in suspension culture as described,[24] were harvested by gravity, washed once with RPMI-1640 (R_0), and resuspended in 2 mL of R_0. Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) was added at a final concentration of 1.2 nmol/10^6 cells from a fresh 10 mM stock in DMSO. After 15 min at 37 °C, the cells were diluted to 10 mL and incubated at 37 °C for 1 h. The cells were then centrifuged and resuspended in HEPES-saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4) at a density of 5×10^6 cells/mL. The cells were kept on ice until used. Fluorescence measurements were performed at 37 °C in an Aminco SPF-500 fluorimeter with a stirred cell attachment. The excitation wavelength was 340 nm and the emission wavelength was 510 nm.

The effect of GRP antagonists on gastrin release in vivo was measured in rats essentially as described.[19,38,39] Female Sprague-Dawley rats (≃150 g) were anesthesized by intramuscular injection of ketamine. One hundred microliters of sample was then injected intraperitoneally. After 5 min, blood samples were taken by cardiac puncture and immediately stored on ice. Gastrin concentration in serum was measured in duplicate by radioimmunoassay (Cambridge Medical Diagnostics, Cambridge, MA). In a pilot time-course experiment, the maximal gastrin response was observed 5 min after injection of GRP (data not shown). Each data point represents the mean value obtained from four separate rats.

Acknowledgment. We are grateful to L. Wassel for determination of amino acid compositions, H. Ramjit for mass spectroscopy analysis, and M. Riemen and R. Freidinger for helpful discussions.

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Determination of Dissociation Constants of High Affinity (pM) Human Renin Inhibitors: Application to Analogues of Ditekiren (U-71,038)

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A fluorescent human renin inhibitor, dansyl-Phe-His-LVA-Ile-Amp (**3**, U-80,825), was synthesized and utilized in a fluorescence energy transfer displacement assay to determine the dissociation constants (k_d's) of a series of ditekiren analogues. These studies have indicated that (1) both the parent ditekiren (**2**) and compounds **8a-3** are *up to 1 order of magnitude more potent* than revealed by their IC_{50}'s, (2) these dissociation constants are in good agreement with the independently determined K_i's for compounds **2**, **3**, and **8d**, and (3) the lower limit of the fluorescence energy transfer displacement assay has been extended beyond the picomolar range. It has therefore been suggested that many examples of underestimation of renin inhibitory activity may exist in the renin literature which could be discovered and rectified by using the methodology described herein.

The renin-angiotensin cascade continues to be a major focus in the search for new treatments for hypertension.[1] The therapeutic success of the angiotensin converting enzyme inhibitors has resulted in extensive efforts to synthesize and develop newer and potentially more selective drugs which act on this system,[2] primarily inhibitors of the aspartic proteinase renin. Indeed, numerous reports have appeared detailing the synthesis of potent (nanomolar) inhibitors of human renin.[3]

Experimental studies of protein inhibition by such high-affinity ligands are complicated by the difficulty in assaying such potent compounds. Specifically, inhibitors having dissociation constants (K_d's) in the nanomolar to subnanomolar range are particularly interesting to explore binding mechanisms and structure-activity relationships and to advance the investigation of the active-site chemistry of a proteins such as enzymes and receptors. This is especially true in research related to the potency assessment of peptide-based inhibitors of human renin (as well as for other enzymes) which are frequently expressed as IC_{50} values,[4] as determined by radioimmunoassay of angiotensin I (ANG I) generated in plasma as a result of angiotensinogen hydrolysis by plasma renin. These (IC_{50}) values are limited in sensitivity both by the accuracy with which the enzyme in the system can be determined and by the difficulty in working at sufficiently low levels of protein in order to be able to deal with very high affinity inhibitors. Thus, as is the case with renin, it is generally not possible to easily and accurately determine the IC_{50} values for large numbers of inhibitors whose activities fall below the 0.1 nM range. It has therefore been of interest to determine if the IC_{50}'s reported for this class of inhibitor are a true reflection of their inhibitory activity.

In an attempt to overcome these aforementioned difficulties, we developed a rapid and sensitive competitive displacement assay for the determination of renin inhibitor dissociation constants (K_d's) using fluorescence energy

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† Physical and Analytical Chemistry.
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∥ Medicinal Chemistry Research.

