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Evaluation of Novel Dipeptide-Bound α,β-Unsaturated Amides and Epoxides as Irreversible Inhibitors of Guinea Pig Liver Transglutaminase

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Abstract—Herein, we report the results of irreversible inhibition of guinea pig liver transglutaminase (TGase) by a series of 24 novel dipeptides containing either an α,β-unsaturated amide or an epoxide functional group. Their inactivation rate constants were measured using a direct continuous spectrophotometric method and were found to vary between 421×10^3 and $3000\times10^3\,M^{-1}\,min^{-1}$. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Transglutaminases (TGases, EC 2.3.2.13) catalyze, in the presence of Ca^{2+}, an acyl-transfer reaction between peptide-bound glutamine residues and the primary amino group of various amines. These enzymes are found in mammalian tissues, plasma factor XIIIa and epidermis. Tissue TGase is implicated in the control of diverse cellular events such as endocytosis,[1,2] apoptosis[3] and the regulation of cell proliferation.[4] Plasma TGase, Factor XIIIa, is formed at sites of blood coagulation and impedes blood loss by catalyzing the cross-linking of fibrin, thus stabilizing blood clots during their formation.[5–7] Epidermal TGase is required for the formation of a cornified cell envelope in epidermal keratinocytes.[8–11] High TGases activities are involved in various disease states such as acne,[12,13] cataracts,[14] immunologic disease,[15] psoriasis[16–18] and Alzheimer's disease.[19–25]

In the interest of developing potential therapeutic agents for the regulation of excess TGase activities, potential specific inhibitors have been synthesized in our laboratories.[26,27] The design of these inhibitors was based on the structure of carbobenzyloxy-L-glutaminylglycine (Z-Gln-Gly), a commonly used dipeptide acyl donor substrate.[28–30] The enzymatic mechanism involves the nucleophilic attack of an active site thiol residue on the (carboxamide moiety of Z-Gln-Gly which leads to the formation of a thiolester intermediate (Fig. 1). In the design of our potential inhibitors, we thus added a functional group capable of forming an irreversible thiol addition product (see Fig. 2)[31] to the end of the dipeptide side chain of Z-Gln-Gly analogues. Herein, we report the inhibition kinetic studies of novel α,β-unsaturated amide (Michael acceptors) and epoxides (Fig. 3).

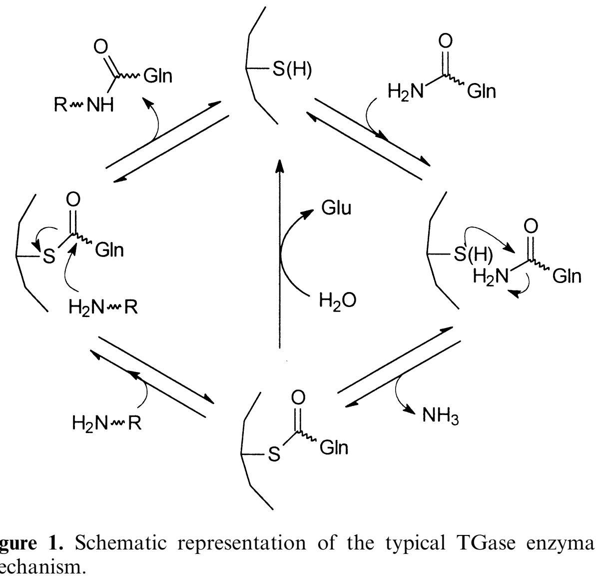

Figure 1. Schematic representation of the typical TGase enzymatic mechanism.

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Results and Discussion

Guinea pig liver was chosen as the source of TGase for this study due to its high natural abundance in TGase compared to rat, mouse and rabbit liver. Furthermore, guinea pig liver TGase is the most widely cited source for tissue TGase and its purification has been well established both elsewhere[29] and in our laboratory.[32] Above all, guinea pig liver TGase shows 80% homology with human tissue TGase thereby validating its use as an animal model of the human enzyme.[35]

In the enzymatic mechanism, the acylation step consists of the attack of the active site cysteine thiol residue on the carboxamide of the side chain of the glutamine unit. Our inhibitors were thus designed with functional groups known to form irreversible thiol addition products. Moreover, since small inhibitor molecules have been observed[36] to show little affinity for TGase active sites and to be ineffective inhibitors by themselves, we have placed these reactive groups on the side chain of the recognized dipeptide in order to benefit from the apparent affinity TGase displays towards Z-Gln-Gly. Furthermore, the lengths of the side chains of these Z-Gln-Gly analogues were varied to include one to four methylene units in order to study the effect of chain length. This design strategy led to the preparation of the 12 Michael-type inhibitors and 12 corresponding epoxide inhibitors shown in Figure 3.[26] All of the compounds studied herein were found to inhibit guinea pig liver TGase in a time-dependent fashion.

