Inhibition Effects of 5-*S*-Glutathionyl-*N*-β-alanyl-L-dopa Analogues against Src Protein Tyrosine Kinase

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Twelve analogues of the antibacterial phenolic peptide 5-*S*-glutathionyl-*N*-β-alanyl-L-dopa (5-*S*-GA-L-D, 1) were synthesized *via* orthoquinone using tyrosinase. Several synthesized compounds inhibited the v-Src autophosphorylation tyrosine kinase reaction with an IC_{50} value comparable to that of herbimycin A. The inhibition of c-Src substrate phosphorylation was much less active than v-Src autophosphorylation inhibition. 5-*S*-GA-L-D (1) and its analogous competed with peptide substrate and non-competed with ATP. The analogues showed no effects on substrate phosphorylation by epidermal growth factor receptor (EGFR), and this selectivity is the most characteristic feature of the 5-*S*-GA-L-D and its analogues (1—12).

Key words protein tyrosine kinase; c-Src; v-Src; EGFR; Raytide

A low molecular peptide 5-*S*-glutathionyl-*N*-β-alanyl-L-dopa (5-*S*-GA-L-D) (**1**) was isolated as an antibacterial compound from the imagoes of flesh fly *Sarcophaga peregrina* when challenged with *Escherichia coli*.[1] This is a good example to demonstrate the difference in the defense system of insects from that of mammals. In fact, the larvae of *S. peregrina* synthesize a series of antibacterial polypeptides upon infection of bacteria or mechanical injury.[2—4] The structure of **1** was confirmed by the synthesis from *N*-β-alanyldopa and glutathione *via* orthoquinone,[1] according to the synthetic method developed for the preparation of 5-*S*-cysteinyl-L-dopa (5-*S*-Cys-L-D, **11**) which possesses a similar structure to **1**.[5] **11** was identified as an intermediate in the biosyntheisis of sulfur containing phaeomelanins, the pigments of hen feathers, animal hairs as well as human hairs,[6] and it was detected in the urine of normal humans irrespective of their skin and hair colours. The levels of **11** in the urine of melanoma patients were significantly higher than healthy controls, in particular those having metastases of malignant melanoma.[7] **11** showed antitumour activity against several tumour cell lines such as murine L1210 leukemia and B-16 melanoma *in vitro* and *in vivo*.[8,9] The mechanism of antitumour activity was proposed to be partly caused by hydrogen peroxide generated in the cells,[10] and the same mechanism was suggested for the antibacterial activity of **1**.[1]

Peptide structure of **1** containing dopa, a derivative of tyrosine, led us to investigate inhibitory effects of **1** against protein tyrosine kinases (PTK).[11] In a previous study **1** inhibited autophosphorylation of v-Src, but no significant inhibition was observed against serine/threonine protein kinases such as protein kinase C.[11] The selective inhibition of PTK reaction was a unique feature of **1** and further investigations on 5-*S*-GA-L-D and its analogues (**1**—**12**) were pursued to obtain quantitative data on their inhibitory activities against PTK reactions. Twelve compounds (**1**—**12**) were synthesized by the method developed by Itoh and Prota for the synthesis of **11** using mushroom tyrosinase with some modification.[5] The reaction of dopa derivatives with tyrosinase generated orthoquinone and the following Michael type condensation of cysteine or glutathione to the orthoquinone afforded **1**—**12** (see Table 1 for the abbreviation of the names of compounds).[12] The modification of the structure was based on the concept to prevent proteinase hydrolysis of amide bond between dopa and β-alanine by replacing natural L-dopa in unnatural dopa derivatives, D-dopa, α-methyl-L-dopa, and dopamine, when their biological activities are evaluated in the experiments with cells and whole animals. In addition to the analogues with β-alanyl group, corresponding compounds without β-alanyl group were also synthesized to determine the effects of *N*-β-alanyl group. The scheme of synthesis of 5-*S*-glutathionyl-β-alanyldopamine (5-*S*-GDA, **4**) is illustrated in Chart 1 as an example of synthesis using mushroom tyrosinase.

In a previous communication, we briefly reported the results of inhibitory effects of 5-*S*-GA-L-D and its analogues (**1**—**12**) against three different PTK reactions, v-Src autophosphorylation, tyrosine phosphorylation of a synthetic peptide (Raytide) by human recombinant c-Src, and tyrosine phosphorylation of angiotensin II by epidermal growth factor receptor (EGFR). The results of the previous investigations showed that 5-*S*-GA-L-D and its analogues (**1**—**12**) are specific inhibitors of Src PTK reactions.[12] This paper describes the detail of the results and discusses the characteristic feature of 5-*S*-GA-L-D and its analogues (**1**—**12**) on their inhibitory activities against three different kinds of PTK reactions.

Results

Synthesis The starting material for the synthesis of 5-*S*-GA-L-D and its analogues containing β-alanyl residue was synthesized by coupling of *tert*-butoxycarbonyl-β-alanyl-*O*-succinimide with corresponding dopa derivatives and dopamine. Removal of the protective group by acid hydrolysis yielded *N*-β-alanyl-L-dopa, β-alanyl-D-dopa, β-alanyl-α-methyl-L-dopa and *N*-β-alanyldopamine, which were subjected to the one pot condensation reaction using tyrosinase. Mushroom tyrosinase generated corresponding orthoquinones and following Michael type addition of glutathione or cysteine to form 5-*S*-GA-L-D and its analogues containing β-alanyl residue (**1**—**4**, **9**, **10**). The other analogues (**5**—**8**, **11**,

