Discovery of Potent Nonpeptide Inhibitors of Stromelysin Using SAR by NMR

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Abstract: With the use of an NMR-based method, potent (IC_{50} < 25 nM) nonpeptide inhibitors of the matrix metalloproteinase stromelysin (MMP-3) were discovered. The method, called SAR by NMR (for structure−activity relationships by nuclear magnetic resonance), involves the identification, optimization, and linking of compounds that bind to proximal sites on a protein. Using this technique, two ligands that bind weakly to stromelysin (acetohydroxamic acid, K_D = 17 mM; 3-(cyanomethyl)-4′-hydroxybiphenyl, K_D = 0.02 mM) were identified. On the basis of NMR-derived structural information, the two fragments were connected to produce a 15 nM inhibitor of this enzyme. This compound was rapidly discovered (less than 6 months) and required only a minimal amount of chemical synthesis. These studies indicate that the SAR by NMR method can be effectively applied to enzymes to yield potent lead inhibitors—an important part of the drug discovery process.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that include the collagenases, gelatinases, and stromelysins.[1] This family of enzymes is involved in matrix degradation and tissue remodeling, and when overexpressed or dysregulated are associated with pathologies such as arthritis and tumor metastases. Although potent peptide-based inhibitors designed on the basis of substrate specificity have been discovered,[1g] many of these compounds exhibit poor bioavailability. Several nonpeptide natural products have been discovered that inhibit this class of enzymes, such as pycnidione[2] and tetracycline derivatives.[3] However, these compounds exhibit only moderate inhibitory activity.

Recently, we described an NMR-based approach for discovering high-affinity ligands called SAR by NMR.[4] The SAR by NMR method involves screening a library of small molecules to identify and optimize compounds that bind to proximal sites on a protein. The compounds are screened by monitoring perturbations of the amide chemical shifts of ^{15}N-labeled protein upon the addition of potential ligands. When two compounds have been identified that bind to the protein, experimentally-derived structural information is used to guide the design of linkers between the molecules. Although the individual fragments may bind only weakly to the protein, tethering the compounds together results in a molecule whose binding energy is the sum of that for each component plus an additional contribution due to linking.[5] Thus, in principle, submicromolar leads can be obtained by linking together two fragments that bind with millimolar dissociation constants.

We have previously applied the SAR by NMR technique to the discovery of high-affinity ligands for the FK506 binding protein (FKBP).[4] By using this method, a ligand with a dissociation constant of 19 nM was produced by tethering two lead ligands which bound to FKBP with dissociation constants of 2 and 100 μM. Here we describe the application of SAR by NMR to the discovery of potent, nonpeptide inhibitors of stromelysin (MMP-3).

Results and Discussion

Identification of a Ligand That Binds to the First Site. In order to apply the SAR by NMR technique to a protease such as stromelysin, a molecule was needed that would inhibit autolytic degradation during screening, be soluble enough to saturate the protein, and be small enough not to occlude nearby binding sites on the protein. Since many of the known MMP inhibitors contain a hydroxamate moiety, acetohydroxamic acid ($CH_3CONHOH$, **1**) was tested for its ability to bind to stromelysin and inhibit autolytic degradation. Although this compound bound only weakly to the protein (K_D = 17 mM), autolytic degradation was effectively inhibited at ligand concentrations >100 mM. Thus, due to its small size and high solubility in aqueous buffers (> 500 mM), acetohydroxamic acid was chosen as the ligand for the first site.

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(1) (a) Liotta, L. A.; Rao, C. N.; Wewer, U. M. *Annu. Rev. Biochem.* **1986**, *55*, 1037−1057. (b) Docherty, A. J. P.; O'Connell, J.; Crabbe, T.; Angal, S.; Murphy, G. *Trends Biotechnol*. **1992**, *10*, 200−207. (c) Walakovits, L. A.; Moore, V. L.; Bhardwaj, N.; Gallick, G. S.; Lark, M. W. *Arthritis Rheum.* **1992**, *35*, 35−42. (d) Wilhelm, S. M.; Shao, Z.-H.; Housley, T. J.; Seperack, P. K.; Baumann, A. P.; Gunja-Smith, Z.; Woessner, J. F., Jr. *J . Biol. Chem*. **1993**, *268*, 21906−21913. (e) Mignatti, P. and Rifkin, D. B. *Physiol. Rev.* **1993**, *73*, 161−195. (f) McCachren, S. S. *Arthritis Rheum.* **1992**, *34*, 1085−1093. (g) Beckett, R. P.; Davidson, A. H.; Drummond, A. H.; Huxley, P.; Whittaker, M. *Drug Discovery Today* **1996**, *1*, 16−26. (h) Niedzwiecki, L.; Teahan, J.; Harrison, R. K.; Stein, R. L. *Biochemistry* **1992**, *31*, 12618−12623.

(2) Harris, G. H.; Hoogsteen, K.; Silverman, K. C.; Raghoobar, S. L.; Bills, G. F. *Tetrahedron* **1993**, *49*, 2139−2144.

(3) (a) Bols, M.; Binderup, L.; Hansen, J.; Rasmussen, P. *J. Med. Chem.* **1992**, *35*, 2768−2771. (b) Suomalainen, K.; Sorsa, T.; Golub, L. M.; Ramamurthy, N.; Lee, H. M.; Uitto, V. J.; Saari, H.; Konttinen, Y. T. *Antimicrob. Agents Chemother*. **1992**, *36*, 227−229.

(4) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1534.

(5) Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046−4050.

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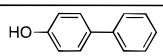
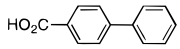
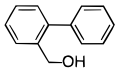
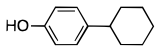
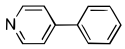
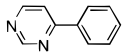
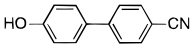
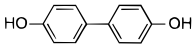
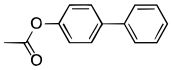
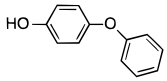
Table 1. Dissociation Constants for the Binding of Biphenyl and Biphenyl Analogs to Stromelysin Identified in the Initial Screen

Compound No.	Compound	K_D (mM)[a]	
2		0.28	(0.12)
3		>10	(-)
4		>10	(-)
5		>10	(-)
6		0.17	(0.03)
7		1.6	(0.31)
8		0.02	(0.01)
9		0.16	(0.15)
10		0.25	(0.08)
11		0.48	(0.27)

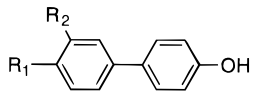
[a] Dissociation constants were derived from an analysis of the changes in amide chemical shifts as a function of the concentration of the compound. [b] Estimated uncertainties in the dissociation constants obtained as described in Methods and Chemical Synthesis. A dash (−) indicates that no uncertainty was determined due to the absence of observed chemical shift changes.

Identification and Optimization of Ligands That Bind to the Second site. The substrate specificity for stromelysin indicates a preference for amino acids with large hydrophobic side chains at the P_1' position.[1g,h] This preference is consistent with the large hydrophobic S_1' binding site observed in structural studies of stromelysin and other matrix metalloproteinases complexed with peptide-based inhibitors.[6a,b,g] To identify ligands that bind to this site, a directed screen was conducted in which hydrophobic compounds were tested for binding to stromelysin in the presence of saturating amounts (500 mM) of acetohydroxamic acid (**1**).

Several biphenyls (e.g., **2**) and biphenyl analogs (see Table 1) were found that bound to stromelysin with dissociation constants in the millimolar range.[7] Although the binding of these compounds to the enzyme is weak, distinct and interpretable structure−activity relationships were observed. For example, the incorporation of a carboxylic acid (e.g., **3**) or substituents *ortho* to the biphenyl linkage (e.g., **4**) resulted in a marked decrease in affinity for stromelysin. The substitution of an aliphatic for an aromatic ring (e.g., **5**) also abrogated binding, while the incorporation of pyridine or pyrimidine rings was tolerated (**6** and **7**). Nearly a 10-fold increase in affinity was observed for a biphenyl which contained a *para* CN substituent (**8**), while only a modest improvement was observed for a *para* OH substituent (**9**).

To further explore the SAR of this class of molecules, 33 compounds containing a variety of functional groups at positions *meta* and *para* to the biphenyl linkage were synthesized (see Table 2). The substituted biphenyls were readily prepared by Suzuki coupling[8] of commercially available aryl bromides and iodides with [*p*-[(*tert*-butyldimethylsilyl)oxy]phenyl]boronic acid. [See Methods and Chemical Synthesis section for a representative procedure for the preparation of 4-fluoro-4′-hydroxybiphenyl (**24**).] Relative to the initial lead compound **2**, biphenyls containing a *para n*-propyl (**20**) or a *meta* CH_2CN substituent (**26**) exhibited a significant increase in affinity for

Table 2. Dissociation Constants for the Binding of Biphenyl Analogs to Stromelysin That Were Synthesized on the Basis of the Initial Biphenyl Leads



compound	R_1	R_2	K_D (mM)[a]
12	$CH{=}CH_2$	H	0.61 (0.17)[b]
13	NH_2	H	>10 (−)
14	$COCH_3$	H	0.43 (0.14)
15	CHO	H	0.14 (0.07)
16	SCH_3	H	5.2 (2.0)
17	OCH_3	H	0.29 (0.07)
18	*t*-Bu	H	>10 (−)
19	*i*-Pr	H	0.53 (0.12)
20	*n*-Pr	H	0.02 (0.02)
21	Et	H	0.15 (0.04)
22	CF_3	H	0.18 (0.06)
23	Cl	H	0.12 (0.03)
24	F	H	0.23 (0.06)
25	CH_3	H	0.29 (0.06)
26	H	CH_2CN	0.02 (0.01)
27	H	CH_3	0.61 (0.18)
28	H	Br	>10 (−)
29	H	Cl	>10 (−)
30	H	F	1.2 (0.35)
31	H	CN	5.2 (2.2)
32	H	$COCH_3$	>10 (−)
33	H	NH_2	0.77 (0.11)
34	H	CH_2OH	>10 (−)
35	H	CH_2Br	0.19 (0.08)
36	H	OCH_3	>10 (−)
37	H	OH	2.0 (0.56)
38	H	CF_3	6.2 (2.1)
39	CH_3	CH_3	0.17 (0.06)
40	OH	CH_3	0.98 (0.15)
41	CH_3	NH_2	0.18 (0.08)
42	Cl	Cl	0.31 (0.07)
43	CH_3	Cl	1.2 (0.19)
44	NH_2	Cl	0.88 (0.19)

[a] Dissociation constants were derived from an analysis of the changes in amide chemical shifts as a function of the concentration of the compound. [b] Estimated uncertainties in the dissociation constants obtained as described in Methods and Chemical Synthesis. A dash (−) indicates that no uncertainty was determined due to the absence of observed chemical shift changes.

