Characterization and Optimization of Selective, Nonpeptidic Inhibitors of Cathepsin S with an Unprecedented Binding Mode†

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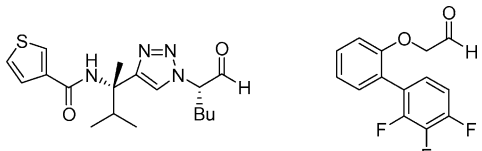
The substrate activity screening (SAS) method, a substrate-based fragment identification and optimization method for the development of enzyme inhibitors, was previously applied to cathepsin S to obtain a novel (2-arylphenoxy)acetaldehyde inhibitor, **2**, with a 0.49 μM K_i value (Wood, W. J. L.; Patterson, A. W.; Tsuruoka, H.; Jain, R. K.; Ellman, J. A. *J. Am. Chem. Soc.* **2005**, *127*, 15521−15527). In this paper we disclose the X-ray structure of a complex between cathepsin S and inhibitor **2** which reveals an unprecedented binding mode. On the basis of this structure, additional 2-biaryloxy substrates with greatly increased cleavage efficiency were designed. Conversion of the optimized substrates to the corresponding aldehyde inhibitors yielded a low molecular weight (304 Daltons) and potent (9.6 nM) cathepsin S inhibitor that showed from 100- to >1000-fold selectivity relative to cathepsins B, L, and K.

Introduction

Recently, we disclosed a new method called substrate activity screening (SAS) for the substrate-based identification of novel fragments that bind to serine or cysteine protease targets.[1,2] This method further enables the subsequent conversion of identified binding fragments to novel and low molecular weight mechanism-based protease inhibitors. In the first application of the SAS method,[1] two distinct nonpeptidic aldehyde inhibitors (Figure 1) were identified to the lysosomal cysteine protease cathepsin S, which is expressed in antigen-presenting cells such as macrophages, dendritic cells, and B cells. Cathepsin S degrades the MHC[a] class II-associated invariant chain that is required for productive loading of antigen onto the MHC class II complex, and therefore, inhibition of cathepsin S may be useful for attenuating antigen presentation in the treatment of autoimmune diseases.[3]

More recently, we reported on further optimization of the potency and selectivity of inhibitor **1**.[4] Here we report that the crystal structure of cathepsin S in complex with **2** establishes that this novel inhibitor, which lacks nitrogen functionality or chiral centers, has an unprecedented binding mode. Moreover, this structural information has provided the basis for the development of a low molecular weight (304 Daltons) inhibitor with a low nanomolar K_i value and from 100- to >1000-fold selectivity relative to the closely related enzymes cathepsins B, L, and K.



1 (K_i = 0.009 μM) **2** (K_i = 0.49 μM)

Figure 1. Cathepsin S inhibitors identified by the SAS method.

Chemistry

Central to the synthesis of analogues of **2** was the preparation of the appropriately substituted arylboronic acids that were needed for Suzuki cross-coupling reactions to prepare the biaryl frameworks. Accordingly, ortholithiation of 1,2-difluorobenzene with *n*-butyllithium in TMEDA/THF followed by addition of the requisite alkyl iodide provided the orthoalkylated difluorobenzenes **3** with high levels of regiocontrol (Scheme 1).[5] A second fluorine-directed ortholithiation followed by reaction with $B(OMe)_3$ and aqueous workup then provided the desired arylboronic acids **4**.

For the synthesis of the 2-arylphenoxy substrates, two approaches were taken.

In the first approach (Scheme 2), Suzuki cross-coupling reactions were performed between arylboronic acids **4a** and **4b** and 2-bromophenol to provide the 2-arylphenols **5a** and **5b**. For the synthesis of **5f** (Scheme 2), Suzuki cross-coupling was performed with **4** (R = OMe), which was prepared by literature methods.[6] The support-bound 7-(chloroacetamido)coumarin **6**[1] was then alkylated with the phenols **5** mediated by the strong base 2-(*tert*-butylimino)-2-(diethylamino)-1,3-dimethylperhydro-1,3,2-diazaphosphorine (BEMP) to provide the support-bound substrates **7**. Cleavage from the support with trifluoroacetic acid in CH_2Cl_2 then provided the desired substrates **8**.

The second method for preparing 2-arylphenoxy substrates **8** was carried out in solution (Scheme 3). Suzuki cross-coupling of **4** with commercially available **9** provided **10**, which either could be taken on to substrate **8** (Scheme 3) or could be readily converted to the corresponding aldehyde inhibitor (vide infra). Saponification of **10** followed by coupling with 7-amino-4-methylcoumarin acetic acid ethyl ester provided **11**, which upon saponification provided the desired substrate **8**. The synthesis

† Data for the X-ray crystal structures have been deposited in the Protein Data Bank, PDB ID 2OP3.

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[a] Abbreviations: AMC, 7-amino-4-(carbamoylmethyl)coumarin; AMCA, 7-amino-4-methylcoumarin acetic acid; E-64, L-*trans*-epoxysuccinyl-Leu-4-guanidinobutylamide; E-64c, L-*trans*-epoxysuccinyl-Leu-3-methylbutylamide; DMPU, 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone; MHC, major histocompatibility complex; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPFC, high-performance flash chromatography; TMEDA, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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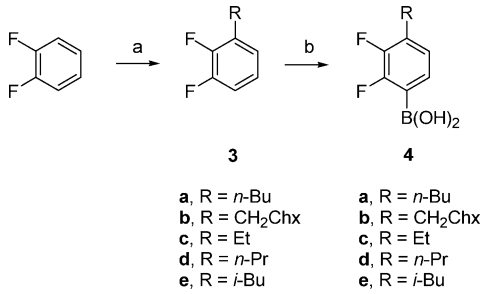
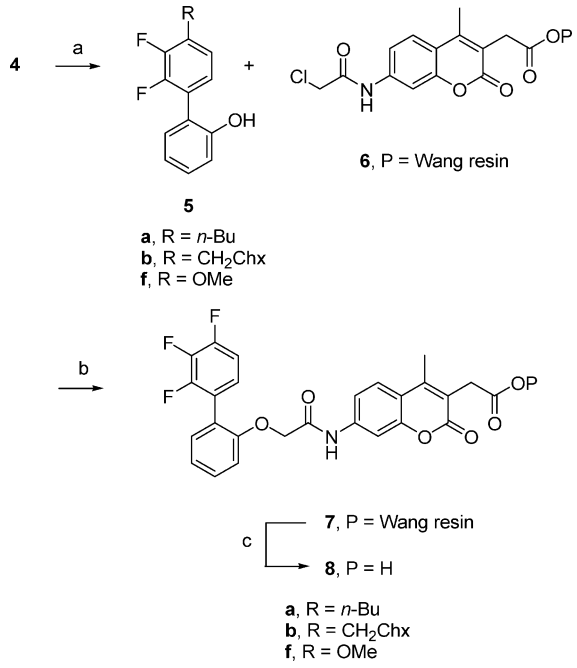
Scheme 1. Synthesis of Arylboronic Acid Derivatives[a]



[a] Reagents: (a) *n*-BuLi, TMEDA/THF, RI, DMPU; (b) *n*-BuLi, TMEDA/THF, $B(OMe)_3$, aqueous HCl.

