Potential Folic Acid Antagonists. III. The Antitumor and Folate Reductase Inhibitory Properties of Some Irreversibly Acting 6-N-Substituted 2,4,6-Triamino-5-(4-carbethoxyphenylazo)pyrimidines

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Received November 24, 1967

The synthesis of some irreversibly acting inhibitors of folic acid reductase is described. These compounds, the 6-N-ω-(N-ethyl-N-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidines (XVI–XX), were tested for antitumor activity against the Ehrlich ascites system. Their ability to reduce the *in vivo* levels of folic acid reductase in mouse tissues was also determined. Only a partial correlation between the *in vitro* effectiveness of these compounds as folate reductase inhibitors and their ability to reduce folate reductase levels *in vivo* in mouse tissues and to inhibit the growth of the Ehrlich ascites tumor was discovered. Some reasons for this discrepancy are discussed. The possible mechanism of action of these compounds at the enzyme level is discussed in terms of Baker's extensive work on pyrimidine binding to folic and dihydrofolic acid reductase.

In continuation of our study of 5-arylazopyrimidines as inhibitors of folic acid reductase[1,2] we have investigated the effect of incorporating alkylating functions at the 6 position of 2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidine. The alkylating function selected was the 2-chloroethylamino group attached to the 6 substituent of the parent pyrimidine by a polymethylene chain. In a preliminary report on these compounds[3] we noted that the extent of inactivation of folic acid reductase by the 6-N-ω-(N-ethyl-N-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidines showed some dependence on the chain length of the 6 substituent and that the relative ineffectiveness of N-2-chloroethyl-N-ethyl-*n*-butylamine (which represents the alkylating side chain of XVIII) was not increased in the presence of 2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidine. These results suggested that the irreversible inactivation of the enzyme is not caused by a random alkylation of a nucleophilic site but is due to the initial formation of a reversible enzyme–inhibitor complex and subsequent alkylation, a process which Baker[4] has termed active-site-directed irreversible inhibition.

In this paper we report the synthesis and a more detailed analysis of the biological activities of the 6-N-ω-(N-ethyl-N-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidines.

Experimental Section

Synthetic Procedures.[5]—The physical constants of compounds used in this study are presented in Tables I and II.

N-Ethyl-N-2-hydroxyethyl-1,ω-alkanediamines (VI–X, Table I).—N-Ethyl-2-hydroxyethylamine (0.5 mole) and N-ω-bromoalkylphthalimide (0.25 mole) dissolved in C_6H_6 (200 ml) were refluxed for 12 hr. The benzene solution of N-ω-(N-ethyl-N-2-hydroxyethyl)aminoalkylphthalimide was decanted from the oily layer of N-ethyl-2-hydroxyethylamine hydrobromide and evaporated *in vacuo* to an uncrystallizable oil a small portion of which was converted to the methiodide (I–V, Table I) for identification purposes. The oily N-ω-(N-ethyl-N-2-hydroxyethyl)-aminoalkylphthalimide was dissolved in concentrated HCl (300 ml, *d* 1.42) and refluxed for 12 hr. H_2O (100 ml) was added to assist the precipitation of phthalic acid and the cooled mixture was filtered. The filtrate was evaporated under reduced pressure to give the oily N-ethyl-N-2-hydroxyethyl-1,ω-alkanediamine dihydrochlorides. These were apparently very hygroscopic and could not be maintained in a crystalline state but were converted to the free bases by addition of the calculated amount of ethanolic sodium ethoxide. Filtration of NaCl and fractional distillation of the filtrate afforded VI–X (Table I) which were further characterized as dipicrates (VIa–Xa, Table I).

6-N-ω-(N-Ethyl-N-2-hydroxyethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidines (XI–XV, Table II) were prepared from 2,4-diamino-6-chloro-5-(4-carbethoxyphenylazo)pyrimidine (3.2 g, 0.01 mole) and N-ethyl-N-2-hydroxyethyl-1-ω-alkanediamine (0.025 mole) in EtOH (75 ml) heated to 100° for 15 hr. On cooling XI–XV crystallized as orange plates in 80–93% yields and were recrystallized to purity.

6-N-ω-(N-Ethyl-N-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidine hydrochlorides (XVI–XX, Table II) were prepared from finely powdered 6-N-ω-(N-ethyl-N-2-hydroxyethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)-pyrimidine (0.005 mole) suspended in anhydrous ether at 0° to which $SOCl_2$ (0.9 g, 0.0075 mole) was added with vigorous stirring; the mixture was maintained at 35° for 1.5 hr. Excess $SOCl_2$ was destroyed by the addition of EtOH and the mixture was filtered, washed (Et_2O), and recrystallized from 90% EtOH to give XVI–XX as the hydrated hydrochlorides in near quantitative yields. Prolonged drying at 100° (0.1 mm) did not remove the water of hydration.

