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FLUORESCENCE LIFETIMES OF BENZOFURAZAN ADDUCTS WITH THIOL GROUPS IN BIOMOLECULES

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Abstract - Fluorescence lifetimes have been measured for the adducts of ammonium 4-chloro-2,1,3-benzoxadiazole-7-sulfonate, which is known as a fluorescence probe specific to thiol groups. Although the adduct with simple molecule such as mercaptoethanol showed a single exponential fluorescence decay, most adducts with amino acid, peptide, coenzyme and protein showed the bi exponential decay. The fluorescence lifetimes and ratio of the short lifetime fraction to long lifetime fraction of the SBD adducts with biomolecules vary with the molecular size, solvent polarity and micro environment around the fluorescence probe.

INTRODUCTION

Thiol groups in biomolecules have much attentions in biochemistry, because of their important roles in the biological functions.[1] There are a lot of efforts to reveal the biochemical functions of the thiol groups by spectroscopic methods. Fluorescence spectra are quite sensitive to micro environments which surround the fluorescence probe molecules.[2-4] Thus, the various fluorescent materials have been used as sensitive probes to investigate the properties of biomolecules.[5-7] There are two types of fluorescence probes; one is the intrinsic probe method in which fluorescent moieties are included in the molecules. The other is extrinsic probe method, in which appropriate fluorescence probe is added to non-emissive

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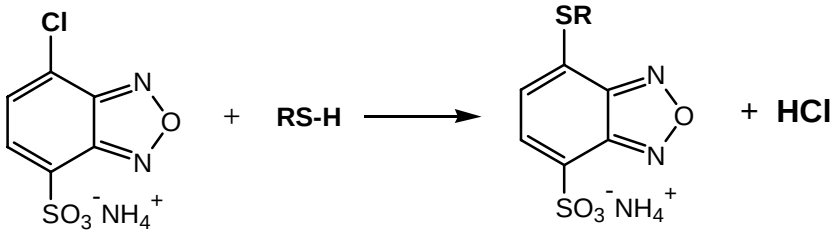
This study is dedicated to Professor Yuichi Kanaoka.

biomolecules. In the case of thiol groups, covalently bonded extrinsic probe method has been employed.[8-12] As for one of the fluorescence probes bound specifically to the thiol group, benzofurazans have been developed.[7-9] The halogenated benzofurazans (benzoxadiazoles) have been used to determine the quantities of the thiol groups in biomolecules by measuring the steady-sate fluorescence intensity, because the halogenated benzofurazan is non-fluorescent while the S-atom substituted one is fluorescent. In the present study, ammonium 4-chloro-2,1,3-benzoxadiazole-7-sulfonate, which was abbreviated as SBD-Cl (Scheme 1), was employed, because of its highly sensitive fluorescence probe the thiols in biomolecules.[6]



SBD-Cl SBD-SR

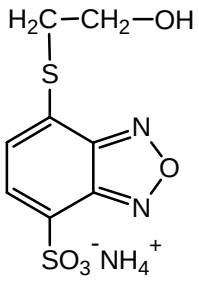
Scheme 1

In general, it would be expected that the fluorescence-lifetime measurements are useful to disclose the dynamic properties of the micro environments near the thiol groups in the molecules.[4] In the present study, we measured the fluorescence lifetimes of the adducts of the benzofurazans with the thiol groups of various biologically important molecules such as amino acid, peptide, coenzyme and protein. Such information would be useful to reveal the biological functions of the thiol group.

RESULTS AND DISCUSSION

Adduct with mercapto-2-ethanol (HS-EtOH)

The adduct formation was followed by the simultaneous appearance of the absorption bands at 382 and 240 nm exhibiting two isosbestic points at 345 and 260 nm with decreasing in the absorption bands of SBD-Cl at 325 and 290 nm after mixing with HS-EtOH (Figure 1). With appearance of the new absorption bands, the intensity of the fluorescence band at 530 nm increases, indicating that adduct SBD S-EtOH was formed.[7] The saturation of the adduct absorbance and fluorescence intensity indicates that the adduct formation finished after 12 h. Figure 2 shows the fluorescence spectra of SBD-S-EtOH in aqueous buffer solution and in 95 % ethanol. The shift of the fluorescence peak was observed by changing the solvent from 95 % ethanol (505 nm) to aqueous solution (530 nm). The fluorescence intensity homogeneously decays in a single exponential giving lifetime (τ_f) of 5.00 ns in aqueous solution



