the synthesized conformationally restricted analogues of arginine, we evaluated them as ligands for three isoforms of NOS: rat nNOS, murine iNOS, and bovine eNOS using known methods.[22]

As a result, the six analogues, **1**, **4**, and ***ent*-1**–***ent*-4**, did not act as substrates for all NOS isoforms but were weak inhibitors with IC_{50} values greater than 300 μM (Table 1). There was very little selectivity among the isoforms of NOS, although their inhibitory effects were slightly different among them (for example, inhibitory effects of **1** at 300 μM were 16% for nNOS; 26% for iNOS; 45% for eNOS).

On the other hand, two analogues, *trans*-L-*syn*-isomer **2** and *cis*-L-*anti*-isomer **3**, did not inhibit any of the NOS isoforms but were found to be substrates for all isoforms of NOS, as shown in Table 2. The K_m values of **2** for all NOS isoforms were only about a factor of three higher than the corresponding K_m values of L-arginine (7.6 μM vs 2.7 μM for nNOS; 20 μM vs 9.5 μM for iNOS; 3.6 μM vs 1.1 μM for eNOS, respectively). The k_{cat}/K_m values of **2** for all NOS isoforms were comparable to those of L-arginine. The selectivities of **2** for nNOS and eNOS over iNOS on the basis of K_m values were 2.6- and 5.6-fold, respectively, and those tendencies were also similar to those of L-arginine (3.5- and 8.6-fold, respectively). These results show that *trans*-L-*syn*-isomer **2**, whose affinities and potencies with each isoform of NOS are comparable to those of L-arginine, is a good substrate for all NOS isoforms, although **2** shows little selectivity.

Compound **3** was identified as a less effective substrate for all three isoforms of NOS than **2**, having K_m values of 29- to 45-times greater than those of **2** and k_{cat}/K_m values less than 33-times those of **2**. The selectivities of **3** for nNOS and eNOS over iNOS on the basis of K_m values were 2.3- and 3.6-fold, respectively, and were both less than those of **2** (2.6- and 5.6-fold, respectively). Thus, *cis*-L-*anti*-isomer **3** was a weak and non-selective substrate for all NOS isoforms. These results suggest that the binding orientation of the side-chain of L-arginine would prefer an extended form to a folded one with all three isoforms of NOS.

While *trans*-L-*syn*-isomer **2** worked as a good substrate, *trans*-L-*anti*-isomer **1** was only a very poor inhibitor of all NOS isoforms. The structural difference between **2** and **1** is only the orientation of the cyclopropane to the carboxylic acid moiety, but their functions and affinities for NOS isoforms are totally changed. When the stereochemistry of the amino acid moiety is in the D-form, all of the analogues (***ent*-1**–***ent*-4**) were very poor ligands irrespective of the orientation of the cyclopropane moiety, that is, *trans*/*cis* and *syn*/*anti*.

As described, only one of these analogues, *trans*-L-*syn*-isomer **2**, was as good of a substrate as L-arginine for all three NOS isoforms. These results indicate that L-arginine binds to all NOS isoforms with the extended form of its side-chain and the stereochemistry of the amino acid moiety in arginine is important to work as the NOS substrate. Thus, the binding sites for L-arginine on all three isoforms of NOS recognize the conformation and configuration of the substrates and the tolerance range of the active sites are very similar. Indeed, the crystal structures of the catalytic domains of all three NOS isoforms show that the substrate binding sites are nearly identical and that the NOS substrate requires both a guanidinium group and an α-amino acid group that make specific H-bonding networks in the first hydroxylation step.[24–28] These results in this study are consistent with the ligand-binding structural information from the crystal structures.

