Efficient Syntheses of Pyrofolic Acid and Pteroyl Azide, Reagents for the Production of Carboxyl-Differentiated Derivatives of Folic Acid

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Abstract: Reaction of folic acid (**1**) with excess trifluoroacetic anhydride provides access to both the previously unknown N^{10}-(trifluoroacetyl)pyrofolic acid (**8)** and pyrofolic acid (**9**). Reaction of either of these materials with hydrazine selectively affords pteroyl hydrazide (**13**), which may be oxidized to pteroyl azide (**27)** on a large scale (62% overall from **1** without the need for chromatography). Treatment of **27** with differentially protected glutamates provides a convenient and high-yielding synthesis of differentially protected, optically pure folates.

A recent trend in cancer chemotherapy is the highly aggressive application of high-dose multiple drug treatment regimens at the earliest point of diagnosis.[1] These protocols are limited by drug toxicity, and severe physiological effects and patient fatalities are not uncommon. This situation has caused several members of the medical community to question whether the benefit/risk boundary has been exceeded with the agents currently available.[2] Enhancement of the differential specificity of anticancer agents by *selective targeting mechanisms* might diminish such problems. The vitamin folic acid (**1**) has attracted considerable attention as a potential means of delivery of covalently bound drug conjugates.[3] Many human cancer cell lines have been found to have highly overexpressed levels of the protein which binds folic acid,[4] a finding which is beginning to be exploited with the preparation of folate−drug conjugates.[5]

Unfortunately, a major impediment in drug design centers around the synthetic aspects of preparing the folate−drug conjugates. Current practice simply involves treatment of the substrate of choice with folic acid (**1**) and a given dehydrating agent, such as DCC. This results in a mixture of both the inactive α-conjugate **2**[5c] and the active γ-conjugate **3**,[5c] often accompanied with the bis-functionalized derivative **4** (not shown) and/or recovered **1** (Scheme 1). Separation of these mixtures is often difficult and some of the exciting biological results have been obtained on mixtures.[5]

Regiospecific functionalization of the γ-carboxylate of glutamic acid is routinely accomplished using urethane derivatives of pyroglutamic acid which exploit the γ-lactam moiety as an acylating agent.[6] A search of the literature surprisingly revealed that pyrofolic acid (**9**) and its N-acylated derivatives are apparently unknown.[7] Nevertheless, treatment of **1** (100 g) with excess trifluoroacetic anhydride in THF for 10 h from 0 to 25 °C produces $N^{2,10}$-bis(trifluoroacetyl)pyrofolic acid (**7**) as an extremely water-labile material which is believed to be a mixture of diacylated anhydride **6** and diacylated carboxylic acid **7**, as judged by ^{19}F-NMR of the crude reaction mixture. In any event, simply stirring the aforementioned material with neutral water affords N^{10}-(trifluoroacetyl)pyrofolic acid (**8**) in a quantitative yield (Scheme 2). Compound **8** can be further transformed to pyrofolic acid (**9**) via deacylation with cesium carbonate and water (87%). Both **8** and **9** are essentially racemic, as determined using an enzymatic assay based on carboxypeptidase G (see Table 3).

Unfortunately, the N^{10}-trifluoroacetyl derivative of azalactone **8′** (not the isomeric pyrofolate **8**) has been alleged to result from reaction of **1** with trifluoroacetic anhydride (Scheme 3).[7a] In addition, the same authors have reported that the N^{10}-acetyl azalactone **Me-8′** results from the analogous acetic anhydride reaction.[7b] Basic hydrolysis of **Me-8′** yields a mixture of **1** and pteroic acid (**10**), entirely consistent with the pyrofolate structure as per our observations in Table 1 (below), while the authors had to invoke an unprecedented[8] hydrolysis of the azalactone imine moiety to explain the production of **10**. This structural misassignment has dire consequences *vis-à-vis* regiospecific functionalization of the two carboxylates of the glutamate

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(2) (a) Dodwell, D. J. *Lancet* **1993**, *341*, 614. (b) Davidson, N. E. *J. Clin. Oncol.* **1992**, *10*, 517. (c) Henderson, I. C.; Hayes, D. F.; Gelman, R. *J. Clin. Oncol.* **1988**, *6*, 1501.

(3) Folic acid and its reduced counterparts enter cells via two unrelated pathways (Dixon, K. H.; Lanpher, B. C.; Chiu, J.; Kelley, K.; Cowan, K. H. *J. Biol. Chem.* **1994**, *269*, 17). The primary intake route is mediated by the reduced folate carrier, a transport protein that exhibits a K_m for folate near 5×10^{-6} M and a strong preference for reduced folates over folic acid. The second path of folate uptake is mediated by a family of membrane receptors termed the folate binding protein (FBP) (Brigle, K. E.; Spinella, M. J.; Westin, E. H.; Goldman, I. D. *Biochem. Pharmacol.* **1994**, *47*, 337. Shen, F.; Ross, J. F.; Wang, X.; Ratnam, M. *Biochemistry* **1994**, *33*, 1209. Leamon, C. P.; Low, P. S. *Biochem. J.* **1993** *291*, 855. Garin-Chesa, P.; Campbell, I.; Saigo, P. E.; Lewis, J. L., Jr.; Old, L. J.; Rettig, W. *J. Am. J. Pathol.* **1993**, *142*, 557). FBP has a K_D for folate that varies from 10^{-9} to 10^{-11} and exhibits a strong preference for folic acid over its reduced counterparts (Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5572).

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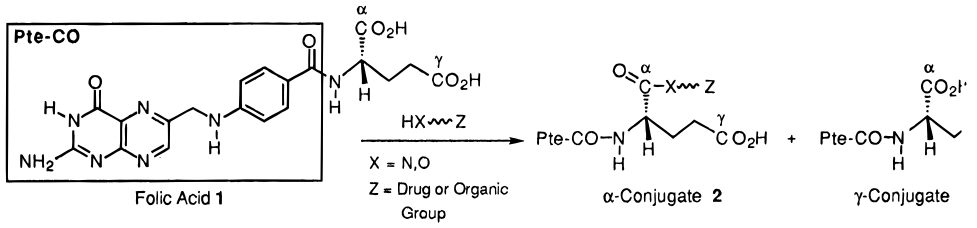
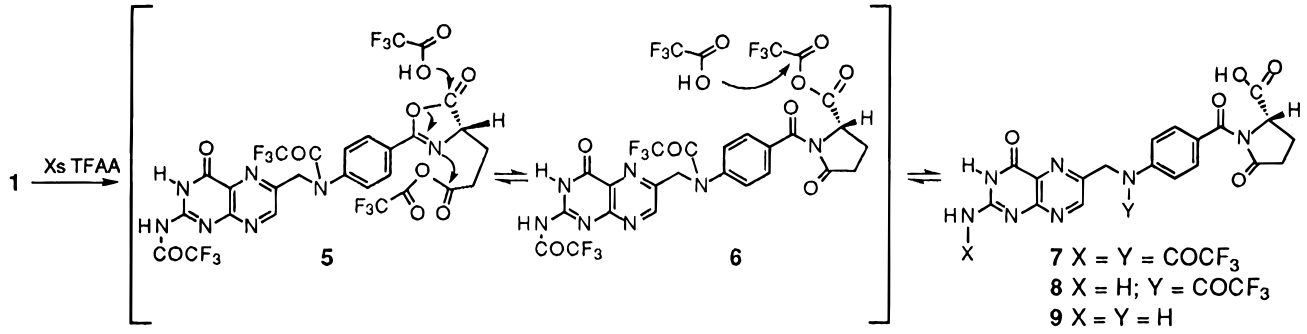
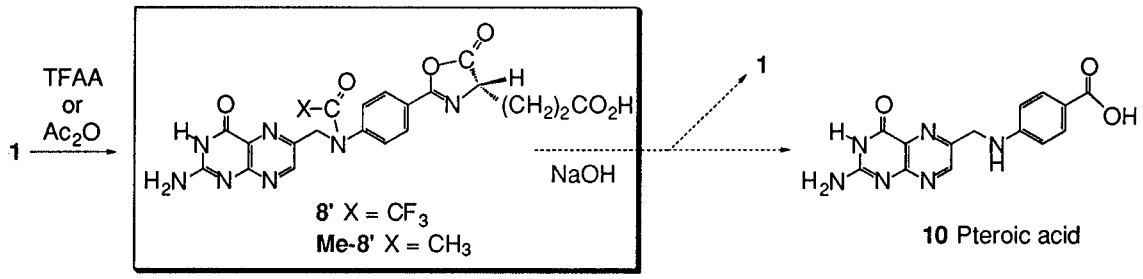
Scheme 1