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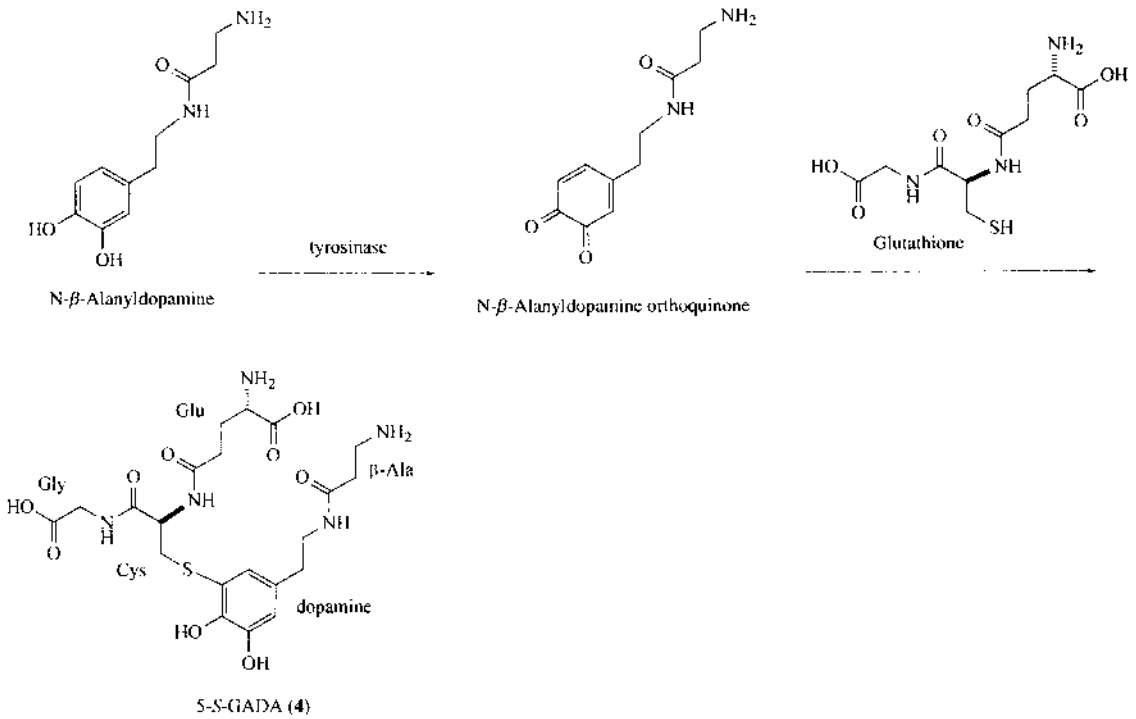

Chart 1. Synthesis of 5-*S*-Glutathionyl-β-alanyldopamine (5-*S*-GADA, **4**)

Table 1. 5-*S*-GA-L-D and Its Synthetic Analogues

Compound	Abbreviation	Name	Yield (%)
1	5-*S*-GA-L-D	5-*S*-Glutathionyl-β-alanyl-L-dopa	58.6
2	5-*S*-GA-D-D	5-*S*-Glutathionyl-β-alanyl-D-dopa	65.3
3	5-*S*-GAMD	5-*S*-Glutathionyl-β-alanyl-α-methyl-L-dopa	40.8
4	5-*S*-GADA	5-*S*-Glutathionyl-β-alanyldopamine	51.5
5	5-*S*-G-L-D	5-*S*-Glutathionyl-L-dopa	50.0
6	5-*S*-G-D-D	5-*S*-Glutathionyl-D-dopa	53.2
7	5-*S*-GDA	5-*S*-Glutathionyldopamine	51.7
8	2,5-*S*,*S*-GDA	2,5-*S*,*S*-Bisglutathionyldopamine	9.8
9	5-*S*-CysA-L-D	5-*S*-Cysteinyl-β-alanyl-L-dopa	56.1
10	5-*S*-CysA-D-D	5-*S*-Cysteinyl-β-alanyl-D-dopa	52.7
11	5-*S*-Cys-L-D	5-*S*-Cysteinyl-L-dopa	44.3
12	5-*S*-Cys-D-D	5-*S*-Cysteinyl-D-dopa	43.5

12) were synthesized from L-dopa, D-dopa and dopamine, and cysteine and glutathione by the one pot reaction with mushroom tyrosinase. The reaction products were purified with preparative HPLC and their structures were confirmed by NMR spectra. A significant amount of 2,5-*S*,*S*-bisglutathionyldopamine (2,5-*S*,*S*-GDA, **8**) was obtained as a by-product in the reaction of dopamine and glutathione in the synthesis of 5-*S*-glutathionyldopamine (5-*S*-GDA, **7**). This indicates that **8** was formed by the second Michael type addition of glutathione to the orthoquinone of **7** generated by further oxidation with tyrosinase. The formation of 2,5-*S*,*S*-bis-substituted derivatives was detected in HPLC analysis of other reaction products, however, the yields were too low to be isolated and characterized as pure compounds.

Inhibition of PTK Reactions by 5-*S*-GA-L-D and Its Analogues (1—12) To learn the structural requirements for the inhibition of different PTK reactions and to find selective PTK inhibitors, twelve synthesized compounds (**1**—**12**) were evaluated in three different PTK assay systems: autophosphorylation of v-Src[13] and substrate phosphorylation by human recombinant c-Src[14] and EGFR.[15] As shown in Table

Table 2. Inhibitory Activities of 5-*S*-GA-L-D Analogues[a]

Compound	v-Src Autophosphorylation	c-Src Phosphorylation of Raytide	EGFR Phosphorylation of angiotensin II
	IC_{50} (μM)[b]	IC_{50} (μM)[b]	Inhibition (%)[c]
1	25	223	2.2
2	25	252	8.2
3	74	285	5.2
4	17	293	4.9
5	28	259	4.4
6	24	213	≒0
7	45	442	1.0
8	56	212	7.6
9	64	264	2.5
10	42	372	≒0
11	66	>1 mM	2.9
12	63	677	≒0
Genistein	>100	44	98.0

a) The three assay methods were modified to measure ^{32}P-labelled phosphorylated proteins or peptides by Fuji Film Bio-image Analyzer BAS 2000. *b*) IC_{50} values are based on three independent determinations. IC_{50} values obtained from repeated experiments varied within <15% from experiment to experiment. *c*) Inhibition % at a concentration of 500 mM.

