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Design and synthesis of a novel enediynyl pentapeptide with predominantly β-turn structural motif and its potential as a fluorescence-based chemosensor

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Abstract—A novel enediynyl pentapeptide in the protected form **1** was synthesized and characterized. It exists predominantly in β-turn structural motif as revealed by variable temperature NMR and CD spectroscopy. In the presence of transition metal ions and gold nanoparticles, the fluorescence intensity of the peptide got enhanced with remarkable quantum yield with the *Z*-enediynyl ω-amino acid acting as a fluorophoric reporter. The interesting photophysical behaviors with alkali and alkaline earth metal ions are also reported.

1. Introduction

Ever since the discovery of naturally occurring enediynes, efforts are ongoing to find analogous rationally designed molecules having less toxicity without sacrificing the antitumor activity of the parent system.[1] Evaluation of the parameters controlling the Bergman cyclization (BC) is another important component that will help in the successful design of such molecules.[1] The enediynes, possessing extended unsaturation, is likely to be perturbed by photoirradiation. However, this aspect has so far been restricted only to the study of BC under photoirradiation.[2] Because of the presence of special structural features, in which a double bond is flanked between two acetylenes in a cis fashion, it occurred to us that the enediynes may serve as a probe for fluorescence spectroscopy provided suitable chelating appendages are incorporated in the two arms. The fluorescence sensing, for example, of metal ions, is of immense interest in biomedical research[3] and as chemical logics.[4] Efforts have also been directed toward the development of biological nanosensors and optoelectronic nanodevices.[5–7] Colloidal gold functionalized with specific binding groups can be used to label a wide variety of biologically active molecules, such as lipids, oligonucleotides, and peptides. Since fluorescence spectroscopy is a very sensitive technique, fluorophore-bound gold nanoparticles are useful probes for biomolecular labeling (e.g., as immunoprobes).[8] Both small peptide (e.g., GHK based ones reported by Leblanc and co-workers[9]) and protein based fluorescence sensors (e.g., mimics for zinc finger motif as developed by Berg and Shi[10a,b] and Imperiali and co-workers[10c,d]) have been designed and their photophysical properties evaluated. In these designs, the fluorophoric activities of dansyl and anthracene moieties have been exploited. As part of our efforts to prepare scaffolds for β-turn peptidomimetics[11] as well as metal ion specific peptides, we have designed and synthesized a pentapeptide **1** where a novel enediynyl ω-amino acid is acting as fluorophoric reporter. To our knowledge, this is the first report of application of the intrinsic fluorophoric property of *Z*-enediyne in monitoring the binding process of metal ions and gold nanoparticles.

2. Results and discussion

2.1. Synthesis of the pentapeptide

The key step in the synthesis of **1** is the preparation of the enediynyl amino acid in carboxy-protected form **2**.

Keywords: Enediyne; Peptide; β-Turn; Fluorescence; Chemosensor.
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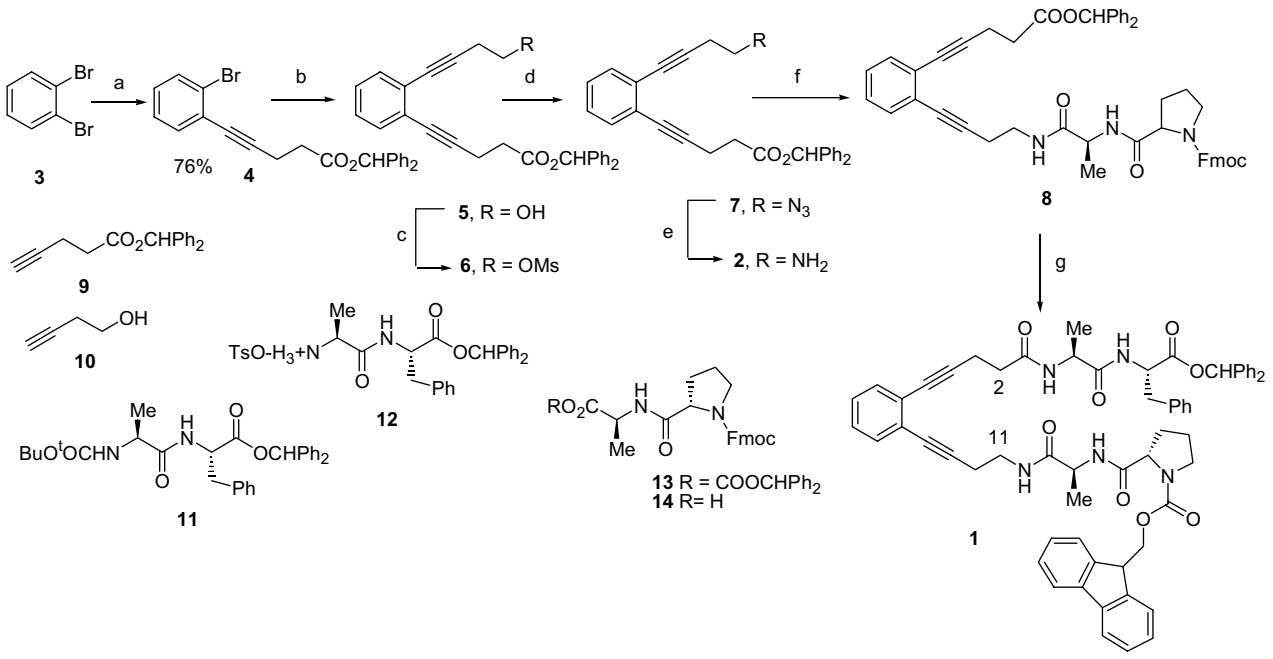