SBD-S-EtOH

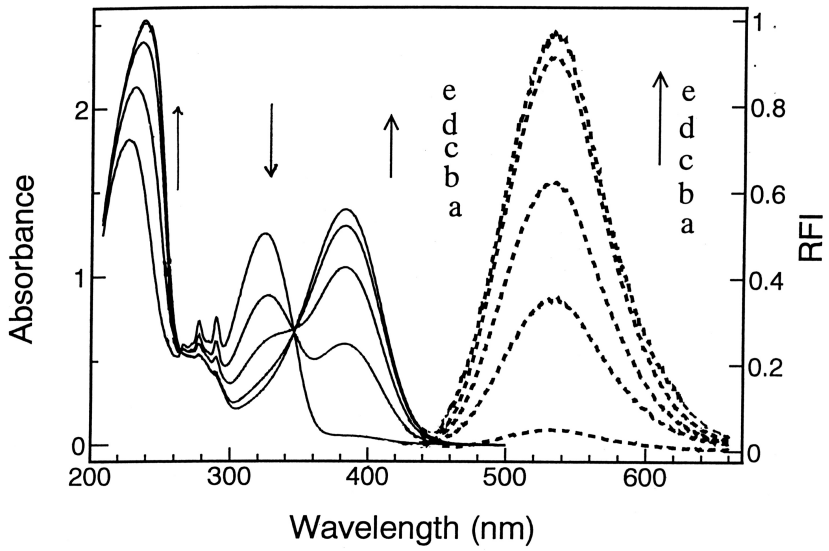


Figure 1. Absorption (solid line) and fluorescence spectra (dashed line, RFI; relative fluorescence intensity, λ_{ex} = 390 nm) of reaction mixture of SBD-Cl (0.2 mM) with HS-EtOH (1.2 mM) in aqueous solution (pH 9). (a) 0, (b) 2, (c) 5, (d) 9 and (e) 12 h.

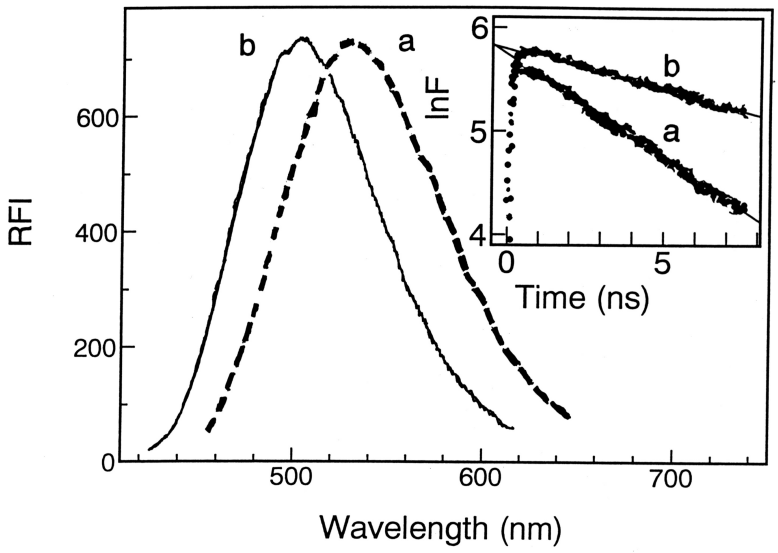


Figure 2. Fluorescence spectra of SBD-S-EtOH (a) in aqueous solution (pH 9) and (b) in 95 % ethanol; intensity is normalized. Insert: ln(fluorescence intensity) vs. time at each peak.

and 12.05 ns in 95 % ethanol. For the SBD adduct with simple small molecule, the observed slight change of the lifetime may be attributed to the solvent polarity effect on the SBD-S-EtOH.[13,14]

Adduct with cysteine (HS-Cys)

The SBD adduct formation with HS-Cys was also recognized by the steady-state absorption band at 382 nm and fluorescence band at 520 nm, although the rate of the adduct formation is slower than that with HS-EtOH. The decay curve of the fluorescence intensity at 522 nm is shown in Figure 3. The decay rate

