Inhibition of Folylpolyglutamate Synthetase by Substrate Analogues with an Ornithine Side Chain [1]

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N^{α}-[4-[[(4-Aminopteridin-6-yl)methyl]amino]benzoyl]-L-ornithine (dAPA-Orn) was synthesized, and its ability to inhibit folylpolyglutamate synthetase from mouse liver was compared with that of the corresponding 2,4-diamino analogue APA-Orn. Also compared were the inhibitory activities of the deaza analogues 5-deazaAPA-Orn, 8-deazaAPA-Orn, and 5,8-dideazaAPA-Orn, as well as those of N^{α}-pteroyl-L-ornithine (PteOrn) and its deaza analogues 5-deazaPteOrn and 5,8-dideazaPteOrn. The inhibition constant K_i of dAPA-Orn was 7-fold greater than that of APA-Orn, indicating that the 2-amino group plays a role in binding to the active site. The binding affinity of the 2,4-diamino compounds increased in the order 5-deazaAPA < APA-Orn < 5,8-dideazaAPA-Orn < 8-deazaAPA-Orn, and that of the 2-amino-4(3*H*)-oxo compounds increased in the order 5-deazaPteOrn < PteOrn < 5,8-dideazaPteOrn. The most potent inhibitor of both groups was 8-deazaAPA-Orn, with a K_i of 0.018 μM, coresponding to an 8-fold and 15-fold increase in affinity relative to APA-Orn and 5-deazaAPA-Orn, respectively. The results suggest (a) that the binding of Orn-containing folylpolyglutamate synthetase inhibitors is affected to a greater degree by replacement of N^8 by a carbon atom than it is by the corresponding change at N^5, (b) that the effect of carbon for nitrogen replacement is greater in the 2,4-diamino derivatives than in the 2-amino-4(3*H*)-oxo compounds, and (c) that the 2,4-diamines are the better inhibitors. Comparison of the K_i values of the Orn-containing inhibitors with the K_m values of the corresponding glutamate-containing substrates revealed that K_m/K_i ratio can vary as much as 100-fold depending on the nature of the heterocyclic moiety, suggesting that caution should be exercised in using K_m values of known substrates to predict K_i values of putative inhibitors.

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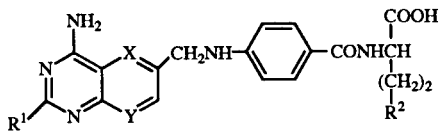
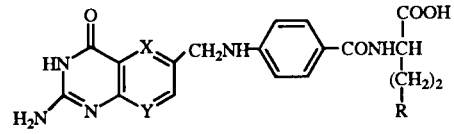
Folylpoly-γ-glutamyl synthetase (FPGS; tetrahydrofolate:L-glutamate γ-lyase, ADP-forming; EC 6.3.2.17) is an important modulator of one-carbon metabolism and cell division and a significant contributor to the potency and therapeutic selectivity of classical antifolates with a gluamate side chain [2-6]. As part of a larger study of the interaction of folate analogues with this enzyme we have reported the synthesis and FPGS substrate activity of 2-desaminoaminopterin (**1**, dAMT), an analogue of aminopterin (AMT, **2**) in which the 2-amino group is replaced by hydrogen [7,8]. The fact that dAMT was almost as good a substrate for murine FPGS as AMT suggested that the 2-amino group is not required for binding to the active site of the enzyme. In view of previous reports that N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine (APA-Orn, **3**), the AMT analogue with an ornithine (Orn) side chain in place of glutamate (Glu), is a potent inhibitor of mammalian FPGS [9-11], as are several other analogues of AMT and folic acid (**4**) with an Orn side chain [10-18], we wished to determine whether the same structural modification would similarly transform dAMT from a substrate into an inhibitor. Accordingly, we synthesized N^{α}-[4-[(4-aminopteridin-6-yl)methyl]amino]benzoyl-L-ornithine (dAPA-Orn, **5**), and determined its inhibitory activity against FPGS from mouse liver. Also compared were the binding affinities of 5-deazaAPA-Orn (**6**) [14], 8-deazaAPA-Orn (**7**) [14], 5,8-dideazaAPA-Orn (**8**) [14], N^{α}-pteroyl-L-ornithine (PteOrn, **9**) [18,19], 5-deazaPteOrn (**10**) [14,15], and 5,8-dideazaPteOrn (**11**) [14]. It was hoped that structure-activity correlations discerned among these AMT and folate analogues would shed light on the larger issue of whether structural features which are favorable for the binding of Glu-containing substrates necessarily have a commensurate effect on the binding of FPGS inhibitors, of which several have been identified in recent years in addition to those with an Orn side chain [15, 20-27].

Chemistry.

