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Design and synthesis of a novel anthracene-based fluorescent probe through the application of the Suzuki–Miyaura cross-coupling reaction

S. Kotha, V. R. Shah, P. P. Mishra, and **A. Datta**

Department of Chemistry, Indian Institute of Technology-Bombay, Powai, Mumbai, India

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Summary. We report on a simple synthetic route to a novel anthracene-based bis-armed amino acid derivative as a useful fluorescent probe. Various photophysical studies of this amino acid derivative are also described. Here, Suzuki–Miyaura cross-coupling reaction has been used as a key step for carbon–carbon bond formation.

Keywords: Bis-armed amino acid – Carbon–carbon bond formation – Cross-coupling – Fluorescent amino acid

Introduction

In the past couple of decades, fluorescent probes have emerged as powerful tools for studying the structure and dynamics of proteins (Lakowicz and Weber, 1973; Eftink and Ghiron, 1977; Guerrier-Takada et al., 1983; Lakowicz, 1983; Beechem and Brand, 1985; Cech and Bass, 1986; Petrich et al., 1987; Sugimoto et al., 1989; Cech et al., 1992; Elove et al., 1992). Fluorophores that can be used as covalent labels of proteins are important, as they can be utilized to report information about particular microenvironments of protein chains. Fluorescent probes containing amino acid groups are particularly attractive in this respect as they are easy to incorporate in proteins via solid-phase synthesis (Kouyama and Mihashi, 1981; Steinhauser et al., 1982). Such extrinsic probes are often preferred to the natural fluorophore in proteins, i.e. tryptophan, because it is extremely difficult to understand the fluorescence properties of multi-tryptophan proteins and besides, tryptophan has a multi-exponential decay of fluorescence, making the situation complicated even for single-tryptophan proteins (Petrich et al., 1983). Moreover, tryptophan absorbs ultraviolet light, which is scattered to a significant extent in heterogeneous media. So, it is desirable to synthesize fluorescent probes with amino acid moieties, which have single exponential fluorescence decays and preferably, absorb light of longer wavelengths, particularly in the range of emission of tryptophan, so that they can be used as energy acceptors from tryptophan. Several researchers are working towards this objective. For example, Hamada et al. (2005) have reported the incorporation of fluorescent amino acid L-2-acridoalanine **1** (Fig. 1) for fluorescence sensing. Brun et al. (2004) have synthesized coumarin based fluorescent amino acid while Vázquez et al. (2004) have synthesized phthalimide based amino acid. Guzow et al. (2001) and Rzeska et al. (2001) have synthesized a highly fluorescent substituted 3-[2-(phenyl)benzoxazol-5-yl]-alanine derivatives **2**. Similarly, Kóczán et al. (2001) synthesized 4-ethoxymethylene-2-[1]-naphthyl-5(4H) oxazolone **3**. A number of unusual amino acids have been incorporated into peptides and the photophysical properties of the corresponding peptides have been studied.

In the present communication, we report the synthesis and structural properties of a functionalized anthracene derivative, which we propose to be a potentially useful fluorescent probe for proteins. Since its discovery, anthracene has found various chemical applications in electroluminicent devices and fluoroscent probes because of its highly fluorescent nature. A number of fluorescent probes with an anthracene unit has been reported by various research groups and found useful applications in different fields. For example, Stack et al. (2002) have synthesized fluorescent probe specific for catechols.

Malakhov et al. (2004) synthesized 1-(phenylethynyl)-pyrene and 9,10-bis(phenylethynyl)anthracene fluorescent

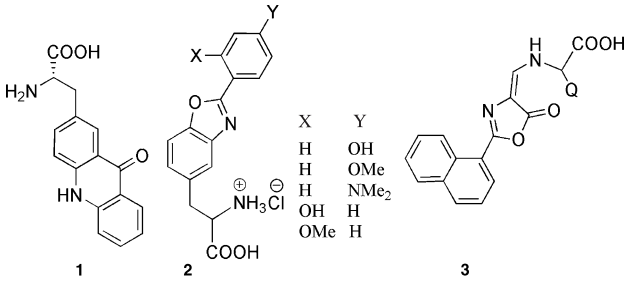

Fig. 1. Fluorescent amino acids

dyes useful for DNA labeling. Swamy et al. (2005) have synthesized anthracene derivative containing two boronic acid groups at 1,8-position and these derivatives are found to be sorbitol-selective fluorescence sensors.

