Fluoro Ketone Containing Peptides as Inhibitors of Human Renin

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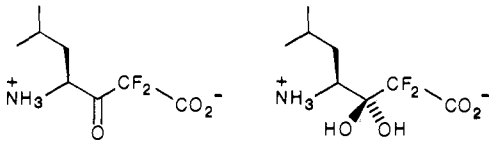
The pentapeptide BOC-Phe-Phe-difluorostatone-Leu-Phe-NH_2 has been prepared and found to be a potent inhibitor of human renin. This compound contains a difluoromethylene ketone group that exists predominantly in the hydrated form in water. The difluorostatone-containing peptide is 7-fold and 22-fold more potent than the analogous statine- and statone-containing peptides, respectively. Structure/activity analysis of the most potent inhibitor was carried out by replacing some of the peptide bonds with *trans*-alkenes. In all cases, a dramatic loss in binding to renin was observed. A number of statine-containing inhibitors of renin have been reported and this work suggests that the replacement of statine with difluorostatone will yield more potent compounds.

We have recently described the inactivation of the aspartyl protease pepsin by peptides containing the amino acid difluorostatone (**1**).[1] This amino acid contains a difluoromethylene ketone that exists predominantly in the hydrated form **2** in water. The rationale behind the use



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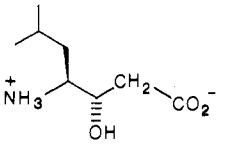

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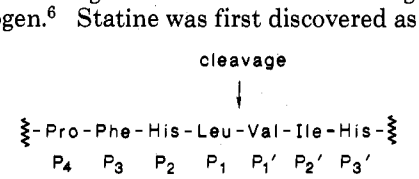
of these compounds as inhibitors of proteolytic enzymes is that the hydrated ketone is a structural mimic of the putative tetrahedral intermediate that forms during the enzymatic cleavage of a peptide substrate. Thus a compound that contains a fluoro ketone as a replacement for the enzymatically cleaved substrate amide bond might act as a "transition state analogue" inhibitor.[2] We have now explored the possibility of using this approach to prepare inhibitors of the aspartyl protease renin. Renin is part of the renin–angiotensin system where it catalyzes the production of the peptide angiotensin I from the circulating protein angiotensinogen as the first step in the production of the vasoconstrictor peptide angiotensin II.[3] Inhibitors of renin have been shown to have antihypertensive activity when given intravenously to animals[4] and are therefore of considerable medicinal interest.

A number of synthetic renin inhibitors have been reported in which a nonhydrolyzable group has been incorporated at the site of cleavage into a peptide sequence derived from angiotensinogen.[5] Some of the most potent renin inhibitors contain the amino acid statine **3** as a dipeptide replacement for the Leu-Val sequence that surrounds the cleavage site in the N-terminal region of angiotensinogen.[6] Statine was first discovered as a compo-



nent of the naturally occurring pepstatin, which is a pentapeptide that is a powerful inhibitor of pepsin.[7]

Peptides containing **2** are expected to be more potent renin inhibitors than those containing **3** since the *gem*-diol of **2** is closer to the structure of the putative tetrahedral transition state for amide hydrolysis than is the hydroxyl group of **3**. Here we describe the preparation of a peptide containing **3** and the evaluation of this compound as an inhibitor of human renin. The potent inhibitory action of the latter peptide prompted us to synthesize and assay two difluorostatone-bearing peptides further modified by the introduction of distal peptide backbone replacements.

Chemistry

The preparation of the new peptide derivatives **8** and **9** is outlined in Scheme I. The preparation of (*tert*-butyloxycarbonyl)difluorostatine ethyl ester **4** has already been reported.[1] This compound has been rigorously characterized as the isomer shown (3*R*,4*S*).[1] Compound **4** was saponified with a slight excess of sodium hydroxide in THF/water to give acid **5**. Coupling of **5** to HCl·H-Leu-Phe-NH_2 was accomplished with dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) to give tripeptide **6**. Removal of the BOC group from **6** with trifluoroacetic acid in methylene chloride produced the salt **7**. Under the conditions employed in the synthesis, compound **7** showed no tendency to undergo intramolecular cyclization by attack of the terminal amino group onto the activated fluorinated amide. Coupling of **7** with BOC-Phe-Phe-OH was carried out with diethylphosphoryl cyanide in the presence of triethylamine.[8] This reaction was also accomplised, albeit in lower yield,

† Department of Chemistry.
‡ Departments of Chemistry and Biochemistry.