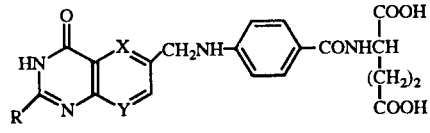
The chemical synthesis of **5** was patterned after our previously described route to **1** [7], using a suitably protected L-ornithine precursor as shown in Scheme 1. N^{δ}-(Benzyloxycarbonyl)-N^{α}-(4-nitrobenzoyl)-L-ornithine (**12**) was obtained in 88% yield by sequential treatment of N^{δ}-(benzyloxycarbonyl)-L-ornithine with trimethylchlorosilane (2 equivalents) and 4-nitrobenzoyl chloride. The silylation step was useful because it transiently esterified the carboxyl group while at the same time activating the amine nitrogen toward acylation and improving solubility in nonpolar solvents. Interestingly, the same reaction with N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithine or N^{δ}-phthaloyl-L-



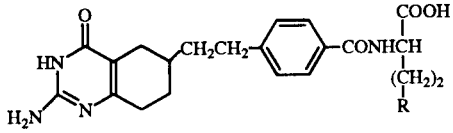


1, X = Y = N; R^1 = H; R^2 = COOH (dAMT)
2, X = Y = N; R^1 = NH_2; R^2 = COOH (AMT)
3, X = Y = N; R^1 = NH_2; R^2 = CH_2NH_2 (APA-Orn)
5, X = Y = N; R^1 = H; R^2 = CH_2NH_2 (dAPA-Orn)
6, X = CH, Y = N, R^1 = NH_2, R^2 = CH_2NH_2 (5-deazaAPA-Orn)
7, X = N, Y = CH, R^1 = NH_2, R^2 = CH_2NH_2 (8-deazaAPA-Orn)
8, X = Y = CH; R^1 = NH_2; R^2 = CH_2NH_2 (5,8-dideazaAPA-Orn)

4, X = Y = N; R = COOH (folic acid, PteGlu)
9, X = Y = N; R = CH_2NH_2 (PteOrn)
10, X = CH, Y = N, R = CH_2NH_2 (5-deazaPteOrn)
11, X = Y = CH; R = CH_2NH_2 (5,8-dideazaPteOrn)



20, X = Y = CH; R = NH_2 (5,8,-dideazaPteGlu)

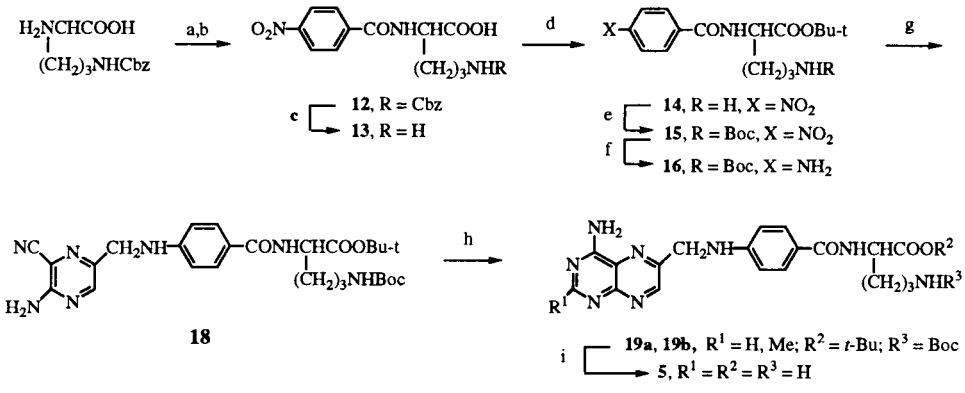


21, R = CH_2NH_2 (5,8,10-trideazaPteOrn)
22, R = COOH (5,8,10-trideazaPteGlu)

ornithine gave complex mixtures. Thus, the N^{δ}-blocking group needs to be chosen with care for N^{α}-(4-nitrobenzoylation) of Orn by this method to succeed. Removal of the benzyloxycarbonyl group in **12** with hydrogen bromide in acetic acid afforded N^{α}-(4-nitrobenzoyl)-L-ornithine (**13**, 98%), and esterification of **13** with *tert*-butyl acetate and 60% perchloric acid afforded the *tert*-butyl ester **14** (70%). Although the methyl ester of **12** was available from work in this laboratory on other Orn analogues [14], a *tert*-butyl ester was chosen here because it would make it possible to deprotect the carboxyl group at the end of the synthesis with acid rather than base, which has been observed to rapidly open the 4-aminopyrimidine ring in **1** [7,8]. Treatment of **14** with di-*tert*-butyl dicarbonate afforded the N^{δ}-(*tert*-butyloxycarbonyl) derivative **15** (94%) and reduction of the nitro group in **15** with hydrogen led to the amine **16** (94%). The acid-labile *tert*-butyloxycarbonyl blocking group was chosen with the idea that it could eventually be cleaved at the same time as the *tert*-butyl ester using mild acid. The overall yield for the four-step sequence from **12** to **16** was 53%.