Scheme 1. Reagents and conditions: (a/b) $Pd(PPh_3)_4$, Et_3N, **9/10**, Cu_2Br_2; (c) MsCl, CH_2Cl_2; (d) NaN_3, DMF; (e) PPh_3,THF–H_2O; (f) **14**, EDC, HOBT; (g) (i) TFA/anisole/CH_2Cl_2, (ii) **12**, EDC, HOBT, CH_2Cl_2–DMF.

This was accomplished by sequential Pd(0)-mediated Sonogashira coupling[12] (Scheme 1). The two arms were then extended using the partially protected dipeptides Ala-Phe **12** and Ala-Pro **14** via standard EDC mediated coupling protocol to furnish the target N,C-diprotected pentapeptide **1** [Fmoc-Pro-Ala-Eda-Ala-Phe-$COOCHPh_2$, where Eda = enediyne amino acid] as a pale brown solid. The reason for choosing such a sequence of amino acids in **1** is its similarity with linear gramicidin S analogues as prepared by Sneider and Kelly,[13] where a dibenzofuran based amino acid is flanked between two hydrophobic amino acids. In our system, the enediyne amino acid is linked between two hydrophobic alanine residues. Mass and extensive 2D NMR experiments confirmed the structure of **1**.

2.2. Conformational studies by CD and VT-NMR

The secondary structure of peptide **1** was estimated by recording its CD spectrum in methanol, which showed a strong maximum at ~198 nm followed by several broad minima at ~205, 212, and 222 nm, indicating that the peptide predominantly adopts a β-turn like structure (Fig. 1) at least in the solvent used for the study. The peptide secondary structure estimation using CD estima program shows a 60% turn like structure, the existence of which implies the possible presence of intramolecular H-bond between the peptide strands on the two arms of the enediyne framework. This could be assessed by determining the variation of chemical shifts of the various NHs with temperature in DMSO-d_6 in which all the four NHs exhibited different chemical shifts. Interestingly, the turn like structure is more or less maintained in the presence of Ca^{2+} ions.

Of the four amide NH's, one alanine NH and the NH belonging to the enediynyl amino acid exhibited $\Delta\delta/\Delta T$ values (Fig. 2) that are within the Kessler limit[14] of −3 ppb/K, indicating strong intramolecular H-bonding and supported the predominant turn like structure of the peptide. The appearance of a crosspeak for the hydrogens attached to C-2 and C-11 in NOESY spectrum also provided further evidence for the turn like conformation of the two peptide arms of the enediyne backbone. The H-bonded conformation was also supported by the semi-empirical AM1 geometry optimization.[15]

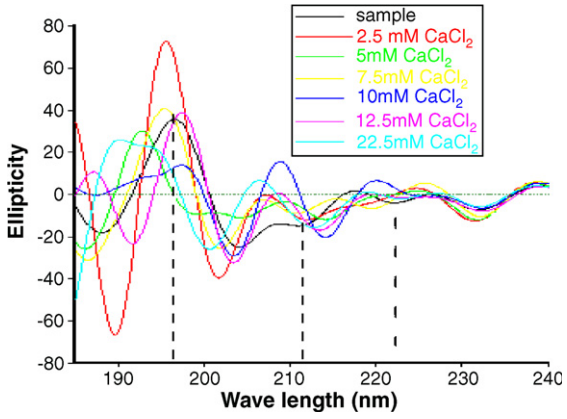

Figure 1. Overlayed deconvulated CD spectra of peptide **1** (333 mM) in MeOH in the absence and presence of various amounts of $CaCl_2$.