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(4) See, for example: Szelke M.; Leckie, B. J.; Tree, M.; Brown, A.; Grant, J.; Hallett, A.; Hughes, M.; Jones, D. M.; Lever, A. F. *Hypertension* **1982**, *4* (Suppl. II), II-59.
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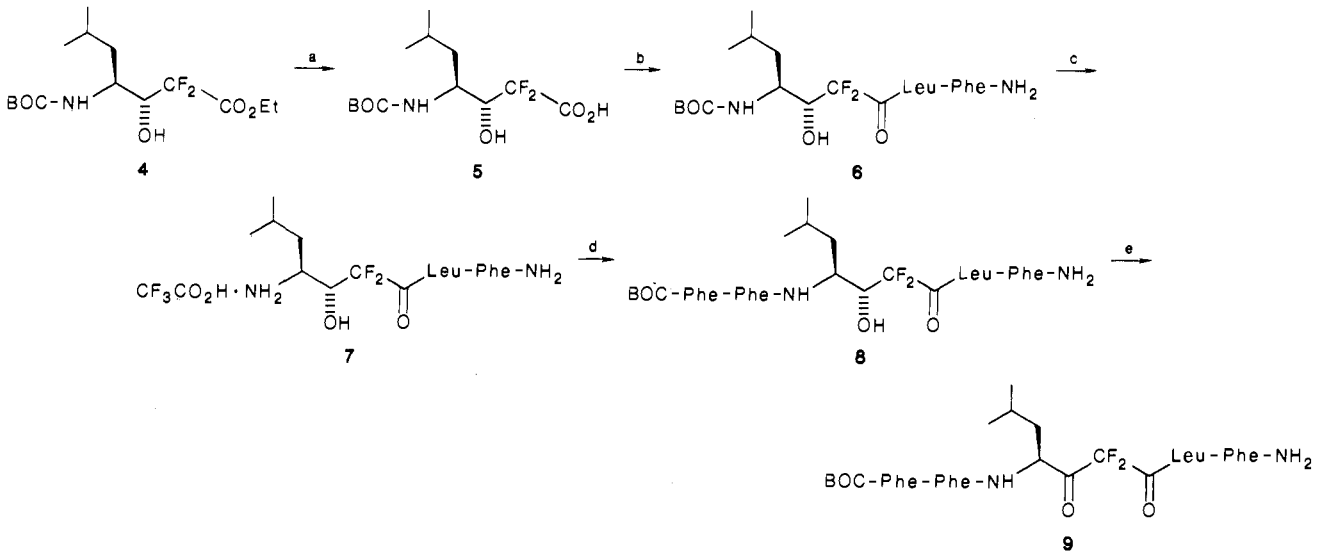
Scheme I[a]

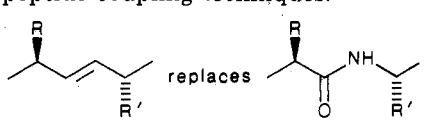


[a] Reagents: (a) 1 M NaOH, THF; (b) HCl·H-Leu-Phe-NH_2, DCC, HOBt, NMM, DMF; (c) CF_3COOH, CH_2Cl_2; (d) BOC-Phe-Phe-OH, diethylphosphoryl cyanide, Et_3N, DMF; (e) DCC, Me_2SO, HCl_2CCOOH, toluene.

with DCC/HOBt. The use of diphenylphosphoryl azide[8] as the coupling reagent resulted in a significant amount of side reaction resulting from Curtius rearrangement of the initially formed acyl azide.

The oxidation of **8** to **9** proved to be difficult due to the diverse functionality of **8** as well as the tendency of fluorinated alcohols to resist oxidation.[9] Attempted oxidations with Me_2SO/oxalyl chloride[10] were not reproducible on small-scale reactions. Furthermore, this reagent resulted in the formation of significant amounts of byproducts and the epimerization of the isobutyl side chain adjacent to the fluoro ketone. Oxidation with Collins' reagent[11] was successful, but large amounts of oxidant (up to 20 equiv) were required and this often resulted in peptide degradation. By far the best method was the use of DCC in Me_2SO/toluene in the presence of dichloroacetic acid.[12] Excess reagent and prolonged reaction times resulted in essentially quantitative oxidation with no detectable peptide degradation or epimerization.

Derivatives of **8** and **9** were prepared that contain *trans*-alkene dipeptide isostere as replacements for the Phe-Phe and Leu-Phe dipeptide units. The chemistry for the preparation of the *trans*-alkene dipeptide isosteres used in this study, which enables the control of the stereochemistry at both chiral centers, has recently been reported.[13] The incorporation of these peptide replacements into peptide renin inhibitors was accomplished by using standard peptide coupling techniques.



All of the final products were judged to be homogeneous by HPLC on both reverse-phase and normal-phase columns. All compounds gave high-resolution NMR spectra and accurate mass liquid secondary ion mass spectra that were consistent with the assigned structures.

Results and Discussion

The hydration state of difluorostatone-containing peptide **9** was investigated by using ^{19}F NMR. In pure Me_2SO, the spectrum of **9** showed a multiplet (AB quartet) centered at −37.84 ppm (relative to a sodium trifluoroacetate internal reference). This signal is assigned to the two diastereotopic fluorines of **9** in the ketone form. In Me_2SO/water (1.9:1), the multiplet shifted upfield to −41.86 ppm, consistent with the formation of the hydrated ketone.[14] Thus, in pure water, **9** is essentially fully hydrated.

The pentapeptide derivatives prepared in this study were tested as inhibitors of human kidney renin as described in the Experimental Section and the results are presented in Table I. Interestingly, **8**, which contains difluorostatine, is a less potent inhibitor than **10**, which contains the nonfluorinated statine. It is not clear whether this difference in potency is due to an increase in steric bulk as a result of fluorine substitution or to the effect of fluorine on the hydrogen-bond-accepting capacity of the adjacent hydroxyl group. In contrast to these results, studies with pepsin and a related series of inhibitors showed no difference in binding affinities for statine- vs. difluorostatine-containing peptides.[1]

It can be seen from Table I that oxidation of **8** to produce **9** results in a 17-fold increase in the potency of inhibition. Compound **9** is the most active inhibitor in the series being 7.4-fold more active than **10**. Compound **9** is seen to bind 22 times tighter to renin than the nonfluorinated ketone containing peptide **11**. Thermodynamic considerations[1] suggest that **9** should bind approximately 1000 times more tightly than **11**, a reflection of the poor tendency of the nonfluorinated ketone to undergo hydration. In a study with pepsin,[1] the peptide containing difluorostatone was found to bind 930-fold tighter than the one containing the nonfluorinated ketone. It is not clear in the present study with renin why the incorporation of fluorine results in a more modest binding enhancement. It is likely that the factors that reduce the binding of **8**

