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A fluorogenic 'click' reaction of azidoanthracene derivatives

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Abstract

Fluorogenic reactions have broad applications in biolabeling, combinatorial synthesis of fluorescent dyes, and materials development. It was recently reported that the highly selective and efficient Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction can be employed in designing new types of fluorogenic reactions. In this study, we report a fluorogenic reaction using anthracene azides as starting materials. The fluorescence of the anthryl core can be greatly inhibited upon introducing electron-donating azido groups in the proximity. Such weakly fluorescent anthracene azides demonstrate high reactivity with a variety of alkynes under the CuAAC conditions producing a strongly fluorescent triazole product with high quantum yields. This reaction can be used in the synthesis and screening of fluorescent dyes combinatorially. Compared with most existing methods, the fluorogenic CuAAC reaction is a much milder and simpler technique to prepare large libraries of fluorescent dyes without further purification. In order to demonstrate the efficiency of using anthracene azides for biolabeling applications, both small molecules and biomolecules including the multialkyne-derivatized cowpea mosaic virus and tobacco mosaic virus had been studied.

1. Introduction

Incorporation of exogenous natural or unnatural tags into proteins and glycans by cellular biosynthetic pathways is an emerging strategy for investigating their cellular activities.[1–3] Since these processes involve multistep enzymatic transformations that prohibit the incorporation of large signaling moieties, chemoselective reactions are often employed for post-labeling.[1,3–6] In this case, a bioorthogonal fluorogenic reaction is invaluable, in which the unreacted reagents show no fluorescent background and the purification can be avoided.[5,7,8]

Being a highly energetic functional group, the organic azide is stable and unreactive with most biomolecules under physiological conditions. Recently, it has been employed as a tag for sequential bioorthogonal labeling via the Bertozzi–Staudinger ligation or the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction.[4,6,9–11] Consequently, the fluorogenic versions of Bertozzi–Staudinger ligation and CuAAC reactions have been reported.[7,12–15] A representative fluorogenic dye developed in our laboratory, 3-azido-7-hydroxycoumarin, has been successfully used for the labeling and visualization of synthesized proteins in different cells and other polymeric systems via the CuAAC reaction.[8,16]

Compared to the coumarin fluorophore, some anthracene derivatives have much higher quantum yields, e.g., 9,10-diphenylanthracene is used as a fluorescent standard in quantum yield determination. In addition, photoinduced electron transfer (PET) process of anthracenes has been widely applied in ion sensing,[17] singlet oxygen detection,[18] screening catalysts,[19] molecular logic gate construction,[20] and cell labeling by recognition of carbohydrates.[21] We therefore exploit the possibility to design a new type of fluorogenic reaction based on the PET process of anthracenes. Due to the high electron density of the α-nitrogen of azido group,[13,14] it was envisioned that the introduction of the azido group close to the anthryl core via a non-conjugated linker would lead to a favorable electron transfer from the azido to the excited anthryl core and induce the quenching of fluorescence. After the CuAAC reaction, the lone pair electrons on nitrogen will become part of the aromatic system and become a much weaker electron donor.

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Therefore, the fluorescence of the anthryl core will be remediated resulting in a fluorogenic phenomenon. In this paper, we report the synthesis of five novel azido derivatized anthracenes and applied the CuAAC reaction to activate their fluorescence. This method was applied to label multiple alkyne organic molecules and large alkyne functionalized biological macromolecules.

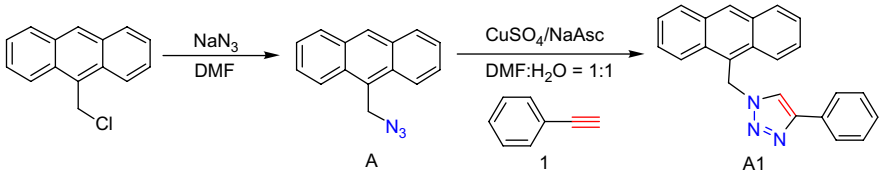
2. Results and discussion

Compound **A** was first synthesized and then reacted with phenylacetylene (**1**) as shown in Scheme 1.[17] The cycloaddition reaction was performed using catalytic amounts of copper sulfate and sodium ascorbate (NaAsc) in a solution of DMF and water (v/v=1:1).[13,22] At the same concentration in pure DMSO, while the triazolyl product **A1** shows almost the same absorption intensity as **A**, its fluorescent emission intensity is 75-fold stronger than that of **A** (Fig. 1). The quantum yield of **A1** is 0.96, much higher than that of **A** (~0.02). Moreover, it should be noticed that there is no shift in emission and excitation wavelength accompanying the change of fluorescence intensity. All these results indicate a PET process between azido group and anthryl core that can be adopted for designing a new type of fluorogenic CuAAC reaction.

In order to examine the scope of the fluorogenic CuAAC reaction of anthracene derivatives, four more anthracene azides (**B**–**E**) were synthesized via easy transformations (Scheme 2). Azido groups were attached to the anthracene core at 1-, 2- or 9-position. In all cases, the fluorescence was quenched efficiently. All azides are quite stable and no decomposition has been found after storing on the shelf or refluxing in toluene for a long period of time. Each azide exhibited high reactivity with a variety of alkynes regardless of the structures under standard CuAAC condition (see Table 1).[22,23] A comparison between the maximum emission intensity of the cycloaddition products **A1**–**E1** and that of the corresponding azides is shown in Figure 2. The fluorescent emission of all triazole products is enhanced dramatically compared to the starting azides except **C**, which shows a noticeable fluorescence and a bathochromic shift of fluorescence due to the introduction of 9-cyano group (Fig. S1).

