

Hydrolysis of N-Substituted Maleimides: Stability of Fluorescence Thiol Reagents in Aqueous Media¹⁾

MINORU MACHIDA,^{2a)} MIKIKO I. MACHIDA, and YUICHI KANAOKA^{2b)}

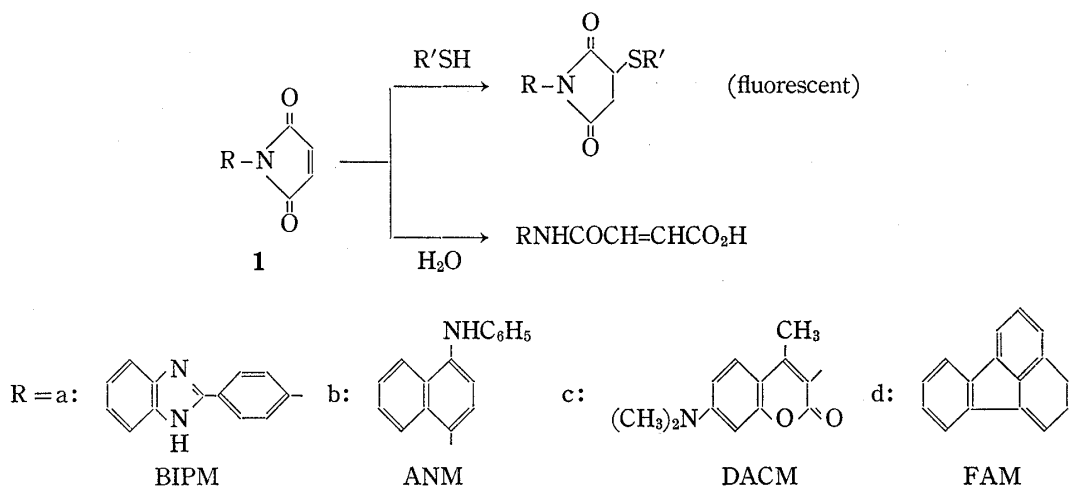
Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University^{2a)} and
Faculty of Pharmaceutical Sciences, Hokkaido University^{2b)}

(Received March 4, 1977)

p-Substituted phenylmaleimides were synthesized and their hydrolytic rates were measured by dual-wavelength method. Hammett plot for the hydrolysis of *p*-substituted phenylmaleimide yields a straight line of $\rho=0.35$ with relative coefficient $r=0.99$. The half-life times of a series of maleimide derivatives including the practical fluorescent thiol reagents such as N-[*p*-(2-benzimidazolyl)phenyl]maleimide, N-(1-anilino-naphthyl-4)-maleimide and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide were compared and their stabilities in aqueous media were discussed.

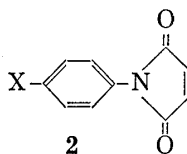
Keywords—fluorogenic group; dual-wavelength method; Hammett plot; half-life time; substitution effect

The chemistry of cyclic imides has been extensively studied in various fields and reviewed up to recent years.³⁾ In particular, since N-substituted maleimides (**1**) react rapidly with thiol groups, they have been widely used in biological fields as thiol reagents.⁴⁾ In previous papers we have reported on the systematic studies of fluorescent thiol reagents which possess both a fluorogenic group and a maleimide moiety in one molecule. For example, BIPM (**1a**),⁵⁾



- 1) a) Fluorescent Thiol Reagents. XIV. Part XIII: M. Machida, M.I. Machida, T. Sekine, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **25**, 1678 (1977); b) The abbreviations used are: BIPM=N-[*p*-(2-benzimidazolyl)phenyl]maleimide; ANM=N-(1-anilino-naphthyl-4)-maleimide; DACM=N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide; FAM=N-(3-fluoranthyl)maleimide; NEM=N-ethylmaleimide.
- 2) Location: a) *Ishikari-Tobetsu, Hokkaido 061-02, Japan*; b) *Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan*.
- 3) M.K. Hargreaves, J.G. Pritchard, and H.R. Dave, *Chem. Rev.*, **70**, 439 (1970).
- 4) For example, see: a) M. Friedman, "The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins." Pergamon Press, Ltd., Oxford, 1973, p. 114; b) P.C. Jocelyn, "Biochemistry of the SH Group." Academic Press, London, 1972, p. 142.
- 5) Y. Kanaoka, M. Machida, K. Ando, and T. Sekine, *Biochim. Biophys. Acta*, **207**, 269 (1970).

