The effect of 2-N-oxide of I in this reaction was affected by the concentration of sulfuric acid and the reaction temperature.

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109. Goro Tsukamoto, Kiyoshi Harada, and Isamu Utsumi : Studies on Thiamine Disulfide. XVI.*1 Geometrical Isomers of Thiaminic Acids.*2

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A number of thiol-type thiamine derivatives are in current clinical use as easily absorbable, long-acting vitamin B_1 preparations. With the development of the massive administration therapy, the curative efficacy has been intensive and the wide indications have been established so far.[1] These thiamine derivatives show the same therapeutic effect despite of their different chemical structure when administered at the large dose which is considerably over the physiological requirement of the vitamin.

In this situation, the therapeutic effect of these derivatives has been considered to be due to their pharmacological one. But no one has yet found the reasonable pharmacological ground for this point. Under the circumstances, the following consideration was made for this problem and led us to carry out the present investigation. These derivatives would be metabolized to a substance which has the pharmacological efficacy, and the metabolite probably would be directly formed from thiamine hydrochloride with difficulty as compared with the formation from the thiol-type thiamine derivatives. Meantime, it is well known that cysteic acid, hypotaurine and taurine appear in the course of metabolism of cysteine and cystine[2~4].

From these points, the authors have supposed that SH group of the various thiamine derivatives would be oxidized to SO_3H to produce 2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-hydroxy-2-pentene-3-sulfonic acid (hereinafter referred to IIb and called thiaminic acid) which had not been reported in any literatures. The oxidation of various thiol-type thiamine derivatives was thus attempted and gave thiaminic acid (IIb) or its O-acyl derivatives such as IIa.[5,6] In this investigation, symmetrical disulfide type thiamine, O-benzoylthiamine disulfide (I), was oxidized to give 2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-sulfonic acid (IIa) (hereinafter referred to O-benzoylthiaminic acid) with the yield of 50~60% under the optimum oxidizing condition by hydrogen peroxide. This low yield implied that there

*1 Part XV. K. Kohno, I. Saito, I. Utsumi : Vitamins, **33**, 340 (1966).
*2 Presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan, Fukuoka, April, 1965.
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1) Japan Vitamin B Research Committee: "Symposium on massive administration of Vitamin B_1 derivatives", Vitamins, **27**, 309 (1963).
2) N. W. Pirie : Biochem. J., **28**, 305 (1934).
3) G. Medes : *Ibid.*, **33**, 1559 (1939); G. Medes, N. Floyd : *Ibid.*, **36**, 259 (1942).
4) J. Awapara : J. Biol. Chem., **203**, 183 (1953); J. Awapara, W. J. Wingo : *Ibid.*, **203**, 189 (1953).
5) I. Utsumi, K. Harada, G. Tsukamoto : J. Vitaminol., **11**, 225 (1965).
6) I. Utsumi, K. Harada, G. Tsukamoto, I. Daira : *Ibid.*, **11**, 234 (1965).

