114095-38-4; 11, 70603-18-8; 12, 586-38-9; 13, 10540-29-1; 14, 1845-11-0; 15, 114095-31-7; 17, 114095-32-8; 18, 114095-33-9; 19, 2159-48-0; 20, 114095-34-0; 3-methoxybenzyl chloride, 824-98-6; 4-nitrophenol, 100-02-7.

Supplementary Material Available: Atomic numbering schemes, tables of atomic coordinates, thermal parameters, bond lengths, and bond angles for compounds **7b** and **10a** (10 pages). Ordering information is given on any current masthead page.

Methotrexate Analogues. 32. Chain Extension, α-Carboxyl Deletion, and γ-Carboxyl Replacement by Sulfonate and Phosphonate: Effect on Enzyme Binding and Cell-Growth Inhibition[1]

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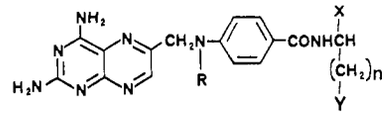
Analogues of methotrexate (MTX) and aminopterin (AMT) with aminophosphonoalkanoic, aminoalkanesulfonic, and aminoalkanephosphonic acid side chains in place of glutamate were synthesized and tested as inhibitors of folylpolyglutamate synthetase (FPGS) from mouse liver. The aminophosphonoalkanoic acid analogues were also tested as inhibitors of dihydrofolate reductase (DHFR) from L1210 murine leukemia cells and as inhibitors of the growth of MTX-sensitive (L1210) and MTX-resistant (L1210/R81) cells in culture. The optimal number of CH_2 groups in aminophosphonoalkanoic acid analogues of AMT was found to be two for both enzyme inhibition and cell growth inhibition but was especially critical for activity against FPGS. Deletion of the α-carboxyl also led to diminished anti-FPGS activity in comparison with previously studied homocysteic acid and 2-amino-4-phosphonobutyric acid analogues. In the aminoalkanesulfonic acid analogues of MTX without an α-carboxyl, anti-FPGS activity was low and showed minimal variation as the number of CH_2 groups between the carboxamide and sulfonate moieties was changed from one to four. In similar aminoalkanephosphonic acid analogues of MTX, anti-FPGS activity was also low, was comparable for two and three CH_2 groups between the carboxamide and phosphonate moieties, and was diminished by monoesterification of the phosphonate group. These effects demonstrate that the α-carboxyl group of folate analogues is involved in binding to the active site of FPGS, and that an α-carboxyl group should be retained as part of the structure of FPGS inhibitors.

Folylpolyglutamate synthetase (FPGS) inhibitors are of potential chemotherapeutic interest because of the importance of this enzyme in cellular folate metabolism.[2] Mutant cell lines lacking FPGS are severely impaired in their capacity to utilize reduced folate cofactors for the synthesis of the nucleotide precursors of DNA and, therefore, are auxotrophic for thymidine and purines.[3,4] Selective disruption of reduced folate metabolism by FPGS inhibitors in tumors as opposed to normal tissues has been proposed as a strategy for the development of new antifolates as cancer drugs.[5]

Considerable work has been done over the past several years to elucidate the general structural features that a compound should have in order to be a good FPGS substrate or inhibitor.[6-8] As part of our overall program of systematic modification of the side chain in methotrexate (MTX, **1**) and aminopterin (AMT, **2**), we have demonstrated that analogues **3–6** are moderately potent inhibitors of FPGS from mouse liver.[9,10] Inhibition of human FPGS by these analogues has also been reported.[11] The sulfonates **3** and **4** were made in both the L and DL configurations, while the phosphonates **5** and **6** were made only in the DL form. The K_i's of **3** and **4** were almost the same as the K_m(app) of MTX, while those of **5** and **6** were comparable to the K_m(app) of AMT.

As part of an ongoing structure–activity study of potential FPGS inhibitors aimed at a more precise delineation of the molecular features that contribute to active-site binding, we were interested in whether (a) the anti-FPGS activity of compounds with a sulfonic or phosphonic acid group on the end of the side chain requires the presence



	R	n	X	Y
1 (MTX)	Me	2	COOH	COOH
2 (AMT)	H	2	COOH	COOH
3	Me	2	COOH	SO_2OH
4	H	2	COOH	SO_2OH
5	Me	2	COOH	$PO(OH)_2$
6	H	2	COOH	$PO(OH)_2$
7	H	1	COOH	$PO(OH)_2$
8	H	3	COOH	$PO(OH)_2$
9	H	4	COOH	$PO(OH)_2$
10	Me	0	H	SO_2OH
11	Me	1	H	SO_2OH
12	Me	2	H	SO_2OH
13	Me	3	H	SO_2OH
14	Me	1	H	$PO(OH)_2$
15	Me	2	H	$PO(OH)_2$
16	Me	2	H	PO(OH)(OEt)
25	Me	2	H	$PO(OEt)_2$

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(1) Paper 31 in this series: Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Freischeim, J. H. *J. Med. Chem.*, in press.
(2) For a useful overview of the folylpolyglutamate synthetase field prior to 1983, see: *Folyl and Antifolyl Polyglutamates*; Goldman, I. D., Chabner, B. A., Bertino, J. R., Eds.; Plenum: New York, 1983.
(3) McBurney, M. W.; Whitmore, G. F. *Cell* **1974**, *2*, 173.
(4) Taylor, R. L.; Hanna, M. L. *Arch. Biochem. Biophys.* **1979**, *181*, 331.
(5) Moran, R. G. In *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates*; Goldman, I. D., Ed.; Praeger: New York, 1985; pp 51–64.