Biological Test Methods.—Toxicity determinations were carried out using random-bred male Swiss mice (20–25 g). The compound dissolved in 10% gum acacia was administered by intraperitonal injection to groups of three to six mice per dose level. Deaths within a 21-day period were recorded. LD_{50} values were calculated using the method of Litchfield and Wilcoxon.[6]

Antitumor activities were determined with the Ehrlich ascites system. Ehrlich ascites cells were taken from mice bearing a 10–11-day-old tumor. The cells were washed with cold sterile saline (0.85%) and mice were inoculated with 10^6 cells. Compounds dissolved in saline or 10% gum acacia were injected intraperitoneally on the day after inoculation once only, or once daily for 5 days. The mice were killed on the 10th day after inoculation and packed-cell volumes were determined. ID_{90} values were calculated according to the method of Litchfield and Wilcoxon.[6]

Folic Acid Reductase Assay.—Folic acid reductase activity was determined as previously described[1,2,7] with the following modifications: in the assay of intestinal enzyme $MgCl_2$ was replaced

(1) J. Hampshire, P. Hebborn, A. M. Triggle, and D. J. Triggle, *J. Med. Chem.*, **8**, 745 (1965).

(2) M. Chadwick, J. Hampshire, P. Hebborn, A. M. Triggle, and D. J. Triggle, *ibid.*, **9**, 874 (1966).

(3) J. Hampshire, P. Hebborn, A. M. Triggle, and D. J. Triggle, *J. Pharm. Sci.*, **55**, 453 (1966).

(4) B. R. Baker, *ibid.*, **53**, 347 (1964).

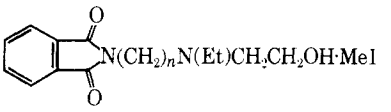
(5) Melting points are corrected and were determined on a Thomas–Kofler hot stage. Analyses were performed by Dr. A. E. Bernhardt, Mülheim, West Germany. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for those elements or functions were within 0.4% of the theoretical values.

(6) J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exptl. Therap.*, **96**, 99 (1949).

(7) W. C. Werkheiser, *J. Biol. Chem.*, **236**, 888 (1961).

TABLE I

PHYSICAL AND ANALYTICAL DATA FOR N-ω-(N'-ETHYL-N'-2-HYDROXYETHYL)ALKYLPHTHALIMIDES AND N-ETHYL-N-2-HYDROXYETHYLALKANEDIAMINES

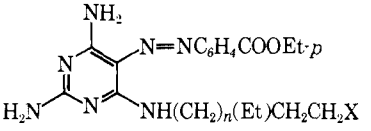


No.	n	Yield, %	Mp or bp (mm), °C	Crystn solvent	Formula	Analyses
I	2		215–216	EtOH–Et_2O	$C_{15}H_{21}IN_2O_3$	C, H, I, N
II	3		268–270	MeOH	$C_{16}H_{23}IN_2O_3$	C, H, I, N
III	4		273–275	MeOH–EtOH	$C_{17}H_{25}IN_2O_3$	C, H, I, N
IV	5		268–271	*i*-PrOH–Et_2O	$C_{18}H_{27}IN_2O_3$	C, H, I, N
V	6		Could not be obtained crystalline			
VI	2	57	70 (2)		$C_6H_{16}N_2O$	N[a]
VII	3	62	81 (2)		$C_7H_{18}N_2O$	N
VIII	4	75	98–102 (2.7)		$C_8H_{20}N_2O$	N
IX	5	51	106 (1.5)		$C_9H_{22}N_2O$	N
X	6	62	95 (0.6)		$C_{10}H_{24}N_2O$	N
VIa	2		174	EtOH–Et_2O	$C_{18}H_{22}N_8O_{15}$	C, H, N
VIIa	3		155–157	EtOH–Et_2O	$C_{19}H_{24}N_8O_{15}$	C, H, N
VIIIa	4		125–127	EtOH–C_6H_6	$C_{20}H_{26}N_8O_{15}$	C, H, N
IXa	5		191–192	*i*-PrOH–C_6H_6	$C_{21}H_{28}N_8O_{15}$	C, H, N

[a] A. R. Surrey and H. F. Hammer, *J. Amer. Chem. Soc.*, **72**, 1814 (1950).

