SYNTHESIS OF THE γ-SULFINIC ACID AND γ-NITRO ANALOGUES OF 5-DEAZATETRAHYDROFOLIC ACID

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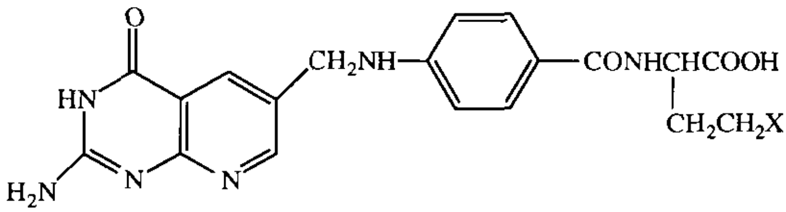
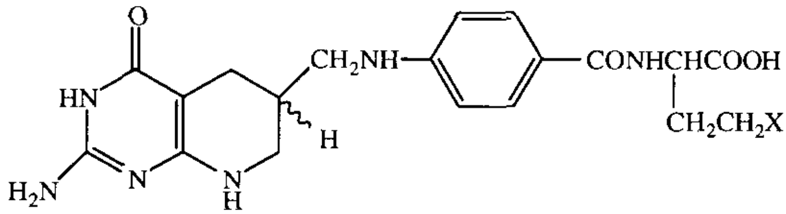
Abstract - Analogues of 5-deaza-5,6,7,8-tetrahydrofolic acid with a γ-sulfinic acid group or γ-nitro group in place of the γ-carboxyl group of the glutamate side chain were synthesized as diastereomeric mixtures, and were tested for their ability to inhibit the growth of CCRF-CEM human leukemia cells in culture. The concentration of the γ-sulfinic acid analogue (**7**) giving 50% inhibition of growth during 120 h of continuous drug treatment was 21 μM versus 93 μM for the γ-nitro analogue (**8**). The K_i of **7** as a competitive inhibitor of the influx of [^{3}H]methotrexate into CCRF-CEM cells via the reduced folate carrier (RFC) was 5.0 μM, a value close to the K_m values typically cited in the literature for MTX and natural reduced folates. Thus, apart from any other mechanistic targets this compound might have, **7** has the potential to deplete endogenous pools of reduced folates in dividing cells by interfering with RFC function.

Analogues of 5-deazafolic acid (**1**) and 5-deazatetrahydrofolic acid (**2**) in which the terminal carboxyl group of the glutamate side chain was replaced by a sulfonic acid group, as in structures (**3**) and (**4**), or a phosphonic acid group, as in structures (**5**) and (**6**), were synthesized by us several years ago as part of a larger effort involving the design and biological evaluation of nonpolyglutamatable antifolates.[1]



1 : X = COOH (L-isomer)
3 : X = SO_2OH (L-isomer)
5 : X = $PO(OH)_2$ (D,L-mixture)



2 : X = COOH (L-isomer)
4 : X = SO_2OH (L-isomer)
6 : X = $PO(OH)_2$ (D,L-mixture)

When tested as mixtures of the 6*R* and 6*S* isomers in cell-free assays using purified enzymes, **4** and **6** were found to be potent inhibitors of the purine biosynthetic enzyme glycinamide ribonucleotide formyltransferase (GARFT), as well as of folylpolyglutamate synthetase (FPGS), a key player in the cellular pharmacology of both antifolates and endogenous reduced folates.[2] The non-reduced compounds (**3**) and (**5**) were considerably weaker inhibitors of both enzymes. Interestingly, the phosphonate (**6**) was a competitive inhibitor of the FPGS reaction, whereas inhibition by the sulfonate (**4**) obeyed noncompetitive kinetics. It was speculated that decreased cellular formation of the long-chain polyglutamates of 10-formyltetrahydrofolate, which are the preferred endogenous substrates for GARFT, might enable these compounds to act as 'self-potentiating' antipurinic drugs.[1] However, despite this dual inhibitory profile, **4** and **6** proved to be virtually noncytotoxic in comparison with the glutamate analogue (**2**). Subsequently it was shown that, while these analogues are actively transported into cells via the reduced folate carrier (RFC), they are less efficiently used in this process than are the natural reduced folates or a number of synthetic antifolates with a glutamate side chain.[3]

In the present paper we report the chemical synthesis of two other side chain analogues (**7**) (Scheme II) and (**8**) (Scheme III), in which the γ-carboxyl group of **2** was replaced by a bioisosteric γ-sulfinic acid group and a γ-nitro group, respectively. It was felt that these functional groups would more closely mimic a carboxyl group than either the γ-sulfonic acid group of **4** or the γ-phosphonic acid group of **6**. In particular, the sulfinate anion and nitro group both have planar trigonal geometry. Moreover, as indicated in Figure 1, the oxygens atoms in one of the canonical resonance forms of a nitro group support a delocalized negative charge. In this sense, therefore, a nitro group can be viewed as being sterically and electronically similar to a carboxylate anion. To our knowledge these are *the first known examples of this type of side chain modification in folate analogues*. Moreover there is no reason in principle why the methods used to obtain **7** and **8** cannot be introduced into a wide range of other antifolate structures.

