Antineoplastic Agents III: Effects of Dibromoethyl and Vinyl Esters of *N*-Benzyloxycarbonyl-L-phenylalanine on Ehrlich Ascites Tumor Cell Metabolism

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Abstract □ Evidence is presented that *N*-benzyloxycarbonyl-L-phenylalanine vinyl ester and 1,2-dibromoethyl ester are inhibitors of Walker 256 carcinosarcoma and Ehrlich ascites carcinoma tumor growth. The major effects of these two agents on Ehrlich ascites cell metabolism were the inhibition of deoxyribonucleic acid and protein synthesis and the alteration of cellular regulatory processes controlling cytokinetics. Deoxynucleotide (purine) kinase enzymes appeared to be the focal site for inhibition of deoxyribonucleic acid synthesis with marginal inhibition of thymidylate synthetase activity. Cyclic adenosine monophosphate levels were elevated by drug treatment whereas chromatin protein phosphorylation, cell respiration, and lysosomal activities were inhibited. *N*-Benzyloxycarbonyl-L-phenylalanine 1,2-dibromoethyl ester was a latent *in vitro* chymotrypsin inhibitor. Some preliminary evidence suggests that these activated esters may inhibit cellular enzymatic activity by alkylating imidazole and lysine residues of proteins.

Keyphrases □ *N*-Benzyloxycarbonyl-L-phenylalanine esters—effect on tumor cell metabolism in mice □ Phenylalanine esters, substituted—effect on tumor cell metabolism in mice □ Antineoplastic activity—substituted phenylalanine esters, effect on tumor cell metabolism in mice □ Metabolism, tumor cell—effect of substituted phenylalanine esters in mice □ Structure–activity relationships—effect of substituted phenylalanine esters on tumor cell metabolism in mice

The antitumor activity of *N*-protected vinyl, 1,2-dibromoethyl, and cyanomethyl esters of amino acids was reported previously (1, 2). Of this series of 50 compounds, the *N*-benzyloxycarbonyl-L-phenylalanine analogs were the most active in the Walker 256 carcinosarcoma and Ehrlich ascites screens. These compounds are structurally related to tosylphenylalanyl chloromethyl ketone (I), which effectively inhibited carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene and growth of Swiss SV-3T3 transformed tissue culture cells (3–6).

At that time, it was postulated that I may inhibit the acquired proteolytic activity associated with cell transformation and increased malignant invasiveness of neoplastic tissue. However, I then was shown to inhibit protein synthesis of tumor cells (7). *N*-Benzyloxycarbonyl-L-phenylalanine vinyl ester (II) and *N*-benzyloxycarbonyl-L-phenylalanine 1,2-dibromoethyl ester (III) were investigated for their effects on tumor cell metabolism, and the preliminary results are now reported.

EXPERIMENTAL

Chemical Synthesis—N-*Benzyloxycarbonyl-L-phenylalanine Vinyl Ester (II)*—The starting material, *N*-benzyloxycarbonyl-L-phenylalanine (IV), was prepared according to the procedure of Bergman and Zervas (8). A mixture of 1.5 g (4 mmoles) of IV, 20 ml of vinyl acetate, and 20 mg of palladium chloride–sodium chloride was refluxed (70–75°) for 5 hr. After cooling, 34 mg of activated charcoal was added in one portion and the mixture was stirred for 5 min. The reaction mixture was filtered, and solvents were removed *in vacuo*. The residue was dissolved in 10 ml of vinyl acetate, then 20 mg of palladium chloride–sodium chloride was added, and the mixture was refluxed for 5 hr.

After the mixture was cooled to room temperature, the procedure was repeated. When the reaction was complete, as demonstrated by TLC, purification of the product was carried out by column chromatography on silica gel [benzene–chloroform (1:1)]. A colorless oily product was obtained in a 75–80% yield; $[\alpha]_D^{24}$ −11.0° (c in $CHCl_3$).

Anal.—Calc. for $C_6H_{16}N_2O_3$: C, 70.13; H, 5.19; N, 9.02. Found: C, 70.07; H, 5.27; N, 9.01.

N-*Benzyloxycarbonyl-L-phenylalanine 1,2-Dibromoethyl Ester (III)*—To a solution of 0.97 g (3 mmoles) of II in 45 ml of benzene, a solution of 0.47 g (3 mmoles) of bromine in 40 ml of benzene was added dropwise over 30 min with vigorous stirring. The reaction mixture was stirred for an additional 3 hr at room temperature, and then the solvent was removed under high vacuum. The product was impure, as shown by TLC with chloroform–ethyl acetate (2:1), and was purified by column chromatography on silica gel eluted with chloroform–ethyl acetate (2:1).

Further purification was carried out by recrystallization from chloroform and low boiling ligroin to yield 1.05 g of product (70–75%), mp 78–80°; $[\alpha]_D^{24}$ −33.5° (c in CH_3OH); IR: 1775 (ester carbonyl) cm^{-1}; NMR: δ 4.05 (2H, d, CH_2Br) and 6.8 (1H, t, OCHBr) ppm.

Anal.—Calc. for $C_{19}H_{19}NO_4$: C, 47.20; H, 3.93; Br, 32.71; N, 2.89. Found: C, 47.00; H, 3.95; Br, 32.88; N, 2.88.

