Ar H), 6.88–7.32 (m, 3 H, Ar H); CIMS, *m/e* 458 ($M^+ + 1$). Anal. ($C_{24}H_{27}NO_6S$) C, H, N.

3,*N*-Bis(ethoxycarbonyl)-3-demethyldeacetylthiocolchicine (15). 3-Demethylthiocolchicine (**2**; 2.01 g, 5 mmol) was refluxed for 16 h in a mixture of 50 mL of methanol and 100 mL of 2 N HCl. Methanol was evaporated under reduced pressure and the residue was made alkaline with concentrated NH_4OH and extracted with $CHCl_3$–MeOH (3:1) (3 × 100 mL). The combined organic layer was washed with brine (2 × 10 mL), dried (Na_2SO_4), and evaporated to yield 1.96 g of 3-demethyldeacetylthiocolchicine as an orange foam (1.37 g, 3.8 mmol), which was treated with ethyl chloroformate by using the standard method. Crude **15** was purified by column chromatography (SiO_2, elution with $CHCl_3$–MeOH, 98:2) to give a yellow solid (1.01 g, 57.6%). A sample was crystallized from EtOAc–Et_2O to yield yellow crystalline **15**: mp 219–221 °C; $[\alpha]^{24}_D$ −130.3° (*c* 0.59, $CHCl_3$); IR ($CHCl_3$) 3460 (NH), 1766 (carbamate), 1721 (C=O), 1615 cm^{-1}; NMR ($CDCl_3$) δ 1.17 (t, 3 H, OCH_2CH_3), 1.4 (t, 3 H, OCH_2CH_3), 2.43 (s, 3 H, SMe), 3.64 (s, 3 H, 1-OMe), 3.96 (s, 3 H,2-OMe), 4.00 (m, 2 H OCH_2CH_3), 4.35 (m, 2 H, OCH_2CH_3), 4.40 (m, 1 H, C_7H), 5.21 (d, 1 H, *J* = 7 Hz, NH) 6.77 (s, 1 H, Ar H), 7.04 (d, 1 H, *J* = 10 Hz, Ar H), 7.29 (d, 1 H, *J* = 10 Hz, Ar H), 7.3 (s, 1 H, Ar H); CIMS, *m/e* 504 ($M^+ + 1$). Anal. ($C_{24}H_{27}NO_6S$) C, H, N, S.

***N*-(Ethoxycarbonyl)-3-demethyldeacetylthiocolchicine (16).** A solution of **15** (806 mg, 1.6 mmol) in methanol (25 mL) and 1 N HCl (25 mL) was refluxed for 24 h. Methanol was removed in vacuum and the residue extracted with $CHCl_3$ (3 × 30 mL), washed with brine (2 × 10 mL), dried (Na_2SO_4), and evaporated. The crude product was purified by column chromatography (SiO_2, elution with $CHCl_3$–MeOH, 98:2) to afford a yellow foam, which was crystallized from EtOAc–Et_2O to yield yellow crystalline solid **16** (460 mg, 66.7%): mp 230–231 °C; $[\alpha]^{24}_D$ −242.3° (*c* 0.65, $CHCl_3$); IR ($CHCl_3$) 3540 (OH), 3460 (NH), 1720 (C=O), 1620 cm^{-1}; NMR ($CDCl_3$) δ 1.19 (t, 3 H, OCH_2CH_3), 2.45 (s, 3 H, SMe), 3.64 (s, 3 H, 1-OMe), 4.03 (s, 3 H, 2-OMe), 4.05 (m, 2 H, OCH_2CH_3), 4.44 (m, 1 H, C_7H), 5.24 (d, 1 H, *J* = 7 Hz, NH), 5.97 (s, 1 H, OH), 6.22 (s, 1 H, Ar H), 7.06 (d, 1 H, *J* = 10 Hz, Ar H), 7.28 (d, 1 H, *J* = 10 Hz, Ar H), 7.34 (s, 1 H, Ar H); CIMS, *m/e432* ($M^+ + 1$). Anal. ($C_{22}H_{25}NO_6S$) C, H, N, S.

Acknowledgment. We thank Mariena Mattson and Dr. Arthur E. Jacobson from our Section on Medicinal Chemistry for having measured and calculated the acute toxicity values of the compounds discussed here.

We also thank Drs. J. Gaignault and P. Bellet from the Roussel Uclaf Co. in Paris, France, for having supported this project with substantial amounts of thiocolchicine and thiocolchicoside.

Registry No. 1, 87424-26-8; **2**, 87424-25-7; **3**, 97042-99-4; 4, 97043-00-0; **5**, 2731-16-0; **6**, 63620-47-3; **7**, 97043-01-1; **8**, 97043-02-2; **9**, 76129-16-3; **10**, 2731-23-9; **11**, 63620-51-9; **12**, 96737-27-8; **13**, 97043-04-4; **14**, 97043-05-5; **15**, 97059-53-5; **16**, 97043-06-6; **17**, 96737-28-9; **18**, 92264-45-4; **19**, 76129-11-8; **20**, 97043-07-7; **21**, 97043-08-8; 3-demethyldeacetylthiocolchicine, 97043-09-9; benzoyl chloride, 98-88-4; butyryl chloride, 141-75-3; ethoxyacetic anhydride, 14521-87-0; ethyl chloroformate, 541-41-3; phenyl chloroformate, 1885-14-9; butyl chloroformate, 592-34-7; vinyl chloroformate, 5130-24-5; ethyl formate, 109-94-4; trifluoroacetic anhydride, 407-25-0; methyl chloroformate, 79-22-1.

Synthesis and Biological Evaluation of Phosphonamidate Peptide Inhibitors of Enkephalinase and Angiotensin-Converting Enzyme

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The effectiveness of phosphonamidate peptide analogues as inhibitors of rat kidney or human brain metalloendopeptidase (enkephalinase, E.C. 3.4.24.11) and angiotensin-converting enzyme (ACE, 3.4.15.1) has been explored with a series of enkephalin analogues in which the scissile Gly^3–Phe^4 amide bond has been replaced with a phosphonamidate moiety. These compounds exhibited good inhibitory potency against enkephalinase with several of the analogues having K_i values in the submicromolar range as contrasted to micromolar or higher toward ACE. Within a series of [(*N*-acylamino)methyl]phosphonamidates there was a dramatic decrease in inhibitory activity against enkephalinase as the *N*-acyl moiety was substituted with larger, more hydrophobic acyl groups. Likewise, the inhibitory activity of the [(*N*-acylamino)methyl]phosphonamidates against ACE was attenuated by larger phenylalkyl acyl functionalities, although not to the same degree as against enkephalinase. However, phosphonamidate pentapeptide analogues of (Leu)enkephalin and (D-Ala^2,D-Leu^5)enkephalin showed good inhibitory potency against both enzymes. Interestingly, these two (Leu)enkephalin phosphonamidate analogues were completely inactive in the electrically stimulated guinea pig ileum and mouse vas deferens preparations. Conformational factors that may be involved in this inactivity are discussed.

The discovery of the enkephalins[1] initiated intensive research into the pharmacological aspects and physiological significance of these neuropeptides. Much of this research has focused on the neurochemical role and metabolic fate of the enkephalins and has led to the discovery and characterization of a membrane-bound zinc endopeptidase (enkephalinase) involved in the in vivo degradation of the enkephalins.[2-5] Extensive studies on the substrate specificity of this enzyme have been complemented by other studies investigating the effectiveness of various inhibitors against this enzyme. These enkephalinase inhibitors have been of great value as pharmacological tools and may have therapeutic application.

