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Analogues of N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) Modified in the Side Chain: Synthesis and Biological Evaluation[1]

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Four heretofore undescribed side chain analogues of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523, **4**) were synthesized via straightforward methods of antifolate chemistry, and their properties were compared with those of PT523 and two related compounds with the aim of defining the contribution of the hemiphthaloyl-L-ornithine moiety to the exceptional *in vitro* antitumor activity of this novel non-polyglutamatable aminopterin analogue. The IC_{50} values of N^{α}-(4-amino-4-deoxypteroyl)-N^{β}-hemiphthaloyl-L-2,3-diaminopropanoic acid (**10**) and N^{α}-(4-amino-4-deoxypteroyl)-N^{γ}-hemiphthaloyl-L-2,4-diaminobutanoic acid (**9**) against A549 human non-small-cell lung carcinoma cells in culture were 23 and 22 nM, whereas those of PT523 and N^{α}-(4-amino-4-deoxypteroyl)-N^{ϵ}-hemiphthaloyl-L-lysine (**8**) were 1.3 and 5.2 nM. A decrease in the *in vitro* activities of **8** and **9** relative to PT523 was also observed against the panel of cell lines used by the National Cancer Institute to screen new drugs. However the potency of **8** and **9** remained several times greater than that of the historical control methotrexate against many of the cell lines in the screening panel. In an *in vivo* tumor model, SCC-VII murine squamous cell carcinoma, **9** and methotrexate were well tolerated as 5-day continuous infusions at doses of 0.52 and 0.75 mg/kg/day, whereas the highest tolerated dose of PT523 on this schedule was 0.19 mg/kg/day, in agreement with its lower IC_{50} in culture. To assess the importance of the hemiphthaloyl group in PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-isophthaloyl-L-ornithine (**11**), N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-terephthaloyl-L-ornithine (**12**), and N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-(4,5-dichlorohemiphthaloyl)-L-ornithine (**13**) were also synthesized. The IC_{50} values of **11** and **12** against A549 cells were 45 and 3300 nM, as compared with 1.3 nM for PT523 and 23 nM for methotrexate. In a clonogenic assay against SCC25 human squamous cell carcinoma cells, the IC_{50} values of **11** and **12** were 2.9 and 72 nM, as compared with 0.3 nM for PT523 and 27 nM for methotrexate. Thus, activity was decreased by moving the aromatic carboxyl group in PT523 to the meta position and was further diminished by moving it to the para position. The IC_{50} of the halogenated analogue **13** against SCC25 human head and neck squamous carcinoma cells was 18 nM, suggesting lack of tolerance for this 4,5-disubstitution in the phthaloyl moiety. Our results suggest that the combination of a hemiphthaloyl group and three CH_2 groups in the side chain are critical determinants of the potent *in vitro* activity of PT523.

The ability of the L-glutamic acid side chain in classical dihydrofolate reductase (DHFR) inhibitors like methotrexate (MTX, **1**), aminopterin (AMT, **2**), and edatrexate (10-EDAM, **3**) to form poly-L-glutamate conjugates of the γ-carboxyl group is a critical determinant of both their potency and therapeutic selectivity.[1−3] The enzyme which catalyzes elongation of the side chain in folates and classical antifolates, folyl γ-polyglutamate synthetase (FPGS),[4,5] is ubiquitous in normal cells and is considered essential for cellular proliferation, since mutant cells lacking this enzyme are auxotrophic for thymidine and a purine.[6] While the mono- and polyglutamates of MTX bind almost equally well to DHFR,[7] polyglutamation causes a substantial increase in binding to two other enzymes of one-carbon metabolism, thymidylate synthase (TS)[8] and aminoimidazolecarboxamide ribotide formyltransferase (AICAR FTase).[9] This enables MTX to act as a multienzyme-targeted drug, blocking purine nucleotide as well as thymidylate *de novo* synthesis. Because cells with low FPGS activity tend to be less sensitive to classical antifolates than those with higher FPGS activity unless they are continuously exposed to drug, the addition of one or more glutamates by FPGS is an important determinant of the potency of not only MTX[10] but also 10-EDAM[2,11] and several of the newer TS[12,13] and AICAR FTase inhibitors[14,15] currently in clinical trial.

In addition to the enhanced binding of classsical antifolates to the enzymes of one-carbon metabolism, polyglutamation plays a critical role in the cellular retention of these drugs. Because of their polyanionic nature, polyglutamates efflux much more slowly than the parent monoglutamates from cells.[16] Within a short time after the end of treatment, typically as little as 1 h, the short-chained species are lost, leaving species containing four to six glutamyl residues. While differences in the baseline activity of DHFR and other enzymes of one-carbon metabolism may likewise play a role in the MTX and 10-EDAM sensitivity of certain cell lines,[11b] it is widely held that the therapeutic *selectivity* of classical DHFR inhibitors (i.e., their cytotoxicity to tumor versus nontumor cells) depends mainly on differential polyglutamation, with FPGS-competent tumors being more sensitive than rapidly proliferating

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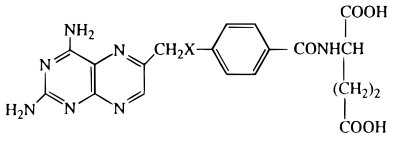
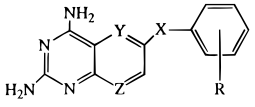
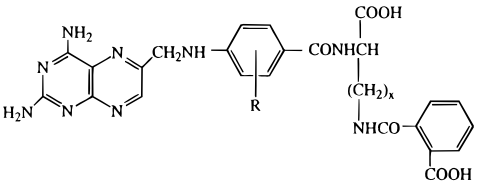


1 (MTX) : X = NMe
2 (AMT) : X = NH
3 (10-EDAM) : X = CHEt



5 : X = CH_2NH, Y = CMe, Z = CH, R = 3',4,'5'-$(OMe)_3$
6 : X = CH_2, Y = CMe, Z = N, R = 2',5'-$(OMe)_2$



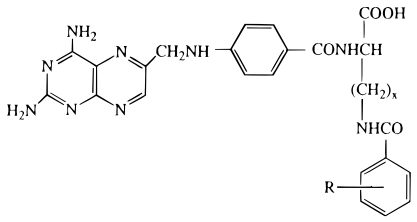
4 (PT523) : x = 3, R = H
7 : x = 3, R = 3',5'-Cl_2
8, **9** : x = 4,2; R = H

host tissues like marrow and gut.[2b] However polyglutamation can be a two-edged sword in that some tumors possess inherently low FPGS activity whereas other tumors acquire this resistance phenotype during treatment. If a patient does not respond to MTX because of low FPGS activity in the tumor, the amount of drug cannot be escalated because of dose-limiting host toxicity.

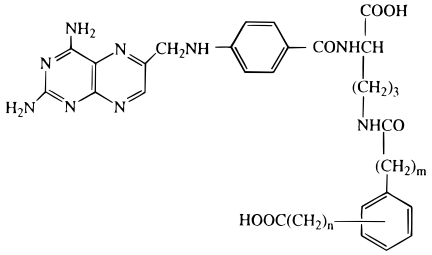
With this hypothesis as the starting point, we became interested some time ago in the therapeutic potential of compounds that combine key pharmacodynamic features of both the classical and nonclassical families of DHFR inhibitors, specifically the ability to be actively transported into cells via the reduced folate carrier without being dependent on polyglutamation as a prerequisite for full antifolate activity.[17] Our basic approach has been to keep the α-COOH group in place and modify the γ-region of the side chain so as to retain tight DHFR binding and the capacity for active transport while preventing polyglutamation. Among the numerous side chain analogues of this type that were evaluated in the course of this work, an unusually promising lead was N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523, **4**).[18,19] Two notable properties of PT523, despite the presence of an additional hydrophobic phenyl ring in the side chain, were its good aqueous solubility and the ability to be actively transported into cells. These properties are undoubtedly due to retention of two COOH groups in the side chain, an advantage which sets PT523 apart from true nonclassical DHFR inhibitors such as trimetrexate (**5**) and piritrexim (**6**), which are not very water soluble even as salts and are not substrates for the reduced folate carrier utilized by MTX and other classical antifolates. A further benefit of the two COOH groups is that PT523 is an unlikely candidate for resistance via the P-glycoprotein multidrug resistance phenotype. This problem has been reported for both **5** and **6**,[20,21] but interestingly is not universal for all lipid-soluble DHFR inhibitors.[22,23]

PT523 proved to be more active than MTX against tumor cells whose level of acquired resistance was in the clinically relevant 10- to 30-fold range and whose resistance was due either to a defect in drug accumulation or to an increase in DHFR activity.[18a] More importantly, in a finding for which there was no precedent in our laboratory, PT523 was more active against the resistant cells than MTX was against the wild-type parental cells. Biochemical studies demonstrated that potent growth inhibition by PT523 was due to a MTX-like effect on reduced folate pools, with thymidine and a purine both needed in the medium to block its effects.[24] More recently, data have been presented which strongly suggest that the enhanced activity of PT523 compared with MTX reflects not only stronger DHFR binding but also more efficient utilization of the reduced folate carrier.[25,26] In addition, it was found that PT523 is synergistic with etoposide *in vitro* and *in vivo* and thus has the potential to be useful in multidrug regimens.[27]

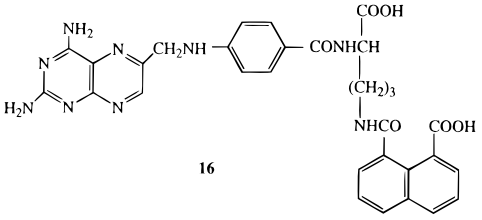
The promising results obtained with PT523 have led us to synthesize second-generation analogues in order to identify the optimal structural features for biological activity. Our initial steps in this direction included the synthesis of the benzoyl ring halogenated analogue **7** and the L-lysine and L-2,4-diaminobutanoic acid analogues **8** and **9**.[28] These three compounds showed similar activity against isolated DHFR, suggesting that binding was not very sensitive to halogenation of the *p*-aminobenzoyl moiety or to small changes in the number of CH_2 groups in the side chain. On the other hand, only **7** was similar to PT523 in its activity against cultured tumor cells, suggesting (a) that the effect of different chain lengths is primarily on cellular uptake and (b) that a side chain with three CH_2 groups, as in L-ornithine, may be optimal. In the present paper we report the synthesis of an additional group of heretofore undescribed side chain analogues consisting of the L-2,3-diaminopropanoic acid analogue **10**, the isophthaloyl



10 : x = 1, R = 2-COOH
11 : x = 3, R = 3-COOH
12 : x = 3, R = 4-COOH
13 : x = 3, R = 4,5-Cl_2-2-COOH

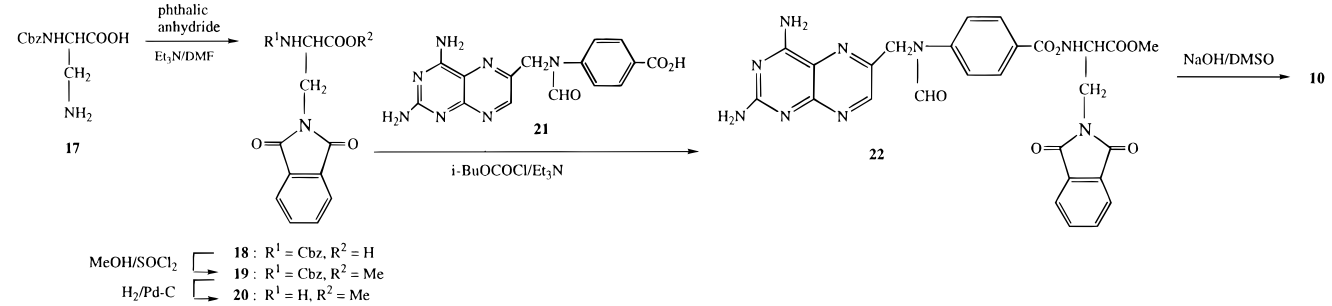


14 : m = 1, n = 0
15 : m = 0, n = 1

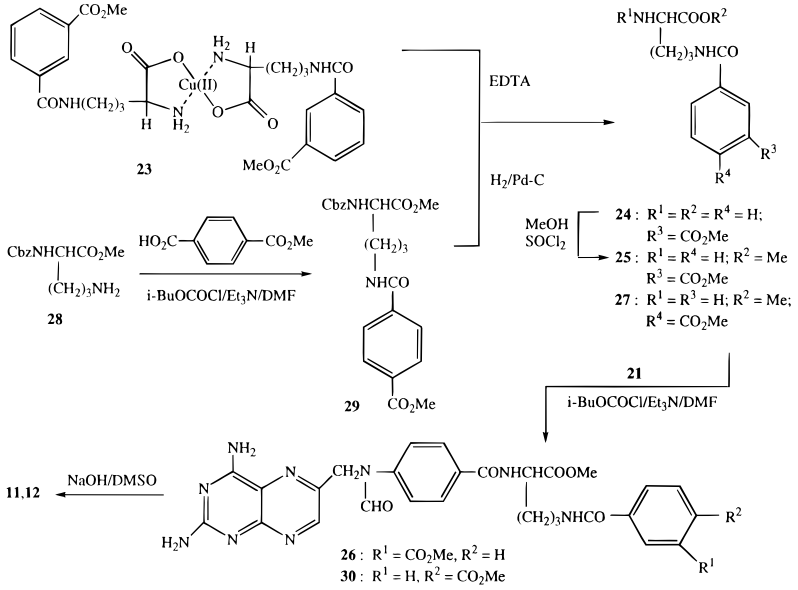


16

Scheme 1



Scheme 2



and terephthaloyl acid analogues **11** and **12**, and the 4,5-dichlorophthaloyl analogue **13**. Experiments directed toward the synthesis of the homophthaloyl analogues **14** and **15** and the 8-carboxynaphthoyl analogue **16** are also described. Compound **10** was made in order to complete the ω-hemiphthaloyl-α,ω-diaminoalkanoic acid series.[28] Its biological properties confirm that the ornithine moiety in PT523 has the optimal chain length. Compounds **11** and **12** were prepared to address the question of whether ortho positioning of the COOH group is optimal. Compound **13** was made to determine whether halogenation is as well tolerated in the phthaloyl ring as it is in the *p*-aminobenzoyl group (cf. **7**).

