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Potent, nonpeptide inhibitors of human mast cell tryptase. Synthesis and biological evaluation of novel spirocyclic piperidine amide derivatives

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Abstract—We have explored a series of spirocyclic piperidine amide derivatives (**5**) as tryptase inhibitors. Thus, **4** (JNJ-27390467) was identified as a potent, selective tryptase inhibitor with oral efficacy in two animal models of airway inflammation (sheep and guinea pig asthma models). An X-ray co-crystal structure of **4** · tryptase revealed a hydrophobic pocket in the enzyme's active site, which is induced by the phenylethynyl group and is comprised of amino acid residues from two different monomers of the tetrameric protein.

The serine protease tryptase (EC 3.4.21.59) is an important mediator of mast cell-dependent allergic and inflammatory responses.[1] This trypsin-like, heparin-stabilized, homotetrameric enzyme is stored in mast cell granules and released upon degranulation.[1] Inhibitors of tryptase have therapeutic potential for treating allergic or inflammatory disorders such as asthma[2] and inflammatory bowel disease.[3]

From our work with a series of α-ketoheterocycle-based tryptase inhibitors,[4] RWJ-58643 (**1**; RWJ-56423 for the L-Arg diastereomer) was selected for proof-of-concept evaluation in humans. In patients with allergic rhinitis, RWJ-58643 demonstrated efficacy when administered intranasally.[5] However, since **1** inhibits both tryptase and trypsin with nearly equal potency, this study was not a pure examination of tryptase inhibition. A similar concern applies to the human clinical studies with APC-366, which is an inhibitor of tryptase, trypsin, and thrombin.[6] Another issue with **1** is its lack of oral bioavailability. Thus, we have continued to search for potent tryptase inhibitors with good enzyme selectivity and oral efficacy.

A nonpeptide series of tryptase inhibitors from Aventis Pharmaceuticals (now Sanofi-Aventis),[7] represented by key compound **2**,[7d] attracted our attention. In particular, we were intrigued by the fact that certain potent derivatives showed favorable enzyme selectivity and good oral bioavailability.[7a] We modeled des-fluoro analogue **3** in the active site of human β-tryptase and determined that a model of a spirocyclic congener, **4**, in a computed low-energy conformation, had good overlap (Fig. 1). Consequently, we decided to explore such spirocyclic piperidine amide derivatives in more detail. Herein, we report on the synthesis and biological assessment of compounds with general structure **5** (Table 1). From

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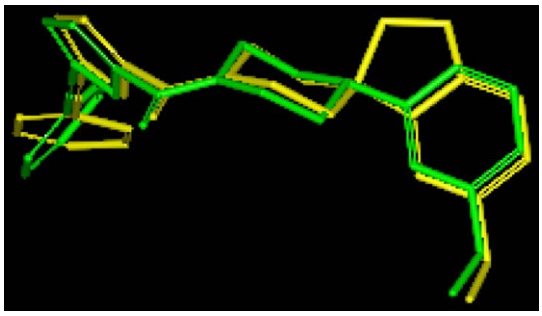


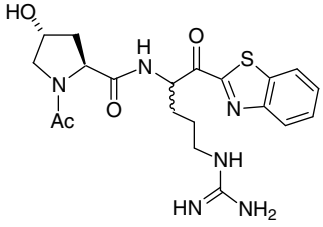
Figure 1. Stick models of three-dimensional structures of **3** (green; enzyme-bound conformation from computer-assisted modeling) and **4** (yellow; energy-minimized conformation).

this study, we determined that **4** (JNJ-27390467) is a potent, selective tryptase inhibitor with oral efficacy in animal models of airway inflammation.

Most of the compounds that we investigated as tryptase inhibitors were spiro-dihydrobenzofuran analogues of **4**, namely **5a**–**k** and **5q**–**s** (Table 1). For comparison, we also examined some dihydroindole analogues, **5l**–**o**, and indane analogue **5p**. The synthesis of **4**, **5e**–**k**, and **5q**–**s** necessitated key intermediate **6**, for which we devised an efficient synthetic route (Scheme 1). Thus, Boc-piperidinone **7** was reacted with phenylsulfinylmethyl lithium to give hydroxyl sulfoxide **8**, which was subjected to a [2,3]-rearrangement[8] according to the procedure described by MacCoss et al.[9] Allyl alcohol **9** was converted to the unstable chloride **10**, which was coupled with 2-bromo-4-cyanophenol to give ether **11**. The crucial radical-based cyclization[10] of **11** to **12** was effected in high yield in 0.02 M solution by using tributyltin hydride and 2,2′-azobis(isobutyronitrile). The nitrile group in **12** was hydrogenated with Raney nickel, the resultant primary amine was protected as a phthalimide (Pht), and the Boc group was removed to yield **6**, which was isolated as a hydrochloride salt. The preparation of **4** serves as a representative example of the end-game. Key intermediate **6** was acylated with 5-(phenylethynyl)furan-2-carboxylic acid by using bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) as an activating agent and the phthalimide was removed with methylhydrazine to give **4**, which was isolated and purified as a benzoic acid salt.

