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Synthesis and Enzymatic Activation of *N*-[N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithiny]-L-phenylalanine, a Candidate for Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

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Abstract—*N*-[N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithinyl]-L-phenylalanine (**1**), a carboxypeptidase A (CPA) cleavable prodrug was synthesized for use in an antibody directed strategy to improve the therapeutic selectivity of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**2**), an extremely potent nonpoly-glutamatable DHFR inhibitor which is also highly cytotoxic. Compound **1** was shown by HPLC analysis to give a >99% yield of **2** upon incubation with bovine CPA (bCPA) for 20 min at 25 °C. In a spectrophotometric kinetic assay with 50 μM dihydrofolate as the competing substrate in the presence of 65 μM NADPH, **1** + bCPA stoichiometrically inhibited recombinant human DHFR (rhDHFR) with a K_i of 0.35 pM. In contrast, **1** without bCPA was a poor inhibitor of rhDHFR (K_i > 10 μM). In a 72 h growth inhibition assay against cultured CCRF-CEM human leukemic lymphoblasts, the growth inhibitory activities of **1** + bCPA, **2** + bCPA, and **2** alone were the same (IC_{50} 1.3–1.4 nM), whereas **1** in the absence of bCPA was >100-fold less potent (IC_{50} 155 nM).

Introduction

Antibody-directed enzyme prodrug therapy (ADEPT)[1,2] is a strategy for improving the specificity of cancer chemotherapy by surrounding tumor cells with a concentrated pool of a diffusible cytotoxic drug.[3] The ADEPT approach utilizes a weakly- or non-immunogenic exogenous macromolecule, typically an enzyme, to catalyze the activation of a prodrug. Optimally, the prodrug should not be a good substrate for human enzymes.[4]

A prokaryotic, genetically altered or chemically modified enzyme may serve as the activating catalyst.[5] In a novel variant of this approach, recent studies have focused on abzymes, which are catalytic antibodies capable of promoting reactions not ordinarily catalyzed by natural enzymes.[6,7] Chemotherapeutic specificity is achieved by conjugating the enzyme or abzyme to a tumor selective antibody or antibody fragment via a linkage that preserves the activities of both the targeting moiety (the antibody) and the activating moiety (the enzyme). Activating and targeting components may be linked by a variety of synthetic chemical methods[8] or assembled by recombinant techniques as bifunctional fusion proteins.[9] The latter approach can provide a reproducibly homogeneous product in high yield.[10]

Some years ago, Huennekens and co-workers[11] synthesized α-L-phenylalanyl MTX (MTX-L-Phe), the first example of an ADEPT based antifolate prodrug. MTX-L-Phe was rapidly cleaved by a CPA-antibody conjugate, giving a high yield of MTX. The cytotoxicity of MTX-L-Phe against UCLA-P3 human lung adenocarcinoma cells improved 350-fold in the presence of conjugated bovine CPA (bCPA).[11] However, the IC_{50} of MTX against these cells, 28 nM, is higher than that of a number of other antifolates that could have been used for this purpose, and also is large in comparison with other drugs that have been used to make ADEPT prodrugs.[12]

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In this paper we describe the synthesis of a derivative of N^{α}-(4-amino-4-deoxypteroyl)-N^{α}-hemiphthaloyl-L-ornithine (**2**, PT523) and its testing as a possible ADEPT prodrug. Compound **2** is a non-polyglutamatable analogue of aminopterin that was discovered in our laboratory and is currently in advanced preclinical testing in preparation for a phase I trial. It is one of the most potent known antifolates, with IC_{50} values in the low to subnanomolar range against a wide variety of tumor cells. In competition with MTX, the affinity of **2** for the reduced folate carrier is 10-fold greater. In the presence of NADPH with dihydrofolate as the competing substrate, its affinity for human DHFR exceeds that of MTX by 15-fold. The inability of **2** to form polyglutamates is due to the nearly absolute specificity of folylpolyglutamate synthetase for substrates with a glutamyl side chain. The 72-h cytotoxicity of **2** toward cultured CCRF-CEM human lymphoblastic leukemia cells surpasses that of MTX by 10-fold.[13]

Of the many possible derivatives of **2** that would be suitable as candidate ADEPT prodrugs, we chose to synthesize and study *N*-[N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithiny]-L-phenylalanine (**1**). Our aim was to obtain a prodrug with CPA substrate activity similar to that of MTX-L-Phe. The structures of **1** and **2** are shown in Figure 1.

Results

Chemistry

Compound **8**, the dipeptide precursor of **1** was synthesized according to Scheme 1. The protected amino acids N^{α}-Cbz-N^{δ}-Boc-L-Orn (**3**), N^{α}-Cbz-N^{δ}-Boc-L-Orn pentafluorophenyl ester (**4**), and L-Phe *tert*-butyl ester (**5**) were prepared in $\geq$85% yield by standard peptide synthesis methods.[14] Condensation of **3** and **4** in the presence of *N*-methylmorpholine (NMM) afforded the blocked dipeptide **6** in 93% yield. Removal of the Cbz group by hydrogenolysis over 10% Pd–C in glacial AcOH for 24 h under 60 psi of H_2 pressure unexpectedly led to reduction of the aromatic ring in the Phe moiety, affording N^{δ}-Boc-L-Orn-L-(β-cyclohexyl)alanine *tert*-butyl ester (**7**) in 85% yield. However, over-reduction could be avoided by diluting the AcOH with two volumes of MeOH, shortening the reaction time to 3 h and decreasing the H_2 pressure to 30 psi. These less vigorous conditions led to N^{δ}-Boc-L-Orn-L-Phe *tert*-butyl ester (**8**) in 86% yield (Scheme 1). Compounds **7** and **8** were clearly distinguishable on the basis of their C,H,N analyses and ^{1}H NMR spectra.

