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A New Fluorometric Method for Latamoxef in Biological Materials Using 2,6-Diamino-3-nitrosopyridine

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A novel fluorometric assay method was established for latamoxef (LMOX), a new type of antibiotic. LMOX was converted into 1, 2,4-dihydroxy-3-[(1-methyltetrazol-5-ylthio)methyl]but-2-enoic acid γ -lactone, in an acid solution. The lactone **1** reacted with 2,6-diamino-3-nitrosopyridine to give the fluorophore, 3-(5-amino-3*H*-imidazo[4,5-*b*]pyridin-2-yl)-2,4-dihydroxybut-2-enoic acid γ -lactone. The fluorescence was measured at 460 nm with excitation at 392 nm. In plasma and urine, 1–15 $\mu\text{g/ml}$ and 20–300 $\mu\text{g/ml}$ of LMOX were determined, respectively, with good precision through a simple technique. The sample volume was 100 μl . The reaction mechanism of the fluorescence reaction is discussed.

Keywords—latamoxef; 2,6-diamino-3-nitrosopyridine; fluorometric determination; 2,4-dihydroxy-3-[(1-methyltetrazol-5-ylthio)methyl]but-2-enoic acid γ -lactone; 3-(5-amino-3*H*-imidazo[4,5-*b*]pyridin-2-yl)-2,4-dihydroxybut-2-enoic acid γ -lactone; fluorescence reaction mechanism; *o*-nitrosoaniline

The assay methods now available for latamoxef (LMOX), a new type of antibiotic developed in our laboratories,¹⁾ are the microbiological method and high performance liquid chromatography (HPLC). These methods were effective for the evaluation of the antibacterial activity²⁾ and the separative determination of *R*- and *S*-epimers of LMOX.³⁾ For further investigations on the distribution, excretion and protein binding of the drug, a more convenient method for analyzing many samples in a short period is required.

In the present work, we aimed to establish a sensitive and simple assay method based on the fluorometric technique. The fluorometric methods for the assay of 1-thiacephem antibiotics^{4,5)} were not applicable to LMOX. We investigated the reactions of LMOX with 2,6-diamino-3-nitrosopyridine (DNP) and *o*-nitrosoaniline to produce the fluorophores **2** and **8**, and found that DNP was suitable for the determination of LMOX. Many samples could be assayed rapidly with good precision. It is also advantageous that the method requires only a small sample, 0.1 ml. This paper describes the application of the assay method to plasma and urine, and the structure of the fluorophore.

Experimental

Apparatus—A Hitachi MPF-2A recording spectrofluorometer was used for fluorescence measurement. Infrared (IR) spectra were measured on a JASCO A-702 spectrometer. Mass spectra (MS) were obtained on a Hitachi M-68 spectrometer. Proton nuclear magnetic resonance (¹H-NMR) and ¹³C-nuclear magnetic resonance (¹³C-NMR) spectra were taken on a Varian EM-360 spectrometer and a Varian XL-100-12A, respectively. Chemical shifts are expressed as ppm downfield from tetramethylsilane as an internal standard. A Sakuma centrifuge, model 90-4, and an Iwaki KM shaker, type V-S, were used for extraction and separation.

Reagents and Materials—All chemicals were of reagent grade. DNP was synthesized by nitrosation of 2,6-diaminopyridine.⁶⁾ 2,4-Dihydroxy-3-[(1-methyltetrazol-5-ylthio)methyl]but-2-enoic acid γ -lactone (**1**) and 2,4-dihydroxy-3-(dimethylaminomethyl)but-2-enoic acid γ -lactone (**3**) were supplied by our laboratories. mp 169 °C (**1**), mp 167 °C (**3**·HCl).

