A Phosphonamidate Dipeptide Analogue as an Inhibitor of Carboxypeptidase A

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Abstract: The application of a new strategy for the design of potential transition-state analogues for carboxypeptidase A (CPA) has led to the development of a potent competitive inhibitor. *N*-[[[(Benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine, dilithium salt, (ZG^{P}P), **4**, is an analogue of the substrate Z-Gly-L-Phe with a tetrahedral phosphonamidate moiety in place of the scissile peptide group. ZG^{P}P is a competitive, reversible inhibitor of CPA, with K_i = 9.0 × 10^{-8} M at pH 7.5 and K_i = 6 × 10^{-9} M at pH 6.0; it is not hydrolyzed by the enzyme. ZG^{P}P resembles the tetrahedral intermediate in the proposed mechanism for CPA-catalyzed peptide hydrolysis, although it lacks a hydrogen-bonding interaction present in this intermediate.

Introduction

The synthesis of biologically active compounds has long captured the interest of organic chemists. When choosing synthetic targets, we have for the most part taken the suggestions of nature and of the structure-activity relationships of medicinal chemistry. However, an increasingly important influence on the design of biologically active compounds is being exerted by mechanistic understanding of enzymatic processes. Suicide inhibitors[2] and transition-state analogues[3] are among the more effective types of enzyme inhibitors to arise from this approach. In this paper, we describe the synthesis and evaluation of an inhibitor of carboxypeptidase A which we designed as a potential transition-state analogue.

The zinc peptidases constitute an important class of proteolytic enzymes, from both the mechanistic and the biological standpoint.[4] Carboxypeptidase A (CPA) is the leading representative of this group. It is specific for cleavage of the C-terminal amino acid from an oligopeptide, favoring those with aromatic side chains. X-ray crystallography has defined the composition of the enzyme active site,[5] and has led to a widely-accepted mechanistic proposal for peptide hydrolysis by zinc-containing enzymes.[5,6] This mechanism involves attack by a water molecule on the scissile carbonyl group, with Lewis acid and general-base assistance by the zinc cation and an active site carboxylate, respectively (Figure 1).

As part of our continued interest in tetrahedral phosphorus derivatives as stable mimics of tetrahedral carbon intermediates,[7] we have prepared a phosphonamidate analogue of a CPA substrate, carbobenzoxyglycyl-L-phenylalanine (ZGP). This analogue, *N*-[[[(benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine, dilithium salt, (ZG^{P}P), **4**, was designed to resemble the tetrahedral adduct resulting from direct addition of water to the scissile carbonyl group of the substrate, as depicted in Figure 1. To our knowledge, this molecule represents the first phosphonamidate employed as an enzyme inhibitor,[8] although amino acid and dipeptide phosphoramidates have been shown to inhibit a number of zinc peptidases.[10]

Materials and Methods[11]

Dimethyl phthalimidomethylphosphonate (1) was prepared as described by Seyferth et al.[12] Triethylammonium bicarbonate was prepared by saturation of an aqueous suspension of triethylamine with CO_2 at 21 °C. Chloroform was rendered ethanol free by washing with concentrated H_2SO_4 and water, drying ($CaCl_2$), and distilling from P_2O_5. Benzoylglycyl-L-phenylalanine (BzGP) (Sigma), carbobenzoxyglycylglycyl-L-phenylalanine (ZGGP) (Sigma), bovine carboxypeptidase A (CPA) (Worthington), and other reagents were used as purchased. The following buffer solutions were employed: pH 8.5 and pH 7.5, 0.025 M Tris/HCl and 0.5 M NaCl; pH 6.2, 0.025 M potassium phosphate and 0.5 M NaCl; pH 6.0, 0.025 M MES/NaOH and 0.5 M NaCl.

Methyl Hydrogen *N*-(Benzyloxycarbonyl)aminomethylphosphonate (2). To a suspension of 20.0 g (74 mmol) of dimethyl phthalimidomethylphosphonate 1 in 128 mL of methanol was added 2.75 mL (78 mmol) of 90% hydrazine, and the resulting solution was kept at 21 °C for 80 h. The precipitated phthalhydrazide was removed by filtration and washed with methanol, and the filtrate was concentrated at 0 °C (10 torr) and finally at 0.1 torr to give the free amine as a pale yellow oil. This material was immediately dissolved in 80 mL of $CHCl_3$ at 0 °C, and 12 mL (84 mmol) of benzyl chloroformate and 15.5 mL (111 mmol) of triethylamine were added. The mixture was stirred at 21 °C for 13 h before it was diluted with 150 mL of $CHCl_3$ and washed with two 250-mL portions of 2 N H_2SO_4. The organic layer was dried ($MgSO_4$) and concentrated to give 20.1 g of the crude carbobenzoxy derivative as an oil.

