Synthesis of N-(9-Acridinyl)maleimide, a Fluorometrical Reagent for Thiol Compounds

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Received October 21, 1977

The authors have reported¹⁾ a new maleimide type fluorescent thiol reagent, N-(9-acridinyl)-maleimide (NAM). In this paper the syntheses of NAM and its coupling products with thiol compounds are presented. NAM was synthesized from 9-aminoacridine and maleic anhydride through dehydratic cyclization in polyphosphoric acid. NAM showed no substantial fluorescence but its coupling products with thiol compounds exhibited strong blue fluorescence. Application of NAM for the fluorometrical analysis of cysteine and glutathione are suggested.

Since Friedman (1952)²⁾ developed N-ethylmaleimide which reacts rapidly and specifically with thiol groups under a mild condition, several derivatives have been synthesized for colorimetric³⁾ and fluorometric⁴⁾ purposes and also for the modification of protein.⁵⁾ N-(9-Acridinyl)maleimide,¹⁾ originally synthesized by us, is one of the maleimide type reagents which has faint fluorescence but gives strong blue fluorescence by the reaction with thiol compounds. In the present paper, the syntheses of the reagent and its thiol derivatives are described.

RESULTS AND DISCUSSION

Synthesis of NAM and NAS

NAM (III) was synthesized through the reactions shown in Scheme 1. The scheme followed the general procedure of the synthesis of N-substituted aromatic maleimides. Aminoacridine (I) and maleic anhydride were reacted to produce N-(9-acridinyl)maleamic acid (II), which was transformed to NAM (III) by dehydratic cyclization with polyphosphoric acid.

Condensation reaction of (I) and maleic anhydride proceeded quantitatively. How-

ever, the yield of the following dehydratic cyclization was only 25.7%. The cyclization with acetic anhydride-sodium acetate was unsuccessful; the yield was only 6%. NAS was synthesized by a similar manner, using succinic anhydride instead of maleic anhydride. The yield was 50.2%.

The spectrometric behavior of NAM and NAS

The IR, PMR, MS and UV spectra of NAM and NAS are shown in Figs. 1~4. Discrimination of the IR spectra of NAM and NAS (Fig. 1) was not clear, although absorptions at 3080, 1715, 1635 (w) and 850 cm^{-1} of NAM seemed to correspond to maleimide ring, and absorptions at 1780, 1705 and 1175 cm^{-1} of NAS seemed to correspond to succinimide ring.⁷⁾ On the other hand, discrimination of the PMR spectra (Fig. 2) was distinct. The spectrum of NAM revealed a singlet of 2 protons at δ 7.28 ppm which was assignable to the double bond protons of maleimide ring. The spectrum of NAS revealed a singlet of 4 protons at δ 3.65 ppm corresponding to the methylene protons of succinimide ring.⁶⁾ The mass spectra (Fig. 3) of NAM and NAS showed large molecular peaks as base peaks. The UV spectra (Fig. 4) of NAM and NAS overlapped. The absorption maximum was 362 nm, and molecular absorption coefficient was 14,000.

Abbreviation: NAM, N-(9-acridinyl)maleimide; NAS, N-(9-acridinyl)succinimide; NAM-Cys, NAM-Et and NAM-GSH, coupling compounds of NAM with cysteine, ethanethiol and glutathione, respectively.

SCHEME 1. Outline of Synthesis of NAM and NAS.

FIG. 1. Infrared Spectra of NAM (a) and NAS (b) (KBr disk).

FIG. 2. PMR Spectrum of NAM.

FIG. 3. Mass Spectra of NAM (a) and NAS (b). Electron-impact Ionization. JEOL JMS 06.

FIG. 4. UV Absorption Spectra of NAM and NAS. Solvent: dioxane, NAM, —; NAS, ----.

FIG. 5. Fluorescence Spectra of a Reaction Mixture of NAM with Cysteine.

Acetone solution of 0.1 mM NAM (2.0 ml) was mixed with HCl-CH₃COONa buffer (0.5 M, pH 3.5) solution of 0.33 mM cysteine (3.0 ml). After 30 min, fluorescence excitation (a) and emission (b) spectra were measured at 426 nm as the monitoring wavelength and at 360 nm as the excitation wavelength. Dotted lines indicate the spectra of the reaction mixture with no cysteine.

