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Communications to the Editor

Difluorostatine- and Difluorostatone-Containing Peptides as Potent and Specific Renin Inhibitors

Sir:

A recent publication by Abeles et al.[1] on fluoro ketone inhibitors of hydrolytic enzymes prompted us to report our own observation in the renin inhibitor area.

Pepstatin is a naturally occurring pentapeptide[2] that is a general aspartyl protease inhibitor. It was proposed that the statine residue, 4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid, acts as a structural analogue of the tetrahedral species during enzymatic hydrolysis of a peptidic bond.[3] More recently, the ketone analogue has been prepared by the oxidation of the 3(*S*)-hydroxyl group of statine in a pepstatin analogue.[4] The ketone carbonyl has been shown by ^{13}C NMR spectroscopy to be converted to a tetrahedral acetal upon binding to pepsin via enzyme-catalyzed addition of water.[5] This pseudo substrate is a weaker inhibitor of pepsin than the corresponding statine-containing peptide. The decreased binding constant might be attributed to the poor tendency of the ketone carbonyl to undergo hydration.

A + H_2O ⇌ B (1)

To facilitate the ease of hydration of the ketone, we proposed to introduce electron-withdrawing fluorine atoms on the methylene carbon adjacent to the carbonyl. In this way, the difluorostatone unit in peptide A should exist predominantly in the hydrated form B. The greater electrophilicity of the carbonyl of difluorostatone over the nonfluorinated statone should also facilitate the enzyme-catalyzed addition of water to form the tetrahedral acetal upon binding to the active site.

We chose to examine this hypothesis on the aspartyl protease renin, which cleaves the protein substrate angiotensinogen into the decapeptide angiotensin I, which in turn is cleaved by converting enzyme into the pressor octapeptide angiotensin II.[6] Highly potent competitive inhibitors of renin have already been reported in which statine was incorporated into renin substrate analogues.[7] Successful renin inhibitors could provide agents for control of cases of renin-associated hypertension.

Hallinan and Fried[8] reported the preparation of 2,2-difluoro-3-hydroxy esters by a Reformatsky reaction. Application of this procedure to Boc-L-leucinal as shown in Scheme I afforded the adducts **1a** and **1b** in good yield, and they were readily separated by chromatography.[9]

Scheme I[a]

(**1a**: R^1 = OH, R^2 = H; **1b**: R^1 = H, R^2 = OH) → (**2a**, **2b**) → (**3a**, **3b**) → **4b**, **4c**; (**4**) → **4f**

[a] Key: (a) BrF_2CCO_2Et, Zn, THF; (b) 1 equiv of NaOH, aqueous THF; L-isoleucyl-2-pyridylmethylamide, HOBT, DCC, CH_2Cl_2; (c) CF_3CO_2H; Boc-His(Ts), $(EtO)_2P(O)CN$, Et_3N, CH_2Cl_2; CF_3CO_2H; Boc-Phe, $(EtO)_2P(O)CN$, Et_3N, CH_2Cl_2; (d) HOBT, CH_3OH; (e) Me_2SO, $(COCl)_2$, CH_2Cl_2; Et_3N.

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(9) **1a**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.96 (d, 3 H, *J* = 6 Hz), 0.97 (d, 3 H, *J* = 6 Hz), 1.39 (t, 3 H, *J* = 7 Hz), 1.46 (s, 9 H), 4.37 (q, 2 H, *J* = 7 Hz), 4.83 (d, 1 H, *J* = 10 Hz); IR (neat) 3390, 1760, 1690 cm^{-1}; $[\alpha]_D$ −11° (*c* 0.615, $CHCl_3$). Anal. ($C_{15}H_{27}NO_5F_2$) C, H, N. **1b**: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.95 (d, 3 H, *J* = 6 Hz), 0.98 (d, 3 H, *J* = 7 Hz), 1.39 (t, 3 H, *J* = 7 Hz), 1.47 (s, 9 H), 4.38 (q, 2 H, *J* = 7 Hz), 4.71 (d, 1 H, *J* = 7 Hz); IR (neat) 3390, 1760, 1695 cm^{-1}; $[\alpha]_D$ −31° (*c* 0.646, $CHCl_3$). Anal. ($C_{15}H_{27}NO_5F_2$) C, H, N.