Compound **1** was obtained from 4-amino-4-deoxypteroic acid (**9**) as shown in Scheme 2. Two strategies were investigated. The first, consisting of five steps, was predicated on the expectation that regiospecific phthaloylation would require N^{10} protection. Thus, **9** was treated with 98% HCO_2H in the presence of a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (DMAP) to obtain 4-amino-4-deoxy-10-formylpteroic acid (**10**), as a tetrahydrate, in 99% yield. This compound was previously obtained in somewhat lower yield by reaction of **9** with a mixture of 98% HCO_2H and Ac_2O.[15] Coupling

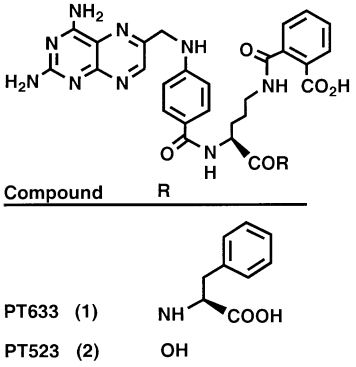
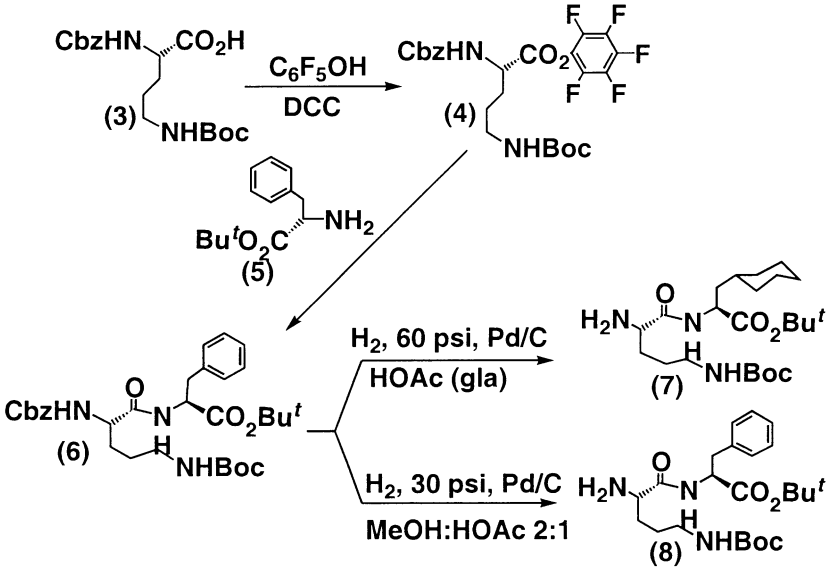


Figure 1. Structures of N^{α}-(4-amino-4-deoxypteroyl)-N^{γ}-hemiphthaloyl-L-ornithinyl-L-phenylalanine (**1**) and N^{α}-(4-amino-4-deoxypteroyl)-N^{∂}-hemiphthaloyl-L-ornithine (PT523, **2**).



Scheme 1. Synthesis of **8**, the dipeptide precursor of N^{α}-(4-amino-4-deoxypteroyl)-N^{∂}-hemiphthaloyl-L-ornithinyl-L-phenylalanine (**1**).

of **8** and **10** in the presence of diethyl phosphorocyanidate (DEPC) and $^{i}Pr_2EtN$[16] led to an 87% yield of **11**, which on cleavage of the Boc group with 1:2 TFA–CH_2Cl_2 was converted to **12** as a TFA salt in 98% yield. Acylation of **12** with phthalic anhydride in DMF and $^{i}Pr_2EtN$ afforded the hemiphthaloyl derivative **13** in 50% yield, but removal of the 10-formyl group by treatment with a stoichiometric amount of NaOH at room temperature for 30 min gave a poor yield of **1**, <7%. Thus the overall yield for the five-step sequence was only 3%. Removal of the 10-formyl group with 10% N_2H_2 in DMF was also tried without success.[14]

The second, and more concise route to **1** was made possible by the subsequent discovery that acylation of unprotected N^{10} did not occur when 1.1 molar equivalents of phthalic anhydride were used. This eliminated the problem of N^{10}-formyl deprotection in the presence of an N^{7}-hemiphthaloyl group. Thus, as indicated in Scheme 2, compound **12** was converted to the fully deprotected dipeptide **14** in 61% yield by a one-pot method in which the formyl group was cleaved with ethanolic HCl and the Boc and *tert*-butyl ester groups were cleaved with TFA. Acylation of **14** with phthalic anhydride occurred in 24% yield. Thus the overall yield of 1 from APA by this improved four-step route was 10%.

Elemental analyses (C, H, N) for **1** and its intermediates were within ±0.4% of calculated values. ^{1}H NMR and FABMS data were consistent with the desired structures. The target compound was >99% pure by HPLC.

