[Chem. Pharm. Bull. 15(11)1738~1743(1967)]

UDC 612.398.1 : 547.96.02.03

# 222. Yuichi Kanaoka,*1 Minoru Machida,*1 Yoshio Ban,*1 and Takamitsu Sekine*2 : Fluorescence and Structure of Proteins as measured by Incorporation of Fluorophore. II.*3 Synthesis of Maleimide Derivatives as Fluorescence-Labeled Protein-Sulfhydryl Reagents.*4

(*Faculty of Pharmaceutical Sciences, Hokkaido University*1 *and Medical School, Juntendo University*2)

A novel approach to studies of structure and function of proteins utilizing fluorescence-labeled specific reagent is proposed. N-[*p*-(2-Benzoxazolyl)phenyl]maleimide (IIIa) and N-[*p*-(2-benzthiazolyl)phenyl]maleimide (IIIb) were synthesized as sulfhydryl reagents of this type and the addition reaction with cysteine ester was investigated. Cyclization of maleamic acids with PPA is demonstrated to be a good method for synthesis of N-substituted maleimides.

(Received February 20, 1967)

The recent studies of emission spectra of proteins have accumulated increasing information about structural aspects of many proteins.[1] Chemical modification method, on the other hand, has become one of the most powerful means for chemical study of structure and function of proteins. Thus, a number of reagents have been found to react specifically with certain functional groups or amino acid residues of peptide backbones, so that advanced elucidation of protein structures may be made.[2] For example, N-ethylmaleimide (NEM) and its derivatives are typical specific reagents for sulfhydryl groups in protein, and have been employed with various enzymes and proteins for labeling and modification purposes.[3,4]

In this series of papers we will present the work in which attempts are made to combine the above two methods by designing certain maleimide derivatives with fluorophore and applying them to proteins with sulfhydryl groups in the hope that this would develop a novel general approach to structural studies of protein. When a fluorescent reagent of this type is reacted specifically with a sulfhydryl group of an enzyme or protein forming a new covalent bond, the reagent is consequently introduced as the fluorophore into the protein molecule at the desired specific site; *i.e.*, the sulfur atom of the reactive sulfhydryl group in this case. Since this incorporated fluorophore may interact with neighboring groups or amino acid residues and with the local environment within or on the surface of the protein molecule, such a system would be expected to provide some information about three-dimensional structures around "the site" by analysis of the spectroscopic behavior, and would play a significant role as a kind of "reporter"[5] in the field of protein research.

---

*1 Kita-15, Nishi-7, Sapporo (金岡祐一, 町田 実, 伴 義雄).
*2 Bunkyo-ku, Tokyo (関根隆光).

*3 The paper entitled "Protein-Sulfhydryl Reagents. I", This Bulletin, **12**, 127 (1964), by Y. Kanaoka, T. Sekine, M. Machida, Y. Soma, K. Tanizawa, Y. Ban, is considered to be the Part I of this series.

*4 Presented in part at the Annual Meeting of the Pharmaceutical Society of Japan, Tokushima, October, 1965.

1) See for example, G. Weber, F. W. J. Teale : "The Proteins (Ed., H. Neurath)." Vol. 3, p. 466 (1965). Academic Press, N. Y.
2) See for example, R. E. Whitfield, W. L. Wasley : "Chemical Reactions of Polymers (Ed., E. M. Fettes)." p. 367 (1964), J. Wiley, N. Y.
3) R. Cecil : "The Proteins (Ed., H. Neurath)" Vol. 1, p. 402 (1963). Academic Press, N. Y.
4) T. Sekine, L. M. Barnett, W. W. Kilelley : J. Biol. Chem., **237**, 2769 (1962).
5) M. Burr, D. E. Koshland : Proc. Nat. Acad. Sci., **52**, 1017 (1964).

Although there have been many reports on emission spectroscopy of protein,[1] they are invariably concerned either with intrinsic fluorescence owing to aromatic amino acid residues or with fluorescence of proteins carrying artificial conjugates or adsorbates. The process proposed here may be in clear distinction from those in that the fluorescence arises from the *specifically introduced fluorophore.*

The present paper describes the synthesis of N-[*p*-(2-benzoxazolyl)phenyl]-maleimide (IIIa) and N-[*p*-(2-benzthiazolyl)phenyl]maleimide (IIIb) as the fluorescent reagents along this line and their reaction with cysteine derivatives, a simple model sulfhydryl system. In a previous work,*[3] 4-methoxybenzimidazole was tested as a fluorophore with preliminary success, but some stronger intensity in fluorescence was still desired for practical purpose. On account of background fluorescence of protein, a reagent of this type is in general required to carry a fluorophore with strong fluorescence having emission maximum at longer than about 360 mμ. Therefore, in the present design, a benzene group is inserted between benzazole ring and maleimide moiety in order to improve the above initial example both in bathochromic and hyperchromic effect by extending the conjugated system of chromophore.

