Structure–Activity Relationships of 2-Aminothiazole Derivatives as Inducible Nitric Oxide Synthase Inhibitor

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Received January 7, 2004; accepted February 10, 2004; published online February 13, 2004

Nitric oxide synthase (NOS) has been divided into two major sub-enzymes, *i.e.* inducible NOS (iNOS) and constitutive NOS (cNOS). Although nitric oxide (NO) plays an important role as host defense mediator, excessive production of NO by iNOS has been involved in the pathology of many inflammatory diseases. Recently, we reported that the 2-imino-1,3-oxazolidine (1a) weakly inhibits iNOS and that introduction of an alkyl moiety on the oxazolidine ring of 1a enhances the inhibitory activity and selectivity for iNOS. In our search for better iNOS inhibitors, we focused our efforts on the 2-aminothiazole scaffold 3 as it possesses a ring similar to that of 1a. In this study, we evaluated the inhibitory activity of a series of 2-aminothiazole derivatives against both iNOS and neuronal NOS (nNOS). Our results show that introduction of appropriately-sized substituents at the 4- and 5-position of the 2-aminothiazole ring improves the inhibitory activity and selectivity for iNOS. We also found that the selectivity of 5a [5-(1-methyl)ethyl-4-methylthiazol-2-ylamine] and 5b [5-(1,1-dimethyl)ethyl-4-methylthiazol-2-ylamine] for iNOS was similar to that of oxazolidine derivative 1b (4-methyl-5-propyl-2-imino-1,3-oxazolidine) and much higher than that of L-NAME. However, we could not enhance the inhibitory activity against iNOS by introducing an alkyl substituent into the 2-aminothiazole ring as we could in the case of oxazolidine one. On the other hand, introduction of bulky or hydrophilic substituent at any position of the 2-aminothiazole ring remarkably decreased or even abolished the inhibitory activity against NOS.

Key words nitric oxide; inducible nitric oxide synthase inhibitor; thiazole; neuronal nitric oxide synthase inhibitor; neuronal nitric oxide synthase (nNOS); 2-aminothiazole

Nitric oxide synthase (NOS) oxidizes one of the two equivalent terminal guanidino nitrogens of L-arginine using NADPH and oxygen as substrates, as well as flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin and haem as cofactors to produce L-citrulline and the biologically active free radical nitric oxide (NO).[1—3] In general, NOS has been divided into two major sub-enzymes, *i.e.* a constitutive NOS (cNOS) which requires Ca^{2+}/Calmodulin for its activation, and an inducible NOS (iNOS) which is independent of Ca^{2+}/Calmodulin for its activation. In addition, cNOS has further been divided into neuronal NOS (nNOS) found mainly in the brain, and endothelial NOS (eNOS) predominantly present in the vascular endothelium. It has been reported that nNOS generates NO that functions as a neurotransmitter regulating neuronal transmission,[4] and that eNOS generates low concentration of NO which lowers blood pressure.[5] On the other hand, iNOS, which is expressed in activated macrophage in response to inflammatory cytokines and lipopolysaccharides (LPS), generates NO that has cytotoxic/cytostatic activity against intracellular pathogens including viruses and therefore plays an important role as host defense mediator.[6] However, excessive production of NO by iNOS has been involved in numerous disease states such as septic shock,[7] rheumatoid arthritis,[8] hypotension,[9] chronic ileitis[10] and autoimmune diabetes.[11]

There was a main approach that could potentially be exploited to inhibit excessive production of NO, and analogues of L-arginine, L-lysine, L-ornithine, L-citrulline and guanidine such as N^G-methyl-L-arginine,[12] N^G-nitro-L-arginine,[13] N^G-nitro-L-arginine methylester (L-NAME),[14] N^6-(1-iminoethyl)-L-lysine,[15] N^5-(1-iminoethyl)-L-ornithine,[16] L-thiocitrulline,[17] *S*-alkyl-L-thiocitrulline[18] and aminoguanidine[19] have been synthesized and investigated in connection with a number of inflammatory diseases. However, these analogues have not been approved for the treatment of inflammatory diseases due to their undesired inhibition of cNOS.