Enzyme studies

Cleavage of **1** by bCPA was performed at 25 °C in pH 7.4 Tris–HCl buffer in the presence of 10 μM $ZnCl_2$ as described in the literature.[11] The reactions were quenched after 20 min by heating for 30 min at 95 °C to denature the enzyme. The extent of cleavage was monitored by reversed phase HPLC. Elution with a 20 min gradient of 5–25% v/v CH_3CN buffered with 0.1 M NH_4OAc at pH 7.5 gave baseline separation of **1** and **2**. As expected from its less hydrophilic character, prodrug **1** had the greater retention volume, 18.8 mL, whereas **2** had a retention volume of 13.6 mL. The co-product, L-phenylalanine was not observed at 370 nm, the detector

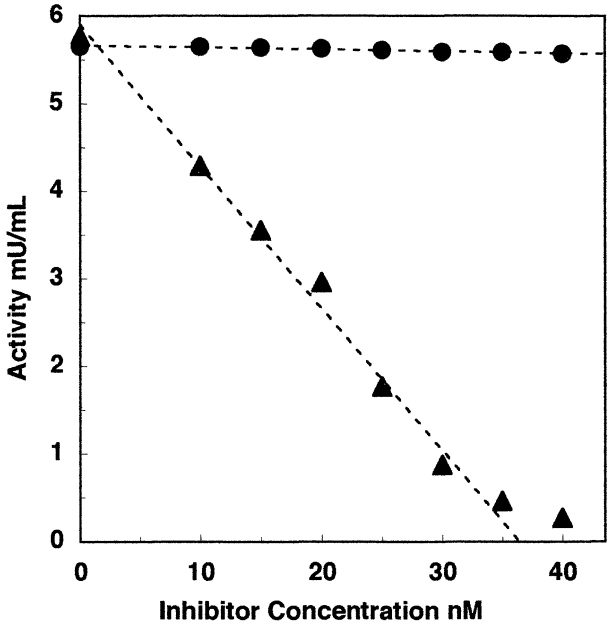
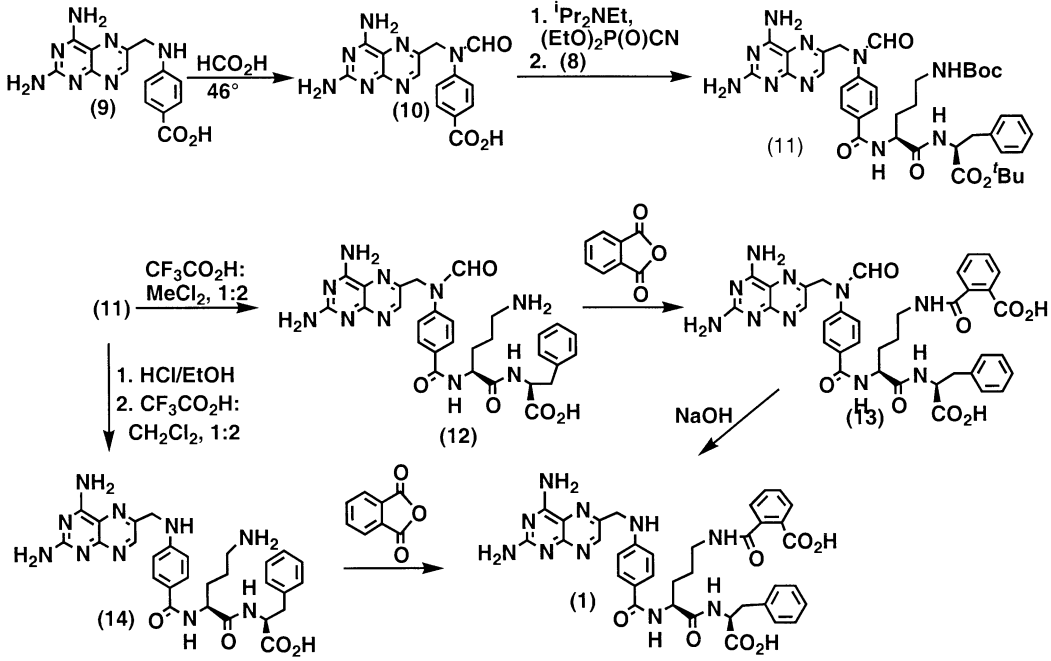


Figure 2. Plot of rhDHFR activity versus concentration of **1** after 20 min incubation in the presence (triangles) or absence (circles) of bovine carboxypeptidase A. DHFR substrates: 50 μM dihydrofolate and 65 μM NADPH. Data obtained by spectrophotometric titration at 340 nm.



Scheme 2. Synthesis of **1** from 4-amino-4-deoxypteroic acid (APA, **9**).

wavelength chosen for its sensitivity to the pteridine nucleus. After 20 min of exposure to bCPA the amount of unchanged **1** was estimated to be $<1\%$. The retention volume of the enzymatic cleavage product was the same as that of compound **2**.

Typical titration curves showing inhibition of rhDHFR activity by **1** in the presence and absence of bCPA are given in Figure 2. The K_i of **1** + bCPA for competitive inhibition of DHFR activity was not significantly different from that of **2**, 0.35 ± 0.15 pM. After incubation with **1** in the absence of bCPA, rhDHFR retained $>98\%$ of its native activity.