Major synthetic route to N-substituted maleimide involves first, preparation of appropriate primary amine having a fluorophore (I); second, addition of I with maleic anhydride to form maleamic acid (II); third, cyclization of II to form maleimide derivative (III) as outlined in the Chart 1. Step A, addition reaction to give II, normally proceeds in inert solvent smoothly. On the other hand, step B, cyclization to give III, often leads to rather poor result owing to side reaction caused probably by high reactivity of the product (III).[6] Therefore, it was necessary to establish a general synthetic procedure of the cyclization (B). The treatment of maleamic acid (II) with polyphosphoric acid (PPA), proposed previously,*[3] is found to be a satisfactory method for this reaction and enabled us to synthesize a variety of maleimide derivatives in this series of projects.

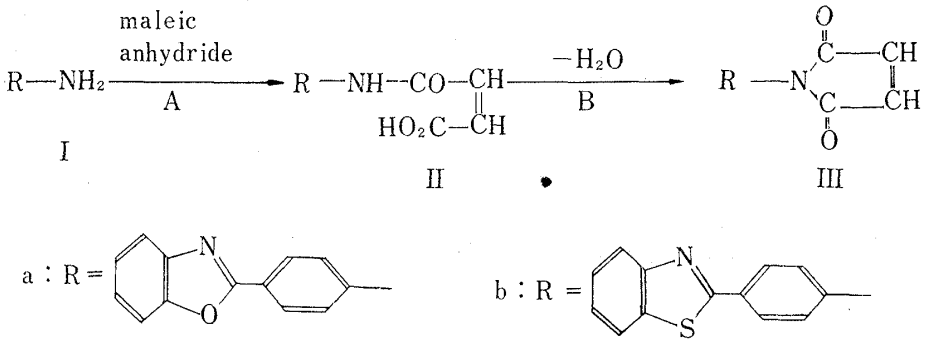



Chart 1.

2-(*p*-Aminophenyl)benzoxazole (Ia), prepared by hydrolysis of N-acetyl compound, was reacted with maleic anhydride in tetrahydrofuran to form N-[*p*-(2-benzoxazolyl) phenyl]maleamic acid (IIa), which was without purification cyclized in PPA at 135~140° to give N-[*p*-(benzoxazolyl)phenyl]maleimide (IIIa) in 74% yield. N-[*p*-(Benzthiazolyl) phenyl]maleimide (IIIb) was also prepared similarly from corresponding amine (Ib) by way of maleamic acid (IIb) in a good yield.

Matsuo studied infrared and ultraviolet spectra of N-substituted maleimides as well as those of other cyclic imide derivatives.[7,8] In confirmation of the structures of IIIa and IIIb, their spectra were now compared with those of the related compounds.

6) cf. papers cited in *[3].
7) T. Matsuo : Bull. Chem. Soc. Japan, **37**, 1844 (1964); *a*) cf. papers cited in ref. 7).
8) *Idem* : *Ibid.*, **38**, 557 (1965).

Cyclic imides generally have two characteristic bands in infrared absorption due to a coupling of the in-phase and out-of-phase vibrations of the two carbonyl groups connected by a nitrogen atom.[7,7a] Comparison shows that the higher-frequency carbonyl bands of maleimides appear normally as a shoulder of low intensity.[7] In going from IIa to IIIa, the carbonyl bands of IIa assigned to carboxylic acid (1710 $cm^{-1}$) and amide (1635 $cm^{-1}$) respectively, changed to the above-mentioned pattern of maleimide having a broad shoulder at higher frequency (1785 $cm^{-1}$) and a strong band at lower frequency (1715 $cm^{-1}$) in accord with imide cyclization. A similar spectroscopic behavior was observed in the case of IIIb. The longest wave-length bands of ultraviolet absorption of N-substituted maleimides were found to be essentially associated with the $n \rightarrow \pi^*$ transition of the carbonyl groups.[8] IIIa and IIIb showed the corresponding band at 308 mμ. These spectral data and the correlation of a series of compounds are summarized in Table I.