In addition to the L-amino acid analogues described above, a number of non-amino acid NOS inhibitors have been investigated.[20—28] Recently, we reported that the 2-imino-1,3-oxazolidine (**1a**) (Chart 1) weakly inhibits iNOS and that introduction of an alkyl moiety on its oxazolidine ring enhances the inhibitory activity and selectivity for iNOS (**1b**, Chart 1, Table 2).[29] In our search for better iNOS inhibitors, we focused our efforts on the 2-aminothiazole scaffold **3** as it possesses a ring similar to that of **1a**. Although the 4,5-di-

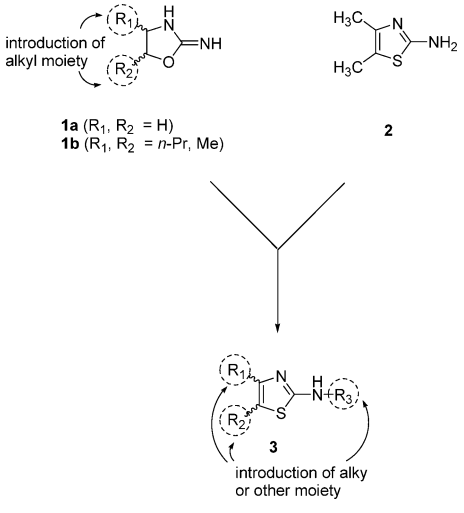

Chart 1. Design of 2-Aminothiazole Derivatives

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methylthiazol-2-ylamine (**2**) has previously been reported to inhibit iNOS[20] to our knowledge, derivatives of 2-aminothiazole scaffold have hardly been studied in connection with iNOS inhibition. Herein, we report the synthesis and structure–activity relationships (SARs) for NOS inhibition of a series of 2-aminothiazole derivatives.

Chemistry

As illustrated in Chart 2, annulation of ketone **4a**—**g** with thiourea using I_2 led to a mixture of the 2-aminothiazole regio isomers **5** and **6**, which were separated by column chromatography with except for **6d** (Table 1).

Next, as shown in Chart 3, the 2,4,5-trisubstituted-2-aminothiazole derivatives were synthesized. Reaction of α-chlorocyclopentanone with phenylthiourea and that of **5a** with isopropylisothiocyanate easily gave the 2-phenylamino derivative **7** and the thiourea derivative **8**, respectively.[30]

Results and Discussion

The inhibitory activity of the synthesized compounds (**5a**—**g**, **6a**—**c**, **g**, **7**, **8**) against iNOS and nNOS was evaluated according to previously reported procedures[31,32] with some modifications and their selectivity was determined from nNOS IC_{50} value/iNOS IC_{50} value ratio.

As shown in Table 2, when a methyl and an isopropyl groups were inserted into the 4-position and 5-position of the 2-aminothiazole ring, respectively, the inhibitory activity against iNOS was improved and the selectivity for iNOS over nNOS was greatly increased (**5a**: IC_{50}=0.6 μM, 4.7-fold stronger than that of **2**; nNOS/iNOS=23, selectivity of **5a** for iNOS 26-fold higher than that of **2**). An additional insertion of a methyl group into the isopropyl group of **5a** significantly decreased nNOS inhibition, thus the selectivity of **5b** for iNOS was the highest among the synthesized compounds (**5b**: nNOS IC_{50} value of 100 μM, nNOS/iNOS=33, selectivity of **5b** for iNOS 37-fold higher than that of **2**). When the length of side chain at the 5-position of the thiazole ring increased, the inhibitory activity against iNOS decreased and that against nNOS was completely abolished (**5c**: iNOS IC_{50}

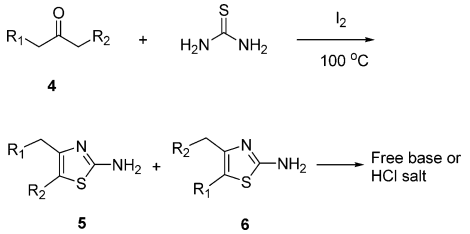


Chart 2. Synthesis of 2-Aminothiazole Derivatives (**5**, **6**) from Ketone **4** and Thiourea

Table 1. Synthesis of Mono and Disubstituted 2-Aminothiazole Derivatives (**5**, **6**) from Ketone **4** and Thiourea

	Starting material	Compound			
		Yield (%)		Salt	
4	(R_1, R_2)	**5**	**6**	**5**	**6**
a	H, ^{i}Pr	19	28	*b)*	HCl
b	H, ^{t}Bu	10	39	*b)*	*b)*
c	H, ^{n}Bu	38	11	*b)*	*b)*
d	Et, Me	13	*a)*		
e	*c*Pn*c)*	32		*b)*	
f	*c*Hex*d)*	76		HCl	
g	H, CH_2CO_2Et	10	3	*b)*	HCl

a) Not isolated purely. *b*) Free amine. *c*) Cyclopentyl. *d*) Cyclohexyl.

