5.3 Hz, $J_{BX}$ = 5.3 Hz, $\Delta\nu_{AB}$ = 14.2 Hz, 2 H, $CH_2OH$), 3.57 (m, 1 H, CHOTBS), 2.05 (m, 1 H, $O_2CCH_2CH_{ax}$), 1.85-1.25 (m, 16 H), 1.18 (d, $J$ = 6.0 Hz, 3 H, $CH_3CHOTBS$), 0.93 (d, $J$ = 7.2 Hz, 3 H, $CH_3CHCH_2CH_2$), 0.91 (t, $J$ = 7.4 Hz, 3 H, $CH_2CH_3$), 0.88 (s, 9 H, $SiC(CH_3)_3$), 0.84 (d, $J$ = 6.9 Hz, 3 H, $CH_3CHCHOH$), 0.05 (s, 6 H, $Si(CH_3)_2$); $^{13}C$ NMR (125.8 MHz, $CDCl_3$) $\delta$ 97.5, 71.2, 69.2, 67.1, 67.1, 64.7, 43.2, 42.1, 38.9, 38.0, 29.8, 29.7, 29.5, 27.4, 26.3, 25.8, 24.3, 23.3, 18.0, 11.0, 10.9, 4.0, -4.5, -4.6. Anal. Calcd for $C_{26}H_{52}O_5Si$: C, 66.05; H, 11.09. Found: C, 66.04; H, 10.95.

**(2*S*,3*S*,4*S*,6*R*,8*S*,9*S*)-3,9-Dimethyl-8-[(*R*)-2-hydroxy-1-propyl]-2-[(*R*)-3-(hydroxymethyl)-1-pentyl]-1,7-dioxaspiro[5.5]undecan-4-ol (4).** A 48% aqueous solution of hydrofluoric acid (157 $\mu$L) was added to a solution of 74 mg (0.157 mmol) of silyl ether **23** in 1.57 mL of $CH_2Cl_2/CH_3CN$ (1:1) at ambient temperature. After stirring for 7 h, 2 mL of saturated aqueous $NaHCO_3$ was added. The resulting mixture was diluted with 10 mL of $H_2O$ and extracted with three 15-mL portions of EtOAc. The combined organic extracts were dried ($Na_2SO_4$) and purified by flash chromatography (19:1 $CHCl_3$/MeOH) to give 51.6 mg (92%) of **4** as a white foam: $[\alpha]^{24}_D$ -95.1° (*c* 0.225, $CHCl_3$) {lit.[1e] $[\alpha]_D$ -88.6° (*c* 1.10, $CHCl_3$)}; IR (neat) 3360, 2970, 1460, 1385, 1100, 1080, 1055, 1030, 970 $cm^{-1}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 4.17 (dt, $J$ = 11.8 Hz, $J$ = 4.8 Hz, 1 H, CHCHOH), 4.07 (br d, $J$ = 10.8 Hz, 1 H, one of CHO), 4.00 (m, 1 H, one of CHO), 3.71 (m, 1 H, one of CHO), 3.55 (AB of ABX, $J_{AB}$ = 10.8 Hz, $J_{AX}$ = 5.8 Hz, $J_{BX}$ = 4.7 Hz, $\Delta\nu_{AB}$ = 46.7 Hz, 2 H, $CH_2OH$), 2.25 (br s, 3 H, OH), 2.13 (m, 1 H, $O_2CCH_2CH_{ax}$), 1.84-1.21 (m, 16 H), 1.21 (d, $J$ = 6.2 Hz, 3 H, $CH_3CHOH$), 0.92 (d, $J$ = 7.0 Hz, 3 H, $CH_3CHCH_2CH_2$), 0.91 (t, $J$ = 7.4 Hz, 3 H, $CH_2CH_3$), 0.84 (d, $J$ = 6.9 Hz, 3 H, $CH_3CHCHOH$); $^{13}C$ NMR (100.6 MHz, $CDCl_3$) $\delta$ 97.5, 71.3, 67.4, 67.4, 64.7, 64.7, 42.5, 42.1, 39.1, 37.8, 30.8, 29.8, 29.2, 26.8, 26.6, 24.5, 23.5, 11.3, 11.2, 3.9. Anal. Calcd for $C_{20}H_{38}O_5$: C, 67.00; H, 10.68. Found: C, 66.83; H, 10.57.

**Acknowledgment.** Support has been provided by the National Institutes of Health, Eli Lilly, and Merck. The NIH BRS Shared Instrumentation Grant Program 1 S10 RR01748-01A1 is acknowledged for providing NMR facilities. D.L.R. and T.K.J. acknowledge support as American Cancer Society and NIH postdoctoral fellows, respectively.

# Potent Inhibition of Pepsin and Penicillopepsin by Phosphorus-Containing Peptide Analogues

Paul A. Bartlett,* John E. Hanson, and Peter P. Giannousis

*Department of Chemistry, University of California, Berkeley, California 94720*

*Received March 7, 1990*

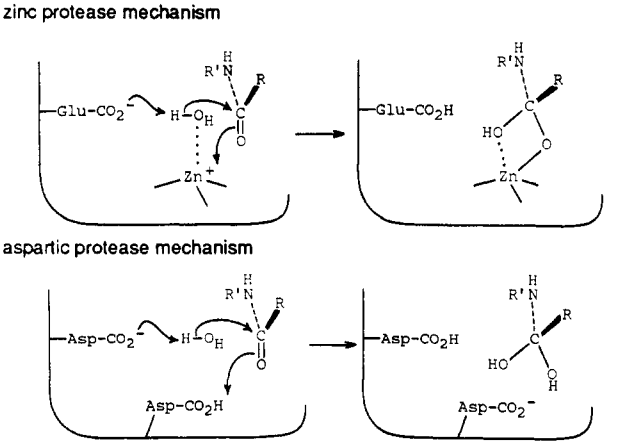
Phosphinic and phosphonic acid peptide derivatives have been evaluated as inhibitors of the aspartic proteases pepsin and penicillopepsin. The most potent of those studied is isovaleryl-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (**4**) (Leu$^P$ represents the phosphonic acid analogue of leucine; (O)Phe represents L-$\beta$-phenyllactic acid, the alcohol analogue of phenylalanine), for which the $K_i$ values for pepsin and penicillopepsin are 0.26 and 0.19 nM, respectively. While this compound binds to penicillopepsin with an association rate constant, $k_{on}$, of $(6.5 \pm 1.5) \times 10^5$ $M^{-1}$ $s^{-1}$, it does not show slow- or two-step binding with pepsin. The binding of Cbz-Ala-Ala-Leu$^P$-(O)Phe-OMe (**1**) to penicillopepsin is strongly dependent on pH: in comparison to pH 4.5, the affinity at pH 3.5 is increased 10-fold and at pH 5.5 it is decreased 40-fold. The two diastereomers of a nonionic phosphinamide analogue (**10A, 10B**) of a statine-containing inhibitor were prepared; however, both are significantly weaker inhibitors of pepsin than the phosphinic acid itself (**7**).

## Introduction

Although the zinc proteases and the aspartic proteases differ in many respects, they are related by mechanism: both catalyze the direct addition of water to the amide linkage, with formation of tetrahedral intermediate as the critical step in hydrolysis of an oligopeptide substrate (Scheme I).

Both mechanisms involve basic catalysis from a carboxylate side chain and acidic or Lewis acidic catalysis from another functional group in the active site. Moreover, both types of enzymes are inhibited by electrophilic keto analogues of substrates[1,2] because of the ability of these compounds to undergo hydration to form a mimic of the tetrahedral intermediate.[3,4] X-ray crystallographic analysis of representative enzymes from both classes provides support for these mechanistic interpretations, as well as detailed pictures of the configurations of the active sites and the orientation of substrates and analogues when

**Scheme I**




(1) Aspartic proteases: (a) Holladay, M. W.; Salituro, F. G.; Rich, D. H. *J. Med. Chem.* **1987**, *30*, 374-383. (b) Rich, D. H.; Boparai, A. S.; Bernatowicz, M. S. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 1127-1133. (c) Szelke, M. In *Aspartic Proteases and Their Inhibitors*; Kostka, V., Ed.; de Gruyter: Berlin, 1985; pp 421-441. (d) Gelb, M. H.; Svaren, J. P.; Abeles, R. H. *Biochemistry* **1985**, *24*, 1813-1817. (e) Tarnus, C.; Jung, M. J.; Rémy, J.-M.; Baltzer, S.; Schirlin, D. G. *FEBS Lett.* **1989**, *249*, 47-50.

