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Design, synthesis and inhibition activity of a novel cyclic enediyne amino acid conjugates against MPtpA

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ABSTRACT

In course of studies towards the discovery of selective inhibitors of MPtpA, a novel cyclic endiyne malonamic acid has been designed and synthesized. The synthesis involves a crucial intramolecular Knoevenagel reaction. The compound displayed a reversible non-competitive inhibition against MPtpA with inhibition constant K_i of 22.5 μM. The enediyne acts as a recognition framework in inducing the inhibition and not as a reactive functional moiety. This was confirmed by comparing the inhibiting activity with that of the corresponding saturated cyclic non-enediyne analogue.

1. Introduction

The enediynes[1] comprise an important class of powerful antitumor agent with unprecedented biological profiles. This is due to their unique molecular architecture and precise mode of interaction with biomolecules. Nearly twenty different enediynes have been tested as potential clinical agent till date. The effort ultimately resulted in the development of the commercial drug Mylotarg[2] for treatment of leukemia. The enediynes themselves have no biological significance until triggered to form cytotoxic diradical (a process known as Bergman cyclization, BC[3]) capable of cleaving DNA backbone at low concentration. Thus the modulation of BC through activation[4] of enediyne moiety becomes an important aspect of the research of enediynes in recent years. However, only few studies[5] have been made where the enediyne skeleton has been utilized as a template for recognition by biomacromolecules. The enediynes, especially the cyclic ones, because of the existence of alkyne moieties, offer special advantage over the corresponding saturated analogues with regard to their interaction with biomolecules like proteins. The conformational constraint coupled with π-electron cloud surrounding the six atoms of enediynes may facilitate binding through stacking interactions with side chains of aromatic amino acids present in the active site of the enzyme. With this in mind, we have undertaken a study of interaction of a cyclic enediyne with the protein MPtpA,[6] a key phosphatase enzyme present in *Mycobacterium tuberculosis*. The genome sequence analysis[7] of *M. tuberculosis* has revealed the presence of two protein tyrosine phosphatases MPtpA and MPtpB[8] both of which are believed to be linked to the pathogenicity of the organism[9]. Therefore the design and application of new molecular framework that acts as a selective inhibitory agent of MPtpA or MPtpB has become an important research topic in medicinal chemistry. Recently, we have reported[10] the moderate inhibition of MPtpA by a new class of cyclic peptides. This prompts us to design and synthesis of a novel cyclic enediyne malonamic acid (CEMA) (**1**) and to evaluate the inhibitory activity against MPtpA. To substantiate the role of enediyne framework, we have also synthesized a cyclic non endiyne malonamic acid (CNEMA) (**2**) for comparison shown in Figure 1.

Due to availability of multiple binding modes in the binding sites, the enediyne malonamic acid (CEMA) may be a good choice which is capable to deliver a number of aspects such as solubility,

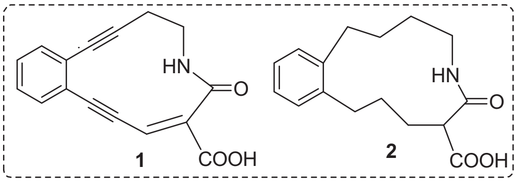

Figure 1. Synthesized cyclic enediyne and non enediyne amino acid conjugates.

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pH, conformational rigidity, recognizable complementary functionality, etc. The probable interaction comprising of multiple binding modes for structure based inhibitor (CEMA) is proposed in Figure 2.

2. Results and discussion

2.1. Synthesis

The synthesis began with Pd-mediated sequential Sonogashira coupling[11] of dibromobenzene with THP-protected propargyl alcohol and homopropargyl alcohol that afforded the alcohol **5**. The latter was converted to amine **8** using standard protocol (Scheme 1). The amine was then acylated with ethylmalonyl chloride, followed by the deprotection of the THP group (PPTS, EtOH)[12] and subsequently oxidized to the primary alcohol by Dess–Martin periodinane.[13] Finally, the crucial intramolecular cyclization was performed in various solvents by varying the reaction condition shown in Table 1. No such intermolecular Knovenegal condensation product was formed; rather the elimination product **12** was obtained as the sole product, confirmed by NMR and Mass spectrometric analysis. Table 1 clearly shows that the cyclization becomes more facile in terms of yield and reaction time when a buffer medium was introduced in THF solvent at high dilution condition. Sole use of base could not complete the elimination; rather a mixture of β-hydroxy ester and the elimination product were obtained. Addition of catalytic amount of acid could complete the elimination process. The structure of elimination product was further confirmed by carrying out Micheal addition reaction with thiophenol and diethyl malonate (Scheme 2). Ester hydrolysis using controlled amount of LiOH in aqueous THF resulted in the formation of a novel 11-membered cyclic enedinyl malonamic acid (CEMA) **1**. The corresponding cyclic non-enedinyl analogue (CNEMA) **2** was derived from compound **1** by catalytic hydrogenation using 10% Pd-charcoal. CEMA was isolated as the only product while CNEMA was a racemic mixture. All the compounds were characterized by NMR, Mass and HPLC technique.