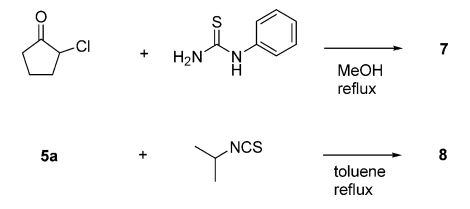
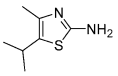
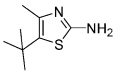
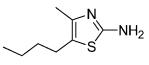
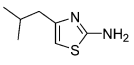
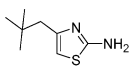
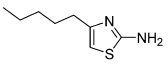
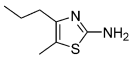
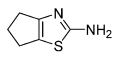
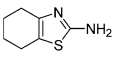
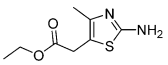
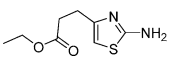
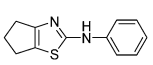
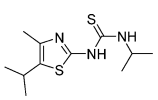


Chart 3. Synthesis of Trisubstituted 2-Aminothiazole Derivatives (**7**, **8**)

Table 2. Inhibitory Activity of Mono, Di and Trisubstituted 2-Aminothiazole Derivatives

Compound		Inhibitory activity*a)*		Selectivity*c)*
		iNOS IC_{50} (μM)*b)*	nNOS IC_{50} (μM)*b)*	nNOS/iNOS
	**5a**	0.6	14	23
	**5b**	3.0	100	33
	**5c**	54	n.s. @100*d)*	—*e)*
	**6a**	6.2	28	4.5
	**6b**	12	25	2.1
	**6c**	21	14	0.7
	**5d**	4.1	5.5	1.3
	**5e**	5.5	17	3.1
	**5f**	9.3	28	3
	**5g**	n.s. @100*d)*	n.s. @100*d)*	—*e)*
	**6g**	n.s. @100*d)*	n.s. @100*d)*	—*e)*
	**7**	n.s. @100*d)*	n.t.*f)*	—*e)*
	**8**	n.s. @100*d)*	n.s. @100*d)*	—*e)*
	1a[29]	7.9	34	4.3
	1b[29]	0.041	0.92	22
	2	2.8	2.4	0.86
	L-NAME	300	0.8	0.0027

a) IC_{50} values for iNOS and nNOS were determined by testing each compound at eight concentrations. *b*) iNOS and nNOS activity was evaluated according to previously reported methods with some modifications.[31,32] *c*) Selectivity was defined as the ratio of IC_{50} value of nNOS to iNOS. *d*) n.s.=no significant effect (<5% inhibition). *e*) Not determined. *f*) Not tested.

value of 54 μM; nNOS IC_{50} value of n.s. @100 μM). Therefore, it is suggested that an increase of the length of side chain at the 5-position can be tolerated in iNOS inhibition but not in nNOS. Monosubstituted compounds, on the other hand, generally displayed moderate inhibitory activity against both iNOS and nNOS (**6a**—**c**). Unexpectedly, the inhibitory activity against iNOS of **5d**, a thiazole analogue of **1b**, was much weaker than that of **1b**. Therefore, it is suggested that a structurally planar ring is not favorable for strong iNOS inhibition.[21)]

Next, we evaluated the inhibitory activity of other 2-aminothiazole derivatives against NOS.

The inhibitory activity of the bicyclic compounds (**5e**, **f**) against iNOS were similar to that of **2**. On the other hand, introduction of substituent at the 2-amino group (**7**, **8**) abolished the inhibitory activity against both iNOS and nNOS. These findings suggest that strong iNOS inhibition requires a non-substituted 2-amino group on the thiazole ring. Hydrophilically substituted 2-aminothiazole derivatives (**5g**, **6g**) also displayed no significant inhibition against both iNOS and nNOS. As iNOS oxygenase haem domain has been reported to form a hydrophobic pocket,[33)] the contrastive hydrophilicity of **5g** and **6g** is assumed to be responsible for the abolishment of inhibitory activity against iNOS.