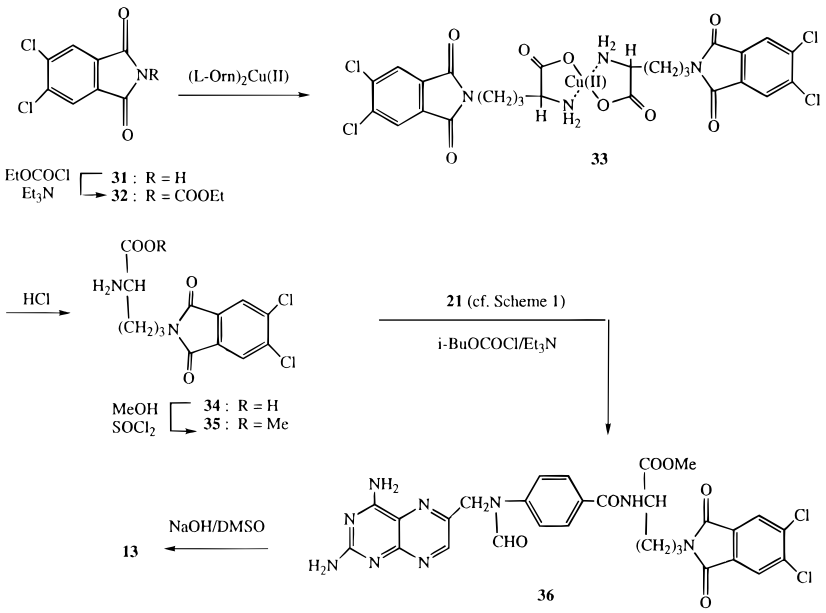
Chemistry

The synthesis of N^{α}-(4-amino-4-deoxypteroyl)-N^{β}-hemiphthaloyl-L-2,3-diaminopropanoic acid (**10**) (Scheme 1) began with N^{α}-(benzyloxycarbonyl)-L-2,3-diaminopropanoic acid (**17**), which we had previously used in connection with other work.[29] Treatment of **17** with phthalic anhydride in DMF solution in the presence of a slight excess of Et_3N at room temperature afforded the phthalimide **18** (52% yield), which was converted sequentially to the methyl ester **19** by reaction with $MeOH/SOCl_2$ and to the hydrochloride salt **20**·HCl by catalytic hydrogenolysis over Pd−C in ethanolic HCl. The overall yield for these last two steps was ca. 66%. Condensation of **20**·HCl with 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA, **21**) in DMF solution by the mixed anhydride method (*i*-BuOCOCl/Et_3N) yielded the protected coupling product **22**, which on treatment with NaOH in DMSO (5 min at room temperature)[18] underwent cleavage of the phthalimide ring and removal of the N^{10}-formyl and methyl ester groups to give **10** in ca. 39% overall yield from **21**.

The synthesis of **11** (Scheme 2) began with the previously unknown chelate bis[N^{δ}-[3-(methoxycarbonyl)benzoyl]-L-ornithinato]copper(II) (**23**), which was prepared in 52% yield from the Cu(II) complex of L-ornithine[30] and 3-(methoxycarbonyl)benzoyl chloride under Schotten−Baumann conditions in water. Removal of the copper from **23** with EDTA gave N^{δ}-[3-(methoxycarbonyl)benzoyl]-L-ornithine (**24**), which on esterification ($MeOH/SOCl_2$) was converted to **25** (65% combined yield, two steps). Condensation with **21** via the mixed anhydride method (*i*-BuOCOCl/Et_3N) then converted **25** to the protected intermediate **26** (33% combined yield, two steps). Treatment of **26** with NaOH in DMSO solution for 5 min at room temperature simultaneously removed the N^{10}-formyl group and cleaved the two ester groups to afford **11** (81%).

The synthesis of the isomeric terephthaloyl derivative **12** from **21** and the previously unknown intermediate methyl N^{δ}-[4-(methoxycarbonyl)benzoyl]-L-ornithinate (**27**) is shown in Scheme 2. N^{α}-(Benzyloxycarbonyl)-L-ornithine was esterified ($MeOH/SOCl_2$) to **28**·HCl (96% yield), and a mixed anhydride reaction (i-BuOCOCl/Et_3N) was used to convert the latter into **29** (83% yield). Hydrogenolysis then gave **27**, which was isolated in 78% yield as the HCl salt. Mixed anhydride coupling with **21**, followed by removal of the protecting groups with

Scheme 3



NaOH in DMSO solution, afforded **30** and **12**, respectively. The overall yield of **12** in the final two steps was 38%.

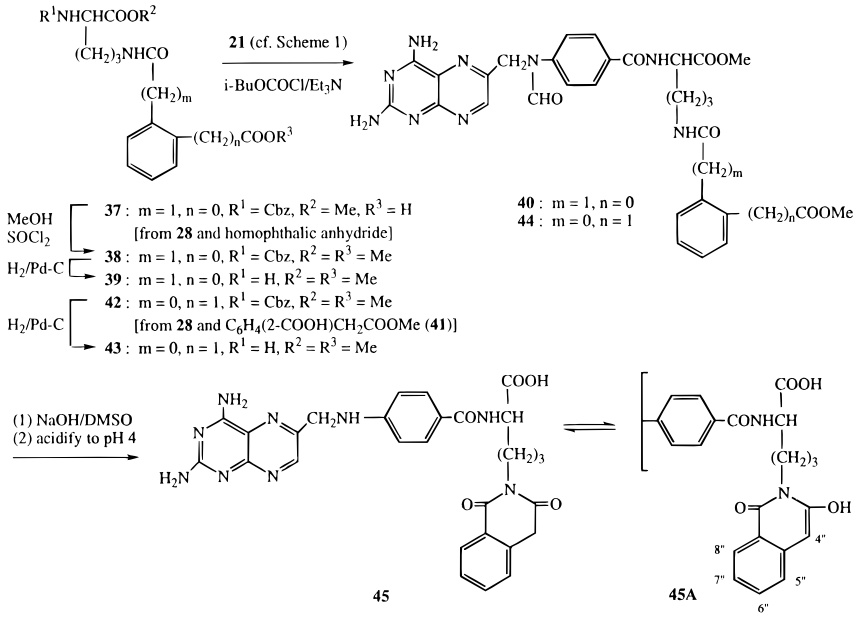
The synthesis of **13** (Scheme 3) began with commercially available 4,5-dichlorophthalic acid, which we converted into the hitherto unknown compound 4,5-dichlorophthalimide (**31**, 44% yield) by heating the dimethyl ester with urea and NaOMe as described for the preparation of phthalimide.[31] Treatment of **31** with ethyl chloroformate in the presence of Et_3N as described for the reaction of phthalimide[32] afforded the *N*-carbethoxy derivative **32** (90%). Acylation of bis(L-ornithinato)Cu(II) with **32** as in the preparation of **23** then gave bis[L-2-amino-5-(4,5-dichlorophthalimido)pentanato]Cu(II) (**33**, 71% yield). Conversion of **33** to L-2-amino-5-(4,5-dichlorophthalimido)pentanoic acid hydrochloride (**34**·HCl) was achieved in 62% yield by treatment with 6 N HCl, a procedure which we found to be more convenient than the use of EDTA. Finally, in a sequence analogous to the one used to obtain **11** and **12**, esterification of **34** with MeOH/$SOCl_2$ gave **35**·HCl (89% yield), mixed anhydride coupling of **35** and **21** gave **36** (21%), and reaction of **36** with NaOH in DMSO gave **13** (67%).

The reaction of dimethyl 4,5-dichlorophthalate with urea and NaOMe proved somewhat capricious, often giving, instead of **31,** a mixture of monomethyl 4,5-dichlorophthalate and a compound we believe to be *N*,*N*′-carbonyldi(4,5-dichlorophthalimide). Interestingly, heating this compound separately with NaOMe in refluxing MeOH converted it to **31**. Similar results were obtained when diethyl 4,5-dichlorophthalate was used instead of the dimethyl ester. Alternative approaches to the synthesis of **31** were also investigated without success, including (a) heating 4,5-dichlorophthalic anhydride with $(NH_4)_2CO_3$ at 260 °C, which gave a sublimate consisting of a mixture of **31** and the starting anhydride; (b) heating dimethyl 4,5-dichlorophthalate in refluxing EtOH saturated with ammonia; and (c) fusion of an intimate mixture of 4,5-dichlorophthaloyl dichloride and NH_4Cl at 130 °C. We concluded that the urea method is workable but requires meticulous optimization of reaction conditions.

In the attempted synthesis of the isomeric homophthaloyl derivatives **14** and **15**, reaction of homophthalic anhydride with **28** (cf. Scheme 2 for structure) in refluxing EtOH yielded **37** (Scheme 4), the product expected from nucleophilic attack on the aliphatic carbonyl group. Esterification of **37** with MeOH/$SOCl_2$ gave **38** (95% yield), which on removal of the Cbz group (H_2/Pd−C) was converted to the amino diester **39** (44% yield). The direction of anhydride ring opening was confirmed by the infrared spectrum of **38**, which contained an aromatic CO_2Me peak at 1710 cm^{-1} in addition to the aliphatic α-CO_2Me peak at 1740 cm^{-1}, and by its ^1H NMR spectrum, which showed an aromatic CO_2Me singlet at δ 3.76 in addition to the aliphatic α-CO_2Me singlet at δ 3.61. Mixed anhydride condensation of **39** and **21** gave the protected coupling product **40** (40%). To obtain the alternative products of ornithine N^{δ}-acylation, homophthalic anhydride was cleaved at the aliphatic carbonyl group with NaOMe, and the resulting monoester (**41**, 80%) was coupled to **28** via a mixed anhydride reaction to obtain **42** (48%). Compounds **42** and **38** had different melting points, and their infrared and ^1H NMR spectra were clearly distinguishable. The infrared spectrum of **42** contained only a single aliphatic CO_2Me peak at 1735 cm^{-1}, and the ^1H NMR spectrum contained, in addition to the usual α-CO_2Me singlet at δ 3.61, a second CO_2Me singlet at δ 3.51 as compared with the aromatic CO_2Me singlet of **38** at δ 3.76. Catalytic hydrogenolysis of the Cbz group in **42** followed by esterification (MeOH/$SOCl_2$) and a mixed anhydride reaction with **21** then gave the expected products **43** (81%) and **44** (44%), respectively. No rearrangements occurred during these steps, as evidenced by clear differences in the infrared and ^1H NMR spectra of **43** and **44** as compared with **39** and **40** (see the Experimental Section).