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Table I. Physical Properties of Dipeptide Derivatives

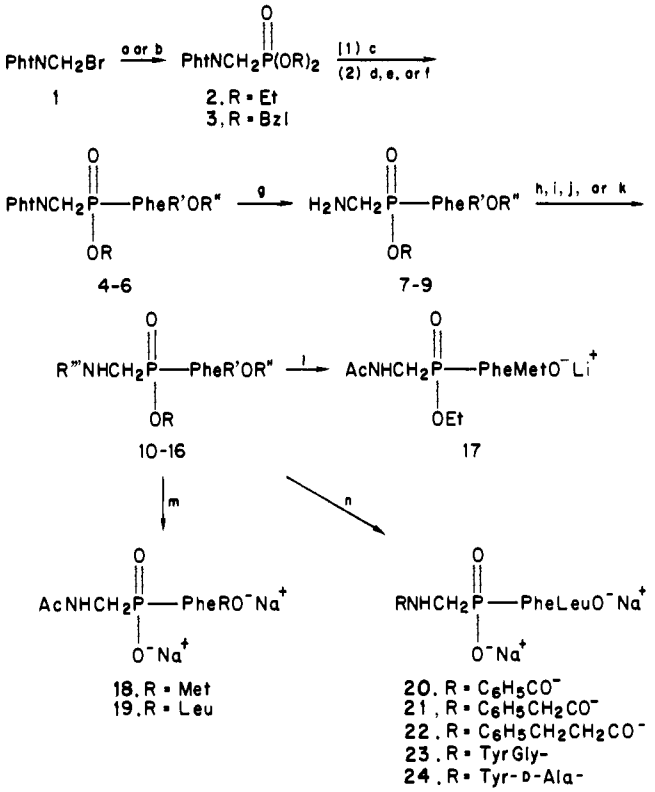
dipeptide	formula[a]	mp, °C	reaction method[b]	% yield	R_f (solvent syst)[c]	$[\alpha]^{25}_D$
BocPheMetOMe	$C_{20}H_{30}N_2O_5S$	85–86[d]	A	97	0.71 (D)	−21.0 (c 1.0, MeOH)[e]
BocPheLeuOMe	$C_{21}H_{32}N_2O_5$	104–105	A	97	0.64 (D)	−26.2 (c 1.0, MeOH)
BocPheLeuOBzl	$C_{27}H_{36}N_2O_5$	84–85	A	83	0.81 (D)	−23.7 (c 1.0, MeOH)
PheMetOMe·HCl	$C_{15}H_{23}N_2O_3SCl$	105–108	B	99	0.69 (H)	−4.6 (c 1.0, MeOH)[f]
PheLeuOMe·HCl	$C_{16}H_{25}N_2O_3Cl$	143–144	B	87	0.77 (H)	−10.6 (c 1.0, MeOH)
PheLeuOBzl·HCl	$C_{22}H_{29}N_2O_3Cl$	161[g]	C	94	0.66 (N)	−21.2 (c 1.0, MeOH)
CbzTyr(OBzl)GlyOMe	$C_{27}H_{28}N_2O_6$	135–136[h]	A	97	0.73 (O)	−22.4 (c 0.96 DMF)[i]
CbzTye(OBzl)-D-AlaOMe	$C_{28}H_{30}N_2O_6$	161–162	A	99	0.71 (O)	−11.8 (c, 1.0 DMF)
CbzTyr(OBzl)Gly	$C_{26}H_{26}N_2O_6$	163–164	D	100	0.32 (P)	−14.4 (c 1.0, MeOH)
CbzTyr(OBzl)-D-Ala	$C_{27}H_{28}N_2O_6$	148–149	D	82	0.37 (P)	−6.7 (c 1.0, MeOH)

[a] Within ±0.4% of calculated values for CHN. [b] See the Experimental Section for reaction procedures. [c] See the Experimental Section for chromatographic solvent system. [d] Lit.[50] mp 84–85 °C. [e] Lit.[50] $[\alpha]^{25}_D$ −20.7. [f] Lit.[50] $[\alpha]^{25}_D$ −4.6. [g] Lit.[51] mp 161 °C. [h] Lit.[52] mp 128–130 °C. [i] Lit.[52] $[\alpha]^{25}_D$ −23.1.

Phosphonamide-containing peptide analogues have been reported to be potent transition-state inhibitors of carboxypeptidase A,[6] thermolysin,[7] and angiotensin-converting enzyme (ACE).[8] In this report we describe the synthesis and biological evaluation of phosphonamidate peptide analogues as enkephalinase inhibitors. In addition, because of the overlapping specificities of enkephalinase and ACE, these compounds have also been tested against ACE. Another facet of this research was concerned with the evaluation of the opioid activity of (Leu)enkephalin analogues that contain a phosphonamidate moiety in place of the carboxamide moiety at the Gly^3 position. Thus, several phosphonamidate enkephalin analogues were screened for opioid activity on guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations.

Chemistry. The synthetic scheme leading to the phosphonamidate inhibitors is outlined in Scheme I. All of the dipeptide precursors needed for the synthesis of the target compounds were synthesized by standard peptide-coupling methods (see Table I and the Experimental Section). *N*-(Bromomethyl)phthalimide (**1**)[9] was readily converted to the diethyl phosphonate **2** with triethyl phosphite[10] or to the dibenzyl phosphonate **3** with the sodium salt of dibenzyl phosphite. Conversion of **2** or **3** to the corresponding phosphonochloridate with PCl_5 followed by treatment with the appropriate dipeptide yielded the phthaloyl-protected phosphonamidates **4–6** in good yield (Table II). Removal of the phthaloyl-protecting group was effected with 1 N methanolic hydrazine to afford the α-aminophosphonamides **7–9**, and conversion of **7** and **8** to the corresponding *N*-acetyl derivatives **10** and **11** was accomplished with use of acetic anhydride and pyridine. The carboxylic methyl ester of **10** was readily removed with 0.25 N LiOH to afford **17**. In contrast, the phosphonamidate ethyl esters of **10** and **11** were much more difficult to remove, requiring an excess of 2 N NaOH over a 24-h period to effect complete removal. Other reagents to effect P–OEt bond cleavage under neutral conditions (including trimethylsilyl iodide,[11] chlorotrimethylsilane–sodium iodide,[12] and lithium iodide[13]) were ineffective.

Scheme I[a]



[a] Key: a, $P(OEt)_3$; b, $NaP(O)(OBzl)_2$; c, PCl_5; d, PheMetOMe/Et_3N; e, PheLeuOMe/Et_3N; f, PheLeuOBzl/Et_3N; g, NH_2NH_2; h, $(Ac)_2O$/py; i, RCOCl/py; j, CbzTyr(OBzl)Gly/DCC-HOBt; k, CbzTyr(OBzl)-D-Ala/DCC-HOBt; l, 0.25 N LiOH; m, 2 N NaOH; n, H_2/Pd-C, $NaHCO_3$.

In agreement with other literature reports,[6,14] the deprotected phosphonamidates **18** and **19** were quite labile in our hands, with considerable decomposition occuring during chromatography (including C-8 reversed-phase chromatography, ion-exchange chromatography, and LH-20 gel filtration). Therefore, no further attempts were made to separate the phosphonamides **18** and **19** from the inorganic salt contaminants, and the degree of contamination was calculated from the phosphorus analysis. However, although a good elemental analysis could not be obtained for **18** and **19**, these compounds were chromatographically pure and showed no signs of organic contaminates by either ^1H NMR or ^{31}P NMR.