(6) (a) Gooley, P. R.; O'Connell, J. F.; Marcy, A. I.; Cuca, G. C.; Salowe, S. P.; Bush, B. L.; Hermes J. D.; Esser, C. K.; Hagmann, W. K.; Springer, J. P.; Johnson, B. A. *Nature Struct. Biol.* **1995**, *1*, 111−118. (b) Van Doren, S. R.; Kurochkin, A. V.; Hu, W.; Ye, Q.-Z.; Johnson, L. L.; Hupe, D. J.; Zuiderweg, E. R. P. *Protein Sci.* **1995**, *4*, 2487−2498. (c) Becker, J. W.; Marcy, A. I.; Rokosz, L. L.; Axel, M. G.; Burbaum, J. J.; Fitzgerald, P. M. D.; Cameron, P. M.; Esser, C. K.; Hagmann, W. K.; Hermes, J. D.; Springer, J. P. *Protein Sci.* **1995**, *4*, 1966−1976. (d) Bode, W.; Reinemer, P.; Huber, R.; Kleine, T.; Schnierer, S.; Tschesche, H. *EMBO J.* **1994**, *13*, 1263−1269. (e) Borkakoti, N.; Winkler, F. K.; Williams, D. H.; Darcy, A.; Broadhurst, M. J.; Brown, P. A.; Johnson, W. H.; Murray, E. J. *Nature Struct. Biol.* **1994**, *1*, 106−110. (f) Stams, T.; Spurlino, J. C.; Smith, D. L.; Wahl, R. C.; Ho, T. F.; Qoronfleh, M. W.; Banks, T. M.; Rubin, B. *Nature Struct. Biol.* **1994**, *1*, 119−123. (g) Lovejoy, B.; Cleasy, A.; Hassell, A. M.; Longley, K.; Luther, M. A.; Weigl, D.; McGeehan, G.; McElroy, A. B.; Drewry, D.; Lambert, M. H.; Jordan, S. R. *Science* **1994**, *263*, 375−377. (h) Spurlino; J.; Smallwood, A. M.; Carlton, D. D.; Banks, T. M.; Vavra, K. J.; Johnson, J. S.; Cook, E. R.; Falvo, J.; Wahl, R. C.; Pulvino, T. A.; Wendoloski, J. J.; Smith, D. L. *Proteins Struct. Funct. Genet.* **1994**, *19*, 98−109.

(7) These biaryl compounds were identified after screening only 125 compounds.

(8) (a) Migaura, N.; Yanagi, T.; Suzuki, A. *Synth. Commun.* **1981**, *11*, 513−519. (b) Migaura, N.; Suzuki, A. *Chem. Rev.* **1995**, *95*, 2457−2483.

Table 3. Structural Statistics and Rmsds for NMR-Derived Structures of Stromelysin Complexes[a]

	ternary complex[b]			binary complex[c]		
	no.	⟨SA⟩	⟨$\underline{SA}$⟩$_r$	no.	⟨SA⟩	⟨$\underline{SA}$⟩$_r$
			Rmsd (Å) from Experimental Distance Restraints[d]			
intraresidue	341	0.007 ± 0.005	0.002	551	0.008 ± 0.012	0.012
sequential	295	0.014 ± 0.003	0.026	382	0.012 ± 0.008	0.008
medium range	115	0.012 ± 0.005	0.008	201	0.015 ± 0.015	0.015
long range	281	0.006 ± 0.003	0.012	563	0.010 ± 0.008	0.008
intermolecular	33	0.012 ± 0.002	0.001	49	0.011 ± 0.005	0.005
hydrogen bonds	118	0.016 ± 0.001	0.016	124	0.014 ± 0.015	0.015
			Rmsd (deg) from Experimental Torsion Restraints			
Φ angles	14	0.15 ± 0.09	0.38	39	0.09 ± 0.05	0.00
			X-PLOR Potential Energies (kcal mol^{-1})			
Etot		294 ± 7	328		301 ± 7	311
Ebond		25 ± 1	25		25 ± 1	26
Eang		214 ± 4	227		214 ± 3	221
Eimp		22 ± 1	27		22 ± 3	26
Erepel		23 ± 2	35		23 ± 3	25
Enoe		8 ± 2	13		15 ± 3	13
Ecdih		0.3 ± 0.3	0.1		0.2 ± 0.1	0.0
			Cartesian Coordinate Rmsd (Å)			
		N, Cα, C′	all heavy		N, Cα, C′	all heavy
⟨SA⟩ vs ⟨SA⟩		1.03 ± 0.09	1.49 ± 0.10		0.75 ± 0.09	1.21 ± 0.08
⟨SA⟩ vs ⟨$\underline{SA}$⟩[e]			0.99 ± 0.40			0.76 ± 0.10

[a] Where ⟨SA⟩ is the ensemble of 20 NMR-derived solution structures of the ternary complex of stromelysin with acetohydroxamic acid (**1**) and **7**, and the binary complex of **50** and stromelysin; ⟨$\underline{SA}$⟩ is the mean atomic structure obtained by averaging the coordinates of the individual ⟨SA⟩ structures following a least-squares superposition of the backbone heavy atoms for residues 93−247; and ⟨$\underline{SA}$⟩$_r$ is the energy minimized average structure. Medium range NOEs are observed between protons separated by more than one and less than five residues in the primary sequence. Long-range NOEs are observed between protons separated by more than five residues. [b] A total of 1065 nontrivial NOE-derived distance restraints were employed. No distance restraint was violated by more than 0.4 Å in any of the final structures. No torsional restraint was violated by more than 5°. [c] A total of 1870 nontrivial NOE-derived distance restraints were employed. No distance restraint was violated by more than 0.4 Å in any of the final structures. No torsional restraint was violated by more than 5°. [d] Rmsd for residues 93−247. [e] Rmsd for the ligand(s).

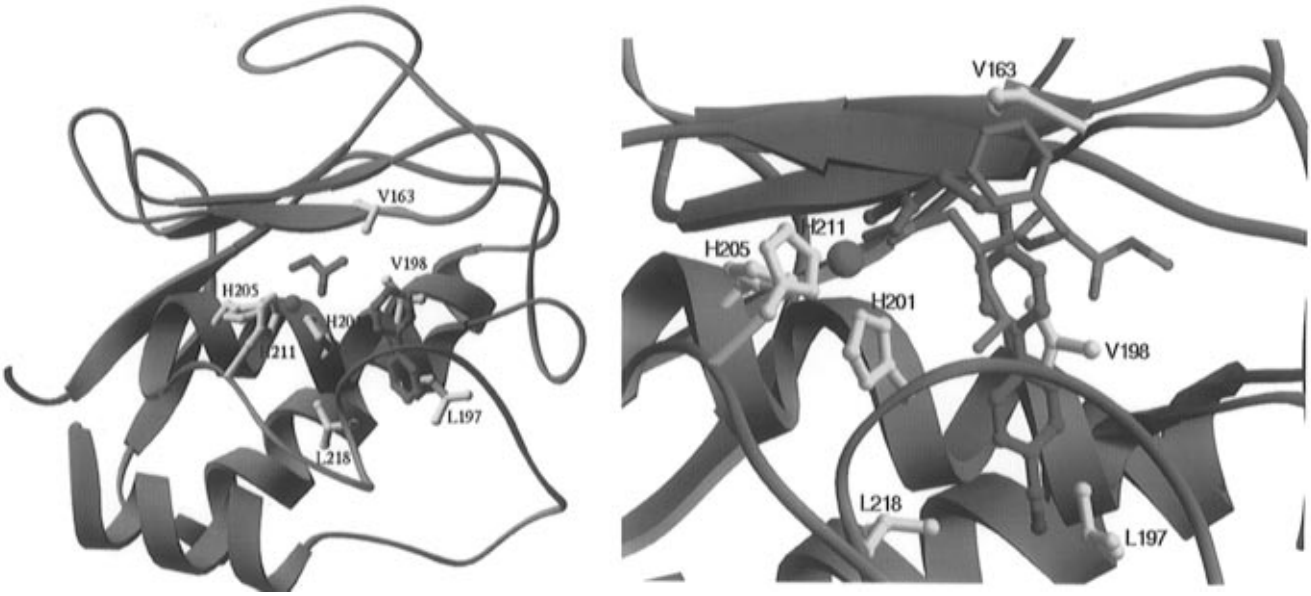
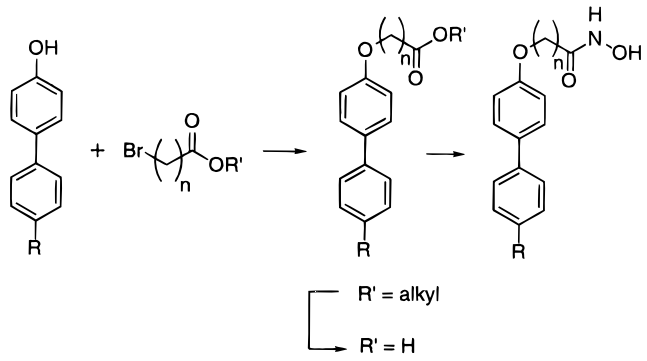


Figure 1. Ribbons[29] depiction of (a, left) the ternary complex composed of stromelysin, **1**, and **7**, and (b, right) the stromelysin/**50** (green carbon atoms) complex superimposed on a collagenase/inhibitor (cyan carbon atoms) complex. Colored in yellow are the three histidines which chelate the catalytic zinc (shown in magenta) and those side chains which make hydrophobic contact with the biphenyl moiety.

stromelysin (>10-fold). Modest improvements in affinity (~2-fold) were observed for biphenyls containing *para* CHO (**15**), ethyl (**21**), CF_3 (**22**), or Cl (**23**) substituents. The majority of the other compounds, however, either decreased or had little effect on the binding affinity.

