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ACKNOWLEDGMENTS

Presented in part at the APhA Academy of Pharmaceutical Sciences, New Orleans meeting, April 1976.

The authors thank E. C. Mora for helpful discussions and J. H. Blalock for skin samples.

Effect of Acylated Amino Acids and Acylated Amino Acid Analogs on Microbial Antitumor Screen

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Received March 10, 1977, from the *Nucleic Acids Section, Laboratory of Pathophysiology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.* Accepted for publication July 26, 1977.

Abstract □ A series of *N*-acetyl, *N*-propionyl, and *N*-chloroacetyl derivatives of amino acids and amino acid analogs was tested for growth-inhibitory activity using a *Lactobacillus casei* system as a prescreen for possible antitumor activity. While none of the acetyl or propionyl derivatives of these amino acids and amino acid analogs caused any remarkable inhibition, certain chloroacetyl derivatives exhibited significant activity. The chloroacetyl derivatives, especially those of essential amino acids and of analogs of essential amino acids, showed modest, but pharmacologically significant, inhibition; those of nonessential amino acids exhibited no activity. When two such inhibitory acyl derivatives were combined in a single assay, the extent of inhibition was neither additive nor synergistic but was that of the more active of the two test components.

Keyphrases □ Amino acids, various acylated—evaluated in microbial antitumor screen □ Acylated amino acids, various—evaluated in microbial antitumor screen □ Antitumor activity—various acylated amino acids evaluated in microbial screen □ Structure–activity relationships—various acylated amino acids evaluated in microbial antitumor screen

In a search for antineoplastic activities among a series of β-hydroxy-α-amino acids, some unsubstituted acids exhibited no growth-inhibitory action in a *Lactobacillus casei* test system, but their chloroacetyl derivatives showed pharmacologically significant activity in this system (1, 2). As a result of that observation, an investigation was initiated to note whether the behavior of other analogs of natural amino acids known to be antimetabolites might not be affected similarly by acylation.

The *N*-acetyl, *N*-chloroacetyl, and *N*-propionyl derivatives of a series of amino acids and amino acid analogs were prepared. These compounds were tested against an *L. casei* system (3) to determine the presence of growth-inhibitory capacity. This microbial system was selected as a prescreen for antitumor activity because of its capability of detecting, very quickly, a high percentage of compounds known to be active against tumors, using very small quantities of test compound (3). A study was also made to determine the existence of additive or synergistic properties when two inhibitory compounds were used simultaneously.

The present paper reports the results of these studies.

EXPERIMENTAL

The free amino acids, except for the isomers of β-hydroxymethoxinine and β-hydroxyhomomethionine which were prepared in this laboratory (4), were obtained commercially. The sources and purity data for the acyl derivatives are shown in Tables I–III. The commercially obtained free amino acids and acyl compounds were recrystallized, and all compounds were checked for purity by elemental analysis, melting-point determination, optical rotation determination, and Van Slyke nitrous acid determination of primary amino nitrogen (5) before use (Tables I–III). In addition, the free amino acids were checked by paper chromatography in at least four different solvent systems (*cf.*, 2).

The chloroacetyl and propionyl derivatives were prepared in this laboratory by acylation of the precursors by the conventional Schotten–Baumann procedure (*cf.*, 6). The chloroacetyl chloride[1] and propionyl chloride[1] used for the acylation and the propionic acid[1] and monochloroacetic acid[2] were freshly redistilled before use. The optically pure amino acids were prepared by asymmetric enzymatic hydrolysis of the racemic chloroacetyl derivatives and subsequent acid hydrolysis of the unsusceptible chloroacetyl-D-amino acid. The optically pure chloroacetyl-L-amino acids were prepared by acylation of the pure L-isomer, and the chloroacetyl-D-isomers were isolated from the resolution mixture as described (11). Hog renal acylase[3] was used for the hydrolysis of the acyl aliphatic amino acids, and pancreatic carboxypeptidase[4] was used for the hydrolysis of the acyl aromatic amino acid (11).

For the microbiological assay, the test compounds were dissolved in a small volume of water and the pH was adjusted to 6 by the addition of an appropriate volume of 0.1 *N* NaOH. L-Tyrosine required equimolar amounts of the base for solution, resulting in a basic solution. In some cases, gentle warming was required for complete solution. The test solutions were sterilized by filtration using an all-glass bacterial filter (ultrafine porosity).

The concentration of the test solution as prepared was 2 mg/ml and was equivalent to a concentration of 1 mg/ml in the assay system. For the experiments comparing the relative activities of the "active" compounds, the concentration of the test solution was 89.4 μmoles/10 ml as prepared and was equivalent to a concentration of 4.47 μmoles of the test compound/ml in the assay system. This concentration is equivalent to 1 mg of *N*-chloroacetyl-β-hydroxy-D-norleucine B/ml, the compound found previously to be the most active isomer of chloroacetyl-β-hydroxynorleucine (2).

All glassware used in the microbiological assay was cleaned in a metal ion-free cleaning agent[5].

[1] Eastman Organic Chemicals, Rochester, N.Y.
[2] Fisher Scientific Co., Silver Spring, Md.
[3] Nutritional Biochemicals, Cleveland, Ohio.
[4] Calbiochem, La Jolla, Calif.
[5] No-chromix, Godax Laboratories, New York, N.Y.

