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Inhibitors of Protein Synthesis. 4.† Studies on the Structure–Activity Relationship of Gougerotin and Some of Its Analogs

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Seventeen structural analogs of gougerotin have been compared with the parent compound as inhibitors of the growth of *E. coli* B and as inhibitors of *N*-acetylphenylalanylpuromycin formation. The analogs comprise compounds with (1) an intact sarcosyl-D-seryl moiety and modifications at various positions of the carbohydrate moiety, (2) compounds which lack the peptidyl residue, and (3) compounds in which the sarcosyl-D-seryl moiety has been replaced by other aminoacyl or peptidyl moieties and in which various sites in the carbohydrate moiety have been modified in addition. These comparisons revealed that (1) the 3′-hydroxyl group makes a strong contribution to inhibitory activity, (2) the introduction of a double bond between the 2′ and 3′ positions of the carbohydrate moiety causes a marked decrease in activity; however, if in addition the sarcosyl-D-seryl moiety is replaced by the ε-*N*-methyl-β-L-arginyl side chain, full activity is restored, (3) there is a marked degree of stereospecificity around the α carbon of the seryl moiety (replacement of the sarcosyl-D-seryl residue with the sarcosyl-L-seryl residue results in greatly reduced activity), and (4) replacement of the carboxamide group at position 5′ with a hydroxymethyl function results in reduced activity which is further decreased by alterations in the sarcosyl-D-seryl moiety. These alterations include (a) replacement of the D-seryl residue of the sarcosyl-D-seryl moiety by the D-alanyl or the D-phenylalanyl residues and (b) replacement of the whole sarcosyl-D-seryl side chain by the D-seryl, D-alanyl, D-phenylalanyl, *p*-methoxy-D-phenylalanyl, or *p*-methoxy-L-phenylalanyl moieties; the replacement of the sarcosyl-D-seryl side chain with the various aminoacyl residues did not lead to acceptor (puromycin-like) activity with *N*-Ac-Phe-tRNA as the donor.

Gougerotin,[1] a nucleoside antibiotic isolated by Kanzaki et al.[2] from *Streptomyces gougerotii*, was shown to be 1-[4-deoxy-4-(sarcosyl-D-seryl)amino-β-D-glucopyranosyluronamide]cytosine (1), both by degradative studies[3] and by total synthesis.[4] Biochemical studies concerning the mode of action of the antibiotic have been rather extensive.[1,5,8] Little is known, however, about the structural features of gougerotin which are essential for its biochemical activity. Since correlation between the structure and activity of gougerotin derivatives would be useful for exploring the site of its action, various structural analogs were prepared, and their effect upon peptide bond formation and cell growth was measured. Some of the analogs, which contained a free aminoacyl residue, were also evaluated as potential acceptor substrates.

The gougerotin analogs which were studied are listed in Chart I. The syntheses of the 6′-hydroxymethyl analogs **2** of gougerotin have been reported[9,10] previously, as have the syntheses of compounds **6** and **7**[11,12] and **2**[9] and **8**.[13,14]

†For parts III and II in this series, see C. Coutsogeorgopoulos, *Arch. Biochem. Biophys.*, **153**, 199 (1972), and ref 6, respectively. This paper is also part 13 of a series entitled "Synthetic Studies on Nucleoside Antibiotics"; part 12 is ref 12.

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Experimental Section

A. Chemistry. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and by Spang Microanalytical Laboratory, Ann Arbor, Mich. Analyses are indicated only by symbols of the elements; for those elements the analytical results were within ±0.4% of the theoretical values. Melting points were determined on a Thomas-Hoover apparatus (capillary method) and are corrected. Optical rotations were determined on a Keston polarimeter attachment to a Beckman DU spectrophotometer set at 589 nm. For biochemical studies, the synthetic gougerotin (1), analogs 4 and 5, and natural gougerotin were purified by paper chromatography (Whatman No. 1 paper, descending, *n*-BuOH–HOAc–H_2O, 4:1:2 v/v/v). The natural gougerotin sample was found to contain a faster migrating, fluorescent impurity which was removed by this process. Paper electrophoretic analyses were performed in pH 7 phosphate buffer at 900 V for 2 hr. The preparation of the hitherto unreported compounds of types **4** and **5** was achieved by procedures analogous to that employed for the total synthesis of gougerotin[4] (Scheme I) and details of these syntheses are as follows.

1-(Methyl 4-amino-4-deoxy-β-D-glucopyranosyluronate)-cytosine Dihydrochloride (8d). C-Substance (**8c**,[14] 1.8 g) was suspended in absolute methanol (150 ml) and saturated with dry hydrogen chloride at 0°. Once a clear solution was obtained, the product crystallized. The hygroscopic crystals (**8d**, 1.6 g) were filtered and dried in a desiccator over phosphorus pentoxide and sodium hydroxide under vacuum overnight: mp 217–223° dec; $[\alpha]^{27}D$ −14° (c 1.0, H_2O). Anal. ($C_{11}H_{16}N_4O_6 \cdot 2HCl \cdot H_2O$) C, H, N.