The syntheses of **5a**–**d** are depicted in Scheme 2. Removal of the Boc group from **12** with trifluoroacetic acid followed by coupling with 5-(phenylethynyl)furan-2-carboxylic acid using BOP-Cl generated nitrile **13**. Pinner reaction of **13** with hydrochloric acid in ethanol at 5 °C, followed by neutralization with 3 N NaOH afforded ethyl imidate **14** as the free base, which was reacted with either anhydrous ammonia, methylamine or hydrazine. Purification by reverse-phase HPLC then yielded **5a**, **5b**, and **5c**, respectively. Carboxamide **5d** was isolated by reverse-phase HPLC as a side-product from the reaction of nitrile **13** with hydroxylamine at 90 °C in a mixture of ethanol/1,4-dioxane/water (3:1:1).

The synthesis of spiro-indoline derivatives **5l**–**o** required key intermediate **15** (Scheme 3). Thus, spiropiperidine **16**[11] was N-formylated to give **17**, which was converted to **18** by a Tscherniac–Einhorn reaction[12] involving *N*-(hydroxymethyl)phthalimide and concentrated sulfuric acid. The *N*-methyl group was eliminated by using α-chloroethyl chloroformate (ACE-Cl)[13] in the presence of Proton Sponge [1,8-bis-(dimethylamino)naphthalene], and the resulting ACE-carbamate was readily cleaved to yield **15**, as a dihydrochloride salt. For **5l**–**o**, key intermediate **15** was acylated with the *N*-succinimidyl ester[14] of 5-(phenylethynyl)furan-2-carboxylic acid to furnish **19** [Ar = 5-(PhC≡C)-2-furyl]. Compound **5l** was obtained by removing the phthalimide with methylhydrazine. In the case of **5m**–**o**, **19** was functionalized on the aniline nitrogen, as shown, and the final product was obtained by removing the phthalimide with methylhydrazine. The four target compounds were purified by reverse-phase HPLC and isolated as trifluoroacetate salts.



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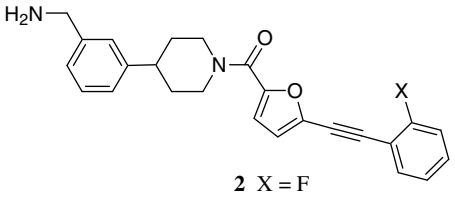
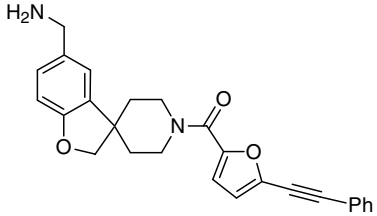


2 X = F
3 X = H



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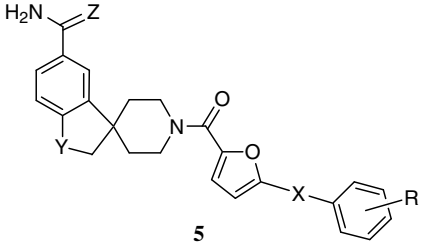

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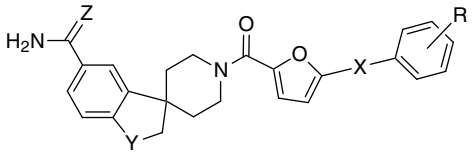
Table 1. Compounds and their inhibition of tryptase and trypsin[a]



Compound	X	Y	Z	R	Tryptase[b] IC_{50} (nM)	Tryptase[b] K_i (nM)	Trypsin[c] IC_{50} (nM)
4	C≡C	O	H_2	H	3.6 ± 0.3[d]	3.7 ± 0.5	18,000
5a	C≡C	O	NH	H	4.5 ± 0.5		1400
5b	C≡C	O	NMe	H	780 ± 220		>50,000
5c	C≡C	O	NNH_2	H	59 ± 2		>50,000
5d	C≡C	O	O	H	29%		39,000
5e	C≡C	O	H_2	2-F	4.3 ± 1.5	3.0 ± 0	12,000
5f	C≡C	O	H_2	2-Cl	5.2 ± 0.3	3.4 ± 0	38,000
5g	C≡C	O	H_2	2-Me	1.2 ± 0.3	4.0 ± 0	12,000
5h	C≡C	O	H_2	2-OMe	13 ± 1		5500
5i	C≡C	O	H_2	4-Me	8.3 ± 1.0		13,000
5j	C≡C	O	H_2	4-*t*-Bu	700 ± 160		13,000
5k	C≡C	O	H_2	4-OH	3200 ± 300		23,000
5l	C≡C	NH	H_2	H	23 ± 3[e]		>50,000
5m	C≡C	NAc	H_2	H	15 ± 2		>50,000
5n	C≡C	NSO_2Me	H_2	H	1.8 ± 0.6	7.1 ± 0	>50,000
5o	C≡C	NSO_2Ph	H_2	H	5.7 ± 2.0	16 ± 0	6700
5p	C≡C	CH_2	H_2	H	3.1 ± 0.2		7700
5q	CH_2CH_2	O	H_2	H	82 ± 27		47,000
5r	S	O	H_2	H	90 ± 40		9100
5s	—[f]	O	H_2	H	28 ± 7		8600
1[g]					13 ± 1	22 ± 5[h]	10 ± 4[i]

[a] Target compounds were purified by reversed-phase HPLC on a Kromasil C-18 column eluting with acetonitrile/water mixtures containing 0.2% trifluoroacetic acid. All compounds were characterized by ESI-MS and 300-MHz ^{1}H NMR.

