7-HYDRO-8-METHYLPTEROYLGLUTAMYLGLUTAMIC ACID, A NEW ANTI-FOLATE FROM AN ACTINOMYCETE

FERMENTATION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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A new anti-folate, 7-hydro-8-methylpteroylglutamylglutamic acid, was isolated from the culture broth of a soil actinomycete. The antibiotic inhibited the growth of *Enterococcus faecium* which requires folic acid. The inhibitory action was reversed by thymidine or excess amounts of folate-related compounds such as pteroic acid, folic acid, dihydrofolic acid and leucovorin. It inhibited thymidylate synthase from *E. faecium*, *Bacillus subtilis* and Ehrlich ascites carcinoma cells.

The authors have reported a screening method for new anti-folates of microbial origin[1]. In the course of the screening for new anti-folates from actinomycetes by the method, a new antibiotic was obtained from the culture filtrate of an actinomycete strain SK-2049, which was isolated from a soil sample collected at Sukumo-shi, Kochi Prefecture, Japan.

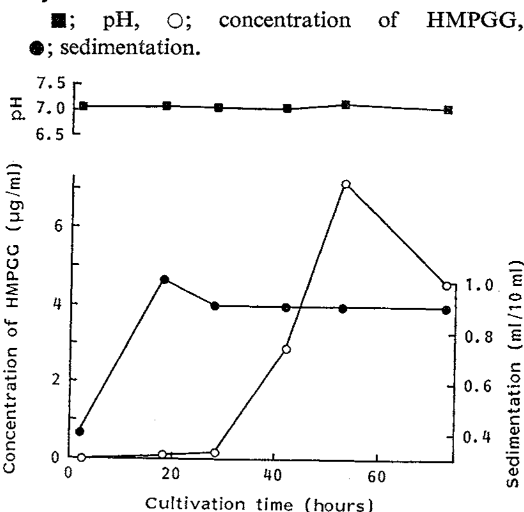
The present paper deals with the fermentation, isolation, physico-chemical and biological properties and structure of the antibiotic, 7-hydro-8-methylpteroylglutamylglutamic acid (HMPGG).

Fermentation and Isolation

A loopful of the aerial mycelia of a slant of strain SK-2049 was transferred into a seed medium (pH 7.0, 100 ml) containing glucose 0.1%, starch 2.4%, meat extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.4% in a 500-ml Sakaguchi flask, and incubated with reciprocal shaking for 3 days at 27°C to give a seed culture for production of the antibiotic. The seed culture was transferred at the rate of 2% into the production medium (pH 7.0, 70 liters) containing dextrin 2.0%, glucose 0.2%, soybean meal 1.5%, yeast extract 0.3% and $CaCO_3$ 0.3% in a 100-liter tank and then incubated at 27°C for 4 days with aeration of 10 liters of air per minute and agitation of 200 rpm. A typical time course of the antibiotic HMPGG production by strain SK-2049 is shown in Fig. 1.

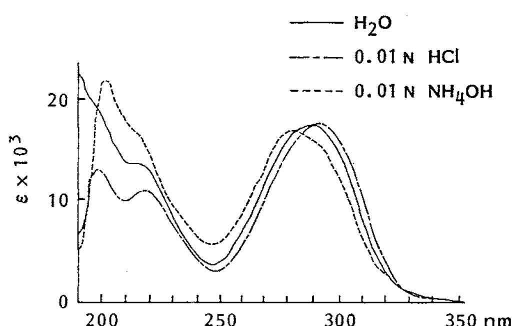
Fig. 1. Time cource of the production of HMPGG by strain SK-2049.

■; pH, ○; concentration of HMPGG, ●; sedimentation.



After 4-day incubation the cultured broth (70 liters) was centrifuged and the supernatant passed through a column of Diaion WA-30

Fig. 2. UV spectrum of HMPGG.



(OH^-, 7.5 liters). After washing with water, the adsorbed material was eluted with 0.5 N HCl. The active eluate was applied to Diaion HP-20 column (1.2 liters). After washing with water, the active material was eluted with 50% MeOH. The eluate was concentrated *in vacuo*, passed through a column of Amberlite IRC-50 (NH_4^+, 400 ml) and applied to Amberlite IR-120 (H^+, 1.0 liter) column. After washing with water, the active material was eluted with 0.5 N NH_4OH. The eluate was concentrated *in vacuo* and washed with butanol. The water layer was concentrated *in vacuo*, applied to Biogel P-4 column (28.1 mm × 560 mm) and developed with water. The active fraction was concentrated *in vacuo*, freeze-dried and applied to a DEAE-Sephadex column (16 mm × 600 mm) previously equilibrated with 10 mM phosphate buffer (pH 6.0) containing 20 mM 2-mercaptoethanol. The adsorbed material was eluted with a gradient from 10 to 800 mM phosphate buffer (pH 6.0) containing 20 mM 2-mercaptoethanol. The active fraction was desalted with Diaion HP-20 column, concentrated *in vacuo* and freeze-dried. The resulting powder was further purified by reversed phase HPLC (Jeol, TRI-MOTER-V, YMC A-343 column). Using a gradient system from 0 to 20% MeOH containing 0.01 N HCOOH, HMPGG appeared as a single peak with a retention time of 34 minutes. After concentration and lyophilization, HMPGG was obtained as a yellowish powder (7 mg, yield 2.2%).

