First Synthesis of 5,6,7,8-Tetrahydro-8-deaza-8-thiafolic Acid

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5,6,7,8-Tetrahydro-8-deaza-8-thiafolic acid (3) was synthesized as a diastereoisomeric mixture *via* thermal condensation of 5-hydroxyisocytosine (6) with diethyl *N*-[*p*-(2-amino-3-mercaptopropyl)aminobenzoyl]glutamate (10) after activation of the C(6)-position in 6 with *N*-bromosuccinimide/ethanol. The corresponding N^5,N^{10}-methylene derivative (5) was also prepared upon treatment of 3 with formaldehyde.

Keywords 5,6,7,8-tetrahydro-8-deaza-8-thiafolic acid; 5-hydroxyisocytosine; *N*-[*p*-(2-amino-3-mercaptopropyl)aminobenzoyl]glutamate; thermal condensation; formaldehyde; N^5,N^{10}-methylene-5,6,7,8-tetrahydro-8-deaza-8-thiafolic acid

Tetrahydrofolic acid (**1**) is an active form of folic acid and plays significant roles as a cofactor for one-carbon unit transfer in the *de novo* synthesis of nucleosides and in amino acid biosyntheses. The functions of **1** as a biological catalyst have been extensively studied in the fields of chemistry and biochemistry.[1] Although catalytic reduction or chemical reduction of folic acid results in the formation of **1**, its tetrahydropyrazine ring is sensitive to autoxidation, particularly in solution,[2] and so its handling during studies on the functions is somewhat troublesome. Recently, deaza analogues of **1** have been shown to be an important class of folate antimetabolites of interest as potential oncolytic agents.[3] Within this series, 5,10-deaza-5,6,7,8-tetrahydrofolic acid has shown exceptionally high activity in a variety of animal models of malignancy.[4]

In a previous paper,[5] we described a new method for the construction of the reduced pyrimido[4,5-*b*][1,4]thiazine-4(3*H*)-one ring system, providing access to 1,5-dihydro-10-deaza-10-thiaisoalloxazines and 5,6,7,8-tetrahydro-8-deaza-8-thiapterins,[6,7] which are fairly stable even in solution[8] compared with the parent 1,5-dihydroisoalloxazines and 5,6,7,8-tetrahydropterins. In this context, we planned to prepare 5,6,7,8-tetrahydro-8-deaza-8-thiafolic acid (**3**) and its N^5,N^{10}-methylene derivative (**5**) by employing the new methodology as a key step.

The tetrahydrofolate analogue (**3**) is anticipated to be a stable material and is of interest as a possible folate antimetabolite and as a viable model compound of **1**. For the construction of **3** based on the condensation of 5-hydroxyisocytosine (**6**) with β-aminothiols,[5] two synthetic approaches are envisaged. Route a involves the condensation of **6** with cysteine ethyl ester (**7**) leading to 6-ethoxycarbonyl-5,6,7,8-tetrahydro-8-deaza-8-thiapterin (**8**), followed by coupling with diethyl *N*-(*p*-aminobenzoyl)-glutamate (**9**). Route b is the direct condensation of **6** with diethyl *N*-[*p*-(2-amino-3-mercaptopropyl)aminobenzoyl]-glutamate (**10**), which is derived from **7** and **9** (see Chart 1, route a and route b).

As reported previously,[5] bromination of **6** with *N*-bromosuccinimide in ethanol followed by treatment with L-cysteine ethyl ester (**7**) resulted in the formation of **8** (47% yield), the C(6)-position of which was racemized to some extent.