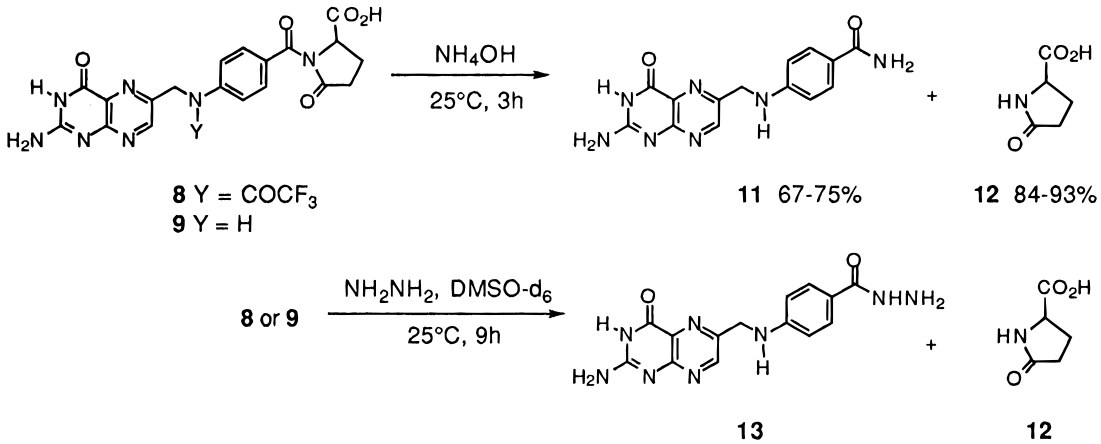
Scheme 2



Scheme 3



Scheme 4



moiety, since nucleophilic attack on the azalactone **8′** would be expected to afford functionalization of the α-carboxyl moiety, while activation of the carboxylic acid should enable regiospecific γ-functionalization. *This is exactly the opposite regiochemistry of that which actually results from functionalization of the correct pyrofolate structure **8** and has resulted in further incorrect structural assignments.*[9]

In order to provide a definitive structural assignment, both **8** and **9** were treated with excess concentrated ammonium hydroxide at 25 °C for several hours to afford pteroylamide (**11**) along with pyroglutamic acid (**12**) in high yield (Scheme 4), a finding which would be exceptionally difficult to rationalize with the alternative azalactone structures **8′** and **9′**. As expected, concomitant deacylation of the N^{10}-trifluoroacetyl moiety occurred during the reaction. The isolated **12** exhibited only ≤5% optical activity, in accord with extensive racemization occurring during the synthesis of **8**. Monitoring a pair of similar reactions of **8** and **9** with hydrazine (10 equiv) in DMSO-d_6 at 25 °C for 9 h unambiguously produces a 1:1 mixture of pteroyl hydrazide (**13**) and (**12**) in near-quantitative yield, as assayed by ^{1}H-NMR and HPLC (Scheme 4 and Table 1, entries 12 and 13).

Spectral evidence strongly supports the pyrofolate structure assigned to **8** and **9**. In particular, the ^{13}C-NMR shift (DMSO-

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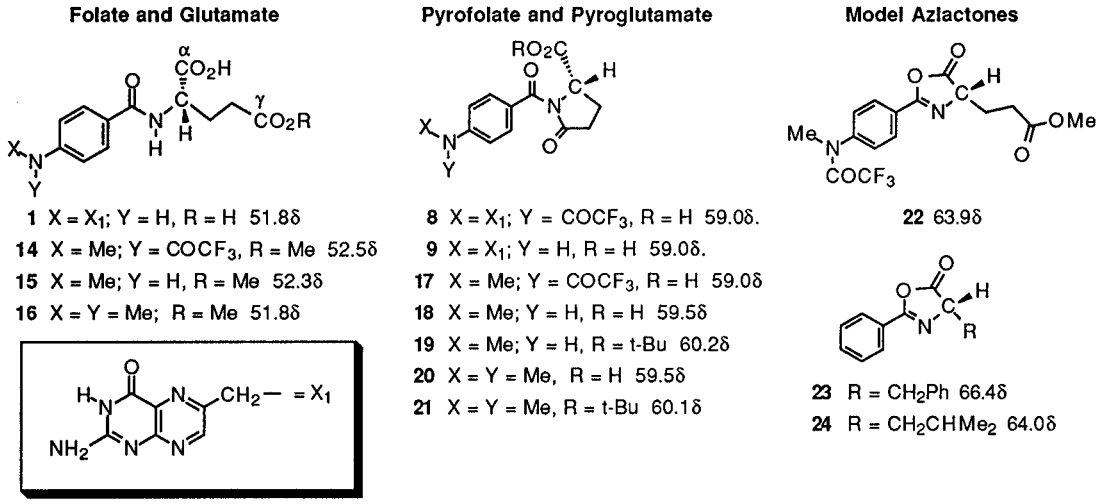


Figure 1.

Table 1. Reactions of Nucleophiles with N^{10}-(Trifluoroacetyl)pyrofolic Acid (**8**) and Pyrofolic Acid (**9**)

run	SM	nucleophile and conditions	folic acid derivative **25** (yield[a] and nucleophile)	pteroic acid derivative **26** (yield[a] and nucleophile)	folate/pteroate (**25**/**26**) ratio[a]
1	**8**	H_2O, NaOH	**25a** ≡ **1** (52%, OH)	**26a** ≡ **10** (48%, OH)	1.1
2	**9**	H_2O, NaOH	**25a** ≡ **1** (68%, OH)	**26a** ≡ **10** (32, OH)	2.1
3	**8**	H_2O, HCl	**25b**[b] (33%, OH)	**26b**[b] (56%, OH)	0.5
4	**9**	MeOH, NaOMe	**25c** (49%, OMe)	**26c** (44%, OMe)	1.1
5	**9**	MeOH, LiOMe	**25c** (81%, OMe)	**26c** (19%, OMe)	4.3
6	**9**	MeOH, DBU	**25c** (58%, OMe)	**26c** (42, OMe)	1.4
7	**8**	MeOH, TlOMe	**25c** (35%, OMe)	**26c** (58%, OMe)	0.6
8	**9**	MeOH, TlOMe	**25c** (69%, OMe)	**26c** (30%, OMe)	2.3
9	**9**	i-PrOH, TlOi-Pr	**25d** (80%, O-iPr)	**26d** (7%, O-iPr)	11.5
10	**9**	t-BuOH, TlOt-Bu	**25e** (nr,[c] Ot-Bu)	**26e** (nr,[c] Ot-Bu)	nr[c]
11	**9**	DEG,[d] NaH	**25f** (48%, DEG′[e])	**26f** (22%,[a] DEG′[e])	2.2
12	**8**	NH_2NH_2, DMSO	**25g** (0.9%, $NHNH_2$)	**26g** ≡ **13** (91%, $NHNH_2$)	0.01
13	**9**	NH_2NH_2, DMSO	**25g** (9.1%, $NHNH_2$)	**26g** ≡ **13** (90%, $NHNH_2$)	0.1
14	**8**	NH_4OH, H_2O	**25h** (33%, NH_2)	**26h** ≡ **11** (67%, NH_2)	0.5
15	**9**	NH_4OH, H_2O	**25h** (60%, NH_2)	**26h** ≡ **11** (40%, NH_2)	1.5
16	**8**	PMBA[f]	**25I** (0.9%, PMBA′[g])	**26i** (75%, PMBA′[g])	0.01

[a] Product ratio assayed by HPLC. [b] This is the only instance in which the N^{10}-trifluoroacetyl moiety survived the reaction conditions. [c] nr = no reaction. [d] DEG = $HOCH_2CH_2OCH_2CH_2OH$. [e] DEG′ = $OCH_2CH_2OCH_2CH_2OH$. [f] PMBA = *p*-methoxybenzylamine. [g] PMBA′ = *p*-methoxybenzylamino.

d_6) of the methine-bearing chiral carbon is highly diagnostic (Figure 1). Folic acid (**1**) and the truncated *p*-aminobenzoyl glutamates **14**−**16**[10] all resonate at ∼52δ. Pyrofolates **8** and **9** and model *p*-aminobenzoyl pyroglutamates **17**−**21** exhibit their methine carbons between 59 and 60δ. This can be contrasted to model azalactones **22**−**24**,[10,11] which have chemical shifts around 64−66δ.

Having secured the pyrofolate structure of derivatives **8** and **9**, we turned our attention to a brief survey of the regiospecificity of reaction of these substrates with oxygen and nitrogen nucleophiles. While the pyrofolate moiety ensures complete regioselection between the α- and γ-carbonyl groups of folic acid, the problem of differentiating between the two imide carbonyl groups (γ-CO and Pte-CO) of **8** and **9** remained to be determined. As can be seen in Table 1, thallium(I)-mediated alcoholysis provides preferential generation of the folic acid derivatives **25c,d**; the selectivity is an important function of the steric environment of the alcohol. Aminolysis is currently unacceptable for *direct* drug conjugation, but, as will be seen in Scheme 5, the acyl hydrazide **26g** (≡**13**) is an ideal intermediate for the *indirect* synthesis of folates via nitrogen acylation of glutamates with pteroyl azide (**27**). As expected, N^{10}-trifluoroacetyl derivative **8** consistently exhibits a smaller folate/pteroate ratio (**25**/**26**) relative to **9**, since increased nucleophilic attack at the benzoate carbonyl moiety is favored when the *p*-amino lone pair is unavailable for resonance deactivation. The full magnitude of this effect cannot be accurately assessed simply by inspection of Table 1, since we did not attempt to determine the rate of deacylation of the N^{10}-trifluoroacetyl group relative to nucleophilic attack at the two competing carbonyl groups (Scheme 5).