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Table I. Inhibition of Mouse Liver FPGS by Sulfonate and Phosphonate Analogues of MTX and AMT

compd	X	*n*	Y	R	% inhibn[a]	$K_{i,s}$,[b] μM
3	COOH	2	SO_2OH	Me	59	188
4	COOH	2	SO_2OH	H	77	45
5	COOH	2	$PO(OH)_2$	Me	53	185
6	COOH	2	$PO(OH)_2$	H	100[c]	8.4
7	COOH	1	$PO(OH)_2$	H	22	900
8	COOH	3	$PO(OH)_2$	H	52	124
9	COOH	4	$PO(OH)_2$	H	44	155
10	H	0	SO_2OH	Me	19	
11	H	1	SO_2OH	Me	22	
12	H	2	SO_2OH	Me	18	
13	H	3	SO_2OH	Me	26	
14[d]	H	1	$PO(OH)_2$	Me	22	
15[d]	H	2	$PO(OH)_2$	Me	16	
16[d]	H	2	PO(OH)(OEt)	Me	9	

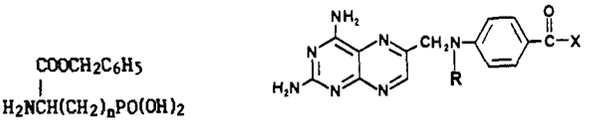
[a] Assays using partially purified mouse liver enzyme were carried out as previously described.[8b] The assay mixture contained 1 mM [^{3}H]-L-glutamic acid, 500 μM folic acid, and 500 μM inhibitor in Tris buffer, pH 8.6, containing 20 mM α-thioglycerol. The reaction was conducted at 37 °C for 1 h. [b] $K_{i,s}$ represents a K_i estimated from a replot of the slopes of lines from a double-reciprocal plot of $1/V$ versus $1/S$, where V = reaction velocity and S = substrate concentration. Compounds 3–7 were competitive inhibitors with respect to the folic acid substrate, whereas 8 and 9 showed a mixed pattern of inhibition. With the exception of compound 7, $K_{i,s}$ values were not determined for analogues that gave <30% inhibition under the conditions of the assay. [c] This compound showed 50% inhibition of FPGS activity at 50 μM. [d] HPLC analysis of the batches of 14–16 used in the FPGS assay showed UV purities of >90% (**14**) and >95% (**15**, **16**), with no single UV-absorbing contaminant comprising more than 5% of the total. As discussed in the Experimental Section, some silica was also present but was unlikely to affect the interpretation of the bioassay results. Molar concentrations were based on empirically estimated formula weights that included the silica.

of an α-carboxyl group and (b) this activity is related to the number of carbons between the terminal sulfonate or phosphonate group and the carboxamide moiety.

To address these questions, we undertook to examine the following compounds as inhibitors of partially purified mouse liver FPGS: **7–9** as analogues of **6**, **10–13** as descarboxy analogues of **3**, and **14–16** as descarboxy analogues of **5**. This paper describes the synthesis of these compounds, and presents data showing that FPGS inhibition by sulfonic and phosphonic analogues of MTX and AMT requires an intact γ-carboxyl group and is stringently dependent on the number of CH_2 groups in the side chain. Experiments were also performed to evaluate the ability of **7–9** to bind to murine dihydrofolate reductase (DHFR) and to inhibit the growth of L1210 mouse leukemia cells in culture. While the chain-shortened analogue **7** was less active than **6** in the latter assay systems, the chain-lengthened analogues **8** and **9** were of comparable potency. The precise length of the side chain thus appeared to be more critical for inhibition of either DHFR activity or cell growth.

Chemistry

In the previously described synthesis of the γ-phosphonate analogues **5** and **6** the α-carboxyl group in DL-2-amino-4-phosphonobutanoic acid was blocked as a methyl ester.[10] The methyl ester was sparingly soluble in DMF, and we therefore elected to use benzyl esters in the hope that these would be more soluble and might give better results. Treatment of DL-2-amino-3-phosphonopropanoic, DL-2-amino-5-phosphonopentanoic, and DL-2-amino-6-phosphonohexanoic acid with benzyl alcohol in the presence of $SOCl_2$ at low temperature afforded the benzyl esters **17–19**, respectively. Treatment of **17** with an



$H_2NCH(CH_2)_nPO(OH)_2$

17 : n - 1
18 : n - 3
19 : n - 4

20 : R - CHO, X - $OC_6H_4NO_2$-p
21 : R - CHO, X - OH
22 : R - Me, X - OH

$H_2N(CH_2)_nPO(OR)(OR')$

23 : n - 2, R - R' - Et
24 : n - 3, R - R' - Et
26 : n - 3, R - H, R' - Et

equimolar amount of *p*-nitrophenyl 4-deoxy-4-amino-N^{10}-formylpteroate (**20**)[8c] and excess Et_3N in DMF at 60 °C for 6 days, followed by alkaline hydrolysis of the benzyl and formyl groups, gave a complex mixture of products including *p*-nitrophenol. After two purifications on a DEAE-cellulose ion-exchange column, a 16% yield of **7** was isolated. Since it appeared that the *p*-nitrophenyl ester method was not very satisfactory, the other two phosphonic acid analogues, **8** and **9**, were synthesized by the modified mixed carboxylic–carbonic anhydride method, which in our earlier work had given **6** from 4-deoxy-4-amino-N^{10}-formylpteroic acid (**21**) in 52% yield after alkaline hydrolysis.[9] Unfortunately, when the same procedure was carried out with **18** and **19**, the products were found to require extensive column purification and were isolated with a final yield of only 14%. Thus, contrary to

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(8) (a) Moran, R. G.; Colman, P. D.; Forsch, R. A.; Rosowsky, A. *J. Med. Chem.* **1984**, *27*, 888. (b) Moran, R. G.; Colman, P. D.; Rosowsky, A.; Forsch, R. A.; Chan, K. K. *Mol. Pharmacol.* **1985**, *27*, 156. (c) Rosowsky, A.; Freisheim, J. H.; Moran, R. G.; Solan, V. C.; Bader, H.; Wright, J. E.; Radike-Smith, M. *J. Med. Chem.* **1986**, *29*, 655.

