Synthesis and Pharmacological Activity of *O*-(5-Isoxazolyl)-L-serine

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A novel isoxazole derivative, *O*-(5-isoxazolyl)-L-serine (OIS, 1), was synthesized by a Mitsunobu reaction of isoxazolin-5-one (4) with *N*-Boc-L-serine *tert*-butyl ester (5) and subsequent deprotection of the coupling product. Its structure was elucidated by spectroscopic analyses. The pharmacological activity of 1 was also examined with cloned glutamate receptors and transporters using a *Xenopus* oocyte-expressing system showing substrate activity on an excitatory amino acid carrier 1 (EAAC 1) as a glutamate transporter.

Key words synthesis; *O*-(5-isoxazolyl)-L-serine; pharmacological activity; excitatory amino acid carrier 1; *Xenopus* oocyte

O-(5-Isoxazolyl)-L-serine (OIS, **1**) is thought to be a structural isomer of naturally occurring isoxazolinone derivatives, β-(isoxazolin-5-on-2-yl)-L-alanine (BIA, **2**) and β-(isoxazolin-5-on-4-yl)-L-alanine (TAN, **3**), but it has not yet been found in nature. BIA (**2**) was identified in the leguminous genus *Lathyrus*, *Lens* and *Pisum*, was confirmed to be the biosynthetic precursor of the neurotoxin 3-*N*-oxalyl-L-2,3-diaminopropionic acid (β-ODAP) in grass pea (*Lathyrus sativus* L.),[1—5] and was also found to have antimycotic activity.[6] BIA was synthesized from *N*-Boc-L-serine (**9**) by Baldwin *et al*.[7] TAN (**3**) was isolated from *Streptomyces platensis* as an antifungal antibiotic,[8] and was also synthesized by Tsubotani *et al*.[9] However, OIS (**1**) has not yet been synthesized, in spite of its stable structure.

During the pharmacological study of isoxazolinone derivatives and such related compounds as neurotoxins, which cause crippling human neurolathyrism, we reported that TAN was a potential agonist for glutamate receptors (Glu R) and glutamate transporters (Glu T) as well as β-ODAP, a major causative agent of neurolathyrism, which is caused by eating the grass pea seeds, whereas BIA had almost no activity.[10—12]

Therefore, we have focused on the synthesis of an isomer, OIS (**1**), to clarify the structure–activity relationship of isoxazolinone compounds. We now report the synthesis and pharmacological activity of **1** in comparison with its two isomers and other related compounds.

Results and Discussion

Synthesis of OIS (1) OIS (**1**) was synthesized by a Mitsunobu reaction[13] of isoxazolin-5-one (**4**) with *N*-Boc-L-serine *tert*-butyl ester (**5**) and subsequent deprotection of the coupling product (**6**), as shown in Chart 1. Its structure was elucidated by spectroscopic analyses. The IR spectrum of **1** showed an absorption band for carbonyl functionality (1689 cm^{-1}) of an amino acid. The ^{1}H-NMR spectrum revealed two doublet signals of aromatic protons of the isoxazole ring at δ 5.46 (d, 1H, J=2.2 Hz, C_4H) and 8.17 (d, 1H, J=2.2 Hz, C_3H), and two multiplet signals of one methine group at the α-position of an amino acid at δ 4.10 (m, 1H) and the adjacent oxymethylene group at δ 4.56 (m, 2H). The ^{13}C-NMR spectrum also showed three signals (δ 79.3, 154.8, 173.2) due to an isoxazole skeleton, together with three signals (δ 54.5, 71.2, 171.7) of serine moiety. A downfield shifted carbon signal at δ 71.2, which does not appear in the spectrum of BIA (**2**) or TAN (**3**), clearly indicated that this carbon is bonded to the isoxazole ring through an oxygen atom (Fig. 1). Information concerning the coupling position was obtained from the heteronuclear multiple bond correlation (HMBC) spectrum of protected OIS (**6**), in which a cross-peak was observed between the oxymethylene proton (δ 4.54) and quaternary carbon (δ 172.9) of the isoxazole nucleus, indicating that a serine moiety is attached to the C-5 position of the isoxazole ring. This structure was reinforced by comparison of the previously observed NMR data for **2** and **3**.[7,9] Finally, the structure of newly synthesized **1** was concluded to be a positional isomer of **2** and **3**, and is represented by the structure formula in Chart 1.

