Synthesis of Nonclassical Quinazolinone Antifolates as Thymidylate Synthase Inhibitors and Their Antitumor Activity *In Vitro*

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Nonclassical quinazolinone analogs **I**, **II**, and **III**, in which the glutamic acid moiety of the classical antifolates is substituted by phenylglycine, phenylalanine or aminobenzoic acid and their methyl esters, were synthesized and evaluated as lipophilic inhibitors of thymidylate synthase (TS). The target compounds were generally potent inhibitors of *L. casei* and human TS with IC_{50} values within the narrow range of 0.2-10 μM and 0.003-0.03 μM, respectively. Further, most of the target compounds showed cytotoxicity against tumor cell lines of murine and human origin with IC_{50} values of as low as 0.050 μM. Substitution of another hydroxyl or carboxylic acid/ester group at the phenyl ring further increased the potency of TS inhibition and cell growth inhibition. Most effective were compounds **If** and **Ic** in which extra carboxylic acid/ester was present at the phenyl ring with nanomolar IC_{50} values of 0.0044 and 0.0093 μM against human TS and submicromolar cytotoxic growth inhibition against all four tumor cell lines.

Key Words : Quinazolinone, Nonclassical antifolates, Thymidylate synthase inhibitors, *In vitro* antitumor activity

Introduction

N^5,N^{10}-Methylenetetrahydrofolate (**1**) is a cofactor for the catalytic action of thymidylate synthase (TS; EC 2.1.1.45), the enzyme involved in the sole *de novo* biosynthesis of deoxythymidine-5'-monophosphate (dTMP, thymidylate, **2**) from deoxyuridine-5'-monophosphate (dUMP, **3**). In the process of this catalytic action, TS forms a ternary complex with N^5,N^{10}-methylenetetrahydrofolate and dUMP, and this complex is cleaved into dihydrofolate (**4**) and dTMP with

simultaneous methylene transfer and hydride reduction, resulting in the methylation of dUMP to dTMP (Figure 1).[1]

Since DNA contains thymine as a base component, rapidly dividing cells such as cancer cells where proliferation is very rapid require an abundant supply of dTMP for DNA synthesis and repair.[2-4] The maximal activity of cellular TS for dTMP synthesis occurs during the S phase of the cell cycle and is 20-fold higher in rapidly dividing cells than in nondividing cells.[5] Thus, the inhibition of dTMP synthesis leads to a "thymineless cell death" particularly in cancer
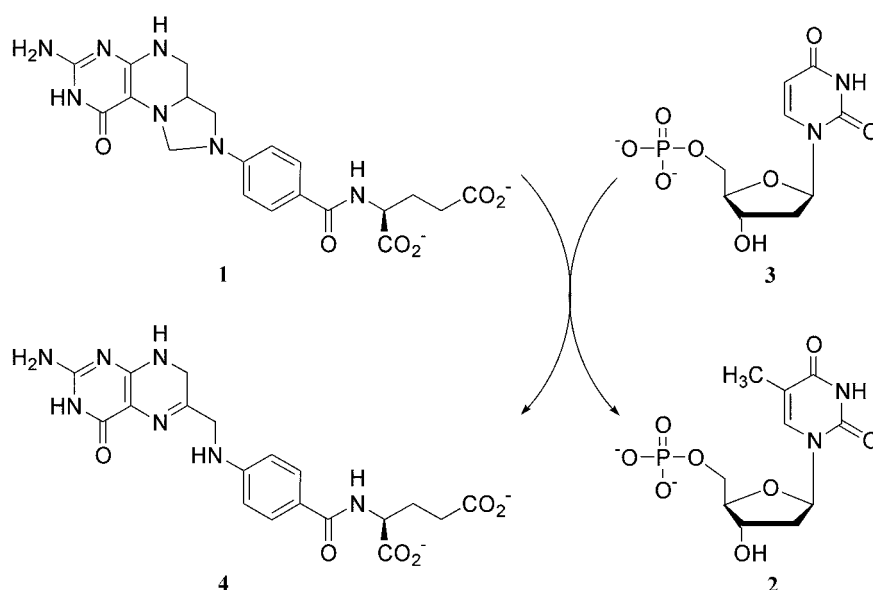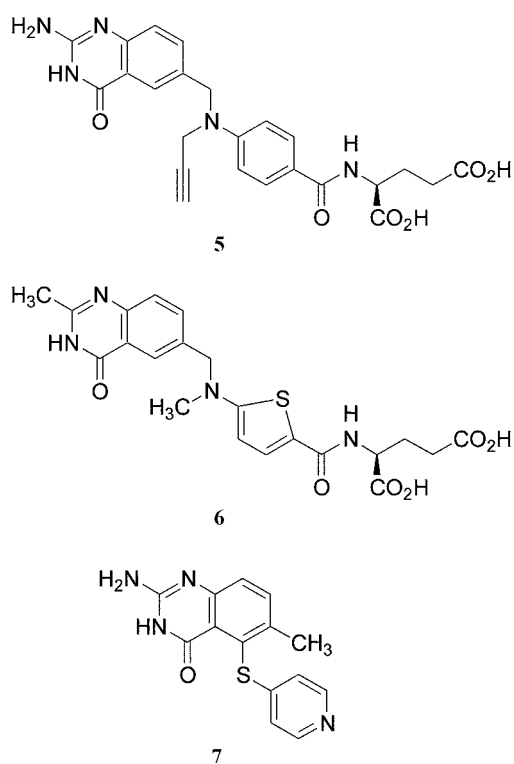

Figure 1

Chart 1

cells, and as a result, inhibition of TS has proven to be an attractive target for anticancer chemotherapy for many years.[6]

One of the first examples of selective, tight-binding inhibitors of TS as classical antifolates is N^{10}-propargyl-5,8-dideazafolic acid (CB3717, **5**) reported by Jones *et al.* in 1981 (Chart 1).[7] The antitumor activity was actually observed in phase 1 and 2 clinical trials with cancer patients.[8] Unfortunately, however, dose-related nephrotoxicity and dose-unrelated hepatotoxicity and malaise limited the clinical utility of **5**.[9] *N*-[[5-[[(1,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]methylamino]-2-thienyl]carbonyl]-L-glutamic acid (ZD1694, raltitrexed, Tomudex®, **6**) has been developed later as a non-nephrotoxic and highly active analog of **5**, and is currently used for cancer treatment clinically.[10]

The potent cytotoxic activity of these classical antifolates is dependent upon the glutamate component, which is essential for the active uptake into cells via the reduced folate carrier and for the binding of antifolate to the active site of TS through hydrogen bonding of α- and γ-carboxylates of glutamate component to the basic amino acid residues of the enzyme.[11] This component is further polyglutamated by folylpolyglutamate synthetase (FPGS) to produce noneffluxing poly-γ-glutamates for the retentive property inside cells for the higher binding affinity and cytotoxic activity.[11,12] The very presence of this glutamate component is, however, detrimental to the activity of these compounds as antitumor agents: Defective cell transport by mutation of the cancer cells themselves induces drug resistance, and unnecessarily long retention inside normal

cells results in toxicity to the host.