The filtrate was evaporated to dryness and the residue was dried by several azeotropic distillations with toluene and dissolved in ab-

Chart I

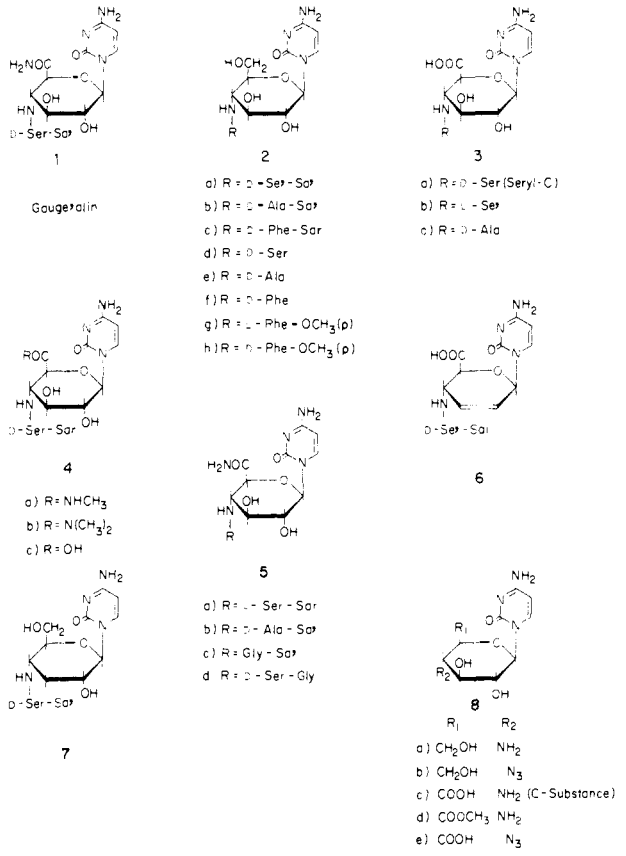
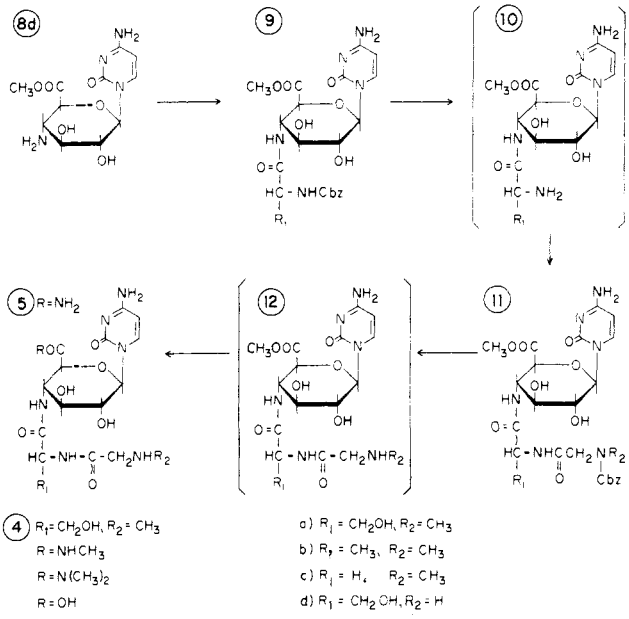


Chart II



solute methanol (50 ml). The solution was saturated with dry hydrogen chloride. Compound **8d** (0.6 g, mp 215–220° dec) precipitated.

1-(Methyl 4-*N*-benzyloxycarbonyl-D-serylamino-4-deoxy-β-D-glucopyranosyluronate)cytosine (9, R_1 = CH_2OH). To a solution of **8d** (782 mg, 2 mmol) and *N*-carbobenzyloxy-D-serine (750 mg, 3 mmol) in absolute methanol (2 ml) was added acetonitrile (2 ml, containing 405 mg of triethylamine) and DCC (620 mg). The mixture was shaken vigorously for 16 hr. Dicyclohexylurea was removed by filtration and washed with methanol (7 ml) and 50% aqueous ethanol (5 ml). The combined filtrate and washings were evaporated to dryness, and the residue was triturated several times with ether. The ether-insoluble solid was dissolved in absolute methanol (20 ml) (three spots were detected on TLC) and treated with a mixture of methanol washed Dowex 50 (H^+) (2 ml) and Dowex 1 (OH^-) (2 ml) for 7 min in an ice bath. The resin mixture was filtered off and washed thoroughly with methanol (50 ml). The combined filtrate and washings (one spot was detected on TLC) were evaporated to dryness. The residue was triturated with ether (50 ml) and then purified by reprecipitation with methanol–ethyl acetate to give 9 (R_1 = CH_2OH) as a colorless powder (1.04 g).

Compounds 9a [1-(methyl 4-*N*-benzyloxycarbonyl-L-serylamino-4-deoxy-β-D-glucopyranosyluronate)cytosine] and 9b [1-(methyl 4-*N*-benzyloxycarbonyl-D-alanylamino-4-deoxy-β-D-glucopyranosyluronate)cytosine] were prepared analogously using the corresponding *N*-carbobenzyloxyamino acids (Table I).