2.2. Study of thermal reactivity by DSC

It has been demonstrated earlier that enediynes with ring size greater than ten does not undergo BC under ambient conditions.[14] Their onset temperature for Bergman Cyclization (BC) is quite high depending upon the ring size. However, in order to know whether endocyclic double bond and amide resonance play any role in lowering the BC temperature, we performed solid phase thermal reaction kinetics by Differential Scanning Calorimetry (DSC).[15] The onset temperature of BC of CEMA was found to be 248 °C (Fig. 3), similar to enediynes of same ring size thus ruling out the involvement of any diradical formation at biological temperature and subsequent inhibition during its interaction with the enzyme. The enediyne thus can provide only a hydrophobic framework around the cyclic amide for better interaction with the enzyme.

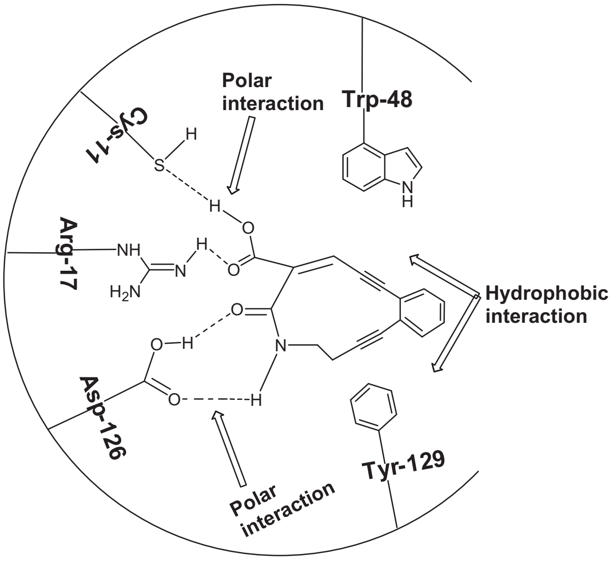


Figure 2. Possible interaction of 1 with the active site residue of MPtpA.

2.3. Inhibition study

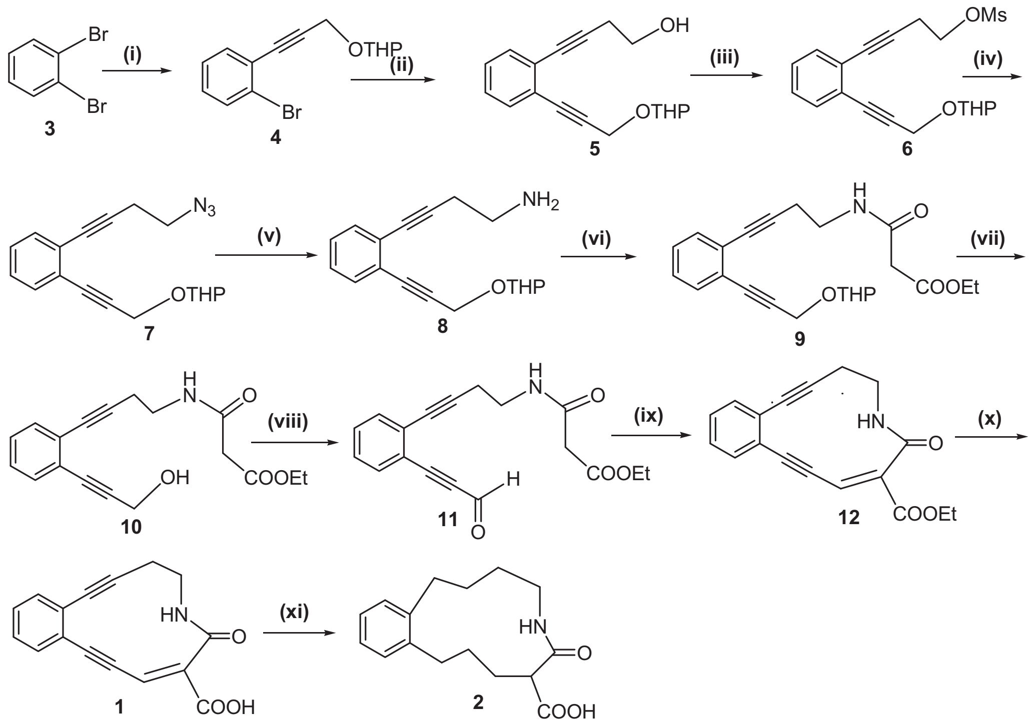
Recombinant MPtpA was purified as described previously.[16] Enzyme purity was assessed by SDS–PAGE and concentration of protein was determined from the absorbance at 280 nm. Buffer containing with 0.1 M Tris–HCl, pH 7.5 with ionic strength 0.15 M of NaCl and 1 mM EDTA was prepared for the kinetic study. All the inhibition studies were performed using *p*-nitrophenyl phosphate (pNPP) as a substrate with a fixed concentration of protein (25 nM). The release of *p*-nitrophenol at 405 nm was quantified both in case of control and in presence of the inhibitors. The effective inhibitor and substrate concentration were varied from 3–7 μm and 10–30 μm for CEMA and 10–15 μm and 10–30 μm for CNEMA, respectively. Each assay was repeated in triplicate.