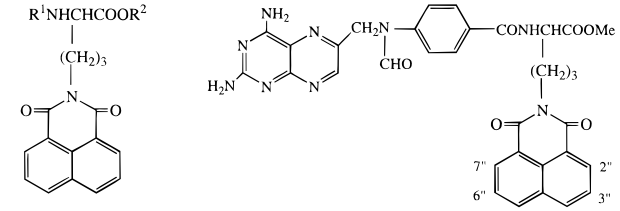
While it had been our aim to convert **40** to **14** and **44** to **15** by treatment with NaOH in DMSO under our usual conditions, we were surprised to find that the identical product formed when either reaction mixture was carefully adjusted to pH 4 and that this product appeared from its ^1H NMR spectrum to be the ring-

Scheme 4



closed structure **45**. Critical to the interpretation of the spectrum of **45** was that the signal assigned to the δ-CH_2 protons was a sharply resolved, highly symmetrical quintuplet at δ 3.86, whereas the δ-CH_2 protons adjacent to the CONH group in the noncyclic precursors **40** and **44** gave broader peaks at δ 3.02 and 3.18, respectively, a difference we ascribe to the fact that **45** is a cyclic imide and thus lacks the NH proton reponsible for the line broadening seen for the δ-CH_2 protons in amides **40** and **44**. The spectrum of **45** also contained two distinctive peaks at δ 4.11 and 7.61. These peaks were absent in the spectra of **40** and **44**, and only the peak at δ 4.11 disappeared on addition of D_2O. We believe these peaks indicate the presence of an enol structure (**45A**) in equilibrium with **45**. This cyclic product apparently forms so rapidly at pH 4 that the open-chain compounds cannot be isolated as free acids. HPLC analysis (C_{18} silica gel, 8% MeCN in 0.1 M NH_4OAC, pH 7.5) of a solution obtained by dissolving this product in base revealed three peaks, one of which coeluted with authentic N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine.[29] The other two products, giving peaks of roughly equal size with retention times of 19 and 21 min, were assumed to be **14** and **15**. Because the retention times on the analytical column were judged to be too similar to allow efficient large-scale separation on a preparative column, and because of the tendency of **45**/**45A** to partially break down to N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine even after a few hours at pH 7.5 (presumably via **14** and **15**), this approach was not pursued further. The facile cleavage of the homophthaloyl group stands in marked contrast with the behavior of the phthaloyl group in PT523, which is stable under these conditions.

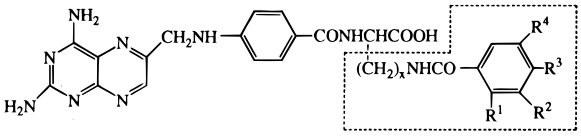
Work was also discontinued on the 8-carboxynaphthoyl derivative **16** because of a severe instability problem. Condensation of 1,8-naphthalic anhydride with N^{α}-(benzyloxycarbonyl)-L-ornithine, followed by the usual sequence of esterification ($MeOH/SOCl_2$), removal of the Cbz group (H_2/Pd-C), and mixed anhydride coupling with **21**, led to the imide **49** via the intermediates **46**−**48**. Unfortunately, when **49** was treated with NaOH under a variety of conditions to remove the formyl group and open the imide ring, the N^{δ}-8-carboxynaphthoyl group was lost even more rapidly than the corresponding group in the homophthaloyl series. Facile cleavage of the amide bond at the *peri* position was presumably due to anchimeric assistance by the 8-carboxy group.



Biological Activity

The ability of **10** to bind to human DHFR and inhibit the growth of human tumor cells in culture was compared with that of PT523 and its previously synthesized chain-lengthened and chain-shortened homologues, **8** and **9**.[28] Our interest in evaluating the analogue with only a single CH_2 group in the side chain was prompted in part by the fact that the spatial separation between the COOH groups in this molecule more closely approximates the distance between these groups in classical antifolates. As shown in Table 1, **10** was a DHFR inhibitor. However, its titration curve was more curvilinear than that of PT523 (results not shown), indicating that a greater concentration would be needed to produce near-stoichiometric inhibition, a critical requirement for complete arrest of *de novo* thymidylate and purine nucleotide synthesis. The results in Table 1 suggest that the location of the aromatic COOH group in PT523 may allow it to interact optimally with some basic residue in DHFR and that altering the number of CH_2 groups diminishes this interaction. This basic residue may lie in the distal part of the active site of DHFR where it lies beyond the reach of the γ-COOH group in the non-polyglutamated form of classical antifolates but may be within striking distance of the

Table 1. Dihydrofolate Reductase Inhibition and *in Vitro* Cell Growth Inhibition by PT523 Analogues Modified in the Hemiphthaloylornithine Moiety



compd	*x*	R^1	R^2	R^3	R^4	rhDHFR activity IC_{50}/[E] (nM/milliunit)[a]	in vitro antitumor activity: IC_{50} (nm) A549 cells[b]	SCC25 cells[c]
8	4	COOH	H	H	H	2.9 (0.69)	5.2 ± 2.2 (0.25)	ND
9	2	COOH	H	H	H	2.5 (0.80)	22 ± 1 (0.059)	ND
10	1	COOH	H	H	H	3.5 (0.57)	23 ± 1 (0.057)	ND
11	3	H	COOH	H	H	2.2 ± 0.4 (0.91)	45 ± 5 (0.029)	2.9 ± 0.7 (0.10)
12	3	H	H	COOH	H	2.8 ± 0.1 (0.71)	3300 ± 580 (0.00039)	72 ± 16 (0.0042)
13	3	COOH	Cl	Cl	H	2.4 (0.83)	ND	18 ± 3 (0.017)
PT523 (**4**)	3	COOH	H	H	H	2.0 ± 0.3 (1.0)	1.3 ± 0.2 (1.0)	0.3 ± 0.1 (1.0)
MTX (**1**)						2.2 ± 0.6 (0.91)	23 ± 5 (0.057)	27 ± 3 (0.011)

[a] Human recombinant DHFR was expressed in *Escherichia coli* and purified as described earlier.[25] Activity was measured at 22 °C by measuring the change in UV absorbance at 340 nm in an assay solution containing 60 μM NADPH, 50 μM dihydrofolate, and 3−6 milliunits of enzyme in 50 mM Tris HCl, pH 7.0. The reaction was initiated by addition of dihydrofolate after preincubation of the other components for 2 min. Results are averaged from a minimum of three experiments on different days for **8**−**10** and **13**, which were assayed once. IC_{50} values are expressed in normalized units of nM/milliunit of DHFR activity to compensate for the fact that precisely the same amount of enzyme was not added to every titration. Values in parentheses are normalized relative to PT523 (1.0). [b] The A459 human non-small lung carcinoma cells (growth assay) were plated at a density of 2000-3000/well in RPMI medium containing 10% fetal bovine serum. After 24 h at 37 °C in a 5% CO_2 humidified atmosphere to allow the cells to adhere to the dishes, they were treated for another 72 h with a range of drug concentrations (0.1−1000 nM), and then stained with Sulforhodamine B as described.[35] Cell growth, expressed as a percent of untreated controls, was plotted as a function of the drug concentration on a log scale. [c] The SCC25 human head and neck squamous carcinoma cells (clonogenicity assay) were grown in tissue culture flasks in Dulbecco's minimal essential medium (MEM) containing 10% fetal bovine serum and hydrocortisone (400 μg/L). Cells in exponential growth cells were preincubated without drug for 48 h, treated with a range of drug concentrations (0.01−5000 nM) for another 72 h, and replated in fresh medium. After 14 days, the colonies were stained with crystal violet and counted. Only colonies containing ≥ 5 cells were counted. The surviving fraction was plotted as a percentage of untreated controls, and the IC_{50} was estimated from a log−log plot. Each experiment was performed in replicate plates and was repeated at least three times on different days. Numbers in parentheses are normalized potencies (PT523 = 1.0). ND = not determined.

α-COOH groups in the second glutamyl residue of a polyglutamate. A residue that could conceivably fulfill this role, most likely via hydrogen bonding through an intervening molecule of water, is Arg-28.[33]

In a growth inhibition assay against human A549 non-small-cell lung carcinoma cells, the IC_{50} of **10** (one CH_2 group) for 72 h of continuous exposure was found to be 23 ± 1 nM, a value essentially identical to that of **9** (two CH_2 groups) or MTX, but 4-fold higher than that of **8** (four CH_2 groups) and 18-fold higher than that of PT523 (three CH_2 groups). It thus appears three CH_2 groups in the side chain are optimal for activity against intact cells as well as for binding to the enzyme. The finding that the IC_{50} differences were greater for growth inhibition than for DHFR inhibition suggested that the length of the side chain affects not only DHFR binding but also cellular accumulation. Since compounds **8**−**10**, unlike MTX, cannot form polyglutamates, their effect on cell growth is not dependent on the level of FPGS activity inside the cell.

The effect of moving the COOH group in PT523 from the ortho to the meta and para position, as in compounds **11** and **12**, was likewise examined (Table 1). The difference in IC_{50} values for DHFR inhibition between PT523 and **11** was not statistically significant (Student's *t*-test). In contrast, the IC_{50} of **12** was 40% higher than that of PT523, suggesting that there is at least some loss of binding when the aromatic COOH group is moved from the ortho to the para position and supporting the view that this group plays an active role in enzyme binding. As with **10**, however, the titration curves for **11** and **12** both showed greater curvilinearity relative to PT523, indicating decreased capacity for near-stoichiometric inhibition. The similarity in the IC_{50} values of **11** and PT523 against DHFR suggests that the meta and ortho COOH groups are able to interact with the same basic amino acid residue in the enzyme complex. However it is also possible that they can interact equally with two different residues. X-ray crystallographic or high-field NMR data for the complexes of these isomeric compounds with DHFR may allow these possibilities to be distinguished.

While the differences in IC_{50} values betwen PT523 and the positional isomers **11** and **12** were relatively minor in the DHFR assay, the ability of these compounds to inhibit cell growth differed substantially. Thus, while the IC_{50} of PT523 against A549 cells after 72 h of continuous drug treatment was 1.3 ± 0.2 nM, the IC_{50} of the meta isomer **11** was 45 ± 5 nM (35-fold decreased activity) and that of the para isomer **12** was 3300 ± 580 nM (2500-fold decreased activity). We were concerned about the possibility that **11** and **12** might be less stable than PT523 under tissue culture conditions (37 °C, pH 7.5), but this proved not to be the case, since the three compounds were completely stable by HPLC after being dissolved in growth medium and kept in the incubator for 48 h. The lower activity of **11** and **12** relative to PT523 can be taken to mean that the aromatic COOH is more than just a passive water-solublizing group on the N^{δ}-aroyl ring and that its position can dramatically influence biological activity. It is worth noting that the ortho COOH group in PT523 can form an internal H-bond to the C═O or NH group in the adjacent amide bond, which is obviously not possible in the meta and para isomers.

The cytotoxicity of **11** and **12** was evaluated also in a clonogenic assay, using human SCC25 squamous cell carcinoma cells. In this assay the IC_{50} of PT523

was 0.3 ± 0.1 nM, whereas that of **11** and **12** was 2.9 nM (10-fold decreased activity) and 72 nM (240-fold decreased activity). Thus, moving the aromatic COOH group to the meta and para position was detrimental to activity in two cell lines representing different histopathologic types and different tissues of origin. Although the differences among the three isomers were less pronounced against the SCC25 cells than against the A549 cells, the same qualitative trend in IC_{50} values was obtained (i.e., ortho < meta ≪ para). Whether the greater differences among these compounds against A549 cells than against SCC25 cells reflect the properties of these cell lines or the assay method (i.e., cell growth versus clonogenicity) was not determined.

The last compound tested in these experiments was the dichlorophthaloyl derivative **13**. In the clonogenic assay against SCC25 cells, its IC_{50} was found to be 18 nM, a 60-fold decrease in activity relative to that of PT523. It thus appears that 4,5-dichloro substitution on the hemiphthaloyl moiety has an undesirable influence on DHFR binding and/or transport. It may be noted that the van der Waals radius of a chlorine atom is 1.8 Å versus 1.2 Å for hydrogen and that the predicted effect of two Cl atoms meta and para to the COOH group would be to increase acidity by approximately 0.6 p*K* unit. Although it is unknown how much each of these factors individually influence DHFR binding and cellular uptake, our results indicate that the net effect of this particular structural modification is a marked decrease in antitumor activity.

Because the *in vitro* activity of **10** was substantially lower than that of the other congeners in the N^{ω}-hemiphthaloyl-α,ω-diaminoalkanoic acid series,[28] this compound was not studied further. However, we did compare the activity of PT523 and its two nearest chain length homologues, **8** and **9**, against the panel of human tumor cell lines currently used by the National Cancer Institute to screen new agents for activity.[35] In addition, we decided to compare the activity of PT523 and its chain-shortened analogue **8** in an *in vivo* tumor growth delay assay using SCC VII squamous cell carcinoma. The latter experiment was done to test the possibility that **8** might have more favorable *in vivo* properties even though it is less active *in vitro*. This could arise, for example, if **8** was cleared more slowly, resulting in a higher plasma AUC. The results of these experiments are summarized in Tables 2 and 3, respectively.

