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Synthesis and Evaluation of Novel Dipeptide-Bound 1,2,4-Thiadiazoles as Irreversible Inhibitors of Guinea Pig Liver Transglutaminase

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Abstract—Herein we report the synthesis and evaluation of 14 novel peptides as potential irreversible inactivators of guinea pig liver transglutaminase (TGase). These peptides were designed to resemble Cbz-L-Gln-Gly, known to be a good TGase substrate, and to include a 1,2,4-thiadiazole group. The side chain length of the amino acid residue bearing the inhibitor group was also varied in order to permit investigation of this effect. Their inactivation rate constants were measured using a direct continuous spectrophotometric method and were found to vary between 0.330 to 0.89 μM^{-1} min^{-1}.

Introduction

Transglutaminases (TGases, EC 2.3.2.13) are calcium-dependent enzymes that catalyze the intermolecular cross-linking of certain proteins by γ-glutamyl-ε-lysine side chain bridges. As shown in Figure 1, the first step of the simplified enzymatic mechanism involves the nucleophilic attack of the active site cysteine thiol on the γ-carboxamide group of the acyl donor substrate. In mammals there are essentially three types of TGases, found in tissue, plasma and epidermis. Tissue TGases are involved in diverse biological processes such as endocytosis,[1,2] apoptosis[3] and cell growth regulation.[4] The plasma-soluble form of TGase, Factor XIIIa, stabilizes blood clots by catalyzing fibrin cross-linking during hemostasis.[5–7] Epidermal TGase plays a key role in the synthesis of the cornified envelope of epidermal keratinocytes.[8–11]

Unregulated high TGase activities give rise to physiological disorders which may be involved in such disease states as acne,[12,13] cataracts,[14] immunologic diseases,[15] psoriasis[16–18] and Alzheimer's disease.[19–25] In order to regulate excess TGase activities, a number of potential TGase inactivators have been developed in recent years, including sulfonamides,[26,27] iodoacetates,[28–31] isocyanates,[32] thioureas,[33] acivicin derivatives,[34,35] sulfonium methyl ketones,[36] thioacetonyl heterocycles[37] and electrophilic glutamine analogues.[38] However, their weak specificity limits their therapeutic utility.

In TGase-catalyzed reactions, an acyl group is transiently transferred from the acyl-donor substrate to an active site cysteine thiol residue of the enzyme (Fig. 1). Previous work has shown that 1,2,4-thiadiazoles are susceptible to nucleophilic attack at sulfur followed by ring opening.[39] Moreover, 1,2,4-thiadiazoles derivatives have been shown to react with active site thiol residues of certain cysteine proteases to form covalent adducts.[40,41] Irreversible inhibition thus occurs by the formation of a disulfide bond upon the opening of the thiadiazole ring (Fig. 2). In this context, we have synthesized several series of novel potential TGase inhibitors.[42] These inhibitors were designed to be analogues of carbobenzyloxy-L-glutaminylglycine (Cbz-Gln-Gly), a well-known TGase acyl donor substrate.[31,43] In this paper, we report the synthesis and kinetic evaluation of peptidic TGase inhibitors containing a pendant 1,2,4-thiadiazole group (Fig. 3).

Results and Discussion

Our inhibitor design strategy was based on the structure of carbobenzyloxy-L-glutaminylglycine (Cbz-Gln-Gly),

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a commonly used dipeptide TGase substrate.[31,43] The acylation step of the enzymatic mechanism involves the attack of the active site cysteine thiol residue on the carbonyl of the γ-carboxamide group of the glutamine side chain of the acyl-donor substrate. We therefore chose to place the reactive thiadiazole units on the end of the γ-glutamyl analogue side chain. The thiadiazole groups were attached to the dipeptide framework through either an amide or amine linkage. Furthermore, we prepared analogues with side chains of different lengths in order to study the effect of this variation.

Synthesis of thiadiazole amide derivatives

The starting point of the synthesis of the amide-linked thiadiazole derivatives was the anhydride of either Cbz-Asp or Cbz-Glu, obtained by reaction of the corresponding protected amino acid with acetic anhydride as described previously.[44,45] In parallel, 5-amino-3-methyl-1,2,4-thiadiazole (**5**) was synthesized from acetamide and potassium thiocyanate in methanol according to a literature procedure.[39,46,47] The pure product **5** was obtained with an excellent yield of 98%.

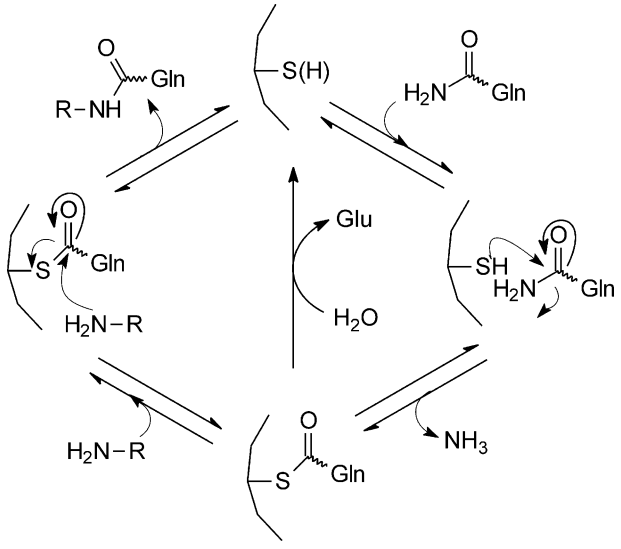


Figure 1. Typical TGase enzymatic mechanism.

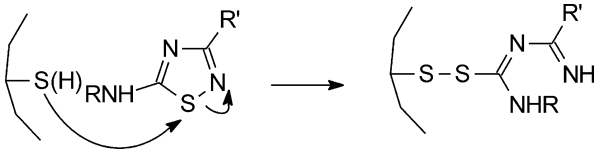


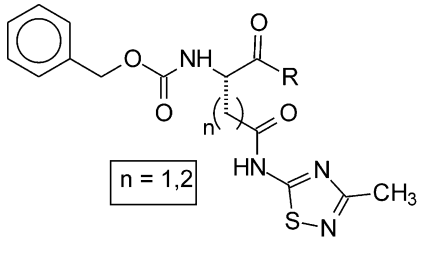
Figure 2. Irreversible inhibition of an active site thiol by a thiadiazole functional group.

Amides **1a**–**2a** were then prepared through a quantitative regioselective opening of the anhydride ring by the nucleophilic attack of the amino group of the thiadiazole on the β or γ position of the corresponding anhydride (Scheme 1). (Note that the compound numbers of these amides also correspond to the number of methylene units contained in their side chains; this numbering system was used for all of the peptide synthetic intermediates described herein.) As shown previously for the formation of anilides,[45,48,49] this nucleophilic attack takes place preferentially on the distal β/γ carbonyl in polar aprotic solvents such as DMSO. In non-polar solvents, nucleophilic attack occurs on the α carbonyl, thus permitting control of the regioselectivity of the reaction through the choice of solvent.