(9) (a) Rosowsky, A.; Moran, R. G.; Forsch, R.; Colman, P.; Uren, J.; Wick, M. *Biochem. Pharmacol.* **1984**, *33*, 155. (b) Rosowsky, A.; Forsch, R. A.; Freisheim, J. H.; Moran, R. G.; Wick, M. *J. Med. Chem.* **1984**, *27*, 600.

(10) Rosowsky, A.; Moran, R. G.; Forsch, R. A.; Radike-Smith, M.; Colman, P. D.; Wick, M. M.; Freisheim, M. M. *Biochem. Pharmacol.* **1986**, *35*, 3327.

(11) Clarke, L.; Rosowsky, A.; Waxman, D. J. *Mol. Pharmacol.* **1986**, *31*, 122.

our hope, benzyl esters gave low yields irrespective of the coupling method.

Compounds **10–13** were prepared from 4-amino-4-deoxy-N^{10}-methylpteroic acid (**22**) by an adaptation of the method used earlier to prepare **3**.[9] The amino sulfonic acids were solubilized by treatment with Me_3SiCl and Et_3N in CH_2Cl_2, **22** was activated with diethyl phosphorocyanidate (DEPC) in DMF, and products were purified by ion-exchange chromatography. Nonoptimized yields were in the 35–70% range.

Because of the low yields encountered in the initial work on compounds **7–9**, we felt that protection of the phosphonate moiety in the form of alkyl esters subsequently removable with Me_3SiBr would be an improvement in the synthesis of phosphonate analogues. Accordingly, diethyl ester **23** was prepared from diethyl vinylphosphonate and ammonia,[12] while diester **24** was prepared by addition of triethyl phosphite to acrylonitrile in the presence of NH_4I,[13] followed by reduction with BH_3, a procedure we found to be more expedient than catalytic hydrogenation. Attempted preparation of the corresponding dimethyl ester from acrylonitrile and trimethyl phosphite led only to dimethyl methylphosphonate. This unexpected outcome was probably due to the fact that methyl iodide, generated from the reaction of trimethyl phosphite with acrylonitrile, had a high degree of P-alkylating activity. Thus, as soon as a methyl iodide molecule was produced, it reacted with trimethyl phosphite to give dimethyl methylphosphonate and another molecule of methyl iodide. The chain reaction continued until all the trimethyl phosphite was consumed. Condensation of **24** with **22** by the DEPC method to form **25** proceeded in 60% yield, demonstrating the importance of phosphonate group protection during the coupling step. To our surprise, however, treatment of **25** with excess Me_3SiBr at room temperature for 6 days, followed by quenching with methanol gave a mixture of the acid **15** and monoethyl ester **16**, along with at least two other products, which have not been identified. Compounds **15** and **16** could be separated conveniently by ion-exchange chromatography as well as HPLC on C_{18}-bonded silica gel. It was reasonable to expect that the compound eluting more slowly from the ion-exchange column and less slowly from the reversed-phase column was **15**, but unequivocal structure proof was required. Accordingly, diester **24** was hydrolyzed with NaOH to a product that analyzed correctly for monoester **26** and showed one ester group in the NMR. Coupling of **26** to **22** by the DEPC method then gave a product that was indistinguishable by chromatography from the one obtained in the Me_3SiBr reaction and assumed to be **16**. To prove that the other product of the Me_3SiBr reaction was **15**, we treated diester **24** with excess Me_3SiBr but omitted the methanol addition in the hope that a bis(trimethylsilyl) phosphonate ester would form. Condensation of the putative silylated intermediate directly with **22** in the presence of DEPC and Et_3N gave, after the usual workup, a product identical with the one to which structure **15** had earlier been provisionally assigned. This technique was also used to prepare **14** from **22** and **23**. Unfortunately, however, the isolation of HPLC-pure products from this sequence was still very laborious, and final yields remained below 10%. Thus, a practical large-scale route to phosphonate analogues of antifolates continues to be elusive.

(12) Pudovik, A. *Dokl. Akad. Nauk SSSR* **1951**, *80*, 65; *Chem. Abstr.* **1956**, *50*, 4143*h*.

(13) Collins, D.; Hetherington, J.; Swan, J. *Aust. J. Chem.* **1974**, *27*, 1759.

Table II. Effect of the Chain Length of *N*-(4-Amino-4-deoxypteroyl)-DL-2-aminoalkanephosphonate Analogues of AMT or DHFR and Cell-Growth Inhibition.

	IC_{50},[a] μM		
		cells	
compd	DHFR activity	L1210	L1210/R81
6[b]	0.063	0.067	240
7	0.55	0.25	390
8	0.083	0.12	>460
9	0.067	0.13	>280

[a] Enzyme inhibition and cell-growth inhibition assays were carried out as previously described.[10] [b] Data from ref 10.

Bioassay Results

Compounds **7–16** were all tested as inhibitors of partially purified mouse liver FPGS according to our previously described procedure.[8b] The effect of each compound on FPGS activity, expressed as a percentage of untreated controls, is presented in Table II. The ω-phosphonoalkanoic acid analogues with one, three, or four CH_2 groups in the side chain were clearly less inhibitory than **6**, whose side chain is of the same length as that of AMT. Moreover, it appeared that a single CH_2 group was more detrimental to binding than three or four. These results suggest that when **5** and **6** bind to FPGS, the PO_3H_2 group is positioned similarly to the γ-COOH group of the MTX and AMT when the latter bind as substrates. We have previously observed in MTX analogues with α,ω-diaminoalkanoic acid side chains that inhibition of mouse liver FPGS is exquisitely sensitive to the number of CH_2 groups, being maximal at three.[8c] McGuire and co-workers[6d] have reported similar results with FPGS from other sources, and a mechanism-based explanation has been put forward to accout for the fact that the best FPGS substrates contain two CH_2 groups while the best FPGS inhibitors of the terminal NH_2 type are those with three CH_2 groups.[14] The present data demonstrate that when the FPGS inhibitor contains an acidic terminal group the optimal number of CH_2 groups is two.

