SYNTHESIS AND BIOLOGICAL ACTIVITY OF ANALOGUES OF DIAZAQUINOMYCIN A, A NEW THYMIDYLATE SYNTHASE INHIBITOR

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Diazaquinomycin A (**1**), a new thymidylate (TMP) synthase inhibitor, is poorly soluble in various solvents and exhibits no antitumor activity, while a series of the analogues prepared from **1** are more soluble in water and chloroform than **1**, and some of them exhibit antitumor activity in mice. Some analogues in which the lactam rings are replaced by pyridine rings did not inhibit TMP synthase. The diethoxy analogue **25** is a 10-fold more potent inhibitor of TMP synthase than **1**. The diacetoxy analogue **23** exhibits significant antitumor activity (T/C: 175%) against Meth-A fibrosarcoma in mice.

Diazaquinomycin A (**1**), possessing an unique 1,8-diazaanthraquinone skeleton, is a new antibiotic found as a folic acid antagonist by a screening method established for inhibitors of folate metabolism.[1~4] Recently, some of the present authors have shown that **1** inhibits thymidylate (TMP) synthase competitively with 5,10-methylenetetrahydrofolate and exhibits effective cytotoxicity against tumor cells, but has no *in vivo* effect on tumors in mice, probably because of its extremely low solubility. In fact, solid residues of **1** were observed when mice were dissected after a suspension of **1** in 0.5% gum arabic was administered intraperitoneally.

This paper describes the synthesis of diazaquinomycin analogues with the aim to improve the solubility of **1** and to evaluate their *in vitro* and *in vivo* activities, including antitumor activity.

Chemical modification of **1** is limited by its very low solubility in organic and aqueous media. However, conversion of the lactam rings of **1**, which are necessary for manifestation of biological activity, to 2-methoxypyridine rings improved the solubility in organic solvents and served to activate the methyl groups, which could then be brominated. Nucleophilic substitution of the aryl bromide by several nucleophiles provided a versatile method to introduce polar groups onto the methyl groups of **1**. Thereafter, acidic treatment reconstituted the 2-oxopyridine rings.

Chemistry

1 was treated with methyl iodide and silver oxide in chloroform to give the imino ether **2** possessing one 2-methoxypyridine and one lactam ring. Refluxing the reaction mixture afforded **3**. Bromination of the two imino ethers gave **4** and **5**. Substituting the bromides by a series of nucleophiles such as cyanide, malonate anion, water, acetic acid, ethanol and morpholine, and then hydrolyzing the imino ethers with H_2SO_4 - EtOH gave the diazaquinomycin analogues as shown in Scheme 1.

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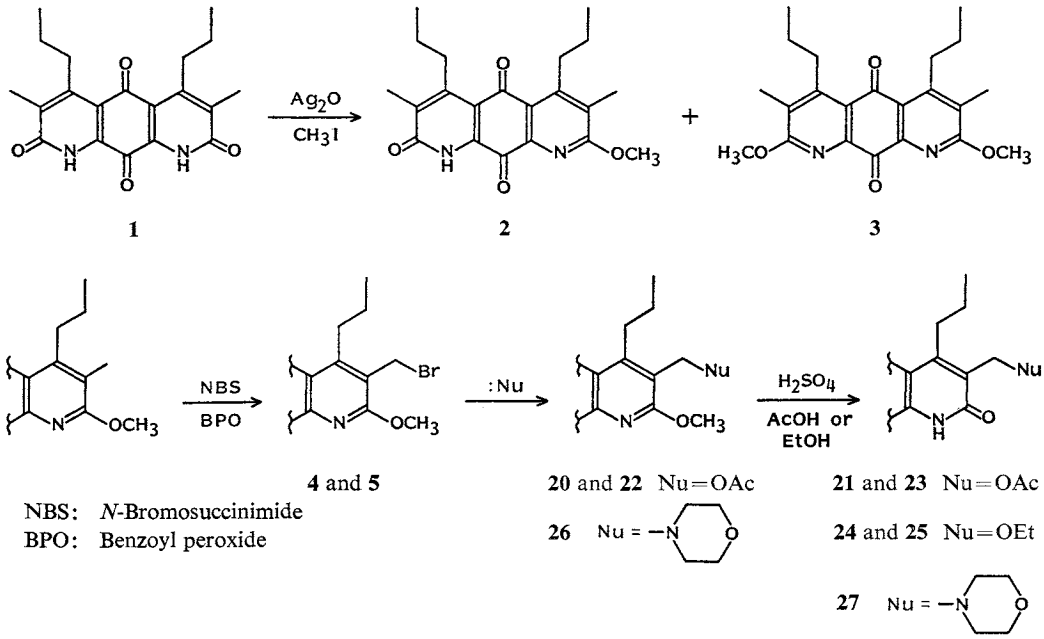
Scheme 1.



