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Substituted 2-Imino-5-arylidenethiazolidin-4-one Inhibitors of Bacterial Type III Secretion

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Abstract

Diverse species of pathogenic Gram-negative bacteria use secretion systems to export a variety of protein toxins and virulence factors that help establish and maintain infection. Disruption of such secretion systems is a potentially effective therapeutic strategy. We developed a high-throughput screen and identified a *tris*-aryl substituted 2-imino-5-arylidenethiazolidin-4-one, compound **1**, as an inhibitor of the Type III secretion system. Expansion of this chemotype enabled us to define the essential pharmacophore for Type III secretion inhibition by this structural class. A synthetic diversity set helped us identify N-3 as the most permissive locus, and led to the design of a panel of novel N-3-dipeptide-modified congeners with improved activity and physicochemical properties. We now report on the synthesis of these compounds, including a novel solid phase approach to the rapid generation of the dipeptide-thiazolidinone hybrids, and their *in vitro* characterization as inhibitors of Type III secretion in *Salmonella enterica* serovar Typhimurium.

Introduction

Diverse species of pathogenic Gram-negative bacteria use Type II (T2SS) and Type III (T3SS) secretion systems to deliver virulence factors to host cells to establish and maintain bacterial infections. Some of the component proteins of these secretory apparatus, such as the outer membrane secretin YscC/InvG, the inner membrane ring protein YscJ/PrgK, and the ATPase YscN/InvC/SpaI, demonstrate primary amino acid sequence conservation.¹ Others demonstrate structural conservation.² A potential advantage of targeting secretion is that it may result in less selection for resistant mutants, because secretion systems are not required for bacterial growth.³ Small molecules that inhibit secretion systems might be indicated for the prevention and/or treatment of infection from a wide variety of Gram-negative bacterial species and be applicable to diverse plant and animal diseases.^{4,5} The concept of secretion inhibition as a potentially effective broad-range therapeutic strategy is supported by the literature reports of *in vitro* activity against *Yersinia*, *Chlamydia*^{6,7} and *Salmonella*⁸ by the salicylhydrazide class of T3SS inhibitors. The potential to disable the virulence processes of diverse Gram-negative pathogens⁹ prompted us to embark on a discovery project for novel T3SS inhibitors.

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A high-throughput screen (HTS) was developed in our laboratory and used to evaluate 92,000 small molecules for inhibition of the *Salmonella enterica* serovar Typhimurium T3SS. This screen produced 89 initial hits, from which 25 were selected for confirmatory and secondary assays. In selecting compounds to advance to the next screens, we placed a priority on compounds likely to be functioning via our target mechanism of action. We rejected frank cytotoxic or cytostatic compounds, or transcriptional inhibitors, and favored those compounds that exhibited synthetic tractability and suitability for chemotype expansion. The results from our secondary assays led us to focus on the *tris*-aryl substituted 2-imino-5-arylidene-thiazolidin-4-one **1** as our candidate hit. We assayed for secretion of SipA, a T3SS-secreted protein, and using both commercially available and our own synthetic compounds, generated a panel of analogs with which we were able to define the essential pharmacophore of this class of secretion inhibitors.¹⁰

Six distinct structural classes of T3SS inhibitors have been described previously. Two classes, the glycolipid caminosides reported by Linington et al^{11, 12} and the pseudotriptide guadinomines recently reported by Iwatsuki et al,¹³ are natural products. Synthetic inhibitor classes include the N-substituted (2-acylamino-4-aryl)-2-pyridones,¹⁴ 2,5-disubstituted oxazole-3-carboxamides,¹⁵ substituted (2-alkylamino)-1,3,5-triazines,¹⁶ and N-acylhydrazones of salicylaldehydes.^{17, 18} Mechanisms of action and molecular targets have not been determined for these compound classes, although currently the acylhydrazones are believed to act by inhibiting transcription of one or more T3SS proteins.^{6, 7} In contrast, our initial studies with the 2-imino thiazolidinones suggest that this class may act directly to disrupt a protein-protein interaction such as the interactions of the secretin protein involved in the assembly and/or stability of the secretion apparatus.¹⁰

The thiazolidinone nucleus appears in drug discovery programs in diverse therapeutic areas. Substituted thiazolidinones are under investigation as anticancer antagonists of the $\alpha_v\beta_3$ integrins,¹⁹ anti-inflammatory agents,^{20, 21} β_3 adrenergic agonists,^{22, 23} antischistosomal agents,²⁴ general antifungal/antimicrobial agents,²⁵⁻²⁷ and inhibitors of the YycG histidine kinase of *S. epidermidis*.²⁸ Many of these thiazolidinones are highly potent and target-specific, suggesting that the substituents impart the high degree of selectivity to the common core from which they are presented. This phenomenon in medicinal chemistry²⁹ may be considered the small-molecule correlate of the observation that diverse proteins often share a similar fold without any primary sequence similarity.

Our initial panel of thiazolidinones was useful for characterizing the target and for supporting the hypothesis that very specific molecular interactions were disrupting a protein-protein interaction, but these compounds were poorly soluble and active only in high micromolar concentrations. In order to enhance solubility and activity, and to gain insight into the ligand-protein interface, we embarked on a synthetic program to first define, and then exploit, the permissive or conserved functional groups on the 2-imino thiazolidinone core. We synthesized a variety of compounds, with particular emphasis on ω -polar groups (Scheme 1), and, subsequently, the dipeptides (Schemes 2 and 3) fused to the thiazolidinone at N-3. We now present the preparation of these more hydrophilic 3-substituted 2-imino-5-arylidene-thiazolidinones and their T3SS inhibitory activity,

Chemistry

Synthetic methods for thiazolidinones are well described in the literature. Our synthesis of the aminomethylphenyl and aminohexyl analogs, shown in Scheme 1, is an adaptation of the method of Klika et al.³⁰

The regiochemical outcome of the cyclization to 2-iminothiazolidinone can be controlled by the choice of reagents and reaction conditions, but unless sufficient differences exist between

the nucleophilic strengths of the two nitrogens the more thermodynamically favored 5-arylidene product will be obtained after the Knoevenagel reaction.^{20, 21} Under the conditions in Scheme 1 we obtained a single compound from the cyclization of the alkyl/aryl thioureas but a mixture of regiomers for all aryl/aryl thioureas. We separated **5** and **7** by preparative reverse phase HPLC at the protected penultimate stage (optimum for this procedure) before cleaving the Boc group to, respectively, **6** and **8**. The 1D and 2D ¹H NMR spectra of **6** and **8** clearly indicated that the two were regiomers, and the mass spectra gave the same parent ions but a different fragmentation pattern for each compound. Absolute assignment of structure was obtained by crystallography of **8** (data not shown). The general method of Scheme 1 was used to generate the compounds in Table 1.

Short peptides are an expeditious way to survey functional groups, stereochemistry, and molecular shape. Significantly for this work, by appending dipeptides to the non-peptidic thiazolidinone scaffold, the hydrophilicities of the ensuing hybrid molecules can be varied across several orders of magnitude. In order to investigate the SAR of the dipeptide component we prepared a diverse set of analogs presenting the dipeptide from R₃ and holding the vinylidene (R₁) and 2-imino (R₂) groups constant. Our selection of amino acids emphasized arginine, tryptophan, and tyrosine in accordance with residues that are statistically overrepresented in protein-protein interactions.³¹ Other residues were selected for their neutrality (alanine), charge (ornithine, glutamic acid), steric demand (valine), or to probe hydrogen-bonding (thiazolidinylalanine, pyridylalanine) or hydrophobic contacts (biphenylalanine, tetrahydroisoquinoline carboxylate). Suitably protected dipeptides were assembled and incorporated into the original synthetic route. With arginine in the terminal position, a protected ornithine was used to provide a common precursor to both the primary amine **20a** and, upon guanylation,³² **22a**. The solution synthesis of dipeptidyl thiazolidinones is illustrated by the preparation of **22a** (Scheme 2).

The linear directionality of the Scheme 2 route, from peptide C-terminus to complete N3-peptidyl thiazolidinone, prompted the investigation of a solid phase route for the rapid generation of dipeptide libraries. A Tentagel Rink resin was loaded with the C-terminal residue, and the Scheme 1 and Scheme 2 methods were adapted to solid phase by increasing the stoichiometry of the reagents (Scheme 3). In the solid phase route, arginine could be added directly as the pentamethylsulfonylbenzofuran (Pbf) protected derivative. Yields and purities were comparable to those obtained in solution chemistry. The chiral integrity was generally preserved for the dipeptides, with the exception of the ValTyr (**42**), AlaTrp (**44**), and OrnDht (**49**) analogs. The D-alanine dipeptides **20b** and **22b** were obtained from D-alanine, the D-biphenylalanine dipeptide **55b** was obtained from D-biphenylalanine.

Results and Discussion

Compounds were evaluated as T3SS inhibitors by measuring secretion of the *Salmonella enterica* serovar Typhimurium effector protein SipA (Tables 1 and 2 and Figure 2).¹⁰ Replacement of the syringyl ring (**28–38**, **40**), with the exception of *p*-thiomethylphenyl (**27**, **39**) or 3,4-dimethoxy (**38**) was generally a poorly tolerated modification. The 2-imino-substituted analog **6** was also somewhat weaker in inhibitory activity than **1**. Amido/imino regiomeric pairs that had proven difficult to separate (**36–40**) were each tested as a mixture with the intention of resolving them only if the inhibitory activity warranted additional effort. Within this data set, none of these compounds qualified. In contrast, multiple analogs with different N-3 groups were active (Table 1), particularly the N-3 ω-carboxy- (**23**) and carbalkoxy- (**24**) analogs that retained a significant degree of SipA inhibition at concentrations of 1 μM or less. We used N-3 as the position of choice for a series of dipeptides with the intention of identifying additional beneficial functional groups.

The inhibitory potencies of the dipeptides are shown in Table 2. Many of the dipeptides, while having improved solubility,³³ retained but did not exceed a level of potency comparable to that of **1**. We selected **1**, **7**, **8**, **52**, and **55a** as representative compounds in the 5-substituted-2-arylimino-thiazolidinone class to test for growth inhibitory activity against *S. typhimurium*, and no effect was observed at concentrations up to 1 mM (data not shown). These data support the conclusion that effects of these compounds are mediated by direct inhibition of the T3SS secretory process and not a result of inhibition of bacterial growth.

The more inhibitory dipeptides, **55a** and **55b**, and to a lesser extent **20a** and **20b**, have a common motif of a neutral residue (alanine or biphenylalanine) in the proximal position coupled to a cationic residue (ornithine or arginine) in the terminal position. It is interesting that other dipeptides that ostensibly share this motif are much weaker: Arginine is in the terminal position of analogs **22a**, **22b**, **50** and **51**, and ornithine is in the terminal position of **56**, but the inhibitory activity of all of these remains comparable to that of **1**. Appending a peptide does not necessarily improve the inhibitory activity of the 3-substituent: glutamyl dipeptide **53**, that might be regarded as a congruent hybrid of two modestly active analogs, acid **25** (66 μ M) and biphenyl peptide **52** (13 μ M), showed no significant improvement over acid **25** and was weaker than the corresponding *t*-butyl ester **24**. Taken together, these data suggest that an interactive relationship between the two residues is likely to be more important than the independent contributions of the individual sidechain functional groups. The two residues of the epimeric active dipeptides might be acting in concert to fold the ligand into the correct pose for the binding epitope, and there may be more than a single solution to the problem of peptide-induced, or peptide-stabilized, ligand folding. The higher activities of **52** vs. **56**, and of **55a/b** vs. **54**, indicate that the dipeptide's effect, however subtle and complex, is the result of specific contacts rather than some general phenomenon such as increased access to the target.

Protein-protein interactions are extended, often over 1000–3000 \AA^2 , but are neither random nor unstructured. The interfaces are frequently coiled coils of helices or other highly defined secondary structures.³⁴ The residues making the most significant contributions to this interface may be buried inside the shape they create, limiting access to external ligands.³⁵ This model describes quaternary structures in the T3SS, including the 3500 \AA^2 surface of the EscJ trimeric interface in which 35% of that interface is buried.³⁶ Strategies to design ligands for protein-protein interfaces have been described. These strategies emphasize the predominance of hydrophobic interactions and aromatic residues in the 'hotspots' that drive the particular protein-protein interaction.^{34, 37} In this work we have identified an epimeric pair of compounds, **55a** and **55b**, that increase the T3SS inhibition of our initial HTS hit more than 10-fold. These dipeptides are characterized by a combination of a cationic group and a functional group having the potential for hydrophobic interactions. Our current effort to determine the optimum consensus pharmacophore takes into account the accumulated data from the Table 1 and Table 2 compounds. From the perspective of future drug evolution, it is encouraging to observe that the more active compounds such as **55a** are an order of magnitude more hydrophilic than **1**, suggesting that potent inhibitors of T3SS can be developed that will have acceptable pharmacokinetic properties.

Conclusion

Virulence targets represent an emerging concept in antibacterial therapy^{3, 38, 39} for which there are examples of compounds that inhibit virulence functions and have demonstrated efficacy in mouse^{40,41, 42} and other⁸ *in vivo* models. The challenge for the further development of anti-virulence therapeutics will require the development of compounds with adequate pharmacokinetic and activity profiles to promote incentive for further development. A key issue when considering a given virulence target is whether drugs successfully directed against it will have sufficiently broad spectrum efficacy to be clinically useful. This work

suggests that dipeptide derivatives of the thiazolidinone scaffold may provide a critical step toward the validation of this strategy and the development of novel therapeutics.

