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# Synthesis and evaluation of peptidic maleimides as transglutaminase inhibitors

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**Abstract**—A series of novel transglutaminase inhibitors was prepared, based on the scaffold of a commonly used peptide substrate and bearing an electrophilic maleimide group. These compounds were evaluated in vitro and shown to lead to irreversible inactivation of tissue transglutaminase. Comparison with inhibitors studied previously provides insight into the steric environment of the enzyme active site.
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Transglutaminases (TGases, EC 2.3.2.13) are $Ca^{2+}$-dependent enzymes that catalyze the formation of isopeptide cross-links between the γ-carboxamide group of a protein- or peptide-bound glutamine residue and a primary amino group, such as the ε-amino group of protein- or peptide-bound lysine residues (Scheme 1).[1–3] Tissue TGase (tTG) has been identified as a contributor to the formation of cataracts and to Celiac disease, and a growing body of evidence suggests that it may be involved in atherosclerosis, inflammation, fibrosis, diabetes, cancer metastases, autoimmune diseases, lamellar ichthyosis, and psoriasis (for a review, see 4). TGase has also been implicated in neurodegenerative diseases associated with an increase in polyglutamine-containing peptides in the brain such as Huntington's disease, Alzheimer disease, Parkinson disease, and supranuclear palsy.[5–7]

A number of potential TGase inactivators have been developed in order to regulate excess TGase activity. These include dihydroisoxazole derivatives,[8] gluten peptide analogs,[9] and dipeptide-bound α,β-unsaturated amides, epoxides, and 1,2,4-thiadiazoles.[10–12] The design of the latter inhibitors was based on the structure of carbobenzyloxy-L-glutaminylglycine (Cbz-Gln-Gly), a commonly used dipeptide acyl-donor substrate.[13] Herein we report the synthesis of a series of novel maleimides based on the same peptidic scaffold, and their in vitro evaluation as irreversible tTG inhibitors (Scheme 2).

The starting points of our syntheses of the peptidic maleimides were the Cbz-protected diamino acids **2a**–**4a**, numbered according to side-chain length. Compounds **3a** and **4a** were commercially available,[14] whereas compound **2a** was obtained from the Hoffmann rearrangement of the corresponding amide, as previously reported.[10] Ensuing protection of the pendant amino group,[15] esterification[16] or peptide coupling[17] and deprotection of the side-chain amine[18] were accomplished by straightforward synthetic routes, affording amines **2c**–**4c** and **2d**–**4d**. Hydrophobic ester groups were incorporated into the inhibitor design both for synthetic simplicity and in consideration of their effect on enzyme affinity as observed previously.[8,11,12] These amines were then transformed into the final maleimide inhibitors by the typical two-step condensation reaction with maleic anhydride.[18,19] Reaction conditions and yields were not optimized, but ample quantities of the final products were obtained for subsequent kinetic analyses.

Recombinant guinea pig liver TGase was expressed in *Escherichia coli* and subsequently purified according to a procedure developed in our laboratories.[20,21] Guinea pig liver tTG was chosen for this study because it can be obtained easily in excellent yield and solubility, and shows high homology with human tTG,[22] thereby validating its use as a model for the evaluation of inhibitors of potential therapeutic utility.