Chromatographic purification (silica gel, 1:19 ethanol/$CHCl_3$) of 236 mg of a sample prepared in this manner afforded 104 mg (44% yield) of purified material as a syrup: ^{1}H NMR ($CDCl_3$) δ 3.68 (overlapping

(1) Fellow of the Alfred P. Sloan Foundation, 1979–1981.

(2) R. R. Rando, *Acc. Chem. Res.*, **8**, 281 (1975); R. H. Abeles and A. L. Maycock, *ibid.*, **9**, 313 (1976); C. Walsh, *Horiz. Biochem. Biophys.*, **3**, 36 (1977).

(3) R. Wolfenden, *Annu. Rev. Biophys. Bioeng.*, **5**, 271 (1976).

(4) Inter alia: (a) carboxypeptidase A, J. A. Hartsuck and W. N. Lipscomb, In "The Enzymes", Volume 3, 3rd ed., P. D. Boyer, Ed., Academic Press, New York, 1977, p 1; (b) carboxypeptidase B, J. E. Folk, *ibid.*, p 57; (c) thermolysin, L. H. Weaver, W. R. Kester, and B. W. Matthews, *J. Mol. Biol.*, **114**, 119 (1977); (d) angiotensin-converting enzyme, M. J. Peach, *Physiol. Rev.*, **57**, 313 (1977); E. G. Erdös, *Am. J. Med.*, **60**, 749 (1976); (e) collagenase, E. D. Harris, Jr., and S. M. Krane, *New Eng. J. Med.*, **291**, 557, 605, 652 (1974).

(5) W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974).

(6) R. Breslow and D. L. Wernick, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 1303 (1977); W. R. Kester and B. W. Matthews, *Biochemistry*, **16**, 2506 (1977); D. S. Auld and B. Holmquist, *ibid.*, **13**, 4355 (1974). But see: M. Makinen, K. Yamamura, and E. T. Kaiser, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3882 (1976).

(7) P. A. Bartlett, J. T. Hunt, J. L. Adams, and J.-C. E. Gehret, *Bioorg. Chem.*, **7**, 421 (1978).

(8) A number of N- and O-protected phosphonamide analogues of di- and tripeptides have been synthesized,[9] but their evaluation as enzyme inhibitors has not been reported.

(9) (a) M. Hoffman and C. Wasielewski, *Rocz. Chem.*, **50**, 139 (1976); (b) K. Yamauchi, Y. Mitsuda, and M. Kinoshita, *Bull. Chem. Soc. Jpn.*, **48**, 3285 (1975), and references cited therein; (c) M. Hariharan, R. J. Motekaitis, and A. E. Martell, *J. Org. Chem.*, **40**, 470 (1975).

(10) (a) T. Komiyama, H. Suda, T. Aoyogi, T. Takeuchi, H. Umezawa, K. Fujimoto, and S. Umezawa, *Arch. Biochem. Biophys.*, **171**, 727 (1975); (b) B. Holmquist, *Biochemistry*, **16**, 4591 (1977); (c) C.-M. Kam, N. Nishino, and J. C. Powers, *ibid.*, **18**, 3032 (1979); (d) N. Nishino and J. C. Powers, *ibid.*, **18**, 4340 (1979).

(11) Melting points are uncorrected. UV spectra were determined with a Cary Model 219 UV spectrophotometer. Routine (60-MHz) ^{1}H NMR spectra were recorded on a Varian Associates T-60 spectrometer; high-field (250-MHz) FT ^{1}H NMR spectra were acquired on an instrument equipped with a Cryomagnet Systems magnet and Nicolet computer. Chemical shifts are reported in parts per million on the δ scale relative to internal tetramethylsilane (no internal reference in D_2O). Data are presented as follows: Chemical shift (multiplicity, number of protons, coupling constants in hertz). ^{13}C NMR spectra were acquired with a Nicolet TT23 spectrometer; chemical shifts are presented on the δ scale, referenced to $CDCl_3$ as 77.0 ppm or dioxane as 66.5 ppm in D_2O. ^{31}P NMR spectra were acquired at 72.9 MHz on an instrument equipped with a Bruker magnet and Nicolet computer; chemical shifts are reported as parts per million downfield from 85% H_3PO_4 (sealed capillary).

(12) D. Seyferth, R. Marmor, and P. Hilberg, *J. Org. Chem.*, **36**, 1379 (1971).

