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A Chromogen Produced in the Reaction of Glycine Derivatives with Acetic Anhydride and Pyridine

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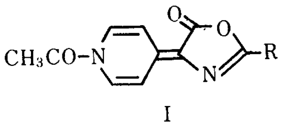
The yellow chromogen produced in the assay of glutathione or glycocholic acid by their reactions with acetic anhydride and pyridine was found to be a conjugated compound containing pyridylidene and 2-oxazoline rings. The structure was confirmed by hydrolysis of the chromogen to 4-aminomethylpyridine. The same chromogen was formed with benzoylpeptides having C-terminal glycine.

Keywords——chromogen; glutathione; glycocholic acid; glycine; spectrophotometry; 2-oxazoline; pyridylidene

The formation of a yellow chromogen in the reaction of benzoylglycine (hippuric acid) with acetic anhydride and pyridine in the presence of 4-(dimethylamino)benzaldehyde provides a sensitive and simple assay for benzoylglycine in urine and liver homogenate.[1] The quantitative conversion of glycine into benzoylglycine was then applied to the determination of glycine in amino acid mixtures and biological samples.[2] In the course of these studies, we found that benzoyl derivatives of di- and tripeptides having glycine at their C-termini showed a similar color reaction, which was then applied to the assay of glutathione in biological samples.[3] The color reaction was also utilized for the assay of glycine-conjugated bile acids in bile or duodenal aspirate.[4]

In the preceding paper,[5] the structure of a yellow chromogen formed in the assay system of benzoylglycine was determined to be 4-(1-acetyl-4(1*H*)-pyridylidene)-2-phenyl-2-oxazolin-5-one (APPO, I with R = phenyl). This compound was quantitatively hydrolyzed to 4-aminomethylpyridine (4-AMP) with hydrochloric acid. According to the mechanism of formation of APPO proposed by us,[5] chromogens derived from benzoylpeptides and from glycine-conjugated bile acids should be APPO analogues with various substituents at the 2-position of the 2-oxazoline ring of I.



I

In this work, the structures of chromogens produced from several glycine derivatives were studied in connection with the structural elucidation of a chromogen produced by benzoylglutathione and glycine-conjugated bile acid, and the products were shown to be I. The structure I was confirmed by the detection of 4-AMP formed by hydrolyzing I, instead of by elucidation of the gross structure as the isolation of I from the reaction mixture was impossible. Furthermore I in the reaction mixture was determined by the quantitative analysis of 4-AMP formed by the quantitative hydrolysis of I. These studies revealed that assay systems containing benzoylpeptides with glycine at their C-termini and glycocholic acid become yellow due to the production of I. Assay of benzoylpeptides without glycine at the C-termini showed no production of 4-AMP, indicating that I was not formed.

Experimental

Chemicals and Instruments——Glutathione, reduced and oxidized form, was supplied by Yamanouchi Pharmaceutical Co. (Tokyo). Other peptides were purchased from Sigma Chemical Co. (St. Louis). 4-AMP was obtained from Tokyo Kasei Kogyo Co. (Tokyo). Glycocholic acid was synthesized by Prof. S. Hayakawa in this university according to the method of Norman.[6] All other reagents were of analytical grade, and were purchased from Wako Pure Chemicals Ind. (Osaka).

Mass spectra (MS) were obtained in a Shimadzu LKB Type 9000 spectrometer at an ionizing energy of 70 eV. A Shimadzu UV-180 spectrophotometer was used for measurements of absorbance. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-4CM with a flame ionization detector.

Preparation of Benzoyl Compounds——*o*-, *m*-, and *p*-Substituted benzoylglycines were prepared as follows. Ring-substituted benzoyl chloride (20 mmol) was added to glycine (1.0 g, 13.3 mmol) in 10% sodium carbonate (50 ml), and the mixture was stirred vigorously at 5 °C for 2 h. After filtration, the filtrate was acidified with 6 N HCl, and the resulting precipitate was collected by filtration. The precipitate was dried over silica gel, washed with *n*-hexane–benzene (1 : 1, v/v), and then recrystallized from ethyl acetate or methanol.

Benzoylpeptides were prepared by benzoylation of peptides by means of a Schotten–Baumann type of reaction.[7] After the reaction, the mixture was neutralized with diluted hydrochloric acid and applied to an acidic ion-exchanger column. The product was eluted with 50% (v/v) methanol from the column, and the eluate was evaporated under reduced pressure. The resulting residue was washed with ether and recrystallized from water.

Thin-Layer Chromatography (TLC) of Benzoylpeptides——The chromatography was performed on a glass plate precoated with Silica gel 60_{254} (Merck 5729). The developing solvent was a solution of chloroform–methanol–acetic acid (13 : 5 : 1, v/v/v). Peptides were detected by spraying a solution of 3% ninhydrin in acetone.