Scheme 2. Synthesis of Substrates on Solid Supports[a]



[a] Reagents: (a) 2-bromophenol, $Pd(PPh_3)_4$, Na_2CO_3, benzene/MeOH/H_2O; (b) BEMP, THF; (c) trifluoroacetic acid, CH_2Cl_2.

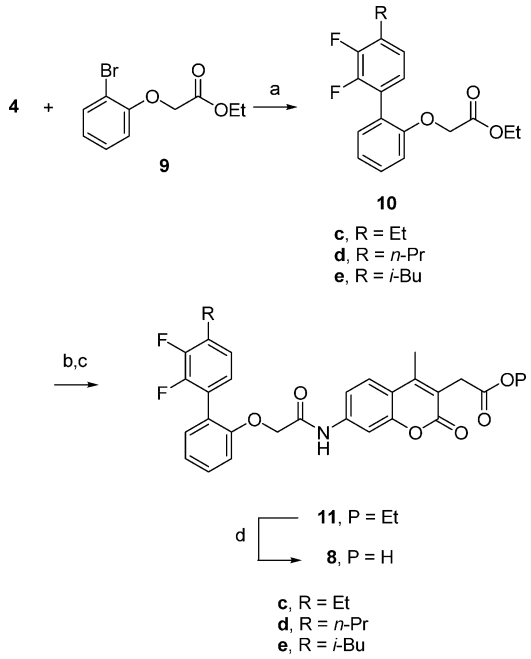
of the aldehyde inhibitors **12** could readily be achieved by diisobutylaluminum hydride (DIBAL-H) reduction of the late-stage intermediate **10** (Scheme 4).

Results and Discussion

Proteases uniformly recognize their substrates as β strands in their active sites.[7] Consequently, peptide backbone hydrogen-bonding is featured prominently in substrate and inhibitor binding interactions. In contrast, the novel inhibitor **2** lacks amide NH groups or alternative hydrogen bond donors. Moreover, H-bond-accepting interactions typically provided by the amide carbonyls or heterocyclic isosteres of protease substrates or inhibitors are also absent. The structure of **2** therefore suggests that its binding interactions with cathepsin S would be quite distinct from that typically observed for peptidic or peptidomimetic inhibitors.

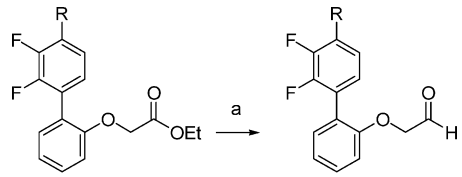
A crystal structure of **2** in complex with cathepsin S was therefore obtained to determine its mode of binding. Crystallographic statistics are summarized in Table 1. Indeed, as illustrated in the crystal structure (Figure 2), inhibitor **2** has a very distinctive mode of binding. While the aldehyde pharmacophore shows the expected tetrahedral adduct with the active site cysteine, the inhibitor does not make any interactions with the S1 and S3 pockets, which typically accommodate the P1 and P3 side chains of peptidic inhibitors. However, the biaryl functionality does make multiple hydrophobic contacts with the three fluorine substituents buried within a hydrophobic cavity,

Scheme 3. Synthesis of Substrates in Solution[a]



[a] Reagents: (a) $Pd_2(dba)_3-CHCl_3$, 2-(dicyclohexylphosphino)-2′,6′-dimethoxybiphenyl, K_3PO_4, dioxane; (b) aqueous NaOH, MeOH/THF; (c) 7-amino-4-methylcoumarin acetic acid ethyl ester, HATU, collidine, DMF; (d) aqueous NaOH, MeOH/THF.

Scheme 4. Synthesis of Aldehyde Inhibitors[a]



[a] Reagents and conditions: (a) DIBAL-H, toluene, −78 °C.

Table 1. Data Collection and Refinement Statistics for Cathepsin S

Data Collection and Processing	
space group	$P4_122$
$a = b$, Å	85.384
c, Å	151.2
wavelength, Å	1.0
resolution, Å	1.6
outer shell diam, Å	1.6−1.66
R_{merge}, % (outer shell)	0.082
$I/\mathrm{sig}I$ (σ) (outer shell)	45.6 (5.0)
no. of unique reflns (obsd)	74679 (994427)
completeness, % (outer shell)	100.0 (100.0)
Refinement	
R_{factor}[a] (R_{free}[b]), %	0.16 (0.18)
no. of protein atoms	3906
no. of water atoms	475
no. of hetero atoms	76
rmsd(bonds), Å	0.012
rmsd(angles), deg	1.310
mean B factor, Å^2	13.328

[a] $R_{factor} = \sum|I_i - \langle I_i\rangle|/\sum|I_i|$, where I_i is the scaled intensity of the ith measurement and $\langle I_i\rangle$ is the mean intensity for that reflection. [b] R_{free} is the same as R_{cryst}, but for 5.0% of the total reflections chosen at random and omitted from refinement.

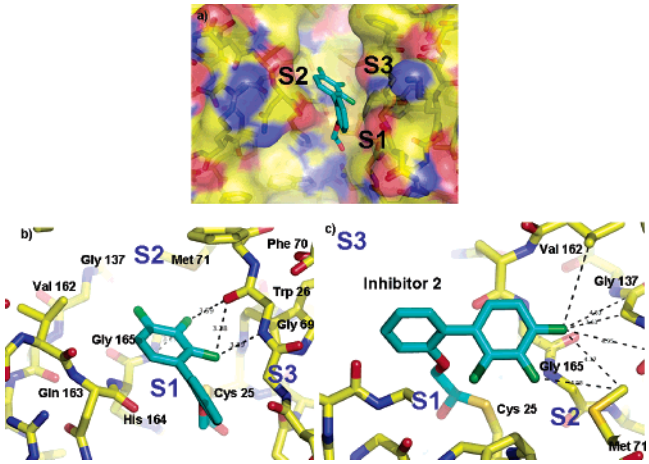


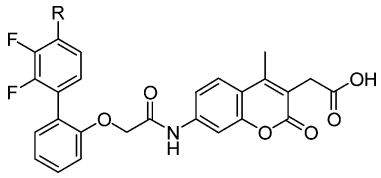
Figure 2. Structure of cathepsin S in complex with inhibitor **2**. Protein carbon atoms are colored yellow, while those from the inhibitor are shaded cyan. Nitrogens are in blue, oxygens in red, and fluorines in green. (a) Surface representation of the cathepsin S active site cleft showing the position of inhibitor **2** in relation to the specificity subsites S1, S2, and S3. (b) Stick representations of inhibitor **2** in the active site. Weak electrostatic interactions between fluorines and main chain atoms under 4.0 Å are represented as dotted lines. (c) Stick representations of inhibitor **2** in the active site oriented toward the hydrophobic S2 pocket. Potential hydrophobic interactions between fluorines and carbon atoms under 5.0 Å are represented as dotted lines. The figure was produced by pymol (www.pymol.org).

defined by residues Phe 70, Met 71, Val 162, and Val 135−Gly 137 (Figure 2c). These contacts are consistent with previously reported substrate and inhibitor SAR, indicating the importance of the binding interactions provided by the fluorine substituents: removal of the 4-fluoro substituent from **2** reduces K_i by 2.5-fold and replacement of all three fluoro substituents reduces K_i by 80-fold.[1] The 2- and 3-fluoro groups are likely to contribute binding interactions and specificity via weak hydrogen bond interactions with the main chain amides of Gly 165 and Gly 69, respectively (Figure 2b).