Our group has earlier reported the synthesis of symmetrical and unsymmetrical 9,10-diarylanthracene derivatives via the Suzuki–Miyaura (SM) (Miyaura and Suzuki, 1995; Kotha et al., 2002) cross-coupling reaction (Kotha et al., 2004). During these studies, some of the 9,10-diarylanthracene derivatives revealed interesting fluorescent properties. Recently, we synthesized various bis-, tris- and tetra-armed amino acid derivatives via the SM cross-coupling reaction of respective aromatic iodides or bromides and DL-4-boronophenylalanine derivative (Kotha et al., 2007). These amino acids can be incorporated in biologically active peptides to deliver ''drug like molecules''.

Materials and methods

Analytical TLC was performed on (10×5 cm) glass plates coated with silica gel G or GF 254 (containing 13% $CaSO_4$ as a binder). Visualization of the spots was achieved either by exposure to I_2 vapor or UV light. Flash chromatography was performed using silica gel (100–200 mesh) and usually eluted with EtOAc and petroleum ether (bp 60–80 °C) mixtures. Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectral data were recorded on Varian VXR 300 or Varian VXR 400 spectrometers using TMS as internal standard and $CDCl_3$ or DMSO-d_6 as solvent. The coupling constants (J) are given in hertz (Hz). High Resolution mass spectral data were recorded on Q-ToF micromass machine. UV spectral data were obtained on Shimadzu UV-2100 or UV-260 instruments or Perkin-Elmer instrument. The fluorescence spectra were recorded on Perkin-Elmer LS-55 spectrofluorimeter. Concentration of bis-armed amino acid derivative **6** was 2.7 μM.

Preparation of bis-armed amino acid derivative ***6***

In a three-necked RB flask attached with reflux condenser and nitrogen inlet–outlet, 9,10-dibromoanthracene **4** (40 mg, 0.12 mmol), boronic acid derivative **5** (154 mg, 0.48 mmol), Na_2CO_3 (50 mg, 0.47 mmol) in H_2O (1 ml) and THF/toluene (1:1, 4 ml) were mixed, stirred and degassed for 20 min. To this mixture, $Pd(PPh_3)_4$ (11.0 mg, 10 mol%) was added. The resulting mixture was refluxed at 80 °C. At the conclusion of the reaction (TLC monitoring), the reaction mixture was quenched with water and usual work-up procedure gave crude product which was purified by the silica gel column chromatography. Elution of the column with 12% ethyl acetate/petroleum ether mixture gave product **6** (59 mg, 68%) as pale yellow solid. Mp: 200–216 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.47$ (s, 18H), 3.34–3.13 (m, 4H), 3.80 (s, 6H), 4.75 (dd, 2H), 5.18 (d, 2H), 7.30–7.32 (m, 4H), 7.36–7.40 (m, 8H), 7.64–7.67 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 28.3$, 38.7, 52.3, 54.6, 80.0, 125.0, 126.8, 129.3, 129.8, 131.4, 135.4, 136.7, 137.6, 155.1, 172.5 ppm. HRMS (QToF): calcd. for $C_{44}H_{48}N_2O_8 + Na$ $[M + Na]^+$: 755.3308; Found: 755.3442.

Results and discussion

Having synthesized the various 9,10-diarylanthracene derivatives and multi-armed amino acid derivatives via SM cross-coupling reaction, we thought to utilize this methodology for the synthesis of bis-armed amino acid derivatives having anthracene moiety which might exhibit interesting photophysical properties. Towards this goal, when 9,10-dibromoanthracene **4** was subjected to the cross-coupling reaction with DL-4-boronophenylalanine derivative **5** (Kotha et al., 2007) as a coupling partner, the corresponding cross-coupling product **6** was isolated in 68% yield (Fig. 2).