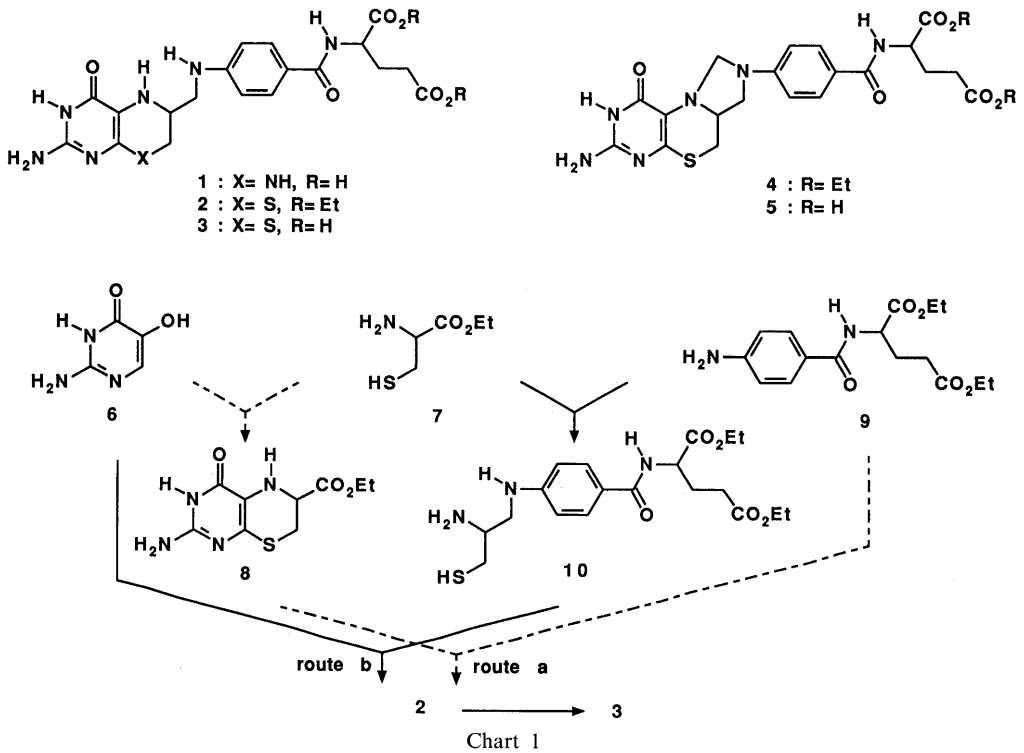

Chart 1

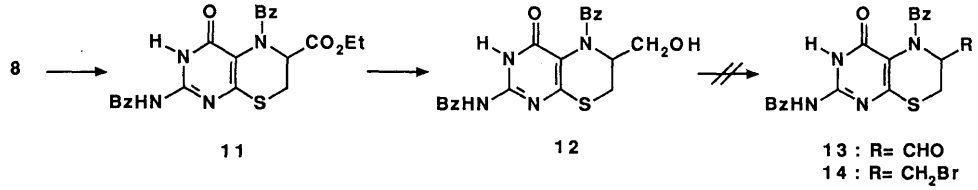

Chart 2

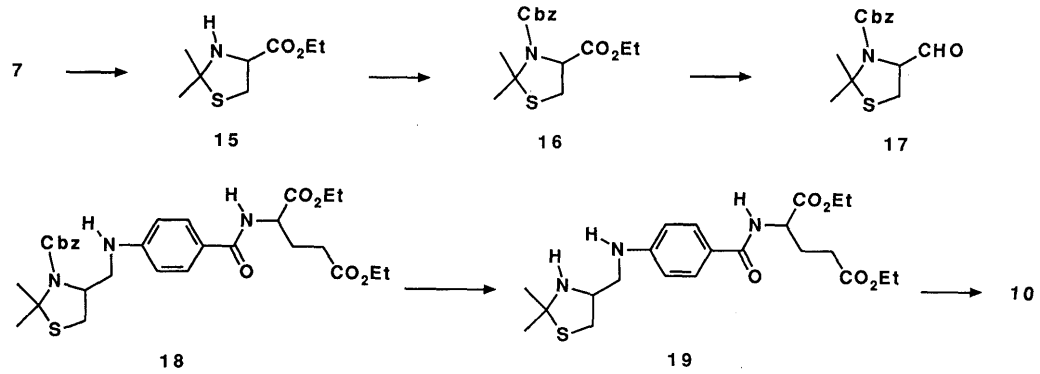

Chart 3

N-Benzoyl protection of **8** gave the N^2,N_5-dibenzoyl derivative (**11**) which was reduced with sodium borohydride to give the 6-hydroxymethyl derivative (**12**) in 63% yield. Attempts to convert **12** into the corresponding aldehyde (**13**) or methylene bromide (**14**), however, were unsuccessful because of the low solubility in the solvent employed or the low stability under the conditions employed. Other attempts to condense **11** or **12** with **9** gave unsatisfactory results. Thus, an alternative route b was investigated in place of route a.

Synthesis of the counterpart **10** for the condensation with **6** in route b was achieved as follows (see Chart 3).

