Identification of Novel Inhibitors of Urokinase via NMR-Based Screening

Philip J. Hajduk, Steven Boyd, David Nettesheim, Vicki Nienaber, Jean Severin, Richard Smith, Don Davidson, Todd Rockway, and Stephen W. Fesik*

Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064

Received May 24, 2000

Using an NMR-based screen, a novel class of urokinase inhibitors were identified that contain a 2-aminobenzimidazole moiety. The inhibitory potency of this family of inhibitors is similar to that of inhibitors containing a guanidine or amidine group. However, unlike previously described guanidino- or amidino-based inhibitors which have pK_a values greater than 9.0, urokinase inhibitors containing a 2-aminobenzimidazole have pK_a values of 7.5. Thus, 2-aminobenzimidazoles may have improved pharmacokinetic properties which could increase the bioavailability of inhibitors which contain this moiety. A crystal structure of one of the lead inhibitors, 2-amino-5-hydroxybenzimidazole, complexed with urokinase reveals the electrostatic and hydrophobic interactions that stabilize complex formation and suggests nearby subsites that may be accessed to increase the potency of this new series of urokinase inhibitors.

Introduction

Urokinase is a potent activator of plasminogen and thus plays a central role in the cascade mechanism leading to basement membrane degradation and tumor metastasis.[1,2] Urokinase is implicated in a large number of malignancies, including cancers of the breast, lung, bladder, stomach, cervix, kidney, and brain, and high levels of urokinase have been correlated with poor patient prognosis.[3,4] Thus, there is great clinical interest in the development of potent and orally bioavailable inhibitors of urokinase that can serve as therapeutic agents for the treatment of cancer.

Small-molecule inhibitors of urokinase have been shown to inhibit tumor metastasis and slow cancer growth. For example, amiloride and *p*-aminobenzamidine reduce the size of tumors in experimental animals,[5,6] and the recently described 4-iodobenzo[*b*]-thiophene-2-carboxamidine (B428) blocks tumor-derived urokinase activity in a mammary carcinoma model in mice.[7] However, all of the reported inhibitors of urokinase contain an amidine or guanidine group, and compounds designed from templates containing these positively charged moieties can be a liability in the search for oral therapeutic agents. Therefore, there is a need for alternative small-molecule inhibitors of urokinase which have favorable pharmacokinetic properties.

Here we describe the NMR-based discovery of 2-aminobenzimidazoles as novel inhibitors of urokinase that may serve as templates for the development of potent inhibitors of this enzyme. In contrast to compounds which contain an amidine or guanidine group, which typically have pK_a values greater than 9.0, the 2-aminobenzimidazoles have pK_a values of approximately 7.5 and thus may improve the bioavailability of urokinase inhibitors which contain this moiety.

* To whom correspondence should be addressed: Dr. Stephen W. Fesik, Abbott Laboratories, D-47G, AP10, 100 Abbott Park Rd., Abbott Park, IL 60064. Phone: (847) 937-1201. Fax: (847) 938-2478. E-mail: stephen.fesik@abbott.com.

Results and Discussion

Preparation of ^{15}N-Labeled Urokinase. To identify low-molecular-weight ligands for urokinase, NMR-based screening employing ^{1}H/^{15}N correlation spectra was applied.[8,9] This approach required ^{15}N-labeled protein that yielded suitable ^{1}H/^{15}N correlation spectra in a short period of time (<30 min). For many proteins, uniform ^{15}N-labeling can be routinely and cost-effectively accomplished by overexpression of the target protein in a bacterial host. However, attempts to produce urokinase in bacterial systems failed. Although it is possible to prepare uniformly ^{15}N-labeled protein from mammalian cells using purified amino acids,[10,11] this is not cost-effective for the large-scale production of protein for NMR-based screening. As an alternative to uniform labeling, we pursued selective ^{15}N-labeling of urokinase in mammalian cells using selected ^{15}N-labeled amino acids as precursors. Hybridoma serum-free medium that did not contain amino acids was obtained from Gibco-BRL, and the medium was enriched with ^{15}N-labeled glycine, alanine, valine, leucine, aspartic acid, lysine, tryptophan, tyrosine, and phenylalanine prior to growth. This allowed the large-scale production of selectively labeled protein (>200 mg). The ^{1}H/^{15}N HSQC spectrum of this protein (Figure 1) contained more than 70 cross-peaks and was sufficient for NMR-based screening.