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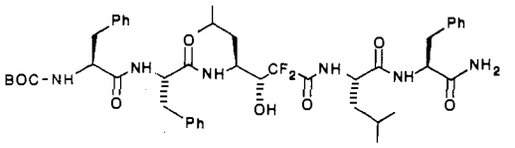
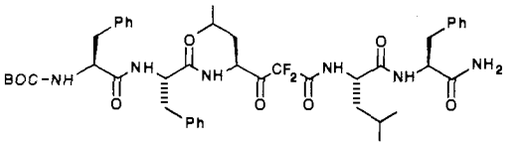
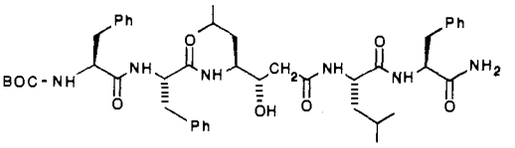
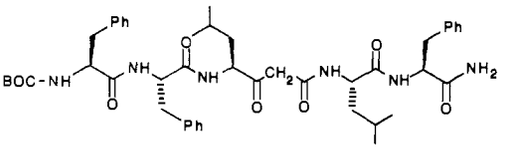
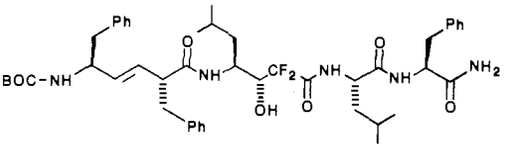
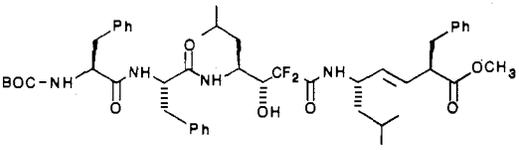
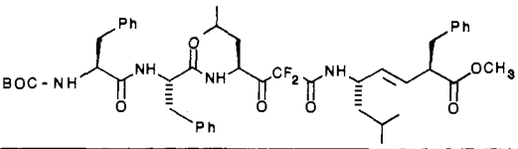
(14) Gelb, M. H., unpublished observations. Guthrie, J. P. *Can. J. Chem.* **1975**, *53*, 898.

Table I. Inhibition of Human Renin

compd	structure	K_i, nM
8		120
9		7
10		52
11		154
12		12000
13		16000
14		1200

relative to **10** are operating to reduce the binding of **9** as well. While this work was nearing completion, a related study appeared on the use of fluorinated statine analogues as inhibitors of human renin.[1] A similar trend in binding affinities was observed in that the difluorostatone-containing peptide bound significantly tighter to renin than peptides containing either statine or difluorostatine.

We have carried out additional structure/function studies on compounds **8** and **9** by replacing some of the amide bonds with *trans*-alkenes. Isosteric replacement of peptide bonds with *trans*-alkenes has been used in the preparation of analogues of peptide hormones that retain variable amounts of biological activity while being resistant to degradation by proteolytic enzymes.[15] The results in Table I show that replacement of either the Phe-Phe or Leu-Phe dipeptides of **8** with a *trans*-alkene dipeptide isostere to produce **12** and **13** results in a dramatic loss of binding affinity to renin. Compounds **12** and **13** are, respectively, 100 times and 133 times less potent than **8**. Oxidation of **13** to produce **14** results in some improvement in binding (13-fold) to renin. With regards to the loss in activity of **12** relative to **8**, it is noted that potent inhibitors of renin have been reported that contain an N-methylated amide as a replacement for the peptide bond linking the two phenylalanines at positions P_2 and P_3.[16] This result suggests that the NH of this amide does not form a hydrogen bond to renin. One interpretation of these results is that the carbonyl portion of the amide linking the two phenylalanines forms a hydrogen bond with the enzyme. The 100-fold decrease in binding of **12** relative to **8** corresponds to a loss in binding free energy of 2.7 kcal/mol, a value close to that observed resulting from the loss of a hydrogen bond in a structurally well characterized enzyme–inhibitor complex.[17] An alternative explanation to these results is that the amide between the two phenylalanines exists in the cis conformation when bound to renin and the reduced binding of **12** is due to the enforced trans geometry of the isostere. The possibility is considered unlikely in light of the report that the N-methylated amide containing peptide binds 3-fold weaker to renin than the

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nonmethylated analogue.[16] If the cis amide were the bound form, it is likely that N-methylation would lead to a dramatic increase in binding since this substitution would promote the formation of the cis rotamer. A definitive explanation of these results must await a full structure determination of renin–inhibitor complexes. The highly attenuated potency of the peptides bearing the *trans*-alkene dipeptide isostere is noteworthy in light of previous studies in which this replacement had little or no effect on potency. Regardless of the precise reasons, it is clear that the utility of this peptide replacement must be judged on a case-by-case basis.

