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Analytical Studies on 1-(2-*o*-Chlorobenzoyl-4-chlorophenyl)-5-glycylaminomethyl-3-dimethylaminocarbonyl-1*H*-1,2,4-triazole Hydrochloride Dihydrate. II.[1] A Fluorometric Method Applicable to Animal Feed

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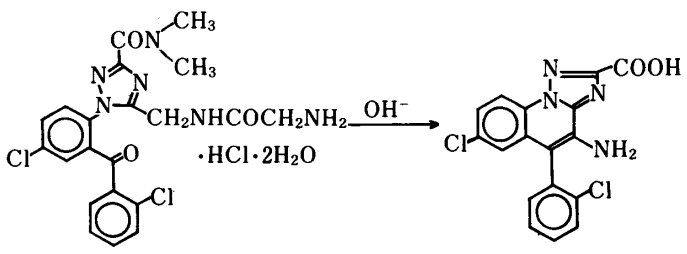
A fluorometric method for the determination of 1-(2-*o*-chlorobenzoyl-4-chlorophenyl)-5-glycylaminomethyl-3-dimethylaminocarbonyl-1*H*-1,2,4-triazole hydrochloride dihydrate (**1**) was established. This highly sensitive method was applied to the analysis of animal feed spiked with the drug. Compound **1** was easily transformed into the fluorophore 4-amino-7-chloro-5-(*o*-chlorophenyl)-[1,2,4]triazolo[1,5-*a*]quinoline-2-carboxylic acid (**2**) in alkaline solution. After the extraction of **1** from the feed, **1** was converted into **2**. The strong fluorescence of **2** allowed the establishment of a method with a quantitation limit of 5 mg/kg.

Compound **1** in feed samples spiked at 10—500 mg/kg was determined with a coefficient of variation of less than 10%. The concentration of **1**, its uniformity in the whole feed and its stability can be evaluated by this method.

Keywords——sleep inducer; 1-(2-*o*-chlorobenzoyl-4-chlorophenyl)-5-glycylaminomethyl-3-dimethylaminocarbonyl-1*H*-1,2,4-triazole hydrochloride dihydrate; 4-amino-7-chloro-5-(*o*-chlorophenyl)-[1,2,4]-triazolo[1,5-*a*]quinoline-2-carboxylic acid; fluorometry; dissociation; animal feed; toxicity test; Good Laboratory Practice Standard

1-(2-*o*-Chlorobenzoyl-4-chlorophenyl)-5-glycylaminomethyl-3-dimethylaminocarbonyl-1*H*-1,2,4-triazole hydrochloride dihydrate (**1**)[2] was developed as a sleep inducer in our laboratories. A highly sensitive method for analysis was required for toxicological studies on **1**. Compound **1** was mixed with animal feed, and this mix was given to animals in the toxicity tests. According to the Good Laboratory Practice Standard, it must be confirmed that the test system is properly exposed to the substance being tested. A proper concentration of the test substance in the spiked feed must be ensured and the uniformity must be evaluated to ensure that the mixing procedures are effective. Further, it must be shown that the test substance is stable during storage. Our previously established colorimetric method[1] could not be adapted for such tests because of its low sensitivity and poor selectivity for **1** in the feed. A fluorometric assay method was therefore developed for this purpose. Compound **1** was easily transformed



1 **2**

Chart 1

into the fluorophore 4-amino-7-chloro-5-(*o*-chlorophenyl)-[1,2,4]triazolo[1,5-*a*]quinoline-2-carboxylic acid (**2**) in alkaline solution. The fluorescent chemical species is the anion of **2**, formed by dissociation. The strong fluorescence allowed the establishment of a method with a quantitation limit of 5 mg/kg. Compound **1** was extracted from the spiked feed and converted into the fluorophore **2**. This paper describes the assay method for **1** in the spiked feed and its application to evaluate the content, the uniformity and the stability.

Experimental

Apparatus——A Hitachi MPF-2A recording spectrofluorometer was used for fluorescence measurement. A Shimadzu UV-190 spectrophotometer was used for absorbance measurement. An Iwaki KM shaker, type V-S, and a Sakuma centrifuge, model 90-4, were used for extraction and separation.