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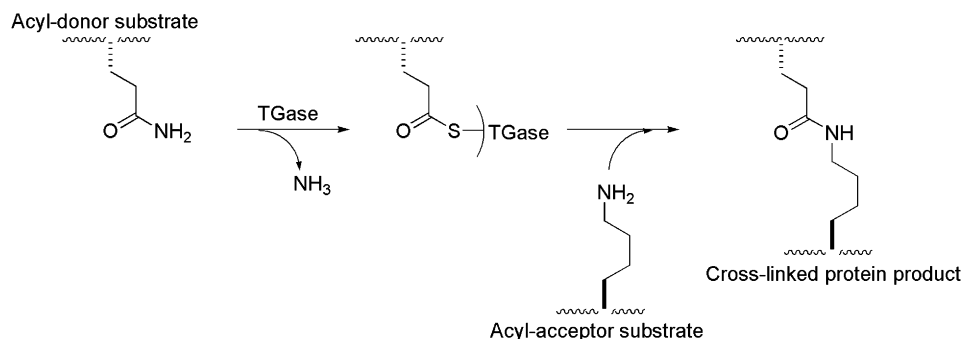
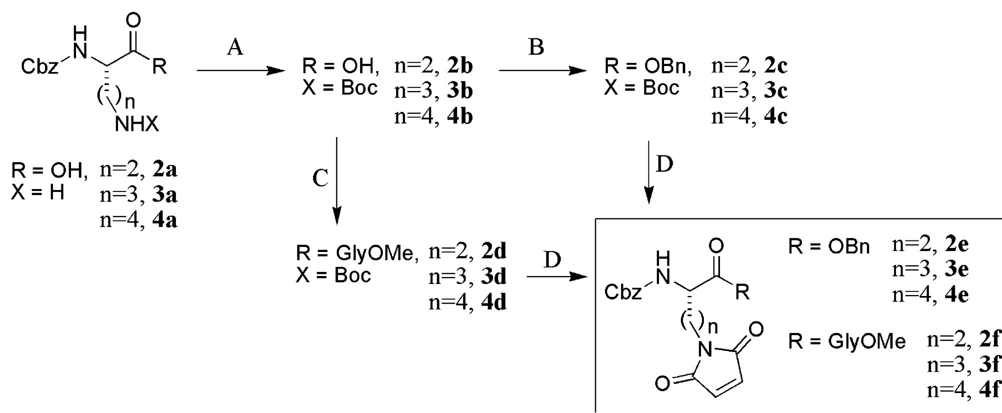



**Scheme 1.** Cross-linking reaction catalyzed by TGase.




**Scheme 2.** Synthesis of inhibitors studied herein. Reagents and conditions: (A) 7 $(Boc)_2O/Et_3N/MeOH$/24 h, rt; (B) Bn–Br/$Et_3N$/DMF/24 h, rt; (C) Gly-OMe/DIEA/TBTU/DMF/24 h, rt; (D) i—TFA/1 h, rt; ii—maleic anhydride/$CHCl_3$/24 h, rt; iii—acetic anhydride/NaOAc/24 h, 95 °C.

All peptidic maleimides demonstrated time-dependent inhibition. Kinetic parameters were determined by incubating TGase with inhibitor and measuring residual TGase activity in the absence of excess inhibitor (upon 40-fold dilution) as a function of time.[23] First-order rate constants of inactivation ($k_{obs}$) were thus measured with respect to inhibitor concentration. However, saturating concentrations could not be attained, owing to the relatively low solubility of the inhibitors with respect to their apparent affinity constants. Therefore, the apparent second-order rate constants for inactivation ($k_{inact}/K_I$, Table 1) were determined from the initial slopes of the plots of $k_{obs}$ versus inhibitor concentration.[23]

**Table 1.** Kinetic parameters determined for inhibition of tTG

| Compound | $k_{inact}/K_I$ ($mM^{-1}$ $min^{-1}$)[a] | $k_{inact}$ ($min^{-1}$) | $K_I$ (μM) |
|---|---|---|---|
| **2e** | 0.67 ± 0.08 | — | — |
| **3e** | 6.26 ± 1.69 | — | — |
| **4e** | 17.08 ± 0.12 | — | — |
| **2f** | 0.28 ± 0.03 | — | — |
| **3f** | 0.43 ± 0.05 | — | — |
| **4f** | 0.83 ± 0.01 | — | — |
| **2g**[b] | 1180 | 0.60 | 0.51 |
| **3g**[b] | 890 | 0.75 | 0.85 |
| **4g**[b] | 2200 | 0.49 | 0.23 |

[a] Std. error from fitting of data.
[b] Kinetic parameters given as '$k_{inact}$', '$k_{cat}$' and $K_I$, respectively, in Ref. 11.