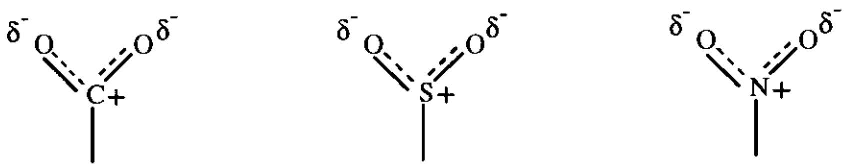
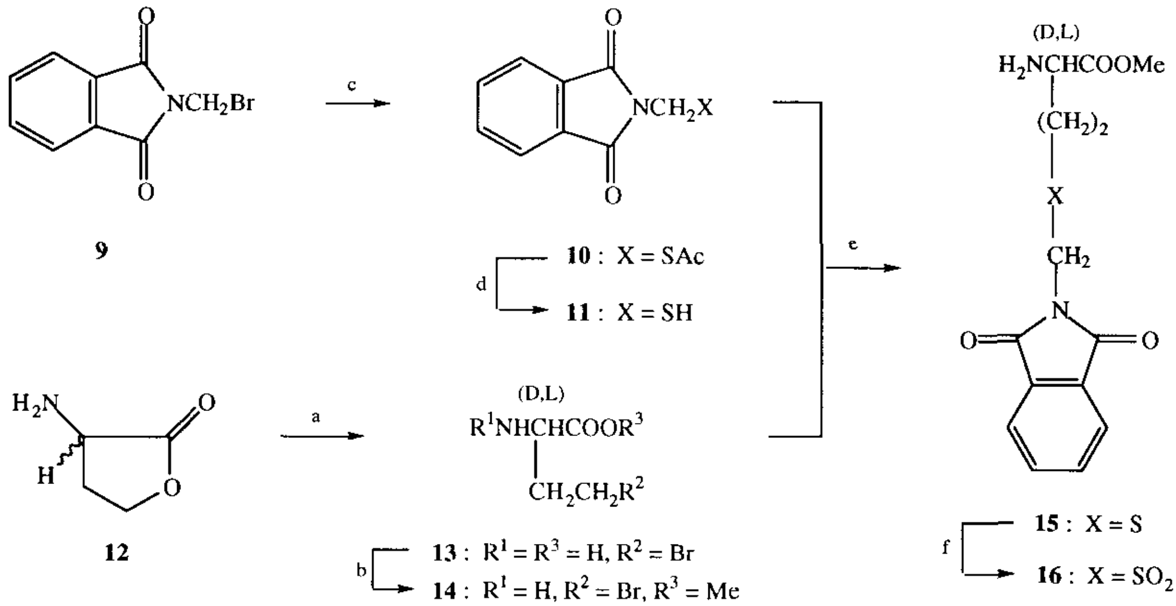


Figure 1. Steric and electronic relationship of carboxyl, sulfinyl, and nitro groups.

The synthesis of the sulfinic acid analogue (**7**) is depicted in Schemes I and II, and was inspired by a recent report of the successful cleavage of a phthalimidomethylsulfone to a sulfinic acid by reaction with a nucleophilic base.[4] The starting point for the synthesis of **7** was *N*-bromomethylphthalimide (**9**), which on treatment with thiolacetic acid in the presence of Et_3N, followed by deacetylation with concentrated HCl, afforded the thiol ester (**10**) and thiol (**11**) in high overall yield. Previously reported syntheses of **11** from **9** and NaSH[5] or from *N*-chloromethylphthalimide and trisodium thiophosphate[6] according to the method of Piper and Johnston[7] were viewed as being undesirable for large-scale work because of the environmental risks posed by H_2S gas, from which NaSH is freshly made before use,[5] and because of the relatively high cost of trisodium thiophosphate. Attempted cleavage of the thiol ester (**10**) with *N,N*-dimethylethylenediamine in methanol was unsuccessful, yielding only an acid-soluble product which we assume came from cleavage of the phthalimide ring. We believe the present method of preparation of **11**, which can be quickly, inexpensively, and safely performed on a multigram scale is markedly superior to the earlier procedures. In the next stage of the synthesis (Scheme I), D,L-2-aminobutyrolactone (**12**) was cleaved with 30% HBr to 2-amino-4-bromobutanoic acid (**13**), which on esterification with methanolic $SOCl_2$ was converted to the amino ester (**14**). As expected from the fact that the starting lactone (**12**) was treated consecutively with HBr and $SOCl_2$, **14** was obtained as a mixture of HBr and HCl salts. This did not materially affect the outcome, since the mixture of salts was neutralized *in situ* during the subsequent reaction. Condensation of **14** with thiol **11** in the presence of K_2CO_3 occurred rapidly in refluxing MeOH, giving **15** in 53% yield, isolated as an analytically pure HCl salt with the expected ^1H NMR features, including singlets at δ 4.72 (2H, CH_2N) and δ 7.92 (4H, aryl) corresponding to the phthalimidomethylsulfenyl moiety.

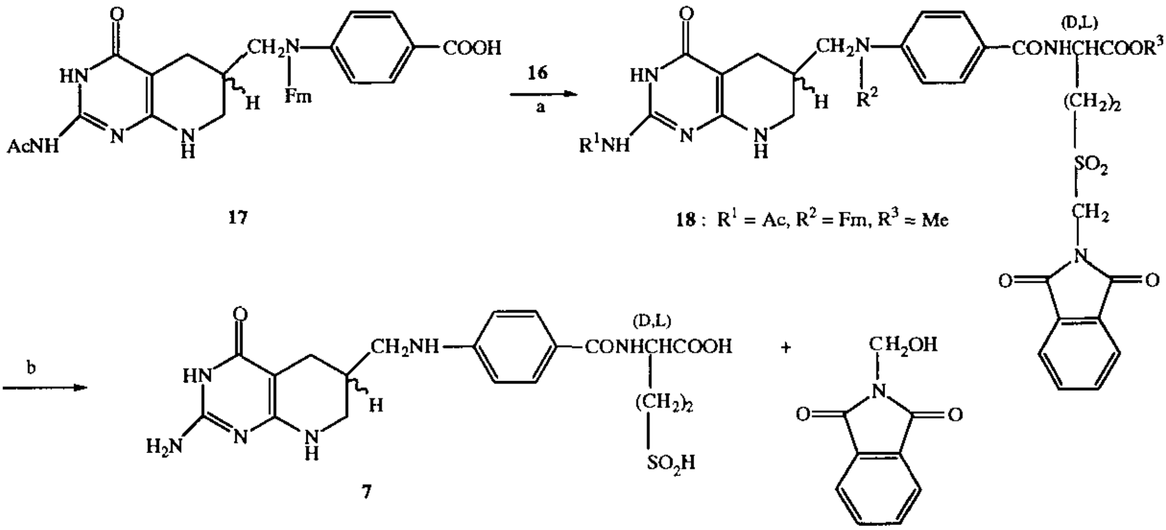
Scheme I



(a) HBr/AcOH; (b) $SOCl_2$/MeOH; (c) MeC(=O)SH/Et_3N; (d) HCl/MeOH; (e) K_2CO_3/MeOH; (f) CF_3CO_3H