Condensation of **16** with an equimolar amount of 2-amino-5-chloromethylpyrazine-3-carbonitrile (**17**) [27], added in small portions to maximize *N*-monoalkylation, led to the desired product **18**. However it was not possible to avoid formation of a second more polar product probably arising *via* *N,N*-dialkylation. Despite several attempts to avoid this problem, the yield of **18** after separation of the byproduct remained only 37%. It would appear that the second *N*-alkylation of the amino group by **17** is more rapid than the first, and can only be prevented by using excess amine [28,29]. The *N*-monoalkyl structure of **18** was firmly established from its microanalysis and from the proton nmr spectrum, in which the phenyl signals (doublets, δ 6.6 and δ 7.6) and the pyrazine signal (singlet, δ 8.15) had the expected 2:2:1 ratio. Heating **18** with a fivefold excess of formamidine acetate in 2-ethoxyethanol at 135° for 45 minutes resulted in the formation of one major product and two minor products which were not identified. The major product (40% yield) was purified on silica gel and confirmed to be the 4-aminopteridine **19a** from its ^{1}H nmr spectrum, which contained downfield singlets at δ 8.77 and δ 9.08 corresponding to the protons on C^2 and C^7 respectively. Similarly, heating **18** with acetamidine acetate afforded the 4-amino-2-methylpteridine **19b**. Deprotection of **19a** was carried out in a 1:2 mixture of trifluoroacetic acid and dichloromethane at room temperature for 3 hours, but was not as clean as the cleavage of the di-*tert*-butyl glutamate side chain in our earlier synthesis of dAMT [7,8]. After extensive purification involving ion exchange column chromatography on Dowex 50W-X2 and DEAE-cellulose followed by C_{18} reversed phase preparative hplc, the yield of dAPA-Orn, isolated as an analytically pure hydrated hemiacetate salt, was only 15%. Two significant side products and several minor contaminants were observed, which together accounted for about 25% of the total material prior to hplc and whose structure was not determined. The acidolysis of **19b** was even more problematic than that of **19a**, yielding too little of the 2-desamino-2-methyl analogue for further work.

Scheme 1



a, Me_3SiCl/Et_3N; **b**, 4-$NO_2C_6H_4COCl/Et_3N$; **c**, HBr/AcOH; **d**, t-BuOAc/$HClO_4$; **e**, $O(CO_2Bu\text{-}t)_2$; **f**, H_2/Pd-C; **g**, 2-amino-5-chloromethylpyrazine-3-carbonitrile (**17**); **h**, $R^1C(=NH)NH_2 \cdot AcOH$ (R^1 = H, Me); **i**, CF_3COOH/CH_2Cl_2 (1:2) (**18** only)

Folylpolyglutamate Synthetase (FPGS) Inhibition.

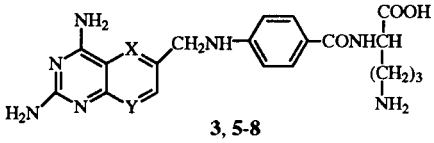
dAPA-Orn (**5**) was tested for its ability to inhibit partially purified FPGS from mouse liver. The K_i values obtained for **5** and seven other previously known Orn-containing analogues **3**, **6-11** are shown in Table 1.

The K_i of dAPA-Orn (**5**) was 1.1 μ*M* as compared with 0.15 μ*M* for APA-Orn (**3**); thus, replacement of the 2-amino group in APA-Orn by hydrogen led a 7-fold decrease in binding. Since the K_i of the corresponding Glu analogues dAMT (**20**) and AMT against this enzyme had been found earlier to be 26 μ*M* [7] and 21 μ*M* [31] respectively, these results indicate that replacing NH_2 by H at the 2-position in this particular pair of Orn inhibitors is detrimental to binding whereas the same change in the corresponding Glu subtrates has only a small effect.

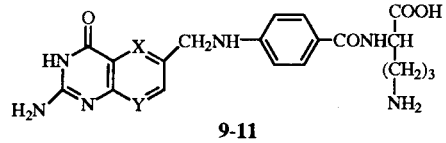
The K_i of 5,8-dideazaPteOrn (**11**) was 0.35 μ*M* as compared with 3.5 μ*M* for PteOrn (**9**); thus, deletion of the carbon atoms at the 5- and 8-positions enhanced binding by approximately one order of magnitude. The K_i of **9** was close to that reported for this compound as an inhibitor of porcine FPGS (5.9 μ*M*) [32], indicating that the interaction of **9** with the active site of the two enzymes is probably not very different. It is of interest that very low values (*ca.* 0.008 μM) were reported for the 5-chloro and 5-trifluoromethyl analogues of **11** [13,16]. Since the binding of **9** to porcine FPGS is greatly enhanced by electron-withdrawing groups at the 5-posi-