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(2) Greenlee, W. J. *Pharm. Res.* 1987, *4*, 367. Ondetti, M. A.; Cushman, D. W. *Annu. Rev. Biochem.* 1982, *51*, 283.
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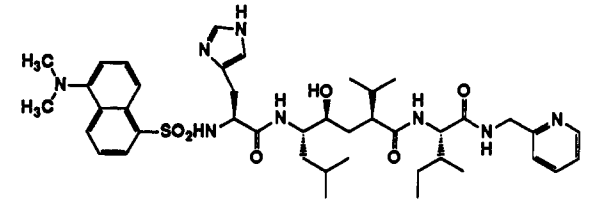
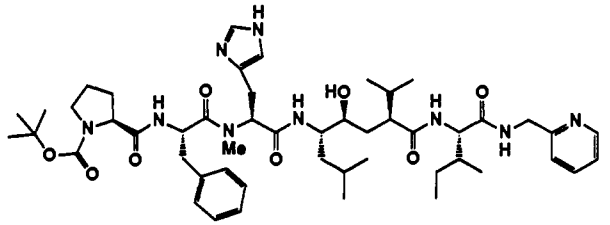
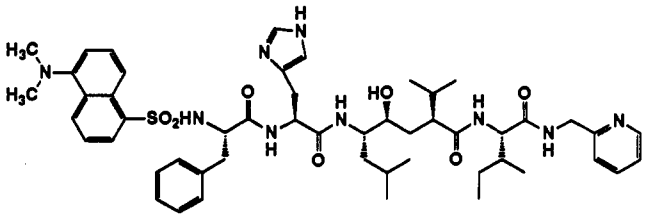


1 (U-80,215)



2 (U-71,038)



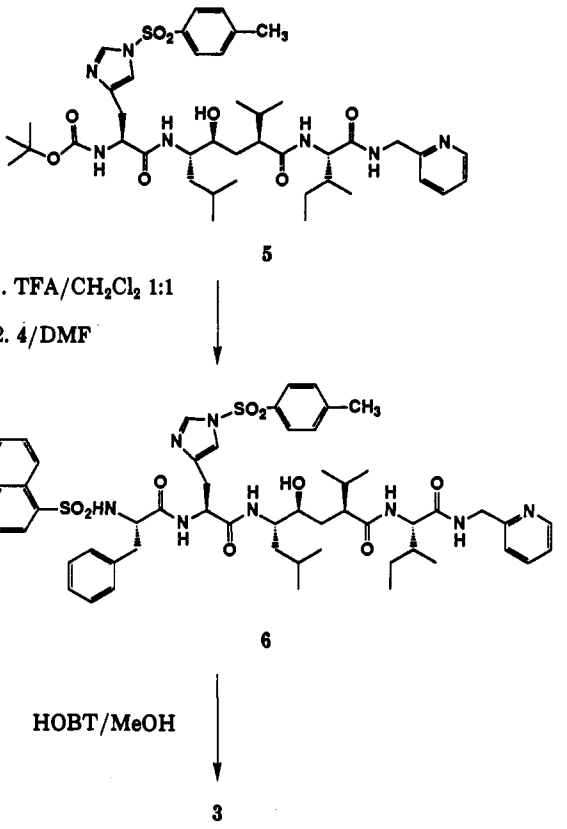
3 (U-80,825)

Figure 1. Fluorescent probes for renin inhibition studies.

transfer from the intrinsic tryptophans of human renin to a dansylated competitive inhibitor peptide.[5,6] Unlabeled competitive inhibitors were subsequently analyzed by displacement, and reproducible K_d values were obtained, which were in good agreement with independently determined K_i values and, in some cases, identical with the IC_{50} values. The dansylated inhibitor described in our previous report, 1 (U-80,215E), was found to have a dissociation constant of 1 μM, which allowed the determination of dissociation constants of unlabeled renin inhibitors to a limiting sensitivity of approximately 0.25 nM. However, we wished to extend the usable concentration range of this procedure to include inhibitors with K_d's in the picomolar to subpicomolar range. This necessitated the development of more sensitive methodology to assay the inhibitory activity of these compounds.

