detect the pyrimidine compounds MU and S-acetyl MU (III) by visual examination (Chromato-Vue, Ultra-Violet Products, Inc.); (2) for the detection of the peptides, glutathione, oxidized glutathione, and S-acetylglutathione, the ninhydrin dipping reagent was used as described by Toennies and Kolb;[19] (3) for the identification of the thiolester compounds the hydroxamate test was employed as described by Stadtman.[20] The R_f values obtained for glutathione, oxidized glutathione, and S-acetylglutathione agree within ±0.06 with those reported.[21]

Acknowledgment.—The authors are grateful to Mr. Michael S. Anderson of the Calasanctius Preparatory School for his technical assistance in this work.

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Cofactor Inhibition of Thymidylate Synthetase. Tetrahydrofolic Acid Analogs[1,2]

MATHIAS P. MERTES AND AI JENG LIN

Department of Medicinal Chemistry, School of Pharmacy, The University of Kansas, Lawrence, Kansas 66044

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Analogs of N^5,N^{10}-methylenetetrahydrofolic acid were designed and synthesized in an effort to define the essential features of cofactor binding and inhibition of the enzyme, thymidylate synthetase. Ethyl 3-methyl-2-pyrazinecarboxylate (**1**), prepared by condensation of ethylenediamine and ethyl 2,3-dioxobutyrate, was reduced (LAH) to the aldehyde **2**. Formation of the Schiff base **3** from **2** and ethyl *p*-aminobenzoate was followed by $NaBH_4$ reduction to give N-(*p*-carbethoxyphenyl)-3-methyl-2-aminomethylpyrazine (**4**). Treatment of **4** with 5-bromomethyluracil gave the N-thyminyl derivative **5**; reduction of **4** gave the piperazine derivative **6**. Condensation of **6** with 5-formyluracil gave 2-*p*-carbethoxyphenyl-3-(5′-uracil)-7-methyloctahydroimidazo-[1,5-*a*]pyrazine (**7**). By the same procedure used in the 3-methylpyrazine series, quinoxaline (**8a–10a**), 2-methylquinoxaline (**8b–10b**, **22**), and 2-methyl-1,2,3,4-tetrahydroquinoxaline (**11b**, **23**) analogs were prepared. An interesting ring enlargement to the symmetrical seven-membered diaza ketone was observed when 1,2,3,4-tetrahydro-2-hydroxymethyl-**1**,4-di-*p*-toluenesulfonylquinoxaline was oxidized using the DCC–DMSO method. The results of inhibition of thymidylate synthetase and dihydrofolate reductase are discussed.

Thymidylate synthetase, in the presence of the cofactor N^5,N^{10}-methylenetetrahydrofolic acid, catalyzes the conversion of 2′-deoxyuridine 5′-monophosphate to thymidine 5′-monophosphate.[3] The mechanism proposed for the one-carbon transfer is by a reductive methylation to give the product thymidine 5′-monophosphate and 7,8-dihydrofolic acid.[3]

Folate analogs have been studied as inhibitors of thymidylate synthetase.[4] In addition to the reduced aminopterin derivatives, tetrahydrohomofolate is an effective inhibitor of this enzyme. The rationale for this approach to inhibition is derived from kinetic studies on the enzyme.[3c] A sequential order of binding is noted; the initial complex of enzyme–cofactor is followed by formation of a ternary complex with the substrate. Stepwise dissociation leads to the products.

The nature of the intermediate proposed for the transfer of the carbon unit from the cofactor to the substrate necessitates the proper spatial positioning of these units on the enzyme. Attempts to bridge the binding sites of the cofactor and substrate in a single inhibitor have been unsuccessful.[2,4p] To achieve this additional studies have been undertaken to determine the essential structural features for cofactor binding to the enzyme. Previous results have suggested that the pyrimidine moiety of the pteridine ring of folic acid, important for dihydrofolate reductase binding, may not be essential for binding to thymidylate synthetase. In addition, relatively basic nitrogens corresponding to N^5 and N^8 of the folic acid model are essential since the piperazine ring is more inhibitory than the pyrazine ring in model compounds.[2a] Further studies in this series have been made to examine the effects of a CH_3 in a position corresponding to C-7 of folic acid since Zakrzewski[5] has involved C-7 of tetrahydrofolic acid as the source of H in the reductive methylation of deoxyuridine 5′-monophosphate. Substitution of a benzene ring for the pyrimidyl moiety of folic acid was also undertaken to access the effect of the aromatic ring on the binding affinity of N^5 and N^8 positions of folic acid analogs.

2,3-Dimethylpyrazine (Scheme I) was synthesized by condensation of ethylenediamine with 2,3-butanedione followed by aromatization; selective oxidation (K-

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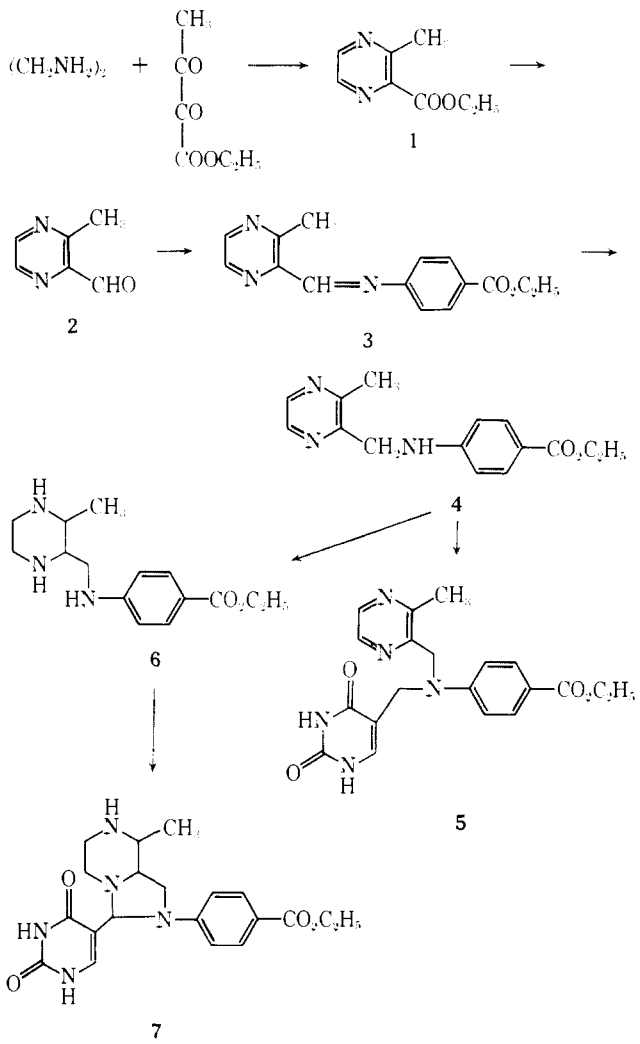
(2) For previous studies in this series see: (a) M. P. Mertes and N. R. Patel, *J. Med. Chem.*, **9** 868 (1966); (b) M. P. Mertes and Q. Gilman, *ibid.*, **10**, 965 (1967).

