S-Alkylcysteinase: Enzymatic Cleavage of S-Methyl-L-cysteine and Its Sulfoxide*

JUNICHI NOMURA,† YASUTOMI NISHIZUKA, AND OSAMU HAYAISHI

From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan

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S-Methylcysteine has been isolated from the nonprotein nitrogen fractions of cabbage, turnips, and several other crucifers, and the structure of the amino acid was established by chemical studies and by comparison with a synthetic material (1–4). Subsequently, this compound has been found in garlic and onion (5), kidney bean seeds (6), Neurospora crassa (7), and the hemolymph of Prodenia eridania (8). It has been isolated also as a peptide with glutamate, γ-glutamyl-S-methylcysteine, from various leguminous plants (9, 10) and garlic (11). Although, in contrast to methionine, S-methylcysteine does not undergo oxidation to the corresponding sulfoxide at room temperature (3), the latter compound was isolated from several plants (1–5).

Wolff, Black, and Downey (12) purified an enzyme from yeast which catalyzed the synthesis of S-methylcysteine from L-serine and methyl mercaptan, but this reaction was shown to be essentially irreversible. The enzymatic degradation of this amino acid has been reported to be catalyzed by another enzyme system or systems occurring in a microorganism (13, 14) and in the seeds of Albizia lebophana (15). Little information is available, however, concerning the mechanism of this reaction(s) or the properties of the enzyme(s) concerned. S-Methylcysteine sulfoxide has been shown to be converted to pyruvic acid, ammonia, and methyl methanethiosulfinate by alliinase purified from garlic (16) and Bacillus subtilis (17).

The present paper describes the partial purification and properties of a new enzyme obtained from cells of a strain of Pseudomonas cruciviae grown with S-methyl-L-cysteine as the major carbon and nitrogen source. This enzyme catalyzes the stoichiometric conversion of S-methyl-L-cysteine to methyl mercaptan, pyruvic acid, and ammonia (Reaction 1), and requires pyridoxal phosphate as a cofactor.

\[
\begin{align*}
\text{CH}_3\text{SCH}_2\text{NH}_2\text{COOH} + \text{H}_2\text{O} & \rightarrow \\
\text{CH}_3\text{SH} + \text{CH}_2\text{COOH} + \text{NH}_3 & \quad (1)
\end{align*}
\]

\[
\begin{align*}
2\text{CH}_3\text{SCH}_2\text{NH}_2\text{COOH} + \text{H}_2\text{O} & \rightarrow \\
\text{CH}_3\text{SSCH}_2\text{H} + 2\text{CH}_2\text{COOH} + 2\text{NH}_3 & \quad (2)
\end{align*}
\]

The enzyme also catalyzes a reaction of S-methyl-L-cysteine sulfoxide (Reaction 2). Analogous reactions occur with other compounds, including S-ethyl-L-cysteine and S-allyl-L-cysteine and their sulfoxides. The enzyme will be referred to as S-alkylcysteinase.

**EXPERIMENTAL PROCEDURE**

**Materials**—S-Methyl-L-cysteine was prepared by reduction of L-cysteine in liquid ammonia with sodium, followed by methylation with methyl iodide (18). The crystallized product gave a rotation of \([\alpha]_D^2 -30.0^\circ (c, 2.5 \text{ in water})\) and decomposed at 248° after becoming brown at 240° (18). S-Methyl-L-cysteine sulfoxide was prepared from S-methyl-L-cysteine by oxidation with hydrogen peroxide according to the method described by Lepp and Dunn (19) for the preparation of methionine sulfoxide, except that the oxidation was conducted at 50–60° for 2 hours. The product of this reaction had no optical rotation. The diastereoisomers were separated by fractional crystallization from ethanol and water (3). The pure (+)-S-methyl-L-cysteine sulfoxide obtained gave a specific rotation of \([\alpha]_D^2 +129^\circ (c, 2.5 \text{ in water})\), and decomposed at 164° (2, 3). S-Ethyl-L-cysteine sulfoxide was obtained from the California Corporation for Biochemical Research. S-Allyl-L-cysteine and its sulfoxide and S-ethyl-L-cysteine sulfoxide were kindly provided by T. Sasaki. S-n-Propyl-L-cysteine, S-n- and S-isobutyl-L-cysteine, and S-n- and S-isomethyl-L-cysteine were kindly donated by Dr. Y. Ishikawa. S-(2-Carboxyisobutyryl)-L-cysteine and other S-(carboxyalkyl) cysteines were kindly provided by Dr. S. Mizukara. Dimethyl disulfide was synthesized from methyl mercaptan by the method of McAllan et al. (20). Methyl methanethiosulfinate was prepared from dimethyl disulfide by the method of Small, Bailey, and Cavallito (21), except that hydrogen peroxide and acetic acid were employed as oxidant and solvent, respectively. S-(Thiomethyl)cysteine was synthesized by the method of Cavallito, Buck, and Suter (22) for the preparation of S-(thioallyl) cysteine. Other amino acids, pyruvic acid, DPNH, and derivatives of pyridoxine were obtained from Sigma Chemical Company and California Corporation for Biochemical Research; protamine sulfate, from Eli Lilly and Company; and methyl mercaptan, from Tokyo Chemical Company. Sephadex G-50 and DEAE-cellulose were products of Pharmacia, Uppsala, Sweden, and Serva Entwicklungs Labor, Heidelberg, respectively. Calcium phosphate gel was prepared according to the method described by Keilin and Hartree (23). Crystalline heart muscle lactic dehydrogenase was obtained from Sigma Chemical Company.