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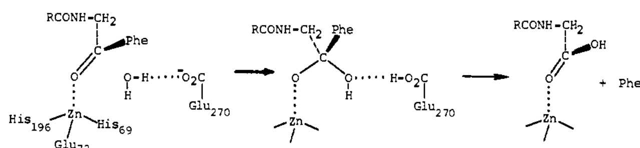

Figure 1. Proposed mechanism of peptide hydrolysis by CPA.[5,6]

d, 8, J_{HP} = 10 Hz), 5.07 (s, 2), 5.8–6.1 (br s, 1), 7.27 (s, 5) (lit.[9a] δ 3.37–3.75, 5.05, 5.87, 7.25).

A mixture of 19.9 g (72.8 mmol) of the crude dimethyl ester from above in 108 mL (0.22 mol) of 2.0 N NaOH was stirred at 21 °C for 45 min, washed with two 130-mL portions of $CHCl_3$ (emulsion), and acidified with 120 mL of 2 N H_2SO_4 in the presence of 250 mL of $CHCl_3$. After the mixture was partitioned, the aqueous layer was extracted twice more with 250 mL of $CHCl_3$, and the combined organic extracts were dried ($MgSO_4$) and evaporated to give 12.9 g (68% yield) of the monomethyl ester **2**, which solidified on standing: mp 101–103 °C; ^{1}H NMR ($CDCl_3$) δ 3.60 (d, 2, J_{HP} = 12 Hz), 3.67 (d, 3, J_{HP} = 12 Hz), 5.10 (s, 2), 7.35 (s, 5). An analytical sample was obtained on recrystallization from benzene; mp 106–106.5 °C. Anal. for C, H, N, and P.

***N*-[[[(Benzyloxycarbonyl)amino]methyl]methoxyphosphinyl]-L-phenylalanine methyl ester (3).** To a solution of 1.40 g (5.4 mmol) of monomethyl ester **2** in 10.5 mL of $CHCl_3$ was added 0.39 mL (5.4 mmol) of $SOCl_2$ at 21 °C. After 4 h, the solvent and volatile byproducts were removed under reduced pressure, and the last traces of SO_2 and HCl were swept out of the residue by dilution with 20 mL of $CHCl_3$ and reevaporation. A solution of the crude product in 10 mL of $CHCl_3$ was cooled to 0 °C and treated with a mixture of 1.16 g (6.48 mmol) of L-phenylalanine methyl ester and 0.90 mL (6.48 mmol) of triethylamine in 10 mL of $CHCl_3$. After 20 min at 21 °C, the mixture was diluted with 70 mL of $CHCl_3$, washed with 80-mL portions of 2 N H_2SO_4 and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated to give 1.79 g of a yellow oil. A 500-mg sample of this material was purified by chromatography on silica gel (8:92 ethanol/$CHCl_3$) to provide 390 mg (62% yield) of phosphonamidate **3** as a 3:2 mixture of diastereomers. Recrystallization from hexane/ethyl acetate did not alter the diastereomer ratio: mp 75–78 °C; ^{1}H NMR ($CDCl_3$) δ 2.98 (br s, 2), 3.38 (d, 1.2, J = 11 Hz), 3.41 (d, 1.8, J = 11 Hz), 3.67 (s, 3), 3.9–4.4 (m, 1), 5.02 (s, 2), 6.1 (br s, 1), 7.13 (s, 5), 7.25 (s, 5); ^{13}C NMR ($CDCl_3$) δ 37.9 (d, J_{CP} = 145 Hz), 39.9, 50.1 and 50.3, 51.5, 54.7 (d, J_{CP} = 7 Hz), 66.3, 126.4, 127.4, 127.9, 128.9, 136.1, 136.2, 155.8 and 156.1, 173.0 and 173.1; ^{31}P NMR ($CDCl_3$) δ 27.2 (major diastereomer), 27.9 (minor). Anal. for C, H, N, and P.

