

SPECIFIC INHIBITION OF GLYCERALDEHYDE-3-PHOSPHATE  
DEHYDROGENASE BY KONINGIC ACID  
(HEPTELIDIC ACID)

AKIRA ENDO\*, KENJI HASUMI, KAORU SAKAI and TOMOMI KANBE

Department of Agricultural and Biological Chemistry, Tokyo Noko University,  
3-5-8 Saiwaicho, Fuchu-shi, Tokyo, 183 Japan

(Received for publication February 8, 1985)

A sesquiterpene named koningic acid has been isolated from a strain of *Trichoderma koningii* as a potent inhibitor of ATP generation in the glycolytic pathway. From experiments with both cultured mouse carcinoma FM3A cells and isolated enzymes, it was shown that koningic acid is a specific inhibitor of glyceraldehyde 3-phosphate dehydrogenase that catalyzes the conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate.

In the course of searching for an active metabolite of microbial origin which inhibits lipid metabolism of animals, an active compound was isolated from culture broth of a strain of *Trichoderma koningii* as an inhibitor of cholesterol biosynthesis in an *in vitro* rat liver enzyme system<sup>1)</sup>. This compound (named koningic acid) was found to be identical to the sesquiterpene lactone heptelidic acid by direct comparison of its physical and chemical properties with those of authentic samples (kindly provided by Dr. H. SAKAI, University of Osaka Prefecture) (Fig. 1). In this communication the authors call this compound koningic acid, since it was independently isolated and characterized as an enzyme inhibitor.

Heptelidic acid was isolated from several fungal strains including *Anthostoma avocetta*<sup>2,3)</sup>, *Chaetomium globosum*, *T. viride* and *Gliocladium virens*<sup>4,5)</sup>. It was shown to have antibiotic activity against anaerobic bacteria such as *Bacteroides* and a cytostatic effect on mouse L1210 cells<sup>4)</sup>.

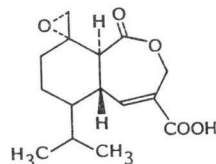
Preliminary experiments showed that koningic acid inhibits ATP-generating system consisting of ATP, glucose 1-phosphate and cytosolic fraction of rat liver homogenate, thereby blocking mevalonate pyrophosphate decarboxylase (EC 4.1.1.33) that catalyzes the conversion of mevalonate pyrophosphate to isopentenyl pyrophosphate in the presence of ATP. Similar inhibition was also observed when ATP and glucose 1-phosphate were replaced by ADP and dihydroxyacetone phosphate, respectively. Since in the glycolytic pathway ATP is generated at the two reactions catalyzed by phosphoglycerate kinase (EC 2.7.2.3) and pyruvate kinase (EC 2.7.1.40), these data indicated that koningic acid was not an inhibitor of enzymes in sterol synthetic pathway but rather an inhibitor of enzyme(s) in the glycolytic pathway.

#### Material and Methods

##### Materials

[3,4,5-<sup>3</sup>H]Leucine (141 Ci/mmol) and [methyl-<sup>3</sup>H]thymidine (2.0 mCi/mmol) were obtained from

Fig. 1. Structure of koningic acid (heptelidic acid).



New England Nuclear. Glyceraldehyde 3-phosphate diethylacetal, dihydroxyacetone phosphate, 3-phosphoglycerate and phosphoenolpyruvate were purchased from Sigma and yeast hexokinase and glucose 6-phosphate dehydrogenase from Oriental Yeast Co. (Japan). Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, triosephosphate isomerase and ATP bioluminescence CLS were products of Boehringer Mannheim. ES medium, designed by Dr. KOYAMA, Cancer Institute, Tokyo, was obtained from Nissui Seiyaku Co. (Japan). All other chemicals were of the highest quality from commercial sources.

Mouse carcinoma FM3A cells (subclonal line F28-7) were kindly supplied by Dr. KOYAMA.

#### Microorganism

The microbial strain M3947, which has been isolated from a soil sample collected at the Ashinoko-lake, Kanagawa Prefecture, Japan and identified taxonomically to be *T. koningii* (data not shown) was maintained on potato dextrose agar slants.