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Table II. Physical Properties of [(*N*-Acylamino)methyl]phosphonamides

compd	R_1	R_2	R_3	R_4	R_5	mp, °C	formula[a]	reaction time, h	reaction solvent[b]	column solvent	% yield	R_f (solvent syst)
4	phthaloyl		Bzl	Leu	Bzl	108–111	$C_{38}H_{40}N_3O_7P$	3,[c] 2[d]	A,[c] B[d]	C	47	0.65 (D)
5	phthaloyl		Et	Met	Me	137–145	$C_{26}H_{32}N_3O_7PS^e$	5,[c] 2[d]	E,[c] B[d]	D	49	0.26 (D)
6	phthaloyl		Et	Leu	Me	155–159	$C_{27}H_{34}N_3O_7P$	5,[c] 2[d]	E,[c] B[d]	D	75	0.67 (F)
7		H	Et	Met	Me	oil	$C_{18}H_{30}N_3O_5SP^f$		H		72	0.32 (G)
8	H	H	Et	Leu	Me	72–76	$C_{19}H_{32}N_3O_5P$	48	H	L	53	0.31 (J)
9	H	H	Bzl	Leu	Bzl	oil	$C_{30}H_{38}N_3O_5P$	72	H	K	58	0.43 (F)
10	H_3CCO	H	Et	Met	Me	137–141	$C_{20}H_{32}N_3O_6PS$	43	L	I	22	0.52 (J)
11	H_3CCO	H	Et	Leu	Me	153–158	$C_{21}H_{34}N_3O_6P$	5	D	I	41	0.57 (J)
12	C_6H_5CO	H	Bzl	Leu	Bzl	143–145[g]	$C_{37}H_{42}N_3O_6P$	17	M	K	49	0.58 (F)
13	$C_6H_5CH_2CO$	H	Bzl	Leu	Bzl	130–133[g]	$C_{38}H_{44}N_3O_6P$	17	M	K	51	0.60 (F)
14	$C_6H_5CH_2CH_2CO$	H	Bzl	Leu	Bzl	135–138[g]	$C_{39}H_{46}N_3O_6P$	17	M	K	50	0.62 (F)
15	CbzTyr(OBzl)Gly	H	Bzl	Leu	Bzl	85–90	$C_{56}H_{62}N_5O_{10}P$	36	L	K	11	0.76 (J)
16	CbzTyr(OBzl)-D-Ala	H	Bzl	Leu	Bzl	188–190	$C_{57}H_{64}N_5O_{10}P$	36	L	K	16	0.62 (F)

[a] Within 0.4% of calculated values for C, H, N, and P unless otherwise indicated. [b] See the Experimental Section for solvent systems. [c] Synthesis of phosphonochloridate. [d] Synthesis of phosphonamide. [e] Within 0.4% for C, H, N, and S. [f] Not fully characterized because of instability. [g] Recrystallized from EtOAc/hexane after chromatography.

Scheme II[a]

[a] Key: a, $HOCH_2CH_2CN/Et_3N$; b, PheLeuOMe/Et_3N; c, 2 N NaOH.

Due to the difficulty in removal of the *P*-ethyl ester and instability of the deprotected phosphonamides toward purification, the synthetic scheme for the target compounds **20–24** was modified, using benzyl esters as protecting groups instead of methyl and ethyl esters—the advantage being that complete deprotection can be accomplished by hydrogenation under neutral, nonracemizing conditions.

Paralleling the synthetic scheme leading to compounds **18** and **19**, the *N*-bromomethyl derivative **1** was converted to the dibenzyl phosphonate **3**, treated with PCl_5 to form the phosphonochloridate, and coupled with phenylalanylleucine benzyl ester to afford the phthaloyl-protected phosphonamidate **4**. Deprotection with hydrazine followed by acylation yielded the fully protected phosphonamidates **12–16**. Hydrogenolysis of **12–16** in the presence of $NaHCO_3$ afforded the target compounds **20–24** in good yields. Although all of the precursors to the target compounds were obtained in analytically pure form, we encountered difficulties in obtaining analytically pure samples of the target compounds **20–24**. The hydrolytic sensitivity of the phosphonamide bond to acidic conditions is well documented;[6,14-16] therefore, care was taken to avoid exposing the deprotected phosphonamidates to acidic conditions. However, even with these precautions a small amount (~10–15% based on ^1H NMR) of decomposition occurred during the hydrogenation and lyophilization procedure, affording products contaminated with the byproducts $RCONHCH_2PO_3H_2$ and PheLeuOH. As mentioned earlier, these compounds appear to be unstable under chromatographic conditions, making it very difficult to obtain these compounds in the pure state. Other groups have also observed decomposition of phosphonamidates during chromatography,[14] and to our knowledge no one has obtained a phosphonamidate in analytically pure form. Apparently, compounds **18** and **19** were protected from any significant decomposition due to the slight excess of NaOH present (which, however, interfered with the elemental analyses). Therefore, these compounds were tested as is, with the degree of contamination determined and corrected for from the phosphorus analyses.

The phosphinothioyl peptide analogue **27** was also desired as a transition-state enkephalinase inhibitor. This compound was synthesized by a route similar to that reported previously for related compounds.[17] Thus, successive treatment of methylphosphonic dichloride (**25**) with 3-hydroxypropanenitrile followed by phenylalanylleucine methyl ester afforded **26** in 30% yield. Deprotection with

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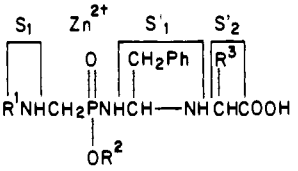
Table III. Inhibitory Potency of Phosphonamidate Peptide Analogues



compd	R_1	R_2	R_3	K_i, μm enkephalinase[a]	K_i, μm ACE[b]	K_i(ACE)/K_i(enkephalinase)
17	H_3CCO	Et	$CH_2CH_2SCH_3$	160	>150	1
18	H_3CCO	Na^+	$CH_2CH_2SCH_3$	0.14	3.4	24.3
19	H_3CCO	Na^+	$CH_2CH(CH_3)_2$	0.85	6.6	7.7
20	C_6H_5CO	Na^+	$CH_2CH(CH_3)_2$	10.6	85	8.0
21	$C_6H_5CH_2CO$	Na^+	$CH_2CH(CH_3)_2$	80.2	120	1.5
22	$C_6H_5CH_2CH_2CO$	Na^+	$CH_2CH(CH_3)_2$	1207	125	0.1
23	TyrGly	Na^+	$CH_2CH(CH_3)_2$	0.11 (0.16 ± 0.06[c])	6.4	58.2
24	Tyr-D-Ala	Na^+	$CH_2CH(CH_3)_2$	0.28 (0.21 ± 0.03[c])	2.8	10.0
27[d]				0.42 (0.83 ± 0.16[c])	11.1	26.4
thiorphan[e]				0.04		
phosphoramidon[f]				0.004		
MK-421[g]					0.0005	

[a] Unless otherwise specified, the K_i values for enkephalinase (metalloendopeptidase, E.C.3.4.24.11) were measured with rat kidney enzyme and are means of two to three determinations, agreeing within 15–20%. [b] The K_i for ACE (3.4.15.1) was measured with rat brain IgG immobilized enzyme or, in the case of analogues **23**, **24**, and **27**, with a soluble affinity-purified preparation (see methods). [c] Human enkephalinase, four to six determinations ±SE. [d] $H_3CP(S)(O^-Na^+)$PheLeuOH. [e] $HSCH_2CH(CH_2C_6H_5)CONHCH_2COOH$. [f] HOP(O)(ORhm)-LeuTrpOH. [g] $C_6H_5CH_2CH_2CH(COOH)$AlaProOH.

2 N NaOH gave the target compound **27** in good yield (Scheme II).

Biological Results. The action of the target compounds as inhibitors was surveyed with use of rat kidney metalloendopeptidase[4] and rat brain ACE (IgG immobilized).[18] Selected compounds were also tested on human brain metalloendopeptidase and a soluble rat brain ACE purified by affinity chromatography (see methods). The potencies of the phosphonamidate inhibitors were compared to thiorphan[19] and phosphoramidon[20] (two potent enkephalinase inhibitors) and to MK-421[21] (a potent ACE inhibitor).

As shown in Table III, several of the phosphonamidate compounds showed good inhibition of rat kidney enkephalinase. Dixon plots indicated that the phosphonamidates acted as competitive inhibitors. The *N*-acetyl derivatives **18** and **19** have K_i values of 1 μM or less, with the methionoine analogue **18** being 5-fold more potent than the leucine analogue **19**. However, the methionine analogue **17**, which has a *P*-ethyl ester group rather than a *P*-hydroxyl group, is over 700 times less potent than **18**. The same rank order for these three analogues was shown for ACE; among these three inhibitors, compound **18** demonstrated the best differentiation between enkephalinase and ACE, having a K_i ratio of 24.

Within the series of leucine tripeptide analogues **19–22** there was a dramatic decrease in inhibitory potency as the *N*-acetyl group was replaced by larger hydrophobic acyl groups, with the 3-phenylpropionyl derivative **22** having a K_i some 1300 times greater than the corresponding *N*-acetyl derivative **19** against enkephalinase and 19 times greater against ACE. Interestingly, ACE appeared to be less sensitive to the detrimental effects of the phenylalkyl acyl groups than was enkephalinase, with the phosphonamidate **22** being almost 10 times more potent against ACE as compared to enkephalinase.