Table 1

Inhibition of Mouse Liver FPGS by Folate Analogues with an Ornithine Side Chain



3, 5-8



9-11

Compound	R	X	Y	K_i, μ*M* [a]
2,4-Diamino type				
APA-Orn (**3**) [b]	NH_2	N	N	0.15 (1)
dAPA-Orn (**5**)	H	N	N	1.1 (0.14)
5-DeazaAPA-Orn (**6**)	NH_2	CH	N	0.46 (0.33)
8-DeazaAPA-Orn (**7**)	NH_2	N	CH	0.018 (8.3)
5,8-DideazaAPA-Orn (**8**)	NH_2	CH	CH	0.072 (2.1)
2-Amino-4(3H)-oxo type				
PteOrn (**9**)	NH_2	N	N	3.5 (0.043)
5-DeazaPteOrn (**10**) [c]	NH_2	CH	N	5.7 (0.026)
5,8-DideazaPteOrn (**11**)	NH_2	CH	CH	0.35 (0.43)

[a] Numbers in parentheses are normalized relative to the previously reported K_i for APA-Orn (**3**) [10]. Assays were performed as described earlier. K_i values listed are means of two or more experiments with a standard deviation of less than ±15%. [b] Data from reference 10. [c] Data from reference 15.

Table 2

Comparison of K_i /K_m Ratios for Selected Pairs of FPGS Inhibitors with an Ornithine Side Chain and FPGS Substrates with a Glutamate Side Chain

Pteridine Ring Substitution	Compounds	K_i (Orn)	K_m (Glu)	K_m/K_i
2,4-Diamino	**3** and **2**	0.15 [a]	21 [b]	140
4-Amino	**5** and **1**	1.1	26 [c]	24
2-Amino-4(3*H*)-oxo	**9** and **4**	3.5	140 [b]	40
2-Amino-4(3*H*)-oxo-5,8-dideaza	**11** and **20**	0.35	6.4 [b]	18
2-Amino-4(3*H*)-oxo-5,6,7,8-tetrahydro-5,8,10-trideaza	**21** and **22**	10 [d]	15 [d]	1.5

[a] Data from reference 10. [b] Data from reference 31. [c] Data from reference 7. [d] Data from reference 35.

tion, we would predict a similar enhancement in the case of the murine enzyme.

Replacement of nitrogen by carbon at the 5- and 8-positions of compounds with an Orn side chain was found to have some surprising effects on FPGS inhibition. As shown in Table 1, replacement of N by CH at the 5-position led to only a 3-fold decrease in binding (**6** *versus* **3**), whereas replacement of N by CH at the 8-position (**7** *versus* **3**) and at the 5- and 8-positions (**8** versus **3**) led to increases of 8-fold and 2-fold, respectively. Replacement of N by CH at the 5-position of PteOrn (**10** *vs.* **9**) likewise had a neglible effect on binding, whereas replacement of N by CH at both the 5- and 8-position led to a 10-fold increase in binding (**11** *vs.* **9**). These data were consistent with the excellent substrate activity of the corresponding glutamate analogues [33,34], and suggested that a nonpolar carbon atom at the 8-position of a 2,4-diamino compound is favorable for binding, giving compounds with K_i values in the 10-100 nanomolar range. By contrast, binding appears to be relatively unaffected by substitution of CH for N at the 5-position. These results suggest that the 5-position of folyl ligands of the 2,4-diamino type may occupy a vacant space in the active site of FPGS whereas the 8-position lies close to a hydrophobic surface. Of further interest was that, in the two pairs of 4-amino and 4-oxo compounds available for comparison in this study (**10** *versus* **6** and **9** *versus* **3**), the 4-amino compounds were 10- to 100-fold more active. This was consistent with the better substrate activity of AMT relative to folic acid, and suggested that FPGS binding may be tighter when the substitution at the 4-position is a hydrogen bond donor (NH_2) rather than a hydrogen bond acceptor (C=O).