Physico-chemical Properties and Structure

HMPGG was obtained as a yellowish powder: MP >196°C (dec); $[\alpha]_D^{20}$ +24° (*c* 0.25, H_2O). The antibiotic is amphoteric and soluble in alkaline water, but practically insoluble in organic solvents such as lower alcohols, ethyl acetate and chloroform. It shows positive color reaction to the Rydon-Smith reagent and negative color reaction to ninhydrin and anisaldehyde - H_2SO_4. The molecular formula ($C_{25}H_{30}N_8O_9$) was established by the combination of elemental analysis (found: C 44.46, H 5.52, N 16.28%; calcd for $C_{25}H_{30}N_8O_9 \cdot 5H_2O$: C 44.43, H 5.96, N 16.56%) and field desorption mass spectroscopy (FD-MS) (*m/z* 609 (M^+ +Na), 587 (M^+ +1)). The UV spectrum (Fig. 2) and IR spectrum (1640 and 1570 cm^{-1}) suggested the presence of a 7,8-dihydropterin and a peptide linkage, respectively. FD-MS (*m/z* 84) also revealed a pterine ring.

In the ^1H COSY 2D NMR spectrum (Fig. 3), a pair of doublets (δ 6.71 (2H, d, J=9.3 Hz) and 7.66 (2H, d, J=9.3 Hz)) and a pair of signals coupled each other (δ 1.72 (1H, m), 1.88 (1H, m), 2.06 (2H, t, J=8.3 Hz) and 4.00 (1H, dd, J=4.6 and 9.0 Hz) and δ 1.95 (1H, m), 2.10 (1H, m), 2.30 (2H, m) and 4.30 (1H, dd, J=4.2 and 9.0 Hz)) indicated the presence of a 1,4-substituted benzene and two pairs of $-\overset{|}{C}HCH_2CH_2-$ moiety. Two singlets (δ 3.30 (2H, s) and 4.05 (2H, s)) out of the unassigned three singlets were quite similar to those of C(7) and C(9)-H_2 of 7,8-dihydrofolic acid (DHF) and the last singlet (δ 2.50 (3H, s)) was assigned to N(8)-CH_3.

HMPGG was hydrolyzed (6 N HCl, 15 hours, 110°C) and the trimethylsilyl (TMS) derivatives of the hydrolysate were analyzed with GC-MS (Jeol JMS-DX 300 JMA-3100, OV-17 column). The main peaks observed were identified as di-TMS-glutamic acid and di-TMS-*p*-aminobenzoic acid in a ratio of 2:1 (Fig. 4). These results correspond to the observations in ^1H NMR analysis described

Fig. 3. ^{1}H COSY 2D NMR spectrum of HMPGG (in D_2O, 400 MHz). DHO was used as an internal standard.