#### Fermentation and Isolation of Koningic Acid

*T. koningii* M3947 was cultured aerobically at 25°C for 7 days in a 30-liter jar fermenter containing 20 liters of a medium consisting of 2% glucose, 0.1% peptone, 3% malt extract and 0.01% Nissan Disform CB442. Under these conditions, productivity of koningic acid was 300~500 µg/ml. The culture filtrate (15 liters) was acidified to pH 3 with HCl and extracted twice with a half volume of EtOAc. The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure to 200 ml. The solution was extracted twice with 200 ml of 5% NaHCO<sub>3</sub>. The aqueous phases were combined, acidified and extracted twice with an equal volume of EtOAc. The solvent layers were dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated *in vacuo* to dryness and applied onto a silica gel column (Wako gel C-200, 100 g). The column was developed successively with benzene-dichloromethane (1:1, 4 liters) and dichloromethane (3 liters). The active fractions were pooled and evaporated under reduced pressure to dryness. The residue was dissolved in benzene containing a small volume of hexane from which koningic acid was obtained as crystals. Recrystallization was carried out in the same solution, yielding 2.6 g of white crystals of koningic acid. The isolated product was identified as heptelidic acid by comparing its molecular formula (C<sub>18</sub>H<sub>20</sub>O<sub>8</sub>), and UV, IR, NMR and mass spectra with those for authentic samples (kindly supplied by Dr. H. SAKAI, University of Osaka Prefecture, Osaka) and with those reported<sup>4)</sup>.

#### Cytosolic Fraction of Rat Liver

From rat liver homogenate obtained as described by KURODA and ENDO<sup>9)</sup>, the cytosolic fraction was isolated after centrifugation at 105,000×g for 60 minutes. This fraction was subjected to ammonium sulfate precipitation, and the precipitate obtained at 40~80% saturation was collected and dialyzed against 0.1 M potassium phosphate buffer, pH 7.4. The dialyzed solution was used for the assay of enzymes involved in glycolysis.

#### Assay for Enzymes in Glycolytic Pathway

Inhibitory effects of koningic acid on enzyme(s) in the glycolytic pathway were assayed by determining ATP produced in the presence of one of the intermediates of the pathway and ADP. The reaction mixture (0.2 ml) contained 222 mM phosphate buffer, pH 7.4, 0.67 mM ADP, 8 mM MgCl<sub>2</sub>, cytosolic fraction of rat liver (0.6 mg protein) and an intermediate (dihydroxyacetone phosphate, 3-phosphoglycerate or phosphoenolpyruvate at 6.7 mM). After incubation at 38°C for 60 minutes, the reaction was terminated by heating in boiling water for 3 minutes and the mixture was centrifuged at 3,000×g for 3 minutes. The resultant supernatant solution was assayed for ATP concentration by the method of ESTABROOK *et al.*<sup>7)</sup> using hexokinase and glucose 6-phosphate dehydrogenase.

#### Assay for Individual Enzymes in Glycolysis

Glyceraldehyde 3-phosphate dehydrogenase was assayed spectrophotometrically according to DUGGLBEY and DENNIS<sup>8)</sup>. Triosephosphate isomerase and phosphoglycerate kinase were determined as described by HARTMAN *et al.*<sup>9)</sup> and SCOPES<sup>10)</sup>, respectively.

#### Growth of FM3A Cells

FM3A cells were maintained in a humidified incubator (5% CO<sub>2</sub>) at 37°C in Pyrex glass dishes

containing ES medium supplemented with 5% bovine serum. Under these conditions, cells grew in suspension with a population-doubling time of 12~14 hours and maximum cell density was  $1.6 \times 10^8$  cells/ml. After incubation for 48 hours when cells were logarithmically growing ( $4 \sim 8 \times 10^8$  cells/ml), cells were harvested, washed twice with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free DULBECCO's phosphate buffered saline and used for various experiments.

#### Lactate Production in FM3A Cells

Cells were transferred to DULBECCO's phosphate buffered saline containing 0.1% glucose at  $6.5 \times 10^6$  cells/ml and incubated in the presence or absence of koningic acid. At intervals 0.1 ml portion of cell culture was transferred to a test tube and mixed with 0.1 ml of 1 N perchloric acid. After 5 minutes, the mixture was neutralized by the addition of 20  $\mu$ l of 5 M  $\text{K}_2\text{CO}_3$  and centrifuged at  $3,000 \times g$  for 5 minutes. The supernatant solution was collected and subjected to the determination of lactate by the method of GAWEHN and BERGMAYER<sup>11)</sup>.