2.3. Photophysical behavior

We then turned our attention to the photophysical properties of **1** and possible perturbation by different metal ions. The peptide itself in TFE showed emission at λ_{max} 380 nm when excited at 320 nm with a large Stoke shift (60 nm), a characteristic emission of the enediyne moi-

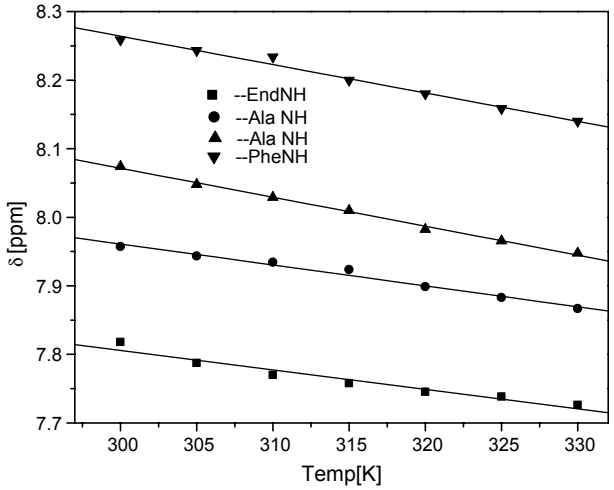

Figure 2. Temperature dependence of NH chemical shifts of peptide **1**.

ety. The intensity of emission increases with increasing concentration of the peptide. For better understanding of the fluorescence phenomenon, we have also observed the emission at low temperature. No phosphorescence could be detected in the solvent (TFE) at 77 K, indicating that the emission arises due to $S_1 \rightarrow S_0$ fluorescence. At 77 K a blue shift of about 12 nm is observed in TFE (Fig. 3). The shape of the band at 77 K in a given solvent also differs from that of the solution spectrum (298 K). Observed changes in the fluorescence spectrum can be rationalized in terms of the modification of the excited state (S_1) of the solute by interaction with the solvent molecule. Significant changes in fluorescence intensity were also observed upon addition of metal ions. Transition metal ions all caused enhancement of fluorescence intensity with the extent of enhancement depending upon the nature of the added metal ion. Although the precise nature of the interactions between the peptide and the metal ions is not known, it is possible that the intrinsic turn like structure allows the accommodation of the metal ions for binding. Generally, transition metal ions are strong quenchers[16] of fluorescence and our results demonstrated, for the first time, the ability of an enediynyl peptide to show fluorescence enhancement upon complexation to various transition metal ions similar to cryptand-based fluorophores.[4c,17] A possible explanation behind the enhancement of fluorescence may be that the distance and orientation of the metal ion entering in relation to the π-system is such that the spin-orbit coupling which facilitates the S–T intersystem crossing is minimum. In other words, the metal ion–fluorophore communication is considerably less than the metal ion–receptor interaction.[18] To quantify the effect of complexation on fluorescence intensity, fluorescence quantum yields (Φ) were determined. The complex formation constants (K) were also calculated using Benesi–Hildebrand[19] equation, which revealed a good correlation between fluorescence quantum yield and their complex formation constant. The order of K and Φ is Co(II) ≅ Zn(II) > Cu(II) > Ni(II). Fluorescence titration experiment (Fig. 4) with Cu^{2+} ion showed that the peptide binds more than one Cu^{2+} ion as is evident from the nonlinear plot of $1/[I - I_0]$ versus 1/[C] (inset). This result was also supported from MALDI TOF mass spectrometry; the expected molecular ion peak, corresponding to the peptide and two Cu^{2+} ions, was observed. For the other metal ions, a linear plot revealed 1:1 complex formation (Table 1).

The fluorescence behavior in the presence of alkali and alkaline earth metal ions is even more interesting. When the ratio of peptide to metal ion was kept at 1:1, fluorescence quenching was observed. At peptide–metal ion ratio of 1:2, enhancement of fluorescence of varying degree was observed. Interestingly, strongest enhancement was shown for K^+ ions while Na^+ and Ca^{2+} showed weak enhancement. This anomalous effect may be due to the fact that at low metal ion concentration, coordination occurs through carbonyl oxygen like in aza-crown ether based cryptand[20] due to well-known hard acid–hard base interaction leaving the lone pair of electrons on the amide N free to interact with the

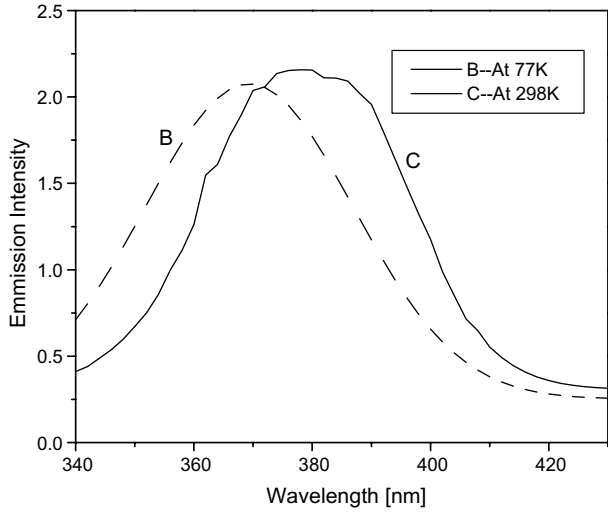

Figure 3. Fluorescence at 77 and 298 K.

