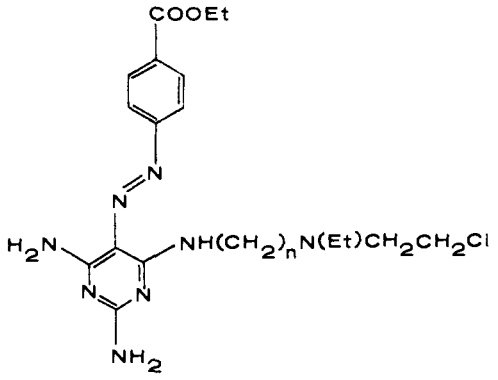
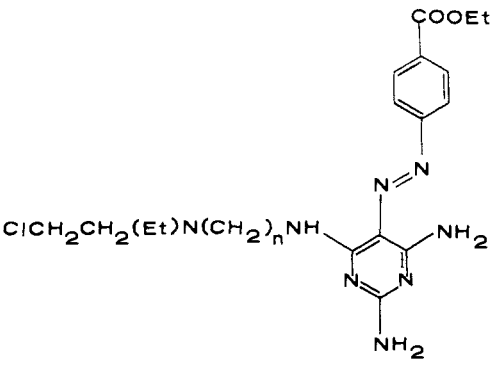
site near the active center the 2,4,6-triamino-5-arylazopyrimidines may be binding in conformation XXIII. In this conformation the alkylating 6 substituent would be able to bridge to the same general area on the enzyme as the corresponding substituent as in XXI. In the case of XXII ionic repulsion between 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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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. Soplina, *Dokl. Akad. Nauk SSSR*, **114**, 1070 (1957); (e) Z. P. Soplina, L. F. Larionov, E. N. Slokodinskaya, O. S. Vasina, and A. Y. Berlin, *Acta Unio Intern. Contra Cancrum*, **20**, 82 (1964).

(4) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 3658 (1960).

(5) V. G. Skulason, C. Piantadosi, B. F. Zambrana, and J. L. Irvin, *J. Med. Chem.*, **8**, 202 (1965).

hydrolysis of the acid chloride to the detriment of the desired coupling reaction. In the cleavage of the phthaloyl group hydrazine hydrate was used in an equimolar concentration. The synthetic routes to peptides of pyrimidine amino acids are illustrated in Scheme I.

SCHEME I

$$\text{III} \xrightarrow{\text{Na-Hg}} \text{IV} \xrightarrow[\text{NaHCO}_3]{\text{II}} \text{V} \xrightarrow{NH_2NH_2 \cdot H_2O} \text{VI}$$

Screening Test.—The compounds were tested *vs.* the Ehrlich ascites carcinoma in Swiss–Webster white mice by a slight modification of procedures described previously.[6,7] Each mouse (initial weight approximately 30 g) received an intraperitoneal injection of 0.1 ml of pooled ascitic fluid, collected from donor mice which had borne the ascites carcinoma for 7–9 days, diluted with 0.9% NaCl to a cell concentration of 10% by volume based upon an initial ascitocrit determination. The 0.1-ml inoculum contained an average of 7×10^6 carcinoma cells. For each assay the mice were divided into a control group of eight mice and several experimental groups of eight mice each. Twenty-four hours after the inoculation, each control mouse received an intraperitoneal injection of 0.2 ml of 0.9% NaCl and each experimental mouse received a suspension of the tested compound in 0.9% NaCl. The intraperitoneal injections of control and experimental mice were continued twice daily for 6 days (total of 11 injections). On the 7th day all surviving mice in control and experimental groups were sacrificed. The volume of ascitic fluid was measured for each animal, and the percentage of cells by volume (ascitocrit) was determined for each sample of ascitic fluid by centrifugation in heparinized capillary tubes. The total packed-cell volume (TPCV) of tumor cells was calculated in each case together with average values and standard deviations. The results of tests of representative compounds are recorded in Table I in which compounds are designated by table number (IV–VI) and compound number (Arabic numerals).