This time-dependent inhibition was found to be cleanly mono-exponential at all concentrations of inhibitor, which is consistent with consumption of the enzyme upon irreversible inactivation. This is 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. Furthermore, when the enzyme was inactivated in the presence of excess inhibitor, which was subsequently removed by filtration over a molecular-weight cutoff membrane, the recuperated enzyme was not found to recover its activity with time,[37] which is again consistent with irreversible inhibition. Finally, this irreversible inactivation by suicide substrates built on Z-Gln-Gly motif has been shown[38] to take place more slowly in the presence of higher concentration of Z-Gln-Gly substrate. This temporary protection from inactivation is consistent with inactivation taking place at the active site.

Irreversible inhibition constants were measured from the mono-exponential curves of the enzymatic reaction followed by a direct continuous spectrophotometric assay. A typical example is shown for compound **1a** in Figure 4. Pseudo-first order inactivation rate constants (k_{obs}) were calculated from these curves. These values were then used to construct a plot of $\frac{1}{k_{\mathrm{obs}}}$ vs $\frac{1}{[\mathrm{I}]}$ as shown in Figure 5. Linear regression of the data from this plot yielded the inhibition constants of k_{cat}, k_{inact} and K_{I}[33,34] that are summarized in Tables 1 and 2.

For the Michael inhibitors shown in Table 1, it is apparent that on passing from compounds **1a**–**4a** to compounds **1b**–**4b** the addition of glycine as a second amino acid to form a dipeptide improves the affinity of the inhibitor. Further examination of the kinetic data also suggests that the removal of the *tert*-butyl group to reveal the free carboxylic acid (in **1c**–**4c**) allows better

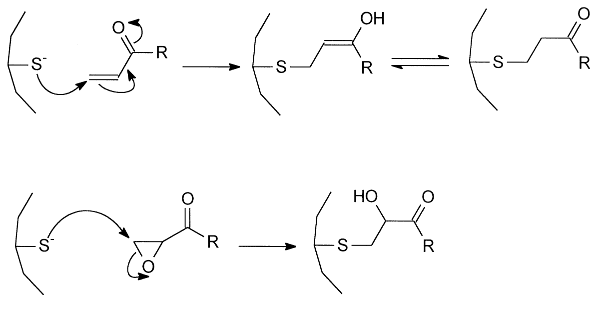
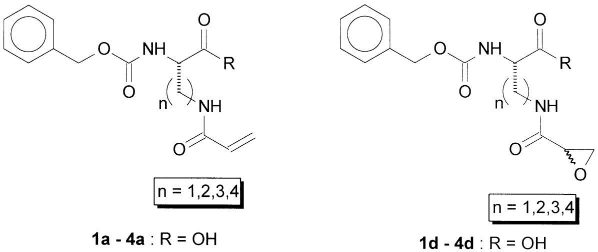


Figure 2. Irreversible inhibition of thiol-dependent enzymes by Michael acceptor or epoxide functional groups.



1a - 4a : R = OH
1b - 4b : R = Gly-O-*t*-Bu
1c - 4c : R = Gly-OH

1d - 4d : R = OH
1e - 4e : R = Gly-O-*t*-B
1f - 4f : R = Gly-OH

Figure 3. α,β-Unsaturated amide and epoxide inhibitors studied herein.