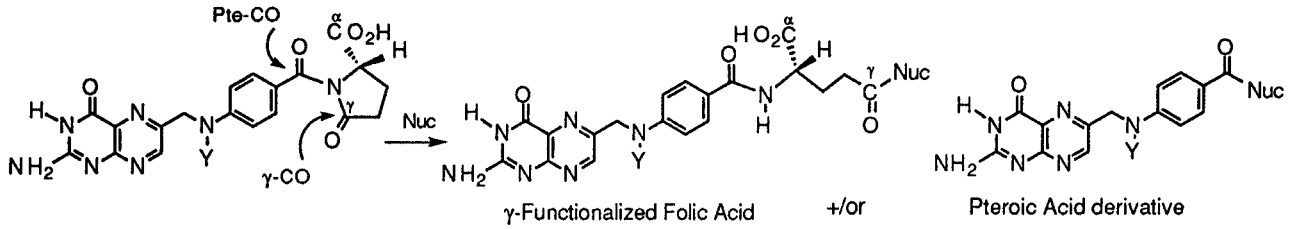
Since we had already determined that pyrofolic acid derivatives **8** and **9** were essentially racemic, we elected to pursue an indirect synthesis of the desired γ-functionalized folic acid derivatives **3** shown in Scheme 1 via exploitation of the exceptionally selective (>99:1) reaction of hydrazine with compound **8** (Table 1, entry 12). To this end, it has been observed that conversion of **13** to **27** can be conveniently effected on a reasonable (28 g) scale simply by treatment with 1.0 equiv of *tert*-butyl nitrite in trifluoroacetic acid containing 5 mol % potassium thiocyanate for 4 h at 10 °C. HPLC analysis reveals that reactions run in the absence of potassium thiocyanate, an N-nitroso transfer catalyst,[12] also produce 20−30% of N^{10}-nitrosopteroyl azide (**28**),[13] along with **27**.[14] In the KSCN-free condition, the remaining **13** slowly reacts with **28**

(10) Synthesis and characterization information for this compound is given in the Supporting Information.

(11) We thank Professor Sih for providing us samples of compounds **22** and **23**. For their synthesis, see: Crich, J. Z.; Brieva, R.; Marquart, P.; Gu, R.-L.; Flemming, S.; Sih, C. J. *J. Org. Chem.* **1993**, *58*, 3252.

(12) Singer, S. S.; Singer, G. M.; Cole, B. B. *J. Org. Chem.* **1980**, *45*, 4931.

Scheme 5

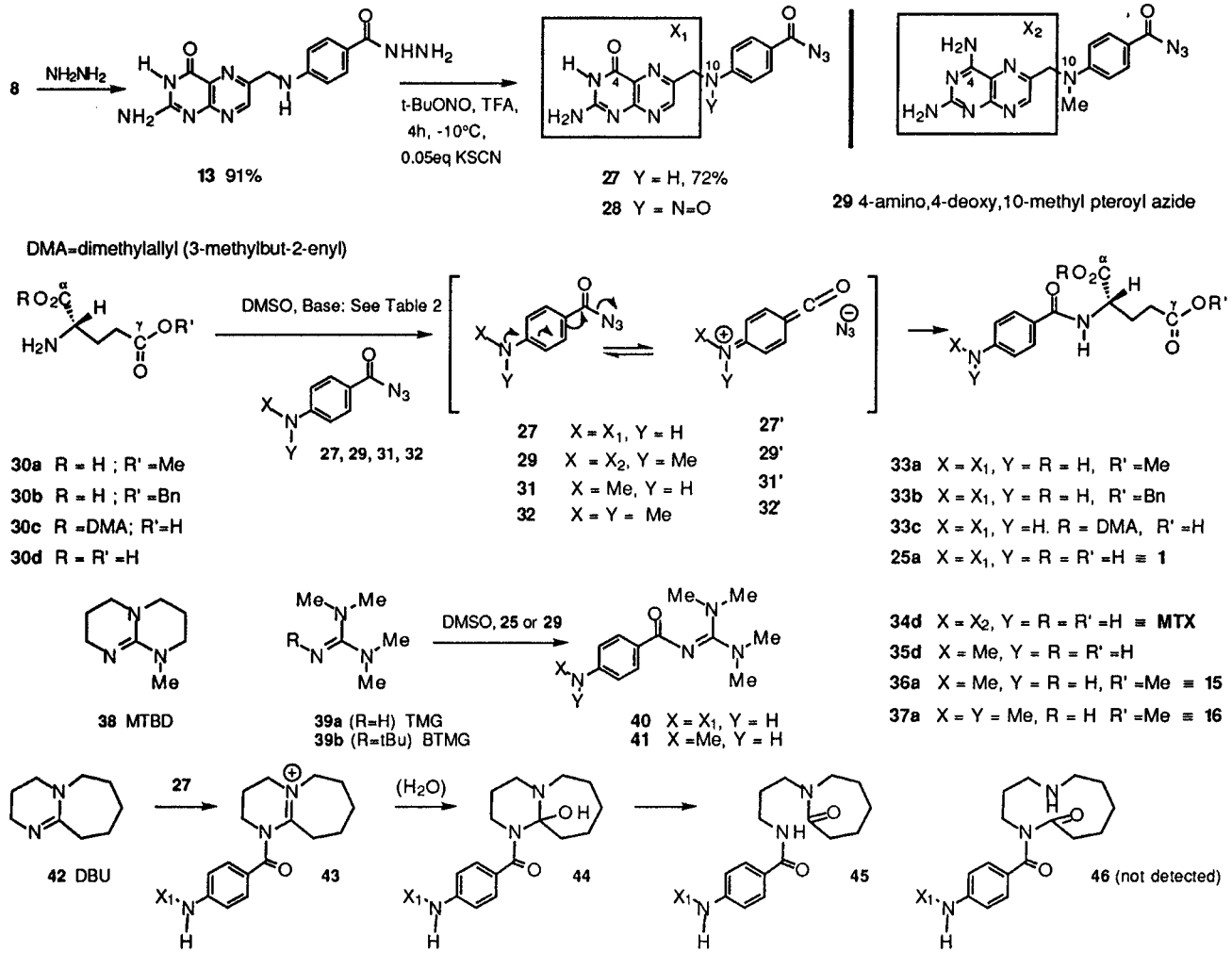


8 Y = $COCF_3$
9 Y = H

25a Y = H, Nuc = OH ≡ **1**
25b Y = $COCF_3$, Nuc=OH
25c Y = H, Nuc = OMe
25d Y = H, Nuc = Oi-Pr
25e Y = H, Nuc = Ot-Bu
25f Y = H, Nuc = OCH_2CH_2OH
25g Y = H, Nuc = $NHNH_2$
25h Y = H, Nuc = NH_2
25i Y = H, Nuc = $NHCH_2C_6H_4OMe$-p

26a Y = H, Nuc = OH ≡ **10**
26b Y = $COCF_3$, Nuc=OH
26c Y = H, Nuc = OMe
26d Y = H, Nuc = Oi-Pr
26e Y = H, Nuc = Ot-Bu
26f Y = H, Nuc = OCH_2CH_2OH
26g Y = H, Nuc = $NHNH_2$ ≡ **13**
26h Y = H, Nuc = NH_2 ≡ **11**
26i Y = H, Nuc = $NHCH_2C_6H_4OMe$-p

Scheme 6



to afford additional amounts of **27**, but the reaction produces unacceptable levels of impurities relative to the optimized procedure (Scheme 6).

Assay of **27** by HPLC reveals a purity of 91%. The known impurities include ~4% of **10**, ~2% of the aniline ($PteNH_2$), apparently resulting from Curtius rearrangement of **27**, ~2% of pteroylamide ($PteCO\text{-}NH_2$, **11**), and <1% of **1**. Pteroyl azide (**27**) may be further purified by dissolution/precipitation from

(13) Nitrosation of pteroic acid derivatives like folic and tetrahydrofolic acid is known to occur at N^{10}: Reed, L. S.; Archer, M. C. *J. Agric. Food Chem.* **1979**, *27*, 995.

(14) Large-scale reactions which use crude pteroyl hydrazide (**13**) may produce small amounts of *N*-nitrosopteroyl azide (**28**) because of the inadvertant use of an excess of *tert*-butyl nitrite. In this instance, treatment of the **27**/**28** mixture with sodium azide (see ref 12) effects smooth reduction of the N-nitroso moiety of **28** to **27**.