The pentapeptide derivatives **23** and **24** also demonstrated good activity against enkephalinase, with the former being only 3 times less potent than thiorphan. However, there was no significant improvement in the activity of **23** or **24** over the *N*-acetyl derivative **19** against ACE, with all three compounds having K_i values in the micromolar range. Compound **23** had the largest K_i ratio (58-fold) of all the inhibitors tested. Interestingly, this selectivity value was reduced to a 10-fold difference upon replacement of the Gly^2 residue with a D-Ala^2 residue.

The phosphinothioyl derivative **27**, in which the oxygen atom of the phosphonamidate moiety was replaced by a sulfur atom, also showed good inhibition against enkephalinase and ACE, with K_i values of 0.42 and 11.1 μM, respectively.

These compounds were also tested for opioid agonist and antagonist activity on the electrically stimulated myenteric plexus of the GPI[22] and the MVD.[23] The antagonist testing was evaluated against morphine in the GPI and against (D-Ala^2,D-Leu^5)enkephalin (DADLE) in the MVD. All inhibitors were inactive up to concentrations as high as 10^{-6} M in both preparations and showed no antagonism toward either morphine or DADLE.

Discussion

Enkephalinase Inhibition. Numerous structure–activity studied with substrates and inhibitors of enkephalinase[19,20,24-28] have delineated the key features needed for

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enzymatic recognition. Primary recognition is dependent on the nature of the amino acid residue at the P_1' and P_2' positions and the C-terminal carboxylic acid. There is also evidence for a hydrophobic S_1 subsite important in the binding of certain inhibitors.[29] Studies with a variety of peptide analogues have indicated that the effectiveness of enkephalinase inhibitors is influenced by the nature of the moiety interacting with the zinc ion in the active site of the enzyme. On the basis of these studies, we have designed and tested enkephalin analogues in which the scissile Gly^3–Phe^4 amide bond has been replaced with a tetrahedral phosphonamidate moiety. These compounds were expected to act as transition-state inhibitors of enkephalinase, mimicking the tetrahedral intermediate formed during amide bond hydrolysis.

Indeed, several of the target compounds are potent enkephalinase inhibitors, being approximately 1 order of magnitude more potent than the most potent dipeptide and tripeptide inhibitors.[27] However, the high K_i values of several of these inhibitors, particularly compounds **17**, **21**, and **22**, indicate that the tetrahedral phosphonamidate moiety is not enough, per se, to ensure potent enzyme inhibition. Evidently, the ethyl ester of the phosphonamidate moiety of compound **17** interferes with the coordination with the catalytic groups in the active site of the enzyme. In addition, there was a dramatic increase in the K_i values of the inhibitors **19–22** as the *N*-acetyl group of **19** was replaced with larger acyl groups. These results were somewhat surprising in view of the potent inhibitory activity of *N*-carboxymethyl enkephalinase inhibitors (RCH(COOH)PheLeuOH) that contain hydrophobic side chains (e.g., R = phenylethyl or *n*-butyl) at the P_1 position.[29] The low inhibition potency of these compounds, particularly compounds **21** and **22**, may be due to an inability of the phosphonamidate moiety to interact properly with the catalytic subunits in the active site of the enzyme. The differences between our findings with the phosphonamidate inhibitors and those with the *N*-carboxymethyl derivatives are interesting, and further studies into these discrepancies are merited.

Although extension of the (aminomethyl)phosphonamide molecule with alkylaryl acyl groups was detrimental to the inhibitory activity of compounds **20–22**, not all [(*N*-acylamino)methyl]phosphonamides are poor enkephalinase inhibitors. Thus, the greater activity of **23** and **24** may be a consequence of favorable interactions between the inhibitor and ancillary binding sites in the enzyme, eg. the tyrosyl side chain and a P_3 binding site. Alternatively, the lower K_i value of **23** and **24** may be ascribable to a better interaction between the phosphonamidate moiety and the catalytic groups in the active site, or possibly a combination of these factors. Also, conformational factors in the improved activities of **23** and **24** cannot be ruled out. The 58-fold greater potency of **23** for enkephalinase as compared to ACE is also of interest. Comparing the K_i ratios of ACE to enkephalinase for **19** and **23**, it appears that the increased selectivity of **23** is due to an increased affinity for enkephalinase rather than a decreased affinity for ACE. Introduction of a D-Ala^2 residue (compound **24**) led to a 6-fold decrease in this selectivity factor, caused by both a decrease in enkephalinase affinity and an increase in ACE affinity. These results indicate that there are differences in the secondary specificity of these two enzymes toward residues at the P_2 and P_3 positions.

Apparently, the enzyme is somewhat insensitive to the nature of the atom X in the tetrahedral moiety RP(X)(O^-)NHR', with **19** and **27** showing almost equal inhibitory potencies against enkephalinase. These similarities may be reflective of an equal affinity of the phosphonamide and phosphinothioyl moieties for the catalytic subunits in the active site of the enzyme.

ACE Inhibition. Extensive structure–activity studies have been performed in an attempt to characterize the active site of ACE.[30-33] These studies have shown that, like enkephalinase, ACE prefers substrates with a free carboxylic acid terminus. The S_2' binding site prefers aromatic amino acids or proline, whereas the S_1' site accepts a wide variety of P_1' residues, including Val, Arg, Ala, and Lys.[30] In addition, the enzymatic activity with various substrates is strongly affected by the nature of the P_1 residue, often preferring phenylalkyl or aminoalkyl substituents at this position. Although the phosphonamidate inhibitors were not specifically designed to inhibit ACE, we felt that (because of the similarities between enkephalinase and ACE) it was important to evaluate the inhibitory potencies of these compounds against both enzymes.

The ethyl phosphonamidate **17** was an equally poor inhibitor of enkephalinase and ACE—due to, in part, the inability of the ethyl phosphonamidate moiety to mimic the electronic and steric characteristics of the transition state. As with enkephalinase, the inhibitory potency of the leucine-containing analogues **19–22** was attenuated upon replacement of the *N*-acetyl group with larger alkylaryl acyl groups. Other groups have evaluated phosphonamidate peptide analogues as ACE inhibitors and have observed the same phenomenon: a wide variation in the K_i values depending on the substituent at the P_1 position.[8,34] It appears that the (*N*-acylamino)methyl moiety of the phosphonamidate inhibitors somehow interferes with optimal binding interactions between the substrate and enzyme, with the phosphonamidate inhibitors containing this functionality having K_i in the micromolar range or higher.[8,34] As mentioned earlier, ACE is less sensitive to these changes at the P_1 position than is enkephalinase. The differing sensitivities of these two enzymes toward the *N*-alkylaryl acyl groups may be of use in the design of even more selective inhibitors.

As might be expected for a dipeptidyl carboxypeptidase, the pentapeptide analogues **23** and **24** were not significantly more active than the *N*-acetyl derivatives **18** and **19**. Also, like enkephalinase, ACE appears to be insensitive to replacement of the P=O moiety with the phosphinothioyl (P=S) moiety, as demonstrated with compound **27**. In general, all of the phosphonamidates were 1 order to magnitude less potent against ACE as compared to enkephalinase and several orders of magnitude less potent

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than MK-421 (which is active at 10^{-11} M).

Evaluation at Opioid Receptors. The complete inactivity of the phosphonamidate peptide analogues **23** and **24** on both the GPI and MVD preparations is of significance. Numerous structure–activity studies on the enkephalins have shown that the Gly^3 position is a very sensitive position in terms of opioid activity, with (in most cases) even slight modifications at this position yielding weakly active or inactive compounds. Thus, replacement of the Gly^3 residue with alanine, β-alanine, AzGly (NHNHC(O)–), proline, D-proline, sarcosine, or glycylglycine or deletion of this residue [(des-Gly^3)enkephalin] affords enkephalin analogues with greatly attenuated activity.[35] In addition, enkephalin analogues in which the Gly^3–Phe^4 amide bond is replaced with either a thiomethylene moiety[36] or with a CH_2NH moiety[37] show greatly reduced opioid activity.