Structure of the Ternary Complex. To aid in the design of linked compounds, the three-dimensional structure of a ternary complex composed of the catalytic domain of stromelysin, acetohydroxamic acid, and biaryl **7** was determined by NMR spectroscopy. This biaryl was chosen for the NMR structure determination because of its good water solubility. The structure was determined by the NMR data to an rmsd of 1.03 Å for the backbone atoms and 1.49 Å for all heavy atoms (Table 3). The overall fold of the protein is similar to previously determined structures of matrix metalloproteinases[6] and consists of a five-stranded β-sheet and three α-helices (Figure 1a). Acetohydroxamic acid (**1**) chelates to the active site zinc, with the methyl group of this compound forming hydrophobic interactions with Val163. Biaryl **7** binds to the large, hydrophobic S_1' subsite and forms hydrophobic interactions with Leu197, Val198, and Leu218. The methyl group of acetohydroxamic acid is located in close proximity to the pyrimidine

Scheme 1



45: R = H, R' = *t*Bu, n = 1 **49**: R = CN, R' = *t*Bu, n = 1

47: R = H, R' = Et, n = 3 **51**: R = CN, R' = Et, n = 3

48: R = H, R' = Me, n = 4 **52**: R = CN, R' = Me, n = 4

ring of **7** (Figure2a) as evidenced by the observation of an NOE between these two groups.

The location of compounds **2**, **6**, and **11** were also examined using two-dimensional isotope-filtered NMR experiments. All of these ligands bound to stromelysin in the S_1' pocket of stromelysin in a similar manner to **7**. The NOE data revealed the relative orientation of the biphenyl rings. For example, NOEs from the methyl group of acetohydroxamic acid to the protons ortho to the phenolic OH of **2** and **11** indicated that the hydroxyl group is located on the same side of the S_1' pocket as the hydroxamic acid.

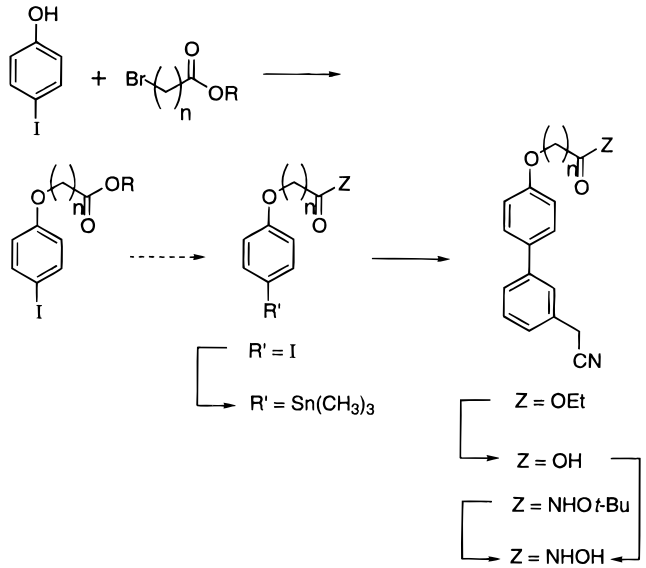
Design and Synthesis of Linked Compounds. On the basis of the NMR-derived structural information of stromelysin/ligand complexes and the relative dissociation constants of the biphenyls measured by NMR, linked compounds were designed. Since compounds containing capped hydroxyl groups (e.g., **10**) exhibited no loss in binding affinity to stromelysin, an ether linkage connecting the phenolic OH of the biphenyl to the hydroxamic acid was incorporated into the design. Compounds containing *para* CN or *meta* CH_2CN substituents on the biphenyl were chosen on the basis of the increase in affinity of biphenyls which contain these substituents. To allow for uncertainties in the ligand position, methylene linkers of varying length were incorporated into the linked compounds.

Compounds in which the biaryloxy and hydroxamic acid fragments are linked with one, three, and four methylene units were readily synthesized by the route shown in Scheme 1. Alkylation of the commercially available 4-hydroxybiphenyl or 4-cyano-4′-hydroxybiphenyl with bromo esters, followed by ester hydrolysis using acid or base as appropriate, provided the (biaryloxy)alkanoic acids in good yield. Hydroxamate formation was carried out by formation of the acid chloride and treatment with hydroxylamine or *O*-(*tert*-butyldimethylsilyl)hydroxyl amine, giving access to compounds **45**, **47**, **48**, **49**, **51**, and **52**.

To prepare the *meta* CH_2CN analogs, the modified approach shown in Scheme 2 was adopted, which is suitable for the introduction of a wide variety of biaryl structures. Alkylation of 4-iodophenol and, optionally, conversion to a protected hydroxamic acid as described above provided aryl iodide intermediates which were converted to the corresponding aryl stannanes by palladium-catalyzed coupling with hexamethyldistannane.[9] Coupling with 1-iodo-3-(cyanomethyl)benzene and deprotection or hydrolysis and coupling as before provided hydroxamates **54** and **55**.

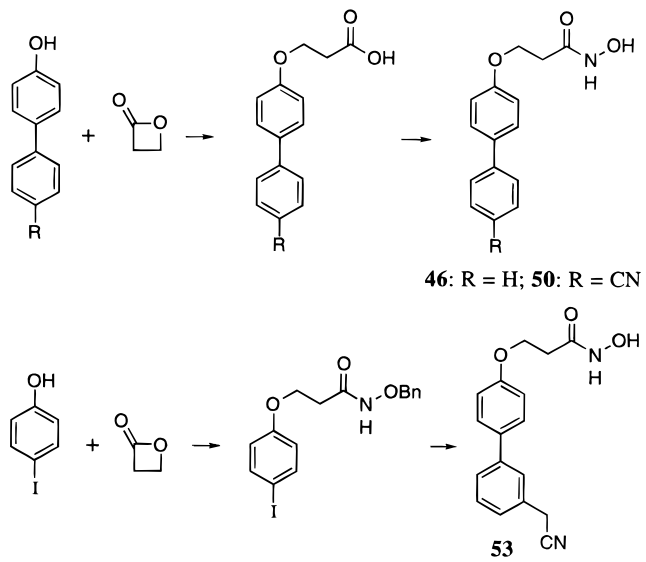
(9) Azizian, H.; Eaborn, C.; Pidcock, A. *J. Organometal. Chem.* **1981**, *215*, 49−58.

Scheme 2



54: R = Et, Z = OEt, n = 3; **55**: R = Me, Z = NHO*t*Bu, n = 4

Scheme 3



The bromoester alkylation routes described above failed to provide access to the 3-(aryloxy)propanoic esters, due to facile β-elimination. Successful alkylation of phenols with β-propiolactone as the alkylating agent has been reported,[10] and this modification to the previous routes provided access to compounds **46**, **50,** and **53** (Scheme 3).

Biological Activity of Linked Compounds. Table 4 depicts 11 of the linked compounds that were synthesized along with their corresponding *in vitro* IC_{50} values as measured in a stromelysin inhibition assay. All of the compounds show an enhancement in activity relative to the untethered compounds. The most potent inhibitors of stromelysin, **50** and **53**, exhibited IC_{50} values of 25 and 15 nM, respectively—a 1000-fold enhancement in activity over the individual components (see Table 1). Within each of the series, the two methylene-linked compounds exhibited the most potent activities, with nearly a

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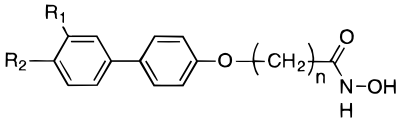
Table 4. Stromelysin Inhibition for Linked Biphenyl Compounds



compound no.	R_1	R_2	*n*	IC_{50} (μM)[a]
45	H	H	1	3.9
46	H	H	2	0.31
47	H	H	3	110
48	H	H	4	100
49	H	CN	1	0.26
50	H	CN	2	0.025
51	H	CN	3	3.4
52	H	CN	4	3.5
53	CH_2CN	H	2	0.015
54	CH_2CN	H	3	1.9
55	CH_2CN	H	4	3.5

[a] IC_{50} values were obtained against the catalytic domain of stromelysin using an enzyme inhibition assay.

100-fold improvement over the three or four methylene-linked compounds and a 10-fold improvement over the one methylene-linked compounds. These results indicate the critical importance of linker length in this system. In addition, a 10-fold improvement in activity was observed for compounds containing *para* CN or *meta* CH_2CN substituents, consistent with the observed increase in affinity for the untethered compounds containing these substituents (see Tables 1 and 2). These results suggest that the SAR observed for the unlinked compounds can serve as a reasonable guide to the SAR in the linked compounds.

Structure of the Stromelysin/50 Complex. To determine whether the linked compounds bind to stromelysin as designed, the three-dimensional structure of stromelysin complexed to **50** was determined by NMR (Table 3, Figure 1b). The structure of the complex was defined by the NMR data to an rmsd of 0.75 Å for the backbone atoms and 1.21 Å for all heavy atoms. The position of the ligand was defined to an rmsd of 0.76 Å for all heavy atoms. The overall fold of the protein is similar to that observed for the ternary complex (rmsd of 1.40 Å for Cα positions for residues 93−247). In addition, the ligand binds to stromelysin in a similar manner to the unlinked compounds, with the hydroxamic acid chelating the zinc and the biphenyl moiety sitting deeply within the S_1' pocket. As in the ternary complex, the biphenyl moiety of **50** forms hydrophobic interactions with Leu197, Val198, and Leu218. The methylene linker forms hydrophobic interactions with Val163. Furthermore, hydrogen-exchange studies suggest that the backbone amide of Leu164 forms a hydrogen bond with the phenolic oxygen of the biphenyl.

