Association Behavior of 1-Pyrenyl Pendants Introduced into the Papain Active Site

Tadamitsu Sakurai,* Kazue Miyata (nee Nojima), Kenichiro Watanabe, Kanji Kubo,† and Hiroyasu Inoue

Department of Applied Chemistry, Faculty of Technology, Kanagawa University, Kanagawa-ku, Yokohama 221-8686

†Institute of Advanced Material Study, Kyushu University, Kasuga-koen, Kasuga, Fukuoka 816-0811

(Received October 8, 1998)

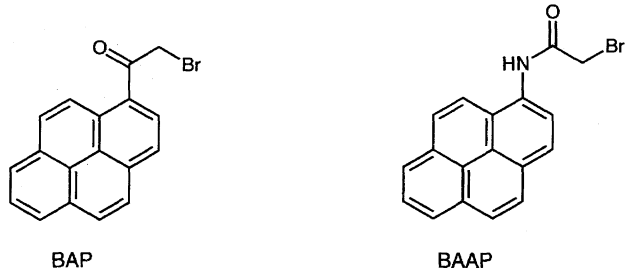
1-(Bromoacetyl)pyrene (BAP) was found to selectively alkylate the cysteine-25 residue (Cys-25) of papain, inactivating the enzyme. A comparison of the spectroscopic behavior of BAP-modified papain with that of the reference compound, 1-{[2-(acetylamino)ethylthio]acetyl}pyrene (TAP), showed that the pyrenecarbonylmethyl pendant (covalently bound to Cys-25 in a 1 : 1 stoichiometric ratio) gives a new absorption around 390 nm and a long-wavelength fluorescence at 465 nm, which are both characteristic of an associated dimer. Effects of solvent and concentration on the absorption and emission spectra of TAP as well as the derivatized enzyme substantiate the dimer formation in the ground state. The finding that the pendant-*carbonyl*-^{13}C carbon signal undergoes a large upfield shift in the papain active site, as compared to the corresponding signal of ^{13}C-labeled TAP, confirms that the pyrenecarbonyl moiety adopts an enolate-type resonance structure (making the pyrene ring electron-deficient) on formation of a strong hydrogen bond to the neighboring amino acid residue(s). It is proposed that the significant contribution of the enolate-type resonance structure is a driving force for promoting the dimerization of the pyrenecarbonylmethyl reporter group attached to Cys-25 mainly through dipole–dipole and hydrophobic interactions. On the other hand, the introduction of the imino spacer between the 1-pyrenyl carbon and the acetyl carbonyl carbon in BAP-derived pendant greatly weakened a tendency to produce a pendant dimer in the ground state, providing a piece of evidence in support of this proposal.

It is well-known that on excitation, a pyrene molecule forms a bimolecular species (excimer) which exists only in the electronically excited state.[1] The strong tendency of pyrene with a high emission efficiency to generate an excited-state dimer has allowed this fluorescent chromophore to become a new probe for unravelling the behavior of many biological systems.[2] There have been also extensive investigations regarding intramolecular excimer-forming systems in which the two pyrenyl groups are separated by various types of spacer chains.[3] These systems provided good means of exploring the dynamic behavior of the chain, which links both chromophores, in the excited singlet state. In recent years particular attention has been called to the examination of the intramolecular interactions between excimer-forming bichromophoric molecules in the ground state.[4] ^{1}H NMR analysis of these bichromophores indicated the presence of a ground-state dimer depending on both the length and the properties of the spacer moiety connecting the two pyrene rings. This strongly suggests that there is possibility of producing the ground-state dimer between two pyrenyl functional groups.

On the other hand, great effort has been devoted to the photochemical control of enzyme activity by the covalent linkage of a photoisomerizable probe molecule to the enzyme surface.[5] Willner and co-workers have succeeded in controlling papain activity through the *cis–trans* photoisomerization of an azobenzene chromophore that had been attached to the lysine residue of this enzyme surface.[5a] However, there are only a few studies concerning the interaction between a probe molecule and an enzyme active-site amino acid residue and/or between reporter groups bound to the enzyme active site.[6] As described above, pyrenyl groups are good probes for examining the dimerization behavior of many systems including biological ones in the ground state as well as in the excited singlet state. Thus, we designed 1-(bromoacetyl)pyrene (BAP) and 1-(bromoacetylamino)pyrene (BAAP, Chart 1) and allowed these reagents to react with papain, hoping to introduce two different types of pyrenyl pendants into its active site and to shed some light on the association behavior of this enzyme through an analysis of the dimerization of the pendants. In this paper we present the results that demonstrate that the pyrenecarbonylmethyl pendant attached to the cysteine-25 residue (Cys-25) of papain has a much stronger propensity to give an associated dimer in the ground state, than the 1-pyrenylcarbamoylmethyl pendant, and becomes an excellent probe for exploring the association behavior of the papain molecule.

Results and Discussion

Association Behavior of the 1-Pyrenecarbonylmethyl Pendant. When activated papain (4.0×10^{-5} mol dm^{-3}) was allowed to react with BAP (2.4×10^{-4} mol dm^{-3}) in an



BAP BAAP

Chart 1.

N_2-purged phosphate buffer (0.1 mol dm^{-3}; pH 7.0) containing 3 vol% dimethyl sulfoxide (DMSO) at room temperature, the papain activity against *N*-benzoyl-L-arginine ethyl ester[7] was completely lost in 120 min, as shown in Fig. 1. The removal of excess BAP by centrifugation and dialysis followed by the purification of a dialyzed enzyme solution by membrane filtration gave the UV absorption spectrum shown in Fig. 2A. The modified papain obtained from the reaction between papain and 2-iodoacetamide, which alkylates Cys-25 selectively,[8] was treated with BAP under the same reaction conditions but showed no indication of the incorporation of a pyrenecarbonyl chromophore into the modified enzyme after centrifugation and sufficient dialysis of the reaction mixture (Fig. 2B). Along with the fact that a bromoacetylisoalloxazine derivative (having a structure related to BAP) reacts selectively with Cys-25,[9] this finding provides definitive evidence for the selective alkylation of Cys-25 with BAP in a 1 : 1 stoichiometric ratio and also eliminates the possibility that there exists a non-covalently associated pyrenecarbonylmethyl pendant on the enzyme surface.