(3) For references on this enzyme see: (a) A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **237**, 3794 (1962); (b) R. Blakley, *ibid.*, **238**, 2113 (1963); (c) P. Reyes and C. Heidelberger, *Mol. Pharmacol.*, **1**, 14 (1965).

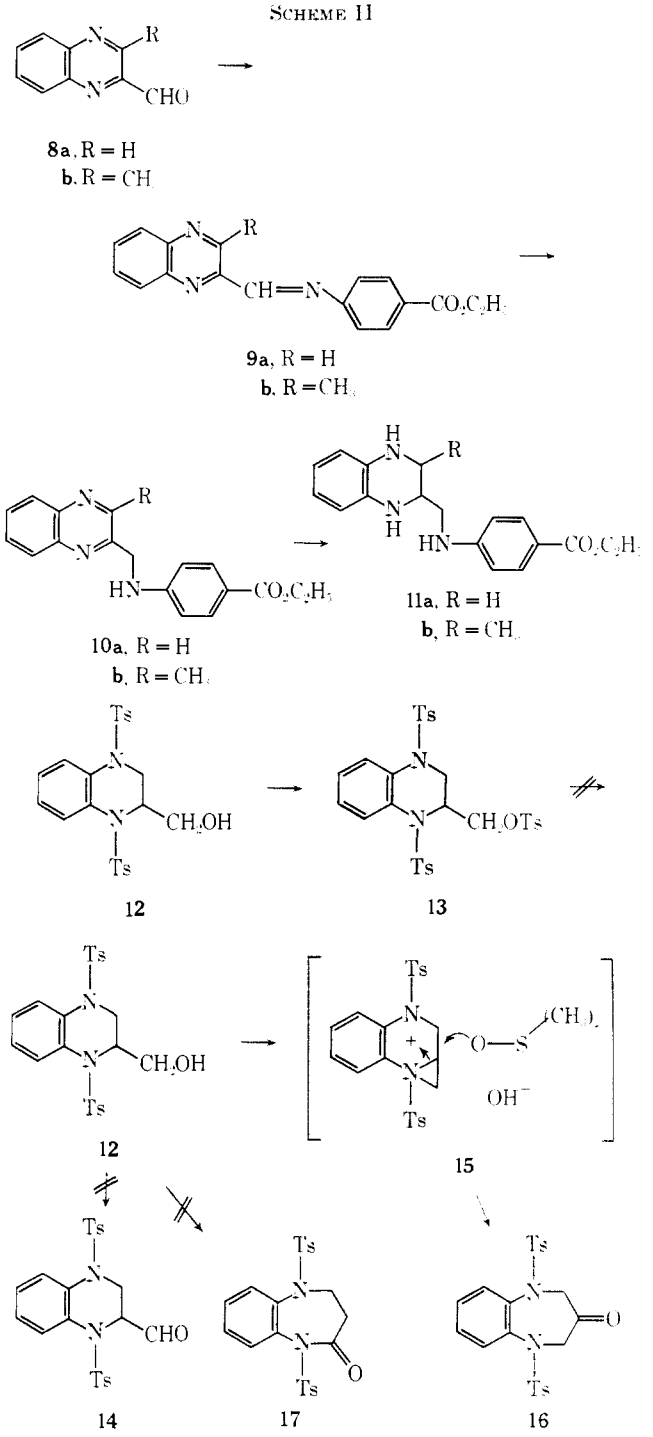
(4) (a) R. L. Kisliuk, *Nature*, **188**, 584 (1960); (b) M. Friedkin, E. J. Crawford, and D. Misra, *Federation Proc.*, **21**, 176 (1962); (c) A. J. Whaba and M. Friedkin, *J. Biol. Chem.*, **236**, PC11 (1961); (d) R. L. Kisliuk and M. D. Levine, *ibid.*, **239**, 1901 (1964); (e) L. Goodman, J. I. DeGraw, R. L. Kisliuk, M. Friedkin, E. J. Pastore, E. J. Crawford, L. T. Plante, A. Al-Nahas, J. F. Morningstar, Jr., G. Kwok, L. Wilson, E. F. Donovan, and J. Ratzan, *J. Am. Chem. Soc.*, **86**, 308 (1964); (f) G. L. Tong, W. W. Lee, and L. Goodman, *ibid.*, **86**, 5664 (1964); (g) B. R. Baker, B. T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964); (h) B. R. Baker B. T. Ho, and G. R. Clheda, *ibid.*, **1**, 88 (1964); (i) J. A. R. Mead, A. Goldin, R. L. Kisliuk, M. Friedkin, L. Plante, E. J. Crawford, and G. Kwok, *Cancer Res.*, **26**, 2374 (1966); (j) L. T. Plante, E. J. Crawford, and M. Friedkin, *J. Biol. Chem.*, **242**, 1466 (1967); (k) V. S. Gupta and F. M. Huennekens, *Biochemistry*, **6**, 2168 (1967); (l) K. Slavik and S. F. Zakrzewski, *Mol. Pharmacol.*, **3**, 370 (1967); (m) D. Livingston, E. J. Crawford, and M. Friedkin, *Biochemistry*, **7**, 2814 (1968); (n) S. B. Horwitz and R. L. Kisliuk, *J. Med. Chem.*, **11**, 907 (1968); (o) L. T. Weinstock, D. E. O'Brien, and C. C. Cheng, *ibid.*, **11**, 1238 (1968); (p) D. V. Santi, *J. Heterocyclic Chem.*, **4**, 475 (1967).

