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Synthesis and evaluation of peptidic irreversible inhibitors of tissue transglutaminase

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Abstract—Herein we report the synthesis and the evaluation of eight novel compounds as irreversible inhibitors of transglutaminase (TGase). These compounds are based on a minimal peptidic scaffold shown previously [*Chem. Biol.* **2005**, *12*, 469–475] to confer affinity for the TGase active site and bear electrophilic groups such as α,β-unsaturated amide, chloroacetamide or maleimide; their general structure being Cbz-Phe-spacer-electrophile. The affinity conferred by the Cbz-Phe scaffold was determined by comparison to *N*-propylacrylamide and the length of the spacer was also varied to evaluate its importance. The inhibitory efficiencies (k_{inact}/K_I) of these compounds vary up to 10^5 M^{-1} min^{-1}, among the highest reported for derivatives based on this simple Cbz-Phe peptidic scaffold.

1. Introduction

Transglutaminases (EC 2.3.2.13) are a family of Ca^{2+}-dependent enzymes that catalyze acyl transfer reactions. Catalytic activity is exhibited toward the γ-carboxamide moiety of a protein- or peptide-bound glutamine residue which serves as acyl donor to either protein- or peptide-bound lysine residues or free amines that serve as acyl acceptors, resulting in new intermolecular amidic cross-links.[2–4]

Tissue transglutaminase 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 Ref. 5). TGase is also suspected to play a role in neurodegenerative diseases associated with an increase in polyglut- amine-containing peptides in the brain such as Huntington's disease, Alzheimer disease, Parkinson disease, and supranuclear palsy.[6–8]

In order to regulate excess TGase activity, a number of potential TGase inactivators have been developed, including dihydroisoxazole derivatives,[1] gluten peptide analogs,[9] and dipeptide-bound α,β-unsaturated amides, epoxides, and 1,2,4-thiadiazoles.[10–12] Herein we report the synthesis and the evaluation of eight novel compounds as irreversible TGase inhibitors. The peptidic scaffold (Cbz-Phe–) of these compounds was similar to those of recently published TGase irreversible inhibitors[1] but bearing functional groups such as α,β-unsaturated amide, chloroacetamide or maleimide, allowing comparison of the relative efficiency of the different electrophilic groups. The chain length of the spacer between the phenylalanine and the electrophilic group was also varied in order to permit investigation of its importance. Finally, *N*-propylacrylamide was also synthesized and evaluated to determine the significance of the Cbz-Phe scaffold on TGase affinity.

2. Results and discussion

The starting point of our parallel syntheses is the commercially available Cbz-Phe (**1**). The corresponding *p*-nitrophenyl ester (**2**) was obtained in 68% isolated yield according to a previously established procedure[13] using *p*-nitrophenyl chloroformate, triethylamine as a base, and catalytic dimethylaminopyridine in acetonitrile (Scheme 1). This activated Cbz-Phe ester (**2**) was then coupled with different diamino alkanes in acetonitrile

Abbreviations: Cbz, carbobenzyloxy; DMAP, 4-dimethylaminopyridine; DMF, dimethyl formamide; GABA, γ-aminobutyric acid; TGase, transglutaminase.

Keywords: Transglutaminase; Enzyme inhibition; Peptide.

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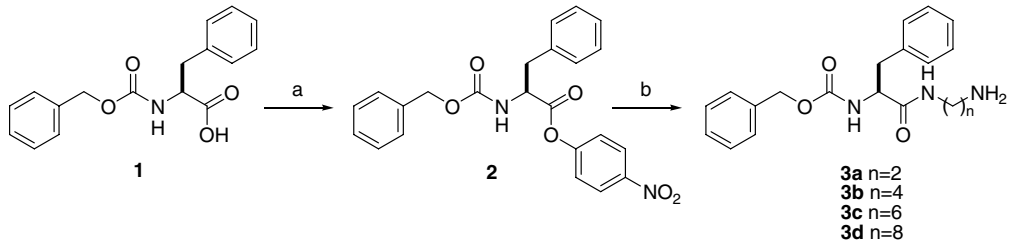
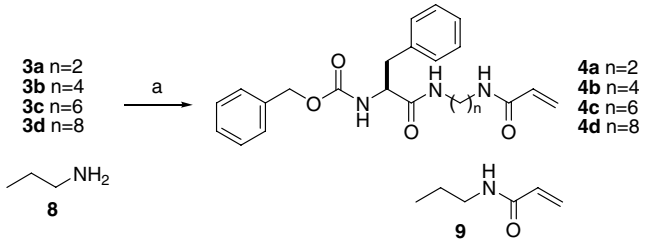


Scheme 1. Synthesis of the common amine scaffolds. Reagents and conditions: (a) *p*-nitrophenyl chloroformate, NEt_3, DMAP, CH_3CN, 50 min, rt. (b) Diaminoalkane, CH_3CN, 1 h, rt.

