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Alkynylpyrenes as Improved Pyrene-Based Biomolecular Probes with the Advantages of High Fluorescence Quantum Yields and Long Absorption/Emission Wavelengths

Hajime Maeda,[a] Tomohiro Maeda,[a] Kazuhiko Mizuno,*[a] Kazuhisa Fujimoto,*[b] Hisao Shimizu,[b] and Masahiko Inouye[b, c]

Abstract: The photochemical properties of various alkynylpyrene derivatives have been investigated in detail with a view to developing a new class of pyrene-based biomolecular probes. The absorption maxima of the alkynylpyrenes were seen to be shifted to longer wavelengths compared with those of the unsubstituted parent pyrene. Fluorescence quantum yields of the alkynylpyrenes dramatically increased up to 0.99 in ethanol, and only slight quenching of the fluorescence occurred even under aerated conditions. The alkynylpyrenes have been successfully introduced into representative biomolecules such as peptides, proteins, and DNAs. The detectabilities of the labeled biomolecules were significantly improved, with the unique photochemical characteristics of the pyrene nucleus being maintained.

Keywords: alkynylpyrenes · biosensors · fluorescence · fluorophores · photochemistry

Introduction

Fluorescent probes are useful in investigations of many aspects of biomolecules, such as their conformations and interactions, in nonradioactive detection and visualization modes.[1] This usefulness is largely due to the ready availability of fluorophores with a variety of fluorescence properties, their specificity and versatility in labeling, and the availability of highly sophisticated fluorescence detection instruments. Among the available fluorophores, pyrene and its derivatives have long been attractive by virtue of their inherent chemical and photochemical characteristics.[2] Indeed, the simple polyaromatic hydrocarbon skeleton does not require any protection when being incorporated into peptides or oligonucleotides. Also inspiring are excimer formation in these systems and their rather long fluorescence lifetimes,[2,3] which have prompted researchers to explore various applications, such as the development of novel strategies for detecting DNA and RNA,[4] observing protein–substrate and protein–protein interactions,[5] demonstrating conformational changes of proteins,[6] and monitoring the real-time dynamics of biological membranes.[7] Furthermore, unlike benzopyrene, pyrene itself is nonmutagenic and has a high LD_{50} (50% lethal dose; 250 mg kg^{-1}, mice), which makes it a more favorable candidate for possible use in applications in vivo.[8] At the same time, however, these fascinating photochemical properties of pyrene also result in serious drawbacks, such as the substantial quenching of its fluorescence by the presence of oxygen[9] and by electron-donating and -accepting molecules that may exist in vivo,[10] and the low fluorescence quantum yield in protic solvents.[4c,11] Moreover, the relatively short absorption wavelengths of pyrene and its simple derivatives are unsatisfactory because several biomolecules and biomolecular segments will also be excited upon irradiation at the same wavelength. To overcome such disadvantages of pyrene-based fluorophores, Berlin et al. have very recently reported the utilization of (phenylethyn-

[a] Dr. H. Maeda, T. Maeda, Prof. K. Mizuno
Department of Applied Chemistry
Graduate School of Engineering, Osaka Prefecture University
1-1 Gakuen-cho, Sakai, Osaka 599-8531 (Japan)
Fax: (+81) 72-254-9289
E-mail: mizuno@chem.osakafu-u.ac.jp

[b] Dr. K. Fujimoto, H. Shimizu, Prof. M. Inouye
Faculty of Pharmaceutical Sciences
Toyama Medical and Pharmaceutical University
Sugitani 2630, Toyama 930-0194 (Japan)
Fax: (+81) 76-434-5049
E-mail: fujimoto@ms.toyama-mpu.ac.jp

[c] Prof. M. Inouye
PRESTO, Japan Science and Technology Agency (JST)