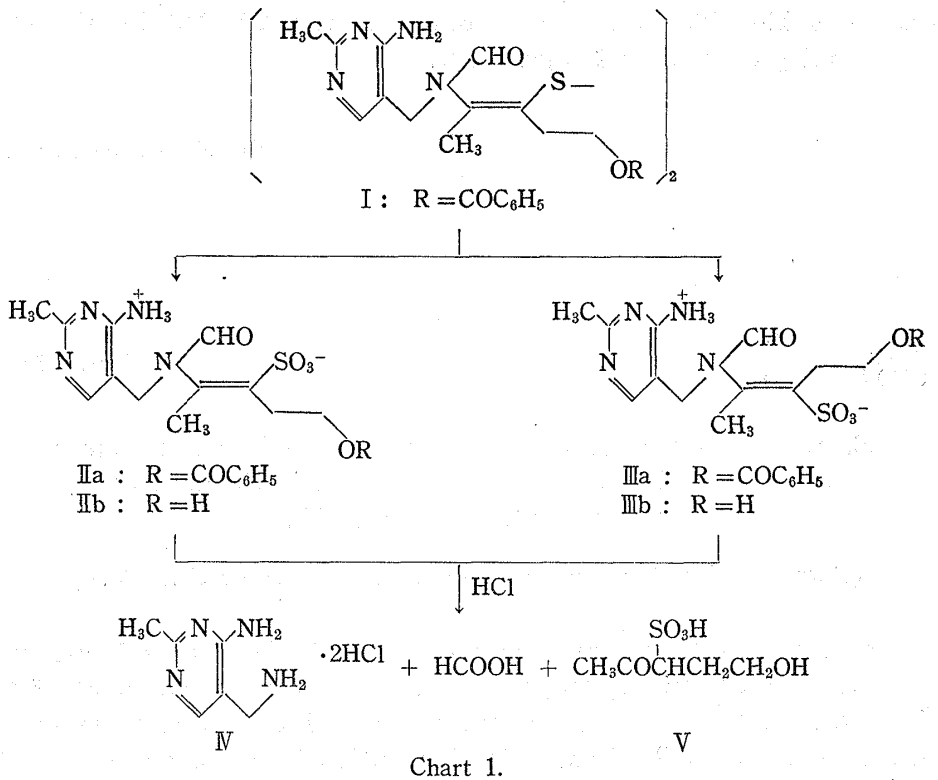


Chart 1.

might be some other oxidized products than O-benzoylthiaminic acid (IIa).

With continuous investigation, a substance (IIIa) which was supposed to be a geometrical isomer of IIa was found to be separated from the reaction mixture. The hydrolysis of IIIa gave IIIb corresponding to a geometrical isomer of IIb. A considerable amount of research has been reported on thiol-type thiamines,[7] but there has been no literature concerning their geometrical isomers. Present paper aims to report the isomers newly found.*4

I was oxidized with amount as much as 6 times molar of hydrogen peroxide in glacial acetic acid at room temperatures until the reaction mixture has shown no response to thiochrome reaction after the reduction by cysteine. The reaction mixture was concentrated to dryness and the residue was separated by the addition of ethanol. The recrystallization from aqueous ethanol afforded colorless prisms, m.p. 234~235° (decomp.), in 50~60% yield which were monohydrate of IIa. This monohydrate losed crystal water by refluxing in absolute ethanol to give colorless needles (IIa), m.p. 237~238° (decomp.).

The mother liquor, from which crude crystals were filtered off, was concentrated to dryness and the residue was treated with water and absolute ethanol to obtain colorless crystals (IIIa), m.p. 233~234° (decomp.). The analytical data of IIIa agreed with the formula of IIa corresponding to $C_{19}H_{22}O_6N_4S$.

The ultraviolet absorption spectra were identical with those of O-benzoylthiaminic acid (IIa) (Fig. 1).

The infrared spectra (Fig. 2) of IIIa and IIa were considerably different in wave shape and number in functional group region, but they were very closely similar to one another in the other region. Especially, noteworthy was that the symmetrical

*4 In a recent communication, M. Murakami, *et al.* have reported on the isomerization of thiol-type thiamine: Yakugaku Zasshi, **85**, 752 (1965).

7) N. Shimazono, E. Katsura: "Review of Japanese Literature on Berberi and Thiamine", 104 (1965). Vitamin B Research Committee of Japan, Kyoto.

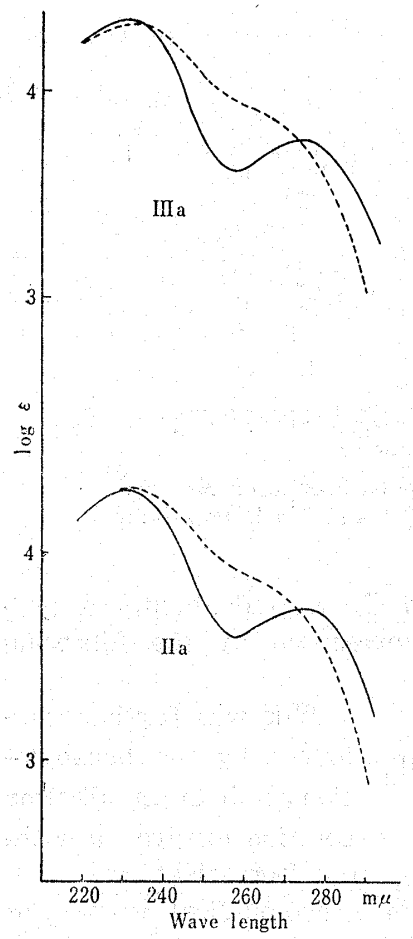


Fig. 1. Ultraviolet Absorption Spectra of *cis*- and *trans*-O-Benzoylthiaminic Acid (IIIa and IIa)
———— pH 7.4
..... pH 3.0