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Table I. Renin Inhibitory Test Results[a]

Boc-Phe-His-X-Ile-NHCH2-(2-pyridyl) (4)[b]

X		IC_{50} (nM)
	4a	1.7
	4b	12
	4c	730
	4d	34
	4e	0.52

[a] Compounds 4a–f were assayed for plasma renin inhibitory activity as follows: Lyophilized human plasma with 0.1% EDTA was obtained commercially (New England Nuclear). The angiotensin I generation step utilized 250 μL of plasma, 2.5 μL phenylmethylsulfonyl fluoride, 25 μL of maleate buffer (pH 6.0), and 10 μL of an appropriate concentration of inhibitor in a 1% Tween 80 in water vehicle. Incubation was for 90 min at 37 °C. Radioimmunoassay for angiotensin I was carried out with a commercial kit (Clinical Assays). Plasma renin activity values for inhibitor tubes were compared to control tubes to estimate percent inhibition. The inhibition results were expressed as I_{50} values which were obtained by plotting three to four inhibitor concentrations on semilog graph paper and estimating the concentration producing 50% inhibition. [b] Physical characteristics were established by high-resolution fast atom bombardment mass spectrometry and reversed-phase high-pressure liquid chromatography.

Two reaction conditions were examined. Under sonicating condition at room temperature, a 70:30 mixture of **1a** and **1b** was obtained in 80% yield, whereas the usual refluxing condition gave the diastereomer **1a** essentially as the only product. When the sonicated reaction mixture was heated to reflux in THF for 30 min, it equilibrated and returned only the thermodynamically favored **1a** isomer. The optical purity of the adduct **1a** was determined via its Mosher ester.[10] The ^{19}F NMR signals from the trifluoromethyl groups of the diastereomeric Mosher esters derived from a racemic sample of **1a** were well-resolved singlets at 89.9 and 90.6 ppm (downfield from hexafluorobenzene). It could be determined that compound **1a** from the refluxing condition was 90% ee and no detectable racemization was observed for compound **1a** from the sonicating condition.

The esters **1a** and **1b** were hydrolyzed with 1 equiv of NaOH and the resulting salts lyophilized. These salts were coupled to L-isoleucyl-2-pyridylmethylamide to give the adducts **2a** and **2b**. Two more amino acids were sequentially added by using diethylphosphoryl cyanide as coupling reagent[11] to afford peptides **3a** and **3b**. Removal of the tosyl protecting group gave the inhibitors **4b** and **4c**, respectively.

Oxidation of the alcohol **3a** or **3b** with dimethyl sulfoxide and oxalyl chloride[12] led to the same epimeric mixture 4. Removal of the tosyl protecting group afforded the inhibitors **4f**, which could be separated into 4e and its C-4 epimer by preparative high-pressure liquid chromatography. These peptides[13] were compared with regards to their renin inhibitory activities in Table I.

The difluorostatine-containing peptide **4b** is a potent renin inhibitor whereas the epimer **4c** is much less effective. This result is in accord with the earlier finding that the 3(*S*)-hydroxyl group of statine in a pepstatin analogue is much better inhibitor of pepsin than the corresponding 3(*R*)-hydroxy analogue.[14] It can be noted that statine-containing peptide **4a** is 1 order of magnitude more potent than the fluorine-containing peptide **4b**. This would

Table II. Comparative Inhibition of Selected Enzymes

	IC_{50} (M)			
	Human Renin	Porcine Pepsin[a]	Bovine Cathepsin D[b]	Rabbit ACE[c]
peptide **4f**[d]	1.4×10^{-9}	4.2×10^{-5}	1.7×10^{-6}	24% at 10^{-3}
pepstatin[e]	6.0×10^{-6}	1.0×10^{-8}	2.8×10^{-8}	—
RIP[f]	9.4×10^{-6}	0% at 10^{-4}	0% at 10^{-3}	1.2×10^{-5}

[a] Inhibition of pepsin was determined as described using porcine pepsin (Sigma), porcine hemoglobin (Sigma), and 0.02 M KCl-HCl buffer (pH 2): Aoyagi, T.; Kunimoto, S.; Morishima, H.; Takeuchi, T.: Umezawa, H. *J. Antibiot.* 1971, *24*, 687. The compounds were dissolved in the buffer, and after incubation for 25 min at 37 °C with the enzyme and substrate, perchloric acid was added and the absorbance of the acid-soluble fractions measured at 280 nm. The percent inhibition was estimated from the net absorbance of inhibited assays in relation to uninhibited control assays. A plot of percent inhibition vs. log inhibitor concentration was constructed with the I_{50} defined as the inhibitor concentration causing 50% inhibition. [b] Bovine cathepsin D (Sigma) inhibition was measured via a slightly modified method of: Aoyagi, T.; Morishima, H.; Nishizawa, R.; Kunimoto, S.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* 1972, *25*, 689. The compounds were dissolved in 0.2 M acetate buffer (pH 3.2) as was the porcine hemoglobin (Sigma) substrate. Incubations for 30 min at 37 °C were terminated in ice, and 1.7 M perchloric acid was added to all mixtures. Extinction at 280 nm was measured and the I_{50} determined as in (a). [c] Inhibition of angiotensin II converting enzyme activity was estimated as described by: Cushman, D. W.; Cheung, H. S. *Biochem. Pharmacol.* 1971, *20*, 1637. Briefly, the enzyme was extracted from rabbit lung. The substrate was hippuryl-L-histidyl-L-leucine (Aldrich), and the buffer was K_2HPO_4 (pH 8.3). The compounds were dissolved in 50% dimethylformamide. Incubations were for 60 min at 37 °C and were terminated via HCl addition. Absorbance of the ethyl acetate extracted hippuric acid was measured at 228 nm. The I_{50} was calculated as in (a). [d] An approximately equal mixture of 4e and its C-4 epimer. [e] Iva-Val-Val-Sta-Ala-Sta. [f] Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys.