When the sulfonic acids **10–13** were compared with the L-homocysteic acid analogue **3** as FPGS inhibitors, they proved considerably less active. Inhibition of the glutamylation of 500 μM folic acid in the presence of an equimolar concentration of the aminoalkanesulfonic acid derivatives was only in the 10–25% range, whereas **3** under the same conditions inhibited activity by >50%. From this it appears that the α-COOH group of **3** has an important role in binding to the active site of FPGS. We have previously shown that deletion of the α-COOH of MTX eliminated activity as a substrate for FPGS.[8a] However, on the basis of substrate activity, it was impossible to distinguish whether such an effect indicated involvement of the α-COOH in binding to enzyme or in induction of catalysis. The data in Table I, which is based solely on relative activities of inhibitors, indicate that the α-COOH of folates and folate analogues is involved in binding to the active site per se.

When FPGS inhibition by the phosphonate analogues was compared, α-carboxyl group deletion was again found to decrease activity, with **14** and **15** inhibiting glutamylation by 22% and 16% while **5** gave >50% inhibition. Esterification of the phosphonate group in **15** to give **16** virtually eliminated FPGS inhibition. An intact α-COOH group is generally considered essential for tight binding to DHFR[15a] and efficient transport into cells.[15b] For this

(14) Rosowsky, A.; Moran, R. G.; Freisheim, J. H.; Bader, H.; Forsch, R. A.; Solan, V. C. *NCI Monographs* **1987**, *5*, 145.

reason, only compounds **7–9**, in which the α-COOH group was preserved, were tested as DHFR inhibitors and cell-growth inhibitors. As shown in Table II, the activity of **7** (one CH_2 group) was 9-fold lower than that of **6**, but not greatly different from that of **8** or **9**. Decreased inhibition of DHFR (with a concomitant change in binding kinetics from "stoichiometric" to competitive) has been reported for the L-aspartate analogue of MTX by Mead and co-workers.[16] It thus appears that placing a second acidic group on the side chain too close to the α-COOH group is detrimental to DHFR binding. It should be noted that IC_{50}'s listed for compounds **7–9** in Table II are for DL mixtures, and that the L-enantiomers might be more potent than the racemates since L-MTX is known to be a better DHFR inhibitor than D-MTX.[17]

The ability of compounds **7–9** to inhibit cell growth was determined against cultured L1210 mouse leukemia cells and the highly MTX- and AMT-resistant mutant L1210/R81.[18] As shown in Table II, the IC_{50}'s of **7** as well as **8** and **9** were 2- to 4-fold higher than the IC_{50} of **6**, demonstrating that two CH_2 groups in the side chain are optimal for both DHFR binding and cell-growth inhibition. The >100-fold loss activity in comparison with AMT, on the other hand, is consistent with the fact that the phosphonate analogues cannot form polyglutamates. In addition, the presence of three negative charges in the side chain may be detrimental to transport across the cell membrane.

In summary, the data reported in this paper suggest that aminophosphonoalkanoic acid analogues of AMT with fewer than, or more than, two CH_2 groups in the side chain are poorer inhibitors of FPGS and DHFR and are less toxic to cells in culture than **6** and that deletion of the α-carboxyl from the homocysteic acid and 2-amino-4-phosphonobutyric acid analogues of MTX leads to decreased FPGS inhibition. Further studies aimed at gaining a better understanding of the molecular pharmacology of this interesting enzyme and designing better inhibitors are in progress.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer. Melting points were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Compounds for which melting points are not specified were found to decompose above 250 °C. TLC was on Eastman 13181 silica gel or Eastman 13254 sheets (fluorescent indicator), and spots were visualized in a viewing chamber under 254-nm illumination or with the aid of ninhydrin spray where appropriate. Adsorption column chromatography was on Baker 3405 silica gel (60–200 mesh) or Brinkmann Avicel microcrystalline cellulose. Ion-exchange column chromatography was on Whatman DE-52 preswollen [(diethylamino)ethyl]cellulose (DEAE-cellulose) or Dowex 50-X2 resin. HPLC was on Waters C_{18} reversed-phase radial compression cartridges (5 μm particle size, 0.5 × 10 cm). A Waters Model 400 instrument equipped with a Model 490 programmable multiwavelength detector and Model 660 programmable solvent gradient system was used. *p*-Nitrophenyl 4-amino-4-deoxy-N^{10}-formylpteroate (**20**), 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA, **21**), and 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA, **22**) were obtained as described earlier.[8c,10] The procedures described by Fujii and Cook[19] were followed to prepare aminomethanesulfonic acid, mp 184–185 °C dec (lit. mp 185–186 °C dec), from 36% formalin, $NaHSO_3$, and 28% NH_4OH (15% yield), and 4-aminobutanesulfonic acid, mp 243–248 °C dec (lit. mp 252–253 °C dec), from sodium 4-bromobutanesulfonate and 28% NH_4OH (76%). Diethyl 2-(cyanoethyl)phosphonate was synthesized from acrylonitrile, triethyl phosphite, and NH_4I by the method of Collins and co-workers.[13] 2-Aminoethanesulfonic acid, 3-aminopropanesulfonic acid, DEPC, Me_3SiCl, and diethylvinylphosphonate were purchased from Aldrich, Milwaukee, WI. DL-2-Amino-3-phosphonopropanic acid, DL-5-phosphonopentanoic acid, and DL-2-amino-6-phosphonohexanoic acid were from Sigma, St. Louis, MO. Et_3N and solvents were dried over Linde 4A molecular sieves (Fisher, Boston, MA). Elemental analyses were by Galbraith Laboratories, Knoxville, TN, and MultiChem Laboratories, Lowell, MA, and were within 0.4% of theory unless otherwise specified. The structures of compounds **14–16** were further supported by fast atom bombardment mass spectra (FAB-MS), which revealed in each case the presence of the parent ion species (MH^+).