We prepared the reference compound 1-{[2-(acetylamino)ethylthio]acetyl}pyrene (TAP, Chart 2) and measured its absorption spectrum in MeOH owing to its negligible solubility in a phosphate buffer solution (Fig. 2C). A comparison of curves A and C in Fig. 2 shows that the absorption maximum of the pendant attached to Cys-25 is red-shifted by about 20 nm with an increase in the absorbance near 390 nm,

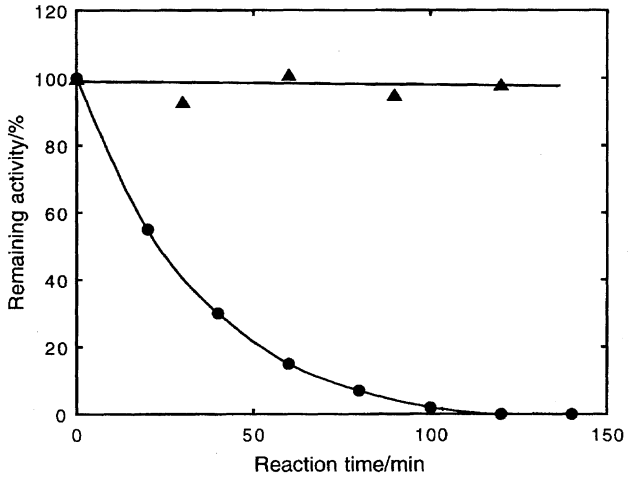


Fig. 1. Remaining activity of BAP-modified papain (●, 4.0×10^{-5} mol dm^{-3}) and native papain (▲, 4.0×10^{-5} mol dm^{-3}) as a function of reaction time.

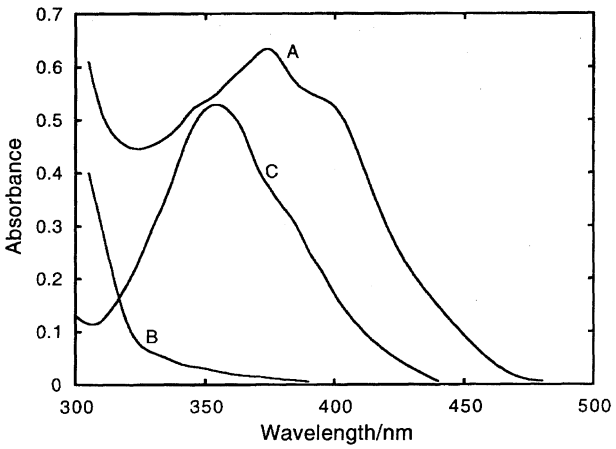
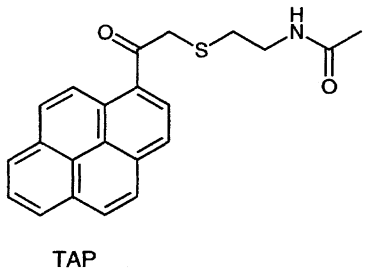


Fig. 2. UV absorption spectra of BAP-modified papain (A, 3×10^{-5} mol dm^{-3}) and iodoacetamide-treated papain (B, 3×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) and TAP (C, 3×10^{-5} mol dm^{-3}) in MeOH at room temperature.



TAP

Chart 2.

compared with that of TAP, although the observed red shift could be ascribed in part to the difference in solvent. This implies the occurrence of interactions between the pendants and/or between the pendant and the amino acid residue(s) at the papain active site. Interestingly, the BAP-derived reporter group on the enzyme had dual fluorescence depending on the excitation wavelength, as seen from Figs. 3A and 3B. In addition, the excitation spectrum (Fig. 3C) for the long-wavelength emission (465 nm; lifetime, τ_f = 2.6 ns) was distinct from that (Fig. 3D) for the short-wavelength one (380 nm; τ_f = 20 ns). These findings suggest that there are two ground-state species. It has been shown that the concentration dependence of the monomer/excimer fluorescence-intensity ratio for the 1-pyrenyl pendant on the derivatized actin subunit can be used as a sensitive technique to detect a shift of the association–dissociation equilibrium induced by dilution.[10] Accordingly, if an associated dimer or an aggregate is formed in the ground state giving the 465 nm fluorescence, we expect that the emission intensity ratio I_f(380 nm)/I_f(465 nm) should decrease with increases in the modified papain concentration. Figure 4A demonstrates that this intensity ratio gradually decreases as the alkylated enzyme concentration is increased, being consistent with our expectation. However, there still remains the possibility of the interaction between the pyrenecarbonylmethyl reporter group and the surrounding amino acid residue(s), owing to

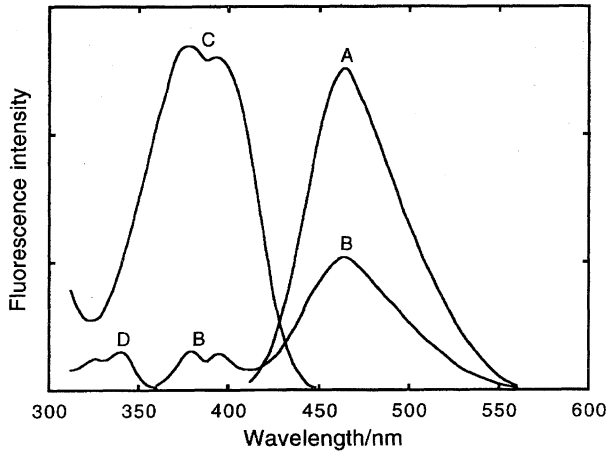


Fig. 3. Fluorescence (A and B) and fluorescence excitation (C and D) spectra of BAP-modified papain (3×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) at room temperature. Excitation and emission wavelengths monitored/nm: A, 400; B, 340; C, 465; D, 380.