Identification of 2-Aminobenzimidazoles. Since urokinase is a protease, screening conditions were required which would inhibit autolytic degradation of the protein. While a number of inhibitors can adequately inhibit the protein, phenylguanidine was chosen since it is small, soluble, and readily available. A phenylguanidine concentration of 1 mM was sufficient to block autolytic degradation for more than several days at room temperature, and this concentration was chosen for use in the NMR-based screen. Under these conditions, more than 3000 compounds were tested for binding to selectively ^{15}N-labeled urokinase by monitoring changes in the amide chemical shifts of the protein upon addition of the test compounds. From an analysis

10.1021/jm0002228 CCC: $19.00
Published on Web 10/03/2000

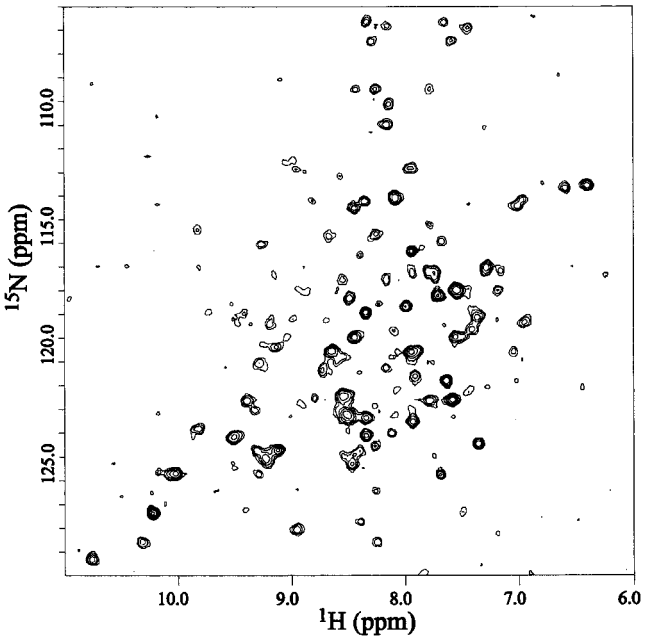


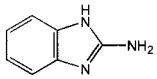
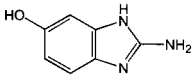
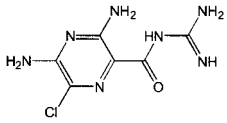
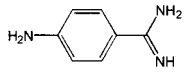
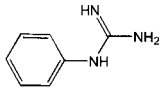
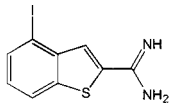
Figure 1. ^{1}H/^{15}N HSQC spectrum of selectively labeled urokinase.

of the pattern of chemical shift changes, it appeared that 2-aminobenzimidazole displaced phenylguanidine. Additional NMR binding experiments confirmed that 2-aminobenzimidazole and phenylguanidine bind competitively to the same site on urokinase. A dissociation constant of 50 μM was obtained for the binding of 2-aminobenzimidazole to urokinase, and enzyme inhibition assays yielded an IC_{50} value of 200 μM for this compound (Table 1). Commercially available analogues, such as benzimidazole, benzoxazole, and benzotriazole, did not bind to urokinase up to compound concentrations of 5 mM, indicating the critical importance of the aminoimidazole moiety for binding to urokinase.

2-Aminobenzimidazole is an attractive template for the design of urokinase inhibitors. In addition to being a novel inhibitor class, the 2-aminobenzimidazoles have pK_a values of 7.5 and will therefore be uncharged at physiological pH. This is in contrast to all of the known urokinase inhibitors that contain a guanidine or amidine moiety that will be positively charged under these conditions. However, the potency of the parent 2-aminobenzimidazole is more than 10-fold less than that of the more traditional inhibitors (Table 1). Therefore, simple analogues were prepared to improve the potency. The most potent of these compounds was 5-hydroxy-2-aminobenzimidazole (**2**), which was prepared from 4-methoxy-1,2-phenylenediamine dihydrochloride using an adaptation of the procedure of Leonard et al. (Scheme 1).[12] 5-Hydroxy-2-aminobenzimidazole exhibited an IC_{50} value of 10 μM for urokinase − similar to that observed for more conventional inhibitor classes which contain a guanidine or amidine functionality (see Table 1). Thus, these aminobenzimidazoles can serve as equipotent replacements for arylamidine or -guanidine moieties with the advantage of improved pK_a values.