Since the CuAAC reaction can tolerate a great diversity of the structural features of azides and alkynes, it is possible to screen the fluorescent properties of the cycloaddition products in relation to the starting alkynes combinatorially. In our study, 5 different anthracene azides **A**–**E** and 34 terminal alkynes **2**–**35** (Table 2) were loaded in microtiter plates. Each well thus represented a unique combination of anthracene and alkyne. The total volume of each reaction mixture was 200 μL (DMSO–H_2O=1:1) containing 1 mM azide and alkyne catalyzed with 2 mM $CuSO_4$ and 5 mM NaAsc. Most reactions were completed in 24 h at room temperature as monitored by TLC or mass spectrometry.

The formation of the fluorescent triazole products is easily visualized upon irradiation at 365 nm with a hand-held UV lamp. Without purification the emission of reaction mixtures was directly evaluated using a Varian Eclipse fluoriphotometer. As shown in Figure 3 and Table S1 in Supplementary data, most triazole products of azides **A** and **B** had distinct enhancement in fluorescent intensity regardless of the structures of alkynes. Although some factors like reaction yield and coordination with Cu^{2+} or Cu^{+} ion would influence the emission intensity of products,[17] we still can get useful information on the fluorescent properties of the final products.



Scheme 1. Synthesis of azide **A** and product **A1**.

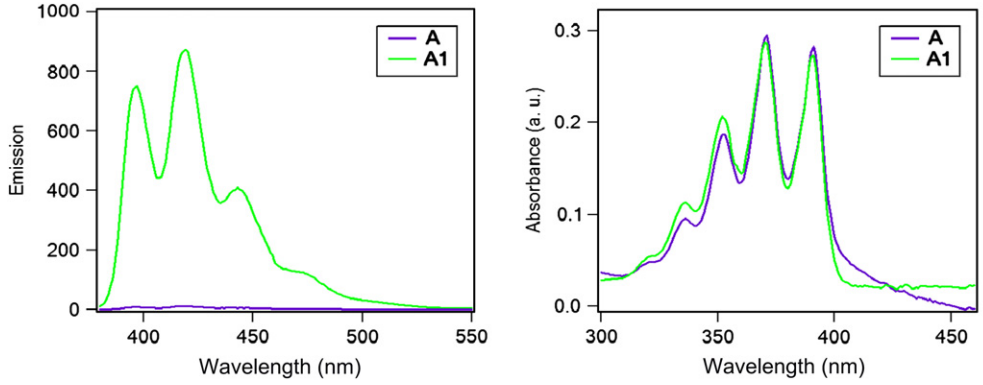
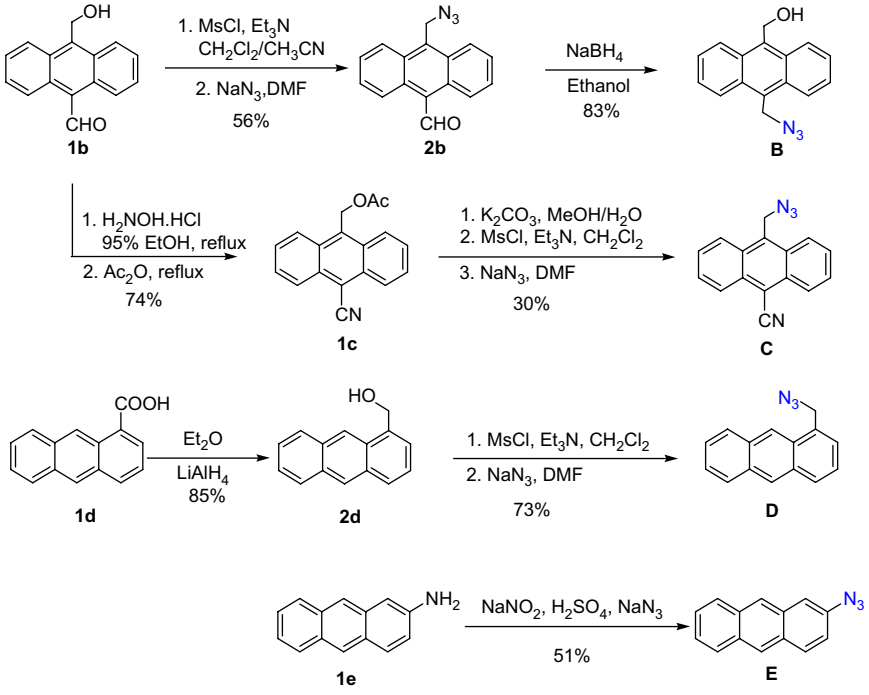


Figure 1. Comparison of fluorescent emission (left, excited at 370 nm) and absorption spectra (right) of **A** and **A1** in DMSO (10 μM for emission spectra and 50 μM for absorption spectra).



Scheme 2. Synthesis of anthracene azides **B**–**E**.

Since CuAAC reaction can tolerate a variety of solvents (including water), pH ranges, and be compatible to most of the functionalities, azide and alkyne groups have been broadly used as biocompatible/chemocompatible tags for biomacromolecules and polymers. The fluorogenic CuAAC reaction reported here can therefore be used in the post-labeling of polyvalent alkyne displayed systems, in particular, in a biological environment.