#### Incorporation of [<sup>3</sup>H]Thymidine and [<sup>3</sup>H]Leucine into Acid-insoluble Fractions in FM3A Cells

Cells were suspended in DULBECCO's phosphate buffered saline containing 10 mM pyruvate at a density of  $5.0 \times 10^5$  cells/ml and preincubated in the presence or absence of koningic acid for 2 hours. Then an aliquot of the suspension (1 ml) was further incubated with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine or [<sup>3</sup>H]leucine at 37°C for 1 hour. The mixture was acidified by the addition of 1 ml of chilled 10% TCA and after 5 minutes the resultant precipitate was collected, washed and then counted in a toluene-based scintillation liquid.

## Results

### Effects of Koningic Acid on Glycolysis at Enzyme Levels

In the experiments shown in Table 1, two intermediates in glycolytic pathway were used as the substrate for ATP generation in the presence of ADP and cytosolic fraction of rat liver homogenate. Thus, ATP formation from dihydroxyacetone phosphate was inhibited by 89% in the presence of 0.1 mM koningic acid, while under the same conditions, koningic acid showed no detectable effects on the production of ATP from phosphoenolpyruvate. These results indicated that koningic acid inhibited the enzymatic step(s) in which dihydroxyacetone phosphate was converted to 3-phosphoglycerate but had no effect on the transformation of the latter intermediate into pyruvate.

Of the three enzymes involved in the production of ATP from dihydroxyacetone phosphate, triose-phosphate isomerase (EC 5.3.1.1.) and phosphoglycerate kinase (EC 2.7.2.3) of rat liver were not significantly inhibited by koningic acid at 1 mM, while glyceraldehyde 3-phosphate dehydrogenase of rat liver was inhibited 50% at 0.3  $\mu$ M (Fig. 2). Similar results were also obtained with glyceraldehyde 3-phosphate dehydrogenase of rabbit muscle (Fig. 2). Considered together, it was concluded that koningic acid specifically inhibited the conversion of glyceraldehyde 3-phosphate dehydrogenase in glycolytic pathway.

### Effects of Koningic Acid on FM3A Cells

The growth of FM3A cells in ES medium supplemented with 5% bovine serum was not affected by koningic acid at 0.36  $\mu$ M, but inhibited 40 to 47% at 3.6  $\mu$ M and over 90% at 36  $\mu$ M, respectively (Fig. 3).

To see the effects of koningic acid on glycolytic pathway, FM3A cells were incubated in DULBECCO's phosphate buffered saline supplemented either glucose or pyruvate in the presence of varying concentrations of koningic acid. As shown in Fig. 4, lactate production from glucose was strongly reduced by koningic acid at concentrations higher than 3.6  $\mu$ M. Conversion of pyruvate to lactate catalyzed by

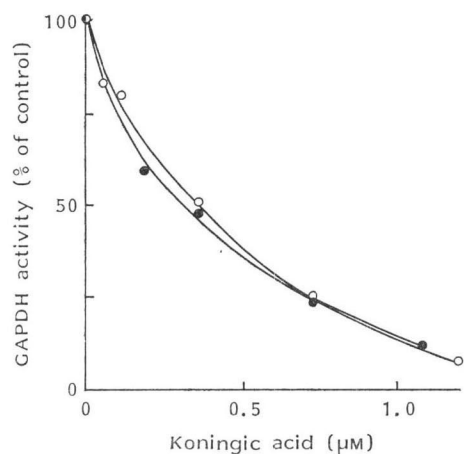
Table 1. Effects of koningic acid on the production of ATP from dihydroxyacetone phosphate and phosphoenolpyruvate by the cytosolic fraction of rat liver.

Experimental conditions are described in Materials and Methods.

Intermediate added	ATP generated (% of control)
Dihydroxyacetone phosphate	11
Phosphoenolpyruvate	108

Fig. 2. Inhibition of glyceraldehyde 3-phosphate dehydrogenase by koningic acid.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of rat liver (cytosolic fraction) (○) and of rabbit muscle (●) were assayed in the presence of varying concentrations of koningic acid.



lactate dehydrogenase was, however, not significantly affected by the inhibitor (data for pyruvate not shown). Under similar conditions, ATP production in the presence of glucose was lowered by koningic acid, while that from pyruvate was not detectably decreased (Fig. 5).

The incorporation of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]leucine into the acid-insoluble fractions in FM3A cells was not significantly affected by koningic acid at concentrations up to 40 μM (Fig. 6).

Considered together, the results obtained with FM3A cells suggested that koningic acid was an inhibitor of glycolytic pathway but not for DNA and protein synthesis.

In the experiments shown in Fig. 7, cell-free extracts, prepared from FM3A cells which had been preincubated with varying concentrations of koningic acid for 2 hours, were assayed for glyceraldehyde

Fig. 3. Effects of koningic acid on the growth of FM3A cells.