(5) S. F. Zakrzewski, *J. Biol. Chem.*, **241**, 2962 (1966).

SCHEME I



SCHEME II



MnO_4) gave 3-methylpyrazine-2-carboxylic acid in poor over-all yield.[6] A better procedure to the desired ester **1** was by condensation of ethylenediamine with ethyl 2,3-dioxobutyrate;[7] aromatization of the unstable intermediate gave ethyl 3-methyl-2-pyrazinecarboxylate (**1**). Reduction of **1** to 3-methylpyrazine-2-carboxaldehyde (**2**) employed the procedure of Rutner and Spoerri:[8] addition of LAH to **1** at −70°. The Schiff base **3**, prepared by condensation of **2** with ethyl *p*-aminobenzoate, was reduced with $NaBH_4$–MeOH to the amine **4**.[2a] The N-thyminyl derivative **5** was synthesized by treating **4** with an equimolar amount of 5-bromomethyluracil[9] and Na_2CO_3 in THF containing a catalytic amount of NaI.

Reduction of the pyrazine ring in **4** by Pt in HOAc to ethyl *p*-N-[2-(3-methylpiperzinyl)methyl]aminobenzoate (**6**) was accompanied by minor hydrogenolysis to give ethyl *p*-aminobenzoate as the side product. Condensation of **6** with 5-formyluracil in MeOH gave 2-*p*-carbethoxyphenyl-3-(5-uracil)-7-methyloctahydroimidazo[1,5-*a*]pyrazine (**7**). 5-Formyluracil[10] can be prepared easily by Ce(IV) oxidation of 5-hydroxymethyluracil[11] according to a known method.[12]

Quinoxaline-2-carboxaldehyde (**8a**)[13] (Scheme II) was condensed with ethyl *p*-aminobenzoate to give the Schiff base **9a** previously reported by Acheson;[14] reduction over Pt in dioxane gave ethyl *p*-2-quinoxalinylmethylaminobenzoate[14] (**10a**) in 35% yield; the low yield is due to hydrogenolysis to give ethyl *p*-aminobenzoate. Reduction of the pyrazine ring of the

(6) (a) T. Ishiguro, M. Matsumura, and H. Murai, *Yakugaku Zasshi*, **80**, 349 (1960); (b) T. Ishiguro, and M. Matsumura, *ibid.*, **78**, 229 (1958).
(7) W. Dennis, *Am. Chem. J.*, **38**, 587 (1907).
(8) H. Rutner and P. E. Spoerri, *J. Org. Chem.*, **28**, 1898 (1963).
(9) J. A. Carlson, *ibid.*, **25**, 1731 (1960).
(10) R. H. Wiley and Y. Yamamoto, *ibid.*, **25**, 1906 (1960).
(11) R. E. Cline, R. M. Fink, and K. Fink, *J. Am. Chem. Soc.*, **81**, 2521 (1959).
(12) (a) W. S. Trahanovsky, L. B. Young, and G. L. Brown, *J. Org. Chem.*, **32**, 3865 (1967); (b) R. Brossmer and D. Ziegler, *Tetrahedron Lett.*, 5253 (1966).
(13) G. L. Leese and H. N. Rydon, *J. Chem. Soc.*, 303 (1955).
(14) (a) R. M. Acheson, *ibid.*, 4731 (1956); (b) S. Tamura, *Yakugaku Zasshi*, **80**, 559 (1960).

quinoxaline **10a** to the tetrahydro derivative **11a** was attempted by catalytic hydrogenation using Pt or Rh–C in a variety of solvents; however, in all cases hydrogenolysis resulted.

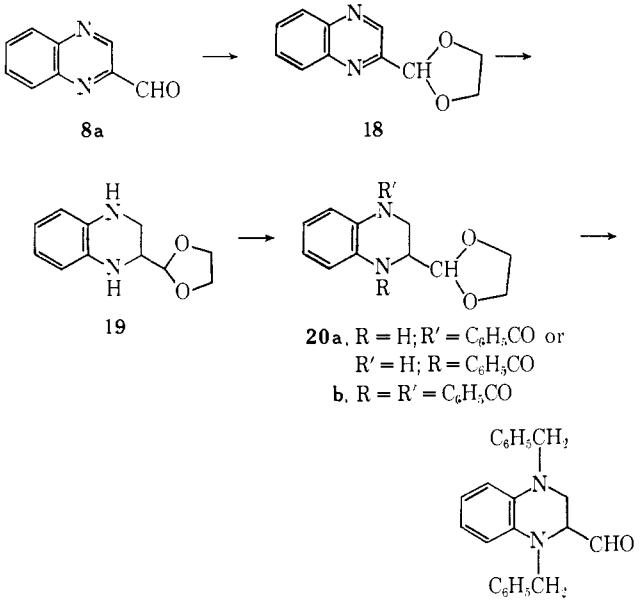
Alternate approaches to **11a** were attempted starting with 1,2,3,4-tetrahydro-2-hydroxymethyl-1,4-di-*p*-toluenesulfonylquinoxaline (**12**).[14] The tritosylate **13** was prepared; however, displacement of the tosyl with ethyl *p*-aminobenzoate failed, probably due to the poor nucleophilicity of the amine. No reaction was observed when oxidation of the hydroxymethyl group of **12** was attempted using Sarett's reagent.[15]

Oxidation of the alcohol **12** using DCC in DMSO according to Moffatt[16] did not give the expected aldehyd **14**. Instead, the product showed a carbonyl in the ir spectrum (1740 cm^{-1}) and four equivalent aliphatic protons in the nmr spectrum (singlet at δ 4.05). Elemental analysis and the nmr and ir data support structure **16**, a seven-membered diaza ketone. Several mechanisms can be formulated for this rearrangement (**12** → **16**); a simple carbonium ion mechanism would most probably lead to the unsymmetrical ketone **17**. The intermediacy of an aziridinium ion (**15**) readily accounts for the product **16**.

