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Synthesis of optically active deuterium-labeled homocysteine thiolactone

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Optically pure (*R*)- and (*S*)-3-aminotetradeutero-2-thiophenone (D- and L-[$^2H_4$]homocysteine thiolactone) for use as substrates for metabolic and pharmacokinetic studies has been prepared. The racemic [$^2H_4$]homocysteine thiolactone hydrochloride was prepared from commercially available and highly enriched DL-[3,3,4,4-$^2H_4$]methionine by intramolecular condensation with hydriodic acid. The racemate was derivatized with (*S*)-(+)-2-methoxyphenylacetic acid to form the diastereomeric amides. The diastereomers were separated by silica gel column chromatography. Hydrolysis of the D- and L-isomer amide in 4 M HCl–ethanol (1:2 v/v) afforded the optically pure D- and L-[$^2H_4$]homocysteine thiolactone, respectively.

Keywords: homocysteine thiolactone; homocysteine; stable isotope; optically active

Introduction

A number of epidemiological studies have confirmed a relationship between an increased plasma homocysteine and the development of vascular disease.[1] Mild hyperhomocysteinemia is prevalent in a general population and results from genetic and nutrient-related disturbances in homocysteine metabolism. Homocysteine is a sulfur non-protein amino acid produced by demethylation of methionine through the intermediates *S*-adenosylmethionine and *S*-adenosylhomocysteine. Homocysteine is then either re-methylated by accepting a methyl group from either betaine or 5-methyltetrahydrofolate to form methionine or catabolized irreversibly to form cystathionine. Homocysteine is also metabolized to the cyclic thioester homocysteine thiolactone when remethylation or transsulfuration reactions are impaired.[2,3] The homocysteine thiolactone is formed by methionyl-tRNA synthetase in an error-editing reaction in protein biosynthesis when homocysteine is mistakenly selected in place of methionine.[4] The accumulation of homocysteine thiolactone has been suggested to be related to cell and tissue damage.[5] However, there is no information on the metabolic and pharmacokinetic characteristics of homocysteine thiolactone.

Stable isotope methodology has provided a useful tool for metabolic and pharmacokinetic investigations for endogenous compounds.[6,7] One of the unique advantages of the use of a stable isotopically labeled compound as a tracer is that an endogenous compound and its exogenous administered labeled analog are separately measured by using gas chromatography–mass spectrometry. Our recent use of stable isotope-labeled methionine ([3,3,4,4,*S*-methyl-$^2H_7$]methionine) has proved a powerful methodology for examining the pharmacokinetic behavior of exogenously administered methionine and studying the remethylation of homocysteine to methionine.[8]

We have decided to characterize the kinetic behavior of homocysteine thiolactone using stable isotope methodology. Successful application of methodology with stable isotopes is always dependent on the availability of compounds that are labeled at predesignated position. We have chosen a deuterium-labeled homocysteine thiolactone at C-4 and C-5 because of their chemical stability.

Several preparations of radioisotope-labeled homocysteine thiolactone have been reported.[9–11] In many cases, the synthesis has been achieved by demethylation of radioisotope-labeled methionine with hydriodic acid. The methods were assumed to give the optically pure L-enantiomer and metabolic studies have been performed by using [^{35}S]-labeled homocysteine thiolactone prepared from L-[^{35}S]methionine by this method.[12–14] However, a recent study by Jakubowski has shown that racemization occurred under the reaction conditions and the rate is similar to the rate of conversion of methionine to homocysteine thiolactone.[15] Hamacher reported the synthesis of [^{35}S]homocysteine thiolactone by demethylation of L-[^{35}S]methionine with sodium in liquid ammonia and subsequent lactonization in 6 M HCl.[10] However, the optical purity was not conformed with synthesized [^{35}S]homocysteine thiolactone.

The present paper describes the preparation of optically pure D- and L-[$^2H_4$]homocysteine thiolactone for use as substrates for metabolic and pharmacokinetic studies.

