Synthesis of a Novel 5-Deaza-5-thia Analogue of Tetrahydrofolic Acid, *N*-(*p*-{[(2-Amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*][1,4]thiazin-6-yl)methyl]amino}benzoyl)glutamic Acid

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N-(*p*-{[(2-Amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*][1,4]thiazin-6-yl)methyl]amino}-benzoyl)glutamic acid **4**, a deaza-thia analogue of tetrahydrofolic acid, was first synthesised as a diastereoisomeric mixture by the thermal condensation of 5-bromo-6-chloroisocytosine **6** with diethyl *N*-{*p*-[(3-amino-2-mercaptopropyl)amino]benzoyl}glutamate **5b** *via* the aliphatic *S*–*N*-type Smiles rearrangement in ethanolic pH 7 phosphate buffer solution followed by smooth alkaline hydrolysis of the ester protecting group.

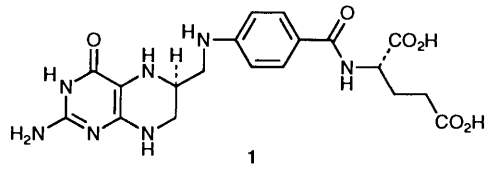
Tetrahydrofolic acid **1**, a primarily active form of folic acid, plays critical roles as a cofactor for cellular one-carbon-unit transfer reactions in the biosynthesis of amino acids, nucleosides, and nucleotides. The functions of the cofactor **1** as a biological catalyst have been extensively studied in both the fields of chemistry and biochemistry.[1] Although the catalytic or chemical reduction of folic acid results in the smooth formation of the cofactor **1**, its tetrahydropyrazine ring is very susceptible to autoxidation, particularly in solution, *e.g.*, the $t_{1/2}$ for the decomposition of the cofactor **1** in the absence of antioxidants is *ca.* 10 min,[2] and handling as a chemical probe for studies on its functions is somewhat troublesome.

On the basis of the above facts, much attention has been paid to the synthesis of deaza analogues of tetrahydrofolic acid **1**[3] which appear to be stable to autoxidation.

Recently, some of the deaza analogues have been shown to be an important class of potential oncolytic agents which inhibit dihydrofolate reductase, thymidylate synthase, or glycinamide ribonucleotide formyltransferase. For example, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid, *N*-{*p*-[2-(2-amino-5,6,7,8-tetrahydro-4-oxo-3*H*-pyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl}-L-glutamic acid, exhibits high antitumour activity against various murine solid tumours, many of which are insensitive to methotrexate, and is currently in clinical trials as an antineoplastic agent.[4]

Along this line, we have synthesised *N*-(*p*-{[(2-amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[4,5-*b*][1,4]thiazin-6-yl)-methyl]amino}benzoyl)glutamic acid **2**, a 8-deaza-8-thia analogue of tetrahydrofolic acid **1**, as a diastereoisomeric mixture *via* the thermal condensation of 5-hydroxyisocytosine with diethyl *N*-{*p*-[(2-amino-3-mercaptopropyl)amino]benzoyl}glutamate **5a**.[5] Compound **2** was found to be very stable to autoxidation even in solution. No appreciable antitumour activity, however, was observed. Thus, our interest was directed to the synthesis of its isomeric thia analogue, *N*-(*p*-{[(2-amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*][1,4]thiazin-6-yl)-methyl]amino}benzoyl)glutamic acid **4**.

To our best knowledge, there has been only a single precedent for the construction of the 2-amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*][1,4]thiazine ring system, *i.e.* Benkovic and coworkers[6] have reported the synthesis of its 6-methyl and 6-phenyl derivatives as thia analogue of tetrahydropterins which involves the *O*-alkylation of the lactam moiety in ethyl 5,6-dihydro-6-methyl (or phenyl)-3-oxo-2*H*-1,4-thiazine-2-carboxylate, prepared by the condensation of diethyl chloromalonate with the corresponding β-mercaptoethylamines, with triethyloxonium tetrafluoroborate and the subsequent thermal