Table I. Spectral Data and Correlation of Cyclic Imides and Related Compounds

| Assignment / Compound | IR[a] | | | UV[b] | | |
|---|---|---|---|---|---|---|
| | Amide | Maleimide | Succinimide | Amide | Maleimide | Succinimide |
| IIa | 1635(s) | | | 332[c] | | |
| IIIa | | 1785(vw; broad)<br>1715(s) | | | 308<br>(4.52) | |
| IVa | | | 1780(w)<br>1703(s) | | | 308<br>(4.51) |
| Va | 1655(s) | | | 318<br>(4.63) | | |
| VIIIa | | | 1780(w)<br>1708(s) | | | 306.5<br>(4.47) |
| IIb | 1635(s) | | | 336[c] | | |
| IIIb | | 1770(vw)<br>1715(s) | | | 308[d]<br>(4.43) | |
| IVb | | | 1780(w)<br>1708(s) | | | 307[c] |
| Vb | 1655(s) | | | 328<br>(4.56) | | |
| VIIIb | | | 1778(w)<br>1708(s) | | | 307<br>(4.40) |

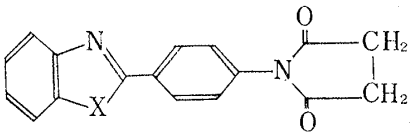
*a*) $\nu_{max}^{Nujol}$ $cm^{-1}$ (carbonyl); s, strong; w, weak; v, very.
*b*) $\lambda_{max}^{EtOH}$ mμ; log ε given in parenthesis. For some detail see Experimental.
*c*) measured qualitatively owing to poor solubility.
*d*) in tetrahydrofuran.

Based on the carbonyl bands of the products such a common side reaction as isoimide formation, for example, is obviously ruled out in this PPA cyclization. Isoimides, which are readily formed from cyclization of maleamic acids with dicyclohexylcarbodiimide or trifluoroacetic anhydride as a reagent, are known to exhibit strong infrared absorption at around 1800 $cm^{-1}$ due to the partial lactone structure.[9a~c]

The reaction of NEM, the parent system of the reagents of this series, with sulfhydryl compounds, have been investigated by several groups,[3] and the kinetics was studied at various pH values.[10~12] In advance of application to protein, however, it seem-

9) *a*) K.-C. Tsou, R. J. Barrnett, A. M. Seligman: J. Am. Chem. Soc., **77**, 4613 (1955); *b*) R. J. Cotter, C. K. Sarers, J. M. Whelan: J. Org. Chem., **26**, 10 (1961); *c*) W. R. Roderick, P. L. Bhatia: *Ibid.*, **28**, 2018 (1963).
10) J. D. Gregory: J. Am. Chem. Soc., **77**, 3922 (1955).
11) C. C. Lee, E. R. Samuels: Can. J. Chem., **42**, 167 (1964).
12) G. Gorin, P. A. Martic, G. Doughty: Arch. Biochem. Biophy., **115**, 593 (1966).

ed advisable to examine the reactivity of Ⅲa and Ⅲb using simple sulfhydryl derivatives of amino acid level as model substrates. Such a model product was needed also with a view to providing useful reference compound for application on a protein level. Thus, Ⅲa or Ⅲb was allowed to react with L-cysteine ethyl ester hydrochloride (Ⅵ) at room temperature to afford S-[N-(*p*-(2-benzoxazolyl)phenyl)succinimido]cysteine ethyl ester hydrochloride (Ⅳa) or S-[N-(*p*-(2-benzthiazolyl)phenyl)succinimido]cysteine ethyl ester hydrochloride (Ⅳb), respectively. In going from Ⅲ to Ⅳ, the carbonyl bands of infrared absorption changed from the characteristic pattern of maleimide to that of succinimide[7]



a : X=O
b : X=S

Ⅷ

having a weak but well-defined band at higher frequency (1780 $cm^{-1}$) and a stronger band at lower frequency (1703~1708 $cm^{-1}$), as assigned in Table I.

