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Discovery of Potent and Specific Dihydroisoxazole Inhibitors of Human Transglutaminase 2

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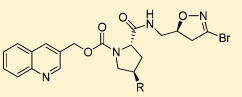
Supporting Information

ABSTRACT: Transglutaminase 2 (TG2) is a ubiquitously expressed enzyme that catalyzes the posttranslational modification of glutamine residues on protein or peptide substrates. A growing body of literature has implicated aberrantly regulated activity of TG2 in the pathogenesis of various human inflammatory, fibrotic, and other diseases. Taken together with the fact that TG2 knockout mice are developmentally and reproductively normal, there is growing interest in the potential use of TG2 inhibitors in the treatment of these conditions. Targeted-covalent inhibitors based on the weakly electrophilic 3-bromo-4,5-dihydroisoxazole (DHI) scaffold have been widely used to study TG2 biology and are well tolerated in vivo, but these compounds have only modest potency, and their selectivity toward other transglutaminase homologues is largely unknown. In the present work, we first profiled the selectivity of existing inhibitors against the most pertinent TG isoforms (TG1, TG3, and FXIIIa). Significant cross-reactivity of these small molecules with TG1 was observed. Structure−activity and −selectivity analyses led to the identification of modifications that improved potency and isoform selectivity. Preliminary pharmacokinetic analysis of the most promising analogues was also undertaken. Our new data provides a clear basis for the rational selection of dihydroisoxazole inhibitors as tools for in vivo biological investigation.



R	k_{inh}/K_i [mM^{-1} min^{-1}] TG1	TG2
phenyl	66.3	48.6
(p-OH)-phenyl	90.7	91.1
(p-OH)-benzamido	31.3	56.4
nicotinamido	20.0	108

INTRODUCTION

The mammalian transglutaminase (TG) family includes nine homologues, eight of which are catalytically competent (TG1−7 and Factor XIIIa), whereas one (band 4.2) is devoid of any known catalytic activity.[1] These enzymes catalyze posttranslational modifications of selected glutamine residues on target peptides or proteins, either through the attachment of small molecule or proteinogenic amines leading to the formation of isopeptide bonds or via hydrolysis resulting in a glutamine (Gln) to glutamic acid (Glu) conversion. Mechanistically, both reactions involve a thioester intermediate in which the substrate is attached to a Cys residue in the enzyme active site (Figure 1A).

The spectrum of biological functions of transglutaminases has been extensively reviewed elsewhere.[1−4] It should be noted that not all of these functions depend upon the ability of these enzymes to modify Gln residues; for example, TG2 is also a G protein.[5] In addition to transcriptional regulation, the activity of TG2 (as well as other mammalian transglutaminases) is also exquisitely regulated by various posttranslational cues, including Ca^{2+}, guanine nucleotides, and intramolecular thiol−disulfide interconversion.[6]

Aberrant transglutaminase activity, most notably in the case of the ubiquitously expressed TG2, has been implicated in the pathogenesis of various human diseases. The role of TG2 has arguably been best studied in celiac disease. In celiac disease, TG2 catalyzes the site-specific deamidation of gluten peptides, which dramatically increases their immunogenic potential in genetically susceptible individuals.[7] TG2 activity has also been implicated in the pathogenesis of Huntington's disease,[8,9] renal fibrosis,[10] and ischemic reperfusion injury.[11,12] Last but not least, studies in TG2 knockout ($TG2^{-/-}$) mice suggest a role for TG2 in lethality due to endotoxic shock.[13] Taken together with the fact that $TG2^{-/-}$ mice appear developmentally and reproductively normal,[14,15] TG2 is thought to be an attractive drug target.

A class of widely used TG inhibitors is based on the mildly electrophilic 3-bromo-4,5-dihydroisoxazole (DHI) moiety. Earlier studies by researchers at Syntex Corporation (Palo Alto, CA)[16,17] as well as our own laboratory[18,19] led to the discovery of (*S*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (ERW1041E, Figure 1.B), a moderately potent inhibitor of TG2. We have shown that ERW1041E (**1**) blocks the catalytic activity of TG2 in cell culture,[20] in polyinosinic−polycytidylic acid (poly(I:C)) induced intestinal injury in mice,[21] and in the hypoxia-induced model of murine pulmonary hypertension.[22] In the latter study, we also demonstrated that twice-daily, intraperitoneal dosing of this inhibitor at 50 mg/kg is well tolerated over the course of several weeks. These studies motivated us to develop an analogue with increased potency

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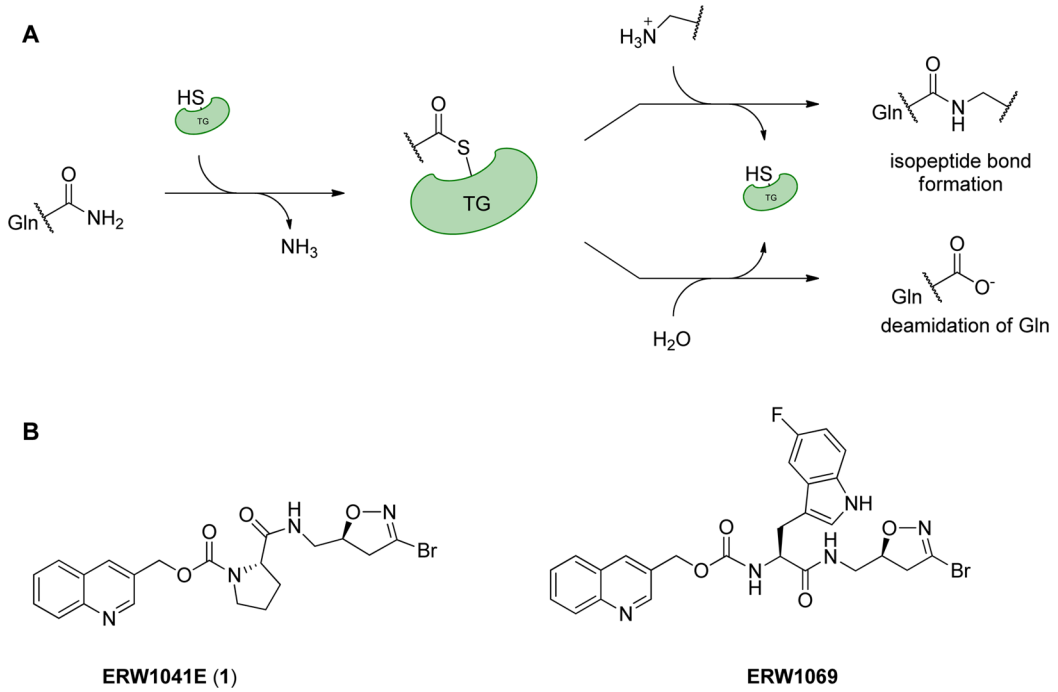

Figure 1. TG catalytic mechanism and structures of known TG2 inhibitors. (A) The active site cysteine of transglutaminases reacts with glutamine residues acyl donor substrates to form an acyl−enzyme intermediate that reacts with lysine side chains or small molecule amines to furnish an isopeptide bond.[1] If water is the acceptor nucleophile, the glutamine donor substrate is effectively hydrolyzed to glutamic acid. (B) Structures of previously published TG2 inhibitors (figure adapted from the literature[6]).

and selectivity. Increased potency would allow us to reduce the dose of the inhibitor, thereby diminishing the risk of off-target toxicity, whereas selectivity for TG2 over other human transglutaminases is important, as their inhibition could give rise to undesired off-target effects. In particular, cross-reactivity with either TG1, TG3, or Factor XIIIa would be most undesirable. The epidermal transglutaminase 1 functions in keratinocyte maturation and mutations in its gene give rise to skin barrier dysfunctions.[23] Plasma Factor XIIIa is activated as part of the blood clotting cascade and is responsible for cross-linking fibrin to form stable clots.[24] As such, loss-of-function mutations in the FXIIIa gene give rise to bleeding disorders in humans.[25] Recently, mutations in the gene encoding for TG3 have been associated with an increased risk of basal cell carcinoma.[26]

To date, little is known about the selectivity of the DHI-based TG2 inhibitors. Schaertl and co-workers profiled a single compound, quinolin-3-ylmethyl ((*S*)-1-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)amino)-3-(5-fluoro-1*H*-indol-3-yl)-1-oxopropan-2-yl)carbamate (ERW1069, Figure 1B) against other transglutaminases and suggested a cross-reactivity with TG1.[27] However, the IC_{50} values determined in this report cannot easily be quantitatively interpreted across different TG isoforms, given that the inhibitors are irreversible and the specificity of the substrate had not been characterized. The present study therefore sought to characterize the selectivity profile of existing DHI inhibitors and to systematically improve their selectivity toward TG2.

RESULTS

Expression and Purification of Transglutaminases. As a first step toward profiling our library of inhibitors against various transglutaminases, we prepared recombinant TG1, TG2, TG3, and Factor XIIIa. The expression of human TG2 from plasmid pJLP4 in *Escherichia coli* and its purification by a sequence of Ni-NTA affinity and anion exchange chromatography has been described previously and yields 2−3 mg of TG2 per liter of culture.[28]

To produce TG1 and TG3, we obtained commercial expression vectors encoding the full-length genes with N-terminal His_6 tags but were unable to obtain useful quantities of soluble protein from the corresponding strains of *E. coli*. We therefore further engineered these expression vectors. In the case of TG1, the N-terminal domain is reported to function as a membrane-anchoring scaffold through posttranslational modification in eukaryotic cells but is dispensable for catalytic activity.[29,30] We therefore prepared a series of N- and C-terminal truncation mutants. As predicted from previous observations,[29] a construct with the first 63 amino acids deleted and appended with an N-terminal His_6-tag (encoded by plasmid pCK16) yielded 1 mg of purified TG1 per liter of culture. The protein had constitutive catalytic activity and did not require posttranslational proteolytic activation.[29]

To produce TG3, the yield of soluble recombinant protein was improved by replacing the N- with a C-terminal His_6-tag in pCK2, resulting in 0.5−1 mg of purified TG3 per liter of culture. This form of TG3 is a zymogen and requires proteolytic cleavage in a loop region. Although previous studies had accomplished this using the bacterial protease dispase,[31] we sought to identify a more plausible physiological candidate for this proteolytic activation step. Cathepsin L has been implicated in this function.[32] We therefore expressed the human cathepsin L gene using plasmid pCK7 in *E. coli*. Cathepsin L was produced as inclusion bodies, denatured, and refolded as described previously.[33]

Initial attempts to produce Factor XIIIa (FXIIIa) using plasmid pGF13A2[34] did not yield sufficient protein for our studies. We therefore replaced the N-terminal glutathione S-

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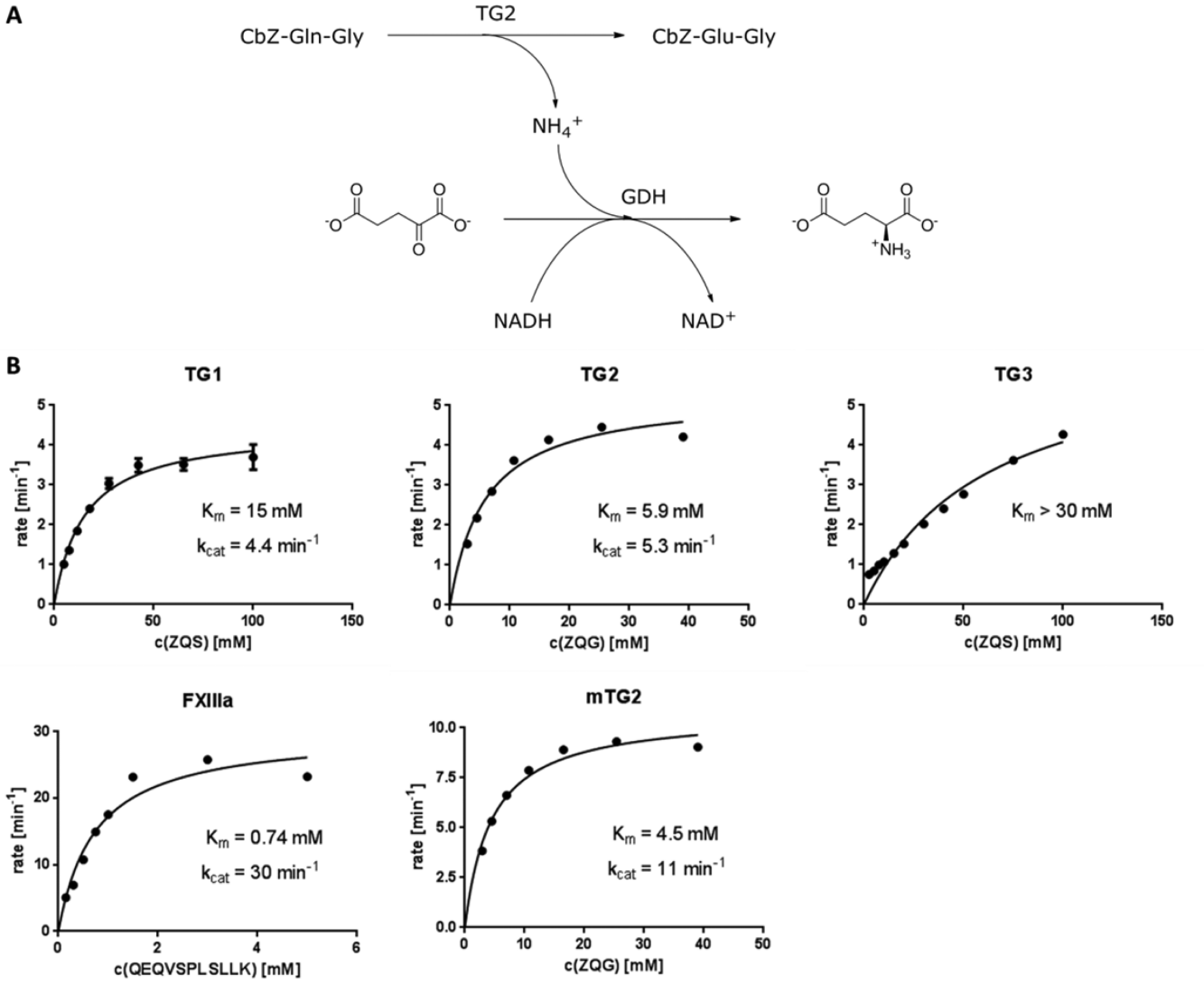

Figure 2. TG assay and substrate specificity of TG isoforms. (A) GDH-coupled deamidation assay for translutaminases, where the transglutaminase (e.g., TG2) reacts with a glutamine-donor substrate (e.g., the dipeptide Z-Q-G) and releases ammonia, which is incorporated into glutamate by the action of glutamate dehydrogenase. The consumption of NADH in the dehydrogenase-catalyzed reaction is followed spectrophotometrically at 340 nm. (B) Michaelis–Menten analyses and the kinetic parameters of the TG isoforms for their respective substrates.

transferase fusion partner with an N-terminal His_6-tag. The resulting plasmid pCK21 furnished improved quantities of recombinant FXIIIa that could be activated with commercial bovine thrombin.

To produce recombinant murine TG2 (mTG2), the gene was synthesized and inserted into an expression vector. Using the same protocol as for its human orthologue, mTG2 was purified in excellent yield.

In Vitro Transglutaminase Assays. With the transglutaminase enzymes at hand, we next implemented assays to assess their catalytic activity and to quantify the potency of candidate inhibitors. Previously, we and others have used the glutamate dehydrogenase (GDH) coupled assay for TG2.[28,35] In this assay, ammonia released by the action of TG2 on its glutamine donor substrate is incorporated into α-ketoglutarate by GDH, consuming one equivalent of NADH in the process and allowing spectrophotometric measurement of the reaction rate at 340 nm (Figure 2A). Typically, the protected dipeptide CbZ-Gln-Gly (ZQG) is used as a TG2 substrate in this setup. We found that this dipeptide was a comparably good substrate for the human and murine forms of TG2 (Figure 2B). For the other transglutaminases, a set of derivatives of this substrate (ZQ-X) were prepared, substituting glycine for other amino acids. Within this set, the serine-containing derivative exhibited the highest turnover at 10 mM for TG1 and TG3 (data not shown) and was therefore chosen as the substrate for these enzymes. FXIIIa did not measurably turn over any of the dipeptide substrates at concentrations up to 100 mM. We therefore synthesized and used the peptide, QEQVSPLSLLK, a derivative of α2-antiplasmin that is widely used in commercial diagnostic applications.[36]

Transglutaminase Specificity of Known DHI Inhibitors. We next assembled a library comprising approximately 60 TG2 inhibitors available in our laboratory from previous published and unpublished studies.[18−20,22] The entire library or a representative subset was profiled against TG1, TG3, FXIIIa, and mTG2. Each compound was assayed at four different inhibitor concentrations and two different substrate concentrations. By fitting these progress curves to exponential decay functions derived from an irreversible inhibition model, the

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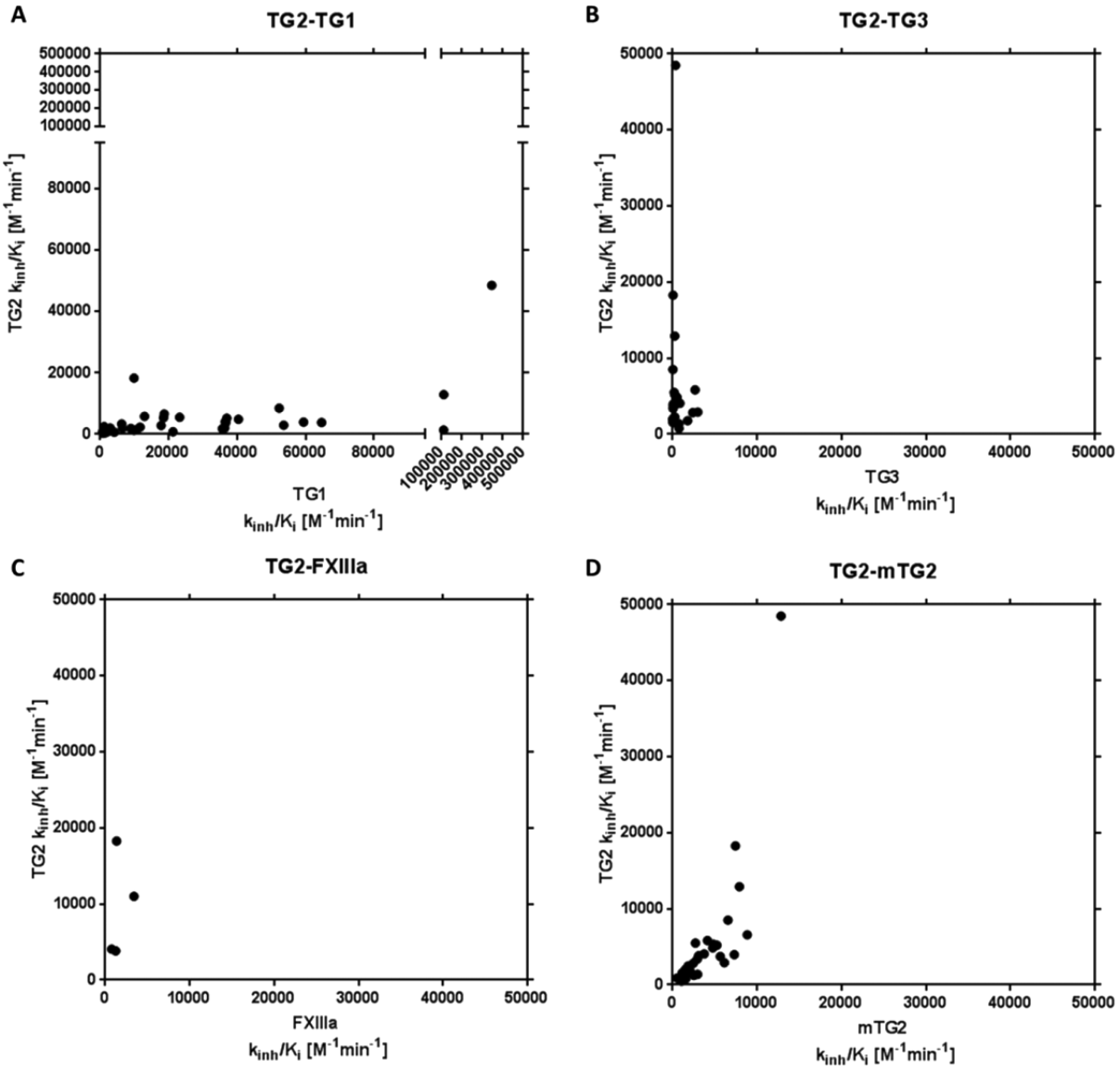

Figure 3. TG isoform specificity profile of our library of TG2 inhibitors. Profiling the specificity of DHI-type transglutaminase inhibitors against individual transglutaminase isoforms furnished inhibition parameter k_{inh}/K_i for a number of previously reported inhibitors. The panels depict pairwise plots of these inhibition parameters for TG2 versus TG1 (A), TG3 (B), FXIIIa (C), and mTG2 (D). Due to limited availability of active FXIIIa, only a subset of inhibitors was assayed against this enzyme.

inhibition parameter k_{inh}/K_i for each compound against each enzyme was estimated.[37,38] In general, the inhibitors were more reactive toward TG2 compared to either TG3 or FXIIIa, as illustrated in the pairwise plots of Figure 3B,C. As anticipated, they were also comparably reactive against the murine orthologue mTG2 (Figure 3D). In contrast, a large number of compounds in our library were also potent inhibitors of TG1. The most promising leads had comparable potency against TG1 and TG2 (Figure 3A).

More detailed analysis of the trends in the structure−activity and −specificity relationships within our library revealed that structures bearing aromatic amino acids or their derivatives were significantly more potent toward TG1 than TG2 and that the enzymes shared a preference for tryptophan derivatives over tyrosine or phenylalanine derivatives. Thus, optimization of the aromatic scaffolds was not deemed to be a productive strategy. In contrast, the proline containing inhibitor ERW1041E had approximately equal reactivity toward TG2 and TG1 even though it was a weaker inhibitor of human TG2 than the (5-fluoro)-tryptophan containing structures. We therefore chose ERW1041E as a suitable starting point for further optimization. Our working hypothesis was that conformational preorganization of the proline ring bestowed specificity, whereas an aromatic side chain increased potency. Accordingly, a suitable combination of the two features was predicted to yield improvements in potency and specificity toward TG2.