In order to circumvent the limits of the IC_{50} assay and to extend the concentration range of our fluorescence energy transfer assay, we designed a second competitive dansylated inhibitor of human renin based on the template of the known high-affinity inhibitor (2, U-71,038; Figure 1) of human renin.[7] This dansylated inhibitor, dansyl-Phe-His-LVA-Ile-Amp (3, U-80,825E), was found by K_i determination, to have an affinity of 43 ± 14 pM and to also undergo slow binding to the enzyme similar to 2.[7]

Scheme I



This report details the chemical synthesis of 3 and specialized procedures for the determination of the K_d and K_i of 3, using fluorescence energy transfer for the K_d, and inhibition of renin catalytic activity for the K_i value. Additionally, 3 was then utilized to evaluate the absolute affinity of a select group of nonlabeled high-affinity renin inhibitors, structurally related to and including 2, by using the fluorescence displacement assay. Using both the fluorescence (K_d) procedure, and the renin catalysis (K_i) method,[8] *we show that these unlabeled inhibitors are, indeed, more potent and in some cases nearly 1 order of magnitude more potent than the their reported IC_{50} values*, and that these procedures are more rapid and less cumbersome than IC_{50} determinations. We also discuss how 3 may be utilized to assess dissociation rate constants of unlabeled inhibitors.

Chemistry

Our previous efforts, the synthetic procedure utilized for the synthesis of 1, suggested that the direct dansylation of Phe-His(Tos)-LVA-Ile-Amp[7] would result in the formation of the penultimate product 6. In practice, this procedure was unsuccessful and we relied on the work of Fricker and Snyder,[9] wherein dansyl-L-phenylalanine was activated as the *N*-hydroxysuccinimidate. Dansyl-L-phenylanine-ONSu (4) was reacted with His-LVA-Ile-Amp, prepared by N-terminal deprotection of Boc-His-LVA-Ile-Amp[7] under acidic (1:1 TFA/CH_2Cl_2) conditions, generating 6 (see Scheme I). The final protecting group, tosyl on histidine, was removed with HOBT/methanol to provide 3 in reasonable overall yield. This dansylated in-

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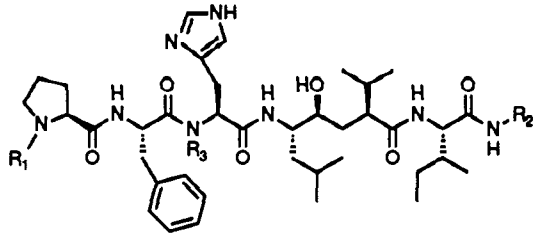
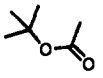
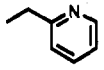
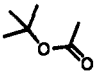
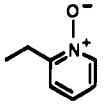
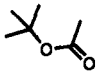
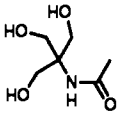
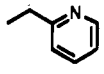
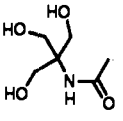
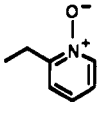
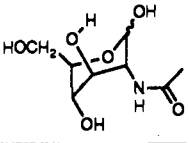
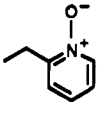
(9) Fricker, L. D.; Snyder, S. H. *J. Biol. Chem.* **1983**, *258*, 10950.

Table I. Potency Comparison of High-Affinity Renin Inhibitors



compd	code	R_1	R_2	R_3	IC^{50}, pM	K_i, pM	K_d, pM
2	U-71,038			CH_3	260[a]	40 ± 11	40 ± 11
3	U-80,825	see Figure 1 for structure			240	43 ± 14	66 ± 11
8a	U-76,394			CH_3	330[b]		131 ± 6
8b	U-79,059			CH_3	260[b]		44 ± 6
8c	U-77,352			CH_3	330[b]		111 ± 32
8d	U-77,436			CH_3	580[b]	51 ± 16	33 ± 8
8e	U-82,590			CH_3	1100[b]		434 ± 30

[a] See ref 7. [b] See ref 13.

hibitor **3** exhibited limited aqueous solubility and was converted to the tris–citrate salt by dissolution of the peptide and citric acid in methanol, removal of the solvent, dissolution in water, and lyophilization. All subsequent experiments in this report were performed with **3** as the tris–citrate salt.

Determination of Dissociation Constants (K_d)

The binding of **3** to human renin was followed by monitoring the change in tryptophan fluorescence at 325 nm after the addition of small aliquots of the dansylated inhibitor by utilizing the previously published procedure,[5] with some modifications (see Experimental Section). Data fitting was accomplished by using a nonlinear least-squares procedure[10] (Figure 2).