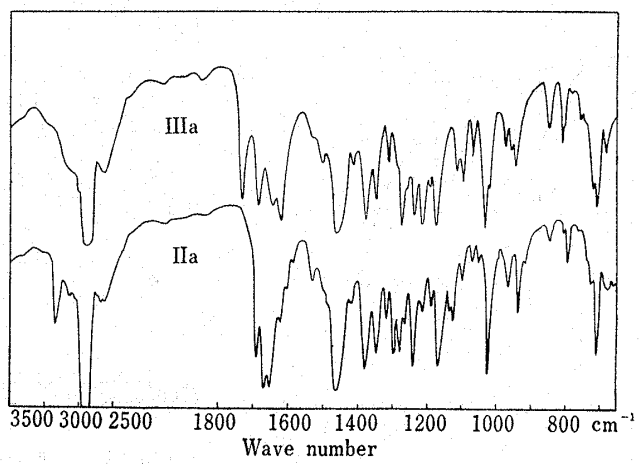


Fig. 2. Infrared Absorption Spectra of *cis*- and *trans*-O-Benzoylthiaminic Acid (IIIa and IIa) in Nujol

stretching vibration due to SO_2 group was observed around the wave number at 1030 cm^{-1} in both IIa and IIIa and the asymmetrical stretching vibration were noted by the existence of some absorptions near 1200 cm^{-1}. From the absorption bands of NH_3^+ around 2650 cm^{-1} and SO_2 described above which frequencies are lower than those of SO_3H group,[8] it may be concluded that IIIa behaves itself like a zwitterion structure as IIa does. Compounds, IIa and IIIa showed the same value by paper partition chromatography and paper electrophoresis and were sparlingly soluble in organic solvent. A little difference between IIa and IIIa is that IIa is sparlingly soluble in hot water while IIIa is readily soluble.

These observations indicated that IIIa would be a structural isomer of IIa. The compound, IIa was hydrolized in an alkaline solution under the same condition as described before when we obtained IIb from IIa,[9] and benzoic acid was produced almost quantitatively. From the mother liquor, colorless crystals (IIIb) were obtained, m.p. 214~215° (decomp.). The elementary analysis of IIIb showed empirical formula of $C_{12}H_{18}O_5N_4S$ corresponding to thiaminic acid (IIb). Decomposition point of IIIb is very close to that of IIb, but the remarkable depression was observed in the mixed melting point determination.

The solubility of IIIb is same as that of IIb, *e.g.*,sparlingly soluble in organic solvent and easily soluble in water.

The ultraviolet spectra of IIIb were in agreement with those of IIb (Fig. 3), but the infrared spectra (Fig. 4) exhibited some differences in the wave number of characteristic absorption except for NH_3^+ absorption band around 2650 cm^{-1} and SO_2 symmetrical stretching vibration at 1030 cm^{-1}.

All these observations seem to suggest that the compound, IIIb is a structural isomer of thiaminic acid (IIb).

8) A. Simon, H. Kriegemann, H. Dutz: Chem. Ber., **89**, 1990 (1956).
9) I. Utsumi, K. Harada, G. Tsukamoto: J. Vitaminol., **11**, 239 (1965).