DNP solution (0.05%): Dissolve 50 mg of DNP in H₂O to make 100 ml. 2-Mercaptoethanol solution (0.04%): Dissolve 40 mg of 2-mercaptoethanol in H₂O to make 100 ml. K₂S₂O₅ solution (0.2%): Dissolve 200 mg of K₂S₂O₅ in H₂O to make 100 ml. Acetate buffer solution (1.1 M, pH 3): Dissolve 44 g of NaOH in H₂O (100 ml) and make up to 1000 ml with AcOH. Phosphate buffer solution (0.1 M, pH 7): Dissolve 5.1 g of KH₂PO₄ and 9.7 g of Na₂HPO₄ in sufficient H₂O to make 1000 ml. Citrate buffer solution (pH 1.5–7.0): Dilute 1 M sodium citrate solution with 1 M citric acid solution and adjust the pH to 1.5–7.0. Acetate buffer solution (pH 2.0–7.0): Dissolve 8.2 g of AcONa in sufficient water, adjust the pH to 2.0–7.0 with AcOH and dilute with H₂O to make 100 ml. LMOX standard solutions: Dissolve 3.0 mg of LMOX in the phosphate buffer to make 100 ml. Pipet 1, 2, 3, 4, and 5 ml of the solution into five 10-ml volumetric flasks and dilute to the mark with the phosphate buffer.

Examination of Conditions for the Fluorescence Reaction: Portions (1 ml) of the phosphate buffer solution of LMOX (0.75 µg/ml) were tested under various conditions (pH, reagent concentration, reaction time and temperature).

Assay Procedure—Pipet 0.1 ml of plasma into a 1-ml volumetric flask and dilute to the mark with the phosphate buffer. Pipet 1 ml of urine into a 500-ml volumetric flask and dilute to the mark with H₂O. Pipet 0.1 ml of each diluted sample solution⁷⁾ into a 12-ml centrifuge tube, and add 6 ml of AcOEt and 0.5 ml of 0.1 N HCl. Shake the tube for 5 min and centrifuge at 2500 rpm for 5 min. Transfer exactly 5 ml of the AcOEt layer to a 12-ml centrifuge tube and add 1.5 ml of the phosphate buffer. Shake the tube for 5 min and centrifuge at 2500 rpm for 5 min. Transfer exactly 1 ml of the aqueous layer to a 10-ml volumetric flask. Add 0.05 ml of 0.2% K₂S₂O₅ solution, 0.05 ml of 0.04% 2-mercaptoethanol, 0.1 ml of 0.05% DNP solution and 3.5 ml of the acetate buffer. Mix well and connect an air condenser⁸⁾ with the neck of the flask. Heat the flask at 118 °C for 45 min, then allow it to cool to room temperature, and dilute to the mark with the acetate buffer. Measure the fluorescence intensity at 460 nm with excitation at 392 nm. Prepare a blank solution in the same manner but without the sample.

Calibration Curve—The calibration curve was made according to the standard assay procedure. A linear relationship between the concentration of LMOX and the fluorescence intensity was obtained in the range of 1–15 µg/ml.

Isolation of the Fluorophore (2)—A mixture of LMOX (10.1 g, 18 mmol) and DNP (5 g, 36 mmol) in 1000 ml of 0.5 M acetate buffer (pH 3) was refluxed for 1 h. The solution was concentrated under reduced pressure to ca. 150 ml. The residue was chromatographed on an HP-20 (Mitsubishi Chem. Co., Ltd.) column (35 i.d. × 400 mm), which was washed with H₂O, and eluted with MeOH–H₂O (1 : 1). The latter eluate was evaporated under reduced pressure. The residue was dissolved in 5% Na₂CO₃ (30 ml), and the solution was chromatographed on a new HP-20 column. The fraction eluted with H₂O was adjusted to pH 2 with hydrochloric acid. Chromatography of the solution on a new HP-20 column with MeOH–H₂O (1 : 1), followed by concentration under reduced pressure gave 46 mg of **2**. mp 270 °C (dec.). IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3320, 3210, 1765 (lactone C=O). ¹H-NMR (DMSO-*d*₆) δ : 5.30 (2H, s, –CH₂–), 6.50 (1H, d, *J*=4.5 Hz, aromatic-H), 7.70 (1H, d, *J*=4.5 Hz, aromatic-H). ¹³C-NMR of **2**·HCl (DMSO-*d*₆) δ : 66.5 (t, –CH₂–), 107.5 (d, aromatic-C), 115.2 (s, aromatic –C), 119.9 (s, C=C–CH₂–), 129.6 (d, aromatic-C), 142.1, 143.4, 143.5 (s, aromatic-2C, C–OH), 153.2 (s, –C $\begin{smallmatrix} \nearrow \text{N} \\ \searrow \text{N} \end{smallmatrix}$), 168.4 (s, C=O). MS *m/z*: 232 (M⁺). *Anal.* Calcd for C₁₀H₈N₄O₃·1/2H₂O: C, 49.78; H, 3.76; N, 23.22. Found: C, 49.53; H, 3.72; N, 22.71.