ANM (**1b**),⁶⁾ DACM (**1c**)^{1a,7)} and FAM (**1d**)⁸⁾ were synthesized as the fluorescent thiol reagents of maleimide-type and applied to various biochemical studies. In the course of design of such fluorescent thiol reagents which contain a variety of groups in various positions in the molecules, the influence of these N-substituents on the reactivities of the maleimide moiety must be considered. For example, since the reaction with thiol compounds is generally carried out in aqueous media, the reaction of a maleimide-type reagent with a thiol compound⁹⁾ must compete with the hydrolytic reaction of the maleimide ring leading to a maleamic acid (Chart 1). Although it has been well known that ring-opening reactions of cyclic imides occur in alkaline solutions under mild conditions,³⁾ there are few reports on quantitative estimations of the influence of N-substitution on the stability of a maleimide ring in aqueous solutions except some limited informations on the hydrolysis of NEM.^{9,10)} Therefore, in order to systematically develop novel fluorescent thiol reagents it was desirable to evaluate the effect of N-substituents (R in **1**) on the hydrolytic behavior of a maleimide moiety. In addition, needs for this study stemmed also from the other fields of biological research. For example, application of enzyme immunoassay for determination of various biochemical entities in clinical studies has recently attracted considerable attention because the method is expected to overcome some drawbacks inherent in the radioimmunoassay technique now commonly employed.¹¹⁾ In the enzyme immunoassay method, the reaction of maleimides and thiols in protein has been proposed to be one of promising means of coupling antigens or antibodies to enzymes.¹²⁾ Thus, the investigation of behavior of maleimides in aqueous media should generally contribute to the understanding of chemical modification of thiols in protein. The present paper describes the hydrolysis of *p*-substituted phenylmaleimides and of the maleimide-type reagents such as BIPM, ANM, DACM and NEM, which are practically employed for studies of thiols.

TABLE I. N-(*p*-Substituted Phenyl)maleimide

X	Maleamic acid mp (°C)	Maleimide		
		Recryst. solvent	mp (°C)	Lit. (mp °C)
NO ₂	190 —192.5	Benzene	168 —169	162 —165 ^{a)}
Cl	196 —198	Acetone- <i>n</i> -hexane	103 —107	109 —110 ^{b)}
H	206 —208	Benzene	88 — 89.5	87.5— 88.5 ^{c)}
CH ₃	192.5—193	Acetone- <i>n</i> -hexane	148.5—150	147 —150 ^{e)}
CH ₃ O	190 —190.5	Acetone- <i>n</i> -hexane	145 —148.5	146 —148 ^{e)}
(CH ₃) ₂ N	202 —203.5	Benzene- <i>n</i> -hexane	148 —150	153 —154 ^{d)}

a) W.R. Roderick, *J. Am. Chem. Soc.*, **79**, 1710 (1957).

b) A.E. Kretov and N.E. Kul'chitskaya, *Zhur. Obshchei Khim.*, **26**, 208 (1956) [*C. A.*, **50**, 13772a (1956)].

c) I. Matsuda, K. Akiyama, T. Toyoshima, S. Kato, and M. Mizuta, *Bull. Chem. Soc. Jpn.*, **48**, 3675 (1975).

d) W.R. Roderick and P. L. Bhatia, *J. Org. Chem.*, **28**, 2018 (1963).