Replacement of the Gly^3 residue with an (aminomethyl)phosphinyl moiety alters the molecule in several ways. First, this substitution changes the geometry from that of the parent compound by virtue of the tetrahedral geometry of the phosphonamide moiety, causing a decrease in the C_3–C_3'–N_4 bond angle from approximately 120° for (Leu)enkephalin to a C_3–P_3'–N_4 bond angle of approximately 109° for the phosphonamidate analogues. Second, the stereoelectronic features of the phosphonamide moiety are quite different from that of a carboxylic amide bond, with the phosphonamide moiety having slightly longer bond lengths[38–40] and a greater electron density than the carboxylic amide. Third, the phosphonamide bond possesses greater flexibility than the corresponding carboxamide bond because of the lack of double-bond character in the P–N linkage. This permits for a greater degree of rotation about this bond and may allow the phosphonamidate analogue to adapt conformations considerably different from those found in leucine enkephalin. Furthermore, additional conformational changes due to the differing hydrogen-bonding capabilities and steric requirements of the phosphonamide moiety as compared to the carboxylic amide are likely.

Studies investigating the solution[41,42] and solid-state conformations[43–45] of the enkephalins have indicated that the molecule can adapt a variety of conformations. These include extended forms without internal hydrogen bonding and folded forms with a β-turn stabilized through internal hydrogen bonds. In fact, the unit cell of one crystalline form of leucine enkephalin contains four different conformers in which the Tyr, Phe, and Leu side chains have different orientations.[45]

Because of the multiple conformations that the enkephalin molecule adopts, it is difficult to draw any definitive conclusions regarding the conformational factors contributing to the inactivity of the phosphonamidate enkephalin analogues **23** and **24**. However, it is likely that, because of the disparate stereoelectronic characteristics of the planar carboxamide moiety and the tetrahedral phosphonamidate moiety, the loss of opioid activity is due to a loss of critical binding interactions between the opioid receptor and essential pharmacophores of the ligand.

Conclusions. The active sites of enkephalinase and ACE have been explored with a series of phosphonamidate peptide inhibitors. These studies have shown that these phosphonamide-containing peptide analogues are effective enkephalinase inhibitors and that within this series of phosphonamidates large alkylaryl acyl groups at the P_1 position are detrimental to inhibitory potency. However, there appear to be ancillary sites in addition to the S_1' and S_2' sites involved in the binding of larger peptides, as suggested by the potent activity of **23** and **24**. Likewise, activity against ACE is attenuated by large alkylaryl acyl moieties at the P_1 position, although to a lesser degree than with enkephalinase. In addition, the inactivity of **23** and **24** on the GPI and MVD preparations demonstrates that, in terms of opioid receptor affinity, a phosphonamidate moiety at the Gly^3-Phe^4 position is not tolerated well.

Experimental Section

Melting points were determined with a Thomas Hoover capillary apparatus and are uncorrected. Infrared spectra were taken on a Perkin-Elmer Model 281 spectrometer. Optical rotations were performed on a Perkin-Elmer 141 polarimeter using a 1-cm polarimeter cell. Elemental analyses were performed by MHW Laboratories, Phoenix, AZ. Mass spectral data were obtained on either an AEI MS-30 or Finnegan 4000 spectrometer. ^{1}H NMR spectra were recorded on a JEOL FX-90 of Nicolet NT 300 spectrometer. Chemical shifts are reported in ppm downfield from internal Me_4Si or DSS standards for ^{1}H NMR spectra and in ppm downfield from an external 85% phosphoric acid reference for ^{31}P NMR spectra. All ^{31}P NMR spectra were acquired at 121 MHz and are proton decoupled. ^{13}C NMR spectra were acquired at 22.5 MHz with complete proton decoupling, and chemical shifts are recorded as ppm downfield from either $CDCl_3$ or dioxane internal standards.

TLC plates (silica gel GF plates, 0.25-mm thickness) were obtained from Analtech, Inc., Neward, DE. Reversed-phase (RP) TLC plates (KC 18F, 200-μm thickness) were obtained from Whatman, Clifton, NJ, and reversed-phase chromatography was performed with Octyl (C8), 40-μm reversed-phase resin from Analytichem International, Harbor City, CA. Silica gel chromatography was performed with either Davisil silica gel (grade 633, 200–425 mesh, Aldrich Chemical Co., Milwaukee, WI) or MN-Kieselgel 60 silica gel (minus 200 mesh ASTM, Brinkman Instruments, Inc., Westbury, NY). Unless otherwise specified, all chromatography was performed on a medium-pressure liquid chromatographic (MPLC) system (Michel-Miller columns) equipped with an RP-SY-1CKC FMI pump (Fluid Metering, Inc., Oyster Bay, NY) and a double-beam UV monitor (Gilson Model 260, Gilson Medical Electronics, Inc., Middleton, WI), monitoring at a 254-nm wavelength.

All chemicals and solvents are reagent grade unless specified otherwise. THF was freshly distilled over sodium prior to use. Amino acids and all chemicals, unless specified otherwise, were obtained from either Aldrich Chemical Co., Milwaukee, WI, or Sigma Chemical Co., St. Louis, MI. All amino acids are the L enantiomers unless otherwise specified.

Solvent Systems: A, CCl_4; B, THF/CH_2Cl_2; C, EtOAc/$CHCl_3$ (1:3); D, EtOAc; E, C_6H_6; F, MeOH/$CHCl_3$ (9:1); G, EtOAc/EtOH/NH_4OH (90:10:5); H, MeOH; I, EtOAc/EtOH (7:1); J,

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EtOAc/EtOH (3:1); K, 2-propanol in $CHCl_3$ gradients; L, THF; M, CH_2Cl_2; N, EtOH; O, acetone/$CHCl_3$ (1:1); P, $CHCl_3$/MeOH/5% AcOH (85:10:5).

Mixed-Anhydride Coupling (Method A). All of the dipeptide derivatives were formed via the mixed-anhydride coupling method as described in an illustrative procedure in ref 46.

Removal of the *tert*-Butyloxycarbonyl Protecting Groups with Methanolic HCl (Method B). To a solution of the *N*-*tert*-butyloxycarbonyl-dipeptide methyl ester (9 mmol) in MeOH (9 mL) were added anisole (12 mmol) and 2.2 N methanolic HCl (25 mL). After the mixture was stirred at room temperature for 2.5–3.5 h, the solvent was removed in vacuo to afford the crude product. Recrystallization from MeOH/ether or EtOAc/ether afforded the product as a solid.

Removal of the *tert*-Butyloxycarbonyl Protecting Group with Trifluoroacetic Acid (Method C). A solution of the *N*-*tert*-butyloxycarbonyl benzyl ester (12 mmol) in trifluoroacetic acid (16 mmol) was stirred at room temperature for 90 min and then diluted with EtOAc (300 mL) and the solution adjusted to pH 8 with saturated $NaHCO_3$ (200 mL). The organic layer was washed with 50% aqueous NaCl (200 mL) and H_2O (200 mL), dried (Na_2SO_4), and concentrated to afford the amine as an oil. This amine was dissolved in ether (100 mL) and treated with saturated ethereal HCl (100 mL) to yield the hydrochloride salt as a solid.

Removal of the Methyl Esters with NaOH (Method D). To a solution of the dipeptide methyl ester (2.45 mmol) in CH_3CN (9 mL) was added 0.25 N NaOH (2.57 mmol). After stirring at room temperature for 90 min, the reaction mixture was diluted with H_2O (30 mL) and the aqueous layer washed with EtOAc (2 × 30 mL). The aqueous layer was then adjusted to pH 2 with 10% citric acid and extracted with EtOAc (4 × 30 mL). These combined extracts were dried (Na_2SO_4) and concentrated to afford the product as a solid.