Synthesis of 2-Amino-ω-phosphonoalkanoate Esters. Benzyl DL-2-Amino-3-phosphonopropanoate (17). A stirred suspension of DL-2-amino-3-phosphonopropanoic acid (673 mg, 4 mmol) in benzyl alcohol (25 mL) in an ice bath was treated dropwise with $SOCl_2$ (4 mL) at a rate that allowed the temperature to remain at or below 12 °C. When addition was complete, the bath was removed, and the mixture was left to stand at room temperature for 18 h. The product could be detected by TLC (cellulose, 1.5% NH_4Cl in 1:1 MeOH–H_2O) as an orange ninhydrin spot, R_f 0.8; minor amounts of starting material remained (R_f 0.5 faint purple ninhydrin spot) after 18 h and did not disappear even on warming. Most of the benzyl alcohol was removed by vacuum evaporation, and the residue was shaken with Et_2O and H_2O. Crystals formed spontaneously and were filtered, washed with ice-cold H_2O, and dried in a lyophilizer to obtain **17** as a white powder (666 mg, 65% yield); mp 212–213 °C; IR (KBr) 3430, 3130, 3030, 1740, 1645, 1595, 1545, 1500 cm^{-1}. Anal. ($C_{10}H_{14}NO_5P \cdot 0.25H_2O$) C, H, N.

Benzyl DL-2-Amino-5-phosphonopentanoate (18) and Benzyl DL-2-Amino-6-phosphonohexanoate (19). The same general procedure as in the preceding experiment was followed, except that the products did not crystallize spontaneously on addition of Et_2O and H_2O to the evaporated reaction mixture but instead dissolved in the aqueous phase. Lyophilization of the H_2O layer then gave a thick gum or foam, which was coupled directly to **20** to **21** without purification.

***N*-(4-Amino-4-deoxypteroyl)-DL-2-amino-3-phosphonopropanoic Acid (7).** A suspension of **17** (104 mg, 0.4 mmol) in dry DMF (10 mL) was treated with **20** (184 mg, 0.4 mmol) and Et_3N (276 μL, 2 mmol) and kept in an oil bath at 60 °C. After 6 days most of the solid had dissolved, and TLC (cellulose, phosphate buffer, pH 7.4) showed a mobile blue fluorescent spot assumed to be the N^{10}-formyl benzyl ester. The solvent was evaporated, the residue was taken up in 0.1 N NaOH (10 mL), and 1 N NaOH was periodically added dropwise until TLC showed no more fluorescent spots. The solution was acidified to pH 6 with 1 N HCl and washed thoroughly with Et_2O to remove *p*-nitrophenol. The pH was then adjusted to 8 with NH_4OH, and the solution was concentrated to a small volume and applied onto a DEAE-cellulose column (1.5 × 20 cm, HCO_3^- form). The column was eluted first with H_2O to remove salts and a dark red-brown impurity and then with 0.4 M NH_4HCO_3 to remove all pteridines. The pooled 0.4 M NH_4HCO_3 eluates were evaporated, and the residue was applied to another DEAE-cellulose column (1.5 × 19 cm, HCO_3^- form), which was eluted with H_2O and then with 0.1 M, 0.2 M (to remove the last of the *p*-nitrophenol), and finally 0.4 M NH_4HCO_3. Fractions containing a single UV-absorbing TLC spot (R_f 0.5) were freeze-dried to obtain a light-yellow solid (32 mg, 16% yield): IR (KBr) 3420, 3170, 1640, 1610, 1515 cm^{-1}. Anal. ($C_{17}H_{19}N_8O_6P \cdot 3H_2O$) C, H, N.

***N*-(4-Amino-4-deoxypteroyl)-DL-2-amino-5-phosphono-**

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pentanoic Acid (8). A suspension of **21** (340 mg, 1 mmol) in dry DMF (20 mL) was treated with Et_3N (139 μL, 1 mmol) and *i*-BuOCOCl (130 μL, 1 mmol). A clear solution formed within a few minutes. To this solution were added successively **18** (4 mL containing a theoretical 0.25 mmol/mL) and Et_3N (417 μL, 3 mmol). The activation and coupling sequence was repeated for 3 cycles with Et_3N (0.5 mmol), *i*-BuOCOCl (0.5 mmol), **18** (0.5 mmol), and Et_3N (1.5 mmol), at which time the TLC (cellulose, phosphate buffer, pH 7.4) showed a major blue fluorescent spot (R_f 0.6, N^{10}-formyl benzyl ester), a small unidentified spot (R_f 0.3), and a small spot at the origin. The solvent was evaporated, and the product was hydrolyzed with 2 N NaOH at room temperature. The course of reaction was monitored by TLC. When the blue fluorescent spot (R_f 0.6) was replaced by an absorbing spot (R_f 0.5), the pH was adjusted to 8 with 2 N HCl. Passage of the product through two DEAE-cellulose columns, with NH_4HCO_3 gradient elution as described above, failed to separate **8** from an unidentified impurity (R_f 0.8). Accordingly, the product was chromatographed twice more on microcrystalline cellulose (1.5 × 19 cm), with 0.1 M NH_4HCO_3 as the eluent. Freeze-drying of TLC-homogeneous fractions afforded **8** as a light-yellow solid (70 mg, 14% yield): IR (KBr) 3430, 3190, 1645, 1615, 1570, 1520 cm^{-1}. Anal. ($C_{19}H_{23}N_8O_6P \cdot 0.4NH_3 \cdot 2.5H_2O$) C, H, N.