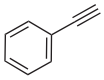
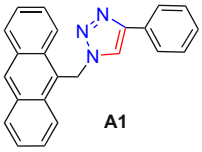
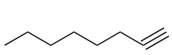
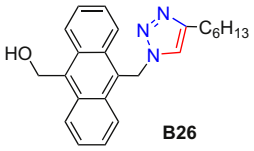
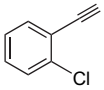
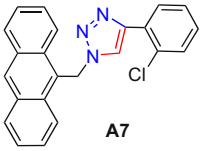
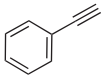
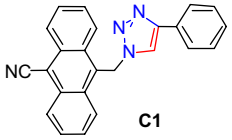
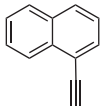
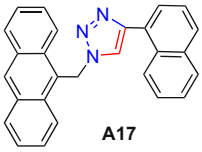
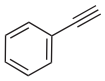
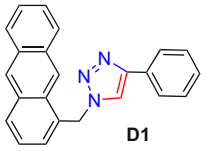
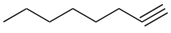
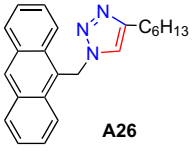
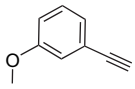
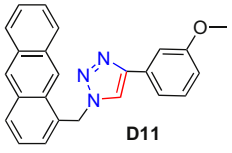
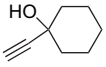
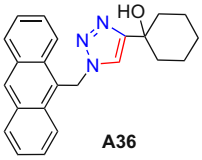
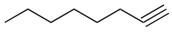
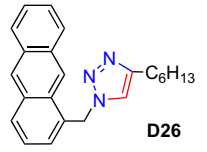
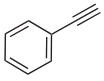
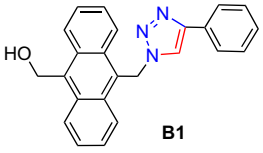
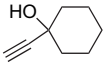
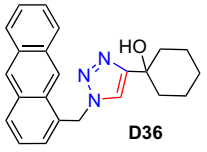
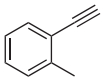
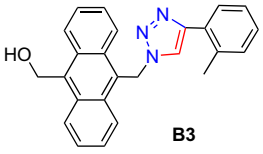
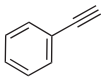
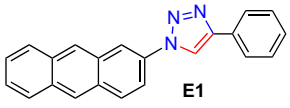
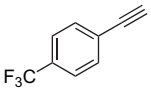
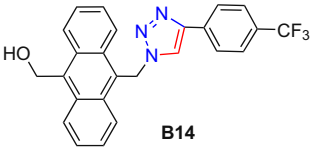
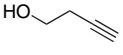
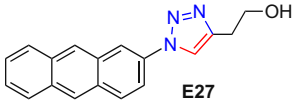
We chose trispropynyloxybenzene (**37**), alkyne-derivatized cowpea mosaic virus (**CPMV**), and tobacco mosaic virus (**TMV**), which have 3, 60, and 2130 terminal alkyne units, respectively, as multivalent model systems to demonstrate the feasibility of this selective post-labeling strategy. Catalyzed with in situ generated Cu(I), **37** can be fluorescently derivatized by **A** in high efficiency (Scheme 3). As previously reported, **CPMV** has been employed as a nano building block for many organic reactions.[24–28] *N*-Hydroxysuccinimidal ester of **34** was synthesized and used to attach terminal alkynes to the reactive lysines of **CPMV**.[29] The newly synthesized **CPMV–Alkyne** was sequentially subjected to the CuAAC reaction with **B** catalyzed through the addition of CuBr and a bathophenanthroline ligand (Scheme 3).[30] Gel electrophoresis confirmed the covalent attachment of fluorescent anthracene units on **CPMV** after cycloaddition reaction (Fig. 4A). The integrity of the **CPMV** products was confirmed by TEM and size-exclusion chromatography (Fig. 4B and C). Due to the strong fluorescence of the cycloaddition product (the quantum yield of the model compound **B34** is 0.51 as compared to azide **B** Φ=0.02), as low as 0.5 nM of the cycloaddition product could be detected without the interference of starting materials.

Similarly, the versatility of this fluorogenic reaction is demonstrated via the CuAAC reaction on the surface of **TMV**, a rod-like particle consisting of 2130 identical protein subunits arranged helically around a single RNA strand.[31] **TMV** is initially subjected to an electrophilic substitution reaction at the *ortho*-position of the phenol ring of tyrosine-139 residues with diazonium salts to insert the alkyne functionality.[32] MALDI-TOF MS analysis indicated that >95% of the capsid monomers were converted into alkyne derivatives (Fig. 5A). The sequential CuAAC reaction is achieved with greatest efficiency using a combination of $CuSO_4$ with NaAsc (Scheme 3).[33] The efficiency of the reaction is easily monitored using MALDI-TOF MS (Fig. 5A) with shift in mass from 17,664 *m/z* (**TMV–Alkyne**) to 17,918 *m/z* (**TMV–Anthracene**) and UV–vis absorption in the 380–420 nm range (Fig. 5C). Due to the often destructive nature of organic reactions on viral particles, the stability of **TMV** was monitored by TEM analysis (Fig. 5B) in conjunction with size-exclusion chromatography (Fig. S9) and was found to remain intact and stable throughout the reaction.[33]

3. Conclusions

In summary, we report here a new bioorthogonal fluorogenic reaction based on the PET principle, which affords an efficient way to link two entities together while the conjugating efficiencies could be reported by the fluorescent emission assays. It should have broad application in the emerging field of cell biology and functional proteomics due to the high reaction efficiency of CuAAC reaction at mild reaction conditions and the distinct fluorescence properties of products. The high reactivity and selectivity between azides and terminal alkynes allow further applications in ligating other biomolecules, polymers, nanoparticles and surfaces, which are under exploration. In addition, since it is generally difficult to construct the

Table 1
CuAAC reactions of anthracene azides **A**–**E** with some typical alkynes

Entry	Azide	Alkyne	Product	Yield[a] (%)	Entry	Azide	Alkyne	Product	Yield[a] (%)
1	**A**		 **A1**	95[b]	9	**B**		 **B26**	93[c]
2	**A**		 **A7**	66	10	**C**		 **C1**	81
3	**A**		 **A17**	71	11	**D**		 **D1**	97
4	**A**		 **A26**	86	12	**D**		 **D11**	93
5	**A**		 **A36**	92	13	**D**		 **D26**	97
6	**B**		 **B1**	69	14	**D**		 **D36**	60
7	**B**		 **B3**	63[c]	15	**E**		 **E1**	66
8	**B**		 **B14**	79[c]	16	**E**		 **E27**	67

[a] Isolated yields.
[b] Quantum yields of azide **A** and the product **A1** are 0.02 and 0.97.
[c] Quantum yields: **B** 0.02, **B3** 0.95, **B14** 0.95, and **B26** 0.97.

highly conjugated fluorophore, only a few reactions have been applied in the combinatorial synthesis of fluorescent compounds including condensation reactions using aldehydes and transition metal catalyzed coupling reactions.[34–39] The CuAAC reaction distinguishes from others by its mild reaction conditions and high transformation efficiency regardless of the structures of azides and alkynes, which make it an ideal reaction to synthesize fluorescent dyes in a combinatorial manner.