The natural tetrahydrofolic acid (**1**) has been shown to adopt the *S*-configuration at the C(6)-position.[9] Employment of D-cysteine ethyl ester (**7**) as a starting material could provide **3** possessing the same 6*S* configuration as that of **1**. In the present conversion into **10** under the conditions employed, however, the occurrence of racemization in the cysteine moiety seems to be unavoidable. Thus, D,L-cysteine ethyl ester (**7**) was used as a starting material, aiming at synthesis of **3** as a diastereo-isomeric mixture to exclude stereochemical ambiguity. Isopropylidene protection of **7** (D,L-, HCl salt) gave 2,2-dimethyl-4-ethoxycarbonylthiazolidine (**15**) in 74% yield as the HCl salt.[10] Since the free thiazolidine (**15**) obtained by base treatment was unstable, **15** was converted into the *N*-benzyloxycarbonyl (Cbz) derivative (**16**) (87% yield). Reduction of **16** with diisobutyl aluminum hydride (DIBAH) in dry toluene at $-78\,^{\circ}C$ resulted in the formation of the corresponding aldehyde (**17**) in 83% yield. Condensation of **17** with the *N*-(*p*-aminobenzoyl)-L-glutamate (**9**) was carried out in dry ethanol containing glacial acetic acid. Without isolation of the intermediary product, its reduction with sodium cyanoborohydride followed by chromatographic purification gave the condensed product (**18**) in 13% overall yield from **7**. Deprotections of the Cbz group using aluminum chloride–anisole[11] and of the isopropylidene group by heating at $60\,^{\circ}C$ in ethanol containing a catalytic amount of hydrochloric acid led to the formation of **10** in a high yield. The fragmentation pattern and the parent peak [*m/z* 411 (M^+)] in its mass spectrum (MS) fully support the structure of **10**, and its proton nuclear magnetic resonance (^{1}H-NMR) spectrum was also consistent with this structure. The specific optical rotation of **10** was totally lost. Thus, **10** is a mixture of four diastereo-isomers which arise from the racemization of the glutamate moiety during the reactions.

The synthetic counterpart (**10**) thus prepared was sensitive to autoxidation and decomposed gradually in an acidic solution. Accordingly, **10** was subjected to condensation with **6** in a neutral medium without purification. After treatment of **6** with *N*-bromosuccinimide in ethanol, the reaction mixture was neutralized with sodium hydrogen carbonate. Then **10** was added to the neutral solution and the mixture was refluxed under argon. Monitoring of the reaction by TLC revealed that a prolonged reaction time of about 40 h was necessary. The desired product **2** was separated by silica gel chromatography in 14% yield. Its ^{1}H-NMR, ultraviolet (UV), and fast atom bombardment-MS (FAB-MS) spectra supported the structure of **2**. Hydrolysis of **2** was achieved almost quantitatively upon treatment with ethanolic caustic alkali to give 5,6,7,8-tetrahydro-8-deaza-8-thiafolic acid (**3**) as a mixture of diastereo-isomers. The structure of **3** was confirmed by ^{1}H-NMR, UV, infrared (IR), and FAB-MS spectral data (see Experimental).

When a suspension of **2** in a 0.025 M formaldehyde solution was adjusted to pH 5 and then stirred, the corresponding N^5,N^{10}-methylene derivative (**4**) was obtained as a precipitate.[12] The structure of **4** was supported by its spectral data, *e.g.*, a C_{11}-methylene signal (δ 4.05, 5.21, $J=4$ Hz) newly appeared in its ^{1}H-NMR spectrum. Analogously, N^5,N^{10}-methylene-8-deaza-8-thiafolic acid (**5**) was prepared from **3** almost quantitatively.

Although the present synthesis of **3** is not satisfactory in terms of the overall yield and stereoselectivity, it is the first synthesis of **3**, which has intriguing chemical and

biochemical properties. Biological evaluation of **3** as a folate antimetabolite is in progress.

Experimental

All melting points (uncorrected) were determined on a Yanagimoto micro hot-stage apparatus. Elemental analyses were performed by the microanalytical laboratory of our university. Spectroscopic measurements for the structural assignment of the reaction products were performed with the following instruments: IR spectra with a Hitachi Model 215 spectrometer; UV absorption spectra with a Shimadzu 260 spectrophotometer; ^{1}H-NMR spectra with a JEOL JNM-GX 270 (270 MHz) Fourier transform-NMR (FT-NMR) spectrometer using tetramethylsilane as an internal standard; MS and high-resolution mass spectra (HR-MS) with a JEOL JMS-D 300 machine operating at 70 eV; FAB-MS with a JEOL JMS-DX 300 machine. Thin-layer chromatographic (TLC) analyses were carried out on precoated Silicagel 60 F_{254} plates (Merck, Art 5715). Column chromatography was accomplished by using silica gel (Wakogel C-300).