The fit of the data to eq 1[5] yields an apparent dissociation constant, K_d(app), which is the K_d of **3** in the presence of the specific concentration of **7** (H-142,[11] U-76,780E) added to the incubation mixture.[12] The actual K_d for **3** is computed from eq 2.

$$[I]_{tot} = K_d\{(F_o - F_x)/(F_x - F_{max}) + [E]_{tot}[(F_o - F_x)/(F_o - F_{max})]\} \quad (1)$$

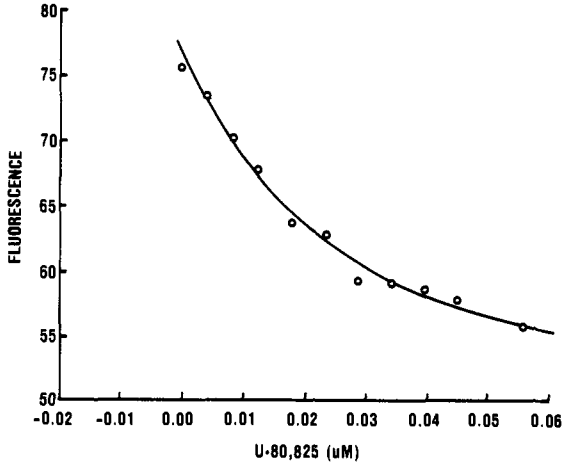


Figure 2. Titration of recombinant human renin by **3** (U-80,825). The titration was performed at 37 °C in 0.01 M Tris/HCl buffer at pH 7.4 with 5 mM β-octylglucoside, 0.2325 μM **7**, and 0.0465 μM recombinant human renin. The curve was fitted by using a nonlinear least-squares procedure.

(10) Yamaoka, K.; Tamigawana, Y.; Nakagama, T.; Uno, T. *J. Pharmacobio-Dyn.* **1981**, *4*, 8.
(11) Webb, D. J.; Manhem, P. J. O.; Ball, S. G.; Inglis, G.; Leckie, B. J.; Lever, A. F.; Morton, J. J.; Robertson, J. I. S.; Murray, G. D.; Menard, J.; Hallett, A.; Jones, D. M.; Szelke, M. *J. Hypertension* **1985**, *3*, 653. Szelke, M.; Leckie, B.; Hallett, A.; Jones, D. M.; Sueiras, J.; Atrash, B.; Lever, A. F. *Nature (London)* **1982**, *299*, 555.

$$K_d(app) = K_d(actual)\{(1 + [7])/K_d(7)\} \quad (2)$$

A series of structurally related and highly active (IC_{50} values) inhibitors were evaluated as validation for this procedure (see Table I). The dissociation constants of these high-affinity inhibitors were determined as follows: fluorescent intensities were determined (see Experimental Section) in the presence of 3 and the inhibitors. The fraction of 3 remaining bound (FB) to the renin was calculated[5] and substituted into eq 3, the numerical solution, along with the other known quantities in order to solve K_H, the dissociation constant of the unlabeled inhibitors. In

$$K_H = H_{tot}(K_L/[L])/(1/f^* - 1 - (K_L/[L]) - R_{tot}f^*(K_L/[L])) \quad (3)$$

this equation, K_L is the K_d of the fluorescent inhibitor, [L], the concentration of fluorescent ligand, f^*, the fraction of fluorescent ligand remaining bound, and R_{tot}, the renin concentration.

The data from a typical experiment in which recombinant human renin was titrated with 3 in the presence of the unlabeled inhibitor 7 (H-142, U-76,780E) (2 nM) is shown in Figure 2 along with the theoretical fit to the data.[12] Three duplicate determinations in the presence of different concentrations of 7 (K_d = 2.0 ± 0.35 nM) yielded a mean K_d of 66 ± 11 pM for 3, the dansylated inhibitor. This value was subsequently used in the determination of the K_d's of the unlabeled inhibitors. The K_d's of the unlabeled inhibitors were determined as described in the Experimental Section, with use of the predicted binding curves shown in Figure 3 or the numerical solution given in eq 3. Mean values (for three duplicate determinations) were calculated from the data at 37 °C.

Determination of Inhibition Constants (K_i)

The ability of 3 and unlabeled inhibitors to inhibit the hydrolysis of RSP, a synthetic substrate of human renin,[8] was used to determine their K_i values. The data (see Experimental Section) was plotted and fitted to an integrated form of the Michaelis–Menton equation or

$$VssI_0/(Vu - Vss) = (1 + K_m/S_0)K_iS_0/K_m + Vss(1 + K_m/S_0)/k_{cat} \quad (4)$$

where K_m and K_i are the Michaelis affinity constants for substrate and inhibitor respectively, S_0 is the initial substrate concentration, k_{cat} is the catalytic rate constant, and Vss and Vu are the steady state and uninhibited reaction velocities. Plots of Vss(×1000) versus I_0[Vss/(Vu − Vss)] yielded straight lines with a slope of $(1 + K_m/S_0)k_{cat}$ and an intercept of $(1 + K_m/S_0)K_iS_0/K_m$ (see Figure 4).