L-Phenylalanine Vinyl Ester Hydrochloride (V)—This compound was prepared by a modification of a reported procedure (9). Trifluoroacetic acid (10 ml) was added gradually at −15° to a mixture of 1.89 g (5 mmoles) of II and 2 ml of anisole. Upon completion of the reaction, the mixture was kept at room temperature for 20 min and then refluxed in an oil bath (70–75°) for 20 min. Most of the trifluoroacetic acid was then distilled off *in vacuo*, and the remaining oil was dissolved in absolute ether. The ester hydrochloride was precipitated from this solution by introduction of gaseous hydrogen chloride at −10°. The yield was 0.95 g (70–75%), mp 155–157° [lit. (9) mp 159–160°].

Tosylphenylalanyl chloromethyl ketone (I), benzaldehyde (VI), acetaldehyde (VII), phenylalanine mustard (VIII), mercaptopurine (IX), fluorouracil, and iodoacetate were purchased.

***In Vivo* Tumor Screens**—In the Ehrlich ascites screen, 10^6 cells were implanted on Day 0. Test compounds were suspended in 0.05% polysorbate 80–water and homogenized to obtain a fine suspension. Each compound was injected intraperitoneally (1 mg/day; *i.e.*, 33.3 mg/kg) into CF_1 male mice (~30 g). On the 7th day, the mice were sacrificed; the total volumes of ascites fluid and packed cells (ascites-crit) were determined, and the percentage inhibition was calculated (1). Phenylalanine mustard and mercaptopurine were used as positive controls.

In the Walker 256 carcinosarcoma screen, 10^6 tumor cells were implanted intraperitoneally into Sprague–Dawley male rats (75 ± 10 g). Test compounds were injected intraperitoneally at 2.5 mg/kg/day, and the day of death was recorded. Treated/control (T/C) values were calculated for the average survival time of each group according to the National Institutes of Health protocol (10). The LD_{50} values were determined in CF_1 male mice by the Litchfield and Wilcoxon method (11).

Macromolecular Synthesis—Unless otherwise stated, all biochemical studies were carried out on 8-day Ehrlich ascites tumor-bearing CF_1 male mice treated with I–III at a subacute dose of 16 mg/kg/day on Days 5–7. ^{14}C-Thymidine incorporation into deoxyribonucleic acid was determined by the method of Chae *et al.* (12). One hour prior to sacrifice of the animal on Day 8, 10 μCi of [*methyl*-^{14}C]thymidine (54 mCi/mmole) in 0.1 ml of isotonic sodium chloride was injected intraperitoneally. The deoxyribonucleic acid was isolated, and the ^{14}C-content was determined by placing an aliquot in 10 ml of scintillation fluid (two parts toluene, one part octoxynol, 0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene).

0022-3549/ 78/ 1200-1726$01.00/ 0
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Table I—Effects of *N*-Benzyloxycarbonyl-L-phenylalanine Esters on Tumor Cell Growth

Compound	n^a	Ehrlich Ascites Tumor, CF$_1$ Male Mice, 33.3 mg/kg/day: Survival at Day 7	Ascites-Crit	Volume	Inhibition, %	Walker 256 Carcinosarcoma, Sprague–Dawley Rats, 2.5 mg/kg/day: Average Days Survived	T/C	LD$_{50}$, CF$_1$ Mice, mmoles/kg
I[b]	6	5/6	0.05	0.01	100.0	—	—	0.21
II	6	6/6	0.40	0.10	99.9	18.1	226	6.15
III	6	6/6	1.40	0.01	100.0	23.0	305	0.15
IV	6	6/6	4.70	0.60	75.0	—	—	0.84
V	6	4/6	27.0	2.20	41.0	—	—	—
VI[c]	6	6/6	49.0	0.80	69.0	—	—	—
VII[d]	6	5/6	27.3	2.04	43.0	—	—	—
VIII[e]	6	6/6	3.00	0.10	99.0	23.0	305	—
IX[f]	6	6/6	0.30	0.70	99.6	—	—	—
X[g]	6	6/6	32.75	41.0	—	7.5	—	—

[a] Number of animals in group. [b] Cyclo chemical. [c] Matheson, Coleman and Bell. [d] Aldrich. [e] 4-[Bis(2-chloroethyl)amino]phenylalanine, Burroughs Wellcome Co. [f] Calbiochem. [g] Water–0.05% Tween 80.

The deoxyribonucleic acid concentration per aliquot was determined by UV spectrophotometry at 260 nm. Uridine incorporation into ribonucleic acid was determined in an analogous manner with 10 μCi of 5-^{3}H-uridine (26.2 Ci/mmole). Ribonucleic acid was extracted by the method of Shibko *et al.* (13). Leucine incorporation into protein was determined by the method of Sartorelli (14) with 1 μCi of 1-^{14}C-leucine (56.9 mCi/mmole). *In vitro* incorporation of 1-^{14}C-acetic acid (57 mCi/mmole) into cholesterol of 8-day Ehrlich ascites cells was determined by the assay of Haven *et al.* (15), and cholesterol was extracted by the method of Wada *et al.* (16) with 6 μmoles of I–III.