6-Ethoxycarbonyl-5,6,7,8-tetrahydro-8-deaza-8-thiapterin (8) *N*-Bromosuccinimide (2.13 g, 12.0 mmol) was added to a suspension of 5-hydroxyisocytosine (**6**) (HCl salt, 1.64 g, 10.0 mmol) in EtOH (50 ml). After stirring of the mixture at room temperature for 1 h followed by the addition of D,L-cysteine ethyl ester (**7**) (HCl salt, 5.57 g, 30.0 mmol), the reaction mixture was refluxed overnight, then concentrated to a small volume (*ca.* 5 ml) under reduced pressure, and diluted with water (5 ml). The aqueous solution was neutralized with $NaHCO_3$ under ice-cooling. The precipitated crystalline mass was collected and recrystallized from EtOH to give **8** (1.20 g, 47.0%). The structure of **8** was confirmed by spectral comparison with the authentic compound previously reported.[5]

N^2,N_5-Dibenzoyl-6-ethoxycarbonyl-5,6,7,8-tetrahydro-8-deaza-8-thiapterin (11) Benzoyl chloride (1.2 ml, 10.3 mmol) was added dropwise to a solution of **8** (128 mg, 0.5 mmol) in pyridine (2.0 ml, 24.7 mmol) under ice-cooling, and the mixture was stirred at room temperature for 2 h. After removal of the solvent under reduced pressure, the residual oil was dissolved in $CHCl_3$ (30 ml). The $CHCl_3$ solution was washed well with dilute HCl, dried over anhydrous $MgSO_4$, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography with $CHCl_3$–Me_2CO (30 : 1) to give **11** (141 mg, 61%) as an oil. Rf=0.6 ($CHCl_3$: Me_2CO = 10 : 1). HR-MS Calcd for $C_{23}H_{20}N_4O_5S$: 464.1181. Found: 464.1206 (M^+). MS *m/z*: 464 (M^+, 2%), 391 ($M^+ - CO_2Et$, 2%), 360 (2%), 287 (9%), 105 (100%). IR ν_{max}^{KBr}: 3200 (NH), 1740 (C=O), 1640 (C=O) cm^{-1}. UV λ_{max}^{EtOH}: 331, 244 nm. ^{1}H-NMR (CD_3OD) δ: 1.38 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 3.35—3.55 (2H, m, C_7-H×2), 4.32 (2H, q, J=7, 14 Hz, $OC\underline{H}_2CH_3$), 4.60 (1H, dd, J=3.9, 4.4 Hz, C_6-H), 7.63—7.73 (6H, m, aromatic H), 8.05 (4H, br d, J=8 Hz, aromatic H).

N^2,N_5-Dibenzoyl-6-hydroxymethyl-5,6,7,8-tetrahydro-8-deaza-8-thiapterin (12) MeOH (0.1 ml) was added dropwise to a suspension of **11** (140 mg, 0.3 mmol) and $NaBH_4$ (46 mg, 1.2 mmol) in dry tetrahydrofuran (THF) (20 ml) at 5 °C, and the mixture was stirred at room temperature until **11** was no longer detectable (monitored by TLC; *ca.* 2 h). After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography and eluted with $CHCl_3$–MeOH (10 : 1) to isolate **12** (80 mg, 63%). mp 251—252 °C (MeOH). Rf=0.27 ($CHCl_3$: Me_2CO = 10 : 1). *Anal.* Calcd for $C_{21}H_{18}N_4O_4S$: C, 59.71; H, 4.30; N, 13.26. Found: C, 59.42; H, 4.38; N, 13.35. MS *m/z*: 422 (M^+, 12%), 300 (7%), 287 (24%), 105 (100%). IR ν_{max}^{KBr}: 3350 (OH), 3150 (NH), 1690 (C=O), 1670 (C=O) cm^{-1}. UV λ_{max}^{EtOH}: 330, 259 nm. ^{1}H-NMR (DMSO-d_6) δ: 3.20—3.70 (5H, m, C_7-H×2, C_6-$C\underline{H}_2$OH, C_6-H), 5.15 (1H, br s, OH), 7.47 (1H, br, NH), 7.5—8.1 (10H, m, aromatic H), 11.93 (1H, br, NH).