Titration of the hydrolysis of RSP by recombinant human renin (see Experimental Section) in the presence of various concentrations of 3 yielded the data plotted in Figure 4. Fits of the data were consistent with the simple competitive inhibitor model and yielded K_i values of 43 ± 14, 40 ± 11, and 51 ± 16 pM for 3, 2, and 8d, respectively. These numbers are in excellent agreement with the independently determined K_d values, but are a factor of 5–6 lower than the corresponding IC_{50} evaluations. The structures, K_d, K_i, and IC_{50} values of 3 and all unlabeled compounds are presented in Table I for comparison.

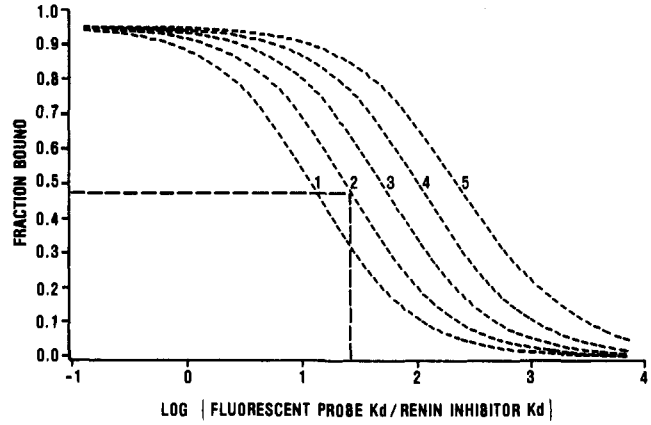


Figure 3. Predicted binding curves utilized for the determination of the dissociation constants of 2 and 7 by the fluorescence competitive binding assay. Predicted binding curves were generated as previously described.[5] The present curves, used in the determination of the k_d's of 2 and 7, were generated where [recombinant human renin] = 33 nM, [3] = 0.227 μM, and [2] or [7] = 33 nM (1), 66 nM (2), 0.13 μM (3), 0.263 μM (4), and 0.526 μM (5), respectively. Note that in the case of this set of predicted binding curves, "0" on the log axis represents 10^{-11} M.

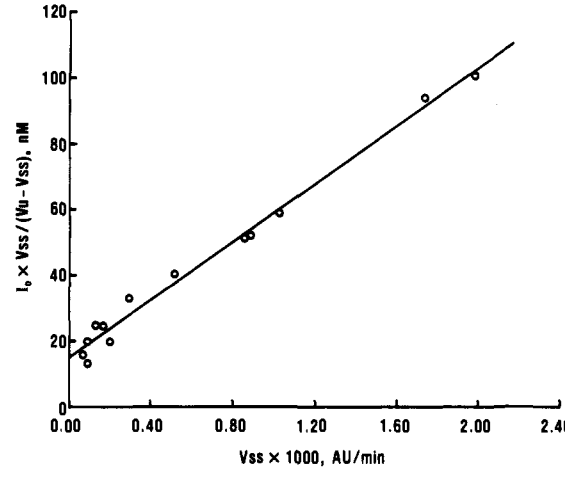


Figure 4. Kinetic determination of the K_i of 2 at 37 °C. The K_i of U-71,038 was determined at 37 °C by inhibition of renin hydrolytic activity as described in the Experimental Section.

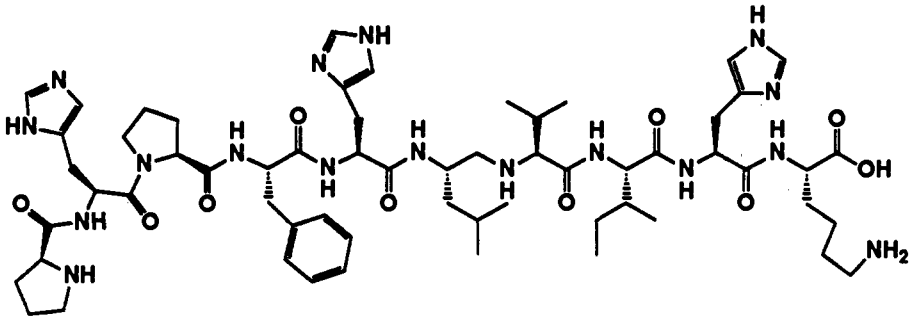
Results

A series of inhibitors, structurally related to 2 (see Figure 5), was chosen to evaluate the experimental methodology as well as to generate a more rational profile of the effects of structural modifications on binding affinity. In general, the structural modifications were designed to improve aqueous solubility and in vivo bioavailability of 2 while retaining high inhibitory activity.[13] As can be seen from the published IC_{50} data, their is little difference between 2 and 8a–d, with the values falling between 200 and 500 pM. This appears to be the lower limit for this assay, as reported.