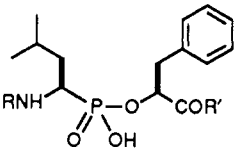
(2) Zinc proteases: (a) Galardy, R. E.; Kortylewicz, Z. P. *Biochemistry* **1985**, *24*, 7607-7612. (b) Grobelny, D.; Goli, U. B.; Galardy, R. E. *Ibid.* **1985**, *24*, 7612-7617. (c) Galardy, R. E.; Kortylewicz, Z. P. *Ibid.* **1984**, *23*, 2083-2087.

(3) Maibaum, J.; Dhaon, M. K.; Schmidt, P. G.; Oka, M.; Rich, D. H. *Proc. Am. Pept. Symp.* **1988**, *10*, 338-340. Rich, D. H.; Bernatowicz, M. S.; Schmidt, P. G. *J. Am. Chem. Soc.* **1982**, *104*, 3535-3536.

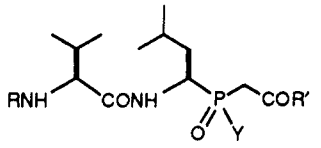
(4) (a) Christianson, D. W.; Lipscomb, W. N. *Proc. Natl. Acad. Sci., U.S.A.* **1985**, *82*, 6840-6844; (b) *J. Am. Chem. Soc.* **1986**, *108*, 4998-5003. (c) Christianson, D. W.; David, P. R.; Lipscomb, W. N. *Proc. Natl. Acad. Sci., U.S.A.* **1987**, *84*, 1512-1515.

0022-3263/90/1955-6268$02.50/0 

they are bound.[5,6] Because of the mechanistic parallels between these classes of enzymes, we investigated the inhibition of representative aspartic proteases by phosphorus-containing peptide analogues, which are potent, transition-state analogue inhibitors of the zinc proteases.[7] The efficacy of these inhibitors against the latter enzymes is rationalized on the basis of their mimicry of the tetrahedral intermediate, and similar arguments suggest that related compounds will be good inhibitors of the aspartic proteases. We have reported previously the synthesis of peptide derivatives incorporating the phosphinic acid analogue of statine (e.g., **7**) and the evaluation of these compounds as inhibitors of pepsin.[8] Allen et al. have recently described the synthesis of analogous and homologous phosphonic and phosphinic acid peptides as renin inhibitors.[9] We now report the extension of these studies in several directions: the synthesis of phosphonic acid derivatives **1–4** that are closely analogous to peptide substrates; the synthesis of the diastereomeric phosphinamides **10A**, **10B** as neutral, hydrogen-bond-donating analogues of **7**;[10] and the evaluation of these compounds as inhibitors of the bacterial protease penicillopepsin as well as the mammalian digestive enzyme pepsin.



**1**: R = Cbz-Ala-Ala, R′ = OMe
**2**: R = Cbz-Ala-Ala, R′ = Ala-Ala-OMe
**3**: R = Iva-Val-Val, R′ = OMe
**4**: R = Iva-Val-Val, R′ = Ala-Ala-OMe



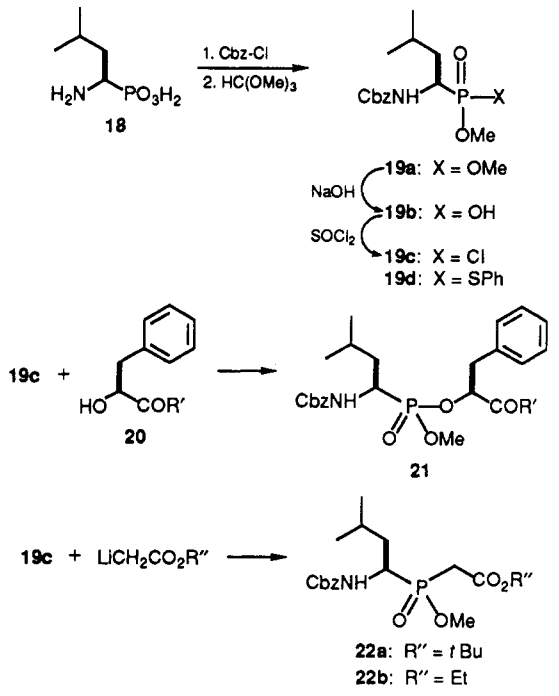
| | | | |
|---|---|---|---|
| **5**: R = Iva, | R′ = OEt, | Y = OH |
| **6**: R = Iva-Val, | R′ = OEt, | Y = OH |
| **7**: R = Iva, | R′ = Ala-Iaa, | Y = OH |
| **8**: R = Iva, | R′ = Ala-Iaa, | Y = $NH_2$ |
| **9**: R = Iva-Val, | R′ = OEt, | Y = $NH_2$ |
| **10A/10B**: R = Iva, | R′ = Ala-Iaa | Y = $NH_2$ |

Iva = isovaleryl; Iaa = isoamylamide

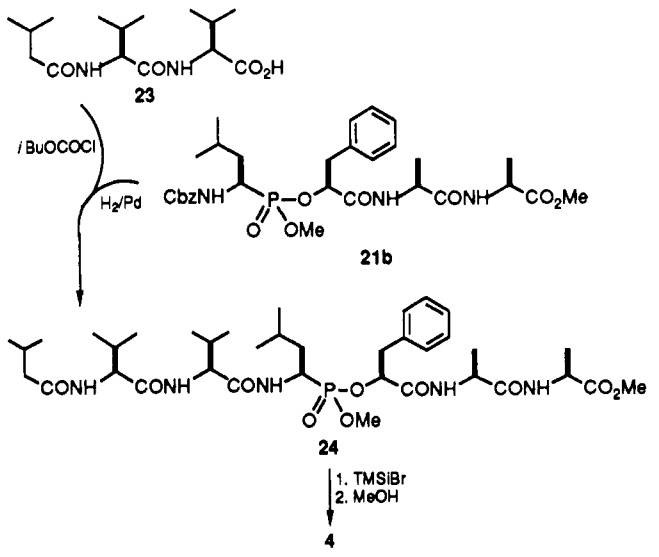
## Results and Discussion

**Synthesis of Inhibitors. Phosphonate Esters.** The optically active Leu$^P$ derivative **19b** served as the key intermediate for synthesis of both the phosphonate ester inhibitors **1–4** as well as the phosphinic acid derivatives **5–10**. The phosphonic acid derivative corresponding to L-leucine, **18**, was synthesized and resolved as described by Oleksyszyn et al. and Kafarski et al.[11] and converted to the N-protected monomethyl ester **19b** in a straightforward manner (Scheme II). This material was converted to the corresponding acid chloride **19c** and coupled either with a derivative of L-β-phenyllactic acid (**20**) for synthesis of the phosphonates **1–4** (yields 60–70%) or with the enolate of an acetate ester to provide the "phosphastatine" analogues **22** (yields 60–90%).[8] The subsequent stages in the syntheses of phosphonates **1–4** were straightforward, culminating in selective deprotection of the phosphonate methyl ester with trimethylsilyl bromide.[12] The full

**Scheme II**



**Scheme III**



(5) Aspartic proteases: (a) James, M. N. G.; Sielecki, A. R. *Biochemistry* **1985**, *24*, 3701–3713. (b) Suguna, K.; Padlan, E. A.; Smith, C. W.; Carlson, W. D.; Davies, D. R. *Proc. Natl. Acad. Sci., U.S.A.* **1987**, *84*, 7009–7013. (c) Cooper, J. B.; Foundling, S. I.; Blundell, T. L.; Boger, J.; Jupp, R. A.; Kay, J. *Biochemistry* **1989**, *28*, 8596–8603. (d) Blundell, T. L.; Cooper, J.; Foundling, S. I.; Jones, D. M.; Atrash, B.; Szelke, M. *Ibid.* **1987**, *26*, 5585–5590. (e) Bott, R.; Subramanian, E.; Davis, D. R. *Ibid.* **1982**, *21*, 6956–6962. (f) James, M. N. G.; Sielecki, A.; Salituro, F.; Rich, D. H.; Hofmann, T. *Proc. Natl. Acad. Sci., U.S.A.* **1982**, *79*, 6137–6141.