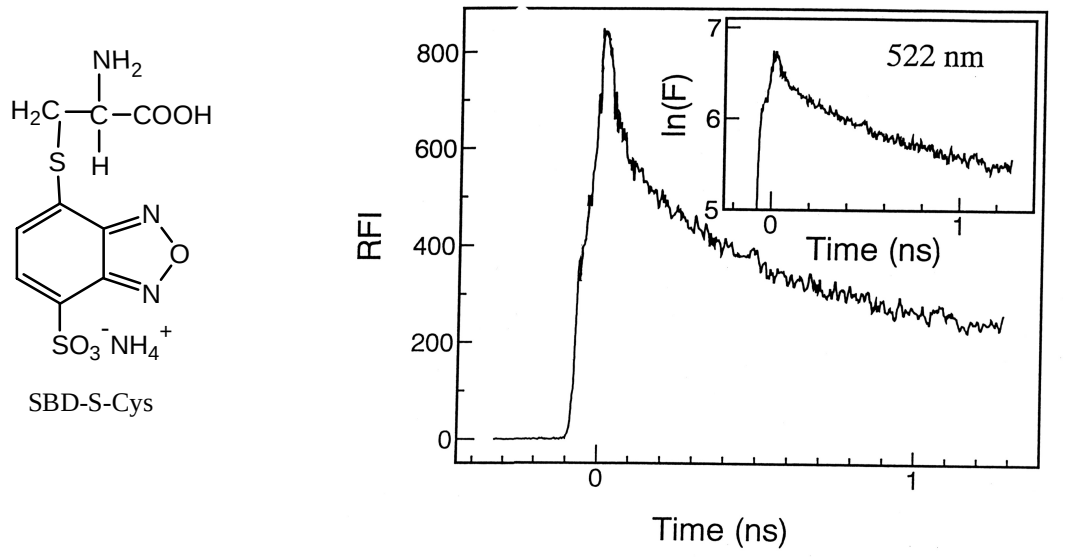
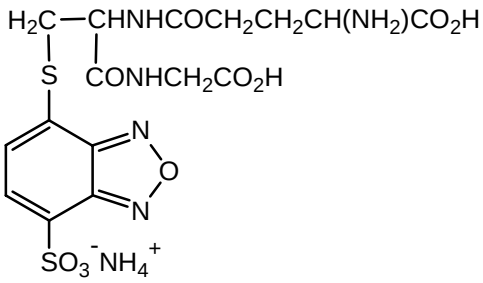


Figure 3. Time profile of fluorescence intensity of SBD-S-Cys in aqueous solution (pH 9). Insert: ln(fluorescence intensity) vs. time.

in the initial part is quite faster than that of SBD-S-EtOH. This was supported by the quite small relative fluorescence quantum yield of SBD-S-Cys reported by Andrews *et al.*[7] As one of the reasons for the short lifetime of SBD-S-Cys adduct, the polar groups such as $-NH_2$ and $-CO_2H$ are present quite near the SBD moiety, which may influence the fluorescence lifetime.[3,13] These decay curves were fitted with bi-exponential with τ_f = 0.17 (40 %) and τ_f = 1.78 ns (60 %) in aqueous solution. In the time-resolved spectra, the fluorescence peak at initial part (0 - 0.2 ns) appears at 520 nm, while the peak shifts to 530 nm in latter time (0.0 - 2.5 ns). This indicates that there are two conformers in the fluorescent adduct. In 95 % ethanol, the τ_f value became short with an increase in the fraction of the fast decay part (τ_f = 0.044 ns (88 %)). This may imply that the interaction between SBD and amino acid groups in SBD-S-Cys increases in ethanol rich solvent.

Adduct with glutathione (HS-Glut)

The adduct formation of SBD-Cl with HS-Glut was also followed by the appearance of the steady state absorption band at 378 nm and the fluorescence peak at 530 nm in aqueous solution.[6] The adduct formation finished within *ca*. 14 h after mixing. The peak of the steady-state fluorescence band at 530 nm in aqueous solution shifts to 503 nm in 95 % ethanol solution. In Figure 4, the fluorescence decay curves in two solvent systems are shown. In 95 % ethanol, the decay rates decreased compared with that in aqueous solution. Each decay curve was also fitted with bi-exponential. The lifetimes of the fast decay part (τ_f = 0.44 ns (52 %) in aqueous solution and τ_f = 0.62 ns (37 %) in 95 % ethanol) are longer