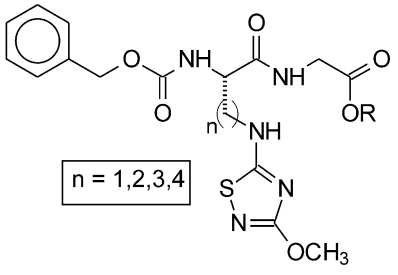
The subsequent coupling of products **1a**–**2a** with glycine *tert*-butyl ester was achieved through activation by reaction with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) in the presence of triethylamine in dichloromethane. Compounds **1b**–**2b** were thus obtained with yields around 85%. The *tert*-butyl esters were then hydrolyzed by bubbling hydrogen chloride gas through dichloromethane solutions to give the corresponding acids **1c**–**2c** in quantitative yields.

Synthesis of thiadiazole amine derivatives

The starting point of the synthesis of the amine-linked thiadiazole derivatives, shown in Scheme 2, was a series of N_α-Cbz-protected diamino acids. These amino acids (derivatives of Cbz-L-Lys ($n=4$), where the number of methylene units of the pendant alkyl amino side chain was varied from $n=1$ to 4) were prepared according to previously described procedures.[50] Protection of the primary amino group by reaction with 9-fluorenylmethyl chloroformate (Fmoc-Cl) yielded compounds **1d**–**4d** in around 95% yields. The fully protected diamino acids were then coupled with glycine *tert*-butyl ester after activation with EDC in the presence of triethylamine in dichloromethane. In this way, dipeptides **1e**–**4e** were achieved in 95–97% yields. The Fmoc



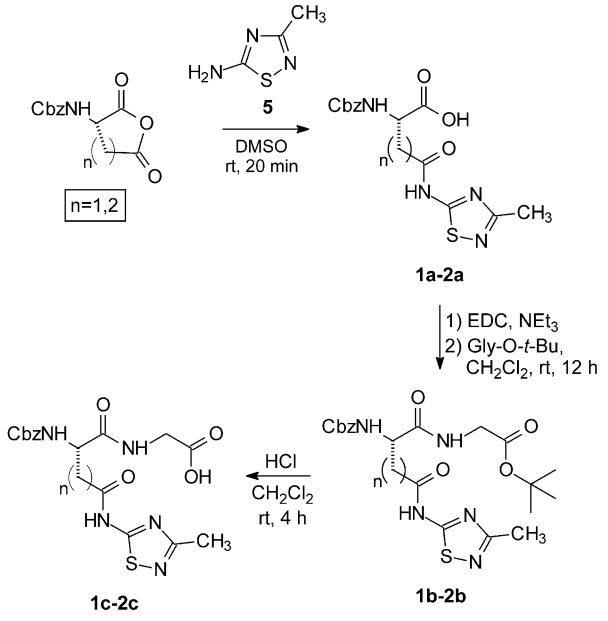
1a - 2a : R = OH
1b - 2b : R = Gly-O-*t*-Bu
1c - 2c : R = Gly-OH



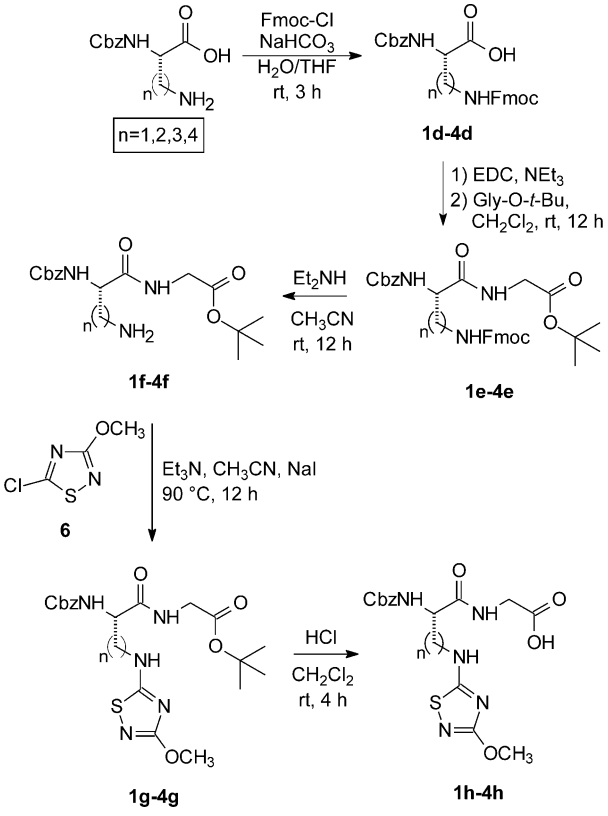
1g - 4g : R = *t*-Bu
1h - 4h : R = H

Figure 3. Thiadiazole inhibitors studied herein.

protecting group was then removed by reaction with an excess of diethylamine in acetonitrile to give compounds **1f**–**4f** in yields around 93%. These primary amines were then coupled with 5-chloro-3-methoxy-1,2,4-thiadiazole (**6**), previously obtained by the reaction of methylisourea hydrogenosulfate with perchloromethylmercaptan.[51] The yield of this coupling reaction to give products **1g**–**4g** varied between 19–26%. This problematic step was optimized by the addition of sodium iodine, the use of a polar aprotic solvent such as acetonitrile and heating. However, in spite of the modest improvements afforded by these modifications, the yields remained low due the instability of the chlorothiadiazole **6**. This instability is in marked contrast to the stability of aminothiadiazole products **1g**–**4g** and aminothiadiazole **5**, which gave no problems during the synthesis of compounds **1a**–**2a**. Finally, the *tert*-butyl esters were hydrolyzed by bubbling hydrogen chloride gas through a dichloromethane solution to provide the corresponding acids **1h**–**4h** in quantitative yields.



Scheme 1.



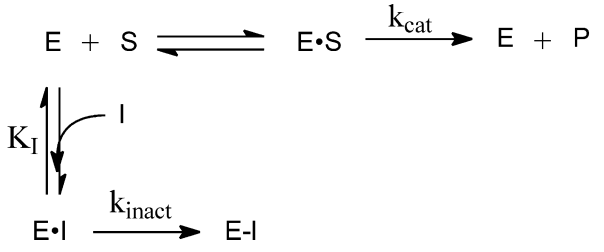
Scheme 2.

Evaluation of thiadiazole peptide inhibitors

Guinea pig liver was chosen as a source of TGase for this kinetic study due to its abundance in TGase in comparison to rat, mouse and rabbit liver. Guinea pig liver is also the most widely used source for TGase and the purification of guinea pig liver TGase has been refined in our labs.[52] Furthermore, guinea pig liver TGase shows 80% homology with human tissue TGase, thereby validating its use as a human model.[53]

Some of the compounds shown in Figure 3 were found to inhibit guinea pig liver TGase and all inhibition was found to be time-dependent. This time-dependent inhibition was found to be cleanly mono-exponential at all concentrations of inhibitor. Mono-exponential time-dependent inhibition is consistent with consumption of the enzyme upon irreversible inactivation and distinctly different than the biphasic (exponential/linear) product release that would be observed if the time-dependent inhibition was due to slow-binding reversible inhibition. In addition, when the enzyme was inactivated in the presence of excess inhibitor, and then filtered over a molecular-weight cutoff membrane to remove excess inhibitor, the recuperated enzyme was found to remain inactive over time, demonstrating the irreversibility of the inhibition. Lastly, this irreversible inactivation was found to take place more slowly in the presence of higher concentrations of substrate. This temporary protection from inactivation afforded by substrate binding is consistent with inactivation taking place at the active site (Scheme 3).