- 6) Y. Kanaoka, M. Machida, M.I. Machida, and T. Sekine, *Biochim. Biophys. Acta*, **317**, 563 (1973).
7) a) M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **23**, 1385 (1975); b) M. Machida, N. Ushijima, T. Takahashi, and Y. Kanaoka, *ibid.*, **25**, 1289 (1977).
8) Y. Kanaoka, T. Takahashi, M. Machida, K. Yamamoto, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **24**, 1417 (1976).
9) J.D. Gregory, *J. Am. Chem. Soc.*, **77**, 3922 (1955).
10) J.R. Heitz, C.D. Anderson, and B.M. Anderson, *Arch. Biochem. Biophys.*, **127**, 627 (1968).
11) For a recent review, see: S.L. Scharpe, W.M. Cooreman, W.J. Blomme, and G.M. Laekeman, *Clin. Chem.*, **22**, 733 (1976).
12) K. Kato, Y. Hamaguchi, H. Fukui, and E. Ishikawa, *J. Biochem.*, **78**, 235 (1975).

Results

p-Substituted phenylmaleimides were synthesized by usual method¹³⁾ (Table I). Kinetic measurements of the imide hydrolysis were carried out by a dual-wavelength method on the basis of the difference of absorbances at two appropriate wavelengths of the N-substituted

TABLE II. Wavelengths (λ_1, λ_2 (nm)) used for Kinetic Measurements

	<i>p</i> -Substituent of phenylmaleimide						BIPM	ANM	DACM
	NO ₂	Cl	H	CH ₃	CH ₃ O	(CH ₃) ₂ N			
λ_1	294.5	340	340	340	350	340	304	330	383
λ_2	327	270	265	270	276	300	340	350	415

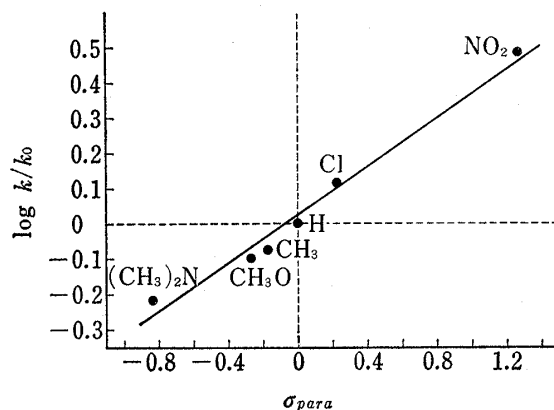
phenylmaleimides and the corresponding phenylmaleamic acids. Two different wavelengths used are listed in Table II. Fig. 1 shows a Hammett plot of $\log k/k_0$ vs the σ value at pH 8.0 (30°).¹⁴⁾ The Hammett plot¹⁵⁾ relative to the *p*-substituents in the N-phenylmaleimide yielded a straight line having a slope of $\rho=0.35$, and correlation coefficient $r=0.99$, as obtained using the least squares method. For the *p*-nitro compound the σ^- value (1.27)¹⁵⁾ provided the most reasonable fit with the data. This ρ value which reflects the nature and the magnitude of the substitution effect indicates that in the hydrolysis of maleimides inductive effects of the substituents are significant and electron-withdrawing groups will enhance the rate of the hydrolysis.

Half-life times of the series of *p*-substituted phenylmaleimides were determined at various pH and listed in Table III. Those of the fluorescent thiol reagents such as BIPM, ANM and DACM were also calculated and included as well as that of NEM for comparison.

TABLE III. Half-Life Times (min) of Maleimide Derivatives in 0.1 M Phosphate Buffers at 30°

Condition	NEM	ANM	DACM	BIPM	<i>p</i> -Substituent(X) of phenylmaleimide					
					X= NO ₂	Cl	H	CH ₃	CH ₃ O	(CH ₃) ₂ N
pH 7.0	25 ^{a)}	3.3 ^{a)}	1.2 ^{a)}	0.6 ^{a)}			1.1 ^{a)}			
pH 8.0				14	7	16	20	24	25	33
pH 9.0				2	1	2	2	3	3	4

a) min $\times 10^{-2}$.