(6) Zinc proteases: (a) Hangauer, D. G.; Monzingo, A. F.; Matthews, B. W. *Biochemistry* **1984**, *23*, 5730–5741. (b) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62–69. (c) Holden, H. M.; Tronrun, D. E.; Monzingo, A. F.; Weaver, L. H.; Matthews, B. W. *Biochemistry* **1987**, *26*, 8542–8553, and references therein. (d) Christianson, D. W.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1988**, *110*, 5560–5565, and references therein.

(7) (a) Hanson, J. E.; Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1989**, *28*, 6294–6305. (b) Bartlett, P. A.; Marlowe, C. K. *Ibid.* **1987**, *26*, 8553–8561, and references therein.

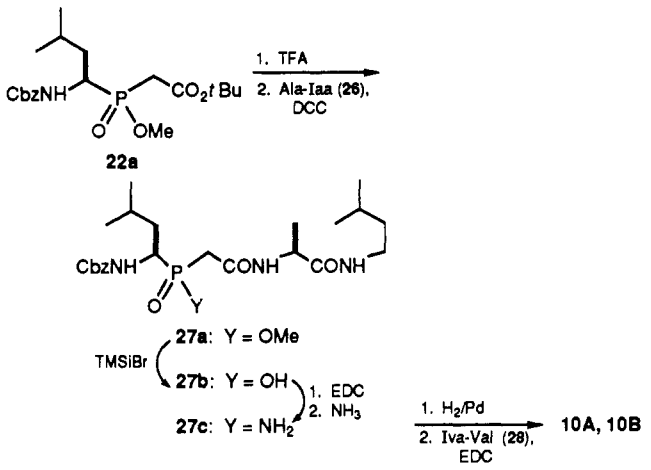
(8) (a) Bartlett, P. A.; Kezer, W. B. *J. Am. Chem. Soc.* **1984**, *106*, 4282–4283. (b) Kezer, W. B. Ph.D. Dissertation, University of California, Berkeley, 1983.

(9) Allen, M. C.; Fuhrer, W.; Tuck, B.; Wade, R.; Wood, J. M. *J. Med. Chem.* **1989**, *32*, 1652–1661.

(10) Bartlett, P. A.; Acher, F. *Bull. Soc. Chim. Fr.* **1986**, 771–775.

(11) (a) Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synthesis* **1979**, 985–986. (b) Kafarski, P.; Lejczak, B.; Szewczyk, J. *Can. J. Chem.* **1983**, *61*, 2425–2430.

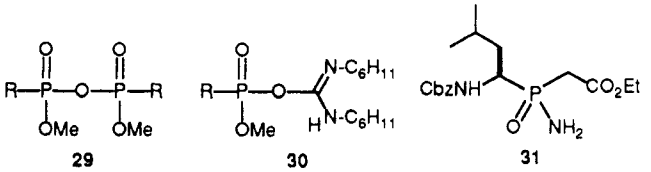
Scheme IV




procedure for synthesis of the hexapeptide analogue 4 (Scheme III) is given in the Experimental Section.[13]

**Phosphinic Acid Derivatives.** The synthesis outlined here for the preparation of the phosphastatine derivatives **22** differs from the route we reported earlier[8] in that we were successful in carrying out the alkylation reaction directly on the phosphonochloridate **19c**, as opposed to the *O*-methyl *S*-phenyl thiophosphonate **19d** and because we employed L- (as opposed to racemic) phosphonate. For the latter reason, we were able to verify the stereochemical assignment of **22a** made previously on the basis of inhibitor potency.[8] The syntheses of inhibitors **5–8** from the phosphastatine diesters **22** were also straightforward.

We have reported previously a synthesis of a mixture of diastereomers of phosphinamide **10**; this route involved reaction of the phosphinic acid **27b** (prepared from racemic phosphonate) with diphenyl phosphorazidate and then ammonia.[10] However, since this transformation proved to be capricious when applied to the less soluble single diasteromer of **27b**, we studied additional methods for accomplishing this conversion. Our earlier work had demonstrated that the phosphinamide moiety is stable to the conditions for removal of a carbobenzoxy group and for aminoacylation, so we focused on the preparation of intermediate **27c**. We found that dicyclohexylcarbodiimide (DCC) and ethyl[(dimethylamino)propyl]carbodiimide (EDC) are effective activating agents for phosphinic and monoalkyl phosphonic acid moieties, producing intermediates that react with ammonia to give the corresponding phosphorus amides in modest yield (Scheme IV). From model studies with racemic methyl phosphonate **19b** and DCC, several pieces of evidence suggest that the activated intermediate is the phosphorus anhydride **29** as opposed to an *O*-phosphorylisourea such as **30**. First, precipitation




of dicyclohexylurea begins during the activation process, prior to addition of ammonia. Second, observation of the reaction mixture by $^{31}P$ NMR at this stage reveals a multitude of peaks in the region 20–22 ppm, which is consistent with the formation of diastereomeric anhydrides and the resultant phosphorus–phosphorus spin–spin coupling. Finally, the maximum conversion observed was 50%, even with a considerable excess of carbodiimide.

Application of this reaction to the phosphinic acid **27b** provided the phosphinamide **27c** in 40% isolated yield. Although we could separate the two diastereomers of this material by reverse-phase chromatography, and therefore carry them on to the two diastereomers **10A** and **10B**, we have not yet been able to assign their configurations at phosphorus.[14] The synthesis of the phosphinamide **9** was similar in that the amide was introduced prior to coupling of the N-terminal moiety. In this case, however, the absence of amide bonds in the phosphinic acid intermediate **22b** allowed us to carry out the activation with thionyl chloride to give phosphinamide **31** in 66% yield after reaction with ammonia. The diastereomers of this derivative were not separated but carried on directly to **9**.

**Affinity of Phosphonate Analogues.** In our previous investigation of phosphorus-containing peptide analogues as inhibitors of pepsin,[8] a phosphinate moiety was incorporated in place of the hydroxymethylene unit found in pepstatin and related inhibitors. Whether statine-containing inhibitors, and the phosphinate and keto analogues, are truly mimics of the tetrahedral intermediate is an arguable point, since statine derivatives are one atom short in comparison to the dipeptide unit they are considered to replace. Indeed, Rich has suggested that pepstatin is a multisubstrate as opposed to transition-state analogue inhibitor, occupying the binding sites of the water molecule and the ground-state form of the peptide substrate.[15] It was therefore of interest to determine whether analogues of normal length containing a tetrahedral phosphorus moiety would be similarly effective as inhibitors of aspartic proteases. A related study of renin inhibitors demonstrated that replacement of a substrate CO-NH group with the phosphinylmethylene ($PO_2^-$-$CH_2$) moiety can result in effective inhibitors.[9]

The results in Table I reveal that the phosphonates are potent inhibitors of pepsin and penicillopepsin, rivaling the analogous phosphinates[8] as well as the statine[16] and hydroxyethylene[1a] analogues that inhibit these enzymes. An interesting difference in specificity is revealed by comparison of the inhibition constants of the four phosphonate inhibitors with these two enzymes. Comparison of the Cbz-Ala-Ala-derivatives **1** and **2** (Table I) demonstrates that removal of the C-terminal alanyl-alanine residues results in a loss of binding affinity that is the same for both enzymes: a factor of 25 or 1.9 kcal/mol. A similar change is found for deletion of the C-terminal alanine residues from the Iva-Val-Val-inhibitors **3** and **4**: a factor of 14.5 or 1.6 kcal/mol. On the other hand, while replacement of the Iva-Val-Val units with Cbz-Ala-Ala leads to only a modest, 2–4-fold loss in affinity for inhibition of pepsin, it decreases the binding to penicillopepsin by 300- to 600-fold (comparison of **1** and **3** with **2** and **4**).[17] These results suggest that the structures of the carboxy terminal

(12) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-E. *Tetrahedron Lett.* **1977**, 155–158. McKenna, C. E.; Schmidhauser, J. *J. Chem. Soc., Chem. Commun.* **1979**, 739.

(13) Full details for the other inhibitors are provided in the supplementary material.

(14) We approached this stereochemical question with a model system, phosphonamidate **19** (X = $NH_2$), by attempting to form cyclic derivatives incorporating the two nitrogen atoms. A variety of routes to cyclic ureas, cyclic imines, and lactams were explored, but without success.

