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Triazolyl-donor–acceptor chromophore-decorated unnatural amino acids and peptides: FRET events in a β-turn conformation†

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The β-turn conformation and FRET process were established in the designed tripeptide containing fluorescent triazolyl donor and acceptor-decorated unnatural amino acids separated by a natural alanine.

In the fast-evolving research area concerned with expanding the genetic code, several unnatural amino acids (UNAAs) have been synthesized and incorporated within the framework of specific proteins, thereby affording novel functionality.[1] Among the various examples of UNAAs, one that has been recently encoded with a 'chemical warhead' is of considerable interest.[1d] However, sensing of protein's microenvironment requires a highly sensitive fluorescence probe, and although reports of such probes exist, these are not sufficient to comprehensively fulfill research needs.[2] Many of the problems associated with extrinsic fluorophore-labeled proteins could be solved by the synthesis of an intrinsically fluorescent amino acid that could be site-specifically incorporated into a protein.[3] Only a very few of such amino acids have been synthesized or encoded genetically.[3] Therefore, the synthesis of unnatural amino acids with novel photophysical properties is currently an emerging area of research.

As part of our ongoing research efforts regarding the installation and modulation of the emission response[4a] and the design of biomolecular building blocks[4b] *via* click chemistry, we disclose herein the conceptual design and synthesis of triazolyl donor–acceptor (Do–Ac) unnatural amino acids with new photophysical properties. We envisioned that incorporation of two such fluorescent UNAAs into the two termini of a tripeptide separated by a natural alanine could result in the formation of a β-turn[5] conformation *via* backbone H-bonding interactions. We also expected that the hydrophobic, π–π stacking and van der Waals interactions in the side chain would stabilize the conformation and allow the two terminal triazolyl aromatics to engage in dipolar photophysical interactions, most likely the Förster resonance energy transfer (FRET) process.[6] FRET, being a distance-dependent phenomenon, is widely utilized in the elucidation of structures, investigating protein dynamics, studying biomolecular interactions and the conformational distribution of unstructured peptides, along with many other sensory applications.[6] Thus, we were interested in a β-turn mimetic peptide capable of showing a FRET interaction between two terminally placed fluorescent unnatural amino acids. The logic behind our choice of triazole unit was its metabolic inertness, its tendency to associate with biological targets and its ability to modulate photophysical properties.[7] Thus, the tripeptide containing fluorescent solvatochromic Do–Ac amino acids adopted a β-turn conformation wherein we observed the FRET process. To the best of our knowledge, the concept of a fluorescent unnatural peptide showing the FRET process in a β-turn conformation is new.

The synthesis of the novel triazolyl amino acids **1–4** was carried out *via* a standard click reaction of the *N*-,*C*-diprotected azido serine **5**, synthesized *via* Weinreb's[8] amide strategy from serine, with the various donor and/or acceptor alkynes **A–D** (Fig. 1 and ESI,† Section 2 and 3). The amino acids were characterized by NMR, and mass spectrometry.

UV-visible spectra of all the amino acids presented here were solvatochromic and significantly red-shifted from those of the parent aromatics (ESI,† Fig. S3–S6). It was also evident from the study of UV-visible spectra of a 1 : 1 mixture and combined spectra of individual amino acids in acetonitrile (ESI,† Fig. S7 and S8) that the two UNAAs, $^{TPhen}Ala^{Do}$ and $^{TCNB}Ala^{Ac}$ (**1** and **3**), could take part in stacking interactions when in close proximity. While amino acid **3** ($^{TCNB}Ala^{Ac}$) exhibited a Stokes shift of 30 nm in its emission behaviour (λ_{em} = 310–360 nm) showing its polarity sensitivity, an intense emission at around 400 nm was observed for amino acid **1** ($^{TPhen}Ala^{Do}$). Increasing the solvent polarity also increased the quantum yields of amino acids **1** and **3**. However, a decrease in quantum yield was observed for amino acids **2** ($^{TNDMAB}Ala^{Do}$) and **4** ($^{TNB}Ala^{Ac}$) which could be due to the polar solvent–solute interaction leading to quenching of fluorescence *via* dipolar or H-bonding interactions.[6c]

We next incorporated two amino acids ($^{TPhen}Ala^{Do}$ and $^{TCNB}Ala^{Ac}$) into tripeptide sequences (a) $^{TPhen}Ala^{Do}$–Ala–$^{TCNB}Ala^{Ac}$ (**6**), (b) $^{TPhen}Ala^{Do}$–Ala–$^{TPhen}Ala^{Do}$ (**7**), and (c) $^{TCNB}Ala^{Ac}$–Ala–$^{TCNB}Ala^{Ac}$