Phthaloyl-Protected (Aminomethyl)phosphonamides (4–6). A solution of the diester phosphonate (50 mmol) in the reaction solvent (45 mL) containing PCl_5 (55 mmol) was refluxed under nitrogen. Upon completion of the reaction, the solvent was frozen and removed in vacuo to afford the crude phosphonochloridate, which was not further purified. A solution of this phosphonochloridate in dry THF (35 mL) was added dropwise over a 20-min period (under a nitrogen atmosphere) to a solution of the dipeptide hydrochloride salt (38 mmol) in CH_2Cl_2 (35 mL) containing Et_3N (150 mmol) at 0 to −5 °C. The reaction mixture was then stirred at room temperature until completion, and then the solvent removed in vacuo to afford an oily residue. This residue was diluted with CH_2Cl_2 (250 mL) and washed successively with 100-mL portions of 5% citric acid, 5% $NaHCO_3$, and H_2O, then dried (Na_2SO_4), and concentrated to yield the crude product. Chromatographic purification (silica) afforded the product as a solid.

(Aminomethyl)phosphonamides (7–9). To a solution of the phthaloyl-protected phosphonamide (5 mmol) in MeOH (10 mL) was added 1 N methanolic hydrazine (5.3 mmol) and the reaction mixture stirred at room temperature. Upon completion of the reaction, the reaction mixture was diluted with MeOH (10 mL), filtered to remove the precipitated phthalhydrazide, and reconcentrated to yield the crude product as an oil. Chromatographic purification (silica) afforded the product as a clear oil.

[(N-Acylamino)methyl]phosphonamides (10–14). To a solution of the (aminomethyl)phosphonamide (1 mmol) in the reaction solvent (2 mL) was added pyridine (1.3 mmol) followed by the acyl chloride (1.1 mmol) and the reaction mixture stirred at room temperature. When the reaction was completed, the reaction mixture was diluted with solvent (50 mL) and washed successively with 30-mL portions of 5% citric acid, 5% $NaHCO_3$, and H_2O. The organic layer was then dried (Na_2SO_4) and concentrated to afford the crude product as an oil. Chromatographic purification (silica) afforded the pure product.

Pentapeptide Phosphonamidate Analogues (15, 16). To a solution of the dipeptide CbzTyr(OBzl)-R-OH (0.5 mmol; R = Gly, D-Ala), the α-aminophosphonamide (0.5 mmol; compounds **7–9**), and HOBt (0.5 mmol) in dry THF (4 mL) was added a solution of DCC (0.5 mmol) in dry THF (4 mL) and the reaction mixture stirred at room temperature. Upon completion of the reaction, the solvent was removed in vacuo and the residue diluted with $CHCl_3$ (65 mL) and washed with 35-mL portions of 10% $NaHCO_3$, 2% citric acid, and H_2O. The organic layer was then dried (Na_2SO_4) and concentrated to give the crude product. Chromatographic purification (silica) afforded the product as a solid.

Dibenzyl (Phthalimidomethyl)phosphonate (3). Dibenzyl phosphite (20.62 mL, 0.0933 mol) in dry THF (100 mL) was added dropwise under a nitrogen atmosphere to a cooled solution (−10 to −15 °C) of sodium hydride (60% mineral oil dispersion; 4.2 g, 0.105 mmol) in dry THF (60 mL) with vigorous stirring over a 15-min period. After the mixture was stirred for 15 min, the resulting sodium salt of dibenzyl phosphite was added dropwise (via a transferring needle) to a cooled solution (−10 to −15 °C) of *N*-(bromomethyl)phthalimide (14.0 g, 0.0583 mmol) in dry THF (60 mL) over a 10-min period. The reaction mixture was stirred at room temperature for 16 h, the solvent was removed in vacuo, and the residue was diluted with $CHCl_3$ (350 mL). The organic layer was washed with 100-mL portions of 5% citric acid, 5% $NaHCO_3$, and H_2O (twice) and dried (Na_2SO_4) and the $CHCl_3$ removed in vacuo to afford the crude product as a yellow oil. Chromatographic purification (silica gel, EtOAc/hexane (1:1)) followed by recrystallization from EtOAc/hexane yielded the dibenzyl phosphonate **3** as a crystalline solid: 17.42 g (71%); R_f 0.30 (EtOAc/hexane (1:1)); ^1H NMR ($CDCl_3$) δ 7.82–7.67 (m, 4, Pht aromatic), 7.30–7.26 (m, 10, benzyl aromatic), 5.16–5.02 (m, 4, OCH_2), 4.16 (d, 2, J = 11.5 Hz, CH_2P); ^{13}C NMR ($CDCl_3$) δ 166.4 (C=O), 135.7, 135.4, 133.8, 131.5, 128.1, 127.8, 123.1 (aromatic), 67.8 (d, $POCH_2$, J = 2.5 Hz), 33.4 (d, CH_2P, J = 156.9 Hz); ^{31}P NMR ($CDCl_3$) δ 21.01; CI–MS (NH_3) m/e 422 ($M^+ + 1$), 421 ($M^+ - 1$). Anal. ($C_{23}H_{20}NO_5P$) C, H, N, P.

***N*-[(2-Cyanoethoxy)methylphosphinothioyl]phenylalanylleucine Methyl Ester (26).** A solution of methylphosphinothioic dichloride (Alfa Chemical Co.; 159.3 μL, 1.520 mmol) in CH_2Cl_2 (5 mL) was cooled to 0 °C under a nitrogen atmosphere and treated with Et_3N (212 μL, 1.520 mmol) and 3-hydroxypropanenitrile (103.8 μL, 1.520 mmol). After the mixture was stirred at room temperature for 3 h, a solution of phenylalanylleucine methyl ester, hydrochloride salt (500 mg, 1.520 mmol), and Et_3N (565 μL, 4.054 mmol) in CH_2Cl_2 (5 mL) was added, and the reaction mixture stirred for 3 h more. The reaction mixture was diluted with CH_2Cl_2 (60 ml) and the organic layer washed with 35-mL portions of 10% citric acid (twice) followed by H_2O (twice), dried (Na_2SO_4), and concentrated to yield the crude product as a solid. Chromatographic purification (silica gel, EtAc/hexane (1:2)) afforded **26** as a clear oil: 210 mg (31%); R_f 0.28 (EtOAc/hexane (1:2)); ^1H NMR ($CDCl_3$) δ 7.40–7.15 (m, 6, aromatic NH), 6.70–6.45 (d.d., 1, NH), 4.65 (m, 1, NH), 4.18 (m, 1 α-H), 4.00 (m, 1, α-H), 3.75–3.30 (m, 2, $POCH_2$), 3.72 (s, 3, OCH_3), 3.20–2.90 (m, 2, Phe β-H), 2.60–2.50 (m, 2, CH_2CN), 1.74–1.50 (m, 3, Leu β,γ-H), 1.66 (dd, 3, CH_3P), 0.98–0.85 (m, 6, Leu δ-H); ^{31}P NMR ($CDCl_3$) δ 86.28, 86.03; CI–MS (CH_4) m/e 440 ($M^+ + 1$), 438 ($M^+ - 1$). Anal. ($C_{20}H_{30}N_3O_4SP$) C, H, N, P, S.

***N*-[[(Acetylamino)methyl]ethoxyphosphinyl]phenylalanylmethionine, Lithium Salt (17).** Compound **10** (60 mg, 0.127 mmol) was treated with 0.25 N LiOH (0.557 mL, 0.139 mmol) and the reaction mixture stirred at room temperature for 1 h. The H_2O was removed by lyophilization and the crude product applied to a C-8 (octyl) reversed-phase column and eluted with H_2O followed by MeOH to afford **17** as a solid: 48 mg (81%); mp 144–146 °C; ^1H NMR (D_2O) δ 7.45–7.25 (m, 5, aromatic), 4.30 (m, α-H; other α-H under HDO peak), 4.15–3.65 (m, 2, $POCH_2$), 3.55–2.80 (m, 4, Phe β-H, CH_2P), 2.45 (m, 2, Met γ-H), 2.10–1.90 (m, 8, Met β,ε-H, $CH_3(O)$–), 1.25–1.10 (m, 3, $POCH_2CH_3$); ^{31}P NMR (D_2O) δ 29.04, 27.35. Anal. ($C_{19}H_{29}N_3O_6PSLi$) C, H, N, P.