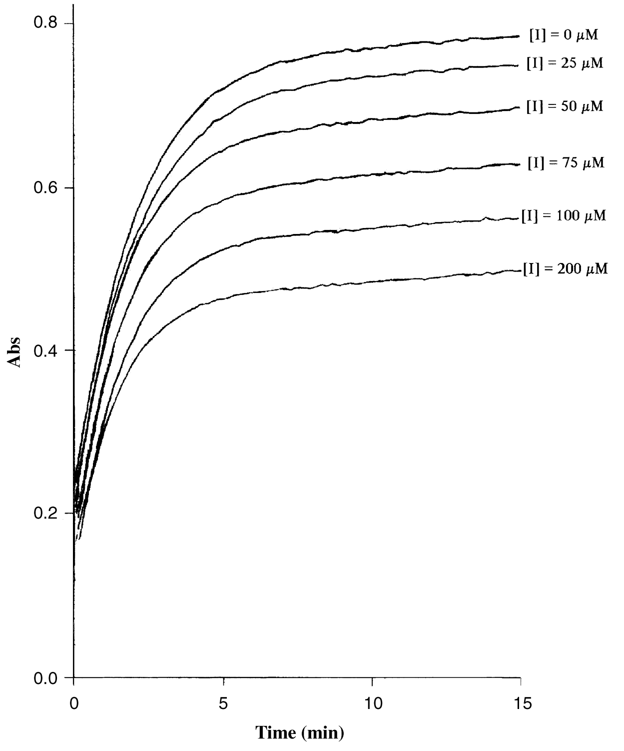


Figure 4. Enzymatic reaction progress in the presence of different concentrations of inhibitor **1a**.

recognition of the inhibitor by the TGase. This may be explained either in terms of the unfavorable steric bulk of the *tert*-butyl ester and/or the favorable formation of a hydrogen bond or electrostatic interactions by the carboxylate group. However, examination of the structure of native protein substrates, where the donor glutamine residue can be located far from the C-terminus would suggest that a free carboxylate at this position is probably not a strict requirement. Current studies in our laboratories[39] aim to determine the effect of the variation of the amino acid(s) (in the form of an ester or free carboxylate) that follow(s) Gln in an attempt to refine our understanding of the preferred amino acid sequence of the acyl donor substrate, following the pioneering work of Folk et al.[28]

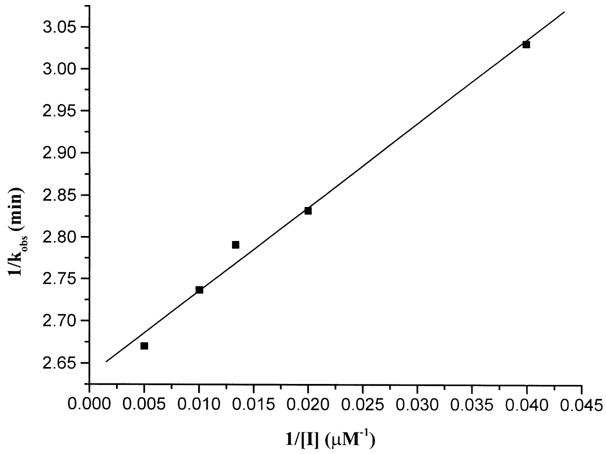
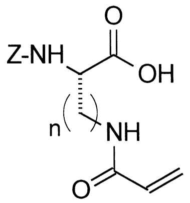
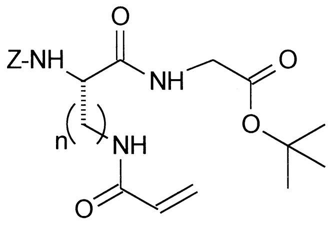
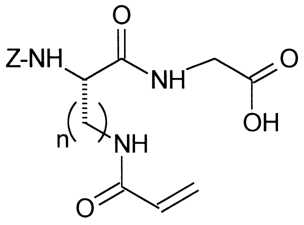


Figure 5. Double reciprocal plot of k_{obs} versus [I] for inhibitor **1a**.

Concerning the effect of the length of the side chain, it is obvious that in each series of Michael-type inhibitors, the compound bearing the longest side chain ($n=4$) is the most efficient. In fact, the best irreversible inhibitor examined is compound **4c** which bears a four methylene unit side chain and a free glycine moiety. It is interesting to consider that TGases are designed to exclude asparagine residues ($n=1$) as acyl-donor substrates, reacting only with glutamine residues ($n=2$). Presumably, this specificity involves the binding of the peptide backbone of potential acyl-donor substrates at a site sufficiently distant from the site of acylation such that asparagine β-carboxamide groups are unable to acylate the active site thiol residue. However, it appears that guinea pig liver TGase is not designed to exclude longer acyl donor substrates ($n>2$) that probably possess the conformational flexibility required to properly position their pendant reactive groups near the active site thiol. Further work is underway to explore the possibility of the use of long chain amides as acyl donor substrates.