Table I—Purity Data of *N*-Acetyl Derivatives [a]

N-Acetyl Derivative	Source[b]	Melting Point, Reported	Melting Point, Observed[c]	Optical Rotation[d], $[\alpha]_D$ 21–24°, Reported	Optical Rotation[d], $[\alpha]_D$ 21–24°, Observed	Empirical Formula	Analysis[e], % Calc.	Analysis[e], % Obs.
DL-Allylglycine	1	114°[f]	116°	—	0° (A)	$C_7H_{11}NO_3$	C 53.49	53.76
							H 7.06	7.35
							N 8.91	8.98
DL-Leucine	2	159°[f]	163°	—	0° (A)	$C_8H_{15}NO_3$	C 55.47	55.76
							H 8.73	8.80
							N 8.09	8.33
L-Leucine[g]	2	185°[f]	193°	−21.3°[f] (A)	−23.0° (A)	$C_8H_{15}NO_3$	C 55.47	55.27
							H 8.73	8.95
							N 8.09	8.27
D-Leucine[g]	3	185°[f]	193°	+21.3°[f] (A)	+23.3° (A)	$C_8H_{15}NO_3$	C 55.47	55.76
							H 8.73	8.82
							N 8.09	8.15
DL-Norleucine[g]	4	105°[f]	99°	—	0° (W)	$C_8H_{15}NO_3$	C 55.47	55.67
							H 8.73	8.98
							N 8.09	8.15
D-Norleucine	2	112°[f]	113°	+20.0°[f] (W)	+20.5° (W)	$C_8H_{15}NO_3$	C 55.47	55.50
							H 8.73	8.92
							N 8.09	8.28
o-Fluoro-DL-phenylalanine	3	151–153°[h]	151–153°	—	0° (A)	$C_{11}H_{12}FNO_3$	C 58.66	58.54
							H 5.37	5.53
							N 6.22	6.12
m-Fluoro-DL-phenylalanine	2	156–158°[h]	153–154°	—	0° (A)	$C_{11}H_{12}FNO_3$	C 58.66	58.37
							H 5.37	5.42
							N 6.22	6.33
p-Fluoro-DL-phenylalanine	3	150–153°[h]	150–153°	—	0° (A)	$C_{11}H_{12}FNO_3$	C 58.66	58.60
							H 5.37	5.49
							N 6.22	6.40
β-2-Thienyl-DL-alanine	1	130°[i]	131–133°	—	0° (A)	$C_9H_{11}NO_3S$	C 50.69	50.67
							H 5.20	5.14
							N 6.57	6.50
β-3-Thienyl-DL-alanine hemihydrate	1	—	125–126°	—	0° (A)	$C_9H_{12}NO_{3\frac{1}{2}}S$	C 48.63	48.17
							H 5.14	5.22
							N 6.30	6.29

[a] Van Slyke nitrous acid determination of primary amino nitrogen (5) made on a 1-ml sample containing an equivalent of 0.3 mg of amino nitrogen (when hydrolyzed) yielded no detectable quantity of nitrogen. [b] Source of compounds: 1, prepared in this laboratory; 2, ICN Pharmaceuticals, Cleveland, Ohio; 3, Sigma Chemical Co., St. Louis, Mo.; and 4, Vega-Fox Biochemicals, Tucson, Ariz. Data on commercial products were obtained from recrystallized materials. [c] Melting points (uncorrected) were determined on a Fisher-Johns melting-point block. [d] Optical rotation was determined on a Rudolph polarimeter (Rudolph Research, Fairfield, N.J.), model 80, sodium lamp, using 100-mm tubes (bore size of 3 mm) and a sample capacity of 0.7 ml. (W) = water, (A) = absolute ethanol, c = 2. [e] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. [f] *Cf.*, Ref. 6. [g] Used as obtained commercially. [h] The Aldrich Catalog-Handbook of Organic and Biochemicals, 1975–1976, p. 10. [i] *Cf.*, Ref. 7.

Table II—Purity Data of *N*-Propionyl Derivatives [a,b]

N-Propionyl Derivative	Observed Melting Point[c]	Observed Optical Rotation[d], $[\alpha]_D$ 22–24°	Empirical Formula	Analysis[e], % Calc.	Analysis[e], % Obs.
L-Alanine	87–88°	−35.4°	$C_6H_{11}NO_3$	C 49.64	49.71
				H 7.64	7.86
				N 9.65	9.55
DL-Allylglycine	85–86°	0°	$C_8H_{13}NO_3$	C 56.12	55.99
				H 7.65	7.44
				N 8.18	8.16
o-Fluoro-DL-phenylalanine	137–138°	0°	$C_{12}H_{14}FNO_3$	C 60.24	60.09
				H 5.90	5.96
				N 5.86	5.82
m-Fluoro-DL-phenylalanine	106–108°	0°	$C_{12}H_{14}FNO_3$	C 60.24	60.50
				H 5.90	6.04
				N 5.86	5.80
p-Fluoro-DL-phenylalanine	117–120°	0°	$C_{12}H_{14}FNO_3$	C 60.24	60.41
				H 5.90	5.96
				N 5.86	5.89
L-Leucine	142–144°	−22.0°	$C_9H_{17}NO_3$	C 57.73	57.80
				H 9.15	9.24
				N 7.48	7.33
DL-Norleucine	84–85°	0°	$C_9H_{17}NO_3$	C 57.73	57.98
				H 9.15	9.14
				N 7.48	7.29
β-2-Thienyl-DL-alanine	115–117°	0°	$C_{10}H_{13}NO_3S$	C 52.85	52.83
				H 5.76	5.58
				N 6.16	6.12
β-2-Thienyl-DL-β-alanine	186–187°	0°	$C_{10}H_{13}NO_3S$	C 52.85	52.92
				H 5.76	5.96
				N 6.16	6.28
β-3-Thienyl-DL-alanine hemihydrate	103–107°	0°	$C_{10}H_{14}NO_{3\frac{1}{2}}S$	C 50.83	50.95
				H 5.97	5.53
				N 5.93	5.86

[a] These compounds were prepared in this laboratory. [b] Van Slyke nitrous acid determination of primary amino nitrogen (5) made on a 1-ml sample containing an equivalent of 0.3 mg of amino nitrogen (when hydrolyzed) yielded no detectable quantity of nitrogen. [c] Melting points (uncorrected) were determined on a Fisher-Johns melting-point block. [d] Optical rotation was determined on a Rudolph polarimeter, model 80, sodium lamp, using 100-mm tubes (bore size of 3 mm) and a sample capacity of 0.7 ml in absolute ethanol, c = 2. [e] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md.