Experimental Procedures

Materials. *N*-(*tert*-Butyloxycarbonyl)-L-leucinal,[18] *N*-(*tert*-butyloxycarbonyl)-L-phenylalanyl-L-phenylalanine,[19] *N*-(*tert*-butyloxycarbonyl)-L-leucinyl-L-phenylalanylamide,[20] 5(*S*)-[(*tert*-butyloxycarbonyl)amino]-6-phenyl-2(*R*)-(phenylmethyl)-3-*trans*-hexenoic acid,[13] and 5(*S*)-[(*tert*-butyloxycarbonyl)-amino]-7-methyl-2(*RS*)-(phenylmethyl)-3-*trans*-octenoic acid[13] were prepared according to the literature procedure cited. Ethyl bromodifluoroacetate was from SCM Specialty Chemicals and diethylphosphoryl cyanide was from Waco Chemicals. Compounds **10** and **11** were generous gifts from Dr. B. Weidmann (Sandoz) and Dr. M. Poe (Merck), respectively.

General Procedures. Proton nuclear magnetic resonance spectra were obtained on a Varian CFT-20 (80 MHz), a Varian VXR-300 (300 MHz), or a Bruker WM-500 (500 MHz) spectrometer. All chemical shift values are reported in δ units (ppm) relative to tetramethylsilane (organic solvents) or D_2O (assigned to 4.8 ppm). Thin-layer chromatography was performed on silica gel plates (0.25 mm, Merck) by using the following detection methods: A, exposed to HCl vapors for 5 min, sprayed with 1% ninhydrin in ethanol, and heated at 140 °C for 2 min; B, exposed to Cl_2 vapors for 5 min, dipped into a solution containing 0.03% *o*-tolidine, 6% acetic acid, and 0.2% potassium iodide in water; C, dipped into a solution containing 1% potassium permanganate and 0.08% sodium hydroxide in water. Flash chromatography was performed with silica gel 60 (230–400 mesh, Merck). Semipreparative HPLC was on a reverse-phase column (column A; Vydac 218TP1010 octadecylsilane, 10 μm, 250 × 10 mm) or on a normal-phase column (column B; Dynamax silica (Rainin Instruments), 8 μm, 250 × 10 mm). The peptides were detected by UV absorbance at 260 nm. Accurate mass liquid secondary ion mass spectra were obtained at the University of California San Francisco mass spectrometry facility. Dry THF was obtained by distillation from sodium/benzophenone under argon. Dry toluene and Me_2SO were obtained by distillation from CaH_2 under argon and were stored over type 4A molecular sieves.

General Procedure for Removal of *tert*-Butyloxycarbonyl Groups. The protected peptide was dissolved to a concentration of approximately 1.25 M in 25% trifluoroacetic acid in dichloromethane and allowed to stand at room temperature for 10 min. The solvent was removed in vacuo and the remaining trifluoroacetic acid was removed by repeated evaporation from benzene in vacuo. Finally, the residue was lyophilized from benzene.

General Procedures for Peptide-Bond Formation. Procedure A. The deprotected peptide (1 equiv) was dissolved to a concentration of 0.5 M in DMF, and N-protected amino acid (1 equiv) and HOBt (2 equiv) were added. The solution was cooled to 4 °C, and *N*-methylmorpholine (1 equiv) was added dropwise, followed by DCC (1 equiv). The reaction mixture was stirred overnight at room temperature. The solid was filtered and washed with ethyl acetate, and the filtrate was concentrated in vacuo. The residue was dissolved in ethyl acetate and washed with 5% citric acid, 5% sodium bicarbonate, and brine. Drying over $MgSO_4$ and concentration in vacuo followed by chromatography on silica gel afforded the desired peptide.

Procedure B. The deprotected peptide (1 equiv) was dissolved to a concentration of 0.2 M in DMF and the N-protected dipeptide (1 equiv) and diethylphosphoryl cyanide (1 equiv) were added. The solution was cooled to 4 °C, the triethylamine (2 equiv) was added dropwise, and the solution was stirred overnight a 4 °C. The DMF was removed in vacuo and the desired peptide was obtained by purification on silica gel.

General Oxidation Procedure. The peptidic alcohol was dissolved to a concentration of 0.06 M in dry Me_2SO/toluene (1:1). DCC (6 equiv) was added, followed by addition of the dichloroacetic acid (0.6 equiv), and the solution was stirred overnight at room temperature in a tightly stoppered flask. Oxalic acid (6 equiv) was dissolved to a concentration of 0.35 M in methanol and was added to the stirring solution. After 10 min the solid was filtered and the filtrate was diluted with ethyl acetate. The organic solution was washed three times with 5% sodium bicarbonate and once with brine and dried over $MgSO_4$, and the solvent was removed in vacuo. Purification on silica gel furnished the desired peptidic ketone.

Ethyl 4(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoate (4). The following procedure is essentially as described previously.[1] Activated zinc[21] (2.4 g, 36.8 mmol) and dry THF (70 mL) were charged to a three-necked flask equipped with two addition funnels and a condensor under argon. The THF was brought to reflux and the ethyl bromodifluoroacetate (7.5 g, 36.8 mmol) was added continuously over 30 s. *N*-(*tert*-butyloxycarbonyl)leucinal (4.4 g, 20.5 mmol), dissolved in THF (10 mL), was added dropwise over 2 min and the solution was refluxed 15 min. The solution was cooled to room temperature, and then equal volumes of 1 M sodium bisulfate and brine were added. The layers were separated, and the water was extracted with ethyl acetate. The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo. Purification on silica gel (10% ethyl acetate/hexanes) afforded 3.1 g (44.9%) of the 3*R* isomer **4** and 0.55 g (8.1%) of the 3*S* compound: Compound **4**: TLC (20% ethyl acetate/hexanes), R_f 0.45, detection method A; ^1H NMR ($CDCl_3$, 80 MHz) δ 0.93 (d, 6 H), 1.37 (t, 3 H), 1.43 (s, 9 H), 1.78–2.05 (m, 3 H), 3.80–4.10 (m, 2 H), 4.30 (q, 2 H), 4.87 (d, 1 H). 3*S* isomer: TLC (20% ethyl acetate/hexanes), R_f 0.38, detection method A; ^1H NMR ($CDCl_3$, 80 MHz) δ 0.92 (d, 3 H), 0.95 (d, 3 H), 1.36 (t 3 H), 1.44 (s, 9 H), 1.80–2.00 (m, 3 H), 3.90–4.20 (m, 2 H), 4.33 (q, 2 H), 4.67 (d, 1 H).