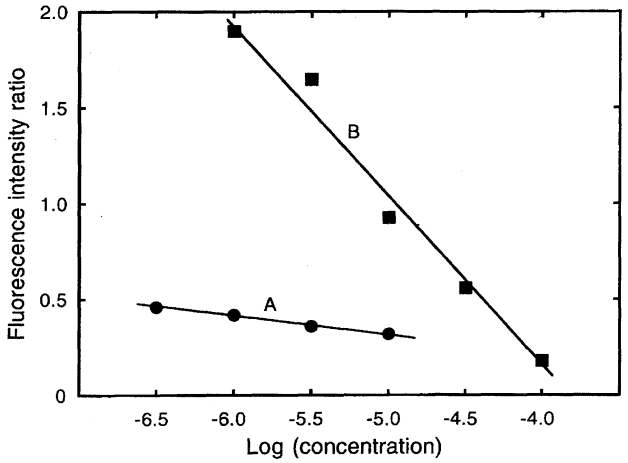


Fig. 4. Effects of BAP-modified papain (●) and TAP (■) concentrations on the fluorescence intensity ratios: ●, I_f(380 nm)/I_f(465 nm) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0); ■, I_f(390 nm)/I_f(456 nm) in MeOH. Excitation wavelength/nm: ●, 340; ■, 337.

the insensitive concentration dependence of the ratio I_f(380 nm)/I_f(465 nm).

TAP in MeOH also showed weak dual fluorescence depending on the excitation wavelength. The long-wavelength emission (456 nm; $\tau_f < 0.6$ ns) is much stronger than the short-wavelength one (390 nm; τ_f = 10 ns) at [TAP] = 1.0×10^{-4} mol dm^{-3} (Fig. 5A), but the decreased concentration of TAP rapidly increases the emission intensity ratio I_f(390 nm)/I_f(456 nm) as demonstrated in Fig. 4B. Additionally, the short-wavelength fluorescence was preferentially observed in MeCN even at [TAP] = 1.0×10^{-4} mol dm^{-3} (Fig. 5B). Taking into consideration that pyrene gives a monomer fluorescence at 390 nm and an excimer one at 470 nm in MeOH[11] and that the 1-pyrenecarbonyl chromophore has a tendency to form a ground-state dimer,[4b,4c] it is suggested from these observations that the protic po-

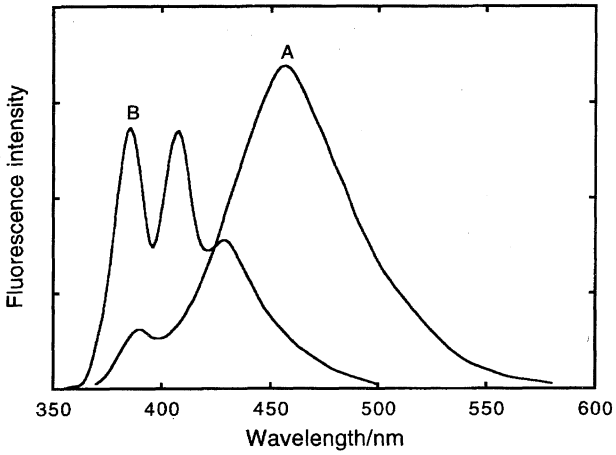


Fig. 5. Fluorescence spectra of TAP (1.0×10^{-4} mol dm^{-3}) in MeOH (A) and MeCN (B) at room temperature. Excitation wavelength/nm = 337.

lar solvent promotes the associated dimer formation of a TAP molecule to a great extent, mainly through hydrogen bonding between the TAP carbonyl oxygen and the solvent hydroxy hydrogen. Since the ground-state associated dimer may show its absorption around 390 nm as judged by the long wavelength-fluorescence excitation spectrum of the reference compound in MeOH, the gradual increase in the molar extinction coefficient (ε) at 390 nm with the TAP concentration (ε = 6500 dm^3 mol^{-1} cm^{-1} at [TAP] = 1.0×10^{-6} mol dm^{-3} and 7600 dm^3 mol^{-1} cm^{-1} at [TAP] = 1.0×10^{-4} mol dm^{-3}) provides evidence in support of this suggestion. There may be, of course, a measurable contribution of the excimer formation to the concentration dependence of the emission intensity ratio provided in Fig. 4B. These results obtained for TAP, therefore, strengthen our argument that the short- and long-wavelength fluorescences (shown by BAP-modified papain) are attributable to the pendant monomer and dimer species, respectively, formed in the ground state. If so, then what are the roles played by the enzyme? One is to significantly increase the dimer-emission intensity and lifetime as compared with those of TAP, reflecting an increase in stability of the associated dimer in the active site. The other is to appreciably suppress dissociation into the monomer pendant, as shown by a comparison of the concentration dependence of the intensity ratio, I_f(short-wavelength)/I_f(long-wavelength), for the BAP-derived reporter group with that for TAP (Fig. 4).

As already described, solvent effects on the fluorescence behavior of TAP make it possible to speculate that hydrogen bonding between the pyrenecarbonyl oxygen and the neighboring amino acid residue(s) increases the dimer stability. An inspection of the UV spectra of TAP and the modified enzyme (Fig. 2) indicates that an increase in the absorbance near 400 nm for the enzyme is more significant than that at absorption maximum wavelength, being consistent with the increased pendant dimer stability at the enzyme active site and, thus, consistent with a great shift of the equilibrium to the dimer on incorporation of the pyrenecarbonylmethyl

moiety into the active site. On the other hand, the finding that upon increasing the ionic strength (I) of a papain solution (pH 7.0) from 2.0×10^{-3} to 1.0 using KCl, the emission intensity ratio I_f(380 nm)/I_f(465 nm) [= 0.26 (I = 2.0×10^{-3}) and 0.72 (I = 1.0)] is increased by a factor of about 3, allows us to interpret the prevention of the pendant dimer from dissociation into the monomer in terms of the high association ability of BAP-modified papain.[12] The relatively small dependence of the intensity ratio on the ionic strength suggests that an electrostatic force and a hydrophobic one both become major factors controlling the extent of association between the modified papain molecules. This suggestion is supported by the finding that the treatment of BAP-modified papain (1×10^{-5} mol dm^{-3}) with guanidine hydrochloride (6 mol dm^{-3}) in phosphate buffer (0.1 mol dm^{-3}; pH 7.0) increases the value of the intensity ratio, I_f(380 nm)/I_f(465 nm), from 0.30 to 0.80.