Table III—Purity Data of *N*-Chloroacetyl Derivatives [a]

N-Chloroacetyl Derivative	Source[b]	Melting Point, Reported	Melting Point, Observed[c]	Optical Rotation[d], $[\alpha]_D$ 22–24°, Reported	Optical Rotation[d], $[\alpha]_D$ 22–24°, Observed	Empirical Formula	Analysis[e], % Calc.	Analysis[e], % Obs.
L-Alanine	1	93°[f]	91–93°	−45.4° (W)[f]	−45.1° (W)	$C_5H_8ClNO_3$	C 36.27 H 4.86 N 8.46	35.97 4.93 8.25
D-Alanine	1	93°[f]	91–94°	+45.4° (W)[f]	+45.8° (W)	$C_5H_8ClNO_3$	C 36.27 H 4.86 N 8.46	36.15 4.95 8.18
L-Aspartic acid	2	144°[f]	140–144°	+4.0° (W)[f]	+3.5° (W)	$C_6H_8ClNO_5$	C 34.38 H 3.84 N 6.68	34.11 3.95 6.69
D-Aspartic acid	2	144°[f]	142–143°	−4.0° (W)[f]	−3.0° (W)	$C_6H_8ClNO_5$	C 34.38 H 3.84 N 6.68	34.09 3.95 6.67
DL-Allylglycine	2	—	87°	—	0° (A)	$C_7H_{10}ClNO_3$	C 43.88 H 5.26 N 7.26	44.15 5.07 7.31
L-Allylglycine	2	—	96–97°	—	+7.5° (W)	$C_7H_{10}ClNO_3$	C 43.88 H 5.26 N 7.26	43.78 5.18 7.38
D-Allylglycine	2	—	95–96°	—	−9.1° (W)	$C_7H_{10}ClNO_3$	C 43.88 H 5.26 N 7.26	43.58 5.32 7.31
o-Fluoro-DL-phenylalanine	2	—	134–136[c]	—	0° (A)	$C_{11}H_{11}ClFNO_3$	C 50.88 H 4.27 N 5.39	50.57 4.33 5.43
m-Fluoro-DL-phenylalanine	2	—	122–123°	—	0° (A)	$C_{11}H_{11}ClFNO_3$	C 50.88 H 4.27 N 5.39	50.77 4.25 5.47
p-Fluoro-DL-phenylalanine	2	—	140–143°	—	0° (A)	$C_{11}H_{11}ClFNO_3$	C 50.88 H 4.27 N 5.39	50.67 4.22 5.38
p-Fluoro-L-phenylalanine	2	—	116–117°	—	+42.3° (A)	$C_{11}H_{11}ClFNO_3$	C 50.88 H 4.27 N 5.39	50.63 4.33 5.32
p-Fluoro-D-phenylalanine	2	—	115–117°	—	−40.8° (A)	$C_{11}H_{11}ClFNO_3$	C 50.88 H 4.27 N 5.39	50.59 4.34 5.22
Glycine	3	100°[f]	99–100°	—	—	$C_4H_6ClNO_3$	C 31.70 H 3.99 N 9.24	31.40 3.84 9.24
β-Hydroxy-DL-homomethionine A	2	—	139–140°	—	0° (W)	$C_8H_{14}ClNO_4S$	C 37.58 H 5.52 N 5.48	37.54 5.61 5.56
β-Hydroxy-DL-homomethionine B	2	—	138–141°[k]	—	0° (W)	$C_8H_{14}ClNO_4S$	C — H — N 5.48	— — 5.11
β-Hydroxy-DL-methoxinine A	2	—	122–124°	—	0° (W)	$C_7H_{12}ClNO_5$	C 37.26 H 5.36 N 6.20	37.11 5.43 6.27
β-Hydroxy-DL-methoxinine B	2	—	152°	—	0° (W)	$C_7H_{12}ClNO_5$	C 37.26 H 5.36 N 6.20	37.46 5.45 6.37
DL-Isoleucine[g]	4	117°[h]	97–103°	—	0° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.24 6.83 6.86
L-Isoleucine	4	74–75°[f]	82°	+25.0° (A)[f]	+25.3° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.20 6.88 6.68
DL-Leucine	1	142°[f]	139–141°	—	0° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.57 7.04 6.66
L-Leucine	2	133°[f]	133–134°	−15.8° (A)[f]	−14.9° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.33 6.63 6.69
D-Leucine	1	134°[f]	133–134°	+16.2° (A)[f]	+15.1° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.51 7.04 6.92
DL-Norleucine	2	116°[f]	112–113°	—	0° (A)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.41 7.03 6.67
L-Norleucine	2	76°[i]	76°	−17.0° (W)[i]	−17.2° (W)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	46.55 7.04 6.55
D-Norleucine	2	76°[f]	75°	+17.0° (W)[f]	+16.2° (W)	$C_8H_{14}ClNO_3$	C 46.27 H 6.80 N 6.75	45.98 6.97 6.63
DL-Norvaline	3	101°[f]	103°	—	0° (W)	$C_7H_{12}ClNO_3$	C 43.42 H 6.25 N 7.24	42.95 6.46 7.50