Experimental

Chemistry

General—All reactions were run under an atmosphere of dry nitrogen. Reagents and solvents were obtained in the highest available purity and used without further purification unless indicated. ^1H NMR spectra were obtained on a 300 MHz (Bruker AV300 or AV301) or 500 MHz (Bruker AV500 or Varian) instrument. ^{13}C NMR spectra were obtained on a 500 MHz Bruker AV500. Identity of the compounds was confirmed by mass spectrometry. The compound solution was infused into the electrospray ionization source operating in positive ion mode. Low resolution spectra were obtained on the Esquire LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Accurate mass measurements were performed on the APEX Qe 47 Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA). LC-MS measurements to determine logP values were obtained on a Waters Quattro Micro mass spectrometer interfaced with a Waters Alliance 2795 liquid chromatography instrument. Normal phase silica gel purifications were done using a Biotage SP4 instrument using the cartridges supplied by Biotage. RP-HPLC was done on a Varian instrument equipped with a diode array ultraviolet detector. For preparative reverse phase chromatography a 10×250 mm C18 5μ column at a flow rate of 4.6 mL/min was used; for analytical reverse phase chromatography a 4.6×250 mm C18 5μ column at a flow rate of 1 mL/min was used. Ultraviolet detection was at 215 and either 254 or 360 nm. Unless otherwise specified, buffer A was 0.05% TFA in H_2O , buffer B was 0.05% TFA in acetonitrile. Thin layer chromatography was done using 0.2 mm polygram SIL G/UV plates (Alltech, Deerfield, Ill) or Si250F (J. T Baker, Phillipsburg, NJ) plates, developed using mobile phases of varying compositions of ethyl acetate/hexane, MeOH/ CH_2Cl_2 , or MeOH/ CHCl_3 , and visualized by UV light supplemented by vanillin, ninhydrin, and other solution stains where appropriate.

Scheme 1—General method A for generation of thioureas is illustrated for the preparation of *t*-Butyl 4-(3-phenylthioureido)benzylcarbamate **2**. To a stirred solution of phenylisothiocyanate (244 μL , 2 mmol) in 50 mL CH_2Cl_2 was added (Boc) 4-aminomethylaniline (244 mg, 2 mmol), and the solution stirred for 5 days until TLC (95:5 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) showed the reaction to be complete. The solvent was removed *in vacuo* and the white solid collected and washed with hexane/diethyl ether to give **2**. Yield: 6.14 mg, 1.72 mmol. ^1H NMR (300 MHz, CDCl_3 , δ): 1.47 (s, 9H), 4.32 (d, $J = 5.9$ Hz, 2H), 4.91 (br, 1H), 7.28–7.86 (m, 9H). MS m/z 358 $[\text{M} + \text{H}]^+$, 380 $[\text{M} + \text{Na}]^+$, 324 $[\text{M} - t\text{-Bu} + \text{H}]^+$, 346 $[\text{M} - t\text{-Bu} + \text{Na}]^+$.

t-Butyl 6-(3-phenylthioureido)hexylcarbamate **9** was prepared by **General method A** to give **9** (593 mg, 1.70 mmol) that was used without further purification. ^1H NMR (300 MHz, CDCl_3 , δ): 1.50 (s, 9H), 1.34–1.58 (m, 8H), 3.10 (dd, $J = 6.3, 6.3$ Hz, 2H), 3.65 (dd, $J = 6.3, 6.3$ Hz, 2H), 7.21–7.57 (m, 5H). MS m/z 374 $[\text{M} + \text{Na}]^+$, 274 $[\text{M} - \text{Boc} + \text{Na}]^+$.

General method B for generation of 2-iminothiazolidinones is illustrated for the preparation of (*Z*)-*t*-Butyl-4-(4-oxo-2-phenylthiazolidin-3-ylideneamino)benzylcarbamate **3** and (*Z*)-*t*-Butyl-4-(4-oxo-3-phenylthiazolidin-2-ylideneamino)benzylcarbamate **4**. To a stirred solution of **2** (435 mg, 1.22 mmol) in 40 mL CH_2Cl_2 were added successively DIEA (424 μL , 2.44 mmol) and methyl bromoacetate (115 μL , 1.22 mmol). The reaction mixture was stirred overnight at ambient temperature, concentrated *in vacuo*, and the residue purified via silica gel chromatography using a gradient from 2 to 20% MeOH in CH_2Cl_2 to give **3** and **4** (484 mg, 1.2 mmol) as a mixture of regioisomers. ^1H NMR (300 MHz, CDCl_3 , δ): 1.48 (s, 9H), 3.99 (s,

2H), 4.29 (br d, $J = 5.1$ Hz, 1H), 4.37 (br d, $J = 5.7$ Hz, 1H), 4.90 (br, 1H), 6.89–7.57 (m, 9H). MS m/z 398 $[M + H]^+$, 420 $[M + Na]^+$, 342 $[M - t\text{-Bu} + H]^+$, 364 $[M - t\text{-Bu} + Na]^+$.

(Z)-*t*-Butyl 6-(4-oxo-2-phenylimino)thiazolidin-3-yl)hexylcarbamate 10 was prepared by **General method B** on a 1.14 mmol scale from **9** to give 471 mg, 1.1 mmol as a colorless oil. ^1H NMR (300 MHz, CDCl_3 , δ): 1.51 (s, 9H), 1.38–1.77 (m, 8H), 3.11 (dd, $J = 6.3, 6.3$ Hz, 2H), 3.81 (s, 2H), 3.86 (dd, $J = 7.2, 7.2$ Hz, 2H), 4.53 (br, 1H), 6.95–7.39 (m, 5H). ^{13}C NMR (500 MHz, CDCl_3 , δ): 26.4, 26.5, 27.1, 285, 29.9, 32.7, 40.5, 43.07, 79.0, 121.0, 124.6, 133.3, 148.26, 154.4, 155.9, 171.8. MS m/z 392 $[M + H]^+$, 414 $[M + Na]^+$, 336 $[M - t\text{-Bu} + H]^+$, 292 $[M - \text{Boc} + H]^+$.

General method C to introduce the 5-arylidene or 5-vinylidene substituent is illustrated by the synthesis of ***t*-Butyl 4-((Z)-((Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-3-phenylthiazolidin-2-ylidene)amino)benzylcarbamate 5** and ***t*-Butyl 4-((Z)-((Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-phenylthiazolidin-3-ylidene)amino)benzylcarbamate 7**. A solution of the mixture of **3** and **4** (484 mg, 1.22 mmol), syringaldehyde (222 mg, 1.22 mmol), and piperidine (163 μL , 1.64 mmol) in 15 mL of EtOH was refluxed for 19 h. The reaction mixture was cooled to ambient temperature and treated with hexane to precipitate the product, which was collected, washed with additional hexane, and dried to give 565 mg of a mixture of **5** and **7**. Preparative reverse phase HPLC using a gradient of 65 to 85% B in A over 15 min enabled the separation of the two pure compounds as well as an overlap band that was held in reserve for further purification. **5** (48.5 mg, 0.09 mmol) ^1H NMR (300 MHz, $\text{DMSO}-d_6$, δ): 1.41 (s, 9H), 3.77 (s, 6H), 4.06–4.14 (m, 2H), 6.90 (s, 2H), 6.95–7.59 (m, 9H), 7.75 (s, 1H), 9.32 (br, 1H). MS m/z 562 $[M + H]^+$, 584 $[M + Na]^+$ **7** (54.2 mg, 0.10 mmol) ^1H NMR (300 MHz, $\text{DMSO}-d_6$, δ): 1.43 (s, 9H), 3.72 (s, 6H), 4.15–4.23 (m, 2H), 6.89 (s, 2H), 7.00–7.64 (m, 9H), 7.76 (s, 1H), 9.31 (br, 1H). MS m/z 562 $[M + H]^+$, 584 $[M + Na]^+$.

***t*-Butyl-6-((2Z,5Z)-6-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)hexylcarbamate 11** was prepared by **General method C** to give 548 mg, 0.95 mmol, as an orange solid. ^1H NMR (300 MHz, CDCl_3 , δ): 1.42 (s, 9H), 1.46–1.54 (m, 6H), 1.71–1.81 (m, 2H), 3.03 (dd, $J = 6.5, 6.6$ Hz, 2H), 3.86 (s, 6H), 3.96 (dd, $J = 4.2, 4.2$ Hz, 2H), 5.28 (br, 1H), 6.81 (s, 2H), 7.07 (d, $J = 7.5$ Hz, 2H), 7.23 (t, $J = 7.4$ Hz, 1H), 7.43 (t, $J = 7.9$ Hz, 2H), 7.66 (s, 1H). ^{13}C NMR (500 MHz, CDCl_3 , δ): 23.6, 24.4, 26.4, 26.5, 27.4, 28.5, 29.5, 42.9, 45.3, 55.7, 56.0, 81.4, 108.0, 121.0, 121.3, 124.5, 129.2, 129.2, 132.6, 148.5, 149.4, 151.0, 167.3. MS m/z 556 $[M + H]^+$, 578 $[M + Na]^+$, 500 $[M - t\text{-Bu} + H]^+$.

General method D for deprotection of acid-labile protecting groups is illustrated by the preparation of **(2Z,5Z)-2-(4-(aminomethyl)phenylimino)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-3-phenylthiazolidin-4-one 6**. Ice cold TFA (0.5 mL) was added to neat **5** (4 mg, 0.007 mmol) cooled in an ice bath. The reaction was complete by HPLC in 15 min, and the reaction mixture was concentrated *in vacuo* to a sticky brown oil. Trituration with diethyl ether gave a solid that was purified on reverse phase HPLC (65 to 85 % B in A over 12 min) to give **6** (3.0 mg, 0.004 mmol) as the TFA salt. ^1H NMR (500 MHz, CD_3CN , δ): 3.83 (s, 6H), 4.25 (s, 2H), 6.85 (s, 2H), 7.02 (d, $J = 8.5$ Hz, 2H), 7.22 (t, $J = 8.2$ Hz, 1H), 7.42 (t, $J = 8.5$ Hz, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.67 (d, $J = 8.4$ Hz, 2H), 7.74 (s, 1H). MS m/z 462 $[M + H]^+$. HRMS (m/z): $[M + H]^+$ calcd for $\text{C}_{25}\text{H}_{23}\text{N}_3\text{O}_4\text{S}$, 462.1488; found 462.1491.

(2Z,5Z)-3-(4-(Aminomethyl)phenylimino)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-2-phenylthiazolidin-4-one 8 was obtained from **7** (6.9 mg, 0.01 mmol) by **General method D**, and purified on reverse phase HPLC (65 to 85 % B in A over 12 min) to give **8** (5.5 mg, 0.009 mmol) as the TFA salt. ^1H NMR (500 MHz, CD_3CN , δ): 3.84 (s, 6H), 4.14 (s, 2H), 6.86

(s, 2H), 7.05 (d, $J = 8.3$ Hz, 2H), 7.46–4.62 (m, 7H) 7.75 (s, 1H). MS m/z 462 $[M + H]^+$. HRMS (m/z): $[M + H]^+$ calcd for $C_{25}H_{23}N_3O_4S$, 462.1488; found 462.1486.

(2Z, 5Z)-3-(6-Aminoethyl)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-2-(phenylimino)thiazolidin-4-one 12 was obtained from **11** on a 0.95 mmol scale to give, after two silica gel chromatographies, the first using a gradient from 0 to 10 % MeOH in $CHCl_3$ and the second from 1 to 50% MeOH in $CHCl_3$, 456 mg (0.80 mmol). 1H NMR (300 MHz, $CDCl_3$, δ): 1.42–1.45 (m, 4H), 1.64–1.66 (m, 2H), 1.77–1.79 (m, 2H), 2.65 (br, 2H), 2.89 (t, $J = 7.5$ Hz, 2H), 3.84 (s, 6H), 3.97 (t, $J = 7.5$ Hz, 2H), 6.71 (s, 2H), 6.99 (d, $J = 8.4$ Hz, 2H), 7.14–7.19 (m, 1H), 7.28–7.38 (m, 2H), 7.63 (s, 1H). ^{13}C NMR (500 MHz, $DMF-d_7$, δ): 30.2, 30.5, 31.3, 43.6, 47.0, 53.0, 60.3, 112.5, 120.6, 122.1, 123.0, 125.5, 129.0, 133.5, 133.6, 135.5, 152.6, 152.8, 154.4, 162.9, 163.1, 163.4, 166.1, 166.4, 166.6, 170.5. MS m/z 456 $[M + H]^+$. HRMS (m/z): $[M + H]^+$ calcd for $C_{24}H_{30}N_3O_4S$, 456.1957; found 456.1946.

Scheme 2—General method E for amidation is illustrated by the preparation of **(S)-benzyl 1-amino-5-*t*-butoxycarbonylamino-1-oxopentan-2-ylcarbamate 13**. To a suspension of *Z*-Orn(Boc)-OH (733 mg, 2.0 mmol) in 3.5 mL THF was added *N*-methyl morpholine (0.48 mL, 4.4 mmol). The reaction mixture was cooled in an ice bath, and isobutyl chloroformate (0.31 mL, 2.4 mmol) was added. After 1 h of stirring on ice, concentrated NH_4OH (0.60 mL, 10 mmol) was added, and the reaction mixture stirred an additional 30 min. The reaction mixture was partitioned between 4:1 $CHCl_3$ /isopropanol and saturated aqueous sodium carbonate. The organic phase was separated, washed with saturated brine, dried over Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified on silica gel using a gradient from 0 to 25% MeOH in CH_2Cl_2 to give **13** 670.2 mg (1.84 mmol). 1H NMR (500 MHz, CD_3OD , δ): 1.46 (s, 9H), 1.51–1.72 (m, 3H), 1.72–1.88 (m, 1H), 2.99–3.16 (m, 2H), 4.07–4.18 (m, 1H), 5.05–5.22 (m, 2H), 7.26–7.46 (m, 5H). ^{13}C NMR (500 MHz, CD_3OD , δ): 30.08, 31.4, 33.3, 43.4, 58.5, 70.3, 82.55, 131.5, 131.6, 132.1, 140.8, 161.1, 180.2. MS m/z 366 $[M + H]^+$, 388 $[M + Na]^+$.