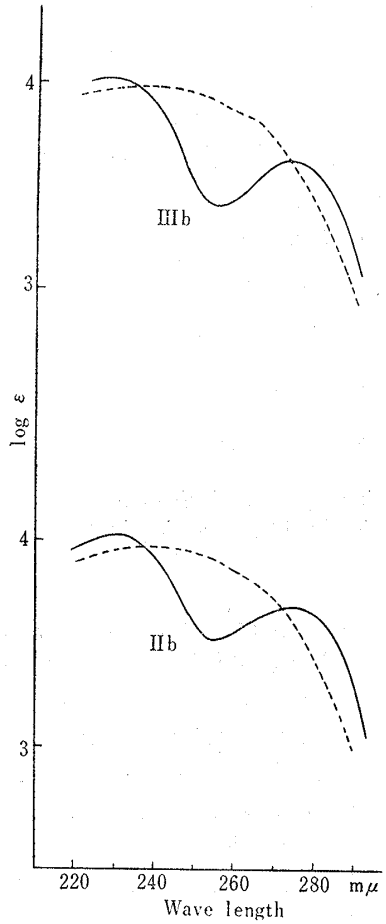


Fig. 3. Ultraviolet Absorption Spectra of *cis*- and *trans*-Thiaminic Acid (IIIb ann IIb)
——— pH 7.4
..... pH 3.0

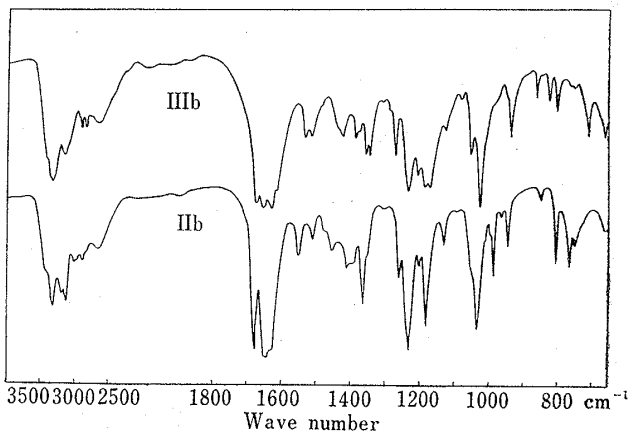
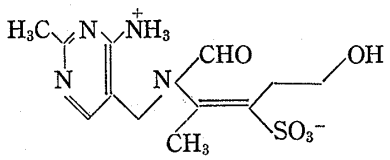


Fig. 4. Infrared Absorption Spectra of *cis*- and *trans*-Thiaminic Acid (IIIb and IIb) in Potassium Bromide

Namely, the compound, IIIb is undoubtedly a geometrical isomer of IIb, represented by the following structure :



This was further confirmed by the benzoylation of IIIb in an alkaline solution to give IIIa without isomerization.

If the compound, IIIb is a geometrical isomer of thiaminic acid (IIb), hydrolysis of IIIb with hydrochloric acid should give the same hydrolysates of IIb.[9] The hydrolysis of IIIb with hydrochloric acid was thus carried out and 2-methyl-4-amino-5-aminomethylpyrimidine (IV) and formic acid were obtained from the reaction mixture. The separation and identification of 1-acetyl-3-hydroxypropane-1-sulfonic acid (V) were not performed, however, barium salt of a substance presumed to be V had the same infrared spectra to that of barium salt of V obtained by hydrolysis of IIb.

The nuclear magnetic resonance spectra (Fig. 5) of IIb and IIIb were measured in deuterium oxide. The spectrum of IIb showed a singlet signal due to protons of olefin-CH_3 at 7.96τ. In IIIb, the spectrum showed an olefin-CH_3 proton at 7.76 which was located in lower field than that of IIb by 0.2 p.p.m. The major contributing factor to this situation seems to be the paramagnetic effect of SO_3^-.

In addition, the proton resonance signals of N-formyl, pyrimidine ring and two methylene of hydroxyethyl in IIb were found almost in the same location as those found in thiamine disulfide (I : R=H)[10]. However, in IIIb the signals due to the proton of N-formyl and pyrimidine ring were found in lower field and those due to the protons of two methylene of hydroxyethyl in higher field than those signals in IIb. This difference might be caused by polar effect.

The infrared absorption spectrum of IIIa showed the absorption band due to carbonyl group of benzoate at 1730 cm^{-1} which is quite identical with that of phenyl benzoate, whereas, that of IIa showed the absorption band at 1690 cm^{-1} which shifted to

10) K. Kotera : This Bulletin, **13**, 440 (1965).

considerable lower frequency. Moreover, the absorption band due to carbonyl group of N-formyl group of IIa lies at 1667 cm^{-1} and that of IIIa at 1690 cm^{-1}.