4. Experimental section

4.1. General methods

Unless otherwise noted, all chemicals and solvents were obtained from commercial suppliers and used without purification. ^{1}H NMR spectra were recorded on Varian 300 NMR spectrometer and ^{13}C NMR spectra were recorded either on Varian 300 NMR or on Varian 400 NMR spectrometer. The

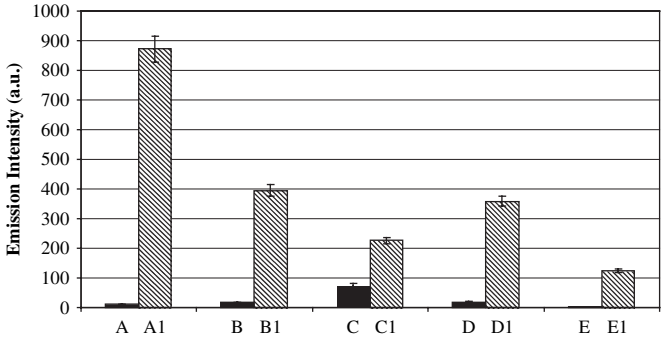


Figure 2. The comparison between the maximum emission intensity of anthracene azides (**A**–**E**) and their respective product with alkyne **1**. All samples were measured in DMSO solution ([*c*]=10 μM). The original spectra are given in Supplementary data.

UV–vis absorption spectra were measured on Agilent 8453 spectrometer. Emission spectra were measured on Varian Cary Eclipse fluorescence spectrophotometer. Ultracentrifugation was performed at the indicated speeds using a Beckman Optima™ L-90K Ultracentrifuge equipped with either SW41 or 50.2 Ti rotors. TEM analyses of viruses were carried out by depositing 20 μL aliquots of each sample at a concentration of 0.1–0.3 mg/mL onto 100-mesh carbon-coated copper grids for 2 min. The grids were then stained with 20 μL of 2% uranyl acetate and viewed with a Hitachi H-8000 TEM electron microscope. MALDI-TOF MS analyses of viral subunit were carried out using a Bruker Ultra-Flex I TOF/TOF mass spectrometer with MS grade sinapinic acid in 70% acetonitrile and 0.1% TFA as the matrix. In general, the viruses were denatured after being treated with guanidinium-HCl (6.0 M, 4 μL) for 5 min at room temperature. The denatured protein was spotted onto a MALDI plate using Millipore ZipTip-C18® tips to remove the salts before the MS analysis. The quantum yields of anthracene derivatives were determined using a literature method.[40]

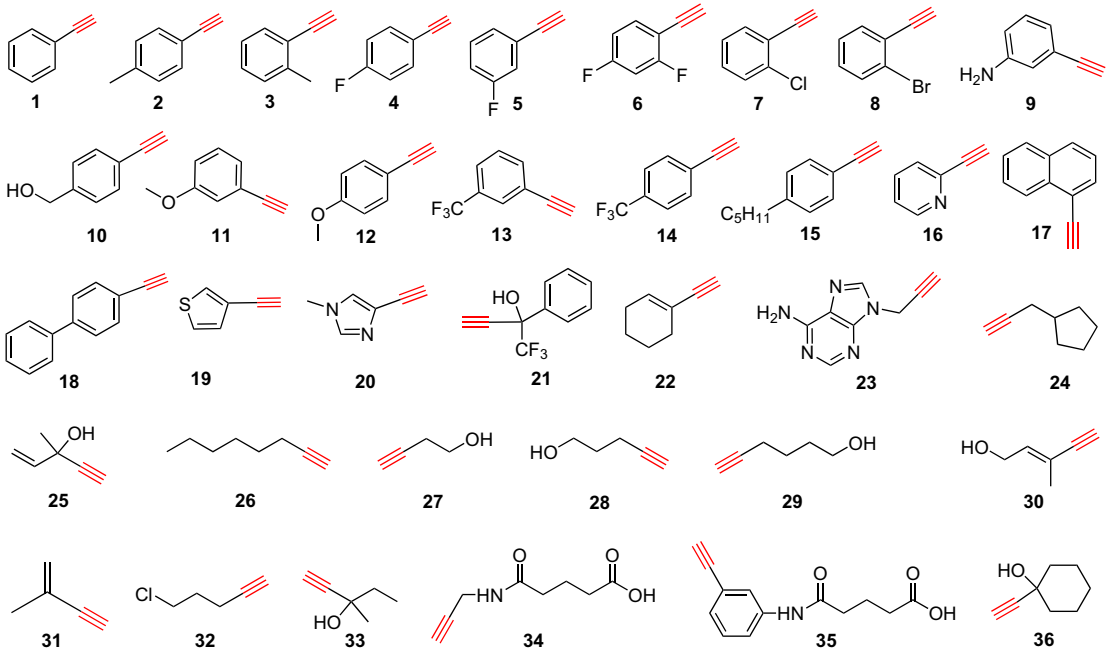
4.2. Synthesis of azides

The preparation of **A**,[17,41] **1b**,[42] and **1d**[43] has previously been described.