Design, Synthesis, and in Vitro Analysis of ERW1041E Analogues. Two synthetic routes have been reported thus far for the synthesis of DHI inhibitors; both were used in this study. Traditionally, DHI inhibitors were prepared by introducing the desired carbamate portion of the inhibitor as a nitrophenyl carbonate precursor (e.g., **S4a**) into an amino acid methyl ester **11**, followed by saponification to yield acid **12** and amide coupling of the DHI moiety, furnishing the final inhibitor **13** (route A in Scheme 1).[18,19] Recently, we reported a convenient multigram synthesis of ERW1041E, where the

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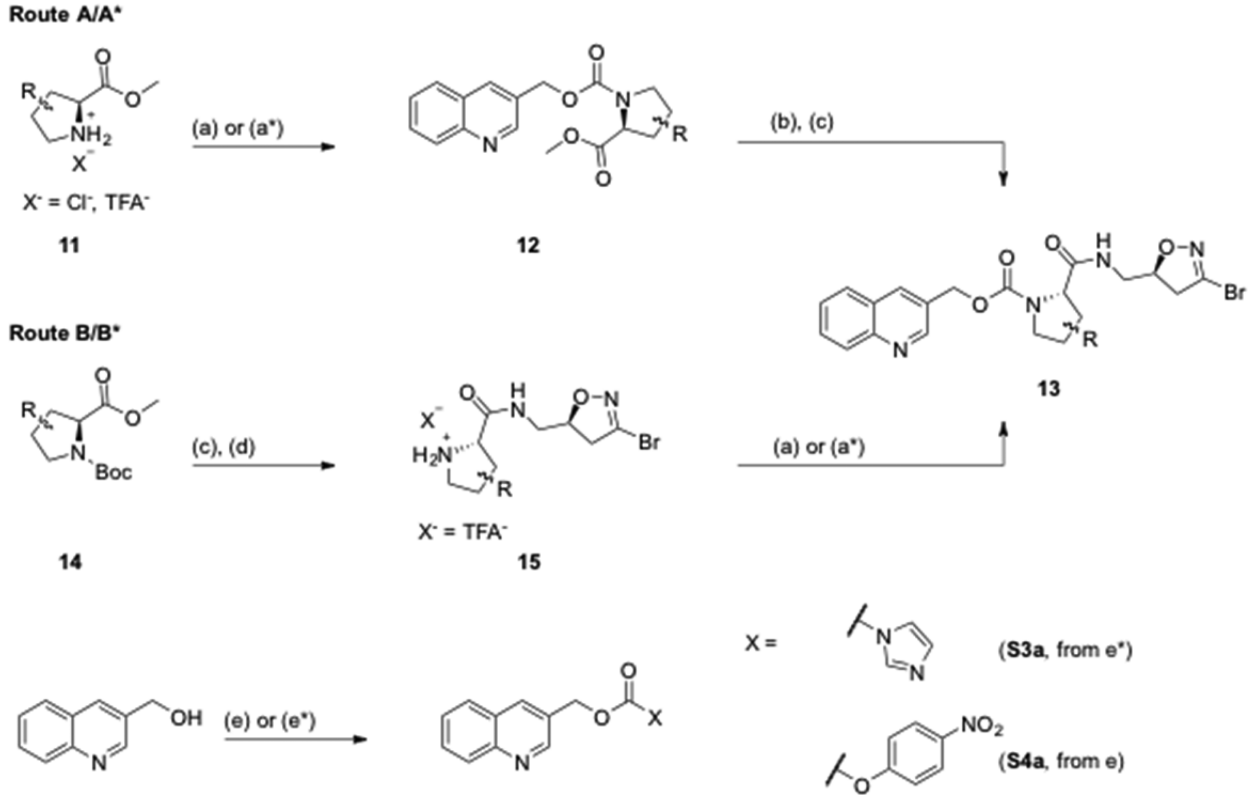
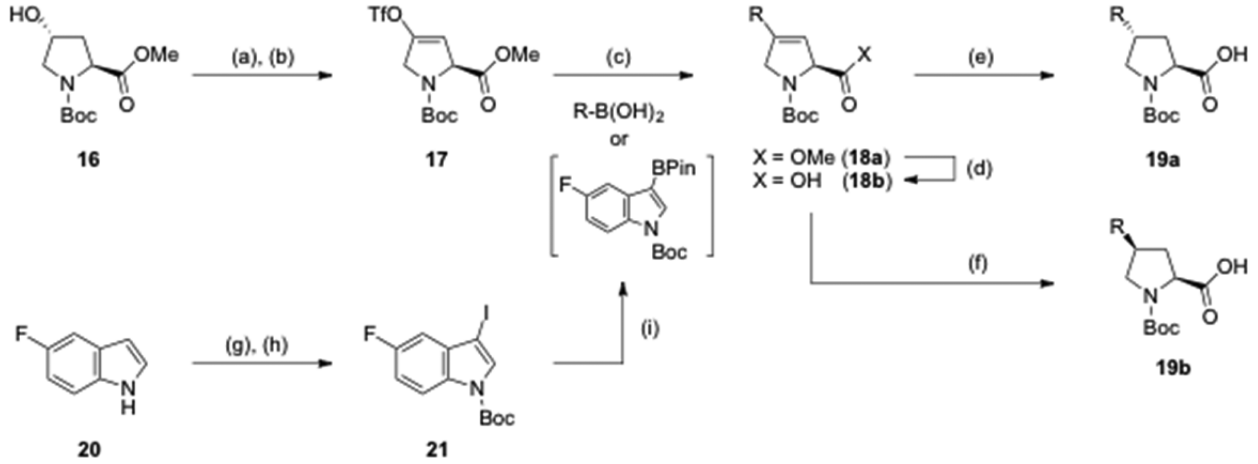
Scheme 1. Preparation of Inhibitors from Commercial or Synthesized Amino Acid Derivatives with C-Terminal Ester Protection (Route A/A*) or N-Terminal Boc-Protection (Route B/B*)[a]



[a]Routes with an asterisk (A*/B*) utilize imidazolyl carbamate building blocks, such as **S3a**, routes without (A/B) utilize the traditional nirophenyl carbonate building block such as **S4a**: (a) (**S4a**), NMM (3 equiv), DMF; (a*) (**S3a**), NEt_3 (1 equiv), DMAP (0.1 equiv), DMF/DCM 3:2, overnight RT; (b) aq LiOH, MeOH/THF; (c) EDCI, HOBt, NMM, (*S*)-DHI, DMF; (d) TFA; (e) *p*-nitrophenyl chloroformate, NMM, DCM; (e*) 1,1′-carbonyldiimidazole, ACN, RT. Figure adapted from the literature.[22].

Scheme 2. Preparation of 4-Aryl Substituted Proline Derivatives[a]



[a](a) $(COCl)_2$, DMSO, DCM, −78 °C, then NEt_3; (b) NaHDMS, Tf_2NPh, THF, −78 °C; (c) $Pd(PPh_3)_4$ (10 mol %), K_2CO_3, dioxane/water; (d) LiOH(aq), MeOH/THF; (e) $Rh(PPh_3)_3Cl$ (10 mol %), NEt_3, H_2, MeOH/THF; (f) Pd/C (10 wt %), NEt_3, MeOH; (g) I_2, KOH, DMF; (h) Boc_2O, DMAP, THF; (i) HBPin, $Pd(PPh_3)_4$ (10 mol %), NEt_3 (10 equiv).

carbamate precursor is an imidazolyl-carbamate **S3a** (route A* in Scheme 1).[22] To utilize the abundance of commercially available Boc-protected amino acids **14** without the need for onerous protecting group manipulations, we recently inverted this sequence, where the DHI moiety is first coupled to the amino acid, followed by deprotection in neat TFA, furnishing intermediate **15** and introduction of the carbamate with either of the previously used precursors **S3a** or **S4a** (routes B/B* in Scheme 1).[22]

Inhibitors bearing substituents at the 4-hydroxy or 4-amino functionality were generally derivatized at the amino acid stage and then carried through the sequence B to assemble the

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Table 1. Activity and Specificity of Proline Derivatives

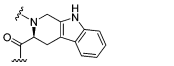
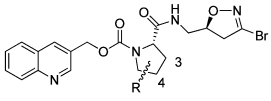
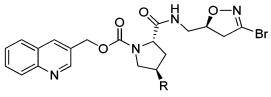
	#	n =	k_{inh}/K_i [mM^{-1} min^{-1}]	
			TG1	TG2
	**2**		26.3	9.1
	**3a**	1	13.6	14.4
	1	2	13.2	16.9
	3b	3	22.2	12.0
	**3c**		25.8	8.8
	**3d**		1.3	1.3

Table 2. Activity and Specificity of Substituted Prolines

	#	R =	k_{inh}/K_i [mM^{-1} min^{-1}]	
			TG1	TG2
	**1**	H	13.2	16.9
	4a	2-Me	2.4	2.0
	4b	4-F (cis)	6.0	13.4
	4c	4-F (trans)	6.7	7.3
	4d	4-NH_2 (trans)	3.1	2.6
	4e	4-OH (trans)	4.8	12.5
	**5a**	-OMe	7.7	10.4
	5b	-OPropargyl	7.1	10.1
	5c	-OBz	5.8	13.8
	6	-Bz	23.1	10.2
	7a	-Ph	66.3	48.6
	9a	-NHC(O)Ph	24.6	25.8

inhibitor. The 4-amino-proline functionality could also be carried through the synthesis as the Fmoc derivative and deprotected (and subsequently functionalized) after assembly of the inhibitor backbone, although Fmoc deprotection conditions were poorly compatible with the active DHI moiety, resulting in bromide displacement of approximately half of the material.

The 4-*trans* aryl substituted proline derivatives in this study were prepared following a literature procedure employing a Suzuki coupling reaction of a vinyl triflate **17** derived from suitably protected L-4-hydroxyproline **16** as the key step, furnishing an intermediate olefin **18** (Scheme 2).[39,40] While the *cis*-substituted L-proline **19b** derivative was readily obtained by heterogeneous catalytic hydrogenation, the literature precedent for preparation of the *trans*-diastereomer **19a** involves single electron transfer reduction in, e.g., Li/NH_3.[41] In contrast to this procedure, we decided to reduce the intermediate olefin by a homogeneous hydrogenation,[42] envisioning that the free carboxylate of the L-proline derivative could serve was a directing group when using Wilkinson's catalyst.[43] Gratifyingly, this procedure furnished the desired *trans*-substituted 4-aryl prolines in good yields and selectivity when the aryl group was electron rich. For electron poor derivatives, conversion was not complete, especially for the 2-chlorophenyl derivative (data not shown).

Structure–Activity Relationships. Using ERW1041E as the reference compound, we first determined the optimal ring size and relative orientation of the N- and C-terminal appendices (Table 1). Contracting the ring to an azetidine (**3a**) core mildly reduced TG2 activity and retained TG1 activity, whereas expansion to the pipecolic acid (**3b**) decreased TG2 activity and specificity significantly. On the five-membered proline scaffold, moving the carboxamide to the β-position (**3c**) also reduced TG2 while increasing TG1 reactivity. In contrast, homo-β-proline (**3d**) was poorly recognized by either enzyme. The results suggested that proline was the preferred cyclic moiety, both from an overall activity and specificity perspective. Next, we sought to explore substituents on this five-membered

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Table 3. Activity and Specificity of 4-Aryl Substituted Prolines

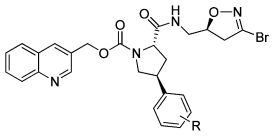
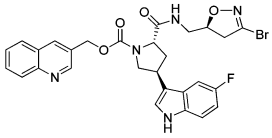
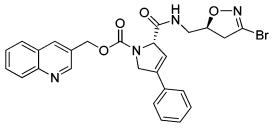
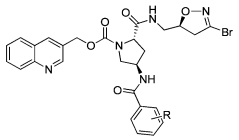
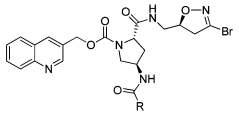
	#	R =	k_{inh}/K_i [mM^{-1} min^{-1}]	
			TG1	TG2
	**7a**	H	66.3	48.6
	7b	H (cis)	17.2	17.7
	7c	o-OH	29.5	33.8
	7d	m-OH	38.7	52.2
	7e	p-OH	90.7	91.1
	7f	m-Cl	41.7	25.3
	7g	p-Cl	15.4	12.0
	**7h**		48.0	18.4
	**8**		18.9	11.1

Table 4. Activity and Specificity of 4-Amido Substituted Prolines

	#	R =	k_{inh}/K_i [mM^{-1} min^{-1}]	
			TG1	TG2
	**9a**	H	24.6	25.8
	9b	o-OH	27.1	37.1
	9c	m-OH	18.1	36.2
	9d	p-OH	31.3	56.4
	**9e**	3-pyridyl	20.0	108
	9f	2-pyrazyl	12.0	15.7
	9g		11.6	14.8

ring (Table 2). It is known, for example, that 2-methyl and 4-fluoro substituents impose a conformational bias.[44,45] While the 2-methyl derivative (**4a**) was a poor inhibitor of either enzyme, 4-*cis* versus 4-*trans*-fluoro substitution (**4b**/**4c**) had a pronounced effect on the specificity, with the *cis*-diastereomer selectively destabilizing TG1 activity. Interestingly, when exploring a set of related inhibitors with hydroxyl groups at the 3- or 4-position (see Table S1 in the Supporting Information), we found the 4-*trans*-hydroxy-derivatives **S1d** or **4e** to possess the highest activity and specificity for TG2, possibly delineating opposing effects of ring conformation versus steric demand.

The 4-*trans*-hydroxy-group presented a functional handle for further elaboration of DHI inhibitors (Table 2). Derivatizing the hydroxyl-group into methyl (**5a**), propargyl (**5b**), and benzyl (**5c**) ethers was well tolerated but offered little to either specificity or potency. Having established that bulky aromatic substituents were tolerated, we moved the aromatic ring closer to the proline core. Surprisingly, whereas 4-benzyl-proline (**6**) significantly elevated TG1 reactivity with little effect on TG2 potency, 4-phenyl proline (**7a**) significantly increased TG2 activity albeit raising TG1 reactivity concomitantly. Introducing a 4-*trans*-amino functionality (**4e**) and thus a positive charge yielded a poor inhibitor itself but offered additional functionalization options. For example, the benzamido derivative **9a** had good TG2 potency, albeit again increased TG1 reactivity.

Guided by our hypothesis that both potency and specificity may be improved by combining conformational preorganization with aromatic side chains, we explored the 4-aryl and the 4-arylamido series further (Table 3). In the 4-aryl series, we first verified that 4-*trans* was indeed the preferred configuration. Both the 4-*cis* derivative **7b** as well as a planar olefin derivative **8** had diminished potency. We next introduced hydroxy (**7c**−**e**) and chloro substituents (**7f**/**g**) on the aromatic ring and found that the phenolic compounds were preferable, both with respect to potency and selectivity. Compound **7e** was particularly promising. Given that earlier studies had shown that tryptophan was the ideal aromatic amino acid and the 5-fluoro substituted derivative a particularly potent inhibitor,[19] we

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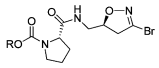
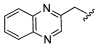
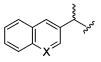
Table 5. Activity and Specificity of Carbamate Derivatives

	#	R=	k_{inh}/K_i [mM^{-1} min^{-1}]	
			TG1	TG2
	10a	tBu	2.3	2.5
	10b	propargyl	0.7	2.4
	10c	H	4.5	4.1
	10d	3-F	5.6	3.9
	**10e**	4-C≡CH	7.6	6.4
	10f	2,3-di(OMe)	1.7	poor
	10g	3-(OBz)	3.9	4.8
	10h	2-pyridyl	1.6	1.2
	10i	3-pyridyl	4.3	4.6
	**10j**	4-pyridyl	10.8	2.6
	1	3-quinolyl	13.2	16.9
	10k	4-quinolyl	12.0	2.0
	**10l**		15.4	1.6
	10m	X = CH, (S)	11.5	9.0
	**10n**	X = CH, (R)	5.1	2.9
	10o	X = N, (S)	8.7	3.7
	10p	X = H	poor	poor
	**10q**	X = Me	0.85	poor

also prepared the 3-(5-fluoro)-indolyl compound **7h**; however, this modification poorly translated from the open chain amino acid to the proline-derived series.

Starting from the 4-benzamido prolyl inhibitor **9a**, we investigated a hydroxyl-substituted series of analogues (**9b−d**, Table 4). An analogous trend to the 4-aryl series was observed where the *p*-hydroxy substituent was optimal, furnishing a potent TG2 inhibitor with modest specificity versus TG1. Moving from the parent phenyl ring to heteroaromatics, we saw a dramatic increase in potency and specificity in the nicotinamido-derivative **9e**. Adding a second nitrogen atom in the ring with the pyrazyl-derivative **9f** yielded a precipitous drop in potency. Given that the change from pyridine to pyrazine or phenyl is accompanied by a significant decrease or absence of hydrogen bond accepting capacity, we speculated that the 3-pyridyl group allows a new hydrogen bond to be established. This was consistent with the observation that an aspartate derivative in our initial library was fairly specific, albeit not potent (data not shown). We further hypothesized that the presence of Lys176 proximal to the active site in the open crystal structure of TG2 (PDB 2Q3Z),[46] but not in a homology model of TG1,[6] was the source of this hydrogen bonding capacity. However, when the negatively charged derivative **9g** was synthesized and evaluated, it was as potent as **9e**.

All new inhibitors presented in this study had the 3-methylquinolinyl carbamoyl substituent, which was determined to be the best within a limited set of variants in a previous study.[19] On the basis of our library screening efforts, we had anticipated that the contributions of the amino acid and carbamate portions were not independent (data not shown). Given that the carbamoyl substituent had previously been optimized on a tyrosine backbone,[19] we sought to explore the structure−activity relationship of this position in the context of the proline core.

A number of analogues were prepared by replacing the carbamate with an amide, but none of these compounds could increase the potency or specificity of the resulting inhibitors (see Table S2 in the Supporting Information). Thus, we explored variations of the parent carbamate motif (Table 5). Whereas propargyl carbamate (**10b**) was 3-fold selective for TG2 over TG1, it was a poor inhibitor. Other simple carbamates, such as *t*-butyl (**10a**) or benzyl carbamate (**10c**), furnished inhibitors with modest potency and no specificity advantage. Introducing substituents on the benzyl group, such as 3-fluoro (**10d**), the previously published "clickable" 4-ethynyl group (**10e**), or methoxy-groups (**10f**) also did not significantly improve these parameters. We next tested whether the flexible positioning of additional aromatic bulk would better fill the hydrophobic pocket observed in the crystal structure,[46] but compound **10g** was a poor inhibitor of TG2. Replacing benzyl by pyridyl revealed a preference of TG2 for 3-pyridyl over the isomers with a 2- or 4- substitution (**10h−j**), but

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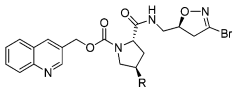
Table 6. Activity and Specificity of Promising Lead Compounds



#	R=	k_{inh}/K_i [mM^{-1} min^{-1}] TG1	TG2	TG3	FXIIa	mTG2
1	H	13.2	16.9	< 1.0	< 0.5	8.3
4b	F (cis)	6.0	13.4	< 1.0	0.43	7.0
4e	OH	4.8	12.5	< 1.0	< 0.5	11.1
5c	OBz	5.8	13.8	< 1.0	< 0.5	15.5
7a	Ph	66.3	48.6	< 1.0	1.5	25.4
7e	(p-OH)-Ph	90.7	91.1	< 1.0	1.4	38.7
9d	(p-OH)-benzamido	31.3	56.4	< 1.0	1.3	9.4
9e	nicotinamido	20.0	108	< 1.0	0.68	10.6

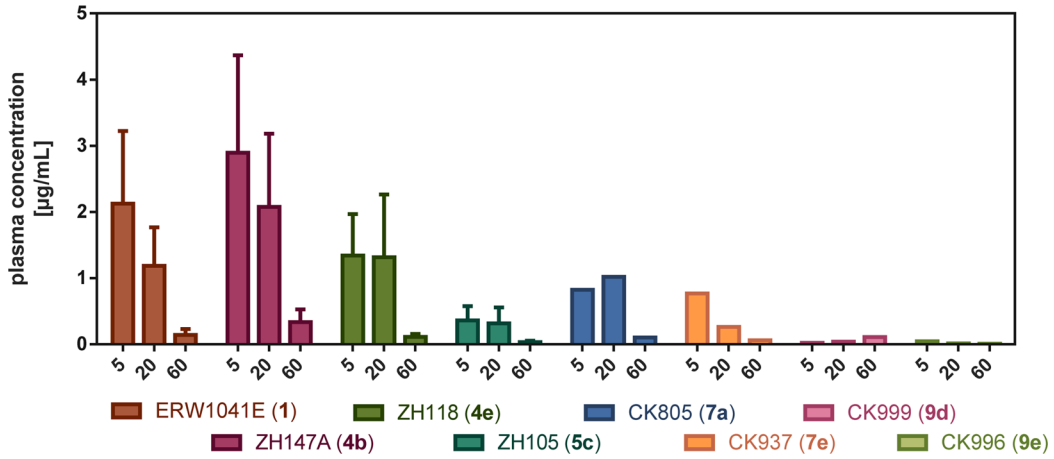


Figure 4. Plasma concentrations of TG inhibitors after oral dosing. Following oral administration of sets of TG inhibitors to mice at 50 mg/kg, blood was sampled at 5, 20, and 60 min time points. The concentration of each compound in plasma was quantified, and the resulting profiles plotted as time versus concentration curves, as depicted. Whereas some inhibitors, such as CK996 and CK999, have negligible systemic availability, others, such as ERW1041E and ZH147A, reach peak concentrations of 2−3 μg/mL.

neither potency nor selectivity was improved. As observed previously, moving to 3-quinolyl increased the potency for TG2 about 4-fold, whereas 4-quinolyl (**10k**) dramatically disfavored binding to TG2 but not TG1. Introducing an additional nitrogen atom in the heteroaromatic system in the form of a 2-quinoxalyl (**10l**) led to a precipitous drop in activity against TG2 and an inhibitor that was 10-fold more selective for TG1. This observation led us to explore the hypothesis that a hydrogen bond to the quinoline nitrogen was important for binding to TG2 but not TG1. Accordingly, we replaced quinoline with the much stronger hydrogen bond accepting 2-benzimidazole (**10p**) or *N*-methyl-2-benzimidazole (**10q**), but these changes abrogated both TG1 and TG2 inhibition. Finally, we introduced methyl substituents on the carbamate methylene with the goal of increasing conformational preorganization. On the naphthyl backbone, the *S* isomer **10m** was preferred by both TG1 and TG2 over its *R* diastereomer **10n**; however, this modification did not yield a tangible benefit when translated to the quinolyl moiety (**10o**).