The shift of carbonyl absorption bands in IIa towards lower frequencies suggested the presence of intra- or intermolecular hydrogen bond, *e.g.* the hydrogen bond between carbonyl oxygen and ammonium (NH_3^+) hydrogen at 4-position of pyrimidine ring. On the other hand, no hydrogen bond is presumed to be present in IIIa from the fact that carbonyl absorption bands were observed at normal frequencies. Thiaminic acids dissociate as chart 2.

If compound IIa have the intramolecular hydrogen bond, there should be some difference between the dissociation

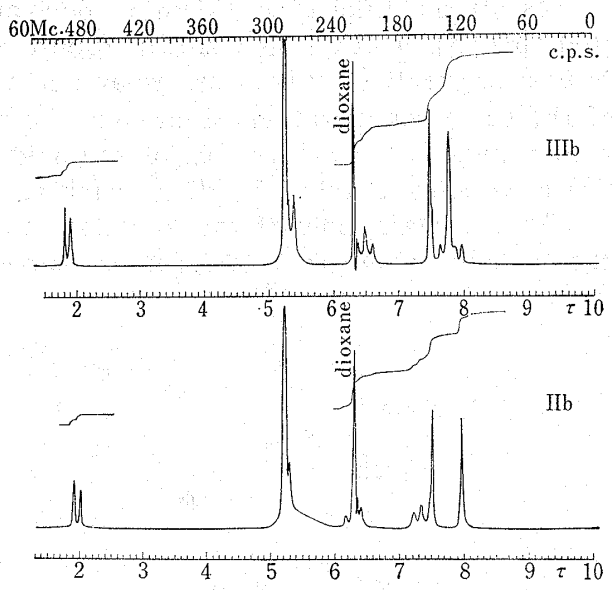


Fig. 5. Nuclear Magnetic Resonance Spectra of *cis*- and *trans*-Thiaminic Acid (IIIb and IIb) in Deuterium Oxide at 60 Mc.p.s.

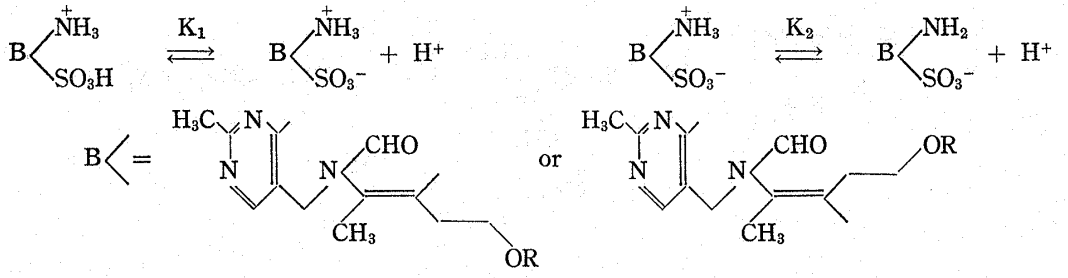


Chart 2.

constant of IIa and IIIa. Acid dissociation constant (pKa) of these compounds were measured by potentiometric titration method. As is shown in the Table I, pK_1 of IIa was identical with that of IIIa, however pK_2 of IIa was 6.1, somewhat larger than that of IIIa, 5.8.

Table I. Dissociation Constants (pKa) of Thiaminic Acids and Their Isomers at 25°

Compd.	pK_1	pK_2
IIIa	2.3	5.8
IIa	2.3	6.1
IIIb	2.2	5.9
IIb	2.2	6.2

From these observations, it is clear that the ammonium group of IIa is more difficult to dissociate than that of IIIa. This result indicates that the ammonium hydrogen of IIa is hard to dissociate owing to the stabilization caused by the intramolecular hydrogen bond. Moreover, a paralleled relation was observed in pK_2 between IIb and IIIb. The characteristic differences in the magnetic resonance spectra and the dissociation constants in their liquid state support that the compounds obtained in this experiments are not polymorphic compounds but geometrical isomers.