Reagents and Materials——The powdered feed and the spiked feed for rats and mice were purchased from Oriental Yeast Co., Ltd. (Charles River CRF-1). The feed was spiked with **1** to the required concentration in a mixer. The standard **1** and the methyl ester of the fluorophore (**2**) were supplied by our laboratories.

10% Tetra-*n*-butylammonium Hydroxide (TBAH) Solution: Dissolve 64 g of tetra-*n*-butylammonium bromide in 370 ml of water (CO_2 free), add 23 g of Ag_2O and shake with a shaker for 20 min. After allowing to stand overnight, filter the solution with a glass filter packed with asbestos at the bottom.

0.2 M Sodium *N*,*N*-Dimethylglycinate: Dissolve 14 g of *N*,*N*-dimethylglycine hydrochloride in 0.4 N NaOH to make 500 ml.

Saturated Sodium Tetraborate Solution: Dissolve 110 g of $Na_2B_4O_7$ in 1000 ml of boiling water. After allowing to stand overnight at room temperature, decant the supernatant.

Standard Solution of **1**: Accurately weigh about 7.4 mg of the standard **1** into a 50-ml volumetric flask, then dissolve it in and dilute to the mark with 0.1 N HNO_3. Pipet 7.5 ml of the solution into a 100-ml volumetric flask and dilute to the mark with 0.1 N HNO_3. Pipet 0.5, 1, 2, 3, 4 and 5 ml of the solution into six 10-ml volumetric flasks and dilute to the marks with 0.1 N HNO_3.

Ethanol was of super special grade purchased from Wako Pure Chemical Ind. Ltd. The other chemicals were of reagent grade.

Assay Procedure——Weigh 1 g of spiked feed into a 20-ml centrifuge tube, add 15 ml of 0.1 N HNO_3 and shake with a shaker for 30 min. After centrifugation at 2000 rpm for 20 min, transfer 7.5 ml of the supernatant fluid to a 50-ml centrifuge tube (dilute to half, one-fifth and one-tenth, when necessary). Add 10 ml of saturated $Na_2B_4O_7$ solution, 5 ml of 0.2 M sodium *N*,*N*-dimethylglycinate and 10 ml of $CHCl_3$. Shake the mixture for 30 min and centrifuge for 10 min. Remove the aqueous layer with an aspirator. Pipet 5 ml of the $CHCl_3$ layer into a 12-ml centrifuge tube. Add 2.5 ml of 0.1 N HNO_3, shake with a shaker for 20 min and cetrifuge for 10 min. Use the aqueous layer as a sample solution. Pipet 1 ml of sample solution into a 10-ml volumetric flask. Add 1 ml of 10% TBAH solution and mix well. Stopper tightly and heat at 100 °C for 1.5 h. After cooling in a water bath, dilute to the mark with EtOH. Measure the fluorescence intensity at 398 nm with excitation at 310 nm. Separately, weigh 1 g of control feed into a 20-ml centrifuge tube. Then proceed as directed for the sample feed. Correct the fluorescence intensity of the sample solution by subtracting that of the solution obtained from the control feed. Separately, pipet 1 ml of each standard solution into a 10-ml volumetric flask and add 1 ml of 10% TBAH solution. Then proceed as directed for the sample solution. Correct the intensities by subtracting that of the blank solution prepared with 1 ml of 0.1 N HNO_3 in the same manner as used for the preparation of the standard solution.

Calibration Curve: Plot the fluorescence intensities against the concentrations of the standard solution and calculate the regression equation. Correct the assay value using the factor which was determined from the recovery test (77.9%) according to the following equation.

$$\text{content of } \mathbf{1} \text{ (mg/kg)} = \frac{\text{found content (mg/kg)}}{0.779} \times \text{dilution factor}$$

Dilution Method and Dilution Factor (f): When the content of **1** is less than 50 mg/kg, use $f=10$. When the content of **1** is from 50 to 150 mg/kg, pipet 5 ml of the acid-extracted solution into a 10-ml volumetric flask and dilute to the mark with 0.1 N HNO_3. Pipet 7.5 ml of the solution and proceed as directed in the assay procedure. Then $f=20$.

In the case of the feed (150—400 mg/kg), dilute it to one-fifth and use $f=50$.

In the case of the feed (300—800 mg/kg), dilute it to one-tenth and use $f=100$.