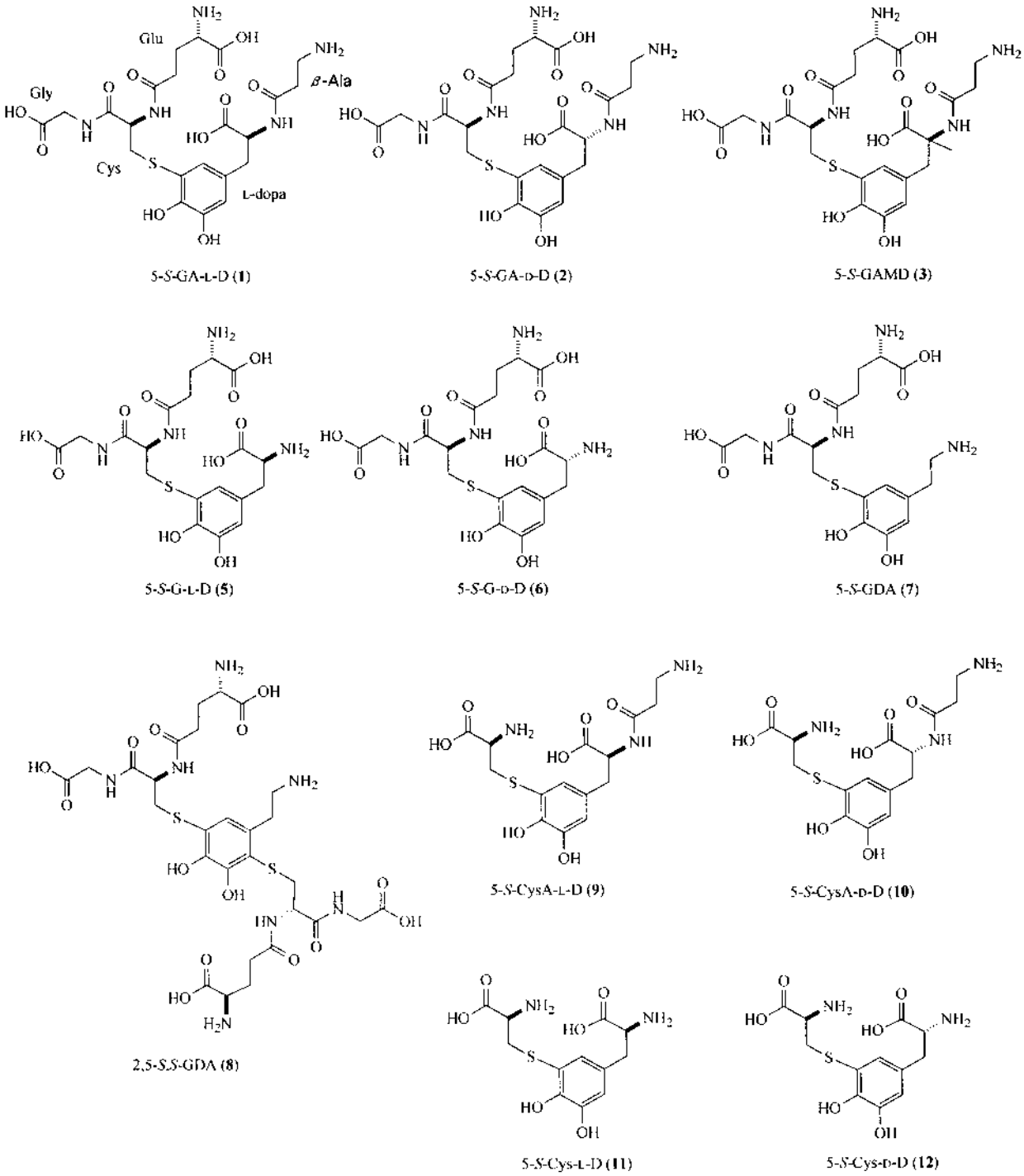

Fig 1. Structure of 5-*S*-GA-L-D (**1**) and Its Analogues

2, the IC_{50} values of 5-*S*-GA-D-D (**1**), 5-*S*-GA-D-D (**2**), 5-*S*-GADA (**4**), 5-*S*-G-L-D (**5**), and 5-*S*-G-D-D (**6**) showed relatively potent inhibitory activities in v-Src autophosphorylation and their IC_{50} values were in the range of 17 to 28 μM. The IC_{50} value of the most active **4** was 17 μM, which is comparable to the reported IC_{50} value of herbimycin A (12 μM) against v-Src autophosphorylation.[16] As reported for 5-*S*-GA-L-D (**1**),[11] synthetic analogues (**2**—**12**) showed no significant inhibition for serine/threonine protein kinases judging from polyacrylamide gel profiles of the ^{32}P labelled proteins in the experiments with *v-src*-transformed NIH 3T3 cells.[13] The introduction of methyl at α of dopa moiety, 5-*S*-glutathionyl-*N*-β-alanyl-α-methyldopa (GAM-L-D, **3**), had the effect of decreasing inhibitory activity in v-Src autophosphorylation.

In general inhibitory effects of 5-*S*-GA-L-D and its analogues (**1**—**12**) on phosphorylation of synthetic peptide substrate (Raytide) were markedly lower than their inhibitory activities against v-Src autophosphorylation. This is evident from the IC_{50} values of genistein known as an ATP competitive inhibitor of PTK (Table 2). Inhibitory effects of 5-*S*-cystenyl-L- and D-dopas, 5-*S*-Cys-L-D (**11**) and 5-*S*-Cys-D-D (**12**), on substrate phosphorylation were markedly lower ($IC_{50}>500$ μM) than the other analogues. 2,5-*S*,*S*-Bisglu-

tathionyldopamine (**8**) inhibited v-Src autophosphorylation with more or less the same potency as the corresponding monosubstituted compound (5-*S*-glutathionyldopamine, **7**), while in substrate phosphorylation with c-Src 2,5-*S*,*S*-bisglutathionyldopamine (2,5-*S*,*S*-GDA, **8**) was twice more potent than monosubstituted 5-*S*-glutathionyldopamine (5-*S*-GDA, **7**). As reported in a previous communication, 5-*S*-cysteinyldopamine was a potent inhibitor for substrate (Raytide) phosphorylation by human recombinant c-Src and its IC_{50} value was 52 μM.[17] Less potent activities of 5-*S*-Cys-L-D (**11**) and 5-*S*-cys-D-D (**12**) are not due to their nonpeptide structure. To clarify the mode of inhibition a conventional kinetic experiment of 5-*S*-GADA (**4**), which was the most active in v-Src autophosphorylation, was carried out with recombinant human c-Src, Raytide and ATP. The apparent mode of inhibition, obtained under fixed concentration of either of the two substrates, was competitive to substrate (Rytide) and non-competitive to ATP (Fig. 1). None of the twelve synthesized compounds inhibited substrate tyrosine phosphorylation by EGFR at a very high concentration of 500 μM. In contrast, a control compound genistein did inhibit phosphorylation 98% at the same concentration. (Table 2)