Oxidation of **15** with peroxytrifluoroacetic acid, prepared in situ from 30% H_2O_2 and TFA, yielded an oily mixture of HCl and TFA salts which was neutralized with $NaHCO_3$, extracted with EtOAc, and treated with methanolic $SOCl_2$ to obtain analytically pure **16**·HCl in 22% yield. The presence of the sulfone group was confirmed by the ^1H NMR spectrum, which showed a downfield shift of the CH_2N singlet from δ 4.72 in **15** to δ 5.12 in **16**. Evaporation of the mother liquor afforded a 79% yield of *N*-hydroxymethyphthalimide. Although the facile cleavage of the carbon-sulfur bond group during workup of **16** was unanticipated, it augured well for our eventual ability to generate sulfinic acid (**7**) (see below). The possibility that **16** was cleaved by solvolysis in the original reaction mixture, rather than during workup, was eliminated by the observation that the ^1H NMR spectrum of a solution of the authentic compound in 80% aqueous TFA remained unchanged after 20 h at room temperature.

Scheme II



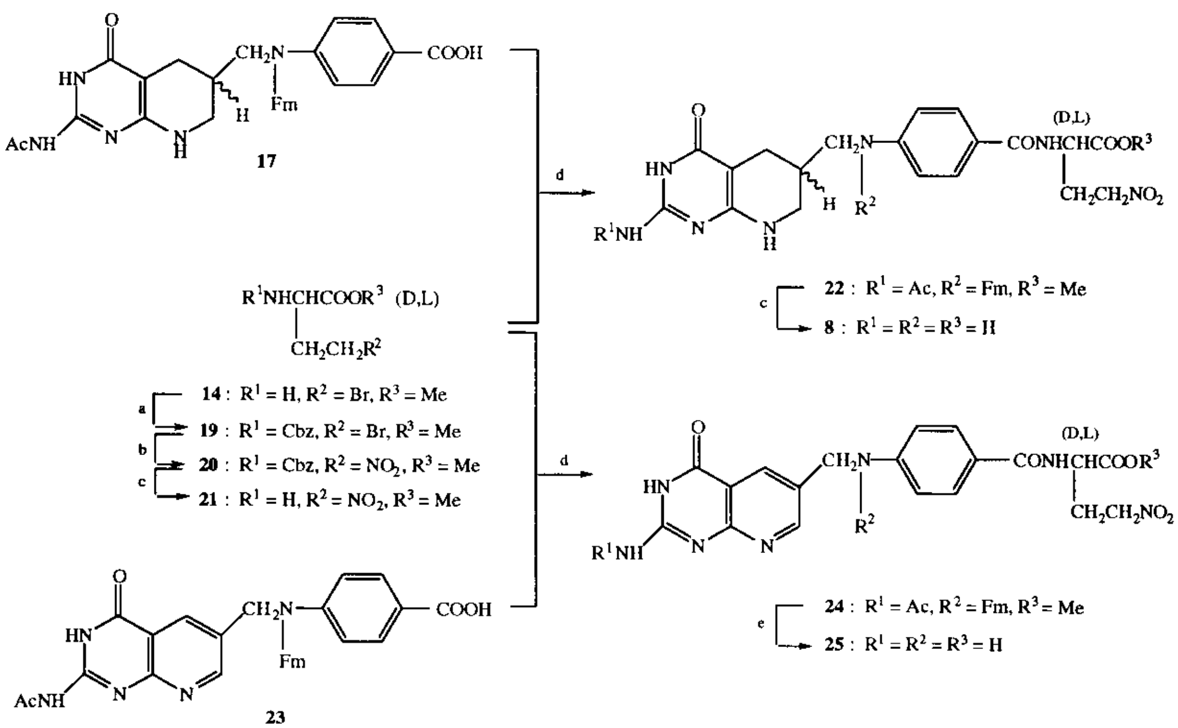
(a) i-BuOCOCl/Et$_3$N/DMF; (b) NaOH/DMSO

To complete the synthesis (Scheme II), **16** was condensed by the mixed carboxylic-carbonic anhydride method with *N*-[4-[(2-acetamido-4(3*H*)-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-formamido]benzoic acid (**17**),[1] and the protected coupling product (**18**) was chromatographed on silica gel and treated directly with NaOH in DMSO at 70 °C for 15 min to remove the ester and amide blocking groups and *simultaneously convert the phthalimidomethylsulfonyl group to a sulfinic acid.* The final product (**7**), isolated in a combined yield of *ca.* 20% after purification by HPLC on C_{18} silica gel followed by ion-exchange chromatography on DEAE-cellulose, had microanalytical values consistent with a trihydrated monoammonium salt. That it was the desired sulfinic acid and not the corresponding sulfonic acid (**4**),[1] which could have formed by air oxidation, was proved by co-chromatographing the two compounds on C_{18} silica gel with 4% MeCN in 0.1 *M* NH_4OAc, pH 6.9, as the eluent. As expected from the more polar nature of **4**, its retention time was 6 min whereas that of **7** was 10 min. It should be noted that both the lactone (**12**) and the heterocycle (**17**) were racemic. Thus the final product (**7**) was assumed to be a mixture of (6*R*,2*R*), (6*R*,2*S*), (6*S*,2*R*), and (6*S*,2*S*) diastereomers even though a single HPLC peak was observed.

However, since the pure 2*R* and 2*S* enantiomers of **12** are both commercially available it should be straightforward to obtain **7** with the side chain in either the L- or D-configuration.

