Bioorganic & Medicinal Chemistry 17 (2009) 3900–3908

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of enediyne–peptide conjugates and their inhibiting activity against chymotrypsin

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ARTICLE INFO

Article history:
Received 27 March 2009
Revised 10 April 2009
Accepted 11 April 2009
Available online 17 April 2009

Keywords:
Enediyne
Amino acid
Trypsin
Proteolytic
Inhibitor
Competitive

ABSTRACT

Novel enediyne–amino acid conjugates **1**–**4** have been synthesized. All of these effectively target the enzyme chymotrypsin inhibiting its proteolytic activity. The conjugate with a directly linked phenyl alanine is the most effective inhibitor with a K_i of 3 μM. The mode of inhibition is mostly competitive or of a mixed type depending on the nature of the inhibitor.

1. Introduction

Proteases are implicated in the pathogenesis of many human diseases including cancer[1] and Alzheimers.[2] Thus modulation of the proteolytic activity offers an attractive potential for chemotherapeutic treatment of these diseases and discovery of agents with selectivity for specific proteases is crucial for the development of useful pharmaceuticals belonging to this class.[3] For the serine proteases, the proteolytic activity depends on a set of amino acid residues in the active site of the enzyme[4]—one of which is always a serine (thus accounting for their name). In mammals, serine proteases perform many important functions, especially in digestion, blood clotting, and the complement system. The enzyme chymotrypsin belongs to the serine protease family. It plays an important role in digestion and performs proteolysis. In the present study, we have attempted to synthesize novel enediyne–peptide or amino acid conjugates[5] which are expected to act as potent inhibitors of chymotrypsin. These compounds consist of two parts: an enediyne moiety and a peptide/amino acid moiety connected via an amide linkage. The enediynes possess powerful DNA cleaving activity,[6] which make them important in anti tumor drug design. However, the lack of correlation between the observed antitumor activity and extent of DNA-damage gave rise to the suspicion that there might be other targets. This was proved to be the case when scientists from Bristol-Meyers-Squibb laboratories reported the degradation of proteins by a synthetic analogue of calicheamicin.[7] The possible mechanism of such protein cleavage has been established by Jones et al. using deuteriated amino acids.[8] These authors have also reported the synthesis and protein degradation capacity of novel types of photoactivated enediynes.[9] In order to attain specificity, enediyne–lysine conjugates have been prepared and have been photochemically triggered to induce DNA double strand cleavage.[10] The peptide/amino acid moiety conjugated to the enediyne system is expected to impart specificity to these compounds for delivery to the target site. In this work our aim is to mimic the substrate of chymotrypsin so that the synthesized enediyne–amino acid conjugates can be recognized by the active site residues of chymotrypsin. The phenylalanine residue linked to the enediyne part in the synthesized compounds is expected to interact with the catalytic triad of the enzyme.

It is expected that the unstable enediyne moiety may be released by the cleavage of the amide bond. There is a possibility of cycloaromatisation[11] of the released enediyne system with a generation of diradical species leading to destruction of the protein framework of the enzyme. Our aim is to increase specificity of the enediyne molecules making it suitable for the use as potent inhibitors of α-chymotrypsin. The enediyne phenyl alanine conjugate may also act as a reversible inhibitor. All these possibilities are shown in Scheme 1.

Based on the above rationale, we have designed and synthesized the enediynes **1**–**4** with the aim to study their inhibition pattern on α-chymotrypsin. The piperidine and the isoquinoline based amino acid conjugates **5** and **6** were prepared for comparison purposes.

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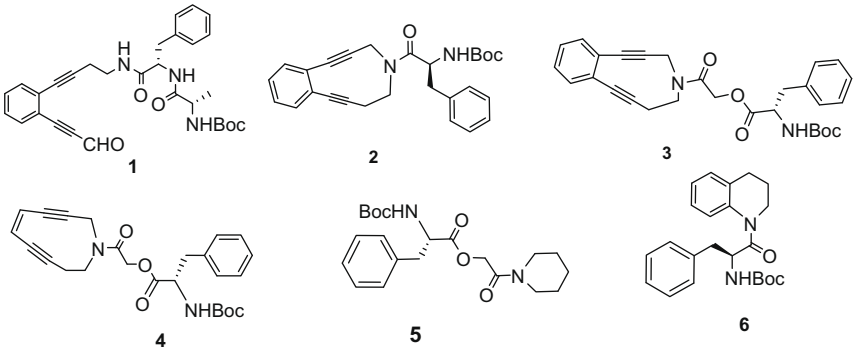
doi:10.1016/j.bmc.2009.04.019



2. Results and discussion

2.1. Synthesis

The synthesis involves the preparation of the common intermediate following the method developed in this laboratory. Deprotection of the amine followed by EDCI mediated coupling[12] with the partially protected *N*-Boc-Ala-Phe-COOH produced the THP protected enediyne peptide. Removal of THP followed by Dess Martin oxidation[13] furnished the target aldehyde (Scheme 2). For the synthesis of the cyclic enediyne based systems, the cyclic amine was directly coupled with *N*-Boc Phenylalanine to produce the target enediyne. Alternately, the amine was acylated with bromoacetyl chloride and the resulting bromide was esterified with *N*-Boc Phenylalanine to furnish the enediyne (Scheme 3). The piperidine based amide and the tetrahydroisoquinoline based amide were also prepared in a similar way (Schemes 4 and 5).

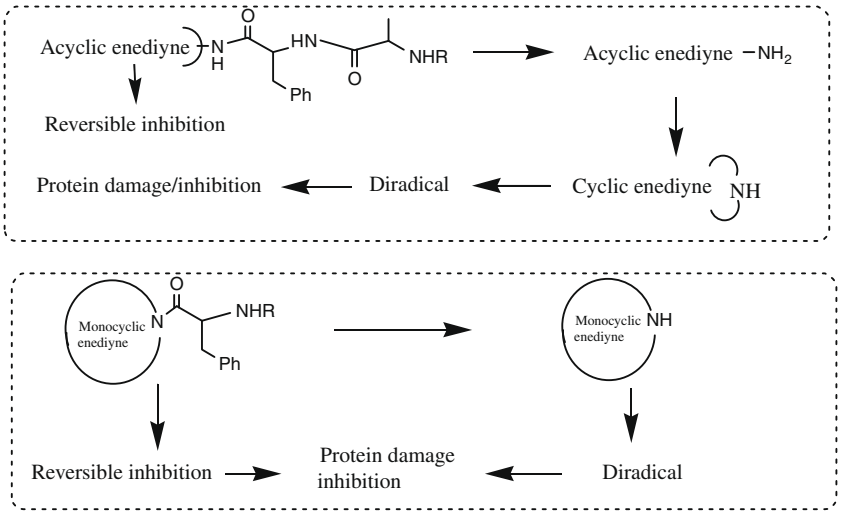
Compound **5** has been synthesized to investigate the role of the enediyne moiety in the synthesized compounds in the inhibition of chymotrypsin. The compound contains a piperidene moiety conjugated to phenylalanine. The compound **6** which corresponds to cycloaromatised form of enediyne–aminoacid conjugates helps us to analyze the contribution the cycloaromatised form of these compounds in the enzyme inhibition.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