4(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-2,2-difluoro-3-(*R*)-hydroxy-6-methylheptanoic Acid (5). Ester **4** (3.1 g, 9.1 mmol) was dissolved in THF (19 mL) and 1 M NaOH (9.5 mL, 1.03 equiv) was added. After the solution was stirred for 2 h, the THF was removed in vacuo and the solution was acidified with 2 N citric acid. The water was extracted twice with ethyl acetate and then the ethyl acetate was washed twice with water and twice with brine. Drying over $MgSO_4$ and evaporation of the solvent in vacuo furnished acid **5** (2.3 g, 80.9%) as a white solid: ^1H NMR ($CDCl_3$, 80 MHz) δ 0.95 (d, 6 H), 1.47 (s, 9 H), 1.80–2.00 (m, 3 H), 3.80–4.14 (m, 2 H), 6.62 (d, 1 H), 7.75 (br s, 1 H).

L-Leucyl-L-phenylalaninamide Hydrochloride. A saturated solution of hydrochloric acid in ethyl acetate (50 mL) was added to *N*-(*tert*-butyloxycarbonyl)-L-leucyl-L-phenylalaninamide (3.1 g, 8.2 mmol) and the mixture was swirled until the peptide dissolved. The mixture was allowed to stand for 45 min at room temperature with moisture protection from a $CaSO_4$ dry tube. The hydrochloride salt of the dipeptide was precipitated with petroleum ether and the resulting solid was filtered, washed with petroleum ether, and dried in a dessicator under vacuum. The solid (2.3 g, 89.8%) was used without further purification: ^1H NMR (D_2O, 80 MHz) δ 1.00 (d, 3 H), 1.10 (d, 3 H), 1.75–1.85 (m, 3 H), 3.14 (m, 1 H), 3.5 (m, 1 H), 3.95–4.10 (m, 1 H), 4.55–4.65 (m, 1 H), 7.45 (s, 5 H).

[4(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-2,2-difluoro-3-(*R*)-hydroxy-6-methylheptanoyl]-L-leucyl-L-phenylalaninamide (6). L-Leucyl-L-phenylalaninamide hydrochloride (0.829 g, 2.65 mmol) and N-protected acid **5** (0.824 g, 2.65 mmol) were coupled by using procedure A. Purification on silica gel (55% ethyl acetate/hexanes) afforded the tripeptide (0.97 g, 64.2%)

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as a light yellow solid. The tripeptide was a 3:1 mixture of the 4*S*,3*R* and 4*R*,3*S* isomers as judged by HPLC (see below). Apparently, 4 is contaminated with a small amount of its enantiomer. The mixture of isomers was used without further purification except where noted below. Compound 6 (4*S*,3*R* isomer): TLC (70% ethyl acetate/hexanes), R_f 0.45, detection method B; HPLC (70% methanol/water), column A, t_R 16.6 min; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.85–0.97 (m, 12 H), 1.45 (s, 9 H), 1.33–1.70 (m, 6 H), 2.95 (m, 1 H), 3.31 (m, 1 H), 3.89 (m, 1 H), 4.19 (m, 1 H), 4.27 (m, 1 H), 4.73 (m, 1 H), 5.52 (br s, 1 H), 6.28 (br s, 1 H), 6.95 (br s, 1 H), 7.25 (m, 5 H), 7.41 (br d, 1 H). 4*R*,3*S* isomer: TLC (70% ethyl acetate/hexanes), R_f 0.45, detection method B; HPLC (70% methanol/water) column A, t_R 19.8 min; ^{1}H NMR ($CDCl_3$, 500 MHz) distinguishing peaks at δ 3.23 (m, 1 H), 4.00 (m, 1 H), 4.33 (m, 1 H), 4.80 (m, 1 H), 5.63 (br s, 1 H), 6.70 (br s, 1 H), 7.12 (br s, 1 H).

[4(*S*)-Amino-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoyl]-L-leucyl-L-phenylalaninamide Trifluoroacetate (7). By the general method for removal of *N*-*tert*-butyloxycarbonyl groups, tripeptide 6 (0.845 g, 1.48 mmol) was converted to trifluoroacetate salt 7 (0.779 g, 90.0%): ^{1}H NMR (1:1 acetone/D_2O, 500 MHz) δ 0.72 (d, 3 H), 0.77 (d, 3 H), 0.83 (d, 3 H), 0.86 (d, 3 H), 1.42 (m, 2H), 1.49 (m, 2 H), 1.64 (m, 2 H), 2.82 (m, 1 H), 3.02 (m, 1 H), 3.57 (m, 1 H), 4.18 (m, 1 H), 4.31 (m, 1 H), 4.52 (m, 1 H), 7.13 (m, 5 H).