2.4. Inhibition kinetics

Both the values of the kinetic parameters and inhibitor constants were obtained from the Lineweaver Burk plot (Fig. 4). For different concentration of same inhibitor, double reciprocal plots were taken into account to conclude the mode of inhibition. Inhibition constant (K_i) was determined from the plot of $\left(\frac{K_m}{V_{max}}\right)$ versus $[I]$ where $[I]$ is the inhibitor concentration, K_m is the Michaelis–Menten constant and V_{max} is the maximum reaction velocity. Both CEMA and CNEMA were found to behave as reversible non-competitive inhibitors (Fig. 4) with K_i values in micromolar range (Table 2). In a previous report[10] we have shown that the increase in hydrophobicity in the cyclic peptides reduces the effective interaction with MPtpA. Here also, CNEMA, being more hydrophobic as compared to CEMA, exhibits weak interaction with MPtpA as reflected in the K_i values and IC_{50} values (Table 2).

2.5. Docking study

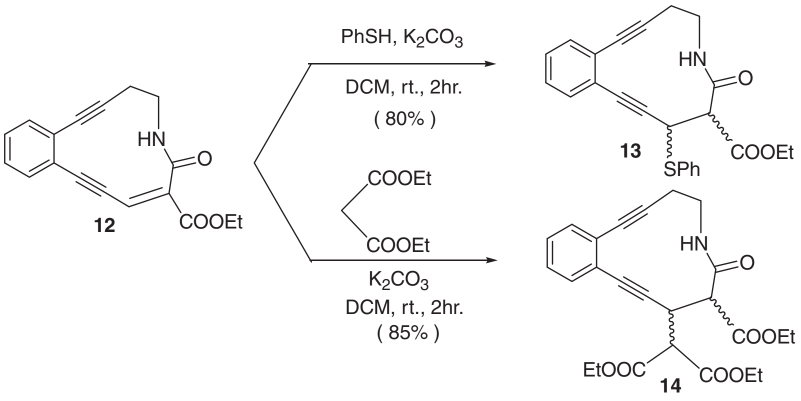
Docking study was performed using Autodock 4.2[17] to throw light on the probable interactions of CEMA with MPtpA. Coordinates of CEMA and CNEMA were generated using PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg/). The surface area of active site groove occupied by CEMA was calculated using PISA server.[18] The docking clearly reflects that the inhibitor binds effectively in the vicinity of the PTP loop by virtue of multiple hydrophobic interactions. CEMA consumes 318.10 $Å^2$ of the active site groove where mostly hydrophobic residues play an important role in determining the probable mode of binding. The PTP loop in MPtpA acts as the key mediator for its flexible accessibility of the substrate to the active site and directs the catalytic mechanism. However, the aromatic ring of CEMA disposes sufficiently close enough to the PTP loop, thereby restricting the flexible movement of the loop. Gly13 and Ile15 are two PTP loop residues involved in strong hydrophobic interactions with aromatic ring of CEMA. Tyr128 is also located within the hydrophobic interacting distance with CEMA whereas Tyr129 interacts with carbonyl group of the enediyne ring via hydrogen bonding interaction. Although Trp48 is involved in H-bonding interaction with CEMA it may indulge



Scheme 1. Synthesis of cyclic enediyne and non-endiyne amino acid conjugates. Reagents and conditions: (i) propargyl-OTHP, Pd$(PPh_3)_4$, *n*-BuNH_2, 85 °C, reflux, 3 h, 66%; (ii) 3-butyne-1-ol,Pd$(PPh_3)_4$, *n*-BuNH_2, 85 °C, reflux, 10 h, 58%; (iii) MsCl, Et_3N, 0 °C, 10 min, 95%; (iv) NaN_3, dry DMF, rt, 3 h, 89%; (v) PPh_3, moist THF, rt, overnight, 78%; (vi) ethylmalonyl chloride, dry DCM, Et_3N, 0 °C, 15 min, 83%; (vii) PPTS, EtOH, 50 °C, 3 h, 68%; (viii) Dess–Martin periodinane, dry DCM, rt, 2 h, 98%; (ix) see Table 1, 65–91%; (x) LiOH, moist THF, rt, 1 h, 81%; (xi) H_2, 10% Pd-charcoal, dry EtOH, rt, 93%.