For further confirmation, model compounds carrying succinimide moiety were prepared. Thus, N-[*p*-(2-benzoxazolyl)phenyl]succinimide (Ⅷa) and N-[*p*-(2-benzthiazolyl)phenyl]succinimide (Ⅷb) were synthesized by cyclization of corresponding succinamic acids (Ⅶa and Ⅶb) by means of PPA. As listed in Table I, infrared spectra of both Ⅷa and Ⅷb exhibit the carbonyl bands similar to that of Ⅳa and Ⅳb, and their ultraviolet spectra of longer wave region are almost superimposable with those of Ⅳa and Ⅳb. This comparison gives strong evidence in support of the structures of Ⅳa and Ⅳb, which resulted from the expected addition reaction of cysteine sulfhydryl group to the olefinic double bond of maleimide moiety of Ⅲa and Ⅲb. Fig. 1 shows the carbonyl region of infrared spectra of Ⅲb, Ⅳb, and the reference compound (Ⅷb) as a typical example.

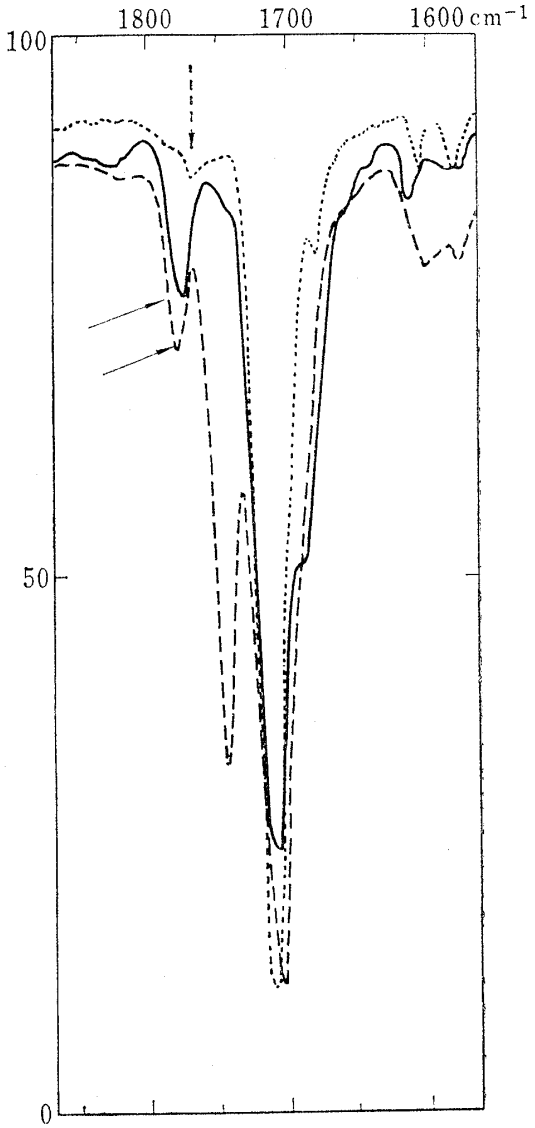



Fig. 1. Carbonyl Region of Infrared Spectra of Some Cyclic Imides (Nujol)
- - - - - - Ⅲb; — — — Ⅳb; ——— Ⅷb.
·····→ maleimide; ——→ succinimide

When Ⅲa was reacted with Ⅵ in the presence of triethylamine, a crystalline compound of m.p. 192~193° (Va) was obtained in place of Ⅳa. Apparently the initially formed adduct (Ⅳa) underwent subsequent opening of the imide ring as the result of intramolecular nucleophilic attack by the neighboring amino group to form a thiazane ring as shown in the Chart 2. Fruton, *et al.* observed similar rearrangement with the cysteine adduct of NEM,[13] while Witter and Tuppy reported also analogous conversion with the adduct of N-(4-dimethylamino-3,5-dinitrophenyl)

13) G.D. Smyth, A. Nagamatsu, J.S. Fruton : J. Am. Chem. Soc., **82**, 4600 (1960).