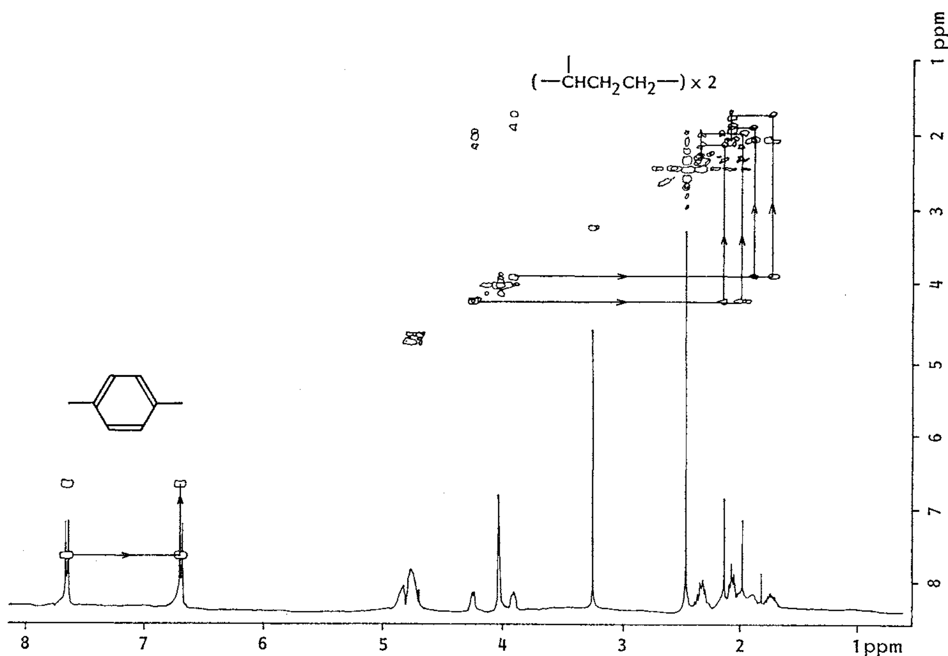
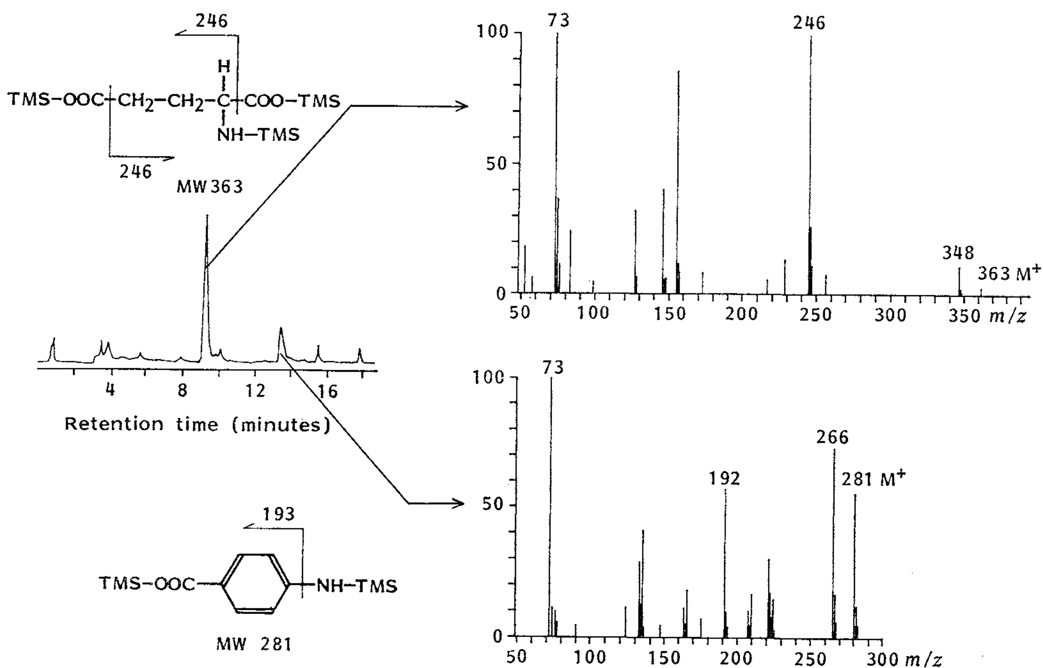


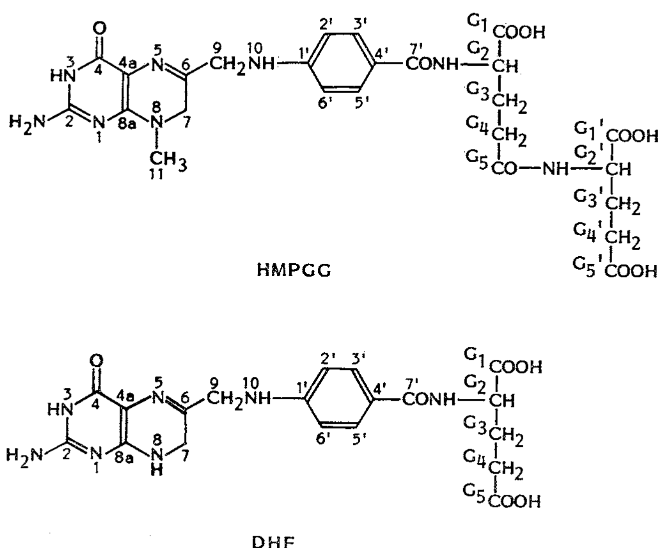
Fig. 4. GC-MS spectrum of TMS derivatives of acid hydrolysate of HMPGG.



above and confirm that two mol of glutamic acid, one mol of *p*-aminobenzoic acid and a 7,8-dihydropterin are involved in the structure of the antibiotic.