TABLE I

RESULTS OF SCREENING TESTS *vs.* THE EHRLICH ASCITES CARCINOMA[a]

					Av TPCV		
Compd	Dose, mg/kg/day	Mortality C	Mortality T	Av wt change T/C, g	T/C, ml	Std dev of T, ml	T as % of C
IV-1	140	1/8	2/8	4.5/5.4	2.4/2.8	0.28	85
VI-1	140	1/8	2/8	4.1/6.8	1.5/2.6	0.33	57
VI-2	65	3/8	5/8	4.6/5.3	1.4/2.6	0.62	54
	90	1/8	3/8	3.8/6.6	1.2/2.9	0.41	41
VI-3	140	0/8	2/8	3.9/5.6	1.6/2.4	0.38	67
VI-5	60	1/8	2/8	5.5/7.1	1.5/2.9	0.51	52
	90	0/8	1/8	4.6/6.4	1.0/2.5	0.49	40

[a] T = treated group, C = controls, TPCV = total packed-cell volume of tumor cells on final day of assay. The average standard deviation for TPCV of cell control groups was ± 0.31 ml.

For control mice the average gain in carcass weight (after removal of ascitic fluid) was 1.3 g for the total assay period, and consequently the total weight changes recorded in Table I can be attributed principally to the weight of ascitic fluid and tumor cells which accumulated in the peritoneal cavities of the mice during the assay period. However, the determination of TPCV of tumor cells is considered to be a more accurate and reliable method for assaying the effectiveness of the compounds.

Comparison of the data of Table I with data reported previously[8] for the parent pyrimidine aldehyde reveals that the amino acid and peptide derivatives are less active than the parent compound. However, the peptides (VI-1–5) are more active than the corresponding pyrimidine amino acids and their phthalimides.

Inhibition of Protein Synthesis.—The effects of the pyrimidine derivatives upon protein synthesis were studied by determining the inhibition of incorporation of L-phenylalanine-1-^{14}C and glycine-1-^{14}C into the proteins of Ehrlich ascites carcinoma cells which were incubated aerobically for 1 hr with the labeled amino acid and the pyrimidine *in vitro* in Krebs–Ringer phosphate buffer at 37 ± 1° by a procedure described previously in detail.[6] Each incubation flask contained 5 ml of a 20% suspension of tumor cells in Krebs–Ringer phosphate buffer (pH 7.1–7.2), 1 ml of Krebs–Ringer phosphate buffer containing the radioactive amino acid (0.1 μmole/ml of L-phenylalanine (0.2 μCi) and 0.11 μmole/ml of glycine-1-^{14}C (0.2 μCi)), and 1 ml of the pyrimidine in Krebs–Ringer phosphate buffer (or 1 ml of Krebs–Ringer phosphate buffer, alone, in the controls). After the incubation of the experimental and control flasks, the total proteins were isolated and freed of lipids and nucleic acids as described previously.[6] Each protein preparation was dissolved in 2.0 ml of 0.3 *N* NaOH, and 0.2 ml of the protein solution was transferred to a glass scintillation vial. To each vial 0.2 ml of Hyamine Hydroxide 10-X and 16 ml of BBOT[9] scintillation fluid were added with shaking. The radioactivity was determined in a Packard 314-EX spectrometer for 5 or 10 min. The background was subtracted from each value. The concentration of protein was determined from the comparison of the optical density of each protein solution in biuret reagent at a wavelength of 540 mμ with serum albumin standards.[10] In Tables II and III radioactivities are

TABLE II

INCORPORATION OF PHENYLALANINE-1-^{14}C INTO PROTEINS OF EHRLICH ASCITES CARCINOMA CELLS[a]

Compd	Dose, μmole	Molar ratio inhib/Phe	Av radioact of total protein, cpm/mg	% of control
Control	...	..	6976	100
IV-1	5	50	5519	79
VI-1	5	50	4968	71
VI-4	2.5	25	4570	65
VI-5·HCl	2.5	25	4356	62

[a] 0.1 μmole of phenylalanine-1-^{14}C was added to each incubation flask.