Discussion

Search for PTK inhibitors of natural origin has been the subject of studies to find potential antitumor compounds and/or useful reagents for use in identifying the role of PTK in intracellular signal transduction. Genistein, emodin,[18] quercetin, piceatannol, erbstatin, radicicol, desmal[19] and herbimycin A were isolated and identified from plants or microbial cultures. PTK inhibitors of natural origin are mostly phenolic compounds except for herbimycin A. They are divided into three groups by the mode of inhibition of PTK. Genistein, emodin and quercetin are competitive to ATP, while piceatannol, erbstatin and desmal are substrate competitive. Herbimycin A is the sole exception which inhibits v-Src autophosphorylation irreversibly by binding SH group of v-Src protein.[16]

Inhibitory effects against the phosphorylation of synthetic peptide Raytide by c-Src were significantly lower in 5-*S*-cysteinyldopas (5-*S*-Cys-L- and -D-dopas, **11** and **12**) than in the other analogues. This seemed to suggest that peptide-like structures were required for potent inhibition against v-Src and c-Src. However, strong inhibition in substrate phosphorylation with c-Src by 5-*S*-cyteinyldopamine is not compatible with this view. As shown in Table 2, no significant inhibition of the EGFR PTK reaction was observed in 5-*S*-GA-L-D and its analogues (**1**—**12**) even at the very high concentration of 500 μM. The inhibition of v-Src autophosphorylation by genistein was lower than that by the synthesized compounds (**1**—**12**), although genistein inhibited c-Src substrate phosphorylation more potently. As expected from the mode of inhibition, genistein inhibited EGFR substrate phosphorylation by 98% at a concentration of 500 μM. It is not appropriate to compare the IC_{50} values with those of genistein, since the latter is ATP-competitive while 5-*S*-GA-L-D and its analogues (**1**—**12**) are substrate competitive. A substrate competitive inhibitor would be advantageous for specific inhibition of tyrosine kinase.

In conclusion, the characteristic feature of 5-*S*-GA-L-D and its analogues (**1**—**12**) is their specific inhibition of v-Src and c-Src PTK reactions, though the inhibition of c-Src substrate phosphorylation was far less potent than the inhibition against v-Src autophosphorylation. This is the first example of phenolic natural products that selectively inhibit a nonreceptor-type PTK (Src), but not receptor-type EGFR PTK, though synthetic heterocyclic compounds were reported to show selectivity among different PTKs.[20] Recent findings have demonstrated that in almost all breast cancers, the level of tyrosine kinase (PTK) activity is elevated up to 25-fold over that in normal breast tissue, and 70% of this elevation is attributed to activated c-Src PTK.[21] Furthermore, c-Src acts as a major signal transducer for various receptors including EGF, platelet derived factor (PDGF), and colony stimulating factor 1 (CSF-1) receptors which play an important driving role in malignant transformations.[21] Therefore, **1** and its analogues provide useful tools in investigating the roles of Src PTKs in signal transduction and cell transformation. Further studies to improve the selectivity and potency of the synthetic analogues and to assess their selectivity toward other PTKs are in progress.

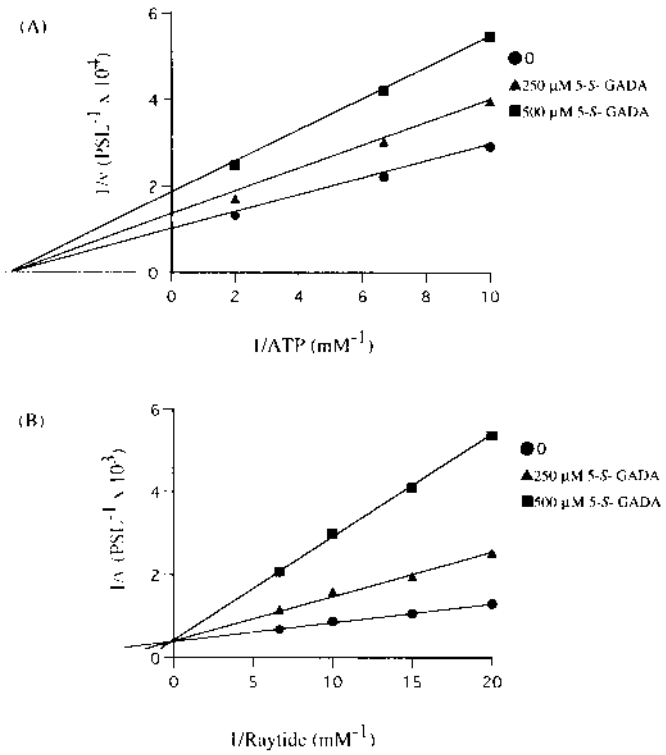

Fig 2. Lineweaver–Burk Plot of c-Src Substrate (Raytide) Phosphorylation with 5-*S*-GADA (**4**)

(A) A Lineweaver–Burk plot showing non-competitive inhibition by 5-*S*-GADA (**4**) against ATP. (B) A Lineweaver–Burk plot showing competitive inhibition by 5-*S*-GADA (**4**) against Raytide.

Experimental

General Notes NMR spectra were obtained on a Varian Unity Plus 500 spectrometer or on a GEMINI 300 spectrometer. High-resolution positive-ion FAB-MS were obtained on a JEOL JMS-HX110. HPLC was carried out using a Tosoh 8020 HPLC system. Shim-Pack PREP-ODS (H) KIT (4.6×250 mm) column was used for analytical purposes and YMC-Pack ODS column (20×250 mm) for preparative scales. Solvents for HPLC were for HPLC grade. Optical rotations were measured with a JASCO DIP-100 using a 1 cm cell. Raytide was obtained from Calbiochem International (Cambridge, MA, U.S.A.) and recombinant human c-Src kinase ($p60^{c\text{-}src}$) from Upstate Biotechnology (Lake Placid, NY, U.S.A.). [γ-^{32}P] ATP was

purchased from NEN Reseach Products. P81 Phosphocellulose ion exchange chromatography paper was the product of Whatman International Ltd. (Maidstone, England). The bovine serum albumin (BSA, initial fraction by heat shock) and angiotensin II were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). β-Mercaptoethanol, L-dopa, D-dopa, α-methyl-L-dopa and dopamine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Drying reagent for SDS–PAGE was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Genistein was purchased from Extraneousness (Gunny, France). Mouse epidermal growth factor (EGF) was purchased from Takara Biomedicals (Tokyo, Japan), and leupeptin, pepstatin A and antipain from Peptide Institute, Inc. (Osaka). All other chemicals were the highest available grade. v-Src-transformed NIH 3T3 and A431 cells were kindly supplied by Drs. Y. Uehara and Y. Murakami of NIH Japan.

