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Irreversible Enzyme Inhibitors. XC. Candidate Active-site-directed

Irreversible Inhibitors of Thymidylate Synthetase. I.

2-Amino-5-(benzenesulfonamidopropyl)-6-methyl-4-pyrimidinol

with Substituents on the Benzene Ring (1,2).

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2-Amino-5-[*p*-(bromoacetamidomethyl)benzenesulfonamidopropyl]-6-methyl-4-pyrimidinol (XV) was synthesized by acylation of 2-amino-5-aminopropyl-6-methyl-4-pyrimidinol (III) with *p*-cyanobenzenesulfonyl chloride followed by catalytic reduction and reaction of the resultant aminomethyl group with *p*-nitrophenyl bromoacetate. A second irreversible inhibitor of thymidylate synthetase, namely 2-amino-5-[*p*-(bromoacetyl)benzenesulfonamidopropyl]-6-methyl-4-pyrimidinol (XVI), was synthesized by acylation of III with *p*-acetylbenzenesulfonyl chloride followed by bromination. Both XV and XVI were good reversible inhibitors of thymidylate synthetase and inactivated the enzyme when the candidate compound was incubated with the enzyme. Iodoacetamide, which does not form a complex with enzyme, could inactivate thymidylate synthetase almost as well as XV; therefore it appears that XV inactivated the enzyme by a random bimolecular mechanism rather than by the desired active-site-directed mechanism via an enzyme-inhibitor complex. Similar conclusions were reached with XVI since phenacyl bromide could inactivate the enzyme somewhat more rapidly than XVI.

Active-site-directed irreversible enzyme inhibitors operate by formation of a complex between the enzyme and the inhibitor, followed by inactivation of the enzyme due to rapid covalent bond formation via a facile neighboring group reaction between a properly positioned leaving group on the inhibitor and a nucleophilic group on the enzyme (3-5). After the first publication on a deliberately designed active-site-directed irreversible inhibitor (6), a spate of publications appeared on this type of inhibition on such enzymes as chymotrypsin (7,8), trypsin (9), dihydrofolic reductase (10,11), adenosine deaminase (12), and other enzymes (3,4). An active-site-directed irreversible enzyme inhibitor for thymidylate synthetase, the enzyme that converts 2'-deoxyuridylate to thymidylate with 5,10-methylene-*l*-tetrahydrofolate as the methyl donor (13), could have considerable utility in chemotherapy (3,4).

It had been previously reported from this laboratory that 2-amino-5-(*p*-tolylsulfonamidopropyl)-6-methyl-4-pyrimidinol (I) (14) and its *N*-butyl derivative (II) (15) are reasonably good reversible inhibitors of thymidylate synthetase; 50% inhibition was observed at a ratio of inhibitor to 5,10-methylene-*l*-tetrahydrofolate of 11 and 17, respectively.

Therefore, our first candidates as active-site-directed irreversible enzyme inhibitors had the leaving group placed on the benzene ring of I; the synthesis and enzymic evaluation of these compounds are the subjects of this paper. The synthesis and enzymic evaluation of candidate irreversible inhibitors with a leaving group on the R-group of I are the subjects of the paper that follows.

The first two candidate irreversible inhibitors of thymidylate synthetase were the *p*-bromoacetamidobenzenesulfonamides, XIII and XXII. The amine intermediate (XII) required for the synthesis of XIII was readily prepared in two steps by reaction of the aminopropylpyrimidine (III) with *p*-nitrobenzenesulfonyl chloride to give VIII in near quantitative yield, as previously described for the synthesis of I (14); catalytic hydrogenation of VIII afforded XII in 62% yield.

The amine intermediate (XXI) required for the synthesis of the bromoacetamide, XXII, was synthesized from *p*-nitrobenzenesulfonilide (XVII) in four steps by the general method previously described (16). Alkylation of XVII with excess 1,3-dibromopropane in dimethyl sulfoxide in the presence of potassium carbonate afforded XVIII in 59%

yield. Condensation of the bromopropyl sulfonamide (XVIII) with sodio ethyl acetoacetate in *t*-butyl alcohol to give XIX followed by reaction with guanidine in *t*-butyl alcohol (17) afforded the pure 2-amino-4-pyrimidinol (XX) in 28% overall yield for the two steps. Catalytic reduction of the nitro group of XX gave XXI in 89% yield.

