Isolation and Some Properties of an Aspartate Aminotransferase Inhibitor, Gostatin

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A novel aspartate aminotransferase inhibitor named gostatin was isolated as crystals from the culture filtrate of *Streptomyces sumanensis* NK-23. The structure has been established to be 5-amino-2-carboxy-4-oxo-1,4,5,6-tetrahydropyridine-3-acetic acid by X-ray crystallographic analysis (K. Ibata *et al.*, manuscript in preparation). The results of chemical and instrumental analyses of the inhibitor are described in this paper.

Gostatin showed time-dependent inhibitory action against aspartate aminotransferases and alanine aminotransferase. Both pyridoxal phosphate- and pyridoxamine phosphate-linked enzymes were sensitive to the inhibitor.

A novel cyclic amino acid named gostatin, produced by *Streptomyces sumanensis* NK-23, shows a strong inhibitory activity against aspartate aminotransferases (EC 2.6.1.1, GOT)* of various origins.^{1,2)} This inhibitor was isolated as crystals and the structure was determined to be 5-amino-2-carboxy-4-oxo-1,4,5,6-tetrahydropyridine-3-acetic acid (Fig. 1) by X-ray crystallographic analysis (K. Ibata *et al.*, manuscript in preparation).

In this paper, the isolation and some chemical and biochemical properties of gostatin are reported.

MATERIALS AND METHODS

Microorganism and culture conditions. *Streptomyces sumanensis* NK-23, which was isolated from soil by the authors,^{1,2)} was used in this experiment. The strain was cultured under the optimum conditions for the production of gostatin in a Marubishi MSJ-U2 jar fermentor.²⁾ The culture filtrate at 114 hr cultivation was used for the preparation of the inhibitor.

Assay of gostatin activity. Gostatin activity was assayed by the method described in the previous paper.²⁾

Isolation of gostatin. The isolation procedure reported in the previous paper was modified.¹⁾ The culture filtrate (35

liters) was adjusted to pH 4.0 with formic acid, and activated charcoal (2%, w/v) was added. After the mixture stood for 30 min, the non-adsorbing fraction was collected by filtration. This solution was applied onto a column (10 × 35 cm) of Dowex 1 × 4 (formate type, 100 ~ 200 mesh) previously equilibrated with 50 mM ammonium formate-formic acid buffer (pH 4.0). Gostatin was weakly adsorbed to the column and eluted with 50 mM ammonium formate-formic acid buffer (pH 4.0). Active fractions thus obtained (36 liters) were then applied onto a column (10 × 35 cm) of Dowex 1 × 4 (OH type, 100 ~ 200 mesh) and eluted with 0.5 M NH₄Cl solution. The fractions containing gostatin were pooled (14 liters), evaporated *in vacuo* to dryness and dissolved in a minimum volume of water (1150 ml). After the solution was adjusted to pH 4.0 with 6N HCl, methanol was added at a final concentration of 50% (v/v) and the precipitate was removed by filtration. Gostatin was precipitated from the supernatant by adding ethanol at a final concentration of 60% and allowing the mixture to stand overnight. The precipitates were collected by centrifugation (10,000 × g, 10 min) and dissolved in 170 ml of water. Gostatin was crystallized from this solution by adjusting the pH (pH 4.0) and adding methanol.

Instrumental analysis. Instrumental analyses were performed using the following instruments: IR, a Hitachi 260-10 infrared spectrometer; UV, a Hitachi 320 spectrophotometer; optical rotation, a JASCO DIP-SL polarimeter; mass spectrum, a Hitachi RMU-7M spectrometer in the electron impact method; ¹H and ¹³C NMR spectra, a JEOL FX-200 spectrometer.

Abbreviations: GOT, aspartate aminotransferase; GPT, alanine aminotransferase; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; DMSO, dimethylsulfoxide; TMS, tetramethylsilane.

Fig. 1. Structure of Gostatin.

5-Amino-2-carboxy-4-oxo-1,4,5,6-tetrahydropyridine-3-acetic acid.