Table 1
Inhibitory activities for three NOS isoforms[a]

Compound	Configuration	Inhibition[b] (%)		
		nNOS	iNOS	eNOS
1	*trans*-L-*anti*	16	26	45
***ent*-1**	*trans*-D-*anti*	5	19	23
***ent*-2**	*trans*-D-*syn*	13	19	4
4	*cis*-L-*syn*	30	22	16
***ent*-3**	*cis*-D-*anti*	10	24	4
***ent*-4**	*cis*-D-*syn*	30	14	27

[a] nNOS from rat, iNOS from murine, eNOS from bovine, respectively: See Ref. 22.
[b] Inhibitory effects of the compounds (300 μM) against L-arginine (10 μM).

3. Conclusion

In summary, we systematically synthesized eight optically active cyclopropane-based conformationally restricted analogues of L- and D-arginine (**1**–**4** and ***ent*-1**–***ent*-4**) with *trans*/*cis*, D/L, and *syn*/*anti* stereochemical diversity using a series of 3,4-methanoamino acid equivalents as key intermediates. Evaluation of the

Table 2
Kinetic constants for substrate activity of compounds **2** and **3** and L-arginine for three NOS isoforms[a]

Compound	Configuration	nNOS		iNOS		eNOS		Selectivity[c]	
		K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	n/i	e/i
2	*trans*-L-*syn*	7.6	317	20	99	3.6	42	2.6	5.6
3	*cis*-L-*anti*	256	8.6	582	3.0	163	0.92	2.3	3.6
L-Arginine[b]	–	2.7	180	9.5	110	1.1	73	3.5	8.6

[a] nNOS from rat, iNOS from murine, eNOS from bovine, respectively: See Ref. 22.
[b] Data taken from Ref. 23.
[c] Ratio of the inverse of the K_m values.

analogues as ligands for NOS isoforms provided useful information of the binding mode of L-arginine for the enzymes. Thus, the stereochemical diversity-oriented approach can be an effective strategy to reveal three-dimensional information of the active site of the target protein in relation to the ligands. Since an arginine residue often occurs in active sites of peptides and proteins due to its effective ionic and hydrogen bond-forming properties, the conformationally restricted analogues of arginine with the stereochemical diversity developed in this study can be effectively used as tools to be introduced into biologically active peptides instead of L-arginine. This strategy, employing a series of 3,4-methanoamino acid equivalents as key intermediates, can be applicable to the side-chain-conformational restriction of various amino acids other than arginine.

4. Experimental section

4.1. General methods

^{1}H NMR and ^{13}C NMR spectra were obtained on JEOL JNM-AL-400 or JEOL JMM-ECA-500 spectrometers with tetramethylsilane as an internal standard and the resonance patterns are reported with notations as followings: br (broad), s (singlet), d (double), t (triplet) and m (multiplet). All ^{1}H NMR assignments described were in agreement with COSY spectra. Mass spectra were obtained using a JEOL JMS-700TZ, JMS-HX110, or JEOL FABmate. Specific rotations were obtained using a JASCO P-1030 polarimeter. Thin-layer chromatography was done on Merck 60F_{254} plates. Silica gel and reverse phase chromatographies were done on silica gel 5715 (Merck) and Fuji Silysia ODS Chromatorex, respectively. HPLC purifications were performed with a JASCO 875-UV (detector), JASCO PU-2087 plus (pump and system controller), and JASCO 807-IT (recorder). Reactions were carried out under an argon atmosphere.

4.1.1. (1*R*,2*R*)-2-*tert*-Butyldiphenylsiloxymethyl-1-[((*S*)-*tert*-butylsulfinyl)iminomethyl]cyclopropane (11)