Despite the lack of saturation kinetics, two lines of evidence suggest that inactivation is taking place through reaction at the active site. First, although homology modeling[24] of guinea pig tTG suggests that Cys229 may be solvent exposed, and therefore theoretically capable of reacting with a maleimide in a second-order fashion, its distance from the substrate binding site (>15 Å) makes its putative reaction with even the longest inhibitor unlikely to inactivate the enzyme. Second, incubation with inhibitor in the presence of substrate afforded temporary protection, slowing inactivation appreciably. For example, in the presence of 12 mM Cbz-Gln-Gly (four times its $K_m$ value) inactivation by 100 μM **2e** was roughly 2-fold slower than in the absence of substrate.

Comparison of the relative efficiencies of benzyl esters **2e**–**4e** and dipeptides **2f**–**4f** reveals the latter series to be ~2- to 20-fold less efficient. Previous docking studies from our group[25] have suggested the peptide backbone of acyl-donor substrates are bound in a shallow, rather hydrophobic groove on the surface of the enzyme. The specificity of tTG with respect to the peptide sequence of its Gln substrates is consistent with the nature of this putative binding site, as noted previously.[26] The greater efficiency of the benzyl esters, compared to the glycine methyl esters, may reflect higher affinity for, or better positioning in, this binding site.

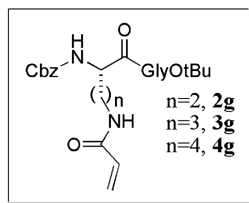



**Figure 1.** Acrylamide inhibitors studied previously.[10,11]

Within each series it can be noted that efficiency increases with chain length. This trend has also been observed for three other series of inhibitors based on the same peptide scaffold.[10,12] Since TGases are designed to exclude asparagine residues as acyl-donor substrates, reacting only with glutamine residues, they are capable of discriminating against substrate analogs having short side chains. However, tTG cannot exclude longer acyl-donor substrates (or irreversible inhibitors) having the conformational flexibility necessary to properly position their pendant reactive groups near the active site thiol.

It is also instructive to draw a comparison with a series of acrylamide inhibitors studied previously. As shown in Figure 1, acrylamides **2g**–**4g** also comprise a dipeptide scaffold having a comparable C-terminal ester, and their pharmacophore differs from maleimide by only one carbonyl completing the heterocycle. Compounds **2g**–**4g** were determined[11] to have $K_I$ values in the range of 0.23–0.85 μM, and their efficiency constants are shown in Table 1. From this comparison it is evident that the maleimides are ~$10^3$- to $10^4$-fold less efficient inhibitors. Although direct comparison of reactivity and affinity constants is not possible, one may presume that the maleimides possess inherently greater reactivity (larger $k_{inact}$ values), given the activation of the double bond toward nucleophilic addition by two conjugated carbonyl groups. By this reasoning, it would appear that the affinity constants of the maleimides are well above those measured for the acrylamides (even larger $K_I$ values). This is consistent with the lack of complete saturation that was observed for the maleimides, for concentrations up to 750 μM. Our modeling of the glutamine substrate binding tunnel of tTG[25] suggests that it is a sterically constrained environment that may not easily accommodate the greater volume of the cyclic maleimide group. Furthermore, this hypothesis is supported by our observation that any substitution for example, by methyl or phenyl, on the γ-carboxamide nitrogen of glutamine results in the complete loss of donor substrate activity.[27]

In summary, the series of novel maleimide inhibitors presented herein confirm the validity of the peptidic scaffold, bearing an electrophilic 'warhead' on a long side-chain, for the design of small molecule inhibitors that target the active site of tTG. The maleimide group itself, although well known for inactivation of thiol-dependent enzymes, appears to be just large enough to decrease the ease of its insertion and productive orientation in the narrow donor substrate binding tunnel of tTG.

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## Supplementary data

Chromatography procedures and spectral data of all synthetic intermediates are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.061.