The synthesis of the γ-nitro analogue (**8**) is shown in Scheme III, and again began with the mixed HBr and HCl salts of **14**. Carbamoylation of the amino group with benzyl chloroformate in the presence of K_2CO_3 afforded the γ-bromo ester (**19**) in 64% yield. Bromide displacement with $NaNO_2$ in DMSO at room temperature gave **20** as a straw-colored oil in 32% yield. This yield was higher than the 13% reported for the same reaction in DMF, and was also higher than the 21% overall yield reported for a two-step process involving successive reactions of the γ-chloro compound with NaI in acetone and $NaNO_2$ in DMF.[8] Although previous workers[8] had reported a melting point of 42-44 °C for this compound, attempts to obtain crystals of **20** consistently failed. However the ^1H NMR spectrum supported the assigned structure: as expected, there was a down-field shift from δ 3.4 to δ 4.5 for the protons on the γ-carbon in **20** versus **19**.

Scheme III



(a) $PhCH_2OCOCl/K_2CO_3$; (b) $NaNO_2$/DMSO; (c) HBr/AcOH; (d) i-$BuOCOCl/Et_3N$/DMF; (e) HCl/AcOH

Treatment of **20** with 30% HBr in AcOH, followed by neutralization with aqueous Na_2CO_3, extraction with EtOAc, and evaporation of the organic layer afforded **21** in 19% yield. A substantial amount of organic material was also recovered from the aqueous layer, but this unfortunately did not appear to be the expected amino ester. Although the low yield of **21** was disappointing, virtually the same result was reported by previous workers[8] when they used base rather than acid to cleave the ester group. After treatment of the purified dicyclohexylammonium salt of the cleavage product with 30% HBr in AcOH acid, the HBr salt of 2-amino-4-nitrobutanoic acid was isolated with an overall yield of only 20%.[8] Although we did not deter-mine the reason for the low yield in the acidolysis of the Cbz group in the presence of a nitro group, aliphatic nitro groups are known to be both acid- and base-sensitive. In acid, they can be converted first to hydroxamic acids and then to carboxylic acids; in strong base, aldehydes are formed *via* the Nef reaction. A better blocking group than Cbz would have been desirable for this purpose.

Condensation of **21** with **17** by the mixed carboxylic-carbonic anhydride method followed by treatment with 48% aqueous HBr in glacial AcOH led to **8**. The intermediate protected adduct (**22**) was too insoluble in solvents typically used for column chromatography on silica gel, and thus was used without purification. After a combination of preparative HPLC on C_{18} silica gel (4% MeCN in 0.05 *M* NH_4OAc, pH 6.9) and ion-exchange chromatography on DEAE-cellulose (0.4 *M* NH_4HCO_3), the combined yield of **8** in the final two steps was 10%. Analytical HPLC showed the twice-chromatographed product to be highly pure, with a single major peak at 14.5 minutes.

Mixed anhydride condensation of **21** with 4-[*N*-[(2-acetamido-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)-methyl]formamido]benzoic acid (**23**) instead of **17** was also carried out, to obtain **24**. Treatment of **24** with an equal mixture of 6 *N* HCl and glacial AcOH then gave **25** with a combined two-step yield of 16% after purification by preparative HPLC on C_{18} silica gel and ion-exchange chromatography on DEAE-cellulose.

The sulfinic acid (**7**) and nitro compounds (**8**) and (**25**) were tested as inhibitors of the growth of CCRF-CEM human leukemic lymphoblasts. The doubling time for this cell line under our cell culture conditions was 28 $\pm$ 1 h. Thus, a drug exposure period of 120 h was chosen in order to allow the untreated cells to undergo approximately four divisions. The concentration required to inhibit cell growth by 50% (i.e. the IC_{50}) was 21 $\pm$ 2 μM for compound (**7**), 93 $\pm$ 16 μM for compound (**8**), and 121 $\pm$ 12 μM for compound (**25**). It is of interest to note that, in our earlier study on γ-modified analogues of (6*R*,6*S*)-5-deaza-5,6,7,8-tetrahydrofolate,[1] the sulfonic acid analogue (**3**) was inactive against CCRF-CEM cells even when used at a concentration of up 100 μM. This lack of activity was tentatively ascribed to a difference in the steric and electronic properties of the SO_2OH group relative to a CO_2H group. An experiment was also performed to determine whether **7** is able to use the reduced folate carrier (RFC) pathway for active transport into cells (reviewed in ref. 9). Using the standard method to measure the ability of a folate analogue to competitively inhibit [^{3}H]methotrexate influx via the RFC,[6] the K_i of **7** was found to be 5.0 μM. The reported K_i of the sulfonic acid **3** is ca. 20 μM.[6] Thus it appears that one reason for the greater cytotoxicity of **7** relative to 3 may be an increased ability to be taken up via the RFC. The greater potency of sulfinic acid (**7**) relative to sulfonic acid **3** is consistent with the idea that the SO_2H group is bioisosterically closer to a CO_2H group than is the SO_3H group.

It should be noted that both **7** and **8** were tested as mixtures of 6*R* and 6S diastereomers whose biological activities individually are not known. However, based on the fact that the K_i values of (6*R*)- and (6*S* (-5,10-dideazatetrahydrofolate are almost the same,[10] as has also been reported for (6*R*)- and (6*S*)-5-formyl-tetrahydrofolate,[11] it is reasonable to assume that the substrate affinity of (6*R*,6*S*)-**7** would be close to that of the 'natural' diastereomer. Moreover, since the ability of (6*R*)- and (6*S*)-5,10-dideazatetrahydrofolate against to inhibit the growth of cultured CCRF-CEM cells is likewise very similar, it can be expected that the IC_{50} values for (6*R*,6*S*)-**7** and (6*R*,6*S*)-**8** would also not have been very different if the individual diastereomers had been tested separately.

Apart from the possibility that prolonged incubation of cells in the presence **7** could induce apoptosis by depletion of endogenous reduced folates[13] by interfering with RFC function, this compound also has the potential to block polyglutamation of reduced folates by FPGS and de novo biosynthesis of purine nucleotides by GARFT. Support for these alternative mechanisms comes from our previous finding that sulfonic acid analogue (**3**) inhibits FPGS with a K_i of 22 μM and GARFT with K_i of 0.19 μM.[1] Thus, experiments to determine how much of the cytotoxicity of **7** is due to inhibition of these enzymes and how much is due to interference with RFC function would be of potential interest.