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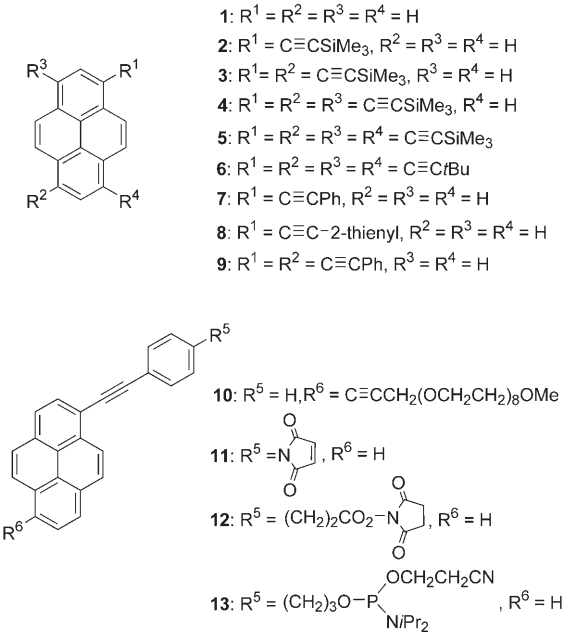
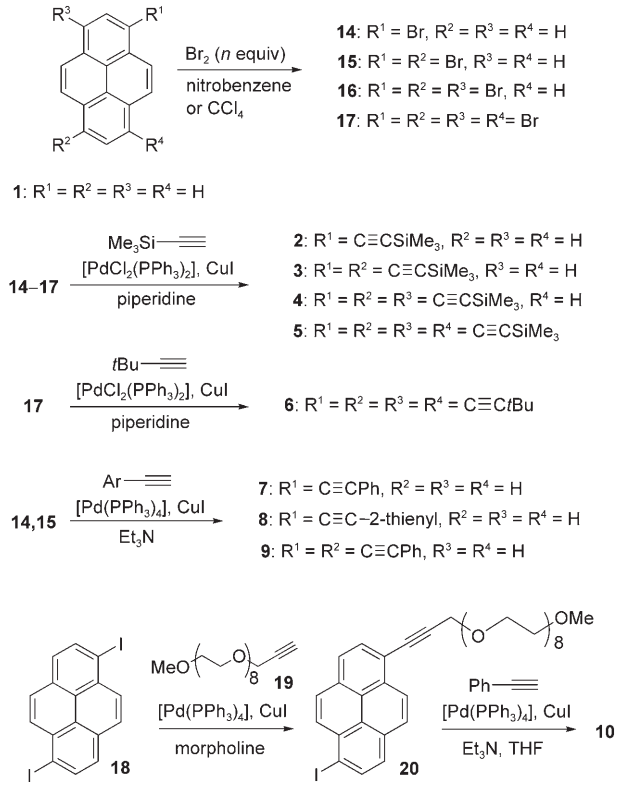
yl)pyrenes as fluorescent dyes for DNA labeling.[12] Although they pointed out that the (phenylethynyl)pyrenes exhibit high fluorescence quantum yields, systematic and quantitative studies of their photochemical characteristics[13] as well as comparisons with those of the parent pyrene remain elusive, as is also the case for many other similar systems. During the course of our studies on pyrene-based biomolecular probes,[4a,14] we have also noted the influence of alkynyl substituents on the photochemical properties of pyrene nuclei. Thus, we report herein in detail the synthesis, photochemical data, and biological applications of various alkynylpyrenes; the results presented here can be expected to lead to fruitful applications in biological investigations.

Results and Discussion

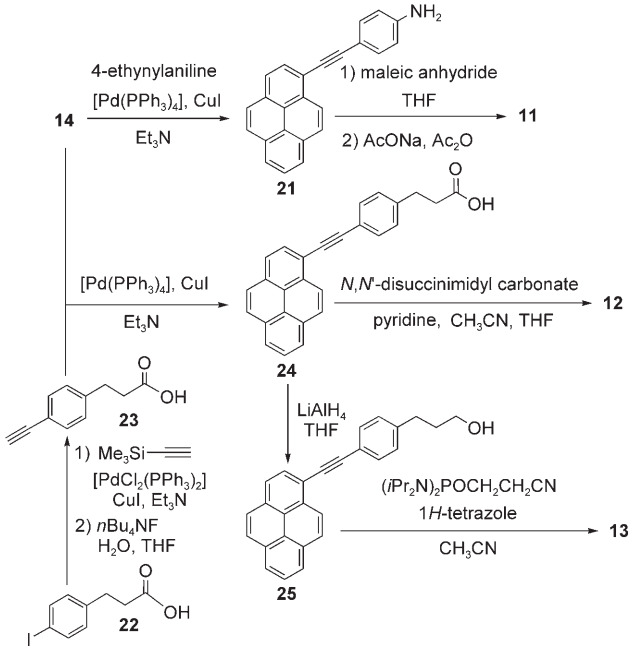
Synthesis of the alkynylpyrenes: Most alkynylpyrenes (pyrene−C≡C−R), where R is pyridyl,[15] bipyridyl,[15] terpyridyl,[15] quinolyl,[16] thienyl,[17] ferrocenyl,[18] or some other group,[19–21] have been synthesized by Sonogashira coupling reactions.[22] We have prepared a series of alkynylpyrenes **2**–**13** according to this method, as shown in Schemes 1 and 2.



Bromination of pyrene (**1**) with one to four equivalents of bromine gave the mono-, di-, tri-, and tetrabromopyrenes, respectively (1-bromopyrene (**14**), 1,6-dibromopyrene (**15**), 1,3,6-tribromopyrene (**16**), and 1,3,6,8-tetrabromopyrene (**17**)).[23] Cross-coupling of these bromopyrenes with (trimethylsilyl)acetylene under the conditions of the Sonogashira reaction afforded mono-, bis-, tris-, and tetrakis(trimethylsilylethynyl)pyrenes **2**–**5**, respectively. Tetrakis(*tert*-butylethynyl)pyrene **6** and (arylethynyl)pyrenes **7**–**9** were synthesized in a similar manner by using the corresponding



Scheme 1. Synthesis of alkynylpyrenes **2**–**10**.