In summary, we determined that significant changes in potency and specificity can be achieved by varying the (hetero)aromatic group in the carbamate moiety, but that none of these were productive toward the goal of this study.

Selectivity and Pharmacokinetic Profiling of the Most Promising Inhibitors. To identify compounds that could be useful for in vivo biological studies, we conducted further selectivity and pharmacokinetic analysis on the most promising compounds from this study. Specifically, a set of eight compounds was selected based on their potency against TG2, selectivity for TG2 over TG1, and chemical diversity. First, their selectivity against a broader range of transglutaminase isozymes was profiled (Table 6). From this analysis, inhibitor **9e** (also designated as CK996) emerged as the most potent and selective inhibitor of human TG2, although compounds **7a** (also designated as CK805) and **7e** (also designated as CK937) may arguably be more effective in mouse studies.

From prior experience with this class of compounds, we anticipated short half-lives ($t_{1/2}$ < 15 min in mice) for all of our selected compounds. We therefore sought to estimate in a resource efficient manner which of these compounds had appreciable oral bioavailability. To do so, we pooled the compounds in two groups of four each, where individual members would not overlap in their masses and adducts in mass spectrometry. We then administered the compound mixtures to mice in a dose of 50 mg/kg for each constituent member by the oral route.

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All compounds were rapidly cleared from circulation (Figure 4). Relative to their plasma concentrations at 20 min, the equivalent values at 60 min were at least 4-fold lower, verifying that their half-lives were shorter than 20 min. No compound appeared to possess a considerably longer half-life than the others; as such, this parameter did not provide any basis for the selection of a specific lead compound. Knowing that this class of compounds achieves high peak concentrations in plasma after intraperitoneal administration (ca. 20−40 μg/mL, data not shown), we sought to establish if any compound could achieve at least 10% of this value range after oral dosing. Compound **4b** (aka ZH147A), harboring the 4-*cis* fluoro moiety, was most promising in this regard, with plasma concentrations of 2.9 ± 1.5 and 2.1 ± 1.1 μg/mL at 5 and 20 min, respectively. The unsubstituted parent ERW1041E (**1**) also had reasonable oral bioavailability, achieving plasma concentrations of 2.1 ± 1.1 and 1.2 ± 0.6 μg/mL at 5 and 20 min, respectively. The plasma levels of the more potent derivatives with a 4-aryl moiety (**7a**/**e**) (aka CK805/CK937) were 3−4-fold lower than that of **4b**, whereas the 4-arylamido derivatives (**9d**/**e**) were much less bioavailable, suggesting that these compounds are either inherently impermeable or that they undergo more rapid presystemic metabolism (possibly even in the intestinal lumen).

In summary, on the basis of all of the above measurements, we propose three improved analogues of ERW1041E for consideration in advanced tissue culture or animal models of diseases where TG2 is believed to play a pathogenic role. Compound **9e** (aka CK996) is the most potent and specific inhibitor of human TG2 and is therefore ideally suited for studies in which isoform selectivity is of paramount importance. Inhibitor **4b** (aka ZH147A) has lower potency but superior oral bioavailability and is arguably most appropriate for "topical" applications such as celiac disease, where TG2-mediated inflammation is thought to be localized to the small intestine and its associated mesenteric lymph nodes.[47] Inhibitor **7a** (aka CK805) combines all of these properties, albeit at an intermediate level, and is therefore also worthy of further consideration.

DISCUSSION AND CONCLUSIONS

The structure−activity relationships of DHI inhibitors of mammalian transglutaminases have been the subject of four reports over the past 25 years.[17−20] Prior to this study, these efforts had led to the discovery of benzyl ((*S*)-1-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)carbamate KCC009, a prototypical nonspecific inhibitor, and ERW1041E, an inhibitor with moderately higher potency. Both compounds have found widespread utility as tools to study the physiological role of TG2 and have aided in defining its role in the pathogenesis of various human diseases.[22,48−51] Our own studies with these tool compounds in animals have highlighted the excellent tolerability of small molecules harboring this weakly electrophilic glutamine isostere and have motivated the development of medicinally useful lead compounds described in this report.

In defining the characteristics of a sufficiently improved compound relative to ERW1041E, we identified potency, selectivity, and pharmacokinetic profile as aspects that warranted attention. The potency of ERW1041E against human TG2 is moderate (k_{inh}/K_i = 17 mM^{-1} min^{-1}). It has comparable activity against human TG1 (k_{inh}/K_i = 13 mM^{-1} min^{-1}). Although it has been successfully used in an intraperitoneal form in studies on a rodent model of pulmonary hypertension,[22] its dose−response characteristics were not explored nor have its pharmacokinetic properties been characterized.

As a first step, we sought to profile the potency and specificity of each member within our existing compound library of DHI inhibitors. It rapidly became apparent that, while the DHI inhibitors had little cross-reactivity against TG3 and FXIIIa, they had strong reactivity toward TG1. This observation might have been anticipated, given that the DHI-based inhibitors were initially developed as TG1 inhibitors.[16,17] However, we were surprised by the extent of cross-reactivity and speculate that it is due to an inherently higher reactivity of the TG1 active site. SAR analysis revealed that aromatic amino acids had high specificity for TG1, whereas the proline-containing inhibitor ERW1041E had the most desirable isoform specificity, being approximately equipotent between TG1 and TG2. This improved specificity, however, was accompanied by a 3−4-fold decrease in potency from ERW1069 to ERW1041E.

We therefore systematically explored cyclic systems, hypothesizing that it may be possible to combine the specificity of a cyclic or conformationally preorganized inhibitor with the gain in potency of inhibitors with aromatic moieties. Proline was the optimal cyclic system, with substitutions at the 4-position being most beneficial for selectivity and potency. Interestingly, the preferred stereochemistry for a fluoro-substituent was *cis*, whereas hydroxy- and larger moieties needed to be attached in the *trans*-orientation, possibly delineating opposing conformational and steric effects. In fact, in the 4-*trans*-orientation, relatively large hydrophobic substituents were tolerated when attached rigidly off the proline ring, either directly or via an amide-bond linkage. Some of these modifications dramatically increased the potency of our inhibitors against TG2. Exploring the ideal nature of the aromatic group, we found that *p*-hydroxy-substitutions were beneficial and, in the arylamide series, attaching nicotinamide increased the selectivity for human TG2 over TG1.

With a number of improved DHI inhibitors at hand, we also sought to profile the pharmacokinetic properties of a representative set of compounds. Whereas all compounds had comparable and short systemic half-lives on the order of 10−15 min, there were marked differences in their oral bioavailability. For reasons outlined above, three compounds, CK996, ZH147A, and CK805, are recommended for further biological evaluation as improvements over the most widely used reference compound of this series, ERW1041E. Their pharmacokinetic profile suggests that one potential area for further biological evaluation is celiac disease. In celiac disease, the pathogenic TG2 activity resides in the gut. A topical agent with low and short systemic exposure might be an appropriate lead for this indication.

In conclusion, we also note that the compounds in this study contain the mildly electrophilic DHI moiety that covalently inactivates TG2. The use of an irreversible inhibition strategy and the presence of even mild electrophiles in medicinal leads has been subject to some controversy due to concerns regarding nonspecific reactivity, haptenization of self-proteins, and the resulting toxicity of these chemotypes.[52,53] In recent years, however, covalent inhibitors have received renewed interest in the form of "targeted covalent inhibition" (TCI).[54] A number of drugs exploiting this strategy have been approved or are currently undergoing clinical trials (such as Afatinib or Neratinib).[55] At the heart of a TCI-based strategy lies a mildly electrophilic functional group that is incorporated into an

inhibitor scaffold, which itself has affinity to a specific binding site and drives the covalent modification of a specific nucleophile in this site through proximity and suitable positioning of the reactive partners rather than blunt reactivity. A widely used reactive moiety is acrylamide.[54] We propose that the 3-bromo-4,5-dihydroisoxazole (DHI) moiety can be a valuable functional group in targeted covalent inhibitors for transglutaminases. The warhead is stable in the presence of millimolar concentrations of glutathione at physiological or above physiological pH and is thus not broadly thiol-reactive.[18] A detailed analysis of the inhibition kinetics also reveals that the rate constant for covalent inactivation k_{inh} is frequently less than 0.1 min^{-1}, underscoring the importance of noncovalent molecular recognition in enzyme inhibition. This is further validated by our observation that lead compounds can be engineered to quite high specificity against closely related proteins, including TG3 and FXIIIa. Perhaps most pertinently, employing a TCI strategy for TG2 appears particularly attractive in light of our current understanding of the pharmacodynamics of the target. In a murine model of hypoxia-induced pulmonary hypertension, we observed that the inhibitory half-life of ERW1041E was about 12 h[22] and such slow (re)activation kinetics seem very amenable to covalent inhibition, where a short period of an inhibitor present drives efficacy for many hours and might thus translate to fewer off-target effects of any chemical matter administered.

EXPERIMENTAL SECTION

Protein Expression. *General Protein Expression Protocol.* All transglutaminases used in this study were expressed in *E. coli*, using standard conditions, similar to the previously published protocol for transglutaminase 2.[28] Briefly, the respective expression vector was inserted into *E. coli* Rosetta 2 pLysS strain by electroporation. Cell stocks were prepared by picking individual colonies, growing them in liquid LB Miller broth with 33 mg/L chloramphenicol and 50 mg/L kanamycin at 37 °C overnight, and freezing aliquots with 20% glycerol at −80 °C until use. Starter cultures of the same LB broth were inoculated with the cell stocks, grown to high cell density at 37 °C overnight, and used to inoculate production cultures in 2 L baffled shake flasks. The production cultures were grown until an OD_{600} of 0.5−0.6 was reached and then transferred to refrigerated shakers at 18 °C. Protein expression from the T7 promoter was induced by the addition of 200 μM IPTG, and shaking continued overnight (usually 12−18 h). Cells were then harvested by centrifugation at 4420*g* at 4 °C and the pellet was either directly used in the extraction and purification steps (vide infra) or flash frozen in liquid nitrogen and stored at −80 °C until workup.

All purification steps were carried out on ice or in a temperature-controlled room at 4 °C. The pellets were suspended in lysis buffer (50 mM phosphate buffer at pH 7.6 with 300 mM sodium chloride, 20 mM imidazole and 20% glycerol), typically using about 25 mL of buffer per liter of production culture. Cells were lysed by sonication on ice (5−8 cycles, each 40−60 s long). The lysate was cleared by centrifugation at 25,000*g* for 45−60 min at 4 °C and then an initial purification carried out by affinity chromatography. The cleared lysate was thus incubated with Ni-NTA resin for 30−40 min and the resin filtered and washed with at least 10 column volumes of lysis buffer. The immobilized proteins were then eluted from the Ni-NTA resin using 20−25 mL of elution buffer (50 mM phosphate buffer at pH 7.6 with 100 mM sodium chloride, 150 mM imidazole, and 20% glycerol). The eluate was diluted to 50 mL with water and then applied to an anion exchange column (HiTrap Q) on an FPLC system. The transglutaminases were eluted with a gradient of sodium chloride in 20 mM Tris buffer at pH 7.8 with 1 mM EDTA and 1 mM DTT. The elution was monitored by UV absorbance, fractions of interest were analyzed by SDS-PAGE, and those containing the desired protein pooled, concentrated, or buffer exchanged on Centricon centrifugal filtration devices.

Human Transglutaminase 1 (TG1). A complete expression vector with codon-optimized human transglutaminase 1 (NM_000359) with an N-terminal His_6-tag in the pQE-T7 expression vector was purchased from Qiagen. A truncated form of this gene, encoding for amino acids 63−817 appended with an N-terminal His_6-tag (MHHHHHHGSG−) was PCR amplified using the primers AAAAAACATATGCACCATCACCATCACCATGGTAGTGG-TCCGGAACCGAGCGATAGCCG and GTGGTGCTCGAGTCT-TACTA. The amplimer was restriction digested with NdeI and XhoI and ligated into the pQE-T7 vector to obtain expression plasmid pCK16. This plasmid was introduced into the *E. coli* Rosetta 2 pLysS strain, and TG1 protein was expressed and purified as described in the general procedure. TG1 eluted from the anion-exchange column at about 230 mM sodium chloride in a yield of about 1 mg per liter of initial culture. The purified protein was concentrated to 4−6 mg/mL in 20 mM Tris buffer at pH 7.8 with 150 mM sodium chloride, 1 mM EDTA, 1 mM DTT, and 20% glycerol and stored at −80 °C.

Human Transglutaminase 2 (TG2). Human transglutaminase 2 was expressed from plasmid pJLP4,[28] which encodes for the V224G variant,[56] in the *E. coli* Rosetta 2 strain and purified using the general procedure outlined above. Purified TG2 was concentrated to 4−5 mg/mL in 20 mM Tris buffer at pH 7.8 with 150 mM sodium chloride, 1 mM EDTA, 1 mM DTT, and 20% glycerol and stored at −80 °C.

Human Transglutaminase 3 (TG3). A complete expression vector with codon-optimized human transglutaminase 3 (NM_003245) with an N-terminal His_6-tag in the pQE-T7 expression vector was purchased from Qiagen Inc. To obtain the C-terminally His_6-tagged (−LEHHHHHH) variant, TGM3 was PCR amplified using primers AAAAAACATATGGCAGCACTGGGTGTTCAGAG and TTTTTTCTCGAGTTCT-GCAACATCAATGCTCA. The amplimer was then inserted between the NdeI and the XhoI sites of the parent pQE-T7 vector, affording plasmid pCK2, and correct ligation was confirmed by sequencing from the T7 promoter and the T7 terminator sites. pCK2 was introduced into the *E. coli* Rosetta 2 pLysS strain as above and TG3 expressed and purified using the general procedure, furnishing about 0.6−1.0 mg per liter of production culture. Because TG3 eluted from the anion exchange column at relatively low ionic strength (approximately 160 mM sodium chloride), the Ni-NTA elution buffer contained no added sodium chloride and only a small volume (approximately 15 mL) of buffer was used to allow for a greater dilution with water prior to loading the FPLC system. The purified protein could be concentrated to approximately 4 mg/mL, but 20% glycerol had to be added prior to this step to avoid precipitation at concentrations greater than 1 mg/mL.

Human Factor XIIIa (FXIIIa). The pGF13A2 plasmid, harboring full length human FXIIIa as an N-terminal GST fusion, originally constructed and published by Greenberg and co-workers[34] was obtained as a gift from Dr. Jeffrey Keillor (University of Ottawa). To generate an N-terminal His_6-tagged (MGHHHHHHGSG−) version of FXIIIa, the gene was PCR amplified from the pGF13A2 vector using primers AAAAAACCATGGGTCATCACCATCAC-CATCACGGTAGTGGTATGGCAGAAACTTCCAGGAC and TTTTTTCTCGAGTCACATGGAAGGTCGTCTTT, the amplimer was restriction digested and finally ligated between the NcoI and XhoI sites of the pET28a(+) vector, furnishing the plasmid pCK21. Correct insertion was confirmed by sequencing the plasmid was then introduced into the *E. coli* Rosetta 2 pLysS strain. Deviating from the general procedure, production cultures of this strain were grown in Terrific Broth. Cells were induced, harvested, and lysed as described above, and the protein purification followed the general procedure. However, it was found crucial that all buffers including the solvents used in the FPLC purification contain 20% glycerol to aid protein stability.[34] Similar to TG3, the elution buffer from the Ni-NTA resin contained no added sodium chloride. During anion-exchange chromatography, Factor XIIIa eluted at approximately 150 mM sodium chloride in about 1 mg overall yield per liter of production

culture. The purified protein was concentrated to 2 mg/mL and stored at −80 °C.

Murine Transglutaminase 2 (mTG2). An expression vector harboring codon-optimized murine TG2 (NM_009373) with a double N-terminal His_6-tag (MGSSHHHHHHSSGLVPRGSHMG-HHHHHHLVPRGS−) was obtained as a gift from Dr. Bana Jabri (University of Chicago). The plasmid was introduced into the *E. coli* Rosetta 2 pLysS strain as described above. Murine TG2 was expressed from this strain and purified using the conditions of human TG2 furnishing approximately 10 mg per liter of production culture.

Human Cathepsin L. A cDNA clone containing full length human procathepsin L (NM_001912) was obtained from Open Biosystems. The gene was amplified via PCR using primers AAAAAAGC-TAGCGCCATGGGCACTCTAACATTTGATCACAG and TTTTTTCTCGAGTCACACAGTGGGGTAGCTGG, restriction digested, and ligated between the NcoI and XhoI sites of the pET28a(+) vector, furnishing plasmid pCK7.

Because it was known that recombinant procathepsin L is expressed as inclusion bodies, we used the following protocol to obtain the purified active enzymes. When the production culture of *E. coli* Rosetta 2 pLysS had reached OD_{600} = 0.6, we induced expression from the T7 promoter with 1 mM IPTG and kept the cells shaking at 37 °C for another 5 h. The cells from 2.4 L of culture were harvested by centrifugation and disrupted by sonication in 50 mL of lysis buffer (100 mM Tris, pH 8.0, 1 mM EDTA) and the lysate cleared by centrifugation as described. The supernatant from this step was discarded, and the pellet with the insoluble inclusion bodies was resuspended in 36 mL of lysis buffer with 1% Triton X-100 followed by centrifugation and removal of the supernatant. After a second cycle of washing, 1.2 g of washed inclusion bodies were obtained from 2.4 L of production culture.

Procathepsin L was solubilized and refolded following a literature procedure.[33,57] Briefly, the inclusion bodies were dissolved to 100 mg/mL in 50 mM sodium acetate buffer at pH 4.5 with 6 M guanidinium hydrochloride, 1 mM EDTA, and 100 mM DTT, and the solution was stored at −80 °C until use. For a small scale refolding setup, 100 μL of denatured procathepsin L was slowly added to a 10 mL aliquot of refolding buffer (192 mM Tris at pH 8.2 with 7.4 mM oxidized glutathione, 8.35 mM reduced glutathione, 400 mM $MgCl_2$, and 0.07% Brij-35) with vigorous stirring. After 3 h, the cloudy mixture was clarified by passing through a 0.2 μM syringe filter, and aqueous 1 M citric acid to a pH of 3−4 was added to activate the proenzyme. After 30 min, 20% glycerol was added. The refolded and activated cathepsin L was concentrated in a Centricon centrifugal filter to 1 mg/mL and stored in aliquots at −20 °C.

In Vitro Activity Assays. *TG Assays.* The activity of transglutaminases was measured via the glutamate dehydrogenase (GDH) coupled assay[28,35] using an assay buffer of 200 mM MOPS, pH 7.2, 5 mM $CaCl_2$, 10 mM α-ketoglutarate, with 300 μM NADH, 36 U/mL bovine GDH, and the respective substrates (ZQG for TG2 and mTG2, ZQS for TG1 and TG3, and QEQVSPLSLLK for Factor XIIIa). TG1, TG2, and murine TG2 were directly diluted from the enzyme stock to typical final concentrations of 0.37, 0.34, and 0.22 μM, respectively. For TG3, an aliquot of the enzyme was diluted with 1.5 volumes of 50 mM MES buffer at pH 6.0 and an appropriate volume of cathepsin L (1 μg per 25 μg of TG3) added. The cleavage reaction was allowed to proceed for 30 min, whereafter 50 μM of the irreversible cathepsin L inhibitor E64 was added (1:200 from 10 mM DMSO stock) and allowed to react for 10 min before the activation mixture was diluted into the assay mixture to a typical TG3 concentration of 0.43 μM. For Factor XIIIa, commercial bovine thrombin was dissolved to 1 U/μL in 1× thrombin dilution/storage buffer from a commercial thrombin cleavage kit (Novagen, specified as 50 mM citrate buffer at pH 6.5, 200 mM sodium chloride, 1 mg/mL PEG-8000, and 50% glycerol). To an aliquot of FXIIIa was added an appropriate volume of 10× cleavage buffer from the same kit (specified as 200 mM Tris buffer at pH 8.4, 1.5 M sodium chloride, 25 mM $CaCl_2$), followed by thrombin (1 U per 80 μg). The cleavage was allowed to proceed for 30 min at room temperature, and the enzyme mixture was diluted into the assay mixture to about 0.092 μM. Transglutaminase inhibitors were dissolved in DMSO, and the final DMSO content was typically 1% (v/v) in the final assay buffer. To allow for accurate dispensing, inhibitors were usually prediluted in a larger volume of assay buffer. Whenever possible, large volumes of premixes were used to decrease assay variability. In assay formats where full progress curves of irreversible inhibitors were to be recorded, the enzymes were allowed to equilibrate and turn over in the final assay buffer for at least 5 min (30 min for TG3) before any inhibitors were added. The progress of the enzymatic reaction was followed colorimetrically at 340 nm in either a quartz cuvette in a UV spectrophotometer or in UV compatible half area microplates in an absorbance plate reader. In the latter, the data was corrected for the volume-dependent path length (typically d = 150 μL/170 μL). To determine the substrate specificity, the assay was conducted with variable concentrations of substrate and the data fit to the Michaelis−Menten equation (Figure 2). For irreversible inhibitors, individual progress curves were fit to exponential decay functions (and corrected for background nonlinearity as for FXIIIa) and the parameters k_{inh}/K_i obtained from an irreversible inhibition model, as described before.[18,28] R^2 values were typically higher than 0.9 (and almost invariably >0.8) for individual fits. To assess the reproducibility of these measurements, the k_{inh}/K_i values for a number of inhibitors (including ERW1041E, CK996, ZH147A, and CK805) were measured via multiple independent experiments. In all such cases, interexperimental variation below 25% was observed.