Compounds **8** and **9** showed reasonably good activity against a number of NCI solid tumor panel cell lines (Table 2) but were consistently less active than PT523, which had mean IC_{50} values in the low nanomolar (1.0−10 nM) or subnanomolar (0.1−1.0 nM) range in 19 (70%) of the 27 cell lines shown. By comparison, **8** and **9** had IC_{50} values in the 0.1−10 nM range against 16/27 (59%) and 9/27 (33%) tumors, respectively, whereas the IC_{50} of MTX was outside this concentration range against all 27 cell lines and was >100 nM in a number of instances. Two additional results in Table 2 are of interest. The first is that MCF-7/ADR cells, which are pleiotropically drug-resistant by virtue of an increase in P-glycoprotein expresssion, were not cross-resistant to PT523. Thus, even though PT523 shares with lipophilic antifolates like trimetrexate (**5**) and piritrexim (**6**) a lack of dependence on FPGS for its activity, it has a potential advantage over these drugs in being impervious to P-glycoprotein-mediated drug resistance. The second interesting result is that, for several of the cell lines against which the IC_{50} (48 h) of PT523 was >10 nM (i.e., NCI-H522, EKVX, SNB-75, OVCAR-3, and PC-3), nanomolar sensitivity was achieved by extending the treatment time to 144 h (cf. Table 2, footnote *c*). For cells whose IC_{50} (48 h) was in the 1−10 nM range, sensitivity was increased to the subnanomolar range in several cases (data not shown). Since PT523 cannot form polyglutamates, differences in FPGS activity among the various cells cannot account for their different sensitivity to this drug. The finding that sensitivity of some of the resistant cell lines is increased dramatically by increasing the duration of exposure suggests that, other things being equal, activity may depend on the time needed for the intracellular drug to reach a critical concentration in excess of the DHFR level. Therefore, in order for *in vivo* treatment with PT523 to be effective,

Table 2. Comparative Activity of MTX with PT523 and Its Nearest Chain Length Homologues **8** and **9** against Selected Human Carcinoma Cell Lines in Culture

		IC_{50} (nM)[a]			
tumor type[b]	cell line	MTX	PT523[c]	**8**	**9**
NSCL	NCI-H460	28	0.5	4.2	14
	NCI-H23	43	4.4	9.2	32
	NCI-H522	229	23	$>10^3$	$>10^3$
	EKVX	$>10^3$	23	36	21
colon	HCT-116	30	0.3	0.6	5.0
	HCT-15	30	1.2	5.2	7.2
	HT29	32	1.2	5.7	9.8
	SW-620	33	1.9	3.4	6.3
	KM12	42	3.2	4.9	$>10^3$
CNS	SF-539	35	3.7	26	57
	SF-268	52	2.5	8.4	$>10^2$
	SNB-75	$>10^4$	$>10^4$		$>10^3$
melanoma	LOX IMVI	26	0.4	0.9	4.4
	UACC-62	28	1.8	$>10^3$	12
	SK-MEL-5	87	6.0	91	85
	MALME-3M	$>10^3$	2.0	$>10^3$	$>10^3$
	SK-MEL-28	$>10^3$	4.6	$>10^3$	$>10^3$
ovarian	OVCAR-8	31	2.4	5.9	8.1
	OVCAR-5	$>10^3$	2.5	$>10^3$	86
	OVCAR-3	398	$>10^2$	$>10^3$	$>10^3$
renal	786-0	33	0.6		9.2
	UO-31	191	5.5	7.8	8.0
	ACHN	40	2.4	5.2	45
breast	MCF7	36	3.6	5.0	$>10^3$
	MCF7-ADR	78	2.7	9.1	$>10^3$
prostate	PC-3	2.0	$>10^2$	5.0	6.7
	DU-145	23	9.5	9.1	34

[a] Assays were performed according to the standard NCI protocol,[33] in which cells are plated in 96-well plates, treated continuously with drug for 48 h, and stained for total protein with Sulforhodamine B. All the data were generously provided by the Division of Cancer Treatment, National Cancer Institute (DCT/NCI). The results for PT523 against all the cell lines except the breast and prostate carcinomas are from two experiments (ID# 9009RC34 and 9510SR81), whereas those for the breast and prostate carcinomas are from a single experiment (ID# 9510SR81). The results for **8** and **9** against all the tumors are likewise from a single experiment (ID# 9407RM60). Although assays were carried out with all the tumors in the NCI solid tumor panel, data are included in the table only for those cell lines whose IC_{50} values (GI_{50} values according to NCI terminology) agreed closely and could be averaged. The IC_{50} values of MTX are the historical averages of >50 assays per cell line and were kindly provided by Dr. J. W. H. Watthey (Starks C. P., Rockville, MD). [b] NSCL = non-small-cell lung. [c] In a separate experiment (ID# 9510LC81) in which cells that were relatively insensitive to PT523 during the standard 48-h exposure were treated for 144 h, the following IC_{50} values were obtained: NCI-H522, 3.7 nM; EKVX, 1.6 nM; SNB-75, 2.9 nM; OVCAR-3, 2.8 nM; PC-3, 0.72 nM.

Table 3. Effect of N^{α}-(4-Amino-4-deoxypteroyl)-N^{γ}-hemiphthaloyl-L-2,4-diaminobutanoic acid (**9**), PT523, and MTX on the Growth of Subcutaneously Implanted SCC-VII Squamous Cell Carcinoma in Mice

			tumor size (mm)[b]						
			day 0		day 7		time to reach 10 mm (days)		
compd	dose[a]	no. of mice	range	mean	range	mean	range	mean	TGD (days)[c]
0.9% NaCl		23[d]	1−2.5	1.3	4.5−15	9.8	4−21	7.2	
9	0.52	5	1−1.5	1.1	1−9	5.1	9−27	9.8	2.6 (35)
PT523	0.19	8[e,f]	1−3	2.1	3.5−9.5	6.8	8−25	12	5 (67)
MTX	0.75	5[f]	1−2	1.6	6−8	7.3	10−14	12	5 (67)

[a] Dose in mg/kg/day infused over 5 days via Alzet osmotic minipumps (Model 2001, volume 200 μL, flow rate 1.0 μL/h). Drugs were injected in water adjusted to pH 7.5−8.0. [b] SCC-VII cells (2×10^6, >90% dye exclusion) were implanted subcutaneously in the hind limb of C3H male mice (21−30 g). Pumps were implanted subcutaneously in the scapular region when the tumor became palpable (day 0) and were removed on day 5. Animals were euthanized in the approved manner when the tumor reached 18-20 mm, or sooner if they appeared moribund. Tumor size was defined as the mean of two orthogonal measurements using calipers. Tumors were generally measured every day, but on a few occasions were measured every second or third day. For calculation purposes, any tumor of <1 mm on day 0 or 1 was scored as being 1 mm in size. [c] Tumor growth delay (TGD) was calculated by subtracting the average time required to reach 10 mm in the control group from the average time to reach this size in the treatment group. Numbers in parentheses represent the percent increase in the time to reach the endpoint. [d] Results of four separate experiments each using five to seven mice. [e] Results of two experiments each using four mice. [f] One mouse died on day 7 in one of the PT523 experiments, and one died on day 10 in the MTX experiment.

a nanomolar concentration of the drug may have to be maintained in plasma for as long as 144 h.

PT523 and **9** were also tested *in vivo* against a subcutaneously implanted murine tumor, SCC VII squamous cell carcinoma.[37] In view of our earlier *in vitro* evidence suggesting that cell killing by PT523 depends markedly on the duration of exposure,[27] the drugs were administered over 5 days from subcutaneously implanted Alzet osmotic minipumps. For comparison purposes MTX was included in the experiment even though we recognized that a 5-day infusion would not necessarily be optimal for a polyglutamated drug. The therapeutic endpoint was taken to be the number of days for the tumor to grow from 1−2 to 10 mm. The SCC VII tumor was chosen because it is relatively unresponsive to MTX and because we wanted to show that, when PT523 and MTX are administered by continuous infusion, a lower dose of PT523 can retard tumor growth to the same degree even though polyglutamation is possible only with MTX. Compound **9** was chosen over **8** because there was a greater difference between its activity and that of PT523 *in vitro*, and we therefore felt that there was a better likelihood of seeing a difference *in vivo*. As indicated in Table 3, an identical tumor growth delay (TGD) of 4.8 days was achieved with PT523 as with MTX, but the dose of PT523 giving this effect was only 0.19 mg/kg/day whereas that of MTX was 0.75 mg/kg/day. This 4-fold difference was consistent with the previously reported 6.5-fold potency difference between PT523 and MTX against SCC VII cells treated continuously for 72 h.[28] Both higher and lower doses of the two drugs were also tested, but the lower doses were less effective whereas the higher doses were toxic (data not shown). Compound **9** was well tolerated at 0.52 mg/kg/day, but gave a somewhat lower TGD of 2.6 days. The most pertinent aspect of these results was that **9** was obviously better tolerated than PT523, in qualitative agreement with their respective *in vitro* activities (cf. Tables 1 and 2). Since we previously found **9** to be 10−20-fold less potent than PT523 in culture depending on the cell line tested,[28] it is conceivable that a dose greater than 0.52 mg/kg/day would increase the TGD beyond 2.6 days without producing toxicity.

In summary, the results in this paper demonstrate that the combination of an unsubstituted hemiphthaloyl group and three CH_2 groups in the side chain are critical determinants of the potent *in vitro* biological activity of PT523. Modifications of other regions of the molecule, such as the B ring and the bridge, are being studied in our laboratory as part of a larger program of structure−activity optimization.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 781 double-beam instrument. With the exception of the homophthaloyl derivatives, for which full spectra are reported in order to validate the structural integrity of the two series, peaks below 1400 cm^{-1} (as well as weak peaks and shoulders) are omitted. Ultraviolet absorption spectra were obtained on a Varian Model 210 spectrophotometer. ^1H NMR spectra were obtained at 60 MHz on a Varian EM360L instrument with Me_4Si as the reference or at 500 MHz on a Varian Model ML500 instrument. TLC analyses were on fluorescent Baker 250F silica gel plates, and spots were visualized under 254-nm UV illumination or with the aid of iodine or ninhydrin. Column chromatography was on Baker silica gel (regular grade, 60−200 or 70−230 mesh; flash grade, 40-μm particle size) or Whatman DE-52 DEAE-cellulose (preswollen). Solvents for moisture sensitive reactions were dried over Linde 4A molecular sieves. Analytical HPLC separations were done on C_{18} silica gel radial compression cartridges (Waters, Milford, MA; analytical: 5-μm particle size, 5 × 100 mm; preparative: 15-μm particle size, 25 × 100 mm). Solid reaction products were generally dried *in vacuo* over P_2O_5 at 50−70 °C, in some instances after preliminary drying in a benchtop lyophilization apparatus. Elemental analysis and ^1H NMR spectra showed that fractional molar amounts of organic solvents were retained in some of the analytical samples even after careful drying. Melting points (not corrected) were measured on a Fisher-Johns hot-stage microscope or in open Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA). Synthetic starting materials and other chemicals were purchased from Aldrich, Milwaukee, WI, and from Fluka, Ronkonkoma, NY. Microanalyses were performed by Robertson Laboratory, Madison, NJ, and were within ±0.4% of theoretical values unless otherwise specified.

2-L-[*N*-(Benzyloxycarbonyl)amino]-3-phthalimidopropanoic Acid (18). Solid N^{α}-(benzyloxycarbonyl)-L-2,3-diaminopropanoic acid (**17**) (477 mg, 2 mmol) was stirred for 24 h in a solution of phthalic anhydride (326 mg, 2.2 mmol) and Et_3N (304 mg, 418 μL, 3 mmol) in dry DMF (25 mL) at room temperature. The solution was evaporated to dryness under reduced pressure, and the residue was redissolved in $CHCl_3$. The solution was washed with 1 N HCl (10 mL), rinsed with H_2O, dried ($MgSO_4$), and evaporated. The oily residue was chromatographed on a silica gel column (30 g, 20 × 2 cm) with 28:12:1 $CHCl_3$−MeOH−28% NH_4OH as the eluent. Fractions were analyzed by TLC with the same eluent, and fractions containing a single spot with R_f 0.53 were pooled and evapo-

rated to a semisolid: crude yield 406 mg (52%); mp 81-83 °C ($CHCl_3$−Et_2O); IR (KBr) ν 2500−3500 br, 1775, 1710, 1660−1680, 1600, 1520, 1470, 1440 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.01 (m, 2H, β-CH_2), 4.40 (m, 1H, α-CH), 4.75 (s, 2H, OCH_2Ph), 6.0−7.7 (m, 9H, aromatic protons). Anal. ($C_{19}H_{16}N_2O_6 \cdot 0.75NH_3 \cdot 0.75\ H_2O$) C, H, N.

Both the solid and the material recovered from the mother liquor were of sufficient purity to be used in the next step.

Methyl 2-L-[*N*-(Benzyloxycarbonyl)amino]-3-phthalimidopropanoate (19). $SOCl_2$ (0.37 mL) was added dropwise to a stirred solution of **18** (137 mg, 0.347 mmol) in MeOH (3 mL) over a period of 3 min while the temperature was kept at −30 to −15 °C. After 18 h at room temperature, the solvent was evaporated under reduced pressure to obtain a solid (142 mg, 100%). Recrystallization from *i*-PrOH afforded colorless needles: mp 139−140 °C; R_f 0.86 (silica gel, 95:5 $CHCl_3$−MeOH); IR (KBr) ν 3420, 3300, 1770, 1750, 1720, 1680, 1530, 1440, 1400 cm^{-1}. Anal. ($C_{20}H_{18}N_2O_6$) C, H, N.