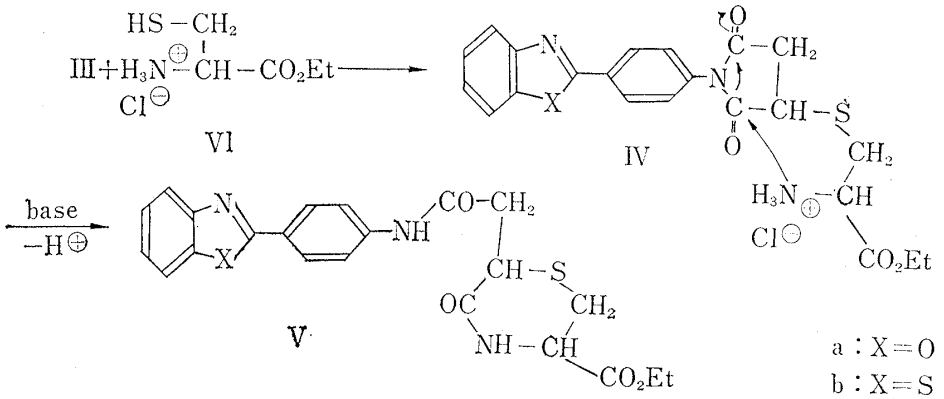


Chart 2.

maleimide (DDPM).[14] Va showed negative ninhydrin test to indicate some participation of amino group of cysteine moiety. In the infrared spectrum of Va, the characteristic imino band (1780 and 1703 $cm^{-1}$) of IVa disappeared and a new amide band (1655 $cm^{-1}$) appeared instead as given in Table I. These data are in good agreement with the thiazane structure assigned to Va.

Likewise, the similar intramolecular transamidation took place in the case of IIIb to give Vb. Upon exposure to base, IVb was transformed into Vb as expected. These observed data together with those in literatures[13,14] suggest that this thiazane formation by rearrangement is a general behavior of cyclic imide ring with appropriate neighboring amino group. The fact, therefore, should be taken into account when maleimide derivatives are applied to sulfhydryl system carrying free amino group.

The fluorescent characteristics of IIIa, IIIb and their derivatives,[15] and the application of them to some proteins containing sulfhydryl groups[16] will be reported elsewhere.

## Experimental*5

**2-(*p*-Aminophenyl)benzoxazole (Ia)**——It was prepared by hydrolysis of 2-(*p*-acetoaminophenyl)benzoxazole*6,17) with sulfuric acid in aq. EtOH following the lit.[17] Colorless needles of m.p. 172~173°(lit.,[17] m.p. 169~170°) from aq. EtOH.

**2-(*p*-Aminophenyl)benzthiazole (Ib)**——It was prepared by the reduction of 2-(*p*-nitrophenyl)benzthiazole[18] with tin and dil. sulfuric acid.[18] Almost colorless needles of m.p. 153~155°(lit.,[18] m.p. 155°) from aq. EtOH.

**N-[*p*-(2-Benzoxazolyl)phenyl]maleamic Acid (IIa)**——To a solution of Ia (1.890 g.; 9 mmoles) in tetrahydrofuran (24 ml.) was added a solution of maleic anhydride (883 mg.; 9 mmoles) in tetrahydrofuran (12 ml.) under stirring and ice-cooling. Yellow powder began to deposit in an hour. After standing overnight the precipitate was collected, washed with tetrahydrofuran and dried to give crude IIa as yellow crystalline powders, m.p. 227~229° (decomp.); 2.58 g. or 93%. UV $\lambda_{max}^{EtOH}$ mμ : 332. IR $\nu_{max}^{Nujol}$ $cm^{-1}$ : 1710 (carboxylic acid), 1635 (amide).

**N-[*p*-(2-Benzthiazolyl)phenyl]maleamic Acid (IIb)**——Ib (4.52 g.; 0.02 mole) in tetrahydrofuran (25 ml.) was reacted with maleic anhydride (1.96 g.; 0.02 mole) in tetrahydrofuran (25 ml.) as above. Precipitation of the product began in few mins. Crude IIb was obtained as yellow crystalline powders of m.p. 239~242° (decomp.); 6.15 g. or 95%. UV $\lambda_{max}^{EtOH}$ mμ : 336. IR $\nu_{max}^{Nujol}$ $cm^{-1}$ : 1710 (carboxylic acid), 1635 (amide).

**N-[*p*-(2-Benzoxazolyl)phenyl]succinamic Acid (VIIa)**——To a solution of succinic anhydride (100 mg.) in tetrahydrofuran (8 ml.) was added a solution of Ia (210 mg.) in tetrahydrofuran (14 ml.) under ice-cooling. After standing overnight the mixture was concentrated *in vacuo* to about 5 ml. and $CHCl_3$ was added to

*5 Melting points are uncorrected.
*6 We are indebted to Mr. T. Hamada for the preparation of this compound.