Scheme 2. Synthesis of alkynylpyrenes **11**–**13**.

acetylene derivatives. Unsymmetrical dialkynylpyrene **10**, designed as a water-soluble alkynylpyrene, was prepared in a stepwise manner via monosubstituted **20** starting from 1,6-diiodopyrene (**18**) and the hydrophilic acetylene **19**.

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Alkynylpyrenes **11**–**13** for biomolecular probes were each prepared from 1-bromopyrene (**14**). Sonogashira reactions of **14** with 4-ethynylaniline and 3-(4-ethynylphenyl)propionic acid (**23**) gave intermediates **21** and **24**, respectively. Condensation of **21** with maleic anhydride afforded the maleimide derivative **11**, which is capable of reacting with the Cys residues of peptides and proteins. The succinimidyl ester **12** was synthesized by treating **24** with *N*,*N*′-disuccinimidyl carbonate. Intermediate **24** was also transformed to the phosphoramidite **13** via the alcohol derivative **25**. Both **12** and **13** were used for labeling DNAs.

UV-visible absorption spectra of the alkynylpyrenes: Table 1 lists the absorption maxima (λ_{abs}) of the alkynylpyrenes **2**–**10** in ethanol, along with their absorption coefficients (ε). The absorption maxima of the (trimethylsilylethynyl)pyrenes **2**–**5** consecutively shift to longer wavelengths, and their absorption coefficients increase with increasing number of (trimethylsilyl)ethynyl groups (Figure 1A). As **5** has an absorption maximum at $\lambda = 434$ nm and its absorption coefficient is 1.32×10^5 mol^{-1} dm^3 cm^{-1}, it exists as an intensely colored orange-red solid. The absorption spectrum of 1,3,6,8-tetrakis(*tert*-butylethynyl)pyrene (**6**), the corresponding carbon counterpart of **5**, also appears in the visible region, but is less bathochromically shifted compared with that of **5**. The effect of the silicon atoms in inducing this extra bathochromic shift might be due to σ–π interaction between the C–Si σ and the acetylenic π bonds.[24] Although the absorptions of (arylethynyl)pyrenes **7** and **8** also reveal bathochromic shifts, their absorption coefficients are near to that of **1** (Figure 1B). On the other hand, the bandwidths of the spectra at longer wavelengths are considerably broadened because of increments due to different vibrational levels of the π-expanded skeletons. The absorption maxima of **9** and **10** bearing two alkynyl groups are further shifted, and their absorption coefficients are larger than those for the monoalkynyl derivatives **7** and **8**.

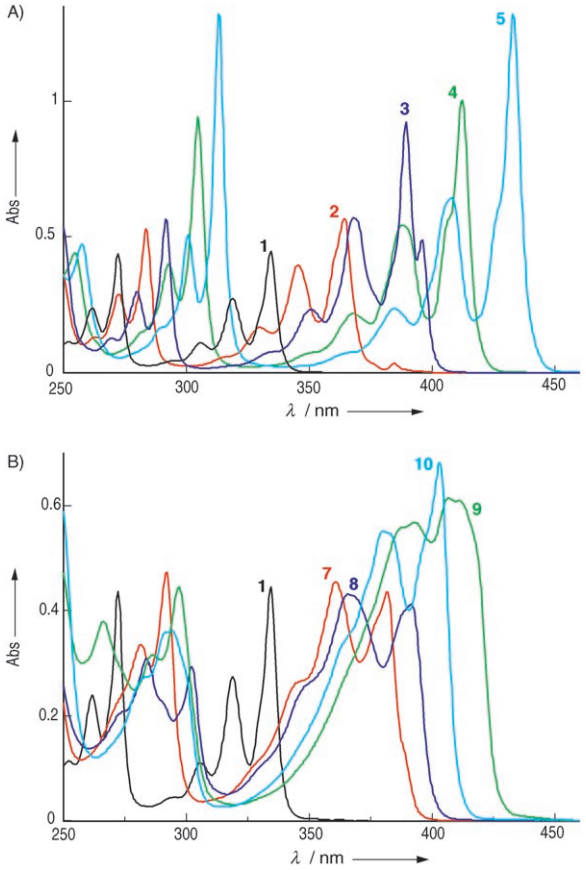

Figure 1. UV-visible absorption spectra of A) **1**–**5** (1.0×10^{-5} M) and B) **1** and **7**–**10** (1.0×10^{-5} M) in aerated EtOH.

Table 1. Photochemical data of compounds **1**–**10**.