(15) Rich, D. H. *J. Med. Chem.* **1985**, *28*, 263–273.

(16) Salituro, F. G.; Agarwal, N.; Hofmann, T.; Rich, D. H. *J. Med. Chem.* **1987**, *30*, 286–295.

(17) Comparison of entries 11 and 13, Table I, reveals another significant difference between pepsin and penicillopepsin with respect to the importance of a group that can occupy the $S_4$-subsite.

**Table I. Inhibition of Pepsin and Penicillopepsin by Phosphonate**

| compd | structure[a] | inhibition constants, $K_i$ (nM) pepsin[b] | penicillopepsin[b] |
|---|---|---|---|
| | Phosphonate Analogues | | |
| 1 | Cbz-Ala-Ala-Leu$^P$-(O)Phe-OMe | 14 | 1600 |
| 2 | Cbz-Ala-Ala-Leu$^P$-(O)Phe-Ala-Ala-OMe | 0.55 | 65 |
| 3 | Iva-Val-Val-Leu$^P$-(O)Phe-OMe | 3.7 | 2.8 |
| 4 | Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe | 0.26 | 0.19 |
| | Phosphinate Pepstatin Analogues | | |
| 5 | Iva-Val-Sta$^P$-OEt | | 213000 (pH 3.5) |
| 6 | Iva-Val-Val-Sta$^P$-OEt | 207 | 22 (pH 3.5) |
| 7 | Iva-Val-Sta$^P$-Ala-Iaa | 3[c,d] | |
| 8 | Iva-Val-Sta$^{P(OMe)}$-Ala-Iaa | 12000 | |
| | Phosphinamide Pepstatin Analogues | | |
| 9 | Iva-Val-Val-Sta$^{P(NH_2)}$-OEt | 730<br>3300 (pH 4.5)<br>16500 (pH 5.5) | |
| 10a | Iva-Val-Sta$^{P(NH_2)}$-Ala-Iaa, isomer A | 19000[e] | |
| 10b | Iva-Val-Sta$^{P(NH_2)}$-Ala-Iaa, isomer B | 1600[b] | |
| | Statine-Containing, Hydroxyethylene, and Ketomethylene Analogues[f] | | |
| 11 | Iva-Val-Val-Sta-Ala-Iaa | 0.1 | 6.5 |
| 12 | Iva-Val-Val-Sto-Ala-Iaa | 10 | 80 |
| 13 | Iva-Val-Sta-Ala-Iaa | 3 | 7600 |
| 14 | Iva-Val-Val-Sta-Phe-OMe | | 1.3 |
| 15 | Iva-Val-Val-Sta-OEt | 10 | 47 |
| 16 | Iva-Val-Leu-[HE]-Ala-Ala-Iaa | 3 | |
| 17 | Iva-Val-Leu-[K]-Ala-Ala-Iaa | 27 | |

[a] Leu$^P$-(O)Phe represents replacement of the Leu-Phe peptide linkage with a phosphonate ester ($PO_2^-$-O); Sta$^P$ represents the phosphinic acid analogue of statine; Sta$^{P(OMe)}$ and Sta$^{P(NH_2)}$ represent the corresponding methyl ester and amide, respectively; Sto represents the keto analogue of statine; Iva = 3-methylbutanoyl (isovaleryl); Iaa = 3-methylbutylamide (isoamylamide); [HE] and [K] represent hydroxyethylene ($CHOHCH_2$) and ketomethylene ($COCH_2$) replacements for the peptide linkage, respectively. [b] Pepsin assays at pH 3.5, 37 °C; penicillopepsin assays at pH 4.5, 25 °C, unless otherwise indicated. [c] $K_i$ value given is $K_D$ of initially formed complex. [d] Previously reported as 7 nm.[8] [e] Value given is considered lower limit. [f] From refs 1a, 15, and 16.

substrate binding sites of pepsin and penicillopepsin are similar and those to the amino side of the scissile linkage are quite different.

**pH Dependence of Inhibition.** The active-site residues carry a net negative charge in the active form of the aspartic proteases; similarly, the phosphonate and phosphinate inhibitors are negatively charged under normal assay conditions, since their p$K_a$ values are ca. 1.5 and 3.0, respectively.[18] Close juxtaposition of a negatively charged phosphinyl moiety and an aspartate carboxylate in the active site would be expected to give rise to unfavorable electrostatic interactions opposing binding. Indeed, this effect has been invoked in explaining the modest affinity that related inhibitors have for renin, an aspartic protease active at neutral pH.[9] The p$K_a$ values of the inhibitors, as well as of the active-site aspartic acid side chains, are close enough to the pH of the assays to suggest that protonation of one or the other is required for binding a phosphorus-containing inhibitor. In view of the fact that the phosphinyl and carboxyl moieties are isolated from solvent in the enzyme–inhibitor complex, it is likely that their p$K_a$ values are significantly increased in comparison to the solution values for these groups.

**Table II. pH Dependence of Penicillopepsin Inhibition by Phosphonate and Phosphinate Peptide Analogues**

| entry | inhibitor | pH (buffer)[a] | $K_i$ (μM) |
|---|---|---|---|
| 1 | 1, Cbz-Ala-Ala-Leu$^P$-(O)Phe-OMe | 3.5 (F) | 0.19 ± 0.01 |
| 2 | | 4.5 (F) | 2.1 ± 0.1 |
| 3 | | 4.5 (A) | 2.7 ± 0.1 |
| 4 | | 5.5 (A) | 107 ± 5 |
| 5 | 6, Iva-Val-Val-Sta$^P$-OEt | 3.5 (A′) | 0.022 ± 0.001 |
| 6 | | 4.5 (A′) | 0.107 ± 0.008 |

[a] F (sodium formate) and A (sodium acetate), buffer concentration of 0.05 M with ionic strength adjusted to 0.05 with NaCl; A′ (sodium acetate), buffer concentration of 0.10 M.

The observed affinity of Cbz-Ala-Ala-Leu$^P$-(O)Phe-OMe for penicillopepsin is strongly dependent on pH (Table II), increasing by an order of magnitude at pH 3.5 and decreasing by a factor of 40 at 5.5, in comparison to the standard assay pH of 4.5 for this enzyme. The phosphinate Iva-Val-Val-Sta$^P$-OEt is also bound significantly more tightly to penicillopepsin at pH 3.5, in comparison to pH 4.5. This effect is not a generalized effect of pH on the binding of peptides to these enzymes, since substrate $K_m$ values are typically invariant[19,20] or increase with decreasing pH.[21] On the other hand, it is consistent with the view that protonation must occur on binding the phosphonate and phosphinate inhibitors.

**Slow-Binding Inhibition.** When pepsin is inhibited by pepstatin and its congeners, including the phosphinate **7**, but not the ketone **13**, a two-step binding process is observed, involving rapidly reversible formation of an initial enzyme–inhibitor complex followed by conversion to a higher affinity complex (eq 1). The time scale for the

$$E + I \underset{K_D}{\rightleftharpoons} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}I^* \qquad (1)$$

latter isomerization ranges from seconds, for pepstatin congeners,[15] to hours, for the phosphinate **7**.[8] The molecular basis for this stepwise binding process is not obvious, although Rich has suggested that it may reflect the difficult displacement of the substrate water molecule, which is deeply buried between the catalytic residues in the active site.[15] Indeed, keto inhibitors, which do not displace but rather combine with this water molecule on binding, do so without discernible intermediates. Whether this two-step binding mechanism is characteristic of tetrahedral inhibitors of pepsin in general, or of pepstatin analogues in particular, provided another point of interest in the investigation of the phosphonic acid analogue inhibitors.

**Phophonate Analogues.** Of the inhibitors that we investigated with penicillopepsin, only for the most potent, Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (**4**), did we determine the on-rate, observing what may be characterized as

(18) Grobelny, D.; Goli, U. B.; Galardy, R. E. *Biochemistry* **1989**, *28*, 4948–4951.

(19) Dunn, B. M.; Valler, M. J.; Rolph, C. E.; Foundling, S. I.; Jimenez, M.; Kay, J. *Biochim. Biophys. Acta* **1987**, *913*, 122–130.