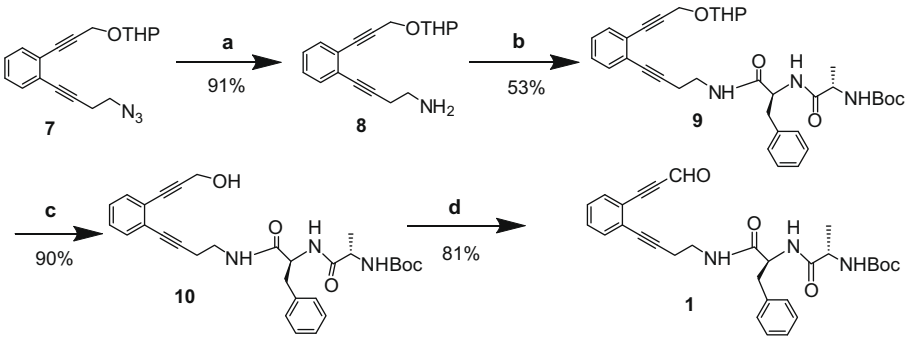
The inhibition of α-chymotrypsin was initially checked by gel based assay. Protease digestive fragments derived from BSA on treatment with chymotrypsin with or without inhibitor were analyzed on SDS–PAGE. The extent of proteolysis of the substrate decreased appreciably when the enzyme was preincubated with the synthesized compounds as is evident from the gel pattern. Analysis of the protease digestive fragments derived from BSA on treatment with chymotrypsin reveals that the band corresponding to BSA disappears with progress of the reaction. However, on preincubation of chymotrypsin with the compounds the extent of proteolysis of BSA is less pronounced as is apparent from the intensity of the BSA band (lanes 3, 5 and 7, Fig. 1A and C; lanes 3, 5 and 8 Fig. 1B; lanes 2, 4, 6 and 8, Fig. 1D). But the effect of the compound **5** and **6** is less prominent as evident for Fig. 1E–H, respectively. The degradation pattern of BSA therefore reflects the inhibitory effect of the enediyne–amino acid conjugates on the cleavage capability of α-chymotrypsin.

2.3. The effect of the enediyne–peptides/amino-acids on protease digestion of BSA with chymotrypsin

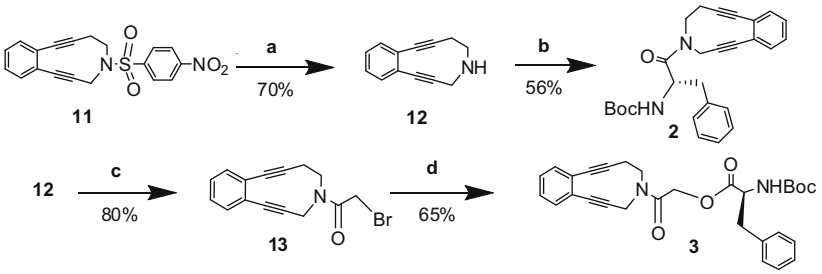
The inhibition of α-chymotrypsin by the enediyne–amino acid conjugates was further assessed spectrophotometrically using



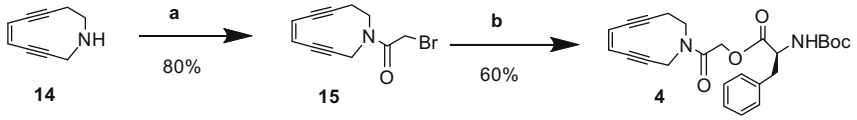
Scheme 1. Possible modes of inhibition of enedyne–amino acid or peptide conjugate.



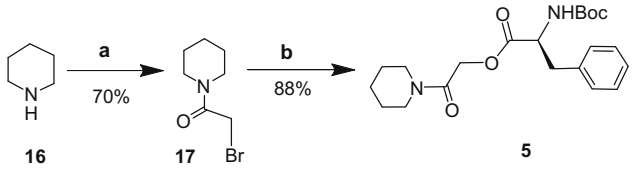
Scheme 2. Synthesis of acyclic enediyne conjugates. Reagents and conditions: (a) PPh_3, moist THF, rt, 12 h; (b) EDC, HOBT, DIPEA, Boc-L-ala.L-phe, DCM, rt,6 h; (c) PPTS, EtOH, 40 °C; (d) Dess Martin reagent, DCM, rt, overnight.



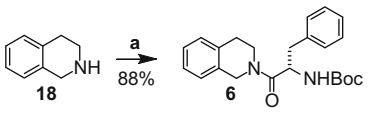
a) PhSH, K_2CO_3, DMF, rt, 30 mins.; b) EDC, HOBT, DIPEA, Boc-L-Phe, DCM, rt, 6h; c) $BrCH_2COCl$, Et_3N, DCM, 0°C, 30 mins; d) Nasalt of Boc-L-Phe, NaI(cat), DMF, rt, overnight



Scheme 3. Synthesis of cyclic enediyne–amino acid conjugates. Reagents and conditions: (a) Et_3N, $BrCH_2COCl$, DCM, 0 °C, 30 min; (b) Na salt of Boc-L-Phe, NaI (cat), DMF, rt, overnight.



Scheme 4. Synthesis of piperidine-amino acid conjugate. Reagents and conditions: (a) Et_3N, $BrCH_2COCl$, DCM, 0 °C, 30 min; (b) Na salt of Boc-L-phe, NaI (cat); DMF, rt, overnight.



Scheme 5. Synthesis of tetrahydroisoquinoline–amino acid conjugate. Reagents and conditions: (a) EDC, HOBT, DIPEA, Boc-L-Phe, DCM, rt, 6 h.

Lowry method. With progress of the reaction for a given time period, BSA was hydrolysed by free chymotrypsin or by chymotrypsin incubated with the inhibitor. The extent of proteolysis is more prominent for free chymotrypsin. On treatment of the enzyme preincubated with the compounds, the appearance of hydrolysed products of BSA is comparatively lower to the control curve (Fig. 2). The pattern of the graphs indicates that protease digestion is hampered with increasing inhibitor concentration. The proteolysis reaction slowed down to a considerable extent in presence of compound **2**.

Thus the above experiment confirms the inhibitory effect of the synthesized compounds on α-chymotrypsin. Compounds **5** and **6** exhibit lesser inhibitory activity compared to the enediyne–peptide conjugates.

2.4. Comparative inhibition of proteolytic activity of α-chymotrypsin by the synthesized enediyne–amino acid conjugates (1–4), cycloaromatised compound 6 and cyclic sulfonamide 11

Comparative inhihition of proteolytic activity of α-chymotrypsin by the synthesized compounds and the compounds **6** and **11** were estimated both by SDS–PAGE and Lowry method.[15] The compound **11** which contains the enediyne moiety and no amino acid part is chosen to ascertain the role of the phenyl alanine which basically mimics the substrate of the enzyme. The differential intensity of the 68 kD BSA band in the lanes 1–7 in Fig. 3B indicate the degree of chymotrypsin inhibition by the synthesized enediyne–amino acid conjugates and the compounds **6** and **11**. The degradation of BSA is observed to be more prominent in case of the enzyme preincubated with compounds **6** and **11**. The hydrolysed fragments are much less produced on treatment of BSA with the enzyme preincubated with the enediyne–amino acid conjugates