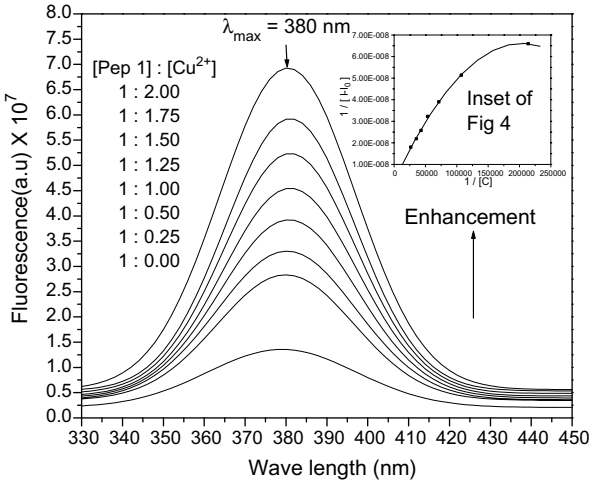

Figure 4. Fluorescence emission spectra of Cu(II) titration of peptide **1**. Excitation wavelength was 320 nm. Initial conc of **1** was 1.87×10^{-5} M in TFE.

Table 1. Summary of complex formation constants and fluorescence quantum yields in TFE at 298 K

Host	Guest ions	Formation constant (K)	Fluorescence quantum yield (Φ)
Peptide **1**	None		0.0087
	Co(II)	1.01×10^5	0.49
	Zn(II)	9.57×10^4	0.40
	Cu(II)	4.52×10^4	0.38
	Ni(II)	2.51×10^4	0.25

fluorophore via the PET mechanism thus causing quenching. With increase in concentration of the metal ion, binding also took place with the amide N; the unavailability of the lone of N for PET caused fluorescence enhancement.

2.4. Fluorescence perturbation by gold nanoparticle

Observing the perturbation of fluorescence behavior of **1** by mono- and divalent metal cations, we became interested to know whether zero valent metal is also able to have similar effect. For this purpose, we studied the changes in fluorescence intensity upon addition of Au(0) nanoparticle[21] in dry THF solvent. Interestingly, we observed enhancement of the fluorescence intensity (Fig. 5) as the concentration of nanoparticle was increased. To the best of our knowledge, uptil now there has been only two reports[22] about the gold-nanoparticle-enhanced emission from the fluorophore.

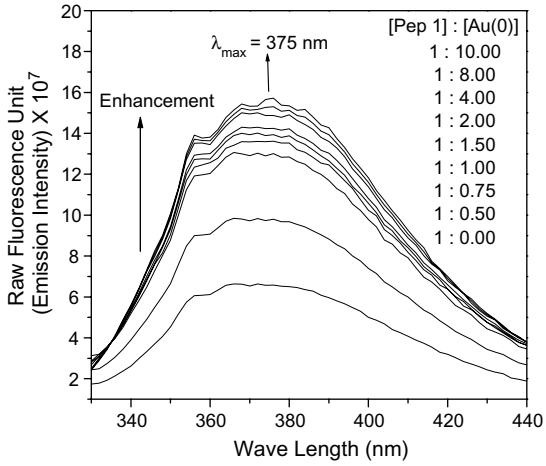

Figure 5. Fluorescence emission spectra of Au(0) titration of peptide **1**. Excitation wavelength was 320 nm. Initial concd of Au(0) was 10^{-3} M in THF.

The gold nanoparticles are nonfluorescent and peptide **1** in THF is moderately fluorescent with emission maxima at 372 nm. The peptide bound to gold nanoparticles exhibits strong emission bands at 375 nm and the intensity increases as the concentration of nanoparticles increases. The red-shift in the emission peaks parallels the shift in absorption bands. These new electronic transitions of the enediyne chromophore become allowed as the amide nitrogens bind strongly to the gold particle. No such spectral shift or enhanced emission could be seen when we added a THF solution of enediynyl peptide containing tetraoctylammonium bromide and treated with $NaBH_4$ (Fig. 6). The fluorescence quantum yield of surface-bound peptide is as high as 0.32 at a gold concentration of 30×10^{-5} mM. On the basis of the absorption as well as steady-state emission, it is concluded that surface binding has a significant effect on the fluorescence yield, but it has no observable effect on the intersystem crossing efficiency and thus the enhancement of fluorescence was observed. The increase in the fluorescence yield reflects the suppression of the nonradiative decay processes upon adsorption of peptide on to the surface of gold nanoparticles. The photoinduced electron transfer between the lone pair of N of amide functional groups and the enediyne moiety competes with the radiative and nonradiative decay of the singlet excited state. Upon binding of the N-lone pairs to the gold surface, the electron-donating ability of the N is decreased and this in turn suppresses the electron transfer from its lone pair to the enediyne moiety. A similar chelation-enhanced fluorescence has been reported earlier by Czarnick[3] and de Silva et al.[23] They were able to demonstrate the suppression of intramolecular quenching by binding metal cations to the amine functional groups of probes (e.g., anthracene).