4.2.1. 10-Azidomethylanthracene-9-carbaldehyde (*2b*)

To the stirred suspension of **1b** (5.22 g, 22 mmol) in 200 mL dry CH_2Cl_2 and 200 mL dry acetonitrile under nitrogen at 0 °C were added dry triethylamine (3.35 g, 33 mmol) and methanesulfonyl chloride (3.15 g, 27.5 mmol). The mixture was stirred at room temperature for 4 h under nitrogen before an aq HCl (1 N, 70 mL) was added to the resulting solution. The organic layer was washed by water (50 mL×2) and brine, and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was dried under vacuum. The resulted solid was dissolved in DMF (250 mL), and NaN_3 (2.1 g, 33 mmol) was added. The mixture was stirred at room temperature overnight. Water (200 mL) and ethyl acetate (EtOAc, 200 mL) were added. The aqueous layer was extracted by EtOAc (50 mL×2) and the combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (EtOAc–hexane=1:9 to 1:4) to give **2b** as a yellow solid (3.1 g, 56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=11.5 (s, 1H), 8.92–8.88 (m, 2H), 8.41–8.38 (m, 2H), 7.74–7.65 (m, 4H), 5.37 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=194.0, 133.7, 131.2, 130.4, 128.6, 127.7, 127.2, 124.6, 124.5, 46.6.

Table 2
The alkyne building blocks used in this study



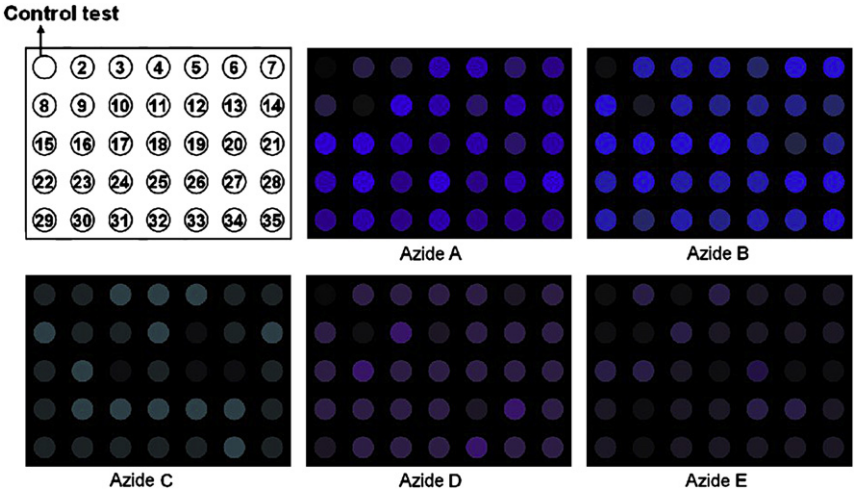


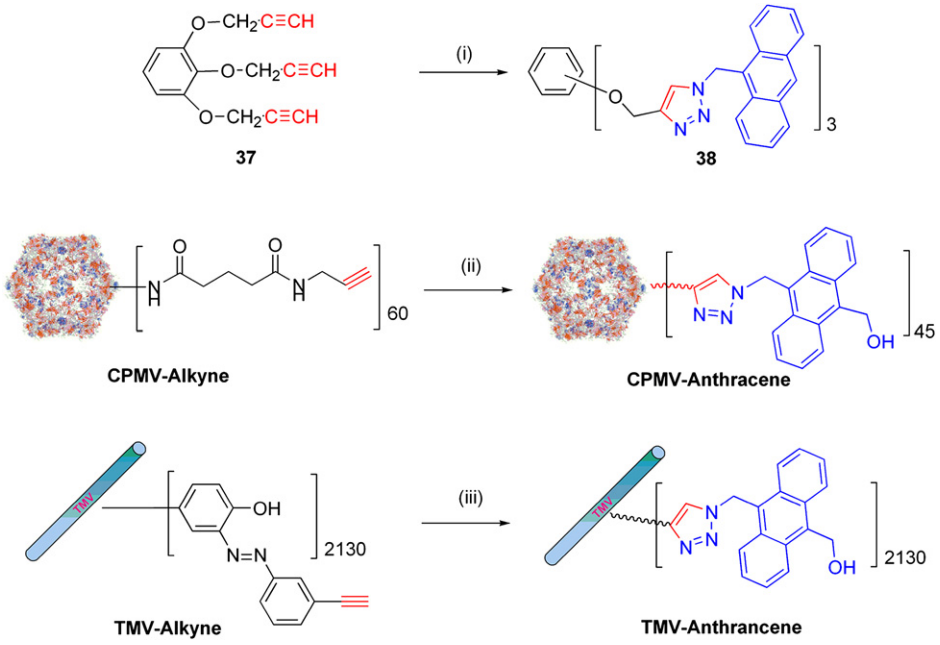
Figure 3. Schematic demonstration of the well plate reactions between azidoanthracenes (**A**–**E**) and alkynes (**2**–**35**). Each cell's color represents the emission property of every compound based on the values of Hue, Sat and Lum in an HSL color model. Hue value represents the maximum emission wavelength of each compound and Lum value represents the maximum emission intensity (more details could be found in Supplementary data).

*4.2.2. (10-Azidomethylanthracen-9-yl)methanol (**B**)*

$NaBH_4$ was added in small portions to the suspension of **2b** (360 mg, 1.38 mmol) in 50 mL ethanol at 0 °C. The reaction mixture was stirred at room temperature for 8 h. Concentrated HCl solution was added dropwise carefully to destroy the unreacted $NaBH_4$ and then water was added until pH was neutral. Dichloromethane (150 mL) was added and the organic layer was separated, washed with brine, and dried over anhydrous Na_2SO_4. The solvents were removed under reduced pressure and the residue was purified by flash chromatography on silica gel (EtOAc–hexane=1:4) to give **B** as a yellow solid (300 mg, 83%). ^{1}H NMR (300 MHz, DMSO): δ=8.57–8.47 (m, 4H), 7.67–7.60 (m, 4H), 5.52 (s, 2H), 5.49–5.41 (m, 3H); ^{13}C NMR (75 MHz, DMSO) δ=135.6, 130.8, 130.2, 127.5, 126.9, 126.3, 125.2, 56.2, 46.3. HRMS calculated for $C_{16}H_{13}N_3O$ (M^+): 263.1059, found: 263.1055.