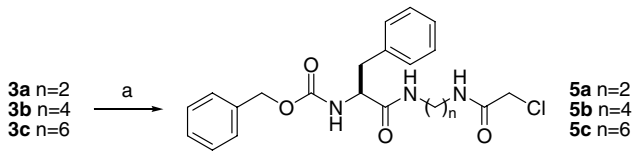
to give Cbz-Phe–NH–$(CH_2)_n$–NH_2 (**3a**–**3d**, $n = 2,4,6,8$) in 27–82% isolated yield.

From this common amine scaffold, compounds **4a**–**4d** were prepared by allowing acryloyl chloride to react with compounds **3a**–**3d** in the presence of triethylamine in acetonitrile and obtained after purification in 29–46% isolated yield. Alternatively, compounds **5a**–**5c** and **9** were prepared by allowing chloroacetic anhydride to react with compounds **3a**–**3c** and **8** in the presence of triethylamine in acetonitrile and obtained after purification in 28–56% isolated yield. Furthermore, a maleimide derivative was prepared by allowing maleic anhydride to react with compound **3b** in the presence of triethylamine in dichloromethane to give the open form of maleimide **6b**, which was then cyclized in the presence of acetic anhydride and sodium acetate to give compound **7b** in 37% isolated yield over the two reaction steps. Although none of the reported yields were optimized, all final compounds were easily obtained in sufficient yield to allow thorough kinetic evaluation as TGase inhibitors (see Schemes 2–4).

Recombinant guinea pig liver TGase was expressed in *Escherichia coli* and subsequently purified according to a procedure developed in our laboratories.[14] In addition to being easy to obtain in excellent yield and solubility, guinea pig liver TGase was chosen for this study because



Scheme 2. Synthesis of α,β-unsaturated amide derivatives. Reagents and condition: (a) acryloyl chloride, NEt_3, CH_3CN, overnight, rt.



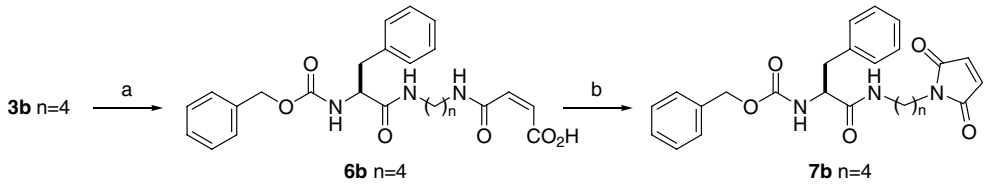
Scheme 3. Synthesis of chloroacetamide derivatives. Reagents and condition: (a) chloroacetic anhydride, NEt_3, CH_3CN, overnight, rt.

it shows 80% homology with human tissue TGase,[15] thereby validating its use as a model for the evaluation of inhibitors of potential therapeutic utility.

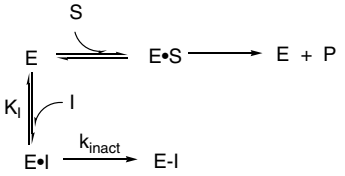
Mono-exponential time-dependent inhibition was observed for all compounds showing inhibitory activity, consistent with consumption of the enzyme upon irreversible inactivation. Irreversibility was confirmed by the lack of activity even after removal of excess inhibitor by dilution. Kinetic evaluation of inhibitors was carried out using direct continuous colorimetric[15] and/or fluorometric[16] assays, giving comparable results. Apparent inactivation rate constants were used to determine the kinetic parameters k_{inact} and K_I (Scheme 5) as described in Section 4.

Shown in Table 1 are the kinetic parameters determined for α,β-unsaturated amides **4a**–**4d** using a fluorogenic substrate. The K_I values were found to vary only slightly across the series, from 3.5 to 13 μM. Khosla has previously reported the synthesis and evaluation of similar inhibitors composed of the same Cbz-Phe scaffold but bearing a dihydroisoxazole electrophile.[1] The affinity constants of these dihydroisoxazole derivatives are ~50-fold higher than compounds **4a**–**4d**, suggesting the larger cyclic electrophilic group may be less easily accommodated by the sterically constrained active site of TGase.[17] We previously reported the synthesis[11] and evaluation[10] of a series of analogous inhibitors where the same electrophilic group was introduced on the side chain of a Cbz-Xaa-Gly peptidic framework (Fig. 1). The affinity constants of those inhibitors are roughly an order of magnitude lower than those of compounds **4a**–**4d**.