1 2 3

4 and 5

20 and 22 Nu=OAc

21 and 23 Nu=OAc

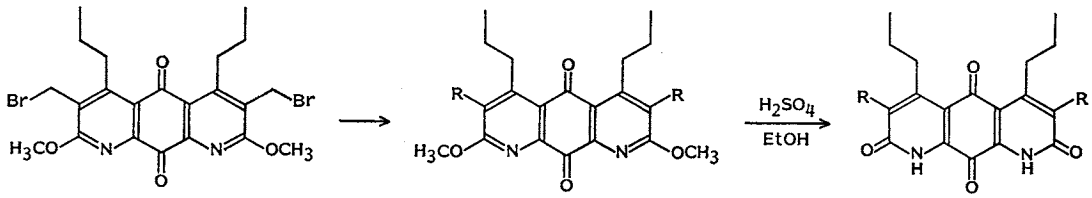
26 Nu = —N O

24 and 25 Nu=OEt

27 Nu = —N O

NBS: *N*-Bromosuccinimide
BPO: Benzoyl peroxide

Scheme 2.



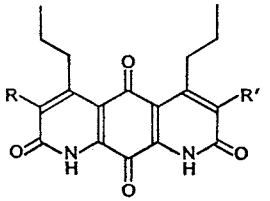
5

6 R=CH_2CN
9 R=$CH_2CH(COOEt)_2$
12 R=$CH_2CH(COOH)_2$
13 R=CH_2CH_2COOH
16 R=CH_2OH
17 R=COOH

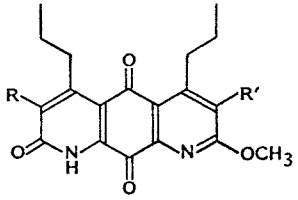
7 R=CH_2COOEt
8 R=CH_2COOH
10 R=$CH_2CH(COOEt)_2$
11 R=$CH_2CH(COOH)_2$
14 R=CH_2CH_2COOEt
15 R=CH_2CH_2COOH
18 R=COOEt
19 R=COOH

Cyanation of the dibromide **5** with tetraethylammonium cyanide[5] gave the dicyanide **6**.[8] The cyano groups were converted to ethyl esters with H_2SO_4 - EtOH and at the same time the imino ethers were hydrolyzed to give the ester **7**. Substitution of **5** by ethyl malonate anion gave the ester **9**, and subsequent hydrolysis with H_2SO_4 - EtOH gave the dimalonate ester **10**. Successive treatments of **9**, such as hydrolysis of the esters with NaOH - aq EtOH, decarboxylation in pyridine and hydrolysis of the imino ethers with H_2SO_4 - EtOH, gave the ester **14**. Hydrolysis of the dibromide **5** with water and THF at reflux without acid and base gave the diol **16**. The hydroxyl groups were oxidized with CrO_3 - H_2SO_4 leading to the dicarboxylic acid **17**. Further treatment of **17** with H_2SO_4 - EtOH afforded the ester **18**. Hydrolysis with NaOH - aq EtOH gave the corresponding carboxylic acids as shown in Scheme 2.

Table 1. *In vitro* activities and solubilities of diazaquinomycin A analogues.



Type I



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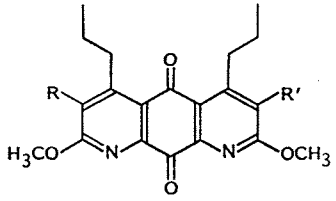
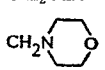
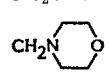


Type III

Compound	Type	R	R′	Inhibitory activity against *Enterococcus faecium* MIC (μg/ml)	Inhibitory activity against *Enterococcus faecium* Reversal with TdR	Inhibitory activity against HeLa cells MIC (μg/ml)	Inhibitory activity against TMP synthase[a] IC_{50} (μg/ml)	Solubility (μg/ml) $CHCl_3$	Solubility (μg/ml) H_2O
1	I	CH_3	CH_3	1.0~2.0	++	0.16~0.31	4.2	640	0.15
2	II	CH_3	CH_3	>500		>5	>20		
3	III	CH_3	CH_3	>500		2.5	>20		
6	III	CH_2CN	CH_2CN	>500		1.25	>20		
7	I	CH_2COOEt	CH_2COOEt	3.9	+	0.31	1.1	5,980	1.8
8	I	CH_2COOH	CH_2COOH	>500		>5	1.5		
10	I	$CH_2CH(COOEt)_2$	$CH_2CH(COOEt)_2$	62.5	+	2.5	1.6	4,360	0.62
11	I	$CH_2CH(COOH)_2$	$CH_2CH(COOH)_2$	>500		5	1.3		
14	I	CH_2CH_2COOEt	CH_2CH_2COOEt	3.9	+	1.25	2.1		
15	I	CH_2CH_2COOH	CH_2CH_2COOH	>500		5	3.4		
18	I	COOEt	COOEt	250		5	1.0		
19	I	COOH	COOH	>500		>5	2.4		
21	I	CH_3	CH_2OAc	2.0	+	0.63	2.0		
22	III	CH_2OAc	CH_2OAc	>500		2.5	>20		
23	I	CH_2OAc	CH_2OAc	3.9	±	0.31	1.0	3,180	0.51
24	I	CH_3	CH_2OEt	1.0	++	0.31	0.7		
25	I	CH_2OEt	CH_2OEt	1.0	++	0.16	0.4	3,500	0.22
27	I			>500		>5	1.0		

[a] Prepared from Ehrlich ascites carcinoma cells.

++: Well reversed, +: moderately reversed, ±: weakly reversed.