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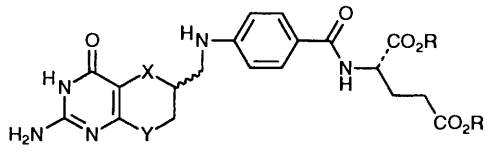
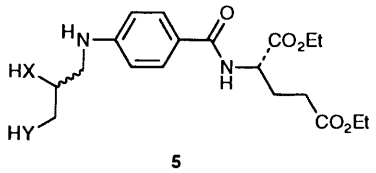


2 X = NH, Y = S, R = H
3 X = S, Y = NH, R = Et
4 X = S, Y = NH, R = H



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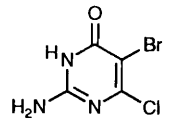
a; X = NH, Y = S
b; X = S, Y = NH



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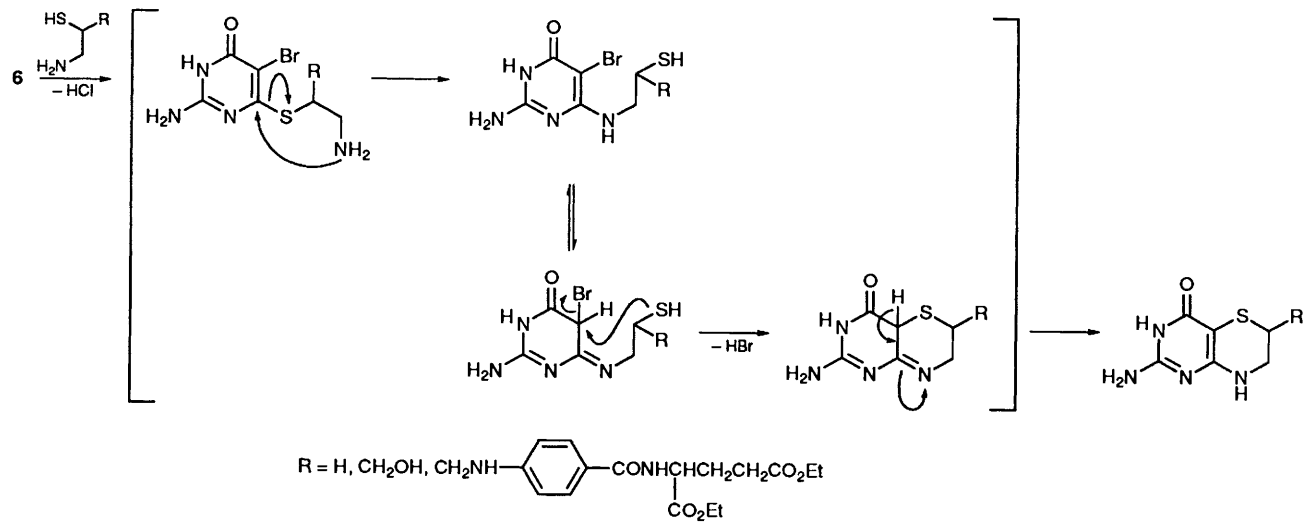
cyclisation in the presence of guanidine. This synthetic methodology, however, appears not to be applicable to the preparation of the thia analogue **4** with a highly functionalised side-chain.†

In a previous paper, we have documented a new and versatile

† According to Benkovic's procedure, we examined the condensation of diethyl chloromalonate with the β-mercaptoethylamine **5b**. The reaction proceeded smoothly even at room temperature, but gave a complex mixture containing acyclic diethyl *N*-{*p*-[(3-amino-2-{[bis(ethoxycarbonyl)methyl]thio}propyl)amino]benzoyl}glutamate (45%), EI-MS *m/z* 567 ($M^+ - 1$, 10%), 522 ($M^+ - EtOH$, 3), 494 ($M^+ - CO_2Et$, 7), 365 (26), 335 (71), 291 (55) and 132 (100); δ_H(400 MHz; $CDCl_3$) 1.22, 1.26, 1.30 and 1.32 (each 3 H, each t, 4 × OCH_2Me), 2.0–2.6 (4 H, m, β- and γ-methylene protons of glutamate), 3.34 (2 H, br d, *J* 5, CH_2NH_2), 3.39 (2 H, br d, *J* 5, $CHCH_2NH$), 3.91 (1 H, br t, NH), 4.10, 4.23, 4.29 and 4.33 (each 2 H, each q, 4 × OCH_2Me), 4.31 (1 H, s, EtO_2CCHS), 4.60 (1 H, br), 4.78 (1 H, m, α-H of glutamate), 6.78 (1 H, br d, *J* 7, CONH) and 6.62 and 7.67 (each 2 H, each d, *J* 9, ArH). Further attempts at the preparation of the 5-thia analogue **4** by the modified Benkovic's approach were unsuccessful.