The reaction of **1** with DNP was performed in a manner similar to that described above. Compound **2** was isolated in 30% yield. The elemental analysis and the spectral data of the product were in good accord with those expected for **2**.

Isolation of the Fluorophore (8)—A mixture of LMOX (6.35 g, 11.2 mmol) and *o*-nitrosoaniline (2.72 g, 22.3 mmol) in 600 ml of 1.1 M acetate buffer (pH 3) was refluxed for 1 h. The solution was concentrated under reduced pressure to ca. 150 ml. The residue was chromatographed on an HP-20 column, which was eluted with H₂O, followed by MeOH–H₂O (1 : 1). The latter eluate was concentrated, giving a pale yellow precipitate. Recrystallization from DMSO–H₂O gave 30 mg of **8** as a pale yellow powder. mp 264–266 °C (dec.). IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3250, 1741 (lactone C=O). ¹H-NMR (DMSO-*d*₆) δ : 5.33 (2H, s, –CH₂–), 7.50 (2H, m, aromatic-H) and 8.00 (2H, m, aromatic-H). MS *m/z*: 216 (M⁺). *Anal.* Calcd for C₁₁H₈N₂O₃: C, 61.11; H, 3.73; N, 12.96. Found: C, 61.02; H, 3.74; N, 13.06.

Results and Discussion

The reaction of LMOX with DNP afforded a main product which had intense fluorescence. The structure of the product was elucidated as **2** (Chart 1).

The assay conditions were established on the basis of the optimum conditions leading to the fluorophore **2** from LMOX.

Physico-Chemical Characteristics of the Fluorophore

Compound **2** was isolated after chromatography on HP-20 as a pale yellow powder, mp 270 °C (dec.), C₁₀H₈N₄O₃·1/2H₂O (*m/z* 232, M⁺). Elemental analysis data suggested that the