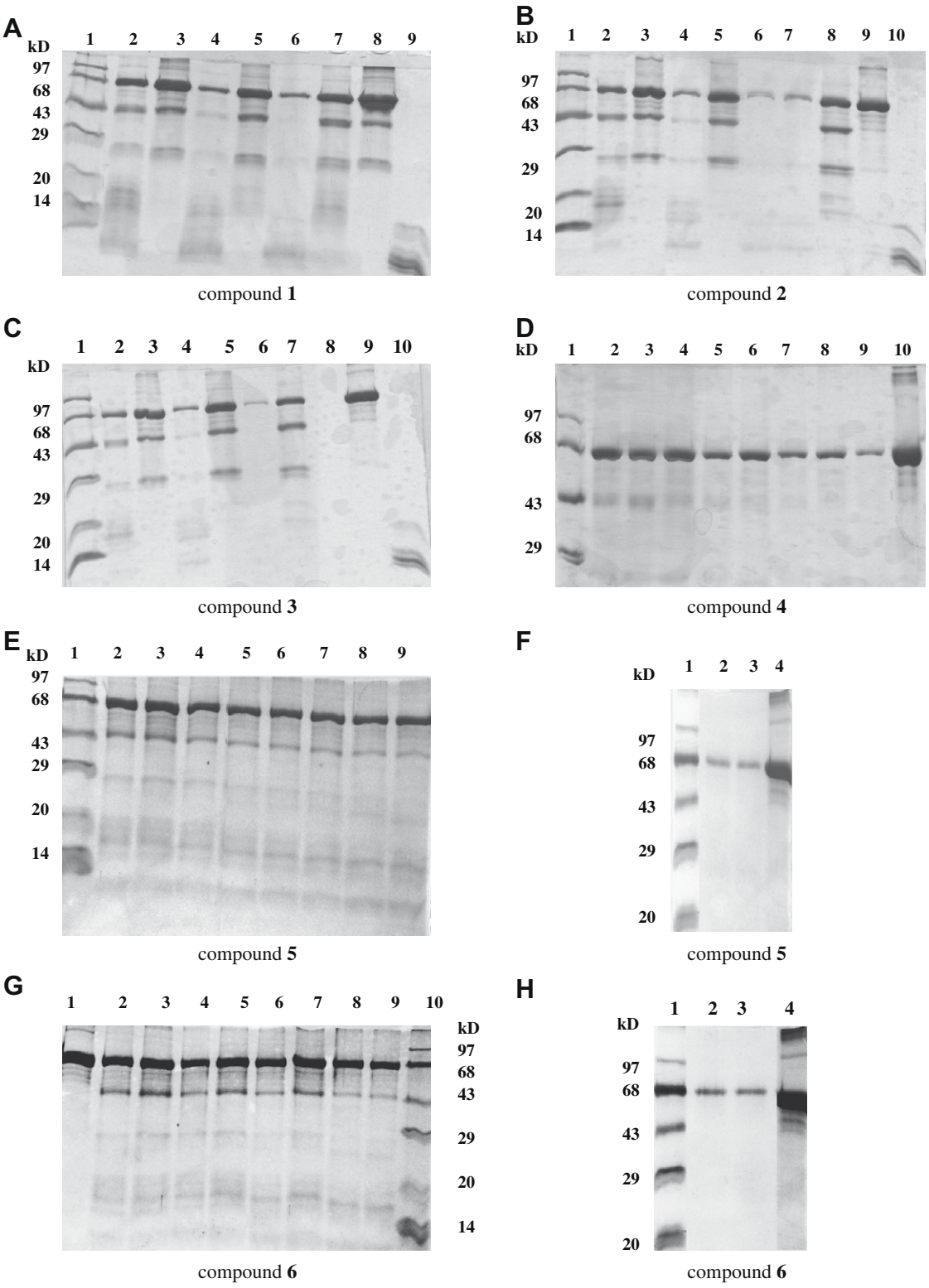


Figure 1. SDS–PAGE patterns of proteolysis of BSA by α-chymotrypsin (α-CT) with or without inhibitor for 60 min-**A**: Lane 1: Mol wt Marker (MWM), Lane 2: BSA + α-CT, 10 min, Lane 3: BSA + α-CT + **1**, 10 min, Lane 4: BSA + α-CT, 30 min, Lane 5: BSA + α-CT + **1**, 30 min, Lane 6: BSA + α-CT, 60 min, Lane 7: BSA + α-CT + **1**, 60 min, Lane 8: BSA, Lane 9: α-CT. **B:** Lane 1: MWM, Lane 2: BSA + α-CT, 10 min, Lane 3: BSA + α-CT + **2**, 10 min, Lane 4: BSA + α-CT, 30 min, Lane 5: BSA + α-CT + **2**, 30 min, Lanes 6 and 7: BSA + α-CT, 60 min, Lane 8: BSA + α-CT + **2**, 60 min, Lane 9: BSA, Lane 10: α-CT. **C:** Lane 1: MWM, Lane 2: BSA + α-CT, 10 min, Lane 3: BSA + α-CT + **3**, 10 min, Lane 4: BSA + α-CT, 30 min, Lane 5: BSA + α-CT + **3**, 30 min, Lane 6: BSA + α-CT, 60 min, Lane 7: BSA + α-CT + **3**, 60 min, Lane 8: empty, Lane 9: BSA, Lane 10: α-CT. **D:** Lane 1: MWM, Lane 2: BSA + α-CT, 10 min, Lane 3: BSA + α-CT + **4**, 10mins, Lane 4: BSA + α-CT, 20 min, Lane 5: BSA + α-CT + **4**, 20 min, Lane 6: BSA + α-CT, 30 min, Lane 7: BSA + α-CT + **4**, 30 min, Lane 8: BSA + α-CT, 60 min, Lane 9: BSA + α-CT + **4**, 60 min, Lane 10: BSA, **E:** Lane 1: MWM, Lane 2: BSA + α-CT, 5 min, Lane 3: BSA + α-CT + **5**, 5 min, Lane 4: BSA + α-CT, 10 min, Lane 5: BSA + α-CT + **5**, 10 min, Lane 6: BSA + α-CT, 20 min, Lane 7: BSA + α-CT + **5**, 20 min, Lane 8: BSA + α-CT, 30 min, Lane 9: BSA + α-CT + **5**, 30 min, **F:** Lane 1: MWM., Lane 2: BSA + α-CT, 60 min, Lane 3: BSA + α-CT + **5**, 60 min, Lane 4: BSA; **G:** Lane 1: BSA, Lane 2: BSA + α-CT, 5 min, Lane 3: BSA + α-CT + **6**, 5 min, Lane 4: BSA + α-CT, 10 min, Lane 5: BSA + α-CT + **6**, 10 min, Lane 6: BSA + α-CT, 20 min, Lane 7: BSA + α-CT + **6**, 20 min, Lane 8: BSA + α-CT, 30 min, Lane 9: BSA + α-CT + **6**, 30 min, Lane 10: MWM, **H:** Lane 1: MWM, Lane 2: BSA + α-CT, 60 min, Lane 3: BSA + α-CT + **6**, 60 min, Lane 4: BSA.