Table III—*(Continued)*

N-Chloroacetyl Derivative	Source[b]	Melting Point, Reported	Melting Point, Observed[c]	Optical Rotation[d], $[\alpha]_D$ 22–24°, Reported	Optical Rotation[d], $[\alpha]_D$ 22–24°, Observed	Empirical Formula	Analysis[e], % Calc.	Analysis[e], % Obs.
DL-Phenylalanine	5	130°[f]	127–130°	—	0° (A)	$C_{11}H_{12}ClNO_3$	C 54.67	54.42
							H 5.01	4.81
							N 5.80	6.03
L-Phenylalanine	3	125°[f]	125°	+50.4° (A)[f]	+48.0° (A)	$C_{11}H_{12}ClNO_3$	C 54.67	55.11
							H 5.01	5.06
							N 5.80	6.01
DL-Serine	2	123°[f]	124–126°	—	0° (A)	$C_5H_8ClNO_4$	C 33.07	33.17
							H 4.44	4.41
							N 7.72	7.90
β-2-Thienyl-DL-alanine	2	128°[f]	126–128°	—	0° (A)	$C_9H_{10}ClNO_3S$	C 43.72	43.51
							H 4.07	4.14
							N 5.66	5.79
β-2-Thienyl-L-alanine	2	120°[f]	120–122°	+46.5° (A)[f]	+47.2° (A)	$C_9H_{10}ClNO_3S$	C 43.72	44.00
							H 4.07	4.17
							N 5.66	5.70
β-2-Thienyl-D-alanine	2	120°[f]	119–120°	−47.2° (A)[f]	−44.0° (A)	$C_9H_{10}ClNO_3S$	C 43.72	43.60
							H 4.07	4.10
							N 5.66	5.67
β-2-Thienyl-DL-β-alanine	2	—	163–166°	—	0° (A)	$C_9H_{10}ClNO_3S$	C 43.72	43.74
							H 4.07	4.17
							N 5.66	5.60
β-3-Thienyl-DL-alanine	2	105–106°[j]	99–103°	—	0° (A)	$C_9H_{10}ClNO_3S$	C 43.72	43.44
							H 4.07	4.13
							N 5.66	5.50
L-Tryptophan	3	158°[f]	158–160°	+32.0° (A)[f]	+29.9° (A)	$C_{13}H_{13}ClN_2O_3$	C 55.63	55.63
							H 4.67	4.68
							N 9.98	10.07
L-Tyrosine	3	155°[f]	150–155°	+59.0° (A)[f]	+60.0° (A)	$C_{11}H_{12}ClNO_4$	C 51.27	151.19
							H 4.70	4.63
							N 5.44	5.57
DL-Valine	4	132°[f]	130°	—	0° (A)	$C_7H_{12}ClNO_3$	C 43.42	43.35
							H 6.25	5.98
							N 7.24	7.32
L-Valine	3	114°[f]	114°	+15.0° (A)[f]	+12.7° (A)	$C_{17}H_{12}ClNO_3$	C 43.42	43.62
							H 6.25	6.17
							N 7.24	7.24

[a] Van Slyke nitrous acid determinations of primary amino nitrogen (5) yielded no detectable quantity of nitrogen (*cf.*, Table I for details). [b] Source of compounds: 1, Cyclo Chemical Corp., Los Angeles, Calif.; 2, prepared in this laboratory; 3, Sigma Chemical Co., St. Louis, Mo.; 4, ICN Pharmaceuticals, Cleveland, Ohio; and 5, Nutritional Biochemicals Corp., Cleveland, Ohio. Data on commercial products were obtained from recrystallized materials. [c] Melting points (uncorrected) were determined on a Fisher-Johns melting-point block. Chloroacetyl-DL-homomethionine B bubbled at 112°. [d] Optical rotation was determined on a Rudolph polarimeter, model 80, sodium lamp (*cf.*, Table I). (W) = water, and (A) = absolute ethanol. For chloroacetyl-L- and chloroacetyl-D-aspartic acid, c = 5. [e] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases (*cf.*, Table I). [f] *Cf.*, Ref. 6. [g] Used as commercially obtained. [h] *Cf.*, Ref. 8. [i] *Cf.*, Ref. 9. [j] *Cf.*, Ref. 10. [k] Crude, melting point not sharp.

Lactobacillus casei 7469[6] was carried on agar[7] and subcultured in a broth[7] for the preparation of the inoculum. The inoculum was a 1:20 dilution in normal saline of the washed bacteria. The assay medium was a riboflavin assay medium[7] supplemented with a minimal amount of riboflavin[4] required to give optimal growth of the organism as determined by construction of a standard curve, plotting concentration of riboflavin in the medium against extent of growth, as measured by turbidity. The minimal riboflavin concentration that supported maximal growth, determined by assay, was 0.03 μg of riboflavin/ml of the final assay system. The pH of the assay system containing the highest level of the test compound was within 0.02 pH unit of a control tube, which contained water in place of the test solution, except that of L-tyrosine, for which the pH of the final assay system was 0.49 pH unit higher than that of the control. The details of the assay procedure were described previously (1).

The extent of growth was measured by determination of turbidity in Klett units on a Klett–Summerson photoelectric colorimeter equipped with a red filter (660 nm). The instrument was set to zero against a tube containing the medium in a concentration equivalent to that of the test system and refrigerated during the incubation of the experimental tubes (reagent blank).

RESULTS

No significant inhibition was observed with either the acetyl (Table IV) or the propionyl (Table V) derivatives of the amino acids and amino acid analogs.

With the exception of *p*-fluoro-DL-phenylalanine and β-3-thienyl-DL-alanine, which showed 48 and 36% inhibition, respectively, at 1 mg/ml, the free amino acids were inactive (Table VI). L-Tyrosine gave strong inhibition at that concentration, but this result was due to the fact that it had to be dissolved in an alkaline system. L-Isoleucine, an inactive compound in the assay system, showed inhibition under similar conditions (Table VII).