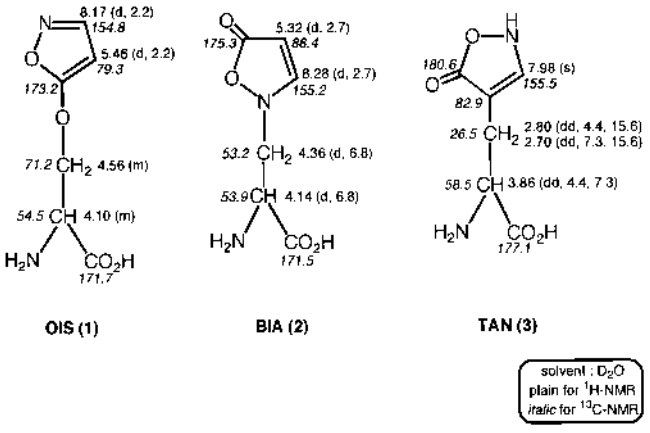

Fig. 1. ^{1}H- and ^{13}C-NMR Spectral Data of OIS (**1**), BIA (**2**) and TAN (**3**)

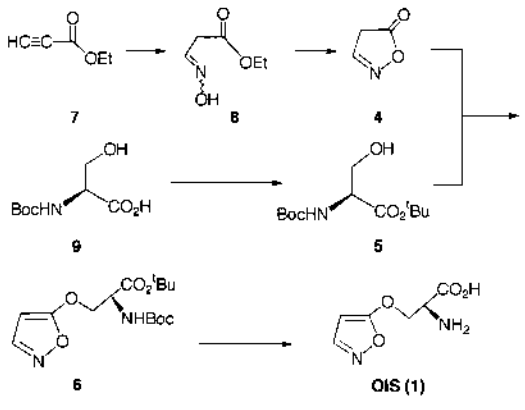

Chart 1. Synthetic Routes of OIS (**1**)

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Table 1. Effects of OIS (**1**) and Related Compounds on the Cloned Glu R and Glu T Expressed in *Xenopus* Oocytes

	NMDA[a]		AMPA[b]	GLAST[b]	EAAC 1[b]	GLT 1[b]
	Glu site[c]	Gly site[d]				
OIS (**1**)	0.4±0.1	9.3±2.1	1.0±0.7	0.4±0.3	7.7±2.0	2.8±1.7
BIA (**2**)	1.5±0.9	3.9±1.0	2.0±0.4	0.5±0.4	4.3±2.5	2.3±1.9
TAN (**3**)	17.3±0.5	15.9±5.3	57.0±3.4	31.6±0.3	18.1±4.0	0.8±0.5
β-ODAP	1.1±0.6	0.9±0.5	81.0±7.6	2.0±0.5	0.8±0.6	0.5±0.5

Inward currents evoked by 0.1 mM **1** and related compounds are expressed as percentage responses of *a*) those with 0.1 mM L-glutamate (Glu) and 0.1 mM glycine (Gly), and of *b*) 0.1 mM Glu. *c*) Glu site agonist activities induced by 0.1 mM compounds and Gly. *d*) Gly site agonist activities induced by 0.1 mM compounds and Glu. Slight responses are observed for each site without an appropriate agonist : *c*) 0.5±0.2, *d*) 0.3±0.05. Data are means±S.E.M. (n=3—7).

Pharmacological Activity of OIS (1) The pharmacological activity of **1** was examined for its activity on Glu R and Glu T by using a *Xenopus* oocyte-expressing system in comparison with its two isomers, **2** and **3**, and the results are summarized in Table 1. Macroscopic currents were recorded using a two-electrode voltage-clamp method as described previously.[11,12] In the voltage-clamp experiments using cloned rat Glu R and Glu T expressed in *Xenopus* oocytes, **1** at 0.1 mM showed weak substrate activity on an excitatory amino acid carrier 1 (EAAC 1), a neuron-type Glu T, while **2** had no activity. OIS (**1**) also had slight agonistic activity on the glycine site of a *N*-methyl-D-aspartic acid (NMDA)-subtype of Glu R composed of 1_A and 2B subunits. However, **1** showed almost no activity on the α-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid (AMPA)-subtype of Glu R composed of α1 and α2 subunits, nor on glutamate/aspartate transporter (GLAST) or glutamate transporter 1 (GLT 1) of Glu T, both being glia-type transporters. As we reported before,[11,12] **3** had a potential activity against all Glu R and Glu T examined, and β-ODAP also showed agonistic activity on the AMPA receptor, while **2** showed no activity toward Glu R or Glu T (Table 1). In summary, **1** had moderate activity toward the glycine site of the NMDA receptor as well as toward the EAAC 1-type Glu T in sharp contrast to its isomer, **3**