Table 2. Acylation of Glutamic Acid Derivatives **30a**−**d** in DMSO with Acyl Azides **27**, **29**, **31**, and **32**

run	acyl azide and glutamate	base (amt); DMSO pK_a	conditions	product, HPLC conversion, and/or (isolated yield)
1	**27** + L-**30a**	Et_3N (3 equiv); 18.5	48 h, 40 °C	L-**33a**, 23%
2	**27** + L-**30a**	Et_3N (5 equiv) ;18.5	48 h, 40 °C	L-**33a**, 25%
3	**27** + L-**30a**	TMG (2 equiv); 20	6 h, 25 °C	L-**33a**, 100% (88%)
4	**27** + L-**30a**	MTBD (2 equiv); 20	12 h, 25 °C	L-**33a**, 100%
5	**27** + L-**30a**	BTMG (2 equiv) 21	9 h, 25 °C	L-**33a**, 100%
6	**27** + L-**30a**	DBU (2 equiv); 19	8 h, 25 °C	L-**33a**, 77%
7	**27** + L-**30b**	TMG (2 equiv); 20	10 h, 25 °C	L-**33b**, 100% (60%)
8	**27** + L-**30c**	TMG (2 equiv); 20	12 h, 25 °C	L-**33c**, 100% (82%)
9	**27** + L-**30d**	TMG (3 equiv); 20	9 h, 25 °C	L-**25a** ≡ L-**1**, 100% (67%)
10	**27** + D-**30d**	TMG (3 equiv); 20	4 h, 25 °C	D-**25a** ≡ D-**1**, 100% (40%)
11	**27** + DL-**30d**	TMG (3 equiv); 20	3 h, 25 °C	DL-**25a** ≡ DL-**1**, 100% (38%)
12	**29** + L-**30d**	TMG (3 equiv); 20	1 h, 25 °C	L-**34d** (100%)
13	**31** + L-**30d**	TMG (3 equiv); 20	1 h, 25 °C	L-**35d**[34] (73%)
14	**31** + L-**30a**	TMG (2 equiv); 20	1 h, 25 °C	L-**36a** ≡ **15** (67%)
15	**32** + L-**30a**	TMG (2 equiv); 20	1 h, 25 °C	L-**37a** ≡ **16** (72%)

trifluoroacetic acid/2-isopropanol, but the known impurities do not interfere, and we routinely use the ∼90% pure material for virtually all coupling operations. Unlike some acyl azides,[15] **27** is quite stable; samples of the lemon-yellow solid may be stored in the dark in a freezer for at least 12 months without appreciable decomposition. Room temperature samples of **27** protected from light appear to have shelf lives of at least a month; while samples of **27** darken appreciably when exposed to light, their HPLC profiles are not substantially degraded.

The traditional approach to the synthesis of differentially functionalized folic acid derivatives relies on the acylation of the readily available monoesters of glutamic acid[16] with pteroic acid (**10**). Unfortunately this highly attractive strategy[17] is compromised by the difficulty of accessing reasonable quantities of the prohibitively expensive **10**[18] (∼$1000/g). Fortunately, **27** now both is easily available and serves as an excellent reagent for nitrogen acylation of differentially protected glutamates. As can be seen in Scheme 6 and Table 2, reaction of **27** in DMSO with L-glutamic acid (**30d**) or glutamates **30a**−**c** is strongly influenced by the nature of the added base, tetramethylguanidine (**39a**, TMG), *tert*-butyltetramethylguanidine (**39b**, BTMG),[19] and the expensive *N*-methyl-1,5,9-triazabicyclo[4.4.0]decene (**38**, MTBD),[20] all giving superb reactions. Presumably, the more basic nature of the guanidine bases, in concert with their ability to form soluble guanidinium carboxylates, is responsible for their ability to foster the acylation reaction. We were, therefore, surprised to note that both Rosowsky et al.[21] and Chaykovsky et al.[22] have reported that the closely related acyl azide **29** is insufficiently reactive (in DMF or DMAC) to acylate glutamates or other α-amino esters.

Our initial hypothesis was that this dramatic reactivity difference between **27** and **29** was a consequence of abstraction of the N-H proton of the *p*-aminobenzoyl azide moiety by the guanidine base, followed by 1,6-elimination of the azide anion to generate the neutral *p*-quinoketene monoimine analog of **27′** (Scheme 6, Y = lone pair; no charge on N). Presumably, such a species would be an exceptionally reactive acylating agent. Two facts mitigate against this intriguing possibility: (1) The pK_a of the N-H proton of **27** should be similar to that of *p*-methylaminomethyl benzoate, having a pK_a of 25.7 in DMSO,[23] which is fairly distant from the pK_a of H-TMG$^+$ at 13.6 in water[24] (∼20 in DMSO); consequently, the concentration of the deprotonated form of **27** would be relatively small, but still attainable; (2) A second observation which argues against **27′** (Scheme 6, Y = lone pair; no charge on N) being a requisite intermediate is that azide **29**,[21,22] which bears no N-H proton, is a perfectly fine acylating agent, providing a high yield of synthetic L-methotrexate (**34d**) upon reaction with L-glutamic acid (**30d**) and tetramethylguanidine (**39a**), provided that DMSO is employed as the reaction solvent (Table 2, entry 12). The fact that **29** reacts more rapidly with **30d** than does **27** rules out the possibility that N-H deprotonation is an integral feature in these acylation reactions.[25] Furthermore, there is no special reactivity conferred to these acyl azides by the pterin moiety, since *p*-(monomethylamino)benzoyl azide (**31**) and *p*-(dimethylamino)benzoyl azide (**32**) both react with γ-methylglutamate (**30a**) in DMSO in the presence of tetramethylguanidine to afford

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(25) Direct competition NMR studies using g-methylglutamate (**30a**, 1 equiv) and TMG (**39**, 3 equiv) in DMSO-d_6 with a 1:1 mixture of model acyl azides **31** and **32** reveal that the *p*-(dimethylamino)benzoyl azide (**32**) reacts 3 times faster with **30a** than does the (monomethylamino)benzoyl azide (**31**).

N-acyl glutamates **36a** and **37a**, respectively.[25] The question as to whether these acylations are simply proceeding via the standard tetrahedral adduct or may progress via the intermediacy of *p*-quinoketene monoiminium ions **27′**, **29′**, **31′**, and **32′** (Scheme 6) still remains to be determined.

Control studies show that in the absence of an added substrate, tetramethylguanidine (TMG, **39a**) slowly reacts with **27** or **31** to afford acylated guanidines **40** or **41**[26] (Scheme 6), but HPLC analyses of the reactions of **27** with glutamates **30a**−**d** show only traces of **40**. By comparison, HPLC analysis of a DMSO solution of **27** with pentaalkylguanidines BTMG (**39b**) and MTBD (**38**) gives no evidence of guanidine acylation or any other reaction after 18 h at 25 °C. This finding requires that, if formed, *p*-quinoketene monoiminium ion **27′** must be in ready equilibrium with the starting acyl azide **27**. DBU (pK_a in DMSO ∼19)[20c] was found to be a less effective base in these reactions (Table 2, run 5), since a portion of the **27** was consumed via an unprofitable acylation−fragmentation sequence,[27] providing byproduct **45** in 20% yield.

Since it had been previously observed that guanidine bases are capable of racemizing acylated glutamates (under more forcing conditions than were employed in the above table),[28] we needed to evaluate the optical purity of the folic acid (=**25a**) and several of its derivatives which had been produced. For this purpose we elected to employ the enzyme carboxypeptidase G,[29] which has been shown to only deacylate the natural L-enantiomer of folic acid (**1**) to L-glutamic acid and pteroic acid (**10**), the yield of the latter material being determined by HPLC. In order to assay the pyrofolates **8** and **9** as well as the α- and γ-monoesters of folic acid L-**33a** and L-**33c**, an initial mild basic hydrolysis was required prior to the enzyme assay. As can be seen in Table 3 (runs 1 and 2), the synthetic folic acid L-**1** (=**25a**) was produced in enantiomerically pure form (within the limits of experimental error). Control reactions on synthetic D-**1** (=**25a**) and DL-**1** (=**25a**) (Table 3, runs 3 and 4) serve to confirm the error limits of the enzymatic method at about ±2−3%.

Synthesis of bis-allylated glutamates **51a,b** was initiated via reaction of L-glutamic acid (**30d**) with allyl chloroformate to afford N-alloc-protected glutamic acid (**47**). This material was not purified but directly reacted with paraformaldehyde under the standard conditions[16] to provide N-alloc oxazolidinone (**48**) in 50% overall yield for the two steps. Reaction of this substrate with slightly less than 2 equiv of sodium metal in the presence of an excess of allyl (**49**) or dimethylallyl alcohol (**50**) provides α-allylated and α-dimethylallylated N-alloc glutamates **51a,b,** in 79 and 71% yield, respectively. Use of an excess of sodium results in partial racemization of glutamate **51b**; in this instance, 2.1 equiv of sodium generated a product which was only 85.9% optically pure (Table 3, run 10a), while employment of the 1.9 equiv conditions afforded material which was 96.7% optically pure as judged by the enzymatic assay (Table 3, run 10b). Since the synergistic value of palladium(0)-mediated deprotection of the allyl and dimethylallyl protecting groups has been convincingly demonstrated for N-alloc esters by the Genet group,[30] we adopted this superb strategy for cleavage of **51a,b**. As can be seen in Scheme 7, reaction of **51a** with diethylamine and 10 mol % palladium tetrakistriphenylphosphine provides a quantitative yield of **30d**, while similar reaction of dimethyl allyl ester **51b** affords a 70% yield of α-DMA-protected glutamate **30c**. As indicated above, both these materials are shown to be essentially optically pure, as assayed after their conjugation (69−82%) with pteroyl azide (**27**), to afford **25a** (=L-**1**) and L-**33c**, respectively (Scheme 7 and Table 3, entries 3 and 10b).