The epoxide-bearing compounds shown in Table 2 also irreversibly inhibit TGase. However, as opposed to what was observed for the Michael-type inhibitors, the addition of glycine as a second amino acid to form a

Table 1. Summary of kinetic parameters for Michael-type inhibitors 1–4a–c[a]

Structure	N	Compd	k_{cat} (min^{-1})	K_I (μM)	k_{inact} (μM^{-1} min^{-1})	[I] (μM)
	1	**1a**	1.08	1.17	0.92	25–200
	2	**2a**	0.73	0.81	0.90	25–200
	3	**3a**	1.33	2.32	0.57	25–200
	4	**4a**	0.67	0.52	1.28	10–100
	1	**1b**	0.95	1.48	0.64	25–200
	2	**2b**	0.60	0.51	1.18	25–200
	3	**3b**	0.75	0.85	0.89	25–200
	4	**4b**	0.49	0.23	2.20	10–100
	1	**1c**	1.34	2.75	0.49	25–200
	2	**2c**	0.59	0.48	1.24	25–200
	3	**3c**	0.54	0.28	1.95	10–100
	4	**4c**	0.46	0.15	3.00	5–50

[a] The experimental error in the kinetic parameters k_{cat}, K_I and k_{inact} is estimated to be 10%.

dipeptide on passing from compounds **1d**–**4d** to compounds **1e**–**4e** decreases the affinity of the inhibitors. Removal of the bulky *tert*-butyl group to give a free carboxylic acid in compounds **1f**–**4f** improves, but only partially restores, their affinity for TGase. Once again, the length of the side chain bearing the reactive group was found to have a significant effect on inhibitor efficiency. Table 2 shows that inhibitors **3e** and **3f** (where $n=3$) display the highest affinity in their series. However, amino acid **4d**, bearing no glycine and a long side chain of four methylene units, is the most efficient of all the epoxides examined, with $k_{\text{inact}} = 2.03\,\mu\text{M}^{-1}\,\text{min}^{-1}$ and $K_{\text{I}} = 276\,\text{nM}$. It should also be noted that the epoxides shown in Table 2 represent a mixture of diastereomers that differ in the absolute configuration of the epoxide groups. Given the chiral nature of the enzyme active site, it is probable that one epoxide stereoisomer should be more efficient than the other, with a potentially higher k_{inact} value.

The epoxide inhibitors may react with TGase through either of their epoxide carbons—the penultimate carbon, or the terminal carbon, as shown in Figure 2. In an attempt to better understand the regiochemistry of their reactivity, the reaction of compound **1d** with 2-aminoethane thiol (Fig. 6) was carried out as a model reaction for the reaction of the epoxide inhibitors with the Cys-His-Asp catalytic triad of the TGase active site. The product of the model reaction was characterized by IR, NMR and mass spectroscopy and found to correspond to the structure shown in Figure 6. This adduct product is consistent with the attack of thiolate on the terminal epoxide carbon, as represented schematically in Figure 2. This suggests that the observed inactivation of TGase by the epoxides studied herein may proceed through the formation of similar covalent adduct products. However, without direct evidence obtained through mass