Association Behavior of the 1-Pyrenylcarbamoylmethyl Pendant. BAAP required longer than BAP (more than 10 h) to completely inhibit papain activity under the same reaction conditions. Figure 6A shows that the BAAP-modified enzyme gives a new absorption band with maxima at 331 and 345 nm. The finding that the reaction of 2-iodoacetamide-treated papain with BAAP afforded no additional absorption band after centrifugation and sufficient dialysis confirms that this reagent also selectively alkylates Cys-25 in a 1 : 1 stoichiometric ratio. For comparison, 1-{2-[2-(acetylamino)ethylthio]acetylamino}pyrene (TAAP, Chart 3) was prepared as the reference compound of the chromophore attached to Cys-25 and its absorption spectrum was measured in MeOH and phosphate buffer (0.10 mol dm^{-3}; pH 7.0) containing 20 vol% DMSO at room temperature (Figs. 6B and 6C). The pendant absorption is rather similar to that obtained in the latter solvent while the absorption due to the pyrenylcarbamoyl chromophore in the enzyme active site is only red-shifted by 4 nm as compared to that of TAAP,

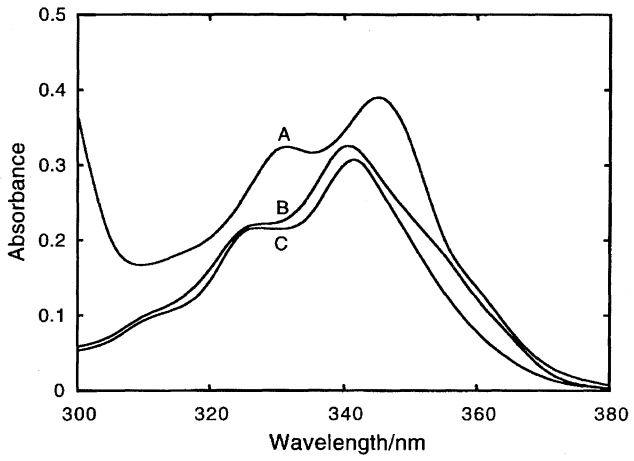
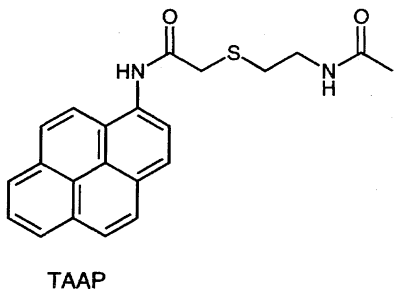


Fig. 6. UV absorption spectra of BAAP-modified papain (A, 3×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) and TAAP (3.0×10^{-5} mol dm^{-3}) in MeOH (B) and 0.1 mol dm^{-3} phosphate butter (pH 7.0) containing 20 vol% DMSO (C) at room temperature.



TAAP

Chart 3.

suggesting no occurrence of appreciable interaction between the pendants in the ground state.

On the other hand, the BAAP-derived reporter group had excitation wavelength-dependent fluorescence with maxima at 382 (short-wavelength emission) and 465 nm (long-wavelength emission) as illustrated in Fig. 7. The dependence of the intensity ratio (of the former emission to the latter) on the modified papain concentration (inset of Fig. 7) is consistent with association of the reporter group in the ground state. In contrast to the fluorescence behavior of TAP in MeOH, TAAP in this protic solvent showed only the short-wavelength fluorescence with a maximum at 386 nm regardless of the excitation wavelength (Fig. 8), thus demonstrating that the 1-pyrenylcarbamoylmethyl group has much less ability to form an associated dimer than does the 1-pyrenecarbonylmethyl. Interestingly, the dual fluorescence of TAAP was obtained in phosphate buffer-20 vol% DMSO irrespective of the excitation wavelength, as shown in Fig. 9. Lower concentration of this reference compound (3.0×10^{-6} mol dm^{-3})

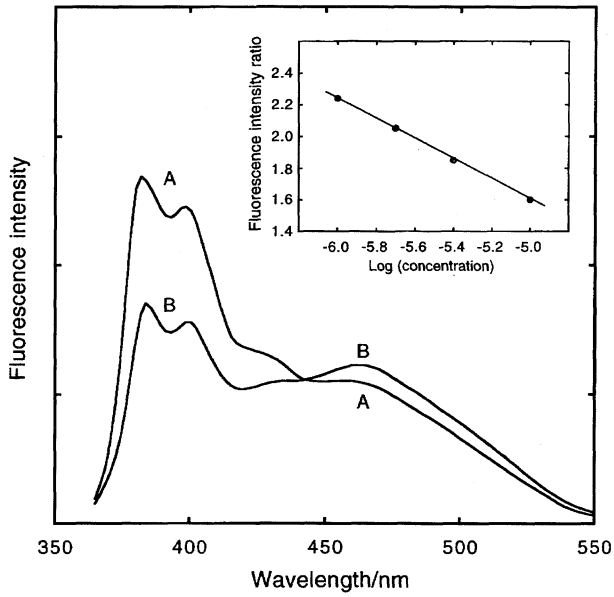


Fig. 7. Fluorescence spectra of BAAP-modified papain (3×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) at room temperature. Excitation wavelength/nm: A, 340; B, 360. Inset: Effects of BAAP-modified papain concentration on the fluorescence intensity ratio, I_f(382 nm)/I_f(465 nm), in 0.1 mol dm^{-3} phosphate buffer (pH 7.0). Excitation wavelength/nm = 360.

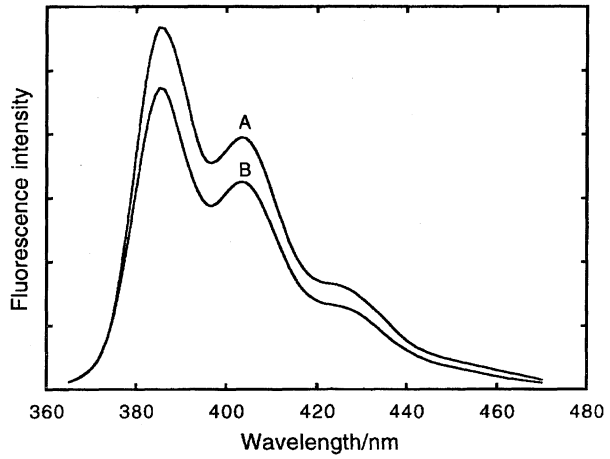


Fig. 8. Fluorescence spectra of TAAP (3.0×10^{-5} mol dm^{-3}) in MeOH at room temperature. Excitation wavelength/nm: A, 310; B, 360.