Fig. 1. A Hammett Plot for the Hydrolysis of *p*-Substituted Phenylmaleimides in 0.1 M Phosphate Buffer (pH 8.0) at 30°

The $\sigma^- = 1.27$ for *p*-nitro compound was used.
The k_0 is the rate constant of unsubstituted phenylmaleimide.

13) M. Machida, M. Bando, Y. Migita, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **24**, 3045 (1976).

14) M.I. Machida, Thesis, Hokkaido University, 1973.

15) J. Hine, "Physical Organic Chemistry," 2nd Ed., International Student Edition, McGraw-Hill Book Co., Inc., New York, Kōgakusha Co., Ltd., 1962, Chapter 4.

Discussion

N-Ethylmaleimide (NEM), a well known thiol reagent in protein chemistry,⁴⁾ has been shown to be stable at pH 7.0 but much less stable under more alkaline condition.¹⁰⁾ Heitz, *et al.* reported that the hydrolytic reaction of NEM is specific base-catalyzed in the pH 8.6—9.4 range, and no noticeable hydrolysis of NEM occurred after 1 hr incubation at pH 7.0 at 20°.¹⁰⁾ It is worth noting that electronic factors introduced by aromatic substitution on the imide nitrogen is reflected in accelerated rate of hydrolysis. Thus as shown in Table III the half-life time of N-phenylmaleimide is more than one order of magnitude shorter than that of NEM suggesting that N-alkylmaleimides are generally more stable for hydrolysis than N-arylmaleimides. These results indicate that the maleimides with aliphatic substituent on the nitrogen may be more suitable than the aromatic counterparts as a reagent in chemical modification particularly when rather long incubation time in aqueous media is required. Therefore, for example, as a cross-linking reagent in the enzyme-immunoassay technique, bifunctional maleimides with aliphatic substituents would be preferred to their aromatic analogs.¹⁶⁾

In N-substituted maleimides resonance structures such as **3** and **4** contribute to the electronic character of the imide carbonyl to be attacked in the course of the hydrolysis. Excluding steric effects introduced by R, the greater the contribution of resonance structure (**4**), the slower the nucleophilic attack by water since the effective positive charge on imide carbonyl is decreased. Introduction of phenyl on the nitrogen provides another contributing structures (**5** and **6**), the influence of which is to increase the net positive character at the reaction center (Chart 2). Placement of electron-withdrawing substituents in the *para* position increases the contribution made by resonance form (**7**) and the rate of hydrolysis is accelerated. A plausible rationalization is dependent upon a rate-determining step involving formation of the solvated, tetrahedral intermediate (**8**) which rapidly collapses to the maleamic acid (**9**) (Chart 3).

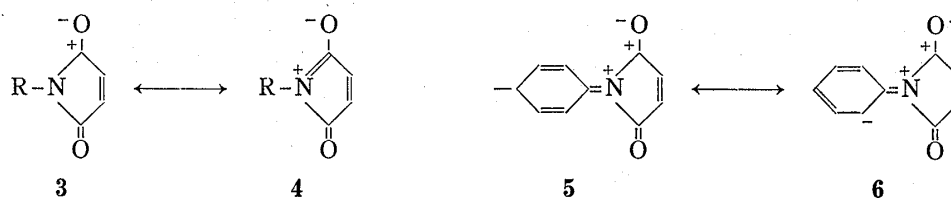


Chart 2

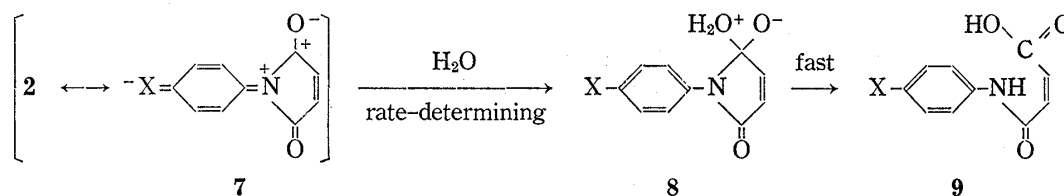


Chart 3

Although the half-life time of BIPM (**1a**) at pH 7.0 is shorter than that of N-phenylmaleimide, **1a** can well be used as a practical reagent because its reaction rate with a thiols¹⁷⁾ is still faster than that of hydrolysis. In addition, it is fortunate for designing this type of fluorescence thiol reagents that the electron-donating group such as a dimethylamino which