Cell growth assay

The ability of **1** to inhibit the proliferation of cultured CCRF-CEM human leukemic lymphoblasts was determined in the presence and absence of bCPA. Median effect curves for cell population growth in the presence of **1** alone, **2** alone, **1** + bCPA, and **2** + bCPA are shown in Figure 3. The IC_{50} values derived from the median effect curves were in the 1.3–1.4 nM range for **2**, **2** + bCPA, and **1** + bCPA. In contrast, the IC_{50} of **1** in the absence of bCPA was 155 nM. These results indicated that the enzyme was able to activate the prodrug quantitatively and did not influence the activity of the released drug as an inhibitor of cell proliferation.

Discussion

Ideally, the use of **1** for repeated cycles of ADEPT chemotherapy in human patients would require that a 'humanized' carboxypeptidase be used in order to prevent immune reactions. A genetically engineered mutant CPA of this type has been reported by Smith and coworkers.[17] They conjugated the mutant enzyme with ING-1, an antibody against Ep-CAM1, an epithelial cell adhesion protein that is overexpressed in colorectal and other tumor cells. The conjugate, ING1-hCPA1-T268G selectively targeted HT-29 human colon adenocarcinoma cells and cleaved terminal Phe and Tyr from MTX[18] and other antifolates.[19] Synthetic adducts with bulky substituents on the aromatic ring of Phe or Tyr were also good substrates.

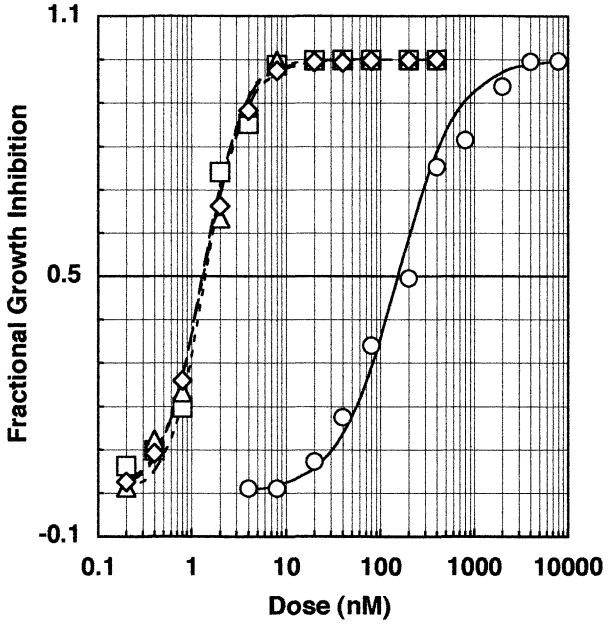


Figure 3. CEM cell growth inhibition curves for **2** incubated in the presence (squares) or absence (diamonds) of carboxypeptidase A and **1** incubated in the presence (triangles) or absence (circles) of carboxypeptidase A.

On an optimal therapeutic schedule, binding of the conjugate to the tumor and clearance of nonbound conjugate should precede administration of the prodrug.[20] Then wherever the circulating prodrug encounters the antibody-enzyme conjugate, a local concentration gradient of cytotoxic metabolite should form[3] and enter the targeted cells. If the activated drug molecule is small and diffusible, it should also enter neighboring cells and produce a 'bystander effect'.[21] This phenomenon has been demonstrated in vivo by ^{19}F NMR imaging with a glucuronic acid acetal derivative of 5-fluorouracil.[22]

Heterogeneous tumors commonly contain some cells that only express targeted antigens sparingly. Surface inaccessibility of targeted epitopes may also occur. Bulky immunotoxins have sometimes failed to achieve multilog cell reduction because of their inability to achieve adequate delivery to the cell surface. This problem may be overcome by the ability of small diffusible drug molecules to elicit a robust bystander effect.[23]

Taking these considerations into account, candidate prodrugs for ADEPT need to exhibit the following characteristics:[12]

1. The cytotoxicity of the prodrug itself must be less than that of the parent drug. As reported here, the cytotoxicity ratio of **1** to **2** (prodrug/activated drug) is similar to those of several other recently reported ADEPT prodrugs,[4,12] though not as high as the 800-fold ratio found with sterically hindered conjugates such as 3-cyclopentyltyrosyl- and 3-cyclobutylphenylalanyl-MTX.[17–19]

2. There should be adequate substrate activity at physiological pH in the presence of the activating enzyme. As reported in this paper, **1** is quantitatively converted to 2, its active product, by bCPA, and in all likelihood would be similarly activated by other enzymes that cleave C-terminal Phe residues.

3. The prodrug should have long-term chemical stability at pH 7.4. We have found that the decomposition of a 1 mM stock solution of **1** after 72 h at 37 °C in pH 7.4 buffer is $<1\%$ (data not shown).

4. The prodrug should have the ability to transit the tumor vasculature and diffuse throughout the tumor. Unlike large macromolecules that have been used in the construction of immunoconjugates for targeted chemotherapy, **1** is a small diffusible molecule.

5. The prodrug should have suitable pharmacological and pharmacokinetic properties. Although the pharmacokinetics and in vivo pharmacology of **1** itself have not been investigated, the pharmacokinetics and tissue distribution characteristics of **2** have been studied in mice and rats,[24,25] and are not likely to differ greatly between **1** and **2**.