General method F for hydrogenolysis, coupling, and subsequent deprotection to the free α -amino dipeptide amide is illustrated by the synthesis of ***t*-Butyl (S)-5-amino-4-((S)-2-aminopropanamido)-5-oxopentylcarbamate 16**. *N*-Protected **13** (664 mg, 1.82 mmol) was hydrogenated over 10% palladium on carbon (650 mg) in 50 mL 4:1 EtOH/DMF for 2.5 h. The catalyst was filtered off and the filtrate concentrated *in vacuo* to give **14** (420 mg, 1.82 mmol) that was used without further purification. (1H NMR (300 MHz, CD_3OD , δ): 1.45 (s, 9H), 1.51–1.65 (m, 3H), 1.65–1.84 (m, 1H), 3.07 (t, $J = 6.2$ Hz, 2H), 3.09–3.24 (m, 1H); ^{13}C NMR (500 MHz, CD_3OD , δ): 29.8, 31.4, 36.3, 43.6, 58.0, 82.5, 158.6, 180.3; MS m/z 232 $[M + H]^+$, 254 $[M + Na]^+$). Tetrahydrofuran (5 mL) was added to a dry mixture of Cbz L-alanine (406 mg, 1.82 mmol) and carbonyldiimidazole (353 mg, 2.18 mmol), and the reaction mixture allowed to stir for 1.5 h. To this was added amine **14**, along with DIEA (350 μ L, 2.00 mmol), and the reaction mixture stirred for 3 days until determined complete by TLC (95:5 CH_2Cl_2 /MeOH). The volatiles were removed *in vacuo*, and a 4% aqueous solution of $NaHCO_3$ (10 mL) was added to precipitate the product. The crude solid was collected and purified on silica gel using a gradient from 0 to 10% MeOH in CH_2Cl_2 to give α -Cbz- δ -Boc dipeptide **15** (226 mg, 0.52 mmol). 1H NMR (300 MHz, CD_3OD , δ): 1.36 (d, $J = 7.2$ Hz, 3H), 1.44 (s, 9H), 1.48–1.74 (m, 3H), 1.76–1.94 (m, 1H), 2.97–3.12 (m, 2H), 4.15 (q, $J = 7.1$ Hz, 1H), 4.27–4.41 (m, 1H), 5.13 (s, 2H), 7.26–7.43 (m, 5H); m/z 437 $[M + H]^+$, 459 $[M + Na]^+$. Hydrogenolysis of the Cbz group over 10% palladium on carbon (225 mg) in 38 mL 14:1 EtOH/DMF for 2 h gave α -amino δ -Boc dipeptide amide **16** (147.8 mg, 0.49 mmol). 1H NMR (300 MHz, CD_3OD , δ): 1.30 (d, $J = 6.9$ Hz, 3H), 1.45 (s, 9H), 1.50–1.75 (m, 3H), 1.77–1.89 (m, 1H), 3.08 (td, $J = 6.6$, 1.7 Hz, 2H), 3.48 (q, $J = 6.9$ Hz, 1H), 4.30–4.49 (m, 1H). MS m/z 303 $[M + H]^+$.

General method G for the formation of N-phenyl-N'-amidodipeptidyl thioureas is illustrated by the preparation of ***t*-Butyl(S)-5-amino-5-oxo-4-((S)-2-(3-phenylthioureido)propanamido)pentylcarbamate 17**. To a solution of **16** (144 mg, 0.48 mmol) in 1.5 mL MeOH was added phenylisothiocyanate (180 μ L, 0.95 mmol) and the reaction mixture stirred overnight, during which time the product precipitated. The volatiles were removed *in vacuo*, hexane was added, and the suspension cooled at 4 °C for 1 h. The crude thiourea was collected and washed with diethyl ether to give **17** (204 mg, 0.47 mmol). ¹H NMR (500 MHz, CD₃OD, δ): 1.45 (s, 9H), 1.46 (d, *J* = 7.4 Hz, 3H), 1.51–1.65 (m, 2H), 1.65–1.76 (m, 1H), 1.85–1.94 (m, 1H), 2.98–3.17 (m, 2H), 4.32–4.44 (m, 1H), 4.97 (q, *J* = 7.0 Hz, 1H), 7.22 (t, *J* = 7.3 Hz, 1H), 7.39 (t, *J* = 7.9 Hz, 2H), 7.45 (d, *J* = 7.9 Hz, 2H). MS *m/z* 438 [M + H]⁺, 460 [M + Na]⁺.

***t*-Butyl (S)-5-amino-5-oxo-4-((S)-2-((Z)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentylcarbamate 18** was prepared by **General Method B** in THF on a 1.77 mmol scale to give, after silica gel chromatography using a gradient from 0 to 10% MeOH in CH₂Cl₂, 603.8 mg (1.27 mmol) product. ¹H NMR (300 MHz, CD₃OD, δ): 1.44 (s, 9H), 1.49–1.78 (m, 3H), 1.66 (d, *J* = 7.0 Hz, 3H), 1.82–2.01 (m, 1H), 2.50–3.10 (m, 2H), 3.97 (s, 2H), 4.33–4.49 (m, 1H), 5.25 (q, *J* = 7.0 Hz, 1H), 6.98 (d, *J* = 7.9 Hz, 2H), 7.14 (t, *J* = 7.4 Hz, 1H), 7.35 (t, *J* = 7.8 Hz, 2H); ¹³C NMR (500 MHz, CDCl₃, δ): 17.7, 31.0, 32.5, 32.8, 36.8, 43.3, 56.5, 56.9, 83.5, 125.1, 129.0, 133.4, 151.3, 157.5, 160.8, 173.0, 175.6, 178.2; *m/z* 478 [M + H]⁺, 500 [M + Na]⁺.

***t*-Butyl(S)-5-amino-4-((S)-2-((2Z,5Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)-5-oxopentylcarbamate 19** was prepared by **General method C** from the corresponding thiazolidinone on a 0.44 mmol scale. The crude solid was purified via silica gel chromatography using a gradient from 0 to 10% MeOH in CH₂Cl₂ to give 224 mg (0.35 mmol) product. ¹H NMR (300 MHz, CD₃OD, δ): 1.41 (s, 9H), 1.47–1.68 (m, 3H), 1.72 (d, *J* = 7.0 Hz, 3H), 1.81–2.02 (m, 1H), 3.03 (t, *J* = 6.5 Hz, 2H), 3.83 (s, 6H), 4.35–4.45 (m, 1H), 5.43 (q, *J* = 7.0 Hz, 1H), 6.81 (s, 2H), 7.07 (d, *J* = 7.4 Hz, 2H), 7.20 (t, *J* = 7.4 Hz, 1H), 7.41 (t, *J* = 7.8 Hz, 2H), 7.71 (s, 1H). ¹³C NMR (500 MHz, CDCl₃, δ): 18.0, 30.82, 32.4, 32.9, 43.3, 56.5, 57.0, 60.4, 83.4, 111.4, 122.3, 125.2, 125.3, 129.1, 129.2, 133.4, 136.3, 141.2, 151.39, 153.3, 160.7, 170.4, 173.2, 178.2. MS *m/z* 642 [M + H]⁺, 664 [M + Na]⁺.

(S)-5-Amino-2-((S)-2-((2Z,5Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentanamide 20a was obtained from **19** (102 mg, 0.16 mmol) by **General method D**, and purified on reverse phase HPLC (10 to 95% B in A over 25 min) to give **20a** (5.1 mg, 0.008 mmol) as the TFA salt. ¹H NMR (300 MHz, CD₃OD, δ): 1.75 (d, *J* = 7.0 Hz, 3H), 1.79–1.92 (m, 3H), 1.96–2.18 (m, 1H), 2.85–3.05 (m, 2H), 3.83 (s, 6H), 4.44–4.57 (m, 1H), 5.46 (q, *J* = 7.0 Hz, 1H), 6.80 (s, 2H), 7.07 (d, *J* = 7.3 Hz, 2H), 7.22 (t, *J* = 7.4 Hz, 1H), 7.42 (t, *J* = 7.8 Hz, 2H), 7.71 (s, 1H). ¹³C NMR (500 MHz, CD₃OD, δ): 16.7, 27.7, 31.9, 42.7, 56.4, 56.6, 59.5, 111.7, 121.5, 125.0, 128.4, 128.9, 133.1, 136.0, 142.5, 151.8, 152.2, 154.0, 170.6, 174.4, 178.7. MS *m/z* 542 [M + H]⁺, 564 [M + Na]⁺. HRMS (*m/z*): [M + H]⁺ calcd for C₂₆H₃₂N₅O₆S, 542.2073; found 542.2070; [M + Na]⁺ calcd for C₂₆H₃₁N₅O₆NaS, 564.1893; found 564.1890.

(S)-5-Amino-2-((R)-2-((2Z,5Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentanamide 20b. Compound **20b** was prepared by **General methods C and D** on a 0.58 mmol scale and purified via silica gel chromatography using a gradient from 1 to 10% MeOH in CH₂Cl₂. Fractions containing protected penultimate intermediate (¹H NMR (300 MHz, CDCl₃, δ): 1.35 (s, 9H), 1.50–1.74 (m, 2H), 1.76 (d, *J* = 7.0 Hz, 3H), 1.97–2.15 (m, 1H), 3.00–3.39 (m, 2H), 3.85 (s, 3H), 3.98 (s, 3H), 4.70–5.10 (m, 1H), 5.34 (br s, 1H), 5.44 (q, *J* = 6.9 Hz, 1H), 6.64 (s, 2H), 6.93 (br s, 1H),

7.00 (d, $J = 7.5$ Hz, 2H), 7.19 (t, $J = 7.0$ Hz, 2H), 7.37 (t, $J = 7.7$ Hz, 2H), 7.65 (s, 1H). MS m/z 642 [M + H]⁺, 664 [M + Na]⁺) were treated with neat TFA for 45 min to give, after silica gel chromatography using a gradient from 1 to 10% MeOH in CH₂Cl₂, **20b** (224 mg, 0.34 mmol) as the TFA salt. ¹H NMR (300 MHz, CD₃OD, δ): 1.74 (d, $J = 7.0$ Hz, 3H), 1.77–1.91 (m, 3H), 1.93–2.13 (m, 1H), 2.93 (t, $J = 6.9$ Hz, 2H), 3.79 (s, 6H), 4.44–4.62 (m, 1H), 5.45 (q, $J = 7.0$ Hz, 1H), 6.73 (s, 2H), 7.06 (d, $J = 7.4$ Hz, 2H), 7.20 (t, $J = 7.4$ Hz, 1H), 7.40 (t, $J = 7.8$ Hz, 2H), 7.68 (s, 1H). ¹³C NMR (500 MHz, CD₃OD, δ): 16.8, 27.7, 32.1, 42.8, 56.4, 56.6, 59.4, 111.7, 121.5, 124.9, 128.4, 128.8, 133.1, 136.1, 142.3, 151.8, 152.1, 153.9, 170.8, 174.3, 178.0. MS m/z 542 [M + H]⁺. HRMS (m/z): [M + H]⁺ calcd for C₂₆H₃₂N₅O₆S, 542.2068; found 542.2064; [M + Na]⁺ calcd for C₂₆H₃₁N₅O₆NaS, 564.1887; found 564.1882.

(S)-5-Guanidino-2-((S)-2-((Z,Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentanamide (21a). General method H for the formation of *bis*-Boc-guanidine and subsequent deprotection to the free guanidine is illustrated by the synthesis of **22a**. 1,3-*bis*-(Boc)-2-methyl-2-thiopseudourea (80 mg, 0.28 mmol), triethylamine (114 μ L, 0.82 mmol), and HgCl₂ (90 mg, 0.33 mmol) were added to a solution of **20a** (150 mg, 0.23 mmol) in 2.8 mL DMF at 0 °C. The reaction mixture stirred for one hour at 0 °C then at room temperature overnight. The reaction mixture was diluted with ethyl acetate, filtered over celite, and the filtrate concentrated *in vacuo*. The crude solid was purified via silica gel chromatography using a gradient from 0 to 50% MeOH in CH₂Cl₂ to give **21a** (80.7 mg, 0.10 mmol). ¹H NMR (300MHz, (CD₃)₂CO, δ): 1.45 (s, 9H), 1.50 (s, 9H), 1.60–1.72 (m, 3H), 1.75 (d, $J = 7.1$ Hz, 3H), 1.83–1.96 (m, 1H), 3.24–3.51 (m, 2H), 3.83 (s, 6H), 4.48–4.62 (m, 1H), 5.40 (q, $J = 7.0$ Hz, 1H), 6.47 (br s, 1H), 6.86 (s, 2H), 7.01 (br s, 1H), 7.08 (d, $J = 7.3$ Hz, 2H), 7.19 (t, $J = 7.4$ Hz, 1H), 7.41 (t, $J = 7.8$ Hz, 2H), 7.67 (s, 1H), 7.69–7.77 (m, 1H), 8.32 (br s, 1H). ¹³C NMR (500 MHz, (CD₃)₂CO, δ): 17.3, 29.7, 31.3, 31.6, 31.7, 44.1, 56.8, 59.9, 60.0, 82.2, 86.8, 112.1, 122.4, 125.3, 128.6, 128.8, 133.3, 135.4, 142.7, 152.0, 152.2, 153.3, 160.2, 167.7, 169.6, 169.9, 172.3, 177.4. MS m/z 784 [M + H]⁺, 806 [M + Na]⁺. **21a** was taken up in 50:50 CH₂Cl₂/TFA (3 mL) and stirred at room temperature for one hour, then concentrated *in vacuo*. The crude solid was purified by preparative reverse phase HPLC using a gradient from 10 to 50% B in A over 30 min to give **22a** (2 mg, 0.003 mmol) as the TFA salt. ¹H NMR (300 MHz, CD₃OD, δ): 1.57–1.73 (m, 3H), 1.74 (d, $J = 7.0$ Hz, 3H), 1.93–2.12 (m, 1H), 3.07–3.28 (m, 2H), 3.82 (s, 6H), 4.43–4.55 (m, 1H), 5.45 (q, $J = 6.9$ Hz, 1H), 6.78 (s, 2H), 7.06 (d, $J = 7.6$ Hz, 2H), 7.21 (t, $J = 7.4$ Hz, 1H), 7.41 (t, $J = 7.7$ Hz, 2H), 7.68 (s, 1H). ¹³C NMR (500 MHz, CD₃OD, δ): 16.8, 29.0, 32.4, 44.5, 56.7, 57.0, 59.4, 111.6, 121.5, 125.0, 128.4, 128.9, 133.1, 136.0, 142.2, 151.7, 152.1, 153.7, 161.2, 170.5, 174.5, 179.2. MS m/z 584 [M + H]⁺. HRMS (m/z): [M + H]⁺ calcd for C₂₇H₃₄N₇O₆S, 584.2286; found 584.2306.