Thin-layer chromatography and paper electrophoresis. Thin-layer chromatography was carried out using cellulose plates (Merck) with the following solvent system: (A), isopropanol-NH₄OH-H₂O (6:1:2); (B), *n*-butanol-acetic acid-H₂O (4:1:2); (C), *tert*-butanol-formic acid-H₂O (70:50:15); (D), water saturated [*n*-butanol-pyridine (2:1)]; (E), isobutanol-acetic acid-H₂O (2:1:2). Paper electrophoresis was performed with the potential gradient of 20 V/cm for 1 hr in 50 mM acetate buffer (pH 5.0).

Assay system for determination of inhibition specificity. Test enzymes were preincubated with gostatin in 50 mM Tris-HCl buffer, pH 8.5 (for GOT and GPT), 50 mM potassium phosphate buffer, pH 7.0 (glutamate decarboxylase and γ -aminobutylate aminotransferase), 0.2 M Tris-maleate buffer, pH 6.8 (phosphorylase), 40 mM imidazole buffer, pH 7.0 (glutamine synthetase) or 0.1 M Tris-HCl buffer, pH 7.9 containing 10% glycerol (glutamate dehydrogenase). GOT (cytosolic enzyme of pig heart and wheat germ) and GPT were assayed by the method of Yatizidis³ and mitochondrial GOT of pig heart was assayed in a system coupled with malate dehydrogenase and NADH.⁴ Glutamate decarboxylase activity was determined in a Warburg apparatus.⁵

Phosphorylase activity was assayed according to Cori *et al.*,⁶ and glutamine synthetase was assayed by the determination of production of γ -glutamylhydroxamate.⁷ Glutamate dehydrogenase was assayed by the method of Fahien *et al.*,⁸ and γ -aminobutylate aminotransferase was assayed in a system coupled with succinic semialdehyde dehydrogenase.⁹

Enzymes. Mitochondrial GOT (pig heart) was kindly donated by Dr. H. Wada (Osaka University Medical School). Wheat germ GOT was prepared by the method of Verjee *et al.*¹⁰ Cytosolic GOT (pig heart), phosphorylase (rabbit muscle), glutamate dehydrogenase (beef liver), γ -aminobutylate aminotransferase (*Pseudomonas fluorescens*) and GPT (pig heart) were purchased from Boehringer Mannheim. Glutamate decarboxylase (type V, *Escherichia coli*) and glutamine synthetase (ovine brain) were obtained from Sigma Chemical Co. Malate dehydrogenase was purchased from Oriental Yeast Co. Transaminases were used after being treated with 2-oxoglutarate and dialysis. Holo GOT in pyridoxamine form was prepared by the method of Jenkins *et al.*¹¹

RESULTS

Isolation of gostatin

A summary of the purification of gostatin from a 35 liters culture filtrate of *S. sumanensis* is shown in Table I. After being recrystallized from aqueous methanol, the total yield was 1900 mg, and colorless needles were obtained. This preparation gave a single spot (with I₂ vapor and ninhydrin) on thin-layer chromatography and paper electrophoresis: *R_f* values, 0.17 (A), 0.22 (B), 0.58 (C), 0.08 (D), 0.51 (E); mobility, +3.5 cm (PLP, +4.6 cm).

Chemical properties of gostatin

Physical and chemical properties of gostatin are summarized in Table II. The molecular formula of gostatin is C₈H₁₀O₅N₂ (MW = 214.17) and was confirmed by mass spectrometry of the tetratrimethylsilyl ester (M⁺, *m/z* 502). Gostatin is an amphoteric compound [*pK_a* $\approx$ 1.5, 4.84 and 8.13, titration equivalent (found) 216] and it is adsorbed to an anion exchange resin (Dowex 1) in alkaline pH and to a cation exchange resin (Dowex 50W) in acidic pH. Gostatin showed a positive color reaction with ninhydrin. The IR spectrum is shown in Fig. 2. It suggested the presence of an amino group (NH₃⁺, 3285 and 1520 cm⁻¹) and a carboxyl group (COOH, 3500–2600 and 1725; COO⁻, 1388). The UV spectra are shown in Fig. 3. $\lambda_{\text{max}}^{\text{pH } 7}$ nm (ϵ): 331 (10,000). $\lambda_{\text{max}}^{0.1 \text{ N HCl}}$: 343 (7470). $\lambda_{\text{max}}^{0.1 \text{ N NaOH}}$: 330 (9170). These properties suggested that gostatin was a novel amino acid. ¹H NMR spectrum was measured in DMSO-*d*₆ at 200 MHz and 50°C. After the structure determination by X-ray crystallographic analysis, the resonances were assigned as follows: $\delta_{\text{TMS}}^{\text{DMSO-}d_6}$ 3.1~3.3 (3H, m, 1'-H, 6-H), 3.6~3.7 (2H, m, 5-H, 6-H), 5.5~6.3 (4H, br, -NH₃⁺, >NH), 7.1~7.4 (1H, br, COOH).