(6) J. E. Wilson, J. L. Irvin, J. E. Suggs, and K. Liu, *Cancer Res.*, **19**, 272 (1959).

(7) J. E. Wilson, J. E. Suggs, and J. L. Irvin, *Cancer Res. Suppl.*, **21**, 692 (1961).

(8) C. Piantadosi, V. G. Skulason, J. L. Irvin, J. M. Powell, and L. Hall, *J. Med. Chem.*, **7**, 337 (1964).

(9) Hyamine Hydroxide 10-X (*p*-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide) and BBOT (2,5-bis[2-(5-*t*-butylbenoxazolyl)]-thiophene) are available from Packard Instrument Co., Inc., Downers Grove, Ill. 60515.

(10) E. Layne, *Methods Enzymol.*, **3**, 447 (1957).

TABLE III

INCORPORATION OF GLYCINE-1-^{14}C INTO PROTEINS OF EHRLICH ASCITES CARCINOMA CELLS[a]

Compd	Dose, μmole	Molar ratio inhib/Gly	Av radioact of total protein, cpm/mg	% of control
Control	...	...	1677	100
IV-1	5	50	1456	87
IV-3	5	50	1576	94
VI-1	5	50	1472	88
VI-2	5	50	1414	86

[a] 0.11 μmole of glycine-1-^{14}C was added to each incubation flask.

expressed as counts per minute per milligram of protein (cpm/mg) after correction for background and quenching. The effects of one of the pyrimidine amino acids (IV-1) and its peptide derivatives (VI-1, VI-4, and VI-5) upon incorporation of L-phenylalanine-1-^{14}C into proteins of the carcinoma cells are recorded in Table II. The peptides are more inhibitory than the parent pyrimidine amino acid, and the phenylalanyl peptide is a stronger inhibitor than the glycyl peptide. On the other hand, the pyrimidine amino acids and their peptides exhibit little or no inhibitory activity against the incorporation of glycine-1-^{14}C into proteins of the carcinoma cells (Table III).

Experimental Section[11]

Phthaloyl Amino Acid (I). Method A.—These compounds were prepared by known procedures[12] from the amino acid and phthalic anhydride by direct fusion.

Method B.—These compounds were also prepared by a known procedure[13] from the amino acid and N-carbethoxyphthalimide.

Phthaloyl amino acid chlorides (II) were prepared by known procedures[12] from the phthaloyl amino acid and $SOCl_2$ or PCl_5.

α-Oximino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic Acid (III-1).—To 24 g (1.04 g-atoms) of Na in 700 ml of absolute EtOH was added a warm solution of 77 g (1.1 moles) of $NH_2OH \cdot HCl$ in 64 ml of H_2O. The solution of $HONH_2$ was filtered from the precipitated NaCl and refluxed with 80 g (0.35 mole) of the crude 2-mercapto-6-oxo-5-methyl-4-(2-carboxy-2-thionoethyl)pyrimidine[5] for 2 hr. At the end of reflux a considerable precipitate formed. The reaction mixture was allowed to cool slowly and left standing in the refrigerator overnight. The precipitate was filtered and washed (EtOH). It was then dissolved in a solution of 10 g of NaOH in 250 ml of H_2O, resulting in the production of free S from Na_2S in the crude product. The solution was filtered to remove free S, cooled in an ice bath, and cautiously acidified to pH 6 with 10% HCl. The acid precipitated immediately, and the mixture was placed in the refrigerator for 1 hr. The precipitate was filtered, washed (H_2O), and air dried. The filtrate was evaporated to small volume to yield additional product. The yield after recrystallization from EtOH-H_2O (1:1) was 70 g (91.5%), mp 190–192°. *Anal.* ($C_8H_9N_3O_4S$) C, H, N, S.