As all the thiol-type thiamine derivatives (Ⅶ) have been derived from thiol-type thiamine (Ⅵ), methyl and sulfur group attached to an olefinic linkage of Ⅶ are in *trans*-configuration. Likewise, thiaminic acid, oxidized form of Ⅵ is presumed to be of the same configuration. However, the compounds obtained in this oxidation were rather unexpected. A portion of the oxidized derivatives was of unknown compound (Ⅷ) in which methyl and sulfur group are in *cis*-configuration.

At a certain step of the reaction, the isomerization of the olefinic double bond should have taken place to form *cis*-isomer from *trans*-thiol-type thiamine derivative (I).

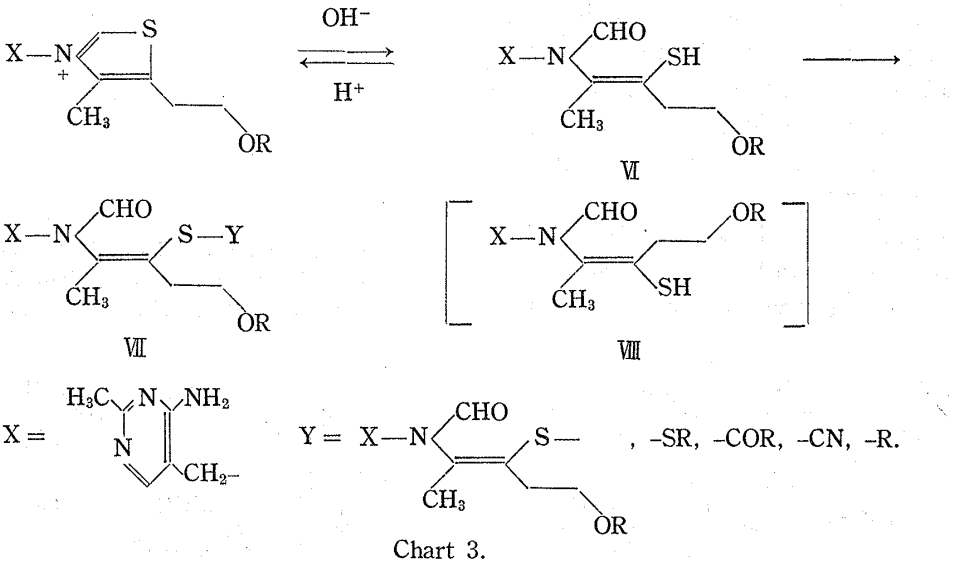
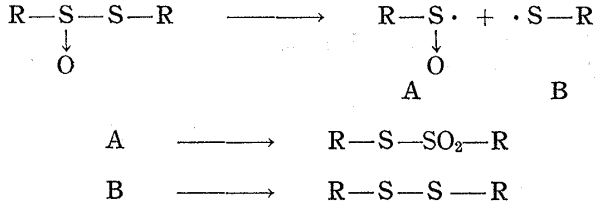


Chart 3.

The initial step of the oxidation is presumed to give the thiolsulfinate[11] which is known to disproportionate readily to form two radicals, 2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-sulfinyl (A) and 2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-mercapto radicals (B).



R = 2-(2-methyl-4-amino-5-pyrimidyl)-methylformamido-5-benzoyloxy-2-pentene-3-

Chart 4.

The mercapto radical thus formed could isomerize olefinic linkage before recombining to return to the original disulfide. Incidentally, olefins are known to isomerize readily in the presence of a minute amount of any mercapto radical.[12,13] Meanwhile the sulfinyl radical would recombine to give the thiolsulfonate. Further oxidation of these substances eventually gives rise to the 2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-sulfonic acid (Ⅱa or Ⅲa).

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12) K. Sugimoto, W. Ando, S. Oae: Bull. Chem. Soc. Japan, **38**, 224 (1965).
13) W. Ando, K. Sugimoto, S. Oae: *Ibid.*, **38**, 226 (1965).

Experimental

All melting points were uncorrected. Ultraviolet absorption spectra were taken with Shimadzu Recording Ultraviolet Spectrophotometer, SV-50A, and Infrared absorption spectra were measured with Hitachi Infrared Spectrophotometer, EPI-S_2 and Perkin-Elmer 21 Infrared Spectrophotometer. Nuclear magnetic resonance spectra were measured with Japan Electron Co. J.N.M-C60 spectrometer. The chemical shifts are given in τ values calibrated using dioxane as an internal standard (τ dioxane=6.33). Dissociation constant data were obtained by Metrohm Potentiograph, E-336.