Determination of irreversible inhibition constants was based on analysis of the mono-exponential curves of the progress of the enzymatic reaction in the presence of the irreversible inhibitors (see Scheme 3 and Fig. 4), followed by the continuous spectrophotometric method previously developed in our laboratory.[54] Pseudo-first order inactivation rate constants k_{obs} were calculated from these curves and used to prepare a plot of $\frac{1}{k_{obs}}$



Scheme 3. Kinetic scheme for irreversible inactivation.

versus $\frac{1}{[I]}$ as shown in Figure 5. According to previously published kinetic analyses,[55,56] linear regression of these data [eq (1)] yields the kinetic parameters of k_{inact} and K_i, summarized in Tables 1 and 2 for the inhibitors studied herein.

For the carboxamido thiadiazole derivatives (Table 1), no significant inhibitory effect was observed for any of the compounds where $n=1$ (**1a**, **1b** and **1c**), at concentrations up to 500 μM. However, inhibition was observed for compounds containing two methylene units in their side chains (i.e., $n=2$: **2a**, **2b**, **2c**). The most efficient inhibitor of this series was compound **2a** with a second order inactivation rate constant k_{inact}/K_i of 0.89 μM^{-1} min^{-1}. Compound **2b** includes a glycine *tert*-butyl ester in its dipeptide backbone and was found to have a similar k_{inact}/K_i value of 0.78 μM^{-1} min^{-1}. The least efficient carboxamido thiadiazole inhibitor of this series was found to be compound **2c**, where the dipeptide backbone contains a glycine residue as its bare carboxylate and whose k_{inact}/K_i was determined to be 0.330 μM^{-1} min^{-1}. Comparison of the inactivation of the homologous series reveals that a side chain containing more than one methylene unit is apparently necessary for the productive orientation of the reactive thiadiazole group with respect to the active site thiol residue. Comparison of k_{inact} and K_i values suggests that while the affinities of **2b** and **2c** are lower than that of **2a**, their rate constants for reaction are higher. This may signify that more productive positioning of the thiadiazole group is possible upon slightly looser binding of the peptide backbone. The result of the competing effects of overall affinity and proper orientation (reactivity) of the thiadiazole groups are reflected in the values of k_{inact}/K_i.

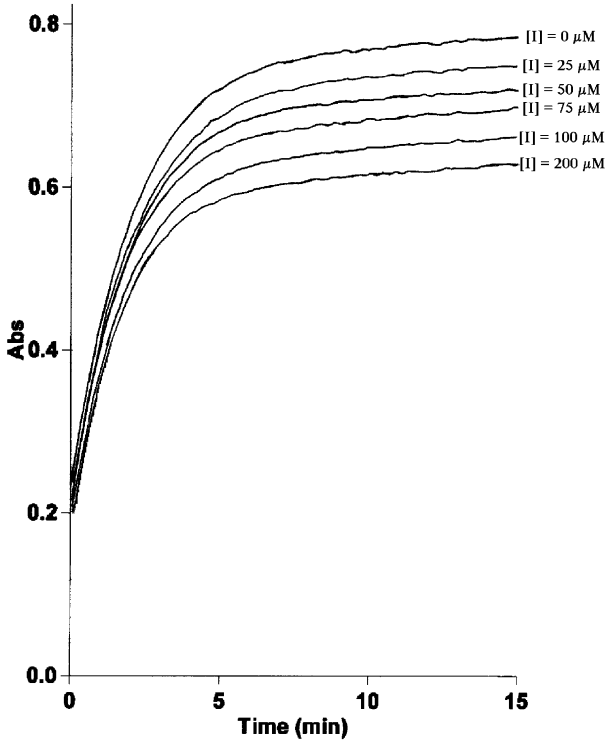


Figure 4. Typical kinetic traces obtained at different concentrations of inhibitor **4h**.

For the amino thiadiazole derivatives (Table 2), no significant inhibition was observed for compounds **1g**–**4g** at concentrations up to 500 μM. On the other hand, although compound **1h** was similarly inactive, compounds **2h** to **4h** were found to inactivate the enzyme with k_{inact}/K_i values between 0.55 μM^{-1} min^{-1} and 0.72 μM^{-1} min^{-1}. The best results were obtained with inhibitors **2h** and **4h**. Based on the inactivity of compounds **1g** and **1h**, it appears that a side chain of a certain length is once again required for the proper orientation of the thiadiazole ring.

Comparison of the carboxamido thiadiazole **2c** to the amino thiadiazole **3h**, both of which contain a four-atom spacer between the peptide backbone and the thiadiazole ring, suggests that the two types of thiadiazole inhibitors may be positioned differently in the active site. For example, the K_i found for **3h**, bearing an amide carbonyl, was 14.3 μM, whereas the value determined for **2c** was 2.25 μM. This difference may be the consequence of the different side chain geometry and binding site interactions arising from the presence of the side chain carbonyl. It is conceivable that the planar sp^2 configuration or the polar nature of the amide linkage may adversely affect the affinity of the enzyme for the side chain of the carboxamido thiadiazole inhibitors. However, it may also be noted that the rate constant of inactivation of carboxamido thiadiazole **2c** is higher than that of amino thiadiazole **3h**, suggesting that while the amide linkage may not interact favorably with the enzyme binding site, it may position the thiadiazole ring in such a way that it reacts more quickly. Of course, the observed differences in the rate constants of inactivation may also be due to the relative electron withdrawing nature of either the carbonyl group or the thiadiazole ring substituent, which would be especially significant if the rate-limiting step of the reaction were the attack of the enzyme thiolate on the thiadiazole sulfur atom. Without an X-ray crystal structure of guinea pig liver TGase and subsequent precise modeling experiments, it is difficult to speculate on the significance and the reasons for the differences of the efficiencies of the inhibitors presented herein. Further experiments are underway to investigate the effect of the ester alkyl group on

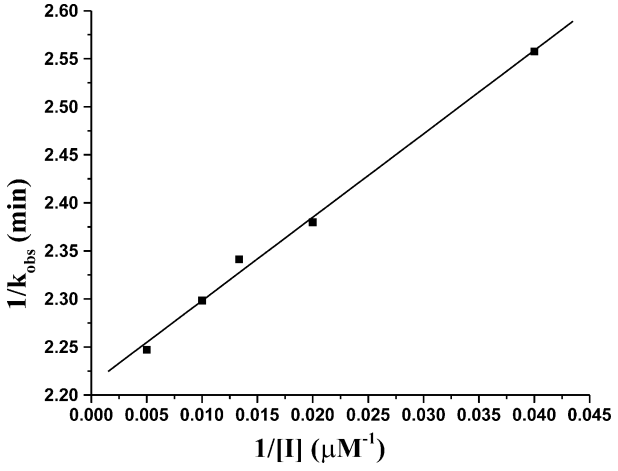
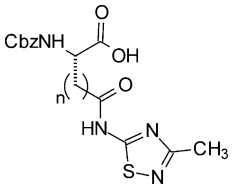
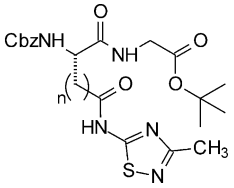
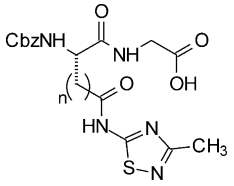


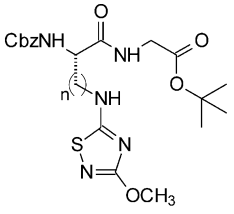
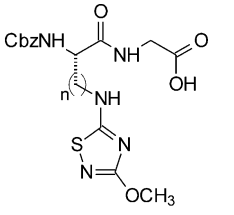
Figure 5. Typical double reciprocal plot of k_{obs} vs [I], shown for compound **4h**.