To further examine the question of whether structural changes favoring FPGS substrate activity in Glu analogues also favor inhibitor activity in Orn analogues, we compared binding data for pairs of Orn and Glu analogues modified in various parts the folyl structure other than at the 2-position (Table 2). The K_i values of 5,8-dideazaPteOrn (**11**) and PteOrn (**9**) against mouse liver FPGS were 0.35 μ*M* and 3.5 μ*M*, whereas the reported K_m values of 5,8-dideazaPteGlu (**20**) and folic acid (**4**) with this enzyme are 6.4 μ*M* and 140 μ*M* [31]; thus, the K_m/K_i ratios for these Orn/Glu pairs were 18:1 and 40:1, which compared reasonably well with the ratio of 24:1 for the pair dAPA-Orn (**5**)/dAMT (**1**). In contrast, the ratio for the pair APA-Orn (**3**)/AMT (**2**) was 140:1, whereas that for the pair 5,8,10-trideaza-5,6,7,8-tetrahydroPteOrn (**21**)/5,8,10-trideaza-5,6,7,8-tetrahydroPteGlu (**22**), based on our previously found kinetic constants of 10 μ*M* and 15 μ*M* for these compounds [35], was only 1.5:1. It thus appears that the proportionality between the K_i of an FPGS inhibitor with an Orn side chain and the K_m of the corresponding Glu analogue as a substrate can vary substantially. Because the quantitative effect of a discrete molecular change on the binding of a folyl ligand to FPGS seems to depend on the *entire structure* of the ligand, we suggest that substrate activity data need to be interpreted cautiously in predicting the behavior of proposed inhibitors.

EXPERIMENTAL

The ir spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer; only peaks above 1200 cm^{-1} are reported. The uv spectra were recorded on a Varian Model 210 instrument, and ^1H nmr spectra on a Varian EM360L spectrometer with tetramethylsilane as the reference. Analyses by tlc were done on fluorescent Eastman 13181 silica gel sheets, Analabs silica gel glass plates, or Eastman 13254 cellulose sheets. Spots were visualized under ultraviolet (254 nm) illumination or with the aid of an iodine chamber. Column chromatography was done on Baker 3405 (60-200 mesh) silica gel or Whatman DE-52 DEAE-cellulose (preswollen). Analysis by hplc was done on Waters C_{18} radial compression cartridges (analytical: 5 μm particle size, 5 x 100 mm; preparative: 15 μm particle size, 25 x 100 mm) on Waters Model 500 and Delta-Prep Model 3000 systems with accessories for variable wavelength uv detection and solvent gradient elution. N^{δ}-(Benzyloxycarbonyl)-L-ornithine was purchased from Bachem, Torrance, CA. Other chemicals were from Aldrich, Milwaukee, WI, and Fisher, Boston, MA. Solvents for moisture-sensitive reactions were dried over Linde 4A molecular sieves. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were performed by Quantitative Technologies,

Whitehouse, NJ. Measurements of the ability of the Orn derivatives listed in Table 1 to inhibit partially purified FPGS from mouse liver [36] were performed according to the method previously reported [37], using AMT as the variable substrate. The enzyme was isolated by ammonium sulfate fractional precipitation, and its activity was determined with 500 μ*M* folic acid as the substrate in *p*H 8.6 Tris buffer [38]. The typical specific activity of the partially purified enzyme was 1.2 nmol/h/mg protein, as in our previous studies [8,15].

N^{α}-(4-Nitrobenzoyl)-N^{δ}-(benzyloxycarbonyl)-L-ornithine (**12**).

A suspension of N^{δ}-(benzyloxycarbonyl)-L-ornithine (11.0 g, 41.3 mmoles) in dichloromethane (250 ml) was treated with triethylamine (11.5 ml, 8.34 g, 82.6 mmoles) and trimethylchlorosilane (10.5 ml, 8.97 g, 82.6 mmoles), and the mixture was stirred at room temperature for 10 minutes. A small amount of material remained undissolved. Additional portions of triethylamine and trimethylchlorosilane (10% excess) were therefore added and stirring was continued for another 10 minutes to obtain a clear solution. 4-Nitrobenzoyl chloride (7.66 g, 41.3 mmoles) was then added as a solid to the stirred solution, which became clear again after 15 minutes. Rotary evaporation, trituration with water, decantation, and recrystallization from methanol gave white crystals (15.1 g, 88% yield), mp 172-173° (lit [39] mp 150-152°; compound synthesized under Schotten-Baumann conditions); ir (potassium bromide): ν 3460 br, 3330, 3310, 3130-2600 br, 1735, 1690, 1655, 1615, 1550, 1545, 1520, 1495, 1480, 1460, 1425, 1345, 1330, 1315, 1295, 1275, 1230, 1205 cm^{-1}; ^1H nmr (2:1 deuteriochloroform + d_6-DMSO): δ 1.80 (m, 4H, CH_2CH_2, 3.5 (m, 2H, CH_2N), 4.57 (m, 1H, α-CH), 5.07 (s, 2H PhCH_2), 6.70 (m, 1H, NH), 7.37 (s, 5H, Ph), 8.23 (m, 4H, aryl protons *ortho* and *meta* to NO_2).

Anal. Calcd. for $C_{20}H_{21}N_3O_7$: C, 57.83; H, 5.10; N, 10.12. Found: C, 57.85; H, 4.80; N, 10.10.

N^{α}-(4-Nitrobenzoyl)-L-ornithine (**13**).

