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FOLATE ANTAGONISTS FROM A CRUDE "X-METHYL FOLATE" PREPARATION

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SUMMARY: A crude folic acid antagonist, previously designated as X-methyl folate was studied. Five components were found to inhibit the growth of *Streptococcus faecalis*. 9-Methyl folic acid was the major bioactive constituent by weight (5%), but because of its low specific activity, contributed only 1% of the biological activity of the crude reaction product. The most active compound accounted for 40% of the activity and constituted 0.04% of the crude product by weight. Spectroscopic data suggest this to be a novel folic acid analogue.

INTRODUCTION

One of the first folic acid antagonists produced was made by coupling 2,4,5-triamino-6-hydroxypyrimidine with p-aminobenzoylglutamic acid and 2,3-dibromobutyraldehyde (instead of 2,3-dibromopropionaldehyde used in preparing folic acid). Since the position of the methyl group in the product was uncertain, the crude product was referred to as X-methyl folic acid (crude XMF) (1) but the active compound was never isolated. However, the crude product was found to function as a folic acid antagonist for microorganisms (2), rats (2), mice and chicks (3), dogs (4), and pigs (5). In all these cases the antagonist produced accentuated symptoms of folic acid deficiency which could be reversed by adding folic acid. The crude product when given together with a low-folate diet produced a remission in a patient with myeloid leukemia (6).

Martin (7) suggested that the product formed by this reaction is 7-methyl folic acid. However, Boothe *et al.* (8) prepared 7-methyl folic acid and isomeric derivatives and found that their activities were too low

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to account for the relatively high biological activity of the crude reaction product.

In view of the activity of the crude X-methyl folate in producing a remission in one case of myeloid leukemia it seemed of interest to identify the active compounds in crude X-methyl folate.

MATERIALS AND METHODS

Purification of XMF was monitored by microbiological assay using *S. faecalis* (ATCC 8043) in which each assay tube (5 ml) contained 2 ng of folate. Activity was expressed as "µg XMF activity" which is *ca.* the amount of crude XMF per tube (1 µg) required to give 50% inhibition in the presence of 2 ng folate.

Fractionation of the crude antagonist. 2.0 g crude XMF was dissolved in 500 ml 1.0 M NH_4OH, excess NH_3 removed *in vacuo*, the solution (pH 6.9) diluted to 1000 ml and 2×10^6 cpm of [^{3}H]PteGlu added as a folic acid position marker. The solution was applied to a DEAE-cellulose (Whatman DE 52) column (5 × 20 cm) and eluted with a series of gradients, as described in Fig. 1.

The fractions from the column separation were pooled according to their biological activities, the pH adjusted to 3 and the samples were applied onto a column (2 × 5 cm) of Amberlite XAD-2 (100-200 µ).

Since the biological activities of Fraction 1 and 2 were not retained by XAD-2, these fractions were not further purified.

The activity of Fraction 3 was eluted from the XAD-2 column with 80 ml of 5% t-BuOH in 1 M HCl. The pH of the eluate was adjusted to 3 with KOH and the resulting precipitate, which contained about 100 mg 9-methyl folic acid, was collected.

Fraction 4, which was strongly retained by the XAD-2 column, was eluted from the resin with 0.1 M NH_4OH and applied onto a QAE-Sephadex column (1.6 × 33 cm). This column was washed with 50 ml of H_2O and eluted with a linear 0-1 M NaCl gradient (pH 6.2) in 5 mM phosphate. The final concentration of NaCl was 0.5 M. 100 fractions of 5 ml each were collected. The activity was eluted in Fraction 51-65. After the pH was adjusted to 3, the solution was passed through an XAD-2 column (1 × 4 cm), and the active compound was eluted with 5 ml 0.1 M NH_4OH. The folate antagonist was finally obtained in pure form (100 µg) by using HPLC. 250 ml aliquots of the previous solution were fractionated on a reversed-phase column (ALTEX ultrasphere-octyl; 5 µ; 1 × 25 cm) by eluting with a 0.05 M citric acid buffer (pH 4) containing 8% dioxane. At a flow rate of 4 ml/min the antagonist eluted after 15 min. Evaporation of dioxane was required to readsorb the pure compound onto XAD-2.

The XAD-2 eluate prepared from Fraction 5a was fractionated by HPLC using the same reversed-phase column as for Fraction 4. By using 15% dioxane in 0.05 M citrate buffer (pH 4), a small UV-absorbing peak appeared after 11 min (4 ml/min), which coincided with the biological activity. The specific activity of this peak corresponded to an activity of about 1000 µg XMF activity per µg of pterin (based on UV-absorption at 254 nm and assuming that 1 absorbance unit equals 20 µg pterin). No activity could be eluted from the column when Fraction 5b was analyzed by HPLC. Usually, Fractions 5a and 5b were coeluted with 5 M HCl, immediately adjusted to pH 3, and the precipitate treated as described in the legend to Table 1.