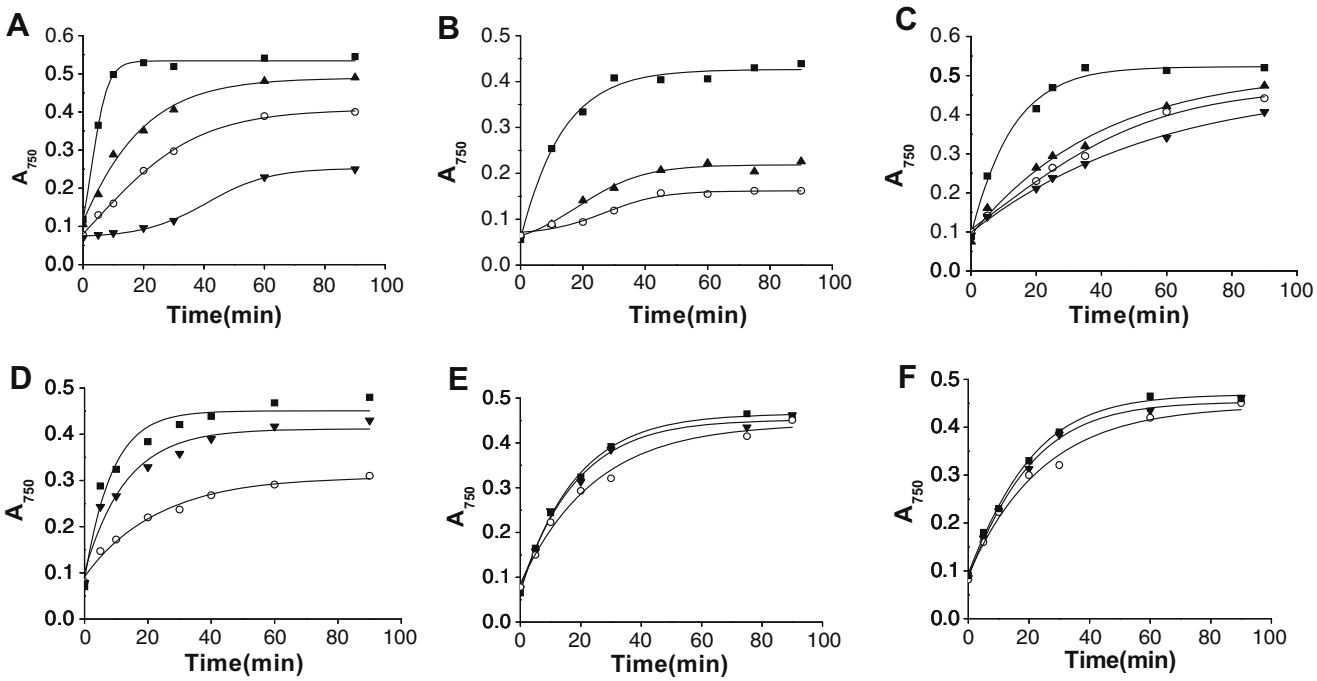


Figure 2. Protease digestion of BSA with free α-chymotrypsin and preincubated α-chymotrypsin With (A) compound **1**:0 μM (■), 8.5 μM (▲), 17 μM (○), 34 μM (▼); (B) compound **2**: 0 μM (■), 4.6 μM (▲), 7.7 μM (○); (C) compound 3: concentrations are 0 μM (■), 5.9 μM (▲), 17.8 μM (○), 35 μM (▼); (D) compound **4**: concentrations are 0 μM (■), 10.23 μM (▼), 40.9 μM (○); (E) compound **5**: concentrations are 0 μM (■), 93 μM (▼), 372 μM (○); (F) compound **6**: concentrations are 0 μM (■), 80 μM (▼), 320 μM (○).

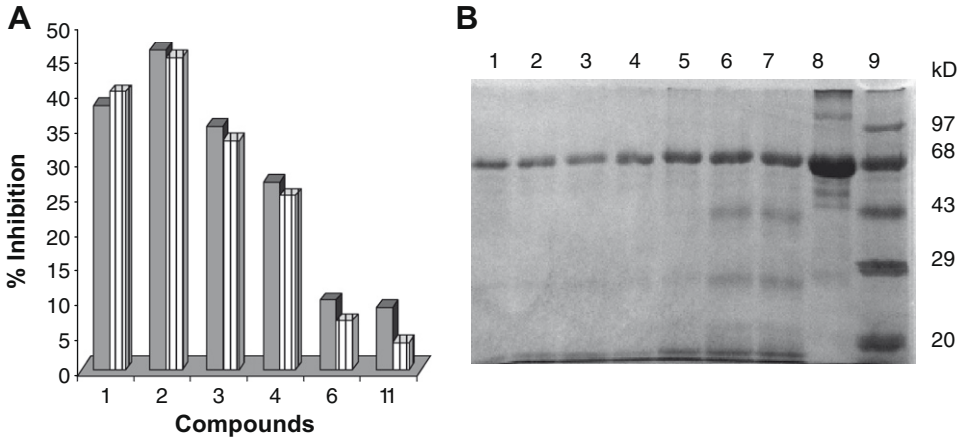


Figure 3. (A) Comparative inhibition of chymotrypsin by the synthesized compounds, % inhibition values obtained from (i) SDS–PAGE shown in grey, (ii) Lowry method shown in stripes; (B) SDS–PAGE patterns of proteolysis of BSA by α-chymotrypsin with or without inhibitor for 30 min; Lane 1: BSA + α-chymotrypsin + compound **11**, Lane 2: BSA + α-chymotrypsin + compound **6**, Lane 3: BSA + α-chymotrypsin, Lane 4: BSA + α-chymotrypsin + compound **4**, Lane 5: BSA + α-chymotrypsin + compound **3**, Lane 6: BSA + α-chymotrypsin, + compound **2**, Lane 7: BSA + α-chymotrypsin + compound **1**, Lane 8: BSA, Lane 9: Mol. wt Marker.

1–**4**. The greater inhibition potency of the enediyne based inhibitors **1**–**4** compared to the compound **11** as evident from Fig. 3A and B supports the fact that the phenyl alanine linked to these compounds helps in their delivery to the catalytic triad of the enzyme. The histogram in Fig. 3A reveals the same as the inhibition is reduced to less than 10% when no phenyl alanine moiety is attached to the enediyne part that is, compound **11**.

2.5. Inhibition kinetics

The type of inhibition and inhibition constants of synthesized compounds were ascertained from kinetic data. The reciprocal of reaction rate is plotted against the reciprocal of substrate concentration with varying inhibitor concentrations following the Lineweaver–Burk plot. Both compounds **1** and **2** behave as mixed type inhibitors with inhibition constants of 12 μM and 3 μM, respectively as obtained from the Lineweaver–Burk plot (Fig. 4). However the type of inhibition for compound **3** is competitive with an inhibition constant of 30 μM as determined from the same plot (Fig. 4). This plot is also indicative of a competitive nature for compound **5** with an inhibition constant of 750 μM. The order of inhibition constants correlates to that of SDS–PAGE and Lowry method. Comparing the inhibition constants of the enediyne–amino acid conjugates with compound **5**, we observe that the inhibitory activity of these compounds is enhanced in presence of the enediyne moiety.