Pyrene and alkynylpyrene	Absorption[a]		Fluorescence (monomer)					Fluorescence (excimer)
	λ_{abs} [nm]	ε ($\times 10^4$) [mol^{-1} dm^3 cm^{-1}]	λ_{em}[b] [nm]	Φ_f[c]	τ_s[d] [ns]	k_f[e] ($\times 10^6$) [s^{-1}]	$k_{isc}+k_{nr}$[e] ($\times 10^6$) [s^{-1}]	λ_{em}[f] [nm]
1	334	4.63	373	0.32[g]	377	0.849	1.80	475
2	365	5.77	386	0.55	72.1	7.63	6.24	505
3	390	9.22	399	0.67	4.68	143	70.5	523
4	413	10.0	421	0.80	3.20	250	62.5	545
5	434	13.2	438	>0.99	3.04	326	n.d.[h]	568
6	424	11.7	428	0.86	3.36	256	41.7	560
7	382	4.47	391	0.78	7.72	101	28.9	509
8	391	4.03	397	0.55	1.96	281	229	509
9	407	6.14	423	0.90	1.65	545	61.1	530
10	403	6.83	407	0.93	1.97	472	35.6	527

[a] 1.0×10^{-5} M in EtOH, λ_{abs} is the absorption band appearing at the longest wavelength. [b] 1.6–2.5 $\times 10^{-7}$ M in EtOH, degassed by bubbling of Ar, λ_{em} is the fluorescence band appearing at the shortest wavelength. [c] Fluorescence quantum yield in EtOH, degassed by bubbling of Ar, measured at room temperature. Standards used were 9,10-diphenylanthracene (for **1**–**4** and **7**–**10**) and coumarin 102 (for **5** and **6**), OD < 0.02. Φ_f (9,10-diphenylanthracene) = 0.95 in EtOH.[25] Φ_f (coumarin 102) = 0.74 in EtOH.[26] [d] Fluorescence lifetime, solution degassed by freeze-pump-thaw cycles, 1.0×10^{-5} M in EtOH. [e] Calculated using the equations $\Phi_f = k_f\tau_s$ and $\tau_s = 1/(k_f+k_{isc}+k_{nr})$. [f] Saturated concentration in CH_2Cl_2. [g] Data from ref. [27]. [h] The corresponding Φ_f value is too large to be used for the estimation.

Fluorescence spectra of the alkynylpyrenes: Fluorescence spectra of the alkynylpyrenes were measured in degassed ethanol (Figure 2, and Figure S1 in the Supporting Information). The fluorescence maxima (λ_{em}) appearing at the shortest wavelengths are listed in Table 1. Monomer emissions of the alkynylpyrenes **2**–**10** are shifted to longer wavelengths compared with those of **1**. As expected from the rigid structures of the alkynylpyrenes, their Stokes shifts are essentially small. It is noteworthy that the fluorescence quantum yields (Φ_f) of **2**–**5** are substantial and increase with increasing number of alkynyl groups

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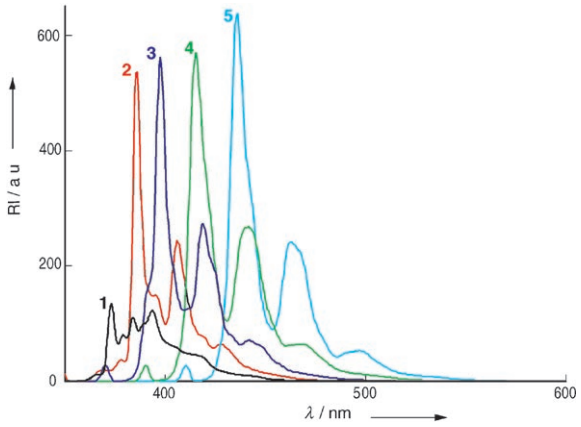

Figure 2. Fluorescence spectra of **1–5** in EtOH degassed by bubbling of Ar (RI = the relative intensity); λ_{ex} = 337, 346, 368, 388, and 408 nm for **1–5**, respectively. Each concentration of **1–5** was adjusted to the condition of OD = 0.01 at the corresponding excitation wavelength.

(Table 1). Especially in the case of **5**, the Φ_f value is >0.99, meaning that the absorbed photons on **5** are exclusively emitted as fluorescence. Conversely, the fluorescence lifetimes of **2–5** decrease with increasing number of attached alkynyl groups. This shortening of the lifetimes is responsible for the high Φ_f values (see below). Excimer emission is the most characteristic photochemical feature of pyrene nuclei, and is also seen for the alkynylpyrenes. Thus, at high concentrations in CH_2Cl_2, **2–10** show excimer emissions in the wavelength region 500–570 nm (Figure 3, and Figure S2 in the Supporting Information). The emission maximum for the excimer of **5** is close to 600 nm, and its spectral band even tails to 700 nm. Therefore, **5** fluoresces with a bright-yellow color under these conditions.

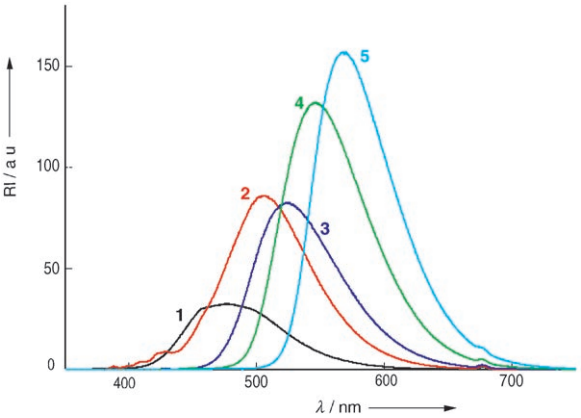

Figure 3. Fluorescence spectra of **1–5** at their saturated concentrations in aerated CH_2Cl_2; λ_{ex} = 337 nm.