[b] Inhibition of human β-tryptase according to the assay described in Ref. 18; ±standard error and $N = 2$ unless noted otherwise. For compounds with very low potency, percent inhibition at 50 μM may be given.

[c] Inhibition of human pancreatic trypsin according to the assay described in Ref. 19; $N = 1$–4.

[d] $N = 6$.

[e] $N = 4$.

[f] Linker X is a direct bond.

[g] Reference standard. Nearly a 1:1 mixture of L-Arg and D-Arg diastereomers, the former of which (RWJ-56423) is the active species ($K_i = 10 \pm 3$ nM) (Ref. 4).

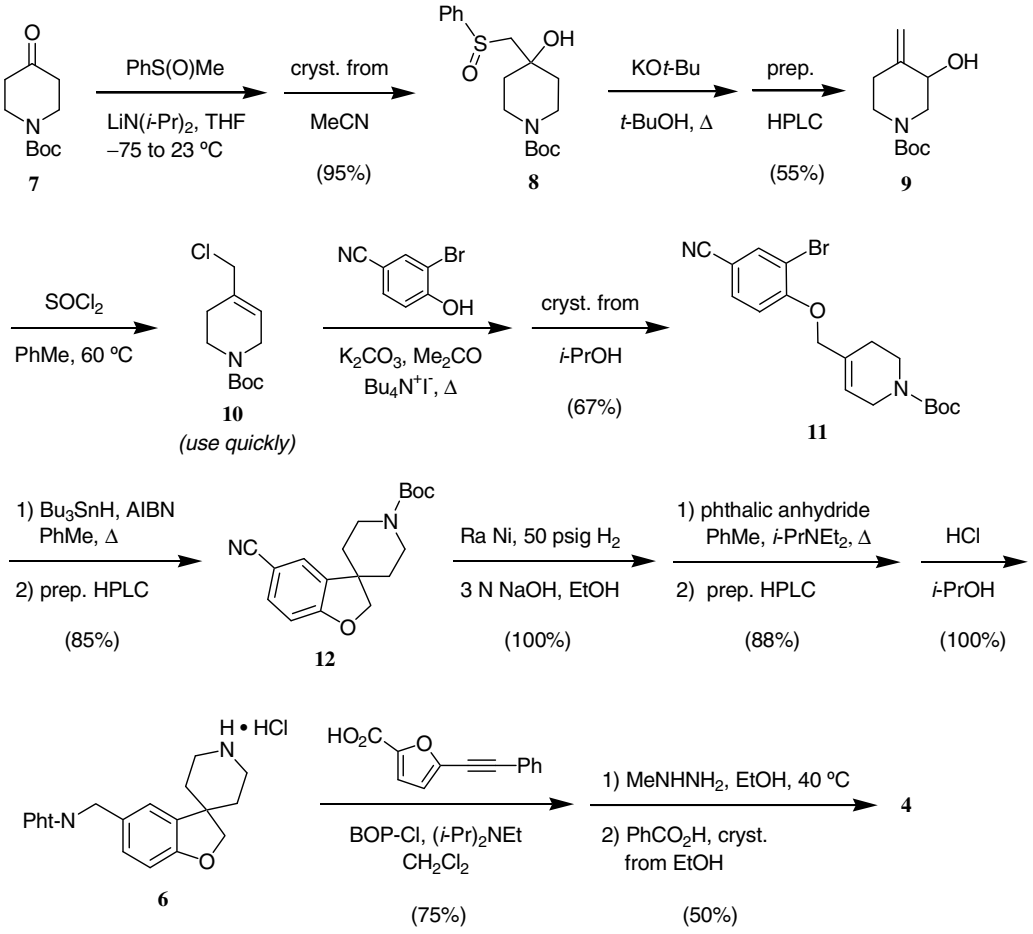
[h] $N = 13$ (Ref. 4).

[i] $K_i = 6.0 \pm 1.0$, $N = 9$ (Ref. 4).