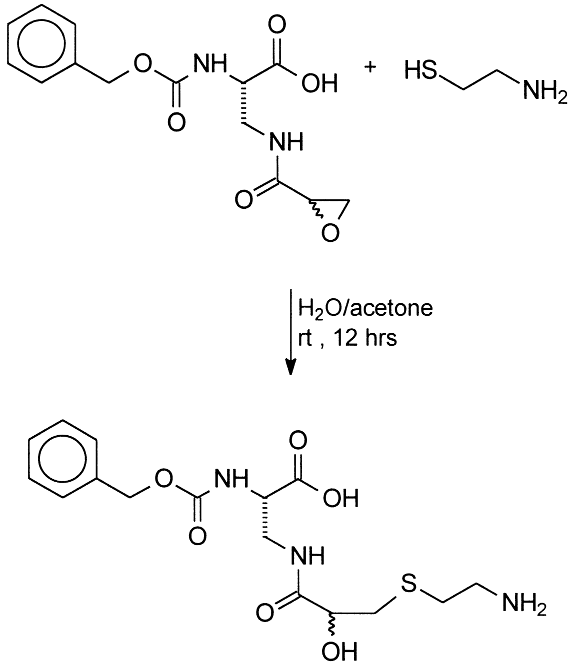
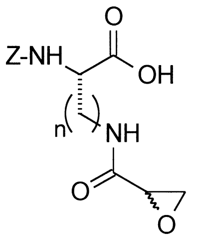
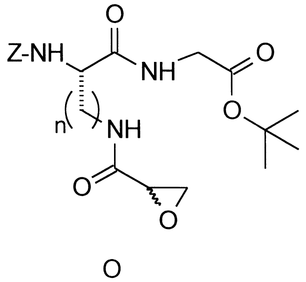
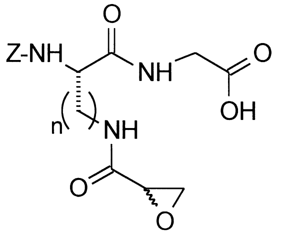


Figure 6. Reaction of epoxide inhibitor **1d** with a thiolamine.

Table 2. Summary of kinetic parameters for epoxide inhibitors 1–4d–f[a]

Structure	n	Compd	k_{cat} (min^{-1})	K_{I} (μM)	k_{inact} (μM^{-1} min^{-1})	[I] (μM)
	1	**1d**	1.39	2.13	0.65	25–200
	2	**2d**	0.61	0.50	1.23	25–200
	3	**3d**	0.50	0.29	1.72	25–200
	4	**4d**	0.56	0.28	2.03	5–50
	1	**1e**	2.49	5.91	0.42	75–300
	2	**2e**	2.02	4.20	0.48	75–300
	3	**3e**	0.87	0.95	0.91	25–200
	4	**4e**	1.45	2.42	0.60	75–300
	1	**1f**	0.60	1.10	0.55	25–200
	2	**2f**	0.81	1.23	0.66	75–300
	3	**3f**	0.63	0.56	1.12	25–200
	4	**4f**	0.59	0.59	1.00	25–200

[a] The experimental error in the kinetic parameters k_{cat}, K_{I} and k_{inact} is estimated to be 10%.

spectrometric or radiolabeling experiments, we cannot exclusively conclude that the observed time-dependent inhibition involves *covalent* modification.

Comparison of the epoxides with the Michael-type inhibitors reveals that the transformation of the double bond into an epoxide significantly changes the volume of the reactive group, not only by the addition of an oxygen atom, but also by removing the planarity of the conjugated α,β-unsaturated amide and changing the hybridization of the carbon adjacent to the carbonyl. These effects may give rise to sufficient steric bulk as to render the reactive group more sensitive to precise positioning and to reduce its overall active site binding affinity. This sensitivity may be more pronounced for compounds possessing greater steric bulk and may explain the greater general decrease in inhibitory efficiency on passing from the Michael acceptors (**1a**–**4c**) to the epoxides (**1d**–**4f**) when the peptide scaffold contains an additional glycine or glycine ester moiety (i.e., **1b**–**4c** cf. **1e**–**4f**) as opposed to a single amino acid residue (i.e., **1a**–**4a** cf. **1d**–**4d**).

Conclusion

In this paper, we report the irreversible inhibition of guinea pig liver TGase by a series of 24 compounds bearing either a α,β-unsaturated amide (Michael acceptor) or an epoxide group. In the series of Michael-type inhibitors, dipeptide **4c** shows the best efficiency, with $k_{\text{inact}} = 3.00\ \mu\text{M}^{-1}\ \text{min}^{-1}$. For the epoxide series, the best effect is obtained with amino acid **4d**, for which $k_{\text{inact}} = 2.03\ \mu\text{M}^{-1}\ \text{min}^{-1}$. Thus, there appears to be evidence that a longer side chain ($n = 4$) can increase the inhibitory effect. The epoxide derivatives display a similar affinity trend with respect to chain length as the Michael-type inhibitors, but display their highest affinity where $n = 3$ or 4. It is possible that the greater steric bulk of the epoxide group is responsible for their decreased active site affinity and different sensitivity to chain length compared to the Michael-type inhibitors.