A further attempt failed to give the aldehyde **14** through condensation of N,N′-di-*p*-toluenesulfonyl-*o*-phenylenediamine with 2,3-dibromoacrolein diethyl acetal using several solvents over a wide temperature range.

The final approach (Scheme III) to **11a** was from the

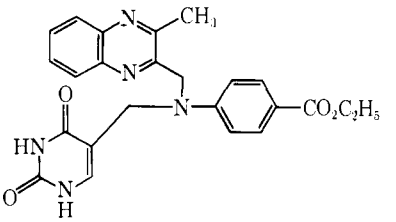
SCHEME III



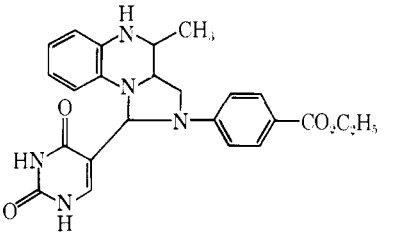
aldehyde **8a**; formation of the acetal **18** followed by LAH reduction[17] gave 1,2,3,4-tetrahydroquinoxaline-2-carboxaldehyde ethylene acetal (**19**). Protection of the amino groups (**20b**) was achieved by heating **19** with an excess of C_6H_5COCl in C_5H_5N–C_6H_6 mixed solvent. Using lower than reflux temperatures and two equivalents of C_6H_5COCl gave the monobenzamide **20a**; the site of benzolation N^1 or N^4 in **20a** was not ascertained. LAH reduction of **20b** gave an excellent yield of the starting acetal **19** through C–N cleavage rather than the expected product of amide reduction, **21**. Diborane[18] reduction of **20b** did yield N^1,N^4-dibenzyl-1,2,3,4-tetrahydroquinoxaline-2-carboxaldehyde ethylene acetal (**21**). Hydrolysis of **21** to the aldehyde was unsuccessful using, among others, the following catalysts: BF_3, $HClO_4$, HCl, H_2SO_4, $AlCl_3$, and HIO_4. It is reported that the dimethyl acetal of pyrazine-2-carboxaldehyde is also resistant to hydrolysis.[19]

The synthesis of **11a** was reported recently by Benkovic and coworkers[20] through reduction of **10a** with $NaBH_4$ in diglyme.

The 3-methylquinoxalines (**10b**, **22**) and the reduced derivatives (**11b**, **23**) were prepared starting with ethyl 3-methylquinoxaline-2-carboxylate.[21] Reduction to the aldehyde **8b** (Scheme II) by inverse addition of LAH at −70°, followed by formation of the Schiff base **9b** and reduction using $NaBH_4$–MeOH, gave **10b**. Catalytic hydrogenation using Pt–HOAc gave a 40% yield of the tetrahydroquinoxaline analog **11b**.



22



23

Alkylation of the quinoxaline **10b** with bromomethyluracil gave the thyminyl derivative **22**. Condensation of the tetrahydroquinoxaline **11b** with 5-formyluracil gave 2-*p*-carbethoxyphenyl-3-(5′-uracil)-9-methylhexahydroimidazo[1,5-*a*]quinoxaline (**23**). The possible formation of a cyclic six-membered condensation product with N^4 instead of N^1 is excluded by the fact that ethyl *p*-N-(3-piperidylmethyl)aminobenzoate failed to give the corresponding cyclic product with 5-formyluracil.[22]

Biochemical Results.—The compounds in this series were assayed for inhibition of thymidylate synthetase and dihydrofolate reductase (Table I) by described procedures.[2a] Both the esters and their saponification products were examined. The results do not show exceptional activity in this series when compared to the

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(16) (a) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, **85**, 3027 (1963); (b) *ibid.*, **87**, 5670 (1965); (c) *ibid.*, **88**, 1762 (1966).

(17) R. C. Deselms and H. S. Mosher, *ibid.*, **82**, 3762 (1960).

(18) H. C. Brown and D. Heim, *ibid.*, **86**, 3566 (1964).

(19) J. D. Dehun and R. Levine, *J. Org. Chem.*, **23**, 406 (1958).

(20) (a) S. J. Benkovic, P. A. Benkovic, and D. R. Comfort, *J. Am. Chem. Soc.*, **91**, 1860 (1969); (b) S. J. Benkovic, private communication.

(21) H. Dahn and H. Hauth, *Helv. Chim. Acta*, **42**, 1214 (1959).

(22) M. P. Mertes and A. J. Lin, in preparation.

marked inhibition of the respective enzymes by 5-trifluoromethyl-2′-deoxyuridine 5′-phosphate and aminopterin; however, structure–activity correlations can be drawn from a comparison of the relative activities.

The effect of basicity at the N's corresponding to N^5 and N^8 of tetrahydrofolic acid is noted when the pyrazine **4** and the quinoxaline **10b** are compared to the reduced analogs **6** and **11b**. The latter are more effective than the aromatic compounds against thymidylate synthetase, whereas no significant difference is noted with the reductase enzyme. This agrees with the reports[4b,c-n] that the reduction of aminopterin to the di- and tetrahydro derivative shows enhancement of inhibition of the synthetase enzyme and loss of inhibition of the reductase.

TABLE I
ENZYME INHIBITION STUDIES[a]

Compound	$([I]/[S])_{0.5}$ for thymidylate synthetase[b]		$([I]/[S])_{0.5}$ for dihydrofolate reductase[c]	
	Ester[d]	Salt of the acid[e]	Ester[d]	Salt of the acid[e]
4	8.4	5.2	31	43
5	5	41	17	325
6	0.9	3.6	40	51
7	3	9	33	70
10b	Insoluble	4.2	Insoluble	8
11b	3	0.9	20	6.4
22	3		52	11
23	1.3	3.2	7	120

[a] For a description of the enzyme sources, isolation procedure, and assay, see ref 2a. [b] $([I]/[S])_{0.5}$ refers to the ratio of the molar concentrations of the inhibitor and substrate (cofactor) measured as *dl*-tetrahydrofolic acid, necessary for 50% inhibition. The assay solution was 2.8×10^{-4} *M* *dl*-tetrahydrofolic acid and 4.2×10^{-5} *M* deoxyuridine 5′-phosphate. [c] $([I]/[S])_{0.5}$ refers to the ratio of the molar concentrations of the inhibitor and substrate, dihydrofolic acid, necessary for 50% inhibition. The substrate was present in 3.3×10^{-5} *M* in the assay solution. [d] The esters were assayed as DMSO solutions using an equivalent amount of DMSO as the control rate. [e] The esters were saponified by heating for several hours in 0.1 *M* KOH and assaying the salt of the acid as an aqueous solution.