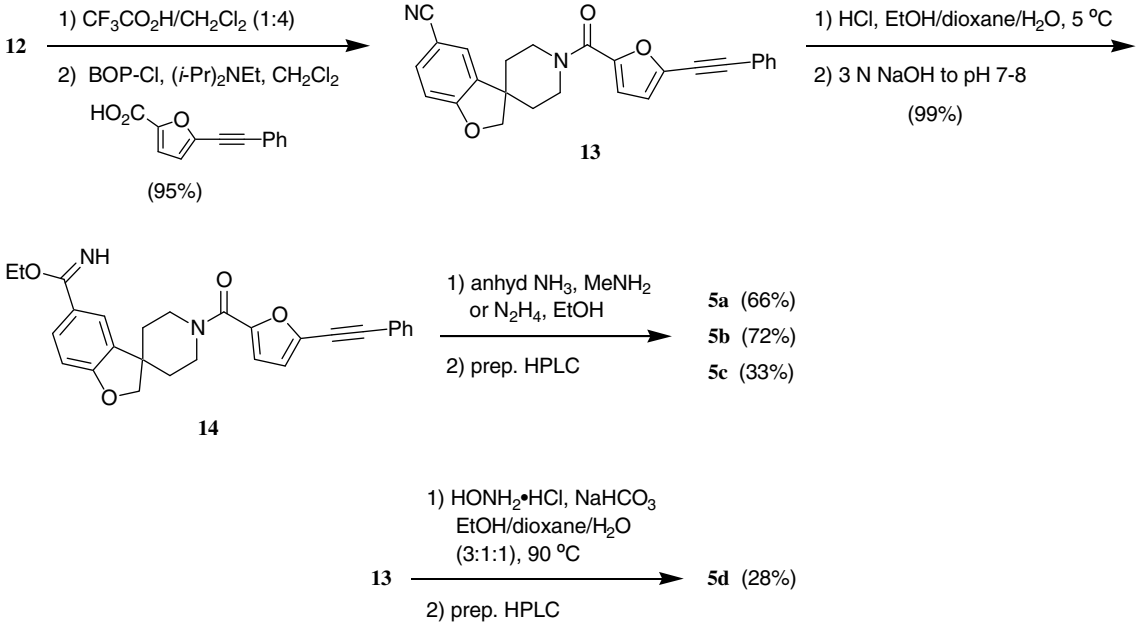
Spiro-indane derivative **5p** was prepared as outlined in Scheme 4. Indene **20**[15] was doubly alkylated to form spirocyclic piperidine **21**,[16] which was hydroformylated with carbon monoxide under Pd(II) catalysis, as shown, to give **22**.[17] Treatment of **22** with hydrazine provided the corresponding hydrazone, which was hydrogenated to the amine, in conjunction with alkene reduction, to yield **23**. The amine was protected as a phthalimide and the Boc group was eliminated to give **24**. Acylation of **24** with 3-ethyl-1-(dimethylaminopropyl)carbodiimide (EDC) and removal of the phthalimide with hydrazine afforded **5p**, which was purified by reverse-phase HPLC and isolated as a trifluoroacetate salt.

The compounds of interest, **4** and **5a**–**s**, were first evaluated for inhibition of human β-tryptase by using a chromogenic assay (Table 1).[18] As a counterscreen for selectivity, we also tested these compounds for inhibition of human pancreatic trypsin by using a chromogenic assay.[19] Prototype **4** turned out to be a very potent tryptase inhibitor, with an IC_{50} value of 3.7 nM; it also had excellent selectivity over trypsin of ~5000-fold. The primary benzylamine of **4** could be replaced by a primary amidine and be virtually equipotent (viz. **5a**), although selectivity vs trypsin was somewhat eroded (~300-fold). Whereas addition of an *N*-methyl substituent to the amidine was unfavorable (viz. **5b**; $IC_{50} = 780$ nM), an amino substituent had less impact on potency (viz. **5c**; $IC_{50} = 59$ nM). By contrast, a primary carboxamide group was not effective (viz. **5d**; $IC_{50} > 50{,}000$ nM). These results are consistent with the ability of this group on the benzene ring to occupy the S1 pocket and interact with the resident Asp-189.

With the primary amine held fixed, we looked at some substituents on the phenyl ring of the alkyne subunit (Table 1). Substitution of the 2-position with fluoro (**5e**), chloro (**5f**), or methyl (**5g**) had a little effect on the single-digit nanomolar potency, although the potency of the 2-methoxy compound (**5h**) was a bit atten-