Library Profiling. To assemble the library of existing TG2 inhibitors, we freshly dissolved solid compound stock (where available) in DMSO to a 10 mM concentration and stored aliquots at −20 °C until use. To obtain enough data points for fitting to an irreversible inhibition model, we assayed each inhibitor at four different concentrations (typically 100/33.3/11.1/3.7 μM) in the presence of two different substrate concentrations (typically 10/20 mM ZQG for TG2 and mTG2, 20/40 mM ZQS for TG1 and TG3, and 0.5/1.5 mM QEQVSPLSLLK for FXIIIa).

For a medium throughput assay method, we plated the compounds in 384-well plates and prepared a 4-point 1:2 dilution series in DMSO as a 100× concentrated stock. We next plated 245 μL of 1× assay buffer into four rows of a 96-well plate and, shortly before commencing the assay, added 7.5 μL of each DMSO dilution stock to obtain enough 3× concentrated inhibitor−master mix for two 96-well plates to be run simultaneously. We then prepared 6 mL of a 1.5× concentrated enzyme−substrate master mix for each substrate concentration and let the mixture equilibrate for 5−10 min (30 min for TG3). To each well in a UV-compatible 96-well plate, we added 50 μL of an inhibitor−master mix and started the assay by adding 100 μL of the pre-equilibrated enzyme−master mix and immediately began data acquisition at 340 nm in an absorbance plate reader (3 reads/min with 3 s shaking in between reads). The full progress curves were analyzed and inhibition parameters computed as described above, using an automation routine implemented in STATA.

In Vivo Pharmacokinetics. *Formulation.* To rapidly profile the pharmacokinetic properties of the novel TG2 inhibitors from this study, eight inhibitors were pooled into two dosing groups, A (compounds **4e**, **5c**, **7a**, and **9d**) and B (compounds **1**, **4b**, **7e**, and **9e**), containing identical amounts of each inhibitor (by mass). For oral delivery, the inhibitors were formulated by dissolving the mixtures to 25 mg/mL (concentration of each compound) in 1 M hydrochloric acid with 10% Tween-80 and 25% absolute ethanol under vortexing and sonication. This solution was rapidly diluted 5-fold into a buffer with 0.94% methylcellulose suspended in 250 mM aqueous trisodium citrate, resulting in a milky suspension that was used within 2 h of preparation and shaken repeatedly to avoid aggregation and precipitation of the very fine suspended particles.

Dosing and Sample Collection. Using the inhibitors formulated as above, cohorts of five male CD-1 mice (6−8 weeks of age) were fasted for approximately 6 h and then each dosed by oral gavage (10 μL/g), equivalent to a dose of 50 mg/kg of each individual inhibitor. Blood was collected in lithium-heparin tubes by sampling from the saphenous vein at 5 and 20 min postdosing and by cardiac puncture at 60 min post dosing after the mice had been euthanized in a CO_2 chamber.

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Sample Workup. Plasma was obtained from whole blood by sedimenting the cells for 20 min in a benchtop microcentrifuge and removing the supernatant liquid. The plasma was stored at −20 °C until analysis. For analysis, the plasma samples were thawed on ice, and 25 μL (group A) or 10 μL (group B and standard curve) were withdrawn. To each sample, an equal volume of internal standard (IS) spiking solution (1 μM **5b** in 95% water, 4.9% acetonitrile, and 0.1% DMSO) was added, followed by an equal volume of blank spiking solution (95% water, 4.9% acetonitrile, and 0.1% DMSO). To generate standard curves, blank plasma was taken and an equal volume of IS spiking solution added, followed by an equal volume of compound spiking solution (24.4 ng/mL to 12.5 μg/mL per compound, separated in the same groups, in 95% water, 4.9% acetonitrile, and 0.1% DMSO). The volumes of all samples were increased to 250 μL by the addition of water and well mixed. Then, 600 μL of ethyl acetate were added and the samples thoroughly shaken and vortexed. The phases were separated by brief centrifugation, and then 500 μL of ethyl acetate were withdrawn to a microcentrifuge tube, 300 μL of new ethyl acetate added, and the extraction repeated. Another 300 μL were then withdrawn, and the combined extracts were evaporated. To reconstitute the residues, 20 μL of methanol were added to each tube and thoroughly vortexed. After brief centrifugation, 80 μL of water with 0.1% formic acid were added and the tubes thoroughly vortexed again. The liquids were withdrawn, passed through 0.45 μm centrifugal filter units, and analyzed by LC-MS (ESI-QTOF).

Analysis. Total ion chromatograms were extracted for the most prevalent masses of each compound ($[M + H]^+$ and its ^{81}Br isotopologues, except for compound **9e**, where $[M + 2H]^{2+}$ and its isotopologue was used) and the peaks integrated. The peak areas of analytes were normalized by the peak area of the internal standard. Standard curves were used to calculate the concentration of analytes in the plasma samples (see Figure S1 in the Supporting Information).

Synthesis. *General Procedure for the Preparation of TG2 Inhibitors.* The inhibitors were prepared analogously to the method reported in the literature and purified to ≥95% as judged by the HPLC chromatogram obtained on an ESI-QTOF LC-MS.[22]

Step 1. The Boc-protected amino acid **14** (1 equiv), EDCI HCl (1.15 equiv), HOBt hydrate (1 equiv), and (*S*)-(3-bromo-4,5-dihydroisoxazol-5-yl)methanamine ((*S*)-DHI, 1 equiv), prepared by our modification[22] of the procedure of Rohloff and co-workers,[58] were dissolved in DMF (to approximately 150 mM) and *N*-methylmorpholine (2 equiv) was added. The mixture was stirred for 30 min but can also be left stirring overnight and then diluted with approximately 10 volumes of water and extracted with 10 volumes of ethyl acetate. The organic layer was washed with 10 volumes of sodium bicarbonate twice, followed by 10 volumes of brine, dried over sodium sulfate, and evaporated. The product was typically obtained as a viscous oil.

Step 2. The crude product was taken up in trifluoroacetic acid (to approximately 170 mM) and stirred for 30 min before the acid was carefully evaporated. The resulting viscous oil was taken up in an equal volume (relative to TFA) of DCM and the volatiles evaporated again. This procedure was repeated with anhydrous methanol. Drying the sample under vacuum furnished the intermediate **15** as an oil or foam, which was used directly in the next step.

Step 3. The intermediate was then dissolved in anhydrous DMF to approximately 150 mM, and triethylamine (1 equiv) and DMAP (0.1−0.2 equiv) were added. Separately, quinolin-3-ylmethyl 1*H*-imidazole-1-carboxylate (**S3a**, 1 equiv), which was prepared as previously described,[22] was dissolved in anhydrous DCM to approximately 300 mM and the two solutions were then combined. The mixture was stirred at room temperature overnight, and then the volatiles were removed under reduced pressure. The residue was diluted with approximately 10 volumes of water (relative to DMF) and extracted with 10 volumes of ethyl acetate. Again, the organic layer was washed with 10 volumes of sodium bicarbonate twice, followed by 10 volumes of brine, dried over sodium sulfate, and evaporated. The crude product **13** was typically purified by silica gel chromatography with a gradient of 80−100% ethyl acetate in pentane, followed by 0−10% methanol in ethyl acetate, by preparative TLC with similar solvent systems or by preparative reverse-phase HPLC in a gradient of acetonitrile in water with 0.1% TFA as acidic modifier.

*(S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**1**, aka ERW1041E).* Compound **1** was prepared according to our recently published scalable synthesis.[22] HRMS (ESI-QTOF) *m/z*: calculated for $C_{20}H_{22}BrN_4O_4^+$ $[M + H]^+$, 461.08189; found, 461.08278.

*(S)-Quinolin-3-ylmethyl 3-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)-3,4-dihydro-1H-pyrido[3,4-b]indole-2(9H)-carboxylate (**2**).* Compound **2** was prepared from (*S*)-2-(*tert*-butoxycarbonyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid similar to the general procedure, with the key difference that after deprotecting the Boc group, the intermediate was extracted from a basic aqueous solution with ethyl acetate to yield the free base and this was coupled to the carbonate building block (**S4a**) instead of the carbamate building block. ^1H NMR (500 MHz, DMSO-d_6) δ 10.87 and 10.78 (2 s, 1H), 9.00 and 8.94 (2 d, *J* = 2.0 Hz, 1H), 8.45−8.32 (m, 2H), 8.07−7.96 (m, 2H), 7.78 (t, *J* = 9.4 Hz, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.32−7.24 (m, 1H), 7.03 (t, *J* = 7.5 Hz, 1H), 6.96 (ddd, *J* = 7.9, 7.1, 1.1 Hz, 1H), 5.48−5.34 (m, 2H), 5.19 (t, *J* = 7.0 Hz, 1H), 4.88 (dd, *J* = 23.6, 16.2 Hz, 1H), 4.70−4.51 (m, 2H), 3.36−3.24 (m, 1H), 3.23−3.07 (m, 3H), 2.99 (d, *J* = 8.5 Hz, 1H), 2.79 (dt, *J* = 17.6, 5.8 Hz, 1H). HRMS (ESI-QTOF) *m/z*: calculated for $C_{27}H_{25}BrN_5O_4^+$ $[M + H]^+$, 562.10844; found, 562.10843.

*(S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)azetidine-1-carboxylate (**3a**).* Commercial (*S*)-1-(*tert*-butoxycarbonyl)azetidine-2-carboxylic acid (500 mg, 2.485 mmol) was coupled to the (*S*)-DHI moiety as described in step 1 of the general procedure, furnishing (*S*)-*tert*-butyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)azetidine-1-carboxylate (891 mg, 2.46 mmol, 99% yield). The Boc-protected intermediate was directly dissolved in neat TFA (5 mL) and stirred for 30 min. The crude mixture was triturated with 20 mL of cold diethyl ether. A sticky oil formed on the walls of the vial which thickened upon standing. The ether was decanted off and the residue washed with more cold diethyl ether and then dissolved in a little methanol. The methanol was evaporated and the product dried under vacuum, furnishing (*S*)-*N*-(((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)azetidine-2-carboxamide, TFA (716 mg, 1.90 mmol, 77% yield), as a sticky yellow oil that was used in the next step without any additional purification. This intermediate was elaborated to the final inhibitor using step 3 of the general procedure, furnishing (*S*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)azetidine-1-carboxylate (148.3 mg, 0.332 mmol, 17.4% yield) as a white solid. ^1H NMR (500 MHz, DMSO-d_6, mixture of rapidly equilibrating rotational isomers) δ 8.96−8.82 (m, 1H), 8.48−8.23 (m, 2H), 8.03 (d, *J* = 8.5 Hz, 1H), 7.97 (d, *J* = 9.7 Hz, 1H), 7.77 (t, *J* = 7.5 Hz, 1H), 7.63 (ddd, *J* = 8.2, 6.8, 1.3 Hz, 1H), 5.35−5.16 (m, 2H), 4.81−4.54 (m, 2H), 4.05−3.80 (m, 2H), 3.43−3.18 (m, 2H, partly obscured by residual water), 3.12−2.95 (m, 1H), 2.50−2.42 (m, 1H), 2.06−1.93 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 171.10, 155.48, 150.54, 147.14, 138.08, 134.64 (d, *J* = 51.4 Hz), 129.84, 129.70, 128.77, 128.10, 127.25, 126.97, 79.96, 63.79, 61.58 (d, *J* = 68.8 Hz), 47.54 (d, *J* = 94.0 Hz), 43.51, 41.29, 20.81. HRMS (ESI-QTOF) *m/z*: calculated for $C_{19}H_{20}BrN_4O_4^+$ $[M + H]^+$, 447.06624; found, 447.06609.

*(S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)piperidine-1-carboxylate (**3b**).* (*S*)-1-(*tert*-Butoxycarbonyl)piperidine-2-carboxylic acid (102 mg, 0.45 mmol), EDCI (111 mg, 0.58 mmol), HOBt hydrate (66 mg, 0.49 mmol), and (*S*)-DHI (80 mg, 0.45 mmol) were dissolved in DMF, and 4-methylmorpholine (49 μL, 0.45 mmol) was added. The mixture was stirred overnight, diluted with 150 mL of ethyl acetate, washed with water (3 × 50 mL) and aqueous sodium bicarbonate (50 mL), and dried over sodium sulfate. The solvent was removed, and the crude intermediate was deprotected in 5 mL of DCM with 1.5 mL of TFA for 1 h before all volatiles were carefully evaporated under vacuum. The crude TFA salt was redissolved in DMF with 4-methylmorpholine (48.9 μL, 0.445 mmol) and coupled to the carbonate building block **S4a** (112 mg, 0.345 mmol) for 20 h. The solution was then again

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diluted with 150 mL of ethyl acetate, washed with brine (2 × 30 mL) and water (30 mL), dried over sodium sulfate, and evaporated. The crude residue was purified by reparative TLC (10% methanol in ethyl acetate), affording (*S*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)piperidine-1-carboxylate (56.7 mg, 0.119 mmol, 26.8% yield over three steps) was an off-white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.97−8.83 (m, 1H), 8.38−8.20 (m, 2H), 8.06−7.94 (m, 2H), 7.80−7.69 (m, 1H), 7.67−7.56 (m, 1H), 5.37−5.20 (m, 2H), 4.80−4.60 (m, 2H), 3.98−3.86 (m, 1H), 3.47−3.36 (m, 1H), 3.25−2.94 (m, 3H), 2.08−1.96 (m, 1H), 1.70−1.51 (m, 3H), 1.41−1.15 (m, 3H). HRMS (ESI-QTOF) *m/z*: calculated for $C_{21}H_{24}BrN_4O_4^+$ [M + H]$^+$, 475.09754; found, 475.09729.

*(R)-Quinolin-3-ylmethyl 3-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate, TFA Salt (**3c**).* Commercial (*R*)-1-(*tert*-butoxycarbonyl)pyrrolidine-3-carboxylic acid (250 mg, 1.16 mmol) was elaborated following the modification to the general procedure reported for compound **3a**, furnishing (*R*)-quinolin-3-ylmethyl 3-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)pyrrolidine-1-carboxylate (199.9 mg, 0.433 mmol, 37.3% yield over three steps) as a white solid after purification by preparative TLC. An aliquot was then further purified by preparative reverse phase HPLC to yield the title compound. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (d, *J* = 2.1 Hz, 1H), 8.69 (s, 1H), 8.34 (q, *J* = 5.7 Hz, 1H), 8.16 (d, *J* = 8.0 Hz, 1H), 8.13 (d, *J* = 8.5 Hz, 1H), 7.92 (ddd, *J* = 8.4, 6.9, 1.4 Hz, 1H), 7.76 (ddd, *J* = 8.1, 6.9, 1.1 Hz, 1H), 5.33 (s, 2H), 4.77−4.67 (m, 1H), 3.60−3.20 (m, 7H), 3.05−2.95 (m, 2H), 2.11−1.98 (m, 1H), 1.98−1.86 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.81 (d, *J* = 12.8 Hz), 153.57, 148.60, 143.19, 138.65, 138.11, 131.70, 130.88, 128.62, 128.20, 127.70, 125.66, 80.22, 63.38, 48.43 (d, *J* = 82.2 Hz), 45.59 (d, *J* = 67.9 Hz), 43.51, 42.72 (d, *J* = 117.2 Hz), 41.48, 29.15 (d, *J* = 105.1 Hz). HRMS (ESI-QTOF) *m/z*: calculated for $C_{20}H_{21}BrN_4O_4^+$ [M + H]$^+$, 461.08189; found, 461.08256.

*(S)-Quinolin-3-ylmethyl 2-(2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)amino)-2-oxoethyl)pyrrolidine-1-carboxylate (**3d**).* Commercial (*S*)-2-(1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl)acetic acid (250 mg, 1.090 mmol) was elaborated to the final inhibitor using the modification to the general procedure described for compound **3a**, furnishing the title compound (87 mg, 0.183 mmol, 16.8% yield over three steps) as a white solid after purification by preparative TLC. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.92 (d, *J* = 2.1 Hz, 1H), 8.36 (d, *J* = 13.0 Hz, 1H), 8.26−8.18 (m, 1H), 8.05−7.97 (m, 2H), 7.77 (ddd, *J* = 8.4, 6.9, 1.5 Hz, 1H), 7.63 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 5.29 (d, *J* = 6.4 Hz, 2H), 4.68 (dh, *J* = 11.0, 5.8 Hz, 1H), 4.23−3.98 (m, 1H), 3.44−3.14 (m, 5H), 3.00 (td, *J* = 17.5, 7.4 Hz, 1H), 2.62 and 2.51 (2 dd, *J* = 13.6, 3.7 Hz, 1H, partly obscured by solvent), 2.22 and 2.15 (2 dd, *J* = 13.7, 10.1 Hz, 1H), 1.97−1.74 (m, 3H), 1.71−1.60 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 170.52 (d, *J* = 11.6 Hz), 153.59 (d, *J* = 13.1 Hz), 150.59 (d, *J* = 15.7 Hz), 147.12 (d, *J* = 5.7 Hz), 138.15, 134.56 (d, *J* = 26.4 Hz), 130.20, 129.67 (d, *J* = 4.4 Hz), 128.75, 128.14, 127.32, 126.95, 80.18, 63.79 (d, *J* = 14.0 Hz), 54.71 (d, *J* = 69.7 Hz), 46.23 (d, *J* = 51.1 Hz), 43.55, 41.28 (d, *J* = 5.1 Hz), 38.75, 29.87 (d, *J* = 96.2 Hz), 22.60 (d, *J* = 106.7 Hz). HRMS (ESI-QTOF) *m/z*: calculated for $C_{21}H_{24}BrN_4O_4^+$ [M + H]$^+$, 475.09754; found, 475.09800.

*(S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-2-methylpyrrolidine-1-carboxylate (**4a**).* (*S*)-1-(*tert*-Butoxycarbonyl)-2-methylpyrrolidine-2-carboxylic acid (100 mg, 0.436 mmol) was elaborated to the title compound following the general procedure and purified by preparative TLC (0.9 mg, 1.9 μmol, 0.44% yield over three steps). ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.95 (s, 1H), 8.28 and 8.20 (2 s, 1H), 8.19−8.10 (m, 1H), 7.86 (dd, *J* = 14.4, 7.1 Hz, 1H), 7.75 (t, *J* = 7.8 Hz, 1H), 7.58 (t, *J* = 7.5 Hz, 1H), 6.84 (br s, 1H), 5.46−5.18 (m, 2H), 4.96−4.93 and 4.86−4.77 (m, 1H), 3.85−3.37 (m, 4H), 3.16 (dd, *J* = 7.9, 3.1 Hz, 1H), 2.45−2.33 (m, 1H), 1.95−1.79 (m, 2H), 1.68−1.47 (m, 5H). HRMS (ESI-QTOF) *m/z*: calculated for $C_{21}H_{24}BrN_4O_4^+$ [M + H]$^+$, 475.09754; found, 475.09826.