TABLE II

PHYSICAL AND ANALYTICAL DATA FOR 6-N-ω-(N-ETHYL-N-2-HYDROXY- (OR CHLORO-) ETHYL)ALKYL-2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES



No.	n	X	Mp, °C	Crystn solvent	Formula	Analyses
XI	2	OH	165	*i*-PrOH	$C_{19}H_{28}N_8O_3$	C, H, N
XII	3	OH	152	*i*-PrOH	$C_{20}H_{30}N_8O_3$	C, H, N
XIII	4	OH	168	*i*-PrOH	$C_{21}H_{32}N_8O_3$	C, H, N
XIV	5	OH	151	*i*-PrOH	$C_{22}H_{34}N_8O_3 \cdot 1.5H_2O$	C, H, N
XV	6	OH	157	*i*-PrOH	$C_{23}H_{36}N_8O_3$	C, H, N
XVI	2	Cl	217–219	EtOH	$C_{19}H_{28}Cl_2N_8O_2 \cdot 1.5H_2O$	C, H, Cl, N
XVII	3	Cl	247–249	EtOH	$C_{20}H_{30}Cl_2N_8O_2 \cdot 1.5H_2O$	C, H, Cl, N
XVIII	4	Cl	248–250	EtOH	$C_{21}H_{32}Cl_2N_8O_2 \cdot 1.5H_2O$	C, H, Cl, N
XIX	5	Cl	240–242	EtOH	$C_{22}H_{34}Cl_2N_8O_2 \cdot 1.5H_2O$	C, H, Cl, N
XX	6	Cl	206	EtOH	$C_{23}H_{36}Cl_2N_8O_2 \cdot 1.5H_2O$	C, H, Cl, N

by 2 μM $MnCl_2$, and glucose 6-phosphate (0.8 mM) and glucose 6-phosphate dehydrogenase (6.4 units/ml) were added to provide an NADPH regenerating system. Nicotinamide (50 μmoles) was present to inhibit NADPase activity. In the assay of folic acid reductase from Ehrlich ascites tumor $MgCl_2$ was replaced by 50 μmoles of KCl and the NADPH regenerating system described above was present.

Preparation of Folic Acid Reductase.—Female mice (ICR/Ha, 40 g) were inoculated intraperitoneally with Ehrlich Ascites cells (10^6). Six days after inoculation the animals were killed and the tumor, liver, and intestine were removed. Tissues from six animals were pooled. The livers were cleaned and homogenized in ice-cold 0.25 M sucrose containing 0.001 M EDTA and the homogenate was centrifuged at 104,000g for 40 min. The supernatant fraction was collected and stored at −20° until used. Intestinal enzyme was prepared similarly from the intestinal mucosa. In the preparation of the tumor enzyme the tumor cells were washed with ice-cold 0.15 M saline, suspended in distilled water for 30 sec to lyse red blood cells, mixed with 2 vol of 0.6 M saline to restore isotonicity, and centrifuged at 5000g for 10 min. The cell pack was washed twice with 0.15 M saline and the suspension was homogenized for 2 min at 45,000 rpm in a Virtis homogenizer. The homogenate was centrifuged at 108,000g for 40 min and the supernatant was removed and stored at −20°. Protein was determined by the method of Lowry.[8]

Determination of Folic Acid Reductase Levels.—The levels of folic acid reductase in the mouse liver, intestine, and tumor homogenates prepared as described above were determined by titration with methotrexate.[9] For the liver and intestine the assay conditions were those described by Werkheiser.[4] For the assay of tumor enzyme $MgCl_2$ was replaced by KCl. In the study of folic acid reductase levels in these tissues following administration of XVI–XX, the compounds were injected intraperitoneally on the sixth day after inoculation with tumor cells, the animals were sacrificed 24 hr later, and the tisses were removed and treated as described above.

Determination of Comparative Alkylating Activities.—The relative chemical reactivities of compounds were determined using a model nucleophile, 4-nitrobenzylpyridine, by the method of Bardos and coworkers[10] as modified by Baker.[11] The compounds were dissolved in 2-methoxyethanol at a concentration of 0.2 mM. Aliquots of solution (0.25 ml) were pipetted into a series of test tubes and 0.25 ml of a 5% w/v solution of 4-nitrobenzylpyridine in 2-methoxyethanol, 0.25 ml of 2-methoxyethanol, and 0.25 ml of 0.05 M potassium phthalate buffer (pH 4.2) were added to each tube. Blank tubes were identical but did not contain the 2-halogenoethylamines. The tubes were maintained at 37°, removed at timed intervals, and placed on ice. Then, 0.1 ml of 0.2 M KOH (80% aqueous 2-methoxyethanol) was added and the contents of the tube was vigorously disturbed

(8) E. Layne, *Methods Enzymol.*, **5**, 27 (1962).