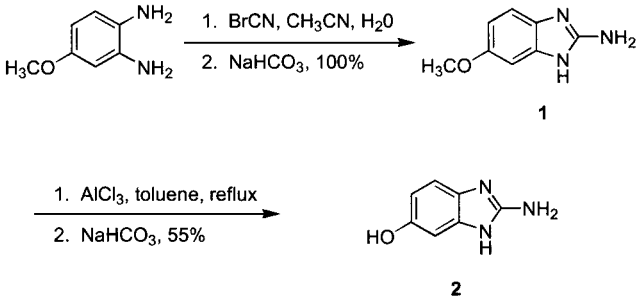
Structural Studies. An X-ray crystal structure of urokinase complexed with 5-hydroxy-2-aminobenzimidazole was obtained (Figure 2, PDB code 1FV9). The structure reveals that the benzimidazole sits deep in the S1′ pocket (Figure 2A), forming hydrogen bonds between

Table 1. Urokinase Inhibition Activity of Selected Compounds

Name	Structure	IC_{50} (μM)	pKa[a]
2-aminobenzimidazole		200	7.5[b,c]
2-amino-5-hydroxy benzimidazole		10	7.4[c]
amiloride		7	8.6
p-aminobenzamidine		7	11.4
phenylguanidine		20	10.9
B428		0.5	9.1

[a] All pK_a values were calculated using the ACD/pK_a package v3.6. Information on this software can be found at http://www.acdlabs.com. [b] The literature value for this compound is also 7.5 (Albert et al. *J. Chem. Soc.* **1948**, 2240). [c] The pK_a values for 2-aminobenzimidazole and 2-amino-5-hydroxybenzimidazole were confirmed by following the UV absorbance of the compounds as a function of pH.

Scheme 1



the 2-amino group of **2** and the side-chain carboxylate of Asp189 and between the N1 imidazole nitrogen and the backbone carbonyl of Gly216 (Figure 2B).[13] These hydrogen bonds are consistent with the binding data that indicated the importance of the aminoimidazole group. The inhibitor is also in close proximity to the side chains of Gln192, Ser195, Val213, and Cys220 and the main-chain atoms of Ser214−Ala221 and Lys224−Val227 (Figure 2B). A comparison of this crystal structure to that of urokinase complexed to B428[14] indicates that the 5-hydroxy-2-aminobenzimidazole sits much deeper in the S1′ pocket than the benzothiophene B428 (Figure 2C). This results in a shift of more than 2 Å in the position of the phenyl rings of the two inhibitors when bound to urokinase. While both inhibitors form hydrogen bonds and make contacts with many of the same residues of urokinase, the S1β subsite (occupied by the iodo group of B428, Figure 2C) is not accessed by the benzimidazole. This site is critical for the potency of B428, as removal of the iodo group results in losses in activity of more than a factor of 10.[15] The crystal