TABLE 1: PURIFICATION OF ACID-SOLUBLE FRACTION FROM CRUDE XMF

Purification step	Total activity (mg XMF)	Total solids (mg)	Specific activity as µg XMF activity per µg solids	Percent overall yield
0) Crude XMF (2 g)	2000	2000	1.0	100
1) DEAE 5 N HCl eluate	1600	400*	4*	80
2) 1st XAD-2 eluate	1500	50*	30*	75
3) Bio Gel P-4 eluate	1400	7.0*	200*	70
4) 2nd XAD-2 eluate	1400	2.2*	400*	45
5) Bio Gel P-2 eluate (homogeneous by HPLC)	400	0.40*	1000*	20

* Solids were estimated from UV-absorption at 254 nm at pH 12 assuming that 1 ml of a solution with an absorbance of 1.0 corresponds to 20 µg of conjugated pterin such as folic acid.

The procedures used to purify the "acid-soluble" folate antagonist and to prepare the fractions described in the table were:

1) 2 g of crude XMF, dissolved in 500 ml 1 M NH_4OH, was applied onto a DEAE-column (4 × 12 cm). After consecutive washing with 200 ml H_2O and 600 ml 1 M KCl, the dark brown layer was removed (4 cm) from the top of the column and eluted with 100 ml 5 M HCl to yield the DEAE 5 N HCl eluate.

2) The DEAE 5 N eluate was adjusted to pH 1 and the activity was adsorbed on an XAD-2 column (2 × 5 cm). It was eluted with 30 ml 0.1 M NH_4OH followed by 20 ml 15% t-BuOH to yield the 1st XAD-2 eluate.

3) The 1st XAD-2 eluate was adjusted to pH 3. The resulting precipitate was collected by centrifuging, redissolved with 0.05 M K_2HPO_4 (15 ml) and applied onto a polyacrylamide (Bio Gel P-4) column (4 × 30 cm). Elution with 0.05 M phosphate buffer (pH 6.3) and selection of the most active fraction yielded the Bio Gel P-4 eluate (800 ml).

4) The Bio Gel P-4 eluate was adjusted to pH 2.5 and added onto a short XAD-2 column (1 × 3 cm). After washing with H_2O, the active compound was eluted with 20 ml 0.1 M NH_4OH (2nd XAD-2 eluate).

5) The 2nd XAD-2 eluate was adjusted to pH 3. The precipitate which formed was separated, redissolved in 1.5 ml 0.05 M K_2HPO_4 and applied to a Bio Gel P-2 column and water applied. Most of the impurities eluted first and the last band contained the active compound. This step was repeated until the active fraction consisted of a compound which appeared homogeneous by HPLC.

RESULTS AND DISCUSSION

The fractionation of crude XMF on DEAE-cellulose, as shown in Fig. 1, revealed four folate-antagonizing components which could be eluted with a KCl gradient. Only strong acid eluted the major active components ("acid-soluble" fraction).

Fraction 1 (probably a pteridine) contained about 3% and Fraction 2 about 1% of the original activity. Fraction 3, accounting for 1% of the original activity and 5% by weight of the crude reaction product, consisted of 9-methyl folic acid which was identified on basis of spectral data (UV; NMR) and by HPLC in comparison with an authentic sample. Folic acid, coeluting with the radioactive tracer and identified by HPLC and biological activity, was present in small amounts (20 µg/g) in the crude XMF. Fraction 4 was further purified by chromatography on QAE-Sephadex followed by HPLC on ALTEX ultrasphere-octyl to yield a folate analogue with a specific biological activity of 20 µg XMF activity per µg solids (based on UV-absorption). The UV-spectrum of the pure compound showed absorption maxima at 317 nm and 400 nm in 0.1 M NaOH and 317 nm and 394 nm in 0.1 M HCl. Fractions 5a and 5b accounted for 60-80% of the biological activity. The observation that these fractions could be eluted only with strong acid is in contrast to common folate derivatives and their polyglutamates which can be eluted by a KCl gradient at pH 7.

The activity of pooled Fraction 5 was further concentrated by the use of Amberlite XAD-2, Bio Gel P-4 and Bio Gel P-2; and a product which appeared homogeneous by HPLC was obtained. The details of these procedures and the purification achieved in the various steps are given in Table 1. The UV-absorption spectrum is shown in Fig. 2. The absorption peak at 398 nm in alkaline solution occurs at a longer wavelength than that of folic acid, 9-methyl- or 7-methyl folic acid which have absorption maxima at *ca.* 355 nm. ^{13}C-NMR spectroscopy indicates the presence of 23 carbons (4 more than folic acid). The detailed NMR spectra and other data relating to structure are being reported separately. The biological activity of the purified acid-soluble fraction expressed as inhibition ratio to folic acid (ratio of