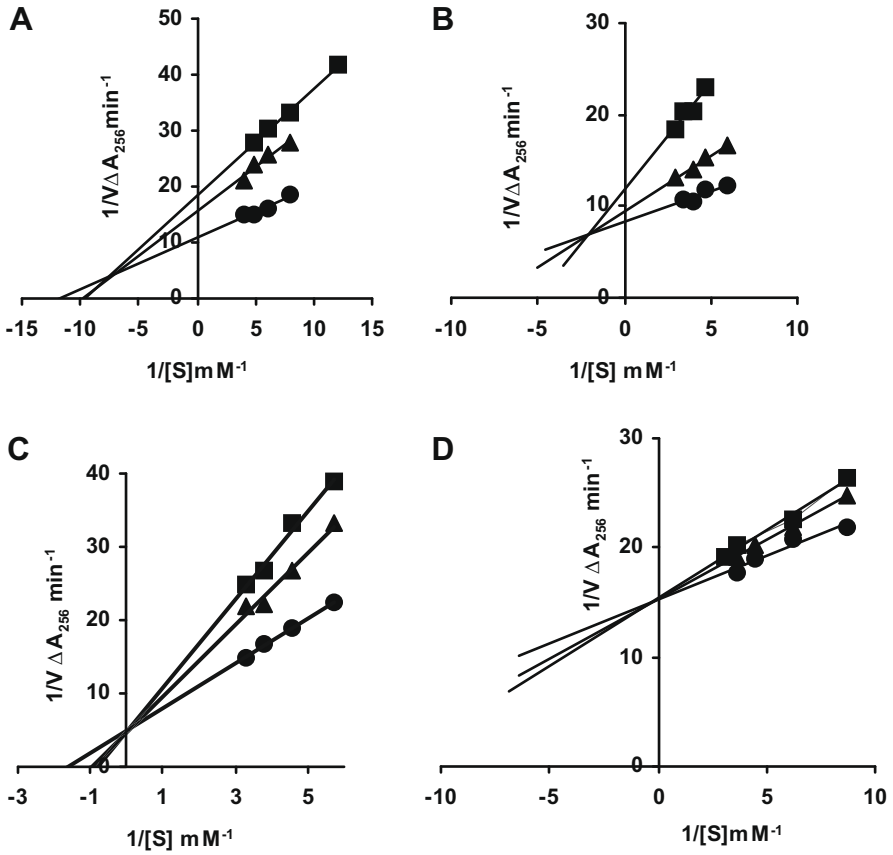


Figure 4. Lineweaver–Burk plot for inhibition of α-chymotrypsin by (A) compound **1**: concentrations are 0 μM (●), 6.4 μM (▲), 12 μM (■); (B) compound **2**: concentrations are 0 μM (●), 4.6 μM (▲), 7.7 μM (■); (C) compound **3**: concentrations are 0 μM (●), 10.5 μM (▲), 42 μM (■); (D) compound **5**: concentrations are 0 μM (●), 265 μM (▲), 397 μM (■).

Compounds	K_i values (μM)
Compound **1**	12
Compound **2**	3
Compound **3**	30
Compound **5**	750

The K_i values indicate that the enediyne–amino acid conjugates have high potential for development as inhibitors of α-chymotrypsin. Compound **5**, which lacks the enediyne moiety has a higher K_i value, thereby establishing the contribution of the enediyne moiety in interference of the normal activity of the enzyme.

2.6. Docking studies

Docking studies of compound **3** with α-chymotrypsin substantiate the experimental findings to some extent. These studies provide some insight about the binding mode of the compounds, which in turn can be correlated with the inhibition of α-chymotrypsin by compound **3**. Docking poses of compound **3** with α-chymotrypsin (PDB id 1YPH)[14] shows that it is in close proximity of the catalytic triad of the enzyme that comprises Ser195, Asp102 and His 57. The compound is within hydrogen bonding distance of Ser195 and His 57 of the catalytic triad and Phe41, which is not an active site residue. The distances measured between the compound and Ser195, His57 and Phe41 are 2.51 Å, 3.12 Å and 2.55 Å, respectively (Fig. 5). Previously reported structures of serine protease inhibitor complexes[15] show strong H-bonding to Ser195. The crystal structure of chymotrypsin-trifluoromethyl ketone inhibitor complexes[16] reveals that the inhibitor is hydrogen bonded to Ser195 with an H-bond distance of 2.7 Å (PDB id 6GCH). The docking poses of compound **3** with α-chymotrypsin resemble the previously reported crystal structures of enzyme inhibitor complexes. Moreover it corroborates our experimental findings that compound **3** is a competitive inhibitor of chymotrypsin. Docking studies indicate that compound **6** does not interact directly with the catalytic triad of α-chymotrypsin (Supplementary data). This accounts for the lower inhibition of compound **6** compared to the enediyne–amino acid conjugates (**1**–**4**).

3. Conclusion

Lack of specificity is the major problem of the enediynes. Here we report enediyne–amino acid conjugates as potent inhibitors of α-chymotrypsin. It is clear that the synthesized enediyne–amino acid conjugates effectively target the enzyme inhibiting its activity. The phenylalanine moiety in the synthesized molecules enhances specificity of these molecules considerably as evident from the assays. The role of enediyne in inhibiting the enzyme is clearly established by the fact that the compounds **5** and **6** lacking the enediyne but having the phenyl alanine moiety are poor inhibitors in comparison to the compounds with enediyne moiety **1**–**4**. However, the enediyne in this case is acting as a recognition element as exemplified by the nature of inhibition (competitive or mixed type). We are yet to get any evidence of protein cleavage expected from the radicals generated from the enediynes. Thus it is not the reactivity but the enediyne framework itself which is recognized by the enzyme and is responsible for the inhibition. Current studies are underway to develop similar strategies but by using more

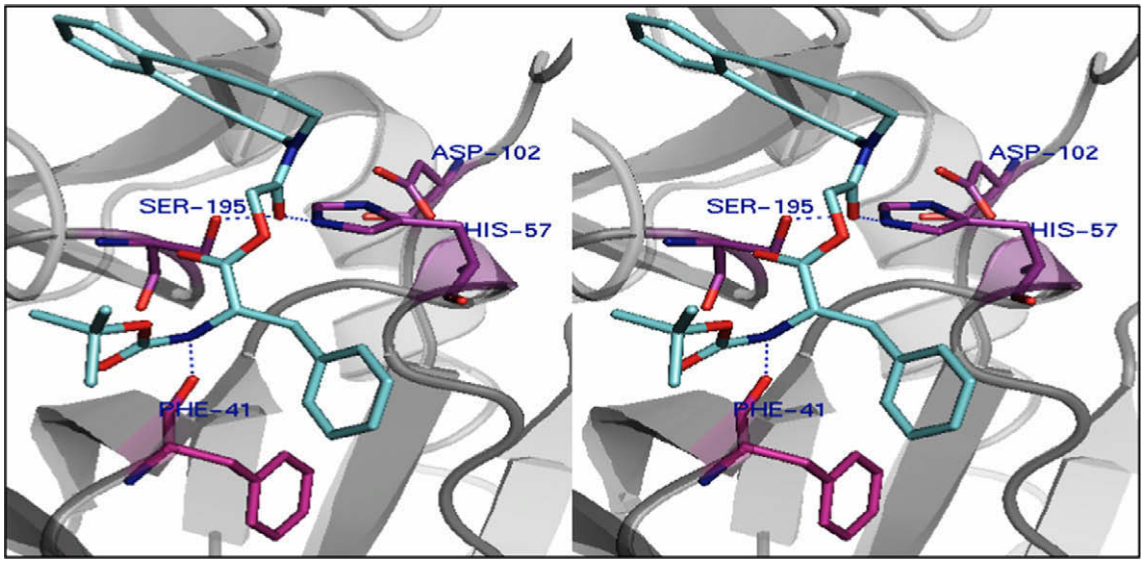


Figure 5. Docking pose of compound **3** with amino acid residues of α-chymotrypsin within hydrogen bonding distance.

reactive enediynes to inhibit proteases connected with pathogenesis of various diseases like AIDS.

4. Experimental

4.1. Characterization

All the new compounds were fully characterized by NMR (taken in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C) and mass spectral data, which are provided below along with the experimental details:

*Compound **1***: The compound **10** (0.0967 mmol) was dissolved in dry CH_2Cl_2 (12 ml) and Dess Martin reagent (3 equiv) was added to it. The reaction mixture was stirred for 5 h. The oxidant was quenched with sodium thiosulfate and the organic layer was washed with saturated sodium bicarbonate solution. It was dried over sodium sulfate, filtered and concentrated under vacuum. The final compound **1** was isolated as pure white solid by column chromatography using 45% ethyl acetate in hexane as eluent; Yield: 81%, mp 105 °C–110 °C; δ_H 9.35 (1H, s, C*H*O), 7.51–7.06 (9H, m, Ar-*H*), 6.80 (1H, br s, N*H*), 6.61 (1H, br s, N*H*), 4.81 (1H, br s, N*H*), 4.59 (1H, m, COC*H*CH_2Ph), 3.98 (1H, m, COC*H*CH_3), 3.44–3.27 (2H, ABX, J_{AX} = 6.4, J_{BX} = 6, J_{AB} = 12.8, COCHC*H*$_2$Ph), 3.02 (2H, m, CH_2CH_2NH), 2.55 (2H, t, J = 6.0 Hz, CH_2CH_2NH), 1.35 [9H, s, NHCOOC(CH_3)$_3$], 1.3–1.14 (3H, m, CH_3CHNHBoc); δ_C 177.5, 172.1, 170.9, 155.6, 136.4, 133.5, 132.2, 132.1, 131.1, 129.3, 128.6, 127.8, 126.9, 121.6, 94.1, 93.8, 91.2, 79.4, 76.8, 53.9, 50.6, 38.6, 28.3, 23.8, 20.3, 18.1; Mass (ESI+) m/z 516.359 (MH^+), 538.348 (MNa^+); HRMS calculated for $C_{30}H_{33}N_3O_5 + H^+$: 516.2500, found: 516.2503.