As in the studies of β-hydroxyamino acids (1, 2), the chloroacetyl derivatives of certain of these amino acids and amino acid analogs exhibited some growth-inhibitory capacity, but no such activity was noted with the corresponding unacylated mother compound (Tables VI and VIII).

Where an increased inhibition was observed, the degree varied (Table

Table IV—Effect of *N*-Acetyl Derivatives of Amino Acids and Amino Acid Analogs on Growth of *L. casei* 7469

N-Acetyl Derivative	Concentration, m*M*	Inhibition[a], %
DL-Allylglycine	6.36	3
DL-Leucine	5.77	+1[b]
L-Leucine	5.77	0
D-Leucine	5.77	0
DL-Norleucine	5.77	0
D-Norleucine	5.77	1
o-Fluoro-DL-phenylalanine	4.44	2
m-Fluoro-DL-phenylalanine	4.44	3
p-Fluoro-DL-phenylalanine	4.44	2
β-2-Thienyl-DL-alanine	4.69	4
β-3-Thienyl-DL-alanine	4.69	4

[a] Growth in inoculated controls (water *in lieu* of test solution), measured turbidimetrically, was 190–210 Klett units for different series of determinations. At least three duplicate determinations were made for each compound. The duplicate values in each determination agreed within ±5 Klett units. The standard deviation of a compound showing a mean value of 20% inhibition, for example, was 2.7, and the standard error was 0.95. Concentration of test compounds was 1.0 mg/ml; the value given is the final concentration in the assay system. For assay conditions, see text. [b] The + indicates stimulation of growth.

[6] American Type Culture Collection, Rockville, Md.

[7] Micro assay culture agar (Difco B319), micro inoculum broth (Difco B320), and riboflavin assay medium (Difco B325), Difco Laboratories, Detroit, Mich.

VIII). The most active chloroacetylated β-hydroxynorleucine isomer, *i.e.*, *N*-chloroacetyl-D-β-hydroxynorleucine B[8], as shown previously (2), exhibited about 50% inhibition at 1.0 mg/ml.

Most of the active chloroacetyl derivatives, when compared on an equimolar basis, showed activity of about 14–30% inhibition (Table IX). Although the extent of inhibition by these compounds was modest, it was dependent on concentration (Table VIII). When the optically pure isomers were tested, little difference in activity was noted, with the L-isomer showing a slightly, if any, greater activity than the D-antipode. With the exception of chloroacetyl-L-alanine, the active compounds generally were the chloroacetyl derivatives of "essential" amino acids or of analogs of essential amino acids. However, the *N*-chloroacetyl-β-hydroxyhomomethionines and the *N*-chloroacetyl-β-hydroxymethoxinines were inactive. When compared on an equimolar basis with other active chloroacetyl amino acids at a concentration equivalent to that of 1 mg of *N*-chloroacetyl-D-β-hydroxynorleucine B/ml or 4.47 μmoles/ml, the activity of chloroacetyl-L-alanine was negligible (Table IX).

When *N*-chloroacetyl-D-β-hydroxynorleucine B and chloroacetyl-β-2-thienyl-DL-alanine were present in equimolar quantities, each at the concentration of 4.47 μmoles/ml, the inhibition observed was that of the more active of the two compounds, *i.e.*, *N*-chloroacetyl-D-β-hydroxynorleucine B (Table X). Similarly, when combinations of chloroacetyl-DL-allylglycine and chloroacetyl-β-2-thienyl-DL-alanine and of chloroacetyl-DL-norleucine and chloroacetyl-β-2-thienyl-DL-alanine were tested, the effect was equivalent to that of the more active compound, namely, chloroacetyl-β-2-thienyl-DL-alanine in both cases (30% for the former combination and 30% for the latter). No additive nor synergistic action was observed.

That the inhibition was not caused by a nonenzymatic hydrolytic release of the constituent chloroacetic acid, which itself has been shown to be active (Table VIII), during preparation of the test solution or during incubation was excluded since no detectable quantity of primary amino nitrogen was observed when a determination of free amino nitrogen was made on the test solution or on the control solution using the Van Slyke nitrous acid method (4) (Table XI).

DISCUSSION

Although more acyl groups should be studied before a generalization is made, the observation that only compounds having one of the three acyl groups tested were active suggested that it was not the acylated configuration in general that caused the activation but, rather, the peculiarity of the chloroacetyl group. Indeed, while acetic acid and propionic acid, the hydrolytic products of two of these acyl compounds, were inactive in the test system, chloroacetic acid, the hydrolytic product of the third, showed remarkable activity (Table VIII). Neither acetyl nor propionyl groups, even when attached to known active antimetabolites such as *p*-fluorophenylalanine, showed any effect. In this connection, Fukushima and Toyoshima (12) observed tumor-inhibitory effects with certain propionyl amino acids.

Apparently, it is not the chloroacetyl moiety alone that contributes to the activity since not all chloroacetyl amino acids exhibit growth-inhibitory action (Table VIII). The nature of the amino acid moiety appears of some importance in the activation, because the chloroacetyl derivatives of only certain amino acids and amino acid analogs showed growth-inhibitory activity. Examination of Tables VIII and IX shows that, in general, the chloroacetyl derivatives of the amino acids considered essential or of the analogs of these essential amino acids were growth inhibitory[9]. Although chloroacetyl-L-alanine showed some activity, its activity appears to be questionable when compared on an equimolar basis with other active chloroacetyl amino acids (Table IX). However, the chloroacetyl derivatives of more amino acids, both of the essential and nonessential categories, should be studied before a more definite statement in this regard is made.