2,2-Dimethyl-4-ethoxycarbonylthiazolidine (15) A solution of cysteine ethyl ester (D,L-**7**) (HCl salt, 11.1 g, 60.0 mmol) in acetone (300 ml) was refluxed for 3 h. After cooling, the precipitated crystalline mass was collected and dissolved in water (20 ml). The aqueous solution was neutralized with $NaHCO_3$ and extracted with $CHCl_3$ (50 ml×3). The collected extract was dried over anhydrous $MgSO_4$ and evaporated to dryness. The resulting residue was purified by column chromatography with $CHCl_3$ to isolate **15** (8.39 g, 74%) as an oil. Rf=0.38 (benzene : EtOAc = 5 : 1). bp 75 °C (4.0 mmHg). *Anal.* Calcd for $C_8H_{15}NO_2S \cdot 1/4H_2O$: C, 49.60; H, 8.07; N, 7.23. Found: C, 49.67; H, 7.95; N, 7.28. MS *m/z*: 189 (M^+, 51%), 174 ($M^+ - Me$, 22%), 142 (28%), 116 ($M^+ - CO_2Et$, 81%), 99 (58%). IR ν_{max}^{KBr}: 3300 (NH), 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 1.53, 1.72 (each 3H, each s, C_2-Me×2), 2.49 (1H, br, NH), 3.03 (1H, q, J=9, 11 Hz, C_5-H), 3.44 (1H, q, J=7, 11 Hz, C_5-H), 4.08 (1H, q, J=7, 9 Hz, C_4-H), 4.25 (2H, q, J=7, 14 Hz, $OC\underline{H}_2CH_3$).

3-Benzyloxycarbonyl-2,2-dimethyl-4-ethoxycarbonylthiazolidine (16) Benzyloxycarbonyl chloride (9.08 ml, 63.6 mmol) was added to a solution of **15** (10.977 g, 58.1 mmol) and $NaHCO_3$ (9.7 g, 115 mmol) in EtOH (120 ml) and water (120 ml) under ice-cooling. The mixture was stirred at room temperature overnight, then extracted with $CHCl_3$ (100 ml×3), and the combined $CHCl_3$ solution was dried over anhydrous $MgSO_4$. After removal of the solvent under reduced pressure, the resulting residue was purified by column chromatography with *n*-hexane–Et_2O (5 : 1) to give **16** (16.3 g, 87%) as an oil. Rf=0.73 (benzene : EtOAc = 5 : 1). HR-MS Calcd for $C_{16}H_{21}NO_4S$: 323.1187. Found: 323.1182. MS *m/z*: 323 (M^+, 2%), 308 ($M^+ - Me$, 11%), 264 (4%), 250 (4%), 91 (100%). IR ν_{max}^{neat}: 1750 (C=O), 1700 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.17 (0.63×3H), 1.27 (0.37×3H) (each t, J=each 7 Hz, $OCH_2C\underline{H}_3$), 1.74 (0.37×3H), 1.81 (0.37×3H), 1.83 (0.63×3H), 1.91 (0.63×3H) (each s, C_2-Me×2), 3.15 (1H, br dd, J=2, 12 Hz, C_5-H), 3.29 (1H, dd, J=7, 12 Hz, C_5-H), 4.0—4.2 (2H, m, $OC\underline{H}_2CH_3$), 4.8—5.2 (3H, m, C_4-H, benzyl protons), 7.3—7.4 (5H, m, aromatic H).