EXPERIMENTAL SECTION

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer. Only peaks above 1200 cm^{-1} are given, and shoulders and weak peaks are omitted. ^{1}H NMR spectra were recorded on a Varian Model EM360L spectrometer with Me_4Si as the reference. Analytical TLC was on fluorescent Baker Si250F silica gel plates or Eastman 13181 silica gel sheets, with spots being visualized under ultraviolet light at 254 nm or with the aid of an I_2 chamber. Column chromatography was on Baker 70-200 mesh silica gel, Baker 'Flash' grade silica gel (40 µm particle size), or Whatman DEAE-cellulose (pre-swollen). HPLC was on Waters C_{18} radial compression cartridges (analytical: 5 µm particle size, 5 x 100 mm); preparative; 15 µm particle size, 25 x 100 mm). Melting points (uncorrected) were obtained in glass capillaries in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA). *N*-[4-[(2-Acetamido-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]formamido]benzoic acid (**16**) and its (6*R*,6*S*)-5,6,7,8-tetrahydro derivative (**23**) were obtained as described.[1] Other chemicals and solvents were from Aldrich, Milwaukee, WI, or Fisher, Boston, MA. The 2-aminobutyrolactone used in the synthesis of 2-amino-4-nitrobutanoic acid and 2-amino-4-sulfinobutanoic acid was a D,L-enantiomeric mixture. Hence these acids were also racemic and the ensuing products (**7**) and (**8**) were presumably mixtures of unresolved diastereomers. Microanalyses were performed by Robertson Laboratory, Madison, NJ. Mass spectral data were provided by the Core Molecular Biology Facility of the Dana-Farber Cancer Institute.

***N*-Mercaptomethylphthalimide (11).** A solution of thiolacetic acid (0.76 g, 10 mmol) and Et_3N (1.39 mL, 1.01 g, 10 mmol) in ice-cold THF (30 mL) was stirred and treated dropwise over 30 min with a solution of *N*-bromomethylphthalimide (**9**) (2.4 g, 10 mmol) in THF (30 mL). After being kept in the refrigerator for 2 days, the mixture was concentrated to dryness by rotary evaporation and the residue was partitioned between EtOAc and H_2O. The organic layer was washed with 1% citric acid and evaporated to obtain *N*-acetylthiomethylphthalimide (**10**) as a light-brown solid (1.7 g, 74%); TLC: R_f 0.5 (silica gel, 3:2 hexane-EtOAc). Recrystallization of a small portion of this solid from hexanes-acetone afforded off-white plates, mp 85-86 °C; ^{1}H NMR ($CDCl_3$) δ 3.27 (s, 3H, CH_3), 5.20 (s, 2H, CH_2), 7.82 (m, 4H, aryl); *m/z* 236 $(M+1)^+$. Because the recrystallized sample appeared to have become less pure than the original light-brown solid, the non-recrystallized material was used directly for the next reaction.

The crude thiol ester (**10**) from a larger-scale reaction [2.44 g (32 mmol) of thiolacetic acid (2.4 g, 32 mmol), and *N*-bromomethylphthalimide (7.7 g, 32 mmol), and Et_3N (4.5 mL) in THF (100 mL)] was added immediately (without being weighed) to MeOH (240 mL), and the solution was cooled in an ice bath and stirred while concentrated HCl (90 mL) was added dropwise without allowing the internal temperature to exceed 15 °C. The mixture was left to stir at rt for 20 h, and the solid which formed during this period was collected, washed with H_2O, and dried *in vacuo* at 65 °C to obtain **11** as a white powder (4.62 g, 75% yield for the two steps), mp 136-138 °C (lit.,[5] mp 138-139 °C; lit.,[6] mp 136-138 °C); IR (KBr) ν 2570 (SH stretch) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.67 (t, J = 9 Hz, 1H, SH), 4.78 (d, J = 9 Hz, 2H, CH_2), 7.83 (m, 4H, aryl).

Methyl 2-Amino-4-bromobutanoate (14). A suspension of **12** (10 g, 0.055 mol) in 30% HBr in AcOH (110 mL) was stirred at 45 °C for 2 days. The resulting clear solution of 2-amino-4-bromobutanoic acid (**13**) was evaporated to dryness, and the solid was taken up in MeOH (100 mL). The solution was stirred in an ice bath and treated dropwise with $SOCl_2$ (10 mL, 16.3 g, 0.137 mol) at such a rate that the internal temperature did not exceed 12 °C. When addition was complete the ice bath was removed and the solution was left at rt for 20 h. The solution was concentrated to dryness by rotary evaporation and the solid kept *in vacuo* at 40 °C to obtain a colorless solid (12 g). After recrystallization from EtOAc-MeOH the product melted at 133 °C. From the method of synthesis and the microchemical analysis the product was assumed to be a 1:1 mixture of **14**·HCl and **14**·HBr. Anal. Calcd for $C_5H_{10}NO_2Br \cdot 0.5HBr \cdot 0.5HCl$: C, 23.57; H, 4.36; N, 5.50. Found: C, 23.50; H, 4.04; N, 5.33.