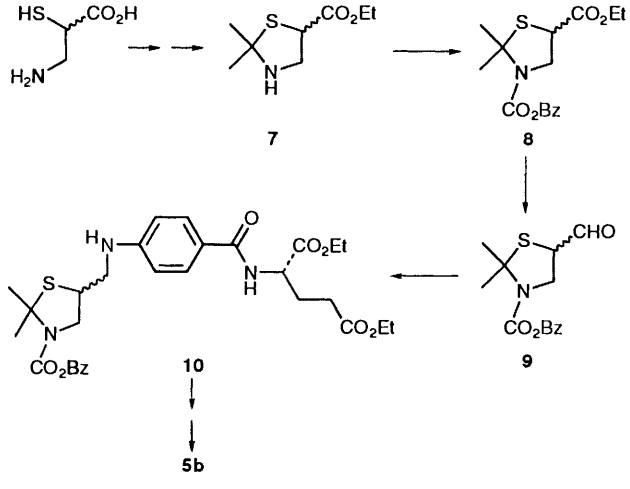


Scheme 1

method for the construction of the 2-amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*][1,4]thiazine ring system involving the thermal condensation of 5-bromo-6-chloroisocytosine **6**[7] with β-mercaptoethylamines in a buffer solution *via* the *S*–*N*-type Smiles rearrangement[8] as depicted in Scheme 1.

Results and Discussion

In this paper, we describe a synthetic approach to the hitherto unknown 5-thia analogue **4** by employing the *S*–*N*-type Smiles rearrangement as a key step. In order to apply our methodology to the preparation of the thia analogue **4**, diethyl *N*-{*p*[(3-amino-2-mercaptopropyl)amino]benzoyl}glutamate **5b** was required as a counterpart for the condensation with the isocytosine **6** (see Scheme 1). The preparation of the mercaptoethylamine **5b** was achieved in seven steps from isocysteine as shown in Scheme 2.



Scheme 2 Bzl = CH_2Ph

Acid-catalysed esterification of (±)-isocysteine[9] followed by isopropylidene protection gave ethyl 2,2-dimethylthiazolidine-5-carboxylate **7**. The thiazolidine **7** was converted into its *N*-benzyloxycarbonyl derivative **8** (79% yield), which was reduced with diisobutylaluminium hydride (DIBAL) to give the corresponding aldehyde **9** (70% yield). Reductive coupling of the aldehyde **9** with diethyl *N*-(*p*-aminobenzoyl)-L-glutamate, by using sodium cyanoborohydride in ethanol containing a small amount of acetic acid, resulting in the formation of diethyl *N*-(*p*-{[(3-benzyloxycarbonyl-2,2-dimethylthiazolidin-5-yl)-methyl]amino}benzoyl)glutamate **10** (57% yield). Deprotection of the thiazolidine-glutamate **10** with hydrogen bromide in acetic acid[10] followed by heating at 60 °C in ethanol containing a small amount of hydrochloric acid led easily to the formation of the desired β-mercaptoethylamine **5b**.