**Growth of Bacterium**—The strain of cells used in the present study was isolated from soil by an aerobic enrichment (24), with...
S-methyl-L-cysteine as the sole carbon and nitrogen source. The organism, which was identified as a strain of Pseudomonas crucivae, was maintained on an agar slant containing 0.5 g of S-methyl-L-cysteine, 1.5 g of KH₂PO₄, 0.5 g of KH₂PO₄, 0.2 g of MgCl₂·6H₂O, 5 g of NH₄Cl, 1 g of polyethylene (Takeda), 1 g of Difco yeast extract, and 40 g of Difco agar per liter. The cells were then harvested with the aid of a Shaples centrifuge and washed three times with 0.9% NaCl solution. The yield of wet packed cells was approximately 2 g per liter of the medium.

**Enzyme Activity**—The enzyme activity was routinely assayed spectrophotometrically by measuring the formation of pyruvic acid in the coupled enzyme reactions with lactate dehydrogenase and DPNH. The reaction mixture contained 0.1 ml of DPNH buffer, pH 8.8, 0.16 μmole of DPNH, 18 pg of crystalline heart muscle lactic dehydrogenase, and the enzyme preparation in a total volume of 1.0 ml.

Experiments with Intact Cells—Oxygen consumption by a suspension of the resting cells grown on S-methyl-L-cysteine was determined manometrically with S-methyl-L-cysteine or S-methyl-L-cysteine sulfoxide as a substrate. As shown in Fig. 1, the cells oxidized both substrates without a lag period, and approximately 1.7 moles of oxygen were consumed per mole of the substrate.

**Results**

The isolated microorganism was identified as Pseudomonas crucivae on the basis of the following properties. The microorganism was a gram-negative rod, 1.0 × 1.0 to 3.0 μ, occurring singly and in pairs, and motile with one to five polar flagella. The agar colonies were grayish white and smooth; no soluble pigment was produced. The organism grew in aerobic media, did not liquefy gelatin, did not hydrolyze starch, and did not produce acids in carbohydrate media. Nitrates were not produced from nitrites.

The enzyme attacked phenol and n-cresol. We are indebted to Dr. K. Tabei and Dr. T. Siomi for the identification of the organism.
To this eluate (40 ml), 9.0 g of ammonium sulfate were added. After approximately 20 minutes, the resulting precipitate was removed by centrifugation, and 5.2 g of ammonium sulfate were added to the supernatant solution. After 20 minutes, the resulting precipitate was collected by centrifugation and dissolved in 12 ml of 0.05 M Tris-HCl buffer, pH 8.8. The solution was again passed through a Sephadex column.

The solution (12 ml) was adsorbed on a DEAE-cellulose column, 1 cm² x 2 cm, which had been equilibrated with 0.02 M phosphate buffer, pH 7.0. After the column was washed with 30 ml of 0.05 M K₂HPO₄ solution, the enzyme was eluted with 30 ml of 0.25 M K₂HPO₄ solution.

The enzyme was purified approximately 25- to 30-fold with an overall yield of 15%, as shown in Table I. The ratio of the specific activity for S-methyl-L-cysteine to that for S-methyl-L-cysteine sulfoxide appeared to be constant (about 3) during the purification procedure. The enzyme is relatively unstable; almost all of the activity was lost within 2 weeks even when it was stored at -20°C.