In order to block the 2-amino group of the pyrimidine, the *p*-nitrobenzenesulfonamide (XX) was

acetylated with boiling acetic anhydride to give XXIII in 60% yield; catalytic reduction afforded XXIV in 61% yield. When VIII was treated with boiling acetic anhydride, as in the preparation of XXIII, the desired XXVI was not obtained; instead a sulfur-free compound was isolated that was not identified. By acetylation of VIII with acetic anhydride in *N,N*-dimethyl formamide at room temperature, the desired 2-acetamidopyrimidine (XXVI) was obtained in

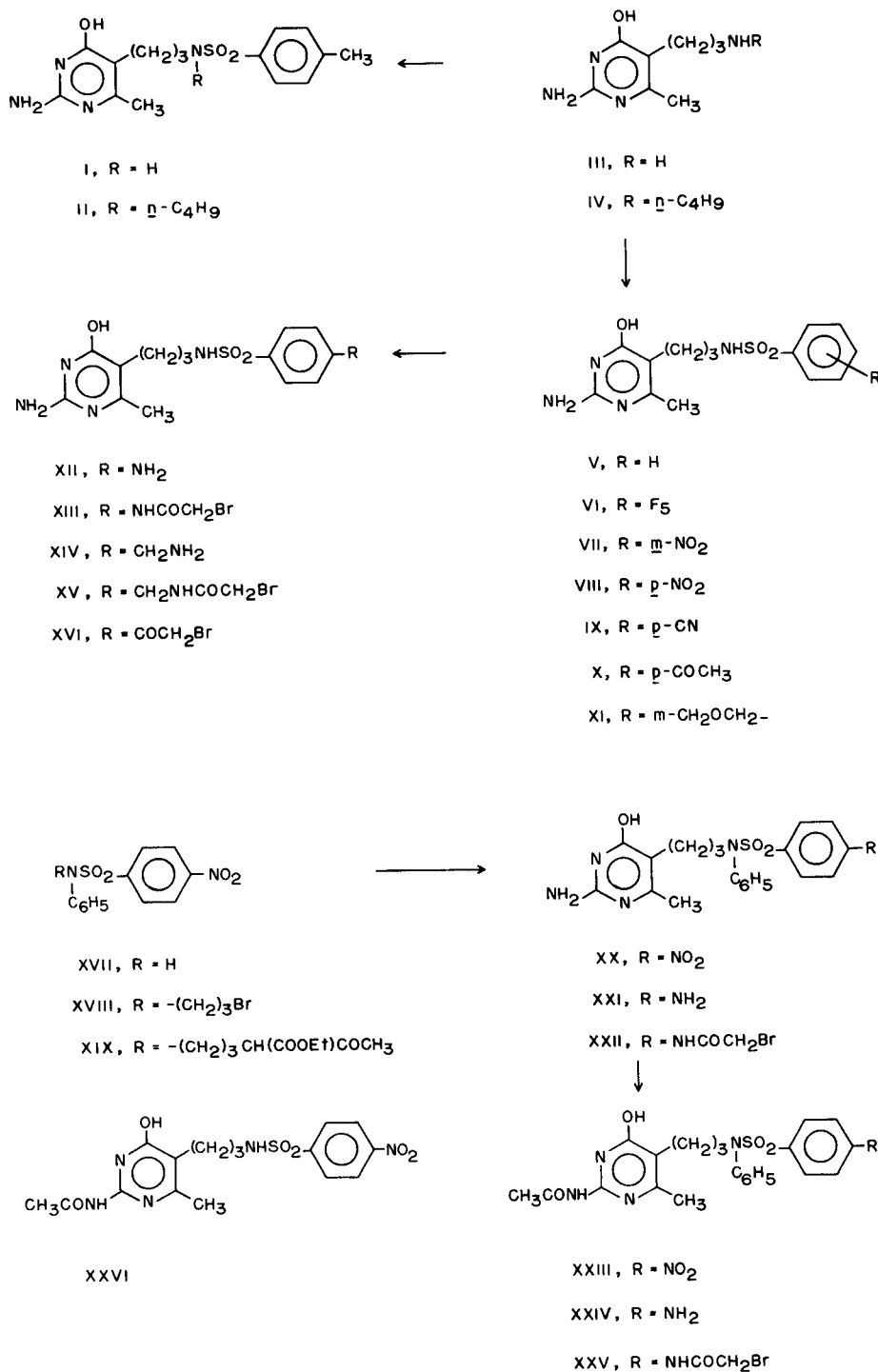
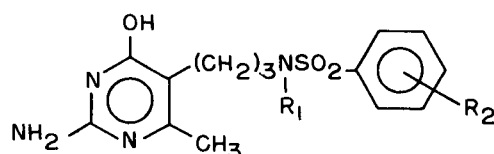