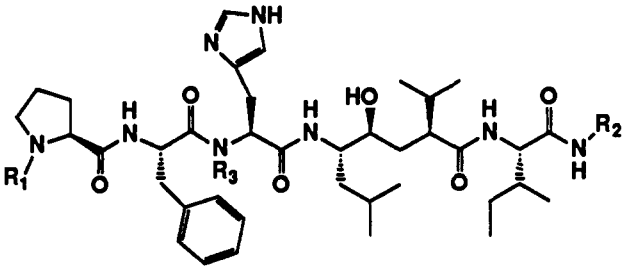
Differences do appear in the displacement assay where 2, 8b, and 8d are comparable, while the activity of 8a and 8c is approximately 2-fold less active. In the case of 8e, both assays provided corroboration of lower inhibitory

(12) The k_d of 3 is extremely low which, experimentally, results in the formation of a 1:1 enzyme–inhibitor complex upon addition to a solution of the enzyme. Depending on the amount of renin employed, the loss of fluorescence after each addition was a significant fraction of the total unless small amounts of the inhibitor were utilized. Better experimental data was obtained when the titrations were performed in the presence of 7, since under these conditions only a portion of the added 3 bound to the enzyme. This did not effect the final data, but extended the range of concentration of 3 over which fluorescence changes occurred, thus minimizing experimental error.

(13) Bundy, G. L.; Pals, D. T.; Lawson, J. A.; Couch, S. J.; Lipton, M. F.; Mauragis, M. A. *J. Med. Chem.* **1990**, *33*, 2276.



7 (H-142)



8

Figure 5. Human renin inhibitors H-142 (7) and 8.

activity. It is extremely important to note that level of activity is *4–10-fold lower* with the data from the displacement assay. These differences were confirmed with selected K_i determinations on **2**, **3**, and **8d**. The results of the K_d and K_i assessments substantiate our contention that it is possible to underestimate the potency of inhibitors if only IC_{50} determinations are utilized.

From a structure–activity standpoint, the results (a lack of change in inhibitory activity) suggest that the modifications at either end of the template, **8**, are outside the large renin binding pocket and solvent accessible.[13]

Discussion

The determination of affinity constants for high potency inhibitors of enzymes has always been an experimental challenge, especially if multiple determinations are to be made. As we have shown within the context of this study, many high-affinity inhibitors may appear to have the same potency because of the limits placed on in vitro (IC_{50}) assays by enzyme concentration. With this imposition of artificial constraints on the determination of inhibitor (drug) affinity, the ability to explore binding mechanisms and structure–activity relationships and to advance the investigation of the active-site chemistry of a proteins such as enzymes and receptors is compromised. This is especially true when interfacing molecular modeling with experimental data where accurate measurement of binding affinities is essential. Whereas the present report deals with the synthesis of a high-affinity inhibitor of human renin and its use in displacement assays to easily determine the K_d's of unlabeled inhibitors, the principles which we have described and the derived equations may be useful in other ligand/receptor systems.

The fluorescence displacement assay was previously shown to have a limiting sensitivity of 250 pM when **1** was used as the fluorescent inhibitor.[5] The competitive fluorescent renin inhibitor which we describe here allows the determination of K_d's of unlabeled inhibitors to a sensitivity less than 0.01 pM. The limiting factor in this method is, therefore, the minimal amount of human renin necessary to give a reproducible fluorescence signal. At 37 °C, the minimal amount of renin required is approximately 0.01 μM under our conditions and with the instrumentation described. Of course if a radioactive rather than fluorescent displaceable inhibitor is used, then the sensitivity could potentially be even greater.