(20) (a) Zeffren, E.; Kaiser, E. T. *J. Am. Chem. Soc.* **1967**, *89*, 4204–4208. (b) Clement, G. E.; Snyder, S. L.; Price, H.; Cartmell, R. *Ibid.* **1968**, *90*, 5603–5610.

(21) Hofmann, T.; Hodges, R. S.; James, M. N. G. *Biochemistry* **1984**, *23*, 635–643.

slow-binding behavior, with $k_{on} = 6.5 \times 10^5$ $M^{-1}$ $s^{-1}$. Although this is a slower rate of association than that often observed with other peptidases and their oligopeptide substrates, it is typical for phosphonate inhibitors of zinc peptidases.[7] No evidence for a two-step binding mechanism was found for this inhibitor over the range of concentrations examined (4.5–38 nM), nor was any kinetic behavior observed that would suggest slow conversion of a reversible E·I complex to a tighter complex, as is found with phosphinic acid inhibitors of pepsin (see below and ref 8).

All of the phosphonate analogues show conventional binding kinetics in their interaction with pepsin, even the most potent inhibitor, 4 ($K_i$ = 0.26 nM). In each case examined, preincubation of the inhibitor with the enzyme for 20 min prior to addition of substrate gave the same initial velocity as that observed without preincubation.

**Phosphinate Analogues.** As reported previously, the inhibition of pepsin by the phosphinate analogue 7 shows unusual kinetic behavior: a two-step binding process is observed, with a dissociation constant for the initial complex ($K_D$) of 3 nM and an apparent half-life for conversion of E·I to E·I* on the order of 2 h. It was therefore of interest to determine both the affinity of the phosphinamide diastereomers **10A** and **10B** for pepsin, as well as the kinetic aspects of their inhibition. Since assay of these neutral phosphinamides is complicated by their low solubility in water, both the conventional assays as well as the preincubations were carried out in the presence of DMF. Corrections for the influence of up to 5% DMF (which was minimal) were made with appropriate controls. With respect to initial binding, both diastereomers are some 3 orders of magnitude weaker as inhibitors than the corresponding phosphinate 7, although they differ from each other by an order of magnitude, with $K_D$ values of 1.6 μM and 19 μM. In view of the significantly higher affinity of the corresponding phosphinic acid, it seems likely that the observed inhibition is due to the presence of a small amount of this hydrolysis product. Relevant in this regard is the observation that, on prolonged incubation of pepsin with the more potent of the phosphinamides, time-dependent inactivation of the enzyme is seen that is similar to the slow phase of the inactivation by the phosphinate. This incubation was carried out in 40% DMF and the progress of the inhibition was monitored by removal of aliquots and dilution into a standard assay mixture. In view of the DMF concentration that was necessary to dissolve the inhibitor, it may be fortuitous that the 2-h half-life for inactivation by the phosphinamide corresponds to that of the phosphinate. Nevertheless, the fact that this conversion is very slow for both the phosphinate and the putative phosphinamide suggests that a similar process is responsible and that it may in fact involve the same species.

Consistent with this interpretation is the observation that the inhibition of pepsin by the phosphinamide Iva-Val-Val-Sta$^{P(NH_2)}$-OEt (**9**, mixture of diastereomers at phosphorus) is also dependent on pH (see Table I), which was not expected for this nonionic derivative. However, if the inhibition observed for this compound is due primarily to the phosphinic acid, considerable hydrolysis must have taken place to account for the calculated value. The low affinity of these inhibitors is surprising in view of the potency of other neutral, tetrahedral analogues of statine-containing inhibitors, such as the difluoro ketone hydrate[1c] and methylcarbinol[22] derivatives.

## Conclusions

The substitution of a phosphonate ester moiety for the scissile peptide linkage in oligopeptide substrates of pepsin and penicillopepsin leads to potent inhibitors of these enzymes. For the most part, conventional binding kinetics were observed for these phosphonate inhibitors, in contrast to the two-step process reported previously for other pepsin inhibitors and seen with the "phosphastatine"-containing analogue. The pH dependence of the binding affinity of the latter compound suggests that protonation of the active-site carboxylate, or possibly of the inhibitor, is required for binding. The phosphinamide analogues, in spite of their potential ability to mimic more closely the transition state for formation of the tetrahedral intermediate, are considerably weaker inhibitors than the anionic phosphinate from which they are derived.

## Experimental Section

**Synthesis. General.** Unless otherwise indicated, all NMR spectra were recorded in $CDCl_3$ or $D_2O$; $^{31}P$ NMR spectra were acquired by using broad-band $^1H$ decoupling; chemical shifts are expressed in ppm (downfield positive) relative to trimethyl phosphate (1% solution in a sealed capillary) at $\delta$ = 3.09 ppm; coupling constants are in hertz. Normal-phase HPLC was performed on Ultrasphere-Si (silica); reverse-phase HPLC was performed on Partisil-10 octadecyldimethylsilane-silica; preparative chromatography was performed according to the method of Still.[23] Anion exchange chromatography was performed on DEAE-Sephadex; the cation exchange resin was Dowex 50W-X8. Triethylammonium bicarbonate (TBK) buffer was prepared by saturating a rapidly stirred mixture of freshly distilled triethylamine and water at 5 °C with $CO_2$ until a pH of 8.6 was reached.

**Phosphonate Peptide Analogues.** The synthesis and characterization of Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (**4**), are described as a representative procedure; full experimental details and characterization of the remaining inhibitors are provided in the supplementary material.

**Methyl Hydrogen [(1*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-3-methylbutyl]phosphonate (19b, Cbz-Leu$^P$-OMe).** A slurry of 4.84 g (29.0 mmol) of [(1*R*)-1-amino-3-methylbutyl]phosphonic acid (**18**),[11b] 4.89 g (58.2 mmol) of $NaHCO_3$, and 6.14 g (57.9 mmol) of $Na_2CO_3$ in 29.5 mL of 2 N NaOH was stirred mechanically and cooled in an ice–water bath. To this mixture was added 4.45 mL (95% pure, 29.6 mmol) of benzyl chloroformate (CbzCl) by syringe. At 1-h intervals, two additional aliquots (4.45 mL each, 29.6 mmol) of CbzCl were added. After stirring at room temperature overnight, 10 mL of 2 N NaOH was added, and the mixture was partitioned between water and ether. The aqueous portion was washed with ether, acidified to litmus red with concentrated HCl, and extracted three times with EtOAc. The EtOAc layer was washed with brine and dried (4Å molecular sieves), and the solvent was evaporated to give 8.84 g (29.3 mmol, quantitative yield) of [(1*R*)-1-[*N*-[(phenylmethoxy)carbonyl]amino]-3-methylbutyl]phosphonic acid as a white foam: $[\alpha]^{23}_D$ = −54° (*c* = 1, 1 N NaOH); $^1H$ NMR ($d_6$-DMSO, 500 MHz) $\delta$ 0.83 (d, 3, *J* = 6.5), 0.87 (d, 3, *J* = 6.6), 1.35–1.65 (m, 3), 1.72 (m, 1), 5.01 + 5.04 (AB q, 2, *J* = 12.8), 7.07 (d, 1, *J* = 9.5), 7.25–7.40 (m, 5); peaks due to a minor conformer were observed at $\delta$ 0.75 (d, *J* = 6.3), 0.83 (d, *J* = 6), and 6.62 (d, *J* = 9); $^{31}P$ NMR ($d_6$-DMSO, 202 MHz) $\delta$ 21.78 (13%, minor conformer), 22.23 (87%, major conformer).

Without further purification, this white foam was mixed with 50 mL (457 mmol) of trimethyl orthoformate. The flask was equipped with a reflux condenser (without coolant) and placed in an oil bath at 110 °C. After 9 h, the volatile products and reactants were removed in vacuo, and the residue was purified by chromatography (0.5% EtOH/EtOAc) to yield 9.08 g (27.6 mmol, 95% yield) of the phosphonate dimethyl ester **19a** as a colorless oil: $[\alpha]^{24}_D$ = −36.4° (*c* = 1, $CHCl_3$); IR (film) 3250, 2970, 1725, 1545, 1460, 1270, 1235, 1040, 835, 750, 705 $cm^{-1}$; $^1H$ NMR

(22) Rich, D. H.; Bernatowicz, M. S.; Agarwal, N. S.; Kawai, M.; Salituro, F. G.; Schmidt, P. G. *Biochemistry* **1985**, *24*, 3165–3173.