These complications may be overcome by deletion or modification of the glutamate moiety from the folate analogs, converting these analogs into nonclassical lipophilic inhibitors of TS. Recently, 2-amino-6-methyl-5-(pyridin-4-ylsulfanyl)-3*H*-quinazolin-4-one (AG-337, Thymitaq®, **7**) which does not bear the appended glutamic acid has been reported as a nonclassical inhibitor of human and *E. coli* TS with the inhibitory binding constants (K_i) of 15 and 49 nM, respectively.[13] This compound further showed high cytotoxic activity against tumor cells in culture,[14] and the clinical trials resulted in regression in tumor size in some treated cancer patients.[15]

Meantime, we wondered whether potent inhibition of TS could be achieved with nonclassical quinazolinone derivatives in which the glutamic acid moiety of the classical antifolates is substituted by a group containing aromatic ring and carboxylic acid/ester. Our strategy was to leave one carboxylic acid/ester group at the α- or γ-position of the glutamic acid with the hope that hydrogen bonding of the carboxylic acid/ester at the active site would be retained for the binding affinity while its lipophilicity attributed by the presence of the aromatic ring would be increased for the passive transport through cell membrane. Thus, this modification of glutamic acid would make it possible to take advantages of both classical and nonclassical antifolates. Toward that purpose, we designed three series of nonclassical quinazolinone analogs as TS inhibitors: Compounds **I**, **II**, and **III** containing phenylglycine, phenylalanine, and aminobenzoic acid moiety, respectively (Chart 2). Herein,
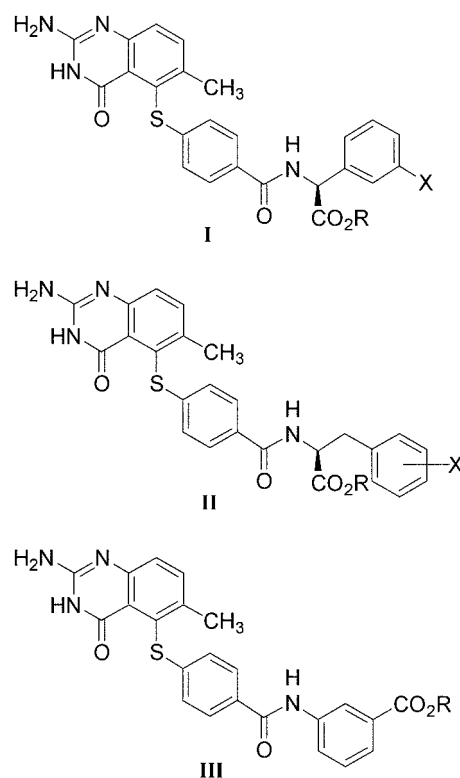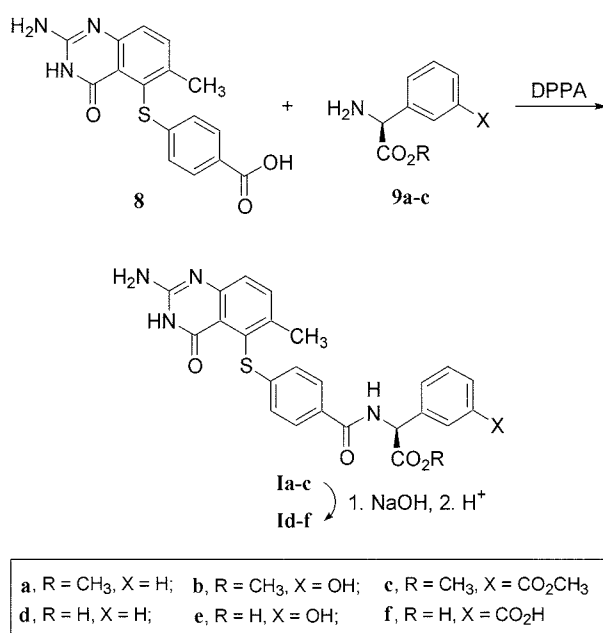

Chart 2

we report on the synthesis of these lipophilic quinazolinone compounds and evaluation for the inhibition against TS and for the cytotoxic growth inhibition of several tumor cell lines of murine and human origin *in vitro*.
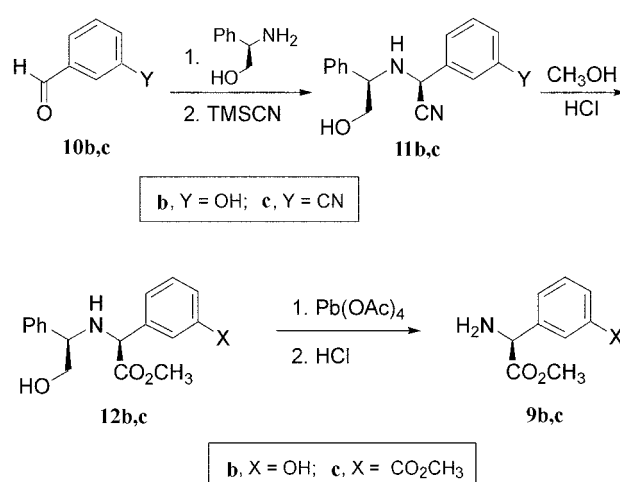
Synthesis

The target compounds **Ia-f** were synthesized according to Scheme 1. First, quinazolinylsulfanyl benzoic acid **8**[13] was reacted with the appropriately substituted phenylglycine methyl esters **9** in the presence of diphenylphosphoryl azide (DPPA) to form amide esters **Ia-c**, and then each of these esters was hydrolyzed to generate carboxylic acids **Id-f**.