Methyl 2-L-Amino-3-phthalimidopropanoate Hydrochloride (20·HCl). A solution of **19** (320 mg, 0.837 mmol) in a mixture of 95% EtOH (70 mL) and 1 N HCl (1.5 mL) was shaken with H_2 and 10% Pd−C for 6 h in a Parr apparatus at 50 lb/in.^{2} initial pressure. The catalyst was filtered, the solution was evaporated to dryness, the residue was taken up in a mixture of $CHCl_3$ and EtOH, and the solution was re-evaporated. The solid residue was stirred in absolute EtOH, a small amount of insoluble material was removed, and the filtrate was concentrated to 10 mL and cooled to 0 °C. Filtration yielded a solid (61 mg): mp 219−220 °C; R_f 0.39 (silica gel, 95:5 $CHCl_3$−MeOH), ninhydrin positive; IR (KBr) ν 3400, 3000, 2900, 1770, 1750, 1710, 1590, 1560, 1500, 1460, 1430, 1410 cm^{-1}. Anal. ($C_{12}H_{13}ClN_2O_4$) C, H, Cl, N.

Evaporation of the mother liquor and crystallization of the residue from *i*-PrOH/MeOH gave another 95 mg of material indistinguishable from the analytical sample; total yield 156 mg (66%).

Methyl 2-L-[(4-Amino-4-deoxy-N^{10}-formylpteroyl)amino]-3-phthalimidopropanoate (22). A suspension of **20**·HCl (183 mg, 0.5 mmol) in dry DMF (10 mL) was treated sequentially with Et_3N (152 μL, 111 mg, 1.1 mmol) and *i*-BuOCOCl (65.3 μL, 68 mg, 0.5 mmol) and stirred for 20 min at room temperature. Solid **21** (142 mg, 0.5 mmol) was then added, and after another 20 min of stirring the reaction was quenched by adding 2 drops of AcOH. The solution was concentrated to dryness by rotary evaporation, the residue was dissolved in 2:5:5 MeOH−MeCN−$CHCl_3$, and the solution was applied onto a flash silica gel column (10 g, 22 × 1 cm), which was eluted with the same solvent mixture. Fractions giving a blue-fluorescent TLC spot (R_f 0.33, silica gel, 2:5:5 MeOH−MeCN−$CHCl_3$) were pooled and evaporated, and the residue was redissolved in $CHCl_3$. The solution was washed twice with H_2O, dried ($MgSO_4$), and evaporated; yield 107 mg (36%). The product was dissolved in $CHCl_3$−MeOH, and the solution was added to Et_2O. The mixture was cooled, and the precipitate was filtered and dried to obtain a yellow powder (86 mg): mp 158−160 °C; IR (KBr) ν 3450, 3350, 3200, 1770, 1740, 1720, 1710, 1660, 1600, 1560, 1530, 1500, 1440 cm^{-1}. Anal. ($C_{27}H_{23}N_9O_6 \cdot 2H_2O$) C, H, N.

Evaporation of the mother liquor yielded a second crop which was indistinguishable from the analytical sample.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{β}-hemiphthaloyl-L-2,3-diaminopropanoic Acid (10). A solution of **22** (90 mg, 0.149 mmol) in DMSO (1.0 mL) was treated dropwise with 2.5 N NaOH (0.4 mL) over a period of 30 s. After 5 min, the solution was diluted with H_2O (5 mL) and adjusted to pH 5.0 with 10% AcOH to obtain a flocculent precipitate. After 1 h at 0 °C, the solid was filtered, washed with a large volume of H_2O, dried in a lyophilization apparatus, and finally dried *in vacuo* over P_2O_5 to obtain a yellow powder (70 mg, 80%): mp >225 °C dec; R_f 0.15 (silica gel, 5:4:1 $CHCl_3$−MeOH−28% NH_4OH); IR (KBr) ν 3340 br, 1635, 1600, 1510, 1450 cm^{-1}. Anal. ($C_{25}H_{23}N_9O_6 \cdot 2.7H_2O$) C, H, N.

Bis[N^{δ}-[3-(methoxycarbonyl)benzoyl]-L-ornithinato]-copper(II) (23). $SOCl_2$ (15 mL) and dry DMF (2 drops) were added to monomethyl isophthalate (5 g, 17.7 mmol), and the reaction mixture was refluxed at 80 °C for 6 h. The $SOCl_2$ was removed *in vacuo*, and the residue was recrystallized from petroleum ether (bp 30−60 °C) to obtain 3-(methoxycarbonyl)-benzoyl chloride (4.5 g, 81%) as a low-melting solid which liquified at room temperature (lit.[36] mp 41−43 °C). The low-melting solid was used directly in the next step.

To a solution of L-ornithine hydrochloride (3.82 g, 22.7 mmol) in H_2O (30 mL) containing NaOH (1.8 g, 45.4 mmol) was added a solution of $Cu(OAc)_2 \cdot H_2O$ (2.26 g, 11.3 mmol) in H_2O (30 mL), followed sequentially by solid $NaHCO_3$ (1.9 g, 22.7 mmol) and 3-(methoxycarbonyl)benzoyl chloride (4.5 g, 22.7 mmol). The reaction mixture was stirred for 1 h and filtered. The solid was washed with hot MeOH and dried, first in a lyophilization apparatus and then overnight over P_2O_5 to obtain a blue powder (3.83 g, 52%): mp 220 °C with darkening; IR (KBr) ν 3260, 2940, 1720, 1660, 1530, 1430, 1400 cm^{-1}. Anal. ($C_{28}H_{34}N_4O_{10}Cu$) C, H, N, Cu.

N^{δ}-[3-(Methoxycarbonyl)benzoyl]-L-ornithine (24). The Cu complex **23** (3.28 g, 5.06 mmol) was treated with 0.12 M EDTA (100 mL, pH 8). A white solid precipitated immediately. The solution was left in the refrigerator overnight, and the precipitate was filtered, washed with ice-cold H_2O (2 × 10 mL), and dried *in vacuo* at 70 °C to obtain a white solid (1.93 g, 65%) of sufficient purity for direct use in the next step: mp 225 °C dec; IR (KBr) ν 3250, 2940, 1720, 1630, 1580, 1540, 1520, 1430, 1400 cm^{-1}; ^1H NMR (TFA) δ 2.1 (m, 4H, β- and γ-CH_2), 3.6 (m, 2H, δ-CH_2), 3.9 (s, 3H, CO_2CH_3), 4.4 (m, 1H, α-CH), 7.6−8.4 (m, 4H, isophthaloyl protons). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

Methyl N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-[3-(methoxycarbonyl)benzoyl]-L-ornithinate (26). A stirred suspension of **24** (1.93 g, 6.6 mmol) in absolute MeOH (100 mL) at −10 °C was treated dropwise with $SOCl_2$ (7.5 mL) while keeping the temperature below 0 °C. When addition was complete, the reaction mixture was left to stir at toom temperature for 18 h. The solvent was evaporated under reduced pressure and the residue taken up in a minimum volume of $CHCl_3$. Excess Et_2O was added, and the precipitated solid was filtered and dried to obtain ester **25** as a white powder (2.25 g, 100%) of sufficient purity for direct use in the next step: mp 70 °C; IR (KBr) ν 3400, 2920, 1740, 1720, 1630, 1530, 1430 cm^{-1}; ^1H NMR (CD_3OD) δ 1.9 (m, 4H, β- and γ-CH_2), 3.5 (m, 2H, δ-CH_2), 3.85 (s, 3H, α-CO_2CH_3), 3.95 (s, 3H, aromatic CO_2CH_3), 4.2 (m, 1H, α-CH), 8.0 (m, 4H, isophthaloyl protons).

To a stirred suspension of **25** (183 mg, 0.5 mmol) in dry DMF (10 mL) were added sequentially Et_3N (140 μL, 1.01 mg, 1 mmol) and *i*-BuOCOCl (65 μL, 69 mg, 0.5 mmol). After 20 min, **21** (190 mg, 0.5 mmol) was added and the reaction mixture was stirred for 40 min. The solvent was evaporated under reduced pressure, and the residue was chomatographed on a silica gel column (15 g, 17 × 2.0 cm), which was packed and eluted first with 98:2 and then with 95:5 $CHCl_3$−MeOH. Fractions giving a single TLC spot (R_f 0.18, silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, and the residue was taken up in a small volume of 95:5 $CHCl_3$−MeOH. This solution was then added dropwise to excess Et_2O, and the yellow precipitate was collected and dried (105 mg, 33%): mp 145 °C; IR (KBr) ν 3350, 2950, 1725, 1650, 1600, 1550, 1500, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6 (m, 2H, β-CH_2), 1.8 (m, 2H, γ-CH_2), 3.3 (m, 2H, δ-CH_2), 3.60 (s, 3H, α-CO_2CH_3), 3.86 (s, 3H, aromatic CO_2CH_3), 4.42 (m, 1H, α-CH), 5.21 (s, 2H, 9-CH_2), 7.56 (m, 2H, 3′- and 5′-H), 7.61 (m, 1H, isophthaloyl CONH), 7.85 (m, 2H, 2′- and 6′-H), 7.58 (m, 1H, proton meta to isophthaloyl CO_2CH_3), 8.05 (s, 2H, proton para to isophthaloyl CONH and proton ortho to isophthaloyl CONH and para to isophthaloyl CO_2CH_3), 8.40 (s, 1H, proton ortho to isophthaloyl COOH and CONH), 8.66 (s, 1H, 7-H), 8.71 (m, 1H, benzoyl CONH), 8.78 (s, 1H, CHO). Anal. ($C_{30}H_{31}N_9O_7 \cdot H_2O$) C, H, N.

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-(isophthaloyl)-L-ornithine (11). NaOH (2.5 N, 0.4 mL) was added to a stirred solution of **26** (95 mg, 0.15 mmol) in DMSO (1 mL) at room temperature, and after 5 min the reaction was quenched by diluting it with H_2O (10 mL) and adjusting the pH to 4.2 with 1 N HCl. The yellow precipitate was filtered immediately, washed with H_2O (70 mL), and dried (78 mg, 81%): mp 220

°C dec; TLC R_f 0.19 (1:4:5 28% NH_4OH−MeOH−$CHCl_3$); HPLC 33.5 min (C_{18} silica gel; 0.1 M NH_4OAc, pH 5.5, with 2.0−35% MeCN linear gradient over 90 min; 1 mL/min); IR (KBr) ν 3400, 2900, 1640, 1600, 1500, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6 (m, 2H, β-CH_2), 1.8 (m, 2H, γ-CH_2), 3.4 (m, 2H, δ-CH_2), 4.3 (m, 1H, α-CH), 4.5 (m, 2H, 9-CH_2), 6.6 (br s, 2H, NH_2), 6.7 (m, 2H, 3′- and 5′-H), 6.80 (m, 1H, 10-H), 7.7 (m, 2H, 2′- and 6′-H), 7.56 (m, 1H, proton meta to isophthaloyl COOH), 8.05 (m, 1H, proton para to isophthaloyl COOH), 8.15 (m, 1H, proton ortho to isophthaloyl COOH and para to isophthaloyl CONH), 8.40 (s, 1H, proton ortho to isophthaloyl COOH and CONH), 8.68 (s, 1H, 7-H), 8.05 (m, 1H, benzoyl CONH), 8.65 (s, 1H, isophthaloyl CONH). Anal. ($C_{27}H_{27}N_9O_6$·1.1H_2O) C, H, N.

Methyl N^{α}-(Benzyloxycarbonyl)-N^{δ}-[4-(methoxycarbonyl)benzoyl]-L-ornithinate (29). A stirred suspension of N^{α}-(benzyloxycarbonyl)-L-ornithine (3 g, 11.3 mmol) in absolute MeOH (100 mL) was treated dropwise with $SOCl_2$ (10 mL) while the temperature was kept below 0 °C. When addition was complete the cooling bath was removed and the reaction mixture left to stir for 18 h. The solvent was evaporated under reduced pressure, and the residue was recrystallized from a mixture of MeOH and EtOAc to obtain methyl N^{α}-(benzyloxycarbonyl)-L-ornithinate hydrochloride (**28**·HCl) as white solid (3.24 g, 96%) which was of sufficient purity for direct use in the next step: mp 139−140 °C; IR (KBr) ν 3340, 2960, 2890, 1735, 1685, 1530, 1470 cm^{-1}; ^1H NMR (CD_3OD) δ 1.85 (m, 4H, β- and γ-CH_2), 3.00 (m, 2H, δ-CH_2), 3.7 (s, 3H, CO_2CH_3), 4.3 (m, 1H, α-CH), 5.1 (s, 2H, benzylic CH_2), 7.3 (s, 5H, aryl protons).