Treatment of the monobromide **4** and the dibromide **5** with sodium acetate and acetic acid gave the acetates **20** and **22**, which were converted to the monoacetate **21** and the diacetate **23**, respectively, with H_2SO_4 - AcOH.

Alcoholysis of **4** and **5** with H_2SO_4 - EtOH gave the ethyl ethers **24** and **25**, respectively, accompanied by hydrolysis of the imino ether. Reaction of **5** with morpholine and a following hydrolysis of the imino ether gave the diamine **27**.

Results and Discussion

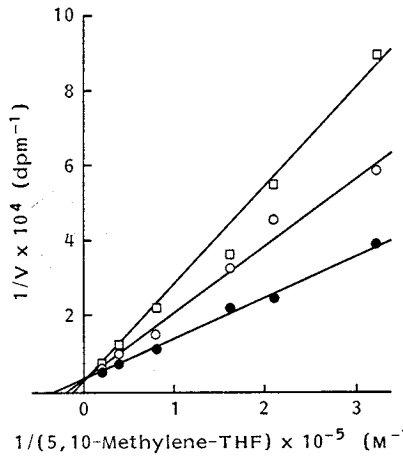
Table 1 summarizes the antimicrobial activities of the synthesized analogues of **1** against the folate requiring *Enterococcus faecium*, their reversal by thymidine (TdR), the cytotoxicities against HeLa cells, the inhibitory activities against TMP synthase prepared from Ehrlich ascites carcinoma cells, and the solubilities in water and chloroform.

All mono- or dimethylated analogues of **1** (Types II and III) exhibited extremely reduced biological activities. The carboxylic acid analogues (**8**, **11**, **15** and **19**) were devoid of activities against *E. faecium* and HeLa cells, but the corresponding esters (**7**, **10**, **14** and **18**) were active. Both the carboxylic acids and their esters, however, were more potent inhibitors of TMP synthase than **1**. These results suggest that the carboxylic acids cannot penetrate the cell membrane. **18** exhibited poor activity against *E. faecium*, but **7** and **14** both containing longer side chains exhibited similar activities against *E. faecium* as did **1**. **7** was most cytotoxic.

Compounds **21** and **24**, which have substituents on one of the two methyl groups of **1**, were only half as potent as **23** and **25**, which have the corresponding substituents on both methyl groups of **1**. All analogues containing two lactam rings (Type I) were more potent inhibitors of TMP synthase

Fig. 1. Mode of inhibitory action of **25** against TMP synthase from Ehrlich ascites carcinoma cells.

● 0 μM, ○ 0.8 μM, □ 1.6 μM.



The results of Lineweaver-Burk plot showed that it inhibits the enzyme competitively with the substrate 5,10-methylene-THF. With the enzyme, *Km* was 45 μM, and *Ki* was 14 μM.

Table 2. Antitumor activities of diazaquinomycin A analogues on Meth-A fibrosarcoma.

Compound	Dose (mg/kg/day × days)	Mean survival days	T/C (%)
1	1×4	12.0	96
	10×4	12.0	96
	100×4	11.5	92
7	1×4	12.3	100
	10×4	12.4	101
	100×4	14.3±0.75	116
23	1×4	12.2	99
	10×4	17.3±2.56	141*
	100×4	21.5±2.06	175*
25	1×4	12.1	98
	10×4	11.8	96
	100×4	12.5	102
5-FU	20×1	17.5	142
Control	—	12.3±0.95	100

* $P<0.05$.

5-FU: 5-Fluorouracil.

than **1**. Among them, **25** was the most potent inhibitor of TMP synthase and inhibited the enzyme competitively with 5,10-methylene-THF as substrate (Fig. 1). The inhibitory activity of **25** was 10-fold stronger than that of **1**.

Among the synthesized analogues, three compounds (**7**, **23** and **25**) exhibited activities against HeLa cells similar to those of **1**, and were more soluble in water and chloroform than **1**. Their antitumor activities against Meth-A fibrosarcoma in mice were examined and the results are summarized in Table 2. The diacetoxy derivative **23** exhibited significant antitumor activity (T/C: 175%) corresponding to that of 5-fluorouracil (5-FU).

Experimental

MP's were determined on a micro melting point apparatus (Yanaco MP-3). They were uncorrected. NMR spectra were run in $CDCl_3$ or MeOH-d_4 on a 90 MHz spectrometer (Jeol FX-90Q). IR spectra were taken on a Jasco A-102 spectrometer. MS were determined by high-resolution electron impact mass spectrometer (JMS-DX300, JMA-3100). Elemental analysis for carbon, hydrogen and nitrogen were determined with a Perkin-Elmer Model 240 elemental analyzer. Column chromatography was performed on Silica gel 60 (Art. No. 7734, Merck).

2-Methoxy-3,7-dimethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-5,8,10(9*H*)-trione (**2**)

A solution of diazaquinomycin A (**1**) (203 mg, 0.57 mmol), methyl iodide (4 ml, 64.2 mmol) and silver oxide (398 mg, 1.72 mmol) in $CHCl_3$ (20 ml) was stirred for 20 hours at room temperature. The mixture was filtered and the filtrate was evaporated. The resulting crude product was purified by column chromatography, eluting with $CHCl_3$. An orange crystalline solid was obtained, 151 mg (72%): MP 288~289°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2960, 1645; MS calcd for $C_{21}H_{24}N_2O_4$ 368.174, observed 368.174.