(S)-5-Guanidino-2-((R)-2-((Z,Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentanamide 22b was prepared by General method H via the free amine **20b** (200 mg, 0.37 mmol), purified via silica gel chromatography using a gradient from 1 to 20% MeOH in CH₂Cl₂ to give the *bis*-Boc guanidine **21b** (226 mg, 0.29 mmol) ¹H NMR (300 MHz, CD₃OD, δ): 1.47 (s, 9H), 1.52 (s, 9H), 1.61–1.85 (m, 3H), 1.76 (d, $J = 6.9$ Hz, 3H), 1.88–2.07 (m, 1H), 3.22–3.46 (br, solvent envelope over CH₂), 3.82 (s, 6H), 4.45–4.56 (m, 1H), 5.37–5.55 (m, 1H), 6.79 (s, 2H), 7.04 (d, $J = 7.7$ Hz, 2H), 7.19 (t, $J = 7.2$ Hz, 1H), 7.39 (t, $J = 7.1$ Hz, 2H), 7.71 (s, 1H); ¹³C NMR (500 MHz, CD₃OD, δ): 16.9, 29.5, 30.9, 30.9, 31.2, 43.9, 56.6, 57.2, 59.5, 83.0, 87.0, 110.6, 111.7, 121.8, 124.9, 128.5, 128.7, 133.0, 135.9, 151.9, 152.2, 156.6, 156.7, 160.3, 167.2, 170.7, 174.2, 179.3; MS m/z 785 [M + H]⁺; 807 [M + Na]⁺; to the free guanidine, purified on reverse phase HPLC (10 to 75 % B in A over 30 min), **22b** (2.0 mg, 0.003 mmol) as the TFA salt. ¹H NMR (300 MHz, CD₃OD, δ): 1.57–1.73 (m, 3H), 1.75 (d, $J = 7.0$ Hz, 3H), 1.90–2.10 (m, 1H), 3.10 (t, $J = 6.3$ Hz, 2H), 3.83 (s, 6H), 4.42–4.58 (m, 1H), 5.45 (q, $J = 7.0$ Hz, 1H), 6.80 (s, 2H), 7.06 (d, $J = 7.4$ Hz, 2H), 7.21 (t, $J = 7.5$ Hz, 1H), 7.41 (t, $J = 7.8$ Hz, 2H), 7.72 (s, 1H). ¹³C NMR (500 MHz,

CD₃OD, δ): 16.8, 29.0, 32.4, 44.5, 56.6, 56.8, 59.5, 111.7, 119.5, 121.9, 124.9, 128.8, 133.1, 136.0, 151.8, 152.3, 153.9, 161.2, 165.7, 166.0, 174.4, 179.1. MS m/z 584 [M + H]⁺. HRMS (m/z): [M + H]⁺ calcd for C₂₇H₃₄N₇O₆S, 584.2286; found 584.2279; [M + Na]⁺ calcd for C₂₇H₃₃N₇O₆NaS, 606.2105; found 606.2115.

2-((2Z,5Z)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)acetic acid 23 (329 mg, 0.70 mmol) was obtained by **General method D**, and purified by silica gel chromatography using a gradient from 0 to 10% MeOH in CH₂Cl₂ to give **23** (235.7 mg, 0.57 mmol). ¹H NMR (300 MHz, DMF-*d*₇, δ): 3.70 (br s, 1H), 4.02 (s, 6H), 4.85 (s, 2H), 7.11 (s, 2H), 7.25 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 1H), 7.62 (t, *J* = 7.8 Hz, 2H), 7.95 (s, 1H), 9.62 (br s, 1H). ¹³C NMR (500 MHz, DMF-*d*₇, δ): 53.1, 60.3, 112.4, 112.6, 122.0, 125.5, 128.3, 129.1, 133.6, 133.7, 126.0, 143.5, 152.2, 152.8, 154.1, 170.2. MS m/z 415 [M + H]⁺, 437 [M + Na]⁺. HRMS (m/z): [M + Na]⁺ calcd for C₂₀H₁₈N₂O₆NaS, 437.0783; found 437.0787.

4-((2Z,5Z)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)butanoic acid 25 (190 mg, 0.38 mmol) was obtained by **General method D**, and purified by silica gel chromatography using a gradient from 0 to 10% MeOH in CHCl₃ to give **25** (89 mg, 0.20 mmol). ¹H NMR (300 MHz, CDCl₃, δ): 1.86–2.14 (m, 4H), 2.62 (br s, 1H), 4.00 (s, 6H), 4.07–4.22 (m, 2H), 6.83 (s, 2H), 7.34 (d, *J* = 6.9 Hz, 2H), 7.47–7.64 (m, 3H), 7.84 (s, 1H). ¹³C NMR (500 MHz, CDCl₃, δ): 13.8, 30.3, 60.6, 68.9, 111.6, 119.8, 128.5, 131.8, 133.9, 134.0, 134.8, 136.5, 137.7, 139.2, 142.0, 151.6, 176.5. MS m/z 443 [M + H]⁺, 465 [M + Na]⁺. HRMS (m/z): [M + Na]⁺ calcd for C₂₂H₂₂N₂O₆NaS, 465.1096; found 465.1106.

(S)-5-Amino-N-((S)-1-amino-3-(biphenyl-4-yl)-1-oxopropan-2-yl)-2-((2Z,5Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)pentanamide 52. Compound **52** was obtained by **General method D** on a 0.052 mmol scale and purified on reverse phase HPLC (10 to 75 % B in A over 30 min) to give **52** (6.0 mg, 0.007 mmol) as the TFA salt. ¹H NMR (300 MHz, CD₃OD, δ): 1.60–1.87 (m, 2H), 2.35 (q, *J* = 7.6 Hz, 2H), 2.94–3.13 (m, 3H), 3.16–3.28 (m, 1H), 3.77 (s, 6H), 4.56–4.71 (m, 1H), 5.35 (t, *J* = 7.5 Hz, 1H), 6.72 (s, 2H), 6.91 (d, *J* = 7.5 Hz, 2H), 7.17 (t, *J* = 7.4 Hz, 1H), 7.21–7.37 (m, 8H), 7.40 (d, *J* = 8.1 Hz, 2H), 7.67 (d, *J* = 7.1 Hz, 1H), 7.69 (s, 1H). ¹³C NMR (500 MHz, CD₃OD, δ): 28.1, 28.2, 40.7, 43.0, 58.6, 59.4, 59.9, 111.6, 120.9, 124.9, 128.2, 128.9, 130.3, 130.8, 130.8, 132.3, 133.0, 133.2, 136.5, 139.9, 142.50, 143.5, 144.4, 151.3, 152.2, 153.5, 170.4, 172.9, 178.8. MS m/z 694 [M + H]⁺. HRMS (m/z): [M + H]⁺ calcd for C₃₈H₄₀N₅O₆S, 694.2694; found 694.2687; [M + Na]⁺ calcd for C₃₈H₃₉N₅O₆NaS, 716.2523; found 716.2513.

Scheme 3—General method I for the solid phase synthesis is illustrated by the preparation of **41**. TentaGel S Ram (Advanced ChemTech, 500 mg, 0.25 mmol/g, 0.125 mmol) was swollen in 15 mL CH₂Cl₂ for 30 min, then deprotected by two 10 min cycles of 20% piperidine/DMF (10 mL) with two DMF washes in between. The resin was washed successively with 10 mL each CH₂Cl₂ (3x), DMF (3x), MeOH (2x), and CH₂Cl₂ (3x). A solution of Fmoc L-alanine (117 mg, 0.375 mmol) in 5 mL CH₂Cl₂ was treated successively with hydroxyazabenzotriazole (HOAt, 51 mg, 0.375 mmol), diisopropylcarbodiimide (DIC, 58 mL, 0.375 mmol), and diisopropylethylamine (109 mL, 0.525 mmol), and the reaction mixture stirred for 5 minutes, then added to the deprotected, washed resin, rinsing with an additional 10 mL CH₂Cl₂. The resin was shaken overnight, drained, and subjected to the above standard washing protocol. A sample of the beads gave a negative Kaiser test. After deprotection as above, the sample of beads gave a positive Kaiser test, and the second Fmoc L-alanine was added by the identical procedure. Overnight shaking gave a resin showing, after washing, a negative Kaiser test. The resin was deprotected, washed, checked, and treated with 30 μ L (0.25 mmol) phenylisothiocyanate in 10 mL CH₂Cl₂. Kaiser test showed the reaction to be incomplete after

overnight shaking, and two additional 150 μL (1.25 mmol) phenylisothiocyanate in 10 mL were added over two days to complete the reaction. After washing, a sample of beads were removed, cleaved with 95:5 TFA/ CH_2Cl_2 for 90 min, and the filtrate analyzed by mass spectrometry to show predominant peaks for the N-amidoAlaAla-N'-phenylthiourea at m/z 317 $[\text{M} + \text{Na}]^+$, 295 $[\text{M} + \text{H}]^+$. The resin was treated with methyl bromoacetate (115 μL , 1.25 mmol) and DIEA (436 μL , 2.5 mmol) in 10 mL CH_2Cl_2 , washed, and an aliquot cleaved and examined by mass spectrometry as above to show a predominant $[\text{M} + \text{H}]^+$ molecular ion of 335. To the resin was added a solution of syringaldehyde (229 mg, 1.25 mmol) and piperidine (173 μL , 1.75 mmol) in 10 mL EtOH, and the suspension refluxed for 9 h. After draining and washing, a cleaved aliquot showed the presence of unreacted starting material, and the charge and reflux were repeated. The drained, washed resin was then cleaved by a 90 min treatment with 95:5:5 TFA/ H_2O /triisopropylsilane (10 mL). After filtering the resin, and two washings with 10 mL MeOH each, the filtrate was concentrated *in vacuo* to give 39 mg of a crude product that was purified on RP-HPLC (20 to 95 % B in A over 18 min) to provide 9.84 mg (0.02 mmol) **41** that was identical to the **41** obtained by solution chemistry under Scheme 2. ^1H NMR (500 MHz, CD_3CN , δ): 1.35 (d, $J = 5.8$ Hz, 3H), 1.67 (d, $J = 7.1$ Hz, 1H), 3.83 (s, 6H), 4.35 (dq, $J = 7.2, 7.2$ Hz, 1H), 5.32 (q, $J = 7.2$ Hz, 1H), 5.7 (br, 1H), 7.01 (s, 2H), 7.05–7.46 (m, 5H), 7.70 (s, 1H). MS m/z 499 $[\text{M} + \text{H}]^+$, 521 $[\text{M} + \text{Na}]^+$ 411 $[\text{M} - \text{Ala} + \text{H}]^+$. HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{26}\text{N}_4\text{NaO}_6\text{S}$, 521.1465; found 521.1467.

An earlier-eluting minor peak was identified as the D-Ala-L-AlaNH₂ diastereomer m/z 499 $[\text{M} + \text{H}]^+$, 521 $[\text{M} + \text{Na}]^+$, 411 $[\text{M} - \text{Ala} + \text{H}]^+$, 428 $[\text{M} - \text{C}(\text{CH}_3)\text{CONH}_2 + \text{H}]^+$.

(S)-2-((S)-3-(Biphenyl-4-yl)-2-((Z,Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)-5-guanidinopentanamide 55a— ^1H NMR (500 MHz, CD_3CN , δ): 1.65–1.70 (m, 2H), 3.12–3.20 (m, 2H), 3.61 (s, 2H), 3.80 (s, 6H), 4.29–4.33 (m, 1H), 5.52 (dd, $J = 6.4, 6.3$ Hz, 1H), 5.86 (br, 1H), 6.75 (s, 2H), 6.85 (d, $J = 7.8$ Hz, 2H), 7.19–7.62 (m, 13H), 7.87 (br, 1H). MS m/z 736 $[\text{M} + \text{H}]^+$. HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{39}\text{H}_{42}\text{N}_7\text{O}_6\text{S}$, 736.2912; found 736.2888.

(S)-N-((S)-1-Amino-3-(biphenyl-4-yl)-1-oxopropan-2-yl)-5-guanidino-2-((Z,Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)pentanamide 54— ^1H NMR (500 MHz, CD_3CN , δ): 1.46–1.50 (m, 2H), 2.91–3.10 (m, 2H), 3.12–3.20 (m, 2H), 3.65 (s, 2H), 4.30–4.45 (m, 1H), 5.10 (t, $J = 7.6$ Hz, 1H), 5.82 (br, 1H), 6.62 (s, 2H), 6.85 (d, $J = 7.8$ Hz, 2H), 6.81–7.56 (m, 13H). MS m/z 736 $[\text{M} + \text{H}]^+$. HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{39}\text{H}_{42}\text{N}_7\text{O}_6\text{S}$, 736.2912; found 736.2884.