Table 1
Reaction parameter of intramolecular Knoevenegal reaction

Solvents	Additives	Concn (M)	Reaction time(h)	% of Yield
THF	0.1(M)Et_3N	4.1×10^{-3}	18	65
THF	0.1(M)Et_3N then .04(M)AcOH	3.8×10^{-3}	2	91
DCM	0.08(M)Et_3N	4.1×10^{-3}	22	55
DCM	0.1(M)Et_3N then .04(M)AcOH	3.8×10^{-3}	4	84
CH_3CN	0.1(M)Et_3N	4.1×10^{-3}	25	59
CH_3CN	0.1(M)Et_3N then .04(M)AcOH	3.8×10^{-3}	5	85
THF	0.1(M)Et_3N then .04(M)HCl	3.8×10^{-3}	3	72
THF	0.1(M)HCl	4.1×10^{-3}	No reaction	



Scheme 2. Michael addition into thiophenol and diethyl malonate.

the molecule to bind via hydrophobic interaction. His49 is also found to exist within the hydrogen bonding distance from CEMA (Fig. 5). No direct interactions between the active site residues (Cys11, Arg17 and Asp126) and CEMA is observed indicating the additional support in favor of non-competitive mode of interaction. It seems that CEMA probably inhibits the reaction by creating a partition between catalytic residues Cys11 and Asp126. The above observations categorically explain the results of inhibition kinetics. Observed interactions between CEMA and MPtpA are shown in Table 3.

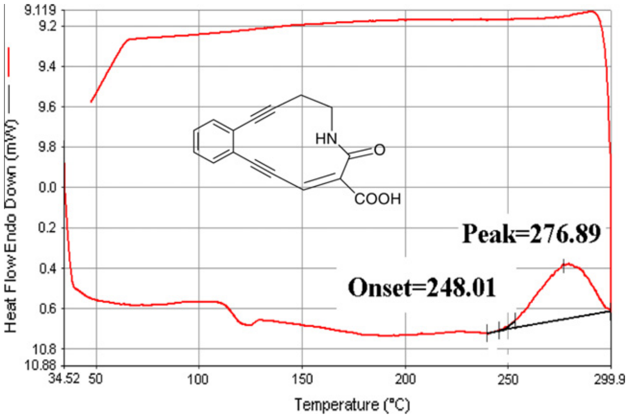


Figure 3. DSC plot of CEMA.

Comparative docking analysis also reveals that unlike CNEMA, CEMA has greater impact on MPtpA mainly because of its conformational preferences controlled by enediyne framework. Actually, the loop undergoes facile conformational change upon CEMA binding that allows associated amino acid residues to form an extensive hydrophobic interaction. Thus, the size, shape and selectivity of the CEMA towards MPtpA have given a new dimension to utilize the enediyne framework for the discovery of potent drug molecules.

3. Conclusion

A novel enediyne malonamic acid conjugates has been identified to be a potential candidate for MPtpA inhibitor and possible protein modulator. The enediyne behaves as a non-competitive inhibitor. Since, the non-competitive type inhibition belongs to reversible inhibition, so the possibility of diradical induced irreversible inhibition has been excluded which was also supported by the very high onset temperature for BC. Thus, a new role of enediyne as an enzyme inhibitor has been comprehensively established after comparison with saturated non enediyne based analogue. Further studies are on the way to bring down the inhibition to nanomolar range as well as deactivation the MPtpA's function by suitable modification in the structure of enediyne framework.

4. Experimental

4.1. General experimental methods

Table 2
Inhibition kinetics parameter of CEMA and CNEMA

Compound	Mode of inhibition	IC_{50} (μM)	K_i (μM)
1	Non-competitive	110.2 ± 10.5	22.5 ± 1.2
2	Non-competitive	235.6 ± 12.4	101.4 ± 10.8

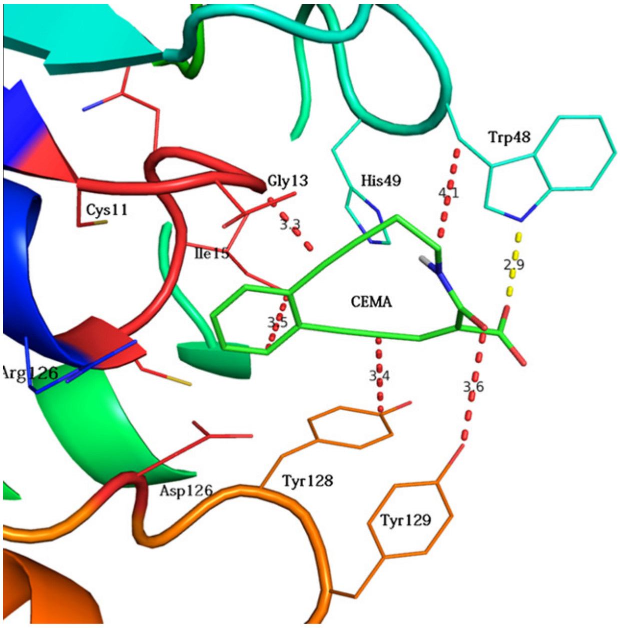


Figure 5. Docking of CEMA on MPtpA is showing the major interactions: PTP loop is shown in red color.