Amino Acid Analysis of Some *Lathyrus* and *Pisum* Species Detection of the newly synthesized **1** in the seedlings and seeds of grass pea (*Lathyrus sativus* L.), sweet pea (*L. odoratus* L.), perennial sweet pea (*L. latifolius* L.) and pea (*Pisum sativum* L.) was attempted using an automatic amino acid analyzer equipped with a UV detector as described previously,[14] since **2** was identified in these plants[1,2] and **1** had not yet been found. However, **1** could not be detected in these plants examined, under standard operating conditions.

In conclusion, a novel isoxazole compound **1** was synthesized from isoxazolin-5-one (**4**) and *N*-Boc-L-serine *tert*-butyl ester (**5**) for the first time, and was found to have substrate activity on EAAC 1 as a Glu T, while **1** could not be found in the *Lathyrus* or *Pisum* species examined. This suggests that it may be a potential lead compound for the development of new Glu T drugs.

Experimental

Melting points were measured on a Yanagimoto apparatus and are uncorrected. IR spectra were recorded on a JASCO FT/IR-300E spectrometer by the diffuse reflection measurement method. NMR spectra were measured on a JEOL GSX-500α (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$ solution and on a JEOL GSX-400α (400 MHz for ^{1}H and 100 MHz for ^{13}C) in D_2O solution, and chemical shifts were reported in δ (ppm) from tetramethylsilane (TMS) as an internal standard. FAB-MS were recorded on a JEOL JMS-HX-110A spectrometer in an *m*-nitrobenzylalcohol (NBA) matrix in the positive ion mode. The electrospray ionization-mass spectra (ESI-MS) were obtained on a JEOL JMS-700T spectrometer in H_2O : CH_3CN : CH_3OH : AcOH=33 : 33 : 33 : 1. TLC : Kieselgel 60 F_{254} 0.25 mm (Merck), Column chromatography (CC) : Kieselgel 60 (70—230 mesh) or Kieselgel 60 (230—400 mesh) (Merck).

BIA (**2**) was obtained from plants as described previously.[1] TAN (**3**) and β-ODAP were purchased from Takeda Chemical Ind., Ltd., and from Tocris Cookson, Ltd., respectively. All chemicals used in the pharmacological study were dissolved in glass-distilled water.

Isoxazolin-5-one (4) **4** was synthesized from ethyl propiolate (**7**) *via* ethyl malonaldehydate oxime (**8**, 400.5 mg, 3.07 mM) as colorless prisms (180.5 mg, 69.2%) in accordance with the method of Sarlo.[15]

***N*-Boc-L-serine *tert*-Butyl Ester (5)**[16] A mixed solution of *N*-Boc-L-serine (**9**, 1.003 g, 4.89 mM) and *N*,*N*-dimethylformamide di-*tert*-butylacetal (DFBA, 6.21 ml, d=0.848, 25.9 mM) in dry benzene (8.0 ml) was refluxed for 19 h under Ar. To the mixture, 5% aq. $NaHCO_3$ was added and stirred for 30 min, then an adequate amount of CH_3OH was added to give one layer. After extraction with AcOEt, its fraction was rinsed 3 times with H_2O and once with sat. NaCl aq., which was dried over $MgSO_4$ and filtered. The AcOEt solution was concentrated *in vacuo* to give a yellow oil (1.427 g), which was purified by CC [SiO_2, Kieselgel Art. 7734, *n*-hexane : AcOEt=2 : 1 (v/v)] to give **5** and its formyl ester (**5′**) as a by-product.