A mixture of *trans*-cyclopropane aldehyde **5**[3] (339 mg, 1.00 mmol), (*S*)-(−)-*tert*-butanesulfinamide (97%, 187 mg, 1.50 mmol), and copper sulfate (638 mg, 4.00 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature until the aldehyde disappeared by TLC. The resulting mixture was filtered through Celite and the filtrate was evaporated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 20/1–15/1) to give **11** (414 mg, 94%) as a colorless oil: $[\alpha]_D^{22}$ +57.59 (*c* 1.03, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.04 (9H, s, *t*Bu), 1.01–1.14 (2H, m, cyclopropyl-CH_2), 1.19 (9H, s, *t*Bu), 1.64 (1H, m, cyclopropyl-CH), 1.92 (1H, m, cyclopropyl-CH), 3.53 (1H, dd, *J* = 6.2, 10.8 Hz, C*H*$_2$OTBDPS), 3.83 (1H, dd, *J* = 4.7, 10.8 Hz, C*H*$_2$OTBDPS), 7.36–7.45 (6H, m, aromatic), 7.53 (1H, d, *J* = 7.7 Hz, –N=C*H*), 7.63–7.65 (4H, m, aromatic); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.0, 19.4, 22.4, 22.9, 24.8, 26.9, 56.8, 64.8, 127.6, 129.6, 133.4, 135.4, 170.3; HRMS (EI) calcd for $C_{25}H_{35}NO_2SSi$: 441.2158 (M^+), found 441.2158 $(M)^+$.

4.1.2. (1*R*,2*R*)-2-*tert*-Butyldiphenylsiloxymethyl-1-[(1*R*)-1-((*S*)-*tert*-butylsulfinylamino)-2-propenyl]cyclopropane (7)

To a solution of **11** (44 mg, 0.10 mmol) in toluene (10 mL) was added vinylmagnesium bromide (1.0 M soln in THF, 0.11 mL, 0.11 mmol) at 110 °C, and the mixture was stirred at 110 °C until **11** disappeared by TLC. After the addition of MeOH, the resulting mixture was evaporated, and the residue was partitioned between AcOEt and aqueous HCl (1 M). The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 3/1) to give **7** (45 mg, 97%) as a light yellow oil: $[\alpha]_D^{18}$ +26.48 (*c* 0.98, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.54 (1H, m, cyclopropyl-CH_2), 0.61 (1H, m, cyclopropyl-CH_2), 0.83 (1H, m, cyclopropyl-CH), 0.98 (1H, m, cyclopropyl-CH), 1.04 (9H, s, *t*Bu), 1.21 (9H, s, *t*Bu), 3.20–3.26 (2H, m), 3.42 (1H, dd, *J* = 7.0, 10.7 Hz, C*H*$_2$OTBDPS), 3.69 (1H, dd, *J* = 5.4, 10.7 Hz, C*H*$_2$OTBDPS), 5.14 (1H, d, *J* = 10.4 Hz, C*H*$_2$=CH–), 5.33 (1H, d, *J* = 17.7 Hz, C*H*$_2$=CH–), 5.90 (1H, m, CH$_2$=C*H*-), 7.35–7.44 (6H, m, aromatic), 7.64–7.66 (4H, m, aromatic); ^{13}C NMR (100 MHz, $CDCl_3$) δ 9.8, 18.7, 19.4, 21.4, 22.7, 27.0, 55.6, 61.7, 66.5, 116.2, 127.5, 129.4, 133.7, 135.4, 138.6; HRMS (FAB) calcd for $C_{27}H_{40}NO_2SSi$: 470.2549 [$(M+H)^+$], found 470.2540 [$(M+H)^+$].

4.1.3. (1*R*,2*R*)-1-[(1*R*)-1-(Benzyloxycarbonylamino)-2-propenyl]-2-hydroxymethylcyclopropane (12)