Furthermore, the inhibitory efficiencies (k_{inact}/K_I) of compounds **4a**–**4d** show the same trends, being 3–20 times lower than the inhibitors developed earlier in our laboratories, and 4–50 times higher than Khosla's corresponding dihydroisoxazole inhibitors. The efficiency of compounds **4a**–**4d** also appears to increase slightly with the length of the spacer. This tendency was observed for three other series of irreversible inhibitors[10,12] and may be explained in terms of the structure of the acyl-donor substrate binding site. TGase is designed to exclude asparagine residues as acyl-donor substrates, reacting only with glutamine residues. Molecular modeling of tissue TGase[17] suggests this specificity involves the binding of peptide backbone of potential acyl-donor substrates at a site sufficiently distant from the site of acylation



Scheme 4. Synthesis of maleimide derivative. Reagents and conditions: (a) maleic anhydride, CH_2Cl_2, 1 h, rt. (b) Ac_2O, NaOAc, 2 h, rt.



Scheme 5. Kinetic scheme used to evaluate irreversible inhibitors.

Table 1. Kinetic parameters determined for α,β-unsaturated amide inhibitors **4a**–**4d**

Inhibitor	n	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}\ min^{-1}$)
4a	2	0.42 ± 0.03	9 ± 2	0.047 ± 0.009
4b	4	0.36 ± 0.03	4.5 ± 1.0	0.080 ± 0.020
4c	6	0.90 ± 0.12	13 ± 3	0.069 ± 0.017
4d	8	0.38 ± 0.02	3.5 ± 1.0	0.109 ± 0.036

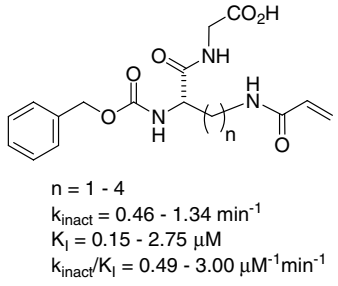


Figure 1. Analogous α,β-unsaturated amides studied previously as irreversible TGase inhibitors.[10,11]

such that asparagine β-carboxamide groups are unable to acylate the active site thiol residue. However, it can be supposed that TGase cannot exclude longer acyl-donor substrates (or irreversible inhibitors) that possess the conformational flexibility required to properly position their pendant reactive groups near the active site thiol. On a practical level, spacers longer than $n = 8$ were not pursued due to difficulties with their preparation (strong emulsion formation) and their kinetic evaluation (low solubility).

The chloroacetamide derivatives **5a**–**5c** were evaluated using a direct continuous colorimetric method[15] and their kinetic parameters are shown in Table 2. Cursory inspection shows that the values determined for these parameters are similar to those measured for the α,β-unsaturated amides **4a**–**4c**. It is noteworthy that compound **5a** does not inhibit the activity of TGase. This may be due to the fact that **5a** is the shortest inhibitor studied, the electrophilic carbon of its chloroacetamide moiety being even nearer to the Cbz-Phe group than in the case of Michael acceptor **4a**. As such, its electrophilic group may not be positioned to react efficiently with the active site cysteine thiol group.

Table 2. Kinetic parameters determined for chloroacetamide inhibitors **5a**–**5c**

Inhibitor	n	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}\ min^{-1}$)
5a	2	No inhibition observed		
5b	4	0.76 ± 0.11	73 ± 17	0.010 ± 0.001
5c	6	0.32 ± 0.04	6 ± 2	0.053 ± 0.017

Compound **7b** was evaluated using a chromogenic substrate[15] and time-dependent inhibition was observed. However, its low solubility and relatively high inhibition constant ($K_I > 160$ μM) did not allow saturating conditions to be achieved, nor its kinetic parameters to be measured precisely. As was discussed above, the volume of the maleimide moiety may lead to steric hindrance in the narrow tunnel of the acyl-donor substrate binding site.[17]

Subsequently, *N*-propylacrylamide **9** was prepared and evaluated to see if the Cbz-Phe moiety was really required for recognition by TGase. Compound **9** was observed to inactivate TGase and the rate of inactivation showed linear dependence on its concentration up to 2 mM, consistent with the second-order reaction of **9** with TGase in solution. Hence it would appear the Cbz-Phe moiety is essential for TGase affinity and specific inhibition, leading to the rapid pre-formation of a discrete Michaelis complex and the saturation kinetics observed for compounds **4**, **5**, and **7**. As suggested previously,[18] the aromatic groups of these and other TGase inhibitors[1] may direct binding to the hydrophobic groove present on the surface of the enzyme, allowing insertion of the pendant electrophilic group into the glutamine side-chain binding tunnel leading to the active site.[17]

Finally, compounds **4c** and **5c** were tested, as representative TGase inhibitors, for their reactivity toward glutathione, as a measure of their resistance to non-specific decomposition upon reaction with other nucleophiles present in living cells. The formation of glutathione adduct upon incubation with a physiological concentration of glutathione at 37 °C (pH 7) was followed quantitatively by mass spectrometry (see Section 4). After 24 h, only ~10% of either inhibitor was consumed by glutathione, in sharp contrast to their rapid reaction with TGase. The specificity of their reactivity with TGase bodes well for the in vivo application of these inhibitors.