Color Reaction and Determination of 4-AMP——The reaction was carried out at a higher concentration than that used for the standard determination of glutathione and glycocholic acid.[3,4] Benzoylpeptide or glycocholic acid (75 μmol) was added to a solution of acetic anhydride (1.0 ml) and pyridine (2.0 ml), and the mixture was incubated with vigorous stirring at 40 °C for 1 h for peptides or at 60 °C for 2 h for glycocholic acid. The reaction solution was evaporated under reduced pressure, and the resulting residue was dissolved in 2 N HCl (5.0 ml). After incubation at 90 °C for 12 h, the solution was again evaporated to dryness under reduced pressure. The resulting residue was dissolved in a solution (0.7 ml) of 60% (v/v) trifluoroacetic anhydride in dichloromethane and allowed to stand at room temperature for 30 min. An aliquot (5—10 μl) was diluted with dichloromethane, and injected into the gas chromatograph. The peak height of the derivatized 4-AMP was measured, and the concentration of 4-AMP was calculated with the use of a calibration curve which was determined for each analysis.

Gas-Chromatographic Conditions——A glass column of 1.5 m × 3 mm i.d. was packed with 5% Thermon 3000 on Chromosorb W, 80—100 mesh. Temperature were maintained at 190 °C for the column oven and at 230 °C for the injector and detector ports. The carrier gas was nitrogen at a flow rate of 40 ml/min. The sensitivity and attenuation were set at 10^2 and 64, respectively. The retention time of the derivatized 4-AMP was 9 min.

Results

Purity of Benzoylpeptide

Benzoylpeptides were prepared by benzoylation of peptides under slightly alkaline conditions; Therefore benzoylated compounds of fragments hydrolyzed during the reaction are possible contaminants. As such impurities contribute to the positive color reaction in these experiments, the purity of each preparation was checked by TLC. For example, benzoylleucylglycylglycine gave a single spot with an *Rf*-value of 0.62, which is different from the *Rf*- values of benzoyl compounds of glycine, and glycylglycine.

Benzoylated glutathione gave two spots with *Rf*-values of 0.53 (major) and 0.89 (minor), which are different from the *Rf*-values of the benzoylated products obtained from the amino acids composing glutathione. Therefore the above product seems to be a mixture of mono- and di-benzoylglutathione reacted either at the amino or sulfhydryl group or at both. Repeated recrystallization of the mixture did not lead to a single spot. Each of the benzoyl compounds except benzoylglutathione provided a single spot on TLC and gave satisfactory elemental analysis results for C, H, and N.

Formation of 4-AMP from Chromogen

Hydrolysis of I to 4-AMP was examined by incubation with 2 N HCl at 90 °C, and a typical time course is shown in Fig. 1. Experiments revealed that the maximal formation of 4-AMP was attained upon incubation for 12 h with all of the compounds. Therefore a hydrolysis time of 12 h was used throughout these experiments.

4-AMP was analyzed by GLC after derivatization to the trifluoroacetyl derivative. Silicone Thermon 3000, one of several column packings tested, provided a good separation with a symmetrical peak of the derivative. In contrast, the use of Silicone OV-17 did not yield accurate determinations because of tailing of the peak.

A product with a retention time identical with that of authentic trifluoroacetylated 4-AMP was present in the yellow reaction mixtures produced by glycocholic acid and by benzoylpeptide with C-terminal glycine. The parent mass number (204 of m/e) of tri-

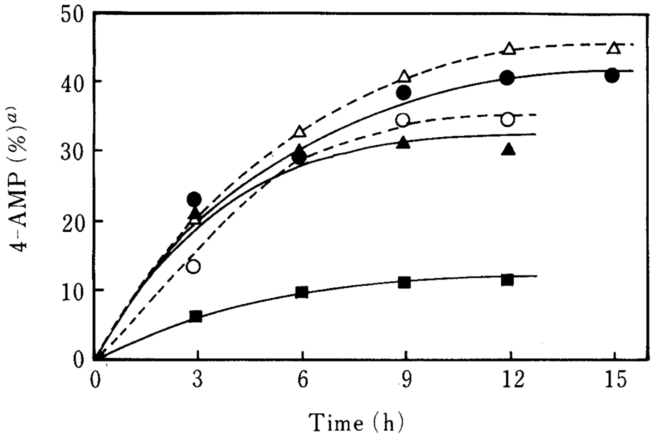


Fig. 1. Typical Hydrolytic Profiles of Several Glycine Derivatives

Each glycine derivative was dissolved in a solution of acetic anhydride and pyridine, and the mixture was incubated. The reaction mixture was evaporated, the residue was hydrolyzed, and 4-AMP liberated was determined. Experimental details are given in the text.

a) Yield based on glycine derivatives.

—■—, glycocholic acid; —▲—, benzoylglycylglycine; —●—, benzoylglycine; —△—, *p*-chlorobenzoylglycine; —○—, benzoylglycylglycylglycine.