Synthesis of *N*-β-Alanyldopa Derivatives and *N*-β-Alanyldopamine *N*-β-Alanyldopa and *N*-β-alanyldopamine were performed according to the procedure reported by Coy *et al.*[22] and Fournier *et al.*[23]

β-Alanyl-L-dopa $[\alpha]_D^{28}$ +20.6° (*c*=0.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{12}H_{17}O_5N_2$ (M^++1): 269.1137. Found: 269.1126. ^{1}H-NMR (D_2O) δ: 2.66 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.90 (1H, dd, *J*=9.2, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.16 (1H, dd, *J*=5.5, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.17 (2H, t, *J*=6.6 Hz, $\underline{CH_2}CH_2NH_2$), 4.65 (1H, dd, *J*=5.5, 9.2 Hz, ArCH$_2\underline{CH}$), 6.73 (1H, dd, *J*=2.1, 8.1 Hz, Ar-6H), 6.81 (1H, d, *J*=2.1 Hz, Ar-2H), 6.87 (1H, d, *J*=8.1 Hz, Ar-5H).

β-Alanyl-D-dopa $[\alpha]_D^{28}$ −22.4° (*c*=0.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{12}H_{17}O_5N_2$ (M^++1): 269.1137. Found: 269.1138. ^{1}H-NMR (D_2O) δ: 2.65 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.90 (1H, dd, *J*=9.2, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.17 (1H, dd, *J*=5.3, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.18 (2H, t, *J*=6.8 Hz, $\underline{CH_2}CH_2NH_2$), 4.62 (1H, dd, *J*=5.3, 9.2 Hz, ArCH$_2\underline{CH}$), 6.74 (1H, dd, *J*=1.9, 8.1 Hz, Ar-6H), 6.82 (1H, d, *J*=1.9 Hz, Ar-2H), 6.87 (1H, d, *J*=8.1 Hz, Ar-5H).

***N*-β-Alanyldopamine** High-resolution positive-ion FAB-MS: Calcd for $C_{11}H_{17}O_3N_2$ (M^++1): 225.1239. Found: 225.1227. ^{1}H-NMR (D_2O) δ: 2.53 (2H, t, *J*=6.8 Hz, $CH_2\underline{CH_2}NH_2$), 2.68 (2H, t, *J*=6.8 Hz, Ar$\underline{CH_2}CH_2$), 3.16 (2H, t, *J*=6.8 Hz, $\underline{CH_2}CH_2NH_2$), 3.39 (2H, t, *J*=6.8 Hz, ArCH$_2\underline{CH_2}$), 6.66 (1H, dd, *J*=1.9, 8.1 Hz, Ar-6H), 6.84 (1H, d, *J*=1.9 Hz, Ar-2H), 6.90 (1H, d, *J*=8.1 Hz, Ar-5H).

β-Alanyl-α-methyl-L-dopa $[\alpha]_D^{28}$ −38.9° (*c*=0.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{13}H_{19}O_5N_2$ (M^++1): 283.1293. Found: 283.1277. ^{1}H-NMR (D_2O) δ: 1.44 (3H, s, CH_3), 2.73 (2H, t, *J*=5.5 Hz, $CH_2\underline{CH_2}NH_2$), 3.02 (1H, d, *J*=13.5 Hz, Ar$\underline{CH_2}$), 3.21 (1H, d, *J*=13.5 Hz, Ar$\underline{CH_2}$), 3.30 (2H, m, $\underline{CH_2}CH_2NH_2$), 6.66 (1H, dd, *J*=2.1, 8.1 Hz, Ar 6-H), 6.73 (1H, d, *J*=2.1 Hz, Ar 2-H), 6.90 (1H, d, *J*=8.1 Hz, Ar 5-H).

Synthesis of 5-*S*-GA-L-D and Its Analogues (1—12) The synthesis of 5-*S*-GA-L-D analogues (**1**—**12**) was based on the method described for that of 5-*S*-cysteinyldopa by Ito and Prota with some modification.[5] The synthesis of 5-*S*-GA-L-D (**1**) is described as a typical example. Reduced glutathione (GSH) (81 mg) and β-alanyldopa (45 mg) were dissolved in 50 mM potassium phosphate buffer pH 6.8 (24 ml), and mushroom tyrosinase (10 mg) was added to the solution. The reaction mixture was deaerated by purging argon. The mixture was kept at room temperature and the reaction was monitored by the disappearance of β-alanyldopa peak in HPLC. Elution of analytical HPLC was performed with 10% acetonitrile in water containing 0.1% tetrafluoroacetic acid (TFA) at a flow rate of 1 ml/min. The eluent was monitored at 280 nm. **1** formed was purified by reverse-phase HPLC. Elution of preparative HPLC was performed with 5% acetonitrile in 0.1% TFA at a flow rate of 5 ml/min. The column eluent was monitored at 280 nm and **1** was obtained by removing solvent by evaporation and lyophilization.

5-*S*-GA-L-D (1) $[\alpha]_D^{28}$ +8.8° (*c*=0.30, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{22}H_{31}O_{11}N_5S$ (M^++1): 574.1819. Found: 574.1829. ^{1}H-NMR (D_2O) δ: 2.20 (2H, m, CH$\underline{CH_2}CH_2$), 2.52 (2H, t, *J*=7.5 Hz, CHCH$_2\underline{CH_2}$), 2.65 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.90 (1H, dd, *J*=8.8, 13.9 Hz, Ar$\underline{CH_2}$CH), 3.11 (1H, dd, *J*=5.3, 13.9 Hz, Ar$\underline{CH_2}$CH), 3.17 (2H, t, *J*=6.6 Hz, $\underline{CH_2}CH_2NH_2$), 3.20 (1H, dd, *J*=8.5, 14.5 Hz, S$\underline{CH_2}$CH), 3.35 (1H, dd, *J*=4.9, 14.5 Hz, S$\underline{CH_2}$CH), 3.87 (2H, s, $\underline{CH_2}$COOH), 4.07 (1H, t, *J*=6.6 Hz, $\underline{CH}CH_2CH_2$) 4.43 (1H, dd, *J*=4.9, 8.5 Hz, SCH$_2\underline{CH}$), 4.66 (1H, dd, *J*=5.3, 8.8 Hz, ArCH$_2\underline{CH}$), 6.79 (1H, d, *J*=1.7 Hz, Ar-2H), 6.89 (1H, d, *J*=1.7 Hz, Ar 6-H).