All reactions were conducted with oven-dried glassware under an atmosphere of argon (Ar). Commercial grade reagents were used without further purification. Solvents were dried and distilled following usual protocols. Purification by Column chromatographic separations were performed using 60–120, 100–200 and 230–400 mesh size silica gel. Further purification was carried out with the help of HPLC. TLC was performed on aluminium-backed plates coated with Silica Gel 60 with F254 indicator. The ^1H NMR spectra were measured on 400 MHz and ^{13}C NMR spectra were measured with 100 MHz using $CDCl_3$ as a solvent in Brucker NMR instruments unless otherwise mentioned. IR spectra were recorded using Thermo Nicolet FT-IR using KBr pellet and the peaks are expressed in cm^{-1}. Finally, Mass spectra were analyzed by Waters LCT mass spectrometer. Enzyme purity was assessed by SDS–PAGE and protein concentration was determined from the absorbance at 280 nm. 0.1 M Tris–HCl pH 7.5 having ionic strength 0.15 M of NaCl and 1 mM EDTA was used for the kinetic study with all the synthesized compounds.

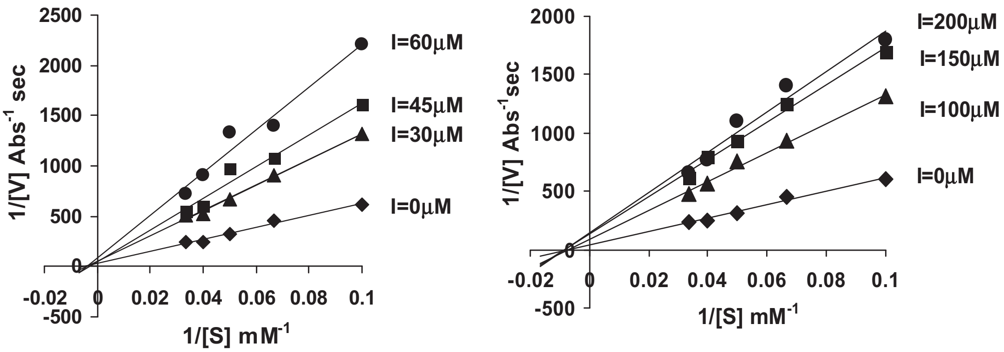


Figure 4. Line Weaver Burk plot of CEMA (left) and CNEMA (right).

Table 3
Docking interaction analysis during complexation between MPtpA and compound **1**

Interacting protein residues	Interacting counterpart of CEMA	Interaction distance (Å)	Remarks on interaction pattern
Gly13	Benzene ring and enediyne moiety	3.3	Hydrophobic
Ile15	Benzene ring and enediyne moiety	3.5	Hydrophobic
Trp48	Cyclic enediyne moiety	4.1	Hydrophobic
Trp48 (OH)	Acid group	2.9	Hydrogen bonding
His49 (NH_2)	Amide hydrogen	3.5	Hydrogen bonding
Tyr128	Cyclic enediyne moiety	3.4	Hydrophobic & π-stacking
Tyr129(OH)	Carbonyl oxygen	3.6	Polar (electrostatic)

4.2. Selected NMR data

4.2.1. Compound (7)

To dry DMF (10 mL) solution of mesylate **6** (250 mg, 0.7 mmol) and sodium azide (68 mg, 1.1 mmol) was stirred 3 h at room temperature. The mixture was partitioned between EtOAc and water (50 mL each). The organic layer was washed thoroughly with brine and dried over Na_2SO_4 and concentrated in vacuo. Finally, the crude residue was purified by column chromatography using 1:7 EtOAc/Hexane ($R_f = 0.6$) as eluent to afford yellowish viscous compound **7** (89%); γ_{max} 1162, 1738, 2131, 2405; δ_H (200 MHz) 1.53–1.86 (6H, m, 3 × C*H*$_2$-THP), 2.76 (2H, t, $J = 7.0$ Hz, NHCH$_2$C*H*$_2$), 3.48–3.61 (3H, m, NHC*H*$_2$CH$_2$, 1 × C*H*$_2$-THP), 3.82–3.92 (1H, m, C*H*$_2$-THP), 4.55 (1H, d, $J = 3.0$ Hz, C*H*$_2$O-THP), 4.98 (1H, m, C*H*-THP), 7.21–7.45 (4H, m, Ar-*H*); HRMS calcd for $C_{18}H_{19}N_3O_2$+H$^+$ 310.1477, found 310.1485.