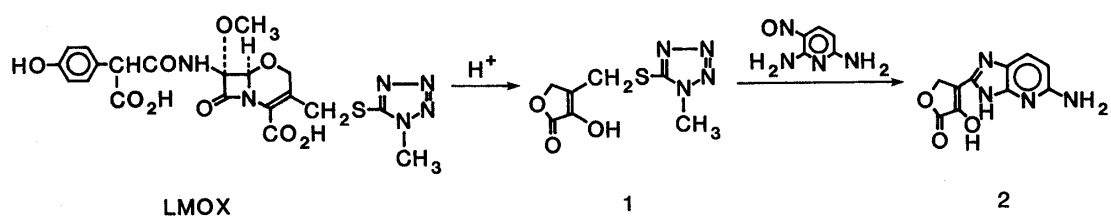


Chart 1

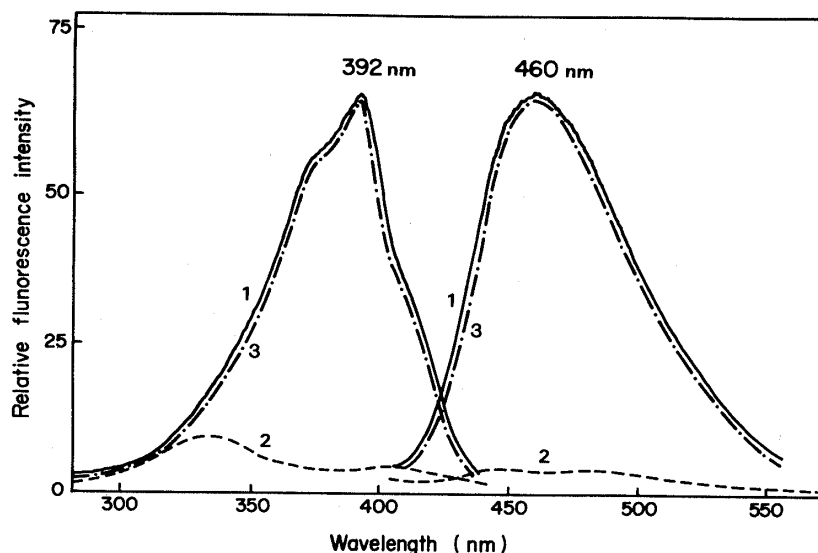


Fig. 1. Excitation and Emission Spectra of the Fluorophore

1, reaction of LMOX with DNP; 2, reagent blank; 3, 2 in acetate buffer (0.03 $\mu\text{g/ml}$). (DNP concentration: 0.05%).

compound contained the DNP moiety. The IR spectrum, which shows characteristic stretching bands at 3210, 3320 (NH_2 and associated OH) and 1765 cm^{-1} ($\text{C}=\text{O}$), suggested the presence of DNP and a γ -lactone ring. The structure **2** was presumed, and was confirmed by the other spectral evidence, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and the fluorescence. The $^1\text{H-NMR}$ spectrum gave a singlet signal (δ 5.30) assignable to methylene protons. The $^{13}\text{C-NMR}$ spectrum showed signals corresponding to each carbon of **2**. The acetate buffer solution of **2** fluoresced at 460 nm on irradiation at 392 nm. Its fluorescence was the same as that observed for the reaction solution obtained in the assay procedure, which is shown in Fig. 1. The acetate buffer solution of the fluorophore obtained in the assay procedure was stable for more than 2 h.

This structure (**2**) was also supported by the fact that the reaction of **1** with DNP gave the same product as that obtained from LMOX. Since compound **1** was isolated by the reaction of LMOX in acid without DNP, it was concluded that **1** was an intermediate in the formation of the fluorophore **2**.

Assay Conditions

Effect of pH on the Fluorescence Development—The reaction was dependent on pH. As shown in Fig. 2, a constant and maximum fluorescence intensity was obtained in the range of pH 2.5–3.0 using the acetate buffer. The formation of the fluorophore decreased at higher pH. The yield obtained in the citrate buffer was about one-half the value in the acetate buffer.

Effect of Time and Temperature on the Fluorescence Development—The reaction was carried out at 100 and 118°C . At 118°C , the solvent was refluxing. The fluorescence intensi-

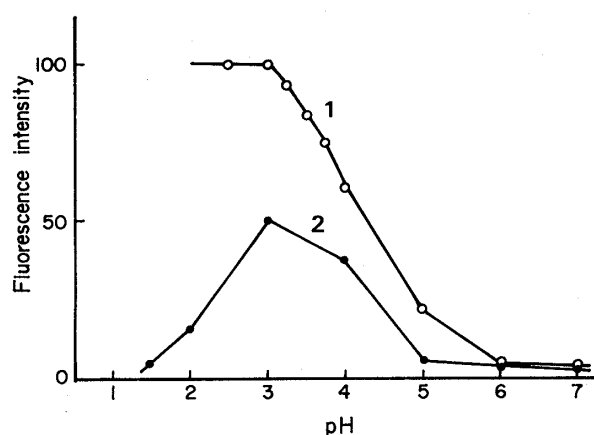


Fig. 2. Effect of pH on the Fluorescence Development

1, acetate buffer; 2, citrate buffer. (DNP concentration: 0.05%).