The fluorescence behavior of NAM was similar to that of the maleimide derivatives presented by Kanaoka *et al.*⁴⁾ Namely these maleimide derivatives were practically non-fluorescent but the addition products with thiol compounds had strong fluorescence. The fluorescence spectra of the reaction mixture of NAM with cysteine is shown in Fig. 5. The excitation maximum was 360 nm and the emission maximum was 426 nm.

Coupling compounds of NAM with thiols

Coupling compounds of NAM with ethanethiol, cysteine and glutathione were synthesized as described in EXPERIMENTAL. By coupling with ethanethiol or glutathione only one compound was obtained, respectively. But by coupling with cysteine, two compounds, NAM-Cys I and II, were obtained.

The IR spectrum of NAM-Et resembled that of NAS, except a slight short wave shift (10~15 cm⁻¹) of CO peak and the appearance of CH stretching at 2940 and 2980 cm⁻¹. The IR spectrum of NAM-Acetylcys (the coupling product of NAM and N-acetyl cysteine) is illustrated in Fig. 6. The spectrum was almost the same as that of NAS, except the appearance of a medium peak at 1655 cm⁻¹ derived from amino acid moiety. The IR spectra of NAM-Cys I and NAM-GSH resembled that of NAM-Acetylcys. But the IR spectrum of NAM-Cys II was different. A strong peak at 1660 cm⁻¹ appeared and the peak at 1780 cm⁻¹ disap-

FIG. 6. IR Spectrum of NAM-Acetylcys.

FIG. 7. Carbonyl Region of Infrared Spectra of NAM Derivatives.

A, NAM-Cys-I; B, NAM-Cys-II; C, NAM-Et; D, NAM-GSH.

FIG. 8. Mass Spectrum of NAM-Cys-I.

peared. The strong peak at 1715 cm^{-1} and the medium peak at 1630 cm^{-1} seemed to disappear or to diminish by being hidden by the strong 1660 peak. A part of these spectra are shown in Fig. 7. The ninhydrin test was positive with NAM-Cys I and negative with NAM-Cys II, indicating the disappearance of the free amino group in the latter. The mass spectrum of NAM-Cys I (Fig. 8) showed peaks at m/e 308 ($307 + \text{H}$), 276 ($275 + \text{H}$) and 274 ($308 - \text{H}_2\text{S}$) indicating the presence of succinimide ring. From these facts the reaction of

SCHEME 2. Reaction of NAM with Cysteine.

NAM with cysteine might probably be illustrated as Scheme 2.

Stability of NAM-thiol coupling compounds

The fluorescence intensity of NAM-Et in a buffer solution was stable more than 2 hr when pH was 3.5~7.0 and then gradually decreased. At pH more than 8.0 the decrease took place more rapidly by increasing alkalinity.

The fluorescence intensity of NAM-Cys-I was stable at pH 2~4 and 9~10, although the intensity was stronger under the latter condition. In the solution of pH 5~8, the intensity gradually increased within 1~2 hr from the acid level to the intensity level of the alkaline condition. The UV-absorption and fluorescence spectra of the solution of NAM-Cys-I and II were different from each other in acid and neutral solutions. But in an alkaline

FIG. 9. UV Absorption Spectra of NAM-Cys.

—, NAM-Cys-I in pH 3.5, 0.5 M HCl-AcONa buffer; ----, NAM-Cys-II in pH 3.5, 0.5 M HCl-AcONa buffer; - · - ·, NAM-Cys-I and II in pH 12.0, 0.25 M phosphate buffer. Concentration, $2.4 \times 10^{-7}\text{ M}$.

FIG. 10. Fluorescence Spectra of NAM-Cys.