3. Conclusion

We have for the first time developed a small peptide fluorescence chemosensor capable of binding strongly the transition metal ions selectively with enhancement of fluorescence intensity, which can be used as small

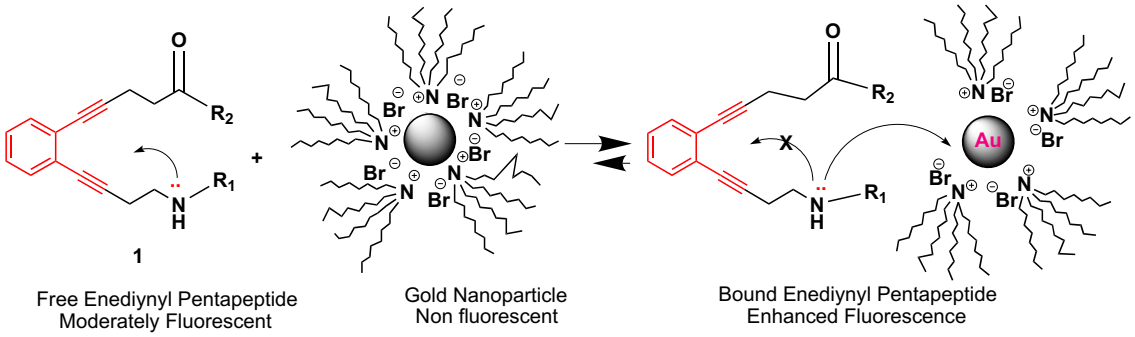

Figure 6. Probable mode of fluorescence enhancement by gold nanoparticle.

molecule receptors for transition metal cations, for example, Zn^{2+} in zinc finger protein, and for molecular recognition study especially for DNA/ATP recognition or continuous DNA-cleavage assay.[24] The design of β-turn receptors for large bio-molecules with bigger residues between the two arms of the enediynyl amino acid is currently under investigation. Efforts are also on toward making water-soluble systems to study possible fluorescence enhancement with transition metal ions.

4. Experimental

4.1. General remarks

Melting points (mp) were recorded on a Toshniwal hot-coil stage melting point apparatus and are uncorrected. Among the spectra, NMR spectra were recorded on a 200 MHz spectrometer (Bruker) unless mentioned otherwise. IR spectra were recorded on Perkin–Elmer 883 using KBr pellet for solids and neat for liquids. Mass spectra were obtained from CRIM (Clinical Research Institute of Montreal), Canada. All solvents for chromatography were distilled prior to use. In most of the column chromatographic purifications, ethyl acetate (EA) and petroleum ether (PE) of boiling range 60–80 °C were used as eluents. Columns were prepared with silica gel (60–120 mesh, S.D. Fine chemicals).

4.2. Synthesis of pentapeptide 1

4.2.1. 5-(2-Bromophenyl)-pent-4-ynoic acid benzhydryl ester (4). To a solution of *o*-dibromobenzene (**3**) (1980 mg, 8.39 mmol) and $Pd(PPh_3)_4$ (291 mg, 0.252 mmol) in dry, degassed triethyl amine (30 mL), 4-pentynoic acid benzhydryl ester **9** (2215 mg, 8.39 mmol) was added followed by Cu_2Br_2 (73 mg, 0.252 mmol) and the solution was refluxed for 4 h under argon. The reaction mixture was concentrated in vacuum, taken in diethyl ether (50 mL) and the organic layer was washed with saturated NH_4Cl (50 mL), then with water (50 mL), and dried over Na_2SO_4. Evaporation of the solvent in vacuum gave oil from which the title compound (**4**) was isolated by column chromatography (Si gel, PE–EA = 30:1) as greenish oil (2109 mg, 60%). ν_{max} (neat): 3452, 2936, 2864, 1726, 1533, 1475, 1443, 1359, 1269, 1122, 1028, 765; δ_H: 2.82 (4H, m, $2\times CH_2$), 6.93 (1H, s, $CHPh_2$), 7.08–7.26 (2H, m, Ar–*H*), 7.43 (1H, dd, $J = 1.8$, 7.5 Hz, Ar–*H*), 7.54 (1H, dd, $J = 1.4$, 7.8 Hz, Ar–*H*); δ_C: 12.35, 30.65, 79.35, 80.14, 91.75, 125.34, 126.33, 127.15, 128.40, 129.82, 130.72, 131.03, 134.35, 143.79, 172.52; Mass (EI) *m*/*z* 419 (M^+).