Enzymatic Studies—Nuclear deoxyribonucleic acid polymerase activity was determined on washed (three times) isolated nuclei from 8-day Ehrlich ascites cells, prepared by the method of Hymer and Kuff (17). The incubation medium was that described by Sawada *et al.* (18), except that 2-^{14}C-deoxyribothymidine triphosphate (45 mCi/mmole) was used and the insoluble nucleic acids were collected on glass fiber paper GF/F by vacuum suction. Thymidylate synthetase activity was determined by the method of Kampf *et al.* (19) with a postmitochondrial supernate (9000$\times g$) from 8-day Ehrlich ascites cells, 1.5 μmoles of test compound, and 5 μCi of 5′-^{3}H-deoxyuridine monophosphate (11 Ci/mmole).

Deoxythymidylate kinase, deoxyguanylate kinase, deoxyadenylate kinase, and deoxythymidylate diphosphate kinase activities were determined by the method of Maley and Ochoa (20) with 1.5 μmoles of test compound. Nucleotide kinase activity was based on the disappearance of 0.1 μmole of reduced nicotinamide adenosine dinucleotide at 340 nm for 20 min. Deoxyribonuclease activity was determined by the method of Hall *et al.* (21) with 1.5 μmoles of test compound.

Nonhistone chromatin (3′,5′-cyclic monophosphate dependent) protein kinase activity was determined on isolated nuclei (17) by the method of Kish and Kleinsmith (22) with 1.5 μmoles of test compounds. Chromatin protein was collected on nitrocellulose membrane filters. 3′,5′-Cyclic adenosine monophosphate levels were determined[1] on 10^6 8-day tumor cells (23). Phosphorus incorporation into histones was determined by the method of Rainer *et al.* (24). One hour prior to sacrifice, 10 μCi of γ-^{32}P-adenosine 5′-triphosphate (8.23 Ci/mmole) was injected intraperitoneally. The nuclear fraction was isolated, and the histones were extracted and counted.

Cathepsin protease activity (pH 5) was determined on 8-day Ehrlich ascites cells by the method of Cho-Chung and Guillino (25). Since I is a chymotrypsin inhibitor, the possibility also existed that *N*-benzyloxycarbonyl-L-phenylalanine esters inhibited chymotrypsin activity. The inhibition of protease activity by II and III was followed with a pH-stat with *N*-acetyl-L-tyrosine ethyl ester ($K_m = 0.83 \times 10^{-3}$ *M*) as the substrate (26). The preincubation medium at 25° contained 0.1 *M* $CaCl_2$, 0.1 *M* tris(hydroxymethyl)aminomethane (pH 7.8), and 50 μmoles of II or III in an ethanolic solution. The reaction was started by adding α-chymotrypsin (in 0.001 *N* HCl) to a final concentration of 3.2 μmoles. At various times (0–390 min), a 4.5-ml aliquot was removed to determine the hydrolysis rate of the substrate by titrating to pH 7.8 with 0.01 *N* NaOH. A Lineweaver–Burke plot was employed to determine chymotrypsin inhibition by II or III.

The *in vitro* aerobic respiration of Ehrlich ascites tumor cell homogenates with succinate, dependent on a flavin adenine dinucleotide-linked dehydrogenase, as the substrate in the presence and absence of adenosine diphosphate was measured in the presence of 0.6 μmole of test compound (27).

[1] Schwarz/Mann radioimmunoassay kit.

UV Spectrophotometric and NMR Studies—UV spectrophotometric binding studies were performed with 75 μg of deoxyribonucleic acid or deoxyguanosine monophosphate, 0.15 μmole of imidazole, or 2 μmoles of lysine after incubation with II, III, VI, or VII in concentrations of 1–5 μmoles in 0.1 *M* phosphate buffer (pH 7.2) over 190–360 nm for 24 hr (23).

Clear solutions for NMR studies were obtained by homogenizing benzaldehyde (0.094 mmole) with imidazole (0.94 mmole) in deuterated chloroform containing 1% tetramethylsilane and adjusting the pH to 6. Benzaldehyde (0.188 mmole) and L-cysteine (0.188 mmole) were dissolved in deuterated water with 1% 3-(trimethylsilyl)-1-propanesulfonic acid, and the pH was adjusted to 9. Benzaldehyde (0.188 mmole) and imidazole (0.188 mmole) were homogenized in deuterated chloroform containing 1% tetramethylsilane. Compound II (0.061 mmole) and imidazole (0.147 mmole) were incubated in deuterated methanol containing 1% tetramethylsilane (pH 6.0).

Compound II (0.061 mmole) and lysine (0.061 mmole) were homogenized in deuterated methanol and deuterated water (pH 11.0) with 1% 2,2-dimethyl-2-silapentane-5-sulfonate as the standard. Compound II (0.061 mmole) and L-cysteine (0.061 mmole) were homogenized in deuterated chloroform with 1% tetramethylsilane (pH 8.0). Compound III (0.092 mmole) was incubated with imidazole (0.147 mmole) (pH 6.0) in deuterated chloroform with 1% tetramethylsilane (pH 6.0). Compound III (0.092 mmole) and lysine (0.092 mmole) were homogenized in deuterated chloroform containing 1% tetramethylsilane and deuterated water (pH 10). Compound III (0.092 mmole) and L-cysteine (0.092 mmole) were reacted in deuterated chloroform containing 1% tetramethylsilane (pH 8.0).