Importantly, the structure suggests numerous opportunities for enhancing the potency of inhibitor **2** by extending into the S1, S2, and S3 pockets. Designing inhibitors that extend into the S2 pocket provides a particularly attractive means to achieve selectivity relative to cathepsin B, and more significantly cathepsins L and K, which have particularly high homology with cathepsin S, because Gly 137 contributes to the wall of the S2 pocket, while for cathepsins B, L, and K the larger Ala residue is located at this site.

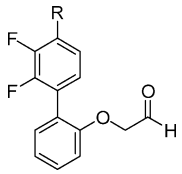
As previously reported in the development of inhibitors **1** and **2**,[1] and in the further optimization of **1**,[4] outstanding correlation between substrate cleavage efficiency and inhibitory activity was observed. Substrate analogues were therefore first prepared to evaluate the effect of introducing substituents that extend into the S2 pocket (Table 2). Replacing the 4-fluoro substituent with alkyl or ether substituents would provide an effective means to append functionality that might interact with the hydrophobic S2 pocket. Two analogues, **8f** and **8c**, with methoxy and ethyl groups at the 4-position, respectively (entries 2 and 3), were first prepared and evaluated as substrates to identify whether an ether or alkyl group would provide the most effective linkage strategy. While the methoxy analogue **8f** showed a 10-fold reduction in substrate cleavage efficiency (entry 2) relative to that of the benchmark substrate **13** (entry 1), the ethyl derivative **8c** was a substrate comparable in efficiency to **13** (entry 3). These results are consistent with the hydrophobic contacts made by the 4-fluoro group in the

Table 2. Cathepsin S-Mediated Substrate Cleavage Efficiency



entry	compd	R	$k_{cat}/K_M \times 10^3$ ($M^{-1}\,s^{-1}$)
1	**13**	F	27.0 ± 0.4
2	**8f**	OMe	2.70 ± 0.04
3	**8c**	Et	25.9 ± 0.2
4	**8d**	*n*-Pr	145 ± 1
5	**8a**	*n*-Bu	85 ± 3
6	**8e**	*i*-Bu	288 ± 30
7	**8b**	CH_2Chx	15.7 ± 0.2

Table 3. Inhibition of Cathepsins B, K, L, and S by Aldehyde Inhibitors



		K_i (nM)			
compd	R	Cat S	Cat L	Cat K	Cat B
2	fluoro	490 ± 50	1540 ± 320	1510 ± 310	>15000
12d	*n*-Pr	48.7 ± 2.0	5190 ± 430	1280 ± 170	>15000
12e	*i*-Bu	9.6 ± 2.4	>15000	1240 ± 280	>15000

cocrystal structure of **2** (Figure 2). Because **8c** was a more efficient substrate than **8f**, an alkyl linkage was employed for all additional analogues designed to extend into the S2 pocket. As shown in entry 4, compound **8d** with R = *n*-propyl was cleaved 5-fold more efficiently than compound **13**, while **8a** with R = *n*-butyl was cleaved only 3-fold more efficiently (entry 5). Introducing branching to provide **8e** with R = isobutyl (entry 6) provided the greatest enhancement in cleavage efficiency (>10-fold) relative to that of **13**. However, branched substrate **8b** incorporating a cyclohexylmethyl substituent showed reduced activity and serves to define the size of the pocket (entry 7).

The aldehyde inhibitors corresponding to the two substrates with the highest cleavage efficiency were prepared, and their inhibitory activities were determined (Table 3). Aldehyde **12d** showed 10-fold greater potency than inhibitor **2**, and aldehyde **12e**, which corresponds to the best substrate, was 50-fold more potent with a 9.6 nM K_i value. On the basis of the crystal structure, the propyl and isobutyl substituents of **12d** and **12e**, respectively, would be expected to interact with Gly 137 in cathepsin S. Because Ala is present at the corresponding site in cathepsins B, L, and K, an increase in selectivity could be anticipated. Indeed, while **2** was only marginally selective for cathepsin S over cathepsin K and cathepsin L, replacing the 4-fluoro group with the alkyl side chains resulted in dramatic improvements in selectivity, with inhibitor **12e** showing >100-fold selectivity for cathepsin K and >1000-fold selectivity for cathepsins B and L (Table 3).

In conclusion, the structure of novel, nonpeptidic inhibitor **2** in complex with cathepsin S has been reported and shows a binding mode that is distinct from that observed for peptidic and peptidomimetic inhibitors. The structural information guided the design of aldehyde inhibitor **12e**, which is both potent (K_i = 9.6 nM) and selective (from 100- to >1000-fold selectivity

relative to cathepsins K, L, and B). The structure of **2** in complex with cathepsin S suggests additional opportunities for enhancing potency and selectivity. The S3 pocket, which is unoccupied, provides a clear opportunity for introducing additional binding interactions. Moreover, the low molecular weight of **12e** enables the aldehyde pharmacophore to be replaced by more metabolically stable covalent[8] or noncovalent[9,10] pharmacophores that extend into the prime binding pockets, a strategy that has proven to be successful in recent cathepsin S and K inhibitor development efforts.