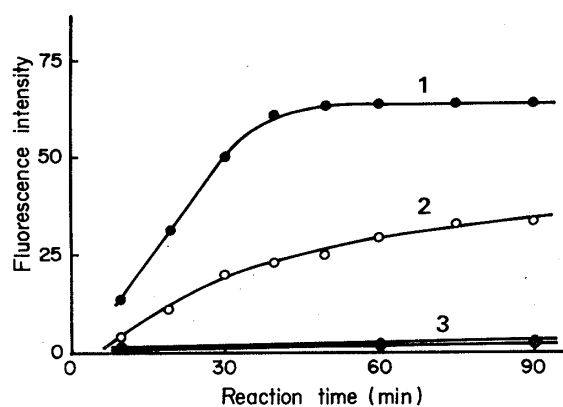


Fig. 3. Effect of Temperature and Time on the Fluorescence Development

1, at 118°C; 2, at 100°C; 3, reagent blank (●, 118°C; ○, 100°C). (DNP concentration: 0.05%).

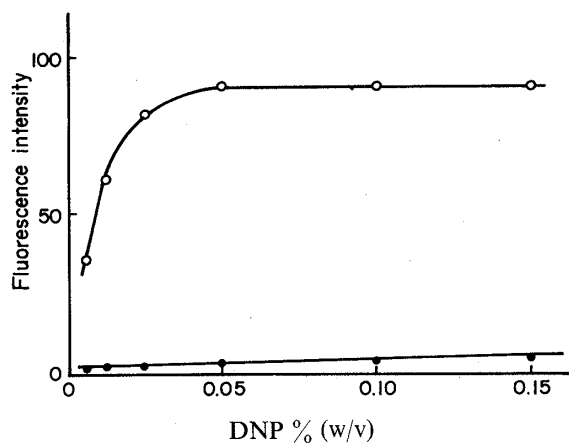


Fig. 4. Effect of the Concentration of DNP on the Fluorescence Development

○, LMOX; ●, reagent blank.

ties were measured, and as shown in Fig. 3, a constant fluorescence intensity was obtained in the range of 45–90 min at 118°C. The reaction rate at 100°C was much slower than that at 118°C.

Effect of DNP Concentration on the Fluorescence Development—Various concentrations of DNP were tested. As shown in Fig. 4, when DNP was present at more than 0.05% (molar ratio, 280-fold), a constant fluorescence intensity was obtained. At concentrations higher than 0.15% (molar ratio, 840-fold), the fluorescence was quenched gradually.

Conditions for the Assay of LMOX in Plasma and Urine—Plasma and urine samples were acidified and extracted with AcOEt. Extracted LMOX was transferred to pH 7 phosphate buffer, then assayed *via* the fluorescence reaction with DNP. Overall recoveries of LMOX through the extraction processes were about 85%.

In order to examine the interference from plasma and urine with the present assay method, another recovery test was also performed. Various amounts of LMOX were added to plasma and urine to prepare two ranges of concentration, 1–15 µg/ml and 20–300 µg/ml, respectively. The recoveries were measured by the standard assay method. A standard curve was obtained by treating phosphate buffer solutions of LMOX according to the assay procedure. Regression analyses for both samples showed no interference in the assay.

Precision and Quantitation Limit

The precision was examined according to the standard assay procedure on replicate runs. In preliminary studies, some anomalous variations of the assay values were noted oc-

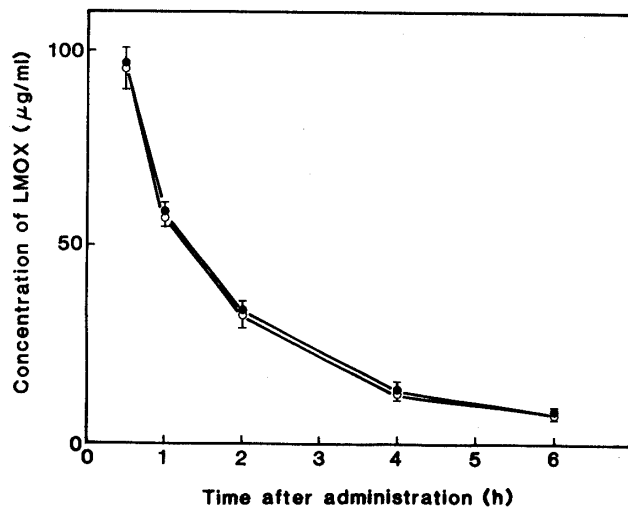


Fig. 5. Plasma Levels of LMOX after Intravenous Administration to Human Subjects (Dose: 20 mg/kg)

○, fluorometry; ●, HPLC.
The data represent the means \pm S.D. of three subjects.