*Compound **2***: To a solution of Boc-L-phe in dry CH_2Cl_2, EDC (1 equiv) and HOBT (1 equiv) were added and the mixture was stirred for 1 h at 0 °C. Then the cyclic amine **12** (0.0819 mmol) dissolved in CH_2Cl_2 (2 ml) was added dropwise followed by DIPEA (2 equiv). The reaction mixture was stirred overnight at room temperature. After partitioning between CH_2Cl_2 and water, the organic layer was washed with sodium bicarbonate, then with dilute hydrochloric acid, brine, dried over sodium sulfate and evaporated to get an oily residue. The compound was isolated pure by column chromatography using 20% ethyl acetate in hexane system. It was further purified by hplc (C-18 reverse phase column, mobile phase: methanol/water 95:5) to get pure white solid; Yield: 56%, mp 80–85 °C, δ_H: 7.32–7.06 (9H, m, Ar-*H*), 5.3 (1H, d, J = 8.4 Hz N*H*), 4.87 (1H, dd, J = 6.8, 14.4 Hz, COC*H*CH_2Ph), 4.46 (1H, d, J = 18.8 Hz, CC*H*HN), 4.1 (1H, d, J = 18.8 Hz, CCH*H*N), 4.0 (1H, d, J = 13.6 Hz, CH_2CHHN), 3.43 (1H, t, J = 10.8 Hz, CH_2CH*H*N), 3.03–2.92 (3H, m, PhCH_2CH, C*H*HCH_2N), 2.64 (1H, m, CH*H*CH_2N), 1.39 [9H, s, NHCOOC(CH_3)$_3$]; δ_C: 171.5, 155.1, 135.9, 129.7, 129.4, 129.2, 128.5, 128.2, 127.8, 127.3, 127.1, 126.7, 98.3, 92.8, 87.8, 82.7, 79.8, 51.9, 51.2, 41.7, 39.1, 28.2, 18.2; Mass (ESI+) m/z 429.30 (MH^+), 451.28 (MNa^+), HRMS calculated for $C_{27}H_{28}N_2O_3Na$: 451.1997, found: 451.1998.

*Compound **3***: The compound **13** (0.0986 mmol) was dissolved in dry DMF (8 ml) followed by the addition of sodium salt of Boc-L-Phe (1 equiv) and catalytic amount of sodium iodide. The reaction mixture was stirred overnight at room temperature. The organic layer was extracted with ethyl acetate, dried over sodium sulfate and concentrated to get brown oil. The compound **3** was isolated as pure white solid by column chromatography (eluent: 35% ethyl acetate in hexane). Yield: 65%, mp 85–90 °C, δ_H: 7.36–7.17 (9H, m, Ar-*H*), 4.95–4.62 (4H, m, N*H*, COCH_2O, PhCH_2C*H*), 4.21 (2H, s, $CH_2NCH_2CH_2$), 3.87–3.75 (2H, m, $CH_2CH_2NCH_2$), 3.31–3.02 (2H, ABX, J_{AX} = 5.2 Hz, J_{BX} = 7.6, J_{AB} = 14.0 Hz, PhCH_2CH), 2.93 (2H, m, $CH_2CH_2NCH_2$), 1.37 [9H, s, NHCOOC(CH_3)$_3$], δ_C: 171.5, 165.9, 155.0, 136.1, 129.8, 129.3, 128.4, 128.2, 127.8, 127.5, 127.4, 127.3, 126.9, 98.0, 92.3, 88.1, 82.5, 79.9, 61.7, 54.3, 51.3, 40.9, 37.9, 26.6, 18.2; Mass (ESI+) m/z 487.34 (MH^+), 509.32 (MNa^+); HRMS calculated for $C_{29}H_{30}N_2O_5 + H^+$: 487.2234, found: 487.2238.

*Compound **4***: The compound **15** (0.1004 mmol) was dissolved in dry DMF (8 ml) followed by the addition of sodium salt of Boc-L-Phe (1 equiv) and catalytic amount of sodium iodide. The reaction mixture was stirred for 6 h at 20 °C. The organic layer was extracted with ethyl acetate, dried over sodium sulfate and concentrated to get brown oil. The target compound was isolated as pale yellow semi solid by column chromatography using 35% ethyl acetate in hexane as eluent. Yield: 60%, δ_H 7.31–7.19 (5H, m, Ar-*H*), 5.93–5.88 (2H, m, CC*H*C*H*C), 5.1–4.69 (4H, m, N*H*, COCH_2, PhCH_2C*H*), 4.14 (2H, s, $CH_2NCH_2CH_2$), 3.74 (2H, m, $CH_2CH_2NCH_2$), 3.28–3.08 (2H, m, PhCH_2CH), 2.89 (2H, m, $CH_2CH_2NCH_2$), 1.3 [9H, s, NHCOOC(CH_3)$_3$]; Mass (ESI+) m/z 437.3038 (MH^+), 459.28 (MNa^+), HRMS (MNa^+) calculated for $C_{25}H_{28}N_2O_5Na$: 459.1886, found: 459.1874.

*Compound **5***: The compound **17** (1.69 mmol) was dissolved in dry DMF (10 ml) followed by the addition of sodium salt of Boc-L-Phe (1 equiv) and catalytic amount of sodium iodide. The reaction

mixture was stirred overnight at room temperature. The organic layer was extracted with ethyl acetate, dried over sodium sulfate and concentrated to get brown oil. The compound **5** was isolated as pure white crystal by column chromatography using 30% ethyl acetate in hexane as eluent; Yield: 88%, mp 115–120 °C, δ_H: 7.31–7.21 (5H, m, Ar-*H*), 4.98 [1H, m, N*H*COOC(CH_3)$_3$], 4.86 (1H, d, *J* = 14.4 Hz, COC*H*HO), 4.71 (1H, d, *J* = 14.4 Hz, COCH*H*O), 4.67 (1H, m, PhCH_2C*H*), 3.55 (2H, m, CH_2CH_2NCH_2), 3.31–3.05 (4H, m, CH_2CH_2NCH_2, PhCH_2CH), 1.66–1.58 (4H, m, NCH_2CH_2CH_2CH_2), 1.38 [11H, s, NHCOOC(CH_3)$_3$, NCH_2CH_2CH_2CH_2]. δ_C: 171.7, 164.0, 155.1, 136.2, 129.4, 128.4, 128.3, 126.7, 79.7, 61.8, 54.3, 45.5, 43.0, 38.1, 28.2, 26.2, 25.2, 24.3, Mass (ESI+) *m/z* 391.225 (MH^+), 413.22 (MNa^+); HRMS calculated for $C_{21}H_{30}N_2O_5 + H^+$: 391.2234, found: 391.2237.