(9) J. R. Bertino, B. A. Booth, A. L. Bieber, A. Cashmore, and A. C. Sartorelli, *J. Biol. Chem.*, **239**, 479 (1964).

(10) T. J. Bardos, N. Datta-Gupta, P. Hebborn, and D. J. Triggle, *J. Med. Chem.*, **8**, 167 (1965).

(11) B. R. Baker and J. H. Jordann, *J. Heterocycl. Chem.*, **2**, 21 (1965).

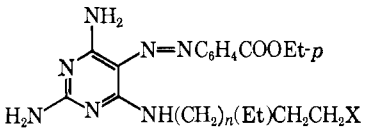
for 20 sec on a vortex mixer; the absorption at 570 mμ was determined in a Beckman DU spectrophotometer.

Results

In Table III are presented values for the subacute toxicities and the antitumor activities of the 6-N-ω-(N-ethyl-N-2-hydroxyethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidines (XI–XV) and their chlorinated counterparts the 6-N-ω-(N-ethyl-N-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)-pyrimidines (XVI–XX). The nonchlorinated com-

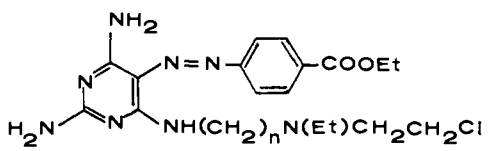
TABLE III

SUBACUTE TOXICITIES AND ANTITUMOR ACTIVITIES OF 6-N-SUBSTITUTED 2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES



Compd	n	X	LD_{50},[a] mg/kg/day	ID_{90},[a] mg/kg/day	Therapeutic index[b]
XVI	2	Cl	24 (20–28)	13 (8–22)	1.8
XVII	3	Cl	43 (38–49)	7.6 (5–12)	5.7
XVIII	4	Cl	30 (18–51)	6.8 (4–13)	4.4
XIX	5	Cl	52 (44–61)	13.0 (5–27)	4.0
XX	6	Cl	30 (24–38)	70 (30–190)	<1.0
XI	2	OH	80 (60–106)	<100	∽1.0
XII	3	OH	>75	>75	...
XIII	4	OH	100 (63–160)	72 (33–155)	1.4
XIV	5	OH	75 (54–104)	>75	<1.0
XV	6	OH	>150	>150	...
$CH_3(CH_2)_3N(Et)$-$CH_2CH_2Cl\cdot HCl$			55 (47–54)	50 (37–78)	1.1
Methotrexate			13 (7–23)	0.9 (0.7–1.3)	13.3

[a] In parentheses, confidence limits, $P = 0.05$. Tumor (10^6 cells) inoculated on day 0 and compound administered intraperitoneally on days 1–5. [b] LD_{50}/ID_{90}.



XVI–XX

pounds XI–XV had insignificant antitumor activities (therapeutic indices <1.0), but the chlorinated analogs XVI–XX had antitumor actions which varied considerably according to the compound (therapeutic indices 1.0–5.5, Table III). N-2-Chloroethyl-N-ethylbutylamine which represents the potential alkylating moiety of XVIII had negligible antitumor activity which is in accord with previous observations[12–14] that monofunctional alkylating agents are generally devoid of antitumor activity unless incorporated in a specific carrier molecule.

The range of therapeutic indices observed with XVI–XX is particularly interesting in view of the minor differences in chemical structure. In Table IV are presented data for the reactivities of these compounds toward 4-nitrobenzylpyridine. These data indicate that the chemical reactivities of XVI–XIX, which contain methylene chains of 2, 3, 4, and 5 units, respectively, are similar, although XX, where the alkylating center is separated from the 6-NH_2 group of the pyrimidine ring by six carbon atoms, is distinctly lower in chemical reactivity. Table V presents acute toxicities and antitumor activities of compounds XVI–XX. Comparison with the data in Table III shows that these compounds are more toxic when administered over a 5-day period but, in contrast with methotrexate, they do not show evidence of producing cumulative toxicity.

TABLE IV

COMPARATIVE CHEMICAL REACTIVITIES OF 6-N-ω-(N-ETHYL-N-2-CHLOROETHYL)ALKYL-2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES TOWARD 4-NITROBENZYLPYRIDINE

Compound	k'_{37}[a]
XVI	55
XVII	46
XVIII	66
XIX	44
XX	20
$CH_3(CH_2)_3N(Et)CH_2CH_2Cl\cdot HCl$	50

[a] $k'_{37} = E_{570}/t$; see ref 9.