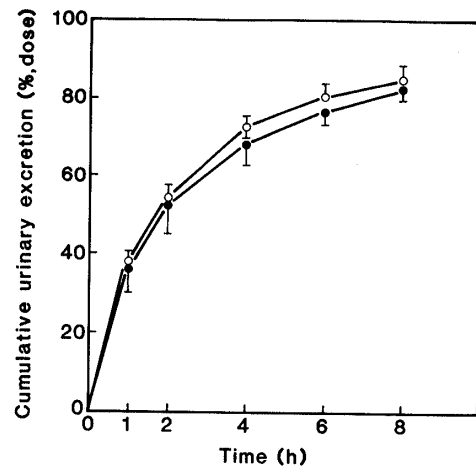


Fig. 6. Urinary Excretion after Intravenous Administration of LMOX to Human Subjects (Dose: 1 g)

○, fluorometry; ●, HPLC.
The data represent the means \pm S.D. of three subjects.

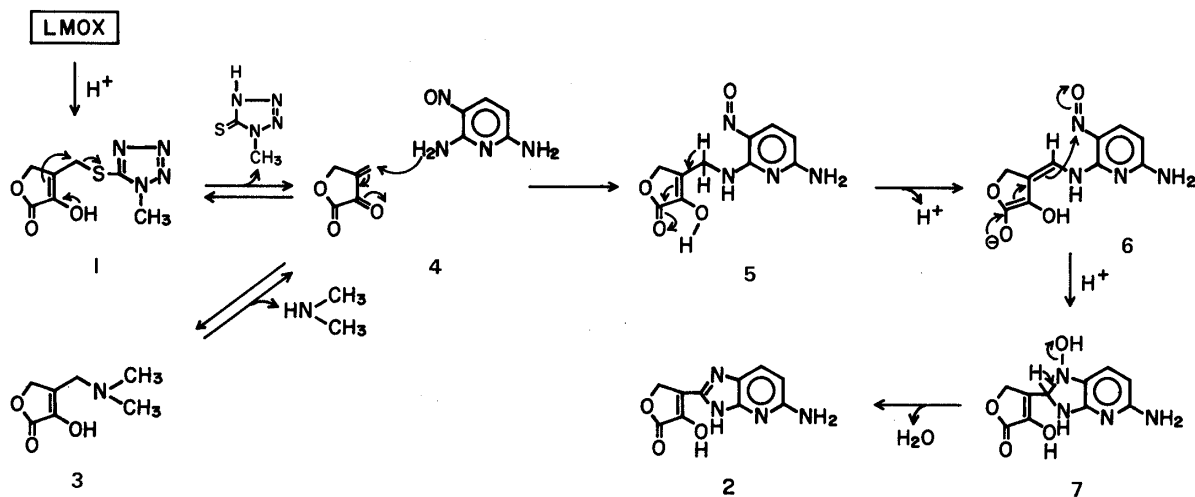


Chart 2

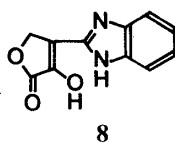
casionaly, but these were eliminated by the additions of potassium metabisulfite and 2-mercaptoethanol in the standard method. Then the coefficients of variation were 3–4% ($n=10$). The quantitation limits were 0.25 and 2.0 $\mu\text{g/ml}$ of LMOX in plasma and urine, respectively.

Plasma Levels of LMOX

Plasma was taken after intravenous administration of LMOX into a healthy volunteer. Figure 5 shows a typical decay curve of plasma LMOX. The assay values were in good accord with those obtained simultaneously by the HPLC method.