3. Conclusions

Eight novel TGase inhibitors were prepared by straightforward synthetic routes, diverging from common amine intermediates based on the aromatic peptidic scaffold Cbz-Phe. The acrylamide (**4a**–**4d**), chloroacetamide (**5a**–**5c**), and maleimide (**7b**) derivatives thus prepared showed varying degrees of affinity and reactivity, similar to the analogous dihydroisoxazole derivatives reported previously by Khosla.[1] The length of the spacer between the Cbz-Phe– group and the electrophilic group seems to require a certain minimal length in order to allow inhibition, probably due to the inherent capacity of TGase to discriminate glutamine from asparagines based in turn on the length of the glutamine side-chain binding tunnel.[17] Further kinetic and structural characterization experiments are underway to verify these hypotheses and to establish guidelines for the design of future TGase inhibitors.

4. Experimental

4.1. Enzyme preparation

Recombinant guinea pig liver TGase was over-expressed in *E. coli* and subsequently purified according to a protocol developed in our laboratory.[14]

4.2. Kinetics

Mono-exponential time-dependent inactivation was observed for all of the inhibitors studied herein. When the inhibition reaction mixture was diluted 100-fold to remove excess inhibitor, no enzyme activity was observed or recovered, relative to the same concentration of uninhibited enzyme used as a positive control, consistent with irreversible inhibition. For kinetic runs, either the chromogenic substrate Cbz-Glu(γ-*p*-nitrophenyl ester)Gly[15] or the fluorogenic substrate Cbz-Phe-GABA umbelliferyl ester[16] was used, at concentrations corresponding to 2× K_M in the presence of 1.25–160 μM of each inhibitor. Stock solutions of all inhibitors were prepared in DMF such that the final concentration of this co-solvent was constant at 5% v/v. Kinetic evaluation was carried out by the method of Stone and Hofsteenge,[19] as described previously.[10–12] Observed first-order rate constants of inactivation were determined from non-linear regression, to a mono-exponential model. These rate constants were in turn fit to Eq. 1 by non-linear regression, providing the kinetic parameters k_{inact} and K_I as defined in Scheme 5.

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

4.3. Glutathione reactivity

Compounds **4c** and **5c** (0.2 mM) were each incubated with 1.6 mM of glutathione and 3.4 mM glutamine as an internal standard in 10 mM ammonium bicarbonate buffer (10% DMSO, pH 7) at 37 °C. The composition of each reaction mixture was determined at time zero and after 1, 2, 6, and 24 h by mass spectrometry, providing the relative proportion of unreacted inhibitor to that of glutathione adduct. Mass measurements were performed by direct injection of 0.2 μL aliquots on a LC-MSD-Tof instrument in positive electrospray mode. The protonated molecular ions $(M+H)^+$ of targeted species and internal standard were integrated and used for quantitation.

4.4. Synthesis

The various diamino alkanes used herein, as well as *N*-Cbz-L-phenylalanine (Cbz-Phe–OH), *p*-nitrophenylchloroformate, acryloyl chloride, chloroacetic anhydride, 1-propylamine, maleic anhydride, and DMAP, were obtained from Aldrich. Triethylamine and DMF were obtained from ACP. Sodium acetate and acetic anhydride were obtained from Anachemia. Acetonitrile and dichloromethane were obtained from EMD. *N*-Cbz-Glu(γ-*p*-nitrophenyl ester)Gly[15] and Cbz-Phe-GABA umbelliferyl ester[16] were synthesized in our laboratory according to published procedures.

^1H and ^{13}C NMR spectra were recorded on a Bruker 300 MHz spectrometer in the solvent indicated in the text. Chemical shifts are reported in ppm with internal reference to TMS. High-resolution mass spectra (HRMS) were recorded on a LC-MSD-Tof instrument from Agilent technologies in positive electrospray mode. Either protonated molecular ions $(M+H)^+$ or sodium adducts $(M+Na)^+$ were used for empirical formula confirmation.

4.4.1. Procedure A: Spacer coupling. Cbz-Phe *p*-nitrophenylester (**2**) (0.5 mmol) was dissolved in 50 mL of CH_3CN. Diaminoalkane (5 mmol) was added in one aliquot and the reaction mixture was stirred for 1 h. The solvent was evaporated under reduced pressure and 40 mL of EtOAc was added. The organic phase was washed with a solution of 1 N NaOH until the aqueous phase was colorless, then dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was precipitated with CH_2Cl_2/hexane to give the desired amide product as a solid.