Conclusion

In this study, we evaluated the inhibitory activity of a series of 2-aminothiazole derivatives against both iNOS and nNOS. Our results show that introduction of appropriately-sized substituent at the 4- and 5-position of the 2-aminothiazole ring improves the inhibitory activity and selectivity for iNOS. We also found that the selectivity of **5a** and **5b** for iNOS was similar to that of oxazolidine **1b** and much higher than that of L-NAME. However, we could not enhance the inhibitory activity against iNOS by introducing an alkyl substituent into the 2-aminothiazole ring as we could in the case of oxazolidine one. On the other hand, introduction of bulky or hydrophilic substituent at any position of the 2-aminothiazole ring remarkably decreased or even abolished the inhibitory activity against NOS.

Experimental

General Melting points were recorded on a Yanagimoto micro-melting point apparatus MP-J3 without correction. ^{1}H-NMR spectra were determined in the indicated solvent on a Varian Jemini (200 MHz) or a JEOL JNM-LA300 (300 MHz) spectrometer. Chemical shifts are expressed as δ (ppm) values downfield from tetramethylsilane as an internal standard. Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (mutiplet), and br (broad). Coupling constants are given in hertz. EI-MS were recorded on a JEOL JMS D-300 or a Hitachi M-80-B mass spectrometer. Column chromatography was carried out on Kieselgel 60 (Merck; 70—230 mesh).

General Procedure for Synthesis of 2-Aminothiazole Derivatives A mixture of ketone **4** (46 mmol), thiourea (93 mmol), and I_2 (46 mmol) was heated at 100 °C with vigorous stirring for 8 h. To this reaction mixture was added hot water (100 ml) and charcoal (5 g). After stirring for 15 min, the charcoal was filtered off and to the filtrate was added 28% aqueous ammonia solution (50 ml). Extraction with two 50-ml portions of ether followed by drying of the combined organic extracts with $MgSO_4$, and evaporation under reduced pressure gave a mixture of **5** and **6**. The mixture was purified by column chromatography on silica gel using MeOH/$CHCl_3$ (1/100) as eluent to give a free amine, or as hydrochloride salt by treatment with ethanolic 4 N hydrochloric acid solution.

The following 2-aminothiazole derivatives (**5a**—**g**, **6a**—**c**, **g**) were synthesized from the corresponding starting materials (**4a**—**g**) according to the general procedure.

4-Methyl-5-(1-methyl)ethylthiazol-2-ylamine (5a) and 4-(2-Methyl)-propylthiazol-2-ylamine (6a) from 4-Methyl-2-pentanone (4a) **5a**: Yield, 19%. mp 65—67 °C (hexane) (lit.[34)] 69—70 °C). **6a**: yield, 28%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.91 (6H, d, J=6.6 Hz), 1.95 (1H, m), 2.39 (2H, d, J=6.6 Hz), 5.11 (2H, br), 6.07 (1H, s). **6a**·HCl, mp 160—162 °C (EtOH/Et_2O). *Anal.* Calcd for $C_7H_{12}N_2S \cdot 0.95HCl \cdot 0.15H_2O$: C, 43.43; H, 6.90; N, 14.47; S, 16.56; Cl, 17.40. Found: C, 43.68; H, 6.81; N, 14.52; S, 16.29; Cl, 17.54. EI-MS *m/z*: 157 (M+1).

5-(1,1-Dimethyl)ethyl-4-methylthiazol-2-ylamine (5b) and 4-(2,2-Dimethyl)propylthiazol-2-ylamine (6b) from 4,4-Dimethyl-2-pentanone (4b) **5b**: Yield, 10%. mp 70—71 °C (hexane). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.35 (9H, s), 2.23 (3H, s), 4.60 (2H, br). *Anal.* Calcd for $C_8H_{14}N_2S$: C, 56.43; H, 8.29; N, 16.45; S, 18.83. Found: C, 56.56; H, 8.55; N, 16.53; S, 19.09. EI-MS *m/z*: 171 $[M+1]^+$. **6b**: Yield, 39%. mp 69—70 °C (hexane). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.95 (9H, s), 2.42 (2H, s), 4.89 (2H, br), 6.08 (1H, s). *Anal.* Calcd for $C_8H_{14}N_2S \cdot 0.1H_2O$: C, 55.84; H, 8.32; N, 16.28; S, 18.63. Found: C, 55.81; H, 8.24; N, 16.27; S, 18.94. EI-MS *m/z*: 171 $[M+1]^+$.