16) a) T. Kitagawa, T. Shimosono, and T. Aikawa, Abstracts of papers, The 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, Apr., 1976, Vol. IV, p. 55; b) T. Kitagawa, Symposium on the Enzyme Immunoassay Method, Kyoto, Nov., 1976.

17) T. Sekine, K. Ando, M. Machida, and Y. Kanaoka, *Anal. Biochem.* **48**, 557 (1972).

serves as an auxochrome with large Stokes shift¹⁸⁾ generally has also the stabilizing effect for the maleimide moiety toward hydrolysis. Thus DACM (1c), which exhibits on reaction with thiols strong fluorescence in a long wavelength region, is relatively stable with a half-life time of 120 min, nearly as long as that of N-phenylmaleimide.

Experimental

Materials—Preparation of N-[*p*-(2-benzimidazolyl)phenyl]maleimide (BIPM),⁵⁾ N-(1-anilinonaphthyl-4)maleimide (ANM),⁶⁾ and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM)⁷⁾ was described in the previous papers. They are also commercially available from Wakō Pure Chemicals, Ltd.

Preparation of *p*-Substituted Phenylmaleamic Acids—To a solution of maleic anhydride in aprotic solvent was added a solution of *p*-substituted aniline under cooling and stirring. *p*-Substituted maleamic acids were used for cyclization without purification.

Preparation of *p*-Substituted Phenylmaleimides (Table I) General Procedure—A mixture of a maleamic acid (1 mmol), acetic anhydride (3 mmol) and anhydrous sodium acetate (0.3 mmol) is heated on an oil bath at 100° for 5–10 min until a clear solution results. The mps of these maleamic acids and maleimides are listed in Table I.

Methods—Kinetic measurements of the imide hydrolysis were carried out in 1 cm cell using a Hitachi 356 two-wavelength double beam spectrophotometer, equipped with a water-jacketed cell holder connector to a circulating constant temperature bath (Komatsu-yamato Coolnics Thermo-Bath CTE-2). Operational mode of the instrument was set at different wavelengths of λ_1 and λ_2 as shown in Table II, and the reaction course was followed by the dual-wavelength method. Changes in the difference of absorbances at two wavelengths of λ_1 and λ_2 vs time were automatically measured and time constants (k_{obs}) were obtained from the slope of the conventional plots of $\log(C_0 - C_t)$ vs time (C_0 =initial concentration and C_t =concentration at time t). A stock solution (0.1–0.2 ml) of *p*-substituted phenylmaleimide (5 mM in monoglyme) was made to 10 ml with various buffer solutions which were warmed prior to each kinetic run. The values of k_{obs} were obtained as the mean from several experiments. The stock solutions of N-phenylmaleimides, BIPM and ANM were concentrations of 10, 0.5 and 5 mM, while that of BIPM contains 7% of dioxane. A mixture was vigorously shaken in a glass-stoppered volumetric flask for several seconds, transferring into 1 cm quartz cell and immediately measured. The half-life times were calculated from $t_{1/2} = 0.693/k$ (Table III). For DACM the parameters were described in the previous paper.^{1a)} The following buffers were used; 0.1 M KH_2PO_4 – Na_2HPO_4 for pH 7–8 and 0.1 M sodium borate– KH_2PO_4 for pH 9.0. The stock solutions of the *p*-substituted phenylmaleimides were stable in a cold room (at 4°) for several months.

Acknowledgement This work was supported in part by grants from the Ministry of Education, Science and Culture.