2,8-Dimethoxy-3,7-dimethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-5,10-dione (**3**)

A suspension of **1** (250 mg, 0.71 mmol), methyl iodide (5 ml, 80.3 mmol) and silver oxide (500 mg, 2.16 mmol) in $CHCl_3$ (25 ml) was heated at reflux for 2 hours. As described for **2**, the mixture was treated and purified to give a yellow crystalline solid, 235 mg (87%): MP 136~138°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2970, 2880, 1695, 1655; MS calcd for $C_{22}H_{26}N_2O_4$, 382.189, observed 382.189.

3-Bromomethyl-2-methoxy-7-methyl-4,6-dipropylpyrido[3,2-*g*]quinoline-5,8,10(9*H*)-trione (**4**)

A solution of **2** (242 mg, 0.66 mmol), *N*-bromosuccinimide (242 mg, 1.36 mmol) and benzoyl peroxide (20 mg, 0.08 mmol) in CCl_4 (93 ml) was heated at reflux for 1 hour. The mixture was cooled at 0°C and filtered. The solids were suspended in CCl_4 and kept at 50°C for 30 minutes. The suspension was filtered, and the solids were dissolved in $CHCl_3$ (100 ml). The solution was washed with water (100 ml) and dried over anhydrous Na_2SO_4. After filtration the solvent was removed to yield a yellow crystalline solid, 224 mg (76%): MP 248~250°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2960, 2925, 1655, 1600; MS calcd for $C_{21}H_{23}BrN_2O_4$, 446.084, observed 446.082.

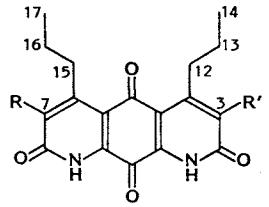
3,7-Dibromomethyl-2,8-dimethoxy-4,6-dipropylpyrido[3,2-*g*]quinoline-5,10-dione (**5**)

A solution of **3** (500 mg, 1.31 mmol), *N*-bromosuccinimide (700 mg, 3.93 mmol) and benzoyl peroxide (10 mg, 0.04 mmol) in CCl_4 (40 ml) was heated at reflux for 2.5 hours. To the mixture was added water (40 ml) and the product was extracted with $CHCl_3$ (3×40 ml). The combined organic extracts were dried (Na_2SO_4) and evaporated. The resulting crude product was purified by column chromatography, eluting with $CHCl_3$. A yellow crystalline solid was obtained 520 mg (74%): MP 226~227°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2975, 1730, 1700, 1670, 1585; MS calcd for $C_{22}H_{24}Br_2N_2O_4$, 538.010, observed 538.007.

2,8-Dimethoxy-4,6-dipropyl-5,10-dioxo-5,10-dihydropyrido[3,2-*g*]quinoline-3,7-diacetonitrile (**6**)

To a solution of **5** (500 mg, 0.93 mmol) in CH_2Cl_2 (50 ml) was added dropwise a solution of tetraethylammonium cyanide (289 mg, 1.85 mmol) in CH_2Cl_2 (50 ml) at 0°C in 4 hours. In a similar

Table 3. ^{1}H NMR parameters (δ) for diazaquinomycin A analogues.[a]



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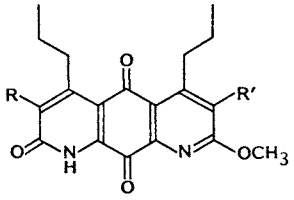

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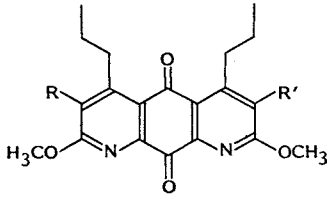
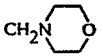
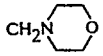
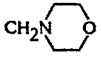
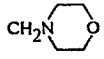


Type III

Compound	Type	R	R′	12-H, 15-H	13-H, 16-H	14-H, 17-H	OCH_3	Other
2	II	CH_3	CH_3	3.07 t	1.59 m	1.10 t	4.13 s	2.27 s (3-CH_3), 2.30 s (7-CH_3)
3	III	CH_3	CH_3	3.09 t	1.57 m	1.09 t	4.15 s	2.28 s (3,7-CH_3)
4	II	CH_3	CH_2Br	2.69 t	1.59 m	1.17 t	4.14 s	2.31 s (7-CH_3), 4.60 s (3-CH_2)
5	III	CH_2Br	CH_2Br	3.20 t	1.69 m	1.16 t	4.22 s	4.62 s (3,7-CH_2)
6	III	CH_2CN	CH_2CN	3.17 t	1.64 m	1.15 t	4.24 s	3.82 s (3,7-CH_2)
7	I	CH_2COOEt	CH_2COOEt	3.00 t	1.54 m	1.08 t		1.27 t ($COOCH_2CH_3$), 3.78 s (3,7-CH_2), 4.19 q ($COOCH_2CH_3$)
8	I	CH_2COOH	CH_2COOH	2.96 t	1.30 m	1.00 t		3.60 s (3,7-CH_2)
9	III	$CH_2CH(COOEt)_2$	$CH_2CH(COOEt)_2$	3.15 t	1.55 m	1.08 t	4.15 s	1.21 t ($CH(COOCH_2CH_3)_2$), 3.34 d (3,7-CH_2), 3.38 t ($CH(COOCH_2CH_3)_2$), 4.15 q ($CH(COOCH_2CH_3)_2$)
10	I	$CH_2CH(COOEt)_2$	$CH_2CH(COOEt)_2$	3.07 t	1.50 m	1.16 t		1.24 t ($CH(COOCH_2CH_3)_2$), 3.29 d (3,7-CH_2), 4.18 q ($CH(COOCH_2CH_3)_2$