1-(4-Deoxy-4-D-serylamino-β-D-glucopyranosyluronic acid)cytosine (Seryl-C, 3a). Compound 9 (R_1 = CH_2OH, 73 mg) was dissolved in 50% aqueous ethanol (10 ml) and hydrogenated for 15 min over 10% Pd/C (25 mg) at an initial hydrogen pressure of 3 atm. The catalyst was filtered off and washed with water (2 ml). The combined filtrate and washings were evaporated to dryness and the residue was dissolved in 0.2 *N* sodium hydroxide (20 ml). After 16 hr, the solution was passed through a column of Amberlite IRC 50 (H^+) (2 × 10 cm). The column was washed with water and the eluate evaporated to dryness. The residue was recrystallized twice from water to give colorless needles of **3a** (20 mg). The physical properties of 3a were found to be identical (uv, ir, and paper electrophoretic and chromatographic mobilities) with a sample of seryl-C obtained by chemical degradation of gougerotin and kindly provided by Dr. Iwasaki.

Compounds 3b and 3c (Table II) were prepared by an analogous procedure from compounds **9a** and **9b**, respectively. These compounds were very insoluble in the common solvents, and their optical rotations could not be determined.

1-[Methyl 4-deoxy-4-(*N*-benzyloxycarbonylsarcosyl-D-seryl)amino-β-D-glucopyranosyluronate]cytosine (11, R_1 = CH_2OH; R_2 = CH_3). Compound 9 (R_1 = CH_2OH, 972 mg) was dissolved in 50% aqueous ethanol (100 ml) and hydrogenated for 15 min in the presence of 10% Pd/C (250 mg) at an initial pressure of 3 atm. The catalyst was removed by filtration and the filtrate was mixed with *N*-carbobenzyloxysarcosine (847 mg). After the solvent was removed by evaporation below 32° in vacuo, the residue was further dried by several coevaporations with toluene. The dried mixture was dissolved in absolute methanol (3 ml), DCC (590 mg) and acetonitrile (3 ml) were added to the solution, and the mixture was shaken vigorously for 16 hr. The sticky reaction mixture was diluted with 50% aqueous ethanol (20 ml) and filtered from dicyclohexylurea. The filtrate was evaporated to dryness, and the residue was triturated several times with ether. The ether-insoluble solid was dissolved in methanol (20 ml) and treated with a mixture of Dowex 1 (OH^-) (2 ml) and Dowex 50 (H^+) (2 ml) for 4 min at 0°. The resin was filtered off, and the filtrate was evaporated to dryness. The residue was coevaporated several times with ethanol until pale yellow crystals (590 mg) were obtained.

Compounds 11a and 11b were synthesized by the same procedure from **9a** and **9b**, respectively. Compound 11c was synthesized analogously.

1-[Methyl 4-deoxy-4-(sarcosyl-D-seryl)amino-D-glucopyranosyluronate]cytosine (12, R_1 = CH_2OH; R_2 = CH_3). Compound 11 (R_1 = CH_2OH; R_2 = CH_3) (513 mg) was dissolved in 50% aqueous ethanol (100 ml) and hydrogenated for 15 min over 10% Pd/C at an initial hydrogen pressure of 3 atm. The catalyst was filtered off, and the filtrate (containing a single compound as evidenced by paper electrophoretic analysis) was diluted with ethanol (1:1). The mixture was evaporated to dryness, below 32°. The residue was coevaporated several times with ethanol until colorless microcrystals were obtained. The product 12 (R_1 = CH_2OH; R_2 = CH_3) (460 mg) thus obtained was homogeneous, as determined by paper electrophoresis.

Compounds **12a**, **12b**, and **12c** were obtained by the same procedure from **11a**, 11b, and 11c, respectively. Compound **12d** was synthesized analogously.

1-[4-Deoxy-4-(sarcosyl-D-seryl)amino-β-D-glucopyranosyluronic amide]cytosine (Gougerotin, 1). Compound 12 (R_1 = CH_2OH; R_2 = CH_3) (148 mg) was dissolved in 26 ml of methanol saturated with ammonia at 0°, and the mixture was kept at room temperature for 16 hr. After evaporation, the residue was dissolved in 0.6 ml of water and a small amount of insoluble material was removed by filtration. The filtrate was diluted with methanol (6 ml) and then evaporated to dryness. Colorless fine needles (130 mg) were obtained, which were homogeneous by paper chromatograph-

Table I. Some Physical Constants of Protected Aminoacyl and Peptidyl Nucleoside Analogs of Gougerotin