Summary

Compound **1** is chemically stable at physiologic pH and temperature, is quantitatively cleaved by CPA at pH 7.4, and is at least 100-fold less cytotoxic than its activated product, **2**. While these are promising preliminary results, and the efficient cellular transport, tight DHFR binding, and potent cytotoxicity of **2** combine to make **1** a promising candidate for ADEPT chemotherapy, additional studies would be needed in order to determine whether **1** would be useful as an prodrug in vivo. These would include an assessment of its pharmacokinetics and toxicology in a suitable animal model.

It may also be noted that, as part of our continuing studies on second-generation analogues of **2**, several compounds modified at positions 5, 8, and 10 have been discovered that are superior to **2** in terms of chemical stability, cellular uptake kinetics, DHFR inhibition, and cytotoxicity.[26,27] Because of the high potency of these analogues, it would seem reasonable to view them as alternatives to **2** in the context of ADEPT chemotherapy.

Experimental

Compounds **1** and **6**–**14** were characterized by elemental analysis (QTI Laboratories, Whitehouse, NJ, USA) and 500 MHz ^{1}H NMR on a Varian model ML500 instrument; **1** by HRFABMS on a Vacuum Generators Ltd instrument, Manchester, UK (resolution ± 2.6 ppm) and **12**–**14** by FABMS on a Perkin-Elmer Voyager system 4036 (resolution ± 8.0 ppm). The purity of compounds **1** and **12**–**14** was confirmed by HPLC with a Waters (Milford, MA, USA) automated gradient controller and two model 510 pumps giving a combined flow rate of 1.0 mL/min. The mobile phase was 1 M NH_4OAc pH 7.5 with a linear concentration gradient of 0–20% v/v CH_3CN for 20 min followed by isocratic 20% CH_3CN for 10 min. The column was a Waters Novapak 8NV4μ C-18 radial compression cartridge. A Waters model 484 tunable absorbance detector was operated at 370 nm. TLC of compounds **6**–**8** and **11**–**13** was performed with MK6F glass plates (Whatman Ltd., Maidenhead, UK). Eluent compositions for TLC and silica gel column chromatography are given below as volume ratios.

Amino acid protection and activation

Standard peptide synthesis methods were used:[14] starting with N^{δ}-Boc-L-ornithine (Bachem Bioscience, Inc., King of Prussia, PA, USA), the benzyloxycarbonyl (Cbz) group of **3** was introduced with *N*-benzyloxysuccinimide, the pentafluorophenyl ester group of **4** with pentafluorophenol and *N*,*N*′-dicyclohexylcarbodiimide and the *tert*-butyl ester group (OtBu) of **5** with isobutylene and dry HCl(g).

N^{α}-Cbz-N^{δ}-Boc-L-ornithinyl-L-phenylalanine *tert*-butyl ester (6). A solution of of NMM (0.225 mL) in DMF (15 mL) was added to L-phenylalanine *tert*-butyl ester hydrochloride (0.516 g, 2 mmol). When the solid dissolved, **4** (1.07 g, 2 mmol) was added. It likewise dissolved rapidly, but a precipitate formed shortly thereafter. After 15 min at 23 °C the mixture was evaporated in vacuo at 27 °C. The residue was triturated for 1h with 0.8 M $NaHCO_3$ (15 mL). The precipitate was filtered and washed with 20 mL each of 0.2 M $KHSO_4$ and H_2O. The filter cake was dried in vacuo at 23 °C to obtain **6** as a white solid (1.06 g, 93%): mp 121–122 °C; TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.70; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.14–1.54 (m, 4H, β- and γ-CH_2), 1.31 (s, 9H, OtBu), 1.35 (s, 9H, Boc), 2.88 (m, 2H, δ-CH_2), 2.93 (m, 2H, PhCH_2CH), 3.93 (m, 1H, α-$CHCO_2$), 4.31 (m, 1H, α-CHCO), 4.99 (s, 2H, $PhCH_2O$), 7.17–7.33 (m, 10H, $2C_6H_5$). Anal. calcd for $C_{31}H_{43}N_3O_7$: C, 65.36; H, 7.61; N, 7.38. Found: C, 65.54, H, 7.67, N, 7.44.

N^{δ}-Boc-L-ornithinyl-L-(β-cyclohexyl)alanine *tert*-butyl ester (7). To a solution of **6** (0.75 g, 1.27 mmol) in glacial AcOH (40 mL) were added 0.3 g of 10% Pd/C. The mixture was shaken for 24 h under 60 psi of H_2 pressure in a Parr apparatus. The mixture was filtered through Celite and the filtrate evaporated under reduced pressure to a volume of 2 mL. It was stirred with Et_2O (10 mL) and chilled overnight at 4 °C. The precipitated solid, consisting of the acetate salt of **7**, was filtered, washed with Et_2O (5 mL) and hexanes (5 mL), and dried in vacuo at 21 °C. The acetate salt was dissolved in CH_2Cl_2 (10 mL) the solution washed with 4 M K_2CO_3 (15 mL) and the aqueous layer back-extracted with CH_2Cl_2 (2×10 mL). The CH_2Cl_2 extracts were pooled and dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave an oil, which was recrystallized from Et_2O–hexanes to obtain the free base **7** as soft white crystals (0.50 g, 85%): mp (dec) 74–75 °C; TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.52; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 0.91–2.52 (m, 17H, $CH_2C_6H_{11}$, β- and γ-CH_2), 1.31 (s, 9H, OtBu), 1.35 (s, 9H, Boc) 2.90 (m, 2H, δ-CH_2), 4.39 (m, 1H, α-CHCO), 3.97 (m, 1H, α-$CHCO_2$). Anal. calcd for $C_{23}H_{43}N_3O_5 \cdot H_2O$: C, 60.10; H, 9.87; N, 9.14. Found: C, 60.13; H, 10.12, N, 9.18.