A mixture of the β-mercaptoethylamine **5b** thus obtained and 5-bromo-6-chloroisocytosine **6** was heated in ethanolic, 0.5 mol dm^{-3} pH 7 phosphate buffer solution under argon at 80 °C for 8 h. After purification by column chromatography, diethyl *N*-(*p*-{[(2-amino-6,7-dihydro-4-oxo-3*H*,8*H*-pyrimido[5,4-*b*]-[1,4]thiazin-6-yl)methyl]amino}benzoyl)glutamate **3** was isolated in 45% yield as a result of the Smiles rearrangement of an initially formed sulfide followed by smooth dehydrobromination (see Scheme 1). Alkaline hydrolysis of the diethyl ester **3** in ethanol gave the thia analogue **4** as a 1:1 mixture of C(6)-epimers, the structure of which was confirmed by microanalytical results and spectral data. Its NMR spectral comparison with the isomeric thiafolic acid **2** showed the C(7)-methylene protons as well resolved signals at δ 3.40 (1 H, dd, *J* 5 and 13) and 3.60 (1 H, br d, *J* 13), at lower field than those of the C(7)-methylene protons in the isomer **2** [δ 2.97 (1 H, dd, *J* 7 and 12) and 3.11 (1 H, br d, *J* 12)], in agreement with previous observations.[8] The occurrence of epimerisation in the glutamate moiety was shown to be negligible by independent experiments using diethyl *N*-(*p*-aminobenzoyl)-L-glutamate as a substrate under the reaction conditions employed during the preparation of compound **4**.

As expected, the thia analogue **4** was quite stable even in solution under aerobic conditions; *e.g.*, no change of this compound was observed after stirring of its ethanolic solution for 1 month.

Although the present synthesis of the tetrahydro-5-deaza-5-thiafolic acid **4** was not optimised, this work provides the synthetic methodology for a new deaza analogue of tetrahydrofolic acid **1**. Compound **4** was found to be very cytotoxic to human epidermoid carcinoma KB cells (IC_{50} 0.63 μg cm^{-3}) and human non-small cell lung carcinoma A 549 (IC_{50} 1.19 μg cm^{-3}). An enzymic study of the thia analogue **4** and a synthesis of its derivatives are now in progress.

Experimental

M.p.s (uncorrected) were determined on a Yanagimoto micro-hot stage apparatus. Elemental analysis was carried out in the Microanalytical Center of our university. Spectroscopic measurements were performed with the following instruments:

IR spectra with Perkin-Elmer 1640 FT-IR spectrometer; UV absorption spectra with Shimadzu-260 spectrophotometer; ^{1}H NMR spectra with a JEOL JNM-GX 270 (270 MHz) and a JNM-EX 400 (400 MHz) FT-NMR spectrometer using tetramethylsilane as internal standard (*J*-values are given in Hz); mass spectra with JEOL JMS-D 300 and Hitachi M-80B machines. Analytical TLC was performed on commercially available plates coated with silica gel 60-F_{254} (Merck Art. 5715, 0.25 mm thick). Column chromatography was carried out by using silica gel (Wakogel C-300). Commercially available diethyl *N*-(*p*-aminobenzoyl)-L-glutamate (Aldrich, 99% purity) was used without further purification.

Ethyl 2,2-*Dimethylthiazolidine*-5-*carboxylate* **7**.—A solution of (±)-isocysteine (4.72 g, 38.9 mmol), prepared from β-alanine according to the modified Schöberl's procedure,[9] in absolute ethanol (500 cm^3) containing an excess of dry hydrogen chloride was refluxed for 8 h. After removal of the solvent under reduced pressure, the corresponding ethyl ester was obtained as the hydrochloride salt. The salt was treated in refluxing acetone (300 cm^3) for 3 h. After cooling, the precipitated crystalline mass was collected, washed well with acetone, and was then dissolved in water (200 cm^3). The aqueous solution was adjusted to pH 7 with sodium hydrogen carbonate and was extracted with chloroform (200 cm^3 × 3). The combined organic layers were dried over anhydrous magnesium sulfate and evaporated to dryness. The resulting residue was purified by column chromatography, and elution with benzene–ethyl acetate (20:1), to give the *thiazolidine* **7** (3.99 g, 54%) as an oil, R_f 0.30 [benzene–ethyl acetate (5:1)]; HR-MS *m/z* 189.0817 ($C_8H_{15}NO_2S$ requires M, 189.0823)]; EI-MS *m/z* 189 (M^+, 32%), 174 (M^+ − Me, 8), 116 (M^+ − CO_2Et, 42) and 70 (100); ν_{max}(film)/cm^{-1} 3320 (NH) and 1727 (C=O); δ_H(270 MHz; $CDCl_3$) 1.27 (3 H, t, OCH_2Me), 1.55 and 1.69 (each 3 H, each s, 2-Me_2), 2.51 (1 H, br, NH), 3.25 (1 H, dd, *J* 6 and 13, 4-H), 3.72 (1 H, dd, *J* 2 and 13, 4-H), 4.04 (1 H, dd, *J* 2 and 6, 5-H) and 4.16 (2 H, q, OCH_2Me).