A solution of monomethyl terephthalate (0.6 g, 3.3 mmol) in dry DMF (50 mL) was treated with *i*-BuOCOCl (428 μL, 452 mg, 3.3 mmol) and Et_3N (459 μL, 330 mg, 3.3 mmol). After 20 min, **28**·HCl (1.04 g, 3.3 mmol) was added, followed by a second portion of Et_3N (459 μL, 330 mg, 3.3 mmol). After 40 min of continued stirring, additional portions of *i*-BuOCOCl (214 μL, 226 mg, 1.65 mmol) and Et_3N (225 μL, 164 mg 1.62 mmol) were added, and after another 40-min period the solvent was evaporated under reduced pressure. The residue was taken up in $CHCl_3$ (120 mL), and the solution was washed with distilled H_2O (2 × 70 mL), dried ($MgSO_4$), and evaporated under reduced pressure. The residue was chromatographed on a silica gel column (60 g, 27 × 3 cm) which was packed and eluted with 98:2 $CHCl_3$−MeOH. Fractions containing a major TLC spot with R_f 0.56 (silica gel, 95:5 $CHCl_3$−MeOH), along with several lesser spots, were pooled and chromatographed on a silica gel column (40 g, 27 × 2.5 cm) which was packed and eluted with $CHCl_3$ followed by 99.5:0.5 $CHCl_3$−MeOH. Fractions giving a single TLC spot (R_f 0.56, 95:5 $CHCl_3$−MeOH) were pooled and evaporated to a white solid (1.21 g, 83%): mp 130 °C; IR (KBr) ν 3330, 2960, 1750, 1725, 1695, 1635, 1540, 1440 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.8 (m, 4H, β- and γ-CH_2), 3.5 (m, 2H, δ-CH_2), 3.75 (s, 3H, α-CO_2CH_3), 3.95 (s, 3H, aromatic CO_2CH_3), 4.4 (m 1H, α-CH), 5.1 (s, 2H, benzylic CH_2), 7.3 (s, 5H, phenyl protons), 7.8−8.2 (dd, 4H, terephthaloyl protons). Anal. ($C_{23}H_{26}N_2O_7$) C, H, N.

Methyl N^{δ}-[4-(Methoxycarbonyl)benzoyl]-L-ornithinate Hydrochloride (27·HCl). A solution of **29** (1.21 g, 2.75 mmol) in a mixture of MeOH (30 mL) and glacial AcOH (9 mL) was shaken under H_2 (2 atm) in a Parr apparatus in the presence of 10% Pd−C (0.17 g) for 14 h. The reaction mixture was filtered through Celite, and the filter pad was washed with MeOH. The combined filtrate and wash were evaporated under reduced pressure, and the residue was treated with CH_2Cl_2 (30 mL) and H_2O (60 mL). The mixture was stirred vigorously while solid $NaHCO_3$ was added in small portions until the pH was 8. After CH_2Cl_2 (30 mL) and H_2O (50 mL) were added, the layers were separated. The aqueous layer was extracted once more with CH_2Cl_2 (30 mL), and the combined organic layers were dried ($MgSO_4$) and evaporated. The residue was dissolved in MeOH (10 mL), and 6 N HCl (2 mL) was added with constant stirring. Excess MeOH and HCl were removed by rotary evaporation, and the solid residue was redissolved in a minimum amount of MeOH. The solution was added to an excess of Et_2O with continuous stirring, and the precipitate was collected, washed with Et_2O, and dried: yield 0.66 g (78%); mp 230 °C dec; IR (KBr) ν 3315, 2960, 1750, 1725, 1650, 1500, 1440 cm^{-1}; ^1H NMR (CD_3OD) δ 1.8 (m, 2H, β-CH_2), 2.0 (m, 2H, γ-CH_2), 3.5 (m, 2H, δ-CH_2), 3.84 (s, 3H, α-CO_2-CH_3), 3.93 (s, 3H, aromatic CO_2CH_3), 4.12 (m, 1H, α-CH), 7.9 (dd, 4H, terephthaloyl protons). Anal. ($C_{15}H_{20}N_2O_5$·HCl·0.1Et_2O) C, H, Cl, N.

Methyl N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-[4-(methoxycarbonyl)benzoyl]-L-ornithinate (30). *i*-BuOCOCl (65 μL, 68 mg, 0.5 mmol) and Et_3N (140 μL, 101 mg, 1 mmol) were added to a stirred suspension of **21** (183 mg, 0.5 mmol) in dry DMF (10 mL). After 20 min, **28**·HCl (190 mg, 0.5 mmol) was added, followed 20 min later by *i*-BuOCOCl (32 μL, 34 mg, 0.25 mmol) and Et_3N (35 μL, 25 mg, 0.25 mmol). After another 20 min, additional portions of **27**·HCl (95 mg, 0.25 mmol) and Et_3N (35 μL, 25 mg, 0.25 mmol) were added, and the mixture was stirred for another 20 min. The solvent was evaporated under reduced pressure, and the residue was dissolved in $CHCl_3$ (100 mL). The solution was washed with distilled H_2O (2 × 50 mL), dried ($MgSO_4$), and applied onto a column of silica gel (20 g, 27 × 2.0 cm) which was packed with 98:2 $CHCl_3$−MeOH and eluted with the same mixture, followed by 95:5 and finally 9:1 $CHCl_3$−MeOH. Fractions giving a single TLC spot (R_f 0.18, silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated. The residue was redissolved in a small amount of 4:1 $CHCl_3$−MeOH and the solution added dropwise to an excess of Et_2O. The yellow precipitate was collected and dried: yield 120 mg (38%); mp 130 °C dec; IR (KBr) ν 3330, 2950, 1725, 1640, 1610, 1565, 1540, 1505, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6 (m, 2H, β-CH_2), 1.8 (m, 2H, γ-CH_2), 3.25 (m, 2H, δ-CH_2), 3.59 (s, 3H, α-CO_2CH_3), 3.85 (s, 3H, aromatic CO_2-CH_3), 4.4 (m, 1H, α-CH), 5.19 (s, 2H, 9-CH_2), 7.57 (m, 2H, 3′- and 5′-H), 7.9−8.0 (m, 4H, terephthaloyl protons), 8.60 (m, 1H, terephthaloyl CONH), 8.63 (m, 2H, 2′- and 6′-H), 8.63 (s, 1H, 7-H), 8.70 (m, 1H, benzoyl CONH), 8.77 (s, 1H, CHO). Anal. ($C_{30}H_{31}N_9O_7$·H_2O).

N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-terephthaloyl-L-ornithine (12). A solution of **30** (72 mg, 0.11 mmol) in DMSO (1 mL) was treated with 2.5 N NaOH (0.4 mL). The mixture was stirred for 5 min, diluted with H_2O (10 mL), adjusted to pH 4.2 with 1 N HCl, and filtered immediately. The yellow solid was washed with H_2O (50 mL) and dried: yield 63 mg (100%); mp 210 °C dec; TLC R_f 0.45 (silica gel, 5:4:1 $CHCl_3$−MeOH−28% NH_4OH); HPLC 33.5 min (C_{18} silica gel; 0.1 M NH_4OAc, pH 5.5, with 2.0−35% MeCN linear gradient over 90 min; 1 mL/min); IR (KBr) ν 3400, 2910, 1640, 1610, 1550, 1510, 1450, 1400 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6 (m, 2H, β-CH_2), 1.8 (m, 2H, γ-CH_2), 3.6 (m, 2H, δ-CH_2), 4.33 (m, 1H, α-CH), 4.47 (s, 2H, 9-CH_2), 6.58 (br s, 2H, NH_2), 6.72 (m, 2H, 3′- and 5′-H), 6.80 (m, 1H, 10-H), 7.70 (m, 2H, 2′- and 6′-H), 7.89−7.97 (m, 4H, terephthaloyl protons), 8.14 (m, 1H, benzoyl CONH), 8.60 (m, 1H, terephthaloyl CONH), 8.67 (s, 1H, 7-H). Anal. ($C_{27}H_{27}N_9O_6$·2H_2O) C, H, N.

4,5-Dichlorophthalimide (31). A stirred solution of 4,5-dichlorophthalic acid (2.0 g, 8.5 mmol) in absolute MeOH (100 mL) was cooled to −10 °C, and $SOCl_2$ (19 mL) was added dropwise while the temperature was kept below 0 °C. After 18 h of stirring, the solvent was evaporated under reduced pressure and MeOH was added several times and re-evaporated to obtain the dimethyl ester, which was used directly in the next step without purification (2.2 g, 100%): ^1H NMR (CD_3OD) δ 4.0 (s, 6H, OMe), 7.9 (s, 2H, aromatic).

Urea (510 mg, 8.5 mmol) was added to a solution of NaOMe prepared by allowing metallic Na (200 mg, 8.5 mmol) to dissolve in absolute MeOH (50 mL). To this solution was added a solution of dimethyl 4,5-dichlorophthalate in a minimum volume of MeOH. The reaction mixture was refluxed for 5 h, and the solvent was evaporated under reduced pressure. The solid residue was triturated with H_2O, and the insoluble portion was filtered off and resuspended in H_2O to which NH_4OH was added dropwise to bring the pH to 10. The material which remained undissolved was again filtered off, and the filtrate was acidified to pH 2 with 1 N HCl. The white solid which precipitated was collected, washed with H_2O, and dried to obtain **31** (0.8 g, 44%): IR (KBr) ν 3320, 3280, 3220, 3080, 3020, 1735, 1725, 1710, 1600 cm^{-1}; ^1H NMR (CD_3OD) δ

8.1 (s, aromatic protons). Anal. ($C_8H_3Cl_2NO_2$) C, H, N; Cl: calcd, 32.82; found, 32.34.

4,5-Dichloro-*N*-carbethoxyphthalimide (32). A stirred solution of **31** (665 mg, 3.08 mmol) and Et_3N (477 μL, 3.42 mmol) in dry DMF (12 mL) was cooled to 5−10 °C and treated with EtOCOCl (327 μL, 3.42 mmol). The reaction mixture was stired for 1.5 h and poured with stirring into H_2O (200 mL). The precipitate was collected, washed with H_2O (100 mL), and dried to obtain **32** as a white solid (0.8 g, 90%): mp 138 °C (EtOH); IR (KBr) ν 3400, 3330, 3080, 3020, 2980, 2925, 1805, 1760, 1720, 1600, 1470, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.4 (t, 3H, CH_2CH_3), 4.5 (q, 2H, CH_2CH_3), 8.3 (s, 2H, aromatic protons). Anal. ($C_{11}H_7Cl_2NO_4$) C, H, N; Cl: calcd, 24.61; found, 24.19.

Bis[L-2-amino-5-(4,5-dichlorophthalimido)pentanato]-Cu(II) (33). A solution of L-ornithine hydrochloride (454 mg, 269 mmol) in H_2O (10 mL) containing NaOH (215 mg, 5.38 mmol) was treated with $Cu(OAc)_2 \cdot H_2O$ (269 mg, 1.34 mmol) in H_2O (15 mL). To the mixture were then added $NaHCO_3$ (226 mg, 2.69 mmol) and **32** (775 mg, 2.69 mmol). The reaction mixture was stirred for 2 h and filtered, and the solid was washed successively with H_2O, EtOH, $CHCl_3$, and Et_2O and then dried to obtain **33** as a blue powder which was used directly for the next reaction (690 mg, 71%): mp 205 °C with darkening; IR (KBr) ν 3450, 3250, 2920, 1770, 1710, 1620, 1430, 1400 cm^{-1}.

L-2-Amino-5-(4,5-dichlorophthalimido)pentanoic Acid, Hydrochloride Salt (34·HCl). The finely pulverized Cu(II) complex **31** (690 mg, 0.096 mmol) was treated with 6 N HCl for 1 h. The solid was filtered, washed with 6 N HCl, air-dried overnight, and redissolved in hot MeOH (7 mL). The solution was added to EtOAc (75 mL), and the mixture left in the refrigerator for 2 h. The white precipitate was collected, washed with EtOAc, and dried: yield 435 mg (62%); mp 230 °C dec; IR (KBr) ν 3550, 3350, 2900, 1770, 1730, 1700, 1600, 1500, 1400 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.65−1.80 (m, 4H, β- and γ-CH_2), 3.58 (m, 2H, δ-CH_2), 4.0 (m, 1H, α- CH), 8.19 (s, aromatic proton), 8.39 (m, 3H, NH_2 and aromatic proton). Anal. ($C_{13}H_{12}Cl_2N_2O_4 \cdot HCl \cdot 0.5MeOH \cdot 0.5H_2O$) C, H, Cl, N.