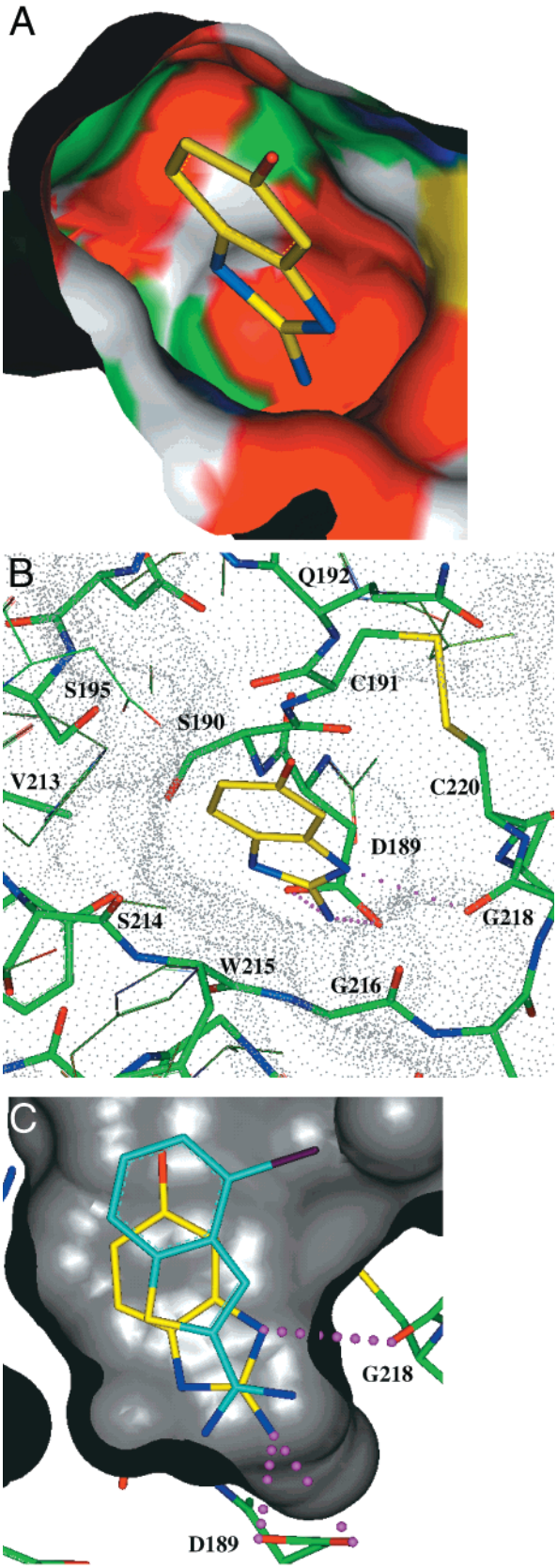


Figure 2. (A) Surface representation of the crystal structure of urokinase (solid surface colored by atom type) complexed to 5-hydroxy-2-aminobenzimidazole (**2**; yellow carbon atoms). (B) Interactions of 5-hydroxy-2-aminobenzimidazole with the S1′ pocket of urokinase (green carbon atoms, heavy rendering), including hydrogen bonds (magenta dotted lines) between the aminoimidazole moiety and Asp189 and Gly218. (C) Comparison of the crystal structures of 5-hydroxy-2-aminobenzimidazole (yellow carbon atoms) and B428 (cyan carbon atoms).[14]

structure of urokinase complexed to **2** indicates that it may be possible to improve the potency of the benzimidazoles by accessing this important structural subsite.[16] The X-ray crystal structure also indicates that substitutions at the 5-position of the benzimidazoles could access additional sites of interaction to further improve the potency of these compounds. On the basis of the crystal structure, substitutions at the 6-position of the benzimidazole should not be tolerated due to steric hindrance by the side chain of Ser195 (see Figure 2A,B). However, the recently reported crystal structure of urokinase complexed with (4-aminomethylphenyl)guanidine inhibitors reveals that the side chains of Ser195 and His57 rearrange to allow access to the S1′ subsite.[17] Similar rearrangements would provide additional sites of interaction for the benzimidazole-based inhibitors.

Conclusions

NMR-based screening utilizing $^{1}H/^{15}N$ HSQC spectra has proven to be a powerful tool for identifying ligands for proteins.[8,18−21] However, all previous reports relied on the production of uniformly ^{15}N-labeled protein from bacteria. The labeling strategy described in this work extends the applicability of NMR-based screening to proteins derived from mammalian systems. Using this strategy, we identified a novel class of urokinase inhibitors that have significantly improved pK_a values compared to inhibitors that contain a guanidine or amidine functionality. These inhibitors bind to the same site on urokinase and make many of the same interactions with the proteins as the more traditional inhibitors. Moreover, crystal structures of these leads complexed to urokinase suggest ways that the compounds may be modified to produce more potent inhibitors of this important therapeutic target.