Compd	Mp, °C	$[\alpha]^{26}D$[a]	Formula	Analyses
9 ($R_1 = CH_2OH$)	270–274 dec	+19	$C_{22}H_{27}O_{10}N_5 \cdot C_2H_5OH \cdot 0.5H_2O$	C, H, N
9a	184–199 eff	−10	$C_{22}H_{27}O_{10}N_5 \cdot C_2H_5OH$	C, H, N
9b	191–201 eff	+35	$C_{22}H_{27}O_9N_5 \cdot 1.5C_2H_5OH$	C, H, N
11 ($R_1 = CH_2OH$; $R_2 = CH_3$)	189–194 eff	+16	$C_{25}H_{32}O_{11}N_6 \cdot C_2H_5OH$	C, H, N
11a	190–200 eff	+10	$C_{25}H_{32}O_{11}N_6 \cdot 1.5C_2H_5OH$	C, H, N
11b	188–202 eff	+24	$C_{25}H_{32}O_{10}N_6 \cdot C_2H_5OH$	C, H, N
11c	144–156 eff	+17	$C_{24}H_{30}O_{11}N_6 \cdot C_2H_5OH$	C, H, N

[a] c 10.0 in methanol.

Table II. Some Physical Constants of Aminoacyl and Peptidyl Nucleoside Analogs of Gougerotin

Compd	Mp, °C	$[\alpha]^{26}D$[a]	Formula	Analyses
3a	230–235 dec	+57[b]	$C_{13}H_{19}O_8N_5$	C, H, N
3b	267–272 dec		$C_{13}H_{19}O_8N_5 \cdot 0.5H_2O$	C, H, N
3c	247–254 dec		$C_{13}H_{19}O_7N_5 \cdot 0.5H_2O$	C, H, N
1	211–217 dec	+53	$C_{16}H_{25}O_8N_7 \cdot H_2O$	C, H, N
4a	197–205 dec	+46	$C_{17}H_{27}O_8N_7 \cdot CH_3OH \cdot H_2O$	C, H, N
4b	220–229 eff	+36	$C_{18}H_{29}O_8N_7 \cdot CH_3OH$	C, H, N
4c	200–210 eff	+39	$C_{16}H_{24}O_9N_6 \cdot H_2O$	C, H, N
5a	238–243 dec	+4	$C_{16}H_{25}O_8N_7 \cdot 0.5H_2O$	C, H, N
5b	235–250 eff	+50	$C_{16}H_{25}O_7N_7 \cdot H_2O$	C, H, N
5c	265–272 dec	+45	$C_{15}H_{23}O_8N_7 \cdot H_2O$	C, H, N
5d	235–250 dec		$C_{15}H_{23}O_7N_7 \cdot H_2O$	C, H, N

[a] c 1.0 in H_2O unless specified otherwise. [b] c 0.12 in H_2O.

ic and paper electrophoretic analyses and identical with gougerotin with respect to uv, ir, and NMR spectra and paper chromatographic and paper electrophoretic behavior.

Compounds **5a–d** were prepared similarly from the corresponding dipeptidyl intermediate **12a–d**.

Compounds **4a** and **4b** were synthesized from 12 ($R_1 = CH_2OH$; $R_2 = CH_3$) by using the corresponding amines instead of ammonia. Compound 4c was prepared as follows. Compound 11 ($R_1 = CH_2OH$; $R_2 = CH_3$) (128 mg) was dissolved in 50% aqueous ethanol (35 ml) and hydrogenated over 10% Pd/C for 15 min at an initial pressure of 2 atm. After the catalyst was removed by filtration, the filtrate was evaporated to dryness. The residue was dissolved in 0.1 *N* sodium hydroxide (40 ml), and the solution was kept at room temperature for 4 hr. The solution was passed through a column of Amberlite IRC 50 (H^+) (2 × 10 cm), and the column was washed with water until the eluate showed no uv absorption. The eluent was evaporated to dryness, and the residue was coevaporated several times with ethanol until colorless microcrystals (102 mg) of **4c** were obtained.

B. Biological Assay. The effect which the synthesized compounds exerted on the growth of *E. coli* B was determined by the procedure described previously[15] and the results are recorded in Table III.

C. Experiments in Cell-Free Systems. Ribosomes washed three times with 0.5 *M* NH_4Cl were prepared from frozen *E. coli* B cells, harvested in the middle of the logarithmic phase of growth as described previously.[16] The fraction FWR (ribosomal wash)[16] was used as a source of initiation factors. *N*-Ac-[^{14}C]- or -[^{3}H]Phe-tRNA was prepared as described elsewhere.[16]