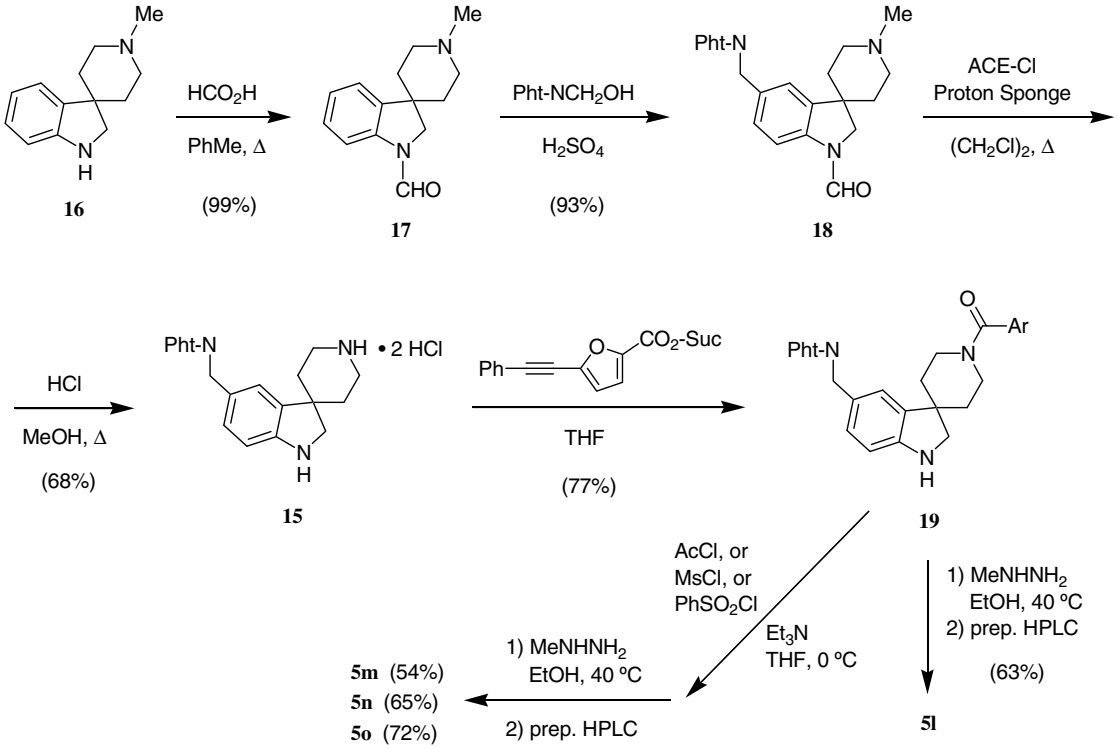


Scheme 1.

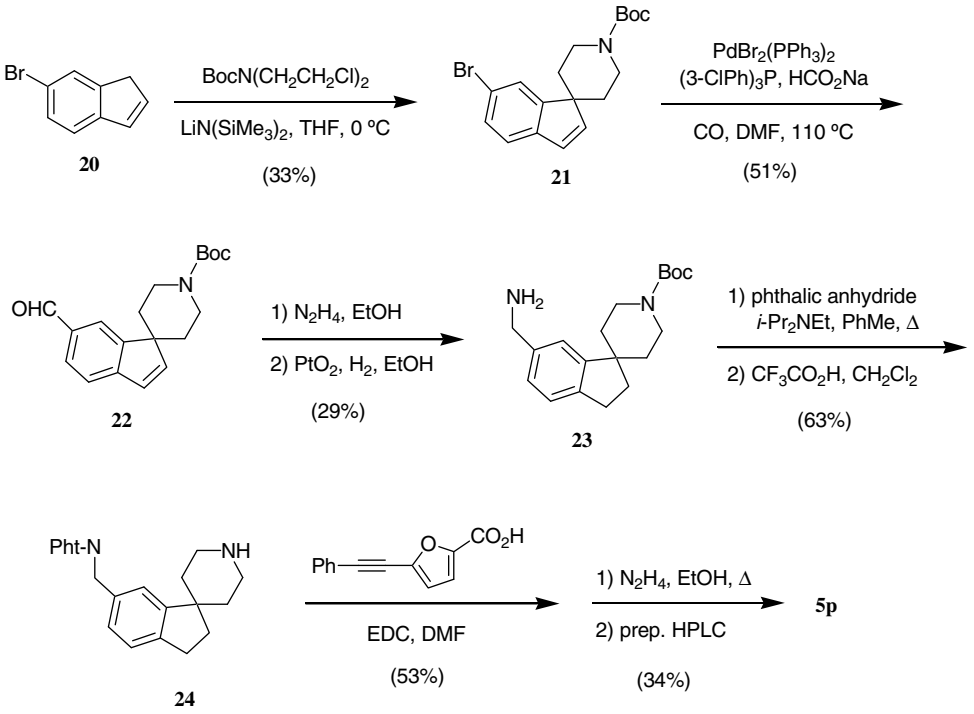


Scheme 2.

uated. Spirocyclic piperidine amides **5e** (K_i = 3.0 nM) and **4** (K_i = 3.7 nM) were equipotent, whereas the values reported[7d] for congeners **3** and **2** (the corresponding piperidine amides) differed by 3.6-fold (K_i's of 8.7 and 31 nM, respectively). 4-Methyl spiropiperidine amide **5i** was in a comparable potency range (IC_{50} = 8.3 nM),



Scheme 3.



Scheme 4.

but the 4-*tert*-butyl (**5j**; IC_{50} = 700 nM) and 4-hydroxy (**5k**; IC_{50} = 3200 nM) analogues were much less potent. The negative effect of a bulky hydrophobic group or a polar group at this phenyl 4-position indicates that there are critical limitations in the tryptase binding mode for this molecular segment (vide infra).