Experimental Section

General Synthesis Methods. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without purification. Tetrahydrofuran (THF), dioxane, CH_2Cl_2, ether, and toluene were passed through a column of activated alumina (type A2, 12 × 32, Purify Co.) under nitrogen pressure immediately prior to use. TMEDA and DMPU were distilled under N_2, and *i*-Pr_2EtN, pyridine, and CH_3OH were distilled under N_2 over CaH_2 immediately prior to use. Low amine content DMF was purchased from Acros, Wang resin and Sieber amide resin were purchased from Novabiochem (San Diego, CA), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) was purchased from PerSeptive Biosystems (Foster City, CA). Fmoc-protected 7-amino-4-methylcoumarin acetic acid (Fmoc-AMCA) was synthesized according to a method analogous to the synthesis of 7-amino-4-(carbamoylmethyl)coumarin (AMC).[11] Compounds **2**, **13**, and Fmoc-AMCA−Wang resin were synthesized as previously reported.[1] All solution-phase reactions were carried out in flame-dried glassware under an inert N_2 atmosphere. Normal-phase HPFC purification was carried out on a Biotage SP1 instrument (Charlottesville, VA) equipped with a Biotage Si flash column. Reversed-phase HPLC analysis and purification were conducted with an Agilent 1100 series instrument. Solid-phase reactions were conducted in polypropylene cartridges equipped with 70 mm PE frits (Applied Separations, Allentown, PA) and Teflon stopcocks and rocked on an orbital shaker. ^{1}H NMR spectra were obtained with Bruker AV-300 and AV-400 spectrometers. Unless otherwise specified, all spectra were obtained in $CDCl_3$, and chemical shifts are reported in parts per million relative to the peak for internal $CHCl_3$. Coupling constants are reported in hertz. Mass spectrometry analyses including high-resolution mass spectrometry analyses were performed by the University of California at Berkeley Micro Analysis and Mass Spectrometry Facilities. GC/MS analyses were performed with an Agilent Technologies 6890N GC system with an HP-5MS column coupled with an Agilent 5973 mass-selective detector.

Synthesis of 1-Alkyl-2,3-difluorobenzenes 3a−e. According to the procedure reported by Reiffenrath,[4] 1,2-difluorobenzene was alkylated with the appropriate alkyl iodide to obtain the title compounds.

1-*n*-Butyl-2,3-difluorobenzene (3a). The general procedure was followed using 0.99 mL (10 mmol) of difluorobenzene to give 1.15 g (6.8 mmol) of product as a colorless oil (yield 68%): ^{1}H NMR (400 MHz) δ 0.93 (t, J = 7.3, 3H), 1.37 (sextet, J = 7.4, 2H), 1.59 (quintet, J = 7.7, 2H), 2.66 (t, J = 7.7, 2H), 6.92−7.02 (m, 3H); MS (EI) m/z 170 (M^+).

1-(Cyclohexylmethyl)-2,3−difluorobenzene (3b). The general procedure was followed using 0.27 mL (2.7 mmol) of difluorobenzene to give 205 mg (0.98 mmol) of product as a colorless oil (yield 36%): ^{1}H NMR (400 MHz) δ 0.90−1.03 (m, 2H), 1.08−1.22 (m, 4H), 1.60−1.75 (m, 5H), 2.54 (d, J = 6.7, 2H), 6.88−7.00 (m, 3H); MS (EI) m/z 210 (M^+).

1-Ethyl-2,3-difluorobenzene (3c). The general procedure was followed using 1.97 mL (20 mmol) of difluorobenzene to give 350 mg (2.2 mmol) of product as a colorless oil (yield 11%): ^{1}H NMR (300 MHz) δ 1.24 (t, J = 7.6, 3H), 2.70 (dq, J = 1.2, 6.4, 2H), 6.92−7.02 (m, 3H); GC/MS (EI) m/z 142 (M^+).

2,3-Difluoro-1-*n*-propylbenzene (3d). The general procedure was followed using 1.97 mL (20 mmol) of difluorobenzene to give 2.98 g (19 mmol) of product as a colorless oil (yield 95%): ^{1}H NMR (400 MHz) δ 0.95 (t, J = 7.4, 3H), 1.64 (sextet, J = 7.5, 2H), 2.64 (dt, J = 1.3, 7.0, 2H), 6.90−7.01 (m, 3H); GC/MS (EI) m/z 156 (M^+).

2,3-Difluoro-1-(2-methylpropyl)benzene (3e). The general procedure was followed using 0.99 mL (10 mmol) of difluorobenzene to give 0.49 g (2.9 mmol) of product as a colorless oil (yield 29%): ^{1}H NMR (400 MHz) δ 0.92 (d, J = 6.7, 6H), 1.91 (m, 1H), 2.54 (dd, J = 1.5, 7.2, 2H), 6.86−7.03 (m, 3H); GC/MS (EI) m/z 170 (M^+).

Synthesis of 4-Alkyl-2,3-difluorobenzeneboronic Acids 4a−e. 4-(Cyclohexylmethyl)-2,3-difluorobenzeneboronic Acid (4b). To a solution of **3b** (1.41 g, 6.7 mmol) and *N*,*N*,*N*′,*N*′-tetrametylethylenediamine (TMEDA; 1.01 mL, 6.7 mmol) in THF (20 mL) was added a solution of *n*-butyllithium in hexane (2.5 M, 3.5 mL, 8.8 mmol) dropwise over 5 min at −78 °C under a nitrogen atmosphere. After the mixture was stirred for 1 h at −78 °C, trimethyl borate (0.97 mL, 8.8 mmol) was added dropwise over 5 min, and then the mixture was stirred for 1.5 h at −78 °C. After the reaction was quenched by adding water (5 mL) at −78 °C, the mixture was allowed to warm to ambient temperature, and then 2 N HCl(aq) (25 mL) was added. The mixture was stirred overnight and extracted with EtOAc. The extracts were washed with brine, dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification using silica gel column chromatography (DCM/MeOH = 50/1 → 20/1 → 10/1) gave the title compound (1.12 g, 4.4 mmol, 66%) as a colorless solid: ^{1}H NMR (400 MHz) δ 0.91−1.05 (m, 2H), 1.10−1.26 (m, 4H), 1.62−1.74 (m, 5H), 2.55 (m, 2H), 5.05 (d, J = 5.6, 1H), 6.89−6.97 (m, 1H), 7.35−7.46 (m, 1H).

4-*n*-Butyl-2,3-difluorobenzeneboronic Acid (4a). Using the same procedure as for **4b**, **3a** (1.70 g, 10 mmol) was converted to 109 mg (0.51 mmol) of the title compound as a colorless solid (yield 5%): ^{1}H NMR (400 MHz) δ 0.91−0.97 (m, 3H), 1.31−1.49 (m, 2H), 1.57−1.69 (m, 2H), 2.65−2.75 (m, 2H), 5.03 (d, J = 5.5, 1H), 6.93−7.09 (m, 1H), 7.37−7.48 (m, 1H).

4-Ethyl-2,3-difluorobenzeneboronic Acid (4c). Using the same procedure as for **4b**, **3c** (350 mg, 2.24 mmol) was converted to 0.28 g (1.51 mmol) of the title compound as a colorless solid (yield 67%): ^{1}H NMR (300 MHz) δ 1.21−1.33 (m, 3H), 2.70−2.82 (m, 2H), 5.00 (br s, 1H), 7.00−7.09 (m, 1H), 7.47 (m, 0.5H), 7.78 (m, 0.5H).

2,3-Difluoro-4-*n*-propylbenzeneboronic Acid (4d). Using the same procedure as for **4b**, **3d** (2.98 g, 19.1 mmol) was converted to 2.34 g (11.7 mmol) of the title compound as a colorless solid (yield 61%): ^{1}H NMR (400 MHz) δ 0.90−1.01 (m, 3H), 1.61−1.74 (m, 2H), 2.62−2.77 (m, 2H), 4.98 (br s, 0.4H), 6.94−7.08 (m, 1H), 7.36−7.47 (m, 0.4H), 7.76 (m, 0.6H).