Assay for Inhibition of Peptide Bond Formation. Peptide bond formation was determined by use of the model reaction between *N*-Ac-[^{3}H]Phe-tRNA and puromycin (puromycin reaction) which we have used before for investigating inhibitors of protein synthesis.[17,18] The amount of *N*-acetyl[^{3}H]phenylalanylpuromycin formed was determined after extraction in ethyl acetate. To the incubation mixture (0.25 ml) were added, in the following sequence, 25 μmol of Tris–HCl buffer (pH 7.2), 25 μmol of NH_4Cl from a solution adjusted with NH_4OH to pH 7.2, 2.5 μmol of magnesium acetate, 20 μg of ammonium polyuridylate, 0.1 μmol of guanosine 5′-triphosphate, 0.025 μmol of puromycin monohydrochloride, gougerotin or its analogs as indicated, 8.0 A_{260} units of washed ribosomes, 80 μg (protein) of fraction FWR (initiation factors), and 3.2 A_{260} units of *N*-Ac-[^{3}H]Phe-tRNA (130,500 cpm, 3450 cpm per pmol of [^{3}H]Phe). After incubation at 25° for 2 or 4 min, the reaction was stopped by cooling at 0° and adding 25 μl of 4 *N* KOH. The amount of *N*-acetyl[^{3}H]phenylalanylpuromycin formed was then determined after adding an aliquot (200 μl) of the incubation mixture to 1.0 ml of 0.1 *M* sodium acetate buffer, pH 5.5, and extracting with ethyl acetate (2.0 ml).[19] To an aliquot (1.5 ml) of the extract was added 15 ml of Bray's solution and the radioactivity determined by counting in a Packard 3000 series liquid scintillation spectrometer. The results are recorded in Table III.

Assay for Acceptor Activity. Some of the gougerotin analogs contain a free amino group as part of the sugar moiety and they could be expected to have acceptor activity by forming an amide bond with the *N*-acetylphenylalanyl moiety of *N*-Ac-Phe-tRNA. In order to assay for possible acceptor activity, these analogs were allowed to react with *N*-Ac-[^{14}C]Phe-RNA in an incubation mixture which was the same as the one used in the "assay for inhibition of peptide bond formation" except for the following differences. (1) Puromycin was omitted when the analog was included. (2) 6.4 A_{260} units of *N*-Ac-[^{14}C]Phe-tRNA (38,500 cpm, 500 cpm per pmol of [^{14}C]Phe, under the conditions of this assay) was used in place of *N*-Ac-[^{3}H]Phe-tRNA. After incubation at 25° for 3 and 6 min, two 0.1-ml aliquots of the incubation mixture were applied to 3 MM Whatman filter paper disks, and the radioactivity of the cold perchloric acid precipitable material was determined as described previously.[20] A control incubation mixture without the gougerotin analogs was included in each determination and gave approximately 15,500 cpm for the cold perchloric acid precipitable radioactive material which corresponds to 0.1 ml of incubation mixture or 2.56 A_{260} units of *N*-Ac-[^{14}C]Phe-tRNA (Table IV).

Results

Three groups of gougerotin analogs were studied for their effect upon in vitro peptide bond formation and cell growth. They comprise (a) analogs with an intact sarcosyl-D-seryl moiety and modifications at various positions of the carbohydrate moiety, (b) analogs which lack an aminoacyl or peptidyl residue at the 4′ position, and (c) analogs in which the sarcosyl-D-seryl moiety was replaced by a different aminoacyl or peptidyl residue and various sites in the carbohydrate moiety were modified as well.

Table III. Inhibitory Activity of Gougerotin Derivatives[a]

Compd	% inhibn of peptide bond formation (at 4×10^{-5} M)	Concn (M) for 50% inhibn of growth of *E. coli* B
Derivatives Bearing the Intact Sarcosyl-D-seryl Moiety		
1	65 ± 0.5	4×10^{-5}
2a	48 ± 1.0	2×10^{-4}
4a	22 ± 1.4	1×10^{-4}
4b	12 ± 1.2	9×10^{-5}
4c	30 ± 1.0	$>10^{-3}$
6	10 ± 1.5	$>10^{-3}$
7	5 ± 2.0	1×10^{-3}
Derivatives Having a Carboxamide Group at the 5′ Position		
5a	9 ± 1.6	$>10^{-3}$
5b	44 ± 0.9	8×10^{-5}
5c	10 ± 0.7	4×10^{-4}
5d	35 ± 0.9	1×10^{-4}
Derivatives Having a Hydroxymethyl Group at the 5′ Position		
2a	48 ± 0.7	2×10^{-4}
2b	20 ± 1.3	9×10^{-4}
2c	4 ± 2.0	$>10^{-3}$
2d	4 ± 1.6	$>10^{-3}$
2e	1 ± 2.0	$>10^{-3}$
2f	5 ± 2.0	$>10^{-3}$
2g	1 ± 1.8	$>10^{-3}$
2h	7 ± 1.8	$>10^{-3}$
3a	2 ± 2.0	$>10^{-3}$

[a] Assays were carried out as described in the Experimental Section (Assay for Inhibition of Peptide Bond Formation). The percent inhibition of peptide bond formation is the average of at least three independent experiments with each compound. In each experiment triplicate samples were included and the standard deviation of the three values was within 2% of the average value. In the absence of inhibitor the formation of *N*-acetyl-[^{3}H]phenylalanylpuromycin proceeded as follows: 17,226 ± 224 (2 min); 33,734 ± 607 (4 min); 50,960 ± 611 (8 min). A blank value from incubation mixtures containing neither puromycin nor gougerotin analogs (775 ± 23 cpm) has been subtracted from these counts. The degree of inhibition determined at 2 and at 4 min did not vary with time. The error shown was calculated by dividing the average control (17,226 for 2 min) into the standard deviation.