14) A. Witter, H. Tuppy : Biochem. Biophys. Acta, **45**, 429 (1960).
15) Y. Kanaoka, M. Machida, H. Kokubun, T. Sekine : in preparation.
16) T. Sekine, K. Kuroda, M. Machida, Y. Kanaoka : in preparation.
17) F.F. Stephenes, J.D. Bower : J. Chem. Soc., **1949**, 2971.
18) M.T. Bogert, F.D. Snell : J. Am. Chem. Soc., **46**, 1308 (1924).

deposit colorless fine needles of m.p. 244~245°(decomp.), 307 mg. or 98%. IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1710 (carboxylic acid), 1670 (amide).

**N-[*p*-(2-Benzthiazolyl)phenyl]succinamic Acid (VIIb)**——Ib (226 mg.) in $CHCl_3$ (6 ml.) was reacted with succinic anhydride (100 mg.) in $CHCl_3$ (6 ml.) as above. The precipitated crude VIIb was collected and washed with $CHCl_3$ to give colorless fine needles of m.p. 236~238°(decomp.); 298 mg. or 91%. IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1695 (carboxylic acid), 1655 (amide).

**N-[*p*-(2-Benzoxazolyl)phenyl]maleimide (IIIa)**——Crude IIa (1.23 g.) was mixed with PPA (6.2 g.; 5 parts of IIa in wt.) and the viscous mixture was heated at 135~140° (oil-bath temp.) for 30 min. After cooling, ice-water and then powdered $NaHCO_3$ was added to decompose excess of PPA and the mixture was extracted with AcOEt. The extract was washed with satd. aq. $NaHCO_3$, water, treated with charcoal followed by filtration, and dried ($Na_2SO_4$). Removal of the solvent *in vacuo* left yellow solid (964 mg. or 83%), which was recrystallized from acetone to give IIIa forming yellow feathers of m.p. 208~210°; 914 mg. or 74%. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 308 (4.52), 322 (sh) (4.45). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1785 (vw, broad), 1715 (s) (imide). *Anal.* Calcd. for $C_{17}H_{10}O_3N_2$ (IIIa): C, 70.34; H, 3.47; N, 9.65. Found: C, 70.55; H, 3.66; N, 9.66.

**N-[*p*-(2-Benzthiazolyl)phenyl]maleimide (IIIb)**——Crude IIb (648 mg.) was cyclized at 120~130° for 30 min. with PPA (5 parts) and worked up as above, using $CHCl_3$ as a solvent. IIIb was obtained forming yellow fine needles of m.p. 231~233.5° from acetone in 79% yield. UV $\lambda_{max}^{THF}$ mμ (log ε): 308 (4.43), 320 (sh) (4.40). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1770 (vw), 1715 (s) (imide). *Anal.* Calcd. for $C_{17}H_{10}O_2N_2S$ (IIIb): C, 66.65; H, 3.29; N, 9.16. Found: C, 66.91; H, 3.43; N, 9.26.

**N-[*p*-(2-Bezoxazolyl)phenyl]succinimide (VIIIa)**——Crude VIIa (155 mg.) was heated with PPA (800 mg.) at 135~140° for *ca* 20 min. and worked up as in the case of IIIa to give VIIIa forming colorless fine needles of m.p. 239~240° from aq. acetone; 117 mg. or 80%. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 296 (sh) (4.45), 306.5 (4.48), 322 (sh) (4.18). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1780 (w), 1708 (s) (imide). *Anal.* Calcd. for $C_{17}H_{12}O_3N_2$ (VIIIa): C, 69.85; H, 4.14; N, 9.59. Found: C, 69.80; H, 4.09; N, 9.72.

**N-[*p*-(2-Benzthiazolyl)phenyl]succinimide (VIIIb)**——Crude VIIb (228 mg.) was cyclized as in the case of VIIIa to give VIIIb forming colorless fine needles of m.p. 259~260°(decomp.) from acetone; 172 mg. or 80%. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 307 (4.40), 331 (sh) (4.15). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1778 (w), 1708 (s) (imide). *Anal.* Calcd. for $C_{17}H_{12}O_2N_2S$ (VIIIb): C, 66.21; H, 3.92; N, 9.09. Found: C, 66.46; H, 4.18; N, 8.94.