*4.2.3. Acetic acid (10-cyanoanthracen-9-yl)methyl ester (**1c**)*

To a suspension of **1b** (0.81 g, 3.43 mmol) in 19 mL of 95% EtOH (19 mL) was added hydroxylamine hydrochloride (280 mg, 4.02 mmol, neutralized with Na_2CO_3) in 4 mL of water. The mixture was heated on a steam bath for 30 min, cooled, and diluted with water. The solid was collected, dried under low pressure, and then acetic anhydride (16 mL) was added. The resulting solution was refluxed for 8 h and then added ice water. Yellow precipitate was collected by filtration,



Scheme 3. Reaction conditions: (i) **A**, $CuSO_4$, NaAsc, DMF–H_2O=1:1, 24 h; (ii) **B**, CuBr, bathophenanthroline, pH 8.5 buffer–DMF=4:1, 24 h; (iii) **B**, $CuSO_4$, NaAsc, pH 8.5 buffer–DMF=4:1, 24 h.

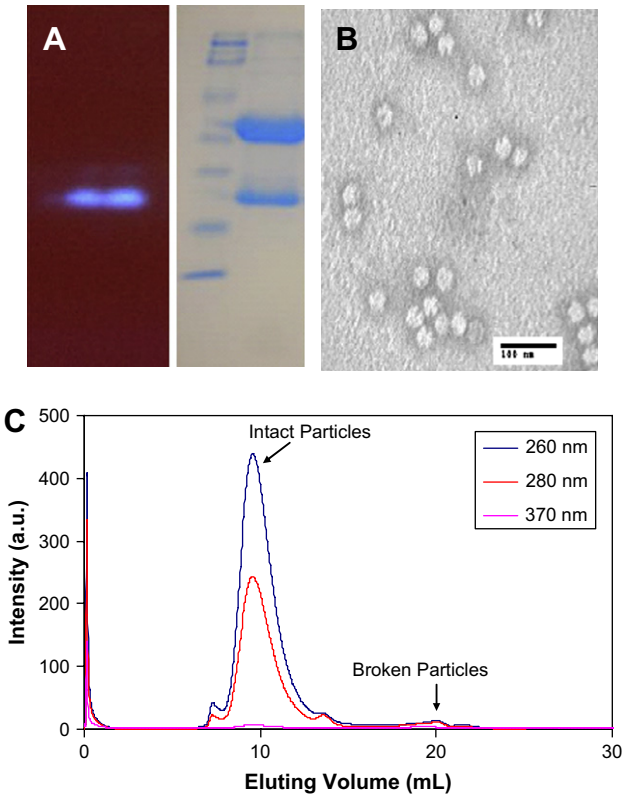


Figure 4. (A) SDS-PAGE of **CPMV–Anthracene** visualized under UV irradiation (left) and upon staining with Coomassie Blue (right). (B) TEM image of **CPMV–Anthracene**. The scale bar is 100 nm. (C) Size-exclusion FPLC analysis of **CPMV–Anthracene**.

washed with water, and finally purified by flash chromatography on silica gel (EtOAc–hexane=1:9 to 1:4) to give **1c** as a light yellow solid (0.70 g, 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.52–8.43 (dm, J=7.2 Hz, 4H), 7.78–7.67 (m, 4H), 6.16 (s, 2H), 2.09 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ=171.1, 133.4, 133.1, 130.4, 128.8, 127.7, 126.4, 125.1, 117.3, 108.3, 58.4, 21.1.

*4.2.4. 10-Azidomethylanthracene-9-carbonitrile (**C**)*

To the stirred suspension of **1c** (220 mg, 0.8 mmol) in methanol (40 mL) and water (10 mL) was added potassium carbonate (237 mg, 60 mmol). The mixture was stirred at room temperature overnight before EtOAc (100 mL) was added into the reaction mixture. The organic layer was washed by aq HCl (1.0 *N*, 50 mL) and brine, and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was dried under vacuum. A similar procedure of preparation of **B** was followed to yield **C** as a yellow solid (60 mg, overall yield 30% from **1c**). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.53 (dm, J=7.8 Hz, 2H), 8.39 (dm, J=7.8 Hz, 2H), 7.80–7.69 (m, 4H), 5.37 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=133.2, 133.0, 130.1, 128.9, 127.9, 126.7, 124.6, 46.4. HRMS calculated for $C_{16}H_{10}N_4$ (M^+): 258.0905, found: 258.0908.

*4.2.5. Anthracen-1-ylmethanol (**2d**)*

To the dispersion of **1d** (4.0 g, 18 mmol) in dry ether (250 mL), $LiAlH_4$ (0.9 g, 26 mmol) was added in portions with vigorous stirring. The mixture was then stirred at room temperature under nitrogen for 30 min and refluxed for additional 8 h. The reaction was quenched with water (10 mL) and concentrated aq HCl (20 mL). The aqueous solution was separated and extracted by EtOAc (50 mL×2). The combined organic solution was washed with aq HCl (1 N, 20 mL), saturated aq $NaHCO_3$ (20 mL), and H_2O (20 mL), and dried on anhydrous Na_2SO_4 to yield crude product **2d** (3.2 g, 85%). ^{1}H NMR (300 MHz, DMSO): δ=8.69 (s, 1H), 8.58 (s, 1H), 8.13–8.05 (m, 2H), 8.00 (d, J=8.4 Hz, 1H), 7.57–7.45 (m, 4H), 5.46 (t, J=5.1 Hz, 1H), 5.12 (d, J=5.1 Hz, 2H); ^{13}C NMR (75 MHz, DMSO): δ=138.5, 132.1, 131.7, 131.5, 129.7, 129.1, 128.5, 128.2, 127.2, 126.3, 126.2, 125.8, 124.1, 122.9, 62.0. HRMS calculated for $C_{15}H_{12}O$ (M^+): 208.0888, found: 208.0889.