With some exceptions the presence of the additional aromatic moiety (quinoxaline series compared to the pyrazine series) enhances the binding to the reductase (**6**, **7**, **11b**, and **23**). Such an increase, however, is not observed for the synthetase enzyme. The addition of the pyrimidine ring to give thyminyl analogs **5** and **22** and the uracil analogs **7** and **23** again did not show enhancement of binding that would be expected from bridging of the enzymatic binding sites for substrate and cofactor. However, the fact that there was no significant change in inhibition (compared to **4**, **10b**, **6**, and **11b**, respectively) suggests bulk tolerance for the pyrimidine ring in this region of the enzyme.[23] It appears from the results in this series that piperazine and tetrahydroquinoxaline analogs should be explored for bulk tolerance and additional modes of binding to thymidylate synthetase, perhaps through hydrophobic binding regions.

This series of compounds was also examined for inhibition of growth of *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. Filter paper disks were saturated with a 0.1 *M* solution of the compound, dried, and placed on inoculated agar plates. Compound **6** showed a slight inhibition of all cultures (~1 mm). Compound **10b** inhibited growth of *B. cereus* and *B. subtilis* (~1 mm). None of the other compounds were inhibitory.

[23] B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Active-Site," John Wiley & Sons, Inc., New York, N. Y., 1967.

Experimental Section[24]

Ethyl 3-Methylpyrazine-2-carboxylate (1).—Ethyl 2,3-dioxobutyrate[6] (10 g, 0.07 mole) in 200 ml of C_6H_6 was added dropwise with vigorous stirring to a solution of ethylenediamine (4.2 g, 0.07 mole) in boiling C_6H_6 (250 ml) with a Dean–Stark trap to remove the H_2O formed. After the addition was complete, the solution was refluxed until the theoretical amount of H_2O was collected. The solution was cooled and 10% Pd–C (1.5 g) was added. The mixture was refluxed for 18 hr with stirring, and filtered. The filtrate was evaporated to dryness under reduced pressure and the dark red residue was extracted several times with 100-ml portions of hot petroleum ether (bp 60–70°). The extracts were combined, cooled to 5° for 1–2 hr, and filtered. The filtrate was concentrated to 100 ml and cooled overnight to give pale yellow needles. More crystals were obtained by again concentrating the filtrate to give a total weight of 3.7 g (30%). Recrystallization from petroleum ether (bp 60–70°) gave yellow needles, mp 49.5–51.5°. For elemental analysis, the compound was sublimed (0.5 mm) to give very pale yellow microcrystals, mp 51.5–52.5°. *Anal.* ($C_8H_{10}N_2O_2$) C, H, N.

3-Methylpyrazine-2-carboxaldehyde (2).—Ethyl 3-methylpyrazine-2-carboxylate (1) (4.3 g, 0.0261 mole) was dissolved in 100 ml of dried THF. The solution was cooled to −70° with a Dry Ice–MeOH bath. To the solution, LAH (0.516 g, 0.0221 mole) in 80 ml of dry THF was added slowly over a period of 30 min. The reaction was stopped by addition of 4 ml of HOAc. The light brown mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in 30 ml of 2.5 *N* HCl and extracted eight times with 50-ml portions of $CHCl_3$. These were stirred with 100 ml of H_2O and $NaHCO_3$ powder was added in small portions until the evolution of CO_2 ceased. The $CHCl_3$ layer was separated, dried ($MgSO_4$), and evaporated to dryness. The pungent oil was purified by solution in small volume of petroleum ether (bp 60–70°) at room temperature, filtration, and overnight cooling to give the colorless needles which melted at room temperature; these were separated by decantation of the mother liquid while cold. The same procedure was repeated twice; trace amounts of petroleum ether were removed under reduced pressure. The purified oil weighed 1.5 g (47%), bp 184–185° (742 mm); the 2,4-DNP prepared by the general method melted at 220–221°. *Anal.* ($C_{12}H_{10}N_6O_4$) C, H, N.

Ethyl *p*-(3-Methyl-2-pyrazalamino)benzoate (3).—The aldehyde **2** (1.20 g, 0.01 mole) and ethyl *p*-aminobenzoate (1.60 g, 0.01 mole) were refluxed in 30 ml of C_6H_6 overnight with removal of H_2O. The solvent was evaporated to dryness, the residue was dissolved in a small amount of EtOAc, petroleum ether was added to turbidity, and the solution was clarified by addition of two drops of EtOAc. The solution was allowed to settle overnight and the precipitates were collected and washed twice with $CHCl_3$ to give an unknown compound (mp 121–123°). The $CHCl_3$ washing solution and the filtrate were evaporated to dryness and the residue was recrystallized from EtOAc–petroleum ether solvent to give **3** as pale yellow microcrystals (1.2 g, 48%), mp 99–101°. *Anal.* ($C_{13}H_{15}N_3O_2$) C, H, N.

N-(*p*-Carbethoxyphenyl)-3-methyl-2-aminomethylpyrazine (4).—The Schiff base **3** (0.8 g, 0.003 mole) was dissolved in 30 ml of anhydrous MeOH, the solution was cooled to 0°, and $NaBH_4$ (0.25 g, 0.006 mole) was added in small portions during 10 min with stirring. The solution was stirred for another 30 min and then refluxed for 50 min. After cooling to room temperature, 30 ml of H_2O was added and the product was collected and washed with H_2O to give 0.7 g (90%) of **4** as white crystals, mp 114–116°.

[24] All melting points were taken on a calibrated Thomas–Hoover capillary melting point apparatus. Analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind., and on an F & M Model 185, The University of Kansas. Spectral data were obtained using Beckman IR-8 and IR-10 Varian A-60 and A-60A spectrometers. The latter used Me_4Si as an internal standard except in D_2O where 3-trimethylpropanesulfonic acid sodium salt was employed. The nmr and ir spectra were as expected. Where analyses are indicated only by symbols of the elements analytical results obtained for those elements are within 0.4% of the theoretical values.