4.2.2. 5-[2-(4-Hydroxy-but-1-ynyl)-phenyl]-pent-4-ynoic acid benzhydryl ester (5). To a solution of **4** (2109 mg, 5.033 mmol) in dry, degassed Et_3N (30 mL), $Pd(PPh_3)_4$ (174 mg, 0.151 mmol), homopropargyl alcohol **10** (419 μL, 5.54 mmol) and Cu_2Br_2 (44 mg, 0.151 mmol) were added in succession and refluxed for 12 h. The reaction mixture was concentrated in vacuum, taken in diethyl ether (50 mL) and the organic layer was washed with saturated NH_4Cl (50 mL), then with water (50 mL), and dried over Na_2SO_4. Evaporation of the solvent in vacuum gave an oil from which the title compound (**5**) was isolated by column chromatography (Si gel, PE–EA = 10:1) as a brown oil (1232 mg, 60%). ν_{max} (KBr): 3454, 2931, 2405, 1731, 1443, 1162, 698; δ_H: 2.69 (2H, t, $J = 9.0$ Hz, CH_2CH_2OH), 2.82 (4H, q, $J = 4.3$, 11 Hz, $CH_2CH_2COOCHPh_2$), 3.77 (2H, t, $J = 8.9$ Hz, CH_2OH), 6.93 (1H, s, $CHPh_2$), 7.20–7.39 (10H, m, Ar–*H*); δ_C: 10.39, 18.86, 28.74, 55.70, 71.21, 85.26, 86.71, 121.90, 122.37, 122.71, 123.29, 126.34, 126.83, 134.80, 165.78; δ_C (DEPT 135): 10.39, 18.85, 28.73, 55.70, 71.12, 121.89, 122.39, 122.73, 123.30, 126.34, 126.84; Mass (EI) *m*/*z* 408 (M^+).

4.2.3. 5-[2-(4-Methanesulfonyloxy-but-1ynyl)-phenyl]-pent-4-ynoic acid benzhydryl ester (6). To a solution of **5** (1200 mg, 2.94 mmol) in CH_2Cl_2 (30 mL), methanesulfonyl chloride (273 μL, 3.53 mmol), and triethylamine (491 μL, 3.53 mmol) were added at 0 °C. The reaction mixture was stirred for 15 min after which it was poured into water and CH_2Cl_2 (50 mL each). The organic layer was washed with water (2×50 mL), dried, and then evaporated. The title compound **6** was then isolated by column chromatography (Si gel, PE–EA = 10:1) as a brown oil (1214 mg, 85%); and subsequently used for the azide formation.

4.2.4. 5-[2-(4-Azido-but-1ynyl)-phenyl]-pent-4-ynoic acid benzhydryl ester (7). To a solution of the mesylate **6** (1200 mg, 2.47 mmol) in dry DMF (20 mL), NaN_3 (241 mg, 3.71 mmol) was added and stirred for 18 h at room temperature. The mixture was then partitioned between EtOAc and water (50 mL each). The organic layer was thoroughly washed with water (3×50 mL), dried, filtered, and then evaporated. The titled compound **7** was isolated by column chromatography (Si gel, PE–EA = 25:1) as a pale brown oil (855 mg, 80%). ν_{max} (neat): 3446, 3063, 2927, 2410, 2102, 1738, 1635, 1158, 754; δ_H: 2.70 (2H, t, $J = 10.3$ Hz, $CH_2CH_2N_3$), 2.81 (4H, q, $J = 4.15$, 10.9 Hz, $CH_2CH_2COOCHPh_2$), 3.44 (2H, t, $J = 10.2$ Hz, CH_2N_3), 6.92 (1H, s, $CHPh_2$), 7.18–7.37 (10H, m, Ar–*H*); Mass (EI) *m*/*z* 433 (M^+).

4.2.5. 5-[2-(4-Amino-but-1ynyl)-phenyl]-pent-4-ynoic acid benzhydryl ester (2). To a solution of the azide **7** (850 mg, 1.96 mmol) in THF (25 mL), PPh_3 (771 mg, 2.94 mmol), and water (250 μL) were added and stirred at room temperature for 24 h. Evaporation of the solvent in vacuum gave oil from which the title compound **2** was isolated by column chromatography (Si gel, 10% CH_3OH in CH_2Cl_2) as a brown oil (678 mg, 85%). ν_{max} (neat): 3025, 2928, 2229, 1735, 1642, 1251, 754; δ_H: 2.76 (6H, m, $3\times CH_2$), 3.13 (2H, t, $J = 9.8$ Hz, CH_2COO-CHPh), 4.01 (1H, br s, NH_2), 6.85 (1H, s, $CHPh_2$), 7.12–7.38 (15H, m, Ar–*H*).