Table 1. Summary of inhibition kinetic parameters for carboxamido thiadiazole derivatives **1a**–**2a**, **1b**–**2b**, **1c**–**2c**

Structure	*n*	Comp.	k_{inact} (min^{-1})	K_i (μM)	k_{inact}/K_i (μM^{-1} min^{-1})	[I] (μM)
	1	**1a**		No significant effect		<500
	2	**2a**	0.69	0.77	0.89	25–200
	1	**1b**		No significant effect		<500
	2	**2b**	1.0	1.3	0.78	25–200
	1	**1c**		No significant effect		<500
	2	**2c**	4.7	14	0.33	100–400

Experimental error is estimated to be around 10%.

Table 2. Summary of inhibition kinetic parameters for amino thiadiazole derivatives **1d**–**4d**, **1e**–**4e**

Structure	*n*	Comp.	k_{inact} (min^{-1})	K_i (μM)	k_{inact}/K_i (μM^{-1} min^{-1})	[I] (μM)
	1	**1g**		No significant effect		<500
	2	**2g**		No significant effect		<500
	3	**3g**		No significant effect		<500
	4	**4g**		No significant effect		<500
	1	**1h**		No significant effect		<500
	2	**2h**	0.88	1.3	0.71	25–200
	3	**3h**	1.2	2.3	0.55	25–200
	4	**4h**	1.2	1.7	0.72	25–200

Experimental error is estimated to be around 10%.

binding (e.g., methyl vs *tert*-butyl) and the effect of electronic nature of substituents on the thiadiazole ring on the reactivity of thiadiazoles with TGase as well as with simple thiols.

Summary

Efficient synthetic pathways have been developed for the preparation of 14 new potential irreversible inhibitors of TGases (i.e., **1a**–**2a**, **1b**–**2b**, **1c**–**2c**, **1g**–**4g** and **1h**–**4h**). All of these compounds are analogues of Cbz-Gln-Gly, a commonly used substrate for TGases. This similarity of structure confers to these thiadiazole derivatives good affinity for TGases.

Six of the 14 thiadiazole peptides synthesized in this work showed significant irreversible inhibitory activity. Comparison of the structures and activities of these compounds revealed the importance of the length and

the electronic nature of the side chains of the inhibitor analogues of Cbz-Gln-Gly. The results of this preliminary study will permit the design of a second generation of thiadiazole inhibitors. For example, it would be worthwhile to synthesize and test the inhibitory effect of various esters of glutamine analogues. Furthermore, work is in progress to determine the effect of thiadiazole-ring substituents on either the affinity or reactivity of future inhibitors. Finally, the X-ray crystal structure of the native enzyme with or without the inhibitor bound in the active site would permit more precise molecular modeling and a better understanding of the orientation and important interactions involved in both the TGase enzymatic mechanism and inhibition.

Experimental

Materials and methods

Enzyme preparation. TGase was isolated from guinea pig livers and purified according to recent modifications[52] to the protocol previously published by Folk and Chung.[43] One unit of TGase was defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of hydroxamate per min (based on the hydroxamate activity assay, where Z-Gln-Gly is used as a γ-glutamyl donor substrate and hydroxylamine is used as an acyl acceptor substrate). All materials were of reagent grade purity and obtained from Sigma-Aldrich Chemical Company. Water was purified using a Millipore BioCell water purification system.

Synthesis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer. Solvents are indicated in the text and the chemical shifts are reported in ppm with internal reference to TMS. Mass spectra (MS) were recorded on a Micromass 1212 spectrometer. Infrared (IR) spectra were recorded in the range 4000–600 cm^{-1} using a Perkin–Elmer 298 infrared spectrometer. Spectra of liquids were taken as films of $CHCl_3$ solutions between NaCl plates and spectra of solids with KBr disks. Melting points (mp) were determined with a capillary tube Thomas Hoover melting point apparatus and are reported as uncorrected values. Flash chromatography was carried out on silica gel (200–430 mesh) obtained from Silicycle. The starting compounds were obtained from Sigma-Aldrich.

General anhydride ring opening procedure A: synthesis of compounds 1a–2a. In 25 mL of anhydrous DMSO was dissolved 10 mmol of either carbobenzyloxy-L-aspartic anhydride or carbobenzyloxy-L-glutamic anhydride. Then, 1.15 g of 5-amino-3-methyl-1,2,4-thiadiazole **5** (10 mmol) was added and the solution was stirred at room temperature for 2 h. The DMSO was removed under reduced pressure to give compounds **1a**–**2a**.

General peptide coupling procedure B: synthesis of compounds 1b–2b. Compounds **1a**–**2a** (6.0 mmol) were activated by reaction with 1.15 g of EDC hydrochloride (6 mmol) and 0.92 mL of triethylamine (6.6 mmol) in dichloromethane (50 mL). After 15 min, 1.00 g of glycine *tert*-butyl ester hydrochloride (6.0 mmol) was added and the mixture was stirred overnight at room temperature. The mixture was washed with HCl (1 M) keeping the pH around 3. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give pure product.

General ester removal procedure C: synthesis of compounds 1c–2c and 1h–4h. Compounds **1b**–**2b** (3.0 mmol) or **1g**–**4g** (1.0 mmol) were dissolved in dichloromethane (50 mL). Gas hydrochloride was bubbled for 4 h at room temperature. The solvent was removed under reduced pressure to give pure product.

General Fmoc protection procedure D: synthesis of compounds 1d–4d. To a solution of 10 mmol of N_α-Cbz-amino acid derivative ($n = 1$–4, Scheme 2) and 4.20 g of sodium bicarbonate (50 mmol) dissolved in water (100 mL) was added a solution of 2.58 g of Fmoc-Cl (10 mmol) in THF (100 mL). The reaction mixture was stirred for 3 h at room temperature. After evaporation of THF, the aqueous phase was extracted with dichloromethane (3×100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give pure product.

General peptide coupling procedure E: synthesis of compound 1e–4e. The compounds **1d**–**4d** were prepared according to procedure B using 9.0 mmol of starting material.

General Fmoc deprotection procedure F: synthesis of compounds 1f–4f. To a solution of 7.0 mmol of compounds **1e**–**4e** dissolved in acetonitrile (100 mL) was added 140 mmol of diethylamine. The mixture was stirred overnight at room temperature. The reaction mixture was then evaporated under reduced pressure and the crude product was dissolved in acetonitrile (100 mL) and washed with hexane (6×100 mL). After evaporation of acetonitrile under reduced pressure, pure product was obtained.

