a nitroamino group are also rapidly excreted, probably by the tubular acid transport system.

It has been possible to design nitrofurans with a high urinary excretion which is probably due to tubular secretion. Their high urinary excretion, which is superior to that of nitrofurantoin, indicates good peroral absorption and slow metabolic inactivation. Excretion by the tubular acid transport system requires that the nitrofurans are acids. However, acidic groups decrease the antibacterial activity, especially against gram-negative bacteria. Although nitrofurantoin is also an acid, it is too weak to be completely dissociated at physiological pH, which may explain its high antibacterial activity. Whether it is possible to design nitrofurans with tubular excretion and a higher antibacterial activity than nitrofurantoin, or whether the optimum has been attained with nitrofurantoin, has yet to be established.

Experimental Section

The structures of all compounds were assigned on the basis of compatible ir, nmr, and mass spectra and satisfactory analyses. The melting points are uncorrected. All compounds were analyzed for C, H, N, and S and the analytical results were within ±0.4% of the calculated values.

Chemistry. Method A. Synthesis of 3a,c and 4a. Starting material for the synthesis of **3a** was 4-methyl-5-(5-nitro-2-furyl)-1,2,4-triazolin-3-one,[8] for **3c** 5-(5-nitro-2-furyl)-1,3,4-thiadiazolin-2-one, and for **4a** 5-(5-nitro-2-furfurylidene)thiazolidine-2,4-dione.[17] The sodium salts of the starting materials were prepared by adding 0.05 mol of sodium methylate dissolved in 30 ml of DMF to a mixture of 0.05 mol of the starting material and 40 ml of DMF. To the sodium salt 5.3 ml (0.05 mol) of ethyl chloroacetate was added and the mixture was heated at 90° for 2 hr. DMF was then evaporated *in vacuo* and the residue was treated with water. A crude product precipitated. **3c** and **4a** were recrystallized from ethanol. Repeated recrystallizations were required to obtain **4a** in pure form. **3a** was purified from the starting material by dissolving in acetone. The starting material was then removed by filtration. The acetone solution was evaporated and the residue recrystallized from water to give pure **3a**.

Method B. Synthesis of 3e and 3g. The starting material for synthesis of **3e** was 5-(5-nitro-2-furyl)-1,3,4-oxadiazolin-2-one[18] and for **3g** 5-(5-nitro-2-furyl)vinyl-1,3,4-oxadiazolin-2-one.[19] The sodium salts of the starting materials were prepared by dissolving 0.025 mol of the starting material in 40 ml of DMF and then adding in portions 1.2 g (0.025 mmol) of NaH (dispersed in oil) over a period of 45 min at 10–15°. To the sodium salt, 4.2 g (0.025 mol) of ethyl bromoacetate was added dropwise. The reaction solution was stirred for 3 hr at room temperature and then poured onto 200 g of ice. The product precipitated and was filtered and recrystallized from ethanol.

Method C. Synthesis of 3b,d,f,h and 4b. **3a,c,e,g** and **4a** were hydrolyzed by adding 0.007 mol of each to a mixture of 0.4 ml of concentrated H_2SO_4 and 18 ml of HCOOH and heating the mixture at 70–80° for 24 hr. The solution was then cooled and **3d,h** and **4b** precipitated. **3b,f** were obtained by evaporating the HCOOH *in vacuo*. **3b,d** and **4b** were recrystallized from water, **3f** from ethyl acetate–CCl_4, and **3h** from acetonitrile.