4.3. General procedure for peptide coupling

To a solution of *N*-protected amino acid or peptide in dry CH_2Cl_2, 1-[3-dimethyl aminopropyl]-3-ethylcarbodiimide hydrochloride (EDC·HCl) (1 equiv) and HOBT (1 equiv) were added and the reaction mixture was stirred for 1 h at 0 °C. The free amine or the tosylate salt of

the *C*-protected amino acid or peptide (1 equiv), dissolved in CH_2Cl_2 or DMF, was added dropwise followed by DIPEA (2 equiv). The reaction mixture was stirred for another 6 h at 0 °C to room temperature. After partitioning between CH_2Cl_2 and water (50 mL each), the organic layer was washed with $NaHCO_3$, then with dil HCl, dried over Na_2SO_4, and evaporated. From the oily residue, the coupled peptides were isolated pure by column chromatography.

4.4. 2-(1-Benzhydryloxycarbonyl-ethylcarbamoyl)-pyrolidine-1-carboxylic acid 1-(9*H*-fluoren-9-ylmethyl)ester (13)

ν_{max} (KBr): 3654, 3034, 2889, 1744, 1681, 1528, 1189, 750; δ_H: 1.40 (3H, d, J = 6.0 Hz, CH_3), 1.93 (2H, m, Pro–CH_2), 2.19 (2H, m, Pro–CH_2), 3.52 (2H, m, Pro–NCH_2), 4.25 (1H, m, Pro–H_α), 4.31(1H, t, J = 11.1 Hz, Fmoc–CH), 4.38 (2H, d, J = 7.5 Hz, Fmoc–CH_2), 4.67 (1H, t, J = 10.77, Ala–H_α), 6.77 (1H, d, J = Hz, NHCO), 6.86 (1H, s, CHPh$_2$), 7.28 (4H, m, Fmoc–Ar–H), 7.57 (2H, d, J = 7.2 Hz, Fmoc–Ar–H), 7.74 (2H, d, J = 7.4 Hz, Fmoc–Ar–H); Mass (EI) m/z 574 (M^+); HRMS calcd for $C_{36}H_{34}N_2O_5$ 574.2469. Found 574.2467.

4.5. Synthesis of 2-(1-carboxy-ethylcarbamoyl)-pyrolidine-1-carboxylic acid 9*H*-fluoren-9-ylmethyl ester (14)

To a solution of **13** (1000 mg, 2.46 mmol) in dry MeOH, 30% Pd–C (30 mol %) was added and stirred for 12 h under H_2 atmosphere. The reaction mixture was filtered and the residue obtained upon evaporation of the filtrate was recrystallized from $CHCl_3$/petrol to yield the desired acid in pure form. ν_{max} (KBr): 3435, 2932, 2240, 1658, 1537, 1122, 752; δ_H: 1.39 (3H, d, J = 6.9 Hz, CH_3), 1.91 (2H, m, Pro–CH_2), 2.19 (2H, m, Pro–CH_2), 3.52 (2H, m, Pro–NCH_2), 4.25 (1H, m, Pro–H_α), 4.31(1H, t, J = 11.1 Hz, Fmoc–CH), 4.39 (2H, d, J = 7.5 Hz, Fmoc–CH_2), 4.52 (1H, t, J = 10.5, Ala–H_α), 6.79 (1H, d, J = Hz, NHCO), 7.27–7.55 (4H, m, Fmoc–Ar–H), 7.73 (2H, d, J = 7.2 Hz, Fmoc–Ar–H), 7.91 (2H, d, J = 7.4 Hz, Fmoc–Ar–H); HRMS calcd for $C_{23}H_{24}N_2O_5$ 408.1686. Found 408.1687.

4.6. 2-(2-*tert*-Butoxycarbonylamino-propionylamino)-3-phenyl-propionic acid benzhydryl ester (11)

ν_{max} (KBr): 3869, 3263, 2973, 1895, 1720, 1662, 1252, 1024, 854; δ_H: 1.24 (3H, d, J = 9.16 Hz, CH_3), 1.41 (9H, s, *t*-butyl–H), 3.13 (2H, t, J = 7.0, CH_2Ph), 4.12 (1H, m, Ala–H_α), 4.93 (1H, m, Phe–H_α), 4.99 (1H, d, J = 7.67 Hz, NHBoc), 6.54 (1H, d, J = 7.8 Hz, NHCO), 6.99 (1H, s, CHPh$_2$), 7.12–7.37 (15H, m, Ar–H); Mass (EI) m/z 502 (M^+).