The spiro-indoline (**5l–o**) and spiro-indane (**5p**) analogues of **4** were also potent tryptase inhibitors, with IC_{50} values in the range of 3–23 nM. Thus, tryptase inhibitory potency is not particularly sensitive to the chemical nature of this spirocyclic ring or to the indole N-substituents, which may be related to the fact that

this portion of the inhibitor ligand is solvent-exposed in the tryptase complex, according to computer modeling and an X-ray co-crystal structure of **4** · tryptase (vide infra).

Isosteres **5q** and **5r** have an ethylidene or a sulfur atom, respectively, in place of the ethynyl linker in **4**. Although the spacing for these two modifications is similar to that for an ethynyl group, the inhibitory potencies for **5q** and **5r** suffered by ~20-fold relative to **4**. This finding suggests that a rigid linear directionality is important for this molecular fragment to achieve optimal binding within the tryptase active-site cleft. Interestingly, different comparisons were found in the reported piperidine amide series (viz. **3**),[7a] in that the 3-(phenylethynyl)benzoyl analogue was 2-fold more potent than the 3-(phenylethyl)benzoyl analogue, and the [5-(phenylethynyl)-3-pyridinyl]carbonyl analogue was 12-fold *less potent* than the [5-(phenylethyl)-3-pyridinyl]carbonyl analogue. Although **5s** has the W linker eliminated, it displayed a 3-fold potency improvement (IC_{50} = 28 nM) compared to **5q** and **5r**.

Compound **4** was found to be a full competitive inhibitor of tryptase (K_i = 3.7 nM) and trypsin (K_i = 2500 nM), with a favorable selectivity ratio of 675 on the basis of K_i values. Enzyme inhibition studies conducted with **4** and additional serine proteases revealed a generally high level of selectivity. At 50 μM, **4** inhibited factor X_a, chymase, and cathepsin G less than 30%; and at 10 μM, **4** inhibited thrombin, plasmin, tissue-type plasminogen activator (tPA), factor VIIa, kallikrein, urokinase-type plasminogen activator (uPA), streptokinase, human leukocyte elastase, and chymotrypsin less than 15%. Since **4** possesses a primary benzylamine group, we examined its inhibition of monoamine oxides A and B. It was a weak inhibitor of MAO-A and MAO-B with IC_{50} values of 23 and 72 μM, respectively. We also evaluated **4** as a substrate for MAO-B and found that it was quite stable: 95% of **4** remaining at 30 min (compared with 13% remaining at 30 min for benzylamine itself).

The pharmacokinetics of **4** in rats and dogs were favorable for oral administration. In rats, **4** had an F value of 100%, an oral C_{max} of 1.1 μM, an oral $t_{1/2}$ of 7 h, and a large volume of distribution (V_d) of 17 L/kg. In dogs, **4** had an F value of 65%, an oral C_{max} of 0.9 μM, an oral $t_{1/2}$ of 27 h, and a volume of distribution of 16 L/kg. The half-life of **4** in a human liver microsome preparation was >100 min, with 100% of **4** remaining after 60 min. Despite having a benzylamine group, **4** was quite stable in the whole blood of humans, rats, dogs, sheep, and guinea pigs. Its inhibition of cytochrome P450 in isolated human liver microsomes was weak, with IC_{50} values of >20 μM for isozymes 1A2, 2C9, 2C19, 3A4, and 2D6.

We evaluated **4** (benzoate salt) for in vivo efficacy in conscious, antigen-sensitized allergic sheep, which served as a useful asthma model.[2b,20] This inhibitor was administered by oral gavage at 30 mg/kg twice daily on Day 1, and on Day 2 at 2 h prior to antigen challenge (*Ascaris suum* delivered by aerosol) and 4 h after antigen challenge (N = 2). Airway resistance (SR_L) was monitored for 8 h following antigen challenge and airway hyper-reactivity to carbachol (PC_{400}) was measured at 24 h. Compound **4** completely blocked the late-phase response (4–8 h post antigen) and the increase in airway hyper-reactivity at 24 h, but had no effect on the acute early-phase response (0-4 h post antigen). Analysis of **4** in the sheep plasma indicated that its levels ranged from 0.4 to 1.0 μM during this efficacy study.

Compound **4** (benzoate salt) was also tested in a model of allergic asthma with ovalbumin-sensitized guinea pigs.[21] Oral doses of **4** (20 mg/kg) were administered twice daily over 3 days, and once on Day 4 at 2 h prior to ovalbumin challenge. Thus, **4** was found to reduce total lung resistance (R_L) by 90% and to increase dynamic lung compliance (C_{dyn}) by 65% relative to vehicle-treated controls. In addition, there was a 20% reduction in the total white blood cell count in the broncho-alveolar lavage (BAL) fluid, which signifies anti-inflammatory activity.