(23) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.

(250 MHz) δ 0.93 (d, 3, J = 6.4), 0.94 (d, 3, J = 6.5), 1.5–1.8 (m, 3), 3.70 (d, 3, J = 10.5), 3.75 (d, 3, J = 10.6), 4.1–4.3 (m, 1), 5.08 + 5.16 (AB q, 2, J = 12.2), 5.15 (br d, 1, J = 9.6), 7.34 (s, 5); $^{31}$P NMR (120 MHz) δ 27.99 (17%, minor conformer), 28.56 (83%, major conformer). [Lit.[8b] for racemate: mp 45–47 °C. Anal. Calcd for $C_{15}H_{24}NO_5P$: C, 54.71; H, 7.35; N, 4.25; P, 9.40. Found: C, 54.79; H, 7.31; N, 4.24; P, 9.52.]

A solution of 9.08 g (27.6 mmol) of the dimethyl ester **19a** in 30 mL of MeOH and 40 mL of 2 N NaOH was stirred for 12 h at 21 °C. The reaction mixture was then diluted with 150 mL of $H_2O$, washed with $CHCl_3$, acidified to litmus red with concentrated HCl, and extracted with $CHCl_3$. The organic layer was dried ($MgSO_4$) and evaporated to give 8.28 g (26.2 mmol, 95% yield) of the monoester **19b** as a white solid: mp 117–119 °C; $[\alpha]^{25}_D$ = −43° (c = 1, $CHCl_3$); IR (KBr) 3310, 2960, 1720, 1540, 1470, 1300, 1255, 1170, 1125, 1060, 1040, 985, 860, 825, 780, 745, 705 cm$^{-1}$; $^1$H NMR (200 MHz) δ 0.93 (d, 6, J = 6.3), 1.35–1.85 (m, 3), 3.71 (d, 3, J = 10.8), 4.05–4.35 (m, 1), 5.12 (s, 2), 5.19 (d, 1, J = 11.0), 7.33 (s, 5), 11.13 (s, 1); peaks due to a minor conformer were observed at δ 0.79 (d, J = 6.5), 0.84 (d, J = 6.7), and 5.60 (d, J = 9.9); $^{31}$P NMR (81.7 MHz) δ 28.6 (23%, minor conformer), 29.3 (77%, major conformer). [Lit.[8b] for racemate: mp 120–121 °C. Anal. Calcd for $C_{14}H_{22}NO_5P$: C, 53.33; H, 7.03; N, 4.44; P, 9.82. Found: C, 53.52; H, 6.87; N, 4.38; P, 9.99.]

**L-2-[[(1*R*)-[1-[*N*-[(Phenylmethoxy)carbonyl]amino]-3-methylbutyl]methoxyphosphinyl]oxy]-3-phenylpropanoyl-L-alanyl-L-alanine Methyl Ester (21b, Cbz-Leu$^P$-(O)Phe-Ala-Ala Dimethyl Ester).** To a stirred solution of 1.00 g (3.18 mmol) of the (*R*)-phosphonic acid monoester **19b** in 5 mL of dry $CH_2Cl_2$ was added 300 μL (3.99 mmol) of $SOCl_2$. After 4 h, the solvent and volatile materials were removed by a stream of dry nitrogen and then in vacuo. To the residue was added with stirring a solution of 1.01 g (3.12 mmol) L-(β-phenyllactylalanyl)alanine methyl ester (**20a**, see supplementary material) and 570 μL (4.09 mmol) of triethylamine in 7.5 mL of $CH_2Cl_2$. After 2 days, the crude reaction product was purified by chromatography (6.5% EtOH/$CH_2Cl_2$) to yield 1.10 g (57% yield) of the coupled diester **21b** (two diastereomers at phosphorus) as a white solid: IR (KBr) 3300, 3070, 3040, 2960, 2880, 1750, 1715, 1695, 1660, 1540, 1445, 1390, 1270, 1230, 1170, 1040, 960, 745, 700 cm$^{-1}$; $^1$H NMR (500 MHz) δ 0.88 (d, 1.5, J = 6.6), 0.89 (d, 1.5, J = 7.0), 0.91 (d, 1.5, J = 6.9), 0.94 (d, 1.5, J = 6.6), 1.17 (d, 1.5, J = 7.0), 1.30 (d, 3, J = 7.2), 1.34 (d, 1.5, J = 7.3), 1.2–1.8 (m, 3), 2.94 (dd, 0.5, J = 7.9, 14.2), 3.13 (dd, 0.5, J = 5.5, 14.2), 3.19 (dd, 0.5, J = 3.0, 15.5), 3.23 (dd, 0.5, J = 4.3, 14.3), 3.28 (d, 1.5, J = 10.7), 3.69 (s, 1.5), 3.70 (s, 1.5), 3.77 (d, 1.5, J = 10.7), 4.1–4.25 (m, 1), 4.46 (dq, 0.5, J = 7.2, 7.2), 4.47 (dq, 0.5, J = 7.1, 7.1), 4.50 (dq, 0.5, J = 7.3, 7.3), 4.55 (dq, 0.5, J = 7.3, 7.3), 4.90 (d, 0.5, J = 12.3), 5.01–5.05 (m, 0.5), 5.04 + 5.07 (AB q, 1, J = 12.2), 5.06 (d, 0.5, J = 12.1), 5.11 (ddd, 0.5, J = 3.4, 7.9, 7.9), 5.46 (d, 1, J = 9.6), 7.1–7.4 (m, 11.5), 7.68 (d, 0.5, J = 7.9); $^{13}$C NMR (125.8 MHz, $CDCl_3$ = 77.0 ppm) δ 17.28, 17.53, 17.70, 18.07, 20.75, 20.79, 23.03, 23.05, 24.16 (d, J = 13.6), 24.25 (d, J = 14.1), 37.16, 37.21, 39.04 (d, J = 2.8), 39.31 (d, J = 4.0), 46.04 (d, J = 156.8), 46.96 (d, J = 158.4), 47.88, 47.99, 48.26, 48.48, 52.15, 52.23, 52.72 (d, J = 7.2), 53.98 (d, J = 6.2), 66.93, 67.17, 76.22 (d, J = 8.4), 76.66 (d, J = 7.4), 126.86, 126.95, 127.63, 127.98, 128.01, 128.17, 128.33, 128.40, 129.65, 129.91, 134.89, 135.61, 135.90, 135.99, 156.09 (d, J = 3.6), 156.31 (d, J = 2.8), 168.55, (d, J = 4.5), 169.33 (d, J = 3.5), 171.36, 171.57, 172.93, 172.97; $^{31}$P NMR (121.5 MHz) δ 26.51, 26.92. Anal. Calcd for $C_{30}H_{42}N_3O_9P$: C, 58.15; H, 6.83; N, 6.78; P, 5.00. Found: C, 57.99; H, 6.82; N, 6.70; P, 4.96.

**L-2-[[(1*R*)-[1-[*N*-(3-Methylbutanoyl)-L-valyl-L-valyl]-amino]-3-methylbutyl]methoxyphosphinyl]oxy]-3-phenylpropanoyl-L-analyl-L-alanine Methyl Ester (24, Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala Dimethyl Ester).** A solution of 314 mg (0.51 mmol) of Cbz-Leu$^P$-(O)Phe-Ala-Ala dimethyl ester (**21b**) and 10% Pd/C (43 mg) in 3.0 mL of EtOAc was stirred under a hydrogen atmosphere for 3.5 h, at which time TLC indicated that the reaction was only half complete. A 2.0-mL portion of MeOH was added to the reaction mixture, which was stirred for an additional 2 h. The mixture was filtered, the solvent was evaporated, and the resulting foam was dissolved in 5 mL of dry EtOAc and cooled in an ice–EtOH bath to ≃ −10 °C.