To a solution of **7** (147 mg, 0.313 mmol) in MeOH (3.1 mL) was added HCl (4 M in AcOEt, 0.31 mL), and the mixture was stirred at rt for 1.5 h. After the mixture was evaporated, the residue was dissolved in THF/H_2O (3/2, 5.0 mL). To the mixture were added benzyloxycarbonyl chloride (2.91 M soln in toluene, 0.118 mL, 0.344 mmol) and sodium carbonate (66 mg, 0.67 mmol) at rt, and the resulting mixture was stirred at rt for 12 h. After dilution with AcOEt, the mixture was washed with H_2O, brine, dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (hexane/AcOEt = 4/1–1/1) to give **12** (62 mg, 75% for two steps) as an amorphous solid: $[\alpha]_D^{21}$ +15.60 (*c* 0.92, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.46 (1H, m, cyclopropyl-CH_2), 0.65 (1H, br s, cyclopropyl-CH_2), 0.85 (1H, br s, cyclopropyl-CH), 1.05 (1H, br s, cyclopropyl-CH), 1.76 (1H, br s, CH$_2$O*H*), 3.38 (1H, dd, *J* = 7.4, 11.4 Hz, C*H*$_2$OH), 3.55 (1H, dd, *J* = 6.3, 11.4 Hz, C*H*$_2$OH), 3.79 (1H, m, –C*H*NH), 5.00 (1H, br s, NH), 5.09–5.24 (4H, m, C*H*$_2$=CH– and CH$_2$ of Cbz), 5.80 (1H, m, CH$_2$=C*H*–), 7.29–7.36 (5H, m, aromatic); ^{13}C NMR (125 MHz, $CDCl_3$) δ 8.0, 18.9, 21.4, 55.7, 65.9, 66.8, 115.4, 128.1, 128.5, 136.4, 137.0, 155.8; HRMS (EI) calcd for $C_{15}H_{19}NO_3$: 261.1365 (M^+), found 261.1350 (M^+).

4.1.4. (3*R*,4*R*)-3,4-Methano-N^{α},N^{δ},N^{ω}-tri-Cbz-L-arginine (14)

A mixture of **12** (80 mg, 0.31 mmol), *N*,*N*′-bis-Cbz-guanidine (200 mg, 0.612 mmol), and triphenylphosphine (96 mg, 0.37 mmol) in toluene (3 mL) was stirred at 0 °C for 10 min. To the mixture was added a solution of diisopropyl azodicarboxylate (72 μL, 0.37 mmol) in toluene (3 mL) at 0 °C, and the resulting mixture was stirred at rt for 2 h. After being evaporated, the residue was purified by short silica gel column chromatography (hexane/AcOEt = 7/1–2/1) to give a crude product of **13** as a colorless oil. To a solution of the crude product of **13** in acetone/H_2O (2/1, 9 mL) were added sodium periodate (326 mg, 1.53 mmol), potassium permanganate (34 mg, 0.21 mmol), and sodium bicarbonate (25 mg, 0.31 mmol) at rt, and the mixture was stirred at rt for 2 h. After dilution with AcOEt, the resulting mixture was washed with aqueous HCl (1 M, four times), brine, dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (0–10% MeOH in $CHCl_3$) to give **14** (141 mg, 78% for two steps) as a colorless amorphous solid: $[\alpha]_D^{17}$ +24.27 (*c* 0.96, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ 0.54–0.60 (2H, m, cyclopropyl-CH_2), 1.27 (1H, m, cyclopropyl-CH), 1.36 (1H, m, cyclopropyl-CH), 3.43 (1H, d, *J* = 9.0 Hz, C*H*NH), 3.77 (1H, dd, *J* = 8.6, 14.0 Hz, C*H*$_2$-guanidine), 4.07 (1H, dd, *J* = 5.4, 14.0 Hz, C*H*$_2$-guanidine), 5.05–5.33 (6H, m, CH$_2$ of Cbz ×3), 7.25–7.45 (15H, m, aromatic); ^{13}C NMR (100 MHz, CD_3OD) δ 9.8, 18.5, 21.1, 58.7, 67.5, 68.2, 70.0, 79.5, 128.8, 128.8. 128.9, 129.4, 129.6, 129.7, 129.7, 136.4, 138.1, 138.5, 157.0, 158.4, 162.2, 164.9, 175.2; HRMS (FAB) calcd for $C_{31}H_{33}N_4O_8$: 589.2298 [$(M+H)^+$], found 589.2303 [$(M+H)^+$].