Urinary Excretion of LMOX

Urine was collected at regular time intervals after administration of LMOX. The accumulated urinary amounts of LMOX are shown in Fig. 6. About 85% of LMOX was excreted. The assay values were in reasonable agreement with those obtained simultaneously by the HPLC method.



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Chart 3

TABLE I. Relative Amounts of Fluorescent Material Formed from Various Compounds

| Compound ^{a)} | λ_{ex} (nm) | λ_{em} (nm) | Relative ^{b)} intensity | |
|----------------------------|-------------------------------|-------------------------------|-------------------------------------|-----|
| 1-Oxa cephem | | | | |
| | | | | |
| R : | LMOX | 392 | 460 | 100 |
| R : | 9 | 392 | 460 | 92 |
| R : NH_2 - | 10 | 392 | 460 | 100 |
| Other antibiotics | | | | |
| Cefamandole | 11 | 392 | 460 | 45 |
| Cephalothin | 12 | 392 | 460 | 54 |
| Cefoperazone | 13 | 392 | 460 | 34 |
| Cefazolin | 14 | 392 | 460 | 31 |
| Cefaclor | 15 | — | — | 0 |
| Cephalexin | 16 | — | — | 0 |
| Carbenicillin | 17 | — | — | 0 |

a) Antibiotics at *ca.* 0.75 $\mu\text{g/ml}$.

b) Fluorescence readings at the same instrument sensitivity.

Reaction Mechanism

The β -lactam ring of LMOX is cleaved in an acidic solution, and this is followed by degradations through several pathways.⁹⁾ Compound **1**, a main degradation product, was isolated. The mechanism of the formation of **2** from **1** may be as follows. The *retro* Michael addition eliminates tetrazole-thiol and dimethylamine from **1** and **3** in the presence of the nucleophile DNP. The resulting **4** will give an adduct **5** by Michael addition. The adduct **5** is converted into **6** with elimination of H^+ . Cyclization then proceeds by means of nucleophilic attack on the nitroso nitrogen by the carbanion to give **7**, followed by dehydration to give the final product **2**. This mechanism seemed to be reasonable since LMOX, **1** and **3** reacted with *o*-nitrosoaniline under the same conditions as used with DNP to give a similar fluorophore **8**.

Other Fluorescence Reactions of LMOX with Aniline Derivatives

The fluorophore **8** has an excitation maximum and an emission maximum at 352 and 422 nm, respectively. The intensity of **8** was one-half the value of **2**. *o*-Phenylenediamine reacted with LMOX in an alkaline solution (pH 9.2) to give a fluorescent product with excitation and emission maximum of 320 and 382 nm, respectively. This reagent gave an interfering fluorescent product by reaction with a plasma component, exhibiting a high blank

value.

Characteristics of the Assay Method

Since the oxygen at the 1-position in the cephem ring provides the ether oxygen in **2**, the method may be applicable to general 1-oxacephems provided that the 3-position is substituted by a methylene group having a leaving group. Compounds **9** and **10** afforded **2** in the same yield as LMOX as estimated fluorometrically (Table I).

Interestingly, several 1-thiacephem antibiotics formed similar fluorophores. The fluorescence characteristics were in accord with those obtained for LMOX. Although the reaction mechanism has not been elucidated, it seems that γ -lactone derivatives are formed as the degradation products and that a similar fluorescence reaction then proceeds. Abraham *et al.*¹⁰⁾ isolated γ -lactones similar to **1** after the acid hydrolysis of cephalosporin.

No fluorophore was produced with other 1-thiacephem derivatives (**15** and **16**) and penicillin, which have no active methylene group at the 3-position.

The present assay method is sensitive and accurate, and is suitable for assaying many samples rapidly. It is also advantageous that only 0.1 ml of samples is required.

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References and Notes

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- 7) Use 0.1 ml of plasma without dilution, when the LMOX concentration is low.
- 8) The air condenser is a glass tube 4 mm in internal diameter and 280 mm in length. One end is fitted with a glass joint attachable to a volumetric flask.
- 9) Unpublished results obtained in Shionogi Laboratories.
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