The (*S*)-phenylglycine methyl esters **9b** and **9c** were not commercially available, thus made from the appropriate benzaldehydes by diastereoselective Strecker synthesis using 2-phenylglycinol as a chiral auxiliary[16] as shown in Scheme 2: Benzaldehyde **10** was reacted with (*R*)-2-phenylglycinol to form imine intermediate, which was reacted *in situ* with trimethylsilyl cyanide (TMSCN) to lead to the stereospecific addition of cyanide to the imine. (*S*)-α-Amino nitrile **11** thus obtained as a major isomer was then treated with saturated methanolic HCl for 5 hr at room temperature to convert the cyano group into methyl ester **12** and subsequently treated with lead tetraacetate and dilute HCl to remove the (*R*)-2-phenylglycinol chiral auxiliary, affording the desired methyl ester **9**.
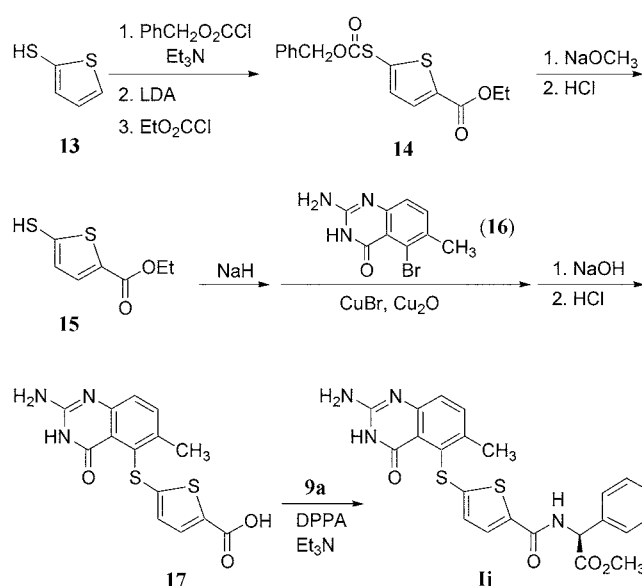
Compounds **Ig-i** were also prepared similarly by the reaction of **8** with the corresponding amines, (*R*)-phenylglycine methyl ester, (*R*)-α-methylbenzylamine, and (*S*)-2-phenylglycinol, respectively. Compound **Ij**, thiophene analog of **Ia**, was synthesized according to Scheme 3. First, thiol **13** was protected as thiocarbonate, and the 5-position of the thiophene ring was carboxylated by lithiation followed

b, Y = OH; **c**, Y = CN

b, X = OH; **c**, X = CO$_2$CH$_3$

Scheme 2

Scheme 3

by the reaction with ethyl chloroformate to produce **14**. Then the thiocarbonate group was deprotected to regenerate thiol **15**, which was coupled with bromoquinazolinone **16**, followed by hydrolysis of the ester group to give thienyl acid **17**, a thiophene analog of **8**. This acid **17** was reacted with **9a** as in Scheme 1, resulting in the formation of **Ij**.

The other series of compounds **II** and **III** were also synthesized analogously from the reaction of **8** with the corresponding phenylalanine methyl esters and aminobenzoic acid methyl esters, respectively.

Biological Activity

The biological activities of the test compounds **I**, **II**, and **III** were evaluated for the inhibition against bacterial (*L. casei*) TS and/or human TS and for the cell growth inhibition of tumor cell lines of murine and human origin *in vitro*. The TS inhibition constants IC$_{50}$ were determined by

a, R = CH$_3$, X = H;   **b**, R = CH$_3$, X = OH;   **c**, R = CH$_3$, X = CO$_2$CH$_3$
d, R = H, X = H;   **e**, R = H, X = OH;   **f**, R = H, X = CO$_2$H

Scheme 1

steady-state analysis against the cofactor N^5,N^{10}-methylene-tetrahydrofolate **1** under conditions of saturating dUMP. Enzyme activity in the *L. casei* TS inhibition assay was measured by following the change in UV absorbance at 340 nm,[17] and enzyme activity in the human TS inhibition assay was measured by the tritium release method.[18] Cell growth inhibition constants IC_{50} were determined by MTT[19] colorimetric assay[20] against four different tumor cell lines: L1210 (mouse lymphocytic leukemia), LY3.7.2C TK−/− (mouse lymphoma, thymidine kinase deficient), CCRF-CEM (human leukemia), and HT-29 (human colon adenocarcinoma). Cell growth was measured spectrophotometrically following a 3- or 4-day incubation in RPMI-1640 medium containing 5% fetal calf serum and a 4-hr treatment with MTT.

Results and Discussion

The results of the biological activities are summarized in Table 1. Most of the target compounds were generally potent inhibitors of *L. casei* and human TS, with IC_{50} values within the narrow range of 0.2-10 μM and 0.003-0.03 μM, respectively. The above data of *L. casei* TS inhibition are comparable to 1.1 μM, that of the reference compound **7**.

In this series of compounds, carboxylic acid analogs were 1.7- to 21-times more potent than the corresponding esters as proved by the comparison of each of the following pairs: **Ia**/**Id**, **Ib**/**Ie**, and **Ic**/**If**. This result suggests that free α-carboxyl acid contributes to the enhanced potency by the increased binding of the quinazolinone derivatives to TS via hydrogen bonding of the carboxylic acid to the basic amino acid residues in the active site of TS. This finding was further supported by the observation that the hydroxymethyl analog **Ii**, in which OH group could be involved in the hydrogen bonding, showed an IC_{50} value of 1.1 μM against *L. casei* TS, which was comparable to those of the carboxylic acid analogs **Id**, **Ie**, and **If**; while the corresponding methyl analog **Ih**, the compound that hydrogen bonding is not possible, had an IC_{50} value of 10 μM, 9-fold less potent than **Ii**. Most effective against human TS were compounds **Ie**, **If**, and **IIb** in which hydroxyl or carboxylic acid group was present at the phenyl ring as an additional hydrogen donor, with nanomolar IC_{50} values of 0.0031-0.0063 μM.

Most of the target compounds were also cytotoxic against tumor cell lines with IC_{50} values of as low as 0.050 μM. Contrary to the TS inhibition, however, esters were generally more active than their corresponding carboxylic acids as shown by the comparison of each of the **Ia**/**Id**, **IIa**/**IIf**, and **IIIa**/**IIIb** pairs in particular, and this inversion of potency between carboxylic acids and esters is due to the likelihood that the more lipophilic esters have greater permeability through the lipophilic cell membrane than the less lipophilic acids. Similar effect was also shown in the comparison between **Ih** and **Ii**: The more lipophilic methyl analog **Ih** showed higher cytotoxicity than the less lipophilic hydroxymethyl analog **Ii** in L1210, LY TK−/−, and HT-29. Among the target compounds, **Ia**, **Ic**, **If**, **IIa**, **IIb**, and **IIIa** exhibited submicromolar cell growth inhibition, and most

effective is compound **If**, which is 2- to 16-times more potent than the reference compound **7** for the cell lines studied in this research.

When the enantiomeric pair **Ia** and **Ig** was compared each other in order to investigate the enantioselectivity in the potency of the inhibition studies, **Ia** was 1.4- to 1.8-times more potent in the TS inhibition and 1.7- to 4.1-times more cytotoxic in the cell growth inhibition than **Ig**. It indicates that (*S*) enantiomer is more potent than the corresponding (*R*) enantiomer against both TS inhibition and cell growth inhibition, even though the enantioselectivity is not very high.

Thiophene analog **Ij** did not increase the potency of both the TS inhibition and cell growth inhibition of the phenyl analog **Ia**.

These results prove that potent inhibition of TS and tumor cell growth can be achieved with nonclassical quinazolinone derivatives containing more lipophilic phenylglycine, phenylalanine, or aminobenzoic acid component, and it is thought that these lipophilic moieties of the target compounds may be quite tolerable in the binding region of the TS active site where glutamate moiety is originally bound in the normal physiological or pathological procedure, and lipophilicity of these moieties may be rather beneficial for the passive transport of these compounds across cell membrane. Substitution of another hydroxyl, carboxylic acid, or ester group at the phenyl ring further increased the potency of TS inhibition and cytotoxic cell growth inhibition. We are presently expanding the scope of this finding in order to discover more potent nonclassical inhibitors of TS.