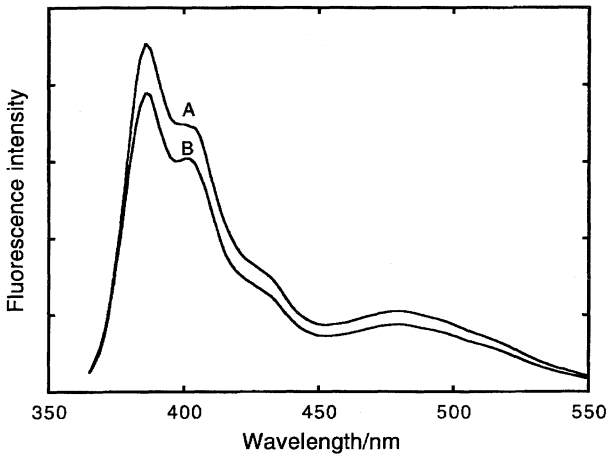


Fig. 9. Fluorescence spectra of TAAP (3.0×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) containing 20 vol% DMSO at room temperature. Excitation wavelength/nm: A, 310; B, 360.

gave the 480 nm emission at a negligible efficiency. In addition, excitation spectra for the short (386 nm)- and long (480 nm)-wavelength fluorescences were very similar, thus allowing us to ascribe most of the 480 nm emission to the excimer. Water molecules are considered to promote the aggregation of the hydrophobic pyrene ring at a given concentration of TAAP.

Associated Dimer and Hydrogen Bonding. Why should a ground-state dimer form between the pyrenecarbonylmethyl pendants covalently bound to Cys-25, despite the fact that papain is a globular protein molecule having a cavity called the active site?[13] It has been demonstrated that ^{13}C NMR spectroscopy provides a good means of detecting the inhibitor–papain covalent adduct.[14,15] ^{13}C NMR analysis of the ^{13}C-labeled peptidyl chloromethyl ketone inhibitor incorporated into Cys-25 of papain showed that the ketone carbonyl oxygen forms strong hydrogen bonds with the neighboring amino-acid residues resulting in a large downfield shift of this carbonyl carbon signal.[15] Based on the X-ray crystal structure of analogous chloromethyl ketone–papain adducts, the backbone amide hydrogen of Cys-25 as well as the amide hydrogens of the glutamine-19 residue was shown to be involved in the hydrogen bonding to the P_1 carbonyl oxygen.[16] Accordingly, it is expected that the carbonyl oxygen of the pyrenecarbonylmethyl pendant might be linked by a hydrogen bond to the amino acid residue(s) described above. To measure the ^{13}C NMR spectrum of BAP-modified papain, BAP-*carbonyl*-^{13}C (99+ atom%; 3.0×10^{-4} mol dm^{-3}) was allowed to react with the enzyme (1.2×10^{-4} mol dm^{-3}) in an N_2-purged phosphate buffer (0.1 mol dm^{-3}; pH 7.0) at room temperature. Centrifugation and subsequent dialysis against a phosphate buffer (0.1 mol dm^{-3}; pH 7.0) and finally membrane filtration of a dialyzed enzyme solution enabled us to record the ^{13}C NMR spectrum of the pyrenecarbonylmethyl pendant attached to Cys-25 in the phosphate buffer containing 10 vol% deuterated water and 0.1 vol% 1,4-dioxane at room temperature. For comparison, we attempted to obtain the NMR spectrum of ^{13}C-labeled TAP under the same conditions but could not because of its negligible solubility. Taking into account the finding that TAP indicates no propensity to form the ground-state dimer in aprotic solvents, we measured its NMR spectrum in [^{2}H$_6$]dimethyl sulfoxide (DMSO-d_6) and [^{2}H]chloroform ($CDCl_3$). The pyrenecarbonylmethyl pendant attached to Cys-25 had its carbonyl carbon signal at 175.5 ppm, the value of which was obtained by converting the observed chemical shift (108.1 ppm) to a tetramethylsilane standard. The appearance of the carbonyl carbon signal in ^{13}C-labeled TAP monomer at 198.9 (DMSO-d_6) and 198.7 ppm ($CDCl_3$), thus, confirms that when the pyrenecarbonyl chromophore is introduced into the papain active site, the labeled-carbon signal is subject to a large upfield shift ($\Delta\delta \approx 23$ ppm). Since the carbonyl oxygen of the pendant attached to Cys-25 may be near a hydrogen bond-donating group (such as Cys-25 or glutamine-19 that is a most likely candidate), it is reasonable to interpret the upfield shift of 23 ppm in terms of the strong hydrogen bonding (to the P_1 carbonyl oxygen) that considerably increases the contribution of the enolate-type resonance structure **I** (Fig. 10). Then, we are led to propose that the strong polarization of π-electrons in the pyrenecarbonyl moiety renders the pyrene ring electron-deficient so that the ring may serve as an electron acceptor. An examination of the three-dimensional structure of the papain active site showed that there are tryptophan, tyrosine, and histidine residues that could be candidates for interaction with the electron-deficient pyrene ring.[16b] The interaction of the naphthoylmethyl probe (having much less association ability than that of the pyrenecarbonylmethyl) bound to Cys-25 of papain with the surrounding aromatic residue(s) caused the appearance of a new absorption band near 500 nm.[17] This makes the ground-state association of the BAP-derived pendant much more likely. Pyrene rings larger and more hydrophobic than naphthalene are deemed to stick out of the enzyme surface (that could be assumed to have a broad and relatively shallow cavity),[18] sterically allowing the association of two pyrenecarbonyl chromophores, as schematically shown in Fig. 11.

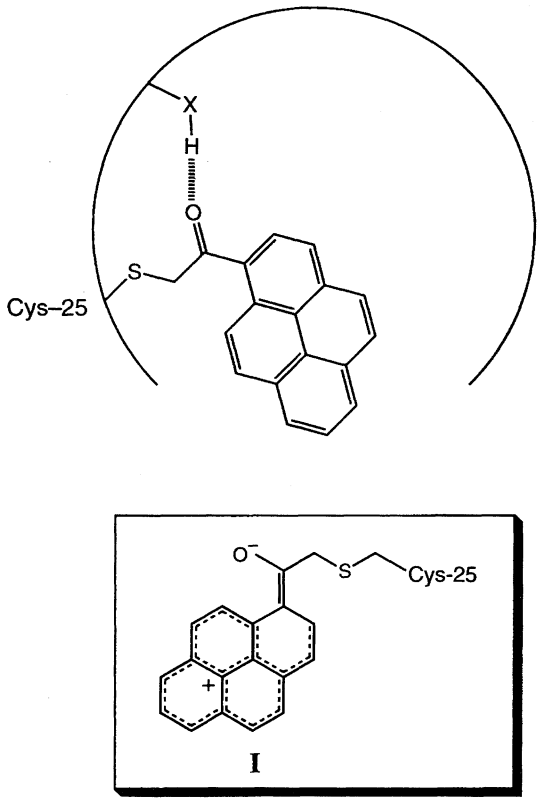


Fig. 10. Schematic illustration for papain-BAP adduct.