Methyl L-2-Amino-5-(4,5-dichlorophthalimido)-pentanoate, Hydrochloride Salt (35·HCl). A stirred solution of **34**·HCl (0.42 g, 1.14 mmol) in MeOH (50 mL) at −10 °C was treated dropwise with $SOCl_2$ (3.2 mL) while the temperature was kept below 0 °C. After addition was complete the mixture was left at room temperature for 18 h. The solvent was evaporated under reduced pressure, the residue was redissolved in a minimum of MeOH, the solution was added to EtOAc (100 mL), and the mixture was kept at 0 °C until a solid precipitated. The white solid was collected, washed with EtOAc, and dried: yield 387 mg (89%); mp 198−199 °C dec; IR (KBr) ν 3450, 3000, 1770, 1750, 1710, 1580, 1500, 1450, 1440, 1400 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.64−1.77 (m, 4H, β- and γ-CH_2), 3.59 (m, 2H, δ-CH_2), 3.70 (s, 3H, CO_2CH_3), 4.05 (m, 1H, α-CH), 8.20 (s, 1H, aromatic proton), 8.50 (m, 3H, NH_2 and aromatic proton). Anal. ($C_{14}H_{14}Cl_2N_2O_4 \cdot HCl \cdot 0.1MeOH$) C, H, Cl, N.

N^α-(4-Amino-4-deoxypteroyl)-N^δ-(4,5-dichlorohemiphthaloyl)-L-ornithine (13). To a stirred suspension of **21** (172 mg, 0.47 mmol) in dry DMF (20 mL) were added sequentially Et_3N (130 μL, 0.94 mmol) and *i*-BuOCOCl (61 μL, 0.47 mmol). After 30 min, **35**·HCl (180 mg, 0.47 mmol) was added, and the mixture was stirred for 1 h. The solvent was evaporated under reduced pressure, and the residue was chromatographed on a silica gel column (20 g, 17 × 2 cm) with 98:2, 96:4, and 95:5 $CHCl_3$−MeOH as the eluents. Fractions giving a single TLC spot with R_f 0.49 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, the residue was redissolved in a small volume of 95:5 $CHCl_3$−MeOH, and the solution was added dropwise to excess Et_2O. The precipitate was collected by centrifugation and dried to obtain **36** as a yellow powder which was of sufficient purity for use in the next step (65 mg, 21%): mp 185 °C dec; IR (KBr) ν 3400, 2900, 1770, 1710, 1640, 1600, 1500, 1470, 1440, 1400 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.65−1.80 (m, 4H, β- and γ-CH_2), 3.56 (m, 5H, δ-CH_2 and α-CO_2CH_3), 4.40 (m, 1H, α-CH), 5.22 (s, 2H, 9-CH_2), 7.0 (br s, 2H, NH_2), 7.55 (m, 2H, 3′- and 5′-H), 7.82 (m, 2H, 2′- and 6′-H), 8.14 (s, 1H, benzoyl CONH), 8.67 (d, J = 2 Hz, 2H, aromatic protons on phthalimide moiety), 8.70 (s, 1H, C_7-H), 8.80 (CH=O).

A stirred solution of **36** (30 mg, 0.045 mmol) in DMSO 0.25 mL) was treated with 2 N NaOH (0.15 mL), and after 5 min the mixture was diluted with H_2O (3.5 mL). The pH was adjusted to 4.2 with 10% AcOH, and the precipitate was collected, washed with H_2O (50 mL), and dried to obtain a yellow powder (19 mg, 67%); mp 215 °C, dec >170 °C; TLC R_f 0.71 (silica gel, 5:4:1 $CHCl_3$−MeOH−28% NH_4OH); HPLC 14 min (C_{18} silica gel, 15% MeCN in 0.1 M NH_4OAc, pH 7.5, 1 mL/min); IR (KBr) ν 3400, 2900, 1640, 1600, 1510, 1450 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.54−1.61 (m, 2H, β-CH_2), 1.73−1.86 (m, 2H, γ-CH_2), 3.19 (m, 2H, δ-CH_2), 4.32 (m, 1H, α-CH), 4.48 (s, 2H, 9-CH_2), 6.71 (m, 2H, 3′- and 5′-H), 6.81 (m, 1H, N^{10}-H), 7.66 (s, 1H, aromatic proton ortho to CONH), 7.71 (m, 2H, 2′- and 6′-H), 7.89 (s, 1H, aromatic proton ortho to COOH), 8.13 (s, 1H, α-CONH), 8.50 (m, 1H, phthaloyl CONH), 8.68 (s, 1H, C_7-H). Anal. ($C_{27}H_{25}Cl_2N_9O_6 \cdot 0.5AcOH \cdot H_2O$) C, N, Cl (H: calcd 4.23, found 3.72).

Methyl N^α-(Benzyloxycarbonyl)-N^δ-[(2-carboxyphenyl)acetyl]-L-ornithine (37). To a solution of **28** (1 g, 3.15 mmol) in absolute EtOH (100 mL) were added *i*-Pr_2NEt (1.1 mL, 6.3 mmol) and homophthalic anhydride (0.510 g, 3.15 mmol), and the mixture was stirred under reflux for 6 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed with 0.1 N HCl (30 mL) and distilled H_2O (2 × 30 mL), dried over $MgSO_4$, and evaporated. The residue was chromatographed on a silica gel column (60 g, 27 × 3 cm), which was packed and eluted with 98:2 CH_2Cl_2−MeOH. Fractions showing a single TLC spot (R_f 0.30, silica gel, 95:5 CH_2Cl_2−MeOH) were pooled and evaporated to obtain a white solid (0.75 g, 54%): IR (KBr) ν 3300, 3060, 2950, 1740, 1690, 1650, 1600, 1540, 1450, 1430, 1350, 1280, 1130, 1075, 1010, 720, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.8 (m, 4H, β- and γ-CH_2), 3.22 (m, 2H, δ-CH_2), 3.61 (s, 3H, α-CO_2CH_3), 3.85 (s, 2H, homophthaloyl CH_2CONH), 4.05 (m, 1H, α-CH), 5.10 (s, 2H, PhCH_2O), 7.2−7.4 (m, 7H, $C_6H_5CH_2O$ and 2 aromatic protons meta to COOH), 7.5 (m, 1H, aromatic proton para to COOH), 8.0 (m, 1H, aromatic proton ortho to COOH). Anal. ($C_{23}H_{26}N_2O_7$) C, H, N.

Methyl N^α-(Benzyloxycarbonyl) N^δ-[[2-(methoxycarbonyl)phenyl]acetyl]-L-ornithine (38). Thionyl chloride (5.2 mL) was added dropwise to a stirred suspension of **37** (0.75 g, 1.67 mmol) in MeOH (100 mL) while the temperature was kept below 0 °C. The reaction mixture was left to stir at room temperature for 18 h and was then evaporated to dryness under reduced pressure. Crystallization from MeOH and EtOAc and drying afforded a white solid (0.73 g, 95%): mp 85−86 °C; IR (KBr) ν 3300, 3060, 2950, 1740 (aliphatic ester), 1710 (aromatic ester), 1690, 1650, 1600, 1540, 1450, 1430, 1350, 1280, 1130, 1075, 1010, 720, 700 cm^{-1}; ^1H NMR (CD_3OD) δ 1.8 (m, 4H, β- and γ-CH_2), 3.09 (m, 2H, δ-CH_2), 3.61 (s, 3H, $CH_2CO_2CH_3$), 3.76 (s, 3H, aromatic CO_2CH_3), 3.81 (s, 2H, CH_2CONH), 4.08 (m, 1H, α-CH), 5.00 (s, 2H, PhCH_2OCO), 7.20−7.34 (m, 7H, $C_6H_5CH_2O$ and 2 aromatic protons meta to CO_2Me), 7.43 (m, 1H, aromatic proton para to CO_2Me), 7.48 (m, 1H, aromatic proton ortho to CO_2Me). Anal. ($C_{24}H_{28}N_2O_7$) C, H, N.

Methyl N^δ-[[2-(methoxycarbonyl)phenyl]acetyl]-L-ornithine Hydrochloride (39·HCl). A solution of **38** (0.4 g, 1.47 mmol) in a mixture of EtOH (30 mL), glacial AcOH (6.5 mL), and 6 N HCl (0.33 mL) was shaken under 30 psi of H_2 in a Parr apparatus in the presence of 10% Pd−C (0.13 g) for 14 h. The catalyst was removed by filtration through Celite, and the filter pad was washed with MeOH. The combined filtrate and wash were evaporated under reduced pressure. Rerystallization from MeOH and *i*-PrOH gave an off-white solid (0.31 g, 44%): mp 132 °C; IR (KBr) ν 3340, 2960, 1745, 1730, 1650, 1580, 1540, 1500, 1450, 1435, 1400, 1380, 1350, 1300, 1280, 1250, 1220, 1140, 1085, 950, 800, 750, 735, 690, 630 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6−1.8 (m, 4H, β- and γ-CH_2), 3.02 (m, 1H, δ-CH_2), 3.72 (s, 3H, α-CO_2CH_3), 3.75 (s, 3H, aromatic CO_2CH_3), 3.78 (s, 2H, CH_2CONH), 4.01 (m, 1H, α-CH), 7.29 (m, 1H, aromatic proton ortho to CO_2Me and meta to CH_2CONH), 7.35 (m, 1H, aromatic proton meta to CO_2Me

and para to CH_2CONH), 7.48 (m, 1H, aromatic proton para to CO_2Me), 7.79 (m, 1H, aromatic proton ortho to CO_2Me), 8.0 (m, 1H, CONH), 8.48 (broad s, NH_2). Anal. ($C_{16}H_{22}ClN_2O_5$·HCl·0.1MeOH) C, H, Cl, N.

Methyl N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-[[2-(methoxycarbonyl)phenyl]acetyl]-L-ornithine (40). To a stirred suspension of **21** (87 mg, 0.23 mmol) in dry DMF (10 mL) at room temperature were added sequentially Et_3N (65 μL, 0.46 mmol) and *i*-BuOCOCl (30 μL, 0.23 mmol). After 30 min, **39** (85 mg, 0.23 mmol) was added, and the mixture was left to stir for 1 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in 9:1 $CHCl_3$−MeOH and chromatographed on a silica gel column (10 g, 17 × 2 cm) with 98:2, 95:5, and 92:8 $CHCl_3$−MeOH as the eluents. Fractions showing a single TLC spot (R_f 0.26, silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated. The residue was taken up in a small volume of 9:2 $CHCl_3$−MeOH, the solution was added dropwise to excess Et_2O, and the precipitate was collected and dried to obtain a yellow powder (60 mg, 40%): mp >130 °C dec; IR (KBr) ν 3320, 2975, 1735, 1715, 1640, 1600, 1550, 1500, 1450, 1350, 1270, 1220, 1130, 1020, 970 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6−1.8 (m, 4H, β- and γ-CH_2), 3.02 (m, 2H, δ-CH_2), 3.60 (s, 3H, α-CO_2CH_3), 3.70 (s, 3H, aromatic CO_2CH_3), 3.76 (s, 2H, CH_2CONH), 4.38 (m, 1H, α-CH), 5.24 (s, 2H, 9-CH_2), 7.28 (m, 1H, aromatic proton ortho to CH_2CONH and meta to CO_2Me), 7.33 (m, 1H, aromatic proton para to CH_2CONH and meta to CO_2Me), 7.46 (m, 1H, aromatic proton para to CO_2Me), 7.75 (m, 1H, aromatic proton ortho to CO_2Me), 7.58 (m, 2H, 3′- and 5′-H), 7.86 (m, 2H, 2′- and 6′-H), 8.70 (m, 2H, C_7−H and CONH), 8.78 (s, 1H, CH═O). Anal. ($C_{31}H_{33}N_9O_7$·H_2O) C, H, N.

Methyl 2-Carboxyphenylacetate (41). Sodium metal (0.142 g, 0.16 mmol) cut into small pieces was allowed to dissolve in absolute MeOH (50 mL), and homophthalic anhydride (1 g, 0.16 mmol) was added. The reaction mixture was stirred overnight at room temperature and then neutralized with 6 N HCl (1.02 mL, 6.16 mmol). The solvent was evaporated under reduced pressure, and the residue was taken up in EtOAc. An insoluble material was removed by filtration, and the filtrate was concentrated to dryness. The product was chromatographed twice on silica gel with 98:2 CH_2Cl_2−MeOH as the eluent. Fractions showing a single TLC spot (R_f 0.36, silica gel, 9:1 CH_2Cl_2−MeOH) were combined and evaporated to obtain a white solid (0.24 g, 80%): mp 87-88 °C; IR (KBr) ν 3420, 3000, 2940, 1725, 1680, 1600, 1580, 1490, 1445, 1400 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.55 (s, 3H, CO_2CH_3), 3.98 (s, 2H, CH_2CO_2Me), 7.32 (m, 1H, 6-H), 7.39 (m, 1H, 4-H), 7.39 (m, 1H, 5-H), 7.90 (m, 1H, 3-H). Anal. ($C_{10}H_{10}O_4$) C, H.