*(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-fluoropyrrolidine-1-carboxylate (**4b**, aka ZH147A).* Slightly modifying the published synthesis of the title compound,[22] (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-fluoropyrrolidine-2-carboxylic acid (400 mg, 1.72 mmol), EDCI HCl (378 mg, 1.97 mmol), HOBt (232 mg, 1.72 mmol), and (*S*)-(3-bromo-4,5-dihydroisoxazol-5-yl)methanamine (307 mg, 1.72 mmol) were dissolved in 12 mL of DMF, and *N*-methylmorpholine (377 μL, 3.43 mmol) was added. The mixture was stirred for 30 min before it was diluted with 120 mL of water and extracted with 100 mL of ethyl acetate. The organic layer was washed with 100 mL of sodium bicarbonate twice and with 100 mL of brine, dried over sodium sulfate, and evaporated. The resulting viscous oil was taken up in 10 mL of trifluoroacetic acid and stirred for 30 min before the acid was carefully evaporated. The resulting viscous oil was taken up in 10 mL of DCM and the volatiles again evaporated. This procedure was repeated with 10 mL of anhydrous methanol. Drying the sample on high vacuum overnight furnished the deprotected intermediate (2*S*,4*S*)-*N*-(((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)-4-fluoropyrrolidine-2-carboxamide as its TFA salt in the form of a viscous oil that was directly used in the next step. For this, the viscous oil was diluted with 12 mL of anhydrous DMF, and triethylamine (241 μL, 1.72 mmol) and DMAP (21 mg, 0.17 mmol) were added. Separately, quinolin-3-ylmethyl 1*H*-imidazole-1-carboxylate (434 mg, 1.72 mmol) was dissolved in 6 mL of anhydrous DCM and the two solutions combined. After stirring the mixture at room temperature overnight, the volatiles were removed under reduced pressure. The residue was diluted with 100 mL of water and extracted with 100 mL of ethyl acetate. The organic layer was washed with sodium bicarbonate solution (2 × 100 mL), followed by brine (100 mL), drying the organic layer over sodium sulfate and evaporating the volatiles. The crude product was purified by silica gel chromatography with a gradient of 80−100% ethyl acetate in pentane, followed by 0−10% methanol in ethyl acetate, furnishing (2*S*,4*S*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)-4-fluoropyrrolidine-1-carboxylate (434 mg, 0.905 mmol, 52.8% yield over three steps) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.95 and 8.87 (2 d, *J* = 2.1 Hz, 1H), 8.38 and 8.28 (2 s, 1H), 8.30 and 8.20 (2 t, *J* = 6.1 Hz, 1H), 8.07−7.93 (m, 2H), 7.81−7.73 (m, 1H), 7.63 (dddd, *J* = 8.2, 6.6, 5.1, 1.3 Hz, 1H), 5.44−5.17 (m, 3H), 4.70 and 4.62 (2 ddt, *J* = 10.7, 7.4, 5.3 Hz, 1H), 4.42 and 4.33 (2 d, *J* = 9.7 Hz, 1H), 3.82−3.58 (m, 2H), 3.43−3.12 (m, 3H, partly obscured by residual water), 2.98 (ddd, *J* = 22.0, 17.6, 7.3 Hz, 1H), 2.60−2.35 (m, 1H, partly obscured by solvent), 2.27−2.10 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, J_{CF} coupled, mixture of rotational isomers) δ 171.67 (d, *J* = 26.6 Hz), 154.07 (d, *J* = 31.4 Hz), 150.54 (d, *J* = 20.6 Hz), 147.15 (d, *J* = 7.0 Hz), 138.16 (d, *J* = 21.0 Hz), 134.53 (d, *J* = 38.2 Hz), 129.90 (d, *J* = 8.5 Hz), 129.69 (d, *J* = 9.6 Hz), 128.78, 128.11 (d, *J* = 12.2 Hz), 127.28 (d, *J* = 5.5 Hz), 126.97 (d, *J* = 2.6 Hz), 92.14 (dd, *J* = 174.3, 124.6 Hz), 80.02 (d, *J* = 6.2 Hz), 64.24 (d, *J* = 14.0 Hz), 59.02 (d, *J* = 63.2 Hz), 53.53 (dd, *J* = 61.6, 23.6 Hz), 43.34, 41.02 (d, *J* = 15.9 Hz), 37.22 (dd, *J* = 117.3, 21.0 Hz). HRMS (ESI-QTOF) *m/z*: calculated for $C_{20}H_{21}BrFN_4O_4^+$ [M + H]$^+$, 479.07247; found, 479.07289.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-fluoropyrrolidine-1-carboxylate (**4c**).* The title compound was prepared as recently described elsewhere.[22] ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.93 and 8.84 (2 d, *J* = 2.2 Hz, 1H), 8.46 and 8.41 (2 t, *J* = 6.0 Hz, 1H), 8.36 and 8.27 (2 d, *J* = 1.3 Hz, 0H), 8.04 and 8.02 (2 d, *J* = 3.1 Hz, 1H), 8.00 and 7.96 (2 d, *J* = 8.3 Hz, 1H), 7.78 (dddd, *J* = 8.4, 6.8, 4.3, 1.5 Hz, 1H), 7.64 (dddd, *J* = 8.1, 6.9, 2.7, 1.2 Hz, 1H), 5.43−5.17 (m, 3H), 4.79−4.65 and 4.55−4.46 (2 m, 1H), 4.39 and 4.30 (2 dd, *J* = 9.0, 7.6 Hz, 1H), 3.89−3.72 (m, 1H), 3.72−3.50 (m, 1H), 3.44−3.32 (m, 1H, partly obscured by residual water), 3.30−3.10 (m, 2H), 3.01 and 2.91 (2 dd, *J* = 17.6, 7.3 Hz, 1H), 2.41 (dd, *J* = 25.4, 11.4 Hz, 1H, partly obscured by solvent), 2.14−1.90 (m, 1H). HRMS (ESI-QTOF) *m/z*: calculated for $C_{20}H_{21}BrFN_4O_4^+$ [M + H]$^+$, 479.07247; found, 479.07212.

(2S,4R)-Quinolin-3-ylmethyl 4-Amino-2-((((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate, Bis-

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*TFA Salt (**4d**).* Adapting the general procedure, (2*S*,4*R*)-4-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-1-(*tert*-butoxycarbonyl)-pyrrolidine-2-carboxylic acid (1.2 g, 2.65 mmol), EDCI HCl (0.585 g, 3.05 mmol), and HOBt hydrate (0.358 g, 2.65 mmol) were dissolved in 5 mL of DMF and stirred for 10 min, and (*S*)-DHI (0.475 g, 2.65 mmol) was added. The mixture was allowed to react for 4 h before 25 mL of water were added, whereby a greasy precipitate formed. The mixture was allowed to sit for 1 h before the liquid portion was decanted off and the greasy solid material dissolved in 40 mL of ethyl acetate. The ethyl acetate was washed with brine and then dried over sodium sulfate before it was evaporated, affording (2*S*,4*R*)-*tert*-butyl 4-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (1.3 g, 2.12 mmol, 80% yield), which was elaborated to the Fmoc protected inhibitor (2*S*,4*R*)-quinolin-3-ylmethyl 4-((((9*H*-fluoren-9-yl)-methoxy)carbonyl)amino)-2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate using steps 2 and 3 of the general procedure. This intermediate was deprotected using piperidine in DCM (1:1) and stirring the mixture for 30 min. All volatiles were then removed under reduced pressure and the residue purified by silica gel chromatography (ethyl acetate/triethylamine 99:1 to ethyl acetate/triethylamine/methanol 89:1:10). The product coeluted with a decomposition product where bromide had been displaced by piperidine (approximately in equimolar ratio by NMR, 670 mg total mass, approximately 30% yield over three steps when corrected for purity). This product was used in the follow-up reactions (vide infra) as it was found easier to remove the decomposition product with the amine further functionalized. For analytical and assay purposes, an aliquot of this mixture was further purified by reverse-phase HPLC, furnishing the title compound as its TFA salt. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.99 and 8.92 (2 d, J = 2.2 Hz, 1H), 8.91−8.86 (m, 1H), 8.45 and 8.39 (2 s, 1H), 8.14 (br s, 3H), 8.08 (d, J = 8.4 Hz, 1H), 8.04 (t, J = 8.5 Hz, 1H), 7.84 (ddd, J = 8.4, 7.0, 1.4 Hz, 1H), 7.69 (ddd, J = 8.1, 6.9, 1.2 Hz, 1H), 5.42−5.21 (m, 2H), 4.78−4.69 and 4.60−4.48 (2 m, 1H), 4.41 and 4.33 (2 dd, J = 9.0, 4.0 Hz, 1H), 3.89−3.81 (m, 1H), 3.77 and 3.72 (2 dd, J = 11.5, 5.9 Hz, 1H), 3.61 and 3.56 (2 dd, J = 11.4, 3.7 Hz, 1H), 3.46−3.16 (m, 3H), 3.03 and 2.94 (2 dd, J = 17.5, 7.4 Hz, 1H), 2.57 (tdd, J = 16.5, 8.7, 4.4 Hz, 1H), 1.90 (tt, J = 14.8, 4.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 173.38 (d, J = 24.4 Hz), 153.42 (d, J = 29.9 Hz), 150.07 (d, J = 22.7 Hz), 146.10 (d, J = 25.3 Hz), 138.19 (d, J = 27.5 Hz), 135.91 (d, J = 17.2 Hz), 130.36, 129.80 (d, J = 11.6 Hz), 128.22 (d, J = 3.9 Hz), 127.94 (d, J = 14.9 Hz), 127.39, 127.36 (d, J = 7.7 Hz), 79.84 (d, J = 20.4 Hz), 64.29 (d, J = 5.5 Hz), 58.27 (d, J = 86.8 Hz), 50.57 (d, J = 46.4 Hz), 48.71 (d, J = 116.5 Hz), 43.51 (d, J = 13.7 Hz), 41.57 (d, J = 18.8 Hz), 33.93 (d, J = 122.4 Hz).

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-hydroxypyrrolidine-1-carboxylate (**4e**, aka ZH118).* Compound **4e** was prepared from commercial (2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (1.0 g, 4.32 mmol) following the general procedure. Gratifyingly, the crude final product precipitated in high purity from a concentrated solution in ethyl acetate, accounting for the bulk of the isolated yield (266 mg), whereas column chromatography of the mother liquor furnished a modest additional yield (120 mg) of the title compound (386 mg overall yield, 0.809 mmol, 18.7% yield over three steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.92 and 8.84 (2 d, J = 2.1 Hz, 1H), 8.39 and 8.32 (2 t, J = 6.0 Hz, 1H), 8.34 and 8.25 (2 s, 1H), 8.03 (dd, J = 8.4, 4.4 Hz, 1H), 8.00 and 7.96 (2 d, J = 8.3 Hz, 1H), 7.77 (dddd, J = 8.4, 6.9, 5.4, 1.4 Hz, 1H), 7.63 (dddd, J = 8.2, 6.9, 3.0, 1.2 Hz, 1H), 5.35−5.17 (m, 2H), 5.10 (br s, 1H), 4.75−4.68 and 4.58−4.51 (2 m, 1H), 4.38−4.21 (m, 2H), 3.58−3.08 (m, 5H), 3.01 and 2.92 (2 dd, J = 17.6, 7.2 Hz, 1H), 2.15−2.01 (m, 1H), 1.88−1.77 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.78 (d, J = 46.3 Hz), 154.00 (d, J = 28.0 Hz), 150.53 (d, J = 23.4 Hz), 147.13 (d, J = 10.0 Hz), 138.03 (d, J = 38.8 Hz), 134.47 (d, J = 59.6 Hz), 130.00 (d, J = 7.7 Hz), 129.68 (d, J = 14.6 Hz), 128.79, 128.09 (d, J = 14.6 Hz), 127.28 (d, J = 5.0 Hz), 126.98 (d, J = 5.1 Hz), 80.14 (d, J = 24.0 Hz), 68.23 (d, J = 85.6 Hz), 64.00, 58.76 (d, J = 76.9 Hz), 55.25 (d, J = 63.9 Hz), 43.36, 41.16 (d, J = 37.0 Hz), 38.92. HRMS (ESI-QTOF) m/z: calculated for $C_{20}H_{22}BrN_4O_5^+$ $[M + H]^+$, 477.07681; found, 477.07752.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-methoxypyrrolidine-1-carboxylate (**5a**).* (2*S*,4*R*)-1-*tert*-Butyl 2-methyl 4-hydroxypyrrolidine-1,2-dicarboxylate (0.347 g, 1.41 mmol) and sodium hydride (0.0865 g, 60% suspension in mineral oil) were individually dissolved in DMF, and the solutions were combined at −10 °C and stirred for 30 min before iodomethane (0.265 mL, 4.24 mmol) was added. After an additional hour, the cooling bath was removed and stirring continued at room temperature for 15 h. The solution was diluted with 50 mL of DCM and washed with brine (4 × 30 mL). The organic phase was dried over sodium sulfate, evaporated, and the residue purified by silica gel chromatography (10−50% ethyl acetate in pentane, R_f = 0.5 in 1:1 ethyl acetate/pentane) to furnish (2*S*,4*R*)-1-*tert*-butyl 2-methyl 4-methoxypyrrolidine-1,2-dicarboxylate (93 mg, 0.359 mmol, 25.4% yield). This intermediate was dissolved in THF and 1 M LiOH added to hydrolyze the methyl ester. Once the reaction was complete, THF was evaporated, the solution acidified to pH 3−4 with 1 M HCl, and extracted with ethyl acetate. The amino acid was then elaborated to the final inhibitor using the procedure described for compound **3b**. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.93 and 8.84 (2 d, J = 2.2 Hz, 1H), 8.40 and 8.33 (2 t, J = 5.9 Hz, 1H), 8.36 and 8.26 (2 s, 1H), 8.07−7.94 (m, 2H), 7.78 (tdd, J = 8.3, 6.2, 1.4 Hz, 1H), 7.69−7.61 (m, 1H), 5.35−5.16 (m, 2H), 4.78−4.66 and 4.60−4.49 (2 m, 1H), 4.29 and 4.20 (2 t, J = 7.9 Hz, 1H), 4.01−3.93 (m, 1H), 3.60−3.44 (m, 2H), 3.43−3.24 (m, 2H, partly obscured by residual water), 3.22 (d, J = 3.4 Hz, 3H), 3.20−3.10 (m, 1H), 3.02 and 2.93 (2 dd, J = 17.6, 7.2 Hz, 1H), 2.31−2.18 (m, 1H), 1.93−1.81 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{21}H_{24}BrN_4O_5^+$ $[M + H]^+$, 491.09246; found, 491.09213.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(prop-2-yn-1-yloxy)pyrrolidine-1-carboxylate (**5b**).* The title compound was prepared as recently described elsewhere.[22] HRMS (ESI-QTOF) m/z: calculated for $C_{23}H_{24}BrN_4O_5^+$ $[M + H]^+$, 515.09246; found, 515.09216.

*(2S,4R)-Quinolin-3-ylmethyl 4-(Benzyloxy)-2-((((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**5c**, aka ZH105).* Compound **5c** was prepared from commercial (2*S*,4*R*)-4-(benzyloxy)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid using the general procedure, furnishing (2*S*,4*R*)-quinolin-3-ylmethyl 4-(benzyloxy)-2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (828 mg, 1.46 mmol, 46.9% yield over three steps) as a white foam after silica gel chromatography. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.92 and 8.84 (2 d, J = 2.1 Hz, 1H), 8.41 and 8.35 (2 t, J = 6.0 Hz, 1H), 8.32 and 8.25 (2 d, J = 1.4 Hz, 1H), 8.08−8.00 (m, 1H), 7.95 (ddd, J = 11.1, 8.2, 1.6 Hz, 1H), 7.80−7.74 (m, 1H), 7.62 (ddd, J = 8.1, 6.9, 1.2 Hz, 1H), 7.36−7.23 (m, 5H), 5.35−5.17 (m, 2H), 4.72 and 4.54 (2 ddt, J = 10.5, 7.4, 5.0 Hz, 1H), 4.47 (s, 2H), 4.36 and 4.27 (2 t, J = 7.8 Hz, 1H), 4.17 (m, 1H), 3.67−3.49 (m, 2H), 3.44−3.10 (m, 3H, partly obscured by residual water), 3.02 and 2.93 (2 dd, J = 17.6, 7.3 Hz, 1H), 2.39−2.26 (m, 1H), 1.98−1.87 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.45 (d, J = 43.5 Hz), 153.91 (d, J = 24.8 Hz), 150.52 (d, J = 23.1 Hz), 147.13 (d, J = 10.4 Hz), 138.22 (d, J = 4.9 Hz), 137.90, 134.47 (d, J = 55.3 Hz), 129.90 (d, J = 6.2 Hz), 129.67 (d, J = 12.7 Hz), 128.78, 128.27 (d, J = 2.7 Hz), 128.07 (d, J = 13.7 Hz), 127.57 (d, J = 5.4 Hz), 127.50 (d, J = 3.1 Hz), 127.26 (d, J = 5.0 Hz), 126.95 (d, J = 3.2 Hz), 80.12 (d, J = 24.0 Hz), 76.13 (d, J = 92.1 Hz), 69.92 (d, J = 3.0 Hz), 64.10, 58.64 (d, J = 74.1 Hz), 52.25 (d, J = 60.3 Hz), 43.38, 41.19 (d, J = 34.4 Hz), 36.42 (d, J = 120.4 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{27}H_{28}BrN_4O_5^+$ $[M + H]^+$, 567.12376; found, 567.12373.

*(2S,4R)-Quinolin-3-ylmethyl 4-Benzyl-2-((((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate, TFA Salt (**6**).* Commercial (2*S*,4*R*)-4-benzyl-1-(*tert*-butoxycarbonyl)-pyrrolidine-2-carboxylic acid (100 mg, 0.327 mmol) was elaborated to the final inhibitor following the general procedure and purified by preparative TLC, furnishing (2*S*,4*R*)-quinolin-3-ylmethyl 4-benzyl-2-

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((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-pyrrolidine-1-carboxylate (112.8 mg, 0.205 mmol, 62.7% yield over three steps). An aliquot of the inhibitor was further purified by reverse-phase HPLC. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.08 and 8.99 (2 d, J = 2.1 Hz, 1H), 8.62 and 8.55 (2 s, 1H), 8.34 and 8.25 (2 t, J = 6.0 Hz, 1H), 8.15−8.06 (m, 2H), 7.91 (ddd, J = 8.5, 6.9, 1.4 Hz, 1H), 7.79−7.72 (m, 1H), 7.32−7.23 (m, 2H), 7.22−7.14 (m, 3H), 5.38−5.20 (m, 2H), 4.70 and 4.58 (2 ddt, J = 10.2, 7.2, 4.9, 11.9, 7.1, 4.9 Hz, 1H), 4.36 and 4.25 (2 dd, J = 8.8, 2.5 Hz, 1H), 3.56 and 3.49 (2 dd, J = 10.2, 7.3 Hz, 1H), 3.42−3.05 (m, 4H), 2.94 (ddd, J = 20.3, 17.5, 7.1 Hz, 1H), 2.68−2.57 (m, 2H), 2.55−2.42 (m, 1H, partly obscured by solvent), 1.94 (ddt, J = 30.7, 12.5, 9.3 Hz, 1H), 1.87−1.76 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.66 (d, J = 37.4 Hz), 153.67 (d, J = 41.4 Hz), 148.66 (d, J = 61.7 Hz), 143.63 (d, J = 47.2 Hz), 140.21, 138.05, 137.85, 131.45 (d, J = 4.2 Hz), 130.65 (d, J = 5.4 Hz), 128.65 (d, J = 5.6 Hz), 128.52, 128.51−128.35 (m), 128.08 (d, J = 8.7 Hz), 127.59 (d, J = 2.6 Hz), 126.14, 125.94, 80.10 (d, J = 15.5 Hz), 63.52 (d, J = 13.5 Hz), 59.78 (d, J = 74.4 Hz), 51.77 (d, J = 73.8 Hz), 43.34, 41.24 (d, J = 27.6 Hz), 38.45 (d, J = 97.8 Hz), 37.96 (d, J = 16.1 Hz), 36.41 (d, J = 109.2 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{27}H_{28}BrN_4O_4^+$ [M + H]$^+$, 551.12884; found, 551.12961.

*(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-phenylpyrrolidine-1-carboxylate (**7a**, aka CK805).* The title compound was prepared from commercial (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-phenylpyrrolidine-2-carboxylic by the general procedure, furnishing (2*S*,4*S*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-phenylpyrrolidine-1-carboxylate (81 mg, 0.151 mmol, 43.9% yield over three steps) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.93 and 8.86 (2 d, J = 2.2 Hz, 1H), 8.41 and 8.35 (2 t, J = 6.1 Hz, 1H), 8.36 and 8.27 (2 d, J = 1.6 Hz, 1H), 8.03 (d, J = 8.3 Hz, 1H), 7.99 (dd, J = 17.0, 8.0 Hz, 1H), 7.77 (ddt, J = 8.1, 6.9, 1.1 Hz, 1H), 7.67−7.60 (m, 1H), 7.35−7.27 (m, 4H), 7.26−7.19 (m, 1H), 5.37−5.20 (m, 2H), 4.80−4.71 and 4.66−4.59 (2 m, 1H), 4.46 and 4.37 (2 dd, J = 8.8, 2.2 Hz, 1H), 3.98 and 3.91 (2 dd, J = 9.8, 7.6 Hz, 1H), 3.54−3.18 (m, 5H, partly obscured by residual water), 3.04 (ddd, J = 26.4, 17.6, 7.2 Hz, 1H), 2.43−2.28 (m, 1H), 2.18−2.08 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.52 (d, J = 33.1 Hz), 153.68 (d, J = 41.6 Hz), 150.50 (d, J = 25.3 Hz), 147.12 (d, J = 7.8 Hz), 140.62 (d, J = 22.0 Hz), 138.02 (d, J = 26.7 Hz), 134.42 (d, J = 52.0 Hz), 129.98 (d, J = 4.6 Hz), 129.65 (d, J = 8.8 Hz), 128.76 (d, J = 3.2 Hz), 128.54 (d, J = 4.4 Hz), 128.09 (d, J = 15.0 Hz), 127.27 (d, J = 3.9 Hz), 127.15 (d, J = 3.3 Hz), 126.86 (d, J = 21.8 Hz), 80.11 (d, J = 14.2 Hz), 64.03 (d, J = 15.0 Hz), 59.93 (d, J = 75.6 Hz), 52.98 (d, J = 61.1 Hz), 43.41, 41.56 (d, J = 59.3 Hz), 40.99 (d, J = 36.4 Hz), 37.82 (d, J = 132.7 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{26}BrN_4O_4^+$ [M + H]$^+$, 537.11319; found, 537.11300.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-phenylpyrrolidine-1-carboxylate (**7b**).* (*S*)-1-(*tert*-butoxycarbonyl)-4-phenyl-2,5-dihydro-1*H*-pyrrole-2-carboxylic acid (**18b**, where R = phenyl) was prepared analogously as steps 1 and 2 of compound **7e**. An aliquot (64 mg, 0.221 mmol) was dissolved in 10 mL of ethanol (200 proof), and palladium 10% on carbon (23.5 mg) was added. The round-bottom flask was capped with a septum and purged with hydrogen gas briefly and kept under a slight positive pressure of hydrogen while stirring overnight. The reaction mixture was then filtered through a small layer of silica gel sandwiched between layers of sand in a pipet column. The matrix was washed with additional ethanol, and the combined filtrate was evaporated under reduced pressure, yielding (2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-phenyl-pyrrolidine-2-carboxylic acid (**19b**, where R = phenyl, 50 mg, 0.172 mmol, 78% yield), which was then elaborated to the final inhibitor using the general procedure (43.3 mg, 0.081 mmol, 47.1% yield over three steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.97−8.84 (m, 1H), 8.45−8.38 (m, 1H), 8.37 and 8.28 (2 s, 1H), 8.11−7.91 (m, 2H), 7.77 (tt, J = 8.2, 1.3 Hz, 1H), 7.67−7.60 (m, 1H), 7.37−7.21 (m, 5H), 5.38−5.19 (m, 2H), 4.79−4.52 (m, 1H), 4.42−4.23 (m, 1H), 4.13−3.97 (m, 1H), 3.53−2.90 (m, 6H), 2.70−2.53 (m, 1H), 1.94−1.79 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{26}BrN_4O_4^+$ [M + H]$^+$, 537.11319; found, 537.11291.