N^{δ}-Boc-L-ornithinyl-L-phenylalanine *tert*-butyl ester (8). To a solution of **6** (1.0 g, 1.75 mmol) in glacial AcOH (40 mL) and MeOH (80 mL) were added 0.3 g of 10% Pd/C. The mixture was shaken for 3 h under 30 psi of H_2 pressure in a Parr apparatus, and was then filtered and evaporated under reduced pressure to a volume 2 mL. After addition of Et_2O (15 mL), the mixture was chilled overnight at 4 °C. The solid was filtered, washed with EtOAc (5 mL), and dried in vacuo at 21 °C for 18 h to obtain the acetate salt of **8** as a white solid (1.74 g, 99%): mp 104–106 °C. The acetate salt was dissolved in CH_2Cl_2 (15 mL), the solution washed with 4 M K_2CO_3

(25 mL), and the aqueous layer back-extracted twice with CH_2Cl_2 (2×15 mL). The CH_2Cl_2 extracts were pooled and dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave an oil, which was dissolved in dry Et_2O (10 mL). The solution was chilled on an ice bath and 6.6 M anhydrous ethereal HCl (0.3 mL) was added. The precipitated solid was filtered, washed immediately with dry Et_2O (5 mL) and hexanes (15 mL) at 25 °C, and dried in vacuo overnight to obtain the HCl salt of **8** as a white crystalline solid (740 mg, 86%): mp (dec) 140–150 °C; TLC ($CHCl_3$/MeOH/AcOH, 90:9:1) R_f 0.15; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.22–1.43 (m, 4H, β- and γ-CH_2), 1.31 (s, 9H, O*t*Bu), 1.35 (s, 9H, Boc), 2.93 (m, 2H, $PhCH_2$), 3.04 (m, 2H, δ-CH_2), 3.11 (m, 1H, α-CHCO), 4.38 (m, 1H, α-$CHCO_2$), 7.17–7.28 (m, 5H, C_6H_5). Anal. calcd for $C_{23}H_{37}N_3O_5{\cdot}HCl{\cdot}H_2O$: C, 56.37; H, 8.23; N, 8.57. Found: C, 56.13, H, 8.12, N, 8.48.

4-Amino-4-deoxy-10-formylpteroic acid (10). A suspension of 1.5 g, 4.0 mmol of 4-amino-4-deoxypteroic acid $3.5H_2O$ (**9**)[28] and DMAP (25 mg) in 98% HCO_2H (100 mL) was stirred at 46 °C for 14 h. The mixture was cooled to 18 °C and poured into rapidly stirred anhydrous Et_2O (900 mL). The white precipitate was collected on a fritted glass funnel. The filter cake was washed with dry Et_2O (4×100 mL) and then dissolved in 0.05 M NH_4OH (760 mL). The pale amber-colored solution was freeze-dried under a 7 μbar vacuum for 120 h to obtain **10** as a yellow solid (1.33 g, 99%): mp (dec) >240 °C; HPLC retention volumes: **10**, 15.3; **9**, 16.4 mL; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 5.23 (s, 2H, 9-CH_2), 7.58–7.92 (m, 4H, C_6H_4), 8.72 (s, 1H, C^7-H), 8.83 (s, 1H, CH=O). Anal. calcd for $C_{15}H_{13}N_7O_3{\cdot}4H_2O$: C, 43.80; H, 5.15; N, 23.83. Found: C, 43.77, H, 5.32, N, 23.60.

***N*$^{\alpha}$-(4-Amino-4-deoxy-10-formylpteroyl)-*N*$^{\delta}$-Boc-L-ornithinyl-L-phenylalanine *tert*-butyl ester (11).** To a suspension of **10** (410 mg, 1.0 mmol) in dry DMF (250 mL) were added ^{i}Pr$_2$NEt (0.45 mL, 2.5 mmol) and DEPC (0.3 mL, 1.7 mmol). The mixture was stirred at 21 °C until the solids slowly dissolved. After 4 h, compound **8** (520 mg, 1.1 mmol) was added and stirring was continued for 3 days. A 400 mg portion of $NaHCO_3$ was then added and the mixture was evaporated under reduced pressure. The residue was stirred for 45 min with CH_2Cl_2/EtOH (50 mL, 9:1, v/v), the mixture was filtered, and the solid on the funnel was washed with the same solvent mixture (2×25 mL). The filtrate and combined washings were evaporated. The residue was purified by flash chromatography on a 38 mm ID×160 mm silica gel column with CH_2Cl_2/MeOH (9:1) as the eluent. Homogeneous product fractions were identified by TLC, pooled and evaporated. The residue was dissolved in acetone (5 mL) and the product was precipitated by stepwise addition of Et_2O (20 mL) and hexanes (20 mL). The precipitate was filtered and dried in vacuo to obtain **11** as a pale-yellow solid (675 mg, 87%): mp (dec) 149–150 °C; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.27; (*n*-BuOH/AcOH/H_2O, 3:1:1), R_f 0.66; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.24–1.67 (m, 4H, β- and γ-CH_2), 1.25 (s, 9H, OtBu), 1.33 (s, 9H, Boc), 2.90 (m, 2H, δ-CH_2), 2.92 (m, 2H, $PhCH_2$), 4.31 (m, 1H, α-$CHCO_2$), 4.41 (m, 1H, α-CHCO), 5.18 (s, 2H, 9-CH_2), 7.12–7.86 (m, 9H, C_6H_5, C_6H_4), 8.63 (s, 1H, C^7-H), 8.78 (s, 1H, CH=O). Anal. calcd for $C_{38}H_{48}N_{10}O_7{\cdot}3/4H_2O$: C, 59.25; H, 6.48; N, 18.18. Found: C, 59.24, H, 6.50, N, 17.89.