(S)-5-Amino-2-((S)-3-(biphenyl-4-yl)-2-((Z,Z)-5-(4-hydroxy-3,5-dimethoxybenzylidene)-4-oxo-2-(phenylimino)thiazolidin-3-yl)propanamido)pentanamide 56— ^1H NMR (500 MHz, CD_3CN , δ): 1.77–1.80 (m, 2H), 1.97–1.99 (m, 2H), 3.06–3.10 (m, 2H), 3.60–3.64 (m, 2H), 3.78 (s, 6H), 4.42–4.55 (m, 1H), 5.64 (dd, $J = 10.3, 5.4$ Hz, 1H), 6.08 (br, 1H), 6.74 (s, 2H), 6.82 (d, $J = 7.6$ Hz, 2H), 7.19–7.62 (m, 16H). MS m/z 694 $[\text{M} + \text{H}]^+$. HRMS (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{38}\text{H}_{40}\text{N}_5\text{O}_6\text{S}$, 694.2694; found 694.2681.

Sip A secretion assay: *Salmonella typhimurium* secretion assays: Bacteria were grown for 18 h in LB broth and bacterial cells were pelleted by centrifugation. Culture supernates were filtered through a 0.2 μm polyethersulfone syringe filter. 10% TCA was added to culture supernates to precipitate proteins and incubated on ice for 30 min and spun at 16K rpm for 20 min. Pellets were washed with acetone and resuspended in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and Western Blotted with polyclonal anti-SipA antibodies.⁴³

MIC determinations: To 30 μL aliquots of a 2 mL overnight culture of *S. typhimurium* (ATCC 14028) 0.1, 1.0, 2.0, 3.0, 6.0, and 15.0 μL of each test compound in DMSO was added. The

treated cultures were incubated overnight and growth determined by reading the OD₆₀₀. Each sample was run in triplicate. DMSO alone was used as a control and gave partial growth inhibition at 20% v/v in LB (comparable to 2 mM compound) and complete growth inhibition at 50% v/v (comparable to 5 mM compound).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ala	alanine
Arg	arginine
ATPase	adenosine triphosphate hydrolase
ATCC	American Type Culture Collection
Boc	<i>tert</i> -butoxycarbonyl
Cbz	benzyloxycarbonyl
Dht	dihydrotryptophan
DIC	diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMSO	dimethylsulfoxide
Fmoc	fluorenylmethyloxycarbonyl
HOAt	hydroxyazabenzotriazole
HTS	

	high-throughput screen
LB	Luria-Bertoni broth
O.D	optical density
Orn	ornithine
Pbf	pentamethylsulfonylbenzofuran
RP-HPLC	reverse phase high performance liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T2SS	Type II Secretion System
T3SS	Type III Secretion System
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TLC	thin layer chromatography
Tyr	tyrosine
Trp	tryptophan
Val	valine
Z	benzyloxycarbonyl

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33. Calculated logP values for the dipeptides, using the algorithm provided by ChemDraw v.9.01, gave a range of values from 0.98 (24, 48) to 4.47 (54, 55a), compared to a calculated logP value of 4.87 for 1. Experimentally derived values were somewhat lower although the ranking did not change. These values were determined for 55a (logP= 2.63) and 1 (logP=3.34 by monitoring the respective molecular ions on LC-MS and using calibration curves ranging from 0.1 to 10 μ M (MeOH) to obtain the concentration of each compound partitioned into octanol and water from the respective dry samples. The LC gradient was 25 to 95% Bin A over 15 minutes, where A was 94:5:1 water:acetonitrile: acetic acid and B was 99:1 acetonitrile:acetic acid. The column was a Zorba SB-C18 (2.1 \times 100 mm) maintained at 30 $^{\circ}$ C. All samples were analyzed in positive ionization mode using the respective specific precursor/product ions 433.1/195.2 (dwell 0.1 min, cone 45, coll energy 35V) for 1 and 736.3/563.2 (dwell 0.1 min, cone 25, coll energy 33V) for 55a.
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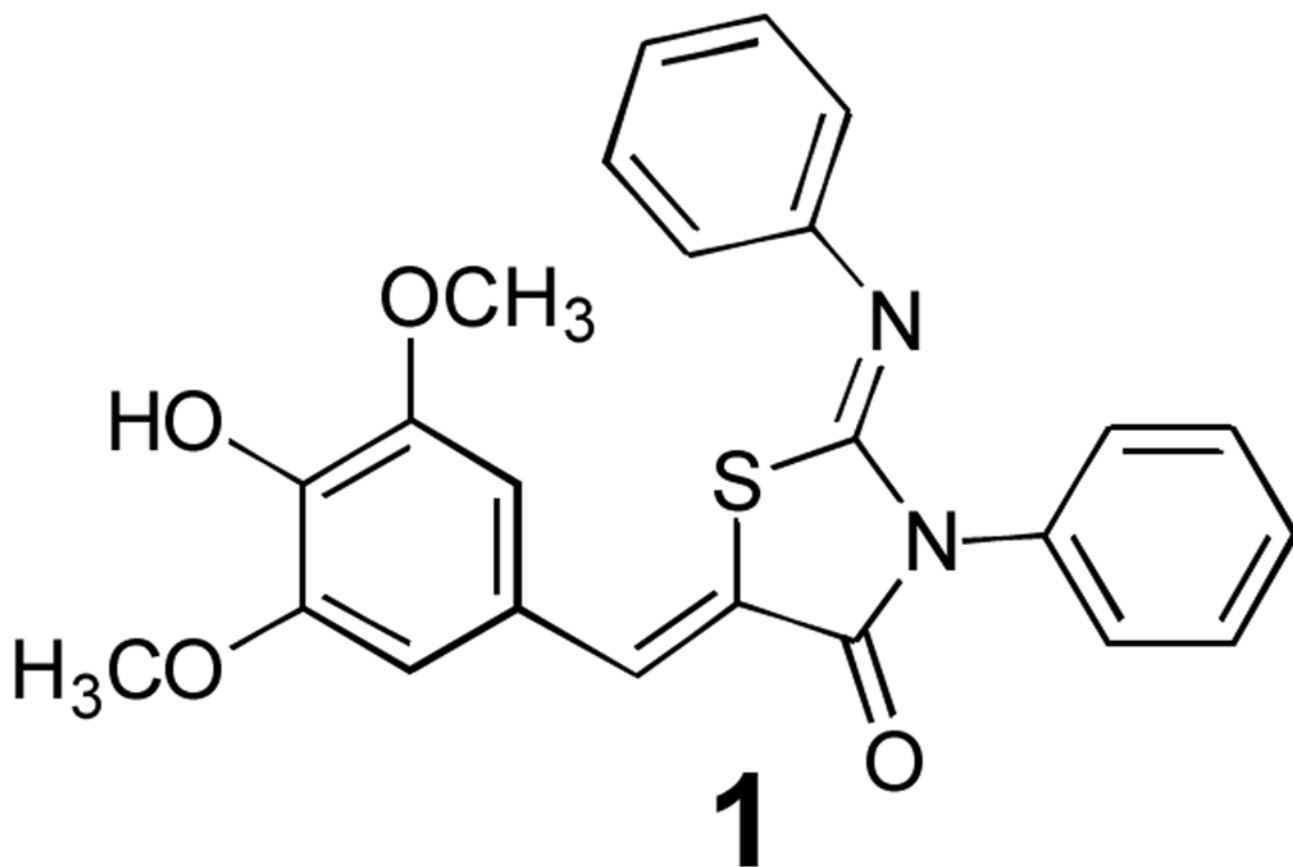


Figure 1.
The lead structure emerging from our high-throughput screen for T3SS inhibitors

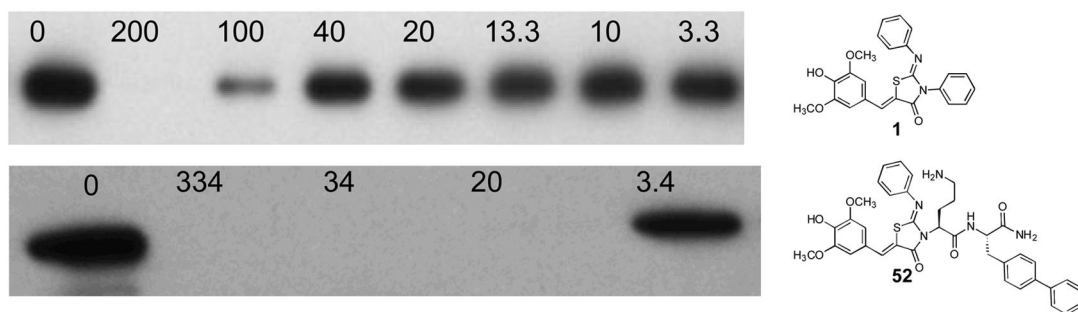
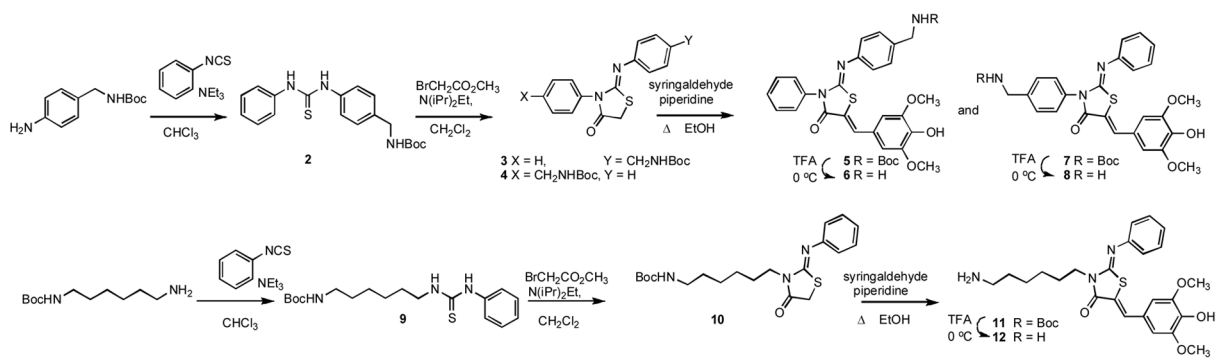
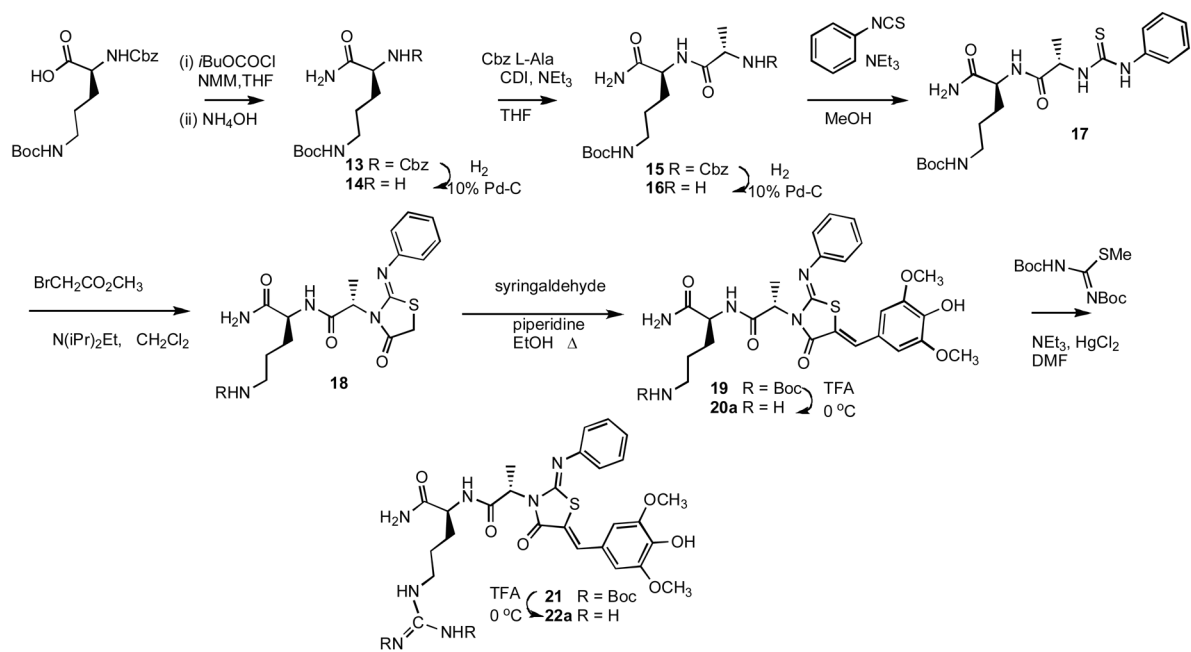


Figure 2. Inhibition of the secretion of the virulence protein SipA into the culture medium by *S. typhimurium*

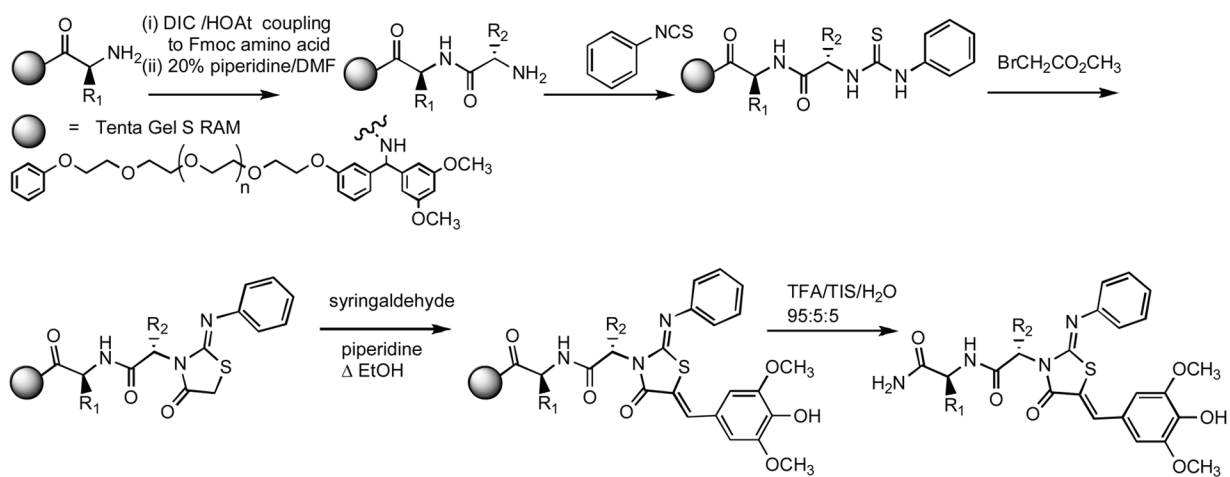
Representative Western Blots of SipA secreted into culture medium from *S. typhimurium* grown in the presence of compound **1** (above) and **52** (below) at the concentrations (in μM) indicated above each blot. The results for **55a** were comparable to those shown for **52**.¹⁰ Secretion in the absence of compound but the presence of 5% DMSO is shown ('0') at the far left.