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14. Starting materials were obtained from Sigma–Aldrich.
15. *Procedure A: Boc protection.* Compounds **2a**, **3a**, and **4a** (1.75 mmol) were dissolved in 10 mL of methanol. After the addition of 1.45 mL of triethylamine (10.5 mmol), 460 mg of $(Boc)_2O$ (2.10 mmol) and a few drops of 1 M NaOH were added. The mixture was stirred overnight at room temperature and then evaporated under reduced pressure. The residue was dissolved in 20 mL of 1 N NaOH. The aqueous phase was washed with 3× 20 mL $CH_2Cl_2$ and acidified to pH ~1.5 by the addition of 6 N HCl. The product was extracted with 3× 25 mL EtOAc and the organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give the final product (36–81% yield).
16. *Procedure B: Benzyl ester formation.* In the dark, compounds **2b**, **3b**, and **4b** (1.15 mmol) were dissolved in 10 mL DMF. After the addition of 0.17 mL of benzyl bromide (1.38 mmol), 0.24 mL of triethylamine (1.73 mmol) was added. The mixture was stirred overnight at room temperature. The reaction mixture was then

evaporated under reduced pressure and the residue was purified by flash chromatography (66–92% yield).

17. *Procedure C: Peptide coupling.* Under nitrogen atmosphere, compounds **2b**, **3b**, and **4b** (3 mmol) were dissolved in 10 mL of anhydrous acetonitrile. After the addition of 2.59 mL DIEA (15 mmol), 744 mg of Gly-OMe (6 mmol) was added. Finally, the reaction was initiated by the addition of 2.39 g TBTU (7.5 mmol). The mixture was stirred overnight at room temperature. The reaction mixture was then evaporated under reduced pressure and the residue was purified by flash chromatography (59–70% yield).
18. *Procedure D: BOC deprotection and maleimide formation.* Compounds **2c**, **3c**, **4c**, **2d**, **3d**, and **4d** (2 mmol) were dissolved in 8 mL TFA. After 1 h of reaction, acetone and cyclohexane were added in 1:5 proportion to make an azeotrope solution. This solution was then evaporated three or four times under reduced pressure. The residue containing the free amine was finally dried under vacuum overnight. Then, under nitrogen atmosphere, it was dissolved in 10 mL of chloroform. Finally, 217 mg of maleic anhydride (2.2 mmol) was added and the reaction mixture was stirred for 20 h at room temperature. The solution was then evaporated under reduced pressure. At this point, the product maleamide can be isolated by flash chromatography, or used without further purification in the ring closure step: Still under nitrogen atmosphere, 10 mL of acetic anhydride and 0.6 mmol of sodium acetate were added to the residue. The mixture was stirred at 95 °C overnight. The final product was purified by flash chromatography (11–46% yield for three steps).
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21. *Enzyme preparation.* Recombinant guinea pig liver tTG was expressed and purified according to the protocol established in our group.[20] 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 Cbz-Gln-Gly is used as a γ-glutamyl donor substrate and hydroxylamine is used as an acyl acceptor substrate[20]). All materials were of reagent grade purity and obtained from Sigma–Aldrich Chemical Company. Water was purified using a Millipore BioCell water purification system.
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23. *Assay procedure.* The progress of the inhibition reactions studied was determined by following the loss of activity as a function of time of incubation with inhibitor. Incubation solutions were composed of 10 μL inhibitor (50–750 μM final concentration), 200 μL TGase (150 μg/mL), 12 μL of 100 mM $CaCl_2$, 168 μL of 200 mM Mops (pH 7), and 10 μL of 20 mM EDTA (total volume 400 μL). At various incubation times a 25-μL aliquot was removed and added to a solution composed of 25 μL stock substrate solution[22] (2.18 mM *N*-Cbz-Glu(γ-*p*-nitrophenyl ester)Gly in DMF), 50 μL water, and 900 μL of buffer (100 mM Mops (pH 7), 3 mM $CaCl_2$, and 0.05 mM EDTA final concentrations). Residual TGase activity was measured at 37 °C as an increase of absorbance at 410 nm. The rate constant for the loss of residual activity as a function of time ($k_{obs}$) was determined from fitting the data to a single exponential equation, for each concentration of inhibitor. Plots of $k_{obs}$ against inhibitor concentration did not show saturation kinetics and were subsequently fitted to a linear equation, the slopes giving the ratios ($k_{inact}/K_I$).
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