Results and discussion

Homocysteine thiolactone has been prepared by refluxing methionine in hydriodic acid.[9,11,13,16] However, the homocysteine

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thiolactone obtained from L-methionine was a racemate.[15,17] Therefore, we have decided to separate the enantiomers of [$^2H_4$]homocysteine thiolactone prepared from commercially available DL-[$^2H_4$]methionine. The synthetic route to D- and L-[$^2H_4$]homocysteine thiolactone from DL-[$^2H_4$]methionine is illustrated in Scheme 1.

Conversion of DL-[$^2H_4$]methionine **1** to DL-[$^2H_4$]homocysteine thiolactone **2** was performed by the method of Baernstein[16] with minor modification. The chemical ionization mass spectra showed that the pseudo-molecular ion $[M+H]^+$ at *m/z* 218 for *N*-trifluoroacetyl derivative of DL-[$^2H_4$]homocysteine thiolactone was four mass units higher than that of unlabeled DL-homocysteine thiolactone (Figure 1). The ^1H NMR spectrum of DL-[$^2H_4$]homocysteine thiolactone showed no proton signals for 4-H and 5-H. Deuterium incorporation can also be monitored indirectly from isotope-induced shifts in ^{13}C NMR spectroscopy.[18] In the proton-decoupled ^{13}C NMR spectrum, the signal for the carbon directly attached to the deuterium shifts its centre of resonance by 0.3–0.6 ppm and spin–spin coupling produces a characteristic multiplet, hence the signals for C^2H, C^2H_2 and C^2H_3 would give a triplet, a quintet and a septet, respectively. The proton-decoupled ^{13}C NMR spectrum of DL-[$^2H_4$]homocysteine thiolactone gave quintet signals at δ 27.21 and 28.18 ppm for 5-C and 4-C, respectively (Figure 2). These results confirmed that the label had been located entirely at the C-4 and C-5 positions in the homocysteine thiolactone.

The racemic amine **2** was optically resolved by derivatization with an optically active carboxylic acid, separation of the resulting diastereomeric amides by silica gel column chromatography and regeneration of the corresponding pure [$^2H_4$]homocysteine thiolactone. Numerous optically active carboxylic acids are available for derivatization of amines, such as 2-phenylpropionic acid, 2-(6-methoxy-2-naphthyl-)propionic acid and 2-methoxyphenylacetic acid. We found that amide derivatives of D- and L-[$^2H_4$]homocysteine thiolactone prepared with (*S*)-(+)-2-methoxyphenylacetic acid are the most suitable for column separation of the stereoisomers. The condensation of DL-[$^2H_4$]homocysteine thiolactone with (*S*)-(+)-2-methoxyphenylacetic acid by the active ester method using 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide afforded the diastereomeric amide, which were separated analytically by thin-layer chromatography, developing with hexane-ethyl acetate (3:2) (*Rf*; **a**: 0.35,