***N*-[[[(Benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine, dilithium salt, Li_2ZG^PP (4).** A solution of 1.86 g (4.42 mmol) of dimethyl ester **3** in 1.6 mL of acetonitrile was mixed with 8.9 mL of 1.5 M LiOH, and the reaction mixture was stirred at 21 °C for 24 h. The resulting paste was lyophilized to give 2.20 g of a white powder. A 400-mg sample of this crude product was applied to a 18.7 × 2.2-cm column of Bio-Rad AG1-X2 ion-exchange resin, 200–400 mesh, HCO_3^- form, and eluted with a 2300-mL linear gradient of 0–2.5 M triethylammonium bicarbonate at 2.0 mL/min. Fractions were monitored by A_{257} and inhibition of carboxypeptidase A. The fractions containing ZG^PP were pooled, 0.84 mL (1.23 × theory, based on UV absorbance) of 1.88 M LiOH was added, and the solution was lyophilized to provide 300 mg of Li_2ZG^PP (**4**) as a white powder: 250-MHz ^{1}H NMR (D_2O) δ 2.72 (AB part of ABX, 2, J_{AB} = 13.4 Hz, $J_{AX} \approx$ 7 Hz, $J_{BX} \approx$ 6 Hz, $\Delta\delta_{AB} \approx$ 0.09 ppm, $PhCH_2CH$), 2.9 (m, 2, NCH_2P), 3.61 (X part of ABX, 1, J_{HP} = 8.1 Hz, $PhCH_2CH$), 4.67 (s, HOD), 4.91 (s, 2, $PhCH_2O$), 7.11 (s, 5, ArH), 7.25 (s, 5, ArH); ^{13}C NMR (D_2O) δ 39.5 (d, J_{CP} = 136 Hz), 41.2 (d, J_{CP} = 6 Hz), 58.3, 67.0, 126.5, 127.8, 128.3, 128.4, 128.7, 129.5, 136.3, 138.5, 157.7, 181.5; UV (H_2O) λ_{max} 257 nm (ϵ 489, based on 86% purity determined by phosphorus analysis); $[\alpha]^{20}_D$ +4.2° (c 2.3, H_2O); ^{31}P NMR (D_2O) δ 18.9.

Acid-Catalyzed Hydrolysis of ZG^PP: Formation of Phenylalanine and *N*-(Carbobenzoxy)aminomethylphosphonic Acid. To a solution of 62 mg of the dilithium salt of ZG^PP (described above) in 5 mL of water was added 0.13 mL (1.7 equiv) of 2 N HCl. After 10 min at 21 °C, enzymatic assay indicated that no inhibitory activity remained. The pH of the solution was adjusted from 2.3 to 7.7 by using 2 N NaOH, and the mixture was separated by ion exchange on Bio-Rad, AG50W-X8, H^+ form (volume of resin bed = 12 mL). *N*-(Carbobenzoxy)aminomethylphosphonic acid was eluted with 25 mL of water and identified by ^{1}H NMR and TLC comparison with an authentic sample prepared by trimethylsilyl bromide cleavage[13] of the dimethyl ester: ^{1}H NMR (D_2O) δ 3.4 (d, 2, J_{HP} = 11.5 Hz), 4.7 (s, HOD), 5.0 (s, 2), 7.28 (s, 5). Phenylalanine was eluted with 25 mL of 4 M NH_4OH and identified with an authentic sample by ^{1}H NMR and TLC.

Hydrolytic Stability of ZG^PP. The hydrolysis of ZG^PP in pH 6.2 buffer at ambient temperature (21 °C) was followed spectrophotometrically at 230 nm. The rates at pH 7.5 and pH 8.5 were determined by enzymatic assay, monitoring loss of inhibitory activity, because the hydrolysis reaction is too slow to be monitored spectrophotometrically at these pHs. At 21 °C, the half-life of ZG^PP at pH 6.2 is 4 h and at pH 7.5 more than 8 days; it is stable indefinitely at pH 8.5 at 5 °C. The presence of 5.5 × 10^{-8} M CPA does not affect the rate of hydrolysis of 1.0 × 10^{-3} M ZG^PP at pH 7.5 (25 °C), as determined spectrophotometrically.

Enzyme Assays. All enzyme assays were performed at 25 °C by the method of Folk and Schirmer.[14] The data obtained by using BzGP as substrate ($\Delta\epsilon_{254}$ = +360) are displayed in Figure 2. The same method was employed with ZGGP as substrate, monitoring the decrease in absorbance at 228 nm ($\Delta\epsilon_{228}$ = −330 at pH 7.5)[15] (see Figure 3). Although data are presented in double-reciprocal form, kinetic parameters were obtained by the method of Eisenthal and Cornish-Bowden.[16] Hydrolysis