Table 3. Carboxypeptidase G Enantiospecific Hydrolysis of Folic Acid Derivatives

run	compound (source)	% hydrolysis
1	L-**1** (commercial[a])	96.7
2	L-**25a** ≡ L-**1** (from Table 2)	98.4
3	L-**25a** ≡ L-**1** (from Table 3)	98.6
4	D-**25a** ≡ D-**1** (from Table 2)	3.0
5	DL-**25a** ≡ DL-**1** (from Table 2)	52.3
6	L-**34d** (NCI MTX[b])	96.1[c]
7	L-**34d** (MTX from Table 2)	99.2[c]
8	DL-**33a** (from Table 1)	54.4
9	L-**33a** (from Table 2)	96.8
10a	L-**33c** (from Table 2)	85.9
10b	L-**33c** (from Table 2 Scheme 7)	96.7
11	**8** (from Scheme 2)	49.5
12	**9** (from Scheme 2)	51.7

[a] Commercial L-1 from Vitamins, Inc. [b] A sample of L-methotrexate (L-**34d**) was provided by the National Cancer Institute. [c] D-**34d** is known not to be hydrolyzed by carboxypeptidase G.[35]

The value of **27** for the synthesis of folic acid analogs is exemplified in the preparation of DTPA folate (γ) (**53**), a metal-binding ligand of an important new imaging agent with outstanding tumor specificity (Scheme 7).[31,32] The initial sample of **52**, the precursor of **53**, was prepared via the direct DCC/NHS coupling of ethylenediamine with **1**, followed by separation of the various reaction components by extensive HPLC, the final yield of the requisite γ-conjugate **52 (**Scheme 1, **3**, X−Z = $NHCH_2CH_2NH_2$) being on the order of 10−15%, on small scale.[32] Our current synthesis of **52,** which is far superior in practical terms, now simply involves the reaction of synthetic γ-methylfolate (L-**33a**, Table 2, run 3, 1.1 equiv) with neat ethylenediamine (50 equiv) for 3 h at 25 °C to afford **52** as a yellow solid in 87% yield, without the need of chromatography. Conversion of **52** to the tumor-specific metal-binding ligand DTPA folate (γ) (**53**) has been previously described (Scheme 8).[31]

In conclusion, we have provided the first efficient syntheses of racemic N^{10}-(trifluoroacetyl)pyrofolic acid (**8**), racemic pyrofolic acid (**9**), pteroyl hydrazide (**13**), and pteroyl azide (**27**). The latter reagent is an effective and economical reagent for the synthesis of differentially functionalized folic acid deriva-

(26) Although simple acetyl and benzoyl tetramethylguanidines show equivalence of the four methyl groups in the ^{1}H-NMR at 25 °C (see (a) Matsumoto, K.; Rapoport, H. *J. Org. Chem.* **1968**, *33*, 552. (b) Kantlehner, W.; Jaus, H.; Kienitz, L.; Bredereck, H. *Liebigs. Ann. Chem.* **1979**, 2096. (c) Kessler, H.; Leibfritz, D. *Liebigs. Ann. Chem.* **1970**, *737*, 53), a Hamett study (see Kessler, H.; Leibfritz, D. *Tetrahedron* **1970**, *26*, 1805) on *p*-substituted *N*-phenyltetramethylguanidine derivatives reveals that electron-releasing substituents increase the NMR colalesence temperature to near 0 °C. This is consistent with our observations on the NMR of pteroyl-substituted tetramethyl guanidine (**40**). The ^{1}H-NMR shows two broad N-methyl singlets, while the ^{13}C-NMR reveals eight closely separated N-methyl absorptions, suggesting the presence of both *E* and *Z* conformational diastereomers (see Supporting Information).

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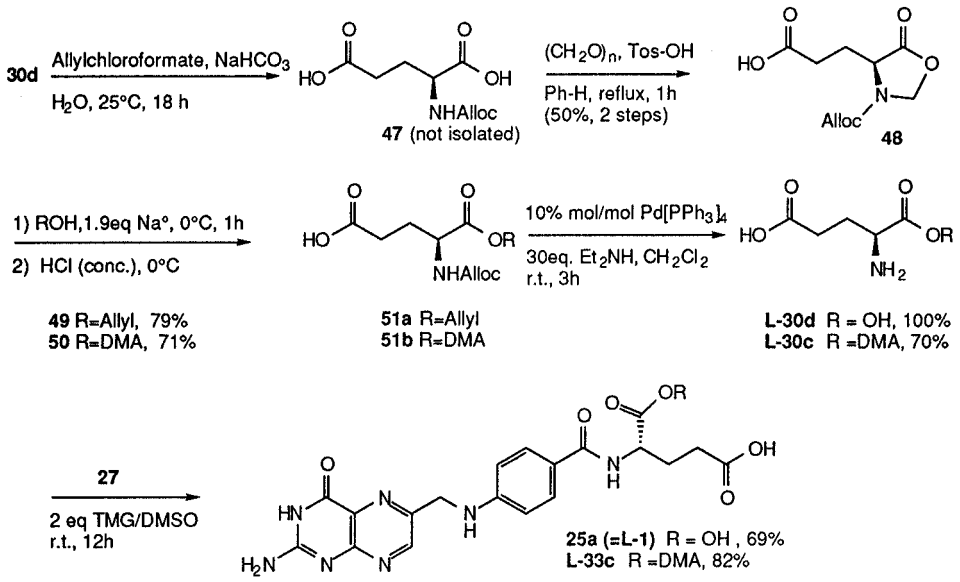
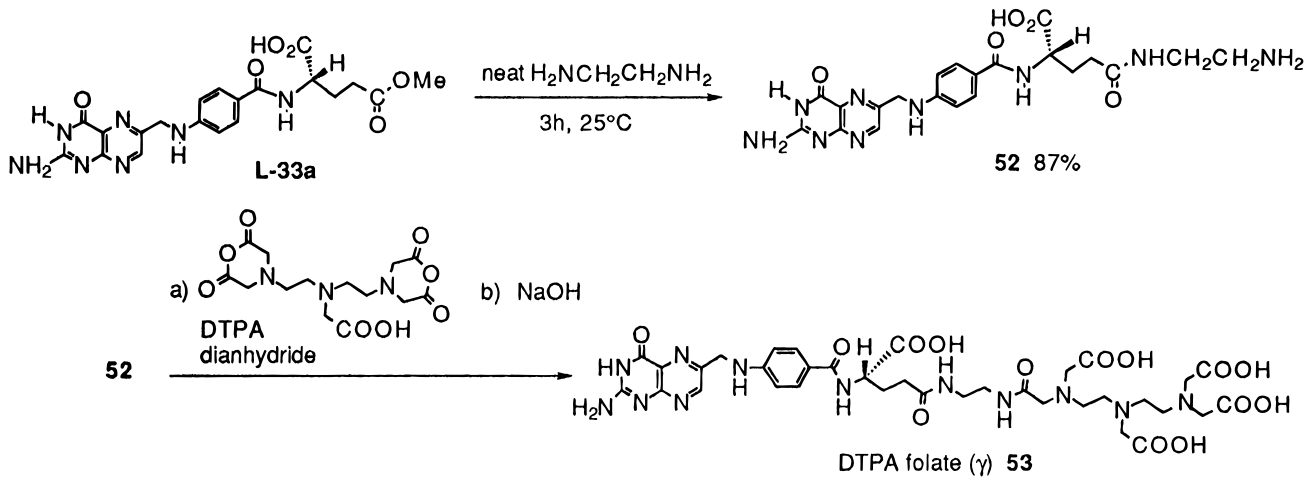
Scheme 7



Scheme 8



tives. Application of this technology for the synthesis of new folate−anticancer drug conjugates is under active investigation.

Experimental Section

General Methods. Melting points were obtained on a MEL-TEMP apparatus and are uncorrected. Unless otherwise stated, reactions were carried out under argon in flame-dried glassware. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone ketyl. Dichloromethane and benzene were distilled from calcium hydride. Cyclohexane was stored over sodium metal. Deuterated NMR solvents ($CDCl_3$ and CD_3CN) were stored over 4 Å molecular sieves for several days prior to use. Flash chromatography on silica gel was carried out as described by Still[33] (230−400 mesh silica gel was used), and reversed phase LC was used for preparative purposes (LiChroprep C-18, 310 mm × 25 mm). ^{1}H- and ^{13}C-NMR spectra were obtained using GE QE-300 NMR and Varian Gemini 200 NMR spectrometers at 300 or 200 MHz and 75 or 50 MHz respectively. ^{1}H-NMR chemical shifts are reported in ppm relative to the residual protonated solvent resonance: $CHCl_3$, δ 7.26; C_6D_5H, δ 7.15. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; br, broadened. Coupling constants (δ) are reported in Hertz. ^{13}C-NMR chemical shifts are reported in ppm relative to solvent resonance: $CDCl_3$, δ 77.00; C_6D_6, δ 128.00. Mass spectral data were obtained on a Finnigan 4000 mass spectrometer (low resolution) and a CEC 21 110 B high-resolution mass spectrometer, with the molecular ion designated as M. All the chemicals were supplied by Aldrich Chemical Co., Inc, Milwaukee, WI, unless otherwise indicated.

Typical Experimental Procedure for Coupling of Pteroyl Azide (27) with Glutamates. To a stirred DMSO suspension of equal molar amounts of pteroyl azide (**27**, 0.02−0.3 M) and a glutamate (Table 2) was added 2 (3 for glutamic acid) equiv of tetramethylguanidine at room temperature. The mixture soon became homogeneous, and the reaction was normally complete within 12 h as indicated by analytical HPLC. The solution was filtered through a pad of Celite to remove any traces of solid residue, and acetonitrile was slowly added to the stirred filtrate. The precipitated solid was centrifuged to give the crude product after washing with diethyl ether and drying 18 h under vacuum. When appropriate, preparative LC was used to provide a purer sample. The procedure is exemplified below by the synthesis of folic acid (L-**1**) and tetramethylguanidinium L-methyl folate (γ) (**33a**).