3-Benzyloxycarbonyl-2,2-dimethyl-4-formylthiazolidine (17) A 1.0 M solution of DIBAH in toluene (43 ml, 43 mmol) was added dropwise to a stirred solution of **16** (8.193 g, 25.4 mmol) in dry toluene (20 ml) at −78 °C over 1 h, and the mixture was stirred for an additional 3 h at −78 °C under an argon atmosphere. The reaction was quenched by slowly adding cold MeOH so as to keep the internal temperature below −65 °C. The resulting white emulsion was slowly poured into a cold 1 N HCl solution and then the aqueous mixture was extracted with EtOAc (100 ml×3). The collected EtOAc solution was dried over $MgSO_4$ and evaporated to dryness. The residue was applied to a silica gel column and eluted with *n*-hexane–EtOAc (10 : 1) to give **17** (5.91 g, 83%) as an oil. Rf=0.26 (*n*-hexane : Et_2O = 2 : 1). MS *m/z*: 264 ($M^+ - Me$, 2%), 250 ($M^+ - CHO$, 11%), 206 (7%), 91 (100%). IR ν_{max}^{film}: 1740 (C=O), 1710 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.79 (0.4×3H), 1.81 (0.4×3H), 1.84 (0.6×3H), 1.90 (0.6×3H) (each br s, C_2-Me×2), 3.07—3.15 (2H, m, C_5-H×2), 4.70 (0.6H), 4.80 (0.4H) (each br s, C_4-H), 5.11 (0.6×2H), 5.21 (0.4×2H) (each s, benzyl protons), 7.2—7.3 (5H, m, aromatic H), 9.51 (1H, s, CHO).

Diethyl *N*-[*p*-(3-Benzyloxycarbonyl-2,2-dimethylthiazolidin-4-yl)methylaminobenzoyl]glutamate (18) A solution of **17** (9.0 g, 32.2 mmol) and diethyl *N*-(*p*-aminobenzoyl)glutamate (10.4 g, 32.3 mmol) in dry EtOH (100 ml)–AcOH (0.5 ml) was stirred at room temperature until **17** was no longer detectable (monitored by TLC; *ca.* 12 h). Then, $NaBH_3CN$ (2.03 g, 32.3 mmol) was added to the reaction mixture and the solution was stirred for 6 h at room temperature. After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography. Elution with benzene–EtOAc (20 : 1) provided **18** (4.54 g, 24%) as an oil. Rf=0.31 (benzene : EtOAc = 5 : 1). MS *m/z*: 585 (M^+, 5%), 539 (1%), 383 (M^+ − glutamate, 11%), 336 (7%), 204 (18%), 148 (44%), 132 (40%), 91 (100%). ^{1}H-NMR ($CDCl_3$) δ: 1.21, 1.30 (each 3H, each t, J=7 Hz, $OCH_2C\underline{H}_3$), 1.81 (6H, br s, C_2-Me×2), 2.1—2.6 (4H, m, β- and γ-methylene protons of glutamate), 2.81 (1H, br d, J=12 Hz, C_5-H), 3.17 (1H, m, C_5-H), 3.3—3.6 (2H, m, C_4-$C\underline{H}_2$NH), 4.10, 4.23 (each 2H, each q, J=each 7, 14 Hz, $OC\underline{H}_2CH_3$×2), 4.53 (1H, br, C_4-H), 4.79 (1H, q, J=8, 12 Hz, C_α-H of glutamate), 5.18 (2H, br s, benzyl protons), 6.45, 6.67 (each 1H, each br, NH×2), 6.6, 7.7 (each 2H, each br, aromatic H), 7.4—7.6 (5H, m, aromatic H).

Diethyl *N*-[*p*-(2,2-Dimethylthiazolidin-4-yl)methylaminobenzoyl]glutamate (19) A suspension of **18** (7.846 g, 13.4 mmol) and $AlCl_3$ (5.3 g, 40 mmol) in anisole (300 ml) was stirred at room temperature overnight. The reaction was quenched by the addition of water and the aqueous mixture was extracted with EtOAc (100 ml×3). The collected EtOAc solution was dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was subjected to column chromatography and elution with $CHCl_3$–Me_2CO (10 : 1) provided **19** (4.54 g, 75%) as an oil. Rf=0.17 ($CHCl_3$: Me_2CO = 5 : 1). HR-MS Calcd for $C_{22}H_{33}N_3O_5S$: 451.2141. Found: 451.2165. MS *m/z*: 451 (M^+, 1%), 436 ($M^+ - Me$, 1%), 361 (10%), 336 (38%), 249 (40%), 204 (100%). ^{1}H-NMR ($CDCl_3$) δ: 1.21, 1.29 (each 3H, each t, J=each 7 Hz, $OCH_2C\underline{H}_3$×2), 1.57, 1.67 (each 3H, each s, C_2-Me×2), 2.0—2.6 (4H, m, β- and γ-methylene protons of glutamate), 2.79 (1H, t, J=10 Hz, NH), 3.2—3.3 (2H, m, C_5-H), 3.4—3.6, 3.65—3.85 (each 1H, each m, C_4-$C\underline{H}_2$NH), 4.09, 4.21 (each 2H, each q, J=each 7, 14 Hz, $OC\underline{H}_2CH_3$×2), 4.0—4.3 (1H, m, C_4-H), 4.54 (1H, br, N_3H), 4.77 (1H, m, C_α-H of glutamate), 6.60, 7.68 (each 2H, each d, J=8 Hz, aromatic H), 6.83 (1H, d, J=3 Hz, CONH).