The natural-abundance ¹³C NMR spectrum of gostatin, measured in DMSO-*d*₆ at 50 MHz, contains resonances ascribable to 8 carbon atoms (Table III). The three resonances at higher field were identified as 5-C, 6-C and 1'-C with the aid of the off resonance proton

TABLE I. SUMMARY OF ISOLATION OF GOSTATIN

Treatment	Volume (ml)	Inhibitory activity (units) $\times 10^3$	$A_{330} \times 10^3$	Specific activity (units/ A_{330})	Recovery (%)
Culture filtrate	35,000	13,900	1580	8.8	100
Activated charcoal	33,200	15,100	413	36.6	109
Dowex 1 $\times$ 4 (formate)	36,000	14,000	355	39.4	101
Dowex 1 $\times$ 4 (OH ⁻)	14,000	13,200	288	45.8	95
Ethanol ppt.	170	7,790	169	46.2	56
Crystallization	100	5,990	126	47.5	43
Recrystallization	1900 (mg)	4,130	86	48.0	30

TABLE II. SOME CHEMICAL PROPERTIES OF GOSTATIN

Elemental analysis

Found: C, 41.86; H, 5.03; N, 12.10

Calcd for $C_8H_{10}O_5N_2 \cdot H_2O$: C, 41.58; H, 5.23; N, 12.12%.

Mass spectrum

502 (M^+ , tetratrimethylsilyl-derivative) $[\alpha]_D^{25} -117^\circ$ ($c=1.0$, 2 M NH_4OH)Color reaction^a

Ninhydrin, +; I_2 vapor, +; potassium permanganate, +; ferric chloride, +; Rydon-Smith, —; Elson-Morgan, —; Morgan-Elson, —; phenol sulfate, —; 2,4-dinitrophenyl hydrazine, —.

Solubility

Soluble in water and DMSO. Slightly soluble in methanol. Insoluble in ethanol, ethyl ether, benzene, chloroform, and ethyl acetate.

$pK_a \approx 1.5, 4.84, 8.13$; titration equivalent = 216 (found)

Melting point

No definite mp.

^a +, positive; —, negative.

FIG. 2. Infrared Spectrum of Gostatin in KBr Disk.

decoupling method. The five resonances of quaternary carbons were also assigned by

FIG. 3. UV Spectra of Gostatin.

The UV spectra were measured in 0.1 N HCl (----), 0.1 M potassium phosphate buffer, pH 7.0 (—) and 0.1 N NaOH (— · —).

TABLE III. CARBON-13 NMR CHEMICAL SHIFTS OF GOSTATIN (IN DMSO- d_6 AT 50 MHz)

Carbon ^a	Chemical shift ^b
6	32.1
1'	44.2
5	50.5
3	97.8
2	159.5
3'	165.6
2'	172.9
4	187.9

^a Numbering of carbon atoms are shown in Fig. 1.^b Chemical shifts are expressed by the δ -value (ppm) from internal TMS reference.

comparison of chemical shifts with those of model compounds.^{12,13)}

The pH and thermal stabilities of gostatin

TABLE IV. INHIBITION SPECTRUM OF GOSTATIN

The enzymes were preincubated with gostatin under the indicated conditions and then residual enzyme activities were assayed. See MATERIALS AND METHODS for details.