2,3-Difluoro-4-(2-methylpropyl)benzeneboronic Acid (4e). Using the same procedure as for **4b**, **3e** (592 mg, 3.48 mmol) was converted to 490 mg (2.29 mmol) of the title compound as a colorless solid (yield 66%): ^{1}H NMR (300 MHz) δ 0.90−0.98 (m, 6H), 1.85−1.99 (m, 1H), 2.52−2.58 (m, 2H), 4.96 (d, J = 5.7, 0.6H), 6.90−7.02 (m, 1H), 7.39−7.48 (m, 1H).

Synthesis of 2-(4-Alkyl-2,3-difluorophenyl)phenols 5a, 5b, and 5f. 2-(4-*n*-Butyl-2,3-difluorophenyl)phenol (5a). A mixture of **4a** (105 mg, 0.49 mmol), 2-bromophenol (57 μL, 0.49 mmol), Pd(Ph_3P)$_4$ (11 mg, 0.010 mmol), sodium carbonate (208 mg, 2.0 mmol), benzene (4 mL), MeOH (0.8 mL), and water (0.2 mL) was heated at 80 °C with stirring for 15 h under a nitrogen atmosphere. The resultant mixture was partitioned between EtOAc (50 mL) and 1 N HCl(aq) (10 mL), and the organic layer was isolated. The organic layer was washed with water (10 mL) and brine (10 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Purification by HPFC (hexane/EtOAc = 100/0 → 88/12) gave the title compound (95 mg, 0.36 mmol, 74%) as a colorless oil: ^{1}H NMR (400 MHz) δ 0.97 (t, J = 7.3, 3H), 1.41 (sextet, J = 7.4, 2H), 1.65 (quintet, J = 7.6, 2H), 2.72 (t, J = 7.7, 2H), 4.96 (br s, 1H), 6.98−7.07 (m, 4H), 7.21−7.33 (m, 2H); MS (EI) m/z 262 (M^+).

2-[4-(Cyclohexylmethyl)-2,3-difluorophenyl]phenol (5b). Using the same procedure as for **5a**, **4b** (178 mg, 0.70 mmol) was

converted to 173 mg (0.57 mmol) of the title compound as a colorless gum (yield 82%): ^{1}H NMR (400 MHz) δ 0.96−1.06 (m, 2H), 1.15−1.28 (m, 3H), 1.60−1.74 (m, 6H), 2.59 (d, J = 7.1, 2H), 5.04 (br s, 1H), 6.98−7.06 (m, 4H), 7.23−7.33 (m, 2H); MS (EI) m/z 302 (M^+).

2-(2,3-Difluoro-4-methoxyphenyl)phenol (5f). Using the same procedure as for **5a**, **4f** (120 mg, 0.64 mmol) was converted to 112 mg (0.47 mmol) of the title compound as a colorless oil (yield 74%); ^{1}H NMR (400 MHz) δ 3.96 (s, 3H), 4.93 (s, 1H), 6.85 (m, 1H), 6.97−7.03 (m, 2H), 7.08 (m, 1H), 7.22 (d, J = 7.4, 1H), 7.30 (m, 1H); MS (EI) m/z 236 (M^+).

Synthesis of [2-(4-Alkyl-2,3-difluorophenyl)phenoxy]acetyl-AMCAs 8a, 8b, and 8f. [2-(4-*n*-Butyl-2,3-difluorophenyl)-phenoxy]acetyl-AMCA (8a). According to the reported procedure,[1] **5a** (89 mg, 0.34 mmol) was reacted with resin-bound AMCA (**6**; 0.10 mmol). Cleavage from the resin followed by reversed-phase HPLC purification yielded 22 mg (0.041 mmol) of the title compound as a colorless solid (yield 41%): ^{1}H NMR (400 MHz) δ 0.95 (t, J = 7.3, 3H), 1.43 (sextet, J = 7.4, 2H), 1.67 (quintet, J = 7.6, 2H), 2.43 (s, 3H), 2.77 (t, J = 7.7, 2H), 3.78 (s, 2H), 4.65 (s, 2H), 6.99 (d, J = 8.2, 1H), 7.09 (m, 2H), 7.16 (t, J = 7.1, 1H), 7.35 (m, 2H), 7.44 (m, 1H), 7.57 (d, J = 8.7, 1H), 7.64 (d, J = 2.1, 1H), 8.43 (d, J = 3.1, 1H); HRMS (FAB+) m/z 536.1892 (MH^+, $C_{30}H_{28}F_2NO_6$ requires 536.1885). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

[2-[4-(Cyclohexylmethyl)-2,3-difluorophenyl]phenoxy]acetyl-AMCA (8b). Using the same procedure as for **8a**, **5b** (91 mg, 0.30 mmol) was converted to 32 mg (0.056 mmol) of the title compound as a colorless solid (yield 56%): ^{1}H NMR (400 MHz) δ 1.00−1.09 (m, 2H), 1.15−1.21 (m, 3H), 1.60−1.76 (m, 6H), 2.43 (s, 3H), 2.65 (d, J = 6.7, 2H), 3.78 (s, 2H), 4.66 (s, 2H), 6.99 (d, J = 8.3, 1H), 7.06 (m, 2H), 7.17 (t, J = 7.2, 1H), 7.35−7.37 (m, 2H), 7.43 (m, 1H), 7.58 (d, J = 8.7, 1H), 7.67 (d, J = 2.0, 1H), 8.45 (d, J = 3.3, 1H); HRMS (FAB+) m/z 576.2198 (MH^+, $C_{33}H_{32}F_2NO_6$ requires 576.2198). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

[2-(2,3-Difluoro-4-methoxyphenyl)phenoxy]acetyl-AMCA (8f). Using the same procedure as for **8a**, **5f** (56 mg, 0.24 mmol) was converted to 21 mg (0.040 mmol) of the title compound as a colorless solid (yield 40%): ^{1}H NMR (400 MHz) δ 2.44 (s, 3H), 3.78 (s, 2H), 4.01 (s, 3H), 4.65 (s, 2H), 6.92 (m, 1H), 6.99 (d, J = 8.0, 1H), 7.10 (m, 1H), 7.16 (t, J = 7.4, 1H), 7.34 (m, 1H), 7.38−7.43 (m, 2H), 7.59−7.62 (m, 2H), 8.41 (br s, 1H); HRMS (FAB+) m/z 510.1371 (MH^+, $C_{27}H_{22}F_2NO_7$ requires 510.1364). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