General thiadiazole coupling procedure G: synthesis of compounds 1g–4g. To a solution of 6.0 mmol of compounds **1f**–**4f** dissolved in acetonitrile (100 mL) was added 1.67 mL of triethylamine (12 mmol) and 0.90 g of sodium iodine (6.0 mmol). After addition of 0.90 g (6.0 mmol) of 5-chloro-3-methoxy-1,2,4-thiadiazole **6**, the mixture was heated to reflux and stirred overnight. The reaction mixture was evaporated under reduced pressure and the crude product was purified by flash chromatography with ethyl acetate/hexane (70:30).

Spectral data

N_α-Carbobenzyloxy-2-amino-N_γ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-asparagine (1a). This compound was prepared using general procedure A to give the product as a pale orange powder (85% yield). IR ν_{max} (cm^{-1}) 3279 (OH); 1749, 1709, 1689 (C=O). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.45–7.25 (m, 5H), 5.10 (s, 2H), 4.24 (t, 1H, $J = 4.1$ Hz), 2.44 (d, 2H, $J = 4.0$ Hz), 2.22 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.32, 170.65, 169.78,

157.19, 138.01, 137.99, 129.29, 128.62, 128.57, 66.80, 54.70, 30.88, 19.01. MS (FAB+) 365.1 (MH+). Mp (°C) 124.

N_α-Carbobenzyloxy-2-amino-N_δ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-glutamine (2a). This compound was prepared using general procedure A to give the product as a dark oil (86% yield). IR ν_{max} (cm^{-1}) 3290 (OH); 1743, 1712, 1675 (C=O). ^1H NMR (CD_3OD, 400 MHz) δ 7.45–7.27 (m, 5H), 5.10 (s, 2H), 4.21 (t, 1H, $J=4.1$ Hz), 2.41 (t, 2H, $J=3.3$ Hz), 2.21 (s, 3H), 1.19 (td, 2H, $J=3.3$ Hz, $J=4.2$ Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.30, 170.60, 169.76, 157.25, 138.04, 138.00, 129.25, 128.66, 128.58, 66.76, 54.74, 31.09, 27.95, 18.91. MS (FAB+) 379.1 (MH+).

N_α-Carbobenzyloxy-2-amino-N_γ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-asparaginylglycine *tert*-butyl ester (1b). This compound was prepared using general procedure B to give the product as a white powder (95% yield). IR ν_{max} (cm^{-1}) 1750, 1747, 1706, 1679 (C=O). ^1H NMR (CD_3OD, 400 MHz) δ 7.42–7.28 (m, 5H), 5.11 (s, 2H), 4.22 (t, 1H, $J=5.6$ Hz), 3.83 (s, 2H), 2.39 (d, 2H, $J=5.7$ Hz), 2.18 (s, 3H), 1.46 (s, 9H). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.31, 174.62, 170.60, 169.77, 158.49, 138.09, 138.02, 129.59, 128.94, 128.89, 82.79, 67.56, 55.09, 42.98, 33.11, 28.54, 19.08. MS (FAB+) 478.2 (MH+). Mp (°C) 94.

N_α-Carbobenzyloxy-2-amino-N_δ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-glutaminylglycine *tert*-butyl ester (2b). This compound was prepared using general procedure B to give the product as a dark powder (94% yield). IR ν_{max} (cm^{-1}) 1753, 1749, 1709, 1691 (C=O). ^1H NMR (CD_3OD, 400 MHz) δ 7.45–7.30 (m, 5H), 5.10 (s, 2H), 4.20 (t, 1H, $J=5.5$ Hz), 3.80 (s, 2H), 2.41 (t, 2H, $J=7.0$ Hz), 2.16 (s, 3H), 1.47 (s, 9H), 1.26 (td, 2H, $J=7.0$ Hz, $J=5.5$ Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.33, 174.66, 170.63, 169.75, 158.52, 138.11, 138.02, 129.60, 128.96, 128.92, 82.89, 67.76, 55.12, 43.00, 33.14, 31.24, 28.51, 19.12. MS (FAB+) 492.2 (MH+). Mp (°C) 84.

N_α-Carbobenzyloxy-2-amino-N_γ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-asparaginylglycine (1c). This compound was prepared using general procedure C to give the product as an orange light powder (100% yield). IR ν_{max} (cm^{-1}) 3291 (OH); 1749, 1712, 1701, 1689 (C=O). ^1H NMR (CD_3OD, 400 MHz) δ 7.44–7.30 (m, 5H), 5.09 (s, 2H), 4.19 (t, 1H, $J=5.4$ Hz), 3.95 (s, 2H), 2.37 (d, 2H, $J=7.1$ Hz), 2.16 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.03, 173.99, 173.22, 169.61, 158.14, 137.90, 129.43, 129.05, 128.91, 67.79, 55.60, 41.71, 32.46, 18.77. MS (FAB+) 422.3 (MH+). Mp (°C) 76.

N_α-Carbobenzyloxy-2-amino-N_δ-(3-methyl-5-[1,2,4]thiadiazolyl)-L-glutaminylglycine (2c). This compound was prepared using general procedure C to give the product as a yellow light powder (100% yield). IR ν_{max} (cm^{-1}) 3301 (OH); 1752, 1715, 1703, 1690 (C=O). ^1H NMR (CD_3OD, 400 MHz) δ 7.36–7.17 (m, 5H), 5.07 (s, 2H), 4.22 (t, 1H, J = 4.9 Hz), 3.90 (s, 2H), 2.43 (t, 2H, J = 7.1 Hz), 2.18 (s, 3H), 2.00 (td, 2H, J = 5.0 Hz, J = 7.1 Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 175.01, 173.94, 173.20, 169.66, 158.37, 139.24, 137.90, 128.43, 129.01, 128.87, 67.85, 55.61, 41.82, 32.49, 28.41, 18.74. MS (FAB+) 436.3 (MH+). Mp (°C) 104.

N_α-Carbobenzyloxy-2-amino-N_β-9-fluorenylmethoxycarbonyl-3-amino-L-propionic acid (1d). This compound was prepared using general procedure D to give the product as a yellow light powder (95% yield). IR ν_{max} (cm^{-1}) 3303 (OH); 1750, 1730, 1672 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, 2H, $J=7.6$ Hz), 7.57 (d, 2H, $J=7.4$ Hz), 7.43–7.29 (m, 9H), 6.43 (s, 1H), 5.62 (s, 1H), 5.10 (s, 2H), 4.48–4.41 (m, 3H), 4.16 (t, 1H, $J=6.3$ Hz), 3.12 (d, 2H, $J=5.9$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.30, 157.69, 156.77, 143.79, 141.30, 136.00, 128.56, 128.21, 127.81, 127.18, 125.19, 124.96, 120.06, 67.37, 64.97, 54.88, 47.02, 42.51. MS (FAB+) 461.2 (MH+). Mp (°C) 95.

N_α-Carbobenzyloxy-2-amino-N_γ-9-fluorenylmethoxycarbonyl-4-amino-L-butyric acid (2d). This compound was prepared using general procedure D to give the product as a white powder (96% yield). IR ν_{max} 3305 (OH); 1764, 1733, 1675 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 2H, $J=7.6$ Hz), 7.58 (d, 2H, $J=7.5$ Hz), 7.48–7.33 (m, 9H), 6.44 (s, 1H), 5.65 (s, 1H), 5.13 (s, 2H), 4.46–4.41 (m, 3H), 4.17 (t, 1H, $J=6.0$ Hz), 3.09 (t, 2H, $J=5.4$ Hz), 1.52 (td, 2H, $J=6.2$ Hz, $J=5.3$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.28, 157.07, 156.79, 143.95, 141.33, 136.11, 128.61, 128.14, 127.79, 127.16, 125.22, 124.95, 120.07, 67.66, 67.31, 51.56, 47.19, 37.55, 332.80. MS (FAB+) 475.2 (MH+). Mp (°C) 106.