TABLE V

ACUTE TOXICITIES AND ANTITUMOR ACTIVITIES OF 6-N-SUBSTITUTED 2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES

Compd	LD_{50},[a] mg/kg	$1D_{90}$, mg/kg	Therapeutic index[b]
XVI	52 (46–58)	56	<1.0
XVII	70 (60–95)	46	1.7
XVIII	61 (51–73)	50	1.2
XIX	160 (110–230)	100	1.6
XX	135 (110–148)	>120	...
$CH_3(CH_2)_3N(Et)$-$CH_2CH_2Cl\cdot HCl$	99 (83–116)	100	1.0
Methotrexate	175 (105–248)	175	<1.0

[a] In parentheses, confidence limits, $P = 0.05$. Compound injected intraperitoneally 24 hr after the inoculation of 10^6 Ehrlich ascites cells. [b] LD_{50}/ID_{90}.

Table VI presents the inhibitory effects of XVI–XX on folic acid reductases prepared from mouse liver, intestine, and Ehrlich ascites cells. Several similarities are immediately apparent: first, optimum binding of these compounds is achieved with XVIII and XIX where the alkylating side chain contains four and five methylene groups, respectively. These compounds are also generally the most effective in inactivating folic reductase from all three tissues as measured by their times for 50% inactivation. Of great interest, also, are the quantitative differences in the behavior of these compounds toward the folic acid reductases from the three tissues. As measured by the $([I]/[S])_{50}$ and K_I values the tumor enzyme is definitely more sensitive to XVI–XX than are the enzymes from the liver and intestine. Furthermore, when concentrations of inhibitor are employed which give approximately the same concentration of reversible enzyme–inhibitor complex there are pronounced differences in the times for 50% inactivation of the enzyme. Thus XVI, at

(12) W. C. J. Ross, "Biological Alkylating Agents," Butterworth and Co. Ltd., London, 1962.
(13) D. J. Triggle, *J. Theoret. Biol.*, **7**, 241 (1964).
(14) P. Hebborn and D. J. Triggle, *J. Med. Chem.*, **8**, 541 (1965).

TABLE VI

INHIBITORY ACTIVITIES OF 6-N-ω-(N-ETHYL-N-2-CHLOROETHYL)ALKYL-2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES ON FOLIC ACID REDUCTASES FROM MOUSE TISSUES

Compd	Tissue[a]	$[I]_i/[S]_{50}$	K_I	Concn, M	Time for 50% inhib, min	$[E \cdots I]$[b]
XVI	Liver	5.0	2.5×10^{-5}	2×10^{-4}	9.0	0.88
XVII	Liver	4.4	2.0×10^{-5}	2×10^{-4}	30.0	0.90
				4×10^{-4}	23	0.96
XVIII	Liver	2.75	7.5×10^{-6}	2×10^{-4}	7.0	0.96
XIX	Liver	1.0	3.0×10^{-6}	4×10^{-5}	7.0	0.90
				8×10^{-5}	5.4	0.96
XX	Liver	3.25	9.0×10^{-6}	2×10^{-4}	10.5	0.96
XVI	Intestine	3.9	3.9×10^{-5}	2.4×10^{-4}	90	0.86
XVII	Intestine	5.0	5.0×10^{-5}	8×10^{-4}	44	0.90
XVIII	Intestine	1.9	1.9×10^{-5}	1.6×10^{-4}	15	0.89
XIX	Intestine	0.6	0.6×10^{-5}	8×10^{-5}	19	0.90
XX	Intestine	1.0	1.0×10^{-5}	1.2×10^{-4}	27	0.90
XVI	Ascites	1.35	2.0×10^{-5}	1.2×10^{-4}	18	0.80
XVII	Ascites	1.6	3.0×10^{-5}	1.5×10^{-4}	35	0.80
XVIII	Ascites	0.67	7.0×10^{-6}	6×10^{-5}	12.5	0.89
XIX	Ascites	0.37	2.5×10^{-6}	2.5×10^{-5}	20	0.90
XX	Ascites	1.0	8.0×10^{-6}	8×10^{-5}	20	0.90

[a] K_m for folic acid, 2.5×10^{-6} M (liver), 1×10^{-5} M (intestine), 7×10^{-6} M (ascites). [b] Fraction of enzyme bound reversibly as enzyme–inhibitor complex calculated from $[E \cdots I] = [E_{tot}]/(K_I/[I]) + 1$ where $[E_{tot}]$ is total enzyme concentration and $[I]$ is concentration of inhibitor: B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, 3, 459 (1962).

concentrations which give from 80–88% EI complex, produced 50% inactivation of the intestinal, liver, and tumor enzymes in 90, 9, and 18 min, respectively. The most potent compound, XIX, employed at concentrations that gave 90% EI complex required 19, 7, and 20 min to produce 50% inactivation of the enzyme from these three tissues.