Scheme 1.

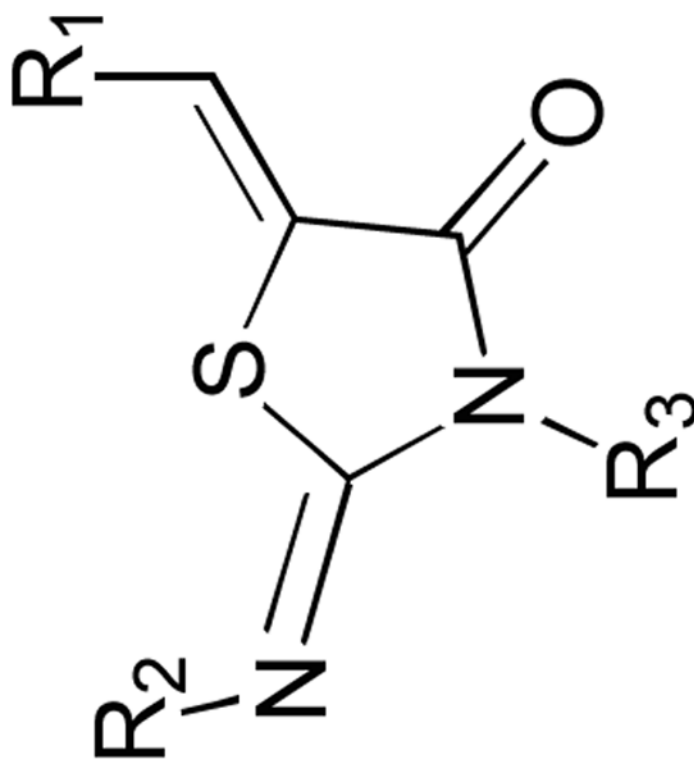


Scheme 2.

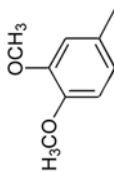
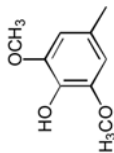


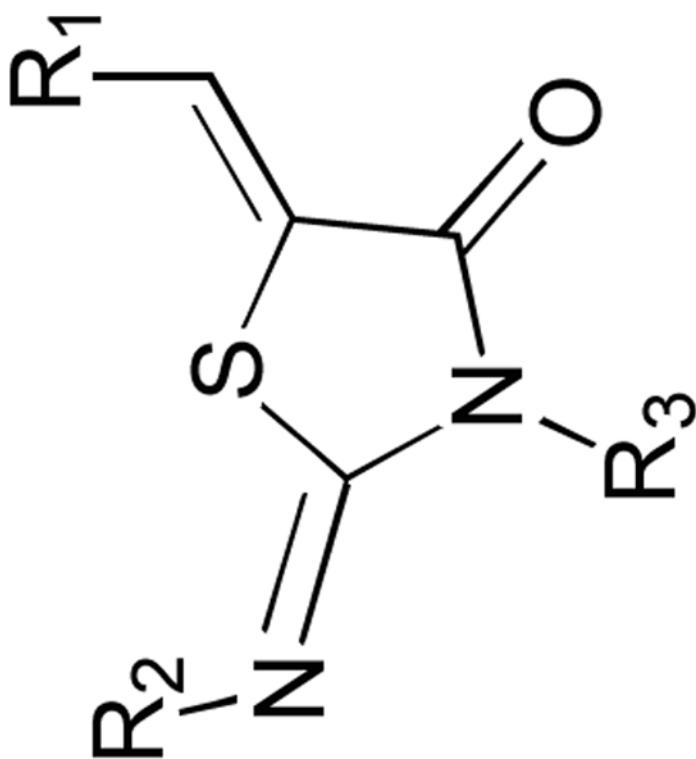
Scheme 3.

Synthetic diversity set generated to evaluate modifications of R₁, R₂, and R₃. The IC₅₀ values are calculated from the percent inhibition of SipA secretion, as determined by Western Blot, using a minimum of 3 concentrations of inhibitor. Compounds **36–40** are regiomeric pairs in which the major isomer has R₂ as *p*-methoxyphenyl and R₃ as phenyl, and the minor isomer has R₃ as *p*-methoxyphenyl and R₂ as phenyl.

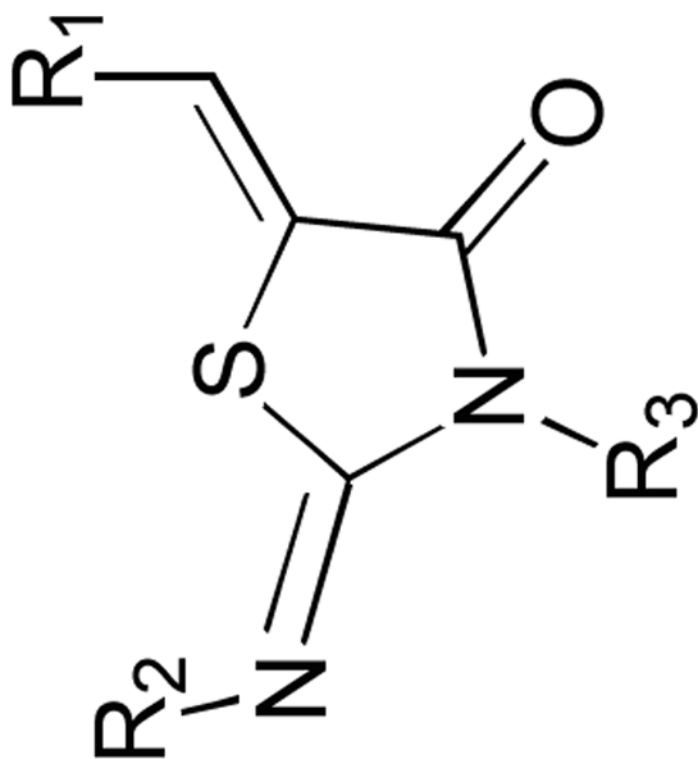


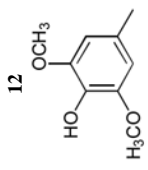

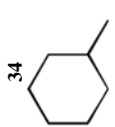
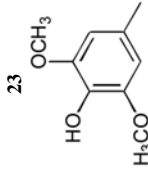

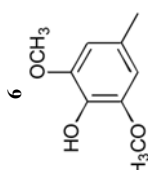
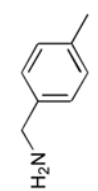
R ₁	R ₂	R ₃	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
1	Ph	Ph	83	31	Ph	Ph	278

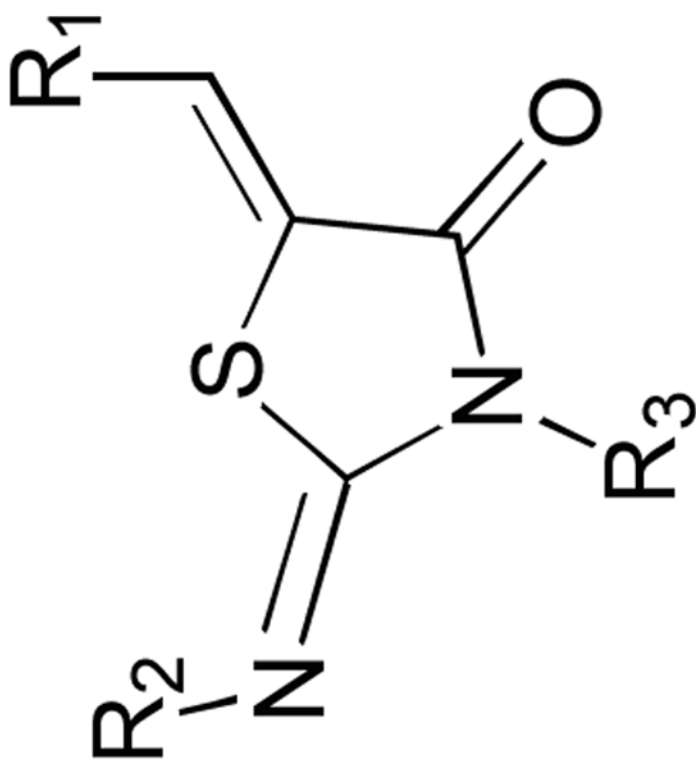




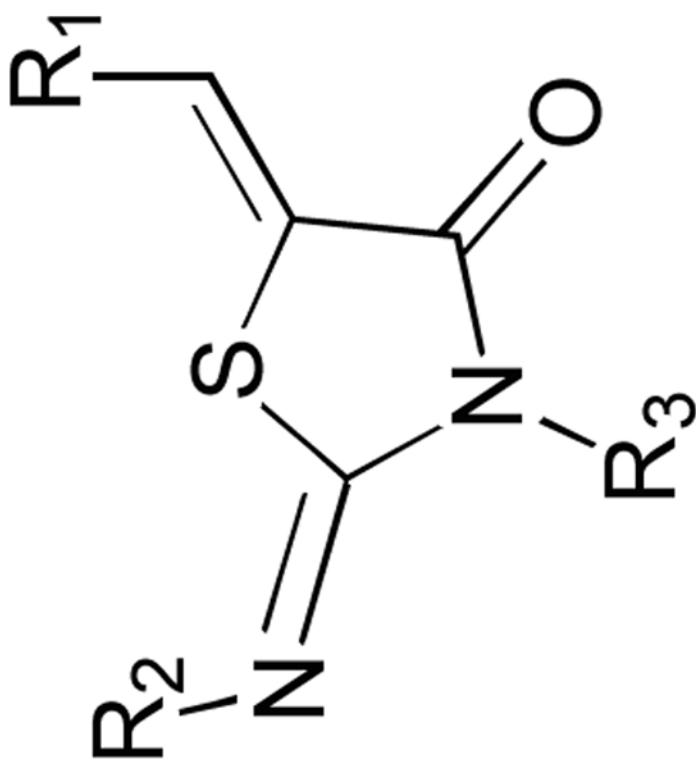
R ₁	R ₂	R ₃	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
	Ph		45		Ph		278
	Ph		52		Ph		160



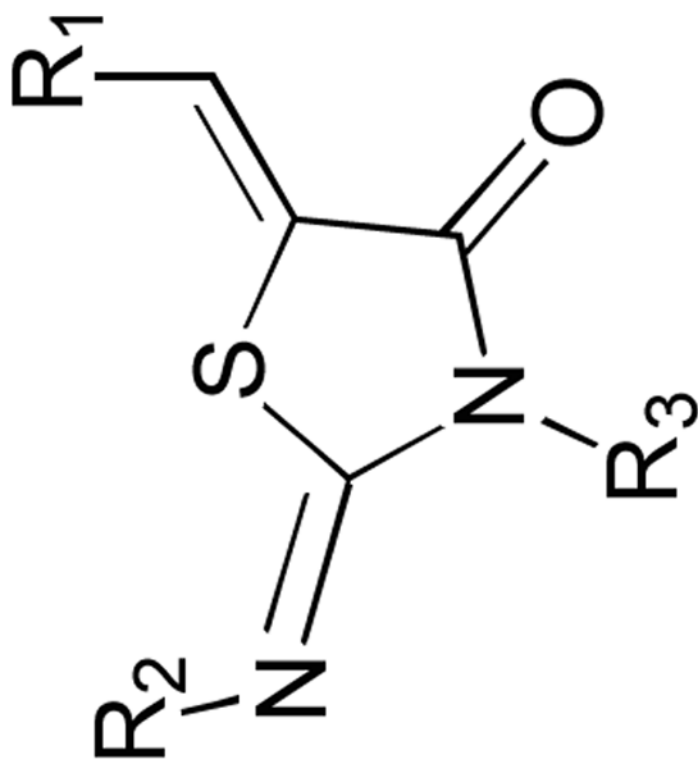
R ₁	R ₂	R ₃	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
 12	Ph	 54	54	 34	Ph	Ph	225
 23	Ph	 36	36	 6	 101	Ph	101

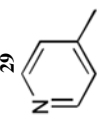
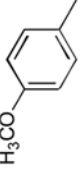
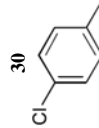


R ₁	R ₂	R ₃	R ₄	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
	Ph			4			Ph	137
	Ph			66			Ph	124



R ₁	R ₂	R ₃	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
	Ph		51			Ph	198
	Ph	Ph	26			Ph	80
	Ph	Ph	244			Ph	52



R ₁	R ₂	R ₃	IC ₅₀ (μM)	R ₁	R ₂	R ₃	IC ₅₀ (μM)
	Ph	Ph	376	40 ^a		Ph	
	Ph	Ph	322				

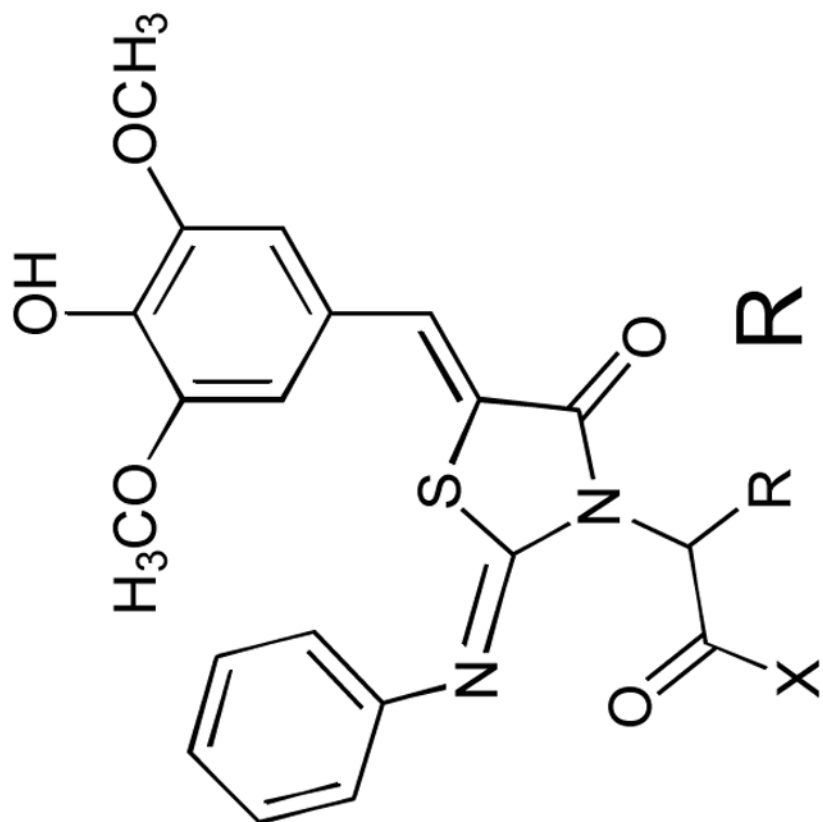
^aR₂ and R₃ were regiomeric mixtures of *p*-methoxyphenyl and phenyl, with the major regiomeric indicated in the column.