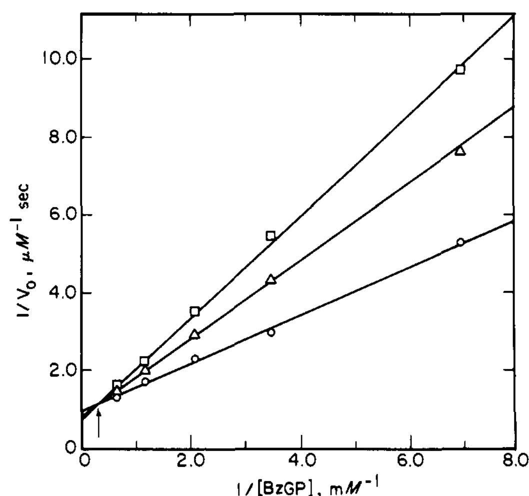

Figure 2. Double-reciprocal plot of the rate of CPA-catalyzed hydrolysis of BzGP at pH 7.5, 25.0 °C, as a function of substrate concentration in the absence (O) and presence of ZG^PP (△, 7.8 × 10^{-8} M; □, 15.1 × 10^{-8} M). Total enzyme concentration is 1.64 × 10^{-8} M. In the absence of ZG^PP, K_m = 0.69 mM and k_{cat} = 67 s^{-1}.

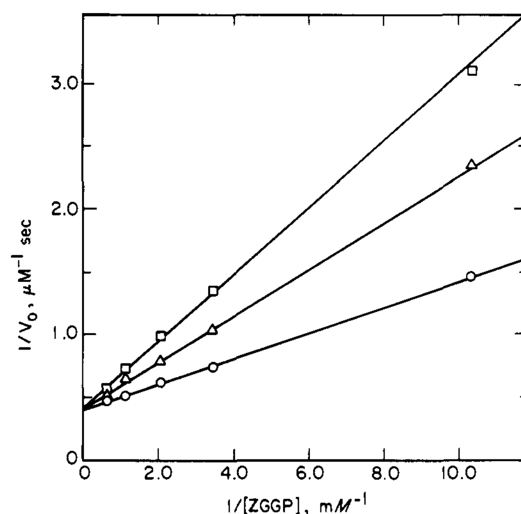

Figure 3. Double-reciprocal plot of the rate of CPA-catalyzed hydrolysis of ZGGP at pH 7.5, 25.0 °C, as a function of substrate concentration in the absence (O) and presence of ZG^PP (△, 7.8 × 10^{-8} M, □, 15.0 × 10^{-8} M). Total enzyme concentration is 1.65 × 10^{-8} M. In the absence of ZG^PP, K_m = 0.24 mM and k_{cat} = 149 s^{-1}.

(13) C. E. McKenna, M. T. Higa, N. H. Cheung, and M.-C. McKenna, *Tetrahedron Lett.*, 155 (1977).

(14) J. E. Folk and E. W. Schirmer, *J. Biol. Chem.*, **238**, 2884 (1963); L. A. Decker, "Worthington Enzyme Manual", Worthington Biochemical Corporation, Freehold, New Jersey, 1977 pp 209–212.

(15) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 602 (1970).

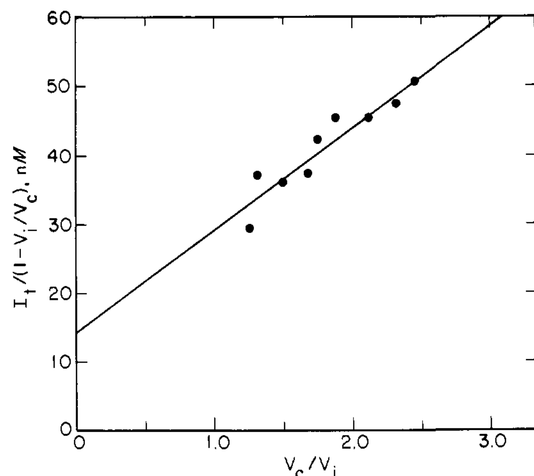

Figure 4. Henderson plot of the inhibition of CPA-catalyzed hydrolysis of ZGGP by ZG^PP at pH 6.0 and 25 °C. I_t is the total concentration of added ZG^PP, V_C is the initial velocity in the absence of inhibitor, and V_i is the initial velocity in the presence of inhibitor. Total enzyme concentration is 1.52×10^{-8} M and substrate concentration is 0.48 mM.

Table I. Reversibility of Inhibition

solns combined[a]	activity obsd[b] (% of control) immediately	after 4 days
EI + S	7.13 (77%)	7.01 (85%)
E + SI	6.88 (74%)	6.46 (78%)
E + S (control)	9.31 (100%)	8.23 (100%)

[a] Solutions described in Materials and Methods. [b] Expressed in units of $\mu M\ s^{-1}$.

of ZGGP at pH 6.0 was followed spectrophotometrically at 228 nm, but because of inhibitor depletion, the method of Henderson[17] was utilized to compute K_i under these conditions (see Figure 4).