Experimental Section

Protein Preparation. The base growth medium was hybridoma serum-free (HSF) medium (Gibco-BRL, no. 94-0016DK) containing 50% NaCl without amino acids or D-glucose. The complete HSF medium for amino acid studies consisted of the base medium plus 10 mL/L 50× NaCl (Gibco-BRL), 10 mL/L 100× D-glucose (Gibco-BRL), 0.25 mL/L 10 mM methotrexate (Sigma), and labeled and unlabeled amino acids. ^{15}N-Labeled amino acids (CIL) were added as powders to the medium at the following levels: ^{15}N-alanine (100 mg/L), ^{15}N-aspartic acid (67 mg/L), ^{15}N-glycine (100 mg/L),^{15}N-leucine (100 mg/L), ^{15}N-lysine (100 mg/L), ^{15}N-phenylalanine (35 mg/L), ^{15}N-tryptophan (16.6 mg/L), ^{15}N-tyrosine (50 mg/L), and^{15}N-valine (67 mg/L). Unlabeled amino acids (Sigma) were added as powders to the medium at the following levels: arginine (175 mg/L), asparagine (50 mg/L), cystine (50 mg/L), cysteine (116 mg/L), glutamic acid (100 mg/L), histidine (45 mg/L), isoleucine (120 mg/L), methionine (45 mg/L), proline (150 mg/L), serine (20 mg/L), and threonine (80 mg/L). The medium was then filtered and stored at 4 °C in the dark. To the final medium were added 3.125 mL/L 100 mM l-glutamine and 10% hyclone fetal bovine serum (Hyclone Laboratories, cat. no. SH30071.03) (which was exhaustively dialyzed and heated for 60 min at 60 °C). Following cell adaptation to the above medium, SP2/0 cells were planted at various densities in Bellco 100-mL microcarrier spinner flasks (Bellco Glass, Inc., stock no. 1065-00100) precoated with Sigmacote (Sigma Aldrich Co., product no. SL-2) according to directions. Incubation was at 37 °C on a Bellco spinner base. The cells were then pelleted by centrifugation at 300 rpm for 30 min at 4 °C. The supernatant was adjusted to pH 5.6 with 1 N HCl and run over an ABx column (Baker)

equilibrated with 10 mM MES, pH 5.6. After loading, the column was washed with equilibration buffer and protein was eluted with 0.5 M sodium acetate, pH 7.0. Aliquots from individual fractions were assayed for total urokinase activity following conversion from single-chain pro-urokinase to active two-chain enzyme by incubation with thermolysin. These fractions were pooled, adjusted to 50 mM Tris (from 500 mM Tris stock), pH 7.5, and applied to a *p*-aminobenzamidine column (Sigma) to remove active serine proteases other than urokinase which may be present. The flow-through was collected, adjusted to 1 mM $CaCl_2$, and incubated with rehydrated thermolysin-agarose beads (Sigma, no. P9040) at 37 °C for 75 min to convert pro-urokinase to active two-chain urokinase. This activation reaction was quenched with 5 mM EDTA, and the thermolysin-agarose beads were removed by filtration through an empty chromatography column. The solution containing active urokinase was then reapplied to a *p*-aminobenzamidine column equilibrated with 50 mM $NaPO_4$, 400 mM NaCl, pH 7.5. Urokinase bound to this column was eluted with 100 mM sodium acetate, 400 mM NaCl, pH 4.5. Fractions containing urokinase were pooled, adjusted to 400 mM arginine, concentrated, and applied to a Superdex-75 (Amersham-Pharmacia) column equilibrated with 100 mM $NaPO_4$, pH 5.0. Active fractions were pooled, concentrated and dialyzed against the appropriate NMR buffer.

NMR Spectroscopy. NMR samples were composed of selectively ^{15}N-labeled urokinase at 0.3 mM in a H_2O/D_2O (9:1) solution containing 50 mM phosphate, 1 mM phenylguanidine (ICN) at pH 7.5. Ligand binding was detected at 30 °C by acquiring sensitivity-enhanced ^{15}N HSQC spectra[22] on 400 μL of protein sample in the presence and absence of added compound. Compounds were added as solutions in perdeuterated DMSO. A Bruker sample changer was used on a Bruker AMX500 spectrometer. Compounds were initially tested at 1.0 mM each in mixtures of 10, with subsequent deconvolution to individual compounds for those mixtures that caused significant perturbations in the spectrum. Perturbations were considered significant if $\Delta\delta > 0.1$ ppm for at least two peaks in the spectrum, where $\Delta\delta = \mathrm{sqrt}[(\delta(^1\mathrm{H,ppm})_{free} - \delta(^1\mathrm{H,ppm})_{test})^2 + 0.04 \times (\delta(^{15}\mathrm{N,ppm})_{free} - \delta(^{15}\mathrm{N,ppm})_{test})^2]$. Spectra were acquired in 30 min with 64 complex points and were processed off-line using in-house written software.

Protein Crystallography. Micro-urokinase consisting of residues Ile16−Lys243 (chymotrypsin numbering system; Ile159−Lys404, urokinase numbering system) and two point mutations, Cys122Ala and Asn145Gln (Cys279Ala, Asn302Gln), was purified and crystallized as described.[14] Briefly, crystals were obtained by the hanging drop vapor diffusion method with a typical well solution of 0.15 M Li_2SO_4, 20% poly(ethylene glycol) MW 4000 in succinate buffer pH 4.8−6.0. On the cover slip, 2 μL of well solution was mixed with 2 μL of protein solution and the slip sealed over the well. Crystallization occurred at 18−24 °C within 24 h. The protein solution was composed of 6 mg/mL (0.21 mM) urokinase in 10 mM citrate, pH 4.0, 3 mM ϵ-amino caproic acid *p*-carbethoxyphenyl ester chloride with 1% DMSO cosolvent.