Method D. Synthesis of 5a–c, 6, and 7. For synthesis of **5a** the starting material was 3-amino-5-(5-nitro-2-furyl)-1,2,4-triazole, for **5b** 3-amino-4-methyl-5-(5-nitro-2-furyl)-1,2,4-triazole, for **5c** 2-amino-5-(5-nitro-2-furyl)-1,3,4-thiadiazole, for **6** 3-amino-2-methyl-5-(5-nitro-2-furyl)-1,2,4-triazole, and for **7** 3-amino-1-methyl-5-(5-nitro-2-furyl)-1,2,4-triazole.[8] The starting material (0.01 mol) was added in small portions to a mixture of 0.7 ml (0.01 mol) of concentrated HNO_3 and 6 ml of concentrated H_2SO_4 at 0° over a period of 20 min. The mixture was stirred for another 60 min at 0° and then poured onto ice. The product was collected by filtration and **5a** and **7** were recrystallized from methanol, **5b** from ethanol, and **6** from ethanol–DMF. **5c** was recrystallized from methanol–DMF and then reprecipitated in water.

***N*-(5-Nitro-2-furoyl)glycine (2).** Glycine (34 mmol) was dissolved in 75 ml of water and pH was adjusted to 8.5. A solution of 6 g (34 mmol) of 5-nitro-2-furoyl chloride in 14 ml of dioxane was added dropwise to the above solution at room temperature over a period of 35 min. The pH of the solution was kept at 8.5 by continuous addition of diluted NaOH. The reaction was completed by stirring for 1 hr. The reaction solution was acidified with concentrated HCl to pH 0.5 and extracted with ethyl acetate. The ethyl acetate phase was collected, dried, and evaporated to give 6.1 g of crude product. This was recrystallized from ethyl acetate: yield, 3.0 g of **2**.

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Potential Anticancer Agents. 10. Synthesis of *N*-[4-[[(2,4-Diamino-7-pteridyl)methyl]-N^{10}-methylamino]benzoyl]glutamic Acid

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N-[4-[[(2,4-Diamino-6-pteridyl)methyl]-N^{10}-methylamino]benzoyl]glutamic acid (I, Methotrexate), synthesized in 1949 at the American Cyanamid Co.,[1] proved to be one of the powerful inhibitors of dihydrofolate reductase[2] and a very effective anticancer agent.[3] In order to explore this structural area, we decided to prepare one of its closely related analogs, the 7-isomer II.

The original procedure for the synthesis of I[1] involved the condensation in aqueous medium (pH 3–3.5) of tetraaminopyrimidine (III) with the sodium salt of *N*-[4-(*N*-methylamino)benzoyl]glutamic acid and 1,2-dibromopropionaldehyde. In alternate routes of synthesis, the use

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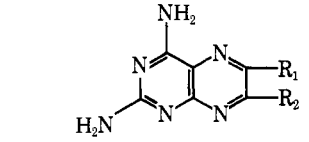


I (6-MTX), $R_1 = X$; $R_2 = H$
II (7-MTX), $R_1 = H$; $R_2 = X$
$X = -CH_2N(CH_3)C_6H_4CONHCHCOOH$
$(CH_2)_2COOH$

of other three-carbon carbonyl derivatives (*i.e.*, 1,1,3-tribromo-[4] and 1,1,3-trichloroacetone[5]) was suggested, the reaction and purification procedures being similar to those described for the preparation of folic acid.

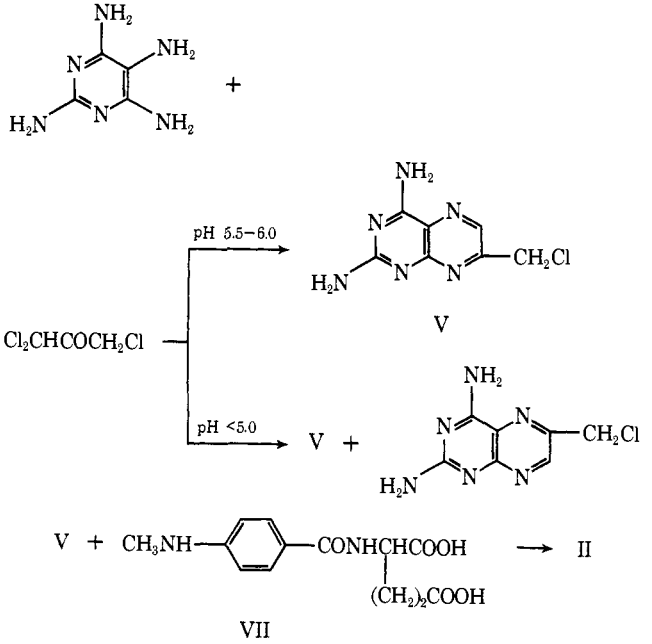
No mention is found in the above-mentioned papers about the isomeric purity of I, although the formation of 6- and 7-pteridyl isomers in the reaction between 4,5-diaminopyrimidines and an asymmetrically substituted polyfunctional three-carbon compound is well known.[6-12]