5-*S*-GA-D-D (2) **2** was obtained from glutathione (45 mg, 0.15 mmol) and *N*-β-alanyl-D-dopa (47 mg, 0.15 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −11.9° (*c*=0.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{22}H_{31}O_{11}N_5S$ (M^++1): 574.1819. Found: 574.1829. ^{1}H-NMR (D_2O) δ: 2.23 (2H, m, CH$\underline{CH_2}CH_2$), 2.56 (2H, t, *J*=7.7 Hz, CHCH$_2\underline{CH_2}$), 2.69 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.90 (1H, dd, *J*=9.0, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.16 (1H, dd, *J*=5.5, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.20 (2H, m, $\underline{CH_2}CH_2NH_2$), 3.22 (1H, dd, *J*=8.5, 14.5 Hz, S$\underline{CH_2}$CH), 3.39 (1H, dd, *J*=4.9, 14.5 Hz, S$\underline{CH_2}$CH), 3.92 (2H, s, $\underline{CH_2}$COOH), 4.10 (1H, t, *J*=6.4 Hz, $\underline{CH}CH_2CH_2$), 4.43 (1H, dd, *J*=4.9, 8.5 Hz, SCH$_2\underline{CH}$), 4.70(1H, dd, *J*=5.5, 9.0 Hz, ArCH$_2\underline{CH}$), 6.82 (1H, d, *J*=1.9 Hz, Ar 2-H), 6.93 (1H, d, *J*=1.9 Hz, Ar 6-H).

5-*S*-GAMD (3) **3** was obtained from glutathione (81 mg, 0.26 mmol) and *N*-β-alanyl-α-methyl-L-dopa (47 mg, 0.15 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −29.2° (*c*=0.051, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{23}H_{34}O_{11}N_5S$ (M^++1): 588.1976. Found: 588.1975. ^{1}H-NMR (D_2O) δ: 1.22 (3H, s, $-CH_3$), 2.01 (2H, m, CH$\underline{CH_2}CH_2$), 2.33 (2H, t, *J*=7.3 Hz, CHCH$_2\underline{CH_2}$), 2.51 (2H, t, *J*=7.1 Hz, $CH_2\underline{CH_2}NH_2$), 2.79 (1H, d, *J*=13.7 Hz, Ar$\underline{CH_2}$) 2.98 (1H, d, *J*=13.7 Hz, Ar$\underline{CH_2}$), 2.99 (1H, dd, *J*=4.9, 14.3 Hz, S$\underline{CH_2}$CH), 3.09 (2H, m, $\underline{CH_2}CH_2NH_2$), 3.18 (1H, dd, *J*=4.9, 14.3 Hz, S$\underline{CH_2}$CH), 3.71 (2H, s, $\underline{CH_2}$COOH), 3.80 (1H, t, *J*=6.6 Hz, $\underline{CH}CH_2CH_2$), 4.22 (1H, dd, *J*=4.7, 8.8 Hz, SCH$_2\underline{CH}$), 6.49 (1H, d, *J*=1.9 Hz, Ar-2H), 6.60 (1H, d, *J*=1.9 Hz, Ar-6H).

5-*S*-GADA (4) **4** was obtained from glutathione (48 mg, 0.16 mmol) and *N*-β-alanyldopamine (33 mg, 0.15 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −6.5° (*c*=0.069, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{21}H_{31}O_9N_5S$ (M^++1): 530.1921. Found: 530.1942. ^{1}H-NMR (D_2O) δ: 2.16 (2H, m, CH$\underline{CH_2}CH_2$), 2.48 (2H, t, *J*=7.5 Hz, CHCH$_2\underline{CH_2}$), 2.57 (2H, t, *J*=6.8 Hz, $CH_2\underline{CH_2}NH_2$), 2.65 (2H, t, *J*=7.1 Hz, Ar$\underline{CH_2}CH_2$), 3.16 (2H, t, *J*=6.8 Hz, $\underline{CH_2}CH_2NH_2$), 3.17 (1H, dd, *J*=8.5, 14.3 Hz, S$\underline{CH_2}$CH), 3.33 (1H, dd, *J*=4.7, 14.3 Hz, S$\underline{CH_2}$CH), 3.37 (2H, m, ArCH$_2\underline{CH_2}$), 3.84 (2H, s, $\underline{CH_2}$COOH), 4.02 (1H, t, *J*=6.6 Hz, $\underline{CH}CH_2CH_2$), 4.39 (1H, dd, *J*=4.7, 8.5 Hz, SCH$_2\underline{CH}$), 6.60 (1H, d, *J*=1.9 Hz, Ar-2H), 6.68 (1H, d, *J*=1.9 Hz, Ar-6H).

5-*S*-G-L-D (5) **5** was obtained from glutathione (34 mg, 0.11 mmol) and L-dopa (20 mg, 0.1 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −5.6° (*c*=0.60, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{19}H_{27}O_{10}N_4S$ (M^++1): 503.1448. Found: 503.1421. ^{1}H-NMR (D_2O) δ: 2.20 (2H, m, CH$\underline{CH_2}CH_2$), 2.53 (2H, t, *J*=7.7 Hz, CHCH$_2\underline{CH_2}$), 3.11 (1H, dd, *J*=7.7, 13.7 Hz, Ar$\underline{CH_2}$CH), 3.19 (1H, dd, *J*=5.4, 13.7 Hz, Ar$\underline{CH_2}$CH), 3.22 (1H, dd, *J*=8.2, 14.3 Hz, S$\underline{CH_2}$CH), 3.35 (1H, dd, *J*=4.9, 14.3 Hz, S$\underline{CH_2}$CH), 3.87 (2H, s, $\underline{CH_2}$COOH), 4.09 (1H, t, *J*=6.6 Hz, $\underline{CH}CH_2CH_2$), 4.30 (1H, dd, *J*=5.4, 7.7 Hz, ArCH$_2\underline{CH}$), 4.46 (1H, dd, *J*=4.9, 8.2 Hz, SCH$_2\underline{CH}$), 6.82 (1H, d, *J*=2.2 Hz, Ar-2H), 6.92 (1H, d, *J*=2.2 Hz, Ar-6H).