RESULTS

Compounds I–III significantly inhibited Ehrlich ascites tumor cell growth. Compounds II and III increased the survival of Walker 256 carcinosarcoma-bearing rats two and three times, respectively (Table I).

Subacute doses of II and III on Days 5–7 inhibited deoxyribonucleic acid synthesis 57 and 49%, respectively. Ribonucleic acid synthesis and content were unaffected by the drug treatment. Protein synthesis was reduced 53% by II and 50% by III. Cholesterol synthesis was inhibited 25% by II and III. Interference with macromolecular synthesis also was reflected in the deoxyribonucleic acid content per milliliter of ascites fluid in that II reduced this content by 85% and III reduced it by 83%. The protein content per milliliter of ascites fluid was lowered by II to 53% and by III to 45% (Table II). The number of ascites cells per milliliter for the control was 233×10^6; treatment with II resulted in 70.4×10^6 cells/ml, and treatment with III resulted in 83.2×10^6 cells/ml.

Initial studies on nuclear deoxyribonucleic acid polymerases demonstrated that treatment with II and III did not reduce significantly the activity of these enzymes. *In vitro* studies of thymidylate synthetase activity showed that II and III inhibited this enzyme 21 and 27%, respectively. Other sites required for deoxyribonucleic acid synthesis also were examined. Deoxythymidylate kinase activity was inhibited 31 and 37%, respectively. Deoxythymidylate diphosphate kinase activity was reduced 20 and 42% by II and III, respectively. Deoxyguanylate kinase activity was suppressed 51 and 53% by II and III, respectively, and deoxyadenylate kinase activity was reduced 46 and 66%, respectively (Table III). The proteolytic inhibitor I significantly reduced these deoxyribonucleotide kinase enzymes also.

Hydrolytic enzymatic activity measured as cathepsin (pH 5) was significantly reduced by I–III. Deoxyribonuclease activity was marginally

Table II—Effects of *N*-Benzyloxycarbonyl-L-phenylalanine Esters on Macromolecular Synthesis of 8-Day Ehrlich Ascites Tumor Cells

		Percent Control, Mean ± *SD*					
Compound[a]	n[b]	^{14}C-Thymidine Incorporation into Deoxyribonucleic Acid	Deoxyribonucleic Acid, mg/ml	^{3}H-Uridine Incorporation into Ribonucleic Acid	^{14}C-Leucine Incorporation into Protein	Protein, mg/ml	^{14}C-Acetic Acid Incorporation into Cholesterol
Control	8	100 ± 26[c]	100 ± 25[d]	100 ± 3[e]	100 ± 5[f]	100 ± 5[g]	100 ± 4[h]
II	6	43 ± 21[i]	15 ± 4[i]	81 ± 25	47 ± 25[i]	46 ± 8[i]	75 ± 8[j]
III	6	51 ± 18[i]	17 ± 5[i]	108 ± 12	50 ± 19[i]	55 ± 9[i]	75 ± 12[j]

[a] One milliliter of control ascites fluid contained 158 × 10^6 cells/ml. Compound II contained 70.4 × 10^6 cells/ml, and III contained 83.2 × 10^6 cells/ml. [b] Number of animals in group. [c] Control value of 263.948 dpm/mg of deoxyribonucleic acid. [d] Control value of 1.3 mg/ml of ascites fluid. [e] Control value of 21,975 dpm/mg of ribonucleic acid. [f] Control value of 814 dpm/mg of protein. [g] Control value of 6.32 mg/ml of ascites fluid. [h] Control value of 132,809 dpm/mg of protein. [i] $p = 0.001$ determined by Student *t* test (62). [j] $p = 0.010$.

Table III—Effects of *N*-Benzyloxycarbonyl-L-phenylalanine Esters on Enzymes Required in Deoxyribonucleic Acid Synthesis of 8-Day Ehrlich Ascites Tumor Cells

		Percent Control Activity, Mean ± *SD*					
Compound	n[a]	Thymidylate Synthetase, dUMP → dTMP	Deoxythymidylate Kinase, dTMP → dTDP	Deoxythymidylate Diphosphate, dTDP → dTTP	Deoxyguanylate Kinase, dGMP → dGDP	Deoxyadenosylate Kinase, dAMP → dADP	Nuclear Deoxyribonucleic Polymerase
Control	8	100 ± 3[b]	100 ± 10[c]	100 ± 11[d]	100 ± 16[e]	100 ± 13[f]	100 ± 18[g]
I	5	81 ± 5	58 ± 12[h]	40 ± 8[h]	45 ± 9[h]	80 ± 8[i]	123 ± 32
II	6	79 ± 7[h]	69 ± 14[h]	80 ± 9[j]	49 ± 8[h]	54 ± 5[h]	95 ± 17
III	6	73 ± 5[h]	67 ± 8[h]	58 ± 14	47 ± 11[h]	34 ± 10[h]	—
VI	4	—	52 ± 9[h]	83 ± 11	43 ± 12[h]	34 ± 12[h]	—
Fluorouracil	5	39 ± 7[h]	—	—	—	—	—
Iodoacetate	5	—	—	—	—	—	41 ± 4[h]

[a] Number of animals in group. [b] Control value of 103,328 dpm/mg of protein. [c] ΔO.D. unit = 0.413/mg of protein. [d] ΔO.D. unit = 0.557/mg of protein. [e] ΔO.D. unit = 0.314/mg of protein. [f] ΔO.D. unit = 0.456/mg of protein. [g] Control value of 47,424 dpm/mg of DNA. [h] $p = 0.001$. [i] $p = 0.025$. [j] $p = 0.010$.