SBD-S-Glut

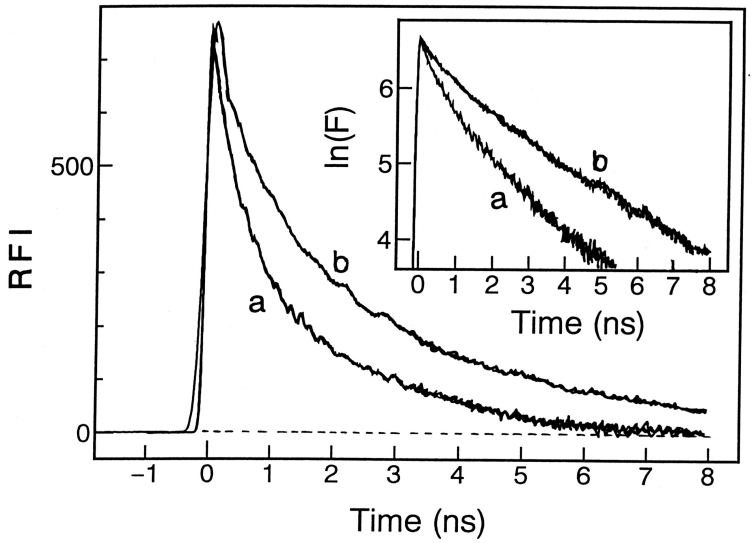


Figure 4. Time profiles of fluorescence intensity of SBD-S-Glut; (a) aqueous solution (at 530 nm) and (b) in 95 % ethanol (at 490 nm). Insert: ln(fluorescence intensity) vs. time.

than those of SBD-S-Cys in each solvent, while those of the slow part are similar (τ_f = 2.05 ns (48 %) in aqueous solution and τ_f = 3.39 ns (63 %) in 95 % ethanol). This suggests that the longer molecular chain of -Glut compared with -Cys slows down the fast decay of the fluorescence of SBD-S-Glut, because -NH_2 and –CO_2H groups are distant from the SBD chromophore.

Adduct with coenzyme A (HS-CoA)

The adduct formation (SBD-S-CoA) was confirmed with the appearance of absorption band at 388 nm and fluorescence band at 530 nm. The rate of the adduct formation becomes further slow down. This finding can be interpreted by the steric reasons that the long side chains of -CoA prohibit the adduct formation reaction. The time-resolved fluorescence spectra for the adduct of SBD-S-CoA are shown in Figure 5. In aqueous buffer solution at pH 9 (spectrum a), the fluorescence intensity decays in a single exponential (τ_f = 1.67 ns (100 %)); the time-resolved spectrum is in agreement with the corresponding steady-state spectrum. This single decay indicates that the major conformer of the excited SBD-moiety is stable within the fluorescence lifetime. In 95 % ethanol, the fluorescence intensity decays with bi-exponential curves; τ_f = 0.34 ns (47 %) and τ_f = 4.47 ns (53 %). The peak of time-resolved spectrum with long τ_f value (spectrum b' = spectrum b'') is shifted to the longer wavelength compared with the peak of spectrum b with short τ_f value. This suggests the presence of some conformers in ethanol rich solvent.