Synthetic L-1 (=25a). To a suspension of **27** (200 mg, 0.590 mmol) and L-glutamic acid (131 mg, 0.890 mmol) in DMSO (30 mL) was added neat tetramethylguanidine (0.222 mL, 1.77 mmol). The stirred mixture soon became homogeneous, and the reaction was complete after 9 h, as indicated by analytical HPLC. The solution was filtered through a pad of Celite to remove any traces of solid residue, and acetonitrile (50 mL) was slowly added to the stirred filtrate. The precipitated solid was then centrifuged to give the crude folic acid (248 mg) after washing with aqueous 1% HCl solution (10 mL × 1), acetonitrile (10 mL × 1), and diethyl ether (25 mL × 2) and drying 18

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h under vacuum. An analytical sample (174 mg, 67%) was obtained using preparative reversed-phase HPLC. ^{1}H-NMR, UV, and analytical HPLC were all the same as those of commercial folic acid. Decomposition point, ~238 °C. $[\alpha]^{25}_D = +17.4°$ ($c = 0.5$ in 0.1 N NaOH); 98.4% of L-folic acid by the enzyme assay. Analytical HPLC: $t_R =$ 7.2 min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

Determination of Optical Purity of Folate Derivatives Listed in Table 3 Using Enzymatic Hydrolysis with Carboxypeptidase G.[29] The folic acid derivative (~2.5 mg) was dissolved in 1 mL of TRIS buffer containing 20 μg of $ZnCl_2$, and 50 microunits of enzyme (Sigma Chemical Co.) was added. The solution was incubated at 37 °C for 2 h. A 20 μL sample was tested by HPLC on a 4.6 mm × 250 mm Microsorb C18 reversed-phase column (eluent A, 5 mM phosphate buffer; eluent B, acetonitrile; flow rate, 1 mL/min; gradient, 0−5 min, 5% B, 10−15, 25% B). Folic acid was eluted at 4.1 min, pteroic acid at 10.6 min, methotrexate at 10.8 min, and amino-N^{10}-methylpteroic acid at 11.9 min. Peak area was used as the standard for analysis.

For folate derivatives, ~5 mg was first dissolved in 1 mL of 0.25 M NaOH and incubated at room temperature for 1 h. The solution was then acidified by 1 M HCl until precipitation. After centrifugation, washing, and drying, the ~2.5 mg yellow pellet was dissolved in 1 mL of TRIS buffer and reacted with enzyme under the conditions described above.

***N*^{2,10}-Bis(trifluoroacetyl)pyrofolic Acid/Anhydride (6/7).** To a mechanically stirred suspension of **1** (100 g, 0.23 mol, U.S.P. grade, supplied from Vitamins Inc., Chicago, IL) and anhydrous tetrahydrofuran (1000 mL) in a three-neck flask was slowly added trifluoroacetic anhydride (256 mL, 1.81 mol) at 0 °C over ~0.5 h before warming the solution to 25 °C. The mixture gradually turned into a dark brown homogeneous phase as the reaction proceeded. After 10 h, analytical HPLC showed that the reaction was complete by the appearance of a number of peaks (presumably a mixture of **7** and other trifluoroacetylated pyrofolic acids) and confirmed the absence of **1**. The solution was filtered through a pad of Celite to remove a small amount of solid residue. Using a rotary evaporator, the filtrate was concentrated to a dark brown viscous liquid (~300 mL), which was slowly transferred with the aid of tetrahydrofuran (~20 mL) to a flask of well-stirred benzene (1500 mL). The precipitated yellowish solid was collected by filtration and washed with diethyl ether (250 mL × 1) to yield crude product **6/7** (151 g). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.89 (s, 1H, C7-H), 7.65 (s, 4H, Ar), 5.25 (s, 2H, C9-H_2), 4.71 (dd, $J = 4.2$, 8.9 Hz, C19-H), 2.59−1.98 (overlap, 4H). ^{13}C-NMR (200 MHz, DMSO-d_6): δ 174.4, 172.6, 170.9, 169.1, 166.0, 165.3, 159.8, 159.1, 159.0, 158.3, 157.5, 156.4, 155.7, 155.1, 149.2, 147.4, 142.2, 135.1, 130.1, 129.4, 128.4, 124.9, 124.8, 119.2, 118.1, 113.5, 112.4, 107.7, 106.6, 58.7, 54.1, 31.4, 21.6. ^{19}F NMR (300 MHz, DMSO-d_6): δ −65.66, −74.13, −80.47 (integration ratio 1.0:0.93:0.13). Analytical HPLC: $t_R = 12.7$ min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

***N*^{10}-(Trifluoroacetyl)pyrofolic Acid (8).** The crude $N^{2,10}$-bis-(trifluoroacetyl)pyrofolic acid/anhydride (**6/7**, 150 g) was dissolved in tetrahydrofuran (500 mL), followed by addition of ice (~100 g) with stirring. Analytical HPLC indicated that all the original peaks converged after ~3 h at 25 °C into a single one (N^{10}-(trifluoroacetyl)-pyrofolic acid (**8**)). The mixture was then slowly transferred to efficiently stirred diethyl ether (2000 mL). The precipitated yellowish powder was collected by filtration, triturated with diethyl ether, washed thoroughly with diethyl ether (200 mL × 3), and dried 18 h under vacuum, giving **8** (123 g) in a quantitative yield from **1**. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.64 (s, H, C7-H), 7.62 (s, 4H, Ar), 5.12 (s, 2H, C9-H_2), 4.70 (dd, $J = 3.2$, 4.9 Hz, 1H, C19-H), 2.54−2.43 (overlap, 4H). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 176.8, 174.7, 172.9, 169.4, 161.2, 156.9, 156.4, 156.0, 155.8, 155.5, 154.5, 149.8, 149.7, 145.1, 142.5, 135.3, 130.3, 128.7, 118.5, 114.7, 110.9, 59.0, 54.4, 31.8, 21.9. ^{19}F-NMR (300 MHz, DMSO-d_6): δ −65.2. Mass spectrum (FAB): m/z 519 (MH^+). HRMS: calcd for $C_{21}H_{16}F_3N_7O_6$, 519.1114; found, 519.1112. Softens and decomposes at 208−214 °C. Elemental analysis calcd for $C_{21}H_{26}F_3N_7O_6 \cdot 0.5H_2O$: H, 3.24; C, 47.73; F, 10.79; N, 18.65. Found: H, 3.04; C, 47.63; F, 10.72; N, 18.64. $[\alpha]^{25}_D = +2.9°$ ($c =$ 0.5 in DMSO) (cf. commercial folic acid from Vitamins, Inc. = +14.4° at the same concentration). Analytical HPLC: $t_R = 11.9$ min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

Pyrofolic Acid (9). To a stirred homogeneous solution of N^{10}-(trifluoroacetyl)pyrofolic acid (**8**, 14 g, 27 mmol) and DMF (250 mL) was slowly added aqueous cesium carbonate (10 mL, 82 mmol) at 25 °C. Analytical HPLC indicated completion of the reaction by disappearance of **8** in 5 h. The mixture was filtered through a pad of Celite, and the filtrate was carefully acidified to pH 4 with 5% aqueous hydrochloric acid. The resulting precipitate was thoroughly washed with water (100 mL × 3) by centrifugation, acetonitrile (100 mL × 1), and diethyl ether (100 mL × 2) by aspirator filtration. The yellowish product **9** (10.2 g, 89%) was obtained after drying for 24 h at 60 °C under vacuum. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.64 (s, H, C7-H), 7.41 (d, $J = 8.3$ Hz, 2H, Ar), 6.58 (d, $J = 8.3$ Hz, 2H, Ar), 4.70 (m, 1H, C19-H), 4.47 (d, $J = 4.7$ Hz, 2H, C9-H_2), 2.57−1.80 (overlap, 4H). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 174.0, 172.8, 169.1, 161.2, 153.9, 152.2, 148.7, 148.4, 132.4, 132.3, 128.1, 120.4, 110.6, 59.0, 45.8, 31.6, 21.6. Mass spectrum (FAB): m/z 424 (MH^+). HRMS: calcd for $C_{19}H_{17}N_7O_5$ 424.1369; found, 424.1365. Mp = ~269 °C dec. $[\alpha]^{25}_D = -1.2°$ ($c = 0.5$ in DMSO). Analytical HPLC: $t_R = 9.0$ min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

Pteroylamide (11) and Pyroglutamic Acid (12). N^{10}-(Trifluoroacetyl)pyrofolic acid (**8**, 540 mg, 1.04 mmol) was dissolved in aqueous concentrated ammonium hydroxide solution (25 mL). As the reaction proceeded, yellowish solid precipitated. After 14 h, the solid powder **11** (217 mg, 67%) was isolated by filtration and washed with water (20 mL × 3), methanol (20 mL × 1), and ether (25 mL × 2) and dried under vacuum. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.63 (s, 1H, C7-H), 7.61 (d, $J = 8.3$ Hz, 2H, Ar), 6.68 (d, $J = 8.3$ Hz, 2H, Ar), 4.44 (d, $J = 5.2$ Hz, 2H, C9-H_2), ^{13}C-NMR (300 MHz, TFA/inserted DMSO-d_6 tube): δ 172.6, 158.6, 151.5, 149.6, 146.7, 145.9, 138.0, 132.6, 130.2, 125.8, 122.9, 53.2. Mass spectrum (FAB): m/z 311 (M^+). HRMS: calcd for $C_{14}H_{13}N_7O_2$, 312.1209 (MH^+); found, 312.1205. Decomposition point, ~293 °C. Analytical HPLC: $t_R = 9.8$ min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm). To isolate pyroglutamic acid **12**, the solvent of the above aqueous filtrate was removed by a rotary aspirator, and the residue was then purified using preparative HPLC to give **12** (112 mg, 84%). ^{1}H-NMR was the same as that one of commercial pyroglutamic acid. $[\alpha]^{25}_D = -0.74°$ ($c = 1.84$ in water). Mp = 154−158 °C. Pyrofolic acid (**9**, 100 mg, 0.236 mmol), in a similar treatment with ammonium hydroxide, yielded **11** (11 mg, 15%) and **12** (23 mg, 92%). For **11**, $[\alpha]^{25}_D = +0.09°$ ($c = 0.46$ in water).