Product of Reaction—The reaction mixture (1.0 ml), containing 20 μmoles of S-methyl-L-cysteine, 40 μmoles of Tris-HCl buffer, pH 8.8, and 0.1 unit of the enzyme (Step 5), was incubated for 1 hour at 37°C. The reaction was stopped by the addition of 0.2 ml of cold 10% HClO₄, and the precipitate was removed by centrifugation. The supernatant solution was neutralized with 4 N KOH, and KClO₄ was removed by centrifugation. The supernatant solution was treated with 1 ml of a saturated 2,4-dinitrophenylhydrazine solution (in 2 N HCl), and the resulting hydrazone was extracted with 5 ml of ethyl acetate. The ethyl acetate layer was removed and extracted with 1 ml of 0.2 M Na₂CO₃. The aqueous layer was then acidified with 4 N HCl. The hydrazine derivative was extracted again with 0.4 ml of ethyl acetate. When an aliquot of the organic layer was subjected to paper chromatography, a new phenylhydrazone spot was detected which coincided well with that of an authentic sample of S-(thiomethyl)cysteine. The Rp value of the phenylhydrazone of pyruvic acid was as follows: with n-butanol-ethanol-0.1 N NaHCO₃ (10:3:10), 0.61; with isopropanol-isooctyl alcohol-pyridine-water (20:4:1:5), 0.58. The phenylhydrazone was also identified as that of pyruvic acid by high voltage paper electrophoresis. The mobility of the hydrazone of pyruvic acid was 8.0 cm to the anode under the conditions described above. Pyruvic acid was also obtained from the incubation mixture that contained S-methyl-L-cysteine sulfoxide in place of S-methyl-L-cysteine, and was identified in the same manner.

Methyl mercaptan was identified by the method of Sogal and Starkey (25). The reaction mixture (5.0 ml), containing 100 μmoles of S-methyl-L-cysteine, 200 μmoles of Tris-HCl buffer, pH 8.8, and 0.5 unit of enzyme (Step 5), was incubated in an air-tight, 2-necked tube for 2 hours at 37°C. After the reaction was stopped by heating for 3 minutes in a boiling water bath, one neck was provided with a capillary and the other was connected by a cock to three trapping chambers, in series, each of which contained 20 ml of 4% mercuric cyanide. The cock was then opened, and CO₂-free air was swept through the capillary into the incubation mixture. The methyl mercaptan gas produced was trapped in the three chambers for 30 minutes. The precipitate formed was collected by filtration and was recrystallized twice from ethyl acetate. The crystals were identified as mercuric dithiomethoxide, (CH₃S)₂Hg, by melting point and by mixed melting point, 174-177°C, which was identical with that of an authentic sample.

When S-methyl-L-cysteine sulfoxide was employed as a substrate, no methyl mercaptan was produced in the reaction mixture, but methyl methanethiol sulfinate was formed. The latter compound was identified as follows. A reaction mixture (1.0 ml) containing 30 μmoles of S-methyl-L-cysteine sulfoxide, 40 μmoles of Tris-HCl buffer, pH 8.8, and 0.4 unit of the enzyme (Step 3) was incubated for 1 hour at 37°C. The reaction was stopped by the addition of 0.2 ml of cold 4% HClO₄. After the precipitate was removed by centrifugation, the supernatant solution was neutralized with 4 N KOH. When an aliquot of the solution was subjected to chromatography on paper, a spot that reacted with an alkaline nitroprusside solution was detected (31, 32). The Rf values of the spot were indistinguishable from those of an authentic sample of methyl methanethiol sulfinate; with methanol-benzene (3:2), 0.55; with methanol-petroleum ether (2:1), 0.77. Further evidence for the identity of the compound was provided by its reactivity with cysteine to produce S-(thiomethyl)cysteine (22). To another 0.5-ml aliquot of the supernatant solution, 0.1 ml of 0.1 M L-cysteine was added, and the mixture was adjusted to pH 6.0. When the mixture was allowed to stand for 30 minutes at 60°C, a new ninhydrin-reactive material was produced which was identified as S-(thiomethyl)cysteine by paper chromatography. The Rf value of the material in n-butanol-acetic acid-water (4:1:5) was 0.48, and it was identical with an authentic sample of S-(thiomethyl)cysteine.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Summary of enzyme purification</th>
</tr>
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<tr>
<td>Fraction</td>
<td>Volume</td>
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<tr>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>1. Crude extract...</td>
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</tr>
<tr>
<td>2. Protamine sulfate</td>
<td>40</td>
</tr>
<tr>
<td>3. First ammonium sulfate</td>
<td>20</td>
</tr>
<tr>
<td>4. Calcium phosphate gel...</td>
<td>16</td>
</tr>
<tr>
<td>5. Second ammonium sulfate...</td>
<td>12</td>
</tr>
<tr>
<td>6. DEAE-cellulose...</td>
<td>30</td>
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</tbody>
</table>