Reversibility of Inhibition. To 2.8 mL of 7.07×10^{-6} M CPA in 10% LiCl was added 0.22 mL of 4.8×10^{-4} M ZG^PP in pH 7.5 buffer; this solution is designated EI ($[CPA]_t = 6.55 \times 10^{-6}$ M, $[ZG^PP] = 3.5 \times 10^{-5}$ M). A similar solution was prepared without inhibitor and designated solution E ($[CPA]_t = 6.55 \times 10^{-6}$ M). Substrate solutions in pH 7.5 buffer were prepared both with and without inhibitor: solution S ($[BzGP] = 1.0 \times 10^{-3}$ M), and solution SI ($[BzGP] = 1.0 \times 10^{-3}$ M, $[ZG^PP] = 2.9 \times 10^{-7}$ M). The degree of inhibition was determined for two combinations: 25 μL of solution EI and 3.0 mL of solution S; 25 μL of solution E and 3.0 mL of solution SI. In both cases the final concentrations were $[CPA]_t = 5.41 \times 10^{-8}$ M, $[BzGP] = 0.99 \times 10^{-3}$ M, and $[ZG^PP] = 2.9 \times 10^{-7}$ M. The rate observed on addition of 25 μL of solution E to 3.0 mL of solution S served as the control. These assays were performed immediately after preparation of solutions E and EI and again after storing them at 5 °C for 4 days. The results are presented in Table I.

Effect of Added Zinc on Inhibition by ZG^PP. Zinc chloride (3.45×10^{-5} M) was added to a solution of substrate or substrate plus inhibitor before the addition of enzyme. The results are presented in Table II.

Results and Discussion

Synthesis of ZG^PP. The steps leading to the dimethyl ester 3 are all precedented, notably in the work of Hariharan, Motekaitis, and Martell[9c] and Yamauchi, Mitsuda, and Kinoshita.[9b] Ester hydrolysis of phosphonamidate peptide analogues and enzymatic assay of the products have not to our knowledge been reported previously, however. Alkaline hydrolysis of 3 involves rapid loss of the carboxyl methyl ester and slower cleavage of the phosphonamidate ester. In a model study, under conditions in which the carboxylic ester of Cbz-phenylalanine methyl ester is completely hydrolyzed in 30 min, the phosphonamidate ester of methyl N-benzylmethylphosphonamidate requires 24 h for 80% cleavage (Me_2SO-d_6, D_2O, LiOD, monitored by ^1H NMR).

ZG^PP was purified by ion-exchange chromatography, eluting with triethylammonium bicarbonate buffer, which can be removed

(16) R. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, **139**, 715 (1974).
(17) P. Henderson, *Biochem. J.*, **127**, 321 (1972).

Scheme I

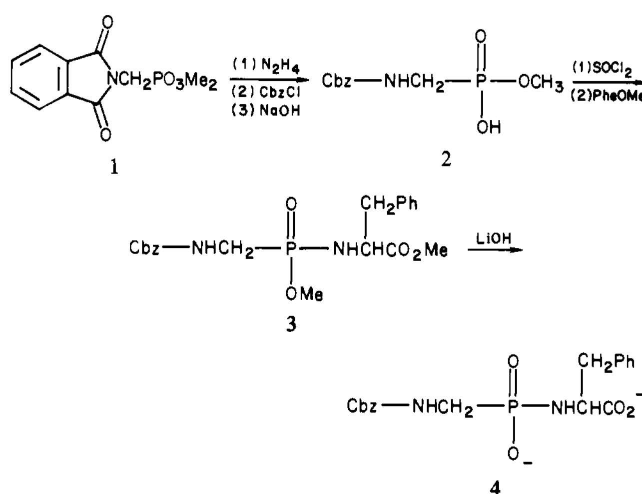

Table II. Effect of Added Zinc on Inhibition

reaction components[a]	activity obsd[b] (% of control) without added Zn	with 3.45×10^{-5} M $ZnCl_2$
CPA + BzGP (control)	1.38 (100%)	1.23 (100%)
CPA + BzGP + 6.9×10^{-7} M ZG^PP	0.565 (41%)	0.513 (42%)
CPA + BzGP + 14×10^{-7} M ZG^PP	0.396 (29%)	0.375 (30%)

[a] $[CPA]_t = 3.6 \times 10^{-8}$ M; $[BzGP] = 1.0 \times 10^{-3}$ M; pH 7.5, 25 °C. [b] Expressed in units of $\mu M\ s^{-1}$.

on lyophilization. We found it necessary to add a slight excess of lithium hydroxide to the product-containing fractions before lyophilization. Otherwise, loss of triethylamine from the phosphonamidate salt results in acidification and subsequent hydrolysis of the phosphonamide linkage during lyophilization. The dilithium salt afforded by our procedure is a nonhygroscopic, white powder. Attempts to prepare a water-insoluble salt for purification of ZG^PP by recrystallization have so far been unsuccessful.