N_α-Carbobenzyloxy-N_δ-9-fluorenylmethoxycarbonyl-L-ornithine (3d). This compound was prepared using general procedure D to give the product as a white powder (96% yield). IR ν_{max} (cm^{-1}) 3299 (OH); 1759, 1730, 1675 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.76 (d, 2H, $J=7.5$ Hz), 7.56 (d, 2H, $J=7.5$ Hz), 7.45–7.32 (m, 9H), 6.42 (s, 1H), 5.61 (s, 1H), 5.11 (s, 2H), 4.47–4.39 (m, 3H), 4.15 (t, 1H, $J=6.2$ Hz), 3.11 (t, 2H, $J=5.5$ Hz), 1.53–1.28 (m, 4H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.54, 156.87, 156.32, 143.88, 141.29, 136.13, 128.53, 128.21, 128.12, 127.70, 125.06, 124.82, 119.99, 67.11, 66.70, 53.43, 47.17, 40.40, 29.53, 25.77. MS (FAB+) 489.3 (MH+). Mp (°C) 121.

N_α-Carbobenzyloxy-N_ε-9-fluorenylmethoxycarbonyl-L-lysine (4d). This compound was prepared using general procedure D to give the product as a yellow light powder (95% yield). IR ν_{max} (cm^{-1}) 3300 (OH); 1760, 1730, 1672 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 2H, $J=7.5$ Hz), 7.56 (d, 2H, $J=7.4$ Hz), 7.43–7.28 (m, 9H), 6.43 (s, 1H), 5.61 (s, 1H), 5.10 (s, 2H), 4.44–4.36 (m, 3H), 4.18 (t, 1H, $J=6.5$ Hz), 3.18 (t, 2H, $J=4.6$ Hz), 1.54–1.24 (m, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.95, 156.97, 156.58, 143.98, 141.33, 136.28, 128.57, 128.21, 128.13, 127.15, 125.15, 125.01, 120.06, 67.09, 66.69, 53.76, 47.24, 40.56, 31.78, 29.26, 22.41. MS (FAB+) 503.4 (MH+). Mp (°C) 139.

N_α-Carbobenzyloxy-2-amino-N_β-9-fluorenylmethoxycarbonyl-3-amino-L-propionylglycine *tert*-butyl ester (1e). This compound was prepared using general procedure

E to give the product as a yellow light powder (95% yield). IR ν_{max} (cm^{-1}) 1751, 1721, 1700, 1659 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 2H, $J=7.6$ Hz), 7.59 (d, 2H, $J=7.6$ Hz), 7.41–7.34 (m, 9H), 6.44 (s, 1H), 5.43 (s, 1H), 5.10 (s, 2H), 4.95 (s, 1H), 4.45–4.37 (m, 3H), 4.24 (t, 1H, $J=6.3$ Hz), 3.90 (s, 2H), 3.04 (d, 2H, $J=5.9$ Hz), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.02, 168.83, 157.40, 156.38, 145.09, 141.13, 137.89, 128.43, 128.28, 127.67, 127.09, 126.88, 125.08, 119.74, 81.29, 66.87, 64.23, 52.94, 47.04, 45.61, 30.34, 27.98. MS (FAB+) 574.2 (MH+). Mp (°C) 118.

N_α-Carbobenzyloxy-2-amino-N_γ-9-fluorenylmethoxycarbonyl-4-diamino-L-butyrylglycine *tert*-butyl ester (2e). This compound was prepared using general procedure E to give the product as a yellow light powder (96% yield). IR ν_{max} (cm^{-1}) 1752, 1720, 1702, 1659 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.77 (d, 2H, $J=7.6$ Hz), 7.61 (d, 2H, $J=7.6$ Hz), 7.41–7.32 (m, 9H), 6.44 (s, 1H), 5.45 (s, 1H), 5.11 (s, 2H), 4.95 (s, 1H), 4.44–4.35 (m, 3H), 4.22 (t, 1H, $J=6.1$ Hz), 3.91 (s, 2H), 3.03 (t, 2H, $J=5.4$ Hz), 1.71 (td, 2H, $J=6.2$ Hz, $J=5.4$ Hz), 1.41 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.01, 168.82, 157.12, 156.40, 143.98, 141.22, 136.35, 128.48, 128.08, 127.95, 127.65, 127.07, 125.17, 119.93, 81.89, 66.90, 64.57, 52.38, 47.16, 42.01, 37.39, 33.49, 27.97. MS (FAB+) 588.2 (MH+). Mp (°C) 90.

N_α-Carbobenzyloxy-N_δ-9-fluorenylmethoxycarbonyl-L-ornithinylglycine *tert*-butyl ester (3e). This compound was prepared using general procedure E to give the product as a yellow light powder (97% yield). IR ν_{max} (cm^{-1}) 1754, 1725, 1700, 1660 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, 2H, $J=7.7$ Hz), 7.59 (d, 2H, $J=7.6$ Hz), 7.40–7.32 (m, 9H), 6.43 (s, 1H), 5.43 (s, 1H), 5.09 (s, 2H), 4.93 (s, 1H), 4.45–4.36 (m, 3H), 4.23 (t, 1H, $J=6.2$ Hz), 3.89 (s, 2H), 3.01 (t, 2H, $J=5.4$ Hz), 1.71–1.45 (m, 13H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.16, 168.75, 156.97, 156.38, 143.94, 141.25, 136.25, 128.49, 128.12, 128.02, 127.65, 127.03, 125.08, 119.93, 82.14, 66.94, 66.60, 53.50, 47.21, 41.92, 39.81, 30.12, 27, 96, 25.99. MS (FAB+) 602.4 (MH+). Mp (°C) 112.

N_α-Carbobenzyloxy-N_ε-9-fluorenylmethoxycarbonyl-L-lysylglycine *tert*-butyl ester (4e). This compound was prepared using general procedure E to give the product as a white powder (97% yield). IR ν_{max} (cm^{-1}) 1750, 1720, 1700, 1658 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.76 (d, 2H, $J=7.6$ Hz), 7.58 (d, 2H, $J=7.5$ Hz), 7.41–7.31 (m, 9H), 6.43 (s, 1H), 5.41 (s, 1H), 5.10 (s, 2H), 4.91 (s, 1H), 4.44–4.36 (m, 3H), 4.21 (t, 1H, $J=6.1$ Hz), 3.92 (s, 2H), 3.08 (t, 2H, $J=4.2$ Hz), 1.70–1.39 (m, 15H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.87, 168.67, 156.56, 156.23, 143.91, 141.15, 136.09, 128.39, 128.04, 127.92, 127.53, 126.92, 124.96, 119.83, 82.16, 66.88, 66.37, 54.58, 47.13, 40.17, 31.84, 29.19, 27.90, 22.14. MS (FAB+) 616.4 (MH+). Mp (°C) 119.