FM3A cells ( $1.0 \times 10^5$  cells) were suspended in 1 ml of ES medium supplemented with 5% bovine serum in the presence of varying concentrations of koningic acid and incubated at 37°C. At intervals, cell density was determined with a Bünker-Türk type haemocytometer.

Koningic acid: 0 μM (○), 0.36 μM (●), 3.6 μM (■), 36 μM (▲).

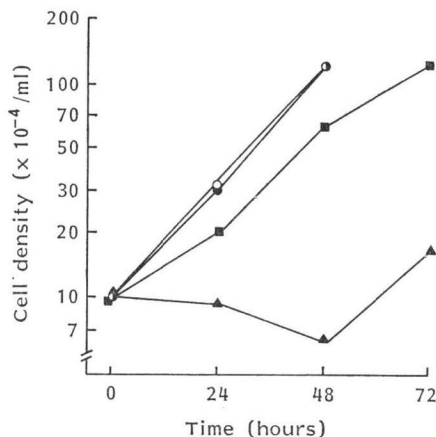


Fig. 4. Inhibition of lactate production from glucose by koningic acid.

Experimental conditions are described in Materials and Methods.

Concentrations of koningic acid: 0 μM (○); 3.6 μM (●); 10 μM (■); 36 μM (▲).

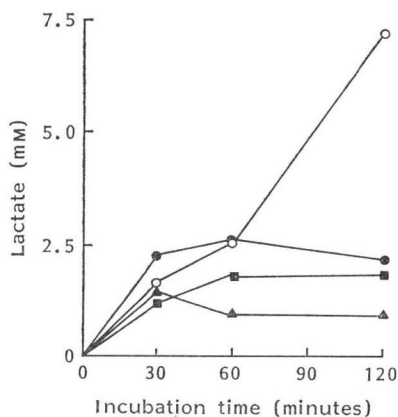


Fig. 5. Effects of koningic acid on ATP production from glucose and pyruvate in FM3A cells.

FM3A cells were suspended in DULBECCO's phosphate buffered saline containing either 5.6 mM glucose (○) or 10 mM pyruvate (●) at a density of  $5 \times 10^5$  cells/ml in the presence of varying concentrations of koningic acid and incubated at 37°C for 2 hours. The perchloric acid-soluble fraction of cells was assayed for ATP concentration using Bioluminescence CLS kit.

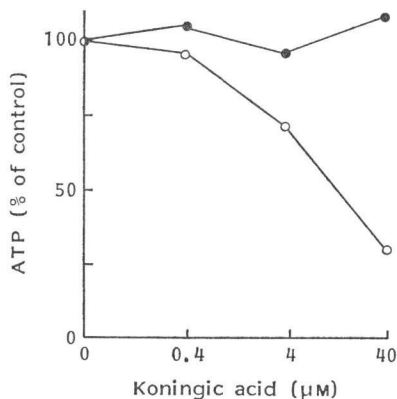
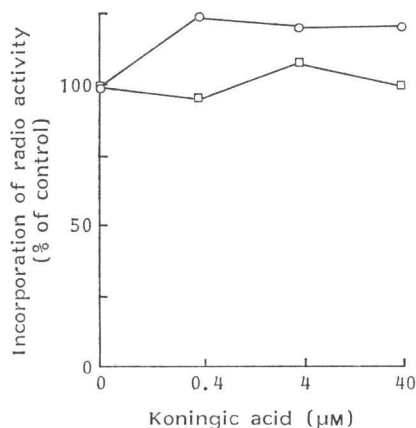


Fig. 6. Effects of koningic acid on the incorporation of [<sup>3</sup>H]thymidine (○) and [<sup>3</sup>H]leucine (□) into the acid-insoluble fraction in FM3A cells.

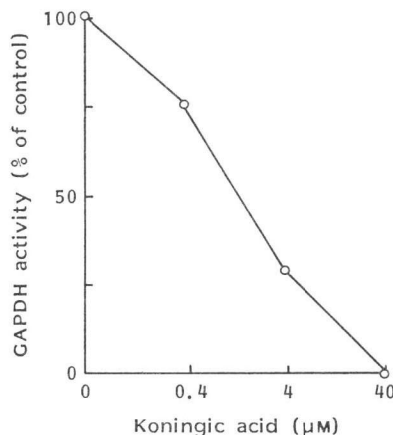
Experimental conditions are described in Materials and Methods.