5-Butyl-4-methylthiazol-2-ylamine (5c) and 4-Pentylthiazol-2-ylamine (6c) from 2-Heptanone (4c) **5c**: Yield, 38%. mp 38—40 °C (hexane) (lit.[35)] 41—42 °C). **6c**: Yield, 11%. mp 36—37 °C (hexane) (lit.[36)] 45—46 °C).

5-Methyl-4-propylthiazol-2-ylamine (5d) from 2-Hexanone (4d) Yield, 13%. mp 58—60 °C (hexane, 4 times). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.92 (3H, t, J=7.2 Hz), 1.55—1.67 (2H, m), 2.19 (3H, s), 2.41 (2H, t, J=7.4 Hz), 4.75 (2H, br). *Anal.* Calcd for $C_7H_{12}N_2S$: C, 53.81; H, 7.74; N, 17.93; S, 20.52. Found: C, 53.82; H, 7.80; N, 17.81; S, 20.31. EI-MS *m/z*: 157 $[M+1]^+$.

4,5,6-Trihydrocyclopenta[*d*]thiazol-2-ylamine (5e) from Cyclopentanone (4e) Yield, 32%. mp 90—92 °C (hexane) (lit.[37)] 92.5—94 °C).

4,5,6,7-Tetrahydrobenzo[*d*]thiazol-2-ylamine (5f) from Cyclohexanone (4f) Yield, 76%. **5f**·HCl, mp 240—243 °C (EtOH/Et_2O) (lit.[37)] 248—251 °C).

(2-Amino-4-methylthiazol-5-yl)acetic Acid Ethyl Ester (5g) and 3-(2-Aminothiazol-4-yl)propionic Acid Ethyl Ester (6g) from 4-Oxopentanoic Acid Ethyl Ester (4g) **5g**: Yield, 10%. mp 114—115 °C (CH_3CN). ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 1.19 (3H, t, J=7.3 Hz), 1.96 (3H, s), 3.56 (3H, s), 4.07 (2H, q, J=7.0 Hz), 6.68 (2H, br). *Anal.* Calcd for $C_8H_{12}N_2O_2S$: C, 47.98; H, 6.04; N, 13.99; S, 16.01. Found: C, 47.63; H, 6.18; N, 13.98; S, 16.10. EI-MS *m/z*: 201 $[M+1]^+$. **6g**: Yield, 3%. **6g**·HCl, mp 103—104 °C (EtOH/Et_2O). ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 1.18 (3H, t, J=7.3 Hz), 2.63—2.82 (4H, m), 4.07 (2H, q, J=7.0 Hz), 6.53 (1H, s), 9.22 (3H, br). *Anal.* Calcd for $C_8H_{12}N_2O_2S \cdot HCl$: C, 40.59; H, 5.54; N, 11.83; S, 13.55; Cl, 14.98. Found: C, 40.95; H, 5.55; N, 11.65; S, 13.85; Cl, 14.88. EI-MS *m/z*: 201 $[M+1]^+$.

***N*-Phenyl-*N*-4,5,6-trihydrocyclopenta[*d*]thiazol-2-ylamine (7)** A mixture of *N*-phenylthiourea (6.4 g, 42 mmol) and 2-chlorocyclopentanone (5.0 g, 42 mmol) in 200 ml of MeOH was heated at 60 °C for 18 h. The solvent was removed *in vacuo* and the residue was partitioned into saturated aqueous sodium hydrogen bicarbonate solution and AcOEt. The organic phase was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure, and then the residue was purified by column chromatography on silica gel using MeOH/$CHCl_3$ (1/100) as eluent to give **7** (0.32 g, 3%), mp 121—123 °C (CH_3CN). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.37—2.46 (2H, m), 2.72—2.85 (4H, m), 7.03 (1H, m), 7.29—7.38 (4H, m). *Anal.* Calcd for $C_{12}H_{12}N_2S \cdot 0.1H_2O$: C, 66.08; H, 5.64; N, 12.84; S, 14.70. Found: C, 66.16; H, 5.59; N, 12.81; S, 14.79. EI-MS *m/z*: 217 $[M+1]^+$.