Ethyl 3-*Benzyloxycarbonyl*-2,2-*dimethylthiazolidine*-5-*carboxylate* **8**.—To an ice-cooled, stirred solution of the thiazolidine **7** (3.99 g, 21.1 mmol) and sodium hydrogen carbonate (3.54 g, 42.1 mmol) in 60% aq. ethanol (150 cm^3) was added dropwise benzyl chloroformate (3.9 cm^3, 27.4 mmol) and the mixture was stirred at room temperature overnight.[11] The reaction mixture was extracted with chloroform (100 cm^3 × 3). After evaporation of the combined extract, the resulting residue was subjected to column chromatography and eluted with hexane–diethyl ether (10:1) to isolate the N-*protected thiazolidine* **8** (5.39 g, 79%) as an oil, R_f 0.31 [hexane–ethyl acetate (4:1)]; HR-MS *m/z* 323.1173 ($C_{16}H_{21}NO_4S$ requires M, 323.1191); EI-MS *m/z* 323 (M^+, 2%), 308 (M^+ − Me, 7), 188 (M^+ − CO_2CH_2Ph, 9) and 91 (100); ν_{max}(film)/cm^{-1} 1737 (C=O) and 1705 (C=O); δ_H(270 MHz; $CDCl_3$) 1.27 (3 H, t, OCH_2Me), 1.77 and 1.82 (each 3 H, each s, 2-Me_2), 3.94 (1 H, dd, *J* 5 and 6, 5-H), 4.04 (1 H, dd, *J* 6 and 11, 4-H), 4.20 (2 H, q, OCH_2Me), 4.33 (1 H, dd, *J* 5 and 11, 4-H) 5.14 (2 H, s, Ph CH_2) and 7.35 (5 H, m, Ph).

Benzyl 5-*Formyl*-2,2-*dimethylthiazolidine*-3-*carboxylate* **9**.—To a stirred solution of the ethyl ester **8** (10.88 g, 33.7 mmol) in dry toluene (200 cm^3) at −78 °C was added dropwise a 1 mol dm^{-3} toluene solution of DIBAL (50 cm^3, 50 mmol) for 1 h under argon and the mixture was then stirred for an additional 3 h at −78 °C. The reaction was quenched by slow addition of cold methanol (10 cm^3) so as to keep the internal temperature below −65 °C. The resulting white emulsion was slowly poured into cold, vigorously stirred, 1 mol dm^{-3} hydrochloric acid (200 cm^3) and then the mixture was extracted with ethyl acetate (100 cm^3 × 3). The extract was washed with cold water, dried over anhydrous magnesium sulfate, and then evaporated to dryness. The residue was subjected to column chromatography [hexane–ethyl acetate (10:1)] to isolate the *aldehyde* **9** (6.59 g, 70%), R_f 0.14 [hexane–ethyl acetate (4:1)]; HR-MS *m/z* 279.0902 ($C_{14}H_{17}NO_3S$ requires M, 279.0928); EI-MS *m/z* 279 (M^+, 2%), 264 (M^+ − Me, 2), 250 (M^+ − CHO, 1), 144 (M^+ − CO_2CH_2Ph, 4) and 91 (100); ν_{max}(film)/cm^{-1} 1730 (C=O); δ_H(270 MHz; $CDCl_3$) 1.74 and 1.76 (each 3 H, each br s, 2-Me_2), 3.70 (1 H, dt *J* 2 and 6, 5-H), 3.87 (1 H, dd, *J* 6 and 11, 4-H), 4.58 (1 H, dd, *J* 2 and 11, 4-H), 5.12 (2 H, s, PhCH_2), 7.2–7.3 (5 H, m, Ph) and 9.49 (1 H, d, *J* 2, CHO).