[4(*S*)-[[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]-L-phenylalanyl]amino]-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoyl]-L-leucyl-L-phenylalaninamide (8). Deprotected tripeptide 7 (0.150 g, 0.257 mmol) and *N*-(*tert*-butyloxycarbonyl)-L-phenylalanyl-L-phenylalanine (0.106 g, 0.257 mmol) were coupled by using procedure B. Purification on silica gel (1.5% methanol/chloroform) furnished pentapeptide 8 (0.138 g, 62.1%). This chromatographic step removed the pentapeptide containing the incorrect enantiomer of difluorostatine. Compound 8 was purified further by normal phase HPLC: TLC (5% methanol/chloroform), R_f 0.40, detection method B; HPLC (4% methanol/chloroform) column B, t_R 9.2 min; ^{1}H NMR (acetone, 500 MHz) δ 0.70 (d, 6 H), 0.80 (d, 3 H), 0.87 (d, 3 H), 1.26 (s, 9 H), 1.30 (m, 2 H), 1.47 (m, 1 H), 1.60 (m, 1 H), 1.68 (m, 1 H), 1.79 (m, 1 H), 2.80–3.20 (m, 6 H), 3.51 (m, 1H), 4.00 (m, 1 H), 4.40 (m, 2 H), 4.73 (m, 2 H), 5.95 (br d, 1 H), 6.09 (br d, 1 H), 6.58 (s, 2 H), 7.20 (m, 15 H), 7.90 (d, 1 H), 7.94 (br d, 1 H), 8.20 (br d, 1 H), 8.39 (br d, 1 H); negative ion MS (calculated for $C_{46}H_{62}F_2N_6O_8$ - 1) 863.45189, obsd 863.45191.

[4(*S*)-[[[*N*-(*tert*-Butyloxycarbonyl)-L-phenyalanyl]-L-phenylalanyl]amino]-2,2-difluoro-3-oxo-6-methylheptanoyl]-L-leucyl-L-phenylalaninamide (9). The general oxidation procedure was used to oxidize 8 (24.7 mg, 0.0286 mmol), which, after purification on silica gel (2% methanol/chloroform), furnished 9 (22.2 mg, 90.1%). The oxidized peptapeptide was purified further by reverse-phase HPLC: TLC (5% methanol/chloroform), R_f 0.43, detection method B; HPLC (65% acetonitrile/water) column A, retention time 9.7 min; ^{1}H NMR (acetone, 300 MHz) δ 0.80 (m, 9 H), 0.88 (d, 3 H), 1.32 (s, 9 H), 1.48–1.75 (m, 6 H), 2.90–3.31 (m, 6 H), 4.25 (m, 1 H), 4.40 (m, 2 H), 4.78 (m, 2 H), 6.09 (d, 1 H), 6.72 (s, 1 H), 7.13–7.38 (m, 15 H), 7.86 (d, 1 H), 8.41 (d, 1 H), 8.68 (d, 1 H); negative ion MS (calculated for $C_{46}H_{60}F_2N_6O_8$ - 1) 861.43624, obsd 861.43626.

[4(*S*)-[[5(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-6-phenyl-2(*R*)-(phenylmethyl)-3-*trans*-hexenoyl]amino]-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoyl]-L-leucyl-L-phenylalaninamide (12). Isomerically pure tripeptide 7 was obtained by deprotection of HPLC-purified 6 by using the general procedure. This material (11.9 mg, 0.0204 mmol) was combined with dichloromethane (1 mL) and 2 M sodium carbonate (1 mL). The layers was separated, and the dichloromethane was washed once more with base. The dichloromethane solution was dried over anhydrous potassium carbonate and concentrated in vacuo to a constant weight. 5(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-6-phenyl-2(*R*)-(phenylmethyl)-3-*trans*-hexenoic acid (6.7 mg, 0.0170 mmol) was reacted with the free amine of 7 following general coupling procedure A except that no *N*-methylmorpholine added. Purification on silica gel (1.7% methanol/chloroform) followed by further purification on reverse-phase HPLC furnished 12 (2.2 mg, 15.3%) and a byproduct (2.0 mg, 13.9%). This byproduct was determined by NMR to contain a conjugated double bond (vide infra). Compound 12: TLC (5% methanol/chloroform), R_f 0.42, detection methods B and C; HPLC (77% methanol/water) column A, t_R 17.4 min; ^{1}H NMR (acetone, 500 MHz δ 0.80 (d, 3 H), 0.85 (m, 9 H), 1.34 (s, 9 H), 1.57 (m, 5 H), 1.72 (m, 1 H), 2.70 (m, 3 H), 2.98 (m, 1 H), 3.12 (m, 1 H), 3.30 (m, 2 H), 4.09 (m, 2 H), 4.24 (m, 2 H), 4.67 (m, 1 H), 5.51 (m, 1 H), 5.66 (m, 1 H), 5.89 (br s, 1 H), 6.35 (br s, 1 H), 6.88 (br s, 1 H), 7.06–7.38 (m, 15 H), 7.78 (br s, 1 H), 8.01 (br s, 1 H); positive ion MS (calculated for $C_{47}H_{63}F_2N_5O_7$ + 1) 848.47739, obsd 848.47647. Byproduct: HPLC (77% methanol/water), column A, t_R 19.8 minutes.