4.2.2. Compound (8)

To a THF (10 mL) solution of compound **7** (225 mg, 0.7 mmol), PPh_3 (288 mg, 1.1 mmol) followed by two drops of water were stirred for overnight. THF was removed by evaporation and the target compound was isolated pure as off-white solid from the crude mixture by column chromatography (100–200 silica gel, yield 78%) using 5% MeOH in DCM ($R_f = 0.2$); δ_H (200 MHz) 1.55–1.80 (6H, m, 3 × C*H*$_2$-THP), 2.72 (2H, t, $J = 6.2$ Hz, NHCH$_2$C*H*$_2$), 3.04 (2H, t, $J = 6.2$ Hz, NHC*H*$_2$CH$_2$), 3.47–3.59 (1H, ddd, $J_1 = 2.4$ Hz, $J_2 = 2.6$ Hz, $J_3 = 3.4$ Hz, C*H*$_2$-THP), 3.80–3.91 (1H, ddd, $J_1 = 2.4$ Hz, $J_2 = 2.6$ Hz, $J_3 = 3.4$ Hz, C*H*$_2$-THP), 4.36 (1H, br s, N*H*), 4.52 (1H, d, $J = 2.6$ Hz, C*H*$_2$O-THP), 4.95 (1H, m, C*H*-THP), 7.20–7.42 (4H, m, Ar-*H*).

4.2.3. Compound (9)

In dry DCM solution (40 mL) of amine **8** (200 mg, 0.7 mmol) and triethyl amine (223 µL, 1.6 mmol), ethylmalonyl chloride (100 µL, 0.7 mmol) was added dropwise at −10 °C for 15 min. The reaction mixture was stirred for further 15 min at 0 °C. The reaction was quenched by adding water and then extracted with DCM after sequential washing with 1 (N) HCl and brine, respectively. The DCM solution was concentrated in rotavapor and dried in vacuo. The crude residue was finally purified by flash column chromatography using 1:1 EtOAc/Hexane ($R_f = 0.3$) as eluent to afford yellowish viscous compound **9** (83%); γ_{max} 1251, 1744, 2229, 2455; δ_H (200 MHz) 1.23 (3H, m, CH$_2$C*H*$_3$), 1.54–1.78 (6H, m, 3 × C*H*$_2$-THP), 2.68 (2H, t, $J = 6.4$ Hz, NHCH$_2$C*H*$_2$), 3.31 (2H, s, COC*H*$_2$CO), 3.53 (2H, t, $J = 6.4$ Hz, NHC*H*$_2$CH$_2$), 3.79–4.21 (4H, m, C*H*$_2$CH$_3$, C*H*$_2$-THP), 4.51 (2H, ABq, $J_1 = J_2 = 2.6$ Hz, C*H*$_2$O-THP), 4.94 (1H, m, C*H*-THP), 7.18–7.42 (4H, m, Ar-*H*); δ_C (50 MHz) 14.0, 19.0, 20.4, 25.3, 30.2, 38.5, 41.5, 54.7, 61.5, 61.9, 84.7, 88.6, 91.2, 96.6, 125.1, 126.0, 127.5, 128.1, 131.8, 132.0, 165.3, 169.0; HRMS calcd for $C_{23}H_{27}NO_5$+H$^+$ 398.1889, found 398.1893.

4.2.4. Compound (10)

To the ethanolic solution (15 mL) of compound **9** (100 mg, 0.2 mmol) and pyridinium *p*-toluene sulphonate (PPTS) (64 mg, 0.2 mmol) were added and the solution was stirred for 2 h at 50 °C. The solution was concentrated and the crude residue was subjected to flash column chromatography using 2:1 EtOAc/Hexane ($R_f = 0.3$) as eluent, afforded off-white semi-solid compound **10** (68%); γ_{max} 1182, 1675, 2395, 3454; δ_H (200 MHz) 1.17 (3H, t, $J = 7.2$ Hz, CH$_2$C*H*$_3$), 2.67 (2H, t, $J = 6.2$ Hz, NHCH$_2$C*H*$_2$), 3.31 (2H, s, COC*H*$_2$CO), 3.50 (2H, t, $J = 6.2$ Hz, NHC*H*$_2$CH$_2$), 4.09 (2H, q, $J = 7.2$ Hz, C*H*$_2$CH$_3$), 4.50 (1H, s, C*H*$_2$OH), 7.16–7.38 (4H, m, Ar-*H*), 7.62 (1H, br s, CH$_2$O*H*); δ_C (50 MHz) 13.9, 20.3, 38.6, 41.6, 51.2, 61.7, 80.7, 83.8, 91.3, 91.5, 125.2, 125.9, 127.6, 128.0, 131.7, 165.8, 169.3; HRMS calcd for $C_{18}H_{19}NO_4$+H$^+$ 314.1314, found 314.1316.