Compound **12** (15.0 g, 36.1 mmoles) was dissolved in 2 *N* hydrogen bromide in glacial acetic acid (100 ml) with the aid of an ultrasonic bath, and after 15 minutes at room temperature the solution was evaporated under reduced pressure (*caution*: lachrymatory benzyl bromide is formed!). The residue was dissolved in a minimum of water, and the solution adjusted to approximately *p*H 6 by dropwise addition of 28% amonium hydroxide. The precipitate was filtered and dried on a lyophilizer to obtain a white solid (9.94 g, 98% yield), mp 227-229° dec; ir (potassium bromide): ν 3460, 3350, 2960, 2930, 2880, 2810, 2660, 2610, 2550, 2160, 1640, 1590, 1535, 1495, 1470, 1410, 1390, 1370, 1350, 1325, 1300, 1270 cm^{-1}; ^1H nmr (deuterium oxide + potassium carbonate): δ 1.77 (m, 4H, CH_2CH_2), 2.95 (m, 2H, CH_2N), 4.38 (m, 1H, α-CH), 7.97 (d, J = 8 Hz, 2H, aryl protons *ortho* to C=O), 8.33 (d, J = 8 Hz, 2H, aryl protons *ortho* to NO_2).

Anal. Calcd for $C_{12}H_{15}N_3O_5$: C, 51.24; H, 5.38; N, 14.94. Found: C, 51.23; H, 5.41; N, 14.87.

tert-Butyl N^{α}-(4-Nitrobenzoyl)-L-ornithinate (**14**).

A suspension of **13** (4.6 g, 16.4 mmoles) in *tert*-butyl acetate (100 ml) was stirred at room temperature and treated dropwise with 60% perchloric acid (3.0 ml, 1.8 g, 18 mmoles). The mixture was stirred for 18 hours at room temperature and then extracted with cold 0.5 *N* hydrochloric acid. The extract was basified with solid sodium carbonate (caution: carbon dioxide evolution), and the precipitate was filtered and redissolved in chloroform. Drying over sodium sulfate and evaporation afforded a light-yellow solid (3.93 g, 71% yield), mp 103-104°; ir (potassium bromide): ν 3440, 3360, 3250, 3120, 3090, 3020, 2990, 2970, 2940, 2880, 1950 w, 1815 w, 1745, 1660, 1610, 1595, 1575, 1525, 1500, 1485, 1465, 1455, 1400, 1400, 1375, 1355, 1325, 1305, 1255, 1220 cm^{-1}; ^1H nmr (deuteriochloroform): δ 1.53 (s, 9H, *t*-Bu), 1.96 (m, 6H, 3 CH_2), 4.72 (m, 1H, α-CH), 8.07 (d, J = 7 Hz, 2H, aryl protons *ortho* to C=O), 8.37 (d, J = 7 Hz, 2H, aryl protons *ortho* to NO_2).

Anal. Calcd. for $C_{16}H_{23}N_3O_5$: C, 56.96; H, 6.87; N, 12.45. Found: C, 56.77; H, 6.72; N, 12.25.

tert-Butyl N^{α}-(4-Nitrobenzoyl)-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithinate (**15**).

A solution of **14** (337 mg, 1 mmole) and di-*tert*-butyl dicarbonate (218 mg, 1 mmole) in chloroform (10 ml) was refluxed for 90 minutes. Evaporation gave an off-white solid (410 mg, 94% yield), mp 123-124°; R_f 0.7 (silica gel, 9:1 chloroform-methanol); ir (potassium bromide): ν 3400, 3000, 2950, 2870, 1740, 1690, 1650, 1610, 1535, 1520, 1495, 1450, 1360, 1350, 1325, 1290, 1250, 1230, 1205 cm^{-1}; ^1H-nmr (deuteriochloroform): δ 1.50 (m, 22H, 2 *t*-Bu and 2 CH_2), 3.13 (m, 2H, CH_2N), 4.67 (m, 2H, α-CH and NH), 7.25 (m, 1H, NH), 8.10 (m, 4H, aryl).

Anal. Calcd. for $C_{21}H_{31}N_3O_7$: C, 57.65; H, 7.14; N, 9.60. Found: C, 57.42; H, 6.94; N, 9.40.

tert-Butyl N^{α}-(4-Aminobenzoyl)-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithinate (**16**).

A solution of **15** (2.24 g, 5.13 mmole) in methanol (100 ml) was shaken with 5% Pd-C (0.2 g) under 3 atmospheres of hydrogen in a Parr apparatus overnight. The catalyst was filtered off and the filtrate evaporated to obtain a white powder (2.01 g, 94% yield), mp 143-145°; ir (potassium bromide): ν 3380, 2990, 2950, 2880, 1735, 1700, 1640, 1620, 1575, 1545, 1520, 1460, 1405, 1380, 1295, 1265 cm^{-1}; ^1H-nmr (deuteriochloroform): δ 1.42 (s, 9H, *t*-Bu), 1.48 (s, 9H, *t*-Bu), 1.70 (m, 4H, CH_2CH_2), 3.15 (m, 4H, CH_2N and NH_2), 4.60 (m, 2H, α-CH and NH), 6.63 (d, J = 9 Hz, 2H, aryl protons *ortho* to NH_2), 7.62 (d, J = 9 Hz, 2H, aryl protons *ortho* to C=O).