4.1.5. (3*R*,4*R*)-3,4-Methano-L-arginine dihydrochloride (1)

Compound **14** (141 mg, 0.239 mmol) was dissolved in a solution of HCl in EtOH (1 M, 15 mL), and Pd/C (10%, 141 mg) was added to the mixture. The resulting mixture was stirred under an atmospheric pressure of H_2 gas at rt for 6 h. The mixture was

filtered through Celite with MeOH, and the filtrate was evaporated. The residue was mixed into H_2O, and evaporated. The residue was purified by C18 reverse phase silica gel column chromatography (10% MeOH in H_2O) to give a crude product of **1** (59 mg) as an amorphous solid. After the crude product of **1** was purified by RP-HPLC (0.1% TFA in H_2O, 10 mL/min, rt, detected at 202 nm) with Mightysil RP-18 (250 × 20 mm, Kanto Chemical Co.), the obtained compound was treated with aqueous HCl (2 M) and the mixture was evaporated (this process was repeated three times.) to give **1** (30 mg, 48%) as an amorphous solid: $[\alpha]_D^{23}$ +33.68 (*c* 0.77, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 0.72–0.79 (2H, m, cyclopropyl-CH_2), 1.13 (1H, m, cyclopropyl-CH), 1.46 (1H, m, cyclopropyl-CH), 2.87 (1H, dd, J = 8.6, 14.5 Hz, CH_2-guanidine), 3.30 (1H, dd, J = 5.9, 14.5 Hz, CH_2-guanidine), 3.35 (1H, d, J = 9.9 Hz, –C*H*NH); ^{13}C NMR (100 MHz, D_2O) δ 9.9, 17.9, 18.1, 44.3, 57.1, 157.3, 172.2; HRMS (FAB) calcd for $C_7H_{15}N_4O_2$: 187.1195 [(M+H)$^+$], found 187.1200 [(M+H)$^+$]. Anal. ($C_7H_{16}Cl_2N_4O_2{\cdot}0.8H_2O$) C, 30.74; H, 6.49; N, 20.48. Found: C, 30.70; H, 6.31; N, 20.52.

4.2. Enzyme and assay

According to the procedures reported previously,[22] all enzymes used were prepared and enzyme inhibition assays were carried out. Enzyme kinetics data were determined using the hemoglobin capture assay as described at 30 °C.[29] A typical assay mixture for nNOS and eNOS contained various concentrations of testing compound, 1.0 mM $CaCl_2$, 600 unit/mL calmodulin (Sigma, P-2277), 100 μM NADPH, 0.125 mg/mL hemoglobin-A_0 (ferrous form, Sigma, H0267), 10 μM H_4B, in 100 mM HEPES (pH 7.5). A typical assay mixture for iNOS contained various concentrations of testing compound, 100 μM NADPH, 0.125 mg/mL hemoglobin-A_0 (ferrous form), 10 μM H_4B, in 100 mM HEPES (pH 7.5). All assays were in a final volume of 600 μL and were initiated by addition of enzyme. Nitric oxide-mediated oxidation of hemoglobin-A_0 was monitored at 401 nm on a PerkinElmer Lamda 10 UV-visible spectrophotometer. The Michaelis constant K_m is the concentration of the substrate required to produce a rate of $V_{max}/2$. The k_{cat} is the rate constant for substrate turnover. Both K_m and k_{cat} values were determined by nonlinear regression analysis of a plot of enzyme activity versus substrate concentration. Curves were fit using the Michaelis–Menten equation in GraphPad Prism 5.0 (GraphPad Software, Inc.).

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A. Supplementary data

Supplementary data (experimental details for the synthesis of **2**, **3**, **4**, ***ent*-1**, ***ent*-2**, ***ent*-3**, and ***ent*-4**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.049.

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