*(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(2-hydroxyphenyl)pyrrolidine-1-carboxylate, TFA Salt (**7c**).* Compound **7c** was prepared analogously to compound **7e**, with the exception that in the amide coupling reaction within step 4, HBTU (1 equiv) was used as the coupling agent in place of EDCI HCl with HOBt. The final product was purified by reverse phase HPLC, furnishing the title compound as its TFA salt (4.9 mg, 0.0073 mmol, 0.92% yield over six steps). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.52 (br s, 1H), 8.97 and 8.89 (2 d, J = 2.1 Hz, 1H), 8.46−8.28 (m, 2H), 8.08−7.97 (m, 2H), 7.81 (ddd, J = 8.5, 6.9, 1.4 Hz, 1H), 7.70−7.61 (m, 1H), 7.09 (dd, J = 7.7, 1.6 Hz, 1H), 7.06−6.98 (m, 1H), 6.79 (ddd, J = 7.2, 5.7, 1.2 Hz, 1H), 6.76−6.65 (m, 1H), 5.38−5.18 (m, 2H), 4.77−4.68 and 4.63−4.53 (2 m, 1H), 4.42 and 4.32 (2 dd, J = 8.7, 3.1 Hz and 8.8, 2.6, 1H), 3.92 and 3.84 (2 dd, J = 10.0, 7.6 Hz, 1H), 3.66 (dt, J = 15.8, 8.0 Hz, 1H), 3.46−3.29 (m, 2H), 3.30−2.91 (m, 3H), 2.46−2.30 (m, 1H), 2.12−1.97 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{26}BrN_4O_5^+$ [M + H]$^+$, 553.10811; found, 553.10750

*(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(3-hydroxyphenyl)pyrrolidine-1-carboxylate, TFA Salt (**7d**).* Compound **7d** was prepared using the procedure of compound **7c** and purified by reverse phase HPLC, furnishing the title compound as its TFA salt (6.7 mg, 0.010 mmol, 1.3% yield over six steps). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.37 (br s, 1H), 8.99 and 8.90 (2 s, 1H), 8.48−8.29 (m, 2H), 8.10−7.94 (m, 2H), 7.85−7.76 (m, 1H), 7.66 (d, J = 8.9 Hz, 1H), 7.09 (q, J = 7.3 Hz, 1H), 6.75−6.56 (m, 3H), 5.40−5.20 (m, 2H), 4.80−4.57 (m, 1H), 4.49−4.31 (m, 1H), 3.98−3.82 (m, 1H), 3.47−3.15 (m, 4H), 3.15−2.95 (m, 2H), 2.38−2.22 (m, 1H), 2.11 (s, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{26}BrN_4O_5^+$ [M + H]$^+$, 553.10811; found, 553.10725.

*(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(4-hydroxyphenyl)pyrrolidine-1-carboxylate (**7e**, aka CK937). Step 1: (S)-1-tert-Butyl 2-Methyl 4-(4-Hydroxyphenyl)-1H-pyrrole-1,2(2H,5H)-dicarboxylate.* Adapting a procedure from the patent literature by Nakai and co-workers,[39] (4-hydroxyphenyl)boronic acid (0.367 g, 2.66 mmol) and tetrakis-(triphenylphosphine) palladium(0) (0.308 g, 0.266 mmol) were placed in a 50 mL round-bottom flask and dissolved in 20 mL of dioxane. Then (*S*)-1-*tert*-butyl 2-methyl 4-(((trifluoromethyl)-sulfonyl)oxy)-1*H*-pyrrole-1,2(2*H*,5*H*)-dicarboxylate (**17**, 1 g, 2.66 mmol), prepared from (*S*)-1-*tert*-butyl 2-methyl 4-oxopyrrolidine-1,2-dicarboxylate (synthesized from **16** by the method of Qiu[40]), as described in the literature reference, was added, followed by potassium carbonate (4.00 mL, 7.99 mmol) as an aqueous solution. The mixture was placed in an oil bath that had been preheated to 80 °C and stirred for 20 min, by which time the solution had turned from orange to dark black, indicating completion. The solution was concentrated under reduced pressure and then diluted with water (50 mL). Aqueous HCl was added to adjust the pH to approximately 5. The mixture was extracted with ethyl acetate (2 × 50 mL), and the combined organic extracts were dried and then filtered through a plug of silica. Removal of the volatiles furnished a gray crude product that was purified by silica gel chromatography in 10−25% ethyl acetate in pentane to yield (*S*)-1-*tert*-butyl 2-methyl 4-(4-hydroxyphenyl)-1*H*-pyrrole-1,2-(2*H*,5*H*)-dicarboxylate (**18a**, where R = *p*-hydroxyphenyl, 0.484 g, 1.52 mmol, 56.9% yield) (R_f in 15% ethyl acetate in pentane approximately 0.15). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.72 (s, 1H), 7.32 (dd, J = 8.7, 6.9 Hz, 2H), 6.79−6.72 (m, 2H), 6.08 (m, 1H), 5.02 (dt, J = 5.2, 2.7 Hz, 1H), 4.44 (ddt, J = 9.5, 4.6, 2.1 Hz, 2H), 3.68 and 3.65 (2 s, 3H), 1.44 and 1.36 (2 s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 170.82 (d, J = 49.7 Hz), 158.05, 152.82 (d, J = 72.9 Hz), 139.91 (d, J = 42.3 Hz), 127.38 (d, J = 5.5 Hz), 123.07, 115.52 (d, J = 16.8 Hz), 114.87 (d, J = 12.8 Hz), 79.44 (d, J = 27.2 Hz), 66.75 (d, J = 33.8 Hz), 53.27 (d, J = 7.6 Hz), 52.11 (d, J = 8.2 Hz), 28.01 (d, J = 25.5 Hz).

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Step 2: (S)-1-(tert-Butoxycarbonyl)-4-(4-hydroxyphenyl)-2,5-dihydro-1H-pyrrole-2-carboxylic Acid. (*S*)-1-*tert*-Butyl 2-methyl 4-(4-hydroxyphenyl)-1*H*-pyrrole-1,2(2*H*,5*H*)-dicarboxylate (484 mg, 1.52 mmol) was dissolved in THF (40 mL) and methanol (20 mL). To the stirred solution, 20 mL of LiOH (1 M in water) was added and the reaction monitored by TLC (50% EtOAc/pentane). Then an equimolar amount of 1 M hydrochloric acid was added and the volatiles were removed under reduced pressure. The residue was taken up in additional water (50 mL final volume), the pH adjusted to 3−4 using hydrochloric acid, and the aqueous phase extracted with ethyl acetate (3 × 50 mL). The combined extracts were dried over sodium sulfate and the solvent evaporated, yielding (*S*)-1-(*tert*-butoxycarbonyl)-4-(4-hydroxyphenyl)-2,5-dihydro-1*H*-pyrrole-2-carboxylic acid (**18b**, where R = *p*-hydroxyphenyl, 431 mg, 1.41 mmol, 93% yield) as an off-white foam that was used without further purification.

Step 3: (2S,4S)-1-(tert-Butoxycarbonyl)-4-(4-hydroxyphenyl)-pyrrolidine-2-carboxylic Acid. Inspired by the report of Zhang and co-workers,[43] (*S*)-1-(*tert*-butoxycarbonyl)-4-(4-hydroxyphenyl)-2,5-dihydro-1*H*-pyrrole-2-carboxylic acid (431 mg, 1.41 mmol) and chlorotris(triphenylphosphine)rhodium(I) (131 mg, 0.141 mmol) were brought under an atmosphere of argon and dissolved in anhydrous THF (24 mL), methanol (24 mL), and triethylamine (198 μL, 1.41 mmol). The atmosphere in the flask was then changed to hydrogen and a slight positive pressure maintained while the solution was stirred overnight. The volatiles were evaporated, the residue suspended in aqueous sodium bicarbonate (100 mL), and the pH adjusted to approximately 10 with 1 M aqueous sodium hydroxide. Ethyl acetate (100 mL) was then added and the mixture partitioned. The organic layer was washed with another 50 mL of sodium bicarbonate solution, and the combined aqueous layers were brought to pH 3−4 using 1 M aqueous hydrochloric acid and the product subsequently back-extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over sodium sulfate and evaporated to furnish (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-(4-hydroxyphenyl)pyrrolidine-2-carboxylic acid (**19a**, where R = *p*-hydroxyphenyl, 384 mg, 1.25 mmol, 89% yield) as an off-white foam in good purity. ^{1}H NMR (400 MHz, methanol-d_4, mixture of rotational isomers) δ 7.08 (d, J = 8.4 Hz, 2H), 6.74 (d, J = 8.5 Hz, 2H), 4.41 and 4.37 (2 dd, J = 8.9, 2.2 Hz, 1H), 3.86 (ddd, J = 12.5, 10.2, 7.7 Hz, 1H), 3.50−3.38 (m, 1H), 3.34−3.26 (m, 1H, partly obscured by solvent), 2.44−2.33 (m, 1H), 2.29 (ddd, J = 13.1, 6.8, 2.6 Hz, 1H), 1.47 and 1.44 (2 s, 9H).

Step 4: (2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(4-hydroxyphenyl)-pyrrolidine-1-carboxylate. An aliquot of the amino acid (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-(4-hydroxyphenyl)pyrrolidine-2-carboxylic acid (250 mg, 0.813 mmol) was elaborated into the final inhibitor using the general procedure and purified by silica gel chromatography (80−100% ethyl acetate in pentane, followed by 0−15% methanol in ethyl acetate), furnishing the title compound as a white foam (264 mg, 0.477 mmol, 58.7% yield over three steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.31 (s, 1H), 8.93 and 8.86 (2 d, J = 2.1 Hz, 1H), 8.40 and 8.33 (2 t, J = 6.1 Hz, 1H), 8.35 and 8.26 (2 d, J = 1.1 Hz, 1H), 7.77 (ddd, J = 8.4, 6.9, 1.5 Hz, 1H), 7.63 (dddd, J = 8.1, 6.9, 4.1, 1.2 Hz, 1H), 7.07 (dd, J = 8.7, 2.7 Hz, 2H), 6.69 (t, J = 8.1 Hz, 2H), 5.35−5.19 (m, 2H), 4.79−4.71 and 4.66−4.59 (2 m, 1H), 4.44 and 4.35 (dd, J = 8.8, 2.0 Hz, 1H), 3.91 and 3.84 (2 dd, J = 9.5, 7.3 Hz, 1H), 3.45−3.18 (m, 5H), 3.03 (ddd, J = 25.1, 17.6, 7.2 Hz, 1H), 2.28 (dtd, J = 24.4, 12.2, 9.0 Hz, 1H), 2.13−2.01 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.61 (d, J = 33.3 Hz), 156.13, 153.70 (d, J = 43.8 Hz), 150.50 (d, J = 26.0 Hz), 147.13 (d, J = 7.7 Hz), 138.05 (d, J = 26.3 Hz), 134.41 (d, J = 52.2 Hz), 130.55 (d, J = 19.4 Hz), 130.02 (d, J = 4.6 Hz), 129.67 (d, J = 9.1 Hz), 128.77 (d, J = 3.0 Hz), 128.17, 128.07, 127.29 (d, J = 3.8 Hz), 126.96, 115.26 (d, J = 4.8 Hz), 80.13 (d, J = 14.1 Hz), 64.02 (d, J = 15.9 Hz), 59.98 (d, J = 73.6 Hz), 53.22 (d, J = 60.6 Hz), 43.42, 41.23 (d, J = 24.8 Hz), 40.16, 38.09 (d, J = 131.5 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{26}BrN_4O_5^+$ [M + H]$^+$, 553.10811; found, 553.10762.

(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(3-chlorophenyl)pyrrolidine-1-carboxylate, TFA Salt (7f). Compound **7f** was prepared using the procedure of compound **7c** and purified by reverse phase HPLC, furnishing the title compound as its TFA salt (8.5 mg, 0.012 mmol, 1.6% yield over six steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.01 and 8.92 (2 s, 1H), 8.51−8.30 (m, 2H), 8.11−7.99 (m, 2H), 7.83 (dd, J = 8.5, 6.7 Hz, 1H), 7.74−7.65 (m, 1H), 7.39 (s, 1H), 7.38−7.24 (m, 3H), 5.43−5.21 (m, 2H), 4.80−4.58 (m, 1H), 4.45 and 4.36 (2 t, J = 9.0 and 8.3 Hz, 1H), 4.03−3.89 (m, 1H), 3.59−3.17 (m, 5H), 3.15−2.94 (m, 1H), 2.45−2.28 (m, 1H), 2.23−2.08 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{25}BrClN_4O_4^+$ [M + H]$^+$, 571.07422; found, 571.07262.

(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(4-chlorophenyl)pyrrolidine-1-carboxylate, TFA Salt (7g). Compound **7g** was prepared using the procedure of compound **7c** and purified by reverse phase HPLC, furnishing the title compound as its TFA salt (8.0 mg, 0.012 mmol, 1.5% yield over six steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.01 and 8.93 (2 d, J = 2.1 Hz, 1H), 8.53−8.32 (m, 2H), 8.14−7.99 (m, 2H), 7.84 (dd, J = 8.6, 6.8 Hz, 1H), 7.70 (td, J = 7.6, 2.8 Hz, 1H), 7.41−7.28 (m, 4H), 5.41−5.22 (m, 2H), 4.82−4.57 (m, 1H), 4.46 and 4.37 (t, J = 9.4 and 8.8 Hz, 1H), 4.04−3.87 (m, 1H), 3.56−3.17 (m, 5H), 3.17−2.94 (m, 1H), 2.35 (ddd, J = 23.1, 19.6, 10.5 Hz, 1H), 2.22−2.07 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{25}BrClN_4O_4^+$ [M + H]$^+$, 571.07422; found, 571.07298.

(2S,4S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(5-fluoro-1H-indol-3-yl)pyrrolidine-1-carboxylate, TFA Salt (7h). *tert*-Butyl 5-fluoro-3-iodo-1*H*-indole-1-carboxylate **21** was prepared from 5-fluoroindole **20** using the procedure reported by Tasch and co-workers[59] and an aliquot (350 mg, 0.969 mmol) then subjected to the Masuda borylation conditions described in the same report, except that 2 equiv of pinacolborane (HBPin) were used. One hour after initiating the borylation reaction, all components for the Suzuki coupling reaction as described in step 1 of compound **7e**, including another aliquot of catalyst, were added to the hot reaction mixture. The bright-red crude material (**18a**, where R = 5-fluoro-1*H*-indol-3-yl) was hydrolyzed as described in step 2 of compound **7e** and briefly purified by extraction and back-extraction, furnishing (*S*)-1-(*tert*-butoxycarbonyl)-4-(5-fluoro-1*H*-indol-3-yl)-2,5-dihydro-1*H*-pyrrole-2-carboxylic acid (**19a**, where R = 5-fluoro-1*H*-indol-3-yl, 159.3 mg, 0.460 mmol, 47.5% yield) as an orange solid that was reduced as described above (step 3) over a period of 42 h. The crude product was purified by reverse-phase HPLC with an isocratic method of 40% acetonitrile in water (+0.1% TFA). An aliquot of (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-(5-fluoro-1*H*-indol-3-yl)pyrrolidine-2-carboxylic acid (15.2 mg, 0.044 mmol) was then elaborated to the final inhibitor using the procedure outlined for compound **7e** with HBTU (1 equiv) as the amide coupling agent instead of EDCI HCl with HOBt (see **7c**). The crude product was purified by reverse-phase HPLC, furnishing the title compound as its TFA salt (14 mg, 0.020 mmol, 45.4% yield over three steps). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotational isomers) δ 11.04 (s, 1H), 9.00 and 8.92 (2 d, J = 2.1 Hz, 1H), 8.48−8.28 (m, 2H), 8.11−7.98 (m, 2H), 7.89−7.78 (m, 1H), 7.68 (q, J = 7.5 Hz, 1H), 7.39−7.27 (m, 3H), 7.01−6.83 (m, 1H), 5.42−5.20 (m, 2H), 4.82−4.59 (m, 1H), 4.53−4.33 (m, 1H), 4.09−3.88 (m, 1H), 3.74−3.57 (m, 1H), 3.55−3.44 (m, 1H), 3.44−3.19 (m, 3H), 3.15−2.95 (m, 1H), 2.47−2.33 (m, 1H), 2.27−2.11 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{28}H_{26}BrFN_5O_4^+$ [M + H]$^+$, 594.11467; found, 594.11403.

(S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)-4-phenyl-2,5-dihydro-1H-pyrrole-1-carboxylate, TFA Salt (8). (*S*)-1-*tert*-Butyl 2-methyl 4-phenyl-1*H*-pyrrole-1,2(2*H*,5*H*)-dicarboxylate was prepared analogously to step 1 of compound **7e** (254 mg, 0.837 mmol, 62.9% yield). The remainder of the synthesis followed a reversed sequence. An aliquot of the intermediate (100 mg, 0.33 mmol) was deprotected using 4 M HCl in dioxane for 30 min. The solvent was carefully evaporated and the residue redissolved in methanol and evaporated again. The HCl salt thus obtained was coupled to quinolin-3-ylmethyl 1*H*-imidazole-1-

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carboxylate analogously to step 3 of the general procedure, furnishing (*S*)-2-methyl 1-(quinolin-3-ylmethyl) 4-phenyl-1*H*-pyrrole-1,2-(2*H*,5*H*)-dicarboxylate, which was purified by preparative TLC. The methyl ester was saponified analogously to step 2 of compound **7e** and the free acid eventually coupled to (*S*)-DHI as described in step 1 of the general procedure but with one equivalent of HBTU in place of EDC and HOBt as the coupling reagent. Reverse-phase HPLC furnished the compound as its TFA salt (5.1 mg, 7.9 μmol, 2.4% yield over four steps). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.06 and 8.94 (2 d, *J* = 2.2 Hz, 1H), 8.54 and 8.43 (2 s, 1 H), 8.53−8.44 (m, 1H), 8.12−8.00 (m, 2H), 7.84 (t, *J* = 7.4 Hz, 1H), 7.69 (t, *J* = 7.5 Hz, 1H), 7.56−7.47 (m, 1H), 7.43−7.29 (m, 2H), 6.32−6.21 (m, 1H), 5.47−5.25 (m, 2H), 5.21−5.04 (m, 1H), 4.78−4.52 (m, 3H), 3.45−2.88 (m, 4H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{26}H_{24}BrN_4O_4^+$ [M + H]$^+$, 535.09754; found, 535.09716.