In our laboratory, the dibromopropionaldehyde procedure gave a low yield (2% based on III) of pure 6-MTX (I). By the procedure of Berezovskii and coworkers[5] we obtained a higher yield, but the product, chromatographically[5,13] identical with an authentic sample of I, exhibited only 60% of its biological activity, together with a higher molar extinction coefficient at 306 nm in 0.1 *N* HCl (as well by direct determination as after elution of the chromatographic spot). Oxidation and hydrolysis of the product to 2-amino-4-hydroxypteridinecarboxylic acids[1,14] provided evidence for the presence of both isomers (6- and 7-MTX).

Since no suitable method for their separation could be found, other approaches for the selective synthesis of the two isomers were undertaken.

Mention should be made that the preparation of 7-isoaminopterin and 7-isofolic acid by T. L. Loo was reported in a review.[3]

The synthesis of II (7-MTX) could be achieved by the following two-step procedure.



VII

The preferential formation of one of the isomers in the closure of the pteridine-ring system is governed by many reaction parameters, one of them being the pH value. Although the amino groups in the 4(6) and 5 positions of the pyrimidine ring have quite different basicities,[15] because of the complex nature of the pH influence both on the pyrimidine and on the carbonyl derivative, no simple general rules can be drawn up. Anyhow, in our case, in the first step, the pH value was critical; V, devoid of 2,4-diamino-6-chloromethylpteridine (VI), could be obtained only when the reaction was carried out in the pH range from 5.5 to 6.0. At pH 5.0 the cyclization led to a mixture of V and VI.

Table I. Comparative Biological Activities of I (6-MTX) and II (7-MTX)

Compd	LD_{50},[a] mg/kg body	Dose, mg/kg/day	Dosage schedule	ILS,[b] %
6-MTX	18	0.3	Daily × 10	130
7-MTX	691	100	Daily × 10	16
		200	Daily × 10	30

[a]Single doses, on Wistar rats: J. Cornfield and N. Mantel, *J. Amer. Statist. Ass.*, **45**, 181 (1950). [b]Determined in L1210 bearing mice BDF_1: R. H. Adamson, S. T. Yancev, M. Ben, T. L. Loo, and D. P. Rall, *Arch Int. Pharmacodyn.*, **153** (1, 2), 87 (1965). The treatment began 24 hr after ip inoculation of 10^6 leukemic cells.

An analytical sample of V could not be obtained but the crude product could be used in the next step without purification. The condensation of V with *N*-[4-(*N*-methylamino)benzoyl]glutamic acid (VII) in Na acetate buffer of pH 3–3.5 gave 7-MTX in an overall yield of 12–16% (based on VII). The purification was achieved by fractional precipitation followed by column chromatography on cellulose and cellulose–charcoal. The standard procedure confirmed the isomeric purity of the compound.

A new procedure[16] was also developed for the synthesis of I devoid of 7-isomer, in an overall yield of about 10% (based on VII).

The comparison between the physicochemical properties and the biological behavior of 6- and 7-MTX confirms the presence of both isomers in the product obtained by the 1,1,3-trichloroacetone procedure.[5] Thus, their uv spectra are slightly different and the molar extinction coefficient is higher for 7-MTX (see Experimental Section). Differences in the ir spectra of the two isomers, difficult to assign, could also be pointed out (see Experimental Section). Due to their extremely low volatility the mass spectra of the two isomers were difficult to perform (when increasing the temperature they decomposed so that no molecular ion appeared) and the few minor differences which appeared at m/e <160 could not be used for structural analysis. In contrast, the nmr spectra of the two compounds (I and II) revealed distinct patterns in the aromatic region.