Recrystallization from MeOH–H_2O solvent raised the melting point to 115–116°. *Anal.* ($C_{15}H_{17}N_3O_2$) C, H, N.

Ethyl *p*-N-[2-(3-Methylpyrazinyl)methyl]-N-thyminylaminobenzoate (5).—The amine **4** (1.0 g, 3.7 mmoles), 5-bromomethyluracil (0.8 g, 3.7 mmoles), anhydrous Na_2CO_3 (0.44 g, 3.7 mmoles), and a catalytic amount of NaI were refluxed in 100 ml of dry THF for 14 hr (drying tube). The solution was filtered while hot, the residue was washed with hot THF, and the filtrates were combined and evaporated to dryness under reduced pressure. The residue was refluxed in 50 ml of absolute MeOH and filtered. The filtrate was cooled at 5° overnight to give pale yellow crystals (0.64 g, 40%). Recrystallization from MeOH gave white needle crystals, mp 206–207°. *Anal.* ($C_{20}H_{21}N_5O_4$) C, H, N.

Ethyl *p*-N-[2-(3-Methylpiperazinyl)methyl]aminobenzoate (6).—The secondary amine **4** (0.5 g, 1.85 mmoles) in 20 ml of HOAc was hydrogenated in a microhydrogenation unit which contained 30 ml of HOAc and 0.5 g of prereduced PtO_2. The hydrogenation was stopped when 3 mole equiv of H_2 had been absorbed. The filtrate from the catalyst was lyophilized. The oily residue was dissolved in 30 ml of H_2O and extracted with Et_2O (20 ml) three times. The H_2O layer was adjusted to pH 10 with 10% NaOH, saturated with NaCl, and extracted several times with $CHCl_3$ (20 ml). The $CHCl_3$ extracts were combined, dried ($NaSO_4$), and evaporated to dryness under reduced pressure to give 0.4 g (80%) of pale yellow oil. The oil was used for further reaction without purification: nmr ($CDCl_3$) δ 1.10 (d, 3, J = 7 Hz, NCCH_3), 1.37 (t, 3, J = 7.5 Hz, OCH$_2$CH_3), 1.70 (s, 2, piperazine NH), 2.3–3.5 (b, 8, NCH), 4.40 (q, 2, J = 7.5, OCH_2), 5.12 (b, 1, Ar-NH), 6.66 (d, 2, J = 9 Hz, phenyl 3,5-H), 8.00 (d, 2, J = 9 Hz, phenyl 2,6-H).

2-*p*-Carbethoxyphenyl-3-(5′-uracil)-7-methyloctahydroimidazo[1,5-*a*]pyrazine (7).—The amine **6** (0.5 g, 1.7 mmoles) and 5-formyluracil (0.25 g, 1.7 mmoles) were refluxed in absolute MeOH (25 ml) overnight. The solution was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was recrystallized from 10% MeOH–H_2O solvent, with charcoal to remove the colored substances, to give 0.25 g (40%) of white powder, mp 240° dec. *Anal.* ($C_{20}H_{25}N_5O_4$) C, H, N.

1,2,3,4-Tetrahydro-2-hydroxymethyl-1,4-di-*p*-toluenesulfonylquinoxaline (12).—A mixture of *p*-toluenesulfonyl chloride (59 g, 0.31 mole), *o*-phenylenediamine (15 g, 0.14 mole), and pyridine (75 moles) was heated to 100° for 1 hr, and poured into 1 l. of H_2O. The precipitate was collected and recrystallized from EtOH to give 27 g of N,N′-di-*p*-tosylphenylenediamine (48%), mp 204° (lit.[14b] 204°). The entire 27 g (0.064 mole) and 2,3-dibromopropanol (14.1 g, 0.065 mole) in EtOH (75 ml) were added to a solution of Na (3.3 g, 0.143 g-atom) in EtOH (300 ml). After refluxing for 6 hr, the solvent was removed and the residual sticky solid was washed with H_2O and dried. It was then refluxed with C_6H_6 (40 ml) and after cooling the residue was collected. Recrystallization from EtOH gave white prisms, mp 190–192° (lit.[14b] 193°).

1,4-Ditosyl-1,2,3,4-tetrahydro-2-quinoxalinylmethyl Tosylate (13).—Compound **12** (11.3 g, 0.024 mole) was dissolved in C_5H_5N (50 ml). The solution was cooled to 10° with stirring and TsCl (0.24 mole) was added gradually to the solution at such a rate that the temperature of the reaction mixture was not over 20°. The mixture was stirred at room temperature for 12 hr. The reaction mixture was then poured into HCl (300 ml of H_2O + 100 ml of concentrated HCl) and the precipitate was collected; recrystallization from EtOH–AcMe gave prisms (10 g, 70%), mp 186–188°. *Anal.* ($C_{30}H_{30}N_2O_7S_3$) C, H, N, S.

N[1],N[5]-Di-*p*-toluenesulfonyl-3-oxo-2,3,4,5-tetrahydro-1,5-benzodiazepine (16).—Compound **12** (2 g, 4.2 mmoles) and DCC (3.1 g, 16.8 mmoles) were dissolved in anhydrous DMSO (10 ml). Phosphorous acid (0.18 g, 2.2 mmoles) in 3 ml of anhydrous DMSO was then added and the mixture was stirred overnight at room temperature. The precipitate was removed by filtration and saturated oxalic acid–MeOH solution was added to the filtrate. The solution was allowed to stand for 1 hr and the precipitated urea was removed by filtration. The collected precipitates were washed with 20 ml of C_6H_6. The filtrate and the C_6H_6 solution were combined, washed (5% aqueous $NaHCO_3$, H_2O), dried ($MgSO_4$), and evaporated to dryness. Recrystallization of the residue from EtOAc gave 0.5 g (25%) of colorless prisms: mp 176–178°; ir (Nujol) 1740 cm^{-1} (C=O); nmr ($CDCl_3$) δ 2.45 (s, 6, CH_3), 4.05 (s, 4, CH_2), 7.50 (m, 12, aromatic H). *Anal.* ($C_{23}H_{22}N_2O_5S_2$) C, H, N, S.