April 1963, J. Nomura, Y. Nishizuka, and O. Hayashi, 1443
Stoichiometry of Reaction—The complete reaction mixture (2.0 ml), containing 50 μmoles of S-methyl-L-cysteine, 250 μmoles of Tris-HCl buffer, pH 8.8, and approximately 0.3 unit of the enzyme (Step 5), was incubated for 1 hour at 37°. For the determination of methyl mercaptan, the same method described above was employed, except that all of the trapping chambers contained 5 ml of 0.1 n iodine solution. The reaction was stopped by heating for 3 minutes in a boiling water bath, and methyl mercaptan produced was trapped in the iodide solution in the same manner described above. The amount of methyl mercaptan was determined by noting the disappearance of iodine, which was measured by titration with 0.1 n Na₂S₂O₃, with starch solution used as an indicator (25). As for ammonia and pyruvate, the reaction was carried out under the same conditions and the reaction was stopped by the addition of 0.3 ml of 10% HClO₄. The supernatant solution was analyzed by the methods described under "Determinations." As shown in Table II, the appearances of pyruvate, ammonia, and methyl mercaptan were well correlated with the disappearance of S-methyl-L-cysteine.

When 50 μmoles of S-methyl-L-cysteine sulfoxide were employed as a substrate and incubated under the same conditions as described above, 6.3 μmoles of pyruvate and 6.1 μmoles of ammonia were produced, with the concomitant disappearance of 5.9 μmoles of the substrate. Methyl methanethiolane was produced during the incubation, and was identified as di- and tri-methyl disulfide was formed during the incubation period.

Effects of pH and Substrate Concentration—The effect of pH on the activity of the enzyme is shown in Fig. 2. An optimal pH of 8.8 was found in Tris-HCl buffer.

The rates of reaction as a function of the concentration of S-methyl-L-cysteine are presented in Fig. 3. The Km values for S-methyl-L-cysteine were calculated to be approximately 2.3 × 10⁻³ M and, for S-methyl-L-cysteine sulfoxide, about 2.4 × 10⁻³ M. A slight inhibitory effect of the substrate, either S-methyl-L-cysteine or its sulfoxide, was observed when the concentration exceeded 1 × 10⁻² M.

Cofactor Requirement—In order to demonstrate the cofactor requirement of the enzyme-catalyzed reaction, the enzyme preparation (Step 5) was dialyzed for 3 hours against 0.005 M Tris-HCl buffer, pH 8.8, containing 0.002 M hydroxylamine, followed by dialysis for 12 hours against a large volume of the same buffer without hydroxylamine to remove the latter from the enzyme solution (33). When the activity of such a dialyzed enzyme preparation was assayed with S-methyl-L-cysteine as substrate, a stimulation of the reaction was demonstrated in the presence of pyridoxal phosphate, as shown in Table III. Prior incubation of the enzyme with derivative of pyridoxine was carried out for 20 minutes at 23°. In Experiments 1 and 2, approximately 400 μg of resolved enzyme and 44 μg (0.04 unit) of unresolved enzyme were used, respectively.

Substrate Specificity—The purified enzyme preparation (Step 6) also catalyzed reactions with several other amino acids, including S-methyl-L-cysteine and its sulfoxide, S-allyl-L-cysteine and its sulfoxide, S-n-propyl-L-cysteine, S-n-butyl-L-cysteine,
Alliinase has been shown to catalyze the degradation of S-allylcysteine sulfoxide (alliin) as well as its alkyl homologues. It is specific for sulfoxides and does not catalyze the cleavage of thioethers (16, 35). The enzyme described in the present paper appears to be unique in catalyzing the degradation not only of several S-alkyl-L-cysteines, but also of their sulfoxides. It is noteworthy that the enzyme can degrade S-(carboxyalkyl)-L-cysteines, one of which, isovalthine, has been recently isolated from human urine (34). Both methionine and cysteine are inert as substrates, indicating that the enzyme is different from cysteine desulphhydrase (36) and methioninase (37). Several experiments were performed to ascertain whether or not a single enzyme was responsible for the degradation of all of these substrates. Although it was not possible to reach a conclusion with regard to the purity of enzyme, the activity could not be separated into more than one component through a 30-fold purification.

The enzyme does not catalyze reactions with related non-sulfur-containing amino acids, including L-serine and L-alanine. Attempts to detect a possible intermediate were unsuccessful. The time courses of the production of pyruvic acid, methyl mercaptan, and ammonia were parallel, and no dissociation was observed in the rates of formation of these products. These results suggest that the mechanism of the enzymatic cleavage of S-methylecysteine to pyruvic acid, ammonia, and methyl mercaptan may be similar to that of tryptophanase of Escherichia coli (38), β tyrosinase (39), and pyrazolealaninase (40).