The urokinase crystal complex was formed by the compound soaking technique and belong to the space group $P2_12_12_1$ with unit cell dimensions of $a = 55.16$ Å, $b = 53.00$ Å, $c = 82.30$ Å, and $\alpha = \beta = \gamma = 90°$. The complex crystal diffracted to 3.0 Å on a Rigaku RTP 300 RC rotating anode source equipped with an RAXISII detector at 160K. Data were processed using the HKL program suite.[23] Data were 99% complete in the 3.0 Å shell with an I/σ of 2 and an R_{sym} of 55%. The overall R_{sym} of the data was 10.1%. An initial electron density map was calculated using the program package XPLOR[24] and the 1.5 Å native model.[14] The electron density map was inspected on a Silicon Graphics INDIGO2 workstation using QUANTA 97, and the orientation of the compound was clearly visualized in the initial $2F_o - F_c$ map. The complex was refined using the program package XPLOR to a final *R* factor of 22.3%.

Enzyme Inhibition Assays. The effects of synthetic inhibitors on the steady-state amidolytic activity of urokinase toward the chromogenic substrate, H-D-pyroglutamyl-Gly-L-Arg-*p*-nitroanilide (S2444, Helena Laboratories, Beaumont, TX), was characterized by the formation of *p*-nitroanaline.[25] Briefly, 0−50 μM concentration of inhibitors were tested against 25 IU/mL (0.14 ng/mL) urokinase and 150 μM concentrations of S2444 in 200-μL volumes in PBS and 0.01% BSA (pH 7.4). Incubations were performed at 37 °C with absorbance at 405 nm recorded every 11 s for 20 min. Data were plotted as percent (%) inhibition versus inhibitor concentration (μM). IC_{50} values were calculated at 50% inhibition from a linear fit slope of the data.[26,27]

5-Methoxy-2-aminobenzimidazole (1). A solution of 4-methoxy-1,2-phenylenediamine dihydrochloride (519 mg, 2.46 mmol) in 5 mL of water was cooled to 0 °C and treated with a solution of cyanogen bromide (0.60 mL, 5 M in acetonitrile, 3.0 mmol) and solid $NaHCO_3$ (414 mg, 4.93 mmol). The deep purple solution was stirred at ambient temperature for 48 h. The mixture was made basic with 1 M aqueous Na_2CO_3; then the solution was concentrated under reduced pressure. The residue was triturated with hot ethanol (3 × 20 mL), and the ethanol solution was filtered and concentrated under reduced pressure to give the title compound as a tan solid (417 mg, 100%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.70 (dd, $J = 2$, 8 Hz, 1H), 6.85 (d, $J = 2$ Hz, 1H), 7.16 (d, $J = 8$ Hz, 1H), 7.52 (br s, 2H); MS (DCI-NH_3) m/z 164 $(M + H)^+$.