An attempt was made to correlate the inhibitory effects of the alkylating compounds XVI–XX on the folic reductase levels *in vivo* with their ability to inhibit growth of the Ehrlich ascites tumor. The data in Table VII record the folic acid reductase levels, expressed as percentage of control values, in mouse tissues following administration of the compounds at dose levels that bracketed the ED_{90}'s determined in acute experiments (Table V). These data indicate that XVI–XX can produce differential effects on the folic acid reductase levels in the three tissues selected which constituted a minimally dividing tissue (liver), a rapidly dividing tissue (intestinal mucosal), and the Ehrlich ascites tumor cells. Thus XVI at a dose of 35 mg/kg can produce a considerable reduction in the level of the tumor enzyme (to 30% of normal) while having little or no effect on the liver and intestinal mucosal enzymes. Similarly, XVII at 70 mg/kg produced a considerable reduction in the tumor enzyme level, a less marked reduction in liver enzyme, and scarcely affected intestinal mucosal enzyme levels.

TABLE VII

EFFECTS OF 6-N-ω-(N-ETHYL-N-2-CHLOROETHYL)ALKYL-2,4,6-TRIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES ON FOLIC ACID REDUCTASE LEVELS *in Vivo* IN EHRLICH ASCITES TUMOR BEARING MICE

Compd	Dose,[a] mg/kg	Enzyme levels in tissues as % of control[b] Liver	Intestine	Tumor
XVI	35	90	100	30
	17.5	90	100	70
XVII	70	54	90	30
	35	100	90	70
XVIII	60	9	45	25
	30	31	66	50
XIX	175	0	14	0
	70	23	29	23
	35	35	56	31
XX	140	54	100	36
	70	68	100	58
	35	100	100	100
$CH_3(CH_2)_3N(Et)$-$CH_2CH_2CL \cdot HCl$	80	100	98	99
	40	100	99	100

[a] Single dose administered intraperitoneally to Swiss mice inoculated 7 days previously with 10^6 Ehrlich ascites cells. [b] 24 hr after drug administration.

Discussion

From the results presented in this paper it is apparent that attempts to correlate the effectiveness of compounds as *in vitro* inhibitors of folic acid reductase with their ability to inhibit the growth of a tumor system *in vivo* are subject to a number of difficulties.

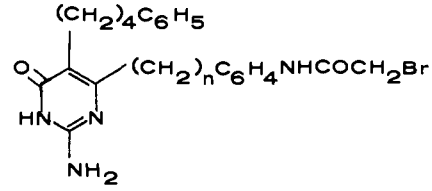
The data in Table III indicate a broad maximum in the therapeutic indices of the alkylating compounds XVI–XX, with XVII being the most effective agent and XVIII and XIX being somewhat less effective. There is only a partial correlation between these results and those from the effectiveness of the compounds as *in vitro* inhibitors of folic acid reductase. These latter results (Table VI) show that XIX is the most effective in reversibly inhibiting the enzyme from the mouse liver, intestine, and ascites tissues. However, when these compounds were compared at concentrations producing essentially the same concentration of reversibly bound enzyme–inhibitor complex, then XIX and XVIII were approximately equally effective in producing 50% irreversible inactivation of the liver enzyme, but XVIII was rather more efficient in producing the same degree of inactivation of the intestinal mucosal and ascites enzymes.

When the abilities of XVI–XX to reduce the enzyme levels in these tissues are compared (Table VII) it is apparent that XVIII and XIX were the most effective

in producing a general reduction in the enzyme levels of all three tissues. These findings accord with the *in vitro* studies (Table VI) on the folic acid reductases from these three tissues which showed that XVIII and XIX were the most effective in producing irreversible inactivation.