*(2S,4R)-Quinolin-3-ylmethyl 4-Benzamido-2-((((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**9a**).* Compound **4d** (30 mg, 0.063 mmol) in the semipure form obtained from silica gel chromatography was dissolved in DMF (0.5 mL). Separately, benzoic acid (23.1 mg, 0.189 mmol), EDCI HCl (36.2 mg, 0.189 mmol), and HOBt (8.5 mg, 0.063 mmol) were dissolved in DMF (0.5 mL), and *N*-methylmorpholine (20.8 μL, 0.189 mmol) was added. The two solutions were combined and stirred for 2 h. Water and ethyl acetate (10 mL each) were then added and the mixture partitioned. The organic layer was washed with aqueous sodium bicarbonate (2 × 10 mL) and brine (10 mL), dried over sodium sulfate, and subsequently evaporated. The crude residue was purified by preparative TLC, furnishing the title compound as an off-white solid (11.5 mg, 0.020 mmol, 31.5% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.93 and 8.86 (2 d, *J* = 2.2 Hz, 1H), 8.65 (t, *J* = 7.3 Hz, 1H), 8.57 and 8.51 (2 t, *J* = 6.0 Hz, 1H), 8.36 and 8.28 (2 d, *J* = 1.1 Hz, 1H), 8.03 (d, *J* = 8.4 Hz, 1H), 8.01−7.93 (m, 1H), 7.84−7.80 (m, 2H), 7.77 (ddd, *J* = 8.4, 6.9, 1.5 Hz, 1H), 7.67−7.60 (m, 1H), 7.56−7.50 (m, 1H), 7.50−7.44 (m, 2H), 5.35−5.20 (m, 2H), 4.75−4.67 and 4.57−4.48 (2 m, 2H), 4.38 and 4.29 (2 dd, *J* = 8.6, 5.7 Hz, 1H), 3.84 and 3.78 (2 dd, *J* = 10.6, 6.5 Hz, 1H), 3.50−3.10 (m, 4H), 3.00 and 2.91 (2 dd, *J* = 17.5, 7.5 Hz, 1H), 2.62−2.45 (m, 1H, partly obscured by solvent), 1.96−1.85 (m, 1H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{27}H_{27}BrN_5O_5^+$ [M + H]$^+$, 580.11901; found, 580.11897.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(2-hydroxybenzamido)pyrrolidine-1-carboxylate (**9b**).* Compound **9b** was prepared from semipure **4d** (30 mg, 0.063 mmol) and 2-hydroxybenzoic acid (8.7 mg, 0.063 mmol) as described for compound **9a** but with only one equivalent of all reagents. Purification of the crude mixture by preparative TLC furnished the title compound (6.9 mg, 0.012 mmol, 18.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 12.33 (d, *J* = 3.0 Hz, 1H), 8.96 (t, *J* = 7.0 Hz, 1H), 8.94 and 8.86 (2 d, *J* = 2.2 Hz, 1H), 8.58 and 8.52 (2 t, *J* = 6.0 Hz, 1H), 8.36 and 8.28 (2 d, 1.5 and 2.2 Hz, 1H), 8.06−7.94 (m, 2H), 7.82−7.73 (m, 2H), 7.63 (tdd, *J* = 6.9, 4.5, 1.3 Hz, 1H), 7.40 (td, *J* = 7.7, 1.7 Hz, 1H), 6.93−6.86 (m, 2H), 5.37−5.20 (m, 2H), 4.75−4.67 and 4.62−4.49 (2 br m, 2H), 4.39 and 4.30 (2 dd, *J* = 8.8, 5.3 Hz, 1H), 3.86 and 3.80 (dd, *J* = 10.7, 6.5 Hz, 1H), 3.46 (ddd, *J* = 31.0, 10.6, 5.1 Hz, 1H), 3.39−3.09 (m, 3H), 2.99 and 2.91 (2 dd, *J* = 17.5, 7.4 Hz, 1H), 2.66−2.51 (m, 1H), 1.97−1.85 (m, 1H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{27}H_{27}BrN_5O_6^+$ [M + H]$^+$, 596.11392; found, 596.11434.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(3-hydroxybenzamido)pyrrolidine-1-carboxylate (**9c**).* Compound **9c** was prepared analogously to compound **9b** (4.6 mg, 7.71 μmol, 12.3% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 10.31 (br s, 1H), 8.93 and 8.85 (2 d, *J* = 2.2 Hz, 1H), 8.67−8.50 (m, 2H), 8.36 and 8.28 (2 d, *J* = 1.5 and 2.2 Hz, 1H), 8.12−7.89 (m, 2H), 7.77 (ddd, *J* = 8.4, 6.9, 1.5 Hz, 1H), 7.67−7.60 (m, 1H), 7.31−7.14 (m, 3H), 6.92 (dt, *J* = 7.2, 2.4 Hz, 1H), 5.36−5.20 (m, 2H), 4.82−4.41 (m, 2H), 4.32 (ddd, *J* = 48.9, 8.6, 5.6 Hz, 1H), 3.79 (ddd, *J* = 31.9, 10.6, 6.5 Hz, 1H), 3.53−3.19 (m, 3H), 3.18−3.09 (m, 1H), 2.96 (ddd, *J* = 43.1, 17.5, 7.3 Hz, 1H), 2.58−2.44 (m, 1H, partly obscured by solvent), 2.03−1.83 (m, 1H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{27}H_{27}BrN_5O_6^+$ [M + H]$^+$, 596.11392; found, 596.11339.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(4-hydroxybenzamido)pyrrolidine-1-carboxylate (**9d**, aka CK999).* Compound **9d** was prepared analogously to compound **9e**. It is noteworthy that the title compound precipitated from an ethyl acetate solution of the crude final material in high purity and satisfactory yield (200 mg, 0.335 mmol, 18.8% yield over 5 steps), obviating the need for a chromatographic purification step. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 10.10 (br s, 1H), 8.93 and 8.85 (2 d, *J* = 2.2 Hz, 1H), 8.56 and 8.49 (2 t, *J* = 6.0 Hz, 1H), 8.38 (t, *J* = 7.3 Hz, 1H), 8.35 and 8.28 (2 d, *J* = 1.3 Hz, 1H), 8.03 (d, *J* = 8.4 Hz, 1H), 7.98 (ddd, *J* = 10.1, 8.3, 1.4 Hz, 1H), 7.77 (ddd, *J* = 8.4, 6.9, 1.5 Hz, 1H), 7.68 (dd, *J* = 8.6, 5.4 Hz, 2H), 7.66−7.60 (m, 1H), 6.79 (dd, *J* = 8.7, 2.9 Hz, 2H), 5.39−5.18 (m, 2H), 4.76−4.66 and 4.56−4.44 (2 m, 2H), 4.36 and 4.27 (2 dd, *J* = 8.6, 5.8 Hz, 1H), 3.82 and 3.75 (2 dd, *J* = 10.5, 6.4 Hz, 1H), 3.52−3.27 (m, 2H, partly obscured by residual water), 3.26−3.10 (m, 2H), 2.99 and 2.91 (2 dd, *J* = 17.5, 7.5 Hz, 1H), 2.57−2.43 (m, 1H, partly obscured by solvent), 1.92−1.80 (m, 1H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{27}H_{27}BrN_5O_6^+$ [M + H]$^+$, 596.11392; found, 596.11359.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(nicotinamido)pyrrolidine-1-carboxylate (**9e**, aka CK996). Step 1: (2S,4R)-1-tert-Butyl 2-Methyl 4-(nicotinamido)pyrrolidine-1,2-dicarboxylate.* Commercial (2*S*,4*R*)-1-*tert*-butyl 2-methyl 4-aminopyrrolidine-1,2-dicarboxylate, HCl (500 mg, 1.781 mmol), nicotinic acid (439 mg, 3.56 mmol), EDC HCl (683 mg, 3.56 mmol), and HOBt (241 mg, 1.781 mmol) were dissolved in 10 mL of DMF, and *N*-methylmorpholine (392 μL, 3.56 mmol) was added. The mixture was stirred at room temperature overnight before it was diluted with 100 mL of water and extracted with 100 mL of ethyl acetate. The organic layer was washed with 100 mL of aqueous sodium bicarbonate solution twice, followed by 100 mL of brine. After drying the organic layer over sodium sulfate, the volatiles were removed under reduced pressure, furnishing the product as yellowish oil.

Step 2: (2S,4R)-1-(tert-Butoxycarbonyl)-4-(nicotinamido)-pyrrolidine-2-carboxylic Acid. To saponify the methyl ester, the intermediate was dissolved in THF (30 mL) and methanol (10 mL) and aqueous lithium hydroxide (1 M) was added in portions while the reaction progress was monitored by TLC. Upon completion, an equimolar amount of aqueous hydrochloric acid (1 M) was added and the volatiles evaporated. The mixture was diluted with water to about 50 mL and the pH adjusted to 4−5. The mixture was then extracted with ethyl acetate (6 × 50 mL). The combined organic fractions were dried over sodium sulfate and evaporated under reduced pressure, furnishing (2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-(nicotinamido)-pyrrolidine-2-carboxylic acid (498 mg, 1.485 mmol, 83% yield over steps 1 and 2).

Step 3: (2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(nicotinamido)pyrrolidine-1-carboxylate. The Boc-protected amino acid thus obtained was used without further purification and elaborated to the final inhibitor using the general procedure and purified by silica gel chromatography (80−100% ethyl acetate in pentane followed by 0−10% methanol in ethyl acetate), furnishing (2*S*,4*R*)-quinolin-3-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(nicotinamido)-pyrrolidine-1-carboxylate (171 mg, 0.294 mmol, 19.8% yield over 3 steps). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.98 (dd, *J* = 8.9, 1.8 Hz, 1H), 8.93 and 8.85 (2 d, *J* = 2.2 Hz, 1H), 8.82 (dd, *J* = 12.4, 6.8 Hz, 1H), 8.72−8.68 (m, 1H), 8.41 and 8.35 (2 t, *J* = 6.0 Hz, 1H), 8.34 and 8.26 (2 s, 1H), 8.16 (ddt, *J* = 10.1, 8.0, 2.0 Hz, 1H), 8.03 (d, *J* = 8.4 Hz, 1H), 7.96 (ddd, *J* = 8.0, 6.2, 1.4 Hz, 1H), 7.77 (ddd, *J* = 8.4, 6.8, 1.4 Hz, 1H), 7.63 (dddd, *J* = 8.1, 6.2, 4.7, 1.2 Hz, 1H), 7.50 (dt, *J* = 8.5, 4.5 Hz, 1H), 5.35−5.18 (m, 2H), 4.77−4.69 and 4.61−4.48 (2 m, 2H), 4.44 and 4.35 (2 dd, *J* = 8.2, 5.5, 8.4, 4.9 Hz, 1H), 3.86 and 3.76 (2 dd, *J* = 10.7, 6.6 Hz, 1H), 3.50−3.28 (m, 2H), 3.28−3.12 (m, 2H), 3.03 and 2.95 (2 dd, *J* = 17.6, 7.3 Hz, 1H), 2.29 (ddt, *J* = 27.8, 12.7, 7.6 Hz, 1H), 2.07 (tt, *J* = 11.4, 5.8 Hz, 1H). ^{13}C

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NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.37 (d, J = 41.8 Hz), 165.14 (d, J = 4.1 Hz), 153.76 (d, J = 32.7 Hz), 152.00, 150.50 (d, J = 23.8 Hz), 148.54 (d, J = 4.0 Hz), 147.13 (d, J = 8.4 Hz), 138.09 (d, J = 32.9 Hz), 135.20 (d, J = 3.0 Hz), 134.44 (d, J = 54.0 Hz), 129.92 (d, J = 5.1 Hz), 129.72, 129.64, 128.78, 128.07 (d, J = 10.8 Hz), 127.26 (d, J = 2.6 Hz), 126.98, 123.42, 80.08 (d, J = 17.9 Hz), 64.09 (d, J = 6.8 Hz), 58.69 (d, J = 70.1 Hz), 51.55 (d, J = 62.0 Hz), 48.20 (d, J = 75.0 Hz), 43.42, 41.23 (d, J = 29.1 Hz), 36.20 (d, J = 140.7 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{27}BrN_6O_5^{2+}$ $[M + 2H]^{2+}$, 291.06077; found, 291.06089.

*(2S,4R)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-4-(pyrazine-2-carboxamido)-pyrrolidine-1-carboxylate (**9f**).* Compound **9f** was prepared analogously to compound **9a** (3.6 mg, 6.18 μmol, 9.8% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.23 (dd, J = 8.8, 5.0 Hz, 1H), 9.18 (dd, J = 4.6, 1.5 Hz, 1H), 8.94 and 8.86 (2 d, J = 2.2 Hz, 1H), 8.87 (d, J = 2.5 Hz, 1H), 8.73 (dd, J = 2.5, 1.5 Hz, 1H), 8.61 and 8.56 (2 t, J = 6.0 Hz, 1H), 8.37 and 8.28 (2 d, J = 2.1 Hz, 1H), 8.03 (dd, J = 8.5, 2.7 Hz, 1H), 7.99 (ddd, J = 12.7, 8.3, 1.4 Hz, 1H), 7.78 (ddd, J = 8.4, 6.8, 1.5 Hz, 1H), 7.67–7.60 (m, 1H), 5.37–5.19 (m, 2H), 4.76–4.64 and 4.60–4.52 (2 br m, 2H), 4.42 and 4.34 (2 dd, J = 9.0, 4.0 Hz, 1H), 3.85 and 3.78 (2 dd, J = 10.9, 6.3 Hz, 1H), 3.59–3.38 (m, 2H), 3.31–3.08 (m, 2H), 3.04–2.85 (m, 1H), 2.67–2.53 (m, 1H), 1.93–1.82 (m, 1H). HRMS (ESI-QTOF) m/z: calculated for $C_{25}H_{25}BrN_7O_5^+$ $[M + H]^+$, 582.10951; found, 582.11011.

*1-(((3R,5S)-5-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)-1-((quinolin-3-ylmethoxy)carbonyl)pyrrolidin-3-yl)-carbamoyl)cyclobutanecarboxylic Acid, TFA Salt (**9g**).* The title compound was prepared analogously to compound **9a** but with a 5-fold excess of all reagents relative to starting material **4d**. The solvent was evaporated from the crude amide coupling mixture under vacuum and the residue directly purified by reverse-phase HPLC, furnishing **9g** as its TFA salt (7.9 mg, 0.011 mmol, 17.5% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.02–8.87 (m, 1H), 8.58–8.33 (m, 2H), 8.09–7.92 (m, 3H), 7.87–7.79 (m, 1H), 7.69 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 5.41–5.19 (m, 2H), 4.80–4.66 and 4.61–4.50 (2 br m, 1H), 4.44–4.17 (m, 2H), 3.73 (ddd, J = 34.3, 10.6, 6.5 Hz, 1H), 3.53–3.09 (m, 4H), 2.96 (ddd, J = 39.5, 17.5, 7.5 Hz, 1H), 2.81–2.70 (m, 1H), 2.47–2.25 (m, 4H), 1.93–1.66 (m, 3H). HRMS (ESI-QTOF) m/z: calculated for $C_{26}H_{27}BrN_5O_7^-$ $[M - H]^-$, 600.10993; found, 600.10961.

*(S)-tert-Butyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)pyrrolidine-1-carboxylate (**10a**).* (*S*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-2-carboxylic acid (2 g, 9.29 mmol), HBTU (3.52 g, 9.29 mmol), and 4-methylmorpholine (1.880 g, 18.58 mmol) were dissolved in 20 mL of DMF and stirred for 2 min. Separately, (*S*)-(3-bromo-4,5-dihydroisoxazol-5-yl)methanamine (1.663 g, 9.29 mmol) was diluted with 3 mL of DMF and added to the reaction. The mixture was stirred for 30 min before it was diluted with 100 mL of saturated sodium bicarbonate solution and 100 mL of water and then extracted with 250 mL of ethyl acetate. The organic layer was washed with additional 200 mL aliquots of saturated sodium bicarbonate solution, water, and brine, dried over sodium sulfate, and eventually evaporated to yield (*S*)-*tert*-butyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (3.373 g, 8.96 mmol, 96% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.19 and 8.10 (2 t, J = 6.0 Hz, 1H), 4.76–4.67 (m, 1H), 4.09–4.01 (m, 1H), 3.45–3.13 (m, 6H, partly obscured by residual water), 3.02 (ddd, J = 17.6, 7.4, 3.6 Hz, 1H), 2.15–2.01 (m, 1H), 1.84–1.67 (m, 3H), 1.39 and 1.32 (2 s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 173.22 (d, J = 41.3 Hz), 153.43 (d, J = 36.6 Hz), 138.16 (d, J = 16.9 Hz), 80.20, 78.51 (d, J = 22.0 Hz), 59.62, 46.56 (d, J = 25.9 Hz), 43.39 (d, J = 22.7 Hz), 41.00 (d, J = 77.0 Hz), 30.77 (d, J = 119.3 Hz), 28.10 (d, J = 15.6 Hz), 23.54 (d, J = 94.0 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{14}H_{22}BrN_3O_4Na^+$ $[M + Na]^+$, 398.06859; found, 398.06837.

*(S)-N-(((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)pyrrolidine-2-carboxamide, TFA Salt (**10a′**).* An aliquot of the crude product from **10a** was then deprotected using neat TFA as described in step 2 of the general procedure, but the deprotection mixture was poured into vigorously stirred cold diethyl ether. A sticky oil separated on the wall of the flask over the course of 1 h. The liquids were decanted off, and the oil was washed with more diethyl ether. The oil was taken up in a little anhydrous methanol and the volatiles evaporated again and dried under high vacuum, furnishing an off-white foam that was used without purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.79 (t, J = 5.8 Hz, 1H), 4.84–4.70 (m, 1H), 4.17–4.10 (m, 1H), 3.50–3.40 (m, 2H), 3.38–3.15 (m, 3H, partly obscured by residual water), 3.00 (dd, J = 17.6, 7.2 Hz, 1H), 2.33–2.22 (m, 1H), 1.95–1.84 (m, 2H), 1.83–1.74 (m, 1H).

To obtain the free base, a modified procedure deprotected **10a** (3 g, 7.97 mmol) in 25 mL of TFA/DCM (3:2) for 1 h, before diluting the mixture with water (60 mL). The mixture was partitioned, the aqueous layer taken, and 10 M sodium hydroxide carefully added until a pH of 12 was reached, before it was extracted (4 × 50 mL EtOAc). The combined extracts were dried over sodium sulfate and evaporated to furnish the deprotected amine in its free base as a viscous oil (1.3 g, 4.71 mmol, 59.0% yield) that was used without further purification.

*(S)-Prop-2-yn-1-yl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10b**).* **10a′** (300 mg, 0.769 mmol) and DMAP (19 mg, 0.16 mmol) were dissolved in 4 mL of anhydrous DMF and triethylamine (322 μL, 2.31 mmol) was added, followed by propargyl chloroformate (182 mg, 1.54 mmol). The mixture was stirred overnight and then diluted with 70 mL of water and 70 mL of ethyl acetate. The organic layer was washed with equal volumes of sodium bicarbonate twice, followed by brine, and then dried over sodium sulfate. The volatiles were evaporated and the crude product purified by preparative TLC, furnishing (*S*)-prop-2-yn-1-yl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)-pyrrolidine-1-carboxylate (79.7 mg, 0.223 mmol, 28.9% yield) as a colorless viscous oil that slowly solidified on standing. LC-MS analysis revealed that under the coupling conditions used herein, chloride had displaced bromide from the DHI moiety in about 13% of the purified product. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.24 (dt, J = 18.2, 6.2 Hz, 1H), 4.71 (ddt, J = 13.3, 9.1, 2.6 Hz, 1H), 4.67–4.49 (m, 2H), 4.15 (ddd, J = 8.6, 5.4, 3.5 Hz, 1H), 3.54–3.16 (m, 6H, partly obscured by residual water), 3.02 (ddd, J = 17.5, 12.2, 7.0 Hz, 1H), 2.11 (m, 1H), 1.91–1.67 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.57 (d, J = 12.1 Hz), 153.16 (d, J = 16.2 Hz), 138.10 (d, J = 6.3 Hz), 80.12 (d, J = 12.5 Hz), 79.19 (d, J = 4.3 Hz), 77.34 (d, J = 25.9 Hz), 59.76 (d, J = 79.2 Hz), 52.28 (d, J = 9.4 Hz), 46.86 (d, J = 83.4 Hz), 43.44 (d, J = 21.3 Hz), 41.12 (d, J = 21.3 Hz), 30.88 (d, J = 125.5 Hz), 23.47 (d, J = 115.3 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{13}H_{17}BrN_3O_4^+$ $[M + H]^+$, 358.03970; found, 358.03971.

*(S)-Benzyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)-carbamoyl)pyrrolidine-1-carboxylate (**10c**).* Commercial (*S*)-1-((benzyloxy)carbonyl)pyrrolidine-2-carboxylic acid (418 mg, 1.676 mmol) was coupled to (*S*)-DHI (300 mg, 1.676 mmol) analogously to step 1 of the general procedure, furnishing the title compound in quantitative yield as a viscous oil which was sufficiently pure as obtained from the extraction step. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.28 and 8.22 (2 t, J = 6.1 Hz, 1H), 7.41–7.25 (m, 5H), 5.12–4.96 (m, 2H), 4.72 and 6.63 (2 ddt, J = 10.5, 7.4, 4.9 Hz, 1H), 4.22 and 4.16 (2 dd, J = 8.5, 3.1 Hz, 1H), 3.51–3.15 (m, 5H, partly obscured by residual water), 3.00 (ddd, J = 20.1, 17.5, 7.2 Hz, 1H), 2.22–2.04 (m, 1H), 1.89–1.71 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.85 (d, J = 27.8 Hz), 153.93 (d, J = 29.2 Hz), 138.09 (d, J = 17.4 Hz), 136.99, 128.36 (d, J = 17.2 Hz), 127.72 (d, J = 25.1 Hz), 127.32 (d, J = 57.4 Hz), 80.15 (d, J = 13.4 Hz), 65.86 (d, J = 15.7 Hz), 59.74 (d, J = 79.0 Hz), 46.82 (d, J = 76.2 Hz), 43.39 (d, J = 8.7 Hz), 41.13 (d, J = 33.8 Hz), 30.92 (d, J = 134.0 Hz), 23.52 (d, J = 104.1 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{17}H_{21}BrN_3O_4^+$ $[M + H]^+$, 410.07100; found, 410.07074.

*(S)-3-Fluorobenzyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10d**).* **10a′** (150 mg, 0.384 mmol) and DMAP (9.4 mg, 0.077 mmol) were dissolved in 2.5

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mL of anhydrous DMF, and triethylamine (53.6 μL, 0.384 mmol) was added. Separately, 3-fluorobenzyl 1*H*-imidazole-1-carboxylate (**S3b**) (85 mg, 0.384 mmol) was dissolved in 1.25 mL of anhydrous DCM. The two mixtures were combined and stirred overnight. DCM was then removed under reduced pressure and the residue diluted with 50 mL of saturated sodium bicarbonate solution and extracted with 75 mL of ethyl acetate. The organic layer was washed with more sodium bicarbonate (2 × 50 mL), water (1 × 50 mL), and brine (1 × 50 mL), dried over sodium sulfate, and evaporated. The crude product was purified by preparative TLC in 70% ethyl acetate in pentane, furnishing (*S*)-3-fluorobenzyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.31 and 8.22 (2 t, *J* = 6.0 Hz, 1H), 7.47−7.35 (m, 1H), 7.23−7.08 (m, 3H), 5.12−4.95 (m, 2H), 4.75−4.61 (m, 1H), 4.25 and 4.16 (2 dd, *J* = 8.5, 3.1 Hz, 1H), 3.54−3.26 (m, 4H, partly obscured by residual water), 3.23−3.16 (m, 1H), 3.00 (ddd, *J* = 17.6, 15.1, 7.2 Hz, 1H), 2.23−2.05 (m, 1H), 1.89−1.71 (m, 3H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{17}H_{20}BrFN_3O_4^+$ [M + H]$^+$, 428.06157; found, 428.06224.

*(S)-4-Ethynylbenzyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10e**).* The title compound was prepared according to the published procedure.[20] HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{19}H_{21}BrN_3O_4^+$ [M + H]$^+$, 434.07100; found, 434.07073.

*(S)-2,3-Dimethoxybenzyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10f**).* The title compound was prepared analogously to compound **10d** using 2,3-dimethoxybenzyl 1*H*-imidazole-1-carboxylate (**S3c**) as the carbamate precursor, furnishing a white foam (107.4 mg, 0.228 mmol, 59.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.26 and 8.21 (2 t, *J* = 6.0 Hz, 1H), 7.09−6.98 (m, 2H), 6.94 and 6.85 (2 dd, *J* = 7.4, 1.8 Hz, 1H), 5.11−4.97 (m, 2H), 4.72 and 4.60 (2 ddt, *J* = 10.5, 7.4, 4.9 Hz, 1H), 4.21 and 4.16 (2 dd, *J* = 8.3, 3.0 Hz, 1H), 3.81 and 3.79 (2 s, 3H), 3.75 and 3.71 (2 s, 3H), 3.50−3.13 (m, 5H, partly obscured by residual water), 3.00 (ddd, *J* = 19.4, 17.6, 7.2 Hz, 1H), 2.20−2.04 (m, 1H), 1.87−1.70 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.80 (d, *J* = 15.2 Hz), 153.90 (d, *J* = 22.6 Hz), 152.22 (d, *J* = 17.1 Hz), 146.33 (d, *J* = 60.1 Hz), 138.05 (d, *J* = 18.4 Hz), 130.28 (d, *J* = 5.1 Hz), 124.00 (d, *J* = 10.3 Hz), 120.43 (d, *J* = 76.6 Hz), 112.65 (d, *J* = 44.3 Hz), 80.16 (d, *J* = 10.9 Hz), 61.37 (d, *J* = 33.1 Hz), 60.34 (d, *J* = 14.2 Hz), 59.71 (d, *J* = 77.2 Hz), 55.69 (d, *J* = 3.0 Hz), 46.81 (d, *J* = 80.7 Hz), 43.38 (d, *J* = 8.3 Hz), 41.11 (d, *J* = 28.0 Hz), 30.92 (d, *J* = 140.1 Hz), 23.48 (d, *J* = 111.6 Hz). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{19}H_{25}BrN_3O_6^+$ [M + H]$^+$, 470.09212; found, 470.09162.