Many peptide inhibitors of MMPs designed on the basis of the observed substrate specificity for this enzyme contain an isobutyl group that binds to the S_1' site.[1] Figure 1b depicts a collagenase/inhibitor crystal structure[6f] superimposed onto the NMR-derived structure of the stromelysin/**50** complex. As can be seen from this comparison, the leucine side chain of the collagenase inhibitor sits at the "top" of the S_1' pocket, whereas the biphenyl moiety of **50** sits deep in the pocket and forms numerous contacts with the protein. This is consistent with the significantly greater affinities of the biaryl compounds as compared to leucine and analogs of leucine (e.g., isobutanol), which exhibited no measurable binding to stromelysin at compound concentrations up to 50 mM.

Conclusions

We have previously reported on the use of the SAR by NMR method for discovering high-affinity ligands that bind to the FK506 binding protein.[4] Here, we extend the applicability of this technique to enzymes as illustrated by the discovery of potent, nonpeptide inhibitors of stromelysin. These inhibitors are small, lack peptide bonds, and exhibit significantly greater potencies than previously reported nonpeptide MMP inhibitors.[1−3]

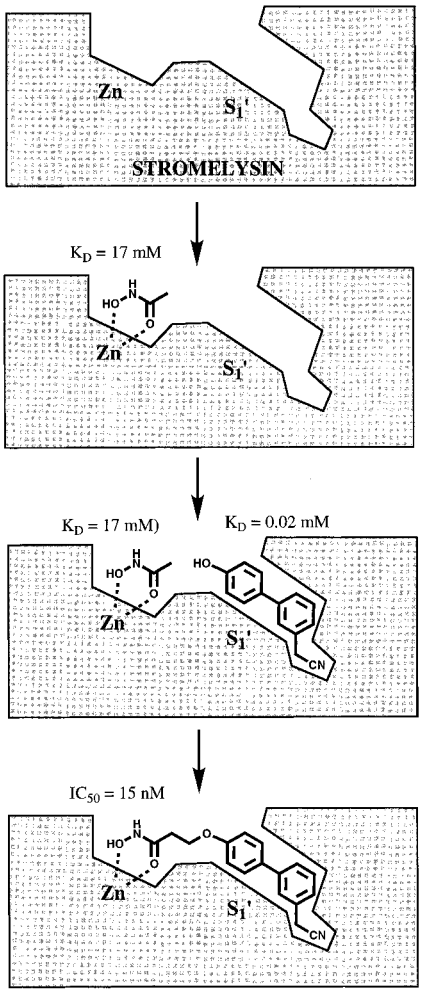


Figure 2. A summary of the SAR by NMR method as applied to discovery of stromelysin inhibitors.

As summarized in Figure 2, the inhibitors were discovered by tethering two ligands that bind weakly to the protein, guided by structural information on how they bind. This approach was successful when other, more conventional, methods failed. For example, in a screen of over 115 000 compounds using an enzyme inhibition assay, no nonpeptide inhibitors were found that exhibited a potency greater than 10 μM. Screens such as this depend on finding a potent molecule that already contains the necessary functional groups in their proper spatial orientation for tight binding to the protein. In contrast, using the SAR by NMR method, pieces that are important for binding are identified and optimized prior to the construction of the linked compounds. Indeed, as demonstrated here, the SAR observed for the unlinked fragments correlates well with that observed for the linked compounds. Analogs of the small fragments are more likely to be commercially available or easier to synthesize than the linked compound. By measuring the binding affinities of these fragments, information on the SAR is obtained prior to linking that will dictate which linked molecules should be prepared. Furthermore, structural studies on the untethered ligands when complexed to the protein can yield information on the relative orientation and proximity of the two ligands. From this information, high-affinity ligands can be rapidly discovered with a minimal amount of chemical synthesis. In the example

described here, only six months were required to identify, optimize, and link together the fragment molecules. These studies as well as our previous work[4] suggest that SAR by NMR will be a useful method for identifying and optimizing high-affinity ligands for proteins and, when applied to protein drug targets, an extremely valuable tool in drug research.

Methods and Chemical Synthesis

Detection of Ligand Binding. Ligand binding was detected by acquiring sensitivity-enhanced ^{15}N-HSQC spectra[11] on 400 μL of 0.3 mM stromelysin 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, and binding was determined by monitoring changes in the ^{15}N-HSQC spectrum. Dissociation constants were obtained for selected compounds by monitoring the chemical shift changes of the backbone amide of Ala199 as a function of ligand concentration. Data were fit using a single binding site model. A least-squares grid search was performed by varying the values of K_D and the chemical shift of the fully saturated protein. Errors in the dissociation constants were obtained using a Monte Carlo simulation of the data[12] assuming a Gaussian distribution for errors in chemical shifts with a standard deviation of 0.01 ppm (the digital resolution of the spectra). 100 Monte Carlo simulations were performed for each dissociation constant, and the reported errors are the standard deviations of the simulated values. Dissociation constants could not be obtained by NMR for compounds **45**−**55** because the spectra exhibited the characteristics of slow- to intermediate-exchange conditions.

NMR Sample Preparation. The catalytic domain of stromelysin (residues 81−256) was generated by PCR amplification from a C-terminal construct of stromelysin (residues 1−256) that was obtained using RT-PCR from human skin fibroblast mRNA. The fragment was cloned into the pET3d expression vector (Novagen) and transformed into *Escherichia coli* BL21 (DE3) pLysS. Uniformly ^{15}N- and ^{15}N-,^{13}C-labeled catalytic domain of stromelysin-1 was produced by growth of bacteria on minimal media containing ^{15}N-labeled ammonium chloride or ^{15}N-labeled ammonium chloride and ^{13}C-labeled glucose, respectively. The protein was purified by using a previously described procedure[13] with minor modifications. The NMR samples were composed of uniformly ^{15}N- or ^{15}N-,^{13}C-labeled stromelysin (0.6 mM) and either acetohydroxamic acid (100 mM) and **7** (10.0 mM) (for the ternary complex) or 0.6 mM **50** (for the binary complex) in a D_2O or H_2O/D_2O (9/1) Tris-buffered solution (50 mM, pH = 7.0) containing $CaCl_2$ (20 mM) and sodium azide (0.05%).

NMR Spectroscopy. All NMR spectra were recorded at 25 and 32 °C on Bruker AMX500, AMX600, or DMX500 NMR spectrometers. In all NMR experiments, pulsed field gradients were applied where appropriate as described[14] to afford the suppression of solvent signal and spectral artifacts. Quadrature detection in the indirectly detected dimensions was accomplished by using the States-TPPI method.[15] The data were processed and analyzed on Silicon Graphics computers using in-house written software.

The ^{1}H, ^{13}C, and ^{15}N backbone resonances of stromelysin were assigned from an analysis of several 3D double- and triple-resonance NMR spectra.[16] The Cα resonances of adjacent spin systems were identified from an analysis of 3D HNCA[17] and HN(CO)CA[18] spectra, and the Cβ signals were determined using a 3D CBCA(CO)NH experiment.[19] The backbone amide assignments made from the triple resonance experiments were consistent with the crosspeaks observed in an ^{15}N-separated 3D NOESY-HSQC[20] spectrum.

The ^{1}H and ^{13}C chemical shifts of the side-chain resonances were assigned from 3D HCCH-TOCSY spectra[21] acquired with a mixing time of 13 ms using the DIPSI-2 sequence[22] for ^{13}C isotropic mixing. An additional 3D HCCH-TOCSY experiment was performed with the ^{13}C carrier at 122.5 ppm to assign the aromatic residues.

Stereospecific assignments of methyl groups of the valine and leucine residues were obtained by using a biosynthetic approach[23] on the basis of the ^{13}C−^{13}C one-bond coupling pattern observed in a high-resolution ^{1}H,^{13}C-HSQC spectrum of a fractionally ^{13}C-labeled protein sample.

A ^{13}C-separated 3D NOESY-HMQC spectrum[20] was recorded using a mixing time of 75 ms. A 3D HNHA-J spectrum[24] was recorded, from which $^3J_{HNH\alpha}$ coupling constants were obtained. To identify amide groups that exchanged slowly with the solvent, a series of ^{15}N-HSQC spectra were recorded at 25 °C at 2 h intervals after the protein was exchanged into D_2O. The acquisition of the first ^{15}N-HSQC spectrum was started 2 h after the addition of D_2O.

NOEs between the ligand and the protein were obtained from a 3D ^{12}C-filtered, ^{13}C-edited NOESY spectrum. The pulse scheme consisted of a double ^{13}C-filter[25] concatenated with a NOESY-HMQC sequence.[20] For the binary complex, a mixing time of 80 ms was used. For the ternary complex, a series of ^{15}N/^{13}C-filtered 2D NOESY experiments were recorded with mixing times ranging from 80 to 350 ms.

Structure Determinations. Protein−protein distance restraints derived from the ^{13}C, and ^{15}N-NOESY spectra were classified into six categories on the basis of the NOE cross peak intensity and given a lower bound of 1.8 Å and upper bounds of 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 Å. Intermolecular distance restraints between the ligands and the protein were given a lower bound of 1.8 Å and an upper bound of 5.0 Å. The large distance bounds for the intermolecular restraints were used to compensate for possible spin diffusion effects. Distance restraints between the two zincs and their histidyl nitrogen ligands and between the catalytic zinc and the hydroxamate were employed as determined from an analysis of several crystal structures of metalloproteinases complexed with hydroxamate inhibitors.[6] Restraints for ϕ torsional angles were derived from $^3J_{HNH\alpha}$ coupling constants. The ϕ angle was restrained to 120° ± 40° for $^3J_{HNH\alpha}$ > 8.5 Hz, and 60° ± 40° for $^3J_{HNH\alpha}$ < 5 Hz. Hydrogen bonds, identified for slowly exchanging amides based on initial structures, were each defined by two restraints: 1.8−2.5 Å for the H−O distance and 1.8−3.3 Å for the N−O distance. Structures were calculated with the X-PLOR 3.1 program[26] on Silicon Graphics computers using a hybrid distance geometry-simulated annealing approach.[27] The X-PLOR F_{repel} function was used to simulate van der Waals interactions with a force constant of 4.0 kcal mol^{-1} and with atomic radii set to 0.8 times their CHARMM values. Distance restraints were employed with a square well potential (F_{NOE} = 50 kcal mol^{-1} Å^{-2}). Hydrogen bonds were included as distance restraints and given bounds of 1.8−2.4 Å (H → O) and 2.8−3.2 Å (N → O). Torsional restraints were applied to ϕ angles with values of −120° ± 40° for those angles with $^3J_{HNH\alpha}$ coupling constants >9.0 Hz and −60° ± 40° for coupling constants <5.5 Hz. Restraints for the latter were applied only in helical regions. Force constants of 200 kcal mol^{-1} rad^{-2} were applied for all torsional restraints.