Synthesis of Ethyl [(4′-Alkyl-2′,3′-difluorobiphenyl-2-yl)oxy]-acetates 10c, 10d, and 10e. Ethyl [(4′-Ethyl-2′,3′-difluorobiphenyl-2-yl)oxy]acetate (10c). A mixture of **4c** (186 mg, 1.00 mmol), ethyl (2-bromophenoxy)acetate (**9**; 130 mg, 0.50 mmol), $Pd_2(dba)_3$−$CHCl_3$ (16 mg, 0.015 mmol), 2-(dicyclohexylphosphino)-2′,6′-dimethoxybiphenyl (11 mg, 0.031 mmol), potassium phosphate (318 mg, 1.50 mmol), and dioxane (3 mL) was heated at 110 °C with stirring for 15 h under a a nitrogen atmosphere. The resultant mixture was filtered through a short silica gel pad and concentrated in vacuo. Purification by silica gel column chromatography (hexane/EtOAc = 20/1 → 10/1) provided the title compound (145 mg, 0.45 mmol, 91%) as a pale yellow oil: ^{1}H NMR (400 MHz) δ 1.24−1.57 (m, 6H), 2.74 (q, J = 7.5, 2H), 4.23 (q, J = 7.1, 2H), 4.63 (s, 2H), 6.87 (d, J = 8.3, 1H), 6.99 (t, J = 7.4, 1H), 7.06−7.12 (m, 2H), 7.30 (d, J = 7.5, 1H), 7.34 (m, 1H); MS (FAB) m/z 536 (M + H^+).

Ethyl [(2′,3′-Difluoro-4′-*n*-propylbiphenyl-2-yl)oxy]acetate (10d). Using the same procedure as for **10c**, **4d** (130 mg, 0.50 mmol) was converted to 164 mg (0.49 mmol) of the title compound as a pale yellow oil (yield 98%): ^{1}H NMR (400 MHz) δ 1.00 (t, J = 7.3, 3H), 1.26 (t, J = 7.1, 3H), 1.69 (sextet, J = 7.5, 2H), 2.67 (t, J = 7.6, 2H), 4.23 (q, J = 7.1, 2H), 4.63 (s, 2H), 6.87 (d, J = 8.2, 1H), 6.97 (t, J = 7.4, 1H), 7.06−7.09 (m, 2H), 7.29−7.42 (m, 2H); MS (FAB) m/z 550 (M + H^+).

Ethyl [[2′,3′-Difluoro-4′-(2-methylpropyl)biphenyl-2-yl]oxy]-acetate (10e). Using the same procedure as for **10c**, **4e** (127 mg, 0.59 mmol) was converted to 101 mg (0.29 mmol) of the title compound as a pale yellow oil (yield 98%): ^{1}H NMR (400 MHz) δ 0.96 (d, J = 6.7, 6H), 1.26 (t, J = 7.1, 3H), 1.95 (m, 1H), 2.57 (d, J = 7.2, 2H), 4.22 (q, J = 7.1, 2H), 4.62 (s, 2H), 6.87 (d, J = 8.2, 1H), 6.93 (t, J = 7.3, 1H), 7.06−7.12 (m, 2H), 7.30−7.36 (m, 2H); MS (FAB) m/z 564 (M + H^+).

Ethyl (7-Amino-4-methyl-2-oxo-2*H*-chromen-3-yl)acetate (AMCA Ethyl Ester). A mixture of [7-(9-fluorenylmethoxycarbonylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl]acetic acid (Fmoc-AMCA; 4.56 g, 10.0 mmol), potassium carbonate (1.52 g, 11.0 mmol), iodoethane (1.20 mL, 15.0 mmol), and DMF (30 mL) was stirred at ambient temperature for 6 h. The resultant mixture was poured into 1 N HCl(aq) (50 mL) and was extracted with DCM (200 mL + 2 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (50 mL), and after dilution with DCM/MeOH = 9/1 solution (500 mL), the organic solution was dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Then the residue was suspended in MeOH, and the insoluble solid was collected by filtration to give as a crude product [7-(9-fluorenylmethoxycarbonylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl]acetic acid ethyl ester (Fmoc-AMCA ethyl ester) as a colorless powder: ^{1}H NMR (400 MHz) δ 1.27 (t, J = 7.1 Hz, 3H), 2.37 (s, 3H), 3.71 (s, 2H), 4.18 (q, J = 7.1 Hz, 2H), 4.29 (t, J = 6.0 Hz, 1H), 4.61 (d, J = 6.3 Hz, 2H), 6.90 (br s, 0.9H), 7.33−7.45 (m, 6H), 7.53 (d, J = 8.7 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.80 (d, J = 7.6 Hz, 2H); MS (FAB) m/z 262 (M + H^+).

Crude Fmoc-AMCA ethyl ester was suspended in DMF (50 mL), and piperidine (5 mL) was added to the suspension at ambient temperature. The mixture was stirred for 20 min at ambient temperature and then concentrated in vacuo. The resultant residue was crystallized in a hexane/DCM solution (90 mL/10 mL) and collected by filtration to give the title compound (2.19 g, 8.34 mmol, 2 steps, 84%) as a pale yellow solid: ^{1}H NMR (400 MHz) δ 1.26 (t, J = 7.1 Hz, 3H), 2.33 (s, 3H), 3.68 (s, 2H), 4.16 (s, 2H), 4.17 (q, J = 7.1, 2H), 6.54−6.58 (m, 2H), 7.38 (d, J = 8.5 Hz, 1H); MS (FAB) m/z 484 (M + H^+).

Synthesis of [(4′-Alkyl-2′,3′-difluorobiphenyl-2-yl)oxy]acetyl-AMCA Ethyl Esters 11c, 11d, and 11e. [(4′-Ethyl-2′,3′-difluorobiphenyl-2-yl)oxy]acetyl-AMCA Ethyl Ester (11c). NaOH(aq) (1 N, 0.60 mL) was added to a mixture of **10c** (61 mg, 0.190 mmol), MeOH (0.6 mL), and THF (1.8 mL), and the mixture was stirred for 6 h at ambient temperature. After 1 N HCl(aq) (2 mL) and water (10 mL) were added, the resulting mixture was extracted with EtOAc (50 mL + 10 mL). The combined extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo to provide [(4′-ethyl-2′,3′-difluorobiphenyl-2-yl)oxy]acetic acid. To a solution of the crude [(4′-ethyl-2′,3′-difluorobiphenyl-2-yl)oxy]acetic acid, AMCA ethyl ester (50 mg, 0.19 mmol), and 2,4,6-collidine (0.050 mL, 0.38 mmol) in DMF (1 mL) was added HATU (108 mg, 0.29 mmol) at ambient temperature, and the mixture was stirred for 17 h at the same temperature. After dilution with DCM (50 mL), the resultant mixture was washed with 1 N HCl(aq) (10 mL), water (10 mL), and brine (10 mL), then dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The obtained crude product was purified by silica gel column chromatography (DCM/EtOAc = 20/1 → 10/1) to give the title compound (90 mg, 0.169 mmol, 89%) as a colorless foam: ^{1}H NMR (400 MHz) δ 1.27 (t, J = 7.1, 3H), 1.33 (t, J = 7.6, 3H), 2.40 (s, 3H), 2.82 (q, J = 7.5, 2H), 3.72 (s, 2H), 4.18 (q, J = 7.2, 2H), 4.65 (s, 2H), 6.99 (d, J = 8.4, 1H), 7.09−7.19 (m, 3H), 7.35−7.44 (m, 3H), 7.55−7.57 (m, 2H), 8.40 (br s, 1H); MS (FAB) m/z 536 (M + H^+).