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(13) FAB MS: $[M + H]^+$ at m/z (calcd (found)): **4a**, 763.4506 (763.4522); **4b**, 799.4318 (799.4305); **4c**, 799.4318 (799.4305); **4d** 761.4350 (761.4357); 4e 797.4161 (797.4169); **4f** 797.4161 (797.4193).

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suggest the importance of the hydrogen bonding of the 3-hydroxyl group.

As anticipated,[4] the statone-containing peptide **4d** is much less potent than the hydroxy analogue **4a**. The notion that substitution of fluorine atoms adjacent to the carbonyl group to increase its propensity toward addition of water and sp^3 hybridization is clearly illustrated in the increased inhibitory potency of the peptide **4e** over that of the nonfluorinated peptide **4d**.

A high degree of enzyme specificity is desirable for a potentially successful therapeutic agent. Pepstatin, for example, is a general aspartyl protease inhibitor and shows poor selectivity. A substrate analogue such a RIP[15] does not discriminate between renin and converting enzyme. The difluoro ketone **4f** does exhibit high renin specificity. It is a very poor inhibitor of converting enzyme and is 3–4 orders of magnitude less effective against pepsin and cathepsin D. Such target specificity is also important for a better understanding of a mechanistic based biological action.

The transition-state analogue concept remains a viable approach in the design of potent enzyme inhibitors as illustrated in aspartyl proteases.[16] The work presented in this report lends support to the value of an understanding of enzymatic mechanism as an aid to create effective inhibitors of therapeutically important enzymes.

Registry No. **1a**, 96056-65-4; **1b**, 97920-08-6; **2a**, 97920-09-7; **2b**, 97995-50-1; **3a**, 97920-10-0; **3b**, 97995-51-2; **4a**, 97920-11-1; **4b**, 97920-12-2; **4c**, 97995-52-3; **4d**, 97920-13-3; **4e** (isomer 1), 97920-14-4; **4e** (isomer 2), 97995-53-4; **4e** tosyl deriv. (isomer 1), 97920-15-5; **4e** tosyl deriv. (isomer 2), 97995-54-5; BrF_2CCO_2Et, 667-27-6; Boc-His(Ts), 35899-43-5; Boc-Phe, 13734-34-4; ACE, 9015-82-1; Boc-L-leucinal, 58521-45-2; L-isoleucyl-2-pyridylmethylamide, 97920-16-6; renin, 9015-94-5; pepsin, 9001-75-6; cathepsin, 9004-08-4.

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Articles

N-(Phosphonoacetyl)amino Phosphonates. Phosphonate Analogues of *N*-(Phosphonoacetyl)-L-aspartic Acid (PALA)

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Michaelis–Arbuzov reaction of *N*-(chloroacetyl)amino phosphonic acids or their esters, followed by acidolysis, gives moderate yields of *N*-(phosphonoacetyl) derivatives of a variety of (aminoalkyl)phosphonic acids, including analogues of the cytostatic agent PALA, in which the α- or β-carboxylic groups in the aspartate moiety are replaced by a PO_3H_2 function. Assay of cytostatic activity with human KB cell lines indicates that the substitution of any of the COOH groups in PALA with PO_3H_2 results in total loss of cytostatic activity. No activity was observed also in the case of other [*N*-(phosphonoacetyl)amino]alkylphosphonic acids described in this report.

N-(Phosphonoacetyl)-L-aspartic acid (PALA (**1**)) is a rationally designed transition-state analogue of aspartate transcarbamylase,[1–3] the second enzyme in the de novo pyrimidine biosynthesis. It also exhibits tumor-inhibitory activity against a number of transplantable solid murine tumors.[4–7] However, in comparison with other antimetabolites, the spectrum of activity of PALA is unique in that leukemias,[6–8] bladder cancer,[9] and hypernoma[9] are relatively or completely insensitive to the drug whereas Lewis lung carcinoma[10,11] and melanomas[4,12,13] are sensitive. The possibility of using PALA in polychemotherapy is also very promising since it was found to be synergistic with several popular anticancer drugs including fluorouracil, alanosine, acivicin, methotrexate, and pyrofurazin.[11,14–20]

Although PALA is a promising anticancer agent, its usefulness in human therapy seems to be limited. It fails to inhibit pyrimidine biosynthesis in vivo as strongly as expected from its effectiveness in vitro, despite an apparently effective concentration of the drug in tissues.[17]

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