Determination of Stromelysin Inhibition. Recombinant truncated stromelysin was assayed by its cleavage of the thiopeptide ester substrate Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly-OEt

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(Bachem).[28] The reported conditions were modified to allow assays to be carried out in a microtiter plate. Upon hydrolysis of the thioester bond, the released thiol group reacts rapidly with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), producing a yellow color which is measured using a Thermomax microtiter plate reader (Molecular Devices) set at 405 nm. The rates of cleavage of the substrate (200 μM) by stromelysin in the presence or absence of inhibitors are measured in a 30 min assay at ambient temperature. Solutions of the compounds in DMSO are prepared, and these are diluted at various concentrations into the assay buffer (50 mM MES/NaOH pH 6.5 with 10 mM $CaCl_2$ and 0.2% Pluronic F-68), which is also used for dilution of the enzyme and substrate. The potency of the compounds [IC_{50}] are calculated from the inhibition/inhibitor concentration data.

Chemical Synthesis. General. Melting points were determined using an Electrothermal digital melting point apparatus and are uncorrected. Infrared spectra were recorded with a Nicolet 5SXC FT-IR spectrometer and are reported in wavenumbers (cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on a GE QE300 spectrometer, and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane as an internal standard. Mass spectra were obtained on a Kratos MS-50 instrument. Elemental analyses were performed Robertson Microlit Laboratories, Inc. of Madison, NJ. Flash column chromatography was carried out using silica gel 60 (E. Merck, 230−400 mesh). THF was freshly distilled from sodium benzophenone ketyl. Et_2O was purchased as "anhydrous" and used as received. Other solvents were HPLC grade when available and were stored over molecular sieves. Unless otherwise noted, all chemicals and reagents were obtained commercially and used without purification. β-Propiolactone was purchased as technical grade (90%) from Aldrich chemical company and used as is. All chemical yields are unoptimized, and generally represent the result of a single experiment.

4-Fluoro-4′-hydroxybiphenyl (24). 4-Bromophenol (50.0 g, 289 mmol) and *tert*-butyldimethylsilyl chloride (47.9 g, 318 mmol) were dissolved in CH_2Cl_2 (300 mL) and imidazole (29.6 g, 434 mmol) was added over a 5 min period. An exotherm was observed and the flask was placed in an ice bath and stirring was continued for an additional 5 min. The reaction mixture was then washed with brine, dried ($MgSO_4$), filtered, and concentrated to provide an off-white solid (82.9 g, 99%). A portion of this material (20.0 g, 69.6 mmol) was dissolved in THF and cooled to −78 °C under N_2. *n*-Butyllithium (1.6 M in hexanes, 47.9 mL, 76.6 mmol) was added over a 45 min period, and the reaction mixture was stirred an additional 15 min. Triisopropyl borate (48.3 mL, 210 mmol) was added, and the reaction mixture was stirred for 10 min at −78 °C and then allowed to warm to room temperature. A 5% solution of HCl (∼75 mL) was added until the aqueous layer became acidic. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated *in vacuo* to provide a tan solid (16.3 g, 97%). A portion of this material (504 mg, 2.0 mmol), 1-fluoro-4-iodobenzene (222 mg, 2.0 mmol), Na_2CO_3 (212 mg, 2.0 mmol), and $Pd(PPH_3)_4$ (37.4 mg, 0.03 mmol) were placed in a flask which was then flushed with N_2. Toluene (4 mL) and water (2 mL) were added, and the reaction mixture was heated to 85 °C under N_2 for 2 h. The mixture was then cooled to room temperature and the organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue (128 mg) was dissolved in THF (5 mL) and tetrabutylammonium fluoride (1.0 M in THF, 0.46 mL) was added. After stirring for 5 min, the reaction mixture was washed with saturated aqueous NH_4Cl, water, and brine, then dried ($MgSO_4$), filtered through a silica plug, and concentrated *in vacuo*. The residue was purified by column chromatography (0−20% EtOAc/hexanes) to provide a white solid (50.8 mg, 27% for two steps): mp 166.5−168 °C; ^1H NMR ($CDCl_3$) δ 4.70 (s, 1H), 6.90 (d, 2H, J = 8.7 Hz), 7.10 (t, 2H, J = 8.6 Hz), 7.42 (d, 2H, J = 8.4 Hz), 7.48 (dd, 2H, J = 5.3, 8.7 Hz); ^{13}C NMR (DMSO-d_6) δ 114.9, 115.1, 115.5, 127.5, 127.6, 130.9, 156.6, 159.8, 163.0. Anal. Calcd for $C_{12}H_9F$: C, 76.58; H, 4.82. Found: C, 76.63; H, 5.15.

2-(4-Phenylphenoxy)ethanohydroxamic Acid (45). To a suspension of 4-phenylphenol (1.71 g, 10.1 mmol) and cesium carbonate (5.22 g, 16.0 mmol) in dry DMF (25 mL) was added *tert*-butyl bromoacetate (1.70 mL, 11.5 mmol). After stirring 18 h at ambient temperature, the mixture was diluted with Et_2O (200 mL) and extracted successively with $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$) and concentrated to give a pale yellow oil (2.65 g). This material was treated for 90 min at 0 °C with 80% TFA/20% CH_2Cl_2 (25 mL). Solvent removal and vacuum drying provided the crude acid as a white solid (2.15g). This material was suspended in thionyl chloride (10 mL) and heated to reflux for 2 h, then cooled, and vacuum dried to provide 2.13 g white solid. The acid chloride (0.251 g, 1.02 mmol) was redissolved in THF (4 mL) and treated with a solution of hydroxylamine generated by dissolving hydroxylamine hydrochloride (0.283 g, 4.07 mmol) in water (4 mL), addition of *N*-methylmorpholine (NMM) (0.55 mL, 5.0 mmol) then dilution with THF (10 mL). After 5 h, the mixture was partitioned between aqueous NH_4Cl and CH_2Cl_2. The organics were dried ($MgSO_4$) and concentrated to give a white solid (0.27 g). Recrystallization from hot CH_3CN (35 mL) provided 0.136 g of the final compound (47% overall yield) as shiny white flakes: mp 190−192 °C; ^1H NMR (DMSO-d_6) δ 4.51 (s, 2H), 7.03 (d, 2H, J = 10 Hz), 7.31 (m, 1H), 7.43 (t, 2H, J = 7 Hz), 7.61 (m, 4H), 8.98 (s, 1H), 10.87 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 65.88, 115.08, 126.19, 126.75, 127.66, 128.81, 133.18, 139.68, 157.40, 164.21; IR (KBr) 3300, 3050, 2840, 1675, 1635, 1520, 1485, 1250 cm^{-1}; MS (DCI/NH_3) 261 (M + NH_4^+, 100). Anal. Calcd for $C_{14}H_{13}NO_3 \cdot 0.8H_2O$: C, 65.26; H, 4.71; N, 5.44. Found: C, 64.90; H, 4.99; N, 5.34.

3-(4-Phenylphenoxy)propanohydroxamic Acid (46). To a solution of 4-phenylphenol (1.86 g, 10.9 mmol) in THF (12 mL) was added potassium *tert*-butoxide (1.22 g, 10.9 mmol). Neat β-propiolactone (0.68 mL, 10.9 mmol) was added dropwise. The resulting white suspension was stirred overnight at ambient temperature and then concentrated *in vacuo*. The residue was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The organic phase was discarded and the aqueous phase acidified and extracted twice with EtOAc. The EtOAc extracts were combined, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give crude 3-(4-phenylphenoxy)propionic acid (0.86 g) as a white solid.

The crude acid was refluxed in thionyl chloride (10 mL) for 2 h. The excess thionyl chloride was removed *in vacuo*, and the resulting acid chloride was dissolved in THF (10 mL). A solution of hydroxylamine was prepared by dissolving hydroxylamine hydrochloride (0.178 g, 2.5 mmol) in distilled water (6 mL) and treatment with NMM (0.3 mL, 2.7 mmol). The solution was diluted with THF (10 mL) and added to the acid chloride solution. After 2.5 h, the reaction mixture was partitioned between CH_2Cl_2 and aqueous NH_4Cl solution, and the organic phase was dried over $MgSO_4$. Solvent removal provided 0.66 g of tan solid containing the hydroxamic acid, carboxylic acid, and 4-hydroxybiphenyl. Preparative reverse-phase HPLC provided the title compound as a white solid (0.014 g, 0.5% overall yield): ^1H NMR (DMSO-d_6) δ 2.44 (t, 2H, J = 7 Hz), 4.22 (t, 2H, J = 7 Hz), 7.01 (d, 2H, J = 6 Hz), 7.31 (t, 1H, J = 5 Hz), 7.42 (t, 2H, J = 5 Hz), 7.61 (m, 4H), 8.88 (bds, 1H), 10.53 (s, 1H). MS (DCI/NH_3) 275 (M + NH_4^+,100). Anal. Calcd for $C_{15}H_{15}NO_3 \cdot 0.25H_2O$: C, 68.82; H, 5.36; N, 5.70. Found: C, 68.78; H, 5.27; N, 5.18.