The C_7 proton of I and C_6 one of II gave singlets at distinctly different chemical shifts (δ 8.51 and 9.01, respectively) in agreement with other literature data.[17] There are also smaller but significant differences in chemical shift in the two pairs of aromatic doublets produced by the proton in the para-substituted benzene ring. Because of their low solubility, nmr spectra of 6- and 7-chloromethylpteridine could not be performed.

Biological Data. The antitumor activity of II was evaluated on L1210 leukemia on mice (see Table I). The change of the side-chain position on the pteridine ring led to striking changes in the biological properties of compound II.[18]

Experimental Section

Melting points were taken on a Boetius apparatus and are uncorrected. The ultraviolet spectra were determined with a CF4 Optica Milano spectrophotometer. The ir spectra were run on an

UR 10 Carl Zeiss Jena spectrophotometer in KBr pellets. The nmr spectra were run on a JEOL 100-MHz spectrometer in DMSO-d_6. Paper chromatograms were run on Whatman No. 1 paper and the spots were detected under uv light.

***N*-[4-[[(2,4-Diamino-7-pteridyl)methyl]-N^{10}-methylamino]benzoyl]glutamic Acid (II).** A solution of tetraaminopyrimidine dihydrochloride (2.1 g, 0.01 mol) (prepared from tetraaminopyrimidine bisulfite[10] and HCl) in H_2O (50 ml) was added dropwise, during 6 hr and at room temperature, to a stirred solution of 1,1,3-trichloroacetone[5,19,20] (IV, 4.8 g, 0.03 mol) in 40 ml of H_2O and 8.2 g of AcONa at pH 6.0 (adjusted with concentrated HCl). NaOH solution (0.5 *M*) was occasionally added to keep the pH of the solution at 5.5–6.0. The reaction mixture was stirred overnight at room temperature and then cooled at 0°. A brown solid was collected by filtration and washed (H_2O, EtOH, EtOH–Et_2O (1:1), Et_2O) to afford 1.7 g of 2,4-diamino-7-chloromethylpteridine (V, 80%).

Paper chromatography in BuOH–EtOH–H_2O (100:35:72, ascending) showed a major spot at R_f 0.57 and a minor impurity at R_f 0.49.

To a warm (45°) solution of *N*-[4-(*N*-methylamino)benzoyl]glutamic acid[21,22] (VIII, 2.8 g, 0.01 mol) in 300 ml of 4 *N* AcONa–AcOH buffer (pH 4) was added dropwise over a period of 1 hr a solution of V (2.1 g, 0.01 mol). The mixture was maintained at 45° for 40 hr and then gradually warmed at 55° for 2 hr, at 65° for 2 hr, and finally at 75° for 1 hr, the pH value being kept at 4 by occasional addition of 1 *N* NaOH. The mixture was cooled and left overnight at 4°. The solid was filtered off, washed (cold H_2O, EtOH, EtOH–Et_2O (1:1), Et_2O; 40 ml, respectively), and dried *in vacuo* at 50° for 24 hr, giving about 3 g of crude II.

The above crude II was suspended in H_2O (150 ml), basified (Na_2CO_3) to pH 11–12, heated at 50°, and the insoluble residue was removed by filtration. The filtrate was neutralized (1 *N* HCl) to pH 7, left overnight at 4°, treated with active charcoal, and filtered. The filtrate was brought to pH 3.5–4.0 (1 *N* HCl), left overnight at 4°. The precipitate was filtered, washed (cold H_2O, EtOH, EtOH–Et_2O (1:1), Et_2O; 20 ml, respectively), and dried *in vacuo* at 50° for 24 hr giving 1.8 g of partially purified II.