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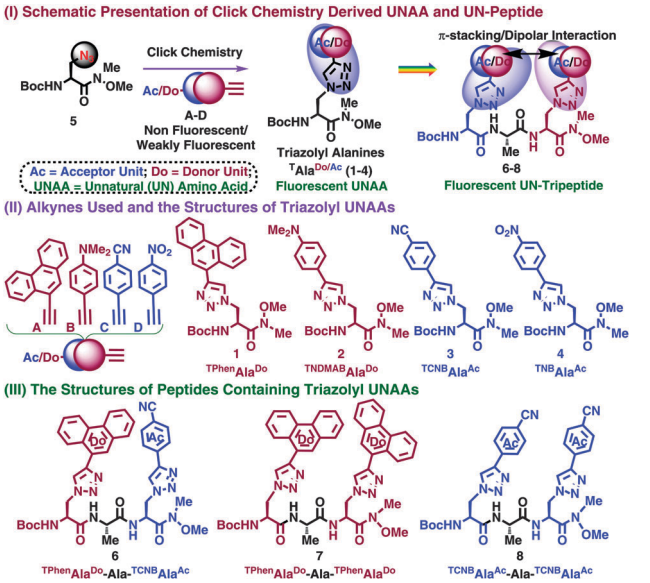

Fig. 1 (I) Schematic presentation of triazolyl UNAAs and peptides. (II) The alkynes used in this study, along with the structures of the amino acids and (III) peptides.

(**8**) following a peptide coupling protocol (Fig. 1, ESI,† Section 2 and 3). All peptides were characterized by NMR and mass spectrometry. While tripeptide **6** was designed to examine the possibility of H-bonding, hydrophobic and/or π-stacking interactions guided formation and stabilization of β-turns, as well as to investigate the photophysical dipolar interaction between the Do–Ac pair, peptides **7** (Do–Do) and **8** (Ac–Ac) were designed to compare the results.

The secondary structure of peptide **6** was estimated by recording its CD spectrum in acetonitrile and methanol, which showed a maximum at ~205–212 nm and a sudden switch from a positive to a negative band at 240–266 nm indicating a β-turn conformation (Fig. 2a).[5c,d] Aromatic absorption or a π–π stacking interaction between two aromatic moieties of terminal amino acids (^{TPhen}**Ala**^{Do} and ^{TCNB}**Ala**^{Ac}) was also evident from the appearance of a signature negative band at 297–301 nm in all solvents studied. The hypsochromic shift in wavelength and hypochromism of the bands at 266 and 301 nm in the variable temperature CD spectrum suggest a weakening of the π–π stacking interaction between the triazolyl-phenanthrene (**TPhen**) unit of ^{TPhen}**Ala**^{Do} and the cyanobenzene (**TCNB**) moiety of ^{TCNB}**Ala**^{Ac} (Fig. 2a, inset).[5e] The other two peptides, **7** and **8**, also showed similar characteristics (ESI,† Section 7.1).

The IR spectrum of peptide **6** showed absorption due to intramolecular H-bonding and free amide –NH stretching at 3320 and 3450 cm^{-1}, respectively.[5f,g] The ratio of absorption was significantly larger in peptide **6** than in peptides **7** and **8**, indicating that the hydrogen-bonded form of amide –NH was more favourable in the case of peptide **6**. Thus, the IR spectrum also supported the formation of a β-turn type structure.[5f,g] The presence of strong intramolecular H-bonding involving the carbamate carbonyl at *i* and the amide –NH (of ^{TCNB}**Ala**^{Ac}) at *i* + 3 was observed from the variable temperature NMR experiment (VT-NMR) where the temperature coefficients of the chemical shifts were found to be closer to the Kessler limit[9], supporting the presence of a β-turn structure in peptide **6** (Fig. 2b, ESI,† Section 7.4).