It appears that the activity is not related to an enzymatic hydrolytic release of chloroacetic acid, itself a remarkably active compound in this test system, since in some compounds showing activity the D-isomers were active to a degree. However, a racemase possibly could be present and be contributing to the activity of the D-isomers. But, to the knowledge of the authors, no such enzyme has been described in this test organism. The possibility of a nonenzymatic breakdown of the test compounds during preparation of the test solution or during incubation, causing a release of chloroacetic acid and the mother amino acid, was excluded in view of the observation that there was no increase of moieties containing free amino nitrogen in the test solution and in the control solution (Table XI), as determined by the Van Slyke nitrous acid determination.

It is possible that the inhibitory effect noted could be due to a small quantity of chloroacetic acid, or of amino acid moiety released, not detectable within the sensitivity of the nitrous acid method. However, this possibility is unlikely; the accuracy of the procedure, even with 0.05 mg of amino nitrogen present, was 1% (5). Moreover, even if the chloroacetyl compound is completely hydrolyzed, the degree of inhibition would be equivalent to the inhibition range noted for the test compound (Table IX).

The fact that both optical isomers were active as inhibitors in many of the active chloroacetyl compounds also suggests that, perhaps, a nonspecific physical property of the test compounds, such as solubility and permeability across the cell membrane, could partly be involved in the mechanism. However, this property could not be the sole mechanism since, although all four isomers of *N*-chloroacetyl-β-hydroxynorleucine were active, their activities were not equal, isomer D-B being appreciably more active than the other three isomers (2).

Although some amino acid analogs, *i.e.*, *p*-fluorophenylalanine and β-3-thienylalanine, showed about equal or somewhat higher activity than their corresponding chloroacetyl derivatives on a molar basis (calculated from Tables VI and VIII), mammalian studies using *p*-fluorophenylalanine (13) and β-3-thienylalanine (14–16) showed these compounds to be highly toxic. Although the chloroacetyl derivative in these cases

Table V—Effect of *N*-Propionyl Derivatives of Amino Acids and Amino Acid Analogs on Growth of *L. casei* 7469

N-Propionyl Derivative	Concentration, m*M*	Inhibition[a], %
L-Alanine	6.89	0
DL-Allylglycine	5.84	0
o-Fluoro-DL-phenylalanine	4.18	3
m-Fluoro-DL-phenylalanine	4.18	4
p-Fluoro-DL-phenylalanine	4.18	5
L-Leucine	5.34	0
DL-Norleucine	5.34	3
β-2-Thienyl-DL-alanine	4.40	3
β-2-Thienyl-DL-β-alanine	4.40	3
β-3-Thienyl-DL-alanine	4.40	9

[a] Growth in inoculated controls (water *in lieu* of test solution), measured turbidimetrically, was 190–210 Klett units. Concentration of test compounds was 1.0 mg/ml; the value given is the final concentration in the test system. For assay conditions, see text. For explanation of extent of variation of values, see footnote of Table IV.

Table VI—Effect of Amino Acids on Growth of *L. casei* 7469

Amino Acid	Concentration, m*M*	Inhibition[a], %
DL-Alanine	11.22	0
L-Aspartic acid	7.51	6
DL-Allylglycine	8.68	4
o-Fluoro-DL-phenylalanine	5.46	3
m-Fluoro-DL-phenylalanine	5.46	0
p-Fluoro-DL-phenylalanine	5.46	48
β-Hydroxy-DL-homomethionine A[b]	5.58	7
β-Hydroxy-DL-homomethionine B[b]	5.58	+3[c]
β-Hydroxy-DL-methoxinine A[b]	6.70	+1[c]
β-Hydroxy-DL-methoxinine B[b]	6.70	+1[c]
L-Isoleucine	7.62	0
DL-Leucine	7.62	+1
L-Leucine	7.62	−5
DL-Norleucine	7.62	+1[c]
DL-Norvaline	8.54	1
DL-Phenylalanine	6.05	+3
β-2-Thienyl-DL-alanine	5.84	8
β-2-Thienyl-DL-β-alanine	5.84	0
β-3-Thienyl-DL-alanine	5.84	36
L-Tryptophan	4.90	5
L-Valine	8.54	0

[a] Concentration of test compounds in the final assay system was 1.0 mg/ml. Growth of the inoculated control (water *in lieu* of test solution), measured turbidimetrically, was 190–210 Klett units. For assay conditions, see text. For explanation of extent of variation of values, see footnote of Table IV. [b] See text footnote 8 for explanation of diastereomeric designations. [c] The + indicates stimulation of growth.

[8] The diastereomeric forms are arbitrarily referred to as Isomers A and B, the former moving faster and the latter slower when chromatographed on paper using methyl ethyl ketone–1-butanol–ammonia–water (3:5:1:1) (*cf.*, 2).

[9] *N*-Chloroacetyl-L-threonine previously (1) showed no appreciable inhibition. The compound used for that experiment since then has become syrupy and could not be used for comparison under the present conditions.

Table VII—Effect of pH on Growth of *L. casei* 7469

Assay System[a]	pH	Inhibition[b], % 0.55 μmole/ml[c]	2.76 μmoles/ml[c]	5.52 μmoles/ml[c]
Water (inoculated control)	6.55	—	—	—
Test solution 1	7.01	0	18	64
Test solution 2	6.85	2	12	55
Test solution 3	6.52	0	0	0

[a] The assay system consisted of 2.5 ml of the test solution plus 2.5 ml of the medium. The inoculated control consisted of 2.5 ml of water *in lieu* of the test solution. The test solutions were as follows: 1, L-tyrosine, 20.0 mg, plus 1.10 ml of 0.1 *N* NaOH in a total volume of 10.0 ml; 2, L-isoleucine, 14.4 mg, plus 1.10 ml of 0.1 *N* NaOH in a total volume of 10.0 ml; and 3, L-isoleucine, 14.4 mg, plus 0.05 ml of 0.1 *N* NaOH in a total volume of 10.0 ml. [b] Growth of inoculated control, measured turbidimetrically, was 201 Klett units. [c] Final concentration of test solution in the assay system.

showed somewhat less activity than the corresponding nonacylated mother compounds, the administration of these antimetabolites as the chloroacetyl derivatives possibly may circumvent this undesirable property.