**Reaction of IIIa with L-Cysteine Ethyl Ester Hydrochloride (VI); S-[N-(*p*-(2-Benzoxazolyl)phenyl)-succinimido]cysteine Ethyl Ester Hydrochloride (IVa)**——To a solution of IIIa (145 mg.; 0.5 mmole) in tetrahydrofuran (10 ml.) was added a solution of VI (93 mg.; 0.5 mmole) in EtOH (3 ml.). Precipitate began to separate in an hour. After standing overnight, the solid product was collected, dried and recrystallized forming colorless fine needles of m.p. 210~212°(decomp.) from MeOH-AcOEt; 178 mg. or 75%. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 296 (sh) (4.48), 308 (4.51), 322 (sh) (4.24). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1780 (w), 1703 (s) (imide), 1745 (s) (ester). *Anal.* Calcd. for $C_{22}H_{21}O_5N_3S \cdot HCl$ (IVa): C, 55.52; H, 4.66; N, 8.83. Found: C, 55.83; H, 4.92; N, 8.72.

**Reaction of IIIb with VI; S-[N-(*p*-(2-Benzthiazolyl)phenyl)succinimido]cysteine Ethyl Ester Hydrochloride (IVb)**——IIIb (154 mg.) in tetrahydrofuran (24 ml.) was reacted with VI (93 mg.) in EtOH (4 ml.) as above. IVb was obtained forming colorless fine needles of m.p. 217~219° (decomp.) from MeOH-AcOEt; 191 mg. or 81%. UV $\lambda_{max}^{EtOH}$ mμ: 307. IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1780 (w), 1708 (s) (imide), 1745 (s) (ester). *Anal.* Calcd. for $C_{22}H_{21}O_4N_3S_2 \cdot HCl$ (IVb): C, 53.70; H, 4.51; N, 8.54. Found: C, 53.80; H, 4.49; N, 8.42.

**Rearrangement of IVa; 6-[*p*-(2-Benzoxazolyl)phenylcarbamoylmethyl]-5-oxo-3-thiomorpholinecarboxylic Acid Ethyl Ester (Va)**——A solution of IIIa (145 mg.; 0.5 mmole) in tetrahydrofuran (5 ml.) was added to a solution of VI (93 mg.; 0.5 mmole) in EtOH (3 ml.) followed by addition of triethylamine (102 mg.; 1 mmole) in tetrahydrofuran (2 ml.) at room temp. IIIa disappeared in 2~3 hr. as checked by thin-layer chromatography with sillica gel. After evaporation of solvent *in vacuo*, acetone was added to the residue and triethylamine hydrochloride was removed by filtration. On addition of water to the filtrate, Va was obtained as crystalline precipitate; 204 mg. or 92%. Recrystallization from AcOEt afforded colorless fine needles of m.p. 192~193°; yield, 88%. Ninhydrin test was negative. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 318 (4.63). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1755 (s) (ester); 1655 (s) (amide). *Anal.* Calcd. for $C_{22}H_{21}O_5N_3S$ (Va): C, 60.12; H, 4.82; N, 9.56. Found: C, 60.08; H, 4.65; N, 9.55.

**Rearrangement of IVb; 6-[*p*-(2-Benzthiazolyl)phenylcarbamoylmethyl]-5-oxo-3-thiomorphlinecarboxylic Acid Ethyl Ester (Vb)**——A solution of IIIb (154 mg.; 0.5 mmole) in tetrahydrofuran (10 ml.) was added to a solution of VI (93 mg.) in EtOH (3 ml.) followed by addition of triethylamine (51 mg.; 0.5 mmole). After an hour, color reaction of sulfhydryl group with nitroprusside turned negative. Solvent was evaporated *in vacuo* and the residue was repeatedly recrystallized from aq. acetone until the band at 1715 $cm^{-1}$ (imide) in the IR of the product disappeared. After treatment with charcoal, Vb was obtained as colorless fine needles of m.p. 187~189° from aq. EtOH; 141 mg. or 62%. Ninhydrin test was negative. UV $\lambda_{max}^{EtOH}$ mμ (log ε): 328 (4.56). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 1755 (s) (ester), 1655 (s) (amide). *Anal.* Calcd. for $C_{22}H_{21}O_4N_3S_2$ (Vb): C, 58.00; H, 4.65; N, 9.22. Found: C, 58.12; H, 4.63; N, 9.34.

Treatment of IVb in EtOH solution with one equivalent of triethylamine afforded the same product (Vb).

We are indebted to Mrs. T. Toma and Miss A. Maeda for elemental analyses.