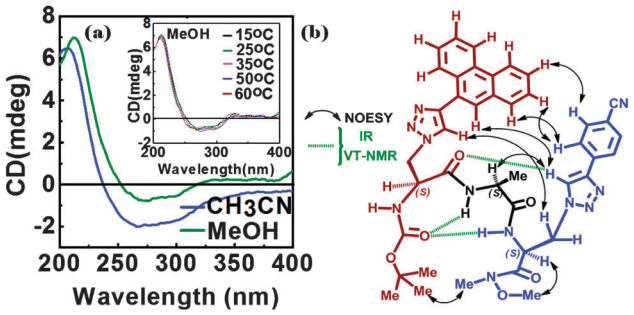

Fig. 2 (a) CD spectra of peptide **6**. Inset: variable temperature CD. (b) Presentation of various interactions revealed from spectroscopic studies.

The presence of strong intramolecular H-bonding involving the triazole sp^2 C–H (of ^{TCNB}**Ala**^{Ac}) and the amide carbonyl (of ^{TPhen}**Ala**^{Do}) in the side chain of the peptide was also evident from both the IR ($\bar{\upsilon}$ = 2973 cm^{-1}) and the variable temperature NMR ($\Delta\delta/\Delta T$ = −1.5 ppb K^{-1}) spectra, and was further supported by a MacroModel optimized geometry study (ESI,† Sections 7 and 11).[10] Two other amide N–Hs were also involved in H-bonding interactions, as seen from the VT-NMR study. The NOESY spectrum of peptide **6** revealed interactions among the aromatic hydrogens of the **TPhen** and **TCNB** units of the two terminal amino acids. It was also evident that the triazolyl C–H of the N-terminal amino acid (^{TPhen}**Ala**^{Do}) interacted with the methylene (–CH_2–) proton of the C-terminal amino acid (^{TCNB}**Ala**^{Ac}). Moreover, the triazolyl C–H of the C-terminal amino acid (^{TCNB}**Ala**^{Ac}) interacted with the aromatic hydrogens (of the **TPhen** unit) of the N-terminal amino acid (^{TPhen}**Ala**^{Do}). Furthermore, ^{t}Boc–H and NMe–H of the two terminal amino acids showed NOESY interactions (ESI,† Section 7). All these interactions in the side chain of peptide **6** possibly played a role in stabilizing the β-turn structure and in bringing the two terminal aromatics closer for a dipole–dipole interaction. However, these side chain interactions were weak in case of other two peptides, probably because of the steric repulsion between two bulky phenanthrenes in peptide **7** and the dipolar repulsion between two **TCNB** units in peptide **8**. Therefore, they also adopted similar but deformed structures, which could be seen in the corresponding CD spectra.

Geometry minimization and molecular dynamics calculations using the Merck Molecular Force Field support the presence of a β-turn conformation with H-bonding between CO(*i*)–NH(*i* + 3) (ESI,† Section 11).[5h,i,10a] Therefore, the spectroscopic evidence and the modelling study support the β-turn conformation of peptide **6**, which primarily derives from backbone H-bonding interactions, and which is stabilized by other side chain interactions such as hydrophobic and π–π stacking interactions between the terminal **TPhen** and **TCNB** moieties.

After establishing the β-turn conformation, we next studied the possibility of photophysical interactions between the terminal Do–Ac amino acid pair in peptide **6**. The UV-visible and fluorescence spectra of the individual donor and acceptor amino acids revealed that the fluorescence spectrum of ^{TCNB}**Ala**^{Ac} overlapped significantly with the absorption spectrum of ^{TPhen}**Ala**^{Do} (Fig. 3a). Moreover, the peptide (**6**) containing these two amino acids can be selectively excited at 272 nm (the maximum absorbance of ^{TCNB}**Ala**^{Ac}) where there is very low absorbance of ^{TPhen}**Ala**^{Do}. Therefore, these two amino acids should form a FRET pair in our designed tripeptide where the conceptual

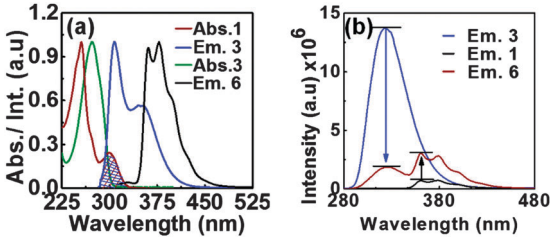

Fig. 3 (a) Overlap of the emission spectrum of $^{TCNB}Ala^{Ac}$ (blue) and the absorbance spectrum of $^{TPhen}Ala^{Do}$ (red). (b) Fluorescence spectra of individual amino acids (black for **1** and blue for **3**) and the tripeptide **6** (red) containing these two amino acids. The mole fractions of triazolyl chromophoric units in the monomers and in the tripeptide were the same. Spectra were recorded in CH_3CN with a 10 μM concentration.

donor amino acid **1** ($^{TPhen}Ala^{Do}$) and the acceptor amino acid **3** ($^{TCNB}Ala^{Ac}$) acted as a FRET acceptor and donor, respectively. With this observation we turned our attention to study the FRET process.