5-*S*-G-D-D (6) Glutathione (34 mg, 0.11 mmol) and D-dopa (20 mg, 0.10 mmol) was converted into **6** by the same procedure as **1**. $[\alpha]_D^{28}$ +5.7° (*c*=0.50, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{22}H_{31}O_{11}N_5S$ (M^++1): 503.1448. Found: 503.1421. ^{1}H-NMR (D_2O) δ: 2.22 (2H, m, CH$\underline{CH_2}CH_2$), 2.54 (2H, t, *J*=7.5 Hz, CHCH$_2\underline{CH_2}$), 3.11 (1H, dd, *J*=7.7, 14.7 Hz, Ar$\underline{CH_2}$CH), 3.13 (1H, dd, *J*=5.6, 14.7 Hz, Ar$\underline{CH_2}$CH), 3.17 (1H, dd, *J*=8.3, 14.5 Hz, S$\underline{CH_2}$CH), 3.36 (1H, dd, *J*=4.9, 14.5 Hz, S$\underline{CH_2}$CH), 3.73 (2H, s, $\underline{CH_2}$COOH), 4.10 (1H, t, *J*=6.4 Hz), 4.33 (1H, dd, *J*=4.9, 8.3 Hz, SCH$_2\underline{CH}$), 4.47 (1H, dd, *J*=5.6, 7.7 Hz, ArCH$_2\underline{CH}$), 6.69 (1H, d, *J*=2.1 Hz, Ar-2H), 6.80 (1H, d, *J*=2.1 Hz, Ar-6H).

5-*S*-G-DA (7) **7** was obtained from glutathione (43 mg, 0.14 mmol) and dopamine (20 mg, 0.13 mmol) by the same procedure as **l**. $[\alpha]_D^{28}$ −5.8° (*c*=0.13, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{18}H_{24}O_8N_4S$ (M^++3): 459.1550. Found: 459.1551. ^{1}H-NMR (D_2O) δ: 2.17 (2H, M, CH$\underline{CH_2}CH_2$), 2.50 (2H, t, *J*=7.5 Hz, CHCH$_2\underline{CH_2}$), 2.83 (2H, t, *J*=7.1 Hz, Ar$\underline{CH_2}CH_2$), 3.19 (2H, t, *J*=7.5 Hz, ArCH$_2\underline{CH_2}$), 3.20 (1H, dd, *J*=8.1, 14.3 Hz, S$\underline{CH_2}$CH), 3.33 (1H, dd, *J*=4.9, 14.3 Hz, S$\underline{CH_2}$CH), 3.82 (2H, s, $\underline{CH_2}$COOH), 4.06 (1H, t, *J*=6.6 Hz, $\underline{CH}CH_2CH_2$), 4.42 (1H, dd, *J*=4.9, 8.1 Hz, SCH$_2\underline{CH}$), 6.79 (1H, d, *J*=1.9 Hz, Ar-2H), 6.88 (1H, d, *J*=1.9 Hz, Ar-6H).

2,5-*S*,*S*-GDA (8) **8** was obtained as a by-product in the reaction of dopamine (20 mg, 0.13 mmol) and glutathione (43 mg, 0.14 mmol) in the synthesis of 5-*S*-GDA (**7**). $[\alpha]_D^{28}$ −5.1° (*c*=0.082, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{28}H_{42}O_{14}N_7S_2$ (M^++1): 764.2231. Found: 764.2264. ^{1}H-NMR (D_2O) δ: 2.12 (4H, m, CH$\underline{CH_2}CH_2$), 2.46 (4H, m, CHCH$_2\underline{CH_2}$), 3.07—3.40 (8H, m), 3.80 (2H, s, $\underline{CH_2}$COOH), 3.84 (2H, s, $\underline{CH_2}$COOH), 3.92 (2H, m, $\underline{CH}CH_2CH_2$) 4.36 (2H, m, SCH$_2\underline{CH}$), 6.96 (1H, s, Ar-6H).

5-*S*-CysA-L-D (9) **9** was obtained from L-cysteine monohydrchloride monohydrate (13 mg, 0.079 mmol) and *N*-β-alanyl-L-dopa (20 mg, 0.075 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ +8.9° (*c*=0.50, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{15}H_{21}O_7N_3S$ (M^++1): 388.1178. Found: 388.1192. ^{1}H-NMR (D_2O) δ: 2.66 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.90 (1H, dd, *J*=8.9, 14.9 Hz, Ar$\underline{CH_2}$CH), 3.15 (1H, dd, *J*=5.8, 14.1 Hz, Ar$\underline{CH_2}$CH), 3.19 (2H, t, *J*=6.8 Hz, $\underline{CH_2}CH_2NH_2$), 3.22 (1H, dd, *J*=8.3, 15.0 Hz, S$\underline{CH_2}$CH), 3.47 (1H, dd, *J*=4.1, 15.0 Hz, S$\underline{CH_2}$CH), 3.90 (1H, dd, *J*=4.1, 8.3 Hz, SCH$_2\underline{CH}$), 4.66 (1H, dd, *J*=5.8, 8.9 Hz, ArCH$_2\underline{CH}$), 6.86 (1H, d, *J*=1.9 Hz, Ar-2H), 7.00 (1H, d, *J*=1.9 Hz, Ar-6H).

5-*S*-CysA-D-D (10) **10** was obtained from L-cysteine monohydrochloride monohydrate (13 mg, 0.079 mmol) and *N*-β-alanyl-L-dopa (20 mg, 0.075 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −15.8° (c=0.40, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{15}H_{21}O_7N_3S$ (M^++1): 388.1178. Found: 388.1192. ^{1}H-NMR (D_2O) δ: 2.61 (2H, m, $CH_2\underline{CH_2}NH_2$), 2.83 (1H, dd, J=8.7, 14.3 Hz, Ar$\underline{CH_2}$CH), 3.04 (1H, dd, J=5.5, 14.3 Hz, Ar$\underline{CH_2}$CH), 3.11 (2H, t, J=6.6 Hz, $\underline{CH_2}CH_2NH_2$), 3.38 (2H, m, S$\underline{CH_2}$CH), 4.11 (1H, t, J=6.6 Hz, SCH$_2\underline{CH}$), 4.58 (1H, dd, J=5.5, 8.7 Hz, ArCH$_2\underline{CH}$), 6.75 (1H, d, J=1.7 Hz, Ar-2H), 6.88 (1H, d, J=1.5 Hz, Ar-6H).