N_α-Carbobenzyloxy-2,3-diamino-L-propionylglycine *tert*-butyl ester (1f). This compound was prepared using general procedure F to give the product as a light yellow viscous solid (93% yield). IR ν_{max} (cm^{-1}) 3099 (NH_2); 1744, 1724, 1670 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.33–7.28 (m, 5H), 6.01 (s, 2H), 5.41 (s, 1H), 5.25 (s, 1H), 5.04 (s, 2H), 3.90 (t, 1H, $J=6.6$ Hz), 3.82 (s, 2H), 2.96 (d, 2H, $J=6.7$ Hz), 1.46 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.74, 168.99, 153.92, 137.49, 128.37, 127.88, 127.76, 81.49, 66.56, 52.91, 45.01, 41.61, 27.92. MS (FAB+) 352.2 (MH+).

N_α-Carbobenzyloxy-2,4-diamino-L-butyrylglycine *tert*-butyl ester (2f). This compound was prepared using general procedure F to give the product as a light yellow viscous solid (92% yield). IR ν_{max} (cm^{-1}) 3054 (NH_2); 1747, 1725, 1666 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.33–7.29 (m, 5H), 6.02 (s, 2H), 5.45 (s, 1H), 5.27 (s, 1H), 5.03 (s, 2H), 3.94 (t, 1H, $J=6.7$ Hz), 3.82 (s, 2H), 2.96 (t, 2H, $J=4.7$ Hz), 1.93 (td, 2H, $J=6.8$ Hz, $J=4.7$ Hz), 1.48 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.67, 168.84, 156.47, 136.45, 128.32, 127.86, 127.76, 81.47, 66.51, 52.81, 44.90, 41.77, 34.88, 27.81. MS (FAB+) 366.2 (MH+).

N_α-Carbobenzyloxy-L-ornithinylglycine *tert*-butyl ester (3f). This compound was prepared using general procedure F to give the product as a light yellow viscous solid (92% yield). IR ν_{max} (cm^{-1}) 3072 (NH_2); 1745, 1723, 1675 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.32–7.28 (m, 5H), 6.03 (s, 2H), 5.44 (s, 1H), 5.27 (s, 1H), 5.05 (s, 2H), 3.91 (t, 1H, $J=6.8$ Hz), 3.83 (s, 2H), 2.99 (t, 2H, $J=4.5$ Hz), 1.94–1.36 (m, 13H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.59, 168.89, 156.40, 136.43, 128.34, 127.89, 127.82, 81.65, 66.53, 54.46, 41.77, 30.19, 28.86, 27.88, 20.96. MS (FAB+) 380.2 (MH+).

N_α-Carbobenzyloxy-L-lysylglycine *tert*-butyl ester (4f). This compound was prepared using general procedure F to give the product as a light yellow viscous solid (93% yield). IR ν_{max} (cm^{-1}) 3104 (NH_2); 1745, 1727, 1671 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.32–7.29 (m, 5H), 6.01 (s, 2H), 5.45 (s, 1H), 5.29 (s, 1H), 5.06 (s, 2H), 3.90 (t, 1H, $J=6.6$ Hz), 3.82 (s, 2H), 2.97 (t, 2H, $J=3.5$ Hz), 1.96–1.37 (m, 15H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.44, 168.56, 156.09, 136.29, 128.09, 127.63, 127.51, 81.84, 66.19, 54.54, 41.50, 32.11, 31.77, 28.51, 27.61, 22.37. MS (FAB+) 393.3 (MH+).

N_α-Carbobenzyloxy-2-amino-N_β-(3-methoxy-5-[1,2,4]thiadiazolylamino)-L-propionylglycine *tert*-butyl ester (1g). This compound was prepared using general procedure G to give the product as a light orange powder (19% yield). IR ν_{max} (cm^{-1}) 1745, 1727, 1670 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.35–7.30 (m, 5H), 6.77 (s, 1H), 5.55 (s, 1H), 5.32 (s, 1H), 5.09 (s, 2H), 4.26 (t, 1H, $J=6.8$ Hz), 3.95 (s, 3H), 3.80 (s, 2H), 3.24 (t, 2H, $J=6.5$ Hz), 1.49 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 183.76, 170.91, 169.23, 168.16, 156.86, 136.47, 128.76, 128.37, 128.20, 82.54, 67.41, 56.35, 53.92, 46.54, 41.40, 27.24. MS (FAB+) 466.2 (MH+). Mp (°C) 82.

N_α-Carbobenzyloxy-2-amino-N_β-(3-methoxy-5-[1,2,4]thiadiazolylamino)-L-butyrylglycine *tert*-butyl ester (2g). This compound was prepared using general procedure G to give the product as a light orange powder (21% yield). IR ν_{max} (cm^{-1}) 1749, 1730, 1674 (C=O). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.34 (m, 5H), 6.79 (s, 1H),

5.54 (s, 1H), 5.32 (s, 1H), 5.10 (s, 2H), 4.27 (t, 1H, $J=6.7$ Hz), 3.96 (s, 3H), 3.80 (s, 2H), 3.24 (t, 2H, $J=4.5$ Hz), 1.93–1.70 (m, 2H), 1.47 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 183.08, 172.19, 168.72, 167.88, 156.48, 136.06, 128.42, 128.05, 127.79, 81.90, 67.01, 55.88, 52.02, 41.88, 41.67, 31.92, 27.97. MS (FAB+) 480.2 (MH+). Mp (°C) 126.

N_α-Carbobenzyloxy-N_δ-(3-methoxy-5-[1,2,4]thiadiazolyl)-L-ornithinylglycine *tert*-butyl ester (3g). This compound was prepared using general procedure G to give the product as a soft white powder (26% yield). IR ν_{max} (cm^{-1}) 1746, 1725, 1675 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35–7.27 (m, 5H), 6.77 (s, 1H), 5.54 (s, 1H), 5.31 (s, 1H), 5.09 (s, 2H), 4.27 (t, 1H, $J=6.7$ Hz), 3.98 (s, 3H), 3.82 (s,2H), 3.25 (t, 2H, $J=4.0$ Hz), 1.90–1.71 (m, 4H), 1.48 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 182.71, 172.47, 168.35, 167.90, 156.80, 136.57, 128.94, 128.61, 128.43, 81.79, 66.50, 55.45, 54.90, 44.76, 41.35, 30.65, 27.71, 26.46, 20.91. MS (FAB+) 494.2 (MH+). Mp (°C) 90.

N_α-Carbobenzyloxy-N_δ-(3-methoxy-5-[1,2,4]thiadiazolyl)-L-lysylglycine *tert*-butyl ester (4g). This compound was prepared using general procedure G to give the product as a soft white powder (25% yield). IR ν_{max} (cm^{-1}) 1745, 1729, 1670 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.34–7.28 (m, 5H), 6.75 (s, 1H), 5.56 (s, 1H), 5.31 (s, 1H), 5.11 (s, 2H), 4.26 (t, 1H, $J=7.0$ Hz), 3.97 (s, 3H), 3.84 (s, 2H), 3.13 (t, 2H, $J=3.9$ Hz), 1.87–1.68 (m, 6H), 1.46 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 183.01, 172.51, 168.75, 168.14, 156.39, 136.19, 128.25, 127.84, 127.69, 81.71, 66.65, 55.74, 54.12, 44.85, 41.79, 29.44, 27.76, 24.71, 22.49. MS (FAB+) 508.1 (MH+). Mp (°C) 89.