Quinoxaline-2-carboxaldehyde Ethylene Acetal (18).—Quinoxaline-2-carboxaldehyde (**8a**) (5 g, 0.031 mole), ethylene glycol (10 ml, excess), and *p*-toluenesulfonic acid (0.1 g) were added to C_6H_6 (60 ml). The mixture was refluxed for 4 hr with stirring and removal of H_2O. The solution was cooled to room temperature and poured into aqueous Et_2O–$NaHCO_3$. The Et_2O layer was separated, washed (H_2O), dried (Na_2SO_4), and evaporated to dryness under reduced pressure to give an oily residue which solidified after standing at room temperature. Recrystallization from petroleum ether (60–70°) gave 5 g (83%) of white prisms, mp 69–70°. *Anal.* ($C_{11}H_{10}N_2O_2$) C, H, N.

1,2,3,4-Tetrahydroquinoxaline-2-carboxyaldehyde Ethylene Acetal (19).—To a stirred solution of **18** (1 g, 0.015 mole) in 100 ml of dry Et_2O was added a solution of LAH (0.36 g, 0.015 mole) in 30 ml of dry Et_2O. The mixture was stirred for 6 hr under an inert atmosphere at room temperature and then decomposed by cautious addition of 5 ml of 10% NaOH solution. The precipitate was removed by filtration and washed twice with 50 ml of Et_2O. The filtrate and washings were combined and dried (Na_2SO_4). Evaporation of the solvent gave an oily residue which was dissolved in 50 ml of C_6H_6. Petroleum ether was then added until the solution became turbid and the solution was cooled for 0.5 hr. The gummy precipitate was removed by decantation. After cooling overnight, 0.6 g (60%) of white crystals were collected, mp 61–63°. *Anal.* ($C_{11}H_{14}N_2O_2$) C, H, N.

1,2,3,4-Tetrahydroquinoxaline-2-carboxaldehyde Ethylene Acetal Monobenzamide (20a).—Compound **19** (0.5 g, 0.0024 mole) was dissolved in 5 ml of dry C_5H_5N and 20 ml of dry C_6H_6. C_6H_5COCl (0.64 g, 0.0048 mole) was added dropwise with continuous shaking; pyridine HCl formed immediately as the reaction proceeded. The suspension was heated at 50–60° for 30 min. H_2O (100 ml) was added after the reaction mixture was cooled to room temperature. The C_6H_6 layer was separated and the H_2O layer was extracted with 20 ml of C_6H_6. The C_6H_6 extracts were combined and washed (1 N HCl, 5% $NaHCO_3$, H_2O). After drying ($MgSO_4$), C_6H_6 was removed under reduced pressure and the residue was recrystallized from petroleum ether (bp 60–70°)–C_6H_6 solvent yielding 0.5 g (66%) of needles, mp 135°. *Anal.* ($C_{18}H_{18}N_2O_3$) (H, N; C: calcd, 69.67; found, 70.08).

1,2,3,4-Tetrahydroquinoxaline-2-carboxaldehyde Ethylene Acetal 1,4-Dibenzamide (20b).—Compound **19** (0.5 g, 0.0024 mole) was dissolved in 20 ml of dry C_6H_6 which contained 5 ml of dry C_5H_5N. C_6H_5COCl (**1** g, excess) was added dropwise with shaking. The pyridine hydrochloride which precipitated was collected and the clear solution was heated under reflux for 5–8 hr. After cooling to room temperature, H_2O (100 ml) was added. The C_6H_6 layer was separated and the aqueous layer was washed once with 20 ml of C_6H_6. The combined C_6H_6 solutions were then washed (1 N HCl, 5% $NaHCO_3$, H_2O) and dried ($MgSO_4$), the solvent was evaporated under reduced pressure, and the residue was recrystallized from petroleum ether–C_6H_6 to give 0.9 g (90%) of white crystals, mp 166–167°. *Anal.* ($C_{25}H_{22}N_2O_4$) C, H, N.

N[1],N[4]-Dibenzyl-1,2,3,4-tetrahydroquinoxaline-2-carboxaldehyde Ethylene Acetal (21).—Compound **20b** (1.5 g, 0.0036 mole) in 25 ml of THF was added slowly under inert atmosphere with stirring to 14.4 ml of 1 M B_2H_6 in THF solution which was maintained at 0° during the addition. The colorless solution was then refluxed for 1 hr. The flask was allowed to cool to room temperature and an adequate amount of 3 M HCl was added to decompose the excess B_2H_6. The solvent was removed by vacuum distillation. NaOH pellets were added to saturate the aqueous solution and extracted three times with 10-ml portions of Et_2O. The Et_2O extracts were combined and dried ($MgSO_4$). Evaporation of the Et_2O gave an oil; attempts to make the picrate salt failed. The solution turned black after the addition of saturated picric acid solution. The ir C=O of **20b** disappeared after the reduction. The product was used for further reactions without purification.

3-Methylquinoxaline-2-carboxaldehyde (8b).—Ethyl 3-methylquinoxaline-2-carboxylate[21] (20 g, 0.096 mole) in dry THF (500 ml) was cooled to −70° in a MeOH–Dry Ice bath. To the solution, LAH (2.00 g, 0.05 mole) in THF (150 ml) was added with stirring over 30 min under N_2. The solution was stirred for another 15 min at −70° and then was decomposed by dropwise addition of HOAc (13 ml). The solvent was evaporated to dryness under reduced pressure and the residue was dissolved in 90 ml of 2.5 N HCl and 60 ml of $CHCl_3$. The H_2O layer was extracted eight times with 60-ml portions of $CHCl_3$ and these were washed with $NaHCO_3$ solution until neutral. The $CHCl_3$ layer was separated, dried (Na_2SO_4), and evaporated to dryness. The residue was recrystallized from C_6H_6 to give 7.5 g (45%) of

yellow needle crystals, mp 142–143°. *Anal.* ($C_{10}H_8N_2O$) C, H, N.