12	III	$CH_2CH(COOH)_2$	$CH_2CH(COOH)_2$	3.28 t	1.50 m	1.09 t	4.14 s	3.40 d (3,7-CH_2), 3.70 t ($CH(COOH)_2$)
13	III	CH_2CH_2COOH	CH_2CH_2COOH	3.08 t	1.55 m	1.05 t	4.16 s	2.60 t (CH_2COOH), 3.08 t (3,7-CH_2)
14	I	CH_2CH_2COOEt	CH_2CH_2COOEt	3.06 t	1.51 m	1.11 t		1.27 t ($CH_2COOCH_2CH_3$), 2.61 t ($CH_2COOCH_2CH_3$), 3.06 t (3,7-CH_2), 4.15 q ($CH_2COOCH_2CH_3$)
16	III	CH_2OH	CH_2OH	3.13 t	1.61 m	1.08 t	4.18 s	4.79 s (3,7-CH_2)
17	III	COOH	COOH	3.11 t	1.65 m	1.06 t	4.16 s	
18	I	COOEt	COOEt	2.91 t	1.60 m	1.06 t		1.20 t ($COOCH_2CH_3$), 4.40 q ($COOCH_2CH_3$)
20	II	CH_3	CH_2OAc	3.10 t	1.59 m	1.10 t	4.14 s	2.09 s (7-CH_3), 2.29 s ($COCH_3$), 5.23 s (3-CH_2)
21	I	CH_3	CH_2OAc	3.02 t	1.57 m	1.10 t		1.26 t ($COCH_3$), 2.29 s (7-CH_3), 5.21 s (3-CH_2)
22	III	CH_2OAc	CH_2OAc	3.17 t	1.55 m	1.11 t	4.24 s	2.10 s ($COCH_3$), 5.28 s (3,7-CH_2)
23	I	CH_2OAc	CH_2OAc	3.11 t	1.60 m	1.17 t		2.08 s ($COCH_3$), 5.22 s (3,7-CH_2)
24	I	CH_3	CH_2OEt	3.15 t	1.60 m	1.11 t		1.25 t (OCH_2CH_3), 2.28 s (7-CH_3), 3.62 q (OCH_2CH_3), 4.58 s (3-CH_2)
25	I	CH_2OEt	CH_2OEt	3.15 t	1.65 m	1.10 t		1.24 t (OCH_2CH_3), 3.63 q (OCH_2CH_3), 4.95 s (3,7-CH_2)
26	III			3.34 t	1.59 m	1.11 t	4.13 s	2.48 t (NCH_2CH_2O), 3.60 s (3,7-CH_2), 3.65 t (NCH_2CH_2O)
27	I			3.18 t	1.75 m	1.10 t		2.55 t (NCH_2CH_2O), 3.65 s (3,7-CH_2), 3.70 t (NCH_2CH_2O)

[a] NMR spectra were run in $CDCl_3$ (compound **8**; MeOH-d_4) on a 90 MHz spectrometer.
Field strengths are expressed in units of δ (ppm).

manner to the preparation of **5**, the mixture was treated and purified to give a yellow crystalline solid, 306 mg (87%): MP 190~193°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2955, 1700, 1665, 1585; MS calcd for $C_{24}H_{24}N_4O_4$ 432.180, observed 432.180.

4,6-Dipropyl-2,5,8,10-tetraoxo-1,2,5,8,9,10-hexahydropyrido[3,2-*g*]quinoline-3,7-diethyl Diacetate (**7**)

A solution of **6** (52 mg, 0.12 mmol) in 30% H_2SO_4 - EtOH (6 ml) was heated at reflux for 2 hours. To the mixture was added water (5 ml) and the product was extracted with $CHCl_3$ (3×5 ml). The combined organic extracts were dried (Na_2SO_4) and evaporated. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (50:1). A red crystalline solid was obtained, 33 mg (55%): MP 267~268°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3360, 1735, 1660; MS calcd for $C_{26}H_{30}N_2O_8$, 498.200, observed 498.201.

4,6-Dipropyl-2,5,8,10-tetraoxo-1,2,5,8,9,10-hexahydropyrido[3,2-*g*]quinoline-3,7-diacetic Acid (**8**)

To a solution of **7** (30 mg, 0.06 mmol) in 80% EtOH - H_2O (12 ml) was added NaOH (30 mg, 0.75 mmol) and the mixture was stirred at 50°C for 30 minutes. After the mixture was acidified with 1 N HCl, the precipitate was filtered and the solid was washed with water (12 ml). This solid was dried *in vacuo* to give a red crystalline solid, 21 mg (82%): MP 300°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3600~2500, 2975, 2945, 2880, 1710, 1645; field desorption (FD)-MS *m/z* 443 (M^++1).