We obtained a co-crystal of **4** and human β_2-tryptase that was suitable for X-ray crystallographic study.[22] In the solved structure (2.0 Å),[23] there was one ligand per monomer unit of the tetrameric protein assembly (Fig. 2). The benzylamine group (lower left) is located in the S1 pocket, as expected, and the amide carbonyl is hydrogen bonded to the Gly-219 Nα. The piperidine ring is in a chair conformation and the 5-membered-ring ether oxygen is directed toward the solvent. Interestingly, the phenyl group of the rigid phenylethynyl moiety disrupted a hydrogen bond found in the original crystal structure reported by Pereira et al.[24] (Fig. 3), between the carboxylate of Glu-217 and the hydroxyl of

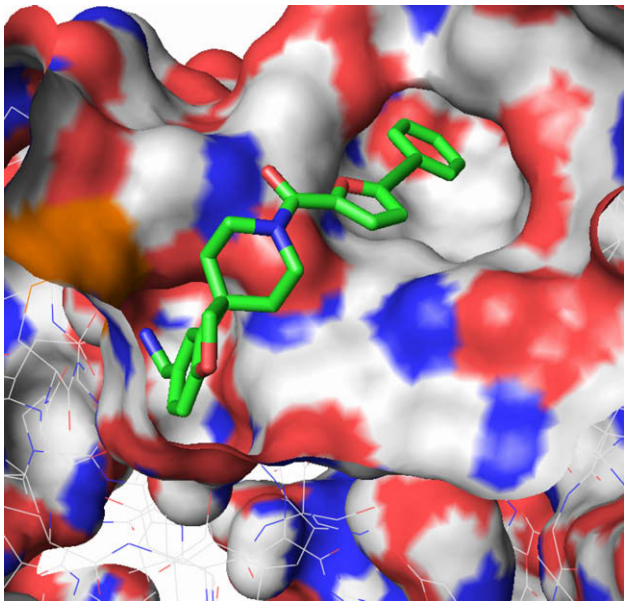


Figure 2. View of one monomer of the **4** · tryptase complex, with **4** (stick model; green with standard coloring scheme for heteroatoms) occupying the tryptase active site (Connelly surface; white with standard coloring scheme for heteroatoms).

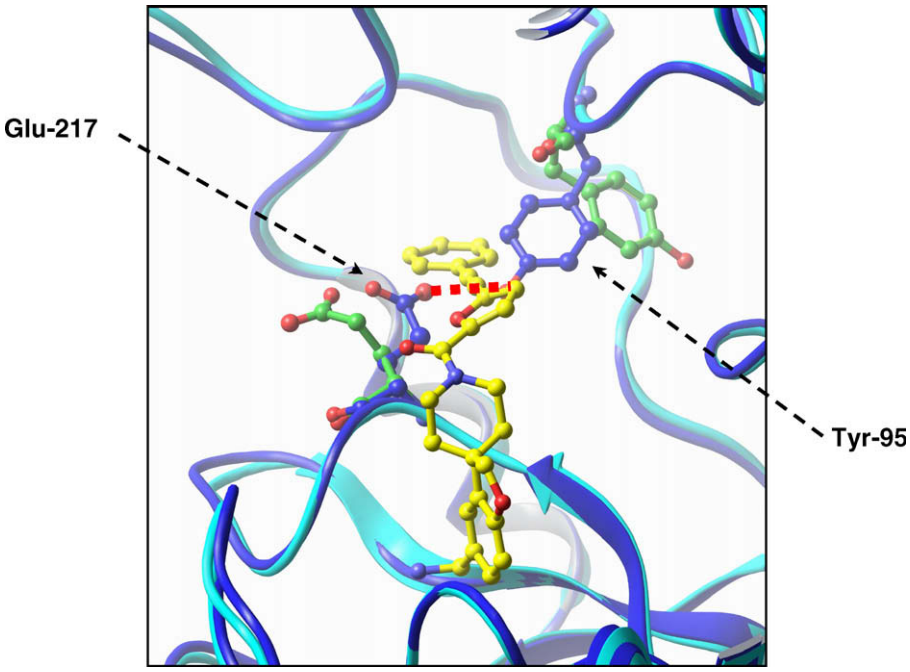


Figure 3. Superimposed active-site views of one monomer of **4** · tryptase and one monomer from the original tryptase X-ray structure reported by Pereira et al.[24] to illustrate the induced-fit hydrophobic pocket. The phenyl substituent of **4** (stick model; yellow with standard coloring scheme for heteroatoms) is situated in tryptase (ribbon representation) such that the side chains of Tyr-95 (green) and Glu-217 (green) are displaced from the position that they adopt in the original X-ray structure,[24] wherein the hydroxyl of Tyr-95 (blue) is hydrogen bonded (red dashed line) to the carboxylate of Glu-217 (blue).

Tyr-95 *in an adjacent subunit of the tetramer* (i.e., in "monomer B"). The displacement of the side chains of Glu-217 and Tyr-95 engendered a shallow hydrophobic binding pocket that is defined by the following residues: Thr-96 and Trp-215 of "monomer A"; Pro-60A and Tyr-95 of "monomer B". This induced-fit binding mode is not evident in the co-crystal structures reported for the related piperidine amide series with different acyl groups.[7a] The much weaker potency of the 4-*tert*-butyl (**5j**) and 4-hydroxy (**5k**) analogues of **4** can be explained by stereoelectronic problems connected with occupancy in this shallow hydrophobic pocket. The linear, rigid phenylethynyl group in **4** may be advantageous relative to the more flexible bioisosteric groups in **5q** and **5r** because of the way these different acyl groups are able to capitalize on this induced pocket.