***N*-(4-Amino-4-deoxypteroyl)-DL-2-amino-6-phosphonohexanoic Acid (9).** The same procedure as in the preceding experiment yielded 72 mg (14%) of light-yellow solid (R_f 0.5): IR (KBr) 3400, 3190, 1645, 1615, 1570, 1515 cm^{-1}. Anal. ($C_{20}H_{25}N_8O_6P \cdot 0.75NH_3 \cdot 3H_2O$) C, N; H: calcd, 5.87; found, 5.27.

[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]alkanesulfonic Acids. General Procedure. A suspension of previously vacuum-dried aminoalkanesulfonic acid (2 mmol) in dry CH_2Cl_2 (10 mL) in a flask protected from moisture was treated with Et_3N (556 μL, 4 mmol) and Me_3SiCl (253 μL, 4 mmol) and stirred at room temperature overnight. If the starting material failed to dissolve, addition of Et_3N and Me_3SiCl in equimolar proportions was continued until a clear solution or homogeneous dispersion was formed. In a separate flask, likewise protected from moisture, **22** (180 mg, 0.5 mmol) was added in small portions over 3 min to a stirred solution of Et_3N (207 μL, 1.5 mmol) and DEPC (245 mg, 1.5 mmol) in dry DMF (20 mL). The mixture was left to stir at room temperature overnight to permit the activation step to go to completion. The CH_2Cl_2 solution in the first flask was then evaporated, and the residue, suspended in a small volume of DMF, was transferred to the second flask. The mixture was stirred at room temperature for several days, and the progress of the reaction was monitored by TLC, either on cellulose (phosphate buffer, pH 7.4), which showed the gradual disappearance of a nonmobile spot (activated intermediate) and appearance of a mobile spot (product), or on silica gel (4:1 $CHCl_3$–MeOH), which showed the gradual disappearance of a mobile spot (activated intermediate) and appearance of nonmobile spot (product). When the reaction was complete, the mixture was concentrated to dryness by rotary evaporation. The residue was stirred in H_2O (25–50 mL), and a few drops of concentrated ammonia were added to obtain a clear solution on gentle warming. The solid that formed on cooling to room temperature was removed by filtration, and the clear solution (pH ca. 8) was applied onto a DEAE-cellulose column (1.5 × 25 cm, HCO_3^- form). The column was eluted first with a large volume of H_2O to remove salts and then with NH_4HCO_3 ranging in concentration from 0.1 to 0.4 M to elute the product. Purity was monitored by HPLC with a 15-min linear gradient (initial, 0.04 M NH_4OAc, pH 7.5, 10% MeCN; final, 0.08 M NH_4OAc, pH 7.5, 20% MeCN). When UV-absorbing impurities with UV absorption indicative of a pteridine structure were detected, the product was rechromatographed on a second DEAE-cellulose column. The desired coupling products gave TLC spots with R_f's of ca. 0.5 on cellulose (phosphate buffer, pH 7.4) and 0.2 on silica gel (15:5:1 $CHCl_3$–MeOH–AcOH).

[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]methanesulfonic acid (10): 67% yield; IR (KBr) 3440, 3180 (br), 1645, 1615, 1560, 1515 cm^{-1}. Anal. ($C_{16}H_{18}N_8O_4S \cdot 0.5NH_3 \cdot 2.5H_2O$) C, N, S; H: calcd, 5.23; found, 4.72.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-2-aminoethanesulfonic acid (11):** 43% yield; IR (KBr) 3420, 3160 (br), 1640, 1610, 1555, 1515 cm^{-1}. Anal. ($C_{17}H_{20}N_8O_4S \cdot 0.5NH_3 \cdot 1.5H_2O$) C, H, N, S.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-3-aminopropanesulfonic acid (12):** 58% yield; IR (KBr) 3440, 3190 (br), 1645, 1555, 1515 cm^{-1}. Anal. ($C_{18}H_{22}N_8O_4S$) C, H, N, S.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-aminobutanesulfonic acid (13):** 37% yield of pure product and another 50% with minor HPLC impurity; IR (KBr) 3440, 3240 (sh), 1660, 1620, 1525 cm^{-1}. Anal. ($C_{19}H_{24}N_8O_4S \cdot 0.5NH_3 \cdot 3H_2O$) C, N, S; H: calcd, 6.07; found 5.37.

Diethyl 2-Aminoethanephosphonate (23). A solution of diethyl vinylphosphonate (1.64 g, 0.01 mol) in concentrated NH_4OH (15 mL) was kept at room temperature for 3 days, diluted with H_2O (30 mL), and extracted thoroughly with $CHCl_3$. Evaporation of the combined $CHCl_3$ extracts gave a colorless liquid (1.8 g, ca. 100%): IR (NaCl) 3430 (br), 2990, 2920, 1655 cm^{-1}; NMR ($CHCl_3$) δ 1.48 (t, J = 7 Hz, 6 H, CH_3), 1.6–2.3 (m, 2 H, CH_2P), 2.6–3.2 (m, 2 H, CH_2N), 4.03 (apparent pentet, J = 7 Hz, 4 H, CH_2O). This compound was coupled directly to **22**.