The influence of oxygen on the fluorescence emissions was investigated. Integrations of the fluorescence spectra of **8–10** in aerated ethanol showed that 90%, 92%, and 89% of the levels observed from the corresponding carefully degassed solutions were maintained. On the other hand, the fluorescence of the parent pyrene was scarcely observed under the same conditions (integral only about 6% of that in the degassed solution; Figure S3 in the Supporting Information). Fluorescence quantum yield can be described by the equation $\Phi_f = k_f\tau_s$, or $\Phi_f = k_f/(k_f + k_{isc} + k_{nr} + k_q[O_2])$, in which k_f, k_{isc}, k_{nr}, and k_q represent the rate constants for fluorescence radiation, intersystem crossing, nonradiative decay, and fluorescence quenching by oxygen, respectively, and τ_s is the lifetime (s = singlet). Table 1 summarizes the values of k_f and $k_{isc} + k_{nr}$ for **1–10**, calculated on the basis of the experimental Φ_f and τ_s values under the degassed conditions, that is, assuming $k_q[O_2] = 0$. As k_{nr} is known to be negligible in aromatic hydrocarbons,[28] k_{isc} should be the major component in $k_{isc} + k_{nr}$.[29] Under aerated conditions, the rate constant for quenching of the fluorescence by oxygen (k_q) is estimated to be nearly diffusion-controlled (up to $3.89 \times 10^{10}\,M^{-1}s^{-1}$ in hexane),[30] and $[O_2]$ is below $2.0 \times 10^{-3}\,M$ in usual solvents. These data imply a $k_q[O_2]$ value of at most $8.0 \times 10^7\,s^{-1}$. As can be seen in Table 1, the k_f values of the alkynylpyrenes, especially **4–6** and **8–10**, are much larger than the $k_q[O_2]$ value. This situation is most likely responsible for the observed high Φ_f values of the alkynylpyrenes even in the presence of oxygen. The fact that the alkynylpyrenes continue to emit their fluorescence in the presence of oxygen represents an additional useful feature in the context of their possible deployment in biological applications.

Application of the alkynylpyrenes as fluorescent probes: As mentioned above, the investigated alkynylpyrenes exhibit bathochromic shifts of their absorption spectra and high fluorescence quantum yields even in the presence of oxygen. These findings encouraged us to apply the alkynylpyrenes as novel hydrophobic fluorescent probes for biomolecules. Because fluorescent probes are usually used in aqueous media, the well-characterized photochemical properties of the alkynylpyrenes in organic solvents must be retained in aqueous buffer solutions. To ascertain this in advance, the water-soluble **10** was chosen, in which the long oxyethylene side chain is expected to render the hydrophobic alkynylpyrene core soluble in such media. Indeed, the solubility of **10** proved to be high enough to permit exact evaluation of its photochemical characteristics at up to $1.0 \times 10^{-3}\,M$ in pure water. Both the monomer (λ_{max} = 407 and 432 nm) and the excimer (λ_{max} = 527 nm) emissions of **10** were clearly observed, and the excimer emission could be detected at concentrations as low as about $1.0 \times 10^{-6}\,M$, conditions far more dilute than could be used for **2**, **7**, **8**, and **9** in CH_2Cl_2. Thus, the strong tendency of **10** to self-associate in aqueous media offers an advantage in that the alkynylpyrenes may be applied to the detection of biomolecule–biomolecule interactions.

As probes for peptides and proteins: We developed a fluorescent probe **11** composed of (phenylethynyl)pyrene and maleimide units as fluorescent and labeling functionalities, respectively. This structure was inspired by that of commer-

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cially available *N*-(1-pyrenyl)maleimide (**26**), in which the maleimide group is attached directly to a pyrene core.[31] The fluorescent probe **26** is used for labeling thiol side chains on Cys residues of peptides and proteins. The inherent fluorescence of the pyrene nucleus in **26** is quenched before the labeling reaction, but fluorescence is then restored by the formation of thiol adducts at the carbon–carbon double bond of the maleimide. This type of off/on switching property is extremely useful for monitoring the labeling reaction, and we expected that the fluorescent probe **11** would behave similarly. Indeed, the fluorescence of **11** itself was found to be so weak that it could hardly be recognized visibly.