Anal Calcd for $C_{22}H_{22}N_2O_8$: C 59.73, H 4.98, N 6.33.
Found: C 59.55, H 4.78, N 6.03.

2,8-Dimethoxy-3,7-dimethyl-4,6-dipropyl-5,10-dioxo-5,10-dihydropyrido[3,2-*g*]quinoline-3′,7′-dimalonic Acid Tetraethyl Ester (**9**)

To a suspension of sodium hydride (60% in oil) (388 mg, 16.2 mmol) in dry THF (100 ml) was added dropwise diethyl malonate (1.8 ml, 11.8 mmol), and the reaction mixture was stirred for 0.5 hour to prepare the anion of diethyl malonate. To the mixture was added a solution of **5** (1.5 g, 2.79 mmol) in dry THF (60 ml), and the resulting solution was stirred for 0.5 hour at room temperature. To the mixture was added EtOH (0.5 ml) to quench the reaction. After concentration *in vacuo*, water (60 ml) was added to the residue. The mixture was acidified with 1 N HCl, and extracted with $CHCl_3$ (3×60 ml). The combined extracts were dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$. A yellow crystalline solid was obtained, 1.3 g (74%): MP 97~100°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3010, 1740, 1705, 1675, 1595; MS calcd for $C_{36}H_{46}N_2O_{12}$, 698.305, observed 698.307.

3,7-Dimethyl-4,6-dipropyl-2,5,8,10-tetraoxo-1,2,5,8,9,10-hexahydropyrido[3,2-*g*]quinoline-3′,7′-dimalonic Acid Tetraethyl Ester (**10**)

A solution of **9** (100 mg, 0.14 mmol) in 30% H_2SO_4 - EtOH (30 ml) was heated at reflux for 12 hours. The mixture was cooled, and concentrated *in vacuo*. To the mixture was added water (5 ml), and the product was extracted with $CHCl_3$. The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (10:1). A red crystalline solid was obtained, 91 mg (95%): MP 89~92°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2975, 1725, 1655; MS calcd for $C_{34}H_{42}N_2O_{12}$, 670.274, observed 670.274.

2,8-Dimethoxy-3,7-dimethyl-4,6-dipropyl-5,10-dioxo-5,10-dihydropyrido[3,2-*g*]quinoline-3′,7′-dimalonic Acid (**12**)

A mixture of **9** (50 mg, 0.07 mmol) in 80% EtOH - H_2O (15 ml) was treated with NaOH (30 mg, 0.75 mmol) and the mixture was heated at reflux for 1 hour. The mixture was acidified with 1 N HCl, and H_2O (15 ml) was added. After extracting with $CHCl_3$, the organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. A yellow crystalline solid was obtained 34 mg (82%): MP 300°C; NMR Table 3; IR (KBr) cm^{-1} 3700~2500, 2975, 1710, 1670, 1585; FD-MS *m/z* 587 (M^++1).

Anal Calcd for $C_{28}H_{30}N_2O_{12}$: C 57.34, H 5.12, N 4.78.
Found: C 57.18, H 5.03, N 5.01.

2,8-Dimethoxy-4,6-dipropyl-5,10-dioxo-5,10-dihydropyrido[3,2-*g*]quinoline-3,7-propionic Acid (**13**)

A solution of **12** (50 mg, 0.09 mmol) in pyridine (10 ml) was heated at reflux for 4 hours. The mixture was concentrated *in vacuo*, and acidified with 1 N HCl. After extracting with $CHCl_3$, the organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$. A yellow crystalline solid was obtained, 34 mg (94%): MP 253~255°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3600~2500, 2960, 1705, 1665, 1585; MS calcd for $C_{26}H_{30}N_2O_8$, 498.200, observed 498.201.

4,6-Dipropyl-2,5,8,10-tetraoxo-1,2,5,8,9,10-hexahydropyrido[3,2-*g*]quinoline-3,7-dipropionic Acid Diethyl Ester (**14**)

A solution of **13** (34 mg, 0.07 mmol) in 30% H_2SO_4 - EtOH (15 ml) was heated at reflux for 4 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **5**, the mixture was treated and purified to give a red crystalline solid, 19 mg (53%): MP 175~177°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3355, 2975, 1730, 1650; MS calcd for $C_{28}H_{34}N_2O_8$, 526.232, observed 526.232.

3,7-Dihydroxymethyl-2,8-dimethoxy-4,6-dipropylpyrido[3,2-*g*]quinoline-5,10-dione (**16**)

A solution of **5** (80 mg, 0.15 mmol) in 33% THF - H_2O (15 ml) was heated at reflux for 2 days. The mixture was concentrated *in vacuo*, and water (30 ml) was added. After extracting with $CHCl_3$, the organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (20:1). A yellow crystalline solid was obtained, 27 mg (44%): MP 230~235°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3400, 2955, 1700, 1670, 1585; MS calcd for $C_{22}H_{26}N_2O_6$, 414.179, observed 414.179.