^bSignificant (≥50%) inhibition only at 1 mM

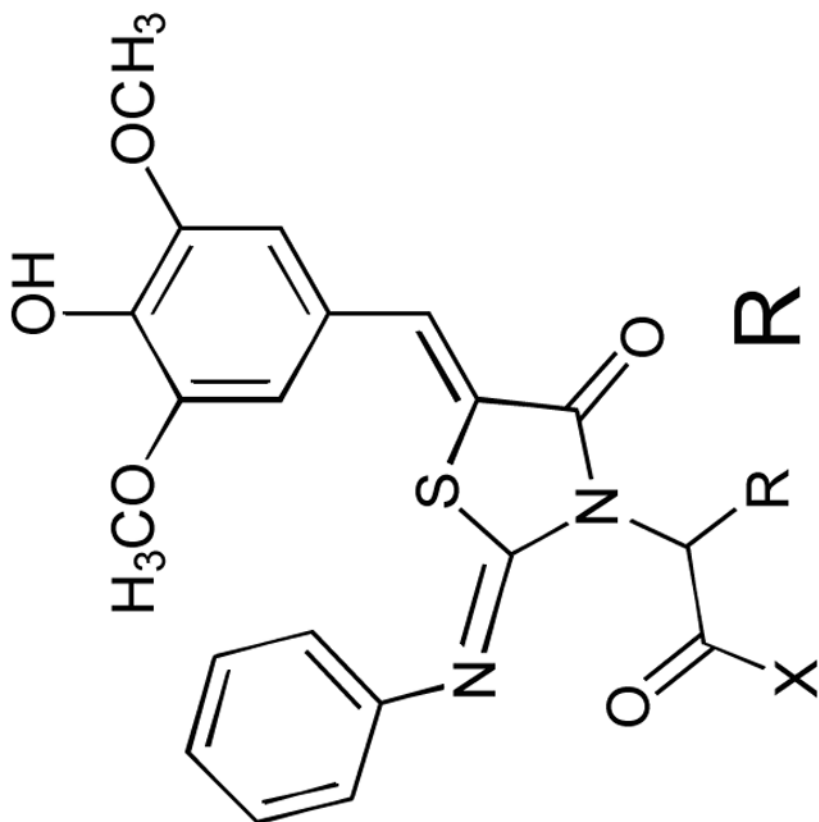
Dipeptides presented from N-3. The IC₅₀ values are calculated from the percent inhibition of SipA secretion, as determined by Western Blot, using a minimum of 3 concentrations of inhibitor. The carbons are all S (retention of the L-amino acid stereochemistry) unless

Table 2

otherwise designated. For the discrete epimers **44a/44b** and **49a/49b** the absolute configurations were not determined. Dihydrotryptophan analogs **49a/49b** and **50**, derived from commercially supplied racemic material, are correspondingly racemic at the γ carbon.



R	X	IC ₅₀ (μM)	R	X	IC ₅₀ (μM;M)
41 CH ₃	AlaCONH ₂	62	49a		52

IC₅₀ (μM)

X

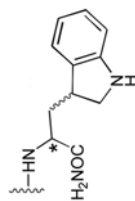
R

IC₅₀ (μM)

X

R

56

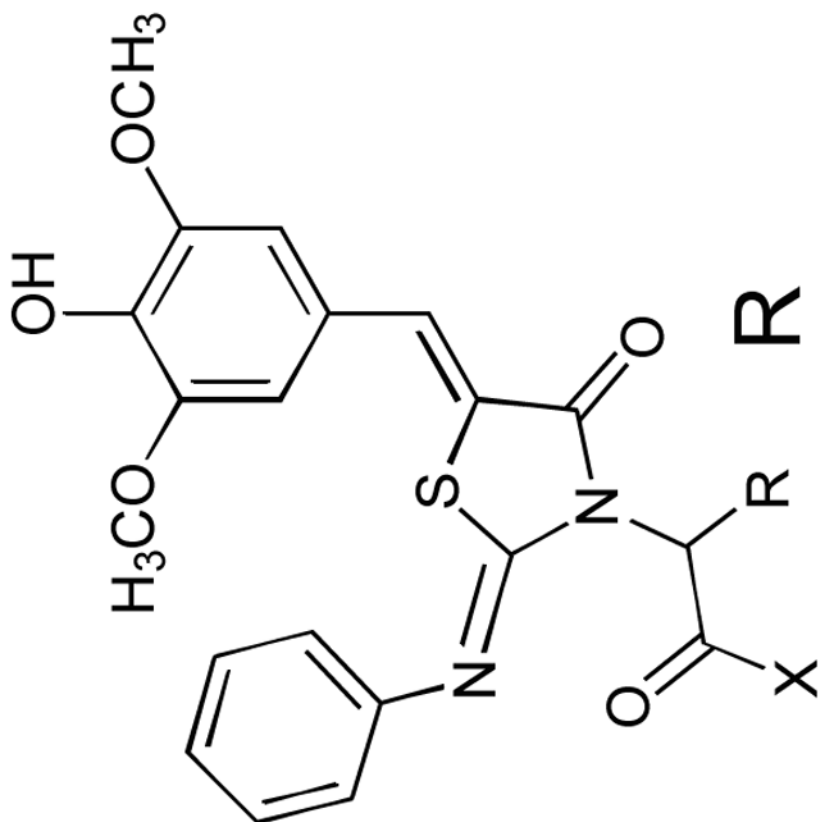


49b



52

TyrCONH₂42 CH(CH₃)₂

IC₅₀ (μM)

X

R

IC₅₀ (μM)

X

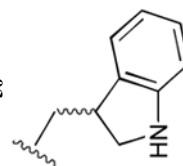
R

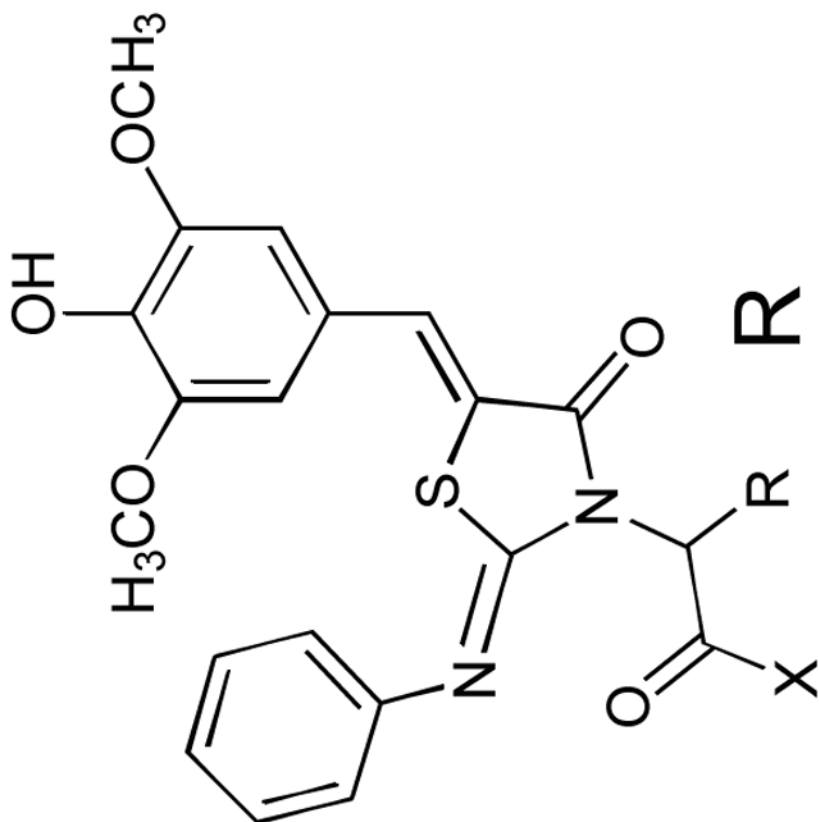
50

ArgCONH₂

50

112

TyrCONH₂43 CH₃

IC₅₀ (μM)

X

R

IC₅₀ (μM)

X

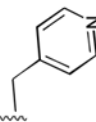
R

109

ArgCONH₂

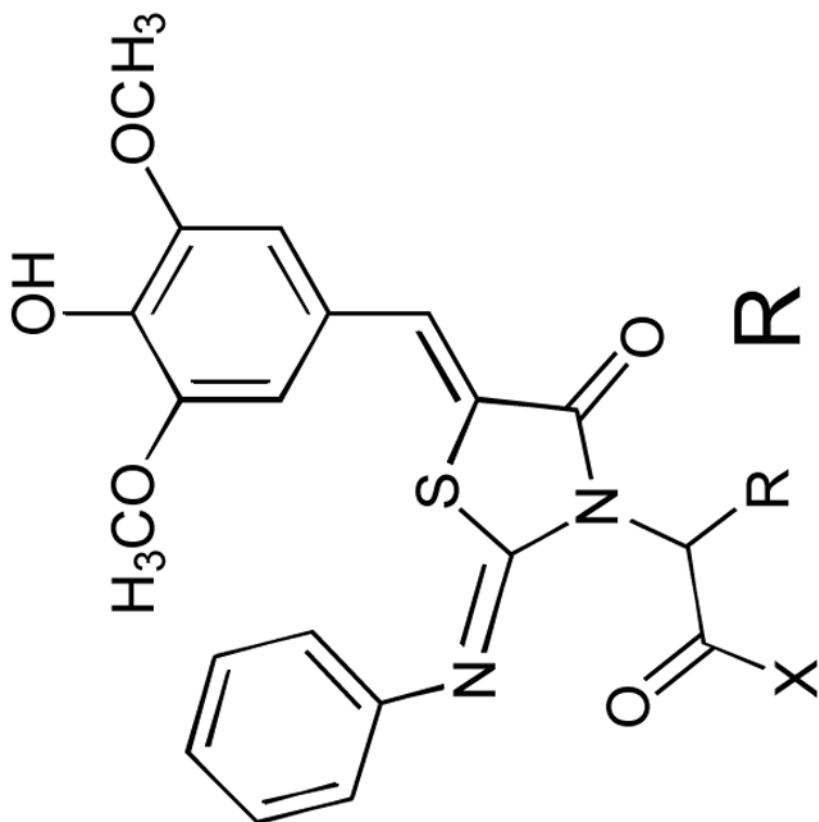
51

30

TrpCONH₂44a CH₃ (isomer A)

29

TrpCONH₂44b CH₃ (isomer B)

IC₅₀ (μM)

X

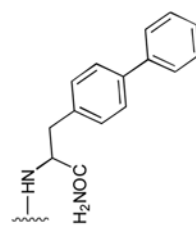
R

IC₅₀ (μM)

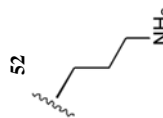
X

R

13

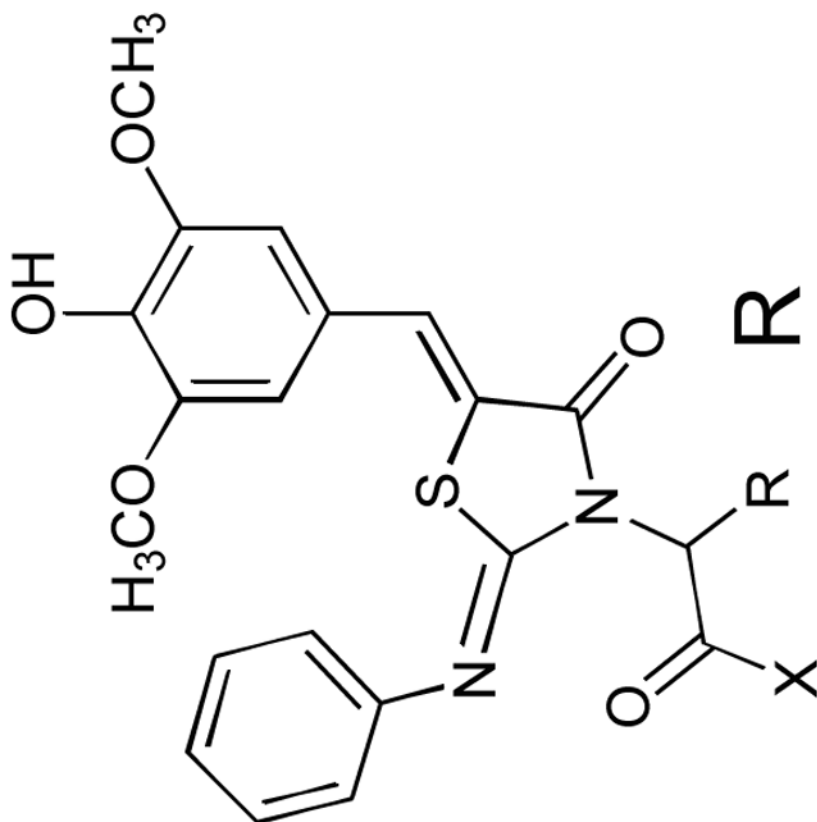


52



16

OmtCONH₂20a CH₃ (S)