It was observed that the fluorescence intensity of $^{TPhen}Ala^{Do}$ increased almost four-fold from that of the monomer emission in the presence of $^{TCNB}Ala^{Ac}$ in peptide **6** when it was excited at the maximum absorbance of $^{TCNB}Ala^{Ac}$ (λ_{max} = 272 nm). On the other hand, the fluorescence intensity of $^{TCNB}Ala^{Ac}$ in the peptide decreased almost seven to eight times that of the monomer fluorescence. This ratiometric change in fluorescence intensity provided visual evidence of the FRET process from $^{TCNB}Ala^{Ac}$ to $^{TPhen}Ala^{Do}$ (Fig. 3b).[6e] The Förster radius and the efficiency of energy transfer were calculated and found to be 20 Å and 85%, respectively. The donor–acceptor distance was found to be r = 15 Å. The time-resolved fluorescence study revealed a decrease in donor lifetime ($^{TCNB}Ala^{Ac}$; λ_{ex} = 308 nm, λ_{em} = 340 nm) from 1.9 ns to 1.2 ns in peptide **6**, indicating the occurrence of the FRET process. More interestingly, the acceptor ($^{TPhen}Ala^{Do}$) exhibited a biexponential profile (λ_{ex} = 308 nm, λ_{em} = 380 nm) having a distinct rise component of 3.2 ns and a decay component of 14.1 ns (ESI,† Tables S8 and S9, Fig. S19). Thus, the lifetime of the acceptor in the presence of the donor in peptide **6** increases from 13.2 ns (in the absence of the donor) to 14.1 ns (in the presence of the donor) which is also clearly an indication of FRET.[11]

While examining the polarity sensitive emission of peptide **6**, we observed to our surprise that FRET-induced fluorescence could be modulated. Thus, as the polarity of the solvents increased, FRET emission from $^{TPhen}Ala^{Do}$ (at 390 nm) decreased slightly (Fig. 4a). This could be due to the influence of the solvent polarity on the FRET parameters and association within the **TPhen–TCNB** pair that led to a slight decrease in FRET emission intensity, which was also supported by a fluorescence lifetime measurement study (ESI,† Section 6 and 10).[6f] A very weak FRET emission was observed in phosphate buffer which could be due to non-radiative deactivation.[6c] However, the FRET signal could be optimized upon addition of DMSO to give a 296% enhancement (Fig. 4b). This addition of DMSO possibly fine-tuned the donor–acceptor interaction to minimize the non-radiative deactivation process, thereby leading to an increase in intensity.[6f]

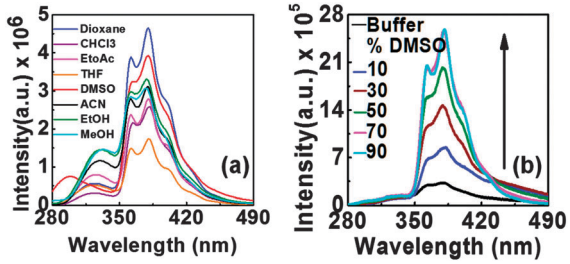

Fig. 4 (a) Solvent-assisted modulation of FRET-induced fluorescence of peptide **6** (10 μM). (b) Amplification of the FRET signal in phosphate buffer (pH 7.0) upon addition of increasing % concentration of DMSO (λ_{ex} = 272 nm).

In summary, Förster resonance energy transfer (FRET) was established in the conceptually designed novel unnatural β-turn peptide containing a new class of fluorescent unnatural donor–acceptor amino acids. The FRET process occurred from the **TCNB** moiety of $^{TCNB}Ala^{Ac}$ (the FRET donor) to the **TPhen** unit of $^{TPhen}Ala^{Do}$ (the FRET acceptor). Our developed FRET pair, $^{TCNB}Ala^{Ac}$–$^{TPhen}Ala^{Do}$, wherein both the partners are unnatural amino acids, is new to the best of our knowledge. As FRET can provide information about peptide and protein conformation, our unnatural amino acid pair (FRET pair) and the FRET peptide could find application in studying the conformational distribution of unstructured peptides in solution and in FRET-based bioassays.

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