The ^{1}H and ^{13}C NMR signals were compared with those of DHF (Table 1). The comparison

Table 1. NMR spectral data of HMPGG, DHF and folic acid.



Position	HMPGG		DHF		Folic acid
	^{13}C NMR[a]	^{1}H NMR[b]	^{13}C NMR[5)]	^{1}H NMR[c]	^{13}C NMR[6)]
2	163.1[d]		162.1		155.0
4	170.5[e]		170.5[f]		166.9
4a	110.1		104.4		126.7
6	163.6[d]		152.1[g]		148.4
7	47.5 (CH_2)	3.30 (2H, s)	43.2	3.4 (s)	148.4
8a	163.7[d]		155.7[g]		153.5
9	49.7 (CH_2)	4.05 (2H, s)	48.5	4.0 (s)	45.8
11	34.1 (CH_3)	2.50 (3H, s)			
1′	151.5		152.4[g]		151.7
2′, 6′	131.1 (2×C)	6.71 (2H, d)	129.9[h](2×C)	7.6 (d)[j]	112.4
3′, 5′	130.1 (2×C)	7.66 (2H, d)	113.0[h](2×C)	6.7 (d)[j]	129.6
4′	123.2		121.9		121.5
7′	171.9[e]		170.8[f]		169.2
G_1, G_1'	179.6, 179.7		183.3[i]		180.1
G_2, G_2'	56.2 (CH)(2×C)	4.00 (1H, dd), 4.30 (1H, dd)	56.8	4.3 (br)	56.9
G_3, G_3'	28.6 (CH_2), 29.3 (CH_2)	1.72 (1H, m), 1.88 (1H, m), 1.95 (1H, m), 2.10 (1H, m)	29.4	1.8~2.3 (br)	29.4
G_4, G_4'	33.4 (CH_2), 35.0 (CH_2)	2.06 (2H, t), 2.30 (2H, m)	35.1		35.2
G_5, G_5'	183.0, 176.0		180.4[i]		183.0

All values show chemical shifts in ppm in D_2O.

[a] Dioxane was used as an internal standard (400 MHz).

[b] DHO was used as an internal standard (400 MHz).

[c] DHO was used as an internal standerd (90 MHz).

[d~g] Assignments may be interchanged.

[h~j] These assignments should be interchanged according to other data[6~8)].

revealed that HMPGG possesses additional *N*-methyl and glutamyl moieties. The *N*-methyl group was assigned to N(8) position as described above. The additional glutamyl group is attached to the first one at either α- or γ-position. Because in the ^{13}C NMR spectrum of HMPGG, signals corresponding to two α-COOH (δ 179.6 and 179.7) and a γ-COOH (δ 183.0) other than an amide (δ 176.0) were observed, the γ-linkage of the second glutamic acid seemed to be preferable to the α-one. The lower field shift of one of the methylene signals (δ_H 2.30) next to the carbonyl group also supports this hypothesis.

From the observations described above it follows that the structure of the antibiotic is 7-hydro-8-methylpteroylglutamylglutamic acid.

Biological Activity

HMPGG showed inhibitory activity only against *Enterococcus faecium* in a Nissui folic acid assay medium[1] supplemented with a limited amount of pteroic acid (1.0 ng/ml). It did not exhibit antimicrobial activity, not even in a minimal medium against bacteria and fungi except *E. faecium* (Table 2).

Table 3 shows that the inhibitory activity of HMPGG against *E. faecium* was reversed by excess amounts of folate-related compounds (1.0 mg/ml). HMPGG exhibited no inhibitory activity in a medium supplemented with thymidine or thymine. These results suggest that HMPGG inhibits thymidylate (TMP) synthase.

The inhibitory effect of HMPGG on TMP synthase was examined. The preparation and assay of TMP synthase from *E. faecium* and Ehrlich ascites carcinoma cells were carried out as previously described[2]. The enzyme from *B. subtilis* was prepared and assayed by a method similar to that used with the enzyme from *E. faecium*. As shown in Fig. 5, HMPGG inhibited

Table 2. Antibacterial spectrum of HMPGG.