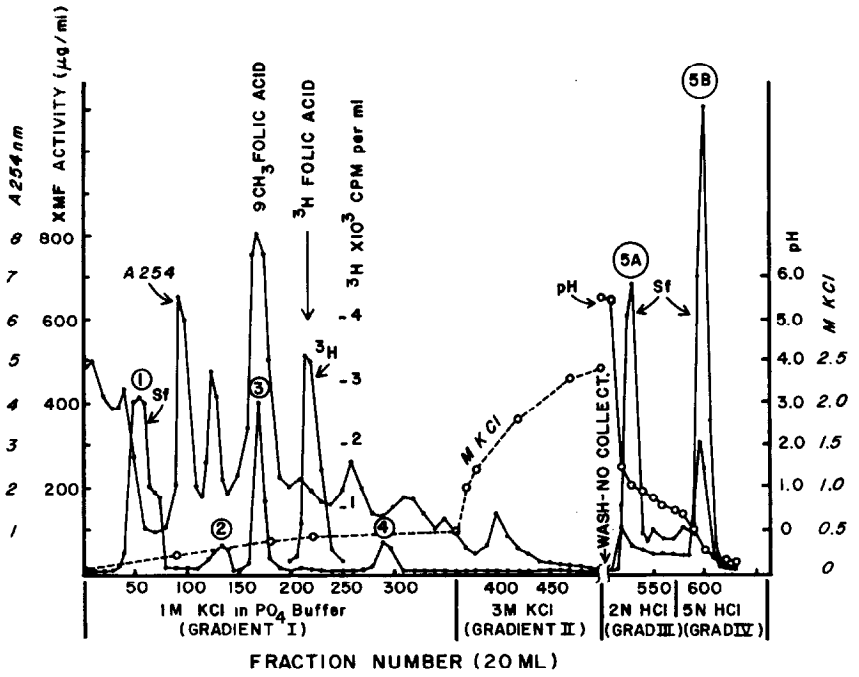

Fig. 1: Chromatographic separation of folate antagonists on a DEAE-column (5 × 20 cm) from 2 g of crude XMF.

Gradient I: This gradient was formed by adding dropwise 7.2 liters of a solution containing 1 M KCl in 5 mM phosphate buffer to a mixing chamber containing 9.0 liters of 5 mM phosphate (pH 7.0). The concentration of KCl at the end of the gradient was 0.55 M.

Gradient II: The solution remaining from gradient I was placed into a 2-liter mixing chamber and an exponential gradient formed by adding 2.0 liters of 3 M KCl in 5 mM phosphate (pH 7.0) from the reservoir. The final concentration of KCl was 2.1 M. After gradient II was applied, the column was washed with 2.0 liters of H_2O (no fractions were collected).

Gradient III: Addition of 2.0 liters of 1 M HCl to 2.0 liters H_2O in the mixing chamber yielded a gradient with a final HCl concentration of 0.63 M.

Gradient IV: 2.0 liters of 5 M HCl was placed into the reservoir and added to the 2.0 liters of HCl remaining from the previous gradient. The concentration of HCl at the end of this gradient was 3.38 M.

antagonist to folic acid to give 50% growth inhibition with *S. faecalis* at a level of 0.4 ng folate per ml) is 1.00 to 1. This may be compared with an inhibition ratio of 100 to 1 for Fraction 4, 500 for crude XMF, 3000 for 9-methyl folic acid, and 20 for 10-methyl folic acid.

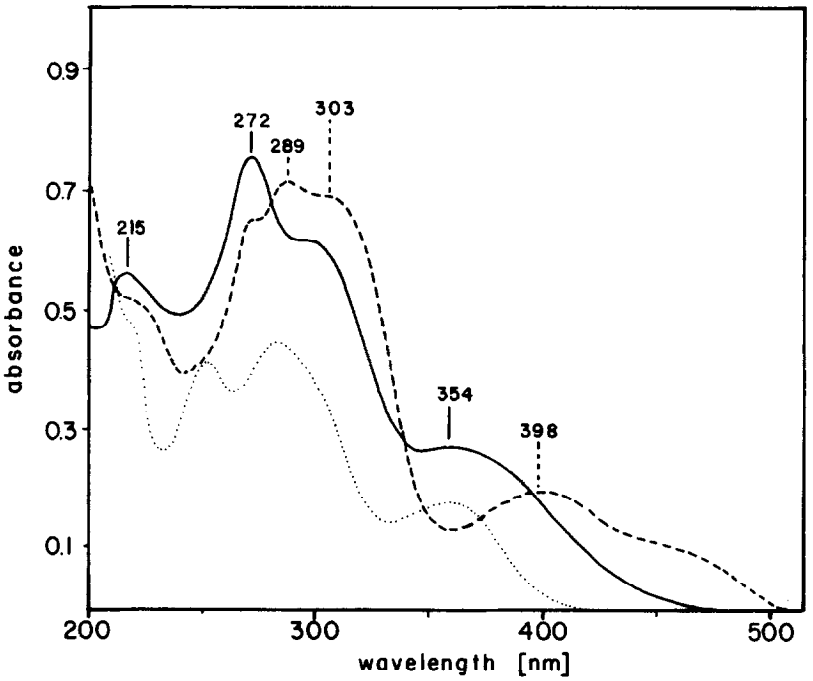

Fig. 2: UV-absorption of XMF in 0.5 M NaOH (——) and in 0.5 M HCl (---) in comparison with 7-methyl folic acid in 0.5 M K_3PO_4 (•••).

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