5-Hydroxy-2-aminobenzimidazole (2). 5-Methoxy-2-aminobenzimidazole (100 mg, 0.613 mmol) was suspended in 6 mL of toluene, and aluminum chloride (332 g, 2.45 mmol) was added. The purple mixture was stirred and warmed to 80 °C for 4 h, at which time starting material had been consumed as determined by reverse-phase HPLC of an aliquot. The reaction mixture was cooled in an ice bath and quenched by dropwise addition of 4 mL of saturated aqueous $NaHCO_3$. The mixture was diluted with 5 mL of ice water containing 1 mL of 1 M aqueous citric acid to aid in dissolving the aluminum salts. The reaction was concentrated in vacuo to a dark solid residue, which was triturated with 3 × 5 mL of EtOH−$CHCl_3$ (1:1). The combined organic phase was concentrated in vacuo, and the residue purified by column chromatography (20% MeOH−1% saturated aqueous NH_4OH−CH_2Cl_2) to produce the title compound as a silverish white solid (50.0 mg, 0.335 mmol, 55% yield): TLC (20% MeOH−1% saturated aqueous NH_4OH−CH_2Cl_2) R_f 0.13; HPLC (Zorbax SB-C_{18}, 4.6-mm i.d., 25-cm length, 0−100% MeCN−H_2O containing 0.1% TFA, 1.5 mL/min flow rate, 20 min elution, 254 nm detection) t_R 5.64 min (96 area %); ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.1−6.3 (br s, 3H), 6.36 (dd, $J = 2$, 8 Hz, 1H), 6.55 (d, $J = 2$ Hz, 1H), 6.87 (d, $J = 8$ Hz, 1H), 8.0−9.0 (vbr s, 1H); HRMS (EI) calcd for $C_7H_7N_3O$ 149.0589, found 149.0591. A sample (28 mg) of this material was purified by preparative reverse-phase chromatography (Zorbax SB-C_{18}, 21.2-mm i.d., 25-cm length, 0−100% MeCN−H_2O containing 0.1% TFA, 15 mL/min flow rate, 70 min elution, 254 nm detection) to provide an analytical sample of the trifluoroacetate salt (15 mg): ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.64 (dd, $J = 2$,8 Hz, 1H), 6.76 (d, $J = 2$ Hz, 1H), 7.14 (d, $J = 8$ Hz, 1H), 8.32 (s, 2H), 9.49 (br s, 1H), 12−13 (v br s, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 98.0, 110.8, 111.7, 122.3, 130.5, 150.5, 154.0. Anal. Calcd for $C_9H_8F_3N_3O_3 \cdot 1.4H_2O$: C, 37.48; H, 3.77; N, 14.57. Found: C, 37.71; H, 3.54; N, 14.56.

Acknowledgment. The authors thank Anne Rueter for assistance in the growth and purification of the protein and Dr. Jack Henkin for useful discussions and support during this project.

References

(1) Duffy, M. J. Urokinase-type Plasminogen Activator and Malignancy. *Fibrinolysis* **1993**, *7*, 295−302.
(2) Mignatti, P.; Rifkin, D. B. Biology and Biochemistry of Proteinases in Tumor Invasion. *Physiol. Rev.* **1993**, *73*, 161−195.