***N*-[[(Acetylamino)methyl]hydroxyphosphinyl]phenylalanylmethionine, Disodium Salt (18).** Compound **10** (30 mg, 0.063 mmol) was treated with 2 N NaOH (96 μL, 0.190 mmol) and the reaction mixture stirred at room temperature. After 1 day the H_2O was removed by lyophilization to afford **18** as a solid: 24.7 mg (82%, 63% pure by P analysis); mp 205 °C (dec); R_f 0.82

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(C-18 RP-TLC plates, MeOH); ^{1}H NMR (D_2O) δ 7.40–7.25 (m, 5, aromatic), 4.22–4.18 (m, 1, α-H), 4.05–3.90 (m, 1, α-H), 3.20 (d, 2, J = 12.9 Hz, CH_2P), 3.2–3.05 (m, 2, Phe β-H), 2.45–2.30 (m, 4, Met β,γ-H), 2.07, 2.01 (6, $CH_3C(O)$–, Met ε-H); ^{31}P NMR (D_2O) δ 18.1. Anal. Calcd for $C_{17}H_{24}N_3O_6PSNa_2$: P, 6.52. Found: P, 4.10.

***N*-[[(Acetylamino)methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (19).** Compound 11 (50 mg, 0.110 mmol) was treated with 2 N NaOH (164 μL, 0.329 mmol) and the reaction mixture stirred at room temperature. After 1 day the H_2O was removed by lyophilization to afford **19** as a solid: 43.2 mg (81%, 74% pure by P analysis); mp 222 °C (dec); R_f 0.83 (C-18 RP-TLC plates, MeOH); ^{1}H NMR (D_2O) δ 7.40–6.25 (m, 5, aromatic), 4.2–4.17 (m, 1, α-H), 4.05–3.95 (m, 1, α-H), 3.08–2.99 (m, 4, Phe β-H, CH_2P), 1.94 (s, 3, $CH_3C(O)$–), 1.58–1.30 (m, 3, Leu β,γ-H), 0.87–0.85 (d, 6, Leu δ-H); ^{31}P NMR (D_2O) δ 18.07. Anal. Calcd for $C_{18}H_{26}N_3O_6PNa_2$: P, 6.77. Found: P, 5.03.

***N*-[[(Benzoylamino)methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (20).** A solution of **12** (50 mg, 0.0762 mmol) in 50% aqueous EtOH (6 mL) containing $NaHCO_3$ (12.8 mg, 0.1524 mmol) and 10% Pd/C (5 mg) was hydrogenated at atmospheric pressure for 90 min. The reaction mixture was then diluted with 50% aqueous EtOH (15 mL) and the solvent removed by lyophilization to afford **20** as a solid: 33 mg (85%, 80.5% pure by P analysis); mp 160 °C (dec); R_f 0.26 (50% aqueous NaCl/MeOH (2:1), C-18 RP-TLC); ^{1}H NMR (D_2O) δ 7.9–7.25 (m, aromatic), 4.2–4.15 (t, 1, α-H), 4.05–3.95 (m, 1, α-H), 3.45 (d, CH_2P), 3.2–3.0 (m, 2, Phe β-H), 1.6–1.4 (m, 3, Leu β,γ-H), 0.9–0.8 (m, 6, Leu δ-H); ^{31}P NMR (D_2O) δ 14.51. Anal. Calcd for $C_{23}H_{28}N_3O_6PNa_2$: P, 5.96. Found: P, 4.80.

***N*-[[[(Phenylacetyl)amino]methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (21).** A solution of **13** (50 mg, 0.0746 mmol) in 50% aqueous EtOH (6 mL) containing $NaHCO_3$ (12.5 mg, 0.1493 mmol) and 10% Pd/C (5 mg) was hydrogenated at atmospheric pressure for 90 min. The reaction mixture was then diluted with 50% aqueous EtOH (15 mL) and the solvent removed by lyophilization to afford **21** as a solid: 36.6 mg (92%, 80.5% pure by P analysis); mp 160 °C (dec); R_f 0.27 (50% aqueous NaCl/MeOH (2:1), C-18 RP-TLC); ^{1}H NMR (D_2O) δ 7.5–7.25 (m, 10, aromatic), 4.2 (m, 1, α-H), 4.1 (m, 1, α-H), 3.65 (s, 2, $CH_2C_6H_5$), 3.3–3.0 (m, 4, CH_2P, Phe β-H), 1.6–1.45 (m, 3, Leu β,γ-H), 0.95–0.80 (m, 6, Leu δ-H); ^{31}P NMR (D_2O) δ 14.62. Anal. Calcd for $C_{24}H_{30}N_3O_6PNa_2$: P, 5.81. Found: P, 4.68.

***N*-[[[(3-Phenylpropionoyl)amino]methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (22).** A solution of 14 (50 mg, 0.0731 mmol) in 50% aqueous EtOH (6 mL) containing $NaHCO_3$ (12.28 mg, 0.1462 mmol) and 10% Pd/C (5 mg) was hydrogenated at atmospheric pressure for 90 min. The reaction mixture was then diluted with 50% aqueous EtOH (15 mL) and the solvent removed by lyophilization to afford **22** as a solid: 34.2 mg (85%, 87% pure by P analysis); mp 160 °C (dec); R_f 0.27 (50% aqueous NaCl/MeOH (2:1), C-18 RP-TLC); ^{1}H NMR (D_2O) δ 7.5–7.2 (m, 22, aromatic, NH), 6.4–6.2 (br s, 1, NH), 4.2 (m, 1, α-H), 4.0 (m, 1, α-H), 3.2–3.0 (m, 4, CH_2P, Phe β-H), 3.0–2.9 (m, 2, $C_6H_5CH_2CH_2$–), 2.65–2.5 (t, 2, $C_6H_5CH_2CH_2$–), 1.6–1.4 (m, 3, Leu β,γ-H), 0.90–0.80 (m, 6, Leu δ-H); ^{31}P NMR (D_2O) δ 14.50. Anal. Calcd for $C_{25}H_{32}N_3O_6PNa_2$: P, 5.66. Found: P, 4.95.

***N*-[[[(Tyrosylglycyl)amino]methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (23).** A solution of compound **15** (30 mg, 0.0301 mmol) and $NaHCO_3$ (5.06 mg, 0.0602 mmol) in 50% aqueous EtOH (5 mL) was treated with 10% Pd/C (10 mg) and hydrogenated at atmospheric pressure for 6 h. The reaction mixture was then filtered through Celite and the solvent removed by lyophilization to afford **23** as a solid: 17.5 mg (92%); mp 157–165 °C (dec); R_f 0.86 (MeOH, C-18 RP-TLC); ^{31}P NMR (D_2O) δ 17.77; ^{1}H NMR (D_2O) δ 7.45–6.85 (m, 9, aromatic), 4.80–3.80 (m, α-H, partially buried under HDO peak), 3.25–2.90 (m, 6, CH_2P, Phe, Tyr β-H), 1.60–1.50 (m, 2, Leu β-H), 1.50–1.40 (m, 1, Leu γ-H), 0.90–0.80 (m, 6, Leu δ-H). Anal. Calcd for $C_{27}H_{36}N_5O_8PNa_2$: P, 4.87. Found: P, 5.13.

***N*-[[[(Tyrosyl-D-alanyl)amino]methyl]hydroxyphosphinyl]phenylalanylleucine, Disodium Salt (24).** A solution of compound **16** (50 mg, 0.0494 mmol) and $NaHCO_3$ (8.30 mg, 0.0989 mmol) in 20% aqueous EtOH (5 mL) was treated with 10% Pd/C (5 mg) and hydrogenated at atmospheric pressure for 6 h. The reaction mixture was then filtered through Celite and the solvent removed by lyophilization to afford **24** as a solid: 10.0 mg (31%, 74% pure by P analysis); mp 130–150 °C (dec); R_f 0.89 (MeOH, C-18 RP-TLC); ^{1}H NMR (D_2O) δ 7.40–6.85 (m, 9, aromatic), 4.80–3.95 (m, α-H, partially buried under HDO peak), 3.75–3.55 (m, 1, α-H), 3.20–2.70 (m, 6, CH_2P, Tyr, Phe β-H), 1.60–1.50 (m, 2, Leu β-H), 1.45–1.35 (m, 1, Leu δ-H), 1.07 (d, 3, Ala β-H), 0.90–0.80 (m, 6, Leu δ-H); ^{31}P NMR (D_2O) δ 17.59. Anal. Calcd for $C_{28}H_{38}N_5O_8PNa_2$: P, 4.77. Found: P, 3.53.