Table IV. Evaluation of the Acceptor Activity of Various Gougerotin Derivatives[a]

Compd	Concn, M	Radioactivity (cpm) in cold perchloric acid precipitate in 3 min	Radioactivity (cpm) in cold perchloric acid precipitate in 6 min
None		15,433 ± 259	15,450 ± 280
Puromycin	10^{-4}	12,192 ± 183	9,106 ± 153
2d	10^{-4}	15,596 ± 166	15,194 ± 187
2e	10^{-4}	15,322 ± 322	15,648 ± 329
2f	10^{-4}	15,254 ± 198	15,267 ± 287
2g	10^{-4}	15,250 ± 233	15,254 ± 163
2g	10^{-3}	15,598 ± 170	15,197 ± 213
2h	10^{-4}	15,596 ± 125	15,582 ± 281
3a	10^{-4}	15,668 ± 204	15,267 ± 244
5d	10^{-4}	15,398 ± 246	15,327 ± 123

[a] Assays were performed as described in the text (Assay for Acceptor Activity). The values given represent the average of at least three independent experiments for each analog. In each experiment, determinations were carried out in triplicates and from each sample two 0.1-ml aliquots were assayed. Thus, from each determination six values for the cold acid precipitable material were obtained and the standard deviation is given next to the average value. In the presence of 1×10^{-4} M puromycin the cold acid precipitable radioactivity (cpm) decreased as follows: 15,433 ± 259 (0 min); 12,192 ± 183 (3 min); 9106 ± 153 (6 min); 4233 ± 106 (12 min); 3087 ± 86 (16 min). The difference from 15,433 ± 259 cpm at each time point represents the amount of *N*-acetyl-[^{14}C]phenylalanylpuromycin formed.

(A) Analogs Bearing an Intact Sarcosyl-D-seryl Moiety and Modifications at Various Sites of the Carbohydrate. To determine the contribution which the carboxamide group at the 5′ position of the sugar makes to the activity of gougerotin, this group was methylated or was replaced by a hydroxymethyl or carboxyl function (Chart I and Table III). As shown in Table III, mono- or dimethylation of the amide group of gougerotin (**4a** and **4b**) decreased its inhibitory activity with respect to both peptide bond formation and cell growth. Replacement of the 5′-carboxamide function of gougerotin with a hydroxymethyl or carboxyl group (**2a** and **4c**) caused a somewhat smaller decrease in the degree of inhibition of peptide bond formation than that effected by dimethylation (**4b**). On the other hand, the analog having a free carboxyl group (**4c**) was more effective in inhibiting peptide bond formation in the cell-free assay than in inhibiting cell growth. This difference possibly reflects a membrane permeability barrier toward the carboxyl group.

When, in addition to a modification at the 5′ position as in **2a**, a change is introduced at the 3′ position by removal of the 3′-hydroxy (**7**), a further decrease in activity results with respect to both inhibition of peptide bond formation and cell growth (Table III), indicating that the 3′-hydroxyl group makes a strong contribution to the activity of gougerotin. Similarly, a double bond between the 2′ and 3′ positions (**6**) further decreases the ability of **4c** to inhibit peptide bond formation.

The absence of 2′- and 3′-hydroxyl groups and the distortion in the conformation of the carbohydrate moiety which results from the introduction of the double bond and which could lead to a relative displacement of the peptide chains are factors which may contribute to decreased binding. It is of great interest, therefore, that replacement of the sarcosyl-D-seryl moiety of **6** with the blastidic acid (ε-*N*-methyl-β-L-arginine) side chain results in blasticidin S which inhibits peptide bond formation to a greater extent than **6**, **4c**, or even gougerotin. Under the conditions given in the legend to Table III blasticidin S at 4×10^{-6} M inhibited peptide bond formation by 62 ± 2% in 2 min. Among the factors possibly responsible for this enhanced activity are the length of the side chain and the greater basicity of the blastidic acid moiety. In any event, the new chain can compensate for both the lack of the 2′- and 3′-hydroxyl groups and the presence of a double bond and a free carboxyl group.

(B) Analogs Lacking the Dipeptide at the 4′ Position. This group includes analogs (**8**) of gougerotin with an amino or azide group at the 4′ position. Under the conditions of assay given in the legend to Table III none of these showed any activity either as inhibitors of peptide bond formation (up to 1×10^{-4} M) or of bacterial growth (up to 1×10^{-3} M) (results not shown), demonstrating the essentiality of the peptidyl residue for eliciting inhibitory activity. Furthermore, at 1×10^{-4} M, compounds **8a**, **8c**, and **8d**, which bear a free amino group at the 4′ position, did not exhibit significant acceptor activity, the results being analogous to those shown in Table IV for other gougerotin analogs devoid of acceptor activity.