Experimental Section

Melting points were determined on a Thomas-Hoover Unimelt apparatus and are uncorrected. Mass spectra were determined on an Extrel ELQ-400 mass spectrometer using electrospray ionization with ammonia as a carrier gas. ^{1}H NMR spectra were determined at 300 MHz on a Bruker AMX-R300 spectrometer in DMSO-d_6 or CDCl$_3$, and chemical shifts are reported in δ scale in parts per million from tetramethylsilane as an internal standard. Peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; and bs, broad singlet. IR absorption spectra were taken on a Perkin-Elmer FTIR 1750 spectrometer from KBr pellets. Elemental microanalysis was performed on a CE Instrument EA1110 and gave results for the elements stated with ± 0.4% of the theoretical values. Thin-layer chromatography (TLC) was performed with E. Merck silica gel 60 F-254 precoated glass plates (0.25 mm). Flash column chromatography was performed using E. Merck silica gel 60 (40-63 μm).[21] Tetrahydrofuran (THF) and dioxane were distilled from sodium benzophenone ketyl under nitrogen, and *N,N*-dimethylformamide (DMF) and methylene chloride were distilled from magnesium sulfate and calcium hydride, respectively. Concentrations were performed on a rotary evaporator at ≤ 45 °C (20 torr) unless otherwise noted.

Table 1. Thymidylate Synthase and Cell Growth Inhibition of Quinazolinone Derivatives

entry			TS inhibition IC$_{50}$, μM		cell growth inhibition IC$_{50}$, μM			
			L. casei	human	L1210	LY TK–/–	CCRF-CEM	HT-29
7			1.1	nd	1.1	1.5	0.81	3.7

	R	X						
Ia	(S)-CO$_2$CH$_3$	H	4.7	0.019	0.39	0.51	0.21	0.60
Ig	(R)-CO$_2$CH$_3$	H	8.3	0.027	0.65	2.1	0.44	1.4
Id	(S)-CO$_2$H	H	0.22	nd	2.0	0.76	5.4	>20
Ib	(S)-CO$_2$CH$_3$	OH	1.3	0.017	2.0	0.30	1.5	8.4
Ie	(S)-CO$_2$H	OH	0.34	0.0031	0.71	0.58	3.4	17
Ic	(S)-CO$_2$CH$_3$	CO$_2$CH$_3$	2.0	0.0093	0.71	0.68	0.24	0.55
If	(S)-CO$_2$H	CO$_2$H	1.2	0.0044	0.52	0.69	0.050	1.2
Ih	(R)-CH$_3$	H	10	nd	1.6	1.7	2.5	3.3
Ii	(S)-CH$_2$OH	H	1.1	nd	>10	>15	1.9	10

Ij			38	0.027	1.8	2.5	0.060	0.20

	R	X						
IIa	(S)-CO$_2$CH$_3$	H	nd	nd	0.76	nd	1.0	nd
IIf	(S)-CO$_2$H	H	nd	nd	3.9	nd	6.9	nd
IIb	(S)-CO$_2$CH$_3$	*p*-OH	6.5	0.0063	0.63	nd	0.59	0.28
IIc	(S)-CO$_2$CH$_3$	*m,p*-(OH)$_2$	1.3	nd	5.5	2.2	4.0	>20
IId	(S)-CO$_2$CH$_3$	*p*-NH$_2$	4.0	nd	4.6	2.3	3.2	8.1
IIe	(S)-CO$_2$CH$_3$		4.4	0.019	1.9	nd	0.80	0.91

	X							
IIIa	CO$_2$CH$_3$		nd	nd	0.45	nd	0.41	nd
IIIb	CO$_2$H		nd	nd	3.8	nd	>30	nd

nd, not determined.

Nonclassical Quinazolinone Antifolates and Antitumor Activity

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Method A: *N*-[4-[[(2-Amino-6-methyl-4-oxo-3,4-dihydro-quinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-phenylglycine methyl ester (Ia). A stirred solution of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-phenylglycine methyl ester HCl salt **9a** (0.135 g, 0.67 mmol) in anhydrous DMF (15 mL) was cooled to 0 °C. To this cold solution was added diphenyl-phosphoryl azide (0.14 mL, 0.67 mmol). After stirring for 30 min, triethylamine (0.19 mL, 1.34 mmol) was added, and the reaction mixture was allowed to stir for 3 hr at room temperature. The solvent was then removed under vacuum, and the residue was purified by flash chromatography on silica using CH_2Cl_2/MeOH (13 : 1) to give **Ia** as a yellow solid (0.124 g, 43%): mp 266-267 °C; IR (KBr) 3340, 3180, 1740, 1670, 1640, 1590 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.29 (s, 3H), 3.64 (s, 3H), 5.63 (t, 1H), 6.49 (s, 2H), 6.95 (d, 2H, *J* = 8.5 Hz), 7.26 (d, 1H, *J* = 8.4 Hz), 7.34-7.47 (m, 5H), 7.57 (d, 1H, *J* = 8.7 Hz), 7.73 (d, 2H, *J* = 8.5 Hz), 9.07 (d, 1H, *J* = 7.1 Hz); Mass *m/e* 474 (M$^+$); Anal. (C$_{25}$H$_{22}$N$_4$O$_4$S) C, H, N, S.

Method B: *N*-[4-[[(2-Amino-6-methyl-4-oxo-3,4-dihydro-quinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-phenylglycine (Id). To a solution of methyl ester **Ia** (0.050 g, 0.105 mmol) in methanol (5 mL) was added an aqueous 1 N NaOH solution (0.5 mL), and the reaction mixture was stirred for 3 hr at 70 °C. The solution was then evaporated to dryness, and the sodium salt was taken up in H_2O (2 mL) and acidified to pH 4 with an aqueous 2 N HCl solution at 0 °C. The precipitated solid was filtered and washed with cold H_2O (5 mL), and dried under vacuum over CaSO$_4$ to give **Id** as a yellow solid (0.041 g, 85%): mp 228.5-230 °C; IR (KBr) 3400, 1705, 1595, 1475, 1380, 1290 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.30 (s, 3H), 5.56 (d, 1H, *J* = 7.5 Hz), 6.88 (bs, 2H), 6.97 (d, 2H, *J* = 8.3 Hz), 7.30-7.37 (m, 5H), 7.47 (d, 2H, *J* = 6.4 Hz), 7.61 (d, 1H, *J* = 8.7 Hz), 7.74 (d, 2H, *J* = 8.6 Hz), 8.89 (d, 1H, *J* = 7.5 Hz); Mass *m/e* 461 (M+1).