4.4.2. Procedure B: Acryloyl chloride reaction. Cbz-Phe–NH–$(CH_2)_n$–NH_2 (0.15 mmol) was dissolved in 3 mL of CH_3CN and NEt_3 (104 μL, 0.75 mmol). The reaction mixture was cooled to 0 °C, acryloyl chloride (30 μL, 0.375 mmol) was added dropwise, and the mixture was then stirred overnight at room temperature. The final product was isolated by filtration in the cases where it precipitated from solution. Otherwise, the pure product was obtained by extraction and flash chromatography.

4.4.3. Procedure C: Chloroacetic anhydride reaction. Cbz-Phe–NH–$(CH_2)_n$–NH_2 (0.15 mmol) was dissolved in 3 mL of CH_3CN and NEt_3 (104 μL, 0.75 mmol). The reaction mixture was cooled to 0 °C, a chloroacetic anhydride (64 mg,0.375 mmol) was added portionwise, and the mixture was then stirred overnight at room temperature. The final product was isolated by filtration in

the cases where it precipitated from solution. Otherwise, the pure product was obtained by extraction and flash chromatography.

4.4.3.1. *N*-Carbobenzyloxy-L-phenylalanine *p*-nitrophenylester (2). *N*-Cbz-L-phenylalanine (**1**) (1 mmol) was dissolved in 10 mL of CH_3CN with NEt_3 (139 μL, 1 mmol) as base and placed in an ice bath, then *p*-nitrophenyl chloroformate (222 mg, 1.1 mmol) was added. The reaction mixture was stirred for 5 min at 4 °C. Then DMAP (12.2 mg, 0.1 mmol) was added and the reaction mixture was stirred another 45 min at 4 °C. The solvent was removed by rotary evaporation and 30 mL of EtOAc was added. The organic phase was washed with 3× 10 mL of 1 N HCl, dried over $MgSO_4$, and filtered and evaporated under reduced pressure. The mixture was triturated with diethyl ether and filtered to obtain a white solid in 68% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (d, 2H, J = 8.9 Hz), 7.33 (m, 10H), 7.10 (d, 2H, J = 8.9 Hz), 5.34 (d, 1H, J = 7.8 Hz), 5.12 (s, 2H), 4.84 (m, 1H), 3.24 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.3, 149.5, 148.6, 140.1, 131.3, 130.5, 125.2, 124.9, 124.5, 124.3, 124.2, 123.6, 121.4, 118.7, 68.1, 56.9, 41.2. HRMS (FAB) calcd for $C_{23}H_{21}N_2O_6$ ($[M+H]^+$): 421.1394, found 421.1396.

4.4.3.2. *N*-Carbobenzyloxy-L-phenylalanine 2-aminoethylamide (3a). This compound was prepared using general procedure A from 1,2-diaminoethane to give the desired product as a white solid in 56% isolated yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.97 (t, 1H, J = 5.5 Hz), 7.49 (d, 1H, J = 8.5 Hz), 7.23 (m, 10H), 4.92 (s, 2H), 4.17 (m, 1H), 3.03 (m, 2H), 2.92 (m, 2H), 2.74 (t, 2H, J = 10.1 Hz), 2.49 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 163.7, 149.8, 132.0, 131.6, 125.3, 124.7, 124.6, 124.3, 124.1, 123.2, 67.9, 58.3, 44.9, 43.9, 42.1. HRMS (FAB) calcd for $C_{19}H_{24}N_3O_3$ ($[M+H]^+$): 342.1812, found 342.1815.

4.4.3.3. *N*-Carbobenzyloxy-L-phenylalanine 4-aminobutylamide (3b). This compound was prepared using general procedure A from 1,4-diaminobutane to give the desired product as a white solid in 82% isolated yield. ^{1}H NMR (300 MHz, DMSO) δ 7.98 (t, 1H, J = 5.5 Hz), 7.47 (d, 1H, J = 8.4 Hz), 7.23 (m, 10H), 4.91 (s, 2H), 4.16 (m, 1H), 2.97 (m, 4H), 2.72 (t, 2H, J = 10.2 Hz), 1.77 (s, 2H), 1.39 (m, 4H). ^{13}C NMR (75 MHz, DMSO) δ 160.6, 146.4, 130.2, 129.2, 122.0, 121.1, 120.9, 120.5, 120.3, 119.2, 63.0, 54.9, 40.9, 38.9, 37.8, 30.8, 27.4. HRMS (FAB) calcd for $C_{21}H_{28}N_3O_3$ ($[M+H]^+$): 370.2125, found 370.2123.