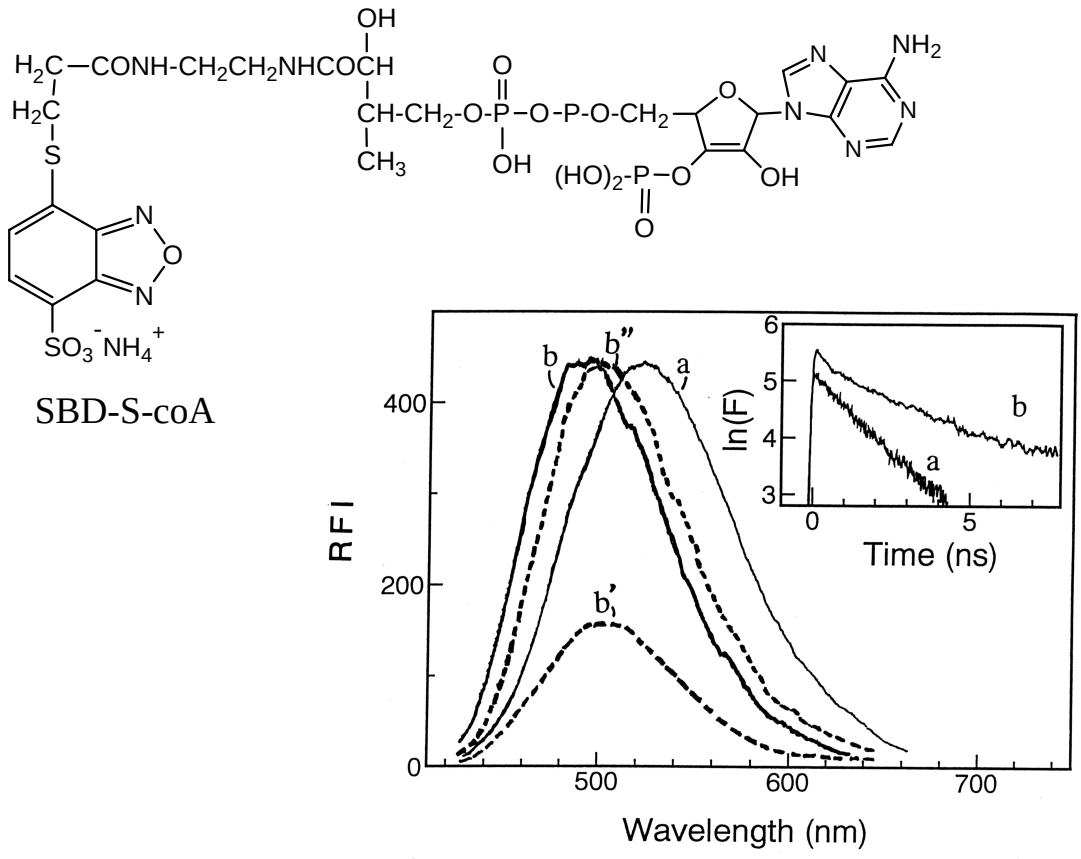


Figure 5. Time-resolved fluorescence spectra of SBD-S-CoA; (a) initial fluorescence spectrum at 0.5 ns in aqueous solution, (b) initial fluorescence spectrum in 95 % ethanol solution at 1.0 ns, (b') at 4.0 ns in 95 % ethanol and (b") intensity is normalized. Insert: ln(fluorescence intensity) vs. time, (a) at 520 nm in aqueous solution and (b) at 500 nm in 95 % ethanol solution.

Adduct with bovine serum albumin (HS-BSA)

The rates of the adduct formation of SBD with bovine serum albumin (HS-BSA) were quite slow. The characteristic fluorescence peak at 505 nm of SBD-S-BSA appeared in aqueous solution.[6] The time resolved fluorescence spectra of SBD-S-BSA are shown in Figure 6, in which spectrum a with short τ_f value is slightly broader than spectra b (=spectrum b') with long τ_f value. The decay time profile at the peak is curve-fitted with bi-exponential decays; the τ_f values in aqueous solution (1.01 ns (51 %) and 7.92 ns (49 %)). The shorter lifetime is the longest among the shorter ones of bi-exponential decays. The longer lifetime is as slow as that of SBD-S-EtOH. These findings suggest that the environment of thiol in BSA is less polar or hydrophobic. This implies that $-NH_2$ and $-CO_2H$ may not be present near the thiol group. It is presumed that the thiol group is present inside of the protein apart from the solvent water.

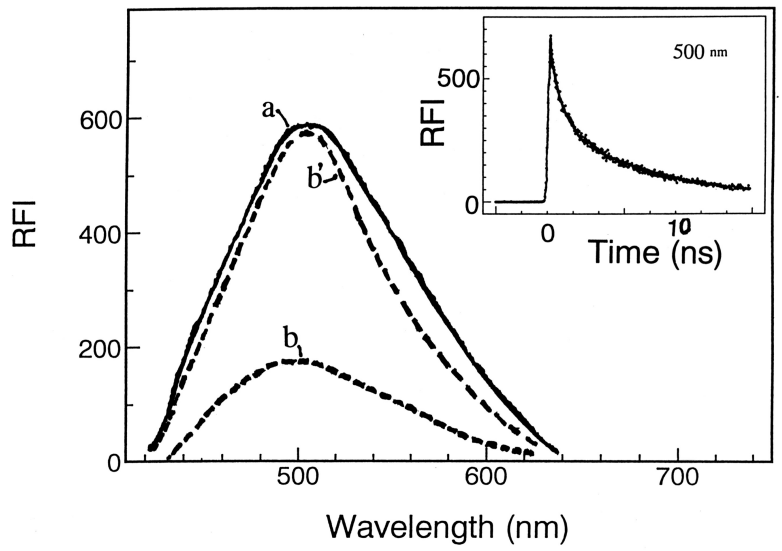


Figure 6. Time-resolved fluorescence spectra of SBD-S-BSA in aqueous solution (pH 9); (a) initial fluorescence spectrum at 1.0 ns, (b) fluorescence spectrum at 6.0 ns and (b') the intensity is normalized. Insert: Time profile of fluorescence intensity at 500 nm.