TABLE I
Reversible Inhibition of Thymidylate Synthetase by



Compound	R ₁	R ₂	mM Conc. of Inhibitor	Percent Inhibition (a)	Estimated [I/S] _{0.5} (b)
I	H	CH ₃ - <i>p</i>	0.28	50	11 (c)
II	<i>n</i> -C ₄ H ₉ -	CH ₃ - <i>p</i>	0.15 (d)	26	17 (e)
V	H	H	0.70	50	27
VI	H	F ₅	0.31	50	12
VIII	H	<i>p</i> -NO ₂	0.25	37	16
IX	H	<i>p</i> -CN	0.72	50	27
XIV	H	<i>p</i> -CH ₂ NH ₃ [⊕]	1.6 (d)	32	130
XV	H	<i>p</i> -CH ₂ NHCOCH ₂ Br	0.27	50	11
XVI	H	<i>p</i> -COCH ₂ Br	0.43	50	17
XX	C ₆ H ₅	<i>p</i> -NO ₂	0.12 (d)	25	12
XXI	C ₆ H ₅	<i>p</i> -NH ₂	0.05 (d)	41	2.8
XXVII	H	<i>p</i> -COO [⊖]	2.0 (d)	26	200 (f)

The technical assistance of Barbara Baine with the assays in Tables I and II is acknowledged. (a) Thymidylate syntheses was a 45-90% saturated ammonium sulfate fraction from *E. Coli* B that was prepared and assayed with 80 μ M 2'-deoxyuridylylate and 51.4 μ M 5,10-methylene-*dl*-tetrahydrofolate in 0.05 M tris buffer (pH 7.4) containing 10 mM mercaptoethanol, 1 mM Versene, and 5% 2-methoxyethanol as previously described (14,20). (b) The ratio of the concentration of inhibitor to concentration of the *l*-isomer of 5,10-methylenetetrahydrofolate giving 50% inhibition. (c) Data from reference 14. (d) Maximum solubility. (e) Data from reference 15. (f) Data from reference 21.

86% yield. That the *N*-acetyl group was on the 2-amino group of the pyrimidine and not on the sulfonamide was indicated by the presence of the sulfonamido NH band at 3290 cm⁻¹ and by the typical ultraviolet spectrum of a 2-acetamido-4-pyrimidinol (14).

Attempts to convert the *p*-aminobenzenesulfonamides, XII, XXI, and XXIV, to the corresponding bromoacetamides, XIII, XXII, and XXV, failed due to the inertness of the *p*-amino group; these attempts have been previously described (18) and these failures were attributed to the inactivation of the *p*-amino group by the strong electron-withdrawing sulfonamide group.

It was reasoned that the higher homolog, XV, should be readily synthesized from the requisite amine (XIV), since the aliphatic amino group is more reactive and, in addition, is insulated by the methylene group from effects of the electron-withdrawing sulfonamide group. The synthesis of the requisite amine (XIV) started with *p*-cyanobenzenesulfonyl chloride, which in turn was prepared from *p*-aminobenzonitrile by the Meerwein modification (19) of the Sandmeyer reaction. Condensation of this sulfonyl