Isolation of *cis*-2-(2-Methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-sulfonic Acid (IIIa)——To a solution of I (6.0 g.) in glacial AcOH (60 ml.) was added 30% hydrogen peroxide (5.3 ml.) and the mixture was allowed to stand for a week. The reaction mixture was concentrated under the reduced pressure. The residue was dissolved in EtOH and the separated crystals were recrystallized from 50% EtOH to give 4.5 g. of colorless prisms (monohydrate of IIa), m.p. 234~235° (decomp.). *Anal.* Calcd. for $C_{19}H_{22}O_6N_4S\cdot H_2O$: C, 50.43; H, 5.35; N, 12.38; S, 7.08. Found: C, 50.18; H, 5.34; N, 12.49; S, 7.04. Refluxing the monohydrate of IIa in abs. EtOH afforded colorless needles (IIa), m.p. 237~238° (decomp.). *Anal.* Calcd. for $C_{19}H_{22}O_6N_4S$: C, 52.52; H, 5.10; N, 12.90; S, 7.38. Found: C, 52.67; H, 4.90; N, 12.78; S, 7.22.

The mother liquor of IIa was again concentrated under the reduced pressure and the residue was dissolved in a little quantity of water. The solution was kept at room temperature for a day to separate white crystals (0.4 g.). The crystals were recrystallized from boiling water repeatedly to give colorless needles (1/2 hydrate of IIIa), m.p. 225~226° (decomp.). *Anal.* Calcd. for $C_{19}H_{22}O_6N_4S\cdot 1/2H_2O$: C, 51.46; H, 5.23; N, 12.63; S, 7.23. Found: C, 51.50; H, 4.92; N, 12.49; S, 7.40. This hydrate was refluxed in abs. EtOH to afford colorless fine crystals (IIa), m.p. 233~234° (decomp.). *Anal.* Calcd. for $C_{19}H_{22}O_6N_4S$: C, 52.52; H, 5.10; N, 12.90; S, 7.38. Found: C, 52.52; H, 5.17; N, 12.77; S, 7.23.

Hydrolysis of IIIa——A solution of IIIa (500 mg.) dissolved in H_2O (9 ml.) and *N* NaOH (1 ml.) was heated at 80° adjusting its pH 9~10 by the dropwise addition of *N* NaOH (1.2 ml.). The cooled solution was adjusted to pH 3.0 by the addition of dil. HCl and extracted with ether. The aqueous layer was concentrated under the reduced pressure. The residue was extracted with hot EtOH to remove inorgnanic salts and then the extract was evaporated to dryness under the reduced pressure. The residue was recrystallized from 90% EtOH to give 250 mg. of colorless plates (1/2 hydrate of IIIb), m.p. 212~213° (decomp.). *Anal.* Calcd. for $C_{12}H_{18}O_5N_4S\cdot 1/2H_2O$: C, 42.47; H, 5.64: N, 16.51; S, 9.45. Found: C, 42.45; H, 5.43; N, 16.60; S, 9.30. This hydrate, after similar treatment to that described for the hydrate of IIa and IIIa, afforded colorless fine crystals (IIIb), m.p. 214~215° (decomp.). *Anal.* Calcd. for $C_{12}H_{18}O_5N_4S$: C, 43.63; H, 5.49; N, 16.96; S, 9.71. Found: C, 43.76; H, 5.48; N, 16.55; S, 9.40.

The ether extract was evaporated to dryness and the residue (130 mg.) was recrystallized from boiling water to give colorless plates, m.p. 122°, which showed no depression in the mixed melting point determination with benzoic acid.