Diethyl 3-Aminopropanephosphonate (24). Diethyl (2-cyanoethyl)phosphonate (19.1 g, 0.1 mol) was added to a stirred solution of 0.98 M BH_3 in THF (102 mL, 0.1 mol) under N_2 in a cold-water bath. After 30 min at room temperature, the reaction was quenched with MeOH and left to stand overnight. The solvent was evaporated, and the residue was partitioned between $CHCl_3$ and saturated aqueous K_2CO_3. Evaporation of the organic layer gave an oil, which partially decomposed on distillation to give 5.98 g (31%) of colorless liquid: bp 114–116 °C (1.5 Torr); IR (NaCl) 3460, 3380, 3310, 2990, 2950, 2920, 2880, 1610 cm^{-1}; NMR spectrum in agreement with the literature.[13] This material was coupled directly to **22**.

Monoethyl 3-Aminopropanephosphonate (26). A solution of crude **24** (1.95 g, 0.01 mol) in 1 M NaOH (13 mL) was heated for 18 h in an oil bath at 100 °C. The solution was cooled to room temperature, a small amount of insoluble material was filtered off, and the filtrate was added onto a Dowex 50W-X2 column (1.5 × 22 cm, H^+ form). The column was eluted with H_2O (ca. 1 L). The eluate was freeze-dried, the residue was swirled with absolute EtOH, and the solid that remained undissolved was collected and dried in vacuo at 100 °C over P_2O_5 to obtain analytically pure white powder (0.16 g, 10%): mp 221–224 °C dec with gas evolution; R_f 0.6 (cellulose, 2:1 EtOH–3% aqueous NH_4Cl); magenta spot with ninhydrin; IR (KBr) 3430, 2990, 2950, 2910, 2210, 1645, 1545, cm^{-1}; NMR (D_2O) δ 1.23 (t, J = 7 Hz, 3 H, Me), 1.4–2.2 (m, 4 H, CH_2CH_2P), 3.03 (m, 2 H, CH_2N), 3.88 (apparent quintet, J = 7 Hz, 2 H, CH_2O). Anal. ($C_5H_{14}NO_3P$) C, H, N, P.

Evaporation of the EtOH gave a second crop of **26** (1.02 g, 61%) whose TLC showed the presence of some unreacted diester (R_f 0.8, purple spot with ninhydrin).

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-2-aminoethanephosphonic Acid (14).** A solution of **23** (312 mg, 1.72 mmol) in CH_2Cl_2 (5 mL) was treated with Me_3SiBr (1071 mg, 923 μL, 7 mmol), stirred at room temperature in a stoppered flask for 18 h, and evaporated to dryness. In a separate flask, a solution of Et_3N (208 μL, 1.5 mmol) and DEPC (245 mg, 1.5 mmol) in DMF (20 mL) was treated with small portions of **22** (180 mg, 0.5 mmol) over 3 min and left to stir at room temperature for 3 h. The residue from the first flask was then added in a small volume of DMF, another portion of Et_3N (973 μL, 7 mmol) was added, and after 12 days at room temperature the reaction was quenched with H_2O. The residue after evaporation was placed on a DEAE-cellulose column (1.5 × 28 cm, HCO_3^- form), which was eluted with H_2O, followed by 0.2 M NH_4HCO_3 (to remove a dark red band), and finally 0.4 M NH_4HCO_3. Fractions were monitored by HPLC, and the product-containing fraction was purified further by passing it twice through a C_{18}-bonded silica gel LPLC column. The column was eluted with 500 mL of 0.1 M NH_4OAc, pH 7.0, containing from 10% to 20% EtOH. Pooled TLC-pure fractions were lyophilized, the residue was redissolved in NH_4OH, a small amount of light-colored solid appearing to be silica[20] was filtered off, and the filtrate was relyophilized. Drying in vacuo at 100

(20) In the purification of 14–16 by LPLC, the product apparently contained silica, some which could not be removed from the analytical sample. However, the correct identity of each of these compounds was satisfactorily established by mass spectrometry.

°C over P_2O_5 gave a yellow-orange solid (18 mg, 7%): IR (KBr) 3430, 3200 (sh), 1640, 1610, 1550, 1515 cm^{-1}; MS calcd for (MH^+) 433, found 433; HPLC t_R 20 min [(solution A, 0.1 M NH_4OAc, pH 7.0, containing 1% MeCN; solution B, 0.1 M NH_4OAc, pH 7.0, containing 15% MeCN; 25–100% B over 20 min; flow rate 0.9 mL/min). Anal. ($C_{17}H_{21}N_8O_4P·0.4C_2H_5OH·0.6SiO_2$) C, H, N; C/N ratio: calcd, 2.23; found, 2.23.

Diethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-3-aminopropanephosphonate (25). To a stirred solution of Et_3N (414 μL, 3 mmol) and DEPC (489 mg, 3 mmol) in dry DMF (40 mL) were added small portions of **22** (360 mg, 1 mmol) over 3 min. The solution was left to stand at room temperature for 18 h, and **24** (390 mg, 2 mmol) was added. After 3 days, the solvent was evaporated, and the residue was partitioned between $CHCl_3$ and dilute NH_4OH. Evaporation of the $CHCl_3$ left a residue, which was purified on a silica gel column (45 g, 2.5 × 40 cm) with 9:1 $CHCl_3$–MeOH as the eluent. After separation of a colored impurity eluting just ahead of the product, the main band was collected and concentrated to a small volume. Addition of Et_2O caused the product to precipitate. The light-yellow solid was dried in vacuo at 60 °C over P_2O_5: yield 312 mg (60%); mp 171–177 °C; IR (KBr) 3430, 2980, 1635, 1615, 1560, 1515 cm^{-1}. Anal. ($C_{22}H_{31}N_8O_4P·0.75H_2O$) C, H, N, P.