The product **6** was fully characterized by ^{1}H NMR, ^{13}C NMR, and QToF-HRMS spectral data. In ^{1}H NMR singlet at $\delta = 1.41$ corresponds to 18 protons of *tert*-butyl group while singlet at $\delta = 3.8$ corresponding to six protons confirmed methyl ester. Multiplet at $\delta = 3.2$ indicated benzylic protons while a doublet at $\delta = 4.75$ revealed the methine protons and a doublet at $\delta = 5.18$ confirmed the presence of two $-$NH protons. Fifteen lines in ^{13}C NMR spectrum further confirmed the two fold C_2-symmetry of the compound **6**. Molecular ion peak in QToF-HRMS at 755.3442 (Calcd. for $C_{44}H_{48}N_2O_8 + Na$: 755.3308) further confirmed the formation of compound. As expected, the product **6** possess interesting fluorescent properties which was visible with naked eye, so detailed studies of absorption and fluorescence were carried out.

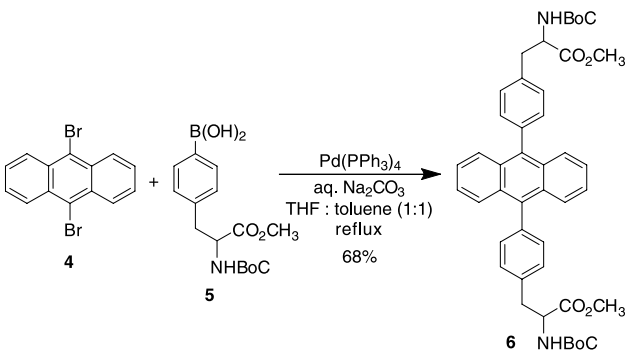

Fig. 2. Synthesis of BAAA derivative **6**

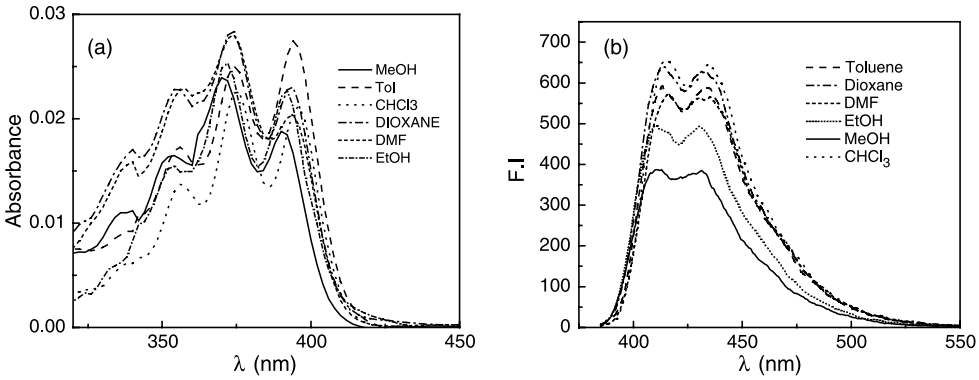

Fig. 3. (**a**) Absorption spectra of compound **6** in different solvents (concentration of **6** is 2.7 μM). (**b**) Fluorescence spectra of compound **6** in different solvents excited at 398 nm

Absorption study

Highest molar extinction coefficient was measured at 398 nm in chloroform. When the same solution was excited at 398 nm, light emission was observed at 414 nm–434 nm. As indicated from Fig. 3a, there is no major effect of the polarity of solvent on the absorption property of the compound **6**.

Fluorescence study

It is well-known that substitutions alter the absorption and fluorescence properties of organic compounds. However, in the present case, we found that the fluorescence quantum yield (ϕ_f) of **6** is almost unaffected by synthetic modification and is rather high (∼1.0 in $CHCl_3$), which is same as the parent 9,10-diphenylanthracene (Du et al., 1998). Polarity of the solvents does not have significant effect on ϕ_f value (Table 1).