Test organism	Inhibitory zone (mm)*
Staphylococcus aureus ATCC 6538P	—
Micrococcus luteus ATCC 9341	—
Bacillus subtilis PCI 219	—
Enterococcus faecium IFO 3181	23.8
Mycobacterium smegmatis ATCC 607	—
Escherichia coli NIHJ	—
Pseudomonas aeruginosa P-3 PCI 602	—
Xanthomonas oryzae KB 88	—
Candida albicans KF 1	—
Saccharomyces sake KF 26	—
Aspergillus niger KF 105	—
Piricularia oryzae KF 180	—

Medium: DAVIS' minimal medium for bacteria (37°C, 20 hours). Glucose-CZAPEK's medium for fungi (27°C, 2 days). Folic Acid Assay Medium "Nissui" for *E. faecium*.
* Paper disk method. Concentration of HMPGG: 100 μg/ml.

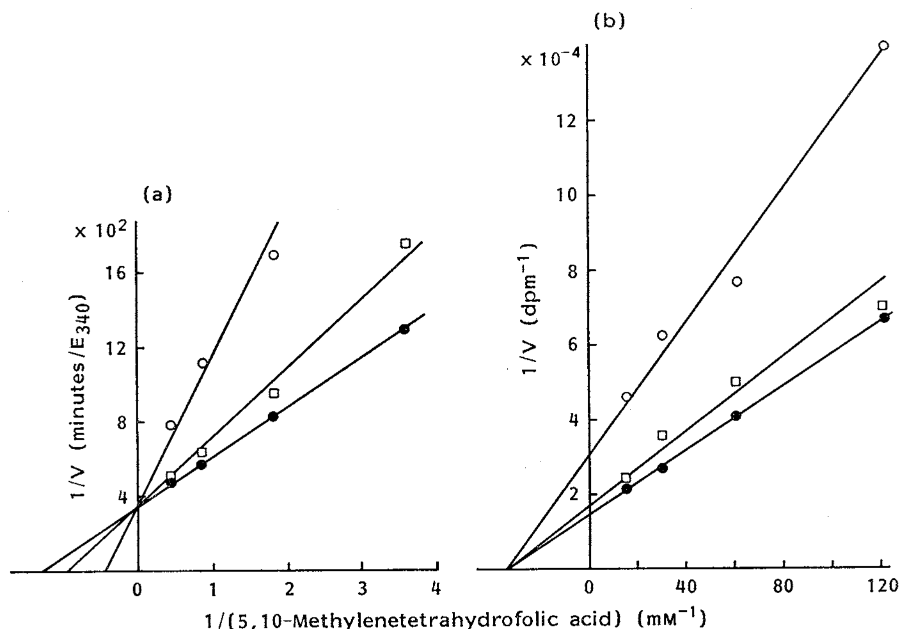
Table 3. Reversal effect of folate-related compounds, and purine - pyrimidine bases and nucleosides on the antibacterial activity of HMPGG against *Enterococcus faecium* in Folic Acid Assay Medium "Nissui".

Reversant	Concentration (μg/ml)		Reversant	Concentration (μg/ml)
	1	1,000		1,000
Pteroic acid	—	+	Adenosine	—
Folic acid	—	+	Guanosine	—
Dihydrofolic acid	—	+	Uridine	—
Leucovorin	—	+	Hypoxanthine	—
Thymidine		+	Adenine	—
Thymine		+	Guanine	—
Inosine		—	Uracil	—

+ Shows reversal. — Shows no reversal.

Fig. 5. Inhibitory action of HMPGG on TMP synthase from *Bacillus subtilis* and Ehrlich ascites carcinoma cells.

(a) *B. subtilis*, Km=180 μM, Ki=10.9 nM. (b) Ehrlich ascites carcinoma cells, Km=30.3 μM, Ki=75.2 nM.



the enzyme from *E. faecium* and *B. subtilis* competitively with 5,10-methylenetetrahydrofolic acid, and that from Ehrlich ascites carcinoma cells non-competitively. Km values for 5,10-methylenetetrahydrofolic acid of the enzyme preparations from *E. faecium*, *B. subtilis* and Ehrlich ascites carcinoma cells were 88.5, 180, and 30.3 μM, respectively and Ki values for HMPGG were 5.7, 10.9 and 75.2 nM, respectively.

No toxicity of HMPGG was observed even when it was intraperitoneally administered to mice at a dose of 200 mg/kg.

Discussion

The structure of HMPGG was determined to be 7-hydro-8-methylpteroylglutamylglutamic acid in which a methyl group substitutes hydrogen in 8-position of dihydrofolic acid and the second glutamic acid is linked to the *C*-terminal glutamic acid through a peptide bond. 5-Formyltetrahydropteroyloligoglutamic acid[3] and pteroyloligoglutamic acid[4] have been reported as folate-related compounds which inhibit TMP synthase. However, no compounds which have the structure of HMPGG are known. Thus, HMPGG is a new folate-related compound which inhibits TMP synthase.

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