Black et al. (41, 42) reported that L-methionine sulfoxide could be reduced to methionine by yeast extract in the presence of TPNH. We also examined the reduction of S-methyl-L-cysteine sulfoxide by this enzyme in the presence of TPNH or DPNH, but no reduction occurred.

The widespread occurrence of S-methylecysteine and its sulfoxide in plants and other organisms directs our interest to the physiological role of these amino acids, especially the role of their methyl group. Horner and Kuchinskas (43) have demonstrated in the rat that the methyl group of S-methyl-L-cysteine was oxidized to CO₂ and was incorporated into choline and creatine, but in a different way from methionine. We have also studied the transfer of the methyl group of S-methyl-L-cysteine and S-methyl-L-cysteine sulfoxide, but cannot reach any conclusion as yet.

**SUMMARY**

1. A new enzyme, S-alkylecysteinase, was isolated from S-methyl-L-cysteine-adapted cells of Pseudomonas crucivirae. The enzyme was purified approximately 30-fold by protamine treatment, ammonium sulfate fractionation, calcium phosphate gel adsorption, and chromatography on a column of diethylaminoethyl cellulose.

2. The enzyme catalyzed the stoichiometric conversion of S-methyl-L-cysteine to methyl mercaptan, pyruvic acid, and ammonia, and of S-methyl-L-cysteine sulfoxide to methyl methanethiol sulfinate, pyruvic acid, and ammonia.

3. The pH optimum of the enzyme is 8.8, and pyridoxal phosphate is required as a coenzyme.

4. In addition to S-methyl-L-cysteine and its sulfoxide, various other S-alkyl-L-cysteines, S-alkyl-L-cysteine sulfoxides, and S-(carboxyalkyl)-L-cysteines could serve as efficient substrates. Both methionine and cysteine were inert as substrates.

**Acknowledgments**—The authors are grateful to Drs. T. Suzuki, T. Kakimoto, and M. Fujiwara for their many helpful suggestions in this experiment, and to Dr. S. Black for his helpful advice and stimulating discussions concerning the preparation of this manuscript. We are indebted to Dr. T. Sasaki for his helpful suggestions and discussions concerning the preparation of this manuscript.

**TABLE IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvic acid formed*</th>
<th>Relative rate</th>
<th>Ammonia formed</th>
<th>Methyl mercaptan formed</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>0.15</td>
<td></td>
<td>0.2</td>
<td>0</td>
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<tr>
<td>S-Methyl-L-cysteine</td>
<td>0.360</td>
<td>100</td>
<td>4.3</td>
<td>4.2</td>
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<tr>
<td>S-Methyl-L-cysteine sulfoxide</td>
<td>0.130</td>
<td></td>
<td>34</td>
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<td>S-Ethyl-L-cysteine</td>
<td>0.400</td>
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<td>0.445</td>
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<tr>
<td>S-Allyl-L-cysteine sulfoxide</td>
<td>0.130</td>
<td>34</td>
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<tr>
<td>S-n-Propyl-L-cysteine</td>
<td>0.213</td>
<td>56</td>
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<tr>
<td>S-n-Butyl-L-cysteine</td>
<td>0.228</td>
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<tr>
<td>S-Isobutyl-L-cysteine</td>
<td>0.160</td>
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<td>S-n-Amyl-L-cysteine</td>
<td>0.100</td>
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<td>S-Isoamyl-L-cysteine</td>
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<td>S-(2-Carboxyisobutyl)-L-cysteine</td>
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<td>S-(2-Carboxy-1,1-dimethylethyl-L cysteine</td>
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<td>S-(2-Carboxy-n-propionyl)-L-cysteine</td>
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<td>0.190</td>
<td>50</td>
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<td>L-Methionine</td>
<td>0.020</td>
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<td>L-Cysteine</td>
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<tr>
<td>L-Alanine</td>
<td>0.010</td>
<td>0.2</td>
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</tr>
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</table>

* Expressed as the decrease in absorbancy at 340 mμ per minute caused by the formation of pyruvic acid.
valuable assistance in the preparation of S-methyl-L-cysteine, S-methyl-L-cysteine sulfoxide, and methyl methanethiosulfinate.

REFERENCES

"S-Alkylcysteinase: Enzymatic Cleavage of S-Methyl-l-cysteine and Its Sulfoxide"

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