The K_d's of **2**, **3**, **8b**, and **8d** are, for all practical purposes, identical within the limits of the experimental data. This is not surprising since they are quite similar in structure. Although long incubation and displacement times were necessary because of the slow binding nature of the compounds, the K_d determinations were not labor intensive, thus making it possible to evaluate numerous high-affinity inhibitors during a normal working day. In addition, if a nonlabeled inhibitor is first bound to renin and then displaced by an excess of **3**, it should be possible to determine the dissociation off rate constants. We feel that the use of a high-affinity fluorescent inhibitor, such as **3**, coupled with our methodology and derivations provides a simple procedure for dealing with high-affinity enzyme inhibitors under nondegenerate conditions.

Experimental Section

Tris base was purchased from Sigma Chemical Co. and *n*-octyl β-D-glucopyranoside was obtained from Calbiochem. Compounds **2**,[7] **7**,[11] and **8a–e**[12] were synthesized at The Upjohn Co. Recombinant human renin was produced in CHO cells and isolated and purified as described previously.[8] Dilute solutions of all inhibitors were prepared in distilled water containing 0.1% sodium azide, and the concentrations of inhibitor and renin solutions were determined by amino acid analysis.

High resolution mass spectra, infrared spectra, ultraviolet spectra, and combustion analyses were obtained by the Physical and Analytical Chemistry Department of The Upjohn Co. High-field ^{1}H NMR spectra at 300 MHz were determined on a Bruker AM-300 and chemical shifts reported as δ units relative to tetramethylsilane. Fluorescence measurements were made in the ratio mode on a Perkin-Elmer MPF 66 spectrofluorometer.

Thin-layer chromatography was conducted with Analtech 0.25-mm glass plates precoated with silica gel GF. For column chromatography, E. Merck silica gel 60, 230–400 mesh, was used. All solvents for chromatography were Burdick and Jackson or

Fisher reagent grade. All nonaqueous reactions were carried out under an inert atmosphere unless otherwise noted.

[1*S*-[1*R,2*R**,4*R**(1*R**,2*R**)]]-*N*-[[5-(Dimethylamino)-1-naphthylenyl]sulfonyl]-1-phenylalanyl-*N*-[2-hydroxy-5-methyl-1-(2-methylpropyl)-4-[[[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]amino]carbonyl]hexyl]-N^{im}-[(4-methylphenyl)sulfonyl]-L-histidinamide.** To a well-stirred solution of Boc-His(Tos)-LVA-Ile-Amp[7] (270 mg, 0.32 mmol) in dichloromethane (5 mL) was added trifluoroacetic acid (5 mL). The reaction mixture was stirred at room temperature for 0.5 h, the solvents were removed in vacuo, and the residue was redissolved in dichloromethane. The solution was washed with saturated sodium bicarbonate solution, water, and brine, dried over anhydrous sodium sulfate, and concentrated. The solid was dissolved in dry dimethylformamide (5 mL), dansyl-Phe-OSu[9] (247 mg, 0.50 mmol) and triethylamine (101 mg, 1.0 mmol) were added, and the reaction mixture was stirred at room temperature for 72 h. The dimethylformamide was removed in vacuo, and the residue was dissolved in dichloromethane and poured into brine. The aqueous phase was extracted with 5% methanol/dichloromethane, and the extracts were combined, dried, (anhydrous sodium sulfate), and concentrated. The residue was chromatographed on silica gel (elution with 5% methanol/dichloromethane) to yield dansyl-Phe-His(Tos)-LVA-Ile-Amp (172 mg, 48%) as an amorphous light green solid: IR (mull, cm^{-1}) 3291, 1648, 1619, 1545, 1173, 1093; MS (FAB) m/z 1106 $[M + H]^+$; UV (MeOH, nm) 236 (sh, 23 630), 252 (sh, 19 260), 259 (sh, 17 500), 266 (sh, 12 8700, 338 (4120).

[1*S*-[1*R,2*R**,4*R**(1*R**,2*R**)]]-*N*-[[5-(Dimethylamino)-1-naphthylenyl]sulfonyl]-1-phenylalanyl-*N*-[2-hydroxy-5-methyl-1-(2-methylpropyl)-4-[[[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]amino]carbonyl]hexyl]-L-histidinamide.** A solution of dansyl-Phe-His(Tos)-LVA-Ile-Amp (42 mg, 0.037 mmol) and 1-hydroxybenzotriazole (15 mg, 0.11 mmol) in methanol (2 mL) was stirred at room temperature for 24 h. The reaction mixture was concentrated and the residue was chromatographed on silica gel (elution with 5% methanol/dichloromethane followed by 5% methanol (saturated with gaseous ammonia)/dichloromethane) to provide dansyl-Phe-His-LVA-Ile-Amp[14] (28 mg, 80%) as an amorphous solid: IR (mull, cm^{-1}) 3288, 1640, 1571, 1545, 1145, 1061; MS (FAB) m/z 952 $[M + H]^+$; UV (MeOH, nm) 255 (15 950), 260 (sh, 15 370), 266 (sh, 11 560), 338 (4080); exact mass calcd for $C_{51}H_{69}N_9O_7S$ 952.5119, found 952.5119.