***N*$^{\alpha}$-(4-Amino-4-deoxy-10-formylpteroyl)-L-ornithinyl-L-phenylalanine (12).** Compound **11** (120 mg, 0.15 mmol) was dissolved in 4 mL of ice-cold CF_3CO_2H/CH_2Cl_2, 1:2 v/v and allowed to warm to 21 °C. After 6 h the solution was evaporated under reduced pressure. TLC (silica gel, *n*-BuOH/AcOH/H_2O, 3:1:1) showed the absence of **11** (R_f 0.66) and formation of **12** (R_f 0.21) as the only product. The residue was dissolved in CH_2Cl_2 (5 mL) and the solution added dropwise to rapidly stirred Et_2O (100 mL). The precipitated solid was filtered and washed with Et_2O (10 mL) and hexanes (10 mL). When exposed to room air the tan powder darkened slightly and appeared to absorb atmospheric moisture. Drying in vacuo at 50 °C afforded **12** as a beige solid (108 mg, 98%): mp (dec) >240 °C; HPLC retention volume: 26.5 mL; HRFABMS *m*/*z* $[M+H]^+$ 601.2616 ($C_{29}H_{33}N_{10}O_5$ requires 601.2635). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.46–1.76 (m, 4H, β- and γ-CH_2), 2.89 (m, 2H, δ-CH_2), 2.92 (m, 2H, $PhCH_2$), 4.39 (m, 1H, α-CHCO), 4.48 (m, 1H, α-$CHCO_2$), 5.32 (s, 2H, 9-CH_2), 7.15–7.70 (m, 9H, C_6H_5, C_6H_4), 8.68 (s, 1H, C^7–H), 8.70 (s, 1H, CH=O). Anal. calcd for $C_{29}H_{32}N_{10}O_5{\cdot}CF_3CO_2H{\cdot}H_2O$: C, 50.82; H, 4.81; N, 19.12. Found: C, 50.72, H, 5.03, N, 18.98.

***N*$^{\alpha}$-(4-Amino-4-deoxy-10-formylpteroyl)-*N*$^{\delta}$-hemiphthaloyl-L-ornithinyl-L-phenylalanine (13).** Compound **12** (74 mg, 0.10 mmol) was stirred in DMF (1.5 mL) containing ^{i}Pr$_2$NEt (0.18 mL, 1.0 mmol) until all the solid dissolved (about 45 min). Phthalic anhydride (18 mg, 0.12 mmol) was then added and stirring was continued for 1.5 h. The solution was evaporated under reduced pressure, and the residue was subjected to flash chromatography on a 24 mm ID×145 mm long column of Baker C18 silica gel. The column was eluted with a 0.5 L gradient of 4–20% EtOH in 0.1M NH_4OAc buffer, pH 8.6, followed by 1 L of 20% EtOH in the same buffer. Fractions (25 mL) were monitored by HPLC with gradient elution as described above. Fractions containing pure product were pooled, and evaporated. The residue was taken up in H_2O (20 mL) and the solution was freeze-dried to obtain the trifluoroacetate salt of **13** as a yellow powder (39 mg, 50%): mp (dec) >240 °C; HPLC retention volume: 24 mL; HRFABMS *m*/*z* $[M+H]^+$ 749.2787 ($C_{37}H_{37}N_{10}O_8$ requires 749.2796; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.46–1.76 (m, 4H, β- and γ-CH_2), 2.89 (m, 2H, δ-CH_2), 3.01 (m, 2H, $PhCH_2$), 4.43 (m, 1H, α-CHCO), 4.53 (m, 1H, α-$CHCO_2$), 5.19 (s, 2H, 9-CH_2), 7.10–8.38 (m, 13H, $2C_6H_4$, C_6H_5), 8.64 (s, 1H, C^7-H), 8.77 (s, 1H, CH=O). Anal. calcd for $C_{37}H_{36}N_{10}O_8{\cdot}CF_3CO_2H{\cdot}3H_2O$: C, 51.09; H, 4.73; N, 15.28. Found: C, 50.83, H, 5.00, N, 15.10.