Assay Conditions——Portions (1 ml) of aqueous solution of **1** (*ca.* 0.5 μg/ml) were tested under various conditions (reagent concentration, reaction time, temperature and solvent effect). To examine the dilution effect, 0.1 N HNO_3 solution (15 ml) containing **1** (*ca.* 0.7—53.3 μg/ml) was added to the control feed and shaken. Then after centrifugation, the supernatant was diluted, followed by determination of **1** as described above.

Determination of pK_a Values——Ionization Constant of **2** as Acid (pK_a^2): The value was measured from the solubility data obtained by Higuchi's method.[3] The concentration was assayed by UV-spectrophotometry (252 nm).

Ionization Constant of **2** and the Methylester of **2** (**3**) as Base (pK_a'): The pK_a values of **2** and **3** wre measured by UV-spectrophotometry in 30% (v/v) ethanolic solution, since the compounds were only slightly soluble in aqueous acid solution. The H_0 values in this medium (1.4 to −3) were determined by the ordinary method.[4] 4-Chloro-2-nitroaniline (pK_a: −1.03[4]) was used as an indicator. The absorbances of the indicator were measured at 426 nm in various concentrations of H_2SO_4 (0.1—9 M).

Isolation of the Fluorophore 2——A solution of **1** (1 g) in 1 N NaOH (100 ml) was heated at 100 °C for 3 h. The yellowish precipitate was filtered off and dissolved in EtOH. Then 0.1 N HCl was added. The resulting precipitate was washed with H_2O. Recrystallization from MeOH–$CHCl_3$ several times gave **2** as a pale yellowish white powder. Yield 0.3 g (41%), mp 231—233 °C. IR_{max}^{Nujol} cm^{-1}: 3377, 3487, 2460, 1735. ^{1}H-NMR (DMSO-d_6) δ: 6.02 (2H, br, NH_2), 6.88 (1H, d, J=2.5 Hz), 7.44—7.79 (5H, m), 8.42 (1H, d, J=9 Hz) (aromatic ring). *Anal.* Calcd for $C_{17}H_{10}Cl_2N_4O_2 \cdot 1.5H_2O$: C, 51.02; H, 3.27; Cl, 17.72; N, 14.00. Found: C, 50.94; H, 3.28; Cl, 17.76; N, 14.20.

Results and Discussion

Fluorescence Spectra

Compound **1** was converted into **2** quantitatively in an alkaline solution. The spectra of the solutions finally obtained in the assay procedure are shown in Fig. 1. The spectra coincided with those of authentic **2**. No materials interfering with the fluorescence measurement were extracted from the feed material. The fluorescence of **2** was stable, no spectral change being observed after 5 h.

Conditions for the Fluorescence Reaction

Effect of TBAH Concentration on Fluorescence Development——The alkali salt of **2** (sodium or potassium) was slightly soluble in H_2O or other solvents, and separated as a precipitate. The salt of the quaternary TBAH was soluble in EtOH. Various concentrations of TBAH (0.1—10%) were used for the reaction at 100 °C for 1.5 h. As shown in Fig. 2, a constant fluorescence intensity was obtained at concentrations of more than 1%.

Effects of Reaction Temperature and Time on Fluorescence Development——Compound **1** was allowed to react with 5% TBAH solution at various temperatures (25—110 °C) for 1.5 h. As shown in Fig. 3, a constant fluorescence intensity was obtained at over 95 °C. The reactions were carried out at 100 °C for various times (0.5—3 h). A constant fluorescence intensity was obtained after 1 h.

Effect of Solvent on Fluorescence Development——After the reaction, the resulting solution was diluted with various solvents. The fluorescence characteristics were affected by the solvents (Table I). Increasing polarity of the solvent resulted in a shift of the emission

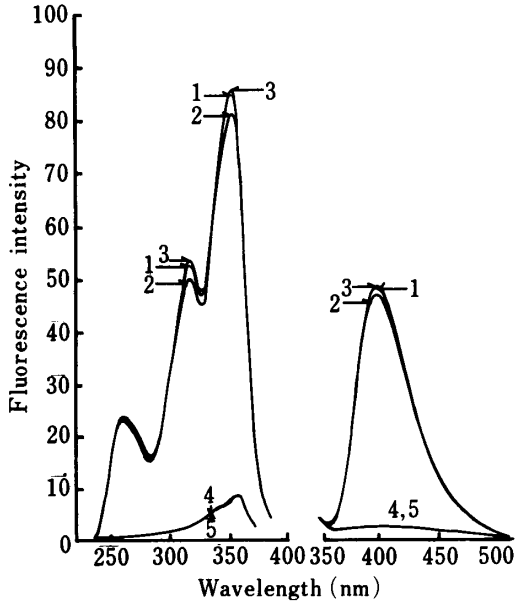


Fig. 1. Excitation and Emission Spectra of the Fluorophore

1) standard solution of **1**, 2) a 0.1 N HNO_3 solution of **1** was added to the control feed, 3) spiked feed, 4) control feed, 5) 0.1 N HNO_3 solution (blank).