Methyl 2-Amino-4-(*N*-phthalimidomethyl)thiobutanoate (15). A mixture of **11** (3.26 g, 17 mmol), the mixed HCl and HBr salts of methyl 2-amino-5-bromobutanoate (**14**) (4.71 g, estimated to be 17 mmol by arbitrarily using the molecular weight of the HBr salt), and K_2CO_3 (4.83 g, 34 mmol) in MeOH (100 mL) was stirred under reflux for 15 min, then cooled and filtered. The inorganic salts were filtered off, the filtrate was concentrated to a small volume, and the residue was taken up into EtOAc. The organic phase was extracted with 0.5 *M* HCl, and the aqueous phase was carefully neutralized with solid Na_2CO_3 (gas evolution!) and re-extracted with EtOAc. The aqueous phase was treated with an additional portion solid K_2CO_3 and extracted once more with EtOAc. The pooled organic layers were evaporated to dryness and the residue was treated with a small volume of MeOH which had previously been cooled in an ice bath and treated with $SOCl_2$ (1.46 mL, 2.38 g, 20 mmol). The solution was kept at 4 °C overnight and the white crystals which formed were filtered, washed with EtOAc, and dried *in vacuo* at 65 °C to obtain **15**·HCl as a white powder which was used without additional purification (3.07 g, 53%), mp 185-186 °C; IR (KBr) ν 3470, 3050, 2950, 1775, 1745, 1720, 1495, 1475, 1445, 1435, 1415, 1385, 1350, 1310, 1295, 1255

cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.23 (m, 2H, β-CH_2), 2.82 (m, 2H, γ-CH_2), 3.72 (s, 3H, OCH_3), 4.08 (t, J = 7 Hz, 1H, α-CH), 4.72 (s, 2H, CH_2N), 7.92 (s, 4H, aryl), 8.70 (br s, 3H, NH_3^+). Anal. Calcd for $C_{14}H_{16}N_2O_4S{\cdot}HCl$: C, 48.77; H, 4.97; N, 8.12; S, 9.30. Found: C, 48.68; H, 4.94; N, 7.88; S, 9.58.

Methyl 2-Amino-4-(phthalimidomethylsulfonyl)butanoate (16). A solution of trifluoroperacetic acid, freshly prepared by diluting 35% aqueous H_2O_2 (3 mL, containing 30.9 mmol) with TFA (6 mL), was added dropwise over 5 min to an ice-cold solution of **15**·HCl (1.72 g, 5.0 mmol) in TFA (5 mL). Gas evolution occurred and the reaction mixture became warm as the ice bath was removed. Excess peroxide was destroyed by adding solid NaI in small portions and testing for oxidizing activity with KI-starch paper. The mixture was then evaporated to dryness under reduced pressure, and H_2O was added. A small amount of insoluble material was removed by filtration, and the solution was carefully neutralized with powdered $NaHCO_3$ (gas evolution!) and extracted three times with EtOAc. The pooled extracts were cooled in ice and treated with a small volume of MeOH which had previously been chilled and treated with $SOCl_2$ (0.22 mL, 3.59 g, 3.0 mmol). The solution was kept in the freezer overnight and the precipitate (187 mg) was collected. The filtrate was reduced in volume and replaced in the freezer to obtain a second crop weighing 226 mg; total yield 413 mg (22%), mp 187-188 °C; IR (KBr) ν 3490, 2920, 2750, 2720, 2650, 2610, 2050, 1785, 1760, 1725, 1610, 1490, 1475, 1455, 1410, 1395, 1320, 1300, 1290, 1275, 1235, 1200 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.35 (m, γ-CH_2, partly covered by the DMSO-d_5 signal), 3.50 (m, γ-CH_2, partly covered by a small water peak), 3.78 (s, 3H, OCH_3), 4.22 (t, J = 6 Hz, 1H, α-CH), 5.12 (s, 2H, CH_2N), 8.00 (s, 4H, aryl), 8.85 (m, 3H, NH_3^+). Anal. Calcd for $C_{14}H_{16}N_2O_6S{\cdot}HCl$: C, 44.63; H, 4.55; N, 7.43; S, 8.51. Found: C, 44.85; H, 4.43; N, 7.29; S, 8.34.

Evaporation of the mother liquor to dryness yielded a solid identified from its ^1H NMR spectrum as *N*-(hydroxymethyl)phthalimide (701 mg, 79%).

2-[*N*-[4-[(2-Amino-4(3*H*)-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methyl]-amino]benzoylamino]-4-sulfinobutanoic Acid (7). A suspension of **17** (91 mg, 0.236 mmol) in dry DMF (5 mL) was treated with Et_3N (36 μL, 26 mg, 0.25 mmol) and isobutyl chloroformate (33 μL 34 mg, 0.25 mmol). Some solid remained undissolved, and an additional 15% of each reagent was therefore added. After nearly all the solid dissolved, the solution was treated with **16**·HCl (113 mg, 0.30 mmol) followed by another portion of Et_3N (41 µL, 30 mg, 0.30 mmol). The mixture was stirred at rt for 20 h and evaporated to dryness under reduced pressure. The residue was triturated several times with ether, the ether was poured off, and the remaining solid was chromatographed on a column of 'Flash' silica gel (10 g, 2 x 8 cm) with 9:1 $CHCl_3$-MeOH as the eluent. Fractions were monitored by TLC (silica gel, 9:1 $CHCl_3$-MeOH), and those containing a spot with R_f 0.3 were pooled, concentrated, and diluted with ether until a solid precipitated. The solid was filtered and dried *in vacuo* over P_2O_5 to obtain a white powder (84 mg). The IR and ^1H NMR spectrum of this material showed that it consisted of **18** along with a small amount of residual triethylammonium chloride which ordinarily would have been removed by washing with water. However, because of the aqueous instability of the phthalimidomethylsulfone group, this material was used directly in the next step without being washed with water.