Because of salts present in this material, a satisfactory combustion analysis was not obtained. Phosphorus analysis indicated that the material we obtained is 86% pure dilithium salt by weight. High-resolution ^1H, ^{13}C, and ^{31}P NMR spectra are entirely consistent with the assigned structure and have confirmed the absence of significant impurities in the preparation. Further characterization involved the hydrolysis of ZG^PP in dilute HCl to give phenylalanine and $CbzNHCH_2PO_3H_2$. This hydrolysis is accompanied by complete loss of inhibitory activity.

Stability of the Phosphonamidate Linkage. Although ZG^PP is hydrolyzed in minutes at pH 2.3, it is sufficiently stable at pHs above 5 that enzymatic assay of its inhibition is not complicated by its decomposition. Moreover, at pH 8.5, stock solutions appear to be stable indefinitely at 5 °C. Not surprisingly, cleavage of the phosphonamide linkage is not catalyzed by carboxypeptidase A.

ZG^PP as an Inhibitor of BzGP Hydrolysis by CPA. Our initial evaluation of ZG^PP as an inhibitor of CPA involved the use of benzoylglycyl-L-phenylalanine (BzGP) as substrate; however, the behavior which we observed is inconsistent with simple competitive, noncompetitive, or mixed inhibition. At moderate inhibitor concentrations, the Lineweaver-Burke plot shows a point of intersection to the right of the vertical axis (see Figure 2); at high inhibitor concentrations ($[ZG^PP] = 4K_i$ and $6K_i$), pronounced curvature is discernible, even in the substrate range within which BzGP is reputed to follow Michaelis-Menton kinetics.[18] To fully characterize this behavior will require further study, but the

(18) B. L. Vallee, J. F. Riordan, J. L. Bethune, T. L. Coombs, D. S. Auld, and M. Sokolovsky, *Biochemistry*, **7**, 3547 (1968), and references cited therein.

Table III. Competitive Inhibitors of CPA

compd	K_i, nM (pH 7.5)	ref
β-phenylpropionate	6200	19
$^{2-}O_3P$-L-Phe	5000	10c
L-benzylsuccinate	450	22
4 (ZG^PP)	90	this work
2-benzyl-3-mercaptopropionate	11	20

existence of ancillary binding sites for both inhibitors and activators of dipeptide hydrolysis is well documented for CPA.[18]

In spite of the anomalous kinetic behavior observed with BzGP, an inhibition constant of 9.4×10^{-8} M can be computed from the data of Figure 2, in good agreement with that obtained with carbobenzoxyglycylglycyl-L-phenylalanine (ZGGP) as substrate (see below). With BzGP we also demonstrated that inhibition caused by ZG^PP is rapid and reversible. Its onset showed no lag time, and an enzyme preparation stored for 4 days at 5 °C in the presence of a saturating concentration of inhibitor exhibited no irreversible inhibition on dilution (see Table I).

ZG^PP is a polydentate ligand, and other phosphonate derivatives are known to be good chelating agents. However, the addition of up to a 50-fold excess of zinc chloride to the substrate/inhibitor solution before addition of CPA did not diminish the inhibition due to ZG^PP (see Table II). This molecule therefore does not simply sequester zinc from the active site of the enzyme.

ZG^PP as an Inhibitor of Carbobenzoxyglycylglycyl-L-phenylalanine Hydrolysis by CPA. In contrast to BzGP, the tripeptide carbobenzoxyglycylglycyl-L-phenylalanine (ZGGP) exhibits Michaelis–Menton kinetics as a substrate for CPA,[15] presumably because it occupies enough of the active site region of the enzyme to preclude nonproductive binding or the simultaneous binding of activators or inhibitors. With ZGGP as substrate at pH 7.5, ZG^PP exhibits potent, competitive inhibition ($K_i = 9 \times 10^{-8}$ M; see Figure 3), with no anomalous behavior. In comparison to other inhibitors of CPA (see Table III), ZG^PP is bound more tightly than all but the thiol-containing compound of Ondetti, Cushman, et al.[20] Of particular interest is the contrast with *N*-phosphoryl-L-phenylalanine, which is bound significantly less tightly than ZG^PP.[10c] The special affinity of ZG^PP for the active site of CPA resides in the ((carbobenzoxy)amino)methyl moiety, which presumably occupies the S_1 and S_2 binding sites. The ability to incorporate such residues in the phosphonamidate structure is a significant advantage of this approach over the use of simple *N*-phosphoryl amino acids.