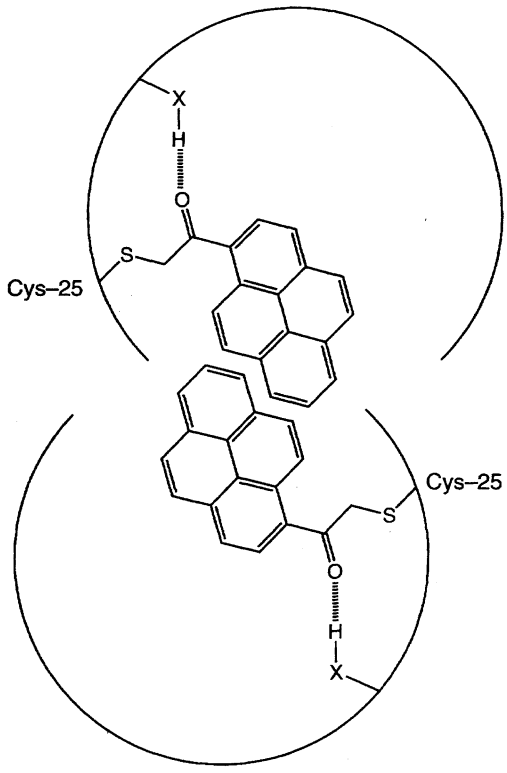


Fig. 11. Schematic illustration for BAP-derived pendant dimer.

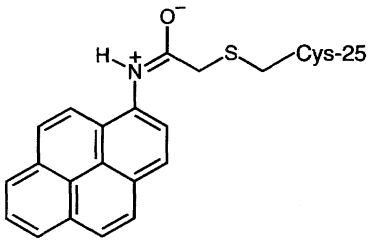
Because the polarization of π-electrons in the strongly hydrogen-bonded pyrenecarbonyl chromophore induces a large dipole moment in this chromophore, the dipole–dipole interaction between two pyrenecarbonylmethyl reporter groups on the enzyme surface (in addition to the hydrophobic interaction) is also a most likely driving force to accelerate the dimerization reaction of the pendant introduced into the active site.

From these consideration we see that the papain active site provides an environment favorable for the association of the pyrenecarbonylmethyl pendant through hydrogen bonding and hydrophobic interactions. If the imino group is introduced as a spacer between the 1-pyrenyl carbon and the acetyl carbonyl carbon in BAP-derived pendant, we can predict that this imino moiety should inhibit the generation of an enolate-type resonance structure and thereby should weaken a tendency to produce a pendant dimer in the ground state, based on the following speculation. Taking into account the possibility that the pyrenylcarbamoyl oxygen in a modified papain molecule is also able to form a hydrogen bond to the neighboring amino acid residue(s), the contribution of the amide resonance structure **II** (Chart 4) should be increased but π-electrons should be polarized in the pyrene ring to only a much smaller extent than the enolate-type resonance structure **I** (Fig. 10) of the pyrenecarbonyl chromophore incorporated into the papain active site. The results obtained for BAAP-modified papain and TAAP are compatible with our prediction and, hence, substantiate our proposal that the significant contribution of the enolate-type resonance structure (caused by the strong hydrogen bonding to the P_1 carbonyl oxygen) is a driving force for increasing the associated-dimer formation of the pyrenecarbonylmethyl probe mainly through dipole–dipole interaction as well as hydrophobic one.

Experimental

Materials and Solvents. Commercially available 1-bromoacetylpyrene (BAP, reagent for fluorescent labeling of organic acids; Wako Pure Chemicals) was used as received. 1-(Bromoacetylamino)pyrene (BAAP) was prepared by *N*-acylation of 1-pyrenamine with bromoacetyl bromide in the presence of pyridine in dichloromethane. 1-{[2-(Acetylamino)ethylthio]acetyl}pyrene (TAP) was synthesized by the reaction of BAP with *N*-(2-mercaptoethyl)acetamide in ethanol containing triethylamine. The reaction between BAAP and *N*-(2-mercaptoethyl)acetamide in the presence of triethylamine under similar conditions afforded 1-{2-[2-(acetylamino)-ethylthio]acetylamino}pyrene (TAAP). The physical and spectroscopic properties of these compounds are as follows.

BAAP: Mp 230—232 °C (recrystallized from acetone); IR (KBr) 3240 and 1660 cm^{-1}; ^1H NMR (200 MHz; DMSO-d_6) δ =



II

Chart 4.

4.35 (2H, s) and 8.05—8.36 (10H, m). Found: C, 63.93; H, 3.97; N, 3.99%. Calcd for $C_{18}H_{12}BrNO$: C, 63.93; H, 3.58; N, 4.14%.

TAP: Mp 138.5—140.5 °C (recrystallized from EtOAc–hexane); IR (KBr) 3280, 1650, and 1590 cm^{-1}; ^1H NMR (200 MHz; $CDCl_3$) δ = 1.93 (3H, s), 2.82 (2H, t, J = 6.3 Hz), 3.51 (2H, dt, J = 6.3, 6.3 Hz), 4.12 (2H, s), 6.20 (1H, t, J = 6.3 Hz), 8.01—8.33 (8H, m), and 8.94 (1H, d, J = 9.5 Hz); ^{13}C NMR ($CDCl_3$) δ = 23.2, 32.6, 38.3, 40.6, 124.0 (2C), 124.2, 124.6, 126.3, 126.58, 126.64, 126.68, 126.71, 127.1 129.9, 130.1, 130.3, 130.4, 131.0, 134.3, 170.2, and 198.7. Found: C, 72.86; H, 5.28; N, 3.75%. Calcd for $C_{22}H_{19}NO_2S$: C, 73.10; H, 5.30; N, 3.88%.