Table VIII—Effect of *N*-Chloroacetyl Derivatives of Amino Acids and Amino Acid Analogs on Growth of *L. casei* 7469[a]

N-Chloroacetyl Derivative	Concentration at 1 mg/ml, m*M*	Inhibition[b], % 0.1 mg/ml[c]	0.5 mg/ml[c]	1.0 mg/ml[c]
L-Alanine	6.04	2	5	14
D-Alanine	6.04	0	3	9
L-Aspartic acid	4.77	0	0	0
D-Aspartic acid	4.77	1	4	3
DL-Allylglycine	5.22	3	9	14
L-Allylglycine	5.22	2	8	16
D-Allylglycine	5.22	1	3	8
o-Fluoro-DL-phenylalanine	3.85	1	10	18
m-Fluoro-DL-phenylalanine	3.85	2	8	15
p-Fluoro-DL-phenylalanine	3.85	6	14	20
p-Fluoro-L-phenylalanine	3.85	6	14	25
p-Fluoro-D-phenylalanine	3.85	4	13	17
Glycine	6.60	4	6	10
β-Hydroxy-DL-homomethionine A	3.91	1	5	8
β-Hydroxy-DL-homomethionine B	3.91	+2[d]	+2[d]	3
β-Hydroxy-DL-methoxinine A	4.43	3	4	7
β-Hydroxy-DL-methoxinine B	4.43	+2[d]	2	11
DL-Isoleucine	4.82	3	14	20
L-Isoleucine	4.82	6	13	20
DL-Leucine	4.82	3	9	13
L-Leucine	4.82	3	11	15
D-Leucine	4.82	2	5	11
DL-Norleucine	4.82	3	12	20
L-Norleucine	4.82	6	14	22
D-Norleucine	4.82	1	7	14
DL-Norvaline	5.16	4	15	18
DL-Phenylalanine	4.14	0	10	20
L-Phenylalanine	4.14	5	10	19
DL-Serine	5.51	3	3	8
β-2-Thienyl-DL-alanine	4.04	10	16	25
β-2-Thienyl-L-alanine	4.04	9	19	34
β-2-Thienyl-D-alanine	4.04	1	13	23
β-2-Thienyl-DL-β-alanine	4.04	3	6	13
β-3-Thienyl-DL-alanine	4.04	3	10	24
L-Tryptophan	3.56	5	15	26
L-Tyrosine	3.88	+1	3	6
DL-Valine	5.16	6	12	18
L-Valine	5.16	6	10	18
Acetic acid[e]	16.65	1	1	4
Monochloroacetic acid[e]	10.58	11	34	54
Propionic acid[e]	13.50	1	2	3

[a] For details of assay procedure, see text. [b] Turbidity readings of the inoculated control tubes (containing no test compound) were 190–210 Klett units. For explanation of extent of variations of values, see footnote of Table IV. [c] Final concentration in assay system. [d] The + indicates stimulation of growth. [e] Free acids.

Table IX—Comparison of Effect of Equimolar Concentrations of *N*-Chloroacetyl Amino Acids and Amino Acid Derivatives on Growth of *L. casei* 7469[a]

N-Chloroacetyl Derivative	Inhibition[b], %
L-Alanine	9
DL-Allylglycine	16
L-Allylglycine	21
D-Allylglycine	9
o-Fluoro-DL-phenylalanine	21
m-Fluoro-DL-phenylalanine	19
p-Fluoro-DL-phenylalanine	16
p-Fluoro-L-phenylalanine	25
p-Fluoro-D-phenylalanine	14
β-Hydroxy-D-norleucine B	51
DL-Isoleucine	18
DL-Leucine	13
L-Leucine	18
D-Leucine	9
DL-Norleucine	20
L-Norleucine	22
D-Norleucine	9
DL-Norvaline	18
DL-Phenylalanine	20
β-2-Thienyl-DL-alanine	27
β-2-Thienyl-L-alanine	26
β-2-Thienyl-D-alanine	18
β-2-Thienyl-DL-β-alanine	15
L-Tryptophan	30
L-Valine	14
Monochloroacetic acid[c]	24
p-Fluoro-DL-phenylalanine[c]	35
β-3-Thienyl-DL-alanine[c]	20

[a] Maximum growth in inoculated control tube containing no test compound, measured turbidimetrically, was 198–205 Klett units. For explanation of extent of variation of values, see footnote of Table IV. [b] Concentration was 4.47 μmoles/ml and was the final concentration in the assay system. For details of assay procedure, see text. [c] Free acids.