To what extent can ZG^PP be considered a transition state analogue? An X-ray crystallographic study of the complex formed between phosphoramidon (the rhamnoside of *N*-phosphoryl-L-leucyl-L-tryptophan[10a]) and the zinc peptidase thermolysin has confirmed that this inhibitor binds in the same location and orientation as an oligopeptide substrate, with the tetrahedral phosphoryl group occupying the position of the scissile peptide carbonyl.[4c] Phosphoramidon and other phosphoramidate inhibitors[10d] of thermolysin are therefore held to be transition-state analogues. In view of the similarity of active sites[21] and inhibitor structures, it is reasonable to expect that CPA binds ZG^PP and *N*-phosphoryl-L-phenylalanine analogously,[10c,d] except with regard to ionization state. Whereas phosphoramidates exist as N-protonated zwitterions at pH 7.5 (pK_a of $PhCH_2N^+H_2PO_3^{2-}$ = 9.1[23]), the phosphonamidate moiety is expected to be a simple monoanion at this pH (for comparison, pK_a of $^+NH_3PO_3^-CH_3$ = 2.5[24]). We found no titratable proton in the pH range 4.5–10 for ZG^PP.

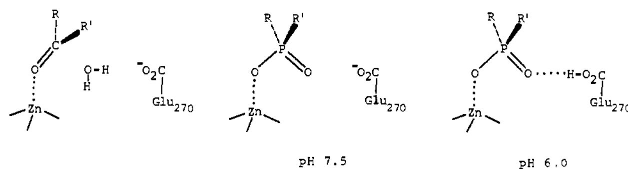

Figure 5. Comparison of substrate and ZG^PP binding at pH 7.5 and 6.0.

Comparison of *N*-phosphoryl-L-phenylalanine with L-benzylsuccinate[22] and β-phenylpropionate[19] (see Table III) indicates that CPA has no special affinity for the tetrahedral phosphorus moiety per se. We suggest an explanation for this by reference to Figure 5. Whereas substrates and inhibitors such as L-benzylsuccinate can bind to the enzyme without displacing the water molecule hydrogen bonded to the Glu_{270}-carboxylate, one of the oxygens of the phosphoryl group must occupy that region of the active site. Replacing this water molecule with the unprotonated phosphoryl oxygen leads to an unfavorable decrease in solvation of this carboxylate.

The pH dependence of CPA-catalyzed peptide hydrolysis shows an inflection at pH 6.2, which has been interpreted as the pK_a of the Glu_{270}-carboxyl group.[25] Protonation of that moiety would convert an unfavorable interaction of two oxygens with negative character in the complex with ZG^PP into a more favorable hydrogen bond, as depicted in Figure 5. In fact, we find that ZG^PP is bound an order of magnitude more tightly at pH 6.0 than it is at pH 7.5: $K_i = 6 \times 10^{-9}$ M at pH 6.0 (Figure 4). A Henderson plot[17] was utilized to determine K_i at pH 6.0, because a significant proportion of ZG^PP is bound to CPA at concentrations appropriate for enzyme assay at this pH.

Although the 15-fold binding enhancement observed with ZG^PP on going from pH 7.5 to pH 6.0 is consistent with the interpretation advanced above, the issue is clearly complicated by the fact that L-benzylsuccinate is also bound more tightly at lower pH.[22] This may be a feature of the binding of all inhibitors to CPA which is unrelated to the interactions postulated in Figure 5. The synthesis of inhibitors which can provide a hydrogen-bonding interaction with the Glu_{270}-carboxylate independently of pH would help to answer this question and is a goal which we are currently pursuing.

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(19) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **178**, 645 (1949).
(20) M. A. Ondetti, M. E. Condon, J. Reid, E. F. Sabo, H. S. Cheung, and D. W. Cushman, *Biochemistry*, **18**, 1427 (1979).
(21) W. R. Kester and B. W. Matthews, *J. Biol. Chem.*, **252**, 7704 (1977).
(22) L. D. Byers and R. Wolfenden, *Biochemistry*, **12**, 2070 (1973).
(23) S. J. Benkovic and E. J. Sampson, *J. Am. Chem. Soc.*, **93**, 4009 (1971).
(24) I. Öney and M. Caplow, *J. Am. Chem. Soc.*, **89**, 6972 (1967).
(25) D. S. Auld and B. L. Vallee, *Biochemistry*, **10**, 2892 (1971).