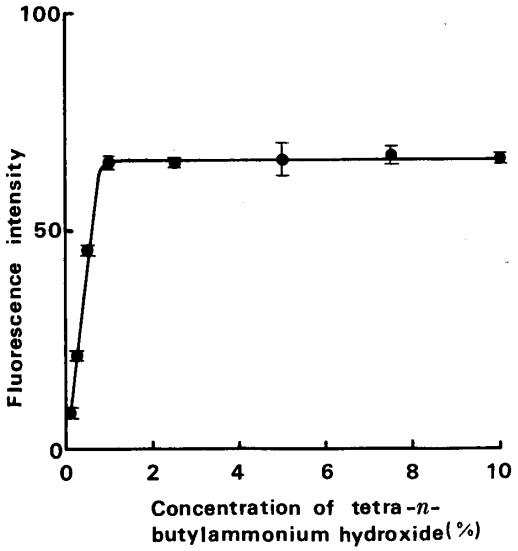


Fig. 2. Effect of TBAH Concentration on Fluorescence Development

●, each point represents the mean ± standard deviation for three replicates.

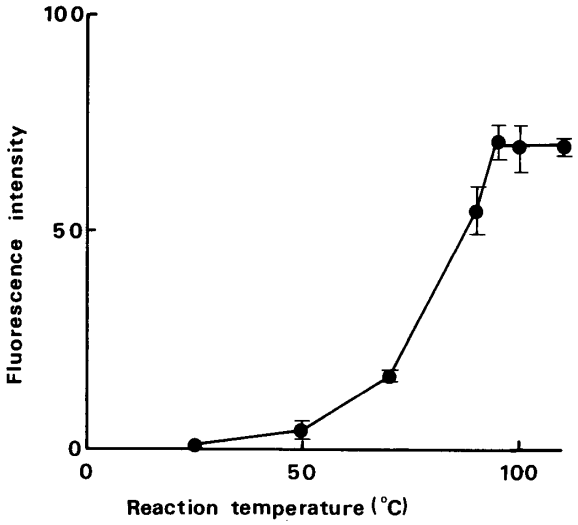


Fig. 3. Effect of Reaction Temperature on Fluorescence Development

●, each point represents the mean ± standard deviation for three replicates.

TABLE I. Fluorescence Characteristics of the Products in Various Solvents

Solvent	Excitation (nm)	Emission (nm)	Fluorescence[a] intensity
H_2O	310	422	0.4
MeOH	310	405	72.9
EtOH	315	398	100.0
MeCN	313	398	91.4
$(Me)_2CO$	342	392	85.7

a) Fluorescence intensities are relative values (100% in EtOH).

spectrum to a longer wavelength. The most intense fluorescence was observed in EtOH.

Fluorescent Chemical Species of 2

The fluorophore (**2**) dissociates in solution to form an anion (**2c**) and becomes protonated to form a cation (**2d**).[5] The pK_a value due to the dissociation as an acid was determined to be 2.86. Fluorescence of **2** was observed in alkaline EtOH under the assay conditions. It seems likely that the fluorescent chemical species is not **2a**, **2b** or **2d**, but the anion **2c** in this medium. The methyl ester of **2** (**3**) did not fluoresce in EtOH or in ethanolic solution containing alkali. This result indicates the nonfluorescent character of **2a**, supporting the above conclusion. Table II shows the effects of solvent, acid and alkali on the fluorescence of **2**. Intense fluorescence was observed in neutral or alkaline solution, but not in an acid solution. In $CHCl_3$ solution, a weak fluorescence was observed. When EtOH was added to the solution, the fluorescence appeared and increased with the amount of EtOH. These results indicate that the fluorescent chemical species is **2c**. Since **2a** is a strong acid, fluorescence developed by dissociation not only in alkaline EtOH, but also in EtOH alone. Weak fluorescence in $CHCl_3$ was due to the depression of the dissociation (**2c**→**2a**) in a low dielectric constant solvent. The dissociation of **2a** to the anion **2c** on addition of EtOH to the $CHCl_3$ solution gave rise to an increasing fluorescence intensity. The presence of a betaine form **2b** was ruled out by the pK_a^1 value. The value in 30% EtOH–H_2O solution was found to be -1.08, which is in good