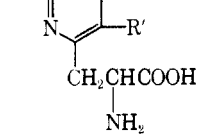
α-Oximino-β-(2-mercapto-6-oxo-1-methyl)-4-pyrimidylpropionic acid (III-2) was prepared by this method; yield 52%, mp 274–276° dec. *Anal.* ($C_8H_9N_3O_4S$) C, H, N, S.

α-Amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic Acid (IV-1).—α-Oximino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic acid (20 g) was dissolved in 800 ml of EtOH. This solution was heated on the steam bath and 800 g of 2% NaHg was added in four 200-g portions with stirring. When all amalgam had been added, enough H_2O (800 ml) was added to effect a solution of the precipitate which had formed. The green reaction mixture was heated gently on the steam bath until H_2 evolution had ceased, and then the solution was cooled to room temperature. The basic solution was passed through a column of 300 g of a weakly acidic ion-exchange resin, Amberlite IRC-50. The white pyrimidine amino acid started to precipitate in the eluate. The product was filtered, and the filtrate was evaporated to a small volume on a flash evaporator, treated with 100 ml of EtOH, and placed in the refrigerator for 1 hr. The resin from the column was poured into H_2O (500 ml). Stirring this mixture resulted in suspension of the product which had been precipitated on the column. The supernatant was decanted, evaporated to a small volume, and treated with EtOH. The white precipitate which formed from both EtOH suspension was filtered and washed (EtOH). Recrystallization from EtOH-H_2O gave 8.4 g (45% yield); mp 286–289° dec; λ_{max} 2.85, 3.2 (OH, NH), 6.03, 6.46, 7.0 μ (NH, CO).

α-Amino-β-(2-mercapto-6-oxo)-4-pyrimidylpropionic acids prepared by this method are listed in Table IV.

TABLE IV

α-AMINO-β-(2-MERCAPTO-6-OXO)-4-PYRIMIDYLPROPIONIC ACID



No.	R	R′	Mp, °C dec	Formula[b]	Yield, %
1	H	CH_3	286–289	$C_8H_{11}N_3O_3S$	45
2	CH_3	H	272–273	$C_8H_{11}N_3O_3S$	32
3[a]	CH_3	CH_3	336–338	$C_9H_{13}N_3O_3S$	38

[a] See ref 5. [b] All compounds were analyzed for C, H, N, S.

Phthaloylglycyl-α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic Acid (V-1).—The solution of 1.117 g (0.005 mole) of phthaloylglycyl chloride in 30 ml of PhH was added slowly with stirring to the ice-cold suspension mixture of 1.145 g (0.005 mole) of α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic acid, 0.84 g (0.01 mole) of $NaHCO_3$, and 15 ml of H_2O. When all the phthaloylglycyl chloride was added, stirring was continued for about 1 hr with the addition of more H_2O (15 ml). The suspension became a clear solution at the end of the reaction. The PhH layer was removed, and the aqueous layer was filtered and acidified with 10% HCl to precipitate the crude white product. This was filtered, washed (H_2O), air dried, and recrystallized (hot H_2O) to give 1.5 g of product (74.5%): mp 235–237° dec; λ_{max} 2.89, 2.93 (OH, NH), 6.10 (amide I), 6.4 (amide II), 8.0, 8.2 (amide III), 13.5, 14.0 μ (C_6H_5).

The phthaloylaminoacylpyrimidine amino acids prepared by this method are listed in Table V.

Glycyl-α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic Acid (VI-1).—Phthaloylglycyl-α-amino-β-(2-mercapto-6-oxo-5-methyl)-4-pyrimidylpropionic acid (1.3 g, 3 mmoles) was dissolved in 40 ml of boiling H_2O. Hydrazine hydrate 85% (0.19 g, 3.1 mmoles) was added to the aqueous solution, and the mixture was refluxed for 30 min. At the end of refluxing some white precipitate was seen. The reaction solution was evaporated to dryness with a flash evaporator. HCl (10 ml, 10%) was added to the white residue, and the mixture was warmed for 15 min on the warm-water bath (40–50°). After cooling the mixture to room temperature, the white precipitate (phthaloyl hydrazide) was filtered, and the filtrate was neutralized to methyl red with morpholine. Absolute EtOH (10 vol) was added slowly with stirring, and a white product started to precipitate. The mixture was put in the ice bath for 1 hr. The product was filtered and was washed (absolute EtOH); yield 620 mg (70%); mp 240–245° dec; λ_{max} 2.85, 3.0 (OH, NH), 5.95 (amide I), 6.45 (amide II), 8.2 μ (amide III).