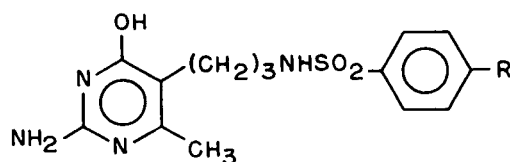
chloride with the aminopropyl pyrimidine (III) afforded the sulfonamide (IX) in 80% yield. Hydrogenation of the nitrile group in 85% aqueous ethanol containing excess hydrochloric acid with a platinum oxide catalyst afforded the *p*-aminomethylbenzenesulfonamide (XIV), isolated as the dihydrochloride in 62% yield. The freed amino group of XIV reacted smoothly with *p*-nitrophenyl bromoacetate in aqueous acetone by the previously described general method (18) to give the desired bromoacetamide (XV) in 70% yield.

Attempts to convert α -methoxy-*p*-toluidine to the corresponding sulfonyl chloride by the Meerwein method was unsuccessful; the reaction was successful with the *m*-isomer which was converted to XI. Attempts to convert XI to a bromomethylbenzenesulfonamide, as previously described for the corresponding bromomethylbenzamide (2), led to facile sulfonamide cleavage with formation of III hydrobromide.

p-Acetylbenzenesulfonyl chloride was readily prepared from *p*-aminoacetophenone as previously described (19b); reaction of this sulfonyl chloride with

TABLE II

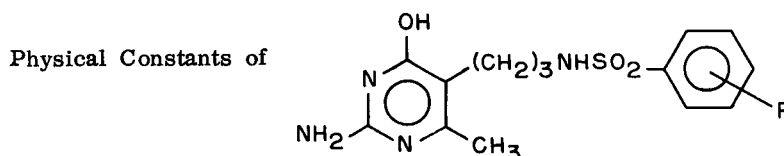
Irreversible Inhibition of Thymidylate Synthetase by



Set No.	Compound	R	mM Conc. For 50% Reversible Inhibition	mM Conc. Inhibitor	Time (min.)	Percent Inactivation (a)
A	XV	-CH ₂ NHCOCH ₂ Br	0.27	0.27	60	32
B	XV	-CH ₂ NHCOCH ₂ Br	0.27	0.27	60	31
	Iodoacetamide		large	0.27	60	23
C	XVI	-COCH ₂ Br	0.43	0.43	60	100
D	XVI	-COCH ₂ Br	0.43	0.10	20	33
	Phenacyl Bromide		large	0.10	20	56
E	XVI	-COCH ₂ Br	0.43	0.033	60	32
	Phenacyl Bromide		large	0.033	60	36

(a) The percent inactivation at 37° is corrected for thermal inactivation of an enzyme control run simultaneously. See experimental for inactivation procedure.

TABLE III



Compound (a)	R	Percent Yield	M. p. °C	C	Analyses				
					Calcd. H	N	C	Found H N	
V	H	93	130-139 (b, c)	52.3	5.63	17.4	52.2	5.50	17.1
VI	F ₅	93	166-169 (b, d)	40.8	3.17	13.6	40.6	2.97	13.3
VII	<i>m</i> -NO ₂	82	137-140 (e)	45.8	4.66	19.1	45.5	4.72	18.9
VIII	<i>p</i> -NO ₂	95	210 (b, d)	45.8	4.66	19.1	45.6	4.54	18.9
IX	<i>p</i> -CN	67	145-147 (b, d)	51.9	4.93	20.2	51.6	5.13	19.9
X	<i>p</i> -COCH ₃	75	145-150 (d)	(f)					

(a) All compounds were prepared by the method described for IX in the experimental. Each compound has ultraviolet and infrared spectra in agreement with its assigned structure and moved as a single spot on TLC. (b) With previous softening. (c) Recrystallized from aqueous acetone. (d) Recrystallized from aqueous ethanol. (e) Recrystallized from aqueous 2-methoxyethanol. (f) The compound moved as a single spot on TLC in 4:3 *t*-butanol-methyl ethyl ketone, but gave erratic combustion value due to difficulty in combustion; it was characteristic as its bromo derivative (XVI).

the aminopropyl pyrimidine (III) afforded the *p*-acetylbenzenesulfonamide (X) in 75% yield. Selective bromination of X to the bromoacetyl derivative, XVI, was achieved in glacial acetic acid containing anhydrous hydrogen bromide to protonate and protect the pyrimidine system; XVI was obtained in 88% yield as its hydrobromide salt.