In conclusion, we have explored a series of spirocyclic piperidine amides, several of which are very potent tryptase inhibitors. In particular, **4** (JNJ-27390467) was found to be a potent, selective tryptase inhibitor with oral efficacy in animal models of airway inflammation. In a sheep asthma model, **4** effectively blocked the late-phase and airway hyperreactivity responses. In a guinea pig asthma model, **4** prevented the increase in airway resistance. An X-ray co-crystal structure of **4** · tryptase revealed an interesting induced-fit binding mode, involving the phenylethynyl group of **4** and a hydrophobic pocket that is formed by amino acid side chains of two adjacent monomers in the tetrameric protein assembly. Compound **4** also possesses many desirable attributes of a potential clinical candidate.

Acknowledgment

We thank Dr. Lei Qiao for the preparation of **5a**.

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18. The β-tryptase assays were performed with human lung tryptase (Cortex Biochem, Inc., San Leandro, CA; #CP3033) in aqueous buffer (10 mM Tris, 10 mM Hepes, 150 mM NaCl, 0.1% PEG 8000, pH 7.4) with the chromogenic substrate H-D-HHT-Ala-Arg-pNa · 2AcOH (American Diagnostica, Inc., Stamford, CT; #238; K_m = 580 μM) by using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The IC_{50} experiments were conducted by fixing enzyme and substrate concentrations (1 nM [E]/500 μM [S]) and varying the inhibitor concentration; the K_i experiments were conducted by fixing the enzyme concentration (1 nM) and varying the inhibitor and substrate concentrations. Changes in absorbance at 405 nm were monitored with the software program Softmax Pro (Molecular Devices), on addition of enzyme without inhibitor at 22 °C for 15 min. Percent inhibition was calculated by comparing the initial reaction velocity of samples without inhibitor to those with inhibitor. Initial reaction velocities were analyzed by using Microsoft Excel. K_i values were calculated in Sigma Plot Enzyme Kinetic Module (SPSS, Inc., Chicago, IL).
19. The trypsin assays were performed with human pancreatic trypsin (Calbiochem #650275; Merck KGaA, Darmstadt, DE) in aqueous buffer [200 mM NaCl, 50 mM Hepes, 0.5% octyl glucoside (Sigma–Aldrich O-9882), pH 7.4] with the chromogenic substrate N-α-Z-D-Arg-Gly-Arg-pNA · 2HCl (Chromogenix, Milan, IT; #S2765; K_m = 25 μM) by using a microplate reader (Molecular Devices). The IC_{50} experiments were conducted by fixing the enzyme and substrate concentrations (1.8 nM [E]/60 μM [S]) and varying the inhibitor concentration; the K_i experiments were conducted by fixing the enzyme concentration (1.8 nM) and varying the inhibitor and substrate concentration. Changes in absorbance at 405 nm were monitored with the software program Softmax Pro, on addition of enzyme without inhibitor at 22 °C for 15 min. Percent inhibition was calculated by comparing the initial reaction velocity of samples without inhibitor to those with inhibitor. Initial reaction velocities were analyzed by using Microsoft Excel. K_i values were calculated in Sigma Plot Enzyme Kinetic Module.
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22. Recombinant human β-tryptase from Promega Corp. (Madison, WI; #G5631) was deglycosylated at 23 °C for 72 h under the following conditions: 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM 1,2-ethylenediamine *N*,*N*,*N*′,*N*′-tetraacetic acid, 0.2 mg/mL of GST-PNGase F (Hampton Research, Aliso Viejo, CA), 5.7 mg/mL of tryptase, and 0.5 mM **4**. The GST-PNGase F was removed by using glutathione–Sepharose 4B (Amersham Biosciences, Piscataway, NJ). For crystallization, this tryptase in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.1) and 1 M NaCl, with 0.9 mM **4**, was concentrated to 6.2 mg/mL. Suitable crystals for X-ray crystallography were grown by the standard vapor-diffusion hanging-drop method.
23. X-ray crystallographic study of **4** · tryptase: The **4** · tryptase complex crystallized in a tetragonal crystal form [space group $P4_1$ with $a = b = 82.68$ Å, $c = 170.28$ Å, and four molecules (one tetramer) in the asymmetric unit], which diffracted X-rays to 2.1 Å resolution. The structure was determined by molecular replacement by using a published tryptase structure (PDB entry 1AOL). Inhibitor **4** could be unambiguously modeled in $2F_0 - F_c$ and $F_0 - F_c$ electron-density maps, and the structure was refined (2.3 Å resolution; R, R_{free} = 0.181, 0.233). Atomic coordinates and structure factors for the **4** · tryptase complex have been deposited with the Protein Data Bank (www.rcsb.org; accession code 2ZA5).
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