4-(4-Phenylphenoxy)butanohydroxamic Acid (47). To a suspension in dry DMF of 4-phenylphenol (4.99 g, 29.4 mmol) and cesium carbonate (14.35 g, 44.0 mmol) was added ethyl 4-bromobutyrate (8.85 g, 45.3 mmol) in a single portion, and the reaction mixture was stirred for 18 h at ambient temperature. The reaction mixture was diluted with Et_2O and extracted with pH 7 buffer. The organic phase was washed twice with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give ethyl 4-(4-phenylphenoxy)butanoate (10.48 g) as a white solid.

A suspension in 1:1 dioxane/water (70 mL) of the ester (10.29 g, 36 mmol) and lithium hydroxide hydrate (2.05 g, 49 mmol) was heated at reflux for 18 h, then was cooled to ambient temperature, and concentrated *in vacuo*. The resulting white solids were shaken with Et_2O and aqueous 1 M NaOH, and the residual solid (4-(4-phenylphenoxy)butyric acid, 3.53 g) was filtered off. The organic phase was discarded, and the aqueous phase was acidified with concentrated HCl. The aqueous phase was extracted with EtOAc. The EtOAc extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give an additional 6.25 g of 4-(4-phenylphenoxy)butanoic acid.

A suspension of 4-(4-phenylphenoxy)butanoic acid (2.42 g, 9.45 mmol) in thionyl chloride (25 mL) was heated at reflux for 90 min,

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during which time the mixture became homogenous. The reaction mixture was cooled to ambient temperature and concentrated *in vacuo*. The residue was taken up in 1:1 CH_2Cl_2/THF. In a separate flask, NMM (3.5 mL, 32 mmol) was added to a solution of hydroxylamine hydrochloride (2.1 g, 30 mmol) in water (10 mL). THF (20 mL) was then added and the hydroxylamine solution was poured into the acid chloride solution, and the reaction mixture vigorously stirred for 2 h. The reaction mixture was partitioned between saturated aqueous NH_4Cl and CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give 4-(4-phenylphenoxy)butanohydroxamic acid (1.45 g, 57% overall yield) as a white solid: mp 131−132 °C; ^{1}H NMR (DMSO-d_6) δ 1.94 (m, 2H), 2.14 (t, 2H, J = 7 Hz), 4.01 (t, 2H, J = 6 Hz), 7.02 (d, 2H, J = 8 Hz), 7.29 (t, 1H, J = 6 Hz), 7.43 (t, 2H, J = 6 Hz), 7.62 (m, 4H), 8.71 (s, 1H), 10.44 (s, 1H); MS (DCI/NH_3) 289 (M + NH_4^+, 100), 272 (M+H^+, 35), 255 (30). Anal. Calcd for $C_{16}H_{17}NO_3$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.55; H, 6.95; N, 4.86.

5-(4-Phenylphenoxy)pentanohydroxamic Acid (48). A mixture in acetone of 4-phenylphenol (0.85 g, 5.0 mmol) and potassium carbonate (0.76 g, 5.5 mmol) was stirred for 30 min. Neat methyl 5-bromovalerate (0.78 mL, 5.5 mmol) was added dropwise via syringe, and the reaction mixture was stirred for 2 h at ambient temperature and overnight at reflux. Catalytic KI was then added, and the reaction mixture was heated overnight at reflux. An additional 10 drops of methyl 5-bromovalerate was then added, and reflux was continued for 8 h. The reaction mixture was cooled to ambient temperature and filtered. The collected solid was washed with acetone, and the combined filtrate and washings were concentrated *in vacuo*. The residue was partitioned between Et_2O and water. The aqueous phase was extracted with Et_2O. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give methyl 5-(4-phenylphenoxy)pentanoate (1.66 g) as a white powder.

A portion of this material (1.42 g, 5.0 mmol) was dissolved in methanol (7.5 mL) and treated with 2 M aqueous NaOH (7.5 mL, 15 mmol). After 7 h, the mixture was evaporated to dryness, and the residues were partitioned between water and Et_2O. The aqueous phase was acidified to pH 2 with concentrated HCl and extracted successively with EtOAc and CH_2Cl_2. The combined organic phases were dried ($MgSO_4$) and evaporated to provide a white solid (1.11 g, 82%).

A portion of this material (0.270 g, 1.0 mmol) was dissolved in CH_2Cl_2 (5 mL) and then treated with DMF (10 μL) and oxalyl chloride (96 μL, 1.1 mmol). After 2 h, a solution of hydroxylamine hydrochloride (0.139 g, 2.00 mmol) and Et_3N (279 μL, 2.0 mmol) in THF/water (1 mL each) was added, and the mixture was stirred a further 2 h at ambient temperature. The mixture was partitioned between 100 mL each of water and of CH_2Cl_2. The organic phase was discarded, and the aqueous phase was acidified to pH 7 with concentrated HCl and extracted with 3 × 100 mL CH_2Cl_2. The combined CH_2Cl_2 extracts were dried (Na_2SO_4) and concentrated to give a white solid (0.090 g, 32% yield): mp 151.5−153.5 °C; ^{1}H NMR (DMSO-d_6) δ 10.36 (S, 1H), 8.66 (d, 1H, J = 1.5 Hz), 7.65−7.55 (c, 4H), 7.46−7.39 (c, 2H), 7.34−7.26 (c, 2H), 4.00 (t, 2H, J = 6 Hz), 2.03 (t, 2H, J = 6 Hz), 1.80−1.58 (c, 4H); IR (KBr) 3200, 3040, 2920, 2860, 1660, 1640, 1620, 1610, 1520, 1490, 1470, 1290, 1270, 1250, 1200, 1180, 1030, 840, 780, 690 cm^{-1}; MS (DCI/NH_3) 269 (M − 16), 286 (M + H)$^+$, 303 (M + NH_4)$^+$. Anal. Calcd for $C_{17}H_{19}NO_3$·0.25 H_2O: C, 70.44; H, 6.78; N, 4.83. Found: C, 70.71; H, 6.82; N, 4.98.

2-[4-(4-Cyanophenyl)phenoxy]ethanohydroxamic Acid (49). To a suspension of 4-cyano-4′-hydroxybiphenyl (2.07 g, 10.6 mmol) and cesium carbonate (5.37 g, 16.5 mmol) in dry DMF (25 mL) was added *tert*-butyl bromoacetate (1.70 mL, 11.5 mmol). After stirring 18 h at ambient temperature, the mixture was diluted with Et_2O (200 mL) and extracted successively with $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$) and concentrated to give an off-white solid (3.26 g). This material was treated for 90 min at 0 °C with 80% TFA/20% CH_2Cl_2 (25 mL), and the mixture was partitioned between brine and CH_2Cl_2. The organics were dried ($MgSO_4$) and concentrated to give a white solid (3.36 g). This material was suspended in thionyl chloride (20 mL) and heated to reflux for 2 h, then cooled and vacuum dried. The resulting acid chloride was redissolved in THF (20 mL) and treated with a solution of hydroxylamine. Hydroxylamine was generated by dissolving hydroxylamine hydrochloride (1.50 g, 21.6 mmol) in water (6 mL), adding of NMM (2.50 mL, 23.0 mmol), and then diluting with THF (20 mL). After 2 h, the mixture was partitioned between aqueous NH_4Cl and CH_2Cl_2. The organics were dried ($MgSO_4$) and concentrated to give a white solid (1.97 g). Recrystallization from hot CH_3CN (100 mL) provided 1.11 g of the final compound (39% overall yield) as a white solid: mp 191−193 °C; ^{1}H NMR (DMSO-d_6) δ 4.54 (s, 2H), 7.08 (d, 2H, J = 8.8 Hz), 7.73 (d, 2H, J = 8.4 Hz), 7.87 (q, 4H, J = 5.5 Hz), 9.01 (s, 1H), 10.88 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 65.85, 109.27, 115.32, 119.02, 126.95, 128.31, 131.07, 132.80, 144.14, 158.44, 164.15; IR (KBr) 3400, 3230, 2960, 2220, 1640, 1600, 1490, 1255 cm^{-1}; MS (DCI/NH_3) 303 (M + $NH_4^+NH_3^+$, 5), 286 (M + NH_4^+, 20), 270 (M + NH_4 − O^+, 100). Anal. Calcd for $C_{15}H_{12}N_2O_3$: C, 67.16; H, 4.51; N, 10.44. Found: C, 67.04; H, 4.51; N, 10.23.

3-[4-(4-Cyanophenyl)phenoxy]propanohydroxamic Acid (50). To a solution of 4-cyano-4′-hydroxybiphenyl (3.30 g, 16.9 mmol) in THF (76 mL) and DMF (10 mL) was added potassium *tert*-butoxide (2.00 g, 16.9 mmol). Neat β-propiolactone (1.30 mL, 18.6 mmol) was added dropwise. The resulting yellow suspension was stirred overnight at ambient temperature and then reduced in volume *in vacuo*. The residue was taken up in EtOAc and extracted twice with 5% aqueous $NaHCO_3$ with NaCl added. The combined aqueous phases were washed with ether and then acidified to approximately pH 2. A white precipitate formed, which was filtered off and dried *in vacuo* to give 3-(4-(4-cyanophenyl)phenoxy)propionic acid (2.48 g, 60%) as a white powder: mp 160−164 °C; ^{1}H NMR (DMSO-d_6) δ 2.72 (t, 2H, J = 5.9 Hz), 4.23 (t, 2H, J = 6.1 Hz), 7.06 (d, 2H, J = 8.5 Hz), 7.72 (d, 2H, J = 8.8 Hz), 7.85 (d, 2H, J = 8.5 Hz), 7.88 (d, 2H, J = 8.5 Hz); IR (neat) 3044, 2976, 2950, 2220, 1702, 1603, 1496, 1254, 1244, 1182, 1043, 824 cm^{-1}; MS (DCI/NH_3) 267 (M + NH_4 − H_2O)$^+$, 285 (M + NH_4)$^+$, 302 (M + NH_4 + NH_3)$^+$. Anal. Calcd for: $C_{16}H_{13}NO_3$·0.10H_2O: C, 71.42; H, 4.94; N, 5.20. Found: C, 71.22; H, 4.75; N, 5.01.