NAM-Cys-I: pH 12, ---; pH 3.5, - · - · -. NAM-Cys-II: pH 12, —; pH 3.5, · · · · ·. Concentration: 4.4×10^{-6} M.

solution (pH 12) both compounds showed identical absorption and fluorescence spectra. These differed from the spectra in the acid and neutral solutions (Figs. 9, 10). In the alkaline solution, the ninhydrin test was negative. The structural change of the product with cysteine has been reported in the case of other maleimide fluorescent derivatives by Kanaoka *et al.*⁴⁾ The fluorescence spectrum of the reaction mixture presented in Fig. 5 was identical with that of NAM-Cys-I. Care must be taken for pH, when NAM is applied to the analysis of cysteine.

EXPERIMENTAL

Synthesis of NAM

9-Aminoacridine (I) was prepared from N-phenylanthranilic acid by the method of Albert *et al.*¹⁰⁾ (Scheme 1), and was recrystallized from acetone. Compound I (5.5 g) and maleic anhydride (20 g) were dissolved each into absolute acetone (420 ml and 80 ml respectively) and mixed. The mixture was stirred at room temperature for 4 hr. The resulting yellow precipitate of N-(9-acridinyl)maleamic acid (II) was collected and dried over P_2O_5 . Yield: 8 g, 96% from (I), mp 152°C.

Compound II (4 g) was mixed with 40 g of polyphosphoric acid. The viscous mixture was heated at 135°C for 1 hr. After cooling to room temperature the reaction mixture was poured on ice. After the precipitate was removed by filtration, the filtrate was neutralized with sodium hydrogen carbonate powder. The resulting precipitate was collected, washed with

water and dried. By recrystallization from acetone, TLC-pure NAM was obtained. Yield: 1 g (25.7% from II), decomp 248°C, *Anal.* found: C, 74.29; H, 3.57; N, 10.08%, calcd. for $C_{17}H_{10}N_2O_2$: C, 74.44; H, 3.68; N, 10.21%. UV (Fig. 4): ϵ_{362} 14,000. IR (Fig. 1): 3450 cm^{-1} (broad), 3050 (m), 1715 (s), 1635 (m), 1560 (w), 1520 (w), 1490 (w), 1430 (s), 1410 (m), 1390 (m), 1320 (m), 1150 (m), 1130 (m), 1050 (w), 1020 (w), 850 (m), 770 (s), 710 (m). PMR (Fig. 2): δ 7.28 ppm (H-3',4'); 8.0 (H-2,3,6,7); 8.35 (H-1,4,5,8). MS (Fig. 3): *m/e* 274 (100%), 192 (41), 218 (36), 220 (32), 164 (22), 204 (20).

Synthesis of NAS

NAS (V) was synthesized by a manner similar to that for NAM, except succinic anhydride was used in place of maleic anhydride. Yield: 1 g (50.2% from IV), decomp. 270°C. UV: ϵ_{362} 14,000. IR: 3450 cm^{-1} (broad), 3050?, 2950?, 1780 (w), 1715 (s), 1560 (w), 1520 (w), 1490 (w), 1430 (m), 1400 (m), 1330 (m), 1300 (m), 1260 (w), 1180 (s), 1020 (w), 960 (w), 770 (s). PMR: δ 3.65 ppm (4 protons, H-3',4'); 8.28 (H-2,3,6,7); 8.53 (H-1,4,5,8). MS: *m/e* 276 (100%), 194 (87), 220 (20).

Synthesis of S-[N-(9-acridinyl)succinimide]ethanthiol (NAM-Et)

To a solution of NAM (140 mg, 0.51 mmol) in 60 ml of acetone, 0.4 ml (0.54 mmol) of ethanthiol and 10 ml of water were added. The mixture was allowed to react for 30 min at room temperature, and then acetone was evaporated. The precipitate was collected by filtration and washed with water. Yield: 170 mg, 99.1%. The crude prepate was purified by silica gel column chromatography (eluent; benzene: ethyl acetate, 3:1). Recrystallization was made from ether-petroleum ether. This preparation was TLC-pure. Yield: 155 mg, mp 106~107°C. *Anal.* found: C, 68.08; H, 4.88; N, 8.18%, calcd. for $C_{19}H_{16}N_2O_2S$: C, 67.84; H, 4.79; N, 8.33%. UV: ϵ_{364} 1.6×10^4 (pH 3.5), ϵ_{360} 1.2×10^4 (pH 10.0). Fluor.: E_{max} 437 nm (pH 8.0). IR: 3420 (broad), 3050 (w), 2980 (w), 2940 (w), 1780 (w), 1720 (s), 1630 (w), 1560 (w), 1520 (w), 1490 (w), 1425 (m), 1395 (m), 1325 (m), 1175 (m), 770 (m).