5(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-7-methyl-2-(*R*)-(phenylmethyl)-3-*trans*-octenoic Acid (15). 5(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-7-methyl-2(*RS*)-(phenylmethyl)-3-*trans*-octenoic acid (61.2 mg, 0.170 mmol) was separated into the two diastereomers by chromatography on silica gel (35% ethyl acetate/petroleum ether). The 2*R* isomer 15 (24.2 mg, 39.5%) was identified by its identical ^{1}H NMR with the known compound.[15]

Methyl 5(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-7-methyl-2(*R*)-(phenylmethyl)-3-*trans*-octenoate (16). Diazomethane was distilled into a solution of 15 (24.0 mg, 0.0665 mmol) in ethyl ether (5 mL) at 0 °C until the solution remained yellow, indicating excess diazomethane. Removal of the solvent in vacuo furnished the desired methyl ester 16 (24.8 mg, 99.5%): TLC (40% ethyl acetate/hexanes), R_f 0.77, detection methods A and C.

Methyl 5(*S*)-Amino-7-methyl-2(*R*)-(phenylmethyl)-3-*trans*-octenoate Trifluoroacetate (17). By the general procedure for the removal of the *N*-*tert*-butyloxycarbonyl group, 16 (24.8 mg, 0.0661 mmol) was converted to 17 (24.1 mg, 93.7%): ^{1}H NMR (acetone, 300 MHz) δ 0.83 (m, 6 H), 1.30 (m, 2 H), 1.58 (m, 1 H), 2.83 (m, 1 H), 3.09 (m, 1 H), 3.45 (m, 1 H), 3.60 (s, 3 H), 4.59 (m, 1 H), 5.60 (m, 1 H), 5.91 (m, 1 H), 7.20 (m, 5 H).

Methyl 5(*S*)-[[4(*S*)-[*N*-(*tert*-Butyloxycarbonyl)amino]-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoyl]amino]-7-methyl-2(*R*)-(phenylmethyl)-3-*trans*-octenoate (18). Compound 17 (24.1 mg, 0.062 mmol) and 5 (19.3 mg, 0.062 mmol) were coupled by using general coupling procedure A. Purification on silica gel (30% ethyl acetate/petroleum ether) furnished 18 (27.5 mg, 78.1%): TLC (25% ethyl acetate/hexanes), R_f 0.43 detection methods B and C; ^{1}H NMR (chloroform, 300 MHz) δ 0.89 (d, 6 H), 0.93 (d, 6 H), 1.40 (m, 3 H), 1.46 (s, 9 H), 1.68 (m, 2 H), 1.82 (m, 1 H), 2.82 (m, 1 H), 3.11 (m, 1 H), 3.30 (m, 1 H), 3.60 (s, 3 H), 3.98 (m, 2 H), 4.47 (m, 1 H), 4.89 (m, 1 H), 5.40 (m, 1 H), 5.72 (m, 1 H), 6.91 (m, 1 H), 7.11–7.31 (m, 5 H).

Methyl 5(*S*)-[[4(*S*)-[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]-L-phenylalanyl]amino]-2,2-difluoro-3(*R*)-hydroxy-6-methylheptanoyl]amino]-7-methyl-2(*R*)-(phenylmethyl)-3-*trans*-octenoate (13). Deprotection of 18 using the general procedure furnished the trifluoroacetate salt. This salt 22.3 mg, 0.0383 mmol) and *N*-[(*tert*-butyloxycarbonyl)-amino]-L-phenylalanyl-L-phenylalanine (15.8 mg, 0.0383 mmol) were coupled by using general procedure B. Purification on silica gel (30% ethyl acetate/petroleum ether) furnished 13 (18.6 mg, 56.3%). The peptide was purified further by reverse-phase HPLC: TLC (40% ethyl acetate/petroleum ether), R_f 0.58, detection method B; HPLC (85% methanol/water), column A, t_R 9.9 min; ^{1}H NMR (acetone, 500 MHz) δ 0.83 (m, 12 H), 1.30 (s, 9 H), 1.33 (m, 2 H), 1.48 (m, 2 H), 1.57 (m, 2 H), 2.88 (m, 3 H), 2.98 (m, 1 H), 3.08 (m, 2 H), 3.31 (m, 1 H), 3.34 (m, 1 H), 3.60 (s, 3 H), 4.08 (m, 1 H), 4.16 (m, 1 H), 4.29 (m, 1 H), 4.41 (m, 1 H), 4.68 (m, 1 H), 5.50 (m, 1 H), 5.73 (m, 1 H), 5.96 (br s, 1 H), 7.10 (br d, 1 H), 7.16–7.26 (m, 15 H), 7.39 (br s, 1 H), 7.67 (br d, 1 H); positive ion MS (calculated for $C_{48}H_{64}F_2N_4O_8$ + 1) 863.47706, obsd 863.47799.

Methyl 5(*S*)-[[4(*S*)-[[[*N*-(*tert*-Butyloxycarbonyl)-L-phenylalanyl]-L-phenylalanyl]amino]-2,2-difluoro-3-oxo-6-methylheptanoyl]amino]-7-methyl-2(*R*)-(phenylmethyl)-3-*trans*-octenoate (14). The general oxidation procedure was used to oxidize 13 (10.8 mg, 0.0125 mmol). Purification by chromatography on silica gel (35% ethyl acetate/petroleum ether) afforded 14 (5.4 mg, 50.1%): TLC (40% ethyl acetate/petroleum ether), R_f 0.54, detection methods B and C; ^{1}H NMR (acetone, 500 MHz) δ 0.84 (m, 6 H), 0.93 (m, 6 H), 1.32 (s, 9 H), 1.37 (m, 1 H), 1.49 (m, 3 H), 1.72 (m, 2 H), 2.81 (m, 2 H), 2.97–3.12 (m,

4 H), 3.32 (m, 1 H), 3.60 (s, 3 H), 4.28 (m, 1 H), 4.45 (m, 1 H), 4.68 (m, 1 H), 5.00 (m, 1 H), 5.49 (m, 1 H), 5.70 (m, 1 H), 6.01 (br d, 1 H), 7.14–7.24 (m, 15 H), 7.40 (br d, 1 H), 7.70 (br s, 1 H), 8.13 (d, 1 H); positive ion MS (calculated for $C_{48}H_{62}F_2N_4O_8$ + 1) 861.46141, obsd 861.46141.