4.4.3.4. *N*-Carbobenzyloxy-L-phenylalanine 6-aminohexylamide (3c). This compound was prepared using general procedure A from 1,6-diaminohexane to give the product as a white solid in 52% isolated yield. ^{1}H NMR (300 MHz, DMSO) δ 7.95 (t, 1H, J = 4.9 Hz), 7.49 (d, 1H, J = 8.6 Hz), 7.23 (m, 10H), 4.92 (s, 1H), 4.16 (m, 1H), 2.97 (m, 4H), 2.72 (m, 2H), 2.48 (s, 2H), 1.23 (m, 8H). ^{13}C NMR (75 MHz, DMSO) δ 160.7, 146.5, 130.3, 129.3, 122.1, 121.2, 121.0, 120.6, 120.4, 119.3, 63.1, 54.9, 41.2, 38.6, 37.9, 33.2, 29.8, 27.3, 27.2. HRMS (FAB) calcd for $C_{23}H_{32}N_3O_3$ ($[M+H]^+$): 398.2438, found 398.2448.

4.4.3.5. *N*-Carbobenzyloxy-L-phenylalanine 8-aminooctylamide (3d). This compound was prepared using general procedure A from 1,8-diaminooctane to give the desired product as a pale yellow solid in 27% isolated yield. ^{1}H NMR (300 MHz, DMSO) δ 7.94 (t, 1H, J = 5.2 Hz), 7.49 (d, 1H, J = 8.5 Hz), 7.23 (m, 10H), 4.92 (s, 2H), 4.16 (m, 1H), 2.97 (m, 4H), 2.72 (m, 2H), 2.48 (s, 2H), 1.20 (m, 12H). ^{13}C NMR (75 MHz, DMSO) δ 160.6, 158.6, 146.5, 130.3, 129.3, 122.1, 121.2, 121.0, 120.6, 120.4, 119.3, 63.1, 54.9, 41.4, 37.9, 33.7, 30.0, 29.8, 29.6, 27.4, 27.3, 23.9. HRMS (FAB) calcd for $C_{25}H_{36}N_3O_3$ ($[M+H]^+$): 426.2751, found 426.2748.

4.4.3.6. *N*-Carbobenzyloxy-L-phenylalanine 2-(acrylamido)ethylamide (4a). This compound was prepared using general procedure B from **3a**. The reaction mixture was evaporated and 20 mL of EtOAc was added. The organic phase was washed with 3× 5 mL of 1 N HCl and 3× 5 mL of 1 N NaOH, dried with $MgSO_4$, and filtered and evaporated. The product was obtained by precipitation in acetone/diethyl ether to give a white solid in 42% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26 (m, 10H), 6.45 (s, 1H), 6.25 (dd, 1H, J = 1.5, 17.0 Hz), 6.22 (s, 1H), 6.03 (dd, 1H, J = 10.2, 17.0 Hz), 5.62 (dd, 1H, J = 1.5, 10.2 Hz), 5.39 (d, 1H, J = 7.2 Hz), 5.07 (s, 2H), 4.36 (q, 1H, J = 7.3 Hz), 3.32 (s, 4H), 3.05 (d, 2H, J = 7.2 Hz). ^{13}C NMR (75 MHz, DMSO) δ 161.1, 154.8, 146.6, 130.3, 129.3, 124.4, 122.0, 121.2, 121.0, 120.7, 120.5, 119.3, 118.3, 63.1, 54.9, 38.3, 37.7. HRMS (FAB) calcd for $C_{22}H_{26}N_3O_4$ ($[M+H]^+$): 396.1917, found 396.1924.

4.4.3.7. *N*-Carbobenzyloxy-L-phenylalanine 4-(acrylamido)butylamide (4b). This compound was prepared using general procedure B from **3b**. The reaction mixture was evaporated and 20 mL of EtOAc was added. The organic phase was washed with 3× 5 mL of 1 N HCl and 3× 5 mL of 1 N NaOH, dried with $MgSO_4$, and filtered and evaporated. The desired product was obtained by precipitation from acetone/diethyl ether to give a white solid in 29% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 6.27 (dd, 1H, J = 1.5, 16.9 Hz), 6.07 (dd, 1H, J = 10.2, 17.0 Hz), 5.96 (s, 1H), 5.86 (s, 1H), 5.62 (dd, 1H, J = 1.5, 10.2 Hz), 5.38 (d, 1H, J = 5.4 Hz), 5.06 (s, 2H), 4.34 (q, 1H, J = 7.4 Hz), 3.24 (m, 6H), 1.38 (m, 4H). ^{13}C NMR (75 MHz, DMSO) δ 160.7, 154.5, 146.5, 130.9, 129.3, 124.5, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 118.0, 63.1, 54.9, 38.7, 38.3, 27.5. HRMS (FAB) calcd for $C_{24}H_{30}N_3O_4$ ($[M+H]^+$): 424.2230, found 424.2232.