A suspension of 172 mg (0.57 mmol) of isovaleryl-valyl-valine (**23**, see supplementary material) and 61 μL (0.55 mmol) of *N*-methylmorpholine in 8 mL of dry EtOAc was stirred and cooled to −10 °C, and 69 μL (0.53 mmol) of isobutyl chloroformate was added. After 3 min, the amine solution described above was added. After 1 h, the cooling bath was removed and the reaction mixture was stirred at room temperature overnight. Purification by chromatography (9% EtOH/$CHCl_3$) yielded 375 mg (92% yield) of the acylated diester **24** as a white solid: IR (KBr) 3280, 3070, 2960, 2880, 1750, 1640, 1540, 1455, 1390, 1370, 1220, 1170, 1040, 965, 745, 700 cm$^{-1}$; $^1$H NMR (300.2 MHz) δ 0.70–1.00 (m, 24), 1.23 (d, 1.5, J = 7.0), 1.35 (d, 1.5, J = 7.1), 1.39 (d, 3, J = 7.2), 1.5–1.7 (m, 3), 1.9–2.2 (m, 5), 3.0–3.3 (m, 2), 3.18 (d, 1.5, J = 11.1), 3.73 (s, 3), 3.73 (d, 1.5, J = 10.4), 4.2–4.7 (m, 5), 5.0–5.1 (m, 0.5), 5.2–5.3 (m, 0.5), 6.6–7.4 (m, 9), 7.85 (br s, 0.5), 8.05 (br s, 0.5); $^{13}$C NMR (125.8 MHz, $CDCl_3$ = 77.0 ppm) δ 17.75, 17.87, 18.11, 18.33, 18.59, 18.70, 18.83, 18.89, 20.64, 20.67, 22.27, 22.31, 22.33, 23.11, 23.20, 24.14 (d, J = 15.2), 24.26 (d, J = 15.5), 26.01, 26.03, 30.68, 30.78, 30.87, 31.08, 36.27, 36.68, 39.33, 39.74, 43.80 (d, J = 156.9), 45.26, 45.48, 47.87, 48.02, 48.32, 48.66, 52.20 (d, J = 7), 52.28, 53.32, 53.82 (d, J = 5.6), 58.12, 58.31, 58.40, 75.99 (d, J = 7.8), 126.93, 127.05, 128.18, 128.35, 129.53, 129.88, 134.86, 135.94, 168.64 (d, J = 3.8), 169.63 (d, J = 4), 171.58 (d, J = 4), 171.57, 171.65, 171.75, 171.84 (d, J = 4), 172.64, 172.86, 173.08, 173.10; $^{31}$P NMR (121.5 MHz) δ 26.53, 26.88. Anal. Calcd for $C_{37}H_{62}N_5O_{10}P$: C, 57.87; H, 8.14; N, 9.12; P, 4.03. Found: C, 57.59; H, 8.03; N, 8.91; P, 4.19.

**L-2-[[(1*R*)-[1-[*N*-[*N*-(3-Methylbutanoyl)-L-valyl-L-valyl]-amino]-3-methylbutyl]hydroxyphosphinyl]oxy]-3-phenylpropanoyl-L-alanyl-L-alanine Methyl Ester (4, Iva-Val-Val-Leu$^P$(O)Phe-Ala-Ala-OMe).** To a solution of isobutylene in 5 mL of $CH_2Cl_2$ (prepared by sparging with isobutylene for 10 min) was added 87 μL (0.66 mmol) of trimethylsilyl bromide. Five minutes later, a solution of 100 mg (0.13 mmol) of the phosphonate diester **24** in 1 mL of $CH_2Cl_2$ was added to the TMSBr solution. After being stirred at room temperature for 24 h, the reaction mixture was evaporated twice from $CHCl_3$, 4 mL of methanol was added, and the white solid that formed was collected and dried in vacuo to yield 63 mg (64% yield) of the phosphonic acid **4**: mp 225–227 °C dec; IR (KBr) 3280, 3080, 2960, 2880, 1750, 1645, 1545, 1455, 1390, 1215, 1160, 1060, 975, 700 cm$^{-1}$; $^1$H NMR (300 MHz, $d_6$-DMSO) δ 0.70 (d, 3, J = 6.1), 0.75–0.90 (m, 18), 0.85 (d, 3, J = 6.0), 1.14 (d, 3, J = 6.7), 1.25 (d, 3, J = 7.3), 1.3–1.65 (m, 3), 1.8–2.1 (m, 5), 2.95–3.15 (m, 2), 4.1–4.2 (m, 5), 4.8–4.9 (m, 1), 7.15–7.3 (m, 5), 7.73 (d, 1, J = 8.6), 7.78 (d, 1, J = 8.8), 7.83 (d, 1, J = 6.9), 8.00 (d, 1, J = 9.0), 8.25–8.35 (m, 1); $^{31}$P NMR (121.5 MHz, $d_6$-DMSO) δ 24.49; MS (FAB$^+$) *m/z* 754 (MH$^+$), 267 (100). Anal. Calcd for $C_{36}H_{60}N_5O_{10}P$: C, 57.36 H, 8.02; N, 9.29; P, 4.11. Found: C, 57.25; H, 7.98; N, 9.19; P, 3.92.

**Enzyme Assays. General.** All solutions were prepared with doubly distilled (or distilled and deionized) water. Stock solutions were filtered through Rainin Nylon-66 0.45-μm filters. Assays were performed on a Cary 219 UV–vis spectrophotometer equipped with an OLIS Model 3820 data acquisition computer or on a Varian DMS-100 UV–vis instrument equipped with a DS-15 data station. Temperature regulation (±0.2 °C) was provided by a Lauda Model RM 20 circulating constant-temperature bath connected to a water-jacketed sample cell holder.

**Penicillopepsin Assays. Buffers.** Unless otherwise indicated, assays were performed at 25 °C in 100 mM sodium acetate at pH 3.5 or 4.5.

**Enzyme Stock Solution.** Penicillopepsin was obtained in the form of a lyophilized powder as a gift of Professor Theo Hofmann (University of Toronto). A 1.4-mg sample of penicillopepsin was dissolved in 1.00 mL of 0.100 M $KH_2PO_4$ containing 10% (v/v) glycerol at 5 °C. The resulting solution was centrifuged for 5 min on an Eppendorf Model 5414 centrifuge. The upper 900 μL of the solution was removed and stored in a plastic tube at 5 °C. The concentration of penicillopepsin in this stock (35 μM) was determined by measurement of the absorbance at 280 nm ($E_{1\,mg/mL}$ = 1.15 cm$^{-1}$, MW = 34 000).[24] A working solution of the enzyme was prepared daily from the above stock by dilution of 100 μL of the stock to 10 mL with 0.10 N $KH_2PO_4$ containing 10% glycerol at 5 °C ([E] = 350 nM). The stock solutions were stored

(24) Professor T. Hofmann, personal communication. See also: Hofmann, T., and Hodges, R. *Biochem. J.* **1982**, *203*, 603–610.

at 5 °C and were stable for at least 1 week.

**Substrate Stock Solution.** The substrate, Ac-Ala-Ala-Lys-(pNO$_2$)Phe-Ala-Ala-Ala-NH$_2$, was provided by Professor Theo Hofmann (University of Toronto) and Dr. Federico Gaeta (Schering-Plough Corporation). The substrate stock was prepared by dilution of the substrate in buffer at the appropriate pH. The substrate concentration was determined from the absorbance at 277 nm ($\epsilon_{277}$ = 10970 M$^{-1}$ cm$^{-1}$).[21]

**Assay Procedure.** Unless otherwise indicated, assays were initiated by addition of enzyme to an appropriate mixture of substrate, inhibitor, and buffer. Typical enzyme concentrations were 2–4 nM. Substrate hydrolysis was followed at $\lambda$ = 306 nm ($\Delta\epsilon$ = −1710 M$^{-1}$ cm$^{-1}$) at pH 3.5 or $\lambda$ = 297 nm ($\Delta\epsilon$ = −1970 M$^{-1}$ cm$^{-1}$) at pH 4.5. Assays were followed for ≤15% of the total reaction and kinetic parameters were obtained by analysis using Cleland's HYPER program.[25]

**Pepsin Assays. Buffers.** The buffer used for all assays was 0.1 M NaOAc, pH 3.5, unless otherwise stated.

**Enzyme Stock Solution.** Chromatographically purified porcine stomach mucosa pepsin (EC 3.4.23.1) was purchased from Sigma Chemical Co. (4000 units/mg; lot no. 117F-8075) and was dissolved and diluted in the assay buffer immediately before use.

**Substrate Stock Solution.** The octapeptide substrate Lys-Pro-Ala-Glu-Phe-*p*(NO$_2$)Phe-Arg-Leu[19] was a gift of Dr. Robert M. Scarborough.