Diethyl *N*-[*p*-(2-Amino-3-mercaptopropyl)aminobenzoyl]glutamate (10) A solution of **19** (3.0 g, 6.65 mmol) in EtOH (70 ml) containing 1 N HCl (6.6 ml) was heated at 60 °C until **19** was no longer detectable (monitored by TLC). After removal of the solvent under reduced pressure, the resulting residue was employed without purification for condensation with **6**. MS *m/z*: 411 (M^+, 4%), 374 (2%), 336 (4%), 322 (5%), 132 (59%), 120 (100%). ^{1}H-NMR (DMSO-d_6) δ: 1.19, 1.20 (each 3H, each t, J=each 7 Hz, $OCH_2C\underline{H}_3 \times 2$), 1.9—2.2 (2H, m, β-methylene protons of glutamate), 2.3—2.6 (2H, m, γ-methylene protons of glutamate), 2.85 (2H, br, $C\underline{H}_2$-SH), 3.04 (1H, t, J=8 Hz, SH), 3.3—3.6 (3H, m, $C\underline{H}C\underline{H}_2NH$), 4.07, 4.11 (each 2H, each q, J=each 7, 14 Hz, $OC\underline{H}_2CH_3 \times 2$), 4.39 (1H, m, C_α-H of glutamate), 6.57 (1H, br, NH), 6.69, 7.69 (each 2H, each d, J=8 Hz, aromatic H), 8.30 (2H, m, NH_2).

Diethyl 5,6,7,8-Tetrahydro-8-deaza-8-thiafolate (2) *N*-Bromosuccinimide (1.43 g, 7.98 mmol) was added to a suspension of **6** (HCl salt, 1.09 g, 6.65 mmol) in EtOH (40 ml), and the mixture was stirred at room temperature for 1.0 h. After the addition of **10** prepared from **19** (6.65 mmol), the mixture was neutralized with $NaHCO_3$ and refluxed under an argon atmosphere for 40 h. After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography. Elution with $CHCl_3$–MeOH (20:1) provided **2** (487 mg, 14%) as an amorphous powder. Rf=0.45 ($CHCl_3$:MeOH=5:1). FAB-MS *m/z*: 519 $[M+H]^+$. IR ν_{max}^{KBr}: 3350 (NH), 1730 (C=O), 1650 (C=O) cm^{-1}. UV λ_{max}^{MeOH}: 298, 218 nm. ^{1}H-NMR (DMSO-d_6) δ: 1.18, 1.21 (each 3H, each t, J=each 7 Hz, $OCH_2C\underline{H}_3 \times 2$), 2.04—2.09 (2H, m, β-methylene protons of glutamate), 2.43 (2H, t, J=7 Hz, γ-methylene protons of glutamate), 3.00 (1H, dd, J=6, 12 Hz, C_7-H), 3.10 (1H, br d, J=12 Hz, C_7-H), 3.26 (2H, br d, J=6 Hz, C_9-H $\times$ 2), 3.51 (1H, br, C_6-H), 4.07, 4.11 (each 2H, each q, J=each 7, 14 Hz, $OC\underline{H}_2CH_3 \times 2$), 4.41 (1H, m, C_α-H of glutamate), 4.63 (1H, br s, N_5-H), 5.86 (2H, br, NH_2), 6.56 (1H, br t, N_{10}H), 6.67, 7.69 (each 2H, each d, J=8 Hz, aromatic H), 8.28 (1H, br d, J=7 Hz, CONH), 10.87 (1H, br s, N_3H).