Renin-Inhibition Studies. The inhibitory potency of the compounds was measured by their ability to inhibit the cleavage of human angiotensinogen by human kidney renin. Human kidney renin (0.02 ng, purified as described[22]) was incubated with angiotensinogen (500 ng, partially purified from outdated human plasma up through the DE-52 chromatography step as described[23]) for 60 min at 37 °C in citrate phosphate buffer (0.1 M, pH 7.20, 0.10 mL) containing inhibitor. The inhibitors were delivered to the assay from stock solutions in methanol. The reactions were stopped by immersion in a boiling water bath for 1 min. The angiotensin I produced was quantitated by radioimmunoassay by competition with ^{125}I-labeled angiotensin I by using a Travenol Labs Angiotensin I radioimmunoassay kit. A plot of renin activity vs. inhibitor concentration was made and the concentration of inhibitor required to inhibit the enzymes by 50% (IC^5_0) was estimated graphically. Since the angiotensinogen concentration in this assay is much lower than its Michaelis constant,[22] the IC_{50} is equal to the inhibition constant (K_i), assuming competitive inhibition. The K_i values determined in this manner are reliable to about ±30% on the basis of multiple repitition of the measurement.

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Registry No. 4, 96056-65-4; (3*S*)-4, 97920-08-6; 5, 108385-36-0; 6, 109064-95-1; (3*S*,4*R*)-6, 109121-33-7; 7, 109064-96-2; 7·TFA, 109064-97-3; 8, 109064-98-4; 9, 108385-34-8; 10, 98858-47-0; 11, 109064-99-5; 12, 109065-00-1; 13, 109065-01-2; 14, 109065-02-3; 15, 109065-03-4; (2*S*)-15, 109065-09-0; 16, 109065-04-5; 17, 109065-06-7; 18, 109065-07-8; 5(*S*)-[(*tert*-butyloxycarbonyl)-amino]-6-phenyl-2(*R*)-(phenylmethyl)-3-*trans*-hexenoic acid, 108385-61-1; ethyl bromodifluoroacetate, 667-27-6; *N*-(*tert*-butyloxycarbonyl)leucinal, 58521-45-2; L-leucyl-L-phenylalaninamide hydrochloride, 74214-38-3; *N*-(*tert*-butyloxycarbonyl)-L-leucyl-L-phenylalaninamide, 33900-15-1; *N*-(*tert*-butyloxycarbonyl)-L-phenylalanyl-L-phenylalanine, 13122-90-2; renin, 9015-94-5.

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Pyridonecarboxylic Acids as Antibacterial Agents. 9.[1] Synthesis and Antibacterial Activity of 1-Substituted 6-Fluoro-1,4-dihydro-4-oxo-7-(4-pyridyl)-1,8-naphthyridine-3-carboxylic Acids

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The title compounds (**7a–e**) with ethyl, 2-fluoroethyl, 2-hydroxyethyl, vinyl, or cyclopropyl groups, respectively, at C-1 were prepared by the method involving the Balz–Schiemann reaction of 2-(4-pyridyl)pyridine- and 7-(4-pyridyl)-1,8-naphthyridinediazonium tetrafluoroborates (**15** and **27**). The 1-ethyl, 1-(2-fluoroethyl), and 1-vinyl derivatives showed in vitro activities as potent as the corresponding 7-(1-piperazinyl) analogues against *Staphylococcus aureus* 209P JC-1 and *Escherichia coli* NIHJ JC-2 but were less active against *Pseudomonas aeruginosa* 12. Among the 7-(4-pyridyl) derivatives having the different C-1 substituent, 1-cyclopropyl derivative **7e** was found to be the most active. In vivo efficacy of **7e** was superior to that of enoxacin against experimental infections due to *S. aureus* 50774. Some aspects of structure–activity relationships associated with the C-1, C-6, and C-7 substituents were discussed.

During the last decade, a new class of pyridonecarboxylic acid antibacterials with much improved potency and broad antibacterial spectrum has been developed. Included in this class are pefloxacin,[2] norfloxacin,[3] enoxacin (**1**),[4] ofloxacin,[5] and ciprofloxacin,[6] which are chemically characterized by having both fluorine and piperazine substituents in each molecule. A combination of the piperazine at C-7 with the fluorine at C-6 on the quinolone or azaquinolone ring system is now well known to be a reliable means for obtaining a potent analogue of this class. Further efforts have been devoted thus far to a search of C-7 substituents that might cause a greater increase in antibacterial activity. We found previously that a 3-aminopyrrolidine substituent, instead of the C-7 piperazine, was efficient for enhancing antibacterial activity in combination with the C-6 fluorine.[7] Lesher et al.[8] reported the synthesis of rosoxacin (**2**), in which a 4-pyridyl group

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