Methyl N^{α}-(Benzyloxycarbonyl)-N^{δ}-[2-[(methoxycarbonyl)methyl]benzoyl]-L-ornithine (42). *i*-BuOCOCl (516 μL, 3.98 mmol) and Et_3N (1.11 mL, 7.96 mmol) were added to a solution of **41** (0.77 g, 3.98 mmol) in dry DMF (50 mL). After 30 min, **28**·HCl (1.2 g, 3.98 mmol) was added, and the reaction mixture was stirred for 1 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed with H_2O (2 × 50 mL), dried ($MgSO_4$), and evaporated. The product was purified by flash chromatography on silica gel, using 70:30 CH_2Cl_2−EtOAc as the eluent. Fractions containing a major TLC spot (R_f 0.32, silica gel, 75:25 CH_2Cl_2−EtOAc) along with a slower-moving impurity were rechromatographed on another flash column with 75:25 CH_2Cl_2−EtOAc as the eluent, and TLC-homogeneous fractions were pooled and evaporated to obtain a white solid (0.86 g, 48%): mp 100−101 °C; IR (KBr) ν 3380, 2950, 1735, 1690, 1530, 1465, 1430, 1290, 1255, 1220, 1130, 1060, 1010, 700 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.8 (m, 4H, β- and γ-CH_2), 3.14 (m, 2H, δ-CH_2), 3.54 (s, 3H, homophthaloyl CO_2CH_3), 3.61 (s, 3H, α-CO_2CH_3), 3.82 (s, 2H, CH_2CO_2Me), 4.03 (m, 1H, α-CH), 5.01 (s, 2H, PhCH_2OCO), 7.20−7.42 (m, 9H, aromatic protons). Anal. ($C_{24}H_{22}N_2O_7$), C, H, N.

Methyl N^{δ}-[2-[(Methoxycarbonyl)methyl]benzoyl]-L-ornithine Hydrochloride (43·HCl). Compound **42** (0.82 g, 1.8 mmol) was hydrogenated in a Parr apparatus as described for **38**·HCl except that, instead of being recrystallized, the product was precipitated from a mixture of i-PrOH and Et_2O to obtain a white solid (0.52 g, 81%): mp 132 °C; IR (KBr) ν 3105, 2960, 1740, 1640, 1600, 1540, 1300, 1260, 1220, 1170, 1100, 1010, 840, 720, 690 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.6−1.8 (m, 4H, β- and γ-CH_2), 3.17 (m, 2H, δ-CH_2), 3.54 (s, 3H, $CH_2CO_2CH_3$), 3.73 (s, 3H, α-CO_2CH_3), 3.83 (s, 2H, CH_2CO_2-Me), 4.05 (m, 1H, α-CH), 7.28 (m, 2H, aromatic proton meta to CH_2NHCO and ortho to CH_2CO_2Me), 7.32 (m, 1H, aromatic proton meta to CH_2NHCO and para to CH_2CO_2Me), 7.39 (m, 1H, aromatic proton para to CH_2CONH), 7.44 (m 1H, aromatic proton ortho to CH_2CONH). Anal. ($C_{22}H_{22}N_2O_5$·HCl) C, H, Cl, N.

Methyl N^{α}-(4-Amino-4-deoxy-N^{10}-formylpteroyl)-N^{δ}-[2-[(methoxycarbonyl)methyl]benzoyl]-L-ornithine (44). This compound was made from **43** (170 mg, 0.66 mmol) and **21** (173 mg, 0.46 mmol) as in the synthesis of **40** to obtain a yellow powder (131 mg, 44%): mp 145 °C, darkening above 125 °C; TLC R_f 0.26 (silica gel, 9:1 $CHCl_3$−MeOH; IR (KBr) ν 3320, 2975, 1740, 1640, 1600, 1550, 1350, 1300, 1220, 1020, 970, 850, 770 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 1.6−1.8 (m, 4H, β- and γ-CH_2), 3.28 (m, 2H, δ-CH_2), 3.48 (s, 3H, $CH_2CO_2CH_3$), 3.61 (s, 3H, α-CO_2CH_3), 3.82 (s, 2H, CH_2CO_2Me), 4.41 (m, 1H, α-CH), 5.24 (s, 2H, 9-CH_2), 7.27 (m, 1H, aromatic proton meta to CH_2CONH and ortho to CH_2CO_2Me), 7.32 (m, 1H, aromatic proton meta to CH_2CONH and para to CH_2CO_2Me), 7.38 (m, 1H, aromatic proton para to CH_2CONH), 7.41 (m, 1H, aromatic proton ortho to CH_2CONH), 7.54 (m, 2H, 3′- and 5′-H), 7.87 (m, 2H, 2′- and 6′-H), 8.32 (m, 1H, CONH), 8.72 (m, 1H, CONH), 8.79 (s, 1H, CH═O), 8.70 (s, 1H, C_7-H). Anal. ($C_{31}H_{33}N_9O_7$·H_2O) C, H, N.

L-2-[N^{α}-(4-Amino-4-deoxypteroyl)amino]-5-(homophthalimido)pentanoic Acid (45). Procedure A. A solution of **40** (40 mg, 0.06 mmol) in DMSO (0.5 mL) was treated for 5 min with 2.5 N NaOH (0.25 mL), diluted with H_2O (5 mL), and acidified to pH 4.5 with 10% AcOH. The precipitate was filtered, washed with H_2O (50 mL), and dried to a yellow powder (30 mg, 86%): mp 185 °C dec; IR (KBr) ν 3350, 2950, 1715, 1660, 1610, 1510, 1540, 1400, 1260, 1335, 1260, 1200, 1150, 850, 770, 750, 700, 650 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 1.6−1.8 (m, 4H, β- and γ-CH_2), 3.86 (m, 2H, δ-CH_2), 4.11 (m, 1H enolic OH of **45A**, disappearing on addition of D_2O), 4.30 (m, 1H, α-CH), 4.47 (s, 2H, 9-CH_2), 6.60 (br s, 2H, NH_2), 6.70 (m, 2H, 3′- and 5′-H), 6.80 (m, 1H, N^{10}-H), 7.36 (m, 1H, 5″-H), 7.44 (m, 1H, 7″-H), 7.61 (m, 1H, 4″-H of **45A**), 7.65 (m, 2H, 2′- and 6′-H), 8.01 (m, 1H, 6″-H), 8.11 (m, 1H, 8″-H), 8.68 (s, 1H, C_7-H). Anal. ($C_{28}H_{29}N_9O_6$·$0.6H_2O$) C, H, N.

Procedure B. A sample of **44** (40 mg, 0.06 mmol) was treated with NaOH in DMSO exactly as in the preceding experiment to obtain a yellow powder (29 mg, 82%); mp 185 °C dec. The IR and ^1H NMR spectra of this material were identical with those of the product obtained from **40**. A microsample of the product via either route, dissolved in dilute NaOH and analyzed immediately by HPLC (C_{18} silica gel, 8% MeCN in 0.1 M NH_4OAc, 1 mL/min), showed a small peak at 11 min, coeluting with authentic N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine, and two larger peaks of ca. equal area at 19 and 21 min, which are presumed to be **14** and **15**.

L-2-[N^{α}-(Benzyloxycarbonyl)amino]-5-(1,8-naphthalimido)pentanoic Acid (46). *N*,*N*-Diisopropylethylamine (544 mL, 3.12 mmol) and 1,8-naphthalic anhydride (0.5 g, 208 mmol) were added to a suspension of N^{α}-(benzyloxycarbonyl)-L-ornithine (0.83 g, 3.12 mmol) in dry DMF, and the reaction mixture was heated for 5 h at an internal temperature of 100 °C (all the solid dissolved after 30 min). The solvent was then evaporated at reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed with 1 N HCl (20 mL), rinsed with H_2O (50 mL), dried ($MgSO_4$), and evaporated. The residue was applied onto a silica gel column (50 g, 27 × 3 cm), packed with $CHCl_3$, and eluted sequentially with $CHCl_3$ and 97:3 $CHCl_3$−MeOH. Fractions containing a major spot at R_f 0.43 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated to a solid (0.54 g, 39%). The analytical sample was obtained by recrystallization from CH_2Cl_2 and cyclohexane: mp 184−185 °C; IR (KBr) ν 3400 br, 1950, 1695, 1655, 1625, 1590, 1535, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.62−1.77 (m, 4H, β- and γ-CH_2), 3.94 (m, 1H, α-CH), 4.08 (m, 2H, δ-CH_2), 4.98 (s, 2H, $C_6H_5CH_2O$), 7.31 (s, 5H, $C_6H_5CH_2$), 7.56 (m, 1H,

CONH), 7.86 (m, 2H, naphthyl 3- and 6-H), 8.46 (m, 4H, naphthyl 2-, 4-, 5-, and 7-H). Anal. ($C_{25}H_{22}N_2O_6$) C, H, N.

Methyl L-2-[N^{α}-(Benzyloxycarbonyl)amino]-5-(1,8-naphthalimido)pentanoate (47). A stirred suspension of **46** (200 mg, 0.45 mmol) in MeOH (12 mL) was cooled to −10 °C and treated dropwise with $SOCl_2$ (0.6 mL) while the temperature was kept below 0 °C. When addition was complete, the reaction mixture was left to stir at room temperature for 18 h and evaporated to dryness under reduced pressure. Recrystallization from CH_2Cl_2 and cyclohexane gave a solid (150 mg, 75%): mp 132−133 °C; IR (KBr) ν 3400, 2900, 1745, 1700, 1660, 1625, 1590, 1535, 1440 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.81−1.98 (m, 4H, β- and γ-CH_2), 3.70 (s, 3H, OMe), 4.21 (m, 2H, δ-CH_2), 4.44 (m, 2H, α-CH), 5.08 (s, 2H $C_6H_5CH_2O$), 7.25 (m, 5H, $C_6H_5CH_2$), 7.75 (m, 2H, naphthyl 3- and 6-H), 8.22 (m, 2H, naphthyl 4- and 5-H), 8.60 (m, 2H, naphthyl 2- and 7-H). Anal. ($C_{26}H_{24}N_2O_6 \cdot 0.1CH_2Cl_2$) C, H, N.

Methyl L-2-[*N*-(4-Amino-4-deoxy-*N*^{10}-formylpteroyl)-amino]-5-(1,8-naphthalimido)pentanoate (49). A solution of **47** (142 mg, 0.3 mmol) in MeOH (30 mL) and 6 N HCl (0.1 mL) was shaken with 10% Pd−C (20 mg) under H_2 (30 psi) in a Parr apparatus for 18 h. The reaction mixture was filtered through Celite, and the filter pad was washed with MeOH. The solvent was evaporated under reduced pressure, the residue was redissolved in MeOH (5 mL), and the solution was added to a large volume of Et_2O with continuous stirring. The precipitate was collected, washed with Et_2O, and dried to obtain **48**·HCl (80 mg, 71%): mp 140 °C, decomposing slowly above 85 °C with gas evolution; IR (KBr) ν 3450, 2950, 1745, 1695, 1655, 1625, 1590, 1510, 1460, 1440 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.70−1.81 (m, 4H, β- and γ-CH_2), 3.70 (s, 3H, OMe), 4.06 (m, 3H, α-CH and δ-CH_2), 7.87 (m, 2H, naphthalimide 3- and 6-H), 8.37 (br s, 2H, NH_2), 8.44 (m, 4H, naphthalimide 2-, 4-, 5-, and 7-H). This material was used directly in the next reaction.

i-BuOCOCl (24 μL, 0.19 mmol) and Et_3N (26 μL, 0.19 mmol) were added to a stirred suspension of **21** (70 mg, 0.19 mmol) in dry DMF (7 mL). After 30 min, the solid obtained in the preceding step (70 mg, 0.19 mmol) was added and stirring was continued for 1 h. The solvent was evaporated to dryness under reduced pressure, the residue was disolved in a minimum volume of 9:1 $CHCl_3$−MeOH, and the solution was loaded onto a column of silica gel (10 g, 27 × 2 cm), which was eluted first with 95:5 and then 9:1 $CHCl_3$−MeOH. Fractions giving a single TLC spot with R_f 0.43 (silica gel, 9:1 $CHCl_3$−MeOH) were pooled and evaporated, the residue was redissolved in a minimum volume of 9:1 $CHCl_3$−MeOH, and the solution was added dropwise with stirring to a large volume of Et_2O. The yellow precipitate was filtered and dried: yield 25 mg (20%); mp 168−170 °C; IR (KBr) ν 3400, 2950, 1735, 1690, 1650, 1620, 1590, 1555, 1500, 1450 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 1.69−1.80 (m, 4H, β- and γ-CH_2), 3.58 (s, 3H, α-CO_2Me), 4.02−4.11 (m, 2H, δ-CH_2), 4.41 (m, 1H, α-CH), 5.17 (s, 2H, 9-CH_2), 6.6 (br s, 2H, NH_2), 7.52 −7.55 (m, 2H, 3′- and 5′-H), 7.80−7.82 (m, 2H, 2′- and 6′-H), 7.82−7.84 (m, 2H, 3″-H and 6″-H), 8.42−8.46 (m, 4H, 2″-, 4″-, 5″-, and 7″-H), 8.62, s, 1H, C_7-H), 8.66−8.67 (m, 1H, CONH), 8.76 (s, 1H, NCH=O). Anal. ($C_{33}H_{29}N_9O_6 \cdot 1.2H_2O$) C, H, N.

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