Other sulfonamides prepared by reaction of III with the appropriate sulfonyl chloride were the parent benzene compound (V), the pentafluoro derivative (VI) and the *m*-nitro derivative (VII).

ENZYMIC EVALUATION

In Table I are listed the results of reversible inhibition of thymidylate synthetase by the candidate inhibitors. The binding of the benzene ring of V is not influenced appreciably by electron-withdrawing groups (VI, VIII, IX, XVI) or an electron-donating group (I); the variation is less than three fold. A striking loss of inhibition occurs with the ionized ammoniummethylene (XIV) groups in the *p*-position; the loss of inhibition by both a cationic and anionic group is taken as evidence that the benzene ring is complexed to a hydrophobic region (22). Contrariwise, as a further study, it would be of interest to see if alkyl, aryl, or aralkyl groups on the benzenesulfonamide moiety of V would give additional hydrophobic bonding to thymidylate synthetase.

The two candidate irreversible inhibitors, XV and XVI, were investigated for inactivation of thymidylate synthetase: the results are tabulated in Table II. The *p*-bromoacetamidomethyl sulfonamide (XV) showed 32% more inactivation in 60 minutes at 37° than the enzyme control (Set A). When XV was incubated simultaneously with another solution containing an equimolar amount of iodoacetamide (Set B), both compounds showed inactivation of the enzyme, but iodoacetamide was somewhat slower. Since iodoacetamide does not form a reversible complex with the enzyme and could inactivate the enzyme almost as well, it is probable that XV inactivates the enzyme by a random bimolecular process and not by the active-site-directed mechanism via a complex between the enzyme and XV (23); however, additional compounds related to XV might be worthy of synthesis and evaluation.

The *p*-bromoacetylbenzene sulfonamide (XVI) showed total inactivation of the enzyme in 60 minutes at 37° at an inhibitor concentration of 0.43 mM (Set C). By reducing the time or concentration (Set E) or both (Set D) less inactivation occurred. Unfortunately, phenacyl bromide, which complexes poorly if at all with the enzyme, inactivated thymidylate synthetase even more rapidly than XVI (Sets D and E). Thus the *p*-bromoacetyl sulfonamide (XVI) clearly inactivates the enzyme by the random bimolecular process which has little chemotherapeutic utility (3, 4, 23).

EXPERIMENTAL

Methods.

Melting points were taken in capillary tubes on a Mel-temp block and are uncorrected. Infrared spectra were determined in potassium bromide pellet with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 spectrophotometer. Thin layer chromatograms (TLC) were run with Brinkmann silica gel GF and spots were detected by visual examination under ultraviolet light. Spot tests for active halogen (as in XV and XVI) with 4-(*p*-nitrobenzyl)pyridine were performed as previously described (18).

p-Cyanobenzenesulfonyl Chloride.

A stirred suspension of 3.54 g. (30 mmoles) of commercial *p*-aminobenzonitrile in 3 ml. of water and 21 ml. of 12 *N* aqueous hydrochloric acid was cooled in an ice-salt bath. A solution of 2.1 g. (30 mmoles) of sodium nitrite in 12 ml. of water cooled to 0° was added dropwise over a period of 15 minutes. The resulting solution of diazonium salt was added dropwise over a period of 10 minutes to 37 ml. of glacial acetic acid previously saturated with sulfur dioxide and containing 150 mg. of cupric chloride. The temperature was maintained at 10-15° during the addition, then the mixture was stirred at 10-15° for additional 30 minutes during which time the product began to separate. The mixture was poured into several volumes of ice-water, the grey precipitate was collected, and washed with water; yield, 1.52 g. (76%), m.p. 100-108°. The crude material was extracted with hot petroleum ether (b.p. 60-110°), then the extracts were cooled to give 1.15 g. (57%) of white crystals, m.p. 111-112°. A m.p. of 111-112° has been recorded for this compound prepared by an alternate method (24). Yields up to 66% of pure material were obtained on a larger scale.

2-Amino-5-(*p*-cyanobenzenesulfonamidopropyl)-6-methyl-4-pyrimidinol (IX).