Synthesis of S-[N-(9-acridinyl)succinimide]cysteine (NAM-Cys)

To a solution of NAM (137 mg, 0.5 mmol) in 50 ml of acetone, cysteine in 2 ml of water (cysteine hydrochloride 95 mg, 0.6 mmol) was added. After 10 min of reaction, most of acetone was removed by evaporation *in vacuo* below 30°C. The residual aqueous solution was allowed to stand in a refrigerator for 3 hr. The precipitated crystal was collected by filtration, washed with water and dried over P_2O_5 . This S-[N-(9-acridinyl)succinimide]cysteine-I was conveniently abbreviated as NAM-Cys-I. Yield: 40 mg (20.2% from

III), mp 159~161°C. Elementary analysis did not agree well with calculation. But the IR spectrum agreed well with that of NAM-Acetyllys (Fig. 6). UV: ϵ_{358} 2.0×10^4 (pH 12.0). IR: 3400~3000 (broad), 1780 (w), 1710 (s), 1630 (m), 1490 (w), 1425 (m), 1390 (m), 1325 (w), 1170 (s), 770 (s) cm^{-1} . MS: no M^+ peak, m/e 179 (100), 194 (84), 211 (82), 220 (41), 166 (41), 168 (41), 276 (35), 308 (32), 204 (29), 192 (29), 247 (27).

The filtrate and washings were combined and allowed to stand in a refrigerator overnight. Another crystals were obtained (55 mg, 26.6% from III) which were recrystallized from diluted HCl. This compound was abbreviated as NAM-Cys-II. Decomp. 203~204°C. Elementary analysis did not agree well with calcd. UV: ϵ_{358} 2.0×10^4 (pH 12.0). IR: 3380, 3240, 1660 (s), 1570 (m), 1510 (m), 1425 (w), 1380 (m), 1320 (m), 1260 (w), 1230 (w), 1190 (w), 1160 (w), 780 (m).

Synthesis of S-[N-(9-acridinyl)succinimide]-N-acetyl-cysteine (NAM-Acetyllys)

To 100 ml of an acetone solution of NAM (164 mg, 0.6 mmol), 8 ml of an aqueous solution of N-acetyl-cysteine (163 mg, 1.0 mmol) was added. After the reaction of 20 min, acetone was evaporated *in vacuo*. The resulting precipitate was collected and was recrystallized from hot 95% ethanol. Yield: 210 mg, 90%. mp 150~152°C. Anal. found: C, 58.44; H, 4.11; N, 9.41%. Calcd. for $\text{C}_{22}\text{H}_{19}\text{N}_3\text{O}_5\text{S} \cdot \text{H}_2\text{O}$: C, 58.01; H, 4.20; N, 9.23%. UV: ϵ_{352} 1.4×10^4 (90% EtOH). IR: 3400 (broad), 3050 (w), 2980 (w), 2940 (w), 1780 (w), 1720 (s), 1650 (m), 1525 (m), 1430 (m), 1400 (m), 1325 (m), 1180 (m), 770 (m).

Synthesis of S-[N-(9-acridinyl)succinimide]-glutathione (NAM-GSH)

To 50 ml of an acetone solution of NAM (137 mg, 0.5 mmol), a solution of glutathione (160 mg, 0.52 mmol, in 0.2 M pH 3.5 $\text{HCl}-\text{CH}_3\text{COONa}$ buffer) was added. After 10 min of reaction, acetone was evaporated. The residual solution was purified by silica gel column chromatography (eluent; water). The fluorescent fraction was collected and was lyophilized. Yield: 110 mg, 80.3%, mp 166.5~169.5°C.

Acknowledgement. We indebted to Mr. Hiroaki Takahashi for the preparation of this manuscript.

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