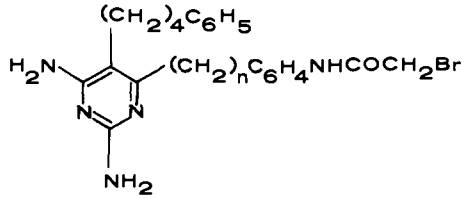
A number of factors probably contribute to the lack of a complete correlation between the antitumor and folic reductase inhibitory effects of XVI–XX. It seems improbable that differences in chemical reactivity alone are sufficient to provide an explanation. In the model system with 4-nitrobenzylpyridine (Table IV), XVI–XIX showed essentially similar reactivities, but XX was distinctly less reactive. This difference is not extended, however, to the folic reductase systems where the times for 50% inactivation of the enzyme by XVIII–XX were comparable. A possible explanation of these findings is that the 6 substituent of XX is sufficiently flexible to offer hindrance (by coiling, partial micelle formation, or internal bonding) to its reaction with 4-nitrobenzylpyridine; however, on the enzyme surface the intermolecular forces exerted on the side chain may cause its extension and realization of its full alkylating potential. In connection with the role of relative chemical reactivities it is relevant to draw attention to the recent work of Schaeffer and his co-workers[15] who have reemphasized that the rate of inactivation of an enzyme by an irreversible inhibitor within the initially formed reversible enzyme–inhibitor complex is dependent both on K_i and k_2. They point out that the study of irreversible inhibitors at only one concentration can lead to erroneous conclusions about their relative effectiveness in producing irreversible inactivation, and inversions in this order can occur if different concentrations are employed. In our study of the *in vivo* effectiveness of XVI–XX in reducing folic reductase levels, it is not known whether equivalent concentrations of the inhibitors reach all tissues or whether the cells of various tissues are equally permeable to an inhibitor molecule. The data of Table VII show that while XVIII and XIX are the most effective in producing a general reduction in reductase content of the three tissues, XVI is able to produce a pronounced reduction of tumor enzyme without affecting liver or intestinal enzymes. The apparently selective action of XVI may be attributable to a more favorable binding to the tumor enzyme or, and this is equally probable, to the operation of the factors discussed above. An additional factor, which may be of equal importance to those already discussed, concerns the relative folic acid levels in the tissues; high folic acid levels will, through competition with the inhibitor, tend to reduce its effectiveness. Work is in progress to determine the relative importance of these factors. Finally, it should be noted that it is most improbable that XVI–XX confine their actions to the inactivation of folic reductase. At lethal dose levels these compounds have marked central effects, probably because of the nature of the alkylating side chain, producing convulsions and death within 30–60 min. This indicates that their toxic effects at these dose levels are not due to inactivation of folic reductase since methotrexate produces deaths within 3–5 days, as a consequence of interference with folate metabolism.

It is of interest to compare our data for the inhibition of folic reductase by the 5-arylazopyrimidines reported in this paper with the extensive data of Baker and his co-workers on the binding of pyrimidine inhibitors to folic and dihydrofolic reductases.[16] Such comparisons must, however, by tempered with the recognition that they are being made between enzymes from different sources and that some of the differences observed may be due to species variation of the enzyme. Baker has synthesized[17,18] irreversible inhibitors of the 2-amino-4-pyrimidinol class (XXI) which are believed to bind in



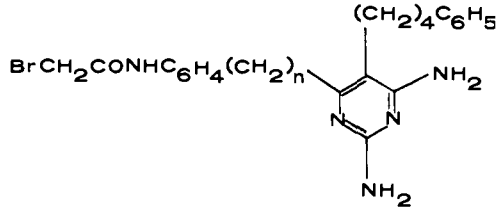
XXI

the conformations depicted in which the hydrophobic interaction between the enzyme and the 5-phenylbutyl substituent determines the binding conformation of the inhibitor molecule. However, the corresponding 2,4-diaminopyrimidine (XXII), while a good reversible



XXII

inhibitor, did not produce irreversible inactivation suggesting that XXII was bound in a conformation in which the alkylating group did not bridge to a nucleophilic site. The reorientation of the alkylating chain of XXII is probably due to repulsion between the 4-amino substituent and the enzyme[19] in conformation XXII leading to its adoption of conformation XXIIa.



XXIIa

Our investigations into the structure–activity relationship of the 5-arylazopyrimidines, while incomplete, suggest that the 5-aryl group is binding to a hydrophobic site[20] and since there is probably only one such

(15) H. J. Schaeffer, M. A. Schwartz, and E. Odin, *J. Med. Chem.*, **10**, 686 (1967).

(16) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley and Sons, Inc., New York, N. Y., 1967, pp 192–266. This book includes a convenient summary of the work of Baker and his colleagues.

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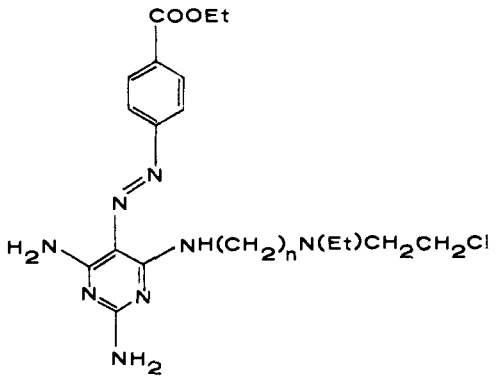
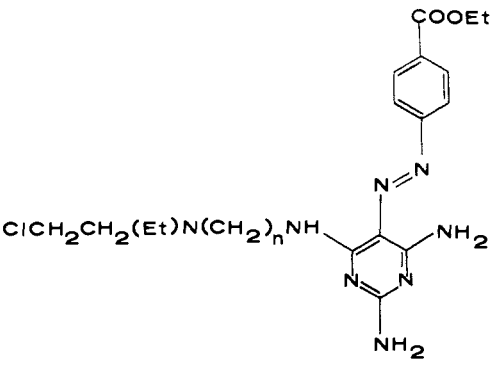
(19) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 314 (1966).