[(2′,3′-Difluoro-4′-*n*-propylbiphenyl-2-yl)oxy]acetyl-AMCA Ethyl Ester (11d). Using the same procedure as for **11c**, **10d** (117 mg, 0.35 mmol) was converted to 125 mg (0.23 mmol) of the title compound as a colorless foam (yield 65%): ^{1}H NMR (400 MHz) δ 1.02 (t, J = 7.3, 3H), 1.27 (t, J = 7.1, 3H), 1.73 (sextet, J = 7.5, 2H), 2.40 (s, 3H), 2.75 (t, J = 7.6, 2H), 3.72 (s, 2H), 4.18 (q, J =

7.1, 2H), 4.65 (s, 2H), 6.94 (d, $J = 8.2$, 1H), 7.09 (m, 2H), 7.17 (t, $J = 7.1$, 1H), 7.34−7.42 (m, 2H), 7.44−7.46 (m, 1H), 7.55−7.57 (m, 2H), 8.40 (br s, 1H); MS (FAB) *m*/*z* 550 ($M + H^+$).

[[2′,3′-Difluoro-4′-(2-methylpropyl)biphenyl-2-yl]oxy]acetyl-AMCA Ethyl Ester (11e). Using the same procedure as for **11c**, **10e** (53 mg, 0.152 mmol) was converted to 75 mg (0.132 mmol) of the title compound as a colorless foam (yield 87%): ^{1}H NMR (400 MHz) δ 0.98 (d, $J = 6.6$, 6H), 1.27 (t, $J = 7.1$, 3H), 2.01 (m, 1H), 2.40 (s, 3H), 2.65 (d, $J = 7.2$, 2H), 3.72 (s, 2H), 4.18 (q, $J = 7.1$, 2H), 4.65 (s, 2H), 6.99 (d, $J = 8.2$, 1H), 7.05−7.12 (m, 2H), 7.17 (t, $J = 7.5$, 1H), 7.37 (m, 2H), 7.44 (m, 1H), 7.56 (d, $J = 8.8$, 1H), 7.59 (d, $J = 2.0$, 1H), 8.42 (br s, 1H); MS (FAB) *m*/*z* 564 ($M + H^+$).

Synthesis of [2-(4-Alkyl-2,3-difluorophenyl)phenoxy]acetyl-AMCAs 8c, 8d, and 8e. [2-(4-Ethyl-2,3-difluorophenyl)phenoxy]-acetyl-AMCA (8c). NaOH(aq) (1 N, 0.50 mL) was added to a mixture of **11c** (88 mg, 0.165 mmol), MeOH (0.5 mL), and THF (1.5 mL), and the resulting mixture was stirred for 2 h at ambient temperature. After 1 N HCl(aq) (5 mL) and water (5 mL) were added, the mixture was extracted with a DCM/MeOH (5/1) solution (once with 50 mL and three times with 10 mL). The combined extracts were dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by reversed-phase HPLC (MeCN/H_2O/TFA) to give the pure title compound (50 mg, 0.098 mmol, 59%) as a colorless solid: ^{1}H NMR (400 MHz) δ 1.33 (t, $J = 7.6$, 3H), 2.43 (s, 3H), 2.82 (q, $J = 7.6$, 2H), 3.77 (s, 2H), 4.65 (s, 2H), 6.99 (d, $J = 8.3$, 1H), 7.11 (m, 2H), 7.17 (t, $J = 7.6$, 1H), 7.36 (m, 2H), 7.44 (m, 1H), 7.56−7.61 (m, 2H), 8.42 (br s, 1H); HRMS (FAB+) *m*/*z* 508.1573 (MH^+, $C_{28}H_{24}F_2NO_6$ requires 508.1572). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

[2-(2,3-Difluoro-4-*n*-propylphenyl)phenoxy]acetyl-AMCA (8d). Using the same procedure as for **8c**, **11d** (123 mg, 0.224 mmol) was converted to 52 mg (0.100 mmol) of the title compound as a colorless solid (yield 45%): ^{1}H NMR (400 MHz) δ 1.02 (t, $J = 7.3$, 3H), 1.72 (sextet, $J = 7.5$, 2H), 2.42 (s, 3H), 2.75 (t, $J = 7.5$, 2H), 3.77 (s, 2H), 4.65 (s, 2H), 6.99 (d, $J = 8.2$, 1H), 7.09 (m, 2H), 7.16 (t, $J = 7.4$, 1H), 7.34−7.38 (m, 2H), 7.43 (m, 1H), 7.56 (d, $J = 8.7$, 1H), 7.59 (d, $J = 2.0$, 1H), 8.42 (br s, 1H); HRMS (FAB+) *m*/*z* 522.1715 (MH^+, $C_{29}H_{26}F_2NO_6$ requires 522.1728). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

[2-(4-Isobutyl-2,3-difluorophenyl)phenoxy]acetyl-AMCA (8e). Using the same procedure as for **8c**, **11e** (72 mg, 0.128 mmol) was converted to 45 mg (0.083 mmol) of the title compound as a colorless solid (yield 65%): ^{1}H NMR (400 MHz) δ 0.98 (d, $J = 6.6$, 6H), 1.99 (m, 1H), 2.43 (s, 3H), 2.64 (d, $J = 7.2$, 2H), 3.77 (s, 2H), 4.65 (s, 2H), 6.99 (d, $J = 8.2$, 1H), 7.07 (m, 2H), 7.17 (t, $J = 7.5$, 1H), 7.35−7.37 (m, 2H), 7.44 (m, 1H), 7.57 (d, $J = 8.7$, 1H), 7.63 (m, 1H), 8.45 (br s, 1H); HRMS (FAB+) *m*/*z* 536.1877 (MH^+, $C_{30}H_{28}F_2NO_6$ requires 536.1885). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