Pteroyl Hydrazide (13). N^{10}-(Trifluoroacetyl)pyrofolic acid (**8**, 49 g, 94 mmol) was dissolved in DMSO (1000 mL) with mechanical stirring. To this homogeneous solution was added hydrazine (30 mL, 0.94 mol) while maintaining the temperature at 25 °C. During the process, the flask was immersed in a water bath at 25 °C, and the hydrazine was added slowly in order to moderate a gentle exotherm. The reaction was complete after 8 h, as indicated by analytical HPLC, and the mixture was filtered through a pad of Celite to remove a trace of solid residue. To the filtrate was then slowly added methanol (1000 mL), and the resulting precipitated solid was collected by aspirator filtration (or centrifugation) and washed thoroughly with methanol (200 mL × 3), followed by diethyl ether (200 mL × 2), to yield crude product **13** (28 g, 91%) after drying for 18 h under vacuum. ^{1}H-NMR (300 MHz, D_2O/NaOD): δ 7.91 (s, H, C7-H), 6.98 (d, $J = 8.5$ Hz, 2H, Ar), 6.47 (d, $J = 8.5$ Hz, 2H, Ar), 4.04 (s, 2H, C9-H_2). ^{13}C-NMR (300 MHz, DMSO-d_6/concentrated HCl ~10/1 v/v): δ 166.3, 158.6, 152.9, 152.8, 152.2, 148.1, 146.9, 130.1, 128.6, 117.9, 112.1, 46.0. Mass spectrum (FAB): m/z 327 (MH^+). HRMS: calcd for $C_{14}H_{14}N_8O_2$, 327.1318; found, 327.1307. Decomposition point, ~291 °C. Analytical HPLC: $t_R = 14.2$ min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; isocratic, 5% B; column, Econosphere C18, 150 mm × 4.6 mm).

DL-Methyl Folate (γ) 25c (=33a)). To a stirred suspension of pyrofolic acid (**9**, 1.1 g, 2.6 mmol) and methanol (150 mL) was added lithium hexamethyldisilazide (1.0 M in THF, 7.8 mL, 7.8 mmol) at −10 °C. The mixture soon became homogeneous, and the reaction was complete in 9 h at this temperature, as indicated by analytical LC. The reaction was quenched by adding acetic acid (5 mL) at −78 °C. The yellow precipitate was isolated by centrifugation and then purified by preparative reversed-phase HPLC to give the methyl folate **25c** (804 mg, 70%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.60 (s, 1H, C7-H), 7.91 (d, J = 7.3 Hz, 1H, N18-H), 7.58 (d, J = 8.4 Hz, 2H, Ar), 7.25 (s, 2H, C2-NH_2), 6.90 (t, J = 5.3 Hz, 1H, N10-H), 6.60 (d, J = 8.4 Hz, 2H, Ar), 4.45 (d, J = 5.3 Hz, 2H, C9-H_2), 4.22 (dd, J = 7.6, 12.3 Hz, 1H, C19-H), 3.51 (s, 3H, OCH_3), 2.47 (t, J = 1.7 Hz, 2H, C21-H_2), 2.36−1.88 (m, 2H, C20-H_2). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 174.9, 173.7, 166.7, 162.1, 156.6, 154.8, 151.2, 149.0, 148.9, 129.4, 128.3, 122.0, 111.8, 52.9, 51.8, 46.4, 30.7, 27.3. LRMS (PDMS) for $C_{20}H_{21}N_7O_6$ (MH^+): calcd, 455, found, 455.5. Decomposition point ~251 °C. Analytical HPLC: t_R = 10.4 min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile, gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

Pteroyl Azide (27) and N^{10}-Nitrosopteroyl Azide (28). To a stirred suspension of pteroyl hydrazide (**13**, 28 g, 86 mmol) and potassium thiocyanate (0.41 g, 4.2 mmol) was added ice-cold trifluoroacetic acid (220 mL). After the solid dissolved, the reaction mixture was cooled to −10 °C, followed by slow addition of neat *tert*-butyl nitrite (10 mL, 86 mmol). Monitoring by analytical HPLC indicated that the reaction was complete in 4 h, after which time the reaction was warmed to 25 °C. In addition to the desired pteroyl azide (**27**), analytical HPLC sometimes showed generation of a small amount (~10%, due to 90% purity of pteroyl azide (**27**)) of N^{10}-nitrosopteroyl azide (**28**), which could be instantly converted into **27** at 25 °C simply by addition of sodium azide (0.5 equiv per equiv of **13** used in the reaction) to the reaction mixture. The solution was then filtered through a pad of celite to remove a trace of solid residue. Slow addition of 2-propanol (250 mL) to the stirred filtrate led to an orange powder, which was collected by centrifugation, washed thoroughly with water (500 mL × 3), acetonitrile (500 mL × 1), and diethyl ether (200 mL × 2), and finally dried for 24 h under vacuum. Multiple cycles of the precipitation process were used in the trifluoroacetic acid−2-propanol combination to obtain material of even higher purity (known impurities include ~4% of pteroic acid (**10**), 2% of the aniline apparently resulting from Curtius rearrangement of **27**, ~2% of pteroylamide (**11**), and <1% of folic acid.). Normally, a purity of >90% (determined by HPLC) was achieved after a single purification cycle. The **27** (21 g, 72%) thus obtained was stored at −15 °C with protection from light.

27. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.63 (s, 1H, C7-H), 7.65 (d, J = 8.7 Hz, 2H, Ar), 7.57 (m, 1H, N10-H), 6.67 (d, J = 8.7 Hz, 2H, Ar), 4.49 (d, J = 5.8 Hz, 2H, C9-H_2). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 171.0, 158.6, 153.9, 152.7, 152.5, 148.3, 146.8, 131.8, 128.3, 117.6, 112.2, 45.8. Mass spectrum (FAB): m/z 338 (MH^+). HRMS: calcd for $C_{14}H_{11}N_9O_2$, 338.1114; found, 338.1110. Decomposition point, ~180 °C. Analytical HPLC: t_R = 18.0 min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

28. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.74 (s, 1H, C7-H), 8.08 (d, J = 8.6 Hz, 2H, Ar), 7.91 (d, J = 8.6 Hz, 2H, Ar), 5.52 (s, 2H, C9-H_2). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 171.6, 160.3, 153.8, 153.3, 149.3, 146.7, 145.1, 131.1, 129.0, 128.5, 120.0, 46.9. Analytical HPLC: t_R = 19.2 min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 50 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 2% B; 25 min, 50% B; column, Econosphere C18, 150 mm × 4.6 mm).

4-{[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methyl]amino}benzoyl Azide (29).[22b] A solution of **56** (100 mg, 0.31 mmol), diphenylphosphoryl azide (0.13 mL 0.46 mmol), and Et_3N (0.09 mL, 0.62 mmol) in DMSO (5 mL) was stirred for 20 h. THF (10 mL) was then added, and the precipitate was filtered to give **29** (92 mg, 83%). The ^{1}H-NMR spectrum is identical to the one in the literature.[22b]

Tetramethylguanidinium L-Methyl Folate (γ) (33a, R = TMG-H$^+$). To a suspension of **27** (5 g, 14.8 mmol) and methyl glutamate (γ) (2.63 g, 16.3 mmol) in DMSO (50 mL) was added neat tetramethylguanidine (3.7 mL, 29.7 mmol). The stirred mixture soon became homogeneous, and the reaction was complete after 6 h, as indicated by analytical HPLC. The solution was filtered through a pad of Celite to remove a trace of solid residue, and acetone (400 mL) was slowly added to the stirred filtrate. The precipitated solid was then filtered to give the crude tetramethylguanidinium salt of methyl folate (γ) (**33a**, R = TMG-H$^+$) (6.1 g, 88%) after washing with diethyl ether (50 mL × 2) and drying for 18 h under vacuum. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.58 (s, 1H, C7-H), 7.55 (d, J = 8.4 Hz, 2H, Ar), 6.87 (t, J = 5.3 Hz, 1H, N10-H), 6.62 (d, J = 8.4 Hz, 2H, Ar), 4.41 (d, J = 5.3 Hz, 2H, C9-H_2), 3.98 (dd, J = 7.6, 12.3 Hz, 1H, C19-H), 3.49 (s, 3H, OCH_3), 2.81 (s, 12H, teramethylguanidine), 2.40−2.18 (m, 2H, C21-H_2), 2.17−1.78 (m, 2H, C20-H_2). Mass spectrum (FAB): m/z 454 (M − H$^+$). HRMS: calcd for $C_{20}H_{21}N_7O_6$, 454.1475; found, 454.1445. Spiking experiments in ^{1}H-NMR and analytical HPLC showed this material to be identical to **25c**, except for the added presence of TMG.