***N*-(1-Methyl)ethyl-*N'*-[5-(1-methyl)ethyl-4-methylthiazol-2-yl]thiourea (8)** A mixture of **5a** (2.0 g, 13 mmol) and isopropylisothiocyanate (1.3 g, 13 mmol) in 30 ml of toluene was refluxed for 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel using MeOH/$CHCl_3$ (1/10) as eluent to give **8** (1.1 g, 35%), mp 186—187 °C (CH_3CN). ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 1.18 (6H, d, J=6.9 Hz), 1.21 (6H, d, J=7.2 Hz), 2.16 (3H, s), 3.13 (1H, m), 4.31 (1H, m), 9.78 (1H, br), 11.32 (1H, br). *Anal.* Calcd for $C_{11}H_{19}N_3S_2$: C, 51.32; H, 7.44; N, 16.32; S, 24.91. Found: C, 51.40; H, 7.57; N, 16.30; S, 24.62. EI-MS *m/z*: 258 $[M+1]^+$.

***In Vitro* Biological Assay. Preparation of Partially Purified iNOS Enzyme and Determination of Inhibitory Activity against iNOS**[31)] RAW 264.7 macrophage cells were grown in Dulbecco's modified eagles medium supplemented by 10% fetal bovine serum under 5% CO_2 atmosphere at 37 °C. To this medium were added lipopolysaccharide and interferon-γ to make a final concentration of 0.2 μg/ml and 100 unit/ml respec-

tively. The grown cells were then collected and mixed with Tris and dithiothreitol to make a final concentration of 50 mM and 100 μM respectively at pH 7.5. The mixture was centrifuged at 10000×***g*** for 30 min, and the supernatant was added to Dowex HCR-W2 at 4 °C and stirred for 30 min at the same temperature. This solution was used as crude enzyme. NOS activity was measured by monitoring the conversion level of L-citrulline from L-arginine. To 70 ml of the crude enzyme solution was added, 20 μl of Tris (pH 7.5) including 1 mM of NADPH, 10 μl of test-compound, 20 μl of 10 μCi/ml L-[H^3]-arginine and 80 μl of Tris (pH 7.5). The resulting mixture was incubated at 37 °C for 30 min, after which 20 μl of 0.1 M (pH 5.0) including 2 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) and 2 mM *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were added. To the resulting solution was added Dowex 50W-8X, and the whole was stirred for 30 min. L-[H^3]-Citrulline in the supernatant was evaluated by scintillation counting.

Preparation of Partially Purified nNOS Enzyme and Determination of Inhibitory Activity against nNOS[32] Male wistar rat (150—170 g) cerebella were homogenized in 50 mM of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulufonic acid (pH 7.1) including 0.1 mM phenylmethylsulfonyl fluoride, 12.5 mM 2-mercaptoethanol and 0.5 mM EDTA, and centrifuged at 10000×***g*** for 1 h. To the supernatant was added Dowex HCR-W2 and the resulting mixture was stirred for 30 min at 4 °C. The final supernatant was used as crude enzyme. NOS activity was measured by monitoring the conversion level of L-citrulline from L-arginine. To 100 μl of the crude enzyme solution was added 60 μl of 50 mM Tris (pH 7.5) including 1 mM NADPH, 2 mM $CaCl_2$ and 300 nM calmodulin, 10 μl of test-compound, 20 μl of 10 μCi/ml L-[H^3]-arginine and 10 μl of Tris (pH 7.5). The resulting mixture was incubated at 37 °C for 30 min, after which 200 μl of 0.1 M sodium acetate buffer (pH 5.0) including 2 mM EDTA and 2 mM EGTA were added. To the resulting solution was added Dowex 50W-8X, and the whole was stirred for 30 min. L-[H^3]-Citrulline in the supernatant was evaluated by scintillation counting.

Acknowledgement The thorough and helpful comments on preliminary versions of the article are gratefully acknowledged to Dr. Nobuhide Watanabe.

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