To a stirred solution of 1.02 g. (4 mmoles) of III dihydrochloride (14) in 2.67 ml. of 3 *N* aqueous sodium hydroxide and 2 ml. of water was added 0.463 g. (4.4 mmoles) of anhydrous sodium carbonate. As soon as solution was complete, 14 ml. of acetone were added followed by 0.884 g. (4.4 mmoles) of *p*-cyanobenzenesulfonyl chloride. After being stirred for 4-8 hours, the mixture was filtered and the product washed with water, yield, 1.12 g. (80%) of crude product. Recrystallization from aqueous ethanol afforded nearly white crystals, m.p. 145-147° (gas) with softening at 130-135°; yield, 0.925 g. (67%). See Table III for analytical data.

2-Amino-5-(*p*-aminobenzenesulfonamidopropyl)-6-methyl-4-pyrimidinol (XII) Hydrochloride.

A mixture of 1.82 g. (5 mmoles) of VIII, 200 ml. of 85% aqueous ethanol, 0.85 ml. of 12 *N* aqueous hydrochloric acid, and 0.20 g. of 5% palladium-charcoal was shaken with hydrogen at 2-3 atm. overnight when reduction was complete. The filtered solution was spin-evaporated *in vacuo*. The residual hydrochloride was dissolved in hot 50% aqueous 2-methoxyethanol, the solution was adjusted to about pH 1 with 3 *N* aqueous hydrochloric acid, then cooled overnight at 5° to give 1.05 g. (62%) of product, m.p. 285-286° dec.

Anal. Calcd. for C₁₄H₁₃N₅O₃S·HCl: C, 45.0; H, 5.37; N, 18.7. Found: C, 44.8; H, 5.30; N, 18.7.

2-Amino-5-[*p*-(aminomethyl)benzenesulfonamidopropyl]-6-methyl-4-pyrimidinol (XIV) Dihydrochloride.

A mixture of 347 mg. (1 mmole) of IX, 100 ml. of 85% aqueous ethanol, 0.17 ml. of 12 *N* hydrochloric acid, and 50 mg. of platinum oxide catalyst was shaken with hydrogen at 2-3 atm. for about 20 hours when reduction was complete. The filtered solution was concentrated *in vacuo* to about 10 ml., then clarified with decolorizing carbon. Addition of acetone gave 264 mg. (62%) of product, m.p. 281-283° dec. Recrystallization from water by addition of acetone gave 200 mg. (47%) of white crystals, m.p. 287-289° dec.

Anal. Calcd. for C₁₅H₂₁N₅O₃S·2HCl: C, 42.5; H, 5.46; N, 16.5. Found: C, 42.4; H, 5.13; N, 16.6.

2-Amino-5-[*p*-(bromoacetamidomethyl)benzenesulfonamidopropyl]-6-methyl-4-pyrimidinol (XV).

To a stirred solution of 212 mg. (0.5 mmole) of XIV dihydrochloride in 0.33 ml. of 3 *N* aqueous sodium hydroxide, 0.50 ml. of water and 2.0 ml. of acetone was added 130 mg. (0.5 mmole) of *p*-nitrophenyl bromoacetate (18). After 90 minutes the yellow solution was poured into 20 ml. of water and cooled at 5° when the product slowly crystallized. The solid was collected on a filter, washed with water, then

with ethyl acetate to remove *p*-nitrophenol; yield, 165 mg. (70%) of a near white powder, m.p. 189-190°. This material moved as a single spot on TLC in 4:3 *t*-butyl alcohol-methyl ethyl ketone and gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen (18).

Anal. Calcd. for $C_{17}H_{22}BrN_5O_4S \cdot H_2O$: C, 41.6; H, 4.94; Br, 16.3. Found: C, 41.6; H, 4.90; Br, 16.7.

2-Amino-5-[*p*-(bromoacetyl)benzenesulfonamidopropyl]-6-methyl-4-pyrimidinol (XVI) Hydrobromide.