**Assay Procedure.** The initial rates of substrate hydrolysis were determined by equilibrating substrate and inhibitor solutions at 37 °C in the cuvette for 3 min and initiating the reaction by addition of 3.0 pmol of pepsin per 1.00 mL final volume. Substrate hydrolysis was followed by observing the decrease in absorbance at $\lambda$ = 310 nm ($\Delta\epsilon$ = −800 M$^{-1}$ cm$^{-1}$). Initial rates were measured from 0.5 min until no more than 10% of the substrate was hydrolyzed.

**Determination of Inhibition Constants.** Representative procedures are provided here for determination of the association rate and $K_i$ value for inhibition of penicillopepsin by the hexapeptide analogue **4**; full descriptions for the other inhibitors are provided in the supplementary material. The compounds that have been synthesized and their inhibition constants with pepsin (pH 3.5, 37 °C) and penicillopepsin (pH 4.5, 25 °C) are given in Table I; the inhibitors are grouped according to structural class.

**Rate of Inhibition of Penicillopepsin by Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (4) at pH 4.5.** Compound **4** (1.12 mg, 1.48 μmol) was dissolved in 2.0 mL of a 1:1 mixture of DMF and buffer to make a 0.741-mM stock from which working solutions were prepared by dilution with buffer. At the high dilutions used for inhibition studies, the concentration of DMF in the assay mixtures was less than 0.5%. Assays were initiated by addition of penicillopepsin to a solution of substrate and inhibitor; the final concentration of substrate was 126 μM, while the concentrations of penicillopepsin and inhibitor were varied from 0.8 to 6.9 nM and 4.5 to 38 nM, respectively (the inhibitor/enzyme ratio varied from 5 to 10). The assays were followed until there was no further change in absorbance (6–120-min assays), and the resulting curves were fit to the equation $y = Ae^{-kt} + C$ using a nonlinear, least-squares program.

A plot of the apparent on-rate ($k_{app}$) versus inhibitor concentration was linear with a slope of (3.43 ± 0.19) × 10$^5$ M$^{-1}$ s$^{-1}$ and yielded an on-rate ($k_{on}$) of (8.4 ± 0.5) × 10$^5$ M$^{-1}$ s$^{-1}$ after multiplication by $(1 + S/K_m)$. However, since the $y$ intercept is negative, this estimate of $k_{on}$ is likely to be too high. Alternatively, an on-rate can be calculated for each run according to eq 2 (with the assumption that $k_{off} \ll k_{app}$, since complete inhibition is observed at the concentrations of inhibitor used); this method yields an average $k_{on}$ of (6.5 ± 1.6) × 10$^5$ M$^{-1}$ s$^{-1}$.

$$k_{app} = k_{off} + \left[\frac{k_{on}}{(1 + [S]/K_m)}\right] \quad (2)$$

(25) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103–138.

**Inhibition of Penicillopepsin by Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (4) at pH 4.5.** After being incubated at 25 °C overnight in the assay buffer, 0.55-nM solutions of penicillopepsin showed no activity. Since bovine serum albumin (BSA, 0.1 mg/mL) prevented loss of activity, it was incorporated in all of the incubation solutions. Solutions containing Iva-Val-Val-Leu$^P$-(O)Phe-Ala-Ala-OMe (**4**) (0, 0.7, 1.0, 1.3, 1.6, and 2.0 nM) and penicillopepsin (0.55 nM) (in 0.1 M sodium acetate, pH 4.5, containing 0.1 mg/mL BSA) were allowed to incubate at 25 °C for >14 h. Aliquots (800 μL) of these solutions were mixed with substrate (200 μL of a 664-μM stock) and the initial velocities were determined by monitoring the absorbance change at 297 nM. Five assays were performed at each concentration of inhibitor; the average of the five assays was determined and used to calculate the slope of the plot of $v_0/v_i$ versus $[I]/(1 - v_i/v_0)$. This procedure yielded a $K_i$ value of 0.19 ± 0.03 nM.

**pH Dependence of Penicillopepsin Inhibition by Cbz-Ala-Ala-Leu$^P$-(O)Phe-OMe (1).** Assays were performed in either 50-mM sodium acetate (pH 5.5 and 4.5) or 50-mM sodium formate buffers (pH 4.5 and 3.5). The ionic strength of all buffers was adjusted to 0.05 by addition of the appropriate amount of NaCl. The kinetic parameters for substrate hydrolysis were determined for each buffer and pH; using a fixed concentration of substrate, the initial rates at various inhibitor concentrations were determined. Plots of $v_0/v_i$ versus inhibitor concentration were linear and the $K_i$ was determined by use of Cleland's COMP program.[24] The results of these experiments are summarized in Table II, along with information for the phosphastatine analogue **6**.

**Acknowledgment.** This work was supported by a grant from the National Institutes of Health (grant no. CA-22747) and by a National Science Foundation Predoctoral Fellowship to J.E.H. We also thank Professor T. Hofmann for generous gifts of penicillopepsin and both him and Drs. R. M. Scarborough and F. Gaeta for gifts of substrates.

**Registry No.** 1, 128901-55-3; 1·Li, 129029-40-9; **2**, 128948-76-5; 2·Li, 128901-49-5; **3**, 128923-36-4; **4**, 128901-50-8; **5**, 128901-51-9; **6**, 128901-52-0; **7**, 90790-47-9; **8** (diastereomer 1), 128901-53-1; **8** (diasteromer 2), 128948-74-3; **9** (diastereomer 1), 128901-54-2; **9** (diastereomer 2), 128948-75-4; **10A**, 110658-46-3; **10B**, 110658-47-4; **18**, 88081-77-0; **18** (*N*-Cbz derivative), 128948-77-6; **19a**, 128948-79-8; **19b**, 128948-78-7; **20a**, 128901-56-4; **21a** (diastereomer 1), 128901-60-0; **21a** (diastereomer 2), 128948-82-3; **21b** (diastereomer 1), 128901-57-5; **21b** (diastereomer 2), 128948-80-1; **22a** (diastereomer 1), 128948-83-4; **22a** (diastereomer 2), 128948-84-5; **22b** (diastereomer 1), 128901-61-1; **22b** (diastereomer 2), 128901-62-2; **22b** (*N*-deprotected, diastereomer 1), 128901-67-7; **22b** (*N*-deprotected, diastereomer 2), 128901-68-8; **22** (R″ = H, diastereomer 1), 128901-63-3; **22** (R″ = H, diastereomer 2), 128901-64-4; **23**, 59452-57-2; **24** (diastereomer 1), 128901-58-6; **24** (diastereomer 2), 128948-81-2; **27a** (diastereomer 1), 128948-85-6; **27a** (diastereomer 2), 128948-86-7; **27c** (diastereomer 1), 110582-34-8; **27c** (diastereomer 2), 110582-35-9; **27c** (*N*-deprotected, diastereomer 2), 110658-53-2; **28**, 39741-06-5; **31** (diastereomer 1), 128901-65-5; **31** (diastereomer 2), 128901-66-6; **31** (*tert*-butyl ester, diastereomer 1), 129029-41-0; **31** (*tert*-butyl ester, diastereomer 2), 129029-42-1; **32** (diastereomer 1), 128901-69-9; **32** (diastereomer 2), 128948-87-8; **33** (diastereomer 1), 128901-70-2; **33** (diastereomer 2), 128948-88-9; **34** (diastereomer 1), 128901-71-3; **34** (diastereomer 2), 128948-89-0; **35** (diastereomer 1), 128901-72-4; **35** (diastereomer 2), 128948-90-3; **36** (diastereomer 1), 128901-73-5; **36** (diastereomer 2), 128948-91-4; Iva-Cl, 108-12-3; H-Val-OMe·HCl, 6306-52-1; Iva-Val-OMe, 128901-59-7; H-Val-Val-OH, 3918-94-3; Cbz-Ala-Ala-OH, 16012-70-7; H-(O)Phe-OH, 20312-36-1; BOC-Ala-Iaa, 72155-58-9; EtOAc, 141-78-6; *t*-BuOAc, 540-88-5; pepsin, 9001-75-6; penicillopepsin, 9074-08-2.

**Supplementary Material Available:** Full experimental details for preparation, characterization, and assay of inhibitors **1–3** and **5–10** (38 pages). Ordering information is given on any current masthead page.