N-Boc-L-serine *tert*-Butyl Ester (**5**): Colorless prisms (837.2 mg, 65.8%) : mp 79.0—83.5 °C. IR ($CHCl_3$) ν_{max} cm^{-1} : 3432, 1709. ^{1}H-NMR (500 MHz): δ 1.38 (s, 9H, *tert*-Bu), 1.41 (s, 9H, *tert*-Bu), 2.62 (s, 1H, OH), 3.82 (d, 2H, J=3.4 Hz, CH_2), 4.18 (br s, 1H, CH), 5.41 (d, 1H, J=1.0 Hz, NH). ^{13}C-NMR (125 MHz): δ 27.9 ($3CH_3$), 28.3 ($3CH_3$), 56.3 (CH), 63.9 (CH_2), 80.1 (O–C), 82.6 (O–C), 156.0 (C=O), 170.0 (C=O). FAB-MS m/z :262 (MH^+), 206 (MH^+−*tert*-Bu), 150 (MH^+−2-*tert*-Bu).

N-Boc-*O*-formyl-L-serine *tert*-Butyl Ester (**5′**): Colorless prisms (23.0 mg) : IR ($CHCl_3$) ν_{max} cm^{-1} : 3438, 1730. ^{1}H-NMR (500 MHz): δ 1.38 (s, 9H, *tert*-Bu), 1.40 (s, 9H, *tert*-Bu), 4.35 (dd, 1H, J=3.0, 11.0 Hz, CH_2), 4.40 (m, 1H, CH), 4.46 (dd, 1H, J=3.0, 11.0 Hz ,CH_2), 5.23 (d, 1H, J=7.1 Hz, NH), 7.98 (s, 1H, CHO). ^{13}C-NMR (125 MHz): δ 27.9 ($3CH_3$), 28.3 ($3CH_3$), 53.2 (CH), 64.1 (CH_2), 80.2 (O–C), 83.0 (O–C), 155.1 (C=O), 160.3 (HC=O), 168.4 (C=O). FAB-MS m/z : 290 (MH^+), 234 (MH^+−*tert*-Bu), 178 (MH^+−2-*tert*-Bu).

Protected OIS (6) Isoxazolin-5-one (**4**, 25.8 mg, 0.30 mM), *N*-Boc-L-serine *tert*-butyl ester (**5**, 79.2 mg, 0.30 mM) and PPh_3 (95.5 mg, 0.36 mM) were dissolved in 1.0 ml of tetrahydrofuran (THF) at 0 °C under Ar, then diisopropyl azodicarboxylate (DIAD, 0.045 ml, d=1.027, 0.36 mM) was added dropwise in accordance with the Mitsunobu reaction.[13] The resulting mixture was stirred at room temperature for 6 h. To the reaction mixture, water was added and extracted with AcOEt. The AcOEt solution was rinsed 3 times with water and once with sat. NaCl aq. After drying over $MgSO_4$, the filtrated solvent was removed *in vacuo* to give a yellow oil (283.5 mg). This was purified by CC [SiO_2, Kieselgel Art. 7734, *n*-hexane : AcOEt=2 : 1 (v/v)] followed by prep. TLC [*n*-hexane : ether=2 : 1 (v/v)] to give a colorless powder (26.1 mg, 26.2%) : mp 58.5—62.0 °C. IR ($CHCl_3$) ν_{max} cm^{-1} : 1707. ^{1}H-NMR (500 MHz): δ 1.43 (s, 9H, *tert*-Bu), 1.44 (s, 9H, *tert*-Bu), 4.42 (m, 1H, CH), 4.54 (m, 2H, CH_2), 4.63 (m, 1H, NH), 5.20 (d, 1H, J=2.0 Hz, C_4-H), 8.05 (d, 1H, J=2.0 Hz, C_3-H). ^{13}C-NMR (125 MHz): δ 27.7 ($3CH_3$), 28.3 ($3CH_3$), 53.7 (C_8H), 66.7 (CH_2), 76.7 (C_4H), 80.4 (O–C), 83.4 (O–C), 152.8 (C_3H), 155.2 (C=O), 167.8 (C_9=O), 172.9 (C_5). FAB-MS m/z: 329 (MH^+).