Keillor et al. have proposed that the off/on switching property in maleimide-attached fluorophores may be due to a reversal of the energy levels between the π orbitals on the fluorophore moiety and the n orbital on the maleimide carbonyl group before and after the labeling reaction.[32] Intramolecular PET (photoinduced electron transfer), however, is considered to be another possible mechanism for this switching, whereby the excited electron on the fluorophore falls to the LUMO of the maleimide group but not to that of the corresponding thiol-added succinimide skeleton. To clarify this aspect, the following experiments were carried out. If the mechanism were to be based on PET, addition of a maleimide molecule (2,5-pyrroledione) to a solution of **7** should cause fluorescence quenching through intermolecular PET. In fact, the fluorescence intensity of **7** (2 μM) decreased to about 10% of the original level in the presence of 0.1 M maleimide in ethanol. On the other hand, no quenching was observed under the same conditions in the case of succinimide. From the Stern–Volmer plot of **7** against the maleimide concentration (see Figure S4 in the Supporting Information), the k_q value was estimated to be about $1.5\times10^{10}\,M^{-1}s^{-1}$, supporting the view that the quenching event is induced by intermolecular PET because the value is larger than the diffusion-controlled rate constant ($6.0\times10^{9}\,M^{-1}s^{-1}$) in ethanol. Thus, this finding suggests that in our alkynylpyrene-based maleimide probe, the off/on switching is most likely controlled by an intramolecular PET mechanism.

L-Cysteine, the simplest biomolecule possessing a thiol group, was chosen as a test target molecule and was allowed to react with **11** in a water/dimethyl sulfoxide mixed solvent. To show that the probe fulfilled the criteria for practical use, fluorescence spectra of the adducts were measured under aerated conditions in water without exclusion of oxygen. Strong fluorescence was observed for the cysteine adduct of **11** at a longer wavelength compared with that of **26** (Figure 4). Thus, the fascinating property of the off/on switching was confirmed to also be operative in our alkynylpyrene-based maleimides. As a next target molecule, glutathione was selected. Glutathione is a biologically active peptide consisting of Cys, Glu, and Gly and is found in most organisms. Glutathione was labeled with **11** and **26**, and the adducts were analyzed under the same conditions as described for L-cysteine. Figure S5 in the Supporting Information shows fluorescence spectra of the glutathione adducts, and a similar off/on switching could again be seen.

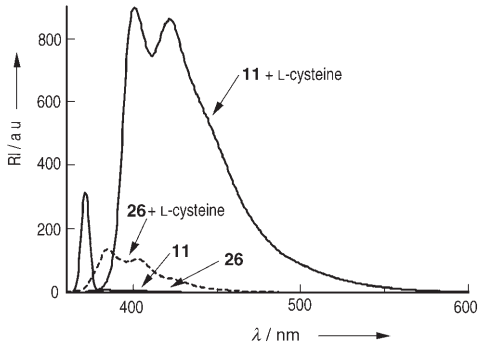

Figure 4. Fluorescence spectra of **11** and **26** before and after the labeling reaction with L-cysteine in 0.07 M phosphate buffer solutions; $[\mathbf{11}]=1.2\times10^{-6}$ M, $[\mathbf{26}]=3.8\times10^{-7}$ M, $[\mathbf{11}+\text{L-cysteine}]=9.3\times10^{-7}$ M, $[\mathbf{26}+\text{L-cysteine}]=5.7\times10^{-7}$ M. $\lambda_{ex}=369$ and 341 nm for **11** and **26**, respectively. Each concentration was adjusted to the condition of OD = 0.01 at the corresponding excitation wavelength.

Comparing the spectra of the adducts of **11** and **26**, we note the following points: the spectra of the adducts of **11** reveal bathochromic shifts (~17 nm) with broadening, and the fluorescence intensities of the adducts of **11** are much more enhanced. To obtain quantitative information, fluorescence quantum yields (Φ_f) were measured in a phosphate buffer solution (pH 7.0) before and after the labeling reactions of L-cysteine and glutathione (Table 2). Both **11** and **26** alone displayed negligible Φ_f values owing to the fluorescence quenching described above. Among the adducts, the Φ_f values for those with **11** were much higher than for those with **26**. Fluorescence lifetimes of the adducts of **11** were determined in order to investigate the reason for these high Φ_f values. The lifetimes were found to be extremely short ($\tau_s=$ 2.05 ns for the cysteine adduct and 2.76 ns for the glutathione adduct), and hence the adducts were able to radiate their fluorescence before collision with oxygen as in the cases of **3**–**10** (see above).[33]

Table 2. Φ_f values for **11** and **26** before and after the labeling reactions.[a]

Adduct	Φ_f for **11**	Φ_f for **26**
before	≈0	≈0
L-cysteine	0.57	0.03
glutathione	0.48	0.02

[a] Quinine sulfate ($\Phi_f=0.55$) in 0.5 M H_2SO_4 used as a standard.

Protein adducts of **11** and **26** were assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The target protein was bovine serum albumin (BSA), which has a molecular weight of around 67 kDa and incorporates 35 Cys residues. After treating BSA (300 μg) with **11** or **26**, the mixture was analyzed by SDS-PAGE. Figure 5 shows gel photographs visualized by Coomassie blue staining (A) and by irradiation with a UV transilluminator (B). In photograph A, bands corresponding to BSA

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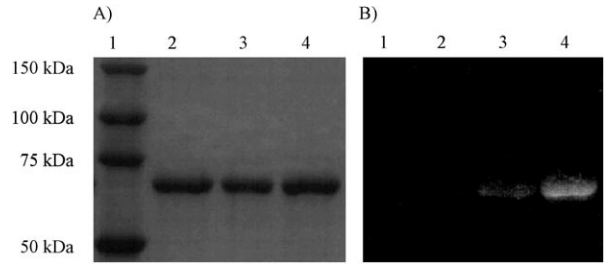