5,6,7,8-Tetrahydro-8-deaza-8-thiafolic Acid (3) A solution of **2** (163 mg, 0.31 mmol) in EtOH (24 ml) containing NaOH (48 mg, 1.2 mmol) was stirred at room temperature until **2** was no longer detectable (monitored by TLC; 4 h). After neutralization of the mixture with dilute HCl, the resulting precipitate was collected and washed with MeOH to give **3** (97.6 mg, 68%) as an amorphous powder. Rf=0.42 ($CHCl_3$:MeOH:AcOH=40:8:1). FAB-MS *m/z*: 463 $[M+H]^+$. IR ν_{max}^{KBr}: 3300 (NH), 1700 (C=O), 1680 (C=O) cm^{-1}. UV λ_{max}^{MeOH}: 300, 231 nm. ^{1}H-NMR (DMSO-d_6) δ: 1.94—2.09 (2H, m, β-methylene protons of glutamate), 2.35 (2H, t, J=7 Hz, γ-methylene protons of glutamate), 2.97 (1H, dd, J=7, 12 Hz, C_7-H), 3.11 (1H, br d, J=12 Hz, C_7-H), 3.24 (2H, br d, J=5 Hz, C_9-H $\times$ 2), 3.48 (1H, m, C_6-H), 4.36 (1H, m, C_α-H of glutamate), 4.63 (1H, br s, N_5-H), 5.87 (2H, br s, NH_2), 6.54 (1H, br t, N_{10}-H), 6.68, 7.69 (each 2H, each d, J=8 Hz, aromatic H), 8.15 (1H, d, J=8 Hz, CONH), 10.88 (1H, br d, N_3H), 12.27 (3H, br, N^+H_3).

Diethyl N^5,N^{10}-Methylene-5,6,7,8-tetrahydro-8-deaza-8-thiafolate (4) An aqueous solution (1.0 ml) of **2** (19.7 mg, 0.04 mmol) containing formaldehyde (25 mmol) was adjusted to pH 5 with 1 N NaOH solution and then the mixture was stirred at room temperature for 5 h. The resulting precipitate was collected and washed with water to give **4** (19.1 mg, 90%) as an amorphous powder. Rf=0.74 ($CHCl_3$:MeOH:AcOH=40:8:1). UV λ_{max}^{EtOH}: 304, 219 nm. ^{1}H-NMR (CD_3OD) δ: 1.21, 1.26 (each 3H, each t, J=each 7 Hz, $OCH_2C\underline{H}_3 \times 2$), 2.07—2.25 (2H, m, β-methylene protons of glutamate), 2.47 (2H, t, J=7 Hz, γ-methylene protons of glutamate), 2.95 (1H, d, J=11 Hz, C_7-H), 3.08 (1H, dd, J=11, 12 Hz, C_7-H), 3.55 (1H, m, C_9-H), 3.65 (1H, m, C_6-H), 3.85 (1H, q, J=6, 9 Hz, C_9-H), 4.05 (1H, d, J=4 Hz, C_{11}-H), 4.10, 4.19 (each 2H, each q, J=each 7, 14 Hz, $OC\underline{H}_2CH_3 \times 2$), 4.59 (1H, q, J=6, 9 Hz, C_α-H of glutamate), 5.21 (1H, d, J=4 Hz, C_{11}-H), 6.59, 7.77 (each 2H, each d, J=9 Hz, aromatic H).

N^5,N^{10}-Methylene-5,6,7,8-tetrahydro-8-deaza-8-thiafolic Acid (5) An aqueous solution (1.0 ml) of **3** (15 mg, 0.03 mmol) containing formaldehyde (25 mmol) was adjusted to pH 5 with 1 N NaOH solution and then the mixture was stirred at room temperature for 5 h. The resulting precipitate was collected and washed with water to give **5** (14 mg, 91%) as an amorphous powder. FAB-MS *m/z*: 475 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6) δ: 2.06—2.24 (2H, m, β-methylene protons of glutamate), 2.49 (2H, t, J=7 Hz, γ-methylene protons of glutamate), 3.13 (2H, m, C_7-H $\times$ 2), 3.57 (1H, m, C_9-H), 3.75 (1H, br s, C_6-H), 3.93 (1H, br s, C_9-H), 4.14 (1H, m, C_{11}-H), 4.52 (1H, m, C_α-H of glutamate), 5.26 (1H, m, C_{11}-H), 6.39 (1H, br s, NH), 6.68, 7.95 (each 2H, each d, J=8 Hz, aromatic H), 8.42 (1H, d, CONH), 11.00 (1H, s, N_3-H), 12.52 (3H, br, N^+H_3).

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References and Notes

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