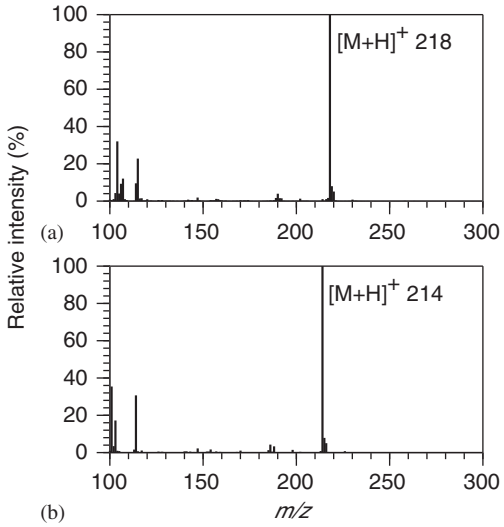
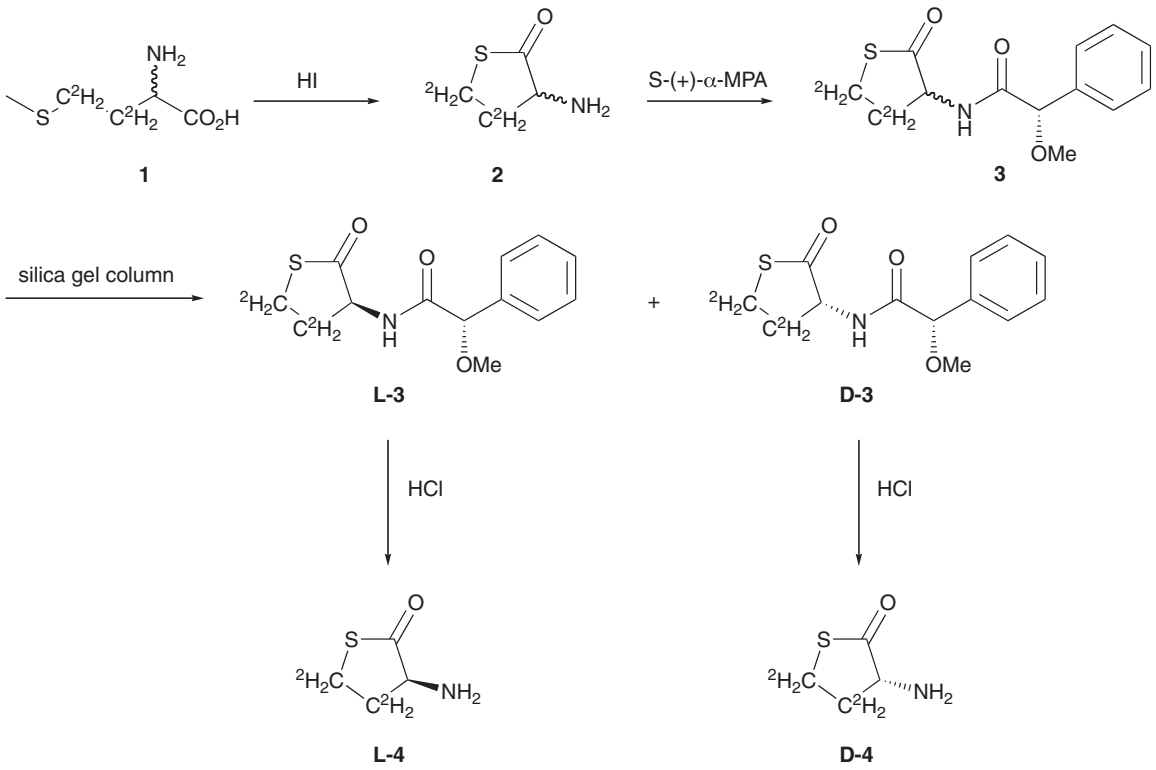


Figure 1. Chemical ionization mass spectra for *N*-trifluoroacetyl derivatives of (a) [$^2H_4$]homocysteine thiolactone and (b) homocysteine thiolactone.



Scheme 1.

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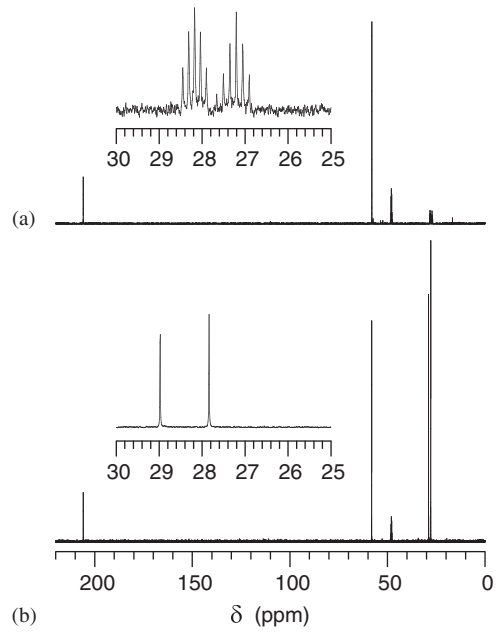

Figure 2. Proton decoupled ^{13}C NMR spectra for (a) [$^2H_4$]homocysteine thiolactone and (b) homocysteine thiolactone in deuterium oxide containing [$^2H_4$]methanol as reference.

b: 0.20). The diastereomers were then separated on a preparative scale by silica gel column chromatography. The two separated crystalline products had a melting point difference of approximately 50°C (**a**: 109–111°C, **b**: 161–163°C). Mass spectrometry showed that both compounds had identical mass spectra with a pseudo-molecular ion ($[M+H]^+$) at *m/z* 270. Reaction of the unlabeled L-enantiomer of homocysteine thiolactone with (*S*)-(+)-2-methoxyphenylacetic acid under the same conditions described above yielded one product, which had an *Rf* value of 0.35 on a thin-layer plate. These results showed that isomers **a** and **b** were identified with L-isomer of **3** and its D-isomer, respectively.