Figure 5. SDS-PAGE photographs of BSA and the adducts. Photographs (A) and (B) were visualized by Coomassie blue staining and by irradiation with light of wavelength 312 nm, respectively; lane 1: molecular weight markers, lane 2: BSA (3.0×10^{-6} g), lane 3: BSA labeled with **26** (1.3×10^{-8} g), lane 4: BSA labeled with **11** (1.8×10^{-8} g).

and the adducts could be confirmed in each of the lanes 2–4. On the other hand, only the fluorescent band due to the adduct of **11** (lane 4) is clearly visible in photograph B, while the band due to the adduct of **26** (lane 3) is faint in the same photograph. This SDS-PAGE experiment demonstrates the favorable photochemical characteristics of the alkynylpyrene skeletons, making **11** a promising practical probe for labeling Cys residues.

As probes for DNAs: We recently reported an excimer-monomer switching DNA probe **27** with two pyrene moieties as fluorophores at both the 3′- and 5′- ends of a single-stranded oligonucleotide (Figure 6).[4a] This DNA probe can

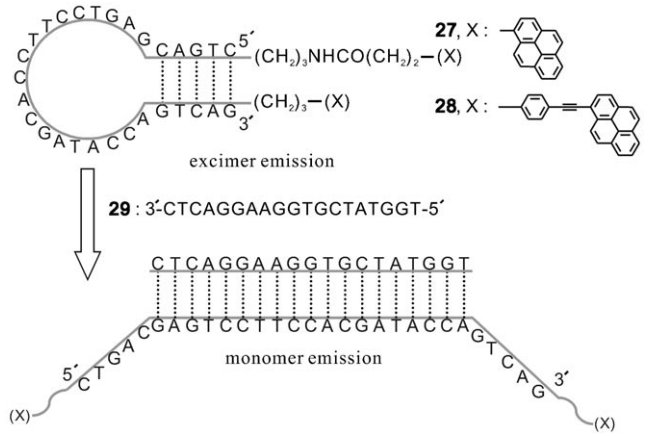

Figure 6. Sequences of a new DNA probe **28** modified with alkynylpyrenes, the previous DNA probe **27** modified with pyrenes, and the fully complementary target DNA **29** for the loop region of **27** and **28**.

be regarded as a so-called molecular beacon, consisting of a stem-and-loop structure. In conventional molecular beacons, the detection of target DNAs is based on the FRET (fluorescence resonance energy transfer) mechanism. Compared with conventional beacons, our DNA probe **27** has the advantages of synthetic simplicity, high signal-to-noise, and unambiguous detectability. Furthermore, the characteristic emission switching of **27** with an isoemissive point allows for ratiometric measurements. Ratiometric measurements on the basis of two emission bands can largely eliminate the uncertainty in the detection that results from individual sample factors such as pH, temperature, the probe concentration, and localization of the probes, as well as from instrumental factors such as the kind of spectrometer or the dependence of excitation intensity on the light source. The fascinating excimer-monomer switching probe **27**, however, suffers from the inherent drawbacks of pyrene fluorophores mentioned in the introduction to this paper. Thus, we decided to introduce alkynylpyrene skeletons instead of the parent pyrenes into our excimer-monomer switching probes in order to improve their efficiency in the context of in vivo applications.

A new DNA probe **28** possessing two (phenylethynyl)pyrene moieties as fluorophores was prepared in a similar manner as described for **27** (Figure 6).[4a] A single-stranded oligonucleotide with a C3-alkylamino linker (3′- end), which consists of the same sequence as that of **27**, was modified at the 3′- and 5′- ends with **12** and **13**, respectively, to give **28**. The melting temperature (T_m) of **28** was found to be about 60 °C on the basis of its electronic absorption spectra, almost the same as that for **27**.

To examine the ability of **28** to act as a switching probe, fluorescence titration experiments were performed with the fully complementary DNA **29** (Figure 7). We ensured that

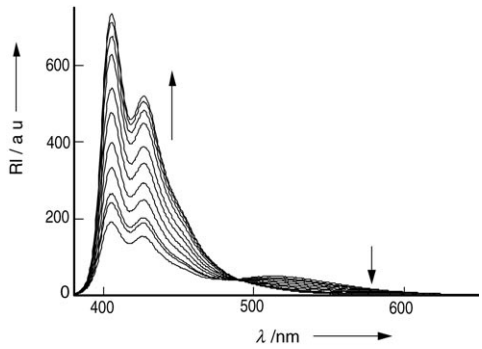

Figure 7. Fluorescence titration spectra of **28** ([**28**], 200 nM; [$MgCl_2$], 5 mM; [KCl], 50 mM; [Tris-HCl], 20 mM, at pH 8.0) in the presence of the fully complementary target DNA **29** (0.1–1.0 equiv); $\lambda_{ex}=370$ nm.