(C) Analogs with an Aminoacyl or Peptidyl Residue

Other Than Sarcosyl-D-serine. Three subgroups can be distinguished, depending upon the nature of the substituent at the 5′ position which is either the carboxamide, the hydroxymethyl, or the carboxyl function.

(1) 5′-Carboxamide Derivatives (5). As shown in Table III, a very marked decrease in activity in both systems resulted when the sarcosyl-D-seryl residue was replaced with the sarcosyl-L-seryl moiety (**5a**). This observation reveals a marked degree of stereospecificity around the α carbon of the seryl moiety and suggests that gougerotin interacts with a ribosomal site in a stereospecific manner. Moreover, when the asymmetry around the α carbon of the seryl moiety is lost, by replacement of the hydroxymethyl group with hydrogen (**5c**), little inhibitory activity is observed. On the other hand, a modification which retains asymmetry, but substitutes the D-seryl with a D-alanyl moiety (**5b**), retains part of the inhibitory activity of gougerotin in both systems. Of course, aside from the asymmetrical aspects, the nature of the substituent groups per se may define the extent of activity.

That other positions in the side chain participate in determining the activity is shown by the finding that removal of the *N*-methyl group from the sarcosyl moiety of gougerotin (compound **5d**) decreases the inhibitory activity of gougerotin in both assay systems. Furthermore, **5d**, which bears a free α-amino group in the peptidyl residue, did not show significant acceptor activity (Table IV).

(2) 5′-Hydroxymethyl Derivatives (2). When the serine hydroxymethyl group of **2a** is replaced by a methyl group, as in the sarcosyl-D-alanyl derivative **2b**, a reduction in activity occurs (Table III) which parallels the decrease seen when the corresponding replacement is carried out in gougerotin (**1** → **5b**).

When a phenyl group replaced the serine hydroxyl function (**2c**), activity was reduced extensively in both assay systems. It is unclear whether this loss is due to restricted space tolerance on the binding site or whether it results from the hydrophobicity of the phenyl group, which exceeds that of the hydrogen in compound **2b**. Removal of the sarcosyl moiety of **2a** results in reduced activity (**2d**). This reduced activity persists when the methyl (**2e**), benzyl (**2f**), or *p*-methoxybenzyl (L- or D-**2g** or -**2h**) group replaces the hydroxymethyl group of the D-seryl residue.

Because these derivatives (**2d** → **2h**) possess an aminoacyl residue with a free α-amino group, they were also evaluated for acceptor activity, with *N*-Ac-Phe-RNA as the potential donor substrate. At 1×10^{-4} *M*, none of the compounds showed acceptor activity which can be considered significant. This conclusion is based on the fact that, as shown in Table IV, the differences in the cold acid precipitable radioactivity of *N*-Ac-[^{14}C]Phe-tRNA, obtained by allowing these compounds to react for 3 and 6 min, fall well within the experimental error of the assay and at these time intervals, the reaction does not proceed to any significant extent. On the other hand, in 6 min puromycin has caused a 41% decrease in the cold acid precipitable radioactivity, denoting extensive *N*-acetylphenylalanylpuromycin formation. Because of the particular nature of **2g** (see Discussion) this compound was also tested at ten times higher concentration (1×10^{-3} *M*) and no significant decrease in cold acid precipitable radioactivity was observed (Table IV).

(3) Aminoacyl Derivatives with a Carboxyl Function at the 5′ Position (3). Unlike the parent compound **4c** which showed some activity in inhibiting peptide bond formation, under the same conditions of assay, the derivative which lacks the sarcosyl moiety (**3a**, seryl-C) at 4×10^{-5} *M* had low activity with respect to inhibition of peptide bond formation and cell growth (Table III), as well as acceptor function (Table IV). Compounds **3b** and **3c** were not evaluated for activity.

Discussion

Previous studies on the mechanism of action of gougerotin have suggested that the antibiotic inhibits the formation of peptide bonds catalyzed by the putative ribosomal enzyme peptidyl transferase.[1] As would be expected on the basis of this mechanism, gougerotin interferes with the formation of peptide bonds between puromycin and model peptidyl-tRNA's (ref 6 and original references therein). In the latter reaction puromycin appears to assume the role of the aminoacyladenylyl terminus of aminoacyl-tRNA, which normally binds to the acceptor site of peptidyl transferase. This assumption is supported by the finding that puromycin inhibits the binding of aminoacyl oligonucleotide fragments to the ribosome.[7] Like puromycin, gougerotin can also inhibit the binding of aminoacyl oligonucleotide fragments to the ribosome and the suggestion has therefore been made[7,8] that gougerotin inhibits peptidyl transferase by competing with the acceptor substrate at the acceptor site. Based on a conformational analysis, the view has been expressed[21] that gougerotin can assume a conformation similar to that of 2′(3′)-*O*-L-phenylalanylcytidine which, like puromycin, exhibits acceptor activity.[22,23] It has been proposed[24] that the cytosine moiety of gougerotin binds to the ribosome at a site at which the adenine moiety of the aminoacyladenylyl terminus of aminoacyl-tRNA binds. By extending the analogy, one might expect that the hexosyl moiety of gougerotin binds where the ribosyl moiety of the aminoacyladenylyl terminus of aminoacyl-tRNA binds and that,[21] if "suitable" aminoacyl residues were present in place of the sarcosyl-D-seryl moiety of gougerotin, the analog would exhibit acceptor activity.