Anal. Calcd. for $C_{21}H_{33}N_3O_5 \cdot 0.5H_2O$: C, 60.56; H, 8.23; N, 10.09. Found: C, 60.88; H, 8.07; N, 9.92.

tert-Butyl N^{α}-[4-[[(2-Amino-3-cyano-5-pyrazinyl)methyl]-amino]benzoyl]-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithinate (**18**).

Aminonitrile **17** (168 mg, 1 mmole) was added in small portions over 5 minutes to a stirred solution of **16** (407 mg, 1 mmole) and diisopropylethylamine (174 μl, 129 mg, 1 mmole) in dry *N,N*-dimethylformamide (10 ml). The solution was left to stand at room temperature for 18 hours and evaporated under reduced pressure, the residue was partitioned between dichloromethane and water, and the dichloromethane layer was evaporated. Tlc (alumina, 50:1 chloroform-methanol) showed spots with R_f 0.7 (**16**), 0.5 (**17**), and 0.3 (probably the corresponding *N,N*-dialkyl derivative, not identified further). The mixture was purified by column chromatography on neutral alumina (30 g, 1.5 x 21 cm) with 50:1 chloroform-methanol as the eluent. Pooled pure fractions (tlc) were evaporated and the residue was dried in vacuo at 60° (phosphorous pentoxide) to obtain a light-yellow solid (202 mg, 37% yield), double mp 83-86°, 98-100°; ir (potassium bromide): ν 3410, 2990, 2940, 2880, 2215 (CN), 1725 sh, 1700, 1635, 1615, 1580, 1520, 1460, 1400, 1375, 1320, 1285, 1260 cm^{-1}; ^1H nmr (deuteriochloroform): δ

1.38 (s, 9H, *t*-Bu), 1.45 (s, 9H, *t*-Bu), 1.65 (m, 4H, β-CH_2, γ-CH_2), 3.10 (m, 2H, δ-CH_2), 4.1-5.1 (m, 6H, α-CH, pyrazine CH_2, NH or H_2O), 5.53 (br s, 2H, NH_2), 6.60 (m, 3H, NH and aryl protons *ortho* to NH_2), 7.60 (d, J = 8 Hz, 2H, aryl protons *ortho* to C=O), 8.15 (s, 1H, pyrazine proton).

Anal. Calcd. for $C_{27}H_{37}N_7O_5 \cdot 0.5H_2O$: C, 59.11; H, 6.98; N, 17.87. Found: C, 59.28; H, 6.98; N, 17.70.

tert-Butyl N^{α}-[4-[[(4-Aminopteridin-6-yl)methyl]amino]benzoyl]-L-N^{δ}-(*tert*-butyloxycarbonyl)-L-ornithinate (**19a**).

A mixture of **18** (300 mg, 0.557 mmole), formamidine acetate (290 mg, 2.8 mmole), and 2-ethoxyethanol (2 ml) was heated in an oil bath at 135° for 45 minutes, during which a clear solution formed. The residue after evaporation under reduced pressure was partitioned between chloroform and water. Tlc (silica gel sheets, 9:1 chloroform-methanol) showed a major spot (R_f 0.5) and two faint spots (R_f 0.0, 0.7). The chloroform layer was evaporated and the residue was purified by column chromatography on silica gel (20 g, 2 x 20 cm), using 19:1 chloroform-methanol to elute the fast-moving minor product and 9:1 chloroform-methanol to elute the major product with R_f 0.5. Pooled tlc-homogeneous fractions were concentrated to a small volume and diluted with a large volume of *n*-hexanes. The precipitate was filtered and dried *in vacuo* at 60° (phosphorus pentoxide) to obtain a yellow powder (125 mg, 40% yield), mp 101-110°; ir (potassium bromide): ν 3440, 2970, 2940, 2870, 1735, 1700, 1640, 1615, 1570, 1560, 1520, 1460, 1375, 1360, 1315, 1270 cm^{-1}; ^1H nmr (deuteriochloroform): δ 1.42 (s, 9H, *t*-Bu), 1.47 (s, 9H, *t*-Bu), 1.6-2.4 (m, 4H, β-CH_2 and γ-CH_2), 3.13 (m, 2H, δ-CH_2), 4.68 (m, 4H, α-CH, pteridine CH_2, NH or H_2O), 6.72 (m, 4H, NH and aryl protons *ortho* to NH), 7.68 (d, J = 8 Hz, 2H, aryl protons *ortho* to C=O), 8.77 (s, 1H, C^2-H), 9.08 (s, 1H, C^7-H).