4.2.5. Compound (11)

To the dry DCM solution (10 mL) of compound **10** (50 mg, 0.2 mmol), Dess–Martin periodinate (100 mg, 0.2 mmol) was added and stirred for 2 h at room temperature. The reaction was quenched by adding of aqueous solution of $Na_2S_2O_3$. It was then washed with $NaHCO_3$ solution followed by brine and finally extracted with DCM. The DCM was concentrated in vacuo and the product was isolated by flash column chromatography using 1:1 EtOAc/Hexane ($R_f = 0.5$) as eluent to afford off-white semi-solid compound **10** (98%); γ_{max} 1245, 1632, 1710, 2805; δ_H (200 MHz): 0.99–1.02 (3H, dd, $J_1 = J_2 = 1.8$ Hz, CH$_2$C*H*$_3$), 2.52 (2H, t, $J = 6.2$ Hz, NHCH$_2$C*H*$_2$), 3.16 (2H, s, COC*H*$_2$CO), 3.32 (2H, t, $J = 6.2$ Hz, NHC*H*$_2$CH$_2$), 3.86–3.94 (2H, ddd, $J_1 = J_2 = J_3 = 1.6$ Hz, C*H*$_2$CH$_3$), 6.97–7.49 (5H, m, Ar-*H*, N*H*), 9.24 (1H, s, C*H*O); δ_C (50 MHz) 14.0, 20.8, 38.5, 41.9, 61.1, 79.4, 91.1, 93.5, 93.7, 121.4, 127.7, 128.1, 128.4, 128.6, 131.0, 165.9, 168.4, 177.2; HRMS calcd for $C_{18}H_{17}NO_4$+H$^+$ 312.1158, found 312.1161.

4.2.6. Compound (12)

The compound **11** (40 mg, 0.1 mmol) was dissolved under high dilution condition in distilled THF (30 mL) in presence of a buffer mixture of Et_3N (35 µL, 2.5 mmol, 0.08 M) and acetic acid (60 µL, 1 mmol, 0.03 M AcOH). The mixture was stirred at room temperature as indicated. The reaction was quenched by adding water. It was then washed with 1 N HCl followed by brine and finally extracted with EtOAc before drying over anhydrous $Na_2S_2O_3$ solution. The organic layer was concentrated in vacuo and crude mixture was then purified in flash chromatography (230–400 silica gel) using EtOAc/Hexane mixture (1:2) solvent ($R_f = 0.4$) yielding **12** as a off-white solid compound (65–91%); mp 143 °C; γ_{max} 1196, 1665, 1764, 2205; δ_H (200 MHz) 1.36 (3H, t, $J = 7.2$ Hz, CH$_2$C*H*$_3$), 2.86 (2H, t, $J = 6$ Hz, NHCH$_2$C*H*$_2$), 3.62 (2H, t, $J = 6$ Hz, NHC*H*$_2$CH$_2$), 4.34 (2H, q, $J_1 = J_2 = 7$ Hz, C*H*$_2$CH$_3$), 6.71 (1H, br s, N*H*), 7.16 (1H, s, CCC*H*), 7.30–7.59 (4H, m, Ar-*H*); δ_C (50 MHz) 14.0, 31.8, 38.5, 61.7, 81.7, 88.1, 93.2, 103.5, 121.4, 125.2, 127.3, 127.7, 129.8, 130.2, 132.1, 137.7, 163.7, 164.2; HRMS calcd for $C_{18}H_{15}NO_3$+H$^+$ 294.1052, found 294.1055.

4.2.7. Compound (1)

To a THF (10 mL) solution of compound **12** (50 mg, 0.2 mmol), LiOH (6 mg, 0.2 mmol) followed by two drops of water were stirred for 1 h. Then THF was removed in rotavapour and the crude mixture was transferred to filtration column chromatography

(230–400 silica gel) and compound **1** was isolated as pure off-white solid (81%) using 4:1 EtOAc/Hexane ($R_f = 0.2$) as eluent; mp 128 °C; γ_{max} 1210, 1678, 2205, 3285; δ_H 2.81 (2H, t, $J = 6$ Hz, NHCH$_2$C*H*$_2$), 3.74 (2H, q, $J_1 = J_2 = 6$ Hz, NHC*H*$_2$CH$_2$), 7.29–7.67 (4H, m, Ar-*H*), 7.93 (1H, s, CCC*H*), 8.65 (1H, br s, COO*H*); δ_C 31.9, 37.6, 83.6, 89.2, 93.0, 110.7, 121.4, 124.2, 127.2, 127.5, 128.5, 130.5, 131.2, 133.3, 165.1, 166.1; HRMS calcd for $C_{16}H_{11}NO_3$+H$^+$ 266.0739, found 266.0741.