To a stirred mixture of 182 mg. (0.5 mmole) of X in 4 ml. of glacial acetic acid containing 10% anhydrous hydrogen bromide was added 0.50 ml. of 1 M bromine in the same solvent; solution soon took place. During 20 hours a cream colored precipitate separated. The mixture was poured into 25 ml. of ethyl acetate containing 46 mg. (0.5 mmole) of phenol to scavenge any remaining bromine. The product was collected on a filter and washed with ethyl acetate; yield, 233 mg. (88%), m.p. 212-217° dec. The product was further purified by solution in warm ethanol containing a few drops of anhydrous 10% hydrogen bromide in acetic acid, then addition of petroleum ether; yield, 180 mg. (70%) of white crystals, m.p. 215-217° dec.

Anal. Calcd. for $C_{18}H_{19}BrN_5O_4S \cdot HBr$: C, 36.7; H, 3.85; Br, 30.5. Found: C, 36.8; H, 4.01; Br, 30.7.

N-(3-Bromopropyl)-*p*-nitrobenzenesulfonanilide (XVIII).

To a stirred solution of 2.77 g. (10 mmoles) of XVII (25) in 10 ml. of dimethyl sulfoxide was added 1.38 g. (10 mmoles) of anhydrous potassium carbonate, which changed the yellow solution to red. After the addition of 8 g. (40 mmoles) of 1,3-dibromopropane, the mixture was stirred for about 18 hours when the color returned to yellow. The mixture was diluted with 100 ml. of water and 100 ml. of benzene. The separated benzene layer was washed successively with 25 ml. of 1 N aqueous sodium hydroxide and two 100-ml. portions of water. Dried with magnesium sulfate, the benzene solution was spin-evaporated *in vacuo*; the residual dimethyl sulfoxide was removed at about 2 mm. pressure in a 90° bath. The residue was extracted with several portions of boiling petroleum ether (b.p. 60-110°). Evaporation of the extracts gave 2.36 g. (59%) of white crystals, m.p. 91-92°.

Anal. Calcd. for $C_{15}H_{15}BrN_2O_4S$: C, 45.1; H, 3.76; N, 7.02. Found: C, 45.3; H, 3.96; N, 7.22.

2-Amino-6-methyl-5-(*N*-phenyl-*p*-nitrobenzenesulfonamidopropyl)-4-pyrimidinol (XX).

To a stirred solution of 585 mg. (4.5 mmoles) of ethyl acetoacetate in 12.3 ml. of *t*-butyl alcohol protected from moisture was added 90 mg. (3.75 mmoles) of sodium hydride as a 50% dispersion in mineral oil. When solution was complete, 1.20 g. (3 mmoles) of XVIII was added, then the solution was refluxed for about 18 hours. The solution was acidified with glacial acetic acid, then spin-evaporated *in vacuo*; residual ethyl acetoacetate was removed at oil pump pressure in a 90° bath; yield, 1.13 g. (84%) of crude XIX that was not further purified.

To a stirred solution of 3.10 g. (6.9 mmoles) of crude XIX in 31 ml. of *t*-butyl alcohol was added 0.62 g. (3.45 mmoles) of guanidine carbonate. The mixture was refluxed for 48 hours during which time the product separated. The cooled mixture was filtered and the product washed with water. Recrystallization from aqueous 2-methoxyethanol gave 1.75 g. (28% from XVIII) of yellow crystals, m.p. 213-214°.

Anal. Calcd. for $C_{20}H_{21}N_5O_5S \cdot 1/2H_2O$: C, 53.1; H, 4.91; N, 15.5. Found: C, 52.8; H, 4.91; N, 15.2.

N-[3-(2-Amino-4-hydroxy-6-methyl-5-pyrimidinyl)propyl]-*p*-amino-benzenesulfonanilide (XXI).

Hydrogenation of XX in 85% ethanol in the presence of a platinum oxide catalyst gave, after recrystallization from aqueous ethanol, a 68% yield of white crystals, m.p. 209-212° dec.

Anal. Calcd. for $C_{20}H_{23}N_5O_5S$: C, 58.1; H, 5.61; N, 16.9. Found: C, 57.8; H, 5.59; N, 16.7.

2-Acetamido-6-methyl-5-(*N*-phenyl-*p*-nitrobenzenesulfonamidopropyl)-4-pyrimidinol (XXIII).