Diethyl N-(p-{[(3-*Benzyloxycarbonyl*-2,2-*dimethylthiazolidin*-5-*yl*)*methyl*]*amino*}*benzoyl*)*glutamate* **10**.—A solution of the aldehyde **9** (1.00 g, 3.6 mmol) and diethyl *N*-(*p*-aminobenzoyl)-L-glutamate (1.27 g, 3.9 mmol) in dry ethanol (50 cm^3) containing acetic acid (0.05 cm^3) was stirred at room temperature for 12 h in the presence of molecular sieves 3 Å (3 g). After complete disappearance of the aldehyde **9** (monitored by TLC; 12 h), sodium cyanoborohydride (0.23 g, 3.6 mmol) was added gradually to the stirred reaction mixture and the mixture was stirred for an additional 6 h. After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography [benzene–ethyl acetate (10:1)] to isolate the thiazolidine-glutamate **10** (1.20 g, 57%) as an oil, R_f 0.35 [benzene–ethyl acetate (3:1)]; EI-MS *m/z* 585 (M^+, 13%), 383 (M^+ − glutamate, 14), 335 (38) and 132 (100); ν_{max}(film)/cm^{-1} 3360 (NH), 1733 (C=O) and 1640 (C=O); δ_H(400 MHz; $CDCl_3$) 1.21 and 1.29 (each 3 H, each t, OCH_2Me), 1.76 and 1.86 (each 3 H, each br s, 2-Me_2), 2.0–2.5 (4 H, m, β- and γ-methylene protons of glutamate), 3.29 (1 H, dd, *J* 7 and 13, 5-CH_2NH), 3.45 (1 H, dd, *J* 6 and 13, 5-CH_2NH), 3.61 (1 H, m, 5-H), 3.90 (1 H, br dd, 4-H), 4.03 (1 H, dd, *J* 6 and 11, 4-H), 4.09 and 4.22 (each 2 H, each q, OCH_2Me), 4.44 (1 H, br, 5-CH_2N*H*), 4.74 (1 H, m, α-H of glutamate), 5.13 (2 H, br s, Ph-CH_2), 6.57 and 7.67 (each 2 H, each d, *J* 9, ArH), 6.83 (1 H, d, *J* 7, CONH), and 7.4–7.6 (5 H, m, Ph) and *benzyl* 5-*hydroxymethyl*-2,2-*dimethylthiazolidine*-N-*carboxylate* (0.30 g, 30%) as an oil, HR-MS *m/z* 281.1081 ($C_{14}H_{19}NO_3S$ requires M, 281.1084); EI-MS *m/z* 281 (M^+, 1%), 266 (M^+ − Me, 11) and 91 (100); δ_H(400 MHz; $CDCl_3$) 1.75 and 1.83 (each 3 H, each s, 2-Me_2), 2.37 (1 H, br, OH), 3.50 (1 H, m, 5-H), 3.64 (1 H, dd, *J* 6 and 11, 4-H), 3.72 (1 H, dd, *J* 7 and 11, 4-H), 3.94 (2 H, d, *J* 4, 5-CH_2OH), 5.11 (2 H, s, CO_2CH_2Ph) and 7.35 (5 H, br s, Ph).