(*S*)-2-(3-Hydroxyphenyl)glycine methyl ester (9b). To a solution of 3-hydroxybenzaldehyde **10b** (0.465 g, 3.81 mmol) in methanol (5 mL) was added (*R*)-2-phenylglycinol (0.52 g, 3.81 mmol) and stirred for 2 hr at room temperature. The reaction mixture was then cooled to 0 °C, and TMSCN (0.81 g, 8.1 mmol) was added over 15 min. After stirring for 12 hr at room temperature, an aqueous 3 N HCl (20 mL) was added to the reaction mixture. The mixture was extracted with ethyl acetate and the organic layer was dried with anhydrous magnesium sulfate, and filtered. The solvent was then removed under vacuum, and the residue was purified by flash chromatography on silica using CH_2Cl_2/MeOH (20 : 1) to give a yellow liquid **11b** (0.92 g, 90%).

To a solution of **11b** (0.92 g, 3.43 mmol) in methanol (30 mL) was added dry ethereal HCl (15 mL) and stirred for 5 hr at room temperature. The reaction mixture was concentrated under vacuum, and the residue was neutralized with phosphate buffer (pH 7) and extracted with ethyl acetate. The solvent was evaporated under vacuum, and the residue was purified by flash chromatography on silica to give **12b** as colorless liquid (0.72 g, 70%).

To a solution of **12b** (0.72 g, 2.39 mmol) in CH_2Cl_2/

MeOH (2 : 1, 20 mL) was added lead tetraacetate (1.3 g, 2.94 mmol) at 0 °C and the reaction mixture was stirred for 5 min at the same temperature. To this reaction mixture was added phosphate buffer (pH 7) and stirred for 30 min at room temperature. After the reaction mixture was filtered, the organic layer was separated and the aqueous layer was extracted with methylene chloride. After concentration of the combined organic layer under vacuum, the residue was dissolved in ether (10 mL), treated with an aqueous 2 N HCl (20 mL), and the reaction mixture was stirred for 15 min at room temperature. The aqueous layer was separated and concentrated to dryness under vacuum. The residue was then neutralized with phosphate buffer (pH 7), and the mixture was extracted with ethyl acetate. The solvent was evaporated under vacuum, and the residue was purified by flash chromatography on silica using CH_2Cl_2/MeOH (9 : 1) to give **9b** as a white solid (0.30 g, 69%): mp 175-176 °C dec; IR (KBr) 3330, 3270, 3230, 1740, 1595, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.16 (bs, 2H), 3.59 (s, 3H), 4.42 (s, 1H), 6.66 (d, 1H, *J* = 8.0 Hz), 6.78 (s, 2H), 7.11 (dd, 1H, *J* = 7.9, 8.0 Hz), 9.39 (bs, 1H); Mass *m/e* 182 (M+1).

***N*-[4-[[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-2-(3-hydroxyphenyl)glycine methyl ester (Ib).** The coupling reaction of benzoic acid **8** (0.060 g, 0.183 mmol) and (*S*)-2-(3-hydroxyphenyl)glycine methyl ester **9b** (0.0365 g, 0.202 mmol) was performed as described in **Method A**, and **Ib** was obtained as a white solid (0.038 g, 42%): mp 280 °C dec; IR (KBr) 3400, 3100, 1740, 1680, 1600 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.30 (s, 3H), 3.65 (s, 3H), 5.51 (d, 1H, *J* = 6.9 Hz), 6.32 (bs, 2H), 6.74 (d, 1H, *J* = 8.5 Hz), 6.86 (d, 2H, *J* = 7.6 Hz), 6.95 (d, 2H, *J* = 8.4 Hz), 7.17 (dd, 1H, *J* = 7.6, 7.8 Hz), 7.25 (d, 1H, *J* = 8.4 Hz), 7.56 (d, 1H, *J* = 8.5 Hz), 7.74 (d, 2H, *J* = 8.4 Hz), 8.97 (d, 1H, *J* = 6.9 Hz), 10.77 (bs, 1H); Mass *m/e* 491 (M+1); Anal. (C$_{25}$H$_{22}$N$_4$O$_5$S) C, H, N, S.

***N*-[4-[[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-2-(3-hydroxyphenyl)glycine (Ie).** Methyl ester **Ib** (0.030 g) was hydrolyzed as in **Method B**, and **Ie** was obtained as a pale yellow solid (0.028 g, 97%): IR (KBr) 3380, 3220, 1720, 1480, 1300 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.33 (s, 3H), 5.45 (d, 1H, *J* = 7.3 Hz), 6.73 (dd, 1H, *J* = 7.8, 1.6 Hz), 6.88 (d, 2H, *J* = 6.2 Hz), 7.01 (d, 2H, *J* = 8.5 Hz), 7.16 (t, 1H, *J* = 9.2 Hz), 7.43 (d, 1H, *J* = 8.4 Hz), 7.75 (t, 3H, *J* = 10.2 Hz), 8.87 (d, 1H, *J* = 7.4 Hz), 9.48 (s, 1H); Mass *m/e* 499 (M+Na); Anal. (C$_{24}$H$_{20}$N$_4$O$_5$S· HCl·2H$_2$O) C, H, N, S.

(*S*)-2-(3-Carboxyphenyl)glycine dimethyl ester (9c). This compound was prepared from 3-cyanobenzaldehyde **10c** using the procedure described to prepare **9b**. ^{1}H NMR (DMSO-d$_6$) δ 2.38 (bs, 2H), 3.61 (s, 3H), 3.87 (s, 3H), 4.67 (s, 1H), 7.52 (t, 1H), 7.69 (d, 1H, *J* = 6.5 Hz), 7.87 (d, 1H, *J* = 4.9 Hz), 8.01 (s, 1H).

***N*-[4-[[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-2-(3-carboxyphenyl)glycine dimethyl ester (Ic).** The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-2-(3-carboxyphenyl)glycine dimethyl ester **9c** (0.135 g, 0.61 mmol) was performed as in

Method A, and **Ic** was obtained as a yellow solid (0.10 g, 31%): mp 216.5-217.5 °C; ^{1}H NMR (DMSO-d_6) δ 2.31 (s, 3H), 3.67 (s, 3H), 3.87 (s, 3H), 5.76 (m, 1H), 6.34 (bs, 2H), 6.98 (d, 2H, J = 8.5 Hz), 7.26 (d, 1H, J = 6.8 Hz), 7.72-7.78 (m, 3H), 7.95 (d, 1H, J = 7.8 Hz), 8.07 (s, 1H), 9.19 (d, 1H, J = 7.2 Hz), 10.82 (bs, 1H); Mass *m/e* 533 (M+1); Anal. ($C_{27}H_{24}N_4O_6S \cdot 0.5H_2O$) C, H, N, S.