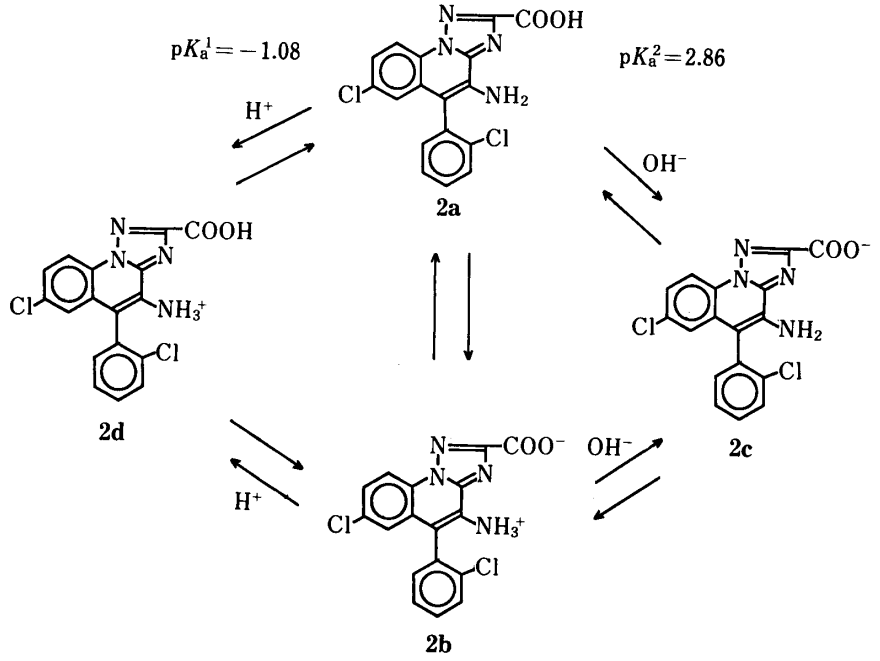


Chart 2

TABLE II. Fluorescence Intensities (%) of **2** and **3**

Solvent	Compound	
	2	**3**
EtOH	84.8	0.6
H_2O–EtOH	70.0	—
10% (v/v) EtOH–$CHCl_3$	31.0	—
50% (v/v) EtOH–$CHCl_3$	48.2	—
0.1 N HCl–EtOH	N.D.	—
0.02 mM Et_3N–EtOH	85.7	0.1
$CHCl_3$	3.9	—

N.D.: not detected.

agreement with that of **3** in the same solvent (−1.17). This result indicates that the fluorophore existed as **2a**, not **2b**.[5]

Conditions for Pretreatment

Extraction Media for the Spiked Feed——Various aqueous solutions were added to the spiked feed (16.9 mg/kg), followed by the extraction of **1** with a shaker. The extract was assayed according to the assay procedure and the recoveries were measured. The recoveries from H_2O or weakly acidic solution (pH 3.6) were less than 44%. Extractions with strong acids gave recoveries of 59—78% of **1**. When HCl solution was employed and shaken with $CHCl_3$ in the following procedure, the $CHCl_3$ extract solidified because of emulsion formation. By repeated centrifugations, the $CHCl_3$ layer was gradually separated. The recovery was 78.3%. Other strong acids did not form emulsions and more than 60% of **1** was recovered. The HNO_3 solution gave a higher recovery (75.5%) than the H_2SO_4 (64.2%) or $HClO_4$ solution (59.4%). Thus, 0.1 N HNO_3 solution was selected, and the feed was shaken with it for 30 min. A constant recovery was obtained over this time. The effect of amount of the acid on the recovery was next examined. When 15 ml of the acid was used, 76% of **1** was

recovered. The recovery was not improved by the use of more acid. However, when the feed after extraction with 15 ml of HNO_3 was reextracted with 10 ml of 0.1 N HNO_3, some residual **1** was recovered and the total yield reached 90%. The recovery (percent) is expressed as the overall value estimated through all the assay procedures before the fluorescence reaction. Since the extraction yield with $CHCl_3$ in the following procedure was determined to be 88%, as described later, this 90% recovery by double extractions with 0.1 N HNO_3 represents almost 100% recovery of **1** from the feed. A single extraction with 15 ml of the acid was selected for the standard procedure in order to establish a simple and rapid method.