Anal. Calcd for $C_{28}H_{38}N_8O_5 \cdot 0.5H_2O$: C, 58.42; H, 6.83; N, 19.46. Found: C, 58.66; H, 6.85; N, 19.70.

tert-Butyl N^{α}-[4-[[(4-Amino-2-methylpteridin-6-yl)methyl]-amino]benzoyl]-L-N^{δ}-(*tert*-butyl-oxycarbonyl)-L-ornithinate (**19b**).

A mixture of **18** (275 mg, 0.51 mmole), acetamidine acetate (301 mg, 2.55 mmoles), and 2-ethoxyethanol (4 ml) was heated in an oil bath at 135° for 75 minutes, during which a clear solution formed. The residue after evaporation under reduced pressure was partitioned between chloroform and water. Although tlc on silica gel sheets failed to distinguish the product and starting material, both compounds giving a spot at R_f 0.5 (9:1 chloroform-methanol), the product gave a clearly distinguishable spot (R_f 0.4) when glass plates were used. Purification essentially as described in the preceding experiment afforded a light-yellow powder (103 mg, 35% yield), mp 115-126°; ir (potassium bromide): ν 3390, 2990, 2950, 2880, 1720 sh, 1700, 1640, 1620, 1575, 1555, 1525, 1465, 1425, 1405, 1380, 1360, 1340, 1285, 1260 cm^{-1}; ^1H nmr (deuteriochloroform): δ 1.42 (s, 9H, *t*-Bu), 1.48 (s, 9H, *t*-Bu), 1.75 (m, 4H, β-CH_2 and γ-CH_2), 2.69 (s, 3H, Me), 3.12 (m, 2H, δ-CH_2), 4.65 (m, 4H, pyrazine CH_2, α-CH, NH), 5.10 (m, 1H, NH), 6.67 (br d, J = 8 Hz, 4H, NH_2 and aryl protons *ortho* to NH), 7.67 (d, J = 8 Hz, 2H, aryl protons *ortho* to C=O), 9.02 (s, 1H, C^7 proton).

Anal. Calcd. for $C_{29}H_{40}N_8O_5 \cdot 0.25H_2O$: C, 59.52; H, 6.98; N, 19.15. Found: C, 59.44; H, 6.81; N, 18.96.

N^{α}-[4-[[(4-Aminopteridin-6-yl)methyl]amino]benzoyl]-L-ornithine (**5**, dAPA-Orn).

Compound **19a** (151 mg, 0.267 mmole) was dissolved in 2:1 dichloromethane-trifluoroacetic acid (6 ml) and the solution was allowed to stand at room temperature for 3 hours and then poured into a separatory funnel containing chloroform (20 ml) and 5% ammonium hydroxide (50 ml). The partitioned aqueous layer was concentrated to 20 ml, at which point a yellow solid formed and redissolved on re-addition of water. The mixture was diluted to 80 ml and applied onto a column of Dowex 50W-X2 (sulfonic acid form, 2 x 27 cm), which was eluted first with water to remove all the trifluoroacetic acid and then 3% ammonium hydroxide to elute the product. Further purification was done on a column of DEAE-cellulose (bicarbonate form), which was eluted with *ca.* 25 ml of 0.2 *M* ammonium bicarbonate adjusted to *p*H 9.5 by dropwise addition of 28% ammonium hydroxide. Several colorless impurities and a minor yellow band preceded the product, and some insoluble material deposited on top of the column during elution and was not examined further. Partial decomposition apparently occurred during this step, probably because of the high *p*H used to move the product through the column. The best fractions were pooled and freeze-dried to obtain a light-yellow solid (26 mg, 21%), mp >300° dec; ir (potassium bromide): ν 3440, 2980, 2950, 1645, 1620, 1575, 1525, 1465, 1400, 1365, 1315, 1295, 1275, 1250, 1210 cm^{-1}; uv (*p*H 7.4): λ_{max} 220 infl (ε 22,100), 247 nm (20,800), 285 (20,400), 338 br (7,400); (0.1 *N* hydrochloric acid): λ_{max} 220 nm (ε 21,600), 292 (17,800), 330 infl (10,700). Analytically pure material was obtained by preparative hplc on C_{18} silica gel (5% acetonitrile in 0.05 *M* ammonium acetate, *p*H 6.7; flow rate 1.0 ml/min). The major fraction eluting at 7.2 minutes accounted for approximately 75% of the total, based on peak height measurement.

Anal. Calcd. for $C_{19}H_{22}N_8O_3 \cdot 0.5CH_3COOH \cdot 3.25H_2O$: C, 48.14; H, 6.16; N, 22.45. Found: C, 48.16; H, 6.01; N, 22.77.

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