Benzoylation of *cis*-2-(2-Methyl-4-amino-5-pyrimidyl)methylformamido-5-hydroxy-2-pentene-3-sulfonic acid (IIIb)——To a solution of IIIb (0.5 g.) dissolved in H_2O (5 ml.) was added dropwise a solution of benzoyl chloride (0.3 g.) in benzene (ca. 0.5 ml.) under adjusting to pH 8~10 by the addition of *N* NaOH and cooling with ice water. After the addition was completed, the reaction mixture was stirred at room temperature for 1 hr., adjusted to pH 3 with dil. HCl, and then concentrated to half volume to give a crystalline product (150 mg.) which was washed with EtOH and ether. The crystals obtained were recrystallized from boiling water to give colorless needles (1/2 hydrate of IIIa), m.p. 225~226° (decomp.) which were identified by infrared spectral comparison.

Decomposition of IIIb by Hydrochloric Acid——IIIb (0.5 g.) was dissolved in 10% HCl (25 ml.) and heated on a boiling water bath for 1 hr. The reaction mixture was submitted to steam distillation, the distillate showing a possitive formic acid reaction by chromotropic acid. The residual solution was concentrated to dryness under the reduced pressure, and to the residue was added ethanol to separate white crystals after being once dissolved. The crude crystals were recrystallized from 80% EtOH to give colorless prisms, m.p. 264~265° (decomp.), which were identified as 2-methyl-4-amino-5-aminomethylpyrimidine dihydrochloride (IV) by the mixed melting point determination and the comparison with infrared absorption spectra.

The mother liquor of IV was concentrated under the reduced pressure and the residue was dissolved in a little quantity of water. To the solution, previously adjusted to pH 7 with *N* NaOH, was added saturated aqueous solution of $BaCl_2$ and then crystals (V), which did not show accurate decomposition point, were obtained. IR ν_{max}^{Nujol} cm^{-1}: 1710, 1215, 1200, 1175, 1045, 1060.

Measurement of Dissociation Constants——A solution of 120 mg. of IIb or IIIb dissolved in a little excess of 0.1*N* HCl and H_2O (50 ml.) was titrated with 0.1*N* NaOH. A solution of 130 mg. of IIa or IIIa dissolved in a little excess of 0.1*N* NaOH was titrated with 0.1*N* HCl. Apparent pKa values obtained from half points of neutralization on potentiometric titration curves were shown in Table I.

The authors express their deep gratitude to Professor S. Oae of Osaka City University for valuable discussions and Dr. M. Fujisawa, Chief of the Manufacturing Technic Headquarter of our Company for his guidance and encouragements. They are also grateful to Dr. K. Kotera and the members of Tokyo Laboratories for NMR spectra and elemental analyses.

Summary

Oxidation of O-benzoylthiamine disulfide (I) with hydrogen peroxide in acetic acid yielded O-benzoylthiaminic acid (IIa) and its stereoisomerical compound (IIIa). It was confirmed that these two compounds were in the relation of geometrical isomers which have not yet been found in thiol-type thiamine derivatives: the compound obtained in this experiment was *cis*-2-(2-methyl-4-amino-5-pyrimidyl)methylformamido-5-benzoyloxy-2-pentene-3-sulfonic acid (IIIa) of which olefin-CH_3 and SO_3^- groups were in *cis*-configuration. Alkali decomposition of IIIa afforded *cis*-thiaminic acid (IIIb) corresponding to geometrical isomer of IIb.

The configuration of these compounds was confirmed by elemental analyses, ultraviolet, infrared, nuclear magnetic resonance spectra and dissociation constants.

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110. Mieko Suzuki (née Saito), Eiko Akaike,*1 Kyosuke Tsuda,*2 and Nobuo Ikekawa*3 : Studies on the Sterol of *Bombyx mori* L. II.*4 Quantitative Analysis of Total Sterol in the Silkworm.

(*The Sericultural Experiment Station,*1 the Institute of Applied Microbiology, University of Tokyo,*2 and the Institute of Physical and Chemical Research*3*)

The sterol nature in insects was investigated by many workers,[1] but only a few workers[2,3] have reported the quantitative analysis of sterols in insects. On the other hand, as it has been demonstrated that cholesterol was one of active ingredients of the brain hormone in the silkworm[4,5] and was also a precursor of ecdysone,[6] the quantity of sterol in the silkworm through all its developmental stages became important.

In the previous report,*4 it was shown by gas chromatographic analysis that silkworm sterols consisted of three sterols; cholesterol, β-sitosterol, and campesterol, and their compositions became different with the progress of age. The present paper

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