Extraction of Free 1——Compound **1** in 0.1 N HNO_3 was alkalized with buffers and extracted with $CHCl_3$. Since **1** was unstable in strong alkaline solution, buffers with lower pH's than 10 were tested. A sufficient yield (87.5%) was obtained with pH 9.3 buffer (0.2 M sodium *N*,*N*-dimethylglycinate–saturated $Na_2B_4O_7$ solution (1 : 2)). A constant recovery was obtained by shaking for more than 30 min.

Back-Extraction of 1 as a Salt——The free **1** in $CHCl_3$ was transferred into 0.1 N HNO_3. Compound **1** was recovered quantitatively by shaking the solution (2.5 ml) for more than 20 min.

Dilution Effect——Samples contained **1** in the range of 15 to 700 mg/kg. When higher concentrations of samples than 50 mg/kg are analyzed, it is necessary to dilute the extracted HNO_3 solution in an appropriate ratio. Samples diluted 2-, 5- and 10-fold with the same HNO_3 solution were assayed according to the standard assay procedure. The recoveries obtained for the diluted solutions were the same as that for the mother extract without dilution.

Recovery Test

The recovery was tested for samples in the range of 100 to 800 mg/kg, which were prepared by adding accurately weighed quantities of **1** to the feed. A straight line relationship was obtained between the amounts of **1** found by this analytical procedure and the added amounts; the line passed through the origin ($y=0.779x$, $s=12.8$). The recovery was estimated from the slope of the line to be 77.9%, and this value was used as the correction factor for the assay value. This factor was reproducible.

Analytical Results on the Spiked Feed

The spiked feed was prepared on a large scale. Two feed packages containing 10 kg of feed were chosen for analysis. One bag containing 2.5 kg of feed was taken from each package and opened. About 10 g of feed was taken from each section (the package was divided crosswise in 4 parts). Table III shows the results of triplicate assays of the feed sampled from

TABLE III. Content of **1** in Spiked Feed

Package	n	Content (mg/kg) Section 1	2	3	4
A	1	11.9	14.5	13.4	13.4
	2	13.5	14.8	13.9	15.2
	3	13.3	14.5	10.5	12.4
B	1	11.8	13.4	13.8	13.6
	2	15.6	12.8	16.1	13.4
	3	12.7	12.2	14.3	12.8

Mean ± S.D.: A, 13.4 ± 1.3; B, 13.5 ± 1.3.

the sections in two different packages. The average contents were similar in the two packages, and the coefficients of variation showed no significant difference. These results show that the drug was well distributed throughout the feed. In the routine work, samples (20, 100 and 500 mg/kg) were taken from one bag per lot and assayed in the same manner.

The feed was stored at 4 °C for 3 or 4 months, and the assays were repeated. No change was observed in the assay values. It was concluded that **1** is stable in the feed.

Precision and Quantitation Limit

The detection limit of the method was 5 mg/kg of **1**. Compound **1** in the animal feed was determined with a coefficient of variation of 3—10%. The spiked feed samples were prepared repeatedly during the toxicity tests. The between-run coefficient of variation did not exceed 10%.

This method confirmed that the content of the drug was as stated, and also confirmed the uniformity and the stability of the drug in the feed.

References and Notes

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4) R. H. Boyd, "Solute Solvent Interactions," ed. by J. F. Coetzee and C. D. Ritchie, Marcel Dekker Inc., New York, 1969, pp. 158—167.
5) The protonation at the NH_2 group was suggested from the IR spectrum of the perchlorate of **2** (IR ν_{max}^{Nujol} cm^{-1}: 3440, 3360 (NH_3^+)). The perchlorate was prepared by adding an equimolar amount of 60% $HClO_4$ to a solution of **2** in $CHCl_3$–EtOH–MeCN, mp 210—217 °C. The elemental analysis data coincided with the calculated values for the monoperchlorate.