Thus, it appears that upon covalent binding with a protein, this fluorophore is likely to behave as a good marker. Degassing the solution does not affect the quantum yield of the fluorophore, indicating the absence of luminescence quenching by oxygen. KI, on the other hand, is found to quench its fluorescence in the methanolic solution quite significantly (from 0.7 to 0.27). The Stern-Volmer constant is found to be 123 l mol^{-1} (Fig. 4).

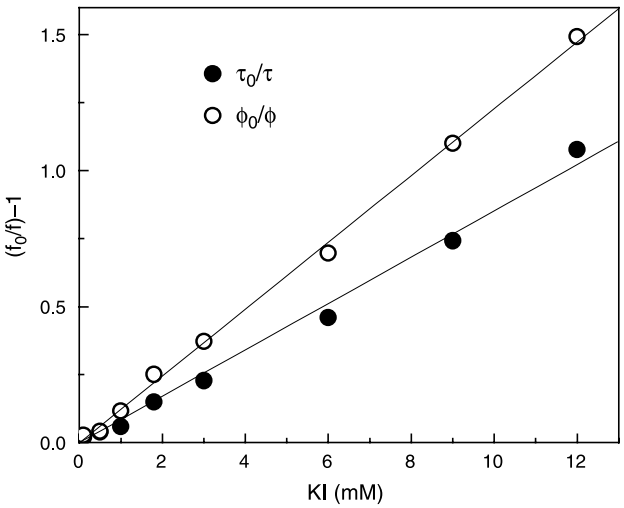

Fig. 4. The Stern-Volmer plot obtained from the steady state and the time resolved experiments with addition of KI solution. The Y-axis represents fluorescence properties

The fluorescence lifetime (τ_f) of compound **6** in different solvents is 5.5 ± 0.5 nsec. With increase in water content in the methanolic solution, there is an increase in τ_f from 5.4 to 6.7 nsec, as the volume of water is increased from 500 to 1500 μl. The bimolecular quenching constant calculated from the Stern-Volmer plot is 851 l mol^{-1}, which is slightly smaller as compared to that of the steady state results (Fig. 4). This could be due to some ultrafast component that is missed in the resolution of our instrument.

Fluorescence of the probe is not quenched by tryptophan. So, it can be used as an excellent fluorocent marker of proteins. Of course, since the ϕ_f does not differ much

Table 1. Variation of ϕ_f with E_T (30)

Solvent	E_T (30)	ϕ_f
Toluene	33.9	0.90
1,4-Dioxane	36.0	0.87
$CHCl_3$	39.1	1.00
DMF	43.2	0.80
t-BuOH	43.3	0.70
EtOH	51.9	0.85
MeOH	55.4	0.70
Ethylene glycol	56.3	0.80