*Compound **6**:* To a solution of Boc-L-phe in dry CH_2Cl_2, EDC (1 equiv) and HOBT (1 equiv) were added and the mixture was stirred for 1 h at 0 °C. Then the tetrahydroisoquinoline **18** (0.78 mmol) dissolved in CH_2Cl_2 (2 ml) was added dropwise followed by DIPEA (2 equiv). The reaction mixture was stirred overnight at room temperature. After partitioning between CH_2Cl_2 and water, the organic layer was washed with sodium bicarbonate, then with dilute hydrochloric acid, brine, dried over sodium sulfate and evaporated to get an oily residue. The compound was isolated pure by column chromatography using 10% ethyl acetate in hexane system. Yield: 88%, δ_H 7.19–6.83 (9H, m, Ar-*H*), 5.53 [1H, m, N*H*COOC(CH_3)$_3$], 4.92 (1H, m, C*H*CH$_2$Ph), 4.76–3.94 (2H, m, CH_2CH_2NCH_2), 3.83–3.11 (2H, m, CH_2CH_2N), 3.05–2.93 (2H, m, PhCH_2CH), 2.79–2.31 (2H, m, CH_2CH_2N), 1.39 [9H, s, NHCOOC(CH_3)$_3$]; δ_C 170.7, 170.4, 155.1, 155.0, 136.5, 136.2, 134.5, 134.0, 132.1, 129.5, 129.3, 128.6, 128.5, 128.3, 128.2, 126.9, 126.8, 126.7, 126.6, 126.5, 126.3, 126.1, 79.7, 76.8, 51.8, 51.6, 47.0, 44.5, 43.0, 40.5, 40.4, 40.1, 31.6, 29.1, 28.4, 28.3; Mass (ESI+) *m/z* 381.21 (MH^+), 403.217 (MNa^+); HRMS calculated for $C_{23}H_{28}N_2O_3 + H^+$: 381.2180, found: 381.2182.

*Compound **7**:* To the solution of the mesylate in dry DMF, NaN_3 (5.49 mmol) was added and stirred for overnight at room temperature. The mixture was then partitioned between EtOAc and water. The organic layer was thoroughly washed with water, dried, filtered and then evaporated. The compound was isolated pure by column chromatography using 10% ethyl acetate in hexane system as pale brown oil. Yield: 88%, δ_H (200 MHz, $CDCl_3$): 7.41–7.32 (2H, m, Ar-*H*), 7.25–7.17 (2H, m, Ar-*H*), 4.95 [1H, t, *J* = 3.1 Hz, OC*H* (pyran)], 4.49 (2H, s, CH_2OTHP), 3.88–3.78 [2H, m, OCH_2 (pyran)], 3.57–3.44 [4H, m, CH_2CH (pyran), CH_2N$_3$], 2.72 (2H, t, *J* = 6.9 Hz, CH_2CH$_2$N$_3$), 1.85–1.51 [4H, m, CH_2CH$_2$ (pyran)]; Mass (ESI+) *m/z* 310 (MH^+); HRMS calculated for $C_{18}H_{19}N_3O_2 + H^+$: 310.1557, found: 310.1560.

*Compound **8**:* The amine **8** was prepared from the corresponding azide**7** via reaction with PPh_3/H_2O. To the solution of azide (0.42 mmol) in THF (15 ml), 1.5 equiv of PPh_3 and water were added and stirred at room temperature for 24 h. The THF was removed by high vacuum pump and the product was obtained by column chromatography 10% methanol in dichloromethane as eluent. Yield: 91%, δ_H (200 MHz, $CDCl_3$): 7.45–7.37 (2H, m, Ar-*H*), 7.25–7.20 (2H, m, Ar-*H*), 4.94 (1H, t, *J* = 3.1 Hz, OC*H* (pyran)), 4.52 (2H, d, *J* = 2.3 Hz, CH_2OTHP), 3.89–3.80 [2H, m, OCH_2 (pyran)], 3.59–3.53 [2H, m, CH_2CH (pyran)], 3.00 (2H, t, *J* = 6.1 Hz, CH_2NH$_2$), 2.67 (2H, t, *J* = 6.1 Hz, CH_2CH$_2$NH$_2$), 1.82–1.56 [4H, m, CH_2CH_2 (pyran)]; Mass (ESI+) *m/z* 284.16 (MH^+), 306.16 (MNa^+).

*Compound **9***: To a solution of Boc-L-ala·L-phe in dry CH_2Cl_2, EDC (1 equiv) and HOBT (1 equiv) were added and the mixture was stirred for 1 h at 0 °C. Then the amine **8** (0.314 mmol) dissolved in CH_2Cl_2 (1 ml) was added dropwise followed by DIPEA (2 equiv). The reaction mixture was stirred overnight at room temperature. After partitioning between CH_2Cl_2 and water, the organic layer was washed with sodium bicarbonate, then with dilute hydrochloric acid, brine, dried over sodium sulfate and evaporated to get an oily residue. From the oily residue the compound was isolated pure by column chromatography using 25% ethyl acetate in hexane as eluent. Yield: 53%, δ_H 7.36–7.09 (9H, m, Ar-*H*), 6.9 (1H, br s, N*H*), 6.55 (1H, br s, N*H*), 4.87 [1H, m, OC*H* (pyran)], 4.8 (1H, br s, N*H*), 4.57–4.41 [3H, m, CCCH_2O (pyran), NHC*H*CH$_2$Ph], 3.99 (1H, m, CH$_3$ C*H*NHBoc), 3.79–3.77 (1H, m, OC*H*HCH$_2$), 3.50–3.42 (2H, m, NHCHCH_2Ph), 3.4 (1H, m, OC*H*HCH$_2$), 3.02–2.99 (2H, m, CH$_2$CH_2 NHCO), 2.56–2.49 (2H, m, CH_2CH$_2$NHCO), 1.56–1.43 [6H, m, CHCH_2CH_2CH_2CH$_2$O (pyran)], 1.32 [9H, s, NHCOOC(CH_3)$_3$], 1.20–1.14 (3H, m, CH_3CHNHBoc); Mass (ESI+) *m/z* 602.32 (MH^+), 624.32 (MNa^+); HRMS calcd for $C_{35}H_{43}N_3O_6 + H^+$ 602.3232, found: 602.3233.

*Compound **10***: To the compound **9** (0.169 mmol) dissolved in dry ethanol (10 ml) PPTS was added and the reaction was stirred for 24 h at 40 °C. The resulting mixture was concentrated in vacuum and the product was obtained pure as white semi solid by column chromatography using 50% ethyl acetate in hexane as eluent. Yield: 90%, δ_H 7.34–7.09 (9H, m, Ar-*H*), 6.9 (1H, br s, N*H*), 6.6 (1H, br s, N*H*), 4.85 (1H, br s, N*H*), 4.7 (1H, m, NHC*H*CH$_2$Ph), 4.57–4.5 (2H, m, CCCH_2OH), 4.109 (1H, m, CH$_3$C*H*NHBoc), 3.6–3.35 (2H, m, NHCHCH_2Ph), 3.1–3.07 (2H, m, CH$_2$CH_2NHCO), 2.57–2.55 (2H, m, CH_2CH$_2$NHCO), 1.4 [9H, s, NHCOOC(CH_3)$_3$], 1.3–1.14 (3H, m, CH_3CHNHBoc); Mass (ESI+) *m/z* 518.33 (MH^+); HRMS calcd for $C_{30}H_{35}N_3O_5 + H^+$: 518.2657, found: 518.2661.