SUMMARY

In Table 1, the fluorescence lifetimes obtained in the present study are summarized. It is revealed that the time-resolved fluorescence measurements afford valuable informations about the fluorescent SBD-thiol adducts. Especially, the circumstance of SBD-thiol moiety in biomolecules can be presumed from the lifetimes of the adducts.

Table 1. Fluorescence lifetimes of SBD-thiol adducts; τ_f is fluorescence lifetime at peak (fraction of bi-exponential decays).

Thiol	SBD-S-EtOH	SBD-S-Cys	SB-S-Glut	SBD-S-CoA	SBD-S-BSA
	τ_f / ns (%)	τ_f / ns (%)	τ_f / ns (%)	τ_f / ns (%)	τ_f / ns (%)
aqueous					
fast decay	--- (0)	0.17 (40)	0.44 (52)	--- (0)	1.01 (51)
slow decay	5.00 (100)	1.78 (60)	2.05 (48)	1.67 (100)	7.92 (49)
EtOH(95%)					
fast decay	--- (0)	0.04 (88)	0.62 (37)	0.34 (47)	insoluble
slow decay	12.05 (100)	3.52 (12)	3.39 (63)	4.47 (53)	insoluble

EXPERIMENTAL

Ammonium 4-chloro-2,1,3-benzoxadiazole-7-sulfonate (SBD-Cl) was purchased from Dojin Laboratory LTD.[6] Commercially available thiols were used without further purification. Solvents were of non-fluorescent spectra-grade. The adducts were prepared in aqueous solution at pH 9 (buffer; 0.1 M H_3BO_3/NaOH) containing 1 mM EDTA by mixing of SBD-Cl (usually 2 mM) with 1 mM of thiols at 40 ^oC.[6]

The lifetimes of fluorescence were measured by a streak scope C4334 (Hamamatsu Photonics Co. LTD) with excitation laser light from a Ti:sapphire-laser pumped with Ar-ion laser (Spectra Physics Co. LTD).

REFERENCES

1 A. L. Fluharty, 'The Chemistry of the Thiol Group,' Vol. 2, ed. by S. Patai, John Wiley & Sons, London, 1974, pp. 589-668.

2 P. Pringsheim, 'Fluorescence and Phosphorescence,' Interscience Publisher Inc., New York, 1949, pp. 315-322.

3 Y. Kanaoka, *Angew. Chem.*, 1977, **89**, 142.

4 J. R. Lakowicz, 'Topics in Fluorescence Spectroscopy,' Vol. 3, Plenum, New York. 1992.

5 D. C. Turner and L. Brand, *Biochemistry*, 1968, **7**, 3381.

6 R. B. Macgregor and G. Weber, *Nature*, 1986, **319**, 70.

7 J. L. Andrews, P. Ghosh, B. Ternai, and M. W. Whitehouse, *Arch. Biochem. Biophys.*,1982, **214**, 386.

8 T. Toyo-oka and K. Imai, *Annal. Chem.*, 1984, **56**, 2461.

9 P. B.Ghosh and M. W. Whitehouse, *J. Med. Chem.*, 1968, **11**, 305.

10 R. A. Kenner and A. A. Aboderin, *Biochemistry*, 1971, **10**, 4433.

11 Y. Matsushita, M. Takahashi, and I. Moriguchi, *Chem. Pham. Bull.*, 1986, **34**, 333.

12 H. Takahashi, Y. Nara, H. Meguro, and K. Tuzimura, *Agric. Biol. Chem.*, 1979, **43**, 1439.

13 G. W. Robinson, R. J. Robinson, G. R. Fleming, J. M. Morris, A. E. W. Knight, and R. J. S. Morrison, *J. Am. Chem. Soc.*, 1978, **100**, 7145.

14 C. Reichardt, 'Solvents and Solvent Effects in Organic Chemistry,' VCH, Weinheim, 1988, pp. 285-338.