Table IV—Effects (Mean ± *SD*) of *N*-Benzyloxycarbonyl-L-phenylalanine Esters on 8-Day Ehrlich Ascites Tumor Cell Metabolism

		Hydrolytic Enzyme Activity					
Compound	n[a]	Cathepsin	Deoxyribonuclease	3′,5′-Cyclic Adenosine Monophosphate Level	Nonhistone Chromatin Protein Kinase Activity	^{32}P-Incorporation into Nuclear Histones	Milligrams of Chromatin Protein per Milliliter
Control	6	100 ± 32[b]	100 ± 8[c]	100 ± 14[d]	100 ± 3[e]	100 ± 18[f]	100 ± 13[g]
I	6	6 ± 6[h]	88 ± 8[i]	—	39 ± 2[h]	—	—
II	6	10 ± 7[h]	73 ± 4[h]	164 ± 11[h]	42 ± 7[h]	48 ± 9[h]	74 ± 13[j]
III	6	20 ± 20	83 ± 13[i]	133 ± 7[h]	49 ± 6[h]	43 ± 12[h]	71 ± 16[j]

[a] Number of animals in group. [b] Control value of 435 μg of protein hydrolyzed per 30 min/mg of protein. [c] Control value of 35 μg of deoxyribonucleic acid hydrolyzed per 30 min/mg of protein. [d] Control value of 3.48 pmoles/10^6 cells. [e] Control value of 97,238 dpm/mg of protein. [f] Control value of 7989 dpm/mg of crude histone protein. [g] Control value of 1.20 mg/ml of ascites fluid. [h] $p = 0.001$. [i] $p = 0.050$. [j] $p = 0.010$.

suppressed by I–III (Table IV). 3′,5′-Cyclic adenosine monophosphate levels were elevated in tumor cells 64% by II and 33% by III. Nonhistone chromatin protein kinase activity, which requires 3′,5′-cyclic adenosine monophosphate for regulation, was suppressed 61% by I, 58% by II, and 51% by III. ^{32}P-Incorporation into crude histones was reduced 52% by II and 57% by III. Chromatin protein also was reduced 26% by II and 29% by III, reflecting the inhibition of protein synthesis observed earlier (Table IV).

Compounds I and III significantly reduced oxidative phosphorylation processes (state 3) of Ehrlich ascites cells. Basal respiration, electron transport system (state 4), was reduced significantly by I and III and uncoupled in the case of II (Table V). These results indicate that I and III interfered with aerobic glycolysis of tumor cells.

Compound II demonstrated no inhibitor activity for the enzyme chymotrypsin. Rather, II appeared to be a reasonably good substrate for chymotrypsin enzymatic activity, resulting in a K_m value of 2.05 × 10^{-3} as compared to 0.8 × 10^{-3} for *N*-acetyl-L-tyrosine ethyl ester. On the other hand, chymotrypsin showed no ability to hydrolyze III. Preincubation periods of longer than 30 min resulted in significant inhibition by III of chymotrypsin activity with the substrate *N*-acetyl-L-tyrosine ethyl ester (Table VI).

UV spectrophotometric studies indicated that there was no interaction between deoxyribonucleic acid or the purine nucleophile guanosine and II, III, VI, and VII. However, when benzaldehyde (VI) was incubated with imidazole, the UV absorption magnitude of benzaldehyde was reduced 50% at 248 nm. Imidazole's maximum peak was at 206 nm, and benzaldehyde had a second peak at 202 nm. This second peak of benzaldehyde also was suppressed in magnitude from the anticipated absorption of two species in solution. Compounds II and III had maximum absorption peaks at 207 and 206 nm, respectively. Interaction with imidazole again resulted in reduction of the expected magnitude of these two independent molecules in solution. When benzaldehyde was incubated with lysine, there

Table V—Effects (Mean ± *SD*) of *N*-Benzyloxycarbonyl-L-phenylalanine Esters of 8-Day Ehrlich Ascites Homogenate Respiration with Succinate as Substrate