(3) Andreasen, P. A.; Kjoller, L.; Christensen, L.; Duffy, M. J. The Urokinase-Type Plasminogen Activator System in Cancer Metastasis: A Review. *Int. J. Cancer* **1997**, *72*, 1−22.
(4) Meyer, T.; Hart, I. R. Mechanisms of Tumour Metastasis. *Eur. J. Cancer* **1998**, *34*, 214−222.
(5) Evens, D. M.; Sloanstakleff, K.; Arvan, M.; Guyton, D. P. Time and Dose Dependency of the Suppression of Pulmonary Metastases of Rat Mammary Cancer by Amiloride. *Clin. Exp. Metastasis* **1998**, *16*, 353−357.
(6) Jankun, J.; Keck, R. W.; Skrzypczak-Jankun, E.; Swierca, R. Inhibitors of Urokinase Reduce Size of Prostate Cancer Xenografts in Severe Combined Immunodeficient Mice. *Cancer Res.* **1997**, *57*, 559−563.
(7) Alonso, D. F.; Tejera, A. M.; Farias, E. F.; Joffe, E. B. D.; Gomez, D. E. Inhibition of Mammary Tumor Cell Adhesion, Migration, and Invasion by the Selective Synthetic Urokinase Inhibitor B428. *Anticancer Res.* **1998**, *18*, 4499−4504.
(8) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering High-Affinity Ligands for Proteins: SAR by NMR. *Science* **1996**, *274*, 1531−1534.
(9) Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering High-Affinity Ligands for Proteins. *Science* **1997**, *278*, 497−499.
(10) Hansen, A. P.; Petros, A. M.; Mazar, A. P.; Pederson, T. M.; Rueter, A.; Fesik, S. W. *Biochemistry* **1992**, *31*, 12713−12718.
(11) Hansen, A. P.; Petros, A. M.; Meadows, R. P.; Nettesheim, D. G.; Mazar, A. P.; Olejniczak, E. T.; Xu, R. X.; Pederson, T. M.; Henkin, J.; Fesik, S. W. Solution Structure of the Amino-Terminal Fragment of Urokinase-Type Plasminogen Activator. *Biochemistry* **1994**, *33*, 4847−4864.
(12) Leonard, N. J.; Curtin, D. Y.; Beck, K. M. Sulfonate Salts of Substituted Benzimidazoles. *J. Am. Chem. Soc.* **1947**, *69*, 2459−2461.
(13) The numbering system is as aligned by Spraggon et al. *Structure* **1995**, *3*, 681−691.
(14) Nienaber, V.; Wang, J.; Davidson, D.; Henkin, J. Re-Engineering of Human Urokinase Provides a System for Structure-Based Drug Design at High Resolution and Reveals a Novel Structural Sub-Site. *J. Biol. Chem.* **2000**, *275*, 7239−7248.
(15) Bridges, A. J.; Lee, A.; Schwartz, E.; Towle, M. J.; Littlefield, B. A. The Synthesis of Three 4-Substituted Benzo[*b*]thiophene-2-Carboxamidines as Potent and Selective Inhibitors of Urokinase. *Bioorg. Med. Chem.* **1993**, *1*, 403−410.
(16) Nienaber, V. L.; Davidson, D.; Edalji, R.; Giranda, V. L.; Klinghofer, V.; Henkin, J.; Magdalinos, P.; Mantei, R.; Merrick, S.; Severin, J. M.; Smith, R. A.; Stewart, K.; Walter, K.; Wang, J.; Wendt, M.; Weitzberg, M.; Zhao, X.; Rockway, T. Structure-directed Discovery of Potent Non-peptidic Inhibitors of Human Urokinase that Access a Novel Binding Subsite. *Structure* **2000**, *8*, 553−563.
(17) Sperl, S.; Jacob, U.; Arroyo de Prada, N.; Stürzebecher, J.; Wilhelm, O. G.; Bode, W.; Magdolen, V.; Huber, R.; Moroder, L. (4-aminomethyl)phenylguanidine Derivatives as Nonpeptidic Highly Selective Inhibitors of Human Urokinase. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5113−5118.
(18) Hajduk, P. J.; Sheppard, G.; Nettesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.; Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.; Morgan, D. W.; Davidsen, S. K.; Fesik, S. W. Discovery of Potent Nonpeptide Inhibitors of Stromelysin Using SAR by NMR. *J. Am. Chem. Soc.* **1997**, *119*, 5818−5827.
(19) Hajduk, P. J.; Dinges, J.; Miknis, G. F.; Merlock, M.; Middleton, T.; Kempf, D. J.; Egan, D. A.; Walter, K. A.; Robins, T. S.; Shuker, S. B.; Holzman, T. F.; Fesik, S. W. NMR−Based Discovery of Lead Inhibitors that Block DNA Binding of the Human Papillomavirus E2 Protein. *J. Med. Chem.* **1997**, *40*, 3144−3150.
(20) Hajduk, P. J.; Dinges, J.; Schkeryantz, J. M.; Janowick, D.; Kaminski, M.; Tufano, M.; Augeri, D. J.; Petros, A.; Nienaber, V.; Zhong, P.; Hammond, R.; Coen, M.; Beutel, B.; Katz, L.; Fesik, S. W. Novel Inhibitors of Erm Methyltransferases from NMR and Parallel Synthesis. *J. Med. Chem.* **1999**, *42*, 3852−3859.
(21) Hajduk, P. J.; Zhou, M.-M.; Fesik, S. W. NMR−Based Discovery Of Phosphotyrosine Mimetics That Bind To The Lck SH2 Domain. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2403−2406.
(22) Kay, L. E.; Keifer, P.; Saarinen, T. Pure Absorption Gradient Enhanced Heteronuclear Single Quantum Correlation Spectroscopy with Improved Sensitivity. *J. Am. Chem. Soc.* **1992**, *114*, 10663−10665.
(23) Otwinowski, Z.; Minor, W. *Processing of X-ray Diffraction Data Collected in Oscillation Mode*; Academic Press: New York, 1997; Vol. 276.
(24) Brunger, A. T. *X-PLOR Version 3.1*; Yale University Press: New Haven and London, 1992.
(25) Barlow, G. H. *Methods Enzymol.* **1976**, *45*, 9−244.
(26) Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; John Wiley and Sons: New York, 1975.
(27) Towle, M. J.; Lee, A.; Maduakor, E. C.; Schwartz, C. E.; Bridges, A. J.; Littlefield, B. A. Inhibition of Urokinase by 4-Substituted Benzo[b]thiophene-2-carboxamidines: An Important New Class of Selective Synthetic Urokinase Inhibitor. *Cancer Res.* **1993**, *53*, 2553−2559.

JM0002228