A solution of **18** (70 mg, 0.1 mmol) in DMSO (2 mL) was treated with 1 *M* NaOH (4 mL) and heated at 70 °C for 15 min. The cooled solution was treated with concentrated $(NH_4)_2SO_4$ to bring the pH to approximately 8, and the product was isolated by preparative HPLC on C_{18} silica gel with 4% MeCN in 0.1 *M* NH_4OAc, pH 6.9, as the eluent. On an analytical column with the same eluent and a flow rate of 1 mL/min the elution time was 10 min as compared with 6 min for the corresponding γ-sulfonic acid (**4**), an authentic sample of which was available from previous work.[1] Pooled fractions containing the desired product were concentrated and freeze-dried, then subjected to final desalting and purification on a DEAE-cellulose column (12 g, 1.5 x 12 cm, HCO_3^- form) which was eluted with a large volume of H_2O followed by 0.2 and 0.4 *M* NH_4HCO_3. Fractions were monitored by HPLC, and those containing a single peak eluting at 10 min were pooled and freeze-dried to obtain **7** as a white solid without further purification (20 mg, 43%), mp >250 °C (decomp) ; IR (KBr) ν 3340br, 2930, 1705, 1660, 1610, 1575, 1545, 1525, 1400, 1345, 1310, 1270, 1225 cm^{-1}; *m/z* 465 $(M+1)^+$. Anal. Calcd for $C_{19}H_{24}N_6O_6S\cdot NH_3\cdot 3H_2O$: C, 42.61; H, 6.21; N, 18.31; S, 5.99. Found: C, 42.60; H, 5.97; N, 18.10; S, 6.35.

2-[*N*-[4-(2-Amino-4(3*H*)-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)methylamino]benzoyl]amino-4-nitrobutanoic Acid (8). A mixture of the HBr and HCl salts of **14** (12 g), synthesized from **12** as described above, was suspended in CH_2Cl_2 (100 mL). Benzyl chloroformate (14.3 mL, 17.1 g, 0.1 mol) and powdered K_2CO_3 (20 g, 0.145 mol) were added with stirring. After 20 h, the reaction was quenched with H_2O (80 mL) and stirring was continued for another 20 min. The organic layer was separated, the aqueous layer was extracted with CH_2Cl_2, the combined organic phases were evaporated, and the solid was chromatographed on silica gel ('Flash' grade, 80 g, 4 x 13 cm) with 2:1 hexane-EtOAc as the eluent. Partial crystallization occurred on the column, requiring the use of a large volume of solvent to completely recover the product. Evaporation of pooled TLC-homogeneous fractions and recrystallization from acetone-hexanes afforded **19** (13.1 g, 64% based on the starting lactone) as a white solid, mp 88-89 °C (lit.,[14] mp 87-89 °C); ^{1}H NMR ($CDCl_3$) δ 2.0-2.5 (m, 2H, β-CH_2), 3.4 (t, 2H, γ-CH_2), 3.75 (s, 3H, OMe), 4.5 (m, 1H, α-CH), 5.1 (s, 2H, OCH_2), 7.35 (s, 5H, aryl).

A solution of **19** (6.60 g, 0.02 mol) and $NaNO_2$ (2.07 g, 0.03 mol) in DMSO (40 mL) was kept at rt for 20 h, then partitioned between EtOAc and H_2O. The organic layer was washed with water and evaporated to obtain a solid (6.2 g) whose TLC (silica gel, 3:2 hexane-*tert*-BuOH) showed spots with R_f 0.3, 0.4, 0.5, and 0.6. The mixture was taken up in EtOAc and the product pre-adsorbed onto silica gel ('Flash' grade, 10 g) by rotary evaporation, and the dried solid was added to the top of a column of wet-packed silica gel (80 g, 4 x 15 cm) which was eluted first with 3:1 hexanes-*tert*-BuOH and then with 1:1 hexanes-EtOAc. Fractions of the 3:1 hexanes-*tert*-BuOH eluent were monitored by TLC, and those containing the R_f 0.5 spot were pooled and evaporated. The resulting solid (3.2 g) was re-chromatographed on another column (60 g, 3 x 15 cm) with the same eluent mixture to obtain **20** as a pale-yellow oil (2.16 g, 32%); IR (neat) ν 3330, 3060, 3030, 2960, 2850, 1720br, 1555, 1455, 1440, 1380, 1330, 1215 cm^{-1}; ^{1}H NMR: ($CDCl_3$) δ 1.25 (s, Me from *tert*-BuOH), 2.03 (m, OH from *tert*-BuOH, exchangeable with D_2O), 2.47 (m, 2H, β-CH_2), 3.73 (s, 3H, OCH_3), 4.45 (t, J = 7 Hz, superimposed on m, 3H, α-CH and CH_2NO_2), 5.10 (s, 2H, CH_2O), 5.67 (br m, 1H, NH, exchangeable with D_2O), 7.33 (s, 5H, aryl). Although this compound has been reported to melt at 42-44 °C,[8] it resisted all attempts to crystallize it and was therefore used directly in the next step an oil.

A solution of **20** (2.14 g, 7.23 mmol) in glacial AcOH (7 mL) was treated with 30% HBr in AcOH (7 mL and after 30 min at rt the reaction mixture was diluted with ether (200 mL) and chilled. The ether was decanted, leaving a gum which was stored for a week in a vacuum desiccator in an unsuccessful effort to obtain crystals. The gummy product was then partitioned between EtOAc and 5% Na_2CO_3, and the organic layer was evaporated under reduced pressure. The residue from the organic layer, consisting of **21** as the free amino ester (0.22 g, 19%), was used without additional purification.