***N*-(Hydroxymethylphosphinothioyl)phenylalanylleucine, Disodium Salt (27).** Compound **26** (107.1 mg, 0.2437 mmol) in MeOH was treated with 2 N NaOH (250 μL, 0.50 mmol) and the reaction mixture stirred at room temperature. After 90 min the reaction mixture was diluted with H_2O (30 mL), the aqueous layer washed with ethyl ether (3 × 30 mL), and the H_2O removed by lyophilization to afford the product as a solid: 83.6 mg; mp 190–192 °C; R_f 0.29 (50% aqueous NaCl/MeOH (1:1), C-18 RP-TLC); ^{1}H NMR (D_2O) δ 7.45–7.20 (m, 5, aromatic), 4.20–4.05 (m, 2, Phe, Leu α-H), 3.25–3.00 (m, 2, Phe β-H), 1.60–1.45 (m, 6, CH_3P, Leu β,γ-H), 0.95–0.80 (m, 6, Leu δ-H); ^{31}P NMR (D_2O) 64.54, 63.85. Anal. ($C_{16}H_{23}N_2O_4SPNa_2 \cdot 1.5H_2O$) C, H, N, P, S.

Biochemical Studies. The enzyme studies were performed on the metalloendopeptidase (enkephalinase) from rat kidney[4] or human brain (postmortem) purified by the same procedure and on ACE from rat brain immobilized on anti-ACE IgG sepharose[18] or a soluble form of the enzyme purified by means of affinity chromatography on MK-521 coupled to sepharose by the method of El-Dorry et al.[47]

The reaction mixture for enkephalinase consisted of 100 μL containing 4 μmol Tris-HCl, pH 7.6, and 1–20 nmol of (Leu)-enkephalin incubated at 37 °C for 15–30 min, then fixed with 20% PCA to yield a final concentration of 4%. Aliquots of the enzymatic incubations (20–100 μL) were analyzed on a C-18 5-μm adsorbosphere reversed-phase column, eluting the cleavage product Tyr-Gly-Gly isocratically with 5% acetonitrile in 0.1 M potassium phosphate buffer, pH 3.0. For ACE, the reaction mixture consisted of 100 μL containing 5 μmol of HEPES, pH 8.1, 30 mol of NaCl, and 5 mol of Hip-His-Leu. The release of His-Leu was measured fluorimetrically following coupling to OPA.[48] In both cases, between 0.01 and 0.02 μg of purified enzyme was employed.

The K_i values for the metalloendopeptidase were calculated from the Dixon plots and are the means of two to three determinations agreeing within 19%; for the human brain, enzyme values are the means of four to six determinations and are given plus or minus the standard deviation. The K_i values for ACE were computed from the $IC_{50} = K_i(1 + S/K_m)$, using a K_m value of 100 μM for Leu(enkephalin) or 3 mM for Hip-His-Leu. Thiorphan was obtained from Ciba-Geigy (Ardsky, NY), MK-421 from Merck, and phosphoramidon from the US-Japan CO-operation Cancer Research Program.

Acknowledgment. The authors thank Myron Benuck for supplying enkephalinase and angiotensin-converting enzyme and Victoria Darrow for technical assistance. We also thank Dr. Ian Jardine of the Mayo Clinic, Rochester, MI, for obtaining the fast atom bombardment mass spectra. This research was supported, in part, by an NIH National Research Service Award (Grant 5T32GM07994-02).

Registry No. 1, 5332-26-3; 2, 33512-26-4; 3, 96914-74-8; 4, 96914-75-9; 5, 96914-76-0; 6, 96914-77-1; 7, 96928-71-1; 8, 96914-78-2; 9, 96914-79-3; 10, 96914-80-6; 11, 96914-81-7; 12, 96914-82-8; 13, 96914-83-9; 14, 96914-84-0; 15, 96914-85-1; 16, 96914-86-2; 17, 96914-87-3; 17 free acid, 96914-88-4; 18, 96914-89-5; 18 free acid, 96914-90-8; 19, 96914-91-9; 19 free acid, 96914-92-0;

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N-(Aminoalkyl)imide Antineoplastic Agents. Synthesis and Biological Activity

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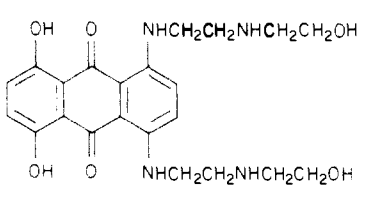
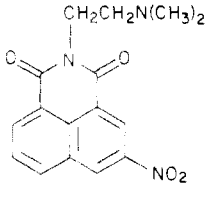
The similarity of the side-chain characteristics of 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-anthraquinone (DHAQ), discovered by us in 1978, and those of the N-substituted imides of 3-nitro-1,8-naphthalic acid, discovered by other investigators recently, led us to conduct a systematic study on the *N*-(aminoalkyl)-substituted derivatives of a variety of imides. Areas of study included (a) selection of the ring system, (b) modification of the side chain, (c) substitution on certain chosen ring systems, and (d) combinations of the aforementioned variants. Preliminary biological activity screening indicated that *N*-(dialkylaminoethyl)imides of the 3,6-dinitro- and 3,6-diamino-1,8-naphthalic acid system possessed prominent antileukemia and antimelanoma activity in both in vitro and in vivo experimental tumor systems.

Since the discovery of the outstanding anticancer activity of 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]ethyl]amino]anthraquinone, DHAQ (1), in 1978,[1-3] we have been interested in introducing its side-chain characteristics to other ring systems[4,5] as well as in the modification of the side chain.[1,5-8] It was found that the basic nitrogen atom in the middle of the side chain of DHAQ is of vital importance to its antineoplastic activity since replacement of this atom by a sulfur,[1] a carbon,[1,5] or an oxygen atom[8] resulted in a total loss of activity. Modification of the terminal chain substituents attached to the pertinent nitrogen atom by other nonbulky substituents, on the other hand, still retains the antineoplastic activity. For example, substitution of the 2-[(2-hydroxyethyl)amino]ethylamine side chain by the 2-(dimethylamino)ethylamine side chain of DHAQ does not nullify the original activity,[1] although compounds containing the former side chain seem to be somewhat superior to the latter in the anthraquinone series.[1,4]

It is, therefore, of interest to note the reported antineoplastic activity of Mitonafide (2) and three related



1



2

N-substituted imides of 3-nitro-1,8-naphthalic acid against mouse Ehrlich ascites and rat Yoshida carcinoma as well as in vitro cytotoxicity against the HeLa cells.[9-11] These naphthalimides did not inhibit the protein synthesis but did inhibit DNA and RNA synthesis,[11] bound to the double-helical DNA by intercalation,[12] inhibited the incorporation of DNA precursor into the acid-insoluble fraction of cultured cells,[13] induced DNA strand break, and increased the frequency of sister chromatid exchanges and chromosome aberration.[14]

The reported structure–activity characteristics of the side chains of these naphthalimides bear a striking resemblance to that of our anthraquinone derivatives. For example, these investigators stated[9-11] that (a) the basic nitrogen atom on the side chain is essential to their activity, (b) substitution of the nitrogen atom by an oxygen, a sulfur, or a carbon atom resulted in inactive compounds, and (c) the activity is maximal when the nitrogen atom is separated from the ring nitrogen by two methylene units.

1,8-Naphthalimides are not the only imides that possess antineoplastic activity. Among the N-unsubstituted imides, the inhibitory activity of glutarimide antibiotics such as cycloheximide (3a) and Streptovitacin (3b) against a number of experimental animal tumors is well-known.[15-19]

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