*4.2.6. 1-Azidomethylanthracene (**D**)*

The similar procedure of preparation of **2b** was followed to prepare **D** as a yellow solid (yield 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.57 (s, 1H), 8.47 (s, 1H), 8.08–7.99 (m, 3H), 7.54–7.40 (m, 4H), 4.88 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=132.2, 131.9, 131.2, 130.1, 129.7, 128.8, 128.2, 127.6, 127.1, 126.1, 126.0, 124.7, 122.6, 53.7. HRMS calculated for $C_{16}H_{15}N_3$ (M^+): 233.0953, found: 233.0945.

*4.2.7. 2-Azidoanthracene (**E**)*

A solution of sodium nitrite (220 mg, 3.13 mmol) in water (8 mL) was added dropwise to a solution of **1e** (450 mg, 2.45 mmol) in water (15 mL) and concentrated sulfuric acid (3 mL) at 0 °C over 5 min. The reaction mixture was stirred at 0 °C for 30 min before a solution of sodium azide (0.3 g, 4.4 mmol) in water (5 mL) was added dropwise over 10 min. The solution was slowly warmed to room temperature and kept stirring for 5 h. The precipitate was isolated by filtration, washed with water, and purified by column chromatography on a silica column to yield a yellow solid **E** (260 mg, 51%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.40 (s, 1H), 8.32 (s, 1H), 8.02–7.96 (m, 3H), 7.59 (m, 1H), 7.63–7.42 (m, 2H), 7.17–7.13 (dd, J=9.0, 2.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO): δ=136.4, 131.8, 131.3, 130.8, 128.9, 128.2, 127.8, 126.4, 126.1, 125.5, 124.9, 119.4, 115.0. HRMS calculated for $C_{14}H_9N_3$ (M^+): 219.0796, found: 219.0799.

4.3. Typical experimental procedure for preparative scale CuAAC reactions

Azide **A** (69 mg, 0.30 mmol) and phenylacetylene (31 mg, 0.30 mmol) were suspended in a 3:1 mixture (v/v) of DMF and water (24 mL). Copper sulfate (7 mg, 0.045 mmol), sodium ascorbate (14 mg, 0.09 mmol), and tris[(*N*-benzyltriazolyl)methyl]amine ligand[9] (8 mg, 0.015 mmol) were added. The mixture was then stirred at room temperature for 16–24 h (monitored by TLC). After the reaction was complete, EtOAc (20 mL×2) was added to extract the product. The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on