4.2.8. Compound (2)

A two-necked RB flask containing a solution of compound **1** (40 mg, 0.2 mmol) in 10 mL dry ethanol was evacuated for 10 min. Then, under stirring condition, hydrogen gas was purged through the solution for 15 min. A pinch of 10% Pd-charcoal was poured quickly into it. The reaction was left stirring at room temperature for 2 h under hydrogen atmosphere. The reaction mixture was filtered through celite bed and the filtrate was concentrated in vacuo yielding compound **2** as pure white solid (93%); mp 161 °C; γ_{max} 1640, 1744, 2358, 3443; γ_{max} 1225, 1520, 2992, 3246; δ_H 1.47–1.71 (6H, m, 2 × ArCH$_2$C*H*$_2$, NHCH$_2$C*H*$_2$), 1.96 (1H, m, COCHC*H*$_2$), 2.12 (1H, m, COCHC*H*$_2$), 2.43–2.71 (4H, m, 2 × ArC*H*$_2$CH$_2$), 3.12–3.34 (2H, m, NHC*H*$_2$CH$_2$), 3.76–3.78 (1H, m, COC*H*CH$_2$), 7.03–7.04 (4H, m, Ar-*H*), 8.54 (1H, br s, COO*H*); δ_C 25.9, 28.4, 29.7, 29.8, 32.0, 32.2, 37.1, 54.8, 125.6, 125.7, 129.4, 129.5, 140.2, 140.3, 169.3, 177.2; HRMS calcd for $C_{16}H_{21}NO_3$+H$^+$ 276.1521, found 276.1525.

4.2.9. Compound (13)

To dry DCM solution (10 mL) of compound **12** (50 mg, 0.2 mmol), thiophenol (20 µL, 0.2 mmol) and dry solid K_2CO_3 (28 mg, 0.2 mmol) were stirred 2 h at room temperature. The mixture was partitioned between DCM and water (50 mL each). The organic layer was washed thoroughly with brine solution and dried over Na_2SO_4 and concentrated in vacuo. Finally, the crude residue was purified by flash column chromatography using 2:1 EtOAc/Hexane ($R_f = 0.5$) as eluent to afford **13** as yellowish viscous compound (**13**, 80%); γ_{max} 1179, 1761, 2291, 2560; δ_H (200 MHz): [Major isomer] 1.37 (3H, t, $J = 7.2$ Hz, CH$_2$C*H*$_3$), 2.71 (2H, dd, $J_1 = J_2 = 4.4$ Hz, NHCH$_2$C*H*$_2$), 3.46–3.63 (2H, dd, $J_1 = J_2 = 4.4$ Hz, NHC*H*$_2$CH$_2$), 4.30 (2H, q, $J_1 = J_2 = 7.2$ Hz, C*H*$_2$CH$_3$), 5.05 (1H, d, $J = 7.6$ Hz, SC*H*CH), 5.75 (1H, d, $J = 7.6$ Hz, SCHC*H*), 7.10–7.39 (9H, m, Ar-*H*); HRMS calcd for $C_{24}H_{21}NO_3S$+H$^+$ 404.1242, found 404.1248.

4.2.10. Compound (14)

To the stirring solution of compound **12** (50 mg, 0.2 mmol) in dry DCM (10 mL), diethyl malonate (12 µL, 0.2 mmol) and dry solid K_2CO_3 (28 mg, 0.2 mmol) were added at 0 °C. The reaction was quenched by water after 2 h and partitioned between water/DCM layers. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated in vacuo. A filtration column chromatography was performed using 3:1 EtOAc/Hexane ($R_f = 0.5$) as eluent resulting yellowish viscous compound **14** (85%); γ_{max} 1640, 1744, 2358, 3443, 1198, 1673, 1515, 2228; δ_H 1.18–1.33 (9H, m, 3 × CH$_2$C*H*$_3$), 2.72–2.83 (2H, m, NHCH$_2$C*H*$_2$), 3.46–3.63 (2H, m, NHC*H*$_2$CH$_2$), 3.81 (1H, d, $J = 6.4$ Hz, COC*H*CO), 3.92 (1H, d, $J = 9.2$ Hz, COC*H*CO), 4.13–4.30 (7H, m, 3 × C*H*$_2$CH$_3$, CHC*H*CH), 7.19–7.33 (4H, m, Ar-*H*), 7.50 (1H, br s, N*H*); δ_C (50 MHz) [Major isomer] 13.9, 14.0, 19.4, 33.9, 37.9, 53.0, 53.9, 54.9, 61.7, 61.9, 62.0, 82.5, 85.0, 88.3, 91.2, 127.8, 128.1, 129.9, 130.1, 130.2, 131.3, 166.0, 167.0, 167.2, 169.3; HRMS calcd for $C_{25}H_{27}NO_7$+H$^+$ 454.1788, found 454.1781.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.024.

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