Diethyl N-{p-[(3-*Amino*-2-*mercaptopropyl*)*amino*]*benzoyl*}-*glutamate* **5b**.—The thiazolidine-glutamate **10** (1.18 g, 2.0 mmol) was dissolved in acetic acid (16 cm^3) containing 25% hydrogen bromide and the solution was kept at room temperature for 1 h. After addition of dry diethyl ether (100 cm^3) to the reaction mixture, the supernatant liquid was decanted and the resulting oily solid was triturated with diethyl ether, filtered off, and washed well with diethyl ether to give the corresponding N-*deprotected thiazolidine* (0.72 g, 68%) as an oily hydrobromide, R_f 0.46 [$CHCl_3$–MeOH (10:1)]; HR-MS *m/z* 451.2110 ($C_{22}H_{33}N_3O_5S$ requires M, 451.2140); EI-MS *m/z* 451 (M^+, 5%), 418 (M^+ − 33, 7), 406 (M^+ − OEt, 3), 361 (78) and 58 (100); ν_{max}(film)/cm^{-1} 3420 (NH), 1734 (C=O) and 1651 (C=O); δ_H(400 MHz; $CDCl_3$) 1.22 and 1.30 (each 3 H, each t, OCH_2Me), 1.59 and 1.66 (each 3 H, each br s, 2-Me_2), 1.89 (1 H, br, NH), 2.0–2.5 (4 H, m, β- and γ-methylene protons of glutamate), 3.24 (1 H, dd, *J* 4 and 13, 4-H), 3.30 (2 H, m, 5-CH_2NH), 3.45 (1 H, dd, *J* 6 and 13, 4-H), 3.91 (1 H, m, 5-H), 4.10 and 4.23 (each 2 H, each q, OCH_2Me), 4.38 (1 H, br, NH), 4.78 (1 H, m, α-H of glutamate), 6.60 and 7.66 (each 2 H, each d, *J* 9, ArH) and 6.80 (1 H, d, *J* 7, CONH).

A solution of the above *N*-deprotected thiazolidine (1.56 g, 3.4 mmol) in ethanol (60 cm^3) containing 0.5 mol dm^3 hydrochloric acid (5 cm^3) was heated at 60 °C until the disappearance of the thiazolidine was complete (monitored by TLC; *ca.* 2 h). TLC analysis of the reaction mixture showed the occurrence of a clean reaction to give the β-mercaptoethylamine **5b** as the sole product. After removal of the solvent under reduced pressure, the resulting residue was used in the next reaction without purification because of its high susceptibility to autoxidation. The structure of the β-mercaptoethylamine **5b** was assigned by its microanalytical and spectral data: R_f 0.2 [$CHCl_3$–MeOH–AcOH (40:8:1)]; EI-MS *m/z* 411 (M^+, 4%), 374 (2), 336 (4), 322 (5), 132 (59) and 120 (100); ν_{max}(film)/cm^{-1} 3420 (NH), 1735 (C=O) and 1637 (C=O); δ_H[400 MHz; $(CD_3)_2SO$] 1.19 and 1.20 (each 3 H, each t, OCH_2Me), 2.0–2.1 (2 H, m, β-methylene protons of glutamate), 2.44 (2 H, br t, *J* 7, γ-methylene protons of glutamate), 2.90 (1 H, m, C*H*SH), 3.03 (1 H, d, *J* 8, SH), 3.23 (2 H, m, CH_2NH_2), 3.33 (1 H, dd, *J* 6 and 14, $CHCH_2NH$), 3.41 (1 H, dd, *J* 7 and 14, $CHCH_2NH$), 4.07 and 4.11 (each 2 H, each q, OCH_2Me), 4.40 (1 H, m, α-H of glutamate), 6.66 and 7.71 (each 2 H, each d, *J* 8, ArH), 7.93 (2 H, br, NH_2) and 8.29 (1 H, d, *J* 8, CONH).