***N*-Alloc-glutamic Acid (47).** To 50 g (340 mmol) of L-glutamic acid in 340 mL of distilled water was added 72 g (857 mmol) of sodium bicarbonate in several portions. After the bubbling ceased, 56 mL (510 mmol) of allyl chloroformate was added all at once and the solution was left to stir for 18 h at 25 °C. The pH was adjusted to 1 using concentrated hydrochloric acid and assayed using pH paper. The solution was transferred to a separatory funnel and extracted 10 times with ethyl acetate. The combined organics were rinsed with brine and dried over magnesium sulfate. The solvent was removed *in vacuo* to give 57 g (73%) of crude **47** as a colorless syrup.

3-[3-((Allyloxy)carbonyl)-5-oxo-1,3-oxazolan-4-yl]propanoic Acid (48). **47** (56.9 g, (246 mmol), paraformaldehyde (16 g 492 mmol), *p*-toluenesulfonic acid (4.7 g (24.6 mmol), and benzene (1.2 L) were combined in a round-bottom flask equipped with a stir bar and a Dean−Stark trap and heated at reflux for 1 h. The solvent was removed *in vacuo*, and the residue was purified by column chromatography (SiO_2 4:1 hexane/ethyl acetate) to afford **48** (40.6 g, 68%) as a white solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.90 (m, 1H), 5.55 (m, 1H), 5.30 (m, 1H), 4.66 (d, J = 5.85 Hz, 2H), 4.41 (t, J = 6.01 Hz, 1H), 2.54 (t, J = 6.95 Hz, 2H), 2.28 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 177.4, 171.6, 152.9, 131.4, 118.7, 77.6, 66.8, 53.7, 28.9, 25.4. Mass spectrum (CI): 244 (M + H$^+$, base peak). HRMS: calcd for $C_{10}H_{13}NO_6$, 244.0821; found, 244.0823.

***N*-Alloc α-Allylglutamate (51a).** Elemental sodium (0.43 g, 18.7 mmol) was added in small pieces to 197 mL of allyl alcohol cooled to 0 °C under argon in a flame-dried round-bottom flask. After all the sodium had dissolved, 2.39 g (9.83 mmol) of oxazolidinone **48** in 20 mL of allyl alcohol was cannulated into the solution of alkoxide. After the solution was stirred for 1 h at 0 °C, the pH was adjusted to 1 using concentrated hydrochloric acid and pH paper. The solution was transferred to a separatory funnel, diluted with an equal volume of water, and extracted 6 times with ethyl acetate. The combined organics were rinsed with brine and dried over magnesium sulfate. The solvent was removed *in vacuo*, and the residue was purified by column chromatography (SiO_2, 4:1 hexane/ethyl acetate) to afford **51a** (2.1g, 79%) as a pale yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.85 (m, 2H), 5.43 (d, J = 7.98 Hz, N-H), 5.26 (m, 4H), 4.64 (d, J = 5.75 Hz, 2H), 4.57 (d, J = 5.33 Hz, 2H), 4.42 (m, 1H), 2.48 (m, 2H), 2.24 (m, 1H), 2.0 (m, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 176.8, 171.4, 155.8, 132.1, 131.0, 118.2, 117.2, 65.5, 65.4, 52.8, 29.4, 26.4. Mass spectrum (CI): m/z 272 (M + H$^+$, base peak). HRMS: calcd for $C_{12}H_{17}NO_6$, 272.1134; found, 272.1142.

***N*-Alloc α-Dimethylallylglutamate (51b).** Elemental sodium (0.54 g, 23.5 mmol) was added in small pieces to 250 mL of allyl alcohol cooled to 0 °C under argon in a flame-dried round-bottom flask. After all the sodium had dissolved, 3.0 g (12.3 mmol) of **48** in 25 mL of allyl alcohol was cannulated into the solution of alkoxide. After the solution was stirred for 1 h at 0 °C, the pH was adjusted to 1 using concentrated hydrochloric acid as monitored using pH paper. The solution was transferred to a separatory funnel, diluted with an equal volume of water, and extracted 6 times with ethyl acetate. The combined organics were rinsed with brine and dried over magnesium sulfate. The solvent was removed *in vacuo*, and the remaining liquid was distilled under vacuum to remove the excess dimethylallyl alcohol (0.10 mmHg, bp 39 °C). The residue was purified by column

chromatography (SiO_2, 4:1 hexane/ethyl acetate) to afford **51b** (2.6 g, 71%) as a pale yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.90 (m, 1H), 5.42 (d, J = 8.0 Hz, N-H), 5.30 (m, 3H), 4.64 (d, J = 7.31 Hz, 2H), 4.57 (d, J = 5.56 Hz, 2H), 4.40 (m, 1H), 2.47 (m, 2H), 2.22 (m, 1H), 1.98 (m, 1H), 1.76, (s, 3H), 1.71 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$), δ 177.0, 171.8, 155.8, 139.5, 132.2, 117.6, 117.4, 65.5, 62.1, 52.9, 29.6, 27.0, 25.3, 17.6. Mass spectrum (CI): m/z 300 ($M + H^+$, base peak). HRMS: calcd for $C_{14}H_{21}NO_6$, 300.1447; found, 300.1459.

2-Aminoethylfolic Acid, γ-Amide (52). To the crude tetramethylguanidinium l-methyl folate (γ) (**33a**, R = TMG-H^+) (2.8 g, 5.95 mmol) was added ethylenediamine (20 mL, 0.3 mol) with stirring at 25 °C. The solid gradually dissolved as it reacted with the diamine. The reaction was complete in 3 h, as indicated by analytical HPLC, and filtration gave a clear solution, which was then transferred to a well-stirred mixture of acetonitrile and diethyl ether (1:1 v/v, 500 mL). The precipitated solid was collected by centrifugation and redissolved in water (500 mL), followed by addition of aqueous 5% hydrochloric acid until pH 7.0, which effected precipitation of the product. Centrifugation was used to collect the precipitate, which was triturated with water and washed thoroughly with water (250 mL × 3) to remove any trace of ethylenediamine (^{1}H-NMR was used to assay for the presence of the diamine). A yellow solid (2.1 g, 88%) was obtained after washing with acetonitrile (100 mL × 1) and diethyl ether (50 mL × 3) and drying for 24 h under vacuum. ^{1}H-NMR (300 MHz, DMSO-d_6/CF_3CO_2D ~10:1 v/v): δ 8.75 (s, 1H, C7-H), 7.66 (d, J = 8.6 Hz, 2H, Ar), 6.63 (d, J = 8.6 Hz, 2H, Ar), 4.57 (s, 2H, C9-H_2), 4.34 (dd, J = 4.0, 9.6 Hz, 1H, C19-H), 3.28~3.13 (m, 2H, C24-H_2), 2.80 (m, 2H, C25-H_2), 2.16 (m, 2H, C21-H_2), 2.15−1.85 (m, 2H, C20-H_2). ^{13}C-NMR (300 MHz, D_2O/NaOD): δ 178.8, 176.0, 173.1, 169.5, 164.0, 155.5, 151.1, 147.3, 147.2, 129.0, 128.2, 121.4, 112.5, 55.3, 45.8, 42.0, 39.8, 32.8, 28.0. Mass spectrum (FAB): m/z 484 (MH^+). HRMS: calcd for $C_{21}H_{25}N_9O_5$, 484.2057; found, 484.2062. $[\alpha]^{25}_D$ = +4.5° (c = 0.4 in 1.0 N NaOH). Decomposition point, ~278 °C. Analytical HPLC: t_R = 15.4 min (flow rate, 0.7 mL/min; eluent A, water and phosphate buffer 5 mmol/dm^3, pH 7; eluent B, acetonitrile; gradient, 0 min, 1% B; 15 min, 10% B; column, Econosphere C18, 150 mm × 4.6 mm).

4-{[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methyl]amino}benzoic Acid 56.[22b] The sodium salt of **34d** (equivalent to 200 mg, 0.44 mmol, from the National Cancer Institute) was dissolved in 5 mL of 0.1 M TRIS buffer. Carboxypeptidase G (~5 units) and $ZnCl_2$ (1 mg) were added, and the pH of the solution was adujsted to 7.2 with concentrated HCl. The solution was shaken in an incubator at 38 °C for 2 days, and the mixture (pH 8.4) was then adjusted to pH 3.5 with dilute HCl. The precipitated yellow solid was filtered, washed with H_2O, EtOH, and Et_2O, and dried to give the known acid **56** (152 mg, 96%). The ^{1}H-NMR spectrum is identical to the one from Aldrich.

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Supporting Information Available: Experimental procedures for the synthesis of compounds: **14**−**22**, **25d**,**f**,**h**,**i**, **26c**,**d**,**f**,**i**, **30c**, **31**, **33b**,**c**, **40**, **41**, and **45**, as well as ^{1}H and ^{13}C-NMR of all new compounds (96 pages). See any current masthead page for ordering and Internet access instructions.

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