TAAP: Mp 169.0—170.0 °C (recrystallized from acetone); IR (KBr) 3260, 1650, and 1520 cm^{-1}; ^1H NMR (200 MHz; DMSO-d_6) δ = 1.84 (3H, s), 2.83 (2H, t, J = 6.6 Hz), 3.37 (2H, dt, J = 6.6, 6.6 Hz), 3.61 (2H, s), 8.05—8.36 (10H, m), and 10.50 (1H, s); ^{13}C NMR (DMSO-d_6) δ = 22.5, 31.6, 35.0, 38.3, 121.9, 122.4, 123.4, 123.8, 124.9, 125.1, 125.3, 126.5, 126.8, 127.2, 127.3, 128.6, 129.9, 130.4, 130.7, 131.5, 168.9, and 169.2. Found: C, 69.94; H, 5.62; N, 7.08%. Calcd for $C_{22}H_{20}N_2O_2S$: C, 70.19; H, 5.35; N, 7.44%.

1-Acetylpyrene-*carbonyl*-^{13}C was prepared from acetylation of pyrene with acetyl-*carbonyl*-^{13}C chloride (Aldrich, 99+ atom%) in the presence of $AlCl_3$[19] and was purified by column chromatography over silica gel (70—230 mesh, Merck) using hexane–EtOAc as the eluent. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ = 30.4 (d, J = 41.4 Hz), 123.95, 123.98, 124.3, 125.0, 126.1, 126.3, 126.4, 127.07, 127.11, 129.5, 129.6, 129.7, 130.5, 131.1, 132.2, 134.0, and 202.1 (^{13}C=O).

BAP-*carbonyl*-^{13}C was obtained (by treatment of ^{13}C-labeled 1-acetylpyrene with Br_2 in the presence of $AlCl_3$) as a crystalline precipitate.[20] ^{13}C NMR (125.7 MHz; $CDCl_3$) δ = 34.1 (d, J = 42.4 Hz), 124.0 (2C), 124.1, 124.7, 126.5, 126.6, 126.7, 126.8 (2C), 127.1, 130.3, 130.4, 130.5, 130.7, 131.1, 134.7, and 194.6 (^{13}C=O).

TAP-*carbonyl*-^{13}C was synthesized in the same manner as that used for making unlabeled TAP. ^{13}C NMR (125.7 MHz; $CDCl_3$) δ = 23.2, 32.7, 38.3, 40.6 (d, J = 42.4 Hz), 123.98, 124.01, 124.2, 124.5, 126.4, 126.58, 126.64, 126.68, 126.71, 127.1, 130.0, 130.2, 130.4, 130.5, 131.1, 134.4, 170.2, and 198.7 (^{13}C=O).

Two-times-crystallized and lyophilized papain (MW 23000) and *N*-benzoyl-L-arginine ethyl ester (L-BAEE) were purchased from Sigma and were used as received. Water was purified by distillation, followed by passage through a Millipore Milli-Q system. MeOH and MeCN were purified according to standard methods.[21] DMSO was of spectroscopic grade. All other chemicals used were obtained from commercial sources and were of the highest grade available.

Measurements. UV absorption and fluorescence spectra were recorded on a Shimadzu UV-2200 spectrophotometer and a Shimadzu RF-5000 spectrofluorimeter, respectively. Fluorescence lifetimes were measured under N_2 at room temperature with a time-correlated single-photon counting apparatus (Horiba NAES-700; excitation wavelength = 320 nm; cut-off wavelength = 360 nm). ^1H and ^{13}C NMR spectra were taken with a JEOL FX-200 or JNM-500 spectrometer. Chemical shifts were measured using tetramethylsilane or 1,4-dioxane as an internal standard.

A given amount of papain was dissolved in a phosphate buffer [0.1 mol dm^{-3}; pH 7.0, ionic strength I = 0.25 (KCl)] containing L-cysteine (5.0×10^{-3} mol dm^{-3}) and EDTA (1.0×10^{-3} mol dm^{-3}) and was activated by leaving the solution for 1—2 h at room temperature. After the enzyme solution had been centrifuged for 30 min at 0 °C, 18000 rpm, the papain concentration was measured by the absorbance of the supernatant solution at 280 nm (ε at 280 nm = 5.75×10^4 dm^3 mol^{-1} cm^{-1}).[7] Activated papain was dialyzed three times against ice-cold and N_2-purged phosphate buffer (0.1 mol dm^{-3}) of pH = 7.0 and I = 0.25 to exclude EDTA and L-cysteine and then allowed to react with 2—6-fold molar excess of BAP or BAAP in an N_2-purged phosphate buffer (pH 7.0) containing 3 vol% DMSO at room temperature. At the same time, a control enzyme solution was prepared under the same conditions except for the exclusion of the alkylating reagents. At suitable times, 100 μl of the reaction mixture was taken and added to a phosphate buffer solution (3 ml; pH 7.0) of L-BAEE (3.2×10^{-4} mol dm^{-3}) to record an increase in the absorbance at 253 nm of this substrate. A control solution was treated in the same way to measure the remaining activity. After the reaction had been completed, a turbidity and/or a precipitate was removed by centrifugation for 30 min at 0 °C, 18000 rpm. The supernatant was then dialyzed at least three times against phosphate buffer (0.1 mol dm^{-3}; pH 7.0) with a given ionic strength (2.0×10^{-3}—1.0) to remove excess alkylating reagents. An alkylated papain solution was stored in a refrigerator and was filtered through a membrane (pore size, 0.22 μm) just before each spectroscopic measurement.

This work was partially supported by a Grant-in-Aid for Scientific Research No. 09680578 (T.S.) from the Ministry of Education, Science, Sports, and Culture.

References

1) J. B. Birks, "Photophysics of Aromatic Molecules," Wiley-Interscience, London (1970).

2) F. M. Winnik, *Chem. Rev.*, **93**, 587 (1993).