Hydrolysis of the N-acyl derivative of the L-isomer (**L-3)** in 4 M HCl–ethanol (1:2 v/v) for 6 h afforded L-[$^2H_4$]homocysteine thiolactone HCl (**L-4**). In a similar way, D-[$^2H_4$]homocysteine thiolactone HCl (**D-4**) was obtained from D-isomer amide. The enantiomeric purity of L- and D-[$^2H_4$]homocysteine thiolactone were determined by HPLC with a chiral stationary column (Crownpak CR) eluting with 0.06% $HClO_4$. Under these conditions, HPLC analysis of DL-[$^2H_4$]homocysteine thiolactone provided baseline separation at the retention time of 11.8 min (**L**) and 14.0 min (**D**) as shown in Figure 3. Both enantiomers obtained were found to be 98% (e.e.), respectively. The high optical purity of each enantiomer showed that the hydrolysis of the N-acyl derivative in ethanol-4 M HCl (2:1) for 6 h gave no racemization.

The present procedure provides a simple method for the synthesis of optically active deuterium-labeled homocysteine thiolactone. The stable-isotope labeled homocysteine thiolactone should be useful for metabolic and pharmacokinetic studies.

Experimental

General

All melting points were determined with a Yanaco (Kyoto, Japan) micromelting point apparatus MP-S3 and were uncorrected.

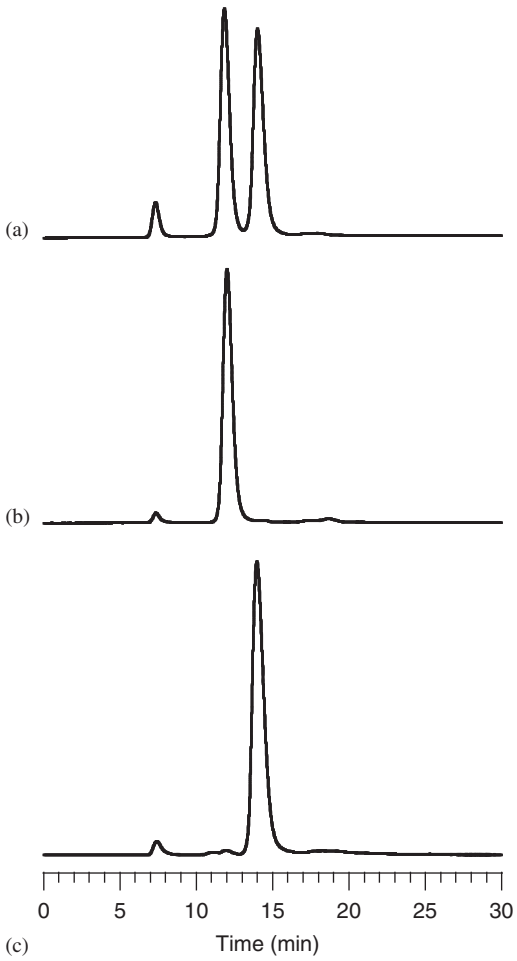

Figure 3. HPLC traces of (a) racemic; (b) L-; and (c) D-[$^2H_4$]homocysteine thiolactone. Column: Crownpak CR (150 × 4 mm id), mobile phase: 0.06% $HClO_4$, flow rate: 0.2 ml/min, temperature: 4°C, UV detector: 200 nm.