The observations reported here would not appear to support these proposals[21,24] although the "suitable" amino acyl residues might not have been part of the analogs used in this study. Compound **2g**, the gougerotin analog with the aminoacyl side chain of puromycin, showed no acceptor activity at a concentration ten times higher than that of puromycin (Table IV). Despite the structural similarity between **2g** and 2′(3′)-*O*-L-phenylalanylcytidine, the latter compound shows acceptor activity,[22,23] whereas the former does not. Thus, it may not be correct to view the nucleoside portion of gougerotin as an analog of 3′-amino-3′-deoxycytidine. It is possible that the cytosine moieties of **2g** and of gougerotin bind to different ribosomal areas than does the cytosine moiety of 2′(3′)-*O*-L-phenylalanylcytidine. It is further possible that the hexopyranosylcytosine moiety of gougerotin may interact at ribosomal sites which normally are involved in binding one of the cytidine residues which adjoin the 3′-terminal adenosine of aminoacyl-tRNA. The present results indicate that it may not be adequate to place gougerotin, and possibly other aminoacylamino nucleosides such as blasticidin S and amicetin, into a conformational pattern which simulates that of puromycin.

The ribosomal sites involved in binding gougerotin or its analogs do not necessarily coincide with those which normally bind the "3′-end" of aminoacyl- or peptidyl-tRNA. Gougerotin or its analogs might induce conformational changes in the ribosomal structure revealing some new binding sites not normally involved in the binding of the "3′-end" of aminoacyl- or peptidyl-tRNA.

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Synthesis and Biological Activity of 10-Thia-10-deaza Analogs of Folic Acid, Pteroic Acid, and Related Compounds†

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The 10-thia analogs of pteroic acid, folic acid, their esters, and their 4-amino analogs were synthesized through a reaction sequence involving, as a key step, the condensation of 2-amino-3-cyano-5-chloromethylpyrazine with appropriately substituted thiols. The abilities of the products to inhibit the growth of methotrexate (MTX)-sensitive and MTX-resistant microorganisms were investigated as were their abilities to inhibit dihydrofolic acid reductase and thymidylic acid synthetase. Several compounds had high activity.

Since the first reports that folic acid antagonists are useful in the treatment of human neoplastic disease,[1,2] a great deal of research has been centered on investigating the functions and interactions of folic acid and its reduced derivatives in the hope that clues for the synthesis of more effective antagonists might be obtained. Further interest has been added to this research area by the usefulness of folic acid antagonists in the treatment of psoriasis[3,4] and their usefulness in suppressing the immune response[5,6] and in treating protozoal disease.[7]

It is well understood that the functions of tetrahydrofolic acid in essential one-carbon transfer reactions require the availability of amine nitrogens in the 5 and 10 positions of the reduced coenzyme.[8] However, in spite of the essential nature of these nitrogens relatively few attempts in preparing antagonists of folic acid or its reduction products have been centered on replacing them with other atoms.

In homofolic acid and related compounds the distance between these nitrogens was lengthened.[9] In the 10-oxa analog of pteroic and folic acid the 10-nitrogen was replaced by an oxygen.[10] In isofolic acid, the positions of the methylene and amine groups separating the pteridine and benzene rings have been reversed.[11] In 10-deazaaminopterin, the 10-nitrogen of the 4-amino derivative of folic acid was replaced by a methylene group.[12,13] Several of these antagonists showed interesting inhibitory activity in systems requiring folic acid or its reduction products.

The present study describes the synthesis of analogs of folic acid and of pteroic acid, as well as that of their 4-amino analogs, in which the nitrogen in the 10 position was replaced with sulfur. It was hoped that these compounds might have useful inhibitory activity in folic acid requiring systems. The synthesis is outlined as shown in Schemes I and II.

4,4′-Dithiobis(benzoic acid) (1) was synthesized by the procedure of Campaigne and Meyer[14] and converted to the diethyl ester **2**. An attempt to synthesize the diethyl ester directly from ethyl *p*-aminobenzoate yielded material contaminated with a highly explosive by-product, possibly a diazonium intermediate. The explosion occurred while the product was being dried. Reduction of **2** with sodium borohydride yielded the thiol **3**; the reaction was monitored by the uv spectrum change[14] from λ_{max} 273 nm to λ_{max} 332 nm. Condensation of **3** with 2-amino-3-cyano-5-chloromethylpyrazine[15] gave rise to the formation of **4** in excellent yield. The 2-amino-3-cyanopyrazine **4** could then be cyclized with guanidine to form ethyl 4-amino-4-deoxy-10-thia-10-deazapteroate (**6**). The methyl ester **5** could be ob-

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