Enzyme (origin)	Preincubation conditions min (°C)	Gostatin concn. (μ M)	Inhibition (%)
GOT (pig heart, mit)	10 (37)	4	100
(pig heart, cyt)	10 (37)	4	95
(wheat germ)	10 (37)	4	32
GPT (pig heart)	10 (37)	4	52
γ -Aminobutylate aminotransferase (<i>Pseud. fluorescens</i>)	30 (25)	20	0
Glutamate decarboxylase (<i>Escherichia coli</i>)	30 (37)	20	0
Glutamate dehydrogenase (bovine liver)	30 (37)	20	0
Phosphorylase A (rabbit muscle)	30 (37)	20	0
Glutamine synthetase (ovine brain)	30 (37)	20	0

FIG. 4. pH and Thermal Stability of Gostatin.

Gostatin was incubated at 37°C for 22 hr and at 100°C for 10 min in various buffers and then residual activity was determined. Buffer system used was 0.1 M acetate-HCl buffer (pH 1.5~3.5), 0.1 M acetate buffer (4.5~5.5), 0.1 M potassium phosphate buffer (6.5~7.0), 0.1 M Tris-HCl buffer (7.5~8.5), 0.1 M glycine-NaOH buffer (10) and 0.01 N NaOH. Open symbols, treatment at 37°C; closed symbols, 100°C.

were determined. The inhibitor (0.1 mg) was dissolved in various buffers and kept at 37°C for 22 hr and 100°C for 10 min, and then residual activity was assayed. As shown in Fig. 4, gostatin was unstable below pH 3.0.

Inhibitory action against various enzymes

The effect of gostatin on various PLP-linked aminotransferases and other enzymes are summarized in Table IV. Pig heart GOTs (mito-

FIG. 5. Time-Dependent Inactivation of GOT in PLP and PMP Form.

PLP (0.03 unit) and PMP (0.025 unit)-linked GOT (pig heart, mitochondrial) were incubated with the indicated concentration of gostatin at 25°C in 50 mM potassium phosphate buffer (pH 7.0). At various time points, residual enzyme activities were assayed in the assay system coupled with malate dehydrogenase and NADH. (A), PLP-linked enzyme; (B), PMP-linked enzyme.

chondrial and cytosolic) were strongly inhibited, and wheat germ GOT and GPT (pig heart) were less effectively inhibited. In addition to these enzymes, gostatin also inhibits yeast kynurenine aminotransferase (76% inhibition after 10 min incubation at 30°C).¹⁴⁾ The inhibitor, however, showed no inhibitory activity against the other pyridoxal-phosphate-dependent enzymes so far tested, such

as γ -aminobutylate aminotransferase, glutamate decarboxylase or phosphorylase.

Gostatin inhibits GOT in both pyridoxal and pyridoxamine forms. Figure 5 shows the time course of inactivation of PLP and PMP-linked GOT by gostatin. Pyridoxamine-linked enzyme showed almost the same sensitivity as that of pyridoxal-linked enzyme. Gostatin required preincubation with the enzyme for its inhibitory action (Fig. 5) and in the presence of substrates it shows no inhibitor activity even against the susceptible enzymes.

DISCUSSION

Certain chemical properties of gostatin, such as the pK_a , color reaction with ninhydrin

Gostatin is a time-dependent irreversible inhibitor of GOT (Fig. 5 and our preliminary experiments) and is considered to be a mechanism-based inhibitor (suicide substrate).¹⁵⁻¹⁸ GOT inhibitors having similar properties have been obtained. These involve β,γ -unsaturated amino acids such as 2-amino-4-methoxy-*trans*-3-butenic acid, allyl chloride and sulfates such as β -chloroalanine, an acetylenic substrate analog (propargylglycine), and compounds containing an isoxazolidine ring such as cycloglutamate.^{15,17,19-21} Gostatin is a derivative of hydrated pyridine (or dehydrated piperidine) and a GOT (and other pyridoxal-dependent enzymes) inhibitor of this type has not been reported.