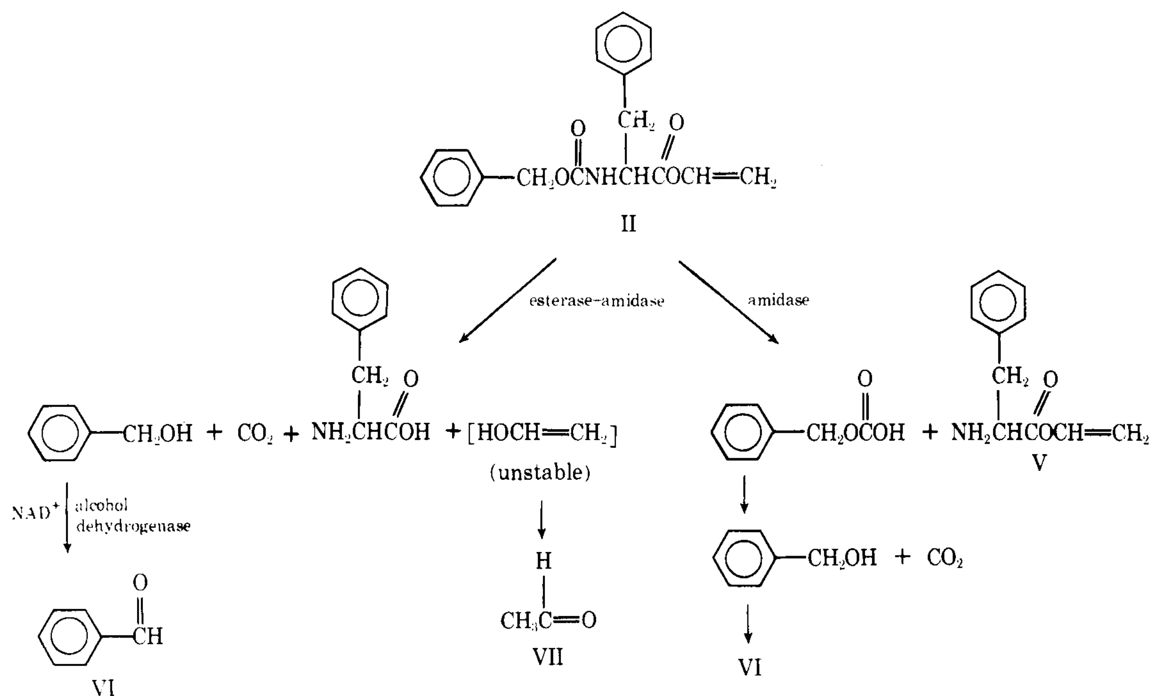
Compound	n[a]	State 4 Basal Respiration	State 3 Adenosine Monophosphate Stimulated Respiration
Control	6	100 ± 10[b]	100 ± 8[c]
I	6	69 ± 12[d]	62 ± 12[d]
II	6	129 ± 16[e]	40 ± 16[d]
III	6	74 ± 14[e]	70 ± 5[d]
VI	6	72 ± 11[e]	63 ± 6[d]

[a] Number of animals per group. [b] Control value of 5.506 μl of O_2/hr/mg of protein. [c] Control value of 6.487 μl of O_2/hr/mg of protein. [d] $p = 0.001$. [e] $p = 0.005$.

Table VI—Effects of III on Hydrolysis of *N*-Acetyl-L-tyrosine Ethyl Ester by Chymotrypsin

Preincubation Time, hr	Percent of Original Activity[a] (V/V_0 × 100)
0	100
1	90
2	48
3	25
4	17
6	5

[a] The initial velocity of the enzyme at zero time and in the absence of the inhibitor for substrate was expressed as V_0 (100% activity), where V represents values of enzymatic activity after specific incubation time. The velocity of the control was not altered over the 6-hr period.



Scheme I

was a reduction of 25% of the benzaldehyde peaks at 240 and 202 nm. Compound II, when incubated with lysine, resulted in a shift of the absorption peak from 207 to 198 nm with a drastic increase in magnitude.

Under the conditions used, NMR studies afforded no evidence that II, III, or VI interacted in any way with the nucleophilic sulfhydryl groups of L-cysteine. Compound VI interacted with lysine, showing clear evidence of a Schiff base formation between the lysine ϵ-amino group and the aldehyde function; *i.e.*, a methinyl (CH=N) proton appeared at δ 8.5 ppm along with a reduction in the intensity of the aldehyde absorption at δ 9.9 ppm. The NMR spectrum of III also was altered in the presence of lysine; however, distinction between amine alkylation and simple dehydrobromination and/or decomposition of III was not possible.

There was no evidence for the interaction of II and lysine under the conditions of incubation. Some spectral changes were observed during the interaction of benzaldehyde and imidazole; however, no assignments were possible. The vinyl ester showed no alteration when incubated with imidazole. However, III appeared to alkylate imidazole through the involvement of the terminal (primary) bromine atom. Changes in the spectrum involved a shift of the absorption assigned to the CH_2Br grouping from δ 3.9 to 3.1 ppm while the absorption of the OCH(Br) was only slightly altered in its splitting pattern but not its chemical shift.

Probable (p) significant difference was determined by the Student t test. Data are expressed in Tables II–V as percent of control and standard deviation; n equals the number of animals per group.

DISCUSSION

Establishment of a molecular mechanism of action for II and III would be premature based on these data. These analogs probably are acted on by tissue kinases, esterases, and proteases once they are introduced into the body. Previous studies indicated that an N-benzyloxycarbonyl-L-phenylalanine ether analog is not active (1). Biotransformation according to Schemes I and II would result in the generation of a number of aldehyde derivatives. Benzaldehyde (VI) has been shown to inhibit Ehrlich tumor growth 69%. N-Benzyloxycarbonyl-L-phenylalanine (IV) was slightly higher in inhibitory activity, *i.e.*, 75%. Since L-phenylalanine is actively transported across membranes, this moiety may facilitate the movement of the benzaldehyde into the tumor cells. L-Phenylalanine vinyl ester (V) also inhibited tumor growth by 41%, which was of the same magnitude as acetaldehyde (VII), 43%. Compounds V and VII are extremely unstable in solution. The low antitumor activity may be due to drug decomposition.