Optical rotations were measured by JASCO (Tokyo, Japan) P1030 automatic digital polarimeter. NMR spectra were obtained on a Bruker (Rheinstetten, Germany) AV600 or DPX400 spectrometer. The samples for ^1H and ^{13}C NMR were dissolved in deuterium oxide or C^2HCl_3. Chemical shifts were expressed in δ (ppm) relative to TMS and H^2HO (4.7 ppm) for ^1H NMR and [$^2H_4$]methanol (48.0 ppm) for ^{13}C NMR. *J*-Values were given in Hertz. Mass spectra were obtained on a Micromass (Manchester, UK) Q-Tof Ultra mass spectrometer by elctrospray ionization. Chemical ionization mass spectra were obtained on a Shimadzu (Kyoto, Japan) QP-2010 quadrupole gas chromatograph-mass spectrameter with isobutane as the reagent gas. HPLC was performed on a Jasco PU-980 instrument equipped with a UV detector operated at 200 nm, a 3-line degasser and a Rheodyne injector with a 20-μl loop. Separation was carried out on a Crownpak CR column (150 × 4 mm id, Daicel Chemical, Tokyo, Japan) coupled with a guard column containing the same stationary phase (10 × 4 mm id) using 0.06% $HClO_4$ as mobile phase. The column temperature and flow rate were optimized to 4°C and 0.2 ml/min, respectively.

DL-[3,3,4,4-$^2H_4$]Methionine (DL-[$^2H_4$]methionine; >99% atom ^2H) was purchased from CDN isotopes (Quefec, Canada). Hydroiodic acid (57% w/v in water), (*S*)-(+)-2-methoxyphenylacetic acid and Wakogel C-300 (silicagel) were purchased from Wako (Osaka, Japan). L-Homocysteine thiolactone hydrochloride was purchased from Sigma (St. Louis, MO, USA). All other

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chemicals and solvents were of reagent grade and purchased from commercial vendors.

DL-3-Aminotetradeutero-2-thiophenone hydrochloride (DL-[$^2H_4$]homocysteine thiolactone HCl) (2)

A solution of DL-[$^2H_4$]methionine **1** (1.00 g, 6.57 mmol) in 57% hydriodic acid (25 g) was heated under reflux for 9 h. After cooling, the solution was evaporated under reduced pressure and remaining hydriodic acid was removed by azeotroping with methanol (10 ml × 2). The residue was dissolved in water (10 ml) and was subjected to anion-exchange column chromatography (Dowex 1 × 8, 50–100 mesh, Cl^--form). The column was eluted with 200 ml of water. The waste and effluent were evaporated under reduced pressure to give DL-[$^2H_4$]homocysteine thiolactone HCl **2** as a pale yellow solid (0.94 g, 91%). Crystallization from ethanol afforded an analytical sample. m.p. 193–197°C (dec.). ^{1}H NMR (600 MHz, ^2H_2O): δ 4.22 (1H, s, 3-H) ^{13}C NMR (150 MHz, ^2H_2O): δ 27.21 (quintet, $J_{C\text{-}D}$ = 22 Hz, 5-C), 28.18 (quintet, $J_{C\text{-}D}$ = 21 Hz, 4-C), 58.04 (s, 3-C), 205.92 (s, 2-C). HR-ESI-MS *m/z* 122.0578 $[M+H]^+$ (Calcd. for $C_4H_4{}^2H_4NOS$: 122.0578). Anal. Calcd. for $C_4H_4{}^2H_4NOSCl$, C 30.47, H(^{2}H) 5.11, N 8.88. Found C 30.54, H(^{2}H) 5.13, N 8.69.

(3*S*,2′*S*)-3-(2′-Methoxy-2′-phenyl-)acetamido-4,5-tetradeutero-2-thiophenone (L-3) and (3*R*,2′*S*)-3-(2′-methoxy-2′-phenyl-)acetamido-4,5-tetradeutero-2-thiophenone (D-3)

To a solution of DL-[$^2H_4$]homocysteine thiolactone HCl **2** (0.94 g, 5.98 mmol) and triethylamine (1.2 ml) in chloroform (100 ml) was added 1-hydroxybenzotriazole (0.97 g, 7.18 mmol) and *S*-(+)-2-methoxyphenylacetic acid (1.19 g, 7.17 mmol). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (1.38 g, 7.20 mmol) was added at 4°C. After being stirred for 4 h at 4°C, the resulting solution was washed with water (50 ml), 5% $NaHCO_3$ (20 ml), 2 M HCl (20 ml) and then saturated brine (40 ml), followed by drying over $MgSO_4$. Evaporation of the solvent under reduced pressure gave the diastereomeric amide **3** (1.20 g) as a colorless solid. The diastereomers were purified and separated using silica gel column chromatography with hexane–ethyl acetate (7:3) as an eluting solvent. It afforded the less polar compound **L-3** (0.55 g, 69%) as colorless crystals and more polar compound **D-3** (0.41 g, 51%) as colorless crystals. Each diastereomer had the following physical characteristics.