A mixture of 222 mg. (0.5 mmole) of XX and 3 ml. of acetic anhydride was refluxed for 90 minutes during which time solution took place. The solution was spin-evaporated *in vacuo*. The residue was recrystallized from ethyl acetate-petroleum ether (b.p. 60-110°) with the aid of decolorizing carbon; yield, 100 mg., m.p. 190-193°. An additional 54 mg., m.p. 185-197°, was isolated from the filtrate. Recrystallization of a similar preparation gave buff-colored crystals,

m.p. 192-195°.

Anal. Calcd. for $C_{22}H_{23}N_5O_6S$: C, 54.4; H, 4.78; N, 14.4; S, 6.59. Found: C, 54.7; H, 5.01; N, 14.2; S, 6.58.

2-Acetamido-6-methyl-5-(*p*-nitrobenzenesulfonamidopropyl)-4-pyrimidinol (XXVI).

A solution of 995 mg. (2.7 mmoles) of VIII in 6.75 ml. of *N,N*-dimethylformamide and 0.27 ml. of acetic anhydride was allowed to stand for 25 hours then diluted with 30 ml. of water. The product was collected on a filter and washed with water; yield, 950 mg. (86%), m.p. 211-216°. Recrystallization of a similar preparation from aqueous 2-methoxyethanol gave light yellow crystals, m.p. 212-213°.

Anal. Calcd. for $C_{18}H_{19}N_5O_6S$: C, 46.9; H, 4.68; N, 17.1. Found: C, 46.7; H, 4.77; N, 16.8.

N-[3-(2-Acetamido-4-hydroxy-6-methyl-5-pyrimidinyl)propyl]-*p*-amino-benzenesulfonanilide (XXIV).

Hydrogenation of XXIII in absolute ethanol on the presence of platinum oxide catalyst gave a 61% yield of product, m.p. 187-190° dec., from ethanol. Recrystallization from aqueous ethanol afforded white crystals, m.p. 195-196°.

Anal. Calcd. for $C_{22}H_{23}N_5O_6S \cdot H_2O$: C, 55.8; H, 5.75; N, 14.8. Found: C, 56.0; H, 5.47; N, 14.6.

Irreversible Inhibition of Thymidylate Synthetase

Buffers.

Buffer A was 0.05 M Tris, pH 7.4. Buffer B had in addition 10 mM mercaptoethanol and 1 mM Versene.

Enzyme Preparation.

The enzyme was isolated as a 45-90% ammonium sulfate fraction from *E. coli* B as previously described (14,20), except Buffer A with no mercaptoethanol was employed.

Incubation Procedure.

The velocity of enzymatic reaction was linear with enzyme concentration when assayed with 214 μ M 2'-deoxyuridylate in Buffer B, as previously described (20).

In each of two centrifuge tubes in a 37° water bath were placed 0.77 ml. of enzyme solution and 0.70 ml. of Buffer A. To the first tube (enzyme control, C) was added 0.075 ml. of 2-methoxyethanol; to the second tube (inhibitor solution, I) was added 0.075 ml. of 2-methoxyethanol containing the inhibitor. After mixing each tube, an 0.70 ml. aliquot was removed within one minute, then chilled in an ice bath until ready for assay, and labeled C_1 and I_1 . The remainder in the two centrifuge tubes (labeled C_2 and I_2) were incubated at 37° for the required time, then chilled in an ice bath until ready for assay.

Prior to assay, the four solutions were centrifuged in a clinical centrifuge at high speed for 2 minutes, then the assay performed with the supernatant.

To assay the remaining enzyme in the solution, 0.14 ml. of incubation mixture, 0.24 ml. of assay mix A (20), and 0.57 ml. of Buffer B were mixed in a 1 ml. cuvette. The velocity of the reaction was then observed after addition of 0.050 ml. of 1.64 mM 2'-deoxyuridylate; the zero incubation time velocities were about 0.006 optical density units per minute at 338 $m\mu$. Since the velocity was proportional to the enzyme concentration, the log of the velocities were plotted against time (23). The inhibitor slope was corrected by the slope of enzyme control, then the percent inactivation noted.

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