N-**[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(*S*)-2-(3-carboxyphenyl)glycine (If).** Dimethyl ester **Ic** (0.07 g) was hydrolyzed as in **Method B**, and **If** was obtained as a solid (0.038 g, 58%): mp 286-287 °C; IR (KBr) 3400, 1715, 1610, 1490, 1305 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 3H), 5.65 (d, 1H, J = 7.3 Hz), 6.47 (bs, 2H), 6.96 (d, 2H, J = 8.4 Hz), 7.26 (d, 1H, J = 8.4 Hz), 7.49 (t, 1H), 7.57 (d, 1H, J = 8.4 Hz), 7.73 (d, 3H, J = 8.4 Hz), 7.89 (d, 1H, J = 7.8 Hz), 8.05 (s, 1H), 9.01 (d, 1H, J = 7.5 Hz); Mass *m/e* 504 (M$^+$); Anal. ($C_{25}H_{20}N_4O_6S \cdot 2H_2O$) C, H, N, O, S.

N-**[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(*R*)-phenylglycine methyl ester (Ig).** The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*R*)-phenylglycine methyl ester HCl salt (0.135 g, 0.67 mmol) was performed as in **Method A**, and **Ig** was obtained as a yellow solid (0.06 g, 32%): mp 243.5-244.5 °C; IR (KBr) 3350, 3170, 1760, 1690, 1600, 1480, 1220 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.22 (s, 3H), 3.57 (s, 3H), 5.56 (d, 1H, J = 7.1 Hz), 6.33 (bs, 2H), 6.87 (d, 2H, J = 8.5 Hz), 7.17 (d, 1H, J = 8.4 Hz), 7.29 (dd, 3H, J = 5.5, 5.4 Hz), 7.39 (dd, 2H, J = 1.9, 1.4 Hz), 7.48 (d, 1H, J = 8.5 Hz), 7.66 (d, 2H, J = 8.5 Hz), 8.99 (d, 1H, J = 7.1 Hz), 10.97 (bs, 1H); Mass *m/e* 474 (M$^+$); Anal. ($C_{25}H_{22}N_4O_4S$) C, H, N, S.

(*S*)-1-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoylamino]-1-phenylethane (Ih). The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-α-methylbenzylamine (0.081 g, 0.67 mmol) was performed as in **Method A**, and **Ih** was obtained as a yellow solid (0.16 g, 62%): mp 308-309 °C; IR (KBr) 3440, 3360, 3160, 1680, 1640, 1600, 1480 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.44 (d, 3H, J = 7.1 Hz), 2.28 (s, 3H), 5.10 (d, 1H, J = 7.5 Hz), 6.30 (s, 2H), 6.94 (d, 2H, J = 8.4 Hz), 7.10 (d, 1H, J = 8.2 Hz), 7.18-7.37 (m, 5H), 7.54 (d, 1H, J = 8.5 Hz), 7.69 (d, 2H, J = 8.4 Hz), 8.63 (d, 1H, J = 7.9 Hz); Mass *m/e* 431 (M+1).

(*S*)-2-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoylamino]-2-phenylethanol (Ii). The coupling reaction of benzoic acid **8** (0.070 g, 0.21 mmol) and (*S*)-2-phenylglycinol (0.029 g, 0.21 mmol) was performed as in **Method A**, and **Ii** was obtained as a solid (0.032 g, 33%): mp 206-207 °C; IR (KBr) 3280, 1670, 1610, 1480 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 3H), 3.60-3.69 (m, 2H), 4.88 (t, 1H), 5.04 (m, 1H), 6.32 (bs, 2H), 6.97 (d, 1H, J = 8.5 Hz), 7.19-7.38 (m, 6H), 7.56 (d, 1H, J = 8.5 Hz), 7.73 (d, 2H, J = 8.5 Hz), 8.55 (d, 1H, J = 8.1 Hz), 10.77 (bs, 1H); Mass *m/e* 446 (M$^+$); Anal. ($C_{24}H_{22}N_4O_3S \cdot 2H_2O$) C, H, N, O, S.

5-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]-2-thiophenecarboxylic acid (17). To a THF

solution (50 mL) of 2-mercaptothiophene (1.0 g, 8.61 mmol) was added triethylamine (1.2 mL, 8.61 mmol) at 0 °C and stirred for 10 min at the same temperature. To this reaction mixture was added benzyloxycarbonyl chloride (1.23 mL, 8.61 mmol) and stirred for 4 hr at room temperature. After concentration of the reaction mixture to dryness under vacuum, the residue was dissolved in ethyl acetate (50 mL) and washed with an aqueous 1 N HCl solution (30 mL) and water (30 mL) successively. The organic layer was dried with anhydrous sodium sulfate and the solvent was evaporated under vacuum to give a yellow liquid (2.03 g, 8.11 mmol, 94%). This liquid product was dissolved in anhydrous THF (100 mL), and a 2 M solution of lithium diisopropylamide in hexane (4.4 mL, 8.8 mmol) was added dropwise at −78 °C under argon atmosphere. After 30 min at the same temperature, ethyl chloroformate (0.84 mL, 8.8 mmol) was added dropwise over 30 min and stirred for additional 2 hr at room temperature. To this reaction mixture was added an aqueous 1 N HCl (100 mL), and extracted with ethyl acetate. The organic layer was evaporated under vacuum, and the residue was purified by flash chromatography on silica to give **14** as a yellow liquid (1.10 g, 42%).

To a solution of **14** (1.10 g, 3.41 mmol) in anhydrous methanol (50 mL) was added 28% sodium methoxide solution in methanol (6.8 mL, 34.3 mmol) and the reaction mixture was stirred for 5 min. This reaction mixture was acidified with an aqueous 1 N HCl, and concentrated under vacuum to remove ethanol. To this residue was added water (50 mL) and extracted 3 times with ethyl acetate. After the organic layer was dried with anhydrous sodium sulfate, the solvent was evaporated under vacuum, and the residue was purified by flash chromatography on silica to give 5-mercapto-2-thiophenecarboxylic acid ethyl ester **15** as a liquid (0.42 g, 65%): ^{1}H NMR (CDCl$_3$) δ 1.38 (t, 3H, J = 7.1 Hz), 4.33 (q, 3H), 7.13 (d, 1H, J = 4.0 Hz), 7.66 (d, 1H, J = 3.9 Hz).

To a suspension of sodium hydride (0.053 g, 2.20 mmol) in anhydrous N,N-dimethylacetamide (10 mL) at 0 °C was added thiol **15** (0.414 g, 2.20 mmol), and the heterogeneous mixture was stirred for 30 min at room temperature. To the reaction mixture was added 2-amino-5-bromo-6-methyl-3*H*-quinazolin-4-one[13] **16** (0.238 g, 0.79 mmol), copper(I) bromide (0.057 g, 0.40 mmol), and copper(I) oxide (0.057 g, 0.40 mmol), and the mixture was heated at 90 °C for 4 hr. The solvent was evaporated to dryness, H$_2$S/methanol solution (20 mL) was added to the residue, and the mixture was stirred for 1 hr. The mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by flash chromatography on silica using CH$_2$Cl$_2$/MeOH (13 : 1) to give a coupling product as a yellow solid (0.163 g, 57%): mp 224-225 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (t, 3H), 2.40 (s, 3H), 4.20 (q, 2H), 6.44 (s, 2H), 7.00 (d, 1H, J = 4.0 Hz), 7.26 (d, 1H, J = 8.5 Hz), 7.54 (d, 1H, J = 8.5 Hz), 7.60 (d, 1H, J = 3.8 Hz), 10.97 (s, 1H).