The amino acid analogs selected in these experiments are known antimetabolites of the corresponding natural amino acids in certain selected systems (*cf.*, 17). For example, allylglycine is believed to be a metabolic antagonist of cysteine, norleucine of methionine, and the fluorophenylalanines of phenylalanine. The chloroacetyl derivatives of analogs that were believed to be antimetabolites of two different classes of antagonists were combined in a single assay system to note if additive or synergistic effects could be observed (Table X). The observation that there was no such effect, coupled with the observation that the activity of the inhibitory compounds (with the exception of *N*-chloroacetyl-D-β-hydroxynorleucine B) fell within a relatively narrow range (14–30%), suggests a common mode of inhibition among these compounds. It may be, for example, that because of sufficient structural similarity of the analogs to the natural amino acid, the difficulty of their metabolism lies not in the activation step of the protein synthetic pathway but in the subsequent utilization step of such activated amino acid analogs. The mode of inhibition by *N*-chloroacetyl-D-β-hydroxynorleucine B is probably different

Table X—Effect of Combination of Active Chloroacetyl Derivatives of Amino Acid Analogs on Growth of *L. casei* 7469

Compound	Inhibition[a], %
N-Chloroacetyl-β-hydroxy-D-norleucine B[b]	50
N-Chloroacetyl-*p*-fluoro-DL-phenylalanine	17
N-Chloroacetyl-β-hydroxy-D-norleucine B plus *N*-chloroacetyl-*p*-fluoro-DL-phenylalanine	53
N-Chloroacetyl-β-2-thienyl-DL-alanine	27
N-Chloroacetyl-β-hydroxy-D-norleucine B plus *N*-chloroacetyl-β-2-thienyl-DL-alanine	49
N-Chloroacetyl-DL-allylglycine	16
N-Chloroacetyl-β-hydroxy-D-norleucine plus *N*-chloroacetyl-DL-allylglycine	54
N-Chloroacetyl-DL-allylglycine plus *N*-chloroacetyl-β-2-thienyl-DL-alanine	30
N-Chloroacetyl-DL-norleucine	19
N-Chloroacetyl-DL-norleucine plus *N*-chloroacetyl-β-2-thienyl-DL-alanine	33

[a] Maximum growth of inoculated control tube, containing no test compound, measured turbidimetrically was 198 Klett units. [b] Concentration of each test compound in assay system was 4.47 μmoles/ml. For details of assay procedure, see text.

Table XI—Determination of Extent of Hydrolysis of *N*-Chloroacetyl-L-isoleucine during Incubation

System	Primary Amino Nitrogen[a], mg
1. Test solution[b], refrigerated 19 hr	0.002
2. Test solution, incubated at 37°, 19 hr	0.002
3. 2.5 ml of uninoculated medium plus 2.5 ml of water, refrigerated 19 hr[c]	0.298
4. 2.5 ml of uninoculated medium plus 2.5 ml of water, incubated at 37°, 19 hr	0.294
5. 2.5 ml of uninoculated medium plus 2.5 ml of test solution, refrigerated 19 hr	0.302
6. 2.5 ml of uninoculated medium plus 2.5 ml of test solution, incubated at 37°, 19 hr	0.305

[a] Determined by Van Slyke nitrous acid method (5). The volumes of solution taken for the analysis were 1.5 ml for Systems 1 and 2 and 1.0 ml for Systems 3–6. For the volumes taken, Systems 1 and 2 should yield 0.201 mg and Systems 3–6 should yield 0.368 mg of primary amino nitrogen when the test compound is completely hydrolyzed. For comments on sensitivity to method, see text. [b] Test solution was chloroacetyl-L-isoleucine, 20.0 mg, plus 0.96 ml of 0.1 *N* NaOH with the volume made to 10.0 ml with water; pH of the test solution was 6. [c] The pH of the assay system (2.5 ml of medium plus 2.5 ml of test solution) was 6.52.

from that of the other chloroacetyl derivatives that showed inhibition in the 15–30% range. In the former, for example, the isomer of chloroacetyl-β-hydroxynorleucine showing the greatest activity was of the D-configuration. However, it is difficult to deduce the mechanism of inhibition in experiments of this type since the system is too complex.

In view of the differences in metabolic behavior between the free amino acids and the *N*-chloroacetylated derivatives, it might prove useful to test these derivatives in suitable mammalian tumor systems; this testing is now being done.

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Thermal and Photolytic Decomposition of Methotrexate in Aqueous Solutions

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Received March 10, 1977, from the *Pharmacy Department, The Clinical Center, National Institutes of Health, Bethesda, MD 20014.* Accepted for publication August 4, 1977.

Abstract □ The chemical kinetics of thermal and photolytic degradation of methotrexate in aqueous solutions were studied. At above pH 7 and 85°, methotrexate hydrolyzed to yield mainly N^{10}-methylpteroylglutamic acid. The hydrolysis rate followed first-order kinetics with respect to methotrexate concentration and increased rapidly above pH 9. When methotrexate solutions were kept under laboratory fluorescent light, the major degradation products were 2,4-diamino-6-pteridinecarbaldehyde, 2,4-diamino-6-pteridinecarboxylic acid, and *p*-aminobenzoylglutamic acid. The photolytic reaction followed zero-order kinetics with respect to methotrexate concentration and was catalyzed by bicarbonate ion. Evidence is presented for a proposed sequential cleavage of methotrexate by a free radical mechanism for the photolysis. Commercial parenteral methotrexate was found to be quite stable as marketed when stored in the original vial at room temperature.

Keyphrases □ Methotrexate—thermal and photolytic decomposition in aqueous solutions □ Thermal decomposition—methotrexate in aqueous solutions □ Photolytic decomposition—methotrexate in aqueous solutions □ Decomposition—thermal and photolytic, methotrexate in aqueous solutions □ Stability—methotrexate in aqueous solutions □ Antineoplastic agents—methotrexate, thermal and photolytic decomposition in aqueous solutions

Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid), a widely used antileukemic agent, is often administered in the form of an aqueous parenteral solution. Although it is recognized that methotrexate decomposes in light and alkaline solutions, the chemical kinetics of methotrexate decomposition have not been reported.

Since commercially available methotrexate is only about 86% pure (1) and contains several impurities (2, 3), it is important to know whether methotrexate degradation accounts for some of the impurities. Furthermore, it is important to ascertain the stability of commercial methotrexate when it is diluted in large-volume intravenous solutions. The present study was undertaken to elucidate the decomposition rate of aqueous solutions of methotrexate under fluorescent light and at high temperatures and to identify the degradation products. Chemical kinetic