***N*$^{\alpha}$-(4-Amino-4-deoxypteroyl)-L-ornithinyl-L-phenylalanine (14).** A solution of **11** (120 mg, 0.15 mmol) in ethanolic 1 M HCl (10 mL) was stirred at 21 °C for 16 h. The mixture was evaporated and the residue dissolved

in 25% v/v CF_3CO_2H in CH_2Cl_2 (4 mL). After another 16 h the solution was evaporated under reduced pressure. TLC (silica gel, *n*-BuOH/AcOH/H_2O, 3:1:1) showed some unchanged **11** (R_f 0.66) along with the desired product **14** (R_f 0.17). The mixture was chromatographed on an 18 mm ID×220 mm long column of cellulose DE-52 (Whatman Ltd., Maidenhead, UK) with a 0.01–0.08 M gradient of $CF_3CO_2NH_4$ buffer, pH 6.5. TLC homogeneous product fractions were pooled and evaporated. The deliquescent residue was dissolved in H_2O (350 mL) and the solution was freeze-dried to obtain **14** as a yellow powder (58 mg, 61%): mp (dec) >165 °C; HPLC retention volume: 27.5 mL; HRFABMS *m/z* $[M+H]^+$ 573.2640 ($C_{28}H_{33}N_{10}O_4$ requires 573.2686). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.54–1.76 (m, 4H, β- and γ-CH_2), 2.89 (m, 2H, δ-CH_2), 2.92 (m, 2H, $PhCH_2$), 4.49 (m, 1H, α-CHCO), 4.50 (m, 1H, α-$CHCO_2$), 5.32 (s, 2H, 9-CH_2), 7.11–8.43 (m, 9H, C_6H_5, C_6H_4), 8.68 (s, 1H, C^7–H), Anal. calcd for $C_{28}H_{32}N_{10}O_4 \cdot 1/4CF_3CO_2H \cdot 2H_2O$: C, 53.72; H, 5.73; N, 21.98. Found: C, 53.63, H, 5.81, N, 21.78.

N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithinyl-L-phenylalanine (1). Method A. Compound **13** (90 mg, 0.11 mmol) was stirred with 0.4 mL of 1 N NaOH (0.4 mL) until it dissolved, H_2O (2.1 mL) was added, and stirring was continued for 30 min at 21 °C. The mixture was chilled to 4 °C and 1 N AcOH (0.5 mL) was added. The precipitated solid was rapidly filtered, washed with ice-cold H_2O (2 mL), and redissolved in 0.05 N NH_4OH (25 mL). The amber-colored solution was freeze-dried for 96 h to obtain **1** as a yellow powder (7.5 mg, 6.8%). The HPLC retention volume of this sample was 25 mL, identical to the sample prepared by Method B.

Method B. Compound **14** (30 mg, 0.047 mmol) was suspended in DMF (0.5 mL) and the mixture was stirred for 45 min. The solid did not fully dissolve, but when phthalic anhydride (7.7 mg, 0.052 mmol) was added a clear solution was obtained. After 1.5 h the DMF was evaporated under reduced pressure, and the residue was chromatographed by the gradient method described for compound **13**. Fractions were monitored by HPLC, and fractions containing pure product were pooled and evaporated. Volatile buffer salts were evaporated by entrainment with H_2O (5×200 mL) at 30 °C. The residue was dissolved in H_2O (20 mL) and the solution freeze-dried to obtain **1** as a yellow powder (8.8 mg, 24%); HRFABMS *m/z* $[M+H]^+$ 721.2827 ($C_{36}H_{37}N_{10}O_7$ requires 721.2846). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 1.46–1.76 (m, 4H, β- and γ-CH_2), 2.89 (m, 2H, δ-CH_2), 3.00 (m, 2H, $PhCH_2$), 4.53 (m, 1H, α-$CHCO_2$), 4.48 (m, 1H, α-CHCO), 5.19 (s, 2H, 9-CH_2), 7.10–8.38 (m, 13H, $2C_6H_4$, C_6H_5), 8.68 (s, 1H, C^7–H). Anal. calcd for $C_{36}H_{36}N_{10}O_7 \cdot 2H_2O$: C, 57.14; H, 5.33; N, 18.51. Found: C, 56.97, H, 5.17, N, 18.54.

Enzymatic cleavage of 1

Reactions were carried out in 1 mL conical freezing tubes. Solutions contained 10 μL of 1.0–1.2 mM **1** or **2** and 80 μL of 0.05M TRIS–HCl pH 7.4 + 10 μM $ZnCl_2$. A fresh 1.3 mU/mL bCPA stock solution was prepared in the same buffer and reactions were initiated by addition of a 10 μL aliquot. For negative controls, 10 μL of buffer were added. After 20 min at 25 °C, the test sample and control tubes were tightly sealed and heated for 30 min at 95 °C to denature the enzyme and stop the reaction. The solutions were analyzed by HPLC as described above. HPLC retention volumes were: **1**, 25; **2**, 21 mL.

DHFR inhibition was analyzed as described previously[29] in a quartz cuvette in which 5.5–6.0 mU of rhDHFR-NADPH were preincubated with 0–45 nM **1** ± bCPA. The reaction was initiated by addition of dihydrofolate (FAH_2) and the rate of decrease of the 340 nm absorption of 50 μM FAH_2 and 65 μM NADPH monitored at 21 °C. The K_i of **2** was calculated by the method of Strauss and Goldstein.[30]

Cell growth inhibition assays were performed to determine the ability of **1** or **2** alone, **1** + bCPA, and **2** + bCPA to inhibit the population growth of CCRF-CEM human leukemic lymphoblasts in a 72 h assay.[26] IC_{50} values were calculated by median effect analysis.[31]

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