3) K. A. Zachariasse and A. Kühnle, *Z. Phys. Chem.*, **101**, 267 (1976); P. Collart, K. Demeyer, S. Toppet, and F. C. De Schryver, *Macromolecules*, **16**, 1390 (1983); R. Goedeweeck and F. C. De Schryver, *Photochem. Photobiol.*, **39**, 515 (1984); P. Collart, S. Toppet, Q. F. Zhou, N. Boens, and F. C. De Schryver, *Macromolecules*, **18**, 1026 (1985); R. Goedeweeck, M. Van der Auweraer, and F. C. De Schryver, *J. Am. Chem. Soc.*, **107**, 2334 (1985); K. A. Zachariasse, G. Duveneck, and W. Kühnle, *Chem. Phys. Lett.*, **113**, 337 (1985); P. Collart, S. Toppet, and F. C. De Schryver, *Macromolecules*, **20**, 1266 (1987); R. Andriessen, F. C. De Schryver, N. Boens, N. Ikeda, and H. Masuhara, *Macromolecules*, **22**, 2166 (1989); D. F. Eaton and B. E. Smart, *J. Am. Chem. Soc.*, **112**, 2821 (1990).

4) a) P. Reynders, H. Dreeskamp, W. Kühnle, and K. A. Zachariasse, *J. Phys. Chem.*, **91**, 3982 (1987); b) P. Reynders, W. Kühnle, and K. A. Zachariasse, *J. Am. Chem. Soc.*, **112**, 3929 (1990); c) P. Reynders, W. Kühnle, and K. A. Zachariasse, *J. Phys. Chem.*, **94**, 4073 (1990); d) D. Declercq, P. Delbeke, F. C. De Schryver, L. Van Meervelt, and R. D. Miller, *J. Am. Chem. Soc.*, **115**, 5702 (1993); e) J. Gelan, P. Adriaensens, D. Vanderzande, D. Declercq, E. Hermans, and F. C. De Schryver, *J. Am. Chem. Soc.*, **116**, 7877 (1994).

5) a) I. Willner S. Rubin, and A. Riklin, *J. Am. Chem. Soc.*, **113**, 3321 (1991), and references cited therein; b) I. Willner and S. Rubin, *J. Am. Chem. Soc.*, **114**, 3150 (1992); c) I. Hamachi, S. Tanaka, and S. Shinkai, *J. Am. Chem. Soc.*, **115**, 10458 (1993); d) T. Hohsaka, K. Kawashima, and M. Sisido, *J. Am. Chem. Soc.*, **116**, 413 (1994); e) I. Willner and E. Zahavy, *Angew. Chem., Int. Ed. Engl.*, **33**, 581 (1994).

6) M. Burr and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **52**, 1017 (1964); M. B. Hille and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **89**, 5945 (1967); D. S. Sigman, D. A. Torchia, and

E. R. Blout, *Biochemistry*, **8**, 4560 (1969); W. C. Groutas, M. A. Stanga, and M. J. Brubaker, *J. Am. Chem. Soc.*, **111**, 1931 (1989); J. Broos and R. M. Kellogg, *Recl. Trav. Chim. Pays-Bas*, **109**, 299 (1990); T. Sakurai, A. Kageyama, H. Hayashi, and H. Inoue, *Bull. Chem. Soc. Jpn.*, **65**, 2948 (1992).

7) J. R. Whitaker and M. L. Bender, *J. Am. Chem. Soc.*, **87**, 2728 (1965).

8) A. N. Glazer and E. L. Smith, "The Enzymes," 3rd ed, ed by P. D. Boyer, Academic, New York (1971), Vol. 3, pp. 515—516.

9) H. L. Levine, Y. Nakagawa, and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **76**, 64 (1977); H. L. Levine and E. T. Kaiser, *J. Am. Chem. Soc.*, **100**, 7670 (1978).

10) T. Ikkai and H. Kondo, *J. Biochem. Biophys. Methods*, **33**, 55 (1996).

11) T. Sakurai, K. Inomata, T. Ishikawa, H. Inoue, T. Hoshi, and J. Okubo, *Bull. Chem. Soc. Jpn.*, **60**, 4099 (1987); T. Sakurai, K. Wada, and H. Inoue, *Nippon Kagaku Kaishi*, **1993**, 728.

12) R. Koren and G. G. Hammes, *Biochemistry*, **15**, 1165 (1965); K. Ikeda, S. Kunugi, and N. Ise, *J. Biochem.*, **92**, 541 (1982); H. Kitano, Y. Maeda, and T. Okubo, *Biophys. Chem.*, **33**, 47 (1989).

13) J. Drenth, J. N. Jansonius, R. Koekoek, and B. G. Wolthers, "The Enzymes," 3rd ed, ed by P. D. Boyer, Academic, New York (1971), Vol. 3, pp. 490—499.

14) N. E. Mackenzie, S. K. Grant, I. Scott, and J. P. G. Malthouse, *Biochemistry*, **25**, 2293 (1986); J.-R. Brisson, P. R. Carey, and A. C. Storer, *J. Biol. Chem.*, **261**, 9087 (1986); J. B. Moon, R. S. Coleman, and R. P. Hanzlik, *J. Am. Chem. Soc.*, **108**, 1350 (1986); T.-C. Liang and R. H. Abeles, *Arch. Biochem. Biophys.*, **252**, 626 (1987); Y. Yabe, D. Guillaume, and D. H. Rich, *J. Am. Chem. Soc.*, **110**, 4043 (1988).

15) R. A. Smith, L. J. Copp, P. J. Coles, H. W. Pauls, V. J. Robinson, R. W. Spencer, S. B. Heard, and A. Krantz, *J. Am. Chem. Soc.*, **110**, 4429 (1988); V. J. Robinson, H. W. Pauls, P. J. Coles, R. A. Smith, and A. Krantz, *Bioorg. Chem.*, **20**, 42 (1992).

16) a) J. Drenth, K. H. Kalk, and H. M. Swen, *Biochemistry*, **15**, 3731 (1976); b) K. Matsumoto, M. Murata, S. Sumiya, K. Kitamura, and T. Ishida, *Biochim. Biophys. Acta*, **1208**, 268 (1994).

17) T. Sakurai, T. Uno, M. Masui, K. Kubo, and H. Inoue, to be published.

18) M.-J. Kim, D. Yamamoto, K. Matsumoto, M. Inoue, T. Ishida, H. Mizuno, S. Sumiya, and K. Kitamura, *Biochem. J.*, **287**, 797 (1992).

19) C. Merritt, Jr., and C. E. Braun, *Org. Synth.*, Coll. Vol. IV, 8 (1963).

20) R. M. Cowper and L. H. Davidson, *Org. Synth.*, Coll. Vol. II, 480 (1950).

21) J. A. Riddick, W. B. Bunger, and T. K. Sakano, "Organic Solvents," Wiley, Chichester (1986).