*Compound **11***: The mesylate dissolved in dry DMF was treated with K_2CO_3 (1.45 mmol) and the mixture was stirred for 3 h at room temperature. It was then partitioned between EtOAc and water. The organic layer was further washed with water, dried and evaporated. From this residue, the title compound was isolated by column chromatography as pure white solid using 25% ethyl acetate in hexane as eluent. Yield: 95%, mp:230–240 °C δ_H 8.25 (2H, d, *J* = 9.2 Hz, Ar-*H*), 8.11 (2H, d, *J* = 8.8 Hz, Ar-*H*), 7.29–7.26 (4H, m, Ar-*H*), 4.34 (2H, s, NCH_2CC), 3.75 (2H, t, *J* = 5.2 Hz, CH$_2$CH_2NSO$_2$Ar), 2.78 (2H, t, *J* = 5.2 Hz, CH_2CH$_2$NSO$_2$Ar); Mass (ESI+) *m/z* 367.07 (MH^+); HRMS calcd for $C_{19}H_{14}N_2O_4S + H^+$:367.0754, found: 367.0757.

*Compound **12***: The compound **11** (0.152 mmol) was dissolved in dry DMF (4 ml) followed by dry potassium carbonate (3 equiv) and thiophenol (1.2 equiv). The reaction mixture was stirred for 30 min at room temperature. The organic layer was extracted with ethyl acetate and concentrated in vacuum. The cyclic enediyne amine **12** was obtained pure by column chromatography as brown oil using 10% methanol in dichloromethane as eluent. Yield: 70%, δ_H 7.24–7.20 (2H, m, Ar-*H*), 7.15–7.12 (2H, m, Ar-*H*), 3.78 (N*H*), 3.58 (2H, s, CH_2N), 3.18 (2H, t, *J* = 5.4 Hz, CH$_2$CH_2N), 2.48 (2H, t, *J* = 5.4 Hz, CH_2CH$_2$N); Mass (ESI+) *m/z* 183 (MH^+).

*Compound **13***: To a solution of compound **12** (0.1365 mmol) in CH_2Cl_2 at 0 °C, Et_3N (1.1 equiv) was added followed by slow addition of bromo acetylchloride (1.0 equiv). The reaction was allowed to stir under N_2 atmosphere for 15 min. Then it was partitioned between CH_2Cl_2 and water, extracted twice with CH_2Cl_2, washed with brine and dried over Na_2SO_4. The compound was isolated pure by column chromatography using 25% ethyl acetate in hexane as eluent. Yield: 80%, δ_H (400 MHz, $CDCl_3$): 7.35–7.31 (2H, m, Ar-*H*), 7.27–7.24 (2H, m, Ar-*H*), 4.39 (2H, s, CH_2Br), 3.94 (2H, s, NCH_2), 3.77 (2H, t, *J* = 5.4 Hz, NCH_2CH$_2$), 2.94 (2H, t, *J* = 5.4 Hz, NCH$_2$CH_2); Mass (ESI+) *m/z* 378 (MH^+).

*Compound **14***: The cyclic sulfonamide (0.126 mmol) was dissolved in dry DMF (4 ml) followed by dry potassium carbonate (3 equiv) and thiophenol (1.2 equiv). The reaction mixture was stirred for 30 min at 20 °C. The organic layer was extracted with ethyl acetate and concentrated in vacuum. The cyclic enediyne amine 15 was obtained pure by column chromatography as brown oil 10% methanol in dichloromethane as eluent. Yield: 89%, δ_H 5.90 (1H, d, *J* = 8.8 Hz, C*H*CH), 5.83 (1H, d, *J* = 8.8 Hz, CCHC*H*C), 3.78

(N*H*), 3.58 (2H, s, CCCH_2N), 3.18 (2H, t, J = 5.4 Hz, CH_2CH_2N), 2.48 (2H, t, J = 5.4 Hz, CH_2CH_2N); Mass (ESI+) m/z 132 (MH^+).

Compound ***15***: To a solution of compound **14** (0.0769 mmol) in CH_2Cl_2 at 0 °C, Et_3N (1.1 equiv) was added followed by slow addition of bromo acetylchloride (1.0 equiv). The reaction was allowed to stir under N_2 atmosphere for 15 min. Then it was partitioned between CH_2Cl_2 and water, extracted twice with CH_2Cl_2, washed with brine and dried over Na_2SO_4. The compound was isolated pure by column chromatography using 25% ethyl acetate in hexane as eluent. Yield: 80%, δ_H: 5.90 (1H, d, J = 8.8 Hz, CC*H*CHC), 5.83 (1H, d, J = 8.8 Hz, CCHC*H*C), 4.33 (2H, s, CH_2Br), 3.90 (2H, s, NCH_2CC), 3.73 (2H, t, J = 5.2 Hz, NCH_2CH_2), 2.89 (2H, t, J = 5.2 Hz, NCH_2CH_2); Mass (ESI+) m/z 253 (MH^+).

4.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The inhibition of chymotrypsin was ascertained qualitatively by the extent of proteolysis of BSA in presence and absence of the synthesized compounds in SDS–PAGE (15% resolving gel). BSA (1 mg/ml) and chymotrypsin stock (1 mg/ml) were prepared in 0.01 M phosphate buffer (pH 7.6). The proteolysis reaction mixture contained 1 ml BSA, 60 μl chymotrypsin and 15 μl inhibitor (1 mg/mlMeOH) and each reaction was monitored for 1hour. 100 μl PMSF (0.03 M stock solution prepared in methanol) was used to quench the reaction at specific time intervals. Gels were stained in Coomassie Brilliant Blue for all the compounds.

4.3. Protease digestion

The protease digestion was carried out by mixing 500 μl of BSA stock (1 mg/ml) in 0.01 M phosphate buffer with 60 μl of chymotrypsin solution (1 mg/ml) in the same buffer. The enzymatic reaction was carried out at 38 °C for a given time period. 500 μl of 4% trichloroacetic acid was used to precipitate out the excess BSA, which was removed by centrifugation. The same protocol was followed with the enzyme preincubated with 10 μl of inhibitor solution at three different concentrations. The hydrolysed products in the supernatants were estimated according to the method of Lowry et al. (1956).[17]

4.4. Inhibition kinetics

The inhibition kinetics of chymotrypsin was studied by UV–vis spectroscopy using *N*-Benzoyl tyrosine ethyl ester as the substrate. The assay was performed in 80 mM Tris HCl buffer (pH 7.8) containing 3 M $CaCl_2$.The substrate concentration was maintained between169 μM and 340 μM whereas the inhibitor concentration was varied from 6.4 μM to 12 μM for compound **1**, 4–8 μM for compound **2** and 10–42 μM for compound **3**. The inhibition constants were determined from initial velocity data. The Lineweaver–Burk plots[18] were obtained from the following equation for all the compounds:

$$\frac{1}{v} = \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

where v is the initial velocity, [S] is the substrate concentration, [I] is the inhibitor concentration, K_m the Michaelis constant and K_i the inhibitor constant, V_{max} is maximum velocity.

4.5. Docking studies

The crystal structure of α-chymotrypsin (PDB entry 1yph) was downloaded from the Protein Data Bank.[14] We have chosen 1yph for the docking studies since this is the structure of the wild type bovine pancreatic α-chymotrypsin. This should be able to appropriately complement our experimental results that have been conducted with the same protein. The 3D structures of the compound was generated by SYBYL6.92 (Tripos, St. Louis) and their energy minimized conformations were obtained with the help of the TRIPOS force field using Gasteiger–Hückel charges with a gradient of 0.005 kcal/mol. The FlexX software as part of the SYBYL suite was used for docking of the compound to α-chymotrypsin. The ranking of the generated solutions is performed using a scoring function that estimates the free binding energy ΔG of the protein–ligand complex. PYMOL[19] was used for visualization of the docked conformations.

Acknowledgements

A. B. thanks DST for a research Grant. SD is grateful to IIT, Kharagpur for a fellowship. DST is thanked for providing funds for 400 MHz NMR facility under the IRPHA program.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.04.019.

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