4.4.3.8. *N*-Carbobenzyloxy-L-phenylalanine 6-(acrylamido)hexylamide (4c). This compound was prepared using general procedure B from **3c**. The reaction mixture was filtered and the solid was purified by flash chromatography (EtOAc) to give pure product as a white solid in 46% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 6.26 (dd, 1H, J = 1.6, 17.0 Hz), 6.07 (dd, 1H, J = 10.2, 17.0 Hz), 6.01 (s, 1H), 5.91 (s, 1H), 5.61 (dd, 1H, J = 1.6, 10.2 Hz), 5.42 (d, 1H,

J = 6.3 Hz), 5.05 (s, 2H), 4.35 (q, 1H, J = 7.5 Hz), 3.27 (m, 2H), 3.12 (m, 4H), 1.46 (m, 2H), 1.23 (m, 6H). ^{13}C NMR (75 MHz, DMSO) δ 160.6, 154.5, 146.5, 130.2, 129.3, 124.5, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 118.0, 63.1, 54.9, 38.5, 37.9, 29.9, 29.8, 27.2, 27.1. HRMS (FAB) calcd for $C_{26}H_{34}N_3O_4$ ([M+H]$^+$): 452.2543, found 452.2532.

4.4.3.9. *N*-Carbobenzyloxy-L-phenylalanine 8-(acrylamido)octylamide (4d). This compound was prepared using general procedure B from **3d**. The reaction mixture was evaporated and 20 mL of EtOAc was added. The organic phase was washed with 3× 5 mL of 1 N HCl, 3× 5 mL of 1 N NaOH and 5 mL of brine, dried with $MgSO_4$, and filtered and evaporated. The product was purified by flash chromatography (EtOAc) to give a white solid in 46% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 6.25 (dd, 1H, J = 1.6, 17.0 Hz), 6.06 (dd, 1H, J = 10.2, 16.9 Hz), 5.66 (s, 1H), 5.65 (s, 1H), 5.60 (dd, 1H, J = 1.6, 10.2 Hz), 5.42 (d, 1H, J = 7.3 Hz), 5.05 (s, 2H), 4.31 (q, 1H, J = 7.0 Hz), 3.28 (m, 2H), 3.09 (m, 4H), 1.49 (m, 2H), 1.24 (m, 10H). ^{13}C NMR (75 MHz, DMSO) δ 160.6, 154.5, 146.5, 130.3, 129.3, 124.5, 122.1, 121.2, 121.0, 120.7, 120.4, 119.3, 118.0, 63.1, 54.9, 38.6, 37.9, 29.9, 29.8, 29.5, 27.5, 27.3. HRMS (FAB) calcd for $C_{28}H_{38}N_3O_4$ ([M+H]$^+$): 480.2856, found 480.2869.

4.4.3.10. *N*-Carbobenzyloxy-L-phenylalanine 2-(chloroacetylamido)ethylamide (5a). This compound was prepared using general procedure C from **3a**. After stirring overnight, the reaction mixture was filtered and the solid obtained was washed with diethyl ether to give the pure product as a white solid in 56% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 7.14 (s, 1H), 6.95 (s, 1H), 5.27 (d, 1H, J = 6.7 Hz), 5.06 (s, 2H), 4.32 (q, 1H, J = 7.2 Hz), 3.95 (s, 2H), 3.29 (s, 4H), 3.02 (m, 2H). ^{13}C NMR (75 MHz, DMSO) δ 161.2, 156.0, 146.6, 130.3, 129.3, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 63.2, 55.0, 42.4, 38.7, 38.2, 37.7. HRMS (FAB) calcd for $C_{21}H_{25}N_3O_4Cl$ ([M+H]$^+$): 418.1528, found 418.1538.

4.4.3.11. *N*-Carbobenzyloxy-L-phenylalanine 4-(chloroacetylamido)butylamide (5b). This compound was prepared using general procedure C from **3b**. After stirring overnight, the solvent was removed and 20 mL of EtOAc was added. The organic phase was washed with 3× 5 mL of 1 N HCl and 3× 5 mL of 1 N NaOH, then dried with $MgSO_4$, filtered and evaporated. The desired product was then purified by flash chromatography (EtOAc) to give a white solid in 28% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 6.60 (s, 1H), 5.78 (s, 1H), 5.33 (d, 1H, J = 6.7 Hz), 5.06 (s, 2H), 4.32 (q, 1H, J = 6.6 Hz), 4.01 (s, 2H), 3.12 (m, 6H), 1.36 (m, 4H). ^{13}C NMR (75 MHz, DMSO) δ 160.7, 155.7, 146.5, 130.3, 129.3, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 63.1, 54.9, 42.4, 38.3, 37.9, 27.4, 27.3. HRMS (FAB) calcd for $C_{23}H_{29}N_3O_4Cl$ ([M+H]$^+$): 446.1841, found 446.1842.