4.7. 2-(1-{4-[2-(4-Benzhydryloxycarbonyl-butynyl)-phenyl]-but-3-ynylcarbamoyl}-ethylcarbamoyl)-pyrolidine-1-carboxylic acid-9*H*-fluoren-9-ylmethyl ester (8)

ν_{max} (KBr): 2928, 2400, 2351, 1655, 1539, 1117, 753; δ_H: 1.38 (3H, d, J = 6.2 Hz, CH_3), 1.58 (2H, m, Pro–CH_2), 1.89 (2H, m, Pro–CH_2), 2.65 (2H, m, CH_2CH$_2$NH), 2.78 (4H, m, CH_2CH_2COOCH$_2$Ph), 3.46 (4H, m, Pro–NCH_2 and NCH_2CO), 4.25 (1H, m, Pro–H_α and Ala–H_α), 4.40 (2H, m, Fmoc–CH, Fmoc–CH_2), 6.78 (1H, d, J = Hz, NHCO), 6.89 (1H, s, CHPh$_2$), 7.14–7.38 (18H, m, Ar–H), 7.56 (2H, d, J = 7.2 Hz, Fmoc–Ar–H), 7.78 (2H, d, J = 7.4 Hz, Fmoc–Ar–H); Mass (EI) m/z 797 (M^+); HRMS calcd for $C_{51}H_{47}N_3O_6 + H^+$ 798.3555. Found 798.3516.

4.8. 2-{1-[4-(2-4-[1-(1-Benzhydryloxycarbonyl-2-phenyl-ethylcarbamoyl)-ethylcarbamoyl]-but-1-ynyl-phenyl)-but-3-ynylcarbamoyl]-ethylcarbamoyl}-pyrolidine-1-carboxylic acid-9*H*-fluoren-9-yl methyl ester (1)

Mp 195 °C; ν_{max} (KBr): 3295, 3068, 2235, 1747, 1639, 1537, 1352, 1120, 753; δ_H (500 MHz): 1.25 (3H, d, J = 12.2 Hz, Ala–CH_3), 1.35 (3H, d, J = 11.8 Hz, Ala–CH_3), 1.8–2.1 (4H, m, Pro–CH_2), 2.45 (2H, m, 2 × H-3), 2.60 (2H, m, H-10), 2.70 (2H, m, 2 × H-2), 3.10 and 3.20 (2H, m, Phe–CH_2), 3.48 (4H, m, 2 × H-11, Pro–NCH_2), 4.24 (2H, m, Fmoc–CH, Pro–H_α), 4.47 (2H, m, Fmoc–CH_2), 4.55 (1H, m, Ala–H_α), 4.90 (1H, m, Phe–H_α), 6.90 (2H, m, β and γ-NH), 7.03 (1H, d, J = 14.5 Hz, δ-NH), 7.10 (1H, t, J = 16.5 Hz, α-NH), 7.18–7.35 (23H, m, Ar–H), 7.6 (2H, d, J = 14.4 Hz, Fmoc–Ar–H), 7.8 (2H, d, J = 14.8 Hz, Fmoc–Ar–H); δ_C (DMSO-d_6, 50 MHz): 8.36, 9.25, 15.14, 18.30, 19.70, 34.07, 36.29, 37.94, 41.14, 45.35, 46.64, 47.72, 48.14, 50.50, 52.50, 62.62, 76.94, 79.24, 79.96, 92.57, 93.58, 120.61, 125.21, 126.42, 126.50, 127.06, 127.65, 128.11, 128.34, 129.01, 131.59, 135.46, 136.27, 136.85, 137.83, 140.12, 140.65, 143.79, 148.24, 172.27, 172.35, 177.35; Mass MALDI TOF m/z 1039.74 (M+Na^+); HRMS calcd for $C_{63}H_{61}N_5O_8 + H^+$ 1016.4601. Found 1016.4581.

4.9. Fluorescence measurement

The fluorescence titration was carried out in 1:1 TFE–water at pH 6.8. A stock solution (1.87×10^{-5} M) of peptide **1** in TFE and various metal salt solutions in water each of 1 mM concentration were prepared. All solutions were centrifuged for 15 mins at 10,000 rpm in order to precipitate all suspended particles. The clear supernatant liquid at the top was used for each fluorescence measurements. The fluorescence measurements were recorded on a scan mode using a FLUOROLOG spectrofluorometer. All studies were conducted in a cuvette of 3 mL capacity. The total volume of each sample was restricted to 200 mL. For metal binding studies the sample solutions containing different concentrations of metal salts were incubated at 37 °C for 12 h. The emission fluorescence was scanned for every 2 nm from 330 to 500 nm with a 1 min shaking before each measurement. To quantify the effect of complexation on fluorescence intensity, fluorescence quantum yield (Φ) were determined by the integration of corrected fluorescence spectra; quinine sulfate in 1 N H_2SO_4 was used for correction of fluorescence spectra as a fluorescence standard (Φ = 0.55). The complex formation constants were calculated using Benesi–Hildebrand equation.

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