These results can serve as a starting point for further modification of TGase inhibitors. For example, the kinetic studies of enantiomeric epoxide derivatives should be investigated for the most efficient racemic epoxide inhibitors. Furthermore, our best inhibitors presented herein will be chosen for co-crystallization studies with TGase in an attempt to clarify the inhibitor–enzyme interactions by X-ray crystallography and concurrent molecular modeling. These experiments will permit us to better understand the mechanism of TGase inhibition and subsequently to define a new generation of inhibitors.

Experimental

Enzyme preparation

TGase was purified from Guinea pig livers according to a recent modification[32] to the purification method previously published by Folk and Chung.[29] One unit of TGase was defined as the amount of enzyme that catalyses 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). Water was purified using a Millipore BioCell water purification system.

Kinetics

Enzymatic assays and inhibition kinetics were carried out as described previously[33] 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 a DMSO solution of inhibitor (whose concentration depends upon its effectiveness) with 20–0 µL of DMSO, to give an intermediate volume of 480 µL. The reaction solution was preincubated for 3 min at 37 °C, prior to initiation of the enzymatic reaction upon the addition of 20 µL of purified guinea pig liver TGase (0.2 units) to give a final volume of 500 µL.

The kinetic parameters K_I, k_{cat} and k_{inact} were determined as described previously[33] according to the method of Tian and Tsu.[34]

Synthesis

Materials and methods. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer in the solvent indicated in the text. Chemical shifts are reported in ppm with internal reference to TMS. Mass spectra (MS) were recorded on a Micromass 1212 spectrometer. The starting compound was obtained from Sigma-Aldrich.

N_α-Carbobenzyloxy-L-2-amino-3-(2-hydroxy-3-(2-aminoethylthio)-ethylcarbonylamino)propionic acid

A 23 mg portion of aminoethanethiol hydrochloride (0.23 mmol) in 5 mL of water was added to 61 mg of N_α-carbobenzyloxy-L-2-amino-3-(oxiranecarbonylamino)-propionic acid **1d** (0.2 mmol) dissolved in 5 mL of acetone. Solution was stirred for 12 h at room temperature. Solvents were removed under reduced pressure to give the product as a light yellow oil. Reaction was quantitative.

^{1}H NMR. (CD_3OD, 400 MHz) δ 7.49–7.34 (m, 5H), 5.09 (s, 2H), 4.31 (t, 1H, $J = 6.4$ Hz), 3.38 (t, 2H, $J = 6.6$ Hz), 3.32 (t, 2H, $J = 6.4$ Hz), 3.14 (t, 2H, $J = 6.5$ Hz), 3.06 (t, 2H, $J = 6.4$ Hz), 2.80 (t, 2H, $J = 6.4$ Hz).

^{13}C NMR. (CD_3OD, 100 MHz) δ 173.83, 171.86, 158.37, 137.98, 129.64, 129.21, 128.93, 67.95, 55.40, 48.42, 43.74, 30.41, 22.66.

MS. (FAB+) 386.1 (MH+).