The product thus obtained was suspended in 20 ml of distilled water and the pH adjusted to 7.0 with 0.5 *N* NaOH. The insoluble impurities were removed by filtration. The solution was passed over a column (ϕ 22 mm) of 100 g of cellulose (Schuchardt) suspended in an aqueous solution of 0.1 *M* Na_2HPO_4 (pH adjusted to 7.0 with concentrated HCl). Elution with the same phosphate buffer was started 1 hr after introducing the sample. The fractions containing II (uv monitoring) were combined, the pH was adjusted to 3.5–4.0 (glacial AcOH) and cooled overnight at 4°, and the precipitate was worked up as above giving 1.2 g of a still impure II with R_f 0.60 (paper chromatography, descending, eluent 0.1 *M* phosphate buffer pH 7.0); additional spots R_f 0.3 and in the start.

The above II (1.2 g) was suspended in 25 ml of distilled H_2O, dissolved by neutralization (1 *N* NaOH) to pH 7.0, and passed over a three-layer column (1 g of cellulose; 12 g of cellulose and 2.5 g of charcoal, and finally 12 g of cellulose, suspended in water) with distilled H_2O as eluent. From the uv, selected fraction II was precipitated and worked up as above giving 0.55–0.75 g of pure II (12–16% based on VII): mp 203°; uv spectra λ_{max} (nm) ($\epsilon \times 10^{-3}$) 0.1 *N* HCl for II (7-MTX) 243.6 (sh), 311.6 (24.1); for I (6-MTX) 240, 306 (21.7); ir (KBr) ν_{max} (cm^{-1}) for II (7-MTX) 767, 811, 832, 924, 946, 1007, 1100, 1208, 1248, 1309, 1368, 1404 (sh), 1451, 1507, 1515, 1557, 1595 (sh), 1609, 1635, 1643; for I (6-MTX) 766, 803, 828, 938, 961, 1018, 1099, 1113, 1206, 1252, 1291, 1385 (sh), 1395, 1452, 1506, 1514, 1551, 1595 (sh), 1608, 1635, 1643; nmr (DMSO-d_6) δ for II (7-MTX) 8.51 (1 H, s), 8.08 (2 H, m), 7.14 (2 H, m) (arom region); for I (6-MTX) 9.01 (1 H, s), 8.18 (2 H, m), 7.14 (2 H, m). *Anal.* ($C_{20}H_{28}N_8O_5 \cdot 2H_2O$) C, H, N; H_2O (K. Fischer).

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In Vitro and *in Vivo* Activity of Certain Thiosemicarbazones against *Trypanosoma cruzi*

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From the time thiosemicarbazones were first prepared as derivatives for the identification of aldehydes, ketones, and quinones,[1] biological activity in many areas has been noted including antibacterial activity (particularly antitubercular[2]), fungistatic activity,[3] antiinflammatory activity,[4] antiparasitic activity (*Trichomonas vaginalis*[5]), antiviral activity (vaccinia,[6-8] herpes, and cytomegalo),[9,10] and antitumor activity.[11] Recent investigations in our laboratory have revealed that certain members of this chemical group possess significant *in vitro* and *in vivo* activity against *Trypanosoma cruzi* infections (Chagas' disease).

Biological Results and Discussion. Of the compounds found active *in vitro* (Table I) against *T. cruzi*, **4**, **5**, **6**, and **10** were examined for *in vivo* activity. All produced significant increases in the mean survival times of infected mice (Table II) although no radical cures were effected at near toxic doses.

General conclusions regarding the structure–activity relationship of thiosemicarbazone structure and *in vitro T. cruzi* activity parallel the conclusions drawn by Bernstein, *et al.*,[12] and Young, *et al.*,[13] with respect to antitubercular structure–activity relationships, namely, (a) the sulfur cannot be replaced by oxygen, (b) substitution at 3 or 4 of the thiosemicarbazone eliminates activity, and (c) thiosemicarbazides and aliphatic thiosemicarbazones are inactive.

Since it has recently been shown[2,9,10] that there are certain parallels between the structure–activity relationships of thiosemicarbazones and antitubercular and antiviral activities, it may be that the antitrypanosomal ac-