A flask containing **17** (100 mg, 0.26 mmol) in dry DMF (5 mL) was treated with Et_3N (40 µL, 29 mg, 0.29 mmol) and isobutyl chloroformate (38 µL, 40 mg, 0.29 mmol) and kept in a sonication bath until a clear solution was obtained (2-3 min). Compound (**21**) (60 mg, 0.30 mmol) followed by a second portion of Et_3N (42 µL, 30 mg, 0.30 mmol) were then added. The solution became clear after 5 min and was left to stand at rt for 20 h. The solvent was evaporated under reduced pressure and the residue was triturated with ether to obtain crude **22**; TLC: major spot with R_f 0.08 along with a number of smaller faster-moving impurities (silica gel, 4:1 $CHCl_3$-MeOH). The product was taken up directly in a mixture of glacial AcOH (6 ml) and 48% aqueous HBr (4 mL), and heated at 70 °C for 15 min. The solvent was evaporated under reduced pressure, the residue was dissolved in water, the resulting solution was neutralized with 28% NH_4OH, and a trace of insoluble material was filtered off. Analytical HPLC (C_{18} silica gel, 4% acetonitrile in 0.05 *M* NH_4OAc, 1.0 mL/min) at this stage revealed mainly one product with a retention time of 14.5 min, along with some very minor impurities eluting at 7 and 21 min. Preparative HPLC using the same eluent system was then performed, and appropriately fractions were pooled, freeze-dried, and applied onto a DEAE-cellulose column (15 g, 1.3 x 18 cm, HCO_3^- form), which was eluted first with a large volume of H_2O and then with 0.4 *M* NH_4HCO_3. Lyophilization of appropriately pooled fractions afforded **8** as a white solid (12 mg, 10%); IR (KBr) ν 3350br, 2930, 1610, 1540, 1480, 1465, 1375, 1345, 1315, 1270, 1225 cm^{-1}; MS: *m/e* 445 (M^+). Anal. Calcd for $C_{19}H_{23}N_7O_6$: C, 51.23; H, 5.20. Found: C, 51.51; H, 5.36. Microchemical analysis using the standard method of combustion failed to give values within acceptable limits (± 0.4% of theory) for nitrogen with two different batches of **8** that had been purified to homogeneity by HPLC. We have occasionally experienced this problem with other nitro compounds whose structure was not otherwise in doubt.

4-[*N*-[(2-Acetamidopyrido[2,3-*d*]pyrimidin-6-yl)methyl]formamido]benzoyl]amino]-4-nitrobutanoic Acid (24). A stirred suspension of **23** (300 mg, 0.79 mmol) in dry DMF (15 mL) at rt was treated with Et_3N (139 µL, 101 mg, 1 mmol) followed by isobutyl chloroformate (117 µL, 123 mg, 0.9 mmol). After 10 min, **21** (224 mg, 1.38 mmol) was added and the mixture was stirred at room temperature for 2 days. At the end of this period the mixture was concentrated to dryness by rotary evaporation, the residue was taken up in warm acetone and MeOH in a sonication bath, and the product was pre-adsorbed onto silica gel (2 g) by rotary evaporation. The silica gel with adsorbed product was applied to the top of a column of wet-packed silica gel (20 g, 2 x 14 cm), which was eluted with acetone. Fractions were monitored by TLC (silica gel, acetone), and a yellow band eluting ahead of the product was discarded. Fractions containing the colorless product (R_f 0.8) were pooled and concentrated to a small volume. Addition of ether caused separation of an oil. Extensive trituration of the oil dissolved a non-aromatic impurity and caused the oil to solidify. Filtration and drying *in vacuo* at 65 °C over P_2O_5 gave **24** as a beige solid, which was used without further purification (142 mg, 34%), mp 141-149 °C; IR (KBr) ν 3260, 3130, 2960, 1740, 1685, 1630, 1605, 1555, 1500, 1460, 1400, 1350br, 1295, 1245 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.18 (s, 3H, CH_3CO), 2.51 (m, β-CH_2, partially covered by DMSO-d_5 signal), 3.65 (s, 3H, OCH_3), 4.68 (t, J = 6 Hz, overlapping a broad m, 3H, CH_2NO_2 and α-CH), 5.28 (s, 2H, 9-CH_2), 7.48 (d, J = 8 Hz, 2H, 3'- and 5'-H), 7.85 (d, J = 8 Hz, 2H, 2'- and 6'-H), 8.25 (d, J = 2 Hz, 1H, 5-H), 8.73 (d, J = 2 Hz, 1H, 7-H), 8.81 (s, 1H, CH=O). Anal. Calcd for $C_{23}H_{23}N_7O_8 \cdot H_2O$: C, 50.83; H, 4.64; N, 18.04. Found: C, 50.52; H, 4.19; N, 17.85.

2-[*N*-[4-[*N*-(2-Amino-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]aminobenzoyl]amino]-4-nitrobutanoic Acid (25). A solution of **24** (108 mg, 0.199 mmol) in glacial AcOH (3 mL) and 6 *N* HCl (3 mL) was heated at 70 °C for 15 min. The solvent was evaporated, the residue dissolved in H_2O with the aid of a few drops of 28% NH_4OH, the pH adjusted to near neutrality with AcOH, and the product isolated by preparative HPLC (C_{18} silica gel; solution A; 1% MeCN in 0.1 *M* NH_4OAc, pH 6.9; solution B: same buffer with 20% MeCN; linear gradient of 30% B to 100% B over 30 min; flow rate 10 mL/min). On the analytical C_{18} silica gel column with the same eluent system and a flow rate of 1 mL/min, four peaks with retention times of 5, 19, 23, and 40 min were obtained. The 19 min peak was collected, concentrated by rotary evaporation, and freeze-dried. The residue was redissolved in dilute ammonia, and the solution was applied onto a DEAE-cellulose column (20 g, 1.5 x 21 cm, HCO_3^- form) which was eluted copiously with H_2O, followed by 0.2 and 0.4 *M* NH_4HCO_3. Because elution of the product was very slow, the NH_4HCO_3 buffer was adjusted to pH 10 with 28% NH_4OH. The total volume of eluent required to achieve complete recovery of the product was *ca.* 500 ml. Appropriate fractions were pooled and concentrated to dryness by rotary evaporation and lyophilization to obtain a white solid (47 mg, 48%); IR (KBr) ν 3270, 2830 br, 1700, 1665, 1605, 1550, 1505, 1400, 1330, 1260 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.50 (m, β-CH_2, partially covered by DMSO-d_5), 4.42 (m, 7H, 9-CH_2, CH_2NO_2, α-CH, NH_2), 6.67 (d, J = 9 Hz, 2H, 3' and 5'-H), 7.63 (d, J = 9 Hz, 2H, 2'- and 6'-H), 8.25 (br s, 2H, 5- and 7-H); MS: *m/z* 442 $(M+1)^+$. Anal. Calcd for $C_{19}H_{19}N_7O_6 \cdot 2H_2O$: C, 47.80; H, 4.86; N, 20.54. Found: C, 47.39; H, 4.46; N, 20.34.

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