This ethyl ester (0.16 g, 0.44 mmol) was hydrolyzed with an aqueous 3 N NaOH solution (10 mL) at 60 °C for 3 hr. After cooling to 0 °C, this mixture was acidified to pH 2 by

slow addition of concentrated HCl, and the precipitate was filtered, washed and dried to give **17** as a yellow solid (0.143 g, 97%): mp 246-247 °C; IR (KBr) 3400, 3200, 2980, 1700, 1620, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.45 (s, 3H), 7.05 (d, 1H, J = 3.9 Hz), 7.51 (d, 1H, J = 8.5 Hz), 7.54 (d, 1H, J = 3.9 Hz), 7.77 (d, 1H, J = 8.5 Hz), 8.29 (s, 2H), 12.94 (bs, 2H); Mass *m/e* 334 (M+1); Anal. (C$_{14}$H$_{11}$N$_3$O$_3$S$_2$·0.4H$_2$O) C, H, N, S.

N-[5-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]-2-thenoyl]-(S)-phenylglycine methyl ester (Ij). The coupling reaction of thiophenecarboxylic acid **17** (0.20 g, 0.60 mmol) and (*S*)-phenylglycine methyl ester HCl salt **9a** (0.133 g, 0.66 mmol) was performed as described in **Method A**, and **Ij** was obtained as a yellow solid (0.064 g, 22%): mp 181-182 °C; IR (KBr) 3400, 3200, 1700, 1620, 1530, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.37 (s, 3H), 3.63 (s, 3H), 5.55 (d, 1H, J = 7.0 Hz), 6.37 (s, 2H), 6.95 (d, 1H, J = 4.0 Hz), 7.21 (d, 1H, J = 8.4 Hz), 7.39 (m, 5H), 7.50 (d, 1H, J = 8.5 Hz), 7.78 (d, 1H, J = 4.0 Hz), 9.09 (d, 1H, J = 7.1 Hz), 10.89 (s, 1H); Mass *m/e* 481 (M+1); Anal. (C$_{23}$H$_{20}$N$_4$O$_4$S$_2$·0.6H$_2$O) C, H, N, S.

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-phenylalanine methyl ester (IIa). The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-phenylalanine methyl ester HCl salt (0.145 g, 0.67 mmol) was performed as in **Method A**, and **IIa** was obtained as a solid (0.135 g, 45%): mp 210-212 °C; IR (KBr) 3420, 1660, 1600, 1560, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.24 (s, 3H), 3.10 (m, 2H), 3.62 (s, 3H), 4.60 (m, 1H), 6.71 (s, 2H), 6.94 (d, 2H, J = 8.4 Hz), 7.19 (d, 1H, J = 8.4 Hz), 7.26 (m, 5H), 7.47 (d, 1H, J = 8.5 Hz), 7.60 (d, 2H, J = 8.3 Hz), 8.73 (d, 1H, J = 7.7 Hz); Mass *m/e* 489 (M+1).

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-phenylalanine (IIf). Methyl ester **IIa** (0.15 g) was hydrolyzed as in **Method B**, and **IIf** was obtained as an off-white solid (0.147 g, 94%): mp 218-220 °C; IR (KBr) 3400, 1720, 1650, 1600, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.38 (s, 3H), 3.05-3.29 (m, 2H), 4.62 (m, 1H), 7.07 (d, 2H, J = 8.4 Hz), 7.27-7.37 (m, 5H), 7.52 (d, 1H, J = 8.4 Hz), 7.69 (d, 2H, J = 8.4 Hz), 7.87 (d, 1H, J = 8.6 Hz), 7.92 (bs, 2H), 8.73 (d, 1H, J = 8.1 Hz), 12.74 (bs, 1H); Mass *m/e* 475 (M+1); Anal. (C$_{25}$H$_{22}$N$_4$O$_4$S·2H$_2$O) C, H, N, S.

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-tyrosine methyl ester (IIb). The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-tyrosine methyl ester (0.131 g, 0.67 mmol) was performed as in **Method A**, and **IIb** was obtained as a yellow solid (0.075 g, 24%): mp 236-238 °C; IR (KBr) 3400, 1750, 1660, 1600, 1530 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.29 (s, 3H), 2.97 (m, 2H), 3.61 (s, 3H), 4.50 (q, 1H), 6.34 (s, 2H), 6.64 (d, 2H, J = 7.7 Hz), 6.94 (d, 2H, J = 7.7 Hz), 7.05 (d, 2H, J = 7.9 Hz), 7.25 (d, 1H, J = 8.5 Hz), 7.55 (d, 1H, J = 8.4 Hz), 7.61 (d, 2H, J = 7.9 Hz), 8.66 (d, 1H, J = 7.4 Hz), 9.19 (s, 1H), 10.78 (s, 1H); Mass *m/e* 505 (M+1); Anal. (C$_{26}$H$_{24}$N$_4$O$_5$S·H$_2$O) C, H, N, S.

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-3-(3,4-dihydroxyphenyl)alanine methyl ester (IIc). The coupling reaction of benzoic acid **8** (0.060 g, 0.183 mmol) and (*S*)-3-(3,4-dihydroxyphenyl)-alanine methyl ester (0.039 g, 0.185 mmol) was performed as in **Method A**, and **IIc** was obtained as a solid (0.017 g, 18%): mp 176.5-177.5 °C; ^{1}H NMR (DMSO-d$_6$) δ 2.28 (s, 3H), 2.94 (m, 2H), 3.60 (s, 3H), 4.49 (m, 1H), 6.30 (bs, 2H), 6.50 (d, 1H), 6.57 (s, 1H), 6.61 (d, 1H, J = 6.7 Hz), 6.93 (d, 2H, J = 8.5 Hz), 7.12 (d, 1H, J = 8.5 Hz), 7.54 (d, 1H, J = 8.5 Hz), 7.61 (d, 2H, J = 8.5 Hz), 8.59 (d, 1H, J = 7.7 Hz), 10.76 (bs, 1H); Mass *m/e* 521 (M+1).

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-3-(4-aminophenyl)alanine methyl ester (IId). The coupling reaction of benzoic acid **8** (0.060 g, 0.183 mmol) and (*S*)-3-(4-aminophenyl)-alanine methyl ester (0.040 g, 0.20 mmol) was performed as in **Method A**, and **IId** was obtained as a yellow solid (0.031 g, 33%): mp 173-174 °C; ^{1}H NMR (DMSO-d$_6$) δ 2.29 (s, 3H), 2.82-2.97 (m, 2H), 3.60 (s, 3H), 4.43-4.50 (m, 1H), 4.84 (bs, 2H), 6.31 (bs, 2H), 6.44 (d, 2H, J = 8.2 Hz), 6.90 (d, 2H, J = 8.4 Hz), 6.94 (d, 2H, J = 8.6 Hz), 7.24 (d, 1H, J = 8.4 Hz), 7.55 (d, 1H, J = 8.5 Hz), 7.61 (d, 2H, J = 8.4 Hz), 8.58 (d, 1H, J = 7.7 Hz); Mass *m/e* 504 (M+1); Anal. (C$_{26}$H$_{25}$N$_5$O$_4$S) C, H, N, O, S.