In addition to its structural characteristics, the inhibitor shows a unique property in its inhibition specificity, *i.e.*, it inactivated both the PLP and PMP-linked GOT at almost the same rate. None of the inhibitors mentioned

and IR spectrum, suggested that gostatin is an amino acid. But the precise structure was difficult to determine from the results of chemical and instrumental analyses and conclusive evidence of the structure was obtained by X-ray crystallographic analysis. The inhibitor contains one asymmetric carbon atom, the carbon at the 5 position. The optical rotation property of gostatin suggested that it is not a mixture of the two possible chiral forms, but its absolute configuration has not yet been established.

Gostatin is a novel heterocyclic amino acid containing one amino and two carboxyl groups. This structure is simply regarded as a derivative of alanine and glutamic acid as follows.

above show the same phenomenon and it is a strange thing for one of these mechanism-based inhibitors.

More detailed investigation of the mechanism of action of gostatin will be published elsewhere.

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REFERENCES

- 1) S. Murao, T. Nishino, N. Katayama and H. Nagano, *Agric. Biol. Chem.*, **45**, 1039 (1980).
- 2) S. Murao and T. Nishino, *Agric. Biol. Chem.*, **47**, 1531 (1983).
- 3) H. Yatizidis, *Nature*, **186**, 79 (1960).
- 4) Y. Morino, S. Tanase, T. Watanabe, H. Kagamiyama and H. Wada, *J. Biochem.*, **82**, 847

- (1977).
- 5) L. P. Sastchenko, E. S. Severin, D. E. Metzler and R. M. Khomutov, *Biochemistry*, **10**, 4888 (1971).
 - 6) G. T. Cori, B. Illingworth and P. J. Keller, "Methods in Enzymology," Vol. I, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1955, p. 200.
 - 7) B. M. Shapiro and E. R. Stadtman, "Methods in Enzymology," Vol. 17A, ed. by H. Tabor and C. W. Tabor, Academic Press Inc., New York, N. Y., 1970, p. 910.
 - 8) L. A. Fahien and P. P. Cohen, "Methods in Enzymology," Vol. 17A, ed. by H. Tabor and C. W. Tabor, Academic Press Inc., New York, N. Y., 1970, p. 839.
 - 9) W. B. Jakoby, "Methods in Enzymology," Vol. V, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1962, p. 770.
 - 10) Z. H. M. Verjee and E. F. Evered, *Biochim. Biophys. Acta*, **185**, 103 (1969).
 - 11) W. T. Jenkins and L. D'Ari, *Biochem. Biophys. Res. Commun.*, **22**, 376 (1966).
 - 12) J. B. Stothers, "Carbon-13 NMR Spectroscopy," Academic Press Inc., New York, N. Y., 1972.
 - 13) L. F. Johnson and W. C. Jankowski, "Carbon-13 NMR Spectra," Wiley-Interscience Publication, New York, N. Y., 1972.
 - 14) Y. Asada, K. Tanizawa, T. Nishino, S. Murao and K. Soda, *Seikagaku*, **53**, 845 (1981).
 - 15) C. T. Walsh, *Horizons in Biochemistry and Biophysics*, **3**, 36 (1977).
 - 16) F. Wold, "Methods in Enzymology," Vol. 46, ed. by W. B. Jakoby and M. Wilcheck, Academic Press Inc., New York, N. Y., 1977, p. 3.
 - 17) R. R. Rando, "Methods in Enzymology," Vol. 46, ed. by W. B. Jakoby and M. Wilcheck, Academic Press Inc., New York, N. Y., 1977, pp. 28, 158.
 - 18) T. Toraya, *Chemistry* (in Japanese), **35**, 87 (1980).
 - 19) A. E. Braunstein, "The Enzymes," Vol. IX (3rd ed.), ed. by P. D. Boyer, Academic Press Inc., New York, N. Y., 1973, p. 379.
 - 20) V. L. Florentiev, V. I. Ivanov and M. Y. Karpeisky, "Methods in Enzymology," Vol. 18A, ed. by D. B. McCormick and L. D. Wright, Academic Press Inc., New York, N. Y., 1970, p. 567.
 - 21) K. Soda, K. Tanizawa and N. Esaki, *Chemistry* (in Japanese), **35**, 97 (1980).
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