N_α-Carbobenzyloxy-2-amino-N_β-(3-methoxy-5-[1,2,4]thiadiazolylamino)-L-propionylglycine (1h). This compound was prepared using general procedure C to give the product as a light orange powder (100% yield). IR ν_{max} (cm^{-1}) 3306 (OH); 1745, 1704, 1671 (C=O). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.36–7.31 (m, 5H), 5.11 (s, 2H), 4.17 (t, 1H, $J=6.9$ Hz), 4.00 (s, 3H), 3.77 (s, 2H), 3.31 (d, 2H, $J=6.9$ Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 183.88, 174.53, 172.79, 161.12, 158.41, 138.00, 129.48, 129.17, 128.87, 67.97, 59.60, 53.77, 48.03, 41.87. MS (FAB+) 410.2 (MH+). Mp (°C) 130.

N_α-Carbobenzyloxy-2-amino-N_γ-(3-methoxy-5-[1,2,4]thiadiazolylamino)-L-butyrylglycine (2h). This compound was prepared using general procedure C to give the product as a light yellow powder (100% yield). IR ν_{max} (cm^{-1}) 3310 (OH); 1750, 1701, 1674 (C=O). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.37–7.32 (m, 5H), 5.09 (s, 2H), 4.15 (t, 1H, $J=6.8$ Hz), 4.01 (s, 3H), 3.75 (s, 2H), 3.28 (t, 2H, $J=5.5$ Hz), 1.53 (td, 2H, $J=6.8$ Hz, $J=5.5$ Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ 183.91, 174.56, 172.82, 161.01, 158.38, 137.91, 129.57, 129.15, 128.94, 67.99, 59.08, 53.75, 48.50, 41.97, 31.98. MS (FAB+) 424.2 (MH+). Mp (°C) 108.

N_α-Carbobenzyloxy-N_δ-(3-methoxy-5-[1,2,4]thiadiazolyl)-L-ornithinylglycine (3h). This compound was prepared using general procedure C to give the product as a soft white powder (100% yield). IR ν_{max} (cm^{-1}) 3308 (OH); 1743, 1699, 1669 (C=O). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.39–7.33 (m, 5H), 5.09 (s, 2H), 4.18 (t, 1H, $J=6.9$ Hz), 3.99 (s, 3H), 3.74 (s, 2H), 3.30 (t, 2H, $J=3.2$ Hz), 1.71–1.50 (m, 4H). ^{13}C NMR (CD_3OD, 100 MHz) δ 183.99, 175.17, 172.61, 160.51, 158.35, 138.06, 129.53, 129.08, 128.88, 67.80, 58.98, 55.80, 47.23, 41.84, 30.40, 25.61. MS (FAB+) 438.2 (MH+). Mp (°C) 108.

N_α-Carbobenzyloxy-N_ε-(3-methoxy-5-[1,2,4]thiadiazolyl)-L-lysylglycine (4h). This compound was prepared using general procedure C to give the product as a soft white powder (100% yield). IR ν_{max} (cm^{-1}) 3301 (OH); 1749, 1700, 1665 (C=O). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.36–7.30 (m, 5H), 5.10 (s, 2H), 4.16 (t, 1H, $J=6.8$ Hz), 3.96 (s, 3H), 3.72 (s, 2H), 3.31 (t, 2H, $J=3.3$ Hz), 1.83–1.47 (m, 6H). ^{13}C NMR (CD_3OD, 100 MHz) δ 184.01, 175.18, 172.65, 160.52, 158.36, 138.11, 129.55, 129.09, 128.89, 67.77, 59.05, 56.21, 47.66, 41.88, 32.79, 28.86, 23.85. MS (FAB+) 452.2 (MH+). Mp (°C) 94. $[\alpha]_D^{20}$ (3.75 mg/mL DMSO) −5.3°

5-Amino-3-methyl-1,2,4-thiadiazole (5). In 150 mL of water was dissolved 12.29 g of acetamidine hydrochloride (0.130 mol). The mixture was cooled to 0 °C and 184 mL of sodium hypochlorite 0.705 M (0.130 mol) was added dropwise over one and a half hours. The reaction was then stirred for 1 h at 0 °C. Next, the aqueous phase was saturated with sodium chloride and the desired product (chloroacetamide) was extracted with ethyl acetate (3×250 mL). Then the organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The chloroacetamide was obtained as 9.86 g of white solid (82% yield).

To 9.25 g of chloroacetamide (0.1 mol) in methanol (500 mL) was added, at 0 °C, 9.72 g of potassium thiocyanate (0.1 mol). The mixture was stirred overnight at room temperature. Then the solvent was removed under reduced pressure. Ethyl acetate was added on the residue and the mixture was filtered. The filtrate was evaporated under reduced pressure to give 11.23 g of pink crystals (98% yield). IR ν_{max} (cm^{-1}) 3250 (N–H), 2050 (N=C–S), 1650 (C=N). ^{1}H NMR (CD_3OD, 400 MHz) δ 2.20 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ 169.94, 133.80, 18.48. MS (FAB+) 116.1 (MH+). Mp (°C) 68.

5-Chloro-3-methoxy-1,2,4-thiadiazole (6). In 50 mL of dichloromethane was dissolved 3.44 g of methylisourea hydrogenosulfate (20 mmol). The mixture was cooled to −4 °C and 2.30 mL of perchloromethyl mercaptan was added. A solution of 4 g of NaOH in 5 mL of water was added dropwise over 1 h to the vigorously stirred mixture. The reaction was then allowed to warm to room temperature over 3 h. Next, the organic layer was decanted, washed with brine (3×50 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting residue of crude product was purified by flash column chromatography using hexane/ethyl acetate (95:5). The final product was obtained as 1.25 g of yellow oil (42% yield). IR ν_{max} (cm^{-1}) 2048 (N=C–S), 1665 (C=N). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.04

(s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ 173.20, 169.47, 56.84. MS (FAB+) 151.1 (MH+).

Kinetics

Enzymatic assays and inhibition kinetics were carried out as previously described[54] at pH 7.0 using stock solutions to give the final concentrations of 120 mM Tris–acetate buffer, 3 mM $CaCl_2$ and 6 mM EDTA. Inhibition constants were measured by performing kinetic assays using 75 μL of 100 mM Z-Gln-Gly, 4 μL of 100 mM DMPDA and 0–20 μL of inhibitors in DMSO (their concentration depending upon their efficacy) with 20–0 μL of DMSO, to give a volume of 480 μL. The reaction solution was preincubated for three min at 37 °C, prior to initiation of the enzymatic reaction upon the addition of 20 μL of purified pig liver TGase (0.2 units) to give a final volume of 500 μL. The inhibition kinetic parameters K_i and k_{inact} shown in Scheme 3 were determined as described previously[54] by treating the pseudo-first order rate constants derived from the enzymatic reaction with substrate in the presence of an irreversible inhibitor according to eq (1):[55,56]

$$\frac{1}{k_{obs}} = \left(K_i + \frac{K_i[S]}{K_M}\right)\frac{1}{[I]} + \frac{1}{k_{inact}} \quad (1)$$

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