5-*S*-Cys-L-D (11) **11** was obtained from L-cysteine monohydrochloride monohydrate (13 mg, 0.079 mmol) and L-dopa (20 mg, 0.10 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ −4.5° (c=0.60, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{12}H_{17}O_6N_2S$ (M^++1): 317.0807. Found: 317.0800. ^{1}H-NMR (D_2O) δ: 3.11 (1H, dd, J=7.7, 14.8 Hz, Ar$\underline{CH_2}$CH), 3.24 (1H, dd, J=5.5, 14.3 Hz, Ar$\underline{CH_2}$CH), 3.48 (2H, m, S$\underline{CH_2}$CH), 4.12 (1H, dd, J=5.5, 6.6 Hz, SCH$_2\underline{CH}$), 4.25 (1H, dd, J=5.5, 7.7 Hz, ArCH$_2\underline{CH}$), 6.90 (1H, d, J=2.2 Hz, Ar-2H), 7.01 (1H, d, J=2.2 Hz, Ar-6H).

5-*S*-Cys-D-D (12) **11** was obtained from L-cysteine monohydrochloride monohydrate (13 mg, 0.079 mmol) and D-dopa (20 mg, 0.10 mmol) by the same procedure as **1**. $[\alpha]_D^{28}$ +21.7° (c=0.40, MeOH). ^{1}H-NMR (D_2O) δ: 3.08 (1H, dd, J=7.1, 14.8 Hz, Ar$\underline{CH_2}$CH), 3.14 (1H, dd, J=5.5, 14.8 Hz, Ar$\underline{CH_2}$CH), 3.40 (1H, dd, J=4.9, 15.4 Hz, S$\underline{CH_2}$CH), 3.41 (1H, dd, J=6.6, 15.4 Hz, S$\underline{CH_2}$CH), 4.10 (1H, dd, J=4.9, 6.6 Hz, SCH$_2\underline{CH}$), 4.23 (1H, dd, J=5.5, 7.1 Hz, ArCH$_2\underline{CH}$), 6.81 (1H, d, J=1.6 Hz, Ar-2H), 6.92 (1H, d, J=1.6 Hz, Ar-6H).

v-Src Kinase Assay The procedures were performed as described previously.[13] In brief, *v-src*-transformed NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in 10 cm dishes for 3 days. The cells were collected and incubated for 10 min on ice in 0.25 ml hypotonic buffer containing 5 mM HEPES, pH 7.4, 5 mM $MgCl_2$, and 25 μg/ml each of protease inhibitors, antipain, leupeptin, and pepstatin A. The swollen cells were homogenized by vortexing the mixture for 2 min at room temperature. Following addtion of HEPES buffer (200 mM, pH 7.4) to adjust the concentration to 20 mM, the homogenate was centrifuged at 500 ***g*** for 5 min to sediment nuclei. The supernatant was used as post-nuclear fraction (PNF). The protein kinase reactions were carried out in a final volume of 15 μl containing 10 μg of PNF, 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM (γ-^{32}P) ATP (185 kBq) with or without inhibitors. The kinase reactions were initiated by the addition of (γ-^{32}P) ATP and incubated for 20 min at 25 °C. The reaction was terminated by the addition of 5 ml four fold concentrated SDS–PAGE sample buffer (0.25 M Tris–HCl, 8% SDS, 20% β-mercaptoethanol, 40% glycerol and 0.16% bromophenol blue). The phosphorylated proteins were separated on a 9% SDS–polyacrylamide gel electrophoresis (6 cm length). The gels were stained with Coomassie brilliant blue, destained in 7.5% acetic acid–5% methanol and then treated with drying reagent and visualized by Fuji Film Bio-image Analyzer BAS 2000. To detect tyrosine-specific phosphorylation, polyacrylamide gels were treated with 1 M KOH at 55 °C for 2 h to remove phosphorylated serine/threonine residues, and then it was neutralized with 10% acetic acid–5% isopropanol. The alkaline treated gels were treated with the drying reagent and subjected to radioactive imaging by BAS 2000.

c-Src Substrate Tyrosine Kinase Assay The protein kinase reactions were carried out in a final volume of 10 μl containing 0.6 unit (each unit of enzyme catalyzes the incorporation of one picomole phosphate per minute from ATP into tyrosyl residues) of human recombinant c-Src, 150 mM Raytide, 35 mM HEPES pH 7.5, 0.1 mM EDTA, 0.01 % Brij 35, 0.03 mg/ml BSA, 0.1% β-mercaptoethanol, 10 mM $MgCl_2$, 50 μM (γ-^{32}p) ATP (37 kBq) with or without inhibitors. The reaction mixture was incubated for 30 min at 30 °C and the reaction was terminated by the addition of 10 ml of 16% phosphoric acid. The sample was spotted onto P81 phosphocellulose ion exchange chromatography paper, and the paper was repeatedly washed with 0.5% phosphoric acid at least four times, once with acetone and dried. The dried paper was submitted for radioactive measurement with BAS 2000. Radioactivity was visualized by a Fuji Film Bio-image Analyzer BAS 2000 as in the case of dried polyacrylamide gel.

EGFR Kinase Assay Human epidermoid carcinoma A431 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS in 10 cm plastic dishes at 37 °C under 5% CO_2 air. The cultured cells were collected and homogenized with a Teflon homogenizer (50 strokes) in sucrose buffer consisting of 20 mM HEPES, pH 7.4, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 250 mM sucrose. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant was re-centrifuged at 50000 rpm for 1 h at 4 °C. The pellet suspended in 20 mM HEPES buffer and centrifuged again at 10000 rpm for 1 min at 4 °C. The supernatant was used as a membrane fraction containing EGFR. Protein kinase reactions were carried out in a final volume of 20 ml containing 20 mM HEPES, pH 7.4, 4 mM $MnCl_2$, 2 mg/ml EGF, 1 mg/ml angiotensin II, 10 mg of A431 membrane fraction, 0.01% BSA, 10 mM (γ-^{32}P) ATP (92.5 kBq) with or without inhibitors. The EGFR fraction was first incubated with EGF for 30 min at 25 °C before assay of kinase activity. The kinase reactions were initiated by the addition of (γ-^{32}P) ATP and continued for 15 min at 0 °C. The reactions were stopped by addition of 10% trichloroacetic acid and then 5 mg/ml BSA 10 μl was added to the reaction mixture. The reaction mixture was centrifuged at 15000 rpm for 15 min and then the supernatant was spotted onto a P81 phosphocellulose ion exchange chromatography paper. It was extensively washed with 0.5% phosphoric acid, once with acetone and dried. The dried paper was submitted for radioactive imaging by BAS 2000.

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