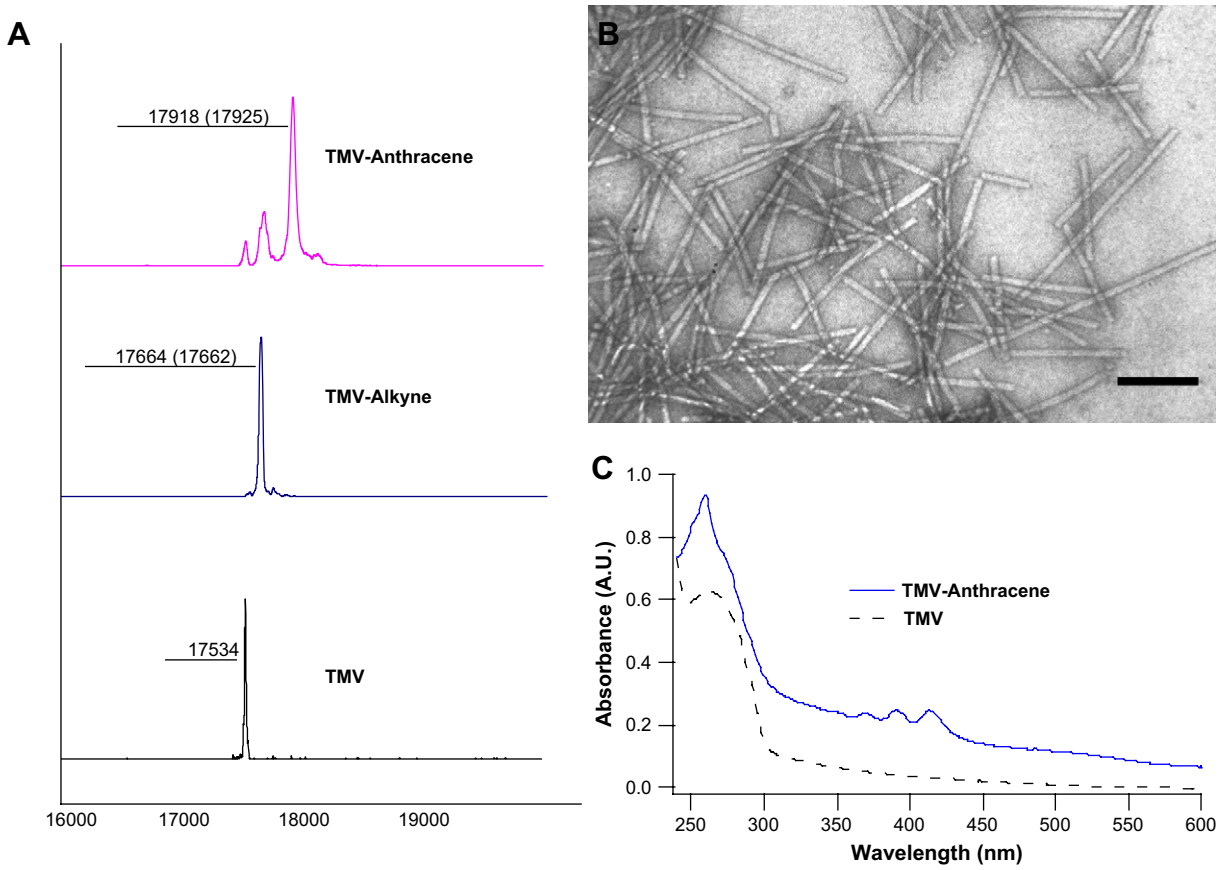


Figure 5. (A) MALDI-TOF MS data of **TMV**, **TMV−Alkyne**, and **TMV−Anthracene** indicating >95% reactivity per subunit. (Numbers in parenthesis refer to the expected masses.) (B) TEM image of **TMV** post-CuAAC functionalization with anthracene. The scale bar is 200 nm. (C) UV−vis spectra of **TMV** and **TMV−Anthracene**.

silica gel (EtOAc−hexane=1:9 to 1:4) to yield compound **A1** as a yellow solid (0.095 g, 95%).

4.4. Typical experimental procedure of combinatorial CuAAC reactions on a 96-microwell plate

The reaction mixture in each well contained a solution of anthracene azides (1.0 mM), alkynes (1.0 mM), copper sulfate (2.0 mM), sodium ascorbate (10.0 mM), and tris[(*N*-benzyl-triazolyl)methyl]amine ligand (0.1 mM) in 200 μL DMSO−water (v/v=1:1). For each of the five anthracene azides one control test was performed, which contained all the above components of the reaction except the alkyne. The reactions including the control test were allowed to incubate at room temperature for 24 h. Then the mixture in each well was diluted 100 times before the fluorescence intensity was measured accordingly at proper excitation wavelength. The excitation wavelength was taken as the maximum of the band in the UV spectrum.

*4.5. Multivalent CuAAC reactions of azidoanthracenes—reaction of alkyne **37** with azide **A***

By following the protocol of preparative scale CuAAC reactions, **38** was obtained as a yellow solid (85%). ^{1}H NMR (300 MHz, DMSO): δ=8.68 (s, 2H), 8.61 (s, 1H), 8.57−8.50 (m, 6H), 8.10 (d, J=8.7 Hz, 4H), 8.05 (d, J=8.4 Hz, 2H), 7.99 (s, 2H), 7.84 (s, 1H), 7.60−7.43 (m, 12H), 6.75 (t, J=7.8 Hz, 1H), 6.59−6.56 (m, 6H), 6.47 (s, 2H), 4.78 (s, 4H), 4.70 (s, 2H); ^{13}C NMR (100 MHz, DMSO): δ=152.5, 144.1, 143.3, 137.4, 131.7, 131.6, 131.0, 130.9, 129.7, 129.6, 127.8, 127.7, 126.5, 126.4, 126.0, 125.9, 124.8, 124.7, 124.6, 124.2, 108.2, 65.9, 62.4, 46.1, 45.9. HRMS calculated for $C_{60}H_{45}N_9O_3$ (M^+): 940.3723, found: 940.3707.

*4.6. Labeling of alkyne-derivatized **CPMV** with azide **B***

Multialkyne-derivatized **CPMV** was synthesized according to the literature protocol.[9] A mixture of **CPMV−Alkyne** (4 mg/mL), bathophenanthroline ligand (4 mM), CuBr (1 mM), and anthracene azide **B** (100 mM) in HEPES buffer (pH=8.5) solution (containing 20% DMF) was incubated at 4 °C for 24 h with nitrogen. **CPMV−Anthracene** was then purified by a sucrose gradient ultra-sedimentation. TEM analysis was performed by depositing 20 μL of purified sample onto 100-mesh carbon-coated grids. After waiting for 2 min, the grids were stained with uranyl acetate and viewed with Hitachi-8000 electron microscope. FPLC analysis was performed with AKTA Explorere (Amersham Pharmacia Biotech) equipment, using Superose™-6 size-exclusion columns. SDS-PAGE analysis was carried out in Bio-Rad MiniPROTEAN® 3 gel electrophoresis cell. **CPMV** (1 μg) was denatured by heating at 95 °C for

5 min with Tris–HCl buffer containing β-mercaptoethanol, bromophenol blue, and glycerol. The proteins were then resolved on a 15% polyacrylamide gel at 200 V for 1 h. **CPMV–Anthracene** was visualized with a UVP Epi Chemi II imager under UV irradiation before staining. For analysis by coomassie blue staining, the gel was stained with Bio-Rad Biosafe® Coomassie Blue for 1 h and destained with distilled water.

*4.7. Labeling of **TMV** with azide **B***

TMV–Alkyne was prepared using reported procedure.[33] Anthracene azide **B** in a 20% DMSO aqueous solution (100 mM, 40 μL) and **TMV–Alkyne** (15 mg/mL, 200 μL) were mixed in Tris buffer (10 mM, pH 8.0, 660 μL). Then $CuSO_4$ (100 mM, 10 μL) and sodium ascorbate (200 mM, 10 μL) were added before the mixture was incubated at room temperature for 18 h. The reaction mixture was then purified via an ultra-sedimentation on a 10–50% sucrose gradient column. The collected modified **TMV** was then pelleted by ultracentrifugation at 160,000*g* for 2.5 h. The pellet was dissolved in 0.01 M PBS buffer and characterized.

Acknowledgements

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Supplementary data

^1H and ^{13}C NMR of all the triazole products, the absorption and emission spectra of compounds in Table 1; the optical data of all products from 96-well plate reaction; and the details of making Figure 3. This material is available free of charge via the internet. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.01.080.

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