Ethyl *p*-N-[2-(3-Methylquinoxalinyl)methylene]aminobenzoate (9b).—The aldehyde **8b** (3.0 g, 0.017 mole) and ethyl *p*-aminobenzoate (3.0 g, 0.018 mole) were refluxed in C_6H_6 (50 ml) for 15 hr, using a Dean–Stark H_2O trap to remove the H_2O formed. The solution was evaporated to dryness under reduced pressure. The residue was dissolved, using a small amount of EtOAc, and cooled to give fine yellow crystals which were collected (0.7 g, 50%) and recrystallized from EtOAc to give yellow needle crystals, mp 123–124°. *Anal.* ($C_{19}H_{17}N_3O_2$) C, H, N.

Ethyl *p*-N-[2-(3-Methylquinoxalinyl)methyl]aminobenzoate (10b).—The Schiff base **9b** (0.35 g, 1.1 mmoles) was suspended in absolute MeOH (20 ml) and cooled in an ice bath. To the suspension, $NaBH_4$ (0.083 g, 2.2 mmoles) was added in small portions within 30 min with stirring. The solution became clear after half of the $NaBH_4$ had been added and the desired product started to precipitate toward the end of the addition. The mixture was stirred at room temperature for another 3–4 hr. H_2O (10 ml) was added and the crystals were collected (0.32 g, 91%). For analysis the sample was recrystallized from MeOH to give white needle crystals, mp 143–144°. *Anal.* ($C_{19}H_{19}N_3O_2$) C, H, N.

Ethyl *p*-N-[2-(3-Methyl-1,2,3,4-tetrahydroquinoxalinyl)methyl]aminobenzoate (11b).—PtO_2 (0.5 g) was prereduced in HOAc (20 ml) in a microhydrogenation unit and a solution of **10b** (0.5 g, 1.57 mmoles) in HOAc (10 ml) was added. The solution was hydrogenated until 2 equiv of H_2 had been absorbed (*ca.* 20 min). The catalyst was removed by filtration and the solvent was removed by lyophilization. The oily residue was dissolved in Et_2O (15 ml), washed with 5% NaOH aqueous solution and H_2O, and dried (Na_2SO_4). The solution was evaporated to 5 ml and was found to contain three major components by tlc. The separation was carried out in a preparative tlc plate (Al_2O_3, Brinkmann, 20 × 20 cm, 1.5 mm thick, petroleum ether–EtOAc 5:1) by multiple-development technique to give 0.2 g (40%) of the desired product as pale yellow flakes after vacuum drying at room temperature. It could not be purified by recrystallization and softened when heated to 50–52°. The ditosylate was prepared according to the general method, mp 196–197°. *Anal.* ($C_{33}H_{34}N_3O_6S_2$) C, H, N.

Ethyl *p*-N-[2-(3-Methyl)quinoxalinyl)methyl]-N-thyminyl-aminobenzoate (22).—The secondary amine **10b** (0.57 g, 1.57 mmoles), 5-bromomethyluracil (0.32 g, 1.57 mmoles), Na_2CO_3 (0.16 g, 1.57 mmoles), and a catalytic amount of NaI were refluxed in dry THF (50 ml) for 14 hr. The solution was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 20 ml of hot MeOH. The white precipitate was collected after cooling and recrystallized from MeOH to give 0.3 g (43%) of white fine crystals, mp 216–217 dec. *Anal.* ($C_{24}H_{23}N_5O_4$) C, H, N.

2-*p*-Carbethoxyphenyl-3-(5′-uracil)-9-methylhexahydroimidazo[1,5-*a*]quinoxaline (23).—The amine **11b** (0.18 g, 0.55 mmole) and 5-formyluracil (0.07 g, 0.55 mmole) were refluxed in 30 ml of absolute MeOH under N_2 for 27 hr. The solution was evaporated to small volume and cooled overnight. The precipitate was collected (0.16 g, 70%) and recrystallized from MeOH–EtOH solvent to give pale yellow fine crystals, mp 237–238°. *Anal.* ($C_{24}H_{25}N_5O_4$) C, H, N.

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Irreversible Enzyme Inhibitors. CLXVI.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 2,4-Diamino-5-(3,4-dichlorophenyl)pyrimidine with 6 Substituents and Some Factors in Their Cell Wall Transport

B. R. BAKER AND NICOLAAS M. J. VERMEULEN[3]

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

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Fourteen 6-substituted derivatives of 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine were synthesized for comparison with the 6-methyl derivative (**1d**) (used as a standard) as reversible inhibitors of L1210 dihydrofolic reductase and for kill of L1210 cell culture; the best compounds were the 6-phenylbutyl (**5**) and 6-(α-naphthylethyl) (**6**) derivatives. However, **5** and **6** were still 100- and 300-fold less effective then the 6-methyl derivative (**1d**) against L1210 cell culture. Even less effective were the 6-phenoxymethyl (**2**) and 6-phenethyl (**3**) derivatives. In order to determine the effect of an SO_2F moiety, three of these 6-substituted pyrimidines were converted to irreversible inhibitors. The 6-(*p*-fluorosulfonyl)phenethyl (**17**) derivative was an excellent irreversible inhibitor of L1210 dihydrofolic reductase which also showed good specificity with no irreversible inhibition of the enzyme from mouse liver; however, **17** was no more effective than the parent **3** against L1210 cell culture. As previously noted in another series of compounds, the SO_2F moiety slows the rate of cell wall penetration, but increases the effect on the target enzyme when the molecule is an active-site-directed irreversible inhibitor.

Among the numerous active-site-directed irreversible inhibitors[4] of dihydrofolic reductase from L1210 mouse leukemia, a few showed specificity with a low amount of inactivation of the enzyme from normal liver, spleen, and intestine of the mouse; among these selective compounds was **1a**.[5,6] Although **1a** was a reasonably specific irreversible inhibitor for the L1210 enzyme, its reversible inhibition of the L1210/DF8 enzyme of $I_{50} = 6K_i = 0.82\ \mu M$ was considered to be too high to be useful *in vivo*.[5] Therefore a series of compounds re-

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(2) (a) For the previous paper of this series see B. R. Baker and M. Cory, *J. Med. Chem.*, **12**, 1053 (1969). (b) For the previous paper on this enzyme see B. R. Baker, E. E. Janson, and N. M. J. Vermeulen, *ibid.*, **12**, 898 (1969).

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