The antibiotic pentalenolactone (arenaemycin) have been shown to be an inhibitor of glyceraldehyde 3-phosphate dehydrogenase of procaryotic and eucaryotic cells<sup>12,13</sup>. The mechanism for the inhibition of glyceraldehyde 3-phosphate dehydrogenase by pentalenolactone has not been reported and remains to be studied further. However, it should be noted that this metabolite, produced by several strains of Actinomycetes, is structurally related to koningic acid in that both compounds are carboxylic acid with an epoxide and lactone ring.

Fig. 7. Inhibition of glyceraldehyde 3-phosphate dehydrogenase of FM3A cells by koningic acid.

FM3A cells were suspended in ES medium containing 5% bovine serum at a density of  $5 \times 10^5$  cells/ml and incubated with varying concentrations of koningic acid at 37°C for 2 hours. Cells were collected by centrifugation, washed with DULBECCO's phosphate buffered saline and disrupted by sonication in 25 mM Tris-HCl, pH 7.5. The suspension was successively centrifuged at  $12,000 \times g$  for 10 minutes and then at  $30,000 \times g$  for 60 minutes. The resultant supernatant was assayed for glyceraldehyde 3-phosphate dehydrogenase.



3-phosphate dehydrogenase. As expected, the results indicated that koningic acid significantly inhibited the dehydrogenase in intact cells at concentrations higher than 0.4 μM.

### Discussion

The present experiments have shown that koningic acid (heptelidic acid) is a specific inhibitor of glyceraldehyde 3-phosphate dehydrogenase, an enzyme involved in glycolysis. Significant inhibition was obtained at 4 μM koningic acid in the experiments with intact cells of FM3A (Figs. 4, 5 and 7), and 0.3 μM was required for 50% inhibition at enzyme levels (Fig. 2).

## References

- 1) ENDO, A. & T. KANBE: Isolation, chemical structure and mechanisms of action of koningic acid, a new inhibitor of cholesterol synthesis. Proc. Jpn. Conf. on the Biochem. of Lipids. 23: 172~174, 1981
- 2) HAGENBACH, A.: Avocetin: Struktur und beiträge Biogenese. Dissertation ETH Prom N.: 4647, 1971
- 3) ARIGONI, D.: Stereochemical aspects of sesquiterpene biosynthesis. Pure Appl. Chem. 41: 219~245, 1975
- 4) ITOH, Y.; K. KODAMA, K. FURUYA, S. TAKAHASHI, T. HANEISHI, Y. TAKIGUCHI & M. ARAI: A new sesquiterpene antibiotic, heptelidic acid producing organisms, fermentation, isolation and characterization. J. Antibiotics 33: 468~473, 1980
- 5) STIPANIVIC, R. D. & C. R. HOWELL: The X-ray crystal structure determination, and biosynthetic studies of the antibiotic, heptelidic acid. Tetrahedron 39: 1103~1107, 1983
- 6) KURODA, M. & A. ENDO: Inhibition of *in vitro* cholesterol synthesis by fatty acids. Biochim. Biophys. Acta 486: 70~81, 1977
- 7) ESTABROOK, R. W.; J. R. WILLIAMSON, R. FRENKEL & P. K. AMITRA: The fluorometric determination of mitochondrial adenin and pyridine nucleotides. Methods Enzymol. 10: 474~482, 1967
- 8) DUGGLEBY, R. G. & D. T. DENNIS: Nicotinamide adenine dinucleotide-specific glyceraldehyde 3-phosphate dehydrogenase from *Pisum sativum*. Assay and steady state kinetics. J. Biol. Chem. 249: 167~174, 1974
- 9) HARTMAN, F. C. & I. L. NORTON: Triosephosphate isomerase from rabbit muscle. Methods Enzymol. 41: 447~453, 1975
- 10) SCOPES, R. K.: 3-Phosphoglycerate kinase of skeletal muscle. Methods Enzymol. 42: 127~134, 1975
- 11) GAWEHN, K. & H. U. BERGMAYER: D-(—)-Lactate. In Methods of Enzymatic Analysis. Vol. 3. Ed., H. U. BERGMAYER, pp. 1492~1495, Academic Press, New York, 1979
- 12) HARTMANN, S.; J. NEEFF, U. HEER & D. MECKE: Arenaemycin (pentalenolactone): A specific inhibition of glycolysis. FEBS Lett. 93: 339~342, 1978
- 13) MANN, K. & D. MECKE: Inhibition of spinach glyceraldehyde 3-phosphate dehydrogenase by pentalenolactone. Nature 282: 535~536, 1979