(20) J. F. Moran, A. M. Triggle, D. J. Triggle, and A. Wayne, unpublished data.

site near the active center the 2,4,6-triamino-5-arylazopyrimidines may be binding in conformation XXIII. In this conformation the alkylating 6 substituent would be able to bridge to the same general area on the enzyme as the corresponding substituent as in XXI. In the case of XXII ionic repulsion between the 4-amino substituent and the enzyme was considered to force its adoption of conformation XXIIa: presumably the same repulsion factor would be operative for the 2,4,6-triaminopyrimidines but since there are



XXIII



XXIIIa

amino groups at the 4 and 6 positions repulsion will probably be approximately equivalent regardless of whether conformation XXIII or XXIIIa is adopted. Such speculation affords an explanation of the irreversible inactivating properties of 6-N-ω-(N-ethyl-2-chloroethyl)alkyl-2,4,6-triamino-5-(4-carbethoxyphenylazo)-pyrimidines and also of their relatively low potency.

Acknowledgment.—This work was supported by a grant (CA 06645) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

Peptides of Pyrimidine Amino Acids[1]

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Received November 22, 1967

The α-amino-β-(2-mercapto-6-oxo)-4-pyrimidylpropionic acids were found to be phenylalanine antagonists. A number of dipeptides of these amino acids have been synthesized and tested as inhibitors of growth and protein synthesis in Ehrlich ascites carcinoma in mice. Glycyl-, DL-phenylalanyl-, and L-phenylalanyl-α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic acid showed an enhanced activity over α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic acid.

Research efforts within recent years have resulted in the synthesis of many antimetabolites of pyrimidines, purines, amino acids, vitamins, and other metabolites as potential anticancer agents. One of the major tasks of cancer chemotherapy is to search for antitumor drugs with diverse and select spectra of action. In order to get better selectivity and transport into the cell interior large numbers of antitumor drugs have been synthesized in which cytotoxic groups have been introduced into natural carriers.[3] Among the natural carriers, the amino acids and peptides have been shown to play important roles. The best example of such a use of an amino acid or a peptide is found in the work of Bergel and Stock,[3a-c] Larionov and Sophina,[3d] and Sophina, *et al.*[3e] They have synthesized DL-, L-, and D-*p*-di(2-chloroethyl)aminophenylalanine and their peptides in order to determine whether any desirable specificity of action can be achieved by attaching a nitrogen mustard group to a natural amino acid or peptide. The L form was found to be much more active than the D form against the Walker rat carcinosarcoma 256. Furthermore, all peptides with a free terminal amino group exerted biological effects comparable with those shown by the L form. The oligopeptides were less toxic than the dipeptides.[4]

As an inhibitor of protein synthesis some pyrimidine-amino acids have been synthesized by the rhodanine method from 2-mercapto-6-oxopyrimidine-4-carboxaldehyde.[5]

The work described in this paper reports the synthesis and biochemical studies of pyrimidine amino acids and their dipeptides with natural amino acids. In the peptide synthesis the phthaloyl group was used as a protecting group for the amino acid. The phthaloyl amino acid chloride and the pyrimidine amino acid were employed in approximately equimolar proportions at 0 to 25°. Higher temperatures tended to favor

(1) This investigation was supported by Public Health Service Research Grants CA-06364-04 and CA-06364-05 from the National Cancer Institute.

(2) To whom inquiries should be sent.

(3) (a) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 2409 (1954); (b) F. Bergel, V. C. E. Burnop, and J. A. Stock, *ibid.*, 1223 (1955); (c) F. Bergel, J. A. Stock, and R. Wade, "Biological Approaches to Cancer Chemotherapy," Academic Press Inc., New York, N. Y., 1961, p 125; (d) L. F. Larionov and Z. P. Sophina, *Dokl. Akad. Nauk SSSR*, **114**, 1070 (1957); (e) Z. P. Sophina, L. F. Larionov, E. N. Shkodinskaya, O. S. Vasina, and A. Y. Berlin, *Acta Unio Intern. Contra Cancrum*, **20**, 82 (1964).

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