the probe **28** retained the characteristic excimer–monomer switching property as in the case of **27**. Thus, upon addition of **29**, the excimer emission ($\lambda=511$ nm) decreased, while the monomer emission ($\lambda=405$ and 427 nm) increased, with an isoemissive point at $\lambda=490$ nm. A significant difference between the spectral changes observed for **27** and **28** is the extent of the increase in the monomer emission, which is probably because of the high fluorescence quantum yield of the alkynylpyrene skeleton on **28** in protic media. Therefore, the probe **28** also exhibits a somewhat "signal-on"-like change in the wavelength region in which the monomer emits, resembling conventional FRET-type molecular beacons. On the other hand, **27** can be regarded as a "signal-off"-type probe: the extent of the decrease in the excimer emission is larger than the increase in the monomer emis-

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sion.[4a] Thus, the probe **28** has the advantages of an inherent switching ability that allows for ratiometric measurements as well as of a simple "signal-on"-type property. Sensitivity is one of the most important criteria for probes directed toward biomolecular detection. The spectral changes of **28** could be followed at a concentration of 1.0×10^{-10} M, which is about 100 times more dilute than with **27** in aqueous buffer solutions.[34] The high sensitivity of **28** further illustrates the usefulness of the alkynylpyrene skeleton as a biomolecular probe.

Conclusion

Direct attachment of alkynyl groups to a pyrene core has been shown to impart photochemical properties that render these systems favorable as fluorescent probes for biomolecules. As the number of alkynyl substituents is increased, the fluorescence intensities of the alkynylpyrene derivatives are enhanced, and this is accompanied by bathochromic shifts in their absorption spectra. Taking advantage of these promising features, we have developed alkynylpyrene-based probes for biological applications. A maleimide-modified probe was designed for labeling Cys residues, and succinimidyl and phosphoramidite derivatives were devised for constructing new DNA probes. These alkynylpyrene-based biomolecular probes have been found to be more practical than conventional pyrene-based ones as a result of excitation at longer wavelengths and the high Φ_f values that are maintained without rigorous exclusion of oxygen.

Experimental Section

Materials and general procedures: Reagents were purchased from commercial sources and were used without further purification. The compounds 1,3,6-tribromopyrene[35] (**16**), 1,3,6,8-tetrabromopyrene[23b] (**17**), 2-ethynylthiophene,[36] octa(ethylene glycol) monomethyl ether,[37] 1,6-diiodopyrene[14a] (**18**), 4-ethynylaniline,[38] 3-(4-iodophenyl)propionic acid[39] (**22**), **2**,[15b] **3**,[20b] **7**,[13a] and **9**[40] have been previously reported and were synthesized according to the published procedures. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively. Melting points are uncorrected. For further experimental procedures, see the Supporting Information.

Spectroscopic measurements: Steady-state absorption and emission spectra were recorded at 298 K using a 1 cm pathlength cell.

Fluorescence quantum yields (Φ_f) of the alkynylpyrenes were determined using 9,10-diphenylanthracence as a standard with a known Φ_f of 0.95 in ethanol.[25] For **5** and **6**, coumarin 102 was used as a standard, assuming its quantum yield to be 0.74 in ethanol.[26] The fluorescence quantum yields were calculated according to the following equation.

$$\Phi_{f(spl)} = \Phi_{f(std)} \times [A_{std}/A_{spl}] \times [I_{spl}/I_{std}] \times [n_{spl}/n_{std}]^2$$

In this equation, $\Phi_{f(spl)}$ and $\Phi_{f(std)}$ are the quantum yields of a sample and a standard, respectively. A_{spl}, I_{spl}, and n_{spl} are the optical density, the integrated emission intensity at the excitation wavelength, and the value of the refractive index of the sample, respectively. A_{std}, I_{std}, and n_{std} are those for the standard.

Fluorescence lifetimes were measured by time correlation, a single-photon counting methodology, using a nanosecond fluorimeter. Sample solutions (1.0×10^{-5} M in EtOH) were carefully degassed by freeze-pump-thaw cycles before the measurements. The excitation wavelength was 337 or 370 nm, and cut-off filters UV-32 for **1** at 337 nm, UV-35 for **2**, **3**, and **7**–**10** at 337 nm, and L38 for **4**–**6** at 370 nm were used. All decay curves were calculated by assuming a single exponential on the basis of the equation $I_{f(t)} = A\exp(-t/\tau)$, in which $I_{f(t)}$ = fluorescence intensity at time t and A = fluorescence intensity at $t=0$.

Determination of the melting temperature (T_m) of 28: Electronic absorption spectra were recorded in the temperature range from 10 to 90 °C. The solution used for the measurements contained 1 μM **28**, 5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl (pH 8.0).

Fluorescence titration: A Milli-Q solution of the complementary DNA **29** (10×0.1 equiv) was added to a 200 nM solution of **28** containing 5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl (pH 8.0) at 25 °C. Each mixture was stirred at 25 °C until no further change in the fluorescence spectra was observed (ca. 15 min). The excitation wavelength was 370 nm, and fluorescence spectra were recorded from 350 to 700 nm following each addition of **29**.

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