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References and Notes

1. Levitzki, A.; Willingham, M.; Pastan, I. H. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2706.
2. Davies, P. J.; Davies, D. R.; Levitzki, A.; Maxfield, F. R.; Milhaud, P.; Willingham, M. C.; Pastan, I. H. *Nature (London)* **1980**, *283*, 162.
3. Fesus, L.; Thomazy, V.; Falus, A. *FEBS Lett.* **1987**, *224*, 104.
4. Birckbichler, P. J.; Orr, G. R.; Patterson, M. K.; Conway, E.; Carter, H. A.; Maxwell, M. D. *Biochim. Biophys. Acta* **1983**, *763*, 27.
5. Matacic, S.; Loewy, A. G. *Biochim Biophys Res. Commun.* **1968**, *30*, 356.
6. Lorand, L. *Ann, N.Y. Acad. Sci.* **1972**, *202*, 6.
7. Pisano, J. J.; Finlayson, J. S.; Peyton, M. P. *Science* **1968**, *160*, 892.
8. Rice, R. H.; Green, H. *Cell* **1979**, *18*, 681.
9. Kim, S. Y.; Chung, S. I.; Steinert, P. *J. Biol. Chem.* **1995**, *270*, 18026.
10. Nemes, Z.; Demeny, M.; Marekov, L. N.; Fesus, L.; Steiner, P. M. *J. Biol. Chem.* **2000**, *275*, 2636.
11. Candi, E.; Tarcsa, E.; Digiovanna, J. J.; Compton, J. G.; Elias, P. M.; Marekov, L. N.; Steinert, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2067.
12. De Young, L.; Ballaron, S.; Epstein, W. *J. Invest. Dermatol.* **1984**, *82*, 275.
13. Dalziel, K.; Dykes, P. J.; Marks, R. *Br. J. Exp. Pathol.* **1984**, *65*, 107.
14. Azari, P.; Rahim, J.; Clarkson, D. P. *Curr. Eye Res.* **1981**, 463.
15. Fesus, L. *Surv. Immunol. Res.* **1982**, *1*, 297.
16. Bernard, B. A.; Reano, A.; Darmon, Y. M.; Thivolet, J. *Brit. J. Dermatol.* **1986**, *114*, 279.
17. Schroeder, W. T.; Thatcher, S. M.; Stewart-Galetka, S.; Annarella, M.; Chema, D.; Siciliano, M. J.; Davies, P. J. A.; Tang, H. Y.; Sowa, B. A.; Duvic, M. *J. Invest. Dermatol.* **1992**, *99*, 27.
18. Wolf, R.; Schiavo, A. L. *Int. J. Dermatol.* **1997**, *36*, 10.
19. Selkoe, D. J.; Ihara, Y.; Salazar, F. *Science* **1982**, *215*, 1243.
20. Selkoe, D. J.; Abraham, C.; Ihara, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6070.
21. Schlaepfer, W. In *Biological Aspects of Alzheimer's Disease*; Katzman, R., Ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1983; pp 107–115.
22. Farmer, P. M.; Peck, A.; Terry, R. D. *J. Neuropathol. Exp. Neurol.* **1976**, *35*, 367.
23. Amendola, A.; Lombardi, G.; Oliverio, S.; Colizzi, V.; Piacentini, M. *FEBS Lett.* **1994**, *339*, 258.
24. Benzinger, T. L. S.; Gregory, D. M.; Burkoth, T. S.; Miller-Auer, H.; Lynn, D. G.; Botto, R. E.; Meredith, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13407.
25. Norlund, M. A.; Lee, J. M.; Zainelli, G. M.; Muma, N. A. *Brain Res.* **1999**, *851*, 154.
26. de Macédo, P.; Marrano, C.; Keillor, J. W. *Bioorg. Med. Chem.*, submitted for publication.
27. Marrano, C.; de Macédo, P.; Gravel, C.; Keillor, J. W. *Bioorg. Med. Chem.*, submitted for publication.
28. Folk, J. E.; Finlayson, J. S. *Adv. Protein Chem.* **1977**, *31*, 1.
29. Folk, J. E.; Chung, S. I. *Methods Enzymol.* **1985**, *113*, 358.
30. Waki, M.; Kitajima, Y.; Izumiya, N. *Synthesis* **1981**, 266.
31. Rich, D. H. In *Proteinase Inhibitors*; Barret, A. J.; Salvesen, G., Eds.; Elsevier: New York, 1986; pp 153–178.
32. Leblanc, A.; Day, N.; Ménard, A.; Keillor, J. W. *Prot. Exp. Purif.* **1999**, *17*, 89.
33. de Macédo, P.; Marrano, C.; Keillor, J. W. *Anal. Biochem.* **2000**, *285*, 16.
34. Tian, W. X.; Tsu, C. L. *Biochemistry* **1982**, *21*, 1028.
35. Aeschlimann, D.; Paulsson, M. *Thromb. Haemostasis* **1994**, *71*, 402.
36. de Macédo, P.; Marrano, C.; Keillor, J. W. Unpublished results.
37. de Macédo, P.; Keillor J. W. Unpublished results.
38. Gagnon, P.; Lapierre, D.; Keillor, J. W. Unpublished results.
39. Gagnon, P.; Huang, X.; Keillor, J. W. Unpublished results.