N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]-(S)-tryptophan methyl ester (IIe). The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and (*S*)-tryptophan methyl ester HCl (0.17 g, 0.67 mmol) was performed as in **Method A**, and **IIe** was obtained as a yellow solid (0.090 g, 29%): mp 171-173 °C; IR (KBr) 3400, 1750, 1660, 1600, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.28 (s, 3H), 3.21 (m, 2H), 3.61 (s, 3H), 4.63 (q, 1H), 6.35 (s, 2H), 6.93 (d, 2H, J = 8.5 Hz), 6.97 (t, 1H), 7.05 (t, 1H), 7.18 (d, 1H, J = 2.1 Hz), 7.24 (d, 1H, J = 8.4 Hz), 7.32 (d, 1H, J = 7.9 Hz), 7.53 (d, 1H), 7.55 (d, 1H, J = 8.5 Hz), 7.63 (d, 2H, J = 8.4 Hz), 8.68 (d, 1H, J = 7.5 Hz); Mass *m/e* 528 (M+1); Anal. (C$_{28}$H$_{25}$N$_5$O$_4$S·2.1H$_2$O) C, H, N, S.

3-[N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]amino]benzoic acid methyl ester (IIIa). The coupling reaction of benzoic acid **8** (0.20 g, 0.61 mmol) and 3-aminobenzoic acid methyl ester (0.093 g, 0.62 mmol) was performed as described in **Method A**, and **IIIa** was obtained as a solid (0.105 g, 37%): mp 258-259 °C; IR (KBr) 3340, 3200, 1660, 1570, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.27 (s, 3H), 3.86 (s, 3H), 6.57 (s, 2H), 7.01 (d, 2H, J = 8.5 Hz), 7.19 (d, 1H, J = 8.4 Hz), 7.38 (dd, 2H, J = 8.2, 8.8 Hz), 7.67 (d, 1H, J = 7.8 Hz), 7.79 (d, 2H, J = 8.4 Hz), 8.02 (d, 1H, J = 8.0 Hz), 8.44 (s, 1H), 10.32 (s, 1H); Mass *m/e* 460 (M$^+$).

3-[N-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)-sulfanyl]benzoyl]amino]benzoic acid (IIIb). Methyl ester **IIIa** (0.15 g) was hydrolyzed as in **Method B**, and **IIIb** was obtained as an off-white solid (0.149 g, 95%): mp 248-249 °C; IR (KBr) 3350, 1720, 1660, 1560, 1480 cm^{-1}; ^{1}H NMR (DMSO-d$_6$) δ 2.33 (s, 3H), 6.45 (s, 2H), 7.02 (d, 2H, J = 8.4 Hz), 7.28 (d, 2H, J = 8.4 Hz), 7.45 (t, 1H),

7.56 (d, 1H, J = 8.5 Hz), 7.66 (d, 1H, J = 7.8 Hz), 7.80 (d, 2H, J = 8.5 Hz), 8.00 (d, 1H, J = 8.2 Hz), 8.39 (s, 1H), 10.31 (s, 1H), 10.99 (bs, 1H), 12.95 (s, 1H); Mass m/e 446 (M$^+$); Anal. ($C_{23}H_{18}N_4O_4S\cdot2H_2O$) C, H, N, S.

Inhibition of *L. casei* TS: Spectrophotometric Assay. TS activity was assayed by steady-state analysis against the cofactor N^5,N^{10}-methylenetetrahydrofolate as a substrate under conditions of saturating dUMP, using a modification of the literature procedure.[17] The assay mixture contained 50 mM Tris (pH 7.4), 25 mM MgCl$_2$, 7.5 mM formaldehyde, 1 mM ethylenediaminetetraacetic acid disodium salt (Na$_2$-EDTA), 75 mM β-mercaptoethanol, 2.5 mM dUMP, and 3 mM N^5,N^{10}-methylenetetrahydrofolate in a final volume of 1 mL. These reactions were either in the absence of inhibitor (control reaction) or in the presence of inhibitor at concentrations ranging from 0.1 to 20 μM to calculate IC$_{50}$. Reactions were started at room temperature by the addition of enzyme, and enzyme activity was measured by following the increase in UV absorbance at 340 nm upon conversion of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate. IC$_{50}$ values were determined from semilogarithmic plots of inhibitor concentration *vs* UV absorbance at 340 nm measured at each concentration relative to that of control reaction.

Inhibition of Human TS: Tritium Release Method. TS activity was measured by assaying the tritium released from [5-^{3}H]dUMP by a modified procedure of Lomax and Greenberg.[18] The composition of the assay mixture was that used in the spectrophotometric assay except for the use of 25 μM [5-^{3}H]dUMP and 300 μM N^5,N^{10}-methylenetetrahydrofolate and for the inclusion of bovine serum albumin at up to 100 μg/mL. The inhibitor concentrations were between 0.3 nM and 100 nM, and the final volume for each assay was 0.1 mL. Reactions were run at room temperature by initiating with the addition of enzyme. After 5 min, the reactions were quenched by the addition of charcoal, the mixture centrifuged to remove charcoal on which unreacted [5-^{3}H]dUMP was adsorbed, and the supernatant counted to determine the release of tritium. IC$_{50}$ values were determined from semilogarithmic plots similar to that described above.

Cell Growth Inhibition Assay. IC$_{50}$ values for the inhibition of cellular growth were measured using a modification of the MTT[19] colorimetric assay of Mosmann[20] using L1210 (mouse lymphocytic leukemia), LY3.7.2C TK–/– (mouse lymphoma, thymidine kinase deficient), CCRF-CEM (human leukemia), and HT-29 (human colon adeno-carcinoma). Cells were seeded at 6,000 (LY TK–/–, HT-29) or 10,000 (L1210, CCRF-CEM) cells per well in 96-well plates, and growth was measured over a range of inhibitor concentrations. Culture medium (RPMI-1640) contained 5% fetal calf serum, 0.5% dimethylsulfoxide (DMSO), and 50 μg/mL penicillin or streptomycin. Plates were incubated for 3 days (L1210, LY TK–/–) or 4 days (CCRF-CEM, HT-29) at 37 °C in a humidified atmosphere containing 5% CO$_2$ in air, and then treated with MTT for 4 hr. Cells were harvested and cell growth was measured spectrophotometrically: The deposited formazan formed from MTT by cells was dissolved in DMSO, and absorbance was measured at 540 nm using microplate reader. IC$_{50}$ values were determined from semilogarithmic plots similar to that described above.

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