Diethyl N-(p-{[(2-*Amino*-6,7-*dihydro*-4-*oxo*-3H,8H-*pyrimido*[5,4-b][1,4]*thiazin*-6-*yl*)*methyl*]*amino*}*benzoyl*)*glutamate* **3**.—To a solution of the β-mercaptoethylamine **5b** (3.4 mmol) in 0.5 mol dm^{-3} pH 7 phosphate buffer (120 cm^3) containing ethanol (60 cm^3) was added 5-bromo-6-chloroisocytosine **6** (136 mg, 0.61 mmol) and the mixture was heated at 80 °C under argon for 8 h. After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography and elution with chloroform–methanol–acetic acid (400:20:1) to give the 5-deaza-5-thiafolate **3** (142 mg, 45%) as an amorphous powder, R_f 0.35 [$CHCl_3$–MeOH (5:1)]; SI-MS *m/z* 519 ($[M + H]^+$ 37%); ν_{max}(KBr)/cm^{-1} 3341 (NH), 1736 (C=O) and 1640 (C=O); λ_{max}(MeOH)/nm 298 and 218; δ_H[400 MHz; $(CD_3SO)_2$] 1.15 and 1.17 (each 3 H, each t, OCH_2Me), 1.9–2.1 (2 H, m, β-methylene protons of glutamate), 2.40 (2 H, br t, *J* 7, γ-methylene protons of glutamate), 3.17 (2 H, m, 6-CH_2NH), 3.23 (1 H, m, 6-H), 3.34 (1 H, dd, *J* 8 and 12, 7-H), 3.54 (1 H, d, *J* 12, 7-H), 4.03 and 4.07 (each 2 H, each q, OCH_2Me), 4.38 (1 H, m, α-H of glutamate), 6.05 (2 H, br, NH_2), 6.48 (1 H, br t, $N^{10}H$), 6.60 and 7.66 (each 2 H, each d, *J* 7, ArH), 6.66 (1 H, br, N^8H), 8.24 (1 H, br d, *J* 7, CONH) and 10.30 (1 H, br, N^3H) and small amounts of undetermined products, accompanied by the starting material **6** and the disulfide (oxidative dimer) of the β-mercaptoethylamine **5b**.

N-(p-{[(2-*Amino*-6,7-*dihydro*-4-*oxo*-3H,8H-*pyrimido*[5,4-b]-[1,4]*thiazin*-6-*yl*)*methyl*]*amino*}*benzoyl*)*glutamic Acid* **4**.—A solution of the diethyl ester **3** (100 mg, 0.19 mmol) in 50% aq. ethanol (20 cm^3) containing sodium hydroxide (30 mg, 0.75 mmol) was stirred at room temperature until the disappearance of the diethyl ester **3** was complete (monitored by TLC; 1 h). After acidification of the mixture with 1 mol dm^{-3} acetic acid, the resulting precipitate was collected, and washed with methanol to give the tetrahydro-5-deaza-5-thiafolic acid **4** (67 mg, 76%) as an analytically pure product, m.p. slowly decomposes above 190 °C (Found: C, 47.2; H, 5.25; N, 16.3. $C_{19}H_{22}N_6O_6S \cdot 2/3CH_3CO_2H \cdot 4/5H_2O$ requires: C, 47.24; H, 5.12; N, 16.26%); R_f 0.30 [$CHCl_3$–MeOH–AcOH (16:6:3)]; SI-MS *m/z* 463 ($[M + H]^+$, 13%); ν_{max}(KBr)/cm^{-1} 1704 (C=O) and 1640 (C=O); λ_{max}(MeOH)/nm 300 and 231; δ_H[400 MHz; $(CD_3)_2SO$] 2.0–2.1 (2 H, m, β-methylene protons of glutamate), 2.35 (2 H, br t, *J* 7, γ-methylene protons of glutamate), 3.2–3.3 (3 H, m, 6-H and 6-CH_2NH), 3.40 (1 H, dd, *J* 5 and 13, 7-H), 3.60 (1 H, br d, *J* 13, 7-H), 4.36 (1 H, m, α-H of glutamate), 6.07 (2 H, br, NH_2), 6.49 (1 H, br, $N^{10}H$), 6.64 and 7.70 (each 2 H, each d, *J* 8, ArH), 6.68 (1 H, br, NH), 8.14 (1 H, br d, *J* 7, CONH), 10.13 (1 H, br, N^3H) and 12.30 (2 H, br, 2 × CO_2H).

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