2,8-Dimethoxy-4,6-dipropyl-5,10-dioxo-5,10-dihydropyrido[3,2-*g*]quinoline-3,7-dicarboxylic Acid (**17**)

To a solution of **16** (550 mg, 1.33 mmol) in acetone (50 ml) was added a solution of CrO_3 - H_2SO_4 in acetone and the resulting mixture was stirred at 0°C for 10 minutes. To the mixture was added 2-propanol and the mixture was concentrated *in vacuo*. Further water (150 ml) was added and the product was extracted with $CHCl_3$. The organic phase was dried (Na_2SO_4), and concentrated *in vacuo*. A yellow crystalline solid was obtained, 510 mg (87%): MP 231~235°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3600~2500, 2980, 1745, 1710, 1670, 1585; MS calcd for $C_{22}H_{22}N_2O_8$, 442.138, observed 442.138.

4,6-Dipropyl-2,5,8,10-tetraoxo-1,2,5,8,9,10-hexahydropyrido[3,2-*g*]quinoline-3,7-dicarboxylic Acid Diethyl Ester (**18**)

A solution of **17** (210 mg, 0.48 mmol) in 30% H_2SO_4 - EtOH (30 ml) was heated at reflux for 5 hours. The mixture was concentrated *in vacuo*, and water (20 ml) was added. After extracting with $CHCl_3$, the organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (25:1). A red crystalline solid was obtained, 53 mg (50%): MP 230~233°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2975, 1735, 1650; MS calcd for $C_{24}H_{26}N_2O_8$, 470.169, observed 470.172.

3-Acetoxymethyl-2-methoxy-7-methyl-4,6-dipropylpyrido[3,2-*g*]quinoline-5,8,10(9*H*)-trione (**20**)

To a solution of **4** (114 mg, 0.26 mmol) in acetic acid (10 ml) was added NaOH (30 mg, 0.75 mmol) and the resulting mixture was stirred at 100°C for 5 hours. After concentration *in vacuo*, to the residue was added water (10 ml) and the product was extracted with $CHCl_3$. The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (25:1). An orange crystalline solid was obtained, 104 mg (96%): MP 188~190°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2945, 1740, 1655, 1575; MS calcd for $C_{23}H_{26}N_2O_6$, 426.179, observed 426.179.

3-Acetoxymethyl-7-methyl-4,6-dipropylpyrido[3,2-*g*]quinoline-2,5,8,10(1*H*,9*H*)-tetrone (**21**)

To a solution of **20** in acetic acid (10 ml) was added conc H_2SO_4 (1 ml), and the resulting solution

was heated at reflux for 5 hours. To the mixture was added water (10 ml), and the product was extracted with $CHCl_3$. After extraction, in a similar manner to the preparation of **5**, the mixture was treated and purified to give a red crystalline solid, 65 mg (65%): MP 271~272°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2945, 1740, 1655; MS calcd for $C_{22}H_{24}N_2O_6$, 412.164, observed 412.163.

3,7-Diacetoxymethyl-2,8-dimethoxy-4,6-dipropylpyrido[3,2-*g*]quinoline-5,10-dione (**22**)

To a solution of **5** (50 mg, 0.09 mmol) in acetic acid (6 ml) was added NaOH (30 mg, 0.75 mmol) and the resulting mixture was stirred at 100°C for 3 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **5**, the mixture was treated and purified to give a yellow crystalline solid, 42 mg (91%): MP 98~100°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2945, 1740, 1660, 1575; FD-MS *m/z* 499 (M^++1).

Anal Calcd for $C_{26}H_{30}N_2O_8$: C 62.65, H 6.02, N 5.62.
Found: C 62.78, H 6.15, N 5.49.

3,7-Diacetoxymethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-2,5,8,10(1*H*,9*H*)-tetrone (**23**)

To a solution of **22** (42 mg, 0.09 mmol) in acetic acid (4 ml) was added conc H_2SO_4 (0.2 ml), and the resulting solution was heated at reflux for 3 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **11**, the mixture was treated and purified to give a red crystalline solid, 25 mg (63%): MP 165~168°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2945, 1740, 1665; MS calcd for $C_{24}H_{26}N_2O_8$, 470.169, observed 470.169.

3-Ethoxymethyl-7-methyl-4,6-dipropylpyrido[3,2-*g*]quinoline-2,5,8,10(1*H*,9*H*)-tetrone (**24**)

A solution of **4** (320 mg, 0.72 mmol) in 30% H_2SO_4 - EtOH (120 ml) was heated at reflux for 10 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **5**, the mixture was treated and purified to give a red crystalline solid, 100 mg (35%): MP 286~288°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2975, 1670, 1630; MS calcd for $C_{22}H_{26}N_2O_5$, 398.184, observed 398.183.

3,7-Diethoxymethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-2,5,8,10(1*H*,9*H*)-tetrone (**25**)

To a solution of **5** (52 mg, 0.09 mmol) in 30% H_2SO_4 - EtOH (8 ml) was heated at reflux for 10 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **5**, the mixture was treated and purified to give a red crystalline solid, 13 mg (32%): MP 233~235°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 3350, 2980, 1655, 1630; MS calcd for $C_{24}H_{30}N_2O_6$, 442.211, observed 442.211.