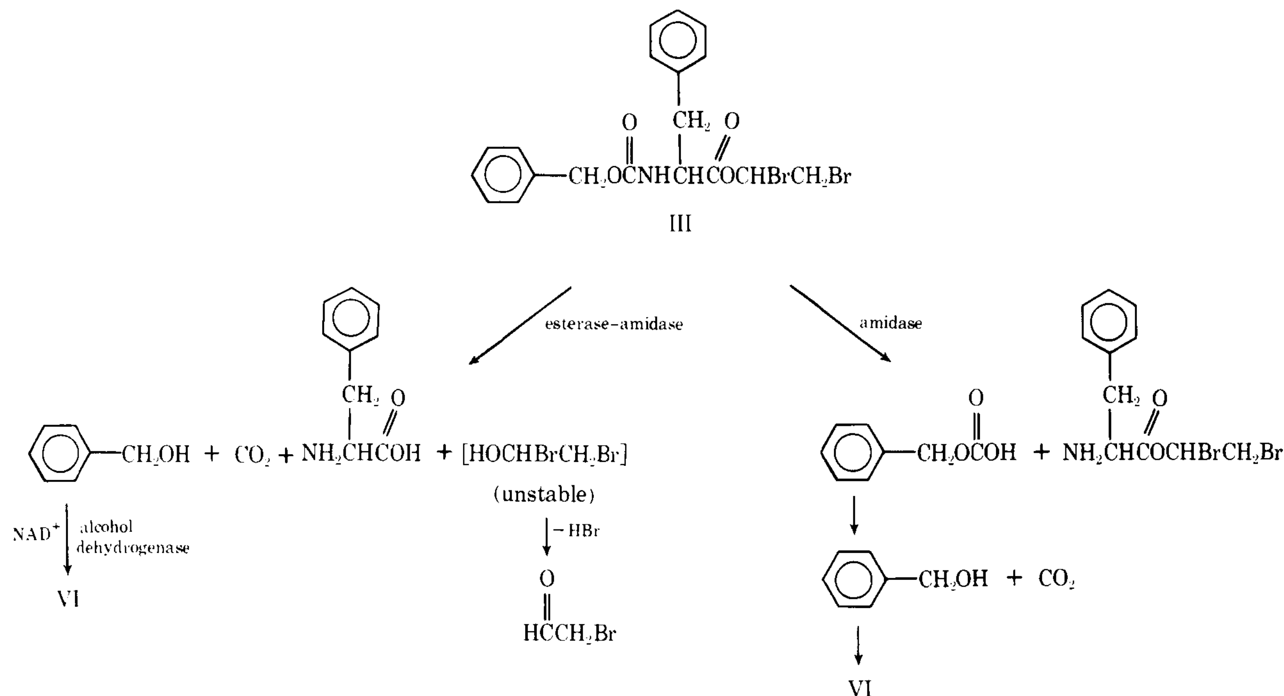
Compounds VI and VII would be converted quickly to the acid analogs when administered *in vivo*, possibly resulting in less activity than anticipated. However, if these metabolites were released in the tumor cells or in close proximity of the tumor cells, they might be more active.

The major effects of II and III on Ehrlich ascites tumor cells were on deoxyribonucleic acid and protein metabolism. Since neither proteolytic nor deoxyribonuclease activity was accelerated by II and III treatment, increased catabolism of protein or deoxyribonucleic acid could not account for the reduced incorporation of labeled precursors into these macromolecules. A number of enzymes required for deoxyribonucleic acid synthesis were examined, and the kinases required to phosphorylate deoxynucleotides to the di- and triphosphate forms were inhibited by the same magnitude as thymidine incorporation into deoxyribonucleic acid. Thymidylate synthetase was inhibited marginally, and nuclear deoxyribonucleic acid polymerase was unaffected by II and III. Both of these enzymes are sulfhydryl bearing.

Furthermore, II and III did not significantly inhibit ribonucleic acid synthesis. Since both polymerase activity and ribonucleic acid synthesis require an intact template of exposed deoxyribonucleic acid to function, the nucleic acids themselves appear unharmed by treatment with II and III. This result was confirmed by the spectrophotometric studies on nucleic acid. Thus, the major effect of II and III on tumor cell metabolism involves inhibition of specific enzymes required for deoxyribonucleic acid synthesis, *e.g.*, nucleotide kinases, and cellular regulatory processes that affect cytokinetics. For a cell to enter into the cell cycle and accelerate nucleic acid synthesis (Go → S), a number of preliminary events occur, *e.g.*, increased proteolytic activity on the cell membrane surface (28), decreased cyclic adenosine monophosphate, increased cyclic guanosine monophosphate (29), phosphorylation of the lysine-rich F-1 fraction of histones, and synthesis of enzymes required for nucleic acid synthesis such as deoxyribonucleic acid polymerases (29).

Treatment with II and III reduced protein synthesis and chromatin protein content. Other protein inhibitors, *e.g.*, hydroxyurea and cycloheximide, inhibited the phosphorylation of histones, regulatory proteins of gene activity (30). Phosphorylation of the F-1 (H-1) fraction of histones during G_1 by a protein kinase dependent on high cyclic guanosine monophosphate and low cyclic adenosine monophosphate concentrations was dependent on the γ-phosphorus of adenosine 5′-triphosphate derived from oxidative phosphorylation in the mitochondria (31–34). Various structurally nonrelated antitumor agents including methotrexate inhibited respiration and energy production by Ehrlich ascites cells (35–38). Protein inhibitors also inhibited respiration (39). Compounds I–III inhibited phosphorylation of histones and oxidative phosphorylation (state 3), processes of Ehrlich ascites cells, and thus reduced the energy available for synthesis.