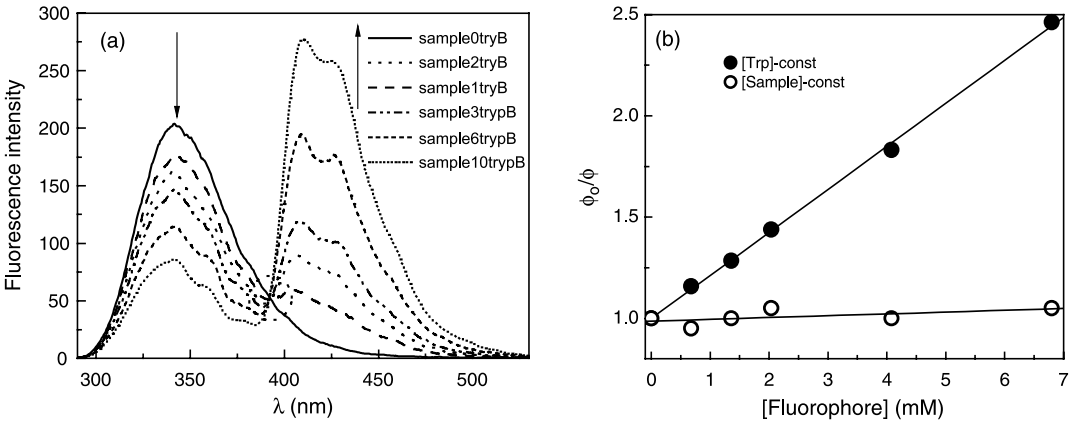

Fig. 5. (**a**) The change in fluorescence spectra with addition of compound **6** (2.7 μM solution, 0–10 μl) to a fix concentration of tryptophan (2.7×10^{-5} M, 2 ml) in $CHCl_3$. (**b**) Stern-Volmer plots representing the fluorescence quenching of tryptophan by compound **6** (●) and compound **6** by tryptophan (○)

from that in water, it can not be used to monitor protein unfolding *per se*. However, the addition of the compound **6** to a fixed concentration of tryptophan quenches the tryptophan fluorescence (Fig. 5). This may be attributed to fluorescence resonance energy trasfer (FRET) from tryptophan to the fluorophore, thus causing the decreasing the quantum yield. This property makes the fluorophore particularly attractive as a covalent label of protein, as there is a possibility of following protein unfolding by a decrease in FRET from tryptophan to the fluorophore. Similar covalent labels have been used conveniently in the recent past to monitor the chain dynamics of labled proteins (Hudgins et al., 2002). Moreover, as described earlier, its fluorescence is quenched by iodide, upon unfolding the fluorophore become more accessible to iodide ions and this can also be used to monitor unfolding.

Conclusions

We have synthesized a highly fluorescent amino acid derivative **6** and studied the photophysical properties. Quantum yield of **6** is found to be 1 in chloroform and no major effect of polarity of solvent was observed. The fluorophore quenches tryptopan fluorescence significantly, presumably by FRET while its own fluorescence is not quenched by tryptophan. However, iodide ion quenches the fluorescence of the fluorophore **6**. So, this highly fluorescent bis-armed amino acid derivative can be used to monitor unfolding of proteins.

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References

Beechem JM, Brand L (1985) Time-resolved fluorescence of proteins. Annu Rev Biochem 54: 43–71

Brun M-P, Bischoff L, Garbay C (2004) A very short route to enantiomerically pure coumarin-bearing fluorescent amino acids. Angew Chem Int Ed 43: 3432–3436

Cech TR, Bass BL (1986) Biological catalysis by RNA. Annu Rev Biochem 55: 599–629

Cech TR, Herschlag D, Piccirilli JA, Pyle AM (1992) RNA catalysis by a group I ribozyme. Developing a model for transition state stabilization. J Biol Chem 267: 17479–17482

Du H, Fuh RA, Li J, Corkan A, Lindsey JS (1998) Photochem CAD: a computer-aided design and research tool in photochemistry. Photochem Photobiol 68: 141–142

Eftink MR, Ghiron CA (1977) Exposure of tryptophanyl residues and protein dynamics. Biochemistry 16: 5546–5551

Elove GA, Chaffotte AF, Roder H, Goldberg ME (1992) Early steps in cytochrome c folding probed by time-resolved circular dichroism and fluorescence spectroscopy. Biochemistry 31: 6876–6883

Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35: 849–857

Guzow K, Szabelski M, Malicka J, Wiczk W (2001) Synthesis of a new, highly fluorescent amino acid derivative: N-[(tert-butoxy)carbonyl]-3-[2-(1H-indol-3-yl)benzoxazol-5-yl]-L-alanine methyl ester. Helv Chim Acta 84: 1086–1092

Hamada K, Kameshima N, Szymańska A, Wegner A, Łankiewicz L, Shinohara H, Takia M, Sisido M (2005) Position-specific incorporation of a highly photodurable and blue-laser excitable fluorescent amino acid into proteins for fluorescence sensing. Bioorg Med Chem 13: 3379–3384