[1*S*-[1*R,2*R**,4*R**(1*R**,2*R**)]]-*N*-[[5-(Dimethylamino)-1-naphthylenyl]sulfonyl]-1-phenylalanyl-*N*-[2-hydroxy-5-methyl-1-(2-methylpropyl)-4-[[[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]amino]carbonyl]hexyl]-L-histidinamide 2-Hydroxy-1,2,3-propanetricarboxylate (1:3).** Dansyl-Phe-His-LVA-Ile-Amp (0.055 g, 0.058 mmol) was dissolved in methanol (2 mL) and citric acid (0.037 g, 0.176 mmol) was added. Upon complete solution, the methanol was removed in vacuo and the residue was dissolved in water (5 mL). The salt (88 mg, 95%) was obtained as a fluffy white solid after lyophilization.

Dissociation Constants. The dissociation constants, K_d's, of the renin inhibitors were determined by the fluorescence displacement assay,[8] with the following modifications: 3 (23 nM, in duplicate) was added to recombinant human renin (33 nM) in 0.01 M Tris, pH 7.4, 5 mM β-octyl glucoside, at 37 °C and incubated for 1.5 h. The fluorescence intensities were recorded prior to (F_o) and after (F_{max}) the addition of dansylated inhibitor. Unlabeled inhibitors (6.6 and 13 nM) were then added, and the incubation was continued an additional 2.5 h, after which the final fluorescence intensity, F_x, was recorded. The fluorescence intensities were normalized to a volume of 2.5 mL.

Inhibition Constants.[8] Recombinant human renin (0.010 77 or 0.0586 μM) was preincubated at 37 °C for 2 h with, for example, 2 (0–0.27 μM) or 3 (0–1.5 μM), respectively. Twenty-five microliters of the renin/inhibitor preincubation mixture was added to 100 μL of RSP, 856 and 1344 μM, respectively in 0.01 M phosphate, 10 mM *n*-octyl β-D-glucopyranoside, pH 6.5, to begin hydrolysis. Cleavage of RSP by renin was followed by quantitation of the products by HPLC. The data was plotted and fitted to an integrated form of the Michaelis–Menton equation (see text).

(14) Amino acid analysis: Ile, 0.994; His, 1.002. The sulfonamide linkage of dansyl-Phe was not hydrolyzed under standard conditions.

Synthesis of a Series of Nitrothiophenes with Basic or Electrophilic Substituents and Evaluation as Radiosensitizers and as Bioreductively Activated Cytotoxins

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A series of 2- and 3-nitrothiophene-5-carboxamides bearing *N*-(ω-aminoalkyl) side chains has been prepared by treatment of the thiophenecarbonyl chloride with the appropriate (protected) ω-aminoalkylamine. Analogous *N*-(oxiranylmethyl)nitrothiophene-5-carboxamides have been synthesized by epoxidation of the corresponding *N*-allylamide. Compounds in both classes were evaluated in vitro both as radiosensitizers of hypoxic mammalian cells and as selective bioreductively activated cytotoxins. The most potent radiosensitizers were those agents with strong tertiary amine bases or oxiranes in the side chain. Studies in vivo showed that 2-methyl-*N*-[2-(dimethylamino)ethyl]-3-nitrothiophene-5-carboxamide caused slight radiosensitization of the KHT sarcoma in mice given 0.34 mmol kg^{-1}. However, administration of this and related tertiary amines at higher doses was precluded by systemic toxicity.

The relative resistance of cells in hypoxic regions of solid tumors to killing by ionizing radiation remains an important reason for failure of local control of cancer by radiotherapy since molecular oxygen is required as an electron acceptor for the manifestation of damage to DNA. Electron-affinic nitroheterocycles can, however, act as mimics of molecular oxygen in this process and thus can be effective as radiosensitizers of hypoxic cells.[1,2] Indeed, a correlation between the one-electron reduction potential (E^1_7) of such compounds and their efficiency as sensitizers of hypoxic cells in vitro to ionizing radiation has been reported.[3,4]

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