2,8-Dimethoxy-3,7-dimorpholinomethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-5,10-dione (**26**)

A solution of **5** (52 mg, 0.09 mmol) in morpholine (2 ml, 22.9 mmol) was stirred for 2 hours at room temperature. The mixture was concentrated to dryness *in vacuo* and to the residue was added 1 N Na_2CO_3 (10 ml). After extracting with $CHCl_3$, the organic phase was dried (Na_2SO_4) and the solvent removed *in vacuo*. The resulting crude product was purified by column chromatography on silica gel, eluting with $CHCl_3$ - MeOH (40:1). A yellow crystalline solid was obtained, 33.3 mg (64%): MP 94~95°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2975, 1700, 1670, 1590; MS calcd for $C_{30}H_{40}N_4O_6$, 552.295, observed 552.293.

3,7-Dimorpholinomethyl-4,6-dipropylpyrido[3,2-*g*]quinoline-2,5,8,10(1*H*,9*H*)-tetrone (**27**)

A solution of **26** (33.3 mg, 0.06 mmol) in 30% H_2SO_4 - EtOH (30 ml) was stirred at 100°C for 1.5 hours. After the reaction was complete according to TLC analysis, in a similar manner to the preparation of **10**, the mixture was treated and purified to give a red crystalline solid, 22.1 mg (67%): MP 210~212°C; NMR Table 3; IR ($CHCl_3$) cm^{-1} 2975, 1660; FD-MS *m/z* 525 (M^++1).

Anal Calcd for $C_{28}H_{36}N_4O_6$: C 64.12, H 6.87, N 10.69.
Found: C 63.97, H 6.69, N 10.82.

Antimicrobial Activity and Reversal Test with TdR

MIC against *E. faecium* IFO 3181 was assayed by agar dilution method with Folic Acid Assay Medium "Nissui" containing 1.0 ng/ml of pteroic acid at 37°C.[2] Reversal of antimicrobial activity

with TdR was tested by conventional counter diffusion method. A compound was dissolved in DMSO at the final concentration of 0.1 μg/ml. The solution, 50 μl, was applied to a sterile paper disk (i.d. 8 mm) and the dried disk was placed onto the medium.

HeLa Cell Culture

A culture of HeLa cells was grown and maintained in EAGLE's minimum essential medium supplemented with 5% calf serum, benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) as monolayer culture. Logarithmic phase cells were harvested by treating with trypsin (0.05%) and EDTA (0.01%) in calcium and magnesium-free phosphate buffered saline solution. After washing with the growth medium described above, cells were incubated in wells of 96-well flat bottom microplates (Corning cell wells). Each well contained 0.1 ml of the fresh growth medium supplemented with the compounds and 5×10^3 cells. The compounds were dissolved in DMSO and added to the medium at the final concentration of 1% of DMSO. The control well contained DMSO at the same rate. The cells were incubated at 37°C in water-saturated atmosphere of 5% CO_2 in air. After incubation for 4 days, the cell growth was observed.

Assay of TMP Synthase

The enzyme preparation was obtained according to the method described by ROBERTS[7] with some modifications. The assay of TMP synthase from Ehrlich ascites carcinoma was performed as described by CALVERT *et al.*[8] with some modifications described previously.

Antitumor Activity

CDF_1 mice were inoculated ip on day-0 with Meth-A fibrosarcoma (1×10^6 cells/mouse). The tumor had been maintained by ip transfer into BALB/c mice. Beginning 24 hours after tumor cell inoculation, the compounds were administered ip daily for four consecutive days. Antitumor activity was evaluated by the increased life span in the treated groups to that in the control group (T/C).

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References

1) ŌMURA, S.; Y. IWAI, K. HINOTOZAWA, H. TANAKA, Y. TAKAHASHI & A. NAKAGAWA: OM-704 A, a new antibiotic active against Gram-positive bacteria produced by *Streptomyces* sp. J. Antibiotics 35: 1425~1429, 1982
2) ŌMURA, S.; M. MURATA, K. KIMURA, S. MATSUKURA, T. NISHIHARA & H. TANAKA: Screening for new antifolates of microbial origin and a new antifolate AM-8402. J. Antibiotics 38: 1016~1024, 1985
3) MURATA, M.; T. MIYASAKA, H. TANAKA & S. ŌMURA: Diazaquinomycin A, a new antifolate antibiotic, inhibits thymidylate synthase. J. Antibiotics 38: 1025~1033, 1985
4) ŌMURA, S.; A. NAKAGAWA, H. AOYAMA, K. HINOTOZAWA & H. SANO: The structures of diazaquinomycins A and B, new antibiotic metabolites. Tetrahedron Lett. 24: 3643~3646, 1983
5) ANDREADES, S. & E. W. ZAHNOW: Anodic cyanations of aromatic compounds. J. Am. Chem. Soc. 91: 4181~4190, 1969
6) SIMCHEN, G. & H. KOBLER: Synthesis of nitriles with tetraalkylammonium cyanide. Synthesis 1975: 605~607, 1975
7) ROBERTS, D.: An isotopic assay for thymidylate synthetase. Biochemistry 5: 3546~3548, 1966
8) CALVERT, A. H.; T. R. JONES, P. J. DADY, B. GRAZELAKOWSKA-SZTABERT, R. M. PAINE, G. A. TAYLOR & K. R. HARRAP: Quinazoline antifolates with dual biochemical loci of action. Biochemical and biological studies directed towards overcoming methotrexate resistance. Eur. J. Cancer 16: 713~722, 1980