A 1.00 g portion of the acid (3.74 mmol) was refluxed in thionyl chloride (10 mL) for 2 h. The excess thionyl chloride was removed *in vacuo*, and the resulting acid chloride was dissolved in THF (10 mL). A solution of hydroxylamine was prepared by dissolving hydroxylamine hydrochloride (0.178 g, 2.5 mmol) in distilled water (6 mL) and treatment with NMM (0.3 mL, 2.7 mmol). The soution was diluted with THF (10 mL) and added to the acid chloride solution. After 2.5 h, the reaction mixture was partitioned between CH_2Cl_2 and aqueous NH_4Cl solution, and the organic phase was dried over Na_2SO_4. Solvent removal, followed by trituration with CH_3CN with 0.1% TFA (20 mL) gave 0.61 g (58%) of a white powder: mp 114−118 °C; ^{1}H NMR (DMSO-d_6) δ 2.45 (t, 2H, J = 5.9 Hz), 4.25 (t, 2H, J = 5.9 Hz), 7.06 (d, 2H, J = 8.8 Hz), 7.72 (d, 2H, J = 8.8 Hz), 7.85 (d, 2H, J = 8.5 Hz), 7.88 (d, 2H, J = 8.5 Hz), 8.87 (s, 1H), 10.56 (s, 1H). IR (neat) 3241, 2244, 2235, 1629, 1606, 1496, 1257, 815 cm^{-1}; MS (DCI/NH_3) 300 (M + NH_4)$^+$, 317 (M + NH_4 + NH_3)$^+$. Anal. Calcd for $C_{16}H_{14}N_2O_3$·0.60 H_2O: C, 65.56; H, 5.23; N, 9.56. Found: C, 65.41; H, 4.85; N, 9.83.

4-[4-(4-Cyanophenyl)phenoxy]butanohydroxamic Acid (51). To a suspension in dry DMF of 4-cyano-4′-hydroxybiphenyl (2.02 g,.10.4 mmol) and cesium carbonate (4.49 g, 14.1 mmol) was added ethyl 4-bromobutyrate (2.66 g, 13.6 mmol), and the reaction mixture was stirred for 18 h at ambient temperature. The reaction mixture was diluted with Et_2O and extracted with pH 7 buffer. The organic phase was washed twice with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give 3.30 g white solid.

The crude ester was treated with lithium hydroxide hydrate (0.635 g, 15.1 mmol) in 2:1 dioxane/water (30 mL) for 18 h at ambient temperature and concentrated *in vacuo*. The resulting white solids were shaken with Et_2O and aqueous Na_2CO_3. The organic phase was discarded and the aqueous phase was acidified with concentrated HCl, and the resulting precipitate collected and vaccum dried to give 2.03 g of the acid.

A portion of the acid (1.00 g, 3.56 mmol) in thionyl chloride (10 mL) was heated at reflux for 90 min. The reaction mixture was cooled to ambient temperature and concentrated *in vacuo*, and the residue was taken up in 1:1 CH_2Cl_2/THF (10 mL). In a separate flask, NMM (0.55 mL, 5.0 mmol) was added to a solution of hydroxylamine hydrochloride (0.345 g, 4.96 mmol) in water (4 mL). THF (10 mL) was then added and the hydroxylamine solution was poured into the acid chloride

solution, and the reaction mixture was vigorously stirred for 1 h. The reaction mixture was partitioned between saturated aqueous NH_4Cl and CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give a white solid (0.85 g, 53% overall yield). An analytical sample was purified by reverse-phase HPLC: mp 170−172 °C; ^{1}H NMR (DMSO-d_6) δ 1.93 (m, 2H), 2.14 (t, 2H, J = 7 Hz), 4.02 (t, 2H, J = 6 Hz), 7.04 (d, 2H, J = 8 Hz), 7.71 (d, 2H, J = 8 Hz), 7.86 (m, 4H), 8.64 (s, 1H), 10.40 (s, 1H); IR (KBr) 3310, 2960, 2220, 1680, 1600, 1490, 1245 cm^{-1}; MS (DCI/NH_3) 314 (M + NH_4^+, 100), 297 (M + H^+, 40), 253 (70) . Anal. Calcd for $C_{17}H_{16}N_2O_3$·0.25 H_2O: C, 67.87; H, 5.53; N, 9.31. Found: C, 68.00; H, 5.41; N, 9.08.

5-[4-(4-Cyanophenyl)phenoxy]pentanohydroxamic Acid (52). To a suspension in 10 mL dry DMF of 4-cyano-4′-hydroxybiphenyl (0.976 g, 5.0 mmol) and cesium carbonate (1.95 g, 6.0 mmol) was added methyl 5-bromovalerate (1.17 g, 6.0 mmol), and the reaction mixture was stirred for 72 h at ambient temperature. The reaction mixture was diluted with Et_2O and extracted with pH 7 buffer. The organic phase was washed twice with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give 1.49 g white solid.

A portion of this material (0.748 g, 2.42 mmol) was dissolved in THF (6 mL) and methanol (3.7 mL) and treated with 2M aqueous NaOH (3.7 mL, 7.4 mmol). After 2 hours, the mixture was evaporated to dryness, and the residues were partitioned between water (400 mL) and Et_2O (100 mL). The aqueous phase was acidified to pH 2 with concentrated HCl and extracted succesively with EtOAc (100 mL) and CH_2Cl_2 (100 mL). The combined organic phases were dried ($MgSO_4$) and evaporated to provide a white solid (500 mg). This material was dissolved in THF (15 mL) and then treated with DMF (10 μL) and oxalyl chloride (163 μL, 1.87 mmol). After 2 h, a solution of *O*-(*tert*-butyldimethylsilyl)hydroxyl amine (0.295 g, 2.00 mmol) and Et_3N (0.251 μL, 1.8 mmol) in THF (2 mL) was added, and the mixture was stirred a further 3.5 hs at ambient temperature. Methanol (5 mL) was added, and the mixture was stirred for 10 min and then evaporated to dryness. The residues were partitioned between 100 mL each of 1M NaOH and of Et_2O, and the aqueous phase was then acidified to pH 2 with concentrated HCl and extracted three times with 100 mL CH_2Cl_2. The combined CH_2Cl_2 extracts were dried (Na_2SO_4) and concentrated to give a white solid (0.46 g) which was recrystalized from hot EtOAc (20 mL) to give the title compound as a white solid (0.243 g, 32% overall yield): mp 141.8−142.6 °C; ^{1}H NMR (DMSO-d_6) δ 1.59−1.84 (c, 4H), 2.03 (t, 2H, J = 7.5 Hz), 4.03 (t, 2H, J = 6 Hz), 7.06 (d, 2H, J = 9 Hz), 7.69 (s, 1H), 7.72 (s, 1H), 7.81−7.92 (c, 4H), 8.71 (s, 1H), 10.39 (s, 1H); IR (KBr) 3200, 3030, 3010, 2860, 2240, 1630, 1600, 1540, 1520, 1490, 1470, 1460, 1390, 1290, 1250, 1180, 1110, 980, 850, 820, 630, 580, 530 cm^{-1}; MS (DCI/NH_3) 294 (M − 16), 311 (M + H)$^+$, 328 (M + NH_4)$^+$. Anal. Calcd for $C_{18}H_{18}N_2O_3$: C, 69.66; H, 5.85; N, 9.03. Found: C, 69.54; H, 5.73; N, 8.99.

3-[4-[3-(Cyanomethyl)phenyl]phenoxy]propanohydroxamic Acid (53) To a solution of 4-iodophenol (5.00 g, 22.7 mmol) in THF (90 mL) was added potassium *tert*-butoxide (2.68 g, 22.7 mmol). Neat β-propiolactone (1.75 mL, 25.0 mmol) was added dropwise. The resulting suspension was stirred overnight at ambient temperature and then reduced in volume *in vacuo*. The residue was taken up in EtOAc (150 mL) and extracted twice with 5% aqueous $NaHCO_3$. The combined aqueous phases were chilled and then acidified with 6 M HCl to approximately pH 2. A precipitate formed, which was filtered off and dried *in vacuo* to give 3-(4-iodophenoxy)propionic acid (1.18 g, 18%) as a beige powder.

A portion of the material (1.00 g, 3.42 mmol) in thionyl chloride (6.0 mL) was heated at reflux for 30 min. The reaction mixture was cooled to ambient temperature, diluted with Et_2O, and concentrated *in vacuo*. The residue was azeotroped three times with Et_2O dried under high vacuum and taken up in CH_2Cl_2 (7 mL) under N_2. *O*-*tert*-Butylhydroxyl amine hydrochloride (1.08 g, 8.60 mmol) in water (10 mL) was treated with aqueous 10% Na_2CO_3 (10 mL), and the solution was extracted with CH_2Cl_2 (20 mL). The organic solution was dried over $MgSO_4$ and added to the acid chloride solution. The reaction mixture was stirred for 2 h at ambient temperature. The reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give a yellow oil which crystallized on standing to give *O*-*tert*-butyl-3-(4-iodophenoxy)-propanohydroxamic acid (1.22 g, 98%).

A portion of this material (0.60 g, 1.6 mmol) was dissolved in toluene (32 mL) under Ar, and hexamethyldistannane (0.58 g, 1.8 mmol) was added, followed by $Pd(PPH_3)_4$ (0.091 g, 0.079 mmol). The reaction mixture was stirred for 10 min at ambient temperature and 40 min at reflux. The reaction mixture was cooled to ambient temperature, diluted with EtOAc (100 mL), and filtered. The filtrate was washed with pH 7 buffer and brine, dried over Na_2SO_4, and concentrated *in vacuo* to give a yellow oil (0.58 g).