Conversion of 25 to 15 and 16 with Trimethylsilyl Bromide. A solution of **25** (50 mg, 0.1 mmol) in dry CH_2Cl_2 (40 mL) was treated with trimethylsilyl bromide (348 mg, 300 μL, 2.26 mmol) and stirred at room temperature for 6 days. The reaction was quenched by addition of MeOH, and the solution was evaporated to dryness. Analysis of the product by gradient HPLC [solution A, H_2O; solution B, 0.05 M NH_4OAc, pH 7.0, containing 15% MeCN; 5–100% B over 30 min; flow rate 0.7 mL/min] showed 4 peaks, A–D, with retention times of 3.8 (peak A), 14.9 (peak B), 19.3 (peak C), and 24.6 min (peak D). Column chromatography on a DEAE-cellulose column (1.5 × 15 cm, HCO_3^- form) with 0.1 M NH_4HCO_3 as the eluent gave four fractions, two of which had UV-absorption features consistent with a 2,4-diaminopteridine structure. Examination of these two fractions by HPLC revealed that they corresponded to peaks C and D and coeluted with authentic samples of **15** and **16** (see below).

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-3-aminopropanephosphonic Acid (15).** A solution of **24** (195 mg, 1 mmol) in CH_2Cl_2 (5 mL) was treated with Me_3SiBr (765 mg, 660 μL, 5 mmol) and stirred at room temperature in a stoppered flask for 18 h. Examination of the reaction by TLC (cellulose, 2:1 EtOH–3% aqueous NH_4Cl) revealed a magenta ninhydrin spot with R_f 0.5 as compared with 0.8 for **23** (purple) and 0.7 for **26** (red). In a separate flask, a solution of Et_3N (840 μL, 6 mmol) and DEPC (489 mg, 3 mmol) in dry DMF (40 mL) was treated with **22** (360 mg, 1 mmol) in small portions over 3 min. After 4 h at room temperature, the residue from evaporation of the first flask was transferred to the second flask with the aid of a small volume of DMF and an additional portion of Et_3N (139 μL, 1 mmol). After 1 week, the reaction was quenched with H_2O, and the solution was evaporated under reduced pressure. The residue was treated with 1 N NaOH (8 mL), diluted with H_2O (50 mL), concentrated under vacuum to remove Et_3N, and finally adjusted to pH 8.5 with 1 N HCl. After 15 h at room temperature, the mixture was filtered, and the filtrate was placed on a DEAE-cellulose column (3 × 24 cm, HCO_3^- form), which was eluted with H_2O, followed by 0.1 M NH_4HCO_3 (1 L), and finally 0.2 M NH_4HCO_3. TLC analysis revealed two spots even after a second pass through the ion-exchange column. Accordingly, the product was further purified by LPLC (twice) as described above. Pooled TLC-pure fractions were freeze-dried and the solid was redissolved in NH_4OH. The solution was made slightly acidic with AcOH, rebasified with NH_4OH, and filtered to remove a small amount of light-colored insoluble material appearing to be silica.[20] The filtrate was lyophilized, and the residue dried in vacuo at 100 °C over P_2O_5 to obtain a yellow-orange solid (40 mg, 9%): IR (KBr) 3410, 1645, 1615, 1560, 1520 cm^{-1}; FAB-MS calcd for (MH^+) 447, found 447; HPLC t_R 20 min (solution A, H_2O; solution B, 0.1 M NH_4OAc, pH 7.0, containing 15% MeCN; 25–100% B over 20 min; flow rate 0.9 mL/min). Co-injection with peak C from the cleavage of **25** with Me_3SiBr (see above) gave a single peak. Anal. ($C_{18}H_{23}N_8O_4P·0.75SiO_2$) C, H, N; C/N ratio: calcd, 2.25; found 2.25.

Monoethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-3-aminopropanephosphonate (16). Compound **22** (180 mg, 0.5 mmol) was added in small portions over 3 min to a stirred solution of Et_3N (414 μL, 3 mmol) and DEPC (245 mg, 1.5 mmol) in dry DMF (20 mL), and stirring was continued overnight. In a separate flask protected from moisture, a suspension of **26** (84 mg, 0.5 mmol) in CH_2Cl_2 (2 mL) was treated with Et_3N (139 μL, 1 mmol) and Me_3SiCl (55 mg, 64 μL, 0.5 mmol). An almost clear solution formed when the flask was placed briefly in a sonication bath, but a small amount of undissolved solid remained. On further addition of 0.5-mmol portions of Et_3N and Me_3SiCl, however, a precipitate formed. After another 5 min of sonication, the mixture was added to the first flask. The resulting almost clear solution was stirred at room temperature for 6 days. Evaporation to dryness left a dark residue, which was applied onto a DEAE-cellulose column (1.5 × 22 cm, HCO_3^- form). The column was eluted first with H_2O to remove salts and then with 0.1 M NH_4HCO_3. Two products were obtained, one with R_f 0.6 (cellulose, phosphate buffer, pH 7.4) and the other with R_f 0.4. Fractions containing mainly the product with R_f 0.6 were pooled and further purified by LPLC on C_{18}-bonded silica gel as described above. Combined TLC-pure fractions with R_f 0.6 were freeze-dried, redissolved in H_2O, filtered to remove a small amount of light-colored material which appeared to be silica,[20] and freeze-dried again to remove all the NH_4OAc. The resulting light-yellow solid (**16**) was dried in vacuo at 100 °C over P_2O_5: yield 36 mg (15%); IR (KBr) 3410, 3190, 2980, 1640, 1610, 1555, 1515 cm^{-1}; FAB-MS calcd for (MH^+) 475, found 475. HPLC t_R 14.8 min (solution A, 0.1 M NH_4OAc, pH 7.0, containing 1% MeCN; solution B, same buffer, containing 15% MeCN; 50–100% B over 20 min; flow rate 0.9 mL/min). Co-injection with peak D from the preceding experiment gave a single peak. Anal. ($C_{20}H_{27}N_8O_4P·SiO_2$) C, H, N; C/N ratio: calcd, 2.50; found, 2.53.

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