Inhibition of histone phosphorylation could also be due to the elevation of cyclic adenosine monophosphate concentration after treatment with II and III. Increased levels of cyclic adenosine monophosphate in cancer cells, according to the Ying-Yang theory of Goldberg *et al.* (40), compete with the elevated levels of cyclic guanosine monophosphate observed in



Scheme II

rapidly dividing cells for the binding sites on nuclear portein kinases that regulate histone phosphorylation. Other protein kinases modulated by cyclic nucleotides phosphorylate nonhistone chromatin protein and also regulate transcription and differentiation. Johnson and Hadden (41) observed that a particular fraction, mol. wt. 52,000, is not phosphorylated in the presence of high cyclic adenosine monophosphate, and this result correlates with a cessation of lymphocyte proliferation. Apparently, this protein kinase is also under the control of the Ying-Yang modulation of cyclic nucleotides. Compounds I–III significantly reduced the activity of nonhistone cyclic adenosine monophosphate-dependent protein kinase.

Compounds I–III were potent inhibitors of cathepsin activity in tumor cells. Proteolytic agents such as trypsin and the protease components of serum mimic virus transformation of cells and result in a decreased cyclic adenosine monophosphate level (42). Proteolytic inhibitors, *e.g.*, I, block the transformation process (43–45), and dibutyryl cyclic adenosine monophosphate inhibits the growth-stimulating action of trypsin and serum (46, 47). Arrest of cancer growth can be achieved by elevating cyclic adenosine monophosphate levels in the cells by administering dibutyryl cyclic adenosine monophosphate (48) or by blocking the enzyme responsible for hydrolysis of the cyclic nucleotide, *i.e.*, phosphodiesterase, with theophylline (49).

Cholesterol synthesis required for newly synthesized membranes, *etc.*, was also impaired by treatment with II and III. Both cholesterol and triglyceride synthesis in the liver was inhibited when the cyclic adenosine monophosphate level was elevated (50, 51). A similar phenomenon also was observed in tumor cells treated with α-methylene-γ-lactone containing antineoplastic agents (27). Cancer patients are hyperlipidemic (52) because of the failure of a cyclic adenosine monophosphate-dependent regulatory feedback mechanism, resulting in the tumor releasing cholesterol into the serum (50, 53).

Based on the premise that II and III are metabolized as outlined in Scheme I, several species are generated that can attack functional groups of specific enzymes like the kinases. Benzaldehyde, bromoacetaldehyde, and acetaldehyde are good candidates. Warburg (54) postulated that glyceraldehyde reacts with the sulfhydryl groups of glycolytic enzymes to form thiohemiacetals, resulting in cancer cell death. Glutaraldehyde forms Schiff bases with lysine residues (55, 56). α,β-Unsaturated glutaraldehydes can form stable Michael-type adducts with the amino groups of lysine (56). Formaldehyde reacts with proteins, enzymes, and nucleic acids (57). Short-term exposure to formaldehyde results in substantial covalent bonding between histones and deoxyribonucleic acids, thus inhibiting gene activity (58).

Compound I is known to alkylate the active site histidine residue of chymotrypsin, thus inhibiting the enzymatic activity. Evidence indicates that active vinyl and dihaloethyl esters of L-phenylalanine containing *N*-protecting groups are latent alkylating agents dependent on catalytic release of a reactive metabolite within the active site of the proteases or esterase enzyme (59), *e.g.*, suicide substrates. As a latent alkylating agent, II would produce acetaldehyde, which reacts with histidine and lysine (60). Compound III would produce bromoacetaldehyde with the potential of reacting with cysteine, lysine, histidine, and guanine (61). Alternatively, these active esters might conceivably function through direct alkylation of a bionucleophilic moiety within the active or allosteric site of enzymes.

In vitro, UV and NMR special data support the thesis that benzaldehyde, possibly arising by catabolism of the *N*-benzyloxycarbonyl group, is capable of interacting with functional groups, *e.g.*, imidazole groups of histidine residues of enzymes, and of forming Schiff bases with lysine residues of enzymes. There is no NMR evidence that sulfhydryl groups are being alkylated by II, III, or VI. UV spectral studies support the idea that II forms Schiff bases with lysine residues, but this hypothesis is not confirmed by NMR spectral data. Compound III interacts with lysine residues according to NMR data, but it is difficult to interpret what chemical species evolve. According to UV data, both II and III can interact with imidazole groups (*e.g.*, histidine); however, NMR evidence indicates a reaction only with III. These spectral studies with II, III, and VI are inconclusive. However, they suggest that a lysine and imidazole nucleophilic attack of activated esters of phenylalanine may be a possible mode of action *in vivo*.

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ACKNOWLEDGMENTS

Supported by a University of North Carolina Research Council Grant and by American Cancer Society Institutional Grant MC157.

The authors thank Ron K. Levy for assistance with the NMR studies.