4.4.3.12. *N*-Carbobenzyloxy-L-phenylalanine 6-(chloroacetylamido)hexylamide (5c). This compound was prepared using general procedure C from **3c**. After stirring overnight, the solvent was removed and 40 mL of EtOAc was added. The organic phase was washed with 3× 6 mL of 1 N HCl and 3× 6 mL of 1 N NaOH, then dried over $MgSO_4$, filtered and evaporated. The pure product was obtained by trituration with EtOAc to give a white solid in 43% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (m, 10H), 6.59 (s, 1H), 5.67 (s, 1H), 5.42 (d, 1H, J = 7.5 Hz), 5.06 (s, 2H), 4.32 (q, 1H, J = 6.8 Hz), 4.01 (s, 2H), 3.25 (q, 2H, J = 6.4 Hz), 3.12 (m, 4H), 1.48 (m, 2H), 1.23 (m, 6H). ^{13}C NMR (75 MHz, DMSO) δ 160.6, 155.7, 146.5, 130.3, 129.3, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 63.1, 54.9, 42.4, 38.5, 37.9, 29.9, 29.8, 27.2, 27.1. HRMS (FAB) calcd for $C_{25}H_{33}N_3O_4Cl$ ([M+H]$^+$): 474.2154, found 474.2157.

4.4.3.13. *N*-Carbobenzyloxy-L-phenylalanine 4-(maleamido)butylamide (6b). The compound **3b** (0.15 mmol) was dissolved in 3 mL of CH_2Cl_2 and maleic anhydride (0.165 mmol) was added. The mixture was stirred for 1 h at room temperature under nitrogen. The precipitated was filtered and washed with CH_2Cl_2 to give the pure product as a white solid in 87% isolated yield. ^{1}H NMR (300 MHz, DMSO) δ 9.1 (s, 1H), 7.99 (t, 1H, J = 5.3 Hz), 7.48 (d, 1H, J = 8.5 Hz), 7.24 (m, 10H), 6.39 (d, 1H, J = 12.6 Hz), 6.23 (d, 1H, J = 12.5 Hz), 4.92 (s, 2H), 4.16 (m, 1H), 3.10 (m, 4H), 2.76 (m, 2H), 1.38 (s, 4H). ^{13}C NMR (75 MHz, DMSO) δ 160.8, 155.4, 146.5, 130.2, 129.3, 125.6, 124.4, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 63.1, 54.9, 38.2, 37.9, 27.5, 26.8. HRMS (FAB) calcd for $C_{25}H_{30}N_3O_6$ ([M+H]$^+$): 468.2129, found 468.2143.

4.4.3.14. *N*-Carbobenzyloxy-L-phenylalanine 4-(maleimido)butylamide (7b). In a flask contained **6b** (0.15 mmol), sodium acetate (0.06 mmol) and 1 mL of acetic anhydride were added. The reaction mixture was stirred for 2 h at 100 °C then 10 mL of 1 N NaOH was added and the precipitate obtained was filtered. The solid was purified by flash chromatography using EtOAc as eluant to give a white solid in 37% isolated yield. ^{1}H NMR (300 MHz, DMSO) δ 7.24 (m, 10H), 6.66 (s, 2H), 5.66 (s, 1H), 5.33 (d, 1H, J = 4.1 Hz), 5.06 (s, 2H), 4.30 (q, 1H, J = 6.7 Hz), 3.42 (t, 2H, J = 6.9 Hz), 3.13 (m, 4H), 1.38 (m, 4H). ^{13}C NMR (75 MHz, DMSO) δ 160.7, 146.5, 130.2, 129.3, 126.9, 122.1, 121.2, 121.0, 120.7, 120.5, 119.3, 63.1, 54.9, 38.1, 37.9, 37.0, 27.3, 26.5. HRMS (FAB) calcd for $C_{25}H_{28}N_3O_5$ ([M+H]$^+$): 450.2023, found 450.2026.

4.4.3.15. *N*-Propylacrylamide (9). This compound was prepared using general procedure B from **8**. The reaction mixture was evaporated and 20 mL of EtOAc was added. The organic phase was washed with 3× 5 mL of 1 N HCl, 3× 5 mL of 1 N NaOH and then 5 mL of brine, dried with $MgSO_4$, and filtered and evaporated. The product was purified by flash chromatography (EtOAc) to give a colorless oil in 65% isolated yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.25 (dd, 1H, J = 1.5, 16.9 Hz), 6.06 (dd, 1H, J = 10.2 Hz, 16.9 Hz), 5.60 (dd, 1H, J = 1.5, 10.2 Hz), 3.28 (q, 2H, J = 6.4 Hz), 1.54 (m, 2H), 0.92 (t, 3H, J = 7.4 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.8, 126.6, 126.0, 48.9, 27.0, 16.7. HRMS

(FAB) calcd for $C_6H_{12}NO$ ($[M+H]^+$): 114.0913, found 114.0912.

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