IC₅₀ (μM)

X

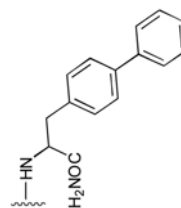
R

IC₅₀ (μM)

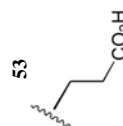
X

R

50

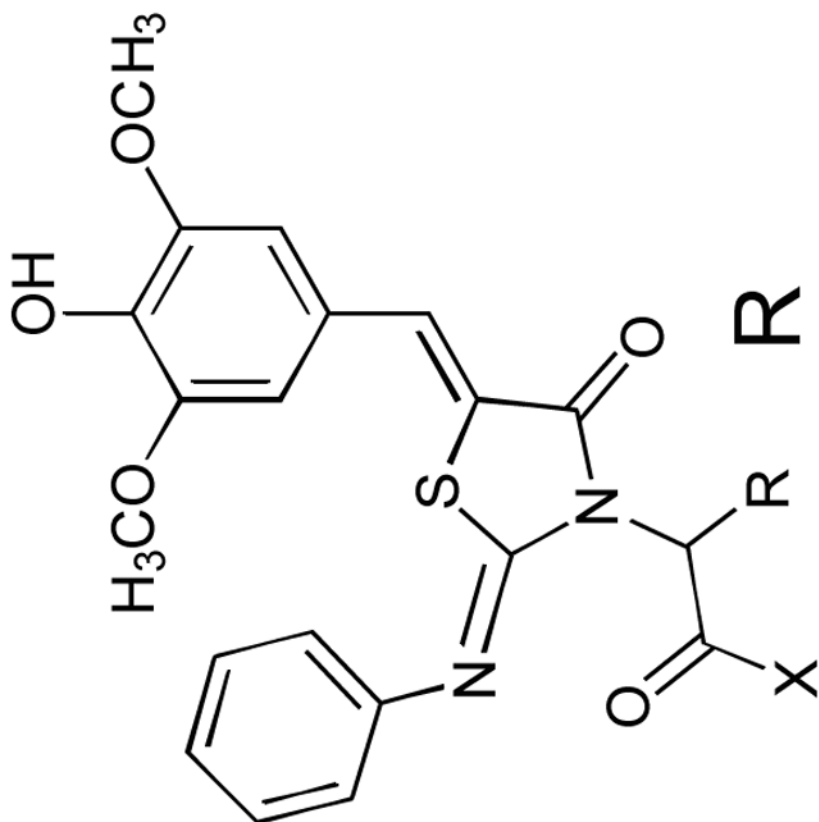


53

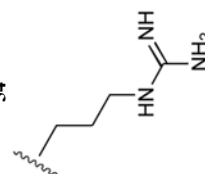
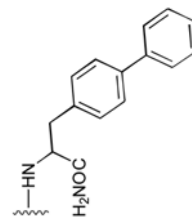


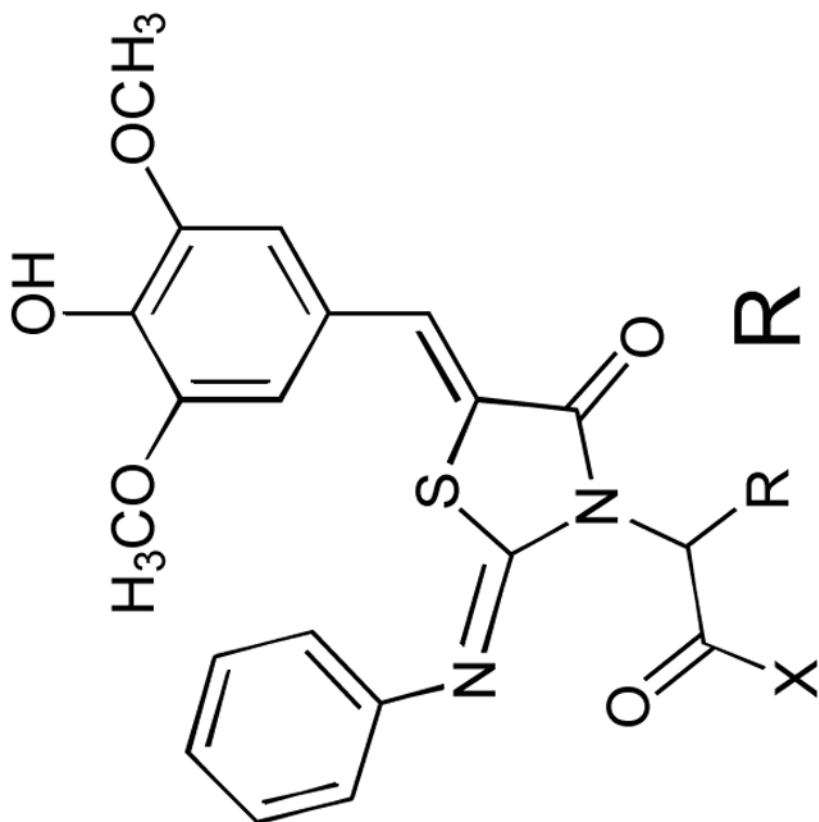
9

O^omCONH₂20b CH₃ (R)



R	X	IC ₅₀ (μM)	R	X	IC ₅₀ (μM)
22a CH ₃ (S)	ArgCONH ₂	77			50
22b CH ₃ (R)	ArgCONH ₂	38			
			54		



IC₅₀ (μM;M)

X

R

IC₅₀ (μM)

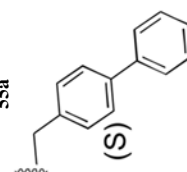
X

R

3

ArgCONH₂

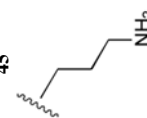
55a

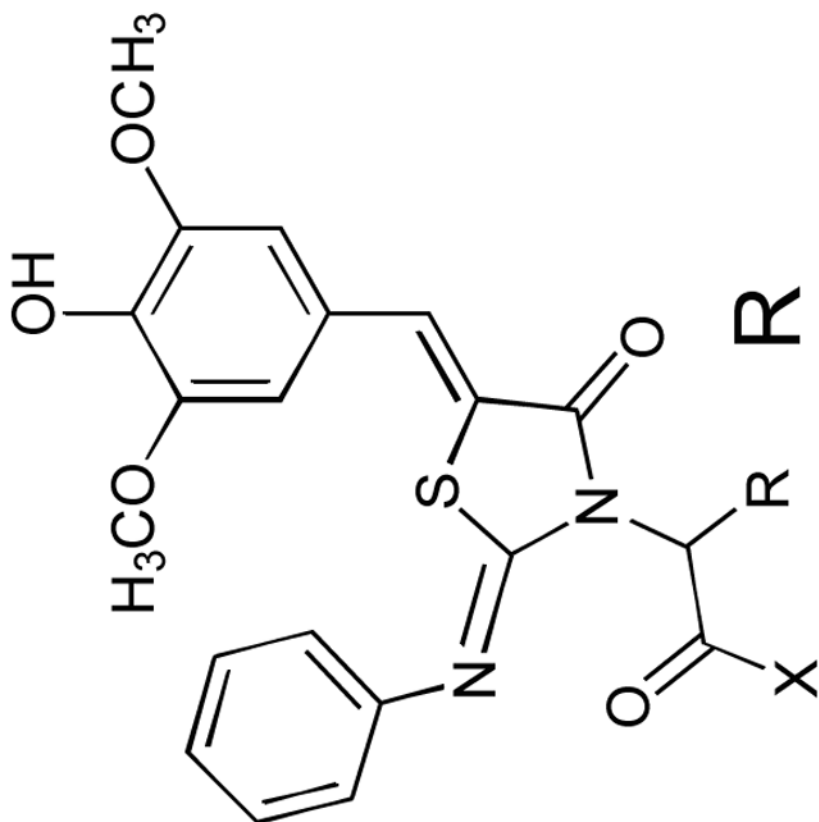


32

AlaCONH₂

45



IC₅₀ (μM)

X

R

IC₅₀ (μM)

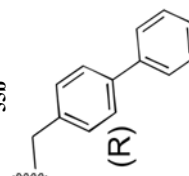
X

R

8

ArgCONH₂

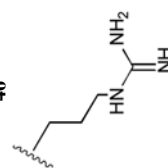
55b

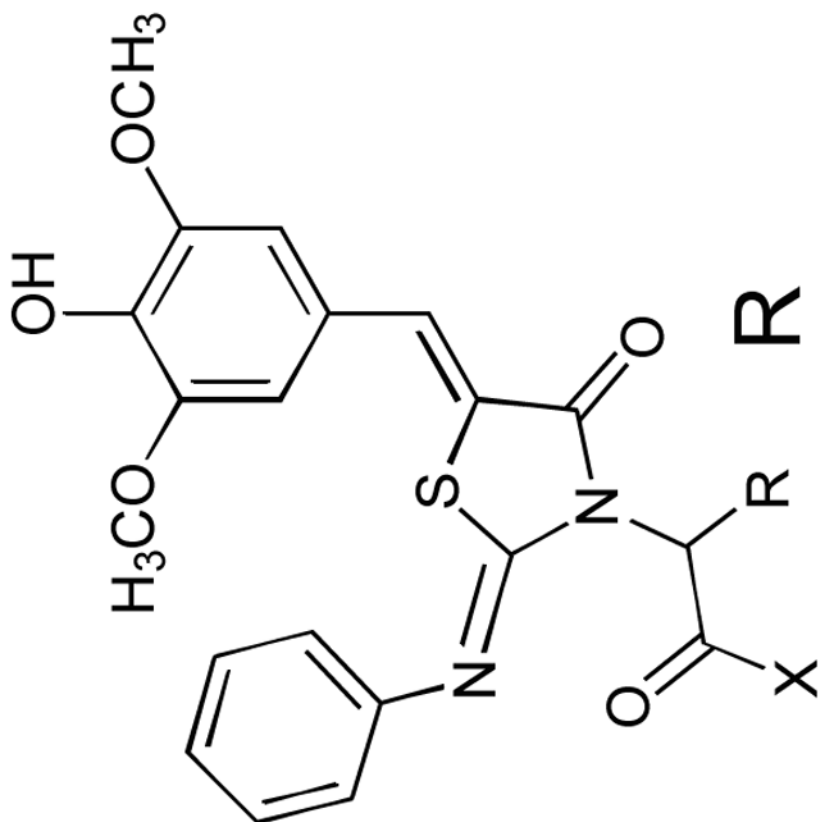


41

AlaCONH₂

46



IC₅₀ (μM)

X

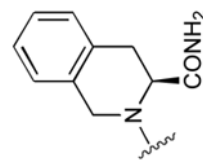
R

IC₅₀ (μM)

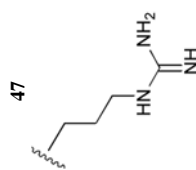
X

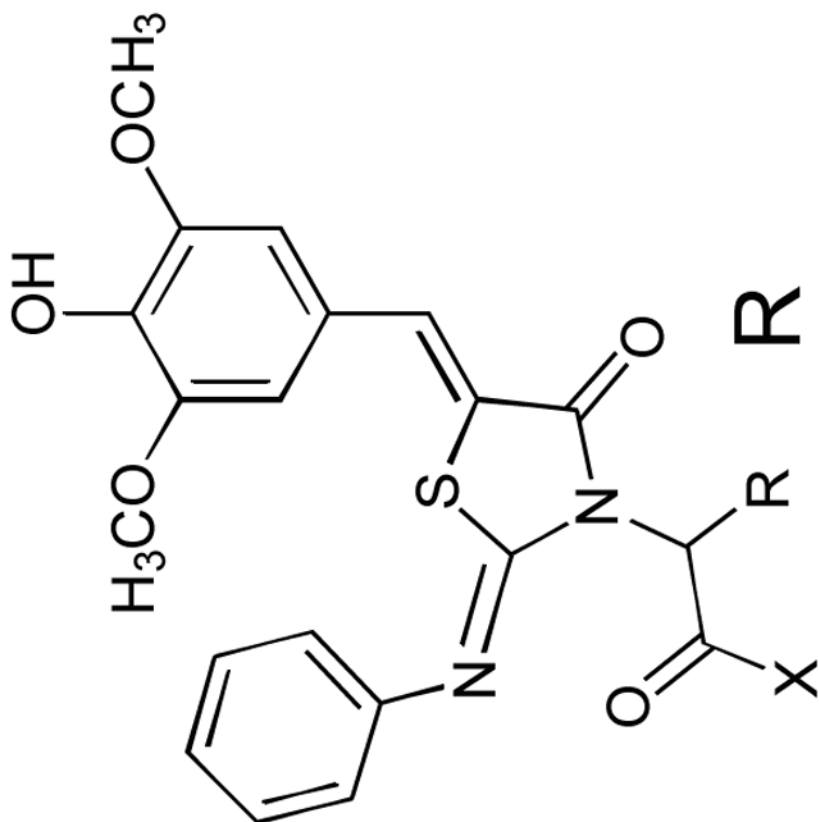
R

115



47



IC₅₀ (μM)

X

R

IC₅₀ (μM)

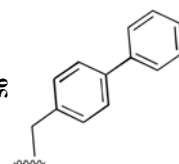
X

R

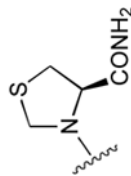
55

OmtCONH₂

56



65



48