Compound **L-3**. m.p. 161–163°C. $[\alpha]_D^{25}$ +25.1 (c 0.05, CH_3OH). ^{1}H NMR (400 MHz, C^2HCl_3): δ 3.40 (3H, s, –OCH_3), 4.55 (1H, d, *J* = 8.0 Hz, 3-H), 4.67 (1H, s, 2′-H), 7.16 (1H, d, *J* = 8.0 Hz, NH), 7.36 (5H, m, Ar-H). HR-ESI-MS *m/z* 270.1107 $[M+H]^+$ (Calcd. for $C_{13}H_{12}{}^2H_4NO_3S$: 270.1102). Anal. Calcd. for $C_{13}H_{11}{}^2H_4NO_3S$, C 57.97, H(^{2}H) 5.61, N 5.20. Found C 58.01, H(^{2}H) 5.53, N 5.31.

Compound **D-3**. m.p. 109–111°C. $[\alpha]_D^{25}$ +68.2 (c 0.05, CH_3OH). ^{1}H NMR(400 MHz, C^2HCl_3): δ 3.34 (3H,s, –OCH_3), 4.47 (1H, d, *J* = 8.0 Hz, 3-H), 4.64 (1H, s, 2′-H), 7.09 (1H, d, *J* = 8.0 Hz, NH), 7.38 (5H, m, Ar-H). HR-ESI-MS *m/z* 270.1101 $[M+H]^+$ (Calcd. for $C_{13}H_{12}{}^2H_4NO_3S$: 270.1102). Anal. Calcd. for $C_{13}H_{11}{}^2H_4NO_3S$, C 57.97, H(^{2}H) 5.61, N 5.20. Found C 58.03, H(^{2}H) 5.64, *N* 5.42.

L-[$^2H_4$]Homocysteine thiolactone HCl (L-4) and D-[$^2H_4$]homocysteine thiolactone HCl (D-4)

A solution of compound **L-3** (0.55 g, 2.06 mmol) in a mixture of ethanol-4 M HCl (2:1; 15 ml) was heated under reflux for 6 h. After cooling, the solution was concentrated to ca. 1 ml under reduced pressure. Water (15 ml) and toluene (10 ml) were added. The aqueous phase was separated, washed with toluene (10 ml × 2) and evaporated under reduced pressure to give L-[$^2H_4$]homocysteine thiolactone HCl **L-4** (0.30 g, 92%). Crystallization from isopropanol afforded an analytical sample. m.p. 176–177°C. $[\alpha]_D^{25}$ +21.3 (c 0.05, H_2O). ^{1}H NMR (400 MHz, ^2H_2O): δ 4.26 (1H, s, 3-H). HR-ESI-MS *m/z* 122.0586 $[M+H]^+$ (Calcd. for $C_4H_4{}^2H_4NOS$: 122.0578). Anal. Calcd. for $C_4H_4{}^2H_4NOSCl$, C 30.47, H(^{2}H) 5.11, N 8.88. Found C 30.72, H(^{2}H) 5.15, N 8.75.

Compound **D-3** (0.35 g, 1.30 mmol) was treated in the same manner as above to give compound **D-4** (0.16 g, 78%). Crystallization from isopropanol afforded an analytical sample. m.p. 177–178°C. $[\alpha]_D^{25}$ −21.7 (c 0.05, H_2O). ^{1}H NMR (400 MHz, ^2H_2O): δ 4.24 (1H, s, 3-H). HR-ESI-MS *m/z* 122.0571 $[M+H]^+$ (Calcd. for $C_4H_4{}^2H_4NOS$: 122.0578). Anal. Calcd. for $C_4H_4{}^2H_4NOSCl$, C 30.47, H(^{2}H) 5.11, N 8.88. Found C 30.63, H(^{2}H) 5.19, N 8.73.

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