OIS (1) Protected OIS (**6**, 12.0 mg, 0.04 mM) and CF_3COOH (10.0 ml) were stirred for 30 min as with the synthetic method of **3** by Tsubotani *et*

al.,[9] then rinsed 2—3 times with ether after removing the solvent. The resulting yellow oil was applied to CC [SiO_2, Kieselgel, ethanol : H_2O=4 : 1 (v/v)], and **1** was obtained as a colorless powder (7.5 mg, quant.): mp 109.5—112.0 °C. IR (nujol) ν_{max} cm^{-1}: 3377, 1689, 1596, 1459. ^{1}H-NMR (400 MHz): δ 4.10 (m, 1H, CH), 4.56 (m, 2H, CH_2), 5.46 (d, 1H, J=2.2 Hz, C_4H), 8.17 (d, 1H, J=2.2 Hz, C_3H). ^{13}C-NMR (100 MHz): δ 54.5 (C_8H), 71.2 (CH_2), 79.3 (C_4H), 154.8 (C_3H), 171.7 (C_9=O), 173.2 (C_5–O). ESI-MS m/z : 173 (MH^+).

Pharmacological Activity Assay This assay of newly synthesized **1** and related compounds was performed using a *Xenopus* oocyte-expressing system as previously described.[11,12]

Amino Acid Analysis Selected *Lathyrus* and *Pisum* seeds were germinated in the dark at 25—26 °C. After 6 or 7 d, the seedlings were collected and extracted in 75% EtOH. Detection of **1** in the seedlings and seeds was attempted using an automatic amino acid analyzer (Hitachi 835-10) equipped with a UV detector (265 nm) under standard operating conditions as described previously:[14] **1** was eluted at about 31 min from the column, and **2** and **3** were eluted at about 23 and 37 min, respectively, at a flow rate of 0.275 ml per min.

References

1) Lambein F., Kuo Y.-H., Van Parijs R., *Heterocycles*, **4**, 567—593 (1976).
2) Lambein F., De Bruyn A., Ikegami F., Kuo Y.-H., "Lathyrus and Lathyrism," ed. by Kaul A. K., Combes D., Third World Medical Research Foundation, New York, 1986, pp. 246—256.
3) Lambein F., Ongena G., Kuo Y.-H., *Phytochemistry*, **29**, 3793—3796 (1990).
4) Kuo Y.-H., Ikegami F., Lambein F., *Phytochemistry*, **49**, 43—48 (1998).
5) Ikegami F., Yamamoto A., Kuo Y.-H., Lambein F., *Biol. Pharm. Bull*., **22**, 770—771 (1999).
6) Schenk S. U., Werner D., *Phytochemistry*, **30**, 467—470 (1991).
7) Baldwin J. E., Adlington R. M., Birch D. J., *Tetrahedron Lett*., **26**, 5931—5934 (1985).
8) Hakoda S., Tsubotani S., Iwasa T., Suzuki M., Kondo M., Harada S., *J. Antibiotics*, **45**, 854—860 (1992).
9) Tsubotani S., Funabashi Y., Takamoto M., Hakoda S., Harada S., *Tetrahedron*, **47**, 8079—8090 (1991).
10) Ikegami F., Kusama-Eguchi K., Sugiyama E., Watanabe K., Lambein F., Murakoshi I., *Biol. Pharm. Bull*., **18**, 360—362 (1995).
11) Kusama-Eguchi K., Ikegami F., Kusama T., Lambein F., Watanabe K., *Environ. Toxicol. Pharmacol*., **2**, 339—342 (1996).
12) Kusama-Eguchi K., Kusama T., Ikegami F., Lambein F., Watanabe K., *Mol. Brain Res*., **52**, 166—169 (1997).
13) Mitsunobu O., Wada M., Sano T., J. *Am. Chem. Soc*., **94**, 679—680 (1972).
14) Ikegami F., Ongena G., Sakai R., Itagaki S., Kobori M., Ishikawa T., Kuo Y.-H., Lambein F., Murakoshi I., *Phytochemistry*, **33**, 93—98 (1993).
15) Sarlo F. D., Dini G., Lacrimini P., *J. Chem. Soc. (C)*, **1971**, 86—89.
16) Widmer U., *Synthesis*, **1983**, 135—136.