TABLE I. Hydrolysis of Chromogens Produced from Glycine Derivatives

Compound	Detection of 204 of m/e	Yield of 4-AMP (%$^{a)}$)
Benzoylpeptide		
Glycylglycine	+	28
Alanylglycine	+	43
Leucylglycine	+	23
Methionylglycine	+	26
Glycylglycylglycine	+	35
Leucylglycylglycine	+	17
Glutathione	+	22
Glycylalanine	−	0
Glycylleucine	−	0
Glycylphenylalanine	−	0
Glycylasparagine	−	0
Glycocholic acid	+	11
Ring-substituted benzoylglycine and benzoylpeptide		
o-Methylbenzoylglycine	−	0
m-Methylbenzoylglycine	+	31
p-Methoxybenzoylglycine	+	12
p-Chlorobenzoylglycine	+	37
p-Nitrobenzoylglycylglycine	+	69

a) Based on glycine derivatives.

fluoroacetylated 4-AMP was observed on GLC-mass analysis (Table I). The yield of 4-AMP was 22—43% for benzoylpeptides and 11% for glycocholic acid. On the other hand, no benzoylpeptide having an amino acid other than glycine at its C-terminus showed either coloration in the reaction or production of 4-AMP upon hydrolysis. *o*-Methylbenzoylglycine produced no 4-AMP, but all other ring-substituted benzoylglycines that were tested produced 4-AMP in yields of 12—37%. Among the compounds tested (Table I), *p*-nitrobenzoylglycylglycine provided the highest yield (69%).

Determination of I

A standard curve, prepared by using a solution of authentic 4-AMP dissolved in 2 N HCl, gave a straight line passing through the origin. The conversion yield of 4-AMP to the derivatized compound was about 70% at each concentration (data not shown). When 4-AMP formed by the hydrolysis of pure crystals of APPO was determined as described above, the same conversion yield based on APPO was obtained. This indicates that APPO was quantitatively hydrolyzed to 4-AMP under the conditions used here and that losses of 4-AMP occur during the two evaporations and the trifluoroacetylation (see Experimental). This finding indicates that the above standard curves can be used for the determination of I.

Discussion

The colorimetric determination of benzoylated glutathione and of glycine-conjugated bile acids is based on the formation of a yellow chromogen produced by the incubation of the compounds with acetic anhydride and pyridine. Attempts to isolate and crystallize these chromogens from the colored solutions failed. It was therefore not possible to determine their structures directly.

These studies revealed that the chromogen, which produced 4-AMP on hydrolysis with hydrochloric acid, is a conjugated compound that includes a pyridylidene ring and a 2-oxazoline ring as seen in I. Benzoylpeptides other than benzoylglutathione with glycine at their C-termini also produced I, but compounds having an amino acid other than glycine at the same position did not. These findings can be accounted for by an APPO formation mechanism proposed by us.[5] *N*-Acetylpyridinium cation (II) formed by the reaction of pyridine with acetic anhydride attacks the 2-oxazoline (III), a dehydrated ring compound formed by the reaction of glycocholic acid or benzoylpeptide with acetic anhydride, nucleophilically to produce a condensation product (IV). Then oxygen dissolved in the reaction mixture oxidizes IV to I. The oxidation of IV is essential to the coloration, which is due to the formation of a conjugated system. Benzoylpeptides having amino acids other than glycine at the C-terminus may produce IV which has the side chain of the amino acid at the 4-position of the oxazoline ring. However, the oxidative conversion to I is then impossible because of the presence of the chain.

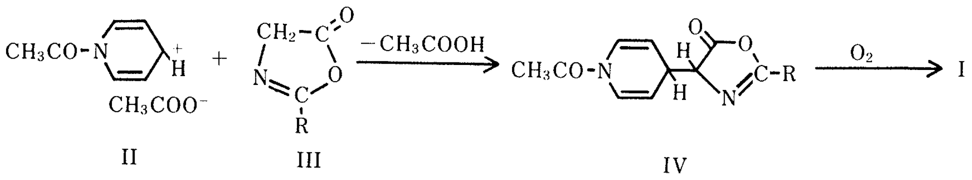


Chart 1

o-Methylbenzoylglycine failed to produce either coloration or detectable 4-AMP, although other ring-substituted benzoylglycines with a methyl, methoxy or chloro substituent at the *m*- or *p*-position were positive in both. These findings indicate that a methyl group at the *o*-position blocks the formation of I owing to steric hindrance with the oxazoline ring in the structure of I (R: *o*-tolyl group). On the other hand, a methyl group at the 3-position of the pyridylidene ring did not hinder the formation of I. A derivative of I having the group at such a position was prepared by us by the reaction of benzoylglycine with 3-methylpyridine and acetic anhydride.[8)]

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