The aminoacylpyrimidine amino acids prepared by this method are listed in Table VI.

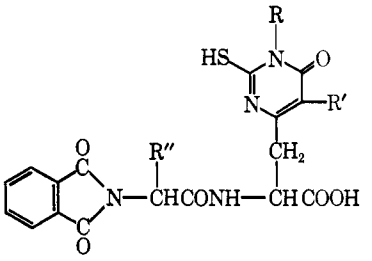
(11) Analyses by Alfred Bernhardt Microanalytical Laboratory, Mülheim (Ruhr), Germany. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values. The melting points were determined with the Mel-Temp apparatus and have been corrected. Ir spectra were determined with a Perkin-Elmer 137 Infracord spectrophotometer in KBr pellets.

(12) (a) J. C. Sheehan and V. S. Frank, *J. Am. Chem. Soc.*, **71**, 1856 (1949); (b) O. E. Emerson, U. S. Patent 2,498,665 (1950).

(13) G. H. L. Nefkens, G. I. Tesser, and R. J. F. Nivard, *Rec. Trav. Chim.*, **79**, 668 (1960).

TABLE V

PHTHALOYLAMINOACYL-α-AMINO-β-(2-MERCAPTO-6-OXO)-4-PYRIMIDYLPROPIONIC ACID

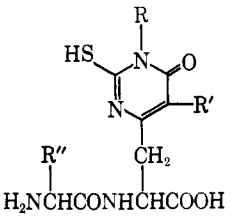


No.	Amino acid	R	R′	R″	Mp, °C	Formula[a]	Yield, %
1	Glycine	H	CH_3	H	235–237 dec	$C_{18}H_{16}N_4O_6S$	74.5
2	Glycine	CH_3	H	H	205–210	$C_{18}H_{16}N_4O_6S$	47
3	DL-Alanine	H	CH_3	CH_3	260–261 dec	$C_{19}H_{18}N_4O_6S$	68
4	DL-Phenylalanine	H	CH_3	$C_6H_5CH_2$	195–200	$C_{25}H_{23}N_4O_6S$	57.5
5	L-Phenylalanine	H	CH_3	$C_6H_5CH_2$	150–160	$C_{25}H_{23}N_4O_6S$	57
6	L-Phenylalanine	CH_3	H	$C_6H_5CH_2$	130–145	$C_{25}H_{23}N_4O_6S$	20

[a] All compounds were analyzed for C, H, N, S.

TABLE VI

AMINOACYL-α-AMINO-β-(2-MERCAPTO-6-OXO)-4-PYRIMIDYLPROPIONIC ACID



No.	Amino acid	R	R′	R″	Mp, °C, dec	Formula[a]	Yield, %
1	Glycine	H	CH_3	H	240–245	$C_{10}H_{14}N_4O_4S$	70
2	Glycine	CH_3	CH_3	H	257–258	$C_{11}H_{16}N_4O_4S$	72
3	DL-Alanine	H	CH_3	CH_3	243–244	$C_{11}H_{16}N_4O_4S$	49
4	DL-Phenylalanine	H	CH_3	$C_6H_5CH_2$	285–290	$C_{17}H_{21}N_4O_4S$	70
5	L-Phenylalanine	H	CH_3	$C_6H_5CH_2$	293–294	$C_{17}H_{21}N_4O_4S$	74

[a] All compounds were analyzed for C, H, N, S.