A portion of this material (0.20 g, 0.50 mmol) was combined with 3-iodophenylacetonitrile in toluene (10 mL). Tetrakis(triphenylphosphine)palladium (0.032 g, 0.028 mmol) was added, and the mixture was stirred for 10 min at ambient temperature and then heated to reflux for 18 h. The solution was cooled and diluted with EtOAc (50 mL) and then extracted with pH 7 buffer and brine. The organic phase was dried (Na_2SO_4) and concentrated, and the residual oil was purified by column chromatography (75% EtOAc/hexanes) to provide a yellow oil (0.043 g, 24%). This material was dissolved in 1:1 TFA/CH_2Cl_2 (1.6 mL) and stirred overnight at ambient temperature. The reaction mixture was filtered, concentrated *in vacuo*, and azeotroped with CH_2Cl_2 and CH_2Cl_2/Et_2O. Chromatography on silica gel (40:1, then 20:1 CH_2Cl_2−methanol, both containing 0.25% acetic acid) gave 3-[4-[3-(cyanomethyl)phenyl]phenoxy]propanohydroxamic acid (0.016 g, 44%): ^{1}H NMR (DMSO-d_6) δ 2.45 (t, 2H, J = 6.0 Hz), 4.08 (s, 2H), 4.23 (t, 2H, J = 6.0 Hz), 7.02 (d, 2H, J = 8.8 Hz), 7.29 (d, 1H, J = 7.5 Hz), 7.46 (t, 1H, J = 8.0 Hz), 7.57 (d, 1H, J = 6 Hz), 7.58 (s, 1H), 7.60 (d, 2H, J = 8.8 Hz), 8.84 (br s, 1H), 10.53 (br s, 1H); IR (microscope) 3245, 2924, 2251, 1643, 1608, 1518, 1483, 1244, 784 cm^{-1}; MS (DCI/NH_3) 314 (M + NH_4)$^+$. Anal. Calcd for $C_{17}H_{16}N_2O_3$·0.70 H_2O·0.20*t*-BuOH: C, 66.04; H, 6.04; N, 8.65. Found: C, 65.93; H, 5.64; N, 8.30.

4-[4-[3-Cyanomethyl)phenyl]phenoxy]butanohydroxamic Acid (54). To a suspension in 40 mL dry DMF of 4-iodophenol (5.00 g, 22.7 mmol) and cesium carbonate (9.25 g, 28.4 mmol) was added ethyl 4-bromobutyrate (5.32 g, 27.2 mmol), and the reaction mixture was stirred for 18 h at ambient temperature. The reaction mixture was diluted with Et_2O and extracted with pH 7 buffer. The organic phase was washed twice with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give 5.52 g of pale yellow plates (73%).

To a solution in toluene (120 mL) under argon of 4-(4-iodophenoxy)-butanoic acid ethyl ester (2.00 g, 5.98 mmol) and hexamethyldistannane (2.35 g, 7.17 mmol) was added $Pd(PPH_3)_4$ (0.35 g, 0.30 mmol). The reaction mixture was stirred for 15 min at ambient temperature and 30 min at reflux. The reaction mixture was cooled to ambient temperature, diluted with EtOAc, and filtered. The filtrate was washed with pH 7 buffer ($NaOH/KH_2PO_4$) and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Chromatography on silica gel (40:1, then 20:1, then 10:1 hexanes/EtOAc) gave 4-[4-(trimethylstannyl)phenoxy]-butanoic acid ethyl ester (1.14 g, 51%) as a clear, colorless oil.

To a solution in toluene (16 mL) under argon of ethyl 4-[4-(trimethylstannyl)phenoxy]butanoate (0.31 g, 0.84 mmol) and 3-(iodophenyl)acetonitrile (0.25 g, 1.0 mmol) was added $Pd(PPH_3)_4$ (0.052 g, 45 mmol). The reaction mixture was stirred for 10 min at ambient temperature and then for 18 h at reflux. The reaction mixture was cooled to ambient temperature and diluted with EtOAc. The organic solution was decanted away from a fine black precipitate, washed with pH 7 buffer ($NaOH/KH_2PO_4$) and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give 0.35 g of crude product. Chromatography on silica gel (10:1, then 5:1, then 3:1 hexanes/EtOAc) gave ethyl 4-[4-[3-(cyanomethyl)phenyl]phenoxy]butanoate (0.12 g, 44%) as white rosettes. This material was treated with lithium hydroxide hydrate (0.016 g, 0.38 mmol) in 2:1 dioxane/water (1.5 mL) for 4.5 h at ambient temperature and concentrated *in vacuo*. The resulting white solids were shaken with water (10 mL) and filtered, and the filtrate was acidified with 1 M HCl. The resulting precipitate was collected and vaccum dried to give a white powder (0.11 g, 100%).

A portion of the acid (0.082 g, 0.28 mmol) in thionyl chloride (1.6 mL) was heated at reflux for 30 min. The reaction mixture was cooled to ambient temperature and concentrated *in vacuo*, and the residue was taken up in THF (2.7 mL). In a separate flask, NMM (0.12 mL, 1.1 mmol) was added to a solution of hydroxyl amine hydrochloride (0.049 g, 0.70 mmol) in water (0.65 mL). THF (4 mL) was then added, and the hydroxyl amine solution was poured into the acid chloride solution,

and the reaction mixture vigorously stirred for 4 h. The reaction mixture was partitioned between saturated aqueous NH_4Cl and CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give a white solid (0.085 g). Trituration with CH_3CN with 0.1%TFA (2.5 mL) gave 0.061 g (70%) of a white powder: mp 140−144 °C; ^{1}H NMR (CD_3OD) δ 2.10 (dt, 2H, J = 7, 14 Hz), 2.31 (t, 2H, J = 7.4 Hz), 3.95 (s, 2H), 4.04 (t, 2H, J = 6.0 Hz), 7.00 (d, 2H, J = 8.5 Hz), 7.28 (d, 1H, J = 7.4 Hz), 7.42 (t, 1H, J = 7.4 Hz), 7.51−7.58 (c, 4H); MS (DCI/NH_3) 328 $(M + NH_4)^+$. Anal. Calcd for $C_{18}H_{18}N_2O_3 \cdot 0.20\ H_2O$ C, 68.86; H, 5.91; N, 8.92. Found: C, 68.92; H, 5.78; N, 8.72

5-[4-[3-Cyanomethyl)phenyl)phenoxy]pentanohydroxamic acid (55). To a suspension in 40 mL of dry DMF of 4-iodophenol (5.00 g,.22.7 mmol) and cesium carbonate (9.6 g, 29.5 mmol) was added methyl 5-bromopentanoate (5.2 g, 27.2 mmol), and the reaction mixture was stirred for 20 h at ambient temperature. The reaction mixture was diluted with Et_2O and extracted with pH 7 buffer. The organic phase was washed twice with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give 7.3 g of pale yellow solid (96%).

This material was treated with lithium hydroxide hydrate (1.1 g, 44 mmol) and 30% aqueous hydrogen peroxide (3.34 g, 88 mmol) in 4:1 THF/water (250 mL) for 18 h at ambient temperature. The solution was quenched with aqueous $NaHSO_3$ and then reduced in volume by rotary evaporation. The solution was acidified with 2 M HCl and then extracted with 3 × 150 mL of CH_2Cl_2. The combined organics were dried (Na_2SO_4) and concentrated to give the acid as a white solid (6.0 g, 85%).

A portion of the acid (4.7 g, 14.6 mmol) in thionyl chloride (20 mL) was heated at reflux for 30 min. The reaction mixture was cooled to ambient temperature and concentrated *in vacuo*, and the residue was taken up in THF (2.7 mL). In a separate flask, 3 M NaOH (10 mL, 30 mmol) was added to a solution of *O*-*tert*-butylhydroxyl amine hydrochloride (4.0 g, 32 mmol) in water (20 mL). THF (80 mL) was then added and the *O*-*tert*-butylhydroxyl amine solution was poured into the acid chloride solution, and the reaction mixture was vigorously stirred for 16 h. The reaction mixture was partitioned between saturated aqueous $NaHCO_3$ and EtOAc. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give an oily solid (5.95 g). Column chromatography (1:1 EtOAc/hexanes) gave 3.9 g (68%) of a white powder.

To a solution in toluene (80 mL) under argon, aryl iodide (3.5 g, 8.0 mmol), and hexamethyldistannane (3.9 g, 11 mmol) was added $Pd(PPH_3)_4$ (0.46 g, 0.40 mmol). The reaction mixture was stirred for 2 min at reflux. The reaction mixture was cooled to ambient temperature, diluted with EtOAc, and filtered. The filtrate was washed with pH 7 buffer and brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Chromatography on silica gel (1:1 hexanes/EtOAc) gave the stannane (2.7 g, 79%) as a clear, colorless oil.

To a portion of the material (1.5 g, 3.5 mmol) and 3-iodophenylacetonitrile (1.1 g, 4.5 mmol) in toluene (20 mL) was added $Pd(PPH_3)_4$ (0.10 g, 0.08 mmol). The reaction mixture was stirred for 18 h at reflux. The reaction mixture was cooled to ambient temperature and diluted with EtOAc. The organic solution was decanted away from a fine black precipitate, washed with pH 7 buffer and brine, dried over Na_2SO_4, filtered, and concentrated. Chromatography on silica gel (1:1 hexanes/EtOAc) yielded 0.66 g of a clear oil (49%). The material was suspended in TFA (20 mL) and stirred for 24 h. The reaction mixture was concentrated and then chromatographed ($CH_2Cl_2/CH_3OH/HCO_2H$ 98:1:1) to give 0.100 g (18%) of a clear oil: ^{1}H NMR (DMSO-d_6) δ 10.38 (s, 1H), 8.16 (s, 1H), 7.60−7.53 (m, 4H), 7.48−7.42 (t, 2H, J = 7.3 Hz), 7.30−7.28 (d, 1H, J = 7.8 Hz), 7.05−7.02 (d, 2H, J = 8.9 Hz), 4.08 (s, 2H), 4.03−3.00 (t, 2H, J = 5.5 Hz), 2.05−1.99 (t, 2H, J = 7 Hz), 1.69−1.64 (m, 4H); MS (DCI/NH_3) 325 $(M + H^+)$, 342 $(M + NH_4^+)$. Anal. Calcd for $C_{19}H_{20}N_2O_3 \cdot 0.25H_20$: C, 69.38; H, 6.28; N, 8.40. Found: C, 69.50; H, 6.46; N, 8.11.

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