Synthesis of [2-(4-Alkyl-2,3-difluorophenyl)phenoxy]acetaldehydes 12d and 12e. [2-(2,3-Difluoro-4-*n*-propylphenyl)phenoxy]acetaldehyde (12d). Diisobutylaluminum hydride solution (1.0 M in toluene, 0.20 mL) was added dropwise over 5 min to **10d** (44 mg, 0.130 mmol) in toluene (1.3 mL) at −78 °C, and the mixture was stirred for 1 h at the same temperature. The mixture was quenched with AcOH (0.12 mL), and then saturated aqueous sodium tartrate solution (8 mL) was added to it. After being stirred for 1.5 h at ambient temperature, the resultant mixture was extracted with EtOAc (50 mL). The extracts were washed with brine (10 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 2/1 → 1/1) and then by reversed-phase HPLC (MeCN/H_2O/TFA) to provide the title compound (12 mg, 31%, 2/3 mixture of the aldehyde and its hydrate) as a colorless gum: ^{1}H NMR (400 MHz, $CD_3CN/D_2O = 3/1$) δ 0.75−0.90 (m, 1.8H), 0.92 (t, $J = 7.4$, 1.2H), 1.39−1.68 (m, 2H), 2.41−2.54 (m, 1.2H), 2.64 (t, $J = 7.7$, 0.8H), 3.86 (d, $J = 5.0$, 1.2H), 4.73 (s, 0.8H), 5.02 (t, $J = 5.2$, 0.6H), 6.80−7.10 (m, 4H), 7.12−7.50 (m, 2H), 9.61 (s, 0.4H); HRMS (EI+) *m*/*z* 290.1117 (M^+, $C_{17}H_{16}F_2O_2$ requires 290.1118). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

[2-(4-Isobutyl-2,3-difluorophenyl)phenoxy]acetaldehyde (12e). Using the same procedure as for **12d**, **10e** (67 mg, 0.193 mmol) was converted to 22 mg (0.074 mmol) of the title compound (1/4 mixture of the aldehyde and its hydrate) as a colorless gum (yield 38%): ^{1}H NMR (400 MHz, $CD_3CN/D_2O = 5/1$) δ 0.80−0.90 (m, 4.8H), 0.90 (d, $J = 6.6$, 1.2H), 1.72−1.90 (m, 1H), 2.32−2.42 (m, 1.6H), 2.55 (d, $J = 7.4$, 0.4H), 3.86 (d, $J = 5.0$, 1.6H), 4.72 (s, 0.4H), 5.01 (t, $J = 0.8$H), 6.80−7.10 (m, 4H), 7.16−7.40 (m, 2H), 9.59 (s, 0.2H); HRMS (EI+) *m*/*z* 304.1275 (M^+, $C_{18}H_{18}F_2O_2$ requires 304.1275). The purity of the compound was checked by RP-HPLC (see the Supporting Information for MeOH/H_2O/TFA and MeCN/H_2O/TFA HPLC traces).

General Assay Procedure. Cathepsins B, K, L, and S were purchased from Calbiochem (San Diego, CA). Cbz-Leu-Arg-AMC, Cbz-Phe-Arg-AMC, l-*trans*-epoxysuccinyl-Leu-4-guanidinobutylamide (E-64), and l-*trans*-epoxysuccinyl-Leu-3-methylbutylamide (E-64c) were purchased from Bachem (Torrance, CA). The proteolytic cleavage of *N*-acylaminocoumarins by cathepsin S was conducted in Dynatech Microfluor fluorescence 96-well microtiter plates, and readings were taken on a Molecular Devices Spectra Max Gemini XS instrument. The excitation wavelength was 370 nm and the emission wavelength was 455 nm with a cutoff of 435 nm for AMCA substrates; the excitation wavelength was 355 nm and the emission wavelength was 450 nm for peptidyl-AMC substrates. The assay buffer consisted of a 100 mM solution of pH 6.1 sodium phosphate buffer with 100 mM sodium chloride, 1 mM DTT, 1 mM EDTA, and 0.001% Tween-20.

Assay Procedure for AMCA Substrates. Assays were conducted at 37 °C in duplicate with and without the enzyme. In each well were placed 38 μL of enzyme solution and 2 μL of a DMSO substrate solution. Assays were performed at substrate concentrations that were at minimum 6 times less than the K_M for that substrate, and assays were performed in duplicate. Relative fluorescent units (RFUs) were measured at regular intervals over a period of time (maximum 15 min). A plot of RFUs versus time was made for each substrate with and without cathepsin S. The slope of the plotted line gave V_{max}/K_M of each substrate for cathepsin S. Using the RFUs per micromolar concentration for AMCA, substrate concentration, and enzyme concentration determined by E-64c titration for cathepsin S,[12] k_{cat}/K_M was determined.

Assay Procedure for Inhibitors. The dissociation constants (K_i) were determined from the IC_{50} values taken from plots of V_i/V_o versus inhibitor concentration, where V_o is the velocity in the absence of the inhibitor and V_i is the velocity with the inhibitor. Because the substrate concentration is significantly below K_M, the IC_{50} values were converted to K_i by the equation $K_i = IC_{50} - E_t/2$, where E_t = enzyme concentration.[13] The cathepsin B concentration in the assays was 2 nM, that of cathepsin K was 10 nM, that of cathepsin L was 0.025 nM, and that of cathepsin S was 0.7 nM. The following concentrations for the fluorogenic substrates were used: (Cbz-Phe-Arg-AMC) 2.5 μM for cathepsins B and K, 0.3 μM for cathepsin L; (Cbz-Leu-Arg-AMC) 2.5 μM for cathepsin S. Assays were conducted in duplicate with and without inhibitor at five inhibitor concentrations to provide 10−90% enzyme inhibition. In each well were placed 180 μL of enzyme solution and 10 μL of a DMSO inhibitor solution. The resulting solutions were incubated for 5 min at 37 °C, then 10 μL of the peptidyl-AMC substrate was added, and generation of AMC was monitored over 5 min.

Crystallization and Data Collection. Cathepsin S was expressed in the baculovirus system and its complex with inhibitor **2**, produced by adding **2** to the protein in a 2/1 molar ratio prior to concentration of the protein to 15 mg/mL. A coarse screen of 480 crystallization conditions was set up in low-profile 96-well Greiner plates at 4 °C in a sitting drop format containing 250 nL of protein combined

with an equal volume of crystallization solution. Small crystals (~20 μm) appeared after 1−2 weeks in a solution of 0.1 M sodium acetate, 20% Peg-8000, and 2.0 M $LiSO_4$. Data were collected at beamline 5.0.3 of the Advanced Light Source (ALS) in Berkeley, CA. The crystal form belongs to space group $P4_122$ and diffracted to 1.6 Å resolution (dataset statistics are presented in Table 1). This tetragonal crystal form was isomorphous with a previously determined crystal form.[9,3] The structure was then iteratively refined until convergence with Coot[14] and Refmac5;[15] all other crystallographic manipulations were carried out with the CCP4 program package.[16] The refined structure exhibited excellent geometry with no residues in disallowed regions of the Ramachandran plot (Table 1). Excellent agreement was observed between the conformations of inhibitor **2** in the two molecules of the asymmetric unit although the presence of an alternative conformation of Cys 25 of molecule B and less defined electron density of the compound suggest that inhibitor **2** was not at full occupancy within this monomer. The structure was deposited with PDB code 2OP3.

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Supporting Information Available: Purity data for substrates **8a**−**8f** and inhibitors **12a** and **12b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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