*(S)-3-(Benzyloxy)benzyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10g**).* The title compound was prepared analogously to compound **10d** using 3-(benzyloxy)benzyl 1*H*-imidazole-1-carboxylate (**S3d**) as the carbamate precursor, furnishing a white foam (121.6 mg, 0.235 mmol, 61.2% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.29 and 8.22 (2 t, *J* = 6.1 Hz, 1H), 7.48−7.43 (m, 2H), 7.42−7.37 (m, 2H), 7.36−7.31 (m, 1H), 7.30−7.22 (m, 1H), 7.02−6.84 (m, 3H), 5.14−4.92 (m, 4H), 4.76−4.68 and 4.64−4.57 (2 m, 1H), 4.23 and 4.16 (2 dd, *J* = 8.5, 3.1 Hz, 1H), 3.51−3.13 (m, 5H, partly obscured by residual water), 2.98 (ddd, *J* = 34.8, 17.6, 7.2 Hz, 1H), 2.22−2.05 (m, 1H), 1.90−1.71 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.85 (d, *J* = 30.4 Hz), 158.38, 153.86 (d, *J* = 29.3 Hz), 138.62 (d, *J* = 5.6 Hz), 138.05 (d, *J* = 22.9 Hz), 137.06 (d, *J* = 3.4 Hz), 129.49 (d, *J* = 17.2 Hz), 128.47, 127.87 (d, *J* = 4.3 Hz), 127.75 (d, *J* = 7.6 Hz), 119.49 (d, *J* = 53.8 Hz), 113.90 (d, *J* = 48.2 Hz), 113.53 (d, *J* = 12.4 Hz), 80.13 (d, *J* = 16.2 Hz), 69.16 (d, *J* = 4.5 Hz), 65.63 (d, *J* = 18.3 Hz), 59.73 (d, *J* = 74.9 Hz), 46.82 (d, *J* = 78.5 Hz), 43.37 (d, *J* = 5.2 Hz), 41.15 (d, *J* = 29.4 Hz), 30.94 (d, *J* = 136.3 Hz), 23.51 (d, *J* = 103.7 Hz). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{24}H_{27}BrN_3O_5^+$ [M + H]$^+$, 516.11286; found, 516.11274.

*(S)-Pyridin-2-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10h**).* The title compound was prepared analogously to compound **10d** using pyridin-2-ylmethyl 1*H*-imidazole-1-carboxylate (**S3e**) as the carbamate precursor and purified by pTLC (100% ethyl acetate, developed twice), furnishing a white solid (72 mg, 0.175 mmol, 45.5% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.54 and 8.50 (2 d, *J* = 4.2 Hz, 1H), 8.34 and 8.22 (2 t, *J* = 6.1 Hz, 1H), 7.85−7.77 (m, 1H), 7.43−7.28 (m, 2H), 5.16−5.01 (m, 2H), 4.75−4.63 (m, 1H), 4.30 and 4.17 (dd, *J* = 8.5, 3.1 Hz, 1H), 3.59−3.16 (m, 5H, partly obscured by residual water), 3.00 (dt, *J* = 17.5, 7.2 Hz, 1H), 2.23−2.08 (m, 1H), 1.92−1.74 (m, 3H). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{16}H_{20}BrN_4O_4^+$ [M + H]$^+$, 411.06624; found, 411.06594.

*(S)-Pyridin-3-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10i**).* The title compound was prepared analogously to compound **10d** using pyridin-3-ylmethyl 1*H*-imidazole-1-carboxylate (**S3f**) as the carbamate precursor and purified by pTLC (100% ethyl acetate, developed twice), furnishing a white solid (38 mg, 0.092 mmol, 24.0% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.62−8.47 (m, 2H), 8.28 and 8.22 (2 t, *J* = 6.0 Hz, 1H), 7.79 and 7.69 (2 dt, *J* = 8.0, 2.0 Hz, 1H), 7.44−7.35 (m, 1H), 5.14−4.98 (m, 2H), 4.75−4.68 and 4.67−4.61 (2 m, 1H), 4.22 and 4.16 (2 dd, *J* = 8.6, 3.3 Hz, 1H), 3.53−3.26 (m, 4H, partly obscured by residual water), 3.24−3.13 (m, 1H), 2.99 (td, *J* = 17.6, 7.2 Hz, 1H), 2.21−2.06 (m, 1H), 1.88−1.70 (m, 3H).

*(S)-Pyridin-4-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (**10j**).* The title compound was prepared analogously to compound **10d** using pyridin-4-ylmethyl 1*H*-imidazole-1-carboxylate (**S3g**) as the carbamate precursor and purified by pTLC (100% ethyl acetate, developed twice), furnishing a white solid (67 mg, 0.163 mmol, 42.4% yield). ^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.54 (ddd, *J* = 12.0, 4.6, 1.5 Hz, 2H), 8.37 and 8.24 (2 t, *J* = 6.0 Hz, 1H), 7.35 and 7.27 (2 d, *J* = 5.2 Hz, 2H), 5.25−5.00 (m, 2H), 4.77−4.64 (m, 1H), 4.30 and 4.17 (2 dd, *J* = 8.3, 3.1 Hz, 1H), 3.60−3.15 (m, 5H), 3.00 (ddd, *J* = 17.6, 7.2, 4.6 Hz, 1H), 2.24−2.06 (m, 1H), 1.92−1.70 (m, 3H).

*(S)-Quinolin-4-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10k**).* **10a′** (241 mg, 0.617 mmol) was dissolved in 2 mL of DMF and *N*-methylmorpholine (203 μL, 1.850 mmol) was added, followed by solid 4-nitrophenyl (quinolin-4-ylmethyl) carbonate (**S4b**, 100 mg, 0.308 mmol). The mixture was stirred at room temperature for 22 h and then diluted with 40 mL of water and extracted with 60 mL of ethyl acetate. The organic layer was washed with additional water (2 × 40 mL), dilute aqueous potassium carbonate (2 × 40 mL), and brine (1 × 40 mL), dried over sodium sulfate, and eventually evaporated. The residue was purifed by pTLC (2 mm plate thickness and developed in 100% ethyl acetate, R_f approximately 0.2), furnishing (*S*)-quinolin-4-ylmethyl 2-((((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)-methyl)carbamoyl)pyrrolidine-1-carboxylate (44.2 mg, 0.096 mmol, 31.1% yield) as a light-orange oil. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.90 and 8.87 (2 d, *J* = 4.4 Hz, 1H), 8.35 and 8.25 (2 t, *J* = 6.0 Hz, 1H), 8.12−7.98 (m, 2H), 7.83−7.77 (m, 1H), 7.66 (m, 1H), 7.53 and 7.45 (2 d, *J* = 4.4 Hz, 1H), 5.68−5.50 (m, 2H), 4.72 and 4.62 (2 ddt, *J* = 10.4, 7.4, 5.0 Hz, 1H), 4.32 and 4.20 (2 dd, *J* = 8.5, 3.0 Hz, 1H), 3.61−3.14 (m, 5H, partly obscured by residual water), 3.00 (td, *J* = 17.7, 7.2 Hz, 1H), 2.23−2.09 (m, 1H), 1.90−1.74 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.73 (d, *J* = 34.3 Hz), 153.52 (d, *J* = 27.7 Hz), 150.34 (d, *J* = 13.3 Hz), 147.39 (d, *J* = 14.4 Hz), 142.32, 138.05 (d, *J* = 19.8 Hz), 129.54 (d, *J* = 9.4 Hz), 129.46, 126.89 (d, *J* = 8.0 Hz), 125.21 (d, *J* = 20.3 Hz), 123.60 (d, *J* = 9.0 Hz), 118.83 (d, *J* = 41.4 Hz), 80.14 (d, *J* = 10.5 Hz), 62.72, 59.79 (d, *J* = 86.0 Hz), 46.94 (d, *J* = 83.2 Hz), 43.37 (d, *J* = 6.7 Hz), 41.18 (d, *J* = 34.0 Hz), 30.97 (d, *J* = 144.3 Hz), 23.49 (d, *J* = 105.2 Hz). HRMS (ESI-QTOF) *m*/*z*: calculated for $C_{20}H_{22}BrN_4O_4^+$ [M + H]$^+$, 461.08189; found, 461.08155.

*(S)-Quinoxalin-2-ylmethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10l**).* Compound **10l** was prepared from 4-nitrophenyl (quinoxalin-2-ylmethyl) carbonate (**S4c**) by the procedure of compound **10k** and purified by pTLC (2 mm plate thickness and developed in 100% ethyl acetate, R_f

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approximately 0.35), furnishing the title compound as a colorless oil (29.8 mg, 0.064 mmol, 41.9% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 9.02 and 8.90 (2 s, 1H), 8.34 and 8.24 (2 t, J = 6.0 Hz, 1H), 8.16−8.03 (m, 2H), 7.92−7.83 (m, 2H), 5.44−5.25 (m, 2H), 4.71 and 4.63 (2 ddt, J = 10.2, 7.2, 5.0 Hz, 1H), 4.33 and 4.18 (2 dd, J = 8.4, 3.2 Hz, 1H), 3.61−3.26 (m, 4H, partly obscured by residual water), 3.25−3.16 (m, 1H), 2.99 (ddd, J = 17.5, 12.3, 7.2 Hz, 1H), 2.25−2.09 (m, 1H), 1.92−1.74 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.70 (d, J = 43.0 Hz), 153.62 (d, J = 35.7 Hz), 152.37, 144.44 (d, J = 16.1 Hz), 141.31 (d, J = 4.7 Hz), 140.85 (d, J = 13.9 Hz), 137.99 (d, J = 28.9 Hz), 130.60 (d, J = 6.4 Hz), 130.16 (d, J = 9.0 Hz), 128.98, 128.79 (d, J = 8.5 Hz), 80.08 (d, J = 15.3 Hz), 65.68 (d, J = 9.5 Hz), 59.87 (d, J = 77.5 Hz), 46.96 (d, J = 74.0 Hz), 43.38, 41.18 (d, J = 26.1 Hz), 30.94 (d, J = 129.8 Hz), 23.54 (d, J = 103.8 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{19}H_{21}BrN_5O_4^+$ [M + H]$^+$, 462.07714; found, 462.07761.

*(S)-(S)-1-(Naphthalen-2-yl)ethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10m**).* (*S*)-1-(Naphthalen-2-yl)ethyl 1*H*-imidazole-1-carboxylate (**S3h**) was elaborated to the final inhibitor adapting the procedure for **10d**, furnishing the title compound in 32.1% yield (58.5 mg, 0.123 mmol). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.35 and 8.18 (2 t, J = 6.0 Hz, 1H), 7.96−7.77 (m, 4H), 7.56−7.40 (m, 3H), 5.89−5.77 (m, 1H), 4.73 and 4.56 (2 ddt, J = 10.2, 7.3, 4.9 Hz, 1H), 4.33 and 4.08 (2 dd, J = 8.7, 3.1 Hz, 1H), 3.61−3.34 and 3.30−3.07 (2 m, 5H), 3.03 and 2.93 (dd, J = 17.6, 7.3 Hz, 1H), 2.24−2.15 and 2.11−2.02 (2 m, 1H), 1.92−1.70 (m, 3H), 1.56 and 1.50 (2 d, J = 6.6 Hz, 3H). HRMS (ESI-QTOF) m/z: calculated for $C_{22}H_{24}BrN_3O_4Na^+$ [M + Na]$^+$, 496.08424; found, 496.08369.

*(S)-(R)-1-(Naphthalen-2-yl)ethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10n**).* (*R*)-1-(Naphthalen-2-yl)ethyl 1*H*-imidazole-1-carboxylate (**S3i**) was elaborated to the final inhibitor adapting the procedure for **10d**, furnishing the title compound in 27.6% yield (50.3 mg, 0.106 mmol). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.43 and 8.17 (2 t, J = 6.0 Hz, 1H), 7.95−7.82 (m, 4H), 7.57−7.47 (m, 3H), 5.88−5.78 (m, 1H), 4.83−4.74 and 4.70−4.62 (2 m, 1H), 4.29 and 4.18 (2 dd, J = 8.3, 3.4 Hz, 1H), 3.58−3.25 and 3.16−3.11 (2 m, 5H, partly obscured by residual water), 3.07 and 2.95 (2 dd, J = 17.6, 7.1 Hz, 1H), 2.22−2.07 (m, 1H), 1.91−1.71 (m, 3H), 1.54 and 1.43 (2 d, J = 6.5 Hz, 3H). HRMS (ESI-QTOF) m/z: calculated for $C_{22}H_{24}BrN_3O_4Na^+$ [M + Na]$^+$, 496.08424; found, 496.08395.

*(S)-(R)-1-(Quinolin-3-yl)ethyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate (**10o**).* 1-(Quinolin-3-yl)ethanone was asymmetrically reduced by a low temperature variation of the CBS reaction.[60,61] Thus, the ketone substrate (200 mg, 1.17 mmol) and (*S*)-1-methyl-3,3-diphenylhexahydropyrrolo[1,2-*c*][1,3,2]oxazaborole (32.4 mg, 0.117 mmol) were dissolved in 10 mL of anhydrous toluene and cooled to −78 °C. Catecholborane (250 μL, 2.34 mmol) was added in a dropwise fashion and the mixture stirred until TLC showed full consumption of the starting material. A few drops of water were added and the mixture allowed to reach room temperature. Ethyl acetate (50 mL) was added and the mixture washed with sodium bicarbonate followed by brine (50 mL each). The organic layer was dried over sodium sulfate, evaporated, and the (*R*)-1-(quinolin-3-yl)ethanol enriched by a brief purification by preparative TLC. The alcohol was dissolved in dry acetonitrile (5 mL), and 1,1′-carbonyldiimidazole (169 mg, 1.039 mmol) was added and the mixture stirred for 30 min. The solvent was then evaporated and the crude residue taken up in ethyl acetate (50 mL) and washed with sodium bicarbonate and brine and dried over sodium sulfate. The volatiles were evaporated and the crude product purified by preparative TLC, furnishing (*R*)-1-(quinolin-3-yl)ethyl 1*H*-imidazole-1-carboxylate (154 mg, 0.576 mmol, 49.3% yield over two steps) as a yellowish solid. An aliquot of the carbamate building block (70 mg, 0.262 mmol) was elaborated to the final inhibitor using the procedure of **10d** and purified by preparative TLC to afford a white solid (38.7 mg, 0.081 mmol, 31.1% yield). ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.96 and 8.92 (2 d, J = 2.2 Hz, 1H), 8.44 and 8.18 (2 t, J = 6.0 Hz, 1H), 8.33 and 8.28 (2 d, J = 2.2 Hz, 1H), 8.05−7.98 (m, 2H), 7.76 (ddd, J = 8.4, 7.0, 1.3 Hz, 1H), 7.63 (ddd, J = 8.0, 6.8, 1.3 Hz, 1H), 5.96−5.85 (m, 1H), 4.83−4.73 and 4.70−4.60 (2 m, 1H), 4.29 and 4.18 (2 dd, J = 8.3, 3.5 Hz, 1H), 3.62−3.23 and 3.15−3.09 (2 m, 5H, partially obscured by residual water), 3.06 and 2.94 (2 dd, J = 17.5, 7.1 Hz, 1H), 2.23−2.08 (m, 1H), 1.90−1.72 (m, 3H), 1.60 and 1.48 (2 d, J = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.91 (d, J = 67.7 Hz), 153.27 (d, J = 41.2 Hz), 149.31, 147.08, 138.06 (d, J = 7.9 Hz), 135.13 (d, J = 14.6 Hz), 132.29 (d, J = 8.0 Hz), 129.50, 128.70 (d, J = 3.6 Hz), 128.18, 127.31, 126.88 (d, J = 2.9 Hz), 80.15 (d, J = 10.1 Hz), 70.39, 59.72 (d, J = 65.9 Hz), 46.77 (d, J = 50.7 Hz), 43.43 (d, J = 26.7 Hz), 41.27 (d, J = 64.9 Hz), 30.85 (d, J = 108.0 Hz), 23.54 (d, J = 85.9 Hz), 22.40 (d, J = 12.4 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{21}H_{24}BrN_4O_4^+$ [M + H]$^+$, 475.09754; found, 475.09802.

*(S)-(1H-Benzo[d]imidazol-2-yl)methyl 2-((((S)-3-Bromo-4,5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate, TFA Salt (**10p**).* (1*H*-Benzo[*d*]imidazol-2-yl)methanol (62.7 mg, 0.423 mmol) and 1,1′-carbonyldiimidazole (68.6 mg, 0.423 mmol) were dissolved in 3 mL of anhydrous acetonitrile/DCM (1:1) and stirred for 15 min. The solvent was then evaporated under reduced pressure at room temperature and the residue suspended in 1 mL of anhydrous DCM. A mixture of **10a′** (150 mg, 0.38 mmol), triethylamine (54 μL, 0.38 mmol), and DMAP (4.70 mg, 0.038 mmol) in 2 mL of anhydrous DMF was then added and stirred overnight. All volatiles were subsequently removed under high vacuum, furnishing a yellow residue. An aliquot of this residue was purified by reverse-phase HPLC, furnishing the title compound as a TFA salt. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.32 and 8.27 (2 t, J = 6.0 Hz, 1H), 7.77 (ddd, J = 9.3, 6.1, 3.2 Hz, 2H), 7.50−7.44 (m, 2H), 5.58−5.31 (m, 2H), 4.70 and 4.62 (2 ddt, J = 10.5, 7.2, 5.0 Hz, 1H), 4.37 and 4.18 (2 dd, J = 8.5, 3.4 Hz, 1H), 3.60−3.15 (m, 5H), 2.98 (ddd, J = 17.6, 7.1, 4.8 Hz, 1H), 2.13 (m, 1H), 1.91−1.73 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6, mixture of rotational isomers) δ 172.40 (d, J = 32.0 Hz), 153.11, 149.57 (d, J = 19.4 Hz), 138.02 (d, J = 16.1 Hz), 132.95 (d, J = 21.1 Hz), 124.98 (d, J = 11.9 Hz), 114.53, 80.04 (d, J = 18.0 Hz), 59.94 (d, J = 67.8 Hz), 57.91 (d, J = 27.2 Hz), 47.05 (d, J = 79.0 Hz), 43.40, 41.28 (d, J = 27.5 Hz), 30.92 (d, J = 106.0 Hz), 23.47 (d, J = 114.5 Hz). HRMS (ESI-QTOF) m/z: calculated for $C_{18}H_{21}BrN_5O_4^+$ [M + H]$^+$, 450.07714; found, 450.07700.

*(S)-(1-Methyl-1H-benzo[d]imidazol-2-yl)methyl 2-((((S)-3-Bromo-4,5-dihydro-isoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate, TFA Salt (**10q**).* (1*H*-Benzo[*d*]imidazol-2-yl)methanol (300 mg, 2.025 mmol) was methylated with methyl iodide following the procedure of Popov, furnishing (1-methyl-1*H*-benzo[*d*]imidazol-2-yl)methanol (205 mg, 1.264 mmol, 62.4% yield) as a white solid by filtration.[62] ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.58 (dt, J = 7.8, 1.0 Hz, 1H), 7.52 (dt, J = 8.2, 1.0 Hz, 1H), 7.24 (ddd, J = 8.0, 7.1, 1.3 Hz, 1H), 7.17 (ddd, J = 8.2, 7.2, 1.3 Hz, 1H), 5.58 (br s, 1H), 4.71 (s, 2H), 3.82 (s, 3H). An aliquot of this solid was reacted with 1,1′-carbonyldiimidazole and in situ coupled to **10a′** as described for compound **10p**. ^{1}H NMR (500 MHz, DMSO-d_6, mixture of rotational isomers) δ 8.28 (dt, J = 16.3, 6.1 Hz, 1H), 7.84 (dd, J = 11.6, 8.1 Hz, 1H), 7.77 (dd, J = 11.5, 8.0 Hz, 1H), 7.54−7.43 (m, 2H), 5.58−5.34 (m, 2H), 4.70 and 4.48 (2 ddt, J = 10.4, 7.2, 5.0 Hz, 1H), 4.26 and 4.17 (2 dd, J = 8.4, 3.1 Hz, 1H), 3.97 and 3.90 (2 s, 3H), 3.56−3.31 (m, 3H), 3.27−3.10 (m, 2H), 2.95 (ddd, J = 35.8, 17.6, 7.1 Hz, 1H), 2.22−2.06 (m, 1H), 1.92−1.70 (m, 3H). HRMS (ESI-QTOF) m/z: calculated for $C_{19}H_{23}BrN_5O_4^+$ [M + H]$^+$, 464.09279; found, 464.09276.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Potency and selectivity of hydroxy-proline and prolyl amide inhibitors. Additional synthetic methods and supplemental compound characterization data (NMR and LC-MS). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): C. Khosla and C. Kloeck are stockholders of Sitari Pharmaceuticals, a company that has licensed a patent application describing some of the compounds from Stanford University.

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ABBREVIATIONS USED

aka, also known as; DHI, 3-bromo-4,5-dihydroisoxazole; FXIIIa, Factor XIIIa; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; TG, transglutaminase; ZQG, ((benzyloxy)carbonyl)-L-glutaminylglycine; ZQS, ((benzyloxy)carbonyl)-L-glutaminyl-L-serine

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