Hudgins RR, Huang F, Gramlich G, Nau WM (2002) A fluorescence-based method for direct measurement of submicrosecond intramolecular contact formation in biopolymers: an exploratory study with polypeptides. J Am Chem Soc 124: 556–564

Kóczán G, Csík G, Csámpai A, Balog E, Bősze S, Sohár P, Hudecz F (2001) Synthesis and characterization of 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone and its fluorescent amino acid derivatives. Tetrahedron 57: 4589–4598

Kotha S, Ghosh AK, Deodhar KD (2004) Synthesis of symmetrical and unsymmetrical 9,10-diarylanthracene derivatives via bis-Suzuki–Miyaura cross-coupling reaction. Synthesis 549–557

Kotha S, Lahiri K, Kashinath D (2002) Recent applications of the Suzuki–Miyaura cross-coupling reaction in organic synthesis. Tetrahedron 58: 9633–9695

Kotha S, Shah VR, Halder S, Ramnathan V, Lahiri K (2007) Synthesis of bis-armed amino acid derivatives via the alkylation of ethyl isocyanoacetate and the Suzuki–Miyaura cross-coupling reaction. Amino Acids 32: 387–394

Kouyama T, Mihashi K (1981) Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. Eur J Biochem 114: 33–38

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York, Chapter 11

Lakowicz JR, Weber G (1973) Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. Biochemistry 12: 4171–4179

Malakhov AD, Skorobogatyi MV, Prokhorenko IA, Gontarev SV, Kozhich DT, Stetsenko DA, Stepanova IA, Shenkarev ZO, Berlin YA, Korshun VA (2004) 1-(Phenylethynyl)pyrene and 9,10-bis(phenylethynyl)anthracene, useful fluorescent dyes for DNA labeling: excimer formation and energy transfer. Eur J Org Chem 1298–1307

Miyaura N, Suzuki A (1995) Palladium-catalyzed cross-coupling reactions of organoboron compounds. Chem Rev 95: 2457–2483

Petrich JW, Change NC, McDonald DB, Fleming GR (1983) On the origin of nonexponential fluorescence decay in tryptophan and its derivatives. J Am Chem Soc 105: 3824–3832

Petrich JW, Longworth JW, Fleming GR (1987) Internal motion and electron transfer in proteins: a picosecond fluorescence study of three homologous azurins. Biochemistry 26: 2711–2722

Rzeska A, Malicka J, Guzow K, Szabelski M, Wiczk W (2001) New highly fluorescent amino-acid derivatives: substituted 3-[2-(phenyl)-benzoxazol-5-yl]-alanines: synthesis and photophysical properties. J Photochem Photobiol A 146: 9–18

Stack DE, Hill AL, Diffendaffer CB, Burns NM (2002) Synthesis of a new fluorescent probe specific for catechols. Org Lett 4: 4487–4490

Steinhauser KG, Woolley P, Epe B, Dijk J (1982) Structure of ribosomal protein L6 from *Escherichia coli*. A fluorescence study. Eur J Biochem 127: 587–595

Sugimoto N, Tomka M, Kierzek R, Bevilaqua PC, Turner DH (1989) Effects of substrate structure on the kinetics of circle opening reactions of the self-splicing intervening sequence from *Tetrahymena thermophila*: evidence for substrate and Mg^{2+} binding interactions. Nucleic Acids Res 17: 355–371

Swamy KMK, Jang YJ, Park MS, Koh HS, Lee SK, Yoona YJ, Yoona J (2005) A sorbitol-selective fluorescence sensor. Tetrahedon Lett 46: 3453–3456

Vázquez ME, Rothman DE, Imperiali B (2004) A new environment-sensitive fluorescent amino acid for Fmoc-based solid phase peptide synthesis. Org Biomol Chem 2: 1965–1966

Authors' address: Sambasivarao Kotha, Department of Chemistry, Indian Institute of Technology-Bombay, Powai, Mumbai 400 076, India, Fax: +91-22-2572 3480, E-mail: srk@chem.iitb.ac.in